

Determination of Methylmercury in Biological Samples Using Dithizone Extraction Method Followed by Purge & Trap GC-MS

Jung-Sub Lee, Yoon-Jung Ryu, Jae-Sung Park, Sung-Hwan Jeon, Sam-Cwan Kim, and Young-Hee Kim*

*Inorganics Analysis Research Division, National Institute of Environmental Research, Environmental Research Complex, Incheon 404-708, Korea. *E-mail: heek89@me.go.kr*

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In this study, a dithizone extraction technique involving purge & trap GC-MS was developed for the determination of methylmercury in biological samples, especially blood and fish. After alkaline digestion, methylmercury in biological samples was extracted into dithizone and back-extracted into aqueous sulfide solution. The extracted methylmercury was converted to the volatile ethyl derivative, purged and trapped onto a solid-phase collection medium, and then introduced into the GC-MS system. The determined MDLs of the established method were $0.9 \text{ ng}\cdot\text{g}^{-1}$ for biological samples and its accuracy and precision were found to be 93% and 3.8%, respectively. The method was validated by analysis of CRMs such as SRM 966, BCR 463 and IAEA 407 and all analytical results were within certified ranges with average RSDs of less than 6%. The analytical results of field-sampled fish also showed that the method can be successfully used as an alternative for commonly used distillation method followed by GC-CVAFS detection.

Key Words : Methylmercury, Dithizone extraction, Ethylation, Purge & Trap GC-MS

Introduction

Among many pollutants, mercury is of particular concern because of its toxicity and accumulative property through food chain.^{1,2} Especially methylmercury contamination in freshwater fish has been known as a problem in Europe and North America because fish consumption is the principal exposure route of methylmercury for human and fish-eating wildlife.^{3,4} Numerous investigations have been conducted to assess the health risks of prenatal exposure to methylmercury.⁵ Further, effects of chronic, low-level exposure to methylmercury, such as increased incidence of heart disease in men⁶ and delayed neurotoxicity⁷ have been recognized. Thus, there is growing need for a more simplified and popularized analytical method for the determination of methylmercury in clinical samples and fish. For methylmercury analysis, a succession of analytical stages is required.⁸ The main steps to speciate mercury are extraction, separation and mercury-specific detection. Coupled techniques including separation by GC or LC and detection by ECD, AAS, AFS and ICP-MS have been widely used.^{1,9-11} For methylmercury extraction a solvent extraction technique and a distillation technique are commonly used. However, the extraction of methylmercury from biological samples, especially in blood has been a difficult task because of severe matrix interferences. The solvent extraction method using toluene or dichloromethane generally showed low extraction efficiencies in certain matrix.¹² Additionally, the distillation technique has a drawback such as co-distillation of a large amount of volatile compounds and these volatile compounds transferred to the distillate can interfere with the ethylation reaction and/or deposit on the GC column leading to inaccurate determinations.¹³ Moreover, the distillation technique may not be feasible for every laboratory condition

as it requires specific distillation apparatus.

Thus, in this study, it was considered appropriate to develop the accurate and simplified methylmercury analytical method using popularized analytical instrument such as purge & trap GC-MS. The GC-MS detection system was combined with dithizone extraction method, which has been successfully used to alleviate matrix interference problems in biological samples (*e.g.*, blood and fish) and to improve extraction efficiencies by the complexation between dithizone and methylmercury.¹² This study showed that the method can be used as an alternative for a commonly used method such as sample distillation followed by CVAFS detection. Additionally, compared to GC-ECD detection, the alternative approach by MS SIM mode detection gives more accurate analytical results without overestimation of methylmercury by interference of impurities.

Experimental Section

Sample collection and preparation. From June to September 2006, 57 freshwater fish samples were collected from the reservoirs and streams in Korea (Figure 1). The fillet of fish samples were cut into small pieces with dissection scissors and homogenized to a pastry state. The samples were kept frozen until further analysis.

