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Micro- scale preparation of isotopically enriched monomethylmercury ($MM^{201}Hg$)

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Abstract

A simple and fast synthesis method for the micro-scale laboratory preparation of isotopically enriched monomethylmercury (MMHg) which is not commercially available has been evaluated and established successfully. This enriched solution is an important standard for species-specific isotope dilution mass spectrometry analysis (SSIDMS) for determination of the true concentration of methylmerury in biological (hair) and environmental samples (soil and sediment). The isotopically enriched MMHg has been synthesised from inorganic standard solution prepared from commercially available mercury oxide (^{201}HgO) using methylcobalamin co-enzyme as methylation agent. The synthesis time required is 1 h at 37°C. The product is highly pure, yielded more than 95% as ^{201}Hg in MMHg. The proposed method is allows control of unintentional formation of health hazard dimethylmercury. It is also allows work on a micro-scale to control the use of expensive enriched isotope standard. The synthesised product was analysed using capillary gas chromatography (CGC) coupled with inductively coupled plasma mass spectrometry (ICP-MS) after derivatisation with sodium tetraethylborate ($NaBPr_4$). The concentration, purity, isotopic composition and stability of synthesized, enriched MMHg have been investigated in order to establish standard protocols for MMHg isotope dilution analysis.

Keywords: Monomethylmercury; species-specific isotope dilution mass spectrometry analysis (SSIDMS); Sodium tetra (n-propyl) borate; mercury oxide (^{201}HgO); Dimethylmercury; methylcobalamin co-enzyme.

Introduction:

Mercury is potentially one of the most toxic metals to organisms as it has strong ability to bioaccumulate and the enrichment of highly toxic mercury (Hg) compounds in aquatic food chain poses a serious environmental problem (1-5). The most important chemical forms of mercury are elemental mercury (Hg°), inorganic mercury (Hg^{2+}), monomethylmercury (Me-Hg, CH_3Hg^+) and dimethylmercury (DMHg, CH_3HgCH_3). In the biogeochemical cycle of mercury, the mercury vapour is released to the environment through anthropogenic sources i.e. human activity including emissions from coal burning in power station, chloro-alkali plants and artisanal gold mining which is oxidized in the upper atmosphere to water-soluble ionic mercury (Hg^{++}), and returned to earth surface (both soil and water) in rainwater(1,6).

The long transport cycle of mercury in the atmosphere; its deposition, bioaccumulation, and the enrichment of highly toxic methylmerury (Me-Hg) compounds in aquatic food chain pose a serious environmental problem, even in remote areas and contaminating, humans(3,4,7-9). MMHg is efficiently adsorbed from the gastro-intestinal tract, and it passes the blood-brain and placenta barriers and it causes disintegration of cells within the brain. The main target of methylmerury in humans is the central nervous system-especially the control sensory, visual and auditory areas involved in coordination.The most severe effects cause widespread brain damage, resulting mental confusion, blindness, coma and death (10-13). On the other hand, the most common forms of exposure to inorganic mercury forms are by inhalation of mercury (Hg) vapour released from chlor alkali plants, dental amalgam and gold mining activities. During the ingestion of inorganic mercury at high concentration, the corrosive effects first of all, damages the digestive tract, causing vomiting and stomach pain, and in severe cases, may result in shock and finally, rental tubule degeneration, and kidneys dysfunction may be seen (14). The exposure of inorganic compounds (In-Hg) (1-5% contents) may cause irritation, contact dermatitis and corrosion of the skin (15).

Regulatory authorities are required to measure mercury (Hg) in a variety of food, industrial, environmental and biological samples for reasons of human being heath. In addition to total mercury (T-Hg) measurement in theenvironmental, food and biological samples, the specific measurement of methylmercury (Me-Hg), noted Me-Hg is of concern to regulatory organisation; as this Hg species has a mammalian LD50 1000 times lower than elemental mercury. Moreover, many foodstuffs such as fish contain the majority of mercury (Hg) as methylmercury (Me-Hg). In addition based on the results of provisional tolerable weekly intakes (PTWIs) from Seychelles, New Zealand and Faroe Island studies, in year 2000 the United States Environmental Protection Agency (U.S. EPA) developed a reference Doses (RfD) for methylmercury of 0.1µg. Me-Hg/kg body weight/day, which were equivalent to a hair mercury concentration of 1.0 µg. Hg/ g (15,16).

