Abstract

It is known that while many elements are considered essential to human health, many others can be toxic. However, because the intake, accumulation, transport, storage and interaction of these different metals and metalloids in nature is strongly influenced by their specific elemental form, complete characterization of the element is essential when assessing its benefits and/or risk. Consequently, interest has grown rapidly in determining oxidation state, chemical ligand association, and complex forms of a many different elements. Elemental speciation, or the analyses that lead to determining the distribution of an element’s particular chemical species in a sample, typically involves the coupling of a separation technique and an element specific detector. A large number of methods have been developed which utilize a multitude of different separation mechanisms and detection instruments. Yet, because of its versatility, robustness, sensitivity and multi-elemental capabilities, the coupling of liquid chromatography to inductively coupled plasma mass spectrometry (LC–ICP–MS) has become one of the most popular techniques for elemental speciation studies. This review focuses on the basic principles of LC–ICP–MS, its historical development and the many ways in which this technique can be applied. Different liquid chromatography separations are discussed as well as the factors that must be considered when coupling each to ICP–MS. Recent applications of LC–ICP–MS to the speciation of environmental, biological and clinical samples are also presented. © 2003 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Inductively coupled plasma mass spectrometry; Elemental speciation; Detectors, LC

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1. Introduction: the need for elemental speciation

It is well known that many elements are involved in the biochemical processes that take place in nature (essential elements) and that some others are considered toxic. However, neither the identification of these isolated elements nor the determination of their concentrations allow for classification in either category. The intake, accumulation, transport, storage and interaction of the different metals and metalloids in nature are strongly influenced by their specific elemental form. Therefore, the complete characterization of a metal in a biochemical system requires the elucidation of properties such as its oxidation state, associated organic ligands and complex forms. Considering these points, elemental speciation has been defined as the analyses that lead to determining the distribution of an element’s particular chemical species in a sample [1].

The instrumentation necessary to perform speciation studies has often been utilized with complementary techniques. These involve the coupling a selective separation with sensitive and element-specific detection. Many different combinations (separation + element-selective detection) have been attempted but high-performance liquid chromatography (HPLC) in conjunction with inductively coupled plasma–mass spectrometry (ICP–MS) has emerged as one of the best combinations. HPLC is a versatile technique, which due to the variety of separation mechanisms developed, can be applied to a great variety of environmentally and biologically important analytes.

Also, ICP–MS is currently the most sensitive and robust commercially available elemental detector and coupling these techniques (HPLC and ICP–MS) is straightforward.

Numerous publications and reviews [2–4] can be found regarding the use of HPLC–ICP–MS. This manuscript addresses the chronological development of the technique rather than a comprehensive summary of all the publications. The different separation mechanisms available in HPLC will be reviewed regarding its suitability for ICP–MS detection, and some applications will be described.

1.1. Historical development of coupling of liquid chromatography with elemental detectors: FAAS, ETAAS, ICP–AES

Initial studies carried out by Suzuki [5] and Van Loon [6] opened the door to speciation by coupling a chromatographic separation and a sensitive element-specific detector. During the 1970s, atomic absorption spectrometry (AAS) using the flame as an atomization source (FAAS) was widely used as a reliable and robust technique to perform total elemental analysis at low concentration levels (detection limits on the high ppb range for most elements). Also, liquid chromatography (LC), in its different modes, was a mature technique by the end of the 1970s, making, the coupling of LC to FAAS a natural first approach for speciation studies [5]. The coupling was simple in terms of instrumentation by directly connecting the exit of the HPLC column to
the nebulizer of the AAS system. Unfortunately, the detection limits were not low enough for most applications and, therefore, most research efforts were focused on improving sample introduction efficiency (e.g. in situ preconcentration [7], on-line hydride generation [8,9], etc.).

Electrothermal atomization atomic absorption spectrometry (ETAAS) overcame the poor detection levels of FAAS. However, ETAAS is not compatible with the continuous flow sample introduction of LC. Although some papers were published in the field of HPLC using ETAAS as detection [10], it was never accepted for speciation studies.

The development of optical emission spectrometry (OES) using the ICP and the microwave induced plasma (MIP) as atomization sources provided two primary advantages over existing elemental detectors: better detection limits (but still in the ppb range) and multi-elemental capabilities. Microwave induced plasmas (MIP–OES) proved to be suitable for coupling to gas chromatography (GC) [11,12], and in the case of HPLC, good results were obtained when coupled to ICP–OES. In addition to the above mentioned advantages, the wide dynamic range of over five orders of magnitude gave way to a number of applications [2]. Still, HPLC–ICP–OES suffered from poor sample transport efficiency (typically 1–5%) and band broadening under certain conditions. In order to overcome such limitations, different nebulizers were developed (e.g. ultrasonic [2]) as well as techniques to generate volatile elemental species [13].

1.2. Commercial introduction of ICP–MS and interfacing to LC

As stated previously, the main limitation of HPLC–FAAS and HPLC–ICP–OES was the lack of sensitivity for analysis of samples of environmental and biological interest. The elements, normally present in nature at trace or ultra-trace levels, are divided among several species of which their contribution to the total elemental weight is minimal. Therefore, when inductively coupled plasma mass spectrometry (ICP–MS) was introduced during the mid-1980s, it offered new horizons for the world of trace elemental analysis. ICP–MS offers the capability of speciation with multi-element detection, of isotope measurements to improve precision and accuracy, excellent sensitivity and detection limits and a wide dynamic range. In coupling with HPLC, the same advantages as those described with ICP–OES are seen: straight-forward connection of the nebulizer to the exit of the HPLC column, compatible liquid flows and ppt detection limits in time resolved analysis mode [14].

However, there are compatibility limitations arising from mobile phase compositions. The presence of organic solvents, salts and buffers in some of the mobile phases employed can produce clogging of the cones and/or changes in sensitivity. The limitations of every type of chromatography relative to the use of ICP–MS as a detector will be described in detail in the next section. The use of micro-HPLC in combination with micro-flow nebulizers is a very attractive approach explored in recent publications, although in this case, the connection between column and nebulizer is not as straightforward [15].

The first paper on the application of ICP–MS as an on-line multi-elemental detector for HPLC was published by Houk et al. [16] in 1986. Since then, the number of publications in the field has been increasing exponentially. For example, in 1989, five papers were published combining a variety of separation techniques for the separation of As [17,18], Sn [19,20] and Cd species [21]. In 1994, the number of published papers increased to 25 and today, the total number of published papers is higher than 100 per year in biological and environmental areas. Some notable applications will be detailed in further sections of the manuscript.

Interfacing HPLC to ICP–MS is strongly dependent on the type of nebulizer employed as the sample introduction device. Most applications make use of the concentric nebulizer (using 1 ml/min for regular bore HPLC flow) connecting the outlet of the column to the liquid sample inlet using a length of inert polymeric or stainless steel tubing. The length of the tubing has to be minimized to avoid peak broadening. The development of micro-nebulizers (e.g. DIN, HEN, DIHEN, Micro Mist, PFA, etc.) has increased the use of narrow bore columns minimizing the mobile phase introduced into the ICP–MS (see Fig. 1 taken from Ref. [22]). Up to 100% transport efficiency can be obtained with some these designs [23] operating at 30–100 μl/min with minimum memory effects and fast wash-out times [1]. The main feature of these nebulizers is that they make possible the introduction of mobile phases that are
typically considered incompatible with ICP–MS, such as those containing acetonitrile, yet may be useful in performing certain separations [24].

