JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

ORIGINAL ARTICLE | DOI: 10.5584/jiomics.v3i2.128

Inorganic mass spectrometry-based metallomics for environmental monitoring of terrestrial ecosystems affected by metal pollution using *Mus spretus* as bioindicator

M.A. García-Sevillano[1,2,3], T. García-Barrera*[1,2,3], J.L. Gómez-Ariza*[1,2,3]

[1]Department of Chemistry and Materials Science, Faculty of Experimental Sciences, University of Huelva, Campus de El Carmen, 21007-Huelva, Spain. [2]Research Center on Health and Environment (CYSMA). University of Huelva. Spain. [3]International Campus of Excellence on Agrofood (ceiA3). University of Huelva. Spain.

Received: 01 February 2013 **Accepted:** 30 May 2013 **Available Online**: 18 July 2013

ABSTRACT

A metallomic approach based on the use of size-exclusion chromatography coupled to inductively coupled plasma-mass spectrometry (SEC-ICP-MS) has been used to characterize the biological response in liver, brain, kidneys, lungs and plasma of the free-living mouse *Mus spretus* in polluted areas located in Doñana National Park (southwest Spain) and the surroundings, mainly affected by agriculture, mining and industry activities, which are responsible for the presence of metallic contaminants. It is remarkable the high presence of Cu, Zn, Cd, As, Pb and Ni in the cytosolic extracts of different organs and plasma, especially in contaminated areas. In liver extracts, high intensity peaks traced by Cu, Zn, Pb and Cd at 7 kDa (matching with metallothionein I standard) are triggered by the presence of contaminants. In kidney, similar Cu and Cd-peaks at 7 kDa were observed but the equivalent Zn-peak was depleted by the competitive interactions of Cu-Cd-Zn for the active sites of these molecules. In addition, peaks traced by Cu and Zn at about 32 kDa in liver extract match with superoxide dismutase standard (Cu,Zn-SOD), which increase in accordance to contamination. An analogous behavior was observed for a Zn,Cu-peak at about 67 kDa that can be related with the bovine serum albumin standard (Cu,Zn-BSA) or other carrier protein such as transferrin (Cu-Tf) present in liver and plasma. Finally, low molecular mass arsenic metabolites were detected in mice captured in MAT site affected by mine waste.

Keywords: *Mus spretus*; SEC-ICP-MS; metal pollution; Doñana National Park; Bioindicators; metallomics.

Abbreviations

AsB: Arsenobetaine; **AsC**: Arsenocholine; **BSA**: Bovine serum albumin; **DMA^V**: Dimethylarsinate; **DNP**: Doñana National Park; **GSH**: Reduced glutathione; **HPLC**: High-performance liquid chromatography; **ICP-MS**: Inductively coupled plasma-mass spectrometry; **ICP-AES**: Inductively coupled plasma- atomic emission spectrometry; **LC**: Liquid chromatography; **MMA^V**: Methylarsonate; **MS**: Mass spectrometry; **MT**: Metallothionein; **PEEK**: Polyether ether ketone; **PMSF**: Phenylmethanesulfonyl fluoride; **PTFE**: Polytetrafluoroethylene; **SEC**: Size exclusion chromatography; **SOD**: Superoxide Dismutase; **TCEP**: Tris-(2-carboxyethyl)phosphine hydrochloride; **Tf**: Transferrin.

1. Introduction

The use of free-living organisms as bioindicators of terrestrial ecosystems has been proposed in numerous papers [1-4]. The interest in monitoring the exposure and associated response to heavy metals on living organisms has increased in the last years, since they can reflect the effect of pollutants on cellular metabolism, trafficking and global homeostasis [5]. In addition, the metabolism of trace elements can not be considered in isolation since different elements and their species act jointly in the cells, tissues or organs, and consequently, it is important to consider their possible synergistic or antagonist interactions [6]. Studies of small mammals, mainly rodents, have been used as bioindicators in numerous environmental studies because they can provide useful information to assess the risk of metals to humans. To this

***Corresponding author:** J.L. Gómez Ariza (ariza@dqcm.uhu.es); T. García-Barrera (tamara@dqcm.uhu.es). José Luis Gómez Ariza, Tamara García Barrera. Department of Chemistry and Materials Sciences. Faculty of Experimental Sciences. University of Huelva. Campus de El Carmen. E21071 Huelva. Phone: +34 959 219962. Fax: +34 959 219942.

end, the aboriginal species *Mus spretus* has been frequently used as bioindicator in the southwest Spain, especially in Doñana National Park (DNP). This important ecological area has a biodiversity unique in Europe, Doñana contains a great variety of ecosystems and shelters wildlife including thousands of European and African migratory bird, fallow deer, Spanish red deer, wild boar, European badger, Egyptian mongoose, and endangered species such as the Spanish Imperial Eagle and Iberian Lynx. However, the Park is affected by agricultural, mining and industrial activities [7,8] which makes necessary the regular monitoring of environment quality based in the analysis of pollutants and the biological responses to them. The application of proteomics for this purpose using the mouse *Mus spretus* as bioindicator has represented a reliable option [9], especially by the demonstrated genetic homology of this mouse with the classical inbred laboratory mouse *Mus musculus* that has been already sequenced. This fact allows the use of databases from *Mus musculus* for the identification of proteins and metalloproteins of *Mus spretus* [5,9] as well as for transcriptomics studies [10], avoiding the cumbersome work associated to *de novo* sequencing. On the other hand, the use of massive information methods, the *-omics*, is a promising alternative to the assessment of metal pollution by using biomarkers in environmental contamination [11,12]. Metallomics is one of the most recent *-omic* [13] which uses metals or metalloids, that are present in one third of biomolecules in cells, as heteroatomic markers or tags to track these molecules in complex biological matrices [14].

Metalloproteins have many different functions in cells, such as enzymatic activity, transport, storage, and signal transduction. Essential elements such as Cu, Zn, Fe, Mn and Se are integral constituents of numerous proteins where they have structural or catalytic roles, as is the case of transferrin [15], serum albumin [16], chaperones [16] and metallothioneins (MTs) [17], among many others. Deficiency or excess of these elements in mammalian organisms can result in metabolic dyshomeostasis which in turn can eventually lead to oxidative stress and toxicity that can ultimately cause disease and even death [18,19]. Carrier or transport proteins are involved in the movement of ion, small molecule, or macromolecules, such as other proteins, across biological membranes [20]. Storage proteins are biological reserves of metal ion and amino acids, used by organisms, as is the case of ferriti for iron, and finally, MTs are an example of signal transduction proteins [21]. For this reason, metallomics provides a good alternative to deep insight into the fate of elements in exposed organisms to metal pollution, and gives information about metals trafficking, interactions and homeostasis.

Metal ions and element species present in living systems are usually present in biological fluids and tissues at low picograms or nanograms per ml or mg concentrations. For this reason, the use of small mammals as bioindicators to evaluate terrestrial ecosystems requires analytical techniques that combine a high resolution separation technique with a sensitive elemental or molecular specific detector [22]. In this sense, the great potential of inductively coupled plasma-mass spectrometry (ICP-MS) for trace metals analysis has been highlighted in recent reviews since it provides great sensitivity, selectivity and precision, and allows the simultaneous detection of multiple heteroatoms in metalloproteins [23,24]. In addition, the use of ICP-MS requires small amount of sample, a very important advantage when working with micro-mammals. On the other hand, the drawback of polyatomic and/or isobaric interferences in the plasma as well as matrix effects can be minimized by the use of high resolution double focusing spectrometers [24] or collision and dynamic reaction cells (ICP-(ORC)-MS) in quadrupole analyzers [25].