In the year 2003, the JECFA, the joint FAO/WHO (Food and Agriculture Organisation of the United Nations and World Health Organisation) Expert Committee on Food Additives recommended that the provisionally human consumption of Me-Hg is limited to 1.6 µg per kg body mass, per week that is equivalent to a hair mercury concentration of about 2.3µg.Hg/g hair(15,16).Therefore, it is essential to measure the exact concentration of methylmercury and inorganic mercury present in biological, environmental and food samples through accurate analytical methods to be used in control laboratories.

Analytical techniques for separation of methylmercury (Me-Hg) are well documented(17). After extraction from solid matrices and derivatization, the methylmercury (Me-Hg) is frequently measured using hyphenated techniques(17). Mercury speciation analysis has been usually performed by resorting to hyphenated techniques, based on the coupling of an effective separation technique to a sensitive element-specific detector. Capillary gas chromatography (CGC), liquid chromatography (LC) or more recently capillary electrophoresis (EC) can be interfaced with specific atomic detection including atomic absorption spectrometry (AAS), atomic fluorescence spectrometry (AFS), electron capture, or inductively coupled plasma mass spectrometry (ICPMS) (10,18-24). recently researches has shown that the coupling of GC to ICP-MS appear to be more suitable hyphenated technique to carry out the mercury speciation analysis because of its high sensitivity, multi isotopic, and multi elemental capabilities(17,18,22). However, the GC-ICP-MS is only suitable for volatile species like mercury species(19,22).

Quality results are sometimes associated with sample pre-treatment, forthe analysis of solids such as biological and environmental samples required a leaching (alkaline or acid) /digestion step to liberate mercury species from sample matrix prior to detection with GC-ICP-MS. However, for ionic mercury species derivatization reaction are required in order to achieve good results. Moreover, there are potential sources of error related to non-quantitative recoveries of mercury species, artefact formation and methylmerurytransformation during solid matrixes sample preparation and derivatisation steps (19,25). For example, Liang and Lazoff reported that the methylmercury artefacts were formed in alkaline digestion step for sediment samples ranging from 0.001 to 0.002% of the total mercury (T-Hg) (spiked plus un-spiked) concentration (26).

To tackle these potential problems of speciation data validation in order to calculate the original level of methylmercury, the use of isotope dilution mass spectrometry techniques offers great potential of very small uncertainties since quantitative recoveries are not required and rearrangement reactions are easily detected(10,24,27). Provided that the enriched isotope can perform the role of ideal internal standard since it is present in an equilibrated and equivalent state to the natural isotope (28). Isotope dilution mass spectrometry technique (IDMS) has been considered to be a best analytical method for speciation and is a well known based on the measurement of isotope ratio in samples where its isotopic composition has been altered by the addition of a known amount of an isotopically enriched element(17,23,29). A main advantage of this technique (IDMS) that chemical separation, if required for accurate ratio determination, need not be quantitative. Moreover, concentrations of chemical species can be measured very precisely because ratios can be measured very reproducibly(17,29).

However, in spite of the benefits of Isotope dilution mass spectrometry technique (IDMS), it is not being used widely as a method of analysis because the lack of suitable enriched internal calibration standards. Due to the isotopically enriched methylmercury standard is not commercially available therefore needs to be synthesized in the laboratory.

According to the severalliterature surveys, there are some proposed methods for the production of organomercury compounds, e.g. the reaction between dimethylmercury (DMHg) and inorganic mercury ($HgCl_2$) the chemically methylate of mercury (II) by methylcobalamin in the absent of cell extract and the reaction of tertramethyltin ($(CH3)_4Sn$) with mercuric chloride ($HgCl_2$) stock solutions(10,28). However, the most often used method for production of organomercury compounds is based on the reaction of methylcobalamin

(CH3 CO, a vitamin B12 analog) with inorganic mercury (In-Hg). In this method, there are two reaction products; monomethylmercury (MMHg) chloride and dimethylmercury (DMHg) chloride were produced with different yield depends mainly on the reaction time, temperature and the mole ratio of the reactants used(10). The major focus of most of these studies was the reaction product of the methylcobalamin ortertramethyltin ($(CH3)_4Sn$) with inorganic mercury (In-Hg), not the synthesis of enriched methylmercurywith high yield and impurity in order to use it as a standard compound. However, only few methods for synthesis of isotopically enriched monomethylmercury chloride have been found in the literature.