2. ICP–MS as detector for HPLC: advantages and limitations

The feasibility of coupling HPLC–ICP–MS is mainly affected by the composition and flow of the mobile phases used to perform the chromatographic separation. This section describes the advantages and limitations of some of the most conventionally employed chromatographic methods to isolate organometallic species: reversed-phase (RP), reversed-phase–ion pairing (RP–IP), size exclusion (SEC), ion-exchange (IEC) and chiral chromatography (CC). Since a comprehensive review [25] of all these techniques has been recently published, more critical comments regarding the use of each separation method with ICP–MS as the detector will be described in this section.

2.1. Reversed-phase chromatography (RP)

Reversed-phase (RP) is probably the most commonly used separation mechanism in liquid chromatography and consists of a non-polar stationary phase
(normally octadecyl, C\textsubscript{18} or octyl C\textsubscript{8} chains) bonded to a solid support that is generally microparticulate silica gel (non-polar). The mobile phases are polar and, therefore, the sample compounds are partitioned between the mobile and the stationary phases (this chromatography was initially called partition chromatography). The separation is normally performed using aqueous mobile phases containing different percentages of organic modifiers (e.g., methanol, ethanol, acetonitrile, or THF) to increase the selectivity between species. Solute retention is also influenced by eluent pH, which affects the dissociation level of the analyte and therefore, its partition between the mobile and the stationary phases.

An advantage of using RP for separation of species prior to detection by ICP–MS is the simplicity of the technique. C\textsubscript{18} and C\textsubscript{8} are stable and well-characterized packing materials provided by a number of companies. However, the main limitation of RP-HPLC–ICP–MS is that most organic modifiers are not ICP–MS compatible. In fact, only low percentages of MeOH or EtOH can be run in ICP–MS instruments using conventional sample introduction devices (such as the concentric nebulizer) without seriously compromising the sensitivity. Recently, the use of micronebulizers has permitted the introduction of other organic modifiers, such as acetonitrile in conjunction with microbore columns [15,24]. The use of ICP–MS as a detector limits the application of RP-HPLC, which is otherwise the most popular separation mode, due to its ability to separate a broad range of compounds with different polarities simply by changing the eluent conditions. The number of applications for protein analysis by RP-ICP–MS is increasing due to the excellent resolution of the technique, enabling the differentiation of species that differ by a single amino acid. Fig. 2 shows the separation of Se-methionine and Se-ethionine diastereomers (previous derivatization) by RP-HPLC–ICP–MS (isocratic run 50% MeOH with 1:1 on-line dilution [26]) as an example.

2.2. Reversed-phase ion pairing chromatography (RP-IP)

This is a special mode of RP-HPLC used for the separation of ionic or ionizable compounds for which an ion-pair is formed between the solute ion and an appropriate ion of the opposite charge (the counter ion present in the mobile phase). The resulting ion-pair is hydrophobic and it is partitioned between the mobile and the stationary phases (it makes use of the same columns, C\textsubscript{18} or C\textsubscript{8}), and therefore, the separation is based on the “ion-pairing” polarity. Mobile phases similar to the ones used in RP-HPLC are employed (water–methanol and water–acetonitrile) but with the addition of an ion-pairing reagent. The ion-pairing reagent is a compound with a polar head and a non-polar tail. Tetraalkylammonium salts and alkylsulfonates are the most commonly used [27].

The principal advantage of RP-IP is the versatility. It permits the analysis of charged and uncharged compounds in a single chromatographic run with great reproducibility and short analysis time. Variations in the size or concentration of the counter ion in the mobile phase allow for the optimization of selectivity in complex mixtures [1] without increasing the percentage of organic modifier present, which is always desirable for ICP–MS detection.

Perfluorinated ion pairing reagents (e.g. TFA, HFBA, etc.) have been widely employed in recent publications [28–30], since they provide excellent chromatographic separation capabilities for Se amino acids. The only limitation of these compounds is the possible degradation of the silica-based columns that can occur (1% TFA results in a pH <2 which can
decrease the lifetime of the column). Fig. 3 shows the change in resolution that can be achieved by varying the perfluorinated ion-pairing reagent for some Se species [31] and maintaining the same MeOH–water ratio.

2.3. Size exclusion chromatography (SEC)

Size exclusion chromatography (SEC), also known as gel permeation chromatography, is used to separate molecules according to their effective size in solution using a stationary phase with pores of a particular dimension. The analyte molecules that are too large to enter the pores of the stationary phase are completely excluded and elute first, while smaller molecules interact more with the stationary phase and elute according to their effective size. This mechanism is especially useful for the separation of fairly large proteins and polypeptides. Smaller species, especially ions with a high charge-to-mass ratio, can get involved in adsorption and ion-exchange effects as secondary separation mechanisms, which can complicate the separation principle. The small number of theoretical plates in SEC leads to relatively poor resolution so each separated fraction may still contain hundreds of compounds. In most cases, further separation by complementary chromatographic techniques is necessary.

The mobile phases utilized for SEC are normally buffered solutions to ensure minimum competition with the stationary phase gel. The wide variety of buffers reported in the literature make it relatively easy to choose one compatible with the detection technique employed [32]. The main advantage of SEC is the correlation that can be established between retention time and molecular mass, which can be used to predict the type of association between the metal and the molecules present in the sample. This property, still largely unexploited, may be of great value when identifying unknown species.

The only limitation of SEC for elemental speciation is due to the high saline content (e.g. NaCl) of some of the mobile phases utilized, which can cause clogging of the cones in the ICP-MS. Alternative mobile phases can be used, as mentioned before, in order to eliminate these problems.

2.4. Ion-exchange chromatography (IEC)

Ion exchange is based on the interactions of charged analytes (anions or cations) with the charged (positively or negatively) functional groups of the stationary phase. When positively charged analytes react with negatively charged sites in the column, it is referred to as cation-exchange, and when negatively charged analytes interact with positively charged sites, it is anion-exchange. Both mechanisms are highly controlled by the pH of the mobile phases because it affects the dissociation of weakly acidic or basic compounds. Packing materials in ion-exchange columns are beads of crosslinked styrene and divinylbenzene. Ionic functional groups are bonded to this rigid structure: typically sulfonic and carboxylic

![Fig. 3. Different resolution between selenium species depending on the perfluorinated ion pairing reagent: (a) heptafluorobutyric acid (10 mM) and (b) nanofluoropentanoic (2 mM). Selenium species separated: trimethyl selenonium (TMSe), Se-methyl Se-methionine (MetSeMet), methyl Se-cysteine (MSeCys) and selenogammaaminobutyric acid (SeGaba). Taken from Ref. [31] with permission of the Royal Society of Chemistry.](image-url)
acid groups as strong and weak cation-exchangers, respectively. Quaternary amines or primary amine groups are commonly used in anion-exchange columns [27]. The mobile phases employed normally consist of an aqueous salt buffer solution, often mixed with a certain amount of organic modifier, such as methanol or acetonitrile. However, most separations carried out using IEC involve the use of gradients to increase ionic strength during the chromatographic run. Gradients allow the separation of complex mixtures but they present two major drawbacks when used in HPLC–ICP–MS separations: they are time consuming (once the run is finished, the column needs to equilibrate with the new mobile phase) and the changes in the composition of the mobile phases during the run may lead to changes in the background or sensitivity. Additionally, high saline concentrations are undesirable for an adequate ICP–MS performance as described in previous sections. Fig. 4 shows an interesting separation of Se amino acids and selenonium species using cation-exchange [33] for the separation mode and pyridinium formate gradient as mobile phase (pH 3).

