Pure Appl. Chem., Vol. 72, No. 8, pp. 1453–1470, 2000.

INTERNATIONAL UNION FOR PURE AND APPLIED CHEMISTRY

ANALYTICAL CHEMISTRY DIVISION
COMMISSION ON MICROCHEMICAL TECHNIQUES AND TRACE ANALYSIS

CHEMISTRY AND THE ENVIRONMENT DIVISION
COMMISSION ON FUNDAMENTAL ENVIRONMENTAL CHEMISTRY

CHEMISTRY AND HUMAN HEALTH DIVISION
CLINICAL CHEMISTRY SECTION, COMMISSION ON TOXICOLOGY

GUIDELINES FOR TERMS RELATED TO CHEMICAL SPECIATION AND FRACTIONATION OF ELEMENTS. DEFINITIONS, STRUCTURAL ASPECTS, AND METHODOLOGICAL APPROACHES

(IUPAC Recommendations 2000)

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Guidelines for terms related to chemical speciation and fractionation of elements. Definitions, structural aspects, and methodological approaches
(IUPAC Recommendations 2000)

Abstract: This paper presents definitions of concepts related to speciation of elements, more particularly speciation analysis and chemical species. Fractionation is distinguished from speciation analysis, and a general outline of fractionation procedures is given. We propose a categorization of species according to isotopic composition of the element, its oxidation and electronic states, and its complex and molecular structure. Examples are given of methodological approaches used for speciation analysis. A synopsis of the methodology of dynamic speciation analysis is also presented.

CONTENTS

1. INTRODUCTION

Trace elements play an important role in the functioning of life on our planet. Some elements can be highly toxic to various life forms; others are considered essential, but can become toxic at higher doses. Many of these effects depend strongly on the particular form in which the element is present in the system. For example, Cr(VI) ions are considered far more toxic than Cr(III) [1]. On the other hand, while both methylmercury and inorganic mercury are toxic, they show different patterns of toxicity. Often these different chemical forms of a particular element or its compounds are referred to as "species". The notion that the distribution among its various species will have a major effect on the behavior of a par-

ticular element has been accepted in such diverse fields as toxicology, clinical chemistry, geochemistry, and environmental chemistry. New developments in analytical instrumentation and methodology now often allow us to identify and measure the species present in a particular system. Because of these possibilities, numerous publications have appeared in which the term "speciation" is employed. However, this term has been used in a number of different ways, including the transformation of species, the distribution of species, or the analytical activity to determine the concentrations of species.

In an attempt to end the present confusion regarding the usage of the term speciation, three IUPAC Divisions (represented by the Commission on Microchemical Techniques and Trace Analysis, the Commission on Fundamental Environmental Chemistry, and the Commission on Toxicology) collaborated to consider the issue. It was agreed that straightforward, standard terminology is important for interdisciplinary communication and also for communication to non-scientists, such as legislators and consumer groups. One of the major goals of this paper is to provide a clear definition of chemical speciation, distinguishing it from fractionation, and where necessary suggest less ambiguous alternative expressions to those in current use. The dynamics of speciation analysis will also be dealt with.

We will discuss the levels at which one can differentiate between various species, from the isotopic composition to the level of macromolecular complexes. Examples will be given to illustrate how the distribution of species can affect the stability, mobility, toxicity, and other properties of a given element. Some examples will also be provided of methodological approaches for speciation analysis and fractionation. The preferred method will, of course, depend on the structural level of interest. We hope that this paper will end the present confusion regarding the use of the term speciation and contribute to a more widespread understanding of the various aspects of speciation, within and outside the chemical sciences community.

2. DEFINITIONS OF TERMS RELATED TO SPECIATION AND FRACTIONATION

In their report on the 1984 workshop on The Importance of Chemical Speciation in Environmental Processes, Bernhard, Brinckman, and Sadler [2] noted that the term speciation has been used in no less than four different ways, and concluded that authors should either avoid the term altogether, or clearly define it. One of these four possible meanings, "reaction specificity", is only very rarely used and will not be further discussed here. Another meaning of the word speciation arises in biology, where it is used to describe the evolution of species. In evolutionary theory, speciation denotes the development of different genetic traits in an isolated subpopulation, leading to a species distinctly different from the original parent population. Referring to this evolutionary concept, geochemists and environmental chemists have often applied the word speciation to describe the transformations taking place during cycling of the elements. An example is the changes that occur between the leaching of trace elements from soil or rock and their subsequent distribution in the aquatic environment. However, in order to avoid confusion, we recommend using the term "species transformation" to describe such processes.

The term "speciation" has often been used to indicate the analytical activity of identifying chemical species and measuring their distribution. Sometimes, it is used to indicate that a method gives more information on the form in which the element is present than other more commonly applied techniques (e.g., measuring distinct organomercury compounds as opposed to a total mercury determination). In order to avoid confusion, we recommend using the term "speciation analysis" when referring to the analytical activity of identifying and measuring species.

Finally, the term speciation is also used to indicate the distribution of species in a particular sample or matrix. For example, one might say, "The toxicity of arsenic depends strongly on its speciation". Speciation in this sense is thus synonymous with "species distribution". We recommend that the term speciation be used exclusively in this fashion.

Having recommended the term species transformation, described speciation analysis as the activity of identifying and measuring species, and restricted the use of the term speciation in chemistry to

species distribution, it becomes necessary to provide a careful definition of the word species in this context. Chemical compounds that differ in isotopic composition, conformation, oxidation or electronic state, or in the nature of their complexed or covalently bound substituents, can be regarded as distinct chemical species. This distinction leads to the following definitions:

i. *Chemical species*. Chemical elements: specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure
ii. *Speciation analysis*. Analytical chemistry: analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample
iii. *Speciation of an element; speciation*. Distribution of an element amongst defined chemical species in a system

Strictly speaking, whenever an element is present in different states in terms of i., it must be regarded as occurring in different species. In practice, however, it will depend on the relevance of the species differences for our understanding of the system under study, and on our ability to distinguish between the various species analytically, whether different species should be grouped or can be measured separately. This practical analytical consideration implies minimum lifetimes and thermodynamic stabilities for detection, the values of which may be expected to change with developments in instrumentation. Among solution chemists studying mixed electrolyte systems such as seawater, weak, short-lived ion pairs such as NaCl can be described either in terms of complexes or in terms of interactions contributing to the activity coefficient of the ions concerned. Both approaches are valid thermodynamically, but in recent years there has been a tendency toward incorporating weak interactions into activity coefficients and treating only stronger, long-lived interactions explicitly as complexes.