Experimental materials and apparatus. All reagents used were of ACS grade and all water was used as doubly distilled and de-ionized water obtained from Barnsted UC/A56220-8 (Iowa, USA). Methylmercury standard stock solution ($1 \text{ mg}\cdot\text{mL}^{-1}$) was prepared by dissolving the appropriate amount of CH_3HgCl (Aldrich, MO, USA) in toluene. Purified 0.02% dithizone solution was prepared by dissolving 0.011 g of diphenylthiocarbazone in 100 mL toluene. Alkaline sodium sulfide stock solution was prepared by

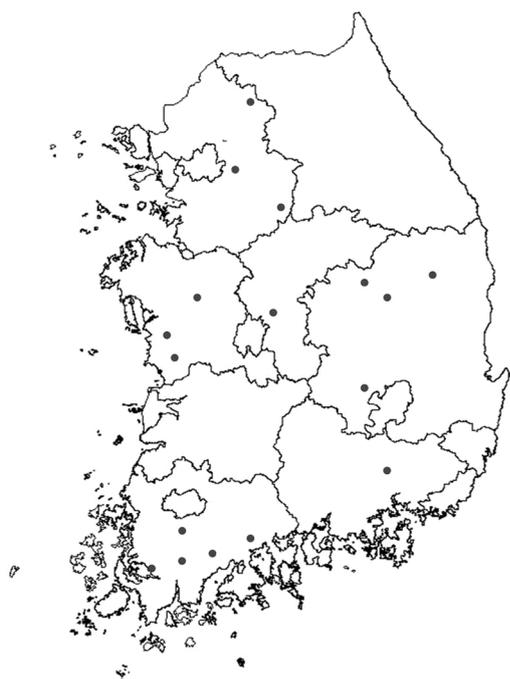


Figure 1. Map of sampling locations.

dissolving 0.15 g of $\text{Na}_2\text{S}_9\text{H}_2\text{O}$ in 10 mL of distilled water. At each use, 0.1 mL of stock solution was diluted with 50 mL of 0.1 N NaOH and 50 mL of ethanol. Walpole's buffer was prepared by mixing 200 mL of 1 M CH_3COONa and about 200 mL of 1 N HCl to adjust to pH 3.0. Sodium acetate buffer (0.2 M) was prepared by dissolving 1.64 g of CH_3COONa in distilled water and added with acetic acid to adjust pH at 4.9. Ethylating reagent, 2% sodium tetraethylborate, was prepared by dissolving with 0.2 g of sodium tetraethylborate [$\text{NaB}(\text{C}_2\text{H}_5)_4$] powder in 10 mL of 1% W/V KOH solution and was kept in ice and darkness after preparation and throughout the analysis.

For purge & trap GC-MS method, the volatile methylmercury were concentrated and injected using Tekmar-Dohmann purge-and-trap (Mason, Ohio, USA) with a Tenax A trap (Suppelco, MO, USA) as adsorbent trap. The sample was purged with helium at $40 \text{ mL}\cdot\text{min}^{-1}$ during 15 min at 40°C and followed by desorption at 200°C for 3 min. Chromatographic analysis was performed with Agilent 6890N GC (CA, USA) equipped with Agilent 5973N MS operating in selected ion monitoring (SIM) mode. The DB-5 MS capillary column (5% phenyl-95% dimethylpolysiloxane; $30 \text{ m} \times 0.5 \text{ mm I.D.}$, $0.25 \mu\text{m}$) was used with helium as carrier gas at a flow rate of $1 \text{ mL}\cdot\text{min}^{-1}$. The column temperature was programmed as follows: 40°C for 4 min, increasing to 280°C at $15^\circ\text{C}\cdot\text{min}^{-1}$ then holding for 5 min. The injection port and detector were operated at 220°C and 230°C , respectively.

