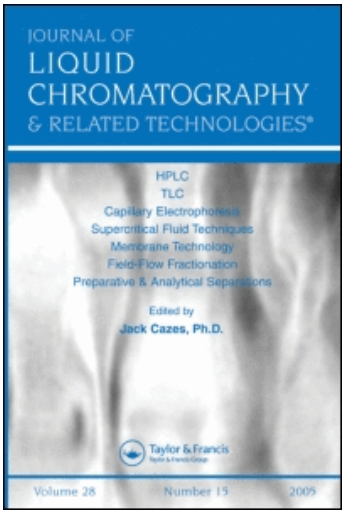

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Liquid Chromatography–Inductively Coupled Plasma Mass Spectrometry (LC–ICP–MS)

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Abstract: The scientific community has realized that the toxicity, bioavailability, bioactivity, transportability in the organism, and the eventual impact of an element on the environment and mankind will be dictated by the particular species or forms of the element, rather than its total concentration. The types of analysis that lead to this information are typically referred to as *speciation*, and involve typically the coupling of a separation instrument with an element specific detector. A large number of publications have been devoted to various separation methods coupled to scores of element-specific detection instruments. Yet, because of the combined virtues of versatility, robustness, sensitivity, and multi-elemental capabilities, the coupling of liquid chromatography to inductively coupled plasma mass spectrometry (LC–ICP–MS) has become the most popular technique for elemental speciation studies. This review will focus on the basics of LC–ICP–MS, its course of development, and its applications in various fields. Liquid chromatography with a variety of separation mechanisms are discussed, as well as the factors that must be considered when coupling each to ICP–MS. Some typical applications of LC–ICP–MS to the speciation of environmental, biological, and clinical samples are also presented.

Keywords: Reviews, LC, Inductively coupled plasma mass spectrometry (ICP–MS), Speciation, Element-specific detectors

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INTRODUCTION

The rapid development of inorganic instrumental analysis after World War II, mainly atomic spectroscopic analysis, enabled the analytical community to look into the role of trace elements in various areas, such as health and environment, geochemistry, and material sciences, to name just a view.

The field of "trace element analysis" enjoyed prompt growth during the three decades following World War II. During this period, it became evident that trace elements play a major role in biological activities, environmental chemistry, or material characteristics. With respect to biological and environmental issues it was also increasingly realized that the distribution, mobility, and biological availability of chemical elements depend not simply on their total concentration normally obtained by one of the trace elemental analysis techniques, but critically, on the chemical and physical forms and/or association which they are in or they undergo in natural systems.

Many elements in the periodic table are thought to be crucial to life, while others to be harmful. Some elements can be either depending on the chemical forms in which they exist. For these elements, the identification and quantification of the total level of elements the analytical chemists used to focus on, do not tell the whole story and thus are not adequate any more in most cases. Since the intake, accumulation, transport, storage, and interaction of these different elements in nature is strongly influenced by their specific chemical form or oxidation state, complete characterization of the element is essential when assessing its benefits and/or risk. As a result, instead of total concentration of an element, scientists are paying more and more attention to the identification and quantification of its oxidation states, ligand associations, and other potential complex forms of the element. This has been strongly supported by the fact that AS^{+3} is much more toxic than arsenobetaine, methylmercury is much more toxic than Hg^{+2}, and tributyltin is a very potent biocide, while Sn^{+4} is not. Generally speaking, elemental speciation has been defined as the analyses that lead to determining the distribution of an element's particular chemical species in a sample.[1,2] During the last 20 years, significant work has been done by academic, industrial, and regulatory communities to identify, rationalize, and promote simple and effective analytical methods and procedures that improve our understanding of metal related issues using speciation analysis. The determination of the chemical form of elements within biomedical, environmental, nutritional, industrial, geochemical, and pharmaceutical samples is essential for the accurate evaluation of their beneficial or toxic effects and, hence, their potential impact on the environment and mankind.

The instrumentation necessary to perform speciation studies has to be one of the hyphenated techniques. This explains the proliferation of standard separation tools as sample introduction devices for inductively coupled plasma mass spectrometry over the last decade. These hyphenated techniques involve the coupling of a selective separation technique (such as gas chromatography (GC), high performance liquid chromatography (HPLC), and

