ARSENIC AND ANTIMONY SPECIES IN THE TERRESTRIAL ENVIRONMENT

by

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ABSTRACT

The determination of arsenic and antimony species in environmental samples can be used to assist in toxicity assessment, as well as to yield information about environmental processes. Such information about samples from the terrestrial environment was sought. Existing methods for speciation were adapted, including high-performance liquid chromatography (HPLC) coupled with inductively coupled plasma-mass spectrometry (ICP-MS), for the determination of both arsenic and antimony species, and hydride generation-gas chromatography (HG-GC) with atomic absorption spectrometric (AAS) detection and mass spectrometric (MS) detection, for the determination of antimony species. Arsenate, when added to mycelial cultures of *Scleroderma citrinum* and *Macrolepiota procera*, was reduced to arsenite, but no further processes (i.e., methylation or formation of arsenosugars or arsenobetaine) were observed. This may indicate that the presence of more complex arsenicals in environmental mushroom specimens is dependent on symbiotic interactions between the fungus and its surroundings, rather than resulting from independent synthesis by the fungus. *Pleurotus flabellatus* oxidized antimony (III) to antimonate ($Sb(OH)_6^-$), and formed an antimony-containing metabolite of unknown identity. Water soluble arsenic species were determined in a host of terrestrial and freshwater biota from a hot springs environment (Meager Creek, BC) and from an area impacted by mining and smelting activities (Yellowknife, NWT). Arsenate and arsenite (the more toxic forms of arsenic) were the predominant species extracted from plants, mosses, microbial mats, algae and lichens. Small amounts of arsenosugars and methylated arsenic were detected as well. Arsenobetaine was discovered for the first time in lichens, and it was also the major form of arsenic in freshwater fish. The majority of detectable arsenic in freshwater mussels and snails was as arsenosugars and the tetramethylarsonium ion, respectively. Large amounts of arsenic, of an unknown

toxicological and chemical nature, remained unextracted or undetected in all samples. A dimethylantimony compound was found in moss samples from Yellowknife, confirming that methylation of antimony takes place in the environment.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

LIST OF ABBREVIATIONS

AB	arsenobetaine
AC	arsenocholine
AsS	extract containing arsenosugars
BCF	bioconcentration factor
BTT	Bill's Toxic Team
CE	capillary electrophoresis
CEPA	Canadian Environmental Protection Agency
CID	collision induced dissociation
CR	Campbell River
CRM	certified reference material
Da	Dalton (atomic mass unit)
DE	digestion efficiency
DMA	dimethylarsinate
DMAA	dimethylarsinic acid
DMAE	dimethylarsinylethanol
DSA	dimethylstibinic acid
dw	dry weight
EDTA	ethylenediaminetetraacetic acid
EE	extraction efficiency
ESI-IT-MS	electrospray ionization-ion trap-mass spectrometry
FAAS	flame atomic absorption spectrometry
FAB	fast atom bombardment
FID	flame ionization detection
fw	fresh weight
GFAAS	graphite furnace atomic absorption spectrometry
HG-GC-AAS	hydride generation-gas chromatography-atomic absorption spectrometry
HPLC	high performance liquid chromatography
i.d.	inner diameter
ICP-MS	inductively coupled plasma-mass spectrometry
IS	ionspray
LOD	limit of detection
M+H	molecular ion + H
M-H	molecular ion - H
M/W	methanol/water (1:1) extraction
m/z	mass to charge ratio
MC	Meager Creek
MeOH	methanol
MMA	methylarsonate
MMAA	methylarsonic acid
MS	mass spectrometry
MSA	methylstibonic acid
MS^n	tandem mass spectrometry
NIST	National Institute for Science and Technology
o.d.	outer diameter

OES	optical emission spectrometry
PC	pixie cups
PD	protease digestion
ppb	parts per billion, ng/g, μg/kg, or μg/L
ppm	parts per million, μg/g, mg/kg, or mg/L
PTFE	poly(tetrafluoroethylene)
R	ratio obtained from (fresh weight mass)/(dry weight mass)
RBF	round bottom flask
SAM	S-adenosylmethionine
SD	standard deviation
SFC	supercritical fluid chromatography
SIDS	Sudden Infant Death Syndrome
TEAH	tetraethylammonium hydroxide
TMA	trimethylarsenic (unspecified structure)
TMAO	trimethylarsenic oxide
TRA	time resolved analysis
TSbO	trimethylantimony oxide
UK	unknown compound
US-EPA	United States Environmental Protection Agency
US-FDA	United States Food and Drug Administration
UV	ultra violet
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organization
X-XIII	arsenosugars X through XIII
YK	Yellowknife

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Chapter 1

INTRODUCTION

"Any wild place is filled with incredible things happening."

David Cavagnaro, *This Living Earth*

The chemistry of the environment is a complex area of study, reaching into the fields of biology, geology, physics, toxicology and medicine, among others. Two crucial aspects of environmental chemistry are the identification of chemical contaminants and the determination of their fate in natural and anthropogenic environments. This knowledge is often used to discover and solve problems caused by the presence of chemical contaminants. Such problems become of utmost concern when they include adverse health effects in humans, animals or plants.

Metals or metalloids are often found in the environment as chemical contaminants. Two examples are arsenic and antimony, which are closely related in chemical behaviour.

1.1. Historical aspects of arsenic and antimony

Both arsenic and antimony have had paradoxical uses throughout history, as poisons and as panaceas.

Arsenic trioxide, known as "white arsenic" is historically one of the most common poisons. Strangely, this same compound was used until recently in a region in Austria by the "arsenic eaters", who attempted to increase their strength, virility and longevity by ingesting arsenic trioxide daily[1]. Hippocrates (460-377 B.C.) recommended the use of a paste of realgar

(As_4S_4) to treat ulcers[2]. In the early part of this century (1900-1950), organoarsenic compounds, such as salvarsan, neosalvarsan and atoxyl, were used for the treatment of syphilis and sleeping sickness[2]. Lewisite ($ClCH=CHAsCl_2$), a severe blistering agent, was developed for chemical warfare purposes. Arsenic trioxide is thought to be the active ingredient in current traditional Chinese treatments of arthritis, skin disorders and even cancer[3].

Antimony also has a fascinating history. It is mentioned in the Bible as a cosmetic and it was commonly used up until the mid-18th century as a medicine to produce sweating, as an emetic, as a purgative, or all three[4]. Many preparations of antimony are described in the well-known "The Triumphal Chariot of Antimony" by Basil Valentine, first published in 1604[5]. Fatalities resulting from its use did not prevent its mention in Martindale's Extra Pharmacopoeia, as recently as 1941[4]. Antimony was of great interest to alchemists, and one of its properties was that it "united with or devoured all the metals then known, with the exception of gold"[6]. Some cases of alleged deliberate antimony poisoning also have been documented[4].

1.2. Chemistry of arsenic and antimony

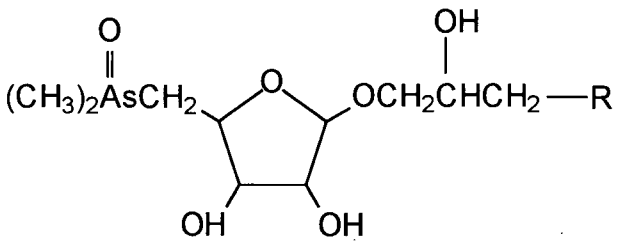
Arsenic and antimony are found in Group 15 of the periodic table. As metalloids, they possess characteristics of both metals and non-metals. Antimony is more metallic than arsenic because it can exist as aqueous cationic species, [SbO^+] and [SbO_2^+] (but not free [Sb^{3+}] or [Sb^{5+}]), and its trioxide is amphoteric, dissolving in both strong acid and strong base. Arsenic, on the other hand, forms oxy-anionic species only. Its trioxide is acidic and hence dissolves in base, which is characteristic of non-metals[7]. Both arsenic and antimony form hydrides, implying that, like non-metals, they can also exist in a negative oxidation state.

Arsenic and antimony possess five valence electrons like other group 15 elements. Their four oxidation states are -3, 0, +3 and +5. An antimony compound with composition Sb_2O_4 has been observed, implying that a +4 oxidation state is possible, but this oxide has been shown to contain only Sb^{V} and Sb^{III} atoms[8].

The arsenic and antimony compounds that are pertinent to this thesis are either hydrides, organometallic compounds (possessing a As-C or Sb-C bond), oxyanions, or complexes of the inorganic cations. Table 1.1 and Table 1.2 show names, abbreviations and structures for some arsenic (Table 1.1) and antimony (Table 1.2) compounds.

Table 1.1. Names, abbreviations and structures of some arsenic compounds.

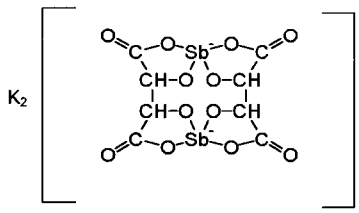
Name	Abbreviation	Structure/Formula
Arsenic acid, arsenate[a]	As (V)	$As(O)(OH)_3$
Arsenous acid, arsenite[a]	As (III)	$As(OH)_3$
Monomethylarsonic acid[a]	MMA	$CH_3AsO(OH)_2$
Dimethylarsinic acid[a]	DMA	$(CH_3)_2AsO(OH)$
Trimethylarsine oxide[b]	TMAO	$(CH_3)_3AsO$
Methylarsine	$MeAsH_2$	CH_3AsH_2
Dimethylarsine	Me_2AsH	$(CH_3)_2AsH$
Trimethylarsine	Me_3As	$(CH_3)_3As$
Tetramethylarsonium ion[b]	Me_4As^+	$(CH_3)_4As^+$
Arsenobetaine[b]	AB	$(CH_3)_3As^+CH_2COO^-$
Arsenocholine[b]	AC	$(CH_3)_3As^+CH_2CH_2OH$
Dimethylarsinylethanol	DMAE	$(CH_3)_2As(O)CH_2CH_2OH$
Arsenosugars[c]	X-XIII	See figure below



Sugar[d]	R
X	-OH
XI	$-OPO_3HCH_2CH(OH)CH_2OH$
XII	$-SO_3H$
XIII	$-OSO_3H$

[a] These compounds are commercially available.
[b] These compounds are available in our lab, having been synthesized according to common methods (see Chapter 2).
[c] These compounds are available in our lab; they are found in a laboratory reference material (kelp powder) and have also been donated (see Chapter 2).
[d] Numbering system (X-XIII) according to Shibata *et al.*[9]

Table 1.2. Names, abbreviations and structures of some antimony compounds.

Name	Abbreviation	Structure/Formula
Antimonate[a]	Sb (V)	$Sb(OH)_6^-$
Pentavalent inorganic antimony	Sb (V)	inorganic ^{V}Sb (complexed)
Antimony trioxide[a]	Sb (III)	Sb_2O_3, $Sb(OH)_{3\ (aq)}$
Antimony trisulfide	---	Sb_2S_3
Antimony potassium tartrate[a]	Sb (III)	
Trivalent inorganic antimony	Sb (III)	inorganic ^{III}Sb (complexed)
Methylstibonic acid	MSA	$CH_3SbO(OH)_2$
Dimethylstibinic acid	DSA	$(CH_3)_2SbO(OH)$
Trimethylantimony oxide	TSbO	$(CH_3)_3SbO$
Trimethylantimony dihydroxide[b]	$Me_3Sb(OH)_2$	$(CH_3)_3Sb(OH)_2$
Trimethylantimony dichloride[b]	Me_3SbCl_2	$(CH_3)_3SbCl_2$
Methylstibine	$MeSbH_2$	CH_3SbH_2
Dimethylstibine	Me_2SbH	$(CH_3)_2SbH$
Trimethylstibine	Me_3Sb	$(CH_3)_3Sb$

[a] These compounds are commercially available.
[b] These compounds are available in our lab, having been synthesized according to common methods (see Chapter 2).

1.3. Toxicity of arsenic and antimony compounds

Although the word "arsenic" is usually immediately associated with poison, the actual toxicity of arsenic in a sample is dependent on the chemical form that it takes. The same principle applies for antimony. Arsenic and antimony compounds in the -3 oxidation state are more toxic than those in the +3 oxidation state, which are more toxic than those in the +5 oxidation state. Organometallic compounds in the +5 oxidation state are less toxic than inorganic ones and some compounds, like arsenosugars and arsenobetaine, have not exhibited any toxic behaviour at all in the systems tested[10,11,12]. The dependence of toxicity on the chemical form (or species) of arsenic and antimony makes their identification (i.e., speciation analysis) necessary.

Trivalent arsenic is thought to exert its toxicological effects by binding with sulfhydryl groups on enzymes[2,13]. Inhibition of pyruvate dehydrogenase takes place for both trivalent arsenic and trivalent antimony[13,14]. Pentavalent arsenic is thought to compete with phosphate during phosphorylation to form unstable arsenyl esters, interfering with bioenergetic processes[2,13]. Trivalent antimony binds easily *in vivo* to sulfhydryl groups, such as those on enzymes, most likely constituting the toxic action of these compounds[15,14]. Pentavalent antimony is excreted rapidly from the body but it has been shown that the liver can reduce Sb (V) to Sb (III)[16].

The International Agency for Research on Cancer (IARC) has determined that there is sufficient evidence to consider arsenic a human carcinogen, and to consider antimony trioxide an animal (but not human) carcinogen[17].

1.4. Environmental chemistry of arsenic

1.4.1. Arsenic sources, uses and exposure to humans

Arsenic is the twentieth most abundant element in the Earth's crust, and is often associated with sulfidic ores such as arsenopyrite (FeAsS), enargite (Cu_3AsS_4), orpiment (As_2S_3), realgar (As_4S_4) and proustite (Ag_3AsS_3)[13,18]. Arsenic is usually recovered as arsenic trioxide from the processing of ores, and its major present uses are in the production of arsenic-containing agricultural pesticides (including disodium methylarsonate, sodium methylarsonate, methylarsonic acid (MMA) and dimethylarsinic acid (DMA)); wood preservatives (including chromated copper arsenate (CCA), ammoniacal copper arsenate (ACA) and fluorchrome arsenate phenol (FCAP)); and animal feed additives (including arsanilic acid and 4-nitrophenylarsonic acid)[13,19]. Arsenic is also used as a doping agent in solid-state products[19].

Arsenic can enter the environment anthropogenically as a consequence of its industrial use, from mining and smelting operations and through the application of arsenic-containing pesticides. Natural inputs of arsenic to the environment can result from the weathering of rocks and geothermal activities, leading to open ocean arsenic concentrations typically ranging from 0.0056-11 ppb[18] and terrestrial soil and rock concentrations ranging from 0.4-100ppm[18,20]. Humans can be exposed to arsenic through inhalation, especially for workers in industries utilizing arsenic, through food (such as fish, shellfish, or marine algae) and through drinking water (e.g., in India, groundwater supplies contain elevated levels of arsenic[21]).

