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## **The role of selenium in mercury toxicity – current analytical techniques and future trends**

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### **Abstract**

Mercury (Hg) and selenium (Se) are trace elements, which are naturally found in the environment. While Hg forms highly potent toxic compounds, which cause irreversible damage to the central nervous system, Se, if present in a correct concentration, is an essential micronutrient needed for the regulation of metabolic processes. The interactions between Hg and Se have been rigorously scrutinized within the past decades particularly due to observed protective effects of Se against Hg toxicity, however very few proposed mechanisms of Hg and Se interactions are supported by empirical data. With mercury rising steadily in the environment, this matter is of urgency, and pushes for the development of new sample preparation protocols and analytical methods, which is a promising approach to study metabolic interactions between Hg and Se. Softer extraction methods should preserve the true chemical speciation, while a combination of chromatographic and direct analytical techniques enable identification and quantitation. This review summarizes the state of the art of current analytical techniques, which focus on the interactions between Hg and Se in biological matrices and outlines the potential future trends that might fill the major gaps in our analytical capabilities.

**Key words:** mercury, selenium, bio-molecules, speciation, extraction, HPLC, XAS,

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## 1. Introduction

### 1.1. Selenium, an essential element

Selenium has an atomic number of 34 and belongs to the group of non-metals. It has an atomic weight of  $78.971 \pm 0.008$  and six stable isotopes of varying abundances (Table 1).<sup>1</sup> It has six valence electrons, two of them being unpaired ( $[\text{Ar}] 3d^{10} 4s^2 4p^4$ ) and it is able to form six covalent bonds. Selenium commonly exists in oxidation states -II in binary compounds, elemental  $\text{Se}^0$ , II, IV and VI in oxygen-containing compounds. The location of Se in the periodic table suggests similarities in the chemical properties between sulfur (S), selenium (Se) and tellurium (Te) that is also supported by formation of analogous compounds found in nature.





Se occurs in the earth's crust mainly as selenide and selenite in association with sulfide minerals. Generally, its concentration varies between 0.05 to 0.09 µg/g in the earth crust. High Se concentrations can be found in volcanic and sedimentary rocks.<sup>2</sup> Its concentration in the soil varies significantly between geographical regions, and the bioavailability of Se in the soil is greatly dependent on the soil's pH, which dictates the chemical form in which Se is present.<sup>3</sup>

Table 1. Selected chemical and physical properties of Se and Hg.

Element	Atomic mass (u)	Isotopes (Abundance)	Oxidation states	1 <sup>st</sup> IP (eV)
Se	78.971(8)	<sup>74</sup> Se (0.86%), <sup>76</sup> Se (9.23%), <sup>77</sup> Se (7.60%), <sup>78</sup> Se (23.69%), <sup>80</sup> Se (49.80%), <sup>82</sup> Se (8.82%)	-II, 0, II, IV, VI	9.752
Hg	200.592(3)	<sup>196</sup> Hg (0.15%), <sup>198</sup> Hg (10.04%), <sup>199</sup> Hg (16.94%), <sup>200</sup> Hg (23.14%), <sup>201</sup> Hg (13.17%), <sup>202</sup> Hg (29.74%), <sup>204</sup> Hg (6.82%)	0, I, II	10.437

Selenium is an essential trace element for many living organisms, including the human body.<sup>4</sup> However, there is a very narrow optimal nutritional window as Se deficiency can be caused by a diet containing less than 0.1 µg/g whereas around 1 µg/g of dietary Se induces toxicity.<sup>5</sup> The recommended dietary allowance (RDA) varies between jurisdictions. In the United Kingdom, the reference nutrient intake (RNI) is 75 µg per day for men and 60 µg per day for women, whereas in the USA, this value is slightly lower at 55 µg per day for both men and women.<sup>6</sup> The minimal intake recommended by the World Health Organization (WHO) is 40 µg and 30 µg per day for men and women, respectively. However, currently the dietary intake in most European countries is less than half of the WHO recommendations.<sup>7</sup>

A Se-deficient diet induces several biological and physiological changes in mammals.<sup>8</sup> In 2012, Rayman<sup>9</sup> published an extensive review on "Selenium and human health". The author concluded that, while an increased risk of mortality, poor immune function and cognitive decline could be correlated with low Se levels, high Se concentration or Se supplementation have shown a reduced risk of autoimmune thyroid disease, antiviral effects and an increase in successful reproduction in males and females. Although there is strong evidence that Se plays an important role in normal brain function, the specific role of brain Se is yet to be discovered. Several human studies have suggested that Se has a role in neuronal diseases, such as



epilepsy,<sup>10,11</sup> coordination disorder,<sup>12</sup> Alzheimer's<sup>13,14</sup> and Parkinson's disease.<sup>12</sup> The contribution of specific Se-proteins to the development of neuronal diseases is poorly understood, but several studies on knockout mouse models have indicated that Se-proteins are not redundant and cannot be substituted by other, non-Se containing proteins.<sup>15,16,17</sup>

The essentiality of Se is attributed to the synthesis of selenocysteine (Se-Cys), the 21<sup>st</sup> essential amino acid. So far, 25 Se-proteins have been determined in the human selenoproteome,<sup>18</sup> of which half have been identified as of biological importance, and all of which contain Se-Cys.<sup>19</sup> The synthesis of Se-proteins goes through a rather complex cycle, and unlike other amino acids, Se-Cys is not recycled but is, instead, degraded to selenide. Newly formed selenide then enters the production sequence of Se-proteins, which starts with *de novo* synthesis of Se-Cys. Although the function of about a third of all Se-proteins is yet to be defined, members of glutathione peroxidases (GPx1 to GPx4, GPx6), thioredoxin reductases (TrxR1- TrxR3), iodothyronine deiodinases (Dio1 to Dio3), methionine sulfoxide reductase (MsrB1) and endoplasmic reticulum selenoproteins (Sel15, SelS, SelK, SelN, SelM and SelT) are well characterised and their functions are summarized in several reviews.<sup>20-21</sup> In general, the enzymatic properties of Se-proteins are mainly involved in antioxidant defense, redox homeostasis and signaling, with exception of SelP and GPx3, which are thought to be exclusively related to Se transport between tissue compartments. Furthermore, SelP can contain between 10 and 17 Se-Cys residues,<sup>22</sup> contributing to more than 50% of total Se in the plasma.<sup>23</sup> It is an extracellular protein, expressed in virtually all tissue; however, its predominant expression is located in the liver, from where it is transported to other organs.<sup>24</sup>

A lot of attention has focused on the role of SelP in the body owing to its structural specificity. But, achieving progress on this issue has proved to be challenging, as bacteria, which are generally used as a simple model organisms cannot express Se-proteins, SelP including.<sup>22</sup> Several studies reported the crucial role of SelP in metal detoxification, most probably attributed to multiple Se-Cys moieties, which are strong nucleophiles as their Se is generally present in an ionized state at physiological pH, making Se-Cys a natural target for electrophilic metals such as Hg, lead or cadmium.

## 1.2. Mercury, a potent neurotoxin



Mercury has the atomic number 80, atomic weight of  $200.592 \pm 0.003$  (Table 1) and belongs to the group of heavy metals.<sup>25</sup> Hg has seven stable isotopes and it naturally occurs in the environment in 0, I and II oxidation states. Owing to the high electronegativity (2.0 on the Pauling scale) and large ionic radius (0.111 nm for  $\text{Hg}^+$  and 0.116 nm for  $\text{Hg}^{2+}$ ), Hg-cations belong to the group of “soft” acids, which predicts their strong reactivity towards thiols and selenols, which are “soft” bases.

Mercury is naturally present in the environment but its extensive use in industrial processes led to a continuous increase of Hg background levels since the beginning of the 20<sup>th</sup> century.<sup>26</sup> It can be found throughout all environmental compartments, however in different molecular forms. The general chemical behaviour of Hg is characterised by its readiness to change its chemical speciation, which is depicted in its biogeochemical cycle, including airborne Hg species. In general, atmospheric Hg species are dominated by elemental Hg ( $\text{Hg}^0$ ), but Hg-halides such as  $\text{HgBr}_2$  and  $\text{HgCl}_2$  were also reported.<sup>27</sup> Inorganic mercury ( $\text{InHg}$ )<sup>+</sup> species in the form of Hg-halides, mercury sulfide (cinnabar,  $\text{HgS}$ ) and mercury selenide (tiemannite,  $\text{HgSe}$ ) dominate soils, sediments and aquatic environments whereas organic Hg species, namely methyl mercury ( $\text{MeHg}$ )<sup>+</sup> and to a lesser extend ethyl mercury ( $\text{EtHg}$ )<sup>+</sup> are the primary species in the aquatic biota.<sup>26, 28</sup>

Mercury (Hg) has a long history of use in the traditional and modern medicine and although it has been known as a neurotoxic substance for decades, its chemical properties are still being exploited in dental amalgams, vaccine preservatives, gold mining and other industrial processes, and its environmental concentrations keep increasing. While the exposure to Hg in the past was worrying mainly in the industrial areas such as vicinities of chlor-alkali plants or gold mining areas, the atmospheric transportation of Hg and global trade of food commodities contributed to human exposure to Hg across broad geographical regions. The most vulnerable to Hg toxicity are developing organisms and therefore, chronic exposure should be minimized in pregnant females and children through regulated uptake of foodstuff such as fish,<sup>29-31</sup> marine mammals<sup>32</sup> or in some instances rice,<sup>33</sup> which can contain elevated levels of Hg.

