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Assessment of methylmercury exposure from seafood consumption

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2017

Thesis Acknowledgements

I would like to thank all the people who contributed to the work described in this thesis. First and foremost, I thank my academic advisors for their kind suggestions to support my work:

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Co- supervisor: Dr. Mineshi Sakamoto
Department of Environmental Science and Epidemiology, National Institute for Minamata Disease

I would like to thank Dr. Minoru Koga (Minamata Environmental Academia, former president of Prefectural University of Kumamoto) and Dr. Atsuhiro Nakano (former department director of Basic Medical Science, National Institute for Minamata Disease) for their guidance and encouragement. I also thank Ms. Hazuki Kashiwagi and Megumi Kudo (National Institute for Minamata Disease) for their technical assistance.

All of my co-authors and colleagues at the National Institute for Minamata Disease, Japan; Graduate School of Environmental and Symbiotic Science, Prefectural University of Kumamoto, Japan; Department of Epidemiology and Preventive Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Japan; National Hospital of Dermatology and Venerology, Vietnam.

I am grateful for the support by the International Postgraduate Scholarship for Research on Mecury from the Prefecture University of Kumamoto, and a grant from the National Institute for Minamata Disease and Kagoshima University.

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Summary

Methylmercury (MeHg) is a well-known environmental neurotoxicant that is absorbed from the gastrointestinal tract and crosses the blood–brain and blood–placental barriers. People are exposed to MeHg mainly through the consumption of seafood; therefore, determining the concentration of MeHg in seafood is important for assessing the risks of MeHg exposure.

For routine analysis of MeHg concentration in seafood, easy and low-cost method has been required. Therefore, we first tried to develop a simple analysis of MeHg in biological samples such as seafood. We developed a simpler method for determining the total mercury (T-Hg) and MeHg concentrations in common biological samples by using MIBK in the degreasing step. This method will be useful for the routine analysis of T-Hg and MeHg in a large number of biological samples such as the tissues of seafood.

Next, this method was applied to determine T-Hg and MeHg in imported and domestic-produced commercial shrimp in Kumamoto and Kagoshima prefecture to obtain information for assessing the risk of MeHg exposure. The T-Hg and MeHg levels in imported and domestically-produced commercial shrimp in Japan were lower than the Japanese regulation level of 300 ng/g for MeHg in fish. In addition, the average selenium/mercury (Se/Hg) molar ratios in the muscles of commercial shrimp were comparatively high in the range of 16–106. Consequently, the shrimp commercially available in Japan will not cause a risk of MeHg exposure to Japanese consumers.

Recently, fish consumption is increasing in Vietnam. However, little information is available on estimating the health risk of MeHg exposure through fish consumption in Vietnam. The association between Hg levels in hair and Se in toenails of 196 Vietnamese people and their fish consumption, using a dietary questionnaire to obtain information pertinent for assessing health risk owing to MeHg exposure was examined. This study has demonstrated that Hg levels in the hair of 98% of the Vietnamese participants were lower than the PTWI for MeHg at 1.6 μg Hg/kg body weight, with an uncertainty factor of 6.4 (Joint FAO/WHO Expert Committee on Food Additives, 2003). A significant difference in the age-adjusted geometric mean of Hg levels in hair from females was related to the frequency of freshwater fish consumption. In addition, Hg levels in hair and Se levels in toenails increased with the increased frequency of marine fish consumption, and showed a significant positive correlation among subjects who consumed marine fish ≥ once/week. This is the first cross-sectional study to investigate the association between hair Hg levels and fish consumption in Vietnam. These findings provide valuable information for future assessments of the health risk of MeHg exposure through fish consumption in Vietnam.

Chapter I. Simple analysis of total mercury and methylmercury in seafood using heating vaporization atomic absorption spectrometry

1.1. Abstract

This study aimed to develop a simpler method for determining total mercury (T-Hg) and methylmercury (MeHg) in biological samples by using methyl isobutyl ketone (MIBK) in the degreasing step. The fat in the samples was extracted by MIBK to the upper phase. T-Hg transferred into the water phase. This was followed by the extraction of MeHg from the water phase using HBr, $CuCl_2$, and toluene. The MeHg fraction was reverse-extracted into L-cysteine-sodium acetate solution from toluene. The concentrations of T-Hg and MeHg were determined by heating vaporization atomic absorption spectrometry. Certified reference materials for T-Hg and MeHg in hair and fish were accurately measured using this method. This method was then applied to determined T-Hg and MeHg concentrations in the muscle, liver and gonads of seafood for the risk assessment of MeHg exposure. The mean T-Hg and MeHg concentrations in squid eggs were 0.023 and 0.022 μg/g, and in squid nidamental glands 0.052 and 0.049 μg/g, respectively. The MeHg/T-Hg ratios in the eggs and nidamental glands of squid were 94.4% and 96.5%, respectively. The mean T-Hg and MeHg concentrations in the gonads of sea urchins were 0.043 and 0.001 μg/g, respectively, with a MeHg/T-Hg ratio of 3.5%. We developed an efficient analytical method for T-Hg and MeHg using MIBK in the degreasing step. The new information on MeHg concentration and MeHg/T-Hg ratios in the egg or nidamental glands of squid and gonads of sea urchin will also be useful for risk assessment of mercury (Hg) in seafood.

1.2. Introduction

Human beings are exposed to MeHg mainly through the consumption of seafood; therefore, determining the concentration of MeHg in seafood is important for assessing the risks of MeHg exposure (World Health Organization, 2008). MeHg is readily absorbed from the gastrointestinal tract and capable of crossing the blood-brain barrier and/or the blood-placental barrier (World Health Organization, 2010). Mercuric toxicity is known to vary according to its chemical form (World Health Organization, 2008). Determining the chemical form and the ratio of mercury compounds in tissues is, thus, also important for understanding the behavior of MeHg and its toxicity *in vivo*.

In many studies, the T-Hg content of biological samples has been commonly analyzed using the atomic absorption method and MeHg by methods such as gas chromatography-electron capture detector (GC-ECD). In this case, it is necessary to prepare two sets of apparatus and two samples for T-Hg and MeHg analysis. One method developed for the selective analysis of inorganic mercury (InHg) and MeHg used reductive vaporization atomic absorption spectrometry (Magos and Clarkson, 1972). This was under the premise that MeHg was the predominant chemical form of organic mercury in most biological samples such as seafood and animal tissues. This experimental procedure was simpler than using gas chromatography. Organic mercury in natural biological samples can be assumed to be MeHg, because only MeHg has been detected in natural biological samples, with the exception of human specimens treated with vaccines containing sodium ethylmercuric thiosalicylate. The method of Magos and Clarkson (1972) has, thus, been modified to become an analytical method for T-Hg and MeHg in biological materials using heating vaporization atomic absorption spectrometry (Miyamoto et al., 2010). In contrast to the reducing-vaporization method, the heating vaporization atomic absorption spectrometry method does not need complex pretreatment of samples, and large volumes of waste liquid such as $SnCl_2$ are not produced. The advantages of this method are that: (1) a single apparatus (heating vaporization atomic absorption spectrometry) is used for both T-Hg and MeHg determination; (2) both T-Hg and MeHg can be measured using the same biological sample in two consecutive steps; (3) only one standard solution needs to be prepared for T-Hg and MeHg in each experiment. Both T-Hg and MeHg can be analyzed using a commercial mercury standard solution (1,000-ppm $HgCl_2$), which has the stability required for a calibration curve; and (4)

the protocol is easy and cost-effective compared with other methods such as gas chromatography-inductively coupled plasma/mass spectrometry or liquid chromatography-inductively coupled plasma/mass spectrometry. These methods require expensive apparatus and conditions (e.g. carrier gas) and higher levels of experimental skills (Rodrigues et al., 2010; Clémens et al., 2012). Thus, there are many limitations to conducting assessments of Hg exposure using these assay systems, especially in developing countries.

In our previous method, chloroform was used to eliminate the fat that inhibited the extraction of the MeHg fraction, because it has a high degreasing ability (Miyamoto et al., 2010). The separated lower chloroform layer was removed using a micro-syringe. However, the boundary between the aqueous layer containing mercury and the chloroform layer containing lipid was not clear. If the upper organic solvent layer containing fat can be eliminated by an aspirator during the degreasing step, the analytical procedure can be expected to be simpler and faster for many routine sample treatments.

1.3. Objective

In the present study, we aim to investigate replacing chloroform with MIBK in the degreasing step. MIBK may make it easier to remove fat using an aspirator because its specific gravity is lower than that of aqueous solutions. It also solubilizes fat components enough to allow the upper phase to separate from the water phase. Furthermore, MIBK has already been used for atomic absorption spectrometry owing to its lower level of contamination by metals (List et al., 1971; Cabrera-Vique et al., 2012; El-Mufleh et al., 2013).

We will apply this modified method to determine the levels of T-Hg and MeHg in tissues of five species of seafood. Although many studies have investigated the concentrations of mercury in edible muscle (Ministry of Health Labour and Welfare, 2010), little information is available concerning mercury levels, especially MeHg, in seafood such as gonads, despite being commonly consumed (Joiris & Holsbeek, 1999; Hammerschmidt et al., 1999; Seixas et al., 2005; Yamashita et al., 2005; Webb et al., 2006; Donald & Sardella, 2010; Atta et al., 2012; Watanabe et al., 2012). In the present study, we will determine the concentration of T-Hg and MeHg in the muscle, liver and gonads of several commercial fish, squid and sea urchins using the currently modified method to obtain information for assessing the risk of MeHg exposure.

1.4. Materials and methods

Reagents

Analytical grade reagents were used in this study. Five M sodium hydroxide (NaOH) solution, hydrogen bromide (HBr), copper (II) chloride dehydrate ($CuCl_2$), nitric acid (HNO_3), methyl isobutyl ketone (MIBK: atomic absorption spectrometry grade), hexane (HPLC analysis grade), toluene (HPLC analysis grade) and L-cysteine (Cys) were perchased from the Kanto Chemical Co. (Tokyo, Japan). Methylmercury chloride (MeHgCl; >98% purity) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Sodium acetate trihydrate (NaOAc: Emsure grade) was purchased from Merck (Darmstadt, Germany). The Hg standard solution (Hg 1,000 mg/L, $HgCl_2$ in 0.1-mol/L HNO_3) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Certified reference materials for MeHg were obtained: hair (NIES CRM No.13) from the National Institute of Environmental Studies (Tsukuba, Japan), cod fish tissue (NMIJ CRM 7402-a No.250) and swordfish tissues (NMIJ CRM 7403-a No.165) from the National Institute of Advanced Industrial Science and Technology (Tsukuba, Japan). Ultra-pure quality water was prepared using a Milli-Q system (Merck Millipore, Tokyo, Japan). Cys (0.1%) solution and Cys (0.2%)-NaOAc (2%) solution were freshly prepared for each experiment.

Fish sample

Fresh marine organisms (red seabream (Pagrus major), n=10; golden threadfin bream (Nemipterus virgatus), n=10; cutlass fish (Trichiurus lepturus), n=10; bigfin reef squid (Sepioteuthis lessoniana),

n=8; and sea urchin (Asthenosoma ijimai), n=6) were purchased in the fish market of Kagoshima, Japan. These marine organisms with gonads were chosen because at least six individuals were available in the fish market at the time. Each marine organism was weighed then dissected to obtain the muscle, liver, testis, ovary, egg and nidamental gland. Photographs of the egg and nidamental gland in bigfin reef squid are shown in Fig. 1-1. Samples (0.6-1.5 g: wet weight) were transferred to 15-mL polypropylene (PP) tubes and were kept at -80^{o}C until use.

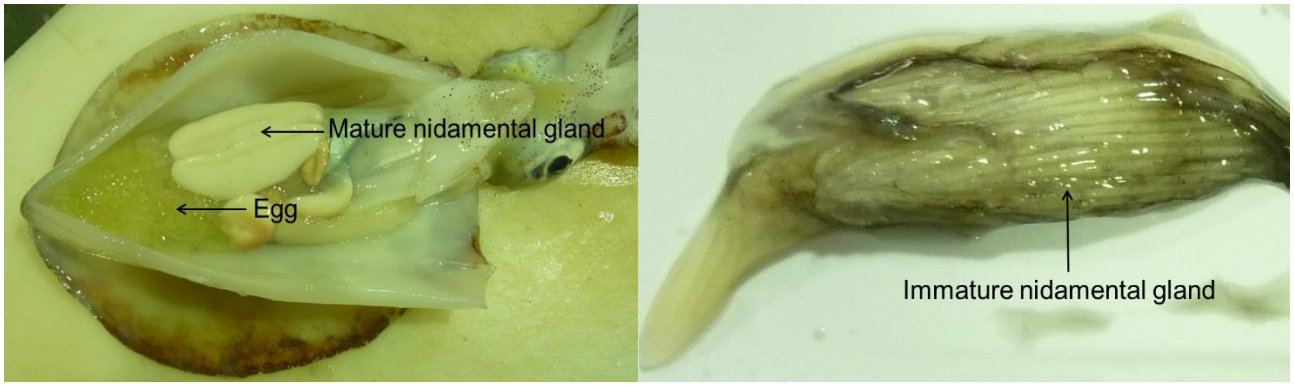

Fig. 1-1. Egg and nidamental gland of bigfin reef squid. Arrows indicate the egg and mature nidamental gland in the left panel and the immature nidamental gland in the right panel.

Extraction of total mercury and methylmercury in biological samples

The basic protocols for extracting T-Hg (Step-1) and MeHg (Step-2) are summarized in Fig. 1-2.

Step-1: 0.1% Cys solution (1 mL) and 5 M NaOH (1 mL) were added to samples (0.6-1.5 g: wet) in 15-mL PP tubes then incubated in a block incubator at 80^{o}C for 1-2 hr with vortexing every 15 min. The resulting degenerated solutions were cooled to room temperature in a water bath and their volumes made up to 5 mL with distilled water (DW). MIBK (6 mL) was added to the solubilized sample solutions to degrease them, followed by vigorous shaking using an SR-1 reciprocal shaker set at 220 rpm for 10 min (Taitec Corp., Nishikata, Japan) and centrifugation (1750 x g, 10 min). After the upper MIBK layer was separated, the remaining solubilized solution was washed with hexane (5 mL) and the hexane layer discarded after shaking (5 min) and centrifugation (5 min).

If the organic and aqueous phases did not separated clearly after MIBK treatment because of biological components such as fat, an additional treatment was applied as follows: (1) The MIBK treatment was repeated twice. (2) During the second treatment with MIBK, the PP tubes were incubated at 60^{o}C for 10-20 min. (3) The solutions were centrifuged to remove the MIBK layer and then treated with hexane, as above. In the present study, red seabream (muscle), golden threadfin bream (muscle) and cutlass fish (muscle, liver and ovary) were degreased using a single MIBK treatment; red seabream (liver) and bigfin reef squid (muscle, liver, egg, nidamental gland) using two MIBK treatments, and red seabream (testis and ovary), golden threadfin bream (liver and testis) and sea urchin (gonad) using two MIBK treatments plus a 60^{o}C treatment.