2.5. Chiral chromatography (CC)

Chromatographic separation of optical isomers or enantiomers has become a routine practice in the pharmaceutical industry in the last decade. However, very few examples can be found in the literature regarding the use of ICP–MS detection utilizing this separation principle. Separation of racemates can be done by direct chromatographic methods (including the presence of a chiral selector in either the mobile or the stationary phase) and by derivatization of the enantiomers to form diastereomers. Chiral separations using chiral stationary phases are based on the formation of transient diastereomeric complexes between the enantiomers and the chiral ligand of the stationary phases. Successful coupling of a crown ether column to ICP–MS for the speciation of L,D Se-methionine in Se nutritional supplements has recently been carried out, demonstrating the potential of this methodology [34]. Similarly, Sanz-Medel et al. [35] performed a similar separation with an antibiotics-based HPLC column and ICP–MS detection.

3. Applications of HPLC–ICP–MS

Several classifications have been developed to define sub-groups in which speciation might be compartmentalized [32,36]. In this work, three main categories have been considered in order to summarize the most recent and significant applications of HPLC–ICP–MS: environmental, biological and clinical applications.

3.1. Environmental applications

3.1.1. Water analysis

LC–ICP–MS has long been used as a tool to speciate potentially toxic substances in environmental samples. Water has been a very common sample for LC–ICP–MS analysis. Not only are many toxins often suspected of being in polluted waterways with potential toxicity to the general public, but such samples are easy to obtain and usually require little preparation.

Mercury is one of the potentially toxic metals most commonly speciated in water samples by LC–
ICP–MS. While techniques such as cold vapor generation flame atomic absorption spectrometry (CVFAAS) and inductively coupled plasma optical emission spectrometry (ICP–OES) have been coupled to LC for mercury speciation, neither method was capable of producing sufficiently low detection limits, but LC–ICP–MS is capable of speciating mercury at the trace levels expected in water samples and has subsequently become a very popular method for doing so.

LC–ICP–MS has been known to yield detection limits of 7 ppm for inorganic mercury and 16 ppm for both ethyl and methyl mercury using a reversed-phase chromatographic column and ion pairing mobile phase and a conventional pneumatic nebulizer [37,38]. Yet, mercury concentrations in water samples may be at significantly lower levels, albeit potentially toxic levels. In fact, unpolluted water samples are expected to contain less than 20 ng/l of mercury. Similarly, many other elements of interest are also present in water samples at concentrations below their LC–ICP–MS detection limits. Therefore, a preconcentration step may need to be employed before analyzing water samples for certain elements.

Preconcentration of water samples can be conducted prior to separation by anion-exchange chromatography merely by employing a guard column of the same packing material. Passing the water sample through the column causes the analyte of interest to become fixed to the column. Cai et al. [39] found that selenium can effectively be retained from up to 100 ml of water. Once the preconcentration was complete, the introduction of an 80 mM (NH₄)₂CO₃–80 mM NH₄HCO₃ mobile phase at a flow-rate of 0.8 ml/min was sufficient to elute the retained selenium species. Complete peak resolution was achieved in less than 4 min by this method. However, it is important to note that this preconcentration step can only be done with ion-exchange chromatography. When using reversed-phase chromatography with an ion-pairing reagent, different mechanisms are involved and the analyte of interest will not be retained on a C₈ or C₁₈ column.

Since reversed-phase ion pair chromatography is the separation technique most commonly applied to water samples, a compatible preconcentration step is desirable. This is easily accomplished by using a guard column packed with a material other than the stationary phase that will allow analytes of interest to be temporarily retained. A slurry of commercially available resin, such as dithiocarbamate resin for mercury speciation, has been added into a guard column, rinsed with water and then rinsed with mobile phase before introducing the sample. Bloxham et al. [40] found that using this technique with a flow-rate of 9 ml/min allowed 1000 ml of sample to be concentrated on the column in less than 2 h. The concentrate can be eluted with 0.15% (v/v) 2-mercaptopethanol and speciated using a C₁₈ column and ion-pairing reagent. Bloxham et al. [40] reports using a Spherisorb S5 ODS2 column with a 0.06-M ammonium acetate, 1.0% (v/v) acetonitrile and 0.005% (v/v) 2-mercaptopethanol mobile phase at a flow of 1.0 ml/min to achieve the separation of methylmercury, ethylmercury, and inorganic mercury in about 13 min. This technique allowed detection limits of 16 ng/l for methylmercury and 17 ng/l of inorganic mercury to be obtained for the seawater samples.

Other LC–ICP–MS techniques have been employed in which better sensitivity is attained and no preconcentration step is necessary. The use of an ultrasonic nebulizer has been shown to give detection limits of 0.4–0.8 ppb mercury [41], which is 10 times better than the detection limits obtained by a conventional nebulizer. The ultrasonic nebulizer allows for the introduction of a larger amount of sample to the plasma as opposed to the 1–3% introduced by conventional nebulizers. This minimizes band broadening caused by large dead volumes. The above detection limits were obtained by Huang and Jiang [42] who, like Bloxham et al. [40], used a C₁₈ reversed-phase column and 0.06 M ammonium acetate as the ion-pairing reagent. Resolution of the mercury species was still seen in less than 15 min, but the use of an ultrasonic nebulizer avoided the need for a 2-h preconcentration step.

Cold vapor generation can also be used in conjunction with LC–ICP–MS for trace analysis of mercury and arsenic in water samples [43–45] as it is able to greatly increase the signal. Wan et al. [45] have used an in situ nebulizer/vapor generator to analyze the mercury species present in seawater and tap water. Again, separation of inorganic, methyl, and ethyl mercury species was conducted using a reversed-phase C₁₈ column. However, 0.5% (m/v)
l-cysteine was used as the ion pairing reagent. This mobile phase permitted the use of 0.1% NaBH₄, and no acid to generate the necessary vapor, and allowed for complete separation of the mercury species in about 6 min. Such a cold vapor method can produce 8–36% signal increase over conventional nebulization with detection limits on the order of 0.04, 0.03 and 0.11 ng/ml for ethylmercury, methylmercury, and inorganic mercury, respectively.

In the same manner, hydride generation can be used with LC–ICP–MS to increase the sensitivity of various other elements, including arsenic, selenium, bismuth, and antimony. Arsenic speciation is of particular interest in environmental samples and has often been conducted by this method [9]. Inorganic arsenic in the form of As(III) and As(V) as well as monomethylarsonic acid (MMAA) and dimethylarsonic acid (DMAA) are often separated in water samples by anion-exchange chromatography [46]. Also, ion-pairing reversed-phase LC using tetra-n-butylammonium salt has been used to achieve resolution of the four arsenic species in less than 12 min. Detection limits were found to be as low as 0.011, 0.018, 0.029, and 0.051 µg/l for As(III), DMAA, MMAA, and As(V), respectively. LC–ICP–MS using a pneumatic nebulizer had previously generated detection limits of 7 µg/l for As(III) and 3 µg/l each for DMAA, MMAA, and As(V) [47], while 1.0–1.2 µg/l of each arsenic species can be detected using direct injection nebulization [23], so a definite increase in sensitivity can be produced by hydride generation.

