Doctoral Thesis

博士論文

Daily exposure to arsenolipids and its associated health risk in the Japanese

（日本人におけるヒ素脂質の一日ばく露量とその健康リスク）

Md Hasan Al Amin

(エムディ ハサン アル アミン)

Acknowledgements

There are several reasons that make this dissertation very special for me- like my first laboratory based scientific research in foreign country and of course the immense effort that I put for this. I owe my gratitude to all those people who have made this dissertation possible and because of whom my doctoral study experience has been one that I will cherish forever.

I owe my immense gratitude to my supervisor, Professor Dr. Yoshikuni Yoshida for his hearty guidance, moral support, constructive advice and help in my study.

My deepest gratitude goes to Professor Dr. Jun Yoshinaga for his constant inspiration, scholastic guidance, immense encouragement, valuable suggestions, timely and solitary instructions, cordial behavior, and providing all facilities for successful completion of the research work as well as preparation of this dissertation.

Especially I would like to thank Professor Dr. Kevin A. Francesconi, Dr. Chan, Dr. Narukawa, and Dr Oguri san for their active help, constrictive advice, and guidance in different stages of my study. In addition, I must thank to Dr Hanamachi san, Dr Tanaka san, Ms. Kano and Ms. Nakamura san for their kind help in food processing and freeze-drying in my study.

I am also indebted to all my lab members particularly, Dr. Nishihama san, Kaneko san, Moriya san, Ameda san, Sadia san, Mizuno san, and Li san for their constant help and motivation in my entire study period.

Most importantly, none of this would have been feasible without the love and patience of my parents, and the immense support of my beloved wife Nahida Arefin Nitu. My family members have been a constant source of love, concern, support and strength all the time during this research. I would like to express my heart-felt gratitude to my family.

The Author

Md Hasan Al Amin

Daily exposure to arsenolipids and its associated health risk in the Japanese

Contents

Chapter 4

Variation of arsenolipids concentration among several fish species and their stability on cooking

Chapter 5

Bioaccessibility of arsenolipids in seafood

Chapter 1

Introduction

1.1. Arsenic

Arsenic is a chemical element having symbol ‘As’ and atomic number 33 in the periodic table. It is classified as metalloid or semi-metal since it has properties of both metals and non-metals. It has various allotropes, but only the grey form is stable at room temperature. Arsenic is found to exist in many minerals, usually in conjunction with oxygen, chlorine, sulphur and metals, and also as a pure elemental crystal with an atomic weight of 74.92. It can exist as powder, amorphous or vitreous forms. Elemental arsenic does not dissolve in water; however some salts of arsenic dissolve in water. Further arsenic trioxide, arsenic pentoxide and other arsenical compounds are soluble, depending on the pH and the ionic environment of the solution. When heated to decomposition, arsenic compounds emit toxic arsenic fumes (HSDB, 2003). Arsenic can exist in four valence states: –3, 0, +3 and +5. Under reducing conditions, +3 valence state as arsenite is the dominant form; +5 valence state as arsenate is generally a more stable form in oxygenized environments (NRC, 1999). Arsenic is found to exist in both organic and inorganic forms in different foods and environmental media such as soil, air, and water. Inorganic arsenic is both highly toxic and readily bioavailable whereas organic arsenic considered as less toxic (NRC, 1999; 2001).

Arsenic is ubiquitous element present in food, soil, water and air. It is released into the environment from both natural and man-made sources, including erosion of arsenic-containing rocks, volcanic eruptions, contamination from mining and smelting ores, and previous or current use of arsenic-containing pesticides. It ranks 20th in natural abundance, 14th in seawater and 12th in the human body (Mandal and Suzuki, 2002). Globally, natural emissions of arsenical compounds have been estimated at about 8,000 tons each year whereas anthropogenic emissions are about 3 times higher (NRC, 1999; 2001).

Among the general people, arsenic is known for its toxicity and often used as poisons in the past (Nriagu, 2002). Arsenic trioxide (As_2O_3), the most toxic and common arsenic compound in commerce is a tasteless and odorless compound. All over the world arsenic is used as an ingredient of a different kind of products in manufacturing industries, e.g., wood preservatives, herbicides, insecticides, pesticides, fungicides, high-emitting diodes, semi-conductors etc. However, arsenicals have also been used in medicine for the treatment of some diseases such as syphilis, psoriasis and leukemia (Gibaud and Jaouen, 2010).

Since arsenic is found naturally in the environment, anyone can be exposed easily to some extent to arsenic through food, drinking water, or breathing air. Arsenic cannot be destroyed in the environment rather it could be changed from one form to another between organic and inorganic, or become absorbed to or separated from particles. Various arsenic forms could be changed to its other forms by reacting with oxygen or other molecules present in air, water, food, soil, or by the action of bacteria that live in soil or sediment.

The general exposure to arsenic is mediated through food followed by drinking water, soil and air. Among these, drinking water and food are usually the largest source of arsenic exposure. Severe human toxicity has been found to occur through the drinking of inorganic arsenic contaminated water (Smith et al., 1998; WHO, 2000; Yoshida et al., 2004). Another predominant source of arsenic is seafood, followed by rice/rice cereals, mushrooms, algae, and poultry. Seafood particularly fish and shellfish contains the large amounts of arsenic, mostly in an organic form called arsenobetaine which is much less harmful than inorganic arsenic. Children are likely to eat small amounts of dust or soil each day, so this could be another way to be exposed to arsenic.

People are mostly exposed to inorganic arsenic through drinking water in areas where arsenic is in naturally high concentration in ground water. In fact, drinking water accounts for the most human arsenic exposures worldwide. Arsenic contamination of ground water became a high-profile problem in recent years due to the use of underground water (tube well water) for drinking purposes, causing serious arsenic poisoning to a large number of people in the world especially in Bangladesh and West Bengal of India (Mandal et al., 1998; Chen et al., 1985; Das et al., 1995; 1996; Dhar et al., 1997).

People can also be exposed to arsenic through the environment of their working place, i.e., occupational exposure. This occupational exposure to arsenic is usually found to occur in several industries including mining, pesticide, pharmaceutical, glass, microelectronics (IARC, 1980; NRC, 1999), optical industries, manufacturing of alloys, leather preservatives, arsenic containing pigments, antifouling paints, poison baits, agrochemicals and of course as well as in environmentally from both industrial and natural

sources. Generally people are exposed to arsenic via the oral route (ingestion), inhalation, dermal contact, and the parental route to some extent. In case of occupational settings, inhalation is the principal route of arsenic exposure. Therefore, workers who produce or use arsenic compounds in their occupation could be exposed to substantially higher levels of arsenic (Jones, 2007; Tchounwou et al., 1999). Another way of exposure to arsenic is the smoking. Exposure of smokers to arsenic arises from the natural inorganic arsenic content of tobacco since tobacco plants essentially take up arsenic naturally present in the soil (WHO, 2000) and this content is increased further where tobacco plants have been treated with lead arsenate insecticide.

1.2. Toxicity of arsenic

Inorganic arsenic (iAs) is classified as a human carcinogen and the ingestion of iAs is associated to cause several adverse human health effects (IARC, 2004). The ground water in different regions of the world is contaminated with arsenic and there are a number of regions including both developing and developed countries where arsenic contamination of drinking water found significant. The uses of arsenic-contaminated ground water for irrigation, bioavailability of arsenic to food crops and subsequent consumption by human population and livestock through the food chain have opened pathways for arsenic exposure all over the world. Millions of people are exposed to elevated levels of toxic inorganic arsenic through the drinking of contaminated ground water and food (Ng et al., 2003; Smith et al., 2000; Meharg, 2003). Organic arsenic, arsenobetaine (AB) are considered to be non-toxic and rapidly excreted by human (Kaise et al., 1985) but some of the other organic arsenic compounds could be a concern, e.g., dimethylarsenic (DMA), and arsenosugars

(Feldmann and Krupp, 2011; Andrews et al., 2004). DMA showed carcinogenicity in recent toxicity study (Arnold et al., 2006). Arsenosugars could be metabolized to AB through the forming of an intermediate of trimethylated arsenosugars (Shibata and Morita, 1988) prior to excretion and the potential exist of their reduction to trivalent arsenosugars appears to exhibit toxicity. Long-term exposure to arsenic is related to severe adverse health effects including dermatitis, cardiovascular diseases, diabetes mellitus, chronic bronchitis, immune disorders, peripheral neuropathy, liver damage, renal failure, adverse reproductive outcomes, hematological effects, and other ailments (Ali et al., 2010; Argos et al., 2010; Chen et al., 2007; Mazumder et al., 1998, 2000; Mazumder 2005; Meliker et al., 2007; Mumford et al., 2007; Tapio and Grosche 2006; Vahidnia et al., 2008; Wang et al., 2002). Recent toxicological study showed that some organic lipid soluble arsenic compounds also exert toxicity to human liver and kidney cells comparable to the toxicity magnitude of inorganic arsenic. As a result, arsenic toxicity has created a major public health concern throughout the world.

1.3. Arsenic in foods

Arsenic is distributed in both the marine and terrestrial environment, whereas its concentration level in marine samples is generally higher than terrestrial samples (Francesconi and Kuehnelt, 2002). It has long been known that various types of foods, including cereals, potatoes, vegetables, fruits, mushrooms, algae, fish, meat, etc. were found to contain arsenic at various levels.

One of the major dietary sources of arsenic is seafood which contains the large amounts of organic arsenic. Among these organic arsenic compounds, most of them are considered as less harmful for human health compared to the inorganic arsenic. But some seaweed may also contain toxic inorganic arsenic. In recent times, exposure to arsenic through food has created an attention since high concentrations of arsenic found in various types of vegetables, dairy products, meats, grains (Rmalli et al., 2005; Grotti et al., 2008) and other food stuffs. Usually food grains and other agricultural products are cultivated by using ground water which is unfortunately contaminated with high concentration of arsenic in some areas of the world. Several studies have already confirmed that use of arsenic contaminated ground water for cultivation of rice and vegetables could be an important pathway of ingesting arsenic (Chakraborti et al., 2004; Rmali et al., 2005). Although it has been established that arsenic enters the food chain but there is great uncertainty about the bioavailability and associated toxicity of arsenic from different foods.

1.4. Arsenic in marine foods

The presence of arsenic in marine organisms were first reported almost a century ago, however, the large number and diversity of arsenic species including lipid soluble arsenicals in marine organisms has been revealed in the last 20-25 years (Francesconi et al., 1998; Vaskovsky et al., 1972; Cooney and Benson, 1978). Arsenic in seafood is primarily present as organic arsenic, whereas in freshwater, mainly as iAs. In the marine food, arsenic is considered to come from the seawater. Arsenic concentration in seawater is usually low and uniform, ranged between 0.5 to 2 µg As/L (Cullen and Reimer, 1989; Andreae, 1978), whereas in the case of rivers and lakes, arsenic concentrations may be variable depending

on the source, availability, and geochemistry (Rahman et al., 2012; Smedley and Kinniburgh, 2002). Although seawater contains lower levels of arsenic, in the marine food webs, much higher concentrations of arsenic are found compared to the freshwater systems. This variability could be explained by the transformation of iAs to organic arsenic compounds at the base of the marine food web, and the accumulation and retention of these organic compounds in marine organisms (Rahman et al,. 2012; Edmonds et al., 1997). Various types of marine algae contain high concentrations of arsenic ranging from 0.1 to 170 mg As/kg, dry weight (dw) (Kuehnelt and Goessler, 2003; EFSA, 2009) and for most of the marine fish tissue, arsenic concentration is usually found bellow 5 mg As/kg (Julshamn et al., 2004; 2012).

Both water soluble and lipid soluble arsenic compounds were also detected in several types of marine organisms (Edmonds and Francesconi, 1993; Feldmann and Krupp, 2011; Rumpler et al., 2008; Taleshi et al., 2010; Amayo et al., 2014). Water soluble arsenic compounds were shown to have many structural forms and to be widely distributed in the marine and fresh water environment. In the marine environment, more than 40 naturally occurring water soluble arsenic species so far have been identified including arsenate (As-V), arsenite (As-III), methylarsonate (MA-V), dimethylarsinate (DMA-V), arsenobetaine (AB), arsenocholine (AC), trimethylarsonium oxide (TMAO), tetramethylarsonium ion (TETRA), different arsenosugars (glycerol sugar, phosphate sugar, sulphonate sugar, sulphate sugar), and others (Francesconi and Kuehnelt, 2002).

AB is the major arsenic species among the water soluble arsenicals in marine organisms (Ballin et al., 1994; Francesconi and Edmonds, 1998). In some marine

organisms such as fish, bivalves and crustaceans, AB constitutes around 90% of the total arsenic (Maher et al., 1999; Foster et al., 2005). Methylated arsenic compounds (methylated of iAs with 1-4 methyl groups) are present in marine foods generally as minor arsenic species where DMA is the most prominent one. In molluscs DMA can be found at higher proportions (3–46%) than are usually found in finfish or algae (Fricke et al., 2004; Cleland et al., 2009; Whaley-Martin et al., 2012). The MA is not commonly found in marine organisms and is usually present in trace amounts only. The TMAO is another minor arsenic compound in seafood but can also occur in higher concentrations in some fish species (Kirby and Maher, 2002; Edmonds et al., 1993). A high proportion of TETRA has been found in clams and gastropods ((Shiomi et al., 1987; Francesconi et al., 1988).

Another minor arsenic compound is AC rarely found in seafood, with some exceptions in sea anemones and jelly fish (Ninh et al., 2008; Hanaoka et al., 2001). AC is not common in marine organisms probably because it is metabolized to AB (Francesconi et al., 1989). There are some other methylated compounds dimethylarsenoethanol (DMAE), dimethylarsenoacetate (DMAA), and dimethyl arsenopropionate (DMAPr) which can also be minor constituents of marine organisms but usually considered as the metabolism products of arsenosugars and arsenolipids (Sloth et al., 2005; Schmeisser et al., 2006a; Rmalli et al., 2005). Some of the As compounds also occur as thiol analogs, where sulfur replaces the oxygen atom in seaweeds and invertebrates (Schmeisser et al., 2004; Maher et al., 2013).

Arsenosugars are the main arsenicals in marine algae and also found in some herbivorous mollusks and gastropods (Edmonds et al 1997; Morita and Shibata, 1990).

Arsenosugars are ribose derivatives, which contain primarily a dimethylarsinoyl [$Me_2As(O)$-] moiety bound to various substituents at C-1 (Edmonds and Francesconi, 1983) via the C-5 of the ribose ring. They can also have a trimethyl As moiety instead of the dimethylated one, although these are far less prevalent (Shibata and Morita, 1988). Over 15 chemical forms of arsenosugars have been identified so far and in different marine organisms (Schmeisser et al., 2004; Maher et al., 2013).

Arsenolipids are another group of arsenic compounds which are lipids soluble and found in several types of seafood. Arsenolipid compounds include fatty acids (AsFAs), hydrocarbons (AsHCs), and glycophospholipids (AsPLs) types. As-containing alcohols, phosphatidylcholines and phosphatidylethanolamines have also been identified in some marine organisms. There is very little information available on the distribution of arsenolipids in seafood, but usually these compounds are associated with oily fish and fish oils.

1.5. Arsenolipids

It has been many years since arsenic was detected in marine foods but in recent years, various types of lipid soluble arsenic compounds have been identified in several marine foods. These compounds, known as arsenolipids, are lipid-soluble arsenicals and hence they have properties very different from arsenobetaine and the other arsenic compounds in marine foods, all of which are water soluble. The presence of arsenolipids was reported for the first time in 1920s when Sadolin identified arsenic in cod liver oil at concentration of 3.0-4.5 µg/g. Further works reported that marine fish and other marine

organisms may usually contain arsenic concentration between 1 to 50 mg/kg in their lipid soluble fractions (Lunde, 1974; 1977). Arsenolipids were usually expected to account for about 10 to 30% of the total arsenic present in marine organisms while some exception with higher relative proportions of arsenolipids have also been found in tuna, ringed seal, herring fillet and others (Lunde, 1977; Taleshi et al., 2010; Lischka et al., 2013). The first identification and characterization of the structure of arsenolipids was that in a brown alga, *Undaria pinnatifida* (wakame), in the classic study of Morita and Shibata (1988). In the two decades following this discovery, only a few attempts were made to identify lipid soluble arsenic species probably due to their chemical complexity and relatively low concentrations. However, in the last few years, identification and characterization of arsenolipids have been carried out on a wide range of marine foods and found often as the major form of arsenic in fish oils (Rumpler et al., 2008; Sele et al., 2012; Lischka et al., 2013), tuna (Taleshi et al., 2010) and other fish (Amayo et al., 2014), and also in some algae (García-Salgado et al., 2012; Raab et al., 2013). There are four major groups of arsenolipids identified so far in marine samples; the arsenic containing hydrocarbons (AsHCs), the arsenic containing fatty acids (AsFAs), the arsenosugar phospholipids (AsSugPLs), and the tri-methylated arsenic fatty alcohols (TMAsFOHs) (Garcia-Salgado et al., 2012; Raab et al., 2013; Amayo et al., 2013; Arroyo-Abad et al., 2013). Chemical structures of natural arsenolipids, representing the four major categories are shown in **Figure 1.1**.

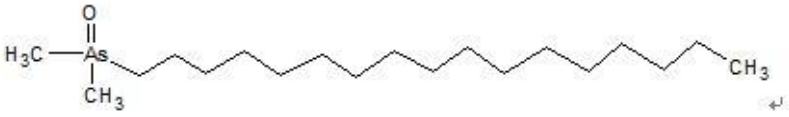
The arsenic containing hydrocarbons (AsHCs) typically comprise of homologous pair of dimethylarsinoyl-alkanes with carbon chains and so far more than 10 different AsHCs of different chemical structure have been identified (Taleshi et al., 2008; García-

Salgado et al., 2012; Raab et al., 2013; Amayo et al., 2013). The presence of AsHCs was reported for the first time in capelin fish oil by Taleshi et al. (2008) and now they have also been found in tuna (Taleshi et al., 2010), fish liver (Arryo-Abad et al., 2010) and in different species of algae (Garcia-Salgado et al., 2012).

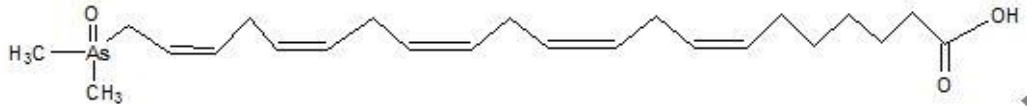
Arsenic containing fatty acids (AsFAs) were first reported in cod liver oil in 2008 (Rumpler et al., 2008) and later have been found in various fish species (Amayo et al., 2011; Lischka et al., 2013; Sele et al., 2014) and in some brown algae (Raab et al., 2013). So far more than 15 different chemical structures of AsFAs were identified in various marine samples including capelin fish, cod liver, herring fillet and in some algae (Amayo et al., 2011; Arroyo-Abad et al., 2010; 2013; Amayo et al., 2014).

The arsenosugar phospholipids (AsSugPLs) have been reported as major arsenolipids in marine algae (Garcia-Salgado et al., 2012; Raab et al., 2013). More than 10 chemical compounds belonging to AsSugPLs have been identified so far in different algae.

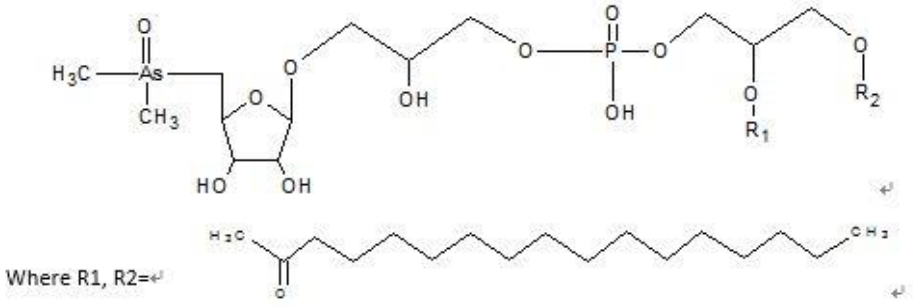
Trimethylated arsenic fatty alcohols (TMAsFOHs) represent another arsenolipid category and are considered as minor arsenolipid species compared to AsHCs, AsFAs and AsSugPLs in marine fish oil. Two chemical structures of cationic TMAsFOHs, comprising fatty alcohols with a positively charged terminal trimethylarsonium group were identified recently (Amayo et al., 2013).



Arsenic containing hydrocarbon

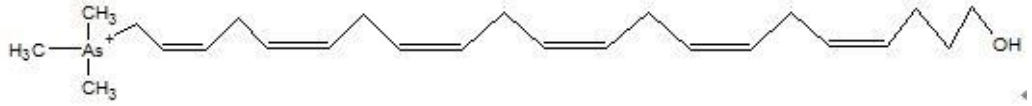


Arsenic containing fatty acid



Where R1, R2=

Arsenosugar phospholipids



Tri-methylated arsenic containing fatty alcohol

Figure 1.1 The chemical structure of arsenolipids, representing four groups of arsenolipids reported so far: arsenic containing hydrocarbons (AsHCs), arsenic containing fatty acids (AsFAs), arsenosugar phospholipids (AsSugPLs), and tri-methylated arsenic containing fatty alcohols. (Taleshi et al., 2008; Arroyo-Abad et al., 2010; Morita and Shibata, 1988; Arroyo-Abad et al., 2013).

1.6. The origin of arsenolipids

Arsenolipids are thought to be produced in marine algae and then transferred through food chain to other organisms. A proposed overview of the origin of arsenolipids described by Sele et al. (2012) is showed in **Figure 1.2**. Wrench et al. (1979) reported that arsenic from seawater is incorporated in phytoplankton which bio-transformed arsenic into lipid soluble arsenic species and then transferred to zooplankton and shrimp. In the case of fish, arsenolipids were considered to originate either from diet and bio-transformation within the fish or from a combination of these processes (Lunde, 1972; Francesconi et al., 1990).