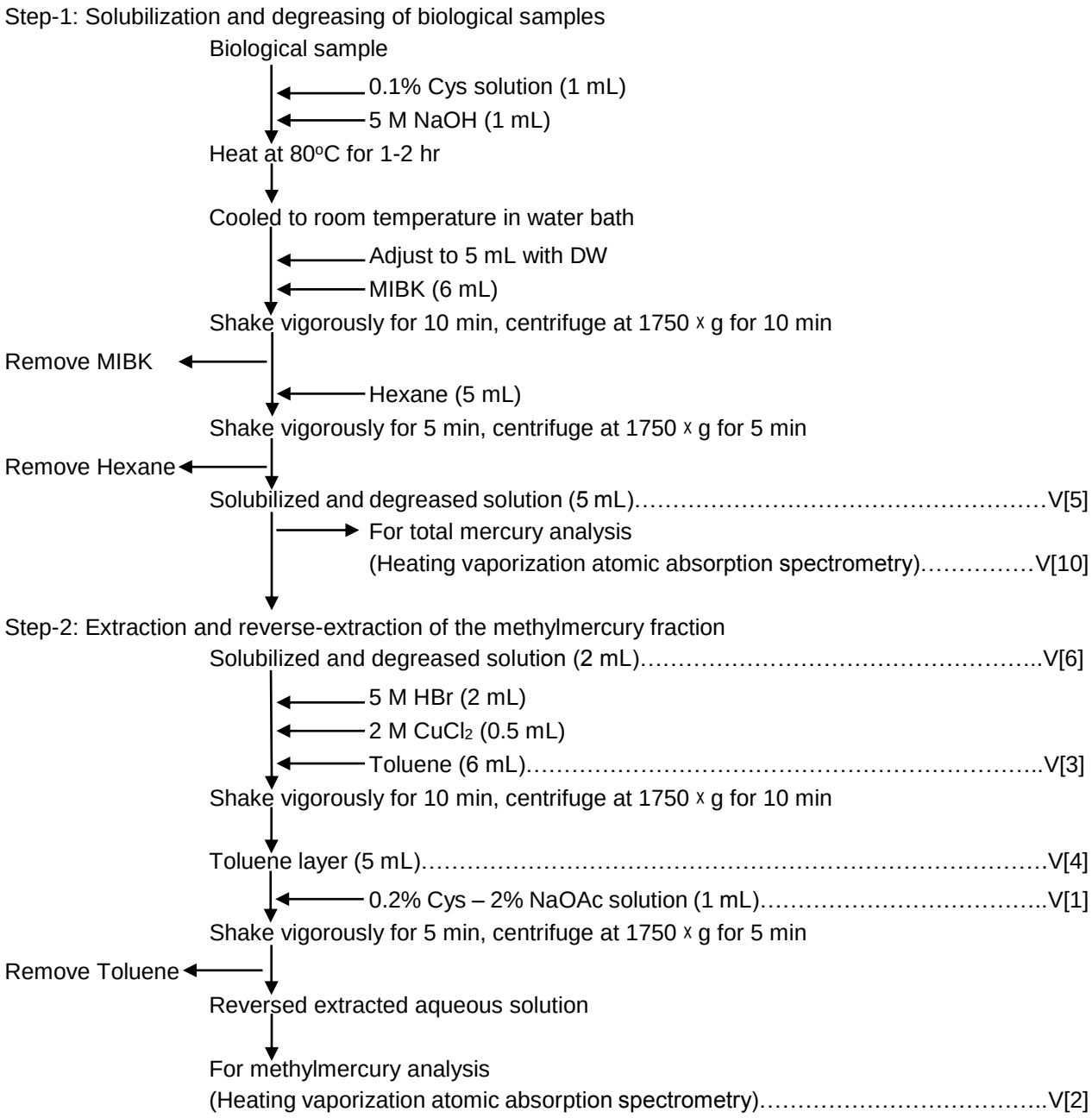

Fig. 1-2. Basic protocol for total mercury and methylmercury analysis. Definitions of V[1~6, 10] are shown in the materials and methods section.

Step-2: The solubilized sample solutions from Step-1 (2 mL) were transferred to new 15-mL PP tubes then 5 M HBr (2 mL), 2 M $CuCl_2$ (0.5 mL) and toluene (6 mL) were added in sequence, followed by vigorous shaking (10 min). After centrifuging the solution (10 min), the upper toluene layer (5 mL) was transferred to a new 15-mL PP tube. Cys (0.2%)-NaOAc (2%) solution was (1 mL) was then added to the toluene extract, followed by vigorous shaking (5 min) and centrifugation (5 min). After the toluene layer was removed using an aspirator, this reverse-extracted Cys (0.2%)-NaOAc (2%) solution was used for MeHg determination. Reagent blank test samples were prepared using the protocol above for each sample in at least duplicate.

Preparation of methylmercury standard solution (MSS) and methylmercury standard working solution (MSWS)

Standard solutions of MeHg were prepared as described previously (Miyamoto et al., 2010). Briefly, standard stock solutions of MeHg (1,000 mg Hg/L) were prepared using MeHgCl (0.125 g) in acetone (50 mL) and diluted using toluene (50 mL). Diluted MeHg standard solutions (MSS: 10 mg/L Hg) were prepared by adding toluene (9.9 mL) to the stock solution (0.1 mL). To remove any traces of InHg, the MSS (3 mL) was shaken with 1 M HBr (3 mL) at 220 rpm for 30 min. After centrifugation of this solution (1750 x g, 5 min), the upper toluene layer containing MSS (2 mL) was transferred to a new 15-mL PP tube. The MSS in toluene (2 mL) was reverse-extracted with Cys (0.2%)-NaOAc (2%) solution (2 mL) by shaking (220 rpm, 10 min), followed by centrifugation (1750 x g, 5 min) to prepare an aqueous MSS.

To prepare the MeHg stand working solution (MSWS: 0.1 mg Hg/L), 0.1% Cys solution (10.89 mL) was added to the centrifuged Cys-NaOAc solution of MSS (0.11 mL).

Recovery of methylmercury from solubilized methylmercury standard working solution and methylmercury-spiked biological sample

The recovery tests were based on previous work with little modification (Miyamoto et al., 2010). In the following formulae, ng is the measured mercury value and V the volume (in mL) of the specified solutions. The specified solutions were as follows: V[1], the total Cys-NaOAc solution used for reverse-extraction of MeHg (1 mL); V[2], the Cys-NaOAc solution containing the Cys-MeHg complex used for measurement; V[3], the total toluene used for extraction of MeHg (6 mL); V]4], the toluene used for reverse-extraction (5 mL); V[5], the total solubilized and degreased sample solution in Step-1 (5 mL); V[6], the solubilized and degreased sample solution used for extraction of MeHg (2 mL); V[7], the half-diluted MSWS used for recovery test (2.5 mL/5 mL); V[8], the total solubilized MSWS used for the recovery test (5 mL); V[9], the total solubilized MSWS used for measurement of the recovery test; and V[10], the solubilized solution used for T-Hg measurement. The sequence of use of the specified solutions (V[1~6, 10]) is shown in Fig. 1-2. The conversion factor for calculating μg from ng is 1/1000.

Recovery test for pure MeHg solution (R1)

MSWS (0.1 mg Hg/L) with any trace of InHg eliminated was used to test the recovery of pure MeHg solution. First, MSWS (2.5 mL; 0.1 mg Hg/L), 0.1% Cys solution (1 mL), 5 M NaOH (1 mL) and DW (0.5 mL) were mixed in 15-mL PP tubes and were solubilized using the same basic protocol as for T-Hg extraction described above (Step-1 in Fig. 1-2). The initial number of solution tubes was determined by the final number of tubes for comparing the variation in each experiment. The solubilized MSWS was mixed in 30-mL PP tubes for equalization of Hg concentration. Reagent blank solutions were prepared using the full amount of 0.1% Cys solution (3.5 mL) instead of MSWS and 5 M NaOH (1 mL) and DW (0.5 mL).

The solubilized MSWS was then divided into 15-mL PP tubes (2 mL/tube) with 1 M NaOH (3 mL) to adjust their alkalinity. These solutions (5 mL) were then extracted and reverse-extracted using the basic protocol for MeHg extraction (Step-1 and -2 in Fig. 1-2). The Hg concentrations in the Cys-NaOAc solutions obtained after extraction/reverse-extraction were compared with those in the solubilized MSWS without extraction/reverse-extraction.

The recovery of MeHg solution alone (R1) was calculated using the following formula:

$$R1\ (\%) = A/B \times 100,$$

where A was the amount of Hg in the solubilized MSWS after extraction/reverse-extraction:

$$A = (ng) \times (V[1]/V[2]) \times (V[3]/V[4]) \times (V[5]/V[6]) \times 1/1000,$$

where B was the amount of Hg in the solubilized MSWS without extraction/reverse-extraction:

$$B = (ng) \times (1/V[9]) \times (V[8]/V[7]) \times 1/1000.$$

Recovery test for MeHg-spiked biological sample (R2)

The MeHg-spiked sample homogenates were prepared as follows: sample (2 g of fresh shrimp muscle in this study), 0.1% Cys (2 mL), DW (2 mL) and 5 M NaOH (1.6 mL) were put into a 30-mL PP tube. The prepared solutions were solubilized and degreased by the basic protocol for T-Hg extraction then each lot of solubilized solutions was collected into one 50-mL PP tube to equalize the mercury concentration. The reagent blank solutions were prepared using the full amount of DW (4 mL) instead of the sample and 5 M NaOH (1.6 mL) and 0.1% Cys (2 mL).

The solubilized sample homogenate was then divided into 15-mL PP tubes (4 mL/tube) and mixed with solubilized MSWS (1 mL) or solubilized reagent blank solution (1 mL). These samples (5 mL each) were then extracted and reverse-extracted by the basic protocol for MeHg extraction.

The recovery of MeHg-spiked sample homogenates (R2) was calculated using the following formula:

$R2\ (\%) = [(C-D_{mean})/B_{mean}/2] \times 100$,

where C was the amount of Hg in the solubilized MSWS-spiked sample after extraction/reverse-extraction:

C= (ng) x (V[1]/V[2]) x (V[3]/V[4]) x (V[5]/V[6]) x (1/1000),

where D was the amount of Hg in the solubilized blank solution-added sample after extraction/reverse-extraction:

D= (ng) x (V[1]/V[2]) x (V[3]/V[4]) x (V[5]/V[6]) x (1/1000).

B was defined as described above.

Calculation of mercury concentration in samples

The concentrations of MeHg and T-Hg in samples were calculated using the following formula as described previously (Miyamoto et al., 2010):

T-Hg (μg/g or μg/mL) = (ng) x (V[5]/V[10]) x (1/1000) x (1/sample-amount in g or mL)

MeHg (μg/g or μg/mL) = (ng) x (V[1]/V[2]) x (V[3]/V[4]) x (V[5]/V[6]) x (100/R1) x (1/1000) x (1/sample-amount in g or mL)

Determination of mercury

The Hg content of the extracts were determined using direct thermal decomposition Hg analyzers SP-3D and MA-3000 (Nippon Instruments, Tokyo, Japan). The detection was based on cold-vapor atomic absorption spectroscopy at a wavelength of 253.7 nm. A standard Hg working solution for the calibration curve was prepared using the Hg standard solution (Hg 1,000 mg/L, $HgCl_2$ in 0.1-mol/L HNO_3) according to the manufacturer's protocol (Nippon Instruments).

Determination of moisture in certified reference materials

The moisture of the certified reference materials was determined after incubating at 85°C for 4 hr for hair (100 mg) and at 105°C for fish tissues (300 mg), according to the procedures from the certificates (National Institute of Advanced Industrial Science and Technology, 2009; 2010; National Institute for Environmental Studies, 2011).

Crosscheck of current method with GC-ECD method for methylmercury analysis

A crosscheck of MeHg concentration in the muscle and ovary of cutlass fish (two samples each) was conducted by Idea Consultants (Shizuoka, Japan) using the GC-ECD method published by the Ministry of the Environment (2004).

Statistical analysis

The Hg concentrations of the certified reference materials were expressed as the mean ± standard deviation (S.D.) and the values from the fish or invertebrate measured in the present study are expressed as the mean ± standard error (S.E.). The Spearman's correlation coefficient was calculated

to assess the correlation of MeHg levels between different organs and body weight using Stata version 13 (StataCorp LP, College Station, TX, USA). A two-sided *p* value of less than 0.05 was considered as statistically significant.

1.5. Results and discussion

In the present study, we have modified the degreasing step used in our previous study on MeHg analysis which used chloroform (Miyamoto et al., 2010), to produce a simpler and easier method using MIBK instead (Fig. 1-2). We conducted the recovery tests for MeHg solution alone and for MeHg-spiked fresh shrimp (Table 1-1).

Table 1-1. Recovery test for methylmercury standard working solution and a methylmercury-spiked biological sample

MeHg standard working solution		MeHg - spiked biological sample solution	
Recovery (%)	CV (%)	Recovery (%)	CV (%)
95.7 ± 0.9	0.9	96.5±0.3	0.3

Recovery of the methylmercury is expressed as the mean ± S.D. n=5

The results indicated that the recovery of MeHg from the MeHg solution alone and MeHg-spiked samples were 95.7 ± 0.9% and 96.5 ± 0.3%, respectively. We also determined the T-Hg and MeHg concentrations in the certified reference materials (cod fish, swordfish and hair) to check the recovery of Hg using this modified method (Table 1-2).

Table 1-2. Recovery test for total mercury and methylmercury from certified reference materials

	T-Hg		MeHg	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Cod fish (NMIJ, CRM 7402-a)	99.5 ± 0.5	0.5	95.1 ± 1.0	1
Swordfish (NMIJ, CRM 7403-a)	101.9 ± 0.7	0.7	95.3 ± 0.6	0.6
Hair (NIES, CRM No.13)	100.0 ± 0.3	0.7	94.6 ± 0.4	0.5
Average	100.5 ± 1.3	1.3	95.1 ± 0.5	0.5

Recovery of the total mercury and methylmercury are expressed as the mean ± S.D. n=5

The recovery of T-Hg from the cod fish, swordfish and hair standards and coefficients of variation (CV) were 99.5 ± 0.5%, 101.9 ± 0.7% and 100 ± 0.3%, and 0.5%, 0.7% and 0.7%, respectively, and for MeHg 95.1 ± 1%, 95.3 ± 0.6% and 94.6 ± 0.4%, and 1%, 0.6% and 0.5%, respectively. The mean values and S.D. were based on five samples. The mean MeHg recovery (95%) was consistent with the ratio in our original method using chloroform in the degreasing step (Miyamoto et al., 2010). Therefore, we used 95% as a correction factor for MeHg extraction of the biological samples in the further experiments.

The measured and certified values of T-Hg and MeHg in the certified reference materials are shown in Table 1-3.

Table 1-3. Values of total mercury and methylmercury in certified reference materials

	Measured value (μg/g)				Certified value (μg/g)	
	T-Hg	CV (%)	MeHg	CV (%)	T-Hg	MeHg
Cod fish (NMIJ, CRM 7402-a)	0.61 ± 0.003	0.5	0.58 ± 0.01	1.0	0.61 ± 0.02	0.58 ± 0.02
Swordfish (NMIJ, CRM 7403-a)	5.44 ± 0.04	0.7	5.02 ± 0.03	0.6	5.34 ± 0.14	5.00 ± 0.22
Hair (NIES, CRM No.13)	4.42 ± 0.03	0.8	3.78 ± 0.02	0.5	4.42 ± 0.20	3.80 ± 0.40

The mercury concentrations are expressed as the mean ± S.D. n=5

The mean values measured for T-Hg from the cod fish, swordfish and hair standard were 0.61, 5.44 and 4.42 μg/g dry weight, respectively, with CV values of less than 1% and for MeHg, 0.58, 5.02 and 3.78 μg/g, respectively, with CV values of less than 1%. All the measured values agreed well with the certified values for each certified reference material. We achieved these consistent results by accurately and precisely determining the sample weight and moisture content of the certified reference materials.