3.1.2. Soils

Speciation of trace elements in other environmental samples, such as soils, by LC–ICP–MS can be more challenging as this technique requires samples to be in solution. Sample preparation, therefore, is of high significance. The need to extract several species as well as prevent any species interconversions that may occur in the process are very important. This is true with any sample, but soil samples can be especially difficult due to the complexity of the matrix and variation from sample to sample. A number of extraction procedures have subsequently been developed in order to bring trace metal species from the soil into solution, allowing for LC–ICP–MS analysis.

By performing various extractions at different pH values, Bissen and Frimmel [48] found that higher pH values allow more arsenic to be extracted from soil samples. However, drastic pH values, namely 1 and 12, result in the interconversion of As(III) to As(V) [49]. Adding 0.3 M sodium carbonate at pH 11 to soil samples and agitating at room temperature extracted the greatest amount of arsenic from the soil. However, this extraction technique only allows the removal of mobile arsenic species from the soil, which were solely inorganic and found to account for only 0.04–20% of the total arsenic in the soil.

While most water samples utilize ion-pair reversed-phase chromatography for speciation, ion chromatography has been shown to generate better results for soil extracts [17,50]. Speciation of the extracts obtained by Bissen and Frimmel [48] was conducted by anion-exchange chromatography with a 50 mM NaOH mobile phase. A flow of 1.0 ml/min with this system was sufficient to separate As(III), As(V), MMA, and DMA within 7 min.

Vassileva et al. [51] employed a similar procedure in their analysis of arsenic in soil samples. However, they found that adding 5% (w/v) methanol to the NaOH mobile phase aided in the nebulization of the sample, allowing it to be more efficiently transported into the plasma. Consequently, sensitivities for the five arsenic species studied increased by a factor of two. Detection limits ranged from 0.46 to 0.81 µg/l for As(III), As(V), MMA, DMA and arsenobetaine, but separation required just over 10 min. One of the simplest extraction processes applied to soil is a single step microwave extraction procedure. Phosphoric acid was used to extract arsenic species from freeze-dried, crushed, and filtered soil, sand and sediment samples [52]. Since such an extraction can remove both the mobile arsenic species and the species more strongly bound to soil constituents into solution for analysis, total recoveries range from 60 to 80%. Separation of these extracts with an anion-exchange column has given way to the separation of As(III), DMA, MMA, and As(V) in about 13 min using a gradient elution of phosphate buffer and methanol mobile phase at a flow-rate of 1.0 ml/min [52]. Detection limits for this method range from 1 to 2 mg/kg for all species, although only inorganic arsenic species were seen in the extracts. Using the anion-exchange column instead of an ion-pair re-
versed-phase column allows the use of mobile phases with low buffer concentrations such as the one employed in this work. These mobile phases are thought to be less susceptible to matrix interferences and minimize salt deposition on the ICP–MS sampling cones.

In order to completely speciate soil samples, multiple extractions, each of which targets differently bound forms of the analyte, should be conducted. A sequential extraction process was developed by Ponce de Leon et al. [53] for the speciation of selenium in soils. A water extraction was able to remove only non-adsorbed selenium from the soil whereas other reagents were used to extract selenium species that were more tightly bound to soil constituents. Phosphate buffer at pH 7 displaces absorbed or exchangeable selenium species; tetramethylammonium hydroxide can be used as an alkaline extractant; and sodium sulfite allows for the removal of elemental selenium. Conducting successive water, buffer, base, and sulfite extractions was reported to result in roughly a 90% recovery of the total selenium from the soil. Speciation of all fractions can be accomplished in roughly 25 min with an anion-exchange column and 6 mM salicylic acid–sodium salicylate mobile phase at a flow-rate of 0.7 ml/min.

A further consideration in the speciation of soil by LC–ICP–MS is the choice of nebulizer. A microconcentric nebulizer (MCN) is designed specifically for the analysis of samples with high mineral acid concentrations or that have high levels of dissolved solids and other inorganic materials. Such a nebulizer has been used in conjunction with an anion-exchange microbore column and low flow-rates to simultaneously speciate the redox species of water soluble arsenic and selenium in soil samples [54]. Woller [54] reports using an ammonium malonate mobile phase at low flow-rates for optimal resolution of the arsenic and selenium redox species. Limits of detection for this LC–ICP–MS system were calculated to be 1 and 4 ng/ml for arsenic and selenium species, respectively.

3.1.3. Other samples

Geological samples can be analyzed for trace elements by LC–ICP–MS. Such samples can be decomposed by alkaline fusion, which involves powdering the rock and fusing it in Pt crucibles with LiBO₂ to form a glass. The glass can be dissolved in a mixture of 1 M nitric acid 0.5% (w/v) hydrogen peroxide and 10% (v/v) glycerol and then diluted with water. Carignan et al. [55] used this technique in their analysis of rock samples for uranium, thorium and a multitude of other rare earth elements (REE) by low pressure liquid chromatography and a flow injection ICP–MS. A column 0.7 cm in diameter and 4 cm long was used in the analysis of REE but a column of 0.2 cm diameter and 1.5 cm length was used for U and Th. Both were packed with El Chrom Thru Spec® resin. The sample was acidified with nitric acid to a concentration of 2 M before being injected onto the column. This allowed the U, Th, and REE to remain fixed on the resin while other elements in the matrix elute out. These elements of interest can then be eluted by the addition of ammonium oxalate. Total analysis takes between 10 and 15 min per sample and detection limits varied from 0.8 to 10 ng/g for the various elements.

3.2. Biological materials

3.2.1. Food and nutritional supplements

Many trace element species can be nutritionally significant while others can have adverse effects on animals and humans. As a result, an important application of LC–ICP–MS has been the speciation of these trace elements in various food substances. For example, the arsenic content in apples has been investigated. Apples have been known to contain up to 64 μg/kg of arsenic. But while As(III) and As(V) are carcinogens, arsenobetaine (AsB) is relatively nontoxic, making it necessary to speciate the arsenic in apples in order to adequately assess the threat to human health. Arsenic species have been extracted from freeze-dried apple samples by a two step process [56]. The freeze-dried sample is first treated with α-amylase in 0.1 M ammonium carbonate at a pH of 7.2 and mechanically shaken at 37°C overnight. Next, 40:60 acetonitrile–water was added and the solution was sonicated for 6 h. Before conducting HPLC–ICP–MS, these extracts were evaporated to 4 ml and then diluted to 10 ml with ultrapure water. Extraction efficiencies for this method range from 79 to 117%. The chromatography that was used to evaluate these apple samples makes use of an anion-
exchange column and 10 mM phosphate:10 mM nitrate mobile phase with a hydride generation system. Separation of four arsenic species can be achieved in less than 9 min, as seen in Fig. 5, with the detection limits being reported as 0.6 µg/kg for As(III), DMAA and MMAA and 1.2 µg/kg for As(V).

