Studies on the analytical method of arsenic compounds in food

Doctoral Dissertation

STUDIES ON ANALYTICAL METHODS OF ARSENIC COMPOUNDS IN FOOD

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Graduate School of Marine Science and Technology
Tokyo University of Marine Science and Technology
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Contents

Abstract

The toxicity of arsenic depends on its chemical form. The relative toxicities of doses of arsenic that are lethal to 50% of a population (LD50) are as follows: As(III) > As(V) > monomethylarsonic(V) acid > dimethylarsinic(V) acid. Arsenobetaine, which is the major arsenic compound in seafood, is considered to be nontoxic. Inorganic arsenic in drinking water is associated with cancer risks in the skin, urinary bladder, and lung, and skin lesions in humans. The Codex Alimentarius Commission of FAO/WHO, Australia/New Zealand, China, and EU has set a regulatory limit for the amount of inorganic arsenic in rice, fish, fishery animals, and seaweed. In 2017, the Codex was amended with an addition regulation for inorganic arsenic in fish oil.

Chapter 1 describes a standardized analytical method that can be used to test for compliance with international regulations regarding the amount of inorganic arsenic in seaweed and seafood. Several arsenic species could be quantitated after heating food samples at 100°C in 0.3 mol/L nitric acid. Arsenic speciation was measured by liquid chromatography-inductively coupled plasma-mass spectrometry (LC-ICP-MS) using an ODS column with a mobile phase containing an ion-pair reagent. Limits of detection (LODs) (0.0023–0.012 mg/kg), limits of quantitation (LOQs) (0.0077–0.042 mg/kg), repeatability (3.0–7.4%), intermediate precision (4.4–7.4%), and accuracy (recoveries of 94–107% based on spikes) of the proposed method were deemed satisfactory.

Inorganic arsenic concentrations were measured in nine seafood samples (muscle of albacore, muscle of rainbow trout, muscle of red-eye round herring, whole body of northern shrimp, mantle muscle of the Japanese common squid, adductor muscle of the Yezo giant scallop, soft tissue of the Japanese oyster, nam pla fish sauce, and oyster sauce). Inorganic arsenic was detected in the soft tissue of the Japanese oyster and in the nam pla and oyster sauces. Since intestinal organs typically contain higher levels of inorganic arsenic than the adductive muscles in oysters and sardines, the inorganic arsenic detected

in the nam pla and oyster sauces is most likely derived from the internal organs of the raw shellfish and fish used in their production.

Inorganic arsenic concentrations were measured in eight dried seaweed samples: kelp, *nori*, *wakame*, sea lettuce, green laver, *mozuku*, boiled *akamoku*, and boiled *hijiki*. High levels of inorganic arsenic were detected in the *hijiki Sargassum fusiforme* and *akamoku S. horneri* dried seaweed products. Inorganic arsenic can be extracted from *hijiki* by boiling with sea salt. Boiling *akamoku* in tap water, or in a 1% sea salt solution, resulted in a dried product containing less than 1 mg/kg of inorganic arsenic.

Chapter 2 describes the validation of an analytical method for quantitating inorganic arsenic in fish oil and fish oil capsules. Inorganic arsenic was extracted from these samples by heating at 80°C in 1.6% tetramethylammonium hydroxide (TMAH)-ethanol. In accordance with the methods described in Chapter 1, the concentration of inorganic arsenic in fish oil was determined by LC-ICP-MS using an ODS column with a mobile phase containing an ion-pair reagent. LODs (0.015, 0.004 mg/kg), LOQs (0.048, 0.011 mg/kg), repeatability (3.4, 3.5%), intermediate precision (4.3, 3.5%), and accuracy (recoveries of 94–109% based on spikes) of the proposed method were deemed satisfactory.

Inorganic arsenic concentrations were also measured in three fish oil samples and four fish oil capsules. Both samples contained less than 0.1 mg/kg inorganic arsenic, which is the regulatory limit given for inorganic arsenic in fish oil, according to the aforementioned Codex. Extraction rates of inorganic arsenic were calculated by comparing the total arsenic concentrations of the extracts and samples, showing that nearly all of the arsenic had been extracted. In addition, As(III) was more soluble in fish oil than was As(V).

Chapter 3 provides a comprehensive discussion of these results.

This study describes a means of analyzing inorganic arsenic in all food groups. The adverse effects of ingesting inorganic arsenic via rice and/or *hijiki* consumption have not been identified. Arsenic in marine products has been a known problem for many years. The health risks and toxicity of arsenic intake via seaweed consumption need to be

characterized.

Introduction

The various chemical forms of arsenic found in marine organisms exhibit vastly different toxicities. The relative concentrations of the different forms are important for people with diets that consist of large proportions of marine products. Kaise *et al.* (1–3) measured the LD50, *i.e.*, the dose that is lethal to 50% of a population, of each arsenic compound found in marine animals. LD50 values in mice following the oral administration of As(III), monomethylarsonic(V) acid [MMA(V)], dimethylarsinic(V) acid [DMA(V)], and trimethlarsine oxide (TMAO) were 34.5, 1,800, 1,200, and 10,600 mg/kg, respectively. Arsenobetaine (AB), the most prominent arsenic compound in seafood, was practically nontoxic, with an LD50 higher than 10,000 mg/kg. These data are quoted in the "Safety Evaluation of Certain Contaminants in Food", by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (4). The JECFA reports relative arsenic toxicities as As(III) > As(V) > MMA(V) > DMA(V). Shiomi *et al.* (5) contributed to the identification of arsenic compounds in marine animals. They reported an LD50 of 890 mg/kg for the tetramethlyarsonium (TeMA) found in the gills of clams (6). Kaise *et al.* (7) examined the cytotoxicity of arsenosugar and reported an acute toxicity between that of AB and MMA.

Hanaoka discusses the cycle of arsenic compounds in marine ecosystems (8). In their hypothesis, inorganic arsenic is sourced from the seafloor by tectonic activity and weathering and is converted to arsenosugars or arsenocholine (AC) by phytoplankton and/or marine algae. These are converted to AB by marine animals, and then converted back to inorganic arsenic by microorganisms. In addition, certain microorganisms may synthesize AB directly from inorganic arsenic.

Hanaoka *et al.* also found TeMA in the roasted muscles of seafood (10). Assuming no contamination had occurred during cooking, a very small quantity of TeMA had been converted from AB. In addition, Yoshida *et al.* (11) reported finding only AB in the fish-based processed food “hanpen”. These data suggest that arsenic does not change form

during food processing.

The *in vivo* chemical reactions of As(III) and As(V) result in different paths to toxicity (12). As(III) can act as a cytotoxin, oxdizing the thiol groups of enzymes or proteins (12). Symptoms of As(III) cytotoxicity include skin darkening and/or cornification, hair loss, weakness in the limbs, amyotrophy, vomiting, diarrhea, and loss of appetite. As(V) cannot combine with thiol groups and is therefore less toxic than As(III). In contrast, *in vivo* studies have shown that As(V) competes with phosphorus, acting as a decoupling agent of oxidative phosphorylation (12).

Inorganic arsenic in drinking water is associated with an increased risk of cancer in the urinary bladder and skin, and with lung and skin lesions in humans (4). In 1988, the JECFA issued a provisional tolerable weekly intake (PTWI) of 0.015 mg/kg body weight for inorganic arsenic. This, however, was withdrawn in 2010 based on the epidemiological findings of a cohort study in northeastern Taiwan. To re-issue a more accurate PTWI, the JECFA continues to collect data related to exposure assessments and the toxicology, analytical methodology, and occurrence of inorganic arsenic in food and drinking water (4).

Ingested inorganic arsenic is rapidly excreted in human urine following biotransformation, *i.e.*, reduction and methylation, to MMA and/or DMA (4). In contrast, organic arsenic, such as AB and various arsenosugars, undergoes very little biotransformation after ingestion and is excreted almost unchanged (4). Kaise *et al.* (1) reported that AB in fish was difficult to metabolize *in vivo*. Fukui *et al.* (13) reported that the arsenosugars found in seaweed are rapidly excreted.

Shiomi *et al.* reported the fractional determination of inorganic arsenic and organic arsenic in marine organisms using a solvent extraction method (14–16). With the exception of the brown alga, *hijiki*, most marine organisms were found to contain very low levels of inorganic arsenic. In general, arsenic was present at much higher levels than other heavy metals, with water-soluble arsenic compounds making up 80% of the total arsenic content.

In 2004, the Food Standards Agency of the UK began advising people not to eat *hijiki*, citing a survey indicating high levels of carcinogenic, inorganic arsenic (17).

Since 2012, many individual countries and international organizations have issued regulatory limits for inorganic arsenic, ranging from 0.1 to 2 mg/kg. Any technique being used to test against these regulations must be able to routinely measure inorganic arsenic levels at or below 0.1 mg/kg. In China, inorganic arsenic is regulated in rice, aquatic animals and products thereof, and supplementary foods for infants and young children (18). Inorganic arsenic levels in crustaceans, fish, mollusks, and seaweed are regulated in Australia and New Zealand (19). In the EU, maximum levels of inorganic arsenic are set for rice and rice-based products (20). In the Codex Alimentarius Commission of the FAO/WHO, maximum levels of inorganic arsenic in polished rice and husked rice were established in 2014 and 2016 (21).