We compared the MeHg concentration making two measurements from single samples of the muscle and ovary of cutlass fish using the GC-ECD method to check for any differences with other MeHg analyses (Ministry of the Environment, 2004). The values of MeHg measured in the muscle using the current method were 0.479 and 0.483 μg/g and using the GC-ECD method 0.486 and 0.491 μg/g (Table 1-4).

Table 1-4. Crosscheck of methylmercury determination in the muscle and ovary of cutlass fish using the GC-ECD (gas chromatography-electron capture detector) method compared with the current method

Tissues	MeHg (μg/g wet)	
	Current method	GC-ECD method
Muscle	0.479	0.486
	0.483	0.491
Ovary	0.052	0.051
	0.055	0.054

The values of MeHg measured in the ovary using the current method were 0.052 and 0.055 μg/g and using the GC-ECD method 0.051 and 0.054 μg/g. These results indicate very similar values for MeHg obtained by the two different methods and that our modified method worked appropriately.

As a result, the procedure for degreasing become simpler and faster than our previous method using chloroform. After confirming the accuracy of the modified method, it was applied to determine the MeHg and T-Hg concentrations and MeHg/T-Hg ratios in tissues from commercially-sourced fish, especially focusing on the gonad, which has been little studied compared with other organs. In some samples, such as liver of golden threadfin bream and the gonads of red seabream, golden threadfin bream and sea urchin, the aqueous phase and the lipid phase with MIBK were not clearly separated, with the aqueous phase remaining turbid after centrifugation. In these cases, a rapid additional treatment using MIBK at 60°C clarified the aqueous phase to provide a better separation. To test whether this additional treatment had any effect on the analyses, we compared the concentration of T-Hg and MeHg in fatty tuna muscles after using the standard MIBK protocol and after using the modified MIBK protocol with additional treatment using MIBK at 60°C: the concentrations of T-Hg using the standard MIBK protocol and additional MIBK modified protocol were 0.289 ± 0.018 (μg/g) and 0.291 ± 0.015 (μg/g), respectively, and for MeHg, 0.284 ± 0.022 (μg/g) and 0.284 ± 0.023 (μg/g), respectively (mean values and S.D.; n=5). These results indicated that the additional treatment using MIBK at 60°C did not affect the recovery of T-Hg and MeHg in this experiment.

The concentrations and ratios of T-Hg and MeHg determined in the tissues of marine fish and invertebrates are shown in Table 1-5. The mean T-Hg and MeHg concentrations in the muscle of cutlass fish were 0.705 and 0.689 μg/g, respectively, indicating that the Hg accumulation was higher than in the other species and exceeded the provisional regulation values for T-Hg (0.4 ppm) and MeHg (0.3 ppm) in Japan (Notice Kannyu No.99, 1973). This high level of accumulation of Hg in cutlass fish has been reported previously (Environment Agency and Kagoshima Prefecture, 1975), so this phenomenon may be caused by the uptake and excretion of MeHg, the habitat or feeding behavior. The MeHg/T-Hg ratios in the muscle of red seabream, golden threadfin bream, cutlass fish and bigfin reef squid were in the range of 96% - 98%, which is was consistent with a previous report by Bloom (1992). The ratios of T-Hg in the liver and muscle were 71.1% (red seabream), 93.8% (golden threadfin bream), 93.6% (cutlass fish) and 87.8% (bigfin reef squid), indicating that the T-Hg concentration in the liver of fish and squid were lower than those in their muscles. For example, the concentrations of T-Hg in the tissues of several freshwater fish have been reported in descending order as: muscle> liver> gonad (Has-Schön et al., 2008). Conversely, others have reported that the T-Hg concentration of tissues from freshwater fish, such as catfish and tilapia, were in descending order as: liver> muscle> intestine> stomach> gonad> gill> swim bladder (Watanabe et al., 2012). These results indicate that the ratio of Hg concentration between muscle and liver depends on the fish species. However, the MeHg/T-Hg ratios in the liver were 63.2% (red seabream), 35.9% (golden threadfin bream), 77.2% (cutlass fish) and 63.9% (bigfin reef squid), indicating that the demethylation activity and excretion of MeHg in the liver varied according to species. Demethylation of MeHg has also been reported to be related to reactive oxygen species (Suda and Hirayama, 1992; Hirayama and Yasutake, 1999; Yasutake and Hirayama, 2001) and to selenium (Se) compounds in mammals (Khan and Wang, 2009) and in fish (Yamashita et al., 2013a). Yamashita et al. (2013b) reported that the selenium-containing compound, selenoneine (2-selenyl-N_{α}, N_{α}, N_{α}-trimethyl-$_{L}$-histidine), accelerated the excretion and demethylation of MeHg, mediated by a selenoneine-specific transporter, OCTN1. This mechanism may work in the liver of the golden threadfin bream, for example. The Hg concentrations in the gonad, egg and nidamental gland were the lowest of all the tissues examined. In the present study, the mercury concentrations in the gonads, eggs and nidamental glands and the MeHg/T-Hg ratios were quite different depending on the species. For example, the MeHg/T-Hg ratio of the ovary has been reported to be 57 ± 8% in *Beryx splendens* (Donald and Sardella, 2010). In the present study, the MeHg/T-Hg ratios in the testis and ovary of red seabream were lower at 46.6% (from seven of 10 samples) and 42.4% (3/10), respectively, but higher in the testis of golden threadfin bream, 80% and in the ovary of cutlass fish, 95.7%. The body weight of the bigfin reef squid and mean MeHg concentration in their eggs and nidamental glands are shown in Table 1-5 and Figure 1-3.

Two of the eight bigfin reef squid (the two groups of data surrounded by a dotted line) had eggs with mature nidamental glands but the remaining six individuals had only immature nidamental glands without eggs. The Hg concentration in the two individual squid (the two groups of data surrounded by a dotted line) with mature nidamental glands was higher than that in the remaining six individuals. Mercury concentration has often been reported to be correlated with body weight (Peterson et al., 1973; Honda et al., 1983; Burger and Gochfeld, 2011). However, the Hg concentration in bigfin reef squid was not significantly correlated with body weight in the present study (Fig.1-3). This indicated that Hg concentration may be correlated with the maturity level of the gonads in bigfin reef squid. The mean T-Hg and MeHg concentrations in the gonads of the sea urchin were 0.043 and 0.001 μg/g, respectively, with a MeHg/T-Hg ratio of 3.5%. In previous reports, the T-Hg concentration in sea urchins has been reported as 0.01 μg/g (Sackett et al., 2013) and 0.007 μg/g (Nishimura et al., 2009), indicating that the gonads might commonly have very low concentrations of MeHg and therefore pose a low risk for human consumption.

Table 1-5. Concentration and ratio of total mercury to methylmercury in tissues of marine fish and invertebrates

Species	Body weight (g)	Total number of samples (male:female)	Tissues	Number of tissue samples analyzed	T-Hg (μg/g)	MeHg (μg/g)	MeHg/T-Hg (%)
Red seabream (*Pagus major*)	747 ± 32	10 (7:3)	Muscle	10	0.083 ± 0.013	0.081 ± 0.013	97.6 ± 0.5
			Liver	10	0.059 ± 0.007	0.038 ± 0.005	63.2 ± 1.8
			Ovary	3	0.024 ± 0.008	0.007 ± 0.001	42.4 ± 18.0
			Milt	7	0.027 ± 0.005	0.013 ± 0.003	46.6 ± 4.3
Golden threadfin bream (*Nemipterus virgatus*)	877 ± 24	10 (10:0)	Muscle	10	0.097 ± 0.006	0.094 ± 0.006	96.8 ± 0.4
			Liver	10	0.104 ± 0.010	0.037 ± 0.004	36.0 ± 1.7
			Milt	10	0.025 ± 0.002	0.020 ± 0.002	80.0 ± 1.5
Cutlass fish (*Trichiurus lepturus*)	534 ± 13	10 (0:10)	Muscle	10	0.705 ± 0.068	0.689 ± 0.067	97.7 ± 0.5
			Liver	10	0.661 ± 0.092	0.522 ± 0.083	77.4 ± 2.1
			Ovary	10	0.161 ± 0.021	0.154 ± 0.021	95.7 ± 0.6
Bigfin reef squid (*Sepiotenthis lessoniana*)	759 ± 56	8 (0:8)	Muscle	8	0.098 ± 0.016	0.096 ± 0.016	97.4 ± 0.5
			Liver	8	0.086 ± 0.008	0.055 ± 0.006	63.9 ± 2.4
			Egg	2	0.023	0.022	94.4
			Nidamental gland	8	0.052 ± 0.013	0.049 ± 0.012	96.5 ± 0.8
Sea urchin (*Asthenosoma ijima Yoshiwara*)	399 ± 39	6	Gonad	6	0.043 ± 0.011	0.001 ± 0.000	3.5 ± 0.7

Values from fish or invertebrates are expressed as the mean ± S.E.

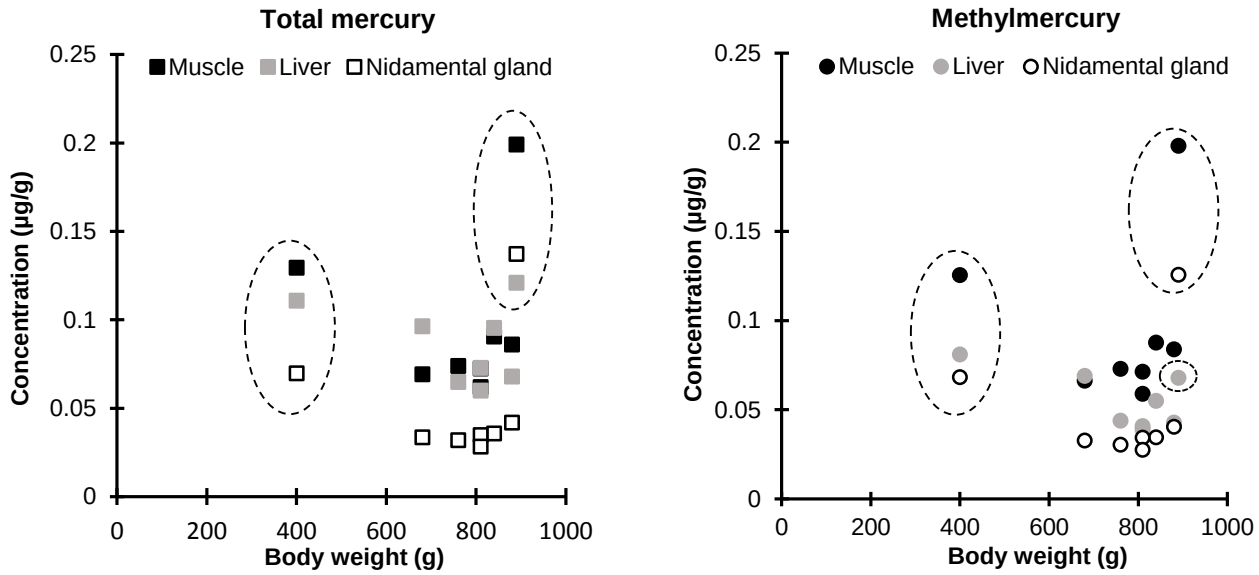

Fig. 1-3. Total mercury and methylmercury concentration in the muscle, liver and nidamental glands and body weight of bigfin reef squid. Sample surrounded by a dotted line indicate the two individuals with a mature nidamental gland.

The correlations between body weight and the MeHg concentration in the muscles and gonads were examined. The results in Figure 1-4 ~ 1-7 indicated moderate or strong correlation coefficients (Spearman's $\rho < -0.6$ or > 0.6).

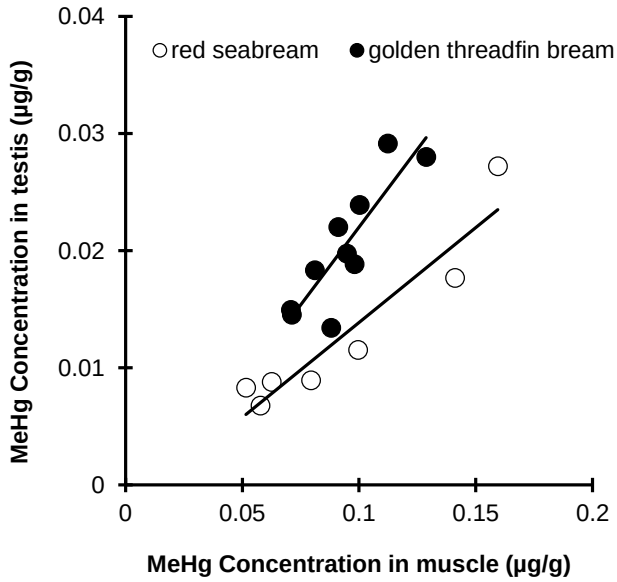

Fig. 1-4. Correlation between methylmercury concentration in the muscle and testis of red seabream and golden threadfin bream

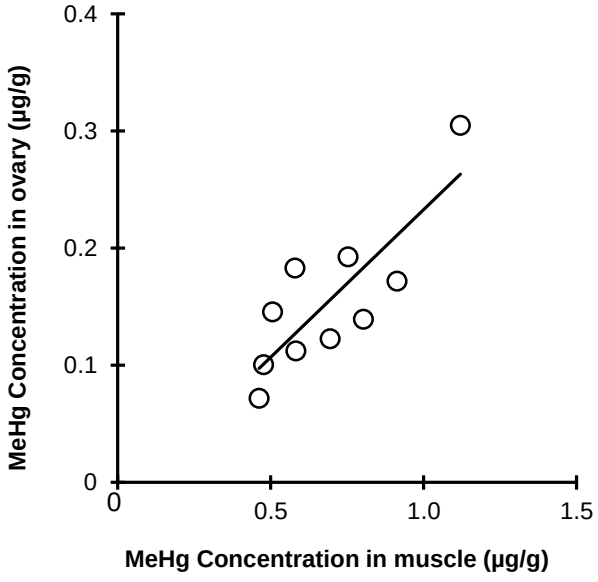

Fig. 1-5. Correlation between methylmercury concentration in the muscle and ovary of cutlass fish

A significant positive correlation between the MeHg concentrations in the muscle and testis of red seabream was observed ($\rho = 0.995$, $p < 0.001$, Fig. 1-4) and similarly for the muscle and testis of golden threadfin bream ($\rho = 0.866$, $p = 0.001$, Fig. 1-4). A significant positive correlation between the MeHg concentration in the muscle and ovary of cutlass fish was also observed ($\rho = 0.697$, $p = 0.025$, Fig. 1-5) and similarly for the muscle and nidamental gland of bigfin reef squid ($\rho = 0.874$, $p = 0.005$, Fig. 1-6).

