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Mercury and selenium binding biomolecules in terrestrial mammals

(*Cervus elaphus* and *Sus scrofa*) from a mercury exposed area

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Highlights

1. Metallomic study in high trophic terrestrial animals from a Hg-polluted area.
2. Sample preparation optimization for Hg and Se-biomolecules extraction in animal tissues.
3. Hg and Se binding biomolecules investigated by size exclusion chromatography-ICP-MS.

Abstract

Mercury (Hg) is likely bound to large biomolecules (e.g. proteins) in living organisms, and in order to assess Hg metabolic pathways and possible toxicological effects, it is essential to study these Hg containing biomolecules. However, the exact nature of most metal binding biomolecules is unknown. Such studies are still in their infancy and information on this topic is scarce because the analysis is challenging, mainly due to their lability upon digestion or extraction from the tissue. New analytical methods that allow complex Hg-biomolecules to be analysed intact are needed and only few very recent studies deal with this approach. Therefore, as an initial step towards the characterization of Hg containing biomolecules, an analytical procedure has been optimized using size-exclusion chromatography (SEC) with inductively coupled plasma mass spectrometry (ICP-MS) detection. We applied this technique to elucidate the distribution and elution profile of Hg and Se, and some physiological important elements such as Fe, Ni, Zn and Cu, to assess metal binding profiles in liver and kidney samples of red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) who roam freely within the largest Hg mining district on Earth, Almadén in Spain. Elemental fractionation profiles of the extracts from different tissues were obtained using two different SEC columns (BioSep-SEC-S2000 GL 300-1 kDa and Superdex 75 10/300 GL 70-3 kDa). Similar profiles of Hg were observed in red deer and wild boar; however, significant differences were evident for liver and kidney. Moreover, the profiles of Se showed a single peak at high-medium molecular weight in all investigated tissues, while co-elution of Hg with Fe, Ni, Zn and Cu was observed.

Introduction

Metals play an important role in the chemistry of life [1]. Paradoxically, some metals are essential in very small amounts, but toxic in high amounts, and some are simply toxic, while others can be assimilated by living organisms even at high levels [2]. The biological processes that depend on metal ions vary both in function and in complexity. For instance, metals such as Zn, Fe, Cu or Ca are not only important catalysts of enzymatic reactions but are also involved in the control and activation of several fundamental biological processes, such as in maintaining the structure and function of cell membranes and walls [3]. Moreover, these metal ions can either be part of the structure of proteins, constituting so-called metalloproteins, or be low molecular weight biomolecules (metallo-biomolecules) which are involved in numerous biological processes that occur in cells and tissues [4, 5]. Therefore, it is important to understand which metalloproteins and/or metallo-biomolecules are involved in the different biological processes to achieve a better understanding of the modes of transport and distribution of metals. For this purpose, metallomics is a relatively new discipline, which tries to answer questions related to the role, assimilation, transport and storage of metals in biological systems.

One special case of interest is mercury (Hg) because it is a very toxic metal with no known biological function. Mercury exists in different chemical forms, both organic and inorganic, but the most worrying is monomethylmercury (MeHg). Monomethylmercury is an important neurotoxin that can bioaccumulate and biomagnify (through the aquatic food chain) leading to dangerous levels for the general population and it is a question of major concern for human health. However, little is known about the mechanism responsible for both accumulation and toxic effects. Mercury in living organisms can adversely affect the intracellular biochemistry even at very low concentrations,

depending on its different chemical forms. This element strongly binds to sulphur- or selenium- containing proteins/biomolecules in biota [6, 7]. Therefore it is very likely to be bound to higher molecular weight compounds in tissues of biota, which also act e.g. as a detoxification mechanism in plants [8]. The study of the Hg-Se interaction is particularly interesting, due to the detoxifying effect of Se with respect to Hg, which has been described within a variety of different organisms, tissues and ecosystems [9]. One of the most important groups of proteins that form coordination complexes with Hg are selenoproteins, which include selenocysteine [10]. Different selenoproteins have been identified and studies have associated Hg mainly with the fractions containing selenoprotein P (SelP, 57 kDa) and glutathione peroxidase (GSH-Px, 80-90 kDa) [11]. In marine animals, a detoxification mechanism involving MeHg has been proposed via selenoprotein interaction, whereby Hg and its various species are transported and/or stored via biomolecule complexes [12]. Other studies have also found the formation of a complex between Hg, Se and SelP in bioaccumulation processes [13]. However, the exact nature of most metal binding biomolecules is unknown, and the identification of biomolecules containing Hg is complicated.

To perform metallomic analysis of biological samples, separation techniques coupled to mass spectrometry are often used. The most favoured tool for screening analysis for the presence of metal-biomolecule complexes in biological samples has been the coupling of size-exclusion liquid chromatography (SEC) and inductively coupled plasma mass spectrometry (ICP-MS) [14, 15]. However, this analysis is not straightforward and precautions must be taken, especially at the stage of sample preparation. Main issues here are the loss of the metal from the biomolecule, metal-exchange with the matrix, change in the molecular configuration and loss of function, or even the rupture of the

entire biomolecule. Therefore, it is necessary to develop methods which preserve the entire biomolecule-metal complex, from sample preparation through to analysis.

