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Simultaneous extraction and determination of methyl mercury and selenomethionine by isotope dilution gas chromatography mass spectrometry

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Abstract

Methyl mercury (MeHg) is a common contaminant worldwide. Because of the recognized toxicity of MeHg, health authorities monitor environmental levels of MeHg, and the corresponding exposure in the population. Several studies have demonstrated the detoxifying role of selenium against mercury species. Among selenium species, selenomethionine (SeMet) is preferentially absorbed from the diet and is, indeed, used in selenium supplements. There is often a need to determine MeHg and SeMet concentrations simultaneously in food and animal samples. Different methods have been used to extract MeHg and SeMet separately. An acidic extraction is required for SeMet, while a basic leaching is preferred for MeHg.

In this study, it was demonstrated that methanesulfonic acid used for SeMet extraction can be used for MeHg, allowing a one-step recovery of both compounds. The method was validated for MeHg determination with dogfish liver certified reference material (DOLT-4). Species specific isotope dilution was employed to achieve accurate results. The derivatized extracts were analyzed with gas chromatography-inductively coupled plasma-mass spectrometry (GC-ICP-MS) and gas chromatography-mass spectrometry (GC-MS). Livers from rats treated with SeMet and/or MeHg were analyzed to demonstrate the applicability of the extraction method to real samples. Quantitative spike recoveries were obtained for both SeMet and MeHg and indicated good extraction efficiency. Methanesulfonic acid can thus be used for extraction of both SeMet and MeHg, thereby significantly simplifying sample preparation when simultaneous determination of both compounds is required.

Keywords

GC-ICP-MS, GC-MS, Isotope dilution, Methanesulfonic acid extraction, Methylmercury, Selenomethionine.

Acid extraction for the determination of methyl mercury in biotissues by isotope dilution gas chromatography inductively coupled plasma-mass spectrometry

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Abstract

Methyl mercury (MeHg) is a common contaminant worldwide. Health authorities keep monitoring MeHg levels in biological and environmental samples to assess the corresponding exposure in the population. So far, a basic leaching has been mostly used for the MeHg extraction. In this study, it was demonstrated that methanesulfonic acid, commonly used for amino acid extraction, can be used for MeHg extraction. Species-specific isotope dilution was employed to achieve accurate results. The method was validated by analysis of dogfish liver certified reference material (DOLT-4). The derivatized extracts were then analyzed with gas chromatography-inductively coupled plasma-mass spectrometry (GC-ICP-MS). Results obtained for MeHg in DOLT-4 are in agreement with the certified value (t -test, $P=0.05$), confirming that methanesulfonic acid extraction is suitable for extraction of MeHg in biological tissues. This new procedure could be of particular interest in biological and toxicological studies where a simultaneous determination of MeHg and certain amino acid is sometimes required.

Keywords

Introduction

Elemental mercury can be released to the environment by human activities, and it can be converted to methyl mercury (MeHg) by bacterial metabolism¹. MeHg bioaccumulates in animal (e.g. predator fish) muscle tissues, and organs such as brain, kidney and liver via the food chain²⁻⁴. MeHg is more toxic than inorganic mercury species and has shown neurotoxic, nephrotoxic and hepatotoxic effects in both animals and humans^{5,6}. The toxic effects of MeHg are of such concern that health authorities have taken action to monitor levels of MeHg in environmental and food samples⁷. The capability of MeHg to bind the thiol groups of certain proteins and the active site of selenoproteins-P affects its toxicity and distribution⁸. Therefore, toxicologists and biologists are often interested to determine the level of MeHg in presence of certain amino acids such as cysteine (Cys) or selenomethionine (SeMet).

Typically, LC/GC combined with MS detection (either molecular or atomic) has been used for the determination of MeHg^{9,10}. Isotope dilution (ID) is often the calibration method of choice to obtain more accurate and precise results for the determination of MeHg⁹. ID requires the detection of two interference-free isotopes of a same element. In ID, the sample is spiked with an isotopically-enriched spike of the selected analyte, and a ratio, instead of an absolute intensity, of the two isotopes is measured. If the enriched spike is added prior to sample preparation and once the isotopic equilibration between the spike and the analyte is reached, it can correct for any subsequent loss or deterioration of the analyte, matrix effects and sampling variations. However, the availability of enriched spikes is often a significant limitation of ID.