Rouleau C and Block M (30)synthesized the radioactive methylmercury chloride ($CH_3{}^{203}Hg(II)$) by methylation of inorganic mercury (^{203}Hg (II)) with methylcobalamin (MeCo) and isolation of $CH_3{}^{203}Hg(II)$ from the reaction mixture in a single extraction step with hexane / benzene (1:1) and the combined organic layers were stirred over sodium carbonate ($NaCO_3$) solution. The synthesized the radioactive methylmercury chloride ($CH_3{}^{203}Hg$ (II)) was left dissolved in ($NaCO_3$) solution in a suitable.The final yield was ≥90% and the time required was less than 4 hours.

On the other hand R. C. Rodriguez Martin-Domimeadioset al.(28)synthesized theisotopically enriched monomethylmercury ($MMe^{201}Hg$) by methylation of commercially available mercury oxide (^{201}HgO) using methylcobalamin co-enzyme as methylation reagent. The mixture was left sitting in the dark place at 37°C in water bath for 1 hour. In order to stop the methylating reaction and to covert the unintentionally formed DMeHg into methylmercury, 1 ml of HCl (conc.) was added to the solution at cooled at 4°C and then the solution was shaken for 5 min.Finally themicro-scale synthesized of isotopically enriched $MMe^{201}Hg$ was extracted three times with 500 ul of toluene and the combination extracts were dried over sodium sulphate. The time required was less than 2 hours and the final yield 90%.

More recently, G. M. MizanurRahman and et al.(31) synthesized the isotopically enriched ($CH_3{}^{201}Hg^+$) by methylation of mercuric oxide (^{201}HgO)with tertramethyltin ($(CH_3)_4Sn$). The resulting reaction mixture was then stirred for one hour at 60 °C in water bath and isolation of $CH_3{}^{201}Hg^+$from the reaction mixture in a triple extraction steps with toluene (4+3+3 ml) after cooled and the extracts combination were then washed with deionised water three times (4+ 3 + 3 ml). Finally 2.5 ml of the toluene extract was dried as same as inR. C. Rodriguez Martin-Domimeadios. et al synthesis method (28). The syntheses time required was one hour and final yield > 90%.

Therefore, the main aim of this study was to produceof isotopically enriched monomethylmercury ($^{201}MMHg$) standard solution with minimum impurities, so as to achieve higher yield and shorter reaction time. For this purpose, the micro- scale preparation of isotopically enriched monomethylmercury ($^{201}MMHg$) procedure described in R. C. Rodriguez Martin-Domimeadios et al. (28) was applied to assess the possibility of producing micro-scale isotopically enriched methylmercury under standard laboratory conditions in order to use it as a standard compound for MMHg analysis in chlor alkali and gold mining hair samplrs. The initial conditions were adapted from available literature methods according to the following criteria (28):

1. The isotopically enriched Mercury oxide (^{201}HgO) standard solution must be used as a starting material; this is the isotopically enriched from that is commercially available.

2. The formation of dimethylmercury (DMHg) must be avoided.
3. Only small quantities of the starting material must be used, so it is necessary to prepare on a micro scale, because the starting material is highly cost.

The reaction product have been analysed and characterized by capillary gas chromatography (GC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) for propylatedspecies.

Experimental

Instrumentation

For speciated isotope dilution analysis (SIDMS) a gas chromatograph (GC) model HP 6850 equipped with a capillary column was coupled to an Agilent model HP-7500 ICP mass spectrometer via a heated steel transfer capillary as shown in **Figure 1**. The heated steel transfer capillary was inserted into the ICP torch injector and connection to the torch was realized by means of a glass T-piece. A conventional Meinhard concentric nebulizer and low volume water cooled cyclonic spray chamber were connected to the heated steel transfer capillary line connected ICP torch and this enabled continuous aspiration of a standard thallium solution ($25\mu gl^{-1}$). This configuration allowed optimization of instrument performance and simultaneous measurement of ^{203}Tl and ^{205}Tl for mass bias correction during the chromatographic run (18).

