Anal Bioanal Chem (2016) 408:2641–2648
DOI 10.1007/s00216-015-9243-8

TRENDS

Pushing back the frontiers of mercury speciation using a combination of biomolecular and isotopic signatures: challenge and perspectives

Zoyne Pedrero[1] · **Olivier F. X. Donard**[1] · **David Amouroux**[1]

Received: 25 September 2015 / Revised: 18 November 2015 / Accepted: 2 December 2015 / Published online: 11 January 2016

Abstract Mercury (Hg) pollution is considered a major environmental problem due to the extreme toxicity of Hg. However, Hg metabolic pathways in biota remain elusive. An understanding of these pathways is crucial to elucidating the (eco)toxic effects of Hg and its biogeochemical cycle. The development of a new analytical methodology based on both speciation and natural isotopic fractionation represents a promising approach for metabolic studies of Hg and other metal(loid)s. Speciation provides valuable information about the reactivity and potential toxicity of metabolites, while the use of natural isotopic signature analysis adds a complementary dynamic dimension that allows the life history of the target element to be probed, the source of the target element (i.e., the source of pollution) to be identified, and reactions to be tracked. The resulting combined (bio)molecular and isotopic signature affords precious insight into the behavior of Hg in biota and Hg detoxification mechanisms. In the long term, this highly innovative methodology could be used in life and environmental science studies of metal(loid)s to push back the frontiers of our knowledge in this field. This paper summarizes the current status of the application of Hg speciation and the isotopic signature of Hg at the biomolecular level in living organisms, and discusses potential future uses of this combination of techniques.

Keywords Biological samples · Metals · Heavy metals · Speciation · Isotopic fractionation · Mercury

Introduction

Mercury (Hg) pollution is considered a major environmental and public health concern. Considering its toxicity, Hg has recently been included in the top ten hazardous chemicals by the World Health Organization (Minamata Convention 2013). In general, fish and seafood consumption is recognized to be the most common pathway leading to human exposure to Hg. This is especially troubling considering a recent report of significantly increased in Hg levels in oceanic waters [1].

Despite its high toxicity, the metabolic pathways of Hg in biota remain elusive. An understanding of these pathways is crucial to elucidating the (eco)toxicological effects of Hg as well as its biogeochemical cycle. The bioavailability, mobility, and toxicity of an element are dependent on its chemical forms (speciation). Considering the ability of Hg to bind thiols [2], interactions of Hg with essential proteins and enzymes appear to be the reason for its high toxicity. However, the unambiguous elucidation of the structures of such complexes in biota in the environment has been achieved in only a limited number of studies [3, 4], as it represents a considerable analytical challenge. Usually, the biomolecules involved are identified based on the chromatographic coelution of Hg and biomolecule standards. Therefore, the development of novel analytical strategies for these Hg complexes is necessary.

Speciation research is undoubtedly a tool with the potential to provide new insights into Hg metabolism and Hg detoxification through the identification of the resulting metabolites.

Published in the topical collection featuring *Young Investigators in Analytical and Bioanalytical Science*, with guest editors S. Daunert, A. Baeumner, S. Deo, J. Ruiz Encinar, and L. Zhang.

✉ Zoyne Pedrero
zoyne.pedrerozayas@univ-pau.fr

[1] Laboratoire de Chimie Analytique Bio-Inorganique et Environnement, Institut des Sciences Analytiques et de Physico-chimie pour l'Environnement et les Matériaux, UMR 5254, CNRS Université de Pau et des Pays de l'Adour, 2 Avenue Pierre Angot, 64053 Pau, France

However, speciation provides a kind of "snapshot," and the elucidation of metabolic pathways also requires dynamic information, especially when attempting to answer questions such as:

Does the Hg in different biomolecules originate from the same source (of pollution)?
Is the identified Hg metabolite a final product of a particular metabolic pathway?
How important are the specific mechanisms?

These and other questions regarding the life history and reactivity of Hg can potentially be answered by observing how the natural isotopic abundance of Hg varies (this is called "isotopic fractionation") in different chemical forms of Hg.

There has recently been a clear trend for investigating Hg in biota via its stable isotope pattern, although so far this has largely been limited to elemental analyses. However, a recent coupling of gas chromatography (GC) to multiple-collector inductively coupled mass spectrometry (MC-ICPMS) allowed for the simultaneous isotopic characterization of different species (iHg, MeHg) [5]. The transition to Hg isotopic characterization at the biomolecular level represents a promising route to a deeper understanding of the metabolism of Hg. The analytical challenges associated with this approach and perspectives on its application in the near future are presented and discussed in this work.

Speciation

The speciation of an element, rather than its total concentration, determines the reactivity of that element in the environment and whether it is essential or toxic to living organisms. Therefore, investigating Hg in biota, principally through the determination of Hg-binding biomolecules, is crucial to assessing its metabolic pathways and toxicological impact. However, most Hg speciation studies have so far been limited to simple discrimination between inorganic mercury (iHg) and methylmercury (MeHg). Fish muscle, rice seeds, and seafood (mussels and oysters) are the matrices that are usually investigated in this context, as they are consumed by humans.

GC-ICP-MS is one of the most popular techniques used for Hg species quantification [6]. Several advanced approaches have improved the accuracy and precision of iHg and MeHg quantification, principally by spiking the sample with isotopically enriched species [7]. In addition, applying isotope pattern deconvolution to the experimental data permits the determination of the extent of Hg methylation/demethylation, which is important as it can affect the initial composition of the species in the sample [7].

