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Methylmercury, carbon, compound specific stable isotopic analysis, GC-C-IRMS, biological materials, standard reference materials, marine biogeochemistry

ABSTRACT:A critical component of the biogeochemical cycle of mercury (Hg) is the transformation of inorganic Hg into neurotoxic monomethylmercury (CH_3Hg). Humans are exposed to CH_3Hg by consuming marine fish, yet the origin of CH_3Hg in fish is a topic of debate. The carbon stable isotopic composition ($\delta^{13}C$) embedded in the methyl group of CH_3Hg remains unexplored. This new isotopic information at the molecular level is thought to represent a new proxy to trace the carbon source at the origin of CH_3Hg. Here, we present a compound specific stable isotope analysis (CSIA) technique for the determination of the $\delta^{13}C$ value of CH_3Hg in biological samplesby gas chromatography combustion isotope ratio mass spectrometry analysis (GC-C-IRMS). The method consisted first of calibrating aCH_3Hg standard solutionfor $\delta^{13}C$ CSIA. This was achieved by comparing three independent approaches consisting of the derivatization and halogenation of the CH_3Hg standard solution. The determination of $\delta^{13}C_{CH3Hg}$values on natural biological samples was performed by combining a CH_3Hg selective extraction, purification and halogenation followed by GC-C-IRMS analysis. Reference $\delta^{13}C$ values were established for a tuna fish certified material (ERM-CE464) originating from the Adriatic Sea ($\delta^{13}C_{CH3Hg}$ = -22.1±1.5 ‰, ± 2SD). This value is similar to the $\delta^{13}C$ value of marine algal derived particulate organic carbon (δ^{13}CPOC = -21‰).

Introduction

The toxicity of mercury (Hg) is directly related to its chemical forms. Biogenic organometallic compounds that are naturally produced in aquatic ecosystems such as methylmercury (CH_3Hg) are known to be potent neurotoxins[1] and to bioaccumulate along aquatic food chains[2]. Marine sea food consumption represents the main source of human CH_3Hg exposure[3], with socio-economical costs estimated to several billions of dollars/year worldwide[4].

Methylmercury is thought to be primarily produced by sulfate reducing bacteria[5-7], yetother anaerobic microorganisms hosting specific methylation genes[8], and possibly complementary abiotic pathways[9]may also be at play[10]. The rapid assimilation of CH_3Hg by primary producers (phytoplankton)[11]and further biomagnification along aquatic foodwebs is well documented[12,13]. Yet, understanding where, how and when CH_3Hg is produced at the base of aquatic ecosystems remains a subject of ongoing debate. Lake, coastal and marine sediments have long been identified as important sites for microbial Hg methylation[14-16]. However, relating elevated open ocean fish CH_3Hg levels to sediment CH_3Hg sources is unrealistic[17]. Recent marine studies on CH_3Hg provide mounting evidence for in situ water column production of CH_3Hg[18-21].

Answering fundamental questions on the origin of CH_3Hg is directly driven by the state of Hg metrology. Traditional Hg speciation techniques mostly involve a gas chromatographic (GC) separation coupled to a sensitive Hg detector such Atomic Fluorescence Spectroscopy (AFS) or Inductively Coupled Plasma Mass Spectrometry (ICP-MS)[22]. These techniques have permitted measurement of CH_3Hg concentrations in a wide range of key compartments of the biogeosphere (biota, oceans, atmosphere, food chain, sediments, soils). The increasing use of mass spectrometry in Hg research has allowed researchers to use enriched Hg stable isotopes in laboratory or field tracer studies to quantity the kinetics of Hg transformations and/or its transfer among compartments[23,24]. More recently, the analysis of the natural stable isotopic variations of Hg by cold vapor multi-collector inductively coupled plasma mass spectrometry (CV-MC-ICPMS) in biogeomatrices provided a new angle on tracking Hg sources and dynamics[25,26]. Until now, most measurements of Hg stable isotope compositions in environmental samples have been performed on total Hg concentrations. Recent analytical developments in the field of Hg compound specific stable isotopic analysis (Hg-CSIA) allow to trace Hg dynamics at the molecular level[27,28]. No attention has been devoted, however, to the isotopic variations of the carbon atom at the molecular level in the CH_3Hg compound. The isotopic composition of this component may be used to better understand the role of organic matter and to trace the carbon sources at the origin of the formation of CH_3Hg.

In this study, we developed a new analytical approach to investigate the unexplored carbon isotope side of the CH_3Hg toxin cycle. Recent analytical developments

permit the CSIA of light elements (C, H, N, O) by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Examples are the determination of the $\delta^{13}C$ values of individual organic compounds: amino acids[29], fatty acids[30], greenhouse gases[31], organic anthropogenic contaminants[32], but also biogenic organometallic arsenic compounds[33]. Here, we present the first method for the determination of $\delta^{13}C$ values of CH_3Hg using CSIA ($\delta^{13}C_{CH3Hg}$)in biological materials.