Methylmercury analysis using GC-ECD was carried out by GC-2010 model from Shimadzu Co. (Kyoto, Japan) fitted with Hg-20A (GL-Science Co., Tokyo, Japan) packed glass column ($1 \text{ m} \times 3.0 \text{ mm}$). The column temperature was kept at 155°C . The injector and detector temperature were set at

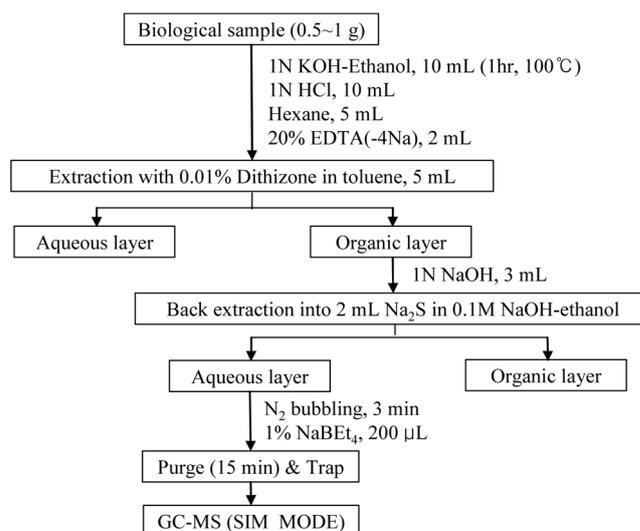


Figure 2. Schematic flowchart of the purge & trap GC-MS method for the analysis of methylmercury in biological samples.

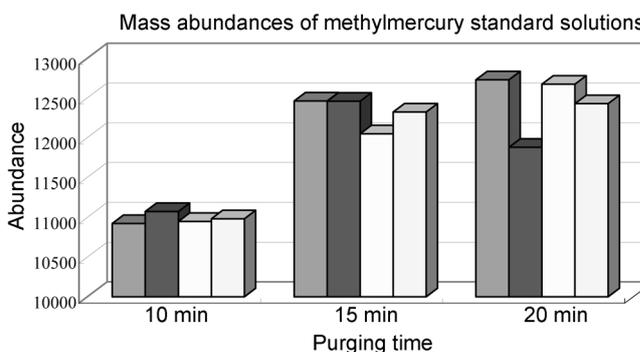


Figure 3. Differences between the analytical signals corresponding to different purging times in purge & trap GC-MS.

180°C and 200°C . Nitrogen gas was used for carrier gas with the flow rate of $40 \text{ mL}\cdot\text{min}^{-1}$. Total mercury analysis was performed using mercury analyzer SP-3DS model from Nippon Co. (Tokyo, Japan). Purified dry air was used for carrier gas with flow rate of $0.5 \text{ L}\cdot\text{min}^{-1}$. The temperature of combustion tube was raised from 250°C to 850°C for 10 min and gold amalgam adsorption temperature was kept at 120°C for 10 min and at 850°C for 1-2 min.

Determination of methylmercury by the purge & trap GC-MS method. Approximately 0.5-1 g of fish or blood sample and 10 mL of 1 N KOH-ethanol solution were placed in a 40-mL screw capped conical centrifuge tube and heated at 100°C for 1 hour. After cooling to room temperature, 10 mL of 1 N HCl was added followed by washing with 5 mL of n-hexane, and then, 2 mL of 20% EDTA-4Na solution was added into the extracted aqueous phase to mask other metal ions contained in the samples. To extract methylmercury, 5 mL of purified 0.01% dithizone-toluene was added and the aqueous phase was discarded. The remaining excess dithizone in toluene phase was removed by washing with 5 mL of 1 N NaOH. A fixed volume of the toluene phase (7 mL) was transferred into 10 mL-centrifuged tube

with glass stopper and 2 mL of Na₂S solution was added to back-extract the methylmercury into aqueous phase, followed by centrifuging at 1,200 rpm for 3 minutes and discarding toluene phase. The solution was acidified with 3-5 drop of 1 N HCl and aerated with N₂ at 50 mL·min⁻¹ for 3 minutes to expel the excess sulfide ions. Lastly, 0.1 mL (0.05-1 mL) of the aerated solution was added into 10 mL of distilled water and 5 mL of sodium acetate buffer in a 20 mL-syringe, followed by adding 0.2 mL of sodium tetraethylborate solution. Blanks and standard solutions for a calibration curve were treated in a similar manner.

