UNIVERSITY OF ALBERTA

BIOLOGICAL MONITORING OF ARSENIC FOR EXPOSURE DETERMINATION

by

KRISTI LEW

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Master of Science

in

Medical Sciences - Laboratory Medicine and Pathology

Edmonton, Alberta

Fall 2008

Your file Votre référence
ISBN: 978-0-494-47293-4
Our file Notre référence
ISBN: 978-0-494-47293-4

ABSTRACT

Arsenic originates from natural and anthropogenic sources; human exposure to arsenic has major health implications. High performance liquid chromatography combined with inductively coupled plasma mass spectrometry (HPLC-ICPMS) was used for the speciation and quantification of arsenic biomarkers in human urine and saliva. Arsenic metabolites were analyzed following exposure to arsenic from dietary or environmental sources. Arsenic speciation was compared in urine and saliva following a seafood meal, and speciation patterns in saliva paralleled those in urine. Children's overall exposure to arsenic on chromated copper arsenate (CCA)-treated wood playgrounds was determined by measuring arsenic speciation in urine and saliva samples. No significant differences in the arsenic concentrations or speciation patterns in the samples from children playing on CCA and non-CCA playgrounds were found. Biological monitoring of arsenic in human urine and saliva is a useful tool for determining overall exposure to arsenic.

ACKNOWLEDGEMENTS

I wish to express my gratitude and appreciation to the following individuals, without whom this thesis could not have been completed:

To Dr. X. Chris Le, my supervisor, for your knowledge, support and your confidence in my abilities. Thank you for sharing your enthusiasm for learning and research, and for encouraging me to keep an open mind.

To Dr. Jason Acker, my co-supervisor. Thank you for your encouragement, expertise and for emphasizing the science behind the research.

To Dr. Donald LeGatt and Dr. Imran Mirza, my committee members, and to Dr. Elaine Leslie, my external examiner, for your insightful comments and your critical review of this work.

To Ms. Xiufen Lu, Dr. Chungang Yuan, Dr. Baowei Chen, and Mr. Anthony McKnight-Whitford for your expertise and technical assistance in the lab.

To the volunteers in each study. These projects would not have been completed without your help.

To the members of the Division of Analytical and Environmental Toxicology and the Acker/McGann Cryobiology Group, for your friendship and for making this an incredible journey.

To Dr. Locksley McGann, thank you for the enlightening conversations and your wisdom and mentorship.

To Mr. Chris Ward, for your mentorship as I completed the University Teaching Program.

To the Medical Laboratory Science staff, for the numerous teaching opportunities and for enabling me to pursue my passion for teaching even further.

To my friends and family, thank you for your smiles and words of encouragement, for being there when a distraction from research or writing was needed, and for putting up with me and my frustrations.

To Andrew, your love, positivity, and belief in me have given me the motivation to achieve my goals.

Most importantly, to my parents. Thank you for your love and support throughout all my endeavors, and for allowing me to create my own path in life. To you I dedicate this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AsB	Arsenobetaine
CCA	Chromated copper arsenate
DMA(V)	Dimethylarsinic acid
HPLC	High performance liquid chromatography
iAs(III)	Arsenite
iAs(V)	Arsenate
ICPMS	Inductively coupled plasma mass spectrometry
MMA(V)	Monomethylarsonic acid

CHAPTER 1

INTRODUCTION

Arsenic is well-known throughout history as a poison. It is naturally occurring and ubiquitously distributed in the earth's crust as the 20th most abundant element. Humans are most commonly exposed to arsenic through their food and drinking water. They may also be exposed to arsenic from occupational, agricultural, or pharmaceutical sources. Acute and chronic exposure to arsenic is linked to adverse effects including neuropathy, renal failure, malignancies, and dermatological conditions.

Because of arsenic's health significance and the numerous ways in which humans can be exposed to arsenic, it is valuable to quantify and biologically monitor their overall exposure to arsenic. Blood and urine are common sample types for monitoring recent exposure while hair and nails are reflective of past and long-term exposure to arsenic. The focus of this thesis work is on arsenic biomonitoring using human urine and saliva samples.

1.1 CHEMICAL AND PHYSICAL PROPERTIES

Arsenic is found in Group 15 of the periodic table of elements and possesses properties of both metals and non-metals. There are four different oxidation states that arsenic can exist in: –3, 0, +3, and +5 [1]. Arsenic can also occur in a multitude of chemical forms, both organic and inorganic. The species

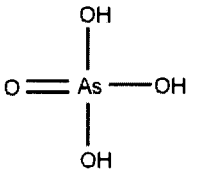
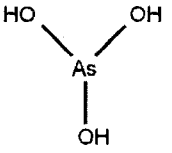
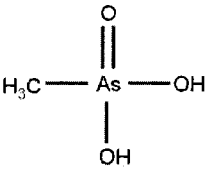
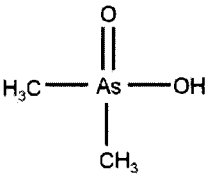
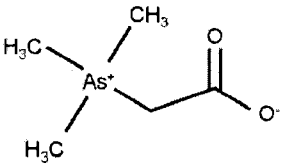
of arsenic plays a role in how it interacts with the environment as well as its toxicity. Table 1 lists the commonly studied arsenic species and their structures.

1.2. ARSENIC IN THE ENVIRONMENT

1.2.1 Natural Sources

Arsenic from natural sources has the potential to be mobilized from soil to water to air and biota. It can change in chemical form as a result of reactions with other compounds or due to the action of bacterial species. The Earth's crustal layer is a source of naturally occurring inorganic arsenic. The bedrock can undergo chemical changes to lead to the formation of smaller rocks, sediment and soil, which aids in the dissemination of arsenic. Rocks and soils can undergo weathering to release arsenic into surface water. Arsenic from the soil and bedrock can also be leached into the surrounding groundwater. Similar to soil, the concentration of arsenic in groundwater is dependent on the geographic location and proximity to anthropogenic sources. In the United States, most of the groundwater contains arsenic concentrations < 10 µg/L while in Bangladesh and West Bengal, India the concentrations range from <10 to >1000 µg/L [2-4]. This is a significant public health concern as many countries rely on groundwater as their source for drinking water. Currently the maximum contaminant level of arsenic in drinking water as recommended by the World Health Organization is <10 µg/L [5].

Table 1.1 Commonly studied arsenic species and their toxicities and chemical structures.

Name of Arsenic Compound	Abbreviation	Toxicity (LD_{50}) in Mice	Chemical Structure
Arsenate	iAs(V)	100 mg/kg [6]	
Arsenite	iAs(III)	10 mg/kg [6]	
Monomethylarsonic acid	MMA(V)	1800 mg/kg [7]	
Dimethylarsinic acid	DMA(V)	1200 mg/kg [7]	
Arsenobetaine	AsB	> 10 000 mg/kg [8]	

Volcanic action and microorganism interaction with arsenic are the most common natural sources for the release of arsenic compounds into the air. Arsenic's presence in the air is usually attributed to adsorption onto particulate matter [9] although it is also possible for microbes to generate volatile arsenic species [10].

The arsenic present in the soil, air and water can end up in our food supply. The amount of arsenic in food is also dependent on the type of food, type of soil grown on, water, use of arsenical pesticides, and processing techniques [1]. Although many foods contain arsenic to some degree, some foods such as seafood, mushrooms, and rice contain more arsenic than other food [11]. It is possible that these foods have a high rate of arsenic uptake, resulting in the higher arsenic concentration. The species present in seafood is predominantly arsenobetaine, while in other food sources is inorganic arsenic [12].

1.2.2 Anthropogenic Sources

Humans are exposed to arsenic as a result of its many uses in industry, from mining to pharmaceutical. In the Earth's crust, arsenic is commonly present in the ores of copper and iron. The mining of these metal ores will release arsenic into the atmosphere via airborne debris and liquid effluents [13]. Arsenic has also been popular in the agricultural industry for several uses: pesticides, herbicides, feed additives, and dessicants. Chromated copper arsenate (CCA) has been applied to wood to protect it from the effects of molds, termites, and

fungi. These wood structures include playgrounds, decks, utility poles, and fences. Arsenic has also been used for medicinal purposes throughout history as a general tonic as well as a treatment for syphilis and trypanosomiasis [14]. Currently, arsenic trioxide is used as an effective treatment for relapsed or refractory acute promyelocytic leukemia [15]. There is also widespread use of arsenic in China as an additive in herbal remedies [16].

1.2.3 Total Arsenic Intake from All Sources

In a population with no occupational exposure to arsenic, the most significant sources of exposure are food and drinking water. In Canada, the average daily intake of arsenic from food and water sources is 38 µg [11]. Fifteen µg of this amount is attributed to inorganic arsenic. However, the total amount of arsenic ingested is dependent on the type of food ingested and the source of the drinking water. The arsenic in the food and water supply in most of Canada is of minimal concern; however in regions of India, Bangladesh, Argentina, and Taiwan, high arsenic concentrations are endemic in the soil and groundwater, creating a major impact on human health [2, 5, 17-19].

1.3 TOXICOKINETICS

1.3.1. Absorption

The most common route of arsenic exposure is through oral ingestion; however inhalation also contributes to a small amount of intake. Exposure to arsenic through the ingestion route is usually in the inorganic form. When

arsenic appears in the stomach through consumption of food, water, or drugs; or intentional or accidental poisoning, it is readily absorbed through the gastrointestinal tract [1]. Arsenic compounds can be inhaled as a result of industrial exposure or cigarette smoking. Since arsenic in the air is most commonly in a particulate mixture, the particles are first inhaled and then deposited onto the lung surface. Smaller particulates can travel to the lower airways thus presenting a greater opportunity for arsenic absorption. Although it is possible for dermal contact with arsenic, absorption of arsenic through this route is very minor in comparison to the oral and inhalation routes [17]

1.3.2 Distribution

Even though arsenic is readily absorbed by the gastrointestinal tract, its bioavailability is dependent on its solubility, other nutritional contents of the stomach, and the food matrix the arsenic is found in [17]. Once in the bloodstream, arsenic is rapidly cleared with a half life of only 1–3 hours [20]. Arsenic is transported to the liver for metabolism and preparation for excretion. Benramdane *et al.* (1999) reported that following a fatal ingestion of 8 g of arsenic trioxide, the greatest accumulation of arsenic was in the liver, followed by the kidney, muscle, heart, spleen, pancreas, lungs, brain, skin, and finally small amounts in hemolyzed blood [21].

1.3.3 Metabolism

Once transported to the liver, inorganic arsenic undergoes a series of methylations and reductions. *S*-adenosylmethionine serves as the methyl donor [22]. This process is also mediated by the enzyme arsenic methyltransferase and the co-factor glutathione [17, 23]. Arsenate becomes reduced to iAs(III), followed by methylation to MMA(V), reduction to MMA(III), and finally methylation and reduction to DMA(V) and DMA(III), respectively (Figure 1) [24, 25]. In bacterial and rat species, DMA(III) can be further methylated to trimethylarsine oxide and trimethylarsine. Conversely, when AsB from marine organisms is ingested and absorbed, it does not undergo metabolism and is thus rapidly excreted [8, 26, 27].

1.3.4 Excretion

The main route of excretion for arsenic and its metabolites is in the urine. Most of the arsenic ingested is excreted in the urine within 2–3 days [26, 28, 29]. The predominant species excreted as a result of exposure to inorganic arsenic is DMA(V). In humans, it comprises between 60–80% of the total urinary excretion of arsenic in urine, while MMA(V) makes up 10–20% and the inorganic species 10–30% [30, 31]. The proportions of these species also vary depending on the arsenic dose since during an acute high-dose exposure to arsenic trioxide, inorganic arsenic accounted for the majority of the arsenic excreted in urine [21]. Other routes of arsenic excretion studied include saliva, feces, bile, and breast milk [32-36].

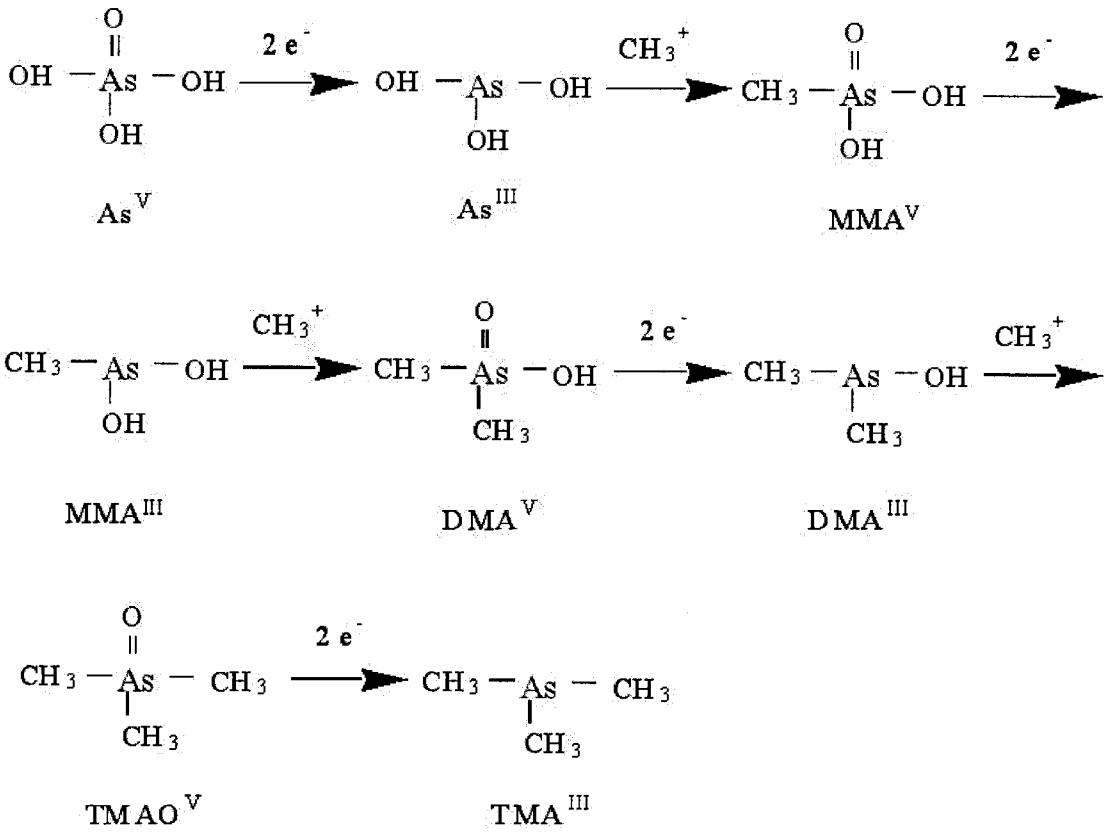


Figure 1.1 Metabolism of inorganic arsenic occurs through alternating reductions and methylations (Adapted from [24]). In humans, the primary metabolite is DMA(V). TMAO(V) and TMA(III) are more commonly detected in rat and bacterial species.

1.4 HEALTH EFFECTS

1.4.1 Acute Exposure

The lethal dose of inorganic arsenic in humans is 1–3 mg/kg [9, 25]. Symptoms of acute arsenic exposure are dose-dependent. With acute arsenic poisoning, gastrointestinal distress is rapid, which includes symptoms of nausea, vomiting, and diarrhea. As arsenic is rapidly absorbed into the bloodstream, it can interact with red blood cells to cause hemolysis. Other consequences of acute arsenic poisoning include cardiac arrhythmias, hypovolemia, shock, renal and respiratory failure, coma, and potentially death [37].

1.4.2 Chronic Exposure

Chronic exposure to arsenic affects many organ systems, including the skin, cardiovascular, nervous, hepatic, hematological, endocrine, and renal systems [2, 3, 5, 9, 36, 38]. Long-term exposure to high concentrations of arsenic in drinking water has been linked to cancers of the lung, bladder, and kidney, as well as hyperkeratosis, hyperpigmentation, and skin lesions. Other adverse effects of chronic arsenic exposure include diabetes mellitus, hypertension, peripheral vascular disease, and impairment of liver function.

1.5 BIOMARKERS OF EXPOSURE

Traditionally, the analysis of total arsenic in human urine has served as a biomarker for arsenic exposure. Another biomarker of interest includes the presence of skin lesions in populations suffering from chronic exposure to arsenic

in drinking water [39]. In addition to urine, other sample types investigated for total arsenic are blood, hair, and nails. The importance of determining the species of arsenic present in the biological sample of interest is now recognized. Speciation provides information of the form of arsenic exposure as well as its metabolism. Each species of arsenic also induces toxicity in different manners. Inorganic arsenite displays an affinity for sulfhydryl groups in proteins and enzymes, thus inhibiting their function [23]. The pentavalent form of inorganic arsenic [iAs(V)] is thought to be similar to phosphorus and thus acts like a phosphate analogue [40]. As a result, it interferes with the synthesis of adenosine triphosphate, a compound required for cellular energy. Finally, DMA(III) has been observed to bind to hemoglobin in rats, which may impede oxygen delivery to tissues [41]. The typical urinary biomarkers for assessing inorganic arsenic exposure include iAs(III), DMA(V), MMA(V), and iAs(V) concentrations. When determining arsenic exposure from seafood sources, AsB in urine is also investigated.

1.5.1 Urine

Urine is suitable for determining recent exposure to arsenic, whose half-life is approximately 2–3 days in humans ([26, 28, 29, 42]). The main excretion route of arsenic is in urine, thus this sample type is appropriate for measuring exposure from the ingestion, inhalation, and dermal pathways. The normal background concentrations of arsenic in urine are between 5 and 50 µg/L [42, 43]. In persons exposed to drinking water concentrations of up to and over 1000

µg/L, the urinary arsenic concentrations can be as high as 3100 µg/L [2, 44]. Studies on the use of urine for biological monitoring of arsenic demonstrate that a high level of arsenic in drinking water is correlated with increased arsenic in urine [44]. Urine is also a useful sample for studying the metabolism and excretion of poorly understood arsenic compounds in originating from seafood sources.

1.5.2 Blood

Determination of arsenic exposure by measuring concentrations in blood is only useful for recent high-dose exposures. This is because arsenic is rapidly cleared from the blood with a half-life of 1–3 hours [20]. The normal blood arsenic levels in a United Kingdom population without increased arsenic exposure was 2.6 ± 1.6 µg/L [43].

1.5.3 Saliva

Little work has been completed on the presence of arsenic biomarkers in saliva. However in the work that has been published, the mean total arsenic concentration in a population exposed to background levels of arsenic is 0.9 µg/L [35]. The authors also demonstrate that there is strong correlation between increased levels of arsenic in drinking water and in saliva. The species that have been identified in saliva are iAs(III), DMA(V), MMA(V), and iAs(V). With drug analysis in saliva, the saliva-to-plasma ratios of the drugs are normally considered [45, 46], however there has not been any work published on the correlation of plasma and salivary arsenic concentrations.

1.5.4 Hair and nails

Arsenic in hair and nails is reflective of past arsenic exposure. Since iAs(III) has an affinity for sulfhydryl groups, keratin is rich in these groups for arsenic to bind to. Hair and nails are less invasive to collect than blood and more convenient than urine; however they are sensitive to external contamination such as when hair is washed in arsenic-contaminated water. More time-consuming extraction procedures are also necessary for arsenic analysis [47].

1.6 BIOLOGICAL MONITORING

1.6.1 Ingestion of Arsenic

The most common manner in which humans are exposed to arsenic is through the ingestion of food and water. The elevated arsenic concentrations in the drinking water of certain regions in the world are a major public health concern. Millions of people are affected by concentrations exceeding the recommended WHO guideline of <10 µg/L. Biological monitoring of arsenic in the residents exposed to concentrations of up to 1800 µg/L in drinking water show that there is an increased prevalence of skin lesions, several types of malignancies, peripheral vascular disease, and diabetes mellitus [35, 38, 39, 48].

Numerous studies have investigated the arsenic excretion patterns in urine following ingestion of controlled amounts of marine species including crab, fish, shrimp, nori, and seaweed [27, 28, 49-52]. This practice contributes to the understanding of the metabolism and excretion profiles of many arsenic species following ingestion exposure. For example, it is now understood that AsB, the

predominant arsenic species in shellfish is not metabolized and is rapidly excreted [8, 26, 27]. These studies also demonstrate that most of the total arsenic ingested is cleared from the body within a few days [26]. Arsenosugars are the predominant forms of arsenic in seaweed [27, 53, 54]. Examining the urine following seaweed ingestion provides information regarding the metabolites of arsenosugar exposure.

1.6.2 Playgrounds

Because arsenic is a component of CCA for wood preservation, the general population may be exposed to arsenic through direct contact with treated fences, playgrounds, decks, and utility poles. It is especially a concern for children who have frequent contact with playgrounds constructed from CCA-treated wood. Children also have an increased hand-to-mouth frequency as well as the increased tendency to place foreign objects such as arsenic-containing soil into their mouths [55-57]. Their capabilities for arsenic metabolism also may not be the same as in adults. Studies have reported that children playing on CCA-treated playgrounds have a higher arsenic load on their hands compared to children playing on non-CCA playgrounds [58, 59]. Further biomonitoring is necessary to determine children's overall exposure to arsenic as a result of playing on CCA-treated playgrounds.

1.6.3 Occupational

Humans can be exposed to high arsenic concentrations in an occupational setting. This is more prevalent in employees of the mining, smelting, glass, semiconductor, and agricultural industries, as well as people who reside near these industrial areas. As a result of directly being involved in or by the mere proximity to these industries, the route of exposure is mainly inhalation of arsenic in particulates. Farmer and Johnson (1990) reported significantly increased concentrations of iAs(III), DMA(V), MMA(V), and iAs(V) in employees of timber treatment firms where arsenic is used in the production of CCA, glass manufacturing firms where arsenic trioxide is used for decolorizing glass, and in employees working for chemical firms who manufacture arsenic-containing compounds [60]. Because of the health effects associated with increased exposure to arsenic, arsenic biomonitoring in an occupational setting is important in creating limits for maximum exposure levels.

1.7 ANALYTICAL TECHNIQUES

While there are numerous methods for arsenic determination in biological samples, high performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICPMS) appears to be the method of choice in several studies [35, 61-63].

1.7.1 Separation of Arsenic Species Using HPLC

Chromatography is a technique for separating various compounds in mixtures; arsenic species in biological samples have been separated using HPLC [61-72]. Small volumes of sample are introduced into the HPLC via manual injection using a syringe, or automatic injection by means of an autosampler [73]. A pump introduces the mobile phase carrying the sample into the chromatography column.

Ion exchange, ion-pairing, and size exclusion chromatographic methods have been used for the separation of a wide range of arsenic species [24, 41, 65, 71, 74, 75].

Ion-exchange chromatography is separation based on the interaction of the sample ions with the counterions on the stationary phase. Anion-exchange chromatography is more commonly used for the separation of arsenic species. In this type of chromatography, negatively-charged anions are retained on the column based on their interactions with the positively charged groups on the stationary phase and thus elute more slowly than cations.

Ion-pair chromatography is performed on a reversed-phase column with the addition of an ion-pairing reagent to the mobile phase. This counterion to the analytes forms an ion-pair, a neutral component that can be retained on the column.

Size exclusion chromatography is based on the separation of compounds of differing sizes through various pore sizes of the column packing material. Larger molecules elute while smaller molecules are retained on the column. This

type of chromatography allows for the separation of arsenic species of different sizes, for example, arsenic species bound to proteins [41].

1.7.2 Detection of Arsenic Species with ICPMS

Once the arsenic species undergo separation through HPLC, they require detection by another source, most commonly by ICPMS. Advantages of ICPMS as a detection technique include its wide elemental coverage, sensitivity, wide dynamic range, ability to control for interferences, and applicability to many sample types [76, 77]. Once the sample exits the chromatography column, it enters the ICPMS via a nebulizer, which uses a flow of gas to convert the liquid sample into an aerosol. The small droplets of sample are then transported to the torch in the interior of the instrument where they interact with high temperature plasma, which ionizes the atoms from the sample aerosol. The ions then pass through a focusing region in which a vacuum aids ion passage to the mass spectrometer. At this point, the ions are separated by their mass-to-charge ratio (*m/z*). The most common type of mass analyzer employed is the quadrupole, which are four rods or parallel surfaces arranged in two rows of two in a rectangular configuration. By modifying the voltages associated with these rods, ions that do not have the selected *m/z* ratio are filtered out and do not reach the detector. The detector is a highly sensitive device due to its electron multiplier capability. If only a single ion hits the first electrode (dynode), electrons are released and multiplied as they hit subsequent dynodes. Eventually, the ion

pulse is sufficiently strong for detection as an ion count, which is translated to a signal on a data handling computer.

1.8 HYPOTHESIS AND OBJECTIVES

The hypothesis of this thesis is that arsenic speciation analysis in human urine and saliva can be used for biomonitoring to determine overall exposure to arsenic. To test this hypothesis, a method needs to be developed to analyze arsenic in human biological samples, specifically urine and saliva. The method would then be used for the speciation and quantification of arsenic in human urine and saliva following exposure to arsenic. First, a controlled seafood ingestion experiment will be performed in which participants eat a controlled amount of seafood. The elimination of arsenic in urine and saliva will be monitored over several days. Next children's overall exposure to arsenic as a result of playing on CCA-treated wood will be monitored. Several urine and saliva samples will be collected following play and analyzed for arsenic.

There are three main objectives for this thesis:

1. To develop a suitable method for performing arsenic speciation and quantification in human urine and saliva.

The method to be used will be HPLC coupled with ICPMS. HPLC will be used for the separation of arsenic species in urine and saliva, and detection of

these species will be completed using ICPMS. Parameters such as specificity, sensitivity, linearity, accuracy, and precision of the method will be evaluated.

2. To determine arsenic speciation and quantification in human urine and saliva following a controlled seafood ingestion experiment.

Participants in this study will ingest a pre-determined amount of crab, which is known to contain large concentrations of AsB. This compound is not metabolized in the human body and is thus excreted unchanged. The excretion of AsB as well as other arsenic species will be monitored in urine and saliva at several time points following ingestion. This study will also look at the relationship between the concentrations of arsenic species in urine and saliva.

3. To perform biomonitoring on children's overall exposure to arsenic following play on playgrounds constructed from CCA-treated wood.

Previous work has shown that children playing on CCA-treated wood playgrounds have approximately four times as much arsenic on their hands as children playing on playgrounds constructed from other materials [58]. Since children's hand to mouth contact is between 42 and 81 times an hour, they have the potential of ingesting any arsenic on their hands [55]. By monitoring the arsenic concentration and speciation patterns in the urine and saliva of children playing on CCA-treated playgrounds, their overall exposure to arsenic can be determined.

1.9 REFERENCES

[1] World Health Organization. Arsenic and Arsenic Compounds. 2 ed. Geneva: World Health Organization 2001.

[2] Rahman MM, Chowdhury UK, Mukherjee SC, Mondal BK, Paul K, Lodh D, et al. Chronic arsenic toxicity in Bangladesh and West Bengal, India--a review and commentary.[see comment][comment]. J Toxicol Clin Toxicol. 2001;39(7):683-700.

[3] Lepkowski W. Arsenic Crisis in Bangladesh. Chem Eng News. 1998 Nov 16;76(46):27-9.

[4] Reid J. Arsenic Occurrence - Usepa Seeks Clearer Picture. J Am Water Works Assoc. 1994 Sep;86(9):44-51.

[5] World Health Organization. Arsenic in Drinking Water. [Fact Sheet 210] 2001 May 2001 [cited 26 Jan 2006]; Available from: http://www.who.int/mediacentre/factsheets/fs210/en/

[6] Schroede.Ha, Balassa JJ. Abnormal Trace Metals in Man - Arsenic. Journal of Chronic Diseases. 1966;19(1):85-&.

[7] Kaise T, Yamauchi H, Horiguchi Y, Tani T, Watanabe S, Hirayama T, et al. A comparative study on acute toxicity of methylarsonic acid, dimethylarsinic acid and trimethylarsine oxide in mice. Appl Organomet Chem. 1989;3(3):273-7.

[8] Kaise T, Watanabe S, Itoh K. The Acute Toxicity of Arsenobetaine. Chemosphere. 1985;14(9):1327-32.

[9] Agency for Toxic Substances and Disease Registry. Toxicological Profile for Arsenic (Update). Atlanta: Agency for Toxic Substances and Disease Registry 2007.

[10] McBride BC, Wolfe RS. Biosynthesis of Dimethylarsine by Methaanobacterium. Biochemistry. 1971;10(23):4312-7.

[11] Dabeka RW, McKenzie AD, Lacroix GMA, Cleroux C, Bowe S, Graham RA, et al. Survey of Arsenic in Total Diet Food Composites and Estimation of the Dietary Intake of Arsenic by Canadian Adults and Children. J AOAC Int. 1993 Jan-Feb;76(1):14-25.

[12] Le SXC, Cullen WR, Reimer KJ. Speciation of Arsenic Compounds in Some Marine Organisms. Environ Sci Technol. 1994 Sep;28(9):1598-604.

[13] Fowler BA, ed. Biological and Environmental Effects of Arsenic. Amsterdam: Elsevier Science Publishers 1983.

[14] Jolliffe DM. A History of the Use of Arsenicals in Man. Journal of the Royal Society of Medicine. 1993 May;86(5):287-9.

[15] Shen Z-X, Chen G-Q, Ni J-H, Li X-S, Xiong S-M, Qiu Q-Y, et al. Use of Arsenic Trioxide (As2O3) in the Treatment of Acute Promyelocytic Leukemia (APL): II. Clinical Efficacy and Pharmacokinetics in Relapsed Patients. Blood. 1997 May 1, 1997;89(9):3354-60.

[16] Garvey GJ, Hahn G, Lee RV, Harbison RD. Heavy metal hazards of Asian traditional remedies. Int J Environ Health Res. 2001;11(1):63-71.

[17] Mandal BK, Suzuki KT. Arsenic round the world: a review. Talanta. 2002 Aug;58(1):201-35.

[18] Mukherjee A, Sengupta MK, Hossain MA, Ahamed S, Das B, Nayak B, et al. Arsenic contamination in groundwater: A global perspective with emphasis on the Asian scenario. J Health Popul Nutr. 2006 Jun;24(2):142-63.

[19] Kile ML, Houseman EA, Breton CV, Smith T, Quamruzzaman O, Rahman M, et al. Dietary arsenic exposure in Bangladesh. Environ Health Perspect. 2007 Jun;115(6):889-93.

[20] World Health Organization. Environmental Health Criteria 18 - Arsenic. Geneva: World Health Organization 1981.

[21] Benramdane L, Accominotti M, Fanton L, Malicier D, Vallon JJ. Arsenic speciation in human organs following fatal arsenic trioxide poisoning - A case report. Clin Chem. 1999 Feb;45(2):301-6.

[22] Thomas DJ, Waters SB, Styblo M. Elucidating the pathway for arsenic methylation. Toxicol Appl Pharmacol. 2004 Aug;198(3):319-26.

[23] Aposhian HV, Zakharyan RA, Avram MD, Sampayo-Reyes A, Wollenberg ML. A review of the enzymology of arsenic metabolism and a new potential role of hydrogen peroxide in the detoxication of the trivalent arsenic species. Toxicol Appl Pharmacol. 2004 Aug;198(3):327-35.

[24] Le XC, Lu XF, Ma MS, Cullen WR, Aposhian HV, Zheng BS. Speciation of key arsenic metabolic intermediates in human urine. Anal Chem. 2000 Nov;72(21):5172-7.

[25] Hughes MF. Arsenic toxicity and potential mechanisms of action. Toxicol Lett. 2002 Jul 7;133(1):1-16.

[26] Freeman HC, Uthe JF, Fleming RB, Odense PH, Ackman RG, Landry G, et al. Clearance of arsenic ingested by man from arsenic contaminated fish. Bull Environ Contam Toxicol. 1979 May;22(1-2):224-9.

[27] Le XC, Cullen WR, Reimer KJ. Human urinary arsenic excretion after one-time ingestion of seaweed, crab, and shrimp. Clin Chem. 1994 Apr;40(4):617-24.

[28] Crecelius EA. Changes in Chemical Speciation of Arsenic Following Ingestion by Man. Environ Health Perspect. 1977;19(Aug):147-50.

[29] Buchet JP, Lauwerys R, Roels H. Urinary excretion of inorganic arsenic and its metabolites after repeated ingestion of sodium metaarsenite by volunteers Int Arch Occup Environ Health. 1981;48(2):111-8.

[30] Goessler W, Schlagenhaufen C, Kuehnelt D, Greschonig H, Irgolic KJ. Can Humans Metabolize Arsenic Compounds to Arsenobetaine? Appl Organomet Chem. 1997;11(4):327-35.

[31] Vahter M. Genetic polymorphism in the biotransformation of inorganic arsenic and its role in toxicity. Toxicol Lett. 2000;112-113:209-17.

[32] Concha G, Vogler G, Nermell B, Vahter M. Low-level arsenic excretion in breast milk of native Andean women exposed to high levels of arsenic in the drinking water. Int Arch Occup Environ Health. 1998;71(1):42-6.

[33] Gregus Z, Gyurasics A, Csanaky I. Biliary and urinary excretion of inorganic arsenic: Monomethylarsonous acid as a major biliary metabolite in rats. Toxicol Sci. 2000 Jul;56(1):18-25.

[34] Cui X, Kobayashi Y, Hayakawa T, Hirano S. Arsenic speciation in bile and urine following oral and intravenous exposure to inorganic and organic arsenics in rats. Toxicol Sci. 2004 Dec;82(2):478-87.

[35] Yuan CG, Lu XF, Oro N, Wang ZW, Xia YJ, Wade TJ, et al. Arsenic speciation analysis in human saliva. Clin Chem. 2008;54(1):163-71.

[36] Winship KA. Toxicity of Inorganic Arsenic Salts. Adverse Drug React Toxicol Rev. 1984;3(3):129-60.

[37] Graeme KA, Pollack CV, Jr. Heavy metal toxicity, Part I: arsenic and mercury. J Emerg Med. 1998 Jan-Feb;16(1):45-56.

[38] Tseng WP. Effects and Dose-Response Relationships of Skin Cancer and Blackfoot Disease with Arsenic. Environ Health Perspect. 1977;19(AUG):109-19.

[39] Chen C-J, Hsu L-I, Wang C-H, Shih W-L, Hsu Y-H, Tseng M-P, et al. Biomarkers of exposure, effect, and susceptibility of arsenic-induced health hazards in Taiwan. Toxicol Appl Pharmacol. 2005;206(2):198-206.

[40] Dixon HBF. The biochemical action of arsonic acids especially as phosphate analogues. *Advances in Inorganic Chemistry, Vol. 44* 1997:191-227.

[41] Lu M, Wang H, Li XF, Lu X, Cullen WR, Arnold LL, et al. Evidence of hemoglobin binding to arsenic as a basis for the accumulation of arsenic in rat blood. Chem Res Toxicol. 2004 Dec;17(12):1733-42.

[42] National Research Council. Arsenic in Drinking Water. Washington, DC: National Academy Press 1999.

[43] Seifert B, Becker K, Helm D, Krause C, Schulz C, Seiwert M. The German Environmental Survey 1990/1992 (GerES II): reference concentrations of

selected environmental pollutants in blood, urine, hair, house dust, drinking water and indoor air. J Expo Anal Environ Epidemiol. 2000 Nov-Dec;10(6):552-65.

[44] Chiou H-Y, Hsueh Y-M, Hsieh L-L, Hsu L-I, Hsu Y-H, Hsieh F-I, et al. Arsenic methylation capacity, body retention, and null genotypes of glutathione S-transferase M1 and T1 among current arsenic-exposed residents in Taiwan. Mutat Res. 1997;386(3):197-207.

[45] Aardal E, Holm AC. Cortisol in saliva - Reference ranges and relation to cortisol in serum. Eur J Clin Chem Clin Biochem. 1995 Dec;33(12):927-32.

[46] Jenkins AJ, Oyler JM, Cone EJ. Comparison of Heroin and Cocaine Concentrations in Saliva with Concentrations in Blood and Plasma. J Anal Toxicol. 1995 Oct;19(6):359-74.

[47] Mandal BK, Ogra Y, Suzuki KT. Speciation of arsenic in human nail and hair from arsenic-affected area by HPLC-inductively coupled argon plasma mass spectrometry. Toxicol Appl Pharmacol. 2003 Jun;189(2):73-83.

[48] Hughes MF. Biomarkers of exposure: A case study with inorganic arsenic. Environ Health Perspect. 2006 Nov;114(11):1790-6.

[49] Lai VWM, Sun YM, Ting E, Cullen WR, Reimer KJ. Arsenic speciation in human urine: are we all the same? Toxicol Appl Pharmacol. 2004 Aug;198(3):297-306.

[50] Heinrich-Ramm R, Mindt-Prufert S, Szadkowski D. Arsenic species excretion after controlled seafood consumption. J Chromatogr B Analyt Technol Biomed Life Sci. 2002 Oct;778(1-2):263-73.

