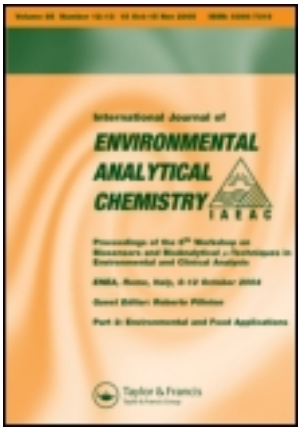

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Characterisation of metal-binding biomolecules in the clam Chamelea gallina by bidimensional liquid chromatography with in series UV and ICP-MS detection

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Characterisation of metal-binding biomolecules in the clam *Chamelea gallina* by bidimensional liquid chromatography with in series UV and ICP-MS detection

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The presence of metal-binding biomolecules has been studied in *Chamelea gallina*, a bioindicator used for environmental contamination monitoring and very popular for human consumption in the Atlantic southwest coast of Spain. This area is affected by metal pollution from mining activities, which can modify biomolecules expression in this bivalve. Total content of elements was determined by ICP-MS and revealed a remarked presence of Fe, Zn, Cu, As and Mn. A metallomics approach has been optimised for this mollusc using size-exclusion chromatography on column Superdex 30 pg HiLoad 26/60 with in series UV and ICP-MS detection. At least four fractions with molecular weight in the range 1540 to 415 Da were observed with UV detection, but the ICP-MS chromatogram showed the presence of metals of interest only in the first two fractions. The apparent molecular weights of these metal-containing fractions were from 1325 to 764 Da. The fractions containing metals compounds were collected and lyophilised for further purification of reconstituted extracts with a second orthogonal chromatographic separation using reverse phase (RP) HPLC with ICP-MS detection. Several peaks were obtained in this second dimension separation which allows the isolation of As-, Cu- and Zn-containing biomolecules.

Keywords: metallomics; *Chamelea gallina*; size-exclusion chromatography; reversed phase high performance liquid chromatography; inductively coupled plasma mass spectrometry

1. Introduction

Bivalve molluscs are extensively used as sentinel organisms for marine pollution monitoring due to both their capacity for contaminants bioaccumulation and their sessile habit. These filter-feeding specimens are widely distributed in sandy coastal areas where pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychloride biphenyls (PCBs) and dioxins induce proteins cross-reaction with CYP1A-antibodies (a subfamily of the CYP system). *Chamelea gallina* has been frequently used as a bioindicator [1] because

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this is a fairly common clam species on the Atlantic coast and constitutes an important economic and alimentary resource. The possible relationship between experimental PCB exposure and oxidative stress [2] as well as the relation with the levels of metallothionein [3] were considered in this clam, and changes in protein expression induced by exposition to four model environmental pollutants: Aroclor 1254, copper (II), tributyltin (TBT) and arsenic (III) were checked [1,4]. Therefore, environmental proteomics have been proposed as a more comprehensive non-biased approach to assess environmental stress situations, as an alternative to traditional biomarkers [5–7].

On the other hand, metals bound to biomolecules and in particular to proteins play key roles in molecular structure stabilisation, catalytic activities and metal transport in cells, but metals may also trigger mechanisms to control their concentration by binding to specific detoxifying biomolecules. Biomolecules that bind metals and metalloids represent a substantial portion of molecules involved in the cell machinery and metabolism, and the identification of a metal cofactor in a protein can greatly facilitate its functional assignment and position in cellular pathways.

Therefore, the study of metallobiomolecules using metal tags for their analysis and identification represents a good alternative in modern bioanalysis. Heteroatom-tagged analysis is based on the use of multidimensional chromatography coupled to ICP-MS for detection, which instrumental platform is becoming a promising tool for massive analysis of these types of biomolecules. This experimental approach can be combined with tandem mass spectrometry identification of cell metallobiomolecules traced by chromatography-ICP-MS constituting the so-called metallomics [8–14]. This approach enables new possibilities in the analysis of cells, tissues or biological fluids, and can be used for environmental stress assessment [5]. The metallomics approach has been applied to characterise metal-binding proteins in many living organisms and tissues, such as *Escherichia coli* [15], liver [16] and biological material [17,18], and it has also been considered in different issues relating to health, food and environmental bioanalysis.