Sample preparation is a crucial step in bioanalysis, efficient and simple digestion methods capable of quantitative recovery of analyte from sample matrices are required to achieve accurate results. In general, a simple leaching is required for MeHg extraction¹⁰. Former reports showed that both hydrochloric and tetramethylammonium hydroxide extracting agents can be successfully used for the recovery of both inorganic and organic mercury¹¹⁻¹⁴. This suggests that an acidic treatment could also be used for the MeHg extraction. In this paper, an acidic extraction/digestion method for MeHg is reported. The method is based on acid hydrolysis typically used in amino acid analysis^{10,15,16}. To avoid the alteration of certain species,

methanesulfonic acid was selected over other acids, such as hydrochloric or nitric, because of its non-oxidizing nature¹⁷.

The proposed MeHg extraction method was validated with DOLT-4 (dogfish liver certified reference material) and ID was the selected calibration method. Results from this study demonstrated that this approach can also be used for the extraction of MeHg, enabling a complete recovery of the organometal. The developed methodology could be further improved and applied whereas a simultaneous extraction of MeHg and other compounds (e.g. SeMet, or other aminoacids) is required.

Experimental

Instrumentation

A Varian 3400 gas chromatograph (Varian Canada Inc., Georgetown, ON, Canada) fitted with a 30 m DB-5 column (0.25 mm id, 0.25 μ m film, Iso-Mass Scientific Inc., Calgary, Alberta, Canada) was used for the separation of methylmercury. An Elan[®] 6000 ICP-MS (Perkin Elmer Sciex, Concord, ON, Canada) equipped with a Gem cross-flow nebulizer was used for mercury detection. Coupling between the GC and the ICP-MS was accomplished by using a home-made interface and transfer line, similar to that reported by Yang *et al*¹⁸. ¹⁹⁸Hg and ²⁰²Hg were the measured spike and reference isotopes, respectively. Instrumental conditions for analysis are outlined in Table 1. A manual SPME device, equipped with a fused-silica fiber coated with a 100- μ m film of polydimethylsiloxane (PDMS, Supelco, Bellefonte, PA, USA) was used for sampling the derivatized methyl mercury in headspace. Details of the SPME sampling are reported below.

Materials

Deionized water was made in-house with a Nanopure mixed bed ion exchange system (MilliQ, Thermo Scientific, Canada). Sodium acetate and glacial acetic acid (99.7%) were obtained from Fisher Scientific (Nepean, Ontario, Canada). Acetate buffer (1 M) was prepared by dissolving the appropriate amount of sodium acetate in deionized water and adjusting the pH to 5 with glacial acetic acid. Ammonium hydroxide (28% v/v) was purchased from Anachemia Science (Montreal, Quebec, Canada). Methanesulfonic acid ($\geq 99.5\%$) was purchased from Sigma-

Aldrich (Oakville, ON, Canada). Sodium tetrapropylborate (NaBPr_4) was obtained from 3B Scientific Corporation (Libertyville, IL, USA). Methanol was purchased from Fisher Scientific (NJ, USA). Hexane was sourced from Caledon Laboratories LTD (Georgetown, Canada). Methyl mercury chloride (MeHgCl , 95%) was obtained from Alfa Aesar (Ward Hill, MA, USA). ^{198}Hg -enriched MeHg (Me^{198}Hg) stock solution (nominal mass fraction, w , of about $3.5 \mu\text{g/g}$) in methanol was synthesized in our laboratory from commercially available inorganic ^{198}Hg ¹⁹. DOLT-4 (dogfish liver CRM) was supplied by the National Research Council (Ottawa, ON, Canada).