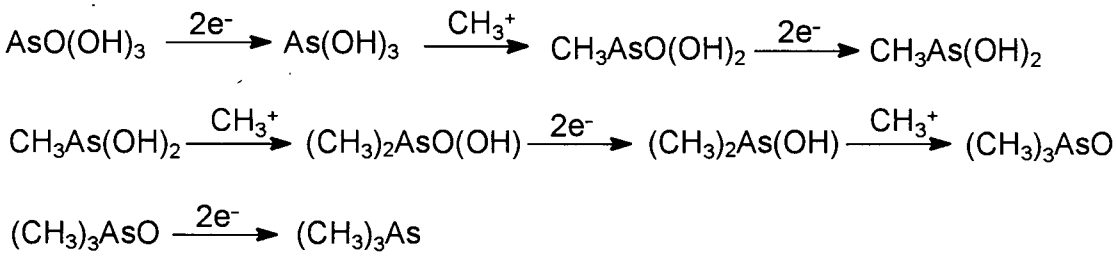
Arsenic is considered to be a priority pollutant by the United States and the Canadian Environmental Protection Agencies (US-EPA and CEPA). US-EPA and CEPA permit drinking water levels to be a maximum of 50[22] and 25 ppb[23] (μg/L), respectively. The World Health

Organization (WHO) recommends a daily limit through food of 0.05 mg total arsenic/kg body weight[22], but for inorganic arsenic a weekly limit of only 15 μg As/kg body weight is suggested[24].

1.4.2. Arsenic species in the marine environment

The major species of arsenic in seawater is As (V)[25,26], although sometimes as much as 10-30% of the total arsenic takes the form of As (III), MMA and DMA in waters from the euphotic zone, in deeper ocean waters and in interstitial waters[20,25,26]. Arsenate is thermodynamically predicted to be the major compound in oxygenated seawater (pH 8)[20] and therefore the presence of As (III) and methylated species imply that biotransformation is taking place. This is indeed observed to be the case because marine algae[27], bacteria and fungi[20] methylate arsenic. The Challenger mechanism[28] (Figure 1.1a), involving alternating steps of reduction and oxidative addition of a methyl group, is considered to be a possible methylation pathway. Studies have shown that S-adenosylmethionine (SAM) (Figure 1.1b) acts as a methyl donor[20].

1.1a



1.1b

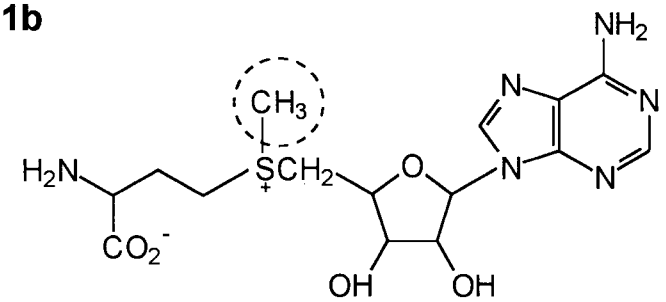


Figure 1.1a. The Challenger mechanism[28], showing alternating reducing and oxidative addition steps.
Figure 1.1b. The structure for S-adenosylmethionine (SAM); the circled methyl group is donated during the methylation steps in the mechanism.

In marine sediments arsenic concentrations can range from 0.4-455 ppm[18]. The majority of arsenic in sediment is associated with Fe and Mn oxides and some is associated with carbonates and organic material[20]. MMA and DMA have been found in environmental marine sediment samples[20]. Laboratory experiments conducted aerobically and anaerobically with cultures from marine sediments, amended with MMA and DMA, suggest that tetramethylarsonium ion may be formed and thus may be present in trace quantities[29]. Reliable evidence for the presence of arsenobetaine (but not AC, TMAO or Me_4As^+) in environmental estuarine waters now exists[30].

Marine algae contain levels of arsenic that are considerably higher than the levels in the surrounding water, ranging from 0.8 to 12.1 ppm wet weight[31]. The major water-soluble forms of arsenic in most species of marine algae are the arsenosugars, some of which are shown in Table 1.1[31,32]. One exception is a Japanese edible algae, hijiki (*Hizikia fusiforme*), which contains 50% of its arsenic as arsenate[33].

Marine animals also contain higher levels of arsenic compared to the levels in the surrounding water[31], although biotransformation studies via the marine food chain indicate that biomagnification of arsenic probably does not take place[34]. In contrast to most previous studies, one study has shown that biomagnification takes place in a short marine food chain (seaweed → herbivorous marine gastropod → carnivorous marine gastropod)[35]. Arsenobetaine is the major arsenic compound in most marine animals, although the tetramethylarsonium ion has been found in marine snails and clams, arsenocholine has been found in gastropods and dogfish muscle[36], trimethylarsine oxide has been found in fish, and arsenosugars have been found in bivalve mollusks[31].

A pathway for the formation of arsenosugars, and, from them, arsenobetaine, has been proposed and is shown in Figure 1.2[32,37].

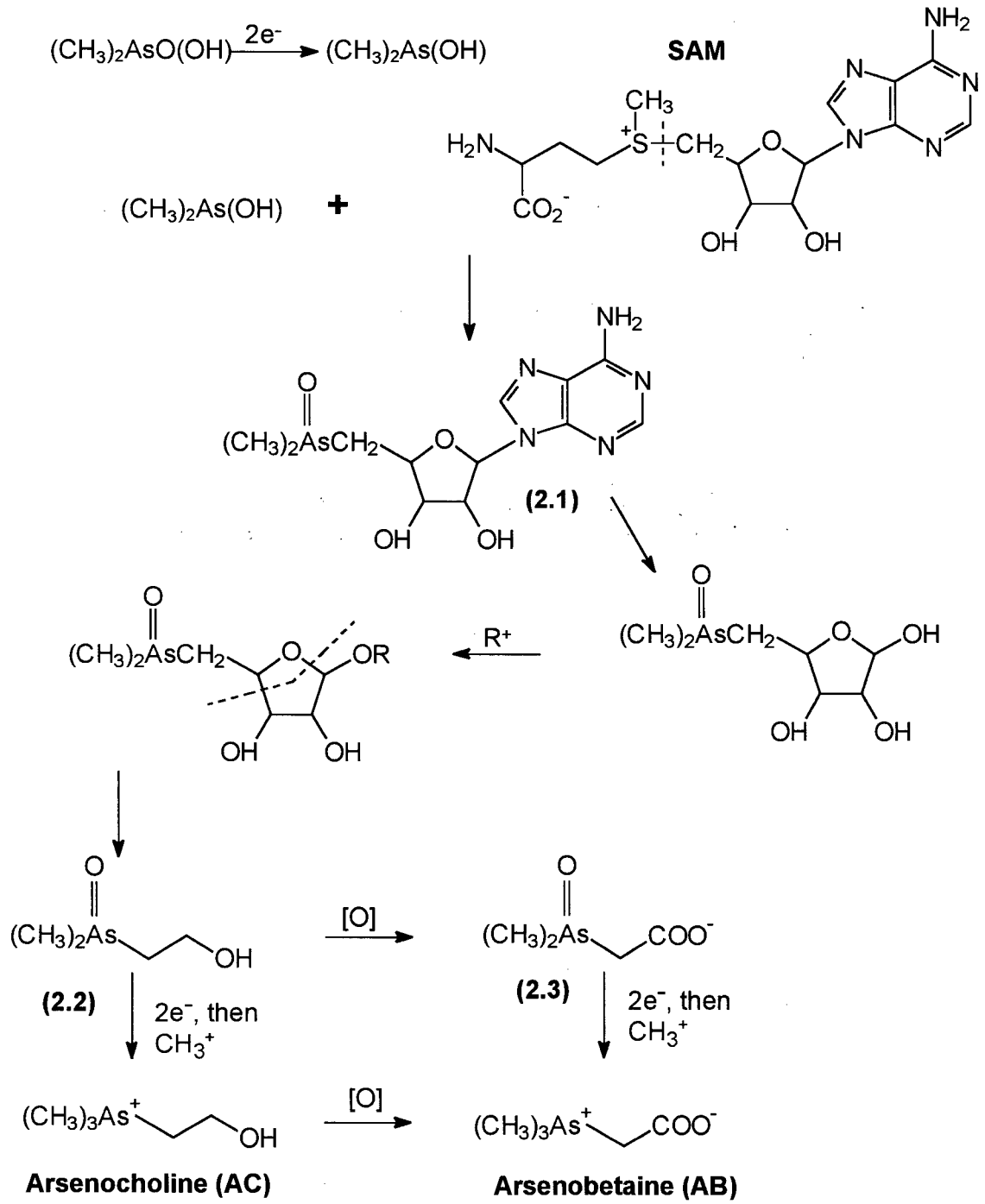


Figure 1.2. Pathway showing the formation of dimethylarsinoribosides and arsenobetaine[30].

The pathway for the synthesis of arsenosugars is similar to the Challenger mechanism up until the formation of DMA (the starting molecule in Figure 1.2). After the reduction of DMA to $(CH_3)_2AsOH$, S-adenosylmethionine (SAM) is proposed to be the donor of the ribosyl moiety. The intermediate **2.1** in Figure 1.2 has been isolated from the kidney of the clam *Tridacna maxima* (symbiotic algae grow in the mantle of the clam and algal products accumulate in the kidney), providing support for this pathway[38]. The formation of arsenobetaine from arsenosugars, as proposed in Figure 1.2, involves an intermediate compound **2.2** (dimethylarsinoylethanol, DMAE), which has not yet been identified as a naturally occurring compound[32]. However, trace amounts of dimethylarsinoylacetic acid (**2.3**), as well as arsenosugars and arsenobetaine, have been reported in a mussel sample[39], which may lend support to one of the routes in the proposed pathway in Figure 1.2.

Small amounts of a trimethylarsonioriboside (Figure 1.3, **3.1**) have been identified in algae[40] leading to the proposal of an alternate pathway for the formation of arsenobetaine, shown in Figure 3. The formation of arsenobetaine from the trimethylarsonioriboside has been demonstrated to be a facile process in laboratory experiments[41,42], which may indicate that although the levels of the starting trimethylarsonioriboside are very low in algae, this pathway may, to some extent, contribute to the formation of arsenobetaine in the marine environment[32].

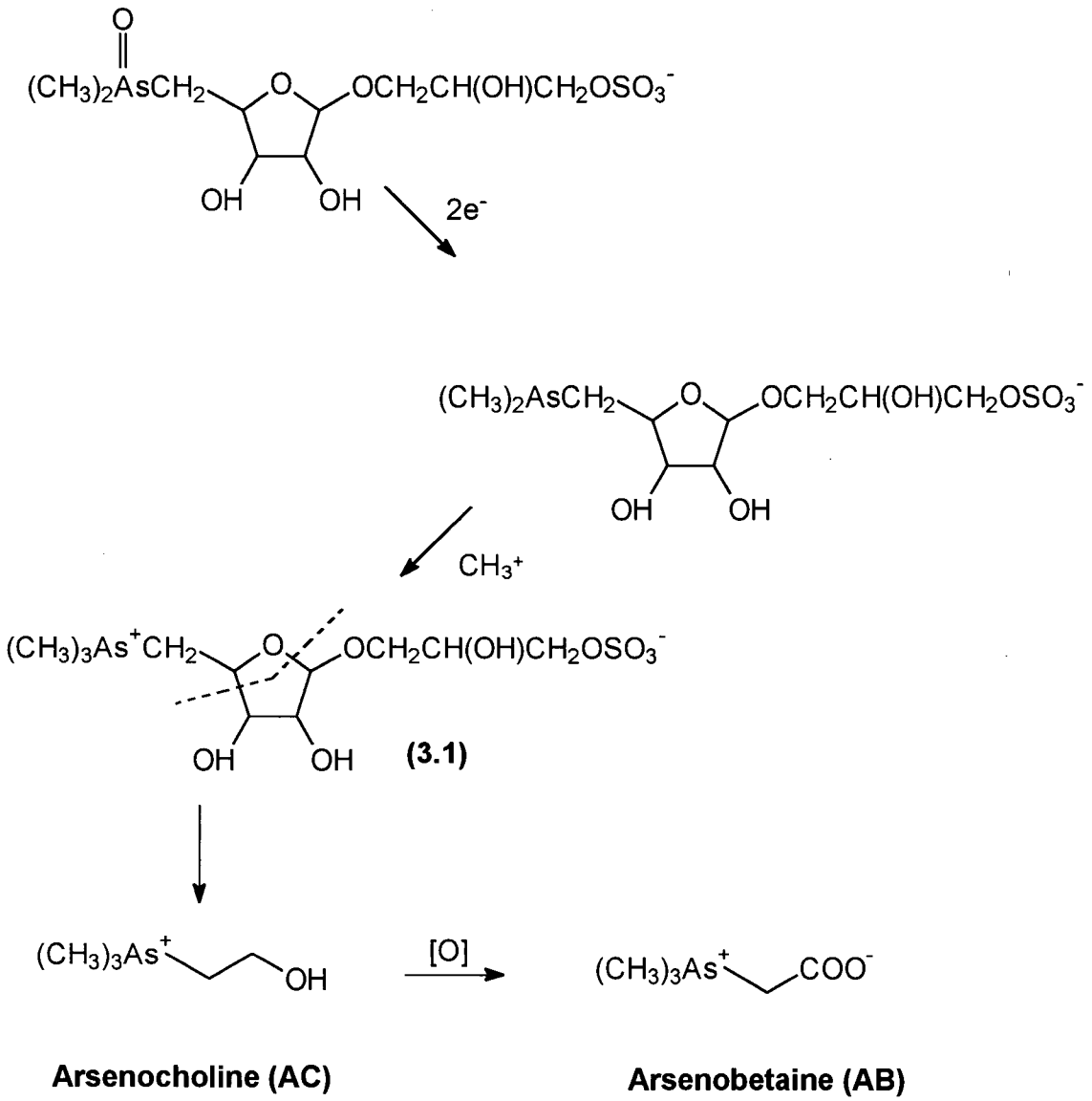


Figure 1.3. Pathway showing the formation of a trimethylarsonioriboside (**3.1**) followed by the formation of arsenobetaine[30].

Much is known about arsenic species in the marine environment, and reviews by Cullen *et al.* [20] and Francesconi *et al.*[32] address this topic in more detail than that given here. Such a wealth of knowledge is available because of the ubiquitous presence of arsenic in marine samples, as well as sufficiently high levels of arsenic for reliable speciation analysis. However, the information that is obtained is dependent on the analytical methods available and as a result, little reliable data exists for arsenic compounds that are not water soluble. Additionally, the presence of trace amounts of crucial intermediates in the formation of organoarsenicals in environmental samples is dependent on the detection limits possible by using current analytical methods.