Maximum levels of food contaminants defined in the Codex must also be applied to commodities being traded internationally. For example, high levels of arsenic have been measured worldwide in Japanese rice. Much of the data regarding the arsenic content of various countries' rice were acquired before maximum permissible levels were established. These levels of Japanese rice were determined using an analytical method that was developed in Japan, consisting of extraction with hot dilute nitric acid and quantitative detection by liquid chromatography-inductively coupled plasma-mass spectrometry (LC-ICP-MS) (22–24). However, there are many methods that have been used for arsenic speciation analyses in rice and rice-base products, including extraction with various solvents, including water (25) and nitric acid (22–24, 26), and enzymes (27, 28). However, given the expense of ICP-MS instrumentation, several other strategies have been developed for measuring inorganic arsenic in foods, including inductively coupled plasma-atomic emission spectrometry (ICP-AES), which relies on the hydrogenation of inorganic arsenic after extraction with dilute nitric acid (29), and atomic absorption spectrophotometry (AAS), which employs a solid-phase separation step following

extraction with dilute nitric acid (30).

Arsenic intake from every food group has been estimated using the domestic market basket method in Japan. Daily arsenic intake was measured to be 98.3 µg from fishery products, 60.8 µg from vegetables and seaweed, and 11.3 µg from rice. The arsenic intake from fishery products, vegetables, seaweed, and rice makes up approximately 96% of the total arsenic intake (31). The JECFA published the arsenic contents of foods obtained from Brazil, France, Japan, and Singapore. The highest total arsenic concentrations were found in seaweed, fish and shellfish, mushrooms and fungi, rice and rice products, and some meat products (4). The food groups containing higher levels of arsenic are common in the Japanese diet. In contrast, relatively little arsenic is sourced from mushrooms and meat products because the average intake of these foods is relatively low in Japan. The JECFA has also reported the levels of inorganic arsenic in foods obtained from Japan, France, and Singapore. Levels of inorganic arsenic in foods and beverages did not usually exceed 0.1 mg/kg. However, seaweed, rice, and some fish and seafood commodities contained relatively high levels of inorganic arsenic (4). Consequently, the concentrations of each chemical form of arsenic in seaweed and seafood need to be taken into account when discussing overall toxicity. The JECFA also states the current need for validated and orthogonal methods for the selective extraction and quantitation of inorganic arsenic in foods.

An analytical method for measuring inorganic arsenic levels in seaweed and seafood remains to be developed and validated. The extraction efficiencies of various solvents, including water (32, 33), nitric acid (34, 35), alkaline reagents (36), enzyme solutions (37, 38), and methanol/water mixtures (39), have been evaluated toward this end. Taylor *et al.* reported that the arsenic extraction efficiency of 1% nitric acid was higher than that of a methanol/water (1:1) solution for seaweed (40). Pell *et al.* (41) used a water extraction method to determine the concentrations of arsenosugars in seaweed but gave priority to maintaining the structure of the arsenosugars over the extraction efficiency. Jia *et al.* (42)

compared the efficiencies of arsenic extraction from seafood using water, methanol-water mixtures, and dilute nitric acid with the latter yielding the highest extraction efficiency. Pétursdóttir *et al.* (43) compared the efficiencies of arsenic extraction from seafood among nine different solvents. The highest extraction efficiency was obtained with a mixed solvent containing 2% nitric acid and 3% hydrogen peroxide. Zmozinski *et al.* (44) reported excellent extraction using 0.2% nitric acid in a solution of 1% hydrogen peroxide.

Nagaoka *et al.* showed that 0.3 mol/L nitric acid was suitable for the extraction of inorganic arsenic from seaweed and seafood in a dry block bath (34). Microwave heating was not required, since the highest extraction efficiency was obtained at 100°C (23, 45). The evaluation of inorganic arsenic must be the sum of As(III) and As(V), because As(III) can change to As(V), or As(V) to As(III), during the extraction process. Since arsenosugars may decompose to MMA or DMA using the extraction method reported previously, the concentrations of organic compounds were only advisory. Extraction rates were calculated from the total arsenic concentration of each sample and each extract to confirm sufficient extraction.

Selection of the column used to separate arsenic species is important in quantitative LC-ICP-MS analyses. Anion exchange columns can separate As(III), As(V), MMA, and DMA from rice matrices. However, AB, which is the primary form of arsenic in seafood, is co-eluted with As(III) at neutral pH. Therefore, an alkaline buffer is often used as a component of the mobile phase with a gradient elution (46). In recent reports detailing arsenic speciation, both anion and cation exchange columns were used for separating and quantitating As(III), As(V), MMA, DMA, AB, AC, and TMAO (40, 41, 44, 47). However, for simplicity, and in accordance with previous reports (34, 48), the current method employs an octadecyl-silica (ODS) column and a mobile phase containing an ion-pair reagent. This strategy is time-consuming and requires that the pH of the mobile phase be carefully adjusted. However, it is rare that peaks corresponding to different arsenic species overlap because the ODS column separates compounds according to their relative

hydrophobicity. While this strategy can be dated back to the 1980s, it is still frequently used in Japan.

The established analytical method can be evaluated using the “Standard Method Performance Requirements (SMPRs) for Quantitation of Arsenic Species in Selected Foods and Beverages” written and adopted by the AOAC (49). The evaluation may be an on-site verification, a single laboratory validation, or a multi-site collaborative study. Suitable foods are rice, rice-based products, fruit juice, fish oil-based supplements, and seafood. The preferred analytical technique is ion chromatography (IC)-ICP-MS or LC-ICP-MS. Acceptance criteria include analytical range, limit of quantitation (LOQ), recovery, repeatability (RSD_r), and reproducibility (RSD_R). The requirement ranges are determined by the analyte concentration. For example, when analyzing a sample containing 0.1 ppm arsenic, the acceptable recovery range is 60–115% with less than 13% and 20% repeatability and reproducibility, respectively.

The Codex defines a maximum total arsenic level of 0.1 mg/kg in fish oil. In 2017, the Codex refined this regulation, stating that inorganic arsenic levels in fish oil must be less than 0.1 mg/kg when total arsenic is present at more than 0.1 mg/kg (21). Fish oil contains low levels of both organic and inorganic arsenic. Therefore, a sensitive analytical method for measuring inorganic arsenic levels in fish oil is required.

Fish oil is produced mainly as a side-product of the fisheries industry, with most being used as feed (50). It also contains relatively high levels of essential fatty acids, such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), and is used as a health supplement for the prevention of arteriosclerosis and myocardial infarction.

Arsenic in fish oil is a result of high arsenic levels in the fish being proccesed. While the major arsenic compound in fish is the non-toxic AB, various forms inorganic arsenic are also present. Given their variability in toxicity level, it is necessary to determine the concentration of every chemical form of arsenic in fish oil.

Several techniques have been reported for measuring arsenic speciation in various oils

(46, 51, 52). Nut oil was dissolved in an organic solvent and arsenic species were analyzed by IC-ICP-MS following their extraction with water (51). Using water to extract arsenic from oils is a relatively common strategy (53). Chu *et al.* (46) analyzed arsenic speciation in soybean oil by IC-ICP-MS following extraction with hot 0.5% nitric acid in 80% methanol. López-García *et al.* (52) screened various oils for water-soluble arsenic species using a 3:1 isopropanol:3% v/v nitric acid extraction solution and electrothermal atomic absorption spectrometry.

It is difficult to confirm the extraction efficiency of arsenolipids from fish oil by the water extraction method (54, 55). To quantitatively extract and separate arsenolipids and other arsenic compounds from fish oil, this study first hydrolyzed the fish oil in accordance with the cholesterol analysis procedure described in Methods of Analysis in Health Science (56), which targets cholesterols, squalene, and other hydrocarbons. In this procedure, cholesterol was hydrolyzed in 56.1 mol/L KOH in ethanol. This study used tetramethylammonium hydroxide (TMAH) as the hydolysis reagent to avoid introducing alkali salts into the ICP-MS. After hydrolysis, the pH of the extracted solution was acidified to match to that of the LC mobile phase (pH 3) and the extracts were filtered to remove insoluble oils. To confirm complete dissolution of arsenic, total arsenic in each extract was measured prior to acidification. Extraction rates were calculated by comparing the total arsenic concentrations of the extracts and samples. Arsenolipids, but not inorganic arsenic, were precipitated during acidification. Since commercially available fish oils have been purified and any arsenic compounds removed, arsenic extraction rates were confirmed using crude fish oils.

Fish oil capsules are eaten as food supplements and fish oil is an ingredient in a variety of functional foods. Therefore, the method described in Chapter 1 for determining levels of inorganic arsenic in seafood and seaweeds was applied to arsenic analyses in fish oil. Fish oil is typically packaged in soft-gel capsules, which are insoluble in ethanol and other organic solvents used for lipid extraction. This study employed a combination of

ethanol and TMAH, which is water-soluble, to dissolve soft-gel capsules and extract fish oils. After extraction, the samples were hydrolyzed by heating in alkaline solution.

LC-ICP-MS was used to quantitate the different arsenic species in fish oil. Samples were separated on an ODS column with a mobile phase containing an ion-pair reagent. Phosphoric acid was included in both the extraction and standard solutions to prevent peak tailing that may result from deterioration of the LC system or the column (57). The solubility of inorganic arsenic in fish oil was also examined by adding As(III) or As(V) to fish oil under acidic conditions.

Chapter 1 describes the validation of an analytical method for the determination of inorganic arsenic in seaweed and seafood. Arsenic speciation was determined by LC-ICP-MS using an ODS column with a mobile phase containing an ion-pair reagent. Inorganic arsenic concentrations were measured in ten seafood samples and eight dried seaweed samples.

Chapter 2 describes the validation of the method for determining the concentrations of inorganic arsenic species in fish oil and fish oil capsules. Arsenic was extracted by heating the fish oil samples at 80°C in 1.6% TMAH-ethanol. As decribed in Chapter 1, inorganic arsenic levels were measured by LC-ICP-MS using an ODS column with a mobile phase containing an ion-pair reagent. Inorganic arsenic concentrations were measured in three fish oil samples and four samples of fish oil capsules. Both types of sample contained less than 0.1 mg/kg inorganic arsenic, which is the regulatory limit for inorganic arsenic for fish oil given by the Codex Alimentarius Commission.