Probably, the key to understanding how arsenic finishes up in the marine organisms lies in the similarities between the chemistry of arsenic and its fellow group 15 members, phosphorus and nitrogen. Arsenic exists primarily as arsenate, or, more specifically, as the di-protonated oxo-anion in normal seawater at pH 8.1, whereas in that same condition, one of the major seawater nutrients phosphate also exists as the di-protonated oxo-anion. To take up the essential phosphate nutrients, marine algae use their membrane transport systems which are insufficiently selective to differentiate against the structurally similar arsenate species from the water. As a result, arsenic can access into the algal cell. After that, it causes toxicity when arsenate is again mistaken by phosphate leading to disruption (decoupling) of oxidative phosphorylation processes (Dixon, 1996). For alleviating the toxicity, algae have developed a process of converting arsenate to arsenosugars through successive oxidative alkylation steps which can be followed by growing algae in sea water with different arsenic concentration with time. This detoxification process in alga *Fucus serratus* was reported by Geiszinger et al. (2001) which relate particularly to water soluble

arsenicals, and they showed that at low arsenic exposure (20 µg As/kg), alga can take up arsenate and convert it to arsenosugars efficiently without accumulation of intermediates in this process but at higher exposure, formation of intermediates methylarsonate and dimethylarsinate was observed, and in the case of the highest exposure (100 µg As/kg), the alkylation or detoxification process was overloaded, and toxic arsenite species accumulated to the fatal level for alga.

Since the presence of arsenolipids in marine organism has been explained by the incompetence of the organisms to discriminate between arsenic containing and non-containing components, based on the similarity in chemical structure of AsFAs and non-arsenic containing fatty acids, the AsFAs were thought to originate from de-novo synthesis like the de-novo synthesis of normal fatty acids (Rumpler et al., 2008).

In case of arsenic containing fatty acids development, the presence of saturated fatty acid C16:0 in position 2" in the AsSugPL, which is typical for bacterial fatty acid synthesis, pointed it towards a bacterial origin of the arsenolipids (Raab et al., 2013; Lipid library, 2014).

In the formation of AsHCs, fatty acids have been considered as start compounds (Taleshi et al., 2008), which were suggested to be reduced to the AsHCs through the bacterial conversion of fatty acids to alkanes and the formation of fatty alcohols (Kaufstad, 1992; Park, 2005).

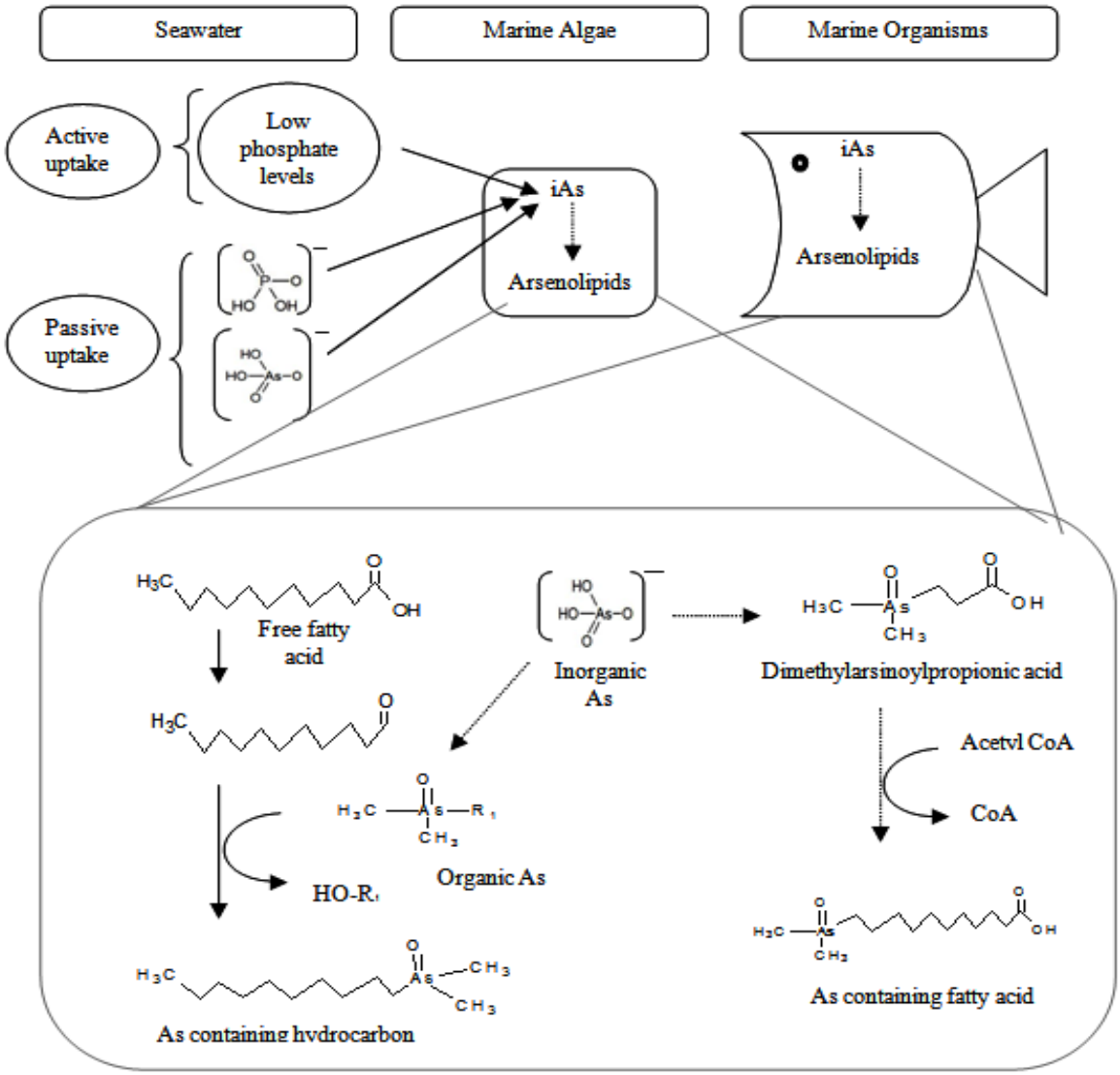


Figure 1.2 A postulated overview of arsenolipids origin in marine organisms (Sele et al., 2012)

1.7. Metabolism and toxicity of arsenolipids

One of the major sources of arsenic exposure to human is consumption of food including fish and other marine foods, whereas in certain parts of the world, increased level of arsenic in drinking water dominates the arsenic intake (EFSA, 2009; JECFA, 2011; Uneyama et al., 2007; Francesconi, 2007). In the metabolism of arsenic, it has been

emphasized that humans metabolize organic arsenicals (including arsenolipids, arsenosugars) and inorganic arsenicals usually to the same major arsenic metabolites; DMA-(V) (Francesconi, 2010; Feldmann and Krupp, 2011). The possible pathway of arsenolipids metabolism in the human body is, firstly conversion to fatty acids, and then further to DMA (Schmeisser et al., 2006).

For evaluating the toxicity of these compounds, a range of naturally occurring organic arsenic compounds including six pure arsenolipids (Taleshi et al., 2014) were synthesized and used in toxicity tests where one group of arsenolipids, the arsenic-containing hydrocarbons (AsHCs), displayed toxicity to human bladder and liver cells to the extent was comparable to that of iAs (Meyer et al., 2014). AsFAs exerted low cytotoxicity in human liver cell, which was 10-20 folds less toxic than the AsHCs (Meyer et al., 2015). When compared with in vitro toxicity of other seafood-relevant organic arsenicals, AsHCs were at least 600 fold more toxic than a glycerol arsenosugar (Andrewes et al., 2004) and 20-25 fold more toxic than DMA (V) (Leffers et al., 2013). Furthermore, in vivo results with whole organisms (Drosophila melanogaster) have also revealed potent toxic effects from exposure to AsHCs (Meyer et al., 2014). These toxicity results raised a concern about the human health risk who consume large amount of seafood containing arsenolipids.

1.8. Aim of the study

The presence of arsenolipids has been studied among several types of fish, algae and other marine foods in recent years. People who consume large amount of marine foods

in their daily life also intake arsenolipids in accordance. Since the toxicity study of arsenolipids showed that some arsenolipids were toxic to human liver and bladder cells, it has become a serious civic concern regarding the safety of foods containing arsenolipids. Hence, there is also a pressing public concern in Japan about arsenolipids because marine foods in both raw and cooked forms represent a significant part of the Japanese diet. With these concerns in mind, in the present study, I focused on the health risk of arsenolipids in marine food in the aspect of exposure assessment of arsenolipids which includes daily intake of arsenolipids, variation of intake, and the bioaccessibility of arsenolipids.

The aim of this study is to estimate the possible health risk of arsenolipids via the ingestion of marine foods by Japanese people. For achieving the aim, information needed to be obtained includes (1) the arsenolipids exposure to the Japanese people, (2) the variation of arsenolipids exposure to the Japanese people, and (3) the bioaccessibility of arsenolipids in marine foods.

Chapter 2

Analytical methods

In the present study, arsenolipids were analyzed in various types of food samples. Extraction of arsenolipids from food samples was performed by using a mixture of dichloromethane (DCM) and methanol (MeOH) (Glabonjat et al., 2014). Total arsenic was determined by using inductively coupled plasma mass spectrometry (ICP-MS), and the identification and quantification of arsenolipids were performed by using high-performance liquid chromatography (HPLC) - ICP-MS / electrospray ionization tandem mass spectrometry (ESI-MS-MS). The methods together with chemicals and reagents, extraction of arsenolipids, and the determination of arsenic species including arsenolipids are described in the following.

2.1. Chemicals and reagents

Water used for the analyses was obtained from a Milli-Q system (18.2 MΩ cm, Millipore GmbH, Vienna, Austria). Ethanol (≥ 99.9%, EtOH), methanol (≥ 99.9%, MeOH), dichloromethane (≥ 99.9%), formic acid (≥ 98%), and ammonia solution (25%) were obtained from Carl Roth GmbH (Karlsruhe, Germany); acetone (≥ 99.5%) was purchased from Sigma-Aldrich (Vienna, Austria); and silica gel 60 was obtained from Merck (Buchs, Switzerland).

For total arsenic determination, single-element standard solutions of arsenic (1,000 mg As/L ± 0.2%, matrix = 2% HNO_3) and germanium (1,000 ± 3 mg Ge/L, matrix = 2% HNO_3) were purchased from CPI International (Santa Rosa, USA).

Standard compounds used for the determination of arsenolipids were the arsenic hydrocarbon AsHC332 and the arsenic fatty acid AsFA362 (**Figure 2.1**), which were available in the laboratory in Austria from a previously reported synthesis (Taleshi et al., 2014).

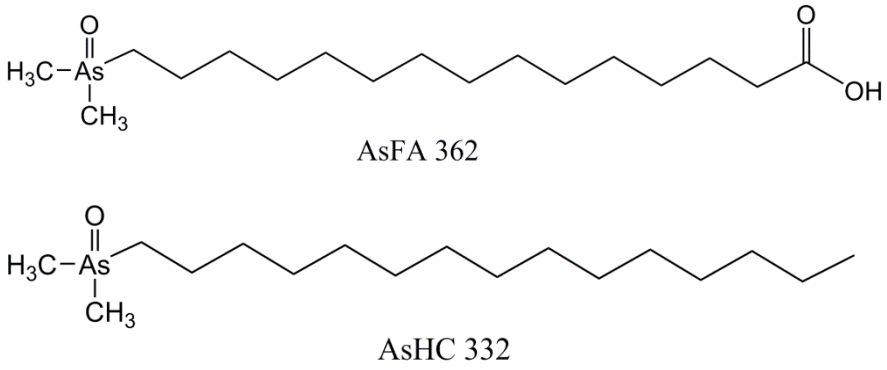


Figure 2.1 The structure of two synthesized arsenolipids, an arsenic-containing fatty acid (AsFA 362) and an arsenic containing hydrocarbon (AsHC 332)

2.2. Extraction procedure for arsenic speciation analysis

Freeze dried food samples (ca 200 mg) were extracted with 6 mL of dichloromethane (DCM)/methanol (MeOH) (2+1, v/v) on a rotatory cross for 2 hours at room temperature (Glabonjat et al., 2014). The mixture was then centrifuged for 10 minute (2100 G). A portion of supernatant (5mL) was transferred to a silica column (glass Pasteur pipet, 150 × 5 mm, filled to a height of 4 cm with silica gel 60), which had been

conditioned with MeOH/acetone (1+1,v/v) containing formic acid (5 mL). After the column was washed by "conditioning" mixture (4 mL), MeOH (2 mL) was added and the eluent was collected in a tube (called "prewash") which is expected to contain matrix components. Then MeOH containing 1% NH_3 (8 mL) was added to the column to remove arsenolipids from the silica and the eluent was collected in another tube (called "NH_3 wash"). Solvent in prewash and NH_3 wash tubes were evaporated completely under vacuum (10 mbar) at room temperature. The residue was re-dissolved in 500 µL EtOH with ultrasonication (15 min) and vortexing (3 minutes) at room temperature. This solution was then centrifuged, and the supernatant was analyzed for arsenolipids.

2.3. Determination of arsenolipid species (HPLC-ICP-MS/ESI-MS-MS)

Determination of arsenolipids was performed by HPLC-ICP-MS/ESI-MS-MS using an Agilent 1260 Infinity HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a degasser (G 4225A), a binary pump (G 1312B), an isocratic pump (G1310A), a thermosstatted auto-sampler (G 7617A), and a thermosstatted column compartment (G 1316A). ICP-MS (Agilent 7900 ICP-MS, Agilent Technologies, Waldbronn, Germany) signals were recorded at m/z 75 (^{75}As and $^{40}Ar^{35}Cl$) and m/z 77 ($^{40}Ar^{37}Cl$, for possible chloride interferences) at dwell times of 300 ms, and for internal standards at m/z 74 (^{74}Ge), m/z 115 (^{115}In) and m/z 125 (^{125}Te) at dwell times of 100 ms. Measurement with ESI-MS-MS (Agilent 6460, Agilent Technologies, Waldbronn, Germany) was performed in the positive ion mode with a precursor ion scan. Product ions at m/z 123 and 105 from the precursor ions of m/z 100-1000 were measured at a fragmentor voltage of 135 V and collision energy of 30 V; product ions at m/z 237 and 409 from the

precursor ions of m/z 400-1200 were measured at a fragmentor voltage of 220 V and collision energy of 50 V. Source conditions were: gas temperature: 100°C, gas flow 12 L/min, nebulizer pressure: 45 psi, sheath gas temperature: 350°C; sheath gas flow: 11 L/min, capillary voltage: 4500 V, nozzle voltage: 500 V.

Separation was performed by reversed-phase HPLC using an ACE Ultra Core super C18 (4.6 × 250 mm, 5 µm particle size). Eluent used was an aqueous solution containing 20 mM ammonium acetate at pH 9.2 and MeOH containing 20 mM ammonium acetate at pH 9.2 with the following gradient: 0-15 min, 20% - 100% MeOH, 15-35 min, 100% MeOH, 35-35.1 min, 100% - 50% MeOH, 35.1-40 min, 50% MeOH. The flow rate was 1 mL/min and the injection volume for ICP-MS and ESI-MS-MS detections was 20 µL. For the measurement, the HPLC effluent was split, whereby 10% was transferred to the ICP-MS unit and 90% to ESI-MS-MS using a passive splitter (Analytical Scientific Instruments, Richmond, USA). To dilute the HPLC eluent, a support flow of water containing 1% formic acid (v/v) and 20 µg/L Ge, Te and In (0.8 mL/min) was introduced through a T-piece after the splitter for ICP-MS measurement. Carbon compensation was performed by continuous addition of H_2O/MeOH (17+3, v/v) delivered with a tygon ISTD tubing (G1820-65220, 0.19 mm id) to ensure a constant introduction of carbon into the plasma. Determination of arsenic in the DCM fraction was carried out by ICP-MS in no gas mode, with 7% optional gas O_2, and with platinum setup.

HPLC-ESI-HR-MS-MS with accurate mass analysis was performed using a Dionex Ultimate 3000 series HPLC system coupled via a heated electrospray ion source to a Q-Exactive Mass Spectrometer (Thermo Scientific). The flow was 0.5 mL/min and the source

parameters were: needle temperature 270 °C and tension 3.5 kV (positive mode); sheath gas and auxiliary gas flow 52 and 13 respectively (arbitrary units), gas temperature 440 °C. Data dependent MS-MS mode was used with the following settings: Full scan at a resolution (FWHM) of 70,000 between m/z 300-1100, Automatic Gain Control set to 10^6 with a Maximum Injection Time of 100 ms, and for the data-dependent MS-MS part: Isolation window 0.4, Resolution: 17,500, Automatic Gain Control: 10^5, Maximum Injection Time: 50 ms, loop count: 5, intensity threshold: 2×10^4, so called Normalized Collision Energy: 15, 30 and 50, Dynamic exclusion time: 10 s and also excluding ^{13}C isotopes.

Identification of arsenolipids for those that had standards were based on retention time matching of both arsenic (ICP-MS) and molecular mass (ESI-MS-MS) chromatograms and for the other arsenolipids those that had no standards, identification was based on ESI-MS-MS data. Quantification was based on ICP-MS peak areas against external calibration with standards AsHC332 and AsFA362; for ICP-MS measurement signal response (peaks) depends on the arsenic content, independent of species. The arsenic-containing compounds eluting before 15 min were quantified based on AsFA 362, and the arsenic-containing compounds eluting after 15 min were quantified based on AsHC332.

ICP-MS data acquisition was performed with chromatographic software MassHunter Version B.01.01 (Agilent Technologies, Waldbronn, Germany). ESI-MS-MS data acquisition was done with chromatographic software Qualitative Analysis Version B.07.00 (Agilent Technologies, Waldbronn, Germany).

2.4. Determination of total arsenic

A portion of food samples and arsenolipids fractions of the sample extracts were analyzed after microwave-assisted digestion (UltraCLAVE IV Microwave Reactor; MLS GmbH, Leutkirch, Germany) for total arsenic. The samples (ca 100 mg) were weighted into quartz tubes (12 mL) and solvent, if present, was evaporated (10 mbar, room temperature; Maxi Dry Plus). Then HNO_3 (2 mL) and water (2 mL) were added to the samples, covered with Teflon caps, and heated according to the temperature programme:- 0 to 10 min, 80°C; 10 to 30 min, 150°C; 30 to 45 min, 200°C; 45 to 65 min, 250°C. After digestion and allowing cooling to room temperature, the samples were then transferred to polypropylene tubes (15 mL) and diluted with water to 9 g. Finally, 1 mL of internal standard (solution containing 100 µg/L of Ge) was added to all digested samples.

Determination of arsenic in the digested samples was performed by using inductively coupled plasma mass spectrometry (ICPMS) (Agilent 7900ce; Agilent Technologies, Waldbronn, Germany) in collision cell mode (He, 5 mL/min) to minimize polyatomic interferences from argon chloride ($^{40}Ar^{35}Cl$) on arsenic (^{75}As). Standards for calibration were prepared in 20% HNO_3 for matrix matching and contained 10 µg/L Ge as the internal standard.

2.5. Reliability of the analysis

As an evaluation of the reliability of the arsenolipids measurement, the trueness and the precision of the analysis were examined. Arsenolipids were measured in a certified reference material (CRM) of algal origin NMIJ CRM 7405-a (Hijiki) (CRM which is a

homogeneous powder made by National Metrology Institute of Japan). **Figure 2.2** shows the analytical result of arsenolipids in hijiki CRM along with literature data on this CRM (Glabonjat et al., 2014).

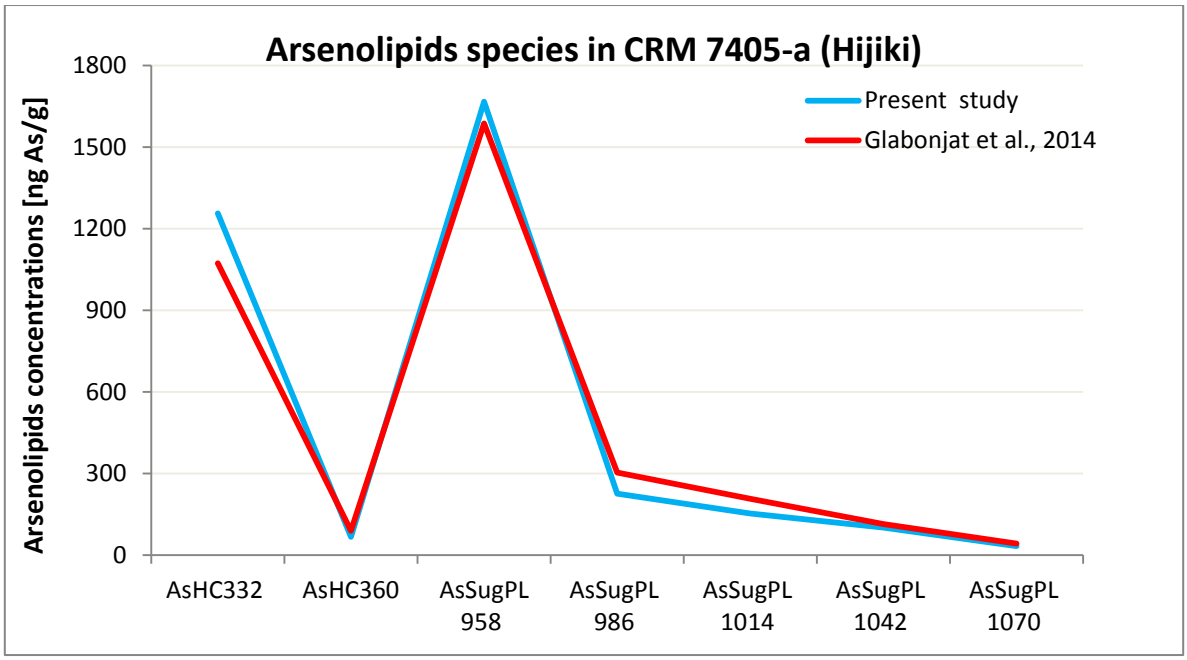


Figure 2.2 Arsenolipids concentrations in CRM 7405-a (Hijiki)

The arsenolipids species including AsHCs and AsSugPLs were detected in hijiki CRM and their relative abundance agreed with those reported in a previous study (Glabonjat et al., 2014) which confirmed the trueness of the present analysis. Since the certified values for arsenolipids are not available in this and other CRMs, I compared the arsenolipids value of present study with the literature value on the same material for the validation of trueness. In this case, there were no significant differences found between the measured values of arsenolipids in the present study and literature values.

