University of Alberta

Arsenic Speciation Analysis in Environmental and Biological Systems

by

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Abstract

My thesis research focused on the development and application of analytical methods that enabled arsenic speciation in biological and environmental samples. A set of complementary chromatographic separation techniques were combined with inductively coupled plasma mass spectrometry and hydride generation. These techniques allowed for the separation and detection of arsenobetaine, arsenite, arsenate, monomethylarsonic acid, and dimethylarsinic acid. The application of these techniques to the determination of arsenic species in human urine has contributed to arsenic exposure measurement in a collaborative pilot epidemiological study. The application of a high performance liquid chromatography – inductively coupled plasma mass spectrometry technique showed that most of the groundwater samples from the Battersea Drain watershed located in southern Alberta had arsenic concentrations below the Canadian drinking water guideline value of 10 μg L^{-1}. A set of complementary chromatographic separation techniques coupled with inductively coupled plasma mass spectrometry was developed to characterize a new arsenic species, Arsenicin A, previously reported for the presence in a marine sponge. These techniques enabled the separation and detection of an Arsenicin A model compound, arsenite, arsenate, monomethylarsonic acid, dimethylarsinic acid, arsenobetaine, and an arsenosugar. The application of these techniques to the determination of arsenic species in marine sponges suggested that arsenic speciation profile may be organism and habitat dependent. A comparative cellular uptake study that used human lung carcinoma A549 cells showed that these cells were able to uptake two

orders of magnitude more Arsenicin A model compound than arsenite. The higher cellular uptake of Arsenicin A model compound was consistent with the higher toxicity of Arsenicin A model compound as compared to arsenite, suggesting that the cellular uptake is an important factor contributing to the toxicity of these arsenic species. My Ph.D. research has provided analytical techniques that are useful to environmental and biological studies.

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List of Abbreviations

APL	Acute Promyelocytic Leukemia
As^{V}	arsenate
As3MT	arsenic (III) methyltransferase
AA	Arsenicin A model compound
As^{III}	arsenite
AsB	arsenobetaine
AsH_3	arsine
AAS	atomic absorption spectrometry
AFS	atomic fluorescence spectrometry
CCA	chromated copper arsenate
CE	collision energy
CXP	collision exit potential
CFOs	confined feeding operations
DP	declustering potential
DMA^{V}	dimethylarsinic acid
DMA^{III}	dimethylarsinous acid
DMDTA	dimethyldithioarsenic acid
DMMTA	dimethylmonothioarsenic acid
DRC	dynamic reaction cell
EP	entrance potential
ESI-MS	electrospray ionization mass spectrometry
EDTA	ethylenediaminetetraacetic acid

GC	gas chromatogrpahy
GF-AAS	graphite furnace-atomic absorption spectrometry
HPLC	high performance liquid chromatography
HPLC-HG-ICPMS	high performance liquid chromatography-hydride-generation-inductively coupled plasma mass spectrometry
HG	hydride generation
HG-AAS	hydride generation-atomic absorption spectrometry
HG-cold trap-AAS	hydride generation-cold trap-atomic absorption spectrometry
HG-QF-AAS	hydride generation-quartz furnace-atomic absorption spectrometry
ICP-AES	inductively coupled plasma-atomic emission spectrometry
ICPMS	inductively coupled plasma mass spectrometry
LDPE	low-density polyethylene
IC_{50}	median inhibitory concentration
LC_{50}	median lethal concentration
µg/L	microgram per liter, equivalent to parts per billion (ppb)
mg/L	milligram per liter, equivalent to parts per million (ppm)
MMA^{V}	monomethylarsonic acid
MMA^{III}	monomethylarsonous acid
MMTA	monomethylthioarsenic acid

PCBs	polychlorinated biphenyls
PAHs	polycyclic aromatic hydrocarbons
US-EPA	United States Environmental Protection Agency
AgDDTC	silver diethylthiocarbamate
$TN_{unfiltered}$	total nitrogen
TP	total phosphorus
WHO	World Health Organization

CHAPTER 1 Introduction and literature review

1.1 Global concerns of arsenic

Arsenic with atomic number 33 and atomic mass 74.92 dalton, belongs to Group VA of the Periodic Table [1–3]. Arsenic is often referred to as a semi-metal or a metalloid, which can be present in four oxidation states (-3, 0, +3, +5) [4, 5]. The trivalent species (As^{III}) dominates in reducing conditions (< 200 mV), while the pentavelent species (As^{V}) prevails under oxygenated environments [6, 7]. Arsenic is a trace element that is less abundant than many of the rare-earth elements in the Earth's continental crust; however, arsenic is ubiquitous in the environment, which can be found in soil, rock, water, and air. [8–12]. Arsenic is present in the earth's crust at an average concentration of around 2–5 mg/kg, which is a similar abundance as uranium, bromine, tin, germanium, tungsten, and molybdenum [1, 10]. There are more than 245 arsenic-containing minerals, in which arsenic is especially concentrated in the presence of iron, copper, lead, cadmium, gold, silver, tungsten, and molybdenum [1, 11].

An estimated one-quarter of atmospheric arsenic has a natural origin (7,900 tonnes per year), mostly from volcanic activity, followed by low temperature volatilization from microorganisms [1]. Alternatively, anthropogenic sources contribute approximately 3 times as much arsenic (24,000 tonnes per year) through mining and ore smelting emission, arsenic pesticides, as well as fossil fuel combustion [13, 1]. It has been estimated that 20–200 ng/day of arsenic may be inhaled in rural areas, whereas 400–600 ng/day of arsenic may be found in cities [13, 1, 2, 14]. Under the appropriate condition (pH, redox potential,

temperature, and solution composition) [15], arsenic may leach from minerals, enter underground water reservoirs, and incorporate into drinking water [4]. Hence, elevated concentrations of arsenic are commonly found in the groundwater of areas that have naturally high arsenic containing mineral deposits, for example, in Bangladesh and India (West Bengal) [2].

In addition to dissolution of rocks and minerals, arsenic may also enter groundwater and soils from many man-made sources, e.g., industrial effluents from mining and ore smelting, poultry and swine feed additives, arsenic containing pesticides, and wood preservatives [16–19]. For example, metal smelting operations may emit arsenic trioxide (As_2O_3) as a by-product. Chromated copper arsenate (CCA) is another major industrial arsenic application. CCA had been a popular antifungal wood preservative that made up to nearly 90% of the total arsenic used by industry, until 2003 when both Canada and the USA voluntary banned its application from residential use [1, 20]. In addition, arsenic had been widely used in the agricultural industry, including cotton desiccants, herbicides, insecticides, and pesticides.

Following the epidemiological studies in Taiwan that showed associations of arsenic with various forms of cancer (skin, lungs, and urinary bladders) [1, 2], and the World Health Organization's recommendation on safe drinking water guideline (1993), the use of inorganic arsenic pesticides in the USA had been phased out [21]. In 2009, the US Environmental Protection Agency (US EPA) announced a cancellation order to eliminate the use of pesticides containing organic arsenic by 2013 [21]. However, organic arsenicals such as Roxarsone and

arsanilic acid and their derivatives are still being used as livestock additives, especially common in swine and poultry farms for growth promotion, improved feed efficiency, pigmentation, and parasite prevention [22–26]. As a result of multiple natural and anthropogenic sources that contributed to an elevated level of arsenic in the environment, as well as arsenic's toxicity and its association with numerous serious human health effects, the US Agency for Toxic Substances and Disease Registry has ranked arsenic as number one on its most current list of priority hazardous substances, followed by lead, mercury, vinyl chloride, polychlorinated biphenyls (PCBs), benzene, cadmium, and polycyclic aromatic hydrocarbons (PAHs) [27].

An estimated 160 million people worldwide are exposed to high concentration of arsenic in drinking water that exceed the guideline value of 10 µg/L recommended by the World Health Organization [2]. There has been an increasing public awareness of the serious health concerns related to exposure to high concentration of arsenic (> 50 µg/L or much greater) in drinking water in various parts of the world, including some areas in Argentina, Bangladesh, Chile, China, India (West Bengal), Mexico, Taiwan, parts of southwestern United States, and some parts in Canada [2] (Table 1.1). Most of these areas that reported on large scale arsenic contamination in drinking water rely heavily on groundwater as their sole water supply. Most of these contaminated sites have geological formations that are rich in arsenic [1]. There are two common types of environment that have been suggested to be more inclined to have large scale elevated arsenic levels in groundwater: closed basins or inland in arid to semi-arid

areas, and secondly, strongly reducing aquifers commonly contained alluvial sediments. The common features shared by these two types of environments are the geologically young sediment formation in low-lying locations where water flow is limited; hence, arsenic is restricted to these areas and may be further concentrated over time with evaporation [15].

Long-term exposure to elevated level of inorganic arsenic (e.g. ≥ 100 µg/L) in drinking water has been reported to have strong associations with cancers of the skin, urinary bladder, and lungs, as well as other non-cancerous health implications, such as skin lesions, diabetes, cardiovascular diseases, and neuropathy [28–32]. However, there is considerable uncertainty of the health effects due to the exposure of lower concentration of arsenic (e.g. < 10 µg/L), [2, 1, 33]. Therefore, there is still much research required to assess the dose-response relationship between exposure to lower level of arsenic and the relevant health outcomes.

Table 1.1 Global concerns of arsenic contamination in groundwater [11, 34].

Country/region	Population exposed[1]	Concentration (µg/L)[2]
Bangladesh	**50, 000, 000**	**< 0.5 – 2,500**
West Bengal, India	**6, 000, 000**	**< 10 – 3,200**
Taiwan, China	**5,600,000**	**10 – 1,820**
Inner Mongolia, China	**100, 000**	**< 1 – 2,400**
Vietnam	**> 1,000,000**	**1 – 3,050**
Thailand	**15,000**	**1 – 5,000**
Hungary and Romania	**29,000**	**< 2 – 176**
Germany	**2,500**	**< 10 – 150**
Argentina	**2,000,000**	**< 1 – 5,300**
Chile	**500,000**	**100 – 1,000**
Mexico	**400,000**	**8 – 620**
Southwestern USA[3]	**350,000**	**< 1 – 2,600**
Alaska, USA	**n/a[4]**	**1 – 10,000**
Alberta, Canada	**n/a[4]**	**<1 – 5,340**

[1] population exposed are estimated based on exposure to > 50 µg/L arsenic in groundwater. [2] arsenic concentrations in groundwater may be from one or both sources: natural processes and anthropogenic activities. [3] Arizona, California, Nevada. [4] n/a = not available.

1.2 Arsenic in water

The concentration of arsenic in natural waters such as open sea water are generally around 1–2 μg/L [19, 14, 3]. However, in the presence of natural sources, such as volcanic sediments, arsenic rich minerals, and geothermal waters, arsenic concentration may significantly increase, as high as 25 mg/L has been reported [14]. Moreover, in the proximity of anthropogenic sources, such as industrial effluents from mineral extraction sites, arsenic containing pesticides, and wood preservatives, arsenic levels may be elevated dramatically as well [16–19].

Although elemental arsenic is sparingly soluble in water, arsenic salts have a wide range of solubilities depending on the pH and the redox environment [1]. For example, arsenite (As^{III}) dominates under reducing condition such as in deep well waters, while arsenate (As^{V}) is the prevalent form in oxygenated water, such as surface fresh water [13, 1, 35–38]. In addition, biological activities also play an important role in the forms of arsenic present in water because there are organisms such as phytoplankton, bacteria, and algae capable of biomethylating that convert inorganic arsenic (As^{III} and As^{V}) to the methylated arsenic forms such as monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}), or the even more complicated arsenosugars and arsenobetaine [7, 39–43].

Arsenic may be released from the arsenic containing minerals and sediments if initiated by one or both potential “triggers”. During natural weathering process, pH may exceed 8.5, such condition may promote desorption of arsenic from the mineral oxides or may even prevent adsorption of arsenic [15].

Secondly, strongly reducing conditions at pH ≈ 7 may enhance desorption of arsenic from mineral oxides and promote iron and manganese dissolution that may ultimately lead to the release of arsenic into the surrounding environment [15]. Moreover, in the presence of competition such as phosphate, bicarbonate, and silicate, arsenic desorption process may accelerate due to the decreasing availability of adsorption sites [15]. Therefore, arsenic may be mobilized in the environment through a combination of natural and anthropogenic sources [13, 1].

In an effort to determine the total arsenic concentrations in water samples, prevention of sample contamination and minimization of specie conversion are two critical considerations to include during the sample collection, transportation, and storage procedures. There are many pre-treatment methods for groundwater samples, of which the most common approaches to preserve arsenic species are through the use of different acids, including sulfuric acid (H_2SO_4), nitric acid (HNO_3), hydrochloric acid (HCl), acetic acid (CH_3COOH), and ethylenediamine tetraacetic acid (EDTA) [44]. Historically, H_2SO_4 and HNO_3 are used to treat water samples to decrease potential arsenic adsorption to the surface of the sample container [2]. Some proposed that EDTA/CH_3COOH pre-treatment method may be especially effective in preserving arsenic species in water samples that had higher iron content (> 6.45 mg/L) by reducing the rate of As^{III} oxidation to As^{V} [42]. To prevent potential adsorption of arsenic caused by the precipitation of iron oxyhydroxides, chelating agent such as EDTA was added to the water samples to sequester Fe^{III} and inhibit the precipitation of iron and arsenic, thereby stabilizing As^{III} and As^{V} [44–48]. In addition, opaque high density polyethylene bottles are

favoured over glass bottle for sample storage because it is less adsorptive for arsenic, and it minimize light exposure, thereby reducing the photo catalyzed Fe^{III} reduction and As^{III} oxidation to As^{V} [45, 2].

In Canada, about 30% of the population rely on groundwater as the main source of drinking water, ranging from the lowest dependence in Alberta (23%) to the greatest dependence in Prince Edward Island (100%) [49]. Canadians generally have access to safe drinking water that contains less than 10 µg/L arsenic, which comply with the most current Health Canada maximum acceptable concentration for arsenic in drinking water [50], and which is also consistent with the guidelines from United States Environmental Protection Agency [51] and the World Health Organization [3]. However, there are some specific locations in Canadian provinces and territories that are known as arsenic "hotspots" (> 10 µg/L), where the weathering process of arsenic rich minerals and sediments is the most common source of arsenic in these areas [34]. Currently, there has been minimum research on the levels of arsenic exposure in Canadian drinking water [34]. Therefore, the determination of arsenic in drinking water is an active area of research that is critical in examining the relevant health outcomes associated with low levels of arsenic exposure.

1.3 Arsenic in food

Except for occupational arsenic exposure, such as individuals who work in coal-fired power plants, pressure-treated wood industry, and glass or electronic manufacturing [16–19], the most common route of human exposure to arsenic is through ingestion of food and drinking water, which include beverages made with

drinking water [3]. It has been estimated 20–300 µg/day of arsenic is ingested through food and beverages [1]. Food typically contains less than 0.25 mg/kg inorganic and organic arsenic [1]. Multiple factors may influence the concentrations and arsenic species found in food, including the type of food (grain, dairy, seafood, or meat), growing conditions (choices of pesticides and soil fertilizer used, sources of water), as well as food-processing techniques [3]. Fish and meat are often the major contributors to the total dietary intake of arsenic, with 0.4–118 mg/kg have been reported in marine fish, while 0.44 mg/kg has been found in meat and poultry [52, 53]. Seafood generally contains the highest amount of total arsenic levels. Depending on the type of seafood, it may range from 3.5 mg/kg in mussels to greater than 100 mg/kg in some crustaceans [52]. The levels of arsenic found in decreasing order in the following food categories are seafood, meats, cereals, vegetables, fruits, and dairy products [13, 1, 2].

Some literatures suggest that approximately 25% of the total dietary intake of arsenic is the more toxic inorganic arsenic species (As^{III} and As^{V}), which have been categorized as human carcinogens by the International Agency for Research on Cancer [1, 54, 55]. However, these data are highly subjective to the type of food that has been ingested [54, 56, 1]. On the contrary, arsenobetaine (AsB), which is commonly found in crustaceans, such as shrimps and lobsters, is essentially non-toxic [57–61]. Some commonly found organic arsenic species in marine organisms may include arsenobetaine, arsenocholine, tetramethylarsonium ion, arsenosugars and arsenic containing lipids; however some of these species have also been identified in terrestrial organisms [1]. Therefore, it is important to

determine both the speciation and concentration of arsenic to more accurately assess the extent of its impact on human health since the toxicity of arsenic is highly dependent on the chemical form in which it exists [62].

There has been a report of an estimated daily intake of arsenic from food and drinking water in a North American pilot study, which found 12–14 µg/L of inorganic arsenic in the dietary intake [63]. In regions where drinking water is contaminated with elevated level of arsenic, assuming a daily water consumption of 2 liters that contained 10 µg/L of arsenic, then the drinking water alone would contribute to 20 µg of daily dietary intake of arsenic, which is a significant contribution. Therefore, it is critical to have early detection of drinking water that may contain elevated arsenic levels, such as the water wells in areas that already have shown high levels of arsenic contamination (Table 1.1) [3].

In addition, arsenic has been used for human consumption for medicinal purposes at least as early as the 15th century [64, 65]. For example, *Fowler's solution* is a potassium bicarbonate-based As_2O_3 (1% wt/vol) solution developed in the 18th century to treat asthma, chorea, eczema, psoriasis, and anemia [66]. *Salvarsan*, also known as arsphenamine, is a mixture of cyclic trimer and pentamer of organic arsenic, and was introduced in 1910 to treat syphilis [67]. Most currently, As_2O_3 in water (Trisenox®, 1mg As_2O_3/mL ampule, Cell Therapeutics, Inc.) was approved by the U.S. Food and Drug Administration to treat Acute Promyelocytic Leukemia [66].

1.4 Arsenic species excreted into urine

Following by ingestion of inorganic arsenics in food and drinking water, arsenate (As^{V}) may be reduced to arsenite (As^{III}) in the blood, then taken up by the liver and subsequently metabolized into methylated arsenic species, and then eventually eliminated from the body through urinary excretion [28, 68]. Following exposure to inorganic arsenic, arsenic speciation analyses of human urine have revealed that dimethylarsinic acid (DMA^{V}) and monomethylarsonic acid (MMA^{V}) are the major metabolites as a result of biomethylation of arsenic, which is a major component of arsenic metabolism in human [69–81]. There has been much debate between two of the most well-known enzymatic pathways to convert inorganic arsenics to the methylated metabolites. In 1945, Challenger proposed an oxidative methylation scheme that involves a multi-step reduction from a pentavalent to a trivalent oxidation state, followed by subsequent transfer of a methyl group from S-adenosyl-L-methionine (SAM) while arsenic is oxidized simultaneously (Fig. 1.1) [82]. This scheme has been widely accepted and has been supported by various experimental data and mechanistic model [83–86]. More recently in 2005, Hayakawa *et al*. proposed a non-oxidative methylation scheme that involves glutathione-bound trivalent arsenics methylated sequentially by SAM and arsenic (III) methyltransferase (As3MT) without undergoing oxidation (Fig. 1.1) [87]. In addition, recent investigations suggest that glutathione may be replaced by other reducing systems and arsenic (III) methyltransferase may still be able to function and catalytically methylate arsenic [88, 89].

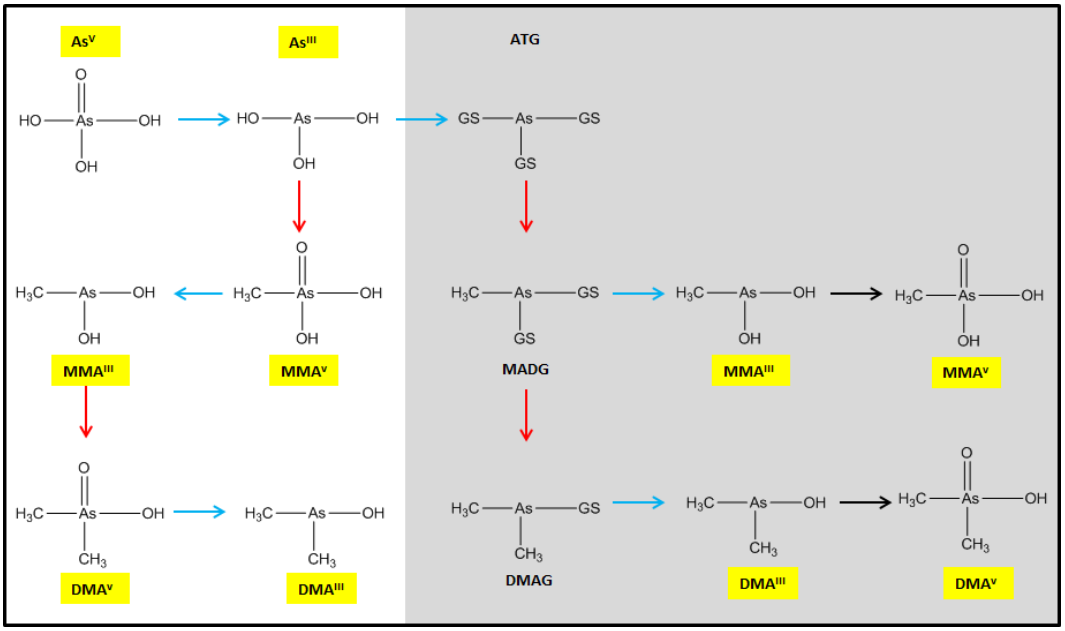


Fig. 1.1 Metabolic pathways of inorganic arsenic in humans [89]. The shaded area represents the more recent pathway proposed by Hayakawa *et al.* [87]. The un-shaded area represents the classical pathway proposed by Challenger [82]. The blue arrows represent reaction assisted by glutathione, while the red arrows represent arsenic methylation by arsenic methyltransferase (As3MT) and S-adenosyl-L-methionine (SAM). (**GS** glutathione; **ATG** arsenic triglutathione; **MADG** monomethylarsonic diglutathione; **DMAG** dimethylarsenic glutathione).

Multiple tissues may be capable of accumulating arsenic; however, not all tissues are suitable candidates as biomarkers of exposure. Some human arsenic exposure biomarkers include hair, nail, blood, and urine. Hair and nails contain large amount of keratins, and have been shown to be reliable indicators as long term exposure biomarkers [90, 91]. Arsenic tends to accumulate in keratin-rich tissues because trivalent arsenic species preferentially targete cellular proteins that have closely spaced cysteine residues and accessible thiol groups [5, 92]. On the other hand, blood and urine are usually used as indicators for recent arsenic exposure because arsenic rapidly passes through the blood stream within a few hours and is excreted out of the body through kidney and urine within a few days either as inorganic arsenic (As^{III} and As^{V}) and/or its methylated metabolites, e.g., MMA^{III}, MMA^{V}, DMA^{III}, and DMA^{V} [93, 94, 28, 95].

Urinary arsenic may be used as an indicator to determine the extent of arsenic exposure in individuals. Generally, the levels of the sum of urinary arsenic species from the general population not exposed to elevated concentrations of arsenic are below 10 μg/L [96, 57, 97]; whereas from individuals exposed to chronic exposure to arsenic, e.g., in Bangladesh and India (West Bengal), the sum of urinary arsenic levels may exceed 1,000 μg /L [1]. Inorganic arsenics (As^{III} and As^{V}) have a residence half-life of 2–40 days [98] and they may accumulate in skin, bone, liver, kidney, and muscle [99], or they may be rapidly eliminated out of the body through urinary excretion [100–102], either in the unchanged inorganic arsenic forms, or as the methylated metabolites [103, 93, 104].

In arsenic speciation analysis, acidification of samples is not always an appropriate sample pre-treatment step, because the addition of acid may change the arsenic species and thereby alter the original urinary arsenic distribution results. Therefore, various sample storage precautions need to be considered to maintain the integrity of arsenic speciation, such as low temperature (4℃ to -80℃) conditions, and the absence of any oxidizing or reducing agent during sample storage and pre-treatment [2].

1.5 Dependence of arsenic's toxicity on arsenic speciation

The finding of the more toxic intermediate arsenic metabolites, namely, the trivalent arsenic species of monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) challenges the previous notion that the arsenic biomethylation scheme is strictly a "detoxification" process. Recent *in vitro* cytotoxicity studies have demonstrated that the trivalent methylated arsenic species (MMA^{III} and DMA^{III}) are orders of magnitude more toxic than their pentavalent counterparts (MMA^{V} and DMA^{V}) as well as the inorganic arsenic species (As^{III} and As^{V}) (Table 1.2) [89, 105–109].

In water samples, inorganic arsenics (As^{III} and As^{V}) are often found to be the major component of the total arsenic, as compared to the methylated species (MMA^{V} and DMA^{V}). Also, the presence of trivalent methylated species (MMA^{III} and DMA^{III}) in natural waters raised the awareness of "hidden arsenic" that was unidentified in fresh waters, which could represent up to 20% of the total arsenic concentrations [110–113].

Table 1.2 LC_{50} values (median lethal concentration) of different arsenic species in human (1T1) and rat (MYP3) urothelial cells [106].

Cell line	LC_{50} (μM)						
	MMA^{III}	MMA^{V}	DMA^{III}	DMA^{V}	As^{III}	As^{V}	TMAO
1T1	1.0	1700	0.8	500	4.8	31.3	1700
MYP3	0.8	1700	0.5	1100	0.4	5.3	4500

Arsenic is present in various chemical forms (Table 1.3 and Fig. 1.2), with different oxidation states and degrees of methylation that significantly influence their cytotoxicity and genotoxicity [28, 114, 108]. Trivalent arsenics can be much more toxic than their pentavalent counterparts, for example, As^{III} is more toxic than As^{V}, MMA^{III} and DMA^{III} are much more toxic than MMA^{V} and DMA^{V} [62, 107, 109]. The order of arsenic toxicity is $DMA^{III} > MMA^{III} > As^{III} > As^{V} > DMA^{V} > MMA^{V} >$ arsenobetaine ≈ non-toxic [108, 106]. Hence, it is necessary to determine both the concentration of the total arsenic, but even more importantly, to determine the individual concentrations of each arsenic species that are present, in order to more accurately assess the level of toxicity exerted by arsenic.

Table 1.3 Arsenic species described in this thesis [1-3].

Name (Abbreviation)	Chemical formula	pKa	Structure
Arsenite (As^{III})	**$As(OH)_3$**	9.23, 12.13, 13.4	1
Monomethylarsonous acid (MMA^{III})	**$CH_3As(OH)_2$**		2
Dimethylarsinous acid (DMA^{III})	**$(CH_3)_2AsOH$**		3
Arsenate (As^{V})	**$AsO(OH)_3$**	2.22, 6.98, 11.53	4
Monomethylarsonic acid (MMA^{V})	**$CH_3AsO(OH)_2$**	4.1, 8.7	5
Dimethylarsinic acid (DMA^{V})	**$(CH_3)_2AsO(OH)$**	6.2	6
Arsenobetaine (AsB)	**$(CH_3)_3As^+CH_2COO^-$**	2.18	7
Trimethylarsine oxide (TMAO)	**$(CH_3)_3AsO$**		8
Arsenic trioxide	**As_2O_3**		9
Roxarsone	**$C_6AsNH_6O_6$**	3.49, 5.74, 9.13	10

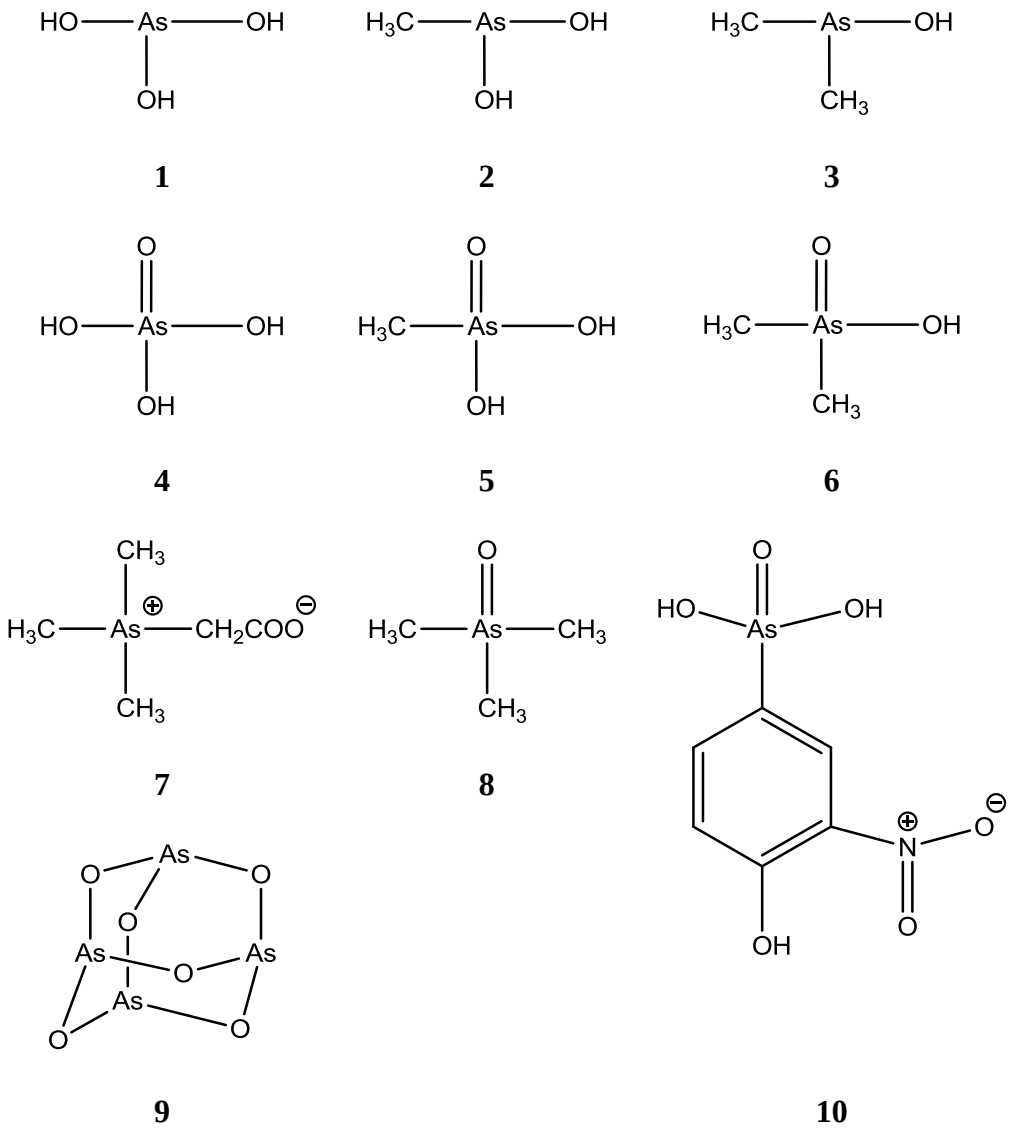


Fig. 1.2 Structures of arsenic species listed in Table 1.3.

1.6 Technologies for arsenic analysis

Analytical techniques capable of determining the total arsenic concentrations and performing arsenic speciation analysis are important prerequisites in the assessment of human exposure to arsenic and its respective human health implications. Since the chemical form of arsenic greatly influences its toxicity, and bioavailability, as well as its behaviour in the environment; there is a need to develop analytical methods that are capable of determining the relative concentrations of individual arsenic species in biological and environmental samples.

1.6.1 Sampling and pre-treatment

Two of the most important considerations to include during sample collection and storage are to prevent contamination and to minimize arsenic speciation changes. The sample container, e.g., high-density polyethylene bottles were pre-washed with acid then with water thoroughly before use, in order to remove any traces of oxidizing or reducing agent that may alter the oxidation state of the arsenic species. It is strongly encouraged to store samples at low temperature (-80℃) to preserve the chemical integrity of the samples by preventing arsenic undergoing speciation changes [74]. Various acids (HCl, HNO_3, H_2SO_4, CH_3COOH, EDTA) have been used alone or in combination to prevent bacterial growth and to preserve arsenic speciation by slowing down the rate of As^{III} oxidation and also by preventing arsenic precipitation [44].

Oxidative digestion is widely accepted as a common sample pre-treatment approach, of which acid digestion [115] and dry ashing [116] are the two most

common methods. In addition, microwave-assisted digestion has been used for a wide variety of samples, e.g, soil, sediments, and marine organisms [117, 118]. Also, solvent extraction is a commonly used sample pre-concentration technique that may require a wide range of solvents, including HCl and chloroform [119, 120], water [121], methanol [122–126], or sodium bis(trifluoroethyl)dithiocarbamate [127, 128]. In addition, another common sample pre-concentration technique used in liquid and gas samples involved the use of different types of filters, such as filter paper [129], cellulose ester [79], glass microfiber [130], and polytetrafluoroethylene [131].

1.6.2 Analytical methods for determining total arsenic

There is a wide range of analytical techniques available for the determination of arsenic. These techniques may include colorimetry/spectrophotometry, atomic absorption spectroscopy (AAS) [132, 96, 133], atomic fluorescence spectrometry (AFS) [134–136], inductively coupled plasma atomic emission spectroscopy (ICP-AES) [137–139], and inductively coupled plasma mass spectrometry (ICPMS) [140, 69, 141].

Colorimetric methods rely on the generation of volatile arsine (AsH_3) to differentiate arsenic from the other interferences that may be present in the sample matrix. These methods are relatively easy to use and have lower operating cost. Hence, they are widely applied to on-site test kits for the determination of total arsenic, especially in developing countries where there is a need for rapid analyses on a large number of samples, which does not require sample transportation, storage, and preservation. However, these methods are limited to

semi-quantitative determination of arsenic, with detection limit ranging from 10 – 100 µg/L under field condition [142–145].

The field test kits currently used in Bangladesh, Vietnam, India, and other areas with large-scale contaminated groundwater are based on a modified method of the Gutzeit' test. Gutzeit's test stemmed from the classic Marsh test, with the modification of detecting arsenic with a strip of filter paper either moistened with silver nitrate ($AgNO_3$) or mercuric chloride ($HgCl_2$) to form a grey or yellow to reddish-brown spot, respectively [146]. In the field test kits, the mercuric bromide ($HgBr_2$) test strip is reacted with zinc dust in an acidified solution to form a yellow [$H(HgBr_2)As$] to brown [$(HgBr)_3As$] to black [Hg_3As_2] stain, whose intensity is proportional to the amount of arsenic present in the water samples, which was then matched to a colour chart [142]. Recently, digital readers have been developed to measure the intensity of the coloured strip [147], and bacterial biosensors have been also been used in the newer test kits to provide improved sensitivity [148, 149].

One of the most popular **spectrophotometric** methods for the determination of arsenic in water is using the silver diethylthiocarbamate (AgDDTC) technique [116, 150, 151]. This technique takes advantage of the generation of AsH_3 through the addition of zinc and HCl, or sodium borohydride ($NaBH_4$) in acid, followed by flushing of the AsH_3 gas with diethylthiocarbamate in pyridine or pyridine/chloroform solution and the subsequent detection at 520 nm. This technique is applied to water sample and tissue samples, with the limit of detection at 40 µg/L [150] and 100 µg/L [116], respectively.

Atomic absorption spectrometry (AAS) is one of the most common methods used to analyze arsenic in biological and environmental samples. Two of the most widely used AAS techniques are **graphite furnace (GF)-AAS** and **hydride generation (HG)-AAS**. GF-AAS requires a small aliquot (5–50 µL) of sample deposited in a graphite tube positioned in the optical path of an atomic absorption spectrophotometer. In addition, matrix modifiers, e.g., palladium and magnesium are added to the sample to prevent premature volatilization of arsenic that may lead to loss of arsenic and the underestimation of the amount of arsenic present in the sample. The graphite tube is heated sequentially by an electrical furnace through the steps of drying, ashing, and atomization. The amount of arsenic that is present in the samples is measured by the amount of light that passes through the graphite tube and is absorbed by the analyte. This technique is widely applied to arsenic analysis in water and various biological samples [152]. Also, it is a United States Environmental Protection Agency (US-EPA) approved analytical method for arsenic analysis in water [2].

On the other hand, HG-AAS technique relies on a continuous flow of sample and the generation of volatile arsine through the addition of zinc and HCl or $NaBH_4$ and acid mixture to produce trivalent arsenic. The gaseous arsine is swept into the flame or quartz cell that is located in the optical path of an atomic absorption spectrophotometer. Then a hollow cathode lamp or a discharge lamp emits a light beam to directly pass through the flame or quartz cell, then into a monochromator, and finally arrive at a detector that determines the amount of arsenic present in the sample by measuring the amount of light absorbed by the

gaseous arsine. HG-AAS may be one of the most commonly used analytical method for the determination of total arsenic in water and various biological samples [144, 153–155]. Also, it is another US EPA-approved analytical method for arsenic analysis in water [2].

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) has been a routine analytical method used by many laboratories mostly due to its multi-element analysis capability. This technique quantifies the target analyte by measuring the intensity of the element-specific atomic emission spectra and compares them to known calibration standards. The use of the axial torch configuration and ultrasonic nebulization can enhance the sensitivity of this method because of the increased path length and the improved analyte transport efficiency [2, 1]. The ICP-AES detection had been coupled to high-performance liquid chromatography (HPLC) and hydride-generation (HG) to perform speciation of arsenic species in marine organisms [156–158]. However in 2002, ICP-AES was no longer approved by the US EPA as an appropriate analytical method for the determination of arsenic because this technique offers a detection limit typically of 30 µg/L which is not sufficient to meet the US-EPA updated drinking water guideline of 10 µg/L [2, 159].

Inductively coupled plasma mass spectrometry (ICPMS) offers a lower detection limit of 0.1 µg/L and a wider linear dynamic range as compared to the ICP-AES technique (Table 1.4). The aqueous sample is delivered by pneumatic nebulization into a high temperature plasma (~8,000K) where the analyte undergoes atomization and then ionization. The analyte ions are extracted into the

ion optic by a series of differentially pumped vacuum and is separated based on the ions' mass-to-charge ratio by a mass spectrometer, of which quadrupole is the most common, but double-focusing magnetic sector or time-of-flight have also been used [159]. This technique is approved by US EPA as a suitable analytical method for the determination of arsenic. Table 1.4 provides a brief summary of the advantages and disadvantages of the aforementioned techniques.

Table 1.4 Analytical method commonly used to determine total arsenic and arsenic speciation in environmental and biological samples.

Analytical methods	Advantages	Disadvantages	L.O.D (µg/L)	T	S
Colorimetry/ Spectrophotometry	easy to use, low cost, on-site field test kits are available	high detection limit	~40	✓	
Inductively coupled plasma-mass spectrometry (ICPMS)*	low detection limit, multi-element, high throughput, wide linear dynamic range, isotopic measurements, easy to interpret spectra	high initial cost for instrument, some spectral interference, required some method development	0.1	✓	
	High performance liquid chromatography (HPLC)-ICPMS can be used for arsenic speciation analysis	Require standards to match the elution time of the analyte	0.01		✓
	(HPLC)-Hydride generation (HG)-ICPMS	can only be applied to limited compounds:	~ 0.001		✓

	can be used to improve sensitivity	As^{III}, As^{V}, MMA^{V}, DMA^{V}, TMAO			
Inductively coupled plasma-atomic emission spectrometry (ICP-AES)	easy to use, multi-element, high throughput, screening ability	high detection limit, some spectral interference, some element limitation	~ 30	✓	
Graphite furnace- atomic absorption spectrometry (GF-AAS)*	low detection limit, small sample size	Slower analysis time, pre-atomization losses, require matrix modifiers, chemical interference, element limitations, no screening ability, limited dynamic range	~ 0.025	✓	
	Hydride generation-quartz furnace-atomic absorption spectrometry (HG-QF-AAS) can be used to improve sensitivity	can only be applied to limited compounds: As^{III}, As^{V}, MMA^{V}, DMA^{V}, TMAO	0.003 - 0.015	✓	✓
Hydride generation-atomic absorption spectrometry	easy to adapt laboratory skills and equipment from furnace or flame-AAS to	can only be applied to limited compounds: As^{III}, As^{V}, MMA^{V}, DMA^{V}, TMAO	0.6 – 6	✓	✓

(HG-AAS)*	HG-AAS				
	High performance liquid chromatography (HPLC)-HG-AAS can be used for arsenic speciation analysis	can only be applied to limited compounds: As^{III}, As^{V}, MMA^{V}, DMA^{V}, TMAO	1 – 4.7	✓	✓
HPLC or solid phase extraction cartridge-hydride generation-atomic fluorescence spectrometry (HPLC-HG-AFS)	fast analysis, low cost	some matrix interferences	0.05 - 0.8		✓

* U.S. Environment Protection Agency approved analytical methods for arsenic analysis in drinking water
L.O.D limit of detection
T total arsenic analysis
S arsenic speciation analysis

1.6.3 Analytical methods for determining arsenic speciation

Hyphenated techniques that combine chromatographic separation methods such as high performance liquid chromatography (HPLC) and gas chromatography (GC) with highly sensitive detection system such as ICPMS have been proven to be particularly useful in providing the selectivity and sensitivity that are required for the determination of arsenic species at trace levels in environmental and biological samples [2, 159]. Common hyphenated techniques may use AAS, AFS, and ICPMS that were previously described in section 1.6.2 as detection methods. Although ICPMS is the most expensive detection system of the three, it offers undisputable advantages: multi-element capabilities, lower detection limit, and wider linear range [159]. Consequently, there have been many methods developed based on the hyphenated techniques of chromatographic separation combined with element-specific spectrometric detection for the determination of trace concentrations of arsenic species [160, 161, 97]. Also, additional sensitivity may be achieved by using a chemical derivatization technique such as hydride generation that is capable of enhancing sensitivity for a limited range of arsenic compounds [162–167].