Consistent with restriction of the use of the term speciation to define chemical species, fractionation has been defined as follows:

iv. *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.

Fractionation is considered in more detail in Section 5 of this paper.

3. STRUCTURAL ASPECTS OF SPECIATION

As the foregoing discussion makes clear, various conformations, excited states, or transient forms of an element, its coordinated atoms, and the molecules of which they are part technically constitute unique species. Nevertheless, analytical methodology and practical considerations dictate that the speciation analysis of a system yields a profile of sufficiently different and measurable species for the desired level of understanding of the system's behavior. Various aspects of structure contribute to identifiable species for a particular element, and may differ in importance depending on the reasons for which a speciation analysis is undertaken. In this section, a systematic approach is taken to describe the levels of structure at which species can be considered distinct, and at which they become important in such fields as environmental chemistry, geochemistry, biochemistry, and toxicology. To be considered are nuclear (isotopic) composition, electronic or oxidation state, inorganic compounds and complexes, organometallic compounds, and organic and macromolecular complexes [3].

3.1 Isotopic composition

The isotopic abundances of an element can vary for several reasons, including radioactive decay (radiogenic) and physical separation (anthropogenic or environmental separation). For example, lead has four stable isotopes (^{204}Pb, ^{206}Pb, ^{207}Pb, ^{208}Pb) but three of these are radiogenic, arising from decay of uranium (^{206}Pb, ^{207}Pb) or thorium (^{208}Pb). Therefore, depending on the geological time of mixing of these elements the isotopic composition of lead will vary with its geological source [4]. Isotopic com-

position has been used to track the movement and deposition of atmospheric lead [5–9] and to fingerprint sources of toxic exposures [4,10,11]. Other isotopic variations of radiogenic origin and geochemical significance include those of osmium [12], strontium, and neodymium [13,14].

Isotopes of elements without radioactive precursors can also become differentially distributed in the environment for several reasons. Differences in mass can lead to separation on both inertial and chemical grounds [15]. When oxygen partitions between two phases where it is bound in different forms, ^{16}O and ^{18}O can be differentially enriched in each phase. This effect is temperature-dependent, and has been measured to assess long-term climatic trends [16] and geochemical cycles [17]. Sulfur-metabolizing bacteria alter the ^{34}S content between sulfates and sulfides upon disproportionation of sulfur [18].

Anthropogenic differences in isotopic composition occur in addition to those arising from radiogenic and environmental factors. For example, large quantities of ^{6}Li are produced for use in regulator rods of thermonuclear reactors, with ^{7}Li as a by-product. These isotopes have different biological behavior, in part due to different rates of transport across cell membranes [19,20] Lithium salts are used in the treatment of manic depressive illness. It is conceivable that future quality surveillance of the production of Li compounds for clinical use will require isotopic speciation analysis.

Only for very light elements do primary kinetic isotope effects become sufficiently large that differential toxicity arises, and in toxicology, isotopic speciation analysis is mainly of interest for identifying sources of exposure. For instance, replacement of 30–40% of body water with D_2O is lethal in rodents but replacement of 60% of body water with $H_2{}^{18}O$ is without effect [21] This can be explained by the difference in kinetics of biochemical reactions involving the O–H and O–D bonds. Based on a simple harmonic oscillator model, the zero-point energy of the bond is proportional to the inverse square root of the reduced mass. The ratio of zero point energies of ^{16}O–H to ^{16}O–D is 1.37, but that of ^{16}O–H to ^{18}O–H is only 1.003. Therefore, for all but the lightest elements, biological interest in isotopic composition rests largely with tracer studies.

3.2 Electronic and oxidation states

The oxidation state of an element can profoundly affect its toxicity. Cr(III) may be an essential element for normal glucose metabolism, but Cr(VI) is genotoxic and carcinogenic [1]. Cr(VI) does not itself bind to DNA but is reduced to Cr(III) which does. The binding of Cr(III) is insufficient to damage DNA *in vitro*, and it is supposed that damage arises from intermediate oxidation states of chromium [22–24]. The case of chromium also illustrates how the oxidation state influences bioavailability. Cr(VI) is taken up by many cells as chromate (CrO_4^{2-}) utilizing anion transporters, whereas Cr(III) ions permeate the lipid membrane with difficulty [1]. In contrast to chromium, the more reduced species of As are the more toxic: arsine (AsH_3) > arsenite (As(III)) > arsenate (As(V)) [25]. Tri- and pentavalent inorganic arsenicals are apparently of comparable bioavailability [26], but they display different biochemistry, in part due to the increased preference of the trivalent forms for binding thiols [25].

The oxidation state can also affect the absorption and elimination of an element. The Fe(II) ion is soluble under physiological conditions and diffuses freely across membranes, whereas Fe(III) does not enter cells readily and is more prone to hydrolysis in aquatic and biological systems [27,28]. Uptake of iron from a number of organic Fe(III) chelates by eukaryotic cells probably involves dissociation of the complex followed by reduction and transport of Fe(II) [29,30]. The Hg(II) ion becomes trapped in cells, but some bacteria possess a mercuric reductase system that reduces Hg (II) to volatile Hg^0, which then diffuses from the cell [31,32]. A similar activity is inducible in human liver [33].