Food analysis is one of the most promising fields in metallomics used in food characterisation, food authentication and provenance, and identification of bioactive metallobiomolecules in food supplements. In this sense, the presence of selenium species in nuts has been characterised by HPLC coupled to ICP-MS [19] and electrospray mass spectrometry (ESI-MS) [20], and yeast enriched in selenium has been studied by SEC-CE-ICP-MS [21]. In addition, the presence of medium and low molecular weight selenium fractions from yeast was confirmed using SEC-HPLC-ICP-MS and MALDI-TOF-MS to identify several selenoproteins in this matrix [22]. The determination of Mn compounds was performed in liver by capillary electrophoresis (CE) coupled to ICP-MS [23] and in human milk by size-exclusion chromatography (SEC)/anion-exchange-ICP-MS [24]. Finally, the fractionation of Ni, Cu, Zn and Mn in nuts [25,26] and soybean flour by SEC-ICP-MS [27] was reported. Multi-elemental fractionation studies were also performed in premature human milk [28,29] using SEC-ICP-MS, and the combination of total metal presence and metal profiles in pine nuts was proposed to establish the unique provenance of this nut from several production areas in Spain and Portugal [30].

The metallomics approach has also been applied in the assessment of environmental issues, environmental metallomics [5], that represents a good alternative to the well-established environmental biomarkers. Several examples can be cited relating to this topic: (a) heavy metal pollution in Brazilian aquatic ecosystems has been monitored by the study of MTs present in white catfish and pearl cichlid using size exclusion-fast protein

liquid chromatography (SE-FPLC) followed by anion-exchange (AE)-FPLC, both coupled with the ICP-(Q)MS [31]; (b) different eel liver MT isofoms have been used as environmental biomarkers coupling anion exchange chromatography with 'postcolumn' isotope dilution analysis (IDA) to ICP-MS [32]; (c) exposition experiments of mussels to Se and Cd has been monitored by analysis with SEC, FPLC and RPC chromatography coupled to a ICP-MS detector, that allows the speciation of metallothionein-like proteins [33]; (d) the map of water-soluble arsenic species in an oyster test reference material has been obtained by using multidimensional (size-exclusion-anion-exchange-cation-exchange) liquid chromatography with ICP-MS detection applying ES-MS-MS to confirm the structure of two arsenosugars [34]; (e) the characterisation of Cd complexes in rat liver tissue was performed using semi-preparative size-exclusion chromatography, followed by a preconcentration step by lyophilisation and desalting for final analysis by micropore reversed-phase HPLC with parallel ICP-MS and ESI-MS detection, which confirmed the existence of two major metallothionein (MT) isoforms [35].

The aim of this paper is to develop a metallomics approach suitable for major metal-binding biomolecules present in cytosolic fractions from the clam *C. gallina*. The metallomics scheme considers the use of bidimensional liquid chromatography with in series UV and ICP-MS detectors to trace molecular weight distribution patterns of metallobiomolecules containing elements such as Fe, Cu, Zn, Mn and As. Semi-preparative SEC coupled to ICP-MS has been used for first dimension fractionation of metallobiomolecules which provide enough metallobiomolecule load for further purification by RP-HPLC-ICP-MS. Problems arising from metallobiomolecules in different fractions and subfractions that progressively reduce the load of targeted species in the successive chromatographic separations and analysis are considered and discussed. The work can be considered as a preliminary study to establish metal profiles in this clam that can be affected by pollution episodes. In this way metals profiles in the bioindicator *C. gallina* can be used as an alternative or complement to conventional biomarkers in environmental stress assessment.

2. Experimental

2.1 *Chemicals and standards*

For total elements determination, the standard solutions were prepared by dilution of a multi-element standard (1000 mg l^{-1} in 1 M HNO_3) obtained from Merck (Darmstadt, Germany). Nitric acid (65%), hydrochloric acid (37%) and perchloric acid (70–72%) of SuprapurR grade (Merck, Darmstadt, Germany) were used for the mineralisation of the samples. Sodium hydroxide was supplied also by Merck. A solution containing Li, Y, Tl and Ce (10 µg l^{-1} each) in 2% nitric acid (Agilent Technologies, USA) was used to tune the ICP-MS relating to the sensitivity, resolution, percentage of oxides and doubly charged ions. The mobile phase solution for SEC was 50 mmoL l^{-1} tris (hydroxymethyl) aminomethane (pH 8.0) (Sigma Aldrich, Steinheim, Germany). A solution containing 0.002 M of ethylenediaminetetraacetic acid disodium salt dihydrate (Sigma-Aldrich, Steinheim, Germany) was used to clean the SEC columns. Dithiothreitol (DTT) and phenylmethylsulphonyl fluoride (PMSF) were supplied by Sigma-Aldrich (Steinheim, Germany) and trifluoroacetic acid (TFA) was obtained from Fluka (Steinheim, Germany).