A promising approach to MeHg quantification without derivatization that has been applied to biological samples has recently been reported [8, 9]. This approach is based on the preconcentration of Hg species followed by their separation and detection via HPLC-CV-AFS (high performance liquid chromatography–cold vapor atomic fluorescence spectrometry). Detection limits of 0.002 mg kg^{-1} and 1.5 ng L^{-1} in marine tissues and urine, respectively, have been obtained using this technique [9].

Obviously, a deep understanding of Hg pathways in living organisms requires investigations of the biomolecules that bind Hg. Several hypotheses have been proposed for these pathways; for example, that methylmercury is transferred from plasma into milk using albumin as a passive carrier [10], that MeHg is transported by hemoglobin after dietary exposure, and that the binding of iHg by metallothioneins (MTs) and Hg detoxification in mammals occur through the formation of an insoluble compound (HgSe, named tiemmanite) (see [11] and references therein). Most of these hypotheses are based on the correlation of the concentration of Hg with that of the corresponding protein, or the chromatographic coelution of Hg and the protein standards (see [11] and references therein). However, these phenomena do not provide a reliable basis for identifying target proteins for Hg, and structural characterization is required. Unfortunately, biomolecules that bind Hg have been studied far less than other metalloproteins containing elements such as Cu, Cd, Zn, and Se. This is principally due to the considerable analytical challenges associated with the structural identification of Hg biomolecules. For instance, the number of Hg-containing biomolecules is relatively small, and there are potential losses/transformations of Hg species during analytical procedures that include multiple chromatographic and preconcentration steps.

Some recent studies have succeeded in identifying Hg biothiols that form in plants [12, 13] and marine mammals [3, 4] and in characterizing the interactions of thimerosal (an organomercury compound) with model proteins [14, 15] by coupling HPLC to ICP-MS and electrospray mass spectrometry (ESI-MS), and have therefore provided valuable information on the behavior of Hg in biota. However, those studies mainly utilized either in vitro experiments with proteins and Hg standards or in vivo studies of organisms exposed in the laboratory to high levels of Hg.

To our knowledge, the only studies that have characterized Hg-binding proteins at levels that occur naturally in the environment were some investigations performed in dolphin liver samples. This characterization was achieved by developing an adequate sample preparation protocol and using state-of-the-art hyphenated techniques (bidimensional HPLC and parallel detection by ICP-MS and sensitive high-resolution electrospray tandem mass spectrometry, ESI-MS/MS). Also, the structures of iHg and MeHg binding to MT [3] and hemoglobin [4], respectively, in bioenvironmental (unenriched) samples were recently characterized for the first time. However, in both of these studies where the Hg species was found

to bind to a major protein, the concentration of the protein without Hg was several orders of magnitude higher than that of the protein containing Hg. This huge difference in abundance resulted in an overwhelming signal from the non-Hg form of the protein in molecular MS which tended to suppress the signal from the Hg-containing species, complicating Hg-binding protein identification, as previously mentioned. For example, in the characterization of MT containing Hg in dolphin liver, a detailed analysis of the mass spectrum indicated the presence of several clusters of the most abundant form of MT (MT-Zn_7) with different adducts (Fig. 1a). The fact that one of these adducts (MT-$Zn_7KH_2PO_4$ at *m*/*z* 1662.38616) has a very similar mass to MT-Zn_6Hg hampered direct identification of the Hg complex due to the overlap between the signals from these two complexes. Comparison of the theoretical and experimental mass spectra at two MT-$Zn_7KH_2PO_4$ to MT-Zn_6Hg ratios (1:1.15 in Fig. 1a and 1:10 in Fig. 1b) unequivocally confirmed the overlap between these species [3].

The crucial role of speciation in the understanding of metabolic processes is indubitable, but it is only capable of providing a "snapshot" of the forms of Hg present. The addition of another dimension that can provide information about the primary source of the Hg (i.e., the source of pollution) present in biomolecules, or can indicate if it is a precursor or an end-product of a specific mechanism, represents a potential route to greatly enhancing our knowledge of the uptake and fate of Hg in biota.

Isotopic signature

The ratio of the natural abundances of particular isotopes—an isotopic signature—is a powerful tool that can provide dynamic and historical data on metal(loid)s, including their origins, modes of transport, degradation mechanisms, and metabolism. Stable isotope composition analysis could represent a complementary technique to speciation, but this combined approach is yet to be exploited, so various interesting applications of it are still to be discovered. Isotopic signatures have traditionally been applied in C, Ca, N, O, and S (bio)geochemical studies. The development of MC-ICP-MS has dramatically increased research into nontraditional stable isotopes that do not tend to be analyzed in geological studies.

Hg is considered a model element for isotopic fractionation studies. It has several stable isotopes that exhibit mass-dependent (MDF) and mass-independent (MIF) fractionation (expressed in δ and Δ notation, respectively, and reported in parts per thousand, ‰) [16]. Combining both types of isotopic fractionation yields precious information on Hg source discrimination, reactivity, and fate in a biogeochemical context. The mechanisms that induce MIF are not fully known. It has been principally associated with photoreactions, and most studies have indicated that MIF does not occur during biological processes [16, 17]. On the other hand, MDF is induced by diverse processes such as evaporation, volatilization, species transformations, and transport and metabolic processes, among others [16, 17].

