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Determination of Methyl- and Inorganic Mercury in Fish Using Focused Microwave Digestion Followed by Cu^{++} Addition, Sodium Tetraethylborate Derivatization, *n*-Heptane Extraction, and Gas Chromatography-Mass Spectrometry

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ABSTRACT

The analytical procedure for analysis of methyl- and inorganic mercury in fish was developed in this study. It involved microwave-assisted digestion with alkaline solution (tetra methyl ammonium hydroxide), addition of Cu^{++}, aqueous-phase derivatization of mercury species with sodium tetraethylborate, and subsequent extraction with *n*-heptane. The various mercury derivatives were desorbed in the splitless injection port of a gas chromatograph and subsequently analyzed by electron impact mass spectrometry. Optimum conditions allowed the sample throughout to be controlled by the instrumental analysis time (about 8 min per sample) but not by the sample preparation step. At the irradiation power of 15-30, 45, and 60-75 W, sample heating times were only 3.5, 2.5, and 1.5 min, respectively. The recoveries of mercury species were 92.3–96.1% and 93.6–95.5% for methyl- and inorganic mercury, respectively. The proposed method was finally validated by the analysis of three biological certified reference materials (BCR CRM 464 tuna fish, NRC DORM-2 dogfish muscle, and NRC DOLT-2 dogfish liver).

Key words: methylmercury, inorganic mercury, microwave-assisted digestion, ethylation, GC-MS, fish

INTRODUCTION

Mercury pollution has become a global problem because of the occurrence from natural and anthropogenic sources and food chain processes. Mercury is a well-known toxic element, especially in the form of methylmercury ($MeHg^+$) compounds, which are considerably more toxic than inorganic mercury (Hg^{2+}). In the environment, $MeHg^+$ is formed by biotic and abiotic methylation of Hg^{2+} and it accumulates in the tissue of fish and other biota[1-2]. Mercury as $MeHg^+$ usually represents more than 85% of total mercury present in fish[3-4]. Mercury poisonings are mainly caused by consumption of contaminated fish through $MeHg^+$ accumulation in the food chain, such as in the case of Minamata disease[5]. As a result, the US Food and Drug Administration (FDA) has set an Action Level of 1 mg/kg (wet mass) for concentration of $MeHg^+$ in fish. Fish containing concentrations of $MeHg^+$ above this level are considered to be hazardous for human consumption and cannot be sold in interstate commerce. Canada and several US states have developed consumption advisories of 0.5 μg/g for $MeHg^+$ in fish[6]. In Taiwan, the guideline level of $MeHg^+$ is set at 2.0 μg/g for migratory fish and 0.5 μg/g for other fish[7]. As public awareness regarding the toxicity and the environmental impact of mercury contamination increases, the demand for the development of an analytical methodology for routine monitoring increases.

The majority of procedures applied in analytical laboratories are based on the classical Westöö procedure[8] that is specific to $MeHg^+$, and on the Magos procedure[9] that implies an operational definition of the inorganic and organic mercury. Despite continuous improvements in the last three decades, the procedures based on these principles have remained time-consuming, tedious, and often unreliable, as comprehensively discussed by Emteborg *et al.*[10]. Nevertheless, they are the basis of the AOAC Official Methods for $MeHg^+$ in fish and shellfish[11].

The most common chromatographic technique used for the determination of mercury species is gas chromatography (GC) employing a variety of different detectors including electron capture detector (ECD)[9], atomic absorption spectrometer (AAS)[12], atomic emission detector (AED)[13-19], atomic fluorescence spectrometer (AFS)[19-23], mass spectrometer (MS)[19,24] and inductively coupled plasma-mass spectrometer (ICP-MS)[23,25]. The main limitations and drawbacks of most detectors used in mercury species analysis are a lack of selectivity and/or sensitivity towards the analytes of interest.

When the GC-MS was used to substitute electron capture detection, the addition of Cu^{2+} in the digestion

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solution would elevate the recoveries of methyl- and inorganic mercury level. Hence, the GC-MS detection after microwave-associated digestion, ethylation and solvent extraction to determine methyl- and inorganic mercury in fish reference material was described in this study.

