The Analyst
CRITICAL REVIEW

Bio-inorganic speciation analysis by hyphenated techniques

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The state-of-the-art of species-selective analysis for trace metals and metalloids in biological materials by chromatographic and electrophoretic separation techniques with element selective detection is critically reviewed. The species of interest include organoarsenic, organoselenium and metal complexes with bioligands such as phytochelatins, metallothioneins, proteins and polysaccharides. The separation mechanisms

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discussed include size-exclusion, anion- and cation-exchange and reversed-phase HPLC and flatbed and capillary zone electrophoresis. Advantages and limitations of various element selective (*e.g.*, AAS, ICP-AES and ICP-MS) and molecule specific (electrospray MS/MS) detection techniques used on- and off-line are discussed. The applications of coupled techniques to the analysis of biological materials are comprehensively reviewed in tabular form. Attention is paid to the sample preparation and sources of error in bioinorganic speciation analysis.

1 Introduction

It has long been known that the proper functioning of life is critically dependent on trace elements in a number of different ways. Some metals (*e.g.*, Hg, Pb) and metalloids (As) are highly toxic whereas others (*e.g.*, Mo, Mn, Fe, Co, Cu, Zn), considered essential, are needed for the accomplishment of life processes.[1] A number of other elements (*e.g.*, V, Cr, Ni) are recognized as being beneficial to life. From the chemical point of view, the intake, accumulation, transport and storage of essential or toxic metals and metalloids are realized by surrounding the element ion by electron-pair donating biological ligands. Sometimes this process is accompanied by the synthesis of specific ligands such as metallothioneins, or by the formation of a metalloid–carbon bond such as in the case of selenoamino acids or organoarsenic compounds. Since the evolution of a metal in a living organism happens by its interaction with the highly complex coordinating environment and involves a number of species with different properties, the information on the total element concentration in a biotissue is not sufficient and may even be misleading to understand the metabolism, bioavailability and toxicity of a metal or a metalloid.

Biochemists and natural product chemists always favoured information on metal species over that on the total element content. Indeed, their concern has been the concentration of haemoglobin, vitamin B_{12} or chlorophyll and not that of total iron, cobalt or magnesium, respectively. The isolation and purification of naturally occurring metal complexes with bioligands and compounds with a carbon–metalloid bond prior to determination of their structures by a variety of spectroscopic methods such as X-ray diffraction, nuclear magnetic resonance (NMR), Mössbauer spectroscopy and electronic, vibration or circular dichroism spectroscopy has been the analytical basis of bioinorganic chemistry.[2–4]

The disadvantage of the above mentioned spectroscopic techniques is the need for a fairly large amount of well purified analyte compounds, typically in the milligram range. Consequently, these techniques apply only to species of the major elements in biological tissues, and are valid only after a tedious purification protocol. Access to information on trace and ultratrace metal and metalloid species, however vital, has been limited by the inadequacy of the analytical methodology. Indeed, total trace element analysis at naturally occurring levels is difficult and the state-of-the-art techniques can still hardly cope with many problems.[5] Species-selective analysis is further complicated by the fact that the trace or ultratrace amount of the analyte element is divided among several species in which the contribution of the metal or metalloid to the total structure is minute in terms of weight. The often unknown molecular identity of the ligand and the sometimes low thermodynamic stability of the complex make the analytical task even more challenging.

In the end of the 1970s, Van Loon[6] and Suzuki[7] pioneered an approach that turned out to open the door to the acquisition of species-selective information at the trace and ultratrace levels. It was based on the coupling of a chromatographic (later also electrophoretic) separation technique with a sensitive and element-specific detector (usually an atomic absorption, emission or mass spectrometer), and came to be known as hyphenated (coupled, hybrid) techniques. The approach gained particular attention in the speciation analysis of environmental organometallic pollutants (organotin, organolead, organomercury) and redox states, and has been the subject of a number of status papers,[8–20] edited works,[21–23] and fundamental and comprehensive reviews, these last being oriented to the separation component of the hyphenated techniques: gas chromatography (GC)[24,25] or liquid chromatography (LC),[26–28] the detection component (ICP-AES and ICP-MS),[13,29–31] or on a particular coupling such as capillary zone electrophoresis (CZE) with ICP-MS.[32] The space devoted to bioinorganic analysis in these reviews was small because of the insufficient detection limits and of the occurrence of many problems with the sample preparation and separation aspects preventing the analysis of real samples.

At the close of the 20th century, environmental speciation analysis, understood as the determination of the particular organometallic species or redox states in the environment, was losing its scientific interest.[33] Not only does reliable methodology based on a hyphenated technique for the routine monitoring of organometallic contaminants exist[34] but also the emissions of potentially hazardous compounds into the environment have been considerably restricted. On the other hand, the continuous progress in microscale isolation and purification techniques, such as analytical HPLC, CZE and capillary electrochromatography (CEC), and the increasing sensitivity of element-selective detectors, especially ICP-MS, have spurred the quest for the detection, identification, characterization and determination of endogenous elemental species in biological systems. Since the report of Gardiner[35] in 1988, the perspectives for this work were recently explored in two edited works[36,37] and in a number of status reports.[38–48] A comprehensive but already somewhat outdated review is available on metal speciation in biological fluids.[49]

Bioinorganic trace analytical chemistry is a rapidly developing field of research at the interface of separation sciences, trace element spectrochemistry and analytical biochemistry. It targets the detection, identification and characterization of substrates and products of reactions of trace metals and metalloids with components of living cells and tissues.[37] Hyphenated techniques based on the coupling of a separation technique (HPLC or CZE) with element- or moiety-selective detection are becoming a fundamental tool for the functional characterization of trace elements or otherwise inconspicuous metal ions in biological systems. The critical evaluation of the state-of-the-art of the field based on a comprehensive review of applications is the topic of this paper.

2 Scope

The choice of references included in this paper was based on a number of strict criteria meant to ensure the comprehensiveness of the coverage of the topic, to facilitate critical analysis and to retain the clarity of the text. These criteria are as follows:

(a) The review covers metal and metalloid species, which are (i) naturally present in biomaterials (tissues of living organisms or products of their processing), (ii) are biosynthesized as a response of an organism to environmental exposure or (iii) result from the metabolism of absorbed environmental pollutants or administered metallodrugs. Consequently, the text ignores notorious organometallic contaminants such as alkyllead,[50,51] butyltin[52,53] and methylmercury,[54] for which a number of excellent reviews have recently appeared.

(b) The above scope limits in a natural way the separation techniques to those employing liquid mobile phases: HPLC, CZE, CEC and gel electrophoresis (GE). According to the

IUPAC guidelines, these techniques may be applied to fractionation (the case when a mixture of several species of the detected element arrives at the detector at a given time) or speciation (a unique species of the detected element arrives at the detector).[55] Techniques based on physical cutoff fractionation (*e.g.*, ultrafiltration) or chemical fractionation (*e.g.*, by selective leaching) without the following chromatographic step are not discussed.

(c) In terms of detection techniques the review is limited to techniques which show elemental specificity (the signal registered by the detector is due to a metal or metalloid contained in a larger analyte species). However, the increased use of molecular ion mass spectrometry (fast atom bombardment, electrospray) needed to be reflected by a number of examples of the use of these techniques for metallocompounds. Most of the examples cited concern on-line detection. However, successfully used off-line detection techniques such as ETAAS, ETV-ICP-MS, laser ablation ICP-MS or PIXE for HPLC, GE and CZE are also discussed.

(d) The review covers stable chemical species. The criterion of stability is operationally defined: a stable species undergoes no change during its migration through the separation medium. The species discussed include both organometallic species in which a carbon–metal (metalloid) bond is present and metal complexes in which the metal is coordinated by a bioligand.

(e) The review covers comprehensively applications of techniques fulfilling the above criteria to real biological samples. The application areas include plant, animal and clinical biochemistry, environmental chemistry, nutrition, pharmacology and medicine. Instrumental analytical developments, demonstrated on standards, synthetic or spiked samples, have not been included with the exception of reviews and some fundamental papers.

(f) The text is based on peer reviewed publications that appeared in the period 1980–1999.

3 Occurrence and classification of metal species in biological materials

A biological tissue represents a highly complex environment, rich in bioligands with a significant coordinating potential. The occurrence of free metal ions, especially of transition elements, is therefore of low probability. The major two classes of elemental species include organometalloid species and coordination complexes. The latter include small simple (*e.g.*, halide) or complex (*e.g.*, citrate, tartrate, oxalate, phytate, amino acids, oligopeptides) ligands, macrocyclic chelating ligands (*e.g.*, porphyrins) or macromolecules (*e.g.*, polypeptides, proteins, DNA restriction fragments, polysaccharides).

3.1 Biosynthesized molecules with the 'true' metal (metalloid)–carbon bond

This category includes selenoamino acids and their higher analogues: selenoglutathione and selenoproteins. They can further coordinate metals with S-affinity using the Se atom as the coordination center.[56] Another important class includes organoarsenic compounds: methylarsonic acids, quaternary compounds (*e.g.*, arsenobetaine) and arsinoylriboside derivatives (arseno sugars).[57,58]

3.2 Complexes with biosynthesized macrocyclic chelating agents

The most important group is the analogues of tetrapyrrole which in their deprotonated form can tightly bind even relatively labile divalent metal cations. The best known compounds of this group include chlorophyll and products of its degradation, cobalamins (the coenzymatically active forms of vitamin B_{12})[59] and porphyrins[60] including the heme group found in hemoglobin, myoglobin, cytochromes and peroxidases.

3.3 Complexes with nucleobases, oligo- and polynucleotides and -nucleosides

Heterocyclic nucleobases, alone or as constituents of nucleosides or nucleotides, offer several different coordination sites for metal ions. Of particular interest is the coordination of metal ions, *e.g.*, CrO_4^- or inert metal complexes to DNA because of the specificity with regard to certain base-pair sequences in the double helix. Metals (Pt, Au, Ru) are components of many therapeutic drugs; their interactions with proteins and DNA are the key to understanding their activity.[61]

3.4 Complexes with amino acids, oligopeptides and polypeptides (proteins)

Metal complexes with proteins, including enzymes, are carriers of biochemical function. Whereas the carboxamide function itself of peptide bonds –C(=O)–N(–H)– is only a poor metal coordination site, peptides contain several functional groups in the side-chains that are particularly well suited for metal coordination. They include especially cysteine ($–CH_2SH$) and methionine ($–CH_2CH_2SCH_3$), which bind metals with sulfur affinity (Cd, Cu, Zn), and histidine, both nitrogen atoms of which become available for coordination after metal-induced deprotonation (*e.g.*, Cu, Zn in superoxide dismutase). Peptide-complexed metal ions are known to perform a wide variety of specific functions (regulatory, storage, catalytic, transport) associated with life processes. The greatest interest is attracted by essential elements associated with ferritin (Fe, Cu, Zn), ß-amylase (Cu), alcohol dehydrogenase (Zn), carbonic anhydrase (Cu, Zn) and other proteins. Homeostatic control, metabolism and detoxification of toxic elements (*e.g.*, Cd, Hg) by their interaction with metallothioneins (MTs) have been in the focus of ecotoxicology and clinical chemistry.[62–64] Detoxification mechanisms of plants exposed to heavy metals involve syntheses of small thiol peptides (phytochelatines) able to chelate heavy metals owing to the high cysteine content in the molecule.[65,66]

3.5 Complexes with other biomacromolecules (polysaccharides, glycoproteins)

Relatively little is known about the relevance of metal coordination to lipids and carbohydrates, although the potentially negatively charged oxygen functions can bind cations electrostatically and even undergo chelate coordination *via* polyhydroxy groups.[67] The complexation of divalent cations with the carboxylic acid groups of uronic acids from plant cell walls polysaccharides (pectins) is well established.[67]

3.6 Exogeneous species: metallodrugs

Platinum (cisplatin, carboplatin), Ru^{3+} [*fac*-$RuCl_3(NH_3)_3$] and gold (auranofin) compounds are well known in cancer therapy and some other gold compounds (aurithiomalate, aurothioglucose) are important antiarthritic drugs.[61] A wide range of Tc compounds (*e.g.*, Tc-labeled antibodies, Tc–mercaptoacetylglycine complex) are used for diagnostic imaging of renal, cardiac and cerebral functions and of various forms of cancer. Gadolinium(III) polyaminopolycarboxylic crown complexes are widely used as magnetic resonance imaging contrast reagents.[68]

The analytical challenges include both the identification of products of metallodrug metabolism and the understanding of the binding of metallodrugs to transport proteins and DNA fragments.

4 Hyphenated techniques for species-selective analysis

The various possibilities for the on-line coupling of a separation technique with an element (moiety, species)-specific detector for bioinorganic speciation analysis include different types of HPLC or electrophoresis for separation, and atomic spectrometry (or molecular MS) for detection. The hyphenated techniques available for species-selective analysis in biological materials are schematically shown in Fig. 1. The presence of a metal bound to a biomacromolecule in a sample is considered to be the prerequisite of using an element-specific detector. Nevertheless, some reports have indicated the possibility of employing a coupled technique to the analysis for a metal-free compound, provided that the latter is derivatized on-column or post-column by saturating the metal binding sites with a metal.[69,70]

The choice of the hyphenated technique depends primarily on the research objective. The separation component of the coupled system becomes of particular concern when the target species have close physico-chemical properties. It may even be necessary to combine two or more separation mechanisms in series to ensure that a unique species arrives at the detector at a given time. The choice of the detector component becomes crucial when the concentration of analyte species in the sample is very small and low limits of detection are required. An important problem is often the interface between chromatography and spectrometry as the separation conditions may be not compatible in terms of flow rate and the mobile phase composition with those required by the detector.

Usually, chromatography and spectrometry can be coupled on-line. However, when a polyacrylamide gel electrophoresis (PAGE) technique is used, the off-line detection of metal species carried out directly in the gel[71–75] or after extraction (blotting) of proteins from the gel[76] is necessary. Also, the preference for a highly sensitive discrete atomization technique such as ETAAS[17,77–86] or ETV-ICP-MS[78] may be the reason for the choice of the off-line method of coupling.

In contrast to the classical speciation analysis,[34] where standards for most of the anthropogenic pollutants are available, the majority of species of interest in bioinorganic trace analysis have not yet been isolated in sufficient purity to be used as retention or migration time standards. Therefore, it is becoming of paramount importance to employ in parallel a molecule (or moiety)-specific detector to establish the identity of the eluted species. Mass spectrometry in the fast atom bombardment (FABMS),[87–90] electrospray (ESMS)[91–93] or matrix assisted laser desorption ionization time-of-flight (MALDI-TOFMS)[94] modes have been the viable choices.

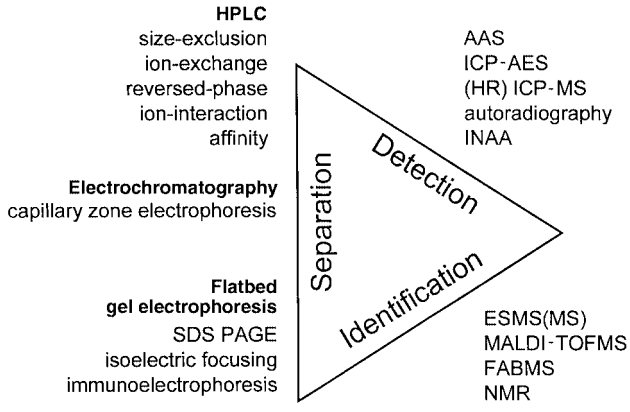

Fig. 1 Hyphenated techniques available for species-selective analysis of biological materials.

4.1 High-performance liquid chromatography (HPLC)

The principal HPLC separation mechanisms used in bioinorganic speciation analysis include size-exclusion, ion-exchange and reversed-phase chromatography. Because of the complex nature of metal–protein interactions, a combination of these separation mechanisms is often needed to identify the trace element species correctly. A number of bioanalytical techniques including ultracentrifugation, dialysis and ammonium sulfate precipitation should often precede the chromatographic separations of metal–protein complexes. Successive ultrafiltration through membranes with molecular weight cut-offs of 30 000, 5000 and 500 u, which was used to study the distribution of metal species as a function of molecular weight,[95–97] is recommended for the simplification of the matrix loaded on an HPLC column. The choice of the pH and ionic strength of the mobile phase are important for optimum separations. The requirements and limitations concerning the interface between HPLC and atomic spectrometry vary as a function of the separation mode and the detector used.

It should be noted that the co-elution of a metal with a particular bio-macromolecule is only an indication and not a proof that they belong together. The confirmation can be obtained by applying affinity chromatography for the particular protein. The presence of the metal attached to the protein bound to the antigen on the column confirms the existence of the metal–protein complex.[41]

The filtration of the cytosol using a 0.45 μm or, better, a 0.22 μm filter before injection on to the chromatographic column is mandatory. A guard column should be inserted to protect the analytical column particularly from the effects of lipids, that would otherwise degrade the separation.

