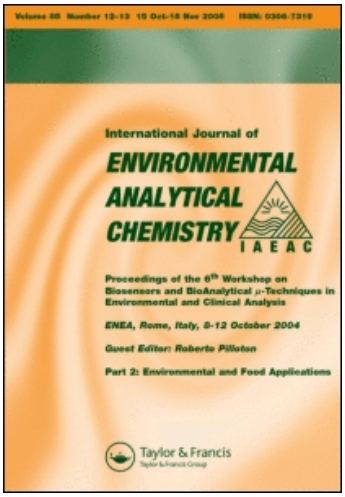

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Analytical characterization of bioactive metal species in the cellular domain (metallomics) to simplify environmental and biological proteomics

José Luis Gómez-Ariza[a]; Tamara García-Barrera[a]; Fernando Lorenzo[a]; Ana Arias[a]
[a] Departamento de Química y Ciencia de los Materiales, Facultad de Ciencias Experimentales, Universidad de Huelva, 21007-Huelva, Spain

To cite this Article Gómez-Ariza, José Luis , García-Barrera, Tamara , Lorenzo, Fernando and Arias, Ana(2005) 'Analytical characterization of bioactive metal species in the cellular domain (metallomics) to simplify environmental and biological proteomics', International Journal of Environmental Analytical Chemistry, 85: 4, 255 — 266
To link to this Article: DOI: 10.1080/03067310412331330776
URL: http://dx.doi.org/10.1080/03067310412331330776

Intern. J. Environ. Anal. Chem.
Vol. 85, No. 4–5, 10 April–20 April 2005, 255–266

Taylor & Francis
Taylor & Francis Group

Analytical characterization of bioactive metal species in the cellular domain (metallomics) to simplify environmental and biological proteomics

JOSÉ LUIS GÓMEZ-ARIZA*, TAMARA GARCÍA-BARRERA, FERNANDO LORENZO and ANA ARIAS

Departamento de Química y Ciencia de los Materiales, Facultad de Ciencias Experimentales, Universidad de Huelva, Campus de El Carmen, 21007-Huelva, Spain

(*Received 16 June 2004; in final form 18 October 2004*)

The speciation approach for the characterization of low-mass metal species including sample treatment and storage is at present a well-established topic based on chromatography-atomic detector coupling. More recently, new endogenous and exogenous metal species from biological systems are attracting considerable interest. Bioactive molecules such as proteins, DNA restriction fragments, phytochelatins, metallothioneins and others are target species of a new generation of analytical tools (bioanalysis) which substitute the traditional atomic detectors based on the use of photons (AAS, FPD, ICP-AES, AFS) by mass detectors (MS and ICP-MS) for ion characterization. Several cases related to biological molecules involving proteins and multiprotein systems, in which frequently metals (metallomics) participate, are described, and a generic metallomic analytical approach is proposed for the identification and quantification of metalloproteins, and other metal molecules present in living systems. In this work, a multiplexed analytical approach (MAA) is described on the basis of three experimental components: (1) a separation technique—*selectivity component*; (2) a highly sensitive elemental detector—*sensitivity component*; and (3) a molecule-specific detector, generally based on mass spectrometry—*structural component*. This approach brings together both elemental and molecular detectors to simplify the identification of metal-tagged biomolecules in environmental, food, and health studies.

Keywords: Metallomics; Metalloproteins; Proteins; Speciation; Mass spectrometry

1. Introduction

Speciation approaches have been developed during the last 15 years; they are now well established and are becoming routinely used in environmental, food, and health-quality-control laboratories. Most speciation procedures are related to elements' oxidation states or the analysis of organometallic compounds [1]. This has been the focal point in environmental studies in previous last decades, especially for butyl- and phenyltins,

*Corresponding author. Fax: +34-959-019942. E-mail: ariza@uhu.es

International Journal of Environmental Analytical Chemistry
ISSN 0306-7319 print: ISSN 1029-0397 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/03067310412331330776

alkyllead species from anthropogenic origin, methylmercury and organoarsenic compounds generated in environmental transformation of inorganic ions, and complexes of essential and toxic metals and non-metals with small molecules, such as selenomethionine and selenocystine [2].

These individual chemical species have a differential behaviour in relation to their toxicity, mobility and bioavailability. Therefore, the higher toxicity of Cr(VI) against Cr(III) has been demonstrated in natural waters, as well as the increasing toxicity connected to element alkylation grade. This is the case of trialkyltins with respect to di- and monoalkyltins and methylmercury against Hg(II), since alkylation, generally biomethylation [3], assists metals in crossing the biological membranes and the subsequent accumulation through the food chain. Otherwise, arsenic presents the opposite situation, since the toxicity of single ions (As(III) and As(V)) decreases, until it is eliminated, when the element is incorporated into a more complex organic molecule, such as arsenobetaine (AsB). Other times, toxicity is associated with the volatile character of some organometallic species, which can be absorbed through the lungs, as in the case of mercury.