3.3 Inorganic compounds and complexes

The distribution of an element among different inorganic compounds profoundly affects its transport and bioavailability by determining such properties as charge, solubility, and diffusion coefficient. The practical importance of inorganic speciation analysis is well illustrated by occupational exposure to Ni

and its compounds. Nickel salts such as chloride and sulfate are water soluble and of low oral toxicity. Nickel oxides and sulfides are highly insoluble in water, but their bioavailability may be influenced by biological ligands. Trinickel disulfide (subsulfide) (Ni_3S_2) is a potent carcinogen in animals. Exposures are seldom to a single species of Ni; for example, in various refining operations, workers may encounter Ni_3S_2, NiO, Ni^0, Ni–Cu oxides, Ni–Fe oxides, $NiSO_4$, $NiCl_2$, and $NiCO_3$ [34,35]. Therefore, an air filter analyzed for exposure monitoring may contain Ni salts deposited from aerosols as well as other inorganic forms of the metal and particulates of different sizes. While a fractionation (for instance, determining soluble and insoluble forms or the particle size distribution) provides more information than measuring total Ni only, additional useful information on species of differing carcinogenic potential can be obtained by speciation analysis.

Complexation reactions with inorganic ligands produce coordination complexes with varying stabilities. The distribution of species in a system containing both metal and ligand will depend on factors such as concentrations, stoichiometry, pH, and ionic strength [36]. This means that such species cannot normally be separated from each other without changes in distribution. However, the lability of inorganic complexes varies within very wide limits from complexes that form and dissociate rapidly, to systems in slow exchange, to complexes that, for most practical analytical purposes, can be considered inert (see Section 7 for further details). A further consequence is that data on the species distribution will normally be presented as two- or three-dimensional plots showing the varying speciation around a set of conditions of interest [36].

Hydrolysis is an important aspect of metal ion chemistry [37]. For many metals, formation of hydroxides in aerobic aqueous environments at neutral pH is a key determinant of their solubility and bioavailability. The reaction $M^{n+} + nH_2O \rightarrow M(OH)_n\downarrow + nH^+$ frequently produces neutral metal hydroxides with extremely low solubility (e.g., $Fe(OH)_3$, $K_S \approx 10^{-38}\,M^4$). Most metals, excluding alkali and alkaline earth elements, form one or more hydroxocomplexes under natural conditions. In this group we can also find kinetically very stable inorganic complexes such as $Al_{13}O_4(OH)_{24}^{7+}$ [37].

The interplay between kinetic and thermodynamic control of speciation can give rise to surprising effects. In the United Kingdom and Norway it was found that the mixing of two waters, one acidic with high concentration of labile monomeric aluminum and the other with high pH, had serious effects on fish. In the actual mixing zone the water was considerably more toxic than any of the components on their own. The cause was found to be the formation of transient polymeric aluminum-hydroxo complexes with high toxicity. Minutes later the extreme toxicity was gone [38,39].

A large variety of inorganic complexes can be found in the environment [40,41]. Anions such as SO_4^{2-} and Br^- generally form weak complexes which are not very important in open fresh waters. However, in certain waters such as well water or soil solutions and in marine waters they may be important. Other anions such as F^- and CO_3^{2-} are important for specific elements. The most commonly occurring type of inorganic complex by far is the hydroxocomplexes as discussed above.

3.4 Organic complexes

Complexation reactions with organic ligands produce coordination complexes with differing degrees of thermodynamic stability and lability, the latter term being related to the reactivity of the species. A more differentiated and quantitative outline of the notion "lability" will be given in Section 7. The distribution of species in a metal-ligand complex also depends on such factors as concentration, stoichiometry, pH, and ionic strength [36]. Whereas some complexes may be *labile* during analysis, some organic metal chelates (e.g., ferrioxamine) are of sufficient thermodynamic stability to be isolated quantitatively under a number of conditions. Organic complexation data will generally be presented in two-dimensional plots, representing conditions varied around a point of interest, and allowing interpolation of the composition in a given sample. Typically, titrations with acid or base monitored with potentiometric pH measurements at different metal-to-ligand ratios are used to derive a set of stability constants for the system. This allows representation of the species distribution as a plot of composition vs. pH [42].

Armed with such information one may deduce the species distribution in a particular sample, knowing its pH, ligand composition, and the total concentration of the metallic element, without actually separating individual metallic compounds.

Concentration-based stability constants that take into account protonation of the ligand (e.g., $\beta_\lambda = [MH_\nu L_\lambda] / [M]\,[H^+]^\nu [L]^\lambda$, where M, H, and L refer to metal, hydrogen, and ligand, respectively (ν and λ are stoichiometric coefficients) can also account for OH^- in the ligand set by allowing the stoichiometric coefficient of the proton (ν) to take negative values. The complete set of stability constants for a system defines the species distribution of the system as a function of pH at the concentrations of metal and ligand studied. Extensive compilations of these stability constants exist [43, www.acadsoft.co.uk] and are useful in predicting the behavior of simple systems by identifying the major species likely to be present. Unfortunately, the large number of N-, O-, and S-ligands available in biological fluids compromises the predictive utility of such data; the "complete" species distribution *in vivo* is never known and predictions of toxicity or bioavailability are successful only when the picture is simplified by exceptionally dominant concentrations (e.g., binding of Cd to the major plasma protein, albumin) or stabilities (e.g., binding of Fe(III) or Al to transferrin).

3.5 Organometallic compounds

Organometallic compounds are defined as compounds containing a covalent bond between a carbon atom and a metal. The practice in nomenclature is to consider any element other than C, H, and the rare gases to be metals if this is useful [44]. Some elements form organometallic compounds with a metal–carbon bond of strong covalent character. The origin of these compounds may be anthropogenic or environmental. In toxicology, hydrophobicity and volatility are important. Toxicity often results from bioaccumulation in fatty tissues and penetration of membrane barriers. Thus, ionic Hg(II) is toxic to the mammalian kidney and corrosive at sites of mucosal absorption, whereas CH_3Hg^+ crosses the placenta and blood–brain barrier, acting as a teratogen and central nervous system toxin [45].