Despite the particularly strong growth in research into Hg species over the last few decades, studies of Hg isotopic composition have mainly focused on tracking sources of pollution and geochemical processes (see [18] and references therein). Recently, however, a clear trend has emerged for investigations of Hg in living organisms that exploit the isotopic signature of Hg. Those studies have provided crucial information on Hg uptake and pathways for bacteria-mediated Hg species transformation, as recently reviewed by Kritee et al. [19]. However, there is one one example of such a study in plants; that research focused on rice (*Oryza sativa* L.) from a Chinese Hg-mining area [20]. The Hg isotopic pattern was used to track Hg sources (via MIF) by estimating the fractions originating from the atmosphere and the soil. Additionally, the MDF signatures in several plant tissues (root, seeds, leaf, and stem) and the soil were used to elucidate the Hg absorption and transport processes that occur in this global crop [20].

The potential of the Hg stable isotopic signature has been also exploited in fish and marine mammals. Such studies can be divided into those focusing on fish species grown in captivity [21, 22] and those studying wild fish collected from specific ecosystems [23–25]. The former employ analyses of Hg MDF and MIF in different fish organs/tissues (such as muscle, kidney, brain, blood, etc.) and provide information about the distribution of the supplied diet in the organs and its metabolization. On the other hand, muscle is usually the only organ that is investigated from fish and marine mammals collected from specific ecosystems [23–25]. Hg isotopic signatures, in some cases combined with those of N and C [24, 25], have revealed that heavier isotopes are enriched (MDF) in predators compared to the heavier isotope abundances found in the trophic level immediately below within the same food web [23–25]. The absolute δ^{202}Hg offset depends on both the characteristics of the ecosystem and the trophic level of the biological species. In contrast to these MDF variations, the MIF signature is usually preserved during trophic transfer, making it a useful tool for characterizing sources of Hg [24, 25]. MIF patterns have recently been used in a similar manner to track MeHg cycling in the Antarctic via a new approach using ornithogenic deposits from marine predators (such as penguins) [26], and to track ice cover in Alaska using the Hg isotopic pattern in seals [27].

The Hg stable isotopic signature is considered a potential biomarker of human exposure to Hg, but has only been applied in analyses of hair [28–30] and urine [30] so far. Analyzing the Hg isotopic pattern in hair permits sources of Hg exposure such as dental amalgams [30], occupational exposure [28–30], and fish in the diet [29, 30] to be discerned. In general, an enrichment of heavier isotopes of approximately 2 ‰ (δ^{202} Hg) is observed in hair as compared to the levels of

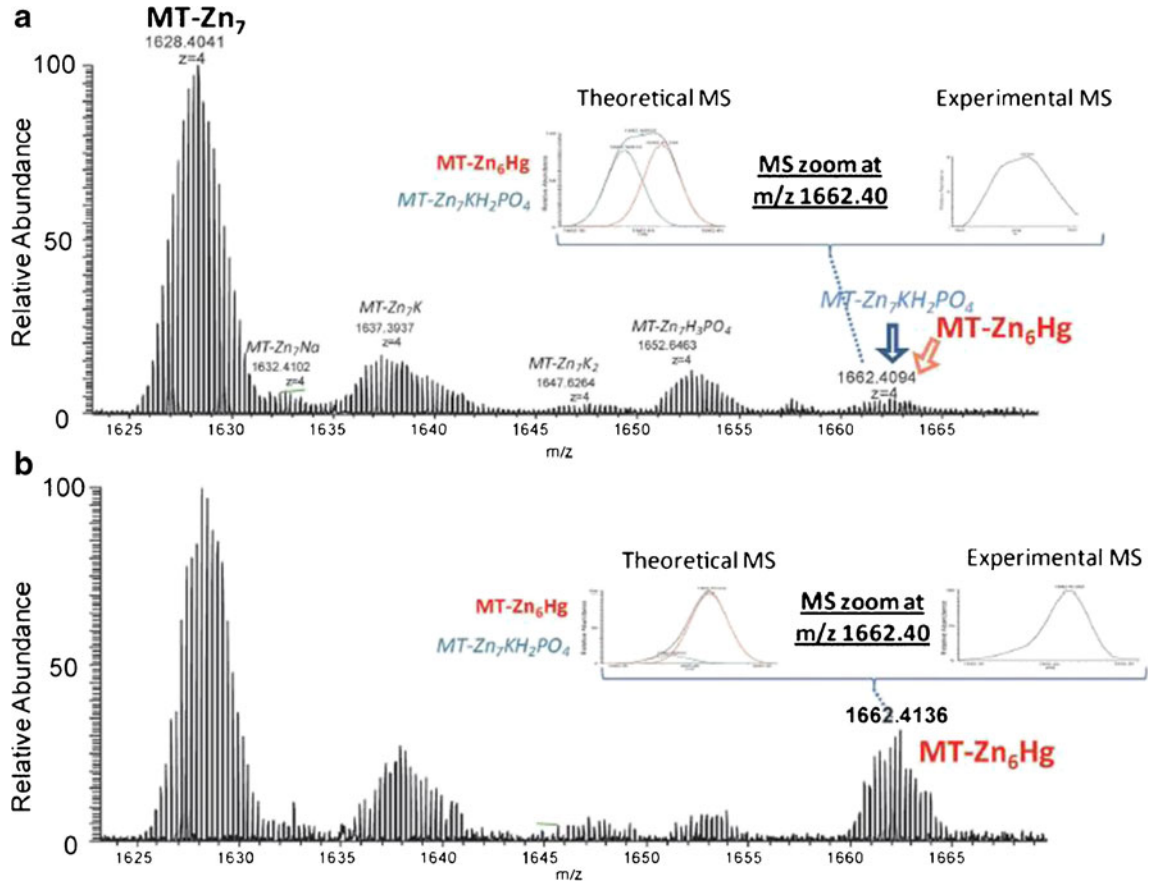

Fig. 1a–b Overlap between the mass spectra of MT–Zn_6Hg and MT–$Zn_7KH_2PO_4$ complexes after HILIC separation of **a** liver extract and **b** liver extract spiked with 100 ng Hg ml^{-1}. Reproduced from [3] with permission from the Royal Society of Chemistry