The molecular weight calibration standards (metallothionein I, gastrin I, vitamin B12, ferritin, aldolase, bovine serum albumin, α-chymotrypsinogen A, ribonuclease A and Blue Dextran) for the SEC columns were supplied by Amersham Biosciences (Uppsala, Sweden). Ultrapure water (18 $\mu\omega$ cm) from a Milli-Q Gradient System (Millipore, Watford, UK) was used throughout. Standard arsenic compounds used for speciation were arsenic trioxide (Panreac, Barcelona, Spain), sodium arsenate (Merck, Darmstadt, Germany), sodium methylarsonate (Carlo Erba, Milano, Italy), dimethylarsinic acid (Sigma, St. Louis, MO, USA) and arsenobetaine (Sigma-Aldrich, Steinheim, Germany). HPLC mobile phase for arsenic speciation was prepared from KH_2PO_4 and $K_2HPO_4 \cdot 3\ H_2O$ (Merck). $NaBH_4$ (Panreac) solution stabilised in NaOH (Merck) was prepared daily. $K_2S_2O_8$ was purchased from Aldrich (Milwaukee, WI, USA).

2.2 *Samples and extracts preparation*

Samples of *Chamelea gallina* were obtained from a local market. The clams were rinsed with Milli-Q water, their shells were discarded and the whole bodies were pooled and stored at -20°C. The frozen tissues of clams were grounded in a mortar with liquid nitrogen, and subsequently homogenised with an Ultraturrax mixer and stored at -20°C until analysis. Portions of frozen sample (about 0.3 g) were weighed and the cytosolic extract was obtained with 10 mL of Milli-Q water containing 0.1 mM of phenylmethylsulfonyl fluoride (PMSF) and 0.1% dithiotreithol (DTT) in an ultrasonic bath for 30 min. The extracts were centrifuged at 2000 rpm for 30 minutes, using a refrigerated centrifuge at 4°C. This procedure was repeated three times. The combined extracts were lyophilised and stored at -20°C for no longer than two weeks.

2.3 *Instrumentation*

Semi-preparative size-exclusion chromatography (SEC) was carried out in both Hiload 26/60 Superdex 30 Prep grade for separations in the molecular weight range lower than 10,000 Da (low molecular weight, LMW) and Superdex 200 Prep grade for separation in the molecular weight range 10,000–600,000 (high molecular weight, HMW) (all from Amersham Biosciences, Uppsala, Sweden), columns volume 319–330 mL. An AKTA-Prime system (pump and UV detector at 280 nm) (Amersham Biosciencies, Uppsala, Sweden) was used as eluent delivery system, equipped with a 2 mL sample loop.

Reversed-phase HPLC was performed in a C18 column Prodigy (Phenomenex, USA), 250×4.6 mm i.d. with 5 µm and 100 Å particle and pore sizes, respectively. Arsenic speciation was performed in a Hamilton PRP-X 100 column, 250×4.1 mm i.d. (Hamilton, Reno, NV, USA). An 1100 Series pump (Agilent Technologies, USA) was used in both cases.

Samples were homogenised with Ultra-Turrax (IKA Werke, Staufen, Germany). Lyophilisation was carried out using lyophiliser BenchTop, Madrid, Spain. A refrigerated centrifuge Eppendorf 5804R (Eppendorf Iberica, Spain) was used throughout. Filters for sample preparation were Millex HV, pore sizes 0.45 µm (Millipore, Belford, USA). The pH was adjusted with a pH meter Micro-pH (Crison Instruments, Barcelona, Spain). Elemental detection was performed using both a Model 4500 ICP-MS system (Hewlett-Packard, USA) to obtain metal-biomolecules profiles and a 7500ce ICP-MS system (Agilent Technologies, Tokyo, Japan) for quantitative measurements.

2.4 *Procedures*

2.4.1 *Quality control of the analyses*

To overcome problems related to contaminations, losses, stability of the species during analysis as well as metallic species identification difficulties, several considerations were taken into account and have been described elsewhere [19]. Briefly, the use of metal pieces (stainless steel) was avoided since this increases signal noise and causes contamination. All the glass material had been previously washed with 20% (v/v) HNO_3 and then with Milli-Q water.