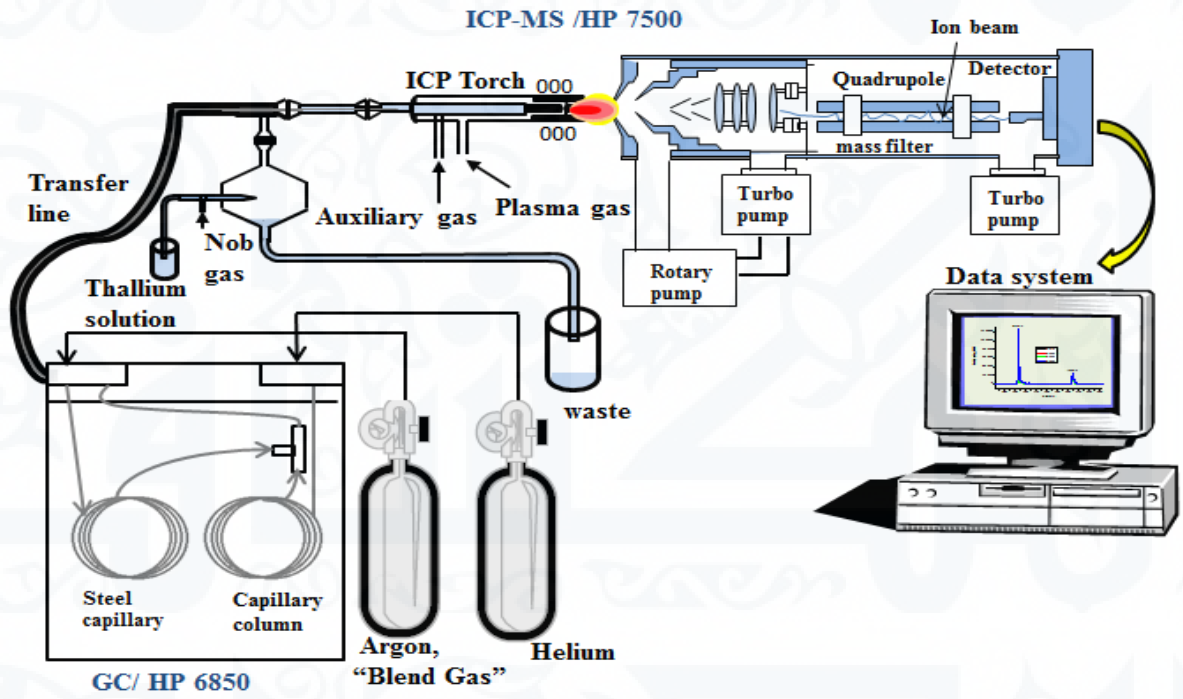

Figure 1 Schematic diagram for Agilent's GC-ICP-MS interface for 6850 GC/HP with 7500 ICP-MS/HP

The optimised operating conditions for the GC-ICP-MS coupling system are listed in Table 1

Table 1:GC and ICP-MS operating parameters.

ICP-MS Instrument	Agilent 7500 series
Hg isotope acquired	196, 198, 199, 200, 201, 202, 204
Acquired mode	Time resolved
Dwell time	0.035 sec/point
RF power	1380 W
RF matching	1.53 V
Sample depth	7.2 mm
Torch-H	1.3 mm
Torch-V	0.5 mm
Carrier gas	Argon / 0.76 l min^{-1}
Makeup gas	Argon / 0.22 l min^{-1}
Extract 1	-2 V
Internal standard	Tl (25 ppb)
Nebulizer pump flow rate	0.20 rps
Spray chamber temperature	2 °C
GC Instrument	**Agilent HP 6850**
Injection	splitless – 1 µl
Oven program	50°C (1 min), 50°C/min →220°C (5 min)
Carrier gas	Helium
Transfer line temp	220°C
GC injector temp	220°C

