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Review

Isotope dilution technique for quantitative analysis of endogenous trace element species in biological systems

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Abstract

The recent developments and trends in quantitative speciation analysis of endogenous trace element compounds in biological systems by ICP isotope dilution MS are discussed. The use of two different isotope dilution techniques is critically evaluated with respect to their advantages and limitations: species-unspecific isotope dilution for the quantification of trace elements bound to unknown biomolecules and species-specific isotope dilution for the quantification of well-defined compounds. Recent data on the quantification of metallobiomolecules by isotope dilution analysis are summarized and future directions in this new field of analytical chemistry are highlighted.

Keywords: Biomolecules; Inductively coupled plasma mass spectrometry; Isotope dilution technique; Speciation

Contents

1. Introduction

In biological systems many life processes are critically dependent on trace elements which can be either toxic or essential for the organism. There are a great variety of biomolecules where metals or metalloids are bound to proteins or peptides, coordinated by nucleic acids or complexed by polysaccharides and small organic ligands such as organic acids [1]. Fig. 1 shows an overview of possible endogenous trace element compounds in biological systems. In recent years the detection, identification and determination of trace element compounds in a biological tissue or body fluid referred to as bioinorganic speciation analysis, has generated much interest in analytical chemistry [2]. The low concentration of the trace element in a biological tissue and the complexity

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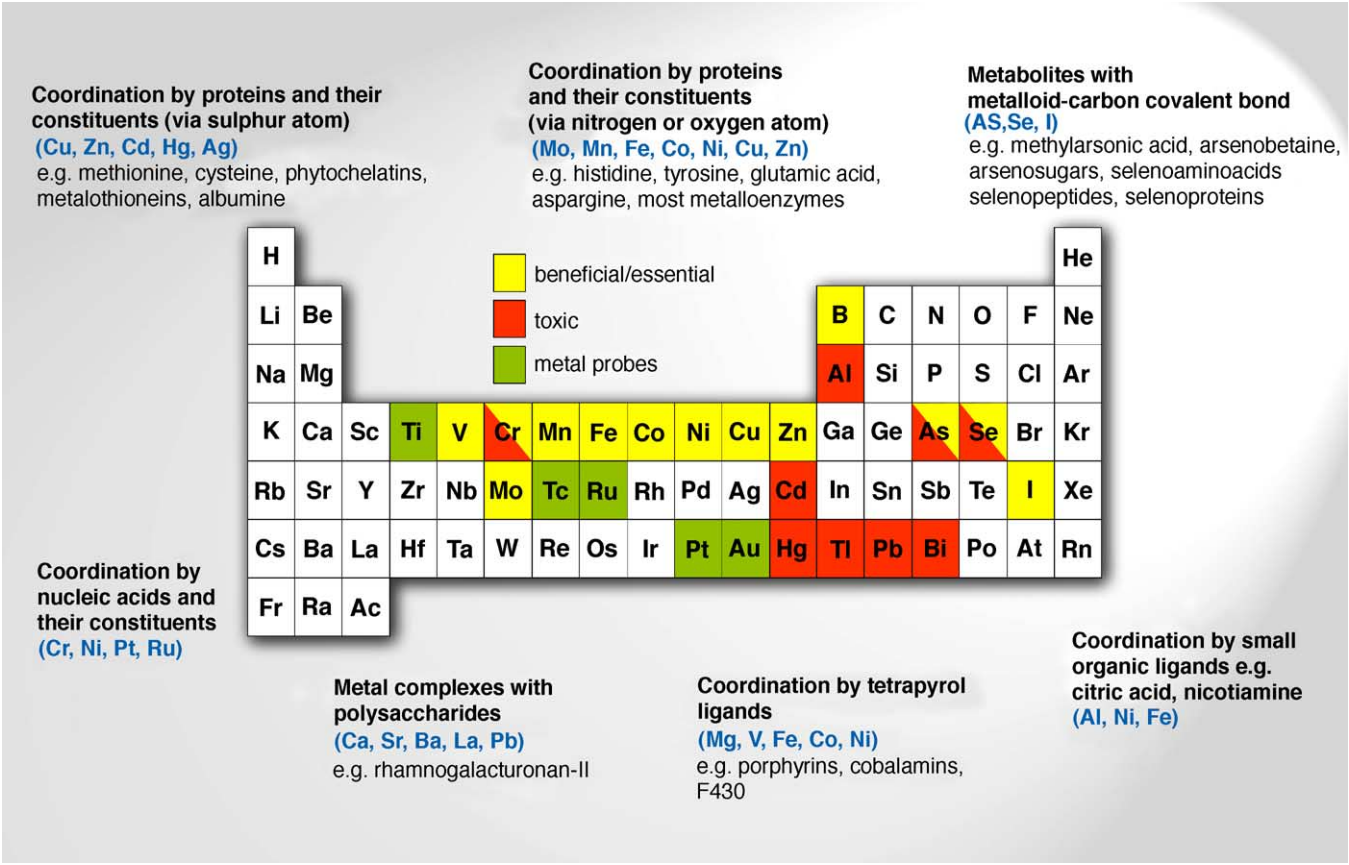