MATERIALS AND METHODS

I. *Reagents*

All chemicals used were of analytical-reagent grade unless stated otherwise. Sodium tetraethylborate ($NaBEt_4$, 98% purity) was purchased from Alfa (Geesthacht, Germany). Tetramethylammonium hydroxide (TMAH, 25% in water) was purchased from Fluka (U.S.A.). Acetic acid (suprepure grade), sodium acetate, copper acetate, potassium hydroxide, *n*-hexane, *n*-heptane, *iso*-octane, and tetrahydrofuran (THF) were purchased from E. Merck (Darmstadt, Germany).

The derivatization solution was prepared by dissolving 1 g of sodium tetraethylborate in 100 mL of 2% potassium hydroxide solution. The solution was stored in a refrigerator and protected from light. Buffer solution was prepared by dissolving 1 M sodium acetate in water and adjusted the pH to 5.0 with concentrated acetic acid. Copper solution was prepared by dissolving copper acetate (4.86 mg/L) in water. Milli-Q quality water (Millipore) was used throughout.

II. *Calibration and Biological Reference Materials*

Methylmercury (II) chloride standard solution ($MeHg^+$, 1,000 μg/mL as Hg) was purchased from Alfa Aesar (U.S.A.). Propylmercury chloride ($PrHg^+$) was purchased from Pfaltz & Bauer (U.S.A.). Mercury standard solution (Hg, 1,000 μg/mL) was purchased from E. Merck (Darmstadt, Germany).

Working calibration solution of methyl- and inorganic mercury was prepared by appropriate dilution of methyl- and inorganic mercury standard solution with water and stored for a maximum of 1 week. Internal standard solution (2 μg/mL as Hg) was prepared by dissolving propylmercury chloride in methanol, then appropriate dilution with water and stored for a maximum of 1 week.

One certified reference material CRM 464 tuna fish (5.50 ± 0.17 μg/g $MeHg^+$) obtained from the Community Bureau of Reference (BCR), and two certified reference materials, DORM-2 dogfish muscle (4.47 ± 0.32 μg/g $MeHg^+$) and DOLT-2 dogfish liver (0.693 ± 0.055 μg/g $MeHg^+$), obtained from the National Research Council of Canada (NRCC), were used to validate the proposed method. The other laboratory tuna fish sample containing 1.98 ± 0.15 μg/g of MeHg+ was detected by the Westöö method[9].

For the recovery test of methyl- and inorganic mercury in fish, a sample of 0.5 g of fish muscle was placed in a microwave vessel and spiked with 1.0 mL of methyl- and inorganic mercury standard solution (250, 500, and 1000 ng/mL). The sample was stored in a refrigerator overnight before analysis.

III. *Devices and Instrument*

Focused microwave digester Microdigest 3.6 (2.45 GHz, maximum power 300 W) was the product of Porlabo (France). Gas chromatograph ion trap mass spectrometer Saturn 2200 was the product of Varian (U.S.A.). Capillary gas chromatograph columns CP-SIL 1 CB (100% dimethyl polysiloxane), and CP-SIL 8 CB (5% diphenyl- 95% dimethyl polysiloxane), and CP-SIL 24 CB (50% diphenyl-50% dimethyl polysiloxane) lowbleed/MS (30 m × 0.25 mm i.d. with a 0.25 μm film) were the product of Chrompack (Netherland).

All volumetric bottles and other glassware were Pyrex brand. Before use, they were washed with detergent and water, soaked with 50% (v/v) nitric acid overnight, rinsed with water, and dried.

IV. *Procedure*

For a sample of 0.5 g tissue, 1 mL of internal standard solution, and 5 mL of TMAH solution were placed in a microwave vessel and a reflux condenser used to prevent evaporation losses, than exposed to the microwave field at 45 W for 2.5 min. After microwave digestion, samples were neutralized by acetic acid and transferred into a 40-mL Pyrex vial with a Teflon cap and diluted with 20 mL of distilled water and 1 mL of 20 mM copper solution. The pH was adjusted to 5.0 using 5 mL of 1 M acetate buffer. One mL of the 1% sodium tetraethylborate/potassium hydroxide solution was added by syringe, mixed and stayed for 10 min at ambient temperature. Then, 2 mL of *n*-heptane was added and shaken for 10 min. An aliquot of the supernatant was analyzed by GC/MS. Instrument parameters used in this study are listed in Table 1.