Reagents and standards

All chemicals and used were of analytical reagent grade unless stated otherwise. Methanol, sodium acetate, acetic acid glacial (super grade) were purchased from VWR (BDH, UK). Sodium tera-n-propylborate ($NaBpR_4$, ≥ 98% purity) was purchased from Chemos GmbH (Germany). Isooctane,Methylcobalamin and methylmerury (II) chloride were purchased from Sigma Aldrich (UK). Natural isotopic composition of inorganic mercury (In-Hg) standard stock solution for ICP (934 ±3.0 mg/kg) was purchased from Fluka (UK). Stock solution of methylmercury chloride (9543 mg/kg as Hg^{2+}) of natural isotopic composition was prepared by dissolving methylmerury chloride (Sigma) in methanol (VWR). Working standard solutions were prepared fresh daily by appropriate dilution of the stock standard solutions in 1% HNO3 and were stored in the fridge. Methylcobalamin (Sigma) used for synthesis was prepared by dissolution in an acetic acid- acetate buffer solution (0.1M, pH 5). Stock of isotopically enriched Mercury oxide (^{201}HgO) standard solution (994 mg/kg as ^{201}Hg) was prepared from mercuric oxide obtained from OaK Ridge National Laboratory

(OaK, Ridge, USA). A part from toluene and isopropanol, which were HPLC grade were used. Double distilled water was used throughout.

Procedures:

Synthesis and primary analysis of ^{201}Hg – enriched methylmercury

A flow chart diagram of enriched monomethylmercury (MM^{201}Hg) synthesis protocol is shown in **Figure 1** below.

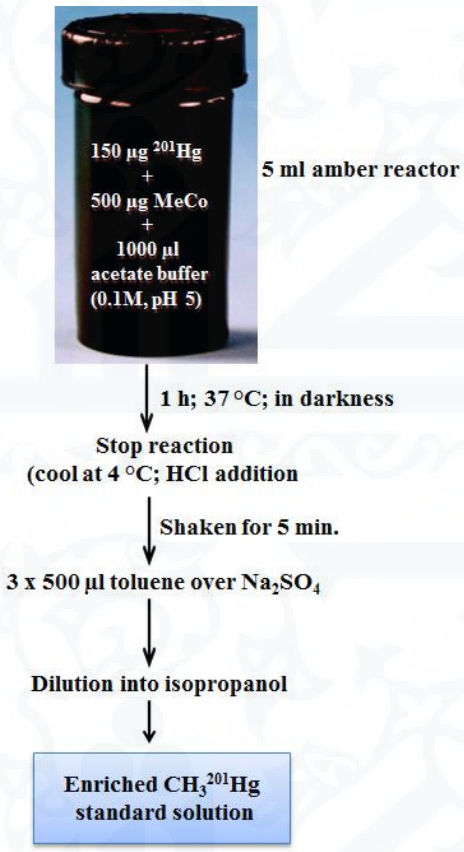

Figure 1 Flow chart formonomethylmercury MMHg isotopically enriched synthesis

Approximately 150 µl from 994 mg/kg ^{201}Hg –enriched inorganic mercury stock solution was transferred to the amber micro-reactors (Supelco) and diluted with 500µl of buffer acetate (0.1M, pH 5). 3.09 mg of methylcobalamin was dissolved in another 3000 µl of buffer acetate (0.1M, pH 5) and 500 µl from this solution was added to the inorganic mercury solution. The pH was adjusted to pH 5 with concentrated sodium acetate 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 1.0 ml of concentrated hydrochloric acid was added and shaken for 5 minute. The MMHg formed was extracted three times with 500 µl of toluene. The combined toluene extracts were dried over sodium sulphate. 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.

Determination of the isotopic abundance and atomic weight concentration in the synthesised $Me^{201}Hg$ enriched standard solution.

In order to calculate the purity of the synthesised enriched $Me^{201}Hg$ standard solution and its atomic weight, 50 ppb of enriched $Me^{201}Hg$ was prepared from stock solution (1.0 mg/kg enriched $Me^{201}Hg$ secondary standard solution). The 1.0 ml of 50 ppb (µg kg^{-1}) was transfer to two clean glass vials and 5.0 ml of buffer acetate (0.1M, pH 3.9) was added in the glass vials and the pH adjusted at about 3.9. After which, 1.0 ml of isooctane was added and propylated with 1.0 ml of 1% $NaBPr_4$. Then, Extraction of derivatised Hg species (peralkylated Hg) was done by vigorous shaking for 5 min and the isooctane extract was afterwards centrifuged for 10 min at 3000 rpm then the extracted Hg species into isooctane layer were transferred to GC vials and analysed with a coupling of GC-ICP-MS.

