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Review

The potential of organic (electrospray- and atmospheric pressure chemical ionisation) mass spectrometric techniques coupled to liquid-phase separation for speciation analysis

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Abstract

The use of mass spectrometry based on atmospheric pressure ionisation techniques (atmospheric pressure chemical ionisation, APCI, and electrospray ionisation, ESI) for speciation analysis is reviewed with emphasis on the literature published in and after 1999. This report accounts for the increasing interest that atmospheric pressure ionisation techniques, and in particular ESI, have found in the past years for qualitative and quantitative speciation analysis. In contrast to element-selective detectors, organic mass spectrometric techniques provide information on the intact metal species which can be used for the identification of unknown species (particularly with MS–MS detection) or the confirmation of the actual presence of species in a given sample. Due to the complexity of real samples, it is inevitable in all but the simplest cases to couple atmospheric pressure MS detection to a separation technique. Separation in the liquid phase (capillary electrophoresis or liquid chromatography in reversed phase, ion chromatographic or size-exclusion mode) is particularly suitable since the available techniques cover a very wide range of analyte polarities and molecular mass. Moreover, derivatisation can normally be avoided in liquid-phase separation. Particularly in complex environmental or biological samples, separation in one dimension is not sufficient for obtaining adequate resolution for all relevant species. In this case, multi-dimensional separation, based on orthogonal separation techniques, has proven successful. ESI-MS is also often used in parallel with inductively coupled plasma MS detection. This review is structured in two parts. In the first, the fundamentals of atmospheric pressure ionisation techniques are briefly reviewed. The second part of the review discusses recent applications including redox species, use of ESI-MS for structural elucidation of metal complexes, characterisation and quantification of small organometallic species with relevance to environment, health and food. Particular attention is given to the characterisation of biomolecules and metalloproteins (metallothioneins and phytochelatins) and to the investigation of the interaction of metals and biomolecules. Particularly in the latter field, ESI-MS is the ideal technique due to the softness of the ionisation process which allows to assume that the detected gas-phase ions are a true representation of the ions or ion–biomolecule complexes prevalent in solution. It is particularly this field, important to biochemistry, physiology and medical chemistry, where we can expect significant developments also in the future.
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1. Introduction

Metals are omnipresent in the environment and in biological systems, and mostly form part of complexes, smaller or larger molecules in which they have specific properties or assume a certain role. The specific form in which an element occurs dictates its physico–chemical properties, its behaviour in the environment, physiological function, its toxicity or essentiality for humans, animals or plants. A number of well-known examples illustrate the importance of speciation analysis, such as the case of chromium which is highly toxic in its valence state Cr(VI), but is considered essential as Cr(III) cation [1], or arsenic whose toxicity is significant in its inorganic forms, As(III) and As(V), but is non-toxic in most of its organic forms such as arsenobetaine or arsenocholine [2]. On the other hand, even for an element whose forms are both considered highly toxic, such as elemental mercury and methylmercury, the patterns of toxicity may vary substantially. These different chemical forms of the same element and its compounds are generally referred to as ‘‘species’’, and the distribution of an element among its different forms (its ‘‘speciation’’ according to the recent IUPAC definition [3]) will be responsible for the behaviour of this particular element observed in the environment, in biochemical or geochemical cycles, in toxicology, clinical chemistry or nutrition and health [4–10].

While the number of publications in this important field is steadily increasing (Welz estimated in 1998 that more than 300 publications per year are published which fall under the closer definition of the term ‘‘speciation’’ [11]), it can be observed that speciation analysis is eventually changing its face and scope: no longer is this subject restricted to the determination of a (metallic) element in its different redox states or to the determination of low-molecular-mass organometallic compounds as it was in the pioneering work in this field [12,13]. Speciation analysis nowadays covers an area that ranges from the determination of isotopic composition, electronic and oxidation states, inorganic compounds and complexes to organic and organometallic complexes, and finally the investigation of macromolecular com-

pounds and complexes [14–17]. While in the ''early'' phases of speciation analysis interest was focused on the development of methods able to differentiate, e.g., between the different redox states of an element (for which titrimetric, gravimetric or electrochemical methods of analysis were often perfectly suitable [14,18]), attention later turned to the determination of anthropogenic, low-molecular-mass organometal compounds, such as the environmentally highly relevant organotin, organolead and organomercury compounds. Hyphenated GC techniques were most frequently employed for the determination of these compounds after derivatisation [19,20]. Having relatively suitable derivatisation techniques at hands [21,22], GC separation was preferred to liquid-phase separation techniques for speciation [23] due to the greater ease of use, and particularly the availability of more sensitive and selective detectors. However, attempts were made already early to circumvent the derivatisation step which may be the least controlled, and sometimes even the least understood step in the entire analytical process [24], and to use instead liquid chromatographic (LC) or capillary electrophoretic (CE) separation. With LC separation in its various modes (reversed phase, ion chromatography or size exclusion) and with CE the range of less volatile analytes became accessible (Fig. 1a), where the reduced volatility is a consequence of either the higher molecular mass or the increased polarity of the analytes. This analytical armoury could be used to respond to the increased interest in the speciation analysis of strongly polar or ionic species such as selenoamino acids, selenoproteins, organoarsenic acids or arsenosugars. And from the analysis of low-molecular-mass organometallic compounds it was only a logical extension to strive now for the characterisation of larger biomolecules incorporating covalently bound or loosely coordinated metals. Typical metal-containing biopolymers are polypeptides (e.g., phytochelatines, metallothioneins or metalloenzymes), macrocycles (e.g., porphyrins, cobalamin or chlorophylls) or glycosides (e.g., glycoproteins) [17,25].

With the focus of speciation analysis shifting from the well-defined, mostly anthropogenic low-molecular-mass compounds to less-defined or in most cases even unknown, biogenic high-molecular-mass species, the choice of detection technique becomes an important issue: no longer will element-specific detectors, such as those commonly used in GC [atomic absorption, atomic emission (pulsed) flame photometric, etc.] be sufficient. Even ICP-MS detection with its unmatched sensitivity and excellent selectivity will only be useful to a limited extent, since all these techniques only provide element specific information. What is needed in this case, however, is molecule specific information, and this can only be obtained with molecular spectroscopic detectors. Of the principally available techniques [UV–Vis and infrared absorption, fluorescence, mass and nuclear magnetic resonance (NMR) spectroscopy], only molecular mass spectrometry provides both the sensitivity and the selectivity to be suitable for this purpose.

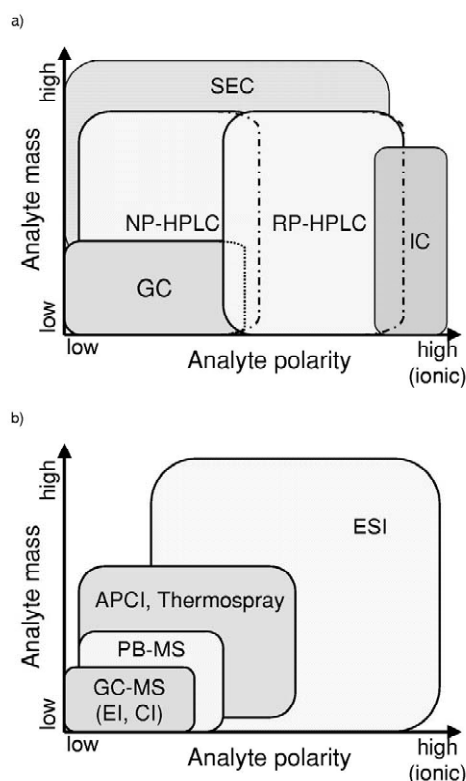


Fig. 1. (a) Application range of the most important separation techniques as function of analyte polarity and molecular mass. (b) Application range of mass spectrometric (ionisation) techniques as function of analyte polarity and molecular mass.

While the combination of GC with MS is a well-

established routine technique in the analytical laboratory and has also widely been used in speciation analysis where sensitivity and selectivity are adequate (e.g., for selenium [26], lead [27] or arsenic [28]), hyphenated LC (or CE) with molecular mass spectrometry has so far been used only to a much lesser extent for speciation analysis, but is now eventually establishing itself as an indispensable technique in the analytical chemist's toolbox for speciation analysis.

The commercial availability of atmospheric pressure ionisation sources for LC–MS (for both electrospray ionisation, ESI, and atmospheric pressure chemical ionisation, APCI) and their robustness and ease of use [29–32] have greatly contributed to their success, and it is to be expected that these techniques will almost completely supplant other interfaces and ionisation techniques that have previously been popular for LC, such as the thermospray or the particle beam interface [33,34]. Considering the range of analyte polarity and molecular mass which is acceptable to electrospray ionisation (Fig. 1b), it becomes evident that this technique outperforms even atmospheric pressure chemical ionisation: ESI is suitable for analytes with higher polarity (or charge) and — this also being an effect of the ability to form multiply charged ions — with significantly higher molecular mass than APCI. Still, both techniques not only complement each other conveniently, but overlap to a certain degree in the range of application as will be apparent from the examples presented later. This justifies a joint review of the application of these two ionisation techniques for speciation analysis.

The use of API techniques for speciation analysis and, in particular, ESI has been the subject of a number of excellent reviews [35–40] and book chapters [41,42] which were entirely or in part [16,17,43–45] devoted to this topic. Particular credit shall be given to the authoritative review of Stewart [37] on the application of ESI-MS to elemental speciation who comprehensively summarised and discussed the literature in this field up to 1999. The present review will thus mainly focus on papers that have appeared after 1999 and include earlier work only where necessary for a comprehensive discussion in an attempt to not duplicate the earlier work. It will mostly consider instrumental developments and applications of hyphenated chromatography (or electrophoresis) and API-MS. However, as molecular mass spectrometry under certain conditions is also able to provide species-specific information without separation (see, e.g., the discussions in Refs. [46–48]), some significant examples of direct speciation by ESI- or APCI-MS without preceding separation shall also be included in this review.

While ICP-MS appears to be the ideal detector for speciation analysis due to its sensitivity and the tolerance of various matrices and analytical conditions, ironically it destroys the molecule whose identity is to be elucidated. In contrast to this, ESI- and APCI-MS mostly preserve the integrity of the analyte, and inevitably also the complexity of the matrix. With the high level of interferences from real-world samples, however, molecular MS techniques can hardly produce reasonable results if applied directly without a preceding separation step. Given the complexity of sample matrices particularly of biological origin, even one-dimensional separation techniques often fail to separate compounds sufficiently well so that they can be identified or quantified by their mass spectra.

A common strategy which has particularly been pioneered by the group of Szpunar and Lobinski (e.g., Refs. [16,17]) is therefore to apply multi-dimensional separation with on-line- or off-line-ESI-MS for identification or confirmation of the species under investigation. The separation power of this multi-dimensional approach increases with increasing difference of the applied separation methods (dimensions). Typical examples for separation techniques which can be considered as non-correlated (orthogonal) in their separation behaviour are size-exclusion-, ion- and reversed-phase chromatography. An additional dimension of information can be added in the detection step by two-dimensional mass spectrometry using, e.g., tandem MS or ion trap MS instruments (Fig. 2).

2. Principles of atmospheric pressure ionisation techniques

2.1. Discussion of atmospheric pressure ionisation techniques

The fundamentals and practical aspects of atmospheric pressure ionisation (API) techniques have

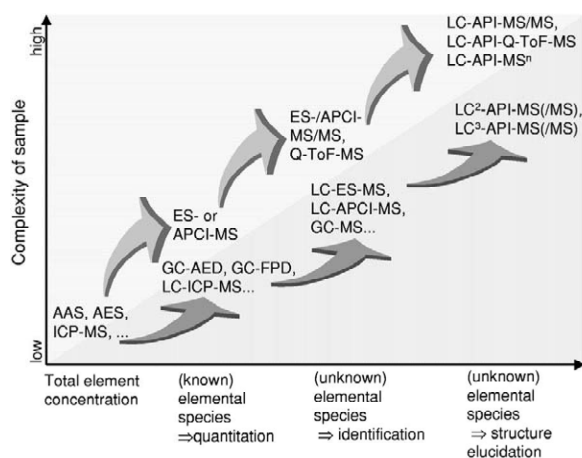


Fig. 2. Schematic of the necessary increase in separation/detection dimensionality as the complexity of a sample increases.

been the subject of a number of books and comprehensive reviews [30–33,49,50]. The reader is thus referred to these sources for a more extensive discussion of API-MS techniques than can be given in this context.

Generally speaking, the two major API techniques, namely ESI and APCI, rely on the formation of a continuous spray from the liquid chromatographic or electrophoretic effluent. Ions are formed or pre-existing ions are extracted in an atmospheric pressure ionisation process and transported through various pumping stages into the vacuum of the mass spectrometer.

There are four significant advantages which have contributed to the overwhelming success of API techniques (according to Voyksner [51]):

(a) API techniques can handle liquid flow-rates that are typically used in LC.
(b) API techniques are suitable for the analysis of non-volatile, medium to highly polar and thermally unstable compounds typically separated in the liquid phase.
(c) API-MS techniques are highly sensitive (for those compounds for which they can be applied) and approach sensitivities known from GC–MS.
(d) API-MS techniques are comparatively robust and fairly easy to use.

One should be aware, however, that ESI and APCI have a number of limitations which may become relevant particularly for speciation analysis. The compatibility of selected liquid-phase separation techniques with API-MS is discussed in Table 1.

2.1.1. Electrospray ionisation

From the earliest reports on the coupling of electrospray ionisation and mass spectrometry which were independently presented by Yamashita and Fenn [52,53] and almost concurrently by Aleksandrov et al. [54], this technique became widely used for the analysis of polar and ionic compounds. It is rather based on the extraction of ions from solution

Table 1
Compatibility of selected liquid-phase separation techniques with API-MS (adapted from Ref. [51])

Mode	ES	APCI	Comments
Reversed phase	+++	++	Formation of ions in solution possible; sample volatility normally limited; mobile phase evaporation depending on water content; only use of volatile mobile phase additives
Normal phase	+	+++	Normally no formation of ions in solution (limited miscibility with non-aqueous phase); sample is more volatile than in RPLC
Size exclusion	+++	+	Buffers used to suppress non-exclusion mechanisms may cause problems; most likely analytes are not volatile and of high molecular mass
Ion pair	++	++	Reagent ions may compete for ion-evaporation process and suppress response; volatility of ion-pairing reagent to be considered
Ion exchange	+	+	High ionic strength may be problematic; limited volatility of mobile phase additives
Hydrophobic interaction	+	+	Use of salt gradients for the elution of biomolecules; involatile salts are not compatible with API-MS
Capillary electrophoresis	+++	+	Very low flow-rate requires often addition of a sheath flow; due to charged analytes ES best suited; involatile buffers problematic

than on their formation under the action of a strong electric field. Particularly the fact that multiply charged ions of high-molecular-mass proteins could be observed and analysed with relatively small (quadrupole) mass spectrometers contributed to the enormous popularity of ESI-MS. Also in speciation analysis, ESI-MS has been a very useful technique due to the often highly polar or ionic nature of the analytes and the softness of the ionisation process which allows to generate gas phase ions which reflect well the ions actually present in the liquid phase. Optimisation of the interface parameters is certainly important to obtain a complete desolvation of the ions without compromising their integrity.

2.1.2. Atmospheric pressure chemical ionisation

APCI is favoured for the analysis of medium to less polar, low- to medium-molecular-mass analytes (Fig. 1b). In contrast to ESI, solvent evaporation and analyte ionisation are two separated processes, the latter being a chemical ionisation at atmospheric pressure with the mobile phase acting as reactand gas. As the evaporation of mobile phase is supported by the application of temperatures of up to 500 °C, this form of ionisation is somewhat less mild than ESI. APCI normally produces singly-charged ions ("quasi-molecular ions") through the addition or abstraction of a proton (e.g., $[M+H]^+$ and $[M-H]^-$, respectively).

2.1.3. Comparison of electrospray and atmospheric pressure chemical ionisation

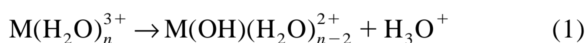
Both ESI and APCI are soft ionisation techniques, however, they have different ranges of applicability in terms of polarity and molecular mass of the analytes. Since most organometallic species of interest are highly polar to ionic and they cover a very large range of molecular masses, starting from low-molecular-mass organometallic compounds to high-molecular-mass proteins, it is evident that ESI is in most cases the better suited ionisation technique. This clearly explains why, although this review discusses the application of both atmospheric pressure ionisation techniques to speciation analysis, most work has been performed by ESI-MS. Due to its principle, ESI also is more likely to preserve the integrity of the particular species than APCI. The disadvantages of ESI compared to APCI however are its lower robustness to matrix interferences and changes of mobile phase conditions, its less favourable quantitative detection ability, and the fact that ESI originally was a low-flow ionisation technique (with sample introduction rates in the low μl min^{-1} range). Compatibility of ESI with typical HPLC flow-rates in the order of 1 ml min^{-1} have been achieved by pneumatically assisted electrospray (often also called ionspray) ionisation.

2.2. Collision-induced dissociation and gas phase chemistry

In both API techniques, solvated ions are formed initially. Desolvation of the solvated ions is normally achieved by the application of heat and a countercurrent stream of drying gas (heated nitrogen). When the ions expand into the vacuum after exiting from the transfer capillary, they are accelerated by a variable potential difference towards the analyser. On their way, the solvated ions collide with residual drying gas molecules present at intermediate pressures (ca. 1 Torr; 1 Torr=133.322 Pa). These collisions not only lead to a complete desolvation of the solvated ions, but may also induce a certain degree of fragmentation which increases with the applied potential difference (fragmentor voltage) as the kinetic energy of the ions increases.

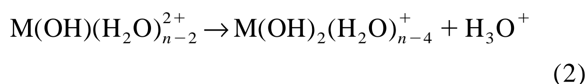
This collision-induced dissociation (CID) is useful to increase the information content of the relatively featureless mass spectra obtained with soft ionisation techniques. Front end-CID produces spectra similar to daughter ion spectra with triple quadrupole or ion trap instruments, however, the selectivity is much poorer since the parent ions are not isolated and the co-eluting matrix compounds may interfere.

The second important process taking place in an API interface is charge reduction. This is observed when a multiply charged ion is no longer able to stabilise its charge as a result of the loss of solvent molecules from its solvation sphere [55–57]. This will eventually lead to charge reduction of the cluster ion by transferring a charge to one of the coordinated water (solvent) molecules according to:



$$M(H_2O)_n^{3+} \rightarrow M(OH)(H_2O)_{n-2}^{2+} + H_3O^+ \qquad (1)$$

Upon further drying, a further charge reduction step may occur:



$$M(OH)(H_2O)_{n-2}^{2+} \rightarrow M(OH)_2(H_2O)_{n-4}^{+} + H_3O^{+} \quad (2)$$

It must be pointed out that charge reduction is in fact achieved by a separation of charges while the oxidation state of the central ion remains constant.

2.3. Electrochemical processes in API interfaces

The analogy between the ESI interface and an electrochemical cell has been recognised by Blades et al. as early as 1991 [58] and was comprehensively discussed in a recent review [59]. As ions are transported from the electrospray capillary tip (biased at a high potential) to the counter electrode (normally grounded), the electrical circuit must be closed by parallel discharging reactions in order to maintain charge balance. In the positive ionisation mode, for example, positive charges are transported from the capillary tip (leaving behind an excess of negative charges) to the spray shield at the entrance of the mass analyser (where a build-up of positive charges will occur). As a consequence, electrochemical conversions will occur at both electrodes with the condition that the observed currents i_{anode} and $i_{cathode}$ equal each other. The reaction at the counter electrode (the cathode in case of positive ion detection) that will most likely take place is either the discharge of a gas phase ion or a reduction of charged species when they hit the electrode surface. The equivalent number of electrons must be supplied from oxidation reactions at the anode (the sprayer capillary). Three types of reactions may therefore occur: (i) discharge of an anion at the electrode surface, (ii) dissolution of the working electrode (e.g., by oxidation of the material of which the sprayer capillary consists, such as $Fe \rightarrow Fe^{2+} + 2e^{-}$), or (iii) oxidation of the mobile phase or the compounds dissolved therein such as the analyte, mobile phase additives or dissolved gases.

For the signal generation process, only the reaction occurring at the working electrode is of importance as it may change the species information and add or remove species from the solution as demonstrated by Blades et al. [58]. Oxidation reactions at the anode will take place to such an extent as needed to maintain a steady state current. It will not necessarily be the analyte which is oxidised at the anode, but rather the most easily oxidisable species in solution in order of increasing redox potentials which also is depending on the concentration and the composition of the medium [60,61].

The phenomenon of oxidation of the analyte as a consequence of the electrolytic nature of the electrospray can advantageously been used to generate ions from neutral compounds and complexes. Particularly Van Berkel and co-workers have published a series of noteworthy papers in which they elegantly made use of on-line oxidation in the electrospray interface to produce charged species from neutral inorganic or metal–organic compounds, such as neutral metalloporphyrin complexes [62–68].

Electrochemical processes have so far not been described for APCI, although interconversion of the oxidation states of organometallic species has been observed. Since these redox processes are normally resulting in a reduction of the oxidation state of the metal, they can be attributed to a gas phase reaction rather than to an electrochemical process in the eluent phase.

