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Application of Isotopically Labeled Methylmercury for Isotope Dilution Analysis of Biological Samples Using Gas Chromatography/ICPMS

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An isotope dilution (ID) procedure for the determination of methylmercury (MMHg) in biological samples using an inductively coupled plasma mass spectrometer as detector after the capillary gas chromatographic separation (CGC/ICPMS) has been developed. For the first time, open-focused-microwave pretreatment has been used in conjunction with ID. Optimum conditions for the measurement of isotope ratios on the fast transient chromatographic peaks have been established. Mass bias was found to be about 1.5%/mass unit and was corrected by using the simultaneously measured thallium signals at ^{203}Tl and ^{205}Tl. After mass-bias correction, deviation of the theoretical mercury ratio values was found to be as low as 0.2%. Isotope ratio precisions based on the peak areas measurements were 0.3% RSD for 20 pg injected (as Hg absolute). The absolute detection limits were in the range of 20−30 fg for ^{202}Hg and ^{201}Hg. Methylmercury enriched in ^{201}Hg has been synthesized by direct reaction with methylcobalamine. The concentration of the MMHg spike has been measured by reverse isotope dilution with a natural MMHg standard. The capabilities of CGC/ICPMS to measure isotope ratios were used to optimize sample derivatization by aqueous ethylation with $NaBEt_4$ with respect to MMHg degradation pathways and quantitative recovery. The accuracy of the method developed has been validated with biological certified reference materials (CRM-463, DORM-1).

Mercury is an element well-known for its toxicity, especially when it occurs in the form of methylmercury (MMHg).[1] Methylmercury may be directly released into the environment from various anthropogenic sources but is also formed by biotic or abiotic methylation of inorganic mercury (Hg^{2+}).[2,3] Then, MMHg can be accumulated in fish or other biota. Especially fish tend to concentrate mercury by a factor of 10^5−10^7, which can lead to dangerous levels in fish even in areas with tolerable aquatic Hg^{2+} concentration.[4] For human and wildlife, fish consumption is the major contributor to mercury risk. This toxicity has led to regulatory agencies to focus on fish as the target organism to protect the health of humans and other sensitive organisms. For example, the U.S. Food and Drug Administration set an advisory standard of 1 ppm wet weight in fish flesh. Finding fish in a body of water that exceed established advisories lead health agencies to issue health warnings regarding fish consumption. The health and economic impacts are evident, creating a demand for sensitive, reliable, and accurate analytical methods to be used in control laboratories.

To date many analytical techniques have been developed for the speciation of mercury, usually combining a powerful separation technique, such as gas chromatography (packed,[5] capillary,[6] or multicapillary column[7]), liquid chromatography,[8] or, more recently, capillary electrophoresis (CE)[9] with specific atomic detection including electron capture,[6] atomic absorption spectrometry (AAS),[5] atomic fluorescence spectrometry (AFS),[10] or inductively coupled plasma mass spectrometry (ICPMS).[11] Among the methods mentioned, the coupling of GC to ICPMS appears to be one of the more promising hyphenated techniques to carry out this type of speciation work because of its extremely high sensitivity and multielemental and multiisotopic capabilities.

Despite significant improvements in instrumentation, the quality of the results is mostly associated with sample pretreatment stages.[12] It must be pointed out that GC/ICPMS is only suitable

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for volatile species, and derivatization reactions are required for ionic compounds.[13,14] Additionally, the analysis of solid samples such as sediments or biotissues requires a leaching/digestion step prior to detection.[15−22] However, there are traditional problems related to nonquantitative recoveries and, more recently, questions have arisen about the artifact formation and transformations of methylmercury during sample preparation.[23−27]

To tackle the present problem of speciation data validation, the use of isotope dilution techniques offers great potential since quantitative recoveries are not necessary and rearrangement reactions are easily detected. Provided the enriched isotope is present in an equilibrated and equivalent state to the natural isotope, it can perform the role of the ideal internal standard. Isotope dilution mass spectrometry (IDMS) has been considered to be a definitive method, offering the potential of small uncertainties. Therefore, IDMS can play an important role as a reference method for MMHg analysis in food stuff for legal purposes.

However, despite the benefits on offer, very few applications of isotope dilution for organometallic compounds analysis have been published.[27−34] Regarding the literature for mercury,[23,24,27,32,33] at the present moment there is a lack of a reliable procedure for the determination of MMHg in fish tissues that could benefit from the accuracy and precision of the ID technique. It could play an important role as a reference method for mercury speciation in fish tissues in control laboratories.