Reversed Isotope dilution mass spectrometry (RIDMS) method

To calculate the actual concentration of synthesised enriched $Me^{201}Hg$ standard solution the RIDMS measurement was done by preparing of 1.0 mg kg^{-1}enriched $Me^{201}Hg$ and 1.0 0 mg kg^{-1} normal abundance Me-Hg from synthesised enriched $Me^{201}Hg$ and normal abundance MeHg stock solutions respectively. Then 100µl from 1.0 0 mg kg^{-1} enriched $MeHg^{201}$was weighed in two clean and dry glass vials and spiked with 300 µl from 1.0 mg kg^{-1} Me-Hg normal abundance and weighed again. 5.0 ml of 0.1 M buffer acetate (pH 3.9) was added in vials and the pH adjusted at about 3.9. Then,the derivatization and the Extraction of derivatised Hg species (peralkylated Hg) were done as same in section 2.3.2 above.

The concentration of the enriched methylmercury in the spiked solution and the reaction yield were calculated using reversed Isotope Dilution Mass Spectrometry (RIDMS) method by applying the isotope dilution equation (19).

$$c = \frac{C' W' A (RY' - X')}{w A' (X - RY)} \qquad (1)$$

Where:

w = weight of the ^{201}Hg enriched solution (g)

c = Hg Concentration of the ^{201}Hg enriched solution (mg/kg)

A = Atomic mass (Hg) of the ^{201}Hg enriched solution (g/mole, from abundance det.)

Y = Isotope abundance of ^{201}Hg in the 201Hg enriched solution (%, from abundance det.)

X = Isotope abundance of ^{202}Hg in the ^{201}Hg enriched solution (%, from abundance det.)

W′ = weight of the sample solution (g)

A′ = Atomic mass (Hg) of the natural standard solution = 200.59 g/mole

Y′ = Isotope abundance of ^{201}Hg in the natural standard solution = 13.2 %

X′ = Isotope abundance of ^{202}Hg in the natural standard solution = 29.8 %

R = Isotope ratio of the integrated peak areas $^{202}Hg/^{201}Hg$ from IDMS measurements

C′ = Hg concentration in the sample solution (µg/kg)

Therefore, the final parameter that requires measurement is the isotope ratio, R($^{202}Hg/^{201}Hg$). A part from R, the other parameters is constants, masses and the concentration of the spike solution (normal abundance MeHg). Thus, the concentration (c) of the synthesised enriched Me201Hg standard solution is ultimately based on the measurement of isotope ratio (R) and the specification of the spiked material (normal abundance MeHg).

Results and discussion

Isotopically enriched MM^{201}Hg synthesis and characterization

Isotopically enriched MMHg standard solution was synthesized from inorganic ^{201}Hg enriched stock standard solution according to the method described by R. C. Rodriguez Martin-Domimeadios et al (28). The analysis of the reaction products are then preformed by CGC-ICP-MS after propylation with $NaBPr_4$. **Figure 3** shows the chromatogram obtained for mercury isotopes as can be seen; very low levels of inorganic mercury (Hg^{2+}) are present in the final product of MMHg. In addition, it can be observed very low levels of MeEtHgartifact from enriched MMHg during derivatization using $NaBPr_4$.