2.4. Quantitative aspects

While the potential of ESI-MS for obtaining qualitative analysis in speciation analysis is indisputable, quantitative analysis has still remained a major challenge (other than for APCI-MS). This is due to the sensitive equilibrium of concurrent and competitive reactions in the ionisation process. A parameter of great importance in this context is the conductivity of the solution which is mostly governed by the concentration of the analyte to be determined. In two fundamental reports, Kebarle and co-workers [69,70] have investigated the non-linearity of the ESI response as function of the electrolyte concentration in detail. In agreement with the findings of Agnes and Horlick [71], they propose that the problem of the inherent non-linearity of the ESI response could be overcome by the use of a supporting electrolyte for the determination of certain metal ions, leading to a linear dynamic range of up to four orders of magnitude. The use of a supporting electrolyte provides two distinct advantages: first, it can be used as internal standard, and second and more important, its presence ensures an overall conductivity that is feasible for the generation of a stable electrospray

("ES stabiliser"). A further, though only rarely realised advantage, is that the addition of an electrolyte with specific chemical or physical properties may alleviate matrix interferences or enhance spray efficiency.

Ideally, the concentration of the supporting electrolyte should be in excess of the concentration of the analyte so that changes between samples do not affect the overall conductivity of the solution significantly. Typical concentrations of the supporting electrolyte are in the 100 μM l^{-1} level, allowing to obtain linear calibration curves down to analyte concentrations of ca. 10^{-8} M l^{-1}. The detection limits of some relevant metal species are summarised in Table 2. Some At the high concentration end of the calibration curve, very often the so-called "roll-off" is observed as a result of non-linear charging efficiency of electrolyte solutions at increasing conductivity for a given set of conditions.

Table 2
Comparison of limits of detection by ESI-MS reported by different groups (adapted from Ref. [42])

Element	Species	Limit of detection (ng ml^{-1})	Ref.
Barium	Ba^{2+}	20	[47]
Cesium	Cs^{+}	2.7	[71]
	Cs^{+}	9	[47]
Chromium	Cr^{3+}	30	[47]
Cobalt	Co^{2+}	4.7	[71]
	Co^{2+}	30	[47]
Copper	Cu^{2+}	100	[47]
Nickel	Ni^{2+}	30	[47]
Rubidium	Rb^{+}	3	[47]
Tin	$Sn(C_4H_9)_3^+$	10	[215]
Uranium	UO_2^{2+}	100	[47]
Vanadium	VO^{2+}	200	[47]
Zinc	Zn^{2+}	300	[47]
Bromide	Br^{-}	0.9	[216]
	BrO_3^-	0.05*	[217]
Chloride	Cl^{-}	35	[216]
	ClO^{-}	1.0*	[217]
	ClO_2^-	0.05*	[217]
	ClO_3^-	0.7	[216]
	ClO_4^-	0.5	[216]
Fluoride	F^{-}	0.8	[216]
Iodide	I^{-}	0.8	[216]
	IO_3^-	1.0	[216]
	IO_3^-	0.5*	[217]

* Limit of quantitation (ng ml^{-1}).

2.5. Coupling of separation techniques with APCI- and ESI-MS

Although APCI- and particularly ESI-MS with direct sample introduction have been used for the analysis of organometallic species [46], this approach is hardly useful for real samples. The interferences which can be expected in real samples make the coupling of API-MS with a suitable separation technique (on-line or off-line) imperative.

What separation technique is considered "suitable" must be decided from case to case. Chromatographic techniques offer a variety of separation modes and principles which can be used, including normal- and reversed-phase HPLC, ion chromatography (IC) and size-exclusion chromatography (SEC) to name but the most important ones which also significantly differ in the resolution power with the order: HPLC>IC>SEC. For this reason, SEC is often used as a clean-up or fractionation technique only rather than as a high-performance separation method.

The mutual combination and the application of a (sufficiently different) separation technique for the heart-cut isolated from a first separation dimension allows to increase the separation power tremendously. Two- and three-dimensional separations have been used to resolve elemental species in complex biological samples. The coupling of API-MS with the different separation techniques has been realised both on-line and, for practical reasons (limited sensitivity, presence of involatile buffers, flow-rate considerations) frequently also off-line.

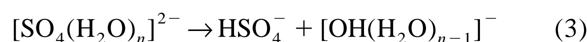
The coupling of LC separation techniques with API-MS detection is straightforward and fully established nowadays, at least for typical flow-rates ($<0.001\ldots1$ ml min^{-1}) and mobile phase compositions that avoid the addition of involatile buffers or additives. For the coupling of CE and API-MS this is not the case yet: commercial interfaces exist, but require a more careful optimisation of the parameters and significantly more expertise in the operation. Consequently, there have been some reports describing the development and construction of dedicated CE–ESI-MS interfaces [72,73]. They normally make use of a sheath liquid flow which is added after the CE separation before the separated

analytes enter the ESI interface and serves both as a make-up flow, and also to close the electrical circuit for the CE high voltage. At the same time, the composition of the mobile phase introduced into the interface may be altered to enhance ion formation by adding suitable modifiers through the make-up flow. Due to the extremely low flow-rates of CE, the capillary length between the junction with the sheath liquid and the ESI interface has to be minimised in order to maintain the achieved separation and to keep total separation times short. Alternatively, the analytes may actively be transported to the ESI interface by applying a positive pressure to the injection side of the CE capillary after completed separation. By doing so, Michalke et al. [138] succeeded in cutting the time for the separation of selenoamino acids from more than 30 min to ca. 10 min, however, the achieved separation was decreasing notably due to the pressure-driven transport of the analytes to the detector. A fundamental limitation of CE remains the low injection volume (as compared to LC) and the resulting low absolute mass of analyte that challenges MS detection which calls for ultrasensitive detection techniques such as provided by ESI-TOF- and Q-TOF-MS.

3. Applications of APCI- and ESI-MS for elemental speciation

3.1. Fundamental speciation studies with ESI-MS with direct sample introduction

The potential of ESI-MS for inorganic speciation was recognised very early and the technique employed even without preceding chromatographic or electrophoretic separation for fundamental speciation studies on model systems. Particularly the important series of papers by Blades and co-workers [58,74–77], by Agnes and Horlick [71,78–81] and by Stewart and Horlick [82–84] established ESI-MS as a versatile technique for the direct speciation of metal ions as well as halogen and oxo-anions in solution. These reports were predominantly studying fundamental aspects: as an example, the investigation of lanthanide spectra under various conditions (solvents, skimmer voltages) allowed one to characterise differences in the solution chemistry, in the strength of the metal–oxide bonds, and the ionisation potential [82]. As a general trend, increasing the skimmer-cone potential difference stripped stepwise water molecules from the hydrated metal ions, $Me(OH)(H_2O)_n^{2+}$, to eventually produce bare charged ions (Me^+ or MeO^+). Charge reduction (either charge separation or actual chemical reduction of the metal ion) was observed, depending on the applied field strength. Although not exactly related to metal speciation, a similar study by Stewart et al. [83] devoted to the speciation of sulfur compounds by ESI-MS is highly instructive. The ESI mass spectra of the sulfate anion at low and high skimmer-cone potential difference are discussed: at low applied voltage, two series of signals are seen: the signals deriving from both the hydrated sulfate anion, $[SO_4(H_2O)_n]^{2-}$, and those of the hydrogen sulfate anion, $[HSO_4(H_2O)_n]^-$. At higher potential difference, the signals from the doubly charged sulfate species disappear completely, and the bare HSO_4^- ion remains the only significant sulfur species that is detected. The authors explain this result with the assumed reaction sequence:



$$[SO_4(H_2O)_n]^{2-} \rightarrow HSO_4^- + [OH(H_2O)_{n-1}]^- \quad (3)$$

which suggests that charge separation takes place at higher potential differences as a consequence of increased CID. Analogous observations were made for the thiosulfate and tetrathionate ions ($S_2O_3^{2-}$ and $S_2O_4^{2-}$), respectively. With thiosulfate acting as a strong complexing agent for Ag, the authors succeeded in observing intact, hydrated $[Ag(S_2O_3)_2]^{3-}$ complexes. In addition to obtaining qualitative information from the use of ESI-MS, the authors were also able to use this technique successfully for quantitative analysis of sulfate in a wastewater sample. Reliable quantitation was achieved by (a) using standard addition, (b) ratioing the sulfate response against an internal standard and (c) running the analysis at high skimmer-cone potential difference which leads to the preferential formation of the bare HSO_4^- ion which was used for quantitative evaluation.

3.2. Structural elucidation of metal complexes by ESI-MS

ESI-MS ideally complements other spectroscopic techniques, such as infrared absorption and ^{1}H- or ^{13}C-NMR spectroscopy, or crystallographic investigations, for the structural characterisation of metal complexes. A particular advantage is that it does not require any particular sample pretreatment other than the dissolution of the sample in a suitable solvent, and that samples can be introduced either directly or after chromatographic or electrophoretic separation. ESI-MS is thus perfectly suited for the characterisation of the elemental species actually present in aqueous solution, provided that these species can be transferred to the gas phase without alteration of their speciation.

As a consequence, the number of reports on structural elucidation or confirmation of metal species and complexes by ESI-MS is increasing strongly. It will be impossible to give a comprehensive account of all those examples in which ESI-MS has been used for the characterisation of inorganic complexes or complexes with small organic ligands. Instead, only some few recent examples have been selected from the literature and are summarised in Table 3. It is important to point out that the data presented in this table has been mostly obtained by ESI-MS with direct sample introduction. It nicely demonstrates that ESI-MS is a versatile technique for speciation analysis even without preceding chromatographic or electrophoretic separation. The individual examples will not be discussed in detail here, since the role of ESI-MS in the majority of these reports is restricted to confirming the identity of the proposed structures, rather than to gain further insight into the ion-formation process or the gas-phase chemistry of a particular species.

3.3. Application of ESI-MS for the speciation analysis of organometallic compounds

ESI-MS has increasingly been applied to the speciation analysis of low-molecular-mass organometallic compounds in recent years [85,86]. Low-molecular-mass organometallic compounds and redox species are of particular importance in environmental analysis (e.g., organotin and organomercury compounds), and in food and nutrition-related studies (e.g., organoselenium and organoarsenic compounds). As the analytes very often are polar or ionic compounds, the advantage of coupling liquid-phase separation and ESI-MS detection becomes evident. In contrast to ICP-MS, ESI-MS is able to positively identify unknown or to confirm the presence of known species based on the molecular mass spectra even when authentic reference compounds are not available. Due to the softness of electrospray ionisation and the comparatively low information content of ESI mass spectra obtained at low fragmentor voltage, this challenging task is mostly achieved by MS^{n} experiments (performed with either a triple quadrupole or an ion trap instrument). A number of convincing examples exists in this field, and they shall be discussed according to the metal the species contains.

3.3.1. Speciation of arsenic by API-MS

The speciation of arsenic compounds is of significant importance, since the toxicity of most of the organic As species [such as arsenobetaine (AsB) and arsenocholine (AsC)] and that of the inorganic As species, As(III) and As(V), differs significantly [87]. Since high amounts of organic arsenic species are contained in seafood, it is important to control the speciation of arsenic therein. Table 4 summarises the analytical methods for speciation of arsenic compounds by ESI- or APCI-MS. Schramel et al. have described both the instrumental developments and the analytical performance of CE–ESI-MS applied to arsenic speciation [88]. Six arsenic species could be separated and detected by this technique in standard solutions and in human urine at high μg l^{-1}-levels (for monomethylarsonic acid, MMA) to low mg l^{-1}-levels [for As(III) and As(V)] in the positive-ion detection mode. The problem of long retention times of the As species due to the particular interface design was alleviated by using a pressure-assisted elution of the species after their complete separation in the electrophoretic separation mode.

The chromatographic separation of arsenic species is very often a two- or multi-step procedure which starts with a low(er) performance separation or fractionation of the sample, e.g., using size-exclusion chromatography. With an element-specific detector

Table 3
Examples for the determination of low-molecular-mass metal species or metal complexes by ESI-MS

Metal/complex	Comments	Ref.
Ni		
Five-coordinate diastereomeric Ni–*N*-glycoside complexes	IT-MS, use of product ion spectra for identification	[218]
Fe		
Fe(III) chelate complexes	ES-IT-MS and laser desorption/ionisation FT-ICR-MS investigation of Fe(III) chelate complexes in the presence of citrate and 3-hydroxy-2-methyl-propyl-1H-pyridin-4-one	[219]
Fe(III) complexes with siderophores	ESI-MS of Fe(III) dihydroxamate siderophore complexes (alcaligin and rhodotorulic acid) and synthetic analogues	[220]
Lanthanides		
$Nd_2(S_2O_6)_3$ and $Pr_2(S_2O_6)_3$	Observed species: $Ln(S_2O_6)^+$ and $Ln(S_2O_6)_2^-$ and their methanol solvates with Ln=Nd, Pr	[221]
Sc, Lu–Nd	Triphenylphosphine oxide–triflate complexes of Sc, and Lu–Nd, observation of extensive ligand redistribution by ES	[222]
Nd, Sm, Y, Tm	Characterisation of organolanthan species $[(C_5Me_5)_2Ln][BPh_4]$ (Ln=Nd, Sm, Y, Tm) in different solvated forms. Also observation of bimetallic compounds	[223]
Transition metals		
Pt, Os, Ru, Pd	Transition-metal halide complexes with Et_2S, PPh_3, pyridine, and bidentate phosphine $Ph_2PCH_2PPH_2$ (dppm)	[224]
Pd	Pd–diimine complex used as catalyst for Ziegler–Natta polymerisation, allows the detection of Pd-adducts of the polymer at different polymerisation degree	[225]
Pd and Pt	Maleonitrile–dithiacrown ether complexes of $PdCl_2$ and $PtCl_2$	[226]
Isopolyoxovandate species	In NI-ESI-MS, two ion series detected: $[H_xV_yO_z]^-$ with $x=0$ to 1; $y=1$ to 10; $z=3$ to 26 and $[H_xV_yO_z]^-$ with $x=0$; $y=3$ to 17; $z=9$ to 44 further: $[H_2VO_4]^-$, $[V_{10}O_{28}]^-$, $[H_5V_{10}O_{28}]^-$; in PI-ESI-MS, three ion series detected: $[H_{m+1}(VO_3)_m]^+$, $[H_{m-1}V_mO_{3m-1}]^+$ and $[H_{m-3}V_mO_{3m-2}]^+$ In the presence of alkali ions ($A=Li^+$, Na^+, K^+): in NI-ESI-MS, $[AV_mO_{3m-2}]^-$ with $m=2$, 4, 6 and in PI-ESI-MS, two ion series detected: $[A_{m+1}(VO_3)_m]^+$, $[A_{m+3}V_mO_{3m+1}]^+$	[227]
Perrhenate species	NI-ESI-MS detection of $[ReO_2]^-$ and $[ReO_3]^-$ as in-source reaction products of $[ReO_3]^-$ and $[ReO_4]^-$	[228]
Isopolyoxorhenate species	PI-ESI-MS shows existence of $[A_{x+1}Re^{VII}O_{4x}]^+$ (for NH_4^+, Na^+, K^+: $x=1$–5) and $[K_{x+2}Re^{V}Re_x^{VII}O_{4x+3}]^+$ for K^+ with $x=0$–4	
Pt Cu Ag	Reaction and redistribution of $[Pt(dppf)(CCPh)_2]$ with $[Cu(NCMe)_4]BF_4$ and $AgBF_4$ with dppf=bis(diphenylphosphino)ferrocene, yielding intense PI-ES mass signals	[229]
Ru	ESI-MS investigation of carbonyl clusters obtained from the reaction of $[Ru_6C(CO)_{17}]$ and $[PPN][M(CO)_4]$ with M=Co or Ir. Observation of energy-dependent stability of clusters	[230]
Ag		
$[Ag(EPh_3)]^+$	Triphenypnictogen ligand complexes EPh_3 with E=P, As, Sb and Bi	[231]
Ag(II)	Coordination complexes of Ag(II) with a broad range of C-, N-, and O-containing ligands e.g., benzene, pyridine, THF	[232]
Cu		
$[Cu(EPh_3)]^+$	Triphenylpnictogen ligand complexes EPh_3 with E=P, As, Sb and Bi	[231]
Cu complexes with zwitterionic buffers	Observation of Cu complexes with some zwitterionic buffers, e.g., 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (HEPPS), and *N*-(2-hydroxyethyl)-piperazine-*N′*-hydroxypropanesulfonic acid (HEPPSO) by ES-TOF-MS	[233]
Cu complexes with 5-Br-PADAP	Observation of binuclear Cu complexes with 5-Br-PADAP [=2-(5-bromo-2-pyriylazo)-5-diethylaminophenol in MeOH–water by ES-TOF-MS	[234]

Table 3. Continued

Metal/complex	Comments	Ref.
Alkali metals		
Li^+, Na^+, K^+, Rb^+, Cs^+	Caged crown-ether complexes of alkali metals ligands: 15-crown-5, 1,7-diaza-15-crown-5, 18-crown-6	[235]
Na^+, K^+	ESI-MS of complexes with diethyldithiocarbamates, diphenylthiocarbazone and glutathione, study of electrochemical and pH effects, complex stability determination	[236]
Various	ESI-MS of complexes with alicyclic and aliphatic diketones with keto groups in close proximity	[237]
Alkaline earth metals		
Ba^{2+}	Cryptand complexes of $[BaL]^{2+}$ with L = cryptand, formed by template polymerisation of tris(3-aminopropyl)amine with 2,6-diformylpyridine	[238]
Ca^{2+}, Mg^{2+}, Mn^{2+}	ESI-MS of complexes with diethyldithiocarbamates, diphenylthiocarbazone and glutathione, electrochemical and pH effects, complex stability determination	[237]
Various	ESI-MS of complexes with alicyclic and aliphatic diketones with keto groups in close proximity	[237]
Manganese		
Mn(II) and Mn(III) complexes with salen	Salen = *N*,*N′*-ethylenebis(salicylidenaminato) ligand	[239]
	Investigation of oxygen transfer to manganese–salen complexes by ES-MS–MS	[240]
	Investigation of salen (*S*) complexes of Me = Cr^{3+}, Mn^{3+}, Co^{3+}, $[SMe]^+$ and particularly the evaporation rate of water from the solvation shell $[SMe(H_2O)_n]^+$	[241]
Chromium		
Polyoxochromate species	Dichromate salts with Li^+, Na^+ and K^+, leading to series of: $[A_{x+1}H_xCr_x^{VI}O_{4x}]^+$ (for Li: $x=1–5$, for Na: $x=1–7$, for K: $x=1–4$) and $[A_{2x-1}Cr_x^{VI}O_{4x-1}]^+$ (for Li: $x=2–3$, for Na: $x=2–4$, for K: $x=2–3$) and $[HCr_x^{VI}O_{3x+1}]^+$ for NH_4^+ with $x=1–5$	[242]
Various		
EDTA-complexes of Al, Cd, Cu, Co, Mn, Ni, Pb, Zn	Separated by IC, detection by NI-ESI-MS detection limits: 0.1–1 *M*	[243]
Alkali, alkaline earth and transition metals (Ag, Ba, Cd, Co, Cs, Cu, K, Mn, Ni, Rb, Sr, Zn)	Determination of alkali, alkaline earth and transition metal ions by post-column addition of 18-crown-6-ether and PI-ESI-MS	[244]
Alkali and alkaline earth metals (e.g., K, Ba)	Assessment of complex cation crown-ether equilibria by ESI-MS with 18-crown-6-carboxylic acid crown ether	[245]
Metal cations, metal complexes and anions	Identification and determination of metal cations, metal complexes (with EDTA, Cl^-, NO^{3-}), and anions by NI-ESI-MS	[246]
Hg(II), Pb(II), Cd(II), Zn(II)	Thiacrown ether macrocycle complexes of Hg, Pb, Cd, Zn formation and selectivity as function of heteroatom, macrocyle ring size and attached cage groups studied by ESI-MS	[247]
Tetraethyl-orthosilicate	ESI-FT-ICR-MS of orthosilicates formed by sol–gel process, ionised by addition of Na^+ or Ni^{2+}	[248]
Silicate oligomers	ESI-MS of silicate oligomers distribution during polymerisation in the presence of TMAH or TEAH	[249]
Ti(IV) catalyst	ESI-MS and NMR investigation of stoichiometry Ti(IV) sulfoxidation catalyst	[250]
Antimony compounds		
Sb(III), Sb(V), $TMSbCl_2$, $TMSb(OH)_2$	PI-ES-TOF-MS of inorganic and organic Sb compounds: $TMSbCl_2$, $[TMSb(OH)_2]$, potassium and NI-ES-TOF-MS hexahydroxyantimonate [Sb(V)] and potassium antimonyl tartrate [Sb(III)]	[251]
Sb(III), Sb(V)	Detection of citrate complex of Sb(III) and Sb(V) by ESI-MS; citrate complexes separated by anion-exchange chromatography and detection by ICP-MS	[252]
$TMSbCl_2$	CE–ESI-MS of metal complexes such as $TMSbCl_2$	[137]
$TMSbCl_2$ and TMSbOH	ESI-MS of $TMSbCl_2$ in aqueous solution	[253]