The combined solution in the syringe was injected into the sparser connected on the purge & trap sampler. During MS detection, the following ions were monitored using SIM mode: m/z 202, 217, 246 for CH₃HgC₂H₅; m/z 202, 231, 260 for Hg(C₂H₅)₂. Between two consecutive analyses, the distilled water was analyzed in order to clean the system and eliminate carryover effects. For Quality Control (QC) purpose, CRMs of IAEA 407 (IAEA, Vienna, Austria) and BCR 463 (ERM, Brussels, Belgium) for fish and Standard Reference Material (SRM) 966 (NIST, MD, USA) for blood were analyzed. The commercially available blood samples were obtained from Centre de Toxicologie du Québec (Québec, Canada). The corrections to dry mass of CRMs (for IAEA 407 and BCR 463) were made from a moisture determination of 100 mg of CRMs which were dried in an oven at 102°C for 3 hours and kept in dark desiccators over 3 days.

Determination of methylmercury by the GC-ECD method. The dithizone extraction/GC-ECD method has been previously reported¹² and used as a control method to compare the results obtained by the newly adopted GC-MS method. The dithizone extraction and clean-up procedures of GC-ECD method were conducted in a similar way to the GC-MS method. The aqueous solution treated with Na₂S solution was added with 2 mL of Walpole's buffer, re-extracted with 0.5 mL of dithizone-toluene and washed with 3 mL of 1 N NaOH. The extracted toluene solution with 2 drops of 1 N HCl was used as an analysis solution for GC-ECD. However, the method needs extra precaution for preventing contamination by impurities from glassware, solvents and oxidized dithizone, which caused interfering peaks on the chromatogram and induced an overestimation of methylmercury concentration.

Results and Discussion

Digestion and extraction procedures. For analysis of biological samples, the samples were digested with KOH/CH₃OH at 100°C and were extracted by dithizone-toluene solution. The hot alkaline digestion was the most efficient pretreatment method for biological samples, especially for blood samples since the digests did not form any emulsion on solvent extraction, due to the breakdown of proteinacious materials in the sample matrix during digestion.¹² The dithizone extraction process and clean-up process by Na₂S were required to remove the interferences in the digests and to

Table 1. Determination of methylmercury in SRM 966 and commercially available blood materials

Materials	Methylmercury Concentrations (ng·g ⁻¹)			
	Certified Value	Determined Value	RSD (%)	Recovery (%)
SRM 966 (n = 5)	16.4 ± 1.4	16.6 ± 1.6	4.9	93-105
M 0605 (n = 3)	7.1* (4.6-9.5)	5.8 ± 0.8	3.3	86-93
M 0618 (n = 3)	26.3* (20.0-32.3)	23.2 ± 1.6	6.4	79-91

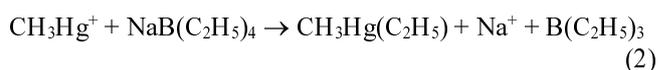
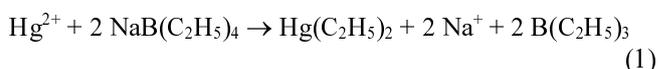
*Data from the total mercury analysis and the materials were spiked with methylmercury.

Table 2. Determination of methylmercury concentrations in CRMs by the purge & trap GC-MS method and the dithizone-extraction/GC-ECD method

CRMs	Methylmercury Concentrations (μg·g ⁻¹)				
	Certified Value	Determined Value		RSD (%)	Recovery (%)
IAEA 407 (n = 7)	0.20 ± 0.012	GC-MS	0.19 ± 0.016	3.9	85-95
		GC-ECD	0.20 ± 0.022	5.7	92-101
BCR 463 (n = 7)	2.83 ± 0.16	GC-MS	2.89 ± 0.26	4.3	98-108
		GC-ECD	2.76 ± 0.32	5.9	91-107

improve the extraction efficiency by metal-ligand complexation. Subsequently, efficient recoveries of methylmercury from CRM analysis were obtained (See Table 1 and Table 2).

