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Variation in Arsenolipid Concentrations in Seafood Consumed in Japan

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ABSTRACT

Variation in arsenolipid concentrations was assessed in 18 seafood samples including fish, shellfish, and crustaceans purchased in Japan. Analyses were performed by high performance liquid chromatography-inductively coupled plasma mass spectrometry/electrospray ionization tandem mass spectrometry. Stable isotope ratios for nitrogen and carbon were also measured in the samples for obtaining trophic level information of the species. Arsenic-containing hydrocarbons (AsHCs) and arsenic-containing fatty acids (AsFAs) were detected in the seafood samples; the toxic AsHCs were found in all of the seafood samples with large variation in the concentrations (83 ± 73 ng As/g fw, coefficient of variation=88%). Our previous point estimate of health risk of AsHCs intake via seafood consumption in Japan, based on average AsHC concentration in seafood, suggested insignificant risk, and the present study supports our previous estimate. AsHC concentrations significantly correlated with lipid content of the seafood samples ($r=0.67$, $p<0.01$), a result expected because of the fat solubility of the compounds. The AsHCs concentrations, however, were not significantly correlated with nitrogen stable isotope ratios suggesting that AsHCs do not biomagnify. The source of the observed large variation in AsHC concentrations will be the subject of further investigation.

INTRODUCTION

Arsenolipids are lipid soluble organic arsenic species with properties very different from arsenobetaine and other organic arsenic compounds commonly reported in marine foods, all of which are water soluble. Immediately after the first identification and characterization of the structure of arsenolipids by Morita and Shibata (1988), there were only a few attempts to identify arsenolipids, probably due to their chemical complexity and relatively low concentrations. In the last decade, however, arsenolipids have been identified and characterized in a wide range of marine organisms, where they are often found as a major form of arsenic especially in fish and fish products (Rumpler et al. 2008; Taleshi et al. 2008, 2010; Arryo-Abad et al. 2010; Lischka et al. 2013; Amayo et al. 2014; Sele et al. 2014;), and in some seaweeds (García-Salgado et al. 2012; Raab et al. 2013). There are several major groups of arsenolipids identified so far in marine organisms, such as arsenic-containing hydrocarbons (AsHCs), arsenic-containing fatty acids (AsFAs), arsenic containing phospholipids (AsPLs), and tri-methylated arsenic fatty alcohols (TMAsFOHs) (Garcia-Salgado et al. 2012; Raab et al. 2013; Amayo et al. 2013; Arroyo-Abad et al. 2013).

In 2014, an *in vitro* toxicity study of arsenolipids revealed that AsHCs were cytotoxic to human liver and bladder cells to a degree comparable with that of toxic inorganic arsenic (III) (Meyer et al. 2014). This result raised concerns regarding the possible health risk of arsenolipids particularly among people, such as the Japanese, who consume large amounts of marine foods that may be rich with lipid-soluble arsenicals.

By considering the reported cytotoxic nature of these arsenolipids to human cells, and their abundance in marine foods, daily intake of AsHCs and its associated human health risk was estimated based on a market basket survey in Japan (Amin et al. 2018). The estimated total daily intake of the reported cytotoxic AsHCs (56 ng As/kg bw/day) was not considered free from health risk and most of the daily intake of those AsHCs by the Japanese came from the food categories of “fish and shellfish” (50 ng As/kg bw/day). Since the estimated daily intake and health risk was a point estimate, it was needed to take into consideration the variation in daily AsHCs intake, as well as its health risk, among the population.

In the present study, we investigated AsHCs and AsFAs concentrations in various fish, shellfish, and crustacean species that Japanese people usually consume in their daily diet to get insight into the variation in daily intake of AsHCs in Japan. The variation was used for the estimation of variation in the associated health risks of

fish consumers. Moreover, the sources of variation in AsHCs levels in the seafood samples were also examined. In this work, stable nitrogen isotope ratios of fish and shellfish samples were measured for assessing if arsenolipids are biomagnified in marine food web. This information would be relevant because it would give us indication if arsenolipids are bio-concentrated or not.

MATERIALS AND METHODS

Sample collection and preparation

Fish, shellfish, and crustacean samples were purchased in supermarkets in Kashiwa, Chiba Prefecture, and Itakura, Gunma Prefecture, Japan. The purchased seafood included salmon, yellow tail, mackerel, tuna, sardine, horse mackerel, flounder, octopus, shrimp, squids, scallop, sea bream, crab, clam, skipjack, pacific saury, and whitebait. These seafoods were selected from the top 20 list of fishes and shellfishes that were purchased by the Japanese in 2016 (FIES 2017 Japan). Purchased seafood samples were individually washed with Millipore purified water (MQ), and the edible portion (mostly muscle tissue) was collected. Whole body was used for whitebait and whole soft tissue was used for scallop and clam because they are eaten as such in Japan. Flounder egg was separated from the fillet and also used as one of the samples. Then the processed samples were individually homogenized in a food processor (Cuisinart, San-ei Co., Ltd, Tokyo, Japan) for 5 minutes. A portion (30 g) of the homogenized sample was freeze dried in a 50 mL polypropylene tube; the weights of the sample before and after freeze drying were recorded and used to assign the moisture content.

