

Automatic Ethylation-Purge and Trap-GC-ICP-MS for Methylmercury Analysis: Method Validation and Application for Isotope Dilution/Tracing

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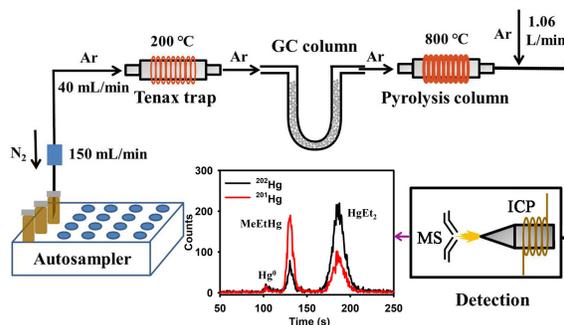
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ABSTRACT: The method of isotope dilution and tracing, based on ethylation-purge and trap-gas chromatography (GC)-inductively coupled plasma mass spectrometry (ICP-MS), has been widely used for the accurate analysis of methylmercury (MeHg) and for tracking its environmental fates (e.g., formation/degradation). However, the tedious ethylation derivatization and purge and trap processes limit analytical throughput. In this work, an automatic ethylation derivatization and purge and trap method, followed by GC separation, pyrolysis, and ICP-MS detection, was developed for MeHg analysis. The throughput and detection limits of this proposed method for MeHg were 7.5 min/sample and 0.03 ng L⁻¹, respectively, with a relative standard deviation of 3.7%. The accuracy of the developed method was validated by the analysis of a spiked water sample and a certified reference material (DORM-4, Fish protein) using isotope dilution (Me²⁰¹Hg). In addition, anaerobic *Geobacter sulfurreducens* PCA-mediated methylation of Hg²⁺ and demethylation of MeHg was monitored by using double-enriched isotope tracing (¹⁹⁹Hg²⁺ and Me²⁰¹Hg). This automatic ethylation-purge and trap-GC-ICP-MS method is promising for routine MeHg analysis with isotope dilution/tracing.



INTRODUCTION

Mercury (Hg) is a global pollutant widely present in the environment due to anthropogenic and natural activities (e.g., emission from coalcombustion and volcanic eruption).¹ In an anoxic environment, inorganic Hg can be methylated into highly neurotoxic methylmercury (MeHg) by anaerobic microorganisms with the *hgcAB* gene cluster,² especially sulfate-reducing bacteria,³ iron-reducing bacteria,⁴ and methanogens.^{5,6} Furthermore, MeHg

is bioaccumulative and can be biomagnified through the aquatic food web,^{7,8} threatening the health of aquatic biota and humans worldwide.^{9,10} Fortunately, MeHg in surface water and sediments can also be demethylated before its incorporation into the aquatic food web through chemical¹¹ and microbial pathways.¹² Therefore, developing a sensitive and accurate method for MeHg monitoring and tracing its methylation/demethylation process is of great significance for understanding the biogeochemical cycle and bioaccumulation of MeHg.

Element-specific detection coupled with chromatographic separation has been widely used for MeHg analysis.¹³⁻¹⁵ In these hyphenated techniques, gas chromatography (GC) coupled with atomic fluorescence spectrometry (AFS) or inductively coupled plasma-mass spectrometry (ICP-MS) is generally more favorable, considering its compatibility with depletion-based injection (*i.e.*, purge and trap).^{14,16} Purge and trap can significantly increase the injection volume, and thus enhance the detection sensitivity following GC-AFS/ICP-MS analysis.^{14,16} An alkylation derivatization process (*e.g.*, ethylation) is required to transform MeHg into volatile Hg species (*e.g.*, methylethylmercury) before purge and trap.¹⁶ However, this derivatization, especially ethylation, suffers from serious matrix effects in which process high levels of Cl⁻ and dissolved organic matter strongly decrease the recovery of the overall method.¹⁷ Therefore, a species-specific isotope dilution¹⁸ or tedious distillation¹⁴ is proposed to correct or remove matrix interferences prior to aqueous ethylation. Compared with AFS, ICP-MS as the detector makes it possible to detect multiple Hg isotopes, facilitating the use of species-specific isotope dilution.¹⁸ In addition, on-line Hg isotope analysis with GC-ICP-MS is also favorable for isotope tracing of the Hg methylation/demethylation process in the environment.¹⁹ Unfortunately, the manual ethylation-purge and trap procedure is time-consuming and labor-intensive, which limits the analytical throughput and application of GC-ICP-MS.