Optimization of purge & trap GC-MS method. Due to polarity of methylmercury compound, adsorption processes of methylmercury occur on the stationary phase during the chromatographic analysis, causing peak broadening and ghost peaks.¹⁴ Polar methylmercury compounds are needed to convert into nonpolar methylmercury compounds before chromatographic separation. In this study, sodium tetraethylborate, NaBEt₄, was used for the derivatization of polar methylmercury compounds to nonpolar ethylated methylmercury compounds. The relative reaction equations are as follows¹⁵:



After the derivatization reaction, the analytes are purged with helium at 40 mL·min⁻¹ to adsorb methylmercury on the trap. In order to obtain an optimum condition for the purging time, the changes of analytical signals were examined using methylmercury standard solutions. As shown in Figure 4, the most consistent sensitivity was obtained from 15 minutes of purging. The concentrated methylmercury in Tenax trap was introduced to GC-MS and was analyzed by using selected ion monitoring (SIM) mode. In the SIM mode the ions of m/z 202, 217, 246 were monitored for CH₃HgC₂H₅

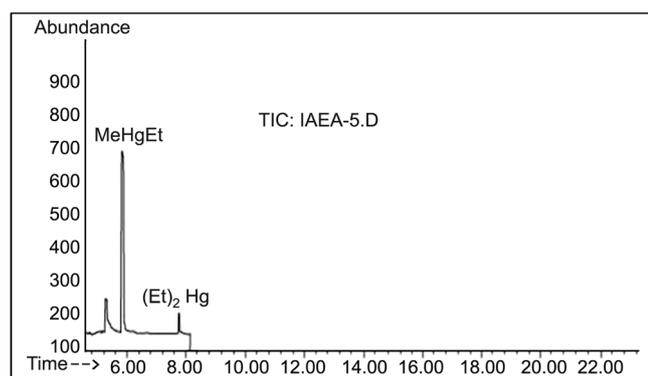


Figure 4. GC chromatogram obtained from CRM IAEA 407 sample by the purge & trap GC-MS method.

and m/z 202, 231, 260 for $\text{Hg}(\text{C}_2\text{H}_5)_2$. The spectrum in Figure 4 is the GC chromatogram of CRM IAEA 407 sample, showing $\text{CH}_3\text{HgC}_2\text{H}_5$ and $\text{Hg}(\text{C}_2\text{H}_5)_2$ peaks which are contributed from CH_3Hg^+ and Hg^{2+} in the sample.

Calibration curve & detection limit. The calibration curve was evaluated in the range from 0.1 to 5 ng (as Hg) and obtained with a determination coefficient, $r^2 > 0.995$ and less than 7% of RSD of calibration factors. The method detection limit was defined as the concentration equivalent to three times standard deviation of concentrations of spiked methylmercury solutions and was found to be $0.9 \text{ ng}\cdot\text{g}^{-1}$ for biological samples. Accuracy and precision of the method, expressed as recovery rates and its RSD of spiked solutions were 93% and 3.8%, respectively.

Analysis of blood samples. The accuracy of the analytical method for methylmercury in blood was evaluating by analysis of CRM, SRM 966. As shown in Table 1, obtained results of SRM analysis were $16.6 \pm 1.6 \text{ ng}\cdot\text{g}^{-1}$ (95% confidence interval with $n = 5$), which were within the certified range and the average RSD was 4.9%. High and low concentrations of commercially available blood samples were also

analyzed. Although not certified for their methylmercury concentrations, these material was expected to be available for methylmercury analysis because they were spiked with methylmercury and the concentration of methylmercury derived from animal was very low. The analysis results were within the ranges with a good precision and confirmed that the developed P&T GC-MS can be applied for the analysis of methylmercury in blood.