[51] Buchet JP, Lison D, Ruggeri M, Foa V, Elia G. Assessment of exposure to inorganic arsenic, a human carcinogen, due to the consumption of seafood. Arch Toxicol. 1996 Sep;70(11):773-8.

[52] Buchet JP, Pauwels J, Lauwerys R. Assessment of Exposure to Inorganic Arsenic Following Ingestion of Marine Organisms by Volunteers. Environ Res. 1994 Jul;66(1):44-51.

[53] Van Hulle M, Zhang C, Schotte B, Mees L, Vanhaecke F, Vanholder R, et al. Identification of some arsenic species in human urine and blood after ingestion of Chinese seaweed Laminaria. J Anal At Spectrom. 2004 Jan;19(1):58-64.

[54] Ma MS, Le XC. Effect of arsenosugar ingestion on urinary arsenic speciation. Clin Chem. 1998 Mar;44(3):539-50.

[55] Tulve NS, Suggs JC, McCurdy T, Hubal EAC, Moya J. Frequency of mouthing behavior in young children. J Expo Anal Environ Epidemiol. 2002 Jul;12(4):259-64.

[56] AuYeung W, Canales RA, Beamer P, Ferguson AC, Leckie JO. Young Children's Mouthing Behavior: An Observational Study via Videotaping in a Primarily Outdoor Residential Setting. J Child Health. 2004;2(3):271 - 95.

[57] Xue JP, Zartarian V, Moya J, Freeman N, Beamer P, Black K, et al. A meta-analysis of children's hand-to-mouth frequency data for estimating nondietary ingestion exposure. Risk Anal. 2007 Apr;27(2):411-20.

[58] Kwon E, Zhang HQ, Wang ZW, Jhangri GS, Lu XF, Fok N, et al. Arsenic on the hands of children after playing in playgrounds. Environ Health Perspect. 2004 Oct;112(14):1375-80.

[59] Shalat SL, Solo-Gabriele HM, Fleming LE, Buckley BT, Black K, Jimenez M, et al. A pilot study of children's exposure to CCA-treated wood from playground equipment. Sci Total Environ. 2006 Aug;367(1):80-8.

[60] Farmer JG, Johnson LR. Assessment of occupational exposure to inorganic arsenic based on urinary concentrations and speciation of arsenic. Br J Ind Med. 1990 May;47(5):342-8.

[61] Larsen EH, Hansen SH. Separation of arsenic species by ion-pair and ion exchange high performance liquid chromatography. Mikrochim Acta. 1992;109(1):47-51.

[62] Londesborough S, Mattusch J, Wennrich R. Separation of organic and inorganic arsenic species by HPLC-ICP-MS. Anal Bioanal Chem. 1999;363(5):577-81.

[63] Lintschinger J, Schramel P, Hatalak-Rauscher A, Wendler I, Michalke B. A new method for the analysis of arsenic species in urine by using HPLC-ICP-MS. Fresenius' J Anal Chem. 1998;362(3):313-8.

[64] Xie RM, Johnson W, Spayd S, Hall GS, Buckley B. Arsenic speciation analysis of human urine using ion exchange chromatography coupled to inductively coupled plasma mass spectrometry. Anal Chim Acta. 2006 Sep;578(2):186-94.

[65] Ritsema R, Dukan L, Navarro T, van Leeuwen W, Oliveira N, Wolfs P, et al. Speciation of arsenic compounds in urine by LC-ICP MS. Appl Organomet Chem. 1998;12(8-9):591-9.

[66] Sloth JJ, Larsen EH, Julshamn K. Selective arsenic speciation analysis of human urine reference materials using gradient elution ion-exchange HPLC-ICP-MS. J Anal At Spectrom. 2004;19(8):973-8.

[67] Yoshinaga J, Chatterjee A, Shibata Y, Morita M, Edmonds JS. Human urine certified reference material for arsenic speciation. Clin Chem. 2000 Nov;46(11):1781-6.

[68] Morton J, Mason H. Speciation of arsenic compounds in urine from occupationally unexposed and exposed persons in the UK using a routine LC-ICP-MS method. J Anal Toxicol. 2006 Jun;30(5):293-301.

[69] Kohlmeyer U, Kuballa J, Jantzen E. Simultaneous separation of 17 inorganic and organic arsenic compounds in marine biota by means of high-performance liquid chromatography/inductively coupled plasma mass spectrometry. Rapid Commun Mass Spectrom. 2002;16(10):965-74.

[70] Hirata S, Toshimitsu H. Determination of arsenic species and arsenosugars in marine samples by HPLC-ICP-MS. Appl Organomet Chem. 2007 Jun;21(6):447-54.

[71] Zheng J, Goessler W, Kosmus W. Speciation of arsenic compounds by coupling high-performance liquid chromatography with inductively coupled plasma mass spectrometry. Mikrochim Acta. 1998;130(1-2):71-9.

[72] Larsen EH, Pritzl G, Hansen SH. Speciation of 8 Arsenic Compounds in Human Urine by High-Performance Liquid-Chromatography with Inductively-Coupled Plasma-Mass Spectrometric Detection Using Antimonate for Internal Chromatographic Standardization. J Anal At Spectrom. 1993 Jun;8(4):557-63.

[73] Skoog D, Holler F, Nieman T, eds. Principles of Instrumental Analysis. 5 ed. Toronto: Harcourt College Publishers 1998.

[74] Le XC, Lu X, Li XF. Arsenic Speciation. Anal Chem. 2004;76(1):26A-33A.

[75] Le XC, Ma MS. Speciation of arsenic compounds by using ion-pair chromatography with atomic spectrometry and mass spectrometry detection. J Chromatogr A. 1997 Mar;764(1):55-64.

[76] Agilent Techologies. ICP-MS Inductively Coupled Plasma Mass Spectrometry - A Primer. USA: Agilent Technolgies 2005.

[77] Perkin Elmer. The 30-Minute Guide to ICP-MS. 2001 [cited 2008 July 10]; Available from: http://las.perkinelmer.com/content/TechnicalInfo/TCH_ICPMSThirtyMinuteGuide.pdf

CHAPTER 2

DEVELOPMENT OF AN HPLC-ICPMS METHOD FOR ARSENIC SPECIATION IN HUMAN BIOLOGICAL SAMPLES

2.1 INTRODUCTION

There are over 20 species of arsenic present in the environment; some species are more toxic than others. Arsenobetaine (AsB) found in marine organisms is fairly non-toxic with an LD_{50} of greater than 10 000 mg/kg in mice, while arsenic trioxide, a trivalent form of arsenic causes greater harm with an LD_{50} of 35 mg/kg in mice [1]. It is also possible for multiple species to co-exist in biological samples. This emphasizes the importance of conducting arsenic speciation, rather than total elemental analysis, when assessing human exposure to arsenic [2].

Numerous analytical tools and techniques have been employed for arsenic speciation analysis. These range from a simple and inexpensive procedure such as spectrophotometry, to more complex and costly methods including electrospray ionization mass spectrometry and hydride generation atomic fluorescence spectrometry [3, 4]. The most commonly used method for arsenic analysis is a coupled technique known as high performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICPMS). The reasons why this method is chosen include its separation capability, compatibility with various environmental and biological samples, and

detector sensitivity. Arsenic compounds in the samples of interest are first separated using HPLC, and then detected using ICPMS.

The objective of this chapter is to develop an HPLC-ICPMS method for the separation and detection of arsenic species in human urine. This method will be used for arsenic biomonitoring applications as a measure of exposure to arsenic.

2.2 EXPERIMENTAL

2.2.1 Reagents and Standards

Laboratory water deionized to 18 MΩ (Millipore Elix UV) was used for all reagent preparation. The arsenic standards prepared include AsB, arsenite [iAs(III)], dimethylarsinic acid [DMA(V)], monomethylarsonic acid [MMA(V)] and arsenate [iAs(V)]. The actual chemical forms used were AsB (Tri Chemical Laboratories; Yamanashi, Japan), monosodium acid methane arsenate sesquihydrate [MMA(V)] (Chem Service; West Chester, PA), sodium *m*-arsenite [iAs(III)], sodium arsenate [iAs(V)], and cacodylic acid [DMA(V)] (all from Sigma-Aldrich; St. Louis, MO). Stock solutions of each species were prepared in deionized water to 10 mg/L and diluted to the necessary concentrations each day for sample analysis (between 0.1 and 100 µg/L). Certified reference material of human urine (No. 18) was obtained from the National Institute for Environmental Studies (NIES) (Tsukuba, Ibaraki, Japan) and also analyzed once per day with each batch of samples.

Ammonium bicarbonate was purchased from Sigma-Aldrich (St. Louis, MO) and used in the preparation of the mobile phase in anion exchange

chromatography. Tetrabutyl ammonium hydroxide (Sigma-Aldrich; St. Louis, MO) and malonic acid (Fisher Scientific; Fair Lawn, NJ) were used in the eluent in ion-pair chromatography. The mobile phases were prepared in 5% HPLC grade methanol (Fisher Scientific; Fair Lawn, NJ). The pH of the mobile phases were adjusted with 10% nitric acid (Fisher Scientific; Fair Lawn, NJ) or 10% ammonium hydroxide (Fisher Scientific; Fair Lawn, NJ).

2.2.2 Sample Processing

Urine samples were frozen at either -20 or -50 °C until analysis, at which point they were thawed at room temperature. To remove particulate matter, 1 mL of each urine sample was filtered through 0.45 µm nylon membrane filters (Whatman; Maidstone, UK) into HPLC sampling vials (National Scientific; Rockwood, TN) for analysis.

Saliva samples were also stored at either -20 or -50 °C. On the day of analysis, they were removed from the freezer and thawed at room temperature. Once thawed, the samples were thoroughly vortexed for 1 min and diluted 1 in 3 with deionized water. The samples were then ultrasonicated (Sonicor Instrument Corporation; Copiague, NY) for 5 min and centrifuged (Sorvall Biofuge Primo, Thermo Scientific; Waltham, MA) at 4000 rpm for 15 min. One mL of supernatant was syringe filtered (Whatman; Maidstone, UK) into HPLC vials (National Scientific; Rockwood, TN) for analysis.

2.2.3 Sample Analysis

Equipment

A PerkinElmer Series 200 HPLC system equipped with an autosampler and a binary pump was used for the separation of the arsenic species. Ion-pair and anion exchange chromatography columns were both used for arsenic separation (Table 2.1). A reverse phase HPLC column (ODS-3, 150 x 4.6 mm, 3 µm particle size; Phenomenex; Torrance, CA) was used for the separation of arsenic species based on ion-pair chromatography. The mobile phase contained 5 mmol/L tetrabutylammonium hydroxide and 3 mmol/L malonic acid in 5% methanol. The pH was adjusted to 5.85 using 10% nitric acid. The column temperature was maintained at 48 °C while using an isocratic elution mode with a flow rate of 1.2 mL/min. The sample injection volume was 50 µL and each sample was analyzed in duplicate. The time required for sample analysis was 7 min per injection.

A 150 x 4.1 mm PRP-X100 column (5 µm particle size) from Hamilton (Reno, NV) along with a guard column was used for the LC separation of arsenic species based on anion exchange properties. The eluent for the separation was 35 mmol/L ammonium bicarbonate in 5% methanol, pH adjusted to 8.2 with 10% ammonium hydroxide. A gradient elution with the following parameters was used: 0–4 min, 0.8 mL/min; 4–10 min, 1.7 mL/min; and 10–16 min, 0.8 mL/min. The sample injection volume was 50 µL and all samples were analyzed in duplicate. The analysis time was 16 min per injection.

The HPLC system was interfaced with the ICPMS (Elan 6100 DRC Plus; PerkinElmer; Norwalk, CT) through polyvinyl chloride tubing. The LC effluent was introduced into the ICPMS through a Meinhard Type A quartz nebulizer coupled to a cyclonic spray chamber (Glass Expansion, Inc.; West Melbourne, Australia). Other ICPMS parameters are summarized in Table 2.2. The instrument was run using the dynamic reaction cell (DRC) technology to monitor arsenic in the form of $^{75}As^{16}O^{+}$ at the *m/z* ratio of 91. If $^{75}As^{+}$ were monitored, chlorine in the samples could potentially interfere through the formation of $^{40}Ar^{35}Cl^{+}$, which has the same *m/z* ratio as $^{75}As^{+}$ [5].

Once the computer received the data, it was in an ICPMS format. The TC Convert option on the Turbochrom Workstation software (version 6.1.2; PerkinElmer; Norwalk CT) was used for the conversion of the ICPMS data into Turbochrom files for chromatogram viewing and peak integration on the same software.

Following completion of analysis, the HPLC column was flushed with 5% methanol for a minimum of 20 min at a flow rate of 0.8 mL/min. The ICPMS was flushed with 1% nitric acid for approximately 15 min followed by deionized water for a minimum of 5 min.

Table 2.1 HPLC Materials and Conditions for the Separation of Arsenic Species in Human Urine and Saliva

Parameter	Ion-Pair	Anion Exchange
Mobile Phase	5 mmol/L tetrabutylammonium hydroxide 3 mmol/L malonic acid 5% methanol pH 5.85	35 mmol/L ammonium bicarbonate 5 % methanol pH 8.2
Column	Phenomenex ODS-3	Hamilton PRP-X100
Injection Volume	50 µL	50 µL
Elution Program	1.2 mL/min	0–4 min: 0.8 mL/min 4–10 min: 1.7 mL/min 10–16 min: 0.8 mL/min
Column Temperature	48 °C	Ambient
Separation Time	7 min	16 min

Table 2.2 ICPMS Operating Conditions and Parameters for the Detection of Arsenic Species in Human Urine and Saliva

Parameter	Setting/Type
Nebulizer	Meinhard Type A quartz
Spray chamber	Glass Expansion cyclonic
RF Power	1350 W
Plasma Gas Flow	15.00 L/min
Nebulizer Gas Flow	0.92 L/min
Auxillary Gas Flow	1.50 L/min
Analyte Monitored	$^{75}As^{16}O^{+}$

Calibration

The performance of the ICPMS was verified by analyzing the Elan 6100 DRC Sensitivity Detection Limit Solution (Perkin Elmer Life & Analytical Sciences; Shelton CT) daily. This solution contains a standard concentration (1 µg/L) each of Be, Co, In, U, Mg, Rh, Pb, Na, Fe, Ca, K, Ba, and Ce. The peak intensities obtained from the ICPMS serve as a measure of the instrument's sensitivity against the expected values for each certified element.

Arsenic standards were prepared to incorporate the range of expected concentrations of arsenic in the samples. The ICPMS was calibrated using eight standard concentrations ranging from 0.1-100 µg/L of a mix each of AsB, iAs(III), DMA(V), MMA(V), and iAs(V). The standards were prepared fresh daily from a stock standard of 10 mg/L and analyzed at the start of each run. A standard from the middle of the calibration range was also analyzed once every five samples to check for instrument drift. Certified reference material of human urine (No. 18, National Institute for Environmental Studies; Tsukuba, Ibaraki, Japan) was quantified once daily for quality control purposes. The measured values of arsenic where compared against the manufacturer's certified values as a determination of the daily accuracy of the method.

2.3 RESULTS

Figure 2.1 shows a chromatogram from the ion-pair chromatographic separation of five arsenic species in a standard calibration solution (10 µg/L of each arsenic species). Although DMA(V), MMA(V) and iAs(V) are well-resolved

(retention times of 2.27, 2.91, and 5.04 min, respectively), AsB and iAs(III) coelute at the void time of 1.45 min.

A chromatogram for the separation of five arsenic species using anion exchange chromatography is shown in Figure 2.2. The elution order and average retention times for each arsenic species was as follows: AsB, 1.86 min; iAs(III), 2.47 min; DMA(V), 3.59 min; MMA(V), 6.29 min; and iAs(V), 13.57 min. This follows an identical elution order as previously described [6]. Anion exchange chromatography was used for the remainder of the arsenic speciation analysis.

Examples of the calibration curves of all five arsenic species analyzed by anion exchange chromatography and ICPMS are shown in Figures 2.3 (a–e). The correlation coefficients for all of the calibration curves were all > 0.99.

Figure 2.4(a) is a chromatogram of an example of a urine sample (1 in 2 dilution). To confirm the identity of each arsenic peak, 5 µg/L of each species was separately added to the urine sample and analyzed under the same conditions. If the standard arsenic spike elutes with the suspected species, then the identity of the arsenic peak is assumed to be the same as the arsenic standard [Figures 2.4(b–e)].

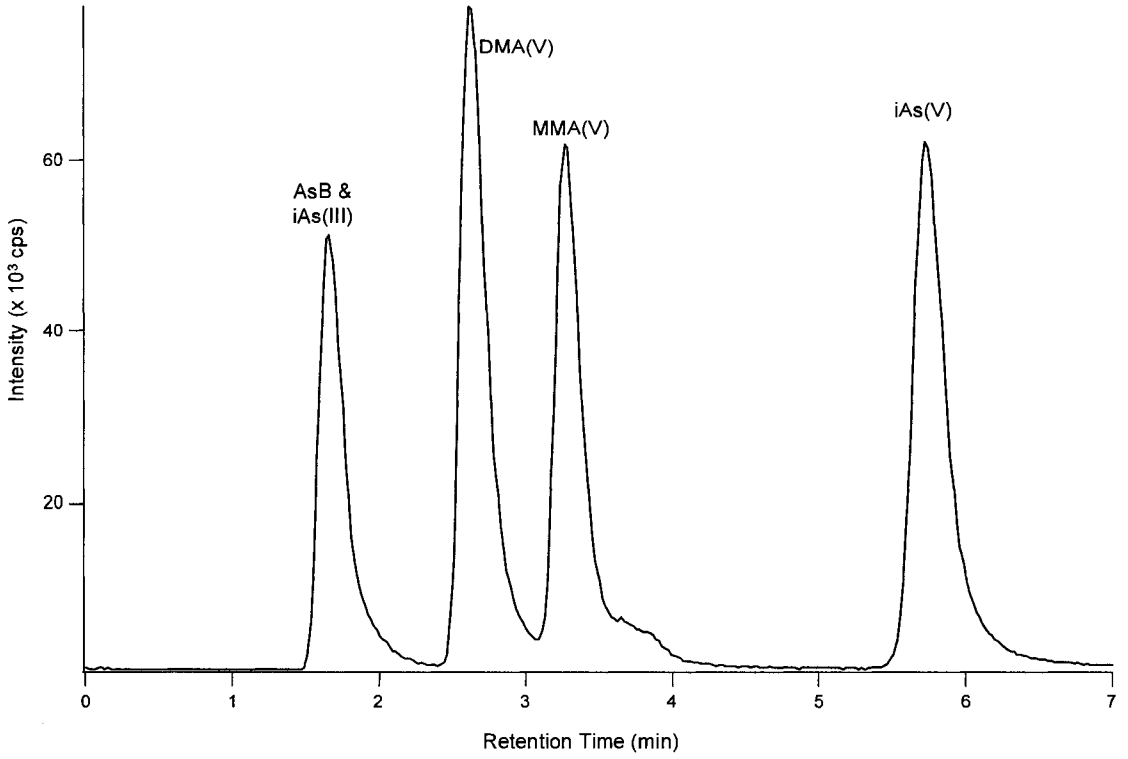


Figure 2.1 Chromatogram showing the ion-pair chromatographic separation of a standard solution of 10 µg/L AsB, iAs(III), DMA(V), MMA(V), and iAs(V) using an ODS C-18 column. AsB elutes together with iAs(III).

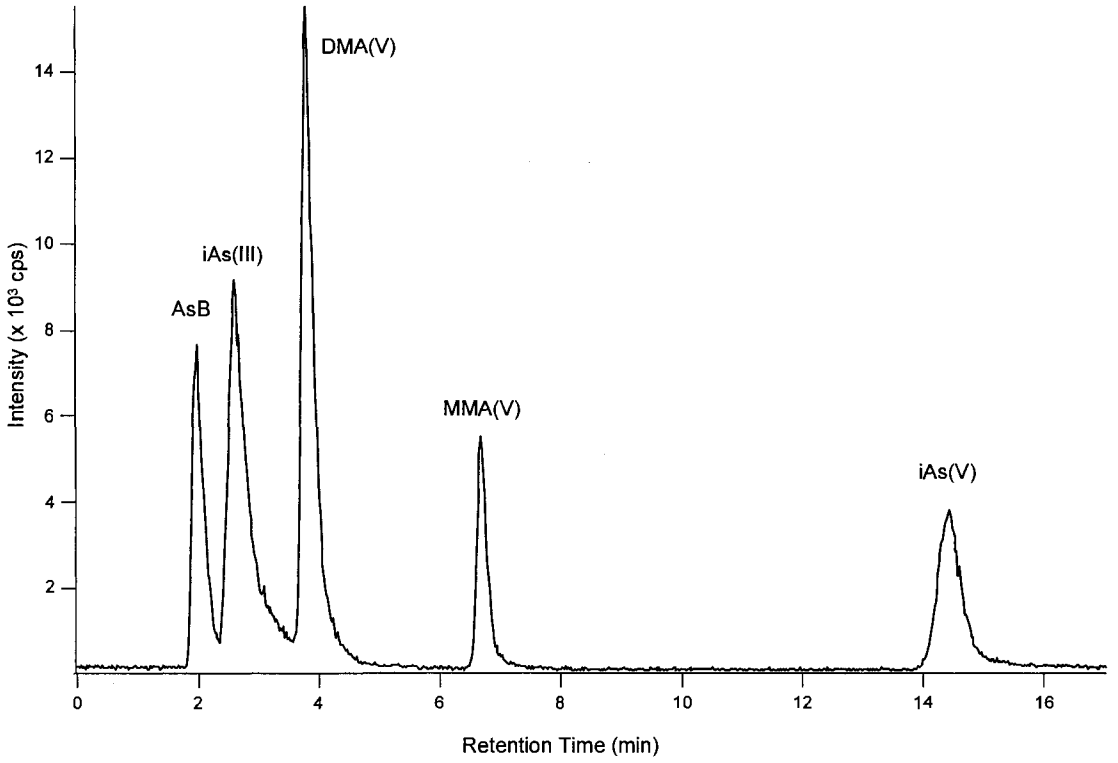


Figure 2.2 Chromatogram demonstrating the anion exchange separation of a standard solution of 10 µg/L AsB, iAs(III), DMA(V), MMA(V), and iAs(V).

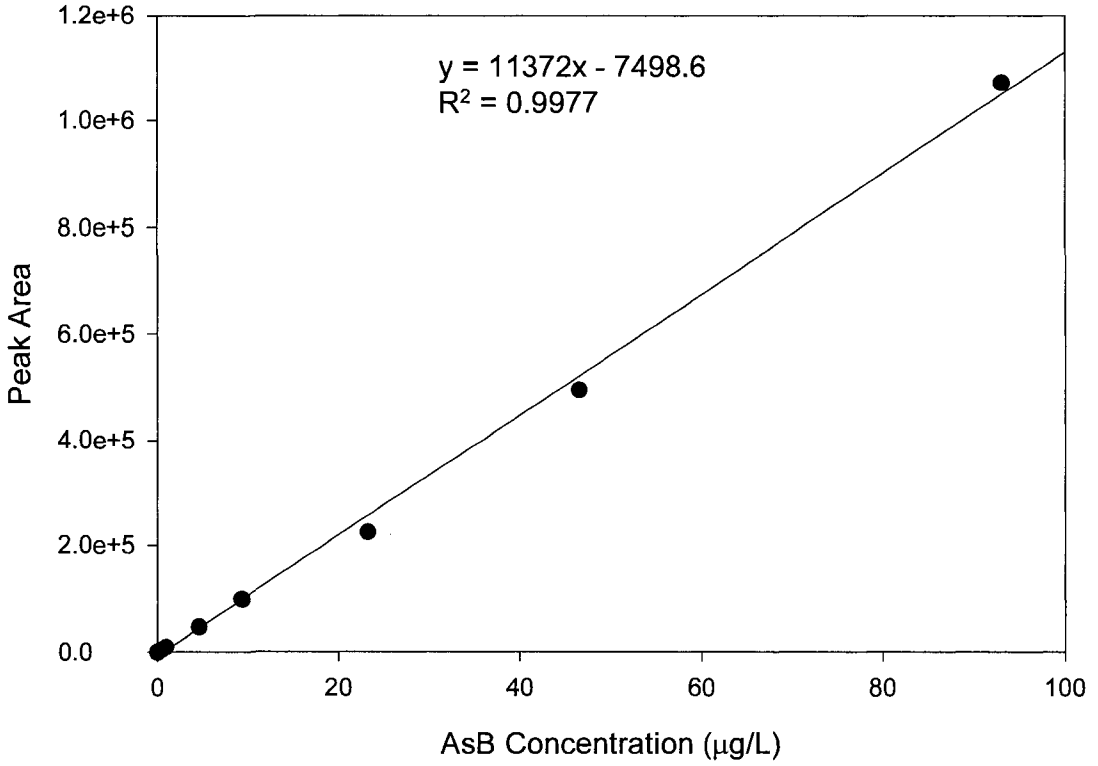


Figure 2.3 (a) Calibration curve of 0.0, 0.047, 0.093, 0.47, 0.93, 4.7, 9.3, 23, 47, and 93 µg/L AsB standards.

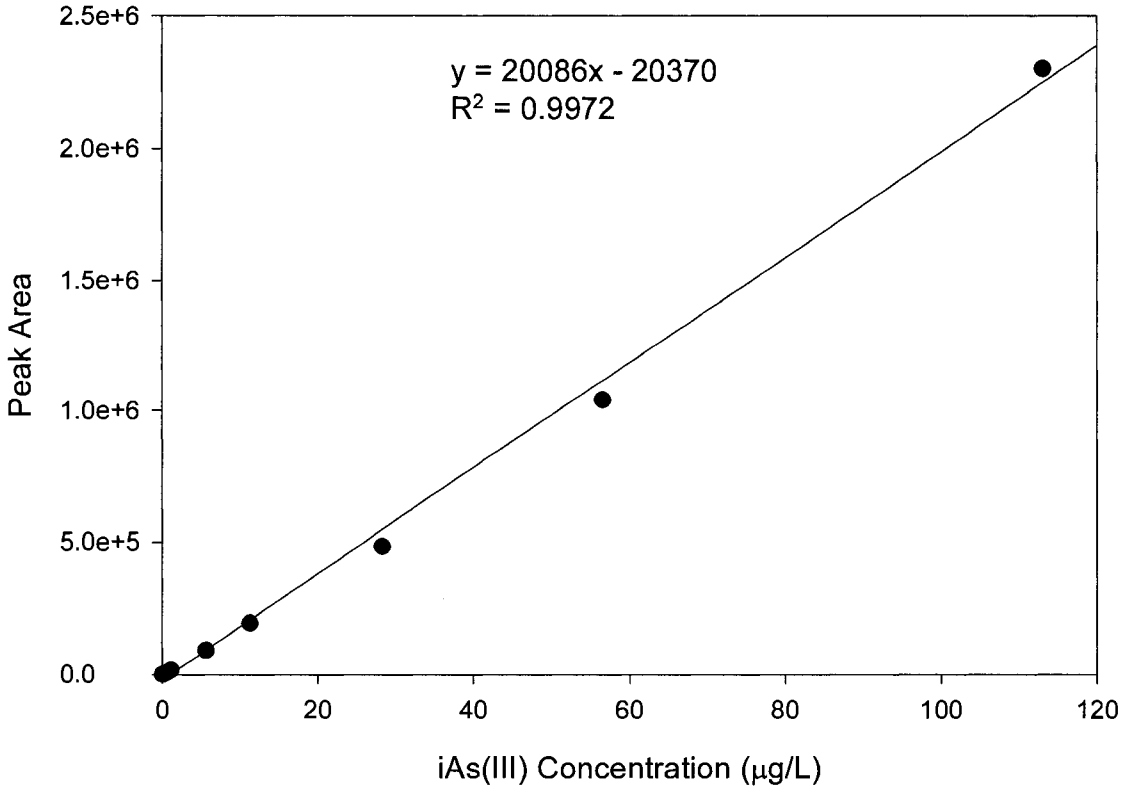


Figure 2.3 (b) Calibration curve of 0.0, 0.057, 0.11, 0.57, 1.1, 5.7, 11, 28, 57, and 113 µg/L iAs(III) standards.

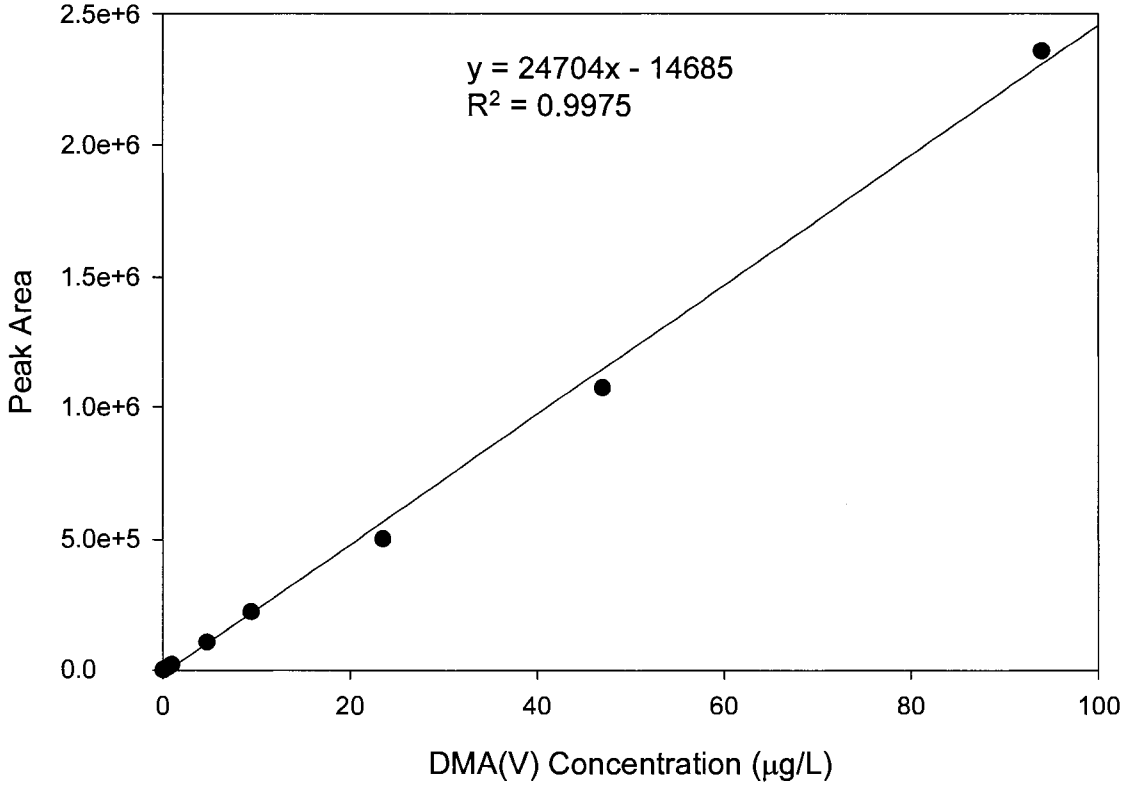


Figure 2.3 (c) Calibration curve of 0.0, 0.047, 0.094, 0.47, 0.94, 4.7, 9.4, 24, 47, and 94 µg/L DMA(V) standards.

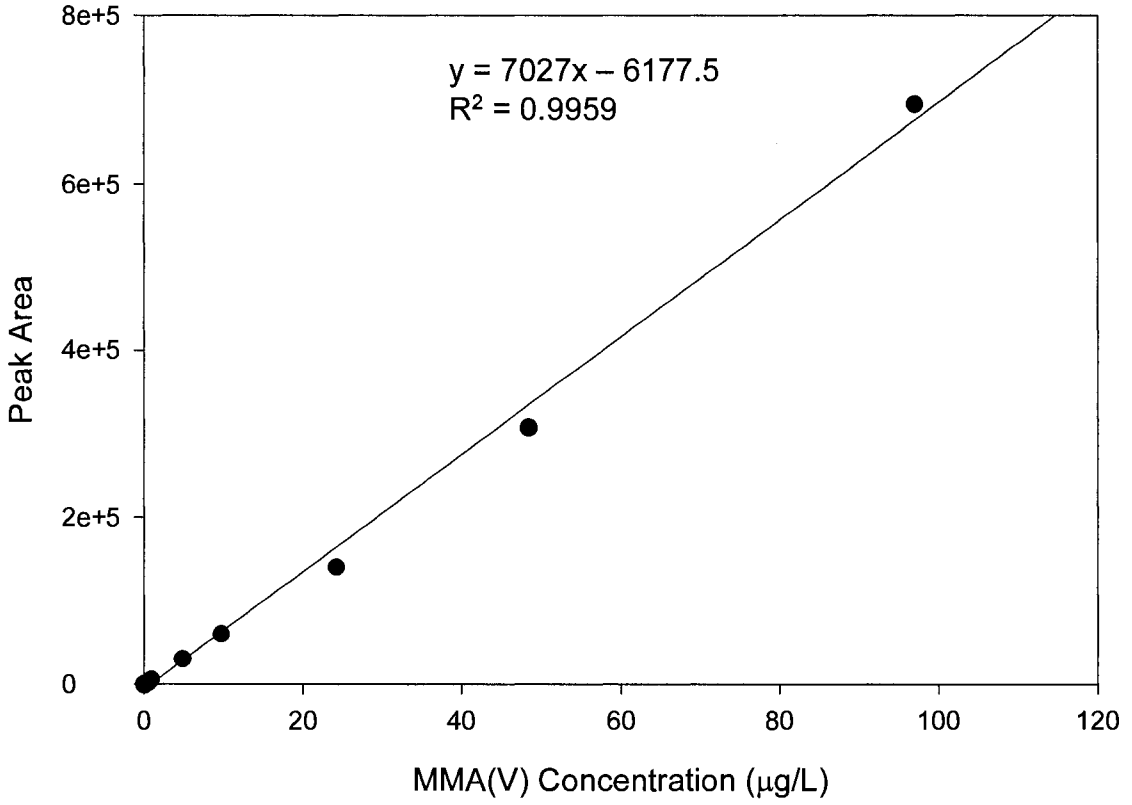


Figure 2.3 (d) Calibration curve of 0.0, 0.049, 0.097, 0.49, 0.97, 4.9, 9.7, 24, 49, and 97 µg/L MMA(V) standards.

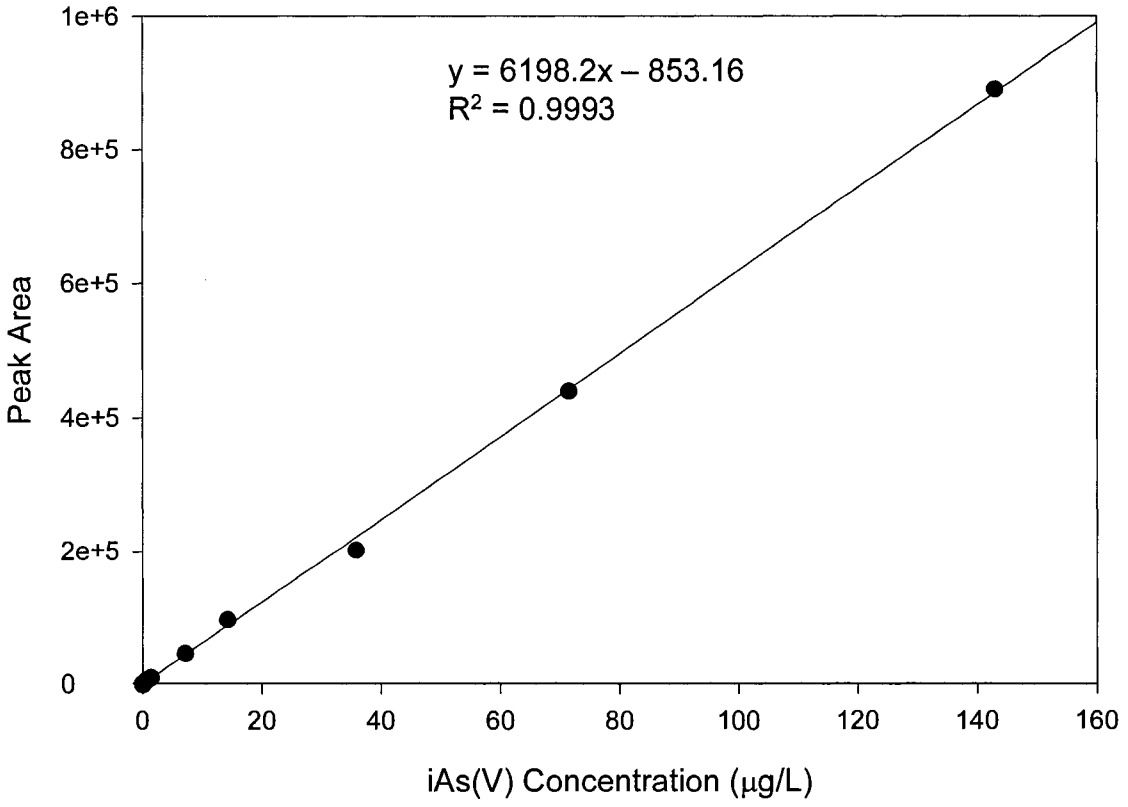


Figure 2.3 (e) Calibration curve of 0.0, 0.072, 0.14, 0.72, 1.4, 7.2, 14, 36, 72, and 143 µg/L iAs(V) standards.