capillary electrophoresis (CE)) with an element-specific detection technique (such as atomic absorption spectrometry (AAS), inductively coupled plasma optical emission spectrometry (ICP–OES), and inductively coupled plasma mass spectrometry (ICP–MS)). Many different hyphenated techniques 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 perhaps the most ubiquitous and versatile separation technique that is coupled to an ICP–MS, principally thanks to the variety of separation mechanisms developed using different mobile and stationary phases. The popularity of this combination can be attributed to a few factors. In the traditional operating mode of an ICP–OES or ICP–MS, sample solutions have to be pumped to a nebulizer where they are turned into fine aerosols, transported to a spray chamber to remove larger aerosol particles, and eventually to the plasma to be ionized. The plasma spectrochemical community has been able to fine tune the spray chamber and nebulizer so that they are extremely robust and flexible and very easy to use. As a result, aqueous liquid sample introduction through the nebulizer and spray chamber is the most preferred and straightforward sample introduction method for both ICP–AES and ICP–MS. In addition, the near universal applicability for the separation of organic, inorganic, and biological analytes regardless of their volatilities is another desirable quality of the HPLC. As such, HPLC systems are much more versatile than the GC methods. Lastly, the eluent flow rate after the separation from a HPLC system (from 0.01 to 2.00 ml/min) is very compatible with the sample introduction system currently used with ICP–OES and ICP–MS instruments. Besides, ICP–MS [3,4] is currently the most sensitive and robust commercially available elemental detector that offers pronounced advantages because of its elemental specificity, wide linear dynamic range, and extremely low detection limits. Coupling these techniques (HPLC and ICP–MS) is straightforward since issues concerning instrument interfacing have mostly been resolved, such that, only solvent compatibility issues remain to be a major concern.

This current review is not meant to be a comprehensive one; it will only present the essence of this hyphenated technique rather than an exhaustive summary of all the publications. The different separation mechanisms available in HPLC will be reviewed regarding their suitability for ICP–MS detection, along with some typical and representative applications.

Liquid Chromatography with an Elemental Detector

Suzuki[5] and Van Loon[6] were the first to couple a chromatographic separation instrument to an element-specific detector. During the 1970s, atomic absorption spectrometry (AAS), using the flame as an atomization source (FAAS), was the only atomic spectroscopic method available for total elemental analysis. High performance liquid chromatography (HPLC) was

already a relatively mature and established technique by the end of the 1970s, thus, making the coupling of a LC to a FAAS a natural choice for speciation studies.[5] The coupling was straightforward, since it is only involved the connection of the eluent of the HPLC column after the separation to the nebulizer of the flame AA system. The downside of this coupling was that the detection limits for the majority of the elements of interest were too high to be useful for most applications and, consequently, most researchers were forced to focus theirs efforts and energies in sample pretreatments, such as preconcentration, and in improving sample introduction efficiencies.[7–9]

The arrival of the graphite furnace (electrothermal vaporization–ETV) atomic absorption spectrometry (GFAAS) changed the landscape and GFAAS improved detection limits for all useful elements dramatically compared to FAAS. There had been published work using HPLC coupled to ETVAAS,[10] the continuously flowing nature of the HPLC eluent, however, is not really compatible with ETVAAS. It was no surprise, that the coupling of HPLC to ETVAAS was never generally accepted by the analytical chemistry community for speciation purposes.

The development of inductively coupled plasma optical emission spectrometry (ICP–OES) was a big step forward for the speciation community. ICP–OES offers several advantages over FAAS and GFAAS: better detection limits for most elements, simultaneous multi-elemental capabilities, and a linear dynamic range of over five orders of magnitudes to boot. ICP–OES has been coupled to HPLC for speciation, and a limited numbers of papers have been published using this combination technique.[11–23] But, ICP–OES was quickly outshone by the advancement of ICP–MS as an element-specific detector for HPLC.

ICP–MS

The main limitation of GFAAS and ICP–OES as an element-specific detector for speciation research was the lack of useful sensitivity for real-life samples. The elements of interest in nature, particularly in a biological system, are normally present at trace or ultratrace levels. To make matters worse, these elements are commonly spread among more than one species, bringing the species of interest to concentrations too low to be detected by GFAA and ICP–OES.

The commercial introduction of inductively coupled plasma mass spectrometry (ICP–MS) during the mid-1980s was a blessing to the analytical chemistry community; it also offered new horizons for the world of speciation. The introduction of samples into the ICP at atmospheric pressure makes it possible to couple various separation techniques to ICP–MS.

The inductively coupled plasma discharge used in ICP–MS is the same as in ICP–OES. Argon is passed through a torch consisting of three concentric quartz tubes, with argon at different flow rates flowing through each tube.

The outer flow has the highest flow rate (15–17 L/min) and is called plasma or coolant gas. Argon flowing through the central tube is called the auxiliary gas, and is used to keep the formed plasma away from the edge of the torch to prevent it from melting. The inner gas flow, commonly known as the nebulizer gas, carries the analyte to the plasma. A copper coil, the load coil, surrounds the top portion of the torch and is connected to a radio frequency (RF) generator. When argon is flowing tangentially through all three tubes of the torch, a spark, normally created by a Tesla coil, produces "seed electrons" which begin to ionize argon: $Ar \rightarrow Ar^{+} + e^{-}$. The RF power typically applied at 0.5 to 2.0 KW and 27 or 40 megahertz (MHz) in the load coil causes RF electric and magnetic fields to be set up and accelerate these electrons. The way of feeding energy through coil to the electrons is known in physics as inductive coupling. These high energy electrons in turn induce further ionization of argon by colliding with other argon atoms. This collisional ionization of the argon gas continues in a chain reaction, breaking down the gas into the plasma consisting of argon atoms, electrons, and argon ions, now forming the so called inductively coupled plasma discharge. The high temperature of this discharge, ranging from 6000 to 10,000 K, results from Ohmic heating (resistance to the moving of charged particles).