Atomic Absorption Spectrometry (AAS)-derived hyphenated techniques may involve modification of the GF-AAS techniques, such as the hydride-generation quartz furnace atomic absorption spectrometry **(HG-QF-AAS)** and another modification method of the HG-AAS technique of the hydride-generation cold trap atomic absorption spectrometry **(HG-cold trap-AAS)**. A quartz furnace replaced the graphite furnace in the HG-QF-AAS method which is

capable of performing both total arsenic and arsenic speciation analysis (Table 1.4) [159]. This method offers detection limits as low as 3 ng/L for As^{III} and As^{V}, and 15 mg/L for DMA^{V} and MMA^{V}. Modifications have been made to the HG-AAS technique to quantitatively determine arsenic species in different environmental and biological matrices [74, 95, 112, 168–173]. The HG-cold-trap-AAS takes advantage of the trapping of volatile arsine at -196℃ (liquid nitrogen temperature), then elutes at room temperature, which enables urinary arsenic speciation with the detection limit in the sub-µg/L range for As^{III}, As^{V}, MMA^{V}, and DMA^{V} [169]. In addition, this technique allowed MMA^{III} and DMA^{III} to be differentiated from their pentavalent counterparts for the first time in natural waters [112]. Moreover, a modified system using a combination of on-line microwave digestion and HPLC was capable of separating arsenobetaine, MMA^{V}, DMA^{V}, As^{III}, and As^{V} with detection limits of 1.0–4.7 µg/L [174, 175].

Atomic fluorescence spectrometry (AFS)-derived hyphenated techniques have been used to detect arsenic hydrides in the ultraviolet region of the spectra. This technique achieves low detection limit (0.05 µg/L) through the use of an intense light source and cold vapour or hydride generation system [132, 134–136]. This technique has been modified with hydride-generation (HG) and high-performance liquid chromatography (HPLC) to achieve detection limits in the range of 0.1–0.8 µg/L for As^{III}, As^{V}, MMA^{V}, and DMA^{V} in water and urine samples [160, 176, 177]. Gas chromatography (GC) coupled to AFS has also been used to detect trace levels of arsenic species with detection limits of 0.5 µg/L in environmental samples [178]. Also, solid-phase extraction cartridge-HG-AFS

offers a detection limit of 0.05 µg/L, which has been used in routine operation for arsenic speciation analysis in drinking water to ensure it fell within the safe drinking water guideline for arsenic [179]. Moreover, the unstable trivalent species of MMA^{III} and DMA^{III} can be identified in urine by using the HG-AFS technique [180].

Inductively coupled plasma mass spectrometry (ICPMS)-derived hyphenated techniques have been widely used in arsenic speciation analysis for environmental and biological samples. A plethora of methods have been developed based on the highly selective separation of high-performance liquid chromatography (HPLC) and the highly sensitive detection using ICPMS [181–188, 118, 160, 165]. Different modes of HPLC separation may be used, including ion pairing [165, 189–191, 137], anion exchange [165, 192, 193, 71], and cation exchange [165, 194, 141].

The HPLC-ICPMS technique (Fig. 1.3) relies on the introduction of an aqueous sample through a nebulizer, which delivers a fine mist of analyte aerosols into a high temperature plasma (~8,000 K). This plasma transfers energy to the analyte which then undergoes the desolvation, atomization, and finally ionization process. The ions are then extracted from the plasma by a series of differentially pumped vacuum interfaces into the ion optic and subsequently entered into the dynamic reaction cell where oxygen (O_2) is introduced to generate arsenic oxide (AsO^+) to avoid the isobaric interference of $^{40}Ar^{35}Cl$ which has the same atomic mass as ^{75}As. The analyte ions will be separated based on the mass-to-charge ratio in the Quadrupole mass spectrometer (Fig. 1.3). The analyte ions transmitted

through the Quadrupole mass spectrometer will arrive at a continuous electron multiplier detector. The mass spectrometer can be operated in two modes: full scan and selective ion monitoring. In the full scan mode, the detector sweeps a wide range of masses, while in the selective ion monitoring mode, only a limited set of masses that are characteristic to the analyte of interest is targeted by the detector. Therefore, in the selective ion monitoring mode, the detector spends more time to monitor a few selections of masses and thereby enhancing the sensitivity of the targeted analyte.

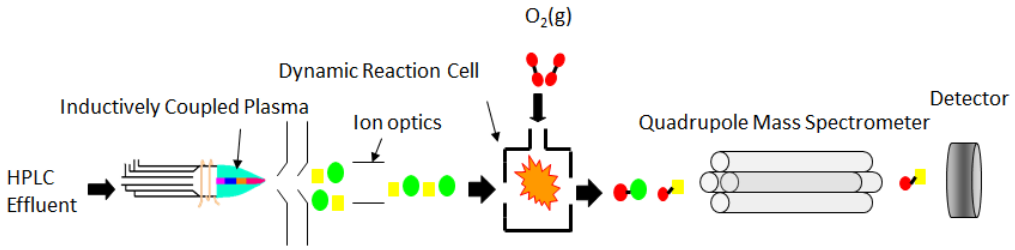


Fig. 1.3 Schematic of separation using high performance liquid chromatography followed by detection with inductively coupled plasma mass spectrometry (HPLC-ICPMS).

The effluent from high-performance liquid chromatography (HPLC) can be introduced to a hydride-generation (HG) system where nitric acid (HNO_3) and sodium borohydride ($NaBH_4$) are being continuously pumped into a mixing chamber. Since there are only certain arsenic compounds that are able to generate arsine (AsH_3), this post-column derivatization technique improves selectivity by removing non-hydride forming arsenicals, such as arsenobetaine from the other hydride forming arsenic compounds such as As^{III}, As^{V}, MMA^{V}, and DMA^{V}. In addition, this technique also enhances sensitivity due to the higher analyte

transport efficiency involved in the introduction of gaseous AsH_3 as compared to the introduction of aerosols from liquid [137, 141, 132, 195, 170, 165]. The effluent from the mixing chamber subsequently enters a liquid gas separator (Fig. 1.4), in which the non-hydride forming arsenicals stay in the liquid phase and elute out to waste, while the gaseous AsH_3 will proceed to the nebulizer, then spray chamber, and finally be detected by ICPMS (Fig. 1.5)

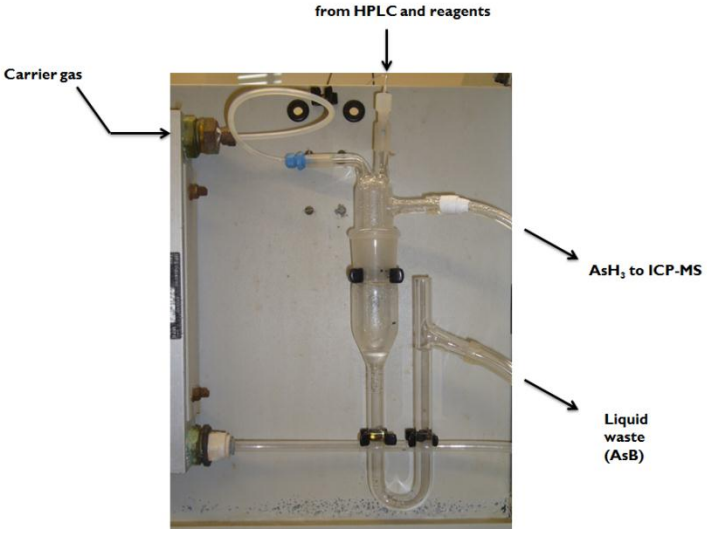


Fig. 1.4 A liquid gas separator that is used in a high-performance liquid chromatography-hydride generation-inductively coupled plasma mass spectrometry (HPLC-HG-ICPMS) system.

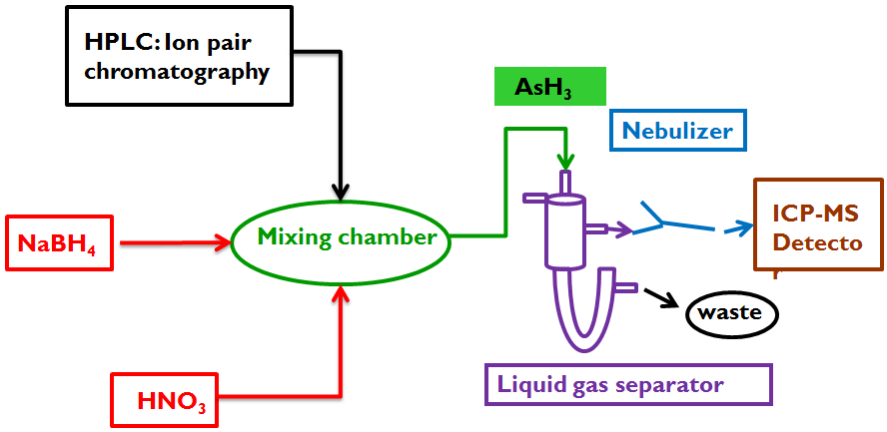
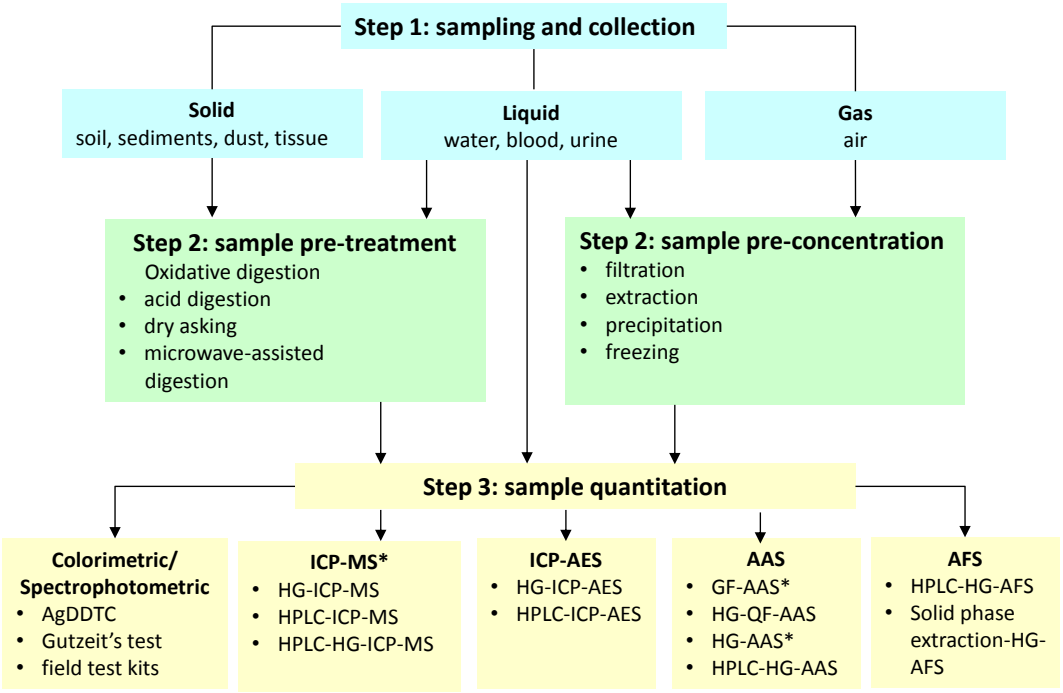


Fig. 1.5 The instrumental set up of a high-performance liquid chromatography-hydride generation-inductively coupled plasma mass spectrometry (HPLC-HG-ICPMS) system.

Depending on the sample types and the specific needs required for a specific analysis, one must choose the method of analysis (total analysis methods or speciation analysis methods) carefully and take appropriate measures to preserve the sample and to prevent contamination during each step of the sample analysis (Fig. 1.6). Although a technique such as HPLC-ICPMS offers excellent sensitivity and specificity, especially when it is coupled with hydride-generation; however, arsenic specie identification relies solely on the comparison between the HPLC retention time of the standards and that of the sample. Therefore, in situations when standards are not available, other structural elucidation techniques, such as electrospray ionization mass spectrometry (ESI-MS) are needed to identify the unknown arsenic species by obtaining additional structural information to provide a more accurate identification of the arsenic species [196–200].



*US EPA-approved analytical method for arsenic analysis in biological and environmental sample

Fig. 1.6 Different analytical procedures for arsenic analysis in various environmental and biological samples.

1.7 Rationale and scope of thesis

Since the data were limited on the health outcomes associated with low level of arsenic exposure, there is a need to develop stringent analytical approaches to measure the magnitude of exposure. Urinary arsenic species can serve as exposure markers to assess the amount of arsenics that have been ingested. Upon ingestion, inorganic arsenic can be eliminated rapidly from the body through urinary excretion as As^{III}, As^{V}, and the methylated metabolites in both trivalent and pentavalent forms [93]. Therefore, urinary arsenic concentrations can reflect the level of arsenic exposure.

The first chapter of my thesis tackles the challenging problem of accurately determine trace concentrations of arsenic in human urines, which were collected from a general population in Quebec, Canada, which was not exposed to elevated level of arsenic in drinking water. Another critical aspect of this chapter was to establish an exposure-effect relationship between the urinary arsenic exposure markers and other biomarker in a pilot epidemiological study. Our collaborator Dr. Patrick Levallois at Public Health Institute of Quebec collected information of the oxidative damage cell in urinary bladder from the same group of the population that provided the urine samples. We aimed to gain a better understanding of the relationship between the low level of arsenic exposure and the associated potential health effects.

This chaper focused on developing a set of separation techniques involving ion pair, cation exchange, and anion exchange chromatography that work together in a complementary fashion to selectively determine the five major arsenic species predominately found in human urine, As^{III}, As^{V}, MMA^{V}, DMA^{V}, and AsB. In addition, a post-column hydride generation technique was used to enhance specificity by discriminating AsB from the other hydride-forming arsenicals, which have more toxicological relevance than that of AsB. Therefore, the combination of high performance separation technique and post-column hydride generation derivatization, hyphenated to the highly sensitive detection system (HPLC-HG-ICPMS) provided a powerful analytical tool to determine the concentrations of major arsenic species present in human urine.

Human populations are more prone to exposure of inorganic arsenic through oral ingestion of drinking-water and less likely through dietary intake of foods [201].There are some areas in southern Alberta that may have elevated levels of inorganic arsenic in groundwater due to high density of confined feeding operations (CFOs) that utilize livestock manure as soil fertilizer. The study area of the second chapter in my thesis focussed on the Battersea Drain watershed study area, which is located 25 km north of Lethbridge in southern Alberta. This land is mostly for agricultural production (86%) to plant barley and other cereals [202]. Despite the advantage of using livestock manure as soil amendment to promote crop growth; there are potential environmental impacts on the water quality as a direct consequence of this recycling effort [203, 204].

Groundwater may be contaminated when heavy precipitation falls on the manure and leach nutrients and bacteria from the manure into the runoff. Also, over-application of the manure, in which manure application rate is higher than the crop's nutrients requirement may also have an environmental impact on the water quality [205, 206]. In addition, the use of septic systems to treat human waste in the Battersea Drain watershed, may introduce additional source of contamination to water [205]. Therefore, the combination of livestock manure and human waste in areas of high density CFOs, such as the Battersea Drain watershed, may have potential environmental impact on the water quality in both surface water and groundwater.

There has been limited research on how the groundwater and surface water quality change with respect to different locations that have varying degree of

CFOs, as well as different time of the year during the sample collection. The emphasis of my second chapter in the thesis was to investigate the total arsenic levels and arsenic species in groundwater and surface water in the Battersea Drain watershed, with respect to geographical and seasonal variation. This is a collaborative effort with two other research groups, Dr. Gary Kachanoski from the Department of Renewable Resources at University of Alberta, and Dr. Norm Neumann from Alberta Provincial Laboratory for Public Health.

The overall water quality assessment would encompass a wide array of parameters: total arsenic concentrations, arsenic speciation, water pH, electric conductivity, major inorganic ions, nutrients, bacteria, and parasites. These diverse components in the water quality analyses would illustrate a more complete picture of how groundwater and surface water quality were influenced by seasonal and geographical factors. Moreover, this collaborative study would determine if the water quality in an area that is predominately irrigated farmland with high density of CFOs, meet the safe drinking water guidelines.

Other than the inorganic arsenic species that are commonly found in drinking water, there are other more complicated forms of arsenic that may exist in nature through various biomethylation processes. For instance, microorganisms (e.g. phytoplankton and bacteria) can uptake As^{V} from their surrounding waters and reducing it to As^{III}, with subsequent methylation to form DMA^{V} and MMA^{V}, or even the more complex organic arsenicals, such as arsenosguars and AsB [207, 40, 41]. The focal point of my third chapter is on yet another natural arsenical, Arsenicin A, ($As_4O_3(CH_2)_3$) that has shown bactericidal and fungicidal properties,

which is isolated from a New Caledonian marine sponge *Echinochalina bargibanti* [208, 209].

Previous studies focused on the structural elucidation aspect of Arsenicin A [209–212], while there was a deficiency in studying the chromatographic separation perspective, which would differentiate Arsenicin A from the other arsenicals that may also be present in the marine sponges. My objectives for this chapter were two folds. I would like to first develop a complementary analytical technique that was capable of differentiating 7 arsenic species including Arsenicin A model compound (AA), arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA^{V}), and dimethylarsinic acid (DMA^{V}), arsenobetane (AsB), and arsenosugar. The second objective was to apply the analytical techniques that I developed using high performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC-ICPMS) to the marine sponges that were collected from Papua New Guinea by our collaborator Dr. Raymond Andersen from the Chemistry Department at University of British Columbia.

The crystal structure of a synthesized Arsenicin A confirms an adamantine-type structure that is analogous to arsenic trioxide (As_2O_3), except three methylene groups were replaced by the oxygen atoms [212]. As_2O_3 (Trisenox®), which solubilizes to As^{III} in aqueous solution, is currently used to treat Acute Promyelocytic Leukemia (APL) [213]. As_2O_3 has shown significant efficacy in treating newly diagnosed and relapsed patients with APL; the complete remission rate are 70 to 90 % and 65 to > 90 % respectively [214, 213]. It would be advantageous to find other arsenicals that are more toxic than As^{III}, such that

lower dosages of the arsenicals could be used in the therapy and to potentially reduce possible drug antagonism. Since it is difficult to isolate a large quantity of AA from the marine sponges, an Arsenicin A model compound (AA) has been synthesized for us by our collaborator Dr. William Cullen from the Department of Chemistry at University of British Columbia to continue further investigation to gain more insight on this new arsenic compound.

Our recent *in vitro* toxicological study using the human lung carcinoma A549 cell line has shown that an Arsenicin A model compound (AA) is more toxic than inorganic arsenite (As^{III}) to mammalian cells. Our group has determined that the IC_{50} values for A549 cells with a 24 h incubation period are 3.5 μM for AA and 76.6 μM for As^{III}. It is not known how AA exerts the higher toxicity and whether the cellular uptake of these compounds could contribute to the differences in toxicity. Therefore, my objective for the final chapter was to compare the concentration and speciation of arsenic in A549 cells after 24 h incubation with either As^{III} or AA at fractional concentrations below their respective IC_{50} values. This study will contribute to a better understanding of the roles of cellular uptake and metabolism of arsenic species in their relative toxicity to the cells.

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CHAPTER 2 Complementary chromatography separation combined with hydride generation – inductively coupled plasma mass spectrometry (HPLC-HG-ICPMS) for arsenic speciation in human urine

2.1 Introduction

Human exposure to high concentration of arsenic in drinking water is a global health concern, e.g., Bangladesh has an estimated 46 – 57 million people drinking water containing elevated arsenic concentration, exceeding the guideline value of 10 µg/L recommended by the World Health Organization [1-2]. Chronic arsenic exposure causes serious health effects, including cancer of the skin, lungs, urinary bladder, and kidney. Some studies have also reported other health implications, such as hyperkeratosis, skin lesions, cardiovascular dysfunctions, diabetes, and reproductive effects [1-3]. Many studies have focused on the exposure of high arsenic concentration in drinking water, yet the health effects due to the exposure to lower concentration of arsenic is still unclear [4-5]. Not only is it analytically challenging to accurately determine trace concentrations of individual arsenic species in such a complex sample matrix as human urine, but reliable data of health effects are rarely available [1, 3]. Health effects of arsenic could be different between individuals; some suggest the differences in genes encoding for enzymes for arsenic metabolism [6-7]. Therefore, it is necessary to develop stringent analytical approaches to enhance the determination of arsenic

metabolties at trace concentrations relevant to environmental exposure to low concentrations of arsenic.

A critical aspect in establishing an exposure-effect relationship is to accurately measure the magnitude of exposure in which urinary arsenic species serve as useful biomarkers to assess the amount of arsenic ingested [4-5]. The most common arsenic species found in human urine are dimethylarsinic acid (DMA^V), monomethylarsonic acid (MMA^V), arsenite (As^{III}), and arsenate (As^V) [6-12]. The toxicity of arsenic varies with different chemical forms, e.g., arsenobetaine (AsB) is essentially a non-toxic form of arsenic [11-18]. If an individual consumes seafood, e.g., crab and lobster, within three days prior to the collection of urine sample, then AsB can be the dominating arsenic species in urine [10, 13]. This presence of AsB in urine along with As^{III}, As^V, MMA^V, and DMA^V renders the measurement of total urinary arsenic not suitable for assessing human exposure to toxic forms of arsenic. Hence, it is critical to separate AsB from the other arsenicals in a speciation analysis to achieve more accurate determination of the individual concentrations of inorganic arsenic and methylated metabolites. In arsenic speciation analysis, many chromatographic separation techniques have been developed, e.g., ion pairing [19-20], cation exchange [21-22], and anion exchange chromatography [8, 21-22], along with various detection methods, e.g., atomic absorption [10, 23-26], atomic emission [20, 25, 27-28], atomic fluorescence [9, 24-25, 29-30], and ICPMS [6, 22, 24, 31]. In particular, the HPLC-ICPMS combination has been used extensively to

perform arsenic speciation analysis because of its wide dynamic range, high selectivity, and low detection limit [22, 24].

This study focuses on the challenging problem of accurately determining trace concentration of arsenic in human urine collected from a general population that is not exposed to elevated level of arsenic (≈ 10 μg/L). We have developed a set of separation techniques involving ion pair, cation exchange, and anion exchange chromatography that work together in a complementary fashion to selectively determine five major arsenic species predominately found in human urine, As^{III}, As^{V}, MMA^{V}, DMA^{V}, and AsB. In this chapter, HPLC is used to achieve differentiation of arsenic species. The effluent from HPLC is then introduced to a hydride generation system where nitric acid (HNO_3) and sodium borohydride ($NaBH_4$) are being continuously pumped into the system. Subsequently, the arsine (AsH_3) generated is detected by the highly sensitive ICPMS. The purpose of hydride generation is to enhance specificity by discriminating AsB from other hydride-forming arsenicals, such that AsB will not interfere with the quantification of other more toxicologically important arsenic species [20, 23, 25]. AsB, unlike inorganic arsenic and its methylated metabolites, does not react with $NaBH_4$ to form a volatile arsine. Furthermore, the use of hydride generation enhances sensitivity because of the higher analyte transport efficiency involved in the introduction of gaseous arsines as compared to the introduction of aerosols from liquid [20, 22, 25]. In addition, we use cysteine as a pre-reduction reagent to pre-reduce pentavalent arsenicals to the trivalent forms. The lower oxidation state of arsenic enables a faster reaction with $NaBH_4$ to

generate arsine [20, 23, 25]. This chapter reports on a set of complementary chromatographic separation techniques along with a post-column hydride generation derivatization step, followed by a highly sensitive ICPMS detection to quantitatively determine the concentrations of major arsenic species in human urine in the sub-microgram per liter range.

2.2 Experimental

2.2.1 Ion pair chromatography

Separation was achieved with a reversed-phase ODS-3 column (150 x 4.60 mm, 3 μm particle size, Phenomenex) and an ODS guard column (4 mm x 3 mm, Phenomenex). The mobile phase contained 3 mM malonic acid (Fisher Scientific), 5 mM tetrabutylammonium hydroxide (Aldrich), and 5% methanol (Fisher Scientific). 10% HNO_3 (Fisher Scientific) was used to adjust the pH to 5.65 (Table 2.1). The mobile phase was filtered through a 0.45 μm membrane and sonicated for 10 minutes before use in HPLC separation. Urine samples were passed through a filter of 0.45 μm pore size, 13 mm diameter (Whatman^{TM}) before being injected into the HPLC. Each sample was analyzed in triplicate. The calibration standards were a mixture of As^{III}, DMA^{V}, MMA^{V}, and As^{V}.

2.2.2 Cation exchange chromatography

Separation was carried out on a PRP-X200 cation exchange column (250 x 4.10 mm, 10 μm particle size, Hamilton) and a guard column (10 μm particle size, 20 mm length, 2.0 mm i.d., Hamilton). The mobile phase contained 2.47 mM pyridine (Aldrich) and 5% methanol (Fisher Scientific). 50% formic acid (Fluka)

was used to adjust the pH to 2.23 (Table 2.1). The mobile phase and urine samples were filtered as described above. Each sample was analyzed in triplicate. The calibration standards were AsB, As^{III}, DMA^{V}, MMA^{V}, and As^{V}.

2.2.3 Anion exchange chromatography

Separation was performed on a PRP-X100 anion exchange column (150 x 4.10 mm, 5 μm particle size, Hamilton) and guard column (10 μm particle size, 20 mm length, 2.0 mm i.d., Hamilton). The mobile phase contained 35 mM ammonium bicarbonate (Sigma) and 5% methanol (Fisher Scientific). 15% ammonium hydroxide (Fisher Scientific) was used to adjust the pH to 8.5 (Table 2.1). The mobile phase and urine samples were filtered as described above. Each urine sample was analyzed in duplicate. The calibration standards were AsB, As^{III}, DMA^{V}, MMA^{V}, and As^{V}.

Table 2.1 HPLC parameters for ion pair, cation exchange, and anion exchange chromatography.

HPLC parameters	Ion pair chromatography	Cation exchange chromatography	Anion exchange chromatography
Column	reversed-phase ODS-3 column: 150 x 4.60 mm, 3 μm particle size	PRP-X200 cation exchange column: 250 x 4.10 mm, 10 μm particle size	PRP-X100 anion exchange column: 150 x 4.10 mm, 5 μm particle size
Column temperature	50^0C	ambient	Ambient
Mobile phase	3 mM malonic acid, 5 mM tetrabutylammonium hydroxide, 5% methanol, pH 5.65 adjusted by 10% HNO_3	2.47 mM pyridine, 5% methanol, pH 2.23 adjusted by 50% formic acid	35 mM ammonium bicarbonate, 5% methanol, pH 8.5 adjusted by 15% ammonium hydroxide
Flow rate	1.2 mL/min	1.0 mL/min	Step gradient: 0-4 min: 0.8 mL/min 4-8 min: 1.7 mL/min
Elution time	7 minutes	7 minutes	12 minutes
Injection volume	50 μL	50 μL	50 μL

2.2.4 Hydride generation

We designed a new sample introduction system, a modified Y-shape device, to replace the commercial nebulizer in order to introduce arsine more efficiently from the liquid-gas separator to the ICPMS detector (Fig. 2.1(a)). A post-column hydride generator was coupled between HPLC ion pair chromatography reversed-phase ODS-3 column and ICPMS detector (Fig. 2.1(b)). This HG system is equipped with a liquid-gas separator, which was fabricated in house, along with two peristaltic pumps to continuously transport HNO_3 and $NaBH_4$ into the system. The optimized condition for the formation of arsine were 0.6% HNO_3 (0.14 M), 1% L-cysteine (0.085 M), 0.8% $NaBH_4$ (0.21 M) in 0.025 M NaOH, reaction coil length 2.5 m, 0.25 mm i.d., water bath temperature 50^0C, argon carrier gas flow is 200 mL min^{-1}.

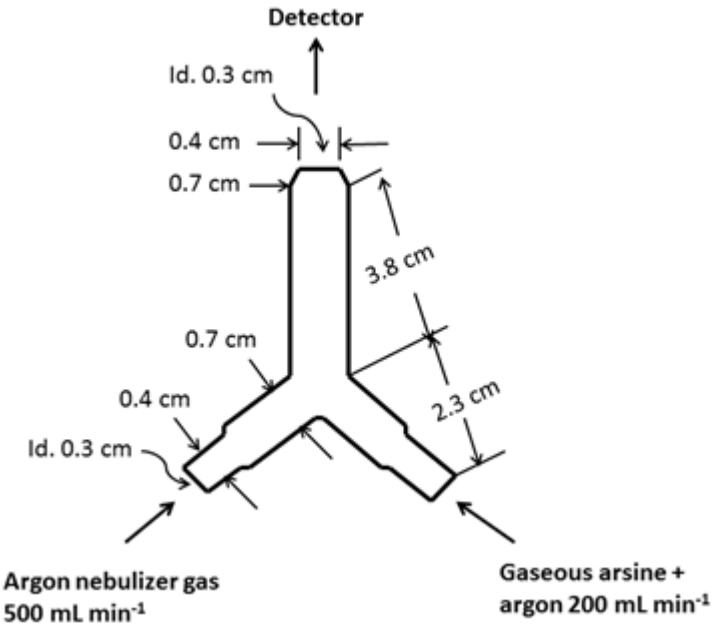

Fig. 2.1 The nebulizer tube and instrument set up for HPLC-HG-ICPMS.
(a) Design of modified glass tube replacing the commercial nebulizer tube. This apparatus improves the introduction of gaseous arsine from a hydride generator to ICPMS.

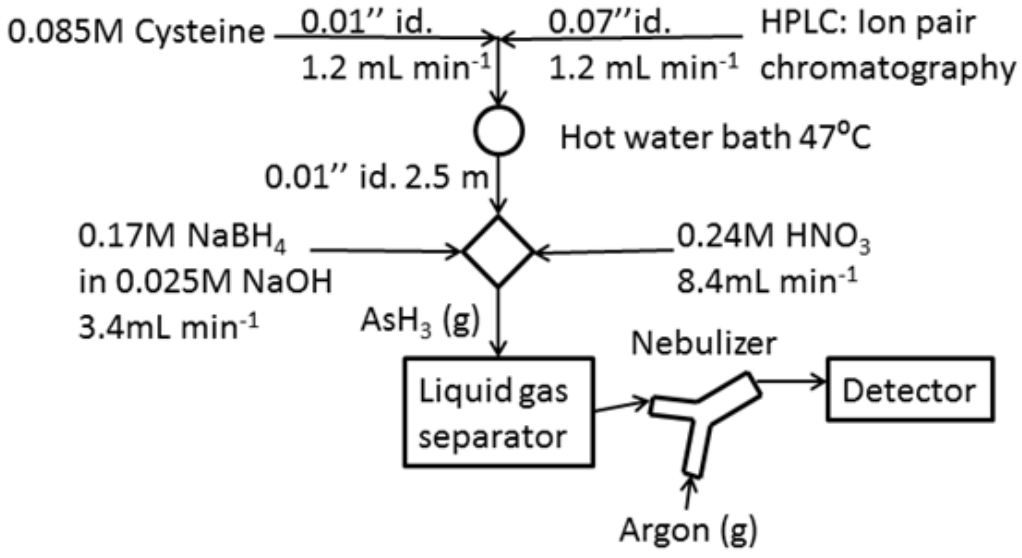

Fig. 2.1 (b) the set up of hydride generation coupled to HPLC and ICPMS.

2.2.5 Total arsenic concentration

The total arsenic concentration was determined in each urine sample to verify the accuracy of the arsenic speciation analysis. Urine samples were acidified with1% HNO_3 before analysis in triplicate for total arsenic. Standard reference material SRM1640 (Trace Elements in Natural Water) from National Institute of Standards and Technology (Gaithersburg, MD) with total arsenic concentration of 26.67 ± 0.41 µg L^{-1} was used to calibrate the instrument. There was a total of 110 human urine samples collected in Quebec, Canada in this pilot epidemiological study based on a general population that has not been exposed to elevated concentrations of arsenic in drinking water (~ 10 µg/L).

2.2.6 Detection and data analysis

An Elan 6000 DRC^{Plus} ICPMS system (PerkinElmer SCIEX) was operated in the dynamic reaction cell (DRC) mode with O_2 as the reaction gas. Peak hopping scan mode was used to monitor AsO^+ at 91 amu. Other optimized parameters are summarized in Table 2.2.

The peak area from chromatograms was used to construct calibration curves from which urinary arsenic species concentration were calculated. Graphic Edit software of Turbochrom Navigator from Perkin Elmer was used to integrate peak area. Concentrations of urinary arsenic species were calculated using Microsoft Office Excel. Chromatograms were plotted using IGOR (WaveMetrics).

Table 2.2 ICPMS operating conditions for arsenic speciation analysis in human urine samples.

ICP		**Mass spectrometer acquisition setting**	
Nebulizer gas flow	**0.5 L/min**	Sweeps/reading	**10**
Auxiliary gas flow	**1.5 L/min**	Readings/replicate	**325**
Plasma gas flow	**15 L/min**	Monitored signal	**AsO 90.9165 amu**
Lens voltage	**6.75 V**	Dwell time	**100 ms**
ICP RF power	**1350 W**	Scan mode	**Peak hopping**

2.3 Results and discussion

2.3.1 Ion pair chromatography

A typical chromatogram obtained from ion pair separation and ICPMS detection of a standard mixture containing five arsenic species in deionized water is shown in Fig. 2.2(a) and that of urine sample in Fig. 2.3(a). The retention time of DMA^V, MMA^V, and As^V were 2.3, 3.1, and 4.8 minutes respectively while As^{III} and AsB co-eluted at 1.5 minutes. This elution order was consistent with the expected retention behaviour based on the respective ionic interactions of each arsenic species with the stationary phase of the column. It had been reported that the pKa of the arsenic species were as follows: As^{III} pKa_1 9.23, pKa_2 12.13, pKa_3 13.4; DMA^V pKa 6.2; MMA^V pKa_1 4.1, pKa_2 8.7; As^V pKa_1 2.22, pKa_2 6.98, pKa_3 11.53, and AsB pKa 2.18 [32-35]. At the mobile phase pH of 5.65, As^{III} was neutral and AsB was a zwitterion. Hence, both As^{III} and AsB were not retained

and were quickly eluted at the void volume. On the other hand, DMA^V, MMA^V, and As^V, having increasing negative charges, exhibited increasing retention on the column as expected from more ionic interaction between the complexes and the stationary phase. The ion pair separation mode was suitable for the detection of DMA^V, MMA^V, and As^V as these three species were well resolved from each other, evident in Fig. 2.2(a). However, As^{III} was unable to differentiate from AsB; this issue occurred in urine sample that contained AsB, as shown in Fig. 2.3(a). To overcome this challenge of As^{III} and AsB co-elution, we explored the use of cation exchange chromatography to specifically focus on the quantification and the separation of AsB from As^{III}. A certified reference material, CRM No. 18 (National Institute for Environmental Studies, Japan Environment Agency), was used to verify the accuracy of this method [11, 36]. The measured value of DMA^V at 35.7 ± 3.5 µg L^{-1} was in good agreement with the certified value of 36 ± 9 µg L^{-1} from CRM No. 18.

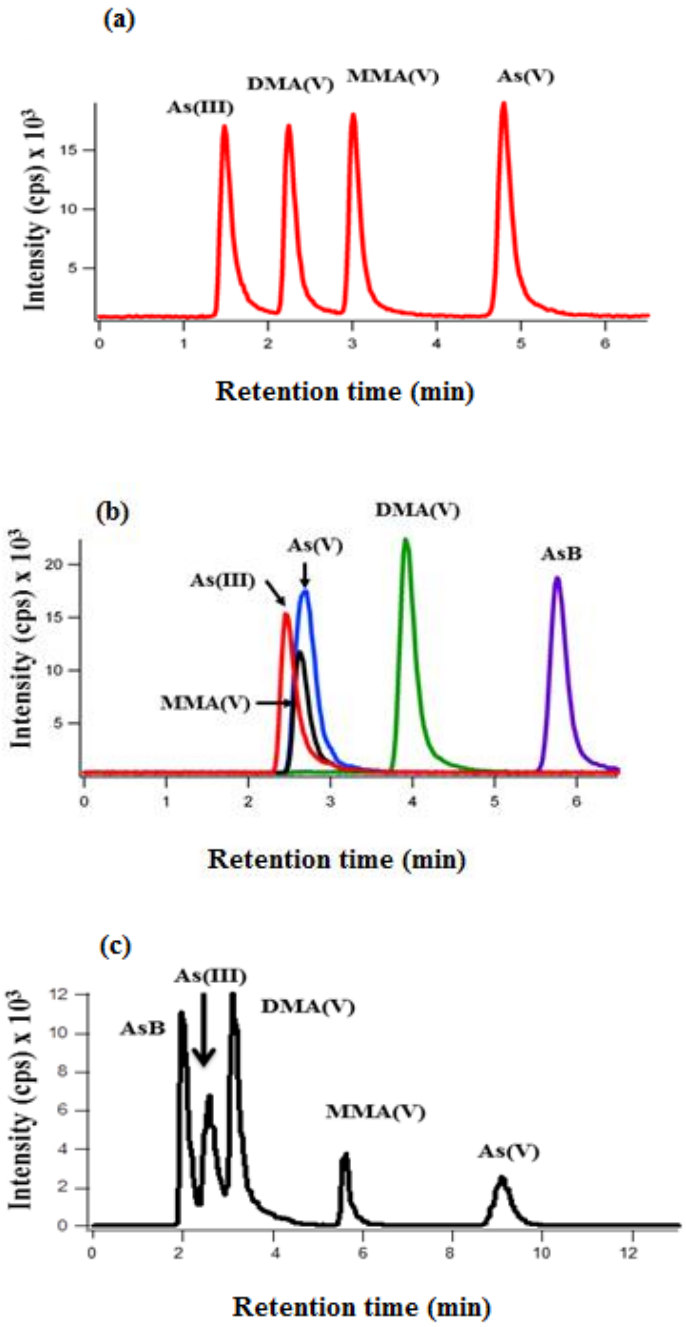

Fig. 2.2 Chromatograms showing HPLC separation and ICPMS detection of arsenic species. (a) Ion pair separation. A calibration standard containing 5 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, and As^{V} in deionized water; (b) Cation exchange separation. Overlay of five chromatograms from analyses of each individual arsenic standard in deionized water; (c) Anion exchange separation. A calibration standard containing 5 µg L^{-1} each of AsB, As^{III}, DMA^{V}, MMA^{V}, and As^{V} in deionized water.

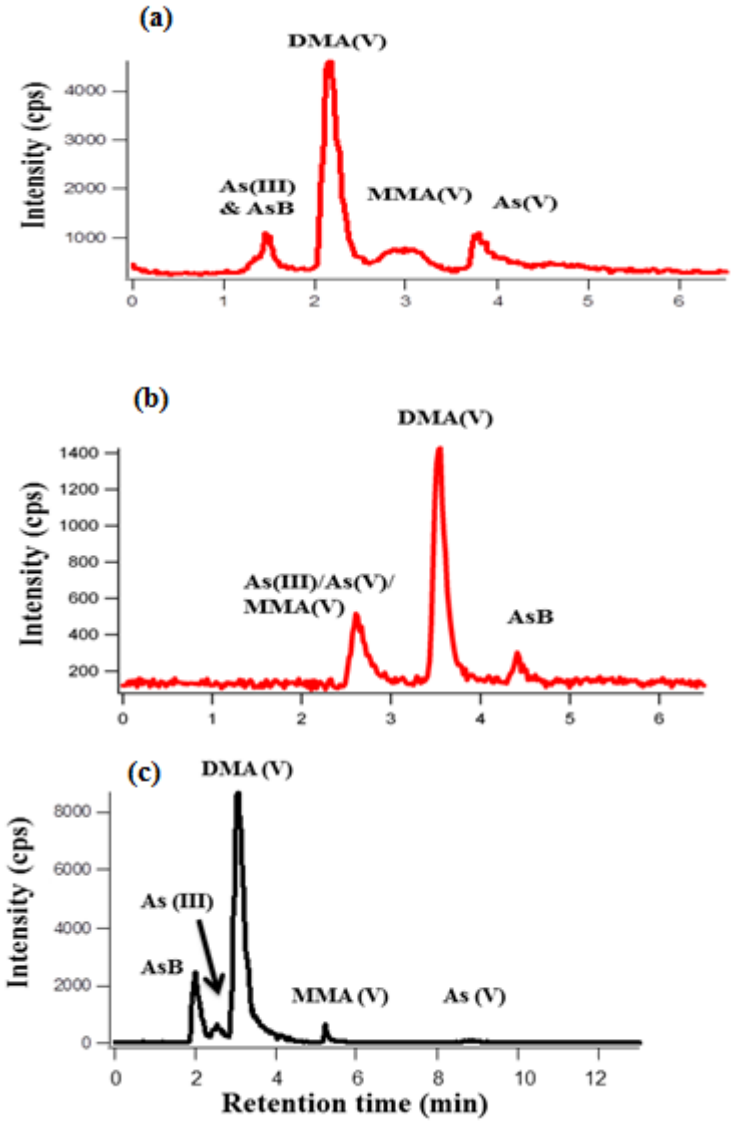

Fig. 2.3 Chromatograms showing separation and ICPMS detection of arsenic species using ion pair, cation exchange, and anion exchange separation. (a) Ion pair separation. A urine sample containing AsB, As^{III}, DMA^{V}, MMA^{V}, and As^{V} at a total arsenic concentration of 3.53 µg L^{-1}; (b) Cation exchange separation. A urine sample containing 0.50 µg L^{-1} of AsB; (c) Anion exchange separation. A urine sample containing AsB, As^{III}, DMA^{V}, MMA^{V}, and As^{V} at a total arsenic concentration of 24.05 µg L^{-1}.