Since accuracy is the combined concept of trueness and precision, I also analyzed arsenolipids in another CRM, TJD (CRM no. 27 Typical Japanese Diet) which represents the duplicate Japanese diet (food matrix) and was prepared by The National Institute for Environmental Studies (NIES), Japan for the evaluation of precision of the present arsenolipids measurement. The arsenolipids concentrations in the TJD CRM obtained by triplicate measurements are shown in **Table 2.1**.

Table 2.1 Arsenolipids in CRM No. 27 Typical Japanese Diet, TJD

Concentrations of arsenolipids (ng/g, dry weight), mean ± SD, n=3	
Arsenolipids	**RP-HPLC-ICPMS/ESIMS**
AsHC332	0.52 ± 0.09
AsHC360	0.31 ± 0.02
AsHC444	1.69 ± 0.54
AsFA362	0.16 ± 0.05
AsFA388	0.16 ± 0.05
AsFA418	0.16 ± 0.05
AsPL958	5.42 ±1.57
Unknown	136 ± 9.66
Total arsenolipids	144 ± 12.6
Total arsenic in arsenolipids fraction	140 ± 13.2

Total arsenolipids concentrations including unknown and the total arsenic concentration in lipids fraction were found to be consistent. The precision of the

arsenolipids measurement was calculated to be about 6-32% throughout the analysis. The trueness and precision examined in the case of Hijiki and TJD CRM indicated that the arsenolipids analysis in the present study was reliable and accurate.

Chapter 3

Exposure assessment of arsenolipids of Japanese people

3.1. Background

Both the organic and inorganic arsenic (iAs) in various foodstuffs have been reported since long. In particular, it has been well known that the levels of arsenic (As) in marine foods can be high (190 µg As/g, dry weight) (Mania et al., 2015). Health authorities, however, have not been concerned by these high concentrations because arsenic in marine foods was generally considered to be present mainly as harmless organic compounds such as AB and arsenosugars (Francesconi, 2005). Organic arsenic species present in fish and shellfish are mostly AB, and those in algae (seaweed) are mostly arsenosugars. Another group of organic arsenic species which usually found to be present in various marine organisms are lipid soluble, and known as arsenolipids. They have properties very different from AB and other organic arsenic compounds in marine foods, all of which are water soluble. Over the last few years, several studies on arsenolipids have been carried out in wide range of marine foods such as fish oils, fish liver, fish muscles of various fish species (Rumpler et al., 2008; Sele et al., 2012, 2014; Lischka et al., 2013; Arryo-Abad et al., 2010; Taleshi et al., 2010; Amayo et al., 2014), and in some algae (García-Salgado et al., 2012; Raab et al., 2013).

Taleshi et al. (2014) chemically synthesized a range of naturally occurring organic arsenic compounds including seven arsenolipids to be used in toxicity tests. These tests revealed that one group of arsenolipids, the arsenic containing hydrocarbons (AsHCs) (**Figure 3.1**) showed cytotoxicity to human bladder and liver cells and that the magnitude of cytotoxicity was comparable to that of arsenite (Meyer et al., 2014) which is known to be one of the most toxic elements. This toxicity result has raised a concern over the human health risk of arsenolipids particularly among populations who consume large amounts of marine foods rich in these arsenolipids.

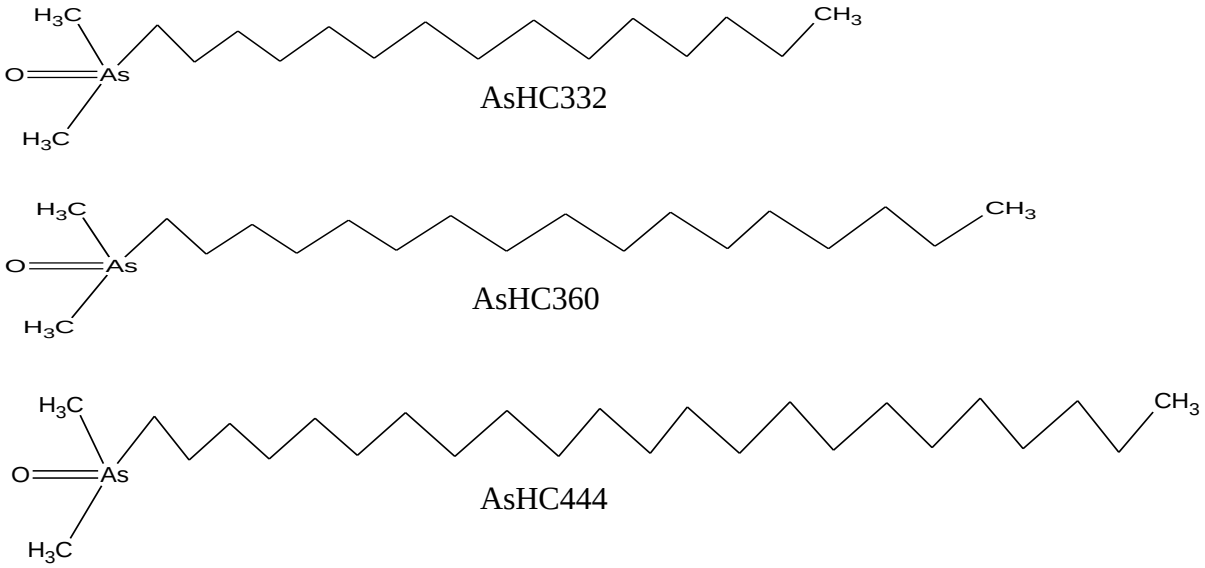


Figure 3.1 Cytotoxic arsenolipids, the arsenic containing hydrocarbon (AsHC) followed by their molecular mass

There is also a serious public concern in Japan about arsenolipids since the marine foods represent a significant part of Japanese diet. The Japanese are known to consume on

average 66 g of fish and shellfish a day (MHLW, 2015) which is probably among the most abundant in the world. The European Food Safety Authority has also requested scientific data on arsenolipids in foods ahead of a re-assessment of safe limits for arsenic in foods (EFSA, 2009). Therefore, the health risk of toxic arsenolipids intake via the consumption of marine foods has to be assessed.

3.2. Objectives

The objectives of this chapter are summarized as follows

(1) To estimate the daily intake of arsenolipids

(2) To determine the major food sources which contribute to the daily intake of arsenolipids for Japanese people

(3) To estimate possible health risk associated with the daily intake of arsenolipids

3.3. Methods

3.3.1. Market basket design and sampling

A market basket survey was performed for this study in which 152 food items of 17 food categories were purchased in one day in December 2015 from supermarkets in Shizuoka city, Shizuoka Prefecture, Japan. This city is located in the central part of Japan, in the Tokai District, being intermediate between two mega cities, Tokyo and Osaka, with different cultural characteristics in many aspects. The average amount of food consumption of 17 food categories in Tokai district is similar to that of entire Japan (MHLW, 2015). The 17 food categories, including cereals, potatoes, sugars and sweeteners, pulses, nuts and seeds, vegetables, fruits, mushrooms, algae, fish and shellfish, meats, eggs, milks, oils and

fats, confectioneries, beverages, and seasonings and spices were based on the Food Categories of the 2013 National Health and Nutrition Survey (NHNS) by the Ministry of Health Labor and Welfare (MHLW) of Japan (MHLW, 2015). Food items in each category were chosen according to the Detailed Environmental Survey (DES) in 2006 (Ministry of the Environment of Japan, 2007). In total, 152 food items were sampled in this survey. Purchased food samples were transferred to the laboratory under refrigerated condition (4°C) overnight and kept in refrigerator (vegetables, fruits, eggs, and some confectioneries), in freezer at -18°C (raw meat, fish and shellfish, etc.) or at room temperature (canned foods, dried foods, oils, nuts and seeds, etc.) until sample preparation within two days after purchase.

3.3.2. Preparation of food composites

Collected food samples were processed (washing or soaking, boiling or baking or frying, etc.) as Japanese people usually do in their households (Ministry of the Environment of Japan, 2007). The food sample preparation methods are briefly listed by food items in **Table 3.1**. An aliquot of each prepared food item in a food category was mixed to prepare a composite for the food category. The weight of each food item in a composite was determined based on food consuming statistics of Tokai District in the 2013 NHNS (MHLW, 2015).

Food composite was homogenized in a food processor (Cuisinart, San-ei Co., Ltd, Tokyo, Japan) for 5 min. A portion (25 g) of the homogenized composite was individually freeze dried in a 50 mL polypropylene tube. The weight of the freeze-dried composite was

measured and the weight loss after freeze-drying was assigned as moisture content. Some composites (sugars and sweeteners, nuts and seeds, oils and fats, and beverages) were not freeze dried for subsequent arsenolipid analysis. The concentrations of arsenolipids in the food composites are expressed as arsenic weight on a fresh weight (fw) basis. The analysis of arsenolipids in food composites was performed according to the procedure presented in "Chapter 2".

Table 3.1 Food items tested (n=152) in this study from a market basket survey

Food categories	N	Sample description	Preparation method	Daily consumption (g/person/day) (MHLW, 2015)
Cereals	16	Rice (n=2), assorted cereal (e.g., barley, millet)	Prewashed and boiled	418 ± 174
		Rice cake, Wheat flour, Noodles, Chinese noodles, Somen,	Baked	
		Precooked Chinese noodles, Spaghetti, Buckwheat noodles,	Boiled	
		White table bread, French bread, Bean jam bun, Soft rolls, Corn flakes	Uncooked	
Potatoes	6	Sweet potatoes, Potatoes, Taro, Konnyaku, Kudzu starch noodles,	Peeled and boiled	53.5 ± 64.4
		Chinese yam	Peeled and grated	
Sugars and sweeteners	1	Sugars	Uncooked	6.40 ± 8.1
Pulses	7	Fried bean curd, Cooked bean curd, Boiled soybean, Boiled red bean,	Boiled	57.4 ± 72.8
		Fermented soy bean, Bean curd, Soy milk	Uncooked	
Nuts and seeds	2	Sesame seeds, Peanuts	Uncooked	1.73 ± 6.2
Vegetables	22	Tomato, Cabbage, Cucumber, Bamboo shoot, Lettuce, Vegetable juice, Picked nozawana, Salted Chinese cabbage,	Uncooked	251 ± 159
		Picked radish, Pickled plum, Celery	Peeled and boiled	
		Carrots, Radish, Onion, East Indian lotus root, Spinach, Pumpkin, Broccoli, Chinese cabbage, Garland chrysanthemum, Bean sprout, Green sweet pepper	Boiled, grilled	

Table 3.1 continued				
Fruits	10	Mandarin, Grapefruit, Banana, Apple, Japanese persimmon, Canned peach, Canned pineapple, Strawberry jam, Orange juice, Apple juice	Peeled Uncooked	104 ± 138
Mushrooms	3	Shiitake, Winter mushroom, Shimeji	Grilled Boiled	15.7 ± 27.8
Algae	4	Hijiki, Wakame, Nori, Kombu	Soaked Uncooked Boiled	10.9 ± 19.5
Fish and shellfish	26	Horse mackerel, Sardine, Pacific saury, Salmon, Tuna, Broiled Japanese eel, Oyster, Scallop, Squid, Octopus, Canned mackerel, Canned tuna, Cooked short necked clam, Fish cake (n=3), Fish sausage, Mackerel, Right eye flounder, Red bream, Yellowtail, Giant tiger shrimp, Salted salmon, Salted cod roe, Dried horse mackerel, Crab	Uncooked Grilled	66.3 ± 71.5
Meats	10	Beef-inside round, Beef chuck, Pork peach, Pork loin, Ham, Pork bacon, Pork sausage, Lamb, Chicken breast, Beef liver	Roasted	88.1 ± 71.7
Eggs	1	Egg	Uncooked	32.4 ± 32.3
Milks	6	Milk, Process cheese, Yogurt, Lactic-acid and bacteria beverages, Condensed whole milk, Coffee creamer	Uncooked	123 ± 139
Oils and fats	6	Butter, Margarine, Vegetable oil, Olive oil, Sesame oil, Lard	Uncooked	10.2 ± 9.0
Confectioneries	8	Sweet red bean paste jelly, Rice crackers, Cake, Custard cream puffs, Crackers, Candy, Chocolate, Potato chips	Uncooked	25.2 ± 50.4
Beverages	8	Sake (rice wine), Beer, Red wine, Green tea (infusion), Black tea, Coffee, Cola drink, Isotonic drink	Uncooked	593 ± 448
Seasonings and spices	16	Worcester sauce, Soy sauce, Salt, Mayonnaise, Miso, Grain vinegar, French dressing, Tomato ketchup, Noodle sauce, Grilled meat sauce, Instant bouillon, Consommé, Curry roux, Demi-glace sauce roux, Ginger paste, Wasabi paste	Uncooked	85.5 ± 84.3

The Japanese daily food consumption weight for each food category was from the national health and nutrition survey, Japan (MHLW, 2015).

3.3.3. Calculation of daily intake of arsenolipids

The daily intake of arsenolipids from each food category was calculated by multiplying the concentrations of arsenolipids in food composites and the average daily consumption weight of the corresponding food category (**Table 3.1**) (MHLW, 2015). When arsenolipids were non-detectable in a food composite, daily intake was estimated by assuming the intake from the category as "0" (zero) ng As/person/day. The daily food consumption data used in this study were collected by MHLW of Japan from 1088 residents in Tokai District where Shizuoka city is located. Along with the large number of study subjects (n=1088) (MHLW, 2015), the food-consumption statistics in Tokai District is close to the national average of Japan making the data representative of whole Japan.

3.4. Results

3.4.1. Concentration of arsenolipids in food composites

Among the 17 food composites, arsenolipids were detected in "algae" and "fish and shellfish" composites (**Table 3.2**) while the concentrations of arsenolipids were below detection limit for all the other food categories. The detection limits of the food samples for arsenolipids varied from 0.8-4 ng As/g depending on the food categories and the moisture content of the initial fresh product.

In "algae" composite, the major arsenolipids by far were arsenosugar phospholipids (AsSugPLs) with concentrations of seven identified compounds ranging from 0.8 to 37.5 ng As/g (**Table 3.2; Figure 3.2**). Two of the cytotoxic arsenic containing hydrocarbons, AsHC332 (3.7 ng As/g) and AsHC360 (10.3 ng As/g) were also found in "algae"

composite, whereas the arsenic containing fatty acids (AsFAs) were not detected. In "fish and shellfish" composite, AsFA362, AsFA388, and AsFA390 were detected at concentrations of 7.7, 0.8, and 10.7 ng As/g, respectively (**Table 3.2; Figure 3.3**). Two arsenic containing hydrocarbons AsHC332 (23 ng As/g) and AsHC360 (4.6 ng As/g) were also found in "fish and shellfish" composite whereas AsSugPLs were not detected.

Table 3.2 Concentrations of arsenolipids in food composites

	Arsenolipids (ng As/g fw)											
Food category	**AsHCs**		**AsFAs**			**AsSugPLs**						
	AsHC 332	AsHC 360	AsFA 362	AsFA 388	AsFA 390	AsSugPL 720	AsSugPL 930	AsSugPL 958	AsSugPL 986	AsSugPL 1014	AsSugPL 1042	AsSugPL 1070
Algae	**3.7**	**10.3**	<0.8	<0.8	<0.8	**37.5**	**4.41**	**29.4**	**5.9**	**5.1**	**0.8**	**2.2**
Fish and shellfish	**23**	**4.6**	**7.7**	**0.8**	**10.7**	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8

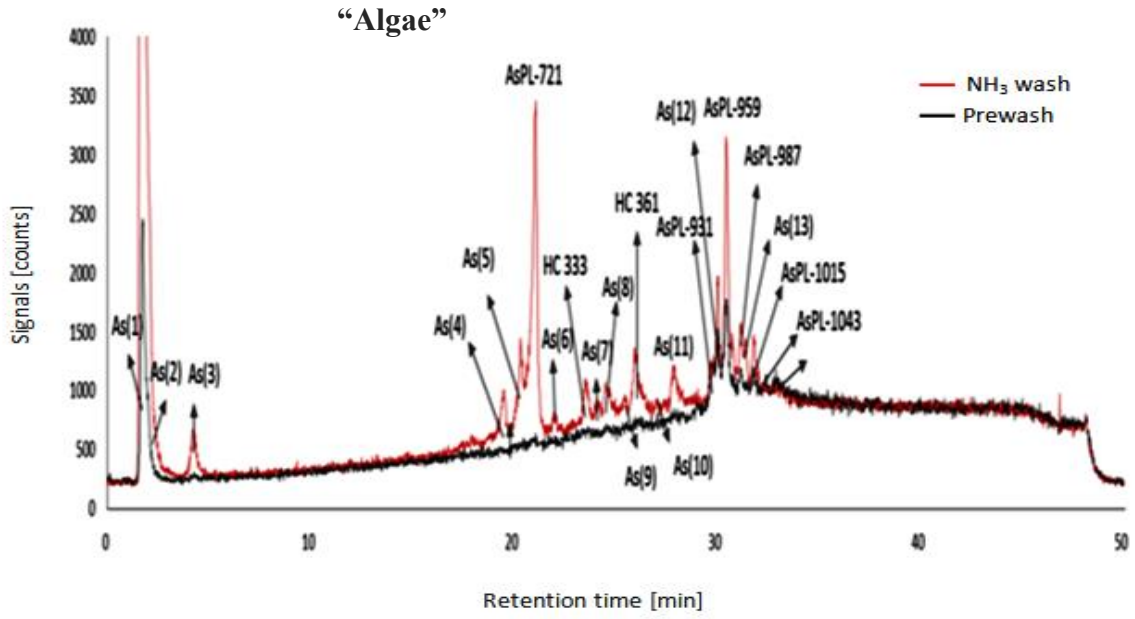


Figure 3.2 HPLC-ICP-MS chromatograms of algae composite after the extraction with DCM/MeOH mixture (2+1, 6mL). Different arsenolipids were detected in NH_3 wash (red) and in prewash eluent (black) obtained in extraction procedure. In chromatograms, arsenic containing hydrocarbons (AsHCs) were expressed as HC and arsenosugar phospholipids (AsSugPLs) were as AsPL in the protonated form. Some unknown arsenic compounds [As (1) to (8)] were also detected in both prewash and NH_3 wash eluents.

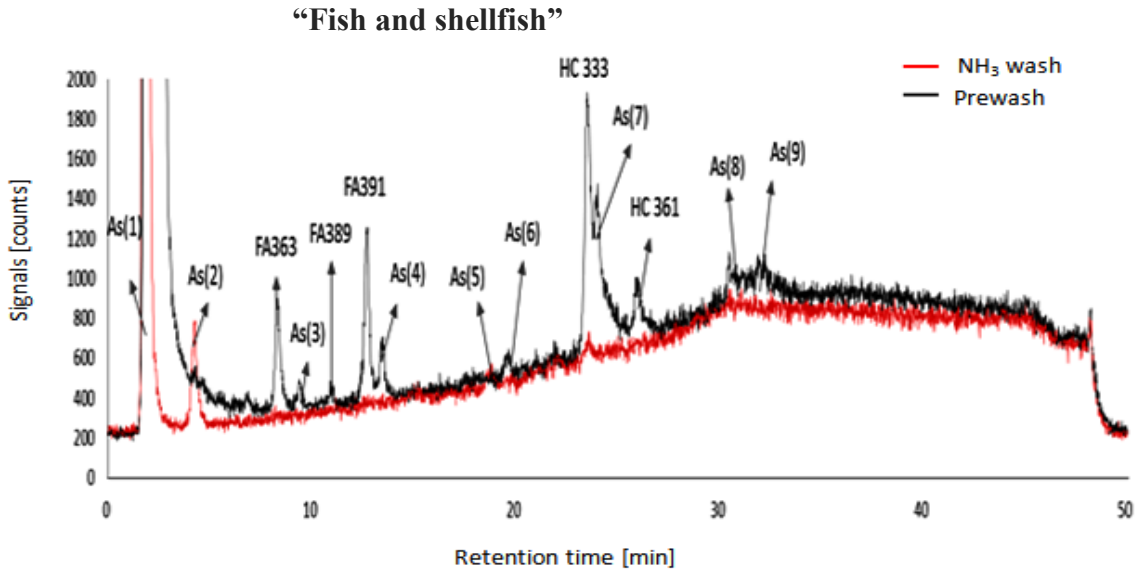


Figure 3.3 HPLC-ICP-MS chromatograms of fish and shellfish composite after extraction with DCM/MeOH mixture (2+1, 6mL). Different arsenolipids detected in NH_3 wash (red) and in prewash eluents (black) were obtained in extraction procedure. In chromatograms, arsenic containing hydrocarbons (AsHCs) were expressed as HC and arsenic containing fatty acids (AsFAs) were as FA in the protonated form. Unknown arsenic compounds [As (1) to (9)] were also detected in both prewash and NH_3 wash eluents.

3.4.2. Estimated daily intake of arsenolipids by Japanese people

The daily intakes of each arsenolipid (ng As/person/day) for Japanese people from the food categories as well as the total daily intake were estimated and shown in **Table 3.3**. From the "algae" food category, the average daily intake of AsHC332 and AsHC360 were 40.4 and 112 ng As/person/day, respectively. The daily intake of AsSugPL 720, AsSugPL

930, AsSugPL 958, AsSugPL 986, AsSugPL 1014, AsSugPL 1042, and AsSugPL 1070 were 409, 48, 321, 64.4, 55.6, 7.64, and 24 ng As/person/day, respectively.