The columns were cleaned between two consecutive injections with one column volume buffer monitoring the signal in the UV and ICP-MS detectors. Each week the SEC columns were washed with one-half to one column volume of 0.5 M NaOH to remove most non-specifically adsorbed proteins to the gel. For a more rigorous cleaning, the columns were washed with four column volumes of 1 M NaOH (removal of hydrophobic proteins and lipoproteins) followed by four column volumes of Milli-Q water. Finally, one-half column volume of 30% isopropyl alcohol (removal of lipids and very hydrophobic proteins) followed by two column volumes of Milli-Q water were passed. The possible retained elements onto the SEC columns were eliminated by passing one column volume of 0.002 M EDTA solution. After cleaning, columns were equilibrated with at least two column volumes of mobile phase until the UV baseline stabilisation was achieved before applying next sample.

In order to obtain an accurate measurement of the relative molecular weight from *Chamelea gallina* extracts, both columns were calibrated with appropriate calibrants. For LMW column, the calibration standards were: metallothionein I (Mr 7000), gastrin I (Mr 2126), vitamine B12 (Mr 1352). For the HMW column, the calibration standards were: ferritin (Mr 440,000), aldolase (Mr 158,000), bovine serum albumin (Mr 67,000), α-chymotrypsinogen A (Mr 25,000) and ribonuclease A (Mr 13,700). The void retention time was determined in the LMW and HMW columns using bovine serum albumin (Mr 67,000) and Blue Dextran 2000 (Mr 2,000,000), respectively.

2.4.2 *Determination of selected toxic and trace elements content by ICP-MS*

The whole bodies of *Chamelea gallina* (without shells) were used for screening total content of selected toxic and trace elements in the sample. Three portions of 1.0000 g of the sample were each weighed in open PTFE vessels. The procedure for the mineralisation of the samples was based in a wet-digestion in open PTFE vessels with 11 mL of a HNO_3, 4 mL of HCl and 5 mL of $HClO_4$. Afterwards, the PTFE vessels were placed in a heated sand bath until dryness, and then the addition of acids and heating was repeated. The obtained recoveries for the elements determined were in the range 90–115% for all the elements except for arsenic (76%) and the relative standard deviations (% RSD, $n = 3$) from 5 to 9%.

Some important elements from an environmental and toxicological point of view were monitored: As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn. The ICP-MS (Model 7500 ec) conditions used for analysis are shown in Table 1.

2.4.3 *Arsenic speciation*

Analysis of arsenic species in whole *Chamelea gallina* tissues was performed using the coupling HPLC-(UV-*photoxidation*)-HG-ICP-MS as described elsewhere [36].

Table 1. ICP-MS, SEC and HPLC instrumental conditions.

SEC conditions	
Columns	Hiload 26/60 Superdex 30 Prep grade, LMW Hiload 26/60 Superdex 200 Prep grade, HMW
Resolution range	Mr < 10,000 (LMW); 10,000–600,000 (HMW)
Mobile phase	50 mmoL l^{-1} tris (hydroxymethyl) aminomethane (pH 8.0)
Flow rate	2 mL min^{-1}
Injection volume	2 mL
HPLC conditions	
Column	C18 Prodigy 250 × 4.6 mm, 5 μm
Mobile phase	0.1 % TFA gradient in methanol
Flow rate	0.75 mL min^{-1}
Injection volume	200 μL
ICP-MS conditions (Agilent 4500)	
Forward power	1177 W
Plasma gas flow rate (Ar)	15.0 dm^3 min^{-1}
Auxiliary gas flow rate (Ar)	0.87 dm^3 min^{-1}
Carrier gas flow rate (Ar)	0.975 dm^3 min^{-1} (with a 6% of O_2, for mobile phase containing methanol)
Sampling depth	6.8 mm
Cones	
for SEC	nickel
For HPLC	platinum
Acquisition mode	TRA[a]
Integration time	0.5s
Dwell time	0.1s per isotope
Monitored isotopes	^{75}As, ^{63}Cu, ^{57}Fe, ^{55}Mn, ^{66}Zn
ICP-MS conditions (Agilent 7500)	
Forward power	1500 W
Plasma gas flow rate (Ar)	15.0 dm^3 min^{-1}
Auxiliary gas flow rate (Ar)	1.0 dm^3 min^{-1}
Carrier gas flow rate (Ar)	1.1 dm^3 min^{-1}
Hydrogen collision gas	
Flow rate gas	3.4 mL min^{-1}
Monitored isotopes	^{57}Fe, ^{78}Se
Helium collision gas	
Flow rate gas	3.2 mL min^{-1}
Monitored isotopes	^{75}As, ^{111}Cd, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{55}Mn, ^{60}Ni, ^{66}Zn, ^{208}Pb
Sampling depth	6.8 mm
Cones	platinum
Acquisition mode	TRA[a]
Integration time	0.5s
Dwell time	0.1s per isotope

[a]Time resolved analysis.