1.4.3. Arsenic species in the terrestrial environment

Arsenic concentrations in freshwater systems vary depending on the geological composition of the area and the input from anthropogenic sources, such as agriculture or mining operations. Levels in rivers and lakes range from 0.1 to 75 ppb in some reports[43], and they can be substantially elevated in hot springs (up to ppm levels)[44]. As (V) is usually the major species, but high proportions (30-75% of total arsenic) of As (III) have been known to occur[21,45], as well as MMA, DMA, TMAO and trivalent methyl- and dimethylarsenic species[46,47]. Methylation of inorganic arsenic by anaerobic microorganisms associated with lake sediments has been observed[48], and biomethylation is expected to take place following the pathway proposed by Challenger (Figure 1.1).

In soils, arsenic is associated with Fe and Al compounds[49]. The amount of arsenic in the fraction that is soluble and hence available to plants has been estimated to be 0.07-0.2% of the estimated total, and the species of arsenic in this fraction were found to be As (V) and As (III)[50]. Another study showed that from 9 soil samples, only one sample contained arsenite in a

proportion greater than 1.38% of the estimated total arsenic, and As (V) was assumed to constitute the remainder[51]. However, arsenic in terrestrial soil and sediment is not limited to inorganic forms only; $(CH_3)AsH_2$, $(CH_3)_2AsH$, $(CH_3)_3As$, $(C_2H_5)(CH_3)_2As$, $(C_2H_5)_2(CH_3)As$ and $(C_2H_5)_3As$ were generated with sodium borohydride from river sediments[52].

As will be discussed in Chapters 4 and 5, few studies have examined the speciation of arsenic compounds in plants and other terrestrial biota, and details of previous studies are given in sections 4.3 and 5.3. A Japanese study concludes that methyl species, of unknown chemical structure, are present in a green alga, a diatom, a freshwater prawn, a marsh snail, freshwater fish and fly larvae, sampled from an area impacted by geothermal waters and containing elevated arsenic levels[53]. Mostly inorganic species, some DMA and trace amounts of MMA and arsenobetaine are present in ants living in an arsenic contaminated area[54]. Vegetables grown in arsenic amended soil were found to contain mostly arsenate, but trace amounts of MMA were present in lettuce, potato and swiss chard[55].

The findings of arsenobetaine and arsenocholine, but not arsenosugars, in mushrooms[56,57,58,59] led to the postulation by some researchers that arsenosugars are not involved in the biosynthesis of arsenobetaine[32]. Since the discovery of arsenosugars in the terrestrial environment[60,61], however, the possibility has emerged that pathways (e.g., Figure 1.2) in the terrestrial environment are similar to those in the marine environment.

Most studies to date indicate that arsenobetaine and arsenosugars are not as common in the terrestrial environment as they are in the marine environment. Among mushrooms, for example, the form of arsenic varies greatly from one species to the next. Some species contain only inorganic arsenic, and others contain only arsenobetaine. Clearly, studies to determine the arsenic compounds present in terrestrial samples are necessary to elucidate the chemical processes taking place in the terrestrial environment.

1.5. Environmental chemistry of antimony

1.5.1. Antimony sources, uses and exposure to humans

Antimony occurs at about one-tenth the concentration of arsenic in the Earth's crust, and is usually found as stibnite (Sb_2S_3), as well as in ores of copper, silver and lead[62]. The major use of antimony is in the form of Sb_2O_3, as a flame retardant in textiles, paper and plastics[62,63]. Antimony compounds are used to a lesser extent in paints and ceramics; as catalysts, glass decolourizers and metal hardeners; and in the semiconductor industry[62,64]. Antimony compounds are also used as a treatment for tropical parasitic diseases[65,66].

Sources of antimony in the environment are usually similar to those for arsenic, since antimony often occurs together with arsenic-containing ores[67]. Hence, weathering of rocks in areas with high arsenic and antimony content, geothermal activities, mining and smelting operations, and industries utilizing antimony can all contribute to the introduction of antimony into the natural environment. For example, high levels of antimony relative to other metalloids have been observed in landfill and sewage sludge fermentation gases[68,69].

Humans are not frequently exposed to antimony, except in working conditions that involve antimony, such as battery charging[64], antimony processing, and welding[66]. Usually, ecological exposure to antimony is also accompanied by exposure to other toxic compounds, such as those of lead, arsenic, cobalt or silica[66]. Nevertheless, the US-EPA considers antimony to be a priority pollutant[70,71] and the threshold limit permitted for antimony and its compounds in work room air is 0.5 mg Sb/m^3 [70]. The United States Food and Drug Administration (US-FDA) tolerates a maximum of 2 ppm of antimony in food[66], and the accepted daily limits for humans

orally exposed to antimony compounds (over an extended period of time) range from 24.5-32.5 μg of antimony compound per day[62].

1.5.2. Antimony species in the environment

Sb (V) species are thermodynamically most favourable under aerobic conditions and hence $Sb(OH)_6^-$ is predicted to be the predominant species of inorganic antimony in oxygenated water[72]. Sb (III) compounds are thermodynamically predicted to be oxidized to Sb (V) at neutral pH and in an oxidizing environment. Sb_2O_3 dissolves to a limited extent in water at neutral pH (<5 ppm), forming an undissociated species, that is, $HSbO_2$ or $Sb(OH)_3$[72]. The structure of organoantimony compounds in aqueous solution is unknown, except for Me_3SbCl_2, Me_3SbBr_2 and Me_3SbI_2, which hydrolyze at neutral pH to form $Me_3Sb(OH)_2$, and possibly Me_3SbO[73,74].

In accordance with the thermodynamic prediction, most studies report predominantly Sb (V) with small proportions of Sb (III), methyl- or dimethylantimony species in seawater[75,76], estuarine waters[75,77], rivers[75,78,79], waste waters[80,81], geothermal waters[82] and condensed water from landfill gas[69]. Levels of antimony in waters are typically less than 1 ppb[62], although polluted waters can contain levels of antimony from 300-800 ppb[75,81].

Sb (V) as $Sb(OH)_6^-$ was the major species extracted from soils[82], and it was postulated that Me_3SbO was present in one such extract[80]. Compounds forming CH_3SbH_2, $(CH_3)_2SbH$, $(CH_3)_3Sb$ and $(C_2H_5)_3Sb$ were found in river sediment that was reacted with sodium borohydride[52], indicating the presence of small, but detectable levels of organoantimony compounds in soils and sediments. Antimony was shown to be associated with iron and aluminum in sediments[83], but in soils it was mostly in a form not extractable by sequential leaching procedures; the highest extractable amount was bound to Fe-Mn oxides[84]. Antimony

concentrations of up to 1489 ppm have been found in soil samples near an antimony smelter[85]. In contrast, the levels of antimony in the Earth's crust are estimated to be between 0.2 and 1 ppm[66]. Less than 1% of antimony can be extracted from soil by using an aqueous extraction method[82], so it is not surprising that levels in human fluids were not significantly elevated, with respect to controls, following internal exposure to antimony contaminated soil[86].

The volatile antimony compounds SbH_3 and Me_3Sb have been found in gases sampled above hot springs, landfills and from sewage[68,69,87]. Very few studies have speciated antimony in biota. Dodd *et al.* found methylated antimony species in aquatic plants collected from an area impacted by mining and hence containing elevated levels of antimony[88]. Kantin reported mostly Sb (V) in extracts of marine algae, with low levels of Sb (III) in some samples[89], and similar results were found in extracts of mollusk shells, with only one sample containing Sb (III)[90]. Total levels of antimony were found to be elevated in biota collected near an antimony smelter with respect to those collected from control areas, and the authors concluded that uptake, but not biomagnification, of antimony takes place[85,91].

1.5.3. Biological and chemical transformations of antimony

The findings of methylated antimony compounds in environmental samples, as mentioned in section 1.5.2, suggest that methylation is taking place in the environment. Recently, it was confirmed that biological methylation of antimony takes place by anaerobic soil cultures[92] and, to a lesser extent, by aerobic cultures of the fungus *Scopulariopsis brevicaulis*[93,94]. The volatile antimony compound formed, Me_3Sb, was found to oxidize rapidly in an aerobic environment[94]; soluble dimethyl- and trimethylantimony compounds were found in liquid culture media[93]. *S. brevicaulis* was capable of converting only 0.001-0.01% of antimony in liquid culture to

Me_3Sb[94], which is much less than its ability to convert arsenic to Me_3As (~ 1%)[20]. The volatilization of antimony by *S. brevicaulis* from mattresses contaminated with the fungus and containing antimony as a flame retardant has been suggested to be a cause of the sudden infant death syndrome (SIDS), or cot death[95,96]. However, stibine, postulated to be the volatile compound responsible for the antimony poisoning, has not yet been found as an antimony metabolite formed by *S. brevicaulis* cultures amended with antimony. This observation, together with the low conversion of antimony to volatile compounds by the fungus, makes it unlikely that antimony is linked to SIDS. Additionally, reliable epidemiological evidence linking the presence of antimony in mattresses to victims of SIDS is lacking[97].

Sb (III) is oxidized to Sb (V) by fungal cultures[98] as well as by the freshwater alga *Chlorella vulgaris*[99]. The antimony that was accumulated by *C. vulgaris* cells appears to be bound to proteins whose molecular weight is around 4×10^4 Da[99]. The bacterium *Stibiobacter senarmontii* is reported to use Sb_2O_3 as a substrate for growth and may also convert this compound to Sb (V). Another bacterium *Thiobacillus ferrooxidans* has been observed to oxidize stibnite[100].

Trimethylstibine is insoluble in water, but it quickly and spontaneously forms the water soluble compound Me_3SbO in the presence of oxygen. This is postulated to contribute to the mobilization of Me_3Sb in the environment[101]. Trimethylstibine was observed to form the water soluble $[Me_4Sb^+]$ ion in the presence of alkyl halides (which may be present in the environment) in polar solvents, leading to the speculation that this may also be a process occurring in the aqueous environment[102]. No evidence exists yet for the presence of the $[Me_4Sb^+]$ ion in the environment. The lack of analytical methodology for the trace detection of such antimony species likely limits the range of compounds found in the environment.

1.6. Objectives and scope of work

The general theme in this work is to expand the knowledge about the species of arsenic and antimony occurring in the terrestrial environment.

The goal of Chapter 2 is to describe improvements and clarifications of some existing analytical methods for arsenic and antimony compounds. These include high-performance-liquid-chromatography coupled to inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) and electrospray ionization-ion trap mass spectrometry (ESI-IT-MS) for the analysis of arsenic compounds, and hydride-generation gas chromatography atomic absorption spectrometry (HG-GC-AAS) for antimony compounds.

Two objectives are sought in Chapter 3; one is to learn more about the pathways to the formation of arsenic compounds in mushrooms, in an attempt to address the uncertainty associated with pathways to the formation of arsenosugars and arsenobetaine, described in section 1.4.2. The other objective is to increase the knowledge of antimony behaviour in the environment by studying controlled laboratory interactions of antimony with organisms. Although some studies concerning the biological transformations of antimony have been carried out (described in section 1.5.3), clearly the number is limited, and nothing is known about the transformations of antimony in biota used for human consumption.

In Chapters 4 and 5, the objective is to increase the knowledge of arsenic speciation in terrestrial ecosystems, including biota such as plants and lichens, which have not previously been studied, by using modern speciation methods. This information may be used to help determine the impact of increased arsenic loading in terrestrial environments.

The speciation analysis of antimony in environmental samples in Chapter 6 contributes to the understanding of antimony in the environment and adds to the limited existing data, especially for biota, as summarized in section 1.5.2.

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Chapter 2

METHODS FOR THE ANALYSIS OF ARSENIC AND ANTIMONY

2.1. Methods for the Analysis of Arsenic

2.1.1. Introduction

Many methods have been proposed for the analysis of total arsenic in environmental samples, but only the most commonly used ones will be summarized here. Usually samples are digested to convert the arsenic in the sample matrix into a water soluble inorganic species. Dry ashing followed by wet digestion, and wet digestion alone are the most commonly used techniques[1]. Arsenic is then quantified in a sample by analyzing the solution. Photometric analysis following arsenic complexation is known[2], but has been replaced with hydride generation (HG) techniques preceding flame atomic absorption spectrometry (FAAS)[3], atomic absorption spectrometry (AAS) with a quartz tube atomizer[4,5,6], graphite furnace atomic absorption spectrometry (GFAAS)[7], inductively coupled plasma (ICP) with optical emission spectrometry (OES)[8], and ICP with mass spectrometric detection (MS)[9]. Direct analysis of solutions containing arsenic (as well as other metals) is very common, using some of the previously mentioned methods: GFAAS[10], ICP-OES and ICP-MS[11,12,13]. Electrochemical methods are known as well[14]. Solid samples (subjected to little or no sample preparation, i.e., no wet digestion) are frequently analyzed by using neutron activation analysis (NAA)[15,16] and X-ray fluorescence methods (XRF)[17].

The speciation of arsenic compounds is based almost exclusively on chromatographic methods coupled to element-specific detection. These methods include gas chromatography

(GC) and high performance liquid chromatography (HPLC), as well as the less commonly used supercritical fluid chromatography (SFC) and capillary electrophoresis (CE).

Arsenic compounds found in most samples are polar or ionic in character, and not volatile. Therefore they are not directly amenable to GC, so that GC methods usually involve the derivatization of the arsenic species. Hydride generation is the most common method, using sodium borohydride as the reducing agent, as shown:

$$3NaBH_{4\,(aq)} + 2As(OH)_{3\,(aq)} + 3H_2O \rightarrow 2AsH_3\uparrow + 6H_2\uparrow + 3NaH_2BO_{3\,(aq)}$$

The aqueous species As(V)/(III), $CH_3AsO(OH)_2$, $(CH_3)_2AsO(OH)$ and $(CH_3)_3AsO$ can be derivatized to the volatile hydrides AsH_3, CH_3AsH_2, $(CH_3)_2AsH$ and $(CH_3)_3As$, respectively, with boiling points of –62.5°C, 2°C, 36°C and 52°C respectively[18]. The inorganic species of As (V) and (III) can be speciated by using different reaction conditions to form AsH_3. More specifically, As (III) can be determined alone at pH>4 and As (III) plus As (V) can be determined quantitatively at pH<2, so that the amount of As (V) is calculated by difference.

An extraction step to selectively complex As (III) compounds can be carried out to differentiate between the normally indistinguishable (by HG) methylarsenic (III) and methylarsenic (V) acids, which are then reacted to form their hydrides, and separated by GC[19]. Methylarsenic(III)thiols, but not methylarsenic (V) compounds, have been selectively derivatized into hydrides by adjusting the pH to 6 during the HG reaction and they were then detected by AAS following GC separation[20].