In the case of "fish and shellfish" food category, the daily intake of AsHC332 and AsHC360 were 1525 and 305 ng As/person/day whereas the daily intakes of AsFA362, AsFA388 and AsFA390 were 511, 53 and 709 ng As/person/day, respectively. For all others food categories, the arsenolipid intake was regarded as 0 ng As/day, although some of the food could have contained very low concentrations of arsenolipids that fell below the detection limits.

Table 3.3 Estimated daily intake of arsenolipids by food categories

	Arsenolipids (ng As/person/day)											
Food category	**AsHCs**		**AsFAs**			**AsSugPLs**						
	AsHC 332	AsHC 360	AsFA 362	AsFA 388	AsFA 390	AsSugPL 720	AsSugPL 930	AsSugPL 958	AsSugPL 986	AsSugPL 1014	AsSugPL 1042	AsSugPL 1070
Algae	**40.4**	**112**	"0"	"0"	"0"	**409**	**48**	**321**	**64.4**	**55.6**	**7.64**	**24.0**
Fish and shellfish	**1525**	**305**	**511**	**53**	**709**	"0"	"0"	"0"	"0"	"0"	"0"	"0"
Total	1565	417	511	53	709	409	48	321	64.4	55.6	7.64	24

Intakes from the food composites with undetectable arsenolipids species were estimated by assuming their concentration as "0" (zero).

3.5. Discussions

3.5.1. Arsenolipids content in foodstuffs

The presence of arsenolipids was reported in various seafood including a range of fish species (Taleshi et al., 2010; Lischka et al., 2013), fish oil (Taleshi et al., 2008), fish liver (Arryo-Abad et al., 2010), various algae (Raab et al., 2013; Garcia-Salgado et al., 2012), and in some marine invertebrates (Vaskovsky et al., 1972). Hence, people who consume high amount of seafood abundantly take up arsenolipids accordingly. From the recent *in vitro* toxicity study of arsenolipids, it was revealed that compounds from one group of arsenolipids, AsHCs are cytotoxic to human bladder and liver cells to a degree comparable with that of toxic inorganic arsenic (III) (Meyer et al., 2014). The AsFAs also exerted cytotoxicity in human liver cells, but at a 10-20 fold lower level than did the AsHCs (Meyer et al., 2015). These preliminary toxicity results have raised concerns about the health risk for people who consume large amounts of seafood.

In view of the cytotoxic nature of arsenolipids to human cells and their abundance in various marine foods, in the present study for the first time, arsenolipids were measured in 17 categories of foods collected in Japan as background information to assess their potential human health risk. Among the food categories, arsenolipids including AsHCs, AsFAs, and AsSugPLs were detected in "algae" and "fish and shellfish" at concentrations ranging from 0.8-37.5 ng As/g (fw), but they were not present at detectable concentrations in any of the other food composites **(Table 3.2)**. The naturally occurring major arsenolipids in fish are AsHCs and AsFAs, where usually arsenic instead of phosphorus binds directly to either a long chain fatty acid or a hydrocarbon (Rumpler et al., 2008; Taleshi et al., 2008).

AsSugPLs are the major arsenolipids found in algae (Raab et al., 2013) where arsenic is usually bound in the form of well-known As−sugar−PO_4 moiety (Morita and Shibata, 1988). The arsenolipids concentration found in "algae" and "fish and shellfish" composite in the present study and their probable variation surely affects the amount of their daily intake for Japanese people. The concentration results were further used for calculating the daily intake of arsenolipids which are very crucial for estimating its health risk.

3.5.2. Average daily intake of arsenolipids

"Algae" and "fish and shellfish" are the food categories in the Japanese diet with an average daily consumption of up to 10.9 and 66.3 g/day/person, respectively (MHLW, 2015). The average daily intake of arsenolipids were estimated for the Japanese (**Table 3.3**) by multiplying the average weight of the consumption of food category (g/day) and the concentrations of arsenolipids in each category (ng As/g). Average daily intake of AsHCs, AsFAs, and AsSugPLs were estimated to be ca 2.0, 1.3, and 1.0 µg As/person/day, respectively. Intake from food categories with undetectable arsenolipids species was estimated by assuming their concentration as "0" (zero) and hence the estimation represents the minimum values. The estimated daily intake of arsenolipids, particularly toxic AsHCs (2 µg As/person/day), could pose some health risk for Japanese people if they could be available for the absorption after human gastrointestinal digestion.

3.5.3. Estimation of health risk associated with the daily intake of arsenolipids

The health risk of toxic AsHC332 and AsHC360 was estimated by using margin of exposure (MOE) to their daily intake of 1.6 and 0.4 µg As/person/day for Japanese people. MOE is the indicator of risk. Usually MOE is calculated by the ratio of No Observed

Adverse Effect Level (NOAEL; unit is mg/kg/day) to Estimated Human Exposure (EHI) but in the case of arsenolipids, there is no NOAEL reported so far. So, I had to use another toxicity indicator which is available for arsenolipids instead of NOAEL. In this study, as a toxicity indicator, I considered the IC_{50} (The half maximal inhibitory concentration) values of 9.2 and 4.8 µM, which were the lowest IC_{50} in the medium for human liver and bladder cells exposed to AsHC332 or AsHC360, respectively (Meyer et al., 2014). For the estimation of MOE, EHI must be converted to body fluid-concentration as of IC_{50} value (µM). In this estimation, couple of assumptions are necessary: the first is that the intact AsHCs in the food are absorbed immediately and completely from the digestive tract; and the second is that the absorbed AsHCs are quickly distributed without metabolism evenly in the blood stream, volume of which is approximately 5 L, and blood concentration is similar to body fluid concentration of AsHCs. Based on these assumptions, body fluid concentration of AsHC332 and AsHC360 corresponding to 1.6 and 0.4 µg As/person/day intake was estimated to be approximately 0.004 and 0.001 µM and then the MOE to the IC_{50} value for AsHC332 and AsHC360 is 2300 (9.2/0.004) and 4800 (4.8/0.001), respectively. Generally health risk is assessed by whether the intake exceeds the tolerable daily intake (TDI) or not. TDI is the product of NOAEL divided by Uncertainty Factors (UFs) and usually UF is set at 100. Therefore, for the health risk assessment, MOE needs to be compared with 100, a typical UF. If MOE<100, then, it implies the presence of risk because intake can exceed the TDI. On the other hand, if MOE>100, then it implies the absence of risk. Since the estimated MOE in the present study is much more than 100, it suggests that the toxic AsHCs intake of the Japanese does not seem to pose significant health risk.

However, IC_{50} values of 9.2 and 4.8 µM is not in fact the minimum concentration level of toxicity since it represents the concentration of inhibitor or toxic chemicals where 50% inhibition of a biological or biochemical function or process required. The actual toxicity of a chemical starts at a much lower concentration than the IC_{50} value and hence the minimum concentration for toxicity of AsHC332 and AsHC360 would be much less. Therefore, the actual health risk would be higher than that estimated in the present study. The estimation of body fluid concentration of 0.004 µM (AsHC332) and 0.001 µM (AsHC360) in the present study was based on a "point estimate" of AsHCs intake without considering the variation of intake. A "point estimate" is not sufficient for the assessment of health risk, which ideally requires "distribution estimates" that include intra- and inter-individual variability of intake within the target population. A large variation of daily intake of arsenolipids is expected from one person to another depending on the variation in frequency and amount of marine food consumed. Variability of arsenolipids concentration within food items due to regional and seasonal variation in marine foods is also expected to be significant and it will result in intra- as well as inter-individual variability. Therefore, it is crucial to estimate the probable range of exposure to arsenolipids for the assessment of the health risk at population levels.

3.6. Conclusion

The present market basket study indicated that only "algae" and "fish and shellfish" food categories significantly contribute to the daily intake of arsenolipids for Japanese people. The estimated daily intakes of AsHCs, AsFAs, and AsSugPLs were ca 2.0, 1.3, and

1.0 μg As/person/day, respectively. The MOE for AsHC332 and AsHC360 to the IC_{50} value was about 2300 and 4800 based on this rough estimation. Although these data do not indicate that exposure to arsenolipids through diet poses significant health risk in the Japanese, the values represent "point estimates" without considering any intra- and inter-individual variability. For the complete evaluation of the health risk of arsenolipids, exposure range of arsenolipids needs to be assessed.

Chapter 4

Variation of arsenolipids concentration among several fish species and their stability on cooking

4.1. Background

In some recent studies, various arsenolipids have been found in a range of marine organisms, especially in various fish and fish products. The presence of AsHCs have been detected in tuna, fish liver, herring fillet, and some commercial fish muscle tissues and fish oils (Taleshi et al., 2010; Arryo-Abad et al., 2010; Amayo et al,, 2014). AsFAs have also been found in various fish species (Lischka et al., 2013; Sele et al., 2014) while AsSugPLs were found exclusively in algae (Garcia-Salgado et al., 2012; Raab et al., 2013). Therefore, people like the Japanese who consume large amounts of fish, fish products, and seaweeds abundantly take up arsenolipids accordingly.

By considering the cytotoxic nature of these arsenolipids to human cells, and their abundance in sea foods, for the first time I measured arsenolipids in Japanese food composites and roughly estimated the health risk of toxic AsHC332 and AsHC360 present in foods for Japanese people. The health risk was evaluated by estimating the margin of exposure (MOE) based on the daily intake of arsenolipids and its available toxicity data (Meyer et al., 2014) (described in Chapter 3). The result suggested that the average AsHCs

intake level of the Japanese did not pose significant health risk though it was a "point estimate" without considering the "distribution estimates" of risk. For more detailed assessment of human health risk of ingesting arsenolipids-containing foods, variation of daily intake in population has to be assessed. Variation of daily intake of arsenolipids can be caused from both variation in food consumption amount and variation in arsenolipids concentrations in foods. Most of the cytotoxic AsHCs (92%) in the Japanese daily intake comes from the food category of fish and shellfish (1.8 μg As/person/day) which was based on the measured AsHCs concentration in composite of "fish and shellfish" prepared by mixing 26 fish species commonly consumed in Japan (Chapter 3). However, it is expected that there is a large variation in arsenolipids concentration among various fish species and it needs to be examined for evaluating the variation of arsenolipids intake for the Japanese.

All of the literature data of arsenolipids reported so far is on raw fish basis, however, human health risk should be evaluated on cooked fish since fish and shellfish are in many cases cooked before eating. The changes in arsenic species during cooking of fish species have been investigated in several studies but all of them have evaluated the water soluble arsenicals (Devesa et al., 2001; 2001; Dahl et al., 2010) and there have been no similar studies which focused on lipids soluble arsenicals. The possibility of changes of arsenic species in foods by cooking would also appear to be specifically relevant to arsenolipids based on the instability of many normal (non-arsenic) lipids (Stephen et al., 2010; Moradi et al., 2011). Therefore, in the health risk estimation, for assessing the variation of arsenolipids daily intake, the concentrations of arsenolipids in various raw fish species and the possible changes for arsenolipids during cooking need to be investigated.

4.2. Objectives

The objectives of this chapter are summarized as follows:

(1) To examine the variation range of toxic AsHCs concentrations in various fish species commonly consumed by Japanese people.

(2) To evaluate if arsenolipids in raw fish are decomposed during cooking.

4.3. Methods

4.3.1. Sample collection and preparation

Nine most commonly consumed fish species by Japanese people available in supermarket including, salmon (*Salmo salar*), mackerel (*Scomber scombrus*), yellow tail (*Gadus morhua*), tuna (*Thunnus thynnus*), sardine (*Sardinops sagax)*, sea bream (*Beryx splendens*), skipjack (Katsuwonus pelamis), pacific saury (*Cololabis saira*), and whitebait (*Galaxias maculatus*) were purchased. These fish samples were then transferred to the laboratory under refrigerated condition (4^{o}C). After washing the fish samples with MQ water, the edible portion was collected as Japanese people usually do in their house. Each of the fish species was then homogenized individually in a food processor (Cuisinart, San-ei Co., Ltd, Tokyo, Japan) for 5 minutes.

For evaluating the arsenolipids changes on cooking, three of the homogenized fish samples (salmon, yellowtail, and mackerel) were divided into three portions. First portion was kept as raw; second one cooked by directly grilling on a pan; and the third portion was grilled on the pan after taking it on an aluminum foil to retain any fish oil which might

otherwise drain out during cooking. Grilling of fish was done at moderate heat of around 200^{o}C. A portion (30g) from all the nine raw homogenized fish species and separately cooked fish (cooked by grilling and cooked by grilling after taking on aluminum foil) was individually freeze dried in a 50 mL polypropylene tube. The weight of the freeze dried fish sample was measured and the weight loss after the freeze drying was assigned as moisture content. Moisture content of nine fish species ranged from 58% to 74%. Lipid content in six of the fish species including tuna (0.40 g/100g of sample), sardine (23.3 g/100g of sample), sea bream (17.6 g/100g of sample), pacific saury (21.3 g/100g of sample), skipjack (4.60 g/100g of sample), and whitebait (4.90 g/100g of sample) was determined by Soxhlet extraction-gravimetric methods in a commercial laboratory and used to evaluate the correlation with arsenolipids. Concentrations of arsenolipids in fish species were determined according to the procedure described in the Chapter 2 and expressed as fresh weight (fw) basis.

4.4. Results

4.4.1. Concentrations of arsenolipids in various fish species

A wide range of concentrations for various arsenolipids including AsHCs and AsFAs (0.84 - 82.1 µg As/kg, fw) were found among fish samples (**Table 4.1**). The toxic AsHC332 and AsHC360 were detected in salmon (6.94 and 29.9 µg As/kg), mackerel (4.34 and 1.55 µg As/kg), yellowtail (12.4 and 6.61 µg As/kg), tuna (0.78 and 2.08 µg As/kg), sardine (69.2 and 22.4 µg As/kg), sea bream (14.2 and 24.3 µg As/kg), pacific saury (9.91 and 21.6 µg As/kg), skipjack (4.67 and 42.6 µg As/kg), and whitebait (71.5 and 56.9 µg

As/kg). Along with AsHCs, AsFA362 and AsFA390 were also detected in most of the fish species at a range of concentrations (**Table 4.1**). The total arsenic concentrations in nine fish samples ranged from 420 to 1350 µg As/kg (fw), where the lowest was found in salmon and highest was in pacific saury.

Table 4.1 Concentrations of arsenolipids measured in edible parts of various fish species

	Concentrations (µg As/kg, fw)					
Fish species	**AsFAs**		**AsHCs**			**Total Arsenic**
	AsFA 362	AsFA 390	AsHC 332	AsHC 360	AsHC 404	
Salmon	<0.8	0.84	6.94	29.9	14.5	420
Mackerel	2.48	2.48	4.34	1.55	5.89	1190
Yellow tail	1.65	2.07	12.4	6.61	11.2	1170
Tuna	<0.8	<0.8	0.78	2.08	3.12	630
Sardine	3.44	9.76	69.2	22.4	24.9	1330
Sea bream	1.91	6.01	14.2	24.3	17.5	640
Pacific saury	8.07	3.67	9.91	21.6	61.3	1350
Skipjack	2.92	3.79	4.67	42.6	14.7	1320
Whitebait	2.71	2.44	71.5	56.9	82.1	500

To examine the source of variation of arsenolipids in various fish species, I analysed the correlation between arsenolipids concentrations and the lipid content in fish species showed in **Figure 4.1** and **Figure 4.2.**

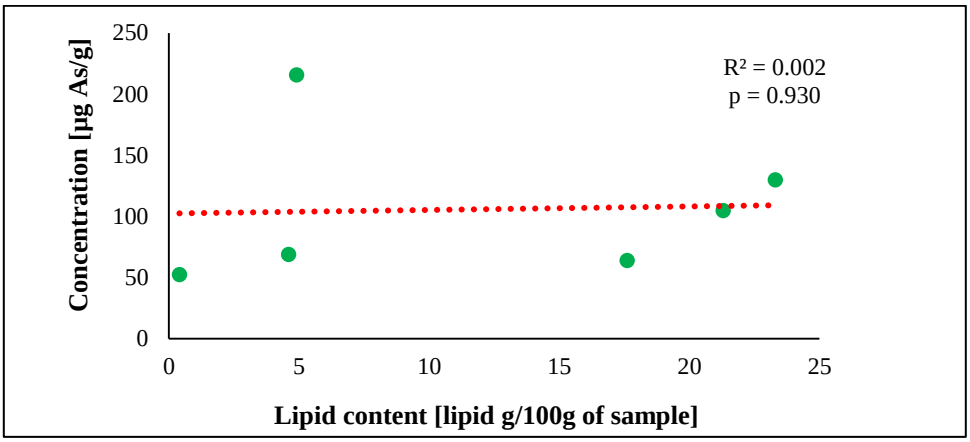


Figure 4.1 Correlation between arsenolipids (AsHCs+AsFAs) concentrations and lipid content in fish species

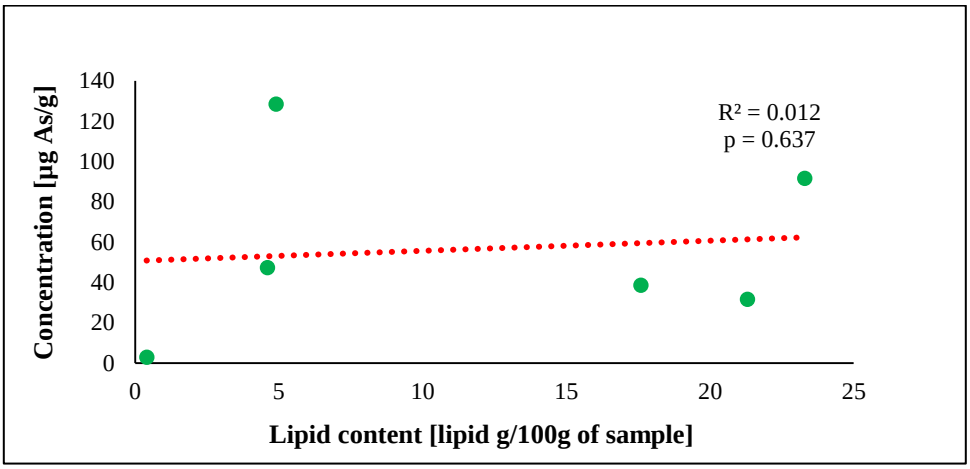


Figure 4.2 Correlation between toxic AsHCs (AsHC332+AsHC360) concentrations and lipid content in fish species

From the **Figure 4.1** and **Figure 4.2**, it can be seen that there was no significant (p=0.930 and p=0.637) correlation found between the concentrations of arsenolipids (AsHCs+AsFAs) and toxic AsHCs, and the lipid content in fishes.

4.4.2. Concentrations of arsenic species in raw and cooked fish samples

The total arsenic concentration in all the three fish samples before and after cooking remained constant (**Table 4.2**). Total arsenic concentration in the cooked (grilled) on aluminum foil did not differ from that of the cooked without aluminum foil, indicating that arsenic was not lost through fluids produced during cooking.

Table 4.2 Total arsenic concentrations in fish samples before and after cooking

Fish samples	Concentrations (mg As/kg, fw) (n=3)		
	Raw	**Cooked (grilled)**	**Cooked (grilled) on Aluminum foil**
Salmon	0.42 ± 0.01	0.42 ± 0.01	0.41 ± 0.01
Mackerel	1.19 ± 0.05	1.19 ± 0.02	1.19 ± 0.06
Yellowtail	1.17 ± 0.07	1.24 ± 0.01	1.13 ± 0.03

Three arsenic containing hydrocarbons (AsHC332, AsHC360, and AsHC404) and two arsenic containing fatty acids (AsFA362 and AsFA390) were detected in salmon, yellow tail and mackerel fish sample of the raw and cooked conditions (**Table 4.1; Figure 4.3; Figure 4.4; and Figure 4.5**). In salmon, toxic AsHC332 and AsHC360 were detected at a concentrations of 8.65 and 29.4 µg As/kg in grilled, and 8.68 and 32.2 µg As/kg in grilled on aluminum foil, respectively, which were close to the values in raw fish (**Table 4.1**; **Figure 4.3**). In yellow tail, the concentrations of AsHC332 and AsHC360 were 12.8 and 7.02 µg As/kg in (cooked) grilled, and 12.8 and 6.61 µg As/kg in (cooked) grilled on aluminum foil, respectively and these values were also close to the values in raw fish

(**Table 4.1; Figure 4.4**). For the mackerel, concentration of AsHC332 and AsHC360 were 4.34 and 1.55 µg As/kg in (cooked) grilled, and 4.65 and 1.86 µg As/kg in (cooked) grilled on aluminum foil, respectively which were not greatly different from the values in raw fish (**Table 4.1; Figure 4.5**). There is no obvious decreasing trend in the concentrations of AsHCs after cooking for the 3 fish species whereas; a small decrease was detected for AsFAs concentrations in the case of yellow tail and mackerel.