Experimental Section

Reagents, standards and reference materials

Millipore 18.6 $M\Omega.cm^{-1}$ ultrapure water, bidistilled nitric and hydrochloric acids were used for preparation of all solutions throughout this study. Sodium bromide (NaBr, Ultra grade 99.5%), potassium iodide (KI, ACS grade ≥99%), anhydrous cupric sulphate ($CuSO_4$, reagent grade 99%+), sodium thiosulfate ($Na_2S_2O_3$, reagent grade 98%), sodium acetate (CH_3COONa, ACS grade 99%+), methanol (CH_3OH, anhydrous 99.8%), toluene (anhydrous 99.8%), hexane (anhydrous ≥95%),sulphuric acid (H_2SO_4, ACS grade 95-98%) and anhydrous 99.8% grade iso-octane were purchased from Sigma Aldrich (Milwaukee, WI, USA). Ultra grade glacial acetic acid (CH_3COOH, 99%) was purchased from Avantor™ (Netherlands). Sodium tetrapropylborate ($NaB(C_3H_7)_4$) and sodium tetraethylborate ($NaB(C_2H_5)_4$) obtained fromMerseburgerSpezialchemikalien (Germany) were prepared daily and stored at -18°C until use. All polypropylene or Teflon labware used in this study were acid cleaned. Glass labwarewas cleaned by pyrolysis before use.

The primary CH_3Hg reference standard stock solution used in this study was prepared from a methylmercury chloride salt (Sigma Aldrich, Milwaukee, WI, USA), dissolved in a 10% (v/v) methanol/water solution. The relative uncertainty of the concentration of the CH_3Hg standard used in this work was 4.1% (n=10) when calibrated by Cold Vapor Atomic Fluorescence Spectrophotometer against NIST SRM3133[28]. Inorganic Hg (iHg) NIST SRM 3133 solution was used in this work. Diluted CH_3Hg and iHg standard solutions were prepared in 0.5% w/w bidistilled HCl or 5mMsodium thiosulfate solution for the derivatization and halogenation experiments respectively. TORT-2 a freeze-dried lobster hepatopancreas tissue was obtained from the National Research Council Canada (NRCC). TORT-2 is characterized by a low certified CH_3Hg concentration of 0.163±0.014 $\mu g.g^{-1}$ (as CH_3Hg), making it suitable to be used as a "CH_3Hg-blank" control matrix material to investigate the absence of matrix effect of the method. The second biological CRM used in this work was ERM-CE464, a tuna fish reference material representative of 322 Kg of tuna collected in the Adriatic Sea. This material was obtained from the Institute for Reference Materials and Measurements (IRMM, Belgium). ERM-CE464 has an elevated certified CH_3Hg concentration of 5.50±0.17 $\mu g.g^{-1}$ (as CH_3Hg).

GC-C-IRMS

All $\delta^{13}C_{CH3Hg}$measurements were performed by GC-C-IRMS (Thermo Scientific), consisting of a Trace GC Ultra coupled to Delta V Advantage IRMS with an Isolink GC coupling interface. Instrumental parameters (GC, combustion reactor and IRMS) are detailed in Table S1. A fused silica Agilent DB-5 column was used for the separation of propylated and ethylated CH_3Hg and iHg-compounds. A fused silica Restek RTX-5 column with a lower film thickness was privileged in the case of halogenated CH_3Hg compounds. Data illustrated in the text, figures and tables correspond to triplicate injections of each sample. Methylmercury $\delta^{13}C$ values measured in this work are expressed relative to a high purity CO_2reference working gas (carbon dioxide N48, Messer France SAS, Puteaux, France), calibrated against reference materials USGS-24, IAEA-CH6, IAEA-600. $^{13}C/^{12}C$ ratios are normalized by the Vienna Pee Dee Belemnite (VPDB) reference standard and expressed in δ notation:

$$\delta^{13}C\ (‰) = \left(\frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{PDB}} - 1 \right) \times 1000$$

CH_3Hg selective extraction method (SEM)

