Characterization of Urinary Arsenic Metabolites for Use as Biomarkers of Susceptibility to Arsenic Toxicity

by

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Abstract

Arsenic is naturally occurring in the environment, and can be found in rocks, soil, water, and air. One main route of human exposure to arsenic is through drinking water from groundwater sources. Chronic exposure to arsenic is associated with cardiovascular, neurological, skin, and cancer- related diseases. However, there is significant variability in susceptibility to arsenic toxicity among humans. Individuals exposed to the same concentration of arsenic can show different clinical symptoms. This variability may partly be because of differences in the metabolism of arsenic to facilitate elimination from the human body.

Urine is the main pathway for the elimination of arsenic; and the arsenic compounds (species) in urine can serve as a biomarker of exposure and metabolism. The primary objective of this research was to determine the concentrations of arsenic metabolites in urine samples collected from an arsenic-affected population in Bangladesh. The participants of this study used well water as their drinking water source, which contained arsenic of various concentrations.

To enable characterization and determination of arsenic metabolites in human urine, I refined and optimized an analytical method that involved high performance liquid chromatography (HPLC) separation and inductively coupled plasma mass spectrometry (ICPMS) detection. The method was able to quantitatively measure five common arsenic compounds in urine within 10 minutes. These arsenic compounds included inorganic arsenic species, arsenite (As^{III}) and arsenate (As^{V}), and their methylated metabolites, monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}). The method also enabled the determination of arsenobetaine, an

arsenic compound commonly found in seafood. The method had a limit of detection of 0.1 μg/L for As^{III}, and 0.05 μg/L for the other four arsenic species.

Using the method that I have developed, I determined concentrations of the five arsenic species in the urine of 879 participants from Bangladesh. The summed concentrations of all arsenic species in the urine were in good agreement with the results of total arsenic analysis. The arsenic speciation analysis showed a significant difference in the arsenic metabolite profiles of different exposure groups. The group of participants who had lower than 50 μg/L of total arsenic in their urine showed a higher proportion of DMA and DMA/MMA ratio as compared to those who had higher than 50 μg/L of total arsenic in their urine. These results will contribute to the identification of biomarkers of an individual's susceptibility to arsenic toxicity.

Preface

The data presented in this thesis is original work by Jagdeesh S. Uppal. This research is a component of a "double blind" collaborative study with Dr. Brandon Pierce's group at the University of Chicago. Our collaborators have provided the "blind" urine samples from a portion of the HEALS cohort, from which they have collected exposure and health information. The University of Chicago has granted ethics approval for this work. Urine samples were provided to our laboratory without any identifiers of study participants. My contribution to the study involved the development of a rapid sensitive assay determination of the arsenic species present in the urine samples.

A part of this thesis (Chapter 1.2) has been published as Uppal J.S.; Zheng, Q.; Le, X.C. Arsenic in drinking water – Recent examples and updates from Southeast Asia. *Current Opinion in Environmental Science & Health*, 7, 126-135 (2019). doi: 10.1016/j.coesh.2019.01.004. I am the primary author of this review paper and wrote the first draft of the manuscript. I revised the manuscript with contributions from my supervisor, Dr. Chris Le, and our collaborator, Dr. Qi Zheng.

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List of Abbreviations

As	Arsenic
As3MT	Arsenic (+3) methyltransferase
AsB	Arsenobetaine
AsC	Arseniocholine
DNA	Deoxyribonucleic acid
DMA^V	Dimethylarsinic acid
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
GST01	Glutathione *S*-transferase omega 1
HPLC	High pressure liquid chromatography
ICPMS	Inductively coupled plasma mass spectrometry
iAs^V	Inorganic arsenate
iAs^{III}	Inorganic arsenite
LOD	Limit of detection
MMA^V	Monomethylarsonic acid
PNP	Purine nucleoside phosphorylase
SAM	*S*-adenosylmethionine
SNP	Single nucleotide polymorphism
TMAO	Trimethylarsine oxide
WHO	World Health Organization

1. INTRODUCTION

Approximately 200 million people have been estimated to consume water with high levels of arsenic (1). This can have the consequence of shortening life expectancy by more than 23% (2). Inorganic arsenic is a naturally occurring element originating from the earth's crust (3–5). Humans may be exposed to arsenic through natural sources such as volcanic eruptions, and human-influenced sources such as mining (6,7). One main source of human exposure to arsenic come from drinking water obtained from groundwater sources (8,9). Additional sources of arsenic include rice, fish products, and air. Irrigation of paddy rice fields with this arsenic-tainted groundwater leads to build-up of arsenic paddy soil and uptake of arsenic by various grains (10). Fish are exposed to arsenic from freshwater and marine waters (11–13). Industrial sources release arsenic, which is absorbed into particulate matters and introduced to humans via air (14,15).

Intake of arsenic leads to accumulation of arsenic in various organs (16), and has been shown to inactivate up to 200 enzymes – mostly those enzymes that are involved in cellular energy pathways, and repair and synthesis of deoxyribonucleic acid (DNA) (17). Arsenic taken up in cells has been shown to cause oxidative stress through generation of nitric oxide and reactive oxygen species (18), increase aggregation of platelets and inflammation (19,20), and lead to apoptosis, necrosis, and loss of conception in the uterus (21,22). Human exposure to high concentrations of arsenic over a long period of time has been associated with cardiovascular, neurological, skin, and cancer-related diseases in humans (16,19,22–25).

However, the toxicity of different arsenic compounds can vary by orders of magnitude. For example, the trivalent state of inorganic arsenic is 60 times more toxic than the pentavalent state of inorganic arsenic. Moreover, organic arsenic has been shown to have little to no toxic effects in humans (17).

Generally, humans metabolize ingested inorganic arsenic into less toxic methylated forms through hepatic biomethylation, in order to facilitate elimination of arsenic from the human body (17). There is significant variability in susceptibility to arsenic toxicity among humans, and this may be due to differences in human metabolism of arsenic. Individuals exposed to the same amount of arsenic can show different clinical symptoms. This variability in susceptibility is exemplified in the Bangladesh population, where high amounts of arsenic in groundwater sources has led to an endemic: however, of the more than 6 million participants of a study population exposed to high amounts of arsenic, only 300,000 show the symptom of skin lesions – a common sign of chronic arsenic exposure (26). There are numerous factors that may affect an individual's susceptibility to arsenic toxicity, including concentrations of arsenic exposure, age, smoking and alcohol consumption, and genetics (27).

Urine is the main elimination pathway for arsenic in humans. Approximately 50% of ingested arsenic is usually excreted within 3-5 days. Most populations show consistent proportions of arsenic compounds excreted: 10-30% of inorganic arsenic, 10-20% of MMA, and 60-70% DMA (28). However, certain populations, such as the native Andean population that has been exposed to high amounts of arsenic over thousands of years, have shown to excrete significantly lower amounts of MMA (29). Conversely, a subsect population in Taiwan show higher averages of 20-

30% MMA in their urine (30). Some research has indicated that subjects with a lower proportion of MMA in urine have faster elimination of arsenic. This suggests that the amount of MMA excreted in urine may be used as a biomarker to measure and characterize an individual's efficiency in metabolizing arsenic (31).

The primary objective of this research was to determine the concentrations of arsenic metabolites in 879 urine samples collected from an arsenic-affected population in Bangladesh. To achieve this, I first refined and optimized an analytical method using high performance liquid chromatography (HPLC) separation and inductively coupled plasma mass spectrometry (ICPMS) detection. I then used this method to determine the concentrations of the five arsenic species in our study population.

1.1 CHARACTERISTICS OF ARSENIC

1.1.1 General Properties of Arsenic

Arsenic (As) is categorized as a metalloid, sharing properties with both metals and non-metals (3). It belongs to Group 15 in the periodic table, and has an atomic weight of 74.92 g/mol (32). Chemical properties of arsenic closely resemble that of phosphorous. Arsenic most commonly exists in three oxidation states: -3, +3, and +5 (4). It can covalently bond with most metals and nonmetals, which results in stable organic compounds. Inorganic arsenic can also be methylated by animals and microorganisms. As a result, arsenic is present in the environment in both inorganic and organic forms (4).

1.1.2 Origination of Arsenic

Arsenic is the 20th most abundant trace element that originates from the earth's crust (3), and is distributed naturally throughout the earth's crust, soil, sediments, water, air and organisms (6). It is a major component of over 245 minerals, and is also present in high concentrations in sulfide deposits (32). Arsenic can be released into the environment, and exposed to humans, through both natural sources, such as volcanic eruptions, and anthropogenic sources (originating from human activity). Examples of anthropogenic sources that result in release of arsenic include disposal of industrial waste chemicals, smelting of arsenic bearing minerals, burning of fossil fuels, and application of arsenic in consumer products (6,7). However, the main route of arsenic exposure to humans comes through contaminated groundwater sources from natural geological sources (33).

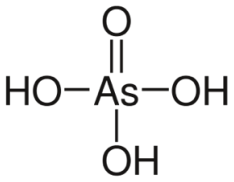
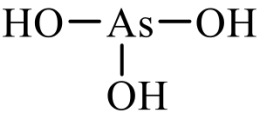
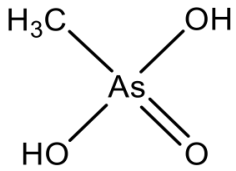
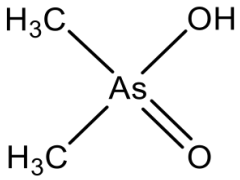
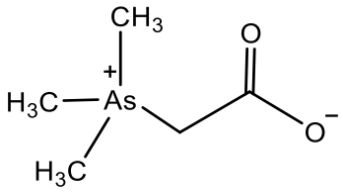
1.1.3 Toxicity of Arsenic

1.1.3.1 Overview

Arsenic compounds vary greatly, with respect to their chemical form, oxidation state, and degree of methylation. Therefore, the toxicity of different arsenic compounds can vary by orders of magnitude (**Table 1.1**). For example, the trivalent form of arsenic (As^{III}) is 10-20 times more toxic than the pentavalent form of arsenic (As^{V}), while AsB – an organic form of arsenic – is relatively harmless to humans. The ranges for the LD_{50} values for As^{III} and As^{V} represent the

lowest and highest concentrations, respectively, that correspond to the lowest observed adverse effect and highest observed adverse effect.

Table 1.1 Difference in toxicities (lethal dose) of various arsenic species in rats

Arsenic Species	Structure	LD_{50}[41]
Arsenate (As^{V})		112-175 mg/kg
Arsenite (As^{III})		15-44 mg/kg
Monomethylarsonic acid (MMA^{V})		960 mg/kg
Dimethylarsinic acid (DMA^{V})		650 mg/kg
Arsenobetaine (AsB)		10,000 mg/kg

1.1.3.2 Mechanisms for Arsenic Toxicity

There are a number of different known mechanisms for arsenic toxicity in humans. Intake of inorganic arsenic has shown to result in inactivation of up to 200 enzymes, including enzymes associated with cellular energy pathways, and DNA repair and synthesis (17). As discussed in section 1.1.1, the chemical properties of arsenic and phosphate are similar. As a result, phosphate may be replaced by arsenic in high energy compounds (such as ATP) (17). Unbound arsenic also has been shown to generate nitric oxide and reactive oxygen species, resulting in oxidative stress in cells (18). Finally, intake of arsenic has also been associated with an increase in platelet aggregation and inflammation in humans (19,20), and cell apoptosis (21).

1.1.3.3 Chronic Arsenic Poisoning

Exposure to arsenic over a long period of time may also result in chronic arsenic poisoning. Studies have shown long-term exposure to arsenic is associated with skin lesions in humans, with the risk of skin lesions being increased 3-fold for those with a significant increase in exposure to arsenic (34). This occurrence of skin lesions (**Figure 1.1**) can also result in social problems for those affected. For example, in countries like Bangladesh where contamination of arsenic in groundwater is most troublesome, people with skin lesions are unable to find partners, and candidates are not offered jobs after their skin manifestations are noticed (9).

Figure 1.1 Patient showing signs of skin lesions due to exposure of arsenic from contaminated drinking water. (35)

Exposure to arsenic may also lead to detrimental neurological effects. One study showed 37.3% of patients exposed to arsenic in West Bengal, India also showed signs of peripheral neuropathy (24). In addition, long term exposure to arsenic in adolescence may result in development of neurological results later in life (36). More broadly, arsenic has also shown to worsen intellectual function proportionally to the concentration of arsenic ingested (23), and negatively affect verbal intelligence quotient and long term memory (18).

As previously stated, arsenic intake can also result in increased aggregation of platelets, and increased inflammation. This increases risk for cardiovascular disease (19). Studies have shown concentration-dependent exposures of arsenic to be associated with manifestations of coronary artery disease, hypertension, cerebral infarction (20).

Exposure to arsenic can have detrimental reproductive effects in humans. Arsenic exposure has been associated with pregnancy complications – including fetal loss and premature delivery. These effects were determined to be proportional to the amount of arsenic the pregnant women

were exposed to (21). In rats, exposure to arsenic also showed higher incidences of apoptosis, necrosis, and loss of contraception in the uterus (22).

Finally, arsenic is also a known carcinogen: ingestion of inorganic arsenic has shown to cause cancer in the lung, bladder, and skin (37). Moreover, smoking cigarettes and ingestion of arsenic were found to have a synergistic effect in developing lung cancer, showing that some people may be at an increased risk due to lifestyle habits (25). Arsenate and arsenite have shown to increase the sensitivity of normal human fibroblasts to UV light (38). Exposure to arsenic in addition to UV radiation showed a 2.4-fold increase in tumor production compared to mice exposed to only UV radiation. In addition, the mice exposed to arsenic showed larger tumors, with an earlier onset (39).

However, the mechanism for arsenic carcinogenicity is still not well known (40). One mechanism for arsenic carcinogenicity may involve inhibition of the DNA repair system. Studies have shown arsenic may inhibit proteins involved in cellular control of DNA repair enzymes (41), and interfere with signal transduction pathways that regulate DNA repair (42). Another mechanism may be through generation of oxidative stress in cells. Arsenic metabolism has shown to generate reactive oxygen species and reactive nitrogen species (43), which can cause direct damage to DNA (44,45).

1.2 ARSENIC IN GROUNDWATER IN SOUTHEAST ASIA*

1.2.1 Overview

Arsenic in drinking water is a global public health issue, and is the main source of arsenic exposure to humans (1,9,46–55). Human exposure to high concentrations of arsenic present in drinking water was responsible for the largest mass poisoning of a population in human history (1,47,52). Despite the tremendous progress made in reducing human exposure to arsenic, more than 200 million people around the world are probably at risk of exposure to water arsenic that exceeds the World Health Organization (WHO) guideline level of 10 µg/L. Assuming an adult's average daily intake of water is 1.5 L, the mean daily intake of arsenic in an adult, according to WHO's guideline, is around 15 µg. Assuming a child's (0.5 – 4 years old) average daily intake of water is 0.7 L, the mean daily intake of arsenic in children, according to WHO's guideline, is 7 µg. The estimates of the arsenic-affected population are incomplete, partly because many water wells that supply drinking water have not been tested for arsenic concentrations (5).

Southeast Asia is among the areas most severely affected by arsenic in drinking water (1,9,50–53,56,57). Advances have been made to the reduction of arsenic exposure, e.g., by removing arsenic from water and by providing residents with alternative sources of drinking water. However, arsenic concentrations in millions of wells were not measured previously, and assumptions and models were used when estimating the magnitude of arsenic-affected

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populations. Due to high population densities and reliance on groundwater as the drinking water source, a large number of people could have been exposed to unknown concentrations of arsenic. With additional analyses of water samples and extensive research on biogeochemistry of arsenic, more information is available, enabling updated estimation of populations exposed to high concentrations of arsenic (including papers published to 2019). The primary objective of this section is to summarize recent studies on drinking water arsenic, with a focus on several arsenic-affected countries in Southeast Asia, namely Bangladesh, Cambodia, China, India, Nepal, Pakistan, and Vietnam.

1.2.2 Bangladesh

Dr. Enamul Karim – the vice president of the Bangladesh public health association – stated, in 2000, that an estimated 60 million people in Bangladesh were exposed to arsenic concentrations over 10 μg/L (58). This estimation was made through surveys of several thousand deep tube wells used for drinking and cooking. However, it is not clear how well this sampling represented the various well depths and geological conditions in Bangladesh, and if the testing was done by a laboratory with a previous track-record in arsenic testing.

In addition, a five-year study from 2000 found 42 districts in Bangladesh (total area of 38,865 km^2 and population of 42.7 million) to be at risk of arsenic concentrations over 50 μg/L (59). It is important to note that this number represents an estimate of the number of people that may be drinking water contaminated with arsenic, and that the actual number of people drinking arsenic contaminated water may differ significantly (60). The researchers used 10,991 water samples to

identify 492 affected villagers in these 42 districts (59). Of these water samples, 59% were found to contain arsenic levels above 50 µg/L. The researchers also surveyed 27 of the 42 districts for arsenic patients, and identified patients with arsenical skin lesions in 25 of the 27 districts. Of the 11,180 patients examined, 24.47% were found to have skin lesions.

The World Bank (61) had stated that approximately 28 to 35 million people in Bangladesh were at risk to be exposed to arsenic in drinking water at concentrations greater than 50 µg/L. This estimate was obtained through a study (62) that collected and analyzed approximately 3,500 samples from shallow and deep tube wells (mostly installed by the government) in Bangladesh during the summers of 1998 and 1999. The locations of the samples were randomly selected to achieve reasonable spatial coverage, and covered a total area of 129,000 km^2 out of a total of approximately 152,000 km^2. The number of people exposed to concentrations of arsenic above 50 µg/L was then estimated by combining the map of arsenic estimates with a map of the population densities. This same study also estimates that 46 to 57 million people are at risk of exposure to arsenic levels above 10 µg/L – although this estimate is less reliable due to a large proportion of the measurements being less than the instrumental detection limit.

In 2012-2013, the Multiple Indicator Cluster Survey (composed of the Government of the People's Republic of Bangladesh, Bangladesh Bureau of Statistics, Statistics and Informatics Division, Ministry of Planning, and UNICEF United Nation and Children's Fund) used a field kit test (Arsenic Econo-QuickTM Test Kit – Industrial Test Systems, USA) to semi-quantitatively measure arsenic in drinking water, and record the results as 0, 10, 25, 50, 100, 200, 300, 500, or 1000 µg As/L (63). These samples were randomly obtained directly from households. A subset

of the field samples was cross-checked in a laboratory using atomic absorption spectrometry for validation. Of the samples tested, 24.8% were found to have concentrations greater than 10 µg As/L, and 12.4% were found to have concentrations greater than 50 µg As/L. In addition, 2.8% of the samples were found to exceed 200 µg As/L. A preceding study done in 2006 showed that 7.7% of tube wells had concentrations of arsenic greater than 50 µg/L, although 37.5% of household respondents said their tube wells had yet to be tested (64). The forthcoming MICS 2019 survey is still in progress and has not yet been released to the public.

More recently, a longitudinal study for arsenic exposure via drinking water was conducted in rural Bangladesh (65), approximately 50 km south-east of Dhaka. The aim of this study was to determine the success of efforts to lower drinking water arsenic levels. Analysis of arsenic in water was performed using inductively coupled plasma mass spectrometry with a limit of detection (LOD) below 0.01 µg As/L. Initial screenings (2002-2003) from 13,000 tube wells in the area showed that arsenic concentrations ranged from 1 to 3644 µg/L, with 70% of the wells containing arsenic concentrations over the WHO guideline of 10 µg/L and 60% of wells containing arsenic concentrations over Bangladesh's own guideline of 50 µg/L.

Drinking water arsenic concentrations were tested regularly, starting in 2002. In a period of ten years (from 2002-2003 to 2013), the researchers observed a decrease in arsenic drinking water concentrations, from a median arsenic concentration of 23 µg/L (ranging from 0.02 - 882 µg/L) to less than 2 µg/L (ranging from <0.01 – 672 µg/L). The researchers attribute this decrease to the use of deeper wells over time.

These tests were done in coordination with tests of arsenic concentrations in urine of pregnant women and their children in order to also determine changes in arsenic concentrations directly in their children over time (65). However, it is important to note that arsenic levels in urine may not all be from arsenic in drinking water. Nevertheless, the results showed a clear decrease in the median arsenic concentrations, from a concentration of 23 μg/L with the pregnant women in 2002 and 2003, to 3.4 μg/L when the children were 5 years of age, and to 1.8 μg/L when the children were 10 years of age.

However, not all children experienced a decrease in As concentrations. Mothers with prenatal water with a concentration greater than 300 μg/L were responsible for 22% of the children who were observed to still have high concentrations of As at 10 years of age. In addition, mothers with an As concentration greater than 50 μg/L were responsible for 49% of the children, and mothers with a As concentration greater than 10 μg/L were responsible for 59% of the children who were observed to have high As concentrations at 10 years of age (65). It is important to note that this increase in arsenic levels may also be due to other sources of exposure to arsenic, such as rice which contains inorganic arsenic and is a main staple in Bangladesh. Nevertheless, this research indicates the importance of ensuring safe drinking water for pregnant women in order to diminish arsenic levels in their offspring.

In another recent study, Kulkarni et al. (66) looked at the impact monsoons had on arsenic in the Holocene (shallow) and Plesitocene (deep) aquifers of the Bengal Basin by sampling 21 tube wells in the Nadia district (representing shallow aquifers) and 10 tube wells from the Hooghly district (representing deep aquifers). They specifically looked at concentrations of arsenic, in

addition to other organic matter, in the pre-monsoon and active monsoon periods using inductively coupled plasma mass spectrometry. Although changes in the total dissolved arsenic were insignificant between the pre-monsoon and active monsoon periods, they found the total dissolved arsenic to range from 50 to 315 µg/L in shallow groundwater. In comparison, deep groundwater contained total dissolved arsenic ranging from 0.5 to 11 µg/L.

1.2.3 Cambodia

Based on the population residing in arsenic-contaminated areas, it was estimated that approximately 2.4 million people in Cambodia are at risk of exposure to arsenic at concentrations greater than 10 µg/L (67). Private tube wells were randomly sampled at a density of one sample per km^2 for a total of 2000 km^2 in Cambodia. Arsenic concentrations in the samples were then determined using atomic fluorescence spectroscopy in parallel with inductively-coupled plasma mass spectrometry, and using atomic absorption spectroscopy. In addition, 20% of the samples were sent to Switzerland to be analyzed by an independent contract laboratory to confirm accuracy of the measurements – the results agreed within 20% deviation.

In addition, at a Regional Workshop in Nepal (World Bank Regional Operational Responses to Arsenic) in 2004, it was reported that a maximum of 320,000 people in Cambodia were at risk of exposure to arsenic at concentrations greater than 50 µg/L (61). However, it is unclear how this number was estimated.

More recently, O'Neill et al. (68) tested arsenic concentrations in tube wells in the province of Prey Vêng. The researchers obtained groundwater data from the Well Database of the Kingdom of Cambodia in order to apply geostatistical methods and create a map displaying the spatial distribution of arsenic levels in Prey Vêng. The database used contained geographical and water quality information of rural tube-wells in Cambodia, and the resulting spatial distribution map showed high (greater than 50 µg As/L), medium (from 10 to 50 µg As/L), and low (less than 10 µg As/L) levels of arsenic in these tube-wells. The results showed arsenic risk zones were concentrated along the south/southwest boundary of the Prey Vêng province, and two villages in this area were selected for further study. Water samples were obtained from tube-wells used as cooking water sources in these villages, and analyzed at the Resource Development International Laboratory in Cambodia.

Out of the 2439 tube wells tested for arsenic in these two villages, the researchers found 76 to be high risk, with concentrations of arsenic over 50 µg/L, and 219 were found to be medium risk, with concentrations of arsenic between 10 µg/L and 50 µg/L.

The high-risk tube wells were found to be mainly concentrated along the East Bank of the Mekong River (along the province's south and southwest boundary). In this high-risk area, two villages with arsenic concentrations over 50 µg/L in tube wells were selected for further studies. These high-risk tube wells were found to have arsenic concentrations ranging from 248 µg/L to 1052 µg/L, with an average of 817 µg/L. Of the two villages tested, only one had access to communal tube wells with concentrations less than the Cambodian Drinking Water Quality Standard (CDWQS) of 50 µg arsenic/L. The other village continued using the high risk tube

wells, falsely believing that boiling the water will purify the water and make it safe to consume (65).

Although the zones where these high-risk areas are located were found to be less populated, it was estimated (using a population map of Prey Vêng, created from data obtained from the National Institute of Statistics Cambodia) that a minimum commune population total of 3500 in these areas may be at risk of arsenic exposure.

Further research in the Makong river basins showed potential for alleviating some of the risk of exposure to high levels of arsenic, by determining that the depth of water may also indicate levels of arsenic, where the level of arsenic increases with depth (69). However, in this case, shallower depths may produce manganese concentrations exceeding safe drinking water guidelines. As such, manganese removal technologies may be needed prior to use or consumption of the water from these shallower sources.

Finally, Kang et al. (70) surveyed the quality of well water in Kandal, located in the province of Prey Vêng, and in Kampong Cham provinces from 2010 to 2012 in order to determine the effectiveness of arsenic removal technologies (using amorphous iron hydroxide adsorbent – which is regenerable and environmentally friendly) that were developed by the researchers. Prior to the start of removal, the researchers determined the initial arsenic levels in 14 tube wells from Kandal, 7 tube wells from Prey Vêng, and 16 tube wells from kampong Cham. Out of the 37 wells tested, they found that 24 exceeded the Cambodian guideline value of 50 µg arsenic /L. In addition, 27 of the 37 wells exceeded the WHO guideline of 10 µg/L. Some arsenic levels of the samples were as high as 1,000 to 6,000 µg/L, showing cause for great concern.

To aid in reducing these levels, Kang et al. (2014) (70) installed 16 arsenic removal tools, and monitored arsenic levels for 10 months. They found the arsenic removal to be successful, with an average removal rate of 93.8%. The results suggest that this arsenic removal technology, and other similar technologies, can be used to treat drinking water and reduce arsenic concentrations to less than 10 µg/L.

1.2.4 China

The National Ministry of Health in China initiated a national survey of arsenic contamination in 1992, which included surveying Jilin, Liaoning, Ningxia Hui Autonomous Region, Qinghai, and Shaxi. After testing 50,000 tube wells in these provinces, wells with concentrations of arsenic greater than 50 µg/L were mapped, and used to estimate that over 2 million people were at risk of exposure to arsenic in these provinces (56). In a 2012 report, the World Bank (61) had estimated 5.6 million people could be at risk of exposure to water arsenic over 50 µg/L in three provinces alone, Inner Mongolia, Xinjiang, and Shanxi. However, it is not clear how well this sampling represented the various well depths and geological conditions in China, and if the testing was done by a laboratory with a previous track-record in arsenic testing.

Inland basins have been shown to have higher concentrations of arsenic compared to river deltas in China (71). As such, Guo et al. (72) tested 223 groundwater samples from the Hetao basin, the Yinchuan basin, and the southwestern Songnen basin; the former two are located along the Yellow River in northwestern China, and the Songnen basin is located in west of the province of

Jilin, in northeastern China. These samples were analyzed by inductively-coupled plasma mass spectrometry.

The researchers found that arsenic concentrations ranged from less than 0.1 to 105 µg/L (average 27.8 µg/L), in the Tinchuan basin, from less than 0.1 to 338 µg/L (average 94.0 µg/L), in the Songnen basin, and from 0.33 to 857 µg/L (average 130 µg/L) in the Hetao basin.

In addition, the Guide basin in northwestern China was tested for arsenic using 97 water samples (73). For this study, 70 of the 97 total water samples tested came from unconfined aquifers (100 to 300 m deep), where arsenic levels were less than 10 µg/L. These low levels for arsenic may be partly due to adsorption of arsenic on iron-oxides under oxic conditions. On the other hand, 20 samples from confined aquifers (100 to 300 m deep and under reducing conditions) had higher arsenic levels, ranging from 9.9 to 377 µg As/L, with an average of 109 µg/L. These samples were analyzed using high performance liquid chromatography hydride generation atomic fluorescence spectrometry, with a relative standard deviation of 5% and analytical precision of 2%.

Xiao et al. (74) also conducted field plot experiments in the Datong Basin in order to determine the effects of irrigation on the mobilization of arsenic in an unsaturated zone. Nine multi-level soil water extractions were made at depths at 0.5-2.0 meters. Total arsenic concentrations were then analyzed using hydride generation atomic fluorescence spectrometry.

It was determined that irrigation indeed played a role in mobilization of arsenic into the unsaturated zone. Infiltration of the As-contaminated irrigation water resulted in oxidation of Fe, which lead to less arsenic being absorbed onto the iron minerals – showing that arsenic, along with other minerals, dissociate after contact with irrigation water. As a result, the authors suggest using drip irrigation methods or irrigation with non-contaminated water to prevent arsenic accumulation in unsaturated zones.

The influence of a large number of arsenic-enriched geothermal springs on river water arsenic levels at the southern Tibetan Plateau was investigated (75). Specifically, Lhasa River and Duilong Qu (Lhasa River's tributary) that are located downstream of the largest geothermal spring in Tibetan Plateau were tested. This plateau is the youngest plateau in the world, and is located in one of the most tectonically active regions in the world – resulting in a vast number of geothermal springs in the area. Water samples were collected at the same location of the Lhasa River and Duilong Qu during the non-monsoon and monsoon seasons in 2010. Inductively-coupled plasma mass spectrometry was then used to analyze for arsenic.

Li et al. (75) found that the geothermal spring caused higher levels of arsenic in the Lhasa river (12.7 μg/L during non-monsoon season) and Duilong Qu (205.6 μg/L during non-monsoon season). During the non-monsoon season, they observed arsenic concentrations approximately 20 times greater than the WHO guideline. The Tibetan Plateau provides water sources to more than one-third of the world's population (76). Therefore, it is important to control and minimize water contamination, including the input of arsenic from geothermal springs. The major

biogeochemical processes for transformation of various arsenic species include oxidation, reduction, methylation, and arsenic sulfide redox cycling (73).

1.2.5 India

From studies published from 1984 to 2002, more than 125,000 water samples and 30,000 urine, hair, nail, or skin bioassays of 100,000 people were used to identify 8500 cases of arsenic-related illnesses in the Lower Gangetic Plain of West Bengal. More than 3000 villages were found to have tube wells that had arsenic concentrations greater than 50 μg/L. Using this, it was estimated that 6 million people from 9 of 18 total districts (total population of 80 million) were exposed to arsenic concentrations greater than 50 μg/L (56).

More recently, new areas that were previously undocumented were tested for arsenic contamination. For example, testing was done on shallow aquifers located on the southern part of the Brahmaputra river along Naga-Hills (Nagaland) (77). Fifty-two groundwater samples were collected from the southern part of the river from depths ranging from 4-62 meters below ground level (representing shallow aquifer systems), and arsenic was analyzed using inductively coupled plasma with an optical emission spectrophotometer. Ninety-two percent of the groundwater samples were found to be high in arsenic, with maximum levels reaching 450 μg/L.