RESULTS AND DISCUSSION

I. *Optimization of Microwave Assisted Digestion*

Table 1. Parameters of the analytical system

Microwave digester	Porlabo Microdigest 3.6
Irradiation power	45 W
Irradiation time	2.5 min
GC-ITMS	Varian Saturn 2200
Column	CP-Sil 8 CB Low Bleed/MS (5% dipheny and 95% dimethylpolysiloxane: 30 m, 0.25 mm i.d., 0.25 μm df
Injection technique	Splitless
Injection volume	1 μL
Injection temperature	260˚C
Temperature program	60˚C(5 min)—20˚C/min—280˚C(10 min)
Carrier gas; flow rate	He; 1 mL/min
Transfer line temperature	280˚C
Ion trap temperature	170˚C
Quantitative ion	Methylmercury: 217 Propylmercury: 274 Inorganic mercury: 231

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Using the conditions in section IV of materials and methods, and changing the microwave digestion condition, 5 mL of TMAH was successively spiked with methyl-, propyl-, and inorganic mercury (each 1 μg as Hg) and exposed to 15-30, 45, or 60-75 W irradiating power for various heating times. After irradiation, the sample solution was diluted with 20 mL of water and mercury species content was determined as described above. Figure 1(A) shows the recovery of methylmercury after different irradiating power and times. Only 73% and 62% of the initial signal were obtained after 3.5 min heating by irradiation at 60 and 75 W, respectively. And 94% and 89% of the initial signal were obtained after 3.0 and 3.5 min, respectively heating by irradiation at 45W. Figure 1(B) shows the recovery of propylmercury after different irradiating power and times. Only 72% and 64% of the initial signal were obtained after 3.5 min heating by irradiation at 60 and 75 W, respectively. And 93% and 90% of the initial signal were obtained after 3.0 and 3.5 min, respectively heating by irradiation at 45W. A 100% methyl- and propylmercury recoveries were achieved after 3.5 min heating by irradiation at 15-30 W, 2.5 min of irradiation at 45 W, or 1.5 min of irradiation at 60-70 W. The analytical signal obtained was strongly dependent on the heating time and irradiating power. The low recoveries of methyl- and propylmercury might be caused by long heating times or high irradiating power. This is similar to the report of Tseng *et al*.[26]. The recovery of inorganic mercury was also dependent on the heating time and irradiating power. The amounts of inorganic mercury obtained exceeded 100%. This might be due to slight degradation of methyl- and propylmercury.

The methylmercury extraction recovery was investigated using one biological certified reference material, DORM-2 dogfish muscle and a laboratory tuna fish sample. Figure 2 shows the recovery of methylmercury after different irradiating power and heating times. The recoveries of methylmercury of DORM-2 dogfish muscle and laboratory tuna fish sample increased with heating times setting irradiation at 15 and 30 W, and decreased with heating times setting irradiation at 60 and 75 W. The highest recoveries of methylmercury were obtained after 2.5 min of irradiation at 45 W. Results showed high irradiation power provided high energy and reduced heating time to extract, but methylmercury might decompose due to high irradiating power. The condition of 45 W irradiation power and 2.5 min heating time could provide the optimal extraction efficiency without decomposing methylmercury.