The CH_3Hg selective extraction method (SEM) used in this study correspond to an upscaled version of the original SEM, initially developed for Hg CSIA[28].Higher CH_3Hg concentrations were required to reach the minimum amountsneeded for precise $\delta^{13}C_{CH3Hg}$measurements by GC-C-IRMS. Briefly, multiple (up to 4) 1g aliquots of the same biological material were extracted separately during 1h (420 rpm lengthwise agitation on a horizontal shaking table) in 50ml centrifuge tubes with 5 ml of acidic sodium bromide (30% w/w NaBr in 4 $mol.l^{-1}$ H_2SO_4), 10 ml of aqueous cupric sulfate (2.5% w/w $CuSO_4$) and 10 ml of toluene. The toluene fractions of each individual sample aliquot were then combined together. The 40mL combined toluene fraction that contains CH_3HgBr was then back-extracted with 4 ml of a 5mM sodium thiosulfate solution to get an aqueous and stable CH_3Hg-thiosulfate complex. Verification of the quantitative extraction and halogenation of CH_3Hg and the absence of analytical degradation, while processing up to 4g of biological material, was verified by Gas Chromatography Sector Field Inductively Coupled Plasma Mass Spectrometry (GC-SF-ICP-MS), before GC-C-IRMS analysis(See Table S2 for details)

Derivatization vs. halogenation conditions

The ethylation and propylation conditions used in this work follow the standard procedures used for Hg speciation by GC-ICP-MS[34]. Briefly, CH_3Hg and iHg standards solutions were derivatized with 0.1 ml of daily prepared $NaB(C_2H_5)_4$ and $NaB(C_3H_7)_4$(20 $mg.ml^{-1}$) in a 5ml acetate buffer solution (0.1 $mol.l^{-1}$, pH=3.9) and in the presence 0.5 ml of hexane. Inorganic Hg concentration levels

were spiked at the same Hg level as CH_3Hg. Samples were hand-shaken for 5 min, before collecting the hexane phase, which was subsequently stored in GC vials at -20°C before analysis. These operating conditions were found suitable to quantitatively derivatize all Hg compounds in the case of standard solutions at the concentration levels needed for GC-C-IRMS analysis (See FigureS1 for details).

The halogenation conditions used in this work are based on published operating protocols found elsewhere[35,36] to form a volatile CH_3HgI compound.Briefly, CH_3HgI is produced by mixing the CH_3Hg standard prepared in a 5mM sodium thiosulfate solution with 0.5 ml of a 3 $mol.l^{-1}$potassium iodide solution. The mixture is vortexed during 30s and then kept in the dark during 10 min. The aqueous CH_3HgI compound is subsequently extracted into 10mL of isooctane by vortexing the solution for 2 minutes. CH_3HgI standard solutions in isooctane were preconcentrated under a stream (0.5 $L.min^{-1}$) of nitrogen at room temperature. Given that CH_3Hg-thiosulfate complexes are also obtained at the end of the SEM, applying the halogenation method to form a CH_3HgI compound was found particularly suitable to determine precise $\delta^{13}C_{CH3Hg}$values for both the CH_3Hg reference standard solution and for biological reference materials.

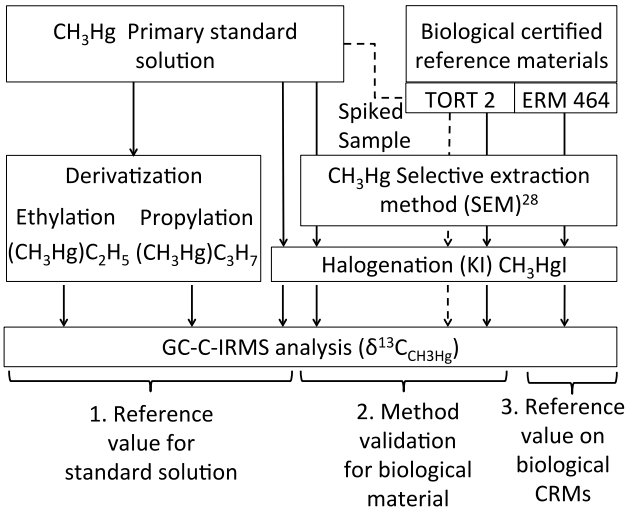

Figure 1. Operating procedure used for validating the analysis of $\delta^{13}C_{CH3Hg}$ on natural biological samples.

Safety considerations

Hg compounds are toxic, and need to be handled only by experienced and well-trained personal, using all safety laboratory measures possible (gloves, glasses, fume hood).

Results and Discussion

Three consecutive steps were taken to develop the method for the determination of $\delta^{13}C_{CH3Hg}$values in reference standard solution and biological certified reference materials (Figure 1). This procedure consisted of:(i) Establishing reference $\delta^{13}C$ values for a CH_3Hg standard solution by comparing different independent derivatization and halogenation procedures; (ii) Validating the application of the selective extraction method[28], by comparing$\delta^{13}C_{CH3Hg}$values of the primary CH_3Hg standard solution processed with, without SEM, and with spiking the CH_3Hg standard into a natural low CH_3Hg reference material (TORT 2) to ensure the absence of matrix effects;(iii) Establishing a $\delta^{13}C_{CH3Hg}$preliminary reference valuefor a natural biological Tuna Fish reference material (ERM CE 464).