In environmental alkylation, an important distinction can be made between biomethylation and addition of a larger alkyl chain. In general, metals undergo only biomethylation, important examples being Hg, Ge, and Sn. *S*-adenosylmethionine and methylcobalamin are methyl donors in bioalkylation reactions [46]. Biomethylation of metals is generally restricted to microorganisms in sediments and soils, with the exception of Co in vitamin B_{12}. Some elements, notably As and Se, also undergo other bioalkylations in addition to methylation [46]. These processes lead to, for example, arsenoylribosides (arsenosugars) and selenoamino acids. Alkyl (including methyl) derivatives of As and Se are metabolites of these elements in most organisms, including humans. Whereas methylation of metals generally increases their toxicity, methylation of arsenic [25] and selenium [47] contributes to their detoxification.

In addition to the products of bioalkylation, manufactured organometallics find their way into the environment, and those of major interest in speciation analysis include compounds of As, Sn, Hg, and Pb. In some cases, introduction into the environment is deliberate, as in the use of various organomercurials to treat seed grains, $(Phenyl)_3Sn$ as a fungicide, and $CH_3As(ONa)_2$ as a herbicide. In other instances it is incidental, as with emission of the antiknock compounds $(CH_3)_4Pb$ and $(C_2H_5)_4Pb$ during gasoline combustion or leaching of various organotin stabilizers, for example, dioctyltin from poly(vinyl chloride) plastics. Alkyl derivatives of lead, tin, and mercury are among the organometallics of major toxicological concern, with $(C_2H_5)_4Pb$, $(CH_3)_3Sn^+$, CH_3Hg^+ and $(CH_3)_2Hg$ being toxic to the central nervous system.

3.6 Macromolecular compounds and complexes

The highest structural level at which speciation analysis is considered here is the macromolecular level. At this level, limits to the definition of unique chemical species must be set. It may be of interest to

describe the distribution of an element among different proteins, or even among isoforms of the same protein, as a distribution among different species (e.g., the distribution of iron among transferrin, ferritin, and heme proteins [48], or even between adult hemoglobin and fetal hemoglobin in an infant). But it is not possible, or useful, to document the state of protonation of every amino acid in a protein. The complex of a metal with a given protein of unique amino acid sequence and a global tertiary structure can be considered a single species even though the sample will contain an ensemble of proteins in different states of protonation and local conformations.

In the environment, binding to polyanions such as humic and fulvic acids and to colloidal particles, contributes to the pattern of transport and availability of elements such as Cd, Cu, and Pb [49,50]. In the organism, polyanionic nucleic acids [51] and glycosaminoglycans [52] bind many metal ions nonspecifically. The nucleic acids may be direct or indirect (through metal-dependent oxygen radical generation) targets for damage leading to mutagenesis, teratogenesis, or oncogenesis [53]. In the association of a metal ion with a glycosaminoglycan in serum or humic substances in aquatic systems, the ligand is both heterogeneous and polydisperse. Determination of the metal associated with heparin or humic acid fractions is not sufficiently refined to be called speciation analysis as strictly defined. However, distinguishing the association of the element with multiple species of the macromolecule from other species possibly present is generally of interest. Analysis of affinity distributions in terms of chemical heterogeneity and polyelectrolytic effects provides continuous distributions of species [54]. However, the significance of such a theoretical speciation for the conditions in a real sample is uncertain. The analytical methods available today are simply not capable of identifying and quantifying all the individual species present in, for instance, the fraction we call metal-humic complexes.

Proteins provide opportunities for an arrangement of ligands to accommodate the coordination requirements of a metal ion. In each case, thermodynamic and kinetic equilibria among aquated and other inorganic species, small organic ligands, and macromolecules affect the speciation of the element and are reflected in its toxicology. On the one hand, binding to albumin renders Cd unavailable for uptake by cells [55]. On the other hand, isomorphous replacement of Ca^{2+} by Cd^{2+} in Ca^{2+}-binding proteins is a part of cadmium's mechanism of toxicity [56]. Removal of Cd from albumin by small organic ligands therefore facilitates its uptake by cells. In contrast, the bioavailability of Ni is decreased by some ligands such as cysteine and histidine and to a much lesser degree by proteins [57].

In human body fluids, a good deal of speciation analysis involves determination of binding of a metal to albumin and transferrin. Binding by albumin is prominent because it is present at far higher concentrations than other proteins in serum (approaching 1 $mmol \cdot dm^{-3}$). Transferrin is the Fe carrier with two Fe(III) ion binding sites of high affinity. It also binds other ions such as those of Al and Ga with reasonable affinity [58]. Chromium in serum of patients on peritoneal dialysis distributes mainly between transferrin and albumin in a time-dependent manner that may influence the kinetics of Cr(III) absorption from the dialysis fluid and the metabolism of Cr in the body [59].

4. METHODOLOGICAL APPROACHES FOR SPECIATION ANALYSIS

The section below provides a brief survey of the methodological approaches available for speciation analysis. Selected examples from each structural level discussed in Section 3 will be given.

4.1 Isotopic composition

Lead is taken as an example. It has four stable isotopes. Their ratios can be measured by mass spectrometric techniques and can be used to determine the environmental origin of the lead. Air samples have been collected on filters, acid digested, and the Pb concentrated by dithizone extraction for analysis by thermal ionization mass spectrometry (TIMS) [5]. The same approach has been used with inductively coupled plasma quadrupole mass spectrometry (ICP-MS), without dithizone extraction [6]. Although of lower precision than TIMS, this approach has the advantage of higher sample throughput since the sam-

ple is introduced as a nebulized aerosol. ICP-MS has been shown to be of sufficient precision for sourcing exposures to Pb using blood [60] and tissue samples [61]. In this approach, the invariant ratio of the adjacent mass peaks $^{203}Tl/^{205}Tl$ can be used to correct for mass bias effects [62]. The precision in ICP-MS can be improved by the recently introduced time-of-flight (TOF) [63] and multi-collector sector field analyzers [64].