To study Hg containing biomolecules in higher trophic terrestrial animals, we used the unique opportunity of animals living on or in the vicinity of the recently closed Almadén mine (Ciudad Real, Spain), which is the largest and oldest Hg mine in the world. Mercury contamination through mining activities in this area has been reported in numerous studies in soils, water, sediments, plants and atmosphere [16-19] but are scarce for terrestrial animals. The area surrounding the Almadén mining district is inhabited by wildlife such as red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*). Mercury exposure of these animals can occur through the food chain (plants or roots) or direct exposure to Hg contaminated soil or water, and, especially in the case of wild boar, through the consumption of small animals by digging in the ground. Berzas et al.[20] carried out a study in this area to evaluate the environmental impact of Hg pollution and to identify adverse effects and defence mechanisms. In their study, tissues of liver, kidney, bones, testis and muscle harvested from red deer (n=168) and wild boar (n=58) were analysed for total Hg and Se. The highest Hg concentrations were found in kidney (0.46 and 0.51 nmol/g d.w. for red deer and wild boar, respectively) and liver (0.065 and 0.115 nmol/g d.w. for red deer and wild boar, respectively). Also, liver and kidney tissues were investigated for Hg species (inorganic Hg and MeHg) and the interaction between total Se and individual Hg species was evaluated by correlation studies [21]. The majority of Hg in kidney was inorganic Hg (more than 81% in all cases) while in liver the percentage of MeHg was higher with highest values of 46 % in some samples. Similar patterns of Hg species distribution were found for both red deer and wild boar. Concerning Hg and Se interaction, similar relationships were found in

kidney for both inorganic Hg and MeHg in both types of animals, but in livers the patterns were different between animals.

These tissue samples are ideally suited for a metallomic approach, and may provide important information regarding biomolecules containing Hg and other elements, such as Se. The main objective of this work is to carry out a screening analysis for the presence of Hg-biomolecules in higher trophic terrestrial animal (red deer and wild boar) tissues (liver and kidney), as an initial step to achieve a better understanding of the transport and distribution of Hg and Se and other essential elements in biota. Size exclusion chromatography coupled to ICP-MS was used to investigate the binding patterns of Hg, Se and other essential elements in liver and kidney and sample preparation was carefully optimized to preserve the biomolecule-metal entity.

Experimental

Instrumentation. Metal biomolecule analysis was performed using SEC coupled to ICP-MS. In this work, we have used two independent SEC-ICP-MS systems and the experimental conditions are given in Table S1. Two SEC columns of different molecular mass range were used: Biosep-SEC-S2000 GL from Phenomenex (separation range of 300-1 kDa, hHigh Molecular Mass, HMM) and Superdex 75 10/300 GL from Sephadex (separation range 70-3 kDa, Low Molecular Mass, LMM).

A UV-Vis spectrometer (Model V-630, Jasco Corporation) for protein detection was used with the HMM-SEC column.

A centrifuge (Digicen 21R Orto Alresa, Madrid, Spain) was used for the separation of the supernatant (soluble fraction) after biomolecule extraction from the tissues.

A freeze drier (Flexi-Dry MPTM, FTS, USA) was used to dry the samples before total element analysis.

For total Hg and Se determination, samples of tissues, extracts (soluble fraction) and residue (insoluble fraction) were digested using a Milestone Ethos plus Microwave system (Milestone, Monroe, USA), equipped with temperature and pressure control. Total analysis was carried out with ICP-MS (Thermo Electron Model XSeries II) using collision cell mode with H₂/He as the collision gas.

Reagents. All reagents were of analytical-reagent grade. Proteins used for the mass calibration of the SEC columns were ferritin (470 kDa), β -amilase (200 kDa), alcohol dehydrogenase (150 kDa), albumin from bovine serum (66 kDa), carbonic anhydrase (29 kDa), citochrome c (12.4 kDa) and vitamin B12 (1.4 kDa). These were all purchased from Sigma Aldrich (Sigma Aldrich, UK).

Tris [tris(hydroxymethyl)-aminomethane] and HCl (37%, v/v) were obtained from Acros organics (Acros, UK) and Scharlab (Scharlab, Spain), respectively.

Standard solution of 1000 $\mu\text{g mL}^{-1}$ of Hg was prepared by dissolving mercury (II) chloride (99 %) (Panreac, Spain) in 5% HNO₃ (69 %, Merck, Germany). Standard solution of 1000 $\mu\text{g mL}^{-1}$ of Se, Rh and Tl were purchased from Inorganic Ventures (Christiansburg, USA).

H₂O₂ (35%, v/v suprapure) and HNO₃ (65% v/v, suprapure) from Scharlab (Spain) were used to digest tissue samples, extracts (soluble fraction) and the residual insoluble fraction.

Ultrapure water (18.2 M Ω ·cm) was obtained from an ELGA Purelab[®] Ultra water purification system (Veolia Water Technologies, UK).