Speciation analysis is a difficult and challenging task, due to problems related to species interconversion and stability, sample storage and conservation, species extraction and treatment, and final species characterization and analysis. Frequently, species have important beneficial or harmful effects at very low concentration levels, thus requiring the use of very sensitive atomic detectors (mainly ICP-MS and AFS). The use of efficient separation techniques (namely, GC and HPLC) coupled to the atomic detectors increases the method selectivity and helps to distinguish between similar species from the same or different elements, for the time-resolved introduction of analytes into the detector [4].

Endogenous and exogenous metallic species in biological systems [5–9] can establish a different perspective, since they get together small size species molecules, such as oxalate, citrate, tartrate, amino acids, and oligopeptides bound to metal, and large metal species including proteins, DNA restriction fragments, or polysaccharides. Many of these elements play important roles in life, although others possess a negative action on the living systems by being toxic, mutagenic or carcinogenic [10].

Generally, many of these systems are not well known, and the trace-metal binding to these organic molecules has to be clarified [11] to assess the essential or toxic character of metals in health and diseases. The new focus, metallomics, considered as an integrated biometal analytical approach [12] denotes metal-assisted function biochemistry that can be considered at the same level as genomic or proteomic [13]. The metallomics approach considers both qualitative information about the identities of individual species and their quantitative evaluation. Metallomics can be considered as a division of speciation analysis centred on the identification and/or quantification of elemental species in the domain of cellular biochemistry [14].

With these new analytical problems related to bioactive molecules, new analytical tools are required for the complementary use of atomic detectors (based in the use of photons) and mass detectors sensitive to ions.

The speciation studies based on atomic detectors require two-dimensional systems, in which species discrimination introduced by a separation device (HPLC or CE) combines with the powerful sensitivity of atomic spectroscopy for metals. However, these systems do not provide structural information, which is essential in the study of unknown bioactive molecules from biological fluids and tissues. Therefore, the innovative use of ICP-MS or AFS coupled to chromatography or capillary electrophoresis is

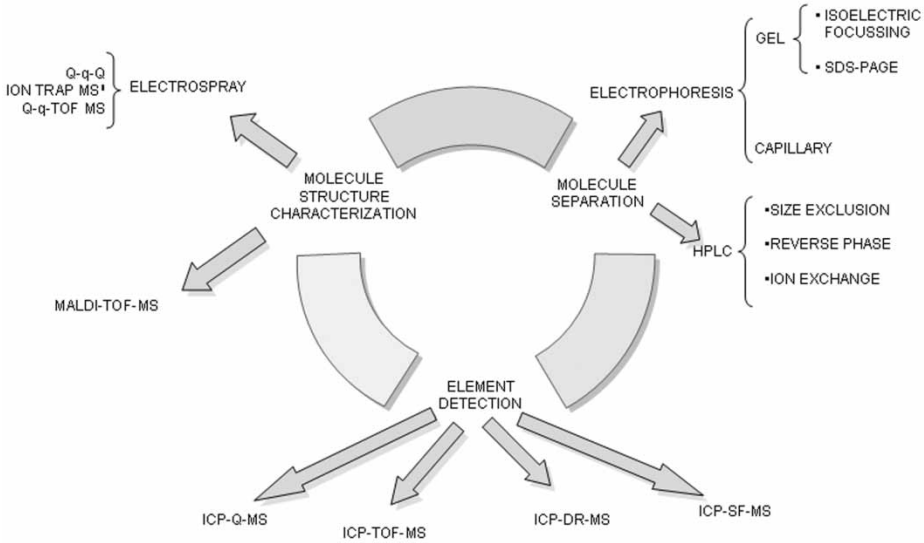

Figure 1. Role of hyphenated techniques in metal bioanalysis (metallomics).

combined with molecular MS techniques, which is producing a new generation of analytical approaches based on the joint use of elemental and molecular mass spectrometry, opening up new possibilities for spotting, identifying and quantifying biomolecules with previously unknown structures [15] (figure 1).

Many biological molecules are proteins, which mainly determine how living organisms work. 'Proteomics' [16] is of great importance in the study of multiproteins systems focused on the interplay of multiple, distinct proteins and their roles as part of a larger system or network. However, a parallel approach can be designed to characterize the entire metal-bioactive molecules participating in organisms' protection from stress (metallothioneins and phytochelatins) or in organisms' response to beneficial or harmful conditions, 'metallomics' [17]. Many of these metallomolecules are proteins, metalloproteins, and in this case metal can be used as a specific tag for metalloprotein identification in complex mixtures with many other non-metallic proteins [10]. Therefore, in this case, metallomics can be considered as metal-tagged proteomics. This concept opens a spectrum of new analytical options and strategies, since these ICP-tags comprise P, S, Se, Si, Cl, Br, I, As and many metals [15]. This approach is, therefore, suitable for molecules with an ICP-tag (e.g. an ICP-sensitive element) in a covalent-like form. Metals weakly coordinated to organic compounds are difficult to analyse by this methodology, and the results should be considered with caution [18].