Arsenic speciation in carrots has also been investigated [57] by accelerated solvent extraction. Vela et al. [57] obtained optimal results when water was used as the solvent and the following conditions were employed: 1500 p.s.i. pressure, 1 min. static extraction time, 5 min heat time, 100°C extraction temperature, 90 s purge time, and 100% flush. The amount of sample used with this procedure ranged from 0.25 to 1.25 g. Sample amounts greater than 1.25 g can also be used with this accelerated solvent extraction in conjunction with a dispersing agent such as acid washed Ottawa sand. The freeze-dried carrots will absorb the water and swell. The dispersing agent causes the formation of channels, allowing water to better flow through the sample and improve extraction efficiency. However, when sample weights below 1.25 g are being used, no benefit is seen from the addition of dispersing agent.

The arsenic in these carrot extracts was speciated with an anion-exchange column and 10 mM ammonium carbonate mobile phase at pH 10.0. Limits of detection were found to be 0.15, 0.11, 0.13, 0.24 and 0.14 ng/ml for As(III), As(V), MMA, DMA and AsB with separation being achieved in just over 5 min. Each of these species were found to be present in carrots at a level of roughly 100 ng/ml when the 68–88% water content of fresh carrots is included in the dilution factor.

As with apples and carrots, the arsenic in mushrooms has also been speciated [58,59]. Bioavailable arsenic can be extracted with water and either sonication [59] or microwave extraction [58]. Larsen et al. [58] found that the best extraction efficiencies were obtained by conducting a microwave assisted extraction using four 8-min treatments at 75 W. The arsenic species in these extracts were separated on a strong anion-exchange column with a 45 mM ammonium carbonate mobile phase in a 97:3 water–methanol solution at a pH of 10.3. Separation of DMA, As(III), MMA and As(V) from cationic species is achieved in less than 13 min when a flow-rate of 1.0 ml/min. Cationic arsenic species including 2-dimethylarsinylacetic acid, arsenobetaine (AsB), arsenocholine (AsC) and trimethylarsine oxide (TMAO) were separated in roughly 10 min with a strong cation-exchange column and 5 mM pyridinium formate mobile phase in the same 97:3 water–methanol solution at a pH of 2.7.

Separation of all these arsenic species, however, can be achieved in only one run with an anion-exchange column. In their work with mushrooms, Londesborough et al. [59] used a nitric acid elution gradient and 0.05 mM benzenedisulfonic acid as an eluent modifier. This particular modifier is di-anionic and forms ion pairs with cationic species, giving them a negative charge and allowing them to separate along with the anionic arsenic species on the anion-exchange column.

Selenium is another element that is frequently investigated in food substances. The selenium species present in nuts have been investigated by Kannamkumarath et al. [60] using both size exclusion chromatography and ion pair reversed-phase chromatography. Brazil nuts, walnuts, cashews and pecans were enzymatically hydrolyzed for HPLC–ICP–MS analysis with proteinase K. Speciation of these extracts followed on a C₈ column with 95:5 5 mM citric acid NaOH, 5 mM hexanesulfonic acid–methanol at pH 4.5 [61].

Onions and garlic have both been found to be particularly high in selenium, so much speciation work has been done on these [28,29,62–64]. The preparation of garlic samples is relatively uncomplicated as 85–95% of the selenium is water soluble. Simple hot water extractions can be conducted once garlic samples have been peeled, cleaned, sliced,
freeze dried and finely ground, but enzymatic digestions have also been reported [28].

Speciation of these extracts has been done with a multitude of different chromatographic systems. Bird et al. [65] have compared the benefits and disadvantages of many of these systems. Their work indicated that the use of a reversed-phase column and ion-pairing agent produced the best separation of the selenium compounds in yeast. Much of the speciation work done with yeast has consequently been conducted by this separation technique. Bird et al. [65] report an optimal mobile phase composition of 98:2 water–methanol with 0.1% TFA, but others have also been used.

Kotrebai et al. [65] report using a 0.1% heptafluorobutanoic acid (HFBA) or 0.1% trifluoroacetic acid (TFA) in 1% methanol mobile phase and reversed-phase column with a polar modifier between the C₈ and silica base. This method allows over 70 selenium species to be separated in 70 min under isocratic conditions.

McSheehy et al. [66] have conducted two-dimensional liquid chromatography with ICP–MS for their work with garlic. This involves using preparative SEC with 1% (v/v) acetic acid eluent at pH 2.97 and off-line ICP–MS detection. Employing a flow-rate of 0.7 ml/min allows this preparative chromatography to be completed in 3.2 min. Fractions from the SEC can be collected, lyophilized, dissolved in water and injected into a reversed-phase column. Separation on the reversed-phase column has been performed with a 0.3% acetic acid mobile phase of pH 3.0.

Selenium enriched yeast was used in the work of Clark et al. [67], which indicated selenium as an anti-tumor agent. Consequently such yeast has been used in commercially available selenium dietary supplements and speciation of this yeast has become important in order to determine the actual chemopreventive selenium species. In fact, the speciation of selenium enriched yeast by LC–ICP–MS has become a popular area of research [33,34,68,69]. Eight different solid–liquid extraction procedures as well as multiple types of HPLC were evaluated for selenium speciation by Casiot et al. [68]. Experiments were conducted with aqueous solutions, non-proteolytic enzymes, sodium doceylsulfate (SDS) and proteolytic enzymes. Roughly 10% of the selenium species in yeast were found to be water soluble and easily extracted by stirring a solution of 5 ml hot water (85–90°C) and 200 mg yeast for 1 h. Using solutions of 10% methanol in 0.2 M HCl and 30 mM Tris–HCl buffer at pH 7.0 produced similar results when sonicated for 1 h. Selenium that was not protein bound was found to be released from yeast cells upon the use of a non-proteolytic enzyme containing laminarinase, xylanase and cellulose. Yet the majority of selenium species present in yeast, up to 80%, are bound to proteins and must be extracted by using proteolytic enzymes. Protease was used to break peptide bonds and allow selenoamino acids to be extracted from the sample.

A variety of chromatographic techniques also can be used to analyze the selenium species once they have been extracted from the yeast. Casiot et al. [68] reported that SEC was particularly useful in the speciation of such extracts. Using a 3 mM Tris buffer mobile phase at pH 7.0 with a size exclusion column permitted the isocratic separation of small water soluble selenium species while still allowing the high molecular mass fractions to be monitored.

Larsen et al. [33] have used both anion and cation-exchange chromatography in their work with selenium enriched yeast. The inorganic forms of selenium are unretained on a cation-exchange column, but many organically bound species that could be present in yeast are able to be separated by this type of column. A cation-exchange column with a pyridinium formate elution gradient was found capable of sufficiently separating the selenoamino acids Se-methylselenocysteine, Se-allylselenocysteine, Se-propylselenocysteine, selenocystine, selenohomocystine, Se-methylselenomethionine, selenomethionine and selenothionine, as well as the selenium species trimethylselenonium ion and dimethylselenonium propionate ion in 30 min.