Sampling and storage. Red deer and wild boar (subadults, >2-3 years old) were hunted in “monterias” (large driven hunts), which usually last 3 hours, at the end of which the animals were butchered. General characteristics of the samples are given in Table S2. Liver and kidney samples were taken from the animals on-site, portioned into plastic zip-lok bags and stored in a portable cooler at 4 °C prior to being transferred into a -80 °C freezer, within 2 hours. Full information about the sample locations is shown in Figure S1. Additional information about the area can be found in Berzas et al. [20].

Total, extractable and residual Hg and Se quantification

To determine the total content of Hg and Se, samples (liver and kidneys from both red deer and wild boar) were lyophilized and homogenized. Approximately 0.2 g of lyophilized sample was microwave digested with 8 mL of 69% HNO₃ and 2 mL of 35% H₂O₂. The digestion was carried out starting from room temperature and ramped to 200 °C in 5 min, held at 200 °C for 5 minutes, then ramped to 220 °C in 5 minutes, and held at 220 °C for a further 5 minutes before it was allowed to cool to room temperature. Digested samples were diluted to an appropriate final volume with ultrapure water. Extracts were kept refrigerated at 4 °C until analysis by ICP-MS. Blanks were processed in each batch of digestions. Solutions used for calibration were prepared from stock standards with 1000 $\mu\text{g mL}^{-1}$ of each element. Rhodium and Thallium (15 $\mu\text{g L}^{-1}$) were used as internal standards for Se and Hg, respectively. The limits of detection (LODs, in ng g⁻¹ dry weight) were 0.49 ng g⁻¹ for Hg and for 0.53 ng g⁻¹ for Se using the Thermo

Electron XSeries ICP-MS. Certified Reference Material (CRM) bovine liver NSC ZC 71001 (NSC, Beijing, China) was analysed and recovery was obtained at $96.9\pm 2.2\%$ for Hg and $99.9\pm 0.5\%$ for Se, $n=3$.

After performing the extraction of biomolecules from kidney tissue of red deer in the optimization section, the extracts (soluble fraction) and the residue (insoluble fraction) were lyophilized and then they were analysed using the conditions described above.

Analysis of biomolecules by SEC-UV-Vis and SEC-ICP-MS

Approximately 0.2 g of tissue (fresh frozen at $-80\text{ }^{\circ}\text{C}$) was de-frosted and immediately homogenised with 400 μL Tris buffer (100 mM Tris-HCl, pH 7.4), using a Potter homogeniser (Schütt homogen plus, Germany). This cell disruption was performed at a cold temperature ($4\text{ }^{\circ}\text{C}$), and then the sample was kept in an ice bath for extraction for 1 hour. The soluble components were thereafter separated by centrifugation for 1 hour at 15,500 g at $4\text{ }^{\circ}\text{C}$, to minimise the risk of degradation. The extracts (soluble fraction) were diluted twofold with the mobile phase and passed through a $0.45\text{ }\mu\text{m}$ nylon filter and were analysed by SEC(HMM)-UV-Vis and SEC(HMM and LMM)-ICP-MS (Table S1).

Size vs retention time calibration of SEC columns

The mass calibration for each column was determined by ICP-MS using metal containing enzyme standards by monitoring the corresponding metal involved: ^{25}Mg (β -amilase), ^{57}Fe (Ferritin, citocrome c), ^{59}Co (Vitamin B12) and ^{66}Zn (Albumin, carbonic anhydrase, alcohol dehydrogenase). Retention time (RT, in minutes) was plotted vs

molecular mass (MW, in kDa) and correlation was determined as $MW = -5.295RT + 115.8$ with $r^2=1.0$ and $MW = 13235e^{-0.561RT}$ with $r^2=0.956$ for LMM- and HMM-SEC columns, respectively.

Results and discussion

a) Optimization of biomolecule extraction conditions

To perform the metal binding molecule analysis in biological tissues, an extraction step is necessary to liberate the molecules from the matrix, and transfer it into the separation and detection system. This step is critical, as the most important goal is to keep the metal-biomolecule integrity, avoiding the rupture of metal-biomolecule bonds or the exchange of metals [22, 23]. Therefore, it is necessary to optimize variables in the stage of sample preparation, such as the extraction agent used, the concentration of extracting agent, extraction volume, homogenization time and pH of the medium, to obtain a maximum extraction efficiency whilst keeping the integrity of the metal-biomolecule entity.

As a first step, we decided to use Tris-HCl buffer as extracting agent, because it is a soft extractant and has often been used to extract the soluble fraction of proteins [23]. A Potter homogenizer was used to facilitate cell disruption and extraction. The amount of sample was approximately 0.2 g of tissue throughout.

The extraction efficiency of Hg and Se into the soluble fraction was performed using a kidney sample from a red deer with known total Hg and total Se concentration content (Hg 0.039 ± 0.001 and Se 0.735 ± 0.007 $\mu\text{g/g}$ (dry weight)). The total Hg and Se content in the extracts and the residues were analysed by ICP-MS after microwave digestion.

The extraction efficiency, expressed as percent, was calculated as the soluble metal relative to the total metal content.

The evaluated variables for extraction of the homogenate were Tris-HCl concentration (30, 50, 80, 100 and 120 mM), pH (from 7.0 to 7.6), extractant volume (300, 400, 600 and 800 μ L) and homogenization time (between 20 and 60 seconds). In Table 1, the extraction efficiencies obtained with different Tris-Cl concentrations (from 30 to 120 mM) are shown. Additional information about the optimization of extraction is given in the Supplementary Material.