Although all Hg species are toxic, MeHg gains the most of the scientific attention due to its low  $\text{LD}_{50}$  (9.3 – 19.6 mg/kg as  $\text{MeHgCl}$  in mice),<sup>34</sup> long half-life (70 - 80 hours)<sup>35</sup> and the ability to cross the blood-brain barrier.<sup>36</sup> The onset of MeHg poisoning is manifested by paraesthesia of the hands, feet and perioral region, ataxia,



deafness and constriction of the visual field, which are followed by death.<sup>37</sup> Strategies for removing MeHg from the organism include exchange transfusions, haemodialysis<sup>38</sup> and chelation therapy using chelating agents such as dimercaptosuccinic acid (DMSA),<sup>39-40</sup> 2,3-dimercapto-1-propanesulfonic acid (DMPS)<sup>41</sup> and *N*-acetylcysteine (NAC).<sup>42</sup> While chelation therapy exploits the strong affinity between Hg and S ( $10^{39}$ ),<sup>43</sup> the bonding between Hg and Se is up to million times stronger ( $10^{45}$ )<sup>43-44</sup> and Se is believed to be a natural antagonist, which can prevent or eliminate symptoms of MeHg intoxication. Experimental studies using laboratory animals provided evidence of a tight metabolic association between Hg and Se, which was also observed in the wild fauna. However, there is a clear divide between what is the role of Se in Hg toxicity. For several decades, it was thought that supplementation of Se promotes the formation of complexes between Hg and bio-selenols, which would immobilize Hg and thereby counteracting its toxicity.<sup>45</sup> However, more recent approaches take into account the essentiality of Se for vital biological functions and argues that binding between Hg and Se leads to withdrawal of Se from Se-proteins synthesis and thus Hg poisoning is in essence displayed through Se depletion.<sup>7</sup> Supplementation of Se simply helps to maintain its essential concentrations imperative to sustain synthesis of Se-proteins. While several molecular structures were proposed and with them the mechanism of Hg's biochemistry,<sup>46</sup> the empirical evidence is still obscure.

The aim of this review is to discuss the recent developments in the applied analytical techniques, which target the interactions between Hg and Se in the biological systems. Having in mind the severity of Hg pollution and thus the impact it may have on the global population, we narrow our review to samples relevant for human analysis, tissues of internal organs, whole blood and its components.

## **2. Overview of analytical methods for Hg and Se speciation**

The first experimental data suggesting protective effects of Se against Hg toxicity were published in the late 60's when necrotic changes on the renal tubules were not observed in a group of rats simultaneously injected with HgCl<sub>2</sub> and sodium selenite.<sup>47</sup> Following this observation, increasing numbers of researchers started to report significant correlations between Hg and Se in a large variety of organisms, soon to be followed by the identification of HgSe as the final product of detoxification mechanisms in marine mammals. This undisputable evidence of Hg and Se interaction





fueled the need for the understanding of the metabolic pathways, which led to formation of HgSe and therefore identification of Hg and Se species, which take part in the reactions. These peculiar interactions between Hg and Se are again getting increasing attention, as new softer sample preparation techniques and sensitive analytical instrumentation are able to preserve and ultimately reveal crucial details of their chemical behavior in the living organism.

## **2.1. Sample preparation**

It is absolutely essential that the extraction method selected for isolation of the chemical species from the matrix conserves their endogenous form and thereby sample preparation for chemical speciation should be generally milder and requires greater understanding of the analytes' molecular structure than it is needed for total elemental analysis. When investigation of the interactions between two analytes is conducted, the selected extraction method should be equally favorable to both analytes and preserve their molecular structures. It appears that the current extraction methods are the cornerstones on which the investigation of Hg and Se interactions falls apart.

### **2.1.1. Extraction of biomolecules from the biological tissue**

A very low oxidation potential of the selenol group of SeCys and some other Se-amino acids poses a great challenge as these molecules suffer of oxidative degradation. As a result of oxidation, formation of (SeCys)<sub>2</sub>, which consists of two SeCys amino acids linked through Se-Se bond is observed. Synthesis of such artificial compounds implies that Se biomolecules extraction very often requires derivatisation of SeCys species, which proceeds usually in two steps. Firstly, the Se-Se bond is cleaved by a reductant such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) resulting in the formation of highly reactive SeH group, which needs to be stabilized against oxidation. Therefore, the reduced Se-Cys is derivatised, most often by using iodoacetamide (IA), which leads to synthesis of chemically stable carboxyamidomethyl-SeCys.<sup>48-51</sup> Additionally, it is important to consider what impact the use of DTT may have on any Hg species present in the sample. It is highly probable, that some Hg species will undergo complexation with DTT due to high affinity of Hg to thiols and/or also undergo reduction.



The extraction of Hg species entails even greater degradation of their molecular structure due to a general acceptance of crude differentiation between  $\text{Hg}^{2+}$  and  $\text{MeHg}^+$ , regardless the evidence that Hg does not commonly exist as free ion. Hg species extraction tends to be more aggressive and despite the strong bonds formed between bio-selenols and thiols, these bonds are rarely preserved. Microwave assisted alkali digestion using either TMAH<sup>49, 52-53</sup> or KOH<sup>54</sup> is the most commonly used method for Hg speciation, which is followed by derivatisation if the samples are to be analysed by gas chromatography (GC). However, an increasing number of researchers began to use high performance liquid chromatography (HPLC) for Hg species separation and with it greater variety of sample preparation method is being reported. Although, these methods are significantly milder they exploit strong binding properties of 2-mercaptoethanol<sup>55-58</sup> or L-cysteine<sup>59</sup> to Hg and thereby formation of new Hg-SR and or MeHg-SR complexes thus the native molecular structure is not preserved.

Clearly, the employment of two different extraction methods while studying the interactions between two analytes creates a conflict because the different sample preparation steps are focused on the analysis of one analyte and discard the information about the other. Therefore, it could be argued that simultaneous extraction preserving the binding between Hg and Se should be the main focus of future research. Some success was achieved by homogenization of the tissue in the water<sup>60</sup> or buffer<sup>61</sup> of low ionic strength, however a low yield of extracted Hg and Se biomolecules prompted a use of more aggressive proteolysis by enzymes<sup>60, 62-66</sup> or mixture of protein inhibitors.<sup>51, 58</sup> To increase the extraction yield, tissues of high lipids content such as liver, kidney or brain can be defatted using binary solvents prior to enzymatic hydrolysis.<sup>49, 67</sup> But, enzymatic hydrolysis, apart of SeCys oxidation also cleaves peptide bonds and therefore it represents a great compromise between the extraction efficiency and the preservation of true chemical species as e.g. Se is very rarely present in the form of free amino acids. Thus, the main drawback of the enzymatic hydrolysis is the destruction of endogenous proteins and obtained results provide although important but only partial information about the identity of the biomolecules. The list of applied extraction methods and identified biomolecules can be found in the Table 2.



Table 2. Overview of extraction protocols used for Se and Hg biomolecules identification.

Identified species	Matrix	Protocol	Reference
SeMet, SeCys	Serum	<b>Serum</b> (450 mg) was mixed with urea (150 $\mu$ L, 6 M) and DTT (15 $\mu$ L, 0.2 M) and mechanically shaken for 1 h. Afterwards, IA was added (20 $\mu$ L, 0.5 M) and sample was shaken for 1 h. Additional DTT was added (30 $\mu$ L) and shaken for another 1 h. Subsequently, the serum was digested with protease/lipase mix (450 mL, 14.7 and 8 mg L <sup>-1</sup> , respectively) for 17 h at 37 °C. To stop the digestion, pH was adjusted to 5 by glacial acetic acid and mixture was centrifuged at 5,800 g for 30 min.	48
SeCys <sub>2</sub> , SeCysCAM, SeDTT, SeMet, S-Me-SeCys, Se-cystathionine MeHg, InHg	Brain, liver	<b>Freeze-dried tissue</b> (100 mg) was defatted with MeOH/DCM (5 mL, 1:2 ratio) sonicated for 5 min. Dry defatted tissue in ammonium acetate buffer (2 mL, 10 mM, pH 7) was derivatised in dark with DTT (0.2 mL, 0.1 M) and IA (0.4 mL, 0.1 M) for 5 min. Afterwards, 2 mL of protease (10 mg mL <sup>-1</sup> ) was added and mixture was incubated at 37 °C for 24 h. The hydrolysate was centrifuged at 12,513 g for 5 min <b>Freeze-dried tissue</b> (50 mg) was solubilised in TMAH (5 mL) using microwave digestion system. 1 mL of the extract was buffered with sodium acetate (5 mL, 0.1 M, pH 3.9) and 1 mL of iso-octane and 1 mL of NaBPr <sub>4</sub> (1% w/w) were added. Mixture was mixed and then centrifuged for 10 min at 3,747 g and organic layer was analysed. <b>Water soluble species: Fresh tissue</b> (50 mg) was homogenised in Tris-HCl buffer (3 mL, 50 mM, pH 7.5) in ice bath. Homogenate was centrifuged at 15,000 g for 30 min.	49
SeCys <sub>2</sub> , SeCys, SeMet	Kidney, liver, muscle, digestive tract, gills	<b>Enzymatic hydrolysis: Lyophilized tissue</b> (75 mg) was mixed with urea (2 mL, 7 M) in Tris-HCl buffer (0.1 M, pH 7.5) and sonicated for 10 min. Sample was then shaken and incubated in dark with DTT (0.2 M) and IA (0.5 M) for 1 h, at each step. Afterwards, more DTT (375 $\mu$ L) was added and sample was shaken for 1 h. Then, sample was diluted with water to reach 1 M urea concentration and digested with protease/lipase mix (450 mL, 14.7 and 8 mg L <sup>-1</sup> , respectively) for 20 h at 37 °C. Digest was centrifuged at 7,500 g for 30 min through 10 kDa cut-off filter. <b>Biomolecules</b> were extracted with solution (3 mL g <sup>-1</sup> ) containing ammonium acetate buffer (20 mM, pH 7.4), TCEP (1 mM, pH 7.4), PMSF (1 mM) at 4 °C using a glass/Teflon homogenizer. Extract was centrifuged at 120,000 g for 1 h at 4 °C.	50
SeLP, SeAlb, HMM, LMM	Liver cytosolic extract, serum,	<b>Freeze-dried tissue</b> (250 mg) was solubilized in TMAH (5 mL) using microwave digestion system. Aliquot of the extract was buffered with acetate buffer (5 mL, pH 4) and NaBPr <sub>4</sub> (0.5 mL, 1% w/v) with iso-octene (1mL) were added. The mixture was shaken for 5 min, vortexed for 5 min, agitated for 7 min and then centrifuged at 2,500 g for 5 min. Organic layer was analysed.	51
MeHg, InHg	Muscle, brain, liver, feces	<b>Freeze-dried tissue</b> (250 mg) was solubilized in TMAH (5 mL) using microwave digestion system. Aliquot of the extract was buffered with acetate buffer (5 mL, pH 4) and NaBPr <sub>4</sub> (0.5 mL, 1% w/v) with iso-octene (1mL) were added. The mixture was shaken for 5 min, vortexed for 5 min, agitated for 7 min and then centrifuged at 2,500 g for 5 min. Organic layer was analysed.	68
MeHg, InHg	Liver, kidney, muscle	<b>Freeze-dried tissue</b> (0.5 g) was solubilised in TMAH (5 mL) using microwave digestion system. 1 mL of the extract was buffered with sodium acetate (5 mL, 0.1 M, pH 3.9) and 1 mL of iso-octane and 0.5 mL of NaBPr <sub>4</sub> (1% w/w) were added. After 30 min the mixture was centrifuged for 10 min at 1,650 g and organic layer was analysed.	53
MeHg		<b>Freeze-dried tissue</b> (0.25 g) was digested with methanolic KOH (25%) at 60 °C for 4 h. Digest was buffered with acetate buffer (2 M, pH 4.9) and derivatised with NaBEt <sub>4</sub> (1%).	54
MeHg, InHg	Brain	<b>Freeze dried tissue</b> (0.1 g) was extracted twice with 5mL mixture of HCl (0.1%) 2-mercaptoethanol (0.1%) and KCl (0.15%) by sonication at 35 °C for 30 min. The digest was then centrifuged at 3000 rpm for 10 min.	56 and 55



