Speciation analysis by small-bore HPLC coupled to ICPMS

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Abstract

Although HPLC-ICPMS has established itself as the analytical technique of choice for elemental speciation analysis, there are some remaining limitations, mainly concerning post-column dispersion effects and the degradation of the ICP stability and ionization efficiency due to organic or saline mobile phases. The application of small-bore columns in conjunction with low dead-volume interfaces proved very useful for overcoming these problems, since they can provide lower matrix load and reduced peak dispersion, along with minimization of sample size and solvent consumption, faster chromatographic separations, better resolution and sensitivity.

Since, more work is expected to be reported in this area, we review recent research in the field of speciation analysis by small-bore HPLC coupled to ICPMS, with the emphasis on significant advances in methods and instrumentation, relevant applications and current limitations.

Keywords: Speciation; Narrow-bore; Micro-bore; Nano-bore; Inductively coupled plasma mass spectrometry; UHPLC;

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Abbreviations: AB, arsenobetaine; AF, Affinity chromatography; AsIII, Arsenite; AsV, Arsenate; Cd-MTs, cadmium metallothioneins; CrIII, trivalent chromium; CrVI, hexavalent chromium; Cu-MTs, copper metallothioneins; DIHEN, Direct injection high efficiency nebulizer; DIN, Direct Injection Nebulizer; DMA, Dimethylarsinate; GP, Gel permeation chromatography; GPx, Glutathione peroxidase; HEN, High efficiency nebulizer; HILIC, Hydrophilic interaction liquid chromatography; HPLC, High performance liquid chromatography; ICPMS, Inductively coupled plasma mass spectrometry; IP-RP, Ion pairing – reversed phase; MA, Methylarsonate; MCN, Micro-concentric nebulizer; MD, Membrane desolvation; MeSeCys, Methylselenocysteine; MeSeMet,

Methylselenomethionine; OCN, Oscillating capillary nebulizer; p-ASA, p-arsanilic acid; PDC, 2,6-pyridinedicarboxylic acid; RP, reverse-phase chromatography; SeIV, selenite; SeVI, selenite; Se-AA, Selenoamino acids; SeAlb, seleno-albumin; SEC, Size exclusion chromatography; SeGaba, Selenogammaaminobutyric acid; SelP, Selenoprotein P; SeMet, Selenomethionine; SF, Sector field; TBAH, tetrabutylammonium hydroxide; TMSe, Trimethylselenonium ion; UHPLC, Ultra high performance liquid chromatography; Zn-MTs, zinc metallothioneins; ICPMS instrument: CRC, Collision/reaction cell; Q, Quadrupole; SF, Sector field (HR, high resolution; LR, low resolution; MR, medium resolution); 4-OH, 4-hydroxyphenylarsonic acid.

1. Introduction

Speciation analysis, defined as the determination of one or more individual chemical species of an element in a sample [1], has grown considerably in recent years and it is now recognised as a major analytical field. In fact, this area of Analytical Chemistry has become an important and demanding issue in several research fields, including green chemistry, eco- and clinical toxicology, medical and nutrition science, food and petrochemical industries, for the reason that the toxicity and reactivity of trace elements strongly depend on their chemical form [2]. For instance, trivalent chromium plays an essential role in the glucose metabolism, while hexavalent chromium is highly toxic and carcinogenic [3].

Therefore, a sensitive and selective technique able to discriminate among species is required. Nowadays, the coupling of high performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICPMS) is recognised as the most effective and widely employed instrumental technique for speciation analysis [4-6]. In fact, liquid chromatography is continuously improving and it offers a very rich tool-box for separating the species of interest – including ion exchange, reverse-phase (RP), ion-pairing reverse-phase (IP-RP) and size-exclusion (SEC) chromatography – while ICPMS allows accurate and sensitive quantification of the eluted compounds. Although the HPLC-ICPMS coupling is generally straightforward, a closer look into this hyphenated system highlighted some potential limitations, such as (i) possible metal contamination deriving from the chromatographic system (pump, valve, tubing) or the stationary and mobile phases, (ii) post-column dispersion effects due to the sample introduction system used as the interface between HPLC and ICPMS and (iii) degradation of the stability and ionization efficiency of the ICP source due to the solvent plasma load, especially in the case of organic mobile phases.

In order to solve the detrimental effects associated to the introduction of high amounts of organic solvents or salts into the plasma, low mobile phase flow rates (*e.g.*, on the order of several tens of microliters per minute) can be employed. In this case, the column dimensions as well as

stationary phase characteristics must be adapted. Small-bore columns are then employed [7]. This miniaturization offers several advantages, such as: (i) lower matrix plasma load; (ii) reduced (up to 100-fold) consumption of mobile phase and consequently, waste minimization [8]; (iii) faster chromatographic separations; (iv) better resolution [7] and (v) improved sensitivity, due to the higher sample introduction efficiency and lower solvent load [9]. In contrast, some reports indicate that small-bore columns do not incorporate significant advantages. For example, Stefánka *et al.* [10] found no improvement of chromatographic resolution, peak width or sensitivity when working with 2.1, 1.0 and 0.32 mm i.d. columns.

There has been a growing interest in the application of small-bore HPLC-ICPMS in the speciation analysis field. Not surprisingly, the most investigated elements were arsenic [11-19] and selenium [13-14, 20-30]. Other important studies dealt with the speciation analysis of potentially toxic elements, such as Cr [3, 8, 31-32], Hg [33-37], Pb [33, 35] and Sn [7], or the identification/quantification of biologically-active compounds, such as metallothioneins (MTs) [38-42], cobalamins [35, 43-44] and phosphopeptides [45-47].

In 1997, Garraud *et al.* [9] reviewed the use of small-bore columns coupled to atomic spectrometry. Considering that significant amount of work has been performed since then and that further research is expected in this promising field of Analytical Chemistry, the main objective of the present paper is to review the use of small-bore columns in HPLC coupled to ICPMS for the elemental speciation, with the special emphasis on the recently achieved results and the remaining limitations.

2. Instrumental aspects

Provided that ICPMS is tuned for optimal sensitivity and robustness, the critical points of HPLC-ICPMS coupling concern the chromatographic system and the interface design.

2.1 Chromatographic system

2.1.1 Column dimensions

Typically, the most important dimensions of chromatographic columns are the length, inner diameter and stationary phase particle diameter.

The column length has a direct effect on peak separation. Thus, an increase in this dimension gives rise to an enhancement in the number of theoretical plates whereas the longer the column the more significant the peak dispersion. For example, selenoamino acids were separated on the multi-mode gel filtration GS320A-M8E column while the same separation on the shorter (and narrower) GS320A-M5D column was not effective [21]. On the other hand, the separation of Hg (II), MeHg$^+$ and PhHg$^+$ by reversed phase micro-HPLC was achieved using a 100-mm length ODS column while coelution between the first two compounds was observed with a 150-mm length column [34].

The column inner diameter is another factor to take into account. However, the development of new columns made the situation to be more complex. Thus, for instance, the modification in the column format from cylindrical to columns with square section implies that the column diameter is not enough for describing its geometry. Furthermore, the geometry of the stationary phase has evolved thus leading to the introduction of monolithic columns or to the so-called fused core particles. This fact has led to consider the particle diameter as an obsolete dimension. Nevertheless, the columns usually employed in the field of elemental speciation are cylindrical, what makes it possible to propose a classification such as that indicated in **Table 1** [10, 26].

Table 1. Classification of the columns according to their inner diameter. Adapted from ref. [48]

Description	Dimension	Approximate typical flow rate at a velocity from 1 to 10 mm/s
Open tubular	< 25 μm id	< 25 nL min^{-1}
Nano-bore column	25 μm ≤ id ≤ 100 μm	25 – 4000 nL min^{-1}
Capillary column	100 μm < id < 1 mm	0.4 – 200 μL min^{-1}

Micro-bore column	1 mm ≤ id ≤ 2.1 mm	50 – 1000 μL min⁻¹
Narrow-bore column	2.1 mm < id < 4 mm	0.3 – 3 mL min⁻¹
Normal-bore column	4 mm ≤ id ≤ 5 mm	1 – 10 mL min⁻¹

The reduction in the column diameter incorporated by small-bore columns while keeping constant the stationary phase particle diameter causes a concomitant reduction in the column dead volume. This variable can take typical values going from 200 to 1000 μl for small bore and conventional columns, respectively. Moreover, as it is stated, frictional (Joule) heating is more easily dissipated when working with columns having low diameters than for conventional ones [49] due to their higher surface area to volume ratio. As a result, lower analyte dispersion is produced, thus increasing the detectability by a factor close to one order of magnitude.