Fig. 1. Endogenous trace element species in biological systems [1]. Reproduced by permission of the Society for Applied Spectroscopy.

of the matrix represent the two major challenges to element speciation analysis in biological systems [1].

The analytical strategy to cope with these challenges usually consists of the combination of high-resolution separation techniques with a highly sensitive element-specific and molecule-specific detector used in parallel [2]. The complexity of a biological matrix demands high-resolution chromatographic or electrophoretic separations. In order to meet this challenge multidimensional separations are often necessary where size-exclusion usually is the first purification step followed by ion-exchange or reversed phase chromatography. However, many metal complexes with biomolecules are too labile to be separated on reversed phase or ion-exchange column. In such cases capillary electrophoresis can be a valuable alternative due to the less interactions between analyte and stationary phase in the capillary [3,4]. The species analysis of labile metal complexes in biological systems is still little explored. In proteomics electrophoresis performed in agarose or polyacrylamide (PAGE) gels is a major technique for protein separation and can be as well applied to metalloproteins, metal–protein complexes and heteroatom (S, Se, P) containing proteins [5]. The downscaling of liquid chromatography has led to the development of capillary and nano-HPLC. These techniques show high separation efficiency combined with low solvent consumption and small sample volumes which opens new applications in bioinorganic speciation analysis [6]. Inductively coupled plasma mass spectrometry (ICP-MS) has been recognized as the most sensitive analytical technique in element analysis allowing also a multielement (multiisotope) determination and accurate quantification by isotope dilution analysis. Recent impressive progress towards lower detection limits and the introduction of collision/reaction cell technology enabling the detection of biologically interesting elements such as sulphur, phosphorus and selenium makes ICP-MS an ideal element-specific detector [7]. Electrospray (ESI) and matrix-assisted-laser desorption ionization (MALDI) mass spectrometry with quadrupole or time-of-flight mass analysers are increasingly used for structural characterization of the biomolecules [8].

The coupling of conventional liquid chromatography with ICP-MS is the most widely applied hyphenated system in bioinorganic speciation analysis. The compatibility of the liquid flow rate used in HPLC with the typical ICP-MS nebulizers allows a simple connection of the HPLC exit to the nebulizer, but the ICP-MS sample introduction system has eventually to cope with high salt concentrations in ion-exchange chromatography or with organic solvents in reversed phase chromatography. Capillary HPLC, nano-HPLC and capillary electrophoresis demand special interface designs for their coupling to ICP-MS due to the

much lower compatibility of the flow-rates [6]. Gel electrophoresis can be interfaced via laser-ablation to ICP-MS [5]. Chromatography and capillary electrophoresis can also be coupled on-line to electrospray MS via conventional ESI and nanoESI sources [8], while MALDI-MS is usually used as an off-line detector after the separation step. During the last years the potential of hyphenated techniques in bio-inorganic speciation analysis could be successfully demonstrated in many cases such as for the identification of organoarsenic compounds in marine biota [9–11], organoselenium compounds in selenized yeast [12,13], garlic [14] and Indian mustard [15], nickel complexes in plant sap [4] and metal–phytochelatin complexes in edible plants [16].