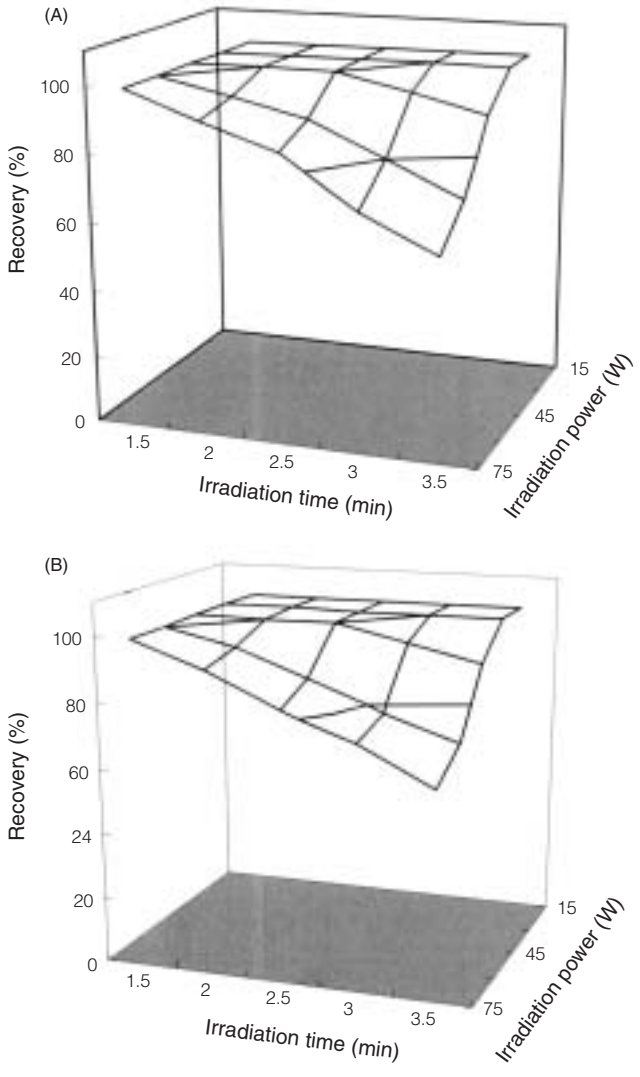

Figure 1. Recoveries of (A) methylmercury (1 μg as Hg) spiked in 25% TMAH solution, (B) propylmercury (1 μg as Hg) spiked in 25% TMAH solution when exposed to microswave irradiation.

II. *Optimization of the Derivatization Parameters*

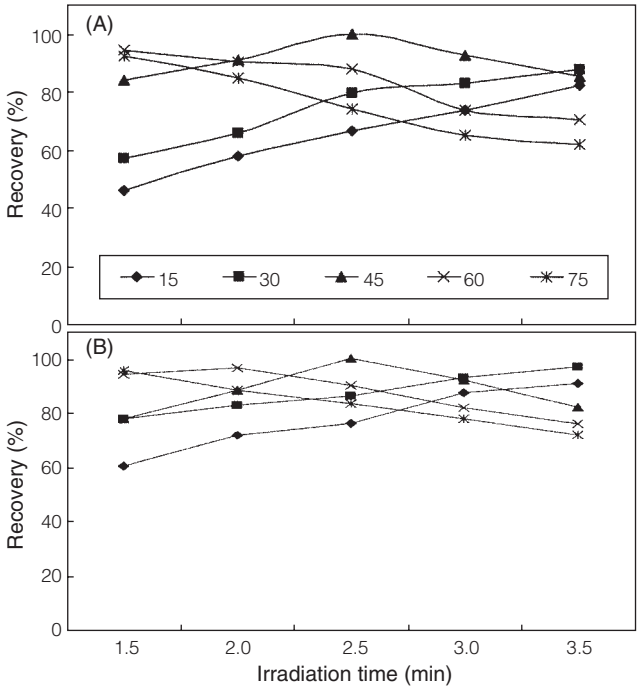

Figure 2. Recoveries of (A) methylmercury in DORM-2 dogfish muscle, and (B) laboratory tuna fish sample exposed to microwave irradiation.

Using the same conditions of section IV of materials and methods, the effect of cupric ion on the derivatization yield of mercury species was examined by adding 1 mL of 0, 10, 20, 40, 60 and 80 mM copper acetate solution. Results show no significant difference in methylmercury detection of methylmercury standard solution. There were no significant differences between 20, 40, 60 and 80 mM copper acetate solution in methylmercury detection of DORM-2 dogfish muscle and laboratory tuna fish sample. A 100% methylmercury recovery was obtained when added 20 mM or higher concentration of copper solution. The recovery was 70% by adding 1 mL of 10 mM copper acetate solution, and 15% by blank. This agrees with the report of Oslon *et al.*[27], who concluded the recoveries of the spiked methylmercury in surface water dropped from 118 to –110% with increasing sulfide in the sample which were not treated with $CuSO_4$. Once the samples were treated with $CuSO_4$, high recoveries were obtained even when the sulfide concentration was as high as 300 μmol/L. Due to a highter affinity of methylmercury with the sulphhydryl group in fish myofibrillar protein, the cupric ion could compete with methylmercury already combined with the sulphhydryl group in fish protein and resulted in release of methylmercury.