Finally, the particle diameter of the stationary phase used in small-bore HPLC-ICPMS ranged from 1.7 to 13 μm, with typical values of 3 – 5 μm. Obviously, the lower the particle size, the higher the chromatographic efficiency and the back pressure required to maintain a given liquid flow rate. This led to the development of a new technique, called "Ultra High Performance Liquid Chromatography" (UHPLC), characterized by the use of sub-2 μm particles and pressures higher than 20,000 psi.

2.1.2 Stationary and mobile phases

Organic species containing a metal or metalloid can be separated according to several chromatographic mechanisms. The present section describes the methods mostly exploited to separate these species using small-bore columns coupled to ICPMS.

Reversed Phase Chromatography (RP) and Ion Pairing Reversed Phase Chromatography (IP-RP)

RPC is one of the most commonly applied chromatographic techniques because of the large choice of eluents that allows the separation of numerous elemental species. The most common RP small bore columns coupled to ICPMS have been C18 and C8, although C4 columns have also been used. The mobile phases in RP chromatography are often aqueous solutions mixed with organic solvents such as methanol, ethanol or acetonitrile. While initially, anion exchange small-bore columns were the most popular to separate chromium, arsenic and selenium species [9], with the introduction of the so-called IP-RP chromatography, it was also possible to separate polar substances on a RP column. This technique is based on the addition of a counter ion to the mobile phase in order to promote the formation of ion-pairs with polar analytes that can be retained on a RP column. **Table 2** shows the main counter ions used with small-bore columns.

Table 2. Studied species, ion pair reagents, stationary and mobile phase used in speciation studies with small-bore columns coupled to ICPMS.

Studied species	Ion pairing reagents	Stationary phase	Mobile phase	Ref.
Et_3Pb^+, Ph_3Pb^+, $MeHg^+$, $EtHg^+$ and $PhHg^+$	Sodium pentanesulfonate	C8	20% ACN	[35]
MeSeCys, SeGaba, TMSe, MeSeMet, SeMet	Perfluorinated carboxylic acids	C8	20 % MeOH	[20]
SeCys, SeMet, SeCA and SeEt	Heptafluorobutyric acid	C18	30% MeOH	[28]
Hg^{2+}, $MeHg^+$, $EtHg^+$, $PhHg^+$, Pb^{2+}, $(Me)_3Pb^+$ and $(Et)_3Pb^+$	Sodium pentanesulfonate	C18	20% ACN	[33]
Arsenite, DMA, MMA, arsenate, *p*-ASA	Tetrabutylammonium hydroxide	C18	0.1 % TFA and 5% MeOH	[19]
Arsenite, DMA, MMA, arsenate, *p*-ASA and 4-OH	Tetrabutylammonium hydroxide	C18	Water	[12]

Reversed phase C18 columns are indicated to carry out the separation of cobalamins [35]. IP-RP micro-bore HPLC has been applied to perform the separation of various cationic species of Hg and Pb [33] as well as anionic species of As [12] and Se compounds [24, 28]. In contrast to normal-bore LC-ICPMS, where the use of high organic solvent flow rates has a detrimental effect on the ICP stability, a C18 nanocolumn (150 mm x 75 μm i.d.) was used obtaining $2-2.5$ times higher intensities

with a mobile phase of 60-70% acetonitrile than with pure aqueous solution [26]. A C18 microcolumn

has also been used in a preliminary study to illustrate the potential of UPLC-ICPMS to contribute to

biomarker discovery via the profiling of sulphur and phosphorus-containing endogenous metabolites

in metabolomic studies [50]. Capillary columns coupled to ICPMS are usually reversed phase C18

columns, with the special characteristic of the small sample volume required (as low as 100 nL),

relevant in some applications in which the available sample volume is low such as forensics, biopsy

samples or single cell analysis [21-22]. Schaumlöffel *et al.* [27] showed that C18 capillary HPLC

could be coupled with ICPMS with sufficient sensitivity and significantly higher chromatographic

resolution and peak capacity than any of the HPLC-ICPMS couplings ever reported. In general,

capillary columns coupled with ICPMS have been used for phosphopeptide identification [45],

selenocompounds speciation [21-22, 27] and Cu and Zn-proteins speciation [41].

C8 columns are RP stationary phases that have less hydrophobic retention than C18 but

comparable selectivity and they are suitable for the determination of small, water-soluble molecules

and peptides. Micro-bore C8 columns have been coupled to ICPMS for the determination of

cobalamins [43], cadmium bio-induced ligands [39], metallothioneins [40], mercury [35], lead [35]

and selenium [20] species, also with the use of an ion pairing reagent in the mobile phase. This type

of column has also been used for the analysis of *cis*-platin adducts with DNA nucleotides [51] and

for the investigation of different metal-containing proteins [41]. Finally, C4 microcolumn has been

suggested for the separation of highly hydrophobic proteins, like Sel P, due to its low hydrophobicity

[24].

Additional chromatographic separation mechanisms

Ion exchange chromatography is one of the most useful chromatographic mechanisms for speciation

analysis of environmental samples [52]. For example, an anion exchange column has been used for

the separation of Cr (III) and Cr (VI) [3, 8, 31, 53], arsenic and selenium compounds [13-16, 18, 54] and metal complexes [52].

Multimode gel filtration columns combine separation mechanisms such as SEC, ion exchange and reversed phase partitioning. Gel filtration nano-columns operating at $2 - 5$ µL min^{-1} have several advantages over other columns for the speciation of biometals [21]: (i) they can be used for biological fluids (tissue supernatants, urine and blood plasma) without requirement for a prior sample treatment; (ii) elution is carried out with salty solutions (similar to physiological conditions) without any detrimental effect on ICPMS due to the low liquid flow rate employed; and, (iii) they can be used for a wide size range of biomacromolecules such as proteins and nucleic acids. This kind of stationary phases was first proposed by Ogra *et. al.* [21] for the separation of selenocompounds. Then, it has been used for biological applications such as metalloproteins separation [30] and copper speciation [42].

Affinity micro-bore columns coupled to ICPMS have been successfully used to carry out the interference-free determination of selenoproteins [23] in limited-size samples. In this study, the simultaneous determination of glutathione peroxidase (GPx), selenoprotein P (SelP) and seleno-albumin (SeAlb) in human serum has been carried out in the shortest analysis time ever reported. This type of small-bore column has also been used for a rapid and effective determination of SelP in human [55] and mouse [56] plasma.

Adsoption chromatography is rarely used in speciation analysis because the high polarity of the stationary phase and the highly non-polar character of the mobile phase can cause irreversible adsorption of species [5].

Multidimensional chromatography allows the separation of complex mixtures by using multiple columns with different stationary phases. These columns are coupled orthogonally, which means that fractions from the first column can be selectively transferred to other columns for additional separation, thus enabling separation of complex mixtures that cannot be separated using a single

column. For example, the separation of hydrophilic Se-metabolites was accomplished by the combined used of hydrophilic interaction liquid chromatography (HILIC) and SEC [25].

2.2 Interfaces

The coupling of HPLC with ICP spectrometry is a relatively easy task. This is because of the compatibility between the mobile phase flow rates used in HPLC and the sample liquid flow rate commonly required when carrying out ICP analyses. When conventional chromatographic columns are adapted, both flow rates are on the order of $0.5 - 1.5$ mL min^{-1}. However, when small-bore columns are employed, the mobile phase flow rate is lowered down to several tens of microliters per minute. In order to obtain good sensitivities and, simultaneously, reduce the analyte dispersion, the interface must be modified [9]. The nebulizer and the spray chamber are one of the weakest points of the process because they contribute significantly to the band broadening. Furthermore, the mass of sample delivered to the plasma and, hence, the sensitivity depends on the quality of the aerosols produced by the nebulizer and on the spray chamber design. This section describes the different instrumental systems used as interface between LC and ICPMS.

2.2.1 Micronebulizers/spray chambers

The use of micronebulizers is the easiest way to match the low separation flow rates needed in micro-bore HPLC to that of ICP. These devices have the capability to efficiently produce a fine and uniform aerosol at low sample uptake rates (e.g., $10 - 100$ µL min^{-1}). The micronebulizers mainly used coupled to micro columns are the micro-concentric nebulizer (MCN) [57], high efficiency nebulizer (HEN) [11, 14, 19]; the oscillating capillary nebulizer (OCN) [58]; and the electrospray nebulizer [59]. Additional pneumatic nebulizers made of plastic materials (PFA) have also been employed for this purpose [18].