Arsenolipid extraction

The extraction of arsenolipids from the seafood samples was performed by using a mixture of methyl tert-butyl ether (MTBE, $\geq 99.5\%$, CarlRothGmbH, Karlsruhe, Germany) and methanol (MeOH, 99.9%, CarlRothGmbH, Karlsruhe, Germany) as described previously (Amin et al. 2018; Glabonjat et al. 2014). This extraction was repeated twice and the combined MTBE/MeOH layer was evaporated to dryness, and then re-dissolved in 500 μ L of ethanol (EtOH, 99.9%, Merck, Buchs, Switzerland) for the arsenolipids analysis.

94

95 **Measurement of arsenolipids concentrations**

96 Arsenolipids including AsHCs and AsFAs were identified and determined by using high-performance
97 liquid chromatography (HPLC) - inductively coupled plasma mass spectrometry (ICP-MS)/electrospray ionization
98 tandem mass spectrometry (ESI-MS-MS). The detailed conditions for the measurements of arsenolipids were
99 described previously (Amin et al. 2018). Our target analytes in the present study were AsHCs because they are
100 reported to be cytotoxic. The three AsHCs, i.e., AsHC332, AsHC360, and AsHC404, were recorded as the sum of
101 AsHCs because for some samples, these peaks were not fully resolved from each other. Analytical quality of the
102 present method had been assessed by the measurement of arsenolipids in a certified reference material (CRM)
103 (NMIJ CRM 7405-a Hijiki) of seaweed origin. Although no values for arsenolipids were given for this CRM, the
104 measured values could be compared with those reported in other publication to find moderate agreement (Amin et
105 al., 2018).

106

107 **Stable carbon and nitrogen isotopes analysis of seafood samples**

108 For the stable isotope analyses, approximately 500 mg of the freeze dried seafood samples was defatted
109 with a mixture of MeOH/chloroform (2+1) and acetone by vigorous shaking. The solvent layer was removed after
110 centrifugation. After evaporating the residual solvent at 55°C, approximately 0.4 mg of defatted samples was
111 enclosed in a tin capsule and analyzed by elemental analyzer- isotope ratio mass spectrometry (Thermo Fisher
112 Scientific Flash 2000 EA coupled to a Delta V Advantage IRMS, Bremen, Germany). Stable isotope ratios were
113 expressed as $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ according to the ordinal notation by the following equation.

114

$$115 \quad \delta^{15}\text{N} \text{ or } \delta^{13}\text{C} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$$

116

117 where R represents $^{15}\text{N}/^{14}\text{N}$ or $^{13}\text{C}/^{12}\text{C}$. Isotope ratios are expressed in per mil (‰) relative to the ratio of
118 international reference standards, i.e., Pee Dee Belemnite for carbon and atmospheric N_2 for nitrogen. Analytical
119 errors (1σ) for nitrogen and carbon were <0.2‰ based on the results of ten replicate analyses of reference glycine,
120 alanine and histidine (SI Science Co., Ltd).

Lipid content analysis in seafood samples

The lipid contents of the 15 freeze dried seafood samples were determined by the Soxhlet extraction-gravimetric method performed in a commercial laboratory (Japan Food and Research Center, Tokyo). Lipid content was expressed as g lipid per 100 g of sample.

Statistical analysis

Calculation of descriptive statistics and Pearson correlation analysis were carried out by using SPSS software (Version 19J, IBM Co.Ltd., Tokyo, Japan). Significance levels was set at $p=0.05$.

RESULTS

Arsenolipid concentrations, lipid content, and $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of the seafood samples analyzed in the present study are shown in **Table 1**. Only the AsHCs and AsFAs concentrations are shown for arsenolipids because other arsenolipids were not detectable by the present analytical method. The limit of detection for the arsenolipids in the seafood samples varied from 0.4 to 1 ng As/g fw depending on the moisture content of the initial fresh sample. **Figure 1** presents a chromatogram of sea bream sample in which the three AsHCs peak are resolved to some extent.

Using HPLC-ICPMS, AsHCs were found in all of the 18 seafood samples with a wide range of concentrations from 6.5 to 241 ng As/g fw (coefficient of variation (CV) =88%) with the lowest being found in tuna and the highest in whitebait. AsFAs were also detected in all of the samples with concentrations ranging from 0.8 to 59 ng As/g fw except for tuna where AsFAs was below the detection limit (0.7 ng As/g fw).