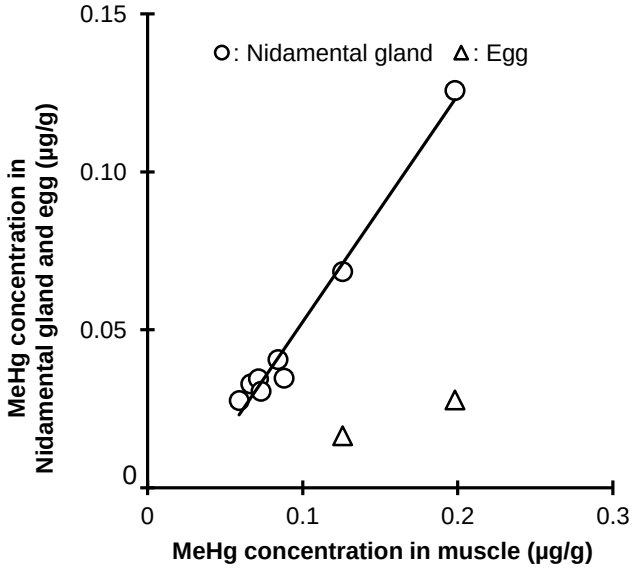

Fig. 1-6. Correlation between methylmercury concentration in the muscle and nidamental gland or egg of bifin reef squid

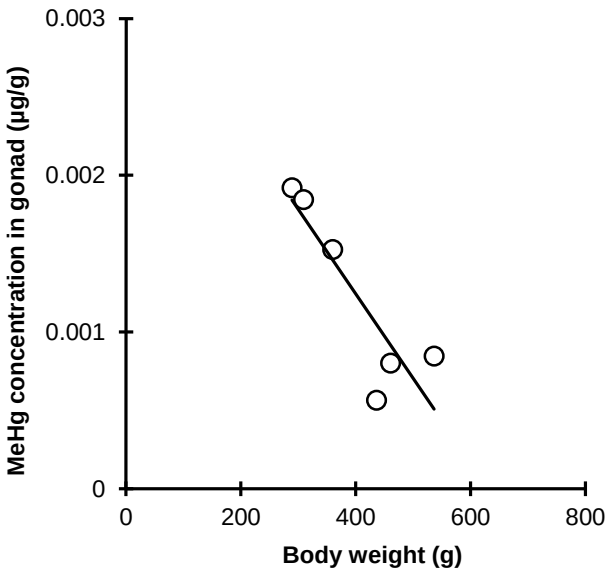

Fig. 1-7. Correlation between body weight and methylmercury concentration in the gonads of sea urchin

The correlation analysis indicated that the MeHg concentration in the gonad was closely correlated with the concentration in the muscle of marternal fish. Hammerschidt et al. (1999) reported that the MeHg/T-Hg in eggs was 91% and that the developing embryo of yellow perch was strongly affected by the material bioaccumulation of MeHg. In addition, a high correlation between egg and muscle Hg levels has been reported in largemouth bass (Yamanouchi et al., 2005). A significant negative correlation was observed between body weight and MeHg concentration in the gonads of sea urchins, indicating that the demethylation activity for MeHg might increase with growth ($\rho = -0.878$, $p = 0.021$, Fig. 1-7).

To summarize the overall advantages of this method: samples for heating vaporization atomic absorption spectrometry need no complex pretreatment; large volumes of waste liquid are not produced; the use of MIBK instead of chloroform speeds up and simplifies sample degreasing; a single apparatus can be used for determining both T-Hg and MeHg in the same biological sample in two consecutive steps; only one Hg standard solution is required for each experiment; and the experimental protocol is easy and cost-effective compared with other methods. In particular, there are several advantages in using disposable PP tubes for analyzing the Hg content of common biological samples; cleaning glass tubes for many analyses requires a considerable amount of labor; new PP tubes are free from contamination by Hg and are also marked with a scale for accurately measuring the liquid volume.

1.6. Conclusion

In conclusion, we report a more efficient analytical method for determining the T-Hg and MeHg concentrations in common biological samples by using MIBK in the degreasing step. This method will be useful for the routine analysis of T-Hg and MeHg in a large number of biological samples such as the

tissues of seafood. In addition, this study provides new information on the MeHg concentration and MeHg/T-Hg ratio in the eggs and nidamental glands of squid and in the gonads of sea urchin. These data will add usefull information to the database for assessing the risk of human exposure to MeHg in seafood.

1.7. Acknowledgements

The authors are grateful to Ms. Hazuki Kashiwagi and Dr. Eriko Motomura of the National Institute for Minamata Disease for their technical assistance. This work was supported by a grant from the National Institute for Minamata Disease.

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Chapter II. Mercury and selenium levels, and their molar ratios in several species of commercial shrimp in Japan regarding the health risk of methylmercury exposure

2.1. Abstract

The Japanese shrimp industry depends on importing shrimp from other countries. However, little information is available on mercury speciation and Se concentrations in commercial shrimp available in Japan. The present study determined the concentrations of T-Hg, MeHg, and Se in the muscle (wet weight) of imported and domestic commercial shrimp from Kumamoto and Kagoshima prefectures to obtain information for assessing the risk of MeHg exposure. The median concentrations of T-Hg, MeHg and Se in shrimp imported from three different countries were, respectively: black tiger shrimp (n = 18), 15.8, 14.4, and 415 ng/g; Vannamei shrimp (n = 25), 11.4, 11.2, and 292 ng/g; and white shrimp (n = 26), 26.8, 26.1, and 396 ng/g. There were significant differences in T-Hg and MeHg concentrations between shrimp imported from different countries. The median concentrations of T-Hg, MeHg and Se in shrimp of Japanese origin were, respectively: Shiba shrimp (n = 10), 15.9, 15.0, and 270 ng/g; Kuruma shrimp (n = 10), 79.9, 75.9, and 390 ng/g; and Ashiaka shrimp (n = 10), 36.1, 34.1, and 303 ng/g. The percentages of MeHg in T-Hg were between 90% and 99%, with MeHg levels in the imported and domestic commercial shrimp lower than the Japanese regulation of 300 ng/g for fish. The mean Se/T-Hg molar ratios (16-160) were comparatively higher than those previously reported in fish. Overall, this survey suggest that shrimp commercially available in Japan will not pose a particularly high risk regarding MeHg exposure to consumers.

2.2. Introduction

MeHg is a well-known environmental neurotoxicant that is absorbed from the gastrointestinal tract and crosses the blood-brain and blood-placental barriers (World Health Organization, 2008; 2010). The primary source of MeHg exposure in humans is from seafood consumption, with the fetus known to be at particularly high risk. Therefore, determining the concentration of T-Hg and MeHg in fish is important for assessing risks from exposure through fish consumption. Many countries and organizations have recommended safe levels of Hg in fish: the World Health Organization (WHO) allows 0.5 mg MeHg/kg in all fish except predatory fish (e.g shark, swordfish, tuna, and pike) (United Nations Environment Programme, 2002), and Japan allows up to 0.4 mg T-Hg/kg and 0.3 mg MeHg/kg in fish (Notice Kannyu No.99, 1973).

Se is essential for human nutrition because it is a component of important enzymes such as glutathione peroxidases and thioredoxin reductase. These are involved in the antioxidant defense mechanism, thyroid hormone metabolism, and the redox control of intracellular reaction (Agency for Toxic Substances and Disease Registry, 2003). Humans consume Se mainly through seafood, with its organic form comprising the greatest proportion of total dietary intake (Agency for Toxic Substances and Disease Registry, 2003; Sharma et al., 2015). Se has also been found to play an important role in the antagonistic effects on Hg toxicity, for example, in various tissues and organs of rodents, fish and marine mammals (Khan and Wang, 2009; Lemes et al., 2011; Sakamoto et al., 2013). However, the bioavailability of Se can be sufficiently reduced by Hg to perturb the normal Se-dependent functions (Sørmo et al., 2011). Others have reported that Se/Hg molar ratios below one may lead to a higher risk from Hg toxicity (Ralston and Raymond, 2010; Burger and Gochfeld, 2013). Therefore, the Se/Hg molar ratio is an important piece of information when assessing the health risk from MeHg exposure due to fish consumption.

Regarding shrimp, Japan is one of its largest consumers and its second largest importer in the world (Food and Agriculture Organization of the United Nations, 2016a). Most commercial shrimp processors in Japan depend on importing shrimp mainly from Vietnam, Indonesia, and India (European Parliament,

2013; Food and Agriculture Organization of the United Nations, 2016b). However, little information is available on the Hg and Se concentrations in commercial shrimp in Japan.

2.3. Objective

In the present study, we aim to determine the concentration of T-Hg, MeHg and Se in the muscles of several species of commercial shrimp available in Kumamoto and Kagoshima prefectures of Japan to obtain information for assessing the health risk from MeHg exposure.

2.4. Materials and methods

Reagents

Analytical grade reagents were described previously in page 3.

Sample collection and preparation

Fresh shrimp were obtained from supermarkets in Kumamoto and Kagoshima prefectures, Japan. The origin of the importing country was specifically shown on the label. The shrimp samples included: black tiger shrimp (*Penaeus monodon*) from Vietnam, n = 5; India, n = 6; and Australia, n = 7; Vannamei shrimp (*Litopenaeus vannamei*) from India, n = 6; Ecuador, n = 9; and Malaysia, n = 10; White shrimp from India, n = 6; Bangladesh, n = 10; and Indonesia, n = 10; from Japan: Shiba shrimp (*Metapenaeus joyneri*), n = 10; Kuruma shrimp (*Marsupenaeus japonicas*), n = 10; and Ashiaka shrimp (*Penaeus semisulcatus*), n = 10; red shrimp (*Pleoticus muelleri*), from Argentina, n = 9; and Irian tiger shrimp (*Penaeus semisulcatus*) from Indonesia, n = 6. We could not provide one scientific name for white shrimp because it could be *Penaeus indicus* or *Penaeus merguiensis*, depending on the habitat. After removing the shell, intestine, head, and tail from the shrimp samples, the muscle were weighed and homogenized for further analysis of Hg and Se. The homogenized shrimp samples (1 g, wet weight) were transferred to 15-mL PP tubes then kept at –30^{o}C until analysis.

Extraction of total mercury and methylmercury

The extraction of T-Hg and MeHg from the shrimp samples was based on the basic protocol described previously (Fig. 1-2) (Miyamoto et al., 2010; Yoshimoto et al., 2016).

We conducted the recovery test using a MeHg standard working solution and MeHg-spiked shrimp homogenates as in our previous study (Yoshimoto et al., 2016) obtaining values for MeHG of 95.7% ± 0.9 with CV of 0.9% and 96.5% ± 0.3 with CV of 0.3%, respectively (n = 5). In addition, the mean MeHg recovery, using CRM of codfish, swordfish and hair, was 95% (Yoshimoto et al., 2016). Therefore, we used 95% as the correction factor for MeHg extraction from shrimp samples to calculate the concentration of MeHg.

Determination of mercury concentration

The Hg content in the extracts of shrimp samples for T-Hg and MeHg analysis was determined using a direct thermal decomposition Hg analyzer MA-3000 (Nippon Instruments, Tokyo, Japan) (described previously in page 7). T-Hg and MeHg were measure in the CRM cod fish (National Institute of Advanced Industrial Science and Technology, 2010) for quality control. The mean values (n = 5) measured for T-Hg and MeHg in cod fish were 0.62 μg/g ± 0.01 and 0.59 μg/g ± 0.01 μg/g, respectively, which were within the certified range of 0.61 ± 0.02 μg/g for T-Hg and 0.58 ± 0.02 μg/g for MeHg. The CV of 0.8% for T-Hg and 0.9% for MeHg were obtained from 5 replicate measurements on the CRM cod fish.

Selenium determination

Se in the sample solution was measured using an inductively-coupled plasma mass spectrometry (ICP-MS) CR system with a collision cell (Agilent 7700x, Agilent Technologies, Santa Clara, CA, USA) by IDEA Consultants, InC. (Shizuoka, Japan). Wet digestion was used to dissolve the shrimp sample (0.4 g wet weight) into its constituent elements with nitric acid (2 mL; Ultrapur-100; Kanto Chemical) and a microwave-accelerated reaction system (Speedwave-4, Berghof Products + Instruments GmbH, Eningen, Germany). After microwave digestion, yttrium (1000 mg/L standard solution for ICP; TraceCERT; Sigma-Aldrich, St. Louis, MO, USA) and acetic acid (Ultrapur grade; Kanto Chemical) were added to the digested solution, and the solution was made up to 100 mL as a test solution. In this test solution, yttrium (1 ng/g) had been added as an internal standard, and 3% (v/v) acetic acid to eliminate the effect of any remaining organic matter derived from the shrimp samples. The calibration standards were prepared using Se standard solution (1000 mg/L for metal analysis grade; Wako Pure Chemical Industries) with yttrium (1 ng/g) and 3% (v/v) acetic acid. The mean values (n = 3) measured for Se in DORM-4 (Fish protein CRM for trace metals, National Research Council Canada) was 3.53 µg/g ± 0.44 µg/g which was within the certified range of 3.45 ± 0.40 µg/g.

Statistical analysis

Values from the shrimp samples in the present study were expressed as the medians and ranges. The non-parametric Kruskal-Wallis test was then used to compare the T-Hg, MeHg, and Se levels of the muscle of different species of shrimps examined in this study from three different countries of origin, for example, a comparison between black tiger shrimps from Vietnam, India and Australia. The associations among the T-Hg, MeHg and Se levels were assessed using the Spearman rank correlation coefficient. Curves were fitted using linear regression plots using GraphPad Prism 7 software (Version 7.00, GraphPad Software Inc., La Jolla, CA, USA). A p value of less than 0.05 was considered as statistically significant.

2.5. Results

Mercury and selenium concentrations in shrimp

Table 2-1 summarizes the concentrations of T-Hg, MeHg, and Se, MeHg (%) in T-Hg, and Se/T-Hg molar ratios in the muscle of shrimp, with the T-Hg and MeHg concentrations being expressed as Hg content.

Figure 2-1 shows the distributions of Hg and Se in the muscle by country and by species of shrimp. There were significant differences ($p < 0.01$) in the Hg concentrations between the muscles of species of shrimps examined in this study from three different countries of origin. The T-Hg and MeHg concentrations were significantly different even in samples from the same species. Of the imported shrimp, the black tiger shrimp from Australia exhibited T-Hg concentrations 6-7 times higher, and MeHg concentrations 6-7 times higher than those of black tiger shrimp from Vietnam and India. For vannamei shrimp, the T-Hg and MeHg concentrations in the muscle of shrimp from Ecuador were 3-6 times higher than those of shrimp from India and Malaysia. Regarding white shrimp, those from Bangladesh exhibited concentrations of T-Hg and MeHg 2 times higher than those of shrimp from Indonesia. The highest median values of T-Hg and MeHg concentration were found in the muscle of Kuruma shrimp from Japan, and the lowest in the muscles of vannamei shrimp from India.

Regarding Se concentration, there were no significant differences in Se levels among the muscle of species of shrimps examined in this study from three different countries of origin (Table 2-1). The medians of Se concentrations were highest in the muscles of red shrimp from Argentina and lowest in the muscle of vannamei shrimp from India.