Among the techniques used for speciation, the coupling of gas chromatography to ICPMS appears to be one of the techniques of choice taking into account the previously stated benefits of the ICPMS detection technique and that capillary gas chromatography provides a good profile for isotope ratio measurements in very short analysis times. With respect to sample pretreatment, the critical step in isotope dilution analysis is the equilibration between the enriched spike and the sample. Long equilibration times are required to ensure that both the sample and spike behave in the same way. In this context, reduction of the time of analysis in the subsequent extraction step is even more important. Among the extraction techniques,[15−22] a microwave-assisted system seems to be the best choice since it drastically reduce time of analysis with respect to classical preparation procedures.[21,22]

In this work, an open microwave-assisted extraction technique was used in combination with an inductively coupled plasma mass spectrometer as detector after the capillary gas chromatographic separation for the isotope dilution speciation of methylmercury in fish samples. With respect to the ID procedure optimization, factors affecting the precision of the isotope ratio measurements have been carefully evaluated. The concentration of the synthesized methylmercury has been calculated by reverse isotope dilution analysis. This solution has been fully characterized, both with respect to purity and isotopic composition. This enriched methylmercury has been used for the species-specific isotope dilution of several certified reference materials and to control different steps during the sample preparation and analysis.

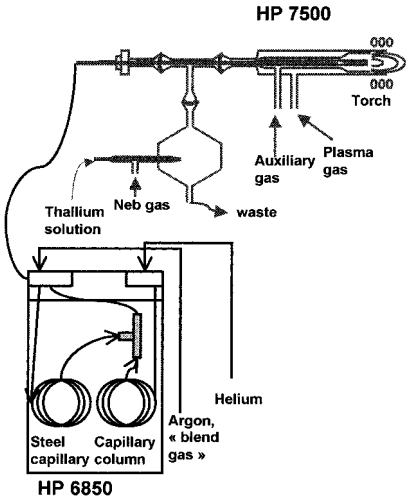

Figure 1. Instrumental setup for mercury speciation.

EXPERIMENTAL SECTION

Instrumentation. A gas chromatograph (HP 6850) equipped with a capillary column was coupled to an Agilent model HP-7500 inductively coupled plasma mass spectrometer via a silcosteel (Restek) transfer capillary. The instrumental configuration is illustrated schematically in Figure 1. This configuration allows one to work under mixed wet and dry plasma conditions. A detailed description of the transfer line design has been previously published.[35] Briefly, the silcosteel capillary was inserted into the torch injector and the connection to the torch was realized by means of glass T-piece. A Scott cooled (2 °C) spray chamber and a conventional Babington nebulizer were connected to this T-piece and enabled continuous aspiration of a standard Tl solution (10 μg/L). This configuration allowed optimization of instrument performance and simultaneous measurement of ^{203}Tl and ^{205}Tl for mass bias correction during the chromatographic run. Operating

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Table 1. Operating Conditions of the CGC/ICPMS

Injector Parameters	
mode	splitless
temp	200 °C
injected vol	2 μL
GC Parameters	
column	MXT silcosteel, 30 m, i.d. 0.53 mm, df 1.0 μm
carrier gas	helium, 25 mL/min
makeup gas	argon, 300 mL/min
GC program	60 °C/ramp 50 °C/min/180 °C
Transfer Line	
length	1 m
inner	silcosteel, i.d. 0.28 mm, o.d. 0.53 mm
oute:	silcosteel, i.d. 1.0 mm, o.d. 1/16 in.
ICPMS Parameters	
rf power	1250 W
gas flow	
plasma	15 L/min
auxiliary	0.9 L/min
nebulizer	0.9 L/min
dweel time	40 ms for ^{201}Hg, ^{202}Hg
	10 ms for ^{203}Tl, ^{205}Tl

conditions and instrumentation are listed in Table 1. GC separation parameters were optimized to obtain symmetrical peaks and to minimize peak integration errors. The raw data of the transient isotope signals for the different mercury species were further processed using chromofile MS software (HP) to obtain the corresponding isotope ratios.

Extraction of mercury species from solid samples was undertaken by a Microdigest A301 (2450 MHz, maximum power 200 W) microwave digestor (Prolabo, Fontenay-sous-bois, France).

Reagents. Stock solutions of Hg^{2+} and MMHg (1000 mg/L) of natural isotopic composition were prepared by dissolving mercury(II) chloride (Strem Chemicals 99.9995% Hg) in 1% HNO_3 (Merck) and methylmercury chloride (Strem Chemicals) in methanol (Merck), respectively. Working standard solutions were prepared fresh daily by appropriate dilution of the stock standard solutions in 1% HNO_3 and stored in the refrigerator. Methylcobalamine (Sigma) used for synthesis was prepared by dissolution in a acetic acid−acetate buffer solution (0.1 M, pH 5). ^{201}HgO was obtained from Oak Ridge National Laboratory (Oak Ridge, TN). The sodium tetraethylborate (98%) was purchased from Strem Chemicals (Bischheim, France). The atomic mass and relative abundance of Tl isotopes used for the mass bias calculations were 202.9723 g/mol and 29.25% for ^{203}Tl and 204.9744 g/mol and 70.48% for ^{205}Tl.

All other reagents were of analytical reagent grade. Ultrapure water (>18 MΩ cm) was obtained from a Milli-Q system (Quantum EX, Millipore).

The reference materials DORM-1 (dogfish muscle) and CRM 463 (tuna fish muscle) were obtained for the Institute for the National Measurement Standard of Research Council of Canada (Ottawa, Ontario, Canada) and the Institute for Reference Materials and Measurements (IRMM) (Geel, Belgium), respectively. An oyster tissue (T-38) used in a recently intercomparison exercise organized by IRMM has also been analyzed.

