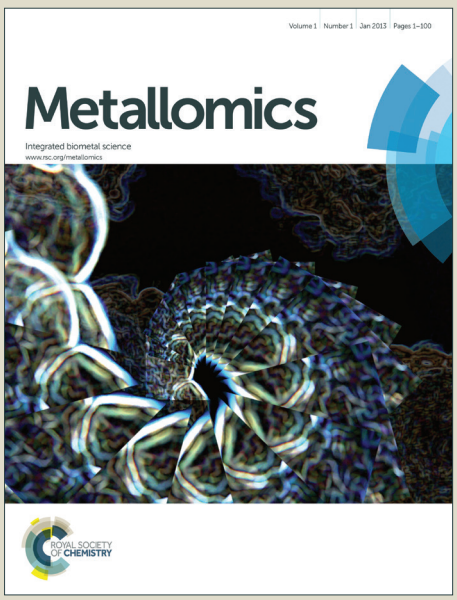
Metallomics

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/metallomics

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Cadmium toxicity in *Mus musculus* mice based on metallomic study. Antagonistic interaction between Se and Cd in the bloodstream

M.A. García-Sevillano[a,b,c], T. García-Barrera[*a,b,c], F. Navarro[d], J.L. Gómez-Ariza[*a,b,c]

[a] *Department of Chemistry and Materials Science, Faculty of Experimental Sciences, University of Huelva, Campus de El Carmen, 21007-Huelva, Spain*
[b] *Research Center on Health and Environment (CYSMA). University of Huelva. Spain*
[c] *International Campus of Excellence on Agrofood (ceiA3). University of Huelva. Spain*
[d] *Department of Environmental Biology and Public Health, Cell Biology. Faculty of Experimental Sciences. University of Huelva. Campus El Carmen. 21007-Huelva, Spain*

AUTHOR E-MAIL ADDRESS: J.L., Gómez-Ariza (ariza@uhu.es); T. García-Barrera (tamara@dqcm.uhu.es)

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Cadmium (Cd) is an important inorganic toxicant in the environment with impact on human health. A metallomic approach based on size-exclusion chromatography (SEC) coupled to inductively coupled plasma-mass spectrometry (ICP-MS) and multidimensional chromatography separation based on SEC coupled to affinity chromatography 2D-SEC-AF-ICP-MS have been applied to achieve a better understanding of the function, detoxification processes and regulation of metals in mice (*Mus musculus*) under controlled exposure to both Cd and Cd plus ^{77}Se. Isotopic dilution analysis (IDA) was performed to quantify selenium containing proteins in mice plasma with ICP-qMS as multielemental detector. Additionally, isotope pattern deconvolution (IPD) was applied to study the fate of enriched ^{77}selenite in mice subjected to cadmium exposure and the effect selenoproteins production in plasma. Moreover, the affinity of Cd for SeP in plasma of mice was corroborated using anion exchange chromatography (AEC) after AF separation and identified by organic mass spectrometry. This work illustrates the high reliability of the integrated use of inorganic and organic mass spectrometry to get a metallomic approximation, which provides a good alternative to deep insight into the fate of elements in exposed organisms, providing information about metals trafficking, interactions and homeostasis.

Introduction

Cadmium is a widespread, highly toxic, environmental pollutant from natural and industrial source, which is known to be accumulated in the human body. Several studies have indicated the carcinogenic potential of Cd in humans[1-3] and experimental animals, such as mice[4-5]. Another major source of Cd is tobacco smoke, resulting in higher blood Cd concentrations in smokers[6]. Cd has a long half-life in the body, and adverse effects of chronic Cd exposures on kidneys and bone are well documented[7-8]. Recently, Cd exposure has been associated with several endocrine effects[9-11]. On the other hand, it is well known that selenium (Se) presents numerous antagonistic interactions with Cd, such as prevention of oxidative stress induced by this element[12], protection against Cd-induced nephrotoxicity and hepatotoxicity[13], and antagonistic action against Cd-induced inhibition of hepatic drug metabolism[14].

Nonetheless, the toxicological effects of Cd in metabolism still remain unclear. For this reason, the use of analytical methods for massive information, such as metallomic and metabolomic approaches is of great interest to evaluate the effects of controlled exposure of *Mus musculus* to Cd and Cd plus ^{77}Se, which has been considered previously[15]. Nowadays, using omics methodologies different authors have obtained a lot of information about the biological function of elements, although most of methods are focused on only one element or very well-defined chemical species related to it. However, actual biological mechanisms and metabolic pathways are based on the interactions of several elements or their species that can counteract the action of others in cooperation or availability mechanisms[16].

Irrespective of the intake route of an element into a living organism, particularly mammals, the bloodstream plays a fundamental role in the transport of species among different organs[17], in which metal assembly with proteins and other

biomolecules occurs. This is the case of cadmium metallothionein (Cd-MT), in which the element is bound to sulfur[18], or the coordination of cadmium and zinc in alcohol dehydrogenase[19].

Metallomic approaches usually combine inductively coupled plasma mass spectrometer (ICP-MS)[20] with a previous chromatographic device (in single or multidimensional arrangements), and mass spectrometry for parallel biomolecules identification in integrated workflows[21].

In addition, isotope dilution analysis (IDA) is a well-known analytical technique based on the measurement of isotope ratios in samples where isotopic composition has been altered by the addition of a known amount of an isotopically enriched element spike[22]. Post-column isotope dilution allows the accurate determination of elemental species even if the structure of the compounds is unknown while multiple isotopically enriched species can be applied for the evaluation and correction of species interconversion[22]. Finally, species-unspecific isotope dilution mode (SUID) is especially useful either when the structure and composition of analysed species is not exactly known or the corresponding isotopically labeled compound is not commercially available[23]. Alternatively, when the isotopically labeled compound is available, species-specific isotopic dilution mode (SSID) is applied[24]. Moreover, enriched stable isotopes are crucial to study the fate of trace elements in biological systems, using an isotope pattern deconvolution approach (IPD) for insolating distinct isotope signatures from mixtures of natural abundance tracers (endogenous) and enriched ones (exogenous)[25].

The aim of the present study is to determine the toxicological effects of Cd in mice *Mus musculus* during a 12-days exposure experiment. For this purpose, cadmium chloride was subcutaneously administered to the mice and liver, kidneys and serum investigated by a metallomic approach. The coupling SEC-ICP-MS was used to characterize the biological response to Cd exposure by the change of metallobiomolecules profiles, and additionally, selenoproteins were quantified by in series coupling of SEC with multiaffinity chromatography (AF) and ICP-MS detection using IDA for quantification. Besides, ^{77}Se (in the form of selenite) was orally administered to evaluate the antagonistic interaction between Cd/Se in the bloodstream.