Previous studies have shown that cessation of arsenic exposure by switching high-arsenic water to arsenic-free water can reverse or minimize arsenic-induced symptoms and diseases in people previously exposed to high levels of arsenic. Recently, the effects of drinking arsenic-safe water

over a prolonged period were also tested in West Bengal (78). A subset of the population (5562 residents in 947 households) was asked about sources of drinking water, diet, water intake, and clinical symptoms in 1995. A general medical examination with careful inspection for arsenic-induced skin lesions was also done on these participants. Water samples were collected from private and public tube wells, and arsenic levels in these samples were analyzed using flow-injection hydride generation atomic absorption spectrophotometry.

This research determined arsenic levels up to 3400 µg/L in drinking water, with a mean of 220 µg/L and standard deviation of 240 µg/L. Examinations with respect to arsenic-induced skin pigmentation showed that 2488 participants had no pigmentation, 131 participants had mild pigmentation, and 1 participant showed signs of moderate pigmentation. Examinations with respect to keratosis showed that 2574 participants had no lesions, and 46 participants had mild cases of keratosis (78).

Subsequent testing was done on 2620 subjects who were drinking safe water for at least 10 years. This testing showed significant improvement with respect to both pigmentation and keratosis of the participants. However, new cases of pigmentation and keratosis observed in participants showed that there is still risk of arsenic contamination present (78).

1.2.6 Nepal

A study from 2003 (79) used testings of 17,000 tube wells in the Terai region of Nepal for arsenic by different agencies, which showed that nearly 31% of the tested tube wells exceeded 10

µg As/L, to estimate that over 3 million people may be at risk to exposure of arsenic at concentrations greater than 10 µg/L. More recently, the worst affected districts in Nepal have been determined to be Nawalparasi, Rautahat, Parsa, Rupandehi, and Bara, where over 29% of more than 20,000 tube wells have concentrations of arsenic greater than 10 µg/L (80). However, it is not clear if field kits or laboratory analyses were used for these testings, and if any validation was done for accuracy.

Yadav et al. (81) collected 48 groundwater samples in two consecutive seasons (pre-monsoon and monsoon seasons in 2010), from three villages (Thylokunwar, Kasia, and Panchgawa) in the Nawalparasi district located in the southwestern Terai region of Nepal. They collected a total of 48 groundwater samples, and performed laboratory chemical analysis using atomic absorption spectrometry coupled with a hydride generator at the laboratory of the Centre of Advanced Study in Botany, Banaras Hindu University. Quality assurance was also performed by inter-laboratory comparisons with the Department of Chemical Engineering at the Indian Institute of Technology in Varanasi, and relative standard deviations lower than 10% were observed.

The researchers found the minimum arsenic concentration to be 60 µg/L during the pre-monsoon season. They determined the mean to be 595 µg/L, with a maximum As level of 3100 µg/L. In comparison, during the monsoon season, the minimum arsenic concentration was 155 µg/L, with a mean of 516 µg/L and a maximum As concentration of 1338 µg/L.

Kayastha, SP (82) also examined deep and shallow groundwater in the Bara District of Nepal, where groundwater is the main source of drinking water. During the pre-monsoon season, 12

random samples from dug wells and 24 from tube wells were collected. The arsenic was analyzed using atomic absorption spectrophotometry with a graphite furnace.

About 50% of the samples were found to exceed the WHO guideline level of 10 µg As/L, and 12.5% of the samples were found to exceed the Nepal Interim Standard of 50 µg/L. In addition, higher levels of arsenic were found in tube wells having depths of 10-20 m, whereas no tube well with a depth greater than 20 m was found to have arsenic levels greater than 50 µg/L. Similar to Yadav et al. (81), it may be useful to compare these concentrations with samples collected from the monsoon season.

1.2.7 Pakistan

Recent estimates for the arsenic-affected population in Pakistan, calculated using geostatistical modeling, ranged from 50-60 million (83). Recent studies incorporating more than 8000 groundwater samples have been used to estimate that approximately 47 million people (84) living in Pakistan are potentially affected by drinking water arsenic (>10 µg/L). These 43 studies incorporated various different methods, sampling depths, and locations. The researchers then used inverse distance weighted interpolation for statistical analysis of this data.

Podgorski et al. (83) performed this modeling by finding statistically significant relationships between arsenic concentrations and environmental predictors. They found most of the Indus Plain in the Sindh province and around Indus tributaries in the Punjab province to have the highest probability of arsenic contamination – especially concerning as these areas have high

population densities. Testing of the Indus River confirmed this to be true, with concentrations greater than 200 µg/L resulting mostly from the southern half of the Indus Plain. Outside of the Indus Plain, the arsenic concentration in groundwater was mostly below 10 µg/L. This relates to another research that has found drinking water wells with arsenic concentrations greater than 10 µg/L to be mainly concentrated within 18 km of the Indus River, and have estimated 13 million people along the course of the river to be exposed to arsenic concentrations greater than 10 µg/L (85).

From this modeling, Podgorski et al. (83) determined that 88 million people live in a hazard area. Assuming that 60-70% of the population in Pakistan relies on groundwater, this results in 50-60 million people potentially affected by arsenic contamination.

Rasool et al. (86) highlighted exposure levels of arsenic in the Mailsi Tehsil area of the Punjab province. They obtained 44 groundwater samples from two villages in the area (Mailsi city and Sargana), and analyzed for arsenic by using atomic absorption spectrophotometry (with reproducibility within 5% and analytical error to be estimated to be less than 10%). They determined that the arsenic contamination in tube well waters that were intended for human consumption ranged from 12 to 448.5 µg/L, with a mean value of 80 µg/L.

A recent review, using data from 43 studies that include a total of over 9800 groundwater samples, has estimated that over 47 million people in Pakistan are at potential risk of As poisoning (84). This number is more conservative from the estimate made by Podgorski, J.E. et al. (83). Shahid et al. (84) found As contamination to be prevalent only in confined areas and

districts – mainly the Lahore and Kasur districts in central Punjab and Muzaffargarh in south Punjab, and Tharparker and Jamshoro regions in Sindh province. In addition, unlike the research by Mueller (80) in the Nepal section, no correlation between As concentrations and sampling depths were able to be made due to the large variation in the reporting of sampling depths for individual studies. Lastly, mean As concentrations for studies with <100 samples were 3 times higher than studies that tested >100 samples, showing that studies with small-scale studies may be prone to overestimation of As concentrations in groundwater.

1.2.8 Vietnam

A previous report in 2012 by the World Bank (61) has estimated approximately 10 million people in Vietnam to be exposed to arsenic in drinking water at concentrations greater than 50 μg/L. However, it is unclear which specific tube wells were used for determining this estimate, and whether arsenic was analyzed using field kit testing or in a laboratory. As such, future testing in Vietnam may allow for a more accurate estimate of the number of people at risk for exposure to arsenic.

Recently, the contamination of arsenic in the province of Ha Nam (Red River Delta) in Northern Vietnam was investigated (87). Samples from 36 tube wells from Chuyen Ngoai and Chau Giang – two villages in the province of Ha Nam – were randomly collected from wells, with ages of the wells ranging from 1 to 26 years old. These samples were then analyzed for arsenic using atomic absorption spectrometry coupled to a hydride vapor generation system. They achieved recoveries

of arsenic in the groundwater samples ranging from 86.6% to 108%, and achieved a limit of detection of 0.2 µg As/L.

Concentrations of arsenic in untreated groundwater were found to range from 12.8 to 884 µg/L, with median arsenic concentrations being 614 µg/L and 140 µg/L and means of 90.14 µg/L and 14.30 µg/L in Chuyen Ngoai and Chau Giang, respectively. Concentrations of arsenic in treated groundwater (such as with the use of sand filtration systems) showed arsenic removal rates greater than 80%. However, Pham et al. (87) determined this reduction to be inadequate in reducing arsenic concentrations to safe levels.

Arsenic contamination of groundwater used for agriculture irrigation was also investigated in Mekong Delta (88). Samples were collected from 16 shallow tube wells in the Thanh Binh district, in the province of Dong Thap. Arsenic content in the samples was then analyzed using inductively-coupled plasma mass spectrometry.

All the samples were over WHO's and the Vietnamese standard for groundwater, with concentrations of arsenic ranging from 60 µg/L to 900 µg/L. These results suggest that arsenic problems in the Thanh Binh district also pose challenges for sustainable agriculture activities (88). Additional testing in the Makong Delta has found thiol-bound trivalent arsenic and arsenian pyrite to be present in the organic matter and sulfur-rich layers, with the highest concentrations ranging from 34-69 ppm (mg/L) at a depth of 16 m (89).

1.2.9 Concluding remarks and perspectives

The issues of drinking water arsenic are not limited to Southeast Asia. Recent studies (46) estimate that that ~560,000 people are potentially exposed to elevated arsenic from groundwater in Burkina Faso, West Africa. Approximately one million people in the UK are served by private water supplies and it is not known how many people are exposed to water arsenic that exceeds the prescribed concentration value for arsenic (90). Many private wells in Canada and the United States have not been tested for arsenic. Approximately nine million people in Canada, representing almost one third of the Canadian population, rely on ground water as their drinking water supply. Most of these wells have not been tested for arsenic, and it is not clear how many people are exposed to high concentrations of arsenic in drinking water (91).

It is also important to note that increases or decreases in estimations of the number of people exposed to arsenic may not entirely be due to worsening of the situation or successful mitigation of arsenic contaminated groundwater; more studies and testings of tube wells results in a more accurate estimate of the number of people at risk of exposure to high concentrations of arsenic.

Food is another major source of human exposure to arsenic species (92,93). Relatively high concentrations of arsenic in rice have drawn much attention (94,95), and much recent effort has devoted to the control and reduction of arsenic in rice (96,97). Some phenylarsenic compounds are still used in some countries as poultry feed additive, and the litter as fertilizer; and as many as 11 arsenic species have been identified in liver and breast meat of broiler chickens fed 3-nitro-4-hydroxyphenylarsonic acid (Roxarsone), although these arsenic levels are very low (98–100).

1.3 HUMAN METABOLISM OF ARSENIC

After ingestion of inorganic arsenic, As^{V} can be reduced to As^{III} and taken up by cells. Generally, humans metabolize ingested inorganic arsenic into less toxic methylated forms through hepatic biomethylation (**Figure 1.2**) (17). Although this may be seen as detoxification of arsenic, it is important to note that the trivalent methylated arsenic species generated through this process, although short-living, are in fact more toxic than the inorganic form of arsenic.

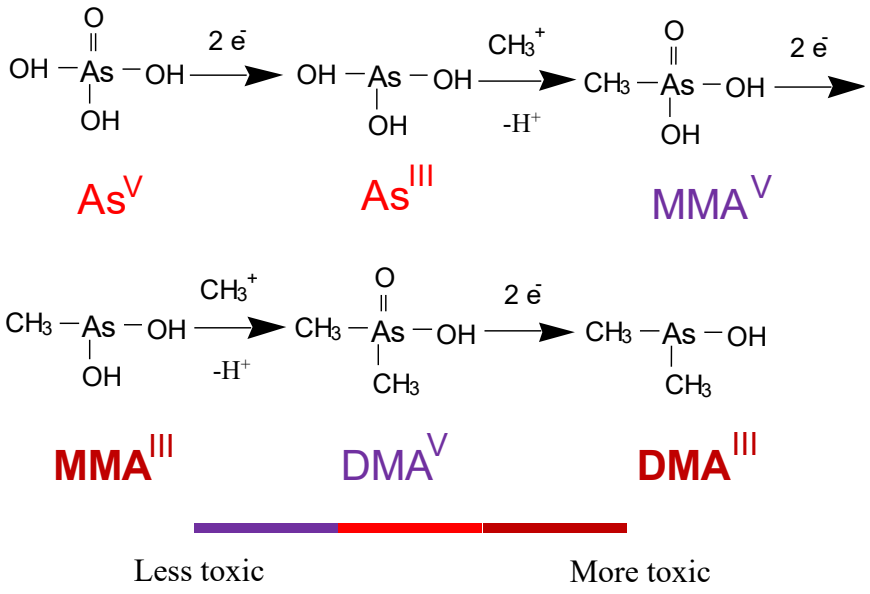

Figure 1.2. Metabolism of arsenic in humans through reduction and methylation reactions

Two different mechanisms for metabolism of arsenic have been proposed (101):

(1): Arsenate (As^{V}) enters the cell through phosphate transporters. It is then metabolized through a series of reduction and methylation reactions to DMA^{V} – the end product which then eliminated from the body.

(2): Arsenic binds to certain proteins or glutathione. It is then metabolized through a series of reduction and methylation reactions to MMA^V and DMA^V – the end products which are then eliminated from the body.

Urinary excretion is the main excretory pathway of arsenic in humans (102). Approximately 58% of ingested arsenic is excreted in urine within 5 days. The majority of the arsenic excreted (average) is DMA (51%), followed by MMA (21%) and inorganic arsenic (27%) (28).

1.4 DETERMINING SUSCEPTIBILITY TO ARSENIC TOXICITY

In order to limit harmful effects associated with arsenic exposure, the World Health Organization (WHO) has recommended guidelines of 10 µg As/L in drinking water obtained from groundwater sources (103). However, approximately 200 million people around the world are estimated to be exposed to concentrations higher than this guideline (103). Moreover, there is significant variability in susceptibility to arsenic toxicity, and it is not clear if this guideline is suitable for protecting the most susceptible populations among us. Further studies are needed in order to determine the effects of low concentrations of arsenic on human health, and determination of populations that are most susceptible to arsenic toxicity. Addressing this knowledge gap will help provide further information on the mechanisms for arsenic toxicity, and help develop guidelines that are most suitable for the health protection of different populations. Three inter-connected sources can be used for elucidation and identification of populations that

may be more-or-less susceptible to arsenic toxicity: urinary arsenic profile, epigenetics, and genetic polymorphisms of individuals.

1.4.1 Urinary Arsenic Profile

The variability in susceptibility to arsenic toxicity may partly be related to differences in metabolism of arsenic among humans, which can be determined through comparisons of urinary arsenic profiles and health outcomes of individuals. There are several enzymes responsible for metabolism of the arsenic species in humans. iAs^{V} is reduced by purine nucleoside phosphorylase (PNP); all pentavalent arsenic forms (iAs^{V}, MMA^{V}, and DMA^{V}) are reduced by glutathione *S*-transferase omega 1 (GSTO1) and glutathione *S*-transferase omega 2 (GSTO2). Arsenic (+3) methyltransferase (AS3MT) methylates the trivalent (reduced) forms of arsenic (iAs^{III}, MMA^{III}, and DMA^{III}) into the methylated pentavalent form. The main methyl donor in this arsenic methylation is *S*-adenosylmethionine (SAM). The different expression levels of these enzymes may play a part in different metabolisms of arsenic among humans. Other factors, such as concentration of arsenic, gender, lifestyle habits – such as smoking and alcohol consumption, and age may also play a part in the variability in arsenic metabolism among humans (27).

Interestingly, most populations have similar urinary arsenic profiles (**Table 1.2**) (28). However, the native Andean population, which has been exposed to high amounts of arsenic through contaminated groundwater over thousands of years, has urinary arsenic profiles that show lower amounts of MMA (29). In contrast, a subset population in Taiwan has urinary arsenic profiles that show higher averages of MMA in their urine (30). This may be indicative of their

susceptibility to arsenic toxicity, as studies have shown that individuals and populations with lower proportions of MMA in their urine have faster elimination of arsenic overall, which may lead to more efficient metabolisms and eliminations of arsenic - resulting in less arsenic retained in the body and decreased incidences of arsenic-related illnesses.

Table 1.2 Percentage of inorganic arsenic, MMA, and DMA in participants' urine

Proportion of arsenic	Average global values (28)	Native Andean Population (29)	Subsect population in Taiwan (30)
Inorganic As (%)	10-30	15-65	-
MMA (%)	10-20	0-11	20-30
DMA (%)	60-70	31-85	-

The urinary arsenic profile provides a valuable resource for the identification of biomarkers that can be used to determine an individual's susceptibility to arsenic-induced diseases. However, there are potential confoundments in using arsenic metabolites in urine as a marker: MMA and DMA can originate from sources additional to metabolism of inorganic arsenic, such as consumption of seafood (104). This may lead to overestimations on the amount of inorganic arsenic an individual can metabolize into MMA and DMA. This confoundment may be overcome by discarding urine samples that show presence of high amounts of arsenic species only found in fish (such as AsB), abnormally high amounts of DMA and MMA, and urine samples from those participants that indicate they regularly consume seafood and seaweed.

1.4.2 Genetic Polymorphisms

Several genetic polymorphisms may affect arsenic metabolism in humans. **Table 1.3** shows the genetic polymorphisms that have been determined to be involved in metabolism of arsenic. In this table, "gene" represents the specific gene in which the polymorphism occurs and "polymorphism (locus)" represents the specific location of the polymorphism. Single nucleotide polymorphisms (SNP) represent a substitution of a single nucleotide at a specific location in the genome. Additional polymorphisms (in relation to DNA repair pathways) have also been shown to play a part in susceptibility to arsenic toxicity. Studying these genetic polymorphisms will allow development of methods that can be used to determine a population's (and individual's) risk to arsenic toxicity through quick genetic tests (i.e. microarrays). Identification of these genetic polymorphisms has an additional advantage of providing further elucidation on proteins and pathways that help alleviate arsenic-related health outcomes (105).

Table 1.3 Polymorphisms in genes associated with arsenic biotransformation; modified from (107)

Gene	Polymorphism (locus)	Genotype	Increased risk	Odds Ratio [95% CI]	p-Value
AS3MT	C10orf32 (rs9527)	G > A	Skin lesions	3.33 [1.81–6.14]	< 0.0001
GSTP1	Ile105Val (rs1695)	A > G	Skin lesions	1.86 [1.15–3.00]	0.01
GSTP1	Ile105Val (rs1695)	A > G	Bladder cancer	5.4 [1.5–20.2]	0.03
EPHX1	Tyr113His (rs1051740)	T > C	Skin cancer	3.74 [1.20–11.66] 2.99 [1.01–8.83]	0.04
PNP	Gly51Ser	G > A	Skin lesions	1.66 [1.04–2.64]	0.04

Thus far, most studies on genetic polymorphisms and arsenic toxicity have focused on genes responsible for arsenic metabolism. One of the main genetic polymorphisms is demonstrated through the arsenic-3 methyltransferase enzyme (As3MT). This enzyme is responsible for the methylation of trivalent arsenic species into the pentavalent methylated form in humans (27). Several single nucleotide polymorphisms (SNPs) have been shown to occur more frequently in the Native Andean population – that had lower proportions of MMA in their urinary excretion and showed less susceptibility to arsenic toxicity (108). These polymorphisms may increase the gene expression for specific enzymes responsible for methylation of arsenic, such as methyltransferase, and allow the Native Andean population to more efficiently metabolize inorganic arsenic into DMA. In fact, the main SNPs discovered in the Native Andean population have been upstream of the As3MT gene which shows that these SNPs may in fact play a regulatory factor in this gene.

Polymorphisms that influence DNA repair pathways have also been determined to play a part in susceptibility to arsenic-induced cancer (107). One study determined a correlation between polymorphisms in the *excision Repairs Cross-complementing rodent repair deficiency, Complementation group 2* protein and arsenic-induced hyperkeratosis (109). This enzyme is involved in nucleotide excision repair, which is the main pathway that mammals use to remove lesions formed by UV light and environmental mutagens (107). Other studies support this notion, and have also showed other polymorphisms that may decrease risk of arsenic-induced cancer (110–112).

1.4.3 Epigenetics

Epigenetics – the study of phenotypic changes not due to any alterations of the DNA sequence - provides another route for determining susceptibility to arsenic toxicity (105). This has been used for identification of arsenic exposure, response, disease, and susceptibility to arsenic-induced illnesses. An additional benefit in the use of epigenetics is the ability to use a bottom-up approach to determine whether changes in significant loci have any effects on protein expression. This allows for identification of adverse health outcomes related to arsenic exposure that would otherwise be difficult to determine (i.e. due to insignificant epidemiological data or consequences from exposure to low concentrations of arsenic).

This has been useful for determining the effects of arsenic exposure to infants. One study has shown how *in utero* exposure to inorganic arsenic may lead to an increased risk for infection, and cancer and non-cancer endpoints (105). The mechanism for this may be from changes (or silencing) in gene expressions via alterations in DNA methylation. These DNA methylation patterns are highly dynamic during embryonic development, and subtle changes can lead to lifelong effects.

Another study investigated the relationship between altered levels of DNA methylation and gene expression, and the health outcomes of newborns exposed to arsenic *in utero* (106). Mother-newborn pairs (n=200) located in an area in Mexico with high amounts of arsenic in groundwater, and in a population that showed symptoms of arsenic toxicity (i.e. skin lesions and diabetes mellitus), were studied. The researchers focused on mRNA expression levels and DNA methylation profiles in cord blood samples in 38 of the participants exposed to different amounts

of arsenic. The levels of arsenic exposure were determined through testing of their drinking water and in maternal urine.

The researchers studied DNA methylation across six different genomic regions:

1. 3’ untranslated region (3’UTR)
2. Gene body (Body)
3. First exon
4. 5’ untranslated region (5’UTR)
5. 200 bp upstream of the transcriptional start site (TSS200)
6. 200 to 1500 bp upstream of the transcription start site (TSS1500)

The results showed that the genomic regions within the first exon, 5’UTR, and TSS200 were the most predictive for arsenic-associated changes in gene expression – 16 genes in these regions showed relationships between DNA methylation and arsenic-related gene expression. Of these 16 genes, 7 genes also showed associations between DNA methylation and birth outcomes – with respect to head circumference, placental weight, and gestational age. This study provides insight into the relationship between DNA methylation, gene expression and health outcomes in newborns exposed to arsenic. However, the study may be repeated with a larger cohort to overcome this study’s limitation of a small sample size (n=38). This can further strengthen the use of epigenetics for identification of populations most susceptible to arsenic toxicity.

1.4.4 Concluding remarks and perspectives

There have been many studies done that showcase that certain individuals and populations may have different susceptibilities to arsenic-induced illnesses. The urinary arsenic profile, epigenetics, and genetic polymorphisms of individuals can be combined with their age, sex, sources of food and drinking water, and health outcomes in order to create profiles of humans with differing susceptibilities to arsenic toxicity. This can then be used to identify new biomarkers for susceptibility to arsenic toxicity, provide a better understanding of arsenic toxicity in humans, and serve as a tool to identify and protect populations that are most susceptible to arsenic toxicity.

1.5 ANALYTICAL TECHNIQUES FOR ARSENIC SPECIATION ANALYSIS IN URINE

My component of our collaborative study involves determination of arsenic species present in a population located in an arsenic-affected area in Bangladesh. Various analytical techniques have been utilized for sensitive detection of trace levels of arsenic in biological and environmental samples (113–117). Different arsenic species have different physiochemical properties that can be utilized in order to achieve efficient separation – even in complex samples. This separation is usually achieved through chromatographic methods. These chromatographic separation methods are usually combined with element-specific spectrometric detection for identification and quantification of the arsenic species present in the sample(s). These techniques can be utilized to

perform arsenic speciation in a large number of urine samples, with good reliability and sensitivity.

1.5.1 Separation of arsenic species

There are various techniques available for separation of arsenic species including liquid chromatography, gas chromatography, and capillary electrophoresis. These techniques make use of the different physiochemical properties of arsenic species in order to differentiate the different arsenic species present in the urine sample. The retention times of specific arsenic standards can then be used to identify the arsenic species present in the samples.

The most common technique for separation of arsenic species is via high pressure liquid chromatography (HPLC) (115). This method provides separation of the different arsenic species based on their interaction with solid adsorbent material (stationary phase). A pump is used to pass pressurized liquid (i.e. sample) through the stationary phase. Species of arsenic that interact more with the stationary phase will be retained longer, and species of arsenic that have less or no interaction with the stationary phase will have a shorter retention. This is the most common separation technique used for separating iAs^{III}, iAs^{V}, MMA, and DMA.

The type of stationary phase used determines the retention, and order of elution, of the arsenic species (113). For example, under neutral pH, the different pKa values of the deprotonated iAs^{V}, MMA, DMA, and the neutral iAs^{III} contribute to different ionic charges for each arsenic species. This difference in the ionic charge can be used to separate the four arsenic species via an anion-

exchange stationary phase. In this case, the order of elution is: iAs^{III}, DMA, MMA, and iAs^{V}. Additionally, a cation exchange stationary phase can be used for separation of positively charged arsenic species, such as arsenobetaine (AsB), arsenocholine (AsC), and trimethylarsine oxide (TMAO).

Gas chromatography can also be used for separation of volatile arsenic species. This method provides excellent separation, but is not very common as most species of arsenic are non-volatile (118). In this case, the sample is vaporized, and separation of the arsenic species is based on their interaction with an inert carrier gas used as the mobile phase (often helium), and the stationary phase.

1.5.2 Detection of arsenic species

There are a number of techniques used for detection of arsenic species, with different advantages and disadvantages. These include flame atomic absorption spectrometry, atomic fluorescence spectroscopy, inductively coupled plasma mass spectrometry (ICPMS), and electrospray ionization mass spectrometry (ESIMS). Of these, ICPMS is the most widely used technique for detection of arsenic due to its high selectivity, high sensitivity, multi-element capabilities, and wide dynamic range (113).

With analysis using ICP-MS, samples are introduced into the ICP-MS as aerosol droplets through a nebulizer and spray chamber (119). The aerosol is dried by a plasma, operating at an extremely high temperature of approximately 6000°C, which ionizes the sample (by removing an

electron and forming single-charged ions). These ions are then directed towards a mass spectrometer, and separated based on their mass-to-charge ratio (m/z). The mass spectrometer only allows ions with a specific mass-to-charge ratio to pass through and enter the detector; for detection of arsenic species, usually ions of a m/z of 75 are selected to reach the detector. In some cases, arsenic may be reacted with oxygen in order to form arsenic oxide, and bypass interferences from other ions that also have the same m/z of 75. In this case, ions of a m/z of 91 are selected to reach the detector. The impact of the ions onto the detector generates a mass spectrum, where the peak intensity is proportional to the number of ions that reached the detector. The peak intensities of known arsenic standards with different concentrations (i.e. calibration standards) can be compared with the peak intensities of the unknown arsenic species in order to determine the concentration of the arsenic species that is present in a sample.

The ICP-MS can be coupled to HPLC in order to achieve sufficient separation and sensitive detection of arsenic species. However, only arsenic species that have the same retention time as known arsenic standards can be identified – and no molecular information of the arsenic species is obtained. This works well for determination of common arsenic species found in urine (i.e. AsB, As^{III}, DMA, MMA, and As^{V}), but does not allow for elucidation of any unknown arsenic compounds present in each urine sample. As such, this method can be complemented by use of electrospray ionization mass spectrometry (ESIMS) to gain molecular information and identify unknown arsenic compounds that have no standards available (120–122).

Similar to the ICP-MS, an ESI-MS is composed of an ion source, mass spectrometer, and detector (123). However, unlike the ICP-MS that utilizes extreme temperatures to achieve "harsh" ionization of the sample, the ESI-MS utilizes a "soft" ionization technique that allows

for retention of the molecular information of the analyte. This is achieved by applying high voltage to the tip of the injection needle to generate aerosols of highly charged electrospray droplets. These droplets undergo solvent evaporation until the charged analytes are released. This "soft" ionization allows the structure of the analytes to remain intact, allowing molecular information to be obtained. This molecular information then can be used to identify unknown or new arsenic compounds.

1.6 RATIONALE AND THESIS OBJECTIVES

My supervisor and I are collaborators with researchers at the University of Chicago on a Health Effects of Arsenic Longitudinal Study (HEALS) in an arsenic-affected population located in Araihazar, Bangladesh to determine whether differences in methylation and excretion of arsenic species may correlate to the incidence of arsenic-induced illnesses.

I have two main research aims for my thesis. My first aim for this study is to refine and optimize a robust method suitable for high volume analysis of arsenic species in urine samples, with good repeatability and a short analysis time. I am using HPLC separation coupled with inductively coupled plasma mass spectrometry (ICP-MS) detection to identify arsenic species present in urine. I aimed to optimize the elution gradient, injection volume, helium gas rate, and mobile phase salt concentration and pH to make this method suitable for rapid analysis of trace concentrations of arsenic species in a high volume of urine samples.

My second aim is to use this method to quantify the arsenic species present in the urine samples from the Bangladesh study population. The urine samples are being tested for the five most common arsenic species found in urine: inorganic arsenite (iAs^{III}) and arsenate (iAs^{V}) as they are the most common forms of arsenic found in drinking water; monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) as the main metabolites of inorganic arsenic; and arsenobetaine (AsB), a common arsenic compound from food sources.