In the present work, a metallomic analytical approach that consisted of high performance liquid chromatography (HPLC) based in size exclusion chromatography (SEC) coupled to ICP-MS has been used to characterize metal containing species (< 70 kDa) in liver, kidney, brain, lungs and plasma of *Mus spretus* captured in Doñana National Park (southwest Spain) and surroundings. For this purpose, Terrestrial ecosystems of this Park affected by differential contamination from mine, industrial and agricultural activities have been considered. The different size exclusion chromatographic profiles traced by the metals have been used to assess environmental contamination and the interactive relationships between metabolically active metals is discussed to explain the mice response against polluted sceneries.

2. Material and Methods

2.1. *Standard solutions and reagents*

For total element determination tissues and plasma, nitric acid (65 mass %), hydrochloric acid (30 mass %), hydrofluoric acid (40 mass %) and hydrogen peroxide (30 mass %) of Suprapur® grade (Merck, Darmstadt, Germany) were used for mineralization of the samples.

All reagents used for sample preparation of cytosolic extracts from the different organs were of the highest available purity. Phenylmethanesulfonyl fluoride (PMSF) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (BioUltra grade, >98%) were obtained from Sigma Aldrich (Steinheim, Germany). Helium used as collision gas in a ICP-ORC-MS system, was of high-purity grade (>99.999%).

Standards used for mass calibration of the analytical SEC column of mass range 70-3 kDa were as follows: ferritin (440 kDa) (purity 95%), bovine serum albumin (67 kDa) (purity 96%), superoxide dismutase containing Cu and Zn (32 kDa) (purity > 70%), metallothionein I containing Cd, Cu and Zn (7 kDa) (purity > 95%) and arsenobetaine (179 Da) (purity > 98%). All these reagents were purchased from Sigma-Aldrich (Steinheim, Germany). On the other hand, the standards used for mass calibration of the analytical SEC column of mass range <10kDa were: bovine serum albumin (67 kDa) (purity 96 %), metallothionein I containing Cd, Cu and Zn

(7 kDa) (purity > 95 %), vitamin B_{12} (1.35 kDa) (purity > 96 %) and reduced glutathione (307 Da) (purity 98–100 %). All these reagents were purchased from Sigma–Aldrich (Steinheim, Germany). Standard stock solutions with a concentration of 10 mg mL^{-1} were prepared by dissolving the respective compound in 20 mM of ammonium acetate at pH 7.4 purchased from Merck (Darmstadt, Germany). The mobile phase solution used in SEC was 20 mM of ammonium acetate (Suprapure grade), which was prepared daily with ultrapure water (18 MΩcm) from a Milli-Q system (Millipore, Watford, UK) and the pH adjusted to pH 7.4 with ammonia solution, this later prepared by dilution of 20% (w/v) ammonia solution (Suprapur, Merck) with ultrapure water.

2.2. *Apparatus*

A cryogenic homogenizer SPEX SamplePrep (Freezer/Mills 6770) was used to prepare the homogenates. Homogenized tissues were subsequently disrupted with a glass/teflon homogenizer. The extraction was followed by ultracentrifugation with an ultracentrifuge Beckman model L9-90 K (rotor 70 Ti). Polycarbonate bottles of 10 ml with cap assembly (Beckman Coulter) were used for this purpose. A microwave oven (CEM Matthews, NC, USA, model MARS) was used for the mineralization of extracts.

Trace metals and metal-linked biomolecules were analyzed with an inductively coupled plasma mass spectrometer Agilent 7500ce (Agilent Technologies, Tokyo, Japan) equipped with an octopole collision/reaction cell. Chromatographic separations were performed using a Model 1100 HPLC pump with detector UV (Agilent, Wilmington, DE, USA) as the delivery system. ICP-MS measurement conditions (Table 1) for collision (He) mode were optimized using a 2% (v/v) HNO_3 aqueous solution of ^{59}Co, ^{7}Li, ^{89}Y and ^{205}Tl (1 mg L^{-1}). The flow of collision gas was fixed at 3.7 mL min^{-1} for He in order to avoid or reduce the polyatomic interferences. Before the SEC-ICP-MS with Superdex-Peptide column the extracts were filtered through Iso-Disc poly(vinylidene difluoride) filters (25-mm diameter, 0.2-μm pore size) to avoid column overloading or clogging and ultrafiltered and preconcentrated (10 times) with AMICON 30K (Millipore) by centrifugation at 10.000 g for 30 min to 4ºC.

2.3. *Sampling area and animals*

Free-living mice (*Mus spretus*) were collected during the autumn 2009 in three sampling areas from Doñana National Park (DNP), southwest Spain. Three areas were considered concerning their differential contamination (Fig. 1): (i) the control was the sampling point located at "Lucio del Palacio" (LDP- green spot) that is a non-contaminated area in the core of the Park; (ii) "La Rocina Stream" (ROC- red spot) with strawberry, citrus fruit and grape fields in the surroundings, that is in addition affected by diffused pollution from the petrochemical and chemical activities from the industrial

Table 1. Operating conditions for ICP-MS and SEC

SEC conditions		
Column	Superdex™-75 (10x300 x13μm)	Superdex™-Peptide (10x300x13μm)
Resolution range	3-70 kDa	<10 kDa
Mobile phase	Ammonium acetate 20 mmol L^{-1} (pH 7.4)	
Flow rate	0.7 mL min^{-1}	
Injection volumen	20 μL	
UV. Visible wavelength	254 nm	
ICP-MS conditions		
Foward power	1500 W	
Plasma gas flow rate	15.0 L min^{-1}	
Auxiliary gas flow rate	1.0 L min^{-1}	
Carrier gas flow rate	0.9 L min^{-1}	
Sampling depth	8 mm	
Sampling and skimmer cones	Ni	
He flow	3.7 mL min^{-1}	
Q_{oct}	-18 V	
Q_p	-16 V	
Dwell time	0.3 per isotope	
Isotopes monitored	^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{75}As, ^{57}Fe, ^{103}Rh, ^{114}Cd, ^{60}Ni, ^{207}Pb, ^{208}Pb.	

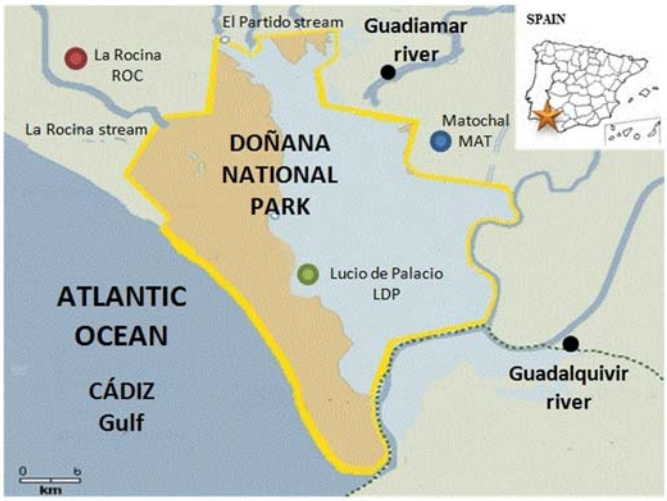

Figure 1. Sampling area in Doñana National Park and surroundings (SW Spain). Localization of sampling point: (i) "Lucio del Palacio" (LDP, green spot) non-contaminated site (control); (ii) "La Rocina" stream (ROC, red spot) and (iii) "Matochal" (MAT, blue spot) contaminated sites.