Procedures.

A flowchart of the simplified ID analytical procedure (i.e., spike, extraction, derivatization) developed is shown in Figure 2.

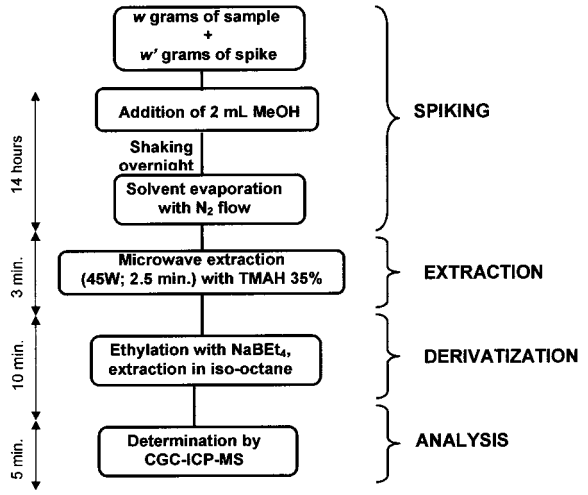

Figure 2. Schematic flow diagram of the isotope dilution protocol for mercury speciation analysis in biomaterials.

Extraction of Methylmercury from Biological Samples. An aliquot of 0.2 g of dry sample was digested in an open-microwave oven as described previously.[22] Briefly, the sample was placed in the extraction tube and 5 mL of 25% TMAH alkaline solution was added and then exposed to microwave radiation at 45 W for 2.5 min (Microdigest A301, Prolabo). After centrifugation, the extract was transferred into a 22-mL Pyrex vial with Teflon-lined cap. Samples were stored in the refrigerator until analysis following the derivatization procedure.

Ethylation of Hg Compounds for GC/ICPMS Analysis. Mixed standards solutions of different mercury compounds were buffered to pH 3.9 with 5 mL of a 0.1 M acetic acid−sodium acetate buffer. For biological samples extracts the pH was previously adjusted by addition of acetic acid (concentrated). Then, 5 mL of 0.5% sodium tetraethylborate and 2 mL of isooctane were added to derivatize and extract the dialkylated compounds formed. After 5 min of manual shaking and 5 min of further centrifugation (2500 rpm), the organic layer was transferred to a glass vial and stored at −18 °C until measurements. All samples were measured during the day of their derivatization.

Synthesis and Analysis of ^{201}Hg-Enriched Methylmercury. The isotopically enriched MMHg has been synthesized from ^{201}HgO using methylcobalamine. Initial conditions have been selected from Filipelli and Baldi's work,[36] but they have been adapted to get the maximum yield of methylmercury, trying to avoid dimethylmercury formation. The optimization of the synthesis conditions will be published elsewhere (Rodríguez et al., manuscript in preparation). Briefly, the optimized synthesis procedure was as follows: a solution of Hg^{2+} was prepared by dissolving 1 mg of ^{201}HgO in 1 mL of HCl (concentrated). Approximately 10 μL of this solution were transferred to amber microreaction reactors (Supelco) and diluted with 500 μL of sodium acetate buffer (0.1 M, pH 5). A 500 μg amount of methylcobalamine was dissolved in another 500 μL of buffer and added to the inorganic Hg solution. The solution was left sitting in the dark at 37 °C for 1 h. To stop the methylating reaction and to convert the

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unintentionally formed dimethylmercury into methylmercury, the mixture was cooled at 4 °C and 1 mL of HCl (concentrated) was added and then shaken for 5 min. The formed $MM^{201}Hg$ was extracted three times each with 500 μL of toluene. The combined toluene extracts were dried over sodium sulfate. A 100 μL volume of this primary solution was diluted with 10 mL of 2-propanol. Working solutions were prepared fresh daily by diluting the secondary 2-propanol stock solution with deionized water as needed.

Isotope Dilution Analytical Protocol. Isotope dilution is based on the addition of a known amount of the enriched isotope to a sample. Equilibration of the spiked isotope with the natural element/species in the sample alters the isotope ratio that is measured and used for calculation. In practice, the achievement of effective equilibrium of the enriched spike and the sample is not easy to obtain, but it is critical for the accuracy of the results.

With this in mind, we have applied the following spiking procedure: Approximately 1 g of sample was spiked with a known weight of the 201-enriched methylmercury solution. A 2 mL volume of methanol was added, and the resulting slurry was shaken mechanically overnight at ambient temperature in the dark. The remaining solvent was evaporated under a stream of nitrogen. An aliquot of 0.2 g of the dry sample was digested in a microwave oven as described previously (Figure 2). This is the first time that an open-microwave digestion system has been used in conjunction with ID analysis.

The methylmercury content of the sample was then calculated by applying the isotope dilution equation:[37]

$$c = \frac{c'w'A_r(RY' - X')}{wA_r'(X - RY)} \qquad (1)$$

For the sample, c is the concentration of sample solution (m/m), w is the mass of sample solution, A_r is the relative atomic mass of the element being determined, X is the isotope abundance (atom %) isotope 1, and Y is the isotope abundance (atom %) isotope 2.