The most common way for introducing a sample into the plasma is through a pneumatic nebulizer, which is a device to turn liquid samples into aerosols. As a consequence, all samples have to be turned into liquid through direct dissolution or sample digestion before they can be introduced into the nebulizer. When sample aerosols produced by the nebulizer are introduced into the plasma, they are desolvated (solvent is removed from the sample aerosols), vaporized, atomized, and ionized before entering the mass analyzer. The ions that are generated by the hot plasma are extracted into the low pressure mass spectrometer interface through a sampler and a skimmer cone, which are metal disks with a small hole ($\sim$1 mm) in the center. Once the ions enter the mass spectrometer, they are separated by their mass-to-charge ratio. The most commonly used type of mass spectrometer is the quadrupole mass filter. In a quadrupole mass filter, alternating AC and DC voltages are applied to opposite pairs of the rods. These voltages are then rapidly switched along with an RF-field. The result is that an electrostatic filter is established that only allows ions of a single mass-to-charge ratio (m/z) pass through the rods to the detector at a given instant in time. The quadrupole mass filter is really a sequential filter, with the settings being changed for each specific m/z in rapid succession. The voltages on the rods can be switched at a very rapid rate. The result is that the quadrupole mass filter can separate up to 2400 amu (atomic mass units) per second. This speed is why the quadrupole ICP–MS is often considered to have simultaneous multi-elemental analysis properties.

Typical quadrupole mass spectrometers used in ICP–MS have resolutions between 0.7–1.0 amu. This is sufficient for most routine applications. However,

there are some instances where this resolution is not sufficient to separate overlapping molecular or isobaric interferences (as a result of equal mass isotopes of different elements present in the sample solution) from the elemental isotope of interest.

To achieve higher resolutions and thereby eliminate or reduce isobaric interferences, e.g., $^{40}Ar^{35}Cl^{+}$ interference with $^{75}As^{+}$, double focusing magnetic sector mass analyzers (commonly known as high resolution ICP–MS, or HR–ICP–MS) must be used. In addition to high resolution, superior sensitivity is another virtue of HR–ICP–MS. In this type of instrument, both a magnetic sector and an electric sector are used to separate and focus the ions. The magnetic sector is dispersive with respect to both ion energy and mass, and focuses all the ions with diverging angles of motion coming from the entrance slit of the spectrometer. The electric sector is dispersive only to ion energy and focuses the ions onto the exit slit. Such an arrangement is called a double focusing high resolution mass spectrometer.

The resolution of high resolution instruments can be changed by adjusting the width of the entrance and exit slits into the spectrometer. Typical HR–ICP–MS instruments have resolving powers up to 10,000 and are typically operated at preset resolution settings for low, medium, or high resolution to make their operation easier for the user. The use of HR–ICP–MS solves many, but not all interference problems.

High resolution instruments also have several limitations. They typically cost 2–3 times that of a quadrupole ICP–MS instrument, and they are also more complex and expensive to operate and maintain.

The ICP–MS spectrometer based on time-of-flight (TOF) mass analyzer, although not popular yet and only accounting for about 1% ICP–MS units sold, also has a great potential for speciation analysis due to its capability for extremely rapid data acquisition, thanks to its multi-channel feature.

Interfacing ICP–MS to LC

The primary reason for coupling an ICP–MS to LC and not using the universal ultraviolet (UV) and refractive index detectors typically used with the LC, is that these detectors are not element-specific and their sensitivity is simply too poor to be useful for speciation purposes. For the majority of the elements, ICP–MS affords detection limits of about a thousand times better than UV and refractive index detectors and it only detects the species of a particular element. Coupling ICP–MS with HPLC is relatively straightforward since only a single connection between the LC discharge eluent from the separation column to the ICP–MS nebulizer is needed. Flow rates ranging from 0.1 to 1 mL/min are needed by a regular ICP–MS nebulizer to function properly.

For certain chromatographic separations, some proportions of organic solvents must be used, resulting in some compatibility issues for some mobile phase compositions. The heat from the plasma will turn the carbons

in the organic solvents into black carbon deposit on the sample injector tube of the plasma torch and on and around the sampler and skimmer cones orifices, leading to 'fouling' and decreased performance. With an extended chromatographic separation with a long run time, the orifices on the injector tube and sampler and skimmer cones can be partially or completely clogged, thus resulting in decreased overall sensitivity or loss of signal completely.

Blending a small portion of oxygen in the nebulizer argon flow will mitigate the problem. The oxygen will react with the carbon in the organic solvents to form carbon dioxide, which will be carried out together with other waste plasma gases with the exhaust gas flow. Chilling the spray chamber between 0°C and 5°C will also reduce the amount of organic solvents that reach the plasma and, thus, also minimizing this problem. Desolvators with membranes are also available with up to almost 100% organic solvent removal to address the same issues.[24]

Another pitfall speciation chemists need to watch out for is that organic content in the sample solution might cause signal enhancement for certain elements of high ionization potential, such as As and Se, two of the most commonly specified elements.