belt of Huelva, as well as by acid waters and metals from north-west mining metallurgical activities of Riotinto village (Huelva); and (iii) "el Matochal" (MAT- blue spot) site next to Guadiamar river that is affected by rice growing fields and suffered the input of metals transported by the Guadiamar river during the rupture of Aznalcollar mine tailing pond in 1998 [26]. A total of 68 *Mus Spretus* mice were caught with Sherman live-traps baited with a hazelnut cream over bread, which were mounted during the evenings and checked the next morning. Adult animals were taken alive to a laboratory at Doñana Biological Reserve, and site/date of capture, sex, weight and external measurements were recorded. In this study, 10 mice of each sampling area were caught. For each mouse, measurements of body size were recorded as body length to the nearest 0.5 mm (calculated by subtracting tail length from total length) and body weight measured to the nearest 0.1 g. On the basis of body weight and colour pattern all mice were considered as adults and they were used in this study. Then, mice were transported to the University of Huelva in other cleaned live-traps and they were sacrificed two hours later for plasma and organs extraction in a laboratory equipped for the manipulation of animals.

Mice were individually anesthetized by isoflurane inhalation and exsanguinated by cardiac puncture, dissected using a ceramic scalpel and finally, the organs were transferred rapidly to dry ice. Individual organs were excised, weighed in Eppendorf vials, cleaned with 0.9% NaCl solution, frozen in liquid nitrogen and stored at -80 ºC, until they were used for sample preparation. Plasma collection was carried out by centrifugation (4000rpm, 30 min, 4ºC) after addition of heparin (ANTICLOT) as an anticoagulant. Mice were handled according to the norms stipulated by the European Community. The investigation was performed after approval by the Ethical Committee of the University of Huelva (Spain).

2.4. *Analytical procedures*

2.4.1. Sample preparation

Livers, brain and plasma from 10 different male mice were treated following a procedure described elsewhere [5]. Briefly, individual organs were disrupted by cryogenic homogenization in a 6770 freezer/mill apparatus (2 min at rate 15) from Spex SamplePrep (Metuchen, NJ, USA). For SEC analysis, three pools of three individual homogenized organs were exactly weighed (0.500 g). After that the metal-biomolecules were extracted with a solution (3 ml of extractant solution per gram of cryhomogenized organs) containing 20 mM of ammonium acetate buffer solution at pH 7.4, 1 mM of tris(2-carboxyethyl)phosphine (TCEP) and 1 mM of phenylmethanesulfonylfluoride (PMSF) using a glass/teflon homogenizer in a cold chamber at a constant temperature of 4ºC. Then the extracts were centrifuged at 120,000 g for 1 h at 4 ºC. Extracts were stored under nitrogen atmosphere to avoid oxidation by air and at -80 ºC until analysis.

2.4.2. Determination of total metals concentration in the tissues and plasma of Mus spretus mice

For this purpose, five pools of two individual homogenized organs each were exactly weighed (0.100 g) in 5-ml microwave vessels and 500 µL of a mixture containing nitric acid and hydrogen peroxide (4:1 v/v) was added. After 10 min, the PTFE vessels were closed and introduced into the microwave oven. The mineralization was carried out at 400 W from room temperature, ramped to 160ºC for 15 min and hold for 40 min at this temperature. Then the solutions were made up to 2 ml and metals analyzed by ICP-MS. Rhodium was added as internal standard (1µg mL^{-1}). In the case of plasma, 100 µL of each mouse was weighed in 5-ml microwave vessels and the mineralization was carried out following the same conditions previously described for tissues digestion. For multielemental determination two replicates from each pool of two organs were carried out. For plasma, two replicates from each mouse specimen were carried out.

2.4.3. Analysis of cytosolic extracts and plasma of Mus spretus mice using SEC-ICP-MS

The SEC-ICP-MS online coupling was performed by connecting the outlet of the chromatographic column to the Micromist nebulizer inlet (GlasExpansion, Switzerland) of the ICP-MS by means of a 30cm PEEK tubing (0.17 i.d. mm). The quality control of the SEC-ICP-MS system was performed as described elsewhere [12] to overcome problems related to contamination, loss and stability of species. The retention times of the mass calibration standards used for the Superdex-75 column are the following: ferritin 12.0 min, bovine serum albumin (BSA) 13.5 min, superoxide dismutase containing Cu and Zn (Cu,Zn-SOD) 15.6 min, metallothionein I containing Cd, Cu and Zn (Cd,Cu,Zn-

MT1) 19.1 min, and reduced glutathione (GSH) 23.6 min. On the other hand, for the Superdex-Peptide column standards and retention times were: bovine serum albumin (BSA) 11.2 min, metallothionein I containing Cd, Cu and Zn (Cd,Cu,Zn-MT1) 14.6 min, vitamin B_{12} 19.3 min and reduced glutathione (GSH) 24.4 min.

3. Results and Discussion

The three sites considered in this study (Fig. 1) were selected by the differential presence of toxic metals. In the stream "La Rocina" (ROC) the presence of remarkable concentrations of nitrate, probably from agricultural sources, has been previously reported [27], and in this area metal levels in pore waters are also high, which suggests a transport of contaminants from the Iberian Pyrite Belt [28]. In addition, in 1998, a part of a 360 ha tailings dam of Aznalcóllar pyrite mine, located 60 km North of DNP, were released to Guadiamar stream, a tributary of Guadalquivir River, that were estimated at four cubic hectometers of acidic water and two cubic hectometers of mud. The high toxic metals content of this mud –35% Fe, 0.8% Zn, 0.8% Pb, 0.5% As, 0.2% Cu, 0.05% Sb, 0.006% Co, 0.005% Tl, 0.005% Bi, 0.0025% Cd, 0.0025% Ag, 0.0015% Hg, 0.001% Se—threatened DNP and the Guadalquivir Estuary [26]. This fact explains the high concentrations of non-essential elements (such as Cd and Hg) in tissues of shrews that can be associated to the pollution from mine activities [29], and high levels of Cu, Zn, Mn, Cd and As in soils and sediments [5].

Biological response of free-living mice *Mus spretus* to contamination at the above described site was studied in organs with high metabolic activity such as liver, brain and kidneys. In addition, plasma has been evaluated using the same procedure. The changes in the levels of metal–biomolecules caused by pollutants were traced by SEC-ICP-MS. The most interesting results were observed in relation to Cu, Zn, Fe and toxic elements such as Cd, As, Ni and Pb.

3.1. *Total metals concentration in the tissues and plasma of Mus spretus mice*

The presence of metals in the different organs and plasma of Mus spretus from the three sampling sites previously described (Fig 1) was evaluated. Recovery experiments were performed by spiking the extracts with 1, 5, 10, or 50 ìg L−1 of metals depending on the relative concentration of each one in the extracts; the results are also shown in Table 2 that confirm quantitative recoveries in all the cases. ICP-MS detection limits are also given in this table.