Briefly, 1.0 g of *Chamelea gallina* was extracted twice with 20 mL of methanol-water (1 : 1) mixture under sonication for 20 min. After centrifugation, supernatants were decanted and methanol removed under nitrogen stream. Both extracts were gathered and water was added to a final weight of 20.0 g. The total recovery of arsenic considering all the species accounts over 95%. Speciation of arsenic species in the final extract was performed by using HPLC with a strong anion exchange column and 25 mM phosphate buffer (pH 5.8)

Table 2. HPLC gradient elution conditions.

Time (min)	0.1% TFA in methanol (%)	0.1% TFA in Milli-Q water (%)
0	0	100
5	5	95
10	10	90
55	45	55

as mobile phase for arsenic species separation and hydride generation (HG) inductively coupled plasma mass spectrometry (ICP-MS, 7500 ec model) for detection. For the analysis of non-hydride generation species such as arsenobetaine (AsB) an optional on-line photooxidation unit was included before hydride generation. This photooxidation unit consists of the 8 m long Teflon tube wrapped around a low pressure Hg lamp that irradiates to 254 nm. In addition, a strong oxidant, 2% m/v $K_2S_2O_8$ in 2% NaOH at a flow rate of 0.3 mL min^{-1}, was also introduced on-line for species oxidation to As (V).

2.4.4 *Analysis of cytosolic extracts by SEC-UV-ICP-MS*

A sample of 50 mg of lyophilised clam extract was dissolved in 4 mL of water and filtered through 0.45 μm filter. A volume of 2 mL of sample was injected onto the preparative size exclusion column HiLoad 26/60 Superdex 30 Prep (Hiload 26/60 Superdex 200 Prep). The elution was performed with 50 mmol l^{-1} tris (hydroxymethyl) aminomethane (pH 8.0) with a flow rate of 2 mL min^{-1}. The UV detection was performed at 280 nm. Elemental detection was carried out using the ICP-MS system. The SEC-UV-ICP-MS coupling was performed connecting the outlet of the UV detector to the nebuliser inlet of the ICP-MS using a PEEK tube with cross-section of 0.8 mm. The instrumental conditions are given in Table 1. Size-exclusion chromatography was repeated and fractions containing metals of interest were collected (as is described in 3.3). Fractions were freeze-dried and stored at −20°C until analysed.

2.4.5 *Analysis of SEC fractions by RP-HPLC-ICP-MS*

Freeze-dried fractions obtained from size-exclusion chromatography were dissolved in 500 μL of Milli-Q water. A sample of 200 μL was injected into the HPLC column with a flow rate of 0.75 mL min^{-1}. The mobile phase was 0.1% TFA in water (A) and in methanol (B) with a gradient described in Table 2. The spray chamber of ICP-MS detector was cooled to 2°C and oxygen (6%) was added to the carrier gas to minimise plasma instability caused by the presence of methanol in the mobile phase The detectors used were UV (at 280 nm) and ICP-MS (the operational conditions are collected in the Table 1).

3. Results and discussion

3.1 *Total metal levels and arsenic speciation*

The total elements content of the sample determined by ICP-MS is shown in Table 4. The results revealed a remarked presence of iron (26.04 μg g^{-1}), which is present at levels higher than other essential elements, such as Zn (9.52 μg g^{-1}) and Cu (6.50 μg g^{-1}). It is

Table 3. Selected toxic and trace elements content determined by ICP-MS[a].