The maintenance of the species integrity during sample preparation in bio-inorganic speciation analysis is a critical issue. Hyphenated techniques are demanding the presence of the species in the liquid phase for the separation step. Metallobiomolecules have to be extracted from a biological tissue under preservation of their original identity. Body fluids or plant saps need to be diluted or the complex matrix has to be separated. For the extraction and dilution aqueous buffers at the physiological pH 7.4 are used to avoid a dissociation of the metal–biomolecule complexes. Furthermore antioxidation agents such as β-mercaptoethanol or dithiotreitol (DTT), antibacterial agents (NaN_3), protease inhibitors and storage at −20 °C prevent uncontrolled species degradation. The drawback of extraction procedures using the simple aqueous buffers is their generally poor recovery (usually 10–20%) of element species. Extraction with protein denaturation reagents and enzymatic digestion of proteins or polysaccharides under controlled species degradation can increase the extraction yield. An overview of sample preparation techniques for speciation analysis of metallobiomolecules was recently published [17].

Despite the impressive progress in detection, identification and structural characterization of trace element compounds in biological systems their accurate quantification remains difficult. Quantitative determination of biomolecules, notably proteins, is an important prerequisite for elucidation of their physiological functions with diagnostic and therapeutic relevance. In particular for peptides and proteins several molecular mass spectrometric techniques may be capable of providing sequence information but there are no solutions to the inherent difficulty to generate quantitative results. For improved protein quantification by electrospray mass spectrometry a number of deuterated tags referred to as isotope-coded affinity tags (ICAT) was developed [18]. However, they show a limited applicability and insufficient sensitivity, and the quantitative results have a relatively poor accuracy.

Metallobiomolecules and heteroatom containing biomolecules, respectively, have the inherent advantage to be detectable by ICP-MS and thus quantifiable by isotope dilution analysis under the condition that the detected element is not monoisotopic. If the metal- or heteroatom is monoisotopic long-lived radioactive isotopes can sometimes be used. Two on-line isotope dilution techniques were proposed in combination with hyphenated systems for the quantification of element species: (i) a species-unspecific post-column isotope dilution for the quantification of elements in compounds of unknown identity, and (ii) a species-specific isotope dilution applicable for element species of known identity if isotopically labelled species are available [19]. In the past the potential of isotope dilution analysis in speciation analysis has been demonstrated for the quantification of inorganic species and organometallic compounds in environmental and biological samples and discussed in several recent review articles [20–22].

This review discusses the current state of the art of quantitative speciation analysis for endogenous trace element compounds in biological systems by ICP isotope dilution MS and highlights future trends in this emerging field of analytical chemistry.

