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Working Methods Paper

Micro-scale preparation and characterization of isotopically enriched monomethylmercury

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A synthesis method for the micro-scale laboratory preparation of isotopically enriched monomethylmercury (MMHg) has been successfully established. This compound is an important standard for species-specific isotope dilution analysis. The isotopically enriched MMHg has been synthesized from commercially available mercury oxide (^{201}HgO) using methylcobalamin coenzyme as methylating agent. The time required is less than 2 h and the final yield is about 90%. The proposed method is faster than those previously reported in the literature. It allows work on a micro scale to control the use of expensive enriched isotope standard. It also allows control of unintentional formation of dimethylmercury. The enriched mercury-containing reaction products were analyzed by capillary gas chromatography coupled to an inductively coupled plasma mass spectrometer after derivatization with sodium tetraethylborate. The isotopic composition, concentration, purity and stability of the synthesized, enriched MMHg have been investigated in order to establish standard protocols for MMHg isotope dilution analysis or isotope labeling incubation experiments. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: monomethylmercury (MMHg); ^{201}Hg; synthesis; isotope dilution analysis

INTRODUCTION

Isotope dilution mass spectrometry (IDMS) has been considered to be a definitive method, offering the potential for very low uncertainties.[1] Provided that 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. For speciation analysis, the use of the isotope dilution technique offers great potential, since quantitative recoveries are not necessary and rearrangement reactions are easily detected. However, in spite of the benefits on offer, IDMS remains an underexploited method of analysis, primarily because of the lack of suitable enriched internal-calibration standards. Isotopically enriched methylmercury is not commercially available and needs to be synthesized in the laboratory.

Several methods are proposed in the literature for organomercury compound production, such as the use of tetramethyltin[2] or an un-symmetrization reaction between mercuric chloride and dimethylmercury (DMHg) stock solutions.[3] The most often used method is based on the reaction of methylcobalamin with inorganic mercury. A few years ago, it was demonstrated that mercury(II) could be methylated chemically by methylcobalamin in the absence of cell extract.[4] The methyl to cobalt bond is stable, but it allows methyl transfer to certain metal species. Different mechanisms are possible, such as direct substitution, radical addition, etc.[5,6] Two reaction products, DMHg chloride and monomethylmercury (MMHg) chloride, were formed with different yields depending on the molar ratio of the reactants and the reaction times.[4] The initial product of this

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reaction was found to be DMHg, especially when equimolar or lesser amounts of mercuric chloride were used. Bertilsson and Neujahr[7] carried out the same experiment, obtaining the same results for MMHg, but DMHg could not be detected by the Westöö method.[8]

More recently, Filippelli and Baldi[9] have carried out a study to determine the organic mercury species formed in the reaction of ionic mercury with methylcobalamin. The reaction products were analyzed by purge-and-trap gas chromatography on line with Fourier transform infrared (PT GC/FTIR) spectroscopy. This method does not require solvent extraction of the organomercurials from aqueous solution. It was found that the yield of DMHg and MMHg formation depended on different parameters: pH, temperature, reaction time, and the methylcobalamin/ionic mercury ratio. The initial reaction product was MMHg, which was further transformed to DMHg. The first methylation reaction rate was two times faster than the second one.

The aim of these studies[2–7,9] was to study the reaction products of methylcobalamin and ionic mercury but not to synthesize methylmercury with the maximum yield and purity in order to use it as a standard compound. This was the aim of a proposed method to synthesize radioactive methylmercury.[10] It is based on the methylation of inorganic ^{203}Hg(II) by methylcobalamin and isolation of the resulting MM^{203}Hg in a single extraction step. The time required was less than 4 h and the final yield was 90%. The reaction conditions were not studied, and no information was provided about such parameters as temperature, pH, and Hg/MeCo ratio, and the reaction product was not characterized with respect to purity and stability.

Therefore, with this in mind, we undertook a series of experiments to assess the possibility of producing isotopically enriched methylmercury under standard laboratory conditions. The initial conditions were adapted from available literature methods according to the following criteria:

1. Mercury oxide must be used as a starting material; this is the isotopically enriched form that is commercially available.
2. DMHg formation must be avoided.
3. Because of the high cost of the starting material, only small quantities are used, so it is necessary to work on a micro scale.
4. For the same economic reason, purification processes involving wasteful separations methods should be avoided.

The reaction products have been analyzed by PT GC coupled to atomic fluoresence spectrometry (AFS) detection after hydride generation and by capillary GC (CGC) coupled to inductively coupled plasma (ICP)-MS for the ethylated species. The isotopic composition, concentration, purity and stability of the synthesized enriched methylmercury have been carefully controlled.