Chapter 3 is a comprehensive discussion relating the results presented in Chapters 1 and 2.

Chapter 1
Determination of inorganic arsenic in seaweed and seafood by LC-ICP-MS: method validation

Seaweed and seafood contain both inorganic and organic arsenic compounds. Since arsenic toxicity depends strongly on its chemical form, risk assessment analyses for arsenic consumption need to account for speciation.

Arsenic intake from every food group in the Japanese diet has been estimated using the domestic market basket method. Estimates of daily arsenic intake were 98.3 μg from fishery products, 60.8 μg from vegetables and seaweed, and 11.3 μg from rice, acounting for approximately 96% of the total arsenic intake (31). However, most of the arsenic in fisheries products and seaweed exists as AB, which is considered non-toxic. Therefore, meaningful measures of arsenic levels in food must account for arsenic speciation and chemical form.

Methods for arsenic speciation analysis in rice and rice products have employed various extraction solvents such as water (25), nitric acid (22–24, 26), and enzyme solutions (27, 28). Extraction solvents for seaweed and seafood have included water (32, 33), nitric acid (34, 35), alkaline reagents (36), enzyme solutions (37, 38), and methanol/water mixtures (39).

Several analytical methods for measuring arsenic speciation in seaweed and seafood have been reported (32–45). One of the most efficient solvents for extracting arsenic from these matrices is hot, diluted nitric acid. A previous study used 0.3 mol/L nitric acid at 80°C for 1 h to extract the arsenic compounds from *hijiki* (34). This strategy was further adapted to extractions of inorganic arsenic from other seaweeds and seafood by heating at 100°C for 2 h (24).

This study validated an analytical method for quantitating inorganic arsenic levels in seaweed and seafood. Arsenic extraction was carried out by heating at 100°C in 0.3 mol/L

nitric acid and arsenic speciation was measured by LC-ICP-MS using an ODS column with a mobile phase containing an ion-pair reagent.

1.1 Method validation for analysis of inorganic arsenic in seaweed and seafood

An analytical method for quantitating levels of inorganic arsenic in seaweed and seafood was validated in a single laboratory setting. The method was evaluated for linearity, limits of detection (LODs), LOQs, recovery, precision, and accuracy. Samples containing low levels of inorganic arsenic, *e.g.*, dried *nori* product (seaweed), Japanese oyster (seafood), and oyster sauce (seafood product), were used to calculate LODs and LOQs.

Experimental

Samples

Dried seaweed products (kelp *Saccharina angustata*; *nori Pyropia yezoensis*; *wakame Undaria pinnatifida*; sea lettuce *Ulva pertusa*; green laver *Ulva prolifera*; *mozuku Nemacystis decipiens*; *akamoku Sargassum horneri (Turner) C. Agardh*; and *hijiki Sargassum fusiforme*), muscles of albacore (*Thunnus alalunga*), rainbow trout (*Oncorhynchus mykiss*), red-eye round herring (*Etrumeus teres*), Japanese sardine (*Sardinops melanostictus*), the edible portion of northern shrimp (*Pandalus eous*), the mantle muscle of the Japanese common squid (*Todarodes pacificus*), Yezo giant scallop (*Mizuhopecten yessoensis*), the edible portion of the Japanese oyster (*Crassostrea gigas*), and processed products (nam pla and oyster sauce) were purchased from a local market in Japan. Seaweed and seafood samples were homogenized in a food processor. NMIJ CRM 7405-a (*hijiki* seaweed) and NRCC DORM-4 (fish protein) were used as reference materials.

Apparatus

(a) *LC system*. 1200 Series LC system (Agilent Technologies, Tokyo, Japan).

(b) *ICP-MS*. 7500ce and 8800 ICP-MS instruments (Agilent Technologies) were used for quantitative analyses of total arsenic.

(c) *Microwave digestion system*. An Ultrawave (Milestone MLS, Leutkirch, Germany) microwave instrument was used for sample digestion prior to total arsenic determination.

(d) *Dry block bath heating system*. An EB-303 (As One, Osaka, Japan) dry block bath was used to heat samples during extraction.

An LC system, coupled to an ICP-MS instrument, was used to quantitate arsenic speciation.

Reagents and standards

a) *Ultrapure water*. Milli-Q system (> 18 MΩ·cm) (Millipore Corp., Billerica, MA, USA) water was used throughout this study.

(b) *Nitric acid* was ultrapure grade, Kanto Chemical Industries, Ltd., Tokyo, Japan

(c) *Methyl orange* was obtained from Kanto Chemical Industries, Ltd.

(d) *Aqueous ammonia* was obtained from Kanto Chemical Industries, Ltd.

(e) *Sodium 1-butanesulfonate* was obtained from Fujifilm Wako Pure Chemical Corporation, Osaka, Japan.

(f) *Malonic acid* was obtained from Fujifilm Wako Pure Chemical Corporation.

(g) *Methanol* was obtained from Fujifilm Wako Pure Chemical Corporation.

(h) *Tetramethylammonium hydroxide (TMAH)* was ultrapure grade and obtained from Tama Chemicals Co., Ltd., Kanagawa, Japan.

(i) *Standard solution of certified reference material (CRM)*. Certified reference material (NMIJ CRM 7912-a), DMA certified reference material (NMIJ CRM 7913-a), and AB certified reference material (NMIJ CRM 7901-a) were purchased from The National Metrology Institute of Japan/National Institute of Advanced Industrial Science and Technology (Ibaraki, Japan).

(j) *Standard solutions of the Japan Calibration Service System*. Standard solutions containing as As(III) and Te (tellurium) (internal standard) were purchased from Kanto Chemical Industries, Ltd.

(k) *Standards for arsenic speciation*. MMA, TMAO, TeMA, and AC were purchased from Tri Chemical Laboratories, Inc. (Yamanashi, Japan) and dissolved in water to prepare arsenic speciation standard solutions.

Preparation of reagents and standard solutions

(a) *0.3 mol/L nitric acid*. Nitric acid (1.92 mL) was added to a 100-mL volumetric flask containing approximately 50 mL of water and diluted to the mark with water.

(b) *0.1% methyl orange solution*. Methyl orange (0.1 g) was weighed into a 100-mL volumetric flask and diluted to the mark with water. The resulting mixture was passed through a membrane filter to remove any precipitate.

(c) *Mobile phase*. 1.458 g of 25% TMAH, 1.602 g of sodium 1-butane sulfonate, 0.416 g of malonic acid, and 0.5 mL of methanol were added to a 1-L beaker with 900 mL of water. The pH of the solution was adjusted to 3.0 with ultrapure nitric acid and confirmed with a pH meter. The solution was transferred to a 1-L volumetric flask and diluted to the mark with water.

(d) *Stock standard solutions of monomethylarsonic acid, trimethylarsine oxide, tetramethylarsonium, and arsenocholine (100 μg/mL as As)*. Stock standard solutions were prepared individually. MMA (0.0474 g) was placed in a 100-mL volumetric flask and diluted to the mark with water. A separate 100-mL volumetric flask was charged with 0.0363 g of TMAO, 0.0350 g of TeMA, and 0.0327 g of AC and diluted to the mark with water.

(e) *Intermediate standard solutions (1 μg/mL as As)*. As(V), As(III), MMA, TMAO, TeMA, and AC stock solutions were prepared by diluting stock standards 100-fold with water. Intermediate standard solutions were prepared immediately before use.

(f) *Intermediate standard solutions (1 μg/mL as As)*. Separate 50-mL volumetric flasks were charged with 3.68 g of DMA and 4.90 g of AB, and diluted to the mark with water. The solutions were stored for 1 month at 4°C.

(g) *Working standard solutions for calibration (from 0.5 to 20 μg/mL as As)*. Separate 50-mL volumetric flasks were charged with 25, 50, 100, 250, 500, or 1,000 μL of intermediate solutions (e) and (f), respectively. To each flask was added 5 mL of 0.3 mol/L

nitric acid and 0.1 mL of 0.1% methyl orange solution. The solutions were adjusted to pH 3 with dilute ammonia (aq) and diluted to the mark with water.

Sample preparation

(a) *Arsenic speciation analysis*

Subsamples (seaweed, 0.1 g; seafood, 0.2 g; seafood products, 0.5 g) were weighed into 10-mL glass test tubes with 2 mL of 0.3 mol/L nitric acid and mixed until the resulting suspension was homogeneous. The glass tubes were then stoppered and placed in a dry block bath at 100°C for 2 h. The tubes were removed from the block bath and allowed to cool to room temperature. Water (3 mL) was added to each tube and the tubes were shaken gently prior to centrifugation for 10 min at 2100 × *g*. The supernatant from each tube was then transferred to a 20-mL volumetric flask. Water (5 mL) was added to each precipitate and the mixture was gently shaken prior to centrifugation for 10 min at 2100 × *g*. The supernatant was transferred again to a 20-mL volumetric flask. This procedure was repeated once. Each supernatant flask was charged with 0.1 mL of 0.1% methyl orange solution and adjusted to pH 3 with dilute ammonia (aq). The flasks were each diluted to the mark with water and filtered through a 0.45-μm membrane filter prior to LC-ICP-MS analysis.