2. Species-unspecific isotope dilution quantification of metallo(id)biomolecules

The species-unspecific isotope dilution technique consists of a separation technique (HPLC or capillary electrophoresis (CZE)) coupled to ICP-MS where an isotopically labelled spike is continuously added after the separation step (post-column). Fig. 2 shows schematically a set-up of a typical HPLC- and CZE-ICP IDMS system. In conventional HPLC a simple T-piece or an inverse Y-junction serves as the mixing unit for effluent and spike flow [23] while in capillary- and nano-HPLC as well as in capillary electrophoresis more sophisticated mixing units are demanded to minimize the broadening of the narrow peaks by keeping the dead volume small [24]. The isotopically labelled spike is not required to have the same chemical form as the analyte, thus allowing the quantification of elements in biomolecules of unknown identity. In order to quantify a biomolecule on the basis of the elemental concentration the knowledge of the empiric formula (stoichiometry of the metal complex) is required. An important prerequisite is that the isotopes of the regarded element in the analyte molecule and the spiked isotope are completely equilibrated in the plasma of the ICP-MS [25]. The spike flow is calibrated by injection of a standard with the natural isotope abundance. This is usually performed via a second injection valve inserted in the system *after* the separation column and before the mixing T-piece. In capillary/nano-HPLC and CZE this calibration standard has to be injected *before* the separation column/capillary to avoid a broadening of the peaks caused by the dead volume of the second valve [24]. The intensities in the chromatogram and electropherogram, respectively, can be transformed into a massflow by applying the basic formula in isotope dilution analysis [23]. The peak area of an analyte in the massflow-chromatogram corresponds then directly to the mass of the analyte. Due to the