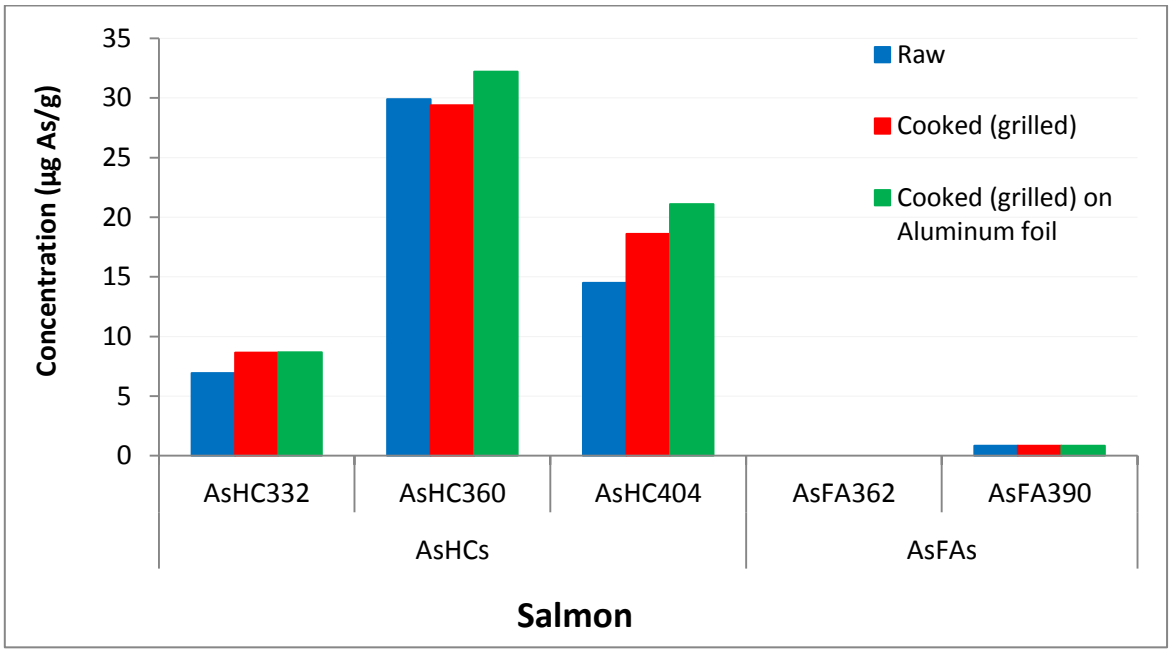


Figure 4.3 Concentrations of arsenolipids species in raw, cooked, and cooked on aluminum foil of salmon

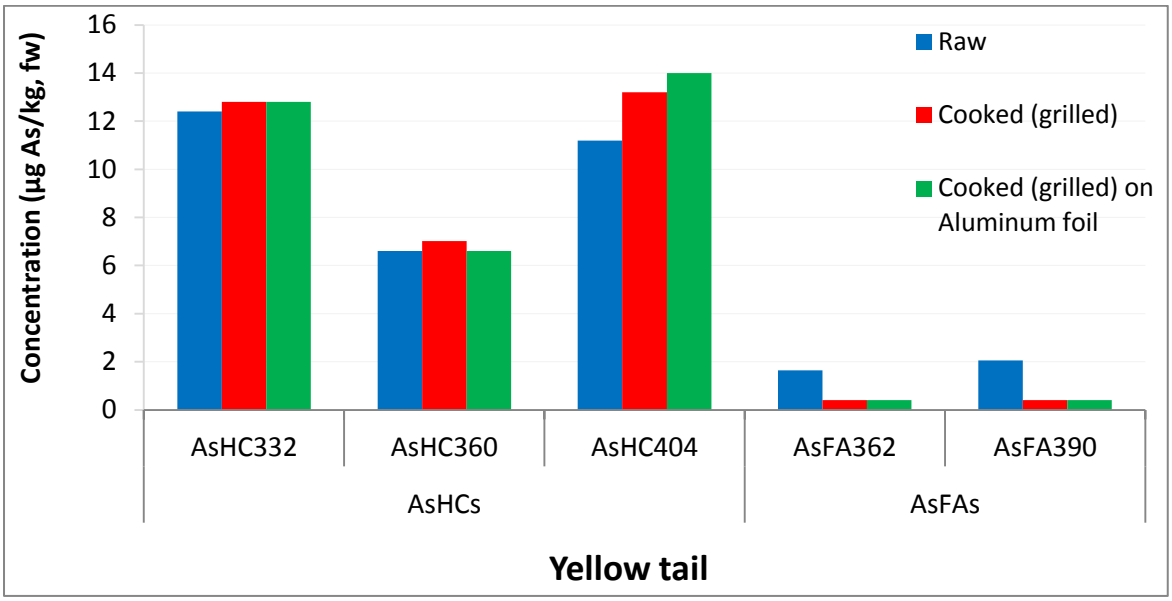


Figure 4.4 Concentrations of arsenolipids species in raw, cooked, and cooked on aluminum foil of Yellow tail

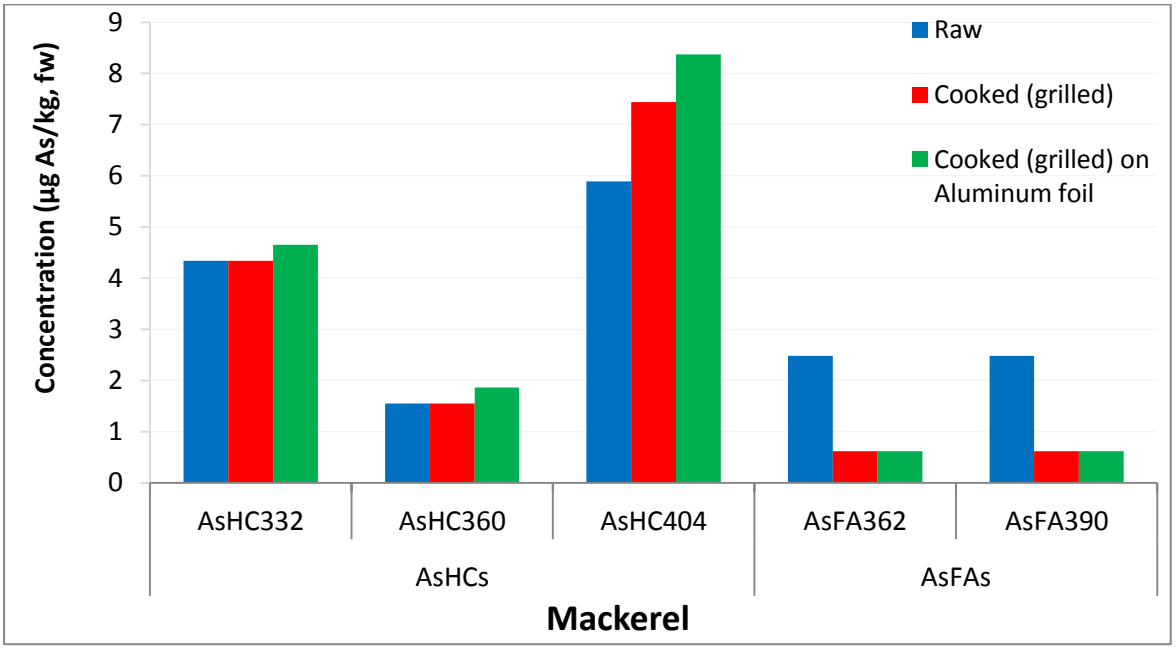


Figure 4.5 Concentrations of arsenolipids species in raw, cooked, and cooked on aluminum foil of Mackerel

4.5. Discussions

4.5.1. Variation of arsenolipids concentrations in various fish species

A probable range of exposure to arsenolipids is required for the complete evaluation of its associated human health risk. In this respect, arsenolipids concentrations were quantified in fish species which are commonly consumed in Japan. Various arsenolipids including three arsenic containing hydrocarbon AsHC332, AsHC360, and AsHC404, two arsenic containing fatty acids AsFA362 and AsFA390 were detected in all the fish species at a wide concentration range from 0.78 to 82.1 µg As/kg whereas the total arsenic concentrations in the fish samples ranged from 420 to 1350 µg As/kg (**Table 4.1**). The concentration range of toxic AsHCs were between 0.78-71.5 µg As/kg among the fish species where the highest concentration was found in whitebait and lowest was in tuna.

No significant correlation found between arsenolipids concentration and lipid content in fish species (**Figure 4.1** and **Figure 4.2**) which indicated that lipid content was not a source of the arsenolipids concentration variations among fish species. Since, arsenolipids are lipid soluble, there was a possibility that lipid content in fishes could be a reason for the variation of arsenolipids concentrations. Therefore, I examined the correlation between the concentration of arsenolipids and lipid content in fishes. Although the results apparently do not support lipid content as responsible for the arsenolipids concentration variation, the samples size was small (n=6), therefore, more data are needed for the further confirmation if lipid content is not associated with arsenolipid concentrations in fish.

Among all the nine fishes, whitebait found to contain highest concentrations of arsenolipids probably because whole body was used for arsenolipids analysis for only whitebait, while the edible muscle tissues for other fish species. In the case of whitebait, whole fish were used because whole fish are usually eaten by people. Arsenolipids may be contained at higher concentrations in internal organs of fish species since in some previous studies, higher concentrations of arsenic compounds (though they were water soluble) were found in internal organs of fish such as fish gills, skins, scales, hearts, livers, kidneys, intestines, brains, bones, and fins (Tyokumbur et al., 2014; Jabeen et al., 2012; Han et al., 2012; Kousar and Javed, 2014; Hamdi et al., 2009).

However, there could be some other sources of the variations in arsenolipids concentration in fish species such as species differences, different food habit, life span, and capacity of metabolism which are needed to be considered for evaluating the detailed sources of variation of arsenolipids concentration in fishes.

Although there were some studies where the presence of toxic AsHCs in some fish species was focused (Taleshi et al., 2008; Arroyo-Abad et al., 2010; Lischka et al., 2013), the quantitative information of their concentration was rarely presented (Amayo et al 2011), which could be used to estimate the variation in the daily intake of AsHCs. The concentrations of AsHC332, AsHC360, and AsHC404 in Amayo et al. (2011), measured in capelin fish meal (Mallotus villosus), were 180, 80, and 60 μg As/kg, respectively. Therefore, from the information on toxic arsenolipids (AsHC332 and AsHC360) concentrations measured in nine fish species in the present study as well as those available from previous literature (Amayo et al., 2014), it was suggested that the variation of

concentrations for toxic AsHC332 and AsHC360 in fish species range from 0.78 to 180 and 1.55 to 80 µg As/kg (fw), respectively. Hence the lowest limit of MOE would be less compared to the rough estimation done in chapter 3 and risk of the intake of toxic arsenolipids would be high.

4.5.2. Changes of arsenolipids during cooking

This is the first study that examined the possible changes in arsenolipids concentrations by cooking, where various arsenolipids including AsHCs and AsFAs were detected in both raw and cooked fish samples of three fish species.

The concentration of toxic AsHC332 and AsHC360 did not substantially change before and after cooking in all the three fish species which suggested that toxic AsHCs were not decomposed by cooking. Another arsenic hydrocarbon, AsHC404 showed a slight increasing trend in salmon and mackerel in cooked fish compared to the raw whereas some decreasing trend was noticed for AsFAs (AsFA362 and AsFA390) (**Figure 4.4; and Figure 4.5**) in yellow tail and mackerel which suggested that some AsFAs could be decomposed by cooking.

However, from these result, it could be tentatively concluded that cooking does not affect toxic AsHCs concentration in fish because it provides only with single measurement data for all the fish species and did not allow statistical evaluation if the change during cooking was present or not. To further ascertain this tentative conclusion, further study with more sample size is needed.

4.5.3. Revised estimated health risk associated with seafood consumption in the Japanese

The possible health risk for AsHC332 and AsHC360 estimated in Chapter 3 based on their daily intake of 1.6 and 0.4 µg/person/day which were calculated by multiplying the arsenolipids concentration present in food composites and the average daily consumption weight of the food categories, and by considering the IC_{50} values of 9.2 and 4.8 µM which were the lowest of IC_{50} values for human cells exposed to AsHC332 or AsHC360, respectively (Meyer et al., 2014). In this chapter, the concentrations range of AsHC332 and AsHC360 in fish were found to be 0.78 - 180 and 1.55 - 80 µg As/kg, respectively, hence the daily intake range of AsHC332 and AsHC360 would be 0.05-11.9 and 0.10-5.30 µg As/person/day. The daily intake was calculated by multiplying the concentration of arsenolipids and the daily consumption amount of fish approximately 66 g by Japanese people (MHLW, 2015).

Hence the body fluid concentrations range of AsHC332 and AsHC360 based on daily intake range (0.05-11.9 and 0.10-5.30 µg As/person/day, respectively) would be approximately 0.0001-0.0317 and 0.0002-0.0141 µM in body fluid based on the assumption I made for converting daily intake to tissue concentration in Chapter 3. The MOE to the IC_{50} value for AsHC332 and AsHC360 would then be approximately 92000-290 and 24000-340, respectively. The lower limit of estimated MOE was 290 whereas MOE <100 implies the presence of risk (Lachenmeier and Rehm, 2015). Therefore, the estimation results of this chapter suggests that the toxic AsHCs intake level of the Japanese does not

pose significant health risk even after the variation range of AsHCs concentration in fish species was taken into consideration.

The variation range of toxic AsHCs concentration in algae food category was not considered in the present study because it is unlikely the variation in algae significantly affects daily intake of AsHCs while considering the fact that small amount of toxic AsHCs come from algae food category whereas most of them come from fish and shellfish.

4.6. Conclusions

The measurement of arsenolipids in nine fish species in the present study and literature data on AsHCs concentrations in raw fish species indicated that the toxic AsHCs in fish samples were found at a concentration ranging from 0.78 to 180 μg As/kg (fw). There were no great changes of toxic AsHCs concentration found during cooking and it suggested that cooking did not affect the concentration of arsenolipids in fish. Based on the AsHCs concentration variation estimated from the present study and literature data, the estimated MOE to AsHC332 and AsHC360 were approximately 92000-290 and 24000-340, respectively. The MOE below 100 is considered to pose health risk and in the present chapter, the minimum estimated MOE was 290. Therefore, the estimated MOE suggested that intake level of AsHCs did not pose significant health risk to the Japanese people when concentration range of arsenolipids were considered although the variation in AsHCs concentration was based only on fish species in this study and it needs to be analyzed for more samples including various algae and more fish specis for further confirmation.

Chapter 5

Bioaccessibility of arsenolipids in seafood

5.1. Background

Arsenolipids (AsHCs) have been reported to be present in various types of marine foods (Taleshi et al., 2010; Lischka et al., 2013) and showed toxicity in the *in vitro* toxicological study on human liver and bladder cells (Meyer et al., 2014). By considering this toxicity concern regarding the food safety and human health risk, in the present study, for the first time, the possible health risk of arsenolipids in the Japanese was estimated in Chapter 3. This risk estimation was based on the assumption that arsenolipids are completely bioaccessible and available for the absorption in the gastrointestinal tract.

The bioaccessibility of a chemical present in food can be defined as the amount or fraction of the chemical which is released from the food matrix into the gastrointestinal tract and becomes available for the absorption (Heaney, 2001; Kelly et al., 2002). In many previous studies, the bioaccessibility of arsenic in various types of foods including, fish, algae, rice, and vegetables (Laparra et al., 2007; 2003; Sun et al., 2012; Koch et al., 2007) has been examined. However, in these previous studies they mainly focused on bioaccessibility of toxic inorganic arsenic and other water soluble arsenic species. At present, there was no study available which has focused to examine the bioaccessibility of toxic arsenolipids. Therefore, to know whether all of the arsenolipids or how much

percentage of arsenolipids in food become available for the absorption in the body for more accurate estimation of health risk, the bioaccessibility of arsenolipids needs to be assessed.

5.2. Objective

The objective of this chapter is to assess the bioaccessibility of arsenolipids in "fish and shellfish" composites and seaweed by an *in vitro* bioaccessibility testing method.

5.3. Methods

5.3.1. Samples collection and preparation

The food samples used for the bioaccessibility test in this study were "fish and shellfish" composite and hijiki seaweed. "Fish and shellfish" was the composite prepared in the market basket survey described in Chapter 3; briefly, most commonly consumed 26 species of fish and shellfish in Japan were mixed after preparing as Japanese people do in their house hold and then freeze dried. Hijiki seaweed used was NMIJ CRM 7405-a (Trace Elements and Arsenic Compounds in Seaweed-Hijiki) purchased from the National Metrology Institute of Japan (Tsukuba, Japan).

The *in vitro* experimental design for the bioaccessibility test performed in this study was that used previously in several studies for examining bioaccessibility of metals in environmental matrices (Rotard et al., 1995; Oomen et al., 2003). This is a static *in vitro* gastrointestinal digestion model based on human physiology where digestive juices were prepared artificially. The digestive juices were introduced to the sample sequentially

according to physiological transit times and were mixed thoroughly with the sample. The arsenolipids fraction that is mobilized from the food into the digestive juice represents the bioaccessible fraction. The gastrointestinal tracts simulated in this experimental scheme include oral cavity, stomach, and small intestines as these compartments are likely to determine the bioaccessibility.

5.3.2. Chemicals and reagents

Water used for the experiment was obtained from a Milli-Q system (18.2MΩ cm, Japan). Other chemicals including KCl, KSCN, NaH_2PO_4, Na_2HPO_4, NaCl, NaOH, $NaHCO_3$, $MgCl_2$, KCl, KH_2PO_4, $CaCl_2$. $2H_2O$, NH_4Cl, HCl, urea, uric acid, alfa-amylase, mucin, glucose, glucuronic acid, glucoseamine hydrochloride, pepsin, bovine serum albumin (BSA), pancreatin, and lipase were used for the preparation of synthetic digestive juices and they were obtained from Wako Pure Chemical Ltd. Tokyo, Japan and bile (porcine pancreas) obtained from Sigma Aldrich, Japan. Various synthetic juices including saliva, gastric, duodenal, and bile were prepared and used in this *in vitro* digestion for the bioaccessibility test of arsenolipids based on the procedure reported by Oomen et al. (2003). Synthetic juices (saliva, gastric, duodenal, and bile) were prepared by mixing specific amount of various organic and inorganic reagents and then made up to 500 mL with milli-Q water followed by addition of some other constituents for each juice shown in **Table 5.1**. The pH of the digestive juices was checked and adjusted to appropriate range by adding 1 mol/L NaOH or concentrated HCl.

Table 5.1 Synthetic digestive juice and their constituents in the *in vitro* digestion

	Saliva	**Gastric juice**	**Duodenal Juice**	**Bile**
Inorganic solution	10 mL KCl 89.6 g/L 10 mL KSCN 20 g/L 10 mL NaH_2PO_4 88.8 g/L 10 mL Na_2HPO_4 57 g/L 1.8 mL NaOH 40 g/L 1.7 mL NaCl 175.3 g/L	15.7 mL NaCl 175.3 g/L 3.0 mL NaH_2PO_4 88.8 g/L 9.2 mL KCl 89.6 g/L 18 mL $CaCl_2.2H_2O$ 22.2 g/L 10 mL NH_4Cl 30.6 g/L 8.3 mL HCl 37%	40 mL NaCl 175.3 g/L 40 mL $NaHCO_3$ 84.7 g/L 10 mL KH_2PO_4 8 g/L 6.3 mL KCl 89.6 g/L 10 mL $MgCl_2$ 5 g/L 180 µL HCl 37%	30 mL NaCl 175.3 g/L 68.3 mL $NaHCO_3$ 84.7 g/L 4.2 mL KCl 89.6 g/L 200 µL HCl 37%
Organic solution	8 mL urea 25 g/L	10 mL glucose 65 g/L 10 mL glucuronic acid 2 g/L 10 mL glucoseamine hydrochloride 33 g/L 3.4 mL urea 25 g/L	4 mL urea 25 g/L	10 mL urea 25 g/L
Constituents added to mixture of organic and inorganic solutions	145 mg alfa-amylase 15 mg uric acid 50 mg mucin	1 g BSA 1 g pepsin 3 g mucin	9 ml $CaCl_2.2H_2O$ 22.2 g/L 1 g BSA 3 g pancreatin 0.5 g lipase	10 ml $CaCl_2.2H_2O$ 22.2 g/L 1.8 g BSA 6 g bile salt (porcine pancreas)
pH	6.5 ± 0.2	1.0 ± 0.7	7.8 ± 0.2	8.0 ± 0.2

For preparing each synthetic juice, organic and inorganic solutions were mixed and made up to 500 ml by milli-Q water and then some further constituents were added to the mixture and dissolved.

5.3.3. Procedure for gastric and duodenal treatment for bioaccessibility test

Schematic representations of *in vitro* digestion in gastric and duodenal phases are shown in **Figure 5.1** and **Figure 5.2**. For the gastric digestion, firstly 9 mL of synthetic saliva (pH 6.5±0.2) was introduced to 0.6 g (freeze dried) of food sample which was then shaken for 5 min at 37^{o}C. Subsequently, 13.5 mL of gastric juice (pH 1.0±0.7) was added and the mixture was shaken for 2 hours at 37^{o}C. The mixture was centrifuged for 10 min at 2000 rpm and then pellet and aqueous phase were separated. After that 20 mL of DCM was added to the aqueous phase and shaken for 1 hour. The DCM layer was separated from aqueous phase by centrifugation and then evaporated to dryness. After re-dissolving the residue in 250 µL of ethanol followed by 15 min ultrasonication, 10 min vortexing, and 15 min centrifugation, the supernatant was used for arsenolipids analysis.

For duodenal digestion, 9 mL of saliva was introduced to 0.6 g of samples followed by shaking for 5 min at 37^{o}C. Subsequently 13.5 mL of gastric juice (pH 1.0±0.7) was added and then shaken for 2 hours at 37^{o}C. After that 27 mL of duodenal juice (pH 7.8±0.2) and 9 mL of bile (pH 8.0±0.2) were added to the mixture and shaken for 2 hr at 37^{o}C. The mixture was centrifuged for 10 min at 2000 rpm to separate the pellet from the aqueous phase. Then 20 mL of DCM was added to aqueous phase and shaken for 1 hour. The DCM layer was separated from aqueous phase by centrifugation and evaporated completely and then followed by the same procedure as in gastric for arsenolipids analysis. For the checking of the breakdown products of arsenolipids in the bioaccessibility test, water soluble arsenic species were also analyzed in water extraction (arsenic extraction by using only mili-Q water), gastric, and duodenal phases.