Most GC separations are carried out by using a packed column, but capillary GC with flame ionization detection (FID), electron capture detection (ECD), AES and MS, has been used as well[21,22]. Derivatization is carried out by using thioglycolic acid methylester

($HSCH_2COOCH_3$) and arsenic compounds that have been analyzed include a series of organoarsenic halides, such as Lewisite ($(ClHC=CH)AsCl_2$), an arsenical considered to be a chemical warfare agent.

The method of HPLC can separate a range of arsenic species and does not require derivatization. The liquid sample can be directly analyzed and the results obtained may be more representative of the arsenic speciation in the original sample or extract. Arsenicals are most often identified and detected by using ICP-MS or ICP-OES for the following reasons. The flow rates from HPLC columns are similar to those used in the ICP uptake and nebulization system (around 1 ml/min) allowing for easy coupling. ICP-MS and ICP-OES are very sensitive (providing low detection limits of pg levels and ng levels, respectively) and are capable of detecting several elements simultaneously.

Ion exchange chromatography is often used for the analysis of arsenic compounds, because arsenicals typically exist as anions or cations, depending on the pH used. The technique depends on the attraction between the ions and the oppositely charged stationary phase. Gel permeation chromatography has also been used for the speciation of arsenic compounds, including a trimethylarsonioriboside[23].

The use of ion-pairing chromatography is very common for arsenic speciation. In this technique, an "ion-pairing reagent", possessing an alkyl chain and an ionic end, is added to the mobile phase and allows the separation to take place on a C18 column, instead of on an ion exchange column. The current theory for ion-pairing chromatography is based on the electric double layer model. In this model, an electric double layer forms when the lipophilic end of the ion-pairing reagent interacts with the C18 column, and a counter ion interacts with the ionic end of the reagent. The resulting layers of charge enable mobility of the analyte through the column

to be a function of coulombic attraction, as well as interaction with the non-polar stationary phase[24,25].

Vesicular chromatography coupled with derivatization of the eluted analytes into their hydrides and microwave induced plasma (MIP)-AES analysis has been used for arsenic speciation[26]. In this technique, a mobile phase containing micelles, formed from a surfactant such as didodecyldimethylammonium bromide (DDAB) in a concentration above the critical micellar concentration (CMC), is used, together with a C18 column. The interaction of the analyte with the chromatographic system is thought to resemble anion exchange.

Supercritical fluid chromatography (SFC) with supercritical CO_2 as the mobile phase has also been used to speciate arsenic compounds. Arsenic compounds were reacted with thioglycolic acid methylester (TGM) and the resulting volatile species were then extracted by using supercritical CO_2 and detected by using capillary SFC with FID[27]. In another study, coupling of SFC to ICP-MS allowed separation of trimethylarsine ($(CH_3)_3As$), triphenylarsine ($(C_6H_5)_3As$) and triphenylarsenic oxide ($(C_6H_5)_3AsO$) with no derivatization[28].

Capillary electrophoresis (CE) has been applied to arsenic speciation. CE has been carried out with UV detection[29,30,31], direct injection nebulizer (DIN)-ICP-MS[32] detection, and HG-ICP-MS detection[33,34]. The use of ICP-MS detection in the aforementioned studies gave better detection limits, but also required coupling devices that are not commercially available. The arsenic compounds As(V), As (III), MMAA and DMAA were separated in all these studies. A study by Schwedt *et al.* incorporated a technique for $HAsO_3S^{2-}$ speciation[31], which allowed the monothioarsenate ion to be determined at ppm levels in soil extracts.

Mass spectrometry has been used as a detector for arsenic compounds, in some cases following HPLC separation. Conventional and microbore HPLC was coupled to liquid secondary ion mass spectrometry (LSI-MS), continuous flow LSI-MS (CF-LSI-MS), and direct

liquid introduction mass spectrometry (DLI-MS) to speciate arsenic animal feed additives[35]. Fast atom bombardment (FAB) combined with MS as well as tandem MS (MS/MS) [36,37], field desorption MS (FD-MS)[36], atmospheric pressure chemical ionization and electrospray ionization combined with a triple quadrupole MS (APCI-MS and ESI-MS)[38], desorption chemical ionization MS (DCI-MS) and matrix-assisted laser desorption ionization/time-of-flight MS (MALDI-TOF-MS)[39,40] are techniques that have been used for the characterization of arsenic compounds as standards, and in some cases, the identification of arsenic species in samples.

The method of electrospray ionization (ESI) with ion trap mass spectrometry (IT-MS) was used in the present study. ESI is a soft ionization technique in which ions in solution are transformed into gas phase ions, suitable for MS analysis. It is especially useful for ionic, heat labile and high molecular weight compounds and allows MS analysis to be performed on compounds present in liquids (e.g., following HPLC separation). Ions are produced when the sample solution is nebulized and the droplets formed are subjected to a high voltage, causing their surfaces to be electrically charged. The solvent evaporates from the droplet, causing the charge density at the surface to increase; the droplet becomes unstable, breaking into smaller droplets; and sample ions are ejected into the gas phase by electrostatic repulsion from the small droplets. The ions can then be analyzed by using a mass analyzer such as an ion trap[41].

The ion trap mass analyzer allows for high sensitivity, simplicity, and tandem techniques (MS^n), among other features[42]. Ions are trapped in the space created by three electrodes: a ring electrode, and two end-cap electrodes. An ac voltage of constant frequency and variable amplitude (an rf voltage) is applied to generate an electric field and its amplitude is kept at a low value initially so as to maintain stability of the ions in the trap. A mass scan takes place when the amplitude of the applied rf voltage is increased, causing instability and subsequent ejection and detection of ions of increasing m/z. MS^n experiments are performed by ejecting all ions from the

trap except for the selected ion, and then applying a supplementary rf voltage to the end caps to translationally excite the ions. Product ions result from collisionally induced dissociation of the excited ions with helium buffer gas.

2.1.2. Experimental

2.1.2.1. Chemicals and reagents

Arsenic standards were obtained as sodium arsenate, $Na_2HAsO_4.7H_2O$ (Aldrich), arsenic trioxide, As_2O_3 (Alfa), methanearsonic acid, $CH_3AsO(OH)_2$ (Vineland Chemical), and cacodylic acid, $(CH_3)_2AsO(OH)$ (BDH), and were dissolved in deionized water to make standard solutions. Extracts of kelp powder (Galloway's, Vancouver, BC) and Nori (*Porphyra tenera*) of known arsenosugar content[43] were used to identify the retention times of arsenosugars; these were verified by comparison to pure arsenosugars generously donated by K. Francesconi and T. Kaise. Arsenobetaine[44], arsenocholine[45], trimethylarsine oxide[46], and tetramethylarsonium iodide[47] had been synthesized previously according to known methods. Methanol (HPLC grade, Fisher), tetraethylammonium hydroxide (TEAH, 20% in water, Aldrich), malonic acid (BDH), concentrated phosphoric acid (Aldrich), ammonium hydroxide (1M, Fluka), pyridine (Fisher), and formic acid (BDH) were used as reagents for mobile phases and extractions.

2.1.2.2. Instrumentation and methods of analysis

2.1.2.2.1. HPLC-ICP-MS

The HPLC apparatus consisted of a Waters 510 double piston pump, a Rheodyne six-port injection valve with a 20 µl loop, in-line filters, a guard column for each analytical column packed with the same stationary phase, and the analytical column. Columns and mobile phases are listed in Table 2.1. A VG Plasmaquad PQ2 Turbo ICP-MS (VG Elemental) was used as a detector. Parameters for the ICP-MS are given in Table 2.2. The m/z monitored were 75, along with m/z 77 and 82 in the case of real samples, to correct for any interference from the Cl-Ar molecular

ion. ^{35}Cl-^{40}Ar gives a m/z of 75; monitoring m/z 77 corresponding to ^{37}Cl-^{40}Ar would allow this interference to be confirmed. However m/z 77 is also a Se isotope, and hence monitoring ^{82}Se allows differentiation from the interference. Chromatographic signals at m/z 77 were not present at the same retention time as those at m/z 75 when they appeared, and hence interference was never a problem. In most analyses, m/z 103 (Rh) was monitored as well, because Rh was added to the mobile phase and used as an internal standard to correct for plasma instability. The HPLC was coupled to the spray chamber of the ICP-MS by using a minimum of PTFE tubing (10 cm × 0.5 mm i.d.) with the appropriate PTFE fittings. Data from the ICP-MS were processed by using chromatographic software[48], and identification of arsenicals in samples was made by comparison of retention times with those of standards by using at least two chromatographic systems. Semi-quantitative concentrations of arsenic compounds were determined by using external calibration curves for each compound corresponding to a matching standard, or to DMA for arsenosugars.

Table 2.1. HPLC conditions for arsenic speciation

Chromatography	Column	Mobile phase	Flowrate (mL/min)
Anion exchange	Hamilton PRP-X100, 150 × 4.6 or 250 × 4.6 mm	20 mM ammonium phosphate, pH 6.0	1.0 or 1.5
Cation exchange	Supelcosil LC-SCX or Whatman SCX Partisil 5, 250 × 4.6 mm	20 mM pyridinium formate, pH 2.7	1.0
Ion-pairing	GL Sciences ODS, 250 × 4.6 mm	10 mM TEAH, 4.5 mM malonic acid, 0.1%-0.5% MeOH, pH 6.8	0.8

Table 2.2. Operation parameters for ICP-MS

Feature	Specific Conditions
Forward radio-frequency power	1350 W
Reflected power	<10 W
Cooling gas flow rate (Ar)	13.8 L/min
Intermediate (auxiliary) gas flow rate (Ar)	0.65 L/min
Nebulizer gas flow rate (Ar)	1.002 L/min
Nebulizer type	de Galan
Analysis mode	Time Resolved Analysis (TRA)
Quadrupole pressure	9×10^{-7} mbar
Expansion pressure	2.5 mbar

2.1.2.2.2. ESI-IT-MS

Arsenosugar standards (diluted to give solutions of about 500 ppb) and the kelp extract were analyzed. The kelp was extracted according to published methods[43] and contained the following approximate concentrations of arsenosugars in the extract: X, 175 ppb; XI, 30 ppb; XII, 70 ppb; XIII, 220 ppb[49].

A Finnigan LCQ™ including a flow injection system consisting of a syringe pump delivering solutions in 50% MeOH at 100 µl/min was used. This instrument can utilize atmospheric pressure ionization (API) in two modes: ESI and atmospheric pressure chemical ionization (APCI), of which the former was used for this study. The ring electrode was kept at 0.76 MHz and the rf amplitude varied from 0 to 8500 V during mass scans. Experiments were carried out in negative and positive ion modes. This was done by changing the polarity of potentials applied to (a) the ion source including the electrospray capillary and the heated capillary tube; (b) the ion optics, specifically the interoctapole lens, situated between two

octapoles; and (c) the conversion dynode in the ion detection system. The partial pressure of helium in the ion trap was kept at 0.1 Pa (1 mTorr). For MS^n experiments, the selected ions were subjected to supplementary voltages (supplied to the endcaps) ranging from 12 to 33 V and further fragmentation took place as a result of collisions with He. Trapping of fragments, fragmentation of daughter ions and changing of voltages were carried out in real time while monitoring the mass spectra.

2.1.3. Results and Discussion

2.1.3.1. Speciation of arsenic compounds by HPLC

2.1.3.1.1. Anion exchange chromatography

The column used in these studies was a resin based column (poly(styrenedivinylbenzene)) with trimethylammonium groups providing the exchange sites (Hamilton PRP-X100, see Table 2.1). The elution order of some arsenic compounds when using 20 mM ammonium phosphate at pH 6 as the mobile phase is shown in Figure 2.1: arsenobetaine (AB)/As (III)/arsenosugar X, DMA, MMA, arsenosugar XI, As (V), arsenosugar XII and arsenosugar XIII. The chromatogram obtained from a mixture of As (III), As (V), MMA and DMA was overlayed with that from a mixture of arsenosugars. Standard cationic compounds were not analyzed by using this system but they are expected to elute in the dead volume, prior to arsenobetaine. ICP-MS was used as a detector in all HPLC experiments.

As (III) is present as a neutral molecule (pK_a 9.3), arsenobetaine is a neutral zwitterion, and arsenosugar X is in the neutral fully protonated form. These neutral species are separable from the cationic species, and to some extent from each other. Their retention times differ by about 10 seconds; therefore, although they are not baseline resolved, if one is present as the major species, a tentative identification can be made based on the retention time. DMA is the next eluting compound and about 50% is in the singly charged anionic form (pK_a 6.28). MMA, which elutes next, is predominantly in the singly charged anionic form (98.6%, pK_{a1} 3.6, pK_{a2} 8.2). The later elution of arsenosugar XI compared with MMA may be a result of the anionic character of the phosphate group in arsenosugar XI (see Figure 1.1, structures of arsenosugars), and possibly enhanced interaction of the sugar with the resin.

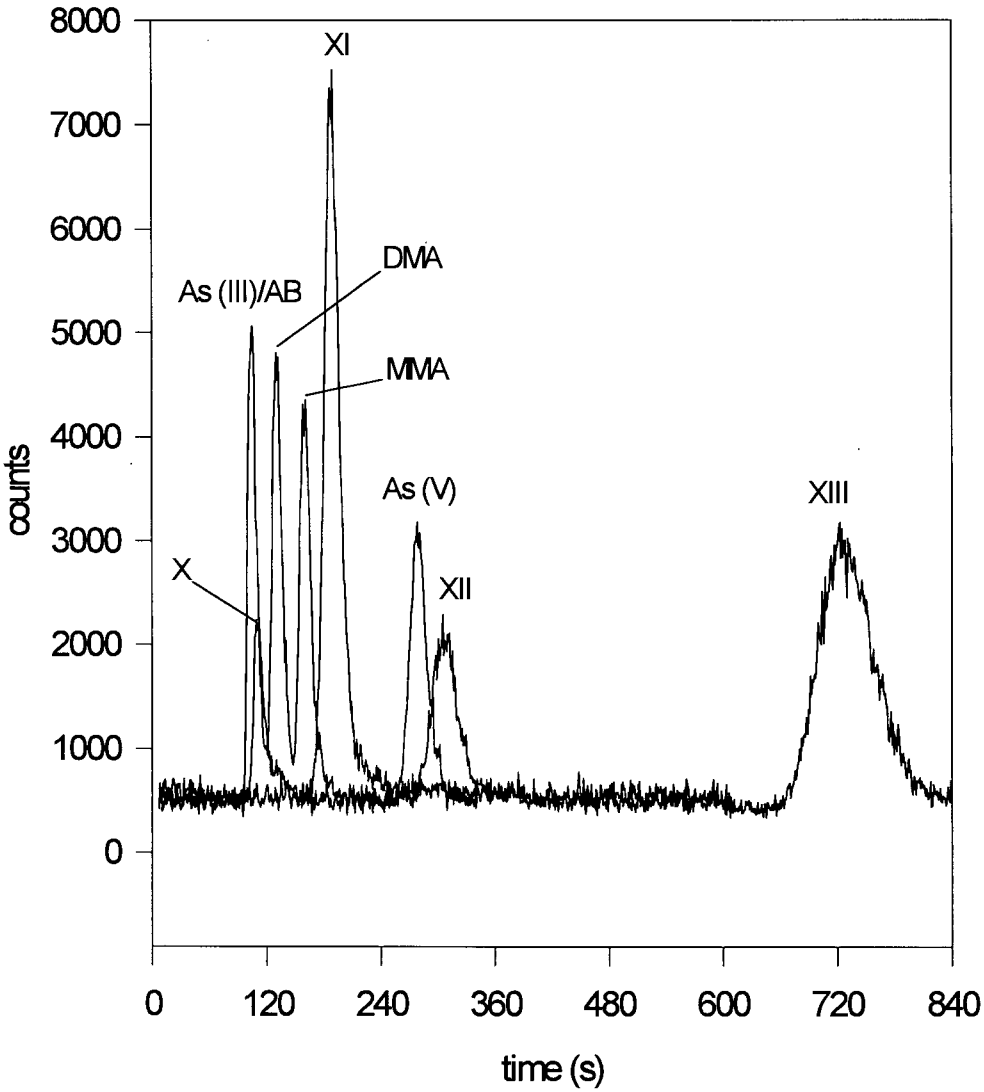

Figure 2.1. Chromatogram of standard arsenic compounds by using Hamilton PRP-X100 anion exchange column (15 cm) with 20 mM ammonium phosphate, pH 6.