The use of smaller bore chromatography columns (smaller than standard 4.6 mm) in combination with microflow nebulizers is an effective way in reducing the amount of organic solvents reaching the plasma, thus minimizing the above mentioned problems that speciation scientists might encounter. However, the interfacing between these smaller columns and nebulizer is not as trivial as with regular columns.[25] The development of micronebulizers, such as direct injection nebulizer (DIN), high efficiency nebulizer (HEN), DIHEN, etc., has made the interfacing an easier job and has increased the use of narrow bore columns, minimizing the mobile phase introduced into the ICP–MS. The typical flow rate of these nebulizers is 25–100 μL/min, which make possible the introduction of mobile phases that are typically considered incompatible with ICP–MS, such as those containing acetonitrile, yet may be indispensable in separating certain species.[26]

Since the first publication on the application of ICP–MS as an element-specific detector for HPLC by Houk et al.[27] in 1986, the number of publications in the field has been increasing steadily.[28–32] The total number of published papers in this field is much more than 100 per year in biological and environmental areas. Some notable publications will be discussed and detailed in further sections of this review.

The Challenges Facing Speciation Scientists

The whole purpose of speciation is to qualitatively and quantitatively determine the original molecular information of the sought particular species. But, it is no easy task. First, it is extremely difficult to preserve the integrity of such species along the whole analytical process, including steps

such as sampling, storage, pretreatment, LC separation, and, finally, the ICP–MS detection. This dictates meticulous sample preparation and storage when not working with species without high thermodynamic stability and kinetic inertness. In this regard, great care should be taken even in LC column selections, since a stationary phase with certain surface modification may alter the nature of the original species. Buffers and organic modifiers might contain chelating agents that can denature the native species through recomplexation. Secondly, the concentration of the element of interest in the real world samples is normally extremely low and becomes much lower when fractionated for speciation. The species in the real world with such low concentration is "buried" in complex matrices, such as soils, urine, serum, animal tissues, etc.

HPLC TECHNIQUES

Reversed-Phase Chromatography (RPC)

RPC is probably the most commonly used separation technique in liquid chromatography, and employs columns with non-polar stationary phase (normally C_{18} or C_8) bonded to a solid support that is generally porous micro-particulate silica gel. The retention mechanism is dependent on compounds with different relative hydrophobicity. These kinds of separation are normally carried out using aqueous mobile phases containing some portions of organic modifiers (e.g., methanol, ethanol, acetonitrile, or THF). With this mechanism, compound retention is also influenced by eluent pH, which affects the dissociation of the metal containing analyte and, therefore, its partition between the mobile and the stationary phases.

One of the major attractions for using RP–HPLC for the separation of species of interest prior to ICP–MS detection is the simplicity of the technique. C_{18} and C_8 columns are stable and well characterized. However, the main limitation of RP-HPLC–ICP–MS is that organic modifiers in the mobile phases are not ICP–MS compatible without additional accessories (such as a desolvation device mentioned earlier) installed or connected to the ICP–MS. Even for MeOH or EtOH, only low percentages of them can be introduced into the ICP–MS instruments using a conventional sample introduction system without extinguishing the plasma and seriously compromising the performance of the instrument.

Reversed-Phase Ion Pairing Chromatography (RPIP)

In the past, chromatographic separation of charged analytes has been achieved by ion suppression (the careful adjustment of the mobile phase pH to result in a nonionized analyte). Determining the optimum mobile phase pH in ion

suppression, however, often requires extensive method development. Samples containing more than one ionizable component were often unusable. The limitations of ion suppression led to the development of a new, more generally applicable approach to separation of ionized components: reversed-phase ion pair chromatography.

RPIP is one particular variation of RP–HPLC for the separation and determination of ionic and ionizable compounds. An ion-paring agent is therefore needed in order to form an ion-pair between the ion-paring agent and the analyte in question. The stationary phase is standard silanised silica packing, such as that used in C_{18} or C_8, and, therefore, the separation is based on the "ion-pairing" polarity. Mobile phases similar to the ones used in RP–HPLC are generally used (water–methanol and water–acetonitrile), but with the addition of an ion-pairing reagent. Tetraalkylammonium salts, triethylalkyl ammonium salts, or anions such as alkylsulfonates are the most commonly used.[33]

RP–IP is an extremely versatile technique. It permits the analysis of charged and uncharged compounds in a single chromatographic run with great reproducibility and reasonably short analysis time. Adjusting the type or concentration of the ion-paring agents in the mobile phase makes the optimization of selectivity in complex mixtures possible, without increasing the percentage of organic modifier present, which is always desirable for ICP–MS detection.