Preparation of solutions

For all standard solutions, the mass fraction (w), instead of the concentration, is reported. The standard stock solution of MeHgCl ($w=46.2232 \mu\text{g/g}$ as mercury) was gravimetrically prepared in methanol. MeHg ($w=2.0514 \mu\text{g/g}$) working standard solution was gravimetrically prepared by diluting the appropriate stock solution in methanol. All standard solutions were stored at $+4^\circ\text{C}$ until use. The ^{198}Hg -enriched MeHg working spike solution (w about $0.73 \mu\text{g/g}$ as mercury) was prepared by gravimetrically diluting the corresponding stock solution in methanol. The concentration of ^{198}Hg enriched MeHg in this spike solution was determined by reverse isotope dilution (RID) using a gravimetrically prepared natural MeHg standard. Four RID samples were prepared by mixing and diluting 0.23 g of MeHg and 0.14 g of enriched Me^{198}Hg working stock solution ($w=2.0514$ and $0.73 \mu\text{g/g}$, respectively) in 10 mL methanol. Gas chromatography requires volatile species. Therefore, derivatization of MeHg with NaBPr_4 was carried out prior to analysis. The NaBPr_4 solution ($1\% \text{ w/v}$) was prepared by dissolving the corresponding salt in deionized water. The solution was then pipetted into single one-mL Eppendorf tubes and stored in a freezer (-80°C) until use.

Optimization of GC-ICP-MS conditions

The design and optimization of the transfer line have been described elsewhere¹⁸. Signal optimization in the ELAN 6000 was carried out by, first, introducing a standard liquid solution into the system. Subsequently, the plasma was switched off and the spray chamber and the nebulizer assembly were replaced by the transfer line and its adapter. Signal optimization was performed manually by SPME injection of the MeHg standard and recording of the signal from

^{202}Hg . In order to determine the mass bias drift, MeHg mass bias standard solutions (prepared as described above) were injected at the beginning of the analysis and after every fourth run.

SPME conditions

The SPME procedure was similar to the one reported by Yang et al⁹. Briefly, 0.5 mL of MeHg standard or RID solutions were diluted in 10 mL of 1 M acetate buffer pH 5, in a glass vial, fitted with a PFTE-silicon septum, and 1 mL of 1% NaBPr₄ was added. The solution was then stirred with a magnetic stirring bar, and a PDMS fiber, placed in the middle of the cone, formed by the vortex to increase collection of analytes, was exposed to the derivatized methyl mercury species in the headspace at room temperature for 10 minutes. Then, the fiber was retracted in the holder, inserted into the GC injection port at 220 °C and desorbed. One minute was the optimal time for complete thermal desorption of the analytes.

Sample preparation for DOLT-4 using isotope dilution

0.5 g of DOLT-4 was weighed into glass flasks and spiked with 0.21 g of the enriched Me¹⁹⁸Hg spike solution (*w* about 0.73 µg/g). Eight mL of methanesulfonic acid and 16 mL of deionized water were added, and the samples were digested under reflux for 16 hours. The level of MeHg in blanks was first estimated with a semi-quantitative analysis. Blanks consisted of a mixture of methanesulfonic acid and water spiked with about 10% of enriched standard solution (0.3674 g, *w*=0.06874 µg/g Me¹⁹⁸Hg) used in the sample. The digested samples were then centrifuged for 10 minutes at 894 g and the supernatant stored at +4 °C until analysis. SPME sampling of DOLT-4 and blanks was performed as described above for the standards and RID solutions. In order to achieve optimum conditions for the derivatization, the extracts were buffered and the pH adjusted to 5-6 with about 1 mL of ammonium hydroxide (28% v/v).

A schematic representation of DOLT-4 sample preparation is given in Figure 1.

Safety consideration

MeHg is highly toxic compound, NaBPr₄ is highly flammable, and methanesulfonic acid is irritant and corrosive. Material Safety Data Sheet must be consulted and safety precautions taken for all manipulations.

Results and discussion

Acidic extraction has been widely used for protein digestion and amino acid analysis, as well as for SeMet determination^{15,16}. Hot concentrated acids easily break down the proteins, by cleaving the peptide bonds. Methanesulfonic acid was selected over hydrochloric or nitric acid, because of its non-oxidizing nature, which prevents the alteration of certain species¹⁷. In the proposed acidic extraction, samples are added with the methanesulfonic/water mixture and digested under reflux for 16 hours. Due to the non-volatile nature of methanesulfonic acid, extraction can be performed in a regular glass flask and no sealing is needed. This procedure is more aggressive than basic leaching, but it is necessary to fully extract and completely release the MeHg from the biological matrix. DOLT-4 certified reference material was selected for method validation to determine whether MeHg can be fully recovered after the acidic digestion. This approach could be of particular interest when simultaneous extraction of MeHg and other species is required.