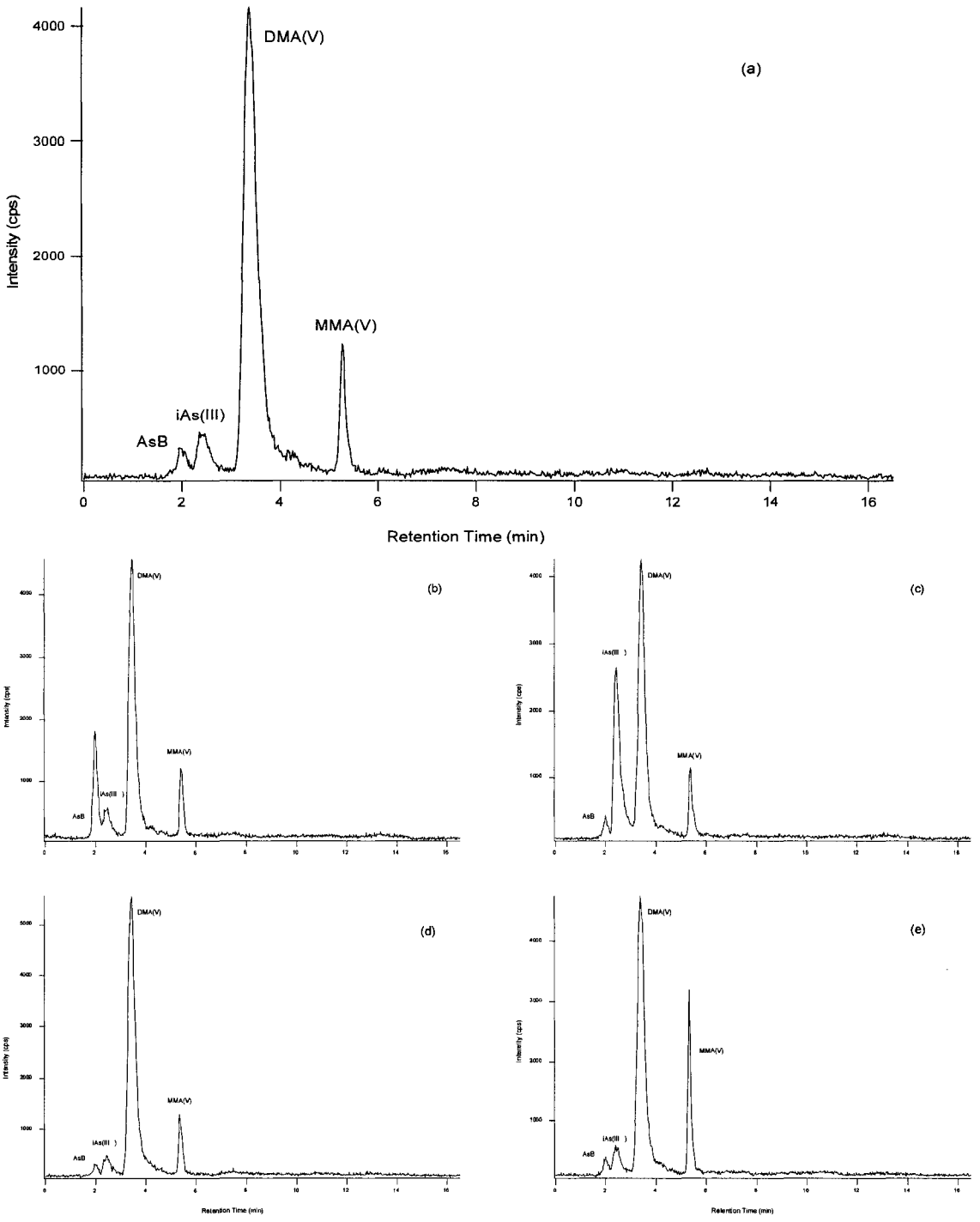


Figure 2.4 Chromatograms of arsenic in a urine sample with co-injections of standard arsenic solutions for identity confirmation. (a) Urine sample diluted 1 in 2. (b) Urine with a 5 µg/L AsB standard spike. (c) Urine with a 5 µg/L iAs(III) spike. (d) Urine with a 5 µg/L DMA(V) spike. (e) Urine with a 5 µg/L MMA(V) spike.

2.4 DISCUSSION

Anion exchange chromatography was able to separate the five arsenic species of interest [AsB, iAs(III), DMA(V), MMA(V) and iAs(V)]. Even though using ion-pair chromatography would have decreased the separation time from 16 min to 7 min, AsB and iAs(III) would co-elute (Figure 2.2). Using ion-pair chromatography and an elution pH of 5.85, both AsB and iAs(III) are neutral and do not form ion pairs [7]. Thus, they are not retained on the column and elute together. Alternatively, the pH for the ammonium bicarbonate mobile phase using anion exchange chromatography is 8.2. At this pH, AsB (pKa = 2.2) contains a positive charge while iAs(III) (pKa = 9.2) is a neutral species [7, 8]. This allows for the separation of AsB from iAs(III).

Human Urine Certified Reference Material (CRM) No. 18 was used as a measure of accuracy and precision [9]. This CRM is pooled urine collected from Japanese males who were not occupationally exposed to arsenic. The manufacturer (NIES) provides certified values for total arsenic, AsB, and DMA(V). Since arsenic speciation is of interest in the evaluation of this HPLC-ICPMS method, the results obtained for DMA(V) and AsB were compared to the certified concentrations. Over the course of 20 separate analyses of the CRM, the measured value of AsB was 73.7 ± 8.2 µg/L (mean ± 2 SD), while the certified value provided by NIES was 69 ± 12 µg/L. The measured concentration of DMA(V) was 36.9 ± 2.9 µg/L. This is also in accordance with NIES' certified value of 36 ± 9 µg/L. Based on these results, the coefficient of variation (CV) for AsB analysis is 11% and for DMA(V) analysis is 8%. These CVs are acceptable

in that they do not exceed the recommended 15% [10]. These results come from multiple days of testing. To determine the within-run variability, the CRM was also repeatedly analyzed five times. The mean concentration obtained for AsB was 69.0 ± 2.7 µg/L and for DMA(V) was 36.0 ± 1.3 µg/L. The % CV for the analysis of AsB and DMA(V) was 4 % for both species.

The detection limits for arsenic species using an HPLC-ICPMS method have been reported to be between 0.2-2.0 µg/L [8, 11, 12]. Presently the detection limit was determined based on the analysis of standard solutions of a mixture of AsB, iAs(III), DMA(V), MMA(V), and iAs(V) at concentrations of 0.0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, and 100 µg/L. The linear range was confirmed between these concentrations tested, as the correlation coefficient for each calibration curve was >0.99. The lowest concentration that produces a detectable response that is three times the level of baseline is 0.05 µg/L for DMA(V), 0.09 µg/L for AsB, and 0.1 µg/L for iAs(III), MMA(V), and iAs(V). The limit of quantification was then based on the detector response that was five times the blank response [10].

Arsenic stability in urine was assessed in several prior studies [13-16]. Feldmann *et al.* (1999) concluded that storage at 4 or -20 °C without the addition of any acid or other preservatives was most appropriate for urine samples [13]. Most urine samples showed minimal changes in arsenic speciation following storage at -20 °C for up to eight months. Similarly, Chen *et al.* (2002) concluded that the arsenic species and concentration in urine remained stable when stored at -20 °C for six months. Thus, the urine samples for our work were stored at -20

or -50 °C for up to eight months. Larsen *et al.* (1993) noted the oxidation of iAs(III) to iAs(V) when iAs(III) standards were prepared in a more complex matrix such as urine [15]. However, in our study this phenomenon was not observed as iAs(V) was not detected in the majority of the samples.

The sample matrix of urine is complex, with a high ionic content that could interfere with arsenic separation [17, 18]. Figure 2.5(a) is a chromatogram of a solution of 10 µg/L arsenic standards. Figure 2.5(b) shows a chromatogram of the separation of arsenic species in a urine sample. Because of other solutes present in the urine, there was an effect on the retention time of the arsenic compounds. Typically, a higher ionic strength reduces the retention time. This was more pronounced in the later eluting species, DMA(V) and MMA(V). Compared to the arsenic standards prepared in deionized water, DMA(V) and MMA(V) eluted earlier when present in the undiluted urine. The addition of a known concentration of a standard solution of each of these species confirmed their identity when the standards eluted with the species in question [Figure 2.5(c)].

Dilution of the urine sample can alleviate this matrix effect [8]. The chromatogram in Figure 2.5(d) demonstrates that the retention times of the arsenic species in the sample were identical to those for the arsenic species in the standard solution [Figure 2.5(a)]. From the samples analyzed in this study, the minimum dilution required ranges from 1 in 2 to 1 in 10.

A limitation of the work here was that only one level of control was used for the measurement of the accuracy of the method. For the wide dynamic range

of arsenic concentrations that were analyzed (0.1–100 µg/L), arsenic concentrations from multiple controls should also have encompassed this range. Dilutions of the NIES' CRM of human urine were prepared instead of analyzing another control.

Although HPLC-ICPMS is a sensitive and robust method for arsenic quantification, there are still limitations. In samples with very low concentrations of arsenic, the dilution of urine samples required for the reduction of the sample matrix effects could potentially decrease the arsenic concentrations to below the detection limits. While ICMPS detects the presence of various arsenic compounds, it provides no information to the identity of each species based on their structural information. Another technique such as electrospray ionization mass spectrometry is required for obtaining structural information when attempting to identify a novel arsenic compound. However, for the purposes of quantifying arsenic metabolites in human urine, HPLC-ICPMS is a robust, sensitive, reproducible, and reliable method.

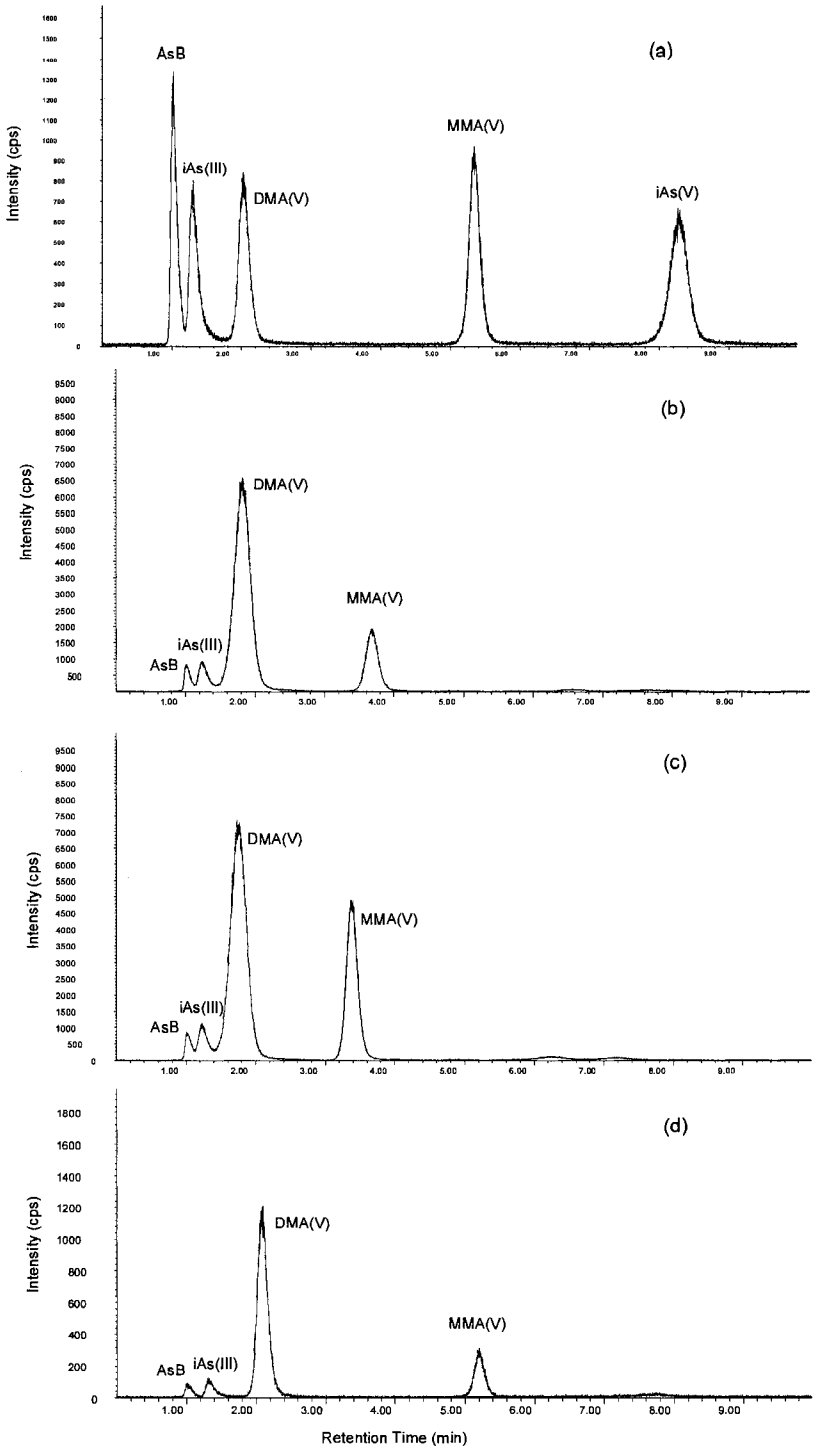


Figure 2.5 Chromatograms demonstrating the shift in retention time due to the urine matrix. (a) A 10 µg/L standard solution of AsB, iAs(III), DMA(V), MMA(V), and iAs(V). (b) A urine sample with MMA eluting more quickly than in the standard solution. (c) Same urine sample with 40 µg/L MMA spike to confirm MMA peak. (d) Urine sample after 1 in 10 dilution with deionized water.

2.5 CONCLUSIONS

The HPLC-ICPMS method is the preferred technique for the separation and detection of arsenic species in various sample types due to its sensitivity, versatility, and reliability

The HPLC-ICPMS method is useful for the purpose of determining the most common species of interest including AsB, iAs(III), DMA(V), MMA(V), and iAs(V) [4, 6-8, 11, 17-19]. Anion exchange chromatography is preferred over ion-pairing since it allows for the successful separation of AsB and the arsenic metabolites. The ICPMS detection method is sensitive enough to offer detection limits of 0.05 µg/L for DMA(V) and 0.09 µg/L for AsB, and 0.1 µg/L for iAs(III), MMA(V), and iAs(V). The availability of dynamic reaction cell technology allows for the monitoring of $^{75}As^{16}O^{+}$ at m/z = 91, rather than 75, thus preventing interference from the formation of $^{40}Ar^{35}Cl^{+}$. For the purposes of quantifying the previously mentioned arsenic species in human urine, HPLC-ICPMS is a suitable method.

2.6 REFERENCES

[1] Kaise T, Watanabe S, Itoh K. The Acute Toxicity of Arsenobetaine. Chemosphere. 1985;14(9):1327-32.

[2] Kales SN, Huyck KL, Goldman RH. Elevated urine arsenic: Un-speciated results lead to unnecessary concern and further evaluations. J Anal Toxicol. 2006 Mar;30(2):80-5.

[3] Francesconi KA, Kuehnelt D. Determination of arsenic species: A critical review of methods and applications, 2000-2003. Analyst. 2004;129(5):373-95.

[4] Gong ZL, Lu XF, Ma MS, Watt C, Le XC. Arsenic speciation analysis. Talanta. 2002 Aug;58(1):77-96.

[5] PerkinElmer. Speciation of Five Arsenic Compounds in Urine by HPLC/ICP-MS. 2003 [cited 2008 July 10]; Available from: http://las.perkinelmer.com/content/applicationnotes/app_hplcicp-msspeciationfivearseniccompounds.pdf

[6] Sloth JJ, Larsen EH, Julshamn K. Selective arsenic speciation analysis of human urine reference materials using gradient elution ion-exchange HPLC-ICP-MS. J Anal At Spectrom. 2004;19(8):973-8.

[7] Larsen EH, Hansen SH. Separation of arsenic species by ion-pair and ion exchange high performance liquid chromatography. Mikrochim Acta. 1992;109(1):47-51.

[8] Ritsema R, Dukan L, Navarro T, van Leeuwen W, Oliveira N, Wolfs P, et al. Speciation of arsenic compounds in urine by LC-ICP MS. Appl Organomet Chem. 1998;12(8-9):591-9.

[9] Yoshinaga J, Chatterjee A, Shibata Y, Morita M, Edmonds JS. Human urine certified reference material for arsenic speciation. Clin Chem. 2000 Nov;46(11):1781-6.

[10] Food and Drug Administration/Center for Drug Evaluation and Research. Guidance for Industry - Bioanalytical Method Validation. 2001.

[11] Londesborough S, Mattusch J, Wennrich R. Separation of organic and inorganic arsenic species by HPLC-ICP-MS. Anal Bioanal Chem. 1999;363(5):577-81.

[12] Le XC, Lu XF, Ma MS, Cullen WR, Aposhian HV, Zheng BS. Speciation of key arsenic metabolic intermediates in human urine. Anal Chem. 2000 Nov;72(21):5172-7.

[13] Feldmann J, Lai VWM, Cullen WR, Ma MS, Lu XF, Le XC. Sample preparation and storage can change arsenic speciation in human urine. Clin Chem. 1999 Nov;45(11):1988-97.

[14] Palacios MA, Gomez M, Camara C, Lopez MA. Stability studies of arsenate, monomethylarsonate, dimethylarsinate, arsenobetaine and arsenocholine in deionized water, urine and clean-up dry residue from urine samples and determination by liquid chromatography with microwave-assisted oxidation-hydride generation atomic absorption spectrometric detection. Anal Chim Acta. 1997 Mar;340(1-3):209-20.

[15] Larsen EH, Pritzl G, Hansen SH. Speciation of 8 Arsenic Compounds in Human Urine by High-Performance Liquid-Chromatography with Inductively-

Coupled Plasma-Mass Spectrometric Detection Using Antimonate for Internal Chromatographic Standardization. J Anal At Spectrom. 1993 Jun;8(4):557-63.

[16] Chen YC, Amarasiriwardena CJ, Hsueh YM, Christiani DC. Stability of arsenic species and insoluble arsenic in human urine. Cancer Epidemiol Biomark Prev. 2002 Nov;11(11):1427-33.

[17] Xie RM, Johnson W, Spayd S, Hall GS, Buckley B. Arsenic speciation analysis of human urine using ion exchange chromatography coupled to inductively coupled plasma mass spectrometry. Anal Chim Acta. 2006 Sep;578(2):186-94.

[18] Lintschinger J, Schramel P, Hatalak-Rauscher A, Wendler I, Michalke B. A new method for the analysis of arsenic species in urine by using HPLC-ICP-MS. Fresenius' J Anal Chem. 1998;362(3):313-8.

[19] Madsen AD, Goessler W, Pedersen SN, Francesconi KA. Characterization of an algal extract by HPLC-ICP-MS and LC-electrospray MS for use in arsenosugar speciation studies. J Anal At Spectrom. 2000;15(6):657-62.

CHAPTER 3

ARSENIC SPECIATION IN URINE AND SALIVA FOLLOWING CONTROLLED SEAFOOD INGESTION

3.1 INTRODUCTION

Assessment of human exposure to arsenic has been performed in many sample types including blood, urine, hair, and fingernails [1-3]. Traditionally, urine has been the specimen of choice for assessing recent exposure to arsenic. This includes exposure to arsenic from food and water sources, as well as occupational settings. While the half-life of arsenic in blood is between 1–3 hours, the half-life in urine is between 2–3 days [4-7]. Hair and fingernails reflect past and long-term exposure to arsenic. Attempts to determine arsenic levels in other biological fluids such as bile, breast milk, and saliva have been reported [8-11]. However, both bile and breast milk may be more difficult and invasive to collect as well as being more complicated to analyze than urine. Saliva however, is readily available and more convenient to collect.

Saliva is released for the purposes of maintaining oral hygiene, food digestion, and protection of the oral mucosa [12]. It is formed though ultrafiltration of the plasma, passive diffusion, active transport, and/or pinocytosis [13]. It is similar in electrolyte and protein content, and osmolality to plasma. Other plasma components of blood can also potentially be released into saliva. Several studies have already investigated the applicability of saliva for other purposes such as diagnosis of viral infections [14, 15], hormone studies [16],

drugs of abuse testing [17], therapeutic drug monitoring [18], and biomonitoring of environmental and occupational exposure to various chemicals [19-21]. These studies have demonstrated strong correlations between the analyte concentrations in plasma and saliva.

To date, only one study has demonstrated the utility of arsenic speciation in human saliva as a method for monitoring arsenic exposure [11]. This group analyzed urine and saliva from volunteers exposed to low levels (< 5 µg/L) of arsenic in drinking water as well as people exposed to concentrations of up to 826 µg/L in drinking water. They detected the presence of arsenite [iAs(III)], dimethylarsinic acid [DMA(V)], monomethylarsonic acid [MMA(V)] and arsenate [iAs(V)] in saliva. Arsenobetaine (AsB) is commonly found in urine as a result of seafood consumption. It is a relatively non-toxic form of arsenic [22] and its presence in urine interferes with total arsenic analysis [23]. The previous study [11] did not measure AsB in saliva. It is not known whether AsB could be present in saliva.

The objective of this study was to compare the arsenic speciation patterns in urine and saliva following controlled seafood consumption, thus determining the potential utility of saliva as an alternative sample type to urine for arsenic biomonitoring. The hypothesis of this study was that saliva would be a suitable biological sample for the determination of biomarkers of arsenic exposure. To test this hypothesis, speciation of arsenic will be compared in urine and saliva samples collected from volunteers following a controlled exposure to arsenic from a single seafood meal.

3.2 MATERIALS AND METHODS

Five healthy participants (three female and two male) between 22 and 30 years of age provided informed consent for their participation in the study. They were not known to be on any medications. The information was collected according to the University of Alberta's Health Research Ethics Board's guidelines. The volunteers refrained from ingesting seafood three days prior to this ingestion study. Live Dungeness crab from a local supermarket was prepared by steaming in white wine, water, and salt. Two participants ingested between 200 and 300 g of crab meat, another two participants ingested the same amount of crab as well as approximately 0.70 g of dried seaweed (nori). The final participant ingested 0.35 g of nori.

3.2.1 Sample Collection

Urine samples were collected into sterile collection containers (Fisher Brand; Nepean, ON) as a first morning void on the day of ingestion. Another sample was collected immediately prior to the consumption of the seafood meal. Following ingestion of the meal, samples were collected approximately once every three waking hours for the next 36 h. In total, approximately 10 urine samples from each volunteer were collected for analysis. Prior to saliva collection, participants thoroughly rinsed out their mouths with water to remove any food particles. Saliva samples (5 mL) were collected in parallel with the urine samples into 50 mL polypropylene conical tubes (Corning, Corning NY). The

method of saliva collection (stimulated or passive) was not recorded. All samples were frozen and stored at -50 °C until analysis.

3.2.2 Sample Processing

Speciation Analysis

Urine samples were placed at room temperature on the day of analysis until thawed. One mL of the defrosted sample was aspirated into 1 mL syringes (BD; Franklin Lakes, NJ) and filtered through 0.45 µm nylon membrane filters (Whatman Int.; Maidstone, UK) into the high performance liquid chromatography (HPLC) sampling vials (National Scientific; Rockwood, TN) for analysis.

Saliva samples were thawed at room temperature the day of analysis. The saliva was diluted 1 in 3 with deionized water (1 mL saliva with 2 mL deionized water) and thoroughly vortexed. The samples were then ultrasonicated for 5 minutes and centrifuged at 4000 rpm for 15 minutes. From the supernatant, 1 mL was syringe filtered as described above.

The steaming liquid from the crab preparation was thawed at room temperature the day of analysis and filtered through a 0.45 µm nylon membrane filter into HPLC vials for analysis. Dilutions were made with deionized water to ensure the arsenic concentrations were within the range of the calibration standards (0.5–100 µg/L).

Total Arsenic Analysis

Samples of saliva, urine, and the crab steaming liquid were thawed and acidified with 1% nitric acid (HNO_3). Based on the speciation results, urine samples were diluted accordingly with 1% HNO_3 to ensure that the arsenic levels of concentrated samples fall within the range of the calibration curve (1–40 µg/L).

Extraction of Arsenic Species from Crab and Nori

Duplicate samples of homogenized crab and nori were weighed and treated with 15 mL of a 1:1 mixture of methanol:deionized water to extract the arsenic species. In addition, sample blanks containing only the methanol and deionized water mixture were prepared in duplicate for analysis. The samples were then ultrasonicated for 20 min, centrifuged at 1350 rpm for 15 min, and the supernatant was removed and kept in a beaker. This procedure was repeated a total of three times. The supernatant was evaporated on a hot plate set at 40 °C until approximately 1 mL of liquid remained. The liquid was then diluted 1 in 3 with deionized water and then filtered through 0.45 µm nylon filters prior to analysis. The crab and nori residues were frozen at -50 °C until acid digestion was performed.

Acid Digestion of Food Samples for Total Arsenic Analysis

Arsenic extracted from food samples using a methanol/water mixture may have a low extraction efficiency [24]. The sum of the arsenic species from the methanol/water extraction may not be equal to the total arsenic concentration

from the acid digestion of the food. There may still be a large amount of arsenic remaining in the residue from extraction. Thus, to obtain a mass balance for the total arsenic in the food samples, the sum of the arsenic species as well as the total arsenic in the residue from the water/methanol extraction must be calculated. Acid digestion was performed on the food residue from the methanol extraction and the original food samples of crab and nori. Standard reference material (SRM) (#1566b, National Institute of Standards and Technology, Gaithersburg, MD) of freeze-dried oyster tissue was also digested and analyzed to ensure the accuracy of the results. The digestions of the food samples were performed in duplicate.

Each of the food samples and reference materials was weighed prior to acid digestion with a solution containing 3 parts concentrated sulfuric acid (H_2SO_4) to one part HNO_3. In a fume hood, 20 mL of HNO_3 was added to the respective food samples in a 100 mL beaker. H_2SO_4 (60 mL) was then gradually added to the beakers, which were then covered with a watch glass and left overnight for digestion to occur. The following day, the samples were further digested by placing the covered beakers on a 200 °C hot plate. The samples were continuously heated until the solutions became transparent. Following digestion, the acid was then evaporated on a hot plate set to 450 °C until approximately 1 mL of liquid remained. The samples were reconstituted with deionized water to either 10 mL (food) or 25 mL (SRM) and frozen at -50 °C until total arsenic analysis.

3.2.3 Sample analysis

Speciation

The arsenic species in urine and saliva were separated on an anion exchange HPLC column (PRP-X100, 5 µm, 150 x 4.1 mm; Hamilton; Reno, NV). A Perkin Elmer Series 200 autosampler was used for the duplicate injections of 50 µL of sample onto the chromatography column. The eluent used was 35 mM ammonium bicarbonate prepared in 5% methanol; pH adjusted to 8.2 using 10 % ammonium hydroxide. The separation was performed on a Perkin Elmer Series 200 high performance liquid chromatograph (HPLC). A gradient elution included the following flow rates: 0–4 min: 0.8 mL/min; 4–10 min: 1.7 mL/min; and 10–16 min: 0.8 mL/min. The separated arsenic species entered an inductively coupled plasma mass spectrometer (ICPMS) for detection (6100DRC Plus, Perkin-Elmer Sciex; Norwalk, CT).

The urine and saliva samples were analyzed along with arsenic standards which include arsenobetaine (AsB), inorganic arsenite [iAs(III)], dimethylarsinic acid [DMA(V)], monomethylarsonic acid [MMA(V)], and inorganic arsenate [iAs(V)]. AsB was obtained from Tri Chemical Laboratories (Yamanashi, Japan), iAs(III), iAs(V), and DMA(V) were obtained from Aldrich (Milwaukee, WI), and MMA(V) came from Chem Service (West Chester, PA). The standards were prepared in concentrations of 0.5, 1, 5, 10, and 100 µg/L and analyzed prior to the urine and saliva samples each day. For quality control, certified reference material of human urine (No. 18, National Institute for Environmental Studies; Tsukuba, Ibaraki, Japan) was quantified once daily prior to the analysis of the

urine and saliva samples. The results were then compared with the certified values from the manufacturer as a check on the accuracy of the method.

Total arsenic

Between 0.5 and 1 mL of sample was injected into the ICPMS (Agilent 7500cs octopole reaction system) at approximately 60 s intervals in duplicate. The octopole reaction in the ICPMS was conducted using helium gas to prevent interference from argon chloride ($^{40}Ar^{35}Cl^{+}$). The mass spectrometer detects ions based on their mass-to-charge (*m/z*) ratio. For arsenic, the *m/z* ratio is 75, which is the same for $^{40}Ar^{35}Cl^{+}$. Helium gas removes this interference through collisions with $^{40}Ar^{35}Cl^{+}$, which decreases its energy and prevents it from entering the mass analyzer [25].

Various concentrations of iAs(III) (1, 5, 10, 20, and 40 µg/L) in 1% nitric acid were prepared for the calibration of the ICPMS. A standard reference material of trace elements in natural water (#1640, National Institute of Standards and Technology; Gaithersburg, MD) was analyzed at the beginning of the sample run for to ensure the daily accuracy of the method.

3.2.4 Creatinine Measurement

The urine samples were sent to the University of Alberta Hospital Laboratory for creatinine analysis. The assay was performed using the UniCel® DxC 800 System (Beckman Coulter; Fullerton, CA). This was a

spectrophotometric method that measured the creatinine-picrate end product of the reaction between creatinine and picric acid [26].

3.2.5 Statistical Analysis

Statistical analysis of the data was performed using Intercooled STATA 10.0 (StataCorp; College Station, TX). The Student's *t*-test was used to compare the arsenic concentrations in urine and saliva prior to and following seafood ingestion. Any *p*-values less than 0.05 were considered statistically significant. The Pearson's Correlation Coefficient was used as a measure for determining the relationship between concentrations of various arsenic species in urine and saliva, and between the total arsenic and sum of arsenic species in both urine and saliva.

3.3 RESULTS

3.3.1 Arsenic in Ingested Crab

The major arsenic species in the Dungeness crab ingested was AsB (Table 3.1). In the crab steaming liquid, AsB was the predominant arsenic species with very small amounts of DMA(V) and iAs(V) present.

The volunteers in the crab group ate between 200 and 300 g of Dungeness crab meat. Assuming ingestion of 250 g of crab, participants consumed 18.2 mg of total arsenic (Table 3.2). There was no record of the crab steaming liquid being ingested, but its total arsenic concentration was 2.5 mg/L. A single participant eating nori consumed 1.0 µg of total arsenic in 0.35 g of nori.

The analysis of 0.1 g of nori showed the presence of MMA(V), DMA(V), and some AsB.

3.3.2 Total Arsenic in Urine Before and After Crab Ingestion

Each participant collected 1 or 2 pre-ingestion urine samples for their baseline arsenic levels. Prior to ingestion, the volunteers' mean total arsenic concentration was 1.7 ± 1.7 µg/L per mmol/L creatinine (range 0.3–4.0 µg/L per mmol/L creatinine) (Figure 3.1). There was a dramatic rise in total arsenic concentration as shown in the first sample collected following ingestion (2–3.5 h post-ingestion). The total arsenic concentration peaks during either the first or second sample collected after ingestion, between 4.5–7.5 h (119.3 ± 47.3 µg/L per mmol/L creatinine; range 51.8–155.9 µg/L; $p = 0.02$ vs. baseline). During the last urine samples collected between 36 and 39 h post-ingestion, the total arsenic concentration was statistically similar to the concentration of the pre-ingestion samples (8.5 ± 3.2 µg/L per mmol/L creatinine; range 5.0–12.4 µg/L creatinine; $p = 0.06$ vs. baseline).

Table 3.1 Arsenic speciation in ingested crab, crab steaming liquid, and nori. The major arsenic species in crab was AsB. In the liquid resulting from steaming of the crab, the major species was AsB, along with small quantities of DMA(V) and iAs(V). In nori, the arsenic species in order of abundance were DMA(V), MMA(V) and an unidentified species.
*n.d. = not detected

Arsenic Species	**Crab (µg/g)**	**Crab Steaming Liquid (µg/L)**	**Nori (µg/g)**
AsB	48418 ± 3473	2499 ± 11	n.d.*
iAs(III)	n.d.	n.d.	n.d.
DMA(V)	n.d.	9.5 ± 0.8	248 ± 37
MMA(V)	n.d.	n.d.	206 ± 119
iAs(V)	n.d.	21.2 ± 0.3	n.d.
Unidentified	n.d.	n.d.	8.9 ± 1.4

Table 3.2 Arsenic content in seafood obtained from the analyses of extracts and acid digestion solutions. Volunteers in the crab group ingested approximately 250 g of crab meat and a single volunteer ingested approximately 0.4 g of nori. There was no record of the amount of crab steaming liquid consumed.

Sample	Sum of Species in the Extract (amount ingested)	Total Arsenic in the Residue (amount ingested)	Sum of Arsenic in the Extract and Residue (amount ingested)	Total Arsenic from Acid Digestion (amount ingested)
Crab	12.1 mg	1.7 mg	13.8 mg	18.2 mg
Nori	0.2 µg	0.6 µg	0.8 µg	1.0 µg
Steaming Liquid	2032.3 µg/L	N/A	2032.3 µg/L	2507.4 µg/L

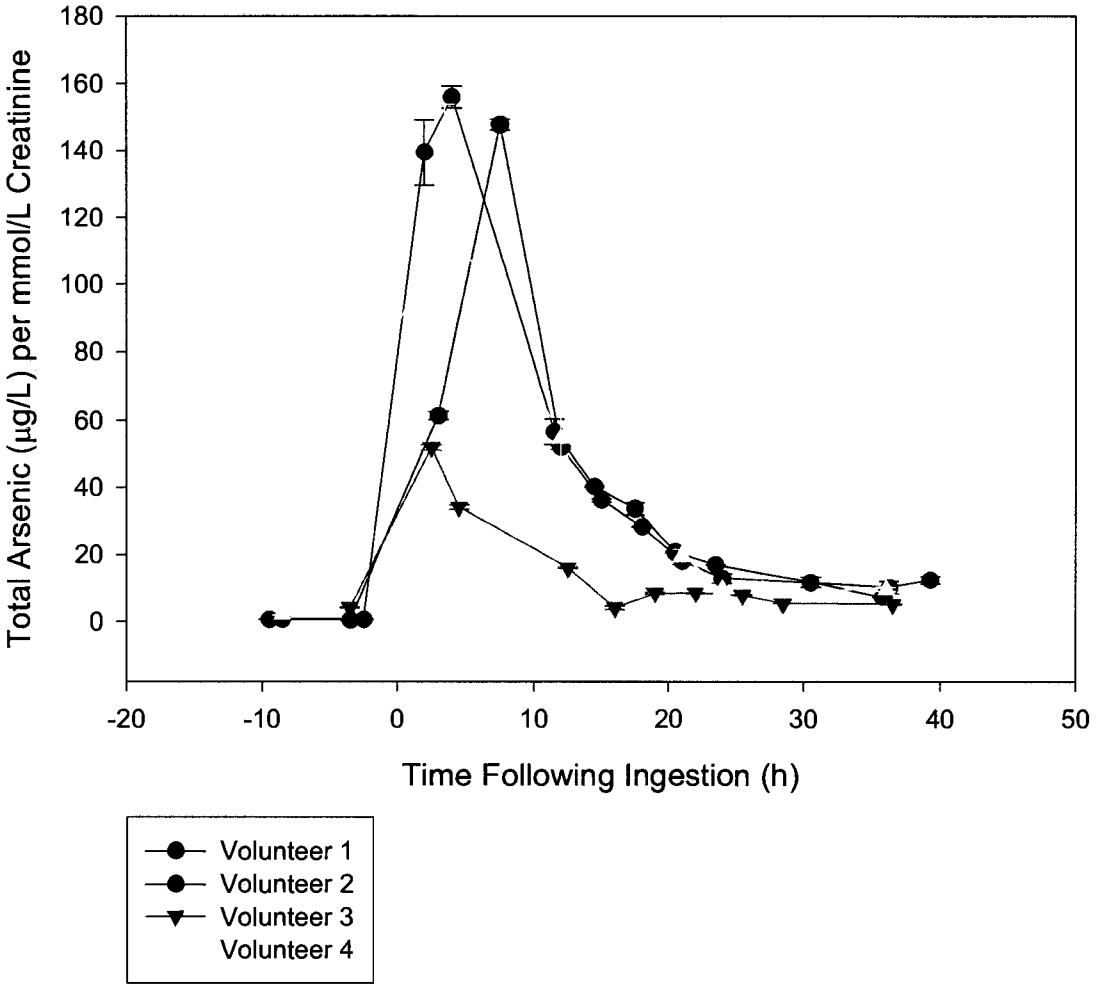


Figure 3.1 Total arsenic concentration in urine before and after crab consumption. A sharp increase in the total arsenic occurred within the first sample collected (2–3.5 h post-ingestion). The maximum total arsenic concentration appeared between 4.5–7.5 h post-ingestion. By 36–39 h post-ingestion the total arsenic in urine returned to concentrations similar to baseline.