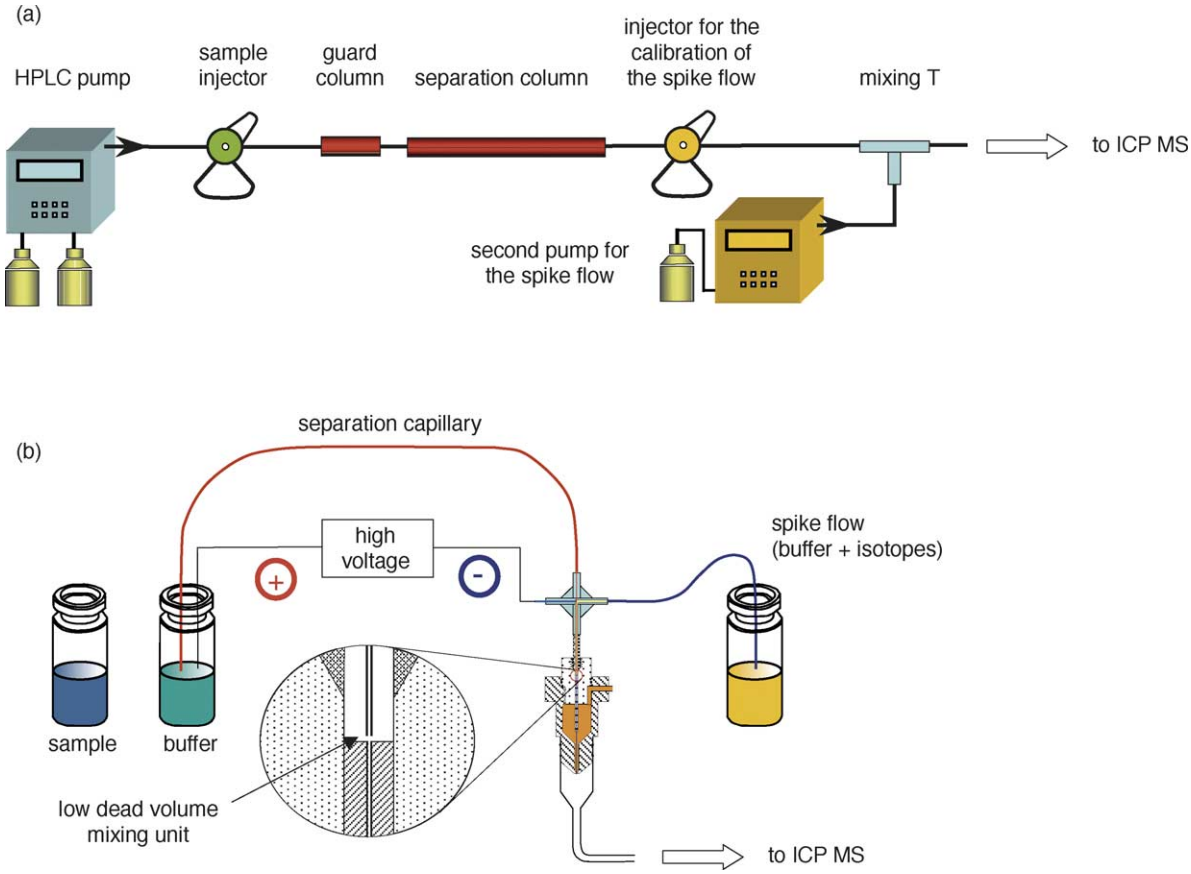

Fig. 2. Instrumental set-up of a typical (a) HPLC- and (b) CZE-ICP IDMS system for post-column isotope dilution with a species-unspecific isotopically labelled spike. A low dead volume mixing unit for the effluent and spike solution in CZE-ICP IDMS is highlighted.

fact that the isotopically labelled spike is added after the separation step, analyte losses during sample preparation and separation are not compensated for [25].

Most applications of post-column isotope dilution analysis for the quantification of metallo(id)biomolecules have been reported using a conventional (column i.d. 4.6 mm) HPLC-ICP-MS system. A major topic in this field is the quantification of selenium species. In selenium detection by ICP-MS the most abundant Se isotopes are interfered by the presence of argon dimer ions. This interference can be eliminated by the use of collision/reaction cells and gases such as He, H_2, O_2, NH_3, CH_4. Hinojosa Reyes et al. quantified the distribution of selenium in human blood serum between glutathione peroxidase, selenoprotein P and albumin separated by affinity chromatography [26]. Selenomethionine was identified and quantified as the main selenium compound in cod muscle [27], yeast and wheat flour by Diaz Huerta et al. [28] In these cases an enzymatic digestion with protease and lipase liberated the selenomethionine incorporated in selenium-containing proteins. Thus not the original seleno-biomolecule was quantified but the digestion product and information on the primary selenium-containing protein was lost. Beside selenomethionine selenite was quantified as a minor selenium compound in yeast and a number of unidentified selenium species in cod muscle were detected likely to be selenium-containing peptides due to incomplete digestion. In a subsequent work from the same group selenomethionine was again quantified as the major compound in three mushroom species but in these cases without applying an enzymatic digestion [29].

The speciation of essential elements in human blood serum was studied by Sariego Muniz et al. [30] who quantified copper, zinc and iron bound to serum proteins such as transferrin and albumin. Serum samples from hemodialysis patients and healthy volunteers were investigated showing differences, particularly in terms of the iron speciation, indicating a potential benefit of quantitative speciation studies for disease diagnostic purposes.

Besides metals also heteroatoms such as chlorine, bromine and iodine can be detected by ICP-MS. The analysis for halogen species of humic substances was reported using HPLC-ICP-MS [31]. Chlorine and bromine isotope dilution is hindered in quadrupole ICP-MS due to interferences on the ^{37}Cl and ^{81}Br nuclides. Although the monoisotopic iodine has only one stable isotope the quantification of iodine species of humic substances could be performed by the use of a spike solution enriched in the long-lived radioactive ^{129}I isotope.

A further field of investigation in bio-inorganic speciation analysis concerned metallothioneins (MT), a low molecular mass (6–7 kDa) metal-binding protein synthesized by animals in response to metal stress. Liquid chromatography hyphenated to ICP-MS was widely applied to the detection of MT and post-column isotope dilution used for the quantification of cadmium, zinc and copper bound to MT in rabbit liver [32], eel liver [33,34] and carp liver [33]. Besides liquid chromatography capillary zone electrophoresis (CZE) is able to separate different MT isoforms with high resolution. Schaumlöffel et al. set up for the first time a CZE-ICP-MS system with post-column isotope dilution and applied it to the quantification of MT isoforms [24]. Stable isotopes of ^{34}S, ^{65}Cu, ^{68}Zn and ^{116}Cd were continuously added via the make-up liquid and mixed with the CZE effluent in the CZE-ICP-MS interface. The set-up was critically dependent on the stability of the spike flow, which was transported by self-aspiration. By using an ICP sector-field MS instrument in medium mass resolution mode sulphur detection was also possible, enabling the quantification of the protein via the number of the cysteine and methionine residues in the amino acid sequence. Cadmium, copper and zinc were simultaneously quantified, allowing a determination of the metal–metallothionein complex stoichiometry by the metal-to-sulphur ratios (Fig. 3). In a subsequent work of this group the developed methodology was applied to metallothionein quantification and characterization of the metal–metallothionein complex stoichiometry in rat liver [35].

