Mercury in environmental samples: Speciation, artifacts and validation

Martine Leermakers, Willy Baeyens, Philippe Quevauviller, Milena Horvat

Speciation of mercury compounds in environmental samples requires rigorous analytical procedures at each stage of sample collection, treatment and measurement. Sampling, the first step, is generally only critical for water samples. The best materials for water-sample storage and processing are Pyrex and Teflon (PTFE or FEP) after a severe cleaning procedure. Extraction of the Hg species from its matrix, the second step, requires an aggressive treatment, such as acid extraction (mostly combined with solvent extraction), distillation or alkaline extraction. Extraction is one of the most critical steps, and, for biota and sediments, almost certainly the most critical, because two conflicting issues need to be addressed – obtaining adequate recovery, and preventing losses. Extraction efficiency and validation are discussed as well as methylation artifacts, especially when the distillation method is applied to sediments. Separation of the Hg species, the third step, includes derivatization followed by gas chromatography (GC), new GC improvements and liquid chromatography (LC), which has, since the development of more sensitive detectors, resulted in wider applications in environmental studies. Detection of the Hg species is the fourth and last step in the analytical procedure. The development of commercial, relatively inexpensive, extremely sensitive, selective cold vapor atomic fluorescence spectrometry (CV-AFS) instrumentation in the late 1980s and 1990s made this the most popular detector for the laboratories working on the biogeochemical cycling of Hg. In recent years, the use of inductively coupled plasma mass spectrometry (ICP-MS) in speciation analysis has increased tremendously. Besides its high sensitivity and selectivity, ICP-MS offers the opportunity to perform speciated isotope dilution mass spectrometry (SID-MS). Finally, as other measurement fields, speciation analysis requires suitable reference materials to be available for the verification of accuracy and to meet quality-assurance needs. We also address these important aspects.

Keywords: Artifacts; Detection; Environmental samples; Extraction; Mercury; Reference materials; Sampling; Speciation; Validation

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1. Environmental and toxicological aspects

Among the toxic trace metals, mercury (Hg) is one of the most hazardous environmental pollutants. Hg exists in a large number of physical and chemical forms with a large variety of properties that determine its complex distribution, biological enrichment and toxicity. The most important chemical forms are elemental Hg (Hg^0), inorganic Hg (Hg^{2+}), monomethylmercury (MMHg, CH_3Hg^+) and dimethylmercury (DMHg, CH_3HgCH_3). In the biogeochemical cycle of Hg, these species may all interchange in atmospheric, aquatic and terrestrial environments. The main exposure pathway of Hg to humans is through the consumption of marine fishery products (fish, shellfish, crustaceans). In most foodstuffs, Hg is predominantly in the inorganic form and in low concentrations (<20 ng/g) in fish and fish products [1]. However, Hg occurs primarily in the methylated form and levels greater than 1200 ng/g have been found in edible portions of shark, swordfish and tuna. Similar levels have also been found in fish of affected freshwater systems and these have led to the introduction of advisory limits for fish consumption in countries such as Canada, Sweden and the USA.

The toxic effects of Hg depend on the chemical form. MMHg compounds are considerably more toxic than elemental Hg and its inorganic salts. MMHg is efficiently adsorbed from the gastro-intestinal tract, and it passes the blood-brain and placenta barriers. MMHg primarily affects the central nervous system. In severe cases, specific

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anatomical areas of the brain are affected, causing irreversible damage.

Well-known outbreaks of Hg poisoning are the contamination of Minamata Bay by an acetaldehyde plant from 1948 to 1960 [2] and the poisoning of bread in Iraq in 1972 after grain seeds had been treated with organomercury fungicides [3]. As a result of these outbreaks, the use of Hg, especially organomercurials, in agricultural and industrial applications has been banned or strongly limited in most countries.

During the last decade, improvements in analytical techniques, speciation and reaction-oriented environmental Hg research has considerably improved knowledge about the biogeochemical cycling of Hg. The main transformation pathways between the various Hg species in the different environmental compartments have been identified (Fig. 1), although uncertainty remains about the reaction mechanisms and/or biological species involved in the interconversion of Hg species in the ocean.

Although all forms of Hg are poisonous, the ecological and human health effects of Hg are generally related to the environmental transformations of inorganic Hg to the toxic and biomagnification-prone MMHg. Despite significant improvements in instrumentation, MMHg determination is hindered by traditional problems related to non-quantitative recoveries and to questions about the possibility of artifact formation and transformations of methylmercury during the sample-preparation and separation steps.

Although DMHg has been found in fish, water and sediments [5–9], most analytical protocols used would not provide reliable results for DMHg. Only very few studies deal with other Hg species (e.g., monoethylmercury, monophenylmercury, methoxyethylmercury, and Thimerosal). The importance of Hg-speciation studies has been highlighted in review articles published in the last decade [6,8,10–15].