2.3.2 Cation exchange chromatography

Fig. 2.2(b) displays a typical chromatogram obtained from cation exchange separation and ICPMS detection of five arsenic species in a standard mixture in deionized water, and in a urine sample (Fig. 2.3(b)). The retention time of DMA^V and AsB were 3.9 and 5.7 minutes respectively while As^{III}, MMA^V, and As^V nearly co-eluted at 2.4, 2.6, and 2.7 minutes. At the mobile phase pH of 2.23, AsB existed in both cation and zwitterion form in approximately equal ratio. The positive charge on AsB's zwitterion enabled stronger retention on the cation exchange column; hence, AsB eluted last. As^{III}, MMA^V, As^V, and DMA^V were either neutral or partially negatively charged; hence, they show minimum retention on the column and eluted earlier than AsB. This separation method successfully differentiated AsB from As^{III} and was sufficient for the quantification of AsB.

By performing two analyses using ion pair and cation exchange chromatography, we were able to separate and quantify AsB, DMA^V, MMA^V, and As^V. The concentration of As^{III} could be obtained by the difference of (As^{III} + AsB) from the ion pair chromatography analysis (Fig. 2.3(a)) and AsB from the cation exchange chromatography analysis (Fig. 2.3(b)). However, this approach was prone to error, especially when the concentration of As^{III} was much lower than that of AsB. In addition, these two separation modes were operated at different days, so the sensitivity of the instrument was likely not identical, thereby adding another variable to the accuracy of quantification. Therefore, we employed anion exchange chromatography to further separate and quantify As^{III}, as well as

the other four arsenic species in a single run. CRM No. 18 was used to verify the accuracy of this method. The measured value of AsB at 63.2 ± 3.6 μg L^{-1} was in good agreement with the certified value of 69 ± 12 μg L^{-1}.

2.3.3 Anion exchange chromatography

A typical chromatogram obtained from anion exchange separation and ICPMS detection of five arsenic species in a standard mixture in deionized water is shown in Fig. 2.2(c) and that in urine sample was shown in Fig. 2.3(c). The retention times of AsB, As^{III}, and DMA^{V} were 2.1, 2.6, and 3.3 minutes respectively, while MMA^{V} and As^{V} were 5.7, and 9.2 minutes. AsB, As^{III}, and DMA^{V} were not baseline resolved, which caused problems in accurately quantifying these species.

A cautionary note regarding anion exchange separation was the overlap between the peaks of AsB and As^{III} in urine samples that contained high concentrations of AsB. The quantification of trace levels of As^{III} eluting immediately after AsB was difficult. Urine samples containing much higher concentrations of AsB than As^{III} were commonly due to seafood consumption. Following the ingestion of seafood high in AsB, e.g., crab and lobster, the concentration of AsB excreted into urine could be orders of magnitude greater than that of As^{III}. The excess AsB would interfere with the accurate determination of As^{III}. To address this challenge, we introduced the effluent from HPLC into a post-column hydride generation system. This post-column derivatization technique aimed to remove AsB interference by discriminating AsB from hydride-forming arsenicals including As^{III}, DMA^{V}, MMA^{V}, and As^{V}. CRM No.

18 was used to verify accuracy and the measured value of AsB at 70.1 ± 4.4 µg L^{-1} was in good agreement with the certified value of 69 ± 12 µg L^{-1}. Standard reference material SRM1640 was also used to validate this method; the measured sum of the arsenic concentration at 26.65 ± 0.13 µg L^{-1} agreed with the total arsenic concentration of 26.67 ± 0.41 µg L^{-1} reported by National Institute of Standards and Technology (Gaithersburg, MD). Table 2.3 summarized the concentrations of individual arsenic species determined by complementary separation methods described in this chapter.

Table 2.3 Concentrations of arsenic species in selected urine samples determined by complementary separation methods.

Sample	AsB ([a])	As^{III} ([b])	As^{V} ([c])	MMA^{V} ([c])	DMA^{V} ([c])	Total
9	0.33 ± 0.03	0.18 ± 0.03	0.4 ± 0.2	0.6 ± 0.2	2.0 ± 0.2	3.5 ± 0.3
25	0.50 ± 0.01	0.10 ± 0.02	0.38 ± 0.04	0.55 ± 0.03	1.7 ± 0.1	3.3 ± 0.1
44	3.92 ± 0.24	1.38 ± 0.01	1.30 ± 0.05	2.6 ± 0.5	14.9 ± 0.6	24.1 ± 0.8
SRM 1640	n/a	n/a	n/a	n/a	n/a	26.65 ± 0.13

[a] determined by cation exchange chromatography
[b] determined by anion exchange chromatography
[c] determined by ion pair chromatography
n/a: not analyzed

2.3.4 Hydride generation

The hydride generation process converts As^{III}, MMA^{V}, DMA^{V}, and As^{V} to volatile arsines (AsH_3), but leaves AsB unchanged in solution. Hence, by separating the gaseous AsH_3 from liquid waste, the interference from AsB could be eliminated. To allow for efficient introduction of gaseous arsines to the ICPMS,

we modified the nebulizer tube by incorporating a Y-shape configuration and by removing the inner concentric tube (Fig. 2.1(a)). Removing the small inner concentric tube reduced the restriction of gas flow. When using HPLC-HG-ICPMS, it was necessary to reduce the nebulizer gas flow to 0.5 L min^{-1} from the original setting of 0.9 L min^{-1} during HPLC-ICPMS, in order to initiate and maintain a stable ICP.

Our experiments using HPLC-HG-ICPMS demonstrated the feasibility of speciating As^{III}, DMA^{V}, MMA^{V}, and As^{V}, without interference from AsB. Chromatograms were shown for a 0.1µg L^{-1} arsenic standard (Fig. 2.4(a)) and also for a urine sample (Fig. 2.4(b)) to compare with and without hydride generation. It was evident that the signal intensity increased by orders of magnitude when HG was used. Although the baseline of the chromatograms with hydride generation were higher, probably due to traces of arsenic present as an impurity in the reagents (cysteine, HNO_3, $NaBH_4$, NaOH) used for hydride generation, the overall signal-to-noise ratio remains higher with hydride generation. In addition, AsB interference was eliminated.

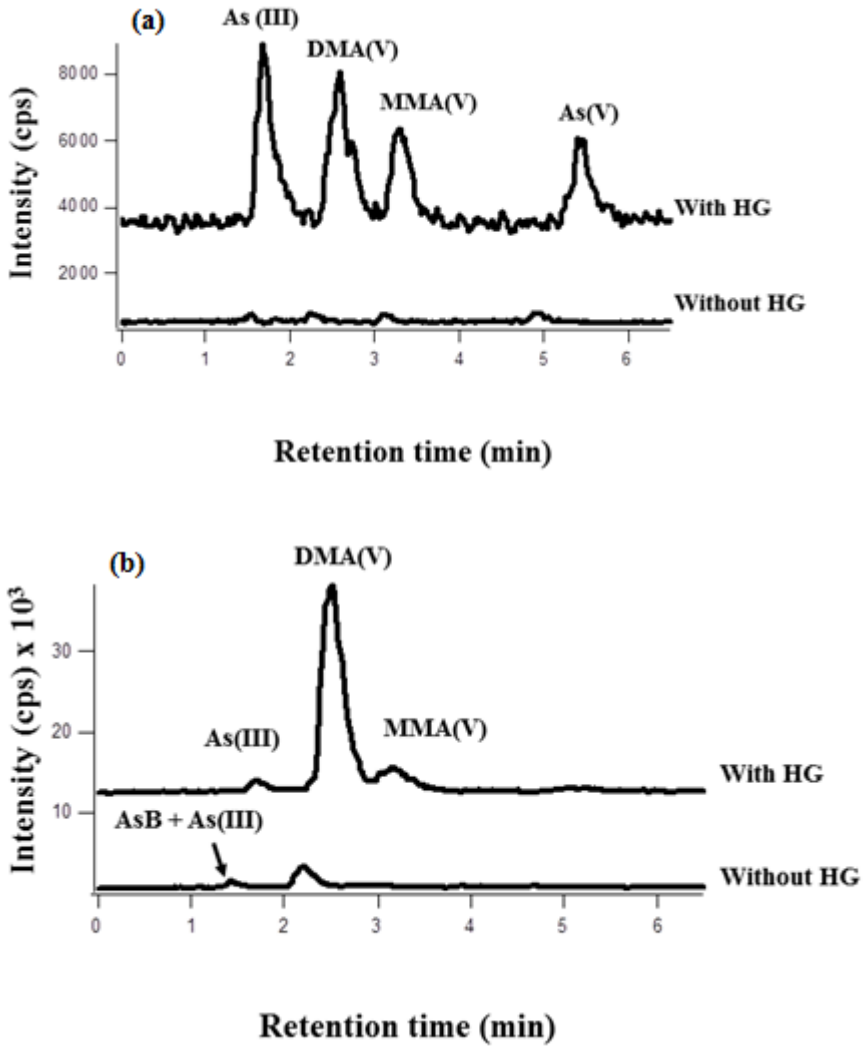

Fig. 2.4 Chromatograms obtained from ion pair separation and ICPMS detection of arsenic species, with and without hydride generation (HG). (a) Analysis of a standard solution containing 0.1 µg L^{-1} of each arsenic species: As^{III}, DMA^{V}, MMA^{V}, As^{V}, and AsB; (b) Analysis of a urine sample containing 0.50 µg L^{-1} of AsB; 0.10 µg L^{-1} of As^{III}; 1.72 µg L^{-1} of DMA^{V}; 0.55 µg L^{-1} of MMA^{V}, and 0.38 µg L^{-1} of As^{V}.

The formation of excess by-products, e.g., H_2, H_2O, and CO_2, may also destabilize ICP and thereby generating higher noise level. L-Cysteine was introduced to reduce the acid requirement without significant tradeoffs with the hydride generation efficiency. This would decrease the formation of by-products, promote ICP stability, and improve detection limits. The limits of detection, based on three times the standard deviation of background noise from repeated blank measurements, were 0.03 µg L^{-1} for As^{III}, 0.03 µg L^{-1} for DMA^{V}, 0.05 µg L^{-1} for MMA^{V}, and 0.04 µg L^{-1} for As^{V}. It is indicated in Table 2.4 that the incorporation of hydride generation into the HPLC-ICPMS system resulted in better detection limit for all four arsenic species due to the improved sample introduction efficiency and the enhanced signal intensity.

Table 2.4 Compare limit of detection (L.O.D.) of arsenic species between HPLC-ICPMS and HPLC-HG-ICPMS. L.O.D. is the concentration which produces a response of 3 x σ_{blank}.

	As^{III}	**DMA^{V}**	**MMA^{V}**	**As^{V}**
L.O.D. [a] (µg L^{-1})	**0.20**	**0.12**	**0.16**	**0.09**
L.O.D. [b] (µg L^{-1})	**0.03**	**0.03**	**0.05**	**0.04**

[a] obtained from HPLC-ICPMS
[b] obtained from HPLC-HG-ICPMS

The concentrations of urinary arsenic species found in the 110 human urine samples were within the single digit microgram per liter range (Table 2.5). These results reflect the lower level of arsenic exposure in drinking water from a

general population in Quebec, Canada. The urinary arsenic distribution after the removal of the AsB interference consisted of 21% As^{III}, 7% As^{V}, 16% MMA^{V}, and 56% DMA^{V} (Fig. 2.5). These data were consistent with the other reported values of arsenic in human urine with distribution typically in the range of 10–30% inorganic arsenic (As^{III} and As^{V}), 10–20% MMA^{V}, and 60–70% DMA^{V} [1-6]. A positive correlation was observed between the levels of arsenic exposure in water and the total arsenic concentrations found in the human urine samples (Fig. 2.6). The concentratinos of arsenic detected in human urine are generally higher than that in the drinking water, which suggested there may be other potential sources of arsenic that may introduce arsenic to human body. Consumptions of food and drinking water, including beverages made in drinking wagter, are the most common pathways of human exposure to arsenic, except for occupational arsenic exposure, e.g., working in coal-fired power plants, glass or electronic manufacturing [1-2]. Studies have shown that food may contribute to < 250 µg/L arsenic, but this is highly dependent on the type of food in the diet, e.g, dairy, meat, or seafood [1-2]. Therefore, total arsenic analysis in the human urine alone is not sufficient to assess the impact of arsenic exposure on human health outcomes. The determination of the concentrations of individual arsenic species that were present in human urine may provide a more complete analysis that is more relevant to assess the environmental exposure of arsenic.

Table 2.5 Urinary arsenic speciation concentrations from 110 human urine samples collected from a general population in Quebec, Canada, (% of sum = % of total arsenic concentration excluded AsB).

	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}
Range (µg/L)	**0.12 – 159**	**0.05 – 76**	**1.02 – 65**	**0.34 – 32**	**0.2 – 7.4**
Median (µg/L)	**1.3**	**0.7**	**4.0**	**1.0**	**0.7**
% of total	**36**	**n/a**	**n/a**	**n/a**	**n/a**
% of sum	**n/a**	**21**	**56**	**16**	**7**

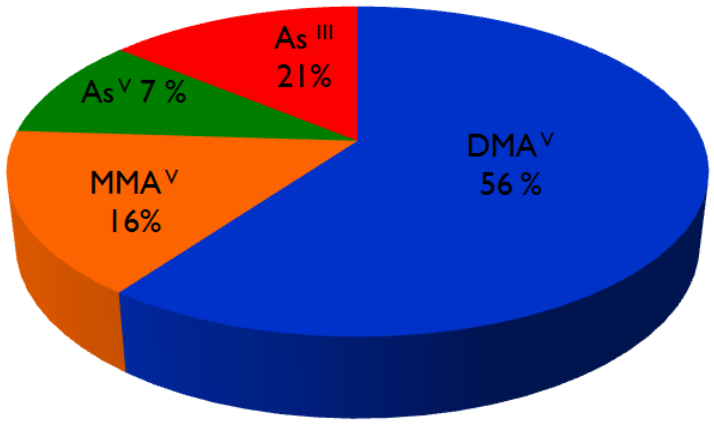

Fig. 2.5 Urinary arsenic speciation distribution (exclude AsB) of 110 human urine samples collected from a general population in Quebec, Canada.

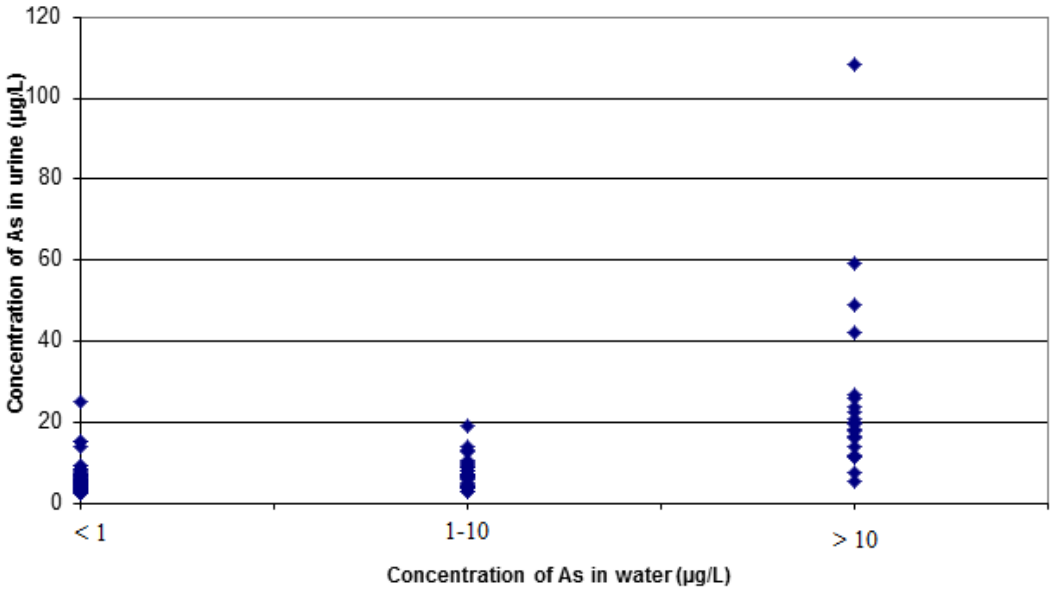

Fig. 2.6 The levels of the total arsenic concentration found in the 110 human urine samples with respect to the levels of arsenic in water.

2.4 Conclusion

To determine the concentrations of As^{III}, DMA^{V}, MMA^{V}, As^{V}, and AsB present in trace levels in the complex matrix of human urine, a set of complementary separation methods was developed. These five arsenic species were subjected to speciation analysis by using three different modes of separation, ion pair, cation exchange, and anion exchange chromatography. Post-column hydride generation derivatization technique was used to remove AsB interference in urine samples that contained elevated concentration of AsB. This developed method combined the advantage of high specificity from the chromatographic separations using HPLC, and the high sensitivity from the detection using ICPMS; a sub-microgram per liter level of detection limit was achieved in human urine. Two analyses of the same sample, one by ion pair chromatography and the other by cation exchange, were able to achieve speciation of the five target arsenic

species, AsB, As^{III}, DMA^{V}, MMA^{V}, and As^{V}. In this case, the concentration of As^{III} was obtained by the difference between the total of AsB and As^{III} from the first analysis and the net concentration of AsB from the second analysis. Strong anion exchange chromatography was able to resolve all five arsenic species, with the sacrifice of a longer elution time. Although anion exchange chromatography is able to achieve speciation of the five arsenic compounds, potential quantitation errors could occur when urine samples contain high concentrations of arsenobetaine which elutes immediately before As^{III}. Hydride generation is useful to differentiate As^{III} from AsB, removing the interference of AsB on the determination of As^{III}. Linking hydride generation between HPLC and ICPMS further improves the sensitivity of the hydride-forming arsenic species. The complementary approach provides options for arsenic speciation analysis for different applications and samples types.

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CHAPTER 3 Battersea Drainage watershed groundwater and drain water quality assessment – arsenic concentrations with temporal and spatial variations

3.1 Introduction

The Battersea Drain watershed (73 km^2) is located 25 km north of Lethbridge in southern Alberta, Canada. This study area is of particular research interest because of its high intensity of confined feeding operations (CFOs), which utilize livestock manure as soil fertilizer. Since the cattle population in Alberta has grown by more than 50% for the past 37 years to 5.51 million in 2010, the amount of manure that may be available for soil amendment also significantly increased [2, 3].The goal of livestock manure application to farmland is to maximize the recycling of nutrients in the manure back to the soil, thereby enhancing crop growth [4–6]. The Battersea Drain watershed is dominated by agricultural production (86%) with planted barley, cereal grains, and irrigated forages [7, 1].

Despite the advantages of livestock waste application, there are potential environmental impacts on the water quality as a direct consequence of this recycling effort [8–11]. Groundwater may be polluted when heavy precipitation falls on the manure and leach nutrients and bacteria from the manure into the runoff. In addition, over-application of the manure may also cause a great impact on water quality, where manure application exceeds the crop's nutrient requirements [12, 4, 13–15]. Moreover, the use of septic systems to treat human

waste in rural areas, such as the Battersea Drain watershed, may also introduce additional source of water contamination. The adsorption field effluent from the septic system may contain bacteria, nitrate, and phosphate, which may pollute groundwater when the water tables are high or when the soil is too saturated to purify wastewater [4]. All the farms on the Battersea Drain watershed have septic systems for waste water treatment [1]. The combination of livestock manure and human waste in areas with a high density of CFOs, such as the Battersea Drain watershed, may have potential environmental impact on the water quality in both drain water and groundwater [7, 4, 12].

Another characteristic that makes the Battersea Drain watershed an interesting study area is that it relies heavily on irrigation as its main water source, due to the low precipitation in this area. In 2010, Alberta accounted for 59% of the greater than 838 million m^3 of water used for agricultural irrigation in Canada [16]. Alberta irrigation farmlands are 356,500 hectares, which make up 67% of the total irrigated land in Canada [16]. About one third of the farms (37%) within the Battersea Drain watershed operate CFOs, with an average of 1430 heads for each CFO [1]. Therefore, it is increasingly important to have a better understanding of how the water quality changes with respect to various sample collection locations (spatial variation) and sampling time (temporal variation) to ensure safe drinking water in areas with high density CFOs. The objective of this chapter is to determine the total arsenic concentrations and arsenic speciation in the groundwater and drain water samples in the Battersea Drainage watershed (73 km^2) and the eastern extension from this area (12 km^2).

3.2 Experimental

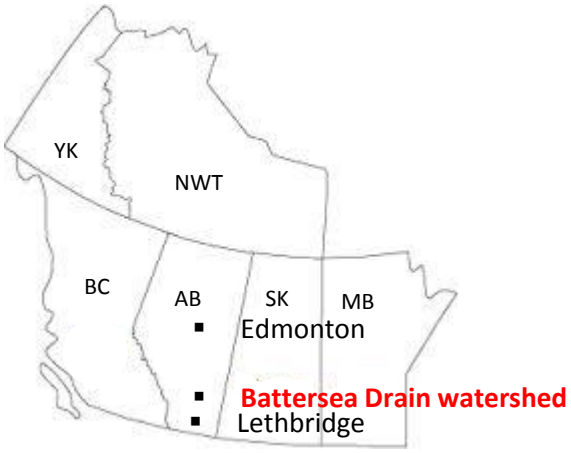
This study is a collaborative effort between three research groups, X.C. Le from the Department of Public Health Sciences at the University of Alberta, G. R. Kachanoski from the Department of Renewable Resources at the University of Alberta, and N.F. Neumann from the Alberta Provincial Laboratory for Public Health. There were five laboratories involved in this water quality assessment. Arsenic concentrations and speciation analyses were determined by L. W. L. Chen (Le's group) in the Division of Analytical and Environmental Toxicology at the University of Alberta. Field parameters (i.e. water pH, electric conductivity) were measured on-site by G. Lilbæk (Kachanoski's group). Major inorganic ions (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, SO_4^{2-}, HCO_3^{-}) and nutrients (total phosphorus, TP; total nitrogen, $TN_{unfiltered}$) were analyzed by the Alberta Agriculture and Rural Development laboratory. Bacteria (total coliforms, *E.coli, Salmonella, Enterococcus*) analyses were conducted by Neumann's group at the Provincial Laboratory for Public Health in Edmonton, while parasites (*Cryptosporidium, Giardia*) analyses were conducted by the Provincial Laboratory for Public Health in Calgary.

3.2.1 Water samples collection and preparation

The Battersea Drain watershed consists of two major water flow paths: an irrigation drain and a groundwater flow path (Fig. 3.1(a)) [1]. In addition, a river to the south of the Battersea Drain watershed, Oldman River was also under investigation to study the effect of the irrigation drain's water on the river water's quality. The Oldman River is a major branch of the South Saskatchewan River,

which is a main source of water supply for agricultural irrigation and domestic drinking water [7]. There were 8 sample collection dates: August 10, 17, 31, September 14, 28, October 12, 26, and November 9, 2009. There were a total of 20 sampling sites, of which 12 were for drain water (BD1 – 12) and the remaining 8 were for groundwater (W2, 5, 7, 9, 13, 11, 21, 22) (Fig. 3.1(b)). Accessibility was the key requirement for the selection of the sampling sites. BD1–10 were located along the drain's main branch (≈20 km) 1–3 km apart; while BD11–12 were by the Oldman River, upstream and downstream of the irrigation drain's outlet [1]. The irrigation drain was turned on from mid-May to mid-October. W2, 5, 7, 9, and 13 were water table wells situated within the basin and the wells water flowed through the groundwater's general flow path from northwest to southeast, determined by a previous study [7]. The topographic slopes eastward to the Little Bow River and southward to the Oldman River [7]. W11, 21, and 22 were water table wells positioned to the east of the basin. More sites were established later as this collaborative study was ongoing (until November 2010). However, within the scope of this paper, we shall focus on the aforementioned 20 sampling sites and 8 sampling dates.

(a)



(b)

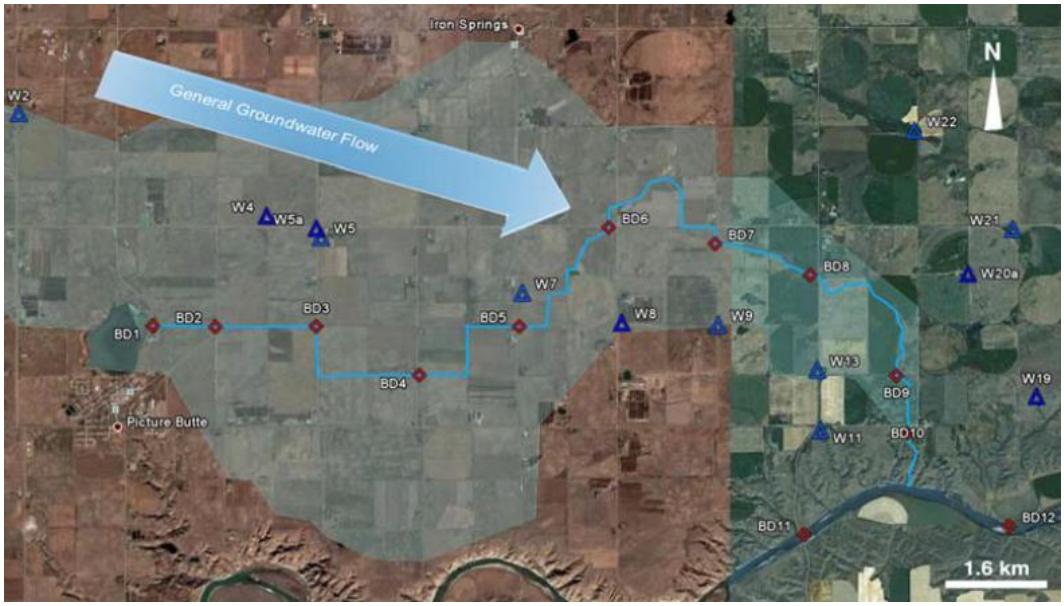


Fig. 3.1 (a) Battersea Drain watershed is located 25 km north of Lethbridge in southern Alberta, Canada; (b) Sampling sites within and around the Battersea Drain watershed. For the purpose of this chapter, we shall focus on the following 20 sampling sites. Groundwater were collected from 8 sampling sites (blue triangle): W2, 5, 7, 9, 13 (within the watershed), and W11, 21, 22 (to the east of the watershed). Drain water samples were collected from 12 sites (red diamond): BD1–10 (drain's main branch), BD11 (upstream of the drain's outlet along the Oldman River) and BD12 (downstream of the drain's outlet along the Oldman River) (Figure taken from Lilbæk, 2012).

Polyethylene bottles (Corning®, sterile, non-pyrogenic, 500 mL) were used to collect 250 mL of untreated water samples [17]. Polyethylene bottles (Fisher, LPDE, 500 mL) were used to collect the treated water samples, in which 4 mL of 0.084 M EDTA and 10 mL of 2.08 M CH_3COOH were added to the samples on site, such that the final volume of each sample was 250 mL. Hence, the treated water samples contained 0.0013 M EDTA and 0.083 M CH_3COOH as the final concentrations. There were 151 different samples collected. Each sample was collected in duplicate in two separate bottles: one bottle for the water sample treated with the preservatives, another bottle for the untreated water sample. Therefore, a total of 302 water samples were analyzed in this chapter.

3.2.2 ICPMS conditions for the determination of total arsenic

The groundwater sample bottles were shaken vigorously for 30 seconds to ensure sample homogeneity. A 10 mL syringe (BD, Luer-lok^{TM} tip) was used to withdraw samples from the bottles, then the samples were passed through a filter (Millex$^{®}$-HA filter, EMD Millipore, 0.45 µm pore size, 33 mm filter diameter, sterile) and transferred to a 15 mL polystyrene centrifuge tube (Corning®, non-pyrogenic, sterile). 10 mL of filtered sample was then diluted in 1% HNO_3. The injection volume for total arsenic analysis was 2 mL. Each sample was analyzed in triplicate.

An Elan 6000 ICPMS system (PerkinElmer SCIEX) was operated in dynamic reaction cell mode using O_2 as the reaction gas. Arsenic species were detected by monitoring AsO^+ at 90.9165 amu (dwell time = 100 ms) to avoid isobaric interference of $Ar^{40}Cl^{35}$ with As^{75}. The ICPMS was operated at 1350 W

rf-power, and 6.75 V lens voltage, with argon flows of 15 L min^{-1} (plasma gas), 0.5 L min^{-1} (nebulizer gas), and 1.5 L min^{-1} (auxiliary gas). Solutions were delivered at a flow rate of 1.2 mL min^{-1} to a cross-flow nebulizer and then finally to a HF-resistant Scott-type spray chamber.

The arsenic calibration standard (10 mg L^{-1},Agilent Technologies, Mississauga, ON, CA) was diluted to 0.2, 1, 5, 10, and 20 µg L^{-1} to construct a calibration plot. Standard reference material 1640a (Trace Elements in Natural Water, National Institute of Standards and Technologies, Gaithersburg, MD, USA) with the total arsenic concentration of 8.075 ± 0.070 µg L^{-1} was used to validate the calibration plot. There was good agreement (≤ 3%) between the certified and the analyzed values for SRM 1640a. The limit of detection was approximately 0.01 µg L^{-1}.

3.2.3 HPLC-ICPMS conditions for arsenic speciation

Standard solutions of arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA^{V}), and dimethylarsinic acid (DMA^{V}) were prepared in deionized water (Milli-Q®, EMD Millipore) (Table 3.1). Sodium m-arsenite ($NaAsO_2$, 98.0 %), sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$, 99.4 %), and dimethylarsinic acid ($C_2H_7AsO_2$, 98.0 %) were purchased from Sigma-Aldrich (Oakville, ON, CA). Monosodium acid methane arsonate (CH_4AsNaO_3, 99.0 %) was purchased from Chem Service Inc. (West Chester, PA, USA).

Based on the results from the total arsenic analysis, the samples that had higher arsenic concentrations were further subject to arsenic speciation. Groundwater and drain water samples were prepared as described above, except

that HNO_3 was not added. Each sample was analyzed in triplicate with an injection volume of 100 µL.

Arsenic species were quantified based on chromatographic peak area by external calibration with an arsenic standard solution (Agilent Technologies) and the quantitation was validated by a standard reference material (NIST). Turbochrom Navigator (PerkinElmer) graphic edit software was used to integrate the peak areas. The limit of detection for HPLC-ICPMS was approximately 0.1 µg L^{-1}.

A reverse-phase C18 octadecylsilane (ODS)-3 column (Phenomenex, Prodigy^{TM} ODS-3V, 150 × 4.60 mm, 3 µm particle size, Torrance, CA, USA) was heated to 50°C for 1 hour before HPLC analysis. The mobile phase consisted of 3 mM malonic acid (Fisher Scientific), 5 mM tetrabutylammonium hydroxide (Aldrich), and 5% methanol (Fisher Scientific). The pH of the mobile phase was adjusted to 5.9 by 10% HNO_3 (Fisher Scientific) and was run isocratically at 1.2 mL min^{-1}.

3.3 Results and discussion

3.3.1 Arsenic concentrations in the treated versus untreated water samples

Generally, the water samples treated with EDTA (0.0013 M) and CH_3COOH (0.083 M) had higher total arsenic concentrations than the untreated water samples. In groundwater, the treated samples had total arsenic concentrations ranging from 0.37 to 27.6 µg L^{-1}, and an overall average of 6.27 ± 6.22 µg L^{-1} (Fig. 3.2(a)) whereas the untreated samples had total arsenic concentrations ranging from 0.24 to 9.6 µg L^{-1}, and an overall average of 2.10 ±

1.95 µg L^{-1} (Fig. 3.2(b)). A similar trend was also observed in the drain water, in which the treated samples had total arsenic concentrations ranging from 0.59 to 7.3 µg L^{-1}, and an overall average of 3.06 ± 1.45 µg L^{-1} (Fig. 3.2(c)) while the untreated samples had total arsenic concentrations ranging from 0.47 to 4.9 µg L^{-1}, and an overall average of 2.24 ± 0.89 µg L^{-1} (Fig. 3.2(d)).

A reagent blank water sample was prepared by adding 4 mL of 0.084 M EDTA (EMD Millipore, 99.0 ~ 101.0% purity, MA, USA) and 10 mL of 2.08 M CH_3COOH (Fisher Scientific, glacial, MA, USA) to a polyethylene bottle (Fisher, LDPE, 500 mL) then diluted it to a final volume of 250 mL with deionized water (Milli-Q®, EMD Millipore). 0.04 ± 0.01 µg L^{-1} total arsenic was found in this reagent blank sample, which is insignificant as compared to the overall higher arsenic concentrations found in the treated water samples. Further speciation analysis of this reagent blank water sample revealed a chromatogram that had a very low arsenic background which resembled the chromatogram of a deionized water sample (Fig. 3.3(a,b)). Four arsenic species, arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA^{V}), and dimethylarsinic acid (DMA^{V}) in the water samples were eluted at approximately 1.6, 2.4, 4.0, and 6.2 minutes respectively (Fig. 3.3(c)).

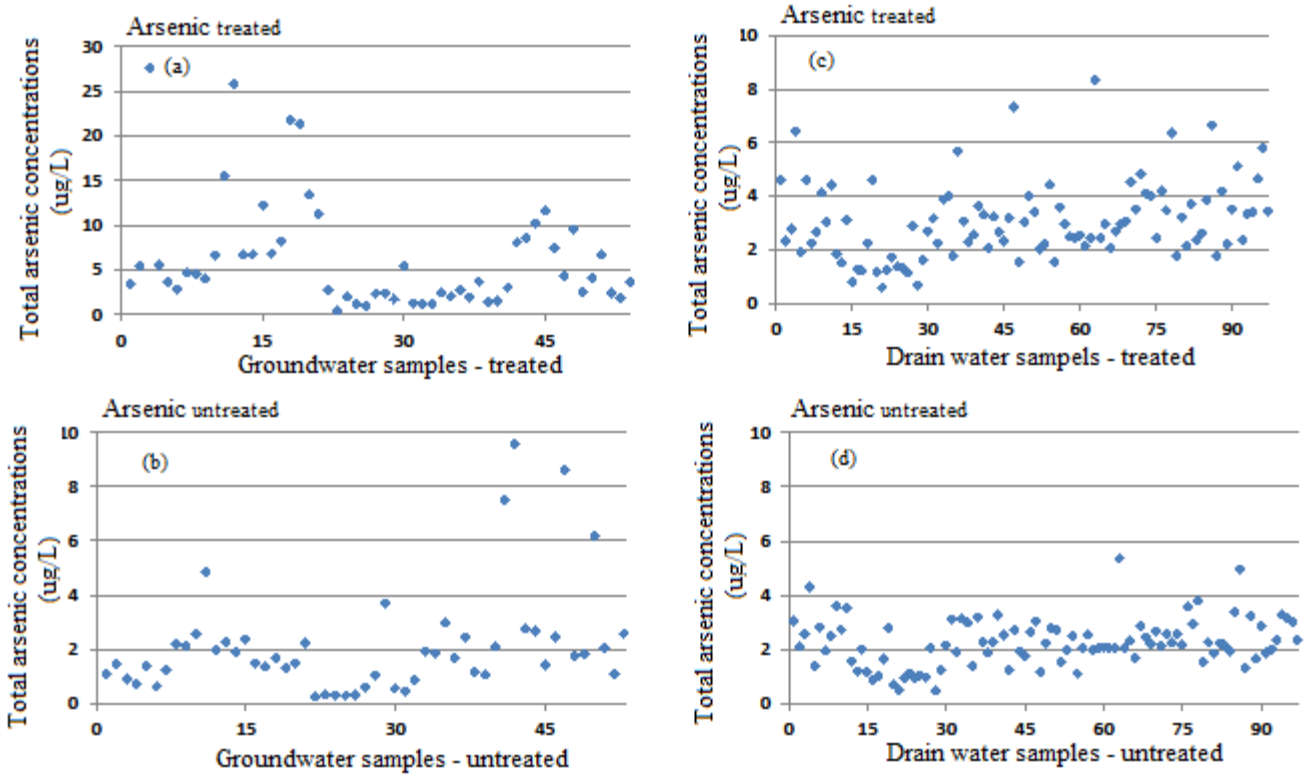


Fig. 3.2 Total arsenic concentrations for 302 water samples. (a) groundwater samples treated with CH_3COOH and EDTA: average 6.27 ± 6.22 µg L^{-1}, median 3.99 µg L^{-1}, range 0.37 to 27.6 µg L^{-1}; (b) untreated groundwater samples: average 2.10 ± 1.95 µg L^{-1}, median 1.66 µg L^{-1}, range 0.24 to 9.6 µg L^{-1}; (c) drain water samples treated with CH_3COOH and EDTA: average 3.06 ± 1.45 µg L^{-1}, median 2.85 µg L^{-1}, range 0.59 to 7.3 µg L^{-1} (d) untreated drain water samples: average 2.24 ± 0.89 µg L^{-1}, median 2.16 µg L^{-1}, range 0.47 to 4.9 µg L^{-1}.

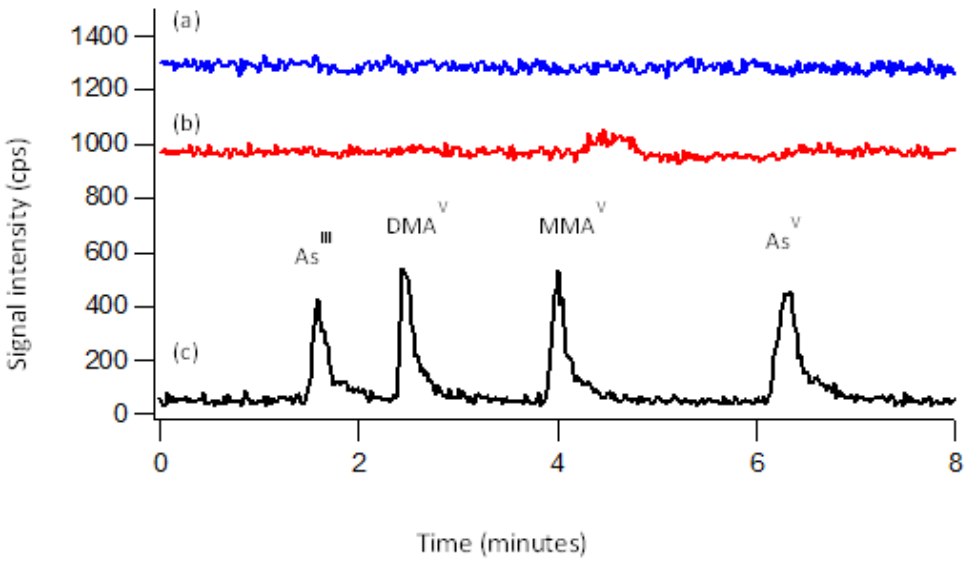


Fig. 3.3 Arsenic speciation analysis of controls (a) deionized water; (b) 0.0013 M EDTA and 0.083 M CH_3COOH in deionized water: the total arsenic concentration was 0.04 ± 0.01 µg L^{-1}; (c) 1 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, As^{V}.

The background total arsenic found in the preservatives (0.0013 M EDTA and 0.083 M CH_3COOH) was so low (0.04 ± 0.01 µg L^{-1}) that it was insufficient to explain why the treated water samples had higher total arsenic concentrations as compared to the untreated water samples. One possibility could be that the addition of EDTA and CH_3COOH prevented arsenic from adsorbing onto the wall of the polyethylene bottle and the sediment particulates. If less arsenic was removed from the filtration step during the sample preparation procedure when sediment particulates were retained, then more arsenic would have remained in the filtrate and eventually led to higher total arsenic levels in the treated samples. Alternatively, if less arsenic was adsorbed on the surface of the sample container, then more arsenic would still be in the liquid phase and consequently higher arsenic concentraitons may be found in the treated water samples. Some

researchers suggested that EDTA and CH_3COOH was especially effective to preserve inorganic arsenic in groundwater samples that had a higher Fe content (> 6.45 mg L^{-1}) [18, 19]. It was proposed that EDTA and CH_3COOH minimize the photocatzlyed Fe^{III} reduction thereby decreasing the oxidation rate of As^{III} to As^{V} [20]. In addition, EDTA and CH_3COOH may sequester Fe^{III} and inhibit the precipitation of Fe and As [21]. In addition, opaque polyethylene bottles (Fisher, LDPE, 500 mL) were used to store the EDTA and CH_3COOH treated water sample as its opaqueness minimized light exposure and thereby reduced the photo-catalyzed Fe^{III} reduction that might cause As^{III} oxidation to As^{V} [18].

The dominant species in the drain water and groundwater samples were As^{III} and As^{V} while methylated species such as MMA^{V} and DMA^{V} were not detected (Table 3.1). A few selected treated water samples were found to consist of predominately As^{V} (79–96%), with minor proportion of As^{III} (4–21%). However, in the untreated water samples, only As^{V} was detected (Table 3.2). For example, the groundwater sample #W2 Sep. 28 contained 0.25 ± 0.03 µg L^{-1} As^{V} in the untreated sample (Fig. 3.4(b)), while the treated sample had 0.2 ± 0.1 µg L^{-1} As^{III} and 1.75 ± 0.02 µg L^{-1} As^{V} (Fig. 3.4(c)). Similarly, in the groundwater sample #W11 Sep. 14, only a trace amount of As^{III} (0.6 ± 0.1 µg L^{-1}) was detected in the untreated sample (Fig. 3.5(b)), while almost 5 times as high an As^{III} concentration (2.8 ± 0.1 µg L^{-1}) were found in the treated sample, along with As^{V} (10.44 ± 0.03 µg L^{-1}) (Fig. 3.5(d)). These data illustrate that only As^{V} was present in the untreated water samples while both As^{V} and As^{III} were in the water samples treated with EDTA and CH_3COOH. These results suggest that the addition of

EDTA and CH_3COOH were able to maintain the integrity of arsenic speciation in water samples, possibly by slowing down the rate of As^{III} oxidation to As^{V} and [19, 18, 22–24].