Materials and methods

Instrumentation

A cryogenic homogenizer SPEX SamplePrep (Freezer/Mills 6770) was used for solid tissues disaggregation. Disaggregated 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 elements and heteroelements-containing 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 delivery system.

Mass spectrometry measurements were performed on a nano-electrospray ionization tandem mass spectrometer (API Qstar XL Hybrid system; Applied Biosystems, Foster City, CA, USA).To acquire MS/MS spectra, nitrogen was used as collision gas.

Standard solutions and reagents

All reagents used for sample preparation in the metallomic approach 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).

Standards used for mass calibration of analytical SEC columns (mass range 70-3 kDa) were: ferritin (440 kDa) (purity 95%), bovine serum albumin (67 kDa) (purity 96%), superoxide dismutase containing Cu and Zn (32 kDa) (purity > 70%), myoglobin (14 kDa) (purity > 98%), 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). The mobile phase used in SEC was 20 mM ammonium acetate (Suprapur grade) purchased from Merck (Darmstadt, Germany), which was prepared daily with ultrapure water (18 MΩcm) from a Milli-Q system (Millipore, Watford, UK). The pH was adjusted at pH 7.4 with ammonia solution, this later prepared by dilution of 20% (w/v) ammonia solution (Suprapur, Merck) with ultrapure water. The void volume was determined using blue ferritin (440kDa).

The human serum certified reference material BCR-637 was purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). Standard solutions of 1000 mg L^{-1} of Se stabilized with 5% (v/v) nitric acid Suprapur and of 1000 mg L^{-1} of Br- stabilized with 5% (v/v) nitric acid Suprapur were purchased from Merck (Darmstadt, Germany). Enriched ^{74}Se and ^{77}Se were obtained from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder and it was dissolved in the minimum volume of nitric acid (Suprapur grade) and diluted to volume with ultrapure water. The concentration of this solution was established by reverse isotope dilution analysis as described elsewhere[23].

Amicon Ultra centrifugal filters (containing a 3000 Da membrane of regenerated celulosa) from Millipore (Billerica, MA). The trypsine (EC 3.4.21.4) TPCK was obtained from Sigma-Aldrich (Steinheim, Germany) and the urea, iodoacetamide and dithiotreithol from Bio-Rad (Madrid, Spain).

Animals and exposure experiments

Mus musculus (inbred BALB/c strain) mice were obtained from Charles River Laboratory (Spain). Mice of 7 weeks of age were fed *ad libitum* with feed selenium deficient pellets. The animals were allowed to acclimate for 5 days with free access to food and water under controlled condition (temperature (25-30ºC) and a 12 h light-dark cycle) before the exposure experiment.

Firstly, 32 specimens of *Mus musculus* mice were divided into two groups, one used as control and the other exposed to Cd(II) (in the form of $CdCl_2$), using subcutaneous injection of 100 µL of a solution of 0.1 mg Cd per kg of body weight per day during a total period of exposure of 12 days. The control mice

were subcutaneously injected with 100 µL of 0.9% NaCl in ultrapure water per day during 12 days. Mice were sacrificed after the 6th and 12th day of exposure.

Secondly, 60 specimens of *Mus musculus* were divided into four groups: *GROUP A* exposed to oral administration of 0.15 mg ^{77}Se per kg of body weight per day during 15 days; *GROUP B* exposed to oral administration of 0.5 mg ^{77}Se per kg of body weight per day during 15 days; *GROUP C* and *GROUP D* both exposed to oral administration of 0.5 mg ^{77}Se per kg of body weight per day during 15 days and 0.1 mg Cd per kg of body weight per day by subcutaneous injection.

Mice were individually anesthetized by isoflurane inhalation and exsanguinated by cardiac puncture, then they were dissected using a ceramic scalpel and finally the organs 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 their use for extract preparation. 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).

Metallomic approaches based on analytical scale SEC-ICP-MS and 2D/SEC-AF-ICP-MS for the analysis of plasma, liver and kidney of mice (*Mus musculus*) exposed to both Cd and Cd/^{77}Se

Pools of organs from male mice of different groups of exposure were treated following a procedure described elsewhere[35]. Plasma collection was carried out by centrifugation (4000 g, 30 min, 4ºC) after addition of heparin (ANTICLOT) as anticoagulant for separation into plasma and red blood cells (RBCs). In addition, 10 mg of 100 mM of PMSF and 100mM of TCEP mixture were added as proteases inhibitor and reductant agents, respectively. Chromatographic conditions are previously by our research in García-Sevillano et al[26].

The retention times corresponding to the peaks of standards used for Superdex^{TM}-75 column calibration were the following: ferritin 11.5 min, bovine serum albumin (BSA) 13.7 min, superoxide dismutase containing Cu and Zn (Cu,Zn-SOD) 16.3 min, myoglobin (an iron containing protein) 18.9 min, metallothionein I containing Cd, Cu and Zn (Cd,Cu,Zn-MT1) 21.0 min, and arsenobetaine (AsB) 26.1 min.

In addition, a metallomic approach based on a multidimensional chromatography SEC-AF-ICP-MS was applied to mice plasma from mice exposed to Cd/^{77}Se during 15 days. To avoid changes in metalloproteins, plasma samples were directly injected into the column, without prior dilution. The fractionation of selenium containing proteins by two-dimensional chromatographic separations, based on SEC prior to the use of a double affinity column, was carried out following a procedure described elsewhere[27]. Briefly, it is based on in series stacking of two 5 ml HiTrap® Desalting Column (GE Healthcare, Uppsala, Sweden), which in turn are in series connected with a dual affinity column arrangement comprising by a 1 ml heparin-sepharose column (HEP-HP) (GE Healthcare, Uppsala, Sweden) and a 1 ml blue-sepharose column (BLU-HP) (GE Healthcare, Uppsala, Sweden), the components of the arrangement are interconnected by a six-way column switching valve. The HiTrap column is based on size exclusion principle allowing separate low molecular mass chemical species (MW<1000Da) from high molecular mass molecules, such as DNA, proteins or peptides (MW>5000Da), the combination of two columns increase the resolution of separation. On the other hand, HEP-HP column is able to retain selectively selenoprotein P (SeP), and numerous non-containing Se-proteins, whereas BLUE-HP column retains both SeP and selenoalbumin (SeAlb), which has been previously described[28]. The isotopes monitored were the same as those discussed in SEC-ICP-MS metallomic approach.