Furthermore, three derivatization agents including water, 2% KOH solution and tetrahydrofuran were tested for effect of solvent on methylmercury detection of DORM-2 dogfish muscle, laboratory tuna fish sample, and methylmercury standard solution. Results show no significant difference in methylmercury detection for mercury standard solution. Water and 2% KOH solution did not affect the ethylation of methylmercury in DORM-2 dogfish muscle, laboratory tuna fish sample, and methylmercury standard solution. Tetrahydrofuran (THF) did not affect the ethylation of methylmercury in standard solution, but reduced the ethylation of methylmercury in DORM-2 dogfish muscle and laboratory tuna fish sample. The reason seemed to be due to the reaction of THF with fish protein to retard the release of methylmercury in fish protein. The actual mechanism needs further study.

The influence of pH on the derivatization of mercury species was examined using 5 mL of pH 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5 buffer solutions in DORM-2 dogfish muscle, laboratory tuna fish sample, and methylmercury standard solution. The highest derivatization yield for all samples was obtained at pH 5.0. This also agrees with the report of Pereiro *et al.*[16].

The influence of acetate concentration on the derivatization of mercury species was checked using 5 mL of 0.01, 0.05, 0.1, 0.5, 1.0 and 2.0 M acetate buffer solutions in DORM-2 dogfish muscle, laboratory tuna fish sample, and methylmercury standard solution. It was found that 100% methylmercury recovery was obtained for all samples when acetate concentration was 0.5-2.0 M. Though the derivatization yield of mercury species decreased significantly when acetate concentration was less than 0.1 M in methylmercury standard solution, it did not vary between 0.01-2 M acatate buffer in DORM-2 dogfish muscle and laboratory tuna fish sample. The pH of DORM-2 dogfish muscle and laboratory tuna fish sample added different concentrations of acetate buffer was about 5.05. The pH of mercury species standard solution was about 5.06 when added 0.50, 1.0 and 2.0 M acetate buffer, but were 11.0, 10.5, and 10.2 when added 0.01, 0.05 and 0.10 M acetate buffer, respectively.

One mL of 0.5, 1.0, 1.5, 2.0 and 2.5% $NaBEt_4$/KOH solutions were proven to affect the derivatization yield of mercury species. Results showed no significant differences between 1.0, 1.5, 2.0 and 2.5% $NaBEt_4$/KOH solutions in mercury species detection of DORM-2 dogfish muscle, laboratory tuna fish sample, and methylmercury standard solution. A 100% methylmercury recovery was obtained when $NaBEt_4$/KOH solution concentration was not less than 1.0%. But only 73% of the peak was obtained by adding 1 mL of 0.5% $NaBEt_4$/KOH solution. One mL of 1.0% $NaBEt_4$/KOH was sufficient enough to derivatize mercury species in DORM-2 dogfish muscle, laboratory tuna fish sample, and methylmercury standard solution. This agrees with the result of Dietz *et al.*[15].

The effect of reaction time on the derivatization yield of mercury species was examined by reacting for 3, 5, 10, 15 and 20 min. Results showed no significant difference between 5, 10, 15 and 20 min reaction in methylmercury detection of DORM-2 dogfish muscle, laboratory tuna fish sample, and methylmercury standard solution. Only 70% of the signal was obtained by reacting for 3 min. This agrees with the result of Pereiro *et al.*[16].

The inter-day derivatization abilities of 1% $NaBEt_4$/KOH and 1% $NaBEt_4/H_2O$ solution were examined using a frozen solution after thawing everyday. Figure 3 shows the mercury derivative was stable within 28 days in 1% $NaBEt_4$/KOH solution, but dropped quickly in 1% $NaBEt_4$/ H_2O solution.

The intra-day derivatization ability of $NaBEt_4$/KOH solution was examined by using freshly prepared solution

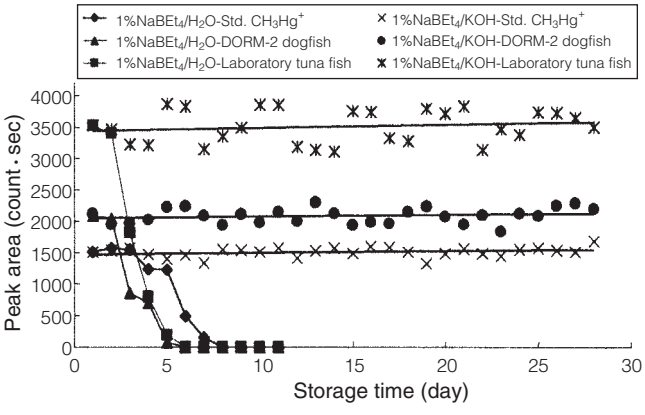

Figure 3. The inter-day derivatization ability of 1% $NaBEt_4$/KOH of DORM-2 dogfish, laboratory tuna fish sample, and methylmercury standard solution, and 1% $NaBEt_4/H_2O$ of DORM-2 dogfish, laboratory tuna fish sample, and methylmercury standard solution. Experimental conditions see Table 1.