3.3.3 Total Arsenic in Saliva Before and After Crab Ingestion

The baseline total arsenic in the participants' saliva was 0.3 ± 0.1 µg/L (range 0.2–0.3 µg/L) (Figure 3.2). Similar to urine, the total arsenic increases as soon as the first collection following crab consumption (2–3.5 h), however the peak concentration was seen anywhere from 3 to 8 h following ingestion (1.8 ± 0.7 µg/L; range 1.2–2.8 µg/L; p = 0.02 vs. baseline). Finally, during the last collection time point (36–39 h post-ingestion), the total arsenic values return to values statistically similar to baseline (0.6 µg/L ± 0.5 µg/L; range 0.2–1.2 µg/L; g/L; p = 0.27 vs. baseline).

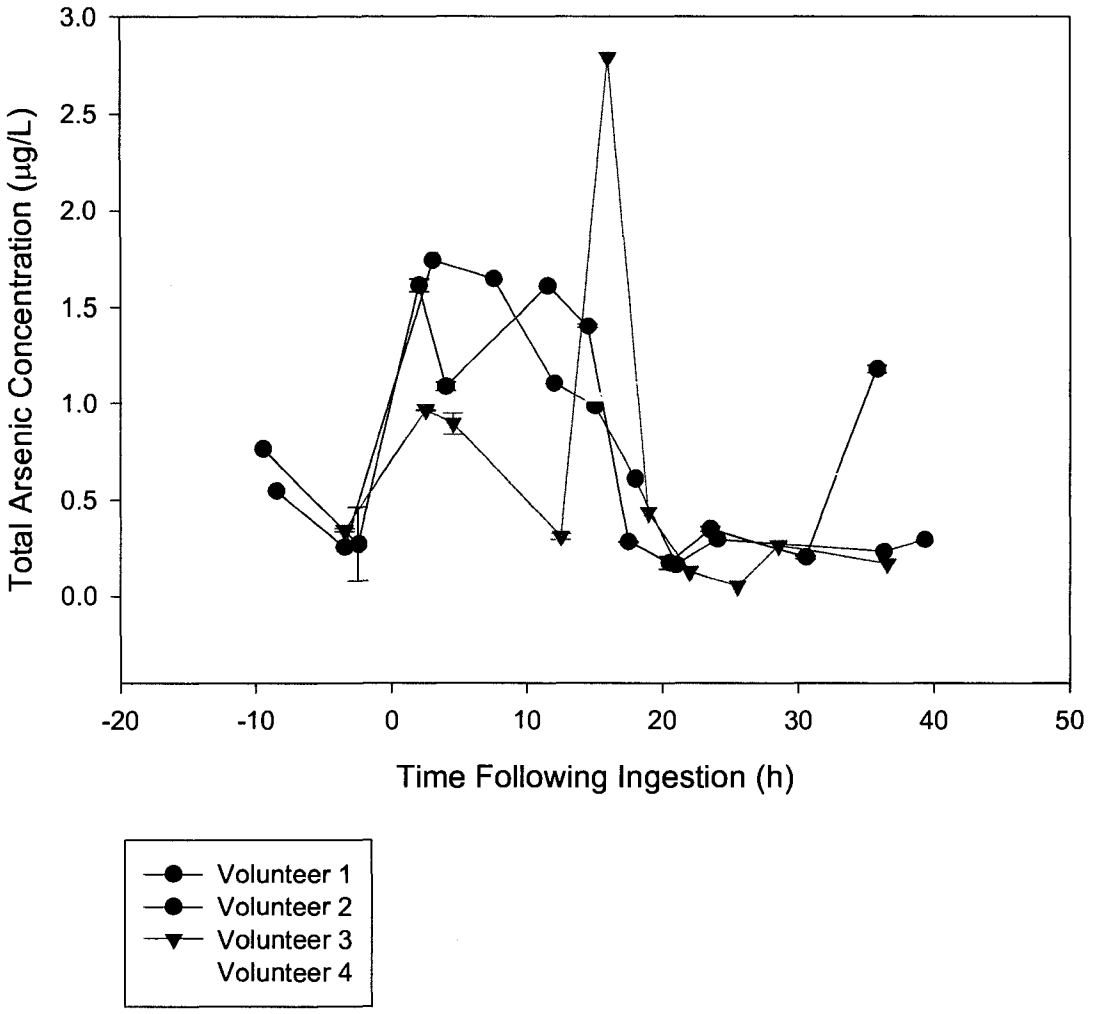


Figure 3.2 Total arsenic concentration in saliva before and after crab ingestion. An increase in the total arsenic was apparent within the first sample collected (between 2 and 3.5 h post-ingestion). The peak concentration appeared between 3 and 8 h following ingestion. There was insufficient sample from Volunteer 4 to perform total arsenic analysis in the first two saliva samples following ingestion.

3.3.4 Separation of Arsenic Species

A chromatogram of the separation of arsenic species in a 5 µg/L standard solution of AsB, iAs(III), DMA(V), MMA(V), and iAs(V) is depicted in Figure 3.3. The five arsenic species of interest were well-separated and the typical retention times of arsenic species are as follows: AsB – 1.87 min; iAs(III) – 2.50 min; DMA(V) – 3.59 min; MMA(V): 6.48 min; and iAs(V): 13.89 min.

Figures 3.4 and 3.5 show typical chromatograms from the analysis of arsenic species in urine (Figure 3.4) and saliva (Figure 3.5) samples collected from a volunteer before and after the ingestion of crab. There was a rapid increase in the arsenic concentration during the first void following crab ingestion (3 h) (Figure 3.4). That particular sample was diluted 1/100 due to the elevated concentration of AsB. The remaining urine samples (representing 15, 24, and 39 h post-crab) did not require the same degree of dilution as the sample collected at 3 h post-ingestion and were diluted 1/50. No arsenic peaks were detected in the saliva sample collected 3.5 h prior to crab ingestion (Figure 3.5). The presence of AsB is detected in the first saliva sample collected at 3 h following crab ingestion. AsB levels decrease for the following samples collected (15 and 24 h post-ingestion). In the chromatogram for the sample collected at 39 h following ingestion, DMA(V) and iAs(V) are also detected.

In both urine and saliva, the baseline samples displayed fairly small peaks relative to the post-ingestion samples. AsB was rapidly excreted into both urine and saliva as there was an increase in peak area at 3 h post-ingestion.

3.3.5 AsB in Urine Before and After Crab Ingestion

Figure 3.6 demonstrates the AsB concentration in urine following crab consumption. Similar to total arsenic, the baseline urinary AsB concentration was relatively low (0.6 ± 1.2 µg/L per mmol/L creatinine; range 0.0–2.5 µg/L creatinine). The rise in AsB appeared as early as 2 h post-ingestion. In all volunteers, the AsB concentration peaked between 2.5–4 h post-ingestion (137.0 ± 75.9 µg/L per mmol/L creatinine; range 48.1–229.0 µg/L per mmol/L creatinine; p = 0.04 vs. baseline). 36–39 h following crab consumption, the AsB concentration was reduced to values not significantly different from baseline (9.1 ± 5.3 µg/L per mmol/L creatinine; range 2.9–14.9 µg/L per mmol/L creatinine; p = 0.08).

3.3.6 AsB in Saliva Before and After Crab Ingestion

AsB in saliva followed a similar excretion pattern (Figure 3.7). The baseline concentration of AsB in saliva was 0.1 ± 0.2 µg/L; range 0.0–0.5 µg/L. Its concentration also peaked between 2.5 and 4 h following crab ingestion (0.8 ± 0.3 µg/L; range 0.6–1.1 µg/L; p = 0.02 vs. baseline). At the last sample collection point in each volunteer, the salivary AsB returned to a concentration similar to baseline (0.1 ± 0.2 µg/L; range 0.0–0.4 µg/L; p = 0.97 vs. baseline).

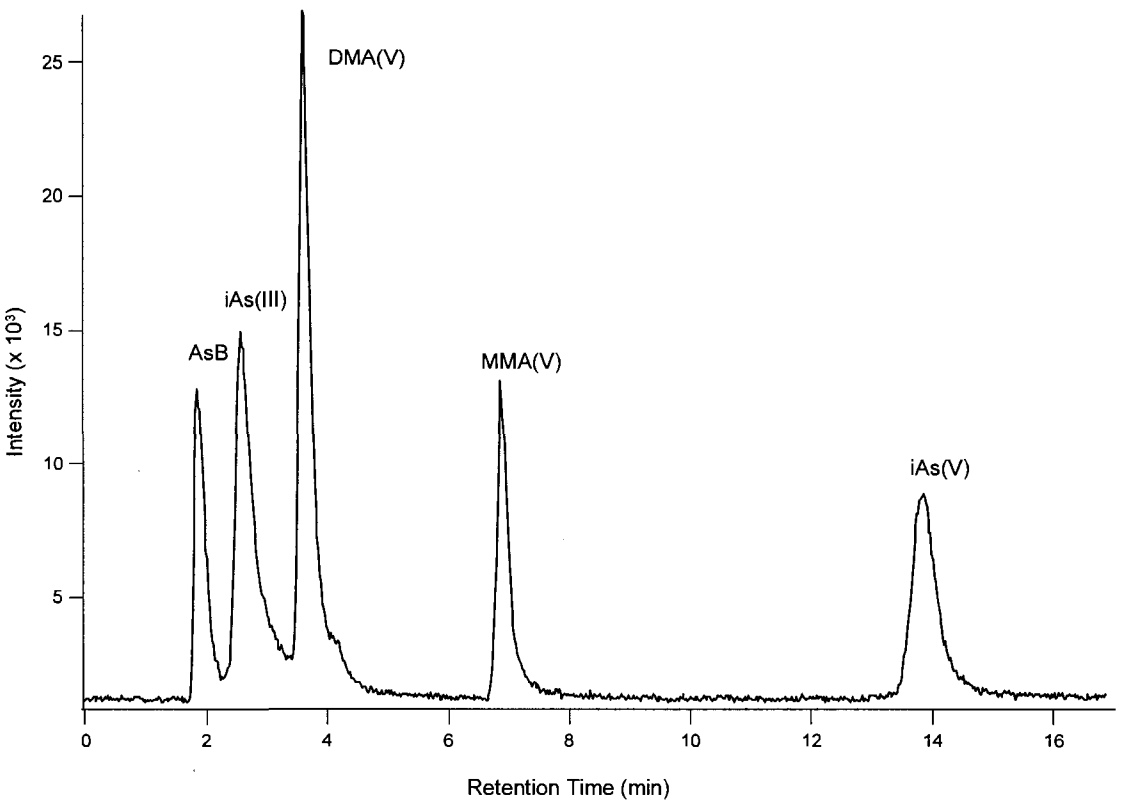


Figure 3.3 Chromatogram displaying the separation of arsenic species in a 5 µg/L standard solution containing AsB, iAs(III), DMA(V), MMA(V), and iAs(V). The chromatographic separation was performed using a Hamilton PRP-X100 column, a 35 mM ammonium bicarbonate mobile phase prepared in 5% methanol, pH 8.2. The flow rate was 0.8 mL/min from 0–4 min; 1.7 mL/min from 4–10 min; and 0.8 mL/min from 10–17 min.

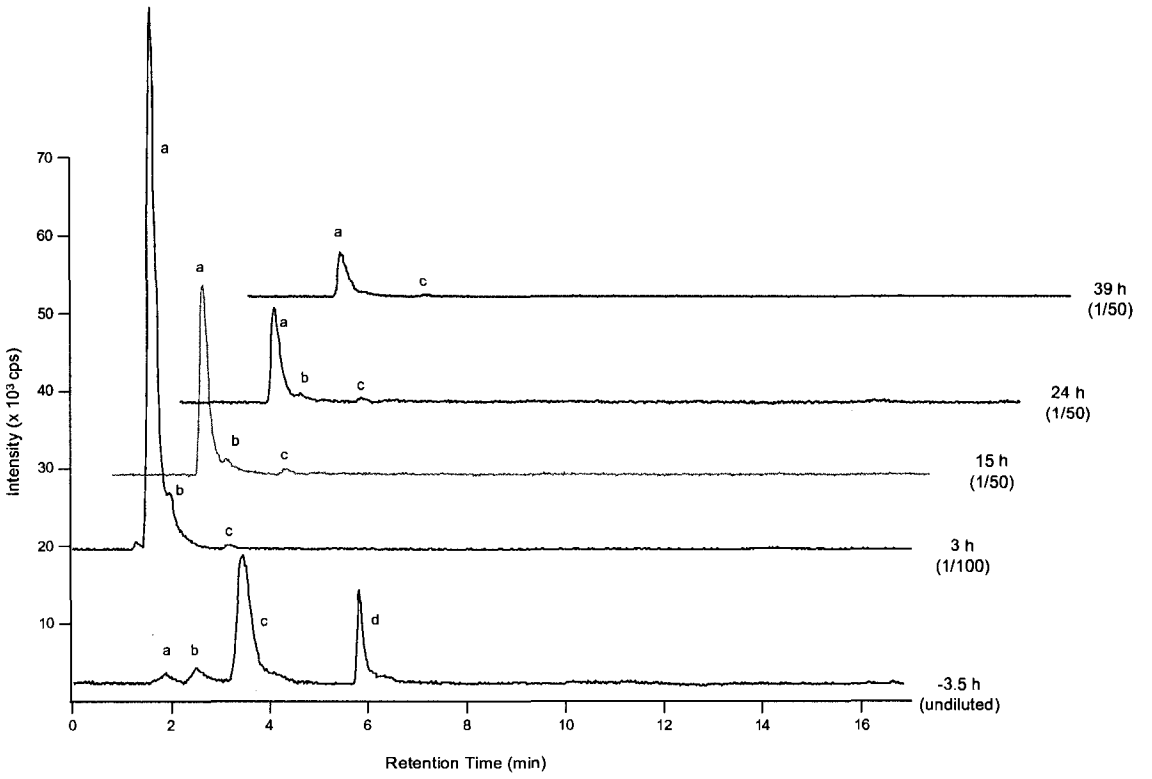


Figure 3.4 Chromatograms of arsenic species in urine of one volunteer prior to and following crab ingestion. Peak (a) corresponds to AsB, (b) iAs(III), (c) DMA(V) and (d) MMA(V). The sample collected prior to crab ingestion did not require dilution prior to analysis. The sample collected 3 h post-ingestion required 1/100 dilution in deionized water and the samples collected at 15, 24, and 39 h were diluted 1/50. The same chromatographic conditions as Figure 3.4 were employed.

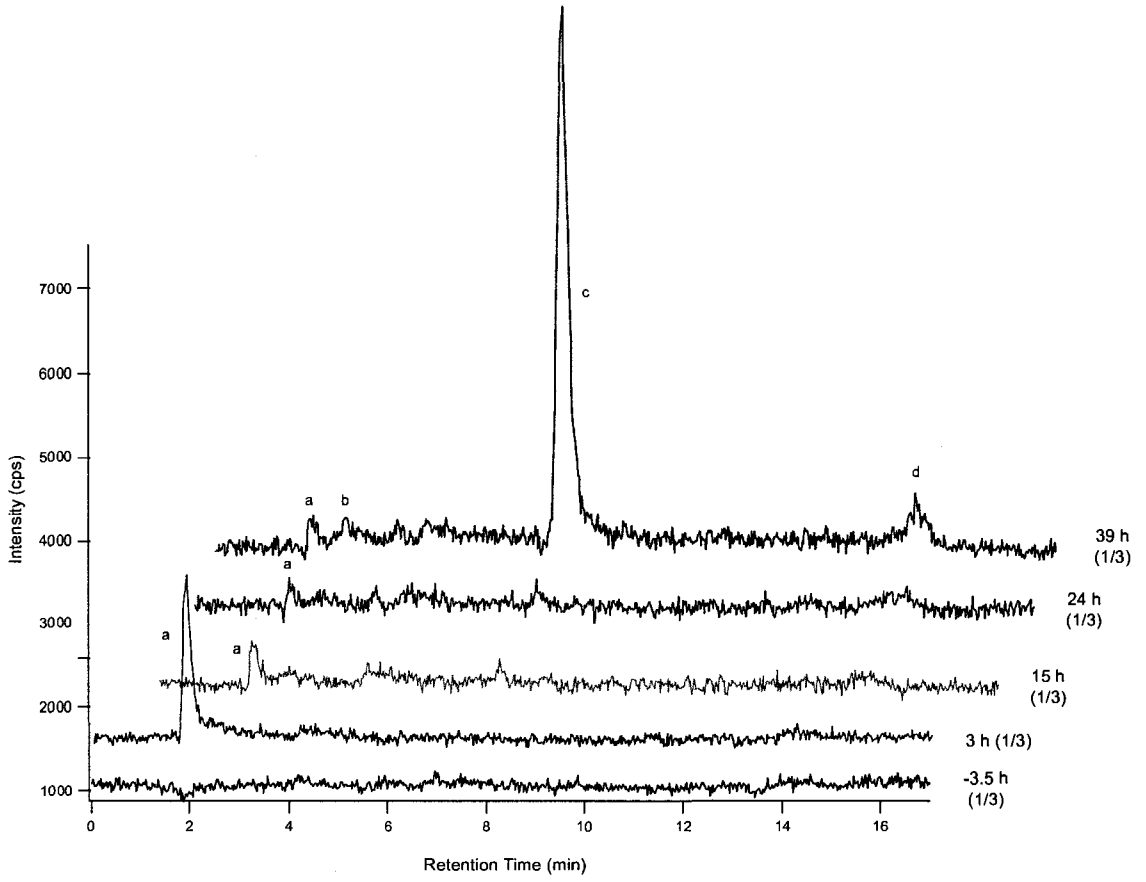


Figure 3.5 Chromatograms of arsenic species in saliva of one volunteer prior to and following crab ingestion. Peak (a) corresponds to AsB, (b) iAs(III), (c) MMA(V) and (d) iAs(V). All samples were diluted 1/3 during sample preparation. The same chromatographic conditions as Figure 3.4 were employed.

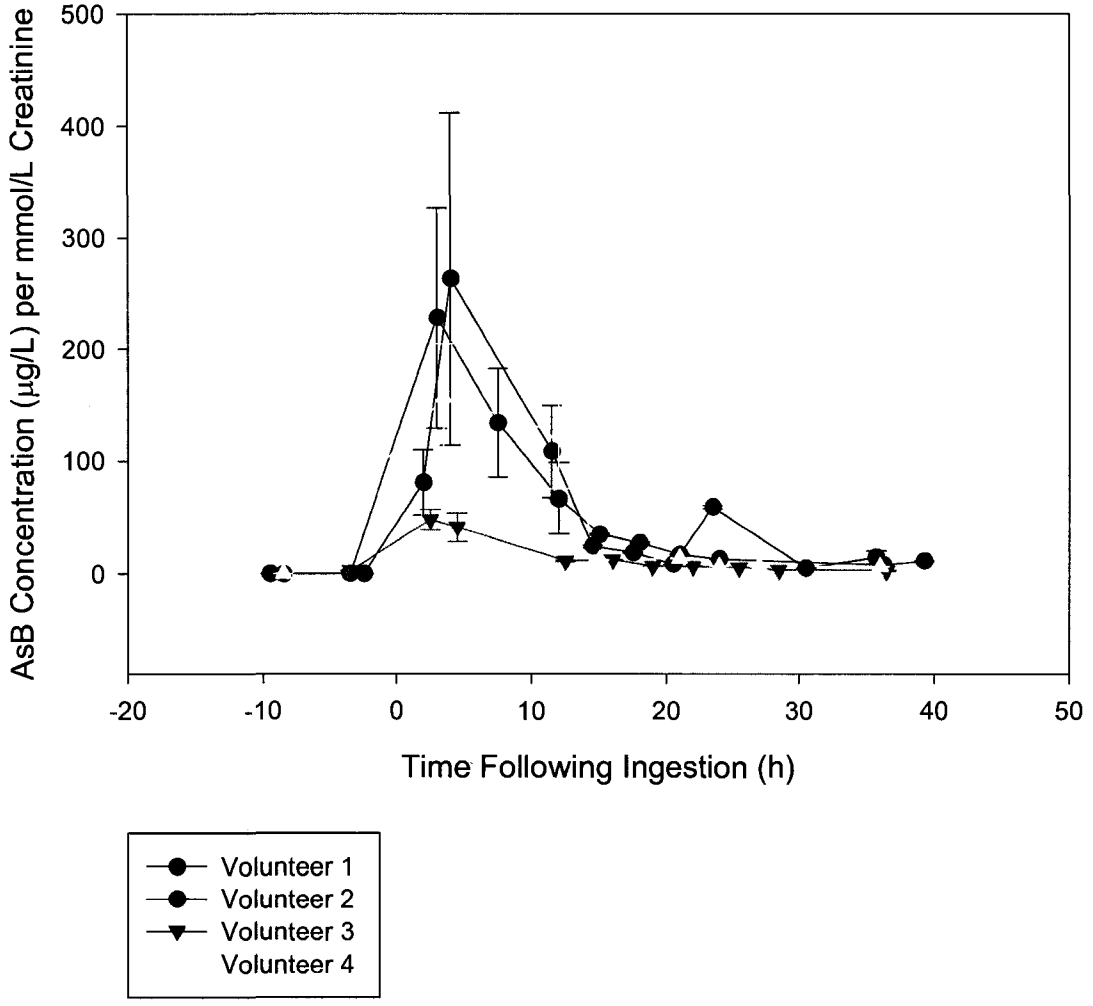


Figure 3.6 AsB concentration in urine prior to and following crab consumption. An increase in AsB appeared as early as 2 h post-crab ingestion. The AsB concentration peaked from 2.5–4 h following the crab meal. At 36–39 h post-ingestion, the AsB concentrations were similar to baseline.

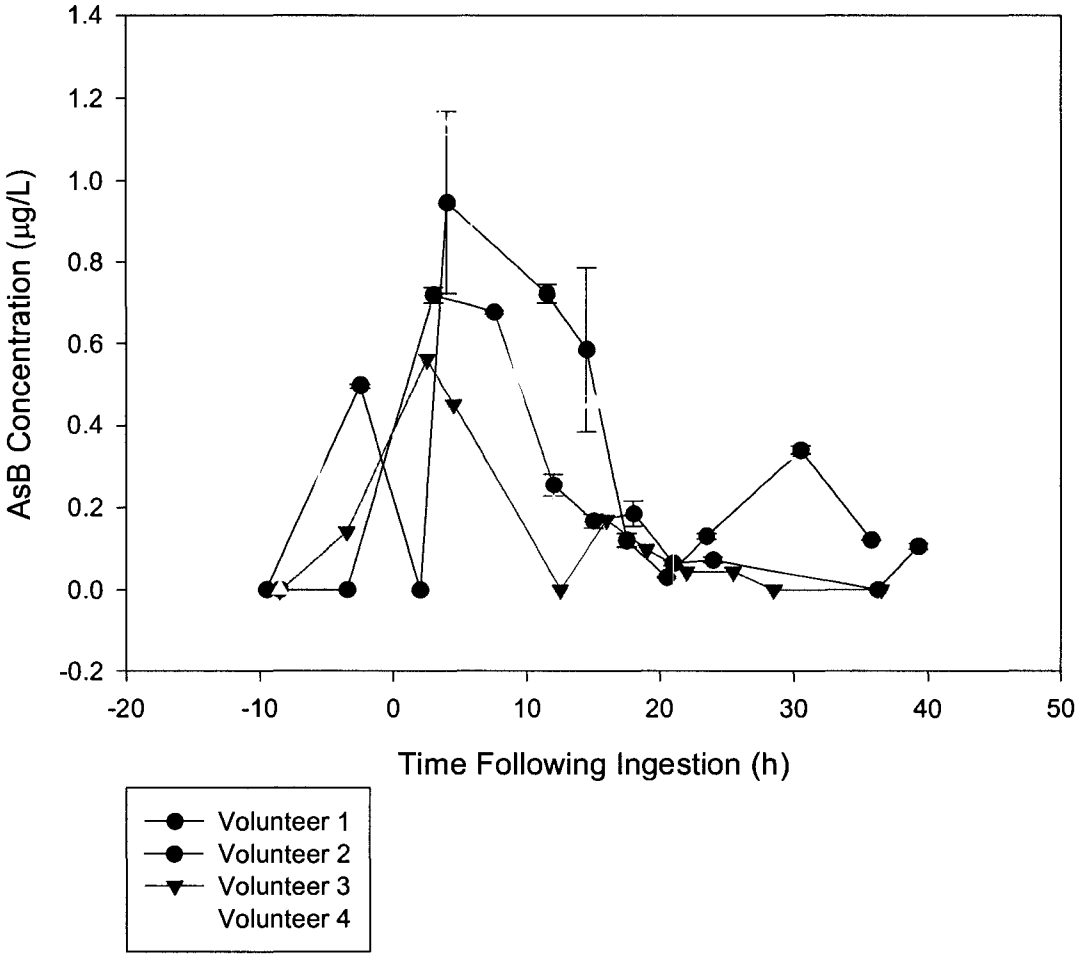


Figure 3.7 AsB concentration in saliva before and after crab consumption. The maximum AsB concentration was seen between 2.5 and 4 h after the crab meal. The last sample collected from each volunteer contained AsB at levels similar to baseline.

3.3.7 Other Arsenic Species in Urine Before and After Crab Ingestion

Arsenite was the only other species to demonstrate a significant increase at its peak compared to baseline (baseline: 0.1 ± 0.1 µg/L per mmol/L creatinine; range 0.0–0.2 µg/L per mmol/L creatinine; peak: 5.9 ± 2.6 µg/L per mmol/L creatinine; range 2.8–8.7 µg/L per mmol/L creatinine; p = 0.02 vs. baseline) (Figure 3.8). Most samples showed an increase in iAs(III) in the first urine sample collected after crab ingestion. Some samples collected at later time points exhibited another rise in iAs(III) levels, but to a lower extent as the initial peak level.

While DMA(V) displayed no considerable difference between the baseline and peak concentrations in urine, the difference between baseline and peak concentrations in saliva was significant (baseline: not detected; peak: 0.5 ± 0.3 µg/L; range 0.2–0.8 µg/L; p = 0.04 vs. baseline) (Figure 3.9).

3.3.8 Arsenic Species in Urine Before and After Nori Ingestion

One individual ate 0.35 g of nori and the arsenic species excreted in their urine are represented in Figure 3.10. There was minor fluctuation in the concentrations of most arsenic species over all time points. DMA(V) was the only species that shows greater fluctuations in concentration over time, however the range is relatively small (0.1–0.5 µg/L). Two arsenic species, DMA(V) and iAs(V) were detected in saliva following nori ingestion only at 15.5 h post-ingestion (Figure 3.11).

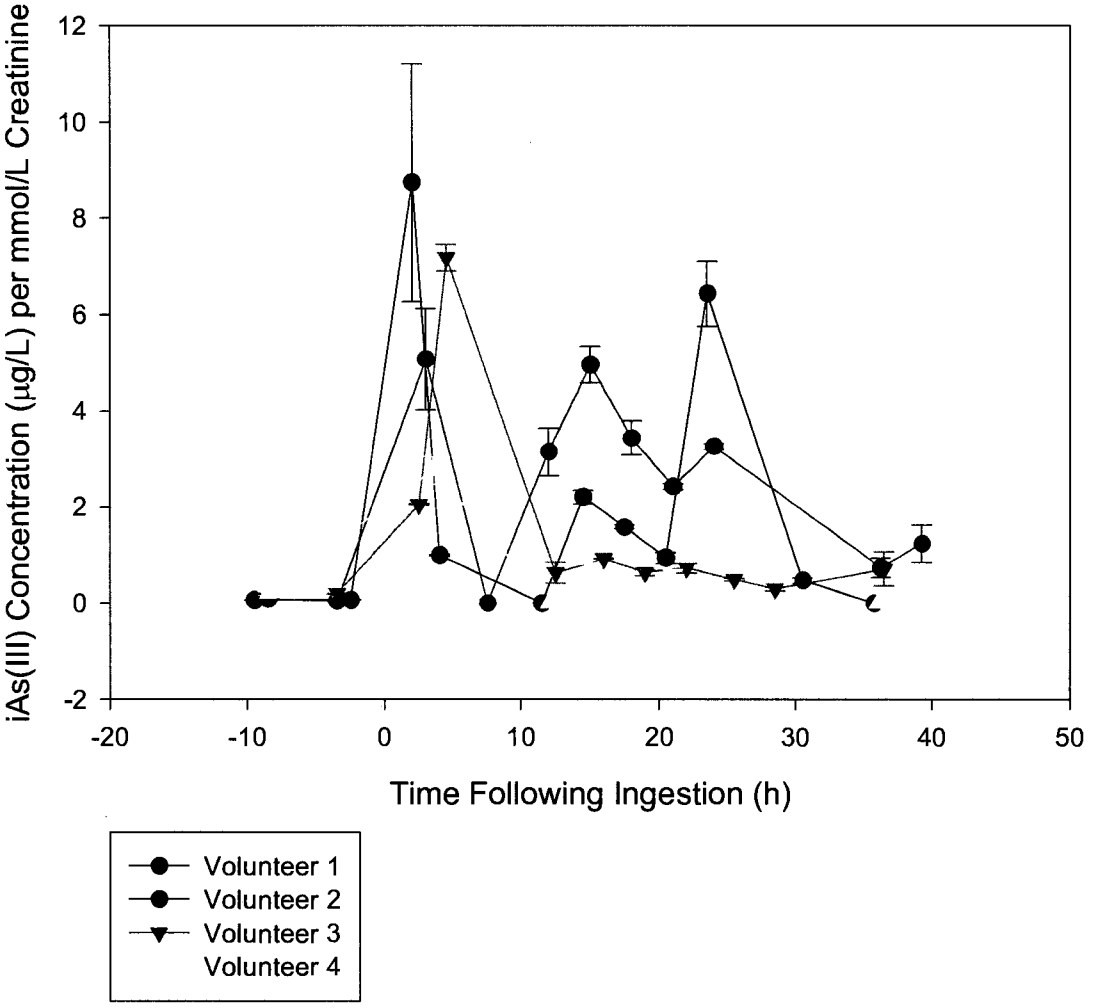


Figure 3.8 Arsenite concentration in urine before and after crab consumption. An increase in iAs(III) appeared within the first sample collected after crab ingestion. In Volunteers 1 and 2 the iAs(III) concentration increased again at later time points but returned to values similar to baseline by the last sample collected.

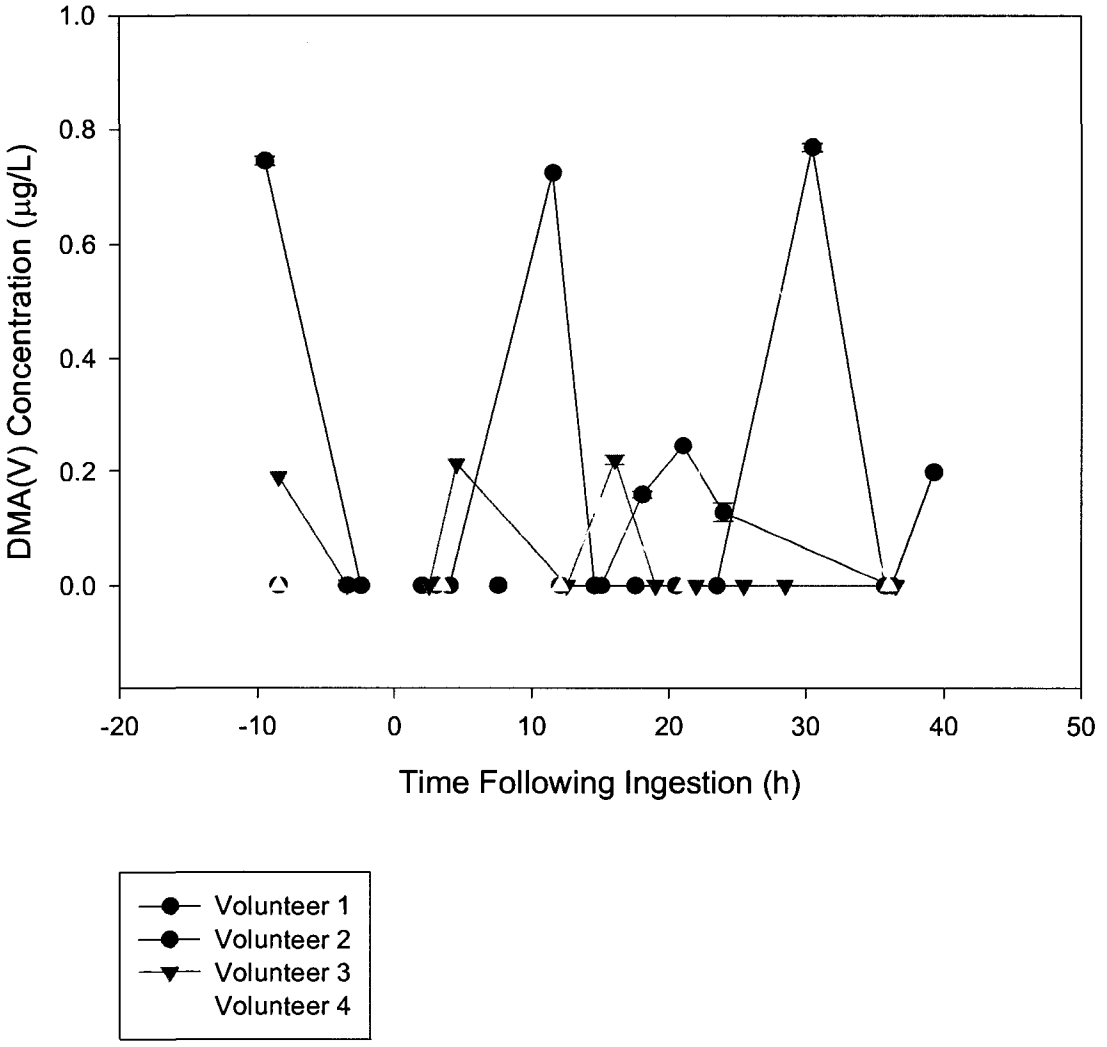


Figure 3.9 DMA(V) concentration in saliva before and after crab ingestion. The peak concentrations appeared at several time points, both prior to and following the crab meal.

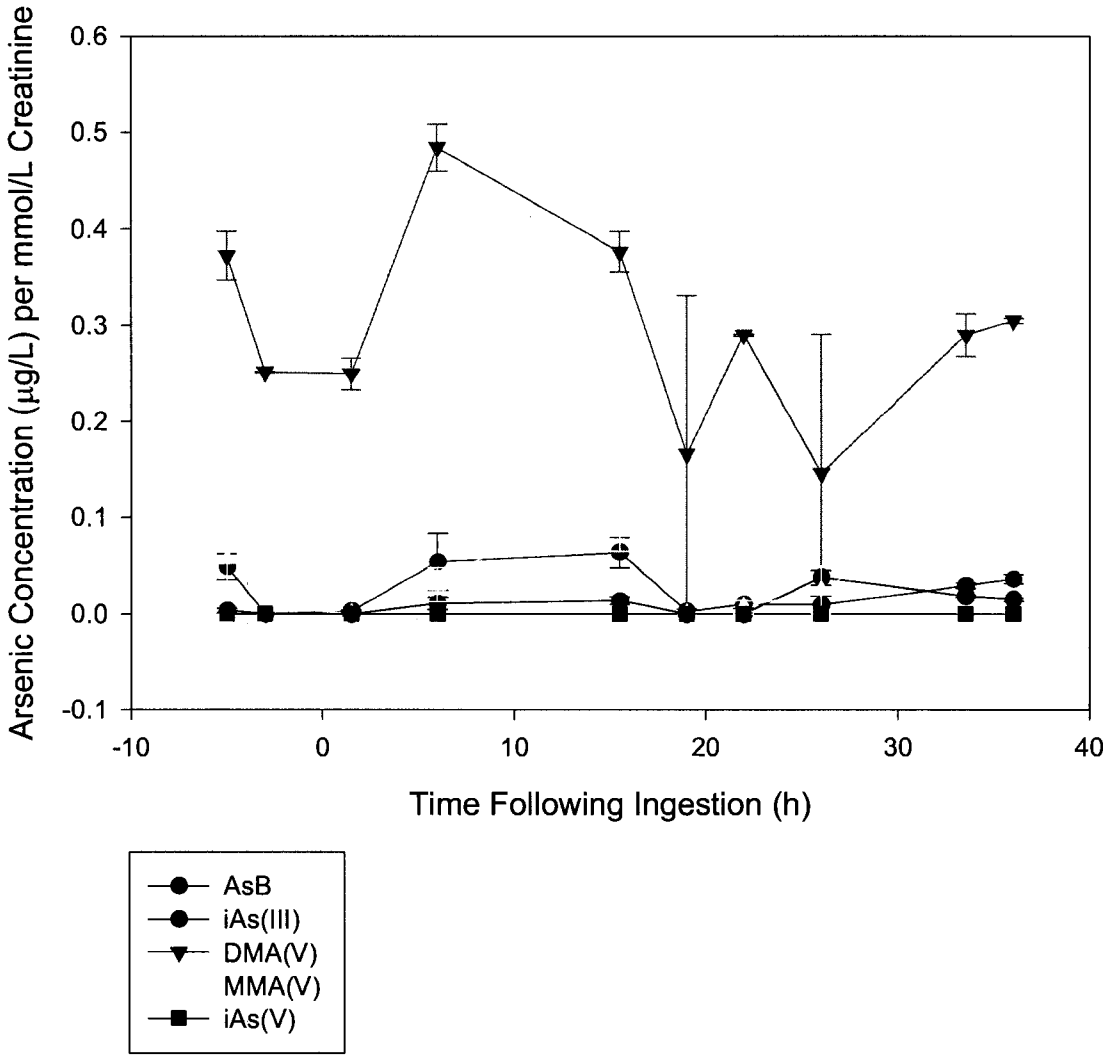


Figure 3.10 Concentrations of arsenic species in urine prior to and following nori ingestion. The concentration of most species [AsB, iAs(III), MMA(V), and iAs(V)] remained stable over the series of urine samples collected.