Despite the type of chromatography used, selenomethionine is consistently identified as the predominant species in selenium enriched yeast and thus the species associated with chemopreventive activity. Although it is possible that this further reacts with other bioselenium species, which might be important in cancer chemoprevention, it is still the most desirable species to have present in selenium dietary supplements and is often listed on the labels of these supplements as being the major species. Speciation work has been conducted on such supplements to
evaluate their selenomethionine content as these supplements are not regulated and much inconsistency has been seen from brand to brand and tablet to tablet [34]. B’Hymer and Caruso [70] found that selenium species could efficiently be extracted from ground supplement tablets by conducting either a microwave extraction at 95 °C with 2 M HCl or enzymatic digestion with protease K at 37 °C over a period of 20 h. Speciation was then done with a C8 column and 89:10:1 (v/v/v) water–methanol–trifluoroacetic acid mobile phase. With a flow-rate of 1.2 ml/min, inorganic selenium, selenocystine, selenomethionine and selenoethionine can be fully resolved in 25 min. With this method, detection limits of 0.7, 0.5, 1.0 and 2.3 μg/kg, respectively, were obtained for these selenium species.

The speciation of organically bound selenium in biological samples was taken a step further by using chiral chromatography coupled to ICP–MS to distinguish the presence of different selenoamino acid enantiomers [63]. A chiral crown ether column and 0.1 M perchloric acid mobile phase was used to separate Se-homocysteine, Se-lanthionine, Se-cystathionine, Se-cystine, Se-ethionine, γ-glutamyl-Se-methylselenocysteine, Se-adenosylhomocysteine, Se-methylselenocysteine and Se-methionine. Some of the enantiomers were separated, however, this column was very temperature dependent. Lower temperatures usually result in better separation, but at the same time may prevent non-polar amino acids from eluting. A temperature of 26 °C was consequently deemed optimal. The selenoamino acids in onions and garlic as well as selenium enriched yeast have been separated by this method following hot water extractions and enzymatic digestions with pepsin, HCl and NaCl solutions. Fig. 6 shows the separation of the four Se-lanthionine enantiomers by this technique.

3.2.2. Speciation in plants

Recent work with phytoremediation has given rise to an interest in the speciation of plants and an important emerging application for LC–ICP–MS. Phytoremediation, or the use of plants to remove pollutants from the environment, represents an inexpensive, uncomplicated and efficient approach to the treatment of sites with high levels of heavy metals. The contamination of these sites has often been assessed by LC–ICP–MS, as described in Section 3.1. The rate of heavy metal decontamination and accumulation into the phytoremediating plants can be assessed a number of ways, but LC–ICP–MS offers the additional benefit of determining which contaminating species are being removed as well as the species into which they are converted and incorporated into the plant.

As was discussed previously, arsenic represents a major environmental toxin. Pityrogramma calomelanos is a species of fern that has been found capable of accumulating relatively large amounts of arsenic and both the plants and the soils where they grow have been speciated for As [71]. The fern was separated into root, stalk and leaf samples, as is commonly the case in the speciation of plant samples. These samples were washed and rinsed followed by either drying at 50–55 °C or freeze-drying and pulverization to a powder. Arsenic species were extracted from both the plant and soil simply by sonication with water for 15 min. Extraction efficiencies for the soil were very poor (6–12%), but ranged from 22 to 93% for the various plant fractions. Dried and freeze dried samples showed no difference in arsenic content or species. Both anion and cation-exchange chromatography was used to separate the arsenic species, in the fern extractions. The mobile phase used with the anion-exchange column consisted of 20 mM NH4H2PO4 adjusted to a pH of 5.6 with NH3. A column temperature of 40 °C was
required in order to achieve separation of As(III), DMA, methylarsonate (MA), and As(V) in less than 6 min. Cation-exchange chromatography with 20 mM pyridine mobile phase at pH 2.6 was also employed for the detection of cationic arsenic species by ICP–MS, but a negligible amount of such As species in the plant samples was seen.

Mung bean seedlings have also been speciated for arsenic [38]. Rather than phytoremediators, these plants have a potential use as bio-indicators for arsenic contamination in soil and water, meaning that they exhibit physical, visual changes in areas of high pollution. Speciation of these plants has been done with an anion-exchange column with a cation-exchange guard column. This allows the cationic arsenic species, AsC, to be retained while As(III), As(V), DMA and MMA are separated on the anion-exchange column with a 80:20 NaH₂PO₄–Na₂HPO₄ mobile phase at pH 6.0. Subsequently, the mobile phase can be changed and the AsC will elute. However, no AsC was in the mung bean seedlings, so this last step was not carried out in the work done by Van den Broeck et al. [38].

Selenium contamination has also become a major concern in recent years. While not as notorious as arsenic, many forms of selenium can be toxic even at low levels. Elevated levels of selenium can have adverse affects on many plants and wildlife. Brassica juncea is just one species of selenium accumulator and phytoremediator on which speciation work has recently been focused. Brassica juncea, or Indian mustard, has been studied and speciated by Montes-Bayon and co-workers [30,72]. In order to acquire the most accurate information on the selenium species present in the plant, several different extractions using HCl, Tris buffer, protease XIV and proteinase K were tested for optimal extraction efficiencies and minimal interconversion of species [30]. An enzymatic digestion, as discussed previously, is able to remove free or weakly bound selenium compounds in addition to some protein-incorporated selenoamino acids, which may not be extracted with HCl or Tris buffer. Despite the fact that selenoproteins have yet to be detected in plants, proteinase K yielded the highest extraction of selenium species. Speciation of leaves, shoots, stems and roots was conducted with a reversed-phase C₈ column and 10% methanol mobile phase using 1% TFA as an ion pairing agent. By using this LC–ICP–MS method, Montes-Bayon and co-workers [30,72] were able to observe a number of selenium species.

As biologists work to create ever better phytoremediating plants through genetic modification, speciation becomes even more important. The mechanism by which the plants are able to accumulate the pollutant can be elucidated by knowing both the species accumulated and incorporated into the plant. This information is necessary when trying to design such a plant, making LC–ICP–MS an invaluable tool.

Considering the selenium speciation of Brassica juncea, the plant recently has been genetically modified to yield increased selenium assimilation. Selenium speciation of this genetically modified version has been conducted and compared to the wild variety [72,73]. In this work, however, a 0.1 M HCl extraction involving 20 h of shaking at 37 °C was employed for the extraction of selenium species, as proteinase K can sometimes be too aggressive a treatment for certain species. Also, HFBA was used in place of the TFA ion-pairing agent for separation on a C₈ reversed-phase column in order to facilitate further studies by ES–MS.

### 3.2.3. Animal tissues

Investigating the trace elemental species in animal tissues is important since many of these animals together with the food substances described in Section 3.2.1 comprise the human diet. Also, an understanding of speciation in animals is useful in assessing the toxicity and/or benefit of these particular elements while shedding light on biological detoxification and metabolic mechanisms.

Because of their significance in human nutrition and because the waterways in which they live are frequently contaminated, fish and other forms of seafood have been extensively investigated by LC–ICP–MS [74–81]. Sample preparation can be complicated when working with such substances due to their oily nature, but lyophilization and homogenization permit the powdering of samples, facilitating many extraction procedures. Depending on the sample, defatting may be necessary. Ackley et al. [79] followed this technique with a microwave assisted extraction, which is less time consuming than conventional solvent extraction methods, in the speciation of arsenic in steelhead salmon, black tip shark and ocean whitefish. Up to 100% extraction of intact
arsenic species was attained when 80:20 (v/v) methanol–water was used with the application of microwave power for 4 min at 65 °C.

