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A simple, highly-sensitive and accurate method is presented for the determination of both inorganic mercury and total mercury in marine biological samples. 0.1 g of sample was completely solubilized in 1 g of tetramethylammonium hydroxide (TMAH) at room temperature. The TMAH-digested samples were spiked with $^{199}\text{Hg}$ enriched isotope and diluted to about 14 mL with deionized water. The spiked sample solutions were then analyzed for inorganic mercury determinations by isotope dilution inductively coupled plasma mass spectrometry with cold vapor generation. The solutions were further 5 times diluted with deionized water for total mercury determinations. SnCl$_2$ reducing agent was used for the inorganic mercury determinations, and NaBH$_4$ for the total mercury determinations. No reagents other than the reducing agents with acid solutions were used for the mercury determinations. The proposed method was validated by the analysis of three certified reference materials, DORM-2 (dogfish muscle), NIST SRM1566b (oyster) and KRISS tuna CRM. The detection limit of the method was found to be 0.018 ng g$^{-1}$.

Introduction

Fish and shellfish are an important part of a healthy diet. Fish and shellfish contain high-quality protein and other essential nutrients. However, some fish contain high levels of the toxic methylmercury that may harm an unborn baby or young child's developing nervous system. The target organ for methyl mercury toxicity is the central nervous system, especially the brain, and damage may occur at doses as low as 3 $\mu$g kg$^{-1}$ in humans. The Food and Drug Administration (USFDA) and the Environmental Protection Agency (USEPA) are advising women who may become pregnant, pregnant women, nursing mothers, and young children to avoid some types of fish, such as shark, swordfish, king mackerel, marlin and tilefish. Since the toxicity of mercury depends on the form of its chemical species, the exact evaluation of its toxicity requires the determination of not only total mercury but also organic mercury content in fish. For the mercury speciation analysis, a chromatographic system has been employed in combination with an element-selective instrument. The chromatography systems, for separation of inorganic and organic mercury species, include gas chromatography (GC),* liquid chromatography (LC)* and ion chromatography (IC).* Since methylmercury is highly toxic and dimethylmercury easily penetrates into rubbers, it is extremely dangerous to treat these organic mercury standards in ordinary laboratories. Thus, for the rapid and safe determination of both inorganic mercury and organic mercury in fish, it is very desirable that a reliable and safe analytical method with high sample throughput must be established which employs neither chromatography systems nor dangerous organic mercury standards.

Cold vapor generation atomic absorption spectrometry (CVAAS) has been one of the most generally used techniques for mercury determination owing to high sensitivity and ease of operation. Tao et al. reported a simple and rapid method which did not require a chromatography technique for the determination of inorganic and total mercury in fish samples. They used different reducing agents and reagents for the determination of inorganic and total mercury following incomplete digestion of biological tissue samples using tetramethylammonium hydroxide (TMAH). The organic mercury in fish, which is mostly methyl mercury,* could be determined by subtracting inorganic mercury from total mercury. Tao et al. had to use reagents such as l-cysteine and KMnO$_4$ for the determination of inorganic mercury and total mercury, respectively. They found that inorganic mercury was not completely reduced to elemental mercury by SnCl$_2$ without l-cysteine, and that organomercury species had to be decomposed with on-line addition of KMnO$_4$ for the determination of total mercury. Another group have also carried out the mercury speciation without using chromatography.

Gelaude et al. employed solid sampling-electrothermal vaporization and an appropriate temperature program for the separation of methylmercury and inorganic mercury. For quantification they used the isotope dilution (ID) method with generation of $^{200}\text{Hg}$ spike isotope vapor using a permeation tube. Though this method has an advantage that no sample preparation is required, it can not be easily implemented in most laboratories due to the complicated instrumentation.

Inductively coupled plasma mass spectrometry (ICP-MS) is a technique with many salient features, such as low detection limits and accurate quantification by the ID method. ICP-MS has another advantage as an element detector in combination with the cold vapor generation device: mercury in a sample does not need to be reduced to its elemental form for detection with...
ICP-MS because any type of volatile mercury compound, such as methyl mercury hydride, can be detected by ICP-MS. This fact suggests that sample dissolution procedure and optimum conditions of the mercury cold vapor generation could be different with ICP-MS from those with CVAAS. In this work, an analytical method employing ICP-MS is proposed for the determination of inorganic and total mercury in marine biological samples. The proposed method provides simple, highly sensitive and accurate determination of inorganic and total mercury using only reducing agents and acids.