these isotopes in consumed fish [28–30]. A lighter MDF signature in human urine (mean δ^{202}Hg value: 1.35 ‰), in which Hg is excreted as iHg, in comparison to that of hair (mean δ^{202}Hg value: 2.35 ‰), in which it is excreted as MeHg, has been reported [30]. This has been associated with in vivo demethylation processes resulting in the enrichment of MeHg in heavier isotopes as opposed to (lighter) iHg excreted in urine [30].

In summary, only a few studies have exploited the advantages of analyzing the Hg isotopic pattern in metabolic processes so far. Those studies focused on bacteria-mediated Hg species transformations [19, 31, 32], Hg transport in plants [20], human excretion of Hg [28–30], and the distribution of Hg in fish organs [21, 22]. In all of the examples mentioned, the MIF signature was used as a classical tool for source identification (a technique that is commonly used in geochemistry). However, it also facilitates the estimation of the original Hg species uptake fractions using models of varying sophistication [21, 28–30], which is extremely useful complementary information in metabolic studies. On the other hand, it has been successfully used in biological systems to estimate the direction and extension of reactions [20, 30, 31], as well as to identify limiting reaction steps [20]. The main reasons that this technique has found such limited use so far in metabolic studies are that Hg concentrations in biological samples are relatively low, which directly affects measurement precision, and that there are only a limited number of laboratories that are capable of performing Hg reseach using MC-ICPMS. Mainly because there is a lack of detailed knowledge about the step-by-step mechanisms for complex biological processes involving Hg, it is a big challenge to deconvolve and interpret MDF isotopic signatures.

Therefore, it is clear why microbe-mediated processes have been the most commonly investigated processes involving Hg so far [19, 31, 32]. The possibility of growing bacteria and incubating them with Hg standards under controlled conditions facilitates the association of MDF with specific processes. One elegant strategy is to use stable isotopically enriched tracers as a starting point. This has recently been applied to investigate the Hg isotope MDF signatures during methylation by sulfate-reducing bacteria in sulfate-containing and sulfate-free environments [31]. This approach was shown to be extremely useful in the deconvolution of the induced (natural) isotopic fractionation and, as a consequence, in data interpretation [31].

The use of aquaria of selected fish species where the diet of the fish is fully characterized and kinetic sampling is carried out represents another example of MDF under laboratory-controlled conditions [21, 22]. An original approach was recently proposed for investigating species-specific pathways in a model fish [21]. The time-dependent Hg species distribution and isotopic fractionation in fish organs (muscle, brain, liver) and feces exhibited different patterns because different Hg species are metabolized differently (Fig. 2). The rapid isotopic re-equilibration observed upon ingestion of the new MeHg food source reflects the high bioaccumulation rate of MeHg [21]. In contrast, in animals exposed to iHg, the enrichment of liver in heavy δ^{202}Hg coincided with the enrichment of brain in lighter isotopes, while the Hg isotopic composition remained stable in muscle. It is well established that transport processes induce MDF, causing enrichment in heavier isotopes [20]. This result can be understood as a release of iHg from liver to the brain after its initial accumulation in the hepatic organ. Electronic microscopy observations, gene response, and MT content were used for the first time as complementary parameters in order to associate metabolic processes with Hg isotopic fractionation [21].

The works described above provide valuable information on the metabolic pathways of Hg and constitute clear evidence of the vast potential of Hg stable isotopic signature analysis in metabolic studies.

Outlook

Recent applications of two complementary approaches, speciation and isotopic signature analysis, demonstrate the importance of coupling the information provided by these two approaches into a unique (bio)molecular and isotopic pattern that can be employed to elucidate the fate of Hg in biota.

Almost all of the studies that have applied isotopic pattern determination to living organisms (as well as geochemical samples) have employed *elemental* isotopic pattern determination. The combined use of speciation and isotopic signature analysis has been limited to just a few recent studies, including some recent investigations that coupled gas chromatography (GC) to MC-ICPMS (Fig. 3) [5, 31, 33, 34]. Another approach, based on a selective extraction method for methylmercury-compound-specific stable isotope analysis (MeHg-CSIA) of biological materials, has also been reported [35]. That method consists of a specific and quantitative MeHg (multistep) extraction and purification process. The MeHg isotopic signature obtained by CV-MC-ICPMS exhibited a fourfold improvement in precision in comparison to online GC-MC-ICPMS measurements, although it was limited to the isotopic characterization of only one species. Malinovsky and Vanhaecke adapted an ion-exchange chromatography separation method [36] for the offline isotopic characterization of Hg species during an abiotic transformation. After quantitatively separating iHg and MeHg, the latter was converted into iHg through the addition of BrCl at 3%, and the resulting iHg solution was separated from the matrix before MC-ICPMS isotopic ratio determination [36].