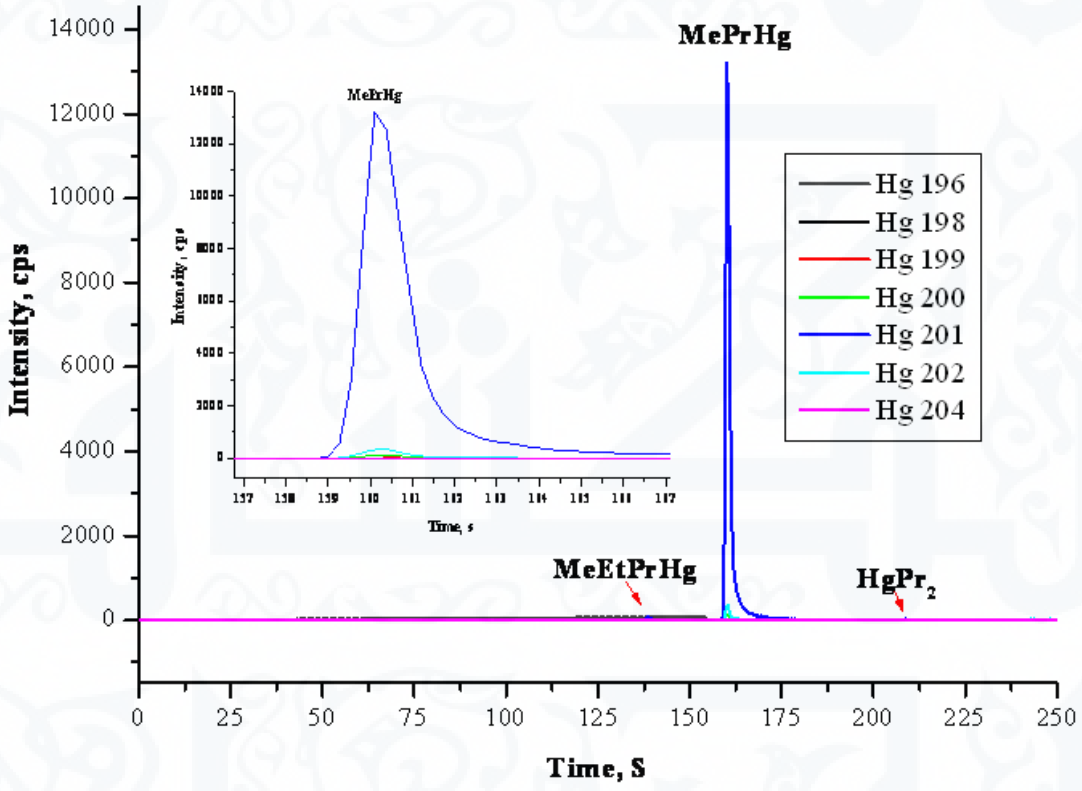

Figure 3 Chromatogram formercury isotopes composition in synthesised enriched monomethylmercury ((MM^{201}Hg).

The isotope composition of the enriched monomethylmercury ($MM^{201}Hg$) is shown in **Table 2**, and was calculated from measured peak area. This standard solution presents a $^{202}Hg/^{201}Hg$ ratio of 0.02510, which is highly different from the natural $^{202}Hg/^{201}Hg$ ratio of 2.2587. Therefore, the synthesised enriched $MM^{201}Hg$ can be used directly as certified isotopically enriched $Me^{201}Hg$ standard solution to perform isotope dilution (IDMS) analysis of MMHg in real samples (spiked solution).

Table 2: The atom fraction composition of enriched $MM^{201}Hg$ aliquot, mass factions and isotope ratio ($^{202}Hg/^{201}Hg$) determined by ICP-MS of the isotopically enriched methylmercury ($Me^{201}Hg$)

Hg isotope	Accurate mass	Abundance (mol at %)	
		Natural (IUPAC)[32]	*Enriched $MM^{201}Hg$
^{196}Hg	195.965	0.1534	0.000
^{198}Hg	197.967	9.9680	0.0604 ± 0.002
^{199}Hg	198.968	16.873	0.1295 ± 0.010
^{200}Hg	199.968	23.096	0.9071 ± 0.030
^{201}Hg	200.970	13.181	**96.3856 ± 0.002**
^{202}Hg	201.971	29.863	**2.4172 ± 0.05**
^{204}Hg	203.974	6.865	0.0994 ± 0.002
Sum (%)		100	100 ± 0.014
Hg atomic weight (g/mole)		200.598	**201.014**
R (202/201)		**2.2587**	**0.02510**

***Mean value ± SD(n = 5)**

Moreover, the actual concentrations of the enriched $MM^{201}Hg$ in the spiked solution and in the reaction yield obtained were calculated using reverse isotope dilution (RIDMS) analysis (100 ul of enriched 1.0 0 mg kg^{-1} $MM^{201}Hg$ standard solution spiked with 300 ul of 1.0 0 mg kg^{-1} normal abundance MeHg standard solution). Two independent IDMS experiments were carried out and the extracted Hg species from each solution was injected five times and the typical chromatogram obtained for mercury isotope showed in **Figure 4**. The average concentration of spiked solution turned out to be 1.31 ± 0.04 µg g^{-1} and the methylation yield is 96.38 %. In addition, the stability of this enriched standard solution has been checked after 8 months and the degradation of about 25.23% was found.