(b) *Total arsenic analysis*

Subsamples (seaweed, 0.1 g; seafood and seafood products, 0.5–0.6 g) were each weighed into microwave digestion vessels with 5 mL of nitric acid and digested at the maximum power of 1500 W as follows: 12-min ramp to 120°C, 13-min ramp to 250°C, 10 min at 250°C. After cooling, the digested subsamples were transferred to 50-mL polypropylene flasks. The internal standard solution (Te) was added to a final concentration of 50 μg/L. Acetic acid (1 mL) was added to each flask to enhance sensitivity (58). The total arsenic concentrations of the sample solutions were measured by ICP-MS. To determine extraction rates, 1–2 mL of each arsenic speciation extract was analyzed for total arsenic in the same manner.

LC-ICP-MS conditions

LC-ICP-MS conditions are shown in Table 1.

Results and discussion

Validation results

(a) *Chromatography*

Example chromatograms are shown in Figure 1.

(b) *Linearity*

In the range of 0.5–20 ng/mL, the correlation coefficient (r) of each calibration curve exceeded 0.999. Residual errors were independent of arsenic concentration.

(c) *LOD/LOQ*

Dried *nori* product (seaweed), Japanese oyster (seafood), and oyster sauce (seafood product) were independently analyzed to determine the LODs and LOQs. All three contained low levels of inorganic arsenic (Figure 1). The LOD and LOQ were defined as 3 and 10 times the standard deviation of six independent analyses (Table 2). Note that the LOQs in Table 2 are significantly lower than the Chinese regulatory limits (18).

(d) *Recovery*

Recovery tests were conducted by adding 20 ng of As(III) and As(V) standard solutions to three samples and an NRCC DORM-4 standard (Table 3). As(III) sometimes converts to As(V), and vice versa, during the extraction process; therefore, the concentration of inorganic arsenic was measured by combining the concentrations of As(III) and As(V). The results were within 90–110% of the actual value and satisfied the requirements of standard method AOAC SMPR 2015.006 for the quantitation of arsenic.

(e) *Precision*

Three samples used in determining LOD and LOQ were used to calculate repeatability (RSD_r) and intermediate precision (RSD_i). Duplicate analyses, separated by 5 days, were performed in accordance with the guidelines of the Ministry of Health, Labour, and Welfare of Japan for the validation of methods for metal analysis in food (59). The

data in Table 2 show that the resulting RSDs did not exceed 8%, thereby satisfying the requirements of AOAC SMPR 2015.006.

(f) *Accuracy*

The test results for NMIJ CRM 7405-a and NRCC DORM-4 standards are shown in Table 4. Total arsenic concentrations were within the certified range: 35.8 ± 0.9 mg/kg for NMIJ CRM 7405-a and 6.80 ± 0.64 mg/kg for NRCC DORM-4. The NMIJ CRM 7405-a reference material was certified with a water-soluble As(V) concentration of 10.1 ± 0.5 mg As/kg and the obtained inorganic arsenic concentrations agreed with the certified value. NRCC DORM-4 was not certified for inorganic arsenic; however, tests with spiked As(III) and As(V) standard solutions yielded 90–110% recovery.

Table 1. HPLC-ICP-MS conditions

HPLC conditions		
Column	CAPCELL PAK C_{18} MG, 4.6 mm i.d. × 250 mm, particle size 5 μm (Osaka Soda Co., Ltd.)	
Mobile phase	10 mmol/L sodium 1-butanesulfonate, 4 mmol/L malonic acid, 4 mmol/L tetramethylammonium hydroxide, 0.05 % methanol, (pH 3.0 adjusted with HNO_3)	
Flow rate	0.75 mL/min	
Injection volume	20 μL	
Column temperature	Room temperature, 20-30°C	
ICP-MS conditions	7500ce	8800
RF power	1.6 kW	1.55 kW
Plasma gas flow rate	Ar 15 L/min	Ar 15 L/min
Carrier gas flow rate	Ar 0.7 L/min	Ar 1.0 L/min
Collision gas flow rate	He 4 mL/min	He 4.3 mL/min
Monitoring ion	m/z 75	m/z 75
Dwell time	0.5 s	0.5 s

Table 2. Limit of detection, limit of quantitation, repeatability and intermediate precision

Sample	LOD (mg/kg)	LOQ (mg/kg)	RSD_r (%)	RSD_i (%)
Dried nori product	0.013	0.042	4.4	4.4
Japanese oyster, soft tissue	0.0031	0.011	3	5.6
Ooyster sauce	0.0024	0.0078	7.4	7.4

Table 3. Recovery rates for samples spiked before extraction[a]

Sample	As(III)		As(V)	
	Recovery (%)	SD (%)	Recovery (%)	SD (%)
Dried nori product	94.8	0.8	100	0.6
Japanese oyster, soft tissue	100.5	1.9	99.2	2.7
Oyster sauce	105	1.9	91.9	0
NRCC DORM-4	92.2	2.4	93.2	2.8

[a] Spike level of 1 ng/g (n = 3)

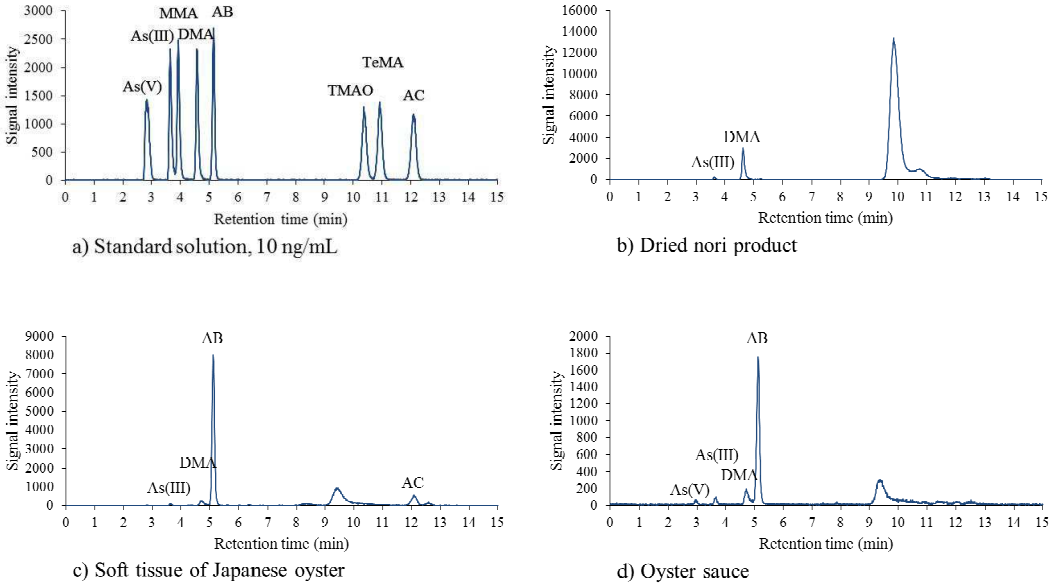

Figure 1. Chromatograms of standard solutions and food samples.

1.2 Total and inorganic arsenic concentrations in seaweed, seafood, and seafood products

This study analyzed the inorganic arsenic content in commercial seaweed and seafood samples using the developed analytical method. The Ministry of Agriculture, Forestry, and Fisheries has already collected data on the aresenic contents of *wakame*, *kombu*, *nori*, and *hijiki* (60). This study examined foods that are eaten daily. Dried seaweed, sliced raw fish, and seafood products were purchased from a local market in Japan.

Results and discussion

The measured concentrations of total and inorganic arsenic in seaweed, seafood, and seafood products are shown in Table 4. Total arsenic was determined for all samples used in this study. Inorganic arsenic was detected in most of the dried seaweed products, but was only marginally detected in seafood. The proportion of inorganic arsenic to total arsenic in seaweed was higher than in seafood. Among the seafoods, inorganic arsenic was detected only in the Japanese oyster samples.

These findings are similar to those of previous reports in that inorganic arsenic was not detected in fish (33, 44, 47, 61).

The *Sargassum* species *akamoku* and *hijiki* contain high levels of inorganic arsenic. Among the brown algae samples examined herein, significant levels of inorganic arsenic were detected in *akamoku*, *hijiki*, and *mozuku*. Pell *et al*. (41) also reported high levels of inorganic arsenic in the Sargassaceae. In this study, inorganic arsenic was not detected in the kelp *S. angustata*. A survey by the Ministry of Agriculture, Forestry, and Fisheries also shows the absence of inorganic arsenic in most kelp species (60). Conversely, Taylor *et al*. (40) and Ronan *et al*. (62) reported high levels of inorganic arsenic in the kelp *Laminoria digitata*. Since the kelp harvested in Japan belongs to the genus *Saccharina*, it is likely that inorganic arsenic is accumulated by the *Laminoria spp*. but not by the *Saccharina spp.*

Extraction rates were calculated as follows: extraction rate (%) = a/b × 100, where a is the total arsenic concentration of the extract solution measured by ICP-MS after microwave digestion and b is the total arsenic concentration of the sample. The extraction rate is reported because all arsenic compounds may not be detected on a chromatogram of seaweed and seafood extracts. The extraction rates of seafood were greater than 80% except for rainbow trout and red-eye round herring, which are listed as containing more than 4% lipids in the standard tables of food composition in Japan (63). Other seafoods

contain approximately 1% lipid. It is possible that rainbow trout and red-eye round herring contained more fat-soluble arsenic that was not extracted.