Size Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC), also known as gel permeation or gel filtration chromatography, is used to separate molecules according to their effective sizes in solution using a stationary phase with pores of various dimensions. The analyte molecules that are larger than the pore size of the stationary phase are not retained by the column and thus elute first, whereas molecules of smaller sizes can diffuse into the pores in the stationary phase and are thereby retained to a greater or lesser extent depending on size, thus elute according to their effective size. This mechanism is especially useful for the separation of compounds of high molecular weight, such as proteins and polymers. 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 many cases, further separation by complementary chromatographic techniques is necessary.

The major advantage of SEC is the easy correlation that can be established between retention time and molecular weight. With molecular weight standards for calibration, separation according to molecular weight is possible.

In contrast to other LC methods, the solvent peak always elutes last, since it is always the smallest molecule and consequently most retained with the porous structure of the stationary phase.

The only limitation of SEC in coupling to ICP–MS comes from the high salt content (e.g., NaCl) in some of the mobile phases utilized, which can cause clogging of the nebulizer, injector tube as well as sampler, and skimmer cones in the ICP–MS. Alternative mobile phases can be used, as mentioned before, in order to eliminate these problems.

Ion-Exchange Chromatography (IEC)

Ion exchange is based on the utilization of exchange equilibria between charged analyte ions (anions or cations) and the oppositely charged (positively or negatively) surface of the stationary phase. When positively charged analytes react with negatively charged sites on the stationary phase, 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 dependent upon the pH and the ionic strength of the mobile phase, and the nature of the ion exchanger.[34]

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 acid groups as strong and weak cation-exchangers, respectively. Quaternary amines or primary amine groups are commonly used in anion-exchange columns.[35] The mobile phases used in IEC are normally aqueous salt buffer solution, often mixed with a certain amount of organic modifier, such as methanol or acetonitrile. Most separations carried out using IEC use gradients to increase ionic strength during the chromatographic run. Gradients improve the separation of complex mixtures, however, they are time consuming and the changes in the composition of the mobile phases during the run may lead to changes in the sensitivity of the elements detected by the ICP–MS. Additionally, high salt content in the mobile phase is undesirable for an adequate ICP–MS performance, as described in previous sections.

An increase in the column temperature may also result in increased column efficiency and dramatic change in selectivity, owing to improved solute diffusion and mass transfer.

Among the most important advantages of the IEC is its high separation efficiency compared to SED, and its wide applicability.[36] However, IEC is mostly suitable for the separation of covalently bound element species of different oxidation states, such as the Cr(III)/Cr(VI) pair, Sb(III)/Sb(V) pair, etc. Interconversion or alterations have been observed for labile metal-protein complexes,[37–39] most often due to the loss of loosely bound metal ions or the original metal ions being replaced by other metals originating from the buffer in the mobile phases.

Chiral Chromatography (CC)

Chirality is a property of a molecule that is not super-imposable on its mirror image, for example, a carbon that has four different groups attached to it. The two mirror image forms of the chiral molecule are termed enantiomers.

It was discovered that the biological activity of chiral substances often depends upon their stereochemistry, since the living body is a highly chiral environment. A large percentage of commercial and investigational pharmaceutical compounds are enantiomers, and many of them show significant enantioselective differences in their pharmacokinetics and pharmacodynamics. The importance of chirality of drugs has been increasingly recognized, and the consequences of using them as racemates or as enantiomers have been frequently discussed in the pharmaceutical literature during recent years. With the increasing evidence of problems related to stereoselectivity in drug action, enantioselective analysis by chromatographic methods has become the focus of intensive research of separation scientists. Most of the pharmaceutical and pharmacological studies of stereoselectivity of chiral drugs before the mid eighties involved precolumn derivatization of the enantiomers with chiral reagents, forming diastereomers. The diastereomers were subsequently separated in the normal or reversed phase mode of chromatography. Chromatographic separation of enantiomers has become a routine practice in the pharmaceutical industry in the last decade.

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 been carried out, demonstrating the potential of this methodology.[40] Medel et al.[41] used a tilcoplanin based column to separate several selenoamino acid enantiometers, and both UV and ICP-MS detections were used and compared in the same injection. Any chiral compounds with an ICP-MS detectable element, or when an ICP-MS detectable element can be incorporated by derivation, can be considered for this application.

APPLICATIONS OF HPLC–ICP–MS

HPLC–ICP–MS has been employed in a wide range of disciplines including environmental, biological, and clinical applications. It represents a broad, multidisciplinary field of study, and a number of recent reviews and books provide an excellent reference to the role of various elements in these fields.[42–44]

Since most studies are related to concerns about human health risks, the majority of the publications have been focusing on only a few toxic element species. About 50% of all papers are dealing with only 5 elements, namely arsenic, selenium, mercury, chromium, and tin. Another 30% of papers are dealing with copper, zinc, lead, cadmium, and iron. All other elements, in total, are in the focus of only 20% of the publications.

Some of the more recent, typical, and important applications in these fields will be cited and discussed in the following sections.