The simultaneous determination of the isotopic compositions of various Hg species demonstrated their divergent isotopic patterns [5], which in turn reflect the different life histories of the species (biogeochemical processes, sources, reactivities, etc.). This is illustrated in Fig. 3, where the different MDF and MIF patterns of iHg and MeHg, obtained via GC-MC-ICPMS, are shown [37]. Until now, this approach has been limited to alkylation/de-alkylation processes occurring under biotic and abiotic conditions. One of the main reasons that it has not been widely applied is the lack of species-specific isotopically certified reference materials, hampering data validation. In an attempt to solve this challenge, studies have reported the isotopic patterns of Hg species in several reference materials (with diverse Hg species contents and biological or environmental origins) and a secondary reference standard commonly used in the validation of elemental isotopic analyses, UM-Almaden Mine (prepared from Hg ores from Almaden, Spain). In contrast to elemental Hg isotopic composition analyses, where NIST 3133 (iHg) is the universally accepted reference standard used for bracketing [16], species-specific characterization via GC-MC-ICPMS requires

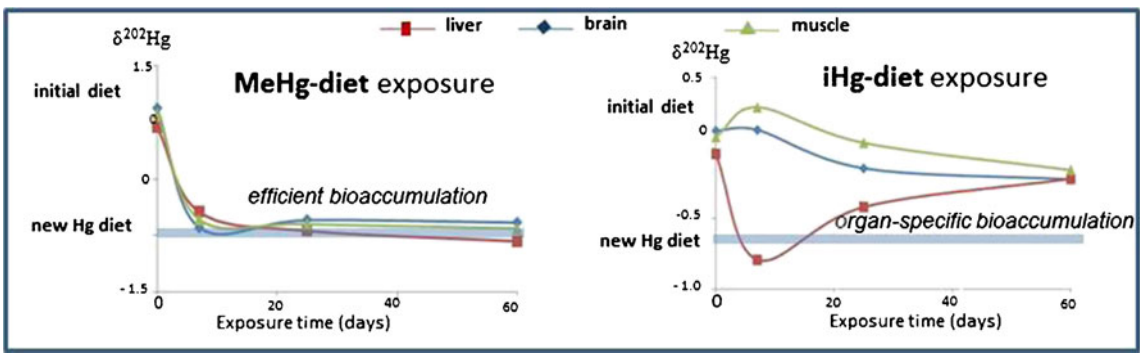

Fig. 2 Kinetic variation in MDF of Hg (δ^{202}Hg) in organs of a fish model (zebrafish *Dario renio*) exposed to dietary MeHg (*left panel*) and iHg (*right panel*), showing the different metabolization paths of these Hg species. Adapted from [23]

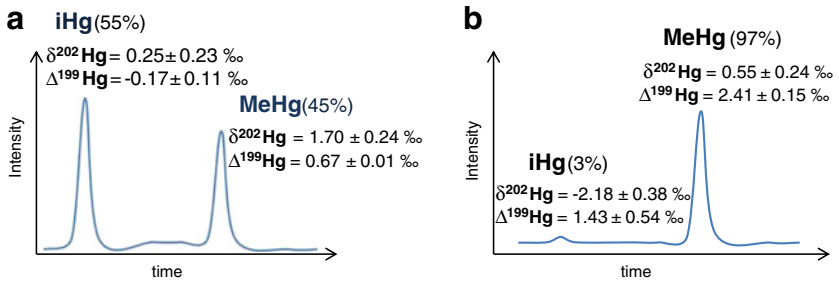

Fig. 3a–b Schematic representation of a typical gas chromatographic separation of iHg and MeHg (arbitrary units). The isotopic signatures corresponding to each species (MDF expressed as δ^{202}Hg; MIF expressed as Δ^{199}Hg and measured relative to the NIST-3133 standard) in **a** human hair and **b** tuna fish muscle are shown. Adapted from [42]

a commercially available stable and homogeneous MeHg reference standard [5, 31].

Just as speciation has evolved from simply discerning between iHg and MeHg to identifying and characterizing proteins and complex biomolecules that bind Hg, further progress in isotopic characterization at the biomolecular level is required to comprehend the metabolism and reactivity of metals in biota. Such progress could be achieved by simultaneously studying the nature of biomolecule-bound metal(loid)s and the specific isotopic signature of the bound element. The application of species-specific isotopic characterization to biomolecules that bind Hg should lead to a greater understanding of metabolic, toxicological, and environmental processes.

The methodology required to realize this goal has not yet been developed, even for other metal(loid)s, probably due to the great analytical challenge it represents. Considering the biological (and environmental) relevance of Hg, and the fact that it is used as a "model" element in isotopic research, Hg would appear to be the best element to target while developing the methodology. The availability of cutting-edge analytical instrumentation and the development of novel strategies are crucial to achieving this aim. In general, HPLC is the state of the art separation technique for the analysis of metal–biomolecule complexes. Therefore, the hyphenation of HPLC with MC-ICPMS would allow the isotopic characterization of the element of interest in biomolecules.