In this paper, the metallomics approach, mainly based on the use of mass spectrometry hyphenated techniques for the characterization of metal-bound to proteins in biological systems, is critically reviewed. This topic is related to the already well-established metal-speciation approach, which is not considered in detail in this appraisal, since it has been covered exhaustively in previous books [19–22] and reviews [5,23–25]. The purpose is to explore the possibilities of the combined use of inorganic and organic mass spectrometry both coupled to liquid chromatography in proteomics

studies related to environmental and biological issues. The use of analytical atomic spectrometry has not been extensively used in metallobiomolecular characterization, although the impressive analytical performance of ICP-MS, with its ability to distinguish between metal (metalloid)-containing species and metal-free species in effluents from a separator device (HPLC or CE), or using laser ablation in a two-dimensional (2-D) gel spot, constitutes a very relevant alternative [10].

2. Analytical experimental approaches for metalloproteins characterization

The low concentration of the trace elements present in biological tissues (generally below 1 μg/g) and the complexity of the matrices make metalloprotein characterization difficult. The occurrence of numerous protein isoforms produced by post-translational changes can also complicate this type of speciation analysis.

Three key instrumental components can be involved in metallomic analysis: (1) a separation device for molecule discrimination; (2) a sensitive atomic spectrometer for metal detection; and (3) a mass spectrometer for molecule information and structure characterization. These instrumental systems can be coupled to obtain information about metal-containing species (figure 2) as well as for their molecular weight and structure identification (figure 3).

Separation can be performed using column devices, together with on-line detection using an atomic spectrometer, ICP-AES or ICP-MS [5,23,25,26]. Off-line characterization is also possible when the species are separated by gradient gel electrophoresis or isoelectric focusing, and the resulting spots previously studied by atomic techniques. Metal species can be excised, and the pieces digested and quantified by ICP-MS, AAS, and AES [27]. Off-line detection of metal species can also be carried out directly in the gel, using a highly sensitive discrete atomization technique for the gel pieces for

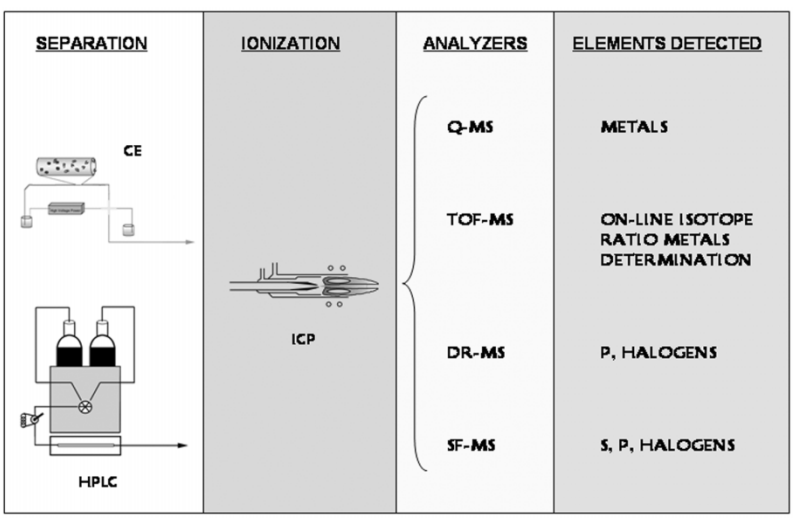

Figure 2. Instrumental couplings for metal-containing species identification.

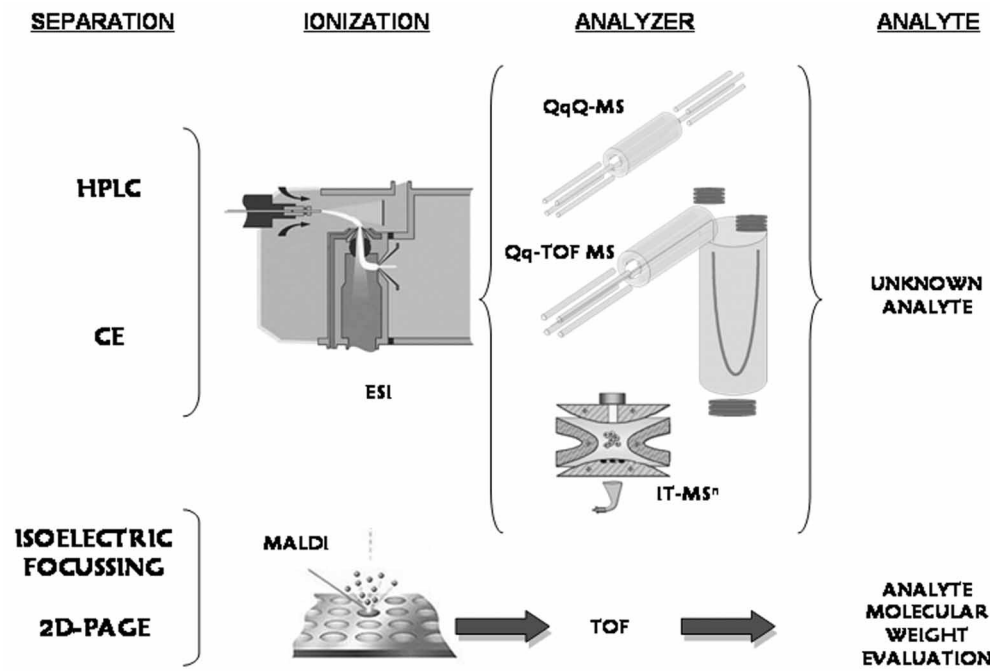

Figure 3. Instrumental couplings for structural characterization and molecular-weight identification of metal-containing species.

solid sample analysis with ETAAS [28] or ETV-ICP-MS [29,30]. Finally, off-line detection of metal species can be directly performed in the whole gel by laser ablation of the spots with ICP-MS detection [31], thus providing excellent detection limits, typically in the μg/L range for pneumatic nebulization (PN), with the additional advantage that several elements can be monitored simultaneously.