Table 4. Inorganic arsenic concentration, total arsenic concentration, total arsenic concentration in extracts, and extraction rates

Sample	Inorganic arsenic		Total arsenic		Total arsenic in extracts		Extraction rate (%)
	Mean[a] (mg/kg)	SD (mg/kg)	Mean[a] (mg/kg)	SD (mg/kg)	Mean[a] (mg/kg)	SD (mg/kg)	
Seaweed product							
Dried kelp	<0.042 [b]	nd[c]	22.9	0.06	20.8	0.38	90.8
Dried nori	0.085	0.003	19.8	0.29	18.9	0.31	95.5
Dried wakame	<0.042 [b]	nd[c]	29.1	0.8	26.2	0.59	90
Dried sea lettuce	0.061	0.001	5.35	0.12	4.93	0.04	92.1
Dried green laver	0.406	0.014	9.23	0.28	7.94	0.08	86
Dried mozuku	6.33	0.072	12.2	0.11	9.1	0.17	74.6
Dried boiled akamoku	17.4	0.011	38.7	0.27	31.3	0.3	80.9
Dried boiled hijiki	38.6	0.405	68.8	0.21	66.3	0.58	96.4
Fish and shellfish							
Albacore, muscle	<0.011 [b]	nd[c]	1.87	0.013	1.79	0.063	95.7
Rainbow trout, muscle	<0.011 [b]	nd[c]	0.24	0.008	0.16	0.002	66.7
Red-eye round herring, muscle	<0.011 [b]	nd[c]	2.51	0.03	1.85	0.103	73.7
Northern shrimp, whole body	<0.011 [b]	nd[c]	8.27	0.144	8.02	0.079	97
Japanese common squid mantle muscle	<0.011 [b]	nd[c]	0.55	0.007	0.53	0.008	96.4
Yezo giant scallop, adductor muscle	<0.011 [b]	nd[c]	1.05	0.023	1	0.031	95.2
Japanese oyster, soft tissue	0.025	0.001	3.32	0.063	3.12	0.079	94
Processed products							
Nam pla	0.011	0.001	0.92	0.026	0.85	0.019	92.4
Oyster sauce	0.011	0	0.33	0.004	0.33	0.012	100
Certificated reference materials							
NMIJ CRM 7405-a	10	0.18	35.8	0.07	30.5	1.76	85.2
NRCC DORM-4	0.249	0.006	6.54	0.05	5.8	0.09	88.7

[a] Triplicate
[b] LOQ
[c] Not detected

Inorganic arsenic was detected in Japanese oyster, oyster sauce, and nam pla. The muscle and internal organs of certain seafoods were subjected to separate analyses to localize the location of inorganic arsenic in the organism. The gut of the scallop has been shown to contain higher levels of cadmium than the surrounding tissues.

Oysters and sardines are the primary raw ingredients in oyster sauce and nam pla, respectively. The amounts of inorganic arsenic in specific tissues of the Japanese oyster, Japanese sardine, and Yezo giant scallop are given in Table 5. Total arsenic was detected in both the internal organs and muscles of these organisms. However, inorganic arsenic was detected only in internal organs. This finding is in agreement with the results of Kirby *et al*. (64). Throughout the organism, the major arsenic compound was AB, although DMA and AC were also detected (Figure 1). A large, unknown peak was observed at a retention time of 10 min in the chromatogram of Japanese oyster extract. Since four types of arsenosugar were co-eluted with arsenosugar glycerol using a heated acidic extraction (40, 62), this peak may be due to arsenosugars.

Since the edible components of the Japanese oyster include the internal organs, inorganic arsenic in our oyster and oyster sauce samples may derive from the internal organs. Likewise, it is likely that the inorganic arsenic found in the nam pla samples was derived from the internal organs of Japanese sardine.

Table 5. Inorganic arsenic concentration, total arsenic concentration, total arsenic concentration in extracts, and extraction rates

Sample		Inorganic arsenic		Total arsenic		Total arsenic in extracts		Extraction rate (%)
		Mean[a] (mg/kg)	SD (mg/kg)	Mean[a] (mg/kg)	SD (mg/kg)	Mean[a] (mg/kg)	SD (mg/kg)	
Japanese oyster	Adductor muscle	<0.011 [b]	nd[c]	2.41	0.036	2.07	0.055	85.9
	Internal organs	0.033	0.004	5.35	0.05	4.71	0.025	88
Japanese sardine	Muscle	<0.011 [b]	nd[c]	4.35	0.16	3.59	0.073	82.5
	Internal organs	0.242	0.016	4.36	0.16	4.18	0.36	95.9
Yezo giant scallop	Adductor muscle	<0.011 [b]	nd[c]	0.91	0.003	0.9	0.002	98.6
	Internal organs	0.16	0.007	2.06	0.028	1.62	0.031	78.6

[a] Triplicate

[b] LOQ

[c] Not detected

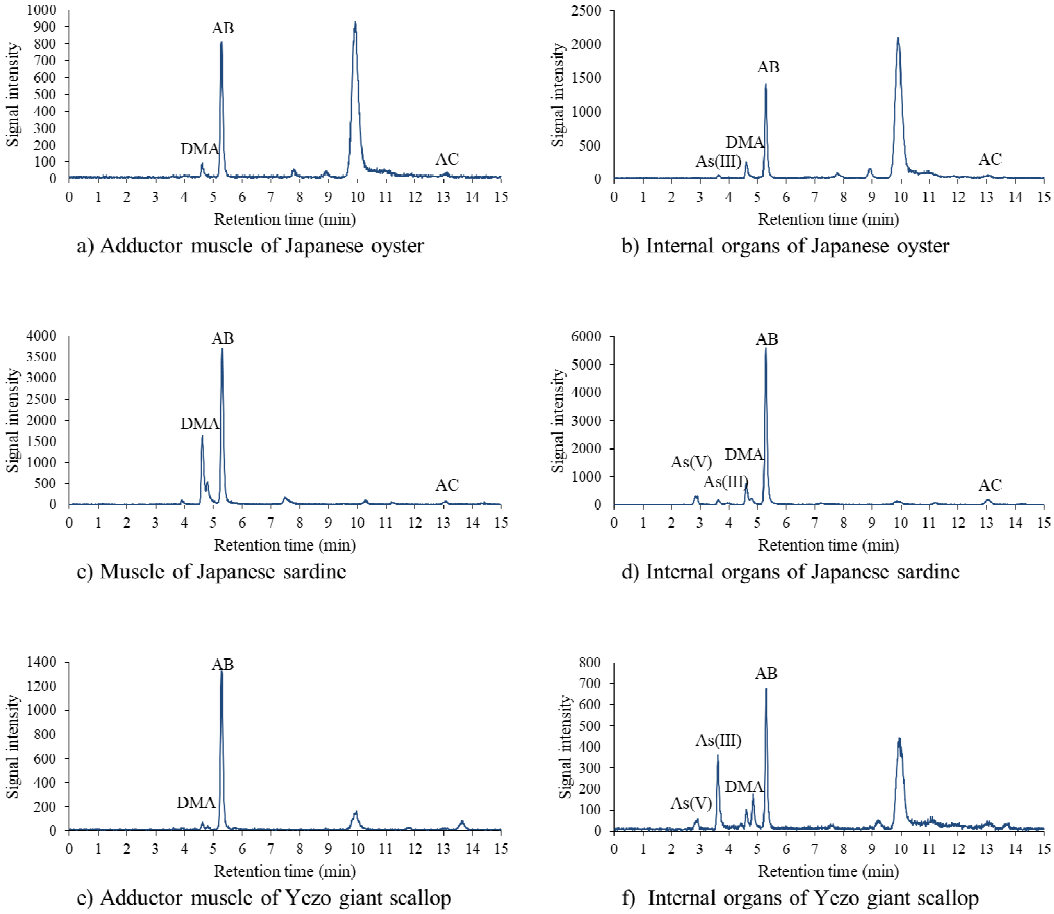

Figure 2. Chromatograms of seafood extract components.

1.3 Total and inorganic arsenic concentrations of *akamoku* after removal of inorganic arsenic by boiling in sea water

Seaweeds such as *akamoku* and *hijiki*, classified in *Sargassum*, contain high levels of inorganic arsenic. In 2004, the Food Standards Agency of the UK advised against eating *hijiki*. Boiling in water or in water containing sea salt has been shown to extract most of the inorganic arsenic in *hijiki* (65). Therefore, this study investigated the levels of inorganic aresenic in *akamoku* before and after repeated boiling.

Experimental

Sample preparation

Removing inorganic arsenic from seaweed

Raw *akamoku* (1.2 kg) was boiled in 10 L of 1% sea salt solution or tap water for 1 min. After boiling, the seaweed (approximately 300 g) was washed with running water. This boiling and washing process was repeated an additional three times and the seaweed was dried in a cold air dryer at 20°C.

Results and discussion

The total and inorganic arsenic concentrations in *akamoku* were measured before and after boiling (Table 6). Samples that had been boiled three times in 1% sea salt or tap water contained less than 0.5 mg/kg inorganic arsenic. Yamashita (65) reported that boiling seawater was more effective than tap water in removing arsenic from *hijiki*. Therefore, the boiling of *hijiki* in a solution containing 1% sea salt was recommended for removing arsenic. In this study, almost all of the inorganic arsenic in *akamoku* was removed by boiling several times in tap water or in a 1% solution of sea salt.

Table 6 Inorganic As concentration and total As concentration of boiled seaweed

Sample	1 % Sea salt solution		Water	
	i-As (mg/kg)	t-As (mg/kg)	i-As (mg/kg)	t-As (mg/kg)
Akamoku before boiling	10.19	14.13	---	---
Once boiled Akamoku	1.31	2.76	0.87	2.93
Twice boiled Akamoku	0.55	2.69	0.32	2.26
Three times boiled Akamoku	0.30	2.03	0.24	3.18
Four times boiled Akamoku	0.37	2.62	0.17	1.87

i-As, inorganic As; t-As, total As.