Table 4
Overview of the use of ESI-MS for speciation analysis of organoarsenic compounds

Element/species	LOD	Matrix	Method	Chromatographic conditions	Ref.
As(III) As(V) MMA DMA AsB AsC	51 mg l^{-1} 1.6 mg l^{-1} 480 g μl^{-1} $<$600 μg l^{-1} $<$600 μg l^{-1} $<$600 μg l^{-1}		CE–ESI-MS	n/a	[88]
Arsenate As (V), dimethylarsinic acid (DMA), arsenobetaine (AsB). Arsenosugars: arsenosugar A, arsenosugar B, arsenosugar C, arsenosugar D. Trimethylarsenic oxide (TMAO) $(CH_3)_3AsO$, tetramethylarsonium ion (TMAs) $(CH_3)_4As^+$ I^-	Used only qualitatively	Chinese edible seaweed (*Laminaria japonica*, *Porphyra crispata* and *Eucheuma denticulatum*)	Anion-exchange chromatography with NI-ESI-MS detection (MRM)	Hamilton PRP-X100; 20 m*M* NH_4HCO_3 (pH 7.7) in 20% MeOH	[89]
Arsenosugars: arsenosugar A, arsenosugar B, arsenosugar C	ca. 150 pg (abs.)	Ribbon kelp extracts	Anion-exchange chromatography with PI-ESI-MS–MS	Hamilton PRP-X100, $(NH_4)_2CO_3$ 20 m*M*, pH 9.0	[90]
Arsenosugars: arsenosugar A, arsenosugar B, arsenosugar C, arsenosugar D and their trimethylarsino analogues	Low pg abs. level	Algal extract (*Sargassum laceriforium*)	PI-ES-Q-TOF-MS (direct infusion)	MeOH–water (1:1)	[93]
Arsenosugars: arsenosugar A, arsenosugar B, arsenosugar C, arsenosugar D	Used only qualitatively	Oysters (*Crassostrea gigas*)	Anion-exchange chromatography with PI-ES-single quadrupole MS	Hamilton PRP-X100, KH_2PO_4–K_2HPO_4, 10 m*M* (pH 5.8)	[94]
Methylarsonic acid 4-Hydroxyphenylarsonic acid *p*-Arsanilic acid Dimethylarsinic acid 3-Nitro-4-hydroxyphenylarsonic acid 4-Nitrophenylarsonic acid Arsenobetaine Trimethylarsine oxide Arsenocholine Tetramethylarsonium ion	305 pg ml^{-1} 250 pg ml^{-1} 107 pg ml^{-1} 72 pg ml^{-1} 150 pg ml^{-1} 145 pg ml^{-1} 2 pg ml^{-1} 40 pg ml^{-1} 21 pg ml^{-1} 15 pg ml^{-1}	None (pure standards)/ urine SRM 2670 (NIST) for AsB	μ-RP-HPLC with ESI-MS–MS detection with PI SRM	Spherisorb 150×1 mm, 3 μm; water–MeOH (80:20)+1% AcOH	[95]
Arsenosugars A–D, MMA, DMA, AsB As(III), As(V)	n/a	Seaweed (*Laminaria*)	ESI-MS–MS (PI detection)	(1) SEC: Superdex Peptide HR 10/30 (2) AEC: Supelcosil SAX1, $NH_4H_2PO_4$–$(NH_4)_2HPO_4$ 5 m*M*	[97]
Unknown 1=5-dimethylarsinoyl-β-ribofuranose, AsC, TMAs$^+$, arsenosugar A, AsB, AsB-CH_2, DMA, unknown 2, arsenosugar B	n/a	Oyster test reference material	ESI-MS–MS (PI detection)	(1) CEC: Supelcosil LC-SCX (250×4.6 mm I.D., 5 μm, pyridine–formate 20 m*M*). (2) RP-HPLC: Inertsil ODS-2 (250×4.6 mm I.D., 5 μm, malonic acid 4 m*M*). (3) SEC: Superdex Peptide HR 10/30 (300×13 mm I.D., 13 μm) AcOH 1%	[98]

Table 4. Continued

Element/species	LOD	Matrix	Method	Chromatographic conditions	Ref.
Standards: 1: As(III), 2: As(V), 3: monomethylarsinic acid (MMA), 4: dimethylarsinic acid (DMA), 5: arsenobetaine, 6: arsenocholine, 7: arsenosugar A, 8: arsenosugar B, 9: arsenosugar C, 10: arsenosugar D, 11: tetramethylarsonium, 12: dimethylethylarsine, 13: dimethylacetylarsine.	n/a	Algal extract (*Hizikia fusiforme*)	Multidimensional HPLC–ICP-MS and ESI-MS–MS	(1) SEC: Superdex Peptide HR 10/30 (300×13 mm I.D., 13 µm) AcOH 1%. (2) AEC: Hamilton PRP-X100 (250×4.6 mm, 10 µm, phosphate buffer, pH 6). (3) CEC: Supelcosil LC-SCX (250×4.6 mm I.D., 5 µm, pyridine–formate, pH 3, 20 m*M*). (4) RP-HPLC: Inertsil ODS-2 (250×4.6 mm I.D., 5 µm, malonic acid, 4 m*M*)	[100]
Standards: 1, arsenosugar D; 2, arsenosugar A; 3, arsenosugar C; 4, As(V); 5, DMAA; 6, MMAA; 7, arsenosugar B; 8, TMA cation; 9, arsenocholine; 10, As(III) 11, Arsenobetaine 12, TMA oxide	n/a	Edible algal food samples (*Hizikia fusiforme*, *Laminaria*, *Sargassum lacerifolium*, etc.)	SEC fractionation; anion-exchange HPLC with ICP-MS and ESI-MS–MS detection	(1) SEC: Superdex Peptide HR 10/30 (300×13 mm I.D., 13 µm) AcOH 1%. (2) AEC: Supelcosil SAX1 (250×4.6 mm, 10 µm, phosphate buffer pH 6);	[101]
$TMAs^+$, DMA, MMA, As(V), AsC, AsB, arsenosugars A–D, four further ribofuranosides, 5-dimethylarsinoyl-2,3,4-trihydroxypentanoic acid	n/a	Kidney of the clam *Tridacna derasa*	Three-dimensional SEC–AEC CE–HPLC–ICP-MS and ES-Q-TOF-MS–MS detection	(1) SEC: Sephadex G-15 preparative column (1% AcOH). (2) CEC: Supelcosil LC-SCX (250×4.6 mm, 10 µm, 5 µm, pyridine–formate, pH 3, 20 m*M*). (3) AEC: Hamilton PRP-X100 (250×4.6 mm, 5 µm, NH_4Ac–AcOH, pH 4, gradient)	[102]
Arsenosugars A–D, 5-dimethylarsinoyl-2,3,4-trihydroxypentanoic acid (and/or its lactone)	n.d.	Kidney of the clam *Tridacna derasa*	AE/CE–HPLC–ESI-MS, partial sample clean-up by SEC	(1) SEC: Sephadex G-15 preparative column. (2) AEC: Hamilton PRP-X100 (150×4.1 mm, 5 µm, water (NH_4HCO_3, 20 m*M*, pH 9)–MeOH (9:1). (3) Ionospher-C: (100×3 mm; pyridine–formate, pH 3, 20 m*M*)	[103]
Arsenosugars A–D, arsenosugars E–G, As(III), As(V), MMA, DMA, TMAO, $TMAs^+$, AsB, AsC, DMAsAc, DMAsEt	n/a	As species in *Laminaria digitata*	LC–ESI-MS with variable fragmentor voltage	(1) SEC: Sephadex G-15 preparative column (1% AcOH), (2) Semi-preparative AEC: Sephadex A-25 [water (NH_4HCO_3, 20 m*M*, pH 9)–MeOH] (9:1). (3) RP-HPLC: Supelcosil LC-SCX (250×4.6 mm, 10 µm, 5 µm, malonic acid 4 m*M*)	[104]
Arsenosugar A Arsenosugar B Arsenosugar C Arsenosugar D AsB, DMA dimethylarsinoylacetic acid	1 µg As l^{-1} 2 µg As l^{-1} 6 µg As l^{-1} 10 µg As l^{-1} (based on a 10 µl injection and $S/N=3$)	Extracts of *Fucus vesiculosis* and *Laminaria digitata*	LC–ESI-MS wih variable fragmentor voltage	AEC: Hamilton PRP-X100 (250×4.1 mm, 5 µm, water (NH_4HCO_3, 20 m*M*, pH 9)–MeOH (90:10)	[105]
Arsenosugars A–D and standards of arsenite, arsenate, methylarsonate (MA), dimethylarsinate (DMA), arsenobetaine (AB), trimethylarsine oxide (TMAO), arsenocholine (AC) and tetramethylarsonium ion	n/a	Algal extract of *Fucus serratus*	LC–ESI-MS (and HPLC–ICP-MS)	AEC: Hamilton PRP-X100 (250×4.1 mm I.D., 10 µm, water (NH_4HCO_3, 20 m*M*, pH 10.3)–MeOH (90:10). CEC: Ionospher C (100×3 mm I.D., 5 µm); aq. pyridine (20 m*M*, pH 2.6 with HCOOH–MeOH (90:10)	[106]
Trimethylarsonium, dimethylarsinic acid, monomethylarsonic acid, arsenobetaine, arsenocholine, trimethylarsinoxide and dimethylarsinoylethanol	<30 pg μl^{-1}	Pure standard solutions	ES-IT-MS^n ($n=1$–6), direct infusion	Not given	[107]

Arsenosugars A–D		Extract of the seaweed *Fucus serratus* and kelp powder	ES-IT-MS^n ($n=2$–4)	RP-HPLC: Eclipse XDB-C_8 (250×4.6 mm I.D., 5 μm; water–acetonitrile, 9:1). AEC: Hamilton PRP-X100 (250×4.1 mm I.D., 10 μm, water (NH_4HCO_3, 20 m*M*, pH 10.3)–MeOH (90:10)	[108]
MMA DMA AsB AsC	1.25 ng ml^{-1} 0.54 ng ml^{-1} 0.18 ng ml^{-1} 0.25 ng ml^{-1}	Standards; AsB in DORM-2 (dogfish CRM) Standard solutions, tap water, DORM-2 dogfish CRM	In tube-SPME–AE–HPLC–ESI-MS	AEC: Supelcosil LC-SAX1 (250×4.6 mm I.D., 5 μm; water (100 m*M* NH_4Ac + AcOH 0.6%)–MeOH (70:30)	[109]
MMA DMA TMAO TeMA AsB	200 (CE) 153 (AE) 78 (CE) 140 (AE) 105 (CE) 68 (AE) 75 (CE) (33 (AE) 13 (CE) 70 (AE) pg As abs.	Rat urine	AE– and CE–ICP-MS and ESI-MS	CEC: Shodex RSpak NN-414 (150×4.6 mm I.D.), HNO_3 (8 m*M*)–NH_4NO_3 (5 m*M*). AEC: Gelpak GL-IC-A15S (175×3 mm I.D.), NH_4–formate buffer, pH 5.5	[110]
Arsenate, methylarsonate, dimethylarsinate, trimethylarsine oxide, tetramethylarsonium ion, arsenocholine, arsenobetaine, dimethylarsinoylethanol, dimethylarsinoylacetate, dimethylarsinoylriboside, trimethylarsonioriboside	n.d.	Metabolites of microbial-enriched seawater	AE– and CE–ICP-MS and ESI-MS	AEC: PRP-X100 (250×4.1 mm I.D., 10 μm) with 20 m*M* $NH_4H_2PO_4$, adjusted to pH 5. CEC: Supelcosil LC-SCX (250×4.6 mm I.D., 5 μm) with 20 m*M* pyridine adjusted to pH 2.6 with HCOOH	[111]
Arsenosugar A, arsenosugar B, DMA, MMA, As(V), As(III)	n.d.	Earthworm *Lumbricus terrestris*	AE– and CE–ICP-MS and ESI-MS	AEC: PRP-X100 (250×4.1 mm I.D., 10 μm) with 20 m*M* NH_4HCO_3, adjusted to pH 10.3 with NH_3	[112]
As(III), As(V), DMA, arsenosugars A–D	n.d.	Seaweed	AE–HPLC–ICP-MS and ESI-MS	AEC: PRP-X100, with 20 m*M* $(NH_4)_2CO_3$, adjusted to pH 9 with NH_3	[113]

(often ICP-MS) those fractions are identified which contain relevant species. These are pooled or analysed individually by a second separation technique of higher separation power such as RP-HPLC or IC. API-MS detection is either coupled on-line or again the relevant fractions are isolated and analysed off-line by ESI- or APCI-MS. If more than one elemental species is suspected in the individual fractions, the respective fractions are subjected to a further separation step using a different separation mechanism. The structural formulas and names of the most relevant organoarsenic compounds are given in Fig. 3.

Van Hulle et al. have applied both ICP-MS and ESI-MS coupled to HPLC for the speciation of arsenic in Chinese seaweeds [89]. Anion-exchange ESI-MS was successful in separating nine different compounds, among which were four arsenosugars (arsenosugars A–D) that were identified by their multiple reaction monitoring (MRM) transitions. ESI-MS was only used for the confirmation of the analytes while their quantitative analysis was performed by HPLC–ICP-MS with separation by cation- and anion-exchange chromatography in order to avoid coelution. [Coelution occurs for arsenosugars B–D on most cation-exchange columns and for arsenosugar B and As(III) and for arsenosugar C and MMA, respectively, on most anion-exchange columns]. Protonated quasi-molecular ions of the arsenosugars were detected, although it was stated that arsenosugars A–D can be detected in the negative ion mode with greater sensitivity. This study confirmed that the arsenosugars are the dominant arsenic compounds in seaweed and account for more than 80% of the total arsenic content. The only other major As species detected is DMA, while three unknown As species could be detected with ICP-MS, however at concentrations too low to be identified by ESI-MS.

The detection of three arsenosugars A–C in kelp extracts was also the focus of the work of Gallagher et al. [90] (see Fig. 4). They used cation chromatography on a PRP-X100 column with an $(NH_4)_2CO_3$ buffer and positive ion detection. In comparison to the ICP-MS measurements on the same column, the buffer concentration had to be reduced to 20 m*M* for better compatibility with ESI-MS detection. Under these conditions, no coelution of the arsenosugars with the other, more toxic arsenic species [As(III), As(V), MMA and DMA] occurred. The three arsenosugars with the molecular masses m/z 328, 392 and 482 have been identified by interpretation of their MS–MS fragmentation patterns. Pertinent to the mass spectra of all three arsenosugars are signals at m/z 97, 195 and 237 which are ascribed to the dimethylarsinoylriboside moiety, common to all the arsenosugars, and its fragments. These assignments are in agreement with the findings of Corr and Larsen [91] (except for a number of peaks that may probably be attributed to a phthalate contamination) and that of Pergantis et al. obtained by FAB-MS [92] and by nanoelectrospray Q-TOF-MS [93]. The detection limits for the three arsenosugars were given by Gallagher et al. as ca. 150 pg and are thus about 30 higher than with ICP-MS in the SIM mode. However, about 22 ng of each compound were required to obtain interpretable mass spectra.

More recently, Sánchez-Rodas et al. have used HPLC–ESI-MS and HPLC–UV–photooxidation–hydride generation (HG)–atomic fluorescence spectrometry (AFS) for the identification and quantitative determination of arsenosugars in oyster extracts [94]. After aqueous extraction, the oyster tissue extract was subjected to gel permeation chromatography followed by anion-exchange chromatography on a DEAE Sephadex column. This rather tedious sample preparation procedure was necessary in order to separate most of the matrix interferences. Still, the authors were only able to confirm the presence of two of the investigated arsenosugars while the presence of the other two compounds was not unambiguously demonstrated. Despite the proposed sample clean-up, signal suppression was observed to be ca. 50% as determined by standard addition experiments for the arsenosugar B.

The total As content in the investigated oysters was found to range from 12.5 to 19.1 mg kg^{-1} of which between 12 and 19% was arsenosugar B (as determined by external calibration and HPLC–UV−HG−AFS).

In an early study on the feasibility of ESI-MS–MS for the determination of organoarsenic compounds, Pergantis et al. [95] reported the use of microbore RP-HPLC with ESI-MS for the determination of ten organoarsenical compounds. The peak shape of the ten compounds achieved on a 150×1.0 mm C_{18}

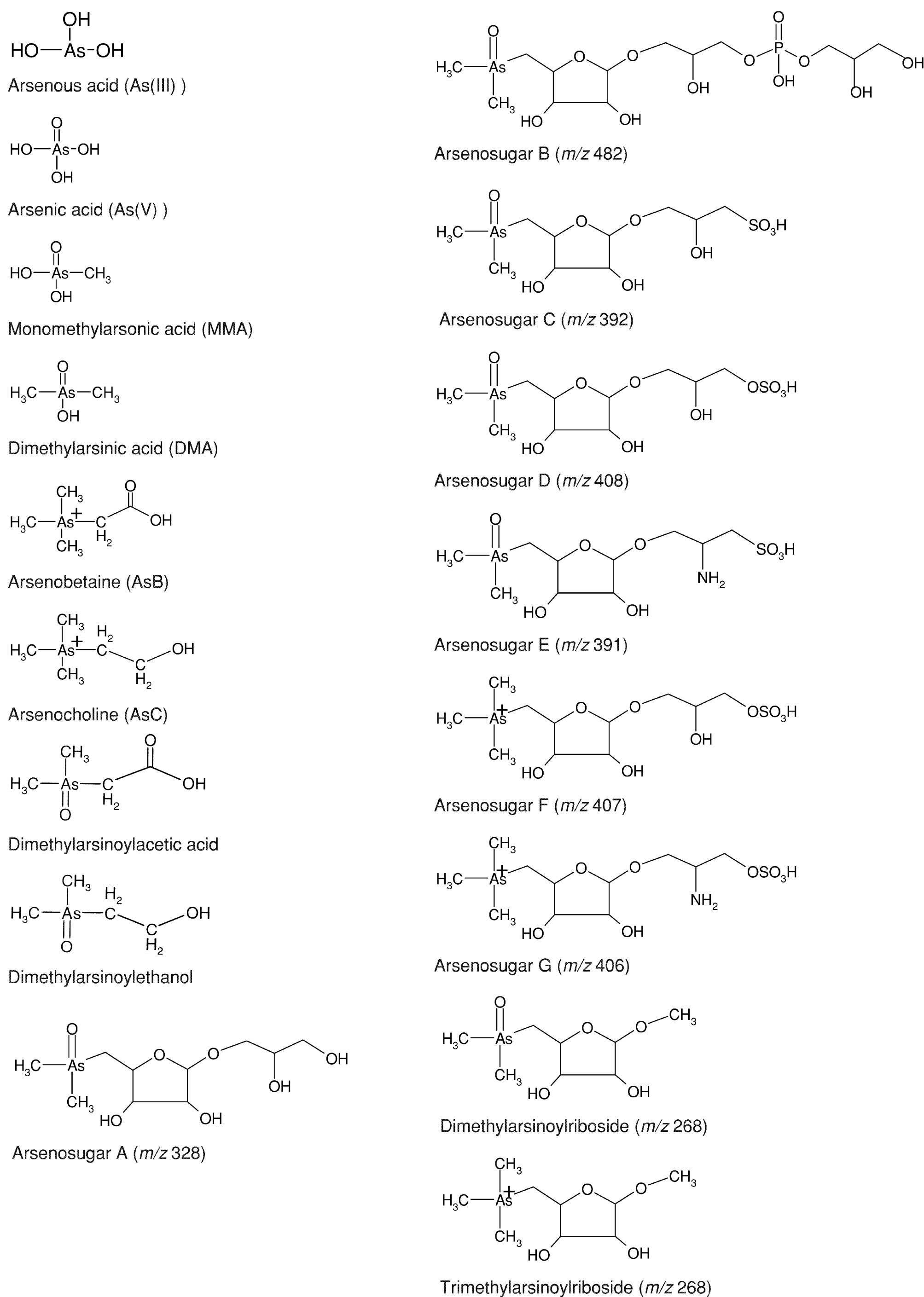


Fig. 3. Structures of important arsenic species. Arsenous acid As(III)]. Arsenic acid As(V)]. Monomethylarsonic acid (MMA). Dimethylarsinic acid (DMA). Arsenobetaine (AsB). Arsenocholine (AsC). Dimethylarsinoylacetic acid. Dimethylarsinoylethanol. Arsenosugar A (*m*/*z* 328). Arsenosugar B (*m*/*z* 482). Arsenosugar C (*m*/*z* 392). Arsenosugar D (*m*/*z* 408). Arsenosugar E (*m*/*z* 391). Arsenosugar F (*m*/*z* 407). Arsenosugar G (*m*/*z* 406). Dimethylarsinoylriboside (*m*/*z* 268). Trimethylarsinoylriboside (*m*/*z* 268).