So far, liquid chromatographic separation has been included as an *off-line* sample preparation step for matrix removal before the isotope ratios of elements such as Zn and Cu are determined [38]. The few recent *on-line* applications of this technique have all focused on elemental analyses (of Sr, Pu, or Si); none of those considered speciation. This can be attributed to the various analytical challenges associated with the hyphenation of chromatographic techniques and MC-ICPMS, as recently reviewed [39, 40]. Nonquantitative recovery from the chromatographic column, which is commonly observed in the elution of metal(loid) species by HPLC, will clearly induce isotopic fractionation [38, 40]. The recurrent use of mobile phase gradients is required for species separation by HPLC. This implies progressive modification of the (organic) composition of the mobile phase, which causes plasma instability and consequently affects the isotope ratio measurements [40, 41]. The use of a desolvation unit (Aridus II) has proven an effective method of removing methanol prior to its introduction into the plasma, thus preserving the sensitivity of sulfur metabolic studies [41].

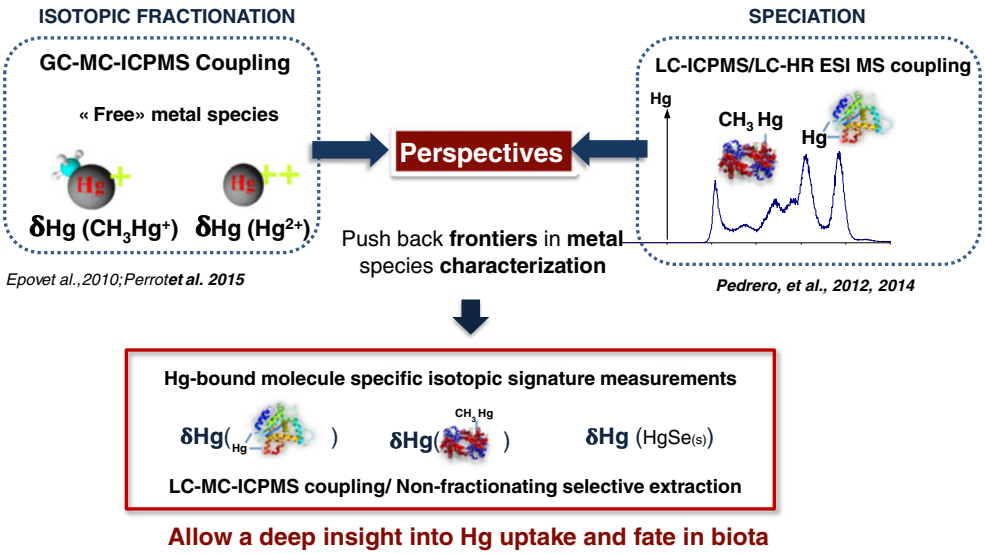

Fig. 4 Illustration of the potential benefits of combining (bio)molecular and isotopic signatures

The coupling of HPLC to MC-ICPMS implies the need to measure isotope ratios in transient signals, leading to reduced performance compared to continuous sample introduction. Several approaches have been developed for the precise and accurate measurement of isotope ratios in transient signals, including peak apex; peak area integration; the average isotope ratio within the peak (point by point); and the linear regression slope method [40]. The latter [42] leads to a significant improvement in accuracy and precision [37, 39, 40, 42]. It has been successfully applied to determine species-specific (iHg and MeHg) isotopic compositions using GC-MC-ICPMS [37].

The chromatographic separation of proteins and biomolecules is generally performed by HPLC, which produces broader signals than GC-ICPMS. In addition, a poor resolution (pronounced peak shoulders/tails) is usually obtained when, for example, Hg-binding proteins are separated by size exclusion chromatography [11]. Even though new techniques such as nano- and capillary chromatography can minimize peak broadening, the smaller injected volume leads to insufficient sensitivity of MC-ICPMS detection.

An alternative method of mitigating any potential alteration of the isotopic signature during chromatographic separation is to perform direct isotopic analyses of the target biomolecules after specific and adequate physicochemical isolation [43]. An example of this is the extraction and purification of Cu metallothioneins and Cu, Zn superoxide dismutase in yeast cells and azurin expressed in *Escherichia coli* and *Pseudomonas aeruginosa*. The isotopic composition of Cu (and Zn in the SOD) was determined and a divergence in the isotopic signatures of two proteins within a single cell was demonstrated, so this method can be used to track biological pathways [43].

A common bottleneck in both approaches (direct analysis after protein purification or HPLC-MC) is the sample preparation stage. This is a crucial stage that usually involves several steps that could induce isotopic fractionation, principally due to (partial) degradation of the target biomolecule and non-quantitative recovery. Another critical issue when determining the isotopic signatures of biomolecule-bound metal(loid)s is the lack of commercially available isotopically characterized standards, which are required for isotopic composition determination and method validation. The synthesis (generally by incubation) of model biomolecules and/or biomolecules (cysteine, glutathione, etc.) and proteins (albumin, hemoglobin, etc.) commonly found in living organisms under controlled conditions with Hg isotopic standards could be a potential solution to this problem with validation. However, it is important to note that such complexes easily degrade, leading to mass-dependent isotopic fractionation.