For the spike, c' is the concentration of the spike solution, w' is the mass of the spike solution, A_r' is the relative atomic mass of the element in the spike, X' is the isotope abundance (atom %) of isotope 1, and Y' is the isotope abundance (atom %) of isotope 2.

The final parameter that requires measurement is the isotope ratio, R (X/Y). Apart from R, the other parameters are constants, masses and the concentration of the spike solution. Thus, the concentration in the unknown sample is ultimately based on the measurement of the isotope ratio R and the specification of the spiked material.

RESULTS AND DISCUSSION

Optimization of the CGC/ICPMS Conditions and Enriched-Methylmercury Synthesis. (1) Optimization of the GC Conditions. GC separation parameters were optimized to obtain symmetrical peaks, which allows one to minimize peak integration error. The solvent elution (2 μL of isooctane) causes severe plasma perturbation due to the high amount of carbon entering the plasma. Therefore, the GC conditions were chosen in a way that MMHg elutes with a retention time sufficiently far away from the disturbed zone, easily determined by monitoring the internal standard signal of continuously aspired Tl solution. First, degradation in the injection port due to high temperature was evaluated. The same solution (50 μg/L, 2 μL = 100 pg absolute as Hg) was injected repeatedly varying the injector temperature between 100 and 200 °C. No effect of injector temperature on elemental mercury formation from ethylated mercury species was found.

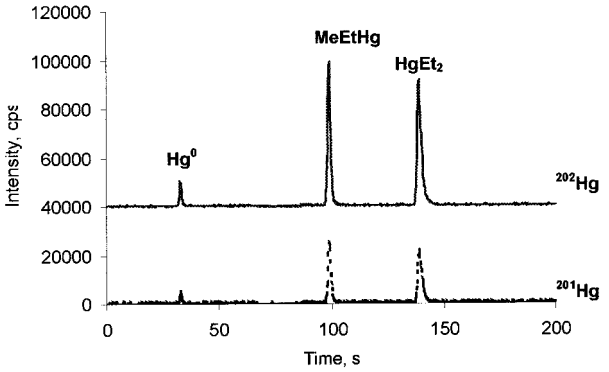

Figure 3. Chromatogram for mercury species (natural isotope abundance) obtained with CGC/ICPMS. (The plot at *m*/*z* 202 has been shifted for clarity.) Derivatization conditions: buffer, 2 M; pH 3.9; $NaBEt_4$, 0.5%.

The temperature program used, indicated in Table 1, gave adequate resolution for all the mercury compounds tested. Heating of the transfer line was not necessary. All compounds are baseline separated within 3.5 min (Figure 3), showing a good chromatographic profile with peak half-width of about 1.2 s. Peak shape is even more important in speciation ID analysis because the isotope ratios are calculated on the basis of the peak areas measurements. A significant improvement is obtained changing from packed to capillary column, with peak width of 1.2 s instead of 60 s for a packed column.[23]

(2) Optimization of the ICPMS Conditions. The configuration of our CGC/ICPMS system allows the aspiration of a solution while the GC is connected. Therefore, plasma and ion lens conditions were optimized daily by aspirating a solution containing 10 ng/g of Li, Y, Ce, and Tl while the helium carrier gas and argon makeup gas flows were the same as during the GC run. No instrument switch off was therefore necessary between ICPMS optimization and GC analysis, allowing optimum condition and continuous monitoring of the analytical performances.

Parameters affecting precision and accuracy of the isotope ratio measurements have been carefully evaluated. Apart from spectral interference and the detector linearity, there are detector dead time, mass bias, and data acquisition parameters.

Detector Dead Time. The detector dead time effect is mathematically corrected by using the equation

$$I_{real} = I_{exp}/(1 - \tau I_{exp}) \qquad (2)$$

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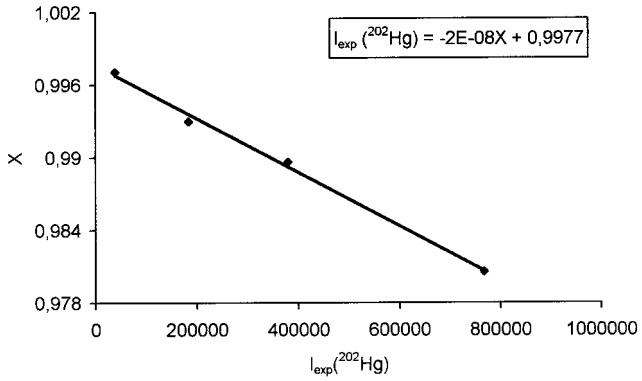

Figure 4. Typical graph obtained for dead time determination on the ICPMS. The resulting dead time is 39.8 ns. $X = R(^{202}Hg/^{201}Hg)_{theo}/R(^{202}Hg/^{201}Hg)_{exp}$.

where I_{real} is the real count rate, I_{exp} is the measured count rate, and τ is the detector dead time in nanoseconds.