Two ablation strategies have been proposed [32]: single-hole drilling, relevant for ablation of spots after 2-D separations, and ablation with translation, i.e. on a line, relevant for one-dimensional (1-D) separations. The ICP-MS detector yields good signals for heavy metals, such as Zn of Cd bound to proteins [33], but quantitative non-metal evaluation, especially P and S, is difficult due to the occurrence of isobaric interferences. This fact is crucial due to the important role of these later elements in many physiological and pathophysiological processes, such as carcinogenic and neurodegenerative diseases [34], that need a double focusing sector field ICP-MS (ICP-SFMS) or ICP-Q-MS with a dynamic reaction cell (ICP-DRCMS) for this purpose [34,35]. These techniques become essential in characterizing post-translational protein modifications, especially in phosphorylation reactions [35].

Mass spectrometry provides structural information about unknown or already known metalloproteins. In this case, electrospray ionization MS (ESI-MS) [36,37], such as ESI-Q-TOF-MS [38], for column separations, and matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) [38,39], for planar separation techniques, provide molecule-specific detection, especially if the MS/MS mode is used.

An analytical speciation scheme for metalloprotein identification and quantification can therefore be proposed, including three main components: (1) a separation technique

(e.g. HPLC, CE, 2DE and 2D-PAGE) for target species isolation from the matrix and the time-resolved introduction into the detector—the *selectivity component*; (2) an high-sensitivity element detector such as ICP-MS, for element quantification—the *sensitivity component*; and (3) a molecule-specific detector, based on mass spectrometry, especially using tandem MS for a more accurate characterization of biomolecules—the *structural component*. This MAA brings together both elemental and molecular detectors, and allows simple and fast metalloprotein identification at very low concentrations (e.g. 10–100 pg/L of protein). The approach strongly simplifies the sample treatment due to the selectivity introduced by the presence of the metal, since the atomic detector (namely, ICP-MS) provides an extra dimension to the separation device in the detection. This fact allows metal-containing peaks to be distinguished from metal-free signals resulting from the separation step, as well as the unambiguous confirmation of the presence of metallocomplexes [26], since artefact peaks from the sample matrix observed with non-selective detectors for metals can be reliably skipped [40]. The ESI-MS detector provides additional information about the peak identity in the case of unknown compounds. Therefore, metal gives a useful tag in heteroatom-containing biomolecules, and this approach can transform the well-established proteomic approach based on whole molecules in other focused on the metal: metallomics [17].

The metallomics approach (M*t*A) can be applied to numerous ligand complexes in biological samples [41]. Metalloproteins in blood and blood plasma, selenoproteins in human and animal body fluids and other biomolecules in which tag elements are present can be the focus of M*t*A.

The core of M*t*A is the use of a highly sensitive atomic detector for the element. Transition metals can be easily quantified in the ng range using the already well-established ICP-MS detector, or alternatively, ICP-DCRMS or ICP-SFMS for non-metals, the latest indispensable technique for the detection of important elements such as S and P. These systems have been used for the determination of metal–sulphur ratios in metallothioneins, with coupling to size exclusion chromatography [42], and for the detection of new selenium compounds in selenized yeast using CE-ICP-MS [43].

3. Metallomic approach in examples

3.1. *Metallothioneins (MT)*

Metallothioneins constitute a typical example of metalloproteins with low molecular mass (6–7 kDa) generally used as indicators of environmental exposure [44]. These cystein-rich metal-binding compounds are of particular interest in connection with organisms exposed to elevated levels of trace metals through both biochemical processes and pollution [23]. Metallothioneins easily link with essential (Cu, Zn) and toxic (Cd, Hg) trace metals [24]. MT are generally present in a mixture of different isoforms separable by anion-exchange chromatography, but each of these fractions still contains a number of sub-isoforms that vary in a small number of amino acids or contain extra residues, whose separation requires high-resolution techniques [1,45]. The presence of high levels of MT in biological tissues is used as a 'biomarker' of environmental exposure to heavy metal, since MT are synthesized by living organisms in the presence of metals as part of a detoxifying process.

The coupling of ICP-MS with size-exclusion chromatography (SEC) has been widely used for the quantification of MT [5] and allows real-time element specific detection of

different metal-containing molecular-weight fractions in unknown cytosols [46]. However, the identification of MT isoforms can only be achieved by further speciation studies of the MT fractions, previously isolated by SEC, using multidimensional chromatographic separation techniques, such as anion-exchange or reverse-phase (RP) HPLC, or CZE [1,47].