5.3.4. Measurement of arsenolipids

Arsenolipids in bioaccessible and in non-treated samples (food sample which is not treated with synthetic gastric juice) were analyzed by HPLC-ICP-MS/ESI-MS-MS. Analytical methods were described in detail in Chapter 2. Bioaccessibility was calculated by the following equation:

$$\text{Bioaccessibility (\%)} = \frac{\text{Arsenolipids in chyme or supernatant (bioaccessible fraction) (ng)}}{\text{Arsenolipids present in non} - \text{treated sample (ng)}} \times 100\%$$

5.3.5. Measurement of other arsenic species (HPLC-ICP-MS)

Determination of water soluble arsenic species was performed by high-performance liquid chromatography (HPLC) - inductively coupled plasma mass spectrometry (ICP-MS) (Narukawa et al., 2012). The ICP-MS used was Agilent 7500c equipped with a Micromist nebulizer (100 μL type) and a Scott spray chamber (2 °C). The typical parameters for the ICP-MS operation were as follows: incident rf power 1600 W, outer Ar gas flow rate 15 L/min, intermediate Ar gas flow rate 0.9 L/min, carrier Ar gas flow rate 0.8 L/min and make-up Ar gas flow rate 0.4 mL/min. To reduce some polyatomic molecular interference helium (He) was used as the collision cell gas (flow rate: 3 mL/min).

HPLC was used for the separation of arsenic species. The exit of the HPLC column (CAPCELL PAK C_{18} MG column, particle size of the filler 3 μm, ID 4.6 mm x 150 mm, polymer-coated type, Shiseido Co., Ltd.) was directly connected to the nebulizer of the ICP-MS by PEEK tubing (HPLC-ICP-MS system). The components of

the eluent were 10 mmol/L sodium 1-butanesulfonate, 4 mmol/L malonic acid, 4 mmol/L tetramethylammonium hydroxide, and 0.05 % methanol (pH 3.0). The flow rate was 0.75 mL/min, and the injection volume was 20 μL.

For the calibration, The Japan Calibration Service System (JCSS) arsenic standard solution (*ca*. 1000 mg/L, Kanto Chemical Co., Inc.) was used. The certified reference materials of As(V) (NMIJ CRM 7912-a), the dimethylarsinic acid (DMA) (NMIJ CRM 7913-a) and the arsenobetaine (AB) (NMIJ CRM 7901-a) obtained from the National Metrology Institute of Japan/National Institute of Advanced Industrial Science and Technology (NMIJ/AIST, Tsukuba, Japan) were used for calibration standards. Standard solutions of the other organoarsenic species such as monomethylarsonic acid (MMA), trimethylarsine oxide (TMAO), tetramethylarsonium ion (TeMA), and arsenocholine (AsC) were prepared from commercially available reagents (Tri-Chemical Laboratories Inc.). An in-house standard solution containing ca 1000 mg As/kg was prepared by dissolving each compound in water. This analysis of water soluble arsenic species was performed by Dr Tomohiro Narukawa from the Research Institute for Material and Chemical Measurement, National Metrology Institute of Japan.

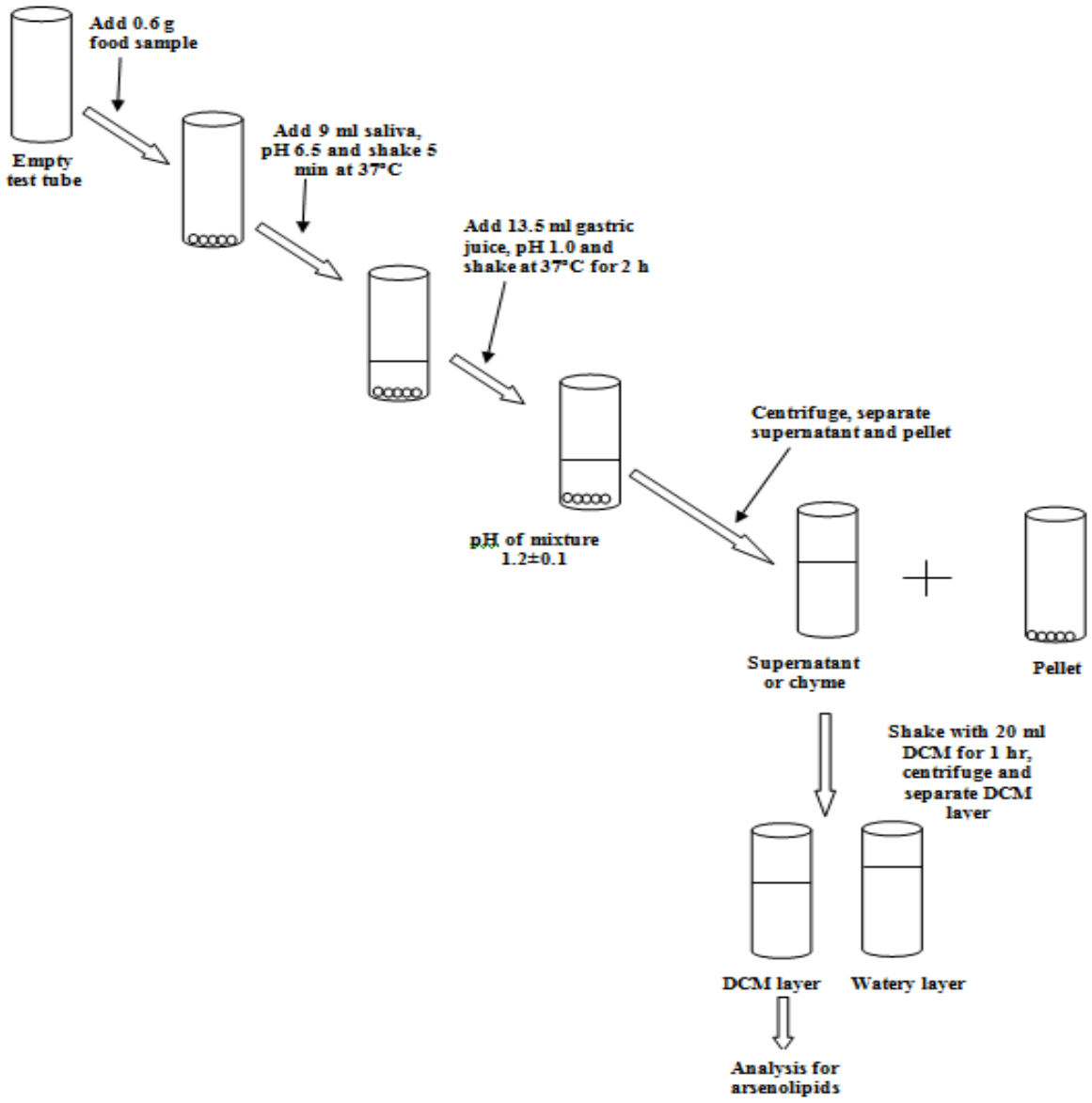


Figure 5.1 The schematic diagram of the in vitro gastric digestion procedure for bioaccessibility test

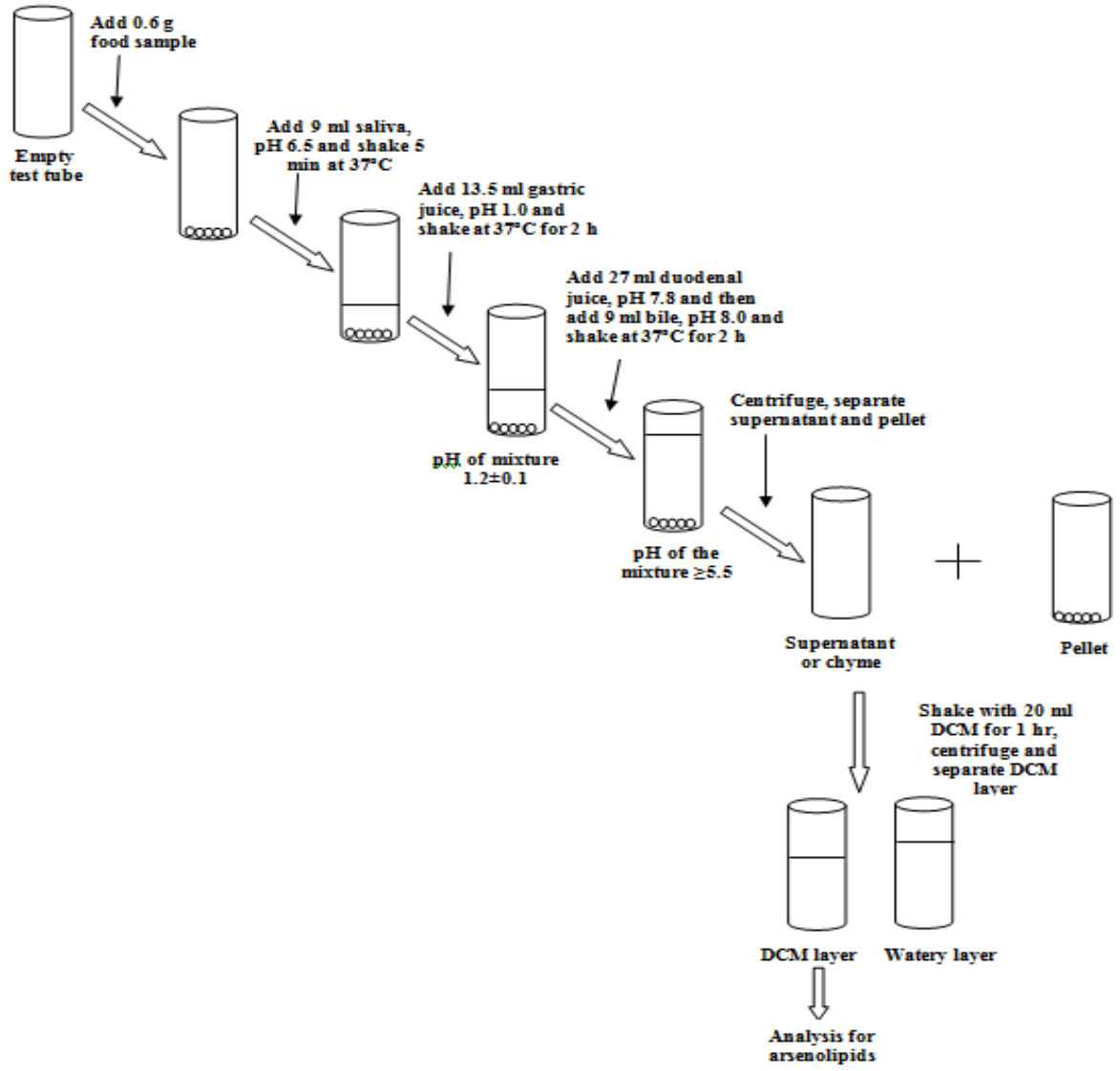


Figure 5.2 A schematic diagram of the in vitro duodenal digestion procedure for bioaccessibility test.

5.4. Results

5.4.1. Concentrations of bioaccessible arsenolipids

Bioaccessible arsenolipids concentrations of "fish and shellfish" composite and hijiki under gastric and duodenal phase are shown in **Table 5.2**. In this table, bioaccessible

concentrations are expressed as a concentration of arsenolipid in food that has leached into respective digestive juice: thus they are expressed as ng As per unit dry weight of food. Arsenolipids concentrations of "non-treated sample" in this table mean arsenolipids concentration in "fish and shellfish" composite and hijiki that were not treated with digestive juices. Five arsenolipids (AsHC332, AsHC360, AsFA362, AsFA388, and AsFA390) were detected in "fish and shellfish" in non-treated sample at concentrations of 73.9, 14.8, 34.6, 2.46, and 34.5 ng As/g (dw) whereas only four arsenolipids (AsHC332, AsHC360, AsFA362, and AsFA390) were found in bioaccessible fraction of gastric phase at a lower concentrations (22, 5.64, 7.82, and 5.09 ng As/g) compared to the arsenolipids in non-treated sample. In duodenal phase, further less concentration of the bioaccessible AsHC332 AsHC360 and AsFA390 (16.3, 5.42, and 14.1 ng As/g) were detected compared to both non-treated sample and gastric phase bioaccessible arsenolipids (**Table 5.2**). In the case of hijiki, the bioaccessible AsHCs (AsHC332 and AsHC360) concentrations in gastric phase were 571 and 63.8 ng As/g, (dw) whereas in non-treated samples concentrations were 1357 and 77.9 ng As/g, respectively. In duodenal phase, bioaccessible AsHC332 and AsHC360 concentrations were 924 and 85.2 ng As/g, respectively. The AsSugPLs (AsSugPL720, AsSugPL958, AsSugPL986, and AsSugPL1014) were also found in bioaccessible fraction in gastric phase at a concentrations (42.5, 38.2, 8.9, and 5.45 ng As/g) much lower than those in non-treated hijiki (1867, 226, 143, and 102 ng As/g). In the case of duodenal phase, only AsSugPL 720 was found at a lower concentration (23.8 ng As/g) compared to the concentration in gastric phase and non-treated hijiki (**Table 5.2**). Some unknown arsenolipids detected in both non-treated samples were also further found at decreased concentrations in both gastric and intestinal phases.

Table 5.2 Concentrations of bioaccessible (gastric and duodenal phase) arsenolipids and their bioaccessibility in "fish and shellfish" composite and hijiki

Type of Sample (n=1)	As-lipids compound	As-lipids in non-treated samples	Gastric phase		Duodenal phase	
			Concentrations (ng As/g dw)		Concentrations (ng As/g dw)	
			Bioaccessible As-lipids	Bio-accessibility of As-lipids (%)	Bioaccessible As-lipids	Bio-accessibility of As-lipids (%)
Fish and shellfish	**AsHC332**	73.9	22.0	30	16.3	22
	AsHC360	14.8	5.64	38	5.42	37
	AsFA362	34.6	7.82	23	36.4	105
	AsFA388	2.46	<0.4	0	<0.4	0
	AsFA390	34.5	5.09	15	14.1	41
	Unknown As-lipids	699	2.40	0.3	4.68	0.7
NMIJ CRM 7405-a (Hijiki)	**AsHC332**	1357	571	45	924	68
	AsHC360	77.9	63.8	82	85.2	109
	AsSugPL 720	556	42.5	7.6	23.8	4.2
	AsSugPL 958	1867	38.2	2.0	<0.4	0
	AsSugPL 986	226	8.91	3.9	<0.4	0
	AsSugPL 1014	143	5.45	3.8	<0.4	0
	AsSugPL 1042	102	<0.4	0	<0.4	0
	AsSugPL 1070	29.1	<0.4	0	<0.4	0
	Unknown As-lipids	170	<0.4	0	<0.4	0

Bioaccessible concentrations of arsenolipids are expressed as As-lipids concentrations in the food (solid) sample that was leached into the synthetic juice.

5.4.2. Concentrations of other bioaccessible arsenic species

In the present study, some bioaccessible arsenic species other than the arsenolipids were also examined in both gastric and duodenal phases for "fish and shellfish" composite and hijiki to see if decomposition products are detectable. In the case of hijiki, the concentration of inorganic arsenic (III and V) did not change much from the water extraction to gastric and intestinal treatment whereas MMA increased in duodenal phase compared to other phases (**Table 5.3**). DMA highly increased in duodenal phase (4224 ng As/g, dw) when compared with gastric (816 ng As/g) and water extraction (850 ng As/g) phase and arsenobetaine did not change much between the phages (**Table 5.3**). Three arsenosugars (As-sugar 328, As-sugar 408, and As-sugar 482) were also found in hijiki, where there were no great changes of concentrations in As-sugar 328 and A-sugar 482 in both of the phases, and for the As-sugar 408, concentrations were found to increase only in duodenal phase.

For the "fish and shellfish" composite, inorganic arsenic was not identified and MMA decreased in gastric phase (24 ng As/g) from the water extraction (42.2 ng As/g) whereas, they were not detected in duodenal phase. There were no substantial changes of bioaccessible DMA in intestinal phases (336 ng As/g) compared to water extraction (306 ng As/g) and gastric phases (287 ng As/g) while arsenobetaine was not changed much between the phases (**Table 5.3**). Arsenosugars were not detected in "fish and shellfish" composite.

Table 5.3 Concentrations of water extracted bioaccessible arsenic species in "fish and shellfish" composites and hijiki.

Arsenic concentrations (ng As/ g, dw), n=2

	Arsenic species		Water extracted arsenic species	Bioaccessible As species in gastric phase	Bioaccessible As species in duodenal phase
Hijiki	**Inorganic arsenic**		9144	8961	9250
	MMA		103	87.9	153
	DMA		850	816	4224
	AB		217	175	181
	Arseno-sugars	As-sugar 328	360	375	340
		As-sugar 408	3591	1244	3845
		As-sugar 482	419	370	347
Fish and shellfish	**Inorganic Arsenic**		ND	ND	ND
	MMA		42.2	24.0	ND
	DMA		306	287	336
	Arsenobetaine		3946	3971	3898
	Arsenosugars		ND	ND	ND

ND= not detected.

5.5. Discussions

5.5.1. Bioaccessibility of arsenolipids

In the present study, for the first time bioaccessibility of arsenolipids (AsHCs, AsFAs, and AsSugPLs) was examined in an *in vitro* digestion model and the results showed that bioaccessible arsenolipids concentrations were found to be low when

compared with the arsenolipids concentration measured in non-treated "fish and shellfish" composite and hijiki (**Table 5.2**). This result indicated that not all the arsenolipids in food are bioaccessible for gastrointestinal absorption after ingestion. It must be noted that the present experimental design did not allow identifying whether the low bioaccessibility was due to decomposition of arsenolipids by digestive juices or to lower leachability of arsenolipids from food matrix. To know this information, gastrointestinal digestions experiment where bioaccessible arsenolipids in gastric and duodenal phase and non-bioaccessible arsenolipids in pellet must be evaluated along with other water soluble arsenic compounds.

In this bioaccessiblity study, arsenolipids were measured in non-treated samples, gastric, and duodenal phases. In the case of toxic AsHCs (AsHC332 and AsHC360), there were no great differences of bioaccessible arsenolipids concentrations found in duodenal phase (16.3 and 5.42 ng As/g) when compared with those in the gastric phase (22 and 5.64 ng As/g) in "fish and shellfish" composite. Moreover, no loss of AsHCs was found in duodenal phase (924 and 85.2 ng As/g) compared to the gastric phase (521 and 63.8 ng As/g) in hijiki. These results suggested that bioaccessible AsHCs in gastric juice were not decomposed in intestinal conditions. AsFAs were detected only in "fish and shellfish" composite where there was no loss of bioaccessible concentrations found in intestinal phase compared to the gastric phase; this suggests that AsFAs leached into gastric juice were not decomposed in intestine as in the case of AsHCs. AsHC332 and AsHC360 were also found at higher concentrations in duodenal phase compared with the gastric phase in the case of Hijiki. Difference in pH in digestive juices (gastric and duodenal phase) can to some extent contribute to the leachability for the bioaccessibility of AsHCs. Some AsHCs can be

extracted in a specific pH range while would not be extracted if the pH has changed; this is why probably some AsHCs were extracted more in duodenal phase than the gastric phase.

Among all the arsenolipids (AsHCs, AsFAs, and AsSugPLs), only the bioaccessible concentrations of AsSugPLs including AsSugPL720, AsSugPL958, AsSugPL986, and AsSugPL1014 were found to be low in duodenal phases (23.8, <0.4, <0.4, and <0.4 ng As/g) compared to the gastric phases (42.5, 38.2, 8.91, and 5.45 ng As/g) (**Table 5.2**). These results suggested that some amount of AsSugPLs leached into gastric juice could be decomposed in intestine. This might be due to the action of lipase in duodenal phase. Lipase generally catalyzes the digestion of the majority of the ingested triglycerides, mainly in the intestine and the products of that reaction are usually free fatty acids and mono-acylglycerols. AsSugPLs have the most similar structural pattern to the triglycerides among the AsHCs, AsFAs, and AsSugPLs. Therefore the result of the present study suggested that some of the AsSugPLs were decomposed to some other arsenic compounds in the duodenal phase.

Water soluble arsenic species were also measured in gastric and duodenal phases for both "fish and shellfish" composite and hijiki in the present study to detect if decomposition of arsenic compounds took place in gastrointestinal conditions. Only the concentrations of DMA among all the other water soluble arsenic species in hijiki increased in duodenal phases compared with both gastric and water extraction phase. The results suggested that probably arsenic compounds present in seafood matrix were decomposed in intestine to produce DMA. The possible decomposition pathway for arsenolipids in the human body is in the first step to arsenic containing fatty acids, and then further to DMA

(Schmeisser et al., 2006). Therefore, AsSugPLs might be one of the arsenic compounds likely to be decomposed in intestine by lipase.

In the present study, the bioaccessibility of toxic AsHC332 and AsHC360 for "fish and shellfish" composite was 30% and 38% in gastric phase, and it was 22% and 37% in duodenal phase (**Table 5.2**). For the hijiki, bioaccessibility of AsHC332 and AsHC360 in gastric phase was 45% and 82% whereas in duodenal phases, it was 68% and 109%. Hence, for the "fish and shellfish" composite, only a fraction of toxic AsHCs in this food matrix was bioaccessible and most part could not be absorbed after gastrointestinal digestion. For hijiki, bioaccessibility of AsHCs was higher than that in the "fish and shellfish" and close to 100%. This means that AsHCs in hijiki are more bioaccessible than those in "fish and shellfish" matrix. The difference in bioaccessibility of AsHCs is maybe due to the differences of food matrix with different amount of lipid content. Higher lipids content might make more complex organization of arsenolipids within food matrix to come to the bioaccessible fraction. The present results indicate that not all of the AsHCs present in foods with fish and shellfish matrix would be available for circulation after ingestion in the body.