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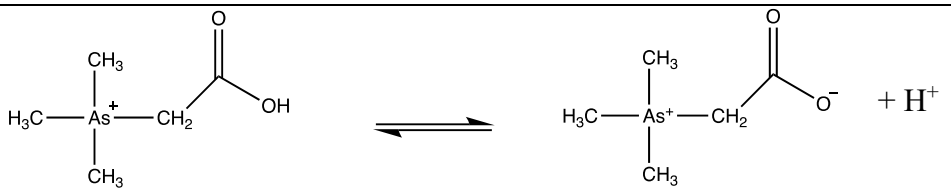
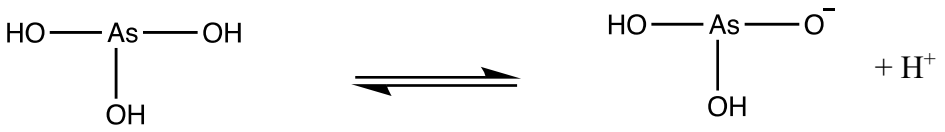
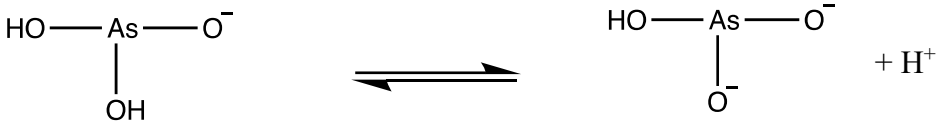
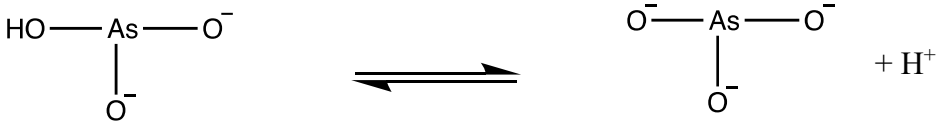
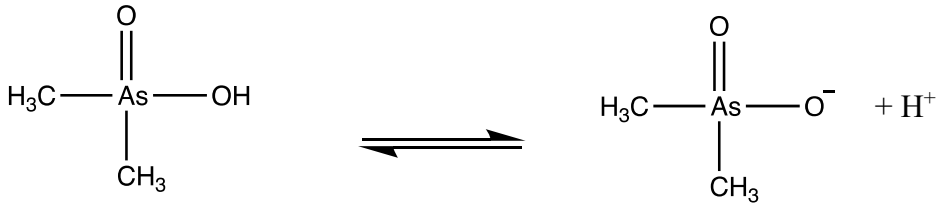
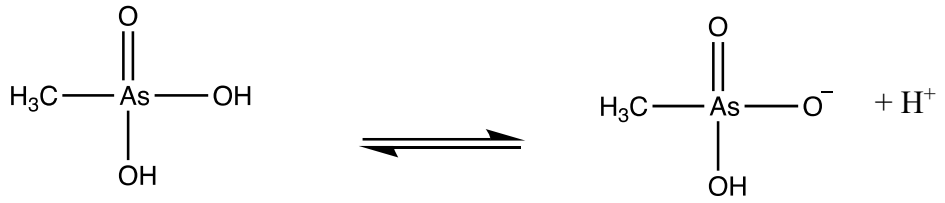
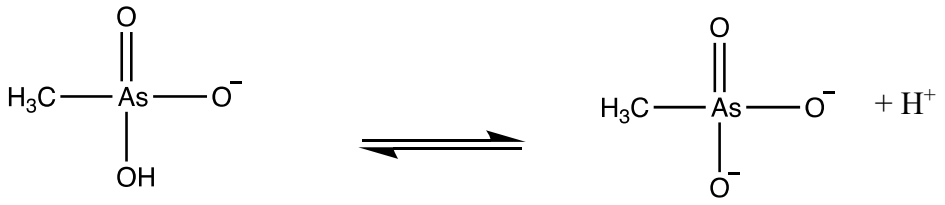
2. DEVELOPMENT OF AN ANALYTICAL METHOD FOR ARSENIC SPECIATION IN URINE

2.1 INTRODUCTION

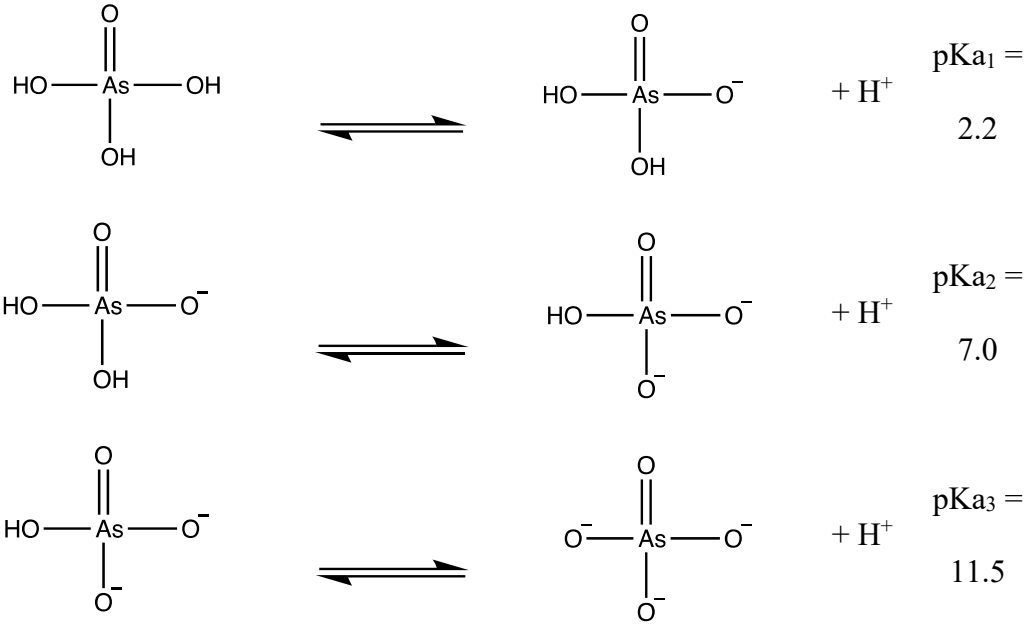
Urine is the main elimination pathway for arsenic in humans, and 58% of arsenic ingested in humans usually excretes within 3-5 days (1). Therefore, a suitable method for arsenic speciation will allow for determination of arsenic urinary profiles of individuals and provide important information on recent exposure to arsenic.

There are pertinent properties of the five main arsenic species that can be utilized to achieve specific determination of each of the compounds in urine. These include both structural differences (size, shape, and charge) and physico-chemical differences (pKa and ionic strength) (2). Of these, differences in the pKa of arsenic compounds (**Table 2.1**) has been commonly used as an effective strategy for separation of the arsenic species.

Table 2.1 Anionic behavior and pKa values for arsenic species

Species	Equilibrium	pKa
AsB		pKa = 2.2
iAs^{III}		pKa$_1$ = 9.2
		pKa$_2$ = 12.1
		pKa$_3$ = 13.4
DMA		pKa = 6.2
MMA		pKa$_1$ = 4.1
		pKa$_2$ = 8.7

iAs^{V}



Chromatographic methods for separation of arsenic are most common, as they provide a variety of separation modes for arsenic separation and allow for different detection techniques (3). For example, high pressure liquid chromatography (HPLC) has been successfully coupled to a variety of detection methods (hydride generation atomic emission spectrometry (HG-AAS), hydride generation atomic emission spectrometry (HG-AES), and inductively coupled plasma mass spectrometry (ICP-MS). These methods have been successful in achieving detection of arsenic species at trace levels in environmental samples.

Anion exchange chromatography was used for separation of the five arsenic compounds, using a PRP-X100 anion exchange HPLC column (150 x 4.10 mm, 5 µm particle size, Hamilton). This column is packed with polystyrene divinylbenzene (PSDVB) and trimethylammonium material – making the column positively charged, with a high surface area and high capacity to interact with

the anionic arsenic species. Compounds that have a greater negative charge will interact with these columns at a greater rate, and thus will be retained in the column for a longer time. Thus, the use of anion exchange chromatography allows for separation of the arsenic species based on their pKa, at a pH of 8, AsB (a zwitter-ion) will elute quickly, as it will have little-to-no interaction with the column, followed by iAs^{III} (neutral charge), DMA (-1 charge), MMA (-1 charge), and iAs^{V} (-2 charge).

I coupled the HPLC separation of arsenic with ICP-MS detection. ICPMS allows for high selectivity and sensitivity for arsenic detection, and a wide dynamic range, and is one of the most commonly used technique for arsenic detection (4). The use of HPLC-ICPMS allowed for detection of arsenic at trace levels, with little interference from the urine matrix (composed of water, chloride, salt, electrolytes, urea, and uric acid).

The objective of this chapter is to develop an analytical method using HPLC-ICPMS that is suitable for determination of arsenic in a high volume of urine samples, with insignificant interference from the aforementioned urine matrix. The mobile phase (pH and salt concentration), HPLC conditions (elution gradient and injection volume), and ICP-MS conditions (helium gas rate) was optimized to achieve optimal arsenic detection in urine. This method was then used for determination of arsenic species in the urine of an arsenic-affected population in Bangladesh in the subsequent chapter.

2.2 MATERIALS AND METHODS

2.2.1 Instrumentation

The separation of arsenic was achieved using a PRP-X100 anion exchange column (150 x 4.10 mm, 5 µm particle size, Hamilton) and installed on an Agilent 1200 series HPLC system (Agilent Technologies, Germany). The temperature of the column was kept constant at room temperature (~30°C). An Agilent 7900 ICPMS system (Agilent Technologies, Japan) was used for detection of arsenic. Various parameters, including elution gradient, injection volume, and helium gas rate were optimized in order to make the method suitable for rapid analysis of trace concentrations of arsenic species in a high volume of urine samples.

2.2.2 Reagents and standards

Stock solutions of 10 mg/L of AsB, iAs^{III}, MMA, DMA, and iAs^{V} were prepared from Arsenobetaine (98% purity, Tri Chemical Laboratories Inc., Japan), sodium m-arsenite (97.0%, Sigma, St. Louis, MO), monosodium acid methane arsonate (99.0%, Chem Service West Chester, PA), cacodylic acid (DMA, 98%, Sigma), and sodium arsenate (99.4%, Sigma). All dilutions were made using deionized water obtained from a Milli-Q water purification system (18.2 M Ω·cm, Millipore, Molsheim, France). Environmental calibration standard (Agilent Technologies, Santa Clara, California, U.S.) was used as a primary standard to calibrate the 10 mg/L arsenic standards. These standards were then serially diluted to 10 µg/L and 1 µg/L, using deionized water. Certified ACS plus concentrated nitric acid (HNO_3) was used to dilute the

standards and urine samples to 1% HNO_3 as the final concentration for determination of total arsenic. Ammonium bicarbonate (NH_4HCO_3) (>99%, Sigma, St. Louis, MO), ammonium hydroxide (NH_4OH) (28% NH_3 in H_2O, Sigma), and HPLC grade methanol (>99.8%, Fisher Scientific, Fair Lawn, NJ) were used to make the mobile phase. Helium gas (99.5-100%, Mississauga, Canada, Praxair) was used to remove interferences. Finally, a negative urine control (Sigma, Merck, Germany) was used to determine any urine matrix effects for optimization of the method.

2.2.3 Standard and certified reference materials

Two standard reference materials (SRM) and one certified reference material (CRM) were used in this study. SRM1640a (trace elements in natural water, National Institute of Standards and Technology (Gaithersburg, MD)) was used for quality control of iAs^{III} and iAs^{V} (8.075 ± 0.070 µg/L) in the calibration standards, and for total arsenic determination. SRM2669 Level I (arsenic species in frozen human urine, National Institute of Standards and Technology (Gaithersburg, MD)) was used for quality control of AsB (12.4 ± 1.9 µg/L), MMA (1.87 ± 0.39 µg/L), and DMA (3.47 ± 0.41 µg/L) in the calibration standards. Finally, CRM18 (human urine, National Institute for Environmental Studies (Tsukuba-City, Japan)) was used for quality control of high concentrations of AsB (0.069 ± 0.012 mg/L) and DMA (0.036 ± 0.009 mg/L) in the calibration standards.

2.2.4 Sample analysis

Table 2.2 shows the optimal instrumental conditions used for operation of the ICP-MS. Arsenic was detected at a m/z of 75, under helium mode, in order to prevent any interference from chloride present in urine samples. Standard and certified reference materials were used prior to analysis of any samples in order to confirm the accuracy of the calibration standards every day. An accurate 1 µg/L and 10 µg/L mixed arsenic standard was prepared in order to develop and optimize a method suitable for analysis of arsenic in a high volume of urine samples.

Table 2.2 ICP-MS instrumental conditions used for analysis

Plasma parameters	
RF power	1550 W
RF matching	1.60 V
Nebulizer gas rate	1.09 L/min
Nebulizer pump speed	0.50 rotations-per-second
Spray chamber temperature	2 °C
Auxiliary gas	0.90 L/min
Plasma gas	15.0 L/min
Reaction/collision cell parameters	
Helium flow rate	3.5 mL/min
Octupole bias	-18.0 V
Quadrupole bias	-13.0 V

RF=radio frequency

The separation of AsB, iAs^{III}, MMA, DMA, and iAs^{V} was tested using a PRP-X100 anion exchange column (150 x 4.10 mm, 5 µm particle size, Hamilton). The initial mobile phase used

was composed of 35 mM ammonium bicarbonate and 5% methanol. The pH of the mobile phase was adjusted to 8.25 using 10% ammonium hydroxide. The temperature of the column was kept at room temperature (30°C). The flow rate was kept at 0.80 mL/min for the first 4.00 minutes, changed from 0.80 mL/min to 1.50 mL/min from 4.00 minutes to 4.01 minutes, then kept at 1.50 mL/min for the next 16.00 minutes. The injection volume was 50 µL.

2.2.5 Human urine sample analysis

A human urine sample (raw sample), from a human not exposed to high levels of arsenic and not related to our study population, was run using the elution gradient and method described in section 2.2.4. Aliquots of the human urine sample were then individually spiked with ~1 µg/L of AsB, As^{III}, DMA, MMA, and As^{V} for identification of the unknown peaks.

2.2.6 Optimization of mobile phase salt concentration and pH

The separation and detection of AsB, As^{III}, DMA, MMA, and As^{V} in the 10 µg/L mixed standards were optimized by adjusting the salt concentration and pH of the mobile phase. The overall run time was set to 20 minutes for lower mobile phase salt concentrations (30 mM and 40 mM ammonium bicarbonate) in order to ensure As^{V} eluted within the run time.

The mobile phase tested ranged from 30 mM to 70 mM ammonium bicarbonate. Each mobile phase also contained 5% methanol. The pH of each mobile phase was adjusted to 8.5 using 10% ammonium hydroxide.

The mobile phase pH tested ranged from 8.2 to 9.0 (adjusted using 10% ammonium hydroxide. The mobile phase salt concentration was kept constant at 50 mM ammonium bicarbonate, with 5% methanol.

2.2.7 Optimization of elution gradient

A mobile phase composed of 60 mM ammonium bicarbonate and 5% methanol, with a pH of 8.5 was used to optimize the elution gradient. Various gradients and flow rates were used in order to assess repeatability (%RSD) of retention time (t_r), peak area and peak height values as measures of separation of arsenic species. The optimization started with use of 10 µg/L mixed standards of AsB, iAs^{III}, DMA, MMA, and iAs^{V}, and then switched to the 1 µg/L.

2.2.8 Optimization of injection volume

Injection volumes of 5 µL, 20 µL, 50 µL, and 100 µL of the 10 µg/L mixed arsenic standards, as well as 50 µL and 100 µL of the 1 µg/L mixed arsenic standards were run with the mobile phase composed of 50 mM ammonium bicarbonate and 5% methanol. The mobile phase pH was adjusted to 8.5 using 10% ammonium hydroxide. Repeatability of retention time, peak area and peak height were assessed for the injection volumes.

2.2.9 Optimization of the flow rate of helium gas into the collision cell of ICP-MS

Direct aspiration of pure 18.2 MΩ-cm water and a 1400 mg/L chloride solution were run through to ICPMS (no HPLC) at various helium gas flow rates ranging from 1.00 mL/min to 4.00 mL/min. The nebulizer pump speed was kept at 0.5 rpm/min. A volume of 50 µL of 1400 mg/L chloride solution, 100 µg/L and 1 µg/L mixed arsenic standards solution were manually injected (the chloride solution was injected multiple times in each run). The optimal helium flow rate was assessed at baseline and the different standard concentrations to determine optimal sensitivity

2.2.10 Statistical Analysis

All urine samples were analyzed in duplicate for arsenic speciation analysis, and in triplicate for total arsenic analysis (unless otherwise stated). The mean values of the arsenic concentrations were reported. Additionally, the accuracy, standard deviation, relative standard deviation, recovery, standard error, error, and residual values of the final method were calculated (equations below).

a. Accuracy: $1 - (\frac{|True\ value - Measured\ value|}{True\ value}) \times 100\%$
b. Standard deviation (SD): $\sqrt{\frac{\Sigma(x_i - \mu)^2}{n-1}}$
c. Relative standard deviation (%RSD): $\frac{\sqrt{\frac{\Sigma(x_i - \mu)^2}{n-1}}}{\mu}\ x\ 100\%$
d. Recovery: $\frac{[Spiked\ As\ (mean)] - [1\ ppb\ As\ (mean)]}{[Spiked\ As]}) \times 100\%$
e. Standard error: $\frac{standard\ deviation}{\sqrt{n}}$

f. Error: Measured value – true value

g. Residual: $Observed\ Y\ value - Predicted\ Y\ value$

2.3 RESULTS AND DISCUSSION

2.3.1 Preliminary analysis of arsenic standards, a urine sample, and individual arsenic standards added into the urine sample

A random urine sample (from an unexposed human) was analyzed to observe the presence of common arsenic species and determine the retention times for each of the 5 arsenic species most commonly found in urine (AsB, iAs^{III}, DMA, MMA, and iAs^{V}). Increases observed for each peak, after spiking with individual arsenic standards, allowed for identification of the peaks – as shown in **Figure 2.1**. The order of elution for the arsenic species was determined to be AsB, iAs^{III}, DMA, MMA, and then iAs^{V}.

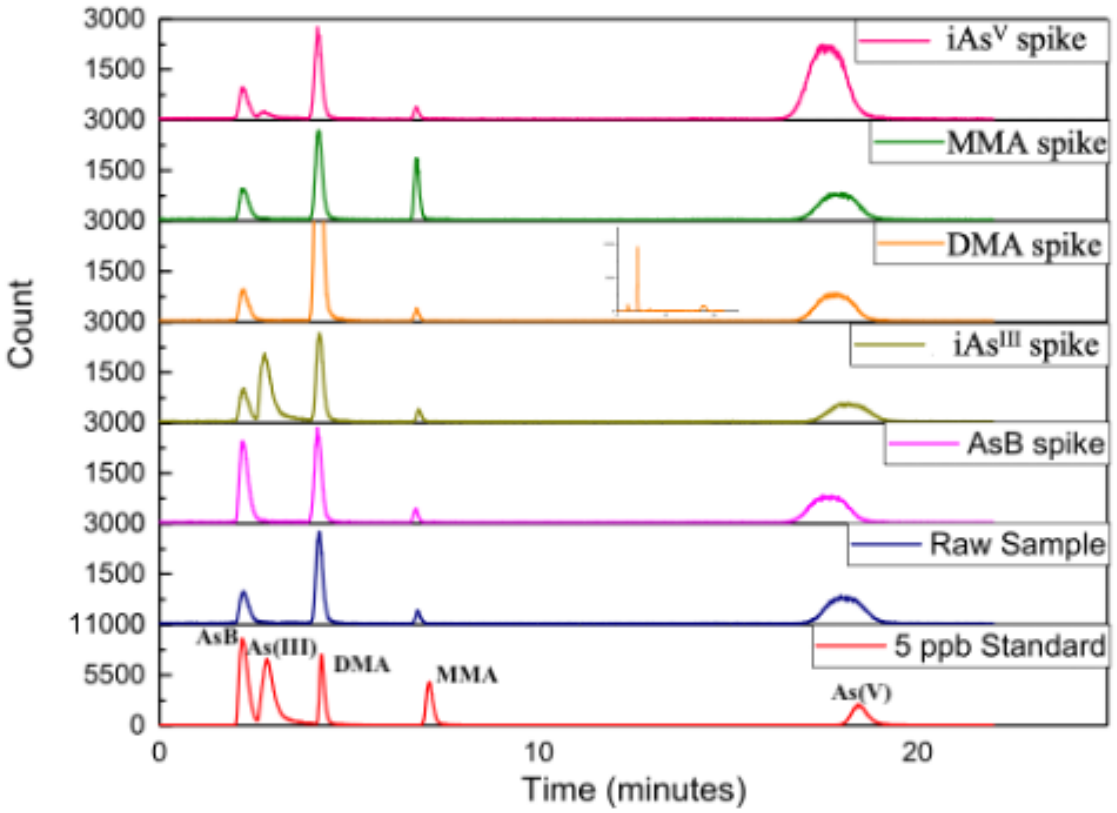


Figure 2.1 Chromatogram of human urine sample with spiked arsenic standards for identification of unknown arsenic peaks. The separation was done using an injection volume of 50 µL, a PRP-X100 anion exchange column, 35 mM ammonium bicarbonate and 5% methanol mobile phase (pH = 8.25), and the flow rate was kept at 0.80 mL/min for the first 4.00 minutes, increased to 1.50 mL/min from 4.00 minutes to 4.01 minutes, and then kept at 1.50 mL/min for the next 16.00 minutes. (5 ppb = 5 µg/L)

2.3.2 Optimization of mobile phase salt concentration and pH

After confirmation of the peak identities, the mobile phase salt concentration and pH were optimized in order to achieve sufficient separation of AsB, iAs^{III}, DMA, MMA, and iAs^{V}, decrease the overall run time, and achieve good peak shape for the arsenic species.

Higher concentrations of ammonium bicarbonate salt compete with, and decrease, the interactions between the anionic arsenic species and the column – resulting in faster elution of the arsenic species. This permits for short run times and makes the method more suitable for high throughput analysis. However, too high concentrations may result in insufficient separations of the arsenic species due to overlapping of different arsenic peaks, broadening of the peak shapes and increases in the baseline. The salt buildup in the column, and salt deposits on the ICP-MS detector may prohibit sensitive and accurate detection of the arsenic species present in the urine samples. A mobile phase ammonium bicarbonate concentration of 30-70 mM was tested and 60 mM was determined to result in the fastest retention of the arsenic species, with good resolution and no increase in baseline (**Figure 2.2**).

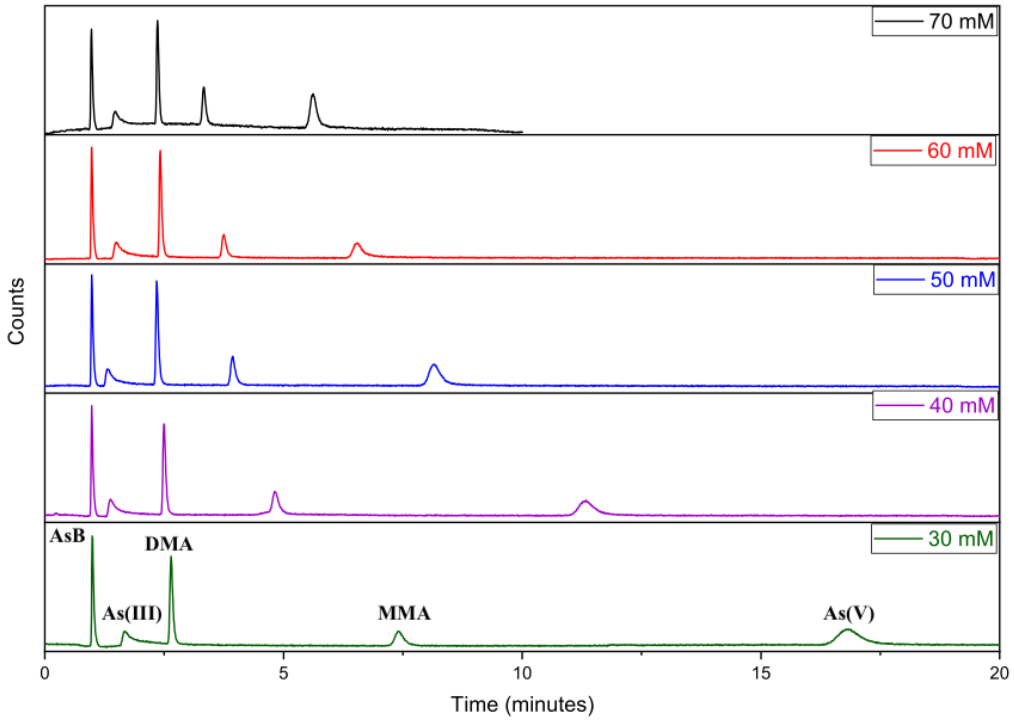


Figure 2.2 Separation of 10 µg/L AsB, iAs^{III}, DMA, MMA, and iAs^{V} using different mobile phase (pH 8.5) ammonium bicarbonate concentrations (from 30 mM to 70 mM).

The pH of the mobile phase determines the anionic characteristics of the arsenic species. Similarities between the mobile phase pH and the pKa of specific arsenic species may result in broader peaks due to greater variability in the charges of the arsenic species. A mobile phase pH of 8.2 was determined to result in the best peak shape and separation of all five arsenic species (**Figure 2.3**).

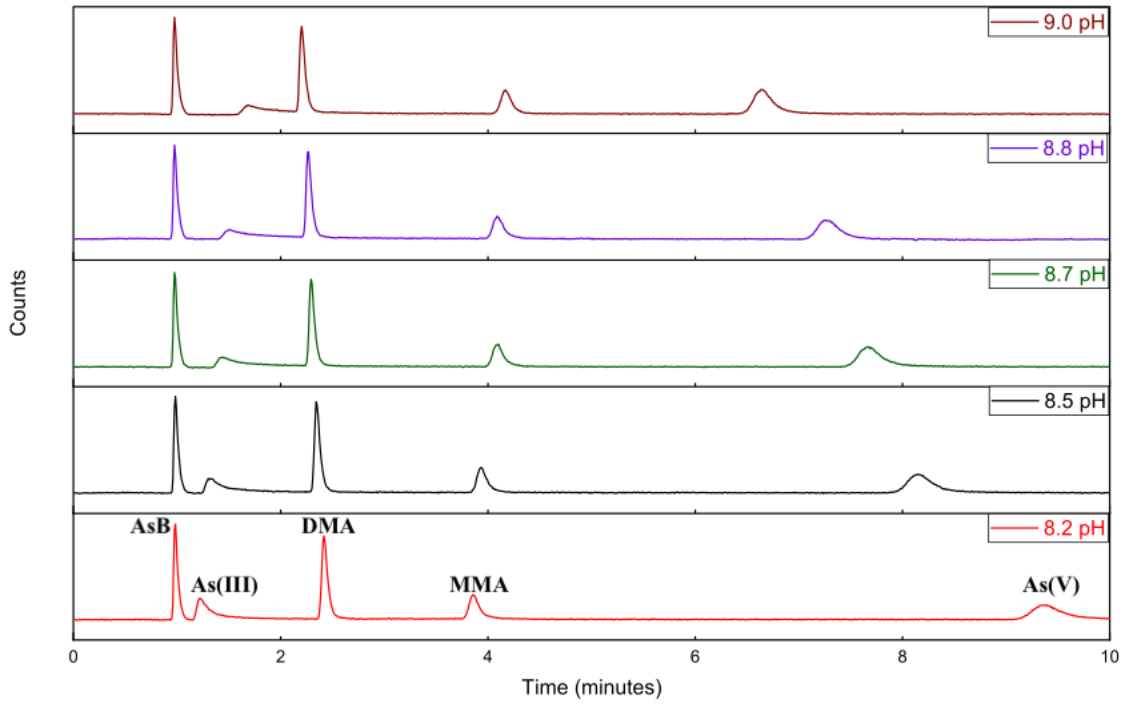


Figure 2.3 Separation of 10 µg/L AsB, iAs^{III}, DMA, MMA, and iAs^{V} using mobile phases (50 mM ammonium bicarbonate) of different pH values (from 8.2 to 9.0) using 10% NH_4OH.

From my experiments and results shown in **Figure 2.2** and **Figure 2.3**, a salt concentration of 60 mM ammonium bicarbonate and pH of 8.2 were shown to achieve the best peak shape, prohibit increases in the baseline, and achieve a fast overall run time.

2.3.3 Optimization of elution gradient

The elution gradient was optimized in order to achieve sufficient separation of the five arsenic species, quick overall run time, and a low %RSD between runs (n=20). An elution gradient allows for use of a lower salt concentration in the beginning of the elution in order to achieve sufficient separation of the first four arsenic species (especially for separation of AsB and iAs^{III}, which elute very close together), and a higher salt concentration in the end of the elution in order

to ensure quick elution of iAs^V and a quick overall run time. The elution gradient was developed to achieve optimal separation of the arsenic species, with the best repeatability (measured by %RSD of the peak areas of each of the peaks).

The initial gradient, (Gradient 1, **Table 2.3**) utilized a ramp from 0% of the 60 mM NH_4HCO_3 mobile phase to 100% of the 60 mM NH_4HCO_3 mobile phase in the first minute, in order to achieve sufficient separation of the AsB and iAs^{III} peaks, which often elute very close together. Then, 100% of the 60 mM NH_4HCO_3 mobile phase was kept for the next 7.00 minutes in order to separate the rest of the arsenic species in a timely manner. Finally, the mobile phase was switched to 0% of the 60mM NH_4HCO_3 mobile phase for the last 2.00 minutes in order to prevent build-up of any salt in my column. This elution gradient resulted in high %RSD values for AsB, iAs^{III}, DMA, MMA, and iAs^V (**Table 2.4**).

Table 2.3 Gradient 1 (initial)

Time (min)	%A	%B	Flow rate
0.00 – 1.00	0	100	Linear increase from 1.00 mL/min to 1.80 mL/min
1.00 – 1.01	Linear increase from 100% B to 100% A		
1.01 – 8.00	100	0	1.80 mL/min
8.00 – 8.01	Linear increase from 100% A to 100% B		1.80 mL/min
8.01 – 10.00	0	100	1.80 mL/min

A = 60 mM NH_4HCO_3, **B** = 5% MeOH

Table 2.4 Gradient 1 repeatability of arsenic species separation (10 µg/L standard)

Standard (10 µg/L)	Retention time (t_r) (min)	t_r RSD (%)	Peak area RSD (%)	Peak Height RSD (%)
AsB	1.0	0.2	10.9	9.6
iAs^{III}	1.5	0.9	58.4	30.1
DMA	2.2	0.6	23.1	16.5
MMA	4.2	0.4	35.8	22.9
iAs^{V}	8.5	0.9	71.4	48.6

This low repeatability may have been due to salt build up in the column over each run, so I increased the overall gradient elution by 2 minutes to allow for elution of 0% of the NH_4HCO_3 mobile phase for 4 minutes total (**Table 2.5** Gradient 2, **Table 2.7** Gradient 3). This resulted in better repeatability for the DMA and MMA peaks, but poor repeatability for inorganic arsenic and AsB (**Tables 2.6 & Table 2.8**).

Table 2.5 Gradient 2 (increased run time)

Time (min)	%A	%B	Flow rate
0.00 – 1.00	0	100	Linear increase from 1.00 mL/min to 1.80 mL/min
1.00 – 1.01	Linear increase from 100% B to 100% A		
1.01 – 8.00	100	0	1.80 mL/min
8.00 – 8.01	Linear increase from 100% A to 100% B		1.80 mL/min
8.01 – 12.00	0	100	1.80 mL/min

A = 60 mM NH_4HCO_3, **B** = 5% MeOH

Table 2.6 Gradient 2 repeatability of arsenic species separation (10 µg/L standard)

Standard (10 µg/L)	Retention time (t_r) (min)	t_r RSD (%)	Peak area RSD (%)	Peak Height RSD (%)
AsB	1.0	0.4	9.0	10.5
iAs^{III}	1.4	0.6	11.3	7.6
DMA	2.3	0.2	1.9	2.1
MMA	4.2	0.1	4.0	3.8
iAs^{V}	8.1	2.4	35.0	29.3

Table 2.7 Gradient 3 (initial calibration of column at time=0)

Time (min)	%A	%B	Flow rate
0.00 – 1.00	0	100	
1.00 – 1.01	Linear increase from 100% B to 100% A		Linear increase from 1.00 mL/min to 1.80 mL/min
1.01 – 8.00	100	0	1.80 mL/min
8.00 – 8.01	Linear increase from 100% A to 100% B		1.80 mL/min
8.01 – 12.00	0	100	1.80 mL/min

A = 60 mM NH_4HCO_3, **B** = 5% MeOH

Table 2.8 Gradient 3 repeatability of arsenic species separation (1 µg/L standard)

Standard (1 µg/L)	Retention time (t_r) (min)	t_r RSD (%)	Peak area RSD (%)	Peak Height RSD (%)
AsB	1.0	0.0	13.1	11.4
iAs^{III}	1.5	0.0	11.6	11.0
DMA	2.2	0.4	8.6	8.8
MMA	4.2	0.2	14.3	11.1
iAs^{V}	8.2	4.2	10.7	36.0

The low repeatability in peak intensity for AsB and iAs^{III} for both the 10 mg/L standards (**Table 2.6**) and 1 mg/L standards (**Table 2.8**) may be due to the drastic change of the mobile phase in the beginning of the method, from 0 mM NH_4HCO_3 to 60 mM NH_4HCO_3. This sudden and large change in mobile phase may have significant variability run-to-run, and result in lower repeatability. To combat this, and calibrate the column for the rest of the run, the first 1.19 minutes of the run only used a mobile phase composed of 50% NH_4HCO_3 (**Gradient 4**, **Table 2.9**). This was done to ensure there was no change in mobile phase salt concentration while AsB and iAs^{III} eluted, and as expected, this change resulted in better repeatability for both peaks (**Table 2.10**). **Gradient 4** (**Table 2.9**) achieved the best overall repeatability of arsenic species separation (**Table 2.10**).