It is remarkable the higher presence of iron, copper and zinc in all organs studied and plasma. In contrast, the levels of arsenic, cadmium, lead and nickel were considerably lower. The lowest concentration of toxic metals was found in brain, lungs and plasma. The highest concentrations of metals such as iron, copper, zinc, arsenic, cadmium and lead have been found in mice captured in MAT, a contaminated area affected by metal pollution in soils and sediments [5], as a consequence of the breakdown of the dams from Aznalcóllar mines and their release from the rice growing fields. ROC presents an intermediate metal contamination between LDP and MAT (table 2). This fact can be explained by metallic contaminants from the Iberian Pyrite Belt and agricultural activities. In addition, the highest concentration of iron in plasma from mice captured in ROC could be related with the high content of this element in the Iberian Pyrite Belt [28], which is a site affected by the acid mine drainage. Numerous transport protein of iron can be found in plasma of mammals, such as transferrin, myoglobin and hemoglobin. On the other hand, the higher copper concentration in lungs from mice captured in LDP and MAT could be related with several agricultural activities developed around, which use pesticides and herbicides containing this element (table 2). The major concentration of arsenic in kidneys from mice sampled in MAT and ROC in comparison with mice captured in LDP could be explained by the proximity of the former to the Guadiamar river, affected by rice growing fields (pesticides and herbicides) that also suffered the input of metals transported by the river during the rupture of Aznalcollar mine tailing pond in 1998 [26],. The concentrations of arsenic in mice kidneys from ROC are also high, probably due to air pollution, since this site is nearest to the industry. In contrast, in ROC lower concentrations of this element in soils and sediments were found [5]. In addition, similar concentration of arsenic was found in lungs from mice captured in both areas (table 2). Finally, it is remarkable the higher concentration of cadmium in kidneys from mice sampled in LDP in comparison with ROC, which are in good agreement with a work previously published work [5].

3.2. *Size characterization of Cu, Zn, Cd, As, Ni and Pb-biomolecules in liver extracts from Mus spretus captured in contaminated and non-contaminated areas*

The study of accumulation and effects of heavy metals in living organisms is very important in connection with global environmental pollution. The induction of Cd and Zn-metallothioneins in *Mus musculus* mice exposed to industrial particles with high content of metals has been reported, and strong interactions between Cu–Zn–Cd-Pb have been confirmed [30]. Antagonistic interactions have been established between several heavy metals, particularly between Cd and Zn. In this sense, Cd replaces Zn from several proteins [31] and high amounts of Cd in the diet leads to deficiency of zinc in living organism [32]. In our study, we can observe potential interactions between these metals in liver of free-living mice from Doñana National Park and surroundings, especially in mice captured in MAT, specially Cu and Cd in the fraction of 7 kDa associated with MTs (Fig. 2).

It is remarkable the high intensities of the peaks traced by Cu, Zn and Cd at 7 kDa in the liver cytosolic extract, that match with the retention time of metallothionein I standard (Fig. 2). The intensity of a MT-Cu-peak from the contami-

Table 2. Concentration of elements (μg g-1) in the different organs and plasma of the mice

		Fe ($\mu g.g^{-1}$)	Cu ($\mu g.g^{-1}$)	Zn ($\mu g.g^{-1}$)	As ($\mu g.g^{-1}$)	Cd ($\mu g.g^{-1}$)	Ni ($\mu g.g^{-1}$)	Pb ($\mu g.g^{-1}$)
LIVER	*Mus Spretus* LDP	8.57 ±0.201	0.369 ±0.0371	4.80 ±0.103	0.00161 ±0.000221	0.0921 ±0.000247	0.0242 ±0.00281	<LOD
	Mus Spretus MAT	14.9 ±0.181	1.328 ±0.0674	10.658 ±0.199	0.00512 ±0.00138	0.00321 ±0.000522	0.0751 ±0.00493	0.00714 ±0.00152
	Mus Spretus ROC	10.3 ±0.220	1.45 ±0.0552	10.3 ±0.208	0.00421 ±0.00181	0.00143 ±0.000321	0.0315 ±0.00373	0.00601 ±0.00211
BRAIN	*Mus Spretus* LDP	1.94 ±0.0912	0.487 ±0.0211	2.94 ±0.0663	0.00102 ±0.000221	0.00112 ±0.000441	<LOD	<LOD
	Mus Spretus MAT	2.96 ±0.111	0.578 ±0.0382	2.96 ±0.0881	0.00622 ±0.00171	0.00944 ±0.00152	<LOD	0.00162 ±0.000511
	Mus Spretus ROC	2.01 ±0.0792	0.492 ±0.0873	2.93 ±0.102	0.00221 ±0.000822	0.00243 ±0.000741	<LOD	<LOD
KIDNEYS	*Mus Spretus* LDP	5.51 ±0.207	0.268 ±0.0232	3.38 ±0.111	0.00192 ±0.000311	0.0277 ±0.00342	0.00284 ±0.000742	<LOD
	Mus Spretus MAT	10.4 ±0.214	0.406 ±0.0214	5.15 ±0.104	0.00332 ±0.00123	0.0324 ±0.0111	0.0122 ±0.00212	<LOD
	Mus Spretus ROC	5.37 ±0.0812	0.467 ±0.0161	3.46 ±0.0972	0.00484 ±0.000942	0.0151 ±0.00922	0.00312 ±0.00121	<LOD
LUNGS	*Mus Spretus* LDP	10.7 ±0.524	0.158 ±0.0213	0.296 ±0.0252	<LOD	<LOD	<LOD	<LOD
	Mus Spretus MAT	13.6 ±0.705	0.201 ±0.0584	0.554 ±0.00612	0.0221 ±0.00182	0.00232 ±0.000914	<LOD	<LOD
	Mus Spretus ROC	9.048 ±0.458	0.147 ±0.0351	0.321 ±0.00344	0.0121 ±0.00122	0.00124 ±0.000522	<LOD	<LOD
PLASMA	*Mus Spretus* LDP	4.69 ±0.251	0.223 ±0.0380	1.641 ±0.0512	<LOD	<LOD	<LOD	<LOD
	Mus Spretus MAT	5.25 ±0.321	1.14 ±0.0842	2.18 ±0.0634	0.00241 ±0.000724	0.00154 ±0.000422	<LOD	0.00122 ±0.000341
	Mus Spretus ROC	10.5 ±0.503	0.553 ±0.0341	1.89 ±0.0607	0.0013 ±0.000411	<LOD	<LOD	<LOD
LOD ($\mu g.L^{-1}$)		**0.0471**	**0.0384**	**0.494**	**0.0272**	**0.00911**	**0.0244**	**0.0322**
Recovery (%)		**117**	**105**	**121**	**99**	**102**	**113**	**97**

LOD: Limit of Detection

nated area MAT is clearly higher than that from other sites, such as ROC and LDP. In addition, the presence of significant peaks of MT-Cd (Fig. 2) and MT-Pb (Fig. 3) in the same sampling site are also remarkable. In contrast, the intensity of this peak traced by Zn for mice captured in MAT presents lower intensity. Metallothioneins (MTs) are synthesized by mammals in response to heavy metal stress [33,34]. Detoxification of heavy metals and scavenging of free radicals are also reported as important actions of MT which are present in mammalian tissues in two major isoforms, MT-1 and MT -2. [35-37]. Due to the relative concentrations of Cd/Cu and Zn in MAT discussed before, is possible to suppose that cadmium and copper can replace the zinc binding to MTs, because these elements have greater affinity for sulfhydryl groups of cysteine present in these proteins [30]. For this reason, up-regulation of MTs triggered in liver by the presence of toxic elements such as Cd and high levels of Cu can be considered as biomarkers of free-living organisms exposure to environmental pollution, in particular to toxic metals.