Environmental Samples

Water

Water has been the most commonly analyzed sample for LC–ICP–MS. Most water samples are easy to obtain and usually require almost no sample preparation at all.

Arsenic is the most speciated element.[45–50] Arsenite (As^{III}) and arsenate (As^{V}) are the most toxic forms, considered and classified as human carcinogen substances and are the predominant forms found in water. Biologically mediated methylation reactions, occurring in terrestrial and marine organisms, convert arsenite and arsenate to methylated compounds of moderate toxicity, such as methylarsonic acid ($MMAA^{V}$) and dimethylarsinic acid ($DMAA^{V}$). Arsenobetaine (AsBet), a more complex arsenic compound, was identified essentially in marine biota or in marine sediments and is considered to be relatively non-toxic.[51]

The five arsenic species are often separated in water samples by anion-exchange chromatography.[52] Also, ion-pairing reversed-phase LC using tetrabutylammonium salt has been used to achieve resolution of the arsenic species in less than 12 min. LC–ICP–MS used in conjunction with hydride generation can increase the sensitivity of arsenic even further.[53]

Great care should be exercised when a quadrupole ICP-MS is used to detect arsenic, since the presence of chlorine in the sample may give rise to the formation of $^{40}Ar^{35}Cl^{+}$ that interferes with the mono-isotopic $^{75}As^{+}$, thus yielding false positive or falsely high arsenic results.[54,55]

Even though the most excessive use in industry and agriculture has ceased due to the poisonous and insidious nature of mercury and mercury compounds, methylmercury may still be found at high concentrations (0.5–1.0 mg/kg) in fish caught in US, Canada, and other parts of the globe. Since most of the mercury found in the atmosphere is in its metallic form, in natural waters as divalent inorganic mercury, and in fish as monomethylmercury, the need for analytical methods able to differentiate between these mercury species is obvious. This makes mercury the other most commonly speciated element in water samples by LC–ICP–MS.

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, however, neither method was capable of delivering usable detection limits, making LC–ICP–MS a much better choice.[56,57]

Mercury concentrations in water samples may be at significantly lower levels than ICP-MS can detect, albeit still 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 even below their LC–ICP–MS detection limits. Therefore, a preconcentration step may be necessary prior to analyzing water samples for certain elements.

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. Bloxham et al.[58] used this technique to preconcentrate 1000 mL of water sample on the column in less than 2 h. With a C_{18} column and ion-pairing reagent or a Spherisorb S5 ODS2 column, they have separated methylmercury, ethylmercury, and inorganic mercury in about 13 min.

Other sample introduction systems for ICP–MS have been reported with much improved sensitivity, such that no labor intensive and time consuming preconcentration steps are needed. The use of an ultrasonic nebulizer[59] has been shown to deliver detection limits of about 10 times better than the detection limits obtainable by a conventional nebulizer.

Cold vapor and hydride generation has also been used in conjunction with LC–ICP–MS for trace analysis of mercury and arsenic in water samples,[60–62] and was proven to be able to greatly enhance the sensitivities for all these elements.

During the last couple of years, platinum in water,[63] iron in natural water,[64] and mercury and tin in natural and sea water[65] have been speciated.

Soils

Speciation of trace elements in soils by LC–ICP–MS can be more challenging, since all samples need to be turned into solution form before they can be injected into the LC system for separation. All species of interest have to be extracted into the solution and adequate precaution has to be taken to prevent any species interconversions that may occur in the process. 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.

It was observed by Bissen and Frimmel[66] 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).[67] Speciation of the

extracts obtained by Bissen and Frimmel[66] was carried out by anion-exchange chromatography with a 50 m 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.[68] employed a similar procedure in their analysis of arsenic in soil samples. In addition, they found that adding 5% (w/v) methanol to the NaOH mobile phase improved the nebulization efficiency of the sample, and consequently, sensitivities were double for the five arsenic species studied. One of the simplest and most efficient extraction processes applied to soil is a single step microwave extraction procedure. Phosphoric acid was used in this case to extract arsenic species from freeze-dried, crushed, and filtered soil, sand, and sediment samples.[69] Separation of these extracts with an anion-exchange column has resulted in 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.

Ochsenkühn-Petropoulou et al.[70] has developed an analytical procedure for selenium speciation of analysis of selenourea (SeU), selenoethionine (SeE), selenomethionine (SeM), Se(VI), Se(IV), dimethylselenide (dMeSe), and dimethyldiselenide (dMedSe), based on two complementary liquid chromatography (LC) techniques coupled with inductively coupled plasma-mass spectrometry (ICP–MS). Specifically, anion exchange chromatography coupled with ICP–MS was used for the separation and quantification of all the earlier mentioned Se compounds, except for the two methyl selenides, which could be separated and determined by reversed phase chromatography coupled with ICP–MS. This procedure was applied to a soil sample from the warm springs area of Thermopyles (Greece). For leaching the Se species from the soil sample, four extraction methods, using water at ambient temperature, hot water, methanol, and 0.5 M HCl, were tested for their efficiency of extracting different Se species.