For our experiments, we determined the detector dead time by measuring mercury solutions in the range of 10−200 μg/L by continuous aspiration, using the isotope ratio acquisition mode of the HP 7500 with an integration time of 5 s/isotope and a total measuring time of 5 min. Detector dead time was then calculated by applying a method proposed by Held and Taylor, using the equation[39]

$$\frac{R(^{202}\mathrm{Hg}/^{201}\mathrm{Hg})_{theo}}{R(^{202}\mathrm{Hg}/^{201}\mathrm{Hg})_{exp}} = 1 + [R(^{202}\mathrm{Hg}/^{201}\mathrm{Hg})_{theo} - 1]\tau I_{exp}(^{202}\mathrm{Hg}) \quad (3)$$

where $R(^{202}Hg/^{201}Hg)_{theo}$ is the theoretical isotope ratio, $R(^{202}Hg/^{201}Hg)_{exp}$ is the measured isotope ratio, $I_{exp}(^{202}Hg)$ is the measured count rate for isotope ^{202}Hg, and τ is the detector dead time. Plotting $R(^{202}Hg/^{201}Hg)_{theo}/R(^{202}Hg/^{201}Hg)_{exp}$ over $I_{exp}(^{202}Hg)$ yields a straight line, from the slope of which ($m = [R(^{202}Hg/^{201}Hg)_{theo} - 1]\tau$) the dead time can be calculated. In Figure 4, the plot of our data is shown. A detector dead time of 39.8 ns was calculated and applied for the subsequent isotope ratio measurements.

Mass Bias. In this work all measurements have been performed under wet plasma conditions with thallium solution simultaneously introduced via a T-piece during the GC measurements. Mass bias for Hg has then been corrected by using the ^{205}Tl/^{203}Tl isotope ratio. The use of the Tl correction system has several benefits on offer, mainly as the analyte in introduced via GC and the solution used for mass bias correction are measured simultaneously, changes during a single measurement or drift occurring during the day are automatically detected and can be corrected for. Additionally, less measurements are required and this allows one to reduce analytical time and cost.

The following correction equation was applied:[40]

$$R(^{202}\mathrm{Hg}/^{201}\mathrm{Hg})_{true} = \frac{R(^{202}\mathrm{Hg}/^{201}\mathrm{Hg})_{exp}}{1 + an} \quad (4)$$

Here a is the bias/mass unit and n is the mass difference between isotopes ^{202}Hg and ^{201}Hg. Factor a was determined by comparison of $(^{205}Tl/^{203}Tl)_{experimental}$ with $(^{205}Tl/^{203}Tl)_{theoretical}$.

Using this Tl correction the accuracy was found to be 0.2%, while without mass bias correction the deviations were usually more than 1.5%. This allows one to demonstrate that Tl is an adequate reference element for mass bias correction of mercury.

Data Acquisition Parameters. For long integration times each point will be well defined, but the chromatographic profile will be ill-defined, which is clearly visible when less than 10 isotopes/chromatographic peak are recorded. Thus, 40 ms for mercury isotopes (201, 202) and 5 ms for thallium isotopes (203, 205) were chosen, resulting a total integration time < 100 ms. With a full peak width of typically about 3 s, the peaks were defined by at least 30 points.

(3) Analytical Performance. Reproducibility of ^{202}Hg/^{201}Hg isotope ratio measurements on peak areas was 0.3% (20 pg, n = 5), and RSD did not improve with more replicates. Calibration curves in the range 5−100 pg for MeHgEt exhibited linear regression slopes and coefficients of 7726.4 counts/pg and r^2 = 0.992, respectively. The respective parameters for inorganic mercury (as $HgEt_2$) were 9423 counts/pg and r^2 = 0.997.

Absolute detection limits (n = 10), calculated using three times the standard deviation of background noise next to the chromatographic peak, were better than 21 fg for ^{202}Hg and 28 fg for ^{201}Hg. However, it is important to note that this is the instrumental detection limit for the CGC/ICPMS. The real limit of detection for isotope dilution analysis depends on the precision with which isotope ratios can be measured. It is not sufficient to detect MMHg with high sensitivity; it is necessary to measure the isotope ratio with optimum precision. This requirement increases the experimental detection limits for isotope dilution analysis significantly and is difficult to quantify. In our experimental conditions, above 5 pg of MMHg, sufficient precision with RSD < 1.0% (n = 5) in the isotope ratio measurement was obtained. Also the detection limit will be affected by the minimum amount of spike and sample that can be weighted with adequate precision, a parameter depending on the precision of the analytical balance used.