Anion-exchange chromatography can be used to separate the As(III), As(V), DMA, and MMA that may be present in fish samples. However AsB and AsC coelute with the void volume. Given that AsB is commonly the major arsenic species present in fish, Ackley et al. [79] utilized a C₈ reversed-phase column and 25 mM citric acid mobile phase with 10 mM 1-pentanesulfonic acid sodium salt as an ion-pairing agent to separate and detect these species.

A microwave-assisted extraction has also been used for mercury speciation in swordfish [78]. However, the chromatographic mobile phase, 0.05% (m/v) L-cysteine 0.05% (v/v) 2-mercaptoethanol, was also used as the extracting solvent, thus minimizing matrix effects. Two minutes of microwave extraction at 60 °C was found sufficient to extract up to 98% of the total mercury with no detectable decomposition of species. As shown in Fig. 7, separation was conducted with a reversed-phase C₈ column and in situ vapor generator. Except for the addition of 2-mercaptoethanol to the mobile phase, the system used was identical to that used for the mercury speciation of water samples done by Wan et al. [45] (Section 3.1).

Fig. 7. Chromatogram from Ref. [94] (with permission of the Royal Society of Chemistry) which depicts the simultaneous detection of the Fe, Zn, and Cu components in undiluted human serum. The separation was achieved with a strong anion-exchange column and ammonium acetate buffer.

Organotin compounds have also been studied in fish. The extraction of these toxic substances from tuna is complex and has been done with the use of supercritical fluids [75]. Supercritical fluids, which are characterized as having both gas-like and liquid-like properties, have been found to be advantageous in speciation because they are associated with better solvating power and faster extractions. Diethylthiocarbamic acid ammonium salt (DDCA) and pyrrolidinecarbothioic acid ammonium salt (PCA) have been used as complexing agents with CO₂, the most common supercritical fluid, forming chelates with the tin. Modifiers such as water and methanol can also be added and have been found to facilitate interaction with the analyte and minimize matrix effects. They also increase the extraction efficiency, although only to about 50%. Supercritical fluid extracts from tuna have been speciated for organotin compounds with reversed-phase ion pair chromatography. Successful separation of inorganic tin, trimethyltin (TrMT), tributyltin (TrBT) and triphenyltin (TrPT) has been achieved in 12 min using 4 mM sodium pentanesulfonate as the ion pair agent in a 94% methanol–5% water–0.046 M acetic acid–0.012 M ammonium acetate mobile phase at pH 6.0.

The speciation of trace elements in animal tissues other than fish has also been reported. For example, livers and kidneys of rats have been speciated for several elements, especially metallothionein associated elements (Se, Fe, Cu and Zn) [82]. After the organ is removed from the animal, intact proteins are extracted from the tissues by homogenization in 50 mM Tris–HCl buffer. The buffer should be within the physiological pH range of about 7.0 to minimize any changes in the protein prior to analysis. After centrifugation, the extractant can be speciated by LC–ICP–MS. The same Tris–HCl buffer can also be used as the mobile phase for SEC to separate the species of the cytosolic tissue fractions. These proteins were extracted from lyophilized liver samples by adding Tris–HCl buffer and centrifuging.

3.3. Clinical samples

3.3.1. Urine samples

Metabolites of many trace elements are present in bodily fluids and are excreted in urine. These metab-
olites give clues as to the biological function of the trace elements and may help to assess their toxicity or benefit to human health. Therefore, speciation of these samples has gained interest. LC–ICP–MS analysis of urine, in particular, has become widespread [31,83–87]. However, urine samples can be very difficult to work with and a number of techniques have been developed to lessen the difficulties associated with them. One of the problems associated with the analysis of “dirty samples” such as urine and other bodily fluids by LC–ICP–MS is the possibility of protein precipitation on the column. Hydro-organic mobile phases are usually to blame, resulting in plugged columns. To minimize this risk, deproteination of urine samples has been carried out by ultrafiltration on membranes with a 10 000 Dalton molecular mass cutoff prior to analysis [85]. But micellar liquid chromatography (MLC) can also be used, without the need for sample pretreatment.

MLC involves the use of surfactants in aqueous solutions at levels above their critical micelle concentration (CMC) as mobile phases for reversed-phase chromatography. These micelles in the mobile phase dissolve any proteins that may be present, causing them to elute in the void volume rather than precipitating in the column. This technique has been applied to the speciation of arsenic in urine with a boric acid buffer mobile phase containing n-propanol as an organic modifier and cetyltrimethylammonium bromide (CTAB) as the surfactant [88]. Although such a technique is capable of simultaneously separating anionic and cationic species, the resolution of As(III), As(V), MMA and DMA was reported in less than 15 min. Also, the separation of Se-aminoacids and the inorganic species can be done using this separation mechanism [84]. No sample preparation, other than filtration, was required prior to injection onto the column. Many papers, however, do not report protein precipitation as a problem with urine samples and often dilution with de-ionized water is the only form of sample preparation employed even when MLC is not performed. Selenium species, for instance, have been detected in urine diluted 1:1 with de-ionized water using cation-exchange chromatography [89]. The mobile phase consisted of 10 mM oxalic acid, 20 mM potassium sulfate and 2% methanol at pH 3.0. Good separation of Se(IV), Se(VI), TMSel + and SeMet can easily be obtained by this system in just over 5 min, but large interference signals were present during actual sample analysis. By simultaneously monitoring with ICP–MS, sodium was discovered to be the interfering ion. Yet, a number of other ions, including Mg, Al, Ca, Mn, Fe, Ni, Co, Cu and Zn, are also present in urine samples and could possibly interfere with LC–ICP–MS analysis.

Chlorine is another possible interference. It is present at particularly high levels in urine, which can be a major drawback when the sample is being speciated for arsenic. Because ArCl + is a major interference for the As signal, these high levels of chlorine in the sample can drastically limit detection. Dilution, however, can still be a viable form of sample preparation if other measures are taken. Hydride generation, for instance, has frequently been used to minimize chloride interference [90]. Ritsema et al. [87] have developed a chromatographic system that causes chloride to elute after all arsenic species of interest, reducing its signal interference [91]. This system consists of an anion-exchange column and gradient elution of ammonium carbonate buffer. Although all cationic arsenic species co-elute with this system, inorganic species can be completely resolved in less than 8 min with detection limits as low as 0.3 µg/l even for undiluted urine samples.

Human urine has been speciated for other trace elements as well, including lead and mercury. These metals have been simultaneously monitored by ICP–MS and speciated on a microbore C 18 column with a 5 mM ammonium pentanesulfonate mobile phase in 20:80 (v/v) acetonitrile–water [83]. EDTA must be added to the sample to ensure that inorganic lead species do not become permanently retained on the column. This enables Pb(II), trimethyl-lead, triethyllead, Hg(II), methylmercury, ethylmercury and phenyl-mercury to be separated in roughly 6 min. However, no organomercury ions could be detected in the urine samples despite the fact that a DIN combined with a microbore column were used, both of which generally lower detection limits. Consequently, preconcentration may be necessary in such situations.