4.2 Electronic and oxidation states

Chromium is given as an example. Cr(VI) can be selectively determined spectrophotometrically after complex formation with 1,5-diphenylcarbazide [65]. Separation of Cr(III) and Cr(VI) is frequently achieved by ion-exchange HPLC with detection by atomic absorption spectrometry (AAS), inductively coupled plasma-atomic emission spectrometry (ICP-AES), or ICP-MS [65]. For extremely low concentrations one can use anodic stripping voltammetry [66].

4.3 Inorganic compounds and complexes

As mentioned above the practical importance of inorganic speciation analysis is well illustrated by occupational exposure to Ni and its compounds. Methods exist for the topochemical and morphological characterization of single particles. Under certain circumstances these methods can provide information on the bonding of elements in the outer layers of the particle that is relevant to speciation [67–69]. Crystal morphology may also affect the chemistry of the compounds.

Hydrolysis of Al^{3+} is an example for inorganic complexes. While one would like to observe the individual products of hydrolysis of a metal ion directly with optical or magnetic spectroscopic techniques, hydrolysis reactions rarely lead to a sufficiently simple speciation to allow individual components to be observed [37]. Generally, potentiometric determination of pH is used to deduce stoichiometries based on the reaction

$$xM^{z+} + yH_2O \leftrightarrows M_x(OH)_y^{(xz-y)+} + yH^+.$$

Supplementary methods are used to deduce the presence of polynuclear complexes, including solubility measurements, ultracentrifugation, and light scattering. This is illustrated by the historical approach to Al^{3+} hydrolysis outlined by Baes and Mesmer [37]. In dilute solutions of Al salts, pH measurements have shown the formation of $AlOH^{2+}$, and competition with OH^- by colored organic ligands has further yielded stability constants for $Al(OH)_2^+$ and $Al(OH)_3$. In alkaline solution, solubility studies of gibbsite ($Al(OH)_3$) have given a stability constant for $Al(OH)_4^-$ and this tetrahedral species was observed by IR and Raman spectroscopies. Ultracentrifugation revealed the occurrence of stable polynuclear species, with a unique species of higher mass with stoichiometric coefficient for Al between 7 and 17. Modeling based on potentiometric data suggested $Al_{13}O_4(OH)_{24}^{7+}$ for this species, as well as bi- and trinuclear species proposed to be $Al_2(OH)_2^{4+}$ and $Al_3(OH)_4^{5+}$.

4.4 Organic complexes

Potentiometric data are widely used to derive stability constants of metal–ligand complexes that allow modeling of the species distribution as a function of pH. Stability constants are derived from titration data by least squares fitting routines [70,42]. An IUPAC database for stability constants is available on the Internet (www.acadsoft.co.uk). The complete set of stability constants $\beta_\lambda = [MH_\nu L_\lambda] / [M][H^+]^\nu[L]^\lambda$ allows calculation of the concentration of each species as a function of pH, provided the total metal concentration, [M], is known. The system of aqueous Ni(II)-glycinate complexes has been thoroughly studied and served as the basis for an interlaboratory comparison that showed standard deviations of the β values among seven laboratories generally less than 0.1 of a log unit [71]. Complexation begins with

$Ni(Gly)^+$ at about pH 4 with $Ni(Gly)_2$ dominating above pH 7 and $Ni(Gly)_3^-$ and Ni(Gly)OH occurring at alkaline pH. The optical spectra of this system are interpretable in terms of these species, strengthening confidence in the potentiometric modeling. The minor species $Ni(Gly)_2OH^-$, $Ni(Gly)_3OH^{2-}$, and $NiOH^+$ are also present in some of the fitted data.

When dissolved species are electroactive at the mercury electrode, electrochemical techniques such as anodic stripping voltammetry (ASV) and adsorptive cathodic stripping voltammetry (ACSV) are the most direct methods for studying trace metal speciation at very low concentrations ($10^{-8} – 10^{-11}$ $mol \cdot dm^{-3}$) [72].

4.5 Organometallic compounds

Speciation analysis for organometallic compounds is usually carried out by hyphenated techniques based on the coupling of chromatography with element-selective detection [73–75].

Thermally stable and volatile species, and those that can be converted into such, are preferably separated by gas chromatography. An example is the speciation analysis for organotin compounds. Environmental residues of butyltin and phenyltin biocides are extracted from the matrix, derivatized by alkylation and determined by, for example, GC with flame photometric detection, GC/MIP-AES, GC/MS, or GC/ICP-MS with absolute detection limits down to the femtogram level. A recent review covers the existing methods for organotin determination in environmental samples [76].

Species not amenable to GC are separated by HPLC or capillary zone electrophoresis. ICP-MS is by far the most widely used detection technique [77]. An important example is organoarsenic compounds; they include mono- and dimethyl arsenoacids, quaternary compounds such as arsenobetaine and arsenocholine, and arsinoyl ribosides [78]. A number of HPLC separation mechanisms have been proposed, anion-exchange [79] and ion-pair reversed-phase HPLC [80] being the most popular.

4.6 Macromolecular compounds and complexes

Chromatographic techniques dominate the analysis for metal–protein complexes [75,81,82]. There is a wide variety of columns available for size-exclusion, ion-exchange, affinity, and reversed-phase chromatographies. When using these techniques it is often assumed that the macromolecule and the metal that are found in the same fraction were associated with one another in the original sample. Before this can be concluded, it is necessary to ensure that dissociation of the metal from the bioligand has not occurred. Unfortunately, such dissociation can be affected by the buffer and the column packing, and this is the rule rather than the exception. When a complex sample such as blood plasma is fractionated, each fraction will still contain numerous potential ligands, and the unequivocal association of a metal with a given component is by no means straightforward [83,84]. The association must be supported by additional evidence, for instance, from mass spectrometry or affinity chromatography. Ionization of metal complexes with macromolecules for mass spectrometry is extremely difficult, and so far only for metallothioneins has some evidence been obtained in this way [85]. If an element binds to a protein bound in turn to an antibody on an affinity column, then the link between the metal and the protein is also convincing.