4.1.1 Size-exclusion chromatography. Size-exclusion chromatography (SEC) is based on the molecular sieve effect and enables species to be separated according to their size and, to a lesser extent, shape. The average time a substance spends in the pores (determined by its size for a given shape) can usually be related directly to its molecular weight. This mechanism is usually valid for fairly large proteins and polysaccharides. For smaller species, especially ions with a high charge-to-mass ratio, secondary adsorption and ion-exchange effects affect the separations. These phenomena, initially considered as a nuisance in SE HPLC, are becoming more and more often employed for the separation of organoselenium[98,99] and organoarsenic compounds.[100–103]

SE HPLC has the advantage over other HPLC techniques of the high tolerance to biological matrices. The flow rates (0.7–1.0 ml min^{-1}) and compositions of the mobile phases used are readily tolerated by flame atomic absorption and ICP atomic emission and mass spectrometers. Another potential advantage of SE HPLC is the possibility of avoiding the buffer salts in the mobile phase,[98,104] and hence the possibility of simplifying the matrix in heartcut and lyophilized fractions. A limitation is the multifold dilution of the sample during chromatography, which is the higher the larger are the column dimensions.

Packing. Separation by SE HPLC should be independent of the analyte's charge but, in practice, the stationary phase surface displays charged properties so that a mixed-mode separation is observed. This makes the choice of packing critical. The two major categories of packing used have included silica and organic polymers. The former were reported to be responsible for metal losses when nanogram amounts of metal species were

separated using low ionic strength eluents.[105–108] Copolymeric styrene–divinylbenzene stationary phases allowed symmetrical peaks to be obtained and prevented losses of Cd during SE HPLC of Cd–metallothionein (MT) complexes.[106,107] The average pore size of packings used varies from 100 to 1000 Å. For a detailed speciation in complex samples such as some body fluids, columns with a separation range up to 250 kDa should be used.[109] The combination of two SEC columns with the different fractionation range may offer an attractive alternative.[109]

Mobile phase. The optimum eluent should ensure the minimum competition between buffer and cytosolic ligands, and between these ligands and the gel. Running chromatography with water as the mobile phase was reported to prevent structural changes, denaturation of proteins and destruction of protein–metal complexes.[110] On the other hand, even dilute buffers, in general, cause adsorption of low molecular weight proteins on the column packing. Therefore, various aqueous mobile phases of fairly high ionic strength have been used to avoid interactions with the packing material. When silica-based packings cannot be avoided, the addition of a non-complexing salt (*e.g.*, 0.1 M NaCl) to the mobile phase is necessary in order to suppress the residual silanol activity of the column packing.[108] Under such conditions no significant exchange of MT-bound Cd occurred but appreciable losses were observed occasionally for other metals (Hg and Zn).[108]

The addition of EDTA, proposed by some workers[111] to minimize metal ion–gel interactions in the chromatography of metal–protein complexes, was found unsuitable by others[112] because of the occasional presence of anomalous Cd peaks in subsequent runs. Polymeric supports suffer from the deposition of excess Cd^{2+} ions which interact with the analytes, often causing severe degradation of peak resolution.[107] Because of the weak complexing properties, TRIS is not sufficient to compete with the polymeric support for Cd^{2+}; the complexation of Cd^{2+} with β-mercaptoethanol is advised.[107]

The wide variety of buffers for SE HPLC reported in the literature makes it relatively easy to chose one compatible with the detection technique. Up to 30 mM TRIS–HCl was found to be well tolerated by ICP-MS whereas 20 mM formate or acetate buffer in 10% methanol is acceptable for ESMS.[48,113] The addition of 0.03% NaN_3 as a retardant of bacterial activity was advised to protect the column from damage resulting from the bacterial growth when real samples are analyzed.[108,114–117]

Analysis time. This is a function of the column size and the flow rate. Although columns up to 120 cm have been used, a standard 300 × 7.6 mm id column is usually a good choice. At a flow rate of 1 ml min^{-1}, the separation requires *ca.* 20 min to complete. The separation of MT-bound and unbound cadmium within 3 min was reported on a 40 mm SE chromatographic column.[118] The availability of small-bore columns with SE packings is still limited, but such columns permit the rapid characterization of various metal-containing molecular weight fractions in microsamples of unknown cytosols (micronebulization ICP-MS is used for detection).[119]

Separation efficiency. The number of theoretical plates in SE HPLC is small; this technique is not only insufficient for the discrimination of the small amino acid heterogeneities in metallopeptides,[113] but also lacks the resolution in frequently encountered problems, such as the separation of serum selenoproteins[120] or the separation of human albumin and transferrin.[121] Each fraction eluted from an SE column may still contain thousands of compounds. In most cases further signal characterization by orthogonal (complementary) chromatographic techniques is necessary.

4.1.2 Ion-exchange chromatography. Ion-exchange chromatography is based on the interactions of analyte cations in the mobile phase with the negatively charged functional groups of the stationary phase (cation-exchange) or of analyte anions with the positively charged functional groups (anion-exchange). In the high-performance version (sometimes called ion chromatography, IC) the polymer beads are coated with an ion exchanger. Both cation and anion exchangers are widely used for the separation of metal species, especially of organoarsenic and organoselenium compounds. In metalloprotein biochemistry the technique has been widely used for the fractionation of metallothionein[122,123] and serum proteins.[80,83,124] Separations on ion exchange resins require drastic elution conditions with acids, bases or high salt concentrations in order to elute multicharged anions and cations in a reasonable time.

A weak anion exchanger with diethylaminoethyl functional groups has been used in the majority of work concerning the separation of metal complexes with MTs. Strong quaternary ammonium anion exchanger was used to separate aluminium carrying serum proteins.[80] It allows the separation of albumin and transferrin leaving the metals attached to these proteins. Strong anion exchangers were also preferred in speciation analysis of arsenic and selenium.

Aqueous buffers with a linear concentration gradient are used as eluents. The concentration of buffers used in anion-exchange (AE) chromatography of proteins often exceeds 0.1 M. Their use may result in variations of the detector's sensitivity because of the clogging of the nebulizer and sampler and skimmer cones. For example, the high potential of separation of the MT-1 and MT-2 isoforms by AE HPLC is not fully exploited in the coupled systems because the common end-concentration of 0.25 M of buffer is not very well tolerated by ICP-MS. Much lower buffer concentrations (3–20 mM) are used in AE chromatography of organometalloid species.[84,125–131]

Cation-exchange HPLC has been less popular. However, since the separations can be carried out in acidic media using several millimolar pyridine–formate buffer[125,132] or simply a few millimolar HNO_3,[133,134] the technique turns out to be attractive prior to ESMS.[135,136]

4.1.3 Reversed-phase chromatography. Analytes in a polar mobile phase, such as water or water–methanol, are chromatographed using a relatively non-polar stationary phase, *i.e.*, silica gel containing chemically (covalently) bonded hydrocarbon (C_4–C_{18}). Polar, uncharged compounds with an M_r < 3000 u are typically analyzed. However, the number of applications for proteins is constantly increasing because of the excellent resolution able to differentiate proteins, such as metallothioneins varying by one amino acid only.[113] The primary applications areas of RP-HPLC in bioinorganic analytical chemistry include the separation of species in 10 kDa ultrafiltrates of samples or sample extracts and the fine characterization of purity of metallothionein fractions isolated by size-exclusion and anion-exchange chromatography. The use of ion-pairing reagents in the mobile phase (ion-interaction chromatography) allows the extension of applications of RP-HPLC to ionic analytes. In particular this mode is employed for speciation of organoarsenic and organoselenium compounds (*cf.*, Tables 1 and 2).

RP-HPLC seems to be superior to SE HPLC and IE HPLC for the separation of metal–biomolecule complexes because the packing material for RPC is principally free of ligands for metals.[137] Since hydrophobicity of a biomolecule primarily dictates its retention in RP-HPLC, the gradual elution of individual species of a mixture is achieved by decreasing the polarity of the mobile phase by the addition of methanol or acetonitrile. The separation of metal complexes with MTs by RP-HPLC has been reviewed.[113,138]

The availability of RP columns with different dimensions makes it possible to adjust the sample introduction flow rate to

that required by the detection system. The flow rate depends strongly on the column geometry. Varying the column inner diameter from 8 to 0.18 mm allows the flow rate to be changed from 10 ml min^{-1} to 2 μl min^{-1} depending on what is necessary. Wide-bore (4–5 mm) 15–25 cm long columns are the most frequently used. The narrow-bore and microbore columns are expected to increase in significance soon because of their higher sensitivity and resolution,[59,139,140] but a microconcentric or direct injection nebulizer (DIN) is required. Microbore RP-HPLC-ESMS has widely been used for the characterization of metallothioneins.[141–146] The presence of 10–50 mM buffer (usually TRIS–HCl) is necessary.

The high concentration of organic modifier makes RP-HPLC poorly compatible with ICP-MS. The use of Pt cones and a desolvation unit and the addition of oxygen to the nebulizer gas are recommended.[147] Decreases in sensitivity cannot apparently be avoided. On the other hand, the RP separation conditions are close to ideal for ESMS detection, which is expected to increase in popularity in the near future.[145] The use of ion-pairing reagents in the mobile phase dramatically reduces the ESMS detection sensitivity.[135,136]

4.2 Element-specific detection in HPLC

The three major atomic spectrometric techniques, flame AAS, ICP-AES and ICP-MS, can be readily used as chromatographic detectors. The basic interface is straighforward: a piece of narrow-bore tubing that connects the outlet of the LC column with the liquid flow inlet of the nebulizer. Typical LC flow rates of 0.5–2 ml min^{-1} are within the range usually required for pneumatic nebulization. Aqueous eluents with a buffer concentration of up to 50 mM are tolerated. The sensitivity is negatively affected by the low (1–5%) transfer efficiency and the losses in the spray chamber. It can be increased by using a hydraulic high-pressure nebulizer (HHPN)[125,148,149] or an ultrasonic nebulizer.[68,150,151]

Organic solvents used in RP-HPLC are fairly well tolerated in flame AAS. In ICP techniques, however, they influence negatively the stability of the plasma (until extinction in extreme cases). Also, they are responsible for the deposition of carbon on the sampler cone and torch. A higher rf power helps to reduce the effect of the solvent load on the plasma whereas the use of a cooled spray chamber limits the amount of solvent vapor arriving at the plasma.[147] A dedicated desolvation unit can cope with the methanol concentrations usually used in reversed-phase chromatography.[152] The addition of oxygen (1–3%) to the nebulizer gas flow can help to minimize carbon deposition and clogging of the sample cone at the expense of the cone's lifetime.[59,147,153–155] Salts can cause short-term signal depression or enhancement and cause blockage of the nebulizer and the sampling cone. Concentrations of 50 mM phosphate buffers caused the rapid erosion and clogging of the nickel sampling cone.[156] Post-column split and makeup with water to lower the concentration of the organic modifier are a possible remedy, but at the expense of the detection power.[157]

In the case of microbore chromatography, the use of a direct injection nebulizer (DIN) interface looks particularly attractive. The DIN is a microconcentric pneumatic nebulizer with no spray chamber; it nebulizes the liquid sample directly into the central channel of the ICP torch. The low dead volume (< 2 μl) and the absence of a spray chamber of the DIN minimize post-column peak broadening and facilitate the use of low flow rates (30–100 μL min^{-1}).[59,119,155,158] Another advantage is the fast sample washout with minimal memory effects.

The need for a nebulizer between an HPLC column and an atomic spectrometer can be eliminated by the post-column conversion of the eluting metal or metalloid into a volatile species (usually hydride) that can be swept into the quartz furnace or plasma. In comparison with pneumatic nebulization, the on-line microwave assisted digestion–hydride generation interface offers a 20–100-fold increase in sensitivity and elimination of interferences from the sample matrix or mobile phase components.[159] This is offset by a higher background noise, reducing the gain in detection limit to a factor of 2–10.[159]

The use of techniques other than AAS, AES or MS for the detection of elemental species in chromatography has been rare. Post-column spectrophotometric reaction with Pyrocatechol Violet was used for the detection of Al in SE HPLC of an extract of a tea sample.[160] Eluates from chromatographic columns have also been analyzed off-line by instrumental neutron activation analysis (INAA)[161,162] or total-reflection X-ray fluorescence (TXRF).[171,172]

4.2.1 Atomic absorption spectrometry (AAS). The primary interest in HPLC-AAS lies in its simplicity and wide availability. AAS is not a truly multi-element technique. Nevertheless, with some instruments, such as the Perkin-Elmer SIMAA 600, up to four elements can be measured simultaneously, which is sufficient for a number of practical applications.

Flame AAS can be coupled with HPLC directly. This technique is compatible both with the flow rates and with the mobile phase composition (including organic solvent) commonly used in HPLC. Taking into account the widespread availability of this technique, it is no wonder that HPLC-AAS was the first hyphenated technique to be used for the determination of metal–protein complexes.[7] The major fields of applications include the detection of complexes with metals that give the most intense response in AAS (Cd, Zn, Cu) or of species that can be converted on-line into volatile hydrides (As, Se, Cd[163]). Calibration graphs in RP-HPLC-flame AAS were reported to be rectilinear up to 20 μg ml^{-1} of Cd and Cu.[137,164] The detection limits for As and Se after post-column hydride generation match those achieved by ICP-MS with direct pneumatic nebulization.

ETAAS is more sensitive than flame AAS but the coupling is not so straightforward. Off-line analysis of MT-bound metals after HPLC by fraction collection and ETAAS analysis has been a common approach.[79,80,82,83,85,86,158,165–167] With an autosampler and flow-through cells a high degree of automation can be achieved, leading to a quasi-on-line coupling.[167] The coupling of microbore HPLC with ETAAS is especially promising.[85,158] The use of ETAAS for metal speciation has been reviewed,[17] with particular attention to protein binding and trace element determination in biological materials.[168]

4.2.2 Inductively coupled plasma atomic emission spectrometry (ICP-AES). Some earlier work used direct-currect plasma AES for the detection of seral proteins in the SEC column effluent[169] but the position of an ICP is well established. HPLC-ICP-AES coupling was the first hyphenated technique used for speciation of arsenic in biomaterials,[170] but the position of ICP-AES has been considerably eroded in favor of ICP-MS.

ICP-AES offers detection limits at the 1 ng ml^{-1} level (continuous infusion), which translates into 10–100 ng ml^{-1} for a transient signal of an analyte eluted from the column.[68,158,162,171–180] They can be decreased by the use of an ultrasonic nebulizer[68] or a direct injection nebulizer (in absolute terms).[158] Because of the absence of cones, ICP-AES offers a higher tolerance to the matrix in terms of salt concentration and, because of a higher rf power, to organic solvents. Methanol or acetonitrile ($> 10\%$) contents may be readily tolerated. The unmatched advantage of ICP-AES is the possibility of the monitoring of sulfur or phosphorus together with metals. A detection limit for S below 10 ng ml^{-1} is common. ICP-AES instruments with axial plasmas, recently commercialized, seem to offer lower detection limits for transition metals.[181] Multi-

element capability is an advantage of instruments equipped with a polychromator. The sensitivity of ICP-AES is generally inadequate to cope with the levels of most elemental species in non-induced samples.

4.2.3 Inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS has been enjoying the largest popularity because of its multi-element capability, low detection limits and the possibility of on-line isotope dilution. Indeed, with its sub-ng l^{-1} detection limits offered by the (most popular) quadrupole analysers, ICP-MS allows the detection of HPLC signals from as little as 0.1 ng ml^{-1} of an element in the injected solution. HPLC-ICP-MS with enriched stable isotopes is a unique analytical method by which speciation of both endogenous elements and external tracers can be achieved in a single experiment.[182] The potential of LC-ICP-MS for trace metal speciation has been widely discussed.[8,29–31]

The sensitivity of ICP-MS can be increased by the use of sector-field analyzers. In the low resolution mode the sensitivity is about two orders of magnitude higher than that of a quadrupole spectrometer; a major problem being keeping blank levels low enough to exploit the extreme sensitivity obtainable in such a low resolution mode.[159] The increase in resolution brings a decrease in sensitivity but allows the easy determination of elements plagued by polyatomic interferences. A wider expansion of high resolution ICP mass spectrometers (with potentially lower detection limits and greater freedom from interferences) is hampered by the prohibitive cost of the instrumentation and the high maintenance costs. An emerging alternative to the determination of elements plagued by polyatomic interferences (Fe, V, Cr, As, Se) is the use of a hexapole collision or a multipole dynamic reaction cells between the plasma and the quadrupole analyser.