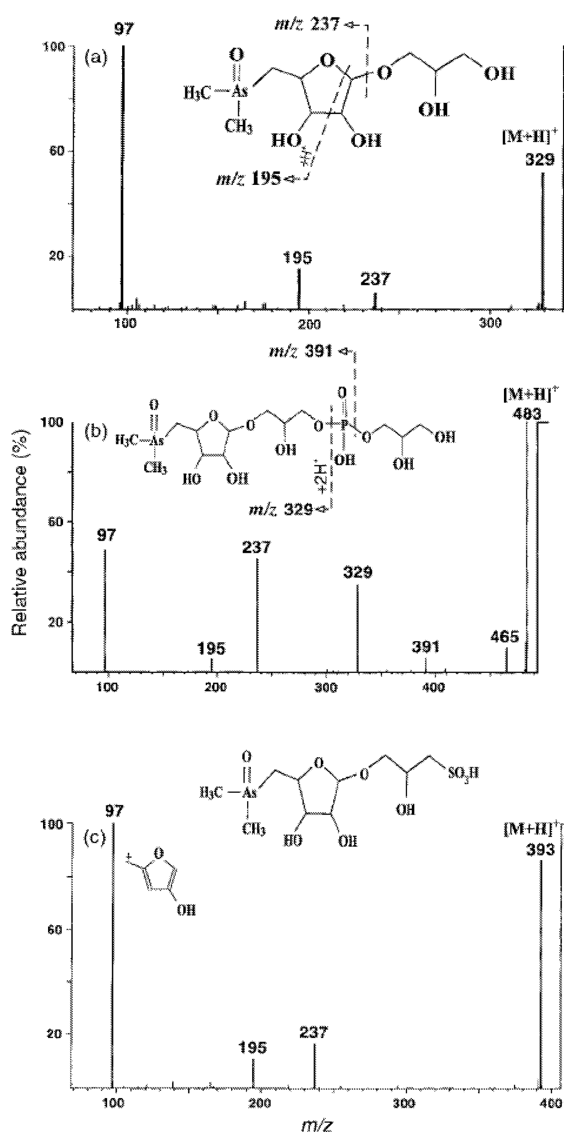


Fig. 4. Characteristic mass spectra of arsenosugars A (a), B (b) and C (c) obtained by ESI-MS–MS with assignment of the most important fragments (reproduced by permission of the Royal Society of Chemistry from Ref. [90]).

column was satisfactory except for the ionic arsenic compounds (tetramethylarsonioum, $TMAs^+$; arsenocholine, AsC and trimethylarsine oxide, TMAO) which exhibited strongly tailing peaks. Since the early eluting peaks could not be resolved chromatographically, their detection was achieved by multiple reaction monitoring of the protonated molecular ion with the triple-quadrupole mass spectrometer. As characteristic CID reactions could be found for all species, the selective and sensitive detection of all 10 organoarsenicals was achieved with detection limits in the 2–300 $pg\,ml^{-1}$ range, based on 1 μl injections. ESI-MS detection was most sensitive for the positively charged ions arsenobetaine and tetramethylarsonium. The authors also point out that some arsenic species can be detected with higher sensitivity in the negative ion (NI) mode, and particularly arsenite and arsenate can only be detected in NI mode but not in the positive ion (PI) mode. The applicability of the method was demonstrated by the analysis of AsB in a urine sample (NIST SRM 2670).

Continuing this work, Pergantis et al. investigated the potential of nanoelectrospray quadrupole time-of-flight mass spectrometry for the identification of arsenosugars at the picogram level [93]. The use of ESI-Q-TOF-MS allowed the unequivocal identification of two series of arsenosugars: in addition to the four dimethylated arsonioribosides (arsenosugars A–D), the mass spectra and fragmentation pathways of the four analogous trimethylated arsonioribosides (arsenosugars 5–8) were reported. The increase of the collision energy produced mass spectra of high information content which enables identification of the arsenosugars even in the absence of standards.

In an extension of earlier work of this group, Pickford et al. [96] made use of ESI-FT-ICR-MS for the identification of arsenic-containing compounds. The high mass resolution and accuracy provided by FT-ICR-MS allows one to assign elemental compositions and to thus differentiate arsenic-containing from non-arsenic containing species. Using this approach, the presence of some fifty arsenic species could be confirmed. As FT-ICR-MS is not widely available, the feasibility of this approach was tested with a more easily available hybrid-Q-TOF-MS instrument. The precision of exact mass determination with the Q-TOF-MS instrument is somewhat lower than with the ICR-FT-MS instrument (in the low ppm level). However, mass accuracy is high enough to allow the identification of As species even in a barely fractionated kelp extract. Although the authors are very optimistic as concerns the ability of this procedure for the identification of As species, it has to be realised that even high resolution MS

measurements do not allow to screen for unknown As species a priori, but rather to identify them a posteriori once they have been recognised as As containing species.

McSheehy et al. proposed pneumatically-assisted ESI MS–MS as a technique for the identification of dimethylarsinoyl-riboside derivatives (arsenosugars) in seaweed (*Laminaria*) [97]. The conditions for the acquisition of MS and MS–MS spectra were optimised. A size-exclusion HPLC (SE-HPLC) step was developed for the purification of algal extracts prior to ESI-MS in which the arsenosugar fraction eluted prior to the majority of other arsenic compounds. The identity of the compounds expected to be arsenosugars was confirmed by CID of the relevant protonated molecule ions. An independent confirmation of the identity of analytes was obtained by two-dimensional (size-exclusion–anion-exchange) HPLC–ICP-MS with signal identification by spiking with the appropriate arsenosugar standards. The combination of size-exclusion and anion-exchange chromatography is not only imperative for removing matrix interferences which would greatly suppress electrospray ionisation, but also since in the anion-exchange separation some of the highly relevant arsenic species [As(III) and arsenosugar B, and MMA and arsenosugar C] coelute in the anion-exchange separation under the given conditions (Supelcosil SAX1 column with 5 m*M* ammonium phosphate buffer). In the SEC separation, however, the arsenosugars, As(III) and MMA elute in different fractions.

In a further study devoted to the investigation of the potential of oyster tissue as a candidate reference material, McSheehy et al. applied both multidimensional HPLC–ICP-MS and ESI-MS–MS for the quantitative analysis of arsenic species as well as for their positive identification [98]. A meticulous procedure was developed for the clean-up of the oyster tissue extract and the fractionation of the arsenic species: in the first step size-exclusion chromatography was used to enrich the arsenosugar fraction and to separate it from high-molecular-mass water-soluble proteins and from the sample matrix containing smaller organoarsenic species. The two SEC fractions containing arsenic (the higher-molecular-mass arsenosugar fraction and the low-molecular-mass fraction) were further separated by anion-exchange LC. Cation-exchange chromatography was applied as the third separation dimension to the void of the anion-exchange separation. The concentration of the arsenic species in the individual fractions was determined by ICP-MS while the purity of each fraction was assessed by the above outlined three-dimensional fractionation/separation procedure. ESI-MS–MS was used to ascertain the identity of the detected organoarsenicals. In addition to confirming the presence of already known compounds such as arsenosugars B and D or DMA, ESI-MS–MS allowed the authors also to identify the presence of two arsenobetaine-like species. Both species exhibit a pronounced signal at m/z 193, and from the CID fragmentation pattern this structure can be attributed to a compound, trimethyl(2-carboxyethyl)arsonium inner salt, which recently was also reported by Francesconi et al. [99]. The second unknown compound which contained the same substructure was tentatively identified as 5-dimethylarsinoyl-β-ribofuranose, and the corresponding fragmentation pattern was given. In this particular tissue material, AsB accounted for more than 60% of the total arsenic, while the two arsenosugars A and B each contributed to less than 10% of the total arsenic content.

A similar analytical approach was applied in a further paper by McSheehy et al. [100] to arsenic speciation in algae. It evolves as a consequence of earlier work in that group in which high-performance size-exclusion chromatography was proposed for the isolation of anionic arsenosugars from an algal extract [101]. The matrix in the isolated fraction was simple enough to allow the identification of two arsenosugars. However, the two-dimensional SEC–AEC followed by HPLC–ICP-MS revealed several other peaks corresponding to minor arsenic species that could not be detected by ESI-MS–MS, probably because of too low concentration. Consequently, McSheehy et al. strived for an in-depth investigation of the chromatographic purity of signals in two-dimensional SE–AE chromatography of algal extracts. They used different separation mechanisms based on different principles, such as reversed-phase and cation-exchange in order to determine the number of arsenic species actually present in the sample extract (of an algae from a popular *Hizikia* family). Particular attention was paid to the optimisation of

the buffer used in the various separations steps to allow its elimination prior to ESI-MS–MS. For this purpose, malonic acid, which shows weak ion-pairing properties but can readily be removed by freeze–drying, was used for RP-HPLC separation instead of the common ion pairing reagents which compromise ESI. The authors also emphasise the danger of misidentifying arsenosugar A when ESI-MS is used without preceding chromatographic separation: The characteristic ion at m/z 329 may arise both from the protonated quasi-molecular ion of arsenosugar A as well as from fragmentation of arsenosugar D (after loss of the SO_3H-group). It appears thus that in the presence of both arsenosugars, non-interfered quantitative analysis of both arsenosugars is only possible after separation of the two compounds.

Increasing the degree of sophistication of their method, McSheehy et al. [102] applied ESI-TOF-MS–MS after multi-dimensional separation for the characterisation of arsenic species in kidney of the clam *Tridacna derasa*. The water-soluble species were isolated by three-dimensional LC (size exclusion–anion-exchange–cation-exchange) as described previously, and the elution of arsenic was monitored by ICP-MS in the individual fractions. The use of tandem Q-TOF-MS detection with the ability to provide exact mass information provided for the identification of in total 15 organoarsenic species. Thirteen of these possessed the dimethylarsinoyl group (eight ribofuranosides, four acyclic compounds, and one dihydroxyfuran). Four of these species were previously unreported. Arsenobetaine and dimethylarsinic acid were also detected. The major species (accounting for up to 50% of the water-soluble arsenic fraction) was 5-dimethylarsinoyl-2,3,4-trihydroxypentanoic acid. Again, particular concern of the authors was focused on the selection of the mobile phase for the size-exclusion chromatographic separation. The use of acetic acid (1%) in the mobile phase not only provided a recovery of 94% of the arsenic species introduced onto the SEC column, but was also practical since the volatile additive could easily be removed in the lyophilisation step prior to ion-exchange chromatographic separation.

This result corroborates the earlier report of Francesconi and Edmonds [103] who have found the same compound in kidney of the same giant clam species, *T. deresa*, and were able to identify it by LC–ESI-MS, although with a less sophisticated method (after cation- or anion-exchange chromatography of the crude aqueous extracts of the clam kidney). They demonstrated that about 50% of the water-soluble arsenic was present as dimethylarsinoylribosides and dimethylarsinate which are common algal metabolites. The major compound in the kidney was identified as a 5-dimethylarsinoyl-2,3,4-trihydroxycarboxylic acid, a new natural product. The authors discuss that the signal at m/z 253 in addition to the expected signal at m/z 271 for the proposed compound may either be due to dehydration of the analyte in the ion source, or due to the formation of a lactone, leading to the ribose ring. It is interesting that the 5-trimethylarsinyl-2,3,4-trihydroxycarboxylic acid which was produced by reduction and methylation of the parent compound with DTT and CH_3I did not lead to the formation of the lactone.

In a further report of McSheehy et al. [104], electrospray MS–MS in tandem with ICP-MS after multidimensional separation (size-exclusion, anion-exchange and reversed-phase chromatography) enabled the detection and identification of a compound in the extract of *Laminaria* algae that was previously unreported. The newly identified compound is an arsenosugar (arsenosugar F), and together with arsenosugars A–D and DMA it accounts for ca. 99% of the extractable arsenic present in the algae. Arsenosugar G is the trimethylarsinyl analogue of arsenosugar C with the alkyl chain hydoxy-group replaced by an amino group, this leading to a molecular mass of 406 (or m/z 407 for the quasi-molecular ion).

A highly interesting approach for the speciation of arsenic compounds was taken by Pedersen and Francesconi [105]: in an attempt to obtain both elemental and molecular information within one chromatographic separation, they used a single-quadrupole ESI-MS instrument (Agilent 1100 series MSD) with programmable fragmentor voltage. The quasi-molecular peaks of the arsenic compounds were recorded at low fragmentor voltage (70–100 V), while the peak at m/z 237 was recorded at intermediate (120–150 V) and the fragment at m/z 75 at high fragmentor voltage (230–250 V). Doing so, the authors demonstrated the ability of ESI-MS

for providing both element specific as well as compound-specific information for arsenic compounds. With this mode of operation, however, the positive ion detection mode becomes imperative, since the bare arsenic ion cannot be detected in negative ion mode. In contrast to this, arsenosugars can produce both positive and negative ions, with the latter claimed to be analytically more useful since they have higher intensity and produce more characteristic product ions [92,93]. The intensity ratio of parent and fragment ions in the variable fragmentor voltage-mass spectra of arsenosugars was a useful indicator for their identity. Based on this criterion, the presence of arsenosugars B–D could be confirmed in extracts of *Fucus vesiculosis* and *Laminaria digitata*, while the ion ratio differed too much to corroborate the occurrence of arsenosugar A. Although not supported by experimental data, the authors claim that the simultaneous elemental/molecular detection mode with ESI-MS will be possible for all organoarsenic compounds containing either the quaternary arsonium group or the dimethylarsinoyl group. However, the two most important inorganic species, arsenite and arsenate, will not be amenable to the detection of As^+ in the positive ion mode.

In a further study, Madsen et al. investigated the speciation of arsenic in a number of algal extracts which were prepared as check samples for arsenic speciation studies [106]. Quantitative analysis of the four major arsenosugars present was performed by HPLC–ICP-MS. HPLC coupled with electrospray MS provided structural verification of the compounds in addition to corroborative quantitative data. The material chosen was an extract of the brown algae *Fucus serratus* which are known to contain the four arsenosugars A–D. From the analytical point of view, the authors used the well-established separation on a PRP-X100 anion-exchange column with a NH_4HCO_3–MeOH mobile phase, or with an Ionospher C cation-exchange column with a mobile phase consisting of pyridine–MeOH, adjusted to pH 2.6 with HCOOH. Quantitation of the 12 arsenic species investigated [arsenite, arsenate, methylarsonate (MA), dimethylarsinate (DMA), arsenobetaine (AB), trimethylarsine oxide (TMAO), arsenocholine (AC) and tetramethylarsonium ion and the four arsenosugars A–D] was achieved by ICP-MS, while ESI-MS was basically used to confirm the identity of the four arsenosugars. The acquisition of ESI-MS chromatograms at different fragmentor voltages allowed to induce increased fragmentation of the arsenosugars, thus leading to the characteristic fragments at m/z 237 and 75. The fact that the intensity ratios observed with pure standards could not be reproduced for all arsenosugars (in particular for the hardly retained arsenosugar A) points to the strong matrix effects which are caused by the algal matrix and which cannot be eliminated by single-quadrupole MS.

While most of the work on ESI-MS speciation analysis of organoarsenicals has been done with single and triple quadrupole-MS instruments, and in some instances also (Q-)TOF-MS has been used, there surprisingly appears to exist only two reports on the utilisation of ion-trap-(IT-) MS for this task. This is the more surprising as ion-trap instruments offer the attractive feature of acquiring multiple mass spectra which greatly simplifies the interpretation of the spectral features. One example is given by Larsen et al. [107] who report on the use of LC–MS for the study of CID fragmentation of seven organoarsenical compounds likely to occur in the environment, i.e., trimethylarsonium, dimethylarsinic acid, monomethylarsonic acid, arsenobetaine, arsenocholine, trimethylarsinoxide and dimethylarsinoylethanol. Although MS–MS spectral interpretations have been proposed before in the literature for all arsenic compounds except dimethylarsinoylethanol (which was custom synthesised) IT-multiple mass spectrometry experiments (MS^n with $n=1$–6) were found useful to confirm these interpretations. In addition, CID data on dimethylarsinoylethanol was presented for the first time.

The authors conclude that the MS^n data are in good agreement with data obtained by triple quadrupole MS–MS analysis. However, as the energy available from resonant excitation collision with helium atoms in the ion trap is significantly lower than that available from collision with argon in a triple quadrupole mass spectrometer, this alters the relative intensities of the observed fragments in the CID mass spectra. Also the formation of reduced fragment ions containing As(I) is not observed. Organoarsenic compounds are mainly fragmented into even electron product ions after elimination of

neutral fragments, although some abundant odd electron fragments are also formed after elimination of radical species possibly driven by redox processes in which As(V) is reduced to As(III).

Continuing their own studies and the report of Larsen et al. [107], Miguens-Roderíguez et al. [108] applied ESI-ion trap-MS to the identification of arsenosugars in seaweed extracts. Both reversed-phase and anion chromatography were coupled on-line with ESI-IT-MS. Diagnostic information on the arsenosugars was obtained from multiple MS experiments (MS^n with $n=2$–4). The spectra not only contained characteristic quasi-molecular and fragment ions for all four arsenosugars, but also provided a signal common to all four arsenosugars at m/z 237 (attributed to the oxonium ion of the diemthylarsinoyl moiety) which is observed either in the MS^2 spectrum (for arsenosugars A and C) or in the MS^3 spectrum (for arsenosugars B and D). The proposed RP-HPLC–IT-MS method is very simple and provides the identification and quantitative analysis of the four arsenosugars. Due to coelution of the arsenosugars on the RP column, selected reaction monitoring has to be applied. The earlier described anion-exchange separation is thus preferred, as it provides baseline separation of all four arsenosugars, although a more thorough sample cleanup has to be performed in this case.

In a further paper reporting improvements for the speciation analysis of organoarsenic compounds, Wu et al. [109] investigated the use of in-tube solid-phase microextraction (SPME) for sample enrichment. In-tube SPME is a relatively new preconcentration technique where hydrophobic analytes are enriched in a short piece of capillary column (60 cm×0.25 mm I.D.) which is internally coated with a polymer suitable for the extraction of the analytes. The advantage of this approach lies in the min amounts of sample required and in the automation which improves reproducibility as compared to off-line sample enrichment. Due to the polarity of the analytes, however, capillaries with (apolar) coatings as typically used in gas chromatography proved to be not as useful as a more polar, in-situ polymerised polypyrrole, which was chosen as a more suitable stationary phase for extraction. Enrichment of the four arsenic compounds under study (MMA, DMA, AsB and AsC) was achieved by aspirating a variable number of sample aliquots (up to 15×30 μl) through the in-tube-SPME column. The elution of the analytes was done on-line directly to the SAX column. For concentrations at the 20 ng ml^{-1} level, extraction yields decreased in the order MMA (22%)>DMA>AsB>AsC (<5%). Despite the rather low recoveries, the RSD values were acceptable (between 3.6 and 7.3% for $n=11$) which made quantitative analysis feasible. This was demonstrated by the analysis of spiked tap water and certified dogfish reference material (DORM-2). Although in the latter sample significant signal suppression was found due to the matrix, results for AsB in good agreement with the certified value were obtained (the other three investigated As species were not detected in DORM-2).