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to stand for 1-10 hr. There were not overall decrease in the reaction yield concerned with duration of the $NaBEt_4$ solution. (Figure 4).

Using 2 mL of *n*-hexane, *n*-heptane and *iso*-octane to examine the influence of extract solvent on the derivatization yield of mercury species, the obtained chromatogram peaks of mercury species were clearly separated from each other by using *n*-heptane and *iso*-octane (Figure 5). The peak presented tailing with *n*-hexane. The extract of *n*-heptane had the highest peak area and S/N ratio of mercury species.

III. *Optimization of the GC/MS Parameters*

After microwave digestion, copper solution and acetate buffer addition, derivatization, and extraction, the optimal temperature condition of using CP-SIL 8CB column in GC-MS was studied. Setting column at 60˚C for 5 min, the temperature of the column was increased at a rate of 5, 10, 15 or 20˚C/min. It was found that methyl-, propyl- and inorganic mercury derivatives were well separated at 20˚C/min. Under this condition, methyl-, propyl- and inorganic mercury derivatives were eluted at 4.21, 6.83 and 7.91 min, respectively (Figure 6). All peaks were narrow and symmetric and well separated within 8 min.

To avoid the decomposition of mercury species derivatives, the optimal temperature of the injection port in GC was investigated. It was found that the peak area of propyl- and inorganic mercury derivatives did not vary between 200 and 260˚C. However, peak area of methylmercury increased from 220 to 230˚C, and kept stable during 230 to 260˚C. The decomposition of methyl-, propyl- and inorganic mercury derivatives were not found even at 260˚C. Hence, the optimal temperature of the injection port in GC was set at 260˚C.

Mass spectra of MeHgEt, PrHgEt and HgEt2 are shown in Figure 7. The quantitative ions were 217, 274 and 231 *m/z*, for methyl-, propyl-, and inorganic mercury derivatives, respectively. The reference spectra were 214-215, 271-272 and 228-229 *m/z* for methyl-, propyl-, and inorganic mercury derivatives, respectively.

Three types of capillary columns were evaluated at the beginning of this study. Three columns, CP-SIL 1 CB, CP-SIL 8 CB, and CP-SIL 24 CB were used. The peaks of mercury species were not separated from each other using CP-SIL 24 CB column, but were clearly and well separated using CP-SIL 1 CB and CP-SIL 8 CB columns. The peak of methylmercury showed tailing when CP-SIL 1 CB column was used. Hence, the CP-SIL 8 CB column was used in this study.

Calibration graphs of methyl- and inorganic mercury derivatives within 0.01-1.0 μg/mL are shown in Figure 8. The calibration curves all were linear with respective slope

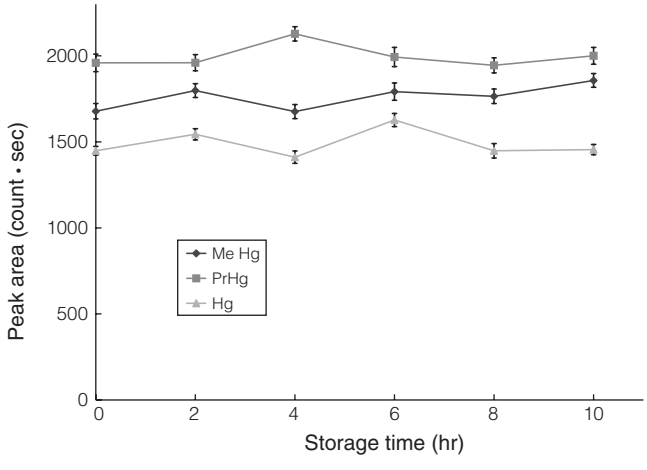

Figure 4. The intra-day stability of 1%$NaBPr_4$/KOH of methyl-, propyl-, and inorganic mercury standard solution. Experimental conditions see Table 1.