Chapter 2

Determination of inorganic arsenic in fish oil and fish oil capsules by LC-ICP-MS: method validation

Previous studies have employed water and/or dilute acid to extract inorganic arsenic from food and various biological samples prior to speciation analysis by LC-ICP-MS (46, 51, 52). However, these strategies have not been evaluated for analyses of fish oil and are not effective for extracting arsenolipids. Instead, arsenolipids can be extracted using water-soluble reagents and hydrolytic methods that are typically used to extract cholesterols. In one study, cholesterol was hydrolyzed in 56.1 mol/L KOH in ethanol (56). For the arsenic analyses, KOH was replaced with TMAH to avoid introducing an alkali salt into the ICP-MS. Alkali salts generally exhibit low ionization potentials and can inhibit the ionization of trace elements in ICP-MS analyses. In addition, the sensitivity of ICP-MS can be strongly affected by sample matrix effects, resulting in unstable or unpredictable results.

Chapter 2 focuses on arsenic speciation analyses in fish oil and fish oil capsules. Oil capsules packaged in water-soluble gelatin were first dissolved in TMAH prior to hydrolysis in ethanol.

2.1 Preparation of fish oil

Fish oil is a common additive in functional foods. Commercially available fish oil is purified and arsenic-free. Therefore, to evaluate the efficiency of inorganic arsenic extraction, this study used freshly prepared oils from fish and squid.

To obtain fish oils for arsenic analyses, this study referred to common industrial extraction techniques. Sardines were homogenized, steamed, and centrifuged to obtain an oil layer. Squid oil was obtained from the internal organs of the Japanese common squid. Krill oil was extracted using a hexane-2-propanol mixture.

Experimental

Sample preparation

Sardine oil

Whole Japanese sardines were homogenized and steamed for 15 min. The resulting liquid was separated from the solids with a strainer and centrifuged to obtain an oil layer.

Krill oil

Frozen Antarctic krill (10 g) was weighed into a 50-mL centrifuge tube. A 3:2 (v/v) mixture of hexane/2-propanol (30 mL) was added and the tube was shaken for 10 min. The hexane layer was separated by centrifugation and evaporated to dryness.

Squid oil

The hepatopancreas of the Japanese common squid (160 g) was homogenized with 320 mL of water and centrifuged to obtain an oil layer.

2.2 Method validation of inorganic arsenic analyses in fish oil

A single laboratory validation was performed on the method for quantitating levels of inorganic arsenic in fish oil. Samples known to contain low levels of inorganic arsenic were used to calculate LODs and LOQs. The maximum permissible level of aresenic in fish oil is 0.1 mg/kg. Inorganic arsenic was not detected in fish oil capsules that were purchased from a local market. Therefore, small amounts of As(III) standard solution were added to oil capsules used for method validation.

Experimental

Samples

Fish oil (Japanese sardine oil, krill oil, Japanese common squid oil) was made from fresh raw materials as described in “2.1 preparation of fish oil”.

Apparatus

(a) *LC system.* An Agilent Technologies 1200 Series LC system (Tokyo, Japan) was used to separate oil samples.

(b) *ICP-MS.* Total arsenic was quantitated with an Agilent Technologies 8800 ICP-MS instrument.

(c) *Microwave digestion system.* An UltraWAVE (Milestone MLS, Leutkirch, Germany) microwave instrument was used for sample digestion prior to total arsenic measurements.

(d) *Dry block bath heating system.* An EB-303 (As One, Osaka, Japan) dry block bath was used for heating the extraction solution prior to arsenic speciation analyses.

(e) *Hot plate.* Digi Prep HT (SCP Science, Quebec, Canada)

(f) *Ultrasonic bath.* ASU-6 (As One, Osaka, Japan)

An LC system coupled to an ICP-MS instrument was used to measure arsenic speciation in fish oil.

Reagents and standards

(a) *Ultrapure water.* A Milli-Q system (>18 MΩ cm) (Millipore Corp., Billerica, MA, USA) was used to purify the water used throughout this study.

(b) *Nitric acid* was of ultrapure grade and obtained from Kanto Chemical Industries, Ltd., Tokyo, Japan.

(c) *Methyl-orange* was obtained from Kanto Chemical Industries Ltd.

(d) *Sodium 1-butanesulfonate* was obtained from Fujifilm Wako Pure Chemical Corporation, Osaka, Japan.

(e) *Malonic acid* was obtained from Fujifilm Wako Pure Chemical Corporation.

(f) *Methanol* was obtained from Fujifilm Wako Pure Chemical Corporation.

(g) *TMAH* was of ultrapure grade and obtained from Tama Chemicals Co., Ltd., Kanagawa, Japan.

(h) *Ethanol* was obtained from Fujifilm Wako Pure Chemical Corporation.

(i) *Phosphoric acid* was obtained from Koso Chemical Co., Ltd., Tokyo, Japan.

(j) *Standard solution of CRM.* As(V) certified reference material (NMIJ CRM 7912-a) was purchased from The National Metrology Institute of Japan/National Institute of Advanced Industrial Science and Technology (Ibaraki, Japan).

(k) *Standard solution of Japan Calibration Service System (JCSS).* Standard solutions of As(III) and Te (internal standard) were purchased from Kanto Chemical Industries, Ltd.

(l) *Hexane* was obtained from Fujifilm Wako Pure Chemical Corporation.

(m) *2-Propanol* was obtained from Fujifilm Wako Pure Chemical Corporation.

Preparation of reagents and standard solutions

(a) *0.3 mol/L nitric acid.* A 100-mL volumetric flask containing approximately 50 mL of water was charged with 1.92 mL nitric acid and diluted to the mark with water.

(b) *0.1 % methyl orange solution.* Methyl orange (0.1 g) was weighed into a 100-mL volumetric flask and diluted to the mark with water. The resulting solution was passed through a membrane filter to remove any precipitate.

(c) *2.5 % phosphoric acid.* A 100-mL volumetric flask was charged with 2.94 g of 85% phosphoric acid and diluted to the mark with water.

(d) *Mobile phase.* A 1-L beaker was charged with 1.458 g of 25% TMAH, 1.602 g of sodium 1-butane sulfonate, 0.416 g of malonic acid, 0.5 mL of methanol, and 900 mL of water. The pH of the mixture was adjusted to 3.0 with ultrapure nitric acid while monitoring with a pH meter. The solution was transferred to a 1-L volumetric flask and diluted to the mark with water.

(e) *Intermediate standard solutions (1 μg/mL as As)*. As(V) and As(III) stock solutions were prepared by diluting stock standards 100-fold with water. Intermediate standard solutions were prepared immediately before use.

(f) *Working standard solutions for calibration (from 0.5 to 20 μg/mL as As).* Volumetric flasks (50 mL) were charged with 25, 50, 100, 250, 500, or 1,000 μL of intermediate solution (e). To each flask, 0.4 mL of 25% TMAH, 1 mL of 2.5% phosphoric acid, and 0.1 mL of 0.1% methyl orange solution were added. The solutions were adjusted to pH 3 with 0.3 mol/L nitric acid and diluted to the mark with water.

Sample preparation

(a) *Arsenic speciation analyses in fish oil*

Fish oil (0.1 g) was weighed into a 10-mL glass test tube. To this, 2.34 mL of ethanol was added and the tube was mixed until the resulting suspension was homogeneous. Then, 0.16 mL of 25% TMAH was added to the tube and the solution was mixed well. The tube was closed with a stopper and placed in a dry block bath at 80°C for 1 h. After heating, the tube was allowed to cool to room temperature before transferring the mixture to a beaker and evaporating the ethanol on a hotplate at 80°C. The resulting solid was dissolved in water by sonication, transferred to a 10-mL volumetric flask, and diluted to the mark with water. An aliquot was removed and placed in a 10-mL of volumetric flask. To this, 0.2 mL of 2.5% phosphoric acid and 0.1 mL of 0.1% methyl orange solution were added. The pH was adjusted to pH 3 with 0.3 mol/L nitric acid and the solution was diluted to the mark with water and filtered through a 0.45-μm membrane filter prior to analysis by LC-ICP-MS.

(b) *Arsenic speciation analysis of fish oil capsules*

Individual fish oil capsules (about 0.4–0.6 g) were added to 10-mL glass test tubes with 0.32 mL of 25% TMAH. The capsules were dissolved by sonicating for about 1.5 h followed by addition of 4.68 mL of ethanol to each tube. The solutions were mixed well and the tubes were stoppered and placed in a dry block bath at 80°C for 1 h. After heating, the tubes were allowed to cool to room temperature before transferring their contents to a beaker and evaporating the ethanol on a hotplate at 80°C. The resulting solids were dissolved in water by sonication, transferred to a 10-mL volumetric flask, and diluted to the mark with water. An aliquot of each sample was removed and placed in a 10-mL volumetric flask. To each sample, 0.2 mL of 2.5% phosphoric acid and 0.1 mL of 0.1% methyl orange solution were added. The pH of each sample was adjusted to pH 3 with 0.3 mol/L nitric acid and the solution was diluted to the mark with water and filtered through a 0.45-μm membrane filter prior to analysis by LC-ICP-MS. An additional capsule

containing granulose shark oil (squalene) was treated in the same way except it was extracted using a double extraction solvent since it was large and difficult to hydrolyze.

(c) *Total Arsenic analysis*

Subsamples (fish oil, 0.1 g; fish oil capsule, 1 drop) were weighed into microwave digestion vessels along with 2 mL of water and 5 mL of nitric acid. Samples were digested in a microwave instrument at maximum power (1500 W) as follows: 15-min ramp to 120°C, 15-min ramp to 250°C, and 10 min at 250°C. After cooling, the sample solutions were transferred to 50-mL polypropylene flasks and the Te internal standard was added to a final concentration of 50 μg/L. Acetic acid (1 mL) was added to enhance sensitivity (58). The total arsenic concentration of each sample solution was measured by ICP-MS. To determine extraction efficiency, 1-mL aliquots of arsenic speciation extracts were analyzed for total arsenic in the same manner.