The MCN is a low-flow pneumatic concentric nebulizer designed to operate at flow rates between 50 and 200 µL min^{-1} [39-40, 50]. The MCN has been used for the analysis of samples containing high concentrations of mineral acids, high levels of dissolved solids and many other types of dissolved and inorganic materials [13]. The installation is easy and no special requirement concerning the gas supply is needed. The MCN has been frequently used with small-bore columns coupled to ICPMS [12, 14, 34, 39-40, 53, 60], and it is commonly associated with a Scott-type double-pass spray chamber [3, 8, 13, 15, 17, 52], giving stable signals and a very low noise level. As organic solvents are often present in the mobile phase, 2-5% of oxygen has to be added to the nebulisation gas or in the spray chamber in order to facilitate the combustion of the residual solvent in the plasma thus avoiding soot deposit at the torch walls [24]. Due to the low liquid flow rate, the MCN can be coupled to a low-volume spray chamber [45, 60]. In fact, conventional chambers have a large dead volume that degrades the resolution [9].

The HEN is another low-flow nebulizer used to efficiently couple HPLC with ICPMS at liquid uptake rates between 10 and 150 µL min^{-1} [9]. For example, this nebulizer was used for the speciation of five arsenic species, providing excellent detection limits and a reduction in solvent waste compared to conventional nebulizers [19]. This nebulizer has a dead volume (about 25 µL) much lower than that of a typical micro-bore column (roughly 200 µL) what that makes the off-column broadening to be negligible [18].

The OCN consists of two concentric fused silica capillary tubes, mounted concentrically with compression fittings. The OCN has been used with both small- and normal-LC-ICPMS interfaces because of its low dead volume and because it operates stably over an exceptionally wide range of liquid flow rates, from 1 µL min^{-1} to 2 mL min^{-1}. It is normally used with a single pass spray chamber. The OCN together with a micro-bore column operating at 50 µL min^{-1} results in an increase in the sensitivity in terms of peak height by one order of magnitude with respect to a traditional column (4.6 mm i.d.) operated at 0.4 mL min^{-1}. This fact can be explained by considering a combination of higher analyte transport efficiency and improved plasma ionization conditions. No drain tube was necessary

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and it was possible to introduce 100 % methanol for at least 2 h at flow rates lower than 50 µL min^{-1} [58].

The *electrospray nebulizer* is based on the process whereby the aerosol is formed by the application of a strong electric field. It operates effectively at extremely low flow rates (≤1-30 µL min^{-1}). Using the electrospray interface it was possible to introduce 100% methanol into the plasma at up to 5 µL min^{-1}. However when this situation was maintained for a long time, the system showed significant carbon build-up on the cones. An electrospray nebulisation interface with a capillary liquid chromatography column has been successfully developed and it might be a good alternative for trace organometallic analysis, providing low detection limits, wide linear range and limited peak broadening [59].

As already anticipated, the spray chamber design and inner volume are very important to minimize the peak dispersion. Thus, Schaumlöffel *et al.* [61] designed a total consumption micronebulizer with a single-pass low-dead-volume spray chamber for selenopeptide mapping. This assembly showed significantly higher chromatographic resolution and detection sensitivity than any of the reported HPLC-ICPMS couplings [27]. This sample introduction system was also used for the speciation of selenocompounds with capillary HPLC [21-22]. The use of low-dead-volume single-pass spray chamber coupled with a designed nanoflow nebulizer [26] allowed the generation of a fine and stable aerosol at nanoliter-per-minute flow rates.

2.2.2 Direct injection nebulizers

The aforementioned interfaces have associated several problems: *(i)* the analyte transport efficiency is low (*i.e.*, typically on the order of 2 – 20%); *(ii)* a waste is generated and *(iii)* memory effects may cause peak overlapping because of the band broadening. Analyte losses are mainly associated with the generation of large droplets by the nebulizer and their subsequent removal from the aerosol in the spray chamber. Finally, especially if organic mobile phases are used, carbon deposits at either the ICPMS sampler cone or the injection torch can be observed. The so-called Direct Injection Nebulizer

(DIN) does not use any spray chamber prior to the plasma [62]. The DIN tip [63-64] is located at a few millimetres below the plasma base, which promotes the use of micro-bore HPLC columns because all the column effluent is introduced into the plasma [30]. Therefore, they offer a 100% analyte transport efficiency, low dead volume, improved precision, minimum peak broadening and enhanced sensitivity [33, 35].

The DIN operates at liquid flow rates from 30 to 120 µL min^{-1}, it has an extremely low dead volume (< 2 µL) and the sample wash-out is fast, leading to minimal memory effects [31, 35, 38, 43]. The main problems of this device are that it is very expensive, fragile and it is difficult to use [43]. Moreover some authors have noted that because of the high solvent load, local plasma cooling is produced [15, 58]. The use of these low-flow rate nebulizers as a micro-bore HPLC-ICPMS hyphenation has been described in several papers [20, 24, 28]. These works showed that mobile phases with high concentrations of organic solvents could be introduced into the plasma with DIN although the signal intensity is influenced by variations in the organic solvent concentration. Chassaigne *et al.* [65] described the use of a DIN with mobile phases up to 50% methanol for the analysis of cobalamins and metallothioneins. Unlike conventional nebulizers, the DIN coupled to micro-bore columns for Cr speciation provides a constant background and improves limits of detection by about 20 times compared with a conventional column [31]. Similar results were found for Pb and Hg speciation [33]. A DIN also shows a good performance to hyphenate UPLC with ICPMS [28]. When using DIN with small-bore columns, the addition of excess oxygen to the aerosol to prevent clogging was not necessary because of the low flow rate [30]. However, attention must be paid when choosing an eluent because the thin capillary of the DIN can be clogged very easily, for instance with samples containing high contents of dissolved salts [30-31, 33].

Direct Injection High Efficiency Nebulizer (DIHEN) appeared with the idea that (1) the HEN-spray chamber combination offers similar detection limits at solution uptake rates of less than 100 µL min^{-1} to those of conventional pneumatic nebulizers at 1-2 mL min^{-1} [9] and (2) the primary aerosols produced by the HEN exhibit droplet size distributions smaller and narrower than those reported for

the DIN [66]. The DIHEN was constructed to combine the simplicity, low cost, and aerosol characteristics of the HEN with the well-documented advantages of the DIN. Therefore, this nebulizer has been used in conjunction with micro-bore HPLC [10, 35, 47]. Acon *et al.* [35] reduced the dead volume of the DIHEN (from 60 to 13 μl) by inserting a Teflon tubing. In this way, the peak broadening attributed to the DIHEN was 4% of the total peak width (*i.e.*, 3 seconds). In other words, the main source of peak dispersion was the column. Another interesting finding was that, at low liquid flow rates, the plasma remained stable and no carbon deposits were found when organically modified mobile phases were used. Very low limits of detection were obtained for the determination of three naturally occurring cobalamin species and five organolead and organomercury species. A modified DIHEN was used for microLC-ICPMS by inserting an additional internal capillary and whereas it was slightly less sensitive than the original one, its signal stability and resolution were superior due to its extremely small dead volume [47]. Absolute detection limits $1 - 2$ orders of magnitude better than those obtained with conventional nebulizers were found for DIHEN, likely due to the complete nebulization of sample into the plasma [9].

A comparison study demonstrated that the nebulizer-spray chamber assemblies with larger dead volumes detrimentally affected the shape of the resulting peaks, generating band broadening and thus degrading the separation efficiency [14-15]. **Figure 1** shows the separation of four arsenic species using a conventional cross-flow nebulizer and a MCN coupled with a Scott spray chamber and DIN [15]. It can be seen that DIN provided higher sensitivities and sharper peaks than the other systems due to the higher sample introduction efficiency and lower dead volume, respectively. In another study, a comparison of HEN and PFA nebulizers resulted in higher ICPMS sensitivities for the former one while no differences were observed in limits of detection and chromatographic resolution [18]. The performance of different nebulizers as the interface between LC and ICPMS were also compared by Castillo *et al.* [34] and Stenfánka *et al.* [10], with similar conclusions.
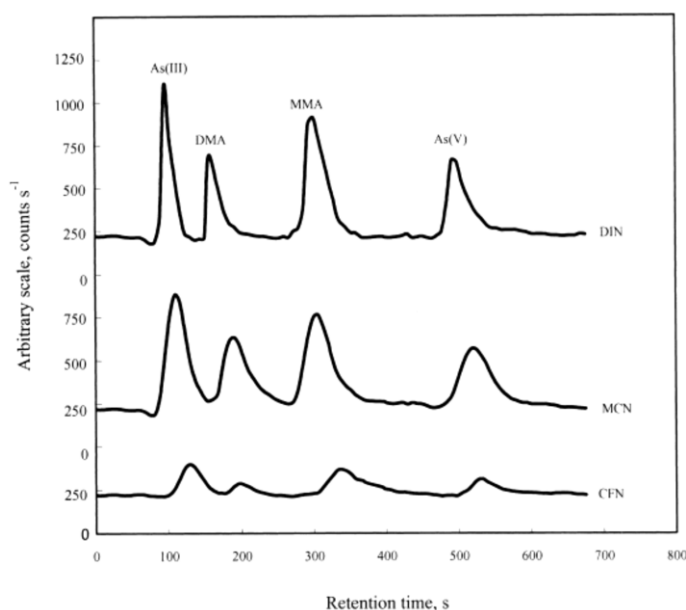
Fig. 1. The chromatograms obtained with use of different nebulizers as interface for the hyphenation of chromatographic separation and ICPMS. From ref. [15]