In general, speciation of Hg species involves the following steps:

(1) sample collection/pretreatment/preservation/storage;
(2) extraction of Hg from the matrix/clean-up/preconcentration;
(3) separation of Hg species of interest; and,
(4) detection.

The appropriate analytical methods depend on the nature of the sample and concentration level. However, the most critical compartments for speciation are still linked to the solid phase – biota and soil/sediments. Extraction is a very subtle step because (1) the whole species content may not be liberated, and (2) artifacts can occur so that some organomercury species can be destroyed or formed (interspecies exchange). Often the extraction step for Hg speciation is applied in combination with a clean-up/preconcentration step (e.g., distillation, solvent extraction, or headspace). Artifacts during the extraction/clean-up phase have been specifically studied with the latter methods. Using isotope-labeled compounds, it is possible to study interspecies

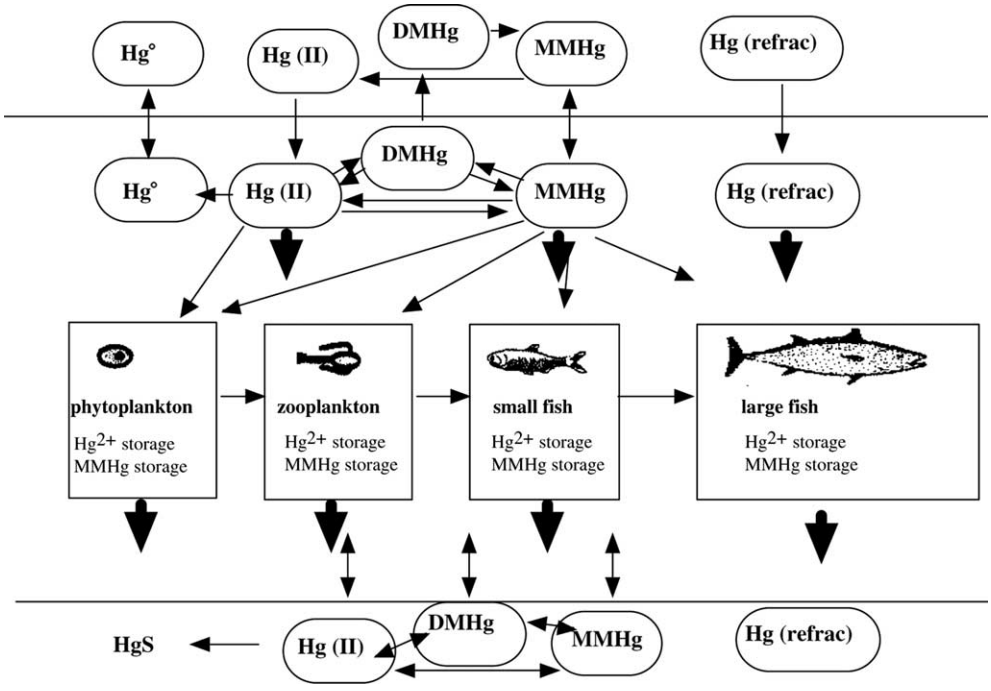

Figure 1. Hg transformation in the aquatic environment (adapted from [4]).

exchange. We will pay special attention to these artifacts in this paper.

2. Sample collection, pretreatment and storage

Rigorous cleaning procedures must be used for all equipment, including sampling equipment, and laboratory ware that comes in contact with the samples, especially for the speciation of Hg at low concentrations, as in water samples. This decontaminated material and the samples are stored under Hg-free conditions. Samples are further processed as soon as possible after collection, and, to avoid contamination, especially by inorganic Hg, cleanroom facilities are required.

2.1. Water

The best materials for sample storage and processing are Pyrex and Teflon (PTFE or FEP). Several cleaning procedures can be used (e.g., aqua regia, chromic acid, nitric acid, and BrCl). A final soaking of Teflon in hot 70°C 1% HCl removes all traces of oxidizing compounds (e.g., chlorine) that can destroy MMHg in solution [14].

The most volatile forms present in water are Hg° and DMHg. They should be removed from the samples immediately after collection by aeration with collection on gold (for total gaseous Hg) and Carbotrap or Tenax (for DMHg). After filtration, samples should be preserved prior to storage. For total Hg, samples can be acidified with HCl or HNO_3 or with the addition of an oxidant (BrCl); whereas, for MMHg, the samples can be acidified with HCl or stored unpreserved deep-frozen [14].