Table 2.9 Gradient 4 (initial calibration of column at time=0)

Time (min)	%A	%B	Flow rate
0.00 – 1.19	50	50	
1.19 – 1.20	Linear increase from 50% A / 50% B to 100% A		Linear increase from 1.00 mL/min to 1.80 mL/min
1.20 – 8.00	100	0	1.80 mL/min
8.00 – 8.01	Linear increase from 100% A to 100% B		1.80 mL/min
8.01 – 12.00	0	100	1.80 mL/min

A = 60 mM NH_4HCO_3, **B** = 5% MeOH

Table 2.10 Gradient 4 repeatability of arsenic species separation (1 µg/L standard)

Standard (1 µg/L)	Retention time (t_r) (min)	t_r RSD (%)	Peak area RSD (%)	Peak Height RSD (%)
AsB	1.0	0.0	3.9	4.5
iAs^{III}	1.4	1.7	6.8	8.7
DMA	2.3	0.3	5.8	4.2
MMA	4.1	0.3	7.5	3.5
iAs^{V}	8.4	0.7	11.3	4.7

2.3.4 Optimization of injection volume

As one may suspect, higher volume injections result in larger amounts of sample being analyzed – and better sensitivity; however, larger injection volumes also increase the probability of peak tailing or fronting. The injection volume was optimized in order to achieve a high sensitivity and low %RSD, without compromising distortion of the peak shape through peak tailing or fronting. The repeatability was tested using injection volumes of 5 µL, 20 µL, 50 µL, and 100 µL. The mobile phase was composed of 50 mM ammonium bicarbonate and 5% methanol. The mobile phase pH was adjusted to 8.5 using 10% ammonium hydroxide. **Gradient 4** (**Table 2.9)** was used for the separation of arsenic species at each injection volume (**Figure 2.4**). Both 10 µg/L and 1 µg/L mixed arsenic standards were used to assess repeatability of retention time, peak area and peak height for the injection volumes (**Tables 2.11 & 2.12**). I found an injection volume of 50 µL provided the best sensitivity without any peak fronting (**Figure 2.4**). An injection volume of 50 µL also achieved the best repeatability (**Tables 2.11 & 2.12**).

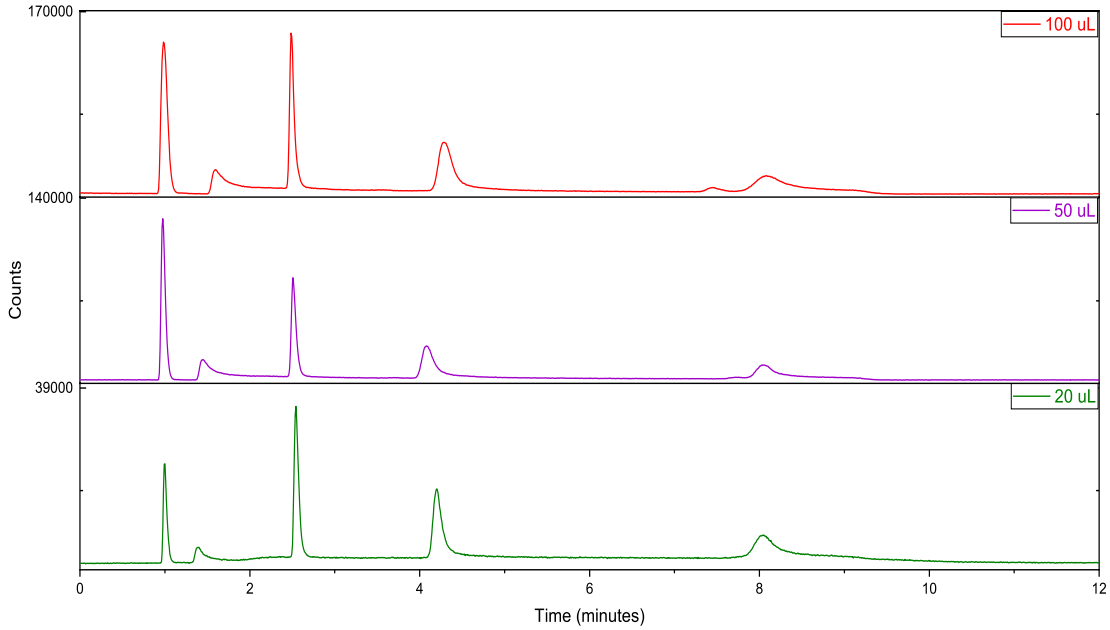


Figure 2.4 10 µg/L mixed standards of AsB, iAs^{III}, DMA, MMA, and iAs^{V}, injected with volumes of 20 µL, 50 µL, and 100 µL.

Table 2.11 Repeatability for varying injection volumes of 10 µg/L mixed arsenic standards (n=10).

Injection Volume	Standard 10 µg/L	t_r RSD (%)	Peak area RSD (%)	Peak Height RSD (%)
5 µL				
	AsB	0.8	4.7	3.9
	iAs^{III}	3.2	49.3	24.5
	DMA	1.9	3.7	4.0
	MMA	1.1	10.1	12.1
	iAs^{V}	1.1	19.3	20.8
20 µL				
	AsB	0.4	9.0	10.5
	iAs^{III}	0.6	11.3	7.6
	DMA	0.2	1.9	2.1
	MMA	0.1	4.0	3.8

	iAs^{V}	2.4	35.0	29.4
50 µL				
	AsB	0.0	5.7	6.6
	iAs^{III}	0.6	5.9	5.0
	DMA	0.2	1.5	1.8
	MMA	0.3	3.1	2.6
	iAs^{V}	0.1	19.2	10.0
100 µL				
	AsB	0.4	5.6	6.6
	iAs^{III}	0.9	15.9	11.8
	DMA	0.1	4.1	4.5
	MMA	0.5	7.3	6.5
	iAs^{V}	1.2	21.9	8.7

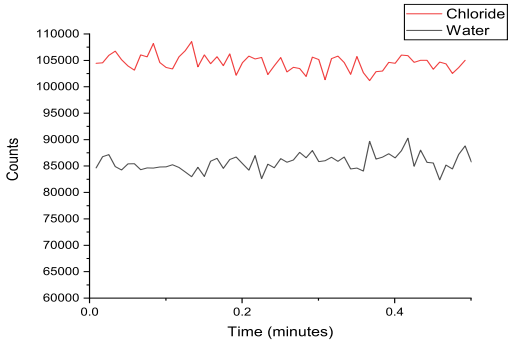
Table 2.12 Repeatability for varying injection volumes of 1 µg/L mixed arsenic standards (n=10).

Injection Volume	**Standard 10 µg/L**	**t_r RSD (%)**	**Peak area RSD (%)**	**Peak Height RSD (%)**
50 µL				
	AsB	0.0	3.9	4.5
	iAs^{III}	1.7	6.8	8.7
	DMA	0.3	5.8	4.2
	MMA	0.3	7.5	3.5
	iAs^{V}	0.7	11.3	4.7
100 µL				
	AsB	0.4	2.6	2.8
	iAs^{III}	0.8	21.0	9.5
	DMA	0.2	3.2	3.5
	MMA	0.2	5.7	6.1
	iAs^{V}	0.4	10.3	8.1

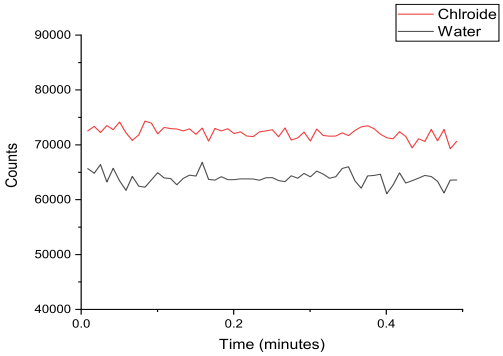
2.3.5 Optimization of the flow rate of helium gas into the collision cell of ICP-MS

Helium gas can also be used in the collision cell of the ICP-MS in order to reduce isobaric interference from potential contaminants. For example, there are large amounts of chloride found in human urine. This chloride can interact with argon gas (used to ignite the plasma for ICP-MS), and form argon chloride ($ArCl^+$), which can interfere with arsenic detection as both compounds have similar m/z values. Polyatomic ion argon chloride ($ArCl^+$) can interfere with arsenic detection at a m/z of 75. Increased concentrations of helium allow for collisions between $ArCl^+$ and helium, preventing $ArCl^+$ to reach the detector. Helium gas rate can be used to remove interferences from chloride in urine, as shown by the decrease in baseline of chloride and the chloride peaks (**Figure 2.5 & 2.6**). However, increased flow rates of helium also result in fewer target ions reaching the detector, and an apparent decrease in sensitivity (**Figure 2.7**). A helium rate of 3.5 mL/min was determined to achieve the best sensitivity while limiting any potential interference that may be present from chloride in human urine (**Figure 2.6**).

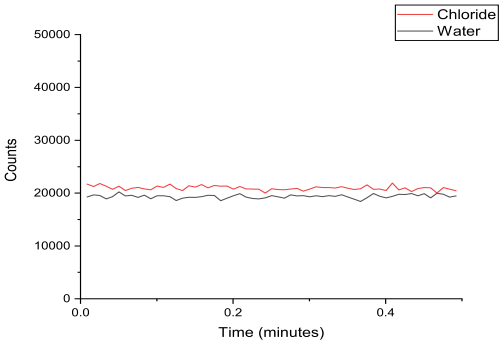
He flow rate = 1.00 mL/min



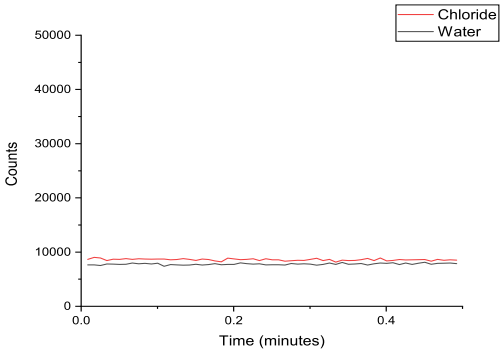
He flow rate = 2.00 mL/min



He flow rate = 3.00 mL/min



He flow rate = 3.50 mL/min



He flow rate = 4.00 mL/min

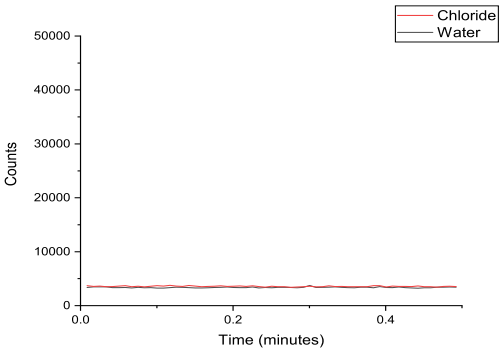


Figure 2.5 Baselines of a pure 18.2 MΩ-cm water and a 1400 mg/L chloride solution through direct aspiration to ICPMS (no HPLC) at various helium gas flow rates ranging from 1.00 mL/min to 4.00 mL/min. The nebulizer pump speed was kept at 0.5 rpm/min.

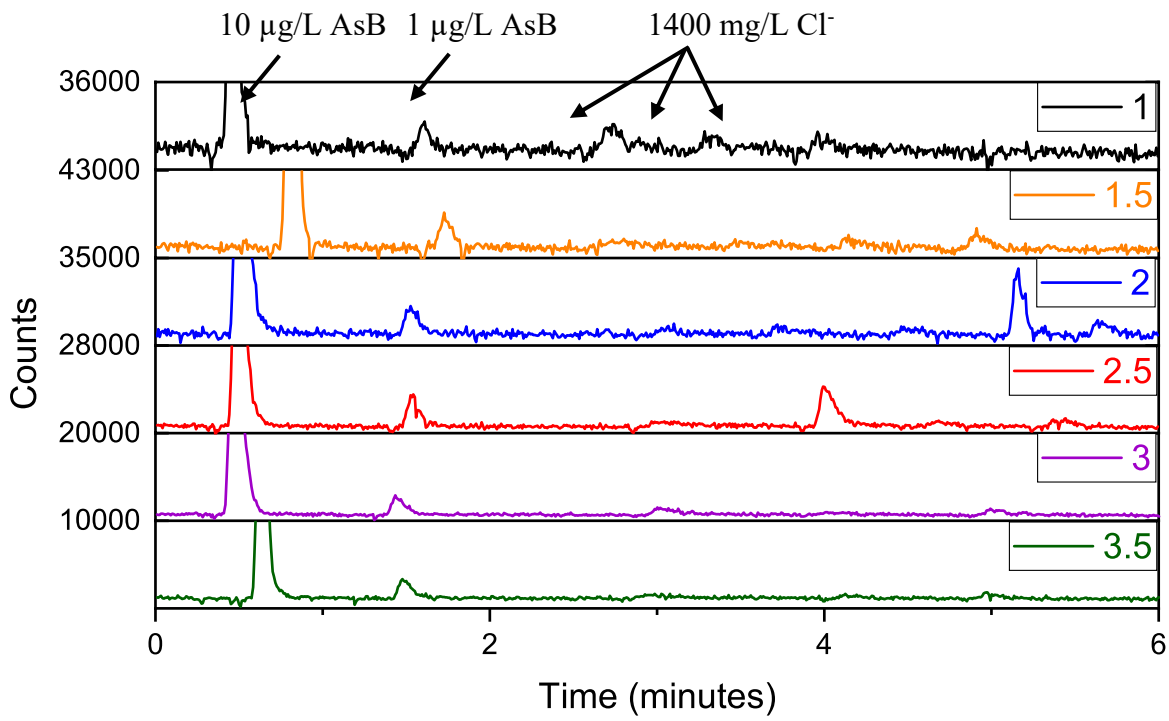


Figure 2.6 Flow injection analysis (50 µL) of, in order of injection, 10 µg/L AsB, 1 µg/L AsB, and multiple injections of 1400 mg/L chloride at various helium gas rates ranging from 1.00 mL/min to 3.50 mL/min. The nebulizer speed was kept at 0.5 rpm/min. A volume of 50 µL of each solution was manually injected (the chloride solution was injected multiple times in each run).

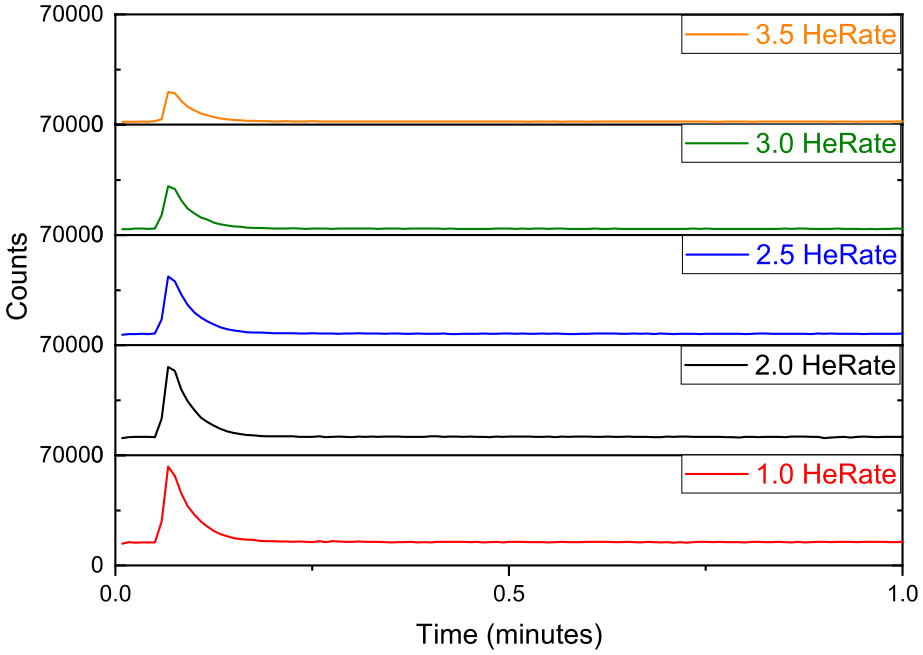


Figure 2.7 Injection of 50 µg/L Mixed As standard (10 µg/L each of AsB, iAs^{III}, DMA, MMA, and iAs^{V}) at helium gas rates ranging from 1.00 mL/min to 3.50 mL/min.

2.3.6 Determination of retention of arsenic species on column

To determine the recovery of the five arsenic compounds from the column, 1 µg/L and 5 µg/L concentrations of each As standard (AsB, iAs^{III}, DMA, MMA, and iAs^{V}) were individually run (one sample per run – ex. 1 µg/L AsB). **Gradient 5** (**Table 2.15**) was used for the run, with a mobile phase salt concentration of 60 mM, and pH of 8.2.

The 1 µg/L and 5 µg/L concentrations of each As standard were then individually run via HPLC-ICPMS, but no column. The resulting peak area was compared to its associated run with the column to determine whether there is any loss of arsenic species due to the column separation.

Multiple injection volumes (20 µL, 30 µL, and 50 µL) were tested to achieve optimal column efficiency (**Tables 2.13 & 2.14**). Longer column washing and a new column were also tested to determine improvements in column efficiency (using an injection volume of 30 µL).

Table 2.13 Agreement between speciation and total arsenic analysis, with increasing injection volume

	% Agreement between Speciation (HPLC-ICPMS) and Total Arsenic (ICPMS)									
Injection volume	**AsB** (µg/L)		**iAs^{III}** (µg/L)		**DMA** (µg/L)		**MMA** (µg/L)		**iAs^{V}** (µg/L)	
(µL)	**1**	**5**	**1**	**5**	**1**	**5**	**1**	**5**	**1**	**5**
20	53	64	69	39	59	65	74	57	50	42
30	66	74	69	57	59	71	69	62	56	58
40	77	70	46	27	43	65	53	60	50	40
50	48	49	26	51	51	63	59	59	47	51

Table 2.14 Agreement between speciation and total arsenic analysis, using an injection volume of 30 µL after cleaning column.

	% Agreement between Speciation (HPLC-ICPMS) and Total Arsenic (ICPMS)									
Injection volume	**AsB** (µg/L)		**iAs^{III}** (µg/L)		**DMA** (µg/L)		**MMA** (µg/L)		**iAs^{V}** (µg/L)	
(µL)	**1**	**5**	**1**	**5**	**1**	**5**	**1**	**5**	**1**	**5**
30	84	87	63	82	59	65	74	57	50	42

Improvement after cleaning the column (with 600 mL of 60 mM ammonium bicarbonate – pH 8.2, 30 µL injection).

To detect any retained arsenic species on our column, a mixed As standard composed of 5 µg/L standards of AsB, iAs^{III}, DMA, MMA, and iAsV. This mixed standard was run using Gradient 5. After each individual run, a blank was run to determine if any species were retained. The absence of peaks in the run with blank water showed that there is no significant retention of arsenic with this method (**Figure 2.8** and **Figure 2.9**).

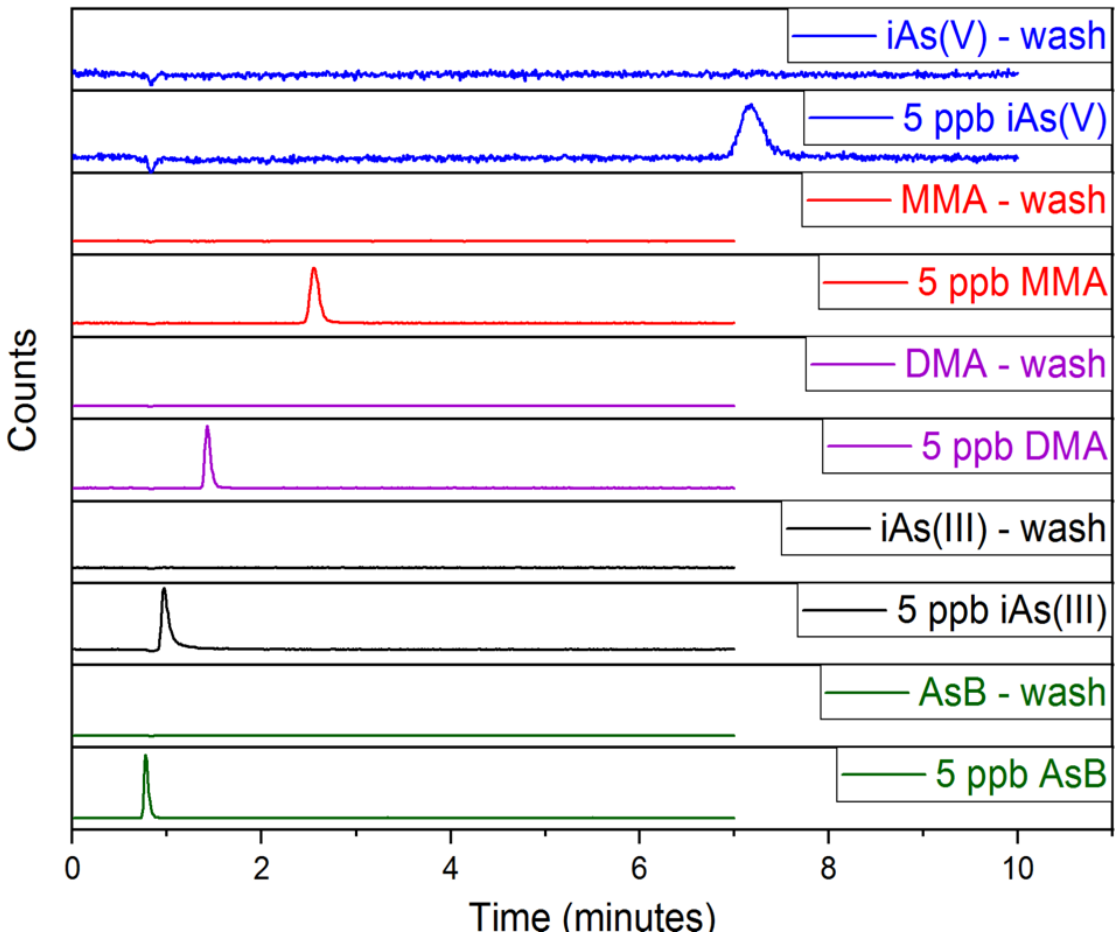


Figure 2.8 Chromatogram showing runs of individual 5 µg/L As standards, and a run of blank water following each 5 µg/L (ppb) As run.

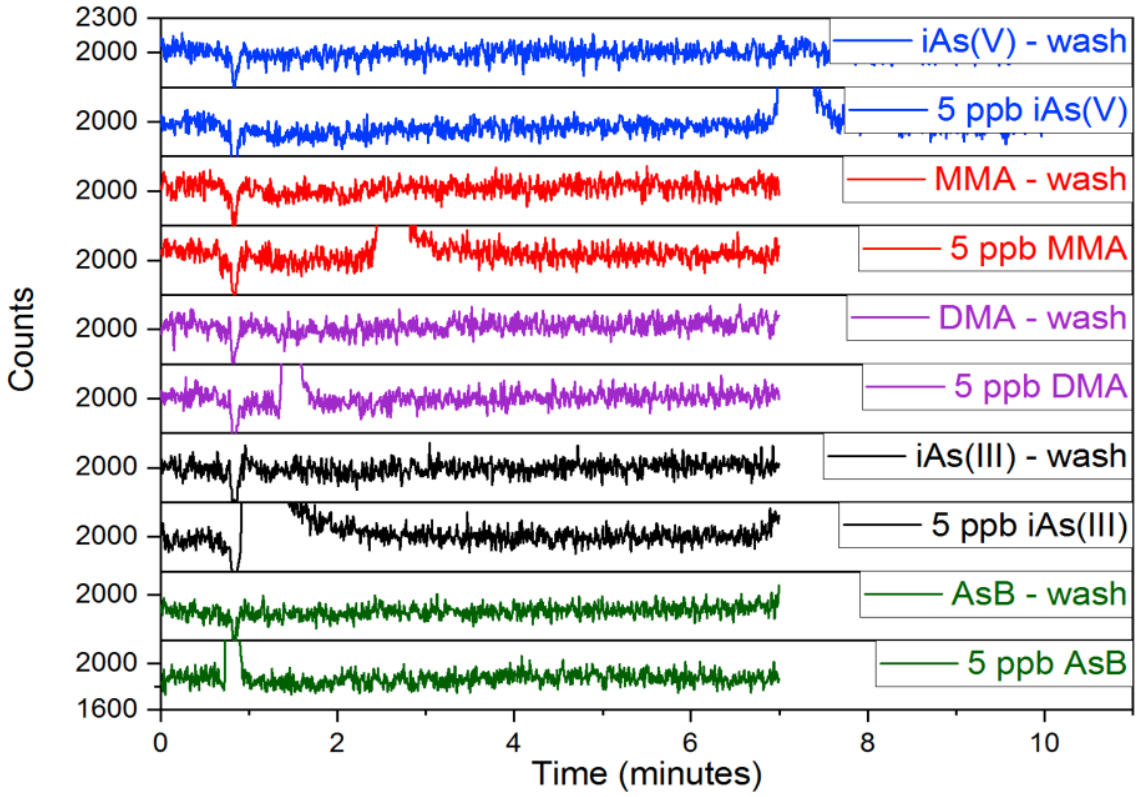


Figure 2.9 Chromatogram showing runs of individual 5 µg/L As standards, and a run of blank water following each 5 µg/L (ppb) As run (zoomed in).

In addition, this experiment was repeated by running a mixed 1 µg/L As standard composed of AsB, iAs^{III}, DMA, MMA, and iAs^{V} using **Gradient 5** (**Figure 2.10 & 2.11**). The wash was collected immediately after the run (~ 10 mL). This wash was then run on the column to further confirm that no arsenic species are retained on the column.

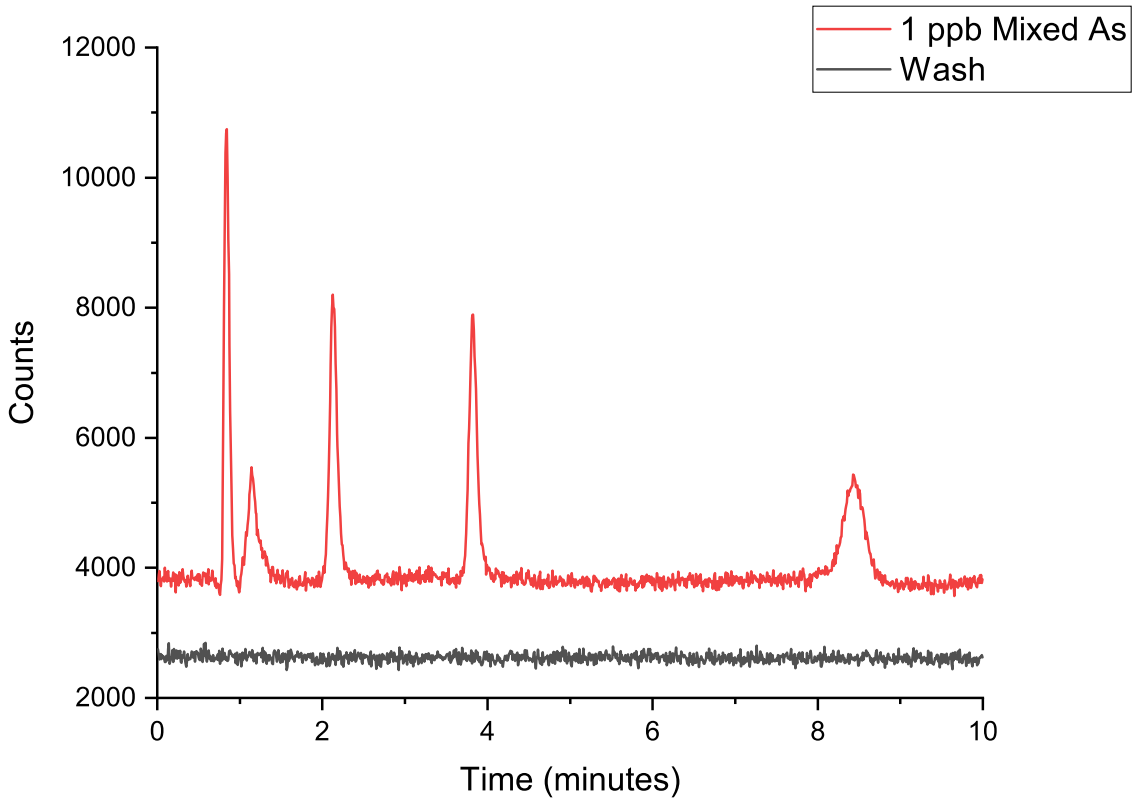


Figure 2.10 Chromatogram showing a run of 1 µg/L (ppb) mixed As, and a run of the wash collected after the 1 µg/L Mixed As run was completed.

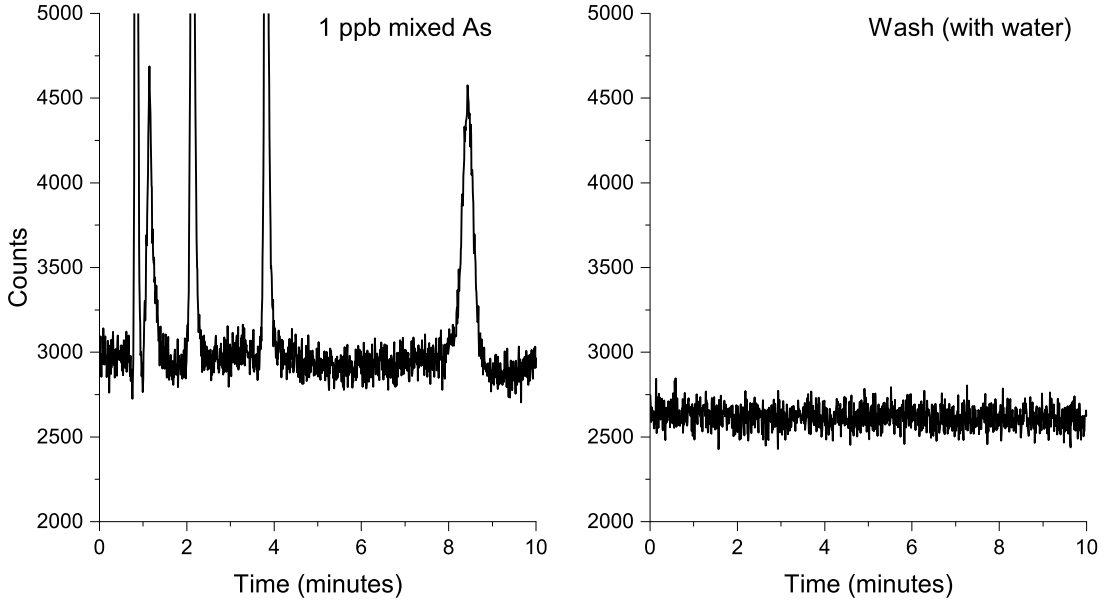


Figure 2.11 Chromatogram showing a run of 1 µg/L (ppb) mixed As, and a run of the wash collected after the 1 µg/L Mixed As run was completed (zoomed in).