For multi-element HPLC-ICP-MS, the elements of interest were divided into groups for the sake of optimal sensitivity. Within each group the replicate time, dwell time and number of sweeps per reading were optimized. A separate injection was made for each group.[154] Eluents should not contain elements that give polyatomic interfering ions in an ICP. Most eluents caused spectral interferences for a number of elements (C, Ca, S), but also non-spectral interferences which result in a decrease in sensitivity or in drift effects.[105,148] In general, ICP-MS requires more dilute buffers to be used and tolerates lower concentrations of organic solvents than ICP-AES. However, even for the detection of iron species, the values were an order of magnitude lower than those obtained by using ICP-AES for detection.[170]

4.3 Electrophoresis

4.3.1 Flatbed gel electrophoresis. Flatbed electrophoresis with its various formats, such as polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF) and immunoelectrophoresis, offers a number of attractive features for the characterization of metallobiomolecules owing to its micropreparative loading and high resolution. Thousands of proteins can be separated in a single run, in two-dimensional systems. Whereas a considerable number of reports on the separation of denatured proteins exist, studies in which the metal–protein binding would have remained intact are scarce.[71–76,83,183–186]

PAGE consists of converting all proteins into similar structures that differ only in molecular weight. It is achieved by attaching a number of sodium dodecyl sulfate (SDS) molecules to a protein which results in its denaturation (breaking the aggregates). As a result, all proteins, regardless of their identity, are imparted the same free-solution mobility so their separation is controlled by molecular weight. Proteins with small mass-to-charge ratio move fastest and furthest, proteins with large mass-to-charge ratio move slowest. The standard SDS-PAGE protocols lead to high resolution and reproducibility but include chemicals such as SDS and dithiothreitol (DDT), which denature the protein structure and cleave protein–metal bonds. Native techniques keep the proteins in their original conformation, but result in poorer resolution. SDS concentrations as low as 0.1% are applied to the purification of proteins under non-denaturating conditions.[184,185]

SDS-PAGE may not ensure the complete dissociation of multimeric proteins into their subunits and thereby may give rise to several labeled bands originating from the same compound. Tissues may contain proteins with similar migration velocities and an orthogonal separation mechanism is necessary. This is achieved by two-dimensional (2D) electrophoresis.

Proteins are separated in the gradient gel according to the mass-to-charge ratio in the first dimension. A second dimension can be added by isoelectric focusing using immobilized pH gradients. Proteins are separated by their isoelectric points in the immobilized pH gradient of the IEF strip gel. The charged proteins migrate within the pH gradient until they reach the pH in the gradient equal to their pI values. At that point their charge is zero and they focus at distinct zones. Two-dimensional electrophoresis is widely used to the characterization of proteins and biomolecules but its application to metal speciation is rare.[184,185]

The second dimension can also be achieved by immunoelectrophoresis in which the second-dimensional gel contains antibodies against the sample (human serum) proteins.[71,187]

4.3.2 Detection of trace elements in gel electrophoresis. The amount of proteins concentrated in the tiny gel volumes is very small and hardly accessible to standard analytical chemical methods. Metal detection by autoradiography is the most elegant approach in the experimental stage but requires a radioactive isotope. The alternatives include analysis of the cut gel by INAA,[72,73] PIXE,[74] HR-ICP-MS[186] or laser ablation ICP-MS,[71] the last method offering quasi-on-line resolution.

Autoradiography requires that the species to be detected contains a radioactive isotope. In bioinorganic analytical chemistry, this technique has been widely applied to selenoproteins.[188–193] The ^{75}Se nuclide ($t_{1/2}$ = 120.4 d and emits γ-rays in the range 10 and 280 keV) is well suited for such experiments. The technique requires labeling an organism *in vivo* by administering ^{75}Se. The tissue homogenate of the labeled animals is separated by SDS-PAGE and the distribution of the tracer on the gel is determined by autoradiography.

The advantage is the very low limit of detection (in the sub-picogram or even femtogram range) that may be controlled by the uptake and retention of the labeled selenium.[188–193] A concern is whether the tracer activity reflects the distribution of the native selenium. The specific incorporation of the labeled element into proteins was found to be the more effective the lower the Se status of the animal and the smaller the amount of tracer administered. In ecotoxicology, reliance on radiotracers is not compatible with the analysis of field-collected samples, which is critical to the understanding of Se biogeochemistry and ecotoxicology.

A number of direct sensitive techniques have been proposed to determine the trace elements in the gel strips obtained after electrophoresis. Particular attention was drawn by INAA because of its high sensitivity (tens of picograms range).[72,161,193] Microbeam X-ray spectrometry energy-dispersive X-ray fluorescence spectrometry and INAA were evaluated for the detection of Se in the glutathione peroxidase previously separated by PAGE.[194] The possible role of ion-beam spectrometric analysis in protein analysis by PAGE or TLC was discussed with emphasis on PIXE.[195,196] Another alternative is sampling the PAGE plate by laser ablation, which offers a rapid high (spatial) resolution.[71] Comparison of the Co

distribution maps thereby obtained with protein distribution maps obtained by staining with Coomassie Brilliant Blue allowed the identification of the main Co-binding proteins in serum.[71]

Protein fractions arising from SDS-PAGE were blotted on to nitrocellulose membranes and analysed for Se contents after digestion of membranes with HNO_3–$HClO_4$.[197] ICP-MS was also applied to detection of trace elements in the gel following the cutting and digesting the gel. The detection limits of the technique are insufficient even when a sector field instrument is used. The trace element concentration should be above 10 ng ml^{-1}, which is seldom the case.[186]

4.3.3 Capillary zone electrophoresis (CZE). CZE offers highly efficient separation, rapid analysis and minute sample size requirements. These features have resulted in a number of applications of this technique to the separation of metallo-biomolecules over a long period, which was discussed in an excellent review.[198] All this work was realized with UV detection, which does not offer elemental specificity.

Attempts to use element-selective detection for CZE turned out to be successful with the description of a CZE-ICP-MS interface by Olesik *et al.*[199] Since then a number of interface designs have been reported. They were based on a (micro) concentric,[200–206] ultrasonic,[207] direct injection nebulizer,[208,209] or a high-efficiency nebulizer,[210] and used a coaxial electrolyte sheath flow (make-up) to establish the electrical connection to the analyte. They have been reviewed in two excellent status reports[32,211,212] that also discussed the potential of a CZE-ESMS coupling. Quadrupole ICP-MS was used in all these studies with the exception of one recent work in which a sector field instrument was applied.[213] Sources of error in CZE-ICP-MS coupling were discussed.[214] Except the work of Michalke and Schramel[215] whose coupling was based on the collection of UV-monitored fractions eluting fom the capillary for ETV-ICP-MS, the CZE-ICP-MS interfaces allowed the continuous detection of analytes.

Applications of CZE-ICP-MS to the analysis of biomolecules have been extremely scarce. Only in two studies was this coupling capable of producing valid information for speciation of an element in a real sample. They concerned the speciation of Se in human milk and blood serum[216] and that of iodine in milk.[217] Some authors used metallothionein standards[200,202,206,207,214] but both the number of these studies and the quality of the electrophoregrams reported are very low in comparison with the data available in the literature for CZE of metallothionein.[113]

The most frequently evoked challenge that has also been blamed for the scarcity of applications is the apparently insufficient concentration detection limits to match the naturally occurring metallocompounds in biological samples. Indeed, the minute amount of sample injected, low transport efficiency and the sheath-flow dilution negatively affect the sensitivity. For example, an absolute detection limit of *ca.* 50 fg for an injection of 50 nl corresponds to a detection limit (DL) of 1 μg l^{-1}, which is the value usually cited for successful CZE-ICP-MS coupling. Paradoxically, this is also the DL of HPLC-ICP-MS, for which a large number of applications have been reported. Note that the peak width of a CZE signal is 10 times smaller than that of an SE HPLC signal, which partly compensates the loss due to the small injection volume. The only work on CZE-HR-ICP-MS coupling does not offer significantly better detection limits for the moment but the potential for improvement has not yet been exhausted.[213]

There is a continuous interest in CZE-ESMS/MS coupling for the structural characterization of organic molecules but for the moment the poor detection limits (also due to highly conductive mobile phases) have virtually prohibited its applications in bioinorganic trace analysis. Nevertheless, a number of reports related to the analysis of metallothionein–metal complexes[145,218,219] and for speciation analysis in standard solutions do exist.[32] Recently, CZE-ESMS was used to identify arsenocholine in urine but the identification (by spiking and migration time matching) does not seem to be convincing in view of the ease of formation of artefacts in the protonated molecule ion mode (no CID mass spectra were acquired).[220]

4.4 Identification of metallocompounds by soft-ionization mass spectrometric techniques

In atomic spectrometry, the signal produced by an atom or by an elemental ion is measured; the species selectivity is a function of the arrival time of the analyte molecule at the atomization/ionization source. Consequently, the identification requires a prior knowledge of the possible species present and the availability of calibration standards (retention or migration time markers). Since these conditions are usually not fulfilled in bioinorganic speciation analysis, a need arises for a technique able to generate a signal that would be inherently specific not to an element but to a species (or its fragment).

Molecular ion MS is an attractive and convenient tool to fulfil the above requirements. Particular attention in the field of speciation analysis has been paid to electrospray (ES) ionization, and its pneumatically assisted modification referred to as Ionspray®. The technique was shown to be capable of producing gas-phase ions of highly labile and non-volatile compounds; in particular, metal complexes of Cd, Zn and Ag were shown to be transferred into the gaseous phase and detected by the mass spectrometer.[141,146,219,221] A fairly strong position, but restricted to organoarsenic species and in the off-line mode, is held by FABMS.[87–90,222] MALDI-TOFMS was investigated for the speciation of As in environmental materials[94] and the identification of selenoproteins from rat muscle.[223] A multi-step purification scheme based on $(NH_4)_2SO_4$ fractionation and subsequent SEC, CE and RP chromatography was proposed to separate four forms of a selenoprotein.[212]

The major advantage of ESMS over the other soft MS techniques is the possibility of its application as the chromatographic detector. The prerequisite is to match the optimum ionization conditions with the optimum separation conditions in terms of the chemical composition and flow rate of the mobile phase. Although different developments with regard to the source (nanospray, micro ionspray or turbo ionspray) allowed different flow rates (from nl min^{-1} to ml min^{-1}) to be handled, the performance of ESMS remains affected negatively by salt concentrations exceeding 10 mM and by the presence of some compounds, such as ion pairing reagents, even if present at the few millimolar level.

4.4.1 Identification of organometallic (metalloid) species by ESMS. Small molecules containing a carbon–metal (metalloid) bond usually readily produce singly protonated ions in the electrospray source that theoretically should allow the identification of the metallocompound on the basis of the molecular mass. However, in the direct infusion mode, the attribution of a signal at a given *m/z* ratio to an elemental species is a daunting and practically impossible task for monoisotopic elements such as arsenic. Sharp *et al.*[224] stated that ionspray MS is sensitive to virtually all the species in a sample, the resulting spectra for real samples are too complex, too likely to contain multiply charged species and too prone to artefact formation in the gas phase to be suitable for speciation without prior chromatography or electrophoresis. However, if an element presents a characteristic isotopic pattern such as Cd,[107] Se[99,130,225–227] or Sn,[228,229] its recognition in the mass spectrum of a sample solution is easier provided that the signal is not suppressed by the matrix.

A deeper insight into the species identity can be gained by the fragmentation of the protonated molecule ion (isolated at the

level of the first mass filter) by collision induced dissociation (CID) followed by MS of the product ions. The MS/MS mode allowed the identification of organoarsenic compounds in algal extracts purified by SE[230] or of the Al–citrate complex in human serum purified by AE HPLC.[231] For species containing an element having more than one stable isotope, such as Se, valuable information can be obtained by fragmenting the two protonated molecule ions containing the adjacent most abundant isotopes (^{78}Se and ^{80}Se). Fragments that contain selenium will still be separated by the distance of two units whereas fragments that do not will remain at the same *m/z* value, thus facilitating the interpretation of the mass spectra.[99]

Interpretation of mass spectra becomes easier when ESMS is used as a chromatographic[135,225,226] or electrophoretic[220] detector. For monoisotopic elements such as As, the use of the MS/MS mode is recommended for the positive identification of signals in HPLC effluents.[135,232] Recently, the use of a source CID process was shown to allow the production of elemental ions from some species in the ES ionization source, and hence the use of ESMS as an element selective detector in HPLC.[136] The elemental ESMS mode is free from many polyatomic interferences present in ICP-MS. The concentration detection limits are, however, 2–3 orders of magnitude higher than in the case of ICP-MS.

4.4.2 Characterization of metal complexes with peptides and proteins. An attractive feature of ESMS is the production of multiply protonated molecular ions from polypeptide molecules and their complexes with metals. This allows a very precise (0.1%) determination of the molecular mass of the species of interest. The formation of ions stable in the gas phase even by the metallated metallothioneins allows one to determine the number and identity of metal atoms bound to the protein by comparison of the spectra for the apoprotein and the metal-saturated protein at acidic and neutral pH, respectively.[107,141,144,221]

Several studies have concerned the characterization of metallothionein complexes with Cd and Zn by microbore HPLC[141–144,146] and CZE[218,219] with ESMS detection. RP-HPLC of metallated isoforms is faster but prone to artefacts because of the number of mixed metal complexes present.[144] A remedy for this is post-column acidification that cleaves the metals bound to MT and allows both the information on the identity of the metal bound[146] and on that of the MT-ligand present to be obtained. The resolution of CZE allows the separation of the metal-free MTs.[218,219]

Peptides of limited molecular weight (up to 2.5 kDa) can be subjected to on-line sequence analysis by MS/MS using CID.[233] Peptides fragment primarily at the amine bonds to produce a ladder of sequence ions. The charge can be retained on the amino terminus (type b ion) or on the carboxy terminus (type y ion). Thus a complete series made of ions of both types allows the determination of the amino acid sequence by subtraction of the masses of adjacent sequence ions. The use of this technique for the identification of phytochelatin ligands in PC–metal complexes detected by HPLC-ICP-MS was demonstrated.[234,235]

5 Analysis for organoarsenic species in biological systems

The metabolism of inorganic arsenic by marine and terrestrial plants and animals gives rise to a range of organic arsenic species that may be considered as naturally occurring compounds.[58] The most widely referred to of this group are quaternary arseno compounds: arsenobetaine and arsenocholine, which is the major organoarsenic compounds in animals, and arsinoyl ribosides (arseno sugars), which are products of the As metabolism in marine plants and some bivalves. Another field of interest is studies of the metabolism of arsenic following its administration to humans and experimental animals that involves a number of simple methylated compounds (methylarsonic acids and tetramethylarsonium salts).[133,134,156,232,236–239]

The literature on the speciation of arsenic by hyphenated techniques is extremely rich, with more than 1000 papers available. In most of these studies, however, commercially available standards, As(III), As(V), mono- and dimethylarsonic acids and later arsenobetaine and arsenocholine, were used with the purpose of optimizing a method, often without the objective of applying it to a biological sample. An overview of these techniques can be found in two recent extensive reviews.[240,241] On the other hand, a number of excellent studies on the speciation of arsenic in marine life are available which are based on multi-step chromatographic purification prior to NMR identification, reviewed by Francesconi and Edmonds.[242] Downscaling and refining of these methods gave rise to many analytical procedures employing the coupling of HPLC with, usually, ICP-MS. These procedures, among others, are summarized in Table 1 and are discussed in brief below.

5.1 Sample preparation

Urine samples were collected in polycarbonate bottles and filtered through a 0.45 μm filter.[237] The storage procedure implies the use of acid-washed PE bottles at −10 °C[156] but storage at 20 °C has also been reported.[237] Urine samples (filtered) can be injected on to a chromatographic column directly or after dilution with diluted acid.[254,257,260,261] A freezing procedure has been commonly used to preserve biosamples; arsenic speciation in fresh and defrosted samples was compared.[243] Arsenobetaine in sample extracts that were stored at 4 °C for 9 months was reported to decompose to trimethylarsine oxide and two other unidentified arsenic species.[243,262]

Defatting (removal of lipids), *e.g.*, by leaching with acetone,[128] diethyl ether or light petroleum[249] is usually the first analytical step in speciation of arsenic in animal tissues and marine foodstuffs. It is necessary to avoid generating an emulsion with the fat, which would make the subsequent clean-up more difficult.[263] Since some samples of seafood products are prepared in oil and generally tend to have a high salt content an additional clean-up step, *e.g.*, on a strong cation exchanger,[128] is required to eliminate the remains of liposoluble compounds not removed during the defatting step. The clean-up also avoids the need for periodically reversing and flushing the chromatographic column in order to overcome pressure build-up due to the accumulation of material on the column. In addition, the efficiency of the subsequent methanol extraction step is apparently higher for defatted than for non-defatted samples.[128] The uncleaned samples generate problems on the levels of ICP-MS cones and ES ion source.