2.2. Air

Although the analysis of total gaseous Hg and particulate-phase Hg in air can be conducted with high accuracy and precision [16], there are still many problems related to the separation of specific Hg compounds in air. In general, two approaches can be used: (1) selective adsorption methods, in which separation is operationally defined, and (2) chromatographic methods, which allow identification of the organomercury compounds. Selective adsorption methods allow the operational separation of Hg°, Hg(II), MMHg and DMHg and have been reviewed extensively in the literature [10,17]. GC techniques are limited to the determination of Hg°, MMHg and DMHg. MMHg and DMHg can be trapped using Carbotrap or Tenax. Hg° is retained by gold amalgamation.

The quantification of total Hg in flue gas (e.g., from electric power plants) depends on a well-characterized, low-blank carbon-based sorbent trap, with historical applications of either KCl/soda lime (MESA Method) or KCl/quartz (FAMS Method) and quartz-fiber filters to separate and determine the speciation, as needed [18].

2.3. Biological samples

An important fraction of the Hg burden in biota, especially fish, can be present as MMHg (e.g., Baeyens et al. [19] found that MMHg averaged 95% in Greater North Sea fish). However, relatively little is known on the effect of storage on the stability of methylmercury in biological samples. Fresh samples are usually stored deep-frozen, lyophilized in darkness or, sometimes, sterilized. For some organisms, methylmercury may decompose with repeated freezing and unfreezing (particularly in bivalves).

2.4. Sediments

In sediments and soils, the percentage of MMHg is usually very low, resulting from equilibrium between methylation and demethylation reactions. Samples are usually analyzed fresh, or, if long-term storage is required, samples should be kept in the dark at low temperatures or lyophilized.

There is still much debate on the effect of sample pretreatment on the MMHg levels obtained. In some cases, no differences were found between fresh sediments and dried (lyophilized) sediments [20,21], whereas, in other cases, much higher results were found in dried sediments compared to wet sediments [21]. Preliminary tests have shown that the presence of oxygen and porewater during sample preparation may also play a role [21]. Further investigation in this field is required.

3. Extraction procedures

The most commonly used procedures for the extraction of organomercury species from environmental samples are acid extraction (mostly combined with solvent extraction), distillation and alkaline extractions. The extraction step is still one of the most critical steps and, for biota and sediments, almost certainly the most critical. Two conflicting issues need to be addressed: obtaining an adequate recovery; and, preventing losses, especially destruction of the compound(s) [22]. Basically, the extraction should be performed in such a way that the analyte is separated from the interfering matrix without loss, contamination, or change of speciation, and with the minimum of interferences.

Acid digestion combined with solvent extraction was first proposed by Westöö [23] for the extraction of MMHg in foodstuffs. The method involved leaching the Hg compounds from the sample using concentrated hydrochloric acid, followed by extraction of the metal chloride into benzene. The Hg species were then taken into an aqueous phase by conversion to the hydroxide using ammonium hydroxide, saturated with sodium sulfate.

Cysteine, thiosulfate or some other thiol-containing reagent are now more commonly used to facilitate phase

transfer [6]. Because GC was used to separate the species, the aqueous phase was acidified with concentrated hydrochloric acid and back-extracted with benzene prior to injection.

Later, many modifications of Westöö's methods were proposed for selective extraction of methylmercury from a mineral acidic medium containing NaCl [24,25], KBr [26–28] and iodoacetic acid [29,30], generally using successive extractions with organic solvents such as benzene [31,32], toluene [33,34], chloroform [26,35] or dichloromethane [36,37]. It seems that benzene is not well suited to MMHg extractions at low concentrations down to 0.5 ng/l [38].

Several authors recommend a back extraction of the Hg species from the benzene or toluene phase to the aqueous phase, in order to clean or preconcentrate the extracted species, using cysteine or sodium thiosulphate [23,34].

Problems derived from solvent background may arise when toluene is used and the detection is carried out by microwave-induced plasma-atomic emission spectrometry (MIP-AES) [39].

In the case of MMHg speciation using chloroform, addition of complexing agents to facilitate the extraction of methylmercury to the chloroformic phase has been proposed [26,35], while addition of $HgC1_2$ [23,40] or $CuC1_2$ [41] solutions has been recommended to release the MMHg from the –SH groups complexing the Hg species in the solid. This methodology is not effective for extracting Hg(II) [42], nor is it efficient for methylmercury from soils and sediments [41]. The breakdown of methylmercury to inorganic or elemental Hg, with subsequent losses due to volatilization, has been documented when heating with concentrated hydrochloric acid [41,42].