2.2.3 Additional systems

Some attempts to design other interfaces for HPLC-ICPMS coupling at low liquid flow rates were aimed at reducing the plasma solvent load. For example, a membrane desolvation system (MD) was suggested for the speciation of selenium by gradient elution. The principle of MD is based on the passive diffusion of solvent vapour through a tubular micro-porous PTFE membrane. In this case, the organic solvent contained in the mobile phase is removed and it does not reach the plasma. However, volatile species, such as some selenium compounds are lost in the membrane [24]. The claimed explanation is the transformation of, for instance, selenious acid in selenium dioxide through dehydration at the high working temperature. Additional volatile compounds (*e.g.*, monomethyl selenic acid) can also be removed by diffusion through the membrane pores after their thermal degradation. Surprisingly, species such as trimethylselenonium can be efficiently determined through this means [24].

An additional desolvation system is based on the use of a heated cyclonic spray chamber followed by a multiple condenser (APEX Q). The system has been employed to interface affinity

microHPLC to ICP-sector field (SF)MS. The achieved sensitivity was more than one order of magnitude higher than that found for a conventional double pass Scott-type spray chamber (2 ºC) for the determination of selenoproteins [23].

A second strategy was to optimize the interface for working at extremely low liquid flow rates. For example, a sheathless interface for capillary HPLC-ICPMS was employed at flow rates of 3-7 µL min^{-1} [26]. This range could be extended down to 0.5 µL min^{-1}, but it resulted in increased signal instability [27, 46]. Similarly, Pröfrock *et al.* [46] tested a modified capillary electrophoresis nebulizer and a specially designed 4-mL spray chamber for nanoHPLC-ICPMS coupling at 0.5 µL min^{-1}. However, this attempt was unsuccessful because it resulted in a loss of chromatographic resolution and sensitivity. Further improvement to reduce dead volume is hence necessary in order to take advantage of the direct hyphenation of nanoLC and ICPMS.

Finally, the use of UV illumination/nano-TiO$_2$ photocatalytic system and an effective and rapid pre-reduction process between the µHPLC system and the Hydride Generation-ICPMS has been used to convert all the arsenic species into As (V) and later to As (III). In this way, an improvement in the analytical sensitivity and the determination of non-hydride-forming arsenic compounds was obtained [16].

2.3. Effect of the mobile phase on ICPMS signal

In order to successfully carry out speciation analysis by HPLC-ICPMS, it is necessary to use a mobile phase that is compatible with the ICPMS system, ideally plain water. However, most of separations, especially in RP chromatography, involve the use of organic solvents. Sometimes (*e.g.* for arsenic), low concentrations of methanol (*e.g.* 1-2%) are added to the mobile phase to enhance the ionization efficiency of the plasma source.

It is well known that ICP shows a low tolerance against organic solvents, due to (i) unsatisfactory long-term stability caused by the formation of carbon deposits onto the torch, injector

or interface cones [10]; (ii) changes in the aerosol generation and transport efficiency and (iii) changes in the ionization processes in the plasma. These phenomena are mainly due to the lower average drop size of the aerosol generated from organic solutions and to their higher evaporation rate, thus leading to high solvent load, cooling of the plasma and reduced ionization efficiency [10, 24, 67]. Also aqueous mobile phases are not completely problem-free. For example, the use of phosphate buffer as the mobile phase may cause salt depositions on the interface cones, thus affecting long-term stability.

The use of small-bore LC columns [44] along with a suitable interface between HPLC and ICPMS provides an excellent solution to the problems caused by organic or saline mobile phases, because of the reduced mobile phase mass to be transported to the plasma. For example, the use of a microconcentric nebulizer in combination with a MD [24] allowed the introduction of concentrations of methanol up to 90 % without observing plasma instability or formation of carbon deposits on the cones, thus obtaining constant ICPMS signal for selenium, irrespective of the methanol concentration. On the other hand, other authors [68] reported significant effects on the ICPMS signal by changing the concentration of organic solvents during the gradient elution.

3. Analytical performances

3.1 Retention times, resolution and efficiency.

Retention times can be shortened by increasing the flow rate or reducing the column length, although these solutions will result in poor resolution [28]. The reduction in particle size (1.7 µm) and the use of high pressures (UPLC and UHPLC) can also reduce the retention times, along with an increase of peak height and a decrease in the peak width [28, 69]. However, there is no obvious evidence of the reduction in retention times directly associated with the use of small-bore columns, due to the fact that both the column length and the liquid flow rate are reduced. Moreover, retention times in small-

bore columns are affected by several factors like in normal bore columns, including the mobile phase composition [32-34, 37, 51], temperature [20], interface design [14, 47] and sample composition [70].

Concerning the chromatographic resolution, the major advantage claimed for small-bore columns comes from the decrease in the analyte transversal diffusion due to the reduced inner diameter of the column, with the consequent reduction of the peak width [9]. However, this is compensated by the lower liquid flow rate usually applied, as well as by the reduction of the number of theoretical plates due to the shortening of the column [10]. On the other hand, the resolution can be enhanced by an increase in the column length, although it causes an increase in the analysis time [35, 71]. **Table 3** reports the separation and detection parameters obtained with conventional and small-bore columns for four selenium compounds [21]. It can be confirmed that the observed plate number is lower when using small-bore columns, likely due to lower column length and liquid flow rate. However the resolution for the separation of the first two compounds was higher for the small-bore columns. If the theoretical plate is calculated with a 100x2.1 mm chromatographic column but at different liquid flow rates (from 60 to 200 μL min^{-1}) the result is an increase in the theoretical plate number with the decrease in liquid flow rate [18].

Table 3. Separation and detection parameters obtained with conventional and small-bore columns for four different selenium compounds (taken from ref. [21]).

Dimensions	300 x 7.6 mm			150 x 0.5 mm			250 x 0.8 mm		
Flow rate	500 μL min^{-1}			2 μL min^{-1}			5.5 μL min^{-1}		
Injection volume	20 μL			100 nL			100 nL		
	N[a]	S/N[b]	R[c]	N	S/N	R	N	S/N	R
gGluMeSeCys	11341	964		5711	1443		6165	400	
MeSeCys	11355	1165	2.66	6209	1627	3.22	7671	459	3.72
MeSeGalNAc	12882	91.2		5785	133.8		4967	92.1	
TMSe	17951	51.6	3.06	8084	90.4	1.88	7992	60.4	2.09

[a]Observed plate number. [b]Signal-to-noise ratio. [c]Resolution between two peaks of the major selenocompounds

Besides column dimensions, the resolution can be modified by different variables such as the concentration of organic solvent in the mobile phase and the interface system. For example, a decrease in the overall resolution was reported by increasing the amount of methanol [11]. On the other hand, the optimization of the gradient conditions resulted in good peak resolution and shape for the analysis of four naturally occurring cobalamines and cobinamide [43]. Concerning the interface, it was observed that DIN and MCN yielded a higher chromatographic resolution in arsenic speciation than that obtained using a conventional nebulizer system [15].

The chromatographic efficiency can be improved by the use of columns with smaller particle size than the conventional ones. In fact, according to the van Deemter equation, the theoretical plate height is reduced when the particle size is decreased [28, 69, 72]. Moreover, beyond the optimum velocity the theoretical plate height varies only slightly with increasing the linear velocity, thereby allowing the use of high flow rates without significantly affecting the resolution. However, special systems (named UHPLC or UPLC) are needed when reducing the particle size to maintain the higher backpressure required. Bendahl *et al.* [72] were the first who coupled UPLC with ICPMS in the speciation field for the analysis of bromine-containing preservatives using a micro-bore column packed with 1.7 μm particle.