Table 3.1 Structures of arsenic species: arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}).

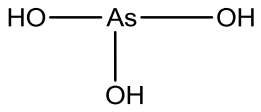
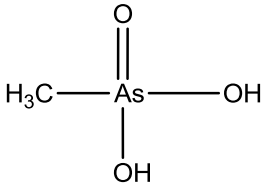
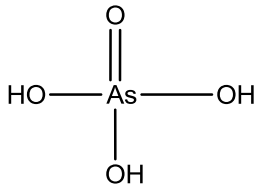
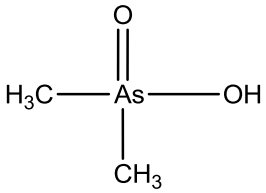
	
Arsenite (As^{III})	**Monomethylarsonic acid (MMA^{V})**
	
Arsenate (As^{V})	**Dimethylarsinic acid (DMA^{V})**

Table 3.2 Concentrations of arsenic species in selectd groundwater samples detrmined by HPLC-ICPMS.

Sample ID	Untreated sample	Treated sample with EDTA and CH_3COOH			
	As^{V}	As^{III}	% of total As	As^{V}	% of total As
W11 Sep. 14	0.6 ± 0.1	2.8 ± 0.1	21	10.44 ± 0.03	79
W13 Sep. 28	1.2 ± 0.1	0.5 ± 0.1	4	11.0 ± 0.6	96
W2 Sep. 14	0.3 ± 0.1	0.7 ± 0.1	9	7.2 ± 0.3	91
W2 Sep. 28	0.25 ± 0.03	0.2 ± 0.1	12	1.75 ± 0.02	88
Average			**12**		**88**

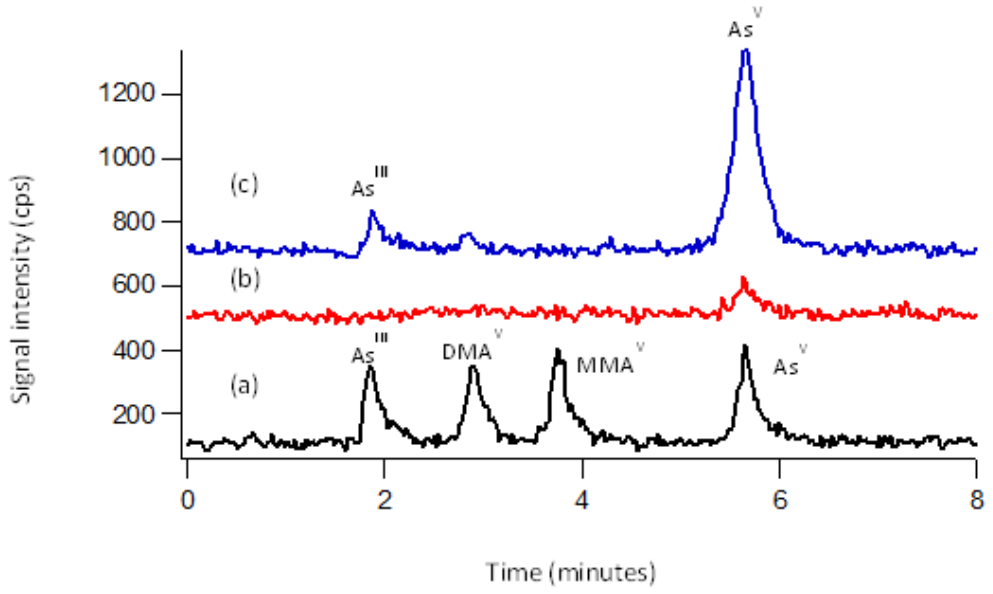


Fig. 3.4 Arsenic speciation analysis using ion pair chromatography of (a) 0.5 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, As^{V}; (b) untreated groundwater sample #W2 Sep. 28: 0.25 ± 0.03 µg L^{-1} As^{V}; (c) treated groundwater sample #W2 Sep. 28 with EDTA and CH_3COOH: 0.2 ± 0.1 µg L^{-1} As^{III} and 1.75 ± 0.02 µg L^{-1} As^{V}.

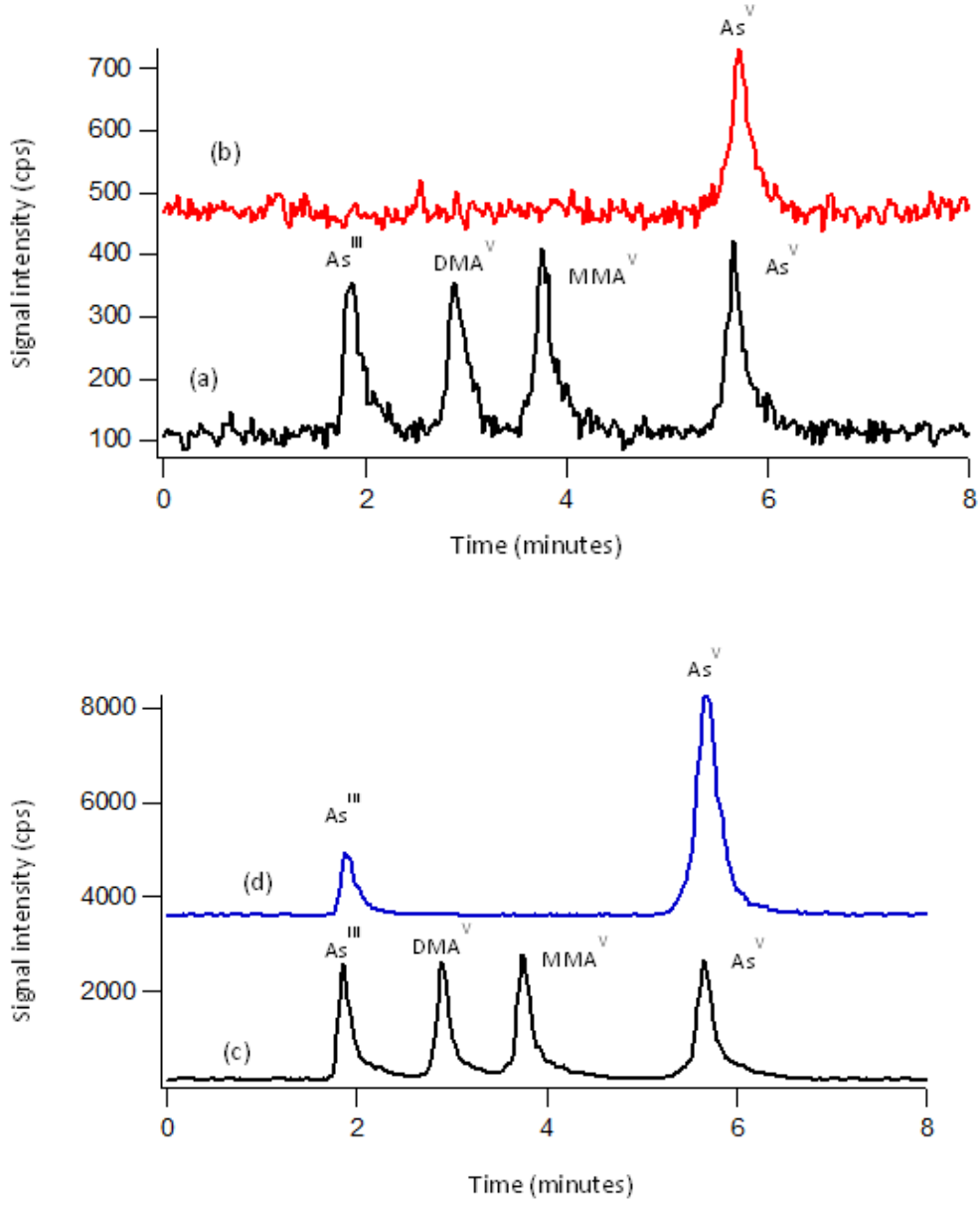


Fig. 3.5 Arsenic speciation analysis using ion pair chromatography of (a) 0.5 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, As^{V}; (b) untreated groundwater sample #W11 Sep. 14: 0.6 ± 0.1 µg L^{-1} As^{III}; (c) 5 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, As^{V}; (d) treated groundwater sample #W11 Sep. 14 with EDTA and CH_3COOH: 2.8 ± 0.1 µg L^{-1} As^{III} and 10.44 ± 0.03 µg L^{-1} As^{V}.

3.3.2 Groundwater – water quality analyses

The total arsenic analysis data from both the untreated and treated water samples were first categorized according to the sampling sites and sampling dates to investigate any spatial and/or temporal trend. Among the treated groundwater samples (53), sample #W11 Sep. 14 had the maximum total arsenic concentration of 27.59 ± 0.47 μg L^{-1}, while the minimum total arsenic concentration 0.37 ± 0.01 μg L^{-1} was obtained from sample #W21 Aug. 31. The overall average of the total arsenic concentration from these treated groundwater samples was 6.27 ± 6.22 μg L^{-1} (Table 3.3). A scatter plot of these 53 samples indicated that in general, higher arsenic concentrations were found in the sampling sites W2, 7, 13, which were located at the western part of the basin (Fig. 3.6(a)and Fig. 3.7). W11, 21, 22, which were positioned in the eastern part of the basin were commonly found to have lower total arsenic concentrations as compared to the rest of the sampling sites. 10 groundwater samples had arsenic concentrations exceeding the Canadian drinking water guideline of 10 μg L^{-1}, which were obtained at sampling sites W2 (5 samples), W7 (2 samples), W13 (2 samples), W11(1 sample). Hence, 7% of the water samples collected did not meet the safe drinking water guideline for arsenic. With respect to various sampling collection dates, Sep. 14 and Sep. 28 generally had the highest total arsenic concentrations as compared to the remaining sampling dates (Fig. 3.6(b) and Fig. 3.8).

In the untreated groundwater samples (53), the maximum total arsenic concentration of 9.55 ± 0.53 μg L^{-1} was found in sample #W7 Aug. 17,while the minimum total arsenic concentration of 0.24 ± 0.01 μg L^{-1} was found in sample

#21 Aug. 31. The overall average total arsenic concentration of the untreated groundwater samples was 2.10 ± 1.95 µg L^{-1} (Table 3.3). Sampling site W7 usually had a higher than the average total arsenic concentration; while sampling site W11, which was located to the east of the study area, always had a lower (1.05 ± 0.32 µg L^{-1}) than the average total arsenic concentration (Fig. 3.6(c) and Fig. 3.9). Most sampling sites did not have an apparent temporal trend (Fig. 3.6(d) and Fig. 3.10). All the untreated groundwater samples had total arsenic concentrations below the Canadian drinking guideline. There was no apparent correlation between the treated and the untreated groundwater samples.

Table 3.3 Total arsenic analysis of 302 groundwater and drain water samples from the Battersea Drain watershed.

Total arsenic analysis	**# [*] of samples**	**Average ± sd. [**] (µg L^{-1})**	**Median (µg L^{-1})**	**Maximum (µg L^{-1})**	**Minimum (µg L^{-1})**
Groundwater – treated	53	**6.27 ± 6.22**	3.99	**27.59 ± 0.47**	0.37 ± 0.01
Groundwater – untreated	53	**2.10 ± 1.95**	1.66	**9.55 ± 0.53**	0.24 ± 0.01
Drain water – treated	98	**3.06 ± 1.45**	2.85	**7.33 ± 0.66**	0.59 ± 0.02
Drain water – untreated	98	**2.24 ± 0.89**	2.16	**4.94 ± 0.31**	0.47 ± 0.02

[*] # = number ; [**] sd. = standard deviation

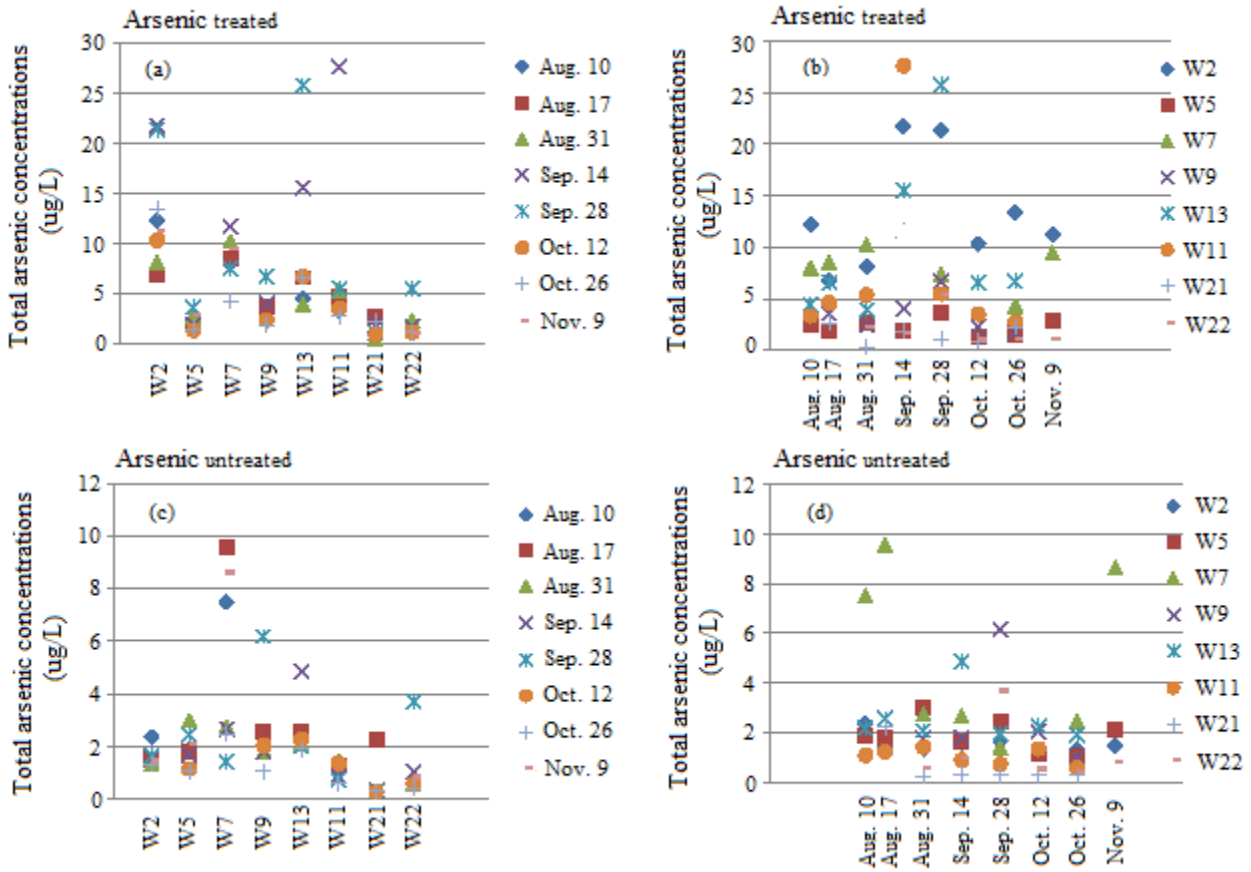


Fig. 3.6 Total arsenic concentrations of groundwater samples. (a) treated groundwater samples at each sampling site; (b) treated groundwater samples at each sampling date; (c) untreated groundwater samples at each sampling site; (d) untreated groundwater samples at each sampling date.

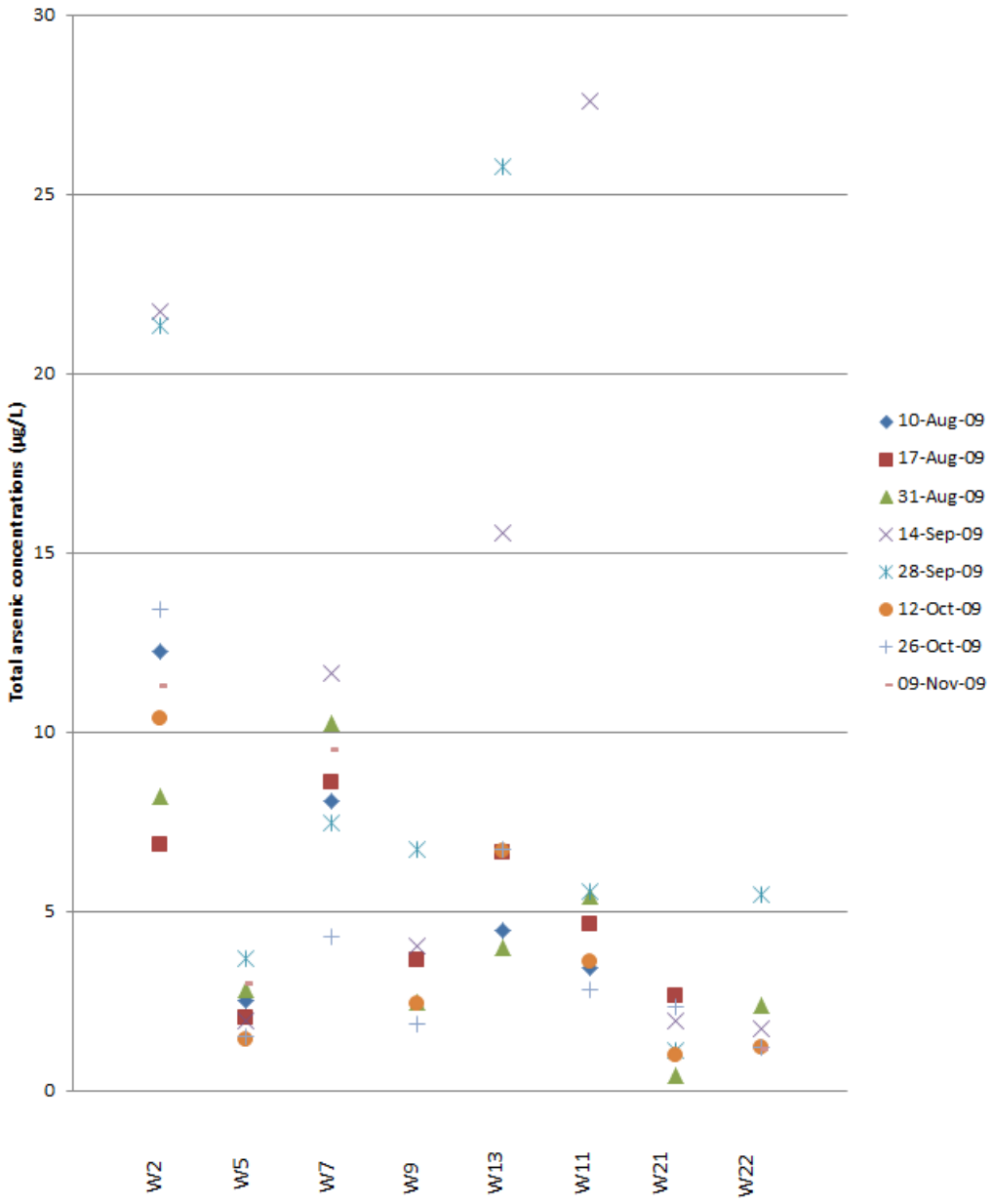


Fig. 3.7 Treated groundwater samples at each sampling site.

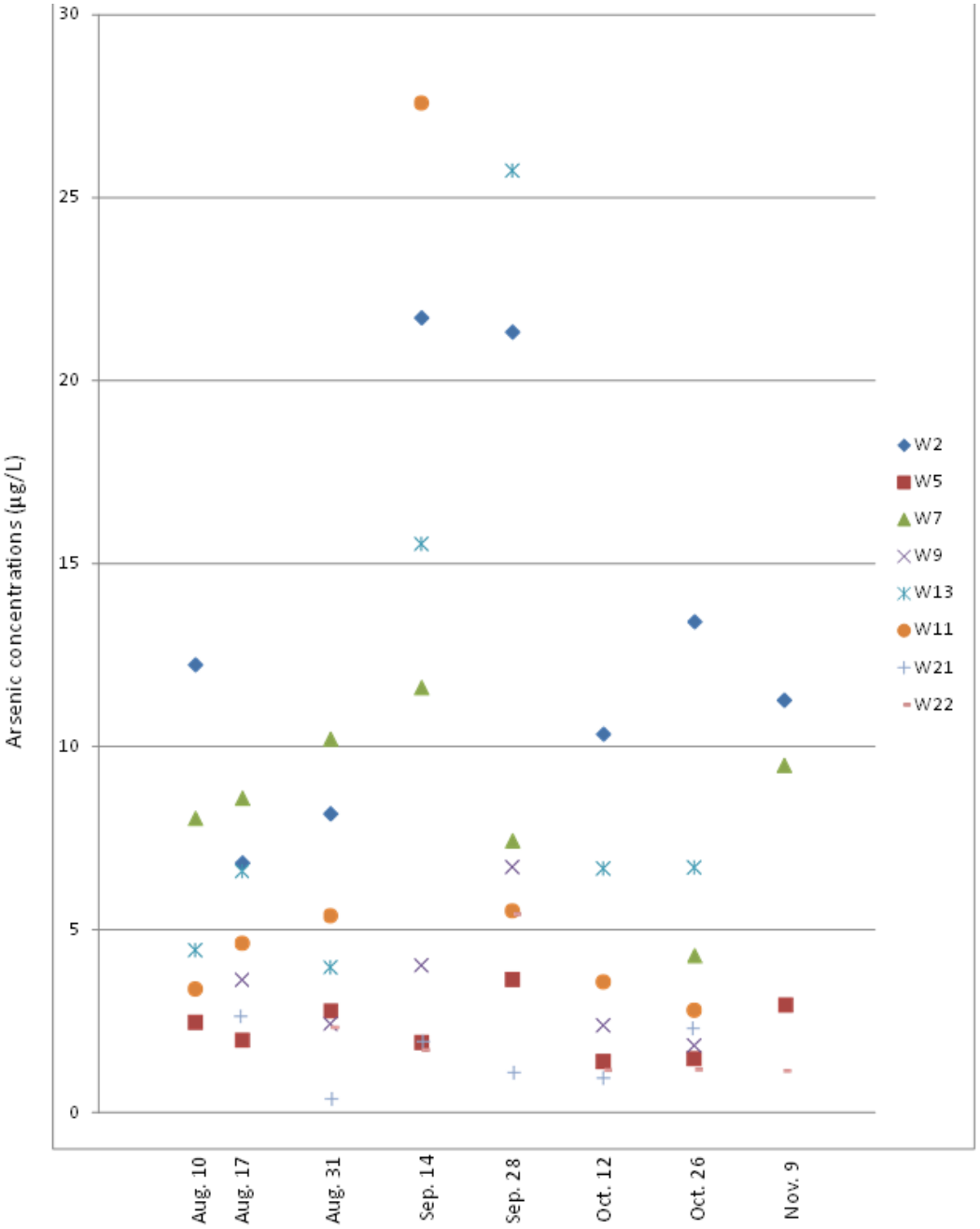


Fig. 3.8 Treated groundwater samples at each sampling date.

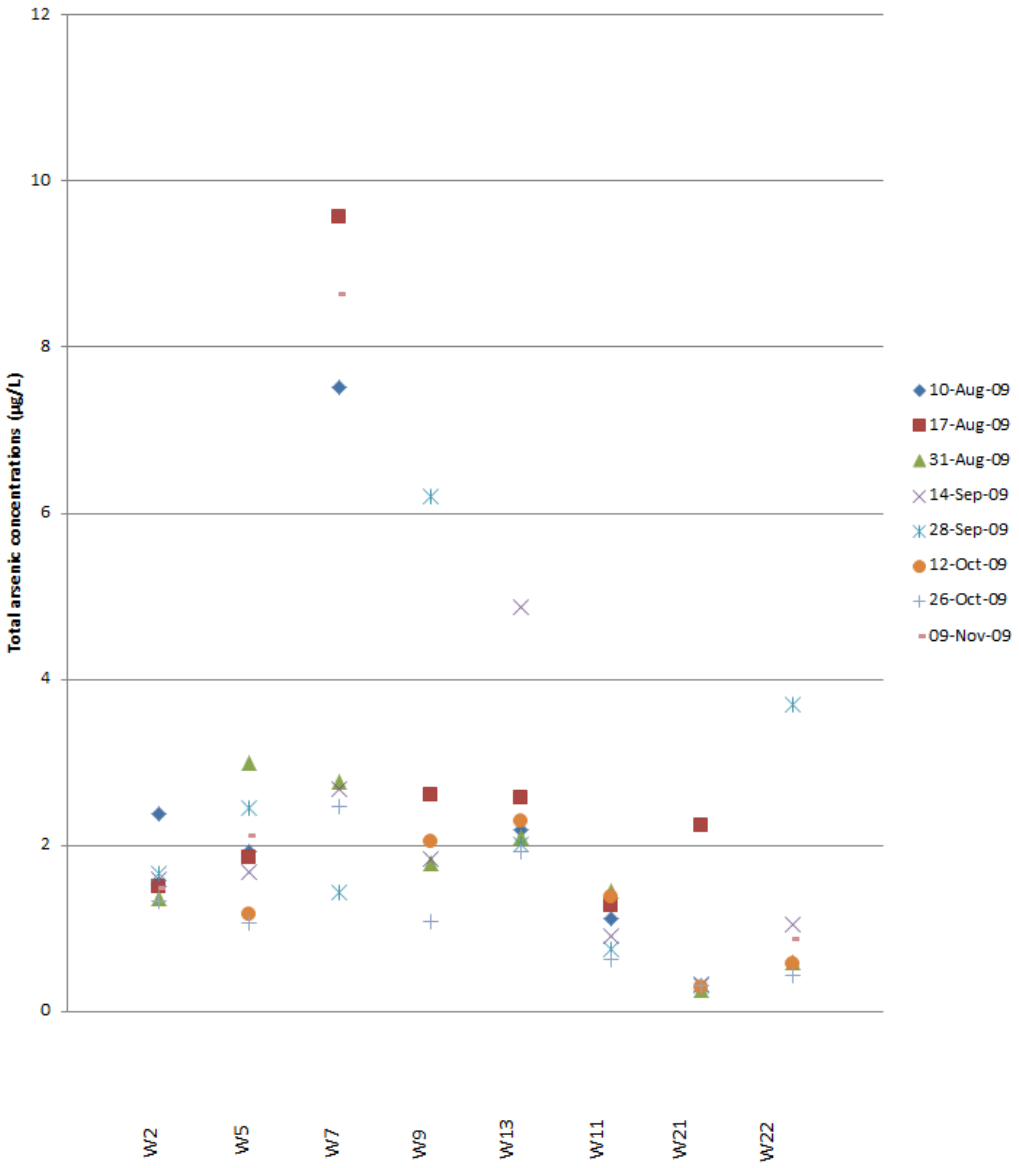


Fig. 3.9 Untreated groundwater samples at each sampling site.

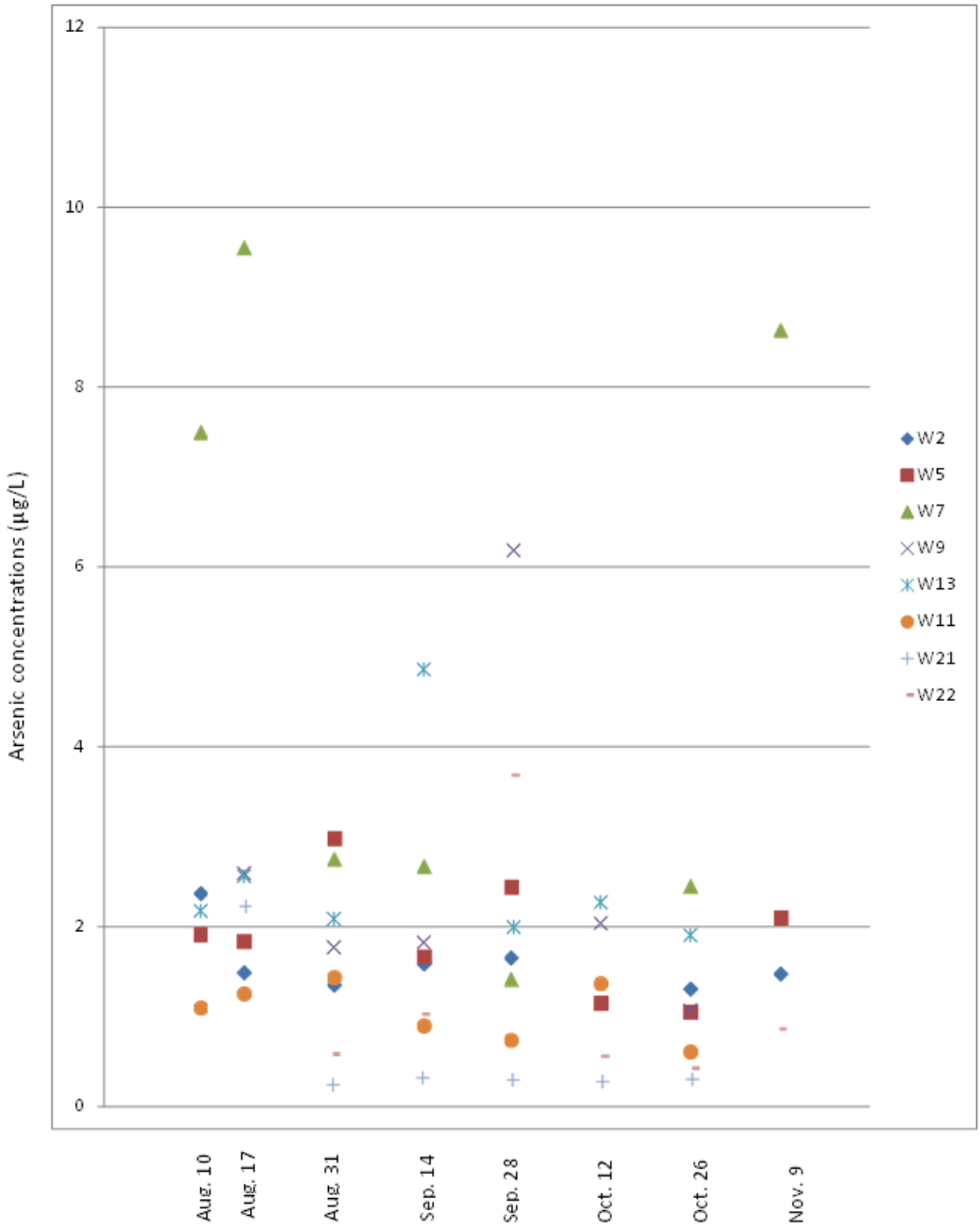


Fig. 3.10 Untreated groundwater samples at each sampling date.

Results from the other laboratories indicated that the majority of the groundwater samples did not meet the safe drinking water guidelines on multiple parameters, including coliforms (coliforms and *Enterococcii*, 82 %) and nutrients (TP, 79 %; $TN_{unfiltered}$, 91%). In general, bacterial concentrations were found to be greater within the watershed, rather than to the east of the watershed [1]. The groundwater samples showed minimum temporal variations in major inorganic ions and nutrients. However, a spatial trend was evident, in which the concentrations of the major inorganic ions and nutrients were much higher in the western part of the Battersea Drain watershed as compared to the eastern part [1]. This spatial trend in the major inorganic ions and nutrients was consistent with that of the total arsenic concentrations. For coliforms, there were no apparent temporal or spatial trends. However, with *E. coli* and *Enterococcus*, the data suggested that there might be point source contamination from a CFO or septic system in the vicinity of a sampling site [1].

3.3.3 Drain water – water quality analyses

The treated drain water samples (98) had a maximum total arsenic concentration of 7.33 ± 0.66 µg L^{-1} from sample #BD4 Sep. 28 while the minimum total arsenic concentration of 0.59 ± 0.02 µg L^{-1} was from sample #BD11 Oct. 26. The overall average of the total arsenic concentration was 3.06 ± 1.45 µg L^{-1} (Table 3.3). Sample site BD12 consistently had a lower (1.53 ± 0.68 µg L^{-1}) than average total arsenic concentration (Fig. 3.11(a) and Fig. 3.12). BD11 and BD12, which were located along the Oldman River, were chosen to evaluate the effect of drain's water quality on the river water's quality. These two sites

were found to have lower arsenic concentrations as compared to the rest of the sampling sites. This suggested that the irrigation drain water's quality had minimal impact on the quality of surface water, namely the Oldman River. Samples collected on Sep. 28 had a slightly higher total arsenic concentration as compared to the other sampling dates. There was no other apparent temporal variation in the arsenic concentration (Fig. 3.11(b) and Fig. 3.13).

In the untreated drain water samples (98), the maximum total arsenic concentration was 4.94 ± 0.31 μg L^{-1}, obtained from sample #BD8 Sep. 28 while the minimum total arsenic concentration was 0.47 ± 0.02 μg L^{-1} found in sample #BD12 Oct. 26. The overall average total arsenic concentration was 2.24 ± 0.89 μg L^{-1} (Table 3.3). All the drain water samples, both treated and untreated, contained total arsenic at concentrations below the Canadian drinking water guideline of 10 μg L^{-1} As. Untreated drain water samples from sampling sites BD11 and BD12 (Oldman River water) usually had lower arsenic concentrations (Fig. 3.11(c) and Fig. 3.14), which were consistent with the treated samples. The arsenic concentrations of untreated samples from most sampling sites were fairly evenly distributed around the overall average concentrations. Similar to the treated samples, higher arsenic concentrations were found on the sampling date of Sep. 28. No other apparent temporal trend was evident in the untreated drain water samples (Fig. 3.11(d) and Fig. 3.15).

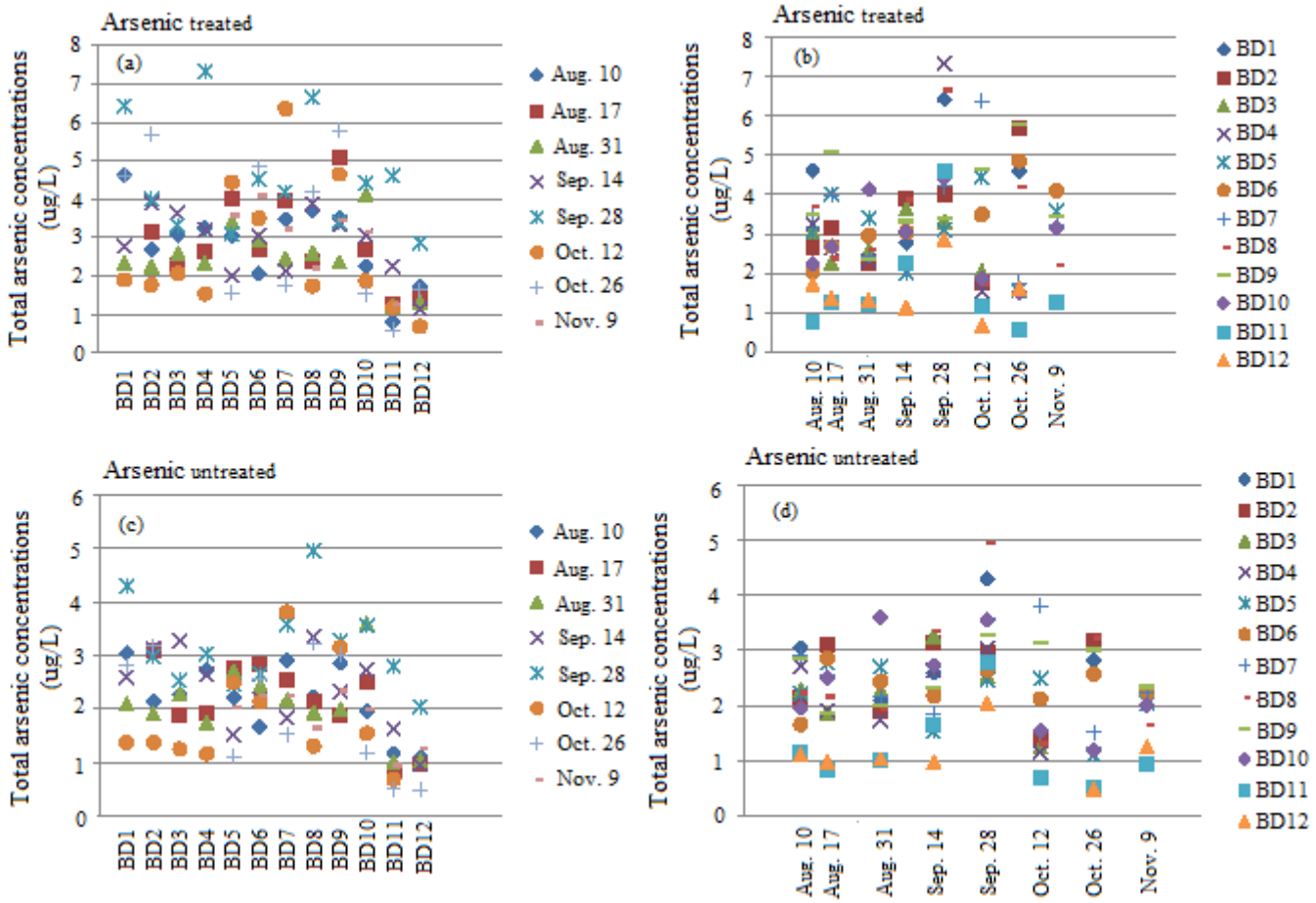


Fig. 3.11 Total arsenic concentrations of drain water samples. (a) treated drain water samples at each smapling site; (b) treated drain water samples at each sampling date; (c) untreated drain water samples at each sampling site; (d) untreated drain water samples at each sampling date.

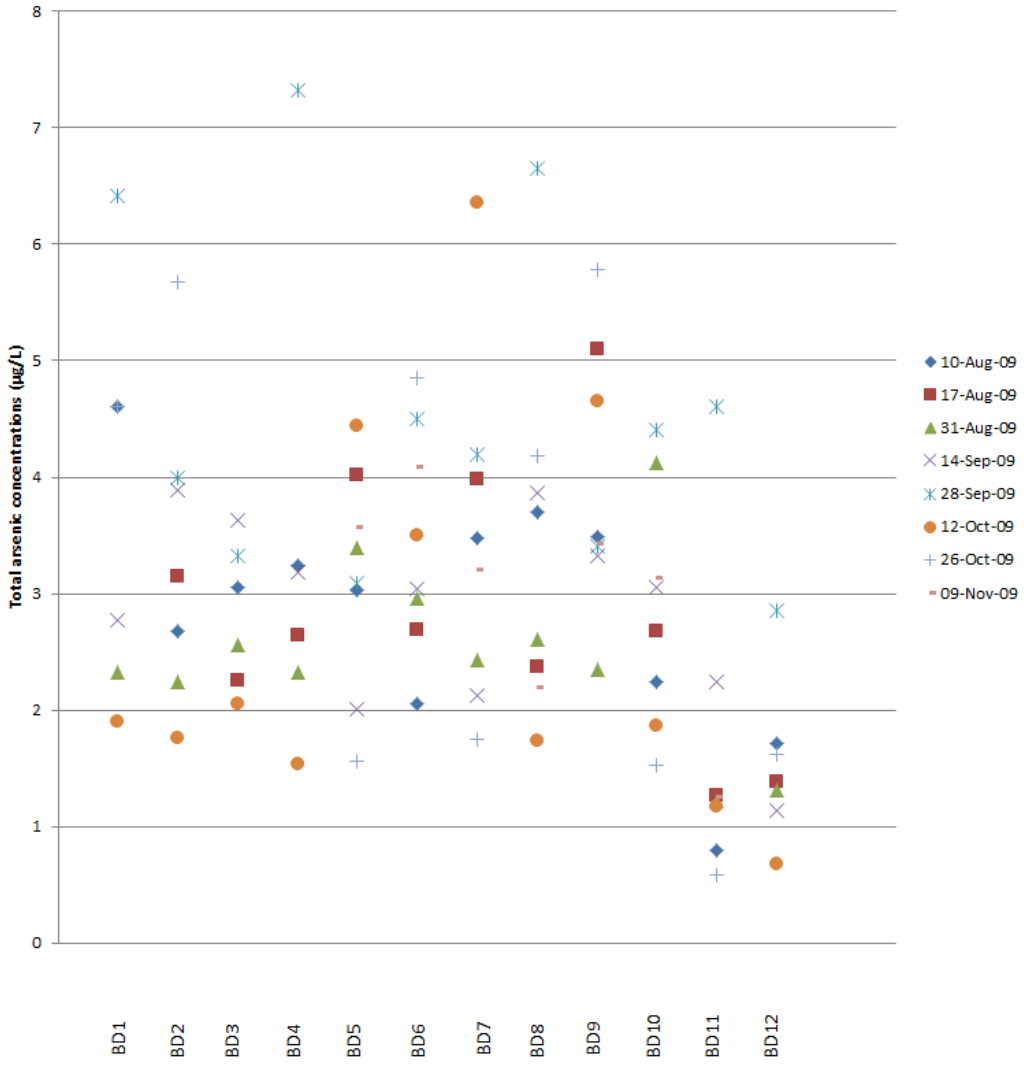


Fig. 3.12 Treated drain water samples at each sampling site.