Quantitation of MeHg using ID and RID

Isotope equilibration is a prerequisite in ID analysis (IDA). If this condition is not achieved, results might be seriously biased. In a double-phase system, isotope equilibration is usually achieved after sample digestion. In order to limit the potential for contamination, samples and standards were handled in a class 10 clean-room environment. Despite these precautions blank correction was necessary in order to obtain accurate results²⁰. For isotope dilution two interference-free isotopes are needed: the reference isotope, which is usually one of the most abundant isotope, as ²⁰²Hg, and the spike isotope, which can be any minor isotope. Since ¹⁹⁸Hg enriched MeHg was synthesized in house and readily available, it was selected as spike isotope for the isotope dilution calibration. Only the nominal concentration of the Me¹⁹⁸Hg enriched standard is available; therefore, quantitation of the spike is necessary. RID solutions were measured on the first day, while ID DOLT-4 and blank samples were measured on subsequent days. A mass bias correction solution was injected between each RID solution and ID sample to evaluate the mass bias drift factor (f)²¹:

$$f = \frac{r_t}{r_0} \quad (1)$$

where r_t and r_0 are the isotope ratios measured at time t and at the beginning of the sequence, respectively. The mass bias drift factor for MeHg was 1.009 ± 0.015 . This value shows that mass bias drift was not significant and there is no influence on the final results. MeHg concentration in DOLT-4 was calculated using the following equation:

$$C_x = C_z \cdot \frac{m_y}{m_x} \cdot \frac{m_z}{m'_y} \cdot \frac{A_y - B_y \cdot R_n}{B_{xz} \cdot R_n - A_{xz}} \cdot \frac{B_{xz} \cdot R'_n - A_{xz}}{A_y - B_y \cdot R'_n} - f_b \cdot C_b \quad (2)$$

where C_x is the concentration of the analyte in dried DOLT-4, C_z is the concentration of the natural abundance standard, m_z is the weight of natural abundance standard used, m'_y is the weight of the enriched spike used to prepare the blend solution of both enriched and natural abundance standard, m_y is the weight of enriched spike used to prepare the blend solution of enriched standard and sample, m_x is the mass of sample used, A_y is the abundance of the reference isotope (^{202}Hg) in the enriched standard, B_y is the abundance of the spike isotope (^{198}Hg) in the enriched standard, A_{xz} is the abundance of the reference isotopes in the sample or in the natural abundance standard, B_{xz} is the abundance of the spike isotope in the sample or in the natural abundance standard, R_n is the measured, mass bias corrected reference/spike isotopic ratio in the blend solution of the spike and the sample, R'_n is the measured, mass bias corrected reference/spike isotopic ratio in the blend solution of the spike and the natural abundance standard, C_b is the concentration of the blank normalized for the sample weight and the factor f_b is given as:

$$f_b = 1 - \frac{m_y}{m'_y} \cdot \frac{A_y - B_y \cdot R_n}{B_{xz} \cdot R_n - A_{xz}} \cdot \frac{B_{xz} \cdot R'_n - A_{xz}}{A_y - B_y \cdot R'_n} \quad (3)$$

Quantitation of MeHg in DOLT-4 with ID after acidic extraction

DOLT-4 (dogfish liver CRM), which is certified for both total Hg and MeHg, was selected as matrix for the method validation. Species-specific ID was chosen over other calibration methods to achieve high precision and accuracy. The enriched Me^{198}Hg spike was added prior to sample digestion and the $^{202}\text{Hg}/^{198}\text{Hg}$ ratio, instead of an absolute intensity value, was used for the calculation. Thus, ID corrects for all possible analyte loss or instrument drift, as well as matrix effects and variation in sampling.