Analysis of fish samples. The accuracy of the method for the analysis of fish samples was also evaluated by analyzing methylmercury concentration in different fish certified reference materials. The results and comparison between the GC-MS and GC-ECD methods are summarized in Table 2. As shown in Table 2, the amounts of methylmercury are $0.19 \pm 0.016 \text{ }\mu\text{g}\cdot\text{g}^{-1}$ for CRM IAEA 407 (95% confidence interval with $n = 7$) and $2.89 \pm 0.26 \text{ }\mu\text{g}\cdot\text{g}^{-1}$ for CRM BCR 463 (95% confidence interval with $n = 7$), which were in good agreement with the certified values. With the GC-MS method, the methylmercury recoveries ranged from 85-108% with RSD of less than 5%. The results suggested that the GC-MS method could successfully used for the determination of methylmercury in biological samples.

Further, the performance of the GC-MS method was tested on various fish samples. Results of total mercury and methylmercury analysis in freshwater fish are given in Table 3. The analysis results of methylmercury concentration by the GC-MS method were compared with those of the GC-ECD method. The ratios of methylmercury concentrations between the methods were in the range of 0.69-1.13, showed two methods were in good agreement.

Total mercury concentrations in fish were in the range of $20.4\text{-}454 \text{ ng}\cdot\text{g}^{-1}$ (mean $175.1 \text{ ng}\cdot\text{g}^{-1}$) and methylmercury concentrations were in the range of $12.9\text{-}424 \text{ ng}\cdot\text{g}^{-1}$ (mean $143.2 \text{ ng}\cdot\text{g}^{-1}$) The proportion of methylmercury to total mercury in all fish samples was in the range of 69.1-103.5% (mean 86.5%) indicating that majority of the total mercury in fish is in the form of methylmercury. This result was in

Table 3. Comparison of total mercury and methylmercury concentrations ($\text{ng}\cdot\text{g}^{-1}$) in freshwater fish

Species	No of sample	T-Hg	MeHg [GC-MS]	MeHg [GC-ECD]	% MeHg [GC-MS]	MS/ECD (ratio)
Mandarin fish	2	413.1 ± 57.8	219.0 ± 45.7	330.3 ± 137.1	53	0.69
Korean piscivorous chub	5	357.9 ± 75.7	254.2 ± 68.2	269.3 ± 73.9	86	0.95
Skin carp	4	220.4 ± 90.3	206.1 ± 159.9	$194.5.1 \pm 95.4$	88	0.99
Catfish	7	216.1 ± 106.2	140.8 ± 82.3	188.2 ± 139.4	68	0.77
Skygager	6	191.8 ± 117.6	175.7 ± 118.7	162.1 ± 97.2	90	1.11
Sharpbelly	1	153.4	77.0	83.7	50	0.91
Northern snake head	6	136.5 ± 62.4	102.3 ± 71.7	101.0 ± 73.5	69	1.01
Largemouth bass	9	116.6 ± 58.8	89.8 ± 53.3	90.7 ± 45.5	84	1.12
Carssius cuvieri	1	151.8	125.1	128.6	82	0.97
Crusian carp	2	59.9 ± 3.0	42.9 ± 0.7	39.0 ± 6.3	74	1.10
Common carp	11	49.2 ± 34.4	50.3 ± 41.1	47.2 ± 37.9	69	1.13
Leather carp	2	35.1 ± 16.7	24.2 ± 10.2	24.8 ± 13.2	72	1.02
Japanese dace	1	183.16	141.4	139.8	77	0.99

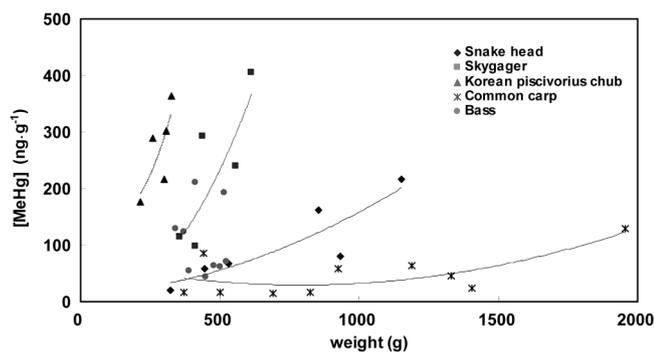


Figure 5. Correlations between methylmercury concentrations and fish weight of specific species.