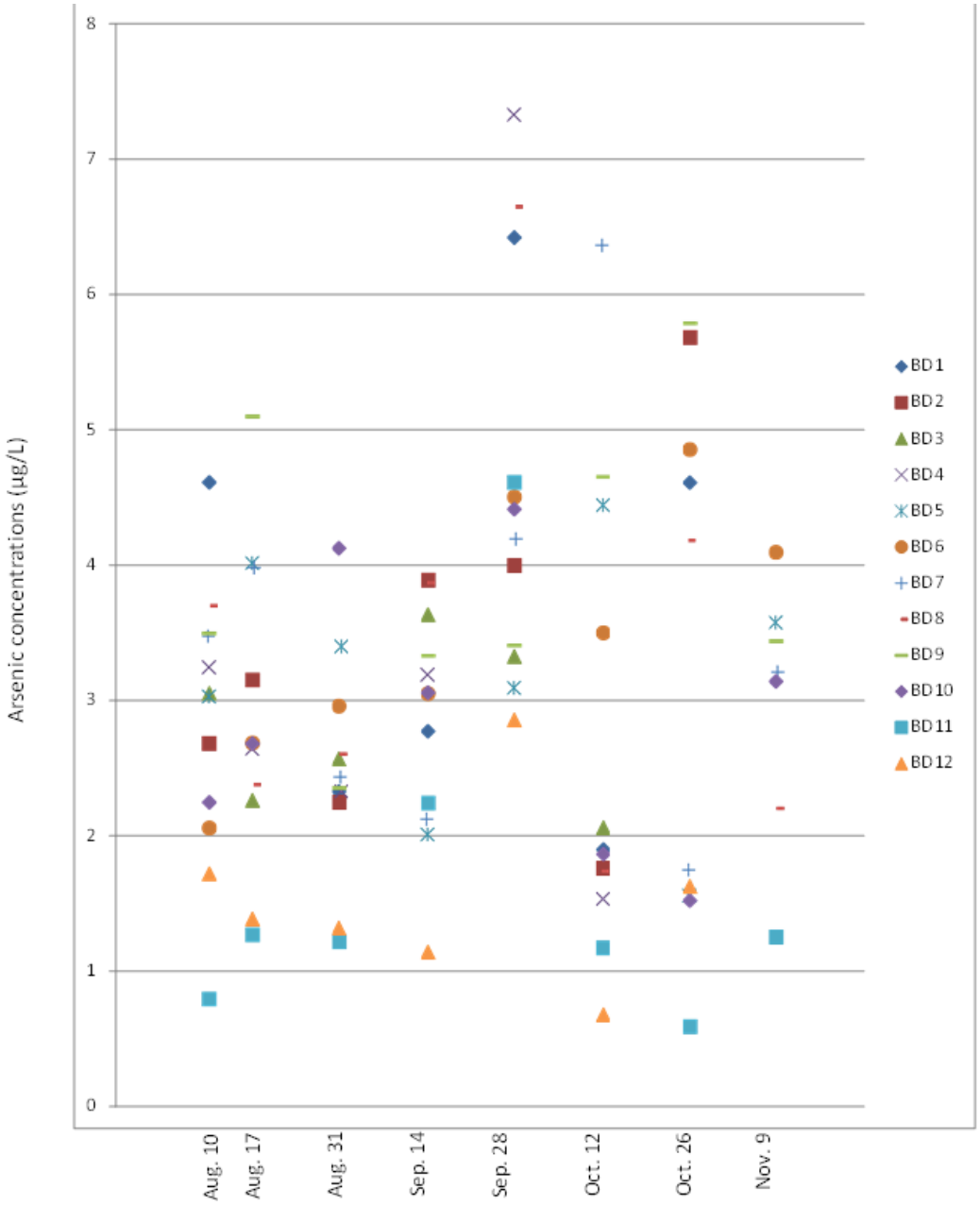


Fig. 3.13 Treated drain water saples at each sampling date.

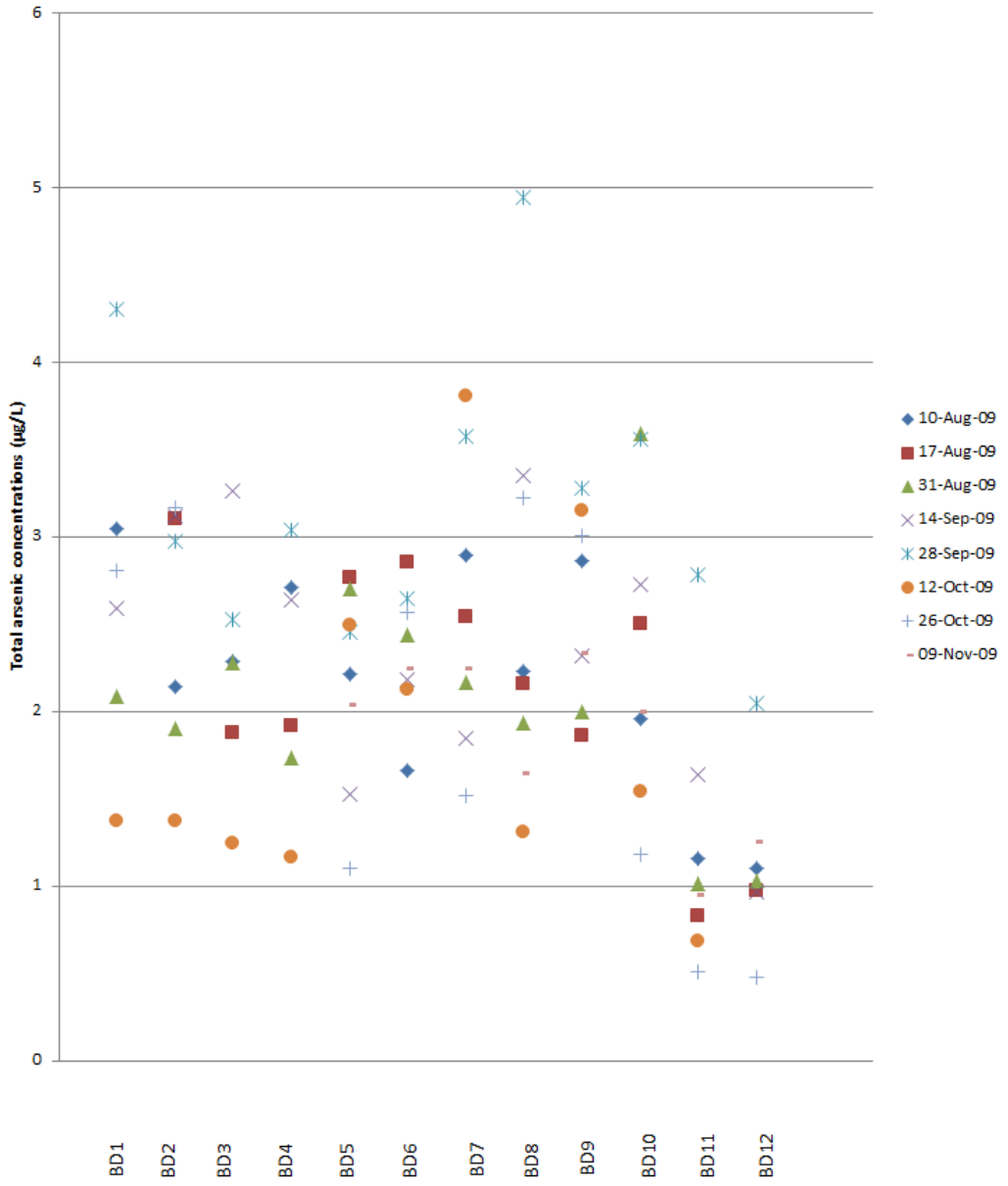


Fig. 3.14 Untreated drain water samples at each sampling site.

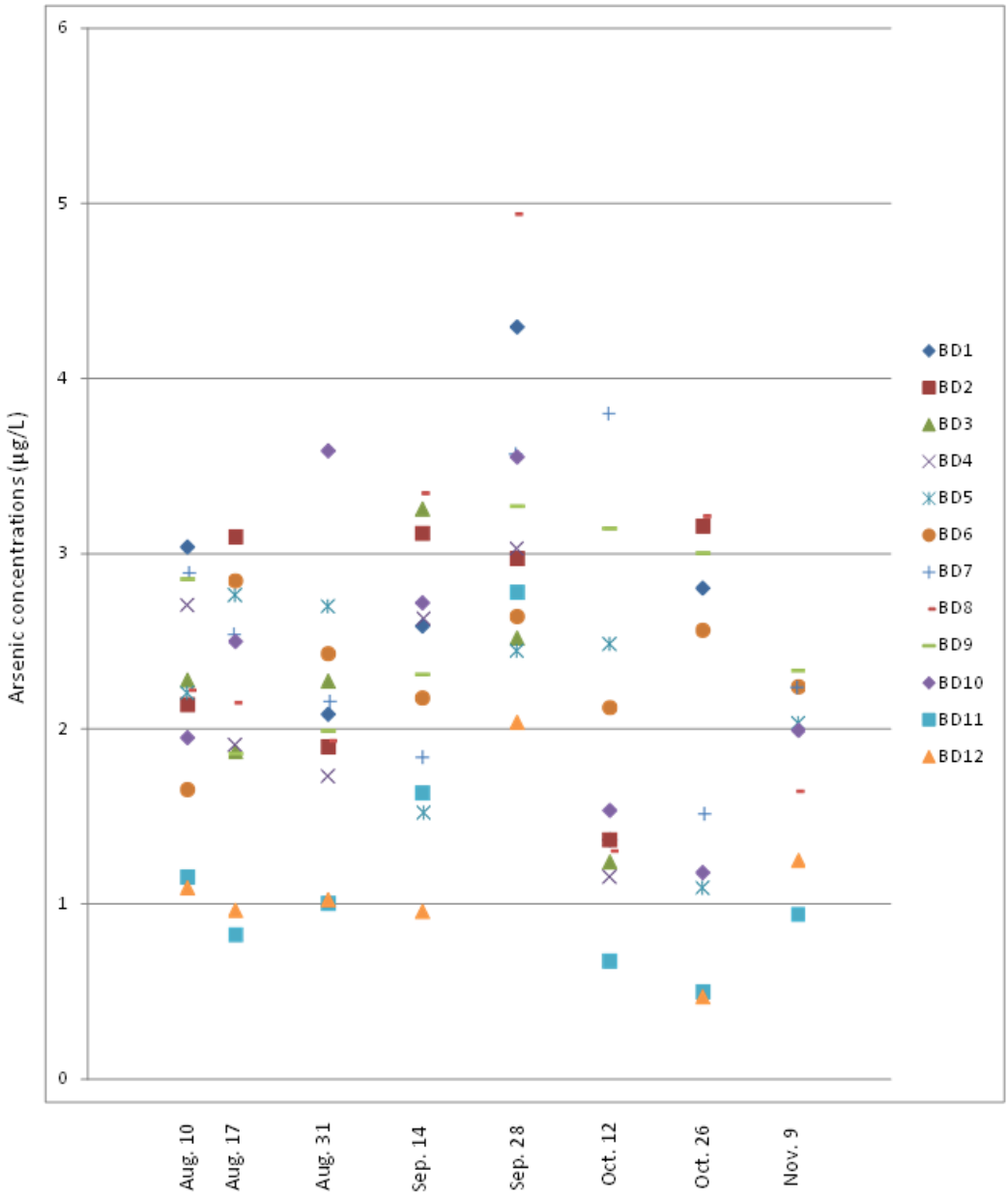


Fig. 3.15 Untreated drain water samples at each sampling date.

Results from the other laboratories showed that the drain water had exceeded numerous safe drinking water guidelines including coliforms (coliforms and *E. coli*, 97 %), parasites (*Cryptosporidium*, 84%), and nutrients (TP and $TN_{unfiltered}$, 89%). The drain water showed little temporal variation in the major inorganic ions, nutrients, bacteria, and parasites during the period when the drain was flowing (mid-May to October) [1]. However, when the drain was shut off (mid-October to April), the flow continued in the latter half of the drain channel, likely because the groundwater permeated into the drain channel there [1]. The data suggested that the groundwater blended with the drain water and caused a significant increase in the concentrations of the major inorganic ions and nutrients while the bacterial concentrations decreased. Upon the turn-on of the drain (mid-May to October), the reverse trend was observed where the concentrations of the major inorganic ions and nutrients dropped while the bacterial content increased [1].

3.4 Conclusions

In general, the water samples treated with EDTA (0.0013 M) and CH_3COOH (0.083 M) had higher total arsenic concentrations than the untreated water samples. This may be due to less surface adsorption of arsenic in the presence of EDTA and CH_3COOH. Only As^V was detected in the untreated water samples while both As^V and As^{III} were found in the treated water samples. In groundwater samples, higher arsenic concentrations were obtained from the western part of the Battersea Drain watershed as compared to the eastern part of the study area. This spatial trend in the arsenic concentration was consistent with

that of the bacterial content, major inorganic ions and nutrients. 7% of the water samples collected exceeded the safe drinking water guideline for arsenic (10 µg L^{-1}). Temporally, late-September usually had higher total arsenic concentrations as compared to the rest of the sampling dates. This temporal trend was also observed in the drain water. The data indicated that the drain water's quality had minimal impact on the water quality in the Oldman River concerning arsenic. Results from our collaborators demonstrated an inverse relationship between the concentrations of the major inorganic ions and nutrient and the bacterial content during the period of drain closure. In conclusion, the water quality in the Battersea Drain watershed exceeded the guidelines for safe drinking water, the conditions required cautionary measures to restore the standard of safe drinking water in this high density agricultural area.

3.5 References

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CHAPTER 4 Arsenic speciation analysis in marine sponges – complementary chromatography separation with inductively coupled plasma mass spectrometry (HPLC-ICPMS)

4.1 Introduction

The speciation, biotransformation, and distribution of arsenic in aquatic environment, especially in marine waters, have been studied extensively [1–4]. Arsenic found in marine organisms can originate from their surrounding water, suspended particles in the water column, sediments, and through their dietary intake. Hence, the accumulation of arsenic is dependent on various biological, chemical, and environmental factors [4, 5]. Microorganisms, such as phytoplankton and bacteria can uptake As^{V} from their surrounding water and reduce it to As^{III}, which then undergoes biotransformation to the methylated arsenicals, such as MMA^{V} and DMA^{V}, or even the more complicated arsenosguars and arsenobetaine (AsB) [2–7]. Other marine organisms such as algae, fish, and shellfish contain mostly organic arsenicals, for example, AsB, arsenosguars, and arsenocholine which are essentially non-toxic; while As^{III} and As^{V} only occur in minor fraction [7–10].

This chapter focuses on the determination of a more recently identified natural organic arsenical, Arsenicin A ($As_4O_3(CH_2)_3$). It was previously isolated from the dichloromethane (CH_2Cl_2) extract of the New Caledonian marine sponge *Echinochalina bargibanti* [11]. This dark red shallow marine sponge belongs to

the *Porifera* phylum, *Demospongiae* class, *Poecilosclerida* order, *Microcionidae* family, and *Echinochalina* genus [12]. Arsenicin A has shown potent bactericidal and fungicidal properties against human pathogens, such as *Staphylococcus aureus* and *Candida albicans* [11]. Researchers have used a variety of techniques to elucidate Arsenicin A's structure, which included ^{1}H and ^{13}C NMR [13], IR and Raman scattering [14, 15]. The crystal structure of a synthesized Arsenicin A confirms its adamantine-type structure that is analogous to arsenic trioxide (As_2O_3) but with three methylene group replacing the oxygen atoms [16] (Table 4.1). As_2O_3 has shown significant efficacy in treating newly diagnosed and relapsed patients with acute promyelocytic leukemia, with the complete remission rate being 70 to 90 % and 65 to > 90 % respectively [17, 18]. A recent *in vitro* toxicological study conducted in our laboratory has shown that Arsenicin A model compound is more toxic than As^{III} (see Chapter 5). Hence, Arsenicin A model compound may have potential therapeutic application.

Although the structure of Arsenicin A has been well characterized in the literature [11, 14–16], there has been limited research on the chromatographic separation technique of Arsenicin A from the other arsenicals. Therefore, the main objective for this chapter is to develop a set of complementary analytical techniques that can differentiate Arsenicin A from the other arsenicals that may also be present in these Papua New Guinean marine sponges. High performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICPMS) was used to differentiate 7 arsenic species: Arsenicin A model compound, As^{III}, As^{V}, MMA^{V}, DMA^{V}, AsB and arsenosugar. Two modes of

separation were used: ion pair and anion exchange chromatography. These separation techniques were combined with ICPMS detection, to offer detection limits in the sub μg L^{-1} range for each arsenic species.

4.2 Experimental

4.2.1 Marine sponge collection and sample preparation

Dr. R. Andersen's group from the University of British Columbia collected marine sponges from the northern coast of Bougainville in Papua New Guinea, near the town of Buka, and at 5–40 m depth. 80–90 % of the samples were confirmed to be sponges, while the rest of the samples were other type of invertebrates. The collected species of the sponges and invertebrates were not identified. Frozen marine sponges were extracted twice with methanol and 2–5 mg of the crude methanol extract of each sample was placed on 96 well plates with 80 samples per plate. We received from Dr. Andersen two 96-well plates, containing a total of 160 samples. Prior to the HPLC-ICPMS analysis, the crude extract in each well was dissolved in a 200 μL mixture of Milli-Q deionized water and methanol (50:50) and then prepared with appropriate dilution with Milli-Q deionized water.

4.2.2 Chemicals

Arsenobetaine was purchased from Tri Chemical Laboratories Inc. (Yamanashi, Japan). Arsenosugar was derived from a brown algae *Fucus serratus* extract, which contains a mixture of four arsenosugars with traces of DMA^V and As^V, provided by Francesconi [19]. Sodium m-arsenite ($NaAsO_2$, 98.0 %), sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$, 99.4 %), and

dimethylarsinic acid ($C_2H_7AsO_2$, 98.0 %) were purchased from Sigma-Aldrich (Oakville, ON, CA). Monosodium acid methane arsonate (CH_4AsNaO_3, 99.0 %) was purchased from Chem Service Inc. (West Chester, PA, USA).

An Arsenicin A model compound (see structure in Table 4.1) was synthesized according to a reaction scheme reported by Marx *et al* [20]. For simplicity, from this point forward, Arsenicin A model compound is abbreviated as AA. As_2O_3 (0.40 g, 2.02 mmol), K_2CO_3 (0.29 g, 2.10 mmol), propionic acid (0.5 mL, 6.68 mmol), and propionic anhydride (2.2 mL, 17.16 mmol) were mixed together in a 50 mL r/b flask and then refluxed at 165°C for 2 h [20]. After the mixture was cooled to room temperature, 0.8 mL of water was added , followed by heating to 88°C for 1 h. Additional water (20 mL) was added to stop the reaction and the reaction product was extracted with CH_2Cl_2 (3 x 30 mL). The combined organic extracts were treated with anhydrous Na_2SO_4 and filtered. The liquid phase was evaporated from the filtrate. The resultant oily solid was dissolved in CH_2Cl_2 (20 mL) and fractionated by silica gel chromatography, eluted with CH_2Cl_2. The solvent was evaporated from the fractions of interest, yielding the Arsenicin A model compound (0.1094 g, 0.26 mmol, 26 % yield).

4.2.3 Total analysis of the marine sponge extracts

An Elan 6000 ICPMS system (PerkinElmer SCIEX) was operated in dynamic reaction cell mode using O_2 as the reaction gas. Arsenic species are detected by monitoring AsO^+ at 90.9165 amu (dwell time = 100 ms) to avoid an isobaric interference of $Ar^{40}Cl^{35}$ with As^{75}. The ICPMS was operated at 1350 W rf-power, and 6.75 V lens voltage, with argon flows of 15 L min^{-1} (plasma gas),

0.5 L min^{-1} (nebulizer gas), and 1.5 L min^{-1} (auxiliary gas). Sample solutions were delivered at a flow rate of 1.2 mL min^{-1} to a cross-flow nebulizer and then finally to a hydrofluoride (HF)-resistant Scott-type spray chamber.

The 10x or 100x diluted marine sponge extracts were further diluted 10 times in 1% HNO_3. The arsenic calibration standard (10 mg L^{-1}, Agilent Technologies, Mississauga, ON, CA) was diluted to 1, 5, 10, 15, and 20 µg L^{-1} in 1% HNO_3 and used to construct a calibration plot. Standard reference material 1643e (Trace Elements in Natural Water, National Institute of Standards and Technologies, Gaithersburg, MD, USA) with the original total arsenic concentration of 60.45 ± 0.72 µg/L was used to validate the accuracy of the method. There were good agreements (≤ 3%) between the certified and the analyzed values for SRM 1643e. The limit of detection was approximately 0.01 µg L^{-1}.

4.2.4 HPLC-ICPMS analysis of the marine sponge extracts

Arsenic species were quantified based on the chromatographic peak area by external calibration against standard solutions of specific arsenic species and further validated by a standard reference material (NIST). Turbochrom Navigator (PerkinElmer) graphic edit software was used to integrate the peak areas. The limit of detection for HPLC-ICPMS was approximately 0.05 µg L^{-1} for most arsenic species.

Ion pair chromatography for the determination of arsenic

A reverse-phase C18 octadecylsilane (ODS) analytical column (Phenomenex, Prodigy^{TM} ODS-3V, 150 × 4.60 mm, 3 µm particle size, Torrance,

CA, USA) was connected to a guard column (Phenomenex, SecurityGuard^{TM}, 4.0 × 3.0 mm cartridges) and both were placed in a column compartment that was maintained at 50℃. The mobile phase consisted of 3 mM malonic acid (Fisher Scientific), 5 mM tetrabutylammonium hydroxide (Aldrich), and 5% methanol (Fisher Scientific). The pH of the mobile phase was adjusted to 5.65 by 10% HNO_3 (Fisher Scientific). The flow rate of the mobile phase was 1.2 mL min^{-1}. The run time was 8 minutes.

Anion-exchange chromatography for the determination of arsenic

An anion-exchange column (Hamilton, PRP-X100, 150 × 4.10 mm, 5 µm particle size) was used to separate AsB from As^{III}. A PRP-X100 guard column (Hamilton, 20 mm length, 2.0 mm i.d., 10 µm particle size) was coupled to the front end of the anion-exchange column. The mobile phase was made of NH_4HCO_3 (Sigma-Aldrich, ReagentPlus®, ≥ 99.0 %, Oakville, Ontario, CA) and 5% methanol (Fisher Chemical, HPLC grade, Ottawa, Ontario, CA) with pH adjusted to 8.5 by NH_4OH (Fisher Chemical, Optima grade, Ottawa, Ontario, CA). A step gradient elution was performed with 10 mM NH_4HCO_3 at 0.8 mL min^{-1} for the first 2 minutes , and 50 mM NH_4HCO_3, at 2.0 mL min^{-1} for the subsequent 6 minutes. Re-equilibration was carried out with 10 mM NH_4HCO_3 at 0.8 mL min^{-1} for 2 minutes.

4.3 Results and discussion

4.3.1 Stability of Arsenicin A

Replicate solutions of Arsenicin A at 10 µg L^{-1} were prepared and then placed on the HPLC autosampler at room temperature. These solutions were

injected sequentially into ion pair HPLC-ICPMS. The resulting chromatograms showed that as time progressed, Arsenicin A, which eluted at approximately 2.0 minutes, converted to the denominated “converted Arsenicin A” that eluted at approximately 4.5 minutes (Fig. 4.1(a)). The peak areas of Arsenicin A and the converted Arsenicin A were plotted against time (Fig. 4.1(b)). It was evident that at room temperature, as time increased, the peak area of the converted Arsenicin A peak increased while the Arsenicin A peak decreased.

Table 4.1 Structures of various arsenic species present in marine sponges.

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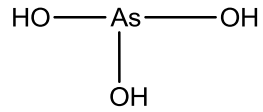
<td> 
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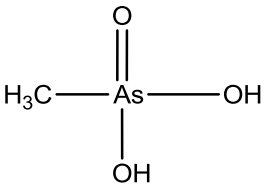
<td> 
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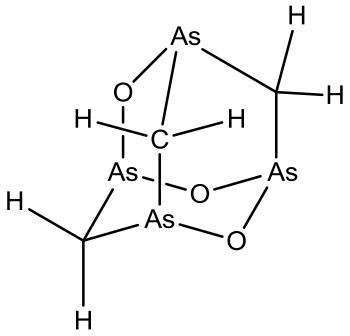
<td colspan="2"> 
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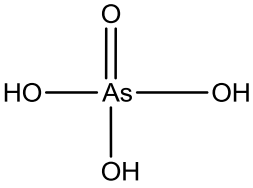
<td> 
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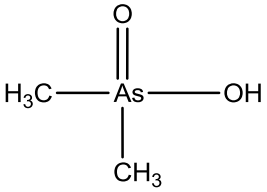
<td> 
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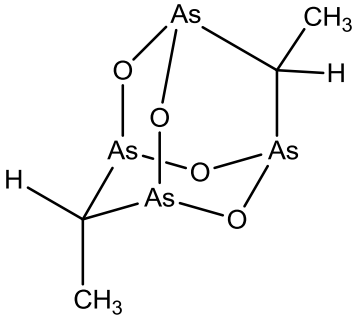
<td colspan="2"> 
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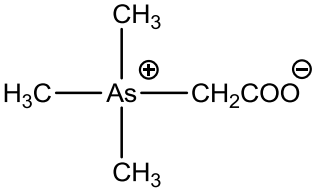
<td> 
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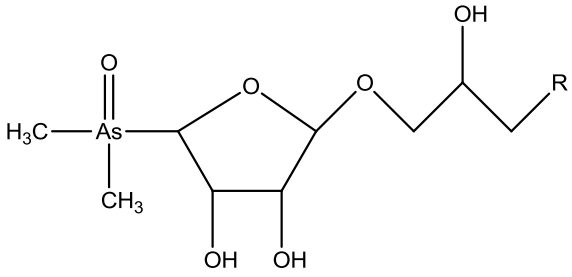
<td colspan="3"> 
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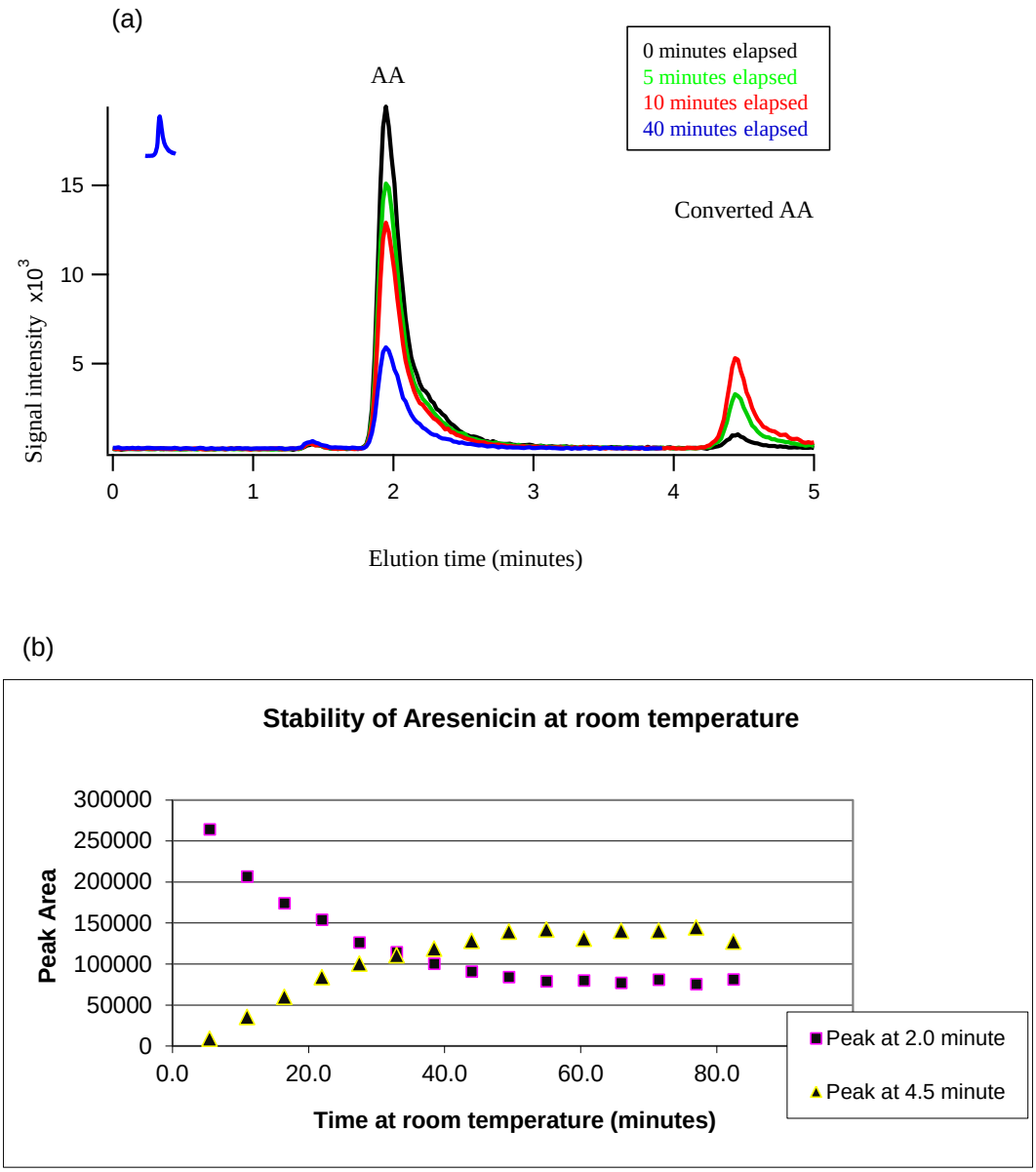
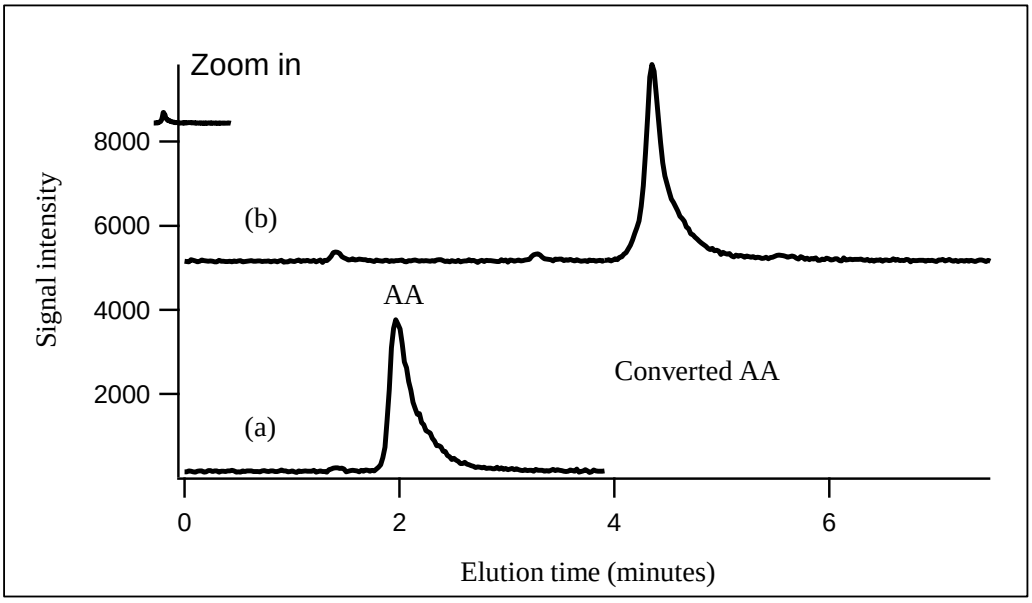


Fig. 4.1 The stability of Arsenicin A model compound at room temperature. (a) chromatograms obtained from ion pair chromatography overlapped when 10 µg L^{-1} Arsenicin A model compound was exposed to room temperature for 0, 5, 10, and 40 minutes; (b) Peak area of Arsenicin A model compound eluted at 2.0 minutes versus the peak area of the converted Arsenicin A model compound eluted at 4.5 minutes.

Different stock concentrations and storage temperatures were tested to determine the appropriate storage condition for Arsenicin A. 86 mg L^{-1} and 10 µg L^{-1} AA were both stored at room temperature for 8 h and diluted to 10 µg L^{-1} immediately before injecting into the HPLC-ICPMS. It was shown that AA had less conversion at 86 mg L^{-1} (Fig. 4.2(a)), while a significant extent of conversion, though incomplete, was observed at 10 µg L^{-1} (Fig. 4.2(b)). When 86 mg L^{-1} AA was stored at 4°C, there was less conversion than when stored at room temperature (Fig. 4.2(c)). This set of experiments suggested that Arsenicin A was more stable when stored at the mg L^{-1} concentration range at 4°C, rather than at the µg L^{-1} concentration range at room temperature.



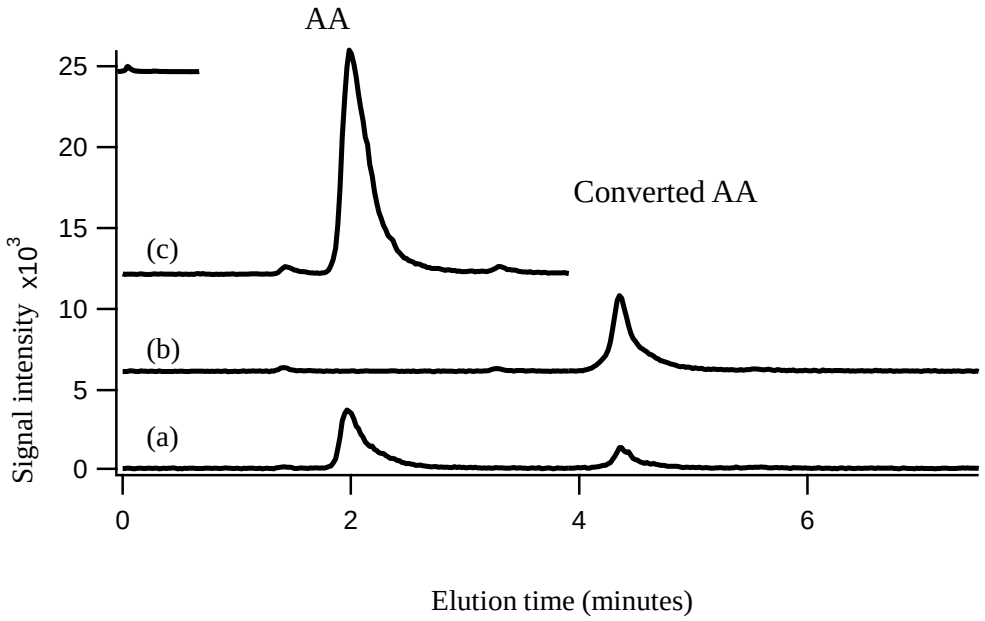


Fig. 4.2 Ion pair chromatography of Arsenicin A model compound at different concentrations and storage temperatures. (a) 86 mg L^{-1} at room temperature for 8 hours, diluted to 10 µg L^{-1} just before injection to HPLC-ICPMS; (b) 10 µg L^{-1} at room temperature for 8 hours; (c) 86 mg L^{-1} at 4°C for 8 hours, diluted to 10 µg L^{-1} just before injection to HPLC-ICPMS.

Additional experiments were conducted to further study how the presence of other arsenicals affected the stability of Arsenicin A at 4°C. 5 μg L^{-1} mixtures of As^{III}, AA, DMA^{V}, MMA^{V}, As^{V} freshly prepared from the stock solutions at the mg L^{-1} concentrations was either injected immediately or after an 8 h storage period at 4°C. The resulting chromatograms illustrated that there was only a slight difference in the speciation profile (Fig. 4.3). Therefore, the result suggested that at 4°C Arsenenicin A was stable enough within the typical time frame of HPLC analysis. The pH effect on the Arsenicin A conversion was also studied and it appeared that the Arsenicin A conversion was unaffected by pH within the range of 4–7 (Fig. 4.4).

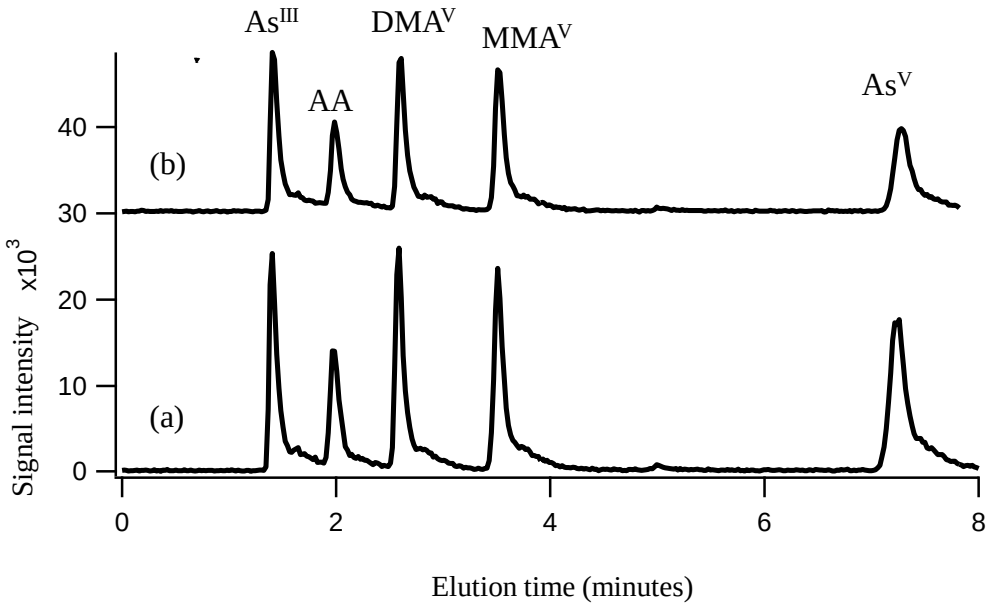


Fig. 4.3 Arsenicin A model compound at the same concentration but different storage periods. (a) 5 µg L^{-1} at 4°C inject immediately after it is made; (b) 5 µg L^{-1} at 4°C for 8 h before injection into HPLC-ICPMS.

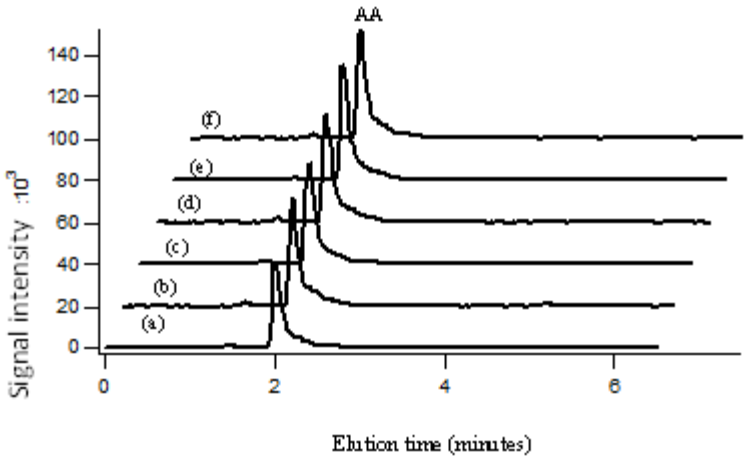


Fig. 4.4 Arsenicin A model compound at 10 µg L^{-1} at various pH values. (a) pH 4; (b) pH 5; (c) pH 5.5; (d) pH 6; (e) pH 6.5; (f) pH 7.

Long-term stability studies showed that after being stored at 4°C for half a year, Arsenicin A model compound consisted of mostly the original Arsenicin A model compound, and a minor fraction of the "converted Arsenicin A model compound" (Fig. 4.5(c)). When stored at room temperature for a year and half, the original AA was converted completely to three different species: As^{III}, As^{V}, and the converted AA (Fig. 4.5(b)).

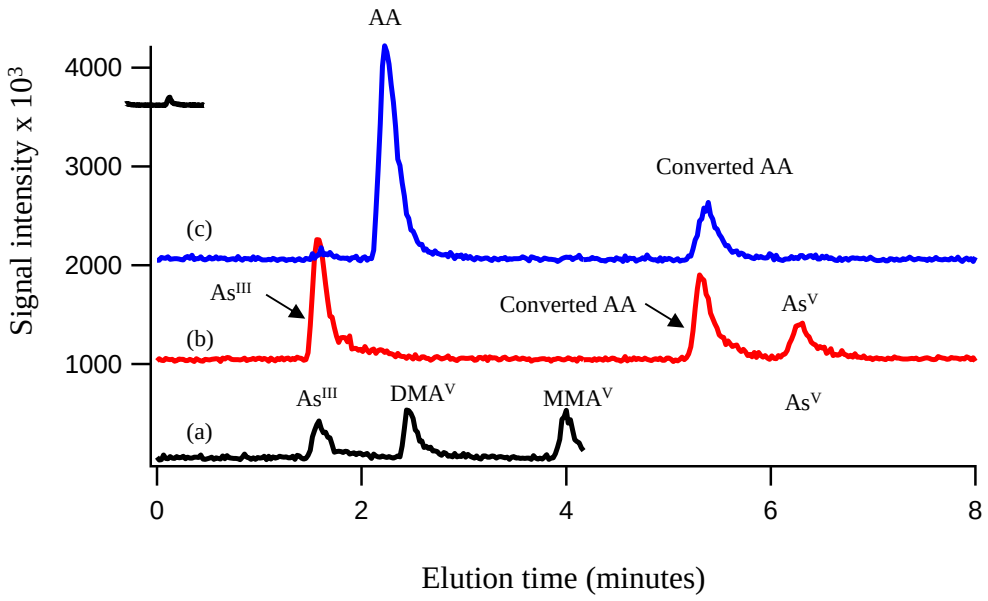


Fig. 4.5 Arsenicin A model compound and its converted species. (a) 1 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, As^{V}; (b)10 µg L^{-1} converted AA: diluted from 86 mg L^{-1} stored at room temperature for 1.5 years; (c)10 µg L^{-1} AA : diluted from 173 mg L^{-1} of AA that was stored at 4°C for 0.5 years.