Adams *et al*. [48] studied Cd and Zn associated with MT1, which showed different retention times from Cu. Absolute detection limits (metal pg) obtained in the characterization of MT in cytosols carp liver and kidney using SE-HPLC-ICP-TOF-MS ranging from 19.9 pg (^{114}Cu) to 158 pg (^{66}Zn). The results showed the possibility of coupling for multi-elemental and multi-isotope detection at pg levels, with a relative standard deviation better than 5%. The two metals are probably bound to the same molecular fraction but probably to molecules with different shapes or bound to different MT molecule clusters. Sanz-Medel *et al*. [49] proposed a two-dimensional separation system combining size-exclusion fractionation of eel-liver cytosol, with SEC-AE-FPLC, and post-column isotope analysis using Cd-, Cu-, and Zn-enriched isotopes. The isotope ratio was monitored on-line by ICP-(Q)MS.

CE-ICP-MS coupling has been proposed as a powerful and versatile analytical approach for the determination of metallothioneins, with a better performance than other couplings based on HPLC [50]. The approach exhibits several advantages such as the small volume of sample required (typically 1–30 nl), high sample throughput, and low reagent consumption. In addition, the disturbance produced by the CE-ICP-MS system does not affect the metal–metallothionein equilibrium. A critical aspect of the coupling is the interface, and different studies have been focused on its design using diverse types of nebulizers. Lobinski *et al*. [40] recommended the parallel identification of metallothioneins by CEZ-ICP-MS and CZE-ES-MS, the first to detect MT-Cd, Cu and Zn complexes, and the second to identify them.

Therefore, the analytical approaches for metallothioneins characterization in environmental, foodstuffs, foods, and biological tissues should use MAA [17] and have to be supported on coupled techniques [24].

3.2. *Metal superoxide dismutase (MSD)*

Besides metallothioneins, MSD have been proposed in the literature as indicators of living organisms' exposure to xenobiotics [51]. The presence of Cu–Zn superoxide dismutase has been tested in rice (*Oryza sativa* L.) under stress by drought [38] or ozone action [52]. A crude protein extract of rice leaves was submitted to 2D-PAGE separation, which detects more than 1000 protein spots in the extracts. The latter densitometric image analysis revealed significant differences in protein abundance under stress. The study was completed with critical proteins characterization using MALDI-TOF-MS and/or ESI-Q-TOF-MS/MS. About 42 protein spots from rice leaves showed a significant change in abundance under stress by drought [38], and the differences between stressed and reference crops were clearly marked by the presence of Cu–Zn superoxide dismutase. Most of these studies denoted the importance of metal bounded to proteins as environmental biomarkers, so the application of the metallomics experimental approach based on the parallel use of atomic and mass detectors could simplify the current proteomic appraisal.

3.3. *Food metalloproteins*

The metallomics approach can also be useful to characterize metals bound to the bioactive molecules present in food, which are frequently the main source of trace elements for the human diet. Size-exclusion chromatography coupled to ICP-MS (SEC-ICP-MS) has been widely used for the speciation of polysaccharides bounded to cations, which are linked by the negatively charged oxygen functions in fresh fruit and vegetables [53]. SEC and cation-exchange separation based on solid-phase extraction cartridges coupled to ICP-MS have been used to obtain complementary information about the metal species present in tea infusion. These techniques confirm that metal-organic ligands in tea infusion are large polyphenolic compounds, which are probably the Al-binding ligands in this food [54].

The composition and structure of Cr(III) bioactive compounds are also the target of speciation studies using HPLC-FAAS for the separation and detection of Cr(III) compounds from Cr-rich yeast biomass. β-Nicotinamide adenine dinucleotide phosphate (NADP) forms a 2 : 1 complex with chromium that exhibits glycaemic activity [55].

3.4. *Seleno and iodoproteins in human tissues and fluids*

In human fluids, the major bioligands are proteins and other small molecules such as ATP, porphyrins and cobalamins. Size-exclusion chromatography with on-line ICP-MS detection has been used to characterize metal covalently integrated into the structure of a metalloprotein (e.g. selenoproteins) or bound in protein complexes (metallothioneins) [56]. However, SEC suffers from poor resolution of the metalloprotein species, and the *metallomics approach*, described previously, could provide a deeper insight into these biomolecules' characterization, especially with the application of multidimensional chromatography.

At present, only a few selenium and iodine proteins have been identified and characterized, such as GPX, selenoproteins P and W, and iodothyroxine deiodinase. Other seleno- and iodoproteins are also present in the biological organisms and help to prevent chronic diseases like cancer and cardiovascular disorders. Therefore, it is critical to investigate the unknown selenoproteins and other metalloproteins present in the biological organisms, as well as to provide dietary supplements in the form of selenized yeast and others. In the case of high-molecular-mass compounds, Se is casually incorporated into the yeast proteins taking the place of sulphur. The absence of any specificity in the Se incorporation motivates the denomination of 'Se-containing proteins', in opposition to selenoproteins [32].