Biological Samples

Food Materials

As stated earlier, some 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. Arsenic species have been extracted from freeze-dried apple samples by a two step process.[71] The sample was freeze-dried before extraction. The extraction efficiencies of the method they used ranged from 79 to 117%. An anion-exchange column and 10 mM phosphate: 10 mM nitrate mobile phase were used, and the eluent was fed to a hydride generation system coupled to the ICP–MS detector. Separation of four arsenic species was achieved in less than 9 min.

Arsenic speciation in carrots has also been performed[72] by accelerated solvent extraction. The arsenic in these freeze-dried carrot extracts was speciated with an anion-exchange column and 10 mM ammonium carbonate mobile phase, and the separation of the five arsenic species was 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.[73,74] Bioavailable arsenic can be extracted with water with a sonicator or with the aid of a microwave digestion system. The arsenic species in these extracts were separated on a strong anion-exchange column with a 45 mM ammonium carbonate mobile phase. Separation of DMA, As(III), MMA, and As(V) from cationic species is achieved in less than 13 min; while 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.

Selenium is another element that is frequently investigated in food substances. There is some indication, that some organoselenium compounds are scavangers of free radicals and as such, may reduce the risk for some types of cancer in humans.

Therefore, it is paramount to identify the active species in order to maximize the potency while reducing the risk for side effects.

The selenium species present in nuts have been investigated by Kannamkumarath et al.[75] using both size exclusion chromatography and ion pair reversed-phase chromatography. Onions and garlic have both been found to be particularly high in selenium, thus, a lot of speciation work has been done on these kinds of samples.[76–80] 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.[76]

McSheehy et al.[77] 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 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.

The speciation of selenium enriched yeast by LC–ICP–MS has become a popular area of research.[78–86] Experiments were conducted with aqueous solutions, non-proteolytic enzymes, sodium docecylsulfate (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.

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.[40]

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.[79] A chiral crown ether column and 0.1 M perchloric acid mobile phase was used to separate Se-homocystine, Se-lanthionine, Se-cystathionine, Se-cystine, Se-ethionine, γ-glutamyl-Se-methylselenocysteine, Se-adenosylhomocystine, Se-methylselenocysteine, and Se-methionine.

Animal Tissues

Because of the world wide consumption and their significance in human nutrition and the fact that the environment in which they live are frequently contaminated with toxins, fish and other forms of seafood have been extensively studied by LC–ICP–MS.[87–93] 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 various extraction procedures. Ackley et al.[91] 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 by this microwave procedure. Anion-exchange chromatography can be used to separate the As(III), As(V), DMA, and MMA that may be present in fish samples.

A microwave assisted extraction has also been used for mercury speciation in swordfish.[90] Two minutes of microwave extraction at 60°C was found sufficient to extract up to 98% of the total mercury with no

detectable interconversion of the species. The separation was conducted with a reversed-phase C_8 column and in situ vapor generator.

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.[88] 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. Diethyldithiocarbamic acid ammonium salt (DDCA) and pyrrolidinecarbothioic acid ammonium salt (PCA) have been used as complexing agents with CO_2, the most common supercritical fluid, forming chelates with the tin. The 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.

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).[94] In the last couple of years, arsenic in oyster tissue SRM (standard reference materials),[95] selenium in chicken muscle, liver, and kidney[96] have also been speciated and reported.

Clinical Samples

Body Fluids

Metabolites of many trace elements are present in bodily fluids and are excreted in urine. These metabolites 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 popularity. LC–ICP–MS analysis of urine, in particular, has become widespread.[97–102] However, urine samples can be very difficult to handle 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.[100]

However, 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 to clog up it.

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.[103] Such a technique is capable of simultaneously separating anionic and cationic species, the resolution of As(III), As(V), MMA, and DMA was accomplished in less than 15 min. Also, the separation of Se-aminoacids and the inorganic species can be done using this separation mechanism.[99] 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.[104] Good separation of Se(IV), Se(VI), $TMSe^+$, 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.

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.[98] 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), trimethyllead, 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.

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.[105]

Fe, Cu, and Zn have been simultaneously speciated in human serum by HPLC–ICP–MS using on-line isotope dilution.[106] 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.

Similarly, zinc binding proteins have been analyzed in human serum with SEC–ICP–MS, as well as anion-exchange chromatography.[107] Zinc containing components of the serum were separated with a 0.025 M Tris–HCl buffer and 0.25 M 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, also contains 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 extremely valuable 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.[108] The only necessary sample preparation is the removal of fat and other insoluble components through centrifugation.

Pharmaceutical Materials

At all stages of drug discovery and development there is a need for information on the metabolism of candidate compounds. This requires the ability to identify and quantify unknown metabolites from in vitro or in vivo drug metabolism studies. Identification is often achieved using HPLC–MS, but for quantitative analysis of metabolites both MS and UV detection are inadequate, as the response of these detectors is dependent on the molecular structure of the metabolites detected. Quantification thus requires authentic standards that are rarely available. To obtain quantitative metabolite data, radiochemical detection is usually applied, but this requires the time consuming and expensive synthesis of isotopically labelled compounds, which are not normally available in the early phases of drug discovery programs. There is, hence, a need for alternative methods of quantification.