EXPERIMENTAL

Instrumentation

For speciated isotope dilution analysis a gas chromatograph (HP 6850) equipped with a capillary column was coupled to an Agilent Model HP-7500 ICP mass spectrometer *via* a Silcosteel (Restek) transfer capillary. A detailed description of the instrumental configuration is published elsewhere.[11] Briefly, the Silcosteel capillary was inserted into the torch injector and the connection to the torch was realized by means of a glass T-piece. A Scott cooled (2 °C) spray chamber and a conventional Babington nebulizer were connected to this T-piece and this enabled continuous aspiration of a standard thallium solution (10 μg l^{-1}). This configuration allowed optimization of instrument performance and simultaneous measurement of ^{203}Tl and ^{205}Tl for mass bias correction during the chromatographic run. The raw data of the transient isotope signals for the different mercury species were further processed using HP software to obtain the corresponding isotope ratios.

Standard measurements were also performed on an automated on-line hydride generation-cryogenic trapping-GC–quartz furnace AFS (HG-CT-GC–QFAFS) when no isotopic information was required. Further information about this analytical method can be found elsewhere, both using atomic absorption spectrometry[12,13] and AFS[14,15] detection systems.

Reagents

Stock solutions of Hg^{2+} and MMHg (1000 mg l^{-1}) of natural isotopic composition were prepared by dissolving mercury(II) chloride (Strem Chemicals 99.9995% mercury) 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 were stored in the fridge. Methylcobalamin (Sigma) used for synthesis was prepared by dissolution in an acetic acid–acetate buffer solution (0.1 M, pH 5). ^{201}HgO was obtained from Oak Ridge National Laboratory (Oak Ridge, USA). The sodium tetraethylborate (98%) was purchased from Strem Chemicals (Bischheim, France).

All other reagents were of analytical grade, apart from the organic solvents, which were HPLC grade. Ultrapure water (>18 MΩ cm) was obtained from a Milli-Q system (Quantum, Ex, Millipore, USA).

Procedures

Hydride generation analysis by CT-GC–QFAFS

Aliquots of 50 μl or 20 μl of the toluene or the isopropanol extract, respectively, were added to 50 ml of Milli-Q water in the 250 ml reaction vessel. The pH was adjusted to 1–2 by adding concentrated HCl. 5 ml of an approximately 4% m/v solution of $NaBH_4$ was added and allowed to react for 0.5 min. The elemental mercury and methylmercury hydride

generated were analyzed by CT-GC–QFAFS.[14,15] Mercury species were purged from the reaction vessel and trapped in the column, which was immersed in liquid N_2 (−196 °C). The column is wrapped with 0.5 mm diameter Nichrome wire, which can be heated by an adjustable DC power supply (Hemitechnic). Then, the column was gradually warmed and the volatile mercury species successively eluted on the basis of increasing boiling points. Atomization took place in the quartz furnace heated to 800 °C and then the species were detected by AFS.

Ethylation of mercury compounds for CGC–ICP-MS analysis

Mixed standards solutions of the different mercury compounds were buffered to pH 3.9 with 5 ml of a 0.1 M acetic acid–sodium acetate buffer. Then, 5 ml of 0.5% sodium tetraethylborate and 2 ml of isooctane were added in order to derivatize and extract the alkylated 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.

Synthesis and analysis of ^{201}Hg-enriched methylmercury

A flow chart diagram of the MMHg synthesis protocol is shown in Fig. 1.

A solution of $HgCl_2$ was prepared by dissolving 1.5 mg of ^{201}HgO in 100 µl of HCl (conc.). Approximately 10 µl of this solution was transferred to the amber micro-reaction reactors (Supelco) and diluted with 500 µl of sodium acetate buffer (0.1 M, pH 5). 500 µg of methylcobalamin was dissolved in another 500 µl of buffer and added to the inorganic mercury solution. The solution was left sitting in the dark at 37 °C for 1 h. In order to stop the methylating reaction and to convert the unintentionally formed DMHg into methylmercury, the mixture was cooled at 4 °C, and 1 ml of HCl (conc.) was added and then shaken for 5 min. The MM^{201}Hg formed was extracted three times with 500 µl of toluene. The combined toluene extracts were dried over sodium sulfate. 100 µl of this primary solution were diluted with 10 ml of isopropanol. Working solutions were prepared fresh daily by diluting the secondary isopropanol stock solution with deionized water as needed. The toluene extracts and the subsequent diluted solutions were stored in the fridge and protected from light until use.