As (V) is anionic, being 90% singly charged and 10% doubly charged, hence its later elution. Arsenosugars XII and XIII, containing sulfonate (XII) and sulfate (XIII) groups (see Figure 1.1, Chapter 1, for structures of arsenic compounds), are most likely singly charged and anionic and they elute later than As (V), when using the chromatographic system described in Figure 2.1. This later elution may be due to the increased interaction between the organic groups on these molecules with the resin.

Arsenosugars have been separated on an anion exchange system in another study[50], where 20mM ammonium carbonate at pH 10.3 was used to give the same elution order for the arsenosugars as that observed under the present conditions. The use of the carbonate mobile phase resulted in co-elution of arsenosugar X with cationic species, and co-elution of arsenosugar XI with DMA. Hence this system using ammonium phosphate as the mobile phase offers some improvement.

2.1.3.1.2. Cation exchange chromatography

Cation exchange chromatography with ICP-MS detection was used to confirm or identify the compounds that co-elute in the anion exchange system described above. The chromatogram in Figure 2.2 shows the elution order of some arsenic species: arsenosugar XI, XIII/MMA, DMA, arsenosugar X, arsenobetaine (AB), trimethylarsine oxide (TMAO), arsenocholine (AC), and Me_4As^+. Chromatographic peaks corresponding to the arsenic species As (V) and As (III) are not shown in Figure 2.2, to provide clarity of presentation, since these compounds elute closely together with other species. Nevertheless, their retention behaviour was observed and is discussed below. The chromatograms for a mixture of standards (MMA, AB, TMAO, AC, and Me_4As^+) was overlayed with the chromatogram for kelp extract, containing arsenosugars X, XI, XII (with unknown retention time) and XIII.

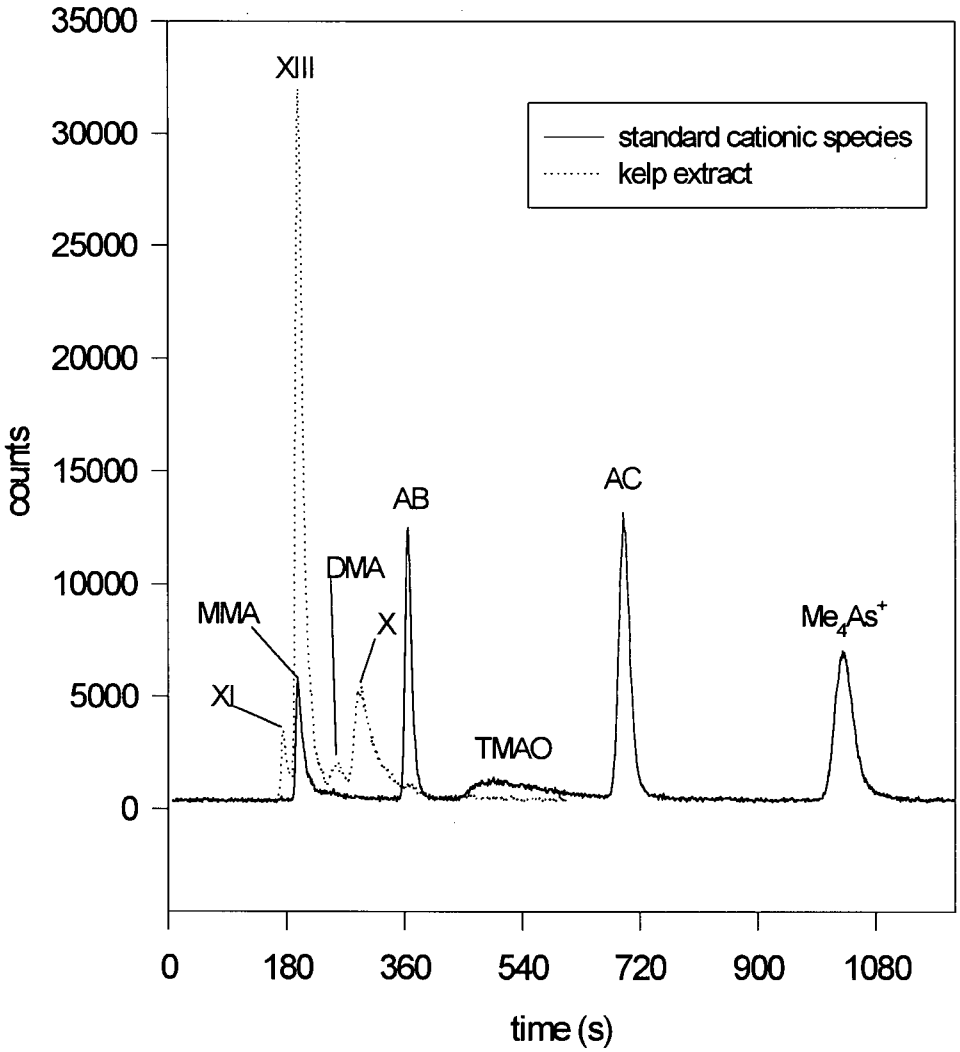

Figure 2.2. Chromatogram of standard arsenic compounds by using Whatman SCX cation exchange column with 20 mM pyridinium formate, pH 2.7.

A silica gel based column containing benzene sulfonic acid functional groups as exchange sites (Whatman SCX or Supelcosil LC-SCX, see Table 2.1) was used with a mobile phase consisting of 20 mM pyridinium formate at pH 2.7, based on methods described in previous studies[51,52]. At this pH, As (V) has 74% singly charged anionic character and is unretained by the column (not shown in Figure 4). pK_a's for the individual arsenosugars are not known but the pK_a for the dimethylarsinoyl moiety on the sugars is estimated to be 3.85[53], indicating that the arsenosugars should be somewhat cationic at this pH. However, the co-elution of arsenosugar XI with As (V) (not shown in Figure 2.2), which is unretained, indicates that arsenosugar XI may be more anionic or neutral in character.

Arsenosugar XIII, on the other hand, was observed to co-elute with the neutral species As (III) and MMA (As (III) not shown in Figure 2.2). DMA is separated from the neutral species, indicating some cationic behaviour, possibly as $Me_2As^+(OH)_2$[54]. The partial protonation of the oxygen in the dimethylarsinoyl moiety in arsenosugar X would give it some cationic character, allowing it to be retained on the column. Arsenobetaine has a pK_a of 2.18[54] and is 20% cationic in nature (80% zwitterionic and neutral at this pH). It is probably more cationic than the previous two species, and hence retained longer on the column. TMAO is thought to exist as $[(Me_3AsOH)^+ (OH)^-]$, from the hydrolysis of Me_3AsO[55], although the peak is very broad under these conditions, which may indicate the presence of more than one species.

Arsenocholine and Me_4As^+ are cations irrespective of pH, and their retention behaviour under these conditions indicate that arsenocholine is less strongly retained. Previous studies have shown the same elution order for these two compounds under the same conditions, but the elution order was reversed when a bare silica column was used[54], and when an ion-pairing system with a reversed phase column and sulfonate mobile phase was used[55]. No explanation was given in the former study, and in the latter study arsenocholine was suggested to be more hydrophobic.

The organic nature of the pyridinium mobile phase may cause the more hydrophobic arsenocholine to be eluted faster from the cation exchange column in the present study. However, other interactions, such as hydrogen bonding between silica OH groups and the OH group on arsenocholine, must be responsible for its longer retention time on the bare silica column.

2.1.3.1.3. Ion-pairing chromatography

When the presence of arsenosugars is indicated by one or both of the two previously mentioned chromatographic systems, it can be confirmed by using a third chromatographic system. The system used here is one that has been developed for the analysis of anions, particularly arsenosugars[56]. Ion-pairing chromatography with ICP-MS detection is used, a technique that combines a mobile phase containing an ion-pairing reagent with a reversed phase column. Tetraethylammonium hydroxide (TEAH) is the ion-pairing reagent, and the mobile phase is adjusted to a pH of 6.8 with malonic acid and nitric acid. A small amount of methanol is added (0.1-0.5% v/v). Methanol can be used to control chromatographic behaviour in reversed phase systems, with increased concentrations resulting in shorter retention times for the arsenosugars. Methanol has also been found to increase sensitivity of arsenic compounds when using ICP-MS detection[57].

The elution order shown in Figure 2.3 for some arsenic species is arsenobetaine (AB), MMA, DMA/As (V), arsenosugar X, XI/XII, and XIII. Chromatograms of AB, MMA, DMA and As (V) standards were overlayed with kelp extract and also with Nori extract. Nori extract is known to contain arsenosugars X and XI as the only arsenosugars[43].

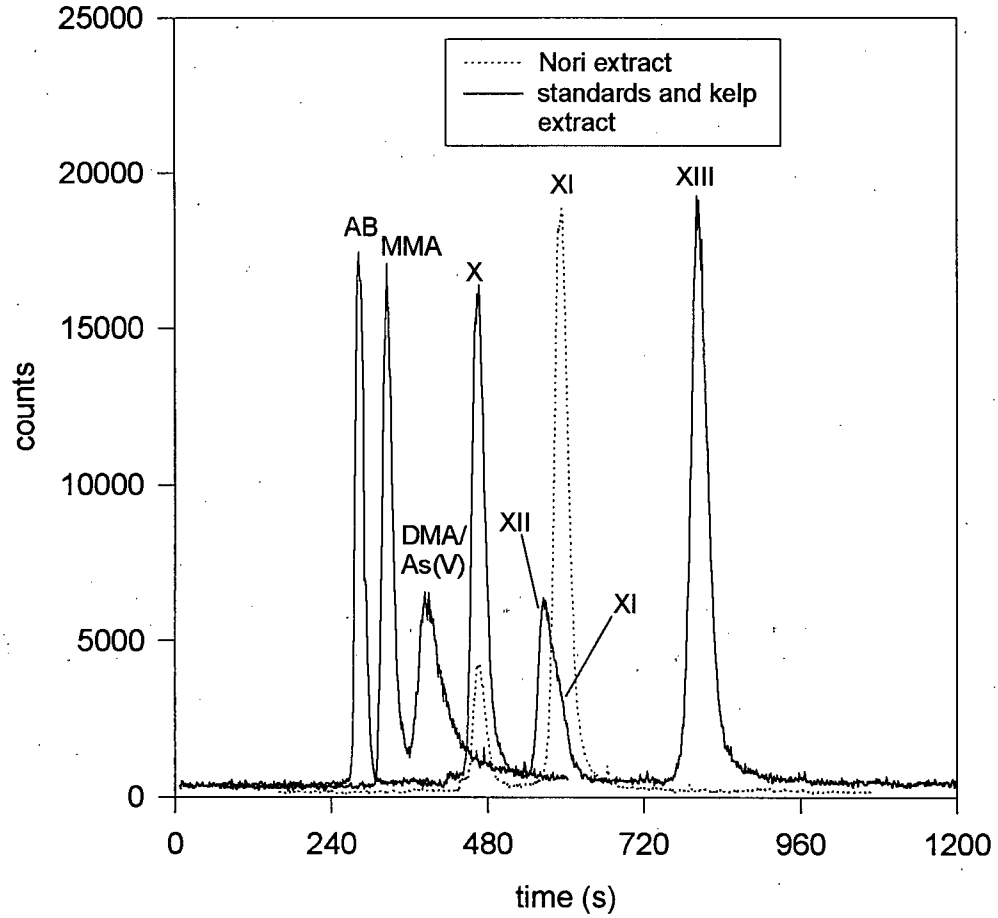

Figure 2.3. Chromatogram of standard arsenic compounds by using ion-pairing reversed phase chromatography, with GL Sciences C18 column and 10 mM TEAH/4.5 mM malonic acid, pH 6.8, 0.1% MeOH.

As (III) elutes closely after arsenobetaine and is not shown on Figure 2.3 to retain clarity. Arsenobetaine and As (III) are expected to be neutral molecules at pH 6.8, and to have short retention times. MMA and DMA elute in the opposite order compared to anion exchange; this may be due to enhanced interaction of DMA (containing 2 methyl groups) with the C18 chains of the stationary phase. DMA co-elutes with As (V), which was also seen by other researchers[58]. Using the present chromatographic system, the arsenosugars are separated from the other ions, although arsenosugars XI and XII are not baseline resolved from each other. Figure 2.3 shows that arsenosugar XI alone (dotted line) elutes slightly later, which allows this compound to be identified in the absence of arsenosugar XII. Likewise, arsenosugar XII can be identified in the absence of arsenosugar XI. The elution order for the sugars most likely indicates increasing anionic character when going from arsenosugar X to XIII.

Although not one of these chromatographic systems can be used alone for the analysis of complex mixtures, such as those found in environmental samples, a combination of anion, cation and ion-pairing chromatography can be used to obtain a satisfactory separation and identification of at least 11 arsenic species. Analyzing a sample by using two or three different chromatographic systems, although time consuming for routine use, is a very useful tool for strengthening the identification of arsenic compounds.

2.1.3.2. Characterization of arsenosugars and compounds in kelp powder extract

Kelp powder has been previously analyzed and it has been suggested that this sample contains the four arsenosugars X, XI, XII, and XIII, based on HPLC retention times[43,49]. As a result of this identification, kelp extract is often used as a reference for the retention times of these arsenosugars, and the retention times are then used to identify arsenosugars in other

samples of unknown arsenic speciation. An attempt was made to identify the arsenosugars in kelp extract by using a mild ionization mass spectrometric technique, possible with the Finnigan LCQ™ ESI-IT-MS instrument, as well as by analyzing pure arsenosugar standards. Such identification would validate other identifications based on retention times of the reference sample.