The quantitative separation of organoarsenic from the matrix is desirable for a chromatographic analysis. Extraction of arsenobetaine, arsenocholine and arsenoribosides has usually been performed using methanol,[128] methanol–chloroform–water[264,265] or methanol–water.[245,246] A comparative study of these methods is available.[266] A methanol–water mixture is recommended for the dry tissues whereas fresh samples could be efficiently leached with pure methanol. With CRM 422 a precipitate of fatty aspect during CH_3OH–H_2O extraction was observed.[251] Recoveries attain 90% for fish and 80% for mussels. No degradation of arsenobetaine to other species was observed when an enzymic (trypsin) digestion procedure was applied to the fish.[248,267] The methanolic extraction is typically repeated 2–3 times followed by preconcentration of the extract by evaporation of methanol using a rotavaporator.

Table 1 Analytical techniques for speciation of organoarsenic compounds

Sample	Species[a]	Column	Mobile phase	Detection (interface)	Ref.
Algae, bivalves, crustaceans	5, 8	RP: Bondclone C_{18} (300 × 3.9 mm × 10 μm)	10 mM sodium heptanesulfonate–4 mM tetramethylammonium hydroxide (pH 3.5, 1 ml min^{-1})	ICP-MS	243
Algae, bivalves, crustaceans, mussel	7	RP: Inertsil ODS-2 (250 × 4.6 mm)	10 mM tetraethylammonium hydroxide–4.5 mM malonic acid (pH 6.8, 0.8 ml min^{-1})	ICP-MS	243, 244
Algae, bivalves, crustaceans, fish	1–8	SE: Asahipak GS-220 (500 × 7.6 mm)	50 mM sodium phosphate buffer (pH 6.8, 1 ml min^{-1})	ICP-AES	100
Algae, gastropods	1–8	AE: Supelcosil SAX1 (250 × 4.6 mm × 5 μm) CE: Supelcosil SCX (250 × 4.6 mm × 5 μm)	AE: 15 mM $NH_4H_2PO_4$ (pH 5.1, 1.5 ml min^{-1}) CE: 20 mM pyridine (pH 2.9)	ICP-MS (HHPN)	125
Algae	4, 5, 7	RP: Inertsil ODS-2 (250 × 4.6 mm)	10 mM tetraethylammonium hydroxide–4.5 mM malonic acid (pH 6.8) in 0.1% MeOH (0.8 ml min^{-1}); 10 mM tetrabutylammonium hydroxide–4.5 mM malonic acid (pH 6.8) in 0.1% MeOH (1 ml min^{-1})	ICP-MS	245
Bivalves, marine CRMs	1–8	SE: Asahipak GS-220 (500 × 7.6 mm) RP: Inertsil ODS-2 (250 × 4.6 mm)	SE: 25 mM malonic acid–25 mM tetramethylammonium hydroxide (pH 6.8, 1 ml min^{-1}) RP: 10 mM tetraethylammonium hydroxide–malonic acid (pH 6.8) in 0.05% MeOH (0.75 ml min^{-1}) RP: 10 mM sodium butanesulfonate–4 mM tetramethylammonium hydroxide–4 mM malonic acid in 0.05% MeOH (pH 3.0, 0.75 ml min^{-1})	ICP-MS	246
Mussel, urine	1–7	RP: Phenomenex C_{18} (250 × 4.6 mm)	10 mM sodium hexanesulfonate in 0.1% MeOH (pH 3.5, 1 ml min^{-1})	ICP-MS	244
Crab	5	RP: Hamilton PRP-1 (250 × 4 mm × 10 μm)	0.05 M sodium heptane-1-sulfonate in 2.5% acetic acid (2 ml min^{-1})	ICP-AES	247
Fish	4, 5	AE: SAX (125 × 4 mm × 10 μm)	50 mM K_2SO_4 (pH 10.5, 1.5 ml min^{-1})	ICP-MS	248
Fish	1, 8	CE: Chemcosorb 7SCX (250 × 4.6 mm)	50 mM pyridine–formate (pH 3.1, 1 ml min^{-1})	ICP-AES	249
Marine mammals	1–6, 8	AE: RPR X-100 (250 × 4.1 mm × 10 μm) CE: Supelcosil SCX-1 (250 × 4.6 mm × 5 μm)	AE: 20 mM $NH_4H_2PO_4$ (pH 6.0, 1 ml min^{-1}) CE: 20 mM pyridine–citric acid (pH 2.65, 1 ml min^{-1})	ICP-MS (HHPN)	126
Seafood	1–6	RP: Hamilton PRP-1 (250 × 4.6 mm × 10 μm)	Tetrabutylammonium dihydrogenphosphate (pH 9.5), gradient elution	ICP-AES QFAAS (HG)	250
Seafood	5	AE: Hamilton PRP X-100 (250 × 4.1 mm × 10 μm)	3 mM phosphate buffer (pH 5.0, 1 ml min^{-1})	QFAAS (MW-HG)	127
Seafood	5	AE: Supelcosil SAC (250 × 4.6 mm × 10 μm)	5 mM phosphate buffer (pH 3.75, 1 ml min^{-1})	ICP-AES	128
Marine CRMs	1–6	AE: PRP X-100 (250 × 4.1 mm × 10 μm)	NaH_2PO_4–Na_2HPO_4 gradient elution (5 to 35 mM) (1 ml min^{-1})	ICP-AES (UVA-HG)	251
Marine CRMs	1–8	AE: PRP X-100 CE: Adsorbosphere SCX 5U (250 × 4.6 mm)	AE: 15 mM KH_2PO_4 (pH 6.1, 1 ml min^{-1}) CE: 2.5 mM pyridine–citric acid (pH 2.65, 1 ml min^{-1})	AFS (UVA-HG)	129
Marine CRMs	1–6	CE: Supelcosil SCX-1 (250 × 4.6 mm × 5 μm)	20 mM pyridine (pH 3.0, 1.5 ml min^{-1})	ICP-MS (HHPN)	132
Earthworm	1–6	AE: PRPX-100 (250 × 4.1 mm × 10 μm)	15 mM tartaric acid (pH 2.91, 1–1.5 ml min^{-1})	ICP-MS	149
Carrots	1, 2	AE: ION 120 anion-exchange column	45 mM $(NH_4)_2CO_3$ in 3% MeOH (pH 10.3, 1 ml min^{-1})	ICP-MS	252
Mushrooms	1–5	AE: Supelcosil SAX (250 × 4.6 mm × 5 μm) CE: Dowex 50W-X8 (240 × 6 mm)	AE: 30 mM NaH_2PO_4–H_3PO_4 (pH 3.75, 1.5 ml min^{-1}) CE: successive elution with different eluents	ICP-MS INAA	253
Urine	1–8	RP: Phenomenex C_{18} (250 × 4.6 mm) or Inertsil ODS-2 (250 × 4.6 mm)	Not given	ICP-MS	238
Urine	1–6, 8	AE: Supelcosil SAX-1 (250 × 4.6 mm × 5 μm)	20 mM phosphate buffer (pH 6.85 or 4.64, 1 ml min^{-1})	QFAAS (UVA-HG)	254
Urine	1–6, 8	AE: PRP X-100 (250 × 4 mm × 10 μm)	Phosphate buffer (pH 6.22, 1 ml min^{-1}) (gradient elution: 12 mM for 0–6 min, then 24 mM for 6–13 min)	UVA-HG)	
Urine	1–5	AE: PolySphere SAW (120 × 4.6 mm)	50 mM phosphate or carbonate buffer (pH 10.3, 1 ml min^{-1})	QFAAS (MW-HG) ICP-MS	255
Urine	1–5	RP: Phenomenex C_{18} (300 × 3.9 mm)	10 mM heptanesulfonate in 0.1% MeOH (pH 3.5, 1 ml min^{-1})	QFAAS (MW-HG) ICP-MS	255
Urine	1–8	SE: Asahipak GS-220 (500 × 7.6 mm) RP: Inertsil ODS-2 (250 × 4.6 mm)	SE: 25 mM malonic acid–25 mM tetramethylammonium hydroxide (pH 6.8, 1 ml min^{-1}) RP: 10 mM tetraethylammonium hydroxide–malonic acid (pH 6.8) in 0.05% MeOH (0.75 ml min^{-1}) RP: 10 mM sodium 1-butanesulfonate–4 mM tetramethylammonium hydroxide–4 mM malonic acid in 0.05% MeOH (pH 3.0, 0.75 ml min^{-1})	ICP-MS	101
Rat urine	1–6, 8	CE: Excelpak ICS-45 (150 × 4.6 mm)	8 mM HNO_3 (1 ml min^{-1})	ICP-MS	133
Rat urine	1–6, 8	CE: Shodex Rspak NN-414 (150 × 4.6 mm) AE: Gelpak GL-IC-A15S (175 × 3 mm)	CE: 8 mM HNO_3–5 mM NH_4NO_3 (0.4 ml min^{-1}) AE: 6 mM $HCOONH_4$ (pH 5.5, 0.4 ml min^{-1})	ICP-MS ESMS	134
Serum	5	AE: Benson BA-X10, 2 × (150 × 4.6 mm)	Ammoniacal potassium sulfate (pH 10, 1.2 ml min^{-1}) (gradient elution: 0.1–100 mM)	ICP-MS	256
Serum	1–4	Dionex Ionpac CS 10 (250 × 4 mm)	50 mM NaH_2PO_4 containing 100 mM HCl (1 ml min^{-1})	QFAAS UVA-HG	258
Serum	4	AE: Supelcosil LC-SAX (250 × 4.6 mm × 5 μm)	30 mM NaH_2PO_4 (pH 4.5, 1 ml min^{-1})	HGAAS	259

[a] Compounds: 1, As(III); 2, As(V); 3, methylarsonic acid; 4, methylarsinic acid; 5, arsenobetaine; 6, arsenocholine; 7, arseno sugars; 8, trimethylarsine oxide (TMAO)

There has been a surge of interest recently in the use of microwave-assisted procedures for the recovery of organoarsenic compounds from biological tissues.[252,268,269] The use of an automated solvent accelerated extractor for the same purpose was found to be efficient.[270] The major advantage of these procedures is that the increased speed of leaching reduces the time of the sample preparation procedure to a few minutes.

5.2 Separation of organoarsenic compounds

The HPLC separation mechanisms have employed the advantage of the fact that organoarsenic compounds are readily and differently ionized at different pH owing to the presence of functional groups such as $Me_2As(O)–$ or $–SO_3H$. The ions formed (cations due to the protonation of the dimethylarsinoyl moiety at low pH and anions due to the dissociation of the $–SO_3H$ groups at neutral and basic pH) are separated by ion-exchange HPLC. Alternatively, they can be made to form hydrophobic compounds with a suitable ion-pairing reagent that are then chromatographed by RP-HPLC.

Anion-exchange HPLC is considered as an established technique for the speciation of arsenic; isocratic elution in neutral media was recommended by Larsen[271] for the separation of the five major arsenic species [As(III), As(V), MMAA, DMAA, and arsenobetaine]. The isocratic elution mode and the low concentrations of organic solvent and dissolved solids in the mobile phases permit stable operation of the ICP with no degradation in sensitivity and little matrix interference. A set of 14 guidelines for performing speciation of arsenic by HPLC-ICP-MS was presented.[271] A 1.5 cm C_{18} guard column placed before the AE column was used to remove most of the organic components from urine that would otherwise bind irreversibly to the packing material.[237]

Cation-exchange HPLC[88,228,234,245–248] was often used in parallel to AE HPLC to confirm the identity of the analyte species, especially for samples that might contain arsenoribosides. Most organoarsenic species are not retained on a reversed-phase column; the addition of an ion-pairing reagent is required to achieve their separation. Both anion-pairing cations such as tetraethylammonium[101,103,244] or tetrabutylammonium[250,272] and cation-pairing anions such as pentanesulfonate,[269] hexanesulfonate,[244] heptanesulfonate[243,247] and dodecyl sulfonate[272,273] were used for this purpose.

Another separation mechanism frequently applied was based on size-exclusion chromatography of ion pairs of organoarsenic compounds including arseno sugars.[100–103] Recently, the use of a size-exclusion column with 1% acetic acid as mobile phase was shown to be capable of the baseline separation of 12 organoarsenic compounds into four fractions.[274]

5.3 Detection of organoarsenic species

ICP-MS coupled *via* a conventional cross-flow or concentric nebulizer is the established detection technique for the speciation of arsenic in biological materials. The signal-to-noise ratio was improved fourfold by the introduction of 3% v/v of methanol into the mobile phase and the use of an ICP power of 1.3 kW.[275] Lower detection limits (0.04–0.6 μg l^{-1}) could be obtained by the use of a hydraulic high-pressure nebulizer[125,149] or by the conversion of the organoarsenic compounds exiting the column into AsH_3.[263,276] This procedure also alleviates the interference from the polyatomic ion $^{40}Ar^{35}Cl^+$ at *m/z* 75 that may occur during the analysis of samples with high chloride content, such as urine.

The interest in routine speciation analysis has resulted in a number of interfaces allowing the detection of organoarsenic compounds by AAS. The prerequisite for success is the conversion of organoarsenic species into AsH_3. This can be achieved thermochemically,[277] by microwave-assisted oxidation[127,267,278] or by UV photo-oxidation.[254,258] The detection limits match those achieved in HPLC-ICP-MS with pneumatic nebulization. They are in the low μg l^{-1} range but are species dependent.

5.4 Identification of organoarsenic species

In most studies, the identification of signals in HPLC with As-specific detection has been achieved by matching their retention times with the standards. The instability of this parameter is well known; retention time irreproducibility is common in the presence of sample matrix, some constituents of which may form ion pairs with organoarsenic compounds, and thus change their behavior. Also, the state of the column plays a role, the efficiency of separation decreasing slowly with the number of chromatographic runs on a given column. Some of these problems can be solved by spiking experiments but a risk occurs that the spike of one compound may match the retention time of another one, leading to signal misidentification. This risk can be reduced by the use of two-dimensional chromatography.[274]

The availability of standards for organoarsenic compounds is still problematic, although the situation has improved with the preparation of pure arsenobetaine and arsenocholine calibrants by the Measurement and Testing Programme[279] and their availability from the National Institute of Environmental Studies (NIES).[103] Regarding arseno sugars, their only source remains isolation from natural samples by preparative chromatography[242] or the use of well characterized reference materials.[103]

A method for the identification of submicroamounts of arseno sugars is molecular ion mass spectrometry. Fast atom bombardment (FAB) MS/MS in the negative and positive modes was proposed for the characterization of arseno sugars and applied to a partially purified *Sargassum lacerifolium* algal extract.[87] A better sensitivity was achieved by pneumatically assisted ESMS,[274] which also offers the possibility of on-line chromatographic detection;[135] an example of a cation-exchange chromatogram showing an arsenosugar peak in the NBS SRM 1566a Oyster Tissue was reported.[135] Electrospray ionization suffers from a matrix suppression effect on the signal and a generally poor compatibility of the chromatographic mobile phase with the ionization conditions. In particular, the use of ion-pairing reagents seems to be disastrous for the sensitivity, requiring an alternative to ion-pairing RP-HPLC approaches.

6 Analysis for organoselenium species

Selenium exists in biological systems in the form of inorganic species such as Se(IV) (SeO_3^{2-}, selenite), Se(VI) (SeO_4^{2-}, selenate) or selenides (*e.g.*, HgSe), or in the form of organic species having a range of molecular masses and charges, starting from the simplest MeSeH and ending at complex selenoproteins.[57] The metabolism of inorganic Se is complex and involves a number of species, the most important of which are selenoamino acids: selenomethionine in microorganisms and plants, and selenocysteine in animals and man. In biological fluids selenium is bound to specific Se-binding proteins, and is a constituent of various selenoproteins (containing covalent C–Se bonds). Both of the common inorganic selenium species (selenate and selenite) are toxic whereas Se-containing amino acids are biologically useful and can be used for the biosynthesis of selenoproteins.

The myriad of selenium species present in biological systems represents a challenge to the analyst. The major fields of interest include the determination of methylselenium species in urine, the species-selective determination of selenoamino acids in microorganisms and plants, and the characterization of seleno-

proteins in mammals. Analytical techniques for the determination of selenium species have been reviewed;[280–283] the coverage of selenoprotein analysis in mammals was reported by Behne *et al.*[189,190] Analytical methods for selenium species in biological samples are summarized in Table 2.