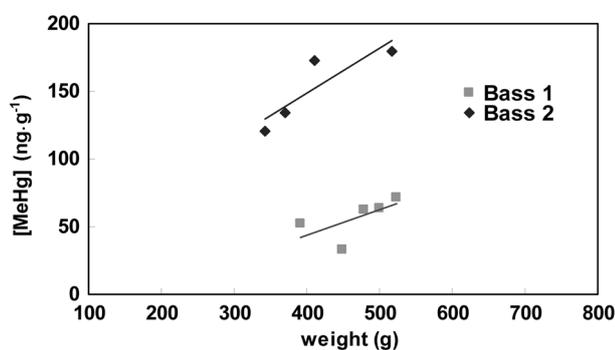


Figure 6. Correlations between methylmercury concentrations and fish weight of Largemouth bass.

good agreement with the previous studies that the mercury in predatory, freshwater fish is found exclusively as methylmercury.^{16,17} As shown in Table 3, relatively high methylmercury concentrations (216 to 424 $\text{ng}\cdot\text{g}^{-1}$) were found in predatory species such as Korean piscivorous chub, while lower concentrations (12.9 to 59.9 $\text{ng}\cdot\text{g}^{-1}$) were found in polyphagia species such as Common carp. Thus, the results clearly showed that methylmercury concentrations of freshwater fish increased with the trophic levels (food chain).

Generally, the methylmercury concentrations in fish are expected to be proportional to its size and weight while methylmercury bioaccumulation is a function of several factors such as uptake (diet) and elimination pathways (excretion, growth dilution).¹⁸ Overall, while methylmercury concentrations increased as fish weight increased, different species showed different patterns (Figure 5). It is interesting to note that Korean piscivorous chub showed statistically high methylmercury concentrations, while their body weight was much less than that of other species. Korean piscivorous chub are actually the top predator and long-lived fish with small body size. Thus, it is likely that Korean piscivorous chub can accumulate methylmercury over their life span with minimal growth dilution, resulting in high methylmercury body burden. As seen in Table 4, methylmercury concentration was significantly correlated ($p < 0.05$) with fish body weight except largemouth bass. Largemouth bass are carnivores and their food preference is crayfish, minnows, and frogs.^{19,20} Despite the small number of samples,

Table 4. The Correlation coefficients of methylmercury concentrations in freshwater species against fish weight

Species	Correlation coefficient
Northern snake head	0.88
Skygager	0.76
Korean piscivorous chub	0.76
Largemouth bass	-0.16
Common carp	0.58

the relationship between methylmercury concentration and body weight were divided into two groups. As seen in Figure 6, the result clearly showed a distinct pattern that methylmercury body burden was much higher in Bass 2, compared to Bass 1, even though their body weights were comparable. Additionally, two groups were collected from different locations, *i.e.* Bass 1 from Ju-Nam reservoir and Bass 2 from Dam-Yang artificial reservoir, which might imply the difference of food availability, methylmercury concentrations in the prey and water chemistry (dissolved methylmercury and dissolved organic carbon).¹⁸

Conclusion

This study showed that the purge & trap GC-MS method provided a reliable measurement of methylmercury in blood and fish samples and was successfully applied to methylmercury analysis in field-sampled fish. Methylmercury concentrations in freshwater fish were found to be correlated with body weight, diet habit and food availability. The current study is preliminary and much more in-depth studies are required in the future to examine and assess important factors controlling methylmercury accumulation in fish and human. In addition, long-term monitoring plans including for not only fish but also water column parameters should be established since mercury level of blood in Korea, (investigated by Korea Ministry of Environment in 2005) was much higher than those in other countries and fish consumption is the major route of methylmercury to human.

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