Three of the samples (salmon, mackerel, and yellow tail) were not analyzed for lipid content and isotopes due to the unavailability of sufficient amounts of samples for the analysis. The lipid contents of the 15 seafood samples ranged from 0.4 to 23 g/100g of samples while $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ranged from -15.4 to -22.6 and 6.2 to 14.3 ‰, respectively.

The associations between AsHCs concentrations and lipid content and that between $\delta^{15}\text{N}$ were analyzed by correlation analysis after log-transformation of AsHCs concentrations and lipid contents since the distributions

of these two skewed positively (skewness>1). Scatter plots of AsHCs concentrations and lipid content (both log-transformed) and that of AsHCs concentrations (log-transformed) and $\delta^{15}\text{N}$ were presented in **Figures 2** and **3**, respectively. Significant positive correlation was found between lipid content and AsHCs concentration (**Figure 2**, $r=0.67$, $p<0.01$). AsHCs concentrations, however, were not correlated with $\delta^{15}\text{N}$ (**Figure 3**, $r=0.21$, $p>0.05$), suggesting that AsHC concentrations do not vary with the trophic level of the organisms.

DISCUSSION

Variation in arsenolipid levels in seafood and associated risk estimate

Although several studies have reported the presence of AsHCs with reported toxicity in marine fish species (Taleshi et al. 2008; Arroyo-Abad et al. 2010; Lischka et al. 2013), only a few studies has determined the concentrations of the compounds in seafood (Amayo et al. 2011; Taleshi et al. 2014). In this study, arsenolipids were quantified in various seafood samples, which are commonly consumed in Japan, to find that AsHCs and AsFAs were detectable with a wide range of concentrations (**Table 1**). On the other hand, other arsenolipids, including AsPLs and TMAAsFOHs, were not detectable.

In our previous study (Amin et al. 2018), the health risk presented by AsHCs intake via the consumption of seafood was estimated by calculating the margin of exposure (MOE, the ratio of No Adverse Effect Level (NOAEL) of a substance to daily intake of the substance). In that study, we used the half maximal (50%) inhibitory concentration (IC_{50}) value for AsHC332 obtained in Meyer et al. (2014) in place of the NOAEL for MOE calculations because NOAEL has not yet been established. The calculated MOE was 1150 suggesting that the health risk was not significant since typically an $\text{MOE}<100$ is regarded as presenting a risk (FAO/WHO 2009). Our MOE calculation was based on the reported cytotoxic AsHC332 intake, which was estimated from AsHC332 concentrations in a composite of 26 fish and shellfish species available on the Japanese market (40 ± 1.1 ng/g fw), because we took a market basket approach in that study (Amin et al. 2018). Therefore, our MOE was a point estimate but it was required a distribution estimate for the evaluation of health risks among Japanese people. The present study results demonstrated that the average concentration of AsHCs, presented as the sum of AsHC332, AsHC360, and AsHC404, of seafood samples was 83 ± 73 ng As/g fw with CV of 88% (**Table 1**). Based on this

result, range of the MOE for AsHC332 could be estimated on the assumption that the variation in the sum of AsHCs (CV=88%) was similar to ~~that~~ the variation in AsHC332; the 95 percentile of AsHC332 concentration in seafood sample could be assumed to correspond to 229 ng/g fw, which was calculated by multiplying average AsHCs concentration 82.9 ng/g fw by 2.76 (1 + 0.88 x 2). Consequently, our estimated MOE for AsHC332 in the previous study would be lowered (or elevated) by 2.76 times, being approximately 420. Therefore, it was suggested that risk can be still considered as insignificant even if we consider the possible variation in AsHC332 concentration among seafood.

It must be noted that the present minimum estimate of MOE (420) does not include variation in daily seafood intake among the population. To further characterize the distribution of AsHC332 intake, and consequently the health risk to a population, this variation needs to be included in the calculation. Such refinements will be addressed in our ongoing studies.

Source of variation in arsenolipid concentrations in seafood

The significant positive correlation (Pearson correlation coefficient, $r = 0.67$, $p < 0.01$) found between the AsHCs and lipid content of the seafood samples (**Figure 2**) indicated that lipid content was a significant reason for the variation in the AsHC concentrations in seafood. This correlation was expected because AsHCs are lipid-soluble and fat tissue would be a storage tissue for lipid soluble compounds. Another reason for this correlation can also be postulated. In the case of fish, arsenolipids could originate either directly from the diet or from bio-transformation of other arsenicals within the fish, or from a combination of these processes (Lunde 1972; Francesconi et al. 1990). Fatty acids have already been suggested as the precursors to AsHCs found in fish (Taleshi et al. 2008). The positive correlation between AsHCs and lipid contents found in the present study was in accordance with the notion that the abundance of lipids facilitated the fatty acids dependent bio-transformation of arsenic to AsHCs.