Concentration of MeHg in DOLT-4 was calculated using equation (2). To minimize the error magnification factor, the spike isotope must be optimized, so that R_n value is neither too close to the natural spike ratio (underspiking) nor too high (overspiking), thus R_n values comprise between 0.1 and 10 are usually recommended²². In our study, an equivalent amount of spike and natural isotopes was selected, so that R_n approaches 1 (Figure 2 and Table 2). The R_n values indicated also that formation of MeHg artefacts was negligible, since their presence would have provoked a significant deviation from that unitary ratio. However, this was not the case, as it was evident from ratio values listed in Table 2. Furthermore, Hintelmann *et al.* reported that MeHg artefacts are really insignificant in DOLT matrix, submitted to hot extraction²³. As showed in Table 2, the measured value for MeHg in DOLT-4 was in a good agreement with the certified value (*t*-test, $P=0.05$), demonstrating the reliability of the method. This result suggests that the methanesulfonic acid sample preparation method is suitable for an efficient extraction of MeHg from tissues, and also indicates that MeHg can well tolerate the acidic hydrolysis. The difference between theoretical and measured $^{202}\text{Hg}/^{198}\text{Hg}$ ratios was negligible (theoretical/measured=1.017), suggesting that there is no significant isobaric interference from the sample matrix. The selection of GC-ICP-MS over GC-MS allows specific detection of mercury species and good sensitivity (S/N up to 80 for MeHg detection in DOLT-4) in a complex matrix as liver. Therefore, the methanesulfonic acid extraction can successfully be applied to recover MeHg from biotissues. With further improvements, this new procedure could simplify the sample preparation, when multiple species must be analysed and their levels compared (e.g. concentration of certain amino acid in presence of MeHg). It is anticipated that the present sample preparation procedure could find applications in bioanalytical and toxicological studies.

Conclusions

In this paper, a sample preparation method was described for the acidic extraction of MeHg in biological samples. Analyses were performed with GC-ICP-MS that enabled a sensitive and specific detection of the target analytes. The MeHg acidic extraction method was successfully validated with DOLT-4 certified reference material. MeHg concentrations obtained were in good agreement with the certified value (*t*-test, $P=0.05$), suggesting that methanesulfonic acid digestion is suitable for MeHg extraction. With further developments, the proposed preparation

method is expected to have useful applications in bioanalytical and/or toxicological studies in which simultaneous extraction of MeHg and other species, e.g. aminoacids, might be required.

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Legends to the Figures:

Fig. 1. Flow chart showing the methanesulfonic acid (MSA) extraction method and derivatization procedure for MeHg in DOLT-4.

Fig. 2. (a) GC-ICP-MS analysis of MeHg in DOLT-4 after species-specific isotope dilution and methane sulfonic extraction. ^{202}Hg and ^{198}Hg were the selected isotopes. (b) Expansion of the MeHg peak in part (a) showing how the isotopic ratio (R_n) value approaches 1.

Fig. 1

Fig. 2

Table 1 GC-ICP-MS conditions for MeHg determination in DOLT-4

GC	
Column	DB-5 (30 m x 0.25 mm i.d. x 0.25 μ m d _f)
Injection mode	Splitless
Injection temperature	220 °C
Oven program	50 °C (2 min) to 250 °C (20 °C/min), 1 min post run
Carrier gas, flow rate	He, 1.5 mL/min
ICP-MS	
Rf power	1100 W
Plasma Ar gas flow rate	15.0 L/min
Auxiliary Ar gas flow rate	1.0 L/min
Nebulizer Ar gas flow rate	0.35 L/min
Dwell time	30 ms
Sweeps per reading	2
Number of replicates	1
Reading per replicate	1700
Dead time	55 ns

Table 2. MeHg concentrations obtained for three samples of DOLT-4 and corresponding blanks after the extraction with methanesulfonic acid. Certified value for MeHg is 1.33 ± 0.12 $\mu\text{g/g}$. LOD for the method was 3.6 ng/g. The concentrations of MeHg in the DOLT-4 samples are blank-corrected.

Sample	R_n	MeHg in samples ($\mu\text{g/g}$)	MeHg in blank ($\mu\text{g/g}$)
DOLT-4 (1)	0.931	1.324	0.079
DOLT-4 (2)	0.993	1.441	0.078
DOLT-4 (3)	1.064	1.358	0.072
Mean	0.996	1.374	0.076
SD	0.067	0.060	0.004