4.3.2 Total arsenic concentrations in marine sponge extracts

The 160 marine sponge samples were prepared with appropriate dilutions in 1% HNO_3 before total arsenic analysis by ICPMS. There were 20 samples with total arsenic concentrations ≥ 5000 μg L^{-1}, 71 samples were 1000–5000 μg L^{-1}, and 69 samples were ≤ 1000 μg L^{-1}. The average total arsenic concentration was 1746 ± 1866 μg L^{-1}, with a median value of 1243 μg L^{-1}. A scatter plot of these 160 marine sponges samples was constructed on a log scale (Fig. 4.6) showing that the total arsenic concentrations in these marine sponges are widely distributed, spanning from tens μg L^{-1} to tens mg L^{-1}. Speciation analyses were subsequently performed on the selected 20 samples from each of the three concentrations sub-ranges (≥ 5000, 1000 – 5000 , ≤ 1000 μg L^{-1}) to obtain an overall picture of the arsenic speciation profiles in these marine sponges.

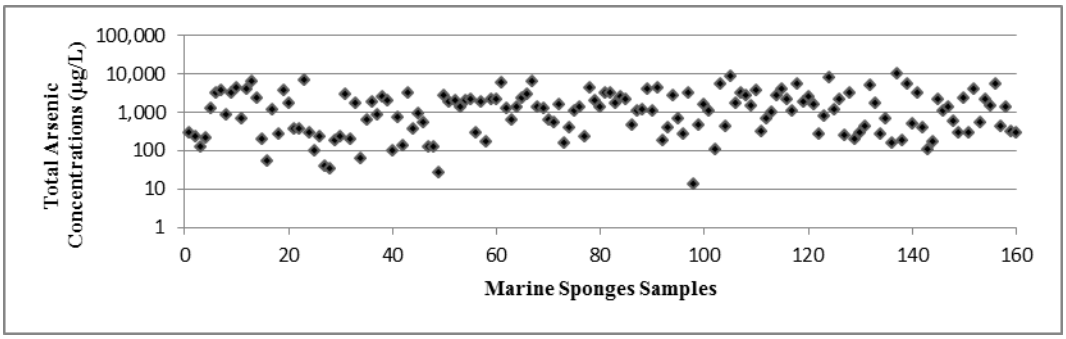


Fig. 4.6 Total arsenic concentrations in 160 marine sponges samples. There were 20 samples with arsenic concentrations ≥ 5000 μg L^{-1}, 71 samples with 1000 – 5000 μg L^{-1}, and 69 samples with ≤ 1000 μg L^{-1}. The average total arsenic concentration is 1746 ± 1866 μg L^{-1} with a median value of 1243 μg L^{-1}. It is estimated that the marine sponges contained approximately 124 μg of arsenic/g of marine sponges.

4.3.3 Speciation analysis in marine sponge extracts

Marine sponges with high concentrations of AsB

Most of the marine sponges in this study contained AsB as the major arsenic species. For illustration, ion pair chromatographic analysis of a 5x diluted sample from marine sponge #1F4 (Fig. 4.7(c)) revealed that it contained mostly As^{III}/AsB (co-eluted ≈ 1.7 minutes), and trace amount of DMA^{V}, MMA^{V}, and an unknown species that eluted around 5.8 minutes. A 10x dilute sample from Marine sponge #1F4 (Fig. 4.8(c)) spiked with AA (Fig. 4.8(b)) or the converted AA (Fig. 4.7(b)) confirmed that the marine sponge matrix did not shift the elution time of AA (Fig. 4.8(d)) and that the unknown species (~ 5.8 minutes) was not the converted AA (~ 5.3 minutes) (Fig. 4.7(d)).

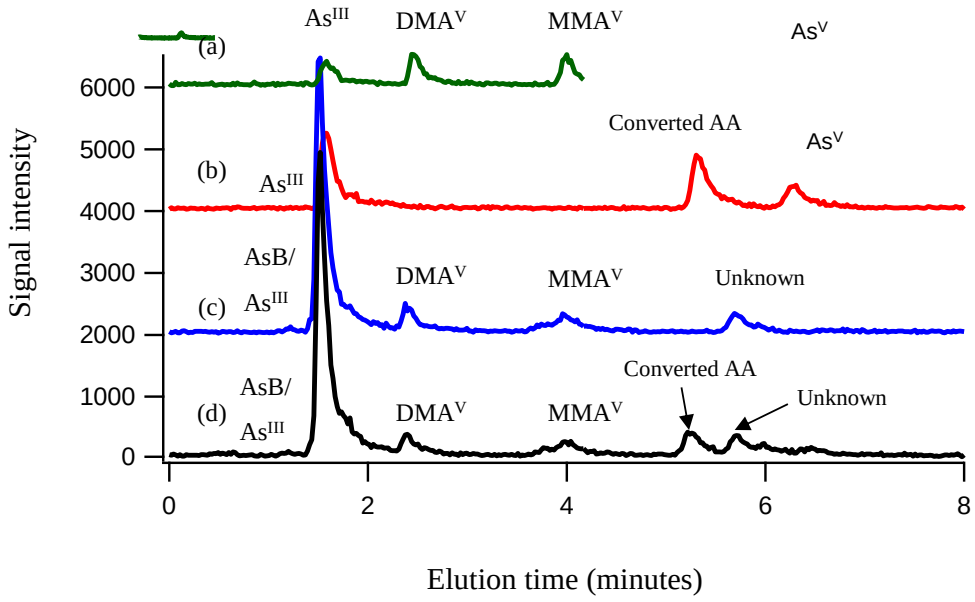


Fig. 4.7 Determination of arsenic species in diluted marine sponge sample #1F4 using ion pair chromatography separation and ICPMS detection. (a) 1 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, As^{V}; (b) 10 µg L^{-1} converted AA; (c) Marine sponge #1F4 after 5x dilution; (d) Marine sponge #1F4 after 5x dilution and spiked with 20 µg L^{-1} converted AA.

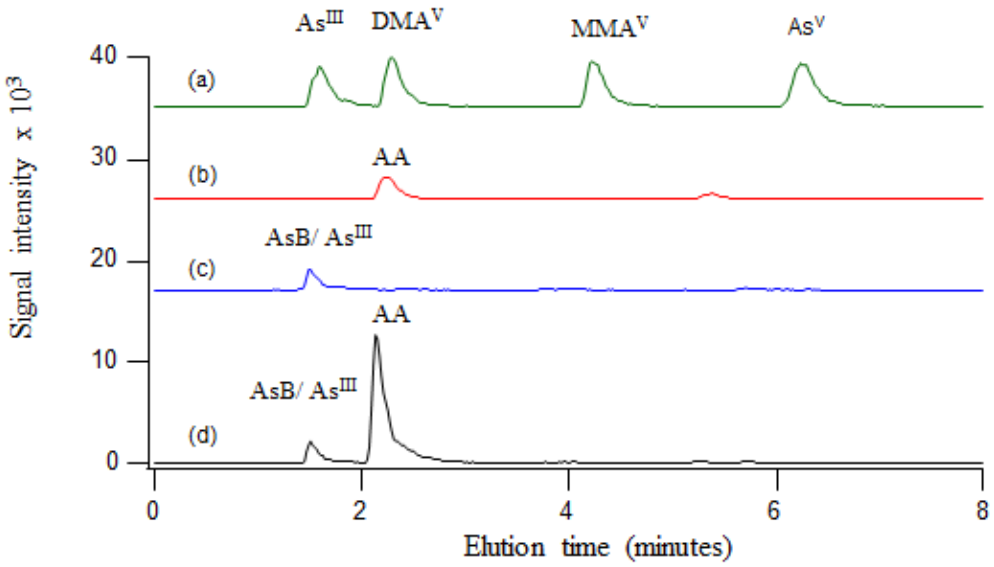


Fig. 4.8 Determination of arsenic species in diluted marine sponge sample #1F4 using ion pair chromatography and ICPMS detection. (a) 10 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, As^{V}; (b) 10 µg L^{-1} AA; (c) Marine sponge #1F4 after 10x dilution; (d) Marine sponge #1F4 after 10x dilution and spiked with 24 µg L^{-1} AA.

The marine sponge #1F4 sample was spiked with the brown algae extracts, which showed that the unknown arsenic specie was unlikely arsenosugars (Fig. 4.9(d)). Brown algae extract (Fig. 4.9(c)) contained 4 arsenosugars (~ 1.4, 3.3, 4.4, 6.3 minutes), DMA^V (~ 2.7 minutes), and As^V (~ 7.2 minutes).

Figure 4.10 illustrated the chromatograms from the HPLC-ICPMS analyses of the diluted marine sponge samples #1F4, after spiking with various concentrations (1, 2, 3, 4, and 5 µg L^{-1}) of MMA^V and As^V. Since the unknown arsenic species in the sponge sample eluted between MMA^V and As^V, MMA^V and As^V were used as standards to estimate the concentration of the unknown arsenic species. The concentration of the unknown was estimated to be 1.30 ± 0.22 and 1.75 ± 0.29 µg L^{-1} using calibration against MMA^V or As^V, respectively (Fig. 4.10).

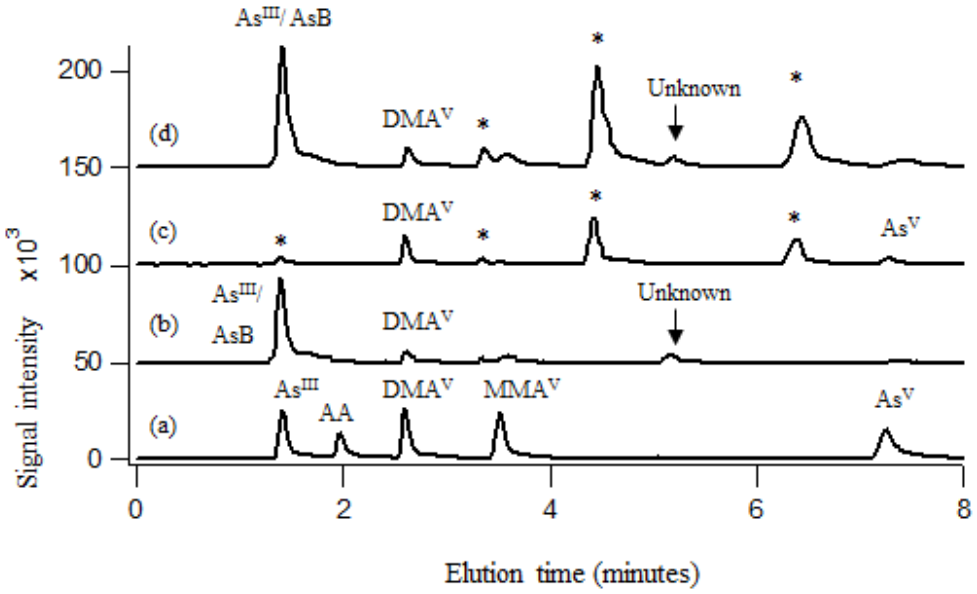


Fig. 4.9 Determination of arsenic species in diluted marine sponge sample #1F4 using ion pair chromatography and ICPMS detection. (a) 5 µg L^{-1} each of As^{III}, AA, DMA^V, MMA^V, As^V; (b) Marine sponge # 1F4 after 10x dilution; (c) Arsenosugars (*) from a brown algae extract; (d) Marine sponge #1F4 after 10x dilution and spiked with the algae extract containing the arsenosugars.

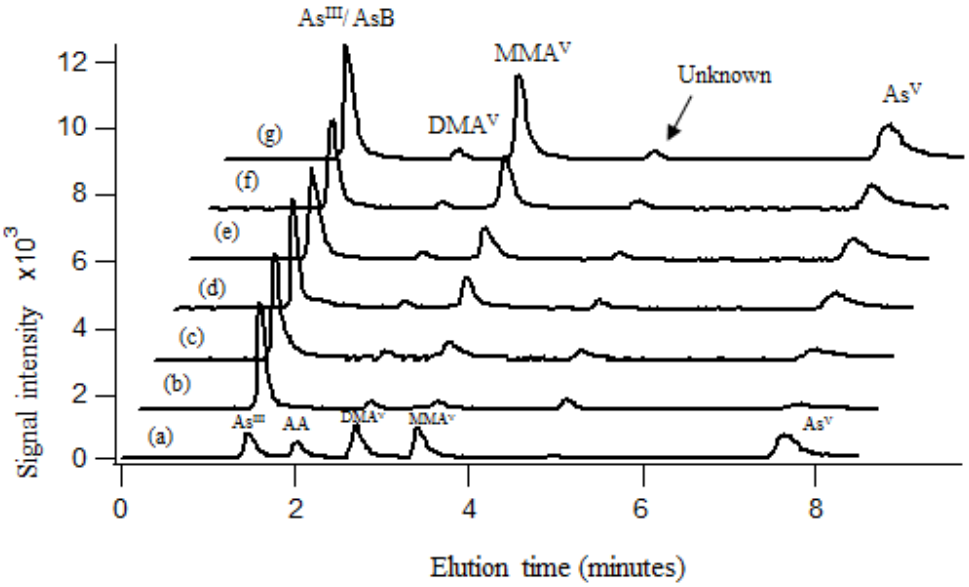


Fig. 4.10 Ion pair chromatography – ICPMS analyses of diluted marine sponge sample #1F4 spiked with various concentrations of MMA^V and As^V. (a) 1 µg L^{-1} each of As^{III}, AA, DMA^V, MMA^V, As^V; (b) Marine sponge # 1F4 after 10x dilution; (c) Marine sponge #1F4 after 10x dilution spiked with 1 µg L^{-1} each of MMA^V and As^V; (d) Marine sponge #1F4 after 10x dilution spiked with 2 µg L^{-1} each of MMA^V and As^V; (e) Marine sponge #1F4 after 10x dilution spiked with 3 µg L^{-1} each of MMA^V and As^V; (f) Marine sponge #1F4 after 10x dilution spiked with 4 µg L^{-1} each of MMA^V and As^V; (g) Marine sponge #1F4 after 10x dilution spiked with 5 µg L^{-1} each of MMA^V and As^V.

Because AsB and As^{III} co-eluted in ion pair chromatography, anion exchange chromatography was used to separate these two species (Fig. 4.11). The concentrations of AsB and As^{III} were quantified using the anion exchange chromatography with ICPMS detection (Table 4.2). The concentrations of Arsenicin A, DMA^V, MMA^V, and As^V were obtained from the ion pair chromatography ICPMS analyses. Hence, the combination of ion pair and anion exchange chromatography successfully achieved the determination of seven arsenic species: AsB, As^{III}, Arsenicin A, DMA^V, MMA^V, As^V, and an arsenosugar (4.4 min).

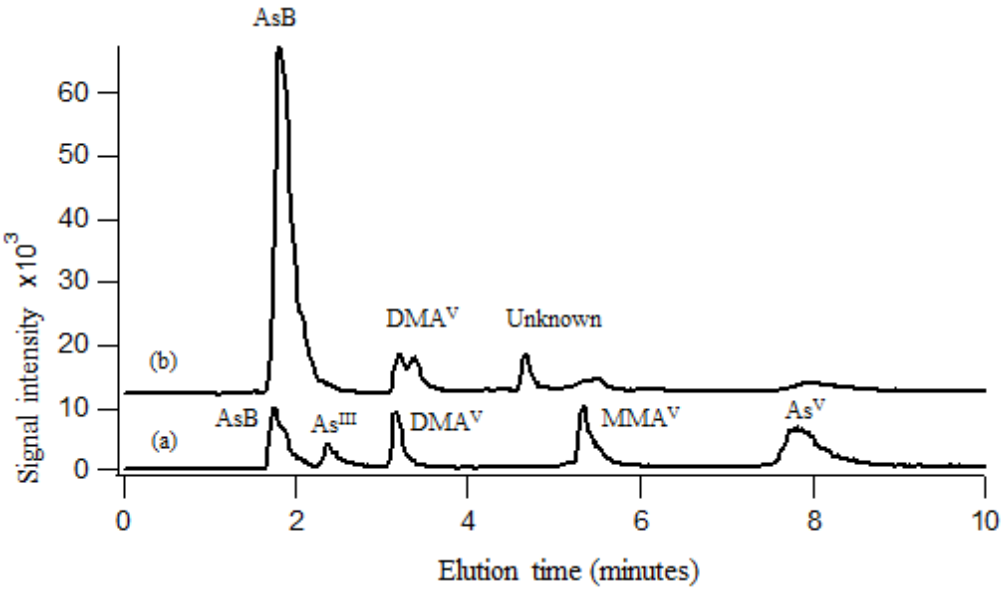


Fig. 4.11 Analyses of diluted marine sponge sample #1F4 using anion exchange chromatography separation and ICPMS detection. (a) 2.5 µg L^{-1} each of As^{III}, AA, DMA^V, MMA^V, As^V; (b) Marine sponge # 1F4 after 10x dilution.

Table 4.2 Concentrations of arsenic species (µg L^{-1}) in selected marine sponge samples, determined by ion pair chromatography and anion exchange chromatography separation couped to ICPMS detection.

Arsenic species	**1F4 after 10x dilution (ug/L)**	**1A6 after 100x dilution (ug/L)**	**2F5 after 10x dilution (ug/L)**
AsB	15.0 ± 0.6	1.38 ± 0.06	12.1 ± 0.5
As^{III}	n.d.	n.d.	n.d.
Unknown	1.3 ± 0.2[a], 1.8 ± 0.3[b]	3.6 ± 0.2[a], 3.3 ± 0.2[b]	n.d.
Arsenosugar 3 (4.4 minutes)	n.d.	n.d.	0.56 ± 0.08[a], 0.7 ± 0.2[b]
DMA^V	0.3 ± 0.1	0.03 ± 0.01	0.8 ± 0.1
MMA^V	0.34 ± 0.04	0.025 ± 0.004	1.3 ± 0.1
As^V	0.10 ± 0.08	0.04 ± 0.01	0.18 ± 0.04

[a] quantified against the MMA^V standard; [b] quantified against the As^V standard; n.d., not detectable

Marine sponges with low concentrations of AsB

Ion pair chromatographic analysis of marine sponge #1A6 showed an unknown arsenic species (~ 5.8 minutes) as the major component (Fig. 4.12(c)). When spiked with AA (Fig. 4.7(b)) or the converted AA (Fig. 4.13(b)), again it was confirmed that the marine sponge matrix did not shift the elution time of AA (Fig. 4.12(d)). The unknown species (~ 5.8 minutes) was not the converted AA (~ 5.3 minutes) (Fig. 4.13(d)).

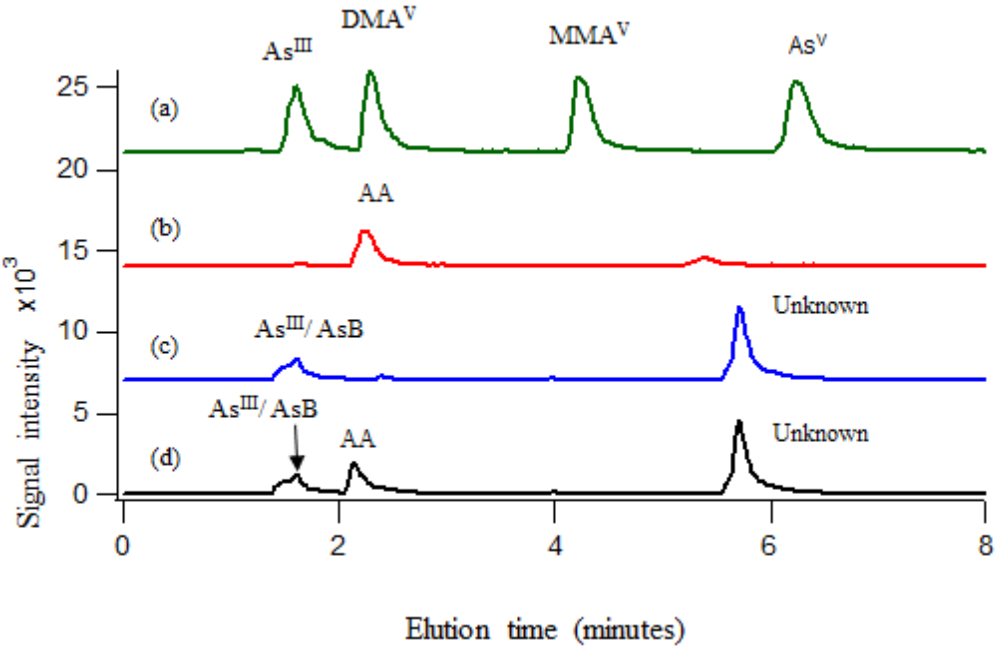


Fig. 4.12 Determination of arsenic species in diluted marine sponge sample #1A6 using ion pair chromatography and ICPMS detection. (a) 10 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, As^{V}; (b) 10 µg L^{-1} AA; (c) Marine sponge # 1A6 after 10x dilution; (d) Marine sponge # 1A6 after 10x dilution spiked with 24 µg L^{-1} AA.

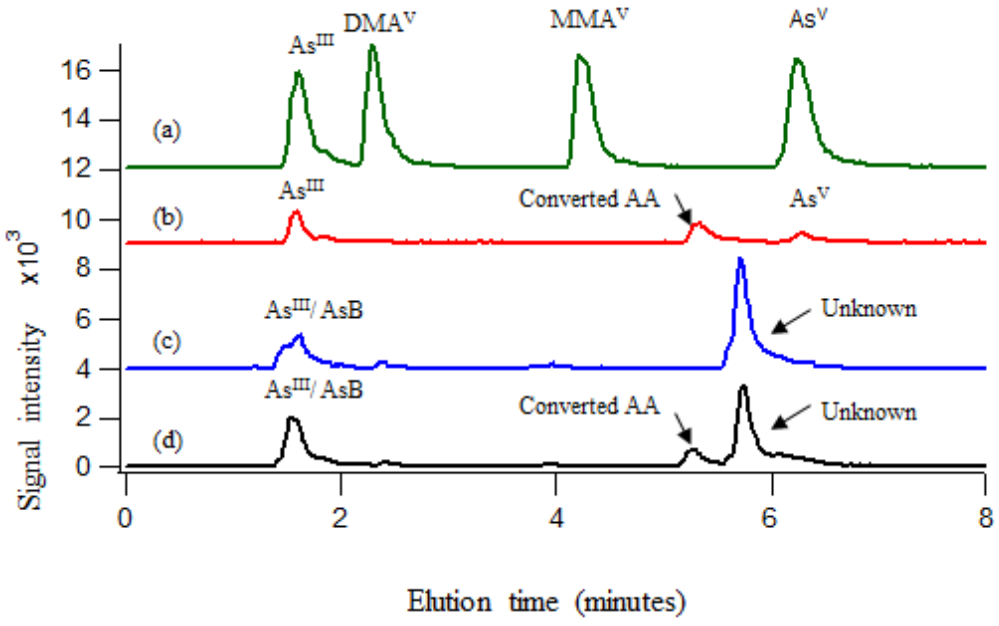


Fig. 4.13 Determination of arsenic species in diluted marine sponge sample #1A6 using ion pair chromatography and ICPMS detection. (a) 10 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, As^{V}; (b) 10 µg L^{-1} converted AA; (c) Marine sponge # 1A6 after 10x dilution; (d) Marine sponge # 1A6 after 10x dilution spiked with 36 µg L^{-1} converted AA.

This unknown species was not an arsenosugar from the brown algae extract as shown in the analysis of marine sponge #1A6 spiked with the brown algae extracts (Fig. 4.14(b)–(d)). The concentration of the unknown was estimated to be 3.56 ± 0.17 µg L^{-1} and 3.27 ± 0.16 µg L^{-1}, respectively, based on MMA^V or As^V standards (Fig. 4.15). Anion exchange chromatographic analysis showed that there was 1.38 ± 0.06 µg L^{-1} of AsB present with non-detectable As^{III} (Fig. 4.16).

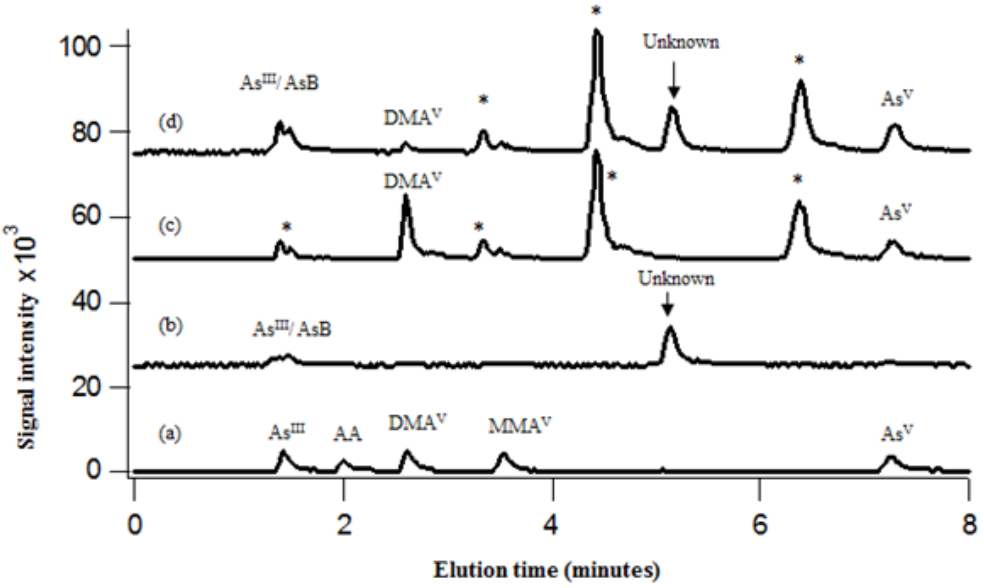


Fig. 4.14 Determination of arsenic species in marine sponge sample #1A6 using ion pair chromatography and ICPMS detection. (a) 5 µg L^{-1} each of As^{III}, AA, DMA^V, MMA^V, As^V; (b) Marine sponge # 1A6 after 100x dilution; (c) Arsenosugars (*) from a brown algae extract; (d) Marine sponge #1A6 after 100x dilution and spiked with the algae extract containing the arsenosugars.

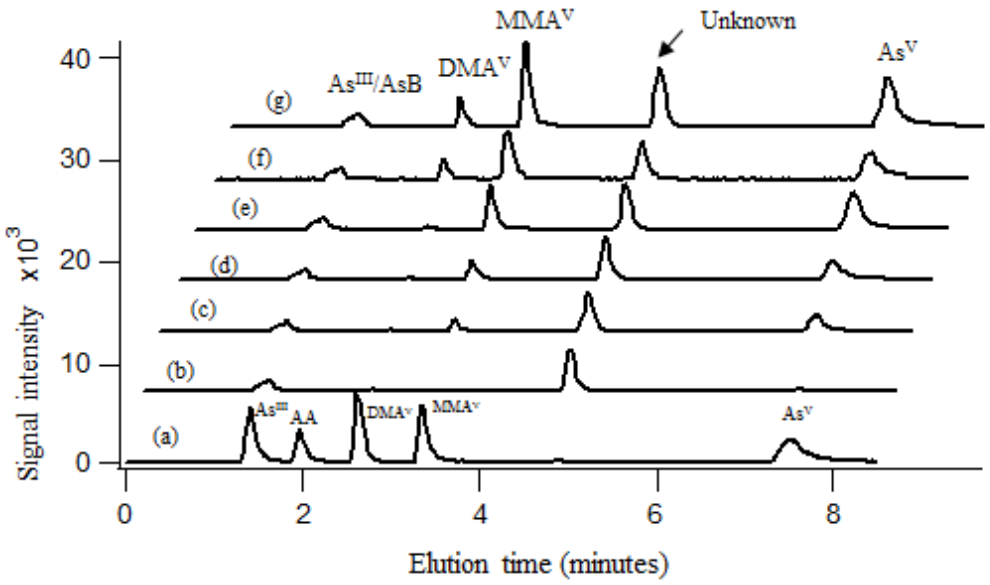


Fig. 4.15 Ion pair chromatography – ICPMS analyses of diluted marine sponge sample #1A6 spiked with various concentrations of MMA^V and As^V. (a) 1 µg L^{-1} each of As^{III}, AA, DMA^V, MMA^V, As^V; (b) Marine sponge # 1A6 after 100x dilution; (c) Marine sponge #1A6 after 100x dilution spiked with 1 µg L^{-1} each of MMA^V and As^V; (d) Marine sponge #1A6 after 100x dilution spiked with 2 µg L^{-1} each of MMA^V and As^V; (e) Marine sponge #1A6 after 100x dilution spiked with 3 µg L^{-1} each of MMA^V and As^V; (f) Marine sponge #1A6 after 100x dilution spiked with 4 µg L^{-1} each of MMA^V and As^V; (g) Marine sponge #1A6 after 100x dilution spiked with 5 µg L^{-1} each of MMA^V and As^V.

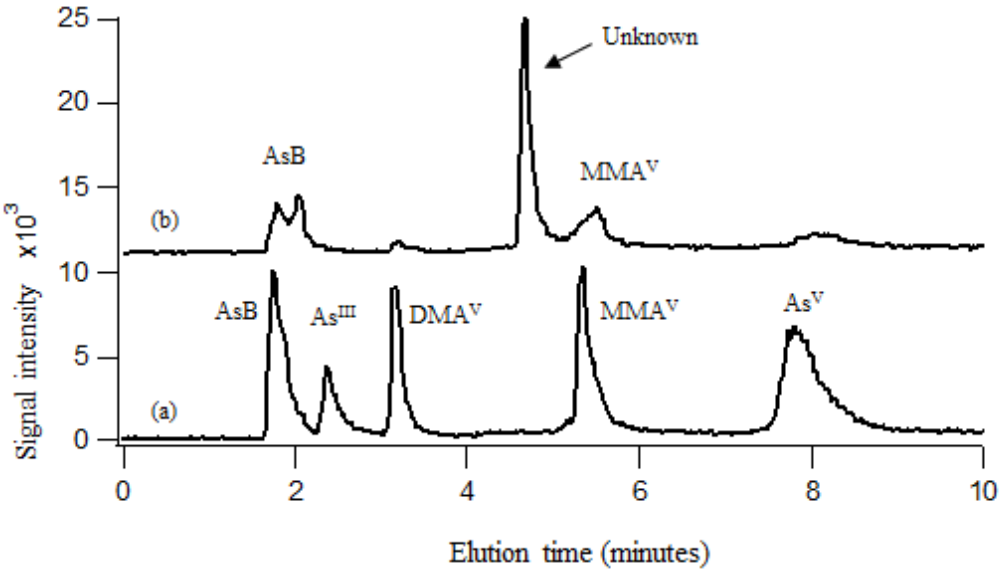


Fig. 4.16 Analyses of diluted marine sponge sample #1A6 using anion exchange chromatography separation and ICPMS detection. (a) 2.5 µg L^{-1} each of As^{III}, AA, DMA^{V}, MMA^{V}, As^{V}; (b) Marine sponge # 1A6 after 100x dilution.

Marine sponges possibly containing arsenosugar

An unknown arsenic species was also detected in sample #2F5 by ion pair chromatography (Fig. 4.17(c)). After spiking with AA (Fig. 4.17(d)), converted AA (Fig. 4.18(d)) or brown algae extract (Fig. 4.19(d)), the unknown arsenic species (~ 4.4 minute) appeared to be one of the arsenosugars in the algae extract. The retention time corresponded to that of arsenosugar 3 (Table 4.1) [19, 21]. The concentration of this arsenic species was estimated to be 0.56 ± 0.08 µg L^{-1} and 0.73 ± 0.21 µg L^{-1}, respectively, based on MMA^{V} and As^{V} as the standards (Fig. 4.20). Anion exchange chromatography was used to determine the concentration of AsB and As^{III}, which were 12.31 ± 0.47 µg L^{-1} and non-detectable, respectively (Fig. 4.21).

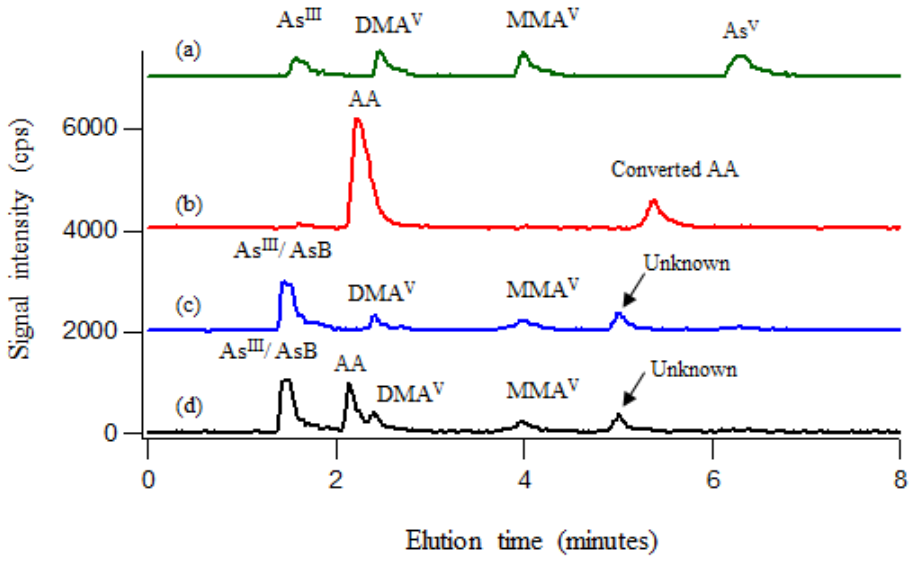


Fig. 4.17 Determination of arsenic species in diluted marine sponge sample #2F5 using ion pair chromatography and ICPMS detection. (a) 10 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, As^{V}; (b) 10 µg L^{-1} AA; (c) Marine sponge # 2F5 after 10x dilution; (d) Marine sponge # 2F5 after 10x dilution spiked with 12 µg L^{-1} AA.

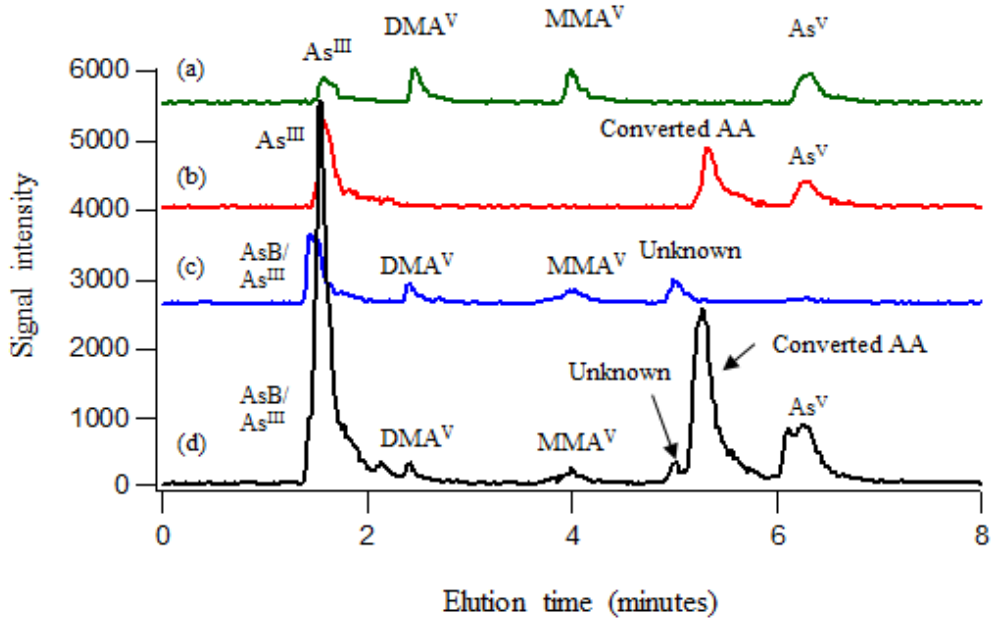


Fig. 4.18 Determination of arsenic species in diluted marine sponge sample #2F5 using ion pair chromatography and ICPMS detection. (a) 10 µg L^{-1} each of As^{III}, DMA^{V}, MMA^{V}, As^{V}; (b) 10 µg L^{-1} converted AA; (c) Marine sponge # 2F5 after 10x dilution; (d) Marine sponge # 2F5 after 10x dilution spiked with 12 µg L^{-1} converted AA.

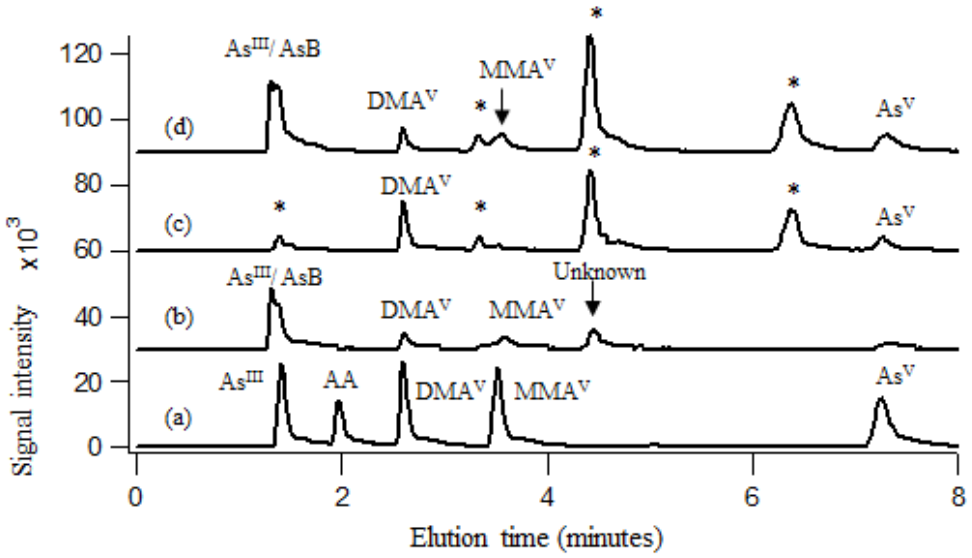


Fig. 4.19 Determination of arsenic secies in diluted marine sponge sample #2F5 using ion pair chromatography and ICPMS detection. (a) 5 µg L^{-1} each of As^{III}, AA, DMA^{V}, MMA^{V}, As^{V}; (b) Marine sponge # 2F5 after 10x dilution; (c) Arsenosugars (*) from a brown algae extract; (d) Marine sponge #2F5 after 10x dilution and spiked with the algae extract containing the arsenosugars.

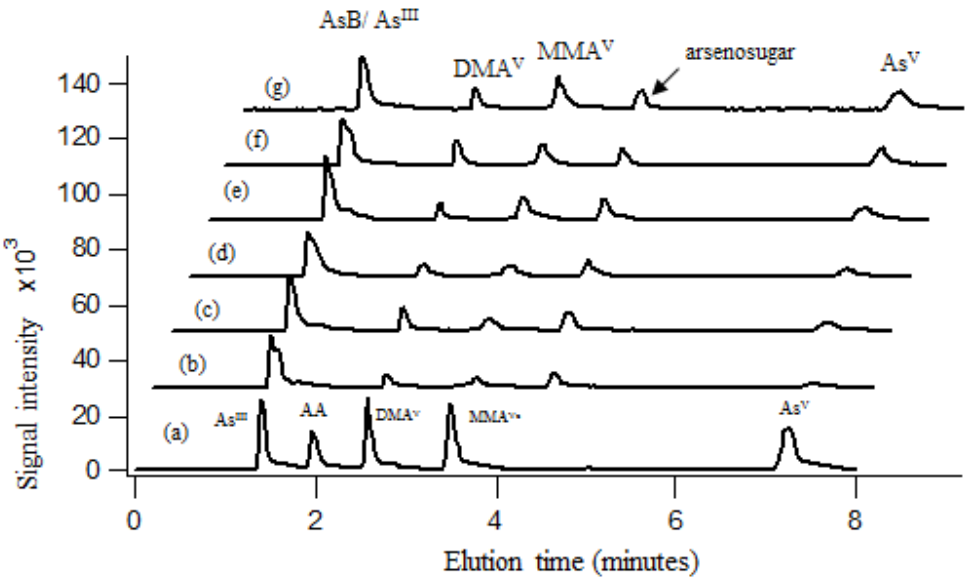


Fig. 4.20 Ion pair chromatography – ICPMS analyses of diluted marine sponge sample #2F5 spiked with various concentrations of MMA^{V} and As^{V}. (a) 1 µg L^{-1} each of As^{III}, AA, DMA^{V}, MMA^{V}, As^{V}; (b) Marine sponge # 2F5 after 10x dilution; (c) Marine sponge #2F5 after 10x dilution spiked with 0.5 µg L^{-1} each of MMA^{V} and As^{V}; (d) Marine sponge #2F5 after 10x dilution spiked with 1 µg L^{-1} each of MMA^{V} and As^{V}; (e) Marine sponge #2F5 after 10x dilution spiked with 2 µg L^{-1} each of MMA^{V} and As^{V}; (f) Marine sponge #2F5 after 10x dilution spiked with 3 µg L^{-1} each of MMA^{V} and As^{V}; (g) Marine sponge #2F5 after 10x dilution spiked with 4 µg L^{-1} each of MMA^{V} and As^{V}.