Fortunately, the sensitivity of ICP–MS is, in principle, independent of the structure of the compound. This makes ICP–MS a promising technique for drug metabolism studies in that quantitative data can be obtained without the need for either standards or radiolabelling. For substances containing any ICP–MS–detectable elements, mostly heteroatoms in pharmaceutical materials and metabolites such as phosphorous,[109] sulphur,[110,111] bromine,[112] and chlorine,[113] HPLC with ICP–MS detection can be applied to the analysis of complex mixtures, including biological fluids, for metabolism studies. Iodine, when present in a molecule, also provides an interesting opportunity for sensitive and specific detection and quantification and a limited number of applications exploiting this element have been reported. Thus, HPLC–ICP–MS, with iodine-specific detection, has been applied to

pharmaceutical substances such as X-ray contrast media[109] or thyroid hormones, either in tablets,[114] enzyme digests of bovine thyroid gland,[115] in urine[116] or serum,[117] and for analysis of iodine containing compounds in human milk.[118] A recent HPLC–ICP–MS study has also demonstrated the use of this approach to obtain metabolite profiles following exposure of 2-fluoro-4-iodoaniline to earthworms.[119] HPLC–ICP–MS and HPLC–ICP–MS/ESI–MS have been used to investigate the metabolic fate and disposition of 2-, 3- and 4-iodobenzoic acids following intraperitoneal administration at 50 mg kg^{-1} to male bile duct cannulated rats.[120]

LC–ICP–MS can also be used to detect low level impurities in the drugs themselves as long as these impurities contain any ICP–MS detectable elements, thanks to the superior sensitivity advantage of the ICP–MS detector. 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.[121] 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 at the time. 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.[122]

LC–ICP–MS can also be a powerful tool for kinetic studies on the catalyst formation process in pharmaceutical research. Tu and Wang et al.,[123] Welch and Tu et al.[124] have used this tool for the identification and quantification of various rhodium species involved in a ligand exchange process leading to formation of Doyles catalyst dirhodium(II) tetrakis[methyl 2-oxopyrrolidin-5(S)-carboxylate], or Rh_2(5S-MEPY)$_4$. ICP-MS was used as the Rh detector following species separation by reversed-phase high performance liquid chromatography (RP–HPLC), and electrospray ionization mass spectrometry (ESI–MS) was used for species identification and confirmation. A variety of reaction intermediates were identified and quantified along the pathway to formation of the desired product, including isomeric di-, tri-, and tetra- substituted species previously believed to be absent. This has provided new insights into the mechanism and kinetics of the reaction.

ELECTROSPRAY IONIZATION MASS SPECTROMETRY (ESI–MS)

Identification of a species by LC–ICP–MS is achieved by the comparison of retention times with a standard. Most often, either peaks obtained do not correspond to a standard or a completely new species is being analyzed.

In these cases, the compounds 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.

Typically, a separation is performed before ES–MS since the clustering of the solvent molecules with the molecules of the sought for species and other gas phase reactions can dramatically complicate the resulting mass spectra to render them impossible to decipher. LC–ICP–MS is often used to identify the retention times of unknown peaks and fractions are collected accordingly, often by conducting another, online or offline chromatographic separation. As a result of its much poorer sensitivity of ES–MS in respect to ICP–MS, preconcentration is often necessary and fractions from multiple chromatographic runs have to be combined in order to get detectable peaks for sought for species. When choosing mobile phase for the separations, care must be taken if ES–MS is to be used. Some mobile phase components may be incompatible with the electrospray source (e.g., high saline mobile phases). As a positive note, 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 information of the sought for species. The molecular mass of the unknown species can be obtained by identifying the molecular ion peak in the spectra and the structure can then be established based on the observed fragmentation pattern. Once the structure is determined or predicted, standards can eventually be made and further molecular confirmation can be obtained by sample spiking or matching retention times.

ES–MS has been used in conjunction with ICP–MS for the characterization of Se species[125,126] or As species[127] with a great success.

CONCLUSION

With high sensitivities, low detection limits, elemental selectivity, isotope ratio capabilities, and wide linear dynamic ranges, ICP–MS is the best detector for elemental speciation at this time.

LC, with its plethora of separation modes, together with ICP–MS, has become the most popular and powerful speciation tool today. This hyphenated technique has been used routinely for elemental speciation studies of samples and matrices ranging from environmental, biological, to clinical. In the recent years, publications focusing on bioanalytical applications rapidly out pace other applications. This can be partly attributed to the overwhelming enthusiasm by the human genome project and subsequent proteomics research. LC–ICP–MS will be the dominating technique for these purposes.

Commercial LC–ICP–MS systems have been available for a couple of years now, this will surely result in more and higher quality publications in this field for years to come.

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