Calibration of a CH_3Hg standard for $\delta^{13}C_{CH3Hg}$analysis

Derivatization (ethylation and propylation) vs. halogenation methods were compared for the same CH_3Hg standard solution (Figure 1). The derivatization method represents an indirect approach for the determination of $\delta^{13}C_{CH3Hg}$values since the isotopic contribution of the exogenous carbon atoms present on the ethyl- and propyl- groups after CH_3Hg derivatization ($CH_3Hg(C_2H_5)$ in the case of ethylation and $CH_3Hg(C_3H_7)$ in the case of propylation) needs to be accounted and corrected for. By adding an inorganic Hg standard to the CH_3Hg standard solution before derivatization, the resulting two derivatizediHg compounds in the case of ethylation are $CH_3Hg(C_2H_5)$ and $Hg(C_2H_5)_2$ (Figure 2a), and $CH_3Hg(C_3H_7)$ and $Hg(C_3H_7)_2$in the case of propylation (Figure 2b).

The $\delta^{13}C$ values of the exogenous carbon atoms of the ethyl and propyl groups can thus be determined on the $Hg(C_2H_5)_2$and Hg $(C_3H_7)_2$peaks. Subsequently a mass balance approach can be considered to estimate the endogenous $\delta^{13}C$ values of CH_3Hg.

In the case of ethylation (Figure 2a), $\delta^{13}C_{CH3Hg}$values can be estimated from the following equation:

$$\delta^{13}C_{CH_3Hg} = 3 \times \delta^{13}C_{CH_3Hg(C_2H_5)} - 2 \times \delta^{13}C_{Hg(C_2H_5)_2}$$

In the case of propylation (Figure 2b), $\delta^{13}C_{CH3Hg}$values can be estimated from the following equation:

$$\delta^{13}C_{CH_3Hg} = 4 \times \delta^{13}C_{CH_3Hg(C_3H_7)} - 3 \times \delta^{13}C_{Hg(C_3H_7)_2}$$

The halogenation method (Figure 2c) represents a direct approach for the determination of $\delta^{13}C_{CH3Hg}$values since no addition of exogenous carbon atoms is required. This approach consists of forming a volatile CH_3Hg-halide compound (CH_3HgI) that can be back-extracted into a solvent and analyzed directly by GC-C-IRMS.

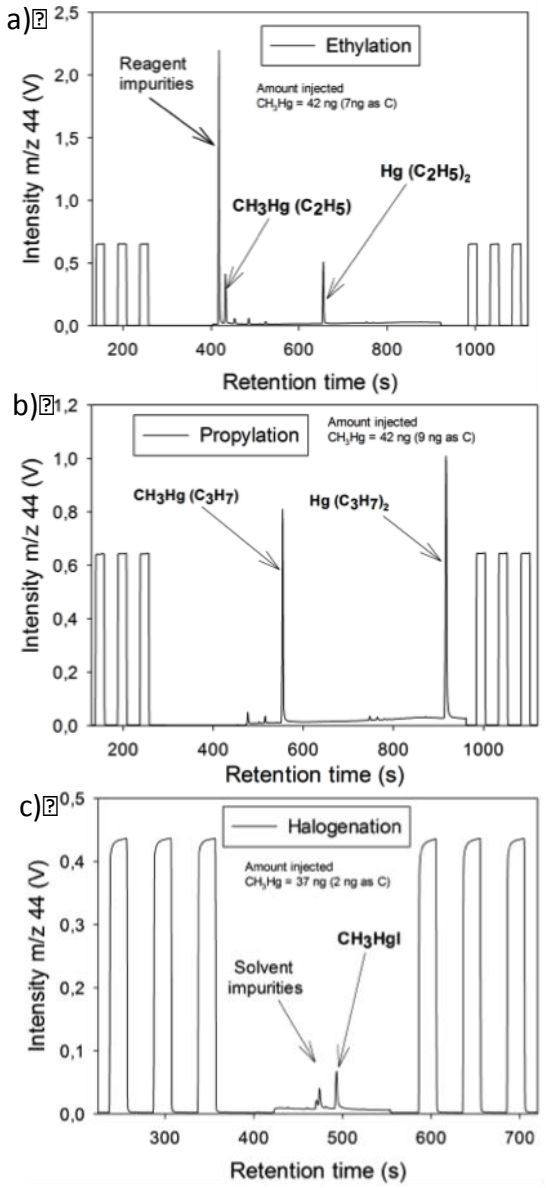

Figure 2. GC-C-IRMS chromatograms of alkylated and halogenated CH_3Hg compounds.a) EthylatedCH_3Hg (($CH_3Hg(C_2H_5)$), b) Propylated CH_3Hg(($CH_3Hg(C_3H_7)$), and (c) halogenated CH_3Hg (CH_3HgI). Note that in the case of derivatized CH_3Hg compounds, an iHg standardwas also spiked into the solution and co-derivatized with CH_3Hg to allow for the correction of the carbon isotopic compositionof the respective ethyl and propyl groups attached to the CH_3Hg molecule. Blank chromatograms of the ethylation, propylation and halogenation experiments are shown in Figure S1.