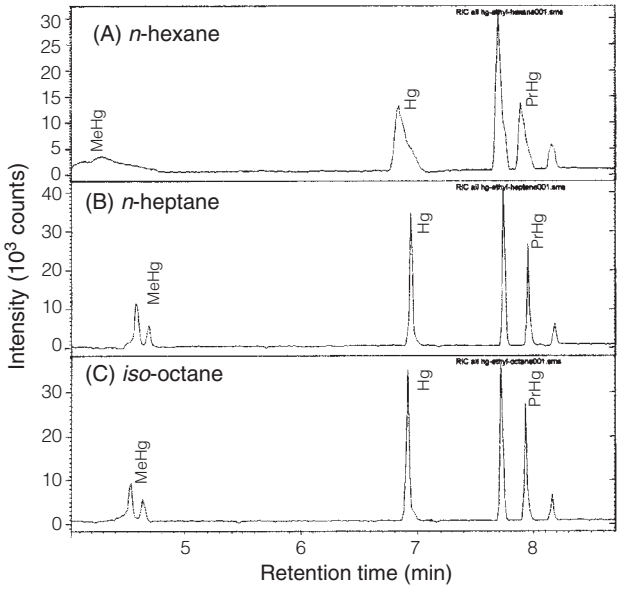

Figure 5. GC-ITMS chromatogram of methyl-, propyl-, and inorganic mercury derivatives of extraction with *n*-hexane, *n*-heptane, and *iso*-octane. Experimental conditions see Table 1.

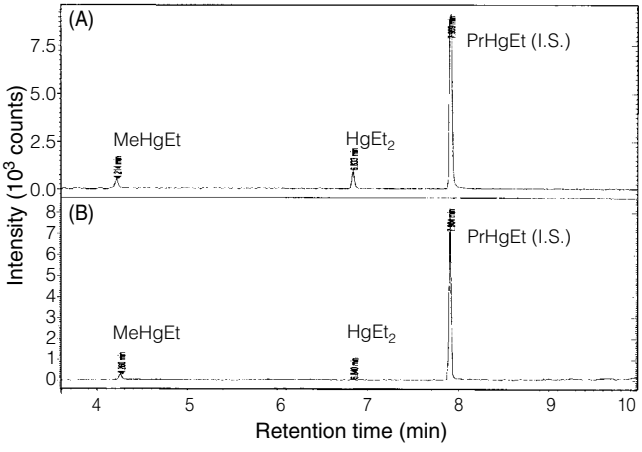

Figure 6. Gas chromatogram of (A) standard solution (1.0 μg/mL as Hg of methyl- and inorganic mercury and 2.0 μg/mL as Hg of propylmercury) and (B) laboratory tuna fish sample (1.98 μg/g methylmercury and 0.20 μg/g inorganic mercury).GC-ITMS conditions see Table 1.

(0.1055 and 0.1164) and intercept (0.0007 and 0.0016) for methyl- and inorganic mercury derivatives, respectively. The regression coefficients of methyl- and inorganic mercury derivatives were 0.9981 and 0.9957, respectively.

The detection limit of methyl- and inorganic mercury derivatives in GC/MS was 10 pg as Hg, which was calculated from three times of the baseline noise peak.

IV. *Validation of the Determination Method*

The recoveries of methy- and inorganic mercury derivatives in fish spiked with 0.5, 1.0, and 2.0 μg/g (as Hg) were tested and were 92.3 ± 4.4%, 93.8 ± 4.6% and 96.1 ± 4.1% for methylmercury, and 93.6 ± 5.1%, 94.9 ± 5.3% and 95.5 ± 4.8% for inorganic mercury, respectively.

The coefficient of variation (CV) of intra-day and inter-day during 1 week calculated from data of seven replicates. The CV values for retention time of methyl-, propyl-, inorganic mercury were 0.1, 0.2 and 0.2% for intra-day, and 0.4, 0.5 and 0.4% for inter-day, respectively. The CV values of peak area of methyl-, propyl-, inorganic mercury were 4.5, 5.1 and 4.9% for intra-day, and 6.5, 5.8 and 7.0% for inter-days up to 7 days, respectively. These small CV values indicate the method has very good reproducibility.