A study of the metabolisation of arsenic in rats was performed by Inoue et al. [110]. They used parallel HPLC–ICP-MS and HPLC–ESI-MS for the identification and quantitation of arsenic metabolites in the urine of rats chronically exposed to DMA. Chromatographic separation was performed alternatively by cation-exchange [with a HNO_3 (8 m*M*)–NH_4NO_3 (5 m*M*) mobile phase] or anion-exchange [with ammonium formate (6 m*M*), adjusted to pH 5.5 as mobile phase]. Cation-exchange chromatography was the preferred separation mode, since it provided baseline resolution for MMA, DMA and AsB and near-baseline resolution for TMAO and TeMA. Still, all arsenic species could be quantitated without mutual interference by using the molecular (for AsB and TeMA) or the quasi-molecular ions (for MMA, DMA and TMAO). It is noteworthy to mention that the authors were also able to observe signals corresponding to $[2M+H]^+$ and $[3M+H]^+$ for MMA and $[2M+H]^+$ for DMA. Detection limits of the method ranged from 33 pg abs. (for TeMA) and 153 pg abs. (for MMA) with ESI-MS for cation-exchange separation and were slightly higher for anion-exchange separation. In general, these values were only 10–20 times higher than with ICP-MS under the same conditions. The investigation of rat urine demonstrated the presence of TMAO as major metabolite of DMA. AsB was also found, but was shown to be already present in the urine of control rats and thus believed to originate from the diet. A peak occurring at the retention time of TeMA in both cation- and anion-chromatographic separations was

interpreted as TeMA being present in the sample, although its mass spectrum was not completely convincing and it is also generally assumed that TeMA is not produced in mammals. If this finding could be further supported, it would have significant toxicological implications. Two further arsenic species were detected in the AE– and CE–ICP-MS chromatograms, however, due to their low concen-

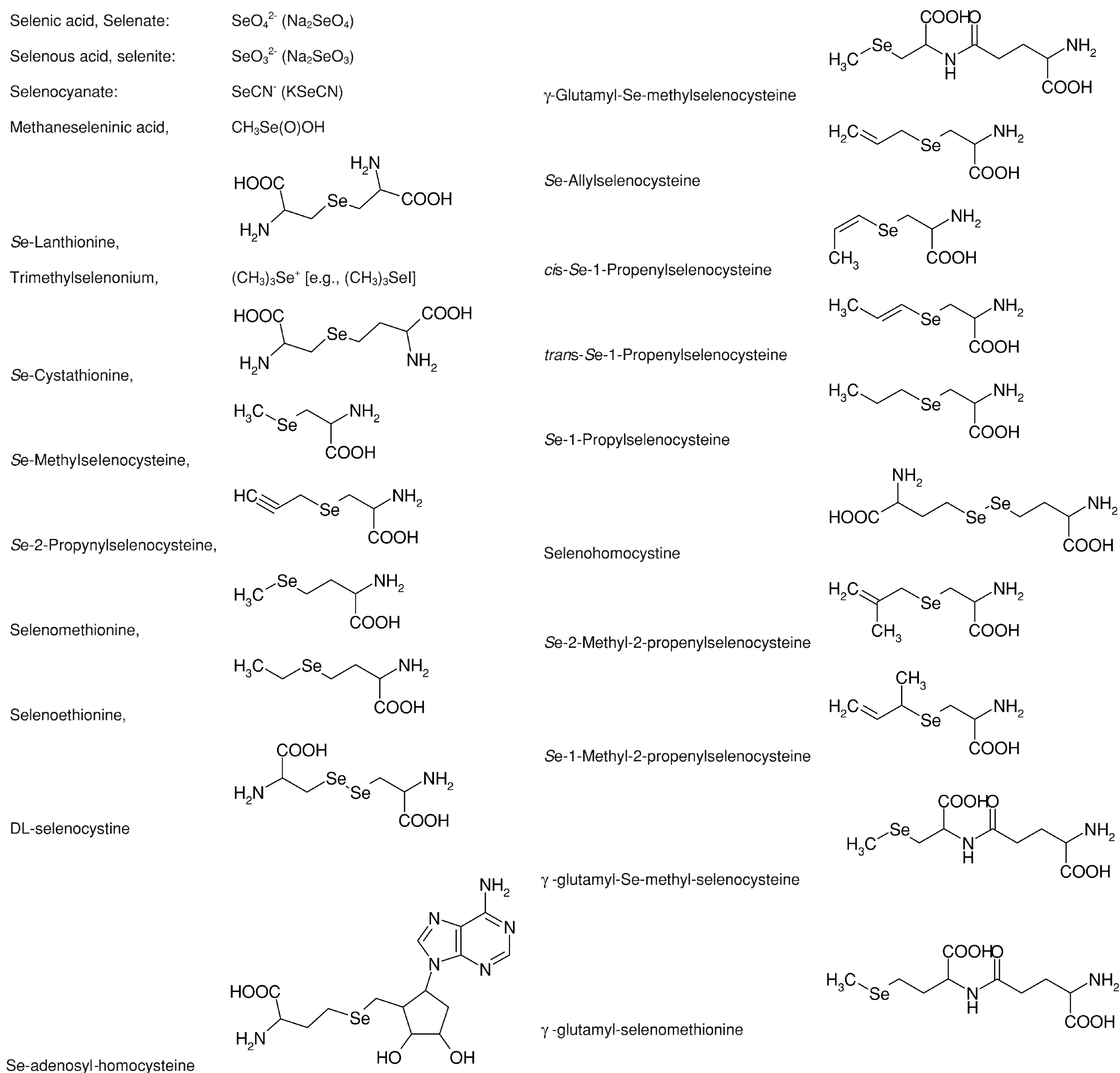


Fig. 5. Structures of important selenium species. Selenic acid, selenate: SeO_4^{2-} (Na_2SeO_4). Selenous acid, selenite: SeO_3^{2-} (Na_2SeO_3). Selenocyanate: $SeCN^-$ (KSeCN). Methaneseleninic acid, $CH_3Se(O)OH$. Se-Lanthionine. Trimethylselenonium, $(CH_3)_3Se^+$ e.g., $(CH_3)_3SeI$]. Se-Cystathionine. Se-Methyiseienocysteine. Se-2-Propynylselenocysteine. Selenomethionine. Selenoethionine. DL-Selenocystine. Se-adenosyl-homocysteine. γ-Glutamyl-Se-methylselenocysteine. Se-Allylselenocysteine. *cis*-Se-1-Propenylselenocysteine. *trans*-Se-1-Propenylselenocysteine. Se-1-Propylselenocysteine. Selenohomocystine. Se-2-Methyl-2-propenylselenocysteine. Se-1-Methyl-2-propenylselenocysteine. γ-Glutamyl-Se-methyl-selenocysteine. γ-Glutamyl-selenomethionine.

Table 5
Overview of the use of ESI-MS for speciation analysis of organoselenium compounds

Species	LOD	Matrix	Method	Refs.
Se-adenosyl-homocysteine	10–100 ng ml^{-1}	Selenised yeast	ESI-MS and ESI-MS–MS in heartcut of RP-HPLC–ICP-MS separation (150 mm×4.6 mm I.D., 5 μm Inertsil ODS-2; 0.1% aq. AcOH)	[119]
Sodium selenate, sodium selenite selenoamino acids: DL-selenomethionine, DL-selenoethionine, DL-selenocystine, Se-methyl-DL-selenocysteine, Se-allyl-DL-selenocysteine, Se-1-propenyl-DL-selenocysteine, Se-propyl-DL-selenocysteine		Hyperaccumulating plants (*Brassica juncea*, *Astragalus praleongus*)	On-line-RP-HPLC–ESI-MS (parallel with HPLC–ICP-MS). (a) RP-HPLC: Zorbax SB-C_8, 150×4.6 mm or (b) Waters Symmetry Shield RP8 150×3.9 mm, 5 μm; (a) water–methanol (98:2) with 0.1% TFA and (b) water–methanol (99:1) with 0.6% TFA	[120]
Se-methionine, Se-adenosyl-homocysteine, γ-glutamyl-Se-methyl-selenocysteine, γ-glutamyl-selenomethionine	1–5 μg ml^{-1} (LOQ)	Selenised yeast, garlic	On-line-RP-HPLC–ESI-MS (parallel with HPLC–ICP-MS) Waters Symmetry Shield RP8 150×3.9 mm, 5 μm, water–methanol (99:1), with 0.6% TFA	[121]
23 selenium containing compounds, including those observed in enriched samples: selenic acid (selenate), selenous acid (selenite), Se-lanthionine, selenocystine Se-cystathionine, Se-methylselenocysteine, selenomethionine, γ-glutamyl-Se-methylselenocysteine, Se-adenosylselenohomocysteine,	ca. 200–5000 ng ml^{-1}	Selenium enriched hyperaccumulators (*Astragalus praleongus*), selenised yeast, ramp (*Allium tricoccum*), onion (*Allium cepa*), garlic (*Allium sativum*)	On-line-RP-HPLC–ESI-MS (parallel with HPLC–ICP-MS)	[123,124]
13 Selenium containing compounds, including those observed in enriched samples: selenic acid (selenate), selenous acid (selenite), Se-cysteine, Se-methylselenocsteine, Se-allylselenocystein, Se-cystine, Se-homocystine, Se-methionine, Se-methyiseienomethionine, Se-ethionine, trimethylselenonioum ion, dimethylselenonium-propionate ion	12 ng ml^{-1} (as Se, for SeMet)	Extracts from selenised yeast, *Chlorella* algae	CE–HPLC–ESI-MS (and AE/CE–HPLC–ICP-MS)	[125]
γ-Glutamyl-Se-methylselenocysteine	n.d.	Extracts from garlic grown on seleniferous soil	Two-dimensional SEC–HPLC–ICP-MS, ESI-MS(/MS) of heartcut fraction	[126]
Se-methionine, Se-adenosylhomocysteine, glutathione *S*-conjugates with selenocompounds	n.d.	Aqueous yeast extracts	Two-dimensional SEC–AE–HPLC–ICP-MS, ESI-MS(/MS) of heartcut fraction	[127]

More than 20 unidentified compounds, only Se-adenosylhomoselenocysteine identified	n.d.	Extracts of selenised yeast	Three-dimensional SEC–AE/CE–ICP-MS and RP-HPLC–ESI-MS–MS	[128]
Selenite, selenocystine, selenoethionine, selenomethionine-Se-oxide, standards, and selenomethionine and three unknown Se-containing compounds of which one potentially is a Se-containing dipeptide	n.d.	Extract from Brazil nuts	RP-HPLC–ICP-MS and ES-Q-TOF-MS	[131]
Se-methionine and a S–Se compound: $HOOCCH_2NHCOCH(NH_2)CH_2SeSCH_2CH(NH_2)CONH_2$	n.d.	*Brassica juncea*	RP-HPLC–ICP-MS and ES-Q-TOF-MS	[132]
Se-methyl-selenocysteine Seleno-L-methionine Seleno-DL-ethionine Selenocystamine Seleno-DL-cystine	2.1 $\mu g\ l^{-1}$ 2.3 $\mu g\ l^{-1}$ 3.1 $\mu g\ l^{-1}$ 2.4 $\mu g\ l^{-1}$ 3.4 $\mu g\ l^{-1}$	Standards	RP-HPLC–ESI-MS–MS and Q-TOF-MS	[133]
Selenomethionine, selenocysteine, methylselenocysteine, dimethylselenonium propionate	n.d.	*Chlorella* algae	GC–MS after silylation, two-dimensional NMR, and ESI-MS	[134]
Selenomethionine, selenocystamine, further four unknown substances	ca. 10 $\mu g\ l^{-1}$	Human urine	Fractionation by RP-HPLC, then ESI-MS–MS	[135]
Se-methyl-*N*-acetylselenohexosamine	n.d.	Human urine	Fractionation by ion-pair chromatography, preparative RP-HPLC, then SEC and ESI-MS–MS detection	[136]
Selenomethionine, selenocysteine, selenocystamine	1–6 $mg\ l^{-1}$	Standards	CE–ESI-MS	[137]
Selenomethionine Selenocystamine Selenocysteine	450 $\mu g\ l^{-1}$ 490 $\mu g\ l^{-1}$ 870 $mg\ l^{-1}$	Standards	CE–ESI-MS	[138]

tration and significant matrix interferences they could not be identified by ESI-MS. However, from their retention behaviour in AE and CE separation it may be assumed that both are anionic species.

A similar methodology was used by Khokiattiwong et al. [111] to monitor the degradation of 11 arsenic compounds in seawater which was enriched with microbes. ICP-MS after anion-exchange (on PRP-X100, with 20 m*M* $NH_4H_2PO_4$, adjusted to pH 5) or cation-exchange separation (on a Supelcosil LC-SCX, with 20 m*M* pyridine adjusted to pH 2.6 with HCOOH) was used to monitor the concentrations of the organoarsenicals during the 10-day experiment. While many of the observed transformation reactions (which were fast for AsB and AsC and comparatively slow for all other arsenic species) were expected and consisting mainly of demethylation of the tri- or tetramethylarsenic moiety, the unexpected finding was that dimethylarsinoylacetate [$Me_2As(O)CH_2COO^-$, whose presence was confirmed by its ESI mass spectrum] is a conversion product of arsenobetaine. It was further discussed by the authors that dimethylarsinoylacetate only intermediately appears as degradation product of AsB before being further converted to DMA. AsC behaved similarly but degraded at a slower rate.

The same analytical procedure was applied by Geiszinger et al. [112] to identify the major extractable arsenic compound in the earthworm *Lumbricus terrestris*. The use of on-line cation-exchange chromatography with ESI-MS detection allowed the unequivocal identification of the phosphate arsenosugar (arsenosugar B) as the main organoarsenical extractable from a terrestrial animal (at the low mg kg^{-1} level). This was a hitherto unreported finding.

A report from another group [113] focuses on the optimisation of the extraction procedure for organoarsenicals from seaweed by accelerated solvent extraction. The analytical methodology applied is in line with previously reported procedures, applying CE–HPLC–ICP-MS and ESI-MS for confirmation of the identity, and so are the results. The main organic arsenic species were found to be DMA, and arsenosugars A–D, however As(III) and As(V) were also detected at surprisingly high fractions of the total As content (up to 8 and 54%, respectively).

An interesting observation was made by Kuehnelt et al. [114] concerning the purity of the nitrogen gas used for the ESI-nebuliser: only when the purity of the nitrogen gas was better than 99.999%, a signal could be seen for the elemental arsenic at m/z 75. With increasing oxygen content in the nebuliser gas (ranging from 10 ppm to 1%), the intensity of the m/z 75 signal was reduced and finally disappeared while at the same time, the signal at m/z 91 (for AsO^+) increased, reflecting the high affinity of the bare As ion to oxygen. The intensity of the signals at m/z 237 and the quasi-molecular ion mass of the four investigated arsenosugars essentially remained unaltered. The authors demonstrated that a nitrogen purity of at least 99.9% is required if elemental information is to be obtained from the ESI-mass spectrum.

Francesconi [115] has recently prepared a review on the determination of arsenic compounds in biological samples using HPLC–ESI-single quadrupole-MS. Specific examples on the quantification of arsenosugars in algae, the confirmation of novel arsenic metabolites from marine microbes and in human urine, and the structural elucidation of two new arsenic compounds (=5-dimethylarsinoyl-2,3,4-trihydroxypenatanoic acid and 5-trimethylarsonio-2,3,4-trihydroxypenatanoic) in marine animals are presented.

3.3.2. Speciation of selenium by API-MS

The increased interest in selenium speciation (see Fig. 5) has been triggered by the finding that it may exert a chemo-preventive effect against certain forms of cancer when administered in doses in excess of the dietary intake level [116]. Many attempts have thus been undertaken to identify the chemical forms in which selenium is present in selenium supplementations, particularly those derived from selenised yeast [117,118]. The hyphenated technique most widely used for this purpose was HPLC–ICP-MS, but in 1999 the first reports on the use of electrospray MS for the structural elucidation of Se-containing species appeared. A summary of the use of API-MS methods for selenium speciation is given in Table 5.

In an approach that was already proposed in the group of Lobinski and Szpunar for the speciation analysis of arsenic compounds, Casiot et al. [119] identified selenium species in yeast extracts by ESI-MS following fractionation. The analytical strategy

was based on heart-cutting the Se-containing fractions of an RP-HPLC chromatogram of the yeast extract, lyophilisation, and analysis by single- and triple-quadrupole MS. With respect to ESI-MS detection, the chromatography was performed with a salt-free mobile phase using dilute (0.01 *M*) acetic acid (as trifluoroacetic acid was feared to suppress electrospray ionisation). Single-quadrupole MS proved already suitable for detecting the presence of Se-containing compounds based on the characteristic isotopic signature of selenium. Using tandem MS in the product ion mode, the identity of an unknown selenium compound could be unravelled as Se-adenosyl-homocysteine. The tentative identification of this compound was supported by the acquisition of product ion spectra of the quasi-molecular ions of the two most abundant selenium isotopes (^{80}Se and ^{78}Se) which had a mass offset of 2 units for the fragments which still contained a Se atom or which were identical when the fragment was a radical no longer containing a Se atom. As a further proof of evidence, the sulfur analogue of Se-adenosyl-homocysteine was also analysed by tandem MS and provided the analogous pattern (with a shift of 48 u for ions that contained ^{32}S instead of ^{80}Se) or identical signals for the adenosyl moiety which did not contain sulfur or selenium.

In their first of a series of papers on the combined use of HPLC–ICP-MS and HPLC–ESI-MS, Kotrebai et al. [120] presented ESI-IT mass spectra obtained on-line from the separation of a number of selenoamino acids, namely DL-selenomethionine, DL-selenoethionine, DL-selenocystine and Se-methyl-DL-selenocysteine. The mass spectra were characteristic for the investigated compounds, and the most intense fragment ions (in most cases produced by the loss of an amino group) could be assigned. The authors claim as the advantage of using ESI-MS detection the potential for identification of unknown compounds in natural sample extracts. However, compound identification in real samples (phytoremediation samples of Se-accumulator plants, *Brassica juncea*, *Astragalus praelongus*) using HPLC–ESI-MS could not be made in the examples presented due to low concentrations of the selenium compounds and interference from the sample matrix.

A second paper in this series [121], again uses the previously reported approach (ESI-IT-MS coupled on-line to RP-HPLC, in parallel to HPLC–ICP-MS detection) with slight modifications. The separation was perfomed on a C_8-RP HPLC column with a mobile phase of water–MeOH (99:1) with 0.1% trifluoroacetic acid. The 1 ml min^{-1} column flow was split 1:5 before introduction into the mass spectrometer. The major selenium compounds were detected by operating the ESI-MS in the selected ion monitoring (SIM) mode at the expected masses of the protonated molecular ions. The identification was based on their mass spectra and the characteristic isotopic pattern of selenium. In addition to selenomethionine and Se-adenosyl-homocysteine, which the authors found as predominant species in selenised yeast, the authors were able to tentatively identify γ-glutamyl-Se-methyl-selenocysteine and possibly γ-glutamyl-selenomethionine in garlic. The compounds identified account for 85 and 90% of the total selenium content of the yeast and the garlic samples, respectively.

A further paper by Ip et al. [122] is mentioned here only for completeness, as it does not add any novel aspects from the methodological point of view, and merely duplicates the ESI-MS data given already earlier.

In their follow-up paper, Kotrebai at al. [123] reported the parallel use of HPLC–ICP-MS and HPLC–ESI-MS for the quantitative and qualitative determination of selenium species in selenium-enriched plants, such as the hyperaccumulating *Astragalus praelongus* or *Brassica juncea*, as well as in the different *Allium* varieties (garlic, *A. sativum*; onion, *A. cepa*; and ramp, *A. tricoccum*). A range of 23 selenium standards was used for the optimisation of the analytical method and for the comparison of retention times. However, only nine of them (selenate, selenite, Se-lanthionine, selenocystine, Se-cystathionine, Se-methyiseienocysteine, selenomethionine, γ-glutamyl-Se-methylselenocysteine, and Se-adenosylselenohomocysteine), could be found in the different samples investigated. The methodological improvement of this paper over the one previously published consists in the use of two different mobile phases [0.1% heptafluorobutryic anhydride (HFBA) and 0.1% TFA in 1% methanol, respectively] for chromatography. TFA was used when a later eluting compound was of interest as the decreased retention time compared with that obtained with HFBA gave

increased peak height and better signal-to-background ratio. HFBA was used when early eluting compounds were of interest.

In an extension of this approach, the same authors reported the HPLC separation and ESI- and ICP-MS detection of more than 20 different selenium compounds [124] using various perfluorinated ion pairing reagents (trifluoroacetic acid, pentafluoropropanoic acid and heptafluorobutanoic acid) to improve the separation. Chromatographic resolution of the more than 20 different selenium compounds seen in the extracts of selenium-enriched yeast, garlic and ramp was achieved in an isocratic run of 70 min. In order to mimic the effect of sample oxidation, γ-glutamyl-Se-methylselenocysteine and Se-methylselenocysteine were oxidised using 30% H_2O_2 solution. Methaneseleninic acid ($MeSeO_2H$) was found to be the principal organic oxidation product in both cases.

While most of the work discussed so far made use of RP-HPLC for the separation of selenium species, Larsen et al. [125] proposed the separation of selenoamino acids, selenonioum ions and inorganic selenium by ion-exchange HPLC with MS detection. Anionic selenium species were separated on a polymeric strong anion-exchange column with isocratic elution by an aqueous salicylate–Tris buffer (pH 8.5) and detected by ICP-MS. Separation of the cationic selenium species was achieved on a silica-based cation-exchange column, followed by either ICP-MS or ESI-MS detection. Using the latter, it was possible to identify one of the unknown compounds in the Se-specific chromatogram as selenomethionine-Se-oxide, the oxidation product of selenomethionine. A second unknown compound, observed in the trichloroacetic acid extract of *Chlorella* algae, was identified as dimethylselenonium propionate by its ESI mass spectrum. Due to the limited sensitivity of ESI-MS (ca. three orders of magnitude less sensitive than ICP-MS), two further unknown selenium compounds in the extract of selenised yeast remained however unidentified.

Applying the same approach as in their work on arsenic, McSheehy et al. [126] used preparative size-exclusion chromatography to fractionate the aqueous extract of garlic grown on naturally seleniferous soil. The only selenium containing fraction was observed at low molecular mass and was subjected to a further RP-HPLC separation with ICP-MS detection which produced one single peak. The heartcut fraction of the SEC separation was then analysed by electrospray MS and tandem MS. Although no reference compound was available, the selenium compound was tentatively identified from the CID mass spectrum as γ-glutamyl-Se-methylselenocysteine in agreement with the findings of Kotrebai et al. [121].