Radioimmunoassay has been proposed for selenoprotein measurement in human serum, [57]. Other selenoproteins have been detected in rat tissues labelled with ^{75}Se and protein separation using gel-electrophoretic methods (2D-SDS-PAGE) for localization of ^{75}Se-containing proteins in the gel by autoradiography [58]. When the *metallomics approach* is applied, attention should be focused on the separation step to obtain the adequate Se-species resolution. Size-exclusion liquid chromatography (SEC) is the fractionation method mostly used in combination with non-radioactive methods, like ICP-MS, but it is limited by the low chromatographic resolution characteristic of size-exclusion separation [59]. The coupling SEC-ICP-MS has been used for glutathione peroxidase characterization in human serum and breast milk. Better results

were obtained with AC, although tandem chromatography combining AC-SEC and ICP-MS detection is the most reliable approach [60].

3.5. *Metallodrugs*

Metallomics can be applied in the study of metallodrug–protein interactions. Anticancer drugs such as cisplatin, ruthenium-based antitumour drugs and others present a differential activity depending on their complexation with proteins. Therefore, fast methods to monitor the interactions of metallodrugs with proteins in human fluids are necessary. SEC-ICP-MS has been proposed to identify complexed 'free' Pt and Ru fractions in drugs containing these elements [61]. The same approach has been used in parallel with nanospray tandem quadrupole time-of-flight (ESI-Q-TOF-MS). SEC-ICP-MS provided the first direct evidence for the co-binding of cadmium and platinum to MT through cysteine binding [62]. CE-ICP-MS has been used in studies of cobalamins in pharmaceutical preparations and food samples [63].

Planar electrophoresis constitutes a powerful approach for good resolution separation of many metalloproteins, in particular selenoproteins. The detection of Se in the spots can be accomplished by ETV-ICP-MS [30], with the advantages of a higher resolution than SEC and sensitive/quantitative Se determination. However, this off-line analysis is too time-consuming if 2-D gel separation is used, and only radioactive detection using the ^{75}Se radioatracer is suitable for this purpose [64]. Recently, laser ablation (LA)-ICP-MS [32] has been successfully applied to the detection of Se-containing proteins in red-blood-cell extracts.

Laser ablation has also been proposed for the identification of Co–human serum protein interactions using 2D gel electrophoresis to obtain a map of metal protein distribution [31]; the consistency and complimentarity of this approach (using laser desorption mass spectrometry for characterizing proteins) are notable.

4. Conclusions

New multidimensional analytical tools are needed for fast, sensitive, quantitative and comprehensive characterization and determination of metals in bioactive molecules, and especially in macromolecules present in living systems. These biometallic systems (metallomic) can be studied using tandem mass spectrometry, with electrospray and MALDI sources, and multidimensional separation techniques, such as multidimensional liquid chromatography and 2D-PAGE, thus providing a deeper insight into environmental stress situations (biomarkers), food quality and authentication assessment, disease diagnosis, and metallodrugs design and action.

Metallomics is based on a multiplexed analytical approach (MAA) that combines three experimental components: separation, metal-sensitive detection, and structure identification, integrated on a multidisciplinary framework. Forthcoming trends are related to the application of more sophisticated instrumental couplings based on MALDI-TOF-TOF and LC-MALDI-TOF-TOF, as well as a closer interface between analytical chemists involved in speciation and molecular biology scientists.

Appendix A: List of abbreviations

2DE:	bi-dimensional electrophoresis
2D-PAGE:	bi-dimensional polyacrylamide gel electrophoresis
AAS:	atomic absorption spectrometry
AC:	affinity chromatography
AE:	anionic exchange
AES:	atomic emission spectroscopy
AFS:	atomic fluorescence spectroscopy
AsB:	arsenobetain
ATP:	adenine triphosphate
CE:	capillary electrophoresis
CZE:	capillary zone electrophoresis
DNA:	deoxyribonucleic acid
DRC:	dynamic reaction cell
ESI:	electrospray ionization
ETAAS:	electrothermal atomic absorption spectroscopy
ETV:	electrothermal vaporization
FAAS:	flame atomic absorption spectroscopy
FPD:	flame photoionization detector
FPLC:	fast protein liquid chromatography
GC:	gas chromatograpy
GPX:	glutation peroxidase
HG:	hydride generation
HPLC:	high-performance liquid chromatography
ICP:	inductively coupled plasma
ICP-MS:	inductively coupled plasma mass spectrometry
ICP-DRCMS:	inductively coupled plasma dynamic reaction cell mass spectrometry
ICP-SFMS:	inductively coupled plasma sector field mass spectrometry
IT:	ion trap
LA:	laser ablation
LC:	liquid chromatography
MAA:	multiplexed analytical approach
MALDI:	matrix-assisted laser desorption ionization
MCN:	microconcentric nebulizer
MeHg:	methyl mercury
MS:	mass spectrometry
MT:	metallotionein
MT1:	metallotionein 1
M*t*A:	metallomics approach
NADP:	β-nicotinamide adenine dinucleotide phosphate
PCR:	polymerase chain reaction
PEPs:	protein expression profiles
PN:	pneumatic nebulization

Q: quadrupole
SDS: sodium dodecyl sulphate
SE: size exclusion
SEC: size-exclusion chromatography
TOF: time of flight

Acknowledgements

The authors would like to thank 'Ministerio de Ciencia y Tenología (MCyT)' for the Grant REN2002-04366-C02-02. T. Garcia-Barrera thanks Universidad de Huelva for a scholarship. F. Lorenzo thanks Junta de Andalucia for a pre-doctoral grant.