Identification of SeP in plasma of *Mus musculus* by AF-AEC-ICP-MS and nanoESI-QTOF-MS after tryptic digestion

The application of two different chromatographic methods was applied to confirm the association of Cd to selenoprotein P. In this sense, the same sample was analysed by anionic exchange chromatography (AEC), after AF separation, because of the presence of other proteins than SeP with high affinity to heparin (HEP-HP column) in the multidimensional approach proposed.

For this purpose, mice plasma was submitted to AF separation (Heparin-Sepharose), and Se containing fraction was collected and loaded onto an AMICON filter to preconcentrate and remove the salts. This procedure was carried out by triplicate using ultrapure water to resuspend the filter content and decrease the concentration of salts. Finally, the fraction excluded from the desalting filter was collected and lyophilized. The lyophilizate was dissolved in ultrapure water and purified by AEC (Protein-Pak DEAE 5PW 7.5 x 75 mm, 10 µm, Waters, Milford, MA, USA) using a buffer of ammonium acetate with a gradient from 2 to 500 mM at flow rate of 1.5 ml min^{-1}, and injection volume of 50 µl. After analysis, 2 mM of ammonium acetate buffer was switched on for 15 min in order to re-equilibrate the column before the next injection. A total volume of 500 µl of the fraction isolated by AEC (retention time between 10.5 min to 11.5 min) was desalted using AMICON filters and lyophilized.

The lyophilized fraction was re-dissolved with 100 µL of 6M urea and 50 mM ammonium bicarbonate (pH 8.3). An aliquot of 50 µL of this solution was separated and 5 µL of 180 mM DTT was added to reduce disulphide bonds in the proteins. After 30 min at 37ºC, 5 µL of 400 mM iodoacetamide (IAA) was added to the reaction mixture and kept it in dark at room temperature for 30 min. Finally, the sample was dissolved in 290 µL of water to reduce the urea concentration to 2 M, to retain the activity of trypsin. Then, 50 µL of trypsin (0.1 µg µL^{-1}) was added and the mixture incubated at 37 ºC overnight. The reaction was then stopped by addition of 10 µL of glacial acetic acid[26]. After tryptic digestion, the peptides were desalted, preconcentrated and purified using ZipTips C18 (Millipore, Massachusetts, USA). The TOF mass analyzer was calibrated immediately prior to sample analysis using glufib as standard. ESI-TOF-MS data acquisition was performed in positive ion mode and the MS spectra of peptides were acquired in the range 400 -1600 *m/z*. The values for ion spray voltage, electron multiplier voltage, curtain gas and declustering potencial were set to: 900 V, 2200 V, 20 psi and 90 V. Data analyses were performed using the Analyst QS software (Applied Biosystems). After recording the MS spectra, doubly charged peptide ions were selected, and MS/MS spectra obtained with collision energy of 15 V. Peptide sequences were then searched by database (NCBI) using MASCOT searching engine.

Quantification of selenium containing proteins by SEC-AF-SUID-ICP-ORS-qMS for selenium speciation of plasma from mice (*Mus musculus*) under Cd exposure.

Species-unspecific isotopic dilution analysis (SUID) was applied to quantified the concentrations of selenium containing proteins a low molecular selenium species using the procedure previously published by García-Sevillano et al[27,29].

Total determination of endogenous and exogenous Se concentration by IPD-ICP-ORS-qMS of plasma from mice (*Mus musculus*) under Cd/^{77}Se exposure

For human serum reference materials (BCR-637), samples of 100 mg were directly weighted into 10-mL glass vials. Then an appropriate amount of the ^{77}Se spike and 800 µL of a mixture 4:1 HNO_3:H_2O_2 were added. The samples were incubated during 8 hours at 80ºC and then diluted with ultrapure water to 5 g. Finally, the digested certified reference materials and samples were filtered using 0.45 µm PTFE syringe filters before the analysis by IPD-ICP-ORS-MS. Mice plasma were digested using the previous procedure but without addition of ^{77}Se spike.

For isotope ratio determination of endogenous and exogenous selenium, individual aqueous solutions enriched in 77, 74, 80 selenium isotopes at concentrations 100 ng g^{-1} were prepared to obtain the maximum signal for these masses. A solution of 5% (w/w) HNO_3 was used to correct the background level caused by polyatomic argon interferences. In addition, the corrections for the formation of SeH^+ and BrH^+ were carried out using mathematical equations, monitoring additionally the signals at masses 76, 82 and 83 (for SeH^+) and 79 and 81 (for BrH^+). On the other hand, the optimum spike to sample ratio was calculated as previously described by García-Alonso[30]. Finally, the concentration of analytes was calculated using the isotope dilution equation described previously[30]. Selenium determination in mice plasma by IPD-ICP-ORS-MS was carried out using the operating conditions summarized in Table 1. All the analyses were performed using two replicates.

Metallomic approach for mice plasma (*Mus musculus*) under Cd/^{77}Se exposure. Use of SEC-AF-IPD-ICP-ORS-qMS

To validate the SEC-AF-HPLC-IPD-ICP-ORS-MS methodology for quantitative selenium speciation in plasma samples, a human serum certified reference material (BCR-637) was used. The CRM was spiked with optimum amounts of ^{77}selenite. After online separation, the quantification of selenium in chromatographic peaks was performed by post-column isotope dilution analysis using a solution of enriched ^{74}Se for quantification of the endogenous and exogenous selenium species in plasma of mice under exposure to both enriched ^{77}Se and Cd. Finally, the IPD technique was applied to each point of chromatogram to obtain the mass flow chromatograms for endogenous and exogenous Se[25].

Table 1. Operating conditions of IPD-ICP-ORS-MS detection

ICP-MS conditions	
Forward power	1500 W
Plasma gas flow rate	15 L min^{-1}
Auxiliary gas flow rate	1 L min^{-1}
Carrier gas flow rate	0.15 L min^{-1}
Sampling depth	7mm
Sampling and skimmer cones	Ni
H_2 flow	4 mL min^{-1}
Nebulizer	Micromist (Glass Expansion)
Torch	Shield (with long life platinum shield plate)
Qoct	-18 V
Qp	-16 V

	IPD Analysis	*IPD Post column*
Points per peak	3	1
Integration time	4 s per isotope	0.3 s per isotope
Replicates	5	1
Isotopes monitored	^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, ^{82}Se, ^{79}Br, ^{81}Br and ^{83}Kr	
Dead time detector	47 ns	

Results and discussion

SEC-ICP-ORS-MS profiles from liver and plasma of mice (*Mus musculus*) under cadmium exposure

To check the presence and potential interactions of metal-biomolecules in organs of *Mus musculus* exposed to Cd the coupling SEC-ICP-MS was used, obtaining Cu, Zn and Cd-traced peaks from cytosolic fractions of liver, kidney and plasma (Fig. 1). The distribution, accumulation and transference of zinc, copper and cadmium in living organisms have been considered in detail in the literature. Several facts such as the modulation of zinc concentration by homeostatic mechanisms[31] and the importance of transport mechanisms on copper distribution[32] are relevant issues to explain the relative presence of metal-binding molecules in the different organs of exposed organisms. In relation to this, the induction of Cd and Zn-metallothioneins in mice (*Mus musculus*) exposed to higher concentrations of Cd has been reported[33], and these experimental data confirm the antagonistic interactions among Cd, Zn, Cu, as well as the differential rate of excretion of these elements from kidney/liver under increasing exposure[34].