Another peak traced by Cu, which matches with the retention time of superoxide dismutase (SOD) at about 32 kDa, shows higher intensity for samples from MAT and LDP (Fig. 2). This fraction has been purified and identified by mass

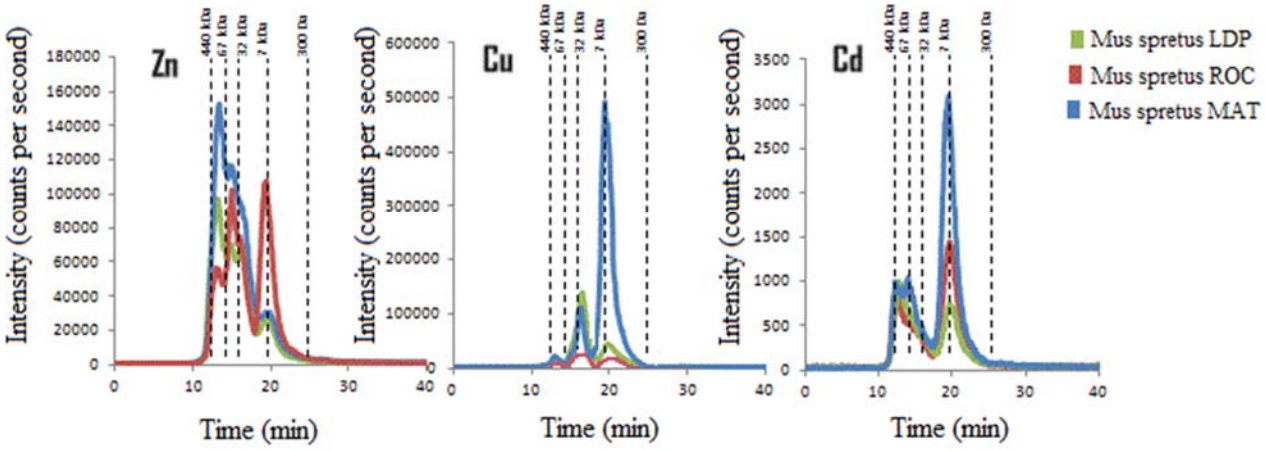

Figure 2. Zn, Cu and Cd-biomolecules complexes in liver of *Mus spretus* from different environmental areas assessed by molecular mass distribution with SEC-ICP-MS. Chromatographic conditions: column, Superdex™-75 (10x300x1.3 µm); mobile phase, ammonium acetate 20 mmol L^{-1} (pH 7.4); flow rate 0.7 ml min^{-1}; injection volume, 20 µL

spectrometry in a previous work [5] demonstrating that peak intensity of the copper can be attributed unequivocally to the enzyme superoxide dismutase (Cu/Zn-SOD), a biomarker used in numerous studies to assess environmental stress [1,8]. In Fig. 2 shows two signals traced by Zn that matches with standards of bovine serum albumin (Zn-BSA) and superoxide dismutase (Cu,Zn-SOD) at about 67 kDa and 32 kDa, respectively. The presence of pollutants in sites such as MAT and ROC enhances the intensity of these peaks, but in the non-polluted LDP the intensity decreases. It is remarkable the results obtained in samples from ROC where despite the significant presence of contaminants, the intensity of putative peaks matching with Zn-BSA and Zn-SOD decreases, which can be explained by zinc homeostasis and Cu, Zn and Cd interactions [30,31].

Finally, it is interesting to observe the presence of arsenic and nickel in liver extracts (Fig. 3). SEC-ICP-MS shows the presence of low molecular mass biomolecules of As, that are less abundant in MAT, and also some small Ni containing compounds, highly abundant in the same site. The high concentration of arsenic in soils and sediments analyzed in previous works [5] led us to conclude that arsenic species to can be bonded to thiol groups of proteins [38] in mice captured in MAT. In addition, the concentration of arsenic found in lung in this area could be related with volatile arsenic species inhaled (table 2). Arsenic and its compounds, especially the trioxide, are used in the production of pesticide, treated wood products, herbicide, and insecticide that maybe are

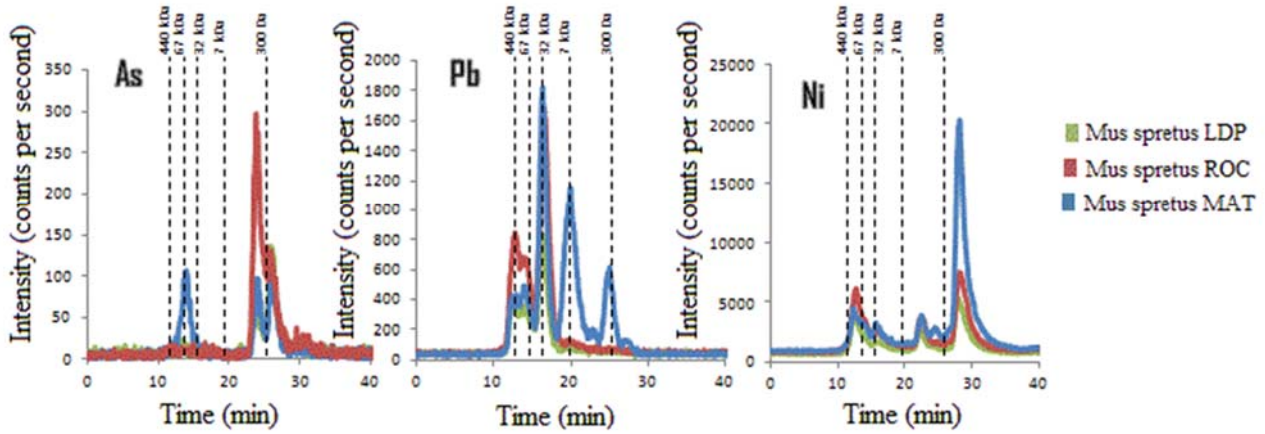

Figure 3. As, Pb and Ni-biomolecules complexes in liver of *Mus spretus* from different environmental areas assessed by molecular mass distribution with SEC-ICP-MS. Chromatographic conditions: column, Superdex™-75 (10x300x1.3 µm); mobile phase, ammonium acetate 20 mmol L^{-1} (pH 7.4); flow rate 0.7 ml min^{-1}; injection volume, 20 µL.

used in the rice and grape fields of MAT and ROC, respectively. When the biomethylation of inorganic arsenic is saturated in liver, the trivalent inorganic arsenic (the most abundant arsenic species in the terrestrial ecosystems) is chemically more reactive than pentavalent species and binds many carrier proteins, such as albumin and transferrin (Tf) (67kDa) [39]. On the other hand, the presence of low molecular mass nickel metabolites and nickel containing metalloproteins is remarkable in liver cytosolic extracts from mice captured in MAT. This element is rarely found in the terrestrial ecosystems; therefore this fact can be explained by the presence of this metal in the leachates from the breakdown of the ponds of Aznalcollar mines in 1998.

3.3. *Size characterization of Cu, Zn, Cd and As -biomolecules in brain extracts from Mus spretus captured in contaminated and non-contaminated areas*

In the case of brain the coupling SEC-ICP-MS was again used to obtain the Cu, Zn, Cd and As-traced peaks in the cytosolic fractions of M. spretus (Fig. 4). These chromatograms show a remarkable intensity of the peaks traced by Cu, Zn, and Cd at 7 kDa as in the case of liver extracts discussed before, which match well with the retention time of metallothionein, although the most abundant MT isoform in brain is MT-3 [35-37]. In MAT the intensities of the 7 kDa peak traced by Cd, Cu and Zn presents higher intensity than ROC and LDP, respectively. In the case of Cu tracing in MAT is not visible in Figure 4, unless it parallels exactly the ROC tracing. On the other hand, the presence of small arsenic-containing metabolites is significantly higher in brain of mice captured in the polluted areas (Fig. 4) that follows the range MAT>ROC>LDP. The presence of this toxic element in the brain can be explained because experiments performed with rats as model organisms have revealed that As can cross the blood brain barrier (BBB) producing an increasing presence of reactive oxygen species (ROS) as well as oxidative stress [40].