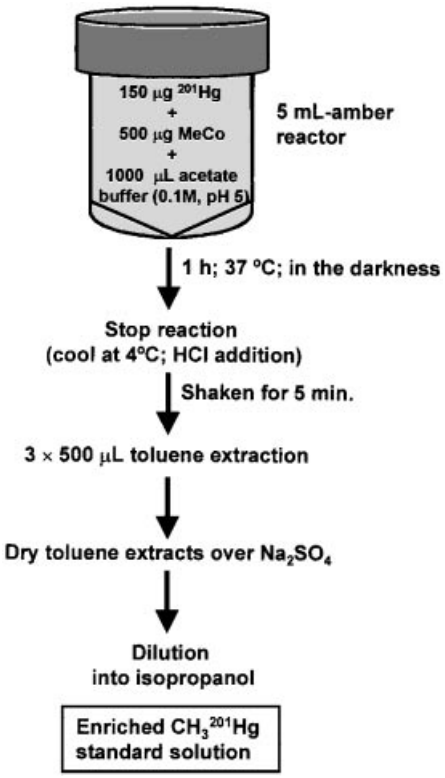

Figure 1. Flow chart for MMHg isotopically enriched synthesis.

Purification procedure: thiosulfate extraction[16]

The MMHg generated was initially extracted in toluene; this organic phase was washed several times with H_2O. To 1 ml of the toluene extract was added 0.1 ml of 1 mM sodium thiosulfate solution. The MMHg in the thiosulfate solution was washed three times with toluene. After addition of 20 µl copper sulfate (1 M) and 50 µl sodium chloride (0.5 M), MMHg was finally extracted into toluene (2 × 0.5 ml). The combined toluene extracts were dried over sodium sulfate. 100 µl of this primary stock solution was diluted to 10 ml of isopropanol.

Purification procedure: cysteine extraction[17]

1 ml of a 1% aqueous solution of cysteine acetate was added to 1 ml of the initial toluene extract. The aqueous layer was then acidified with 1 ml of 6 M hydrochloric acid and back-extracted twice with 1 ml of toluene. The combined toluene extracts were dried over sodium sulfate. 100 µl of this primary stock solution was diluted with 10 ml of isopropanol.

RESULTS AND DISCUSSION

Synthesis conditions

The most critical parameters affecting inorganic mercury alkylation by methylcobalamin are: reaction time, temperature, pH and Hg/MeCo ratio. In our case, special attention was paid to conditions affecting DMHg production, since our aim was to produce MMHg standard solution with minimum impurities.

Preliminary tests were performed using natural mercury oxide and the reaction products both before and after toluene extraction were analyzed by automated on-line (HG-CT-GC–QFAFS).[14,15] Table 1 shows the results for the different conditions tested (reaction time, buffer concentration, Hg/MeCo ratio and stop methylation procedure). Before

Table 1. Reaction conditions tested for the small-scale preparation of isotopically enriched methylmercury. Analysis by HG-CT-GC–QFAFS before and after toluene extraction

Reaction conditions[a]	MMHg yield before extraction (%)	After toluene extraction				
		Extraction efficiency (%)			Final concentration (ng g^{-1})	
		MMHg	Hg^{2+}	Area (2.71 min)	MMHg	Hg^{2+}
1	94.1	82.7	22	266027	399.3	78.6
2	56.2	124	39.4	291707	445	75.7
3	57.6	89.2	52	74959	293.6	264.3
4	81.9	73.9	37.5	88956	345.6	140
5	89.5	89.1	36.6	134849	455.7	165.4
6	79.2	66.1	22.4	79169	300	95
7	57.0	90.2	84.0	62868	257.1	80.7
8	61.5	55.9	49.8	65088	171.4	59.8

[a] 1: **1 h**; HCl 20 min; buffer 0.5 M; 2:1 Hg/MeCo. 2: **3 h**; HCl 20 min; buffer 0.5 M; 2:1 Hg/MeCo. 3: 1 h; HCl 20 min; buffer **0.1 M**; 2:1 Hg/MeCo. 4: 1 h; HCl **2 min**; buffer 0.1 M; 2:1 Hg/MeCo. 5: 1 h; **without HCl**; buffer **0.38 M**; 2:1 Hg/MeCo. 6: 1 h; HCl 5 min; buffer 0.1 M; 2:1 Hg/MeCo. 7: 1 h; HCl 5 min; buffer 0.1 M; **1:1** Hg/MeCo. 8: 1 h; HCl 5 min; buffer 0.1 M; **1:1** Hg/MeCo. MeCo = methylcobalamin.

toluene extraction, only MMHg yield is given. Afterwards, both MMHg and inorganic mercury efficiency extraction are evaluated; the final concentrations are also reported. Additionally, peak area values of an 'interfering peak' are given.