6.1 Methylselenium species

The reduction of Se followed by its methylation to methylselenol, dimethyl selenide and the trimethylselenonium ion is the primary pathway of the metabolism of selenite by animals and man. Some of these and other unidentified compounds of this type are excreted in urine.[295,298]

Trimethylselenonium accounts for a few per cent of Se in urine in normal subjects but it becomes predominant if the nutritional intake of selenium increases, probably owing to detoxification in the kidney. Monomethylselenium was found in untreated rats but rats to which selenate was administered by injection excreted trimethylselenonium. Three signals were observed in RP-HPLC chromatograms of basal human urine but their identification by retention time matching was unconvincing.[159] At least five species have been found in urine but only two organoselenium compounds, trimethylselenonium and monomethylselenol, have been identified so far.[298]

In view of the low concentrations involved, the use of an ultrasonic nebulizer to interface HPLC with ICP-MS[151] or the use of an HR-ICP mass spectrometer[159] was advised. Detection limits at the sub-ng ml^{-1} level can be obtained.

Table 2 Analytical methods for species-selective analysis of seleno compounds by HPLC with element-selective detection

Sample	Column	Mobile phase	Detection	Ref.
Seagull eggs	RP: 5 μm C_{18} Nucleosil	30 mM $HCOONH_4$ in 5% aq. MeOH (pH 3) (1.2 ml min^{-1})	HR-ICP-MS	148
Nutritional supplements	CE: Supelcosil LC-SCX (250 × 4.6 mm)	20 mM pyridine (pH 4.71) + 5 mM citric acid (pH 5.95) (1.5 ml min^{-1})	ICP-MS	284
Nutritional supplements	RP: Nucleosil (200 × 4 mm i.d. × 5μm)	0.05% triethylamine (0.65 ml min^{-1})	HGAAS	285
Enzymic extract of cooked cod	AE: Polysphere IC AN-2 (120 × 4.6 mm × 10 μm)	5 mM salicylate –TRIS (pH 8.5) (0.75 ml min^{-1})	ICP-MS	130
Corn and rice, hydrolyzed selenoprotein	Dionex DC6A	Not given	Radioactivity	286
Se-enriched garlic, onion and broccoli	Hamilton PRPX-100 (150 × 4.6 mm)	5 mM ammonium citrate buffer (pH 4.8) containing 2% MeOH	ICP-MS	287
Se-enriched yeast	SE: Superdex-200 HR (300 × 10 mm × 13 μm) AE: Hamilton PRPX-100 (250 × 4.1 mm × 10 μm) RP: Inertsil ODS-2 (150 × 4.6 mm × 5 μm)	SE: 30 mM TRIS–HCl (pH 7.0) AE: gradient elution with ammonium phosphate buffer RP: 0.1% TFA in 2% MeOH	ICP-MS	98
Se-enriched yeast	RP: Inertsil ODS-2 (150 × 4.6 mm × 5 μm)	0.1% TFA in 2% MeOH	ICP-MS ESMS/MS	99
Se-enriched yeast and garlic	AE: Hamilton PRPX-100 (250 × 4.1 mm × 10 μm) RP: Brownlee C_{18} (220 × 2.1 mm) IRP: Zorbax SB-C8 (150 × 4.6 mm)	AE: 5 mM ammonium citrate buffer (pH 4.8) containing 2% MeOH (1 ml min^{-1}) RP: 0.034% TFA in 60% MeOH IRP: 0.1% TFA in 2% MeOH	ICP MS ICP-MS	131
Se-enriched yeast, garlic	IRP: Symmetry Shield RP8 (150 × 3.9 mm × 5 μm)	0.1% heptafluorobutanoic acid in 1% MeOH	ICP-MS	226
Se-enriched yeast, garlic	IRP: Symmetry Shield RP8 (150 × 3.9 mm × 5 μm)	1% MeOH with 0.1% TFA (1.0 ml min^{-1})	ICP-MS ESMS	225, 288
Se-enriched yeast	IRP: Zorbax SB-C8 (150 × 4.6 mm)	0.1% TFA in 2% MeOH (1 ml min^{-1})	ICP-MS	289
Se-enriched yeast enzymic hydrolyzate	IRP: Hamilton PRP-1 (250 × 4.1 mm)	Sodium heptanesulfonate in 10% CH_3CN (pH 2.4)	ETAAS	290
White clover CRM 402	AE: Polysphere IC AN-2 (120 × 4.6 mm × 10 μm)	6 mM salicylate in 3% MeOH (1 ml min^{-1})	FAAS, ICP-MS	84
White clover CRM 402	AE: PAX-100 or Ion-Pac AG10 (50 × 2 mm)	Ammonium carbonate (pH 10) containing 2% MeOH (80 μl min^{-1})	DIN-ICP-AES, ETAAS	158
White clover CRM 402	IRP: Hamilton PRP-1 (150 × 4.1 mm)	1% acetonitrile–10 mM tetraammonium bromide (pH 4) (0.4 ml min^{-1})	ETAAS	291
White clover CRM 402	AE: PAX 100 (50 × 2 mm)	2% MeOH containing 15 mM ammonium carbonate (pH 10) (80 μl min^{-1})	ETAAS	85
Rat urine	SE: Asahipak GS520 or GS320	50 mM TRIS–HCl buffer (pH 7.4) (1.0 ml min^{-1})	ICP-MS	292, 293
Urine	IRP: Hamilton PRP-1 (150 × 4.6 mm × 5 μm)	5 mM tetrabutylammonium phosphate in 3% MeOH (pH 7.6) (1.7 ml min^{-1})	ICP-MS	151
Serum, urine	IRP: Hamilton PRP-1 (150 × 4.1 mm × 5 μm)	0.1 mM sodium pentanesulfonate in 2% MeOH (pH 4.5) (1 ml min^{-1})	ICP-MS	294
Human erythrocyte lysate	SE: G3000 SWXL (300 × 7.8 mm × 10 μm)	10 mM TRIS buffer (pH 7) containing 0.1 M NH_4NO_3 (0.25 ml min^{-1})	On-line ETAAS	167
Serum, breast milk SEC fraction	CZE and CIF		ICP-MS	216
Rat plasma	SE: Asahipak GS520 or GS320	50 mM TRIS–HCl buffer (pH 7.4) (1.0 ml min^{-1})	ICP-MS	56, 295
Human liver	Sephadex 200 (300 × 10 mm × 13 μm)	Not given	INAA	296
Human plasma	SEC	10 mM sodium phosphate buffer (pH 7.0) (0.6 ml min^{-1})	ICP-MS	297
Se-proteins in human and mouse plasma	Tandem HPLC: heparin affinity (AFPak) and SE (Asahipak GS520HQ)	Sodium heparin in 20 mM phosphate buffer (pH 7.5) containing 0.01% EDTA	ICP-MS	120
Se-exposed cyanobacterium	CE: Nucleosil 100-5SA (250 × 4.6 mm)	A: 25 mM $NH_4H_2PO_4$ (pH 3.0) B: 25 mM $(NH_4)_2HPO_4$ (pH 7.0) Gradient elution: 0–20 min 0–67% B	ICP-MS	153

6.2 Selenoamino acids

A number of selenoamino acids have been identified in microorganisms and plants, in addition to the most common selenate and selenite species. In the most widely investigated sample, Se-enriched yeast, more than 20 Se compounds including selenocysteine, selenomethionine, methylselenocysteine, and inorganic forms seem to be present.[98,99,131,289,299] Five selenium species and several unknown peaks were observed in selenium-enriched garlic, onion and broccoli.[225,287] Several seleno analogues of sulfur-containing amino acids and their derivatives have been identified in terrestrial plants, especially in so-called 'selenium accumulator plants'.[226,288] Among the six organoselenium compounds detected in seagull eggs the major compounds were selenocysteine and selenocystamine.[148] Another area of interest in species-selective determination of amino acids is their being the product of enzymolysis of selenoproteins.[130,227,286,290]

6.2.1 Extraction of selenoamino acids. Since selenoamino acids are water-soluble, leaching with hot water has been judged sufficient to recover selenium species not incorporated into larger molecules. The sample is homogenized with water, sonicated or heated and ultracentrifuged. The typical recovery of selenium extracted in this way is *ca.* 10%,[98,131,284,289,290] but it can be 100% in the case of some selenized yeast samples. Free selenoamino acids were separated by ultrafiltration (breast milk)[216] or dialysis (algal extract).[227] Selenocysteine and some other selenoamino acids are highly susceptible to oxidative degradation, because the selenol group has a significantly lower oxidation potential than its sulfur counterpart. The carboxymethyl derivative was synthesized (by addition of iodoacetic acid) to stabilize selenocysteine and thus to prevent its degradation.[153]

The low yields of the aqueous leaching procedure for some species and samples promoted more aggressive leaching media to be used by some workers. A trade-off is always necessary between the recovery of Se from a solid matrix and the preservation of the original Se species. As shown by Casiot *et al.*,[98] the addition of SDS to the leaching mixture increases the yield of Se by releasing selenoamino acids bound in selenoproteins. The recovery of selenoamino acids can increase to above 95% by degrading the species originally present with a mixture of proteolytic enzymes.[290]

Care is advised in the interpretation of literature data since the results depend on the way in which the sample was prepared. This applies in particular to the frequently used statement 'the majority of Se is present as selenomethionine', when describing the result of a procedure involving an enzymic digestion. Actually, selenomethionine usually constitutes a part of a larger stable selenoprotein that had been destroyed during the sample preparation procedure.

6.2.2 Identification and determination of selenoamino acids. A plethora of methods claimed to be useful for the speciation of Se(IV), Se(VI), selenomethionine and selenocysteine exist, as can be seen from review papers.[280–283] The reason for this choice of the analytes seems to be the commercial availability of standards for these compounds, since many of the methods developed have never been applied. Paradoxically, the opposite approach, *i.e.*, one based on the screening of a sample for the presence of stable selenium species by HPLC with Se-specific detection, leads not only to the conclusion that the actual number of species present in selenized yeast exceeds 20 but also that none of the four above-mentioned standard compounds is apparently the dominant species.[98,99,131,289,299]

As demonstrated in Table 2, the direct coupling of HPLC to ICP-MS is an established approach to the determination of Se species in biological materials. The separation mechanisms employed have included ion-exchange (anion and cation) and cation-pairing reversed-phase chromatography. The last approach (using perfluorinated carboxylic acids as cation-pairing reagents) offers the highest resolution and should be given preference for the characterization of samples containing many seleno compounds.[226,288] In the cases when the interest is limited to the quantification of inorganic selenium species or selenomethionine (enzymic extracts), anion-exchange chromatography seems to be the choice. The chromatographic mobile phase should possess a pH-buffer effect in the range of the pK_a values of the anionic Se compounds and, in order to prevent salt build-up on the sampler and skimmer cones, the eluting molecular species should preferably be organic in nature.[84] The application of CZE with ICP-MS and ESMS detection was demonstrated recently for the analysis of selenoamino acids in breast milk.[216,300,301]

Retention time matching with the commercially available standards has usually been used for the identification of selenium species, usually with little success. The breakthrough can be achieved by the synthesis of a significant number of selenium compounds expected to be found in the analyzed samples[226,288] or by using ESMS[130,225] or ESMS/MS[99] for the identification of Se species eluted from an HPLC column.

6.3 Selenoproteins

In mammals, speciation of Se usually involves the determination of the different Se-containing proteins.[120,165,188,297,302–305] The most important seem to be selenoprotein P, a major protein which is sometimes used as a biochemical marker of selenium status,[302] selenoenzymes such as several glutathione peroxidases and type 1 iodothyronine deiodinase[165,188,302] and albumin.[165,304,305] More than 25 Se-containing proteins or protein sub-units were detected in rat tissues labeled *in-vivo* with ^{75}Se.[188] Two types of analytical approaches have been developed for the identification and determination of selenoproteins: one based on LC (size-exclusion and affinity) and the other on flatbed electrophoresis.

Despite the fact that SE HPLC was the first method to produce evidence on the presence of selenoprotein P in the human plasma, it is considered to lack adequate resolution and cannot allow the separation of the major selenoproteins, and the large dilution factor limits the detection sensitivity. SE HPLC-ICP-MS of a human serum sample yielded three signals, none of which, however, co-eluted with the glutathione peroxidase activity.[297] SE HPLC of human breast milk whey produced four Se signals corresponding to species with the apparent molecular weights of 15, 60, 1500 and > 2000 kDa.[162]

The principal speciation approach for selenoprotein in serum is based on affinity chromatography; column packing materials are available that allow the separation of serum selenoproteins.[165,304,305] Affinity chromatography using a heparin–Sepharose CL-6B column in series with a column of Reactive Blue 2-Sepharose CL-6B with off-line detection was proposed for the separation of selenoproteins in serum.[165,304] Recently, a combination of affinity chromatography with SE HPLC with on-line ICP-MS detection was reported to separate the three major Se-containing proteins (albumin, glutathione peroxidase and selenoprotein P) found in human plasma.[120]

An enriched stable isotope (^{82}Se) was used to study the fate of injected and endogenous Se by HPLC-ICP-MS.[293,298,306] An unambiguous identification of Se peaks was not possible; the identity of some of them was postulated on the basis of the molecular size estimated from the SE HPLC chromatograms.[182,293,298,306]

Flatbed electrophoresis offers a much better resolution than HPLC for the separation of selenoproteins. The fact that Se is bound *via* a covalent bond allows the use of SDS-PAGE without the risk of Se losses.[188–193,307,308] The principal approaches were based on *in vivo* labeling of rats with ^{75}Se (by injection of

the tracer to Se-depleted animals) which was then detected in the gel strips by autoradiography,[188–193] INAA[193,307,309] and XRF.[194] The sensitivities of the techniques were 0.02 pg, 0.1 ng, and 2–30 ng, respectively.[194,307] A few tens of different selenoproteins could be identified, as discussed in a review paper[188] and in the above cited studies. Differences between different tissues were observed indicating specific functions of some of the Se-containing proteins in certain tissues. Differences were also observed between the subcellular fraction indicating that some selenoproteins were involved in different intracellular processes.[190] SDS-PAGE was also successfully used for the detection of selenoproteins in soybean radiolabeled with ^{75}Se.[309,310] Prior to PAGE, the isolation of the protein fraction by anion-exchange chromatography was recommended.[310]

7 Analysis for metal complexes in microorganisms, plants and food of plant origin

Microorganisms and plants have developed a number of internal mechanisms to control the homeostasis of essential elements and to cope with the stress induced by toxic elements. They include high turnover of organic acids such as phytate, malate, citrate, oxalate, succinate and others, induction and activation of antioxidant enzymes, such as superoxide dismutase or glutathione peroxidase, or induction of phytochelatins or heat-shock proteins.[311] Metals complexed by uronic acid derivatives are components of cell walls complexed by pectin subunits. The complexation of metals leads to a number of relatively poorly characterized metal complexes. The understanding of mechanisms controlling the detoxification is possible only by the availability of analytical data on the species formed.