3.4. *Size characterization of Cu, Zn, Cd and As -biomolecules in plasma from Mus spretus captured in contaminated and non-contaminated areas*

The most interesting results obtained by the analysis of plasma samples by SEC-ICP-MS are related with Cu, Zn, Cd and As-biomolecules (Fig. 5). In the chromatogram traced by Cu we can observe a peak of about 67 kDa of high intensity. This peak is can be related with the transport proteins Cu/Zn-BSA and Cu-Tf of 67 kDa and 79kDa, respectively. The same 67 KDa-matched peak is also traced by Zn which agrees with this hypothesis (Fig. 5). Higher intensities of both metals were detected in plasma from mice captured in MAT which agrees with the increased presence of these metals in the sampling area both in soils and sediments [5]. The profile traced by Cd presents differences between mice captured in MAT with those captured in LDP and ROC (Fig. 5),

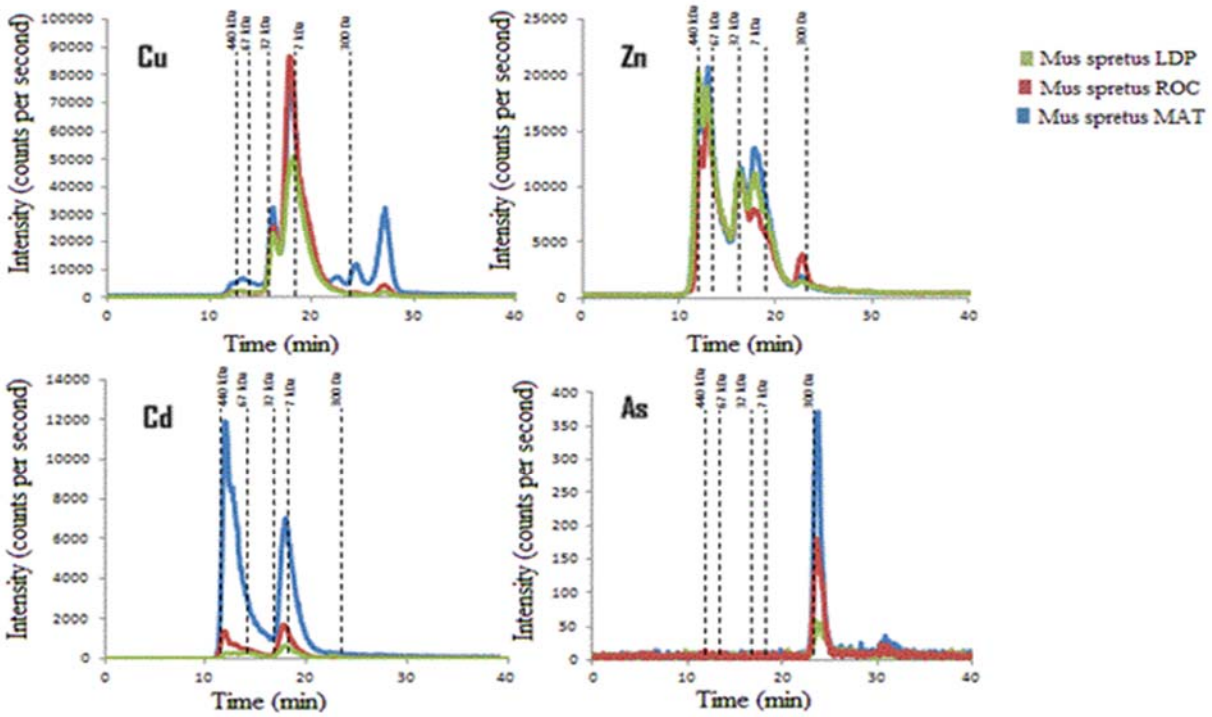

Figure 4. Cu, Zn, Cd and As-biomolecules complexes in brain of *Mus spretus* from different environmental areas assessed by molecular mass distribution with SEC-ICP-MS. Chromatographic conditions: column, Superdex™-75 (10x300x1.3 µm); mobile phase, ammonium acetate 20 mmol L^{-1} (pH 7.4); flow rate 0.7 ml min^{-1}; injection volume, 50 µL.

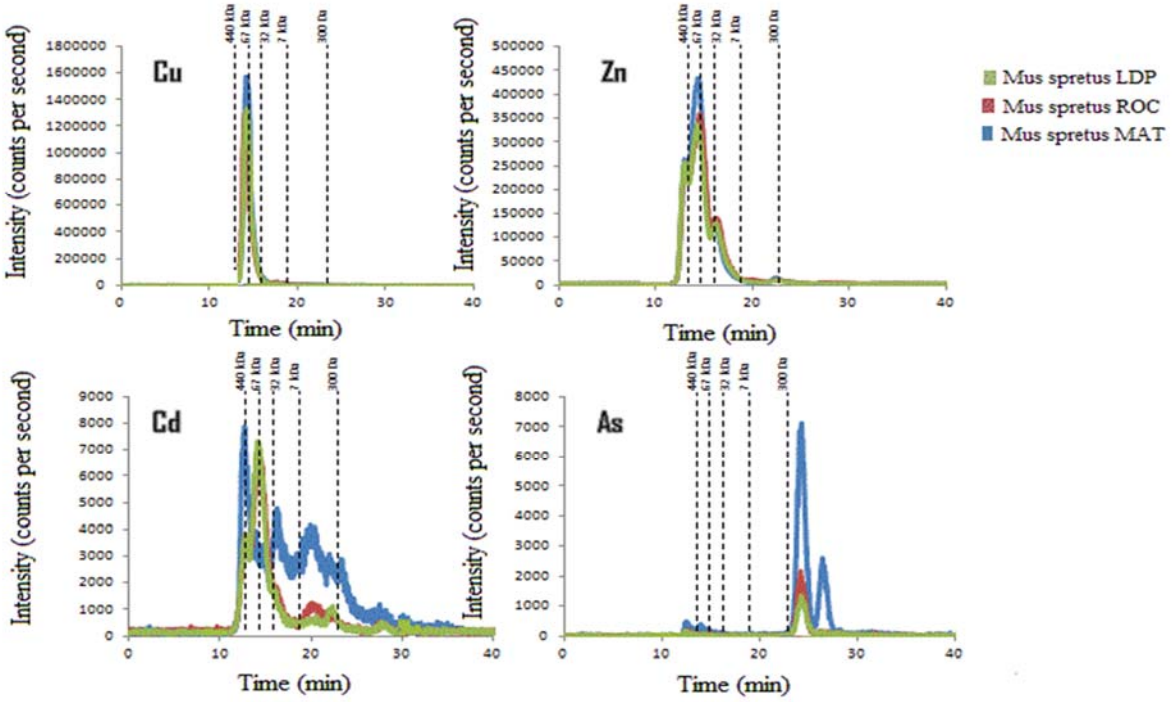

Figure 5. Cu, Zn, Cd and As-biomolecules complexes in plasma of *Mus spretus* from different environmental areas assessed by molecular mass distribution with SEC-ICP-MS. Chromatographic conditions: column, Superdex™-75 (10x300x1.3 μm); mobile phase, ammonium acetate 20 mmol L^{-1} (pH 7.4); flow rate 0.7 ml min^{-1}; injection volume, 50 μL.

and the chromatograms show several peaks that can be explained by the high affinity of this element for thiol groups of proteins as stated by other authors [41]. Finally, in the chromatogram obtained for As-biomolecules (Fig. 5) is also observed the high intensity obtained in plasma samples from mice captured in MAT that confirm the assertion of Suzuki et al [41] about the potential use of arsenic presence in blood and plasma as biomarker of exposure to this element.