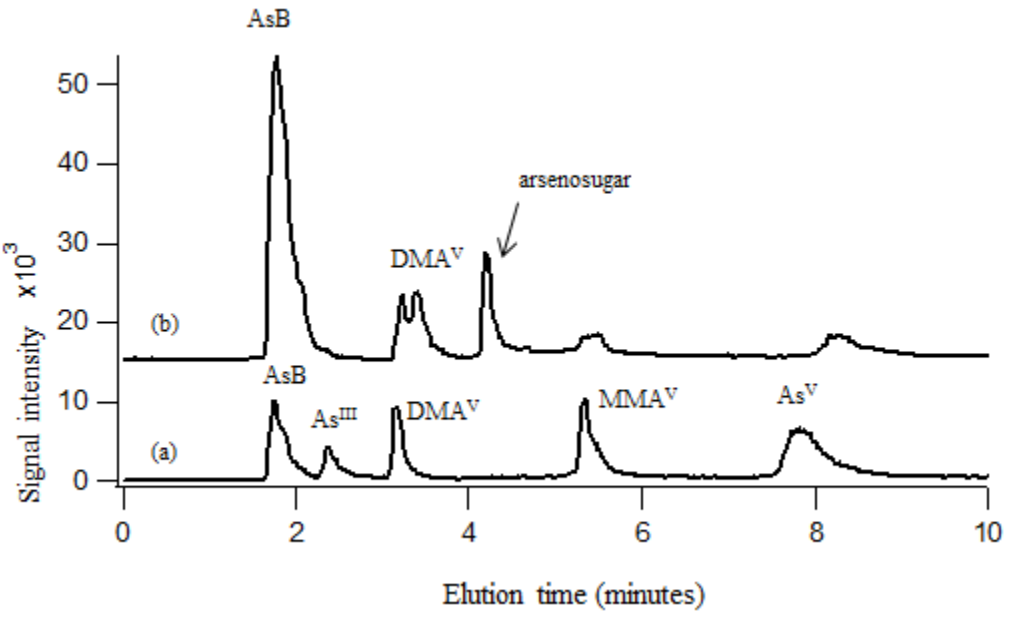


Fig. 4.21 Determination of arsenic species, including an arsenosugar, in diluted marine sponge sample #2F5 using anion exchange chromatography separation and ICPMS detection. (a) 2.5 µg L^{-1} each of As^{III}, AA, DMA^V, MMA^V, As^V; (b) Marine sponge # 2F50 after 10x dilution.

The arsenic speciation was determined in 20 selected samples (Table 4.3). The representative speciation results of marine sponge samples #1F4, 1A6, and 2F5 suggested no Arsenicin A was present in these samples. However, an unknown arsenic species was found in the ng L^{-1} range (Table 4.2). The identity of the unknown species has yet to be determined. These marine sponges were found to consist of AsB (28–98%), DMA^V (0–10%), MMA^V (0–22%), As^V (0–18%), arsenosugars (0–16%), and unknown species (0–70%). This distribution coincided with other reports on marine animals where inorganic arsenic and the simple methylated arsenicals were the minor species, while the more complicated organic arsenicals such as AsB was the major component (70 – 98%) [22, 23].

The median total arsenic concentration from the 160 marine sponges samples was approximately 124 mg of As/kg of marine sponges, which was comparable to 100 mg of As/kg of algae reported by another research group [24].

The total arsenic concentration and arsenic speciation profile are highly dependent on the class of organisms, dietary intake, and habitats in which they were found [23, 25]. This may provide an explanation to the absence of Arsenicin A in these marine sponges, as they were collected from Papua New Guinea instead of New Caledonia where the natural Arsenicin A was originally reported [11]. In addition, these marine sponges may not necessary be *Echinochalina bargibanti*; hence, the sponges may have different biotransformation efficiency of converting As^{III} to different methylated arsenic species [10]. Moreover, Mancini *et al*. were able to isolate Arsenicin A from the CH_2Cl_2 extract of the sponges, whereas in this study, methanol (CH_3OH) extract was used in the analysis. Therefore, the possible combination of differences in marine sponge species, harvest locations, and organic extracts, may contribute to the absence of Arsenicin A in the marine sponges from this study.

Table 4.3 Concentrations of arsenic species in 20 selected marine sponge samples, determined by ion pair chromatography and anion exchange chromatography separation coupled to ICPMS detection.

Sample	**Dilution factor**	**AsB**	**DMA^V**	**MMA^V**	**As^V**	**Arseno-sugar 3**	**unknown**
1A6	100	1.38 ± 0.06	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	n.d.	3.4 ± 0.2
1B4	100	5.03 ± 0.09	n.d.	0.65 ± 0.04	n.d.	n.d.	n.d.
1B5	100	4.1 ± 0.3	0.09 ± 0.01	1.0 ± 0.1	n.d.	n.d.	n.d.
1B9	10	6.7 ± 0.4	n.d.	1.57 ± 0.01	0.03 ± 0.01	n.d.	n.d.
1C4	100	6.3 ± 0.1	n.d.	2.2 ± 0.4	n.d.	1.6 ± 0.5	n.d.
1C7	10	7.7 ± 0.4	0.59 ± 0.01	0.29 ± 0.01	0.04 ± 0.01	n.d.	n.d.
1D3	10	10.8 ± 0.2	0.06 ± 0.01	0.13 ± 0.08	0.05 ± 0.01	n.d.	n.d.
1E9	10	20.1 ± 0.1	n.d.	0.83 ± 0.07	n.d.	n.d.	n.d.
1F4	10	15.0 ± 0.6	0.27 ± 0.06	0.34 ± 0.04	0.10 ± 0.08	n.d.	1.5 ± 0.3
1G2	100	4.2 ± 0.4	n.d	1.17 ± 0.04	n.d	n.d.	n.d.
1G5	100	5.8 ± 0.1	0.71 ± 0.09	0.17 ± 0.02	1.4 ± 0.3	n.d.	n.d.
1G8	100	5.4 ± 0.1	n.d.	1.4 ± 0.2	n.d.	n.d.	n.d.
2C3	10	0.35 ± 0.02	0.05 ± 0.01	0.11 ± 0.01	n.d.	n.d.	n.d.
2C4	100	6.4 ± 0.4	n.d.	2.02 ± 0.07	n.d.	1.1 ± 0.3	n.d.
2D9	100	29.6 ± 0.7	n.d.	2.8 ± 0.4	n.d.	n.d.	n.d.
2E5	100	62.2 ± 0.9	n.d.	5.6 ± 0.1	n.d.	n.d.	n.d.
2F5	10	12.3 ± 0.5	0.8 ± 0.1	1.3 ± 0.1	0.18 ± 0.04	0.7 ± 0.2	n.d.
2F8	100	6.8 ± 0.4	n.d.	n.d.	0.29 ± 0.01	n.d.	n.d.
2F10	100	35.1 ± 0.8	n.d.	8.7 ± 0.1	n.d.	n.d.	n.d.
2H7	100	20.0 ± 0.9	n.d.	1.4 ± 0.2	n.d.	n.d.	n.d.

n.d., not detectable

4.4 Conclusion

The developed method of high performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC-ICPMS) with complementary separation techniques involved ion pair and anion exchange chromatography. The method successfully differentiated 7 arsenic species: Arsenicin A model compound, As^{III}, As^{V}, MMA^{V}, DMA^{V}, AsB and an arsenosugar. Arsenicin A model compound has to be stored at 4℃, and dilute solutions are prepared on the same day as the analyses in order to minimize species conversion. Analysis of 160 Papua New Guinean marine sponges samples revealed a wide range of total arsenic concentrations, which spanned 3 orders of magnitude (μg L^{-1} to mg L^{-1}), with an average of 1746 ± 1866 μg L^{-1}. The median total arsenic concentration in these sponges was approximately 124 μg of As/g of marine sponges. Speciation analyses of 20 selected samples showed that these sponges consisted of AsB (28–98%), As^{V} (0–18%), MMA^{V} (0–22%), DMA^{V} (0–10%), arsenosugar (0–16%), and unknown (0–70%). Although our developed complementary chromatographic technique was capable of separating Arsenicin A model compound from the other arsenicals, Arsenicin A model compound was not detected in these marine sponges. The identity of the unknown arsenic species have yet to be determined. Further research using electrospray tandem mass spectrometry could contribute to the characterization of these arsenic species. Future work could also involve establishing any correlations between the arsenic speciation profile with the species of the marine sponges and other invertebrates.

4.5 References

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CHAPTER 5 A comparative study of cellular uptake and speciation between an Arsenicin A model compound and arsenite in A549 human lung carcinoma cell line

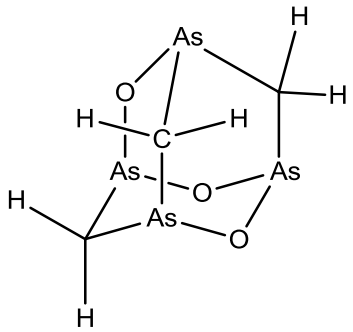
5.1 Introduction

Arsenicin A is a natural arsenical, initially isolated from the marine sponge, *Echinochalina bargibanti* [1], and subsequently a model compound was synthesized [1– 4]. Studies have shown that Arsenicin A possesses bactericidal and fungicidal activity on several human pathogenic strains, [1]. We are interested in exploring the toxicity of this natural organoarsenical, as compared to inorganic arsenite (Fig. 5.1).

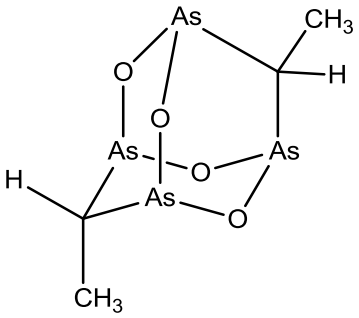
Since it is difficult to isolate a large quantity of Arsenicin A from the marine sponge as evident in Chapter 4, an Arsenicin A model compound (AA, Fig. 5.1(b)) has been synthesized for us to conduct our investigation. Our recent *in vitro* toxicological study using human lung carcinoma A549 cell line has shown that AA is more toxic than As^{III}. A549 cells are human lung carcinoma epithelial cells that are relatively easy to grow with a doubling time around 20–22 h [5]. Our group has determined that the IC_{50} values for A549 cells with a 24 h incubation period are 3.5 μM for AA and 76.6 μM for As^{III} [6] Arsenic trioxide (As_2O_3, Trisenox®), which solubilizes to As^{III} in aqueous solution, is currently used to treat Acute Promyelocytic Leukemia (APL) [7] . As_2O_3 has demonstrated significant efficacy for APL, with complete remission rates of 70 to 90% in newly diagnosed patients, and 65 to > 90% in relapsed APL patients [7–9]. Studies

confirmed that As_2O_3 at 0.06–0.2 mg/kg of body weight is needed to induce complete remission in 85–92% APL patients [10, 11]. It would be advantageous to find other arsenicals that are more toxic than As^{III}, such that lower dosages of the arsenicals could be used in the therapy and to potentially reduce possible drug antagonism.

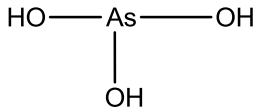
It is not clear how AA excerts a higher toxicity than As^{III} and if cellular uptake of these arsenicals plays a role in the differences in toxicity. Our objectives of this chapter are two folds: to compare the intracellular arsenic concentrations and to determine the speciation of arsenic in A549 cells after 24 h of incubation with either AA or As^{III} at their fractional concentrations below the respective IC_{50} values. High performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICPMS) was used to analyze cells and medium samples that were incubated with either AA or As^{III}. This research will contribute to a better understanding of the roles of cellular uptake and metabolism of arsenic species in their relative toxicity to the cells.



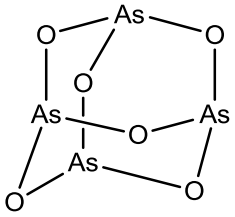
(a) Arsenicin A



(b) Arsenicin A model compound



(c) inorganic arsenite (As^{III})



(d) arsenic trioxide

Fig. 5.1 Structures of (a) Arsenicin A, (b) Arsenicin A model compound, (c) inorganic As^{III}, (d) arsenic trioxide.

5.2 Experimental

5.2.1 Cell cultures

A549 cells were obtained from American Type Culture Collection [5]. These adherent cells originated from human lung carcinoma epithelium [5]. These cells were cultivated in RPMI Media 1640 [+] $_{L}$-Glutamine (Gibco at Life Technologies, Burlington, ON, CA) with 10% Fetal Bovine Serum (Sigma,

Canadian origin, sterile filtered, Oakville, ON, CA) and 1% penicillin/streptomycin (10,000 µg/L, Gibco at Life Technologies, Burlington, ON, CA) at 37.0°C and 5.0% CO_2. 11 µL of cell suspension was put on a counting chamber of a hemocytometer (Bright-line at Hausser Scientific, Horsham, PA, USA). Cell count consisted of an average of the counts from four quadrants in a counting chamber. The cells were seeded at a density of 4.0×10^4 cells/mL in a 6 well cell culture plate (Greiner Cellstar, Okaville, ON, CA). These cells were subsequently treated with AA or As^{III} at the desired concentrations followed by incubation for 24 h. 0.05% Trypsin-EDTA (Gibco at Life Technologies, Burlington, ON, CA) was used to detach cells from the plate during cell harvesting. Reagents and arsenic species were stored in a 4°C fridge. All cell culture procedures were done in a Biological Service Cabinet Forma Class II, A2 (See Supporting information)

5.2.2 Chemicals and reagents

The Arsenicin A model compound was synthesized according to an reaction scheme developed by Marx *et al*. [12]. As_2O_3 (0.40 g, 2.02 mmol), K_2CO_3 (0.29 g, 2.10 mmol), propionic acid (0.5 mL, 6.68 mmol), and propionic anhydride (2.2 mL, 17.16 mmol) were mixed together in a 50 mL round bottom flask then refluxed at 165°C for 2 h. Water (0.8 mL) was added to the cooled room temperature reaction mixture, followed by heating to 88°C for 1 h. Additional water (20 mL) was added to the cooled room temperature reaction mixture, followed by extraction with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were treated with Na_2SO_4, filtered and the liquid phase evaporated from the

filtrate. The oil and solid mixture product was dissolved in CH_2Cl_2 (20 mL) and loaded onto a silica gel column, eluted with CH_2Cl_2. The solvent was evaporated from the fractions containing the Arsenicin A model compound. An overall yield of 26% (0.1094 g or 0.26 mmol) was achieved. ^{1}H NMR was used to characterize the Arsenicin A model compound.

Sodium m-arsenite ($NaAsO_2$, 98.0%), sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$, 99.4%), and dimethylarsinic acid ($C_2H_7AsO_2$, 98.0%) were purchased from Sigma-Aldrich (Oakville, ON, CA). Monosodium acid methane arsonate (CH_4AsNaO_3, 99.0%) was purchased from Chem Service Inc. (West Chester, PA, USA). Monomethylmonothioarsenic acid (MMMTA), dimethylmonothioarsenic acid (DMMTA), and dimethyldithioarsenic acid (DMDTA) were synthesized by our lab and were prepared fresh just before speciation analysis by dissolving 0.0085 g, 0.0073 g, and 0.0090 g, respectively, in three separate aliquots of 1 mL of Milli-Q deionized water. The concentrations of these arsenic species were standardized against As^{III} standard.

5.2.3 ICPMS conditions and method for the determination of arsenic

An Elan 6000 ICPMS system (PerkinElmer SCIEX) was operated in the dynamic reaction cell mode using O_2 as the reaction gas. Arsenic species were detected by monitoring AsO^+ at 90.9165 amu (dwell time = 100 ms) to avoid an isobaric interference of $Ar^{40}Cl^{35}$ with As^{75}. The ICPMS was operated at 1350 W rf-power, and 6.75 V lens voltage, with argon flows of 15 L min^{-1} (plasma gas), 0.5 L min^{-1} (nebulizer gas), and 1.5 L min^{-1} (auxiliary gas). Solutions were

delivered at a flow rate of 1.2 mL min^{-1} to a cross-flow nebulizer and then to a hydrofluoride (HF)-resistant Scott-type spray chamber.

Standard reference material 1640a (Trace Elements in Natural Water) from the National Institute of Standards and Technologies (Gaithersburg, MD, USA) with total arsenic concentration of 8.075 ± 0.070 µg L^{-1} was used for quality control. Calibration solutions at concentrations of 0.1, 0.5, 1, 5, and 10 µg L^{-1} were prepared by serial dilutions from a 10 mg L^{-1} stock of the calibration standard (Agilent Technologies). The R^2 value of the calibration plot was at least 0.9999. There was good agreement (≤ 3%) between the certified and the analyzed values for SRM 1640a. The limit of detection was approximately 0.03 µg L^{-1} and was calculated as three times the standard deviation of blank.

5.2.4 HPLC-ICPMS conditions and method for the separation of arsenic species

A reverse-phase octyldecylsilane (ODS) column with 3 µm particle size, 100 Å pore size, and 150 × 4.60 mm column size (Phenomenex, Prodigy™ ODS-3V, Torrance, CA, USA) was placed in a column compartment that was maintained at 50°C. The mobile phase consisted of 3 mM malonic acid (Fisher Scientific), 5 mM tetrabutyl ammonium hydroxide (Aldrich), and 5% methanol (Fisher Scientific). The pH of the mobile phase was adjusted to 5.9 with 10% HNO_3 (Fisher Scientific). The mobile phase was filtered through a 0.45 µm pore size membrane and sonicated for 10 min before use. Isocratic elution at 1.2 mL min^{-1} was used for all analyses. Each sample was analyzed in triplicate with 30 µL/injection. Arsenic species were quantified based on external calibration

against standard solutions of specific arsenic species and validated by a standard reference material (NIST). Turbochrom Navigator (PerkinElmer) graphic edit software was used to integrate peak areas. Microsoft Office Excel was used to calculate the concentrations of arsenic species present in the samples. Finally, the IGOR (WaveMetrics) plotting program was used to convert text files obtained by Turbochrom Navigator software to a graphical representation illustrated throughout this chapter. The limit of detection for HPLC-ICPMS was approximately 0.1 μg L^{-1} for most arsenic species.

5.2.5 Preparation on cell samples

On the day of cell harvesting, the media were collected in 15 mL plastic centrifuge tubes (Corning, Fisher Scientific, Toronto, ON, CA) for later analysis. The cells were washed twice with 200 μL phosphate buffered saline solution (Gibco at Life Technologies, Burlington, ON, CA) per well to remove any residual arsenic on the cell surface and the plate surface. The cells were incubated with 200 μL of 0.05% Trypsin-EDTA (Gibco at Life Technologies, Burlington, ON, CA) per well for 3 min in order to detach cells from the bottom of the plate. 2 mL media per well was added to neutralize the Trypsin and the cell suspension was transferred to 15 mL plastic centrifuge tube. An IEC Centra CL2 Centrifuge (Thermo Electron Corporation, Asheville, NC, USA) was used to spin down cells at 1700 rpm for 3 min. Then the cell pellet was washed with 3 mL cold PBS to remove the last trace of arsenic that adhered to the surface of the cells. A hemocytometer was used to perform cell counts for each well. The cell counts would be used subsequently to normalize the concentrations of arsenic in the cells.

All of the data presented here were normalized against the number of cells in each well. The number of viable cells after incubation with As^{III} and AA further confirmed the previously determined IC_{50} values (Fig. 5.2). For total arsenic analysis, the cell pellet was re-suspended in 2% HNO_3 followed by 30 min sonication. After centrifugation at 2500 rpm for 30 min, the supernatant (cell lysate) was collected and diluted in 1% HNO_3 (typically10 times). Each treatment used 3 wells and each well had 3 determinations by ICPMS. Hence, the value reported was an average of 9 determinations for each treatment. The reported arsenic concentration of each treatment was the average of 3 wells (n = 3), where each well's arsenic concentration was normalized with respect to the cell number in the well. (Table 5.1, Fig. 5.3–5.4).

For speciation analysis, the cell pellet was re-suspended in 400 µL Milli-Q deionized water for the As^{III} treated samples. For the case of AA exposure, the cell pellet was re-suspended in 100 µL H_2O_2 (30%, Fisher Scientific, Canada) and 300 µL Milli-Q deionized water for 24 h. Appropriate dilutions were made with Milli-Q deionized water prior to HPLC speciation. For As^{III} treatment, the cell culture medium and the medium without cells incubation as the negative control were both diluted by 500 times; while the cell lysate were not diluted (Fig. 5.5). For AA treatment, the cell culture medium and medium without cells incubation as the negative control were diluted 50 fold (Fig. 5.6 and 5.8) while the cell lysate was not diluted (Fig. 5.7 and 5.8).

5.3 Results and discussion

5.3.1 Comparative uptake of As^{III} and AA in A549 cells

To better understand if there was a difference in the capabilities of A549 cells to take up AA and As^{III}, we treated A549 cells with 0.3–3.5 µM AA and 1.0–76.6 µM As^{III}. The uptake of AA was reported in unit of 10^6 arsenic atoms per cell (Table 5.1 (a)). A graphical representation of these data (Fig. 5.3(a)) illustrated that A549 cells were able to take up AA in a near-linearity fashion , particularly in the range of 1.8–3.5 µM. However, below 1.8 µM the analyses were challenged by the limit of detection offered by ICPMS. Therefore, it was difficult to distinguish the uptake between treatment with ≤1.8 µM AA and the controls. Similarly the uptake of As^{III} also resembled an analogous linear trend, especially around 38.3–76.6 µM range (Table 5.1 (b) and Fig. 5.3(b)). The analysis approached the limit of detection for ICPMS when the cells were incubated with $As^{III} \leq 38.3$ µM; hence, the uptake appeared to be non-linear below this concentration.

Table 5.1(a) Concentrations of total arsenic in A549 cells incubated with different concentrations of Arsenicin A model compound (AA) for 24 hours. The choices of AA were based on its IC_{50} value (3.5 μM) and the fractions of IC_{50} values.

Fraction of IC_{50} value	**[AA] (μM)**	**Equivalent [As] (μM)**	**Number of cells survived after AA treatment ($\times 10^4$ cells)**	**As atoms/ cell ($\times 10^6$)**
	0.3	1.2	26.3 ± 3.6	0.6 ± 0.1
2/8	0.9	3.5	24.4 ± 3.4	77 ± 5
4/8	1.8	7.1	19.2 ± 2.1	313 ± 23
5/8	2.2	8.8	17.7 ± 1.5	516 ± 33
6/8	2.6	10.6	17.5 ± 2.2	732 ±185
7/8	3.1	12.3	13.5 ± 1.5	814 ±127
1	3.5	14.1	13.6 ± 4.0	986 ±150

Table 5.1(b) Concentrations of total arsenic in A549 cells incubated with different concentrations of arsenite (As^{III}) for 24 hours. The choices of As^{III} were based on its IC_{50} value (76.6 μM) and the fractions of IC_{50} values.

Fraction of IC_{50} value	**[As^{III}] (μM)**	**Number of cells survived after As^{III} treatment ($\times 10^4$ cells)**	**As atoms / cell ($\times 10^6$)**
	1.0	26.4 ± 3.5	0.4 ± 0.1
2/8	19.2	22.0 ± 4.4	3 ± 1
4/8	38.3	19.3 ±1.3	59 ± 21
5/8	47.9	17.4 ± 1.0	110 ± 19
6/8	57.5	17.4 ± 1.6	169 ± 9
7/8	67.0	15.4 ± 0.2	205 ± 10
1	76.6	12.7 ± 2.8	255 ± 9

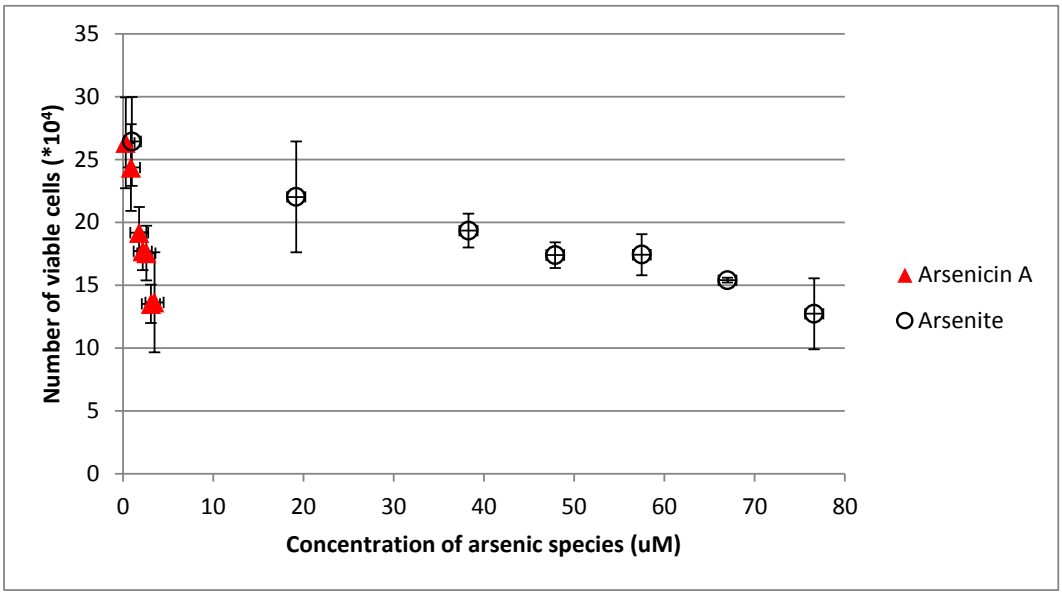


Fig. 5.2 (a) Number of cells survived after incubation with varying concentrations of either Arsenicin A model compound (AA) or arsenite (As^{III}) for 24 hours. The choices of AA and As^{III} were based on their IC_{50} value (3.5 µM for AA and 76.6 µM for As^{III}) and the fractions of IC_{50} values.

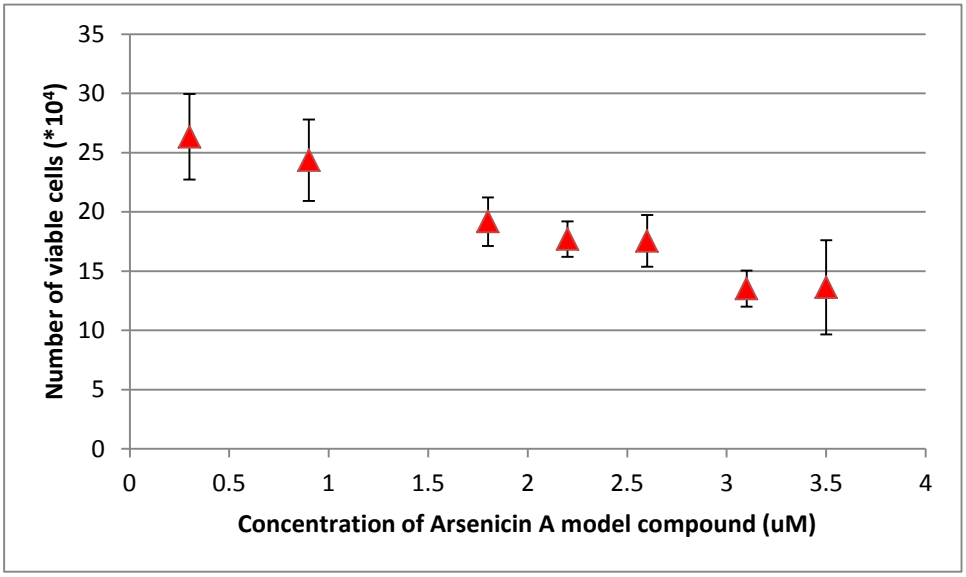


Fig. 5.2 (b) Zoom in on the Arsenicin A model compound data.

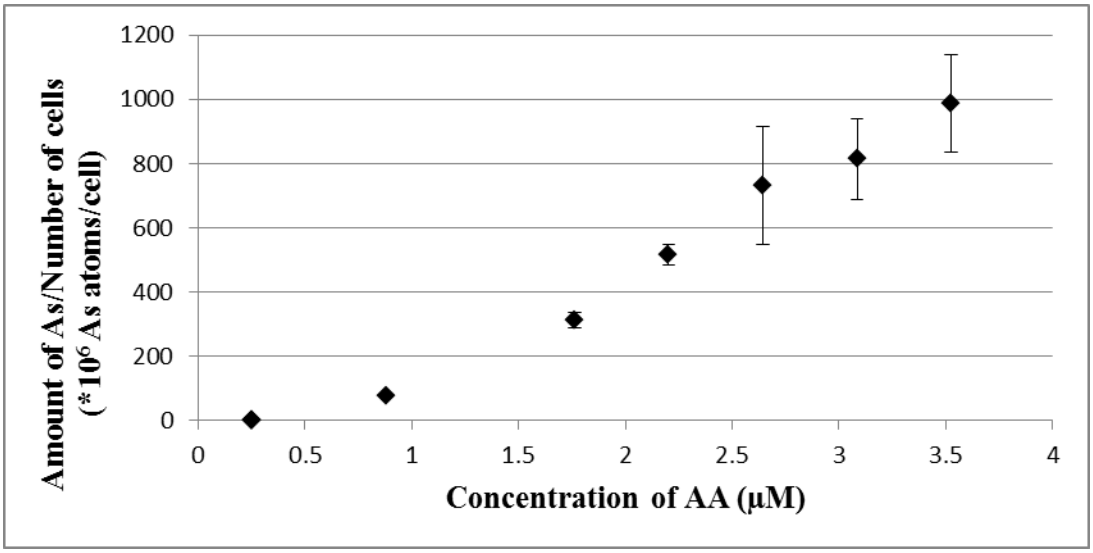


Fig. 5.3 (a) Concentrations of arsenic in A549 cells after incubation with varying concentrations (0.3–3.5 µM) of Arsenicin A model compound (AA) for 24 hours. The concentrations of arsenic were measured using ICPMS, and they were normalized against the number of cells. So the data were expressed as the number of arsenic atoms per cell.

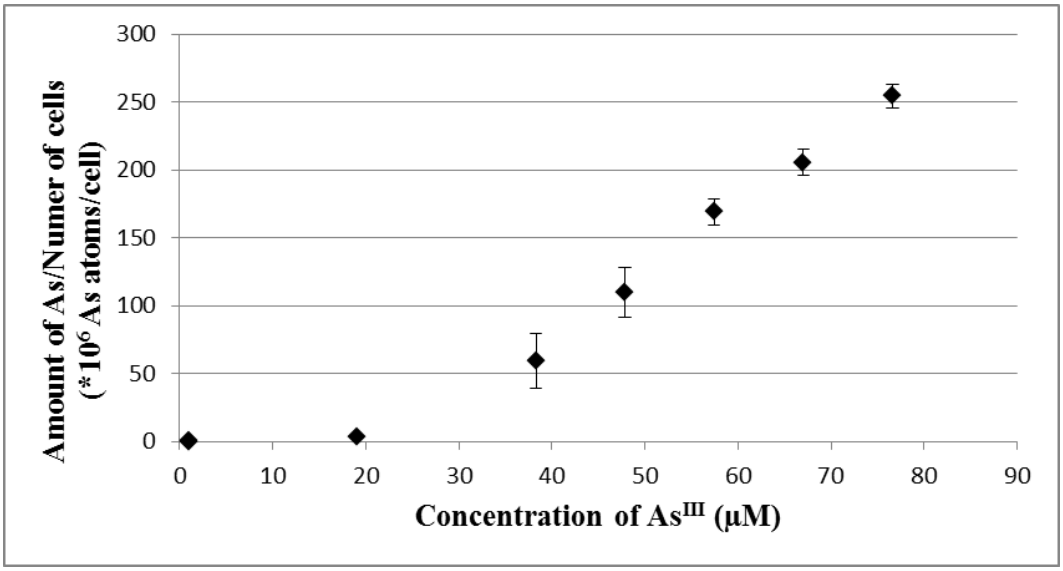


Fig. 5.3 (b) Concentrations of arsenic in A549 cells after incubation with varying concentrations (1.0–76.6 µM) of arsenite (As^{III}) for 24 hours. The concentrations of arsenic were measured using ICPMS, and they were normalized against the number of cells. So the data were expressed as the number of arsenic atoms per cell.

In order to compare the uptake of AA and As^{III} more efficiently, we paired up the results according to their respective fractions of IC_{50} values (Table 5.2). This table presented the cellular uptake of AA and As^{III} in unit of AA or As^{III} molecules per cell. A graphical illustration of these data depicted an interesting relationship between the cellular uptake and the chemical formulae of the two arsenic species (Fig. 5.4). It was evident that similar numbers of AA and As^{III} molecules were found in the cells, even though the cells were incubated with As^{III} at concentrations approximately 20 times higher than with AA. For example, at their respective IC_{50} values, $247 \pm 38 \times 10^6$ AA molecules per cell and $255 \pm 9 \times 10^6$ As^{III} molecules per cell were detected in A549 cells. These data verified that AA is more toxic than As^{III} to A549 cells after 24 h of incubation. There are four arsenic atoms per AA molecule (Fig. 5.1(b)); while there is one arsenic atom per As^{III} molecule (Fig. 5.1(c)).

Table 5.2 Concentrations of total arsenic in A549 cells incubated with different concentrations of either AA or As^{III} for 24 hours. Data were paired up according to the fractions of their respective IC_{50} values.

Fraction of IC_{50}	**Cell treatment (μM)**		**AA or As^{III} molecules/cells ($\times 10^6$)**	**As atoms/cell ($\times 10^6$)**
2/8	**AA**	**0.9**	**19 ± 1**	**77 ± 5**
	As^{III}	19.2	3 ± 1	3 ± 1
4/8	**AA**	**1.8**	**78 ± 6**	**313 ± 23**
	As^{III}	38.3	59 ± 21	59 ± 21
5/8	**AA**	**2.2**	**129 ± 8**	**516 ± 33**
	As^{III}	47.9	110 ± 19	110 ± 19
6/8	**AA**	**2.6**	**183 ± 46**	**732 ± 185**
	As^{III}	57.5	169 ± 9	169 ± 9
7/8	**AA**	**3.1**	**203 ± 32**	**814 ± 127**
	As^{III}	67.0	205 ± 10	205 ± 10
1	**AA**	**3.5**	**247 ± 38**	**986 ± 150**
	As^{III}	76.6	255 ± 9	255 ± 9

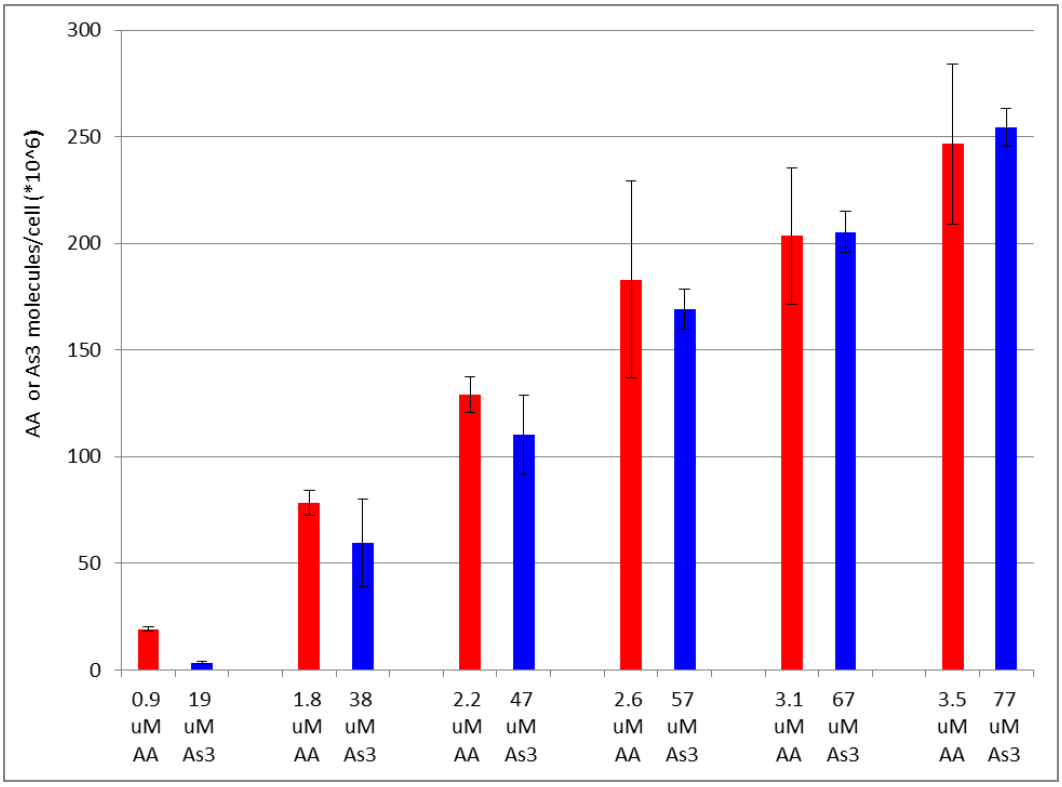


Fig. 5.4 (a) Comparison of Arsenicin A model compound (AA, red) and arsenite (As^{III}, blue) concentrations in A549 cells incubated with either AA or As^{III} at varying concentrations that were equivalent to their respective fractions IC_{50} values. The arsenic concentrations are expressed as the number of AA molecules or As^{III} molecules per cell.

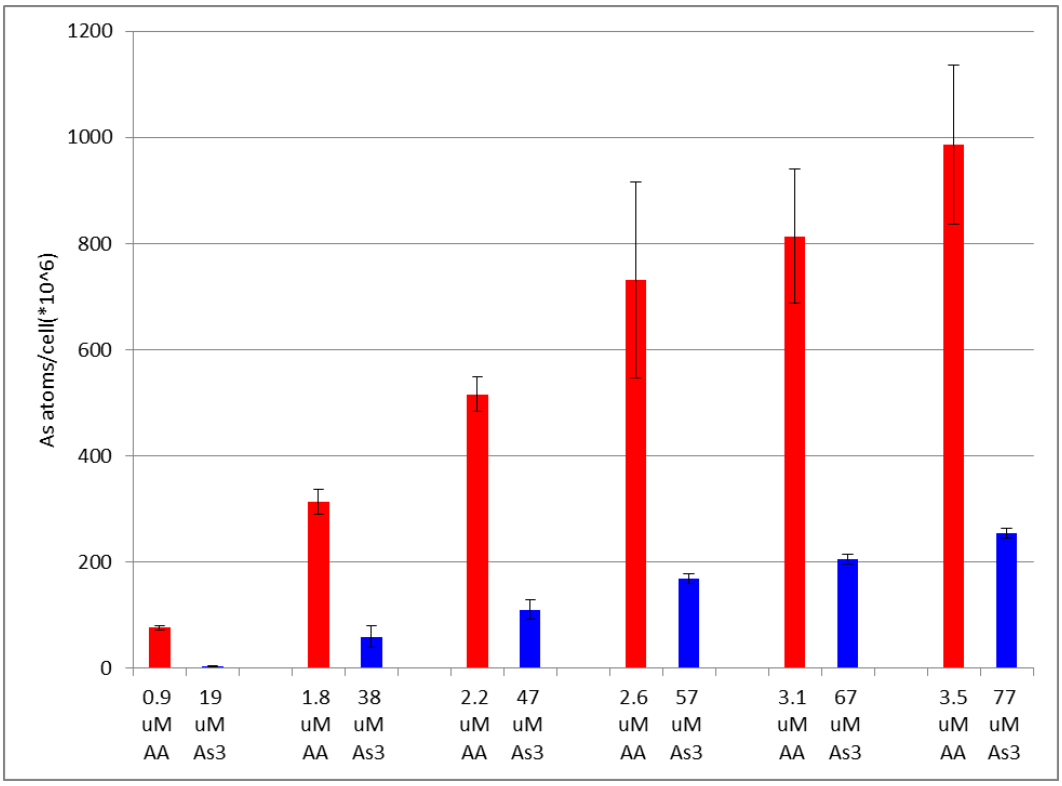


Fig. 5.4 (b) Comparison of Arsenicin A model compound (AA, red) and arsenite (As^{III}, blue) concentrations in A549 cells incubated with either AA or As^{III} at varying concentrations that were equivalent to their respective fractions of IC_{50} values. The arsenic concentrations are expressed as number of arsenic atoms per cell.

If the cells were treated with AA and As^{III} at the same concentrations, it would become even clearer that AA was taken up more readily than As^{III}. For example, if we were to examine the results when cells were treated with similar concentrations of elemental arsenic, namely 19.2 μM As^{III} versus 14.1 μM atomic arsenic in AA (equivalent to 3.5 μM AA at the molecular concentration), there were 3×10^6 As^{III} molecules/cell and 986×10^6 AA molecules/cell, respectively. Theoretically, it would be rational to conduct an experiment that had identical arsenic atomic concentrations for both AA and As^{III}. However, in reality, it was very challenging to select a concentration that was high enough for As^{III} to be detected, but low enough for AA not to kill most cells. Therefore, we chose to use concentrations of As^{III} and AA relevant to their respective IC_{50} values. Our results showed that cellular uptake was two orders of magnitude higher for AA than for As^{III} when treated with AA and As^{III} at concentrations to kill similar fractions of cells. This higher cellular uptake of AA coincided with the observed higher toxicity of this compound. These results suggest that the difference in cellular uptake plays an important role in contributing to the higher toxicity of AA as compared to As^{III}.