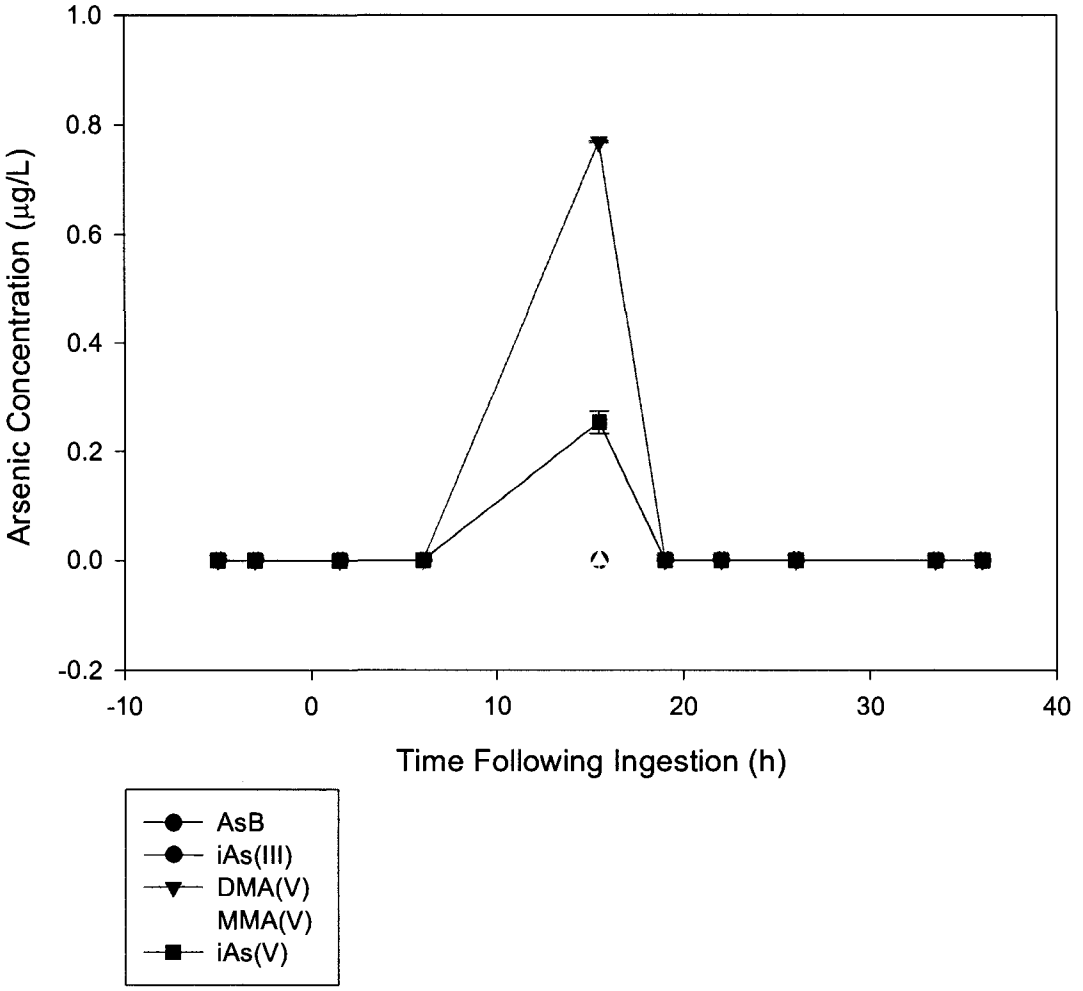


Figure 3.11 Concentrations of arsenic species in saliva before and after nori ingestion. Only DMA(V) and iAs(V) were detected at 16 h after eating nori.

3.3.9 Relative Concentrations of Arsenic Species in Urine and Saliva

In 4 out of 5 volunteers, the major arsenic species in urine prior to seafood ingestion was DMA(V) (Figure 3.12). Following seafood ingestion, the major arsenic species in urine was AsB in the volunteers who ate crab, and DMA(V) in the volunteer who ingested nori (Figure 3.13). In some saliva samples, arsenic species were not detected in the pre-ingestion samples. In the samples that contained detectable concentrations of arsenic, the predominant species were AsB and DMA(V) (Figure 3.14). Following seafood ingestion, the main arsenic species in saliva was AsB in the crab group (Figure 3.15). The predominant species was DMA(V) in the volunteer consuming nori.

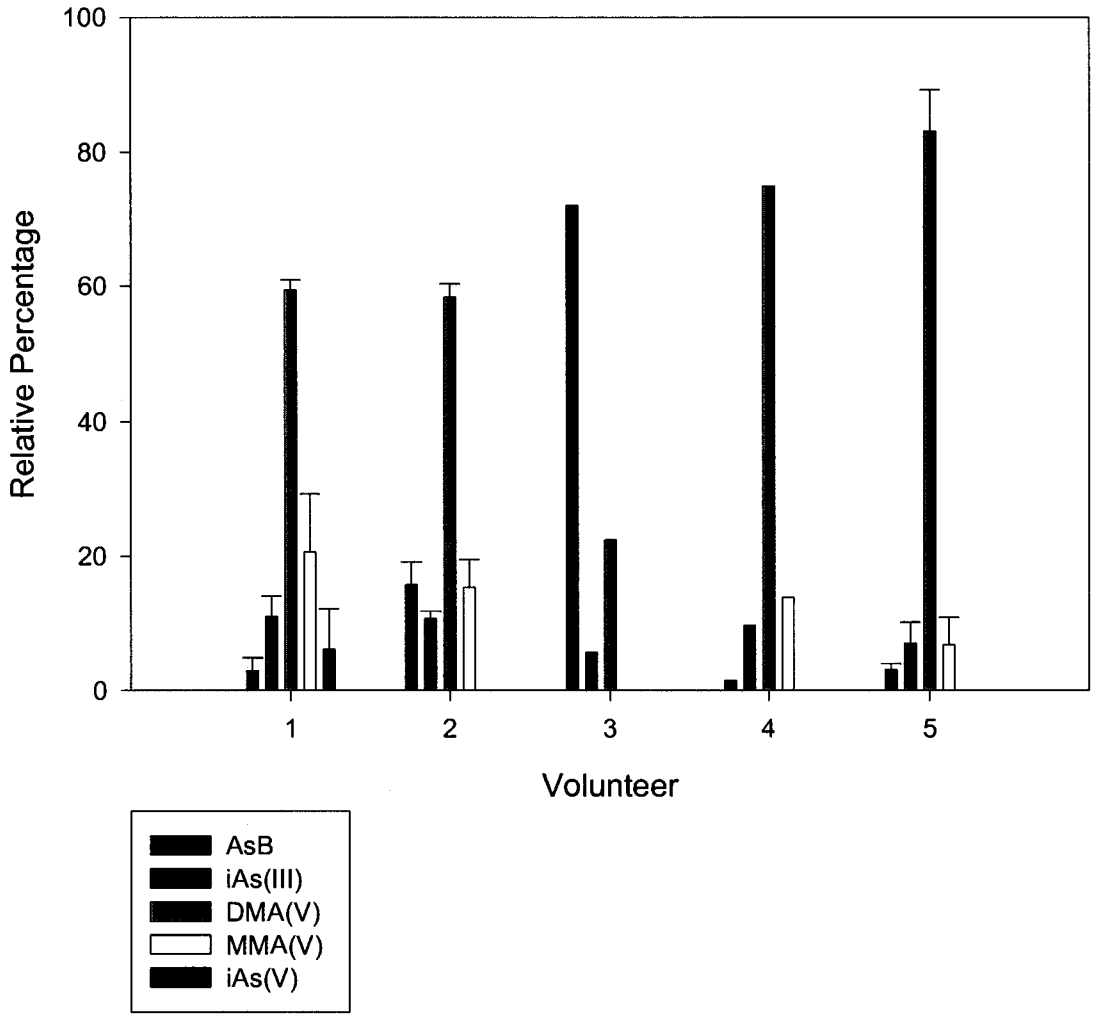


Figure 3.12 Relative percentage of arsenic species in urine prior to seafood ingestion. Volunteers 1–4 consumed crab while Volunteer 5 ingested nori. DMA(V) was the predominant species in all volunteers with the exception of Volunteer 3. This volunteer did not knowingly consume any seafood within the three days prior to the crab ingestion experiment.

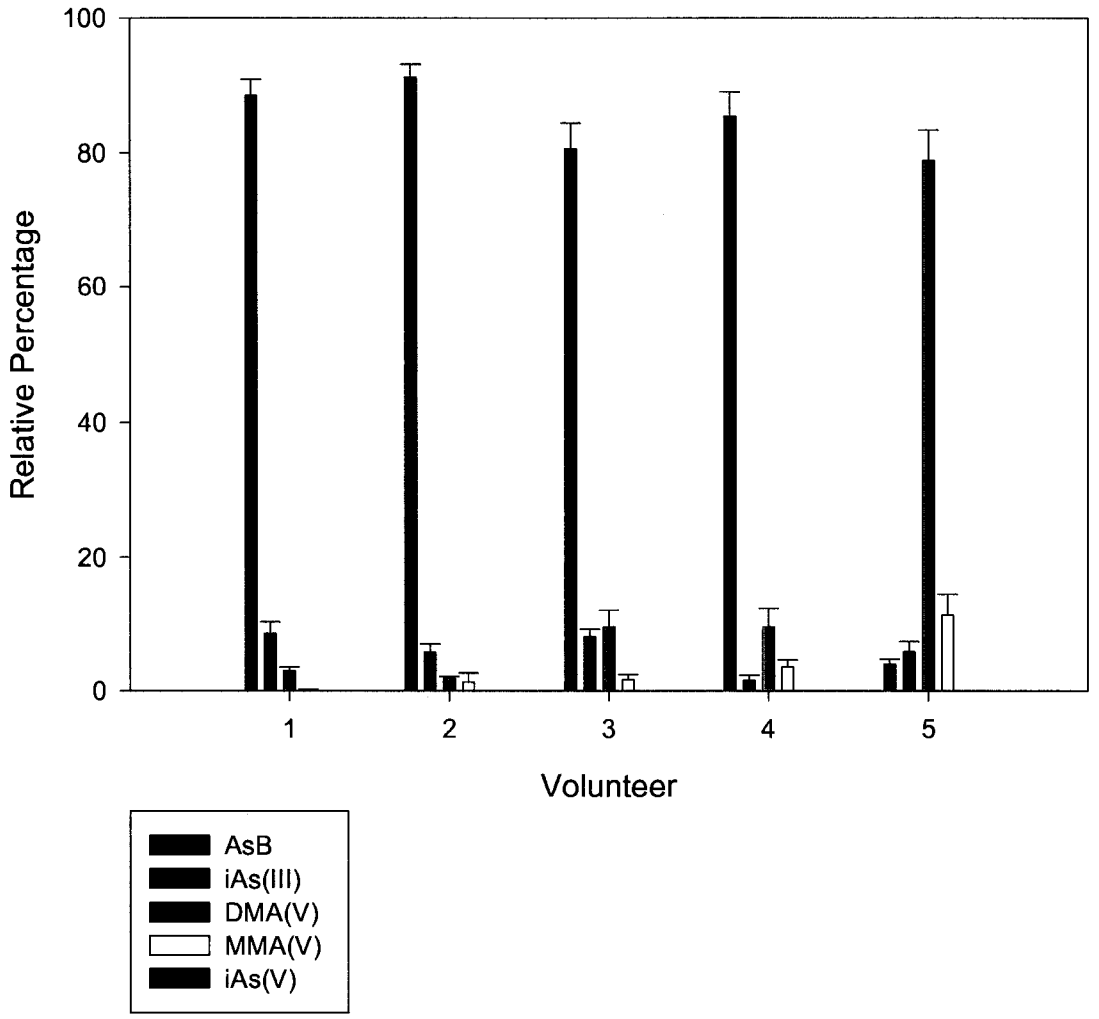


Figure 3.13 Relative percentage of arsenic species in urine following seafood ingestion. In the participants who ate crab (Volunteers 1–4), AsB was the predominant species in urine. DMA(V) was the predominant species in the urine of the volunteer who ate nori (Volunteer 5).

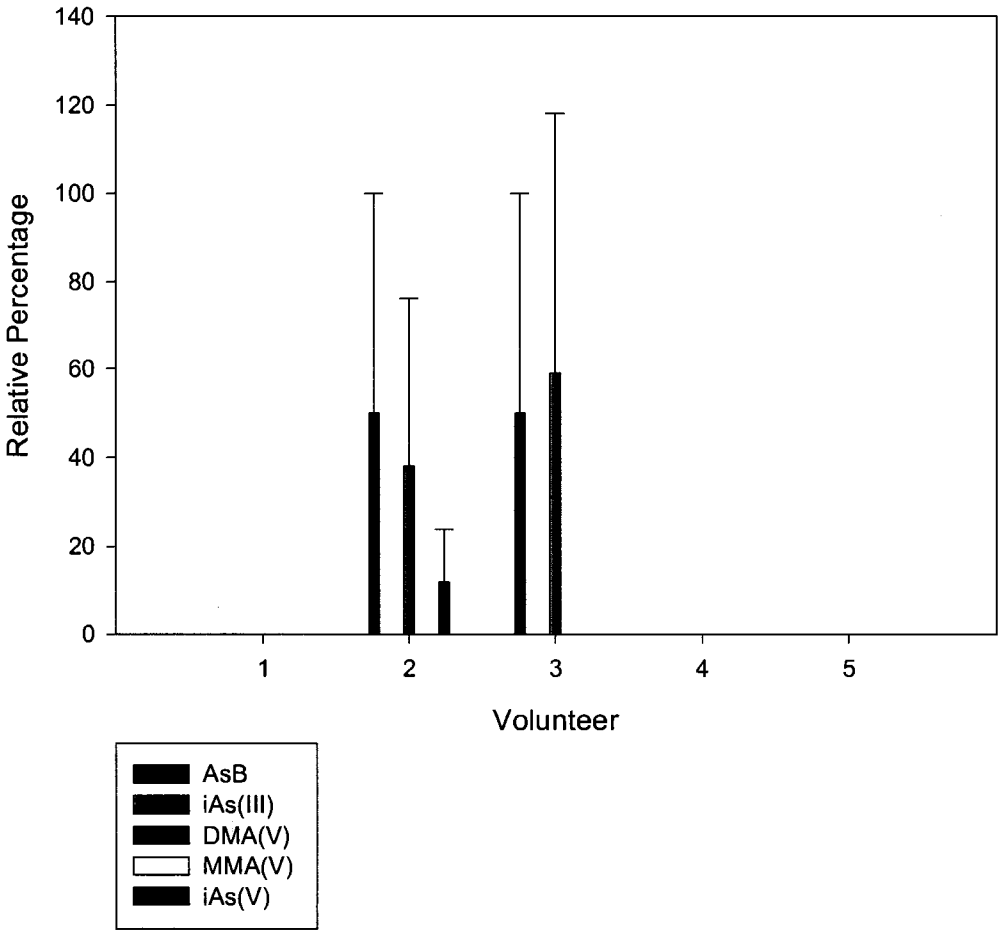


Figure 3.14 Relative percentage of arsenic species in saliva prior to seafood ingestion. Many arsenic species were not detected in saliva prior to ingestion. In Volunteers 2 and 3, the major species were AsB and DMA(V).

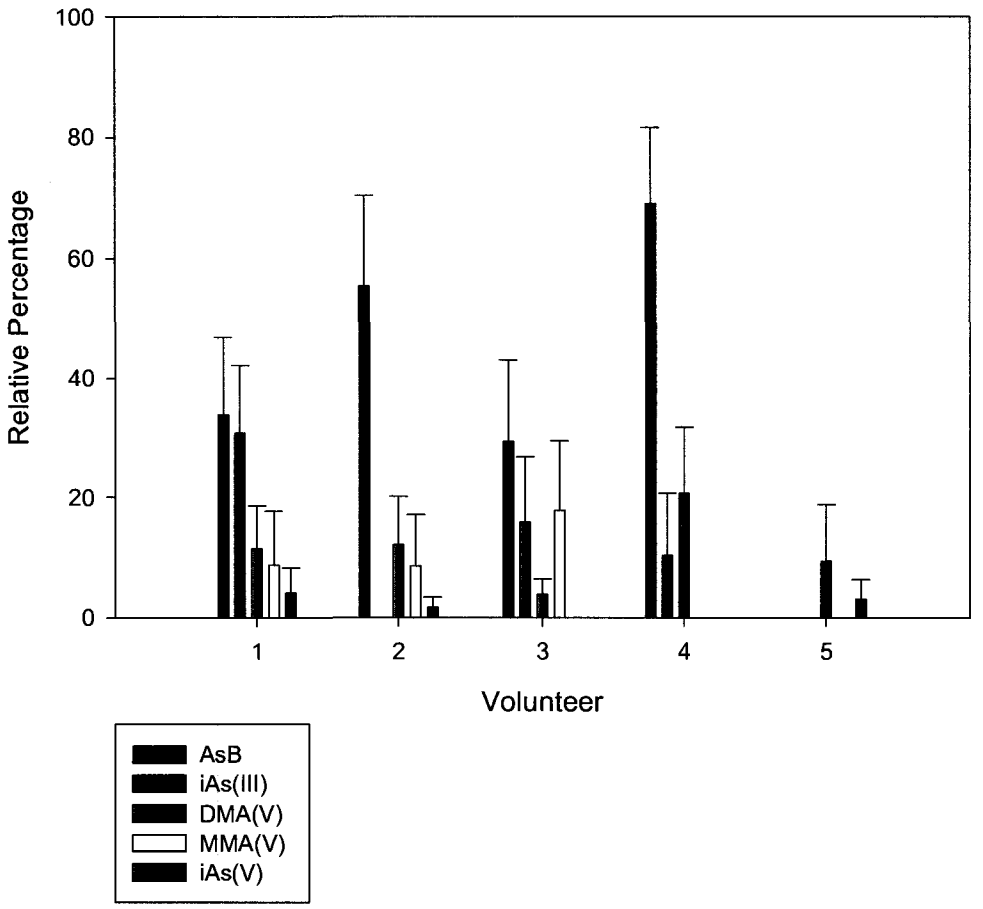


Figure 3.15 Relative percentage of arsenic species in saliva following seafood ingestion. Following crab ingestion, the predominant species was AsB (Volunteers 1–4). Following nori ingestion, the major species was DMA(V) with some iAs(V) (Volunteer 5).

3.3.10 Correlation Between Arsenic in Urine and Saliva

Figure 3.16 displays the correlation between total arsenic concentrations in urine and saliva. There was a strong correlation between total arsenic excretion in urine and saliva ($r = 0.55$, $p < 0.001$). There was also a good correlation between the concentrations of AsB in urine and saliva ($r = 0.47$, $p = 0.001$) (Figure 3.17). For all remaining species, there was an absence of correlation between the concentrations in urine and saliva (iAs(III): $r = 0.22$, $p = 0.14$; DMA(V): $r = 0.02$, $p = 0.92$; MMA(V): $r = 0.04$, $p = 0.77$; iAs(V): no correlation because undetected in urine). The correlation between the total arsenic and sum of species in urine was strong ($r = 0.79$, $p < 0.001$) (Figure 3.18). This suggests that most of the arsenic in urine was attributed to the five species of interest analyzed [AsB, iAs(III), DMA(V), MMA(V), and iAs(V)]. However, there was poorer correlation between the total arsenic and sum of species in saliva ($r = 0.26$, $p = 0.07$) (Figure 3.19).

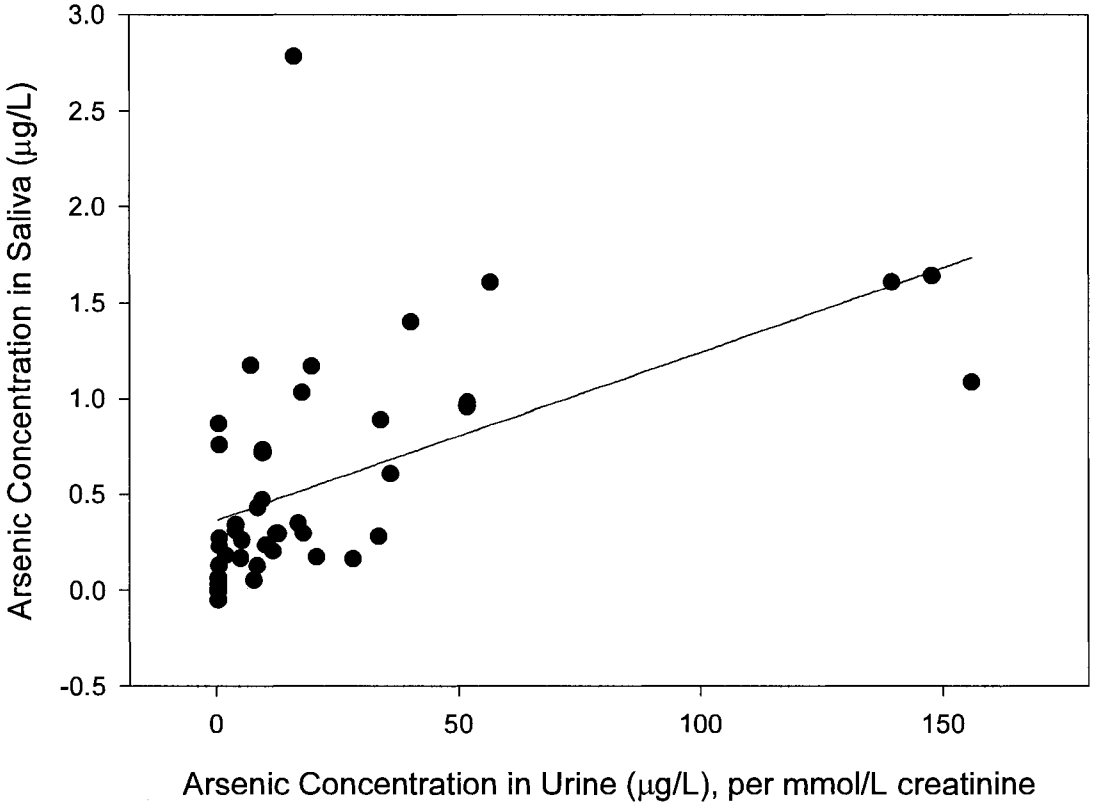


Figure 3.16 Correlation between total arsenic in urine and saliva. There was a strong correlation between the arsenic concentration in urine and in saliva ($r = 0.55$; $p < 0.001$).

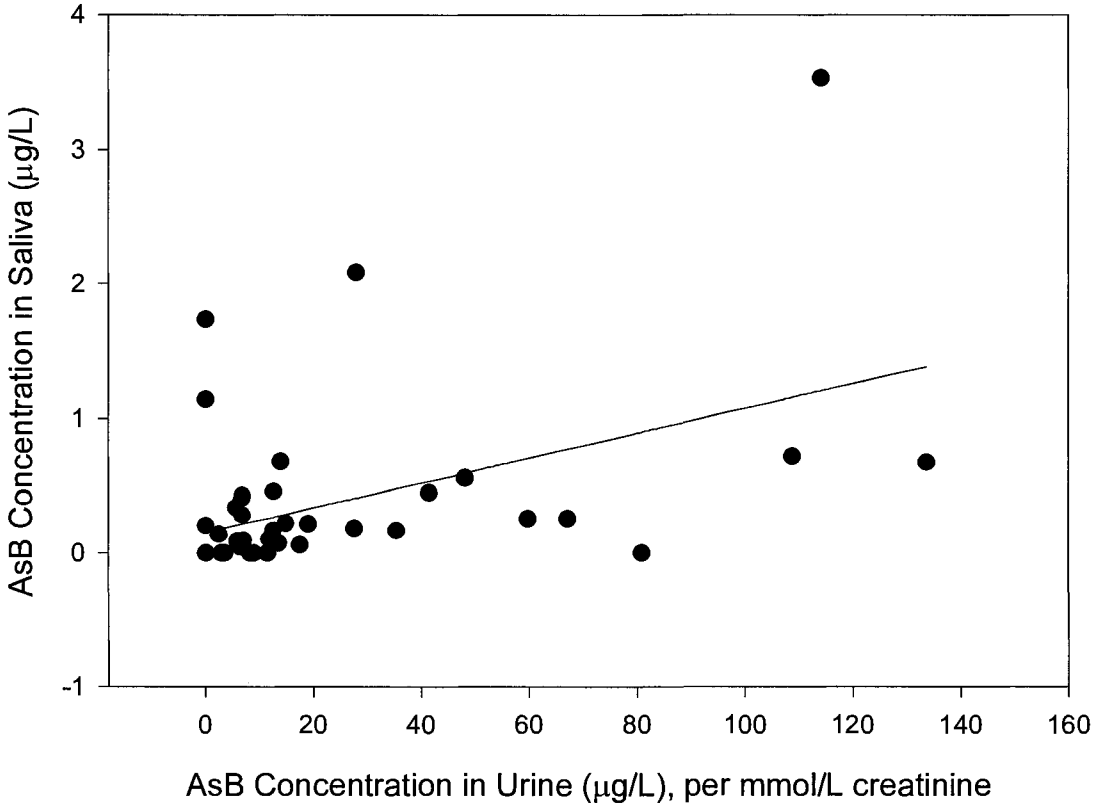


Figure 3.17 Correlation between AsB in urine and saliva. There was a good correlation between the AsB concentration in urine and in saliva (r = 0.47; p = 0.001).

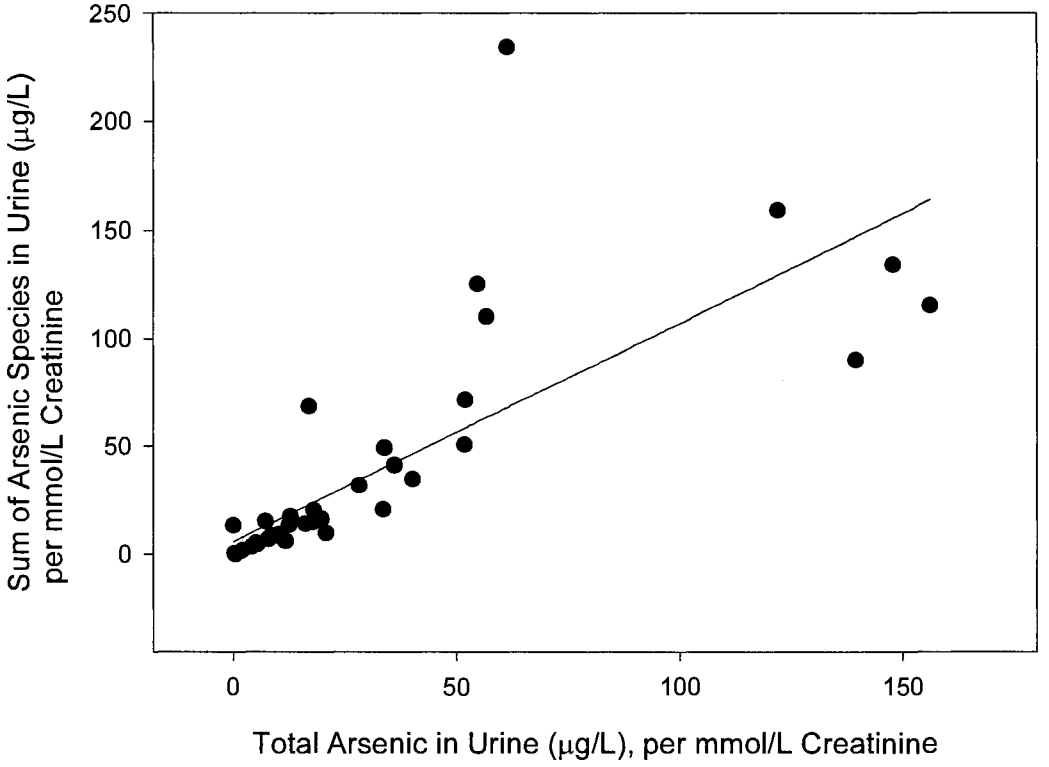


Figure 3.18 Correlation between total arsenic and the sum of arsenic species in urine. There was a good correlation between the total arsenic in urine and the sum of the arsenic species in urine ($r = 0.79$, $p < 0.001$).

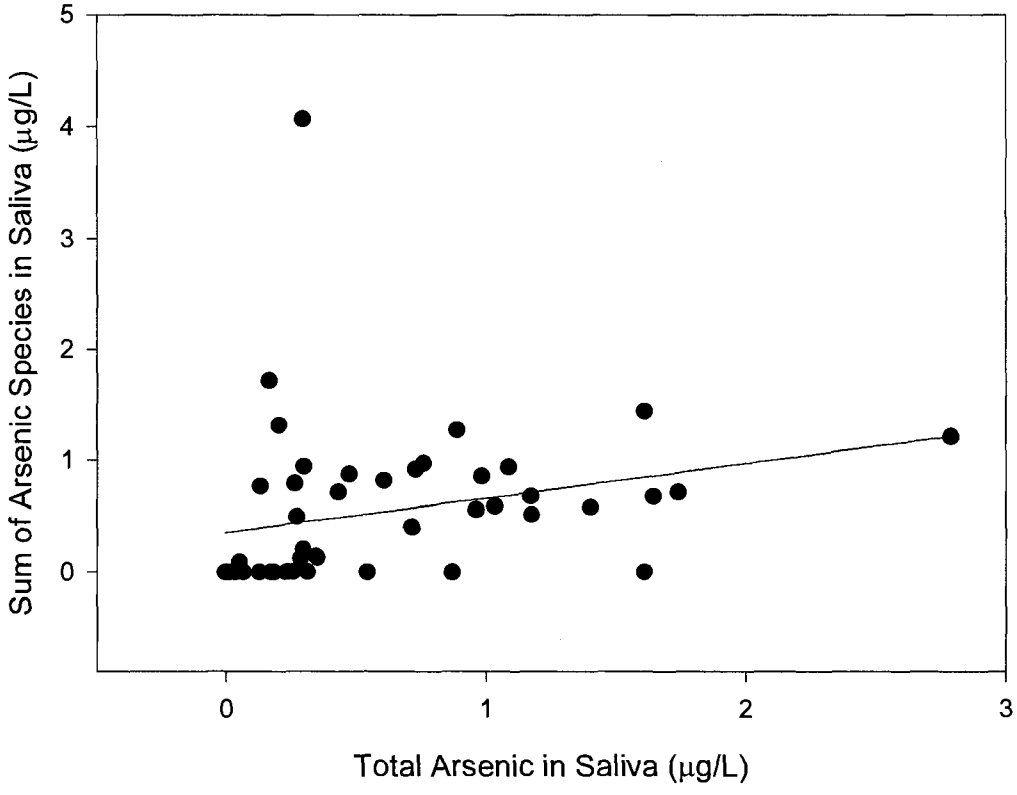


Figure 3.19 Correlation between total arsenic and the sum of arsenic species in saliva. The correlation between the total arsenic and the sum of species in saliva was good, however not as strong as in urine (r = 0.26, p = 0.07).

3.4. DISCUSSION

3.4.1 Crab Ingestion

Urinary Excretion of Arsenic

Prior to seafood ingestion, in most volunteers the major arsenic species in urine was DMA(V), accounting for between 58 and 83% of all species. Following crab ingestion, there was a rapid increase in AsB, demonstrated at 2–3.5 h post-ingestion. This finding is similar to what is presented in the literature; AsB has been found to be excreted from the human body rapidly and unchanged [6, 22, 27-29]. Here, AsB contributed to over 80% of all arsenic species present following crab consumption, thus it was a major contributor to the early and substantial increase in total arsenic. The AsB (and hence total arsenic) concentrations peaked between 2.5 and 4 h after ingestion. Both total arsenic and AsB gradually returned to concentrations similar to pre-ingestion by 39 h post-ingestion (Figures 3.1 and 3.6). Freeman *et al.* (1979) observed that most of the arsenic consumed as a result of fish ingested was excreted within 2 days [6]. In one study Le *et al.* (1994) reported a total arsenic peak at 8 h following ingestion of crab [28] and in another study, the peak concentrations appeared between 4 and 17 h after seafood ingestion [30]. A final study reported the greatest amount of AsB excretion half a day following mussel ingestion [27]. The variation in when the peak levels are detected is likely due to differences in the time of urine collection. Whether the AsB comes from a crustacean or fish source, it appears to follow the general trend of being excreted rapidly and unchanged.

The pre- and post-ingestion concentrations of iAs(III) in urine showed statistically significant differences (Figure 3.8). Prior to crab ingestion, the baseline iAs(III) was 0.1 ± 0.1 µg/L per mmol/L creatinine, while post-ingestion, the peak concentration was 5.9 ± 2.6 µg/L per mmol/L creatinine (p = 0.02). In two participants, there appears to be a second increase in iAs(III) concentrations at later time points, suggesting a biphasic excretion pattern for iAs(III). This was not seen in saliva, as there was no significant change in iAs(III) concentrations compared to baseline.

Arsenic compounds are excreted into urine in the following percentages of the total concentration: iAs(III) and iAs(V): 10–30%; DMA(V): 60–80%; and MMA(V): 10–20% [31, 32]. Excluding AsB, the relative percentage of each arsenic species in urine prior to seafood ingestion are: iAs(III) and iAs(V): 13%; DMA(V): 74%; and MMA(V): 13%. These percentages agree with the published values. Following seafood ingestion, the relative percentages of arsenic in urine changes to: iAs(III) & iAs(V): 42%; DMA(V): 50%; and MMA(V): 8%. It appears that with crab ingestion, the increased excretion of AsB in urine interferes with the normal excretion patterns of iAs(III), DMA(V), MMA(V), and iAs(V). The altered ratios of these arsenic compounds may affect the interpretation of speciation results and create clinical concern. That is, seafood ingestion may confound the arsenic analysis if exposure determination from other sources such as drinking water or occupational settings were to be assessed. If arsenic biomonitoring were to be performed, an accurate history of any potential

exposure to arsenic should be documented and the amount of exposure from seafood sources should be restricted [23, 33-36].

Salivary Excretion of Arsenic

Only one report on arsenic determination in saliva has been published by Yuan *et al.* [11]. This study reported the main arsenic species in the saliva of a group exposed to low concentrations of arsenic (< 5 µg/L) in drinking water were iAs(III), DMA(V), and iAs(V). In a group exposed to arsenic concentrations up to 826 µg/L in their drinking water, the predominant species in saliva were iAs(III) and iAs(V). Here, the crab ingestion participants were from the same demographic location as the group exposed to low levels of arsenic in drinking water. Of the two participants with detectable levels of arsenic in her saliva (Volunteers 2 and 3), the major species were DMA(V) and AsB (Figure 3.14). Volunteer 2 also had some iAs(V) in their saliva. Yuan *et al.* (2008) did not report the presence of salivary AsB in their study. Following crab ingestion, AsB levels in saliva increased similar to urine, however the relative percentage was lower than in urine (29-69%). It appeared in saliva within 2.5 h after ingestion and also peaks between 2.5 and 4 h. Within 39 h following the crab meal, AsB concentrations also returned to baseline (Figure 3.7). The elimination profile of AsB in saliva demonstrates that this compound is also rapidly excreted into saliva and follows a similar pattern to its excretion in urine. Since its presence in saliva was at a lower relative percentage than other species, this indicates that urine is the key route of AsB excretion.