Various analytical approaches have been proposed to study metal speciation in plants (Table 3). Except for a few, their common feature is the analyte recovery procedure based on sample homogenization with an aqueous buffer, sonication, ultracentrifugation and the analysis of the supernatant. Studies on trace element speciation in the solid residue have been scarce.[312]

7.1 Metal complexes with phytochelatins and soluble proteins

Phytochelatins (PCs) are a class of oligopetides composed only of three amino acids, cysteine (Cys), glutamic acid (Glu) and glycine (Gly), and in which glutamic acid is linked to cysteine through a γ-peptide linkage. Their general formula is (Glu-

Table 3 Hyphenated techniques for the analysis of metal complexes with biomacromolecules of bacterial and plant origin

Sample	Elements	Column	Mobile phase	Detector	Ref.
Bacillus cereus, *Pseudomonas aeruginosa*	Pb, Zn	SE: Eurogel GFC (300 × 7.5 mm × 8 μm)	50 mM TRIS–HCl buffer (pH 7.5) with 0.2% sulfobetaine SB-12 (1 ml min^{-1})	ICP-MS	313
Cyanobacterium *Anacystis nidulans*	Cd, Zn	RP: Capcellpak C_8 (150 × 4.5 mm)	CH_3CN–50 mM TRIS–HCl (pH 7.5) (9:91) and 0.1 mM EDTA (1 ml min^{-1})	ICP-MS	314
Cyanobacterium *Anacystis nidulans*	Zn, Se	SE: Asahipak GFA-30F (300 × 7.6 mm)	50 mM TRIS–HCl (pH 7.5) and 0.2 M $(NH_4)_2SO_4$–0.1 mM EDTA (0.8 ml min^{-1})	ICP-MS	153
Cyanobacterium *Anacystis nidulans*	Cd, Cu, Zn, Hg	SE: DuPont GF-250 (250 × 9.4 mm)	50 mM TRIS–HCl (pH 7.5) and 0.2 M $(NH_4)_2SO_4$	ICP-MS	111
Yeast	Cr	RP: LiChrosorb C_8 (250 × 4 mm × 7 μm)	1% methanol (0.5 ml min^{-1})	ETAAS	315
Yeast	Cr	RP: LiChrospher C_{18} (150 × 3 mm)	Gradient elution: A, 0.2 M KH_2PO_4–50 mM TBAHS[a] (pH 5.2); B, A in 50% MeOH	RAD	316
Tea	Metals	SE: Superdex 75 HR (300 × 10 mm)	0.1 M CH_3COONH_4 (pH 5.5) (1 ml min^{-1})	ICP-MS	154
Tea	Al	SE: Superose 12 HR (300 × 10 mm)	0.12 M TRIS–HCl (pH 5.5)	VIS	160
Soybean flour	Many	SE: Fractogel EMD BioSEC(S) (600 × 16 mm)	0.2 M TRIS–HNO_3 (pH 7.5) (0.4–2 ml min^{-1})	ICP-MS	73
Soybean flour	Cu, Ni, Zn	SE: Fractogel TSK-HW 50 (S) (300 × 16 mm); PAGE	SEC: H_2O (0.95 ml min^{-1})	FAAS	76
Vegetables	Many	SE: Sephadex G-50 (70 × 3 cm)	20 mM TRIS–HCl, 1 mM NaN_3 (pH 8.0), (0.2 ml min^{-1})	TXRF	114
Vegetables	Cd, Zn	SE: Sephadex G-50 (70 × 3 cm)	20 mM TRIS–HCl, 1 mM NaN_3 (pH 8.0, 0.2 ml min^{-1})	ETAAS	115
Plants	Pb, Ba, Sr, REE	SE: Superdex-75 HR (300 × 10 mm × 13 μm)	30 mM $HCOONH_4$ (pH 5.8), (0.8 ml min^{-1})	ICP-MS	312
Radish roots	B	SE: YMC-Pack Diol-120 (300 × 8 mm)	0.2 M $HCOONH_4$ (pH 6.5), (1 ml min^{-1})	ICP-MS	317
Sugar beet, bamboo	B, Mg, Cu, Zn, Pb, Sr	SE: YMC-Pack Diol-120 (300 × 8 mm)	0.2 M $HCOONH_4$ (pH 6.5), (1 ml min^{-1})	ICP-MS	318
Grass (< 10 kDa fraction)	Pt, Pb, Ca	SE: Bio-gel 20XL (300 × 7.8 mm)	25 mM NaCl (0.7 ml min^{-1})	ICP-MS	105, 148
Grass	Pt	SE: Sephadex G-25,G-50, Sephacryl S-400 HR (100 × 16 mm)	25 mM TRIS–HCl (pH 8.0), (12 ml h^{-1})	ASV	319
Grass (< 10 kDa fraction)	Pt	SE: Sephadex G-15 (40 × 2.6 cm) + ITP	25 mM TRIS–HCl (pH 8.0), (12 ml h^{-1})	ASV	320
Grass	Pt	SE: Sephadex G-25 (700 × 16 mm) followed by ITP SE: Sephacryl S-300 HR (100 cm × 16 mm), followed by ITP	25 mM TRIS–HCl (pH 8.0), (12 ml h^{-1})	ASV	321
Fern (REE)	REE	Sephadex 200	Not given	INAA	296
Wine	Pb	SE: Superdex-75 HR (300 × 10 mm × 13 μm)	30 mM formate buffer (pH 5.8)	ICP-MS	322
Wine, juices	Fe	RP: Spherisorb S5 ODS-2 (250 × 4 mm)	$(NH_4)_2SO_4$–H_2SO_4 (pH 2.5), (0.5 ml min^{-1})	EC, FAAS	323

[a] Tetrabutylammonium hydrogensulfate.

Cys)$_n$Gly where n = 2–11.[65,66,324] PCs can detoxify these metals by forming a metal–PC complex in which the metal is bound to the thiol group of the cysteine unit. The general structure of phytochelatins is conservative in a wide variety of plants but some modifications may occur on the C-terminal amino acid leading to the replacement of glycine by β-alanine, serine or glutamic acid.[66,324] Some microorganisms (cyanobacterium) were reported to bioinduce higher metal-complexing polypeptides referred to as metallothionein-like proteins.[111,153,314,325]

HPLC-ICP-MS was proposed as a convenient technique for the monitoring of Cd, Cu and Zn complexes in aqueous extracts of microorganisms and plants that had been submitted to metal stress.[111,153,234,312,314,326,327] Under carefully optimized conditions this technique allows the separation of the complexes with PC ligands having a different length of the peptide chain.[312] However, the formation of mixed-ligand complexes is common, which leads to chromatograms showing multiple peaks of unknown identity. ESMS/MS of fractions (Cd peaks) was proposed for the identification of these complexes; CID MS offered possibility of verification of the PC sequence.[235]

Higher molecular weight compounds, referred to as protein complexes with Cd or Ni, were extracted into warm H_2O. The extracts were centrifuged and the proteins were fractionated by successive filtration through membranes with molecular weight cut-offs of 30 000, 5000 and 500[95–97] prior to off-line ETAAS. Guenther *et al.*[114,115,328] used low-pressure semi-preparative chromatography to separate water-soluble Cd and Zn species. AAS,[95–97] total reflection XRF,[114] γ-ray spectrometry[329] and ICP-AES or ICP-MS[330] were used to determine the metal concentration off-line in the eluted fractions.

Metalloenzymes and metalloproteases with Pb and Zn as the central metal ions were investigated.[313] Iron and nickel complexes in bacteria with myoglobin and algal superoxide dismutase were examined by SDS-PAGE with PIXE detection.[74,75,195,196,331] The use of concentrated surfactants and dithiothreitol (DTT) is necessary for the solubilization of high molecular weight proteins and metalloenzymes.[313]

7.2 Metal complexes with polysaccharides

Plants contain significant concentrations of polysaccharides of which the potentially negatively charged oxygen functions can bind cations electrostatically and even chelate them *via* polyhydroxy groups.[67] In comparison with proteins, however, little is known about the relevance of metal coordination to carbohydrates that are the most abundant (by weight) class of compounds in the biosphere.[332] Recently, attention was attracted by a structurally complex pectic polysaccharide rhamnogalacturonan-II (RG-II).[333–336] This ubiquitous component of primary plant cell walls forms dimers cross-linked by 1:2 borate diol esters (dRG-II) that were found to complex *in vitro* specific divalent cations[335] and the majority of Pb, Sr, Ba and REEs in wine and vegetables.[312,322]

SE HPLC with the parallel ICP-MS and refractometric detection was the primary technique to investigate metal complexes with polysaccharides in plants and related samples.[312,322] The same technique was used to characterize water-soluble boron compounds in radish roots.[317]

Aqueous extracts of plants contain the dRG-II complex but the presence of water-soluble polysaccharide species with higher molecular weights was also demonstrated.[312] The latter are probably of pectic origin since they can be readily decomposed by enzymic hydrolysis with a mixture of pectinase and hemicellulase to release the dRG-II complex.[312] The same mixture of enzymes was reported to be efficient to extract the dRG-II–metal complexes from water-insoluble residues of vegetables owing to the destruction of the original pectic structure.[312]

7.3 Other metal species in plant tissues

The above-discussed cases concern reports in which the authors achieved the identification of the metal–biomolecule complexes eluted from a chromatographic column or at least claimed to have done so. There have been, however, a number of studies of the screening of plant extracts for the presence of stable metallospecies without the unambiguous identification of the latter.

Several studies discussed the speciation of platinum found in plant materials as the consequence of the use of this metal in automotive catalysts.[105,148,319–321] Platinum was found to form a number of compounds in the M_r 400–800 Da range but the determination of the molecular weight was highly speculative.[148] The strong correlation of the Pt concentration with the intensity of the pulsed amperometric detection (selective for carbohydrates) signal enabled the Pt-binding ligands to be identified as carbohydrate oligomers (1000 Da).[320] From UV absorbance and electrochemical data, these species could be characterized as partly oxidized oligosaccharides (about 2–5 monomeric units of aldonic, aldaric and uronic acids) that could originate from the hydrolysis of biopolymers such as pectin (polygalacturonic acid).[320] A Pt-binding fraction of 180–195 kDa could be isolated from native grass. In the grass grown on Pt-rich soil, seven species with M_r ranging from 19 to 1000 kDa could be observed; most Pt was bound to the low molecular weight species.[319]

Speciation of trace elements, especially aluminium, in tea infusions has attracted some attention.[154,160] The metal was found to be bound to the same range of organic molecules in the infusions, regardless of the origin of the tea.[160] The combination of SE and CE HPLC allowed the identification of the large polyphenolic compounds present in tea as the principal metal-binding organic ligands.[154]

The distribution (speciation) of some metals (Cu, Zn, Ni) and non-metals (P, S) in soybean flour extracts was studied.[76,337,338] Different flatbed electrophoretic techniques, including PAGE and isoelectric focusing, were investigated to study the separation of protein-bound Zn, Ni and Cu in soybean flour extracts and were compared with SE HPLC. Gradient gel electrophoresis afforded a better separation of the protein fractions, but could not detect proteins of M_r < 6500; the latter could be detected by SE HPLC.[76] PAGE offers a high resolving power and provides information on molecular size, charge and stability. Isoelectric focusing is useful for further characterization in terms of isoelectric points of separated fractions and for checking peak purity.

The possible presence of a Cr(III)–β-NADP complex in extracts of Cr-enriched yeast was investigated.[315] Two Cr samples were detected by HPLC, one of which was subsequently identified as the Cr(III)–NADP complex by spiking the yeast extract with a standard.[315] Two low molecular weight anionic Cr species were detected in cytosolic yeast extracts by ion-pair RP-HPLC.[316] The coordinative bonds of compounds from trivalent cations are prone to hydrolysis and easily deteriorate by ligand exchange reactions.

Iron species in beverages were investigated.[323] The predominant species in apple juice were Fe(II)-malate and Fe(III)-citrate, whereas Fe(II) and Fe(III) tartrates were the most abundant in white wine.[323]

8 Analysis for metal complexes in animal tissues and related foodstuffs

Most studies of the distribution of trace elements in animal tissues concerned three metals, Cd, Zn and Cu, usually in the context of their association with metallothioneins. The analytical methods developed for the fractionation of metal complexes

in animal tissues are summarized in Table 4. Studies on organoarsenic quaternary compounds (arsenobetaine, arsenocholine) and on selenoproteins have been discussed earlier.

8.1 Metal complexes with metallothioneins

Metallothioneins (MTs) are a group of non-enzymatic, low molecular mass (6–7 kDa), cysteine-rich, metal-binding proteins, resistant to thermocoagulation and acid precipitation.[62–64] They are considered to intervene in the metabolism, homeostatic control and detoxification of a number of essential (Zn, Cu) and toxic (Cd, Hg, As) trace elements. The most widely studied mammalian MTs have been isolated from kidney, liver and brain samples; MTs isolated from mussels have also been reported. Analysis for the MT complexes with metals using coupled techniques was reviewed by Lobinski *et al.*[113]

Soluble extracts of tissues and cultured cells were prepared by homogenizing the sample in an appropriate buffer. Neutral buffers were usually used for the extraction since Zn starts to

Table 4 Analytical techniques for the analysis of metal complexes with proteins in animal tissues

Sample (tissue)	Elements	Column	Mobile phase	Detector	Ref.
Mussel (digestive gland)	Cd, Cu, Zn, S	SE: Progel TSK G3000 SW (300 × 7.8 mm)	50 mM K_2HPO_4–KH_2PO_4 (pH 7.5), (0.7 ml min^{-1})	ICP-AES	177, 178
Mussel (digestive gland)	Cd, Zn, S	SE: ProteinPack 125 Sephadex (100 × 1.5 cm)	50 mM K_2HPO_4–KH_2PO_4 (pH 7), (0.7 ml min^{-1})	ICP-AES	176
Mussel, osprey blood	Cd, Cu, Zn	SE: TSK, G3000PWxL, (300 × 7.8 mm)	30 mM TRIS–HCl (pH 8.6), (0.8 ml min^{-1})	AAS, ICP-MS	106, 107
Mussel (*M. galloprovincialis*)	Cd, Cu, Zn	SE: TSK G3000PWxL (300 × 7.8 mm)	30 mM TRIS–HCl (pH 7.5), (0.8 ml min^{-1})	ICP-MS	118
Mussel (*Anodonta grandis*)	Cd, Hg, Zn	SE: TSK 2000SW (300 × 7.5 mm)	10 mM TRIS–HCl–0.1 M NaCl–0.03% NaN_3 (pH 7), (0.5 ml min^{-1})	RAD	108
Mussel	Cd, Zn	AE: DEAE-5PW (75 cm × 7.5 mm)	0.1 M TRIS–HCl (pH 7.2), (1 ml min^{-1})	TSAAS[a]	339
Mussel	Zn, Cd	SE: Sephadex G-50 and Sephacryl S400		ETAAS	340
Mussel	V	RP: LiChrospher RP-8 (250 × 4.6 mm × 5 μm)	90% MeOH (0.8 ml min^{-1})	ICP-AES	179
Meat liver	Co	RP: Spherisorb ODS-2 (150 × 4.6 mm × 5 μm)	MeOH–0.05 M phosphate buffer (pH 4.2) (from 26:74 to 50:50 over 8 min), (1.5 ml min^{-1})	ETAAS	86
Crab meat	Cd	RP: PRP-1 (250 × 5 mm × 10 μm)	0.05% H_3PO_4 (1.5 ml min^{-1})	ETAAS	79
Fish (bream)	Cd, Cu, Zn	RP: Purosphere C_{18} (250 × 4 mm × 5 μm)	A, 30 mM TRIS–HCl (pH 7.0); B, A in CH_3CN; linear gradient, 0–20 min, 0–12% B	ICP-MS	118
Polychaete worm *Neanthes arenaceodentata*	Cd, Zn, Cu	SE: Spherogel SW 2000 (600 × 7.5 mm × 10 μm)	60 mM TRIS–HCl (pH 7.5) or 0.25 M NaCl–60 mM TRIS–HCl (pH 7.5) (+ 0.05% NaN_3), (1 ml min^{-1})	ICP-MS	116
Mouse liver	Cu, Se, Zn, Fe	SE: Asahipak GS 520 (500 × 7.6 mm × 9 μm)	50 mM TRIS–HCl (pH 7.4), (1.0 ml min^{-1})	ICP-MS	341
Rat liver and kidney	Fe, Cu, Zn, Se	SE: Asahipak GS 520 (500 × 7.6 mm × 9 μm)	50 mM TRIS–HCl (pH 7.4), (1 ml min^{-1})	ICP-MS	182,293, 298
Rat liver	Cd, Zn	AE: DEAE-5PW (75 × 7.5 mm)	Gradient elution with 0–40% of 0.2 M TRIS–HCl (pH 7.4) in 10 mM TRIS–HCl (1 ml min^{-1})	AAS	123
Rat liver	Cd, Zn	AE: DEAE-Sepharose Fast Flow (40 × 3 cm) SE: Sephacryl S-100 (120 × 1.6 cm)	AE: 2 l linear salt gradient (limiting buffer, 250 mM TRIS–HCl, pH 8.6) SE: 10 mM $(NH_4)_2CO_3$	AAS, EC	122
Rat and bovine liver	Cd, Cu, Zn	RP: LiChrospher 100 RP-8 (125 × 4 mm × 5 μm)	20 min linear gradient from 0.5 to 30% MeOH in 50 mM TRIS–HCl buffer (pH 7.0), (1.5 ml min^{-1})	FAAS	137
Rat liver and kidney	Cd, Cu, Zn	RP: PLRP-S (150 × 4.6 mm × 8 μm)	Gradient elution with 10 mM $(NH_4)_2HPO_4$ (pH 8.2)–CH_3CN	FAAS	164
Rat kidney	Hg	SE: Sephadex G-75 (70 × 2.6 cm); Protein-Pack 125 (300 × 7.8 mm)	20 mM ammonium formate buffer (pH 7.65), (1 ml min^{-1})	RAD	342
Rat hepatoma cells	Cu, S, Zn	AE: DEAE-5PW (75 × 7.5 mm)	Gradient elution: from 20 to 250 mM TRIS–HCl (pH 7), 0.02% NaN_3 (1 ml min^{-1})	RAD	117
Rat neuroblastoma cells	Al	SE: Superdex-75 HR (300 × 10 mm)	30 mM TRIS–HCl (pH 7.2) (0.9 ml min^{-1})	ICP-MS	343
Rabbit kidney and liver	Cd	AE: DEAE-5PW, (75 × 7.5 mm)	Gradient elution with TRIS–HCl buffer (0.01 to 0.25 M; pH 8.6), (1 ml min^{-1})	ETAAS	344
Pig kidney	Cd	Pharmacia Superose-12 (20 × 5 mm)	0.12 M TRIS–HCl (pH 7.5)	ICP-MS	112
Bovine kidney	Cd, Zn, Fe, Mg, Cu, Ca, Mn, Mo, Se, V, Zn	SEC: Sephadex G-150 AE: DEAE Sephadex A-50 IEF, ITP	SEC: not given AE: 0.1 M TRIS–acetate with increasing concentration of NaCl	INAA	345
Chicken meat simulated gastrointestinal digest	^{64,66,67,68}Zn	SE: Pharmacia Superose-12 (300 × 10 mm)	0.1 M CH_3OONH_4–0.1% TFA (pH 6.0)	ICP-MS	346
Guinea pig stomach (soluble fraction)	Cu, Zn, Al, Mn, Sr, Rb	SE: Progel TSK-HW55S (25–40 μm)	0.12 M TRIS–HCl (pH 8.2), (0.75 ml min^{-1})	ICP-MS	347
Bovine thyroglobulin enzymic digest	I	RP: Shiseido C_{18} SG120 (350 × 4.6 mm × 5 μm)	0.1 M $(NH_4)_2HPO_4$–0.1 M H_3PO_4 in MeOH–H_2O (1:1 or 9:1)	ICP-MS	348