2.3.7 Optimized parameters and analytical features of the improved method for As speciation analysis

The mobile phase composition changes from 100% of the 60 mM NH_4HCO_3 to 0% of the 60 mM NH_4HCO_3 when iAs^V is eluted. This significant change in mobile phase composition may lead to low repeatability for the iAs^V peak. To improve the repeatability and improve calibration of the column, the end of the elution was adjusted so that the column is washed with 0% of the NH_4HCO_3 mobile phase for 1.9 minutes starting at 7.30 minutes, and then increased to 50% NH_4HCO_3 for the rest of the run (**Table 2.15** Gradient 5). This elution gradient allows 1.9 minutes for washing the column with 0% NH_4HCO_3. Because the injection process takes 2

minutes, and involves running the last used mobile phase through the column for the duration of the injection, this elution gradient also allows 4 minutes total for calibration of the column.

The procedure (consisting of running a 1 µg As/L solution 10 times) described in section 2.3.3 was repeated with the optimized parameters of the method (**Table 2.15**); a mobile phase composed of 60 mM ammonium bicarbonate and 5% methanol was used, with a pH of 8.2; a helium gas rate of 3.5 mL/min was used; and an injection volume of 30 µL was used. A 1 µg/L mixed arsenic standard was then used to determine analytical features of the final method (**Tables 2.15 & 2.16**, and **Figures 2.13 & 2.14**).

Table 2.15 Gradient 5

Time (min)	%A	%B	Flow rate
0.00 – 1.19	50	50	Linear increase from 1.00 mL / min to 1.60* mL / min
1.19 – 1.20	Linear increase from 50% A / 50% B to 100% A		
1.20 – 7.30	100	0	1.60* mL / min
7.30 – 7.31	Linear increase from 100% A to 100% B		1.60* mL / min
7.31 – 9.20	0	100	1.60* mL / min
9.20 – 9.21	Linear increase from 100% B to 50% A / 50% B		1.60* mL / min
9.21 – 10.0	50	50	1.60* mL / min

A = 60 mM NH_4HCO_3 **B** = 5% MeOH; **Injection volume** = 30 µL; **He rate** = 3.5 mL/min

*Flow rate needed to be reduced to 1.6 mL/min as pressure was too high when using the guard column.

Table 2.16 Repeatability of retention time, peak area, and peak height for the five arsenic species

	Retention time (t_r)	t_r (n=10) RSD (%)	Day-to-day t_r* RSD (%)	Peak area RSD (%)	Peak height RSD (%)
AsB	1.0	0.4	0.5	5.9	4.0
iAs^{III}	1.4	0.3	0.5	5.4	5.2
DMA	2.2	0.7	0.4	2.6	2.1
MMA	4.2	0.2	0.7	3.9	3.2
iAs^{V}	8.4	0.2	0.4	3.1	3.1

*To calculate day-to-day variation in t_r, 1 µg/L mixed arsenic solutions were run (n = 20) on two separate days. The retention time was averaged for both days. The %RSD then was calculated using the two averages for each arsenic species.

Running arsenic standards in a water matrix, and arsenic standards in a urine matrix, showed that there was little difference in peak shape, and overall matrix effect, for arsenic detection in water and urine matrices (**Figure 2.12**).

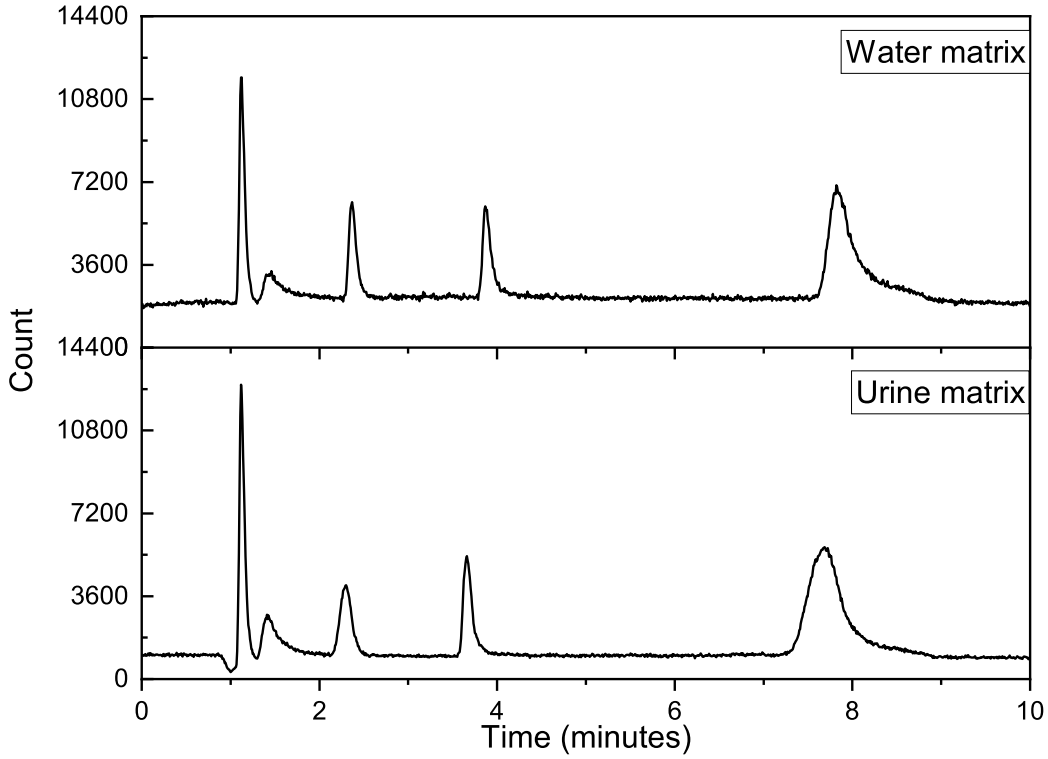


Figure 2.12 1 µg/L concentration of arsenic standards in water matrix and urine matrix. The two matrices were compared to determine if the more acidic pH of urine, and addition of other contaminants found in urine (e.x. chloride, salt, uric acid, urea) could affect the detection of arsenic.

The calibration curves showed a linear relationship from 0 µg As/L to 20 µg As/L (**Figure 2.13**).

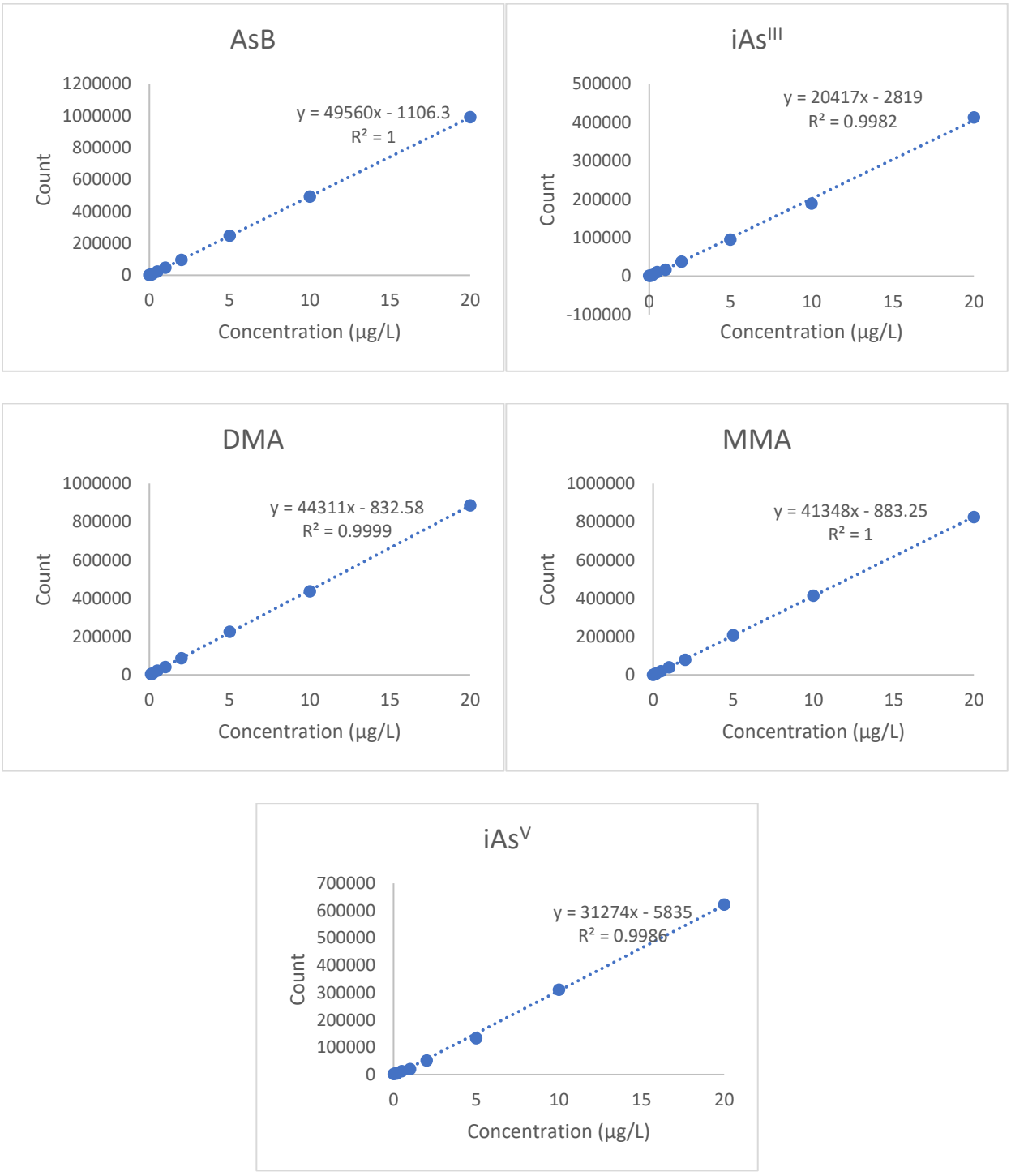


Figure 2.13 Calibration curves used to quantitate arsenic species present in sample, with concentrations of 0 µg/L, 0.1 µg/L, 0.2 µg/L, 1 µg/L, 2 µg/L, 5 µg/L, 10 µg/L, and 20 µg/L used for the calibration standards.

The accuracy of the method was determined by comparing the measured and certified/standard arsenic concentrations in reference materials, and the accuracy ranged from 86% to 97% for the different arsenic species (**Table 2.17**).

Table 2.17 Accuracy of measured concentrations of certified arsenic standard reference materials

Standard	True value (µg/L)	Measured value (µg/L)	Accuracy (%)
AsB (SRM2669)	12.4 ±1.9	11.4	92
AsB (CRM18)	69 ±12	59.4	86
DMA (SRM2669)	3.47 ±0.41	3.02	87
DMA (CRM18)	36 ±9	33.6	93
MMA (SRM2669)	1.87 ±0.39	1.64	88
iAs^{III} + iAs^{V} (SRM1640a)	8.075 ± 0.070	7.80	97

2.3.8 Applying final method to real human urine sample

The final method described in section 2.3.7 was used to measure arsenic concentrations in a real human urine sample. The human urine sample (undiluted) was filtered using a 0.45 µm filter and run 10 times using Gradient 5 (**Table 2.15**), which was described in section 2.3.7. Peaks corresponding to AsB, iAs^{III}, DMA, and MMA were detected (**Figure 2.14**) and their concentration was determined (**Table 2.18**).

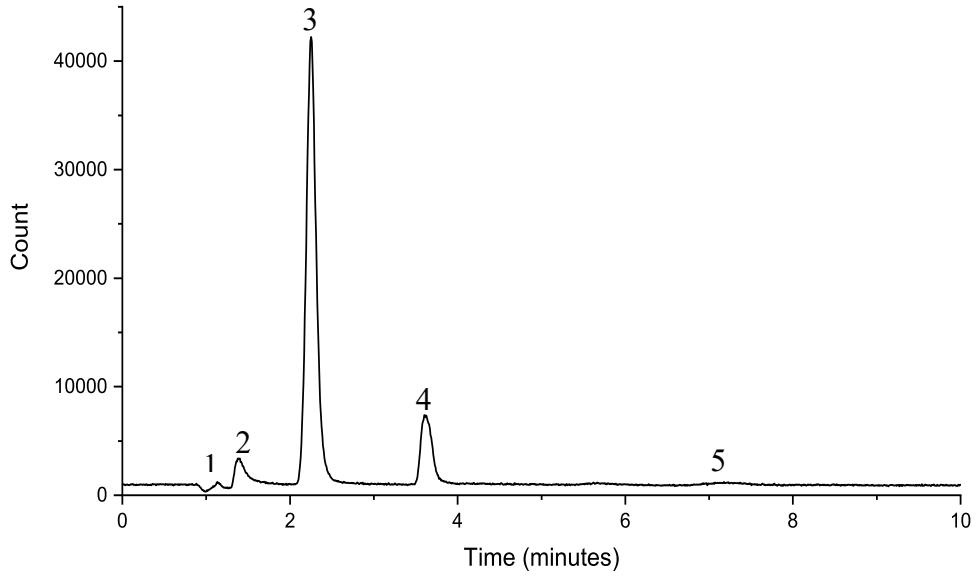


Figure 2.14 Arsenic species in a human urine sample.

Table 2.18 The concentration, standard deviation, and relative standard deviation of the arsenic species present in the real human urine sample (n=10).

Arsenic species	Concentration (µg/L) (mean)	Standard Deviation (n = 10)	RSD (%) (n = 10)
AsB (1)	0.03 (below LOD)	± 0.01	15.7
iAs^{III} (2)	1.73	± 0.14	7.9
DMA (3)	14.0	± 0.5	3.4
MMA (4)	2.15	± 0.06	2.7
iAs^{V} (5)	0.02 (below LOD)	± 0.01	26.6

2.3.9 Figures of merit for final method

The calibration sensitivity for the five arsenic species was determined (**Table 2.19**) using the calibration curves from **Figure 2.13**.

Table 2.19 Calibration sensitivity calculated to determine the change in analytical signal (Y-axis) per unit change in analyte concentration (X-axis).

Standard	Sensitivity (CPS/µg/L)
AsB	49,560
iAs^{III}	20,417
DMA	44,311
MMA	41,348
iAs^{V}	31,274

Using a signal-to-noise ratio of 3, the detection limit for AsB, DMA, MMA, and iAs^{V} was determined to be 0.05 µg/L (**Figure 2.15**).

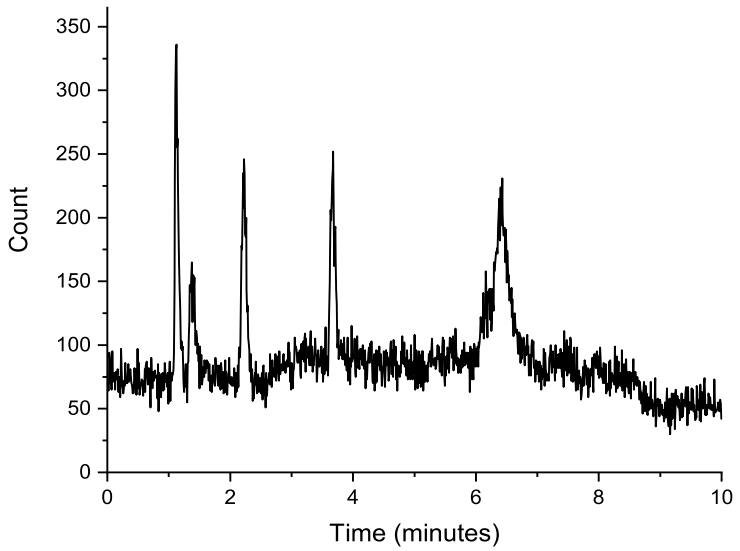


Figure 2.15 Detection limits of AsB, DMA, MMA, and iAs^{V} using 0.05 µg/L arsenic standard

Using a signal-to-noise ratio of 3, the detection limit for iAs^{III} was determined to be 0.1 µg/L (**Figure 2.16**).

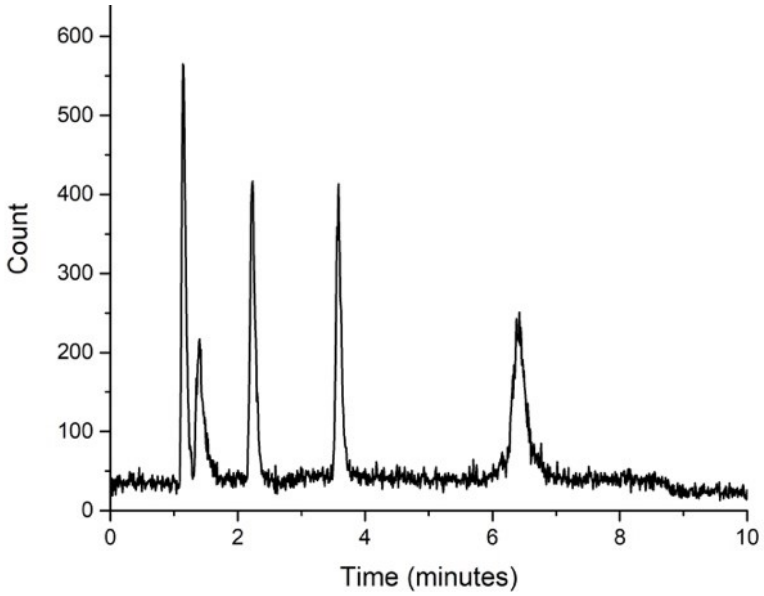


Figure 2.16 Detection limit of iAs^{III} using 1 µg/L arsenic standard

Detection limits for the As species were determined via signal-to-noise ratios of 3 (**Table 2.20**).

Table 2.20 Detection limits determined for the five arsenic species

Arsenic Standard	Detection limit (µg/L)
AsB	0.05
iAs^{III}	0.1
DMA	0.05
MMA	0.05
iAs^{V}	0.05

The As species were found to have a linear relationship from 0 µg/L to 200 µg/L (**Figure 2.17**).

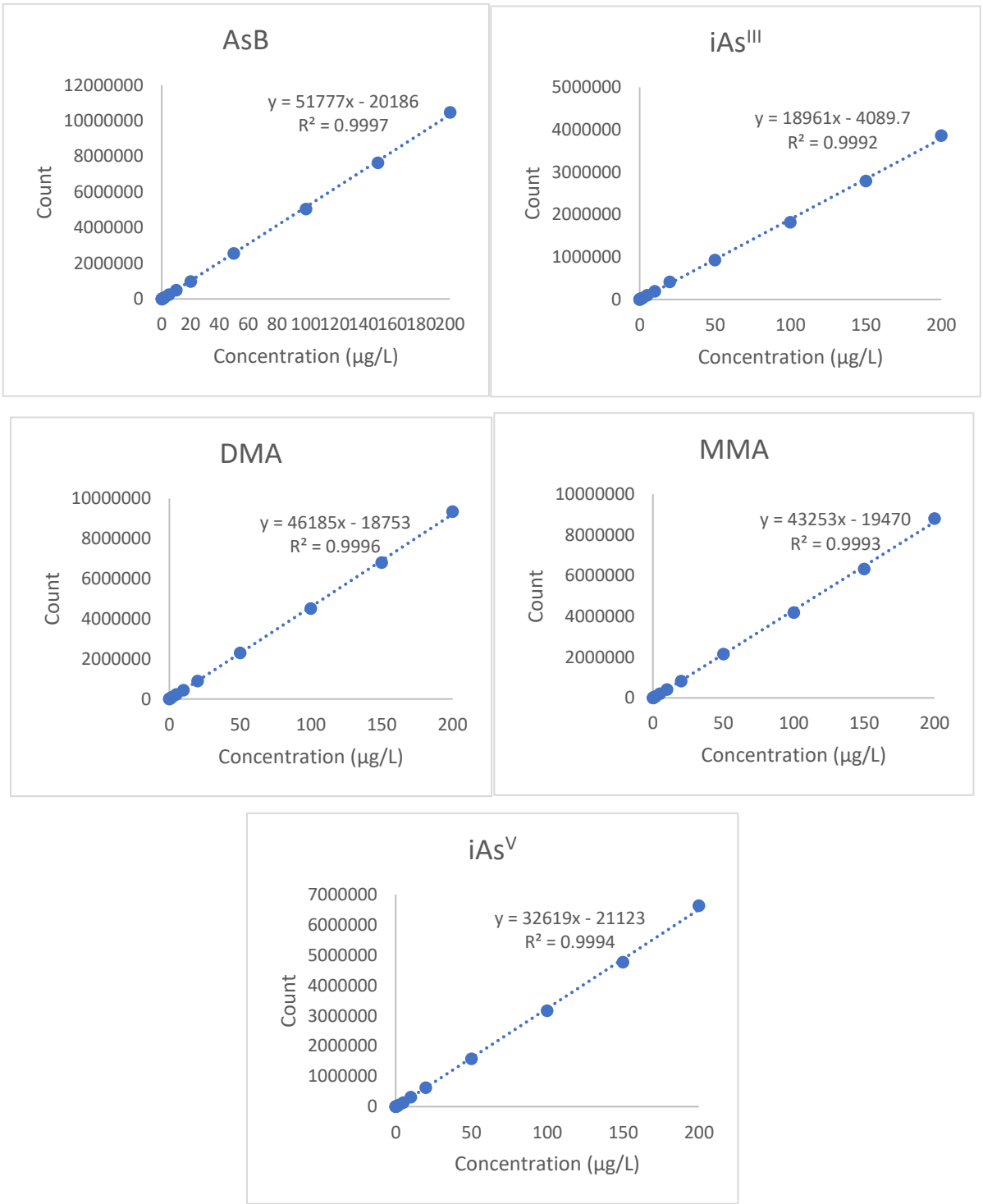


Figure 2.17 Linearity of our method tested using arsenic concentrations from 0.1 µg/L to 200 µg/L.

To determine the recovery of the arsenic compounds, a 1 µg/L mixed arsenic standard was run using **Gradient 5** (n = 10) (**Figure 2.18A**). A 1 µg/L mixed arsenic standard spiked with 1 µg/L mixed arsenic standards (AsB, iAs^{III}, DMA, MMA, iAs^{V}) was then run using **Gradient 5** (n=10) (**Figure 2.18B**). The concentration values for each of the runs, and each of the arsenic species, were averaged and the recovery for each standard was calculated (**Table 2.21**).

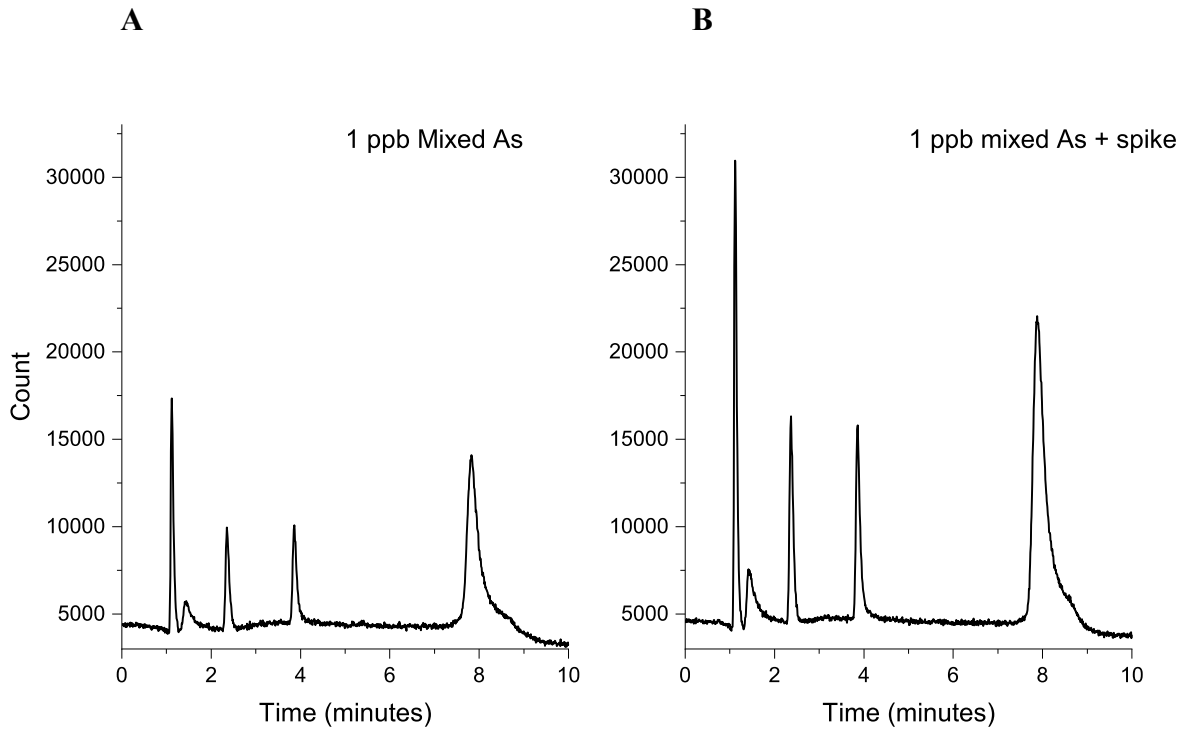


Figure 2.18 1 µg/L (ppb) arsenic standard (A), and 1 µg/L arsenic standard spiked with an additional 1 µg/L (B), used to determine if detection of the As species is affected by differences in the matrix due to dilutions.

Table 2.21 Recovery values for the five arsenic species

Standard (1 µg/L)	Recovery (%) (day 1)	Recovery (%) (day 2)	RSD (%) (n = 2)
AsB	104	99	4
iAs^{III}	96	96	0
DMA	108	99	6
MMA	103	102	1
iAs^{V}	93	92	1

The standard error was found to range from 0.01 to 0.02 µg/L for the As species (**Table 2.22**).

Table 2.22 Standard error of the five arsenic species (n = 10)

Standard (1 µg/L)	Standard error (µg/L)
AsB	0.02
iAs^{III}	0.02
DMA	0.01
MMA	0.01
iAs^{V}	0.01

The error of the measurements of As species ranged from -9.6 µg/L to +2.4 µg/L (**Table 2.23**).

Table 2.23 Standard deviation of the measurements of reference materials to their true value.

Standard	Measured value (µg/L)	True value (µg/L)	Error (µg/L)
AsB (SRM2669)	11.4	12.4 ±1.9	-1.0
AsB (CRM18)	59.4	69 ±12	-9.6
DMA (SRM2669)	3.02	3.47 ±0.41	-0.45
DMA (CRM18)	33.6	36 ±9	+2.4
MMA (SRM2669)	1.64	1.87 ±0.39	-0.23
iAs^{III} + iAs^{V} (CRM1640a)	7.80	8.075 ±0.070	-0.28

The residual values of the five arsenic species were also determined (**Table 2.24**.)

Table 2.24 Summary of the residual values for the five arsenic species, with concentrations ranging from 0.1 µg/L to 20 µg/L.

Arsenic Species	Concentration (µg/L)	Observed Y value (CPS)	Predicted Y value (CPS)	Residual (CPS)
AsB	0.1	1943.3	1106.3	+837.0
	0.2	4161.7	6062.3	-1900.6
	0.5	9012.4	11018.3	-2005.9
	1.0	23359.8	25886.3	-2526.5
	2.0	46947.7	50666.3	-3718.6
	5.0	96107.2	100226.3	-4119.1
	10.0	247686.8	248906.3	-1219.5
	20.0	492785.4	496706.3	-3920.9
As^{III}	0.1	1333.6	-777.3	+2110.9
	0.2	3249.0	1264.4	+1984.6
	0.5	10886.8	7389.5	+3497.3
	1.0	16508.9	17598.0	-1089.1

	2.0	37297.1	38015.0	-717.9
	5.0	95066.0	99266.0	-4200.0
	10.0	188566.2	201351.0	-12784.8
	20.0	412971.0	405521.0	+7450.0
DMA	0.1	4924.5	3598.5	+1326.0
	0.2	7717.5	8029.6	-312.1
	0.5	22022.3	21322.9	+699.4
	1.0	41599.6	43478.4	-1878.8
	2.0	86797.6	87789.4	-991.9
	5.0	225109.6	220722.4	+4387.2
	10.0	437666.2	442277.4	-4611.3
	20.0	886767.2	885387.4	+1379.8
MMA	0.1	3494.7	3251.6	+243.2
	0.2	7650.2	7386.4	+263.8
	0.5	19729.3	19790.8	-61.4
	1.0	39145.8	40464.8	-1318.9
	2.0	78901.0	81812.8	-2911.7
	5.0	208071.7	205856.8	+2215.0
	10.0	413721.5	412596.8	+1124.7
	20.0	825310.8	826076.8	-765.9
As^V	0.1	-2707.6	+6456.6	3749.0
	0.2	419.8	+3564.1	3983.9
	0.5	9802.0	+2690.6	12492.6
	1.0	25439.0	-5114.2	20324.9
	2.0	56713.0	-4866.9	51846.1
	5.0	150535.0	-17367.6	133167.4
	10.0	306905.0	+4210.6	311115.6
	20.0	619645.0	+2850.6	622495.6

These residual values represent the vertical distance between the data points and regression line, and provide information on how well the regression line fits an individual data point.

2.4 CONCLUSION

A method for analysis of arsenic in urine was successfully developed with an analysis time of 10 minutes, and the resulting analytical features show that this method is suitable for analysis of the high volume of urine samples our collaborators have obtained from an arsenic-affected population in Bangladesh. There is little variation run-to-run, with relative standard deviations ranging from 2.6% to 5.9% for peak area, and 2.1% to 5.2% for peak height. In addition, there is little day-to-day variation in the retention time for each of the arsenic species (less than 1% relative standard deviation). The accuracy and validity of this method is also shown in **Table 2.20**, with good comparability between our measured values and the true values of various standard and certified reference materials. Finally, a limit of detection below 0.1 μg/L demonstrate that this method is suitable for determination of the most commonly found concentrations of arsenic in human urine.