5.5.2. Revised estimated health risk corresponding to bioaccessibility of arsenolipids

The possible health risk for AsHC332 and AsHC360 were estimated in Chapter 3 by assuming that bioaccessibility of arsenolipids is 100%. In this chapter, for "fish and shellfish" composite, bioaccessibility of AsHC332 and AsHC360 was 22% and 37% whereas for hijiki, it was 68% and about 100%, respectively. By considering these bioaccessibility results, daily intake of AsHC332 and AsHC360 from "fish and shellfish"

composite and hijiki would be 0.36 and 0.22 µg As/person/day. Hence, the body fluid concentration of AsHC332 and AsHC360 are expected to be about 0.0009 and 0.0006 µM and then the margin of exposure to the IC_{50} values for AsHC332 and AsHC360 would be approximately 10000 (9.2/0.0009) and 8000 (4.8/0.0006), respectively. Therefore, the estimation results based on bioaccessibility of arsenolipids suggest that the possible health risk of AsHCs ingestion via seafood consumption for Japanese people would be less than that in the Chapter 3 (2300 for AsHC332 and 4800 for AsHC360).

Although this study examined bioaccessibility of arsenolipids in foods for the first time, there is a limitation: the number of sample for the measurement of bioaccessible fraction of arsenolipids was just one (**Table 5.2**). Therefore, it was not possible to detect possible small decrement of bioaccessibility with a statistical method; however, in this particular study where MOE is estimated, statistically detectable small decrement would not be relevant. Where small decrement of bioaccessibility is relevant, further sample analyses would be necessary.

5.6. Conclusions

The *in vitro* bioaccessibility test for arsenolipids in the present study performed for "fish and shellfish" composite and hijiki, showed that 22-37% of AsHCs in "fish and shellfish" and 68-100% in hijiki were bioaccessible. Therefore, it was indicated that the toxic AsHCs present in "fish and shellfish" and seaweed food categories would not be 100% available for humans. Taking this lower bioaccessibility into consideration, the estimated margin of exposure for AsHC332 and AsHC360 to the IC_{50} value, approximately 10000 and 8000, respectively, was greater than those estimated in Chapter 3. Therefore, the

possible health risk for AsHCs intake of the Japanese would in fact be less than that estimated in Chapter 3, although the bioaccessibility results in the present study was based on one sample analysis. For further confirmation of the present results, bioaccessibility test with more samples is necessary.

Chapter 6

Summary and overall evaluation of health risk

The possible health risk of toxic arsenolipids for Japanese people was estimated in the present study for the first time by considering their toxic nature and abundance in marine foods. The significant points of this study including exposure assessment of arsenolipids along with variation range in various fish species, stability of arsenolipids during cooking, and bioaccessibility of arsenolipids was summarized first, and then the overall health risk was estimated as follows.

(1) Arsenolipids in foodstuffs and their daily intake through food consumption in Japan

Among the 17 food categories in Japan, arsenolipids were found to be present in only “algae” and “fish and shellfish” composites. Various arsenolipids including AsHCs, AsFAs, and AsSugPLs were detected at concentrations of 0.8-37.5 ng As/g (fw) in “algae” and “fish and shellfish” composites while they were not present at detectable concentration typically <0.8 ng As/g for other food categories. “Algae” and “fish and shellfish” are major food categories in the Japanese diet with the average daily consumption of about 11 and 66 g/day/person, respectively (MHLW, 2015). The average daily intakes of toxic AsHC332 and AsHC360 for Japanese people were estimated to be ca 1.6 and 0.4 μg As/person/day by multiplying AsHC concentrations in food and the daily consumption amount that food.

Based on the toxicity data (IC_{50} values of 9.2 and 4.8 µM for AsHC332 and AsHC360) (Meyer et al., 2014), calculated daily intake (1.6 and 0.4 µg As/person/day), and an assumption of 100% bioaccessibility of arsenolipids, point estimates of the MOE for AsHC332 and AsHC360 to Japanese people were 2300 and 4800, respectively, which are much larger than 100. This "point estimate" suggests that intake of toxic AsHCs of Japanese does not pose significant health risk, although it requires "distribution estimates" that includes both intra- and inter-individual variabilities of intake within target population for the full estimation of health risk.

(2) Concentration variation of arsenolipids and their stability during cooking

Most of the daily intake of arsenolipids for the Japanese comes from "fish and shellfish" category (92%, Chapter 3) and a large variation of arsenolipids concentrations was expected to be present among various fish species. Arsenolipids were analyzed in 9 fish species that are commonly consumed in Japan and both AsHCs and AsFAs were found to be present at a wide range of concentrations from 0.78 to 82.1 µg As/kg fw. It is also observed that the toxic AsHCs were not decomposed during cooking of fish. The quantification data of toxic AsHCs (AsHC332 and AsHC360) in various fish species in the present study and from previous literature (Amayo et al., 2011) suggest that the concentration variation ranges of toxic AsHC332 and AsHC360 were 0.78-180 µg As/kg and 1.55 - 80 µg As/kg fw. The concentrations of these two AsHCs measured in "fish and shellfish" composite were 23 and 4.6 µg As/kg, respectively (Chapter 3) which was between the concentration ranges of AsHCs estimated among fish species. Therefore, the daily intake range of AsHC332 and AsHC360 for the Japanese would be estimated to be

0.05-11.9 and 0.10-5.30 µg As/person/day, respectively, although the concentration variation range of AsHCs was determined only in fish species. The arsenolipids concentration variation among various algae was not considered since small amount of daily intake of AsHCs comes from algae food category (8%, Chapter 3).

(3) Bioaccessibility of arsenolipids

The *in vitro* bioaccessibility test for arsenolipids performed for "fish and shellfish" composite and hijiki indicates that all of the arsenolipids present in food are not completely bioaccessible after ingestion. The bioaccessibility of toxic AsHC332 and AsHC360 was about 22% and 37% for "fish and shellfish" whereas about 68% and 100% for hijiki, although the bioaccessibility results in this study were based on single sample analysis and need to be further investigated in larger number of samples for further confirmation of the present result.

Overall evaluation of health risk

The estimated daily intake of AsHCs could further be variable by variation in the consumption weight of food among the Japanese people; therefore, it also needed to consider this variation. According to the data from National Health and Nutrition Survey of Japan (MHLW, 2015), the daily consumption of "algae" and "fish and shellfish" is 10.9±19.5 and 66.3± 71.5 g/person/day, respectively. By assuming the normal distribution in consumption amount, the 95 percentile of the population will consume approximately 50 and 209 g/person/day of "algae" and "fish and shellfish", respectively (where the lower

limit of consumption amount is 0 g/person/day). Therefore, by considering the food consumption variation of Japanese people, the daily intake of AsHC332 and AsHC360 would be (by multiplying the consumption amount to the concentration range upper limit, since here upper limit is relevance to the health risk) up to 37.8 and 17.2 µg As/person/day, respectively. Then by considering the estimated bioaccessibility of toxic AsHC332 (22-68%) and AsHC360 (37-100%), the body fluid concentration range of AsHC332 and AsHC360 is expected to be up to approximately 0.022 - 0.069 and 0.017 - 0.045 µM. Therefore, the MOE to the IC_{50} value (9.2 and 4.8 µM) for toxic AsHC332 and AsHC360 would be as a minimum approximately 420 - 130 and 280 - 110, respectively. The MOE <100 implies presence of risk whereas the estimated minimum MOE in the present study was 110, which is close to 100. Further study on the estimation of health risk is needed to confirm whether health risks of arsenolipids for Japanese people is actually present or not since there are some sources of uncertainty not considered in the present study that could make MOE more variable.

The intra-species variation in AsHCs concentrations in fish (regional, seasonal, etc.) could make concentration range greater. The concentration variation in "algae" food category was not also considered (Chapter 4) even though small amount of the AsHCs daily intake comes from "algae" (0.04 µg As/person/day for AsHC332 and 0.11 µg As/person/day for AsHC360). By obtaining all these data, intake variation range of toxic AsHCs would be larger both to lower and higher directions and consequently, will make the lower limit of MOE lower.

The daily intake of arsenolipids estimated using the Japanese food consumption data (MHLW, 2015) by assuming the normal distribution of consumption (95 percentile), due to the unavailability of sufficient information on the distribution was very rough. In future, the arsenolipids analyses within a large number of inter- and intra-species of fish and seaweeds as well as duplicate diet study among many Japanese people are necessary for evaluating the variations range of arsenolipids for further confirmation.

Due to the unavailability of toxicity data like NOAEL for toxic arsenolipids, corresponding IC_{50} values were used as a toxicity indicator to estimate the MOE for the toxic AsHCs intake level (Chapter 3). The NOAEL represents the maximum concentration of a chemical at which it causes no adverse effect even if the chemical is exposed for a long time whereas the IC_{50} value represents the concentration of a chemical at which it causes 50% inhibition of a biological or biochemical process. Therefore the estimated MOE for toxic AsHCs by using IC_{50} value surely represents higher MOE value than by using NOAEL. If the NOAEL data for toxic arsenolipids becomes available in future, then MOE would be calculated which makes health risk estimation greater. Various toxicity tests for arsenolipids in the most sensitive and relevant animals with subacute (one-month), subchronic (three month), and/or chronic (two-year) exposures will be needed for determining the NOAEL for arsenolipids.

For estimating the MOE, the bioaccessibility data obtained in the present study for toxic arsenolipids was used due to the unavailability of bioavailability data for arsenolipids. The quantity or fraction of a chemical which is released from the food matrix in the gastrointestinal tract and becomes available for the absorption is known as bioaccessibility.

On the other hand, the fraction of ingested chemical that reaches the systemic circulation and is utilized is called bioavailability. All the bioavailable compounds are bioaccessible but all the bioaccessible arsenolipids are not bioavailable. In future when the bioavailability data for toxic arsenolipids would be available, it will make the MOE larger and consequently risk will be estimated less. Pharmacokinetic and pharmacodynamics studies will be necessary for determining the bioavailability data of arsenolipids in future.

However, despite all these uncertainties, the combined MOE estimated in the present study suggested for the first time that intake level of arsenolipids through the consumption of marine foods could pose health risk for Japanese people. This study on the health risk estimation of consuming arsenolipids containing seafood comprised some original and new data such as daily intake of arsenolipids for the Japanese, variation range of arsenolipids in fish species, bioaccessibility of arsenolipids in seafood, and the possible health risk of toxic arsenolipids which will have great significance in the respect of the food safety concern. This will certainly help the health authorities to get a notion about the presence of toxic arsenolipids in fish and algae, and also their possible health risk for people who consume large amount of marine foods all over the world. It will also trigger the necessity of further research on arsenolipids particularly on their toxicity, degradation pathway, and the estimation of their health risk for people in other countries, etc.

References

Ali, N., Hoque, M.A., Haque, A., Salam, K.A., Karim, M,R., Rahman, A., Islam, K., Saud, Z.A., Khalek, M.A., Akhand, A.A., Hossain, M., Mandal, A., Karim, M.R., Miyataka, H., Himeno, S., Hossain, K., 2010. Association between arsenic exposure and plasma cholinesterase activity: a population based study in Bangladesh. Environ Health 9:36. doi: 10.1186/1476-069X-9-36

Amayo, K. O., Petursdottir, A., Newcombe, C., Gunnlaugsdottir, H., Raab, A., Krupp, M.E., and Feldmann, J., 2011. Identification and quantification of arsenolipids using reversed phase HPLC coupled simultaneously to high resolution ICPMS and high resolution electrospray MS without species-specific standards. Anal Chem. 83, 3589-3595.

Amayo, K.O., Raab, A., Krupp, E. M., Gunnlaugsdottir, H., and Feldmann, J., 2013. Novel Identification of Arsenolipids Using Chemical Derivatizations in Conjunction with RP-HPLC-ICPMS/ESMS. *Analytical Chemistry*. 85(19): 9321-7.

Amayo, K.O., Raab, A., Krupp, E.M., Feldmann, J., 2014. Identification of arsenolipids and their degradation products in cod-liver oil. Talanta. 118, 217–223.

Andreae, M.O., 1978. Distribution and speciation of arsenic in natural waters and some marine algea. Deep Sea Research. 25: 391-402.

Andrewes, P., Demarini, D.M., Funasaka. K., Wallace, k., Lai, V.W.M., Sun, H., Cullen, W.R., Kitchin, K.T., 2004. Do arsenosugar pose a risk to human health? The comparative toxicities of a trivalent and pentavalent arsenosugar. Environ. Sci. Technol.

38, 4140-4148.

Argos, M., Kalra, T., Rathouz, P.J., Chen, Y., Pierce, B., Parvez, F., Islam, T., Ahmed, A., Rakibuz-Zaman, M., Hasan, R., Sarwar, G., Slavkovich, V., van Geen, A., Graziano, J., Ahsan, H., 2010. Arsenic exposure from drinking water, and all-cause and chronic-disease mortalities in Bangladesh (HEALS): a prospective cohort study. Lancet 376:252–258.

Arroyo-Abad, U., Lischka, S., Piechotta, C., Mattusch, J., and Reemtsma, T., 2013. Determination and identification of hydrophilic and hydrophobic arsenic species in methanol extract of fresh cod liver by RP-HPLC with simultaneous ICP-MS and ESI-Q-TOF-MS detection. *Food Chemistry*. 141(3): 3093-102.

Arroyo-Abad, U., Mattusch, J., Mothes, S., Moder, M., Wennrich, R., Elizalde-Gonzalez, M.P., Matysik, F.M., 2010. Detection of arsenic-containing hydrocarbons in canned cod liver tissue. Talanta. 82, 38-43.

Ballin, U., Kruse, R., and Russel, H. A., 1994. Determination of total arsenic and speciation of arseno-betaine in marine fish by means of reaction headspace gaschromatography utilizing flame-ionization detection and element-specific spectrometric detection. *Fresenius Journal of Analytical Chemistry*. 350(1-2): 54-61.

Borak, J., and Hosgood, H.D., 2007. Seafood arsenic: Implications for human risk assessment. Regulatory Toxicology and Pharmacology. 47(2): 204-212.

Chakraborti, D., Sengupta, M.K., Rahman, M.M., Ahmed, S., Chowdhury, U.K., Hossain, M.A., Mukherjee, S.C., Pati, S., Saha, K.C., Dutta, R.N., Quamruzzaman, Q., 2004. Groundwater arsenic contamination and its health effects in the Ganga-Meghna-Brahmaputra plain. J Environ Monit 6:74–83.

Chen Y, Santella RM, Kibriya MG, Wang Q, Kappil M, Verret WJ, Graziano JH, Ahsan H. 2007. Association between arsenic exposure from drinking water and plasma levels of soluble cell adhesion molecules. Environ Health Perspect 115:1415–1420.

Chen, C.J., Chuang, Y.C., Lin, T.M., Wu, H.Y., 1985. Malignant neoplasms among residents of a blackfoot disease-endemic area in Taiwan: high-arsenic artesian well water and cancers. Cancer Res 45:5895–5899.

Cleland, B., Tsuchiya, A., Kalman, D.A., Dills, R., Burbacher, T.M., White, J.W., Elaine M. Faustman, E.M., and Mariën, K., 2009. Arsenic Exposure within the Korean Community (United States) Based on Dietary Behavior and Arsenic Levels in Hair, Urine, Air, and Water. *Environ Health Perspect*; DOI:10.1289/ehp.11827

Cooney, R.V., Mumma, R.O., Benson, A.A., 1978. Arsoniumphospholipid in algae. Proc. Natl. Acad. Sci. USA. 75, 4262-4264.

Cullen, W.R., and Reimer, K.J., 1989. Arsenic speciation in the environment. *Chemical Review*. 89: 713-764.

Dahl, L., Molin, M., Amlund, H., Meltzer, H. M., Julshamn, K., Alexander, J., & Sloth, J. J., 2010. Stability of arsenic compounds in seafood samples during processing and storage by freezing. Food chemistry, 123, 720–727.

Das, D., Chatterjee. A., Mandal, B.K., Samanta, G., Chanda, B., Chakraborti, D., 1995. Arsenic in ground water in six districts of West Bengal, India, The biggest arsenic calamity in the world. Part-2. Arsenic concentration in drinking water, hair, nail, urine, skin-scale and liver tissue (biopsy) of the affected people. Analyst 120:917–924.

Das, D., Samanta, G., Mandal, B.K., Chowdhury, T.R., Chanda, C.R., Chowdhury, P.P., Basu, G.K., Chakraborti, D., 1996. Arsenic in ground water in six districts of West Bengal, India. Environ Geochem Health 18:5–15.

Devesa, V., Macho, M. L., Jalón, M., Urieta, I., Muñoz, O., Súñer, M. A., López, F., Vélez, D., & Montoro, R., 2001. Arsenic in Cooked Seafood Products: Study on the Effect of Cooking on Total and Inorganic Arsenic Contents. Journal of Agricultural and Food Chemistry, 49, 4132–4140.

Devesa, V., Martínez, A., Súñer, M. A., Vélez, D., Almela, C., & Montoro, R., 2001. Effect of Cooking Temperatures on Chemical Changes in Species of Organic Arsenic in Seafood. Journal of Agricultural and Food Chemistry, 49, 2272–2276.

Dhar, R.K., Biswas, B.K., Samanta, G., Mandal, B.K., Chakraborti, D., Roy, S., Jafar, A., Islam, A., Ara, G., Saiful Kabir, S.A., Wadud Khan, W.S., Akther A., and Hadi, S.A., 1997. Groundwater arsenic calamity in Bangladesh. Curr Sci 73:48–59.

Dixon, H., 1996. The biochemical action of arsnonic acids especially As phosphate analogues. Adv. Inorg. Chem. 44, 191-227.

Edmonds, J.S., and Francesconi, K.A., 1983. Arsenic-containing ribofuranosides: isolation from brown kelp *Ecklonia radiata* and nuclear magnetic resonance spectra. J Chem Soc perkin Trans 1. (0), 2375-2382.

Edmonds, J.S., and Francesconi, K.A., 1993. Arsenic in seafoods -Human health aspects and regulations. *Marine Pollution Bulletin*. 26(12): 665-674.

Edmonds, J.S., Francesconi, K.A., and RV, S., 1993. Arsenic compounds from marine organisms. *Natural Products Report*. 10: 421-428.

Edmonds, J.S., Francesconi, K.A., Cannon, J.R., Raston, C.L., Skelton, B.W., and White, A.H., 1977. Isolation, crystal structure and synthesis of arsenobetaine, the arsenical constituent of the western rock lobster *Palinurus- lonipes-cygnus* George. *Tetrahedron Lett.*18

Edmonds, J.S., Shibata, Y., Francesconi, K.A., Rippingale, R.J., and Morita, M., 1997. Arsenic transformations in short marine food chains studied by HPLC-ICP MS. Applied Organometallic Chemistry. 11(4): 281-287.

Edmonds, J.S., Shibata, Y., Prince, R.I.T., Francesconi, K.A., and Morita, M., 1994. Arsenic compounds in tissues of leatherback turtle Dermochelys coriacea Journal of Marine Biological Association of the United Kingdom. 74: 463-466.

European Food Safety Authority (EFSA), 2009. Panel on contaminants in the food chain. Scientific opinion on arsenic in food. The EFSA J. 7(10) 1351. http://www.efsa.europa.eu/en/scdocs/doc/1351.pdf.

Feldmann, J., and Krupp, E.M., 2011. Critical review or scientific opinion paper: Arsenosugars-a class of benign arsenic species or justification for developing partly speciated arsenic fractionation in foodstuffs? Analytical and Bioanalytical Chemistry. 399(5): 17351741.

Foster, S., Maher, W., Taylor, A., Krikowa, F., and Telford, K., 2005. Distribution and speciation of arsenic in temperate marine saltmarsh ecosystems. *Environmental Chemistry*. 2: 177-189.

Francesconi, K. A., and Edmonds, J.S., 1998. Arsenic species in marine samples. *Croatica Chemica Acta*. 71: 343-359.

Francesconi, K. A., and Kuehnelt, D., 2002. Arsenic compounds in the environment In: Environmental chemistry of arsenic. Frankenberger, W.T. (Ed.). Marcel Decker, Inc., New York pp. 51-94.

Francesconi, K.A., 2005. Current perspectives in arsenic environmental and biological research. Environ. Chem. 2, 141-145.

Francesconi, K.A., 2007. Toxic metal species and food regulations making a healthy choice. Analyst. 132(1): 17-20. 103.

Francesconi, K.A., 2010. Arsenic species in seafood: Origin and human health implications. Pure and Applied Chemistry. 82: 373-381.

Francesconi, K.A., Edmonds, J.S., Stick, R.V., 1889. Accumulation of arsenic in yelloweye mullet (*Aldrichetta forsteri*) following oral administration of organoarsenic compounds and arsenate. Sci Total Environ. 79 (1), 59-67.

Francesconi, K.A., Edmonds, J.S.E., Hatcher, B.G., 1988. Examination of the arsenic constituents of the herbivorous marine gastropod *Tectus pyramis*: Isolation of tetramethylarsonium ion. Comp Biochem Physiol C. Pharmacol. 90 (2), 313-316.

Francesconi, K.A., Stick, R.V., and Edmonds, J.S., 1990. Glycerylphosphorylarsenocholine and phosphatidylarsenocholine in yelloweye mullet (*Aldrechetta forsteri*) following oral administration of arsenocholine. *Experienta*. 46: 464-466.

Fricke, M.W., Creed, P.A., Amy N. Parks, A.N., Shoemaker, J.A., Schwegel, C.A., and Creed, J.T., 2004. Extraction and detection of a new arsine sulfide containing arsenosugar in molluscs by IC-ICP-MS and IC-ESI-MS/MS. Anal. At. Spectrom. 19**,** 1454-1459.

Garcia-Salgado, S., Raber, G., Raml, R., Magnes, C., and Francesconi, K.A., 2012.

Arsenosugar phospholipids and arsenic hydrocarbons in two species of brown macroalgae.*Environmental Chemistry*. 9(1): 63-66.

Geiszinger, A., Goessler, W., Pedersen, S.N., Francesconi, K.A., 2001. Arsenic biotransformation by the brown macroalga *Fucus serratus*. Environ. Toxicol. Chem. 20, 2255.