It is well known that Cd exposure causes change in the distribution of endogenous Zn and Cu in tissues and biological fluids, since both metals play a protective role against Cd toxicity, due to their contribution in MTs induction (Fig. 1A)[35-37]. Therefore, in Fig. 1B can be seen that Cd intake causes increasing intensity of Cu-traced peak in plasma at about 67 kDa, that can be related with two important copper transport proteins in bloodstream, such as BSA and transferrin (Tf) with 67kDa and 79kDa molecular mass, respectively[38-39].

The Figs. 1C and 1D show peaks traced by Cd and Zn bound to MT fraction in liver, which increases consistently with Cd exposure. The induction of MTs is accompanied by a small increase of the intensity of Zn associated with MT fraction (Fig. 1D), fact previously observed by Srivastava *et al*[40]. The same effect was observed for Zn associated with the HMM protein fractions.

On the other hand, higher intensity of signals from Cd associated to MT is observed in kidney when compared with liver and plasma (Fig. 1E). This fact confirms the role of urine

as main route for excretion of MTs[41]. Finally, it is interesting to observe the presence of Cu-peaks (Fig. 1F) in kidney extract at about 7 kDa which is in contrast with the absence of the equivalent peak for Zn (data no shown). Decreased levels associated to this fraction are observed along the exposure time (Fig. 1F). Possibly, the difference in binding affinity of MTs for metal ions can contribute to this result, since Cd, Hg or Cu can displace Zn from metal-MT complex[42]. This hypothesis is supported by the experiment of Liu *et al*[43] that exposes rats to Cu after subcutaneous injection of Cd-MT, which results in decreasing concentration of Cd-MT in kidney, this effect is not observed when the experience is repeated with exposure to Zn.

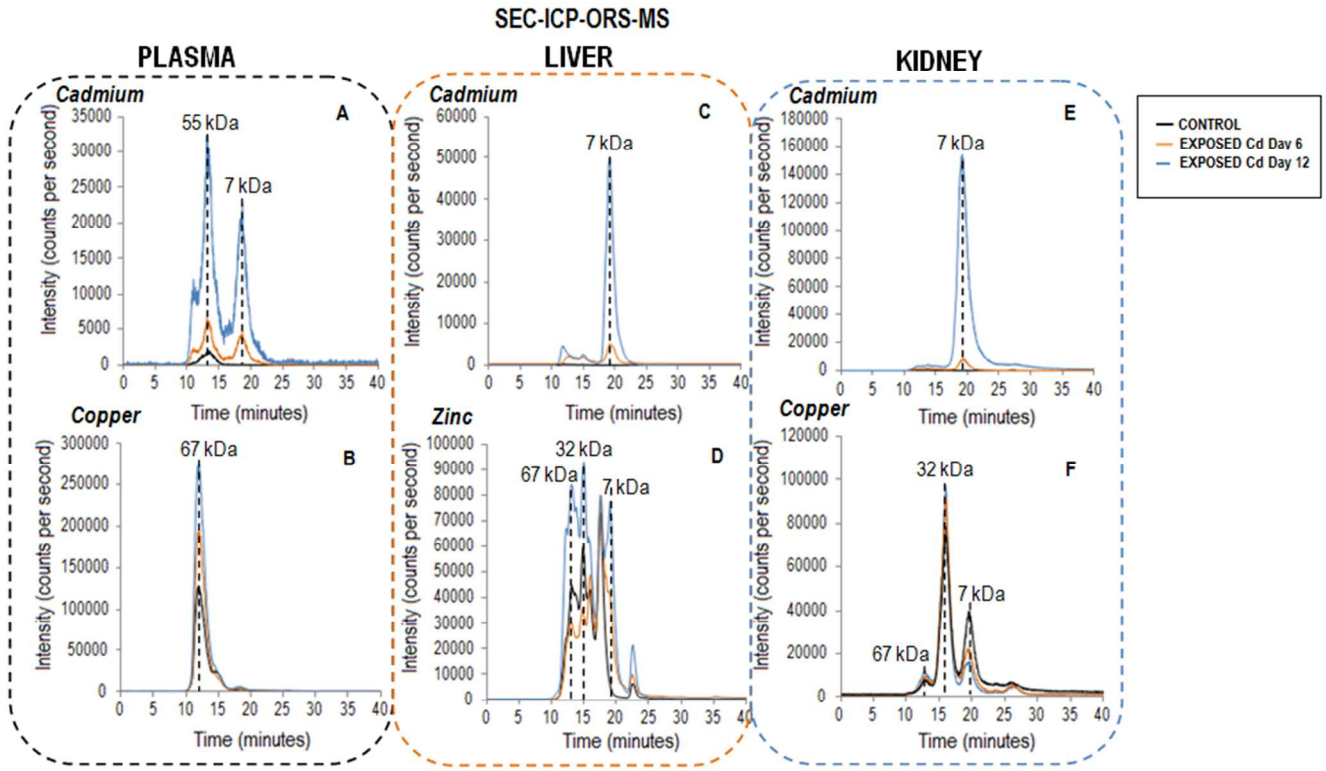


Fig.1. Up/down regulation of metal-biomolecule complexes in plasma (a, b), liver (c, d) and kidney (e, f) of *Mus musculus* exposed to cadmium assessed by molecular mass distribution with SEC-ICP-MS. Chromatographic conditions: column, Superdex™-75 (10x300x13 µm); mobile phase, ammonium acetate 20 mM (pH 7.4); flow rate 0.7 ml min^{-1}; injection volume, 20 µL.

Additionally, multidimensional chromatography 2D-SEC-AF-ICP-MS have been applied to achieve a better understanding of the function of SeP, detoxification processes and regulation of Cd in mice (*Mus musculus*) under controlled exposure to Cd using the chromatography conditions previously published by García-Sevillano et al[27]. The Fig. 2 shows the 2D-SEC-AF-ICP-MS chromatogram obtained for Cd and Se in plasma from mice exposed to Cd.