There are certain inherit limitations with this method. As previously described in the introduction section, this method is suitable for identification and quantification of the five most commonly found arsenic species in human urine: AsB, iAs^{III}, DMA, MMA and iAs^{V}. However, additional instrumentation (ESI-MS) and/or standards may be needed to determine any unknown compounds present in the sample. In addition, the duration for speciation analysis is approximately 12 hours. Therefore, there is interconversion between iAs^{III} and iAs^{V}, making determination of the total inorganic arsenic more appropriate. Overall, the analytical features show that this method is robust, sensitive, and suitable for high volume analysis of arsenic in urine samples.

2.5 REFERENCES

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3. CHARACTERIZATION OF URINARY ARSENIC METABOLITES IN HUMAN URINE OF ARSENIC-AFFECTED POPULATION IN BANGLADESH

3.1 INTRODUCTION

Arsenic is a naturally occurring toxic element that is toxic to humans, and can shorten life expectancy in humans by more than 23% (1). Intake of arsenic has shown to result in platelet aggregation and inflammation in humans (2–4), and inactivation of up to 200 enzymes – such as those involved in cellular energy pathways, and DNA repair and synthesis (5). Exposure to arsenic over a long period time is associated with skin lesions, cardiovascular disease, neurological diseases, and cancer (2,6–10).

However, there is significant variability in susceptibility to these illnesses associated with arsenic exposure, and this may partly be due to differences in human metabolism of arsenic. Different people that are exposed to the same concentration of arsenic may show different clinical symptoms (11–13). There are many factors that may affect an individual's susceptibility to arsenic toxicity. Some of these factors include level of exposure to arsenic, gender, age, smoking and alcohol consumption, and genetics (12).

In order to limit harmful effects associated with exposure to arsenic, the World Health Organization (WHO) has set guidelines of 10 µg As/L in drinking water. However, it is not clear if this guideline is suitable for protecting the most susceptible populations. In addition, it is not clear what the effects of low exposure to arsenic is on human health. Addressing these low

concentration effects, and determining populations most susceptible to arsenic toxicity, allowed for development of more suitable guidelines for health protection of different populations.

Urinary excretion is the main arsenic excretory pathway in humans (14), and provides a simple method for determining an individual's susceptibility to arsenic toxicity (13,15,16). Generally, humans metabolize ingested inorganic arsenic into less toxic methylated forms through biomethylation (5). Approximately 58% of ingested arsenic is then excreted within 3-5 days (14). The proportions of arsenic compounds excreted are usually consistent between most populations: 10-30% of inorganic arsenic, 10-20% of MMA, and 60-70% of DMA (15). However, studies have indicated that lower proportions of MMA (and higher proportions of DMA) in their urinary excretion are correlated with faster elimination of arsenic. Therefore, individuals that have lower proportions of MMA in their urine may be less susceptible to arsenic toxicity (17).

Determining the profiles of arsenic metabolites in the urine of individuals may allow for estimations of their susceptibility to arsenic toxicity. A higher proportion of inorganic arsenic species in an individual's urine may indicate an increased susceptibility to arsenic toxicity, possibly because of a decreased ability to metabolize inorganic arsenic. Similarly, a higher proportion of DMA in an individual's urine may suggest decreased susceptibility to arsenic toxicity.

The aim of this chapter is to use the method that was refined and optimized in Chapter 1 to quantify the arsenic species present in urine samples from an arsenic-affected Bangladesh study

population. In future studies this data will then be incorporated into statistical models, with the addition of the genetic information, age, sex, and health outcomes of our study participants (collected by our collaborators), to determine the efficacy of using the urinary arsenic profile to estimate susceptibility to arsenic toxicity.

3.2 MATERIALS AND METHODS

3.2.1 Sample population

The Health Effects of Arsenic Longitudinal Study (HEALS) population is located in Araihazar, Bangladesh (18). This specific population has been exposed to diverse concentrations of arsenic, and was selected to evaluate the concentration-response relationships between arsenic exposure and health outcomes. The study population is located 25 km southeast of the capital city Dhaka, and was chosen based on their homogenous sociocultural characteristics, diverse arsenic exposure levels, consistent levels of arsenic exposure due to little-to-no mitigation activities, and population level of approximately 70,000 residents of which 12,000 were recruited for the study in 2000-01. Various statistics, including the name, age, sex, and primary drinking water well of the participants were recorded. In addition, every well used by the residents as sources for drinking water (n = 6000) were tested for arsenic levels.

3.2.2 Sample pre-treatment

The urine samples (total volume = ~1.5 mL) were shipped on dry ice, stored in 2 mL plastic vials, and kept in a -80°C freezer. A small subset of the samples (n = 40) was thawed at room temperature every morning for that day's analysis. Each sample was vortexed for 20 seconds at high speed. An approximate volume of 500 µL was syringe-filtered using a 0.45 µm membrane and aliquoted into pre-labelled 1.5 mL Eppendorf tubes. These aliquots were then used for total and speciation analysis.

3.2.3 Total arsenic analysis

The accuracy of the arsenic speciation analysis was verified by comparing the sum of the arsenic species (via arsenic speciation analysis) with the total arsenic concentration (via total arsenic analysis). A 1:9 dilution was performed using 300 µL of the total 500 µL sample aliquot. The samples were acidified with 1% HNO_3 before analysis in triplicate for total arsenic. An external calibration ranging from 0.1 µg/L to 50 µg/L was prepared using the environmental calibration standard (Agilent technologies, U.S.). The calibration curve was validated using SRM1640a (trace elements in natural water, National Institute of Standards and Technology (Gaithersburg, MD)). The analysis was performed at a m/z of 75 in helium mode to detect arsenic, and a 10 µg/L As standard was run after every 10 samples to check for any instrumental drift.

3.2.4 Arsenic speciation analysis

The results of the total arsenic analysis were used to determine if any samples had very high arsenic concentrations and needed to be diluted prior to arsenic speciation analysis; the samples with total arsenic concentrations over 50 µg/L were diluted to a final concentration less than 50 µg/L. The samples were then transferred to HPLC glass vials for analysis (in duplicate) using the aforementioned method for arsenic speciation (see Chapter 2). Two standard reference materials and one certified reference material were used for verifying the accuracy of the method every day (**Table 3.1**): SRM2669 Level I (arsenic species in frozen human urine, National Institute of Standards and Technology (Gaithersburg, MD)), SRM1640a (trace elements in natural water, National Institute of Standards and Technology (Gaithersburg, MD)), and CRM18 (human urine, National Institute for Environmental Studies (Tsukuba-City, Japan)). A 10 µg/L mixed arsenic standard was run after every 10 samples as a quality control to ensure there was no significant instrumental drift (characterized by changes in signal intensity greater than 10%).

Table 3.1 The verified concentrations for arsenic in standard and certified reference materials used in this study

Reference Material	Arsenic Species	Certified value (µg/L)
SRM2669	AsB	12.4 ± 1.9
	MMA	1.87 ± 0.39
	DMA	3.47 ± 0.41
SRM1640a	$iAs^{III} + iAs^{V}$	8.075 ± 0.070
CRM18	AsB	0.069 ± 0.012
	DMA	0.036 ± 0.009

3.2.5 Statistical Analysis

All urine samples were analyzed in duplicate for arsenic speciation analysis, and in triplicate for total arsenic analysis. The mean values of the arsenic concentrations were reported. In addition, standard deviation, relative standard deviation, Kruskal-Wallis, and ANOVA tests were used for analysis. Statistical significance was identified through p-values less than 0.05.

3.3 RESULTS AND DISCUSSION

3.3.1 Comparison of total arsenic analysis and arsenic speciation analysis

The purpose of total arsenic analysis was two-fold: to determine if any dilutions were necessary prior to arsenic speciation analysis, and to confirm validity of the arsenic speciation analysis. **Figure 3.1** shows the comparisons of the total arsenic analysis and arsenic speciation analysis for all the samples analyzed (n = 879). Generally, any difference less than 20% in the total arsenic values was considered acceptable. If any of the samples had a larger difference than 20% between total and speciation analysis, the sample was re-analyzed at a later date. As can be seen in **Figure 3.1**, there was good agreement between total arsenic analysis and arsenic speciation analysis.

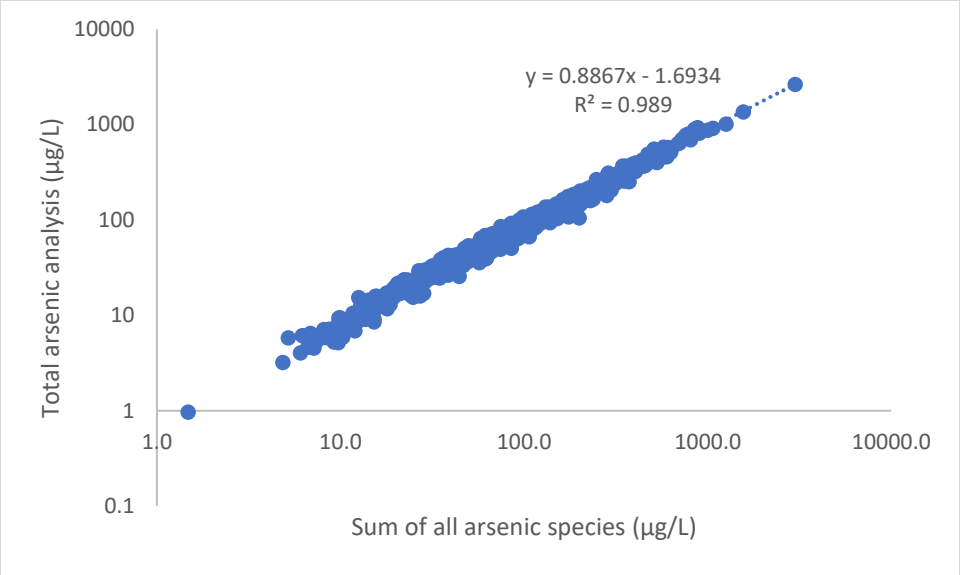

Figure 3.1 Comparison of total arsenic analysis and arsenic speciation analysis (sum of all arsenic species) (logarithmic scale).

3.3.2 Validation of our analysis using collaborator data

Our collaborators have also performed total arsenic analysis on the urine samples. In order to further confirm the validity of our speciation analysis, we compared the sum of inorganic and methylated arsenic species from our data with the total arsenic analysis analyzed by our collaborators in University of Chicago (for the first 100 samples analyzed). There was good agreement between most of our analyses (**Figure 3.2**). Some reasons for the discrepancy between the two analyses (3 outliers) may be due to inaccurate dilutions, contamination, or mis-labelling of the samples.

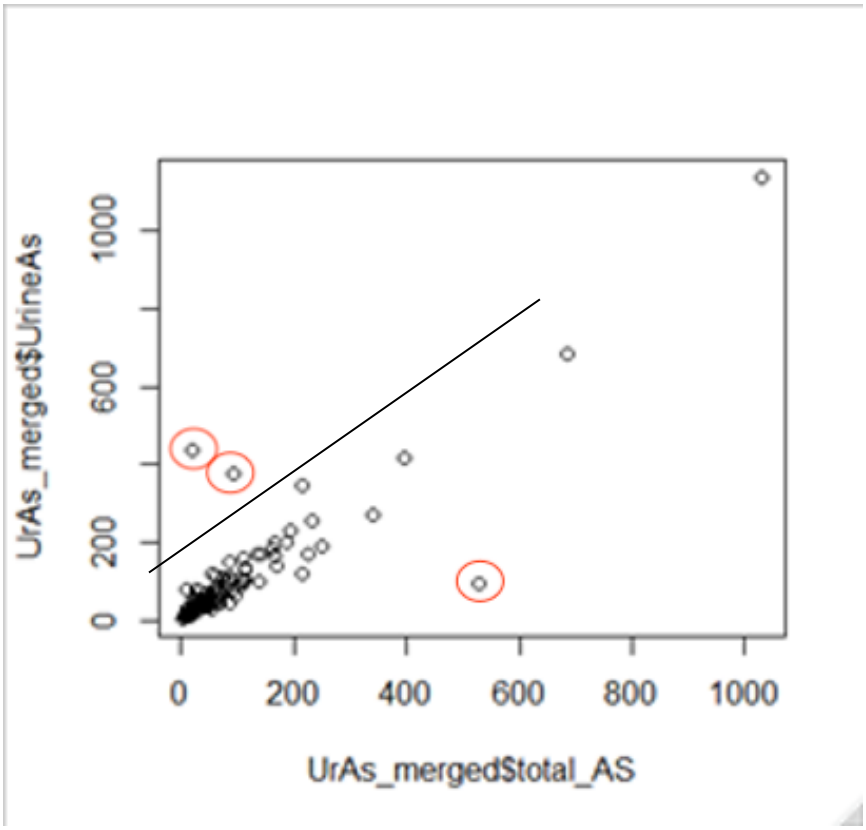

Figure 3.2 Comparison of our speciation analysis and our collaborator's total arsenic analysis

3.3.3 Proportions of arsenic in urine of participants

Previous studies have shown that higher proportions of DMA may correspond to faster arsenic excretion, and less susceptibility to arsenic toxicity in humans (14,15,17). Here, histograms are used to show the general distribution of each of the five arsenic species and summarize the general arsenic urinary profile of our study population. The majority of the arsenic excreted in urine was DMA, which is consistent with other studies. Interestingly, the distribution for iAs^{V} is comparatively broader than AsB, iAs^{III}, and MMA. One reason for this may be due to interconversion of iAs^{III} to iAs^{V} during the sample storage and analysis processes. For better accuracy, I have reported the values of iAs^{III} and iAs^{V} as total inorganic arsenic.

DMA was found to make up the majority of the arsenic found in the urine of the study population, with and without exclusion of AsB (**Figure 3.3 & 3.4**).

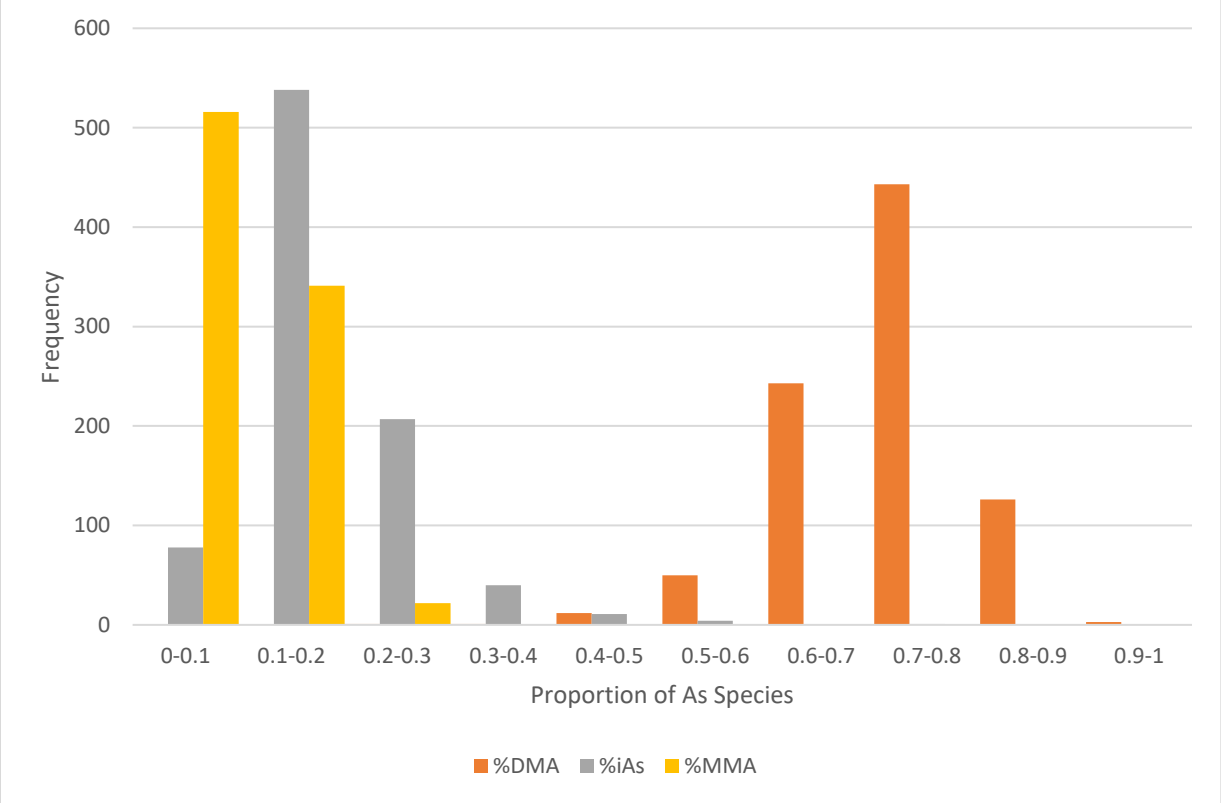

Figure 3.3 The frequency of the proportions of each arsenic species found in the urine of our study population (excluding AsB)

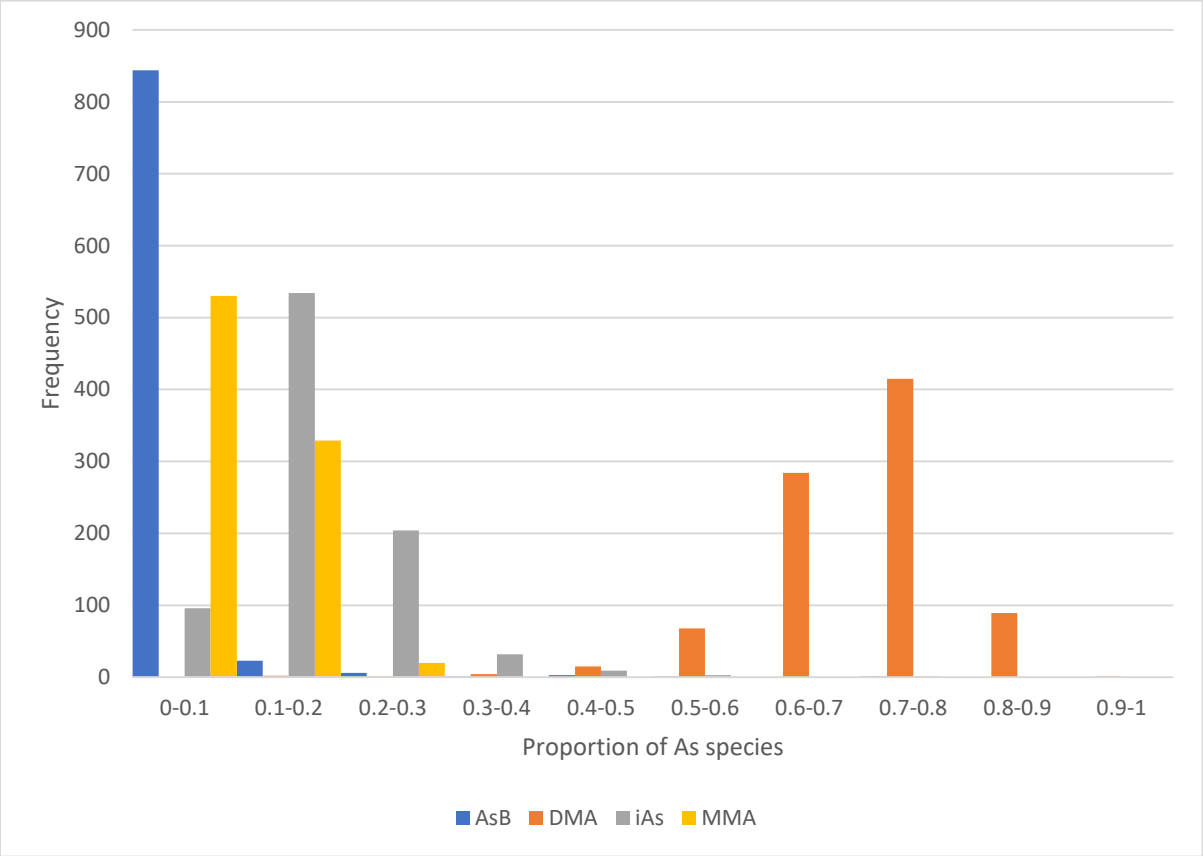

Figure 3.4 The frequency of the proportions of each arsenic species found in the urine of our study population (including AsB)

Overall, 71% of the arsenic found in the urine of the participants was in DMA form, 9% was in MMA form, 13% was in iAs^{V} form, 2% in AsB form, and 5% in iAs^{III} form (**Figure 3.5**).

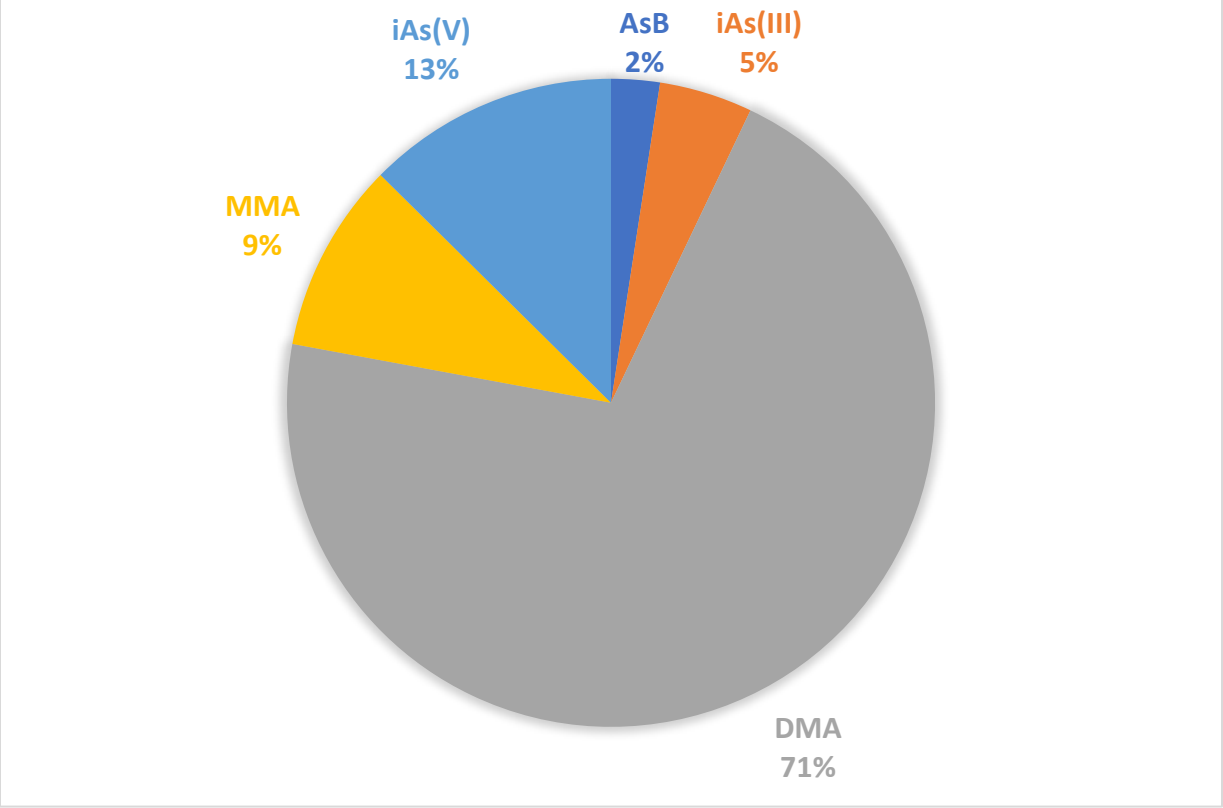

Figure 3.5 Overview of the mean percentage of arsenic species in the urine of our study population

The AsB in the majority of the urine samples made up 0-10% of the total arsenic (**Figure 3.6**).

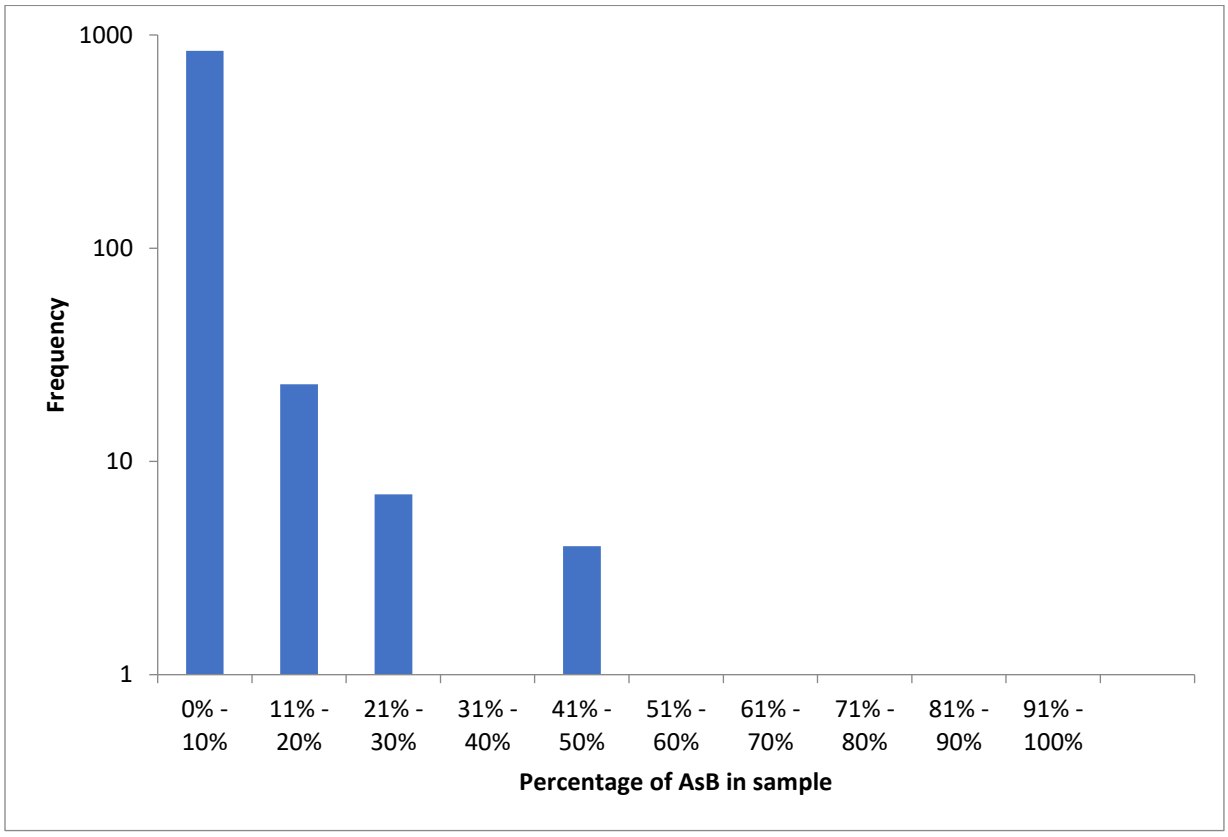

Figure 3.6 Distribution of AsB in the urine samples in relation to the sum of total arsenic

The iAs^{III} in the majority of the urine samples made up 0-20% of the total arsenic (**Figure 3.7**).

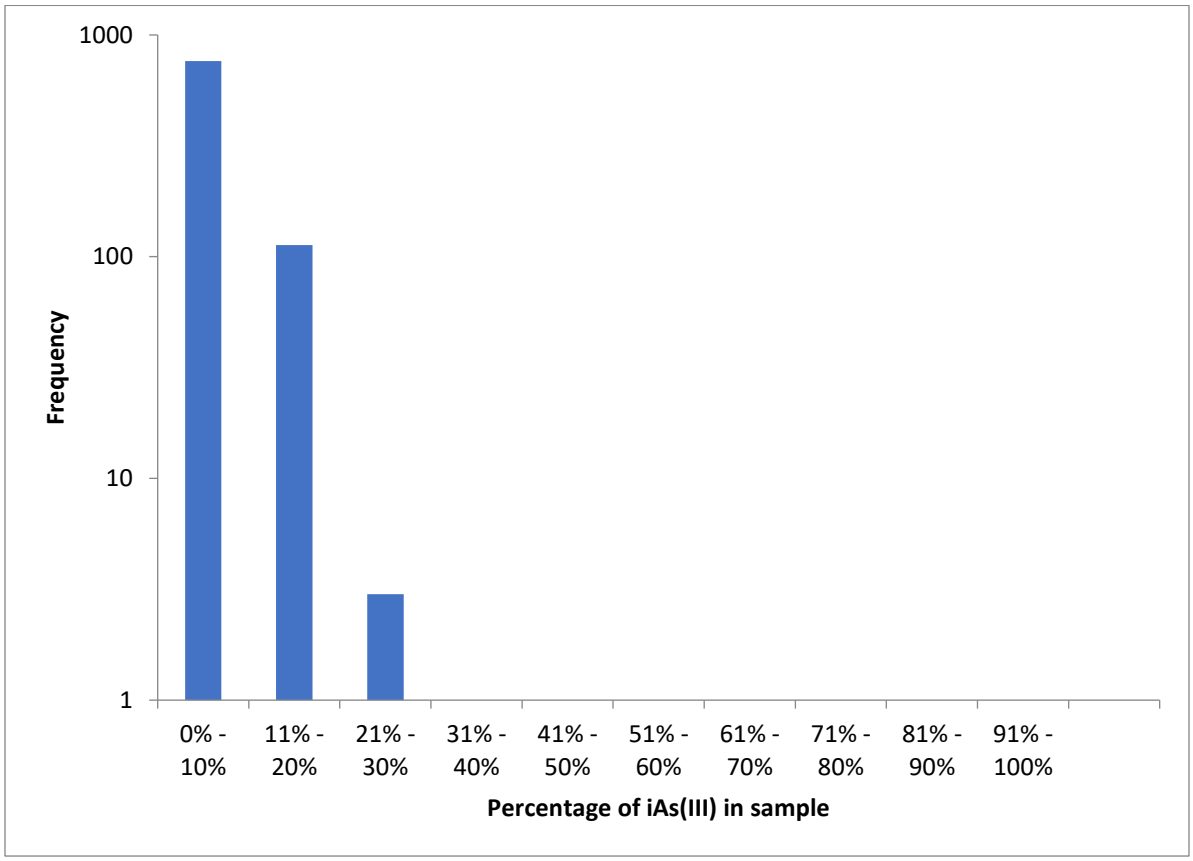

Figure 3.7 Distribution of iAs^{III} in the urine samples in relation to the sum of total arsenic

The DMA in the majority of the urine samples made up 60-80% of the total arsenic (**Figure 3.8**).