(4) Characterization and Analysis of Isotopically Enriched Methylmercury by Reverse Isotope Dilution. First, the differences between the supplier's recommended atom fraction composition certify and actual abundances of the individual mercury isotopes in the initial inorganic mercury tracer solution were evaluated. This study has been performed directly with mercury liquid solution aspirated continuously. Measured values correspond quite well with the recommended ones; the most significant difference was found for the less abundant isotope. As expected, significant enrichment in ^{201}Hg (found, 97.31 ± 0.06%;

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supplier's value, 98.11%) is found with respect to the natural isotopic distribution (13.18%).[41]

The ^{201}Hg-enriched MMHg was synthesized by reaction with methylcobalamine as described in the procedures. The isotopic analysis of the spike has been performed by CGC/ICPMS after ethylation. The isotopic composition has been calculated from the measured peak area. This solution has a ^{202}Hg/^{201}Hg ratio of 0.0146 greatly different from the natural ^{202}Hg/^{201}Hg ratio of 2.2655.[41]

There are also some traces of inorganic mercury, typically less than 5%, in the enriched methylmercury solution. From the isotope abundance, it is evident that there are two sources of this inorganic mercury, one coming from the excess of ^{201}Hg that has not reacted and another part derived from the reagent blank. The ^{202}Hg/^{201}Hg ratio of this inorganic mercury is 0.157 instead of 2.265 for natural or 0.0146 for enriched MM^{201}Hg.

The concentration of the enriched MMHg in the spiked solution and the obtained reaction yield were calculated using reverse isotope dilution analysis (this final solution was spiked with natural methylmercury of known concentration and purity). Two independent isotope dilution experiments were carried out. Each solution was injected four times. Average concentration of the spike solution turned out to be 468.4 ± 1.4 ng/g and this value corresponds to a reaction yield of 87.8%.

The stability of this enriched solution has been checked during a period of 3 months. During the first 1 month it was quite stable, but in the whole 3 months period a degradation of about 20% was found. Therefore, if this solution is going to be used as a standard, concentration should be frequently checked.

Optimization and Validation of the Isotope Dilution Procedure. (1) Derivatization Conditions. *Degradation of MMHg.* One of the most popular derivatization approaches to convert inorganic mercury and methylmercury into species amenable for GC separation is ethylation using $NaBEt_4$. However, effective interference during the ethylation reaction have been observed especially with halide ions.[26,27,42] The degradation of MMHg into Hg^0 during the derivatization step has been reported. These statements have lead to propose alternative derivatization reagents for aquatic and biological samples, such as hydride generation or more recently propylation.[22,27,42] Although these methods have been successfully applied, ethylation has been found to be suitable for a large number of applications after proper optimization of the derivatization reaction conditions.[26]

In consequence, derivatization conditions were carefully evaluated. Using the coupled CGC/ICPMS technique and isotope ratio measurements, the potential occurrence of chemical transformation during sample treatment can be evaluated by analyzing a mercury solution with altered isotope abundance.[23] Isotope ratio calculations for each mercury species allow the identification of possible degradation reactions during sample treatment.

To evaluate such possible degradation effects during derivatization, a mercury solution of MMHg enriched with the 201 isotope was derivatized as by aqueous ethylation. Initial experiments have been carried out working at pH 4.9 using buffer acetate (2 M) and $NaBEt_4$ concentration of 0.06% w/v. Under these conditions, an additional peak appears and corresponds to elemental mercury, as confirmed by mercury vapor injection. The peak at mass 201 for elemental mercury was much higher than that at mass 202, indicating its provenance from the enriched spike.

Table 2. Isotope Ratio (^{202}Hg/^{201}Hg) Measurements of the 201-Enriched Methylmercury Standard ("Unspiked") and the 201-Enriched Methylmercury Spiked with Hg^{2+} of Natural Isotope Abundance ("Spiked") (Mean Value ± SD)

species	natural Hg	unspiked MM^{201}Hg	spiked MM^{201}Hg
Hg^0	2.265	0.0165 ± 0.0002	0.1226 ± 0.0042
MMHg	2.265	0.0141 ± 0.0002	0.0145 ± 0.0005
Hg^{2+}	2.265	0.0882[a] ± 0.0028	0.7519[a] ± 0.0193

[a] Impurities of Hg^{2+} in the enriched MM^{201}Hg originated in both excess of none reacting ^{201}Hg and reagent blank during sample preparation.

Additionally, to test for possible degradation also of Et_2Hg derived from Hg^{2+}, the enriched MM^{201}Hg was spiked with natural inorganic mercury. Isotope ratios were measured in both the spiked and unspiked enriched MMHg for all three species (peaks of Hg^0, MeEtHg, and Et_2Hg) on the basis of peak-area ratios. These results are summarized in Table 2. The isotope ratio of the peak representing methylmercury changed accordingly. The isotope ratio of the Hg^0 peak is different when natural, unspiked, or spiked enriched methylmercury is processed. This is evidence that Hg^0 is formed from the degradation of both ethylated methyl and inorganic mercury. No attempt to quantify these effects has been undertaken as the aim of this study was to determine optimum derivatization conditions to prevent MMHg degradation.