In Fig. 2 can be observed, with the use of affinity column (2D-SEC-AF-ICP-MS), that Cd is present in plasma in two remarkable peaks, one close to void volume and other more intense at retention time matching with SeP peak, which increase along the exposure time. Additionally, the chromatogram obtained by SEC-ICP-MS (Fig. 1) shows a Cd-peak with high molecular mass proteins (HMM) (about 55 kDa), corresponding to the molecular mass of SeP, and other peak eluting with the MTs fraction at 19 min, as has been previously discussed. However, peaks traced by Cd are not observed in control samples or their intensities are significantly lower (Fig. 1A and Fig. 2). Therefore, these results show that the accumulation of Cd in plasma along the exposure time in the fraction of 55kDa is related with selenoprotein P due to its high affinity for Cd[44].

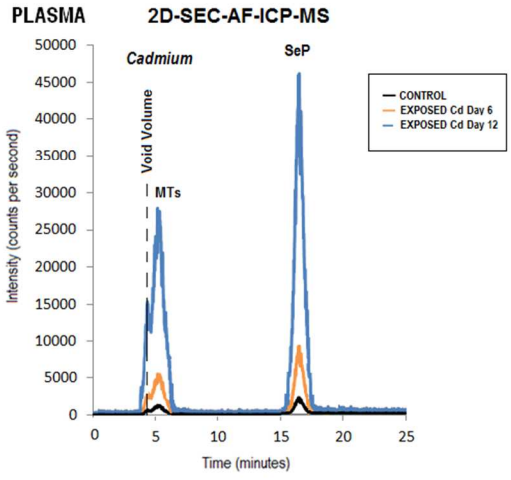


Fig. 2. Up/down regulation of Cd-biomolecule complexes in plasma of *Mus musculus* exposed to cadmium assessed by molecular mass distribution with 2D-SEC-AF-ICP-MS.

To confirm the association of Cd to SeP-peak, it was collected from plasma of *Mus musculus* mice using an affinity column as first chromatography dimension (Fig. 3A-3B). Then, the fraction was desalted, lyophilized, and analyzed by a second chromatographic dimension based on AEC (Fig. 3C). In this later chromatogram it is possible to observe a predominant peak traced by exogenous ^{77}Se and cadmium about 10 min, which can be collected with an analytical AEC column, lyophilized, desalted and submitted to tryptic digestion for identification by nESI-QqQ-TOF/MS. The mass spectrum of peptides (Fig. 4) was used for protein identification, using the doubly charged peptide ions of m/z 528.75 and 915.94 for MS/MS analysis and protein identification in MASCOT database. The results confirm the presence of SeP associated to Se and Cd containing peak, Fig. 3C.

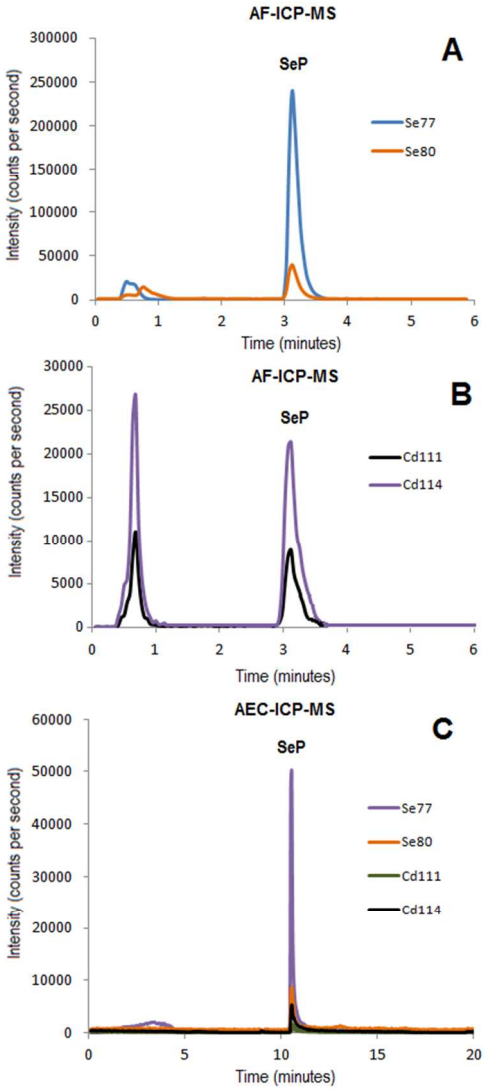


Fig. 3. A) Se-biomolecule complexes in plasma of *Mus musculus* exposed to ^{77}Se and Cd assessed by AF-ICP-MS; B) Cd-biomolecule complexes in plasma of *Mus musculus* exposed to ^{77}Se and Cd assessed by AF-ICP-MS; C) Se/Cd-biomolecule in plasma of *Mus musculus* exposed to ^{77}Se and Cd assessed by AEC-ICP-MS after AF separation.

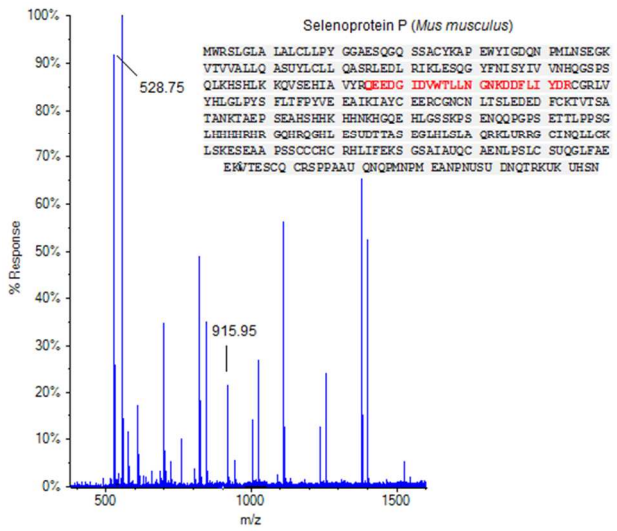


Fig. 4. Mass spectrum obtained by nano-ESI-MS of the fraction collected by AEC after tryptic digestion.

Speciation of selenium in plasma of mice (*Mus musculus*) under cadmium exposure by SEC-AF-HPLC-SUID-ICP-ORS-MS

Quantification of Se containing proteins and low molecular weight Se species has been performed in mice plasma using the proposed speciation method. Selenium concentration in selenoproteins is in good accordance with total Se concentrations determined by IDA-ICP-ORS-MS after acid digestion (table 2).