As cellular uptake is one of the essential requirements to cause toxic effects, the transport of AA and As^{III} molecules across cell membranes is an important prerequisite for cellular uptake [13, 14]. Earlier literature has shown that the rate of arsenic uptake is highly dependent on both cell types and arsenic species [13, 15–17]. For instance, DMA^{III} was found to be the most membrane-permeable arsenic compound, which may contribute to its high toxicity [13, 15–

18]. There are membrane-spanning proteins, such as aquaporin that can transport water, glycerol, and uncharged solutes across cell membranes [20, 21]. Rosen *et al*. demonstrated that As^{III} transported through aquaporin 7 and 9 [19]. Other channels, such as the phosphate transporter, can also play a significant role in transporting some arsenicals across cell membranes [22, 23]. However, the uptake mechanism of AA is unknown, and future research is required to answer this enquiry.

5.3.2 Speciation of arsenic in A549 cells incubated with As^{III} or AA

An arsenic standard mixture containing 5 µg/L each of As^{III}, AA, DMA^{V}, MMA^{V}, and As^{V} was subjected to HPLC-ICPMS analysis and the arsenic species eluted as follows 1.8, 2.2, 2.6, 4.0, and 6.2 min, respectively (Fig. 5.5(a)). After cells were incubated with 76.6 µM As^{III} for 24 h, only As^{III} was found in the cell culture medium (Fig. 5.5(b)), diluted medium without incubation with cells (Fig. 5.5(c)), as well as in the cell lysates (Fig. 5.5(d)). Many processes are involved in the fate of arsenic during arsenic incubation: uptake or influx, efflux, biotransformation in the cells, and interaction with cellular proteins [14, 24]. The absence of As^{III} metabolites in A549 cells when treated for 24 h at the IC_{50} value of As^{III} possibly suggested that the methylating capacity of A549 is insignificant. To the best of our knowledge, there is little evidence as to whether A549 cells can metabolize As^{III}.

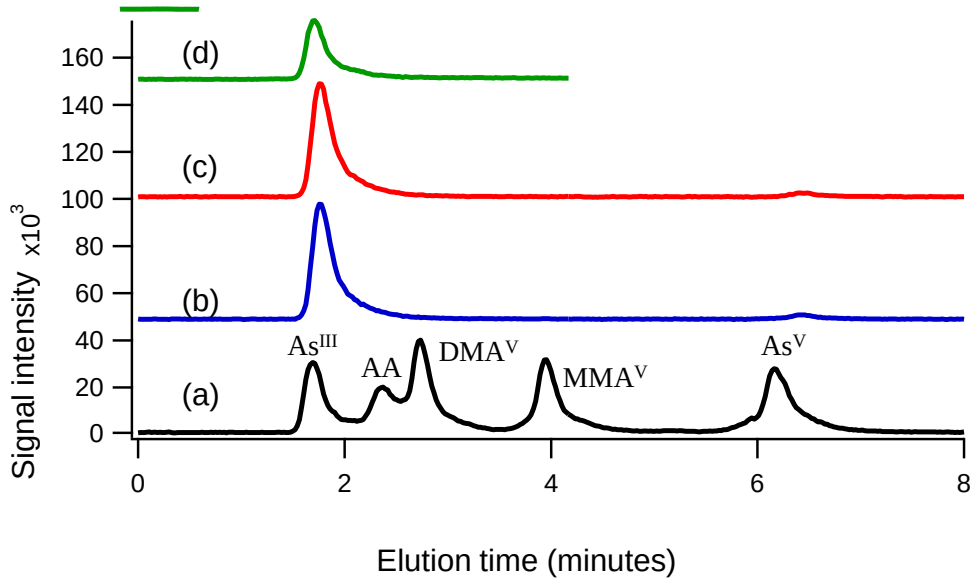


Fig. 5.5 Representative chromatograms from the HPLC-ICPMS analyses of (a) arsenic standard solution containing 5 µg/L each of As^{III}, AA, DMA^{V}, MMA^{V}, and As^{V}, (b) diluted cell culture medium, (c) diluted medium without incubation with cells, (d) lysate of 12.7 ± 2.8 ($\times 10^4$) A549 cells that were incubated with 76.6 µM As^{III} (IC_{50} value) at 37.0 °C and 5.0 % CO_2 for 24 hours. Cells were washed with phosphate buffered saline (PBS) for 3 times, and then lysed with Milli-Q deionized water.

In a comparative speciation study on AA, two peaks were observed from the HPLC-ICPMS analysis of the diluted medium without cells incubation: AA at around 2.2 min and the "converted AA" at around 5.2 min (Fig. 5.6(c)) Spiking fresh AA standard to this diluted medium without cell incubation confirmed the identity of the peak at around 2.2 min as AA (Fig. 5.6(d)). This conversion was abiotic because there was no cell in this diluted solution. Only the "converted AA" peak was observed when analyzing the diluted cell culture medium (Fig. 5.6(b)). These results suggested that the conversion of AA was more pronounced in the cell culture as compared to the medium alone.

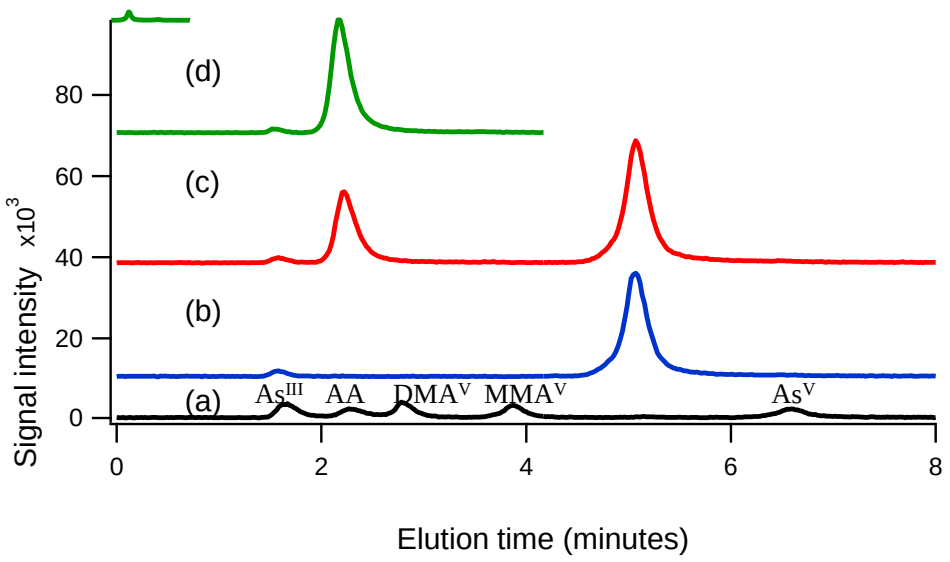


Fig. 5.6 Representative chromatograms from the HPLC-ICPMS analyses of (a) arsenic standard solution containing 1µg/L each of As^{III}, AA, DMA^{V}, MMA^{V}, and As^{V}, (b) diluted cell culture medium, (c) diluted medium without incubation with cells, (d) spike AA to sample (c).

The lysates from cells treated with 3.5 µM AA were initially prepared by re-suspending the cell pellets in 400 µL water, followed by sonication and centrifugation. However, the speciation analyses of these samples yielded arsenic concentrations below the limit of detection (Fig. 5.7(b)). When 100 µL H_2O_2 and 300 µL water solution was used to re-suspend the cell pellets, the speciation analysis of the cell lysates yield a converted AA peak eluted around 5.2 min (Fig. 5.7(d)). In our experiment, the 100 µL H_2O_2 and 300 µL water solution was also analyzed to ensure low arsenic background and no contamination of H_2O_2 by any arsenic species with a retention time around 5.2 min (Fig. 5.7(c)). H_2O_2 was previously used to release arsenic species from proteins or other biomacromolecules [25, 26] at the risk that information on the oxidation state of the arsenicals present in the samples may be lost because H_2O_2 can oxidize trivalent arsenic species to pentavalent arsenic species.

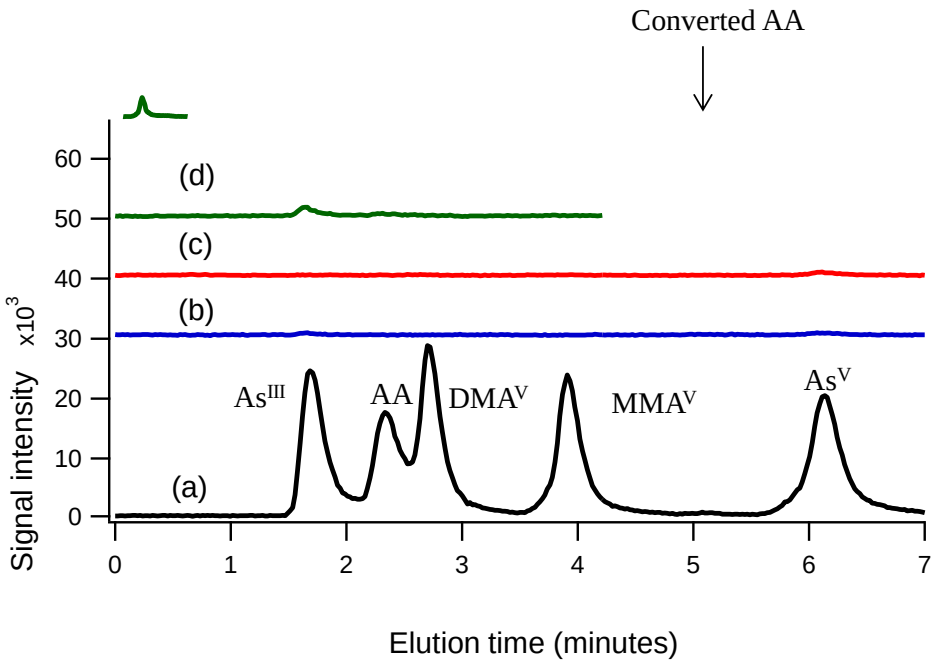


Fig. 5.7 Representative chromatograms from the HPLC-ICPMS analyses of (a) arsenic standard solution containing 5 µg/L each of As^{III}, AA, DMA^{V}, MMA^{V}, and As^{V}, (b) lysate of 13.6 ± 4.0 ($\times 10^4$) A549 cells that were incubated with 3.5 µM AA (IC_{50} value) at 37.0 ˚C and 5.0 % CO_2 for 24 hours. Cells were washed with phosphate buffered saline (PBS) for 3 times, and then lysed with 400 µL Milli-Q deionized water, (c) 100 µL H_2O_2 and 300 µL Milli-Q deionized water, (d) lysate of 13.6 ± 4.0 ($\times 10^4$) A549 cells that were incubated with 3.5 µM AA (IC_{50} value) at 37.0 ˚C and 5.0 % CO_2 for 24 hours. Cells were washed with phosphate buffered saline (PBS) for 3 times, and then lysed with 100 µL H_2O_2 and 300 µL Milli-Q deionized water.

The identity of the converted AA remained unknown. Since this arsenic species in cell lysates was only observed after the addition of 100 µL H_2O_2 and 300 µL water to re-suspend the cell pellets, it was likely that this species bound to biomacromolecules such as proteins [25, 26]. Since trivalent arsenic preferentially targets cellular proteins that have closely spaced cysteine residues and accessible thiol groups [9, 27] several thiol containing arsenicals were included in speciation analysis to help identify the H_2O_2 releasable converted AA species. Standards of monomethylthioarsenic acid (MMTA), dimethylmonothioarsenic acid (DMMTA), and dimethyldithioarsenic acid (DMDTA) were revealed to have retention times around 3.9, 4.3, and 6.0 min, respectively (Fig. 5.8(b)–(d)), which did not match the retention time of the converted AA (~ 5.2 min). Therefore, it will be intriguing to identify this new arsenic species.

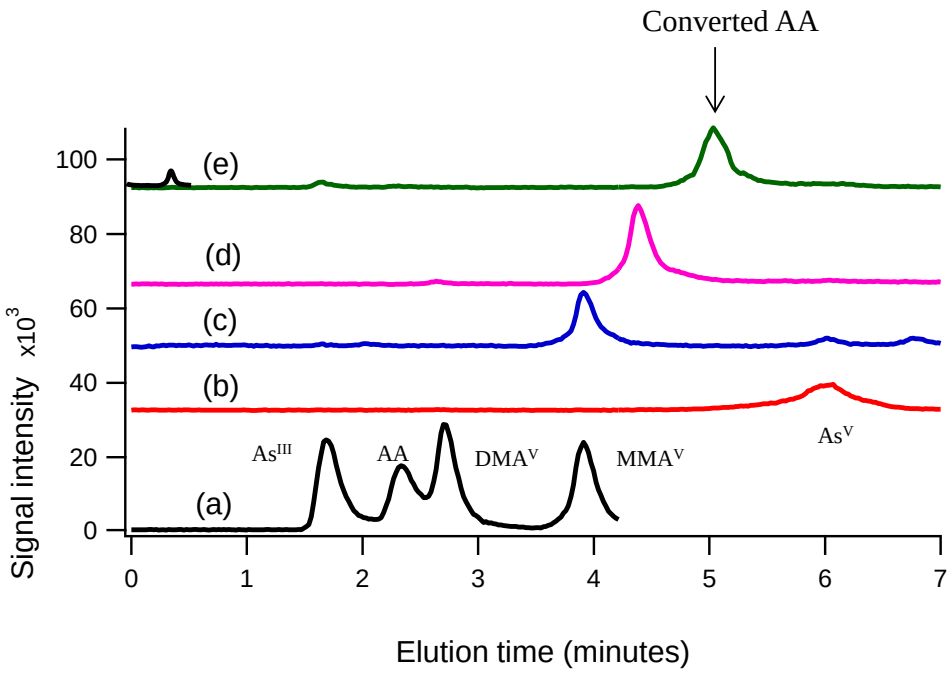


Fig. 5.8 Representative chromatograms from the HPLC-ICPMS analyses of (a) arsenic standard solution containing 5 µg/L each of As^{III}, AA, DMA^{V}, MMA^{V}, and As^{V}, (b) diluted dimethyldithioarsinic acid (DMDTA), (c) diluted monomethylthioarsonic acid (MMTA), (d) dimetylmonothioarsinic acid (DMMTA), (e) lysate of 13.6 ± 4.0 ($\times 10^4$) A549 cells that were incubated with 3.5 µM AA (IC_{50} value) at 37.0 °C and 5.0 % CO_2 for 24 hours. Cells were washed with phosphate buffered saline (PBS) for 3 times, and then lysed with 100 µL H_2O_2 and 300 µL Milli-Q deionized water.

Electrospray ionization mass spectrometry (ESI-MS) was used in an attempt to elucide sturcutral information on this unknown arsenic speices (Table 5.3–5.4). Although the HPLC-ICPMS technique was able to separate six arsenic species in a standard mixture containing AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, and Arsenicin A, the result obtained from HPLC-ESI-MS showed an absence of the Arsenicin A peak (Fig. 5.9). These chromatograms suggested that Arsenicin A may be challenging to undergo ionization, thereby rendering it not detected by the ESI-MS. In addition, the chromatograms from a cell lysate that had been incubated with 3.5 µM Arsenicin A for 24 h also revealed that HPLC-ESI-MS was unable to detect Arsenicin A (Fig. 5.10). A spike study to the cell lysate indicated the matrix did not shift the retention time of the arsenic species, which eliminate the possibility that the absence of Arsenicin A peak in the cell lysate may be due to matrix effect (Fig. 5.11). Hence, the identity of this arsenic species, namely the converted Arsenicin A, remained unknown.

Table 5.3 QTRAP 5500 ESI-MS operating conditions.

Arsenic species	Q1 mass (Da)	Q3 mass (Da)	Mode	DP* (V)	EP* (V)	CE* (V)	CXP* (V)
AsB	179.000	105.000	Positive	71	10	37	9
	179.000	120.000	Positive	71	10	28	11
As^{III}	125.000	107.000	Negative	-10	-10	-18	-15
DMA^{V}	137.000	107.000	Negative	-70	-10	-30	-11
	137.000	102.000	Negative	-70	-10	-18	-13
MMA^{V}	139.000	107.000	Negative	-40	-10	-40	-43
	139.000	124.000	Negative	-40	-10	-24	-7
As^{V}	141.000	107.000	Negative	-15	-10	-58	-13
Arsenicin A model compound	Scan 100.00 – 400.00	na.	na.	na.	na.	na.	na.

DP, declustering potential; EP, entrance potential; CE, collision energy; CSP, collision cell exit potential; na., not available

Table 5.4 Comparison of the separation conditions in HPLC-ICPMS and HPLC-ESI-MS.

Methods	**HPLC-ICPMS**	**HPLC-ESI-MS**	
	Ion pair chromatography	Anion exchange chromatography	
Column	Phenomenex, Prodigy^{TM} ODS-3V	Hamilton PRP-X110	
Column size	4.60 × 150 mm	4.1 × 150 mm	
Particle size	3 µm	7 µm	
Pore size	100 Å	100 Å	
Mobile phase	3 mM malonic acid, 5 mM tetrabutylammonium hydroxide, 5% methanol, pH 5.9 (adjusted by HNO_3)	ammonium bicarbonate (10 mM, mobile phase A; 60 mM, mobile phase B), 5% methanol, pH 8.75 (adjusted by NH_3·OH)	
Isocratic or gradient elution	1.2 mL min^{-1} isocratic elution	Time (min)	Mobile phase
		0 – 2	0 % A → 50 % A
		2 – 5	50 % A
		5 – 8	50 % A → 100 % A
		8 – 15	100 % A
		15 – 20	100 % A → 0% A

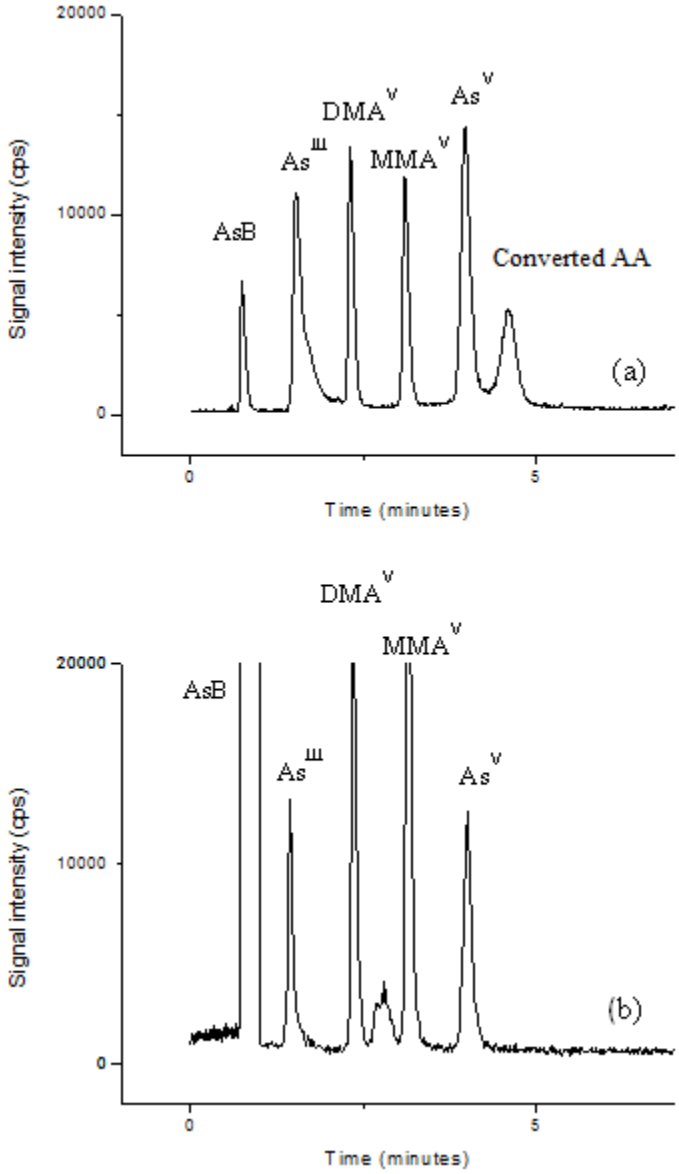


Fig. 5.9 (a) HPLC-ICPMS analysis of a mixture containing 0.5 µg/L AsB, 20 µg/L As^{III}, 30 µg/L AA, 10 µg/L each of As^{V}, DMA^{V}, MMA^{V}; **(b)** HPLC-ESI-MS analysis of a mixture containing10 µg/L each of AsB, As^{III}, AA, As^{V}, DMA^{V}, MMA^{V}.

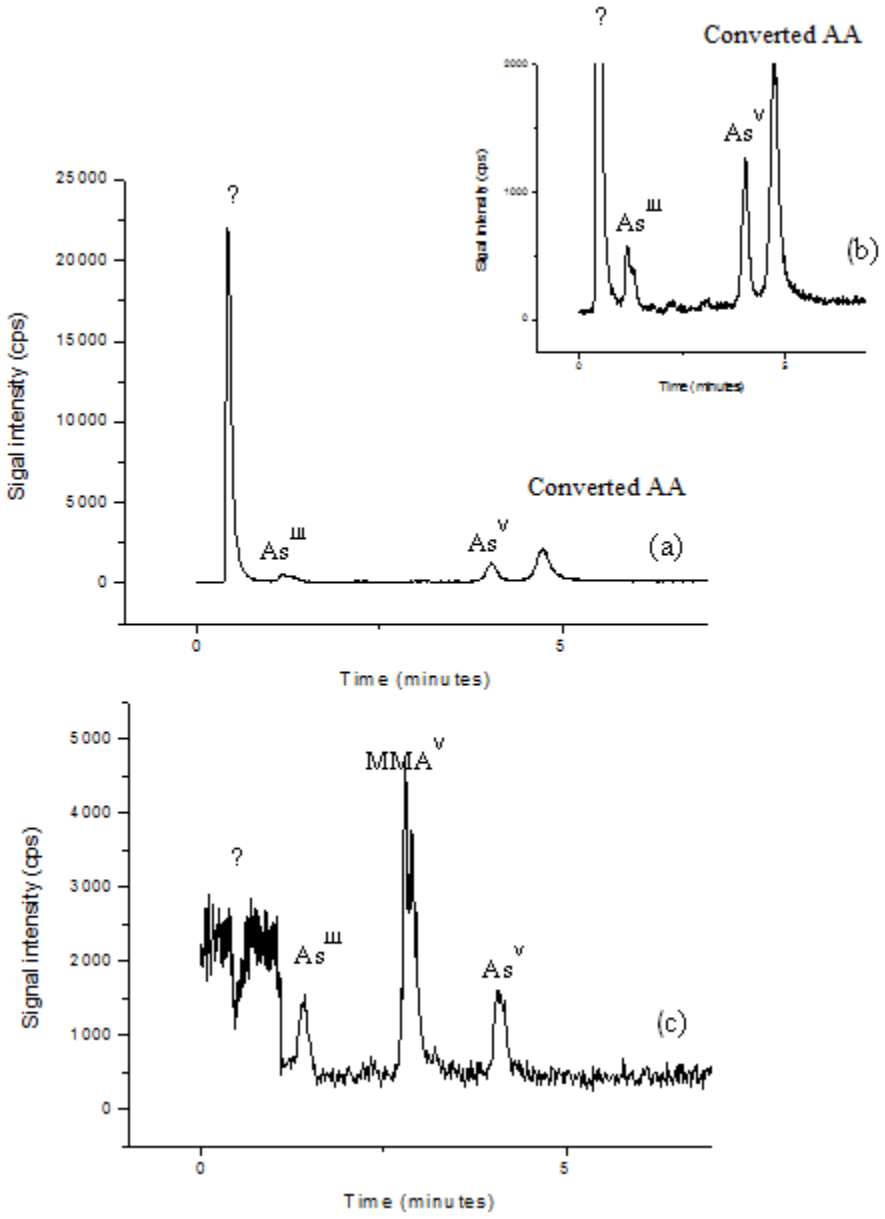


Fig. 5.10 HPLC-ICPMS and HPLC-ESI-MS analyses of lysate with 100 µL deionized water and 300 µL H_2O_2 from A549 cells after incubation with 3.5 µM Arsenicin A for 24 h. **(a)** HPLC-ICPMS; **(b)** HPLC-ICPMS zoomed in; **(c)** HPLC-ESI-MS.

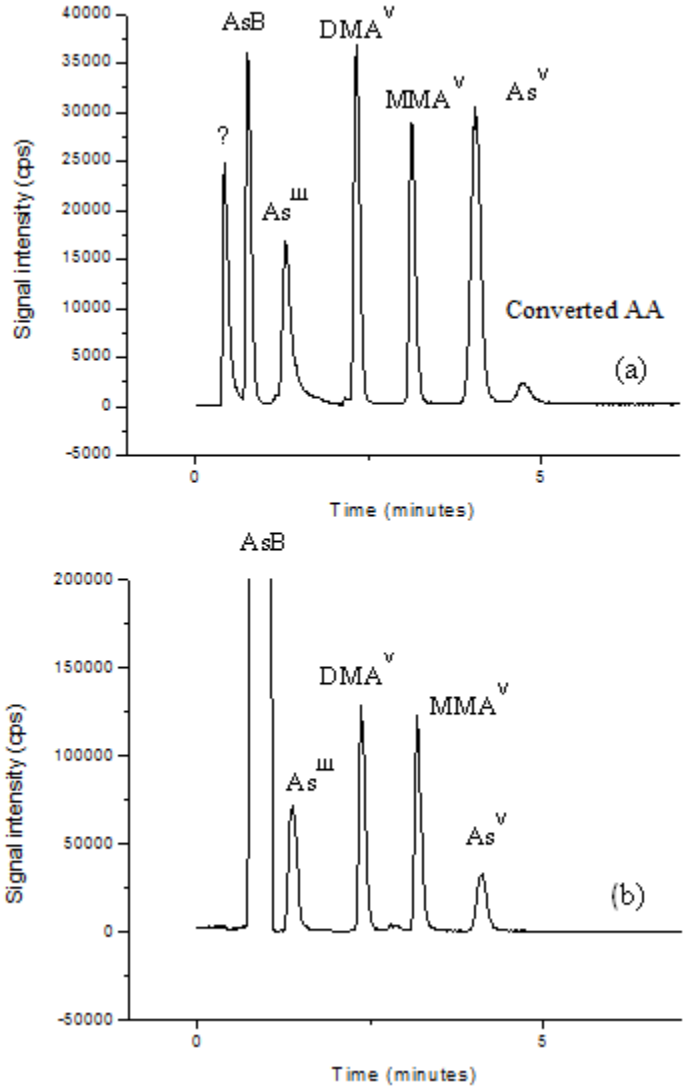


Fig. 5.11 HPLC-ICPMS and HPLC-ESI-MS analyses of cell lysate. The same cell lysate sample as shown in Fig. 5.10 was used and spiked with AsB, As^{III}, AA, As^{V}, DMA^{V}, MMA^{V} (a) HPLC-ICPMS; (b) HPLC-ESI-MS.

5.4 Conclusions

This chapter compares A549 cells incubated with either As^{III} or AA with the objectives to determine the cellular uptake and to identify arsenic species in the cells. The cellular uptake results clearly showed that A549 cells took up two orders of magnitude more AA than As^{III}, which is consistent with the observed higher toxicity of AA. Therefore, the difference in cell uptake is an important factor contributing to the higher toxicity of AA as compared to As^{III}. In addition, a new arsenic species in A549 cells incubated with AA was detected using HPLC-ICPMS; however, its identity is yet to be determined.

5.5 References

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CHAPTER 6 Conclusions

6.1 Conclusions and future works

The central theme of my thesis was to develop analytical methods to obtain arsenic speciation information from a variety of biological and environmental samples, including human urine, human lung carcinoma cells, marine sponges, groundwater and surface water.

Chapter 2.

Chapter 2 focussed on the application of analytical techniques to determine arsenic speciation in human urine samples that were collected from a general Canadian population exposed to low level of arsenic (≈ 10 μg/L) in drinking water. A set of complementary separation methods was developed to determine trace levels of the five major arsenic species found in human urine: As^{III}, DMA^{V}, MMA^{V}, As^{V}, and AsB. These five arsenic species were differentiated by using three different modes of separation that include ion pair, cation exchange, and anion exchange chromatography. These three techniques each had their advantage and disadvantage, but by working together in a complementary fashion, they achieved successfully separation of the five major arsenic species found in human urine.

It was challenging to quantify trace levels of As^{III} that eluted immediately after AsB that may be orders of magnitude greater in concentration than that of As^{III}. This elevated level of AsB was commonly due to seafood consumption, e.g., lobster and crab [1]. Hence, we introduced the effluent from HPLC into a post-column hydride generation system. This post-column derivatization technique

was used to remove AsB interference. The hydride generation process converts As^{III}, DMA^{V}, MMA^{V}, As^{V} to volatile arsines, but leaves AsB unchanged in solution [2–4]. Thus, enhanced specificity was achieved by separating the gaseous arsine from the liquid waste, and the interference from AsB was eliminated. Moreover, the use of hydride generation improved sensitivity due to higher analyte transport efficiency from the introduction of gaseous arsine as compared to the introduction of aerosols from the liquid [2–5].

This developed method combined the advantage of high specificity from the chromatographic separations using HPLC, and the high sensitivity from the detection using ICPMS. The concentrations of the arsenic species determined by using this complementary method were in good agreement with the certified value reported in the certified reference material CRM No. 18; and the sum of the arsenic species was also in good agreement with the reference value provided by the standard reference material SRM 1640. Therefore, I successfully developed complementary chromatographic separations techniques that applied HPLC-HG-ICPMS to quantitatively determine concentrations of As^{III}, DMA^{V}, MMA^{V}, As^{V}, and AsB in the sub-µg/L range in human urine.

Future work of this first chapter would involve combining our urinary arsenic species exposure data with the oxidative damage urinary bladder cell biomarker data from our collaborator Dr. Patrick Levallois from the Public Health Institute of Quebec. We would like to establish an exposure-effect relationship, if one does exist, from this pilot epidemiological study to gain a better

understanding of the potential health effects from exposure to low level of arsenic in drinking water in a Canadian population.

Chapter 3.

My second component investigated the groundwater and surface water quality in a study area located in southern Alberta, Canada, which was primarily irrigated farmland with high density of confined feeding operations. This water quality assessment was a joint effort by five laboratories. My contribution to the overall study was the determination of total arsenic concentration and arsenic speciation in the water samples with respect to spatial and temporal variation in the Battersea Drain watershed.

Each water sample was divided into two aliquots at the site of collection: one containing EDTA (0.0013 M) and CH_3COOH (0.083 M) as treated sample, and another for untreated sample. The treated water samples were determined to consist of predominately As^{V} with a minor fraction of As^{III}, while the untreated water samples only contained As^{V}. This trend was consistent with previous literatures, where EDTA /CH_3COOH treatment was proposed to decrease the oxidation rate of As^{III} to As^{V} and thereby preserved the integrity of arsenic species in water samples [6, 7]. Also, the total arsenic concentrations in the treated water samples were greater than that of the untreated samples. One possible explanation may be that in the presence of EDTA /CH_3COOH, arsenic was less likely to adhere to surfaces, e.g., sample collection container and sediment particles. Hence, more arsenic was present in the soluble component of

the treated water samples. Literatures have also suggested that the EDTA /CH_3COOH treatment may inhibit the precipitation of iron and arsenic [8, 9].

In groundwater samples, higher arsenic concentrations were obtained from the western part of the Battersea Drain watershed as compared to the eastern part of the study area. This spatial trend was consistent with that of the bacterial content, major inorganic ions and nutrients determined by our collaborators. The groundwater samples had exceeded the safe drinking water guideline set by Health Canada on multiple parameters, including arsenic (7%), bacteria (82%), total nitrogen (91%), and total phosphorus (79%) [10]. Temporally, late-September generally had higher total arsenic concentrations as compared to the rest of the sampling dates. This temporal trend was again observed in the drain water samples. The drain water samples did not meet the safe drinking water guidelines with respect to bacteria (97%), total nitrogen and/or total phosphorus (89%), as well as parasites (84%) [10].

Also, the data indicated that the drain water's quality had minimal impact on the water quality in fresh water system, namely, the Oldman River. In short, the water quality in the Battersea Drain watershed exceeds several guidelines for safe drinking water, which would require cautionary intervention to restore the standard of safe drinking water in this high density agricultural area.

Future work would include multi-elements analyses on the water samples for their antimony and selenium concentrations. It would be intriguing to establish correlation, if any, between arsenic, antimony, and selenium in the water samples.

Chapter 4.

My third component was sparked from a recent discovery of a natural arsenic species, Arsenicin A. Arsenicin A has been isolated from a New Caledonian marine sponge, *Echinochalina bargibanti*. This arsenical has shown bactericidal and fungicidal properties. A set of complementary separation techniques, involving ion pair and anion exchange chromatography, were developed to differentiate seven arsenic species: Arsenicin A model compound (AA), As^{III}, As^{V}, MMA^{V}, DMA^{V}, AsB, and arsenosugar. A main challenge was that AA was found to be unstable at room temperature that it rapidly converted to another arsenic species, referred to as "converted Arsenicin A", within minutes of exposure at room temperature. Hence, it is important to prepare and store AA at 4°C on the same day of the analyses in order to minimize species conversion.

Although the developed complementary chromatographic separations were capable of differentiating AA from the other arsenicals, AA was below the limit of detection in these marine sponges. Instead, we found an unknown arsenic species that eluted close to AA from a RP-C18 column. Considering that the total arsenic level and arsenic speciation profile were specific to the class of the organisms, dietary intake, and habitats in which they were found [11, 12], the following factors may contribute to certain degree to the absence of AA in the sponges used in this study: difference in marine sponge species, harvest locations (New Caledonia [13] versus Papua New Guinea), and the types of organic solvent (CH_2Cl_2 [13] versus CH_3OH) used in the extraction process of the sponges. Since we have not obtained the sponge species information from our collaborator Dr.

Raymond Andersen, there is a possibility that these sponges under this investigation was not *Echinochalina bargibanti* [13]. Different sponge species may have different biotransformation efficiency of converting As^V from their surrounding water to various forms of methylated arsenic species [14]; hence, we may not expect to find the identical arsenic species in our marine sponges as compared to that in the literature, more specifically, Arsenicin A.

The arsenic speciation profile from selective marine sponge samples showed they consisted of predominantly AsB (28–98%) and minor fractions of As^V (0–18%), MMA^V (0–22%), DMA^V (0–10%), arsenosugar (0–16%), and unknown species (0–70%). This speciation profile was consistent with the literatures, which reported that in marine animals, inorganic arsenic species and simple methylated arsenic species were only minor fractions as compared to the more complicated organic arsenic species, e.g., AsB (70 – 98%) [15, 11]. The median total arsenic concentration from the 160 Papua New Guinean marine sponges was ≈ 124 mg of As/kg of marine sponges, which was similar to other literature's reported value of 100 mg/kg of arsenosugar found in algae [16].

Future work would be to identify the unknown arsenic species found in the marine sponges. Also, it may be interesting to establish any correlation between the arsenic speciation data that we obtained, with the marine sponges and invertebrates species information from our collaborator, Dr. Raymond Andersen from the Department of Chemistry at University of British Columbia.

Chapter 5.

My final component was a cellular uptake study that compared the concentration and speciation of arsenic in A549 human lung carcinoma cell line incubated with either As^{III} or AA at their respective IC_{50} values (76.6 µM for As^{III} and 3.5 µM for AA). The results clearly indicated that A549 cells had cellular uptake two orders of magnitude higher for AA than for As^{III}. The higher uptake of AA by A549 cells was consistent with the observed higher toxicity of this compound. Therefore, the difference in cell uptake capabilities is an important factor contributing to the higher toxicity of AA as compared to As^{III}.

High performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICPMS) detection revealed a new arsenic species in cells incubated with AA. The identity of this new arsenic species, "converted AA", remained to be determined. This unknown species was likely to bind to biomacromolecules because H_2O_2 was used in the cell lysing step, which had also been suggested previously in the literature, to release arsenic species from biomacromolecules such as proteins [17, 18]. Since trivalent arsenic species preferentially target cellular proteins that have closely spaced cysteine residues and accessible thiol groups [19, 20], several thiol containing arsenicals were included in the speciation analyses to help identify this H_2O_2 releasable converted AA species. However, the retention time of monomethylthioarsenic acid (MMTA), dimethylmonothioarsenic acid (DMMTA), and dimethyldithioarsenic acid (DMDTA) did not match with that of the converted AA. Therefore, future

research would likely to pursue in the direction of identifying this unknown converted AA specie.

Cellular uptake is one of the essential requirements to cause toxic effects. Similarly, the transport of AA across cell membrane is an important prerequisite for cellular uptake [21, 22]. Aquaporin 7 and 9 are capable of transporting As^{III} across cell membranes [23]. Other channels, such as the phosphate transporter, may also play a role in moving arsenicals across the cell membranes [24, 25]. However, the uptake mechanism of AA remains a mystery. Therefore, I would be curious to investigate how AA was transported across cell membranes.

In conclusion, my Ph.D. thesis has contributed to the scientific community by providing alternative analytical techniques to achieve arsenic speciation analyses in different biological and environmental samples that encompassed human urine, human lung carcinoma cells, marine sponges, and groundwater.

6.2 References

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Appendix

The ranges of concentrations used in chapter 5 to treat the A549 cells were intentionally chosen to cause negligible cell death as the lower limit of the concentrations to be examined; hence 0.3 µM AA and 1.0 µM As^{III} were adopted. For the upper limit of the concentrations, we opted for those that would cause 50% cell death, which were the IC_{50} values that were determined by our earlier work; consequently 3.5µM AA and 76.6µM As^{III} were selected. The concept behind treating A549 cells with concentrations that would cause 0% and 50% cell death was to subject cells under similar degree of stress. By doing so, we could minimize confounding factors such as different uptake and efflux mechanisms when cells experienced varied stress level [12, 15, 16]. Therefore, we could be more confident that if there was a difference in the intracellular arsenic concentrations, it would be more likely arsenic species dependent, instead of concentrations dependent.

Cell culture protocol

At day 1, cells were grown in cell culture media RPMI Media 1640 [+] L-Glutamine (Gibco at Life Technologies, Burlington, ON, CA) with 10% Fetal Bovine Serum (Sigma, Canadian origin, sterile filtered, Oakville, ON, CA) and 1% Penicillin Streptomycin (Gibco at Life Technologies, Burlington, ON, CA). 9 mL of media was placed in a cell culture plate (100 mm x 20 mm, treated polystyrene, non-pyrogenic, sterile, Corning Incorporated, NY, USA) and incubated for 20 min in an IR Autoflow Direct Heat CO_2 incubator (Pacific Sciences, Torrance, CA, USA) at 37.0°C and 5.0% CO_2. Cells were then passaged

into this warm cell culture media and incubate for 20–22 h or until cells covered about 60–80% of the cell culture plate.

At day 2, 2449 µL media was placed in each well of a 6 well cell culture plate (Greiner Cellstar, Okaville, ON, CA) and incubated for 20 min. The media incubated with cells were removed and the cells were washed twice with 200 µL phosphate buffered saline solution (Gibco at Life Technologies, Burlington, ON, CA) per well. Cells were incubated with 200 µL of 0.05% Trypsin-EDTA (Gibco at Life Technologies, Burlington, ON, CA) per well for 3 min in order to detach cells from the bottom of the plate. Cells were then collected with 5 mL media per plate and transferred to 15 mL plastic centrifuge tube. 11 µL of cell suspension was put on a counting chamber for cell counting. Cells were counted with a Microsc Optics IV900 microscope (Oakland Country, MI, USA). The cells were seeded at a concentration of 4.0×10^4 cells/mL. Cell count consisted of an average of the counts from four quadrants in a counting chamber of a hemocytometer (Bright-line at Hausser Scientific, PA, USA). 4.9 mL of cell suspension were added to the warm media and incubated for 20–22 h. At day 3, media was removed from each well, then cells were treated with 9796 µL of AA and As^{III} at the desired concentrations made with media and incubated for 24 h. Cell control samples were cells incubated with only media for 24 h. Media control samples had no cell but only media that was incubated for 24 h. AA and As^{III} control samples had no cell but only arsenic species incubated for 24 h.

At day 4, cells were harvested. The media that were incubated with cells were collected in 15 mL plastic centrifuge tubes (Corning, Fisher Scientific,

Toronto, ON, CA) for later analysis. The cells were washed twice with 200 µL phosphate buffered saline solution (Gibco at Life Technologies, Burlington, ON, CA) per well to remove media and any residual arsenic on the cell surface and the plate surface. Cells were incubated with 200 µL of 0.05% Trypsin-EDTA (Gibco at Life Technologies, Burlington, ON, CA) per well for 3 min in order to detach cells from the bottom of the plate. Cells were then collected with 2 mL media per well and transferred to 15 mL plastic centrifuge tube. An IEC Centra CL2 Centrifuge (Thermo Electron Corporation, Asheville, NC, USA) was used to spin down cell suspension at a speed of 1700 rpm for 3 min. Then cell pellet was washed with 3 mL cold PBS to remove the last trace of arsenic adheres to the surface of the cells. A hemocytometer was used to perform cell counts for each well. The cell counts would be used subsequently to normalize the concentration of arsenic. Cell pellet was re-suspend in 20 µL of 2% HNO_3 (total analysis) or 400 µL milliQ deionized water (speciation analysis), followed by 30 min sonication. Cell suspension was then centrifuged at 2500 rpm for 30 min. Finally, the supernatant (cell lysate) was collected for further analysis. The pipets used in this study were 20, 100, 200, 1000 µL (Eppendorf Research, Mississauga, ON, CA). A Vortex-gene 2 was used to mix the media with arsenic species (Scientific Industries, Bohemia, NY, USA). Cell culture procedures were done in a Biological Service Cabinet Forma Class II, A2. Reagents and arsenic species were stored in a 4°C fridge.