Despite the analytical challenges associated with the characterization of unique isotopic and biomolecular fingerprints, the development of such an approach is crucial to obtaining exceptional results that could greatly enhance our understanding of Hg in biota (Fig. 4). It is unquestionably an analytical approach that will provide revolutionary insights into the Hg biogeochemical cycle at the molecular scale. In the long term, this highly innovative methodology could also be applied to other metal(loid)s, leading to a much deeper understanding of their roles in biological and environmental processes.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. Lamborg CH, Hammerschmidt CR, Bowman KL, Swarr GJ, Munson KM, Ohnemus DC, et al. A global ocean inventory of anthropogenic mercury based on water column measurements. Nature. 2014;512(1):65–8.
2. Harris HH, Pickering IJ, George GN. The chemical form of mercury in fish. Science. 2003;301(5637):1203.
3. Pedrero Z, Ouerdane L, Mounicou S, Lobinski R, Monperrus M, Amouroux D. Identification of mercury and other metal complexes with metallothioneins in dolphin liver by hydrophilic interaction liquid chromatography with the parallel detection by ICP MS and electrospray hybrid linear/orbital trap MS/MS. Metallomics. 2012;4(5):473–9.
4. Pedrero Zayas Z, Ouerdane L, Mounicou S, Lobinski R, Monperrus M, Amouroux D. Hemoglobin as a major binding protein for methylmercury in white-sided dolphin liver. Anal Bioanal Chem. 2014;406(4):1121–9.
5. Epov VN, Rodriguez-Gonzalez P, Sonke JE, Tessier E, Amouroux D, Bourgoin LM, et al. Simultaneous determination of species-specific isotopic composition of Hg by gas chromatography coupled to multicollector ICPMS. Anal Chem. 2008;80(10):3530–8.
6. Leermakers M, Baeyens W, Quevauviller P, Horvat M. Mercury in environmental samples: speciation, artifacts and validation. TrAC–Trend Anal Chem. 2005;24(5):383–93.
7. Clémens S, Monperrus M, Donard OFX, Amouroux D, Guérin T. Mercury speciation in seafood using isotope dilution analysis: a review. Talanta. 2012;89:12–20.
8. Brombach CC, Chen B, Corns WT, Feldmann J, Krupp EM. Methylmercury in water samples at the pg/L level by online preconcentration liquid chromatography cold vapor-atomic fluorescence spectrometry. Spectrochim Acta Part B. 2015;105:103–8.
9. Brombach CC, Gajdosechova Z, Chen B, Brownlow A, Corns WT, Feldmann J, et al. Direct online HPLC-CV-AFS method for traces of methylmercury without derivatisation: a matrix-independent method for urine, sediment and biological tissue samples. Anal Bioanal Chem. 2015;407:973–81. doi:10.1007/s00216-014-8254-1.
10. Sundberg J, Ersson B, Lönnerdal B, Oskarsson A. Protein binding of mercury in milk and plasma from mice and man—a comparison between methylmercury and inorganic mercury. Toxicology. 1999;137(3):169–84.