Mass flow chromatograms of selenium species in plasma from each group of Cd exposed mice are plotted in Fig. 5. It has been documented that Cd and Se interact in the body of mammals, and the co-administration of both elements reduces the toxicity of each other[45]. On the other hand, it is well known that Se level in plasma decreases under Cd exposure in rats subjected to oral administration[45].

In mammalian plasma, Se is incorporated mainly into three selenium containing proteins -SeP, eGPx (especially abundant in plasma[46]) and SeAlb. However, SeP is the unique selenoprotein that contains several selenocysteine (SeCys) and cystine (Cys) residues, which increase its availability to transport Se that can be bound to cadmium for excretion. For this reason, increased levels of SeP have been found with the exposure (Fig. 5). In addition, the peak of Cd at about 55 kDa in Fig. 1A shows a significant increase of this element with exposure, since as previously commented, SeP plays an important role in the traffic of cadmium between the organs.

Nevertheless, Se metabolites and SeAlb are required for the synthesis of selenoproteins in liver that are then transported to plasma[47]. Therefore, decreased levels of selenium metabolites have been observed in mice after the administration of Cd, as well as a little reduction of SeAlb levels along the exposure (Table 2), which support this hypothesis (Fig.5). In addition, eGPx are a family of antioxidant enzymes that reduce the

presence of lipid hydroperoxides in plasma[48], therefore, the increased levels of eGPx in plasma observed in our results can be related with the transport of this enzyme from liver to plasma to neutralize lipid peroxidation.

Table 2. Quantification of selenium species in mice plasma (*Mus musculus)* under cadmium exposure.

Selenium Species	*Plasma from Mus musculus mice*			
	CONTROL MICE (n=5)	*Cd EXPOSED MICE Day 6 (n=5)*	*Cd EXPOSED MICE Day 12 (n=5)*	**Detection limits** (LD, ng g^{-1})
GPx (ng g^{-1})	5.5±0.3	14±0.6	6.5±0.4	0.2
SeP (ng g^{-1})	132±9	146±8	154±10	0.7
SeAlb (ng g^{-1})	16.2±0.4	10.4±0.7	8.8±0.6	1.3
Se metabolites (ng g^{-1})	38.4±8	<LOD	<LOD	0.8
Se content in species (ng g^{-1})	193	170.4	169.3	-----
Total selenium[a] (ng g^{-1})	196±5	172±3	171±5	0.1

[a] Quantification of total selenium by IDA-ICP-ORS-MS.

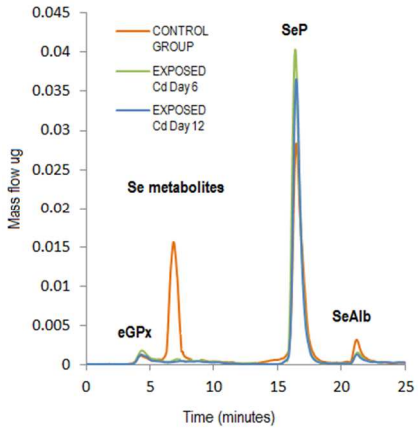


Fig. 5. Mass flow chromatograms of mice plasma after Cd exposure traced by $^{78}Se/^{74}Se$ isotope ratios using 2D-SE-AF-HPLC-IDA-ICP-ORS-qMS

Antagonistic interaction between Cd/Se in plasma mice (*Mus musculus*) under ^{77}Se and cadmium controlled exposure

When mice is only exposed to cadmium during 12 days results show increase of Se requirement in plasma (Table 2), since Se levels are reduced about 20 % along the exposure time. This fact is in agreement with previous results by other authors in rats under exposure experiments to cadmium[45], as the study of Suzuki et al[47] about *in vitro* experiments in blood, using the SeP fraction isolated from rat serum[41].

On the other hand, IPD was applied to blood samples to deep insight into the mechanism underlying the *in vivo* interactions between Cd and Se in mice bloodstream. For this purpose, ^{77}Se was orally administrated to evaluate the behavior of natural (endogenous) and enriched Se (exogenous) in mice, simultaneously exposed to cadmium, and to check the effect of this element on plasma selenoproteins biosynthesis. Thus, the analytical methodology for Se speciation was validated using a CRM of human serum spiked with enriched ^{77}selenite (Table 3).

The results show the increase of exogenous Se in bloodstream when Cd is simultaneously supplied to mice (Fig. 6A), this effect is more pronounced for higher doses of selenium (Fig. 6A, Group D). In addition, the concentration of cadmium in the bloodstream decreases when high levels of enriched Se are administrated (Table 3). Additionally, when the instrumental coupling SEC-AF-IPD-ICP-ORS-qMS is applied to serum of exposed mice, an increase in the concentration of ^{77}SeP is observed (Fig. 6B and 6C) under enriched Se exposure. Similar results were obtained by Kobayashi et al[49] by intravenous injection of enriched ^{82}selenite to rats. Nevertheless, this fact is more pronounced when Cd is simultaneously administrated (Fig. 6C), possibly induced by this toxic element. In summary, the results confirm that SeP plays a fundamental role in Cd detoxification. Correlatively, decline in the concentration of endogenous ^{80}SeP is obtained, especially when Cd is simultaneously ingested, since endogenous ^{80}SeP is consumed in the interaction with Cd but not replaced by the intake of ^{77}SeP. The Figs. 6A, Group B, support this hypothesis.