The optimised conditions were: 0.2 g of sample, 400 μ L of Tris-HCl (100 mM, pH 7.4) and a homogenization time of 30 s, with an extraction efficiency of 19.1 ± 0.1 % for Hg and 5.1 ± 0.1 % for Se, respectively, and were applied in all further experiments.

b) Analysis of extracts by SEC coupled with UV-Vis spectroscopy and ICP-MS

In order to establish distribution patterns in biomolecules depending on size, extracts of kidney and liver of both red deer and wild boar were analysed by SEC with UV-Vis or ICP-MS detection.

b.1. SEC(HMM)-UV-Vis profiles for red deer and wild boar kidney

The extracts of red deer and wild boar kidney were analysed by SEC(HMM)-UV-Vis at 280 nm, with a linear separation range between 300 and 1 kDa. An example for the SEC(HMM)-UV-Vis chromatographic profile of red deer kidney extract is shown in Figure 1. The elution profile shows peaks along the entire separation range indicating the presence of proteins in the extract.

Most of the extracted biomolecules were found in the high-medium molecular weight region (>300-40 kDa). The first peak (major peak) corresponds to high molecular weight biomolecules (>300 kDa). The second and third peaks corresponded to 98 and 45 kDa, respectively. The following peaks represent compounds with low molecular weight (below 12 kDa).

b.2. SEC(HMM)-ICP-MS element profiles: evaluation in terms of reproducibility and stability

In a first step, the reproducibility of the sample preparation in terms of biomolecule profiles was evaluated. For this, four independent extracts were prepared from the same red deer kidney sample and analysed on the same day with the HMM-SEC column. For all extracts, the chromatographic profiles of all elements monitored were found to be very similar in both profile and peak areas. As an example, the Se-biomolecule profiles are shown in Figure S2 (Supplementary material) and the coefficient of variation for the peak areas was 1.8 % (n=4). Therefore, we concluded that the reproducibility in the sample preparation was adequate.

A study of the stability of the extracts was performed to evaluate possible changes to the extracted metal compounds with time. For this, extracts of kidney were analysed by SEC(HMM)-ICP-MS on day 0, 7 and 14. The overall chromatographic profiles for all the elements did not change during the time studied, but a decrease in peak intensity was observed. As an example, the elution profiles for Se compounds in red deer kidney extracts are shown in Figure S3 (Supplementary material). Thus, we may infer that while quantification is compromised, however the nature of the still existing metal-biomolecules remains unchanged.

b.3 Evaluation of Hg binding biomolecules with LMM and HMM-SEC-ICP-MS

Analysis of kidney extracts from red deer and wild boar was performed using both LMM and HMM-SEC-ICP-MS. The molecular size distribution profiles of Hg with the HMM-SEC column are shown in Figure 2. In both cases (red deer and wild boar), the chromatographic profiles obtained were similar and the Hg-containing fractions were detected in molecular mass range from 250 to 20 kDa. Then, the extracts of kidney were analysed using the LMM-SEC column, the Hg-containing biomolecules were detected in the molecular mass range from 70 to 20 kDa (Figure 3). The patterns obtained for Hg with two columns reflect the binding of this element to high-medium molecular mass protein, probably due to high affinity of Hg to sulfhydryl groups of proteins [6, 7]. This corresponds to the Chen et al. [9] finding, who reported that Hg was redistributed to the high molecular weight proteins (80-100 kDa) in porcine kidney.

The extracts of liver of both animals were analysed using the LMM-SEC column (Figure 4). Mercury was detected over a large molecular weight range, and a big shoulder corresponded to medium molecular weight (70-40 kDa), followed by a fraction with apparent relative molecular weight of 30 kDa and another fraction was detected in the molecular region below 20 kDa. A clear difference in chromatographic profiles and overall Hg signal intensity between liver (Figure 4) and kidney (Figure 3) extracts of both animal tissues can be seen. The overall Hg signal intensity in kidney is around 3% of what was detected in liver extracts, although the total overall content of Hg is higher in kidney (0.991 ± 0.019 and 0.195 ± 0.002 $\mu\text{g/g}$ for red deer and wild boar, respectively) than in liver (0.010 ± 0.001 and 0.046 ± 0.003 $\mu\text{g/g}$ for red deer and wild boar, respectively). This can be interpreted in two ways: either Hg is bound very tightly to

e.g. cell membranes in kidney, and is thus unavailable for the very soft extraction used, or Hg may be bound to a very small molecule that is not eluted from the column.