This study aims at developing an automatic ethylation-purge and trap-GC-ICP-MS method for accurate, precise, and high-throughput MeHg analysis. The analytical merit of this automatic hyphenated system was evaluated. The recovery of MeHg in environmental water and biological samples was determined, and species-specific isotope dilution (Me²⁰¹Hg) was further applied to correct interference from complex matrices. In addition, this automatic hyphenated system, combined with double isotope tracing (¹⁹⁹Hg²⁺ and Me²⁰¹Hg), was used for tracking the

methylation and demethylation process mediated by the Hg-methylating model strain *G. sulfurreducens* PCA.

EXPERIMENTAL

Reagents and standards. Methylmercury chloride (MeHgCl) was purchased from the National Institute of Metrology, P.R. China. Enriched stable Hg isotopes of ¹⁹⁹HgCl₂ and ²⁰¹HgCl₂ were obtained from Trace Sciences International (Canada). Me²⁰¹Hg was chemically synthesized from ²⁰¹HgCl₂ using methylcobalamin.²⁰ The stock standard solution of MeHgCl and Me²⁰¹Hg was dissolved in methanol (CH₃OH) and stored in dark brown glass bottles at 4 °C. CH₃OH (99.9%) was purchased from Fisher Chemical (USA). Sodium tetraethylborate (NaBEt₄, 1%, w/v), as an ethylating reagent for Hg species, was prepared monthly by dissolving NaBEt₄ (98%, J&K Chemicals, P. R. China) in 2% KOH solution and stored at -20 °C before use. Acetate buffer (2 mol L⁻¹, pH 4.7) was prepared by dissolving sodium acetate (Sigma-Aldrich, USA) and acetic acid (Merck, Germany) in ultrapure water. The 25% KOH/CH₃OH solution was prepared by dissolving 25 g KOH (Merck) in 100 mL CH₃OH to digest the cell pellet. CuSO₄ (1 mol L⁻¹)-KBr (18%, m/v)-H₂SO₄ (5%, v/v) was prepared for digestion of the cell supernatant. Dichloromethane (CH₂Cl₂), as the extraction reagent, was obtained from Fisher Chemical (USA). Ultrapure water (>18.2 MΩ), obtained with a Milli-Q Element system (IQ 7000, Millipore Corporation, USA), was used throughout this work. Other reagents used were of analytical grade or higher.

Instrumentation. The instrumental configuration is illustrated in Fig. 1. The automatic alkyl Hg analyzer (MMA72, Polytech Instrumental Co., Ltd, Beijing, P. R. China) consists of autosampler, purge and trap device, and GC column (AFS detector was dismantled). The volatile Hg species formed by ethylation were purged with nitrogen gas and trapped on a Tenax column,

Fig. 1 Instrumentation for automatic purge and trap-GC-ICP-MS for MeHg determination.

which was filled with 100 mg of Tenax TA (mesh 35/60, Dikma, Beijing, P. R. China). The traps were dried with nitrogen gas and coupled to a GC column filled with 15% m/m OV-3. The GC oven was operated at 45 °C. After going through a quartz tube heated at 800 °C, the organomercury compounds were pyrolyzed to elemental Hg and introduced into an Agilent 7900 ICP-MS (Agilent Technologies, Inc., USA). The mass-to-charge ratios ($m/z=199, 201, \text{ and } 202$) were monitored in peak transient mode. The operating conditions for the purge and trap-GC-ICP-MS coupling system are listed in Table 1. When the automatic alkyl mercury analyzer desorbed the sample, the Agilent 7900 ICP-MS instrument began to collect the chromatographic raw data. Then all data were imported into and processed with the MassHunter 4.5 Workstation software for further analysis. The peak areas were used for calculations. It should be noted that, during the experiments, GC column in the automatic alkyl Hg analyzer was repacked, resulting in a slight difference in retention times of Hg species.

Bacterial culture conditions. *G. sulfurreducens* PCA (ATCC 51573) was cultured anaerobically at 30 °C in a modified NBAF medium,^{21,22} with 10 mmol L⁻¹ acetate as the electron donor and 50 mmol L⁻¹ fumarate as the electron acceptor.²² The cells were harvested at the late exponential growth phase by centrifugation (1500 g for 10 min at 23 °C). Following centrifugation, the culture supernatant was decanted. The cells were resuspended in deoxygenated phosphate-buffered saline (PBS) solution, washed three times, and finally exposed to ¹⁹⁹Hg²⁺/Me²⁰¹Hg in deoxygenated PBS at pH 7.0 for the Hg²⁺ methylation and MeHg degradation assays. The exposed cell density was validated by direct cell counting using a hemocytometer under a microscope.²³