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

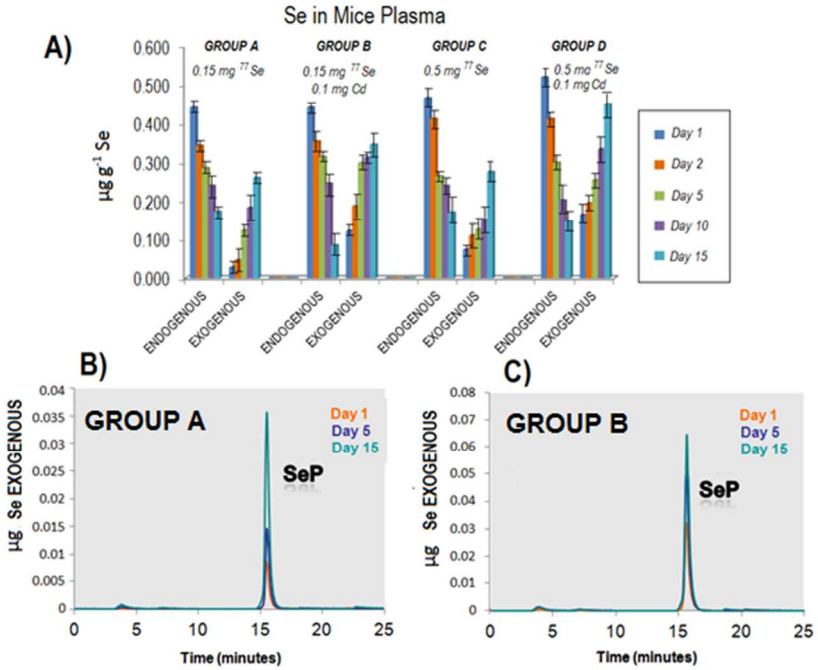


Fig. 6. A) Endogenous and exogenous selenium in plasma of mice under ^{77}selenite and cadmium administration by IPD-ICP-ORS-MS; B) Mass flow chromatogram of Se exogenous (^{77}Se supplemented) of plasma from *Mus musculus* mice exposed to 0.5 mg ^{77}Se during 15 days (GROUP C), studied by SE-AF-HPLC-IPD-ICP-ORS-MS; C) Mass flow chromatogram of Se exogenous (^{77}Se supplemented) using of plasma from *Mus musculus* mice exposed to 0.5 mg ^{77}Se and 0.1 mg Cd simultaneously during 15 days (GROUP D), studied by SE-AF-HPLC-IPD-ICP-ORS-MS.

Table 4. Quantification of cadmium in mice plasma under ^{77}Se/Cd exposure by ICP-ORS-MS and selenium species in human serum (BCR-637) and BCR-637 spiked samples (50 ng of ^{77}Se g^{-1}; as sodium selenite) by IPD-ICP-ORS-MS.

Selenium Species	Certified Human serum BCR-637 (ng g^{-1}± SD) Endogenous Se	SD (n=5)	Human serum BCR-637 (SEC-AF) (ng g^{-1}± SD) Endogenous Se	SD (n=5)	Human serum BCR-637 (SEC-AF) (ng g^{-1}± SD) Exogenous Se	SD (n=5)	Cadmium concentrations	Plasma GROUP B 0.15 mg of ^{77}Se and 0.1 mg Cd (ng g^{-1}± SD)	SD (n=5)	Plasma GROUP D 0.50 mg of ^{77}Se and 0.1 mg Cd (ng g^{-1}± SD)	SD (n=5)
EGPx	15	± 4	12	± 2	< LOD		Day 1	12.36	± 1.1	11.72	± 0.9
SeP	60	± 7	53	± 2	< LOD		Day 2	38.24	± 5.4	24.66	± 4.9
SeAlb	13	± 4	14	± 3	<LOD		Day 5	52.22	± 3.7	38.51	± 3.2
^{77}Selenite spiked	-----	-----	<LOD		51	± 3	Day 10	132.2	± 7.8	60.54	± 7.1
Se content in the species	79	± 3	79	± 2	51	± 3	Day 15	162.2	± 11	88.21	± 9.4
TOTAL Se	-----	-----	82	± 1	52	± 1					
Certified value (ng.mL^{-1})	81	± 7	81	± 7	50	-----		LOD (ng. g^{-1}) 0.012			

Conclusions

This work illustrated the high reliability of inorganic and organic mass spectrometry based on metallomic approach to study the biochemical effects induced by cadmium chloride in exposed mice and their effects on liver, kidney and plasma metals homeostasis. The application of size exclusion chromatography coupled to ICP-MS to cytosolic extracts of metabolic active organs and biological fluids from the

laboratory mice *Mus musculus* exposed to Cd, allows deciphering the changes of metal-binding biomolecules induced by this metal, such as induction of Cd-MT and Cd-transporting proteins, as well as the traffic of this element from liver to kidney mediated by the bloodstream for final excretion as Cd-MT by urine. In addition, selenium speciation in plasma by SEC-AF-HPLC-SUID-ICP-ORS-MS confirms the role of SeP in Cd detoxification/excretion and the need for selenium intake after Cd exposure. In addition, the use of enriched stable isotopes is crucial to study the fate of trace elements in biological systems, employing IPD measurements, which shows unequivocally the importance of SeP for Cd protection.

On the other hand, the study of metals interactions with proteins, such as SeP and Cd, require the combined use of complementary chromatographic separations as is the case of affinity and anion exchange chromatography. Therefore, these multidimensional metallomic approaches allow to deep insight into homeostasis and interactions of metals in living organisms involving metal-biomolecules. This provides a valuable experimental approximation to get overall information and conclusions in relation to toxicological studies.

Acknowledgements

The authors thank the projects CTM2012-38720-C03-01 (Ministerio de Economia y Competitividad-Spain), P08-FQM-03554 and P09-FQM-04659 (Consejería de Innovación, Junta de Andalucía). Miguel Ángel García Sevillano thanks the Ministerio de Educación for a predoctoral scholarship. We want to thank the group of Prof. Sanz-Medel at the University of Oviedo, in particular to Dr. M.L. Fernández-Sánchez and S. Fernández, for introducing and training in isotope dilution analysis and isotope pattern deconvolution techniques.