The positive-ion ionspray (IS) tandem mass spectra (i.e., MS-MS) of pure arsenosugars X, XI, XII and XIII have been presented by others[59]. Positive and negative-ion fast atom bombardment (FAB) tandem mass spectra have been produced for arsenosugars XI, XII and XIII, as well[60]. It was of interest to compare the fragmentation behaviour of arsenosugars in the present MS-MS experiments by using low-energy, low pressure (1mTorr) CID conditions in the ion trap, with that observed by using low-energy, higher pressure (4-18mTorr)[61] CID MS-MS conditions (IS with a triple-quadrupole mass filter). Because Corr *et al.*[59] observed only positive ions, comparisons were also made between the present results and the results obtained by Pergantis *et al.*[60] (high-energy CID conditions, with FAB reverse-geometry four-sector mass analyzer) for negative and positive-ion mass spectra.

A summary of MS and MS-MS analyses obtained from pure arsenosugars is given in Table 2.3.

Table 2.3. ESI-IT-MS experiments and fragments for pure arsenosugars.

Arsenosugar (mode)	Precursor ion m/z selected	Product ion m/z	Relative abundance (%)
X, MW = 328	329	329	46
(positive)		311	52
		237	100
		195	26
	329 → 311	311	100
		209	30
	329 → 237	237	100
		219	75
XI, MW = 482	483	391	65
(positive)		329	100
		237	13
	483 → 329	329	100
		237	55
XI, MW = 482	481	389	100
(negative)		245	27
	481 → 389	389	100
		267	20
		223	49
		193	33
XII, MW = 392	393	392	100
(positive)		281	53
XII, MW = 392	391	391	52
(negative)		373	100
		269	48
	391 → 269	269	100
		225	90
		197	17
XIII, MW = 408	407	408	24
(negative)		389	100
		285	62
		171	52
	407 → 389	171	100
	407 → 285	285	21
		241	100
		213	35
		153	75
		137	28
		97	27
		87	30
	407 → 389 → 171	171	35
		97	100

Only low concentrations of arsenosugars were available and the mass spectrometer was contaminated by previous samples, which caused high background levels during the arsenosugar analyses. Hence, the MS^n capability of the ion trap mass analyzer in the mass spectrometer was useful because it allowed isolation of specific ions of the sugars and subsequent fragmentation. Only the two sugars XI and XII could be analyzed by both negative and positive modes to give useful information. Molecular type ions were not observed in large enough abundance for trapping and subsequent MS-MS analysis for the other two sugars, X and XIII, in the negative and positive modes, respectively.

As mentioned earlier, arsenosugar X (MW = 328) was characterized in the positive ionization mode by Corr *et al.*[59] by using an ionspray source and triple quadrupole mass spectrometer with MS-MS capabilities. They found a single product ion forming at m/z 237, corresponding to the fragment containing arsenic and the ribose group. Other previous studies showed the formation of fragments at m/z 311, 221 and 177 for this compound[47]. In the present work (Figure 2.4), the fragments at m/z 311 (loss of H_2O), 237 (with the structure shown in Figure 2.4) and 209 are produced when the $(M+H)^+$ ion at m/z 329 is trapped and subsequently fragmented. The fragments obtained when m/z 311 ($[(M+H)-H_2O]^+$) was trapped and fragmented are m/z 209 and 195, which have not yet been identified. The product ion obtained when m/z 237 was trapped and fragmented is m/z 219 (resulting from loss of water). Hence these results show similarity to both of the previous studies, where the fragment of m/z 237 is common with the study by Corr *et al.*[59] and the fragment of m/z 311 is common with the study by Cullen *et al.*[47] Although Pergantis *et al.*[60] did not analyze standard arsenosugar X, they were able to isolate a precursor ion at m/z 329 in the positive-ion mode from an algal extract, and found fragments at m/z 311 and 237 (similar to the present study), as well as at m/z 208, 176, 165, 122 and 97.

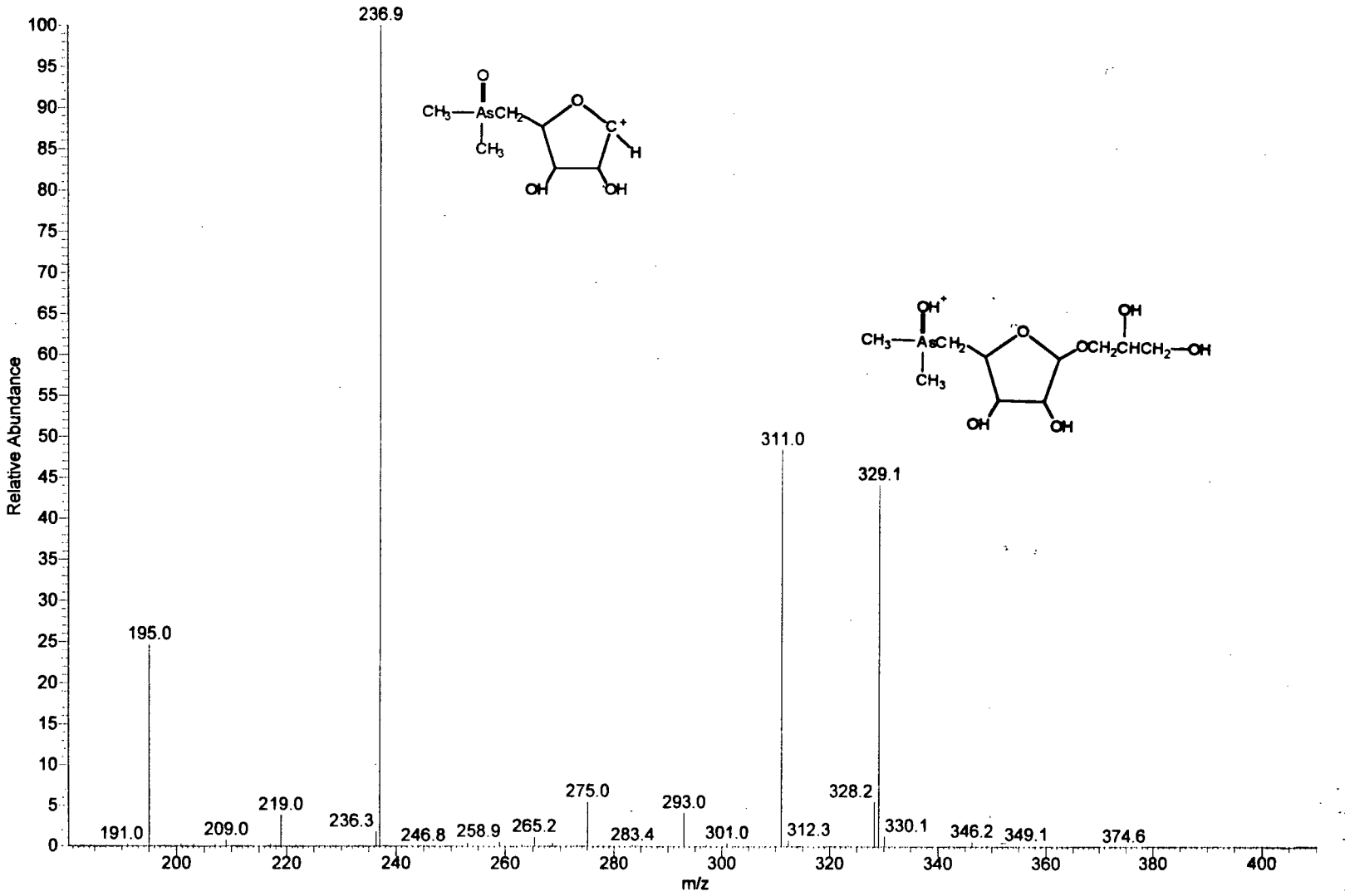

Figure 2.4. Mass spectrum of arsenosugar X standard, MS-MS with m/z 329 selected, positive mode.

Both positive and negative modes of ionization were successful for arsenosugar XI (MW = 482), as mentioned before. When $(M+H)^+$ (m/z 483) was trapped and fragmented (see Figure 2.5), m/z 391 corresponding to loss of $(OH)CH_2(CHOH)(CH_2OH)$ is observed. A fragment of m/z 329, corresponding to arsenosugar X is observed, as well as m/z 237, corresponding to the dimethylarsenic-ribose moiety. These fragments were also observed in the previous studies by Corr *et al.*[59] and Pergantis *et al.*[60]. The higher energy CID conditions led to additional ions in those studies.

When arsenosugar XI was analyzed in the negative ionization mode, and $(M-H)^-$ was trapped and fragmented (see Table 2.3 for these results, and Figure 2.6 for the structure of $(M-H)^-$), m/z 389 is observed as the negative ion analogous to the ion of m/z 391 described above. A product ion of m/z 245 is also observed, corresponding to the fragment remaining when the dimethylarsenic-ribose moiety is lost, with the structure $[(CH_2CH(OH)CH_2OH)_2PO_4^-]$. When m/z 389 was trapped and fragmented (Figure 2.6), m/z 267 is produced, having the structure shown in Figure 2.6, after loss of the dimethylarsenic group. The fragment at m/z 223 may indicate loss of CH_2=CHOH (44 amu) from the m/z 267 fragment. The fragment at m/z 193 is unidentified. Pergantis *et al.*[60] observed m/z 389 and 245 as well, but not 267 or 223.

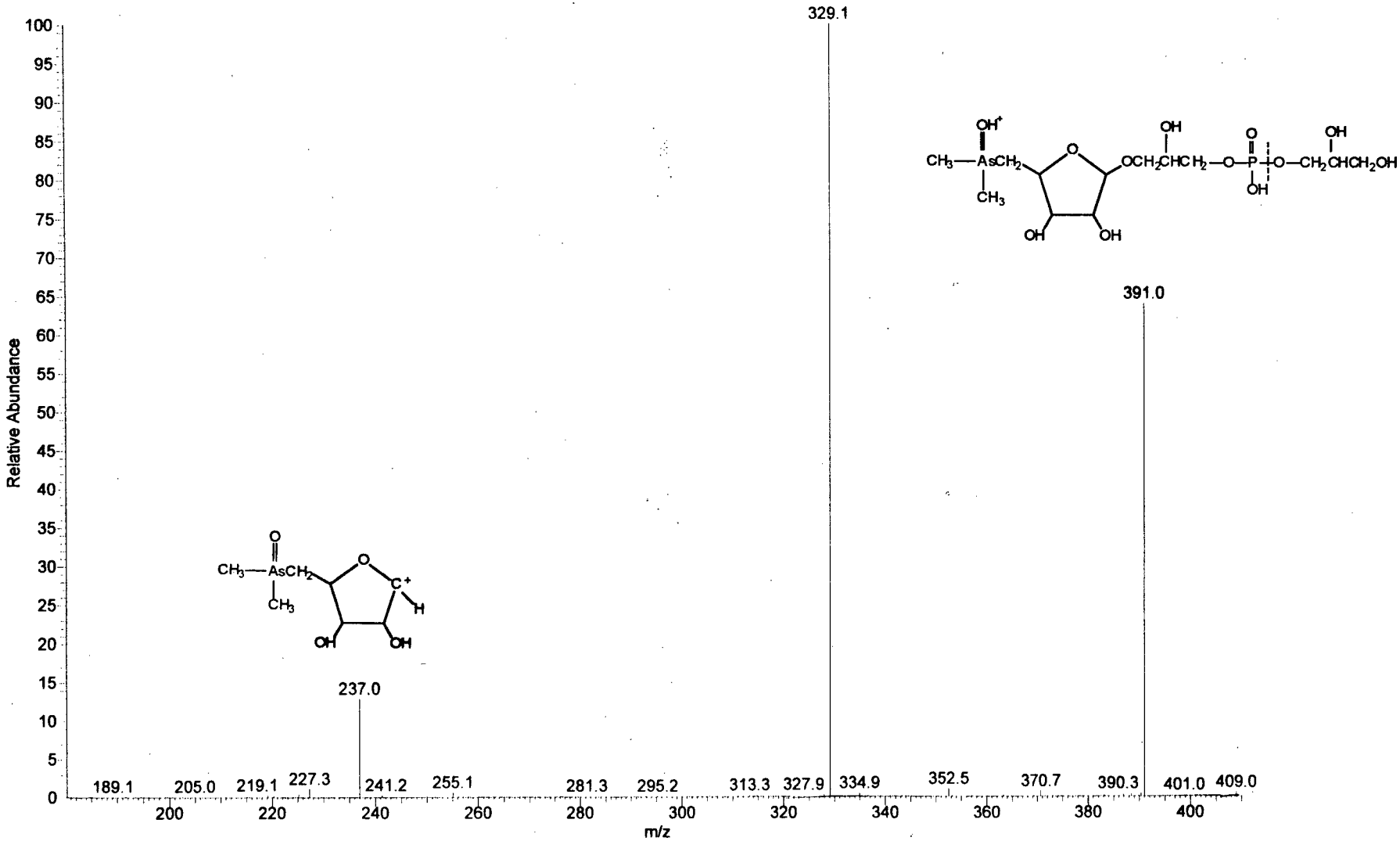

Figure 2.5. Mass spectrum of arsenosugar XI standard, MS-MS with m/z 483 selected, positive mode.