Table 2-1. Median values and ranges of T-Hg, MeHg and Se concentrations (ng/g wet weight) and mean Se/T-Hg molar ratios in the muscle of shrimp marketed in Japan

Species	Country	n	Muscle weight (g)	T-Hg (ng/g wet weight)		MeHg (ng/g wet weight)		MeHg/T-Hg (%)	Se (ng/g wet weight)		Se/T-Hg Molar ratio
			Mean ± SD	*Median*	*Range*	*Median*	*Range*	*Mean ± SD*	*Median*	*Range*	*Mean ± SD*
Black tiger shrimp (Penaeus monodon)	Vietnam	5	15.1 ± 1.7	8.5	6.7–14.4	8.2	6.5-14.0	98.1 ± 1.8	322	267–438	99.9 ± 31.1
	India	6	14.0 ± 1.9	10.8	4.3–22.5	9.1	4.3–21.7	91.3 ± 7.7	343	301–480	106.1 ± 60.6
	Australia	7	17.7 ± 1.0	62.1	36.7–91.2	58.6	35.0–83.8	93.8 ± 3.3	446	363–630	19.9 ± 5.9
	Total*	18	15.8 ± 2.2	15.8	4.3–91.2	14.4	4.3–83.8	94.2 ± 5.4	415	267–630	70.9 ± 55.5
Vannamei shrimp (Litopenaeus vannamei)	India	6	10.1 ± 1.6	5.3	4.3–5.8	5.3	4.4–5.8	99.9 ± 2.1	219	175–248	106.4 ± 21.3
	Ecuador	9	10.9 ± 0.7	31.1	25.1–39.8	30.6	24.2–39.9	98.2 ± 1.2	289	250–320	22.7 ± 3.1
	Malaysia	10	12.2 ± 1.3	11.2	10.0–12.1	11	9.9–11.9	98.1 ± 2.0	305	250–340	69.9 ± 9.2
	Total*	25	11.3 ± 1.4	11.4	4.3–39.8	11.2	4.4–39.9	98.6 ± 1.9	292	175–340	61.6 ± 35.1
White shrimp (*Penaeus indicus* or *Penaeus merguiensis*)	India	6	26.8 ± 2.2	26.8	23.6–40.9	26.1	23.1–39.6	97.9 ± 1.0	391	244–448	32.9 ± 12.2
	Bangladesh	10	25.0 ± 2.4	39.1	31.6–49.0	35.4	29.1–43.3	90.6 ± 3.5	412	340–560	27.8 ± 6.4
	Indonesia	10	15.3 ± 1.0	16	8.9–23.1	14.6	8.5–21.7	91.7 ± 3.1	385	318–490	64.4 ± 16.5
	Total*	26	21.7 ± 5.5	26.8	8.9–49.0	26.1	8.5–43.3	92.7 ± 4.1	396	244–560	43.1 ± 21.1
Shiba shrimp (Metapenaeus joyneri)	Japan	10	3.2 ± 0.4	15.9	11.4–19.0	15	10.9–17.7	95.9 ± 2.6	270	170–370	46.7 ± 12.6
Kuruma shrimp (Marsupenaeus japonicus)	Japan	10	27.0 ± 9.8	79.9	32.5–151.0	75.9	30.4–140.8	94.1 ± 3.1	390	280–829	16.2 ± 8.6
Ashiaka shrimp (Penaeus semisulcatus)	Japan	10	21.6 ± 2.3	36.1	22.0–48.4	34.1	20.8–45.7	94.7 ± 0.5	303	180–420	22.0 ± 4.6
Red shrimp (Pleoticus muelleri)	Argentina	9	12.9 ± 2.0	14.5	6.5–40.9	13.5	5.9–38.5	92.8 ± 1.5	480	390–550	97.8 ± 54.9
Irian tiger shrimp (Penaeus semisulcatus)	Indonesia	6	24.2 ± 0.7	62	24.3–140.6	55.9	23.1–117.2	90.0 ± 4.4	402	345–550	22.6 ± 15.6

**Total medians and ranges of values for individual black tiger shrimp, vannamei shrimp and white shrimp.*

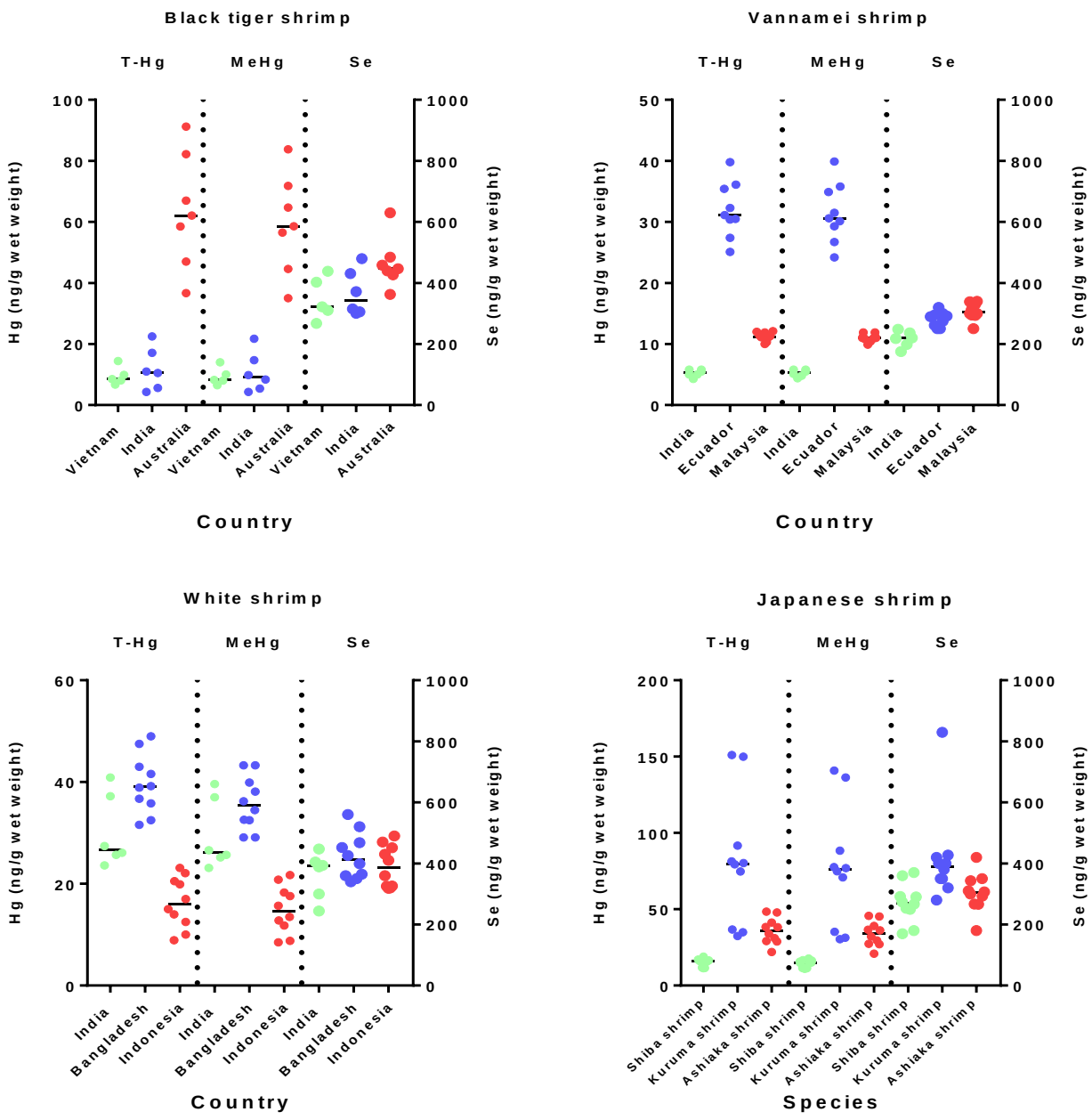

Fig. 2-1. Distribution of T-Hg, MeHg and Se concentration in the muscles of shrimp marketed in Japan. T-Hg and MeHg concentrations expressed as Hg content. Black tiger shrimp form Vietnam (n = 5); India (n = 6) and Australia (n = 7); Vannamei shrimp from India (n = 6); Ecuador (n = 9) and Malaysia (n = 10); white shrimp from India (n = 6); Bangladesh (n = 10) and Indonesia (n = 10); Japan Shiba shrimp (n = 10), Japan Kuruma shrimp (n = 10), Japan Ashiaka shrimp (n = 10). Transverse lines indicate the median values.

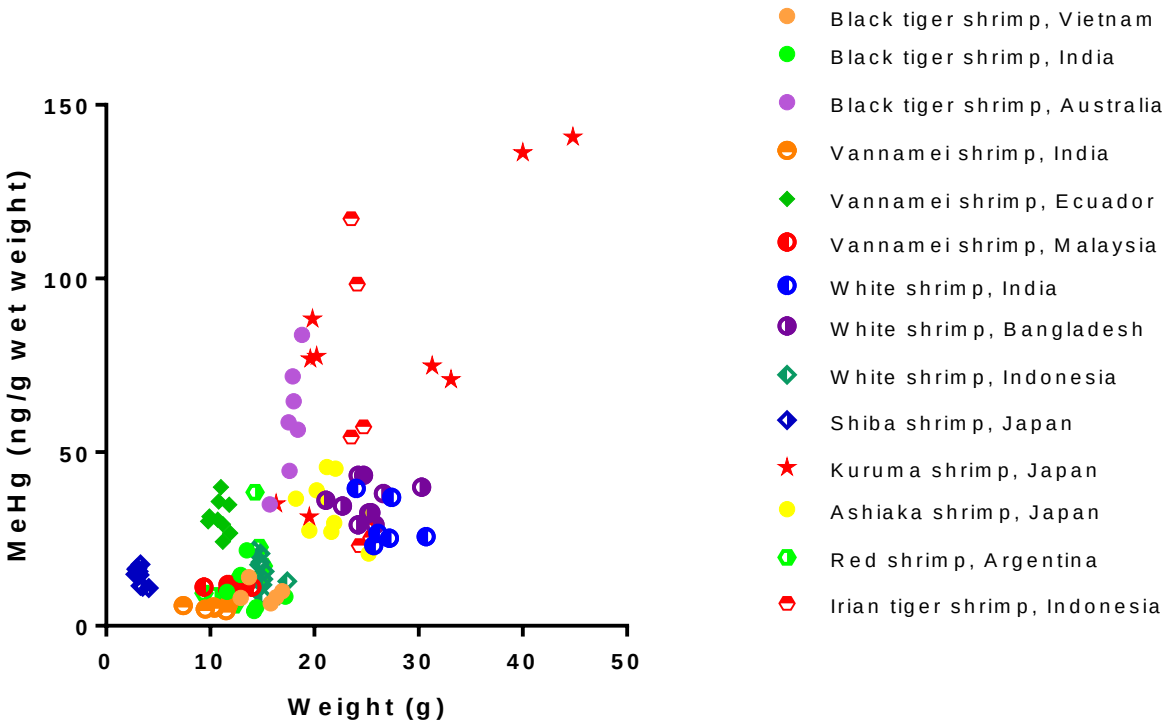

Fig. 2-2. Distributions of MeHg concentration by source and muscle weight of shrimp marketed in Japan. Black tiger shrimp from Vietnam (n = 5), India (n = 6), and Australia (n = 7); Vannamei shrimp from India (n = 6), Ecuador (n = 9), and Malaysia (n = 10); white shrimp from India (n = 6), Bangladesh (n = 10), Indonesia (n = 10); Japan Shiba shrimp (n = 10); Japan Kuruma shrimp (n = 10); Japan Ashiaka shrimp (n = 10); Argentina red shrimp (n = 9); Indonesia Irian tiger shrimp (n = 6).

Figure 2-2 shows the distribution of MeHg in the muscles of shrimp by muscle weight. The MeHg concentrations in the muscles tended to increase with muscle weight. The Kuruma shrimp from Japan with the highest muscle weight exhibited the highest MeHg levels, whereas vannamei shrimp from India with the lowest muscle weight (except for Shiba shrimp from Japan) exhibited the lowest MeHg levels.

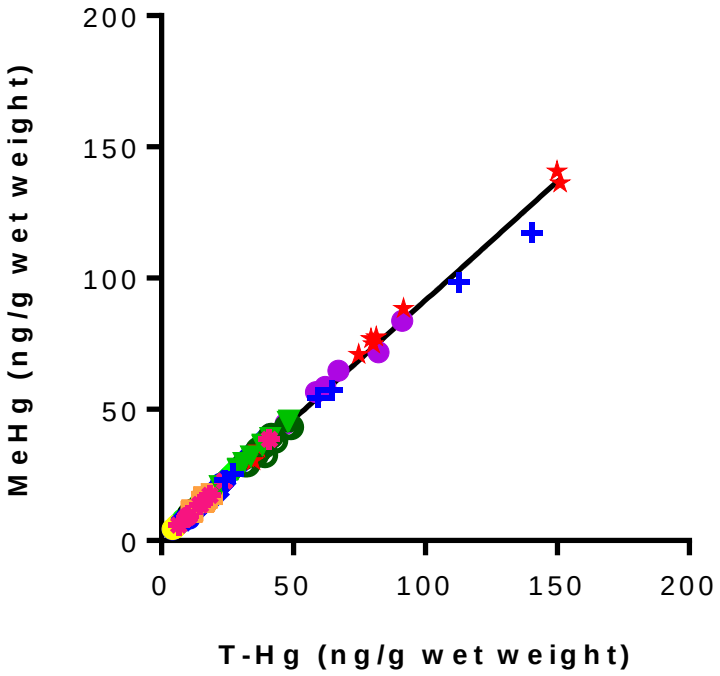

Fig. 2-3. Relationship between T-Hg and MeHg concentrations in the muscles of shrimp marketed in Japan. Linear regression formula: Y = 0.9055 x X + 0.9393, $r = 0.99$, $p < 0.01$. n = 114.

The relationships between T-Hg and MeHg concentrations in the muscle of shrimp are shown in Figure 2-3. The T-Hg and MeHg concentrations in the shrimp muscles showed a significant linear correlation ($r = 0.99$, $p < 0.01$). Table 2-1 and Fig 2-3 showed that the values of MeHg as a percentage of T-Hg ranged from 90% to 99%.

Relationship between total mercury and selenium molar concentrations

Fig. 2-4 shows the relationship between the T-Hg and Se molar concentrations (nmol/g) in the muscles of shrimp. The T-Hg and Se molar concentrations showed a significant linear correlation ($r = 0.31$, $p < 0.01$). The average Se/T-Hg molar ratios in the muscle of shrimp ranged from 101 to 16 with individual ratios ranging from 215 (highest: Red shrimp, Argentina) to 6.5 (lowest: Kuruma shrimp, Japan). All data points were above the dotted line for Y (Se) = 1 x X (T-Hg).