The application of two-dimensional HPLC with ICP-MS and electrospray MS–MS detection allowed the same group to more comprehensively analyse selenium species in selenised yeast [127]. This analytical approach enabled the detection of previously unreported glutathione S-conjugates with selenocompounds in water extracts of nutritional yeast supplements. It was based on the use of two-dimensional size-exclusion and reversed-phase HPLC for the separation of selenium species, ICP-MS for the monitoring of the eluting selenium, and electrospray MS–MS for the identification of the eluted compounds. The presence of six compounds with molecular mass of 197, 603, 562, 584, 372 and 432 (in the elution order from reversed-phase HPLC) was identified on the basis of the selenium isotopic pattern. The identity of the species with protonated molecular ions at m/z 198 and 433 was confirmed, by collision-induced dissociation MS, to be selenomethionine and Se-adenosylhomocysteine, respectively. The compounds with larger molecular mass (m/z 562, 584, 604) were demonstrated, by reaction with dithiothreitol followed by HPLC–ICP-MS and ESI-MS–MS, to contain an Se–S bridge between the tripeptide glutathione (Glu–Cys–Gly) and a selenocompound that did not respond in the positive ion ESI-MS mode. The MS–MS analysis of the original compound confirmed the presence of a selenium-containing fragment that could be fragmented only at high fragmentation energies. A similar Se-containing moiety (m/z 227), resistant to collision induced dissociation, was found in the m/z 372 compound attached to a glutamine (Gln) residue as demonstrated by MS–MS.

In an extension of their previous work, McSheehy et al. [128] tried to overcome the drawbacks of limited peak capacity of the two-dimensional separation by resorting to a three-dimensional separation based on orthogonal separation mechanisms. Additionally, the first two separation steps were scaled up and the mobile phase composition was optimised

with respect to ESI-MS. The analytical strategy was based on preparative scale SEC, followed by semi-preparative anion-exchange chromatography. The buffer used for AE separation was triethylamine–acetic acid (2:1) and provided sufficient volatility for subsequent ESI-MS measurements. The measurements performed on extracts of selenised yeast indicated the presence of three selenium-containing compounds in two different fractions of the SE–AE chromatographic separation. However, only Se-adenosylhomoselenocysteine with $[M+H]^+$ at m/z 433 could be identified by its CID mass spectrum. The two other mass spectra, tentatively attributed to selenium compounds based on their characteristic isotopic pattern (with putative protonated molecular ions of the ^{80}Se isotope at m/z 532 and 534), could not be identified. The authors speculate on the occurrence of a selenium compound containing an adenosyl moiety, however, it has two hydrogen atoms less in the selenocysteine moiety, indicative for the presence of a double bound. Allyl-Se derivatives have been reported earlier [129,130], however, not in selenised yeast.

The importance of a suitable sample preparation procedure as a prerequisite for the successful identification of selenium containing compounds was underlined by Vonderheide et al. [131]. In their study of selenium species in Brazil nuts, they investigated various sample preparation procedures after defatting of the sample, including microwave or enzymatic extraction of which enzymatic hydrolysis using proteinase K was found to be the most effective. After hydrolysis, residual proteins and excess of the proteinase K were removed by precipitation with TCA or ethanol. The so prepared sample extract was chromatographed on a C_8 RP-HPLC column and detected by either ICP-MS or ESI-MS using a Q-TOF instrument (mobile phase: water (5 m*M* citric acid + 5 m*M* methanesulfonic acid, pH 3.5)–methanol, 90:10). With HPLC–ICP-MS, four selenium-containing peaks could be detected in the chromatogram of the Brazil nut extract. Only one of these — the most intensive — was identified as selenomethionine, while the identity of the three other selenocompounds remained undisclosed. Attempts to identify the earliest eluting of the three unknowns as selenomethionine-Se-oxide by retention time matching was not successful. The ESI-MS–MS spectrum of the third unknown compound indicated a selenium-containing dipeptide with molecular mass of 360 (protonated $[M+H]^+$ at m/z 361 for ^{80}Se) where the characteristic Se isotopic pattern could be observed for the quasi-molecular peak cluster. The proposed structure of the dipeptide is p-$HO(C_6H_4)$-$CH_2CH(NH_2)CONHCH(COOH)CH_2CH_2SeCH_3$, although this assignment is only tentative due to the poor signal-to-background ratio of the measurement in the highly complex nut extract.

The same group published in a subsequent report the application of LC with ICP-MS and ESI-MS detection for initial studies on selenium species in *B. juncea* used for phytoremediation [132]. The analytical methodology was similar to the one developed in their previous publication, with minor adjustments of the chromatographic parameters for the ESI-Q-TOF-MS measurements. HPLC–ICP-MS revealed the presence of numerous Se-containing compounds in different relative proportions, depending on which enzyme (protease XIV or proteinase K) had been used for hydrolysis. Except for one compound which could be attributed to Se-methionine according to its spectrum and retention time matching with an authentic standard, no other peak matched the retention times of standards available in that laboratory. As one of the early eluting peaks was however found to contain both S and Se, it was assumed that this compound includes a S–Se bond which could be cleaved by the addition of dithiothreitol (DTT). This was in fact possible, and the authors were able to propose a structure for the protonated molecular peak at m/z 345 (for ^{80}Se) which was consistent with the observed fragmentation pattern and the two compounds induced by DTT-induced cleavage of the Se–S bond.

Different from the previously cited studies, the work of Lindemann and Hintelmann [133] focused on methodological aspects: the performance of a triple-quadrupole instrument (Quattro LC) and a time-of-flight instrument (Q-TOF-2) were compared and the superiority of the Q-TOF instrument in terms of mass resolution ($m/\Delta m$ 250 and 5000, respectively) was demonstrated. On the other hand, the signal intensities decreased by four orders of magnitude when changing from the triple-quadrupole to the Q-TOF instrument. For the chromatographic separation of selenoamino acids, a porous graphitic

carbon (PGC) column was employed. Using this column, baseline separation of the five selenoamino acids (Se-methyl-selenocysteine, seleno-L-methionine, seleno-DL-ethionine, selenocystamine and seleno-DL-cystine) was achieved with a gradient of 7.5 m*M* HFBA and a relatively high fraction of MeOH which was claimed to improve the formation of a stable electrospray. Only standards were investigated, with detection limits multiple reaction mode of ca. 2–3 μg l^{-1}. The authors investigated both APCI and ESI in their work and gave preference to electrospray ionisation for the detection of the above set of compounds since they observed signal suppression for APCI by a factor of 10–50 due to the addition of the ion pairing reagent while ES ionisation was hardly affected. This is an interesting observation, as it is in contradiction to common understanding.

Three recent studies were concerned with the identification and determination of selenium metabolites. The first of these, performed by Fan et al. [134] applied a multi-method approach to characterise Se-containing metabolites in the acid digest of the proteinous fraction of a *Chlorella* culture grown on Se-enriched medium. The applied methods included GC–MS after silylation for the determination of selenomethionine, selenocysteine and methylselenocysteine, and electrospray MS analysis of selenomethionine, methylselenocysteine, and dimethylselenonium propionate (DMSeP), which was custom synthesised in order to have an authentic standard for comparison. Additionally, two-dimensional-multinuclear NMR measurements confirmed the identity of these analytes, and thus probably for the first time the presence of DMSeP in extracts of a *Chlorella* culture.

Cao et al. [135] described a method for the speciation and identification of organoselenium metabolites in human urine samples using HPLC–ICP-MS and MS–MS. Reversed-phase chromatographic separation (on a Hypersil BDS C_{18} column with a water (2% acetic acid)–MeOH–i-PrOH gradient) was used for the fractionation of the sample where the ICP-MS served as an element-selective detector. Six well-separated peaks of selenium-containing species were detected in a human urine sample. Fractions were then collected and analyzed using an ESI-triple quadrupole-MS. Structural information was obtained by collision-induced dissociation. Only the compounds in the first two fractions were positively identified as selenomethionine and selenocystamine, estimated to be present at approximately 11 and 40 ppb, respectively.

With a similar aim, Gammelgaard et al. [136] have investigated the separation, purification and identification of the major selenium metabolite from human urine by multi-dimensional HPLC–ICP-MS and APCI-MS in a recent study. For this purpose, they have investigated urine samples from six male volunteers who have been supplied with selenium-containing nutritional preparations, leading to an increase of the concentration of this particular metabolite. Analysis was performed by ion-pair chromatography with ICP-MS. The fractions containing the metabolite were pooled and subjected to solid-phase extraction for the removal of ionic substances. The extract was purified and preconcentrated by twofold preparative-scale reversed-phase chromatography. The selenium-containing fractions were collected and further purified by size-exclusion chromatography. Since it was not possible to ionise the selenium metabolite by electrospray ionisation, atmospheric pressure chemical ionisation, was used instead. The molecular mass of the selenium metabolite was 300 for the ^{80}Se isotope, and MS–MS experiments indicated that the metabolite was a selenosugar. It is therefore proposed that the selenium metabolite is a Se-methyl-N-acetylselenohexosamine.

While most of the work on selenium speciation has been performed by one- or multidimensional liquid chromatographic separation, there exist a few noteworthy exceptions which report the use of CE–ESI-MS. One of these is the report by Schramel et al. [137] on the construction and preliminary evaluation of a custom-built CE–ESI-MS interface. In addition to the investigation of several metal complexes, they also reported on the determination of three Se-amino acids (selenocystine, SeC; selenocystamine, SeCM; selenomethionine, SeM). The use of an alkaline buffer system (Na_2CO_3–NaOH) which was adapted for CE–ESI-MS for selenium speciation from an existing ICP-MS method led to unsatisfactory results. The non-volatile electrolyte suppresses electrospray ionisation dramatically. As a consequence, a volatile acidic background electrolyte (2% acetic acid) was chosen for the separation of the three

organoselenium species SeC, SeCM and SeM. The Se species were sufficiently separated from each other and the run time of a complete separation was ca. 21 min. The method proved however unsuccessful for the determination of the inorganic selenium species Se(IV) and Se(VI). Detection limits were calculated as 1–6 mg l^{-1} for the organic Se species.

Two further reports by the same group are detailing some further developments in the analytical methodology. In the two companion papers [138,139], the use of the pressure-driven detection mode for CE–ESI-MS was presented: In order to shorten the prohibitively long separation times for the selenoamino acids with the chosen supporting electrolyte, Michalke et al. applied moderate (350 mbar) or high pressure (8 bar) to the capillary to drive the analytes to the detector after an initial electrophoretic separation phase. By this means, separation was completed within 13 min (medium pressure) or 9 min (high pressure), however, at the price of loosing resolution. The two late eluting selenoamino acids (SeM and SeC) were only marginally separated in the low pressure mode while they completely coeluted when a high pressure was applied to the CE capillary. Still, they could be separated based on their different masses.

Completely different from the previously summarised work is the one from Raymond et al. [140], discussing the speciation of polyselenide species by electrospray mass spectrometry.

By applying ESI-MS the authors observed that the speciation of the polyselenide anion in aqueous solution can be controlled by the countercation and by pH adjustments. The countercation was found to affect the degree of disproportionation or comproportionation. Solutions of sodium tetraselenide displayed numerous Se_n^{2-} and HSe_n^- species in neutral and basic solutions. In potassium solutions, the dominant species was $Se_4^{2-} \cdot H_2O$. Solutions of caesium pentaselenide showed preference also for higher-order polyselenides. The pH dependence has been analysed in terms of the percent Se_n^{2-} ($n=2$–5) present. Di-, tri-, tetra-, and pentaselenide species were present over the entire pH range investigated, and trends in selenide species versus pH were developed. It appears that with increasing pH, n of the most abundant species gradually increases from 2 to 4, for example, diselenide species have their highest relative abundance at pH 6–7, triselenide species are most abundant at pH 8.5–9.5 and tetraselenide species at pH$>$10. Pentaselenide species are of lower abundance in the investigated pH range (5–11).

3.3.3. Speciation of lead and tin species by API-MS

Organometallic compounds of the heavy metals tin, lead and also mercury which is not discussed in this context probably have the highest environmental impact of all organometallic species introduced by men into the environment. As a consequence of their high toxicity and environmental relevance (some organotin compounds, for example, are also known as endocrine disrupting compounds), numerous methods have been developed for their analysis. Dominant among these are gas chromatographic techniques, coupled with suitable, that is, sensitive and selective detectors, such as atomic emission, mass spectrometric or ICP-MS [141]. A common drawback to all these methods is the necessity to derivatise the mostly ionic analytes to make them amenable to GC separation [142].

Separation in liquid phase circumvents the problems associated with the derivatisation of involatile analytes. A number of reports have thus aimed at the development of liquid-phase separation techniques for small organometallic compounds [143]. Table 6 presents a synopsis of API-MS techniques for the speciation analysis of organolead and organotin compounds.

In two sequel papers by Mester and Pawliszyn the determination of trialkylated organolead compounds (trimethyllead, TML; and triethyllead, TEL) was described. The first of these investigates the potential of ESI-MS for the determination of TML and TEL [144]. At low fragmentor voltages, the signals of the TML and TEL cations, respectively, could be observed at m/z 253 and 295. At increasing fragmentor voltage, the alkyl groups were sequentially stripped off the molecule, leading to signals at m/z 238, 223 and 208 (for TML^+) and at m/z 266, 237 and 208 (for TEL^+), based on the ^{208}Pb isotope. It is worth noticing that the loss of alkyl groups from the trialkyllead cation is accompanied by a stepwise reduction of Pb(IV) to the bare Pb^+ cation. This is an excellent demonstration of the parallel use of

Table 6
Overview of the use of API-MS for speciation analysis of organometallic (lead and tin) compounds

Element/species	LOD	Matrix	Method	Ref.
Lead				
Trimethyllead	n.d.	Aqueous standards	In-tube-SPME–ESI-MS	[144]
Triethyllead				
Trimethyllead	11.3 ng ml^{-1}	Aqueous standards	In-tube-SPME–HPLC–ESI-MS	[145]
Triethyllead	12.6 ng ml^{-1}			
Bu_3PbOAc, Ph_3PbCl, Pr_3PbOAc, i-Bu_3PbOAc	ca. 3 ng ml^{-1}	Standards, fuel	Direct injection ESI-MS	[85]
Tin				
Tributyltin	~5 pg abs. as Sn	Standards, PACS-1 sediment CRM	Direct injection-ESI-MS–MS	[146]
butyl- and phenyltins	n.d.	Standards	Direct injection-ESI-MS	[150]
Di- and tributyltin	ca. 50 ng ml^{-1} for DPhT and TPhT	Standards, harbour sediment sample	RP-HPLC–APCI-MS Kromasil-100 C_{18} 150 mm, 5 μm ACN–AcOH–water (65:10:25)+0.05% triethylamine	[154]
Di- and triphenyltin	ca. 100 ng ml^{-1} for DBT and TBT			
Dibutyltin	~1600 pg (abs.)	Standards, PACS-2 harbour sediment CRM	RP-HPLC–APCI-MS Zorbax SDB C_{18}, 30 mm×2 mm, 3 μm, water–MeOH gradient+1% TFA	[155]
Tributyltin	~ 750 pg (abs.)			
Diphenyltin	~ 2000 pg (abs.)			
Triphenyltin	~ 1000 pg (abs.)			
Triphenyltin	~ 100 pg (abs.)	Standards	RP-HPLC–ESI-MS	[159]
Tributyltin	~ 200 pg (abs.)			
Tributyltin	780 pg	Standards	RP-HPLC–ESI-IT-MS	[160]
Dibutyltin	970 pg			
Monobutyltin	1 ng			
Dibutyltin	970 pg	Aqueous leachate of PVC tubing	RP-HPLC–ESI-IT-MS	[161]
Tributyltin	0.05 ng ml^{-1}	Standards and PACS-2 harbour sediment CRM	In-tube-SPME–HPLC–ESI-MS	[162]

ESI-MS in both molecular and elemental mode. In-tube SPME was used as sample enrichment technique where up to 15 sample aliquots were aspirated through the SPME capillary. The analyte was enriched in the coating of the capillary and in a subsequent step eluted directly in a small volume of mobile phase into the mass spectrometer without chromatographic separation.

In the second paper [145], the method was improved by integrating a separation of the two analytes on a C_{18} RP-HPLC column. The use of water (0.1% TFA)–MeOH (88:12) as mobile phase enabled complete separation and detection of TML and TEL in less than 5 min. Precision is better than 5% and estimated limits of detection are 11.3 and 12.6 ng ml^{-1}, respectively, for TML and TEL.

In an earlier report of Zoorob et al. [85], the determination of organic (Bu_3PbOAc, Ph_3PbCl, Pr_3PbOAc, i-Bu_3PbOAc) and inorganic lead species was investigated. ESI-MS was used in the ‘‘bare metal ion’’ or ‘‘elemental’’ mode at high fragmentor (skimmer-sampling plate) voltage. This provided a surprisingly high degree of compound-independent response, as was demonstrated by the analysis of a certified reference material (NIST SRM 215, certified for lead in fuel) which was quantified by using a Pb^{2+} standard.

The reports on the determination of organotin compounds by HPLC–MS are more abundant. The potential of atmospheric pressure ionisation techniques for this task has been recognised as early as 1989 when Siu et al. presented a method for the determination of tributyltin in a sediment reference material (PACS-1) based on ionspray tandem mass spectrometry in the selected reaction monitoring mode with direct-injection [146]. Surprisingly, electrospray (or ionspray) MS was hardly used initially for the determination of organotin compounds despite its demonstrated ability for this purpose. Instead, a number of reports on the usage of thermospray LC–MS [147,148] and particle beam LC–MS [149] were published. Later, electrospray

MS was revisited by Lawson et al. [150] and found to be highly suitable for obtaining structural information on organotin compounds based on their characteristic fragmentation pattern. Unfortunately, most of these studies are focusing only on the mass spectrometric detection of organotin compounds but not on their liquid chromatographic separation. The various aspects of ES-MS detection of organotin compounds are, for example, also discussed in great detail by Betowski and Jones [151]. However, the difficulty of obtaining a good chromatographic resolution for all environmentally relevant organotin compounds (which includes the mono-, di- and trisubstituted butyl- and phenyltin compounds) has been tackled only by few authors. The reason is that the bi- and trivalent organotin cations (R_2Sn^{2+} and RSn^{3+}) are hardly retained on the typical reversed-phase HPLC columns. An improvement can be achieved by using complexing agents as mobile phase additives, but due to their limited volatility they have not been popular with ESI- or APCI-MS detection [152,153]. As a consequence, the monosubstituted compounds are hardly eluting from the column (if typical silica based RP-HPLC columns are used), or, if a high percentage of mobile phase additive is used, they are eluting just in the dead volume of the column. This explains why in two studies on the potential of LC–APCI-MS for the determination of organotin compounds [154,155], the range of compounds has been restricted to di- and trisubstituted compounds for quantitative analysis. In both papers, however, the APCI mass spectra of the organotin compounds are discussed in detail. For the trisubstituted organotin compounds, White at al. [154] observed the molecular ion (at m/z 291 for TBT and m/z 351 for TPhT) as dominant signal at low fragmentor voltage. Some adducts with the solvent (acetonitrile, $[M+ACN]^+$) are also seen, as well as fragments obtained by the loss of one or two butyl- or phenyl groups. As for the fragments, as well as for the dibutyl- or diphenyltin compounds being originally present, the charge of a divalent cation cannot be sufficiently stabilised, the charge is typically reduced by complex formation with an ion present in solution. This phenomenon has more extensively been investigated by Rosenberg et al. [155] who have recorded the mass spectra of organotin compounds at various fragmentor voltages. In accordance with theory, at low fragmentor voltages the molecular ion can be seen for the trisubstituted organotins, while the compounds break down to finally produce the bare atomic ion at high fragmentor voltage (e.g., the $^{120}Sn^+$). A number of observations are remarkable in this context: first, APCI is not able to stabilise ions with charges greater than one under these conditions. That means that for the breakdown products of the trisubstituted organotins or for the mono- and disubstituted compounds, ligands have to be coordinated from the solution in order to reduce the net charge of the ion to one. For this reason, a number of ions with mixed ligands can be observed, e.g., the ions $SnBuAc_2^+$ for MBT or $SnPh_2Ac^+$ for DPhT in acetic acid solution.

Second, it will depend on the ligands present in solution and their complex stability which complexes are detected in the APCI mass spectrum. In this context, it was surprising to see that with the exception of TPhT for all other ions also mixed chlorine containing complexes were detected, obviously indicating a relatively high stability of the Sn–Cl bond in the compounds used as alkyl- or aryltinchlorides for the experiments.

Third, at increased fragmentor voltage a stepwise reduction of the substituted Sn(VI) compounds could be detected: ions that were attributed to $PhSn^+$, $SnAc^+$ or $SnCl^+$ (at m/z 197, 179 and 155, respectively) have Sn in the valence state (+2), and the bare Sn^+ ion has the oxidation state (+1). It is thus demonstrated that reductions during the atmospheric pressure ionisation process are not only of electrochemical nature as in the electrospray ionisation process [156,157], but may as well happen in the gas phase in the atmospheric pressure chemical ionisation process.

Organotin halides and their hydrolysed species were studied in solution by positive and negative ion electrospray mass spectrometry by Henderson and Taylor [158]. The most intriguing observation is that in the positive ion mode high-molecular-mass clusters for the trisubstituted compounds are observed: for TPhT $[(Ph_3Sn)_n(OH)_{n-1}]^+$ with $n=1$–5, and for TBT and TMT $[(Bu_3Sn)_n(OH)_{n-1})]^+$ and $[(Me_3Sn)_n(OH)_{n-1})]^+$, respectively, with $n=1$–3 are formed in water–acetonitrile (50:50) as mobile phase. In the presence of (excess) halide ions (Cl^-, Br^-), the organotin compounds can also be detected

in the negative ion mode. In that case, the anions $[R_3SnCl_2]^-$ or $[R_3SnBr_2]^-$ are the dominant species.