In saliva, the excretion of AsB did not parallel the total arsenic concentrations as it did in urine. While AsB peaked in saliva between 2.5 and 4 h, the total arsenic reached its upper limit between 3 and 8 h post-ingestion (Figure 3.2). In urine, AsB comprised of a greater percentage of total arsenic than in saliva. The increased presence of the other arsenic species in saliva was likely contributing to the delayed peak in total arsenic concentrations.

While there was no significant change from baseline DMA(V) concentrations in urine, there were differences between the peak and baseline DMA(V) concentrations in saliva (Figure 3.9). In two participants (Volunteer 1 and 3), there appeared to be one increase DMA(V) concentration per day, even prior to ingestion. This may suggest a diurnal variation in the DMA(V) concentrations in saliva. However, further exploration into this hypothesis is necessary since this trend was demonstrated in only two volunteers.

The chromatogram of the sample collected at 39 h post-ingestion (Figure 3.5) also showed that both DMA(V) and iAs(V) were present even though they were not in high concentrations in saliva samples collected earlier in the experiment. This may indicate a delay of arsenic excretion in saliva or the participant may have consumed other arsenic-containing foods other such as mushrooms close to that sample collection time point. If it is due to a delay in the elimination of arsenic in saliva, further studies on the arsenic excretion patterns in saliva would be helpful for the interpretation.

Relative Concentrations of Arsenic in Urine and Saliva

In the study by Yuan *et al.* (2008), the relative concentrations of arsenic species in the saliva of the population exposed to low levels of arsenic in drinking water were: iAs(III): 20%; iAs(V): 40%; DMA(V): 23%; and MMA(V): 1% [11]. Not including AsB, the pre-seafood ingestion saliva samples of this study yielded relative concentrations of: iAs(III): 0%; iAs(V): 3%; DMA(V): 22%; and MMA(V): 0%. While these percentages do not entirely agree with Yuan *et al.* (2008), the number of participants used for calculating these percentages is low (n=5). Following seafood ingestion the relative concentrations (excluding AsB) changed to: iAs(III): 14%; iAs(V): 2%; DMA(V): 18%; and MMA(V): 9%.

The variation in the relative percentages of each species excreted may be explained by individual variation in metabolism, reliability of the method, or sensitivity of the instrument, as the concentrations of each arsenic species in saliva was quite low. Studies have reported inter-individual variability in the arsenic excretion profile in urine following seafood ingestion [3, 11, 27, 28, 32, 35, 37-39]. Some have attributed these differences to genetic polymorphisms, sex, and ethnic background [3, 27, 32, 35, 37, 39]. For a general population, the arsenic concentrations in saliva are fairly low . Here, prior to seafood ingestion, arsenic species were detected in the saliva of only in a couple of participants. However, most samples exhibited detectable, but low levels of total arsenic (0.3 ± 0.2 µg/L). Because of these low concentrations, use of the HPLC-ICPMS method for species separation and detection may not be optimal. It is possible that some of the arsenic may be retained on the column thus preventing all

arsenic from entering the mass spectrometer for detection. It would be valuable to optimize the method to ensure that low concentrations of arsenic can efficiently be separated and detected whether it is in the instrument itself or in sample preparation [40].

3.4.2 Nori Ingestion

Urinary Excretion of Arsenic

The excretion of arsenic species in urine following nori ingestion did not follow an expected excretion profile (Figure 3.10). The main arsenic compounds present in nori are arsenosugars. In the body, these become metabolized to primarily DMA(V) [28, 38, 41]. Following nori ingestion, there was a peak in salivary DMA(V) and iAs(V) at 16 h after the meal (Figure 3.11). This was the only time point where any arsenic species in saliva are detected. Arsenosugars take a longer time than AsB to be excreted in humans. The reason for this is that AsB does not undergo metabolism prior to excretion in the urine, while arsenosugars are metabolized to DMA(V), thus requiring a longer transit time prior to excretion [5, 28, 38]. Ma and Le (1998) found that subjects who ingested 10 g of yakinori excreted maximum arsenic concentrations 22–38 h following ingestion [38]; while Le *et al.* (1994) reported maximum arsenic concentrations between 10 and 60 h following ingestion of 9.5 g of nori. The participants in the nori ingestion group ate only 0.35 or 0.70 g of dry nori. Other ingestion studies report that their participants consumed upwards of 9.5 g of nori or seaweed [3, 28, 38, 42, 43]. The maximum concentrations of total arsenic in urine (without

creatinine correction) ranged from approximately 30–100 µg/L in the study by Le *et al.* (1994). It is possible that the amount of nori consumed in this study was not sufficient to result in any major differences in the arsenic excretion profile in comparison to baseline. This may also explain why the two participants who ingested both 0.70 g of nori and 200–300 g of crab showed an arsenic excretion profile more similar to what is seen when only crab is ingested, thus their data were analyzed as part of the crab ingestion group.

3.4.3 Correlation Between Arsenic in Urine and Saliva

There was a good correlation between the total arsenic concentration in urine and in saliva (Figure 3.16). Since AsB was the species that contributed to a large percentage of the total arsenic in both urine and saliva, the correlation between the urinary and salivary AsB levels was investigated (Figure 3.17). Although the correlation was not as strong as with total arsenic, the urinary and salivary AsB concentrations did show evidence of correlation ($r = 0.47$, $p = 0.001$) All remaining arsenic species demonstrated weak correlation coefficients for their concentrations in both urine and saliva. This may be due to AsB levels being higher in both urine and saliva, thus allowing for greater chances of detection. The concentrations of the other species in saliva were low relative to AsB and therefore correlation between the concentrations in urine and saliva could not be sufficiently demonstrated. If steps were taken to further concentrate the saliva samples, sensitivity in species detection and quantification would be enhanced,

and a better correlation between the concentrations in urine and saliva may be seen.

When comparing the sum of all arsenic species against the total arsenic concentrations, the correlation in urine was strong ($r = 0.79$, $p < 0.001$) (Figure 3.18). However, the correlation between the sum of species and the total arsenic in saliva was weaker ($r = 0.26$, $p = 0.07$) (Figure 3.19). Compared to urine, the concentrations of total arsenic as well as the individual species are much lower in saliva. Any retention of species on the chromatography column or sample dilution will have a far greater impact on the salivary arsenic concentrations rather than the urinary arsenic. The method used for total arsenic analysis involves direct injection of the sample into the ICPMS. This prevents the loss of any arsenic in the sample. Figure 3.19 reveals that most of the saliva samples showed a higher total arsenic concentration compared to its corresponding sum of species. Determining a method to improve the separation technique and column efficiency in HPLC would be beneficial for the quantification of low levels of arsenic, an example of which would be investigating the use of micro chromatography columns for arsenic separation.

3.4.4 Arsenic Species in Ingested Seafood

To confirm the species of arsenic ingested from the seafood meal, both crab and nori were analyzed for their arsenic content. The most abundant arsenic species in Dungeness crab legs was AsB. Other studies confirm that AsB accounts for a substantial percentage of total arsenic [40, 44-46]. In the

steaming liquid from crab preparation, there was also both AsB and iAs(V) present, however the percentage of AsB in the cooking liquid is lower than in the actual crab meat. In nori, the arsenic species detected in order of abundance were DMA(V), MMA(V) and some AsB (Table 3.1). Arsenosugars should have been detected in the nori, however there was only a small amount of nori available for arsenic analysis (0.1 g). As a result, the extraction procedure may not have been very effective at removing the arsenosugars in the small nori sample.

Table 3.2 demonstrates the arsenic concentrations from acid digestion (total arsenic), and methanol extraction (speciation and total arsenic). The mass balance with regards to the Dungeness crab does not entirely add up to the total arsenic. It is possible that the extraction procedure using methanol and deionized water, followed by ultrasonication and centrifugation may not fully extract all of the arsenic present in the sample. Other studies have also noticed low extraction efficiencies when comparing the sum of arsenic species in food to the total, however the methanol extraction method appeared to work most efficiently [41]. It is also possible that arsenic species other than the five species of interest were present in the crab sample.

3.5 CONCLUSIONS

This study investigated the excretion profile of arsenic compounds into urine and saliva following ingestion of seafood. AsB was the major arsenic species in crab and was demonstrated to be present in saliva while following a

similar timeline of excretion in urine. Its concentrations were increased in urine and saliva within 2 h following crab ingestion. AsB was the main contributor to the increased total arsenic concentrations in urine and saliva. Both AsB and total arsenic concentrations returned to levels similar to baseline by the last sample collection point at 36–39 h.

The small quantity of nori ingested did not allow for the detection of any significant differences in the post-ingestion arsenic levels compared to baseline. Future studies of nori ingestion should consider consumption of a larger quantity of nori for assessing the arsenic excretion profile.

There was a good correlation between total arsenic in both urine and saliva, as well as AsB in urine and saliva. However, there was lack of evidence of correlation between urinary and salivary concentrations of other arsenic species due to the lower concentrations of these species in saliva. Similar to plasma, arsenic concentrations in saliva are magnitudes lower than the concentrations in urine [47]. With better techniques for concentrating the saliva during sample preparation and with more sensitive analytical methods, the analysis of salivary arsenic could be a more useful biomarker for biomonitoring purposes.

This study contributes to the understanding of arsenic metabolism and excretion in the human body according to the type and route of arsenic exposure. Saliva should not yet be deemed a reliable alternative sample to urine for arsenic analysis; further work in understanding arsenic species excretion into saliva is warranted. Future studies on arsenic compounds in saliva should include an

analysis of the relationship between arsenic concentrations in saliva and plasma, as well as urine, and efforts should be made to further concentrate analytes in saliva prior to analysis.

3.6 REFERENCES

[1] Hindmarsh JT. Caveats in hair analysis in chronic arsenic poisoning. Clin Biochem. 2002 Feb;35(1):1-11.

[2] Mandal BK, Ogra Y, Anzai K, Suzuki KT. Speciation of arsenic in biological samples. Toxicol Appl Pharmacol. 2004 Aug;198(3):307-18.

[3] Van Hulle M, Zhang C, Schotte B, Mees L, Vanhaecke F, Vanholder R, et al. Identification of some arsenic species in human urine and blood after ingestion of Chinese seaweed Laminaria. J Anal At Spectrom. 2004 Jan;19(1):58-64.

[4] Buchet JP, Lauwerys R, Roels H. Urinary excretion of inorganic arsenic and its metabolites after repeated ingestion of sodium metaarsenite by volunteers Int Arch Occup Environ Health. 1981;48(2):111-8.

[5] Crecelius EA. Changes in Chemical Speciation of Arsenic Following Ingestion by Man. Environ Health Perspect. 1977;19(Aug):147-50.

[6] Freeman HC, Uthe JF, Fleming RB, Odense PH, Ackman RG, Landry G, et al. Clearance of arsenic ingested by man from arsenic contaminated fish. Bull Environ Contam Toxicol. 1979 May;22(1-2):224-9.

[7] World Health Organization. Environmental Health Criteria 18 - Arsenic. Geneva: World Health Organization 1981.

[8] Concha G, Vogler G, Nermell B, Vahter M. Low-level arsenic excretion in breast milk of native Andean women exposed to high levels of arsenic in the drinking water. Int Arch Occup Environ Health. 1998;71(1):42-6.

[9] Gregus Z, Gyurasics A, Csanaky I. Biliary and urinary excretion of inorganic arsenic: Monomethylarsonous acid as a major biliary metabolite in rats. Toxicol Sci. 2000 Jul;56(1):18-25.

[10] Cui X, Kobayashi Y, Hayakawa T, Hirano S. Arsenic speciation in bile and urine following oral and intravenous exposure to inorganic and organic arsenics in rats. Toxicol Sci. 2004 Dec;82(2):478-87.

[11] Yuan CG, Lu XF, Oro N, Wang ZW, Xia YJ, Wade TJ, .et al. Arsenic speciation analysis in human saliva. Clin Chem. 2008;54(1):163-71.

[12] Chiappin S, Antonelli G, Gatti R, De Palo EF. Saliva specimen: A new laboratory tool for diagnostic and basic investigation. Clin Chim Acta. 2007;383(1-2):30-40.

[13] Hold K, de Boer D, Zuidema J, Maes R. Saliva as an Analytical Tool in Toxicology. Int J Drug Test. 1996;1(1):1-36.

[14] Yaari A, Tovbin D, Zlotnick M, Mostoslavsky M, Shemer-Avni Y, Hanuka N, et al. Detection of HCV salivary antibodies by a simple and rapid test. J Virol Methods. 2006 Apr;133(1):1-5.

[15] Schramm W, Angulo GB, Torres PC, Burgess-Cassler A. A simple saliva-based test for detecting antibodies to human immunodeficiency virus. Clin Diagn Lab Immunol. 1999 Jul;6(4):577-80.

[16] Aardal E, Holm AC. Cortisol in saliva - Reference ranges and relation to cortisol in serum. Eur J Clin Chem Clin Biochem. 1995 Dec;33(12):927-32.

[17] Jenkins AJ, Oyler JM, Cone EJ. Comparison of Heroin and Cocaine Concentrations in Saliva with Concentrations in Blood and Plasma. J Anal Toxicol. 1995 Oct;19(6):359-74.

[18] Al Za'abi M, Deleu D, Batchelor C. Salivary free concentrations of Anti-Epileptic Drugs: An evaluation in a routine clinical setting. Acta Neurol Belg. 2003 Mar;103(1):19-23.

[19] Denovan LA, Lu C, Hines CJ, Fenske RA. Saliva biomonitoring of atrazine exposure among herbicide applicators. Int Arch Occup Environ Health. 2000;73(7):457-62.

[20] Silva M, Reidy J, Samandar E, Herbert A, Needham L, Calafat A. Detection of phthalate metabolites in human saliva. Arch Toxicol. 2005;79(11):647-52.

[21] Ferrari M, Negri S, Zadra P, Ghittori S, Imbriani M. Saliva as an analytical tool to measure occupational exposure to toluene. Int Arch Occup Environ Health. 2008 Aug;81(8):1021-8.

[22] Kaise T, Watanabe S, Itoh K. The Acute Toxicity of Arsenobetaine. Chemosphere. 1985;14(9):1327-32.

[23] Kales SN, Huyck KL, Goldman RH. Elevated urine arsenic: Un-speciated results lead to unnecessary concern and further evaluations. J Anal Toxicol. 2006 Mar;30(2):80-5.

[24] Le XC, Lu X, Li XF. Arsenic Speciation. Anal Chem. 2004;76(1):26A-33A.

[25] Agilent Techologies. ICP-MS Inductively Coupled Plasma Mass Spectrometry - A Primer. USA: Agilent Technolgies 2005.

[26] Burtis C, Ashwood E, eds. Tietz Fundamentals of Clinical Chemistry. 5 ed. Philadelphia: W.B. Saunders Company 2001.

[27] Lai VWM, Sun YM, Ting E, Cullen WR, Reimer KJ. Arsenic speciation in human urine: are we all the same? Toxicol Appl Pharmacol. 2004 Aug;198(3):297-306.

[28] Le XC, Cullen WR, Reimer KJ. Human urinary arsenic excretion after one-time ingestion of seaweed, crab, and shrimp. Clin Chem. 1994 Apr;40(4):617-24.

[29] Vahter M. Species differences in the metabolism of arsenic compounds. Appl Organomet Chem. 1994;8(3):175-82.

[30] Le XC, Cullen WR, Reimer KJ. Determination of Urinary Arsenic and Impact of Dietary Arsenic Intake. Talanta. 1993 Feb;40(2):185-93.

[31] Goessler W, Schlagenhaufen C, Kuehnelt D, Greschonig H, Irgolic KJ. Can Humans Metabolize Arsenic Compounds to Arsenobetaine? Appl Organomet Chem. 1997;11(4):327-35.

[32] Vahter M. Genetic polymorphism in the biotransformation of inorganic arsenic and its role in toxicity. Toxicol Lett. 2000;112-113:209-17.

[33] Kalman DA, Hughes J, Belle Gv, Burbacher T, Bolgiano D, Coble K, et al. The Effect of Variable Environmental Arsenic Contamination on Urinary Concentrations of Arsenic Species. Environ Health Perspect. 1990;89:145-51.

[34] Edmonds JS. Can humans metabolize arsenic compounds to arsenobetaine? Appl Organomet Chem. 1998 Jul;12(7):515-7.

[35] Hata A, Endo Y, Nakajima Y, Ikebe M, Ogawa M, Fujitani N, et al. HPLC-ICP-MS speciation analysis of arsenic in urine of Japanese subjects without occupational exposure. J Occup Health. 2007 May;49(3):217-23.

[36] Buchet JP, Lison D, Ruggeri M, Foa V, Elia G. Assessment of exposure to inorganic arsenic, a human carcinogen, due to the consumption of seafood. Arch Toxicol. 1996 Sep;70(11):773-8.

[37] Lindberg A-L, Ekström E-C, Nermell B, Rahman M, Lönnerdal B, Persson L-Å, et al. Gender and age differences in the metabolism of inorganic arsenic in a highly exposed population in Bangladesh. Environ Res. 2008;106(1):110-20.

[38] Ma MS, Le XC. Effect of arsenosugar ingestion on urinary arsenic speciation. Clin Chem. 1998 Mar;44(3):539-50.

[39] Loffredo CA, Aposhian HV, Cebrian ME, Yamauchi H, Silbergeld EK. Variability in human metabolism of arsenic. Environ Res. 2003 Jun;92(2):85-91.

[40] Le SXC, Cullen WR, Reimer KJ. Speciation of Arsenic Compounds in Some Marine Organisms. Environ Sci Technol. 1994 Sep;28(9):1598-604.

[41] Madsen AD, Goessler W, Pedersen SN, Francesconi KA. Characterization of an algal extract by HPLC-ICP-MS and LC-electrospray MS for use in arsenosugar speciation studies. J Anal At Spectrom. 2000;15(6):657-62.

[42] Matsuura H, Asano M, Hasegawa T, Umemura T, Haraguchi H. Speciation and excretion patterns of arsenic metabolites in human urine after ingestion of edible seaweed, Hizikia fusiforme. Bull Chem Soc Jpn. 2005 Nov;78(11):1977-81.

[43] Francesconi KA, Tanggaard R, McKenzie CJ, Goessler W. Arsenic metabolites in human urine after ingestion of an arsenosugar. Clin Chem. 2002 Jan;48(1):92-101.

[44] Francesconi KA, Micks P, Stockton RA, Irgolic KJ. Quantitative determination of arsenobetaine, the major water-soluble arsenical in three species of crab, using high pressure liquid chromatography and an inductively coupled argon plasma emission spectrometer as the arsenic-specific detector. Chemosphere. 1985;14(10):Pages: 1443-53.

[45] Norum U, Lai VWM, Pergantis SA, Cullen WR. Arsenic compounds in the haemolymph of the Dungeness crab, Cancer magister, as determined by using HPLC on-line with inductively coupled plasma mass spectrometry. J Environ Monit. 2005;7(2):122-6.

[46] Larsen EH, Pritzl G, Hansen SH. Arsenic Speciation in Seafood Samples with Emphasis on Minor Constituents: an Investigation Using High-Performance Liquid Chromatography with Detection by Inductively Coupled Plasma Mass Spectrometry. J Anal At Spectrom. 1993 Dec;8(8):1075-84.

[47] Seifert B, Becker K, Helm D, Krause C, Schulz C, Seiwert M. The German Environmental Survey 1990/1992 (GerES II): reference concentrations of selected environmental pollutants in blood, urine, hair, house dust, drinking water and indoor air. J Expo Anal Environ Epidemiol. 2000 Nov-Dec;10(6):552-65.

CHAPTER 4

ARSENIC BIOMONITORING IN CHILDREN PLAYING ON CHROMATED COPPER ARSENATE-TREATED PLAYGROUNDS

4.1 INTRODUCTION

Chromated copper arsenate (CCA) protects wood products from the decaying effects of fungi and insects, and therefore has been used as a wood preservative. Since the beginning of 2004, the wood preservation and manufacturing industries in both Canada and the United States have voluntarily discontinued the use of CCA in residential wood products [1, 2]. The reason for the ban is because of the observed adverse health effects resulting from exposure to high levels of arsenic in drinking water [3-6]. The presence of CCA in the existing playground structures is a topic of debate.

Numerous assessments have been performed to establish children's exposure to arsenic as a result of contact with CCA-treated structures and materials surrounding them. Many of these included the use of mathematical models based on factors such as hand surface area, the amount of skin in contact with the CCA-treated wood, and the frequency of hand-to-mouth activity [7-10]. Other studies have investigated the amount of arsenic dislodged from CCA-treated structures as a result of contact with human hands or absorbent materials [7, 11-14] and quantified the amount leached from the pressure-treated wood into the surrounding sand or soil [11-13, 15].

There is direct evidence of increased arsenic levels on the hands of children following play on CCA playgrounds [11, 13]. Kwon *et al.* (2004) have previously measured the total arsenic on the hands of children playing on eight CCA-treated and eight non-CCA-treated playgrounds across the city of Edmonton [11]. The mean total arsenic (soluble arsenic on hands plus the arsenic in the sand residue of the hand wash samples) was 561 ± 552 ng (range 8–3865 ng) from the hands of the children in contact with CCA playgrounds. Children playing on non-CCA playgrounds had a mean of 143 ± 95 ng (range 23–475 ng) of arsenic in their hand wash samples. Similar differences between the amounts of arsenic in the hand rinses also existed between the children playing on CCA and non-CCA playgrounds in the study by Shalat *et al.* (2006) [13]. One group incorporated experimental data generated by Kwon *et al.* (2004) into the Stochastic Human Exposure and Dose Simulation (SHEDS) Model for estimating children's exposure to arsenic [16]. This study suggests that the use of data from actual studies, rather than information from the United States Environmental Protection Agency's Consolidated Human Activity Database, is more accurate in estimating children's overall exposure to arsenic.

Since arsenic is easily transferred to children's hands upon contact with the pressure-treated wood, it is beneficial to study the amount of arsenic that children may ingest from hand-to-mouth contact. Children 24 months of age and under place their hands in their mouths an average of 13–18 times per hour [17, 18]. In contrast, children over 24 months of age average a hand-to-mouth frequency of 11–16 times per hour. Analyzing urine or saliva for biomarkers of

arsenic exposure will provide information on children's actual exposure to arsenic as a result of playing on playgrounds constructed from CCA-treated wood and ingesting the arsenic from hand-to-mouth contact.

There is limited quantitative evidence regarding the amounts of arsenic children potentially ingest or absorb while in contact with CCA-treated structures. The pilot study by Shalat *et al.* (2006) measured urinary arsenic levels in children with access to CCA-treated playgrounds [13]. In the 11 urine samples analyzed, they did not find any relationship between contact with CCA playgrounds and total arsenic concentrations in urine. However, arsenic speciation was not performed on the urine samples. As a result, it is difficult to discern the differences between the presence of non-toxic arsenic species from dietary sources and the more clinically significant inorganic arsenic species in the urine.

The objective of this study was to perform biomonitoring on children to assess their overall exposure to arsenic from oral ingestion as result of playing on CCA-treated playgrounds. Kwon *et al.* (2004) previously reported the mean total arsenic on children's hands after play on CCA-treated playgrounds to be 561 ± 552 ng [11]. If this amount were ingested, it would be lower than the average daily intake of arsenic from dietary sources. Canadian children, on average, ingest 15 µg of arsenic daily, with 5–11 µg as inorganic arsenic [19, 20]. Since arsenic is naturally occurring in food and water, all children we test would have a baseline level of arsenic in their urine and saliva. We predicted that any ingestion of arsenic from playground sources would not contribute considerably to the daily arsenic intake; thus we hypothesized that there would be no

significant difference in the speciation and concentrations of arsenic in the urine and saliva samples of children exposed to CCA-treated and non-CCA-treated playgrounds.

4.2 MATERIALS AND METHODS

4.2.1 Sample Collection

The same 16 playgrounds as previously described were selected for sampling [11]. One playground per day was visited between August 21, 2006 and September 12, 2006 in an alternating pattern of CCA and non-CCA each day. Parents or adult guardians were provided with information regarding the study and gave informed consent for their children to participate in the study. The information was collected according to the University of Alberta's Health Research Ethics Board's guidelines. Demographic information which included the children's frequency of play at the playground of sampling, age, and gender were also collected. The length of time the children spent on the playground on the day of the study was also documented.

Urine Samples

The parents were provided with three urine collection containers (Fisher Brand, Fisher Scientific; Nepean, ON) for collecting urine samples from their children at home. The parents were asked to assist their children in collecting the urine samples. The first sample was to be collected during the evening of the day of play. Another sample was to be collected during the first urination the

following morning, and a final sample was to be collected later that same morning or during the early afternoon following the day of play. The parents were asked to store the urine samples in the refrigerator until we picked them up the afternoon after the day of play. They were taken to the laboratory for storage at -20 °C until analysis.

Saliva Samples

A pre-play saliva sample from each child who was not already playing on the playgrounds was collected. Children were provided with approximately 50 mL of bottled water (Nestle Pure Life; Hope, BC) and asked to thoroughly rinse out their mouths in order to remove any food particles. Children were asked to pretend to chew on a piece of chewing gum in order to stimulate saliva flow. The saliva was then collected into 50 mL polypropylene conical tubes (Fisher Brand, Fisher Scientific; Mexico). Parents were provided with another two 50 mL conical tubes for the collection of saliva at home. They were asked to aid their children in collecting one saliva sample later that same afternoon of play and another one the next morning. The samples were stored as described above.

4.2.2 Sample Processing

Urine Sample Processing

The urine samples were removed from the freezer on the day of analysis and thawed at room temperature. One mL of the defrosted sample was aspirated into 1 mL syringes (BD Biosciences; Franklin Lakes, NJ) and filtered

through 0.45 µm nylon membrane filters (Whatman Int.; Maidstone, UK) into the high performance liquid chromatography (HPLC) sampling vials (National Scientific; Rockwood, TN) for analysis.

Saliva Sample Processing

On each day of analysis, saliva samples were removed from the freezer and stored at room temperature until thawed. Once thawed, they were vortexed for 1 min and diluted 1 in 3 with deionized water. The samples were then ultrasonicated for 5 minutes and centrifuged at 4000 rpm for 15 min. From the supernatant, 1 mL was syringe filtered as described above.

4.2.3 Sample Analysis

Analysis of Urine Samples

HPLC was used to separate the arsenic species in the urine with an anion exchange column (PRP-X100, 5 µm, 150 x 4.1 mm; Hamilton; Reno, NV). The mobile phase for the separation was 35 mmol/L ammonium bicarbonate in 5% methanol, pH adjusted to 8.2. A gradient elution included the following flow rates: 0–4 min: 0.8 mL/min; 4–10 min: 1.7 mL/min; and 10–16 min: 0.8 mL/min. The sample injection volume was 50 µL. All samples were analyzed in duplicate.

The arsenic species were detected using an inductively coupled plasma-mass spectrometer (ICPMS) (6100DRC Plus; Perkin-Elmer Sciex; Norwalk, CT). The species of arsenic investigated included arsenobetaine (AsB), inorganic trivalent and pentavalent arsenic [iAs(III) and iAs(V)], dimethylarsinic acid

[DMA(V)], and monomethylarsonic acid [MMA(V)]. The ICPMS was calibrated using one arsenic standard containing 10 µg/L each of AsB, iAs(III), DMA(V), MMA(V) and iAs(V). AsB was obtained from Tri Chemical Laboratories (Yamanashi, Japan), iAs(III), iAs(V), and DMA(V) were obtained from Sigma-Aldrich (Milwaukee, WI), and MMA(V) was from Chem Service (West Chester, PA). This standard was measured once at the beginning of each day as well as once every three samples. Certified reference material (No. 18, National Institute for Environmental Studies; Tsukuba, Ibaraki, Japan) was quantified once daily prior to the analysis of the playground saliva samples as a quality control measure of accuracy.

Analysis of Saliva Samples

A reverse phase HPLC column (ODS-3, 150 x 4.6 mm, 3 µm particle size; Phenomenex; Torrance, CA) was used for the separation of arsenic in the saliva samples. The mobile phase contained 5 mmol/L tetrabutylammonium hydroxide and 3 mmol/L malonic acid in 5% methanol, with the pH adjusted to 5.85, as previously described [21, 22] The temperature for the separation was maintained at 48 °C while using an isocratic elution mode with a flow rate of 1.2 mL/min. The sample injection volume was 50 µL. Each sample was analyzed in duplicate.

Arsenic detection was conducted using ICPMS. The instrument was calibrated using three concentrations of arsenic standards (1, 5, and 10 µg/L of iAs(III), DMA(V), MMA(V), and iAs(V)). These samples were measured at the

beginning of each run. The 10 µg/L standard was analyzed again once every five samples. Certified reference material (No. 18, National Institute for Environmental Studies; Tsukuba, Ibaraki, Japan) was analyzed for quality control as described above.

4.2.4 Creatinine Measurement

The urine samples were sent to the University of Alberta Hospital Laboratory for creatinine analysis. The assay was performed using the UniCel® DxC 800 System (Beckman Coulter; Fullerton, CA). The principle was based on a spectrophotometric method that measured the creatinine-picrate end product of the reaction between creatinine and picric acid (see reference 26 in Chapter 3).

4.2.5 Statistical Analysis

The data were analyzed using Intercooled STATA 9.2 (StataCorp; College Station, TX). An analysis of variance (ANOVA) was used to compare the arsenic concentrations across the various time points. To compare the concentration of the arsenic species among CCA and non-CCA playgrounds, the Student's *t*-test was used. The Student's *t*-test was also used for the comparison of the children's age, frequency of play, and duration of play between the CCA and non-CCA groups. The Pearson's Correlation Coefficient was used to determine the relationship between the arsenic concentration in urine and saliva, and each of the following factors: the age, frequency of play, and duration of play of the children. Any *p*-values less than 0.05 were considered statistically significant.

4.3. RESULTS

4.3.1 Arsenic Concentrations in Urine and Saliva

Urine

In the urine samples, AsB, iAs(III), DMA(V), and MMA(V) were detected, but iAs(V) was not (Figure 4.1). The mean concentrations of AsB, iAs(III), DMA(V), and MMA(V) in urine samples of children playing on CCA playgrounds were 6.5 ± 12, 1.2 ± 2.0, 13 ± 23, and 1.4 ± 3.3 µg/L, respectively (Table 4.1). From the urine of children playing on non-CCA playgrounds, the AsB, iAs(III), DMA(V), and MMA(V) concentrations were 14 ± 25, 2.6 ± 5.4, 9.8 ± 20, and 0.7 ± 0.6 µg/L, respectively. The sum of the urinary iAs(III), DMA(V), and MMA(V) concentrations in the urine of children playing on CCA playgrounds was 15 ± 28 µg/L, and in that of children playing on non-CCA playgrounds was 12 ± 23 µg/L (Table 4.1). As each participant was requested to collect three post-play urine samples, ANOVA was used to determine whether any differences existed between the sums of each arsenic species across the three time points. In both the CCA and non-CCA playgrounds, the differences were not significant (p = 0.92 and p = 0.83, respectively). Thus, the various time points were grouped together when comparing the sums of iAs(III), DMA(V), and MMA(V) in the urine of children playing on CCA and non-CCA playgrounds. Using the Student's t-test, the sum of iAs(III), DMA(V), and MMA(V) between children's urine from the CCA and non-CCA playgrounds was not statistically significant (p = 0.60) (Figure 4.2).

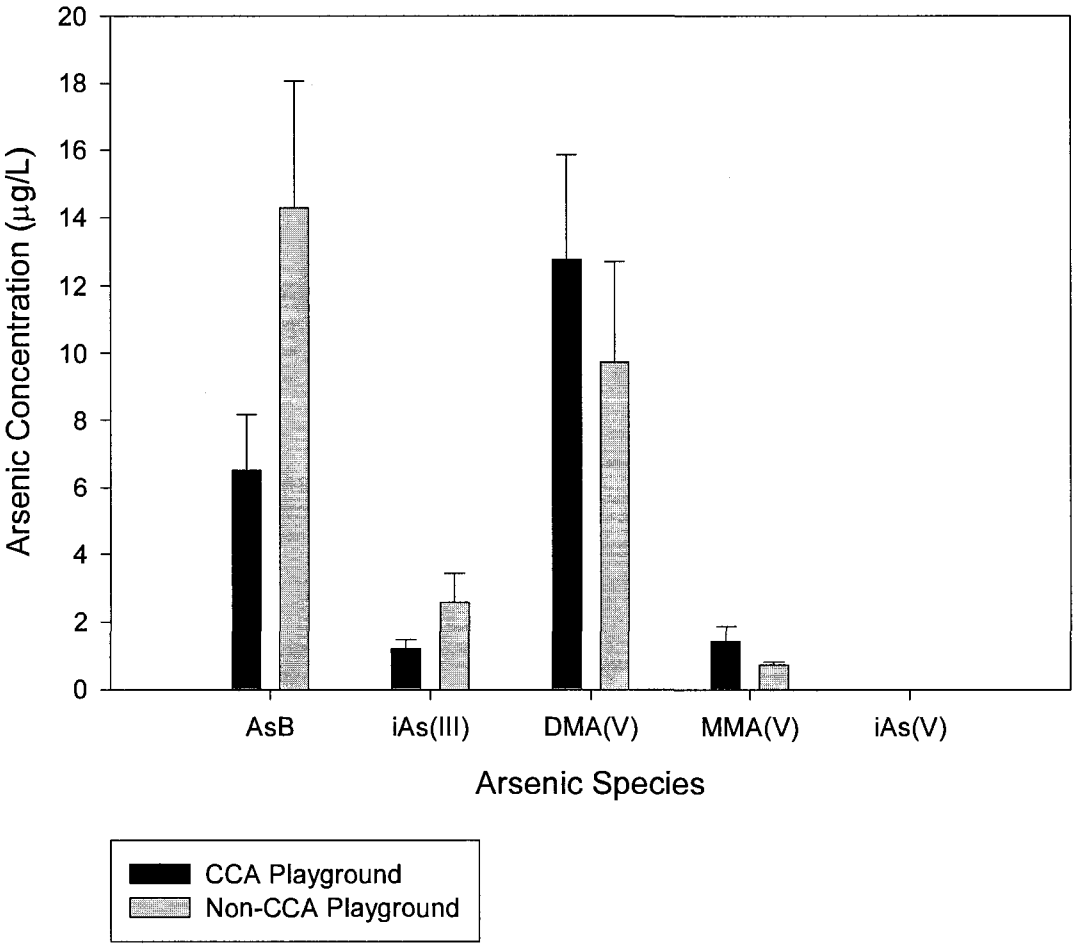


Figure 4.1 Mean concentrations of AsB, iAs(III), DMA(V), MMA(V), and iAs(V) in the urine of children playing on CCA and non-CCA playgrounds. Inorganic pentavalent arsenic (iAs(V)) was not detected in the urine samples from children playing on both CCA and non-CCA playgrounds.

Table 4.1. Concentrations of arsenic species detected in the urine and saliva samples of children playing on CCA and non-CCA playgrounds. *n.d. = not detected.