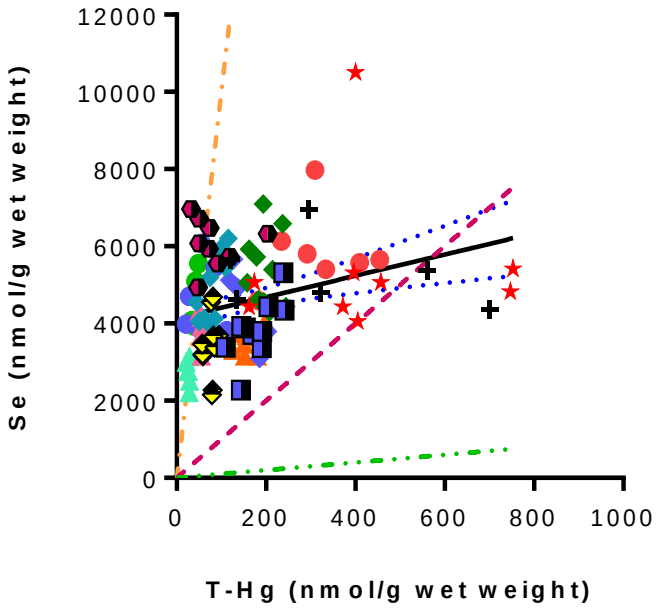

Fig. 2-4. Relationship between T-Hg (X) and Se (Y) molar concentrations in the muscles of shrimp marketed in Japan. Linear regression formula: Y = 2.76 x X + 4132, r = 0.31, p < 0.01, n = 114.
——— Linear regression line
····· Lines indicating 95% confidence interval
—·—·— Line indicating Y = 1 x X
- - - - - Line indicating Y = 10 x X
-·-·-·- Line indicating Y = 100 x X

2.6. Discussion

Regarding imported shrimp, significant differences in T-Hg and MeHg levels were observed in the shrimp muscles imported from different countries, possibly because of differences in MeHg levels in the diet during commercial shrimp culture (Fig. 2-1). The natural food sources for shrimp are micro-algae, phytoplankton, and zoo-plankton but the ingredients used in commercial feed for shrimp contain fish ingredients (fish meal, shrimp shell meal, squid meal, and trash fish) (New, 2002). In addition, MeHg levels in the aquatic environment used for commercial production will affect the Hg levels in the shrimp.

We also found that MeHg levels tended to increase with increasing muscle weight so that large shrimp may have higher Hg levels (Fig. 2-2). Hg concentrations have been reported to correlate with fish body weight (bw) (Simonin et al., 2008; Burger and Gochfeld, 2011). In the present study, the T-Hg and MeHg concentrations in the muscles of shrimp were also observed to be positively related (Fig. 2-3), a finding agreeing with other studies on fish (World Health Organization, 2010). The MeHg (%) of T-Hg in the muscles of shrimp ranged from 90% to 99%, a result consistent with that in the muscles of fish and squid provided in previous studies (Bloom, 1992; Yoshimoto et al., 2016). The T-Hg levels in the muscles of shrimp in this study were similar to those in shrimp and small fish, such as sardine, salmon, and horse mackerel from Japan, provided in a previous report and in surveys in other countries such as the US (50 ng/g), Taiwan (10 ng/g), and Mexico (10 ng/g) (Sunderland, 2007; Ministry of Health Labour and Welfare, 2010; Fang et al., 2011; Basu et al., 2014). The Hg concentrations in the muscles of shrimp examined in this study were much lower than those permitted by Japanese regulations of 400 ng/g for T-Hg and 300 ng/g for MeHg (Notice Kannyu No.99, 1973) and than the maximum level allowed by WHO of 500 ng/g for MeHg in all types of fish (United Nations Environment Programme, 2002).

In the present study, the Kuruma shrimp from Japan exhibited the highest concentrations of MeHg so to calculate a level of MeHg intake as a 'worst-case-scenario', we used data on the level of Kuruma shrimp, consumption per capita in Japan in 2015 (Fisheries Agency of Japan, 2015) and the average bw of a Japanese woman in the late stage of pregnancy (Japan Food Safety Commission, 2005). This calculation estimated the MeHg intake as 0.01 μg/kg bw/week, a level much lower than the tolerable weekly intake of 2.0 μg/kg bw/week for MeHg regarded as safe for pregnant and potentially pregnant women in Japan (Japan Food Safety Commission, 2005). This suggests that the shrimp commercially available in Japan will not pose a health risk for women of childbearing age from MeHg exposure.

In the present study, the Se levels in the muscles of shrimp were similar among the same species of shrimp (Table 2-1). The Se levels in the muscles of shrimp were similar to those found in other fish species in previous studies: shortfin mako (260 ng/g), and Atlantic bluefin tuna (430 ng/g) (Burger and Gochfeld, 2011). Se levels are known to occur in a narrow range between doses that lead to deficiency or toxicity. Thus although Se is an essential element, it can be toxic at elevated levels (Sharma et al., 2015), so may be regulated to a constant level in the body of the shrimp.

Large differences in the Se/T-Hg molar ratios were observed in the muscles of the shrimp examined in this study (Table 2-1). These differences in Se/T-Hg molar ratios may have been caused by differences in Hg levels in the muscles of the shrimp. Burger and Gochfeld (2013) and Eisler (2000) have also reported that Se/Hg molar ratios reflected differences in the Hg levels of the edible portions of fish. In addition, the Se/T-Hg molar ratios in the muscles of the shrimp were comparatively higher than in fish reported in other studies, such as yellow fin tuna (2.9), and blue fish (4.9) (Burger and Gochfeld, 2013) with average ratios ranging from 106 to 16 and individual ratios from 215 (highest; Red shrimp, Argentina) to 6.5 (lowest; Kuruma shrimp, Japan). All data points were above the dotted line for Y (Se) = 1 × X (T-Hg), with the Se/Hg molar ratios still being greater than one, even at the highest T-Hg molar concentration exhibited by Kuruma shrimp from Japan (Fig. 2-4). Other studies have reported that Se/Hg molar ratios above one might increase the protective effects of Se against MeHg exposure (Peterson et al., 2009; Ralston and Raymond, 2010). The Se/Hg molar ratios in shrimp determined in the present study have indicated that the shrimp examined would be a low-risk seafood because of their low MeHg and high Se concentrations.

The strengths of the present study can be summarized as analyzing the T-Hg, MeHg and Se concentrations in the eight species of imported and domestic shrimp that are consumed in Japan, and demonstrating that the Se/THg molar ratios in the shrimp examined were comparatively higher than those in other species of fish. The limitations of this study were the limited numbers of shrimp of similar commercial size for each species available for analysis and the unavailability of detailed information on the contamination levels of MeHg in the aquatic environment for the commercially-produced shrimp.

2.7. Conclusion

In conclusion, the present study has demonstrated that T-Hg and MeHg levels in imported and domestically-produced commercial shrimp in Japan were lower than the Japanese regulation level of 300 ng/g for MeHg in fish. In addition, the average Se/Hg molar ratios in the muscles of commercial shrimp were comparatively high in the range of 16–106. Consequently, the shrimp commercially available in Japan will not cause a risk of MeHg exposure to Japanese consumers.

2.8. Acknowledgements

The authors are grateful to Dr. Yasuhiro Ishibashi (Prefectural University of Kumamoto), Dr. Minoru Koga (Minamata Environmental Academia) and Dr. Atsuhiro Nakano (former department director of Basic Medical Science, National Institute for Minamata Disease) for their guidance and encouragement. We also thank Ms. Hazuki Kashiwagi and Megumi Kudo (National Institute for Minamata Disease) for their technical assistance. This work was supported by the International

Postgraduate Scholarship for Research on Mercury from the Prefectural University of Kumamoto and a grant from the National Institute for Minamata Disease.

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Chapter III. Hair mercury levels in relation to fish consumption among Vietnamese in Hanoi

3.1. Abstract

People are exposed to MeHg mainly through fish consumption, which is increasing in Vietnam. However, little information is available on estimating the health risk of MeHg exposure through fish consumption in Vietnam. The present study examined the association between Hg levels in hair and Se levels in toenails of 196 Vietnamese people and their fish consumption, using a dietary questionnaire to obtain information pertinent for assessing health risk owing to MeHg exposure. The geometric mean of Hg levels in the hair of males and females was 617 ng/g and 575 ng/g, respectively. We found that Hg levels in the hair of 98% of the Vietnamese study subjects were lower than the provisional tolerable weekly intake for MeHg (1.6 μg Hg/kg body weight; which is equivalent to a hair Hg concentration of approximately 2300 ng/g, with an uncertainty factor of 6.4). There were significant differences in the age-adjusted geometric mean of Hg levels found in hair from females related to their frequency of freshwater fish consumption. The levels of Hg in hair and Se in toenails increased with an increased frequency of marine fish consumption, and both showed a significant positive correlation in subjects who consumed marine fish ≥ once/week. This is the first cross-sectional study to investigate the association between hair Hg levels and fish consumption in Vietnam. These findings provide valuable information for future assessments of the health risk of MeHg exposure through fish consumption in Vietnam.

3.2. Introduction

MeHg is a well-known neurotoxin (World Health Organization, 2008; 2010). The target organ for MeHg toxicity is the brain, and the developing brain is especially sensitive to MeHg exposure. Hence, fetuses, newborn and young children are especially susceptible to MeHg exposure. Parents, pregnant women, and women of childbearing age should be aware of the potential risk of MeHg. People are primarily exposed to MeHg through consumption of fish and marine mammals (World Health Organization, 2008; 2010). Consequently, assessment of MeHg exposure is an important public health issue and evaluation of associated health risks is needed, especially for people who consume large amounts of fish and other seafood containing high concentrations of Hg.

Se is an essential trace nutrient in humans that participates in antioxidant defense mechanisms, thyroid hormone metabolism, and the redox control of intracellular reactions (Agency for Toxic Substances and Disease Registry, 2003). People consume Se mainly through seafood, with its organic form comprising the greatest proportion of total dietary intake (Agency for Toxic Substances and Disease Registry, 2003; Roman et al., 2014). Selenium plays an important role providing an antagonistic effect on Hg toxicity in various tissues and organs of rodents, fish and marine mammals (Peterson et al., 2009; Khan and Wang, 2009; Sakamoto et al., 2015). The correlation between Se concentrations in human specimens and health risk has been widely studied in the environmental health science of MeHg (Mozaffarian and Rimm, 2006).

Recently, fish consumption is increasing in Vietnam (Food and Agriculture Organization of the United Nations, 2004). The rise in demand for fish and fishery products in Vietnam is caused by two main factors, population and income growth (Food and Agriculture Organization of the United Nations, 2004). Vietnam is ranked sixth place in eight Southeast Asian countries for the consumption of fish and fish products as determined by household surveys (Needham and Funge-Smith, 2015). Most commercial fish and seafood in Vietnam is locally harvested (Food and Agriculture Organization of the United Nations, 2005). Food and Agriculture Organization of the United Nations (2004) reported that a strong and sustained demand for fish and fishery products was expected from the Vietnamese population. However, little information is available on estimating the health risk of MeHg exposure through fish consumption in Vietnam.

Hair is the preferred biological sample for the assessment of MeHg exposure in many studies because it provides a simple, integrative, and non-invasive sample for estimating long-term average exposure. Hair contains a higher concentration of Hg than blood or urine, allowing easier detection of Hg (Srogi, 2007). T-Hg in hair is about 250 to 300 times higher than the blood concentration of Hg at the corresponding moment of hair formation (World Health Organization, 2008; 2010). The MeHg usually constitutes at least 80% of T-Hg analyzed in hair from fish consumers (McDowell et al., 2004). Nail clippings also have advantages compared with other biological materials and are thought to reflect long-term exposures (Slotnick and Nriagu, 2006; Koriyama et al., 2008).

3.3. Objective

In the present study, we examined the association between Hg levels in hair or Se levels in toenails and fish consumption using a dietary questionnaire for subjects recruited in Hanoi, to obtain information pertinent for assessing the potential health risk from MeHg exposure in Vietnamese. In addition, T-Hg and MeHg concentrations in several commercially canned seafood products in Hanoi were determined for a preliminary survey.

3.4. Materials and methods

Sample collection and preparation

A total of 196 Vietnamese people with no occupational exposure to Hg compounds were selected from staff (n = 98), and outpatients with acne (n = 29), tinea (n = 29) or sexually transmitted infections (n = 40), at the National Hospital of Dermatology and Venereology in Hanoi from 2015 to 2016. To recruit subjects from wide areas and with various characteristics, we selected a national referral hospital (National Hospital of Dermatology and Venereology), which had outpatients from Hanoi and other provinces in northern Vietnam. All subjects agreed to participate and gave their consent after an explanation of study objectives and methods. Subjects were 16 to 55 years old (mean 27.8 ± 7.4). For hair collection, approximately 50 full-length strands of hair were cut close to the scalp in the occipital area and stored in clean polyethylene bags. Clipped toenail samples were stored in a separate plastic envelope with the identification number of the corresponding subject. Hair and toenail samples were transferred to the National Institute for Minamata Disease, Japan, and stored at room temperature before analysis. This study was approved by the ethics committees of the National Institute for Minamata Disease, Kagoshima University, Prefectural University of Kumamoto (Japan) and National Hospital of Dermatology and Venereology (Vietnam).

Frequency and amount of fish consumption

General information (age, gender, bodyweight, height, occupation, education, marital status, hair treatment, and habits of smoking, alcohol drinking and eating out), frequency of fish consumption and amount of fish consumed per time were estimated for each subject using a food frequency questionnaire (FFQ). The FFQ was used to evaluate the frequency of freshwater and marine fish consumption during the past year. There were three alternatives for each species of fish: ≥ 4 times/week, 1–3 times/week, never or < 1 time/week. Regarding the amount of fish consumed per time, there were two alternatives for each species of fish: ≥ 50 g/time and < 50 g/time. We used food models of fish (50 g and 100 g) and pictures of fish commonly consumed in Hanoi to increase the accuracy of the survey.

Reagents

Analytical grade reagents were used described previously in page 3

Determination of total mercury concentration in hair

The T-Hg in the 3 cm proximal end of scalp hair was determined using direct thermal decomposition Hg analyzers MA-3000 (Nippon Instruments, Tokyo, Japan) (described previously in page 7). The certified reference material (CRM) of hair (NIES CRM No.13) was measured as a quality control, and the determined T-Hg level of 4.41 μg/g ± 0.03 μg/g (n = 5) was within the certified range of 4.42 ± 0.20 μg/g. A coefficient of variation (CV) of 0.7% was obtained from five repeated measurements using standard hair.

Determination of total mercury and methylmercury in canned fish

Canned fish were obtained from supermarkets in Hanoi, Vietnam. Using company names shown on the label, canned fish samples included: sardines in tomato sauce from companies A, B and C; mackerel in tomato sauce from company D; tuna in oil from companies E and F; yellowfin tuna chunks in vegetable oil from company G, light tuna chunks in oil from company H; tuna in vegetable oil from company I; tuna chunks in brine from company J (n = 1 for each). Muscles of canned fish samples were weighed (1 g, wet weight) and transferred to 15 mL polypropylene (PP) tubes then kept at −30°C until analysis.

Extraction of T-Hg and MeHg from canned fish samples followed the protocol described previously in Fig. 1-2 (Miyamoto et al., 2010; Yoshimoto et al., 2016). The T-Hg and MeHg concentrations in extracts of canned fish samples were determined using direct thermal decomposition Hg analyzers MA-3000 (Nippon Instruments, Tokyo, Japan) (described previously in page 7). Total Hg and MeHg were measured in cod fish CRM (National Institute of Advanced Industrial Science and Technology, 2010) for quality control. The mean values (n = 5) measured for T-Hg and MeHg in cod fish were 0.62 μg/g ± 0.01 and 0.59 μg/g ± 0.01 μg/g, respectively, which were within the certified range of 0.61 ± 0.02 μg/g and 0.58 ± 0.02 μg/g, respectively. A CV of 0.8% and 0.9% was obtained for T-Hg and MeHg, respectively, after five replicate measurements using cod fish CRM.

Determination of selenium concentration

A toenail sample was washed by sonication in 0.3 % polyoxyethylene lauryl ether and subsequently dried for 12 hr at 80°C. The sample was digested in 10 ml of 35% HNO_3 in a Teflon polytetrafluoroethylene tube in a closed vessel microwave system (Ethos Easy, Milestone Srl, Sorisole, BG, Italy). The concentration of Se was determined with an inductively coupled plasma mass spectrometer (Agilent 7500cs, Agilent Technologies, Tokyo, Japan). Accuracy of the method was assessed using CRM for hair (NIES CRM No.13). Recovery of Se ranged from 92–98% (n = 3).