3.2 Sensitivity and limits of detection.

As the micro-bore columns operate at lower flow rates than conventional ones, the relative peak concentration is higher for the former and better sensitivities are hence expected. Furthermore, the analyte transport efficiency grows as the liquid flow rate drops. As a constant sample volume is injected into the system, the net mass of analyte delivered to the plasma is lower at high than at low liquid flow rates. This is clearly shown in **Table 3**, where a significant increase in the absolute sensitivity may be observed for the small-bore columns considering the 200-fold reduction in the injection volume.

Sensitivity also depends on other factors, mainly the interface employed. For example, Bendahl *et al.* [24] reported a 50% lower sensitivity using the MCN/cyclonic spray chamber system compared to MCN/membrane desolvator and DIN systems for the speciation of selenium compounds. In the arsenic speciation field, Todolí and Grotti [18] demonstrated that HEN nebulizer provided a 50% higher sensitivity than PFA, irrespectively of the arsenosugars considered. However, the improvement in sensitivity by HEN over the PFA decreased with lowering the flow rate (about 30% at 100 μL min^{-1} and about 10-20% at 60 μL min^{-1}). These results were explained taking into account the higher solvent evaporation inside the spray chamber when working at low liquid flow rates.

As regards limits of detection, it has been shown that small-bore columns generally provide figures comparable to normal-bore columns, despite the former ones work at much lower liquid flow rates. For instance, Castillo *et al.* [14] demonstrated that detection limits as low as those obtained by conventional HPLC-ICPMS can be achieved for the speciation of arsenic and selenium compounds, provided that the HEN is used. Similarly, Bendahl *et al.* [72] analyzed bromine-containing preservatives by UPLC-ICPMS at 50 μL min^{-1}, reporting detection limits comparable with those previously obtained by conventional HPLC-ICPMS. The detection limits can be further improved by increasing injection volumes [32, 41], although this leads to degradation in the chromatographic separation.

3.3. Column recovery

Speciation analysis with HPLC-ICPMS usually comprises three steps: extraction, separation and selective detection of species, and reliable quantitative results would require the knowledge of mass balance on the distribution of the element during each step. Surprisingly, not all studies clearly report the relevant data and it is unclear if and where losses occur [73]. Extraction efficiency is a major concern also in the speciation analysis by small-bore HPLC-ICPMS and it is merely related to the sample preparation procedure. Column recovery may be defined as the ratio between the sum of the

eluted species and the total elemental concentration in the injected solution. For example, Jitaru *et. al.* [23] determined the column recovery for the determination of selenoproteins in human serum by comparison of the total AF-HPLC peak area (GPx+SelP+SeAlb) with that of the peak obtained by the analysis of the same amount of serum in flow injection mode (no column used). A recovery of ~100% was obtained for the HEP column, whereas only ~80% of selenoproteins was recovered from the BLUE column, likely due to the low recovery of SelP. The mass balance between the sum of the quantified species and the total concentration in the sample was also evaluated by Chen and co-workers [36] for mercury speciation in seawater and by Vanhaecke and co-workers [3] for chromium speciation in industrial process solutions, both reporting quantitative column recoveries.

4. Applications

The main applications of narrow-bore HPLC coupled to ICPMS in the field of arsenic speciation analysis are summarized in **Table 4**. A narrow-bore reversed-phase HPLC column with ion-pairing was used for the first time by Pergantis *et al* [11] to determine phenylarsonic compounds used as animal feed additives in environmental (water and soil extracts) and biological (urine) reference samples. The developed method provided a good separation of the target analytes from the naturally occurring arsenic compounds, with low detection limits, small sample size (<1 μl) and reduced solvent consumption (40 μL min^{-1}). Later, a similar chromatographic system was used by Wangkarn and Pergantis [12] to accomplish a fast (< 3 min) determination of As^{III}, As^{V}, MA and DMA in wine samples and of arsenosugars in kelp powder extracts. However, a wider column (2.1 mm vs 1 mm i.d.) and much higher mobile phase flow rate (700 μL min^{-1} vs 40 μL min^{-1}) were used in this occasion. Woller *et al.* [13] reported the simultaneous speciation of redox species of arsenic (As^{III}, As^{V}) and selenium (Se^{IV}, Se^{VI}) in natural water samples and polluted soil leachings, using an anion-exchange micro-bore column. The chromatographic run time was about 4 min and the instrumental limits of detection at the ppb-level. However, possible interconversion of As^{V} to As^{III} and Se^{VI} to Se^{IV} in the real samples was highlighted. Similarly, Castillo *et al.* [14] achieved the simultaneous separation of arsenic (As^{III}, As^{V}, MA and DMA) and selenium (Se^{IV}, Se^{VI}) species by anion-exchange chromatography, using a PRP-X100 1mm-ID column along with mobile phases based on a mixture of $NH_4H_2PO_4$ and NH_4NO_3. Detection limits were in the range of 0.03–0.04 μg/l for arsenic species and 0.35 μg/l for selenium species. The method was applied to different spiked environmental water samples, showing good accuracy and repeatability, although the analysis of the high salinity samples required the mathematical correction of ArCl-interference on MMA determination. Sun *et al.* [15-16] reported the determination of As^{III}, As^{V}, MA and DMA in urine samples by anion-exchange narrow-bore HPLC, using pneumatic nebulisation [15] or UV photooxidation/Hydride Generation [16] as the interface between the chromatographic system and ICPMS. The achieved detection limits were in the

0.2-0.4 µg/l range. Narrow-bore ion chromatography coupled to ICP-SFMS was applied by Ammann [17] to determine As^{III}, As^V, MA, DMA and AB in groundwater samples. The use of NH_4NO_3 as the mobile phase allowed an easy selection of the pH to enhance the selectivity without compromising the eluent ionic strength and the 50–100 sample dilution drastically reduced the matrix influences on the separation. Possible reduction of As^V to As^{III} due to column deposits during the gradient run was eliminated by an appropriate PO_4-excess in the injected sample, thus preventing artefacts in As^{III}/As^V ratios. Finally, Todolí and Grotti [18] reported the fast determination of arsenosugars in algal extracts by narrow bore HPLC-ICPMS in conjunction with a low sample consumption system. Accurate and precise determination of the major arsenosugars in algae was obtained, with comparable sensitivity as the conventional system, but with strongly reduced analytical times, lower sample and mobile phase volumes and minor matrix loading into the ICP and interface regions of the ICP mass spectrometer.

Selected applications of narrow-bore HPLC coupled to ICPMS for speciation analysis of selenium are compiled in **Table 5**. Gammerlgaard *et al.* [20] reported the determination of selenium metabolites in urine samples by ion-paring chromatography with perfluorinated carboxylic acids and ICPMS detection. The separation was performed on a micro-bore C8 column, connected to the ICPMS via a laboratory-made direct injection nebuliser, which allowed the introduction of high methanol concentrations at the flow rate of 50 µL min^{-1}. The detection limits in urine ranged between 0.8 and 1.7 µg/l. Urinary selenium metabolites were also determined by Ogra and Suzuki [21] using capillary HPLC coupled to ICPMS through a total consumption sample introduction system. Two major selenometabolites (1-β-methylseleno-*N*-acetyl-D-galactosamine and TMSe) were successfully measured in the urine of a Se-toxicosis rat using a 0.5mm-ID gel-permeation column, with 50-75%-higher signal-to-noise ratios, 30%-shorter analytical time and 200-fold lower sample consumption compared to conventional HPLC. Similar improvements were also obtained for two major selenocompounds (γ-glutamyl-Se-methylselenocysteine and MeSeCys) in selenized garlic. Ruiz-Encinar and co-workers [22] reported a method for the accurate determination of SeMet and SeCys

in human serum using reverse-phase capillary HPLC-ICPMS with species-specific isotope dilution calibration. The serum sample was enzymatically digested and the selenoamino acid fraction was isolated by SEC, followed by the separation of SeMet and the carboxymethylated SeCys on a 0.3mm-ID C18 column. The interference-free determination of [77]Se and [80]Se was achieved by using the reaction cell pressurized with hydrogen. The method detection limit was below 0.5 ng/g for a 450-mg serum sample and the precision was better than 5%. Jitaru *et al.* [23] reported the speciation analysis of selenoproteins in human serum using micro-bore affinity HPLC hyphenated to ICP-SFMS. Simultaneous determination of GPx, Sel P and seleno-albumin in only 5µl of human serum was carried out in less than 7 minutes. Due to the use of high mass resolution, no correction equations or preliminary sample preparation steps for removing the Cl- and Br-based interferences were necessary. In order to compensate for the loss in sensitivity due to the high-resolution mode and the very low amount of sample taken for analysis, a high-efficiency sample nebulisation/desolvation system was employed. The analysis of human Sel P was also performed by Bendhal *et al.* [24] using micro-bore reversed phase chromatography with methanol gradient elution. By the application of a micronebulizer in association with a membrane desolvation unit, increasing amounts of methanol (up to 90%) were introduced into the ICPMS instrument without any significant effect on the analytical signal, thereby allowing the quantification of human Sel P isoforms on the basis of an internal TMSe standard. A similar chromatographic system was applied for the analysis of the water soluble fraction of [77]Se-enriched yeast and more than 30 selenium compounds were separated using a linear gradient from 5% to 95% methanol. Unfortunately, none of those peaks was identified. Far and co-workers [25] achieved the separation of a number of hydrophilic selenium compounds in selenium-rich yeast extracts using micro-bore HILIC-ICPMS after fractionation by SEC. Elution was carried out isocratically with ammonium acetate buffer in 80% acetonitrile and a total consumption sample introduction system was used as the interface to ICPMS. Out of 15 peaks observed with the Se-specific ICPMS detection, 12 were identified by ESI Q-TOF MS/MS. Finally, downscaling of HPLC in the selenium speciation field is exemplified by the paper by Giusti *et al.* [26] that reported the