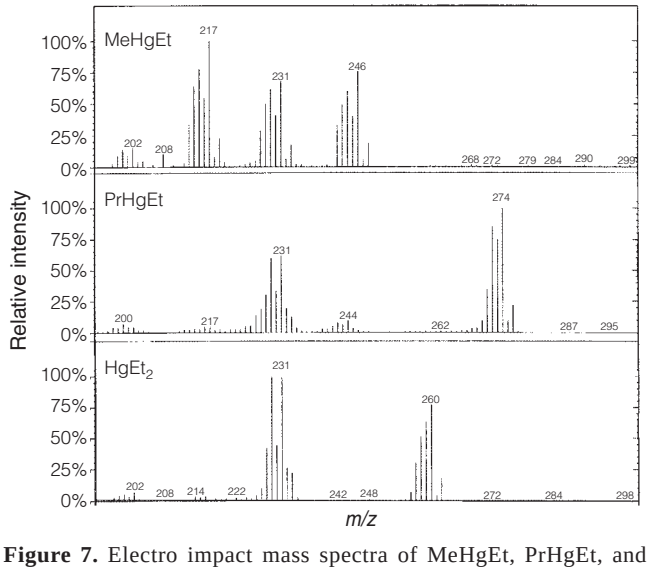

Figure 7. Electro impact mass spectra of MeHgEt, PrHgEt, and $HgEt_2$ obtained at 70 eV. GC-ITMS conditions see Table 1.

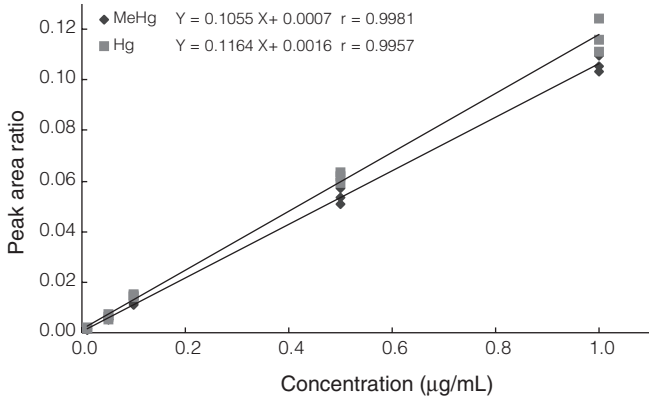

Figure 8. Calibration curves of methyl- and inorganic mercury (propylmercury as internal standard).

Table 2. Analysis of methylmercury contents from various reference materials

Reference material	Result* (μg/g)	Certified value (μg/g)
Dogfish muscle (NRC DORM-2)	4.40 ± 0.34	4.47 ± 0.32
Dogfish liver (NRC DOLT-2)	0.663 ± 0.061	0.693 ± 0.053
Tuna fish (BCR CRM 464)	5.31 ± 0.32	5.50 ± 0.17

*Mean ± S.D. (n = 3).

The developed method was validated by determination of three reference materials: CRM 464 tuna fish, DORM-2 dogfish muscle, and DOLT-2 dogfish liver. The tested methylmercury concentration was 5.31 ± 0.32, 4.40 ± 0.34 and 0.663 ± 0.061 μg/g for CRM 464 tuna fish, DORM-2 dogfish muscle, and DOLT-2 dogfish liver (Table 2), respectively. The obtained levels also agree with the certified values 5.50 ± 0.17 μg/g, 4.47 ± 0.32 μg/g and 0.693 ± 0.055 μg/g for CRM 464 tuna fish, DORM-2 dogfish muscle, and DOLT-2 dogfish liver, respectively. It indicates that the developed method was suitable to quantify methyl- and inorganic mercury in fish

CONCLUSION

A procedure for detecting methyl- and inorganic mercury in fish has been developed. Microwave-assisted digestion with TMAH solution, cupric ion addition, pH adjusting, derivatization with 1% $NaBEt_4$/KOH solution, *n*-heptane extraction, and GC/MS analysis were performed stepwise. Optimal condition for microwave-assisted digestion of mercury species from fish with TMAH was found to be 3.5 min at 15-30 W, 2.5 min at 45 W, or 1.5 min at 60-70W. Adding 20 mM cupric ion could perfectly release the mercury species. Optimal condition for ethylation with 1% $NaBEt_4$/KOH solution was adjusting pH to 5.0. The recoveries were 92.3–96.1% and 93.6–95.5% for methyl- and inorganic mercury, respectively. The combination of microwave-assisted digestion, aqueous phase derivatization and GC/MS analysis has resulted in a rapid, safe and accurate method for determining methyl- and inorganic mercury in fish.

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