For sediments, several acids have been proposed. Bloom et al. [43] used 5% H_2SO_4 in combination with $CuSO_4$ and KBr. 4 M HNO_3 and 4 M HCl have been used by Tseng et al. [42] and Leermakers et al. [44]. Room-temperature procedures [43] or procedures at elevated temperatures using either conventional heating or microwave-assisted heating have been used [42,44]. Microwave-assisted extraction has been shown to be an efficient improvement in the extraction of various organic and organometallic compounds from the environmental matrices [45]. Both microwave-assisted acid extraction [42,44] and microwave-assisted organic solvent extraction [46] have been used for the extraction of MMHg from sediments. Both open vessel (working at atmospheric pressure) and closed vessel (working under controlled pressure) microwave ovens have been used.

In water samples, MMHg complexed to organic ligands may be extracted by $HCl/KCl/CH_2Cl_2$ followed by back extraction in water [36]. Recently, an alternative method was proposed for the simultaneous extraction of Hg^{2+} and MMHg in natural waters at pg/l levels. Hg^{2+} and MMHg are extracted into toluene as dithiozonates after acidification of the water sample, followed by back extraction into an aqueous solution of Na_2S, and removal of H_2S by purging with N_2 [47].

Vapor distillation, in a stream of air or nitrogen at 150°C, of a homogenate of the solid sample in diluted H_2SO_4 or HCl with excess of NaCl was first proposed by Nagase et al. [48] and Horvat et al. [49] for the non-chromatographic separation of inorganic Hg and MMHg. The more volatile CH_3HgCl compound formed is distilled and collected in a closed tube. This tube is water-cooled and stored in the dark in order to keep extracted MMHg degradation at a minimum before its final determination. The use of HCl alone for this distillation is not advisable, because it may not be able to release completely the MMHg contained in the sediment. In combination with the ethylation technique, Carbotrap or Tenax preconcentration, GC separation and AFS detection [36,41], this became the method of choice for the extraction of MMHg in sediments because of its high efficiency (MMHg recoveries practically 100%), elimination of inorganic Hg in the extract and formation of clean aqueous extracts that eliminate interferences in the ethylation step. However, investigations in the mid 1990s showed that the distillation procedure used to separate methylmercury from both water and sediment samples artificially generates MeHg aided by the presence of natural organic substances (see below).

3.1. Alkaline digestion and extraction

Extractions in KOH-methanol [5] and tetramethyl ammonium hydroxide (TMAH) [50] have been proposed to release MMHg from biological samples and sediments while maintaining original Hg–C bonds. This is the most efficient method for extraction of MMHg from biological samples, but, for sediments, serious problems are encountered in subsequent steps (preconcentration, separation or detection) due to the high levels of organic matter, sulfides or ferric ions co-extracted with the methylmercury species being sought using this sample treatment [41].

Supercritical fluid extraction (SFE) has also been used to extract MMHg from sediments [39,51]. Lorenzo et al. [51] compared manual, microwave-assisted techniques and SFE for extracting Hg from aquatic sediments. Higher recoveries were obtained with microwave-extraction techniques compared to manual extraction techniques and SFE.

Not all the available methods extract the Hg species from solid samples (soil, sediment or biological material) with acceptable efficiency. The procedure giving the best recovery for methylmercury from soil (95 ± 4%) is the distillation method [41]; from fish tissue, it is alkaline digestion using TMAH with focused microwave power (95–105%) [42].

3.2. Extraction efficiency and validation

There is no standardized method to assess the extraction efficiency of a particular method. However, a recent overview of the certification of total Hg and methylmercury in estuarine sediment recommended the use of standard additions as the best means to establish the efficiency of the method. This is achieved by spiking the material under investigation at three different levels prior to extraction [52]. However, this method does not guarantee that there is no loss (e.g., degradation) or complete recovery of a given compound from the matrix (aqueous solution, biological tissue or sediment). It is current practice to apply compensation for these losses by correcting the results with a recovery factor in order to achieve a better approximation to the true value in a material. These correction factors are established after undertaking recovery studies, which are an essential component of the validation of extraction-based techniques. As described below, this practice is not without problems, and the most critical aspect is the lack of common strategies for the assessment of recovery and the way in which corrections have to be applied. Recovery evaluation using surrogates or spikes implies the assumption that the extraction of the spike is equivalent of the native analyte. In practice, it is often difficult to demonstrate that equivalence so that the only solution is to accept the above assumption (extraction of spike is equivalent to that of native compound). A special form of this method is the standard addition method where spiking at different levels is performed. Depending on the number of levels (i.e., two, three or more) and/or the concentration jump chosen for the spiking experiment, a different recovery evaluation can be obtained. The best method is to use the same organometal compound but containing a strongly enriched metal isotope, different from the major natural one. Recoveries can be assessed as long as the native analyte and the spike come into equilibrium. The latter is impossible to verify so this approach, although the best of the state of the art, may still yield a biased recovery estimate.