Derivatization method

The use of typical Sodium Tetraethylborate ($NaB(C_2H_5)_4$)and Sodium Tetrapropylborate ($NaB(C_3H_7)_4$)quantities as in Gas Chromatography Inductively Coupled Plasma Mass Spectrometry (GC-ICP-MS) studies(20 $mg.mL^{-1}$, 0.1mL) was found sufficient to quantitatively derivatize about 21 µg of CH_3Hg (1.2 µg as C) and 20 µg of iHg (back-extracted into 0.5mL hexane) for precise determination of $\delta^{13}C_{CH3Hg}$values of the CH_3Hg standard solution (see Figure S2 for details).

The analysis of the same CH_3Hg standard solution derivatized over a wide rangeof ethylation and propylation reagent quantities lead to similar $\delta^{13}C_{CH3Hg}$ values of -54.5±1.5‰ (±2SD, n=6), and -54.6±1.9‰ (±2SD, n=6) respectively (See Figure S2 for details). This results showed the absence of a significant effect of the amount and type of derivatization agent used on the determination of $\delta^{13}C_{CH3Hg}$ values. A second derivatization of the remaining aqueous solutions did not reveal any residual peaks indicating the quantitative derivatization and back-extraction of CH_3Hg into hexane.

We subsequently used the referencederivatizationprotocol as determined above (20 $mg.mL^{-1}$, 0.1mL), and investigated $\delta^{13}C_{CH3Hg}$values over an analyte mass range of 5-80 µg of CH_3Hg (0.28-4.45 µg as C) while keeping the iHgconcentration constant (20 µg, Figure 3a and 3b). This corresponds to a concentration range of 6-112ng of CH_3Hg injected into the GC-CIRMS,representing 1-19 ng and 1-25ngof carbon in the case of ethylation and propylation respectively. In terms of isotopic composition, homogenous $\delta^{13}C_{CH3Hg}$values were observed over theconcentration range considered. The highest precision and reproducibility of the measurements were obtained when iHg/CH_3Hg concentration ratios were in the range of 0.25 to 4, and with a minimum peak intensity of approximately 0.3 Volts. Under these conditions, $\delta^{13}C_{CH3Hg}$ measurements obtained for the same standard solution were found in good agreement using either ethylation -55.1±0.9‰ (±2SD, n=5) and propylation -54.9±1.7‰ (±2SD, n=5). These results indicate that in the case of quantitative derivatization yields and using iHg as an internal standard for correcting the isotopic contribution of the alkyl groups added to CH_3Hg during the derivatization reaction, accurate, precise and reproducible $\delta^{13}C_{CH3Hg}$ measurementscan be achieved.

Halogenation method

With only a single carbon atom present in CH_3HgI, a significantly higher concentration of the CH_3Hg standard was required to reach the sensitivity level required for precise measurements (123 µg of CH_3Hg in a 5mM sodium thiosulfate solution quantitatively back-extracted as CH_3HgI into 10 mL isooctane (see [35],[36], and experimental section for details). The injection of this solution (37 ng of CH_3Hg; 2 ng as C) led to a $\delta^{13}C_{CH3Hg}$ value of -53.7±0.8‰ (±2SD, n=3).

The evaporation of the isooctane solution under N_2 to different preconcentration levels analyzed by GC-C-IRMS showed a linear relationship (Figure 3c, R^2=0.99) between CH_3HgI peak intensity and the corresponding amount of carbon injected. Identical $\delta^{13}C_{CH3Hg}$ values were also measured for the different preconcentrated sub fractions (Figure 3c).These results confirmed the absence of CH_3HgI loss and isotope fractionation artifact during the solvent preconcentration step.