Sample pretreatment. The certified biological reference material DORM-4 (Fish protein) (within certified MeHg concentration of 0.355 ± 0.028 mg kg⁻¹) from the National Research Council Canada was used to validate the developed method. DORM-4 (0.01 g) was weighed into a 15-mL plastic digestion vial, and 0.35 ng Me²⁰¹Hg was added as an isotope dilution reagent for calculating the Me²⁰²Hg concentration. MeHg in DORM-4 was digested with 5 mL 25% KOH/CH₃OH at 200 rpm for 4 hours at room temperature.^{18,24} After digestion, the supernatant was transferred into the acetate buffer and analyzed via automatic ethylation-purge and trap-GC-ICP-MS method.

Fresh water and seawater samples were collected at the Olympic Park (116°40' E, 40°00' N) and taken from the coastal Yellow Sea (120°29' E, 36°05' N), P. R. China, respectively. The samples were filtered through 0.22 μm membrane filters and then spiked at known concentrations of Me²⁰¹Hg as an isotope dilution reagent for calculating the Me²⁰²Hg concentration in the real samples. The samples were then stored at 4 °C until analysis.

Bacterial samples were prepared in an anaerobic chamber (AW400SG, Electrotek, UK) containing a mixture of 10% H₂, 10% CO₂ and 80% N₂. The Hg solution was prepared in

Table 1. Operating Conditions for The Automatic Purge and Trap-GC-ICP-MS System

Purge and trap device	
Gas flow	N ₂ 150 mL min ⁻¹
Column	100 mg Tenax TA (mesh 35/60) in quartz tube (10 × 0.4 cm)
Trapping time	300 s
Drying time of the trap	180 s
Thermal desorption temperature	200 °C within 12 s
GC	
Column	6 g 15% m/m OV-3 in PTFE tube (340 × 1.59 mm)
Argon flow	40 mL min ⁻¹
Temperature	45 °C
ICP-MS	
Sampler and skimmer cone	Platinum
Forward power	1200 W
Cooling gas	14 L min ⁻¹
Auxiliary gas	0.9 L min ⁻¹
Measured isotopes	¹⁹⁹ Hg, ²⁰¹ Hg, and ²⁰² Hg
Dwell time	100 ms

deoxygenated PBS from the concentrated stock solution (10 mg L⁻¹ ¹⁹⁹HgCl₂ in 1% HCl and 1 mg L⁻¹ Me²⁰¹Hg in 1% HCl), and then immediately mixed with the cell suspension (1 mL each). The final cell density was 10⁸ cells mL⁻¹, and the added ¹⁹⁹Hg²⁺ and Me²⁰¹Hg concentrations were 5 μg L⁻¹ and 0.5 μg L⁻¹, respectively. At 0, 4, 8 and 12 h, three parallel samples were taken out of the anaerobic chamber and immediately centrifuged to separate the supernatant and cell pellet for MeHg determination. MeHg in supernatant was isolated by an acidic KBr/H₂SO₄/CuSO₄ (1.5/1.8/1 M) solution followed by extraction of MeHg into CH₂Cl₂.²⁵ Five mL of CH₂Cl₂ extract were pipetted into a 15-mL plastic vial containing 10 mL of de-ionized water. MeHg in CH₂Cl₂ layer was back-extracted into the aqueous solution in a water bath at 45 °C.²⁶ MeHg in cell pellet was digested directly with 25% KOH/CH₃OH at 200 rpm for 4 h at room temperature,^{18,24} and added the supernatant to the acetate buffer. The concentration of MeHg in acetate buffer was analyzed via automatic ethylation-purge and trap-GC-ICP-MS method.

Calculation of ¹⁹⁹Hg²⁺ methylation and Me²⁰¹Hg demethylation rate constants in bacterial samples. The Hg²⁺ methylation (k_m) and MeHg demethylation rate constants (k_d) in the bacterial samples were estimated according to the equations as published by Y. B. Li, *et al.*²⁷:

$$\ln([\text{Me}^{201}\text{Hg}]_t) = \ln([\text{Me}^{201}\text{Hg}]_0) - k_d t \quad (\text{Eq. 1})$$

when $k_d > 0$, $[\text{Me}^{199}\text{Hg}]_t$ is given by Equation (2)

$$[\text{Me}^{199}\text{Hg}]_t = \frac{k_m [^{199}\text{Hg}^{2+}]_0 - (k_m [^{199}\text{Hg}^{2+}]_0 - k_d [\text{Me}^{199}\text{Hg}]_0) e^{-k_d t}}{k_d} \quad (\text{Eq. 2})$$