[a] Thermospray enhanced flame AAS.

dissociate from protein complexes at pH 5; Cd and Cu are removed at lower pH values. A 10–50 mM TRIS–HCl buffer at pH 7.4–9 was the most common choice. For cytosols containing Cd-induced MTs, dilution factors up to 10 were used, whereas for those with natural MT levels equal amounts of tissue and buffer were considered to be suitable. The washing of cells with a TRIS–HCl buffer (pH 8) containing 1 M EDTA was recommended to remove metal ions reversibly bound to the cell wall.[314]

Metallothioneins are prone to oxidation owing to their high cysteine content. Disulfide bridges are then formed and MTs either copolymerize or combine with other proteins to move into the high molecular weight fraction. Therefore, the homogenization of tissues and the subsequent isolation of MTs should normally be performed in deoxygenated buffers and/or in the presence of a thiolic reducing agent. β-Mercaptoethanol was usually added as an antioxidant. Other components added during homogenization included 0.02% NaN_3 (an antibacterial agent) and phenylmethanesulfonyl fluoride (protease inhibitor).

The homogenization step is followed by ultracentrifugation at 4 °C. As a result, two fractions, a soluble one (cell supernatant, cytosol) and a particulate one (cell membranes and organelles), are obtained. The heating of the supernatant at 60 °C for 15 min allows the precipitation (and removal) of the high molecular weight proteins, leaving MTs (which are heat stable) in the supernatant. This procedure allows the protein load on the HPLC column to be decreased not only improving the separation of MT isoforms but also prolonging the column lifetime. Several workers, however, have preferred gel filtration to heat treatment for the isolation of the metallothionein fraction from the tissue cytosol. Guidelines for the preparation of biological samples prior to quantification of MTs were proposed.[349] The typical extraction efficiency from experimental rats and mice tissues was estimated to be 50–80% of Cd.[112]

As can be seen from Table 4, SE HPLC-ICP-MS has been the most widely used technique for the quantification of MTs. The selectivity in terms of MT-1 and MT-2 classes could be achieved by anion-exchange HPLC with AAS[122,123,339,344] or radioactivity detection[117] or RP-HPLC-FAAS.[164] The highest resolution was reported to be given by microbore RP-HPLC with DIN-ICP-MS and ESMS detection[141] and CZE-ESMS[219] which were shown to be able to separate the particular subisoforms within each of the classes of MT isoforms.

8.2 Other metal species

Enzymic digests of foodstuffs were analyzed in the quest for molecular information to contribute to the knowledge on the bioavailability of essential and toxic elements. Enzymolysis in simulated gastric and gastrointestinal juices was proposed for meat samples.[346] The soluble fraction of the stomach and upper intestinal contents of guinea pigs on different diets were investigated for the species of Al, Cu, Zn, Mn, Sr and Rb. The effect of citrate on each of these elements was also assessed.[347] An enzymic digest of bovine thyroglobulin was analyzed for iodine species.[348] A combination of the separation techniques was applied to isolate a 30 kDa Cu protein from bovine kidney prior to INAA.[161]

Vanadium from oil spills enters the environment in the form of vanadyl porphyrins, a group of macrocyclic aromatics consisting of a porphyrin ring in which a metal ion is bound. They were proposed for environmental monitoring because of the much higher resistance to biodegradation and weathering than the hydrocarbon fraction.[179]

9 Analysis for metal complexes in human body fluids and tissues

The most common body fluids include blood [subdivided by centrifugation into plasma (serum) and red cells (erythrocytes)], amniotic fluid, breast milk and urine. Liver and kidney have been the most widely studied organs because of their crucial function in the metabolism of metals. The major bioligands include proteins and small anions with specific functions, such as citrate, ATP, porphyrins or cobalamins. The greatest interest has been enjoyed by some essential metals such as Fe, Cu and Zn, and toxic metals such as Al, Cr, Pb, Cd and Hg. The metals can be the integral part of metalloproteins and metalloenzymes or bind less firmly to transport proteins (albumin, transferrin).

Sampling, sample preservation and preparation prior to chromatography are particularly critical in clinical chemistry because of the low concentrations involved (risk of contamination), the thermodynamic instability of some species and the complexity of the matrix. Problems that occur during sampling, storage and sample preparation were discussed.[38,41,46] The methods for species-selective analysis of clinical samples are summarized in Table 5.

9.1 Blood plasma (serum)

Serum is obtained from whole blood by centrifugation. It is a complex matrix but has the advantage that all the components are soluble in aqueous buffers. Dilution with the chromatographic buffer (1 + 5) and preventive filtration through a 0.2 μm filter are the usual steps prior to injection on the column.

SE HPLC with ICP-MS[119,352] or ICP-AES[173] detection is the most convenient method for the fractionation of the metal-bearing serum proteins. Multi-element detection is readily feasible but this technique was usually used for speciation of a particular element. Albumin, which is a major serum protein, complicates the identification of other proteins. Anion-exchange chromatography was preferred where the separation of albumin and transferrin was required.[80,82,83,124] When low molecular weight compounds are of interest, ultrafiltration on a 10 kDa filter is required.[171,353,354] The filtrate is protein free and can be analyzed, *e.g.*, for drug metabolites or porphyrins. Reversed-phase chromatography with ICP-MS detection is the preferred technique for the metal species in the filtrate.[171,353,354]

9.1.1 Toxic elements: aluminium, chromium and lead.

Aluminium build-up in patients with chronic renal failure results in a number of diseases. Efforts have been made to elucidate the mechanisms of this build-up by the investigation of Al speciation in serum.[80–83,124,166,168,350,366,367] The techniques used for speciation studies of Al in biological fluids have been reviewed.[168,367] Transferrin seems to bind ~90% of the total serum Al;[46] the remaining 10% is likely to form a complex with citrate.[231] The addition of a desferioxamine (DFO) drug to serum led to the displacement of Al from transferrin and to the formation of an Al–DFO chelate.[80] The interaction between Al and Fe for binding to transferrin and the effect of citrate was studied.[168] As the Al level increased in occupationally exposed individuals, more Al was bound to the high molecular weight fractions.[350]

Anion-exchange chromatography with off-line ETAAS detection was the most widely used technique[80,82,83] SEC being incapable of separating albumin from transferrin. The use of an HR-ICP-MS detector enabled Al speciation to be performed at the basal levels in human serum.[124] Binding of serum proteins to Al and Si was studied by further separating the HPLC column fractions using SDS-PAGE.[83] The low molecular weight Al complexes were separated by ultrafiltration using a 10 kDa

Table 5 Species-selective analysis of metal complexes with proteins in biological fluids

Sample	Metals	Column	Mobile phase	Detection	Ref.
Serum	Al	SE: TSK G4000 SW (600 × 7.5 mm)	100 mM NaH_2PO_4–0.2 M NaCl–0.05% NaN_3 (pH 7.4)	ETAAS	350
Serum	Al	AE: Mono-Q HR (50 × 5 mm × 10 μm)	Gradient: A, 10 mM $NaHCO_3$ in 20 mM TRIS–HCl (pH 7.4) to A + 0.25 M NaCl (1 ml min^{-1})	ETAAS	80
Serum	Al, Si	AE: DEAE-5PW (75 × 7.5 mm × 10 μm)	Gradient: A, 10 mM $NaHCO_3$ in 20 mM TRIS–HCl (pH 7.4) to A + 1 M NaCl (1.2 ml min^{-1})	ETAAS	83
Serum	Al, Fe	AE: Bio-gel TSK-DEAE-5PW (100 × 7.5 mm)	Gradient: 0.05 M TRIS–HCl (pH 9.2) to 0.05 M TRIS-NaCl (pH 9.2)	ETAAS	82
Serum	Cd, Cu, Fe, Pb, Zn	SE: Synchropak GPC 300 (250 × 2 mm × 5 μm)	100 mM TRIS–HCl (pH 6.9)	ICP-MS	119
Serum	Cu, Fe, Mg, Pb, Zn	SE: TSK 3000 SW (300 × 7 mm)	100 mM TRIS–HCl (pH 7.2)	ICP-MS	77
Serum	Pt, Ru	SE: Progel TSK G3000 PWxL (60 × 7.8 mm)	30 mM TRIS-HCl (pH 7.2), (0.9 ml min^{-1})	ICP-MS	121
Serum	Mg	AE: TSK-gel DEAE-5PW	Gradient: 10 mM TRIS-HCl (pH 7.4)–0.5 M acetic acid in 0.01 M TRIS–HCl	ETAAS	77
Serum	I	SE: TSK HW 40F (59 × 2 cm)	20 mM $NaHCO_3$ (3 ml min^{-1})	ICP-MS off-line	351
Serum	Cu, Fe, Mn, Zn	Fractogel EMD BioSEC 650 (S) (600 × 16 mm)	20 mM NaH_2PO_4 + 0.3 M NaCl (pH 6.8)	ICP-AES	173
Serum	Ce, Fe, Na, Zn	SEC: TSK G3000SW_{xl} (600 × 7.5 mm) Asahipak GS-520 h (250 × 7.5 mm)	100 mM TRIS (pH 7.4)	γ-Radio-activity	352
Serum	Al	AE: Mono-Q HR (50 × 5 mm × 10 μm)	Gradient: 0–250 mM ammonium acetate in 50 mM TRIS–HCl buffer (pH 7.4), (1 ml min^{-1})	HR-ICP-MS	124
Serum, seminal fluid, milk whey	Cu, Co, Fe, Zn	SE: TSK G3000SW	100 mM HEPES in 100 mM NaCl (pH 7.4), (0.75 ml min^{-1})	ICP-AES	172
Serum	Au auranofin metabolites	AE: Alltech WAX 300 (150 × 4.6 mm)	AE: gradient: 20–200 mM TRIS buffer (pH 6.5)	ICP-MS	353
		SE: Bio-Sil TSK 250 (300 × 7.5 mm)	SE: 25 mM TRIS buffer (pH 7.7)		
Serum 10 kDa ultrafiltrate	Pt	RP: PLRP-S (250 × 4.6 mm)	Gradient: 30–95% MeOH (0.6 ml min^{-1})	ESMS, AAS	354
Serum (human and rat) 10 kDa ultrafiltrate	Pt	Hypersil ODS (150 × 4.6 mm × 5 μm)	Gradient: 0–5% propan-2-ol in 10–60 mM phosphate buffer (pH 2.8) containing 1 mM Na octane-1-sulfonate, (1 ml min^{-1})	ICP-AES	171
Blood	Co, Fe, Zn	RP: Hypersil SAS C (250 × 4.6 mm × 5 μm)	68% MeOH (pH 4.5)	ICP-MS	355
Erythrocytes		SEC: Superdex 200 HR (300 × 10 mm × 13 μm)		ICP-MS	356
Erythrocytes	Cu, Fe, Mn, Zn	Fractogel EMD BioSEC 650 (S) (600 × 16 mm)	20 mM NaH_2PO_4 + 0.3 M NaCl (pH 6.8)	ICP-AES	173
Erythrocytes	Pb, Fe, Cu, Zn, Mg	SEC: TSK G3000 SW (300 × 7 mm)	100 mM TRIS–HCl (pH 7.2)	ICP-MS	357
Amniotic fluid	Pb, Cu, Zn	SEC: TSK G3000SW_{xl} (300 × 7.8 mm)	100 mM TRIS–HCl with 0.1% of propan-2-ol (0.8 ml min^{-1})	ICP-MS	358
Breast milk whey	Zn, Rb, Fe, Cu, Co, Mn, Pb, I, Cd, Se, Mo	SEC: Asahipak GS 620 (500 × 7.6 mm × 9 μm) + Asahipak GS 520 (500 × 7.6 mm × 9 μm)	100 mM TRIS (pH 7.1)	ICP-MS	180
Breast milk whey	19 elements	SEC: TSK G3000SW_{XL} (300 × 7.6 mm × 9 μm) + TSK G4000SW_{XL} (300 × 7.6 mm × 9 μm)	0.1 M HEPES + 0.3 M NaCl (pH 7.0)	ICP-AES ICP-MS	359
Breast milk whey	Zn	SEC: Asahipak GS 620 (500 × 7.6 mm × 9 μm) + Asahipak GS 520 (500 × 7.6 mm × 9 μm)		ICP-AES	180
Breast milk whey	Se	SE: Toyo Pearl TSK HW 40, followed by CZE	H_2O (3 ml min^{-1})	UV, ICP-MS	205, 301, 300
Breast milk whey	I	SEC: Superdex-75 (300 × 10 mm × 13 μm)	30 mM TRIS–HCl buffer (pH 7.5)	ICP-MS	360
Breast milk whey	Cu	SEC: Superdex-75 (300 × 10 mm × 13 μm)	30 mM TRIS–HCl buffer (pH 7.5)	ICP-MS	70
Breast milk whey	Fe, Se, Zn	SEC: Asahipak GS 620 (500 × 7.6 mm × 9 μm) + Asahipak GS 520 (500 × 7.6 mm × 9 μm)	20 mM NaH_2PO_4 + 50 mM NaCl (pH 7.4)	ICP-AES	162
Urine	Cd	AE: Protein-Pak DEAE-5PW (75 × 7.5 mm × 10 μm)	Gradient: 2–200 mM TRIS–HCl (pH 7.4)	HG-ICP-MS	163
Urine, blood	Au (drug metabolites)	IPRP: Spherisorb ODS-2(250 × 4.6 mm × 5 μm) with a 5 cm guard column of the same material	MeOH–25 mM ammonium formate (pH 6) (1:1) containing 10 mM tetrabutylammonium chloride	ICP-MS	361, 362
Urine	Au (drug metabolites)	150 × 4.6 mm × 7 μm with WAX–diethylmethylamine packing material (Alltech)	Gradient: 100% 20 mM TRIS (pH 5.5) to 100% 200 mM TRIS over 15 min	ICP-MS	363
Cisplatin, products of its hydrolysis and incubation with amino acids	Pt	ODS C_{18} (150 × 4.6 mm × 5 μm) with a guard column Spherisorb ODS-2 (50 × 4.6 mm × 5 μm)	5 mM heptanesulfonate–10% MeOH–0.1% formic acid or 10 mM trichloroacetic acid (pH 2.6)	ICP-MS	364
Breast milk ultrafiltrate	Zn, Ni, Cu citrate, orotate and hippurate	CZE		UV	365

membrane.[350] The presence of citrate was identified by AE chromatography and confirmed by ESMS/MS.[231]

The interest in chromium in clinical studies results from markedly elevated plasma Cr concentrations in dialysis patients and workers exposed to elevated Cr levels in the workplace. Chromium in the plasma of healthy persons is known to be mainly bound to transferrin and to a lesser extent to albumin.[368,369] These proteins can be separated by AE chromatography.[51] Cr(III)-labeled plasma was used for the studies.[368,369]

Lead in human serum was found to be present in at least three molecular weight fractions. The major part of Pb was coincident with Cu and was found to be bound to ceruloplasmin. Of the protein-bound Pb recovered, 80% was reported to be contained in protein with an apparent molecular weight of 240 kDa, and 20% in protein with an apparent molecular weight of 45 kDa.[356]

9.1.2 Essential elements: copper, iron and zinc. Cu, Fe and Zn are essential elements and constituents of important metalloproteins such as ferritin (Fe, Cu, Zn), myoglobin (Fe), cytochrome (Fe) and metalloenzymes: β-amylase (Cu), alcohol dehydrogenase (Cd, Zn) and carbonic anhydrase (Cu, Zn). They have usually been determined in a multi-element array by SE HPLC with ICP-MS or ICP-AES detection (Table 5). The binding of Fe to transferrin was studied by HPLC-ETAAS.[82] Immunoaffinity chromatography compared favorably with SE HPLC for the isolation of proteins binding Zn in human serum.[370] The Zn-containing serum proteins were separated by PAGE; the metal was detected by INAA.[72] In the low molecular weight fraction, Cu, Fe and Zn exist as chelates with porphyrins. The separation and quantification of these complexes are of biological and clinical significance because abnormal species and/or excessive amounts of porphyrins are found in a variety of disorders.[60]

Methods for the separation and determination of porphyrins, their precursors, their isomers and their free acid and ester derivatives, metalloporphyrins and hematoporphyrin and its derivatives in biological matrices were reviewed.[60] The majority of methods were based on HPLC with spectrophotometric or fluorescence detection but the use of the HPLC-ICP-MS coupling allowed the simplification of the sample preparation procedure.[355] The high detection sensitivity made a single extraction step prior to chromatography sufficient and rendered derivatization unnecessary.