Electrophoretic techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [86] and 2-D electrophoresis [87] are useful for separating proteins. However, for selenoproteins or metal–protein complexes the low ratio of one to thousands in mass of metal to mass of protein means it may be difficult to detect the element. Radioisotopes are often used to improve detection limits.

Because of the complexity of the system, obtaining information on metal–macromolecule complexes involves a number of steps. Figure 1 illustrates the sequence of steps in the fractionation and speciation analysis for cadmium from rabbit liver. The first step (Fig. 1a) is a fractionation by SEC-ICP-MS that separates the Cd-metallothionein (Cd-MT) fraction from other Cd species potentially present.

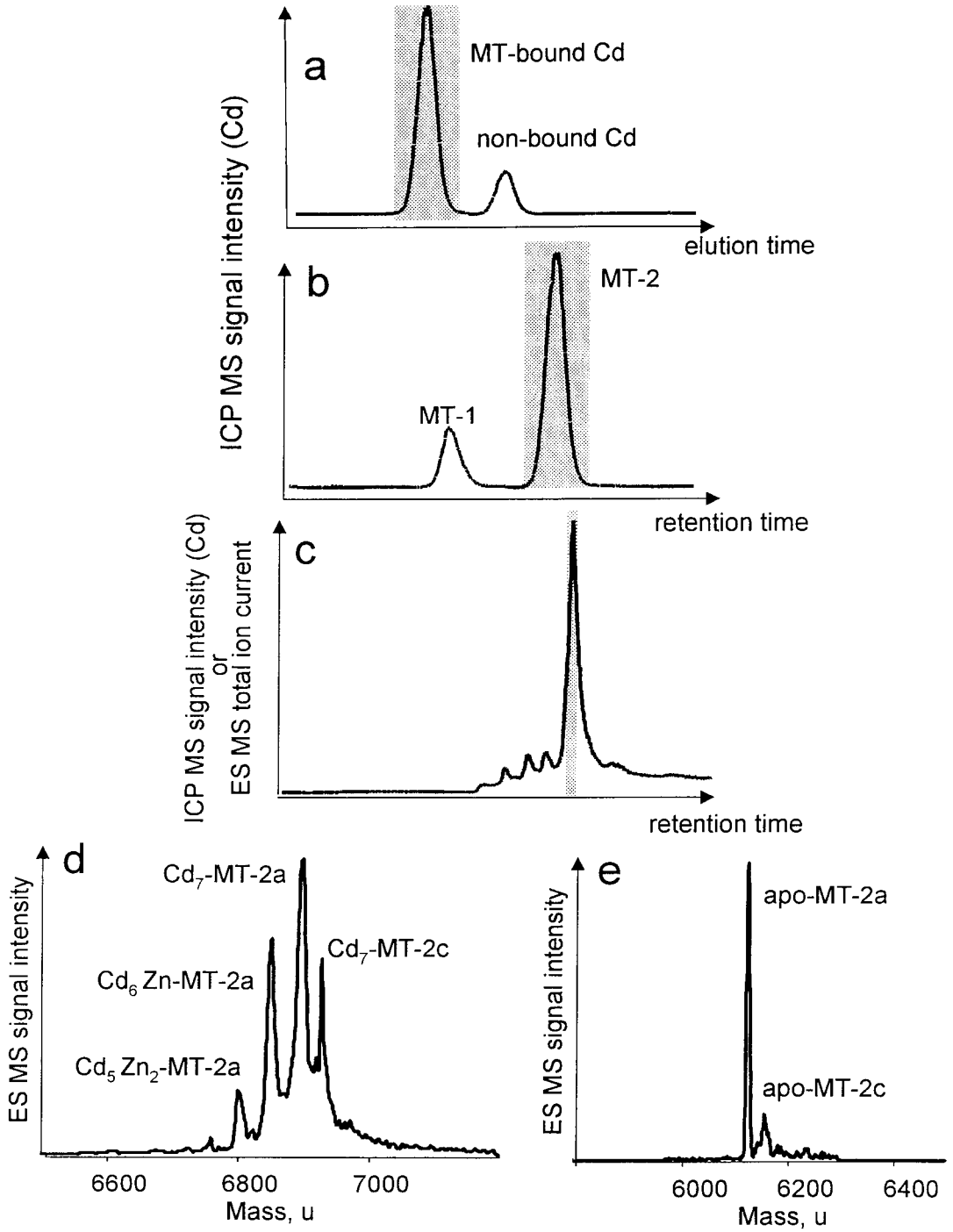

Fig 1 Illustration of a strategy for fractionation and speciation analysis using cadmium-metallothionein (Cd-MT). 1a) Fractionation of Cd by SEC/ICP-MS. 1b) Further fractionation of the indicated fraction of 1a by anion-exchange chromatography. 1c) Further fractionation of the indicated fraction of 1b by reversed-phase HPLC. 1d) Electrospray mass spectrum of the indicated fraction of 1c. 1e) Electrospray mass spectrum of the same fraction as in 1d following acidification to dissociate the metal.

In a second step (Fig. 1b), anion-exchange HPLC coupled to ICP-MS further separates the Cd-MT fraction into MT-1 and MT-2 isoforms according to the sequence of the ligand. A third step (Fig. 1c) combines elements of both fractionation and speciation analysis; reversed-phase HPLC is applied to the Cd-MT-2 anion-exchange fraction. This illustrates either speciation analysis, provided that a peak contains one species, or fractionation if a signal arises from a mixture of species. A technique for the verification of the chromatographic purity is necessary. The mass spectrum (Fig. 1d) of one of the peaks identifies it as representing a mixture of complexes. It is possible to attribute each molecular mass to a single species. Three of the peaks correspond to different Cd-Zn MT-2 complexes with the same amino acid sequence (MT-2a). As a confirmation, Fig. 1e shows a mass spectrum of the apoprotein stripped of its metal, demonstrating that we had different Cd complexes of the same ligand. The fourth peak corresponds to the Cd_7 complex of a ligand with a different amino acid sequence (isoform MT-2c).