To test the assumption that trophic level could be another source of AsHC variations in seafood samples we measured $\delta^{15}\text{N}$ of seafood samples as an indicator of trophic level (Wada et al. 1991). Significant positive correlation with $\delta^{15}\text{N}$ has been reported for the concentrations of organochlorine compounds (Elliot et al. 2005; Bodin et al. 2008) and mercury (Yoshinaga et al. 1992; Muir et al. 2005) in organisms, consistent with the view that these chemicals are biomagnified along a food chain. However, we found no significant correlation ($r = 0.21$,

p>0.05) between AsHCs and $\delta^{15}\text{N}$ (**Figure 3**) suggesting that AsHCs did not accumulate along the trophic level of the organism, though the seafood samples in this study did not belong to a single food web.

The correlation between lipid content and AsHC concentrations ($r=0.67$) suggested that approximately 45% of the variation in AsHCs concentration among seafood samples could be explained by the lipid content of the sample. However, the insignificant correlation coefficient indicated that the variation was not explained by the trophic level. This result indicates that there are other sources that contribute to the variation in AsHCs concentration in seafood. Possible other sources of variation may include food habit and life span of organisms, and metabolism of species. Further studies are needed to clarify the sources of variation in arsenolipid levels in different marine organisms, and the results from such studies will be valuable from a food safety viewpoint.

CONCLUSION

The present study found large variation in AsHC concentrations (83 ± 73 ng As/g fw, CV=88%) in seafood samples including fish, shellfish. By considering the variation of AsHCs in seafood species, our previously found point estimate of MOE (1150) for toxic AsHCs would be lowered to 420 but it still does not pose a significant health risk to the Japanese consumers. Lipid content of seafood was identified as a source of variation in AsHC concentrations among seafood, but because trophic level was not, AsHCs appear not to biomagnify in food webs. Identification of other sources of variation in AsHC levels in marine organisms is warranted from the viewpoints of biology and food safety.

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FIGURE LEGEND

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313 **Figure 1** The HPLC-ICPMS chromatograms of sea bream sample. The vertical axis depicts ion count at $m/z=75$.

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315 **Figure 2** Scatter plot of log-transformed AsHC concentrations and lipid contents of the seafood samples.

316 Correlation coefficient $r=0.67$ was significant at $p=0.01$.

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318 **Figure 3** Scatter plot of log-transformed AsHC concentrations and crude $\delta^{15}\text{N}$ of the seafood samples. Correlation
319 coefficient $r=0.21$ was not significant.

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323 **Figure 1**

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326 **Figure 2**

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331 **Figure 3**

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Table 1 Arsenolipid concentrations (ng As/g, fw), lipid content (g/100g of sample), and stable isotopes ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) (‰) in 18 seafood samples

Seafood	AsHCs ^a	AsFAs ^b	Lipid content	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$
Tuna	6.50	<0.7	0.4	13.6	-17.2
Sea bream	80.2	16.2	18	12.8	-16.8
Pacific saury	109	35.9	21	7.70	-20.9
Squids	23.1	1.95	4.4	10.0	-16.6
Skipjack	211	21.5	4.6	9.90	-19.5
Sardine	196	59.2	23	9.10	-17.8
Scallop	50.9	6.46	4.2	7.80	-19.6
Horse mackerel	80.6	26.5	16	11.6	-17.8
Flounder	45.4	11.8	2.6	13.7	-16.0
Flounder eggs	171	5.2	3.3	14.0	-17.1
Octopus	19.4	5.70	2.2	14.0	-15.4
Clam	75.8	12.1	2.7	9.20	-17.6
Crab	63.8	5.60	2.2	14.3	-20.6
Shrimp	25.4	2.88	0.7	6.20	-22.6
Whitebait	241	5.13	4.9	9.80	-18.0
Salmon	51.5	0.84	NA ^c	NA ^c	NA ^c
Mackerel	11.8	4.96	NA ^c	NA ^c	NA ^c
Yellow tail	30.2	2.72	NA ^c	NA ^c	NA ^c
Mean $\pm$ SD ^d	83 $\pm$ 73	13. $\pm$ 15	7.4 $\pm$ 7.8	10.3 $\pm$ 2.3	-18 $\pm$ 2.

^a The sum of three arsenic hydrocarbon, AsHC332, AsHC360, and AsHC404

^b The sum of two arsenic containing fatty acids, AsFA362 and AsFA390

^c Not analyzed

^d Standard deviation