There is very little information in the literature about Hg-biomolecules in terrestrial mammal tissues and the comparison is very difficult because different analytical conditions are used. Thereby, in the investigation on Hg-biomolecules in liver porcine from Hg-contaminated and non-Hg contaminated areas Chen et al. [9] found that Hg was mostly bound to the proteins with high molecular weight (>70 kDa) and minority with medium molecular weight (40-60 kDa) [9]. On the other hand, Cabañero et al. [23] investigated the binding patterns of many trace elements in liver from chicken supplemented with Hg (II), MeHg and Se. In the study, they observed that Hg was associated to high and medium molecular weight proteins (<300-45 kDa), along with low molecular weight species (<10 kDa). In addition, they found that the UV (280 nm) and S chromatogram were similar to Hg chromatogram, suggesting that Hg attaches to sulphhydryl groups and therefore, binds to proteins. As well as, García-Sevillano et al.[24] studied by SEC-ICP-ORS-MS (SEC column mass range between 10 and 600 kDa) the biochemical response of mouse that had been exposed to Hg and Se for 10 days. The protein binding pattern distribution of Hg in liver showed a first Hg-containing peak that matches with dead volume of the column indicating high molecular mass protein (>440 kDa) and a second and third peaks match with bovine serum albumin (66 kDa) and superoxide dismutase (32 kDa) standards, respectively.

Therefore, the results found in the present work suggest that Hg preferential binds to high molecular weight biomolecules in kidney and high-medium in liver and they are similar to those found in the literature.

b.4 Study of Hg-Se binding biomolecules with HMM and LMM-SEC-ICP-MS

The molecular mass distribution profiles of Se obtained for red deer and wild boar kidney and liver extracts using HMM- (Figure 2) and LMM-SEC-ICP-MS (Figures 3 and 4) show a single chromatographic peak. Using SEC(HMM)-ICP-MS, a Se-peak was observed in red deer and wild boar kidney extracts with molecular mass close to 80 kDa. However with LMM-SEC-ICP-MS, in kidney and liver extracts the Se-peak was close to 60 kDa. According to literature, this Se-peak could correspond to selenoproteins, such as GSH-Px or SelP [11, 24, 25].

Glutathione peroxidase (80-90 kDa) is an antioxidant in the cell cytosol of mammals and it can be used as a bioindicator of the Se status in the organism [25]. Daun et al. [11] investigated the role the Se compounds in muscles from different animal species (chicken, duck, turkey, ostrich and cattle) by SEC-ICP-MS using a column with mass resolution ranged between 10 and 600 kDa. The patterns of Se distribution among animal species were similar. Four Se-peaks were found in the muscle extracts, where second and third Se-peaks could correspond to GSH-Px and selenoprotein W (9.5 kDa), respectively.

On the other hand, SelP is mainly produced in heart, lung and liver but its function is not confirmed. One function could be as selenocysteine transporter for selenoproteins synthesis [25]. Moreover, the SelP could play a significant role in Hg-Se interaction. Thus, in 1997 Suzuki's group [26] identified the plasma protein to which a structurally unknown Hg-Se entity bound as SelP. Later, the same group concluded [13, 27] that Hg-Se entities containing around 100 atoms of Hg and Se bind to SelP. Moreover, several authors have proposed that Se sequentially binds to Hg and SelP in the bloodstream, to form a non-toxic complex [28-30]. It seems that the redistribution of Hg

in the organism or the reduction in the absorption of Hg takes place by the formation of an Hg-Se complex with SeIP.

In our work is interesting to observe in all cases that the Se-peak co-eluted with the Hg-containing fraction at high-medium molecular weight. Comparisons of results obtained for Hg-Se biomolecules in terrestrial animal tissues are limited, as the bibliography is quite scarce. To our knowledge a previous work on Hg-biomolecules in similar terrestrial animal tissues was carried out by Chen et al. [9], who studied the Hg and Se binding biomolecules in porcine kidney and liver. The cytosolic fraction was measured by SEC (separation range 150-4 kDa). They report that Hg and Se were redistributed into the high MW proteins (80-100 kDa) in the Hg-exposed group and only a minor fraction was found in the soluble cytosolic fraction, which is in agreement with the main findings of our work. However, it should be pointed out that a comparison between these two studies is difficult because very different experimental conditions were used (i.e. different extracting reagent, centrifugation or SEC column range). Nevertheless, Naganuma et al. [31] investigated the mechanism of formation of high molecular weight compounds containing Hg and Se in rabbit blood. They demonstrated that reduced glutathione (GSH) is essential for formation of the Hg-Se complex.

The present study provides new information about approximate molecular weight of Hg-Se compounds in kidney and liver of higher trophic terrestrial mammals such as red deer and wild boar. However, further studies are required for the identification of the selenocompounds in the peaks using complementary techniques such ESI-MS-MS.

b.5. Study of other metal biomolecules by HMM and LMM-SEC-ICP-MS

The use of ICP-MS detection allows the simultaneous measurement of different elements; therefore the physiologically important elements Fe, Ni, Cu and Zn were recorded along with Hg and Se.

Figure 5 and Figure S4 depict the molecular mass fraction profiles of Fe, Ni, Cu and Zn obtained using SEC(LMM)-ICP-MS and SEC(HMM)-ICP-MS, respectively. In general, elution profiles were similar between red deer and wild boar but not between liver and kidney, and exhibit a higher intensity signal in profiles of liver.