Temperature and light

In the literature,[2–7,9] there is a general agreement about maximum reaction yield at 37°C and in absence of light. Therefore, all experiments in the present study were performed in amber micro-reaction reactors incubated at 37°C in the dark.

pH and buffer concentration

Optimum formation has been reported at acid pH values, with a peak at pH 5 for MMHg.[9] Therefore, it was considered to be advisable to carry out all the experiments at pH 5, controlled by the presence of buffer acetate. This buffer addition was previously discarded by Filippelli and Baldi[9] to avoid interference by presumed complexes with mercury compounds in the aqueous solution. Different acetate buffer concentrations (0.1, 0.5 and 0.4 M) were tested (Table 1) without affecting the mercury methylation yield.

Mercury/methylcobalamin ratio

The formation of MMHg also depends on the ratio between Hg^{2+} and methylcobalamin. Filippelli and Baldi[9] have reported that at lower ratios, i.e. with more methylcobalamin than ionic mercury, the rate of DMHg formation was favored, whereas at higher concentration of inorganic mercury with respect to the cofactor it was MMHg that was favored. From experiments 6, 7 and 8 (Table 1), the maximum MMHg yield is obtained with mercury/MeCo ratio of 2:1.

Reaction time

Methylmercury, as the intermediate compound between Hg^{2+} and DMHg, was formed quickly. Therefore, short reaction times were tested (Table 1: experiments 1 and 2). No significant differences in the yield of MMHg after toluene extraction were observed with a reaction time of 1 or 3 h, so 1 h was finally retained.

Recovery and formation of DMHg

Under the synthesis conditions tested, no DMHg formation was found. In the present study DMHg was measured in the liquid phase under closed conditions. This DMHg could be underestimated due to the chemical equilibrium reached between DMHg concentrations in the aqueous and gaseous phases. A percentage of the volatile DMHg could be lost during procedures of mercury speciation, mainly when pouring the sample from the tinted vials to the purge-and-trap reaction vessel. Therefore, although DMHg has not been found under our experimental conditions, possible traces cannot be discarded. Imura *et al.*[4] have reported that methylation of mercury is stopped by the addition of concentrated HCl to the reaction mixture, and that DMHg was quantitatively changed into methylmercury chloride by this acid treatment. Therefore, we stopped the reaction by cooling at 4°C and by addition of concentrated HCl, keeping the minimum shaking time (5 min; Table 1: experiments 3, 4 and 5).

Purification procedure

Purification under toluene extraction was carried out, as it does not form ligands to inorganic mercury salts. Up to 90% extraction efficiency was found for MMHg, whereas values

Table 2. Supplier's recommended atom fraction composition of the ^{201}Hg-enriched mercury aliquot and mass fractions determined by ICP-MS of the ^{201}Hg tracer and isotopically enriched methylmercury. Natural abundance are included for comparison

		Enriched ^{201}Hg ($n = 9$)		Enriched MM^{201}Hg ($n = 5$)	
Mass	Natural abundance	Certified	Determined	MMHg	Hg^{2+}
196	0.15	<0.05	<0.05	–	–
198	10.02	0.08	0.159 ± 0.005	0.131 ± 0.005	3.4 ± 0.3
199	16.84	0.10	0.31 ± 0.02	0.21 ± 0.01	5.8 ± 0.2
200	23.13	0.45	0.64 ± 0.01	0.56 ± 0.02	8.1 ± 0.6
201	13.22	98.11	97.31 ± 0.06	97.6 ± 0.1	69.3 ± 1.4
202	29.86	1.18	1.44 ± 0.02	1.43 ± 0.06	10.9 ± 0.3
204	6.85	0.08	0.134 ± 0.006	1.299 ± 0.008	2.5 ± 0.3
R (202/201)	2.2655	0.0120	0.0147	0.0146	0.157

as low as 10% were obtained for Hg^{2+}. An additional peak at a retention time of 2.71 min appears when the isopropanol solution is measured by HG-CT-GC–QFAFS, along with the inorganic and methylmercury peaks. Initially it was thought to be another mercury species produced as a secondary product during the reaction, for instance MeEtHg as a consequence of the acetate buffer presence. However, this hypothesis was finally discarded, and this 'additional' peak seems more likely to be an optical interference due to the AFS detector system. This is supported by the following facts: when the reaction product is analyzed before the toluene extraction this peak is not observed; also, when toluene is directly diluted in isopropanol, without the synthesis procedure, this peak also appears.