References

1. L.N. Kolonel, *Cancer*, 1976, **37**, 1782.
2. *G.* Bako, E.S.O. Smith, J. Hanson, R. Dewar, *Can. J. Public Health*, 1982, **73**, 92.
3. C.G. Elinder, T. Kjellstrom, C. Hogstedt, K. Andersson, G. Spang, *Br. J. Ind. Med.*, 1985, **42**, 651.
4. A. Haddow. F.J.C. Roe, C.E. Dukes, B.C.V. Mitchley, *Br. J. Cancer*, 1964, **18**, 667 .
5. S.A. Gunn, T.C. Gould, W.A.D. Anderson, *J. Nati. Cancer Inst*., 1963, **31**, 745.
6. I.M. Olsson, I. Bensryd, T. Lundh, H. Ottosson, S. Skerfving, A. Oskarsson, A., *Environ. Health Perspect*., 2002, **110**, 1185.
7. A. Åkesson, T. Lundh, M. Vahter, P. Bjellerup, J. Lidfeldt, C. Nerbrand, G. Samsioe, U. Stromberg, S. Skerfving, *Environ. Health Perspect.*, 2005, **113**, 1627.
8. A. Åkesson, P. Bjellerup, T. Lundh, J. Lidfeldt, C. Nerbrand, G. Samsioe, S. Skerfving, M. Vahter, *Environ. Health Perspect*., 2006, **114**, 830.
9. A. Åkesson, B. Julin, A. Wolk, A., *Cancer Res*., 2008, **68**, 6435.
10. M.D. Johnson, N. Kenney, A. Stoica, L. Hilakivi-Clarke, B. Singh, G. Chepko, R. Clarke, P.F. Sholler, A.A. Lirio, C. Foss, R. Reiter, B. Trock, S. Paik, M.B. Martin, *Nat. Med*., 2003, **9**, 1081.
11. M. Piasek, M. Blanusa, K. Kostial, J.W. Laskey, *Reprod. Toxicol*., 2001, **15**, 673.
12. I. Messaoudi, J. El Heni, F. Hammouda, K. Said, A. Kerkeni, *Biol. Trace Elem. Res*., 2009, **130**, 152.
13. S.J.S. Flora, J.R. Behari, M. Ashquin, S.K. Tandon, *Chem. Biol. Interactions*, 1982, **42**, 345.
14. J.L. Early et al., *Toxicol. Appl. Pharmacol*., 1981, **58**, 57.
15. M.A. García-Sevillano, R. Jara-Biedma, M. González-Fernández, T. García-Barrera, J.L. Gómez-Ariza, *Biometals*, 2013, **26**, 651.
16. T. García-Barrera, J.L. Gómez-Ariza, M. González-Fernández, F. Moreno, M.A. García-Sevillano, V. Gómez-Jacinto, *Anal. Bioanal. Chem.*, 2012, **403**, 2237.
17. J.L. Gómez-Ariza, E. Zeini-Jahromi, M. González-Fernández, T. García-Barrera, J. Gailer, *Metallomics*, 2011, **3**, 566.
18. C. Cobbett, P. Goldsbrough, *Annu. Rev. Plant Biol.*, 2002, **53**, 159.
19. M. Rob, A. Hans-Werner, D. Zbigniew, S.W. Keith, V.S. Lamzin, E.S. Cedergren-Zeppezauer. *Biochem.*, 2007, **46**, 5446.
20. J. Bettmer, M. Montes Bayón, J. Ruiz Encinar, M.L. Fernández Sánchez, M.R. Fernández de la Campa, A. Sanz Medel, *J. Proteomics*, 2009, **72**, 989
21. J.L. Gomez-Ariza, T. Garcia-Barrera, F. Lorenzo, V. Bernal, M.J. Villegas, V. Oliveira, *Anal. Chim. Acta*, 2004, **524**, 15.
22. K.G. Heumann, L. Rottmann and J. Vogl, *J. Anal. At. Spectrom.*, 1994, **9**, 1351.
23. L. Hinojosa-Reyes, J.M. Marchante-Gayón, J.I. García-Alonso, A. Sanz-Medel, *J. Anal. At. Spectrom*., 2003, **18**, 1210.
24. J.R. Encinar, D. Schaumlöffel, Y. Ogra, R. Lobinski, *Anal. Chem.*, 2004, **76**, 6635.
25. H. González Iglesias, M.L. Fernández Sánchez, J.A. Rodríguez-Castrillón, J.I. García-Alonso, J. López Sastre, A. Sanz-Medel, *J. Anal. At. Spectrom*., 2009, **24**, 460.
26. M. A. García-Sevillano, M. González-Fernández, R. Jara-Biedma, T. García-Barrera, J. López-Barea, C. Pueyo, J. L. Gómez-Ariza, *Anal. Bioanal. Chem*., 2012, **404**, 1967.
27. M.A. García-Sevillano, T. García-Barrera, J.L. Gómez-Ariza, *J. Chromatogr. A*, 2013, **1318**, 171.
28. P. Jitaru, H. Goenaga-Infante, S. Vaslin-Reimann, P. Fisicaro, *Anal. Chim. Acta*, 2010, **657**, 100.
29. M.A. García-Sevillano, T. García-Barrera, J.L. Gómez-Ariza, Anal. Bioanal. Chem., 2014, In press.
30. J.I. García-Alonso, *Anal. Chim. Acta*, 1995, **312**, 57.
31. C.T. Walshe, H.H. Sanddstead, A.S. Prasad, *Health Perspect*., 1994, **102**, 5.
32. B. Sarkar, J.P. Laussac, S. Lau S (Ed.) Raven Press, 1983, New York, pp. 23–40.
33. R. Jara-Biedma, R. González-Dominguez, T. García-Barrera, J. López-Barea, C. Pueyo, J.L. Gómez-Ariza, *Biometals*, 2013, **26**, 639.
34. S. Teodorova, R. Metcheva, M. Topashka-Ancheva, *Environ. Res*., 2003, **91**, 85.
35. C.D. Klassen, J. Liu, S. Choudhuri, *Ann. Rev. Pharmacol. Toxicol*., 1999, **39**, 267.
36. A. Martelli, E. Rousselet, C. Dycke, J.M. Moulis, *Biochimie*, 2006, **88**,1807.
37. R. Shimoda, W.E. Achanzar, W. Qu, T. Nagamine, H. Takagi, M. Mori, M.P. Waalkes, *Toxicol. Sci*., 2003, **73**, 294.
38. G.M. Bogdan, A. Samgayo-Reyest, H.V. Aposhian, *Toxicol.*, 1994, **93**, 175.
39. L. Meiling, W. Hailin, L. Xing-Fang, L.L. Arnold, S.M. Cohen, X.C. Le, *Chem. Res. Toxicol*., 2007, **20**, 27.
40. R.C. Srivastava, I. Ahmad, G. Kaur, S.K. Hasan, *J. Environ. Sci. Health A*, 1988, **23**, 95.
41. Y. Suzuki, H. Yoshikawa, *J. Toxicol. Environ. Health*, 1981, **8**, 479.
42. I. Sabolic, B. Breljak, M. Slarica, C.M. Herak-Kramberger, *Biometals*, 2010, **23**, 897.
43. X. Liu, T. Jin, G.F. Nordberg, M. Sjöström, Y. Zhou, *Toxicol. Appl. Pharmacol*., 1994, **126**, 84.
44. C. Sasakura, K.T. Suzuki, *J. Inorg. Biochem*., 1998, **71**, 159.
45. L. Said, M. Banni, A. Kerkeni, K. Said, I. Messaoudi, *Food Chem. Toxicol.*, 2010, **48**, 2759.
46. F.L. Muller, M.S. Lustgarten, Y. Jang, A. Richardson, H. Van Remmen, *Free Radic. Biol. Med*., 2007, **43**, 477.
47. L.V. Papp, J. Lu, A. Holmgren, K.K. Khanna, *Antioxid. Redox Signal*, 2007, **9**, 775.
48. R. Brigelius-Flohé, *Free Rad. Biol. Med*., 1999, **27**, 951.
49. Y. Kobayashi, Y. Ogra, K.T. Suzuki, *J. Chromatog. B: Biomed. Sci. Applications,* 2001, **760**, 73.