5. FRACTIONATION

For the reasons given briefly above, an assessment of the impact of an element cannot be made based solely on its total concentration. It is, however, often not possible to determine the concentrations of the different chemical species that sum up to the total concentration of an element in a given matrix. Often, chemical species present in a given sample are not stable enough to be determined as such. During the measurement process the partitioning of the element among its species may be changed. This behavior can be caused by, for example, a change in pH necessitated by the analytical procedure, or by intrinsic properties of measurement methods that affect the equilibrium between species. Very common but less obvious is the situation where the analyte must be transformed into a complex to be measured or separated from the sample matrix. Even in the idealized situation that the complexation reaction is highly selective, errors can occur if the sample contains other species that can be transformed into the target analyte within the time frame of the analysis.

In some cases, species are stable enough to be determined as such (e.g., tetraethyllead, arsenobetaine, cyanocobalamin). In a given situation the determination of the concentration of such a species may be what is actually desired. However, this does not mean that the speciation of the element has been determined, only the concentration of one or a few species. The direct determination of labile species at their natural levels requires noninvasive methods such as direct spectroscopic or potentiometric measurements that do not perturb the sample.

In many cases the large numbers of individual species (e.g., in metal-humic acid complexes or metal complexes in biological fluids) will make it impossible to determine the speciation. The practice has been to identify various classes of species of an element and to determine the sum of its concentrations in each class [88]. This practice is useful and will continue. Such fractionations can be based on many different properties of the chemical species, such as size, solubility, affinity, charge, and hydrophobicity. Fractionation may involve an actual physical separation (e.g., filtration, size-exclusion chromatography). In some instances, fractionation may be refined by supplementary speciation analysis. For example, for copper in natural waters a fractionation can be performed to determine organic and inorganic copper concentrations. With further analyses and calculations the inorganic fraction can be subdivided into individual species. It is also desirable to measure the total concentration of the element in order to verify the mass balance.

Many existing methods are claimed to give concentrations for “free” or “available” elements. Most of these methods are based on electrochemical and/or kinetic discrimination. In order to produce information on the chemical speciation of the elements the measured fraction is factored into chemical species concentrations using an equilibrium model. These attempts at indirect measurements of speciation basically suffer the same shortcomings as do fractionations according to size. The discrimination inherent in the method of measurement can be more or less selective but it is not absolute. Further, the equilibrium model applied is based on a choice of complexes and their stability constants, which makes the results questionable.

Although a direct determination of the speciation of an element is often not attainable, the available methods can still provide much useful information. An evaluation of the environmental impact of an element may sometimes be made without determining its speciation and even though the fractions are only operationally defined.

6. METHODOLOGICAL APPROACHES FOR FRACTIONATION

A typical example of an operationally defined fractionation procedure is that for aluminum in natural waters [89]. The method is based on kinetic discrimination, measuring only those aluminum species that react within the timeframe defined by the analytical procedure. In order to achieve consistency between results obtained using different methods and by different analysts it is important to validate procedures as far as possible against solutions of relevant species with known concentrations and known labilities. The procedure for "reactive" aluminum can be validated based on the interaction between aluminum and fluoride in synthetic solutions [90].

Another example is fractionation using membranes of different pore sizes. The most obvious fractions are of dissolved species and particulates, but fractionations according to molecular size are also possible using appropriate membranes. However, one should keep in mind that the distinction between dissolved and particulate fractions is arbitrary and that the results obtained with a given set-up are critically dependent on operating conditions. For instance, the retention of iron colloid particles on a standard 0.45-μm membrane filter is strongly dependent on the filter load. When the filter clogs it starts retaining much smaller particles as well [91,92]. Further, the molar mass cut-off given for a certain type of membrane is based on measurements using a certain set of calibrants, often with globular shape, and this cut-off does not necessarily apply to all the chemical species fractionated.

Another way of fractionating is to use field-flow fractionation (FFF). Suspended particles are separated into specific size fractions by sedimentation FFF [93]. Using on-channel preconcentration, the cross-flow version of FFF coupled on-line with ICP-MS has been applied to the study of element distributions in colloids in, for example, deep seawater [94] and creek water [95]. Sedimentation FFF has also been used on-line with ICP-MS for the characterization of larger particles [96]. These techniques need further refinement but can be of great help in studies of element transport in rivers, estuaries, and aquifers.

Many research groups have worked on the design and study of extraction schemes aiming at the sequential solubilization of metals bound to the specific substrates making up sediments and known to undergo changes in the yearly life cycle of lakes or rivers [40]. Typical substrates are:

- carbonates of calcium, magnesium and iron, which dissolve upon decrease in pH
- iron and manganese compounds present in the sediments and changing their adsorption capacities drastically according to the redox conditions (presence/absence of oxygen), creating either iron sulfides or iron-oxy-hydrates setting free co-precipitated or adsorbed metals at every change
- organic matter present in sediments undergoing slow degradation, thus setting free the incorporated metals
- silicates and other refractory minerals, which might contain high metal concentration, but will not, under any environmental conditions, release them to the aquatic environment

Measurement of such functionally defined metal fractions will allow some forecasting of metal release from sediments under certain conditions and constitutes therefore a valuable tool in lake and river management. A harmonization of methods was necessary because the wide variety of procedures led to incomparability of results even for single aquatic systems. A collaborative study led to the development of a three-step sequential extraction procedure, yielding information on four metal fractions [97]: 1) exchangeable and associated with carbonates; 2) associated with easily and moderately reducible iron and manganese compounds; 3) associated with organic matter and sulfidic phases; and 4) residual frac-

tion. This information is essentially based on the Tessier procedure [98] which was later modified by Förstner and co-workers [99].