In a recent study, González-Toledo et al. [159] used liquid chromatography–electrospray mass spectrometry for the determination of the trisubstituted organotin species tributyltin and triphenyltin. Tripropyltin was used as an internal standard, and the di- and monosubstituted organotin compounds have not been included in this study for the reasons discussed above. After optimisation of the chromatographic conditions, the authors propose an isocratic separation using isocratic mobile phase of 0.02% trifluoroacetic acid in acetonitrile–water (50:50, v/v) and a Kromasil C_{18} column. Quantitation was based on the positive ion detection of the molecular ions, and absolute detection limits of 100 and 200 pg were obtained for TPhT and TBT, respectively, with standards. With a preceding SPE step on disposable C_{18} cartridges, the sensitivity of the method can be brought to the environmentally relevant low ng l^{-1} concentration range.

Two papers by Jones-Lepp and co-workers extended the work on organotin compound analysis by the application of micro-LC–MS: in the first of these [160], the method development is discussed which is based on a μ-HPLC separation using a 10 cm×160 μm I.D. column packed with C_{18} material at a flow-rate of 4 μl/min. The mobile phase consists of methanol–water–acetic acid (80:14:6) and 0.1% (w/v) tropolone. Without the addition of tropolone, the authors claim that the analytes do either not elute at all, or they elute in the dead volume, depending on the species. MS detection is essential for this method, first to provide the required sensitivity, and second to allow separation of the very closely eluting mono- and disubstituted organotin compounds based on their characteristic ions. The most significant signals observed in the mass spectra are the molecular ion for the trisubstituted compound and adducts with the tropylium cation and/or solvent molecules. The sensitivity for the detection of the phenyltin compounds ranges from ca. 0.8 ng (for tributyltin) to 1 ng (for dibutyltin and monobutyltin). No quantitative data is given for the phenyltin compounds. Although the method appears advantageous over current methods for the determination of organotin compounds, it is pointed out by the authors that the electrospray signal is relatively unstable and requires normalisation to a suitable internal standard. Organotin compounds are extracted from water samples with Empore SPE disks, for which also relatively large variations have been observed.

The same group described in a subsequent paper the application of their previously developed method to the determination of organotins from PVC pipe [161]. The use of μ-HPLC–MS allowed the direct determination of DBT (and, presumably, also MBT) in water samples that were in contact with polyvinyl chloride (PVC) tubing for an extended period of time (24 to 96 h) at the low μg l^{-1} level. It appeared that the initial concentration (1 μg l^{-1}) decreased somewhat after 48 h and returned close to the initial value (0.8 μg l^{-1}) after 96 h of contact time for the investigated specimens. Although the chosen experimental design clearly demonstrates that dibutyltin is leached from PVC tubing (or from the glue used for connecting PVC pipes), the determined concentration may be considered as an upper limit, since the contact time of water with PVC tubing is likely to be less than the ones chosen for this experiment (24 to 96 h).

Wu et al. [162] used their earlier developed technique based on automated in-tube solid-phase microextraction for the determination of tributyltin. Both the enrichment on various commercially available capillary columns and the parameters for ESI mass spectrometric detection of TBT were optimised, and the Supelco Q-PLOT capillary column was demonstrated to show the highest extraction efficiency for TBT. The in-tube SPME–HPLC–ESI-MS method provided a linear working range of 0.5–200 ng ml^{-1} TBT, with a detection limit of 0.05 ng ml^{-1}. The method performance was evaluated by determining the content of TBT in a sediment reference material (PACS-2).

3.4. Analysis of metalloproteins by LC–ESI-MS

A task even more challenging than the determination of low-molecular-mass (LMW) organometallic compounds is the analysis of metalloproteins by LC–MS. Wile in the former case the analytes are usually well-defined, thermodynamically stable and standards are commercially available, the situation is different for high-molecular-mass (HMW) organometallic compounds. They are — in contrast to

the mostly anthropogenic, organometallic compounds — of biogenic origin, and the number of potentially occurring compounds is extremely large, while the difference between compounds may be small. For instance, an amino acid in a protein may be substituted by a homologue or a hydroxy or amino group may be inserted or missing. Still, these subtle differences may already change the complexation of metal ions, and consequently the structure and physiological function of the biomolecule. Biomolecules also tend to have the metal ions complexed only by coordinative bonds instead of the covalent carbon–metal bonds seen for LMW organometallic compounds. This not only has consequences for the sample preparation procedure which has to be carefully monitored for the occurrence of possible artefacts, but as well on the separation and detection: size-exclusion chromatography which offers minimal physico–chemical interaction with the analytes is therefore the most suitable separation technique for labile metalloproteins [163]. However, as size-exclusion chromatography is not a highly efficient separation technique, the use of multi-dimensional separation, employing also other (orthogonal) modes of separation will normally be required. The concept of a such multi-dimensional separation is shown in Fig. 6: it is advantageous to use SEC as the first separation dimension, as a fractionation of the sample can be achieved in which the relevant portions of the sample are isolated and subjected to the next separation step. For practical reasons, orthogonal separation techniques are hardly coupled on-line due to a mismatch in the mobile phase composition. Also, the separation in the first dimension mostly serves as a fractionation technique only. Relevant fractions containing the metallocompounds of interest (which is normally monitored by an elemental analysis technique) are pooled and preconcentrated, normally by lyophilisation, before the fraction is subjected to the second chromatographic dimension. Ion chromatography is very often used as second dimension, followed by reversed-phase HPLC in the third dimension. Alternatively, the order of RP-HPLC and ion chromatography may be reversed, or RP-HPLC may be used in the second dimension and CE as third separation dimension. Due to the very low absolute sample amounts handled by CE, this technique can only be used as separation technique in the final dimension. ICP-MS and ESI-MS(-MS) are normally used for detection of metalloproteins after multidimensional separation, the former technique providing adequate sensitivity, but no compound specific information, while ESI-MS provides compound-specific information at the price of significantly lower sensitivity. This has often precluded the on-line use of ESI-MS for detection, making it necessary to detect relevant species off-line, often after preconcentration. Except for the limited sensitivity, however, ESI-MS is the ideal detection technique for HMW metallocompounds: due to the softness of ionisation, even labile complexes are likely to be transferred intact into the gas phase. Furthermore, polypeptides and proteins are able to form various multiply charged (protonated) ions which produce a characteristic envelope in the electrospray mass spectrum at masses normally well below m/z 2000, where the transmission of a quadrupole mass spectrometer decreases already significantly. From the signals of multiply charged ions, the exact molecular mass of the ion can conveniently be calculated, provided that only protonated species are formed. If we assume that a biomolecule of mass M_r produces a signal of a multiply charged ion at $m/z_1 = p_1$, then the equation:

$$p_1 z_1 = M_r + m_H z_1 \tag{4}$$

holds true where m_H is the mass of a proton. And for the signal of the adjacent peak at higher m/z ratios (which carries one proton less), there is:

$$p_2 \cdot (z_1 - 1) = M_r + m_H \cdot (z_1 - 1) \tag{5}$$

From these two equations the charge state may be calculated from:

$$z_1 = (p_2 - m_H)/(p_2 - p_1) \tag{6}$$

and the molecular mass from the rearranged Eq. (4):

$$M_r = p_1 z_1 - m_H z_1$$

ESI-MS with CID may be used successfully to determine the sequence of oligopeptides. Peptides fragment preferably at the amine bonds, producing thereby a ladder of sequence ions. The charge can be retained on either the amino-terminus (type B ions) or the carboxy terminus (type A ions). This gives rise to two concurrently occurring ion series which

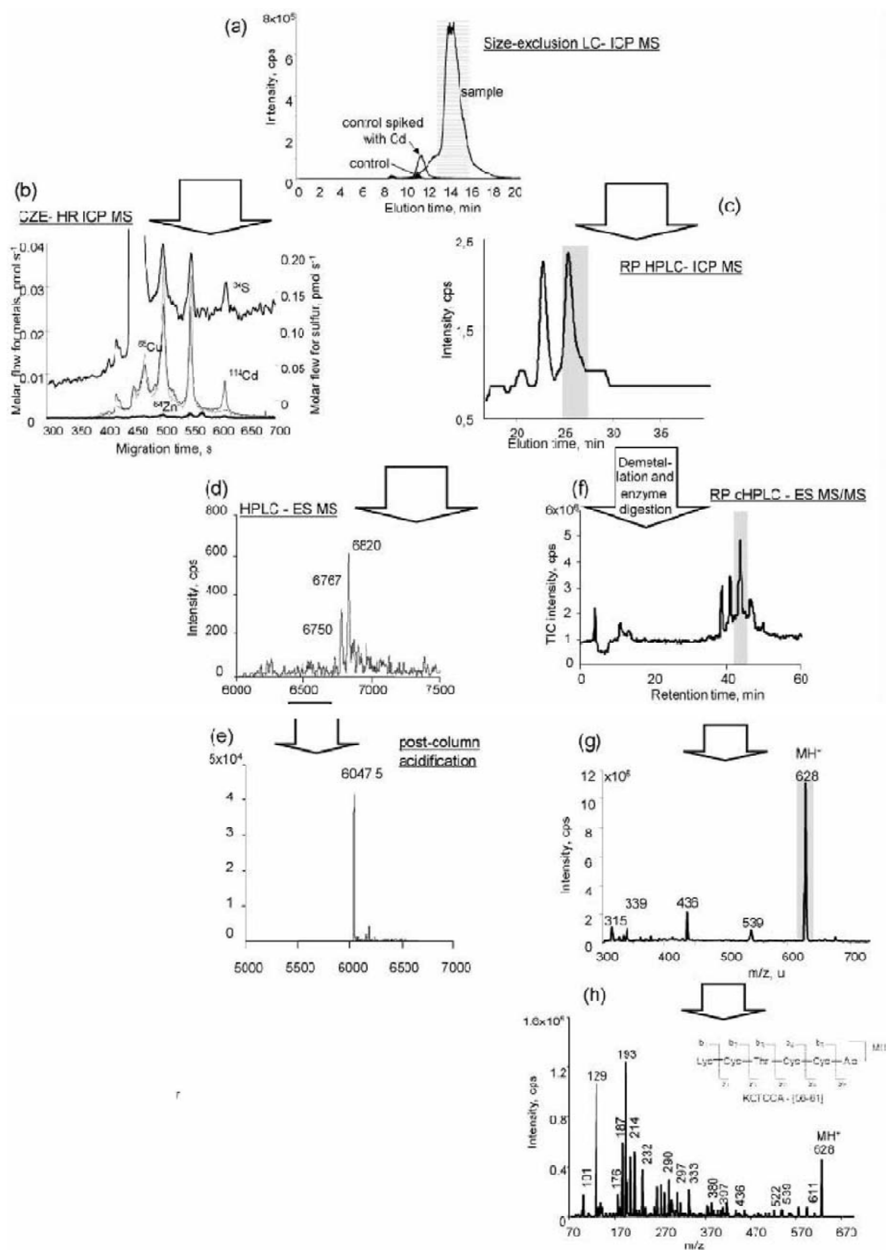


Fig. 6. Multidimensional analytical strategy, exemplified here for the identification of metallothioneins induced in animal exposed to Cd. (a) Size-exclusion chromatogram with ICP-MS detection. The shaded heart cut fraction is subjected to further analysis. (b) CZE-ICP sector field MS of the heart cut fraction in (a). The simultaneous determination of the ^{34}S trace allows the calculation of stoichiometry for each peak from the metal:sulfur molar ratios and its concentration from the absolute peak signal. (c) Reversed-phase HPLC–ICP-MS of the heart cut fraction in (a). (d) Measurement of the M_r of the complex: ESI mass spectrum taken at the apex of the shaded peak in (c). (e) Measurement of the M_r of the ligand: ESI mass spectrum taken at the apex of the shaded peak in Fig. 3c after post-column acidification. Sequencing of the peptide ligand by reversed-phase HPLC–ESI-MS–MS: (f) RP-HPLC–ESI-MS chromatogram (TIC) of the fraction shaded in (c) after demetallation and tryptic digestion. (g) M_r measurement of oligopeptides resulting from the tryptic digestion of metallothionein: mass spectrum taken at the apex of the shaded peak in (f). (h) Sequencing of oligopeptides resulting from the tryptic digestion of metallothionein: CID mass spectrum of the peak in (g). Reproduced by permission of Springer-Verlag from Ref. [17].

allow the determination of the sequence of amino acids by subtraction of the masses of subsequent sequence ions. However, as fragmentation patterns become very complex even for comparatively simple oligo- and polypeptides, this method is limited to proteins up to ca. M_r 2500 molecular mass.

Table 7 compares the characteristic features of ESI-MS analysis of low-molecular-mass and high-molecular-mass organometallic compounds.

Successful examples for the application of this multi-dimensional approach — with ICP-MS or parallel ICP-MS and ESI-MS detection — have already been discussed earlier for selenium speciation in human milk [164], garlic [126] and yeast [127,128] and for arsenic speciation in algae [97,100,102] and oysters [127].

This approach has also been used successfully for the separation and characterisation of metalloproteins in human brain [165] and rat tissue [166,167].

3.4.1. Metallothioneins

Metallothioneins (MTs) are a group of non-enzymatic, medium-molecular-mass (typically 6000–7000 Da) proteins that are cysteine-rich and therefore able to coordinatively bind metals. They are resistant to thermocoagulation and acid precipitation [168]. They are believed to play a significant role in the metabolism and the homoeostatic control of a number of essential (Zn, Cu) and toxic (Cd, Hg) trace metals. The formation of metallothioneins is a response to the exposure of an organism to high metal concentrations, however, as this response is non-linear, initial attempts to use the formation of metallothioneins as bioindicators for the exposure of organisms to metals turned out to be unsuccessful.

The analytical strategy for metallothioneins normally requires the use of multi-dimensional separation techniques (Table 8) [169–171], starting with an SEC separation (normally with on-line or off-line ICP-MS detection). The heart-cut of the metal-containing fraction is then subjected to a RP-HPLC separation, again with ICP-MS detection. Once the relevant peak has been located, it is further analysed by ESI-MS for identification of the metalloprotein. As the metal binding capacity of metallothioneins is strongly pH-dependent, a step-wise or complete demetallation can be achieved by lowering the pH to a value below 2. Both the disappearance of the different isoforms of the metallothionein (with the formation of the corresponding apo-metallothionein) and the higher degree of protonation lead to a significant increase in sensitivity for the detection of the protein at low pH. If the sequence of the ligand (=the apo-metallothionein) is already known and existing in a protein database, the determination of its molecular mass is already sufficient for its identification. In case of a previously unknown protein, in-source or more favourably, CID with a tandem MS instrument can be performed for the elucidation of its amino acid sequence. A number of examples for the structural elucidation of various metallothioneins and their isoforms were given by

Table 7
Comparison of speciation analysis for low-molecular-mass (organometallic) compounds and for high-molecular-mass metalloproteins

Criterium	Low-molecular-mass compounds	Metalloproteins
Molecular mass	Low (<1000)	Medium to high (1 . . . 70 000)
Stoichometry	Well defined (mostly)	Not always known or stable (due to coordinative binding of metals)
Structure known	Yes	Often not a priori, can be determined after tryptic digest or CID fragmentation by comparison with protein databases
Ions formed	Mostly singly charged	Mostly multiply charged
Matrix	Moderate complexity	High complexity
Complexity of method	Low to medium (one-dimensional separation often sufficient)	Medium to high (mostly two- to three-dimensional separations required)
Availability of standards	Yes, mostly	Limited availability to unavailable
Availability of reference materials	For few species and matrices	Hardly any
Thermodynamic stability	Yes, mostly	Not always given
Nature of carbon–metal bond	Covalent	Coordinative
Volatility	High to medium (sometimes derivatisation required)	Low to very low, cannot be separated in the gas phase

Table 8
Use of ESI-MS coupled to CZE and HPLC for the analysis of metallothionein isoforms

Sample	Separation	Detection	LOD (mg l^{-1})	Refs.
Rabbit liver MT, MT-1, MT-2 (Sigma)	CZE	ICP-QMS and ESI-MS–UV	n.d.	[173,254]
Rat tissue	CZE	ICP-QMS, ICP-SFMS and ESI-MS	80 (for MT) 3.5 (for ^{114}Cd)	[174]
Rabbit liver MT-2 (Sigma)	RP-HPLC (Vydac C_8 microbore column)	UV, ICP-QMS and ESI-MS	n.d.	[255]
Rabbit liver MT-1 (Sigma)	RP-HPLC (Vydac C_8 microbore column)	ICP-QMS and ESI-MS	n.d.	[254]
Rat liver (induced MTs)	RP-HPLC (Vydac C_8 microbore column)	ICP-QMS and ESI-MS	n.d.	[166]
Rabbit liver (Sigma)	RP-HPLC (Synchrom C_{18})	UV and ESI-MS	n.d.	[256]
Sheep liver	RP-HPLC (C_{18})	UV and ESI-MS	n.d.	[257]
Rabbit liver MTs (Sigma)	RP-HPLC (Vydac C_4)	ESI-MS	n.d.	[258]
Rabbit liver MT, MT-1, MT-2 (Sigma)	RP-HPLC (Vydac C_8)	UV and ESI-MS	n.d.	[259,260]
Rabbit liver MT-1, MT-2 (Sigma)	RP-HPLC (Vydac C_8)	UV and ESI-MS	n.d.	[172]
Horse kidney MT (Sigma)	RP-HPLC (Vydac C_8)	UV and ESI-MS	n.d.	[261]
Cd complexes in plant tissue	RP-HPLC (Nucleosil C_{18})	Nano-ESI-MS	n.d.	[262]
Rabbit liver MT-2a (Sigma) human bladder T24 tumour cells	RP-HPLC (Aquaphore RP-300 C_{18})	UV and ESI-MS	n.d.	[263]
Rabbit MT-1 and recombinant human MT-3	RP-HPLC	Off-line ESI-TOF-MS	n.d.	[264]
mouse liver MT-1	CZE	ESI-MS	n.d.	[265]
Rabbit liver MT 2 (MT2a and 2c, Sigma)	SEC (BioSep-SEC-S-2000)	Nano-ESI-Q-TOF-MS	n.d.	[266]
Recombinant human MT		ESI-MS	n.d.	[267]
MT isoforms in rat kidney and liver	SEC (Superdex-75) and microbore RP-HPLC (Vydac C_8) or CZE	ICP-MS and ESI-MS	n.d.	[268]
α- and β-domains of human MTs		ESI-MS	n.d.	[269]
Rabbit liver MT-2 (Sigma)	Off-line RP-HPLC (PrimeSphere C_{18})	UV and ESI-MS	n.d.	[270]

Chassaigne and Lobinski (characterisation of Cd-metallothionein isoforms [170,172]), by Mounicou et al. (rabbit liver metallothioneins [173]) and by Polec and co-workers (metallothioneins in rat liver [174,175]).

Lobinski and co-workers also included several examples of metallothionein analysis by ESI-MS in some of their reviews on the use of multi-dimensional separation techniques and electrospray MS in biochemical analysis [17,16,39]. A comprehensive review on the use of hyphenated techniques for the characterisation and quantification of metallothionein isoforms has recently been prepared by Prange and Schaumlöffel and discusses in detail the analytical strategies (again, mostly based on multidimensional separation) and the underlying instrumental developments, particularly in capillary zone electrophoresis interfacing technology (CZE–ESI-MS and CZE–ICP-MS) [171].

3.4.2. Phytochelatins

Plant cells resist to the cytotoxic effect of heavy metal ions by sequestering them in stable, intracellular macromolecular complexes. Different types of metal-chelating compounds have been developed by living organisms to regulate the intracellular metal ion concentration. Out of these, phytochelatins, the short metal-induced sulfhydryl-rich peptides possessing the general structure: (γ-GluCys)$_n$Gly with $n=$ 2–11, have attracted the biggest attention [41].

Phytochelatins (PCs) are a class of comparatively low-molecular-mass oligopeptides ($M_r<1000$) composed only of the three amino acids cysteine (Cys), glutamic acid (Glu) and glycine (Gly) where glutamic acid is linked to cysteine through a γ-peptide linkage. Their general formula is (γ-GluCys)$_n$Gly where n typically is between 2 and 11 [176]. They are synthesised from glutathione (GSH) during a reaction catalysed by an enzyme called PC-synthase in the presence of some heavy metals. The complexation of heavy metals by the sulfhydryl-rich peptide (from cysteine) allows the plant to control the intracellular concentration level of metal ions and imparts a certain degree of tolerance to the heavy metal stress. The general structure of PCs is conservative in a wide variety of plants, and only some modifications may occur on the C terminal amino acid. These modified PCs are called iso-PCs.