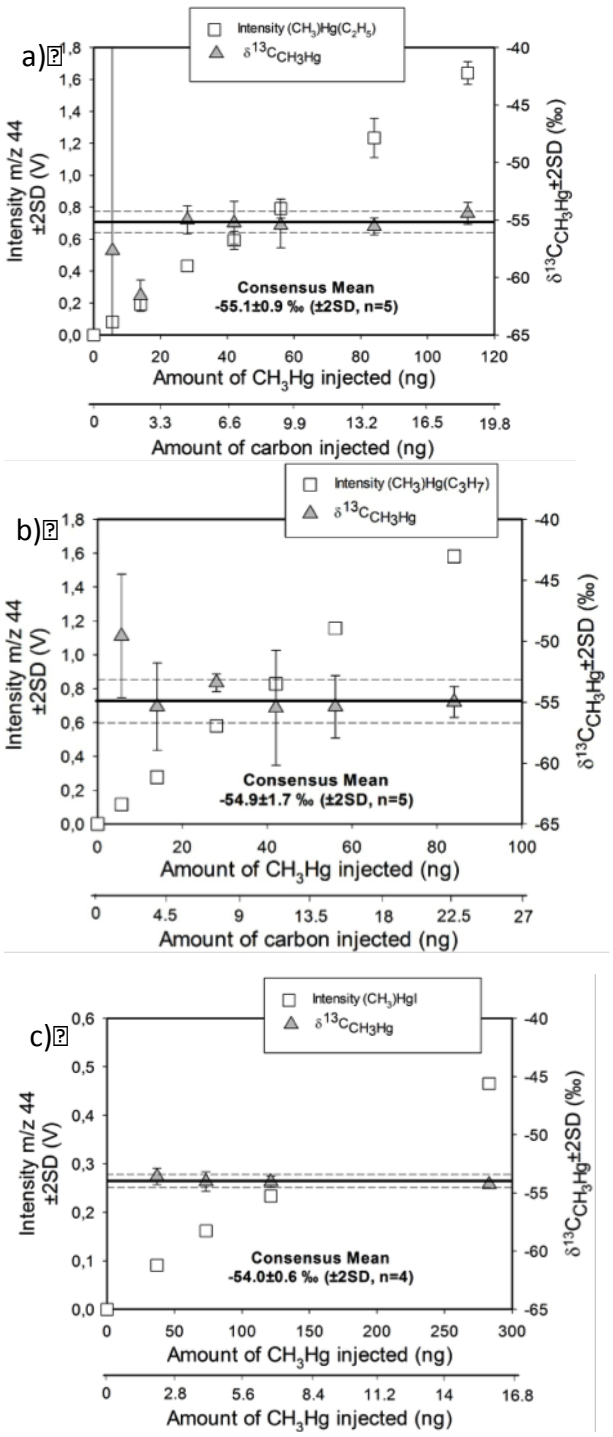

Figure 3. Influence of the concentration of CH_3Hg on $\delta^{13}C_{CH3Hg}$values obtained after a) ethylation, b)propylationand c) halogenation of the same CH_3Hg standard. Consensus mean values (plain line) and their uncertainty(dashed lines (±2SD)) are based on all measurements in the case of direct $\delta^{13}C_{CH3Hg}$ determinations (CH_3HgI), and at peak intensities starting 0.3V In the case of estimated$\delta^{13}C_{CH3Hg}$ determinations by derivatization.

Further, a second halogenation step performed on the remaining aqueous phase after the initial halogenation of the CH_3Hg standard solution did not reveal any residual CH_3HgI peak, also confirming the quantitative transfer of CH_3HgI into the organic phase prior to GC-C-IRMS analysis. The similar $\delta^{13}C_{CH3Hg}$values obtained over a CH_3HgI concentration range of 37 to 283 ng of CH_3Hg (2-16 ng as C) led to a mean value of -54.0±0.7‰ ((±2SD, n=4), Fig 3c) for the CH_3Hg reference standard.

In summary, the comparison of the $\delta^{13}C_{CH3Hg}$ valuesobtained for the same CH_3Hg standard solution showed similar valuesgiven uncertainties between indirect ethylation (-55.1±0.9‰ (±2SD)) and propylation (-54.9±1.7‰ (±2SD)) approaches and the direct halogenation (-54.0±0.7‰ (±2SD)) method. The precision of $\delta^{13}C_{CH3Hg}$ values by the halogenation method appeared also to be significantly better relative to the propylation and ethylation methods. This difference is related to the larger uncertainty budgets of the indirect propylation and ethylation methods where carbon CSIA of two individual Hg compounds needs to be combined for estimating the endogenous composition of the carbon atom present in CH_3Hg. These results were also confirmed during a long-term reproducibility experiment (See TableS4for details). Given all uncertainties and the long-term $\delta^{13}C_{CH3Hg}$ measurements obtained by the three independent methods, a $\delta^{13}C_{CH3Hg}$ reference value of -53.8±1.1‰ (±2SD) was proposed for the CH_3Hg standard solution used in this study.