In kidney (Figure 5a and 5c) we observed a Hg-containing fraction that co-eluted with the Fe-, Cu-, and Zn-containing fractions in the high-medium molecular weight (70-40 kDa), but does not coincide with Ni (similar results were found using SEC-HMM column). Nevertheless, in liver (Figure 5b and 5d) it is observed that the samples have an important Fe-containing fraction in the molecular mass range from 70 to 50 kDa and another important Ni-containing fraction below 25 kDa coinciding with Hg-biomolecules. Some important fractions were observed in the molecular mass range from 70 to 35 for Cu and Zn coinciding with Hg biomolecules and albumin standard (66 kDa). Fractions of Cu and Zn were also observed below 20 kDa where important biomolecules could elute, such as metallothioneins (MTs) (~7-6 kDa), rich in cysteine [32, 33]. They are associated both with detoxication of heavy metals (e.g. Cd or Hg) and with homeostasis (e.g. Zn and Cu) [33]. Metallothioneins are able to bind Hg and seem to play a protective role in Hg toxicity in mammals [34, 35]. Das et al. [36] detected MTs in livers and kidneys of different marine mammals species and investigated the participation of this protein in metal detoxification such Hg and Cd. They suggested that those animals can mitigate at least in part the toxic effect of Hg and Cd through binding to MTs. González-Fernández [37] investigated by SEC-ICP-MS the molecular mass distribution of various metals in liver and brain from free-living and laboratory mice

and they observed that the molecular mass distribution profiles of Cu and Zn were remarkably abundant in liver tissue from mice captured in the polluted area. They showed also a fraction of about 7 kDa, which matches with MT standard. On the other hand, laboratory mice were exposed to Cd to investigate about the metabolic effects of this contaminant. The Cu-, Zn- and Cd- chromatograms obtained from liver extract showed a peak chromatographic, whose retention time similar to the MT standard, which increased considerably with the Cd dose administrated to mice.

Conclusions

The work presented here on biomolecules binding Hg and other elements in higher trophic terrestrial animal tissues is a first approach, but it revealed interesting results about the molecular size distribution patterns in liver and kidney of red deer and wild boar from a Hg polluted area. To perform this study, it was necessary to develop an extraction procedure for biomolecules which keeps their integrity prior to SEC-ICP-MS analysis. This extraction procedure has been optimised and checked in terms of total and extractable content. The reproducibility of sample preparation in terms of elemental chromatographic profile has also been demonstrated. In general, a great likeness is found between the chromatographic profiles obtained for red deer and wild boar in the same tissue type, whereas the chromatographic profiles between kidney and liver are very different. In general we found that Hg and Se are bound to high-medium molecular weight biomolecules.

It must however be stressed that biomolecule analysis is a difficult task, and many precautions must be taken in order to prevent metal exchange or breaking of metal-biomolecule bonds, and to date no method exists that can address all these issues. An

isotope tracer experiment performed by Pedrero et al. [38] investigated Hg biomolecules in dolphin liver. This study was performed on lyophilised tissue, which was subsequently spiked with isotopically labelled Hg species, and proves that Hg is easily interchangeable revealing the most important problem of such studies: for our measurements, the biomolecules need to be in an accessible form, and any Hg mobilised through sample preparation may subsequently react with other biomolecules into stable complexes. A metal exchange as such cannot be ruled out either. This is stressed by our investigations on phytochelatin complexes in plants [39] where oxidation (formation of S-S bonds) or metal replacement, e.g. by “free” Se in the extract [40] was evidenced after lyophilisation [41].

In addition further studies are necessary for the identification of these compounds, especially to establish a molecular understanding of the Hg-Se antagonism. Different sampling extraction procedures are necessary keeping the integrity of biomolecule. Complementary separation techniques applied sequentially are needed for identifying all different metal-containing biocompounds [42, 43], as shown in the approach of defining the complete metallo-proteome [2]. Confirmation of the molecular nature of metal-biomolecule cannot be solved with one method only, but complementary methods, like molecular organic mass spectrometry or NMR must be employed.

The knowledge of how exactly toxic metals like Hg are bound to biomolecules in biota will open up new insights into the understanding of uptake, transport and toxic behaviour in organisms. This field of research is at present still under-investigated and we hope that this work will be another step towards a more holistic understanding, which will be beneficial for our modern society.

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Dedication

The authors dedicate this paper to the memory of the late Prof. J.J. Berzas Nevado with all our love and respect.

Legends to figures

Figure 1. SEC(HMM)-UV-Vis chromatogram (280 nm) of red deer kidney extracts. The values 200, 66 and 12.4 correspond to the molecular weights in kDa of calibration standards.

Figure 2. SEC(HMM)-ICP-MS chromatograms of ^{202}Hg and ^{78}Se for red deer (a) and wild boar (b) kidney extracts. The values 200, 66 and 12.4 correspond to the molecular weights in kDa of calibration standards.

Figure 3. SEC(LMM)-ICP-MS chromatograms of ^{202}Hg and ^{82}Se for red deer (a) and wild boar (b) kidney extracts. The values 66 and 1.4 correspond to the molecular weights in kDa of calibration standards.

Figure 4. SEC(LMM)-ICP-MS chromatograms of ^{202}Hg and ^{82}Se for red deer (a) and wild boar (b) liver extracts. The values 66 and 1.4 correspond to the molecular weights in kDa of calibration standards.