## Experimental

### Reagents and enriched isotope

Enriched 199Hg (97.4% enrichment) metal was obtained from US Services Inc. (Summit, NJ, USA). A stock standard solution of mercury was prepared by dissolving 1 g of mercury metal (99.999995%, Alfa, Ward Hill, MA, USA) in 5% (v/v) HNO3 solution. Working standard solutions were made by serial dilution of the stock solution. TMAH (25% in water) was bought from Aldrich (Milwaukee, WI, USA). SnCl2 solution (1% m/v) was prepared daily by dissolving 0.5 g of SnCl2 powder (99.99%, Aldrich) in 50 mL of 10% (v/v) HCl solution. The SnCl2 solution was purified before use by purging it with argon gas for a few hours to remove mercury impurity in the solution. NaBH4 solution (1% m/v) was daily prepared by dissolving 0.5 g of NaBH4 powder (99.99%, Aldrich) in 50 mL of deionized water. Methylmercury chloride was bought from Aldrich. Three certified reference materials (CRM), analyzed for the method validation, are oyster (SRM 1566b) from National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA), dogfish (DORM-2) from National Research Council of Canada (NRCC, Ottawa, Canada) and tuna from Korea Research Institute of Standards and Science (KRISS, Daejeon, Korea). Deionized water was obtained from a Mill-Q Plus water purifier (Millipore, Bedford, MA, USA). High-purity HNO3 was prepared by subboiling distillation of electronic grade HNO3 purchased from Dongwoo Pure Chemicals (Iksan, Korea).

### Instrumentation

All measurements were carried out on a Thermo X-series II ICP-MS instrument (Thermo Electron, Bremen, Germany). A continuous flow cold vapor generation system is described in Fig. 1. A sample solution and reducing agent (either SnCl2 or NaBH4) are mixed in a Teflon tube (i.d. = 0.07 cm and length = 30 cm) and mercury in the sample solution is converted to its volatile form (Hg⁰ or CH₃HgH). The volatile mercury vapor is introduced into the plasma following extraction into argon carrier gas stream through a membrane tube inside a gas–liquid separator. Methylmercury hydride was identified using a gas chromatograph mass spectrometer (GC-MS, Agilent 5973). GC was equipped with a 5% phenylmethylpolysiloxane fused silica capillary column (length = 60 m, i.d. = 0.25 mm, Agilent), and operated isothermally at 100 °C with He carrier flow rate of 0.8 mL min⁻¹.

### Sample preparation

For each CRM, a group of five samples were weighed for replicate analyses together with one extra-sample for moisture correction. The extra-sample was dried for more than five days inside a desiccator filled with P₂O₅ to find moisture content. 0.1 g of sample was dissolved for DORM-2 and tuna CRM, while 0.5 g was solubilized for oyster CRM due to its low mercury content. Each sample was weighed into a 15 mL PFA (perfluoroalkoxy) vessel with addition of 1 g of TMAH and 4 g of deionized water for oyster sample). The samples were completely solubilized at room temperature, and then they were diluted to 14 mL with deionized water after spiking with an appropriate amount of 199Hg enriched isotope. The solutions were analyzed for the inorganic mercury determinations, and 4 mL of the solutions were diluted a further 5 times with deionized water for the total mercury determinations. When the sample was not completely solubilized, analytical results showed poor repeatability. When the sample was heated using a microwave oven or heater, the determined total mercury concentration was much lower than the certified value, indicating that there was some mercury loss during the heating.

### Cryogenic trapping for desorption chromatogram

It is generally known that SnCl₂ reduces only inorganic mercury into elemental mercury (Hg⁰), and that NaBH₄ reduces both inorganic and organic forms of mercury, but with a slower reduction rate of organomercury than that of inorganic mercury. Puk and Weber, however, reported that NaBH₄ did not reduce methyl mercury to elemental mercury. Quite a few research groups also confirmed that NaBH₄ converted methyl mercury into stable and volatile methylmercury hydride...
In this work, the same kind of experiment was carried out as those done by the research groups in order to clarify the role of NaBH₄ in the mercury cold vapor generation. A U-shaped Pyrex tube (40 cm length × 4 mm id) was packed with about 1 g of molecular sieve (40/60 mesh, Alltech Associates, Deerfield, IL, USA) and wrapped with Nichrome wire of 0.5 mm in diameter. The mercury cold vapor generation device was connected to the Pyrex tube immersed in a bath of liquid N₂, while introducing a sample solution and a reducing agent with He (99.99%) carrier gas flow. After the cryogenic trapping, the Pyrex tube was slowly heated to get a desorption chromatogram of mercury species by applying 30 V to the two ends of Nichrome wire using a variac. A platinum temperature sensor (100 Ω, Heraeus, North Brunswick, NJ, USA) was attached to the Nichrome wire to record temperature.