Sample Type	Playground Type	Arsenic Species	Urinary Arsenic Concentration (µg/L)		
			Mean ± SD	Median	Range
Urine					
	CCA				
		AsB	6.5 ± 12	1.7	n.d.*-49
		iAs(III)	1.2 ± 2.0	0.5	n.d.-12
		DMA(V)	13 ± 23	3.5	0.6-126
		MMA(V)	1.4 ± 3.3	0.6	0.1-24
		iAs(V)	n.d.	n.d.	n.d.
		Sum	15 ± 28	4.4	1.0-161
	Non-CCA				
		AsB	14 ± 25	2.6	n.d.-98
		iAs(III)	2.6 ± 5.4	0.7	n.d.-30
		DMA(V)	9.8 ± 20	4.6	n.d.-118
		MMA(V)	0.7 ± 0.6	0.5	n.d.-2.6
		iAs(V)	n.d.	n.d.	n.d.
		Sum	12 ± 23	6.1	0.9-124
Saliva					
	CCA	iAs(III)	0.8 ± 1.4	0.3	n.d.-5.4
		DMA(V)	0.3 ± 0.1	0.2	n.d.-0.4
		MMA(V)	n.d.	n.d.	n.d.
		iAs(V)	0.9 ± 1.6	0.5	n.d.-11
		Sum	1.1 ± 2.1	0.6	n.d.-14
	Non-CCA				
		iAs(III)	1.0 ± 0.4	1.0	n.d.-1.3
		DMA(V)	0.5 ± 0.2	0.4	n.d.-0.9
		MMA(V)	0.4 ± 0.2	0.4	n.d.-0.5
		iAs(V)	1.2 ± 0.8	0.9	n.d.-2.9
		Sum	1.4 ± 1.1	1.0	n.d.-3.9

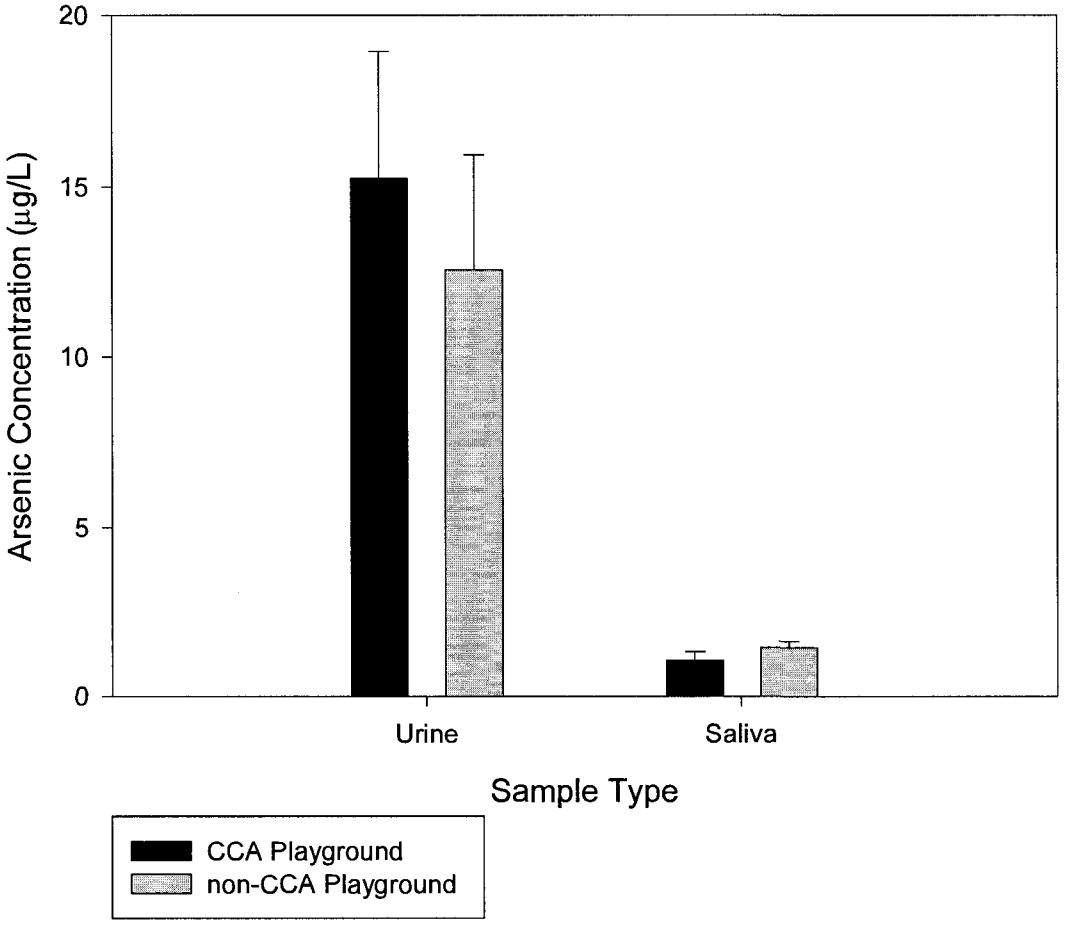


Figure 4.2 Sum of iAs(III), DMA(V), MMA(V), and iAs(V) in the urine and saliva samples from children playing on CCA and non-CCA playgrounds. There were no significant differences in the sum of species in urine (p = 0.60) and saliva (p = 0.32) samples from the CCA and non-CCA groups.

Between the CCA and non-CCA playgrounds, the relative percentages of each species were similar in that DMA(V) was the major arsenic species in urine, with some iAs(III) and MMA(V) present as well. In the urine samples from children playing on CCA playgrounds, DMA(V) comprised 76 ± 11 % of the total arsenic (excluding AsB), with iAs(III) at 10 ± 8 %, and MMA(V) at 14 ± 9 %. The relative concentrations of DMA(V), iAs(III), and MMA(V) in the urine of children playing on non-CCA playgrounds were 73 ± 20 %, 17 ± 22 %, and 10 ± 7 %, respectively.

Saliva

In the saliva samples from children playing on CCA playgrounds, iAs(III), DMA(V), and iAs(V) were detected (Figure 4.3), and their mean concentrations were 0.8 ± 1.4, 0.3 ± 0.1, and 0.9 ± 1.6 µg/L, respectively (Table 1). In the saliva samples of children playing on non-CCA playgrounds, iAs(III), DMA(V), MMA(V), and iAs(V) were detected with concentrations of 1.0 ± 0.4, 0.5 ± 0.2, 0.4 ± 0.2, and 1.2 ± 0.8 µg/L, respectively.

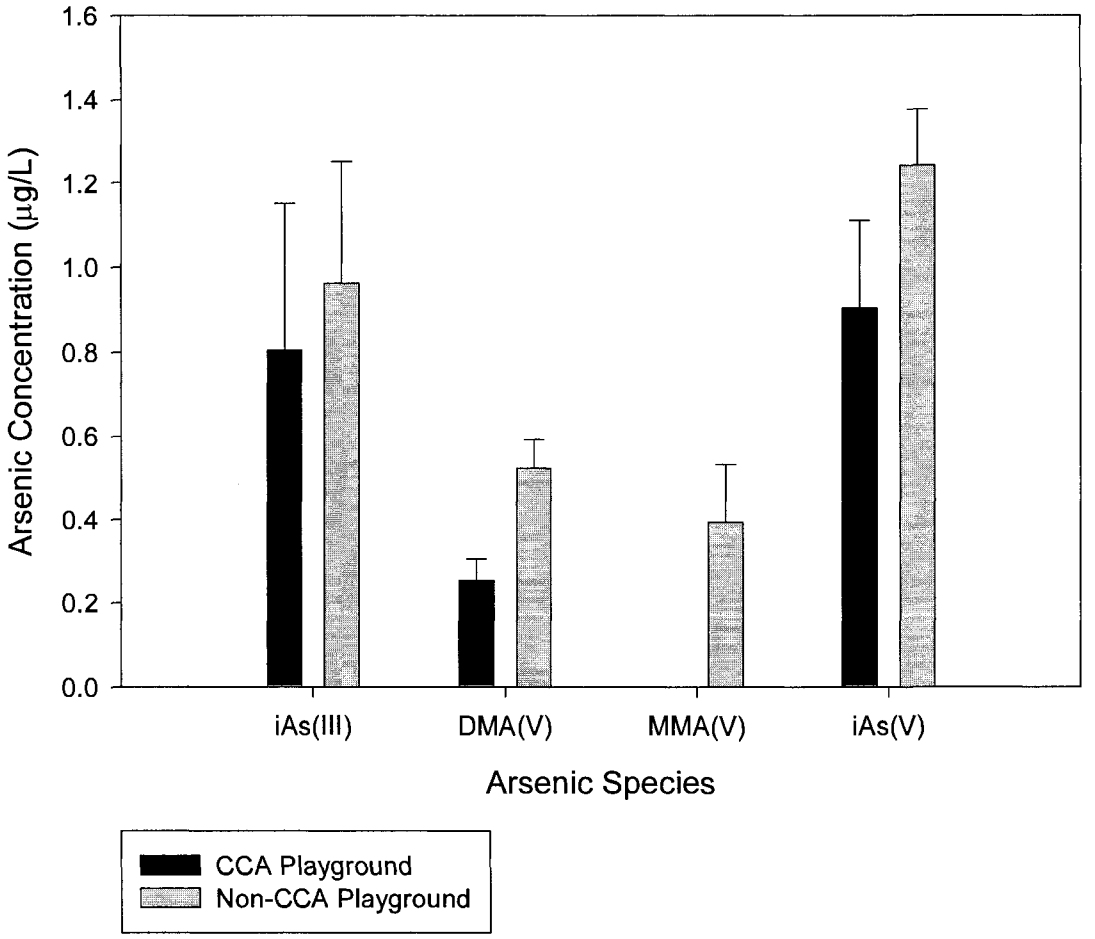


Figure 4.3 Mean concentrations of iAs(III), DMA(V), MMA(V), and iAs(V) in the saliva of children playing on CCA and non-CCA playgrounds. From the saliva of children playing on CCA playgrounds, iAs(III), DMA(V), and iAs(V) were detected. All four arsenic species were detected in the saliva of children playing on non-CCA playgrounds.

In the majority of the saliva samples, the concentrations of iAs(III), DMA(V), and MMA(V) were undetectable (Table 1). However, iAs(V) was detected in over 80 % of the samples from children playing on both CCA and non-CCA playgrounds. From the concentrations detected, the major arsenic species in the saliva samples from both the CCA and non-CCA groups was iAs(V) (Figure 4.3). In the CCA group, the relative concentrations of iAs(III), DMA(V), MMA(V), and iAs(V) were 11 ± 24 %, 2 ± 7 %, 0 ± 0 %, and 88 ± 25 %, respectively. In the non-CCA group, the relative concentrations of iAs(III), DMA(V), MMA(V), and iAs(V) were 2 ± 11 %, 4 ± 10 %, 1 ± 3 %, and 92 ± 15 %, respectively.

No child submitted any pre-play saliva samples from the non-CCA group, thus ANOVA was used to compare the salivary arsenic concentrations in the three sets of post-play samples collected at the playground and at home in both playground groups. In both the CCA and non-CCA groups, there were no differences in the sum of all arsenic species across the three time points (p = 0.56 and p = 0.21, respectively). Since the sums of each arsenic species in saliva were similar across the three time points, the data gathered from the different time points were grouped together to compare the differences in the salivary arsenic among children playing on CCA and non-CCA playgrounds. The mean sum of the arsenic species was 1.1 ± 2.1 µg/L in the saliva samples from the CCA group and 1.4 ± 1.1 µg/L in the non-CCA group (Table 1). According to the Student's t-test, the difference between these concentrations was not statistically significant (p = 0.32) (Figure 4.2).

4.3.2 Demographics of the Participating Children

Since there were no significant differences between the total arsenic concentrations in urine and saliva in both the CCA and non-CCA playgrounds, the demographic data were examined to determine if any of these variables may have confounded the results. The descriptions of the demographic factors for the urine and saliva samples were separated as not all children who participated in the saliva collection submitted urine samples and vice versa.

Urine

A total of 45 children participated in the urine collection (Table 4.2). Twenty-six of these children played on CCA-treated playgrounds, while the remaining 19 children played on non-CCA playgrounds. From the children in the CCA group, 56 urine samples were collected. Forty-five urine samples were collected from children in the non-CCA group.

Urine was collected from 17 males (65 %) and 9 females (35 %) playing on CCA playgrounds, and from 10 males (53 %) and 9 females (47 %) playing on non-CCA playgrounds. The average age of the children who played on CCA playgrounds was 6.7 ± 3.7 years (Table 4.2). In the group who played on non-CCA playgrounds, the average age was 5.3 ± 3.2 years. The difference between the ages of these two groups was not significant (p = 0.14).

Table 4.2 Age, frequency of play and duration of play of the children playing on CCA and non-CCA playgrounds.

Playground Type	Sample Type	Number of Children	Number of Samples		Age (years)	Frequency of Play (times per week)	Duration of Play (min
CCA							
	Urine	26	56	Mean ± SD	6.7 ± 3.7	1.3 ± 1.5	68.8 ± 33.6
				Median	6.0	1.0	62.5
				Range	1.0-14.0	0.02-7.0	25.0-135.0
	Saliva	41	78	Mean ± SD	7.3 ± 3.2	1.5 ± 2.1	68.8 ± 41.6
				Median	6.0	0.5	60.0
				Range	2.0-14.0	0.02-7.0	15.0-150.0
Non-CCA							
	Urine	19	45	Mean ± SD	5.3 ± 2.0	4.9 ± 4.3	46.3 ± 28.1
				Median	5.0	4.0	45.0
				Range	3.0-10.0	3.0-10.0	10.0-100.0
	Saliva	24	47	Mean ± SD	5.2 ± 2.0	5.0 ± 4.2	51.5 ± 26.3
				Median	5.0	5.0	60.0
				Range	3.0-10.0	0.8-14.0	10.0-100.0

Saliva

Saliva samples were obtained from 64 children. Of these, 40 children played on CCA-treated playgrounds and 24 children played on non-CCA playgrounds. In total, 83 saliva samples were received from children in contact with CCA playgrounds and 52 saliva samples from children playing on non-CCA playgrounds. Five saliva samples from the CCA group and 5 samples from the non-CCA group were not included in the sample analysis due to an insufficient sample volume, leaving 78 CCA saliva samples and 47 non-CCA saliva samples for arsenic speciation and quantification. As a result, this disqualified 3 children from the CCA group and one child from the non-CCA group from participating in the saliva analysis portion of the study.

From the CCA-treated playgrounds, 22 males (59 %) and 15 females (41 %) participated and submitted 50 and 29 samples, respectively. There were 13 males (58 %) and 10 females (42 %) who participated from the non-CCA playgrounds. The mean age of the children participating from the CCA-treated playgrounds was 7.3 ± 3.2 years, and the mean age of the children participating from the non-CCA playgrounds was 5.2 ± 2.0 years (Table 4.2). There was a significant difference (p = 0.01) between the ages in these two groups due to the participation of 7 children older than 10 years on the CCA playgrounds. There were no children older than 10 years of age playing on the non-CCA playgrounds.

4.3.3 Duration of Play

Urine

The mean length of time children spent on the CCA playgrounds was 68.8 ± 33.6 min (Table 4.2). On non-CCA playgrounds, children played for a mean time of 46.3 ± 28.1 min. There was a significant difference in the duration of play between the CCA group and non-CCA group (p = 0.02). This difference was attributed to three children who played for 135 min on a CCA playground, while no children played for more than 100 min on a non-CCA playground.

Saliva

The mean length of time children played on CCA playgrounds was 68.8 ± 41.6 min (Table 4.2). On the non-CCA playgrounds, the duration of play was 51.5 ± 26.3 min. There was no significant difference in the duration of play between the CCA and non-CCA groups (p = 0.08).

4.3.4 Frequency of Play

Urine

On the CCA playgrounds, children's mean frequency of play was 1.3 ± 1.5 times per week (Table 4.2). The mean frequency of play on a non-CCA playground was 4.9 ± 4.3 times per week. The frequency of play on a CCA playground was statistically lower than on a non-CCA playground (p < 0.01). This was the result of several children (n = 6) with infrequent visits to CCA playgrounds (1–2 times per year).

Saliva

The mean frequency of play for children playing on CCA-treated playgrounds was 1.5 ± 2.1 times per week (Table 4.2). On non-CCA playgrounds the mean frequency of play was 5.0 ± 4.2 times per week. The difference between the frequency of play between the two groups was also statistically significant ($p < 0.01$), for the same reasons as stated above.

4.4 DISCUSSION

4.4.1 Arsenic Concentrations in Urine and Saliva

The normal background levels of arsenic in urine are 5–50 µg/L [23]. The mean urinary arsenic concentrations [sum of iAs(III), DMA(V) and MMA(V)] from this study are 16 ± 28 µg/L in the CCA group and 13 ± 23 µg/L in the non-CCA group. These concentrations are within the normal background arsenic levels in the urine. Populations exposed to arsenic concentrations higher than the World Health Organization's maximum contaminant level of 10 µg/L in drinking water may have total urinary arsenic concentrations exceeding 150 µg/L [4, 24]. Shalat *et al.* (2006) did not find a relationship between contact with CCA-treated wood and the arsenic concentrations in urine; this is also in accordance with our data [13].

Arsenite, DMA(V), MMA(V), and iAs(V) were detected in the saliva samples with a mean sum of 1.1 ± 2.1 µg/L in children playing on CCA playgrounds and 1.4 ± 1.1 µg/L in children playing on non-CCA playgrounds. The major arsenic species in the saliva samples of children playing on CCA and

non-CCA playgrounds was iAs(V). This species was detected in 83 % of the samples in the CCA group and 81 % of the samples in the non-CCA group. In contrast, MMA(V) was not detected in any of the samples from the CCA group and in 4 % of the samples in the non-CCA group. These results agree with the data from Yuan *et al.* (2008) [25]. In the saliva collected from 32 adult volunteers living in Edmonton, Canada, iAs(V) was found in the most samples, whereas MMA(V) was found in the least. In this population, the arsenic concentration in the drinking water was less than 5 µg/L, and the mean sum of iAs(III), DMA(V), MMA(V), and iAs(V) was 0.79 µg/L. In comparison, saliva samples collected from 301 volunteers living in inner Mongolia contained iAs(III) and iAs(V) as the major species. The mean sum of iAs(III), DMA(V), MMA(V), and iAs(V) in saliva was 11.9 µg/L. As the Mongolian population was exposed to high concentrations of arsenic in their drinking water (up to 826 µg/L), there is a higher detection rate of each arsenic species in saliva, as well as in higher concentrations than from the Edmonton volunteers.

Three urine samples and two saliva samples were to be collected at the participants' homes, in addition to any saliva samples collected at the playgrounds. Arsenic has a short half-life of approximately 1–3 h in blood; its metabolites are excreted in the urine with a half-life between 2 and 3 days [23, 26-29]. Therefore, we asked that urine be collected at three time points following play as a potential increase in the concentrations of arsenic in the urine may be attributed to exposure to playgrounds constructed using CCA-treated wood. The ANOVA test determined that there were no significant differences between the

urinary arsenic concentrations across the three time points. This is true for both CCA and non-CCA playgrounds (p = 0.92 and p = 0.83, respectively). Several arsenic ingestion studies report that an increase in arsenic occurs in the urine within 24 hours following consumption [26, 30, 31]. Since arsenic concentrations were measured in urine collected on the same day of play, and on the morning and early afternoon of the day following play (up to 24 hours post-play), no differences were detected in the total arsenic concentrations after this time. Therefore, children playing on the CCA-treated playgrounds were not ingesting a considerable amount of arsenic from the treated wood.

The concentrations of the arsenic species in each individual are presented in Tables A.1 and A.2 in the Appendix. Although on average there were no statistical differences between the sum of arsenic species in the CCA and non-CCA groups there are certain individuals with very high concentrations of DMA(V). However, the relative percentage of DMA(V) in the CCA group is 76% and in the non-CCA group is 73%. There were no statistical differences between the ratio of DMA(V) to the sum of arsenic species across the two playground groups (p = 0.28). This suggests that there were no differences in the arsenic ingestion between the two groups.

Any significant differences in the total salivary arsenic concentrations in the children playing on CCA and non-CCA-treated playgrounds were also investigated. Since pre-play saliva samples from children in contact with non-CCA-treated wood could not be obtained, ANOVA test was performed only on the post-play saliva samples and the two samples collected at home. In the

saliva samples from children playing on CCA playgrounds, there was no difference in the total arsenic concentrations across the three time points (p = 0.56). There was also no significant difference in the total salivary arsenic concentration in the children playing on non-CCA treated playgrounds across the three time points (p = 0.21). The half-life of arsenic in saliva is not well-known, but with the samples collected at three time points: post-play, afternoon of the day of play, and morning following the day of play; there were no significant differences in the arsenic concentrations reported.

4.4.2 Demographics of the Participating Children

Urine samples were obtained from children playing on 5 CCA and 5 non-CCA playgrounds and saliva samples from children playing on 6 CCA and 6 non-CCA playgrounds dispersed throughout the city of Edmonton. Samples were not obtained from all eight CCA and eight non-CCA playgrounds because of the lack of children at some playgrounds on those days. This may have been due to poor weather conditions on the days we visited or the result of children being in class, since the sampling period overlapped with the commencement of the school year.

Variables that were maintained between the CCA and non-CCA playgrounds included the age and the manufacturer of the playgrounds. The weather conditions documented for each playground site were similar between the two types of playgrounds. On each sampling day, the playground visits were alternated between CCA and non-CCA. Demographic factors such as age and

sex, or the duration and frequency of play were not controlled for to refrain from limiting the number of children participating in the study. In the saliva collection, there were statistical differences in the mean ages and frequency of play between the CCA and non-CCA group. In the urine collection, there were also significant differences in the mean frequency of play and the duration of play between the children playing on the CCA playgrounds and the children playing on non-CCA playgrounds. However, the distributions of the children's ages and durations of play were similar between the two types of playgrounds for both the urine and saliva collections [Figures 4.4 (a-b) and Figures 4.5(a-b)]. The distributions of the frequency of play showed that there were more children who played less frequently on the CCA playgrounds [Figures 4.6 (a-b)]; however the number of participants in the CCA group was also higher. No correlation was found between these demographic factors and the arsenic concentrations in urine and saliva. In both the urine and saliva samples, there was an absence of a strong correlation between the age of the child and arsenic concentration (r = 0.26 and -0.17, respectively) [Figures 4.7(a-b)]. There was also a lack of a relationship between the duration of play and arsenic concentrations in the urine and saliva (r = -0.25 and -0.06, respectively) [Figures 4.8(a-b)]. When comparing the frequency of play and the arsenic concentration in urine and saliva, there was also an absence of any relationship (r = -0.19 and -0.07, respectively) [Figures 4.9(a-b)].

The mean ages of children in contact with CCA playgrounds were 6.7 ± 3.7 years (urine collection) and 7.3 ± 3.2 years (saliva collection). From the

children playing on non-CCA playgrounds, the mean ages were 5.3 ± 2.0 years (urine) and 5.2 ± 2.0 years (saliva). Although studies suggest that young children demonstrate the most frequent hand-to-mouth behavior [17, 18, 32], this activity can still occur in older children and adults [33, 34]. Thus, older children are also at risk of ingesting arsenic from CCA-treated playgrounds if they do not wash their hands between contact with CCA-treated wood and hand-to-mouth activity.

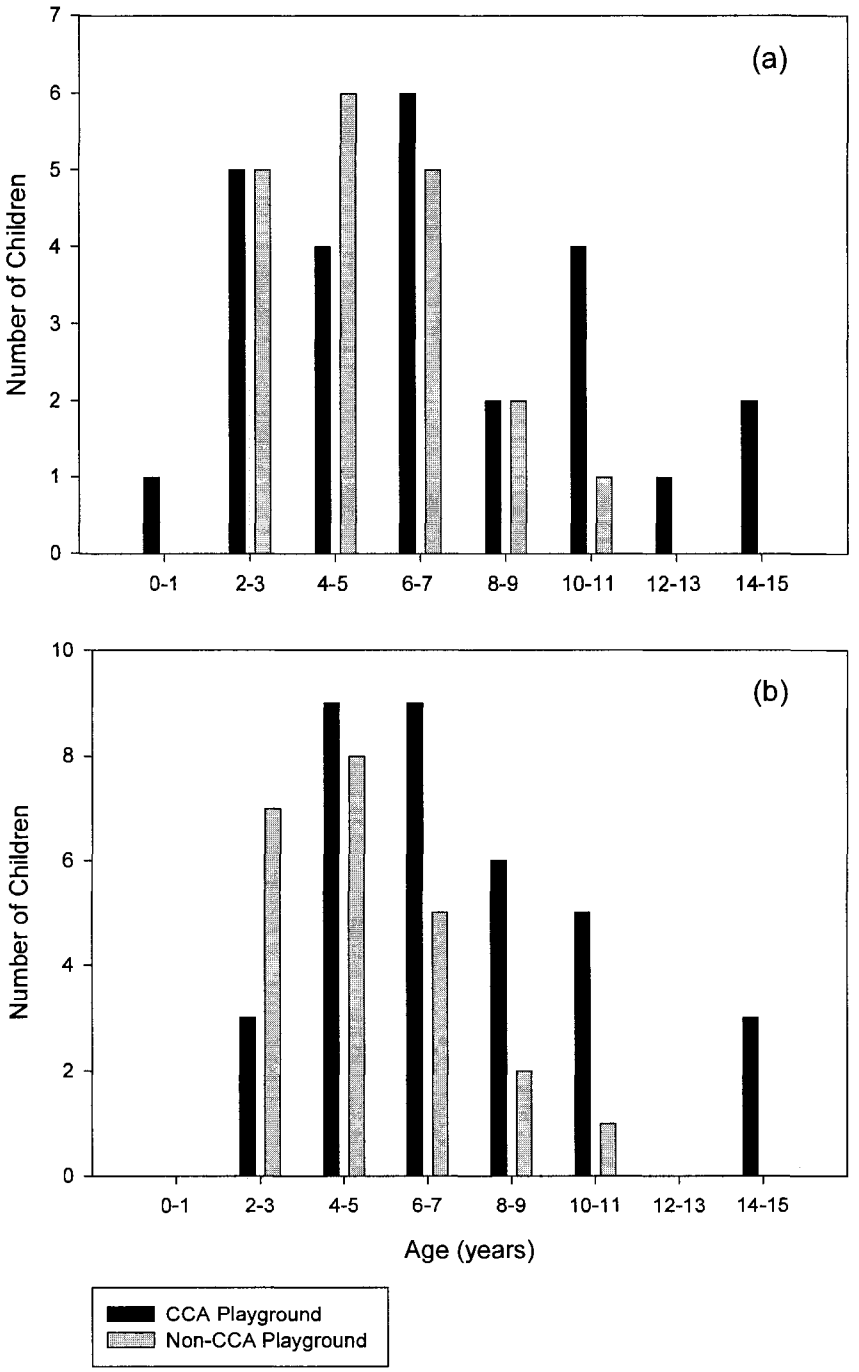


Figure 4.4 Distribution of children's ages playing on CCA and non-CCA playgrounds who submitted (a) urine and (b) saliva samples. The median age of children submitting urine and saliva samples from the CCA group was 6 years and 5 years from the non-CCA group.

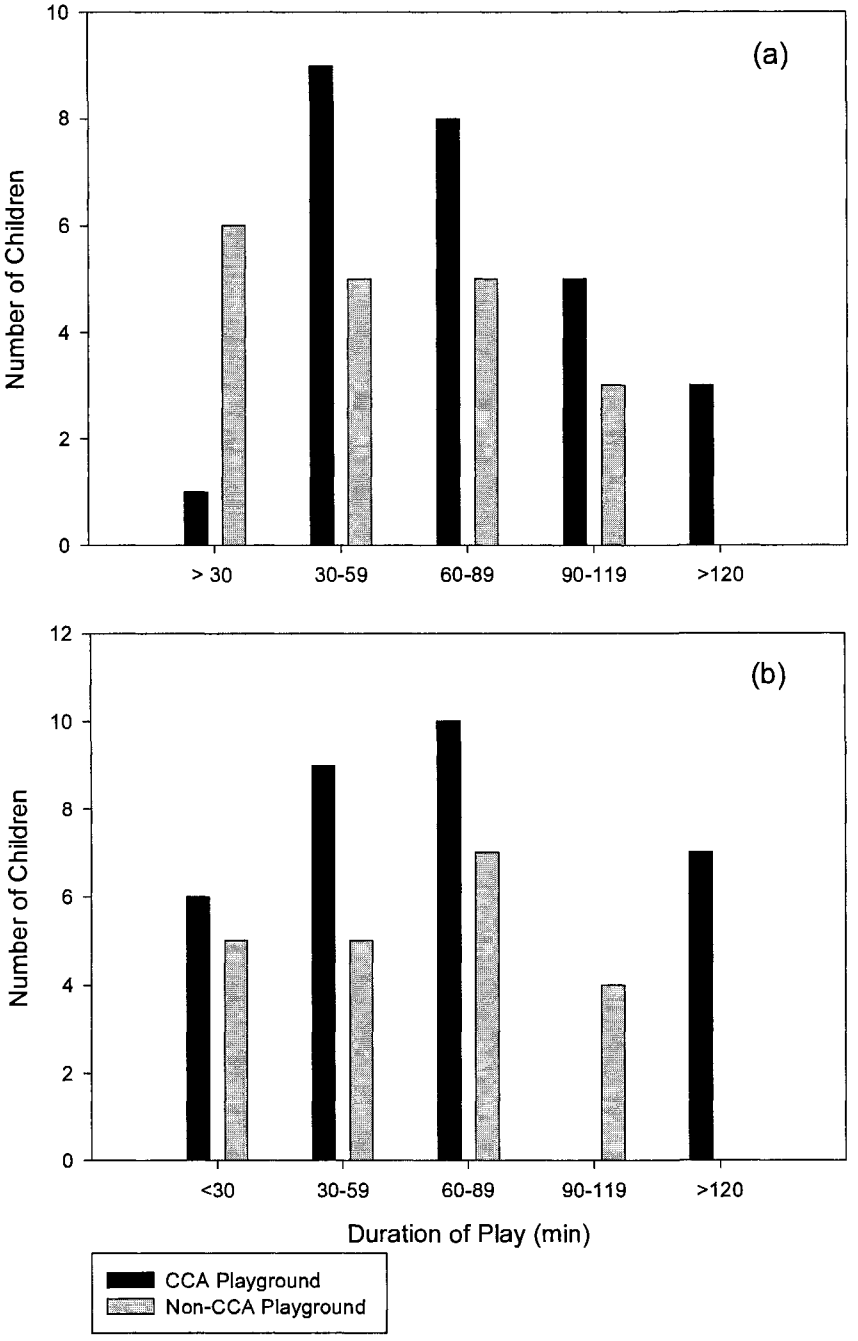


Figure 4.5 Amount of time children spent on the CCA and non-CCA playgrounds and submitted (a) urine and (b) saliva samples. In the urine collection, the median duration of play was 62.5 min in the CCA group and 45.0 min in the non-CCA group. For saliva collection, the median duration of play was 60.0 min in both the CCA and non-CCA groups.

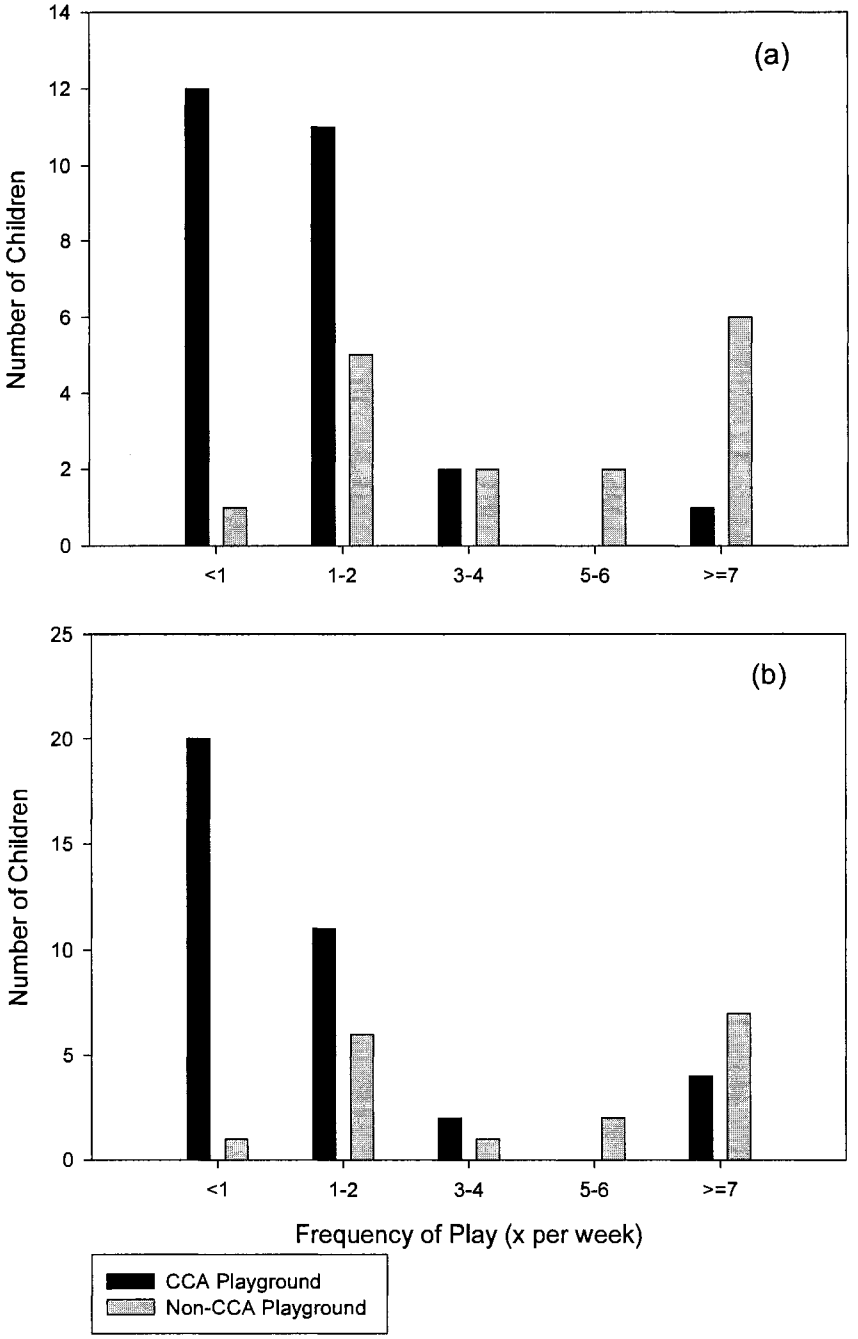


Figure 4.6 Frequency of children's playground activity on CCA and non-CCA playgrounds who submitted (a) urine and (b) saliva samples. In the children who submitted urine samples, the median frequency of play on CCA playgrounds was 1.0 times per week and on non-CCA playgrounds was 4.0 times per week. Children who submitted urine samples had a mean frequency of play of 0.5 times per week in the CCA group and 5.0 times per week on the non-CCA playgrounds.

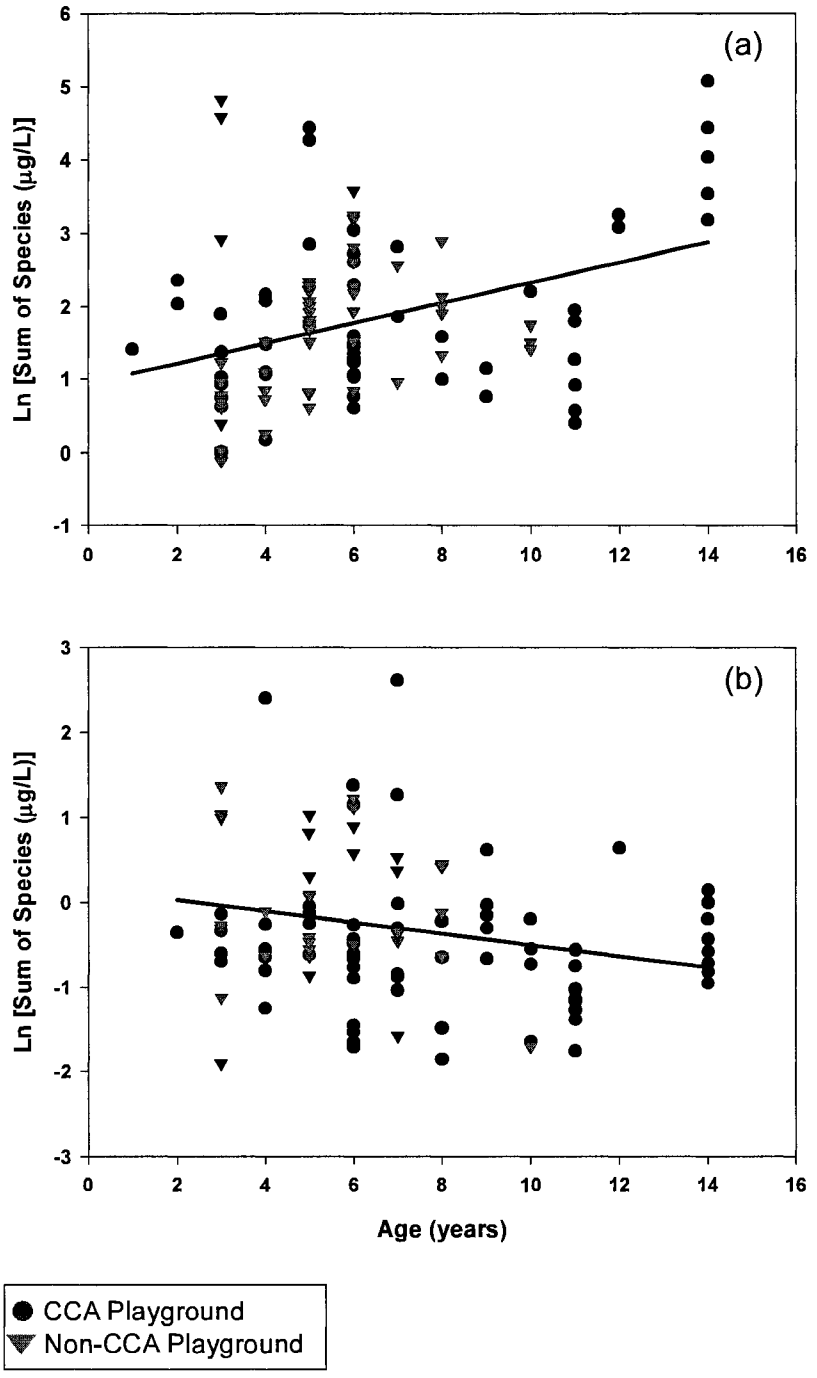


Figure 4.7 Correlation between the age of the children and the (a) sum of iAs(III), DMA(V) and MMA(V) in urine (r = 0.35) (b) sum of iAs(III), DMA(V), MMA(V) and iAs(V) in saliva (r = -0.22).