Other purification procedures, apart from toluene extraction, were tried. As proposed by Hintelmann and Evans[16] to remove trace quantities of inorganic mercury, Hg^{2+} extraction with thiosulfate was tested. An 80% of recovery for both MMHg and inorganic mercury was found, so no purification was obtained by this procedure. Clean-up of the extract was also tried by complexation of MMHg with an aqueous solution of cysteine acetate.[17] Again, the extraction was nearly quantitative, both for MMHg and inorganic mercury (91% and 85% recovery respectively). Therefore, purification only under toluene extraction was finally retained.

Working under the optimized conditions (i.e. 37 °C, in the dark, pH 5 controlled by acetate buffer (0.1 M), 1 h reaction time, Hg/MeCo ratio of 2:1, concentrated acid treatment and toluene extraction) the yield of MMHg was about 80%. A flow chart of the MMHg synthesis protocol is shown in Fig. 1.

Isotopically enriched MMHg synthesis and characterization

Once the synthesis conditions had been optimized, the definitive synthesis using ^{201}Hg-enriched mercury oxide was carried out.

Firstly, the differences between the supplier's recommended atom fraction composition certified and the actual abundance of the individual mercury isotopes in the initial inorganic mercury tracer solution were evaluated. This study was performed directly with the mercury liquid solution aspirated continuously. The values are shown in Table 2, together with the natural mercury isotope abundance.[18] The measured values correspond quite well with the recommended ones; the most significant difference corresponds with the less abundant isotopes. As expected, significant enrichment in ^{201}Hg is found with respect to the natural isotopic distribution.

Isotopically enriched MMHg was synthesized from ^{201}Hg-enriched solution according to the conditions previously selected. The analysis of the reaction products are then performed by CGC–ICP-MS after ethylation with $NaBEt_4$. Figure 2 shows the chromatogram obtained for mercury isotopes. As can be observed, very low levels of Hg^{2+} are present in the final product.

The isotopic composition of the ^{201}Hg-enriched methylmercury is also shown in Table 2, and was calculated from the measured peak area. This solution presents a

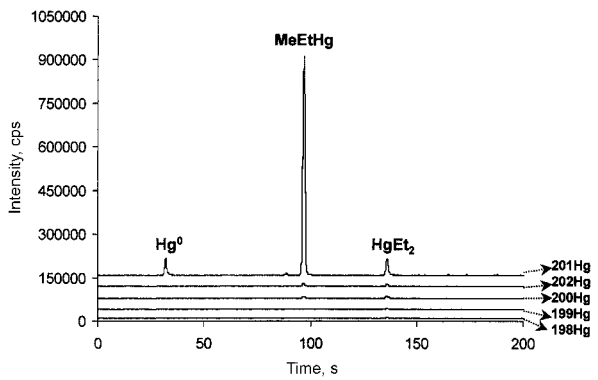

Figure 2. Chromatogram obtained for the synthesized ^{201}Hg-enriched methylmercury. Chromatograms obtained at different masses were shifted for clarity.

$^{202}Hg/^{201}Hg$ ratio of 0.0146, which is greatly different from the natural $^{202}Hg/^{201}Hg$ ratio[18] of 2.2655. It can therefore be used directly to perform isotope dilution analysis of MMHg in real samples (spiked solution).

There were also some traces of inorganic mercury. From the isotope abundance, it is evident that there are two sources of this inorganic mercury: from the excess ^{201}Hg that has not reacted, and from contamination by natural mercury during the preparation of the solution. 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 concentrations of the enriched MMHg in the spiked solution and in the reaction yield obtained were calculated using reverse isotope dilution analysis (this final solution was spiked with natural methylmercury of known concentration). Two independent isotope dilution experiments were carried out. Each solution was injected four times. The average concentration of the spiked solution turned out to be 468.4 ± 1.4 ng g^{-1}. This value indicates that, under our synthesis conditions, the methylation reaction yield is 87.8%.

The stability of this enriched solution has been checked over a period of 3 months. During the first 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, the concentration should be checked frequently.

This enriched methylmercury solution has been successfully used for isotope dilution analysis of methylmercury in biological samples.[11]

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