**Results and discussion**

**Desorption chromatogram of mercury species**

Thermal desorption chromatograms were obtained for the mercury species generated with the introduction of tuna CRM solution dissolved in TMAH and two different reducing agents (SnCl₂ and NaBH₄). Fig. 2 shows typical chromatograms of mercury species generated with 1% SnCl₂ and 1% NaBH₄ as reducing agents. Approximately 10% of mercury species in the tuna solution are inorganic mercury (refer to Table 2, 3). The chromatogram generated with SnCl₂ shows that SnCl₂ reduces only inorganic species into elemental mercury to generate one peak in Fig. 2. The chromatogram generated with NaBH₄ shows two peaks. The first peak appears at the same retention time (about 40 s) as the chromatogram peak generated with SnCl₂, and it corresponds to elemental mercury from inorganic species. Judging from the previous chromatogram reported by Tseng *et al.*,¹¹ the second peak is believed to correspond to methylmercury hydride generated from methylmercury species in the tuna solution. The ratio of the two peak heights is about 1 : 10 and it does not change much when the reaction time of the sample solution and reducing agent is increased by lengthening the mixing tube from 30 cm to 200 cm. This result indicates that NaBH₄ reduces only inorganic mercury species to elemental mercury, and that NaBH₄ reacts with methylmercury to yield methylmercury hydride. ICP-MS detects the two volatile mercury species (Hg⁰ and CH₃HgH) equally, and hence it is not necessary to decompose organic mercury in the sample solution into Hg²⁺ for the total mercury determinations by ICP-MS.

**Identification of methylmercury hydride**

In order to confirm that the second peak of the desorption chromatogram in Fig. 2 was from methylmercury hydride, GC-MS analysis was carried out. Since the sensitivity of GC-MS is quite lower than that of ICP-MS, a synthetic solution was prepared by spiking 10 µg of methylmercury chloride into the TMAH-digested tuna solution. Vapor generated by reaction of the synthetic solution with 1% NaBH₄ was cryogenically trapped in the U-shaped tube. Both ends of the tube were subsequently blocked with septa and the tube was heated to 100 °C. The trapped vapor was collected in a syringe through a septum, and injected into the GC-MS to obtain a mass spectrum. Fig. 3(a) shows two clusters of peaks and it closely resembles the previous GC-MS spectrum reported by Filippelli *et al.*¹² The first cluster of peaks from m/z 198–204 corresponds to Hg isotopes and the second cluster from m/z 214–220 corresponds to CH₃HgH. In order to further verify that the second cluster of peaks was from CH₃HgH, 2 µg of methylmercury chloride was directly injected into GC-MS to get a mass spectrum representing CH₃Hg. The mass spectrum in Fig. 3(b) also shows two clusters of peaks and the second cluster shows a shift of 1 mass toward lower side in comparison with the second cluster in Fig. 3(a). Thus, the GC-MS spectra in Fig. 3(a) and (b) clearly identifies that the second...
peak of the desorption chromatogram in Fig. 2 is from methyl-mercury hydride.

**Optimization of cold vapor generation**

Various parameters of the mercury vapor generation were optimized individually while others were kept at fixed values. The optimized parameters included concentrations of SnCl₂ and HCl in SnCl₂ solution for inorganic mercury determinations, and concentrations of NaBH₄ and HNO₃ solutions for total mercury determinations. Fig. 4 shows the effect of HCl and SnCl₂ concentrations on the inorganic mercury signal. It can be observed from Fig. 4 that the inorganic mercury signal goes up as the concentrations of HCl and SnCl₂ are increased. 1% SnCl₂ in 10% HCl solution was chosen as a reducing agent compromised between sensitivity and reagent consumption. Fig. 5 shows the effect of concentrations of HNO₃ and NaBH₄ solutions. The total mercury signal in Fig. 5(a) stays almost constant at HNO₃ concentration interval between 2 and 15%, but the signal in Fig. 5(b) steadily goes up as the NaBH₄ concentration is increased. Thus, 1% NaBH₄ and 10% HNO₃ were chosen as optimum concentrations for the total mercury determinations.

The length of the mixing tube determines the reaction time for which the cold vapor generation takes place. The mercury signal showed no significant difference as the mixing tube length was increased from 30 cm to 200 cm. This means that the volatile mercury species are formed rapidly, and that they are pretty stable in the gas phase. The length of the mixing tube was fixed to 30 cm.

**Isotope dilution method**

All the analytical data reported here were quantified by the ID method, which provides accurate results even when the mercury vapor generation is not steady. The ID method is based on addition of a known amount of an enriched isotope to a sample. It is necessary in the ID method to know an approximate content of the analyte so that an appropriate amount of the enriched isotope can be added to minimize uncertainty. In this work, the ¹⁹⁹Hg spike isotope in an inorganic form was used for both inorganic and total mercury determinations, and the spike isotope concentration was determined by the reverse ID with an inorganic mercury standard solution. The two isotopes of Hg employed for the ID are ¹⁹⁹Hg and ²⁰⁰Hg, and the two isotope ratios (¹⁹⁹Hg/²⁰⁰Hg) of sample and standard solution are assumed equal. The moisture-corrected sample mass was used in the ID calculation.