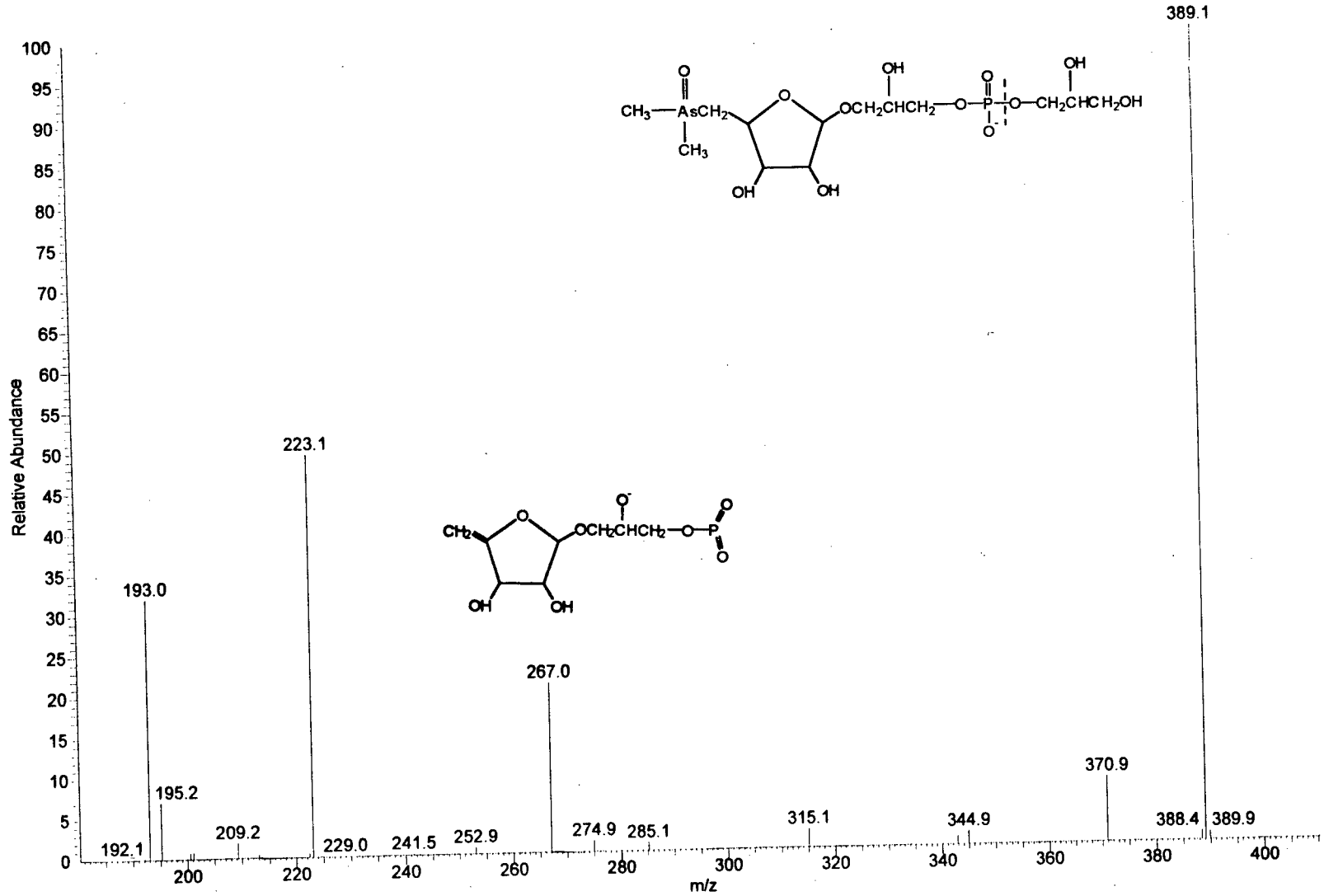

Figure 2.6. Mass spectrum of arsenosugar XI standard, MS-MS-MS with m/z 481 selected, then m/z 389, negative mode.

Arsenosugar XII (MW = 392) was successfully fragmented in both the positive and negative ionization modes. In the positive mode, trapping of m/z 393 ($M+H^+$) ion and MS-MS analysis (Figure 2.7) gives a large abundance of m/z 392 ($M\bullet^+$) and no m/z 393 is observed. A fragment having m/z 281, which may correspond to loss of CH_2-SO_3H and OH (see Figure 2.7) is also observed. These ions (m/z 393 and 281) were not observed when this compound was subjected to ionspray MS in the positive mode by Corr *et al.*[59] or to FAB-MS by Pergantis *et al.*[60] Those authors observed m/z 375, 296 and 237[59], and 375, 237, 165 and 122[60], fragments that were found only in very small abundance or not at all in the present study.

More structural information is obtained when arsenosugar XII was analyzed in the negative mode. The $[M-H]^-$ ion of m/z 391 was trapped and fragmented (Figure 2.8) and loss of H_2O gives m/z 373, and the dimethylarsenic moiety (m/z 269, see Figure 2.8 for structure) is lost. When m/z 269 was subsequently trapped and fragmented, loss of CH_2=CHOH (269-44) followed by loss of CO (225-28) may have occurred to form the fragments at m/z 225 and 197 (structures (C=O)CH(OH)CHOR and (OH)CH=CH(OR), where R is $CH_2CH(OH)CH_2SO_3^-$). Interestingly, m/z 197, but not m/z 225 was observed by Pergantis *et al.*[60]; m/z 197 was assigned a similar structure to the one proposed in the present study.

Arsenosugar XIII (MW = 408) was ionized successfully only in the negative mode, indicating greater stability of the molecule in the anionic sulfate form compared to its stability in the cationic protonated form. This enhanced stability of the anion corresponds well to the more anionic character of this sugar as observed in its HPLC behaviour in the previous section. The anion $(M-H)^-$ at m/z 407 was trapped and fragmented (Figure 2.9) to produce the following ions: m/z 389, corresponding to loss of H_2O; m/z 285, corresponding to loss of the dimethylarsenic moiety as described above; and m/z 171, corresponding to $[HO\text{-}CH_2\text{-}(CHOH)\text{-}CH_2\text{-}O\text{-}SO_3^-]$ (i.e., loss of the dimethylarsenic-ribose moiety). Subsequent trapping of m/z 389 produced the

aforementioned fragment at m/z 171. Trapping of m/z 285, (Figure 2.10) produced fragments at m/z 241 and 213, which seem to follow the same pattern as the fragments at m/z 225 and 197 for the ion at m/z 269 in arsenosugar XII, which may again indicate loss of CH_2=CHOH and then CO. Other product ions from m/z 285 are found at m/z 153 (CH_2=C(OH)-CH_2-O-SO_3^-), 137 (not easy to identify, but possibly resulting from loss of O from m/z 153), 97 (HO-SO_3^-) and 87 (unidentified). The fragmentation of m/z 171 produced m/z 97 (HO-SO_3^-). Pergantis *et al.*[60] observed some of the same fragments: m/z 389, 285, 213 (but not 241), 171, 153 and 97.

Useful structural information was obtained in the negative ion mode for all sugars but arsenosugar X. The loss of the 122 amu fragment was observed for sugars XI, XII and XIII in the negative mode and can be considered diagnostic of this group of compounds[60]. In general, fewer fragments were observed in the positive mode of analysis. Characteristic arsenic containing fragments were observed in arsenosugars X, XI and XII in the positive mode, however. The previous studies reported a greater number of fragments compared with those in the present study, resulting from the use of higher energy CID conditions in those studies. MS-MS-MS experiments were conducted in the present study, providing new information about the behaviour of some of the arsenosugars in this mode.

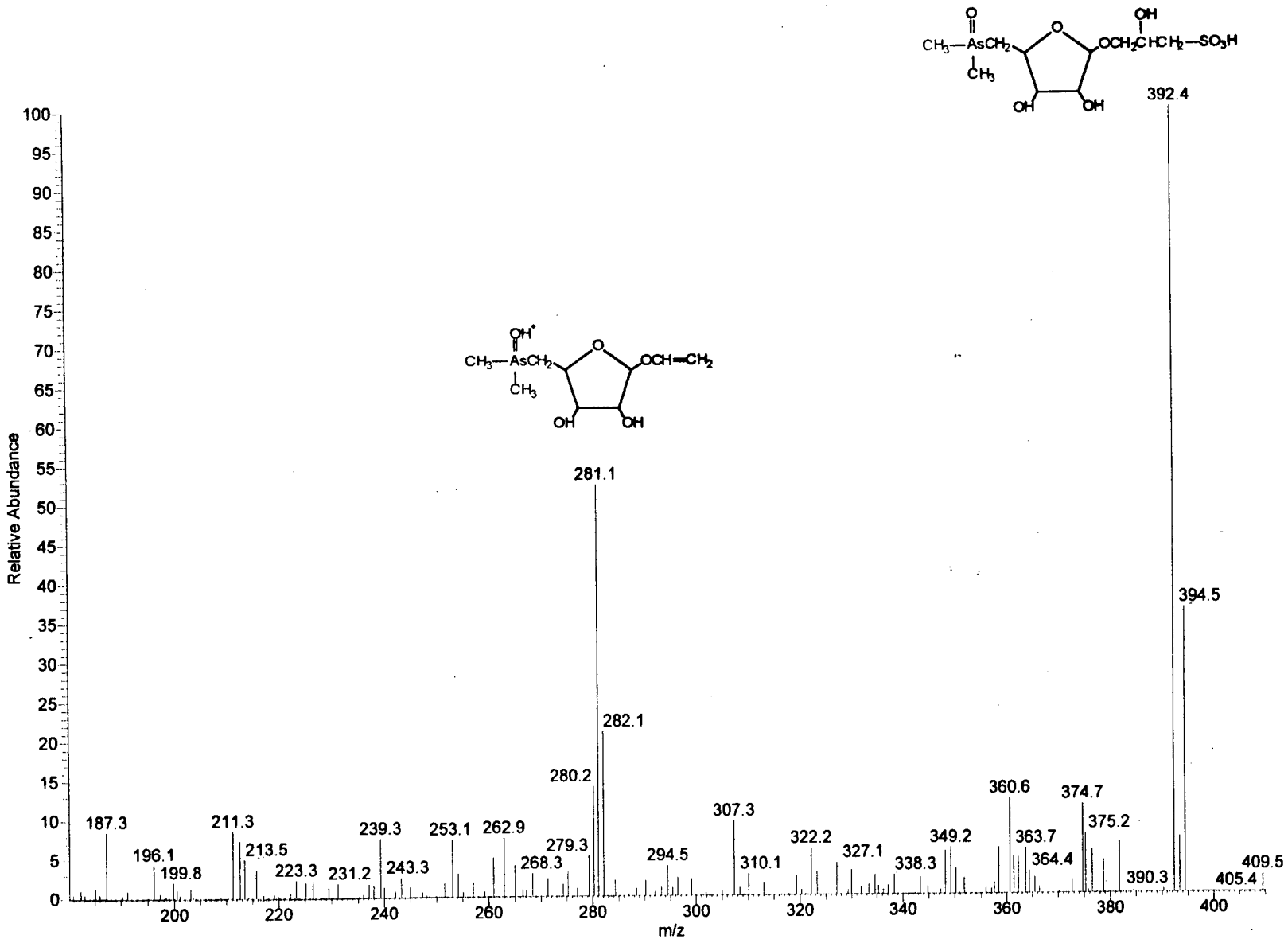

Figure 2.7. Mass spectrum of arsenosugar XII standard, MS-MS with m/z 393 selected, positive mode.

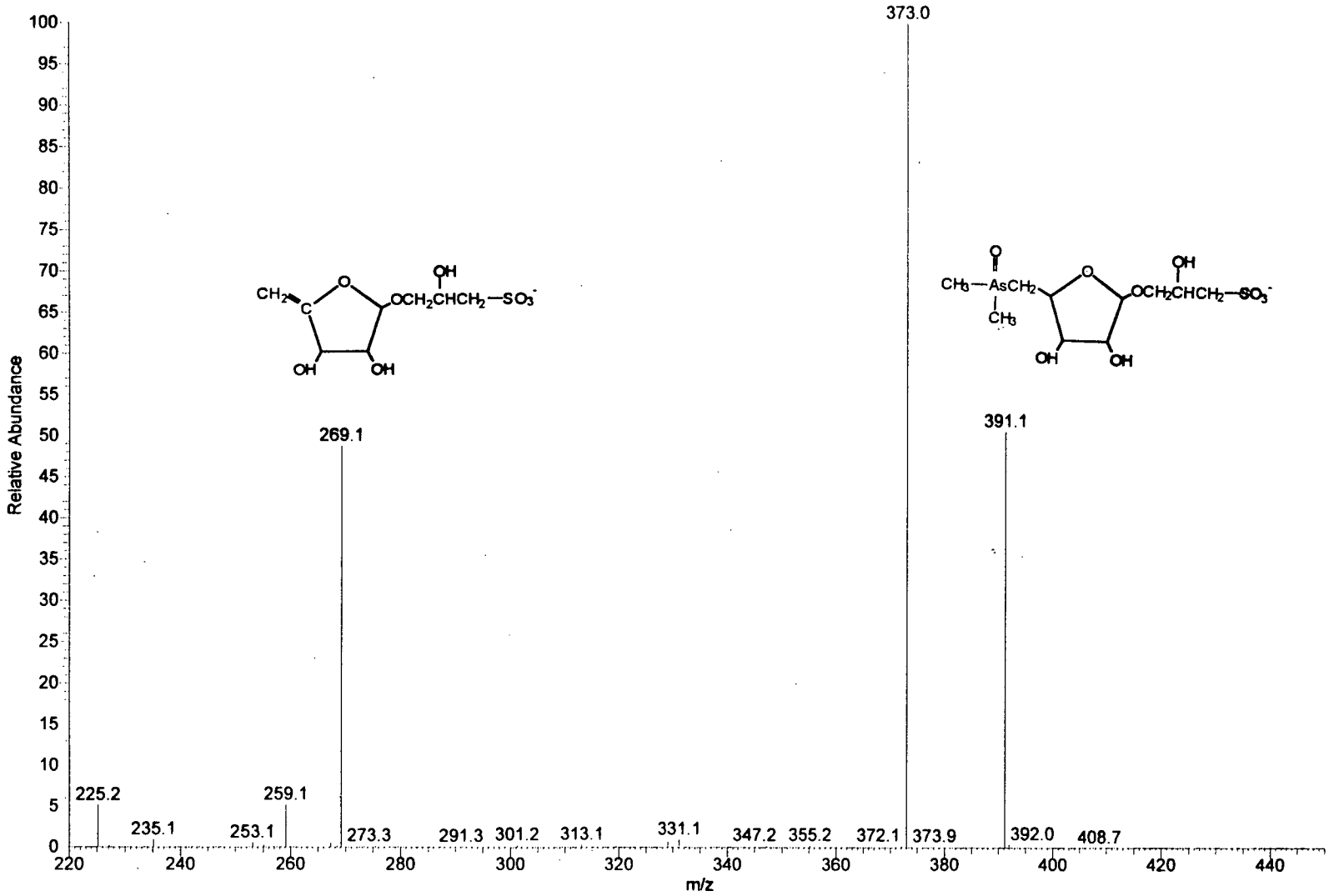

Figure 2.8. Mass spectrum of arsenosugar XII standard, MS-MS with m/z 391 selected, negative mode.