Gibaud, S., and Jaouen, G., 2010. Arsenic-Based Drugs: From Fowler's Solution to Modern Anticancer Chemotherapy, In: Medicinal Organometallic Chemistry. Jaouen, G. and MetzlerNolte, N. (Ed.). pp. 1-20.

Glabonjat, R.A., Raber, G., Jensen, K.B., Ehgartner, J., Francesconi, K.A., 2014. Quantification of arsenolipids in the certified reference material NMIJ 7405-a (Hijiki) using HPLC/mass spectrometry after chemical derivatization. Anal. Chem. 86, 10282–10287.

Grotti, M., Soggia, F., Lagomarsino, C., Goessler, W., Francesconi, K.A., 2008. Arsenobetaine is a significant arsenical constituent of the red Antarctic alga Phyllophora Antarctica. Environ Chem 5:171–175.

Hamdi, M., Sanchez, M.A., Beene, L.C., Liu, Q., Landfear, S.M., Rosen, B.P., Liu, Z., 2009. Arsenic transport by zebrafish aquaglyceroporins. BMC Mol Biol 10(1):104.

Han, Z., Li, J., Zhang, M., Chunxia, L.V., 2012. Effect of montmorillonite on arsenic accumulation in common carp. African J Biotech 11(22):6160–6168.

Hanaoka, K., Tanaka, Y., Nagata, Y., Yoshida, K., Toshika, Kaise, T., 2001. Water-soluble arsenic residues from several arsenolipids occurring in the tissues of the starspotted shark *Musterus manazo.* Appl Organomet Chem. 15 (4) 299-305.

Heaney, RP., 2001. Factors influencing the measurement of bioavailability, taking calcium as a model. J. Nutr. 131(4):1344–8.

HSDB (Hazardous Substances Data Bank), 2003. Sodium Arsenate. Canadian Centre for Occupational Health and Safety. U.S. National Library of Medicine. Available: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB

IARC (International Agency for Research on Cancer), 1980. Monographs on the evaluation of carcinogenic risk of chemicals to humans. In Some Metals and Metallic Compounds. IARC Press, Lyon 23:139–141.

IARC (International Agency for Research on Cancer), 2004. Some drinking-water disinfectants and contaminants, including arsenic. IARC Monographs on the evaluation of carcinogenic risks to humans. 84:139–141.

Jabeen, G., Javed, M., Azmat, H., 2012. Assessment of heavy metals in the fish collected from the river Ravi. Pakistan. Pak Vet J 32(1):107–111.

JECFA, 2011. Safety evaluation of certain contaminants in food in WHO Food Additives Series: 63, FAO/JECFA Monographs 8. pp. 153-316. Jones FT, 2007. A broad view of arsenic. Poult Sci 86:2–14.

Julshamn, K., Lundebye, A.K., Heggstad, K., Berntssen, M.H.G., and Boe, B., 2004. Norwegian monitoring programme on the inorganic and organic contaminants in fish caught in the Barents Sea, Norwegian Sea and North Sea, 1994-2001. Food Additives and Contaminants. 21: 365-376. 39.

Julshamn, K., Nilsen, B.M., Frantzen, S., Valdersnes, S., Maage, A., Nedreaas, K., and Sloth, J.J., 2012. Total and inorganic arsenic in fish samples from Norwegian waters. Food Additives & Contaminants Part B-Surveillance. 5(4): 229-235.

Kaise, T., and Fukui, S., 1992. The Chemical Form and Acute Toxicity of Arsenic Compounds in Marine Organisms. Applied Organometallic Chemistry. 6: 155-160.

Kaise, T., Watanabe, S., Itoh, K., 1985. The acute toxicity of arsenobetaine. Chemosphere 14 (9), 1327–1332.

Kaufstad, P., 1992. Uorganisk kjemi: en innføring i grunnstoffenes kjemi. 3 utgave. Kaufstad, P. (ed.).Aschehoug, Oslo, Norway.

Kelly, M. E., Brauning, S. E., Schoof, R. A., and Ruby, M. V., 2002. Assessing oral bioavailability of metals in soil. Columbus:Battelle Press.

Kirby, J., and Maher, W., 2002. Tissue accumulation and distribution of arsenic compounds in three marine fish species: relationship to trophic position. Environ Biol Toxicol. 16 (2), 108-115.

Koch, I., Mcpherson, K., Smith, P., Easton, L., Doe, K. G., Reimer, K.J., 2007. Arsenic bioaccessibility and speciation analysis in clams and seaweed from a contaminated marine environment. Mar Pullut Bull. 54:586-94.

Kousar, S., Javed, M., 2014. Heavy metals toxicity and bioaccumulation patterns in the body organs of four fresh water fish species. Pak Vet J 34(2):161–164.

Kuehnelt, D., and Goessler, W., 2003. Organoarsenic compounds in the terrestrial environment, In: Organometallic Compounds in the Environment. Craig, P.J. (Ed.). John Wiley & Sons Ltd, Chichester, England. pp. 223-275

Lachenmeier, D. W., and Rehm, J., 2015. Comparative risk assessment of alcohol, tobacco, cannabis and other illicit drugs using the margin of exposure approach. Sci. Rep. 5:8126.

Laparra, J.M., Velez, D., Montoro, R., Barbera, R., 2003. Estimation of arsenic bioaccessibility in edible seaweed by an in vitro digestion method. J. Agric Food Chem. 51:6080-5.

Laparra, J.M., Velez, D., Montoro, R., Barbera, R., Farre, R., 2007. Bioaccessibility and transport by caco-2 cells of organoarsenical species present in seafood. J Agric Food Chem.55:5892-7.

Leffers, L., Ebert, F., Taleshi, M.S., Francesconi, K.A., and Schwerdtle, T., 2013. In vitro toxicological characterization of two arsenosugars and their metabolites. Mol. Nutr. Food Res. 57, 1270-1282. DOI: 10.1002/mnfr.201200821.

Lipid Library. The AOCS Lipid Library. Lipid chemistry, biology, technology and analysis. http://lipidlibrary.aocs.org/index.html [retrieved 01.02.2014].

Lischka, S., Arroyo-Abad, U., Mattusch, J., Kuehn, A., Piechotta, Ch., 2013. The high diversity of arsenolipids in herring fillet (Clupeaharengus). Talanta 110, 144–152.

Lunde G.., 1968. Analysis of arsenolipids in marine oils by neutron activation. Evidence of arseno organic compounds. J. Am. Oil Chem. Soc. 45, 331-332.

Lunde, G., 1972. The absorption and metabolism of arsenic in fish. *Fiskeridirektorates skrifter serie Teknologiske undersøkelser*. 5(12): 1-15.

Lunde, G., 1974. The Analysis and Characterization of Trace Elements, in Particular Bromine, Selenium and Arsenic in Marine Organisms, in Central Institute for Industrial

Research (CIR). University of Oslo, Oslo, Norway. pp. 38.

Lunde, G., 1977. Occurence and transformation of arsenic in the marine environment. Environmental Health Perspectives. 19: 47-52.

Maher, W., Goessler, W., Kirby, J., and Raber, G., 1999. Arsenic concentrations andspeciation in the tissues and blood of sea mullet (*Mugil cephalus*) from Lake Macquarie NSW, Australia. *Marine Chemistry*. 68: 169-182.

Maher, W.A., Foster, S., Krikowa, F., Duncan, E., St John, A., Hug, K., and Moreau, J.W., 2013. Thio arsenic species measurements in marine organisms and geothermal waters. *Microchemical Journal*. 111: 82-90.

Mandal, B.K,, Chowdhury, T.R,, Samanta, G., Basu, G.K,, Chowdhary, P.P., Chanda, C.R., 1996. Arsenic in groundwater in seven districts of West Bengal, India–the biggest arsenic calamity in the world. Curr Sci 70:976–986.

Mandal, B.K., Suzuki, K.T., 2002. Arsenic around the world: a review. Talanta 58: 201–235.

Mania, M., Rebeniak, M., Szynal, T., Wojciechowska-Mazurek, M., Starska, K., Ledzion, E., Postupolski, J., 2015. Total and inorganic arsenic in fish, seafood and seaweeds-esposure assessment. Rocz Panstw Zakl Hig. 66, 203-210.

Mass, M.J., Tennant, A., Roop, B.C., Cullen, W.R., Styblo, M., Thomas, D.J., and Kligerman, A.D., 2001. Methylated trivalent arsenic species are genotoxic. Chemical Research in Toxicology. 14(4): 355-361. 108.

Mazumder, D.N.G., 2005. Effect of chronic intake of arsenic-contaminated water on liver. Toxicol Appl Pharmacol 206:169–175.

Mazumder, D.N.G., Haque, R., Gosh, N., De, B.K., Santra, A., Chakraborty, D., Smith, A.H., 1998. Arsenic levels in drinking water and the prevalence of skin lesions in West Bengal, India. Int J Epidemio 27:871–877.

Meharg, A.A., Rahman, M.M., 2003. Arsenic contamination of Bangladesh paddy field soils: implications for rice contribution to arsenic consumption. Environ Sci Technol 37: 229–334.

Meliker, J.R., Wahl, R.L., Cameron, L.L,, Nriagu, J.O., 2007. Arsenic in drinking water and cerebrovascular disease, diabetes mellitus, and kidney disease in Michigan: a standardized mortality ratio analysis. Environ Health 2:4–6.

Meyer, S., Matissek, M., Müller, S.M., Taleshi, M.S., Ebert, F., Francesconi, K.A., Schwerdtle, T., 2014. In vitro toxicological characterization of three arsenic-containing hydrocarbons. Metallomics. 6, 1023–1033.

Meyer, S., Raber, G., Ebert, F., Leffers, L., Müller, S.M., Taleshi, M.S., Francesconi, K.A., Schwerdtle, T., 2015. In vitro toxicological characterization of arsenic containing fatty acids and three of their metabolites. Toxicol. Res. 4, 1289.

Ministry of Health, Labour and Welfare of Japan 2015. The National Health and Nutrition Survey in Japan, 2013. http://www.mhlw.go.jp/bunya/kenkou/eiyou/dl/h25-houkoku.pdf (in Japanese).

Ministry of the Environmental of Japan, 2007. The detailed environmental survey (market basket) in 2006. http://www.env.go.jp/chemi/kurohon/2007/sokutei/pdf/02_05_27.pdf (in Japanese)

Moradi, Y., Bakar, J., Motalebi, A. A., Syed Muhamad, S. H., & Che Man, Y., 2011. A Review on Fish Lipid: Composition and Changes During Cooking Methods. J AQAT FOOD PROD T. 20, 379–390.

Morita, M., and Shibata, Y., 1990. Chemical form of arsenic in marine macroalgae. *Applied Organometallic Chemistry*. 4: 181-190.

Morita, M., Shibata, Y., 1988. Isolation and identification of arseno-lipid from a brown alga, *Undaria pinnatifida* (Wakame). Chemosphere. 17, 1147-1152.

Mumford, J.L., Wu, K,, Xia, Y., Kwok, R., Yang, Z., Foster, J., Sanders, W.E., 2007. Chronic Arsenic Exposure and Cardiac Repolarization Abnormalities with QT Interval Prolongation in a Population-based Study. Environ Health Perspect 115:690–694.

Naranmandura, H., Ogra, Y., Iwata, K., Lee, J., Suzuki, K.T., Weinfeld, M., and Le, X.C., 2009. Evidence for toxicity differences between inorganic arsenite and thioarsenicals in human bladder cancer cells. Toxicology and Applied Pharmacology. 238(2): 133-140.

Narukawa, T., Hioki, A., and Chiba, Koichi., 2012. Aqueous extraction of water-soluble inorganic arsenic in marine algae for speciation analysis. Analytical Sciences. Vol. 28.

Ng, J.C., Wang, J., Shrai, A.. 2003. A global health problem caused by arsenic from natural sources. Chemosphere 52:1353–1359.

Ninh, T.D., Nagashima, Y., Shiomi, K., 2008. Unusual arsenic speciation in sea anemones. Chemosphere. 70 (7), 1168-1174.

NRC (National Research Council), 1999. Subcommittee on Arsenic in D rinking Water. Arsenic in Drinking Water. National Academy press; Washington, DC. Available at : http://www.nap.edu/openbook.php?isbn=0309063337

NRC (National Research Council), 2001. Arsenic in Drinking Water: 2001 Update. National Academy press, Washington DC. Available at: http://www.nap.edu/catalog.php?record_id=10194

Nriagu, J.O., 2002. Arsenic Poisoning Through the Ages, In: Environmental Chemistry of Arsenic. Frankenberger, W.T. (Ed.). Marcel Dekker, Inc., New York, USA. pp. 1-26.

Nriagu, J.O., Azcue, J.M., 1990. Arsenic in the environment. Part I: cycling and characterization. New York: John Wiley and Sons, Inc 1–15.

Oomen, A.G., Rompelberg, C.J.M., Bruil, M.A., Dobbe, C.J.G., Pereboom, D.P.K.H., Sips, A.J.A.M., 2003. Development of an in vitro digestion model for estimating the bioaccessibility of soil contaminants. Arch Environ Contam Toxicol. 44, 281-287.

Park, M.O., 2005. New pathway for long-chain n-alkane synthesis via 1-alcohol in *Vibrio furnissii* M1. *Journal of Bacteriology*. 187(4): 1426-1429.

Raab, A., Newcombe, C., Pitton, D., Ebel, R., and Feldmann, J., 2013. Comprehensive Analysis of Lipophilic Arsenic Species in a Brown Alga (*Saccharina latissima*). *Analytical Chemistry*. 85(5): 2817-2824.

Rahman, M.A., Hasegawa, H., and Lim, R.P., 2012. Bioaccumulation, biotransformation and trophic transfer of arsenic in the aquatic food chain. Environmental Research. 116: 118-135. 37

Rmalli, Al., S.W., Haris, P.I., Harrington, C.F., Ayub, M., 2005. A survey of arsenic in foodstuffs on sale in the United Kingdom and imported from Bangladesh. Sci Total Environ 337:23–30.

Rotard, W., Christman, W., Knoth W., Mailahn, W., 1995. Bestimmung der resorptionsverfuugbaren PCDD/PCDF aus Kieselrot. UWSF-Z Umweltchem Okotx 7:3-9.

Rumpler, A., Edmonds, J.S., Katsu, M., Jensen, K.B., Goessler, W., Raber, G., Gunnlaugsdottir, H., Francesconi, K.A., 2008. Arsenic-containing long-chain fatty acids in cod-liver oil: A result of biosynthetic infidelity? Angew. Chem. Int. Ed. 47, 2665–2667.

Sadolin, E., 1928. Untersuchungen uber das Vorkommen des Arsens im Organismus der Fische. Biochemische Zeitschrift, 201, 323–331.

Schmeisser, E., Goessler, W., Francesconi K. A., 2006. Human metabolism of arsenolipids present in cod liver. Anal Bioanal Chem. 385:367-376.

Schmeisser, E., Raml, R., Francesconi, K.A., Kuehnelt, D., Lindberg, A.L., Soros, C., and Goessler, W., 2004. Thio arsenosugars identified as natural constituents of mussels by liquid chromatography mass spectrometry. *Chemical Communications*: 1824-1825.

Sele, V., Sloth, J.J., Holmelid, B., Valdersnes, S., Skov, K., Amlund, H., 2014. Arsenic containing fatty acids and hydrocarbons in marine oils- determination using reversed-phase HPLC-ICP-MS and HPLC-q TOF-MS. Talanta. 121, 89–96.

Sele, V., Sloth, J.J., Lundebye, A.K., Larsen, E.H., Berntssen, M.H.G., Amlund, H., 2012. Arsenolipids in marine oils and fats: A review of occurrence, chemistry and future research needs. Food Chem. 133, 618-630.

Shibata, Y., and Morita, M., 1988. A novel, tri-methylated arseno-sugar isolated from the brown alga Sargassum Thunbergii. Agricultural and Biological Chemistry. 52:4, 1087-1089.

Shiomi, K., 1994. Arsenic in marine organisms: Chemical forms and toxicological aspects, In: Arsenic in the Environment, Part II: Human Health and Ecosystem Effects. Nriagu, J.O. (Ed.). John Wiley & Sons, Inc., New York, USA. pp. 261-282.

Shiomi, K., Kakehashi, Y., Yamnaka, H., Kikuchi, T., 1987. Identification of arsenobetaine and a tetramethylarsonium salt in the clam. Appl Organomet Chem. *1(2), 177-183.*

Sloth, J.J., Larsen, E.H., Julshamn, K., 2004. Report on three aliphatic dimethylarsinoyl compounds as common minor constituents in marine samples. An investigation using high-performance liquid chromatography/inductively coupled plasma mass spectrometry and electrospray ionisation tandem mass spectrometry. Rapid Commun Mass Spec. 19 (2) 227-235.

Smedley, P.L., and Kinniburgh, D.G., 2002. A review on the sources, behaviour and distribution of arsenic in natural waters. Applied Geochemistry. 17(517-568).

Smith AH, Lingas EO, Rahman M. 2000. Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. Bull World Health Organ 78:1093-103.

Smith, A.H., Goycolea, M., Haque, R., Biggs, M.L., 1998. Marked increase in bladder and lung cancer mortality in a region of northern Chile due to arsenic in drinking water. Am J Epidemiol 147:660–669.

Stephen, N. M., Jeya Shakila, R., Jeyasekaran, G., & Sukumar, D., 2010. Effect of different types of heat processing on chemical changes in tuna. Journal of food science and technology, 47, 174–181.

Styblo, M., Del Razo, L.M., LeCluyse, E.L., Hamilton, G.A., Wang, C.Q., Cullen, W.R., and Thomas, D.J., 1999. Metabolism of arsenic in primary cultures of human and rat hepatocytes. Chemical Research in Toxicology. 12(7): 560-565. 109.

Styblo, M., Del Razo, L.M., Vega, L., Germolec, D.R., LeCluyse, E.L., Hamilton, G.A., Reed, W., Wang, C., Cullen, W.R., and Thomas, D.J., 2000. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. Archives of Toxicology. 74(6): 289-299.

Sun, G. X., Van de wiele, T., Alava, P., Tack, F., Du Laing, G., 2012. Arsenic in cooked rice: effect of chemical, enzymatic and microbial processes on bioaccessibility and speciation in human gastrointestinal tract. Environ Pollut. 162:241-6.

Taleshi, M.S., Edmonds, J.S., Goessler, W., Ruiz-Chancho, M.J., Raber, G., Jenson, K.B., Francesconi, K.A., 2010. Arsenic-containing lipids are natural constituents of sashimi tuna. Environ. Sci. Technol. 44, 1478-1483.

Taleshi, M.S., Jensen, K.B., Raber, G., Edmonds, J.S., Gunnlaugsdottir, H., Francesconi, K.A., 2008. Arsenic-containing hydrocarbons: Natural compounds in oil from the fish capelin, *Mallotus villosus*. Chem. Commun. 39, 4706-4707. DOI:10.1039/b808049f.

Taleshi, M.S., Seidler-Egdal, R.K., Jensen, K.B., Schwerdtle, T., Francesconi, K.A., 2014. Synthesis and characterization of arsenolipids: Naturally occurring arsenic compounds in fish and algae. Organomet. 33, 1397-1403.

Tapio, S., Grosche, B., 2006. Arsenic in the aetiology of cancer. Mutat Res 612:215–246.

Tchounwou, P.B., Wilson, B., Ishaque, A., 1999. Important considerations in the development of public health advisories for arsenic and arsenic containing compounds in drinking water. Rev Environ Health 14:211–229.

Tyokumbur, E.T., Okorie, T.G., Umma, B.S., 2014. Bioaccumulation of arsenic and uranium in two fish species (Clarias lazera and Tilapia zilli) from Alaro stream in Ibadan, Nigeria. Health Sciences Research 1(4):68–71.

Uneyama, C., Toda, M., Yamamoto, M., and Morikawa, K., 2007. Arsenic in various foods: Cumulative data. Food Additives and Contaminants. 24(5): 447-534.

Vahidnia, A., Romijn, F., van der Voet, G.B., de Wolff, F.A., 2008. Arsenic-induced neurotoxicity in relation to toxicokinetics: Effects on sciatic nerve proteins. Chem Biol Interact 176:188–195.

Vaskovsky, V.E., Korotchenko, O.D., Kosheleva, L.P., Levin, V.S., 1972. Arsenic in the lipid extracts of marine invertebrates. Comp. Biochem. Physiol. Part B: Comp. Biochem. 41, 777–784.

Wang, C.H., Jeng, J.S., Yip, P.K., Chen, C.L., Hsu, L.I., Hsueh, Y.M., Chiou, H.Y., Wu, M.M., Chen, C.J., 2002. Biological gradient between long-term arsenic exposure and carotid atherosclerosis Circulation 105:1804–1809.

Whaley-Martin, K.J., Koch, I., Moriarty, M., and Reimer, K.J., 2012. Arsenic Speciation in Blue Mussels (Mytilus edulis) Along a Highly Contaminated Arsenic Gradient**.** Environ. Sci. Technol., *2012, 46 (6), pp 3110–3118*

WHO (World Health Organization), 2000. Towards an Assessment of the Socioeconomic Impact of Arsenic Poisoning in Bangladesh. Available at: http://www.bvsde.ops-oms.org/bvsaca/i/fulltext/impact/impact.pdf

Wnek, S.M., Jensen, T.J., Severson, P.L., Futscher, B.W., and Gandolfi, A.J., 2010. Monomethylarsonous Acid Produces Irreversible Events Resulting in Malignant Transformation of a Human Bladder Cell Line Following 12 Weeks of Low-Level Exposure. Toxicological Sciences. 116(1): 44-57. 111.

Wrench, J. J., Fowler, S. W., and Ünlü, M.Y., 1979. Arsenic Metabolism in a Marine Food Chain. *Marine Pollution Bulletin* 10: 18-20.

Yoshida, T., Yamauchi, H., Fan, S.G., 2004. Chronic health effects in people exposed to arsenic via the drinking water: dose-response relationships in review. Toxicol Appl Pharmacol 198:243–252.