MeHg, InHg	Liver, muscle	Freeze-dried tissue (0.1 g) was extracted with 5 mL extractant solution containing HCl (0.1%), 2-mercaptoethanol (0.1%) and KCl (0.15%) during 12 h shaking at RT.	57
MeHg, InHg	Brain	Fresh tissue was suspended with 3 volumes of 6 mL of Tris-HCl buffer (20 mM, pH 7.4) containing sucrose (0.3 M), DTT (5 mM), PMSF (40 µg mL <sup>-1</sup> ), leupeptin (5 µg mL <sup>-1</sup> ), pepstatin (0.7 µg mL <sup>-1</sup> ), trypsin inhibitor (5 µg mL <sup>-1</sup> ). The homogenate was centrifuged at 50 g for 7 min and non-disrupted cells were removed. Then the supernatant was centrifuged successively at 800 g for 10 min, 9,000 g for 10 min, 30,000 g for 25 min and 100,000 g for 2 h at 4 °C.	58
MeHg-Hb	Liver, kidney	Fresh tissue was homogenized with 4 volumes of 6 mL of Tris-HCl buffer (20 mM, pH 7.4). The homogenate was centrifuged at 50 g for 7 min and non-disrupted cells were removed. Then the supernatant was centrifuged successively at 800 g for 10 min, 9,000 g for 10 min, 30,000 g for 25 min and 100,000 g for 2 h at 4 °C.	59
EtHg, InHg	Blood, brain, heart, liver, kidney	Tissue was extracted in 2 mL ammonium acetate buffer (0.2 M, pH 7.4) with ultrasonic probe (at 100 W, 30 s) in the ice bath. Extract was then centrifuged at 100,000 g for 20 min at 4 °C. Obtained cytosol was fractionated by SEC Superdex 200 (10 x 300 mm, 13 µm). Desired fraction was hydrolyzed with trypsin (10 mg mL <sup>-1</sup> ) at 37 °C for 16 h and then passed through 10 kDa filter membrane.	60
Se-MeSeCys, Se <sup>IV</sup> , Se <sup>VI</sup> , L-SeMet, D-SeMet, MeHg, InHg	Serum	Tissue and blood were extracted with solution containing HCl (0.1%), L-cysteine (0.05%) and 2-mercaptoethanol (0.1%) during sonication for 15 min.	59
Se-sugar, TMSe, HMM-Hg	Liver	Cold blood was let to clot for 30 min at RT and centrifuged at 2,500 g for 10 min	70
TMSe, SeCys <sub>2</sub> , SeMet, SeEt	Mussle and tuna tissue	Fresh tissue (1 g) was homogenized in 10 mL of Tris-HCl buffer (50 mM, pH 7.6) under N <sub>2</sub> atm. Cytosol was separated by ultracentrifugation at 100,000 g for 90 min at 4 °C. A portion of the cytosol fraction was heated in a water bath for 20 min at 95 °C and centrifuged for 20 min at 12,000 rpm.	61
SeCys, Se-Me-SeCys, SeMet, Se <sup>IV</sup> , Se <sup>VI</sup>	Muscle, liver, kidney, blood	Freeze-dried tissue (0.25 g) was defatted with MeOH:TCM (6 mL, 2:1) and then MeOH:TCM (6 mL, 1:2) in ultrasonic bath for 5 min. Dry tissue was then hydrolyzed using non-specific protease (5 mg mL <sup>-1</sup> ) in phosphate buffer (0.1 M, pH 7.5) in the incubator at 37 °C for 24 h. Then, another portion of protein was added (5 mg mL <sup>-1</sup> ) and tissue was incubated for another 24 h at 37 °C. The digest was passed through Bond-Elute C18 cartridge and eluted with phosphate buffer (5 mL, 3.5 mM). The eluate was diluted to 10 mL with water.	62
MeHgCys, MeHgGlu, MeHgX, HgX	Fish muscle	Homogenized tissue (0.1 g) or blood was sonicated with 1 mL of water for 30 min and centrifuged for 15 min at 14,000 g. Both, the supernatant and tissue residue were hydrolyzed with Pronase E (10 mg mL <sup>-1</sup> ) in Tris-HCl buffer (0.1 M, pH 7.5) at 37 °C for 48 h. The digest was centrifuged at 14,000 g for 20 min and passed through 10 kDa cut-off filter.	63
Hg-biomolecules	Muscle, brain, liver, kidney	Freeze-dried tissue (0.1 g) was extracted with 10 mL of extractant containing ammonium acetate (50 mM, pH 8) and trypsin (1 mg mL <sup>-1</sup> ). The extraction was carried out at 37 °C during constant rotation at 20 rpm for 24 h in dark. Afterwards the solution was centrifuged, filtered and diluted 10 folds.	64
MeHg-Cys, MeHg-SG	Liver	Freeze-dried tissue was solubilized in 0.5% SDS solution at 37 °C for 3h.	65
InHg-MT2, Hg-biomolecules	Liver	Freeze-dried tissue (0.1 g) was hydrolyzed with trypsin (2 mg mL <sup>-1</sup> ) in acetate buffer (50 mM, pH 8) containing SDS (0.5 %) at 37 °C for 4 h during constant rotation (20 rpm) in dark. Afterwards, the extract was centrifuged and filtered.	71
		Freeze-dried tissue (0.7 g) was extracted in 2 mL ammonium acetate buffer (0.2 M, pH 7.4) with ultrasonic probe (at 100 W, 30 s) in the ice bath. Extract was then centrifuged at 100,000 g for 20 min at 4 °C. Solid residue was extracted	





MeHgCys, MeHgSG	Brain	with 3 mL of SDS (4% w/v) during constant shaking for 1 h at RT. Sample was centrifuged at 4,000 rpm for 10 min.	66
SeMeSeCys, SeMet, Se <sup>IV</sup> , Se <sup>VI</sup> , SeCys <sub>2</sub>	Liver, muscle	<b>Freeze-dried tissue</b> (10 mg) was 3 x extracted in solution containing HCl (0.1%), 2-mercaptoethanol (0.1%) and KCl (0.15%) during 30 min of sonication at 35 °C. Afterwards, extract was centrifuged at 3000 rpm for 10 min at 30 °C. The sedimented tissue was rinsed again with the extractant and centrifuged at 4000 rpm for 10 min at 24 °C. <b>Freeze-dried tissue</b> (150 mg) was 3 x defatted using diethyl ether (30 mL) for 2 h during agitation at 200 rpm. Dried tissue and lipids were hydrolyzed using Protease XIV (7.5 mg mL <sup>-1</sup> ) in water at 37 °C for 24 h. Afterwards, the mixture was centrifuged at 11,000 rpm for 60 min at 4 °C.	67
SeAlb, GPX, SeIP	Blood plasma	<b>Blood</b> was mixed with Na <sub>2</sub> -EDTA and centrifuged at 1,500 g for 10 min and supernatant was collected.	72
EtHg-Cys, EtHg-GS	Blood	<b>Blood</b> was spiked with Liquemin® (100 µL) and centrifuged at 5,285 g for 15 min. Plasma was collected and diluted with ACN (1:1) and proteins were removed by centrifugation for 15 min at 5,285 g. Plasma liquid was collected and proteins were re-suspended in ACN: water (1 mL, 1:1) and vortexed for 15 min and centrifuged.	73
MeHg-CA, EtHg-CA	Blood hemolysate  CA stock sol.	<b>Blood</b> was spiked with Li-Heparin and centrifuged at 2,000 g for 10 min. Sedimented erythrocytes were washed twice with NaCl (0.9%) and centrifuged at 2,000 g for 10 min. Afterwards, DI water was added (0.5 mL) to erythrocytes and sample was incubated for 30 min at RT to obtain hemolysate. <b>Protein</b> was heated for 30 min at 90 °C and CaCl <sub>2</sub> (2 mM) and ammonium bicarbonate (50 mM) were added followed by addition of trypsin and chymotrypsin at enzyme/protein ratio 1:30 (w/w). The mixture was incubated at 37 °C overnight.	74
SeMet, TMSe, MeSeCys, Se-sugars, Se <sup>IV</sup> , Se <sup>VI</sup> , EtHg-β-LGA, EtHg-HSA	Serum	<b>Blood</b> sample was coagulated and centrifuged at 1,800 g for 10 min to separate serum. Serum proteins were pre-concentrated via precipitation in ACN, centrifuged at 1,800 g for 10 min and re-dissolved in the mobile phase.	75
	Synthetic β-LGA and HSA proteins	<b>Proteins digestion:</b> Protein in ammonium formate solution (20 mM, pH 7.4) was digested with trypsin (2.3 mg mL <sup>-1</sup> ) at 37 °C for 90 min. The digestion was stopped by addition of ice-cold ACN (200 µL) containing FA (1%).	76