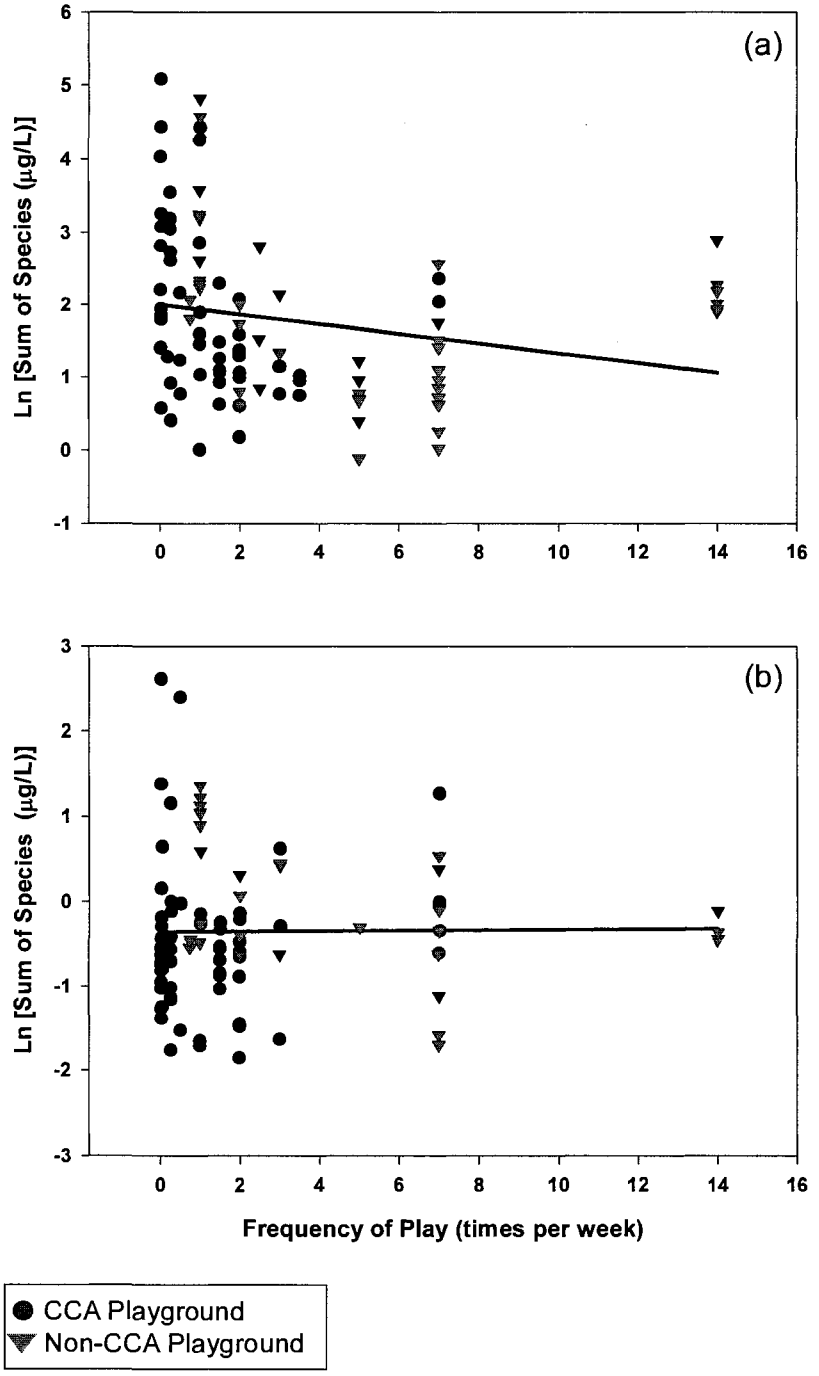


Figure 4.8 Correlation between the frequency of play and the (a) sum of iAs(III), DMA(V) and MMA(V) in urine (r = -0.18) (b) sum of iAs(III), DMA(V), MMA(V) and iAs(V) in saliva (r = 0.01).

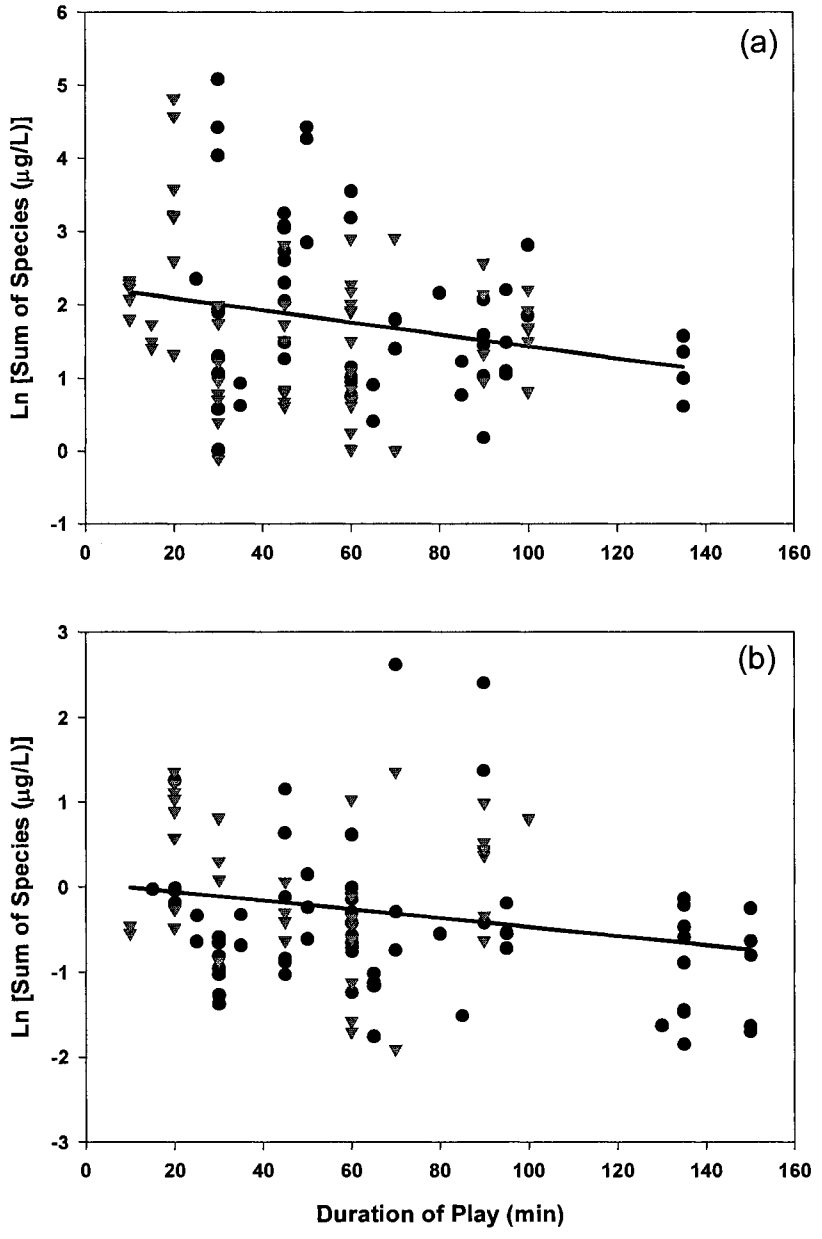


Figure 4.9 Correlation between the duration of play and the (a) sum of iAs(III), DMA(V) and MMA(V) in urine (r = -0.25) (b) sum of iAs(III), DMA(V), MMA(V) and iAs(V) in saliva (r = -0.23).

4.4.3 Limitations

The children's arsenic intake from dietary sources was not controlled. Normally, participants in arsenic speciation analysis studies are asked to refrain from ingesting food high in arsenic compounds (such as seafood) three days prior to sample collection and during the sample collection period. Arsenobetaine is present in seafood such as shellfish, and is excreted from the human body unchanged [29, 31, 35]. When measuring total arsenic in urine, the presence of AsB can confound the quantification [36]. Thus it is important to perform arsenic speciation in urine samples to ensure that the arsenic measured is from a significant source. We did not report total arsenic, but rather the sum of the species detected, which included iAs(III), DMA(V), and MMA(V).

Following consumption of foods such as seaweed, mussels, and clams, which are high in arsenosugars, the DMA(V) concentration increases as it is a product of arsenosugar metabolism [30, 37, 38]. The presence of DMA(V) from seafood sources may falsely increase the arsenic speciation results. Normally, the relative concentration of DMA(V) in urine is 60–80% of the total arsenic levels [39]. Consumption of arsenosugars may increase the relative percentage of DMA(V) in the urine. In this study, the percentage of DMA(V) in the urine of children who were in contact with CCA-treated playgrounds was 76 ± 11 % (Table A.1). In the urine samples of children playing on non-CCA playgrounds, the relative DMA(V) concentration was 73 ± 20 % (Table A.2). Using the Student's *t*-test, there was no significant difference between the relative percentage of DMA(V) between the CCA and non-CCA group (p = 0.29).

However, if arsenic speciation analysis were to be performed again in the future, it is recommended that participants refrain from ingesting seafood for at least three days prior to urine sample collection, as well as during the duration of the urine collection period [36, 38]. This will provide the most accurate assessment for biomarkers of arsenic exposure in the urine.

The urinary arsenic concentrations were not adjusted for creatinine concentration, however the results of the creatinine measurement are available in Tables A.1 and A.2 in the Appendix.

4.5 CONCLUSIONS

Kwon *et al.* (2004) have previously reported a mean of 561 ± 552 ng of arsenic on the hands of children in contact with playgrounds constructed from CCA-treated wood [11]. If this entire amount were consumed, there would still be more arsenic ingested in the daily diet (~ 15 µg) than from the dermal arsenic on the children's hands [19]. The results supported the hypothesis as there was no significant difference in the arsenic concentration and speciation patterns between the urine and saliva samples of children playing on CCA-treated and non-CCA-treated playgrounds. Children from the CCA group did not have higher total concentrations of arsenic in their urine and saliva in comparison to children in the non-CCA group. Even though children frequently place their hands in their mouths, they did not appear to be ingesting a significant amount of arsenic that ended up on their hands following play on CCA playgrounds. The sum of iAs(III), DMA(V), and MMA(V) in the urine of children playing on CCA and non-CCA

playgrounds were 15 ± 28 µg/L and 12 ± 23 µg/L, respectively. These concentrations were within the normal background levels of arsenic in urine in the general population. Exposure to arsenic from the diet would have been more substantial than through contact with CCA playgrounds. Any ingestion of arsenic as a result of being in contact with CCA-treated wood would have likely been a minor contribution to the children's overall arsenic exposure.

Here the information gap regarding children's overall exposure to arsenic as a result of being in contact with CCA-treated playgrounds is filled. Biomonitoring of children's exposure to arsenic was performed by speciating and quantifying arsenic in the urine and saliva samples of children playing on CCA and non-CCA playgrounds. No significant differences in the arsenic concentrations and speciation patterns in these samples between the two groups of playgrounds were found. Thus, children's exposure to CCA-treated playgrounds does not considerably contribute to their total ingested arsenic. However, since arsenic can become dislodged onto the hands of children in contact with CCA-treated wood, they should still wash their hands thoroughly following play to reduce exposure to arsenic.

4.6 REFERENCES

[1] Health Canada. Fact Sheet on Chromated Copper Arsenate (CCA) Treated Wood. In: Agency PMR, ed. 2005.

[2] United States Environmental Protection Agency. Whitman Announces Transition From Consumer Use of Treated Wood Containing Arsenic. EPA Environmental News 2002 [cited June 13, 2007]; Available from: http://yosemite.epa.gov/opa/admpress.nsf/b1ab9f485b098972852562e7004dc686/1a8cfb4970823b3885256b5e006ffd67?OpenDocument

[3] Lepkowski W. Arsenic Crisis in Bangladesh. Chem Eng News. 1998 Nov 16;76(46):27-9.

[4] Rahman MM, Chowdhury UK, Mukherjee SC, Mondal BK, Paul K, Lodh D, et al. Chronic arsenic toxicity in Bangladesh and West Bengal, India--a review and commentary.[see comment][comment]. J Toxicol Clin Toxicol. 2001;39(7):683-700.

[5] Tseng WP. Effects and Dose-Response Relationships of Skin Cancer and Blackfoot Disease with Arsenic. Environ Health Perspect. 1977;19(AUG):109-19.

[6] World Health Organization. Arsenic in Drinking Water. [Fact Sheet 210] 2001 May 2001 [cited 26 Jan 2006]; Available from: http://www.who.int/mediacentre/factsheets/fs210/en/

[7] Hemond HF, Solo-Gabriele HM. Children's exposure to arsenic from CCA-treated wooden decks and playground structures. Risk Anal. 2004 Feb;24(1):51-64.

[8] Hatlelid KM, Bittner PM, Midgett JD, Thomas TA, Saltzman LE. Exposure and Risk Assessment for Arsenic from Chromated Copper Arsenate (CCA)-Treated Wood Playground Equipment. J Child Health. 2004;2(3):215 - 41.

[9] United States Environmental Protection Agency. A Probabilistic Risk Assessment for Children Who Contact CCA-Treated Playsets and Decks; 2003 November 10, 2003. Report No.: Draft Preliminary Report.

[10] United States Environmental Protection Agency. A Probabilistic Exposure Assessment for Children Who Contact CCA-Treated Playsets and Decks Using the Stochastic Human Exposure and Dose Simulation Model for the Wood Preservative Exposure Scenario (SHEDS-Wood). Draft Preliminary Report ed 2003.

[11] Kwon E, Zhang HQ, Wang ZW, Jhangri GS, Lu XF, Fok N, et al. Arsenic on the hands of children after playing in playgrounds. Environ Health Perspect. 2004 Oct;112(14):1375-80.

[12] Nico PS, Ruby MV, Lowney YW, Holm SE. Chemical speciation and bioaccessibility of arsenic and chromium in chromated copper arsenate-treated wood and soils. Environ Sci Technol. 2006 Jan;40(1):402-8.

[13] Shalat SL, Solo-Gabriele HM, Fleming LE, Buckley BT, Black K, Jimenez M, et al. A pilot study of children's exposure to CCA-treated wood from playground equipment. Sci Total Environ. 2006 Aug;367(1):80-8.

[14] Levenson MS, Thomas TA, Porter WK, Cobb DG, Davis D, Midgett JD, et al. A Field Study of Dislodgeable Arsenic from CCA-Treated Wood Using Human-Hand and Surrogate Wipes. J Child Health. 2004;2(3):197 - 213.

[15] De Miguel E, Iribarren I, Chacon E, Ordonez A, Charlesworth S. Risk-based evaluation of the exposure of children to trace elements in playgrounds in Madrid (Spain). Chemosphere. 2007 Jan;66(3):505-13.

[16] Barraj LM, Tsuji JS, Scrafford CG. The SHEDS-wood model: Incorporation of observational data to estimate exposure to arsenic for children playing on CCA-treated wood structures. Environ Health Perspect. 2007 May;115(5):781-6.

[17] AuYeung W, Canales RA, Beamer P, Ferguson AC, Leckie JO. Young Children's Mouthing Behavior: An Observational Study via Videotaping in a Primarily Outdoor Residential Setting. J Child Health. 2004;2(3):271 - 95.

[18] Tulve NS, Suggs JC, McCurdy T, Hubal EAC, Moya J. Frequency of mouthing behavior in young children. J Expo Anal Environ Epidemiol. 2002 Jul;12(4):259-64.

[19] Dabeka RW, McKenzie AD, Lacroix GMA, Cleroux C, Bowe S, Graham RA, et al. Survey of Arsenic in Total Diet Food Composites and Estimation of the Dietary Intake of Arsenic by Canadian Adults and Children. J AOAC Int. 1993 Jan-Feb;76(1):14-25.

[20] Hughes K, Meek ME, Burnett R. Inorganic Arsenic: Evaluation of Risks to Health from Environmental Exposure in Canada. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 1994;12(2):145-59.

[21] Le XC, Lu X, Li XF. Arsenic Speciation. Anal Chem. 2004;76(1):26A-33A.

[22] Le XC, Lu XF, Ma MS, Cullen WR, Aposhian HV, Zheng BS. Speciation of key arsenic metabolic intermediates in human urine. Anal Chem. 2000 Nov;72(21):5172-7.

[23] National Research Council. Arsenic in Drinking Water. Washington, DC: National Academy Press 1999.

[24] Harrington JM, Middaugh JP, Morse DL, Housworth J. Survey of a Population Exposed to High-Concentrations of Arsenic in Well Water in Fairbanks, Alaska. Am J Epidemiol. 1978;108(5):377-85.

[25] Yuan CG, Lu XF, Oro N, Wang ZW, Xia YJ, Wade TJ, et al. Arsenic speciation analysis in human saliva. Clin Chem. 2008;54(1):163-71.

[26] Buchet JP, Lauwerys R, Roels H. Comparison of the urinary excretion of arsenic metabolites after a single oral dose of sodium arsenite, monomethylarsonate, or dimethylarseinate in man. Int Arch Occup Environ Health. 1981;48(1):71-9.

[27] World Health Organization. Arsenic and Arsenic Compounds. 2 ed. Geneva: World Health Organization 2001.

[28] Crecelius EA. Changes in Chemical Speciation of Arsenic Following Ingestion by Man. Environ Health Perspect. 1977;19(Aug):147-50.

[29] Freeman HC, Uthe JF, Fleming RB, Odense PH, Ackman RG, Landry G, et al. Clearance of arsenic ingested by man from arsenic contaminated fish. Bull Environ Contam Toxicol. 1979 May;22(1-2):224-9.

[30] Heinrich-Ramm R, Mindt-Prufert S, Szadkowski D. Arsenic species excretion after controlled seafood consumption. J Chromatogr B Analyt Technol Biomed Life Sci. 2002 Oct;778(1-2):263-73.

[31] Le XC, Cullen WR, Reimer KJ. Human urinary arsenic excretion after one-time ingestion of seaweed, crab, and shrimp. Clin Chem. 1994 Apr;40(4):617-24.

[32] Xue JP, Zartarian V, Moya J, Freeman N, Beamer P, Black K, et al. A meta-analysis of children's hand-to-mouth frequency data for estimating nondietary ingestion exposure. Risk Anal. 2007 Apr;27(2):411-20.

[33] Cherrie JW, Semple S, Christopher Y, Saleem A, Hughson GW, Philips A. How important is inadvertent ingestion of hazardous substances at work? Ann Occup Hyg. 2006 Oct;50(7):693-704.

[34] Woods DW, Miltenberger RG. Are persons with nervous habits nervous? A preliminary examination of habit function in a nonreferred population. J Appl Behav Anal. 1996 Sum;29(2):259-61.

[35] Kaise T, Watanabe S, Itoh K. The Acute Toxicity of Arsenobetaine. Chemosphere. 1985;14(9):1327-32.

[36] Kales SN, Huyck KL, Goldman RH. Elevated urine arsenic: Un-speciated results lead to unnecessary concern and further evaluations. J Anal Toxicol. 2006 Mar;30(2):80-5.

[37] Buchet JP, Lison D, Ruggeri M, Foa V, Elia G. Assessment of exposure to inorganic arsenic, a human carcinogen, due to the consumption of seafood. Arch Toxicol. 1996 Sep;70(11):773-8.

[38] Ma MS, Le XC. Effect of arsenosugar ingestion on urinary arsenic speciation. Clin Chem. 1998 Mar;44(3):539-50.

[39] Vahter M. Genetic polymorphism in the biotransformation of inorganic arsenic and its role in toxicity. Toxicol Lett. 2000;112-113:209-17.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 REVIEW OF THESIS OBJECTIVES

The objective of this thesis was to perform arsenic biomonitoring in human biological samples as a measure of exposure determination. The hypothesis of this work was that urinary and salivary arsenic were suitable biomarkers for assessing exposure to arsenic. In Chapter 2, a method using high performance liquid chromatography combined (HPLC) with inductively coupled plasma mass spectrometry (ICPMS) was developed for the separation and detection of arsenic species in human biological samples. Once the method was developed, it was used in two biomonitoring applications. First, HPLC-ICPMS was used to determine arsenic speciation and quantification in urine and saliva following a controlled seafood ingestion experiment (Chapter 3). Second, the method was used in an environmental study assessing children's overall exposure to arsenic following activity on pressure-treated wood playgrounds (Chapter 4).

5.2 SUMMARY OF RESULTS

HPLC-ICPMS is a suitable method for arsenic speciation and detection because it is sensitive, robust, has a dynamic linear range, and is versatile in that many sample types can be analyzed using this method. HPLC-ICPMS was successfully used for the separation and quantification of five arsenic species: arsenobetaine (AsB), arsenite [iAs(III)], dimethylarsinic acid [DMA(V)],

monomethylarsonic acid [MMA(V)], and arsenate [iAs(V)]. AsB is the predominant arsenic species in seafood and is relatively non-toxic, while DMA(V) and MMA(V) are metabolites of inorganic arsenic [iAs(III) and iAs(V)] exposure. The following biomonitoring studies used HPLC-ICPMS to measure the arsenic speciation and concentrations in urine and saliva following ingestion and environmental exposure.

Following ingestion of crab, AsB was present in urine and saliva within a few hours. AsB accounted for a large portion of the total excreted arsenic, thus explaining the rapid increase in total arsenic following the crab meal. Both AsB and total arsenic concentrations peaked within eight hours of ingestion and returned to levels similar to baseline by 39 h post-ingestion. There was a strong correlation demonstrated between total arsenic in urine and saliva as well as AsB in urine and saliva.

Children's exposure to arsenic as a result of activity on playgrounds constructed from chromated copper arsenate (CCA)-treated wood was also monitored. Urine and saliva samples were collected following play and analyzed. Children playing on CCA-treated playgrounds did not appear to have higher arsenic concentrations or different speciation patterns compared to children playing on non-CCA playgrounds.

5.3 RELEVANCE TO ENVIRONMENTAL TOXICOLOGY

The arsenic ingestion project contributed to the understanding of arsenic metabolism and excretion in humans. Prior to this study, only one report had

been published pertaining to arsenic species present in human saliva [1]. However, the relationship between the arsenic excretion profile in urine and saliva was not well-understood. The seafood ingestion study in this thesis monitored arsenic excretion in urine and saliva before and after eating crab or nori. Following crab ingestion, arsenic concentrations were at their maximum levels at similar time points in both urine and saliva. The presence of AsB in saliva had not previously been addressed. Since AsB comes from seafood sources, its presence in the diet may confound biomonitoring results. The preliminary work in this thesis determined that AsB is present in saliva and follows a similar excretion profile as in urine.

An earlier work published indicated that children playing on playgrounds constructed from chromated copper arsenate (CCA)-treated wood have approximately four times as much arsenic on their hands as children playing on playgrounds built from other materials [2]. However, it was not known how much of the arsenic on the children's hands was potentially ingested. The biomonitoring study in this thesis determined children's overall exposure to arsenic as a result of activity on CCA-treated playgrounds. Urine and saliva samples were analyzed to measure inorganic arsenic and the associated metabolites. The results of this work indicated that the children playing on CCA-treated playgrounds did not have significantly higher concentrations of arsenic species in their urine and saliva compared to children playing on non-CCA playgrounds. Playing on CCA playgrounds is not a major contributor to children's

overall arsenic ingestion, rather food and water sources of arsenic are more significant to their overall daily intake [3-5].

5.4 LIMITATIONS OF RESEARCH

While the seafood ingestion study provided some insight on the excretion of arsenic in saliva, further work is required in order to improve this understanding. It has been shown that saliva contains a small amount of arsenic. With the sample preparation and separation procedures used, it is possible that there was some loss of arsenic along the way. Improved methods for concentrating saliva samples or increasing the amount of sample reaching the detector will allow for greater accuracy in the results of low analyte samples.

Biomonitoring is a useful method for determining exposure to arsenic through occupational and environmental settings. Recent exposure to arsenic from dietary sources may confound biomonitoring results. Even though arsenic speciation results may provide a clearer understanding of the potential sources of exposure, it would be most beneficial if participants in biomonitoring studies refrained from ingesting foods with high arsenic content three days prior to as well as during the sample collection period. An accurate history of any other potential sources of arsenic exposure in addition to the source of interest would also assist in the interpretation of biomonitoring results.

5.5 FUTURE DIRECTIONS

The collection of saliva is easier and more convenient to collect than urine. Saliva appears to be a suitable sample type for arsenic determination. In this thesis, preliminary results are presented relating to the excretion of arsenic in saliva following seafood ingestion and metabolism as well as exposure to CCA-treated playgrounds. However, further research into the understanding of arsenic excretion into saliva is required. This includes determining the biological half-life of arsenic in saliva, and whether or not arsenosugars and trivalent methylated arsenic species are present in saliva. Both urine and saliva should be analyzed simultaneously in future biomonitoring applications in order to further understand the correlation between the excretion of arsenic species in these two sample types.

Even though this thesis work determined that children playing on CCA playgrounds are not internally exposed to more arsenic than children playing on non-CCA playgrounds, arsenic is still a major health concern. Millions of people are still affected by high levels of arsenic in their drinking water and others may be occupationally exposed to arsenic. It is important to continue the practice of arsenic biomonitoring to determine overall human exposure to arsenic.

5.6 REFERENCES

[1] Yuan CG, Lu XF, Oro N, Wang ZW, Xia YJ, Wade TJ, et al. Arsenic speciation analysis in human saliva. Clin Chem. 2008;54(1):163-71.

[2] Kwon E, Zhang HQ, Wang ZW, Jhangri GS, Lu XF, Fok N, et al. Arsenic on the hands of children after playing in playgrounds. Environ Health Perspect. 2004 Oct;112(14):1375-80.

[3] Dabeka RW, McKenzie AD, Lacroix GMA, Cleroux C, Bowe S, Graham RA, et al. Survey of Arsenic in Total Diet Food Composites and Estimation of the Dietary Intake of Arsenic by Canadian Adults and Children. J AOAC Int. 1993 Jan-Feb;76(1):14-25.

[4] Tao SSH, Bolger PM. Dietary arsenic intakes in the United States: FDA total diet study, September 1991-December 1996. Food Addit Contam. 1999 Nov;16(11):465-72.

[5] Yost LJ, Tao SH, Egan SK, Barraj LM, Smith KM, Tsuji JS, et al. Estimation of dietary intake of inorganic arsenic in US children. Hum Ecol Risk Assess. 2004 Jun;10(3):473-83.

APPENDIX

A.1 Supplementary Data to Section 4.4.1

Table A.1 Concentrations of Arsenic Species (µg/L), the Sum of iAs(III), DMA(V), MMA(V), and iAs(V) (µg/L), Relative % DMA, and Creatinine Concentrations (mmol/L) in Urine of Children Playing on CCA Playgrounds. Samples coded a, b, and c denote the first, second, and third samples collected, respectively, following activity on playgrounds. Samples coded with x, y, or z were not labelled with respect to time.
*n.d. = not detected, N/A = not analyzed

Playground	Sample	AsB	iAs(III)	DMA(V)	MMA(V)	iAs(V)	Sum	% DMA	Creatinine
A	A1a	4.2	0.5	3.4	0.2	n.d.*	4.1	83	3.7
	A2b	4.5	0.7	14.5	1.4	n.d.	16.7	87	9.7
	A2c	4.5	0.2	5.7	0.5	n.d.	6.4	89	11.2
	A3a	11.1	0.7	7.7	0.6	n.d.	9.1	85	10
	A5a	0.5	0.9	9.0	2.1	n.d.	12.1	75	2.9
	A15a	2.4	0.3	0.6	0.1	n.d.	1.0	56	1.4
	A15b	28.3	2.8	3.5	0.3	n.d.	6.7	53	4.5
I	I1a	6.8	11.9	126.0	23.5	n.d.	161.5	78	15.7
	I1b	4.2	5.6	68.8	9.3	n.d.	83.8	82	5.5
	I1c	6.4	3.8	48.3	4.3	n.d.	56.5	86	5.6
	I2a	0.9	0.8	5.3	0.9	n.d.	7.0	76	13.1
	I2b	0.4	n.d.	1.3	0.5	n.d.	1.8	72	2.8
	I2c	0.7	n.d.	3.0	0.6	n.d.	3.6	82	7.0
	I3a	44.4	5.2	15.3	0.6	n.d.	21.1	73	3.5
	I3b	21.5	4.4	10.5	0.5	n.d.	15.4	68	8.0
	I3c	24.1	2.7	10.3	0.6	n.d.	13.6	76	4.6
	I4a	49.5	1.6	22.6	1.6	n.d.	25.9	88	8.0
	I4c	44.0	1.7	17.9	2.2	n.d.	21.7	82	8.3
	I5b	2.8	0.6	6.8	0.4	n.d.	7.7	88	3.8
	I5c	6.5	1.0	19.8	0.3	n.d.	21.1	94	2.2
	I6a	1.5	0.6	16.1	0.6	n.d.	17.2	93	4.9
	I6b	1.9	0.6	69.3	1.4	n.d.	71.3	97	4.6
	I6c	13.3	1.4	80.7	1.7	n.d.	83.7	96	N/A*
N	N1a	0.6	0.1	1.9	0.6	n.d.	2.6	73	6.0
	N1b	0.3	0.2	0.9	0.4	n.d.	1.5	62	3.5
	N2a	3.1	1.3	30.7	2.6	n.d.	34.6	89	9.7

Playground	Sample	AsB	iAs(III)	DMA(V)	MMA(V)	iAs(V)	Sum	% DMA	Creatinine
	N2b	2.9	1.9	30.4	2.3	n.d.	34.6	88	6.6
	N2c	6.7	2.1	42.7	3.5	n.d.	48.4	88	9.3
	N4a	0.5	0.2	1.9	0.4	n.d.	2.6	76	3.3
	N4b	0.4	0.2	1.4	0.2	n.d.	1.9	77	9.5
	N5a	0.8	0.3	2.7	0.5	n.d.	3.5	75	5.0
	N5b	0.7	0.4	3.5	0.6	n.d.	4.4	78	8.9
	N5c	0.8	1.0	7.7	1.3	n.d.	10.0	78	8.2
	N6a	6.4	0.5	2.1	0.2	n.d.	2.9	73	7.9
	N6b1	7.4	0.7	2.6	0.4	n.d.	3.7	70	11.0
R	R7a	0.6	0.4	1.5	0.7	n.d.	2.6	58	1.8
	R7b	0.5	0.3	1.9	0.6	n.d.	2.8	68	2.7
	R7c	0.4	0.2	1.5	0.5	n.d.	2.1	70	2.3
	R9b	0.1	0.9	6.5	1.3	n.d.	8.7	75	4.6
	R10b	0.1	0.2	2.6	0.6	n.d.	3.4	77	3.2
	R10c	0.1	0.1	1.7	0.3	n.d.	2.2	78	2.2
	R13a	0.1	n.d.	3.5	0.8	n.d.	4.3	80	3.9
	R13b	0.2	0.3	3.6	1.1	n.d.	4.9	73	4.5
	R13c	0.1	0.1	2.2	0.6	n.d.	2.9	76	4.1
	R14x	2.4	0.2	0.9	0.1	n.d.	1.2	71	1.2
	R14b	16.8	0.5	13.6	1.7	n.d.	15.9	86	N/A
	R17a	0.1	0.3	3.5	0.6	n.d.	4.4	79	3.0
	R17b	0.1	0.2	2.2	0.4	n.d.	2.9	77	6.5
	R17c	0.1	0.3	2.4	0.4	n.d.	3.0	79	4.5
	R19a	1.1	0.5	2.5	0.9	n.d.	3.9	64	10.3
	R20a	0.4	0.2	1.1	0.5	n.d.	1.8	62	2.8
	R20b	0.7	0.2	2.9	0.7	n.d.	3.9	76	5.8
	R21a	0.7	0.4	1.5	0.9	n.d.	2.7	55	4.8
	R21b	1.3	0.5	3.2	1.2	n.d.	4.9	66	5.8
F	F4a	1.2	0.2	1.7	1.3	n.d.	3.2	54	6.8
	F4c	0.6	0.4	1.3	0.5	n.d.	2.2	59	2.6
Average		**6.1**	**1.1**	**13.5**	**1.5**	**n.d.**	**16.1**	**76**	**5.8**

Table A.2 Concentrations of Arsenic Species (µg/L), the Sum of iAs(III), DMA(V), MMA(V), and iAs(V) (µg/L), Relative % DMA, and Creatinine Concentrations (mmol/L) in Urine of Children Playing on non-CCA Playgrounds. Samples coded a, b, and c denote the first, second, and third samples collected following activity on playgrounds. Samples coded with x, y, or z were not labelled with respect to time.
*n.d. = not detected, N/A = not analyzed

Playground	Sample	AsB	iAs(III)	DMA(V)	MMA(V)	iAs(V)	Sum	% DMA	Creatinine
B	B6x	1.0	0.2	4.4	1.0	n.d.*	5.6	78	8.7
	B6y	1.1	0.5	5.7	1.1	n.d.	7.4	77	12.3
	B8a	0.3	0.2	1.1	0.2	n.d.	1.5	75	2.5
	B8b	0.3	0.1	1.6	0.3	n.d.	2.0	79	4.9
	B8c	0.2	n.d.	0.8	0.1	n.d.	0.9	81	2.5
H	H2b	0.5	0.6	15.9	1.9	n.d.	18.4	86	5.1
	H3a	1.3	0.4	3.6	0.5	n.d.	4.5	80	7.2
	H3b	1.8	0.3	4.6	0.4	n.d.	5.3	87	9.3
	H3c	1.0	0.4	4.5	0.5	n.d.	5.4	84	6.8
	H4b	26.2	1.7	4.8	0.8	n.d.	7.4	65	12.0
K	K2a	0.3	0.2	3.7	0.4	n.d.	4.3	87	4.9
	K2b	0.2	0.1	2.4	0.4	n.d.	3.0	81	6.2
	K2c	0.3	0.2	3.8	0.5	n.d.	4.5	84	7.9
	K3a	0.4	0.2	1.4	0.4	n.d.	2.0	69	4.1
	K3b	0.2	0.1	1.2	0.6	n.d.	1.9	63	3.9
	K3c	0.1	0.1	0.7	0.3	n.d.	1.1	65	2.0
	K4a	3.4	0.7	7.9	1.1	n.d.	9.6	82	4.9
	K4b	5.5	0.9	6.2	0.9	n.d.	7.9	78	N/A
P	P4a	2.7	0.5	3.4	0.6	n.d.	4.5	75	2.7
	P4b	2.4	0.5	2.9	0.7	n.d.	4.1	70	3.9
	P4c	3.3	0.1	4.5	1.1	n.d.	5.7	80	N/A
Q	Q1x	2.4	16.1	0.5	0.1	n.d.	16.6	3	N/A
	Q1y	7.6	0.7	1.4	0.2	n.d.	2.3	59	4.2
	Q1z	9.4	0.9	3.3	0.3	n.d.	4.5	73	9.8
	Q2x	8.4	n.d.	1.7	0.3	n.d.	2.0	84	4.7
	Q3a	6.7	0.9	6.2	0.4	n.d.	7.5	83	4.2
	Q3b	6.2	1.3	15.4	1.4	n.d.	18.1	85	9.2
	Q3c	4.6	0.9	5.3	0.5	n.d.	6.7	79	4.3
	Q4a	6.6	1.0	5.4	0.5	n.d.	6.9	78	3.8

Playground	Sample	AsB	iAs(III)	DMA(V)	MMA(V)	iAs(V)	Sum	% DMA	Creatinine
	Q4b	3.8	0.9	8.0	0.8	n.d.	9.7	83	5.3
	Q4c	6.2	1.0	7.2	0.7	n.d.	8.9	81	5.5
	Q9a	0.2	0.4	8.3	0.6	n.d.	9.3	89	10.3
	Q9b	0.2	0.7	8.9	0.7	n.d.	10.3	87	8.6
	Q9c	0.2	0.5	8.6	0.7	n.d.	9.8	88	10.4
	Q12a	0.1	0.2	2.2	0.2	n.d.	2.6	85	N/A
	Q12b	0.1	9.6	3.1	0.3	n.d.	13.0	24	N/A
	Q13a	84.6	3.8	n.d.	n.d.	n.d.	3.8	0	3.9
	Q13b	50.0	2.3	5.9	0.2	n.d.	8.4	70	3.3
	Q13c	22.6	1.2	2.5	0.1	n.d.	3.8	66	21.4
	Q14b	17.9	7.7	15.3	1.4	n.d.	24.4	63	11.0
	Q14c	10.7	6.1	6.9	0.5	n.d.	13.5	51	6.4
	Q15a	74.8	4.9	29.4	1.5	n.d.	35.8	82	9.1
	Q15b	57.6	3.3	20.7	1.4	n.d.	25.4	81	9.4
	Q16a	48.4	3.4	117.9	2.3	n.d.	123.6	95	8.6
	Q16c	20.8	30.1	64.4	2.6	n.d.	97.0	66	15.0
Average		**11.2**	**2.4**	**6.6**	**0.7**	**n.d.**	**12.7**	**73**	**7.0**