3.5. *Size characterization of Cu, Cd and As-biomolecules in kidneys extracts from Mus spretus captured in contaminated and non-contaminated areas*

In order to evaluate potential interactions between Cu and Cd, the cytosolic extracts of kidneys were ultrafiltered using microcentrifugal filters with a cut-off mass of 30kDa and directly analyzed by SEC-ICP-MS using a Superdex-Peptide column (with mass range <10kDa). The results obtained shown different expression profiles for Cu, Cd and As-biomolecules in kidneys ultrafiltered extracts from mice captured in the different areas of study (Fig. 6). As previously discussed in liver (Fig. 2), potential interactions between Cu and Cd are observed in kidney from mice captured in MAT and ROC in the fraction of 7 kDa related with MTs. In MAT, the higher presence of cadmium in the ecosystem and the greater affinity of this element for MTs explain the high intensity of the cadmium peak about 7 kDa against the lower intensity of copper in the same peak (Fig. 6).

On the other hand, the presence of low-molecular-mass As species in kidney cytosolic extracts analyzed by SEC-ICP-MS (column Superdex-Peptide) can be seen in Fig. 6. The higher intensity of the signal was obtained from ROC, in which total As concentration in kidney organs is also the highest (Table 2). High concentrations of arsenic in kidneys have been explained by other authors by the diet [42], while in contrast, the major concentration of arsenic in plasma, liver and brain could be related with the inhalation of this element [43] arsenic. In addition, inorganic arsenic exposure in humans, by the inhalation route, has been shown to be strongly associated with lung cancer [44], while ingestion of inorganic arsenic in humans has been linked to a form of skin cancer and also to bladder and kidney cancer [45].

4. Concluding Remarks

The importance of monitoring the exposure and studying the effects of heavy metals in living organisms has increased in the last decades. Studies of small mammals, mainly free-living mice (Mus spretus), have been used as bioindicators in numerous environmental studies because they can provide useful information for assessment of risk of metals to humans. The use of SEC-ICP-MS coupling is a good choice to

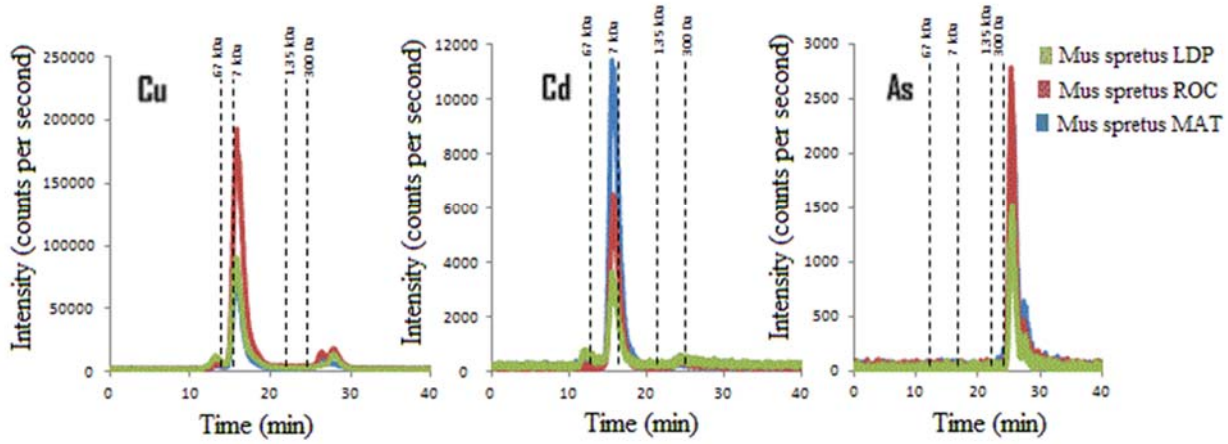

Figure 6. Cu, Cd and As-biomolecules complexes in kidneys of *Mus spretus* from different environmental areas assessed by molecular mass distribution with SEC-ICP-MS. Chromatographic conditions: column, Superdex™-Peptide (10x300x1.3 μm); mobile phase, ammonium acetate 20 mmol L^{-1} (pH 7.4); flow rate 0.7 ml min^{-1}; injection volume, 20 μL.

assess changes in the profiles of metal-binding biomolecules in environmental bioindicators (e.g. Mus spretus) caused by metal pollution. The study of liver, brain, kidneys, lungs and plasma from mice captured in contaminated and non-contaminated areas in Doñana National Park (southwest Spain) and the surroundings reveals differences in the expression of Cu, Zn, Cd, As, Pb and Ni-biomolecules that can be related with contamination episodes. These results, confirm the potential metallomics for environmental issues assessment and the characterization of metal interactions in organisms exposed to contamination. Future works are necessary to identify by organic mass spectrometry the metal-biomolecules traced by SEC-ICP-MS as well as the identification and quantification of the arsenic containing peaks by HPLC-MS. In addition, the use of micro-mammals as bioindicators to evaluate terrestrial ecosystems requires analytical techniques with sensitive elemental or molecular specific detectors. In this sense, the potential of inductively coupled plasma-mass spectrometry (ICP-MS) joining great sensitivity, selectivity, precision and multiple heteroatoms detection of metalloproteins makes essential its use in this type of environmental studies. On the other hand, the intensities of SEC-ICP-MS signals from metal-containing biomolecules show a complex metal-profile that cannot be well resolved, and for this reason, multidimensional chromatographic approaches will be used to overcome this problem in further studies, such as ionic exchange chromatography and, finally, the altered metalloproteins will be identified using organic mass spectrometry.

Acknowledgements

This work was supported by the project CTM2009-12858-C02-01 from the Ministerio de Ciencia e Innovación, and the projects P08-FQM-3554 and P09-FQM-4659 from Consejería de Innovación Ciencia y Empresa (Junta de Andalucía). M. Angel García Sevillano thanks the Ministerio de Educación for a predoctoral scholarship.

References

[1] D. Bonilla-Valverde, J. Ruiz-Laguna, A. Muñoz, J. Ballesteros, F. Lorenzo, J.L. Gómez-Ariza, J. López-Barea, Toxicology 197 (2004) 123–138. DOI: 10.1016/j.tox.2003.12.010

[2] B. Sure, Aquatic Ecology 35(2) (2001) 245-255. DOI: 10.1023/A:1011422310314

[3] R. Montes Nieto, T. García-Barrera, J.L. Gómez-Ariza, J. López-Barea, Environmental Pollution 158 (2010) 401-408. DOI: 10.1016/j.envpol.2009.09.005

[4] A. Romero-Ruiz, J. Alhama, J. Blasco, J.L. Gómez-Ariza, J. López-Barea, Environmental Pollution 156 (2008) 1340–1347. DOI:10.1016/j.envpol.2008.02.02

[5] M. A. García-Sevillano, M. González-Fernández, R. Jara-Biedma, T. García-Barrera, J. López-Barea, C. Pueyo and J. L. Gómez-Ariza, Analytical and Bioanalytical Chemistry 404 (2012) 1967-1981. DOI: 10.1007/s00216-012-6274-2