LC-ICP-MS conditions

The LC-ICP-MS conditions are shown in Table 1.

Results and discussion

Validation results

Chromatography

Example chromatograms are shown in Figure 3.

Linearity

Correlation coefficients (r) exceeded 0.999 for all samples within the concentration range of 0.5 to 20 ng/mL. Residual errors were independent of arsenic concentration.

LOD/LOQ

This study analyzed Japanese sardine oil (fish oil) and oil capsules containing EPA and DHA (fish oil capsules) to determine LODs and LOQs. Each of the samples was spiked with a standard solution to attain a low level of inorganic arsenic. LODs and LOQs were defined as the concentrations corresponding to signal strengths three and ten times the standard deviation of six independent analyses (Table 7). Note that the calculated LOQs were significantly lower than the maximum permissible level of arsenic defined in the Codex (21).

Recovery

Recovery tests were conducted with fish oil samples and fish oil capsules by adding 10 and 20 ng, respectively, of As(III) and As(V) standard solutions (Table 8). Due to possible interconversion between As(III) and As(V) during the extraction process, the concentration of inorganic arsenic was calculated as the combined concentration of As(V) and As(III). The results were within 90–110% of their nominal values, thereby satisfying standard method AOAC SMPR 2015.006 for the quantitation of arsenic.

Precision

Two samples that had been used in analyses of LOD and LOQ were used to calculate the RSD_r and RSD_i. The tests were carried out using duplicate analyses separated by 5 days in accordance with the guidelines set by the Ministry of Health, Labour, and Welfare of Japan for the validation of methods for metal analysis in food (59). The data in Table 7 show that the RSDs did not exceed 5%, thereby satisfying the requirements of standard method AOAC SMPR 2015.006.

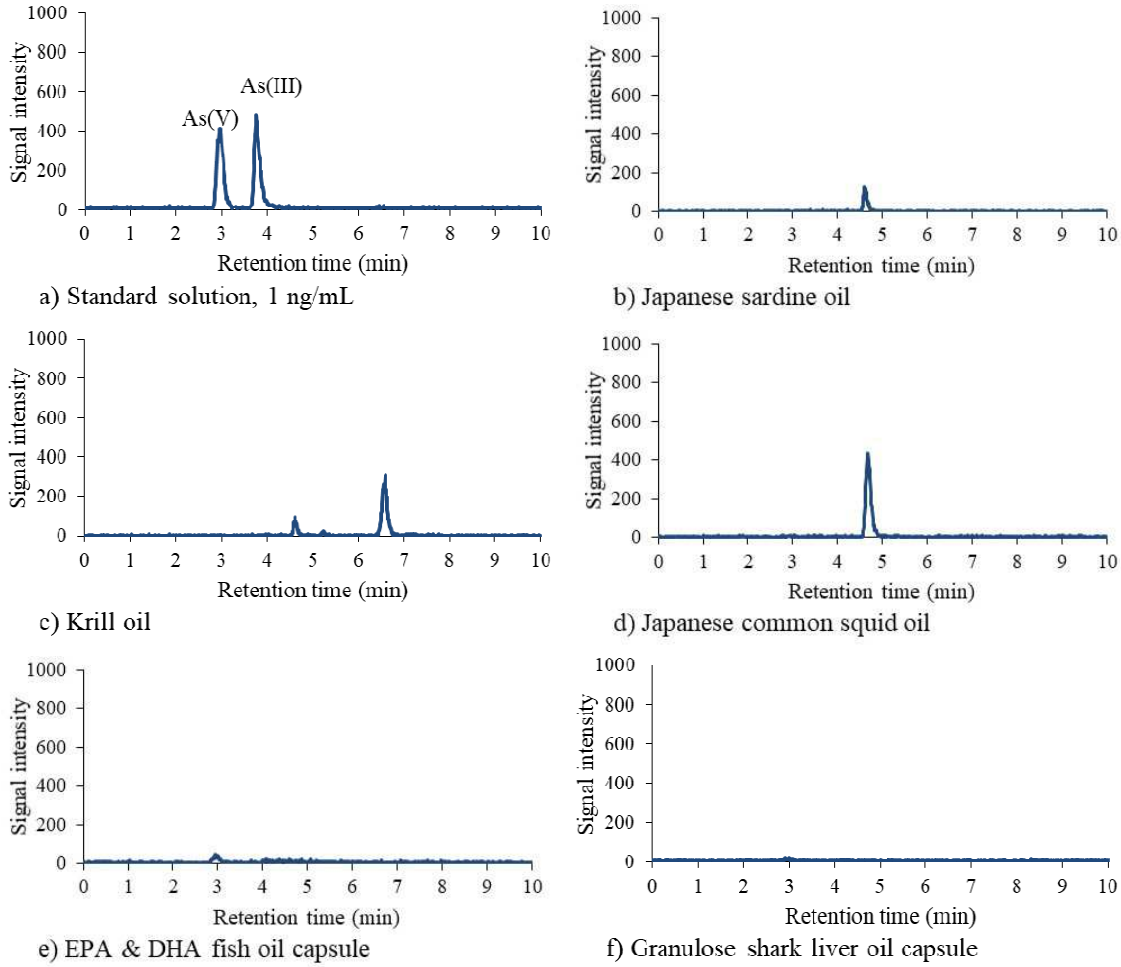

Figure 3. Chromatograms of standard solutions and fish oil samples.

Table 7. Limit of detection, limit of quantitation, repeatability and intermediate precision

Sample	LOD (mg/kg)	LOQ (mg/kg)	RSD_r (%)	RSD_i (%)
Japanese sardine oil	0.015	0.048	3.4	4.3
EPA & DHA capsule 1	0.004	0.011	3.5	3.5

Table 8. Recovery rates for samples spiked before extraction[a]

Sample	As(III)		As(V)	
	Recovery (%)	SD (%)	Recovery (%)	SD (%)
Japanese sardine oil	97.3	3.2	98.3	4.0
EPA & DHA capsule 1	106	3.2	102	1.5
EPA & DHA capsule 2	102	4.7	97.3	2.1
EPA & DHA capsule 3	100	4.6	103	1.0
Granulose shark liver oil capsule	103	1.0	107	1.7

[a] Spike level of 0.5 ng/g (n = 3)

2.3 Total and inorganic arsenic in fish oil and fish oil capsules

In accordance with the method described above for cholesterol hydrolysis, the fish oil samples were completely disolved in alkaline solution containing TMAH. After hydrolysis, each extract was adjusted to pH 3 to match the pH of the HPLC mobile phase, resulting in the formation of a precipitate. Recovery tests showed that no inorganic arsenic was in the precipitate, which was removed by centrifugation to avoid damaging the HPLC column.

Three fish oil capsules containing EPA and DHA and an additional caspule containing granulose shark liver oil (squalene) were analyzed for arsenic. The fish oil capsules weighed between 0.4 and 0.5 g and contained approximately 0.3 g of fish oil. The shark oil capsule weighed 0.6 g and contained 0.4 g of oil.

Results and Discussion

Total and inorganic arsenic concentrations in fish oil

The concentrations of total and inorganic arsenic in fish oils are shown Table 9. Total arsenic was measured for each sample used in this study. Inorganic arsenic was not detected in the fish oil samples. López-García *et al.* (52) reported significant concentrations of inorganic arsenic in the laboratory products of fish oil, fish oil nutritional supplements, and olive or sunflower oil used in the processing of canned fish. In this study, inorganic arsenic was found only in laboratory products of fish oil. Fish liver oil contained significantly high inorganic arsenic concentrations of 10.2 mg/kg. The intestinal organs of fish and shellfish, such as Japanese sardine, contained higher levels of inorganic arsenic than did muscle tissue (64). Squid oil prepared from the hepatopancreas of Japanese common squid did not contain inorganic arsenic. This suggests that inorganic arsenic may exist only in the water layer of the squid hepatopancreas extract.

Extraction rates were calculated as follows:

extraction rate (%) = a/b × 100

where a is the total arsenic concentration of the extracted solution measured by ICP-MS after microwave digestion, and b is the total arsenic concentration of the sample. The total arsenic concentrations of the extracts were measured before acidification. The extraction rate is provided in terms of total arsenic concentration because all arsenic compounds may not be evident on a chromatogram of fish oil extract.

Total and inorganic arsenic concentrations in fish oil capsules

The data in Table 9 show that no arsenic was detected in fish oil capsules. These findings are similar to those of a previous study in which inorganic arsenic was found at very low levels in fish oil nutritional supplements (52). It is likely that arsenic is removed from the raw materials prior to extracting and packaging fish oil into capsules.

Table 9. Inorganic arsenic concentration, total arsenic concentration, total arsenic concentration in extracts, and extraction rates

Sample	Inorganic arsenic		Total arsenic		Total arsenic in extracts		Extraction rate (%)
	Mean[a] (mg/kg)	SD (mg/kg)	Mean[a] (mg/kg)	SD (mg/kg)	Mean[a] (mg/kg)	SD (mg/kg)	
Fish oil							
Japanese sardine oil	<0.048[b]	nd[c]	9.68	0.29	9.70	0.01	100
Krill oil	<0.048[b]	nd[c]	5.57	0.04	5.57	0.02	100
Japanese common squid oil	<0.048[b]	nd[c]	19.6	0.28	19.4	0.23	99
Fish oil capsule							
EPA & DHA capsule 1	<0.011[b]	nd[c]	<0.01	nd[c]	---[d]	---[d]	---[d]
EPA & DHA capsule 2	<0.011[b]	nd[c]	<0.01	nd[c]	---[d]	---[d]	---[d]
EPA & DHA capsule 3	<0.011[b]	nd[c]	<0.01	nd[c]	---[d]	---[d]	---[d]
Granulose shark liver oil capsule	<0.011[b]	nd[c]	<0.01	nd[c]	---[d]	---[d]	---[d]

[a] Triplicate
[b] LOQ
[c] Not detected
[d] Not calculated

2.4 Solubility of inorganic arsenic in fish oil

Inorganic arsenic was not detected in fish oil in this study. This may be because water-soluble inorganic arsenic species are insoluble in oils. This study therefore examined the solubility of As(III) and As(V) in oil.