7. DYNAMIC ASPECTS OF SPECIATION ANALYSIS

Dynamic speciation is not only concerned with the equilibrium distribution of different species, but also with the kinetics of their interconversion. Knowledge of dynamic speciation is necessary to understand nonequilibrium properties such as transport processes and consumptive processes such as chemical reactions or biological uptake. Such understanding of a complex system is also a prerequisite for the proper interpretation of analytical data obtained from nonequilibrium techniques. This section is intended to stress the meaning of the dynamic or nondynamic nature of existing methods for speciation analysis, and to establish the relation between the operational timescale of a given technique and the dynamic response of the complex system in terms of the rate parameters of the responsible association/dissociation reactions. In doing so, it is helpful to define ranges of values of parameters such as thermodynamic equilibrium constants, and ranges of rate constants covered by certain techniques. Such ranges, often denoted as "windows", are useful for the quantitative understanding of analytical data.

In dynamic metal speciation analysis, parameters relating to volume reactions and interfacial reactions should be considered in addition to thermodynamic and kinetic windows. The underlying theory can be found in the specialized literature [100–103] and is summarized below.

Dynamic nature of volume reactions: Let us consider the simple scheme of a metal ion M that can associate with a ligand, L, to form the complex species ML

$$M + L \leftrightarrows ML \ (\rightarrow k_a ; \leftarrow k_d)$$

The rate constant for the association reaction is denoted as k_a, and that for the dissociation reaction as k_d. The ratio k_a/k_d equals the thermodynamic stability constant K. For ligand concentrations in sufficient excess over the metal concentration, the association reaction becomes pseudo-first order with a rate constant k_a', equal to $k_a c_L$. This is, however, a simplification that is not always valid.

The metal species involved in complex formation reactions have characteristic lifetimes that derive from the reaction rate constants, $1/k_a'$ and $1/k_d$ for M and ML, respectively. On a timescale t much larger than the lifetimes of M and ML, each individual metal ion frequently changes from M to ML and vice versa. Such a situation is denoted as "dynamic" and obeys the condition

$$k_a' t \text{ and } k_d t >> 1$$

The other limiting case is where the lifetimes of M and ML are much larger than the operational timescale. Changes in species concentrations are then not followed by significant (re)equilibration. The case is defined by

$$k_a' t \text{ and } k_d t << 1$$

which is referred to as "static" and the complex species are frequently called "inert". The above conditions for dynamic or static behavior are concerned with the rate characteristics of a volume reaction. They not only determine if the system is capable of attaining equilibrium within a certain time, but also what is detectable by consumptive bulk analytical methods like exchange or titration techniques. Even if one is only interested in equilibrium speciation, and applies nonconsumptive methods of analysis, it is useful to consider the above conditions since they are helpful in confirming whether or not equilibrium exists at all.

It has been shown by Eigen [100] that in aqueous systems the reaction rate of complex formation is determined by the rate of dehydration of the inner hydration shell of the metal ion. This is true for a wide range of ligands and allows us to make *a priori* estimations of the dynamic character of complex systems from tabulated values of k_a and the stabilities K (from which k_d values derive for given k_a).

Dynamic nature of interfacial reactions: Next we have to consider what happens if, for example, the free-metal M is taken up at an interface that is in contact with a homogeneous solution containing both M and ML. The interface may be the surface of some analytical sensor like an electrode, a species-selective membrane, a size-exclusion interface, the surface of an organism, etc. In the limiting case where the interface acts as a perfect sink for M, the transfer processes at the interface are much faster than transport processes and/or kinetics of the volume reaction in the solution. The scheme is:

$$ML \leftrightarrows L + M \rightarrow \text{ fast interfacial transfer}$$

The resulting flux of M can be found by solving the conservation equations for the different species under the appropriate initial and boundary conditions. For the case of excess ligand ($c_L >> c_M + c_{ML}$), the result in terms of the interfacial flux J_M can be derived analytically [101]. The derivation shows that there are some interesting limiting cases. The most important is found in the category of dynamic systems and concerns the definition of lability. Within the context of an M-selective interfacial reaction, a complex ML is considered 'labile' if its association/dissociation kinetics is so fast that the flux J_M is purely controlled by the coupled diffusion of M and ML. For the case of semi-infinite linear diffusion and pseudo-first order reaction kinetics, the lability condition is

$$\frac{k_d^{1/2}(\varepsilon^{-1} + K')t^{1/2}}{\varepsilon^{1/2}K'(1 + K')^{1/2}} >> 1$$

where ε is the diffusion coefficient ratio D_{ML}/D_M and K' is k'_a/k_d [102]. The steady-state equivalent of this lability criterion contains a diffusion layer thickness instead of the time t. It is important to emphasize that the notion of lability depends on experimental conditions. In the present context of an interfacial reaction, it refers to a combination of two conditions: one on the volume reaction and another one on the flux towards an interface.

Methods for speciation analysis may be based i) on bulk reactions involving chemical competition (e.g., ligand exchange) or ii) on interfacial reactions (e.g., electrode measurements). One can also distinguish between nonconsumptive species-selective detection in the bulk (e.g., direct spectrophotometry) or the same at an interface (e.g., ion-selective electrode potentiometry). For all bulk methods, equilibrium requires that the condition of dynamic behavior be fulfilled. This means that the operational timescale must be sufficiently large compared to the reciprocal rate constants of the volume reaction. In stepwise experiments like titrations, the condition applies to the time between two steps. For nonequilibrium interfacial methods, speciation analysis is more complicated and requires consideration of the lability.

8. CONCLUSIONS

This paper is meant to clarify the use of the terms speciation and fractionation in the different chemical disciplines. A recommendation is made to restrict the use of the term speciation to the *distribution* of an element among defined chemical species and to use the terms speciation analysis and fractionation to refer to analytical *activities*. Examples of methodological approaches give insight into the current practice of speciation analysis. When speciation analysis is impractical, fractionation may still be useful. Analysis of labile species requires an understanding of the dynamic properties of the system, as well as the timescale of the analytical methodology.

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