interfacing of nanoHPLC with ICPMS, through a nanoflow nebulizer coupled to a low-volume evaporation chamber and its application to the analysis of a tryptic digest of SIP18 protein. The sample volume was only 11 nl and the employed liquid flow rate, as low as 300 nL min^{-1}, enabled the stable introduction into the ICPMS of aqueous mobile phases containing up to 95% acetonitrile. The achieved absolute detection limit was 25 fg, which allowed the detection of low-abundant selenopeptides at the femtomole level. Identification of selenopeptides was carried out by parallel nanoHPLC-ESIMS.

Another topic of great interest in the bioinorganic speciation analysis field pertains to the characterization of heavy metals complexes with bioligands, mainly cystein-rich polypeptides termed metallothionein (MTs). Most relevant applications of narrow-bore HPLC coupled to ICPMS for the analysis of Cd-, Cu- and Zn-MTs are summarized in **Table 6**. Chassaigne and Lobinski [38] discussed the speciation of MT-bound cadmium by reversed-phase HPLC with ion-spray and ICPMS detection. A micro-bore C8 column with a gradient of up to 50% methanol in acetate buffer was successfully applied to separate the Cd-MT isoforms in the rabbit liver MT-2. The coupling of HPLC with ICPMS was realised by a direct injection nebulizer, allowing the specific detection of cadmium down to the 10 μg/l level. Similarly, Polec *et al*. [39-40] investigated the identification of metal complexes with MTs in rat liver and kidney using parallel HPLC-ICPMS and HPLC-ESIMS. The mixture of MT complexes was isolated from the tissues by SEC and further preconcentrated by lyophilization and desalting. The resulting sample was finally analysed by micro-bore reversed-phase HPLC coupled to ICPMS or ESIMS. The analytical approach allowed the detection of two major MT isoforms in liver and one MT isoform in kidney. Montes-Bayon *et al*. [41] reported the application of capillary HPLC coupled to collision cell ICPMS for the determination of Cu- and Zn-proteins extracted from red blood cells of seal samples. The selected column was a 0.3mm-ID C8 and the separation conditions involved a gradient up to 80% methanol in 10mM ammonium acetate buffer. A new total consumption sample introduction system permitted to work at very low flow rate (3 μL min^{-1}) with high methanol content, while maintaining the sensitivity throughout the chromatographic run. In

order to investigate the Cu metabolism in mutant mouse neonates, Miyayama and co-workers [42] analysed micro-amounts of tissue supernatants by narrow-bore HPLC coupled with ICPMS. Cu species were separated on a gel- permeation 2mm-ID column using Tris-HCl as the mobile phase and the relationship between the amount of Cu in the MT-bound form and MT mRNA expression was evaluated and discussed.

Determination of metal species showing various degree of toxicity is a major issue in the environmental and toxicological field. Representative applications of narrow-bore HPLC-ICPMS for the speciation of Cr, Hg and Pb are presented in **Table 7**. Zoorob *et al.* [31] achieved the separation of Cr^{III} (pre-complexed with 2,6-pyridinedicarboxylic acid) and Cr^{VI} on a micro-bore AS11 column, using 7 mM 2,6-pyridinecarboxylic acid and 0.6 mM lithium hydroxide at pH 7.7 as the mobile phase. A direct injection nebulizer was designed and used to introduce the eluent into the mass spectrometer at 150 µL min^{-1}. The method was applied to the analysis of the certified urine SRM 2670, obtaining accurate results for Cr^{III} at m/z 52. On the other hand, at m/z 53 the Cr^{III} peak was overlapped by a large peak due to Cl-based interferences. Moreover, the analytical time was quite high (20 min). Vanhaecke and co-workers [3] reported the fast (5 min) determination of Cr^{III} and Cr^{VI} in industrial process solutions by micro-bore anion exchange HPLC coupled to ICP-SFMS. As the mobile phase consisted of 60mM HNO_3, matrix effects often encountered in HPLC-ICPMS (e.g. reduction of ion transmission due to clogging of the sampler cone) were minimized. The spectral interferences due to co-eluting C- and Cl-containing species were eliminated by the application of medium mass resolution (R=3000), although this mode led to a 15-fold reduction in transmission efficiency (sensitivity). Nevertheless, for both species involved, sub-µg/l limits of detection were achieved. Speciation of mercury and lead compounds by narrow-bore HPLC-ICPMS was investigated by Shum *et al.* [33]. Various cationic species of mercury (Hg^{2+}, $MeHg^+$, $EtHg^+$, $PhHg^+$) and lead (Pb^{2+}, $(Et)_3Pb^+$, $(Me)_3Pb^+$) were separated by IP-RP chromatography and determined in spiked urine samples, with limits of detection of 0.1 µg/l and 4-8 µg/l for Pb for Hg species, respectively. Finally, Castillo *et al.* [34] developed a method based on the microHPLC-ICPMS coupling for the

determination of four mercury species in spiked environmental waters, including groundwater, depurated urban wastewater and seawater. Gradient elution using methanol and L-cysteine at pH 3.0 allowed the chromatographic separation of all species in about 15 min. Detection limits ranged from 8 to 32 ng/l. However, low recovery for high saline samples was observed.

According to the enlargement of the speciation concept to the analysis of compounds of interest by monitoring an elemental tag, narrow-bore HPLC-ICPMS was also applied to identify and/or quantify other species than those previously reported. Representative examples are reported in **Table 8**. Wind *et al.* [45] described a method for phosphopeptide identification by capillary liquid chromatography interfaced alternatively to ICPMS (^{31}P detection) and ESIMS. The separation was performed on a 0.2mm-ID C18 column using acetonitrile gradient (up to 80%) elution. The method was applied to the analysis of a complex mixture of synthetic phosphopeptides and to tryptic digests of three phosphoproteins, namely β-casein, activated MAP kinase and protein kinase A catalytic subunit. Similarly, Profrock and co-workers [46] coupled capillary- and nano-HPLC with collision-cell ICPMS for phosphorylation profiling of tryptic protein digests. Sar *et al.* [51] determined *cis*-platin adducts with DNA nucleotides using narrow-bore HPLC-ICPMS with elemental specific detection of ^{31}P and ^{195}Pt. A 2-1mm-ID C8 column was used with a mobile phase containing 60 mM ammonium acetate (pH = 5.8) and 7.5% methanol, allowing an easy separation and detection of free nucleotides (by monitoring phosphorus) from the synthesized adduct (containing phosphorus and platinum in the same molecule). Structural characterization of adducts was accomplished by ESI-Q-TOF-MS. Chassaigne and Lobinski [43] reported the determination of cobalamins in pharmaceutical preparations by narrow-bore HPLC with ICPMS detection. Five cobalamins and cobinamide dicyanide were separated in 30 min on a 1mm-ID C8 column, using methanol linear gradient from 10 to 40% in 25mM ammonium acetate buffer (pH 4). Detection limits ranged from 10 to 50 μg/l. Finally, Nowak and Seubert [70] accomplished the ultra-trace determination of bromate in drinking waters by means of micro ion chromatography coupled to ICPMS. The method employed a 2mm-ID column filled with a self-made high-capacity anion exchanger and elution with NH_4NO_3, enabling

the direct analysis of almost every water sample without matrix elimination, at concentration levels ranging from 0.05 to 10 µg/l. However, it was observed that retention behaviour of bromate as well as background levels were influenced by the sample composition.

Aqueous mobile phases have many advantages for ICPMS, but they have also many disadvantages as to the types of compound that can be separated in this way, Smith *et. al.* used a micro-bore column with a isotopically enriched methanol as the organic modifier with the advantages of low flows then the quantity of methanol that arrives to the plasma is reduced and the solvent consumption is also reduced [74].