The formation of Hg^0 as a breakdown product of Et_2Hg or EtMeHg has been previously reported working with a purge-and-trap systems.[26,27,42] De Diego et al. and Demuth and Heumann have demonstrated that halide ions (e.g., Cl^-) are responsible for the degradation of MMHg in seawater samples. Tseng et al. reported that pH and the amount of $NaBEt_4$ affected the Hg^0 formation in the presence of large amounts of inorganic mercury. In our case, the halide interference mechanisms is not very likely to be significant as the degradation is found in standards prepared in Milli-Q water where high chloride content not expected. The effect of the buffer solution pH and concentration was tested (Figure 5). At the lower pH and concentration, MMHg degradation is reduced and can be maintained below 10% yield. This is consistent with the results of Tseng et al., who found the lower was the pH, the less Hg^0 formation was induced. Below pH 3, the derivatization of Hg species will be diminished owing to the rapid decomposition of the ethylation agent.

All the investigations agreed with the fact that transformations of MMHg take place during the derivatization step. However, the mechanism is still unresolved. It seems to be significantly affected by the combined effect of several variables, pH, buffer, and $NaBEt_4$ concentrations, presence of halide ions, etc., but further experiments are still needed to fully understand the pathways of these degradation processes.

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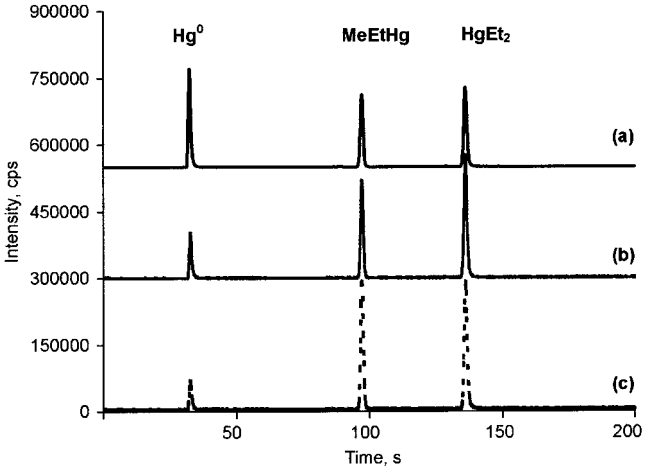

Figure 5. Hg^0 formation as a function of the buffer solution pH and concentration for 100 pg MMHg and Hg^{2+} aqueous standards: (a) 2 M, pH 4.9; (b) 2 M, pH 3.9; (c) 0.1 M, pH 3.9. (The plot at mass 202 is shown.)

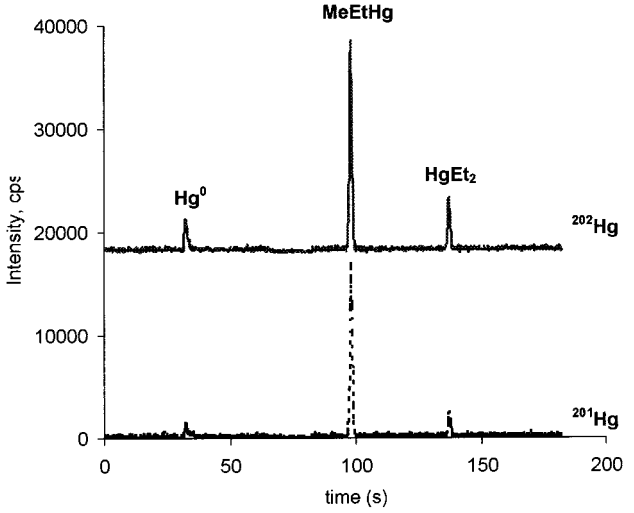

Figure 6. Chromatogram obtained for the DORM-1 extract spiked with ^{201}Hg-enriched methylmercury solution. The chromatogram at mass 202 was shifted.

In our case, the ethylation reaction step has been just optimized for quantitative analysis by setting proper pH conditions and reducing interference mechanisms.

Recovery of MMHg. Optimized derivatization reaction conditions (pH 3.9; 0.06% w/v $NaBEt_4$) were applied to a hydrolysate of biomaterial (DORM-1) (0.2 g of dry tissue in 5 mL of TMAH, prepared as described in the procedures) and yielded quantitative recoveries (>95%) after direct determination by CGC/ICPMS (i.e., external calibration). On the contrary, after the biomaterial was spiked with the $MM^{201}Hg$ by following the procedure previously described, the yield calculated by direct measurement was as low as 20%. This important loss of total recovery produces a significant diminution of sensitivity that cannot be accepted for trace analysis. However, the quantitative results provided by isotope dilution calculation were in good agreement with the certified values. In this case, speciated isotope dilution is again demonstrating the full potential for environmental samples determination when spike equilibrium is achieved.

The possible reasons for this signal depression in the spiked sample were studied. The spiking procedure includes the addition of the 201-enriched MMHg, which is dissolved in 2-propanol, and the addition of methanol for homogenization. Methanol is eliminated by a N_2 stream before MW extraction. The possibility of methylmercury loss during the solvent evaporation was tested. Similar signal diminution was found when methanol was left out and the spike was added directly to the solid before MW extraction without solvent evaporation. No loss by volatilization during the MW extraction related to the low boiling point of 2-propanol (boiling point 82.4 °C) was observed. Similar results were obtained when the spike was diluted in ethylene glycol (boiling point 198 °C) just before MW heating.