Method validation for $\delta^{13}C_{CH3Hg}$measurements on biological samples

Accurate $\delta^{13}C_{CH3Hg}$measurements in biological samples require a quantitative extraction and purification of CH_3Hg from the initial sample matrix to the intermediate sodium thiosulfate fraction, but also a quantitative transfer of CH_3Hg from the sodium thiosulfate fraction to the final organic phase prior to GC-C-IRMS analysis.Complementary experiments conducted by GC-SF-ICP-MS showed that the CH_3Hg selective extraction method (SEM) we intially developped for Hg CSIA[28]was able to answer these needs (see Table S2 for details). While our standard SEM protocol[28]was found to quantitatively extract and preconcentrate CH_3Hg from several grams of biological material, preliminary attempts to derivatize the intermediate CH_3Hg-thosulfate complex were found unsuccessful. The strong CH_3Hg-S bond is known to inhibit the derivatizationprocess[37], leading to low transfer yields towards the organic phase. On the contrary, the halogenation method hadpreviously been shown to successfully extract CH_3Hg in the presence of sodium thiosulfate and to quantitatively form a CH_3HgI compound that can be back extracted into an organic solvent[35].

Table 1. Validationand application of the SEM for $\delta^{13}C_{CH3Hg}$measurements in biological materials.

Biological reference material* (mass extracted (g))	CH_3Hg spike concentration	SEM replicates** (n)	SEM CH_3Hg recovery (%±SD)***	Amount of CH_3Hg injected (ng), (ng. as C)	Peak Intensity m/z 44 (mV)	$\delta^{13}C_{CH3Hg}$ ±2SD (‰)****
-	20 µg	3	107±7	306 (17)	488	-53.8±0.4
TORT-2 (0.4 g)	20 µg	1	108±5	391 (22)	567	-54.2±0.4
TORT-2 (3 g)	20 µg	1	93±7	378 (21)	527	-54.4±0.4
ERM-CE464 (4g)	-	4	103±6	253 (15)	492	-22.1±1.5

* TORT-2: 0.163 μ g.g^{-1}(asCH_3Hg) certified concentration, ERM-CE464: 5.50 μ g.g^{-1} (asCH_3Hg)certified concentration

** Number of independent SEM replicates performed

*** Determined by external calibration based on a CH_3HgI standard calibration curve (non-processed by the SEM)

**** Average values and their uncertainties are based on the triplicate injection of each SEM replicate

The quantitative halogenation of CH_3Hg in 5mM thiosulfate solution was also confirmed in this study by GC-SF-ICP-MS measurements (See Table S2 for details), performed on the same solvent fractions as those analyzed by GC-C-IRMS. For these reasons, the halogenation method has been chosento process the biological SEM extracts of CH_3Hg in thiosulfate prior to$\delta^{13}C_{CH3Hg}$measurements by GC-C-IRMS.

We subsequently tested first the combined influence of the SEM and halogenation methods on the reference CH_3Hg standard previously calibrated for $\delta^{13}C_{CH3Hg}$measurements. Results in Table 1 show that extracting 20 µg of CH_3Hg by the SEM, followed by halogenation and preconcentration of the isooctane phase resulted in a $\delta^{13}C_{CH3Hg}$value of -53.8±0.4‰ ((±2SD), n=3). This value is similar, given uncertainties with the reference value obtained for the same CH_3Hg standard not processed by the SEM (-53.8±1.1‰). CH_3Hg standard recovery obtained after the SEM was 107±7% (n=3). This confirmed the quantitative extraction, purification, halogenation and preconcentrationof CH_3Hg prior to GC-C-IRMS analysis. These results also indicated the absence of carbon isotope fractionation artifacts associated with the SEM when processing matrix-free CH_3Hg standard solutions.

In a second step, we investigated the ability of the SEM-halogenation method to handle the same CH_3Hg amount as processed above, but in the presence of a virtually “MeHg-blank” sample tissue matrix. Variable amounts (0.4 and 3 g) of the NRCC-TORT-2 certified reference material, for which no CH_3Hg peaks could be detected (<15mV) were spiked with 20 µg of the previously $\delta^{13}C$isotopically characterized CH_3Hg standard. As shown in Table 2, no matrix effects were observed to influence CH_3Hg spike recovery (93-108%) during the extraction, halogenation, and evaporation steps. The uncertainty budget of the CH_3Hg recovery values reflects the combined influence of the uncertainty on the CH_3Hg concentration in both the standard solution and the biological reference materials (See experimental section), but also theslight increase in CH_3Hg signal due to the progressive evaporation of the few µL of CH_3HgI hexane solution occurring in the GC vial during the triplicate injection and analysis of each sample. This source of uncertainty could be eventually reduced by using a Programmed Temperature Vaporizing Injector (PTV) allowing to increase the sensitivity by injecting more sample and evaporating the hexane solution directly into the injector. However, and because of the absence of isotope fraction during the evaporation and preconcentration of CH_3HgI in the hexane phase (Figure 3c), this source of uncertainty has no influence on the precision and accuracy on the $\delta^{13}C_{CH3Hg}$ measurements. Methylmercury $\delta^{13}C$ analysis performed for the different tissue sample masses to CH_3Hg spike concentrationratio conditions yield to similar$\delta^{13}C$ values relative to the reference CH_3Hg standard solution. This results confirmed the absence of isotope fractionation bias associated with the SEM when processing biological matrices for accurate $\delta^{13}C$measurements.