Recently the major urinary metabolite of Se in rats has been characterized by LC–ICP–MS and ES–MS as Se-methyl-N-acetyl-selenohexosamine by Ogra et
al. [92] using ammonium formate at pH 7.4 as mobile phase.

3.3.2. Blood, breast milk and other bodily fluids

The ability to detect trace elements in bodily fluids not only allows for the elucidation of metabolic and detoxification pathways, but also aids in the identification and characterization of certain proteins. With proteomics becoming a surging area of research, the use of LC–ICP–MS in this manner has become more common. For instance, Al and Fe, both present in blood, bind to serum transferrin. The chemical forms of these coexisting metals have been investigated and with the use of HPLC-HR–ICP–MS preferential binding of Al (as Al-citrate) to the N-lobe of Apo-transferrin was demonstrated [93].

Fe, Cu and Zn have been simultaneously speciated in human serum by HPLC–ICP–MS using on-line isotope dilution [94]. Separation was performed in an anion-exchange column (Mono-Q HR 5/5) with a mobile phase gradient of ammonium acetate at pH 7.4 (Fig. 7). Quantification has been carried out by adding a solution of enriched $^{57}$Fe, $^{65}$Cu and $^{67}$Zn on-line at the exit of the column after protein separation.

Similarly, zinc-binding proteins have been analyzed in human serum with SEC–ICP–MS, but anion-exchange chromatography has also been utilized [95]. Zinc containing components of the serum were separated with a 0.025 M Tris–HCl buffer and 0.25 NaCl buffer gradient within 13 min at a pH of 7.4. This system represents an uncomplicated way to monitor the partitioning of trace elements between various constituents in human blood, especially since the only sample preparation involved was the dilution of serum in the Tris–HCl solution.

Breast milk, like any other bodily fluid, will also contain trace elements. The speciation of these elements is particularly important since breast milk is the sole source of dietary nutrition for infants and can be useful in the design of infant formulas. Speciation of all elements of nutritional interest can be done with LC–ICP–MS. Calcium, potassium, magnesium, phosphorus, sulfur, cobalt, copper, iron, iodine, manganese, molybdenum, selenium, and zinc are just some of the essential elements that have been monitored. As most of these are in some way protein bound, SEC is the best separation technique with a mobile phase such as 0.1 M Tris buffer at a physiological pH [96]. The only necessary sample preparation is centrifugation to remove fat and insoluble components.

3.3.3. Pharmaceuticals

Yet another emerging LC–ICP–MS application has been the analysis of drug substances. As alluded to previously, the metabolism of certain drugs can be screened in bodily fluids. However, LC–ICP–MS can also be used to detect impurities in the drugs themselves. Cimetidine is a sulfur containing drug that has been analyzed by RP-HPLC–ICP–MS with an elution gradient from 0.05 M ammonium acetate to 40% methanol [97]. More than one peak in the chromatogram indicates an impurity when sulfur is monitored. One problem associated with this, however, is the polyatomic interference of $^{16}$O$^{16}$O$^+$ with the major sulfur isotope of $^{32}$S$^+$. High resolution ICP–MS has subsequently been utilized as regulations require that impurities as low as 0.1% of the main component must be characterized.

Cisplatin is a cytotoxic Pt compound used in the treatment of several solid tumors. This compound and its metabolite (monohydrated cisplatin) are responsible for side effects like nephrotoxicity. Therefore, several studies have been conducted on the use of LC–ICP–MS to determine these species in clinical samples such as urine or serum [98].

4. Trend in speciation by LC–ICP–MS: complementary information

4.1. Use of ES–MS for complementary information

One limitation of LC–ICP–MS is that it is sometimes unable to identify the species of a particular element. Speciation by LC–ICP–MS is achieved by the comparison of retention times with a standard. But, when peaks are seen that do not correspond to a standard, the compound cannot be positively identified and further information is needed. electrospray mass spectrometry (ES–MS) has become the most common method of obtaining complementary information in order to identify unknown peaks. ES–MS is a soft ionization technique in which a high voltage is applied to the injected liquid in a capillary
tube. Highly charged droplets are formed which shrink as solvent evaporates, eventually rupturing when the Coulombic forces come close to the cohesion forces. A mass spectrum can thereby be obtained based on the mass to charge ratio of these ions.

ES–MS has been used for elemental speciation [99], but solvent clustering and gas phase reactions can cause spectra to be quite complex. Therefore, separation techniques such as LC are typically done beforehand. Most effectively, LC–ICP–MS is done, the retention times of unknown peaks are noted and fractions are collected by conducting another, offline, chromatographic run. Because of the reduced sensitivity of ES–MS in respect to ICP–MS, preconcentration is often necessary and fractions from multiple chromatographic runs are combined. The mobile phase used to achieve this separation, however, must be taken into consideration when ES–MS is to be used. Some mobile phase components may be incompatible with the electrospray source (e.g. high saline mobile phases). Also, methanol–acetonitrile can be added to the mobile phase in order to facilitate ionization of the fractions by electrospray.

While ICP–MS can only provide elemental information, the mass spectra obtained by ES–MS can provide structural and molecular verification of compounds. First, the molecular mass of the unknown species can be determined by identifying the molecular ion peak in the spectra. The structure can then be established based on the observed fragmentation pattern, which can be interpreted using information obtained from fragmentation studies. Once the structure is predicted, standards can eventually be made and further molecular confirmation can be obtained by sample spiking or matching retention times.

Recently, ES–MS has been employed in conjunction with ICP–MS for the characterization of Se species [32,72] or As species [100] with a great success. LC–ICP–MS is useful to monitor the element and determine fractions to be further pre-concentrated to perform ES–MS.

4.2. Need for reference materials

Elemental speciation by LC–ICP–MS can be highly dependent on matrix effects. Both extraction and separation techniques can be affected by differences in sample composition. Typically, reference materials are used to ensure the effectiveness of these procedures. However reference materials are not always available as the application of LC–ICP–MS continues to expand to more and more sample types. Therefore, in order to ensure the legitimacy of the speciation work being done on these emerging classes of environmental, biological and clinical samples, reference materials need to be created and made readily available.

5. Conclusion

LC separations with ICP–MS detection represent the most popular combination of separation and detection for elemental speciation studies today. LC has multiple separation modes associated with it allowing one or more separations prior to final detection. Multidimensional chromatography, for example with SEC, followed by RP is close to routine prior to ICP–MS detection. LC may also be used as a follow-up from ICP–MS detection by combining with other types of mass spectrometry for molecular/structural information following elemental screening with LC–ICP–MS.

ICP–MS provides ultra-trace elemental detection, which also allows elucidating temporally overlapping chromatographic peaks. With high sensitivities, low detection limits, mass (elemental) selectivity, isotope ratio capabilities, and wide dynamic ranges, ICP–MS is the best detector for elemental speciation at this time. Through about 15 years of development, problems with buffer matrices, organic solvents, time-resolved detection, various interferences, and interfacing have been adequately resolved, if not eliminated so that LC–ICP–MS can now be used routinely for elemental speciation studies of samples ranging from environmental to biological. To these ends more recent studies show high potential for an elemental technique to inform complex molecular systems. ICP–MS detection and ES–MS have great potential working in combination to help unravel the proteomics challenge, especially where metalloproteins or peptides are involved. With commercial instrumentation now available with metal speciation
“packages” as options, years of research have produced the harvest.

References