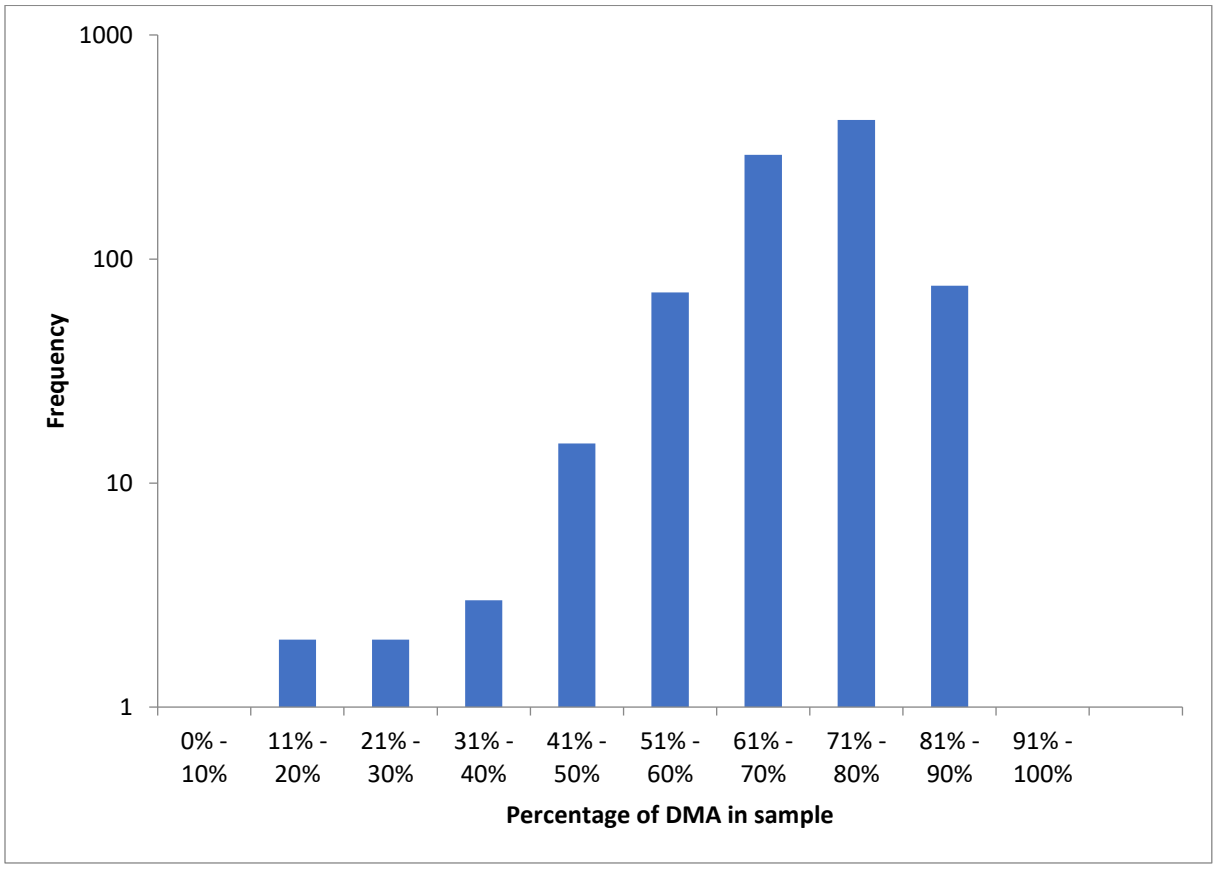

Figure 3.8 Distribution of DMA in the urine samples in relation to the sum of total arsenic

The MMA in the majority of the urine samples made up 0-20% of the total arsenic (**Figure 3.9**).

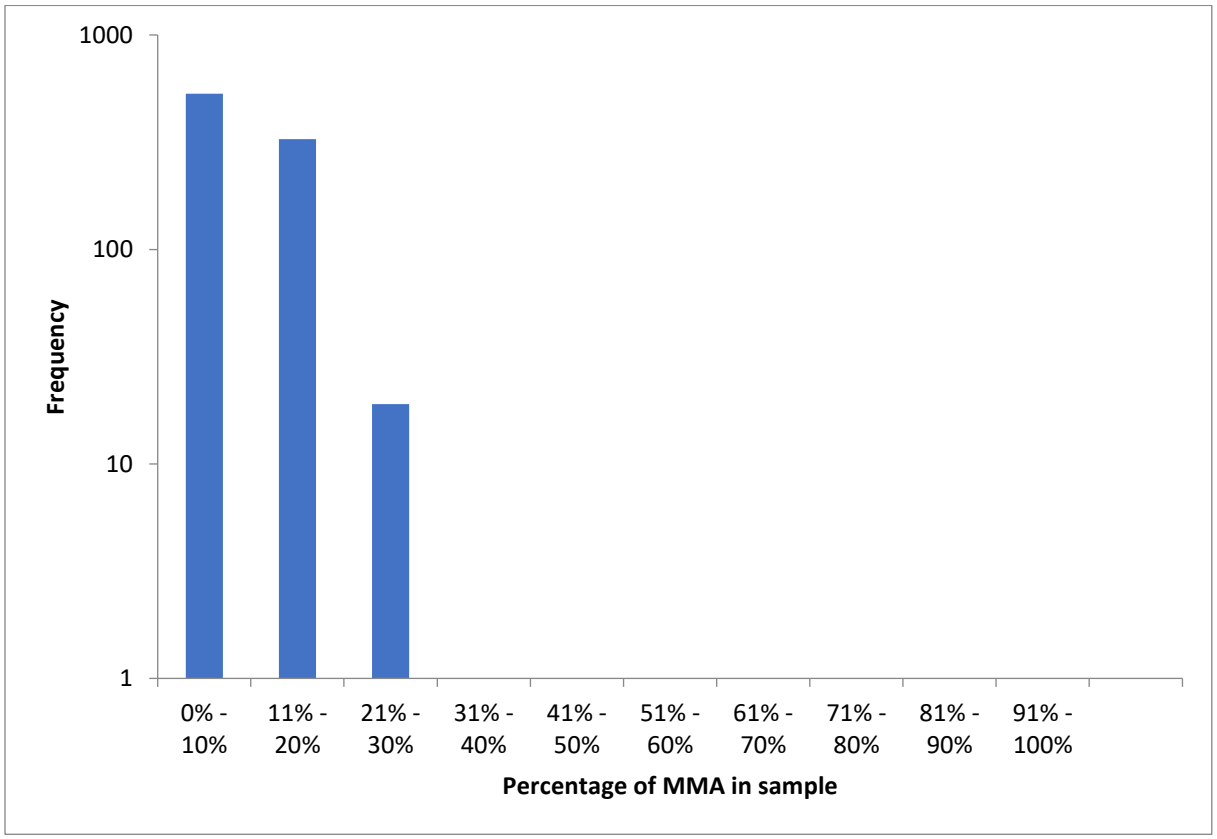

Figure 3.9 Distribution of MMA in the urine samples in relation to the sum of total arsenic

The iAs^V in the majority of the urine samples made up 0-30% of the total arsenic (**Figure 3.10**).

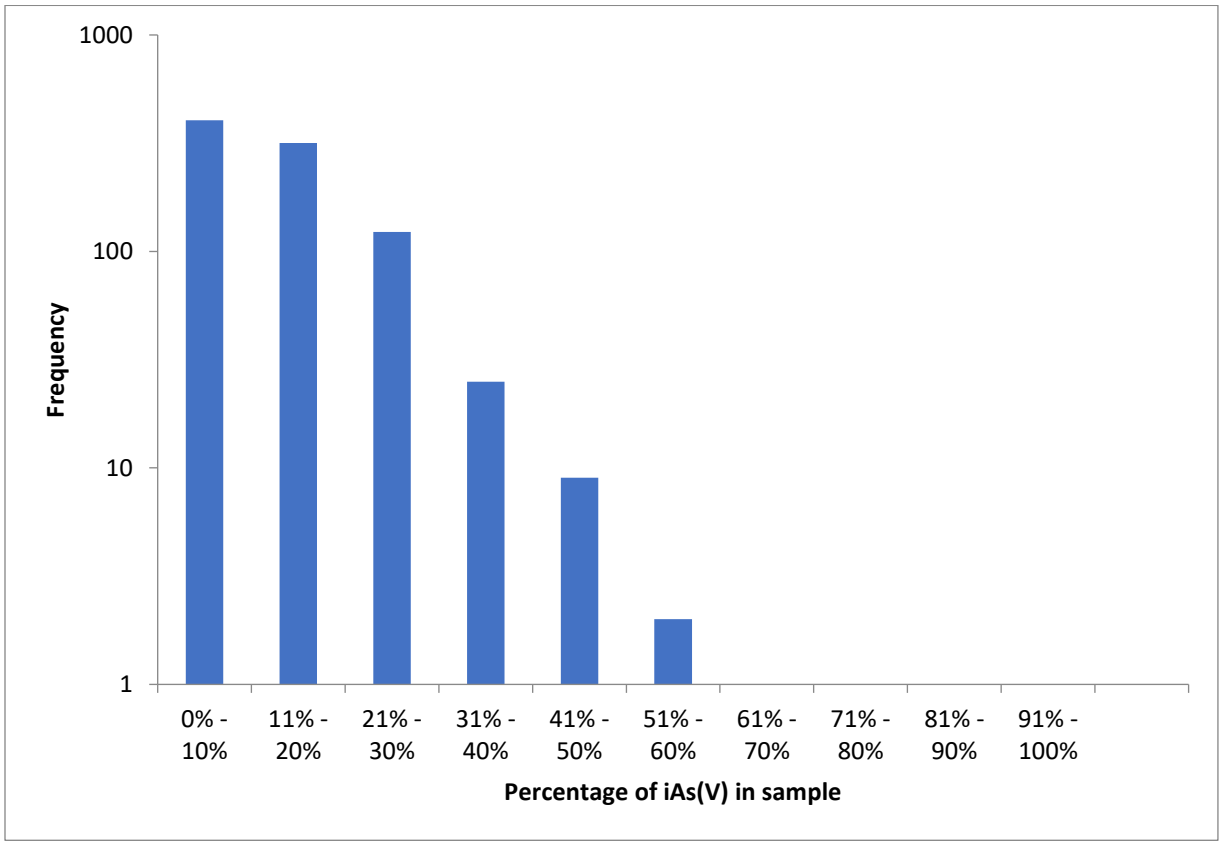

Figure 3.10 Distribution of iAs^V in the urine samples in relation to the sum of total arsenic

The major arsenic excreted in the urine of the participants was in DMA form, and the minority was in the form of AsB, iAs^{III}, MMA, and iAs^{V} (**Figure 3.11**).

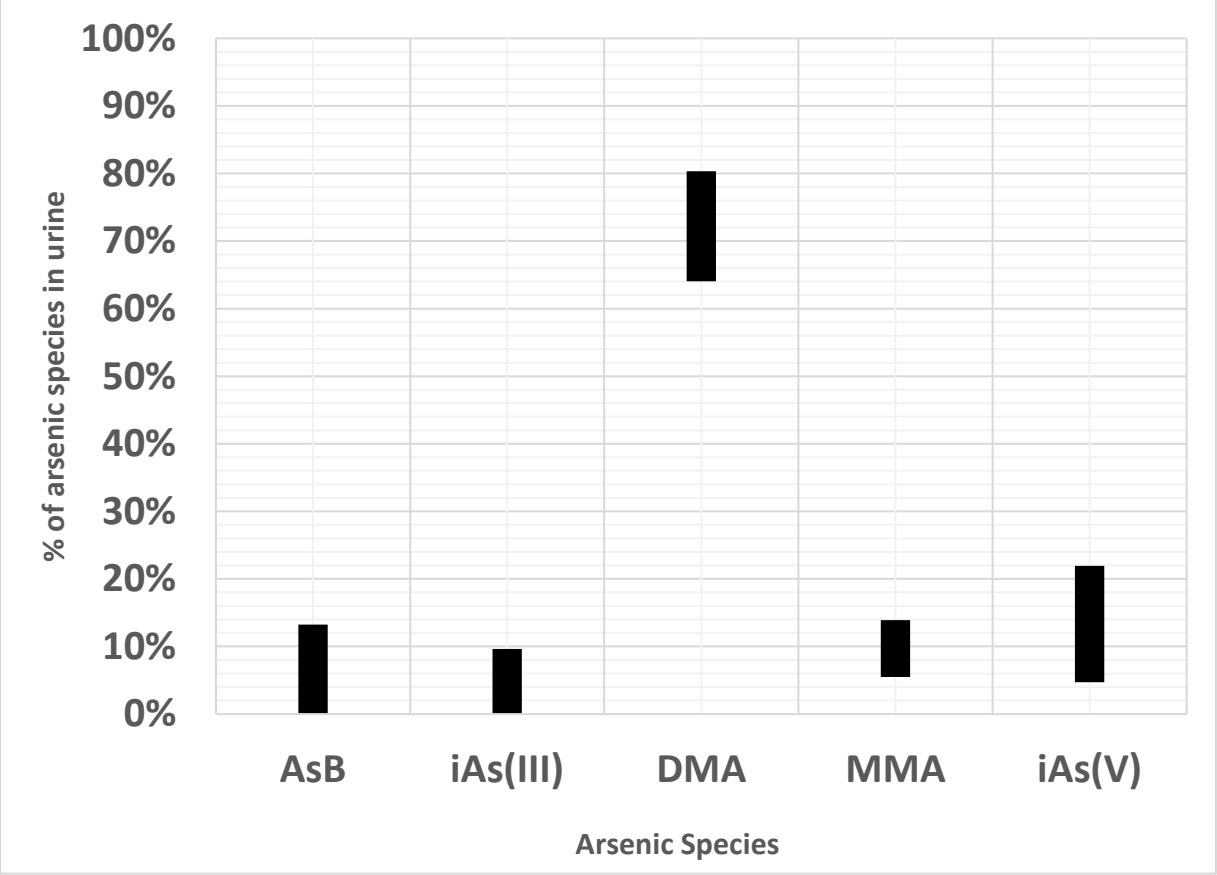

Figure 3.11 Summary of the distribution of the five arsenic species in our study population (data from Table 3.2)

The mean value of the percentage of arsenic species calculated using the sum of arsenic, was calculated to be 2% for AsB, 5% for iAs^{III}, 70% for DMA, 9% for MMA, and 13% for iAs^{V} (**Table 3.2**).

Table 3.2 The mean value percentages and standard deviations of the proportions of arsenic species excreted in the urine of our study population

Arsenic Species	Mean value of percentage of arsenic species in relation to sum of arsenic (%) (including AsB)	Standard deviation (%)
AsB	**2**	**4.5**
As^{III}	**5**	**4.8**
DMA	**70**	**8.7**
MMA	**9**	**4.2**
As^{V}	**13**	**8.4**

The percentage of DMA (majority As species excreted) was 70%, with inclusion of AsB (**Figure 3.12**).

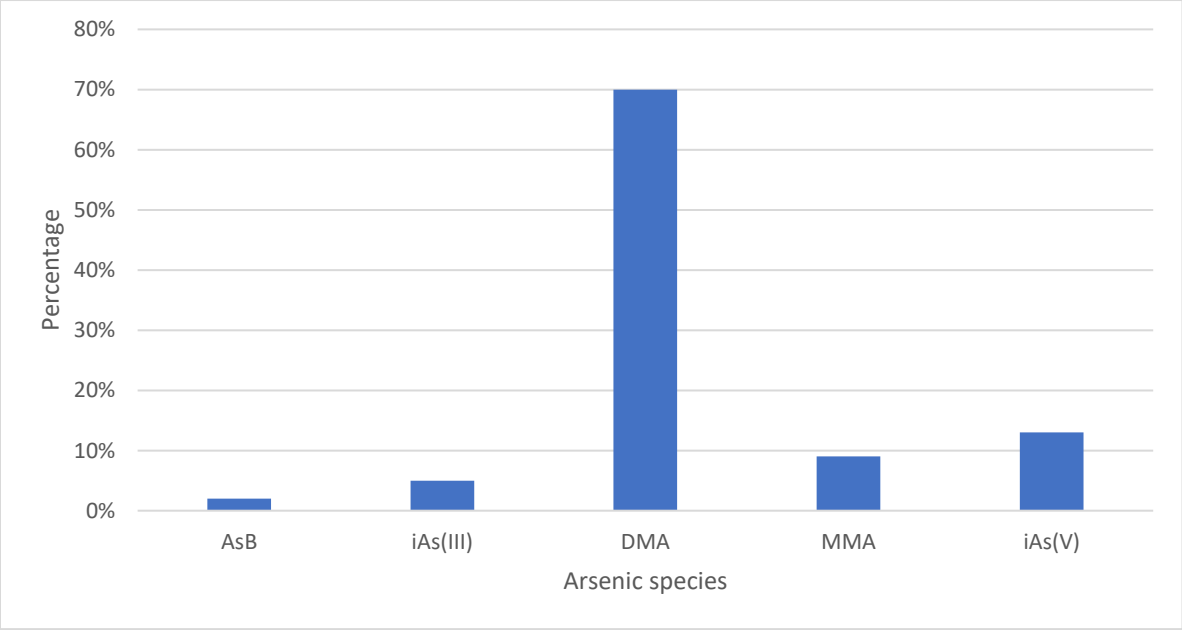

Figure 3.12 Percentage of arsenic species present in the urine of our study population

The percentage of DMA (majority As species excreted) was 72%, with exclusion of AsB (**Figure 3.13**).

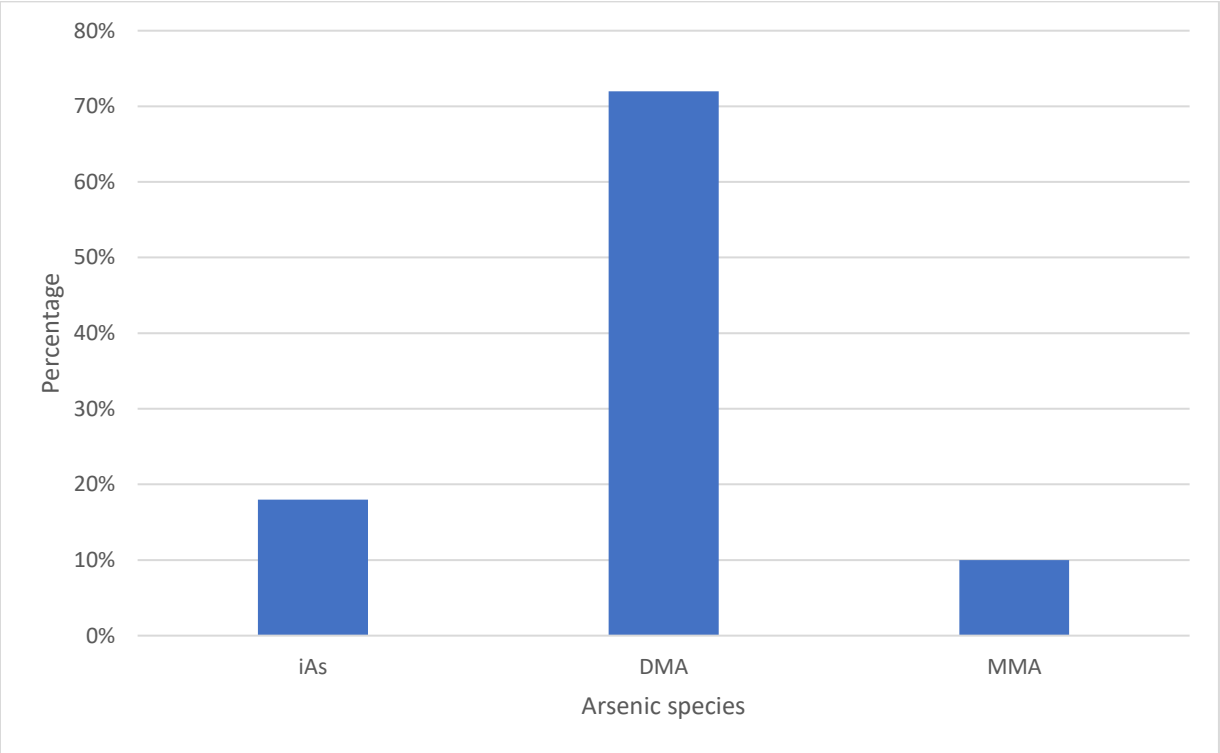

Figure 3.13 Percentage of arsenic species present in the urine of our study population (excluding AsB)

3.3.4 Determining potential confoundments in our data

Metabolism of inorganic arsenic results in methylated arsenic in humans. However, methylated arsenic can also originate from other sources, such as consumption of seafood (19). This potential confoundment may lead to overestimations of the amount of inorganic arsenic an individual converts to the methylated form. One way to overcome this limitation is by discarding

urine samples that show presence of high amounts of arsenic species only found in fish – such as AsB. Individuals with high amounts of AsB in their urine may have higher ingestions of other arsenic species, such as arsenosugars, commonly found in seafood. These compounds can also be metabolized by humans into methylated arsenic. Figure 3.14 shows the proportions of AsB in relation to the total arsenic for each of the arsenic compounds. Only five urine samples were found to have had higher-than-normal levels of AsB (over 30%), indicating that the majority of individuals in this study population are not avid consumers of seafood.

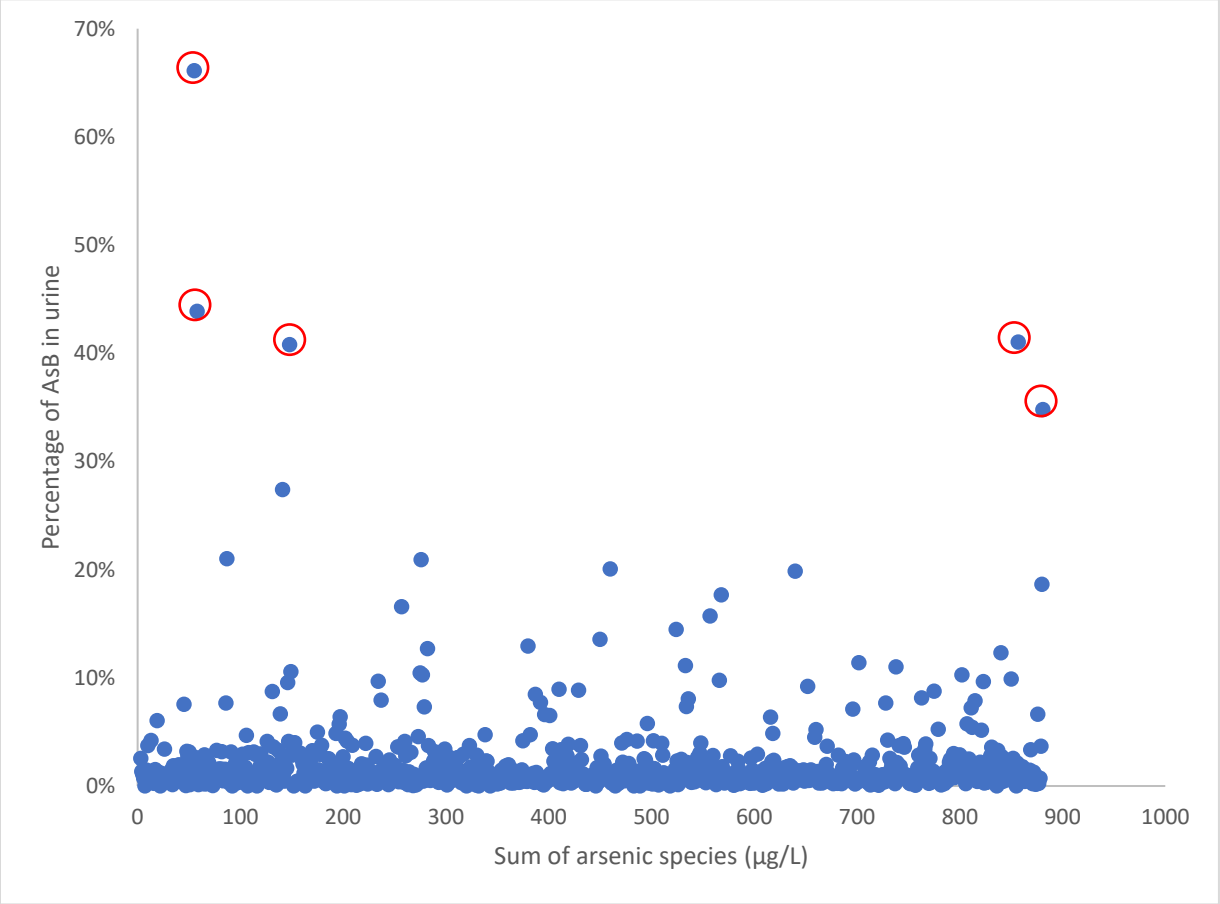

Figure 3.14 Percentage of AsB in relation to the total amount of arsenic present in the urine samples

3.3.5 Arsenic urinary profile

Statistical values of the urinary profile of our study population, such as the DMA/MMA ratio, methylated/inorganic arsenic ratio, and the percentages of inorganic arsenic and methylated arsenic were determined (Table 3.3). These values give an overview of the arsenic metabolism characteristics of our study population. These statistical values may also allow for identification of biomarkers to estimate susceptibility to arsenic toxicity. Identification of such biomarkers will allow for identification and protection of the most susceptible populations to arsenic toxicity.

Table 3.3 Urinary profile of our study population (n = 879)

	Minimum	Maximum	Median	Mean
Primary methylation index (MMA/iAs)	0.0	2.9	0.5	0.6
Secondary methylation index (DMA/MMA)	0.0	81.7	8.1	9.3
Total As (µg/L)	1.1	2387.2	66.7	114.2
Methylated As / iAs	0.3	22.5	4.9	5.4
MMA (%)	0.0	28.1	9.1	9.7
DMA (%)	20.4	91.5	73.0	72.2
iAs (%)	4.3	77.0	17.0	18.1
Methylated As (%)	23.0	95.7	83.0	81.9
[AsB] (µg/L)	0.0	57.3	0.7	1.8
[iAs] (µg/L)	0.4	611.2	10.1	18.8
[DMA] (µg/L)	0.6	1598.7	46.9	81.9
[MMA] (µg/L)	0.0	226.3	6.0	11.7
Sum of As species (iAs, DMA, and MMA) (µg/L)	1.1	2384.2	64.5	112.4
Total arsenic (from ICPMS analysis) (µg/L)	1.0	2633.6	68.1	116.6

As expected, statistical values for our study population showed that the majority of the arsenic excreted was in the methylated form – the majority of which was DMA. In addition, similar to other populations, the secondary methylation index (conversion of MMA to DMA) was much greater than the primary methylation index (conversion of iAs to MMA), hinting that the conversion from MMA to DMA may be more efficient than the conversion from iAs to MMA.

Despite the diverse levels of arsenic humans are exposed to around the world, and various genetic and lifestyle differences, the average distribution of urinary arsenic metabolites stays fairly constant among various populations (20). The average percentages of MMA, DMA, and iAs of our study population were compared with the values consistently found for average populations around the world (17) (**Table 3.4**). Interestingly, the proportions of our study population's urinary DMA levels are towards the higher end compared to the average urinary DMA levels. In addition, the proportions of our study population's urinary MMA levels are lower than those found in the average population. As studies have indicated that lower proportions of MMA are correlated with faster elimination of arsenic, and because DMA is the least retained arsenic species in humans, this may indicate that our study population is more efficient at metabolizing arsenic than the average population.

Table 3.4 The proportions of arsenic metabolites in our study population's urine, compared to those consistently found in average populations

	Study population (average)	Average global range (17)
MMA (%)	9.5	10 - 20
DMA (%)	70.2	60 - 70
iAs (%)	17.6	10 - 30

Certain populations have displayed significant differences in their urinary arsenic profile compared to the average population. For example, the native Andean population – which has been exposed to large amounts of arsenic over many generations – have very low urinary excretions of MMA, while also displaying significantly less incidences of arsenic-induced illness (13). This indicates that the native Andean population have genetic polymorphisms of arsenic methylating enzymes, which makes them less susceptible to arsenic toxicity. On the other hand, a population located in the northeast of Taiwan show very high amounts of MMA excretion in their urine, suggesting that they may be more susceptible to arsenic toxicity (16).

Figure 3.15 compares the MMA excretion of our study population with other populations that have been exposed to arsenic via drinking water. There are significant variations for urinary MMA levels among populations. There can also be large variations in proportions of urinary arsenic metabolites found within a population, as seen in the San Pedro population. Our population's MMA excretion levels overlap with the native Andean population (S. Antonio) MMA excretion levels.

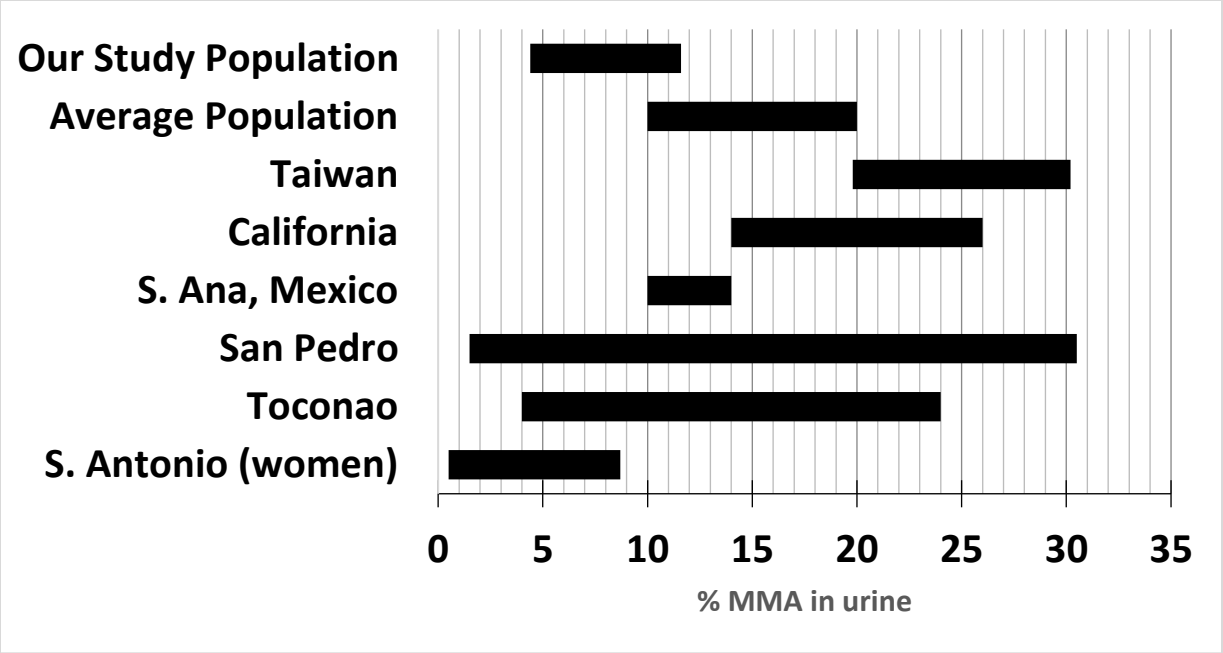

Figure 3.15 Comparisons of the excretion of MMA via urine in our study population, and other populations that have been exposed to arsenic. Modified from Vahter, M. (2000) (20)

3.3.6 Associations between various biomarkers among our study populations

A Pearson correlation test was used to determine if there are any associations between each individual arsenic species and the total arsenic content in the urine samples (**Table 3.5, Figures 3.16-3.20**). As expected, there was negligible correlation between AsB and all other variables, as AsB is not metabolized by humans, and made up a very small amount of the total arsenic found in the urine of our study population. Conversely, a very strong positive correlation was found between DMA excretion and total arsenic excretion – as DMA is the major arsenic metabolite excreted via urine in humans.