Finally, the effect of the amount of ethylation reagent on the recovery of methylmercury was examined. The higher the $NaBEt_4$ quantity, the higher the recovery; an 8-fold increase in the concentration of ethylation reagent was required. Consequently, when an organic solvent is present during the MW extraction, interfering precursors are probably generated and consume the bulk of the derivatization reagent before it reacts with the target species. Taking into consideration that the reagent will be consumed to various degrees depending on the composition of matrix components, including other trace metals present in the sample, the amount of reagent added should be in excess of the minimum requirement. These conditions were also tested with an other reference material (CRM 463). For such material, addition of 5 mL of 0.5% $NaBEt_4$ was also found to be sufficient for a maximum ethylation yield (>95%).

The two optimization experiments performed have allowed us to set the derivatization conditions by adjusting the pH and $NaBEt_4$ amounts for MMHg ethylation. Additionally, a complete samples preparation protocol (i.e., spike, extraction, derivatization) has been validated for MMHg determination by speciated isotope dilution in biological materials.

(2) Validation of the Isotope Dilution Analysis of MMHg in Certified Biological Samples. Speciated isotope dilution analysis was applied to two fish muscle certified reference materials (DORM-1 and CRM 463) and an oyster tissue obtained from an intercomparison exercise (T-38). Biological samples were analyzed by following the procedures of spiking, extraction, and derivatization described in the Experimental Section. Figure 2 represents a flowchart of the different steps along with the time. Long equilibration times of the spike and the sample (hours) are used in combination with very short extraction times (minutes) by microwave-assisted preparation. Full equilibration is the main requirement of isotope dilution technique.

Optimum of the sample-to-spike ratio is also important for minimizing errors in the final determination. Most of the analytical uncertainties, except mass bias, can be avoided when the spiked isotope concentration is matching the sample concentration to be determined. In our case, preliminary determination was performed using conventional techniques to obtain an estimation of the MMHg concentration in the sample. Then, speciated isotope dilution was subsequently performed to obtain an isotope ratio of spike isotope to analyte isotope close to unity. At least five independent spiking experiments were carried out to analyze each reference material, and each sample solution was injected four times into the CGC/ICPMS system.

Table 3. Concentration of Methylmercury in Reference Materials Determined by Speciated Isotope Dilution Analysis (Enriched Solution Spike before and after MW Extraction)[a]

material	concn of MMHg, μg/g		
	determined		
	before	after	certified
DORM-1 (fish muscle)	0.791 ± 0.030 (n = 5)	0.766 ± 0.036 (n = 3)	0.786 ± 0.060
CRM 463 (fish muscle)	2.96 ± 0.07 (n = 6)	2.93 ± 0.06 (n = 3)	3.03 ± 0.15
T-38 (oyster tissue)	0.061 ± 0.001 (n = 5)	0.060 ± 0.001 (n = 4)	0.057[b]

[a] n, number of independent determinations. [b] "Recommended value".

A chromatogram obtained for DORM-1 spiked with ^{201}Hg-enriched methylmercury solution is shown in Figure 6 (for masses 201 and 202). As can be seen, the isotopic composition of methylmercury has been altered drastically; a measured 202/201 isotope ratio of 1.21 instead of the natural value of 2.26 was determined. Final results for the speciation of methylmercury by isotope dilution analysis (with the spike performed before the microwave heating) are presented in Table 3. As can be seen, the results show both good accuracy and precision.

The isotope dilution procedure provide excellent results independent from the total recovery, as long as spike and analyte are in equilibrium prior to sample preparation. To study the efficiency of the microwave extraction procedure with TMAH another experiment was performed. Here, the labeled methylmercury was added to the extract after the microwave extraction. The obtained results are also shown in the Table 3. Results within the analytical uncertainty between the certified and determined concentrations were obtained for both CRM 463 and DORM-1, and values close to the "recommended value" were found for the intercomparison material. These results indicated that the extraction procedure is adequate for various biological materials and provide good accuracy when combined with ethylation, as previously reported by other methods.[22]

CONCLUSIONS

An accurate and precise isotope dilution method for MMHg in biological samples by CGC/ICPMS has been developed. Taking into account that ID is considered to be an absolute method of analysis, the proposed procedure could be used as a reference method in control laboratories. The combination of microwave-assisted extraction with CGC/ICPMS has been demonstrated to be a consistent approach for species specific isotope dilution analysis. Systematic errors seriously affecting the accuracy of the attainable isotope ratio measurement have been carefully evaluated. The use of wet plasma conditions have allowed, for the first time for mercury, to perform mass bias correction by using continuously and simultaneously added aqueous thallium solution. Another advantage of this configuration it is the lack of requirement for oxygen, which is necessary to avoid carbon deposition from the organic solvent under dry plasma conditions.

A ^{201}Hg-labeled MMHg has been prepared and characterized by reverse isotope dilution analysis to be used for isotope dilution analysis of MMHg in different reference materials. However, the application of stable isotopes in environmental studies is not limited to the classical isotope dilution analysis. Stable enriched tracers can be applied to study transformations processes during the different steps of the analysis. Isotopically labeled species have an even greater potential in investigating dynamic environmental processes.

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