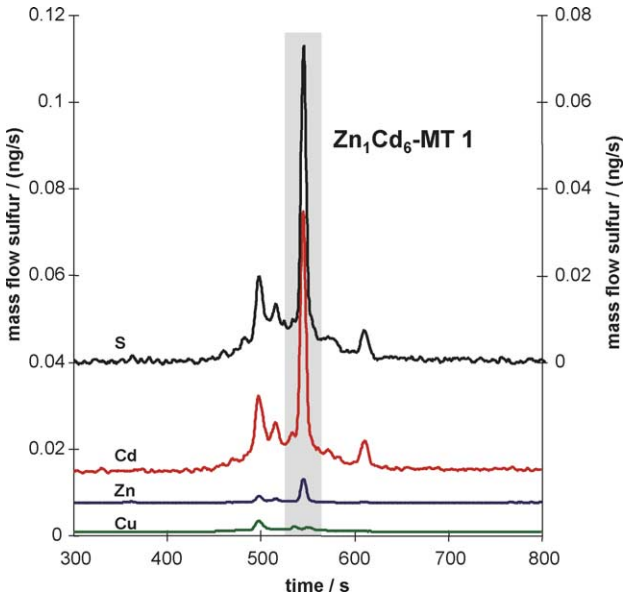

Fig. 3. Quantification and characterization of the complex stoichiometry of rabbit liver metallothionein-1 by species-unspecific isotope dilution in CZE-ICP-MS. The shaded area shows the MT 1 peak in the mass-flow electropherogram. The complex stoichiometry is obtained from the sulphur-to-metal ratio [24].

3. Species-specific isotope dilution quantification of metallo(id)biomolecules

Species-specific isotope dilution analysis has become an increasingly popular method to improve the precision and accuracy of speciation analysis in complex biological and environmental matrices [22,36]. In this method an isotopically labelled analyte species is added to the sample and is supposed to co-elute from a chromatographic column with the analyte species after an entire analytical procedure. Since the basis of quantification is the measurement of the isotope ratio of the speciated element in the mixture producing a chromatographic peak, incomplete recoveries and matrix effects can be corrected for [22]. Important prerequisites are that the analyte and the spiked species are completely equilibrated before the separation step and that no isotope exchange occurs between the different species [25].

Application of the species-specific isotope dilution analysis to large biomolecules is a challenging task. This requires first of all the structural characterization of the analyte biomolecule followed by the synthesis of the isotopically labelled spike compounds. Thus species-specific isotope dilution is not applicable for unknown biomolecules. However, in contrast to post-column isotope dilution no dedicated instrumental set-up is necessary.

To date species-specific isotope dilution methods developed for biomolecules have been based on molecular mass spectrometry, with fast atom bombardment (FAB) or electrospray ionization. Synthetic peptides which incorporate a stable isotope (^{2}H, ^{13}C, ^{15}N, ^{18}O) or are labelled by a stable-isotope containing tag are ideal internal standards to mimic native peptides formed by proteolysis and can be employed for absolute quantification [37]. This can provide an attractive complement to the relative quantification strategies in proteomics based either on growing the cells in media enriched in stable isotopes [38] or on chemically labelling proteins by isotope tags [39]. The sensitivity of these methods is critically dependent on the ionization of a given molecule and the quality of purification from the matrix. The poor linearity of the response as a function of concentration leads to accuracies of an order of 30%. However, the use of isotopically labelled peptides to generate quantitative data in molecular mass spectrometry on the absolute amount of the parent protein has been scarce.