Statistical analysis

Sheehan et al. (2014) suggested that population Hg biomarker distributions are often skewed, so the central tendency is best captured by geometric means or medians. As the distribution of Hg concentrations in hair was skewed and had a long upper tail, we calculated the geometric means for hair Hg concentrations and their corresponding 95% confidence intervals (CIs). We used log-transformed Hg values for statistical analyses. A multivariable regression model was used to examine the association of Hg levels with fish consumption and other personal characteristics after adjusting for the effect of age. The associations between log-transformed Hg in hair and age, and log-transformed Hg in hair and Se levels in toenails were assessed using the Spearman rank correlation coefficient. Stata version 14 software (StataCorp LP, College Station, TX, USA) was used for all statistical analyses. Figures were drawn using GraphPad Prism 7 software (Version 7.00, GraphPad Software Inc., La Jolla, CA, USA). *P* values for heterogeneity were obtained using the likelihood ratio test, and *p* values for trend were obtained using the multivariable regression model. All *p* values presented are two-sided and a *p* value of less than 0.05 was considered statistically significant.

3.5. Results

Relationship between mercury concentrations in hair and personal characteristics

Figure 3-1 shows the distribution of Hg concentrations in hair for males and females. The distributions of Hg concentrations in hair from both males and females were skewed and had long upper tails.

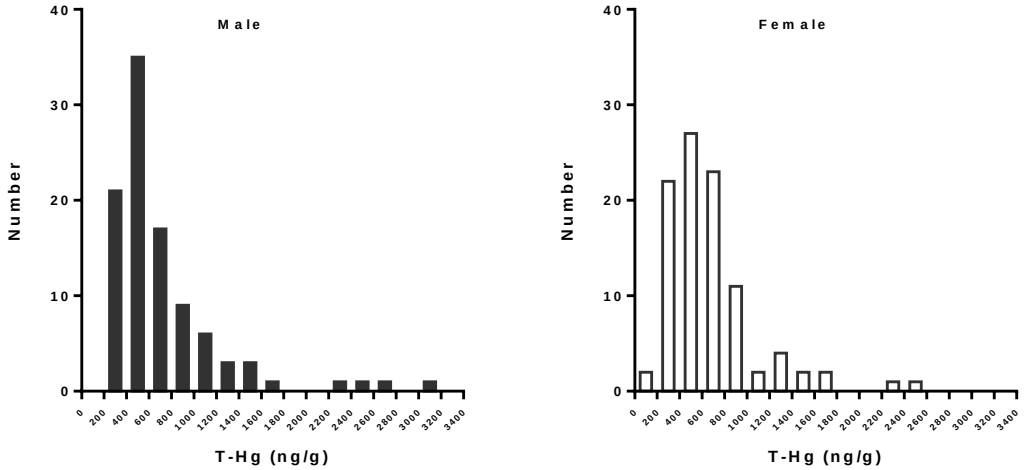

Fig. 3-1. Distribution histograms of mercury concentrations in hair of males and females.
Males: n = 99; Females: n = 97

The geometric means of Hg levels in males and female were 617 ng/g and 575 ng/g, respectively. The present study found no significant difference in Hg concentrations in hair from males and females. In addition, there was no significant difference in hair Hg concentrations among staff and outpatients with acne, tinea or sexually transmitted infections (data not shown). The highest geometric mean of Hg levels in hair was found in staff (603 ng/g), and the lowest Hg level was found in outpatients with acne (562 ng/g).

Figure 3-2 shows the relationships between age and log-transformed Hg concentrations in hair for males and females. There were significant positive correlations between log-transformed Hg concentrations in hair and age for males ($r = 0.37$, $p < 0.01$) and females ($r = 0.33$, $p < 0.01$). The log-transformed Hg concentrations in hair tended to increase with age.

The age-adjusted geometric means of Hg levels in hair from males and females grouped by personal characteristics are shown in Table 3-1. In the male population, there were no significant associations between the age-adjusted geometric mean of Hg levels and other characteristics. In contrast, female samples exhibited significant differences in the age-adjusted geometric means of Hg levels grouped by occupation and the frequency of eating out. Females grouped as “factory workers and house makers” and “farmers and craftspeople” had higher Hg levels in hair than those grouped as “state officers and retired state officers” or “lecturers and students”. A multivariate regression model adjusting for the effect of age showed that females who ate out < 3 times/week had higher Hg levels in hair than those who ate out ≥ 3 times/week.

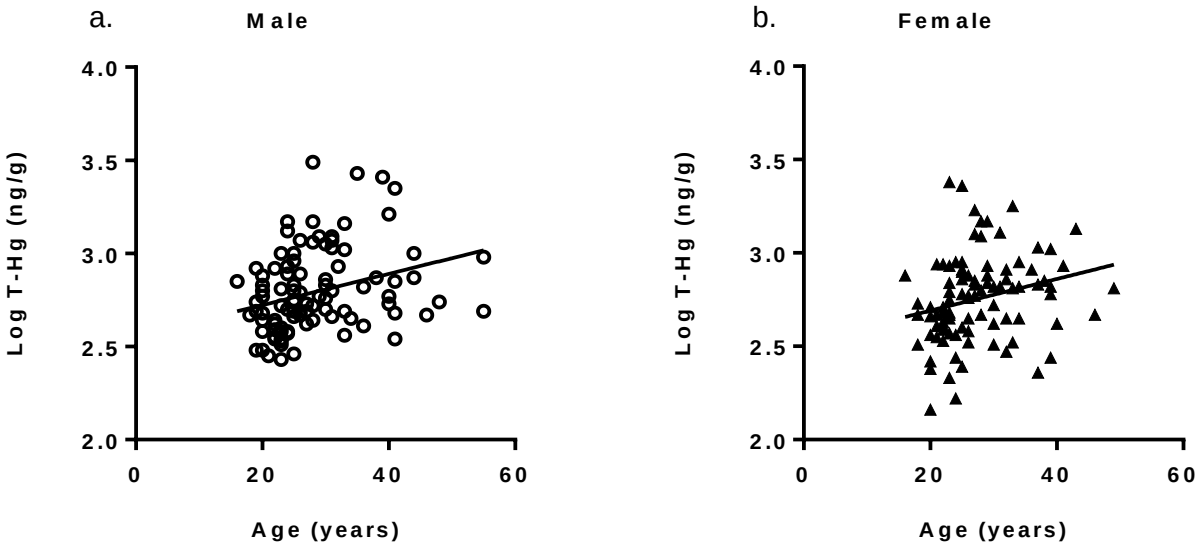

Fig. 3-2. Relationship between age and log-transformed mercury concentrations in hair of males and females.

a. Relationship between age and log-transformed mercury concentrations in hair of males.
○: Male: n = 99, r = 0.37, $p < 0.01$;

b. Relationship between age and log-transformed mercury concentrations in hair of females.
▲: Female: n = 97, r = 0.33, $p < 0.01$

Relationship between mercury concentrations in hair and fish consumption

Table 3-2 shows the age-adjusted geometric mean of Hg levels in hair from males and females grouped by frequency and amount of fish consumption. The female population showed a significant difference in the age-adjusted geometric mean of Hg levels among all subgroups: frequency of freshwater fish consumption (p = 0.027); frequency of marine fish consumption (p = 0.017); amount of freshwater fish consumption (p = 0.022) and amount of marine fish consumption (p = 0.033). The male population showed significant differences in the age-adjusted geometric mean of hair Hg levels related to their frequency of freshwater fish consumption (p = 0.019).

To investigate the effect of freshwater fish consumption on hair Hg levels, the age-adjusted geometric mean of Hg levels in hair from males and females was grouped by a frequency of marine fish consumption of "never or < 1 time/week", as shown in Table 3-3. After limiting the subjects who had a frequency of marine fish consumption grouped as "never or < 1 time/week", there were no significant differences in the age-adjusted geometric means of hair Hg levels between males grouped according to the frequency or amount of freshwater fish consumption. However, there were significant differences in the age-adjusted geometric mean of Hg levels in hair from females grouped by the frequency of freshwater fish consumption (p = 0.016). Females who had a higher fish consumption frequency showed higher age-adjusted geometric mean Hg levels in hair.

Table 3-1. Age-adjusted geometric mean of mercury levels (ng/g) in hair from males and females in relation to personal characteristics

Item	Male				Female			
	N	GM** (ng/g)	95% CI	p	N	GM** (ng/g)	95% CI	p
Total	**99**	**617**	**550–676**		**97**	**575**	**513–631**	
Occupation								
State officer and state officer retired	23	603	501–692		38	562	479–646	
Lecturer and student	23	603	537–692		24	562	501–631	
Factory worker and house maker	18	617	562–692	0.534	20	575	501–646	0.009*
Farmer and craftspeople	12	631	562–724		5	589	490–692	
Others	23	646	537–776		10	589	457–759	
Age								
16–24	40	490	372–646		39	479	355–661	
25–29	25	617	257–676	0.051	28	575	513–631	0.011*
30–39	21	759	589–955		25	676	479–955	
40–55	13	933	575–1479		5	794	427–1514	
Education								
Less than high school	13	617	490–794		6	525	372–741	
High school	19	617	550–708	0.356	15	550	468–646	0.896
Degree/diploma	67	617	550–692		76	575	513–646	
Marital status								
Single	50	589	501–692	0.433	46	537	447–646	0.460
Married	49	646	550–759		51	589	501–708	
Smoke								
Smoker	32	589	501–708	0.570	1	562	195–1585	N/A
Non-Smoker	67	631	562–708		96	562	513–631	
Hair treatment								
Treatment	4	513	302–851	0.431	62	550	479–631	0.468
Non-treatment	95	631	562–692		35	603	501–708	
Alcohol								
Drinker	84	617	550–692	0.844	11	457	339–631	0.158
Non-drinker	15	631	490–813		86	589	525–646	
Eat out								
Less than 3 times/week	65	603	525–676	0.322	74	617	550–692	0.001*
3 or more than 3 times/week	34	661	562–794		23	437	355–537	

**Significant difference.*
Males: n = 99; Females: n = 97
*** Geometric mean*

Table 3-2. Age-adjusted geometric mean of mercury levels (ng/g) in hair from males and females grouped by frequency and amount of fish consumption

Item		Male					Female				
					P values for					P values for	
		N	GM** (ng/g)	95% CI	heterogeneity	trend	N	GM** (ng/g)	95% CI	heterogeneity	trend
Frequency of consumption	**Freshwater fish**										
	Never or < 1 time/week	16	513	417–617			7	417	324–537		
	1–3 times/week	62	617	550–676	0.019*	0.028*	70	550	490–603	0.027*	0.009*
	≥ 4 times/week	21	741	617–891			20	724	589–871		
	Marine fish										
	Never or < 1 time/week	69	589	525–661			58	525	457–603		
	1–3 times/week	24	676	589–776	0.163	0.117	36	631	537–724	0.017*	0.062
	≥ 4 times/week	6	776	575–1023			3	759	550–1023		
Amount of consumption	**Freshwater fish**										
	Never or < 50 g/time	25	617	513–759	0.963		14	427	324–562	0.022*	
	≥ 50 g/time	74	617	550–692			83	603	537–661		
	Marine fish										
	Never or < 50 g/time	59	603	537–692	0.642		50	513	437–589	0.033*	
	≥ 50 g/time	40	631	550–741			47	631	550–741		

**Significant difference.*
Males: n = 99; Females: n = 97
*** Geometric mean*

Table 3-3. Age-adjusted geometric mean of mercury levels (ng/g) in hair from subjects with a frequency of marine fish consumption ≤ once/week

Item		Male					Female				
					P values for					P values for	
		N	GM** (ng/g)	95% CI	heterogeneity	trend	N	GM** (ng/g)	95% CI	heterogeneity	trend
Frequency of consumption	**Freshwater fish**										
	Never or < 1 time/week	13	513	407–646			7	380	282–501		
	1–3 times/week	45	589	525–661	0.218	0.180	39	501	447–575	0.016*	0.015*
	≥ 4 times/week	11	676	537–851			12	676	525–871		
Amount of consumption	**Freshwater fish**										
	Never or < 50 g/time	18	617	501–776	0.507		11	417	302–562	0.098	
	≥ 50 g/time	51	575	501–646			47	550	468–631		

**Significant difference; n = 127*

***Geometric mean*

Relationship between mercury concentrations in hair and selenium concentrations in toenails

Figure 3-3 shows the relationships between log-transformed Hg concentrations in hair and Se concentrations in toenails in groups defined by the frequency of freshwater fish and marine fish consumption. There was no significant correlation between log-transformed Hg concentrations in hair and Se concentrations in toenails in subjects that consumed freshwater fish. However, log-transformed Hg concentrations in hair and Se concentrations in toenails tended to increase with the increased frequency of marine fish consumption, and showed a positive correlation in subjects that consumed marine fish ≥ once/week ($r = 0.37$, $p = 0.047$).

Species of consumed fish and mercury concentrations in canned fish

Table 3-4 summarizes the species of fish commonly consumed by males and females examined in Hanoi. During one year, 93 males and 96 females consumed freshwater fish. The freshwater fishes consumed by males and females were common carp, roho labeo and tilapia. During one year, 56 males and 63 females consumed marine fish. Males typically consumed mackerel, salmon and scad, whereas females usually consumed basa, mackerel and scad. Tuna was the second lowest and least consumed fish species in the male and female populations, respectively.

Table 3-5 summarizes the Hg concentrations determined in the muscle of canned fish purchased in Hanoi. The highest Hg concentration was found in yellowfin tuna chunks in vegetable oil (T-Hg: 662.9 ng/g; MeHg: 589.5 ng/g) and the lowest Hg concentration was found in the muscle of sardines in tomato sauce from company C (T-Hg: 5.1 ng/g; MeHg: 5.1 ng/g). The values of MeHg as a percentage of T-Hg in all canned fish ranged from 90% to 100%.