When made into halogen salts, certain elements can be extracted into organic solvents. For example, the chloride salt of As(III) is soluble in hexane (53). This study therefore examined the relative partitioning of inorganic arsenic between an organic solvent and an aqueous solution of 9 mol/L hydrochloric acid.

Results and Discussion

To examine the solubility of inorganic arsenic in fish oil, As(III) or As(V) was added to fish oil under acidic conditions (Figure 4). As(III) was detected at 0.1 or 2.9 µg/g in fish oil in the absence or presence of 9 mol/L hydrochloric acid, respectively. As(V) was only detected (0.27 µg/g) in fish oil in the presence of the hydrochloric acid solution.

These findings show that the majority of As(III) and a small amount of As(V) are transferred into fish oil under acidic conditions. This strategy can therefore be used in the extraction and analysis of fish oils containing inorganic As(III) and As(V).

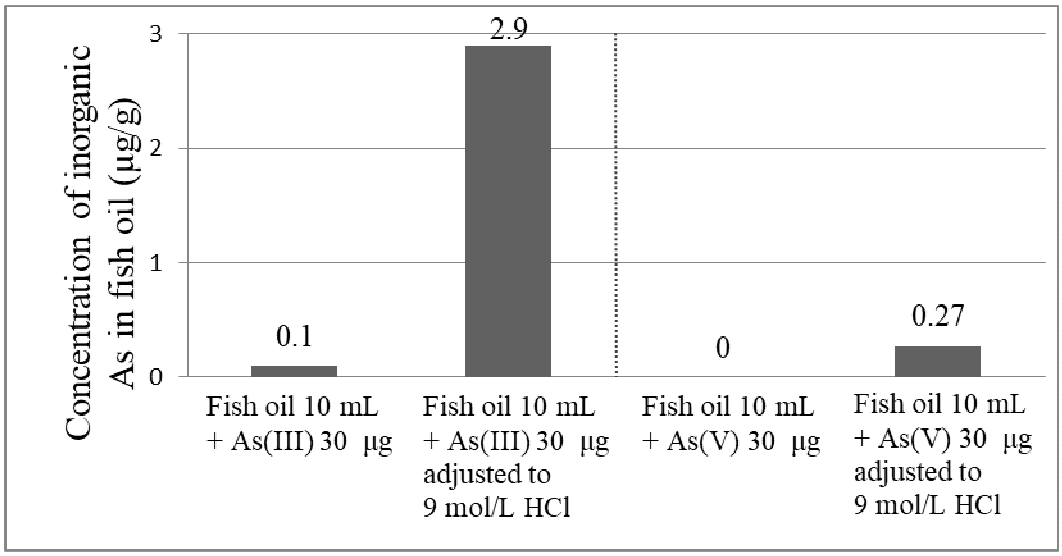

Figure 4. Inorganic arsenic concentrations of fish oils spiked with As(III) or As(V) in the presence or absence of an acidic aqueous phase.

Chapter 3
Comprehensive discussion

This study developed an analytical method for the quantitation of inorganic arsenic in seaweeds and seafood. Inorganic arsenic was extracted from food samples by heating at 100°C in 0.3 mol/L nitric acid. For simplicity, arsenic speciation was determined by LC-ICP-MS using an ODS column with a mobile phase containing an ion-pair reagent. In validating this method, LOQs were less than 0.05 mg/kg and recovery tests and precisions satisfied standard method AOAC SMPR 2015.006 for arsenic analysis. Therefore, an analytical method for inorganic arsenic as As(III) and As(V) in seaweeds and seafood containing high levels of inorganic and/or organic arsenic compounds was established in this study.

Significant inorganic arsenic concentrations have been found in the brown algae *akamoku*, *hijiki*, and *mozuku*. This study applied the present analytical method to the determination of inorganic arsenic concentration during the processing of *akamoku* by boiling. Inorganic arsenic was removed from the raw seaweed by repeated boiling.

This study also measured inorganic arsenic levels in the internal organs of Japanese oyster, nam pla sauce, and oyster sauce. Inorganic arsenic was not detected in the other seafoods used in this study.

This study also developed an analytical method for the quantitation of inorganic arsenic in fish oil and fish oil capsules. Inorganic arsenic was quantitatively extracted from fish oil samples by heating at 80°C in an alkaline solution containing 1.6% TMAH following lipid extraction with ethanol. In validating this method, LOQs were less than 0.05 mg/kg, and recovery tests and precisions satisfied standard method AOAC SMPR 2015.006 for arsenic analysis. Therefore, an analytical method for measuring the amounts of inorganic arsenic in fish oils and fish oil capsules was established in this study.

Furthermore, the developed analytical method can be applied to all food groups.

International databases on the inorganic and organic arsenic contents of various foods will be established using the analytical methods described herein. With data collected using this method, the intake of inorganic arsenic from seafood, seaweeds, fish oils, and other types of foods may be estimated, allowing a more accurate estimate of consumer health risks. The ingestion of inorganic arsenic in drinking water is known to cause cancer, skin lesions, developmental defects, cardiovascular disease, neurotoxicity, and diabetes in humans (4). Inorganic arsenic contributions from food and related toxicities will be clearly elucidated in the future. In particular, the adverse effects of inorganic arsenic on human health by the ingestion of rice and *hijiki* in Japan need to characterized.

Summary

The toxicity of arsenic depends on its chemical form (1–4, 6, 7). Regulatory limits for highly toxic forms of inorganic arsenic have been set by the Codex, Australia/New Zealand, China, and EU (18–21). The foods that are targeted for regulation include rice, seaweed, and seafood. While a method for determining inorganic arsenic levels in rice has already been established (24), a means of analyzing for inorganic arsenic in seaweed and seafood is in need of development.

In 2017, the Codex added an additional regulation stating that the inorganic arsenic concentration in fish oil must be less than 0.1 mg/kg when the fish oil contains a total arsenic concentration of more than 0.1 mg/kg (21). Therefore, an established method for quantitating inorganic arsenic levels in fish oil will be required.

Chapter 1 describes the validation of an analytical method for quantitating inorganic arsenic levels in seaweed and seafood via extraction at 100°C in 0.3 mol/L nitric acid and LC-ICP-MS analysis with an ODS column and a mobile phase containing an ion-pair reagent, as described by Nagaoka *et al.* (34). Samples purchased from a local market were used in our analyses. Among the dried seaweed products that were evaluated, significant levels of inorganic arsenic were detected in the seaweeds *akamoku*, *hijiki*, and *mozuku*. The *Sargassum* species *akamoku* and *hijiki* contained high levels of inorganic arsenic. This study also confirms that inorganic arsenic can be removed from seaweed by repeated boiling, a method described by Yamashita (65).

Taylor *et al.* (40) and Ronan *et al.* (62) reported high levels of inorganic arsenic in the kelp *L. digitata*. Since the dried kelp products produced in Japan belong to the genus *Saccharina*, it is likely that inorganic arsenic is accumulated by the *Laminoria spp*. but not by the *Saccharina spp.*

Inorganic arsenic was detected in the Japanese oyster, oyster sauce, and nam pla. The muscle and the internal organs of the oyster were analyzed separately to examine the

distribution of inorganic arsenic throughout the organism. Inorganic arsenic was detected in the internal organs of the oyster and in oyster sauce, the latter being derived from the internal organs of the Japanese oyster. Likewise, the inorganic arsenic found in nam pla likely results from the arsenic found in the internal organs of the sardine, which are used in the production of nam pla.

Chapter 2 describes the adaptation of the above method to quantitate inorganic arsenic in fish oil and fish oil capsules. There are relatively few articles that describe the analysis of inorganic arsenic in oils (46, 51, 52). In these, inorganic arsenic was extracted with water or dilute acid. To determine the extraction rates of inorganic arsenic from fish oils containing arsenolipids, this study employed a hydrolysis technique used in the analysis of cholesterols (56).

The validation results were satisfactory for inorganic arsenic in fish oil and fish oil capsules. Although López-García *et al.* (52) reported that fish liver oil contained high levels of inorganic arsenic, inorganic arsenic was not detected in fish oil samples extracted from fish or squid, nor in commercially available fish oil capsules.
Since As(III) is known to be soluble in hexane in the presence of chloride ion (53), this study examined the solubility of inorganic arsenic in fish oil by spiking As(III) or As(V) into fish oil under acidic conditions. In the presence of 9 mol/L hydrochloric acid, most of the inorganic arsenic was transferred to the organic phase of fish oil.

Chapter 3 provides a comprehensive discussion relating these results. International databases on the inorganic and organic arsenic contents of foods will be established using the analytical methods described in this study. These will allow better estimates of arsenic ingestion and provide more accurate estimates of consumer health risk.

Acknowledgements

The author wishes to express sincere gratitude to Professor Michiaki Yamashita for helpful advise and discussions. The author also wishes to express appreciation to Professor Ikuo Hirono, Associate Professor Hidehiro Kondo, and Professor Toshiyiki Suzuki at Tokyo University of Marine Science and Technology for helpful assistance. The author is also grateful to all members of the Japan Food Research Laboratories for their encouragement and support.

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