**Species:** SeCyCAM - carbamidomethylated selenocysteine, SeDTT - seleno-dithiothreitol, S-Me-SeCys - thiol-methyl-selenocysteine, SeAlb - seleno-albumin, HMM - high molecular mass fraction, LMM - low molecular mass fraction, Se-Me-SeCys - selenol-methyl-selenocysteine, MT2 - metallothione-2, GPX- glutathione peroxidase, EtHg-GS - EtHg bound to glutathione, CA - carbonic anhydrase, TMSe - trimethylselenonium ion, SeCM - selenocystamine, β-LGA - β lactoglobulin A, HSA - human serum albumin

**Chemicals:** DTT - dithiothreitol, IA - iodoacetamide, MeOH - methanol, DCM - dichloromethane, TMAH - tetramethylammonium hydroxide, NaBPr<sub>4</sub> - sodium tetrapropylborate, TCEP - tris(2-carboxyethyl)phosphine, KOH - potassium hydroxide, NaBEt<sub>4</sub> - sodium tetraethylborate, HCl - hydrochloric acid, KCl - potassium chloride, RT - room temperature, PMSF - phenylmethylsulfonyl fluoride, TCM - trichloridomethylene, SDS - Sodium dodecyl sulfate, Na<sub>2</sub>-EDTA - Disodium ethylenediaminetetraacetate dehydrate, ACN - acetonitrile, NaCl - sodium chloride, CaCl<sub>2</sub> - calcium chloride, FA - formic acid



### **2.1.2. Extraction of biomolecules from the blood**

Liquid samples such as whole blood can be directly analysed by injection on the separation column and thus the analysis of biomolecules in these matrices benefits from very minimal sample preparation and thereby maximum preservation of the native chemical species. Various extraction strategies from the whole blood and its components with identified Hg and Se biomolecules are summarized in the Table 2. Analysis of Se-proteins in blood samples is generally focused on the specific blood fraction, most often serum and plasma. To obtain plasma for analysis, collected blood samples are treated with an anticoagulant such as Na<sub>2</sub>-EDTA,<sup>72</sup> Liqueenim<sup>73</sup> or Li-Heparin<sup>74</sup> whereas for the collection of serum, the whole blood samples are left to coagulate either at room temperature<sup>70</sup> or in an incubator.<sup>51</sup> The desired fractions are obtained by centrifugation at low temperature (4 °C) and collected supernatant should be stored at -80 °C. For the collection of particular sizes of Se-proteins, several researchers implemented the use of molecular sieve filters during the centrifugation steps, which increases the specificity of the sample preparation.<sup>77</sup> Additionally, preconcentration of the plasma and/or serum proteins can be achieved by their precipitation using organic solvents and subsequent re-suspension in the mobile phase.<sup>75</sup> However, such an aggressive sample treatment should be used only if Hg is covalently bound to the proteins as denaturation of the proteins may result in cleavage of weak bonds.

### **2.2. Chromatographic techniques**

Chromatographic separation of the analyte's species from the plethora of matrices can provide a vast amount of vital information about the biochemistry of the analyte in the studied biological systems. It is strongly dependent on the chemical and physical properties of the analyte's species which chromatographic technique can be implemented for the separation. Gas chromatography (GC) has been used for the past several decades to investigate Hg speciation due to the ease of chemical derivatisation of Hg species and subsequent formation of highly volatile and stable compounds. On the contrary, the benefits of versatile choice of mobile and stationary phases in liquid chromatography (LC), this technique found its pursuit in separation of the large family of Se species. Thus, following non-compatible sample preparation steps, the



separation of Hg and Se species also very often focuses on the species of one analyte at the time.

#### **2.2.1. Gas chromatography**

Gas chromatography coupled to ICP-MS or atomic fluorescence spectroscopy is extensively used for quantification of InHg and MeHg as it provides extremely low detection limits ( $\text{pg kg}^{-1}$ ), which can be enhanced through variety of preconcentration methods. But, as large biomolecules are not volatile, this technique requires invasive Hg species derivatisation, which destroys their native molecular structures. Similarly, GC coupled to various detectors was used for analysis of methylated Se species<sup>78</sup> but majority of Se-biomolecules are not volatile and require derivatisation, which is not compatible with simultaneous detection of Hg species. Thus GC provides very limited and not fit for purpose information when it comes to identification of intact Hg-biomolecules what ultimately determined its role in investigation of Hg and Se interactions.

#### **2.2.2. Liquid chromatography**

High performance liquid chromatography (HPLC) has almost universal applicability and in terms of separation of large bio-molecules is superior to GC. It is the most versatile separation technique, which benefits from a wide array of stationary phases providing different separation modes. A broad range of compounds from non-polar to ionic can be separated principles of normal or reverse-phase high-performance liquid chromatography (HPLC or RP-HPLC), affinity chromatography (AF), size exclusion chromatography (SEC), hydrophilic interaction chromatography (HILIC) and ion chromatography (IC). Application of HPLC in Hg and Se speciation seen significant growth as the sample preparation methods are generally softer, thus preserve the molecular structure of the existing complexes, molecules do not require derivatisation, which reduces the potential risk of contamination or a loss of unstable analytes and there is significantly larger choice of mobile and stationary phases which may aid the separation.

##### **2.2.2.1. Reversed-phase chromatography**

Despite the low hydrophobicity of organo-Se compounds to be retained and separated on the reversed-phase (RP) columns, several research groups investigating the



interaction of Thimerosal with proteins<sup>73, 76</sup> and its metabolism<sup>59</sup> achieved sufficient separation on C5, C8 and C18 columns. Thimerosal is Hg-containing compound, which is used as a preservative in the vaccines and decomposes into thiosalicylic acid and EtHg in aqueous media.<sup>79</sup> Thimerosal was found to form adducts with human serum albumin,  $\beta$ -lactoglobulin A<sup>76</sup> and with L-glutathione<sup>73</sup> (GSH) in the plasma surrogate samples. Identification of adducts was done through retention time matching and coupling of HPLC with ESI-MS. Obtained exact masses of the formed adducts (Table 2) confirmed decomposition of Thimerosal into EtHg and its subsequent binding to the blood proteins such as carbonic anhydrase (CA),<sup>74</sup> the second most abundant protein of the erythrocytes. Interestingly, it was also shown that although EtHg of Thimerosal binds to cysteine (Cys) if present in the solution alone, however, under physiological conditions when other amino acids are present, EtHg predominantly binds to GSH.<sup>73</sup> It appears that the biological distribution of EtHg might be very similar to that of MeHg and despite larger molecular size of EtHg, it was found to cross the blood-brain barrier in mice injected with Thimerosal.<sup>59</sup> Subsequent increase of InHg concentration in the brain suggests that EtHg undergoes de-alkylation but more interestingly, authors observed complete Hg detoxification within 80 days following the exposure as most Hg had been excreted. However, the mechanism of Hg transport across the blood-brain barrier was not discussed.

Nonetheless, it can be argued that RP chromatography suffers from numerous problems when used for separation of proteins. Mainly, the conditions under which the separation is performed, low pH and high and variable organic solvent concentration may result in denaturation of the proteins. Investigation of Thimerosal binding to Cys and GSH encountered proteins precipitation under applied chromatographic conditions when human blood was used and therefore, plasma surrogate substituted the authentic samples.<sup>73</sup> Hg species decomposition due to low pH during the chromatographic separation was observed in the study of CA proteins adducts, which resulted in increased Hg background.<sup>74</sup> Thereupon the majority of the studies that employed RP chromatography are using synthetic, highly purified proteins<sup>73-74, 76, 80</sup> or report limited structural information about Hg species.<sup>59</sup> Typically, RP chromatography of proteins suffers from peak broadening and poor peak asymmetry, which results from conformational heterogeneity and weak kinetics. Poor recovery and high on column carry over may also arise from strong and/or irreversible binding of proteins to the stationary phase. For these reasons elucidation





of Hg-biomolecules' structure with RP chromatography may be a challenging task and typically structural elucidation is performed on the protein subunits, amino acids.

Table 3. Detected and calculated exact masses of reported Hg adducts analysed by HPLC-ESI-MS

Adduct	Sum formula	Mode	Detector	Detected m/z	Calculated m/z	$\delta$ [ppm]	Ref.
EtHg-GS	C <sub>12</sub> H <sub>20</sub> HgN <sub>3</sub> O <sub>6</sub> S	-ve	ESI-ToF-MS	536.0787	536.0779	1.49	<sup>73</sup>
Cys-Hg-GS	C <sub>13</sub> H <sub>22</sub> HgN <sub>4</sub> O <sub>8</sub> S <sub>2</sub>	-ve	ESI-ToF-MS	627.0480	627.0502	-3.51	<sup>76</sup>
GS-Hg-GS	C <sub>20</sub> H <sub>32</sub> HgN <sub>6</sub> O <sub>12</sub> S <sub>2</sub>	-ve		813.1109	813.1142	-4.08	
(DMSA-Hg) <sub>2</sub>	C <sub>8</sub> H <sub>8</sub> Hg <sub>2</sub> O <sub>8</sub> S <sub>4</sub>	-ve		762.8391	762.8431	-5.23	
GS-Hg-NAC	C <sub>15</sub> H <sub>24</sub> HgN <sub>4</sub> O <sub>9</sub> S <sub>2</sub>	-ve		669.0578	669.0607	-4.35	
NAC-Hg-NAC	C <sub>10</sub> H <sub>16</sub> HgN <sub>2</sub> O <sub>6</sub> S <sub>2</sub>	-ve		525.0094	525.0072	4.06	
GS-MeHg	C <sub>11</sub> H <sub>19</sub> HgN <sub>3</sub> O <sub>6</sub> S	-ve		522.0617	522.0617	3.81	
DMSA-MeHg	C <sub>5</sub> H <sub>8</sub> HgO <sub>4</sub> S <sub>2</sub>	-ve		396.9485	396.9487	-0.38	
DMSA-(MeHg) <sub>2</sub>	C <sub>9</sub> H <sub>10</sub> Hg <sub>2</sub> O <sub>4</sub> S <sub>2</sub>	-ve		612.9356	612.9349	1.14	
NAC-MeHg	C <sub>6</sub> H <sub>10</sub> HgNO <sub>3</sub> S	-ve		378.0069	378.0082	-3.6	
T19 + MeHg	[C <sub>217</sub> H <sub>316</sub> N <sub>46</sub> O <sub>60</sub> SHg] <sup>4+</sup>	+ve	ESI-ToF-MS	1190.0633	1190.0603	2.6	<sup>74</sup>
T19 + EtHg	[C <sub>218</sub> H <sub>318</sub> N <sub>46</sub> O <sub>60</sub> SHg] <sup>4+</sup>	+ve		1194.0682	1194.0647	2.9	
C40 + MeHg	[C <sub>66</sub> H <sub>118</sub> N <sub>16</sub> O <sub>24</sub> SHg] <sup>2+</sup>	+ve		876.3946	876.3966	-2.3	
C40 + EtHg	[C <sub>67</sub> H <sub>120</sub> N <sub>16</sub> O <sub>24</sub> SHg] <sup>2+</sup>	+ve		883.4044	883.406	-1.8	