Malavolta *et al.* [60] used a gradient anion exchange microcolumn coupled to a ICPMS though MicroMist nebulizer and a Cinnabar Cyclonic spray chamber for the speciation of different metals Fe, Cu and Se in biological samples. They obtained the chromatograms in less than 10 min without waiting for column wash and regeneration after each run, with very limited amount of biological material. With this technique is possible the screening of changes of trace elements distribution in serum among individuals caused by various diseases or treatment or physiological changes.

Table 4. Selected applications of small-bore HPLC coupled to ICPMS for speciation analysis of arsenic.

Analytes[a]	Sample	HPLC[b]	Interface	ICPMS[c]	Flow rate (μL min^{-1})	Volume (μl)	Time (min)	LOD[d] (μg/l)	Notes	Ref
As^{III}, As^{V} p-ASA, 4-OH	Spiked water, soil and urine CRMs	IP-RP (C18 150x1 mm, 3 μm) 0.5%MeOH+5mM TBAH[e] (pH 5.8)	Modified HEN+ Scott-type spray chamber [19]	Q	40	0.5-1	10	0.6-0.9	Co-elution of As^{III} and AB in the urine sample	[11]
As^{III}, As^{V} MA, DMA Arsenosugars	Wines and kelp powder extract	IP-RP (C18 150x2.1 mm, 4 μm) 5 mM TBAH (pH 6.2)	Micromist+ impact bead water-cooled (5°C) spray chamber	Q	700	1	2.7	0.3-1.3	4-OH was used as the internal standard for wine analysis	[12]
As^{III}, As^{V} Se^{IV}, Se^{VI}	Spiked groundwater; polluted soil leaching	AE (ANX-1606As; 100 x2 mm) 5 mM CH$_2$(COONH$_4$)$_2$ (pH 8.5)	MCN-100+ Scott-type spray chamber	Q	100	10	4	1 (As) 6 (Se)	Possible interconversion of redox species	[13]

[a] As^{III} = arsenite; As^{V} = arsenate; *p*-ASA = *p*-arsanilic acid ; 4-OH = 4-hydroxyphenylarsonic acid; AB = arsenobetaine; MA = methylarsonate; DMA = dimethylarsinate; Arsenosugars = dimethylarsinoylribosides; Se^{IV} = selenite; Se^{VI} = selenate.

[b] HPLC system: AE = anion-exchange chromatography; RP = reverse-phase chromatography; IP-RP = ion-pairing reverse-phase chromatography; IC = ion chromatography.

[c] ICPMS instrument: Q=quadrupole; SF=sector field (LR=low resolution, MR=medium resolution, HR=high resolution); CRC=collision/reaction cell.

[d] LOD = limit of detection. Values, unless otherwise stated, refer to instrumental LOD.

[e] TBAH = tetrabutylammonium hydroxide.

Analytes	Matrix	Column	Interface	ICP-MS				LOD	Comments	Ref.
As^{III}, As^{V} MA, DMA Se^{IV}, Se^{VI}	Spiked surface water; spiked water treatment plant	AE (PRP-X100; 150x1 mm, 3 μm) $NH_4NO_3/NH_4H_2PO_4$ gradient (pH 8.3)	HEN+ Scott-type cooled (2°C) spray chamber	Q	100	15	5	0.03-0.04 (As) 0.35 (Se)	Correction equation needed for MA in high salinity samples	[14]
As^{III}, As^{V} MA, DMA	Urine CRMs	AE (Nucleosil SB; 150x1 mm, 5 μm) 20 mM $NH_4H_2PO_4$ / $(NH_4)_2HPO_4$ (pH 5.25)	MCN-100+ Scott-type spray chamber	Q	100	5	13	0.2-0.3		[15]
As^{III}, As^{V} MA, DMA	Natural and spiked urine	AE (Nucleosil SB; 150x1 mm, 5 μm) 20 mM $NH_4H_2PO_4$ / $(NH_4)_2HPO_4$ (pH 5.2)	UV nano-TiO2 photooxidation +HG	Q	100	10	15	0.17-0.37		[16]
As^{III}, As^{V} MA, DMA AB	Diluted groundwater	IC (AG-11/AS-11; 50x2 mm / 250x2 mm) NH_4NO_3 gradient (pH 7.6-8.3)	PFA-50+ Scott-type cooled (2-5°C) spray chamber	SF (LR)	300	25	10	0.005-0.010	PO_4-excess in the injected sample to eliminate reduction of As^{V} to As^{III}	[17]
Arsenosugars	Algal extract	AE (Nucleosil SB 100×2.1 mm, 5μm) 60mM $NH_4H_2PO_4$ (pH 5.9)	PFA-ST+low volume cyclonic spray chamber	CRC (no gas)	200	2	5	0.2		[18]

Table 5. Selected applications of small-bore HPLC coupled to ICPMS for speciation analysis of selenium.

Analytes[a]	Sample	HPLC[b]	Interface	ICPMS[c]	Flow rate (μL min^{-1})	Volume (μl)	Time (min)	LOD[d] (μg/l)	Notes	Ref
SeMet, TMSe MeSeMet MeSeCys SeGaba	Urine	IP-RP (C8; 100x1 mm, 3μm) 20% MeOH + HFBA	Home-made direct injection nebulizer [75]	Q	50	3	10-20	0.8-1.7[e]		[20]
Se-AA and urinary Se-metabolites	Selenized garlic; rat urine	GP (GS320A; 150x0.5 mm) NH$_4$Ac (pH 6.5)	Home-made micronebulizer+ evaporation chamber [27]	Q	2	0.100	15-20	-		[21]
SeMet SeCys	Human serum	RP (C18 BDS; 150x0.3 mm, 3 μm) CH$_3$CN gradient + TFA	Home-made micronebulizer+ evaporation [27]	CRC (H$_2$)	4	0.2	20	0.1-0.5 ng/g[e]	Prior proteolytic digestion and fractionation by SEC;	[22]

[a] SeMet = selenomethionine; TMSe = trimethylselenonium ion; MeSeMet = methylselenomethionine; MeSeCys = methylselenocysteine; SeGaba = selenogammaaminobutyric acid; Se-AA = selenoamino acids; GPx = glutathione peroxidase; SelP = selenoprotein P; SeAlb = seleno-albumin.

[b] HPLC system: RP = reverse-phase chromatography; IP-RP = ion-pairing reverse-phase chromatography; GP = gel permeation chromatography; AF = affinity chromatography; HILIC = hydrophilic interaction chromatography.

[c] ICPMS instrument: Q=quadrupole; SF=sector field (LR=low resolution, MR=medium resolution, HR=high resolution); CRC=collision/reaction cell.

[d] LOD = limit of detection. Values, unless otherwise stated, refer to instrumental LOD.

[e] Method detection limit.

			chamber						quantification by speciated ID	
Se-proteins GPx, SelP, SeAlb	Human serum	AF (HEP and BLUE; 50x1 mm, 34 μm) NH$_4$Ac gradient (pH 7)	High-efficiency nebulisation/ MD APEX	SF (HR)	300	5	7	2.5^e	Automated exclusion of the BLUE column during the elution of SelP; quantification by on-line ID	[23]
SelP	Human plasma (purified protein fraction)	RP (C4; 150x1 mm, 5 μm) MeOH gradient +TFA	MCN+ heated (100°C) spray chamber/MD	Q	50	3	25	150		[24]
≈ 15 Se compounds	Water extract of Se-rich yeast	HILIC (Amide-80; 150x1 mm, 5 μm) 80% CH$_3$CN +NH$_4$Ac / HAc (pH .7±0.2)	Home-made micronebulizer+ evaporation chamber [27]	Q	50	5	40	-	Prior fractionation by SEC; peak identification by ESI Q-TOF MS/MS	[25]
Se-peptides	Tryptic digest of SIP18 protein	RP (C18 PepMap 100; 150x0.075 mm, 3 μm) CH$_3$CN gradient + TFA	nanoflow nebulizer nDS-200+evaporation chamber	CRC (H$_2$)	0.300	0.011	20	2.3	NanoHPLC/ESI-MS used for selenopeptide identification	[26]

Table 6. Selected applications of small-bore HPLC coupled to ICPMS for speciation analysis of Cd, Cu and Zn.