9.1.3 Metallodrugs: binding to proteins and metabolites. The use of hyphenated techniques in metallodrug research has focused on two major approaches: identification of the drug transport proteins and investigation of the binding kinetics,[121,184] and identification of the drug degradation and metabolism products.[171,353,354,361,371] Consequently, two basic types of analytical approaches have been developed: one (for the high molecular weight compounds) based on SE HPLC-ICP-MS[121,353] or PAGE-ICP-MS[184] of serum, and the other (for the low-molecular weight compounds) based on the ultrafiltration of serum followed by the analysis of the filtrate by RP-HPLC with ICP-MS[171,353,361,371] or ESMS[354] detection. A mini-review on the determination of Pt species in plasma ultrafiltrates and standard solutions using analytical techniques with other than plasma sources is available.[372]

Metal complexes with DNA have been studied by HPLC and CZE. Isomers of the platinated oligonucleotides that differ by the binding site of the Pt complex on the single GG sequence were separated.[373]

9.1.4 Miscellaneous. Vanadium is considered as an environmental hazard but it is also regarded as a therapeutic agent in diabetes treatment. The binding of vanadium(V) is very weak and very sensitive to changes in its environment. The original pH value of the fluid should be maintained during chromatography. RP-HPLC with mobile phases containing acetonitrile seems to be impossible. Very mild separation conditions are necessary to avoid the formation of artefact species.[374] The separation of protein-bound vanadium in incubated serum samples was discussed.[186]

The speciation of Si in biological fluids was reviewed.[168,367] Si was not bound specifically to any serum protein[83] but adsorbed *via* weak interactions of silicic acid.[46] Ultrafiltrable Si constitutes 15–45% of this element.

Magnesium speciation in human serum was studied by AE HPLC-ETAAS;[77] the metal was found to be associated with both the albumin and globulin fractions but not with transferrin.

Free iodine was determined in serum fractionated by SE HPLC.[351] An SE HPLC-ICP-MS chromatogram showing four iodine species in human serum was reported.[343]

9.2 Erythrocytes

Erythrocytes (packed cells, red blood cells) need to be lysed to free their content prior to chromatographic separation. Three freeze–thaw cycles to lyse the cells were proposed followed by a 10-fold dilution with a buffer and centrifugation to remove fragments of membranes.[356,357] An alternative procedure recommended by Cornelis *et al.*[41] was based on mixing one part of packed cells with one part of toluene and 40 parts of ice-cold water, followed by centrifugation and 0.45 μm filtration of the lysate.

The distribution of metals in erythrocytes was studied by SE HPLC with ICP-MS or ICP-AES detection. The major lead binding site in erythrocytes was identified as hemoglobin.[357] The latter, which represents up to 94% of the total amount of proteins in packed cells complicates the identification of other proteins, however.[356]

9.3 Breast milk

Milk is a single source of nutrients for infants. Since speciation of trace elements affects their bioavailability, they should be present in milk not only in appropriate amounts but also in specific forms. The interest in speciation is stimulated by the need to match the elemental species present in breast milk with those in infant formula. Speciation of trace elements in human milk has been the subject of three fairly comprehensive multi-element studies.[162,180,359] A number of other studies concerned specific elements such as Cd,[110] Zn,[104,365] Fe[109] and I.[360] Comparisons between elemental speciation in human and cow milk were made.[360,365]

Milk is analyzed after the elimination of lipids by centrifugation at 3000 rpm for 30 min at 4–5 °C. The resulting whey is injected on to the column; the precipitation of casein with 1 M acetate is optional.[109] SE HPLC with ICP-MS and ICP-AES detection were the principal analytical techniques used to investigate the trace element speciation in milk.[70,162,180,360] Dialysis and purification by SE HPLC are required if further separation by RP-HPLC is to be undertaken.[109] Ultrafiltration using a 10 kDa cut-off filter is an alternative.[365]

Casein (a mixture of α-, β- and κ-casein) is the major milk protein. Despite its molecular weight of *ca.* 20 kDa, it elutes in the void since it is present in milk in the micellar form.[359] Such micelles are spherical polyspread colloidal aggregates with molecular weights exceeding 1000 kDa. Lactoferrin exists in four molecular forms in nature, as does transferrin, and is dominant in human milk.[109] It may elute together with casein, as do immunoglobulins.[359] Another metal-complexing protein is albumin, but the signal identification is tricky because of the possible presence of disaggregated micelles of casein.[359] Free

amino acids, small complexes such as citrate and ions such as iodide elute in or close to the total volume of the column. The use of enzymes to destroy the protein edifice excluded from the column into smaller proteins that would elute in the middle of the chromatogram was proposed; the preliminary investigations however, did not allow the identification of the product species.[70]

9.4 Amniotic fluid

Amniotic fluid is a urine-like fluid inhaled and swallowed by the human fetus. Some heavy metals, *e.g.* Pb, can cross the placenta and end up in amniotic fluid. Metal-binding ligands are important in amniotic fluid because of their potential of being transporters to the neurological system.[358] Prior to analysis, an amniotic fluid sample is usually centrifuged and the supernatant is stored frozen at −20 °C. Most of the Pb was found to be bound to ceruloplasmin or to a 5 kDa Zn peptide.[358] The Cu–ceruloplasmin peak was used as an elution volume marker.[358] The poor resolution of SE HPLC prevents the accurate assignment of elements bound to either albumin or transferrin,[358] the two important bioligands in amniotic fluid.[358]

9.5 Urine

Despite its apparent simplicity, urine is a complex matrix but its constituents are water-soluble, which allows direct chromatography. It may be troublesome to handle because of its higher salt concentration, in comparison with the concentrations of metallo-compounds present. Urine contains *ca.* 1% NaCl, but the actual concentration varies greatly. The urine matrix causes column overloading, which results in peak broadening. High dilution may be recommended.[236] Peak broadening or splitting due to the high salt concentration was reported.[156]

The application of hyphenated techniques to other than organoarsenic and organoselenium metabolites discussed earlier, and drug metabolites[361–363] is scarce. The notable exception is the determination of Cd proteins by AE HPLC with ICP-MS detection.[163]

10 Quality control and assurance in bioinorganic trace analysis

During species-selective analysis by hyphenated techniques, the major obstacles are contamination, break-up of the original metal–protein bond during the separation process and insufficient detection limits of the analyte element in the eluate.

10.1 Contamination risk

The risk of contamination at the level of sampling is at least as acute as in the trace analysis for total metals and the same precautions should be applied. The contamination hazard consists not only of the addition of an exogeneous analyte element to the sample but also the fact the contaminant element may exchange analyte metals in species present in the sample and hence change the initial speciation.

Protocols for sampling of milk and standard specifications for cleaning the material used were discussed in detail.[109] The procedure seems likely to be suitable when extended for other biofluids. Chromatographic buffers were cleaned by elution over Chelex 100 and subsequently checked for contamination.[109,359] A silica-based scavenger placed in the proximity of the injection valve was proposed to retain any Al and Fe originated from buffer solutions and recipients.[168] A column of C_{18} silica impregnated with Chelex 100 placed between the chromatographic pumps and the injection valve was proposed to minimize exogenous contamination.[80] An AE column was used as a scavenger to retain contaminating elements from the eluent.[313] A metal-free HPLC-ETAAS method for the separation of the proteins and of the inorganic/organic metal species of interest was described.[168] Trace metal recoveries were quantitative.[168]

Platinum released from the electrodes in PAGE can apparently react with biomolecules and give rise to contaminating artefacts.[186]

10.2 Stability of species and acid–base equilibria

Biomolecules are easily subject to deterioration. As part of an investigation into the stability of metal–protein complexes in human milk, different buffers (varying in molarity and pH) were compared with water as the mobile phase in SE HPLC in order to investigate differences between the Zn distribution pattern and the stoichiometry of the complex. H_2O was found to be a more suitable mobile phase than the buffers with regard to contamination. A method was developed to investigate the possibility of the transfer of Zn among the proteins during SEC; casein and metallothionein were chosen as competitive Zn ligands.[110] No change in the Zn status of the protein was detected, indicating a stable protein–metal complex under the experimental conditions.[110]

Since the complexation equilibria between complexes of metals with biomacromolecules and ‘free’ metals are strongly pH dependent, the control of the pH of the mobile phase is crucial, irrespective of which separation technique is going to be used. An acidic pH is responsible for the depletion of metals, leading gradually to apo forms. For instance, Zn is lost from MT at pH 5, at pH 3.0 Cd_4 adducts of the isoform are present and at pH 2 Cu still remains attached.[113,138] Various intermediate partially metallated forms occur in various pH ranges. The buffer chosen should therefore ensure that speciation of the analyte is not altered during its passage through the column. In the analysis of body fluids a buffer solution (pH 7.4) corresponding to the physiological conditions is selected in order to avoid denaturation of the proteins during the chromatographic separation.

The native form of the proteins in the separation system must be preserved. Polymeric supports or a silica gel matrix carrying different polymeric chains with active ends were investigated.[173] The saline concentration of the mobile phase is an important parameter affecting the elution volume of proteins. It affects the protein conformation and their hydrodynamic radius and needs to be carefully optimized.[359] Transport proteins are the most influenced by the separation conditions; in the case of metalloenzymes the metal ion is strongly bound within the structure of the proteins. The non-aggressive separation conditions in SEC do not denature these complexes.

10.3 Recovery

SEC columns are generally used to separate biological macromolecules. The recovery with SEC was reported to be problematic because the result tended to suffer from poor protein recovery caused by adsorption of proteins on the stainless steel tubing.[110] Spiking cannot be regarded as an aid to the verification of recovery since a selective enhancement of metal concentrations is possible owing to the free metal-binding capacities of certain proteins.[338] Also, depletion of metal ions added to the mobile phase was observed in the separation of proteins with free metal-binding capacities.[338] The addition of metal salts can result in severe modifications of the intrinsic metal speciation.[338]

Non-specific binding of metal ions or proteins to the column stationary phase is common. The possible ways to limit them

include a reduction in the number of non-specific binding sites by using $NaBH_4$ or the continual saturation of all binding sites by adding the relevant metal ion to the buffer system. Stabilization of the elution pattern may be achieved by methylation of the binding sites.[174] The use of organic vinyl copolymers is recommended.[352] In order to prevent changes in metal binding, the separation conditions must be optimized, and the use of radioactively labeled isotopes offers a sensitive way to do this. The complex of interest can be labeled *in vivo* for animal subjects but for humans only *in vitro* labeling is possible.

The optimum eluent should be chosen for the minimum competition between buffer and cytosolic ligands and for the reduction of chemical interactions between these ligands and the gel.[108] Dilute buffers were not acceptable because of adsorption of low molecular weight compounds by the column packing. Metal ions were proposed to be added to the chromatographic mobile phase to prevent the dissociation of the intrinsic element species and to increase the recovery. The formation of new complexes with high binding stability may result.[338]

10.4 Standardization and accuracy

The issues of quality control and assurance in bioinorganic analysis have some important peculiarities in comparison with the chromatographic analysis and with total trace element analysis. In classical speciation analysis the analyte is usually precisely defined and calibration standards are generally available. There has also been progress regarding the availability of CRMs.[34]

A different situation is faced by an analyst interested in naturally occurring species in biological tissues. Not only reference materials but even simple standards are unavailable for the majority of metal species of interest. Some metal–protein complexes have been identified, but the majority, especially those at lower trace and ultratrace levels, have remained undiscovered so far. To date, the applications of hyphenated techniques to bioinorganic chemistry have been exploratory (looking for new species) rather than confirmatory (determining an expected-to-be-found compound).[157] The two acute problems associated with the reliability of biochemical speciation analysis include the question whether a signal produced by the detector belongs to one particular compound and the identification of this compound.[157]

Most data were obtained by matching the retention time with the available standards. However, in addition to the above-mentioned limited availability of standards, this method may lead to a number of errors.[159] The use of different (complementary, orthogonal) separation mechanisms to ensure the chromatographic purity of the species arriving at the detector and to minimize the risk of misidentification was advocated.[20,157,159,375] A recent study on the speciation of arsenic showed a considerable risk of signal overlaps in the case of the analysis of algal samples containing arseno sugars.[230]

The problem of artefact formation should be addressed. Any organic species that adheres to the column can also bind inorganic species, giving rise to anomalous peaks in subsequent runs.[112] The use of a new guard column for each injection and an extensive column clean-up were recommended.[112]

As discussed above, molecular mass spectrometric techniques such as ESMS or MALDI-TOF will certainly play an increasingly important role in the identification of trace element metal and metalloid species in bioinorganic chemistry, but multi-step fractionation and purification procedures are the hampering factor at the moment.

In many applications SE HPLC-ICP-MS has been used as a semi-quantitative technique to monitor relative changes in analytical signals in a well defined series of samples. Quantification of signals has usually been done using peak area calibration either by converting (by removing the column) the measurement system into the flow injection mode after completing the chromatographic run,[322,325] or by using a calibration graph if standards were available.[107,118,45] ICP-MS instrumental instability due to the clogging of the plasma torch and sampling and skimmer orifices is common. Correction is possible by the post-column addition of an internal standard.

11 Conclusions

It is essential that the analytical capabilities in the area of the biological speciation analysis be improved if we are to understand in more detail the role of trace metals in nutrition, health and disease. Hyphenated techniques are an attractive tool for the rapid, sensitive and comprehensive characterization and quantitative determination of metal–macromolecule complexes in biological samples. The progress in ICP-MS and electrospray (tandem) mass spectrometry, and the increasing speed and efficiency of modern separation techniques, are expected to offer attractive analytical approaches in the near future.

There is an urgent need to create an interface between biochemists, whose studies on the role of biomacromolecular complexes are still hampered by the tediousness and labour-intensity of the analytical procedures applied, and analytical chemists, ready to demonstrate the usefulness of their newly developed instrumental techniques. The availability of standards and certified reference materials would permit the validation of hyphenated techniques against the classical methods, which would facilitate their entry into biochemical and clinical laboratories.

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13 Appendix

List of abbreviations

AE	anion-exchange
AES	atomic emission spectrometry
ASV	anodic stripping voltammetry
CE	cation-exchange
CEC	capillary electrochromatography
CID	collision-induced dissociation
CZE	capillary zone electrophoresis
DDT	dithiothreitol
DMAA	dimethylarsonic acid
EC	electrochemical (detection)
ES	electrospray
ET	electrothermal
ETV	electrothermal vaporization
FAAS	flame atomic absorption spectrometry
FAB	fast atom bombardment
GC	gas chromatography
GE	gel electrophoresis
HG	hydride generation
HHPN	hydraulic high-pressure nebulization
HPLC	high-performance liquid chromatography

HR	high resolution
ICP	inductively coupled plasma
IE	ion exchange
IEF	isoelectric focusing
INAA	instrumental neutron activation analysis
LC	liquid chromatography
MALDI	matrix assisted laser desorption/ionization
MMAA	monomethylarsonic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MT	metallothionein
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PC	phytochelatin
PIXE	proton induced X-ray emission
QF	quartz furnace
RAD	radioactivity (detection)
REE	rare earth element
TOF	time-of-flight
SDS	sodium dodecyl sulfonate
SE	size-exclusion
SEC	size-exclusion chromatography
TLC	thin-layer chromatography
TXRF	total-reflection X-ray fluorescence
UVA	ultraviolet assisted
XRF	X-ray fluorescence

14 References

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