GS – adduct containing glutathione peroxidase, DMSA – 2,3-dimercaptosuccinic acid, NAC – *N*-acetylcysteine, T19 – tryptic peptide (#174-213) after digestion of carbonic anhydrase, C40 – chemotryptic peptide (#210-223) after digestion of carbonic anhydrase

#### 2.2.2.2. Size exclusion chromatography

Applications of size exclusion chromatography (SEC) significantly improved the understanding of Se's role in Hg biochemistry, as contrary to RP chromatography, SEC is soft separation technique during which the molecules generally don't interact with the stationary or mobile phase but are separated on the bases of their hydrodynamic radius. This allows for use of mild mobile phases such as low ionic strength buffers of neutral pH that preserve the intact protein structures.

Application of SEC provided the first evidence of a specific Se-protein, which appeared to bind significant portion of an equimolar complex between Hg and Se, following *in vivo* and *in vitro* exposure studies.<sup>81</sup> Subsequently, SelP was identified as the major Se-protein binding Hg and Se equimolar complex in the rats' serum.<sup>82</sup> Additionally, analysis of mice serum and cytosolic extract following HgCl<sub>2</sub> exposure identified binding of Hg to the high molecular mass fraction and albumin.<sup>51</sup> However,



SEC generally lacks the needed resolution for separation of the large group of Se-proteins present in the biological samples and therefore, the separation is usually enhanced by affinity chromatography. Heparin affinity column in tandem with SEC allows separation of SelP, GPx and albumin in serum<sup>72</sup> and the application of this chromatographic method identified SelP as the primary Se-protein in human plasma, which responds to MeHg intoxication.<sup>83</sup> Another way to improve the resolution of SEC is to simplify the complexity of the crude tissue extracts by adopting the proteomics approach of subcellular fractionation of the internal tissues, thus maintaining the integrity of the proteins. Following this approach, Hg in the cytosolic fraction of the brain was found to bind to metallothionein-3<sup>58</sup> whereas in the dolphin hepatic cytosolic extract binding of InHg to metallothionein-2 was observed with MeHg being associated with biomolecules of larger molecular weight.<sup>71</sup> From Se species, selenoneine was identified in the hepatic cytosolic extract of turtles<sup>61</sup> and dolphins but interestingly, while no reactivity of selenoneine towards InHg or MeHg was observed in the cytosolic extract<sup>84</sup> other researchers suggested possible role of selenoneine in MeHg detoxification in larger marine mammals.<sup>69</sup>

The increasing number of studies implementing the separation principle of SEC underlines its great potential, as identification of the Hg binding Se-proteins is performed in their natural state. However, to maintain the native conditions the analyses are often carried out in the media of 100% aqueous solutions and high salt content, which in effect can be problematic. Bacterial contamination can arise within a few hours in the aqueous solution when antibacterial agents, such as sodium azide are not used. On the other hand, high salt concentration in the mobile phase and extraction solutions increases the potential of particles formation thus affecting system and column performance. Additionally, highly ionic solutions are not compatible with ESI-MS and therefore structural elucidation by hyphenation of the HPLC system is not possible. This is particularly troublesome due to limited availability of analytical standard for identification of Se-proteins. Furthermore, proteins are prone to interact with the charged surface of the column packing material that may result in either ion exclusion, when protein is not allowed to enter the pore, or adsorption leading to increased retention of the protein, thereby peak broadening and overlapping.

#### **2.2.2.3. Ion exchange chromatography**



The concept of separation using IEC is based on the cations (CEC) and anions (AEC) exchange between the stationary and mobile phase. Because amino acids are zwitterionic molecules their overall charge depends on the pH of the mobile phase and thus the type of ionic interaction between amino acids and the stationary phase. This technique generally offers high separation efficiency but low specificity and is commonly used for separation of inorganic Se and small molecules. AEC was previously employed for quantification of inorganic Se, SeMet and SeCys in the human serum of individuals exposed to high Hg concentrations.<sup>85</sup> But to enhance separation specificity of IEC the columns are often being used in tandem or conjunction with other chromatographic columns. Combination of AEC and RP chromatography was used for identification of Hg and Se biomolecules in the liver and brain of beluga whales.<sup>65-66</sup> However, as the elution of species from IEC column is pH dependent, the variation of pH might have a negative impact on the species integrity. Additionally, buffers of higher ionic strength, which generally provide better chromatographic separation are not compatible with molecular detectors such as ESI-MS and could cause salt deposition on the cones of ICP-MS.



Table 4. Summary of published Hg and Se biomolecules separation methods.

Identify species	Matrix	Column	Preparative SEC: Superdex Peptide HR 10/30	Mobile phase	Flow rate	Elution	Detector	Ref
SeMet, SeCys	Serum	RP: C18 Hypersil BDS (0.3 x 150 mm, 3 μm)	10 mM ammonium acetate (pH 9.5)	A: 5% ACN, 0.1% TFA, B: 95% ACN, 0.1% TFA A: 0.1% FA in water, B: 0.1% FA in MeOH	0.7 mL min <sup>-1</sup> 4 μL min <sup>-1</sup> Not reported	isocratic 0-15 min 10-60% B, 15-20 min 60% B 0-8 min 100% A, 8-15 min 100% B, 15-22 min 100% A	ICP-MS ICP-MS ICP-MS/ESI-MS	48  49
SeCys <sub>2</sub> , SeCysCAM, SeDTT, SeMet, S-Me-SeCys, Se-cystathionine MeHg, InHg	Brain, kidney	RP: C18 Zorbax Elixir (4.6 x 150 mm, 5 μm)	GC: DB-1 100% PDMS (0.59 mm x 30 m x 1 μm) AEC: Hamilton PRP X-100 (4.1 x 250 mm)	NA Ammonium acetate (10 mM, pH 5)	Helium 16 mL min <sup>-1</sup> 1 mL min <sup>-1</sup>	50 °C (1 min), 50 °C/min up to 250 °C isocratic	ICP-MS ICP-MS	50
SeCys <sub>2</sub> , SeCys, SeMet	Kidney, liver, muscle, digestive tract, gills	SEC/IEC: Shodex Asahipak GS-220 HQ (7.5 x 300 mm) SEC: Biosep SEC 2000 (7.8 x 300 mm) RP: Symmetry Shield C18 (3.9 x 150 mm, 5 μm) SEC: Superdex™-200 (10 x 300 mm, 13 μm)	Ammonium acetate (25 mM, pH 6.7) Tris-HCl (50 mM, pH 6.8) A: 0.1% HFA, B: 1% HFBA, 2% MeOH, pH 2.1 Ammonium acetate (50 mM, pH 7.4)	0.6 mL min <sup>-1</sup> 1 mL min <sup>-1</sup> 0.8 mL min <sup>-1</sup> 0.7 mL min <sup>-1</sup>	isocratic isocratic isocratic isocratic	ICP-MS   ICP-MS	51	
SeIP, SeAlb, HMM, LMM	Liver cytosolic extract, serum,	SEC-AF: HiTrap® Desalting column (5 mL), heparin-Sepharose (1 mL), blue-Sepharose (1 mL) RP: Vydac C8 (1 x 250 mm, 5 μm)	A: ammonium acetate (50 mM, pH 7.4), B: ammonium acetate (1.5 M, pH 7.4) A: ammonium acetate (5 mM, pH 6.0), B: ACN	1.3 mL min <sup>-1</sup> Not reported	0-7 min 100% A, 7-18 min 100% B, 18-20 min 100% A 0-40 min 50% B, 40-48 min 80% B, 48-50 min 80% B, 50-55 min 1% B, 55-60 min 1% B 0-5 min 10% B, 5-20 min 50% B, 20-25 min 100% B	ICP-MS/ESI-MS	69	
MeHg-Hb	Liver	HILIC: TSK gel amide 80 (1 x 250 mm, 5 μm)	A: ACN, B: ammonium acetate (10 mM, pH 5)	50 μL min <sup>-1</sup>				
Selenonine								