Hence, there is an increasing interest in the use of hyphenated systems with ICP-MS detection for species-specific isotope dilution quantification of biomolecules owing to the high sensitivity of ICP-MS, its linearity, quasi-independence of the signal of the coordination environment of an element within a molecule, and very limited matrix effects [7,40,41]. However, examples of application are to date rather scarce mainly due to the lack of isotopically labelled biomolecules.

Polatajko et al. described for the first time the use of a ^{77}Se labelled selenopetide (Asp-Tyr-^{77}SeMet-Gly-Ala-Ala-Lys) for the quantification of 12 kDa heat-shock protein in selenized yeast by capillary HPLC-ICP collision cell MS [42].

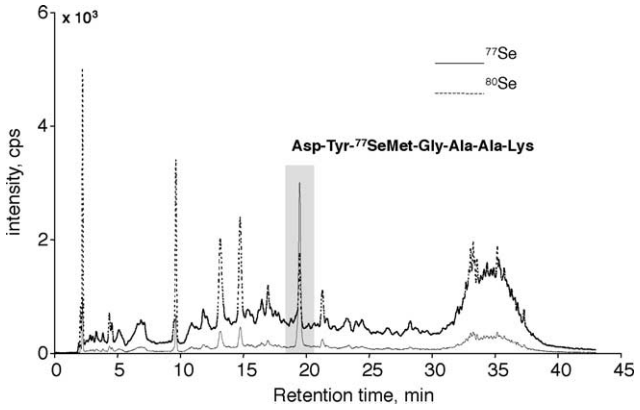

Fig. 4. Capillary HPLC-ICP IDMS analysis of aqueous yeast extract with a species-specific spike by a ^{77}Se labelled peptide (shaded area) [42].

The heat-shock protein was previously identified to contain one selenomethionine residue which results in one selenopeptide after tryptic digestion. Species-specific isotope dilution analysis allowed the accurate determination of the Asp-Tyr-SeMet-Gly-Ala-Ala-Lys peptide directly in a tryptic digest of an aqueous extract of selenized yeast (Fig. 4) and thus the quantification of the heat-shock protein. The ^{77}Se labelled peptide had been purified by 2D liquid chromatography from yeast grown on $^{77}SeO_4$-rich culture.

A further application was recently reported by Ruiz Encinar et al. on the determination of selenomethionine and selenocysteine in human blood serum [43]. A serum sample was enzymatically digested with a mixture of lipase and protease after derivatization of the selenocysteine residues with iodoacetamide. Species-specific isotope dilution was performed using a ^{77}Se-labeled selenomethionine spike in capillary HPLC-ICP collision cell MS and the accurately determined selenomethionine was used as an internal standard for the selenocysteine determination from the same chromatogram. Impressive procedural detection limits at the low ng g^{-1} level could be achieved. A critical issue in this method was the equilibration of the ^{77}Se labelled selenomethionine spike with the selenomethionine in the sample, which is incorporated in proteins. Before the protein-bound selenomethionine is liberated by proteolytic digestion the selenomethionine spike may react with iodoacetamide which had to be prevented. Once equilibration has been achieved, isotope dilution compensates for all analyte losses. The developed method is meanwhile routinely used in the authors' laboratory for selenomethionine determination in complex biological matrices such as blood, milk and eggs.