Table 3.5 Intercorrelations between individual arsenic species and total arsenic excreted in urine

	AsB	iAs^{III}	DMA	MMA	iAs^{V}	$\sum iAs^{III} + iAs^{V}$	Total As
AsB	1						
iAs^{III}	0.12	1					
DMA	0.19	0.80	1				
MMA	0.11	0.73	0.87	1			
iAs^{V}	0.10	0.60	0.77	0.68	1		
$\sum iAs^{III} + iAs^{V}$	0.13	0.90	0.88	0.79	0.91	1	
Total As	0.20	0.84	0.99	0.90	0.81	0.92	1

Strong positive correlations were also found between individual arsenic species. Urinary excretion of inorganic arsenic had a strong positive correlation with DMA, and a moderate to strong positive correlation with MMA. There was also a strong positive correlation with MMA and DMA. This is expected as larger amounts of inorganic arsenic ingested will result in a higher number of methylated species being created.

However, very high concentrations of arsenic species may result in saturation of the arsenic methylation enzymes. As the relationship between the sum of inorganic arsenic and sum of methylated arsenic species shows some qualitative signs of saturation (Figure 3.20), it is possible that there was some saturation of arsenic methylation enzymes present for one individual in our study population. However, more data is needed to reach a conclusion.

There was only a moderate positive correlation between iAs^{III} and iAs^{V}. As the main source of inorganic arsenic comes from the same source (contaminated drinking water from groundwater sources), it is interesting that there was only a moderate positive correlation between the two inorganic arsenic species. One possible reason for this lower positive correlation may be due to inter-conversion between the two species, as oxidation of iAs^{III} to iAs^{V} occurs readily.

There was little to no correlation between AsB and total arsenic (**Figure 3.16**).

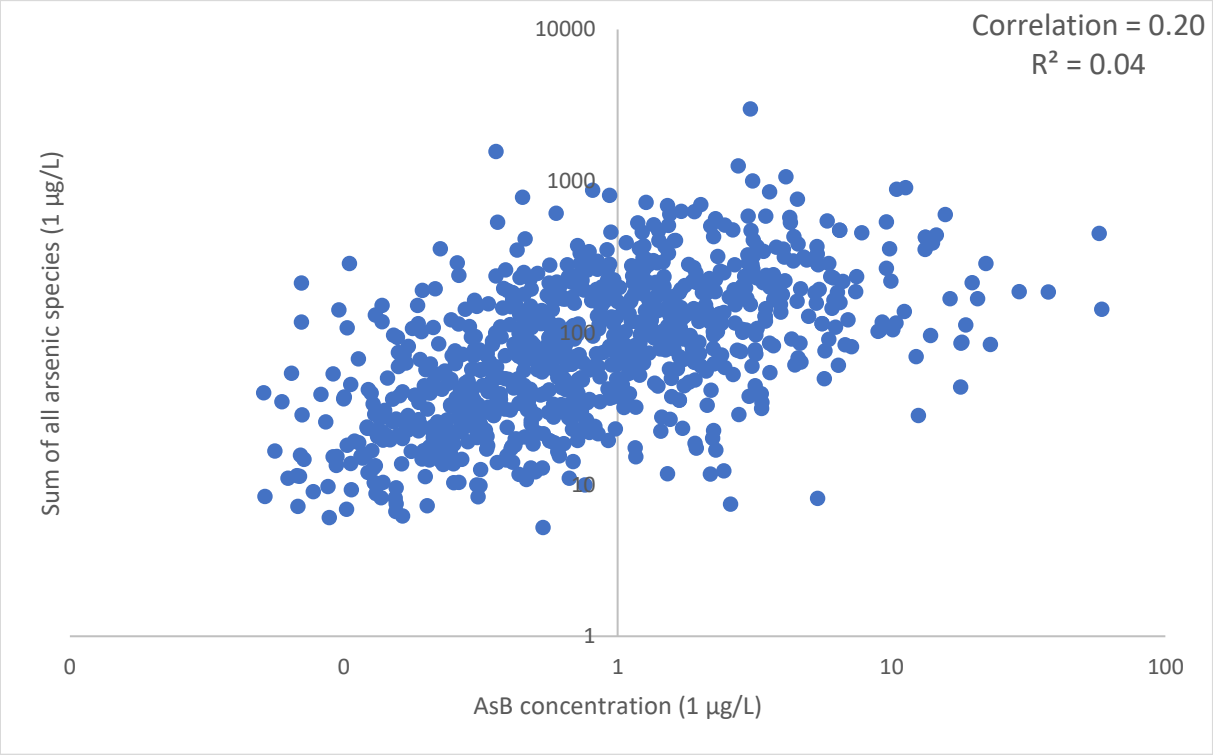

Figure 3.16 Comparisons in the excretions of AsB and total arsenic (n = 879) (logarithmic scale)

There was positive correlation between iAs and total arsenic (**Figure 3.17**).

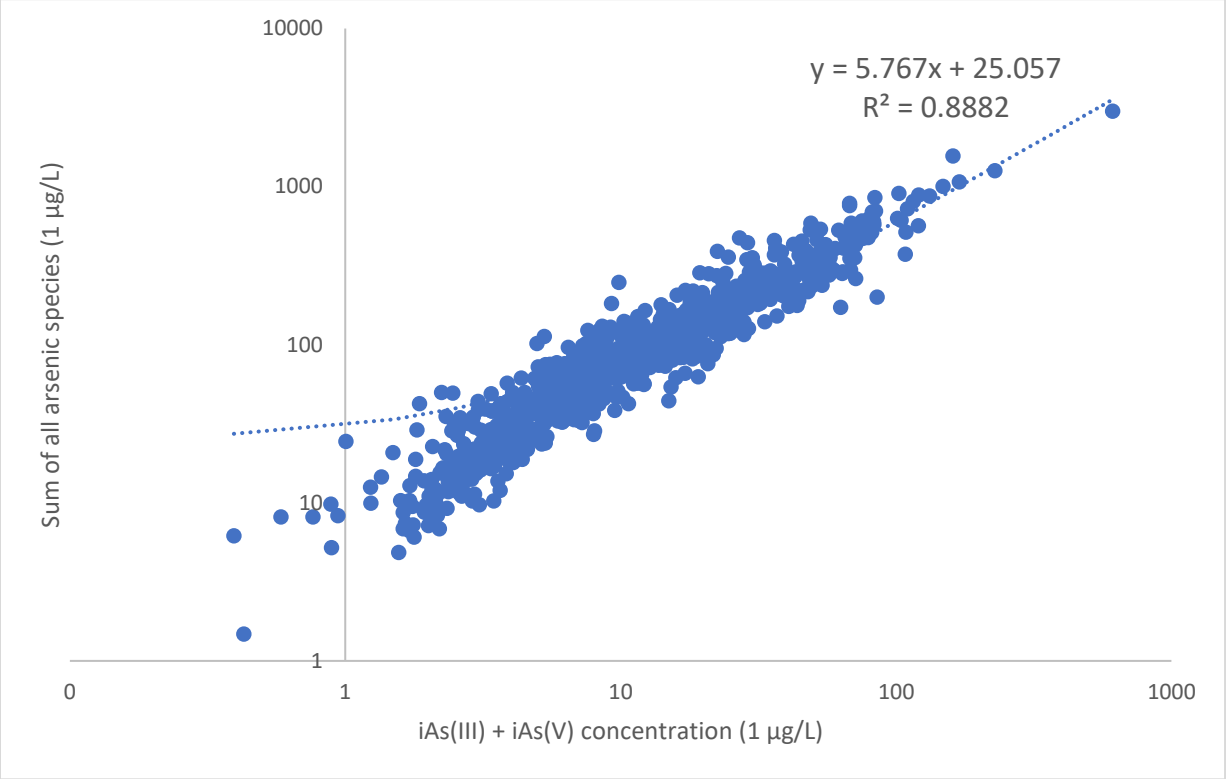

Figure 3.17 Comparisons in the excretions of inorganic arsenic and total arsenic (n = 879) (logarithmic scale)

There was positive correlation between DMA and total arsenic (**Figure 3.18**).

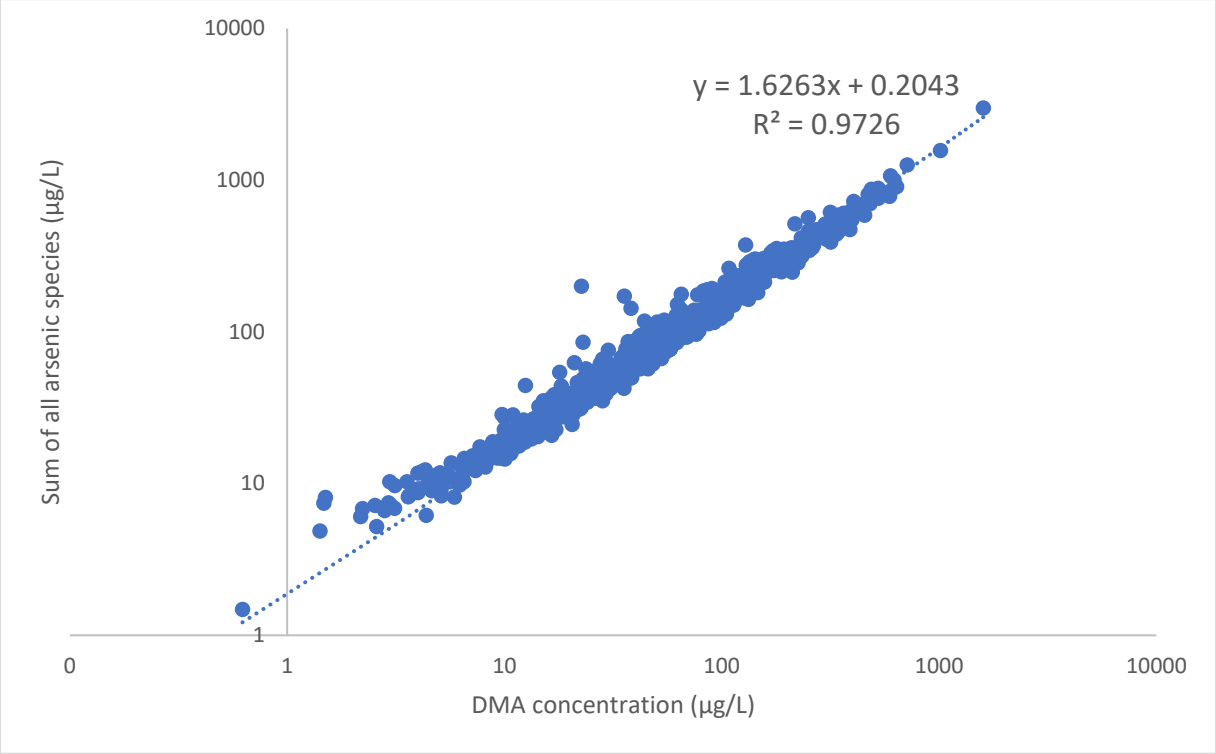

Figure 3.18 Comparisons in the excretions of DMA and total arsenic (n = 879) (logarithmic scale)

There was positive correlation between MMA and total arsenic (**Figure 3.19**).

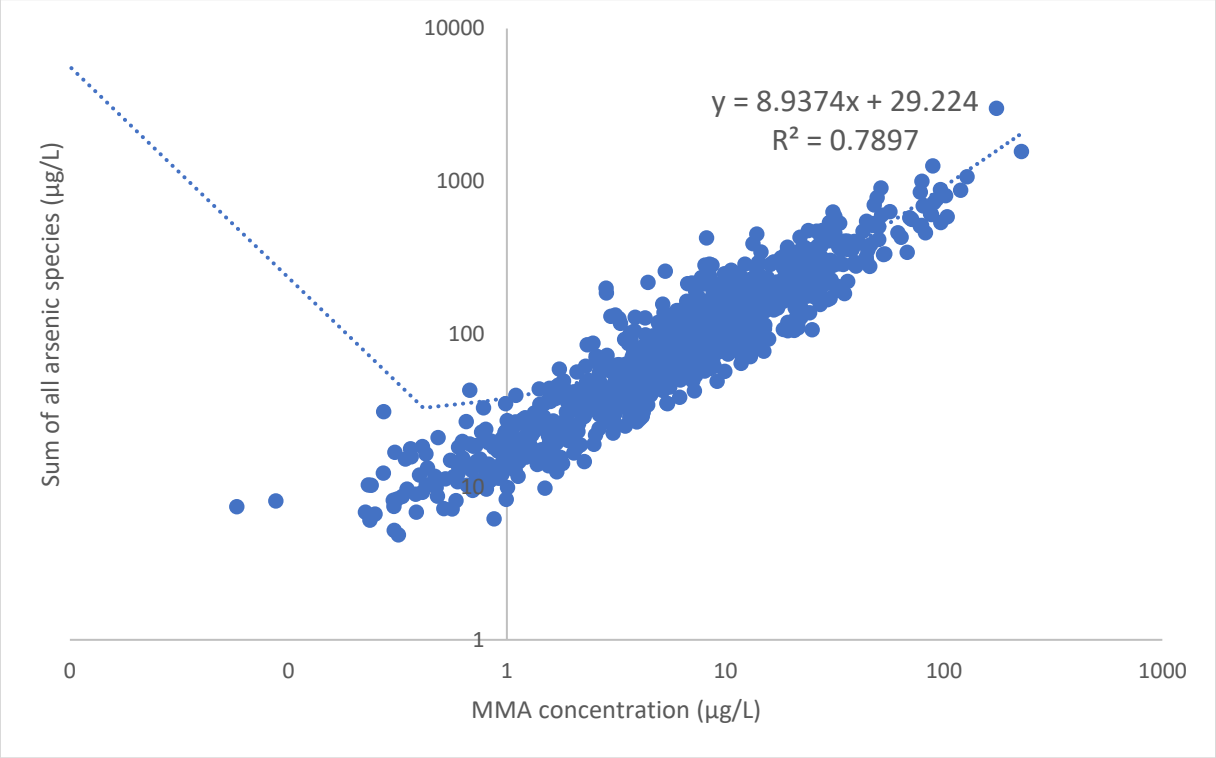

Figure 3.19 Comparisons in the excretions of MMA and total arsenic (n = 879) (logarithmic scale)

There was positive correlation between iAs and methylated arsenic (**Figure 3.20**).

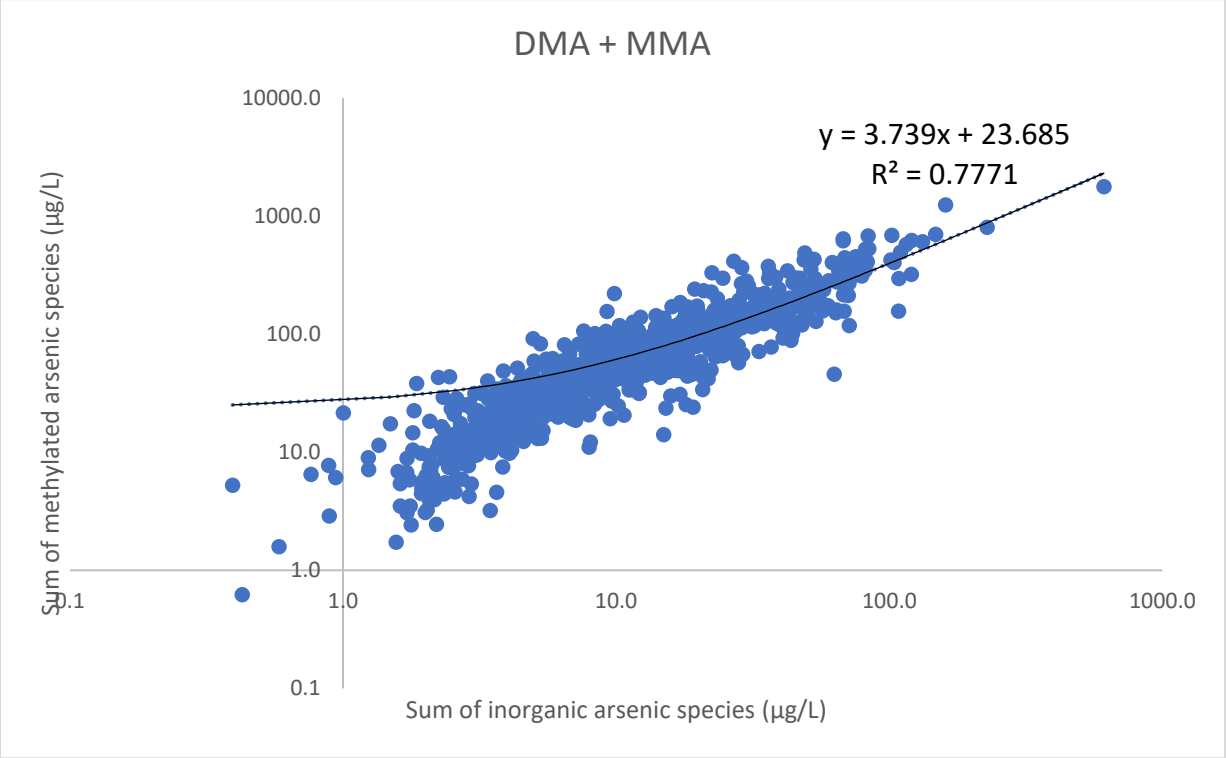

Figure 3.20 Comparisons in the excretions of inorganic arsenic species and methylated arsenic species (logarithmic scale)

3.3.7 Differences in arsenic metabolism among three exposure groups

Values for individual arsenic species and various methylation values were determined for different exposure groups within our study population. A Kruskal-Wallis test was used to compare the mean of these values among the different exposure groups. The difference was considered significant at $P<0.05$. Comparisons between different exposure groups within our study population allows us to investigate whether the concentration of arsenic exposure may affect methylation efficiency in humans.

Significant differences were observed among the three exposure groups for all the median values except inorganic arsenic and methylated arsenic (**Table 3.6**).

Table 3.6 Urine profile for individuals excreting <50 µg/L, 50.1-150 µg/L and >150 µg/L of arsenic

	Urine Arsenic Concentration			
	≤ 50.0 µg/L (n = 322) (Median)	**50.1 – 150.0 µg/L (n = 305) (Median)**	**≥ 150.0 µg/L (n = 252) (Median)**	**p-value**
Primary methylation index (MMA/iAs)	0.43	0.63	0.61	<0.001
Secondary methylation index (DMA/MMA)	8.67	7.64	7.63	0.001
Methylated As / iAs	4.30	5.41	5.15	<0.001
MMA (%)	8.2	9.6	9.9	<0.001*
DMA (%)	72.0	73.6	73.1	0.003*
iAs (%)	18.9	15.6	16.3	1
Methylated As (%)	81.1	84.4	83.7	1

*ANOVA test; p-values by Kruskal-Wallis test (calculations for percentages excluded AsB).

There was no significant difference in the median values of the urine profiles of the >150 µg/L versus the 50.1-150.0 µg/L exposure groups (**Table 3.7**).

Table 3.7 Urine profile for individuals excreting 50.1-150 µg/L and >150 µg/L of arsenic

	Urine Arsenic Concentration		
	50.1 – 150.0 µg/L (n = 305) (Median)	**≥ 150.0 µg/L (n = 252) (Median)**	**p-value**
Primary methylation index (MMA/iAs)	0.63	0.61	0.746
Secondary methylation index (DMA/MMA)	7.64	7.63	0.554
Methylated As / iAs	5.41	5.15	0.273
MMA (%)	9.6	9.9	0.518
DMA (%)	73.6	73.1	0.824
iAs (%)	15.6	16.3	1
Methylated As (%)	84.4	83.7	1

p-values calculated using a Kruskal-Wallis test; calculations for percentages excluded AsB).

Interestingly, the percentage of inorganic arsenic was greater, and the percentage of methylated arsenic was less, in the group exposed to less than 50 µg/L of arsenic compared to the groups exposed to arsenic greater than 50 µg/L (**Table 3.8**). However, the group exposed to less than 50 µg/L of arsenic had a greater secondary methylation index, suggesting that this group may convert MMA to DMA more efficiently.

Table 3.8 Urine profile for individuals excreting ≤50 µg/L and >50 µg/L arsenic

	Urine Arsenic Concentration		
	≤ 50.0 µg/L (n = 322) (Median)	**> 50.0 µg/L (n = 557) (Median)**	**p-value**
Primary methylation index (MMA/iAs)	0.43	0.62	<0.001
Secondary methylation index (DMA/MMA)	8.67	7.64	0.001
Methylated As / iAs	4.30	5.27	<0.001
MMA (%)	8.2	9.6	<0.001
DMA (%)	72.0	73.6	<0.001
iAs (%)	18.9	15.6	1
Methylated As (%)	81.1	84.4	1

p-values calculated using a Kruskal-Wallis test

After additional comparisons between the three exposure groups, it is clear that the group with excretions of arsenic less than 50 µg/L had significantly different median values for their urinary arsenic profiles compared to the group with excretions of arsenic greater than 50 µg/L. These significant differences between the two exposure groups remained after correcting for arsenic species that were below my detection limit (by substituting values below my LOD by LOD/2 in order to prevent biases that may over- or underestimate the actual concentrations), which may have been artificially changing the urinary profile values. This is important to note as it may be difficult to compare the urinary arsenic profiles of our study population with their health data if there are significant differences between the exposure groups. Additional measures will need to be taken to control for the exposure group.

3.4 CONCLUSION

This chapter characterized urinary arsenic metabolites in the urine of an arsenic-affected population in Bangladesh. The results of the arsenic speciation analysis showed good comparability with my total arsenic analysis, and the total arsenic analysis performed by our collaborators. Our results were consistent with other studies on arsenic speciation, showing DMA is the major arsenic metabolite found in urine. The arsenic urinary profile of our study population shows that this population has lower proportions of MMA and higher proportions of DMA compared to the average population. In addition, there is a significant difference in the arsenic urinary profile of different exposure groups; the group of participants who had lower than 50 μg/L of total arsenic in their urine showed higher proportions of DMA and DMA/MMA ratios as compared to those who had higher than 50 μg/L of total arsenic in their urine. The results of this study can be combined with additional data collected by our collaborators, including health outcomes of the participants, to determine the efficacy of using urinary arsenic profiles to estimate susceptibility to arsenic toxicity.

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4. CONCLUSIONS AND FUTURE DIRECTIONS

The method developed in Chapter 2 was able to achieve quantification of the five most relevant arsenic compounds, including inorganic arsenic compounds and their metabolites, in an analysis time of 10 minutes. Analytical features this method include a limit of detection below 0.1 µg/L (**Table 2.20**), relative standard deviations of the peak area (used for quantitation) below 6% (**Table 2.16**) and day-to-day relative standard deviations of the retention time (used for identification) below 1% (**Table 2.16**).

In addition, the summed concentrations of all arsenic species in the urine samples were in good agreement with the results of total arsenic analysis (**Figure 3.1**), and the results of the first 100 urine samples tested show good comparability with the total arsenic results from our collaborators (**Figure 3.2**). These analytical features show that this method is sensitive, robust, and suitable for analysis of a large number of urine samples.

After refinement, optimization, and validation of this method, I used this method to determine the concentrations of five arsenic species in 879 urine samples from our study population. The results obtained agree with other research, showing that DMA is the major arsenic metabolite excreted in urine in humans.

The arsenic urinary profile of our study population shows that compared to the average population, our population has lower proportions of MMA and higher proportions of DMA. There was also a significant difference in the arsenic urinary profile of the different exposure

groups. The group of participants who had concentrations of total arsenic less than 50 µg/L showed higher proportions of DMA and DMA/MMA ratios compared to the group of participants who had concentrations of total arsenic greater than 50 µg/L. These results can be combined with additional data, such as the health outcome of the participants, in order to determine the efficacy of using urinary arsenic profiles to estimate susceptibility to arsenic toxicity.

There are certain limitations present with this research thesis. As previously discussed in the introduction section, the analytical method used is suitable for identification and quantification of the five most common arsenic species in human urine: AsB, iAs^{III}, DMA, MMA, and iAs^{V}. However, additional instrumentation and/or standards will be needed to identify and quantify any unknown arsenic species that are present in the urine sample. Additionally, the duration for speciation analysis of one batch (40 urine samples) is approximately 12 hours. Therefore, there is interconversion between iAs^{III} and iAs^{V}, making it difficult to accurately measure concentrations of the two species independently. There are also some inherit limitations with the overall cohort study, including difficulties in determining relationships between arsenic exposure and rare diseases and in determining the harmful effects of exposure to smaller concentrations of arsenic (less than 10 µg/L).

There are still remaining research needs for this project. There are ~2,100 urine samples that remain to be analyzed for arsenic speciation. Once the urinary arsenic profile of the ~3,000 participants is complete, this data can be incorporated with incidence of arsenic-induced diseases collected by our collaborators, and statistical models can be used to analyze for any associations

between urinary arsenic profiles and health outcomes of the study participants. These arsenic-induced illnesses include skin lesions, premalignant and malignant tumors, pregnancy outcomes, and total mortality. The urinary arsenic metabolite marker will allow us to determine whether more efficient methylation of arsenic corresponds to reduced susceptibility to arsenic-induced illnesses.

This collaborative study will identify biomarkers that can be used for determining an individual's susceptibility to arsenic-induced diseases. This will allow for identification and health protection of populations most susceptible to arsenic-induced diseases. The results will also provide a better understanding of how arsenic metabolism relates to detoxification of arsenic. Sixty-percent of our study cohort is exposed to arsenic levels less than 100 μg/L from their drinking water sources; investigation of associations between genetics, arsenic speciation in urine, and health outcomes in this cohort will provide information on human health effects from exposure to lower amounts of arsenic. Finally, our focus on one study population may limit the scope of this study. Consequently, this study may be replicated in other populations as well as provide a more complete picture of the numerous factors that could influence susceptibility to arsenic toxicity.

There is also need for additional research for further elucidation of the harmful effects to arsenic exposure in humans. More testings are needed of private water supplies to determine, and protect, those that may be exposed to high concentrations of arsenic in drinking water. In addition, humans are exposed to a wide variety of arsenic species in addition to inorganic arsenic. For example, broiler chickens fed 3-nitro-4-hydroxyphenylarsonic acid (Roxarsone) were found to have as many as 11 arsenic species identified in their liver and breast meat in very

low concentrations (98-100). It is important to determine and classify the additional arsenic species humans may be exposed to and determine their health risks. Finally, additional epidemiological studies are needed for populations exposed to low concentrations of arsenic (less than 10 μg/L). This will help elucidate the health effects of human exposure to low exposures of arsenic, and determine whether the current guidelines are sufficient for health protection of humans.

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APPENDIX – SUPPORTING INFORMATION

5.1 STANDARD OPERATING PROCEDURE (SOP) FOR URINE ANALYSIS WORKFLOW

Afternoon prior to analysis:

a. Preparing centrifuge tubes for total analysis:
    a. Label 10 mL centrifuge tubes (n = 40) “1” to “40”
    b. Fill the labelled 10 mL centrifuge tubes with 3 mL of water (using 5 mL pipet)
    c. Remove 330 µL of the water from each centrifuge tube
b. Labelling other vials:
    a. Label 1.5 mL plastic vials (n = 40) “1” to “40”
    b. Label HPLC vials (n = 40) “1” to “40”, and add 300 µL inserts into each HPLC vial
    c. Label HPLC vials used for the calibration curve: “0”, “0.1”, “0.5”, “1”, “5”, “10”, “20”, “50”
c. Other
    a. Check level of argon gas to confirm level is at least 25% full
    b. Restock 1 mL pipet tips

Day of analysis

Sample preparation:

a. Remove 40 urine samples from sample box (kept in -80°C freezer), place them in a sample rack, and let them defrost at room temperature (~20-30 minutes)
b. In notebook, record the sample ID that corresponds to each labelled vial
c. Prepare calibration curve for total analysis: 0 µg/L, 1 µg/L, 5 µg/L, 10 µg/L, 20 µg/L, 500 µg/L (in 50 mL centrifuge tubes, final volume = 20 mL in 1% HNO_3)
d. Prepare 1640a standard reference material (trace elements in natural water, National Institute of Standards and Technology (Gaithersburg, MD)) (diluted 1:1, with final solution containing 1% HNO_3) to validate the calibration curve
e. Prepare for sample filtration by gathering 0.45 µm filter tips, syringes, 40 square pieces of parafilm, and a waste beaker (the urine samples should be defrosted by this time)

Sample filtration:

a. Vortex sample at high speed for 20 seconds
b. Filter ~500 µL of the sample (using a syringe and 0.45 µm filter tip) into the corresponding labelled 1.5 mL plastic vial
c. Label the used 0.45 µm filter tip with the sample ID, wrap with parafilm, and store in a zip-lock bag labelled with the analysis date (and place in -80°C freezer)
d. Discard syringe into waste beaker
e. Begin warming up ICPMS instrument after filtering sample #20, so that the instrument may be ready in time

Total analysis:

a. Add 30 µL of HNO_3 to each labelled 10 mL centrifuge tube (so that the final concentration is 1% HNO_3)
b. Add 300 µL of each sample to the corresponding labelled 10 mL centrifuge tube (remainder of sample in each vial will be used for speciation analysis)
c. Vortex the previously prepared 50 mL centrifuge tubes containing the calibration curve standards for 10 seconds
d. Vortex the 10 mL centrifuge tubes containing the diluted samples for 10 seconds
e. Add calibration curve standards and diluted samples to autosampler
f. Create new batch using template "BLOCKLIST-TotalAnalysis-Oct102019.icpms.template.icpms.template" located in: Computer -> Data (D:) -> Jagdeesh -> Templates
g. Update sample list, so that the sample ID's correspond to that day's analysis
h. Run total analysis
i. Further dilute samples that have concentrations that don't fit the calibration curve for total analysis

Speciation analysis:

a. While total analysis is being performed:
    a. Prepare mobile phase and speciation calibration standards (0 µg/L, 0.1 µg/L, 0.5 µg/L, 1 µg/L, 5 µg/L, 10 µg/L, 20 µg/L, 50 µg/L)
    b. Record the total concentration values for each sample, and determine dilutions needed to bring total arsenic concentration for each sample to less than 50 µg/L
    c. Filter water using 5 syringes and 0.45 µm filters into 5 HPLC vials to serve as the method blanks (run prior to calibration curve during speciation analysis)
b. Dilute filtered samples with water, so that the final concentration is less than 50 µg/L (using results from total analysis)
c. Defrost and/or prepare standard reference materials: SRM2669 Level I (arsenic species in frozen human urine, National Institute of Standards and Technology (Gaithersburg, MD)), SRM 1640a (trace elements in natural water, National Institute of Standards and Technology (Gaithersburg, MD)), and CRM18 (human urine, National Institute for Environmental Studies (Tsukuba-City, Japan)).
d. Create new batch using template "BLOCKLIST-Jagdeesh-OptimizedMethod.icpms.template.icpms.template" located in: Computer -> Data (D:) -> Jagdeesh -> Templates
e. Update sample list, so that the sample ID's correspond to that day's analysis
f. Run speciation analysis overnight