MeHg, InHg	Muscle, brain, liver, feces	GC: MIXT Silcosteel (0.53 mm x 30 m x 1 $\mu$ m)	NA	Helium 25 mL min <sup>-1</sup>	50% B, 25 – 27 min 65% B, 27 – 30 min 65% B, 30 – 35 min 10% B, 35 – 40 min 10% B	ICP-MS	68
MeHg, InHg	Liver, kidney, muscle	GC: DB-1 100% PDMS (0.59 mm x 30 m x 1 $\mu$ m)	NA	Helium 16 mL min <sup>-1</sup>	75 °C to 250 °C at 120 °C/min 50 °C (1 min), 50 °C/min up to 250 °C	ICP-MS	53
MeHg, InHg	Brain	RP: C18 Zorbax Eclipse (2.1 x 50 mm, 5 $\mu$ m)	4% MeOH, 0.1% 2-mercaptoethanol, 60 mM ammonium acetate	0.45 mL min <sup>-1</sup>	isocratic	ICP-MS	56 and 55
MeHg, InHg	Liver, muscle	RP: Symmetry Shield C18 (3.9 x 150 mm, 5 $\mu$ m)	5% MeOH, 0.1% 2-mercaptoethanol, 60 mM ammonium acetate	1 mL min <sup>-1</sup>	isocratic	ICP-MS	57
MeHg, InHg	Liver, kidney, brain	RP: Symmetry Shield C18 (3.9 x 150 mm, 5 $\mu$ m)	5% MeOH, 0.1% 2-mercaptoethanol, 60 mM ammonium acetate	1 mL min <sup>-1</sup>	isocratic	ICP-MS	
InHg-MT2, Hg-biomolecules	Liver	SEC: TSK-GEL G3000 SWxl (1.8 mm x 300 mm) SEC: Superdex 200 HR (10 x 300 x 13 $\mu$ m) and Superdex peptide HR 10/30 (10 x 300 mm, 13 $\mu$ m)	Tris-HCl (0.1 M, pH 7.4) Ammonium acetate buffer (0.1 M, pH 7.4)	0.5 mL min <sup>-1</sup> 0.7 mL min <sup>-1</sup>	isocratic isocratic	ICP-MS ICP-MS	71
EtHg, InHg	Blood, brain, heart, liver, kidney	RP: Brownlee C8 (4.6 mm x 33, 3 $\mu$ m)	3% MeOH, 0.5% 2-mercaptoethanol, 0.05% FA in water	1.2 mL min <sup>-1</sup>	isocratic	ICP-MS	59
Se-MeSeCys, Se <sup>IV</sup> , Se <sup>VI</sup> , L-SeMet, D-SeMet, MeHg, InHg	Serum	RP: Phenomenex Luna C18 (4.6 x 250 mm, 5 $\mu$ m)	A: 0.075% TEAC at pH 4.5 water B: 5% MeOH, 0.1% 2-mercaptoethanol in 60 mM ammonium acetate buffer	Not reported	0 - 13 min 100% A, 13 - 25 min 100% B	ICP-MS	70
Se-sugar, TMSe, HMM-Hg	Liver	Astec Chirobiotic T column (4.6 x 250 mm, 5 $\mu$ m) SEC: GS-520 HQ (7.5 mm x 300 mm)	Tris-HCl (50 mM, pH 7.4)	1 mL min <sup>-1</sup>	isocratic	ICP-MS	61



		SEC: GS-320 HQ (7.5 x 300 mm)	Ammonium acetate (50 mM, pH 6.5)	0.6 mL min <sup>-1</sup>	isocratic	
		AEC: DEAE-2SW (4.6 x 250 mm)	Ammonium acetate (50 mM, pH 4.0)	0.7 mL min <sup>-1</sup>	isocratic	
		SEC: GS-320A-2E (2 x 250 mm)	Ammonium acetate (10 mM, pH 6.5)	40 µL min <sup>-1</sup>	isocratic	ESI-MS
TMSe, SeCys <sub>2</sub> , SeMet, SeEt, Se <sup>IV</sup> , Se <sup>VI</sup>	Mussel and tuna tissue	RP: Phenomenex Spherisorb C18 (4.6 x 250 mm, 5 µm)	A: Phosphate buffer (3.5 mM, pH 6.0) B: phosphate buffer (7.0 mM, pH 6) Phosphate buffer (5.0 mM, pH 2.8)	1 mL min <sup>-1</sup>	0 - 5 min 100% A, 5 - 6 min 100% B, 6 - 17 min 100% B isocratic	ICP-MS
TMSe, SeCys <sub>2</sub> , SeMet, SeEt	Muscle, liver, kidney, blood	RP: Zorbax RX-C8 (4.6 x 250 mm, 5 µm)	5% MeOH in ammonium citrate buffer (5.5 mM, pH 4.8)	1 mL min <sup>-1</sup>	isocratic	ICP-MS
SeCys, Se-Me-SeCys, SeMet, Se <sup>IV</sup> , Se <sup>VI</sup>	Fish muscle	RP: Phenomenex Luna C18 guard column (4 x 3 mm, 5 µm) and Luna C18 (3.0 x 50 mm, 5 µm)	7.5% MeOH, 2.5% ACN, 90% MSA (pH 5) with 100 µg L <sup>-1</sup> Au	1 mL min <sup>-1</sup>	isocratic	ICP-MS
MeHgCys, MeHgGlu, MeHgX, HgX Hg-biomolecules		SEC: Sephadex G-75 (3.0 x 97 cm)	Tris-HCl buffer (10 mM, pH 7.5) with glycerol (10 mM)	Not reported	isocratic	UV
MeHg-Cys, MeHg-SG	Muscle, brain, liver, kidney	RP: Phenomenex Luna C18	7.5% MeOH, 2.5% ACN, 90% MSA (2 mM, pH 5) with 100 µg L <sup>-1</sup> Au	Not reported	isocratic	ICP-MS
MeHgCys, MeHgSG	Brain	RP: Zorbax Exlipse XDB-C18 (2.1 x 50 mm, 5 µm)	94% of 2-mercaptoethanol (0.1%) in ammonium acetate (60 mM) and 6% MeOH	0.45 mL min <sup>-1</sup>	isocratic	ICP-MS
Se-biomolecules		AEC: IonPac® AG18 (4 x 250 mm) with guard column	KOH (23 mM)	1 mL min <sup>-1</sup>	isocratic	ICP-MS
SeMeSeCys, SeMet, Se <sup>IV</sup> , Se <sup>VI</sup> , SeCys <sub>2</sub>	Liver, muscle	AIC: Hamilton PRP-X 100 (4.1 x 250 mm, 5 µm)	2% MeOH in citrate buffer (3 mM, pH 4.8)	0.5 mL min <sup>-1</sup>	isocratic	ICP-MS
		CIC: Zorbax 300-SCX (4.6 x 250 mm, 5 µm)	2% MeOH in pyridine solution (3 mM, pH 2.1)			
SeAlb, GPX, SeLP	Blood plasma	AF: AFPak Heparin	A: Na2-EDTA (0.1 g L <sup>-1</sup> ) in phosphate buffer (20 mM, pH 7.5) B: as A with added heparin (500 units mL <sup>-1</sup> )	0.6 mL min <sup>-1</sup>	0 - 15 min 100% A, 15 - 20 min 100% B, 20 - 60 min 100% A	ICP-MS
		SEC: Asahipak GS 520-HQ				



Ethg-Cys, EtHg-GS	Blood surrogate	RP: Shim-Pack XR-C18 (2.0 x 30 mm, 2.2 $\mu$ m)	A: 0.1% FA in water B: MeOH	0.3 mL min <sup>-1</sup>	0 - 0.5 min 5% B, 0.5 - 2 min 60% B, 2 - 3.5 min 60% B, 3.5 - 4.5 min 5% B, 4.5 - 9 min 5% B	ICP-MS, ESI-ToF-MS	Trumpler
MeHg-CA, EtHg-CA	Blood hemolysate	RP: BioBasic C18 (2.1 x 150 mm, 5 $\mu$ m) with guard column (4 x 4 mm, 300 Å)	A: 0.1% FA in water, B: ACN	0.3 mL min <sup>-1</sup>	0 - 2 min 35% B, 2 - 20 min 42% B, 20 - 24 min 70% B, 24 - 27 min 70% B, 27 - 29 min 35% B, 29 - 35 min 35% B, 0 - 2 min 10% B, 2 - 19 min 80% B, 19 - 23 min 80% B, 23 - 28 min 10% B, 28 - 30 min 10% B, 0 - 2.5 min 100% A, 2.5 - 30 min 100% B	ICP-MS, ESI-ToF-MS	74
Se <sup>IV</sup> , Se <sup>VI</sup> , TMSe, SeCys, SeUr, SeMet, SeCM	Liver cytosolic extract, serum, urine	RP: Discovery BioWidePore C5 (2.1 x 150 mm, 5 $\mu$ m) with guard column (4 x 4 mm, 300 Å)					
SeMet, Se-sugars	Serum	RP: Symmetry Shield C18 (3.9 x 150 mm, 5 $\mu$ m) with C18 guard column (3.9 x 20 mm)	A: 0.1% HFBA, 0.3% MeOH in water, B: .1% HFBA, 2% MeOH in water	Not reported		ICP-MS	77
TMSe, MeSeCys, Se-sugars, Se <sup>IV</sup> , Se <sup>VI</sup>		RP: Atlantis dC18 (4.1 x 150 mm) CEC: PRP-X200 (4.1 x 200 mm)	3% MeOH in ammonium formate buffer (20 mM, pH 3.0) Ammonium acetate buffer (20 mM, pH 3.0)	1 mL min <sup>-1</sup>	isocratic	ICP-MS, ESI-MS	75
Ethg- $\beta$ -LGA, EtHg-HSA	Synthetic $\beta$ -LGA and HSA proteins	AEC: PRP-X100 (4.6 x 150 mm) RP: Discovery BioWidePore C5 (2.1 x 150 mm, 5 $\mu$ m)	Malonate (20 mM, pH 9.5) A: 0.1% FA in water, B: ACN	0.3 mL min <sup>-1</sup>	0 - 2 min 20% B, 2 - 12 min 60% B, 12 - 19 min 60% B, 19 - 21 min 25% B, 21 - 23 min 25% B	ICP-MS, ESI-ToF-MS	76

**Species:** SeCyCAM - carbamidomethylated selenocysteine, SeDTT - seleno-dithiothreitol, S-Me-SeCys - thiol-methyl-selenocysteine, SeAlb - seleno-albumin, HMM - high molecular mass fraction, LMM - low molecular mass fraction, Se-Me-SeCys - selenol-methyl-selenocysteine, MT2 - metallothioneine-2, GPX- glutathione peroxidase, EtHg-GS - EtHg bound to glutathione, CA - carbonic anhydrase, TMSe - trimethylselenonium ion, SeCM - selenocystamine, SeUr- selenourea,  $\beta$ -LGA -  $\beta$  lactoglobulin A, HSA - human serum albumin



**Chemicals:** ACN – acetonitrile, TFA – Trifluoroacetic acid, FA – formic acid, MeOH – methanol, Tris-HCl – **Tris**(hydroxymethyl)aminomethane hydrochloride, HFA – hydrofluorosilicic acid, HFBA – heptafluorobutyric acid, PDMS – polydimethylsiloxane, TEAC – tetraethylammonium chloride, MSA – methanesulfonic acid, KOH – potassium hydroxide, Na<sub>2</sub>-EDTA – disodium ethylenediaminetetraacetate dehydrate





### 2.3. Detection and identification of Se and Hg biomolecules

Although HPLC systems are generally equipped with absorbance detectors such as UV/VIS and GC with flame ionization detector, these are very rarely used in Hg and Se speciation. Instead, for determination of Hg and Se interactions, HPLC and GC are almost always hyphenated with mass-specific detector, such as inductively coupled plasma mass spectrometer (ICP-MS) and/or electrospray ionization mass spectrometer (ESI-MS).