when $k_d = 0$, $[\text{Me}^{199}\text{Hg}]_t$ is given by Equation (3)

$$[\text{Me}^{199}\text{Hg}]_t = k_m [^{199}\text{Hg}^{2+}]_0 t + [\text{Me}^{199}\text{Hg}]_0 \quad (\text{Eq. 3})$$

where the k_m and k_d are the Hg^{2+} methylation and the MeHg demethylation rate constants, respectively. $[\text{Me}^m\text{Hg}]_t$ and $[\text{Me}^m\text{Hg}]_0$ ($m = 199, 201$) were the MeHg concentrations of m isotope at t and 0 hour (ng L^{-1}), respectively. $[\text{}^{199}\text{Hg}^{2+}]_0$ was the concentration of $^{199}\text{Hg}^{2+}$ at 0 hour (ng L^{-1}).

Statistical analysis. All data processing and statistical analysis were conducted using Microsoft Excel 2010, while all figures were plotted using SigmaPlot 12.5. Analysis of variance (ANOVA) test was performed to test the differences for methylation and demethylation rate constants at different incubation time. Statistical significances were defined at $P < 0.05$ and $P < 0.01$, indicating the “significant” and “highly significant” differences, respectively.

RESULTS AND DISCUSSION

Interface configuration. The home-made interface used in this study allowed enhanced performance of the GC-ICP-MS system. Fig. 1 details the configuration of an in-house designed interface for coupling the GC with the ICP-MS. The analyte from the GC is directly mixed with the argon gas flow in the ICP-MS through a PTFE three-way valve, and a perfluoroalkoxy alkane (PFA) tube with a customized quartz adapter was used to make a gas-tight connection between the GC and the ICP-MS instruments. The analyte from the GC does not need to go through the spray chamber but enters the ICP torch directly. This connection is deemed to be more convenient and the hyphenation of GC to ICP-MS is simplified. Heating of the transfer line was not necessary because after the GC separation, the organomercury compounds were pyrolyzed to elemental mercury. The developed configuration is satisfactory due to its low cost, flexible assembly and disassembly, and easy access to additional argon gas flow.

Analytical merits and method validation. The developed analysis system was evaluated in terms of the classic analytical parameters (Table 2). Precision, based on six consecutive determinations of MeHg (10 pg as Hg), and measurement of peak area showed a relative standard deviation (RSD) of 3.7%. The detection limit, calculated as three times the standard deviation of the blank signals (as total ion current of Hg) in seven replicates, was found to be 0.03 ng L^{-1} for MeHg when 25 mL of aqueous sample was analyzed. In addition, the calibration curves for the Hg isotopes (^{199}Hg , ^{201}Hg , ^{202}Hg) from MeHg ($0\text{--}0.8 \text{ ng L}^{-1}$, as Hg)

Table 2. Analytical Merits of The Automatic Purge and Trap-GC-ICP-MS System

Equation ^a	$y^b = 12429.7x + 372.2$
R^2	0.996
LOD ^c (ng L^{-1})	0.03
RSD ^d ($n=6$)	3.66 %
Analytical time	7.5 min

^a Calculation based on the peak area of ^{202}Hg ; ^b y : peak area, x : Hg (ng L^{-1}); ^c Limits of detection; ^d At MeHg mass 10 pg.

Fig. 2 Chromatogram of ethylated Hg species from standard samples obtained by automatic purge and trap-GC-ICP-MS. The colored solid lines represent different MeHg concentrations, respectively.

Fig. 3 Chromatogram obtained for DORM-4 extract spiked with ^{201}Hg -enriched MeHg solution.

were all linear with correlation coefficients of 0.996 or better (Table 2 and Fig. 2).

To validate the accuracy of the automatic ethylation-purge and trap-GC-ICP-MS method, the reference material DORM-4 (Fish protein) was analyzed by using isotope dilution (Fig. 3). The mean ($\pm s$, $n = 3$) MeHg concentration of $0.357 \pm 0.009 \text{ mg kg}^{-1}$ statistically shows no difference ($P > 0.05$) from the certified value of $0.355 \pm 0.028 \text{ mg kg}^{-1}$. The results suggest that this automatic ethylation-purge and trap-GC-ICP-MS method is reliable for MeHg analysis.