By using a spiked solution containing the different organomercury species that are isotopically labeled with different isotopes (for Hg, there are sufficient isotopes), it is possible to evaluate species interconversion or degradation [53]. However, this procedure is limited by the availability and the cost of isotopically enriched organomercury compounds and instrumentation for their determination.

3.3. Methylation artifact

Significant artificial methylmercury production during analysis was first highlighted at the Fourth Conference on Mercury as a Global Pollutant, which took place in Hamburg in 1996 [43,54]. The production of artificial MMHg during the analytical procedure is a problem reported especially when the distillation method is applied on sediments, and it may result in a significant bias in measurements. Natural sediments often contain very low amounts of MMHg, representing only 0.1–1.5% of total Hg, so, even if artificial Hg methylation occurs in the small proportion of 0.02–0.03% of inorganic Hg only, this can result in 30–80% overestimation of MMHg concentrations in sediment.

In the aftermath of these early investigations, critical comments concerning the certified MMHg values in sediment reference materials were made by a small group of laboratories, which based their conclusions on limited scientific evidence [55]. The controversy led to a Workshop financed by the European Commission; the Workshop's conclusions were summarized in a special issue of *Chemosphere* published in 1999. The causes and factors involved in methylmercury formation during analysis were systematically evaluated. A series of different techniques commonly used to extract MMHg from various matrixes were screened and tested to evaluate their potential to accidentally generate MMHg from inorganic Hg^{2+} during sample preparation. The results highlighted the assumption that, in certain conditions, Hg-species transformations could occur during the sample-pretreatment step, especially with distillation-based methods.

The magnitude of the artificial methylation using the distillation procedure:

- increased linearly with both total Hg content and DOC content (in water samples);
- was highest in the presence of carboxylic acids, humic materials, degraded terrestrial leaves or particles with large surface area;
- was not present in fresh plant material; and,
- was not observed in the presence of biological tissues and showed a time–temperature dependence.

The observation was not limited to sediment distillates. Methylation artifacts were also noticed during hot alkaline digestion and supercritical fluid extraction (SFE). Acid leaching with H_2SO_4/KBr/$CuSO_4$ at room temperature or with diluted HNO_3 (short microwave-extraction procedure) followed by CH_2Cl_2 extraction and back extraction in water did not give rise to methylation artifacts [42–44,56]. Later experiments showed that the methylation artifact was linked to the amount of reactive Hg in the leachate or distillate [57].

Using speciation isotope dilution ICP-MS coupled to capillary GC (CGC), Rodriguez et al. [58] concluded that the amount of inorganic Hg present in the final derivatization and extraction step is the determining factor for the methylation artifacts and that transalkylation reactions in the final organic phase are the most plausible mechanisms. In their work, the derivatized compounds were extracted in an organic phase for

injection in the CGC in contrast to the other studies that used: Tenax collection, thermosorption, GC, pyrolysis and CV-AFS [57]; ICP-MS [54]; headspace injection of the derivatized compounds [44,56]; or, cryogenic trapping–GC-AAS [59]. For example, in the case of headspace injection [44,56], methylation artifacts were never observed and analyses of certified reference materials (CRMs) agreed very well with the proposed MeHg levels. Therefore, the controversial thesis about certified MeHg contents in sediment reference material does not seem to be supported by strong scientific evidence. Moreover, experts participating in the 1998 Workshop agreed that existing CRMs fulfill the purposes of verifying the accuracy of current methods and achieving data comparability [55].

Methylation artifacts have also been shown to occur during derivatization due to the presence of small impurities of methyl groups in the derivatization reagents [41] as well as during separation due to the silanizing agent (dimethyldisilizane) used to prepare the GC column [59].

4. GC separation methods

Apart from the problems associated with the extraction of organomercurials mentioned above, problems also exist with the chromatography of the organomercury halides. The different packed and capillary columns used have been reviewed by Baeyens [10]. In order to prevent ion-exchange and adsorption processes on the column (which cause undesirable effects such as tailing, change of the retention time and decrease of peak areas/heights), passivation of packing material is needed with the Hg(II)-chloride in benzene (or toluene). Moreover, the more common GC detectors may lack the required selectivity to be used for the speciation of Hg in environmental samples. For instance, electron capture detection has commonly been used for methylmercury speciation in biological samples. Its unselective response required laborious clean-up processes of the extract in the organic phase.

4.1. Derivatization

In order to overcome these problems, alternative methods were developed involving precolumn derivatization of Hg species. The non-polar derivatives can then be separated on non-polar packed [29,36,60] or capillary columns [25]. Iodation with acetic acid [29,30], hydration with $NaBH_4$ [42,61–63], aqueous phase ethylation with $NaBEt_4$ [36,41] and derivatization with a Grignard reagent (e.g., ethylation, butylation, and propylation) [25] are the most commonly used methods.