11. Pedrero Z, Mounicou S, Monperrus M, Amouroux D. Investigation of Hg species binding biomolecules in dolphin liver combining GC and LC-ICP-MS with isotopic tracers. J Anal At Spectrom. 2011;26(1):187–94.
12. Krupp EM, Mestrot A, Wielgus J, Meharg AA, Feldmann J. The molecular form of mercury in biota: identification of novel mercury peptide complexes in plants. Chem Commun. 2009;28:4257–9. doi:10.1039/b823121d(28):4257-4259.
13. Krupp EM, Milne BF, Mestrot A, Meharg AA, Feldmann J. Investigation into mercury bound to biothiols: structural identification using ESI-ion-trap MS and introduction of a method for their HPLC separation with simultaneous detection by ICP-MS and ESI-MS. Anal Bioanal Chem. 2008;390(7):1753–64.
14. Trümpler S, Lohmann W, Meermann B, Buscher W, Sperling M, Karst U. Interaction of thimerosal with proteins—ethylmercury adduct formation of human serum albumin and β-lactoglobulin A. Metallomics. 2009;1(1):87–91.
15. Trümpler S, Meermann B, Nowak S, Buscher W, Karst U, Sperling M. In vitro study of thimerosal reactions in human whole blood and plasma surrogate samples. J Trace Elem Med Biol. 2014;28(2):125–30.
16. Bergquist BA, Blum JD. Mass-dependent and -independent fractionation of Hg isotopes by photoreduction in aquatic systems. Science. 2007;318(5849):417–20.
17. Blum JD, Sherman LS, Johnson MW. Mercury isotopes in earth and environmental sciences. Ann Rev Earth Planet Sci. 2014;42:249–69.
18. Yin R, Feng X, Li X, Yu B, Du B. Trends and advances in mercury stable isotopes as a geochemical tracer. Trend Environ Analyt Chem. 2014;2:1–10.
19. Kritee K, Blum JD, Reinfelder JR, Barkay T. Microbial stable isotope fractionation of mercury: a synthesis of present understanding and future directions. Chem Geol. 2013;336:13–25.
20. Yin R, Feng X, Meng B. Stable mercury isotope variation in rice plants (*Oryza sativa* L.) from the Wanshan Mercury Mining District, SW China. Environ Sci Technol. 2013;47(5):2238–45.
21. Feng C, Pedrero Z, Gentès S, Barre J, Renedo M, Tessier E, et al. Specific pathways of dietary methylmercury and inorganic mercury determined by mercury speciation and isotopic composition in zebrafish (*Danio rerio*). Environ Sci Technol. 2015;49:12984–93. doi:10.1021/acs.est.5b03587.
22. Kwon SY, Blum JD, Carvan MJ, Basu N, Head JA, Madenjian CP, et al. Absence of fractionation of mercury isotopes during trophic transfer of methylmercury to freshwater fish in captivity. Environ Sci Technol. 2012;46(14):7527–34.
23. Gantner N, Hintelmann H, Zheng W, Muir DC. Variations in stable isotope fractionation of Hg in food webs of Arctic lakes. Environ Sci Technol. 2009;43(24):9148–54.
24. Perrot V, Pastukhov MV, Epov VN, Husted S, Donard OFX, Amouroux D. Higher mass-independent isotope fractionation of methylmercury in the pelagic food web of Lake Baikal (Russia). Environ Sci Technol. 2012;46(11):5902–11.
25. Senn DB, Chesney EJ, Blum JD, Bank MS, Maage A, Shine JP. Stable isotope (N, C, Hg) study of methylmercury sources and trophic transfer in the northern Gulf of Mexico. Environ Sci Technol. 2010;44(5):1630–7.
26. Zheng W, Xie Z, Bergquist BA. Mercury stable isotopes in ornithogenic deposits as tracers of historical cycling of mercury in Ross Sea, Antarctica. Environ Sci Technol. 2015;49(13):7623–32.
27. Masbou J, Point D, Sonke JE, Frappart F, Perrot V, Amouroux D, et al. Hg stable isotope time trend in ringed seals registers decreasing sea ice cover in the Alaskan Arctic. Environ Sci Technol. 2015;49(15):8977–85.
28. Laffont L, Sonke JE, Maurice L, Monrroy SL, Chincheros J, Amouroux D, et al. Hg speciation and stable isotope signatures in human hair as a tracer for dietary and occupational exposure to mercury. Environ Sci Technol. 2011;45(23):9910–6.
29. Li M, Sherman LS, Blum JD, Grandjean P, Mikkelsen B, Weihe P, et al. Assessing sources of human methylmercury exposure using stable mercury isotopes. Environ Sci Technol. 2014;48(15):8800–6.
30. Sherman LS, Blum JD, Franzblau A, Basu N. New insight into biomarkers of human mercury exposure using naturally occurring mercury stable isotopes. Environ Sci Technol. 2013;47(7):3403–9.
31. Perrot V, Bridou R, Pedrero Z, Guyoneaud R, Monperrus M, Amouroux D. Identical Hg isotope mass dependent fractionation signature during methylation by sulfate-reducing bacteria in sulfate and sulfate-free environment. Environ Sci Technol. 2015;49(3):1365–73.
32. Rodŕiguez-Gonźalez P, Epov VN, Bridou R, Tessier E, Guyoneaud R, Monperrus M, et al. Species-specific stable isotope fractionation of mercury during Hg(II) methylation by an anaerobic bacteria (*Desulfobulbus propionicus*) under dark conditions. Environ Sci Technol. 2009;43(24):9183–8.
33. Krupp EM, Donard OFX. Isotope ratios on transient signals with GC-MC-ICP-MS. Int J Mass Spectrom. 2005;242(2–3):233–42.
34. Perrot V, Jimenez-Moreno M, Berail S, Epov VN, Monperrus M, Amouroux D. Successive methylation and demethylation of methylated mercury species (MeHg and DMeHg) induce mass dependent fractionation of mercury isotopes. Chem Geol. 2013;355:153–62.
35. Masbou J, Point D, Sonke JE. Application of a selective extraction method for methylmercury compound specific stable isotope analysis (MeHg-CSIA) in biological materials. J Anal At Spectrom. 2013;28(10):1620–8.
36. Malinovsky D, Vanhaecke F. Mercury isotope fractionation during abiotic transmethylation reactions. Int J Mass Spectrom. 2011;307(1–3):214–24.
37. Epov VN, Berail S, Jimenez-Moreno M, Perrot V, Pecheyran C, Amouroux D, et al. Approach to measure isotopic ratios in species using multicollector-ICPMS coupled with chromatography. Anal Chem. 2010;82(13):5652–62.
38. Maréchal CN, Télouk P, Albarède F. Precise analysis of copper and zinc isotopic compositions by plasma-source mass spectrometry. Chem Geol. 1999;156(1–4):251–73.
39. Epov VN, Berail S, Pécheyran C, Amouroux D, Donard OFX. Isotopic analysis via multi-collector inductively coupled plasma mass spectrometry in elemental speciation. In: Vanhaecke F, Degryse P, editors. Isotopic analysis: fundamentals and applications using ICP-MS. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2012. p. 495–517.
40. Rodríguez-González P, Epov VN, Pecheyran C, Amouroux D, Donard OFX. Species-specific stable isotope analysis by the hyphenation of chromatographic techniques with MC-ICPMS. Mass Spectrom Rev. 2012;31(4):504–21.
41. San Blas OG, Marchante Gayón JM, García Alonso JI. Evaluation of multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) for sulfur metabolic studies using 34S-labelled yeast. J Anal At Spectrom. 2015;30(8):1764–73.
42. Fietzke J, Liebetrau V, Günther D, Gürs K, Hametner K, Zumholz K, et al. An alternative data acquisition and evaluation strategy for improved isotope ratio precision using LA-MC-ICP-MS applied to stable and radiogenic strontium isotopes in carbonates. J Anal At Spectrom. 2008;23(7):955–61.
43. Zhu XK, Guo Y, Williams RJP, O'Nions RK, Matthews A, Belshaw NS, et al. Mass fractionation processes of transition metal isotopes. Earth Planet Sci Lett. 2002;200(1–2):47–62.