$\delta^{13}C_{CH3Hg}$values for ERM-CE464 tuna fish material

The ERM-CE464 tuna fish freeze dried reference material was prepared in 1989 by the Institute for Reference Materials and Measurements (IRMM) from 322 Kg of dorsal muscle orignating from several tuna fish individuals collected in the Adriatic basin of the Mediterranean Sea. ERM-CE464 is characterized by a high CH_3Hg content (5.50±0.17 µg.g^{-1} as CH_3Hg), and represents an ideal candidate material for applying the method developed in this study to establish a representative CH_3Hg $\delta^{13}C$ value. We prepared and analyzed 4 g (n=4) of ERM-CE464 (representing approximately 22 µg of CH_3Hg), in the same way as the CH_3Hg reference solution and TORT-2 spiking experiments (Table 2). Recovery of CH_3Hg from ERM-CE464 was 103±6%. A $\delta^{13}C_{CH3Hg}$ value of -22.1±1.5‰ (±2SD) was obtained for the n=4 independent SEM-halogenation extractions.

Conclusions

In this work, we tested three techniques (propylation, ethylation, halogenation) to determine $^{13}C/^{12}C$ isotopic ratios of the carbon atom present in the methyl group of the CH_3Hg compound in a commercial CH_3Hg salt. A CH_3Hg $\delta^{13}C$ reference value of -53.8±1.1‰ (±2SD) was established for the commercial CH_3Hg salt. The halogenation method was shown to yield the highest precision. Following our previously developed CH_3Hg selective extraction method (SEM), we further show that the CH_3Hg SEM is not only relevant for Hg-CSIA[28] but also now for C-CSIA of CH_3Hg in biological samples. We found a $\delta^{13}C_{CH3Hg}$value of -22.1±1.5‰ (±2SD) for the Adritic Sea tuna fish ERM-CE464 certified reference material. The tuna fish $\delta^{13}C_{CH3Hg}$ value apperared closely related to Adriatic and Atlantic derived particulate organic matter $\delta^{13}C_{POC}$ of -21/-22‰[38-41], but different from that of Terrestrial Organic Matter (-27/-28‰)[42]and from sediments of the Adriatic Sea[38]. Assuming that CH_3Hg $\delta^{13}C$ fractionation is probably limited during CH_3Hg biomagnification in marine food chains since CH_3Hg is mostly preserved in fish tissues and efficiently transferred from one trophic level to the next, this would suggest that fresh marine algal organic matter is the most likely carbon source at the origin of bioaccumulatedCH_3Hg in tuna fish from this region.

We previously found Hg-CSIA derived $\delta^{202}Hg$ and $\Delta^{199}Hg$ compositions of 0.62±0.11‰ and 2.34±0.11‰ for ERM-CE464[28]. These combined two dimensional (2D) C and Hg isotope compositions open up new opportunities to improve our knwoledge of the mercury cycle and to investigate the question of the origin of CH_3Hg in marine ecosystems.

ASSOCIATED CONTENT

Supporting Information

The Supporting information file containsTable S1: GC-C-IRMS instrumental conditions, Table S2: Quantitative extraction and halogenation efficiency of the SEM on natural biological material determined by GC-SF-ICP-MS, Table S3: GC-SF-ICP-MS instrumental conditions, Table S4: Summary of $\delta^{13}C_{CH3Hg}$ values obtained for the CH3Hg primary standard solution, comparing short term and long term measurements,Figure S1. GC-C-IRMS blank chromatograms, Figure S2: Influence of the amount and type of derivatizing agent in solution on the derivatization efficiency of CH_3Hg and iHg, and associated influence on $\delta^{13}C_{CH3Hg}$ values. “This material is available free of charge via the Internet at http://pubs.acs.org.”

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Author Contributions

‡These authors performed the measurements and supervised the method development.The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

This work is supported by the CNRS MI « Instrumentation aux limites » Project 2D ORGANOMETAL grant and the Observatoire Midi Pyrénées (OMP) internal stable isotope funding to DP. JM is grateful to the “Ministére de l'EnseignementSupérieuret de la Recherche” for his Doctoral Fellowship (EcoleDoctorale ED SDU2E/UPS). A. Baya is thanked for technical assistance on the CH_3Hg sample preparation and extraction.

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