Aqueous phase ethylation, room-temperature precollection and separation by GC with CV-AFS detection has become the most frequently used method in laboratories involved in studies of the biogeochemical cycle of Hg. The ethylated species are volatile as are elemental Hg and DMHg, so they can be purged from solution at room temperature and collected on sorbents, such as Carbotrap or Tenax. After thermal release, the Hg compounds are transferred to a (packed) GC column (OV3 on Chromosorb W). Individual Hg compounds are separated by cryogenic [36], isothermal [64] or temperature-programmed GC [44]. Instead of collection on Carbotrap or Tenax, the ethylated compounds may be injected directly on the GC column by headspace injection [44,56] or cryotrapped on a fused silica column and desorbed by flash heating [50,65]. As the Hg species are eluted from the column, they are thermally decomposed in a pyrolytic column (900°C) before being measured by a Hg-specific detector (e.g., CV-AFS, CV-AAS, QF-AAS, MIP-AES, or ICP-MS). Very low limits of detection (LODs) can be achieved, particularly if methylmercury is pre-separated by distillation (6 pg/l for water and 1 pg/g for biota and sediment samples) [41].

The critical part of this procedure is sample preparation prior to ethylation. Methylmercury compounds must be removed from their binding sites to facilitate the ethylation reaction, and interfering compounds, such as chlorides and sulfides, must also be removed [36,41]. Both inorganic Hg and MMHg can be determined simultaneously. But, the sample-preparation technique determines the species that can be measured.

However, we need to mention that ethylation cannot be used for the determination of other organomercurials and is not specific in cases where ethylmercury compounds are present in the original sample. As a result, the usefulness of other derivatization agents has been investigated. Sodiumtetrapropylborate ($NaBPr_4$) has been proposed by De Smaele et al. [66] and has been used in several Hg-speciation studies [67–69]. Phenylation using sodiumteraphenhylborate ($NaBPh_4$) has also been used by several authors [69–71]. Sodium borohydride may also be used to form volatile methylmercury hydride, which is then quantified by GC in line with a Fourier transform infrared (FT-IR) spectrophotometer [62].

Especially when using Grignard derivatization, sample preparation may be laborious and time consuming, and extraction of the organometallic compounds from the concomitant matrix, derivatization and further clean-up are required.

4.2. Derivatization efficiencies and validation

If derivatization of the native species is carried out, derivatization yields should also be assessed. In aqueous samples, these yields are relatively easy to assess when a derivatized standard similar to the derivatized organomercury compound is available. Using the standard addition method allows the yield of derivatization to be determined. Especially with sediments and biota, other

compounds in the sample compete for the derivatization agent in the solution. In such cases, the amount of derivatization agent may not be sufficient for a total derivatization of the organomercury compound, so a double experiment can be carried out:

(1) the same organomercury compound containing a strongly enriched Hg isotope different from the natural one is added before extraction and derivatization; and,
(2) the same organomercury compound, but now derivatized, is added before extraction and derivatization [53].

The higher the recovery of the isotopically labeled organomercury compound in experiment (2) compared to (1), the lower the derivatization yield.

4.3. GC improvements

Several techniques have been used to overcome the problem of low column loadings on capillary columns. Capillary columns have also been used after preconcentration of the alkyl derivatives on a wide-bore fused silica column [65] or by solid phase microextraction (SPME) [69]. Large-volume injection (LVI) techniques have also been applied with a capillary column coated with 0.25 μm DB-5 [72].

Multicapillary GC (MCGC) (919 capillaries, 1 m × 40 μm i.d. coated with 0.2 μm SE 30 stationary phase (Alltech)) coupled to ICP-MS [65,73] allows column loadings and carrier-gas flow rates to approach those of packed columns. Basic, unique features are:

- the high speed of separation at large sample injection volumes; and,
- the exceptionally wide range of volumetric velocities of the carrier gas at which the column retains its high efficiency.

This makes plasma source detection ideally suited for MCGC, leading to a coupled technique with a tremendous potential for separation analysis. Several applications involve the coupling of MCGC with MIP-AES [74] or with ICP-MS [73].

Solid-phase microextraction capillary GC (SPME-CGC) has also been proposed. SPME can be used for the extraction of organometallic compounds after they have been derivatized to a sufficiently volatile form. A silica fiber coated with polydimethylsiloxane (PDMS) is brought into the (headspace) of the sample, while the latter is magnetically stirred and heated. At equilibrium, the analyte concentrations in the three phases (aqueous, the headspace and the fiber coating) depend on the volume of the phases and the partitioning coefficient, which depends on the volatility of the compounds. After exposure, the fiber is inserted into the GC injection port and the compounds are thermally desorbed for subsequent analysis. This method has a much higher sensitivity compared to the injection of solvent on a capillary column (usually 1 μl) but requires the use of standard addition as a calibration method. After derivatization with tetraethylborate, tetrapropylborate or tetraphenylborate, the ethylated compounds are extracted by SPME. SPME can be performed either in the aqueous phase or in the headspace. After SPME extraction, species were separated by GC and analyzed by furnace atomization plasma emission spectrometry (FAPES) [69].