[6] T. García-Barrera, J.L. Gómez-Ariza, M. González-Fernández, F. Moreno, M.A. García-Sevillano, V. Gómez-Jacinto, Analytical and Bioanalytical Chemistry 403(8) (2012) 2237-2253. DOI:10.1007/s00216-012-5776-2

[7] V. Funes, J. Alhama, J. López-Barea, J. Peinado, Journal of Environmental Pollution 139 (2006) 214-223. DOI: 10.1016/j.envpol.2005.05.016

[8] A. Vioque-Fernández, E. Alves de Almeida, J. López-Barea, Science of the Total Environment 407 (2009) 1784-1797. DOI: 10.1016/j.scitotenv.2008.11.051

[9] R. Montes-Nieto, C.A. Fuentes-Almagro, D. Bonilla-Valverde, M.J. Prieto-Álamo, J. Jurado, M. Carrascal, J.L. Gómez-Ariza, J. López-Barea, C. Pueyo, Proteomics 7 (2007) 4376–4387. DOI: 10.1002/pmic.200700409

[10] J. Ruiz-Laguna, N. Abril, T. Garcia-Barrera, J.L. Gómez-Ariza, J. López-Barea, C. Pueyo, Environmental Sciences & Technologies 40 (2006) 3646-3652. DOI: 10.1021/es060056e

[11] J. López-Barea, J.L. Gómez-Ariza, Proteomics 6 (2006) 51-62. DOI: 10.1002/pmic.200500374

[12] M. González-Fernandez, T. García-Barrera, J. Jurado, M.J. Prieto-Álamo, J. López-Barea, C. Pueyo, J.L. Gómez-Ariza, Pure Applied Chemistry 80 (2008) 2609-2626. DOI: 10.1351/pac200880122609

[13] S. Mounicou, J. Szpunar, R. Lobinski, Chemical Society Reviews 38 (2009) 1119–1138. DOI: 10.1039/b713633c

[14] A. Sanz-Medel, Analytical and Bioanalytical Chemistry 390 (2008) 1–2. DOI: 10.1007/s00216-007-1705-1

[15] R.R. Crichton, M. Charloteaux-Wauters, European Journal of Biochemistry 164(3) (1987) 485-506. DOI: 10.1111/j.1432-1033

[16] J.R. Prohaska, American Journal Clinical Nutrition 88(3) (2008) 826–829.

[17] A. Sigel, H. Sigel, R.K.O. Sigel,Editors, ,Metal Ions in Life Sciences, Volume 5, Metallothioneins and Related Chelators (2009) The Royal Society of Chemistry (RSC),Cambridge 536 Hard cover, 266 (1-3), 55-56.

[18] D.P. Giedroc, A.I. Arunkumar, Dalton Transactions 2007, 3107–3120. DOI:10.1039/B706769K

[19] W. Zheng, M. Aschner, J. F. Ghersi-Egea, Toxicology Applied Pharmacology 192 (2003) 1–11. DOI: 10.1016/S0041-008X (03)00251-5

[20] D. Sadava et al. Life, the Science of Biology, 9th Edition. Macmillan Publishers, 2009.

[21] T. Kimura, N. Itoh, Journal of Health Science, 54 (3) (2008) 251-260.

[22] J. Bettmer, M. Montes Bayón, J. Ruiz Encinar, M.L. Fernández Sánchez, M.R. Fernández de la Campa, A. Sanz Medel, Journal of Proteomics 72 (2009) 989-1005. DOI: 10.1016/j.jprot.2009.05.003.

[23] 23. S. Mounico, J. Szpuna, R. Lobinsk, European Journal of Mass Spectrometry 16(3) (2010 243-253. DOI: 10.1255/ejems.1059.

[24] J.M. Marchante-Gayón, C. Sariego Muñíz, J.I. García Alonso, A. Sanz-Medel, Analytical Chimica Acta 400 (1999) 307–320. DOI:

[25] S.D. Tanner, V.I. Baranov, D.R. Bandura, Spectrochimica Acta Part B: Atomic Spectroscopy, 57(9) (2002) 1361-1452. DOI: S0584-8547(02)00069-1

[26] J.O. Grimalt, M. Ferrer, E. Macpherson, Science of the Total Environment 242 (1999) 3-11.

[27] G. Tortosa, D. Correa, A.J. Sánchez-Raya, A. Delgado, M.A. Sánchez-Monedero, E.J. Bedmar, Ecological Engineer 37 (2001) 539–548.

[28] A. Tovar-Sanchez, M.A. Huerta-Diaz, J.J. Negro, M.A. Bravo, S.A. Sañudo-Wilhelmy, Journal Environmental Manag 78 (2006) 286-293.

[29] A. Sánchez-Chardi, C.A. Oliveira Ribeiro, J. Nadal, Chemosphere 76 (2009) 387–394.

[30] S. Teodorova, R. Metcheva, M. Topashka-Ancheva, Environmental Research 91 (2003) 85–94.

[31] H.M. Perry, E.F. Perry, J.E. Putifoy, J.N. Erlanger, Trace Subst. Environ. Health 7 (1973) 281–288.

[32] A.W. Voors, M.S. Shuman, P.M. Galagher, Trace Subst. Environ. Health 6 (1972) 215–222.

[33] M. J. Stillman, C. F. Shaw and K. T. Suzuki, VCH, New York, 1992.

[34] J.F. Riodan, B.L. Valee, Methods in Enzymology, vol. 205, Academic Press, New York, 1991.

[35] M. Sato, K.T. Suzuki, Biomedical Research on Trace Element, 6 (1995) 13.

[36] Y. Uchida, Neurosignals 3 (1994) 211-215. DOI: 10.1159/000109547

[37] C.J. Quaife, S.D. Findley, J.C. Erickson, G.J. Froelick, E.J. Kelly, B.P. Zambrowicz, R.D. Palmiter, Biochemistry 33 (1994) 7250.

[38] Aposhian, H.V., Aposhian, M.M., Chemical Research in Toxicology, 19 (2006) 1-15. DOI: 10.1021/tx050106d

[39] M.G. Bogdana, A. Samgayo-Reyest, H.V. Aposhian, Toxicology 93 (1994) 175-193. DOI: 10.1016/0300-483X(94) 90077-

[40] F. Piao, N. Ma, Y. Hiraku, M. Murata, S. Oikawa, F. Cheng, L. Zhong, T. Yamauchi, S. Kawanishi, K. Yokoyama, Journal of Occupational Health 47 (2005) 445–449. DOI:10.1539/joh.47.445

[41] A.Y. Aoki, K.T. Suzuki, Journal of Biochemical Toxicology 2 (1) (2006) 67–71, DOI: 10.1002/jbt.2570020107

[42] 42. E.M. Kenyo, L.M. Del Raz, Toxicological Sciences 85 (1) (2005) 468-475. DOI: 10.1093/toxsci/kfi107

[43] S.W. Burchiel, L.A. Mitchell, F.T. Laver, X. Sun, J.D. McDonald, L.G. Hudson, K.J. Liu, Toxicology & Applied Pharmacology, 241 (2009) 253-259. DOI: 10.1016/j.taap.2009.09.019.

[44] H.J. Lubi, L.E. Moor, J.F. Fraumeni, K.P. Canto, Health Perspectives 116(12) (2008) 1661–1665. DOI: 10.1289/ehp.1151

[45] C.J. Chen, C.W. Chen, M.M. Wu, T.L. Kuo, British Journal of Cancer 66 (1992) 888-892.