\[
C_s = C_p m_p m_s R_s / (m_p R_p - R_f R_s)
\]

where \(C_s\) = analyte concentration in the sample (ng g⁻¹); \(C_p\) = concentration of the primary standard solution (ng g⁻¹); \(m_p\) = mass of the primary standard solution (g) used for the spike-primary standard mixture; \(m_s\) = mass of sample (g) used for the sample-spke mixture; \(m_sp\) = mass of the spike (g) used for the spike-sample-spke mixture; \(m_sp\) = mass of the spike (g) used for the spike-primary standard mixture; \(R\) = isotope ratio (¹⁹⁹Hg/²⁰⁰Hg) measured from the sample-spke mixture; \(R_f\) = isotope ratio (¹⁹⁹Hg/²⁰⁰Hg) measured from the spike-primary standard mixture; \(R_s\) = isotope ratio (¹⁹⁹Hg/²⁰⁰Hg) in the sample; and \(R_sp\) = isotope ratio (¹⁹⁹Hg/²⁰⁰Hg) in the spike.

**Total mercury determination**

At first, attempts were made to decompose organomercury species into inorganic mercury with on-line addition of strong oxidizers such as KMnO₄ and H₂SO₄ into the TMAH-solubilized.
Inorganic mercury determination

Tao et al.6 added L-cysteine to sample solutions by on-line merging in order to liberate mercury bound to proteins or other molecules in the TMAH-digested solutions. In this work, however, the L-cysteine addition did not enhance the inorganic mercury signal, and hence SnCl₂ reducing agent acidified with 10% HCl was the only reagent for the generation of mercury vapor from inorganic mercury in the sample solutions. For each CRM, five replicate samples were separately prepared, spiked and analyzed using the ID method. Analytical results of the three CRM’s are presented in Table 3 together with reference values. Inorganic mercury content is not certified in the CRM’s, and contents of total mercury and methyl mercury are certified in DORM-2 and NIST oyster CRM. Since the organomercury in fish is mostly methylmercury, the difference between the certified values of total and methyl mercury is given in Table 3 as a reference value for the inorganic mercury content. The measurement repeatability (relative standard deviation) estimated from the five different sample-spike mixtures is shown to be 2.8% for DORM-2, 3.5% for oyster SRM 1566b and 3.4% for tuna CRM. The determined inorganic mercury concentrations of dogfish and oyster CRM’s in Table 3 agree well with the reference values.

Detection limit

A blank concentration was determined by the ID analysis of the TMAH solution without sample. It was almost the same for the two different reducing agents, and calculated to be 0.2 ± 0.006 ng g⁻¹ based on 0.5 g of sample. The detection limit of this method, determined as the concentration corresponding to three times the standard deviation of the blank was 0.018 ng g⁻¹.

Uncertainty

The standard uncertainty due to systematic effects in ID calculation was estimated using the EURACHEM Guide.46 The ID eqn (1) was transformed into eqn (2) to take into account the mass bias correction (Kᵣ for R; Kᵣ for R; Kᵣ for R, and Kₛ for Rₛ) and blank subtraction.

\[
C_i = \frac{m_{sp}}{m_{sp}m_{sp}} \left( \frac{K_{sp}R_{sp} - K_{sp}R}{K_{sp}R_{sp} - K_{sp}R} \right) - \text{Blank} \tag{2}
\]

For each parameter of eqn (2), a relative standard uncertainty contribution to the sample concentration was estimated, and the combined relative standard uncertainty was calculated by taking the square root of the sum of squares. The measurement repeatability (as relative standard deviation) is less than 4% for all three certified reference materials (CRMs). For each CRM, the standard deviation from random effects was combined with the standard uncertainty from systematic effects.17 Finally, expanded uncertainty at 95% confidence level was estimated by multiplying the combined uncertainty with the coverage factor 2.

Conclusion

An analytical method has been developed for the determination of both inorganic and total mercury in marine biological samples by ICP-MS following the cold vapor generation of the TMAH-digested sample. The method provides important information on
the toxic organomercury content in biological samples without using the dangerous organic mercury standards. Both inorganic mercury and methylmercury contribute to the cold vapor generation by reaction with NaBH₄ but with different reaction products of Hg⁰ and CH₃HgH, respectively. Extra-reagents such as L-cysteine and KMnO₄ are not required for the determination of inorganic mercury and total mercury. The mercury signal was independent of the mixing tube length from 0.3 to 2 m. This fact, together with good agreement between the determined and certified values, suggests that methyl mercury hydride is generated quickly and at almost the same rate as the reduction rate of Hg²⁺ into Hg⁰ when sample solutions containing both Hg²⁺ and CH₃Hg⁺ are in reaction with NaBH₄.

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