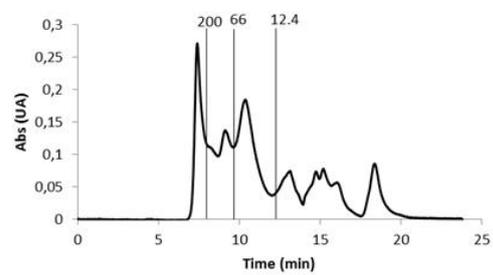
Figure 5. Superimposed SEC(LMM)-ICP-MS multielemental chromatographic profiles (Hg in a different scale): (a) wild boar kidney, (b) wild boar liver, (c) red deer kidney, (d) red deer liver. The values 66 and 1.4 correspond to the molecular weights in kDa of calibration standards.

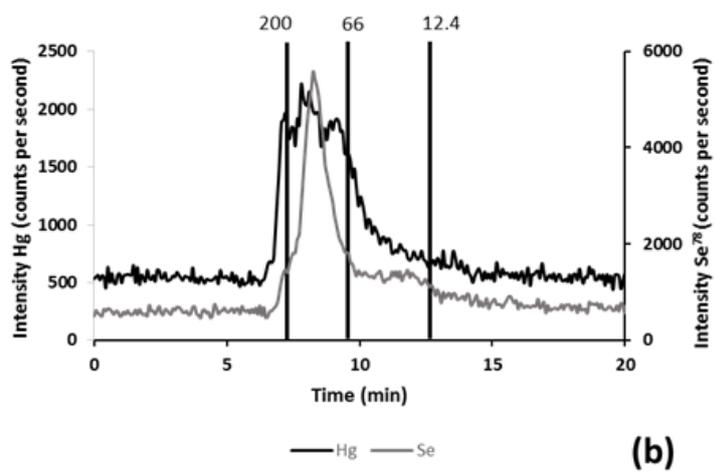
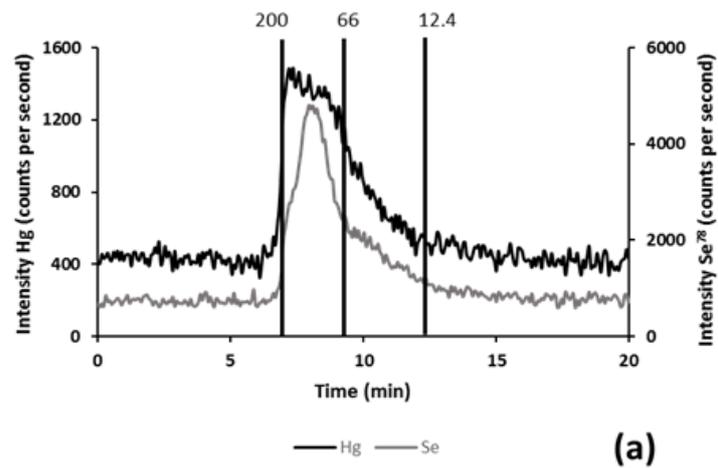
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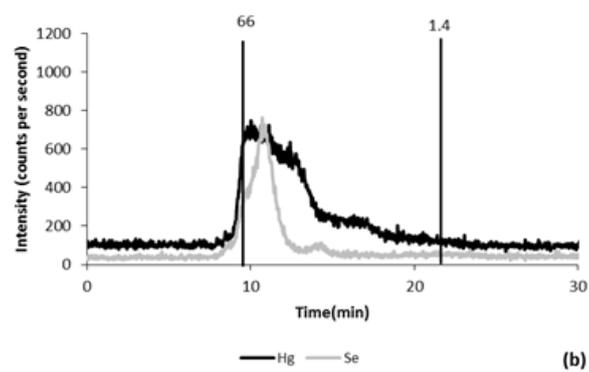
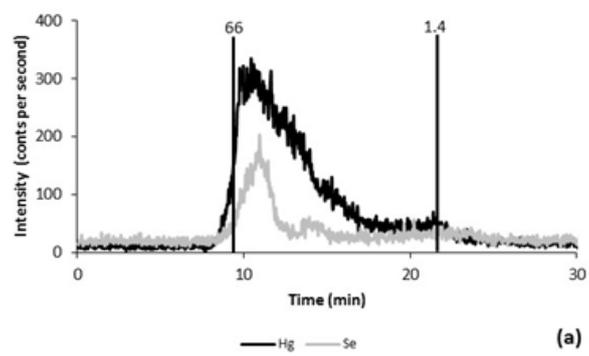
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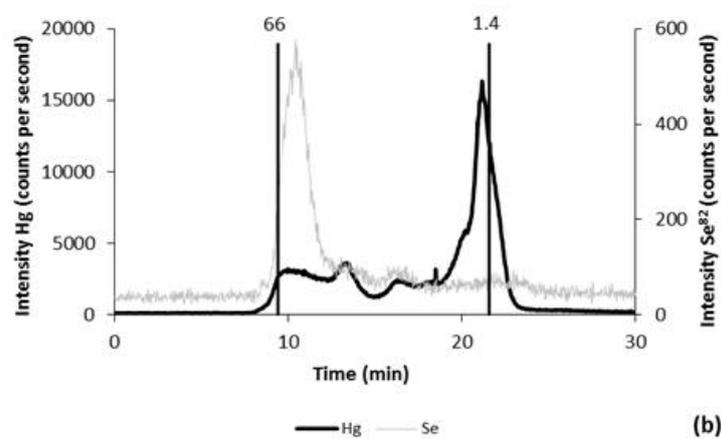
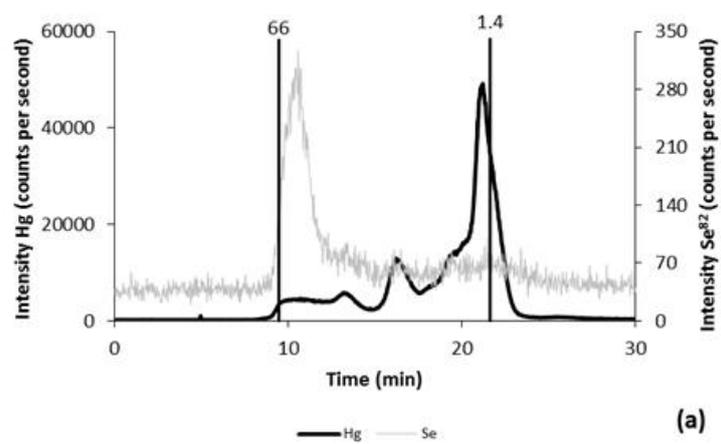
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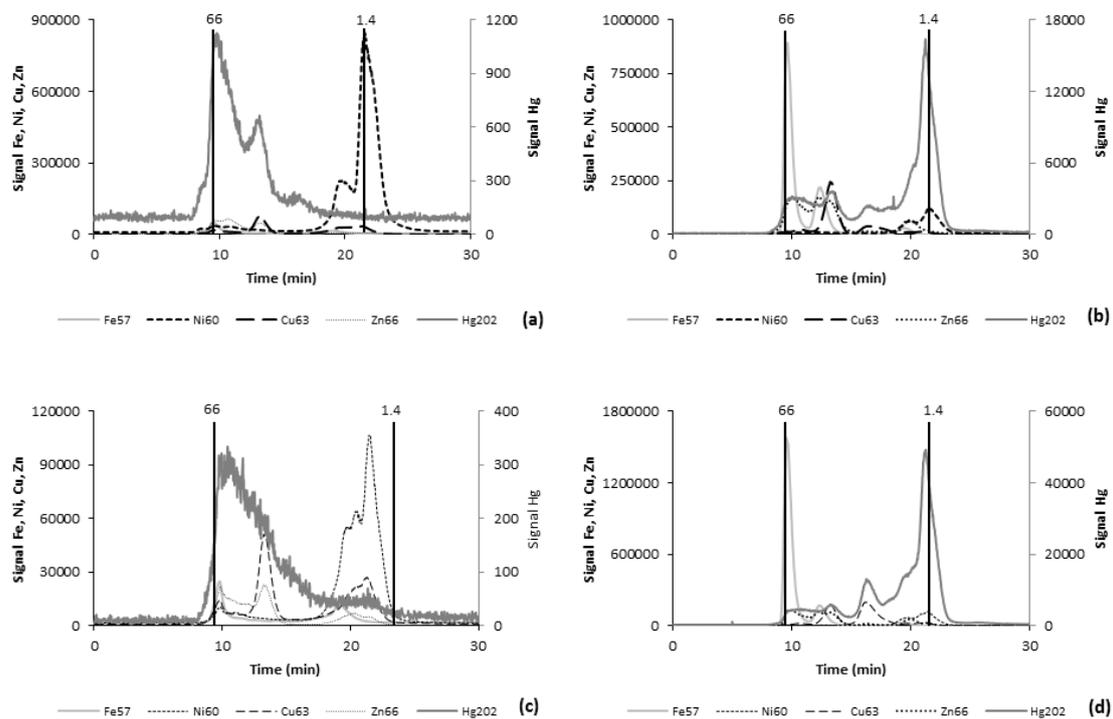


Table 1. Hg and Se concentration (mean±SD) in extract and residue (µg/g) of red deer kidney and percentage of extraction with different concentrations of Tris-HCl

[TrisHCl] mM	Hg				Se			
	µg/g extract	% extraction	µg/g residue	% residue	µg/g extract	% extraction	µg/g residue	% residue
30	0.00641±0.0019	16.4±0.3	0.0292±0.0041	75.2±1.4	0.021±0.004	2.9±0.2	0.543±0.010	73.9±1.8
50	0.00564±0.0011	14.5±0.2	0.0287±0.0080	73.6±2.8	0.033±0.009	4.6±0.3	0.568±0.008	77.3±1.5
80	0.00602±0.0024	16.1±0.4	0.0267±0.0045	68.5±1.7	0.024±0.002	3.3±0.1	0.465±0.006	63.2±1.2
100	0.00745±0.0007	19.1±0.1	0.0271±0.0032	69.2±1.2	0.037±0.004	5.1±0.1	0.539±0.004	73.3±0.8
120	0.00611±0.0021	15.7±0.4	0.0289±0.0060	74.1±2.3	0.026±0.008	3.5±0.3	0.544±0.014	74.0±2.5