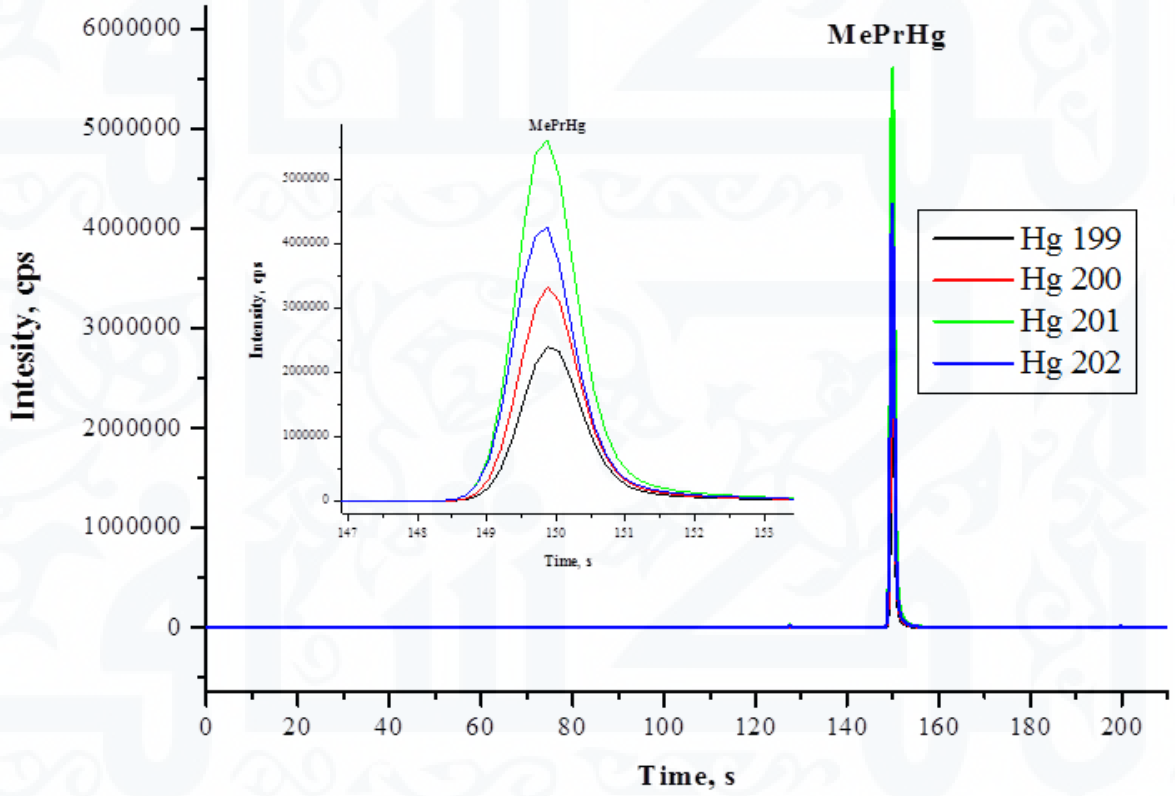

Figure 4 Chromatogram obtanied fromReversed Isotope dilution mass spectrometry (RIDMS) experiment of synthesised ^{201}Hg – enriched monomethlmercury

Conclusion

A highly pure isotopically enriched monomethylmercury (MM^{201}Hg) has been synthesis from inorganic mercury (^{201}HgO) standard stock solution and methylcobalamin (MeCo) with a yield of more than 95% in a synthesis procedure lasting less than 1.5 hour at 37°C. The product is highly pure and stays stable for long time in cold conditions (-20 °C). However, if this enriched solution is going to be used as standard, the concentration should be checked frequently. This isotopically enriched monomethylmercury (MMHg) standard solution has been successfully used for isotope dilution analysis of methylmerury in biological (hair) samples from contaminated area (chlor alkali plant and gold mining areas), environmental and food samples.

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