Element	Concentration $\pm \sigma$ ($\mu g\,g^{-1}$)	RSD (%)
As	1.91 ± 0.07	4
Cd	0.07 ± 0.01	9
Co	0.17 ± 0.01	6
Cr	0.55 ± 0.03	5
Cu	6.50 ± 0.25	4
Fe	26.04 ± 0.91	3
Mn	1.30 ± 0.04	3
Ni	0.18 ± 0.06	6
Se	0.35 ± 0.00	1
Zn	9.52 ± 0.95	10
Pb	0.28 ± 0.01	2

[a]Average of three replicates.

remarkable the presence of about $2\,\mu g\,g^{-1}$ of arsenic (wet weight), which is in accordance to previous studies in this coastal area [37,38]. The presence of arsenic in this bivalve is not surprising; because the Atlantic southwest coast of Spain receives inputs of pyrite metals from an important mine area. Further, speciation analysis of arsenic reveals that this element is mainly presents in *Chamelea gallina* as organic species, possibly due to the transformation of toxic inorganic arsenic by the bivalve metabolism to organic species, mainly arsenobetaine that represents about 85% of arsenic content in the bivalve. The non-toxic character of arsenobetaine points out the occurrence of active detoxification mechanisms in *Chamelea gallina*. Other toxic elements such as Cd, Pb and Ni were also detected at lower concentrations ($<0.2\,\mu g\,g^{-1}$).

3.2 *SEC-UV fractionation profiles*

The SEC-UV chromatograms of *Chamelea gallina* extracts are shown in the Figure 1(a) LMW column and 1(b) HMW column. The chromatographic run times were extended to 250 min to ensure the elution of all fractions. However, no peaks were observed beyond 210 min in any column. As can be seen in Figure 1(a), most of fractions obtained with LMW column using UV detection, are located between 120 and 180 min, where we can distinguish four peaks. However, it is also possible to observe two low intensity peaks next to the void volume. Figure 1(b), for HMW column, shows the six peaks previously mentioned, but resolution is poorer, and the main fraction represented by four peaks cluster were located between 150 and 190 min (elution volumes from 300 mL to 380 mL, respectively).

3.3 *SEC-ICP-MS fractionation profiles*

The SEC-ICP-MS analysis of the extracts from *Chamelea gallina* using the LMW column showed that elements of interest (iron, zinc, copper, arsenic and manganese) can be traced at the first and second peaks of SEC-UV chromatogram (Figure 1(a)), with retention times between 115 min and 145 min that can be associated to molecular weight between 1358 and 705 Da (Figure 2). Using these retention times as reference, four fractions were collected

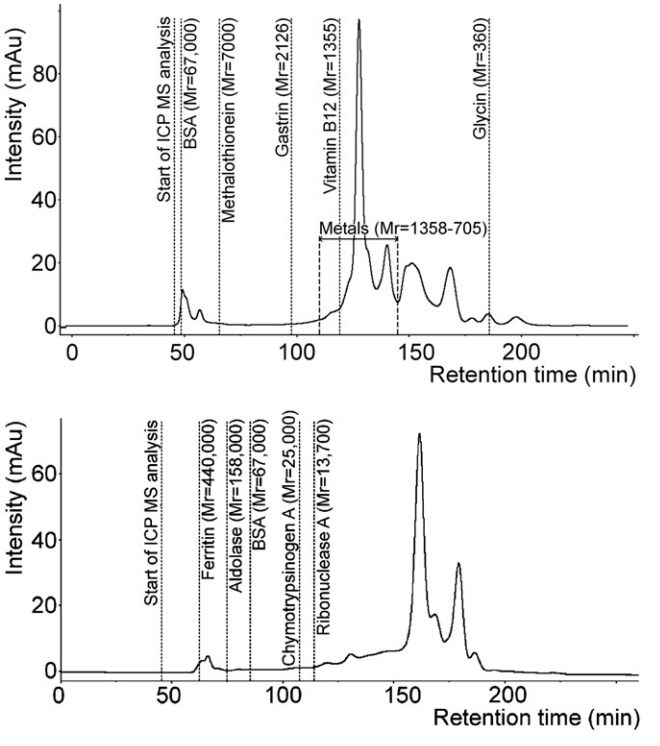

Figure 1. SEC-UV chromatograms of *Chamelea gallina* extract: (a) LMW and (b) HMW.

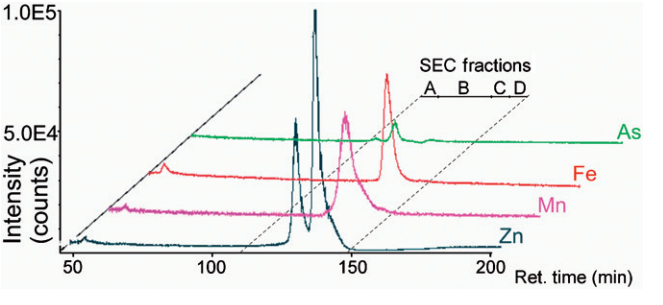

Figure 2. SEC-ICP-MS chromatograms of *Chamelea gallina* extract.