Analytes[a]	Sample	HPLC[b]	Interface	ICPMS[c]	Flow rate (μL min^{-1})	Volume (μl)	Time (min)	LOD[d] (μg/l))	Notes	Ref
Cd-MTs	Rabbit liver MT-2	RP (C8; 150x1 mm, 5 μm) MeOH gradient +NH$_4$Ac (pH 6)	DIN (+O$_2$ in the nebulizer gas)	Q	40	5	48	$\approx$ 10	Preconcentration step of 2 min before gradient elution	[38]
Cd-MTs Cu-MTs Zn-MTs	Rat liver and kidney tissues	RP (C8 150x1mm, 5 μm) CH$_3$CN gradient +NH$_4$Ac (pH 6)	MicroMist+ cooled (2°C) spray chamber (+O$_2$ in the nebulizer gas)	Q	40	5	40	-	Prior fractionation by SEC, liophilization and desalting; peak identification by parallel ESI triple-quadrupole MS	[39-40]
Cu-proteins Zn-proteins	Red blood cell extracts	RP (C8 150x0.3 mm, 3.5 μm)	Modified CEI 100 nebuliser +	CRC (H$_2$)	3	0.01-2	60	0.43 (Cu) 2 (Zn)		[41]

[a] Cd-MTs = cadmium metallothioneins; Cu-MTs = copper metallothioneins ; Zn-MTs = zinc metallothioneins.
[b] HPLC system: RP = reverse-phase chromatography; GP = gel permeation chromatography.
[c] ICPMS instrument: Q=quadrupole; CRC=collision/reaction cell.
[d] LOD = limit of detection. Values, unless otherwise stated, refer to instrumental LOD.

		MeOH gradient +NH$_4$Ac (pH 7.4)	home-made evaporation chamber [46]							
Cu-MT	Mouse tissue supernatants	GP (KW802.5; 250x2 mm) Tris-HCl (pH 7.2)	AriMist+ cyclonic spray chamber	CRC	40	5	20	-		[42]

Table 7. Selected applications of small-bore HPLC coupled to ICPMS for speciation analysis of Cr, Hg and Pb

Analytes[a]	Sample	HPLC[b]	Interface	ICPMS[c]	Flow rate (μL min^{-1})	Volume (μl)	Time (min)	LOD[d] (μg/l)	Notes	Ref
Cr^{III}, Cr^{VI}	Urine CRMs	IC (AS-11; 250x2 mm, 13μm) 7 mM PDC[e] + 9.6 mM LiOH (pH 7.7)	DIN	Q	150	2.5	20	1.1-1.2	Cr^{III} was pre-complexed with PDC[s] by warming to 65°C for two hours	[31]
Cr^{III}, Cr^{VI}	Industrial process solutions	AE (ANX-3202; 20x2 mm) 60 mM HNO$_3$	MCN-100+ Scott-type spray chamber	SF (MR)	100	20	5	0.05-0.12		[3]
Hg^{2+}, $MeHg^+$ $EtHg^+$, $PhHg^+$ Pb^{2+}, $(Et)_3Pb^+$ $(Me)_3Pb^+$	Natural and spiked urine	IP-RP (C18 50x1.6 mm / 150x1.6 mm) 20% CH$_3$CN + 5mM C$_5$H$_{11}$SO$_3$NH$_4$ (pH 3.4)	DIN	Q	100	2	5-11	0.1 (Pb)[f] 4-8 (Hg)[f]		[33]
Hg^{2+}, $MeHg^+$, $EtHg^+$, $PhHg^+$	Spiked groundwater, depurated urban	RP (C18; 100x1 mm, 3 μm) MeOH/NH$_4$Ac/L-cystein gradient (pH 3)	MicroMist+	Q	70	20	15	0.008-0.032	Low recovery for high saline samples	[34]

[a] Cr^{III} = trivalent chromium; Cr^{VI} = hexavalent chromium.
[b] HPLC system: IC = ion chromatography; AE = anion exchange chromatography; RP = reverse-phase chromatography; IP-RP = ion-pairing reverse-phase chromatography
[c] ICPMS instrument: Q=quadrupole; SF=sector field (LR=low resolution, MR=medium resolution, HR=high resolution).
[d] LOD = limit of detection. Values, unless otherwise stated, refer to instrumental LOD.
[e] PDC = 2,6-pyridinedicarboxylic acid.
[f] Method detection limit.

	wastewater and seawater		Scott-type cooled (2°C) spray chamber							

Table 8. Selected applications of small-bore HPLC coupled to ICPMS for the determination of other species.

Analytes	Sample	HPLC[a]	Interface	ICPMS[b]	Flow rate (µL min^{-1})	Volume (µl)	Time (min)	LOD[c] (µg/l))	Notes	Ref
Phospho-peptides	Tryptic digests of β-casein, activated MAP kinase and protein kinase A catalytic subunit	RP (C18 ; 25x0.3mm, 5 µm) CH$_3$CN gradient + TFA	PFA-100+ low-volume spray chamber	SF (MR)	4	5	35-40	≈ 0.1 pmol	Assignment of the chromatographic peaks by LC/ESI-MS	[45]
Phospho-peptides	Tryptic digest of $\boldsymbol{\beta}$-casein	RP (C18 150x0.3 mm, 3.5 µm or 150x0.1 mm, 3.5 µm) CH$_3$CN gradient + TFA	Modified CEI 100 nebuliser + home-made evaporation chamber	CRC (He)	0.5-4	0.01-8	110	1.95		[46]
Cis-Platin adducts with	Calf thymus DNA samples	RP (C8 250x2.1 mm, 5 µm)	PFA micronebulizer	CRC (no gas)	200	20	35	-	Structural	[51]

[a] HPLC system: IC = ion chromatography; AE = anion exchange chromatography; RP = reverse-phase chromatography; IP-RP = ion-pairing reverse-phase chromatography
[b] ICPMS instrument: Q=quadrupole; SF=sector field (LR=low resolution, MR=medium resolution, HR=high resolution); CRC=collision/reaction cell.
[c] LOD = limit of detection. Values, unless otherwise stated, refer to instrumental LOD.

DNA nucleotides		60 mM NH$_4$Ac (pH = 5.8) + 7.5% MeOH							characterization of adducts by ESI-Q-TOF-MS	
Cobalamins	Pharmaceutical preparations	RP (C8; 150x1 mm, 5 μm / 100x1 mm, 7 μm) MeOH gradient +25 mM NH$_4$Ac (pH 4)	DIN (+O$_2$ in the nebulizer gas)	Q	40	5	30	10-50		[43]
Bromate	Drinking waters	IC (home-made high-capacity anion exchanger; 100x2 mm) NH$_4$NO$_3$ (pH 6)	V-groove nebulizer	Q	500	885	8-15	0.05[a]	Retention time and background level depended on the sample composition	[70]
Fe, Cu, Se	Human plasma and serum samples	AE (SAX1-S; 50 x 1 mm) Buffer A: 10 mM Tris (pH 7.4) Buffer B: 1 M ammonium acetate in 10 mM Tris (pH 7.4)	MicroMist nebulizer / low-volume Cinnabar Cyclonic spray chamber	CRC (8% H$_2$ in He)	200	100	< 10	-	Tandem LC mode was used to save the time needed for washing and regenerating the columns.	[60]

[a] Method detection limit.

5. Future trends.

Future trends in the use of small-bore HPLC columns coupled to ICPMS lie in the accomplishment of lower limits of detection and faster separations as compared with normal bore HPLC. The need of the reduction in detection limits is noticeable in the bibliography because of the intensive search to reduce extra column contribution and dead volumes in the whole system. This is achieved by the optimization of the system components; use of special micronebulizers, chromatographic columns with small particle sizes, minimizing tubing length and id.

The use of UHPLC coupled to ICPMS is an approach to get faster separations with the use of stationary phases with small particle size (1.7 μm) but working at high pressures. In addition, the use of capillary columns in fast LC gives us the advantage of the absence of additional band broadening due to frictional heating (use of very low nano-flow rates) [76].

The benefit of temperature in liquid chromatography is evidenced [77] and it could be another approach to achieve faster analysis and lower detection times with small-bore columns coupled to ICPMS. The influence of column temperature in these columns has only been employed in two works with a maximum temperature of 65 ºC, being the main reason the challenge to find an adequate high temperature resistant stationary phase. Gammelgaard *et al.* examined temperatures between 20 and 60 ºC in selenium speciation, noticing that retention times decreased for all species with increasing temperature, but due to the peak shapes and resolution deteriorated at high temperatures, the temperature was kept constant at 25 ºC to avoid any possible decomposition of the species [20]. Porous graphitic carbon stationary phase is composed by layers of carbon arranged as hexagons and it supports temperatures as high as 250 ºC, it has the capability of separating organic as well as pure inorganic species and it could be an option to carry out determinations at high temperatures. It has been used as stationary phase to carry out the separation of arsenic compounds [78] at room temperature.

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