ICP-MS is highly sensitive, multi-elemental and isotopic analytical instrument with a wide linear dynamic range (4 – 11 orders of magnitude) and a high sample throughput. Nevertheless, it also suffers of isobaric and spectral interferences, which has to be addressed particularly during Se detection. Se isotopes ( $^{76}\text{Se}$ ,  $^{77}\text{Se}$ ,  $^{78}\text{Se}$ ,  $^{80}\text{Se}$ ) suffer from interferences caused by  $^{36}\text{Ar}^{40}\text{Ar}$ ,  $^{38}\text{Ar}^{38}\text{Ar}$ ,  $^{38}\text{Ar}^{40}\text{Ar}$ ,  $^{40}\text{Ar}_2$ ,  $^{79}\text{Br}^1\text{H}$  and  $^{40}\text{Ar}^{37}\text{Cl}$ , which are formed in the plasma during the ionization process. Such interferences can be resolved by using mathematical correction,<sup>86</sup> collision or reaction gas<sup>86-87</sup> if quadrupole instruments are used otherwise, high-resolution sector field ICP-MS<sup>88</sup> should be employed. Another setback is caused by high ionization potential of Se, which results in low ionization efficiency in the plasma. Due to interferences on the  $m/z$  of most abundant Se isotope ( $^{80}\text{Se}$ ) the detection of  $^{77}\text{Se}$  and  $^{82}\text{Se}$  is generally preferred. However, the already hampered sensitivity by the low ionization efficiency is further effected by low abundance of these isotopes what has a significant influence on the sensitivity. To enhance the Se signal, low percentage of MeOH could be added into the post column flow, which slightly increases the plasma temperature causing Se signal enhancement.<sup>89</sup> Contrary to Se, Hg does not suffer of significant interferences and thus is generally analysed without addition of reaction or collision gas or in the low resolution.

Identification of Hg and Se biomolecules using ICP-MS relies solely on the retention time matching with authentic standards as the hard ionization of ICP results in the complete elemental ionization of analytes and no molecular information can be obtained from it. However, retention time matching is extremely limited because it requires prior knowledge of the possible molecular structure of the compound in order to purchase or synthesise the standard, which are often not commercially available. Additionally, pure synthetic standards might exhibit retention time shift as the



interaction of biomolecules with the mobile and stationary phase when present in the sample might be affected the sample matrix.

ESI-MS is often used either in tandem with ICP-MS or as a complementary detector as its soft ionization mode preserves the molecular form and thus this instrument is invaluable for structural elucidation of the molecular species.<sup>90</sup> Identification of novel Se and Hg biomolecules is exclusively done by ESI-MS with high molecular mass precision when high-resolution instruments such as Orbitrap, ESI-TOF-MS or ESI-MS/MS are used. Nonetheless, ESI-MS is not compatible with all liquid chromatographic techniques, as buffers of higher ionic strength tend to precipitate in the introduction system and cause carry over between the samples. Additionally, analysis of complex mixtures like biological samples produces complicated mass spectra, which deconvolution is not always possible. Therefore, such sample require several clean up and purification steps that ultimately might result in modification of the molecular structure.

#### **2.4. Quantification methods**

Under ideal conditions, quantification should be performed by comparison of the instrument's response to the analysed species with a response provided by calibration standards of the identical species. However, such quantification is practically impossible as the availability of synthesis molecular standards is greatly limited. Therefore, the quantification of various molecular species using HPLC-ICP-MS is usually performed by external calibration using a single molecular species of the desired analyte. As ICP is a hard ionization technique, it doesn't differentiate between individual analyte species and therefore the ionization efficiency can be considered to be species independent. There are several other assumptions, which need to be contemplated before species independent external calibration is performed. The behaviour of individual chemical species during the chromatographic separation is assumed to be identical and any possible species conversion, decomposition or retention on the chromatographic column must be excluded. If gradient elution is applied to aid the chromatographic separation then the plasma conditions should be monitored by continuous addition of an internal standard. This is particularly important when the gradient with organic solvent is used as it is during RP chromatography, because the varying concentration of the organic solvent may cause changes in the plasma temperature and thereby the ionization efficiency of the



monitored analytes. Depending whether the change in plasma carbon content enhances or suppresses the ionization, the quantification can be affected by false positive or false negative errors.<sup>#</sup> There are two methods described in the literature that can be adopted for accurate quantification under such conditions. One method uses continuous addition of the organic solvent, usually MeOH to the spray chamber, thus oversaturating the spray chamber with MeOH so that any changes in MeOH concentration in the plasma caused by the gradient will be negligible.<sup>91</sup> The other method applies mathematical correction in a form of response factor, which is calculated from a separate chromatographic run.<sup>92</sup>

Another approach used for quantification of Se-proteins is to calculate the percentage proportion of the area under chromatographically separated peak against the sum of all peaks' areas in the chromatogram, which is then multiplied by the total Se concentration.<sup>83</sup> Although this method does not require external calibration, it suffers from the same drawbacks as previously described.

Another problem during quantification may arise when analyte's species are a product of derivatisation reaction with non-quantitative reaction yield. In such circumstances quantification using isotope dilution mass spectrometry (IDMS) method seem to be a unique method that can provide accurate results. IDMS can be performed as species-specific or species-unspecific analyses and the principles behind these techniques are described in details elsewhere.<sup>93-94</sup> The former involves spiking the sample with an enriched species of interest prior to chromatographic separation in order to alter the natural isotope ratio of the analyte. As the isotope ration between the endogenous and enriched species is the only measured unknown, any species conversion during the extraction or derivatisation process can be detected if the equilibration with spike was achieved. The main advantage of species-specific IDMS is its independence from extraction or derivatisation efficiency during sample preparation. Additionally, this method offers an alternative for quantification when matrix-matched standards are not available. But species-specific IDMS can only be used if the identity of an analysed species is known.

On the other hand, in species-unspecific IDMS the spike is mixed with the eluting compound after chromatographic separation via a T connector before it reaches the plasma. In this way, the structure and composition of the species is not relevant, but its accurate quantification is still possible by calculating the isotope ratio between endogenous isotope in the eluting peak and the spike. This quantification method was



applied in several studies of Se-proteins quantification which standards are very rarely available.<sup>95-97</sup>

## **2.5. Direct analytical techniques**

### **2.5.1. XAS and LA-ICP-MS**

An advantage of synchrotron X-ray absorption spectroscopy (XAS) is that it requires minimal sample preparation as it probes all elements in any physical or chemical state. During measurement, ionising radiation of the X-ray, which contains sufficient energy to excite a core electron of targeted atoms, irradiates the sample and the absorbed energy is detected. The absorption spectrum can be divided into two regions: X-ray absorption near-edge spectroscopy (XANES), which is sensitive to formal oxidation state and coordination number; and extended X-ray absorption fine-structure spectroscopy (EXAFS), which can be used to determine the proximity, coordination number and chemical environment around the absorbing atom.<sup>98</sup> Thereby the speciation of larger molecular structures can be rather ambiguous however, XAS proved to be exceptionally important tool for identification of Hg and Se containing aggregates in environmental samples.

HgSe was found to be a dominant species of Hg in the liver and pituitary gland of beluga whale,<sup>99</sup> in the muscle of bottlenose dolphin<sup>100</sup> and widely distributed in the majority of internal organs of striped dolphins.<sup>101</sup> Similarly, analysis of human brains after both low and high exposures to MeHg, found the dominant Hg species to be present as inert HgSe.<sup>102</sup> However, while Se-containing intermediate species were not found, a portion of Hg was bound to thiols as it was observed in other studies<sup>103</sup> which led to suggestions that formed particulate species of Hg may not be pure HgSe clusters.<sup>104-105</sup> It is possible that in the biological media of high biothiol concentrations, a random addition of S takes place during formation of Hg containing clusters and thus mixed chalcogenide  $\text{HgS}_x\text{Se}_{(1-x)}$  can be formed. The lack of observed Se-containing intermediates in the reported studies could also result from the insensitivity of the technique to the low levels at which particular Se-proteins can be found.

X-rays can also be used to excite a core electron of an atom, which, upon return to the ground state, emits X-ray fluorescence (XRF); this X-ray spectrum can then be used for analysis. Synchrotron based XRF technique is typically used for bio-imaging of Hg distribution in tissue and to map its possible co-localization with other elements.