in which the relative concentration of metals is different: (a) fraction A (115–120 min; 1358–1200 Da) contained most of As species; (b) fraction B (120–135 min; 1200–863 Da) with remarkable presence of Zn and Fe; (c) fraction C with Mn and Fe (135–140 min; 863–779 Da); and (d) Fe and Zn in Fraction D (140–145 min; 779–705 Da). Therefore, Fe and Zn are distributed among several fractions. The metal profiles in Figure 2 show two Zn-containing peaks while only one peak is detected for As, Fe and Mn. The Fe-containing fraction traced by ^{57}Fe has a molecular weight in the range 940–764 Da,

^{55}Mn is detected in the fraction at 1010–789 Da, and ^{75}As at 1325–1131 Da. Finally, ^{66}Zn-contaning peaks are located at 1176–874 Da and 874–764 Da. However, Cu-containing compounds were not detected by SEC-ICP-MS, although total concentration of Cu in the sample was significant ($6.50 \pm 0.25\,\mu g\,g^{-1}$), which points to the unsuitability of the SEC-ICP-MS system to trace Cu-containing molecules present in these samples. This fact is confirmed by the detection of important signals of Cu when a RP-HPLC-ICP-MS separation is subsequently applied to SEC-ICP-MS fractions (as is described in 3.4).

3.4 *Second chromatographic dimension by RP-HPLC-ICP-MS of SEC fractions*

The four fractions A to D isolated by SEC with the LMW column (see Section 3.3) were analysed by RP-HPLC with ICP-MS detection. This second chromatographic purification intends isolate metallospecies present in the SEC fractions, because the orthogonal combination of SEC and RP-HPLC markedly increase the resolution of global separation. Results can be seen in Figure 3, which shows that relative peak intensities and chromatographic profiles change from fraction A to D. The RP-HPLC-ICP-MS analysis of SEC fraction A (Figure 3(a)) showed that Cu is the element most abundant in the subfraction A1 and Mn in the subfraction A2. Arsenic can be associated to the isolated peak that constitutes subfraction A3. In fraction B (Figure 3(b)) Cu is the element predominant in subfraction B1 and Zn in subfraction B3. Fractions C and D exhibit similar metal profiles with predominance of Cu in subfractions C1 and D1 and Zn and Mn in subfractions C2 and D2. The combined use of SEC and RP-HPLC fractionation is shown in Table 4.

3.5 *Mass balance of metals in the different fractions*

In order to carry out a quality control of the analytical procedure, the concentration of several metals was measured in the SEC fractions collected from the preparatory column. The data for Zn, Fe, Mn and As are shown in Table 5. The recoveries are over 75%, which confirm the suitability of the chromatographic separation for fractions isolation. A similar mass balance can not be applied to the RP-HPLC fractionation due to the small volume of fraction recovered.

4. Conclusion

The molecular size distribution patterns of elements in *Chamelea gallina* has been obtained for the first time which contributes to the knowledge of metallome of this mollusc, very useful in studies related to environmental concern and used for human consumption. The use of an ICP-MS detector allows high sample throughput since multi-elemental profiles are obtained in only one run, although the combined use of SEC and RP chromatography with this detector involves a long period of analysis. This fact represents a drawback if the approach is intended to be used routinely in environmental studies involving many samples. However, this approach can be very useful to obtain massive information about metallospecies related with environmental stress in a selected number of individual or polled samples. Therefore, the approach based on SEC-UV-ICP-MS is a powerful tool in the metallomics appraisal of *Chamelea gallina* or other bioindicators, and the resulting