4. Conclusions and future trends

Isotope dilution analysis in combination with high-resolution separation techniques and ICP-MS detection offers an accurate quantification of trace element compounds in biological systems. All biomolecules containing a metal or heteroatom are theoretically accessible by this method if at least two isotopes (stable isotopes or long-lived radionuclides) of the regarded element exist and their detection in ICP-MS is not interfered. To date only few applications have published in the field of bio-inorganic speciation analysis

Table 1
Applications of isotope dilution analysis for metallo(id)biomolecule quantification

Analyte and sample	Hyphenated system	Separation column/capillary	Reference
(a) Species-unspecific isotope dilution			
Se species in human blood serum	AF HPLC-ICP (ORC) MS	Pharmacia HiTrap-heparin and HiTrap-blue sepharose	[26]
Se species in yeast and wheat flour	AE HPLC-ICP (ORC) MS	Hamilton PRP-X-100 (250 mm × 4.1 mm, 10 μm)	[28]
Se species in cod muscle	RP HPLC-ICP (ORC) MS	Waters Spherisorb ODS2 (250 mm × 4.6 mm, 5 μm)	[27]
	SEC-ICP (ORC) MS	TSK G 2000 SW_{XL} (300 mm × 7.8 mm)	
Se species in mushrooms	AE HPLC-ICP (ORC) MS	Hamilton PRP-X-100 (250 mm × 4.1 mm, 10 μm)	[29]
	RP HPLC-ICP (ORC) MS	Waters Spherisorb ODS2 (250 mm × 4.6 mm, 5 μm)	
	SEC-ICP (ORC) MS	Pharmacia Superdex Peptide (300 mm × 7.5 mm)	
Fe, Cu and Zn in human blood serum	AE HPLC-ICP (SF) MS	Pharmacia Mono-Q HR 5/5 (50 mm × 5 mm, 10 μm)	[30]
Cl, Br and I species of humic substances	SEC-ICP (Q) MS	TSK Gel 3000 PW and HEMA-SEC BIO 300	[31]
MT-1 isoform in rabbit liver	RP HPLC-ICP (Q) MS	Vydac C8 VHP 259 (150 mm × 4.6 mm)	[32]
	RP HPLC-ICP (TOF) MS		
	RP HPLC-ICP (SF) MS		
MT isoforms in eel liver	AE HPLC-ICP (Q) MS	Pharmacia Mono-Q HR 5/5 (50 mm × 5 mm, 10 μm)	[34]
	AE HPLC-ICP (SF) MS		
MT isoforms in eel and carp liver	SEC-ICP (TOF) MS	TSK Gel 3000 PWxL (300 mm × 7.8 mm, 6 μm)	[33]
MT isoforms in rabbit liver	CZE-ICP (SF) MS	Fused silica capillary (75 μm i.d × 70 cm)	[24]
MT isoforms in rat liver	CZE-ICP (SF) MS	Fused silica capillary (75 μm i.d × 75 cm)	[35]
(b) Species-specific isotope dilution			
Selenopeptide in yeast	Capillary HPLC-ICP (ORC) MS	LC Packings Hypersil C18 BDS (150 mm × 0.3 mm, 3 μm)	[42]
Selenomethionine in human blood serum	Capillary HPLC-ICP (ORC) MS	LC Packings Hypersil C18 BDS (150 mm × 0.3 mm, 3 μm)	[43]

Abbreviations: MT, metallothionein; AF, affinity; AE, anion-exchange; RP, reversed-phase; SEC, size-exclusion chromatography; ORC, octapole reaction cell (collision cell); Q, quadrupole; SF, sector field; TOF, time-of-flight.

(summarized in Table 1) which can be regarded as a starting point of the future developments.

Recent impressive progress towards high-resolution separation with the development of capillary and nano-HPLC and capillary zone electrophoresis and towards lower detection limits in ICP-MS is likely to spur the research in this emerging field. Especially the introduction of the collision/reaction cell technology in ICP-MS opens the way for the detection of heteroelements such as sulphur, selenium and phosphorus in biomolecules playing important roles in life processes. The quantification of proteins and peptides are in the recent focus of research to elucidate physiological processes with therapeutical and pharmaceutical relevance. The sensitive detection and quantification of sulphur as a widely spread element in many proteins bears the potential for a new general approach of a quantitative proteomics based on isotope dilution analysis.

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