5. LC separation methods

Until recently, the main disadvantage of LC was the poor sensitivity of the detectors. Development of more sensitive detectors, such as a reductive amperometric electrochemical, ultraviolet (UV), ICP-AES, ICP-MS, AFS and AAS, has resulted in wider applications in environmental studies. The main advantage of LC over a number of methods is the possibility of separating a great variety of organomercury compounds. Applications of HPLC for Hg-speciation studies have been reviewed by Harrington [15]. Practically all HPLC methods for Hg speciation reported in the literature were based on reversed phase separations, involving the use of a silica-bonded phase column and a mobile phase containing an organic modifier, a chelating or ion-pair reagent and, in some cases, a pH buffer.

The interface to couple HPLC columns with the atomizer can be very simple, with the exit of the column directly connected to the nebulizer of the AAS or plasma detector. Unfortunately, nebulizer efficiency is very low (1–3%) and limits sensitivity, especially for flame AAS. Generally, a way out of this lack of sensitivity is post-column derivatization to form cold vapor of Hg. However, generation of a cold vapor from organomercury species requires an extra step to convert to Hg(II); otherwise, the response will depend on the species present. This conversion is usually on-line and has been facilitated using a number of different approaches, including:

- oxidation with potassium dichromate on its own [75] and in the presence of copper sulfate [76] or cadmium [77];
- the use of UV light on its own [78] and with hydrogen peroxide [79]; and,
- acidic potassium persulfate in the presence of copper sulfate [80].

In an effort to analyze low levels of Hg species, some workers have developed on-line and off-line sample preconcentration methods [75,76,81].

Besides reversed phased HPLC, ion chromatography (IC) has also been used to separate Hg species [82,83]. IC provides the possibility of separating more polar and ionic species directly, so that sample pretreatment can be simplified. The coupling of IC with CV-ICP-MS allows very low LODs to be obtained [83].

6. Detection methods

The analytical sensitivity and selectivity requirements for reliable Hg-speciation analysis can be achieved only by using hyphenated techniques, coupling chromatographic separation methods on-line to Hg-specific detectors. Most chromatographic detectors incorporated in commercial instruments are either universal or selective but lack the necessary specificity for Hg.

The first work on Hg speciation was performed using GC with ECD detection. The non-specific character of the detector favored the use of GC-MIP-AES because of its high element specificity towards Hg [25,30,39,63,84–86]. The availability of a commercial instrument and its higher sensitivity compared to direct nebulization in an ICP-AES has made it very popular. Furnace atomization plasma emission spectrometry (FAPES) [39,69,87,88] and quartz furnace atomic absorption spectrometry [50] have also been used.

The development of a commercial, relatively inexpensive, extremely sensitive and selective CV-AFS instrumentation in the late 1980s and 1990s [64,89,90] made this the most popular detector for the laboratories working on the biogeochemical cycling of Hg. In recent years, the use of ICP-MS in speciation analysis has increased tremendously; this is evident from the large number of publications devoted to the use of ICP-MS in the speciation of Hg (e.g., see [91] for a review).

Besides its high sensitivity and selectivity, ICP-MS offers the opportunity to perform speciated isotope dilution mass spectrometry (SID-MS) [54,58,92]. Not only is this technique highly accurate and precise, but it can also check for species transformations and extraction recoveries by using isotopically enriched isotopes as tracers. The use of species-specific enriched stable isotopes could greatly assist in the testing and diagnostics of analytical methods. The isotope-dilution approach has the potential to provide several types of information not available with other techniques by not only spiking inorganic Hg but also labeling Hg species with enriched Hg isotopes. Based on SID-MS, Gelaude [93] recently reported the separation and the quantification of inorganic Hg and MMHg in solid samples after thermal liberation of the compounds with an electrothermal graphite furnace.

The detection systems used with HPLC can be broadly divided into three: photometry; plasma techniques (ICP-AES, ICP-MS); and, cold vapor atomic absorption and fluorescence spectroscopy (CV-AAS, CV-AFS). The method with the lowest LODs with sample introduction via a direct injection nebulizer used ICP-MS [94]. An HPLC system coupled to atmospheric pressure chemical ionization (APCI) MS was used to identify methyl Hg spiked into a fish tissue CRM (DORM-1, NRCC) [95]. This type of system has a significant advantage over elemental detection methods because identification of the species present is based on their structure, rather than on matching the analyte retention time to that of a standard.