Analysis of real water samples. Isotope dilution was further applied to determine MeHg in real water samples (Fig. 4). Species-specific isotope dilution (Me^{201}Hg) was used to correct interference from complex matrices, especially for seawater. The concentration of MeHg in the sample from the Olympic Park, determined by the isotope dilution method, was $0.082 \pm 0.005 \text{ ng L}^{-1}$, which was close to the directly measured value of $0.074 \pm 0.002 \text{ ng L}^{-1}$ (without isotope dilution). Since the concentration of

Fig. 4 Chromatogram of MeHg from fresh water (a) and spiked seawater (b) samples obtained by automatic purge and trap-GC-ICP-MS using isotope dilution method (Me^{201}Hg).

MeHg in the seawater from the Yellow Sea was below the detection limit (0.03 ng L^{-1}), a known concentration of MeHg was then spiked into these samples. However, the total recovery of MeHg in the seawater using direct ethylation (without isotope dilution) was 31.9% ($n = 4$) after spiking with 0.24 ng L^{-1} MeHg, indicating that incomplete recovery occurred from the Cl^- interference during the ethylation procedure.¹⁷ When adopting isotope dilution, the recovery of the spiked MeHg was 106.1%. Compared with direct ethylation, the isotope dilution protocol provides excellent results for samples with complex matrices (e.g., Cl^- interference). In addition, the tedious pretreatment procedures (e.g., extraction/back-extraction or distillation) of traditional methods decrease the total recovery and also may induce artifact formation of MeHg. The isotope dilution protocol solves these problems of losses and chemical transformation of MeHg in sample cleanup and derivatization.^{28,29}

Double isotope tracing for methylation and demethylation mediated by *G. sulfurreducens* PCA. Double isotope tracing was used to track the methylation and demethylation mediated by the Hg-methylating model strain *G. sulfurreducens* PCA. The processes of methylation and demethylation were analyzed by mixing $^{199}\text{Hg}^{2+}$ and Me^{201}Hg with the *G. sulfurreducens* PCA cell suspensions. The results showed that Me^{199}Hg gradually increased

Fig. 5 Automatic purge and trap-GC-ICP-MS chromatograms of mercury species obtained after aqueous phase ethylation, where (a) and (b) represent the effect on $^{199}\text{Hg}^{2+}$ methylation and Me^{201}Hg demethylation by *G. sulfurreducens* PCA at 0, 4, 8, 24 h, respectively.

within 24 hours of incubation, while Me^{201}Hg decreased within 24 hours of incubation (Fig. 5 and Fig. 6a). Equations (1)–(3), which consider the contributions from both the Hg^{2+} methylation and the MeHg demethylation in the function describing the variation of MeHg concentration, were applied to calculate the methylation rate constants (k_m) and demethylation rate constants (k_d).²⁷ From 0–4 h to 8–24 h, the k_m values of $^{199}\text{Hg}^{2+}$ significantly ($P < 0.05$) reduced from $(1.90 \pm 0.19) \times 10^{-3} \text{ h}^{-1}$ to $(0.26 \pm 0.04) \times 10^{-3} \text{ h}^{-1}$ (Fig. 6b). However, the k_d values of Me^{201}Hg showed a trend of first increasing and then decreasing (Fig. 6c). For instance, during 0–4 h, the k_d value of $^{201}\text{MeHg}$ was $(6.4 \pm 0.46) \times 10^{-3} \text{ h}^{-1}$, while significantly ($P < 0.05$) increasing to $(12.5 \pm 1.02) \times 10^{-3} \text{ h}^{-1}$ during 4–8 h, and then decreasing to $(5.8 \pm 2.1) \times 10^{-3} \text{ h}^{-1}$ during 8–24 h.

CONCLUSIONS

In this study, the automatic ethylation-purge and trap-GC-ICP-MS system largely solves the drawbacks of manual sample preparation (ethylation and purge and trap are cumbersome and time-consuming), and improves the analytical throughput and application of the traditional GC-ICP-MS method. The developed method not only offers sufficient precision, accuracy, and repeatability with respect to the measurements obtained for the

Fig. 6 The concentrations of produced Me¹⁹⁹Hg and Me²⁰¹Hg (a), as well as the methylation rate constants (k_m , h⁻¹) of ¹⁹⁹Hg²⁺ (b) and the demethylation rate constants (k_d , h⁻¹) of Me²⁰¹Hg (c) in *G. sulfurreducens* PCA.

environmental water samples and the biological certified reference material (DORM-4), but also corrects interference from complex matrices by species-specific isotope dilution. In addition, the developed method can be applied to monitor the Hg methylation and demethylation processes and double isotope tracing. It is expected that this automatic ethylation-purge and trap-GC-ICP-MS method will be routinely used for MeHg analysis with isotope dilution/tracing.

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Notes

The authors declare no competing financial interest.

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