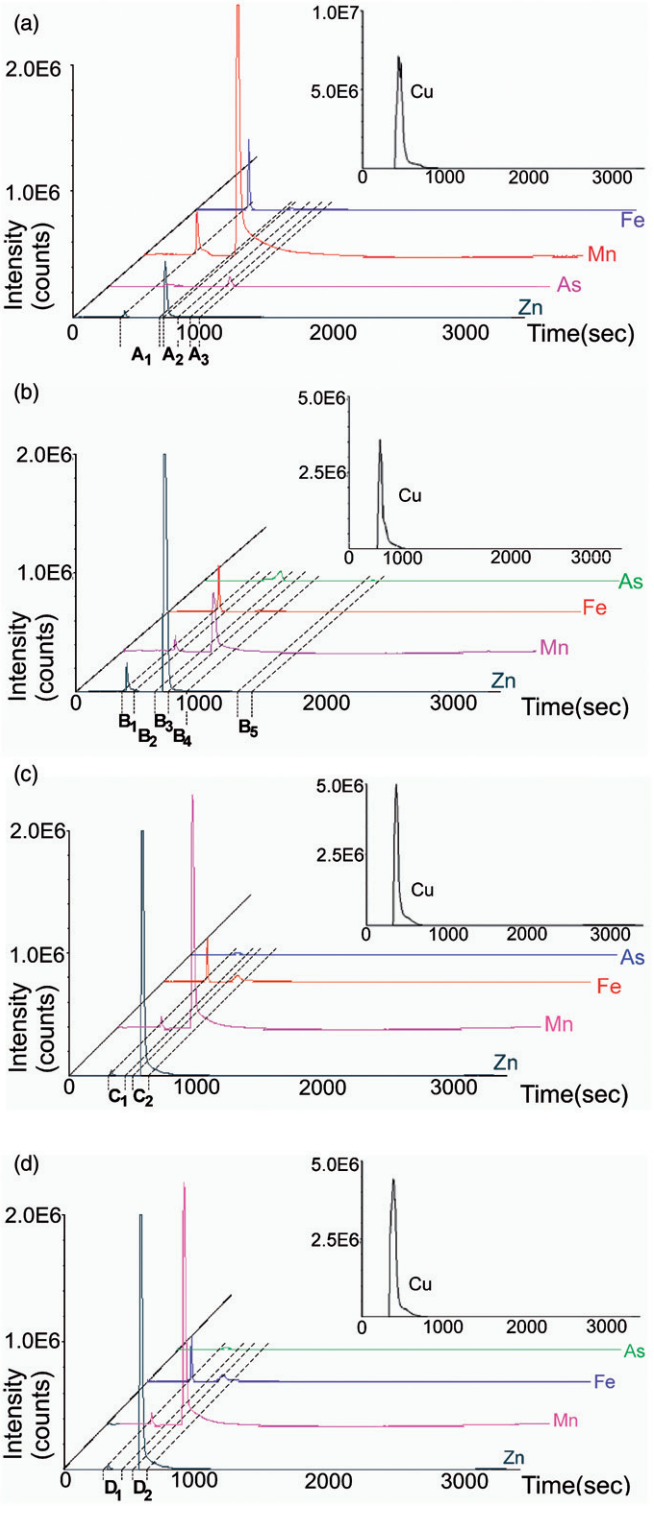

Figure 3. RP-HPLC-ICP-MS chromatograms from of SEC fractions: (a) fraction A; (b) fraction B; (c) fraction C; (d) fraction D.

Table 4. Metalbiomolecule fractionation by combined SEC and RP-HPLC.

SEC fraction	Molecular weight	RP-HPLC	Fraction time (s)	Elements in the fraction
Fraction A	1358–1204	A1	360–660	Fe, Zn, Cu, As, Mn
		A2	690–800	Mn, Zn
		A3	880–950	As
Fraction B	1204–863	B1	350–440	Mn, Fe, Cu, Zn, As
		B2	450–560	As
		B3	600–660	Zn, Mn
		B4	700–840	As
		B5	1230–1340	As
Fraction C	863–779	C1	310–440	Mn, Fe, Cu, As
		C2	500–620	Mn, Fe, Zn
Fraction D	779–705	D1	320–460	Mn, Fe, Cu, Zn, As
		D2	550–660	Mn, Fe, Zn

Table 5. Mass balance monitoring of metals (% efficiency).

	Fractions	Fe	Zn	As	Mn
Total metal in the sample (μg^{-1})		26.04	9.52	1.91	1.30
Metals (μg^{-1}) in SEC fractions	A	4.2	1.3	1.2	–
	B	18.3	5.7	0.3	0.3
	C	1.1	1.1	–	0.7
	D	–	0.7	–	0.1
Total extraction recovery (%)		90.6	92.4	78.5	84.6

fractionation profiles of elements make possible a deep insight into the interactions of elements with biomolecules as well as their possible connection with the metabolic pathways in these species. The use of an ICP-MS detector allows outlining multi-elemental profiles in complex biological tissues and it is an excellent metal-tags tracer for further studies based on molecular weight spectrometric elucidation of targeted metallospecies. Therefore, additional studies are necessary for identification of individual metallobiomolecules isolated in the bidimensional SEC-RP-ICP-MS fractions using MALDI-TOF and/or n-ESI-Qq-TOF-MS, combined with tryptic digestion of extracts, when necessary.

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