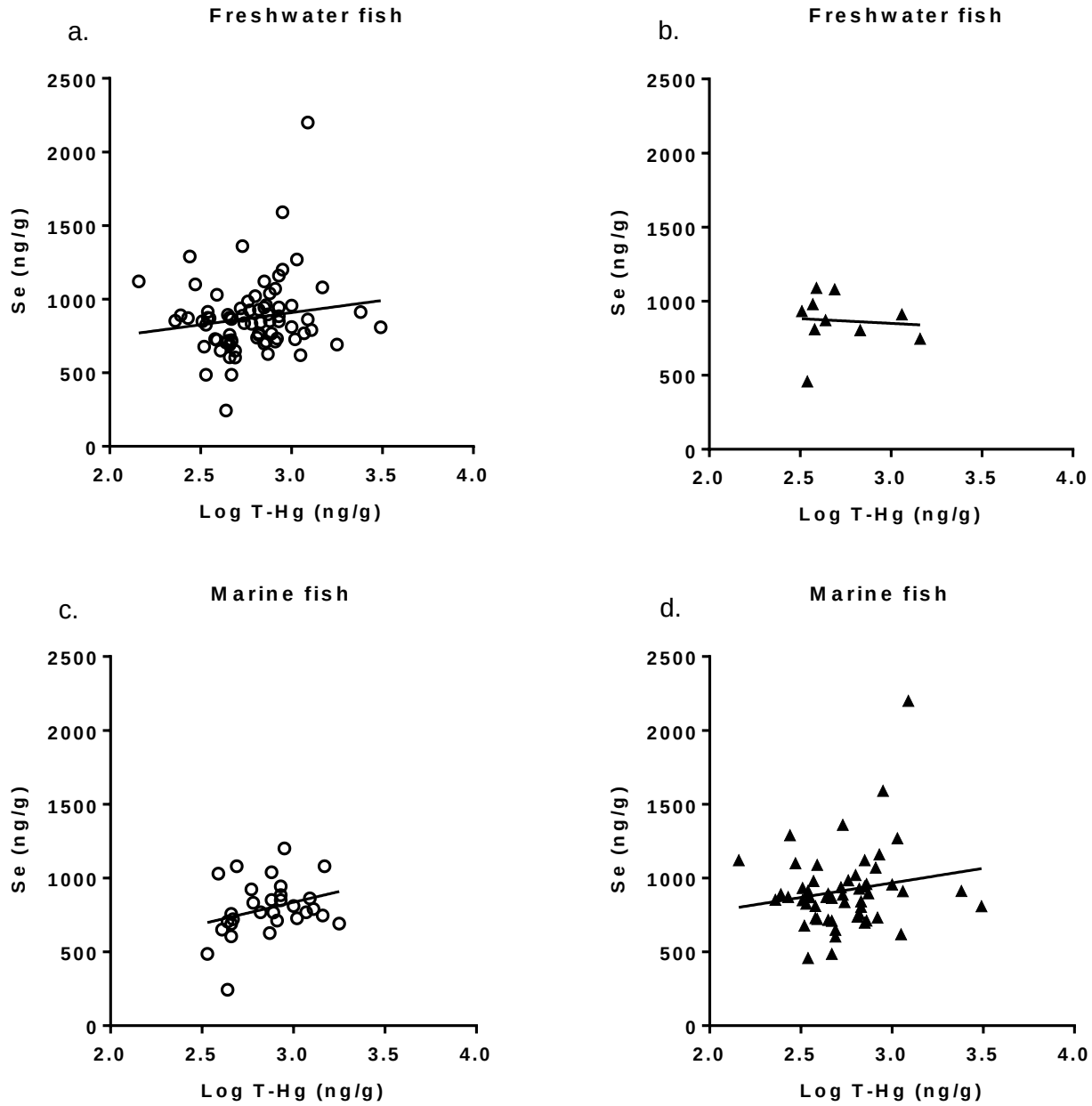

Fig. 3-3. Relationship between selenium concentrations in toenail and log-transformed mercury concentrations in hair.

Frequency of freshwater fish consumption: n = 86

a. ○: ≥ 1 time/week: r = 0.15, $p = 0.20$;

b. ▲: Never or < 1 time/week: r = 0.15, $p = 0.68$

Frequency of marine fish consumption: n = 86

c. ○: ≥ 1 time/week: r = 0.37, $p = 0.047$;

d. ▲: Never or < 1 time/week: r = 0.13, $p = 0.36$

Table 3-4. Fish species mostly commonly consumed by males and females in Hanoi, Vietnam

Species		Scientific name	Male		Female	
			N	%	N	%
Freshwater fish	**Total**		**93**	**100**	**96**	**100**
	Tilapia	*Oreochromis mosambicus*	72	77.4	76	79.2
	Common carp	*Cyprinus carpio* Linnaeus	71	76.3	70	72.9
	Roho labeo	*Labeo rohita*	51	54.8	53	55.2
Marine fish	**Total**		**56**	**100**	**63**	**100**
	Mackerel	*Scomberomorus maculatus*	38	67.9	39	61.9
	Scad	*Decapterus*	22	39.3	25	39.7
	Salmon	*Oncorhynchus spp*	17	30.4	16	25.4
	Tuna	Thunnini	13	23.2	8	12.7
	Basa	*Pangasius bocourti*	9	16.1	20	31.7

Table 3-5. Mercury concentrations (ng/g) in canned fish marketed in Hanoi, Vietnam

Type	Company	T-Hg (ng/g wet weight)	MeHg (ng/g wet weight)	MeHg/T-Hg (%)
Sardines in tomato sauce	A	81.6	73.8	90.4
Sardines in tomato sauce	B	7.1	6.8	95.8
Sardines in tomato sauce	C	5.1	5.1	100
Mackerel in tomato sauce	D	8.1	8.1	100
Tuna in oil	E	76.5	76.1	99.5
Tuna in oil	F	54.4	52.9	97.2
Yellow fin tuna chunks in vegetable oil	G	622.9	589.5	94.6
Light tuna chunks in oil	H	63.2	62.5	98.9
Tuna in vegetable oil	I	29.7	29.6	99.7
Tuna chunks in brine	J	78.3	76.1	97.2

n = 1 for each type of canned fish examined

3.6. Discussion

To the best of our knowledge, this is the first cross-sectional study to demonstrate the association between hair Hg levels and fish consumption using a dietary questionnaire to obtain information pertinent for assessing the health risk from MeHg exposure in Vietnam. In the present study, Hg levels in the hair of 98% of Vietnamese participants were lower than the provisional tolerable weekly intake (PTWI) for MeHg (1.6 μg Hg/kg body weight, which is equivalent to hair Hg levels of approximately 2300 ng/g, with an uncertainty factor of 6.4) (Joint FAO/WHO Expert Committee on Food Additives, 2003; Sakamoto et al., 2012). There was no significant difference in Hg concentrations in hair from males and females. Males and females also had no significant difference in the frequency of fish consumption, which may explain the similar levels of Hg in hair from both sexes.

In the present study, we calculated daily average MeHg intakes using the age-adjusted geometric mean of Hg levels in hair from males and females. The calculated daily average MeHg intakes for males and females (0.062 and 0.058 μg/kg per day, respectively) were lower than the EPA reference dose for MeHg of 0.1 μg/kg per day (United States Environmental Protection Agency, 1997; National Research Council, 2000). This suggests that males and females in the present study will not have an appreciable risk of deleterious effects from MeHg exposure through fish consumption.

We found that Hg levels in hair tended to increase with age for both males and females, consistent with the previously reported correlation between Hg levels in hair and age (National Research Council, 2000; McDowell et al., 2004). Fish consumption differs with age and older age groups of both males and females eat more fish than younger age groups (Weichselbaum et al., 2013; Otsuka et al., 2014). The present findings were consistent with the reported Hg exposure through the consumption of freshwater and marine fish in Pakistan (Shah et al., 2016) and Hg contamination in human hair and fish in Cambodia (Agusa et al., 2005).

In the present study, the age-adjusted geometric means for Hg levels in hair were significantly different between participants grouped by distinct occupations, and between females grouped by the frequency of eating out. Mercury levels in hair were previously correlated with the frequency of fish consumption (McDowell et al., 2004) and species of fish consumption (Burger, 2000). Hence, differences in the frequency and species of fish consumption may explain the different Hg levels in hair found in different occupation groups and in females that ate out more frequently.

Yasutake et al. (2003) reported that the treatment of hair samples with a lotion for artificial waving caused a 30% reduction in Hg levels. In the present study, we found no significant difference between age-adjusted Hg levels in hair from subjects who had hair treatment compared with non-treatment in both males and females. This finding agreed with the report that no difference was found in hair Hg levels among hair treatments and untreated groups in research on hair Hg levels in U.S children and women of childbearing age (McDowell et al., 2004).

In the present study, there was a significant difference in hair Hg levels between freshwater and marine fish consumption. However, inhabitants of Hanoi eat both freshwater and marine fish. Therefore, when subjects were limited to those who consumed marine fish ≤ once/week, we found higher Hg levels in the hair of females who had a higher frequency of freshwater fish consumption. Other confounding factors, such as the pattern of fish consumption and dietary interactions (Chapman and Chan, 2000; Bradley et al., 2017), may also explain differences in the hair Hg levels of females in this study. However, the current questionnaire did not include details of such factors, and further studies are necessary to fully explain the current observations.

We found a significant positive correlation between hair Hg levels and toenail Se levels in subjects who consumed marine fish ≥ 1 time/week. Past studies showed that levels of Hg in hair and Se in toenails

were correlated with blood Hg and Se levels, respectively (Thomson, 2004; World Health Organization, 2008). Furthermore, levels of both Hg and Se in human blood were related to fish consumption (Burger and Gochfeld, 2013). Thomson (2004) reviewed the interaction between Se and heavy metals and found a strong interaction between Se and Hg in marine foods. Burger and Gochfeld (2013) concluded that the mean and median of Se/Hg molar ratios were higher for marine fish than for all freshwater fish examined. Therefore, a significant positive correlation between hair Hg levels and toenail Se levels may relate to consumption of marine fish.

In the current study, freshwater fish commonly consumed by males and females included common carp, roho labeo and tilapia, whereas basa, mackerel, salmon and scad were the marine fish typically consumed by both males and females. These fish species are reported to have low levels of Hg (< 100 ng/g), except for carp with Hg levels in the intermediate range 100–500 ng/g (Ministry of Health, Labour and Welfare, 2003; World Health Organization, 2010; U.S. Food and Drug Administration, 2014). In contrast, tuna have high levels of Hg (World Health Organization, 2010) and was one of the least consumed fish by Vietnamese males and females. Therefore, low Hg levels in the hair of Vietnamese participants may reflect the consumption of fish with low Hg levels, and relatively low consumption of high Hg fish such as tuna.

We determined T-Hg and MeHg concentrations in commercial canned seafood purchased in Hanoi to survey the Hg concentration in seafood in Vietnam. Canned seafood is a known source of MeHg exposure (Boadi et al., 2011; Siedlikowski et al., 2016), and canned tuna contains high concentrations of Hg (Alcala-Orozco et al., 2017). In all samples tested, only Hg concentrations in the muscle of yellowfin tuna exceeded the maximum level allowed by WHO, 500 ng/g for MeHg in all types of fish (United Nations Environment Programme, 2002), and permitted by Japanese regulations, 400 ng/g for T-Hg and 300 ng/g for MeHg (Notice Kannyu No.99, 1973). The Hg concentrations in remaining tuna products were lower than the range (100–200 ng/g) reported by the FDA (Burger and Gochfeld, 2004; U.S. Food and Drug Administration, 2001; 2014). The Hg concentrations in the muscle of mackerels were lower than the levels previously reported (jack mackerel, Orleans: 61 ng/g and jack jurel mackerel, Chicken of the Sea: 50 ng/g) by Shim et al. (2004). Similarly, Hg concentrations in sardines were lower than the Hg levels in canned sardines (117 ng/g, 141 ng/g, and 143 ng/g) reported by Boadi et al. (2011). The current study found that the percentage of MeHg in T-Hg in canned fish samples ranged from 90% to 100%, which was consistent with levels found in fish, squid and shrimp in previous studies (Bloom, 1992; Yoshimoto et al., 2016; Hoang et al., 2017).

Fish processing in Vietnam plays an important role in producing high value products and contributing to export development. The main Vietnamese fish products include fresh, frozen, dried and canned, as well as fish sauce or paste; frozen fish are the main product, followed by dried fish and fish sauce or paste (Food and Agriculture Organization of the United Nations, 2004). In addition, fish supplies in Vietnam moved from freshwater fishing to freshwater aquaculture or cage culture, which made fish more available for consumption (Food and Agriculture Organization of the United Nations, 2004). Thong and Olsen (2012) reported that fish consumption in Vietnam was related to the quality, price and availability of fish. Therefore, the increased fish consumption in Vietnam may be related to the increased availability, better quality and reduced price of fish.

Recently, we reported that T-Hg, MeHg and Se concentrations in commercial shrimp in Japan, which included Vietnamese black tiger shrimp, were lower than the Japanese regulated level (Notice Kannyu No.99, 1973) of 300 ng/g for MeHg in fish (Hoang et al., 2017). Moreover, the average Se/Hg molar ratios in the muscle of commercial shrimp were comparatively high (in the range of 16–106). Other studies reported that Se may counteract Hg toxicity and Se/Hg molar ratios above one may increase the protective effect of Se against MeHg exposure (Peterson et al., 2009; Khan and Wang, 2009). We showed

that shrimp commercially available in Japan do not pose a particularly high risk regarding MeHg exposure to consumers. Further survey analysis of MeHg and Se concentrations in commercial fresh seafood in Vietnam is warranted for proper assessment of health risk due to MeHg exposure.

The strengths of the present study are: (1) this is the first cross-sectional study of the association between hair Hg levels and Vietnamese fish consumption, using a dietary questionnaire to obtain information for assessing the health risk from MeHg exposure in Vietnam; (2) we demonstrated that the age-adjusted geometric mean level of Hg in hair from Vietnamese females was related to freshwater fish consumption; (3) we found significant positive correlations between hair Hg levels and toenail Se levels among subjects who consumed marine fish ≥ once/week. Limitations of this study are: (1) we did not find the main reason for the significant difference in the age-adjusted Hg levels in hair of Vietnamese females related to the frequency of freshwater fish consumption; (2) we have not analyzed Hg levels in fresh fish and other seafood from Vietnam, and further studies are required to fully explain the current findings.

3.7. Conclusion

In conclusion, the present study has demonstrated that Hg levels in the hair of 98% of the Vietnamese participants were lower than the PTWI for MeHg at 1.6 μg Hg/kg body weight, with an uncertainty factor of 6.4 (Joint FAO/WHO Expert Committee on Food Additives, 2003). A significant difference in the age-adjusted geometric mean of Hg levels in hair from females was related to the frequency of freshwater fish consumption. In addition, Hg levels in hair and Se levels in toenails increased with the increased frequency of marine fish consumption, and showed a significant positive correlation among subjects who consumed marine fish ≥ once/week. The values of MeHg as a percentage of T-Hg in canned fish purchased in Vietnam ranged from 90% to 100%.

3.8. Acknowledgement

The authors are grateful to Drs. Le Huu Doanh, Le Huyen My, Tran Cam Van, Dinh Huu Nghi, Hoang Thi Ngoc Ly, Nguyen Thi Tuyen, Than Trong Tuy, Nguyen Thi Huyen Thuong, and Tran Thi Thanh Tam (National Hospital of Dermatology and Venereology) for their supports in the collection of human samples. We also thank Ms. Hazuki Kashiwagi and Megumi Kudo (National Institute for Minamata Disease) and Ms. Yukiko Uchiyama (Prefectural University of Kumamoto) for their technical assistance. This work was supported by the International Postgraduate Scholarship for Research on Mercury from the Prefectural University of Kumamoto, and a grant from the National Institute for Minamata Disease and Kagoshima University.

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Chapter IV. Future study

In the thesis research in Japan, I have learnt the analytical method for T-Hg and MeHg determination in biological samples, the survey study concerning T-Hg and MeHg concentration in seafood for risk assessment regarding MeHg exposure to consumers, and epidemiological study concerning MeHg exposure through seafood consumption. Based on these studies, I will continue to study with following direction and would like to contribute to Vietnam:

- Distribution of this analytical method for determining Hg concentration in food and human samples in Vietnam
- Survey of T-Hg and MeHg concentration in food in Vietnam.
- Epidemiological study concerning MeHg exposure through seafood consumption in Vietnam
- Contribution to prepare a guidance of fish and seafood consumption for MeHg exposure assessment of women at childbearing age in Vietnam