References

[1] J.L. Gómez-Ariza, E. Morales, I. Giráldez, D. Sánchez-Rodas, In: *Trace Element Speciation For Environmental, Food and Health*, L. Ebdon, L. Pitts, R. Cornelis, H. Crews, O.F.X. Donard, Ph. Quevauviller (Eds.), pp. 51–80, Royal Society of Chemistry, Cambridge (2001).
[2] R. Lobinski, *Appl. Spectrosc.*, **51**, 260A (1997).
[3] J.S. Thayer, *Appl. Organomet. Chem.*, **16**, 677 (2002).
[4] R. Lobinski, I.R. Pereiro, H. Chassaigne, A. Wasik, J. Szpunar, *J. Anal. At. Spectrom.*, **13**, 859 (1998).
[5] J. Szpunar, *Analyst*, **125**, 963 (2000).
[6] R. Cornelis, J. De Kimpe, *J. Anal. At. Spectrom.*, **9**, 945 (1994).
[7] R. Cornelis, J. De Kimpe, X. Zhang, *Spectrochim. Acta B*, **53**, 187 (1998).
[8] A. Sanz-Medel, *Spectrochim. Acta B*, **53**, 197 (1998).
[9] J. Szpunar, R. Lobinski, *Pure Appl. Chem.*, **71**, 899 (1999).
[10] N. Jakubowski, R. Lobinski, L. Moens, *J. Anal. At. Spectrom.*, **19**, 1 (2003).
[11] J. Szpunar, *Anal. Bioanal. Chem.*, **378**, 54 (2004).
[12] H. Haraguchi, *J. Anal. At. Spectrom.*, **19**, 5 (2003).
[13] H. Haraguchi, H. Matsuura, In: *Proceedings of International Symposium on Bio-Trace Elements 2002 (BITRE 2002)*, S. Enomoto, Y. Seko (Eds.), pp. 3–8, The Institute of Physical and Chemical Research (RIKEN), Wako, 2003.
[14] D. Templeton, F. Ariese, R. Cornelis, L.G. Danielsson, H. Muntau, H.P. van Leeuven, R. Lobinski, *Pure Appl. Chem.,* **72**, 1453 (2000).
[15] M. Wind, W.D. Lehmann, *J. Anal. At. Spectrom.*, **19**, 20 (2003).
[16] M.R. Wilkins, J.C. Sanchez, A.A. Gooley, R.D. Appel, I. Humphery-Smith, D.F. Hochstrasser, K.L. Williams, *Biotechnol. Gen. Eng. Rev.*, **13**, 19 (1996).
[17] J.L. Gómez-Ariza, T. García-Barrera, F. Lorenzo, V. Bernal. M.J. Villegas, V. Oliveira, *Anal. Chim. Acta*, **524**, 15 (2004).
[18] R. Lobinsky, J. Szpunar, *Anal. Chim. Acta*, **400**, 321 (1999).
[19] L. Ebdon, L. Pitts, R. Cornelis, H. Crews, O.F.X. Donard, Ph. Quevauviller, *Trace Element Speciation for Environmental, Food and Health*, Royal Society of Chemistry, Cambridge (2001).
[20] C.C. Chéry, *Handbook of Elemental Speciation, Techniques and Methodology*, R. Cornelis, J. Caruso, H. Crews, K. Heumann (Eds.), pp. 224–240, Wiley, Chichester, UK (2003).
[21] S. Caroli (Ed.), *Elemental Speciation on Bioinorganic Chemistry*, Wiley-Interscience, Hoboken, NJ (1996).
[22] A.M. Ure, C.M. Davidson, *Chemical Speciation in the Environment,* 2nd ed., Blackwell Science, Edinburgh (2002).
[23] J. Szpunar, R. Lobinski, A. Prange, *Appl. Spectrosc.*, **57**, 102A (2003).
[24] R. Lobinski, H. Chassaigne, J. Szpunar, *Talanta*, **46**, 271 (1998).
[25] S.S. Kannamkumarath, K. Wrobel, K. Wrobel, C.B′. Hymer, J.A. Caruso, *J. Chromatogr. A*, **975**, 245 (2002).
[26] K. Polec, J. Szpunar, O. Palacios, P. González-Duarte, S. Atrian, R. Lobinski, *J. Anal. At. Spectrom.*, **16**, 567 (2001).
[27] J. Messerschmidt, F. Alt, G. Tölg, *Electrophoresis*, **16**, 800 (1995).
[28] U. Sidenius, B. Gammelgaard, *Fresenius' J. Anal. Chem.*, **367**, 96 (2000).
[29] K. Wrobel, E. Blanco González, K. Wrobel, A. Sanz-Medel, *Analyst*, **120**, 809 (1995).
[30] C.C. Chéry, H. Chassaigne, L. Verbeeck, R. Cornelis, F. Vanhaecke, L. Moens, *J. Anal. At. Spectrom.*, **17**, 576 (2002).