Such an application of XRF was used to map spatial distribution of Hg and Se in the larvae of zebrafish exposed to MeHg and InHg.<sup>104</sup> 2D maps showed clear pattern of co-accumulation of Hg and Se when the fish were exposed to InHg, but interestingly the co-localisation was not observed under MeHg exposure. Using high spatial resolution the authors were able to localize nano-sized structures smaller than 250 nm containing both Hg and Se. In another study, XRF helped to identify significant differences in the distribution of various organic and inorganic Hg species in exposed zebrafish larvae indicating that individual organic and inorganic Hg species may possess different toxicities.<sup>106</sup> Similarly, laser ablation (LA) coupled to ICP-MS has been used for bio-imaging of Hg distribution and co-localization in the tissue. During the analysis, the laser beam is focused on the surface of sample tissue and by firing the laser beam small particles are ablated and transported to the plasma of an ICP-MS for elemental analysis. Spatial resolution of the LA-ICP-MS system is dependent on the laser setting but generally it can go down to 5  $\mu\text{m}$ . Both of the techniques require very minimal sample preparation, usually cryogenic thin sectioning which is directly analysed. Combination of LA-ICP-MS and XRF was used for co-localization of Hg and Se in melano-macrophage aggregates in yelloweye rockfish<sup>107</sup> and in the internal organs of pilot whales.<sup>49</sup> However, both techniques can provide only elemental information and thus the identity of Hg and Se species has to be investigated by other complementary techniques.

### **3. Future perspectives**

#### **3.1. Tandem chromatography**

Chromatographic separation of the targeted group of proteins from the plethora of biological matrices may require a combination of several chromatographic principles, which often results in enhanced peak resolution. In the recent years affinity chromatography (AFC) became increasingly popular for tandem separation with RP or SEC columns. Similarly as IEC, AFC is based on the interaction between the stationary phase and the target molecule but AFC possesses greater specificity with respect to the binding partner. The stationary phase materials of AFC exploit proteins' specific structure or function for binding and thus often provide high resolution and selectivity. In particular, heparin-sepharose and blue sepharose columns are specifically designed to interact with Se-proteins. The former one is able to retain



SelP, whereas the latter one strongly binds SelP and albumin, however, other Se-proteins like GPx are eluted without retention.

This principle was used for investigation of the impacts of MeHg rich diet on the levels of SelP, albumin and GPx in the human plasma.<sup>83</sup> Heparin-sepharose and SEC columns connected in tandem helped to identify increased biosynthesis of SelP upon increased dietary MeHg uptake but on the contrary, MeHg had very weak impact on the GPx concentrations in the human plasma. Garcia-Sevillano et al.<sup>108</sup> took the tandem chromatography a step further by combining previously developed methods of double AFC<sup>109</sup> and SEC<sup>110</sup> to study Hg and Se interaction in mice under controlled Hg exposure conditions. Sera and cytosolic extracts were first pass through two HiTrap (SEC) columns in order to remove low molecular mass fractions and then, were carried onto heparin-sepharose and blue-sepharose columns, which retained SelP and albumin, respectively. For sequential elution of the Se-proteins, a 6-port valve was used between the two AF columns, which allowed firstly elution of SelP from heparin-sepharose by bypassing blue-sepharose column and afterwards the valve was switched for elution of albumin from blue-sepharose column.<sup>95</sup> The authors found that Hg without Se supplementation has negative impact on Se in the serum, which decreased under experimental conditions, however, caused increase of Se concentration in the liver. The speciation analysis showed a decrease in albumin concentration in the serum and its increase in the liver, which is indicative of increased Se-Cys biosynthesis through biodegradation of Se-Met in albumin. Upregulation of SelP expression in the liver and transport of Se containing low molecular mass fraction was also observed in this study. To reduce the complexity of hepatic cytosolic extract, Pedrero et al.<sup>69</sup> used SEC to separate Hg-biomolecules by their size and then collected fractions were analysed  $\mu$ RP chromatography following a tryptic digestion. This approach was successful in identification of significant fraction of MeHg binding to haemoglobin  $\beta$  through Cys moiety, indicating that haemoglobin  $\beta$  might be the major carrier of MeHg from the blood to the liver.

Tandem chromatography can provide another dimension to the separation of desired proteins as a combination between two or more separation principles and can improve the peaks resolution and thereby enhances the qualitative and quantitative potentials of liquid chromatography.

### 3.2. Analysis without borders



The slow pace in identification of Hg and Se biochemistry is without doubts caused by the complexity of the chemical processes involved, relatively late identification of Se's importance in the biochemical pathways of Hg's toxicity and in part, by researchers' selection of very narrow spectrum of analytical techniques. It is becoming gradually apparent that applied analytical techniques have very limited capabilities in providing complete answers to such complex questions and the future progressive approach will require close collaboration between various fields of analytical and bio-analytical chemistry. An original combination of several powerful analytical techniques was recently adapted to identify the impact of Hg bioaccumulation on Se species in the liver and brain of pilot whales.<sup>49</sup> The authors complemented GC-ICP-MS and HPLC-ICP-MS/ESI-MS analysis of Hg and Se species, respectively with solid-state analytical techniques of XANES, XRF and LA-ICP-MS to explore *in vivo* formation of Hg-Se particles, which size was determined for the first time by single particle ICP-MS analysis. The study showed that while Se-Cys in the brain and liver of adult and juvenile whales is maintained constant regardless of increasing Hg concentration in these tissues, Se-Met, the actual pool of Se for Se-Cys synthesis is being depleted in the adult whale. It was suggested that such depletion might be a response to upregulation of Se-Cys synthesis, which is being targeted by dietary MeHg and incorporated into the formation of Hg-Se particles and clusters. The study disputed previous suggestions of required minimal Hg concentration in the biological systems needed for initiation of detoxification mechanism as Hg-Se particles were also found in the juvenile whales. Despite of the exhaustive investigation of the Hg-Se formed particles, the authors were not able to confirm their identity, however suggested that the core of Hg rich clusters consists of HgSe nanoparticles that are bound to Se-rich proteins, most probably Selp.

### 3.3. Nanoparticles measurements

Although chromatographic speciation is a very powerful tool, it is not able to quantify all Hg species present in environmental samples. Hg forms solid particles with sulfur (HgS) and selenium (HgSe), which must be removed from samples prior to chromatographic separation and are usually excluded from the quantification. In biological samples, Ostertag *et al.*<sup>66</sup> operationally defined this fraction as "strongly bound Hg<sup>2+</sup>", which is not susceptible to extraction. While the chemical species of nanoparticles in the biological samples is thought to be HgSe based on the evidence



provided by XAS techniques, there is a pressing urgency for the application of nanoparticles targeted analysis. In the field of engineered nanoparticles, single particle ICP-MS and field flow fractionation were found to be particularly useful, yet so far their application for the analysis of naturally formed Hg particles was very rare.<sup>49</sup> It is assumed that HgSe nanoparticles are formed as a result of detoxification mechanism however, high reactivity of nanoparticles predicts formation of larger clusters, which may further reduce the bioavailability of Se. Additionally, similarities between Hg and Cd biochemistry, which can also cross the blood-brain barrier,<sup>111</sup> indicate that formed particles may contain more elements than Hg and Se. Therefore, it is absolutely essential for the understanding of HgSe formation and its role in Se bioavailability that future research focuses on the development of improved methods for quantitative and qualitative measurements of particulate forms of Hg.

### 3.4. Isotopic signature

Isotope ratio (IR) measurements dominated by C, N and O has a long tradition in geochemical disciplines, however owing to the multi-isotopic properties of Hg, there is a recent surge of studies focusing on the changes of Hg isotopic signature as a result of bioaccumulation.<sup>68, 112-117</sup> From isotope fractionation perspective, Hg is a unique element, which undergoes both, mass dependent (MDF) and mass independent fractions (MIF). The former one results from biological processes, including species transformation during detoxification, metabolism and biological transport, whereas the latter one can be caused by photoreactions, although the process of MIF is not fully understood. The changes in MIF isotope signature can provide information about the direct pathway of Hg from the exposure source to the target organ<sup>68</sup> or geographical origin.<sup>118</sup> On the other hand, transport processes are causing MDF<sup>119</sup> and with it enrichment of the source in the heavier isotopes, hence the destination organ is enriched in the lighter isotopes, what provides means to monitor Hg cycling in the biological system. Based on the evidence provided by MDF, Feng et al.<sup>68</sup> suggested that after the initial accumulation in the liver, InHg is transported to the brain, although the biochemical pathway was not elucidated. Clearly, IR measurements and with it MDF and MIF of Hg isotopes can serve as valuable complementary information in broader understanding of Hg's fate in the biological systems. Unfortunately, the IR measurements by multi-collector ICP-MS suffer from poor sensitivity that restricts the range of samples in term of required minimum Hg





concentration. Perhaps for the same reason, Se's isotopic signature in the biological matrices didn't receive much attention, despite having six stable isotopes. Unlike Hg, Se undergoes only MDF and thereby isotopic signature of Se can provide only limited information. Nonetheless in combination with the data obtained from Hg isotopic signature, IR measurements certainly provide new and exciting perspectives into understanding of the nature of the interactions between these two elements.

#### 4. Conclusions

There is ample evidence of common metabolic pathways of Hg and Se through their significant correlations in numerous internal organs of animals after short and long-term exposure, which gave grounds for more in depth investigations. In the past several decades analytical techniques evolved from determination of the total concentration of the analyte towards differentiation between its chemical species, implementing chromatographic methods. However, currently we are seeing a shift towards molecular identification of Hg containing bio-selenols and with it improvements in sample preparation steps. It appears that the extraction of the molecular species is the most crucial step as regardless of the strong affinity between Hg and Se, decomposition of Hg containing bio-selenols is very often observed. Nonetheless, the lack of suitable extraction method is increasingly being compensated by information obtained through combination of various chromatographic and/or direct analytical techniques.

#### 5. Notes

<sup>‡</sup> (CH<sub>3</sub>)Hg-X, (CH<sub>3</sub>CH<sub>2</sub>)Hg-X and HgX<sub>2</sub> are abbreviated as MeHg, EtHg and InHg, respectively as so far majority of the sample preparation techniques fail to preserve the bond between Hg and its heterogeneous binding ligand, Hg-X. Where the full molecular structure was preserved, Hg species abbreviation contains the corresponding binding ligand. Abbreviation of Hg species as MeHg<sup>+</sup> and Hg<sup>2+</sup> may be seen as misleading as this connotation fundamentally suggests that these Hg species could exist in the environment as free ions what is probably a very rare occurrence.

<sup>#</sup> False positive and false negative errors are rooted in the statistical hypothesis testing, where the former one incorrectly retains false null hypothesis and the latter one incorrectly rejects a true null hypothesis.<sup>120</sup>



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