The analytical strategy for PCs is similar to the one outlined for MT isoforms. The presence of metal-complexing phytochelatins can easily be detected by the on-line coupling of SEC with ICP-MS detection in neutral medium [177,178]. If RP-HPLC separation under the same is to be used as second separation dimension, the mobile phase has to be acidified in order to provide reasonable retention for the analytes. Under these conditions, however, the metal–peptide complex is dissociated, for which reason ESI-MS detection has to target the ligand. When the molecular mass determined is not sufficient to positively identify the phytochelatine, its unequivocal confirmation can be achieved by fragmentation of the molecular ion. As the fragmentation of peptides is well understood and produces, as already mentioned before, two characteristic series of fragments, depending on which moiety the charge remains, the peptide can be identified from the CID mass spectrum of the protonated molecule ion [179,180].

3.5. Interaction of metals and proteins

From the very early stages of electrospray ionisation with the pioneering work of Fenn et al. [181] on electrospray ionisation, great interest was directed towards the use of ESI-MS for the investigation of noncovalent complexes which are of greatest importance in biology, biochemistry and biological research [182–187]. Most proteins and other biomolecules consist of functional subunits, and their coherence as well as the interaction with substrates, ligands, activators or inhibitors occurs through weak forces such as electrostatic, hydrophobic and hydrogen bonding interactions. The determination of the molecular mass of a complex can be used to study molecular assemblies and their individual building blocks. The relative signal intensity observed may be used as an indicator for the strength of interaction, and complex stability constants may be calculated from these data [188]. The investigation of complexes formed between proteins or oligonucleotides and their potential inhibitors is a valuable tool, e.g., for drug screening [189]. Of particular importance are metal–protein complexes. Metal ions very often have the role of activators or cofactors in biological systems, and the function of an enzyme system may be triggered or switched off by the presence or absence of certain metal ions. Additionally, a number of important proteins are known (such as the heme) for which the coordination of metal ions is essential to fulfill their physiological role. These metal ions (e.g., Fe^{2+}/Fe^{3+}) are only bound by weak coordinative bonds, and they may change their redox states, thus challenging the analyst with a particularly difficult task of speciation. For this reason we will include the discussion of the use of ESI-MS techniques for the study of metal–protein interactions in this review. For obvious reasons, electrospray ionisation is exclusively used for this task, while APCI cannot be used for the study of comparatively labile, high-molecular-mass complexes.

An important question in this context is whether the complex structure and conformation as present in solution (normally, in aqueous phase) is preserved without alteration when transferred into the gas phase. Particularly for the study of biomolecules, a compromise has to be found here: while from the theoretical point of view the investigation of aqueous solutions and near-neutral pH would be desirable, it is from the practical point of view preferable to study protein solutions at a pH of 3–4 and with the addition of organic solvents to the mobile phase in order to improve sensitivity and electrospraying behaviour. In addition to solution pH and temperature, complexes are also very sensitive to gas phase dissociation. For this reason, instrumental parameters such a the energy of collisional dissociation, the capillary interface temperature, and the countercurrent gas flow and temperature have to be optimised to enable the observation of the intact complex. As many of the observed complexes are rather labile, but on the other hand it is essential to desolvate the gas phase complex, the stripping of solvent molecules from the molecular complex prior to mass spectrometric determination has to be balanced with the forces that keep the complex together.

The desolvation of ESI-generated droplets is an issue of paramount importance for the direct observation of non-covalent complexes. When measuring complexes in highly aqueous, neutral pH solutions, solvent adducts to the multiply charged ion are typically observed. This is not desirable, since it reduces the overall sensitivity as the total ion current is distributed over a great number of heterogeneously

solvated ionic species. Broader peaks also complicate the exact determination of the molecular mass. While modern instrument designs include a number of possibilities for removing or reducing analyte solvatisation, they have to be carefully adjusted to preserve the integrity of the complex. It here becomes evident that every instrument, and more, every biochemical system has its own set of optimum conditions. The recent application of ESI-TOF and Q-TOF instruments for the study of biomolecules has, for instance, led to the observation that an increased pressure in the ESI interface and downstream prior to the detector significantly improves the detection of very large noncovalent protein complexes [190,191]. The increase of pressure generally enhances the performance for detection of most complexes, but the enhancement is most noticeable for very large assemblies observed at the high m/z accessible by TOF-MS. It is at the moment still under debate whether the observed effect is a result of collisional focusing or rather efficient desolvation or the both.

The factor contributing most importantly to the popularity of ESI-MS for the study of noncovalent complexes is the assumption that the observed gas phase ions are a true representation of the ions present in solution. This assumption may not be valid completely, but it may serve as a good enough approximation to allow the study of complex formation and stability. The strongest correlation between gas and solution phase behaviour can be found for the determination of complex stoichiometry from the relative abundance of the signals in the ESI mass spectra. The positive correlation between the mass spectrometric observations and the solution phase chemistry has prompted the application of ESI-MS for the determination of solution equilibrium binding constants. Particularly for protein–ligand interactions, this has been a viable approach as was demonstrated by Lim et al., who as an example, have used ESI-MS data to construct Scatchard plots from which the binding constants of vancomycin antibiotics with peptide ligands could be determined [192]. ESI-MS is of particular value in this type of investigations, since the types of interaction that govern noncovalent interaction in solution can at least in part be distinguished from the observed MS results. For example, electrostatic forces are greatly strengthened in a solventless environment, and thus complexes held together by electrostatic forces are very stable in the gas phase. Electrostatic interactions are decreased in solution by its dielectric constant. In contrast to this, interactions that are mainly governed by hydrophobic interactions in solution appear to be weakened in vacuum. This may explain why apparent relative affinities measured by ESI-MS for the hydrophobic binding of small molecules not necessarily correlate with their affinities in solution. The different relative stabilities of gas phase interactions have certain implications for the determination of absolute and relative binding affinities in solution: If ligands bind to a target molecule with similar binding mechanisms (and thus may have similar gas phase stabilities), the determination of relative binding affinities from ESI-MS measurements is likely to be reasonable. If, however, hydrophobic interactions are mainly or at least to a significant part responsible for complex formation in solution, the lability of the gas phase complex may compromise the conclusions drawn on binding affinity from the MS data. Bearing this in mind, ESI-MS offers an extremely valuable tool for studying weak interactions between metals and biomolecules, such as peptides, nucleotides and proteins which range from studies on the mass spectrometric behaviour of biomolecule–metal complexes to the study of the interaction mechanisms and sites of metals and biomolecules.

In a study on the fragmentation behaviour of photomodified oligodeoxynucleotides, Wang et al. [193,194] report that metal ion adduct formation favours the fragmentation of the oligodeoxynucleotides within the photomodified moiety while the DNA strand fragment remains largely unaffected compared to the oligodeoxynucleotide form that carries protons on the backbone phosphates. The authors interpret this finding in the way that the energy required for strand cleavage increases for oligodeoxynucleotide–metal ion adducts as compared to its protonated form. This observation is in line with the data presented by Pramanik et al. in their tutorial review on ESI-MS for the study of non-covalent complexes [195]. They discuss that the adduct formation of the hepatitis C virus (HCV) with Zn ions (which is coordinated to the three cysteines and one histidine molecules of HCV) is stabilising its tertiary structure which can be directly derived

from the envelope of the ESI-MS spectrum of the multiply charged ions. An indirect proof for the stabilisation of proteins by adduct formation was given by Guy et al. [196]. They demonstrate that betabellin 15D, a 64-residue, disulfide-bridged homodimer, is able to bind a divalent metal ion in each of its clusters. When incubated with Cu^{2+}, Zn^{2+}, Co^{2+} or Mn^{2+}, metal complexes are observed by ESI-MS with the binding affinity decreasing in the above given order (consistent with the solution-phase affinity for nitrogen- and sulfur-containing ligands). In the presence of Cu^{2+} ions, the protein shows increased stability towards cleavage by pepsin which is attributed to the stabilisation of the tertiary structure of the betabellin and thus decreased accessibility to pepsin attack.

Loo [197] investigated the binding of Mg^{2+} and Mn^{2+} to enolase and nucleocapsid protein (NCp7) as example for the interactions of metals and proteins. The presence of the divalent cations reduces gas-phase dissociation of the enolase which is observed in metal free solutions or solutions containing only alkali metal ions. For the Zn-binding NCp7, a study of the metal binding site was performed by controlled proteolysis with trypsin in conjunction with ESI-MS monitoring of the metabolite.

The formation of mercuro–polypeptide complexes after deprotection of the protected cysteine residues in synthetic polypeptides was studied by ESI-MS by Boysen and Hearn [198]. Very stable mercury–polypeptide complexes were formed while Zn^{2+} and Co^{2+} were shown to possess lower affinity as concluded from the evaluation of the relative spectral intensities of competitive exposure experiments.

The binding of Zn to engineered haemoglobin was studied by ESI-MS by Lippincott et al. [199] and allowed the binding sites for Zn to be identified. It is interesting to note that differences in the mass spectra of different haemoglobin species were only seen in the spectra of intact proteins, however not in the spectra of the denaturated proteins. The observed mass difference corresponded to the binding of a Zn atom to the haemoglobin. At increased fragmentor voltage, the loss of a fragment of 96 Da could be seen which was attributed to the loss of ZnS. This indicates that the Zn is bound to a cysteine residue which was also confirmed by other techniques.

The metal–protein binding abilities were also investigated by Troxler et al. [200] in an ESI-MS study of the Ca^{2+} binding properties of human recombinant α-parvalbumin and nine mutant forms. ESI-MS allowed the determination of the complex stoichiometry which was 2 mol Ca^{2+}/mol protein for the native protein and the binding of 1 or 0 mol Ca^{2+} per mol protein for the mutant forms.

Nousiainen et al. [201] report on the use of ESI-FT-ICR-MS for the study of Ca^{2+}, Mg^{2+} and La^{3+} binding to bovine bone osteocalcin (OCN). The OCN was shown to bind 3 mol Ca^{2+} per mol protein. While Ca^{2+} induced the dimerisation of OCN, both Mg^{2+} and La^{3+} did not induce dimerisation, although binding to a similar extent to OCN. This was attributed and experimentally demonstrated to be due to the binding of Ca and the other metals at different sites of the OCN molecule.

Studies of the interaction of low-molecular-mass biomolecules and metals often have model character to deduce information on the actual form of interaction in larger biomolecules. As an example, the interaction of the amino acid L-histidine with various metal ions (Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}) has been studied as model for the active binding site of many metalloproteins by ESI-MS–MS [202]. The fragmentation patterns of metal–histidine (1:1) complexes were studied and appeared to differ depending on the bound metal ion [e.g., loss of the (neutral) coordinated metal, charge reduction reactions of the ion, or fragmentation of the organic residue].

On a related issue, electrospray mass spectrometric studies on L-carnosine complexes with Cu(II) and Zn(II) ions in aqueous solution were investigated [203]. Both the stoichiometry and the stability of the complexes of L-carnosine (=β-alanyl-L-histidine) with Zn and Cu were probed and results indicated the formation of *bis*-complex species for both copper and zinc, and for zinc also the existence of oligomeric species. It is pointed out that ESI-MS is able to produce data at high pH or low concentrations where other techniques, such as potentiometric measurements, already fail.

The interaction of biomolecules and metals can also be used to increase detectability of weakly responding analytes by ‘‘metallation reactions’’. Prominent examples are carbohydrates which normally show a very poor response in ESI-MS detection. If bivalent cations ions are added to the

sample, metal adducts are readily formed which can be detected with good sensitivity [204]. The abundance of the doubly charged adduct ion for the investigated carbohydrates (maltoheptaose and several N-linked glycans) decreased in the order Ca > Mg > Mn > Co > Cu. Singly charged ions were also observed as a consequence of charge reduction reactions.

Other biomolecules of interest are flavonoids that are known for its antioxidant behaviour. This behaviour may be related to its chelation ability for iron and copper. Fernandez et al. [205] have investigated the stoichiometry of flavonoid–metal complexes of iron and copper with flavonoids from different classes. The stoichiometry of metal:flavonoid complexes was found to range from 1:1 to 2:3 with the stoichiometry of 1:2 being the preferred one. Redox reactions were observed to take place between the flavonoid molecule and the metal ion.

Related work is reported by Ross et al. who characterised the metal-ion complexes of dissolved tannins [206]. Complexes of this biogeochemically important compounds with copper and other metal ions were studied and allowed their stoichiometry and oxidation state to be determined. Using tandem MS, the principal binding site of one of the tannins for copper was identified.

Metal–oligonucleotide interactions have also been observed by Kobayashi et al. for Fe(III) complexes and a DNA chain [207]. ESI-MS gave clear evidence for the formation of a such complex as well as of a Fe(III) peroxide adduct to a DNA chain.

Metal complexes of mycotoxins and related compounds have been investigated by Woodcock and co-workers [208,209]. The mycotoxins and their metabolites Sporidesmin A and D, gliotoxin and dimethylgliotoxin form *bis*-ligand complexes with Ag^{+} and Cu^{+} of remarkable stability which do not dissociate even at high fragmentor voltage. The complexes with divalent cations (Zn^{2+}, Cd^{2+}, Co^{2+} and Mn^{2+}) are of significantly lower stability.

Hormones also show considerable metal binding affinities. This was demonstrated by Wei et al. [210] with an ESI-MS study of the binding of the nonapeptide hormone oxytocin and bivalent Cu, Ni, Mn, Zn and Pd ions at different pH values. At low pH ($pH \approx 2$), no complexes are formed. At pH 5, a number of metal–oxytocin complexes are observed, and with increasing pH both the stability and the coordination site of the metals are changing. Complex formation is accompanied by significant conformational changes of the oligopeptide.

The interaction of the anticancer drug cisplatin and the chemopreventive agent selenomethionine have been studied by Liu et al. [211]. Both ESI-MS and NMR spectroscopy were used for the characterisation of reaction intermediates and products. Stable Pt-selenomethionine complexes were identified depending on the stoichiometry, and this finding may be relevant for the prevention of cisplatin-caused intoxication for patients that are under treatment by this platinum anticancer drug.

Complexes of cysteine, homocysteine and glutathione with bismuth have also been investigated by ESI-MS: Burford et al. [212] report that the three sulfur-containing amino acids most likely form ligand–Bi (2:1) complexes in solutions containing Bi^{3+} as $BiCl_3$ or $Bi(NO_3)_3$, while no complexation has been observed with methionine, despite an earlier report of a such complex [213]. This suggests the importance of the thiolate group in the formation of bismuth complexes.

The study of interaction of drugs, metals and proteins with oligonucleotides by ESI-MS is comprehensively reviewed by Beck et al. [214]. Widest attention is paid in this review to platinum complexes with DNA which is justified by their relevance for chemotherapy. Complexes of DNA or oligonucleotides with other metal ions, including alkali and transition metal ions, are also discussed, but according to their less frequent appearance in the literature in less detail. The authors point also out that, despite the known carcinogeneity of Cr(VI) there exist so far only very few studies on the mechanism of interaction of chromium and DNA or DNA fragments.

4. Conclusion

API-MS techniques coupled with liquid-phase separation are analytical tools of increasing importance for speciation analysis. A key factor for this success is that they provide molecule specific detection which allows the confirmation of known and the identification of unknown analytes. This is an important advantage over the element specific de-

tection techniques used in speciation analysis, with HPLC–ICP-MS being the most prominent example.

The preservation of the integrity of molecular species is an issue of increasing importance, as the focus of speciation analysis is eventually shifting from the (quantitative) analysis of small, well defined, and often anthropogenically produced elemental species towards the identification and structural elucidation of metal proteins and complexes in medical and biochemistry. Since metal ions are often bound only through coordinative bonding (weak interactions) to proteins and biomolecules, electrospray ionisation (in contrast to APCI) particularly suited for the characterisation of these metal–biomolecule complexes due to the softness of the ionisation process and the ability to measure even very high-molecular-mass compounds as multiply charged ions. This advantage is probably only paralleled by matrix-assisted laser desorption-ionisation (MALDI) MS, however, this technique can hardly be coupled on-line to a chromatographic separation.

The attractiveness of API-MS techniques (compared to other ionisation techniques) derives to a great extent from the fact that they can be easily and conveniently be coupled to liquid chromatographic separation techniques: robust and easy-to-use interfaces are available for LC–MS and, to a lesser degree also for CE–MS. Only few restrictions apply to the use of mobile phase flow-rates or additives and allow to optimise chromatographic separation in the various modes of liquid chromatographic separation.

For the study of model systems or relatively simple samples, direct infusion or the introduction of the sample after one-dimensional separation may prove adequate. However, due to the fact that APCI- and ESI-MS are non-destructive ionisation techniques, not only the integrity of the analytes is preserved, but also the complexity of the matrix. This makes selective signal detection significantly more difficult than in elemental MS and either calls for a tedious sample clean-up before (one dimensional) chromatographic separation, or for a chromatographic separation in two or three dimensions, ideally with orthogonal separation techniques. This approach has successfully been adopted by a number of groups which have presented convincing examples on the separation power achievable with three-dimensional separation, e.g., by SEC, IC and HPLC. At the present time, it appears that three-dimensional separation is the practical limit. This is mostly due to the fact that it will be difficult to combine more than three separation techniques that are sufficiently different (ideally, ''orthogonal'') to provide a notable improvement in separation power or peak capacity.

Increasing the dimensionality (and thus the information content) of the hyphenated technique may also be achieved by using multi-dimensional detection. This can be achieved, e.g., by a triple-quadrupole MS instrument that provides MS–MS spectra, or by an ion trap instrument that could provide mass spectra of higher order, MS^n. Alternative routes to increase the information content of the detector is to use high resolution which has become more easily accessible to the analytical community through the introduction of TOF- and Q-TOF-MS instruments. Not only that the sensitivity of TOF-MS detectors surpasses that of all other common MS detectors, but at the same time high mass resolution and accuracy in the determination of the exact mass is provided. These advantages have already been recognised and exploited in elemental speciation studies.

But even with the increase in sensitivity brought about by the new generation of TOF mass spectrometers, ESI- and APCI-MS will not make ICP-MS obsolete for speciation studies, but molecular and elemental mass spectrometry will remain a complementary pair of analytical tools for speciation analysis.

What may however be expected in the near future is that API-MS techniques gain so much in sensitivity due to the improvement in mass analyser technology that the alternative or even parallel use of an instrument in ''elemental'' and ''molecular mode'' becomes feasible. Where ultimate sensitivity is not indispensable, this may be an attractive possibility to achieve both element and compound specific information in one run.

A trend that eventually will also be recognised in speciation analysis by API-MS techniques is the use of isotopically labelled standards for method validation and quality assurance. Although the use of isotopically labelled compounds may probably not appear as attractive with API-MS than with inorganic MS (ICP-MS or thermal ionisation MS) due to the inherently lower precision of isotope ratio determi-

nation, it may still be a valuable tool in the method development and validation phase.

In conclusion, both APCI- and ESI-MS will continue to be valuable tools for speciation analysis, with a strong bias in favour of ESI. This situation is already reflected in the number of speciation applications discussed in this review for electrospray and atmospheric pressure ionisation, and it is expected to be even more biased towards ESI in the future due to the particular suitability of this ionisation technique for the study of metal–biomolecule complexes and their interactions which is believed to become increasingly important in the future.

5. Nomenclature

ACN	Acetonitrile
AE(c)	Anion-exchange (chromatography)
AFS	Atomic fluorescence spectroscopy
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
CAD	Collision-activated dissociation
CE(c)	Cation-exchange (chromatography); capillary electrophoresis
CID	Collision-induced dissociation
Cys	Cysteine
DBT	Dibutyltin
DMA	Dimethylarsinic acid
DEAE	Diethylaminoethyl (resin)
DNA	Desoxyribonucleinic acid
DMSeP	Dimethylselenoniumpropionate
DPhT	Dipenyltin
DTT	Dithiothreitol
ES	Electrospray
ESI	Electrospray ionisation
FT-ICR-MS	Fourier-transform ion cyclotron resonance mass spectrometry
Glu	Glutamine
Gly	Glycine
GSH	Glutathione
HMW	High molecular mass
HG	Hydride generation
IC	Ion chromatography
IP	Ion pairing
IT	Ion trap
LC	Liquid chromatography
LMW	Low molecular mass
LOD	Limit of detection
LOQ	Limit of quantitation
MALDI	Matrix-assisted laser desorption-ionisation
MBT	Monobutyltin
MMA	Monomethylarsonic acid
MPhT	Monophenyltin
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MT	Metallothionein
NI	Negative ion(isation)
NMR	Nuclear magnetic resonance
PB	Particle beam
PC	Phytochelatin
PGC	Pyrolytic graphitic carbon
PI	Positive ion(isation)
Q-TOF	Quadrupole time-of-flight
RPLC	Reversed-phase liquid chromatography
SAX	Strong anion-exchange
SEC	Size-exclusion chromatography
SIM	Selected ion monitoring (mode)
SPME	Solid-phase microextraction
SRM	Selected reaction monitoring (mode); standard reference material
TBT	Tributyltin
TCA	Trichloroacetic acid
TEAH	Tetraethylammonium hydroxide
TeMA	Tetramethylarsonium (iodide)
TFA	Trifluoroacetic acid
$TMAs^{+}$	Trimethylarsonium ion
TMAH	Tetramethylammonium hydroxide
TMAO	Trimethylarsine oxide
TMT	Trimethyltin
TOF	Time-of-flight
TPhT	Triphenyltin

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