[31] J. L. Neilsen, A. Abildtrup, J. Christensen, P. Watson, A. Cox, C. W. McLeod, *Spectrochim. Acta B*, **53**, 339 (1998).
[32] C.C. Chéry, D. Günther, R. Cornelis, F. Vanhaecke, L. Moens, *Electrophoresis,* **24**, 3305 (2003).
[33] M.R.B. Binet, R. Ma, C.W. McLeod, R.K. Poole, *Anal. Biochem.*, **318**, 30 (2003).
[34] J.S. Becker, S.F. Boulyga, J.S. Becker, C. Pickhardt, E. Damoc, M. Przybylski, *Intern. J. Mass Spectrom.*, **228**, 985 (2003).
[35] J.S. Becker, S.F. Boulyga, C. Pickhardt, J. Becker, S. Buddrus, M. Przylbylsky, *Anal. Bioanal. Chem.,* **375**, 561 (2003).
[36] G. Zoorob, F.B. Brown, J. Caruso, *J. Anal. At. Spectrom.*, **12**, 517 (1997).
[37] H. Chassaigne, R. Lobinski, *Analysis*, **25**, M37 (1997).
[38] G.H. Salekdeh, J. Siopongco, L.J. Wade, B. Ghareyazie, J. Bennett, *Proteomics*, **2**, 1131 (2002).
[39] S.A. Pergantis, W.R. Cullen, G.K. Eigendorf, *Biol. Mass Spectrom.*, **23**, 749 (1994).
[40] S. Mounicou, K. Polek, H. Chassaigne, M. Potin-Gautier, R. Lobinski, *J. Anal. At. Spectrom.*, **15**, 635 (2000).
[41] B. Sas, E. Peys, M. Helsen, *J. Chromatogr. A*, **864**, 179 (1999).
[42] S. Hann, G. Koellenspeger, C. Obinger, P.G. Furtmüller, G. Stingeder, *J. Anal. At. Spectrom.*, **19**, 74 (2004).
[43] L. Bendhal, B. Gammelgaard, *J. Anal. At. Spectrom.*, **19**, 143 (2004).
[44] M. Nordberg, *J. Trace Elem. Exp. Med.*, **13**, 97 (2000).
[45] M.J. Stillman, C.F. Shaw, K.T. Suzuki, *Metallothioneins Synthesis, Structure and Properties of Metallothioneins, Phytochelatins and Metalthiolate Complexes*, VCH, New York (1992).
[46] V. Vacchina, K. Polec, J. Szpunar, *J. Anal. At. Spectrom.*, **14**, 1557 (1999).
[47] C.N. Ferrarello, M.M. Bayon, R.F. de la Campa, A. Sanz-Medel, *J. Anal. At. Spectrom.*, **15**, 1558 (2000).
[48] H.G. Infante, K. Van-Campenhout, R. Blust, F.C. Adams, *J. Anal. At. Spectrom.*, **17**, 79 (2002).
[49] A. Rodríguez-Cea, M.R. Fernández de la Campa, E. Blanco González, B. Andón Fernández, A. Sanz-Medel, *J. Anal. At. Spectrom.*, **18**, 1357 (2003).
[50] K.A. Taylor, B.L. Sharp, D.J. Lewis, H.M. Crews, *J. Anal. At. Spectrom.*, **13**, 1095 (1998).
[51] F. Gil, A. Pla, *J. Appl. Toxicol*, **21**, 245 (2001).
[52] G.K. Agrawal, R. Rakwal, M. Yonekura, A. Kubo, H. Saji, *Proteomics*, **2**, 947 (2002).
[53] J. Szpunar, P. Pellerin, A. Makarov, T. Doco, P. Williams, R. Lobinski, *J. Anal. At. Spectrom.*, **14**, 639 (1999).
[54] K. Odegard, W. Lund, *J. Anal. At. Spectrom.*, **12**, 403 (1997).
[55] M. Beran, R. Stahl, M. Beran, Jr, *Analyst*, **120**, 979 (1995).
[56] A.N. Richarz, C. Wolf, P. Brätter, *Analyst*, **128**, 640 (2003).
[57] M. Persson-Moschos, W. Huang, T.S. Srikumar, B. Akesson, *Analyst*, **120**, 833 (1995).
[58] D. Behne, C. Weissnowak, M. Kalcklosch, C. Westphal, H. Gessner, A. Kyriakopoulos, *Analyst*, **120**, 823 (1995).
[59] R. Lobinski, J.S. Edmonds, K.T. Suzuki, P.C. Uden, *Pure Appl. Chem.*, **72**, 447 (2000).
[60] H. Koyama, K. Omura, A. Ejima, Y. Kasanuma, C. Watanabe, H. Satoh, *Anal. Biochem.*, **267**, 84 (1999).
[61] J. Szpunar, A. Makarov, T. Pieper, B.K. Keppler, R. Lobinski, *Anal. Chim. Acta*, **387**, 135 (1999).
[62] R. Mandal, G. Jiang, X.-F.- Li, *Appl. Organometal. Chem.*, **17**, 675 (2003).
[63] S.A. Baker, N.J. Miller-Ihli, *Spectrochim. Acta B*, **55**, 1823 (2000).
[64] C.C. Chery, E. Dumont, R. Cornelis, L. Moens, *Fresenius' J. Anal. Chem.*, **775**, 375 (2001).