The use of CV generation coupled to ICP-MS lowers the LOD by a factor of about 15, facilitating the detection of Hg species in ocean-water samples [96]; this is impossible with conventional nebulization. The use of CV-AAS allows for the detection of Hg compounds down to 0.1–1 ng for Hg(II), methylmercury and ethyl mercury (depending on the system).

In comparison with HPLC-ICP-MS, CGC-ICP-MS offers a higher resolving power and 100% introduction efficiency, allows more stable plasma, gives rise to fewer spectral interferences as the result of the plasma being dry and, finally, leads to less sampling cone and skimmer wear [91]. The coupling is somewhat more complicated. Usually a heated transfer line is used to avoid condensation of the species [97] for multi-element speciation. For Hg, this is not mandatory. Simultaneous speciation analysis of Hg and tin in biological samples using CGC-ICP-MS has been performed by Monperrus et al. [98].

Time of flight MS (TOF-MS) is an alternative to scanning-based mass analyzers. Coupled to ICP, TOF-MS can produce a complete mass spectrum in less than 50-μs. CGC combined with ICP-TOF-MS has been developed for the speciation of Hg [99] and later improved by MCGC-ICP-TOF-MS [100] allowing complete chromatographic separation within a chromatographic run-time of less than 1 min.

7. Quality control

As other measurement fields, speciation analysis requires the availability of suitable reference materials for the purpose of verification of accuracy and quality assurance needs [101]. Measurements that represented a real start in speciation science were those that had a link with identified toxicity risks, namely the determination of methylmercury in biological tissues and organotins in environmental matrices. This awareness was naturally associated with the needs expressed by laboratories with respect to quality control tools, and organizations, such as the National Institute for Environmental Sciences (NIES, Japan) and the National Research Council Canada (NRCC), started work on interlaboratory studies and CRM production for these compounds during the 1980s. Later (1988 and afterwards), the European Commission, through the BCR programme (French acronym for the Community Bureau of Reference), launched a series of projects aiming to improve the quality of speciation measurements for chemical forms of Al, As, Hg, Pb, Se and Sn in various biological and environmental matrices, along with extractable forms of trace metals in soils and sediments [102].

Table 1. Examples of reference materials certified for their contents in methylmercury

CRM	Compounds and matrices	Producer
SRM 1974a	Total Hg and methylmercury in mussel tissue	NIST
SRM 2974	Total Hg and methylmercury in mussel tissue	NIST
SRM 2976	Total Hg and methylmercury in mussel tissue	NIST
IAEA-142	Total Hg and methylmercury in mussel tissue	IAEA
BCR-710	Methylmercury, tributyltin and Asbetaine in oyster tissue	BCR
DORM-1	Methylmercury in fish muscle (dogfish)	NRCC
DORM-2	Total Hg and methylmercury in fish muscle (dogfish)	NRCC
DOLT-1	Total Hg and methylmercury in dogfish liver	NRCC
DOLT-2	Total Hg and methylmercury in dogfish liver	NRCC
CRM 463	Total Hg and methylmercury in fish muscle (tuna)	BCR
CRM 464	Total Hg and methylmercury in fish muscle (tuna)	BCR
IAEA-350	Total Hg and methylmercury in fish muscle (tuna)	IAEA
TORT-1	Trace elements and methylmercury in lobster tissue	NRCC
LUTS-1	Trace elements and methylmercury in lobster tissue	NRCC
IAEA-140	Total Hg and methylmercury in sea plant	IAEA
CRM 580	Total Hg and methylmercury in sediment	BCR
IAEA 356	Total Hg and methylmercury in sediment	IAEA
IAEA-085	Total Hg and methylmercury in human hair (spiked)	IAEA
IAEA-086	Total Hg and methylmercury in human hair	IAEA
NIES 13	Total Hg and methylmercury in human hair	NIES

The table lists CRMs available at BCR (European Commission), IAEA (Austria), NIES (Japan), NIST (USA) and NRC (Canada). This list is not exhaustive.

With regard to Hg species, the production of CRMs has essentially focused on methylmercury. Table 1 gives a (non-exhaustive) list of available CRMs for the quality control of methylmercury determinations in various environmental matrices. Many of these CRMs are the results of improvement schemes and/or have been produced in the frame of interlaboratory studies involving expert laboratories. They therefore represent the best of the state-of-the-art in Hg-speciation analysis and internationally recognized references to establish traceability of measurements of the chemical forms of elements in various matrices. However, much remains to be done to produce CRMs for other chemical species [103]. Also, materials certified for a range of chemical forms of elements are strongly needed. In this respect, a recent project has permitted joint certification of butyltins, MeHg and As species in an oyster reference material [104].

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