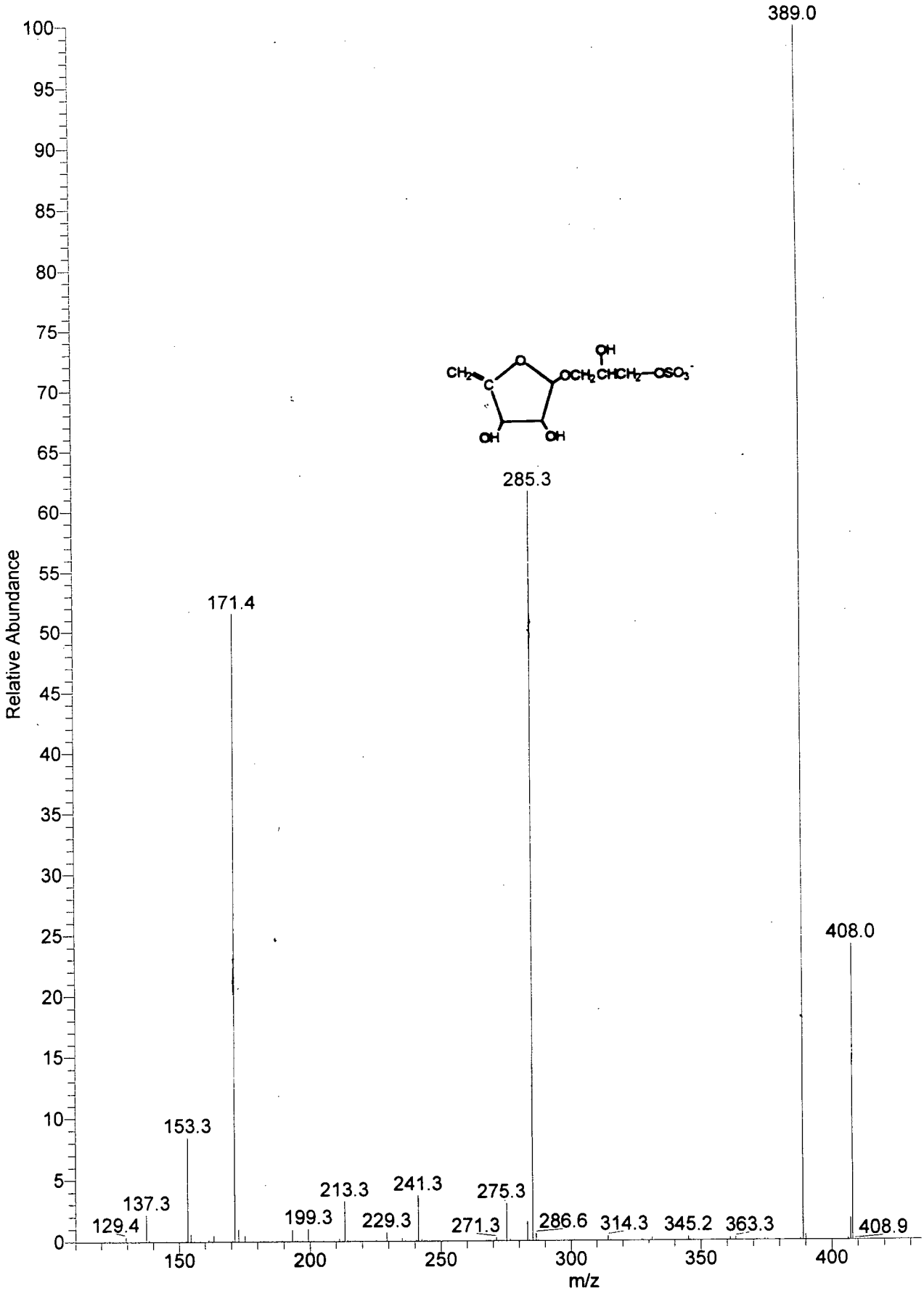

Figure 2.9. Mass spectrum of arsenosugar XIII standard, MS-MS with m/z 407 selected, negative mode.

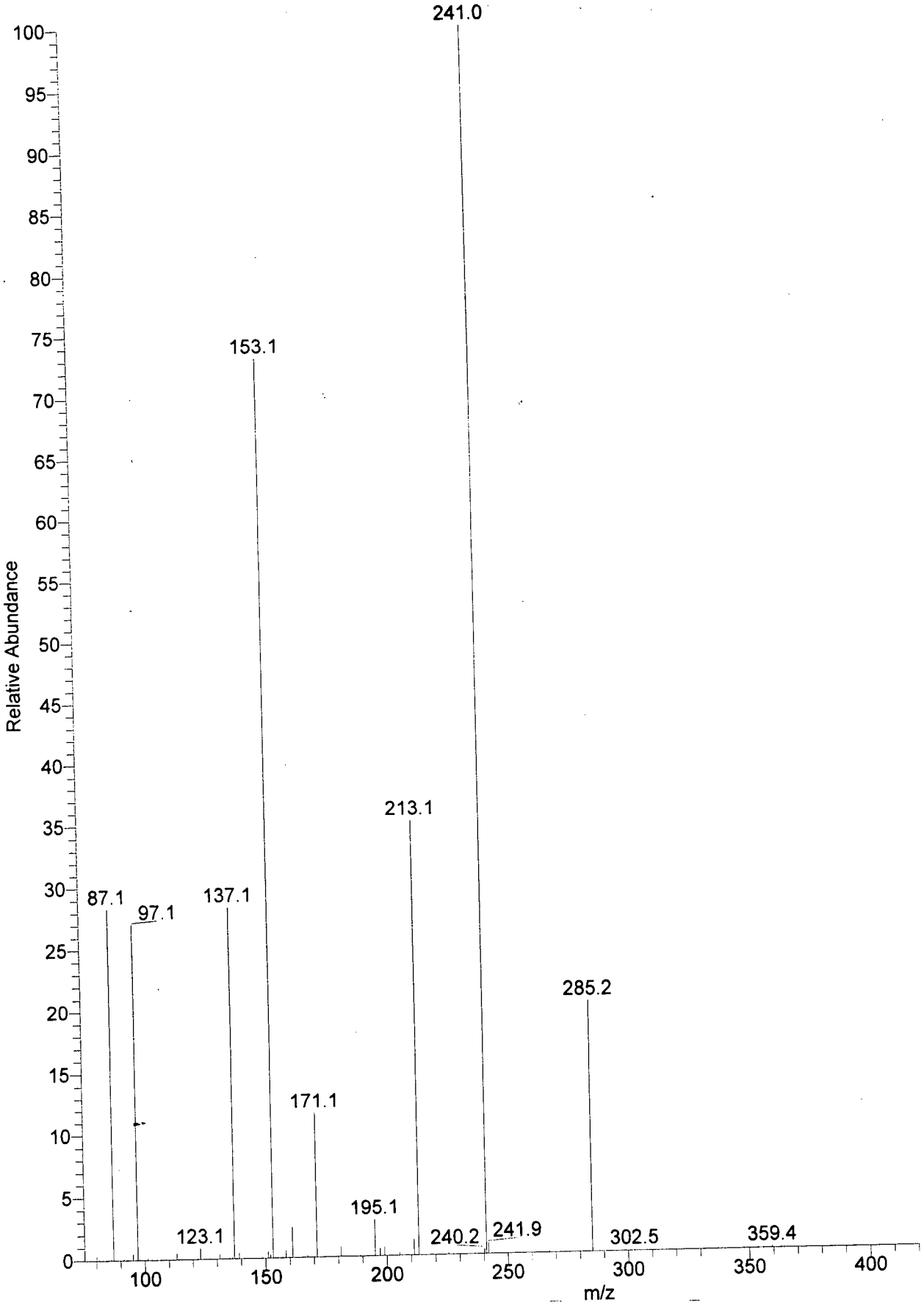

Figure 2.10. Mass spectrum of arsenosugar XIII standard, MS-MS-MS with m/z 407 selected, then m/z 285, negative mode.

A full mass scan in both the positive and negative modes of the crude kelp powder extract did not show the arsenosugars as major ions. However, the MS^n capability of the LCQ™ instrument proved valuable since the ions characteristic of the individual arsenosugars (m/z 329 (positive mode) for arsenosugar X, m/z 481 (negative mode) for arsenosugar XI, m/z 391 (negative mode) for arsenosugar XII and m/z 407 (negative mode) for arsenosugar XIII) could be selected from the complex matrix and then fragmented to ascertain if the characteristic product ions were observed.

Some of the fragments that were produced during the MS and MS-MS experiments are summarized in Table 2.4. The fragments listed are ones that also appear in the pure arsenosugar standards.

Table 2.4. ESI-IT-MS experiments for kelp powder extract

Arsenosugar (mode)	Precursor ion m/z selected	Product ion m/z	Relative abundance (%)
X, MW = 328	329	329	100
(positive)		328	82
		311	7
		237	11
XI, MW = 482	481	481	17
(negative)		463	100
		389	30
		245	27
XII, MW = 392	391	391	100
(negative)		373	85
		225	6
XIII, MW = 408	407	407	80
(negative)		389	100
		285	30
	407 → 285	285	100
		241	25

The fragments that might be considered characteristic occur in very low abundance and in some cases are no more abundant than the other peaks that appear in the mass spectra. This is the case for the precursor ion of m/z 329 (arsenosugar X) and m/z 391 (arsenosugar XII); the characteristic m/z 237 for arsenosugar X, and m/z 225 for arsenosugar XII have abundances of 11 and 6%, respectively (Table 2.4).

Another problem is that other fragments are observed in addition to those obtained from MS-MS experiments with standard compounds and these are probably due to the impurity of the

sample (i.e., precursor ions not with the structure of the arsenosugar may be trapped). Interestingly, some of the other fragments that occur when m/z 481 is trapped and dissociated in the negative mode (Figure 2.11) were also observed by Pergantis *et al.*[60] (e.g., m/z 407 and 287), but not when the standard compound was analyzed in the present study. The ion occurring at 100% abundance, m/z 463, indicates loss of water, but it is also not observed for the standard. Again, the characteristic ions at m/z 389 and 245 are at an abundance not significantly different from the other fragments whose structures are unknown. Low concentrations for arsenosugars XI and XII (about 15 and 35 ppb, respectively, after 1:1 dilution of the extract with methanol), made further confirmation by MS-MS-MS experiments impossible.

In the positive mode, only the $(M+H)^+$ ion (m/z 329) for arsenosugar X could be isolated from the complex mixture, and in addition to the low abundance of the characteristic m/z 237, the presence of $M\bullet^+$ ion at m/z 328, a fragment that was not observed previously, can be seen (Table 2.4).

When the $(M-H)^-$ ion for arsenosugar XIII in the kelp extract (m/z 407) was trapped, the characteristic fragments of m/z 389 (loss of water) and 285 (loss of dimethylarsenic group) are observed. When m/z 285 was subsequently trapped, the fragment of m/z 241, observed in the same experiment with the standard, is seen (Figure 2.12). Another fragment of appreciable abundance is present in Figure 2.12a at m/z 363, indicating either loss of 44 amu (CH_2=CHOH or CO_2) from m/z 407, or loss of 26 amu (C_2H_2) from m/z 389. This fragment was not observed in the mass spectra generated for the standard compound, nor in any other reports. The mechanisms are not obvious for the suggested mass losses and they may involve ring cleavage or molecular rearrangement. However, a similar mass loss is observed from m/z 481/463 (sugar XI) and 391/373 (sugar XII). Dissociation processes are apparently taking place that are specific to these compounds in the kelp extract.

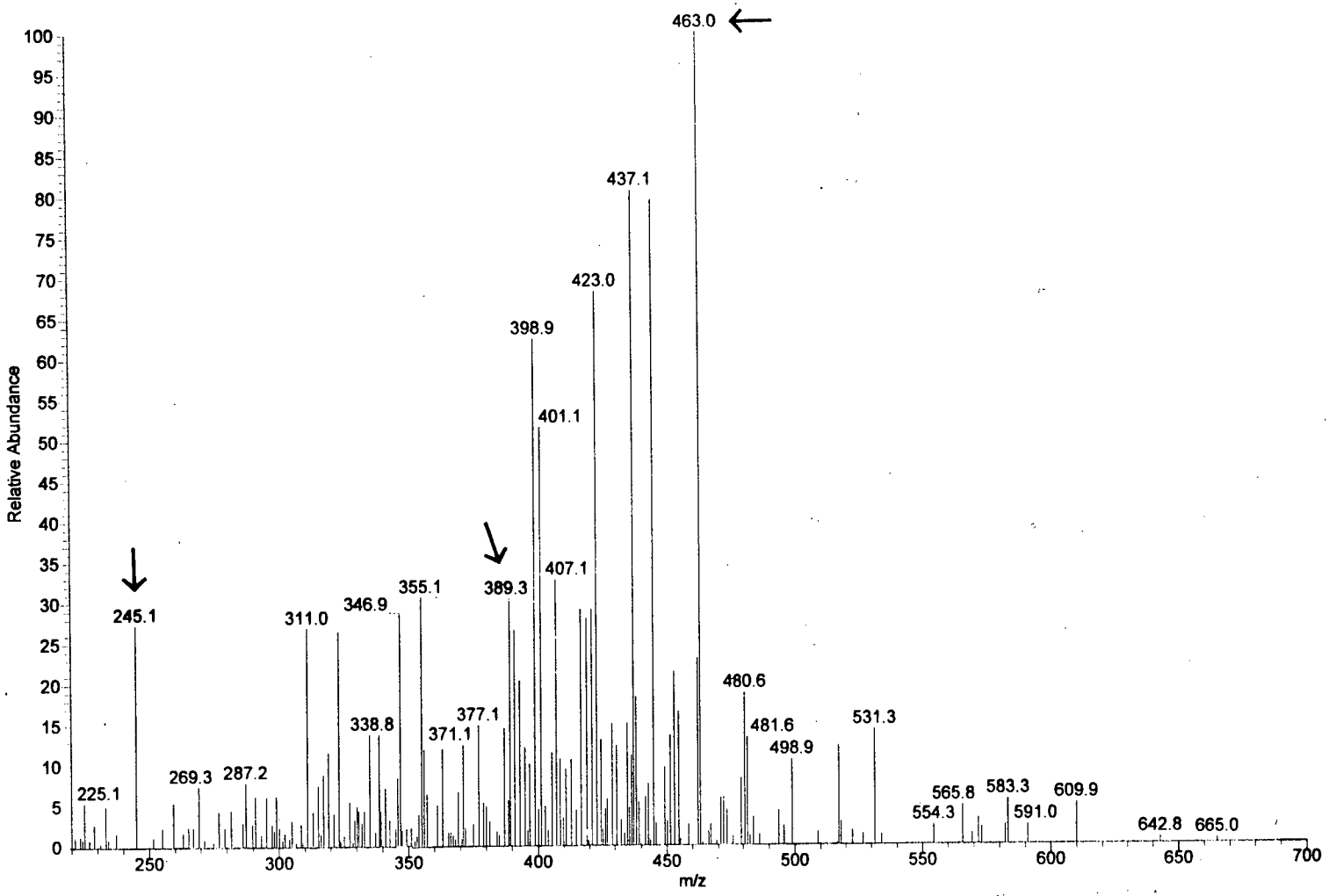

Figure 2.11. Mass spectrum of precursor ion m/z 481 in kelp extract subjected to CID, negative mode. The fragment at m/z 463 indicates loss of water, and at m/z 389 and 245 suggest the presence of ions with structures shown in Figure 2.8.

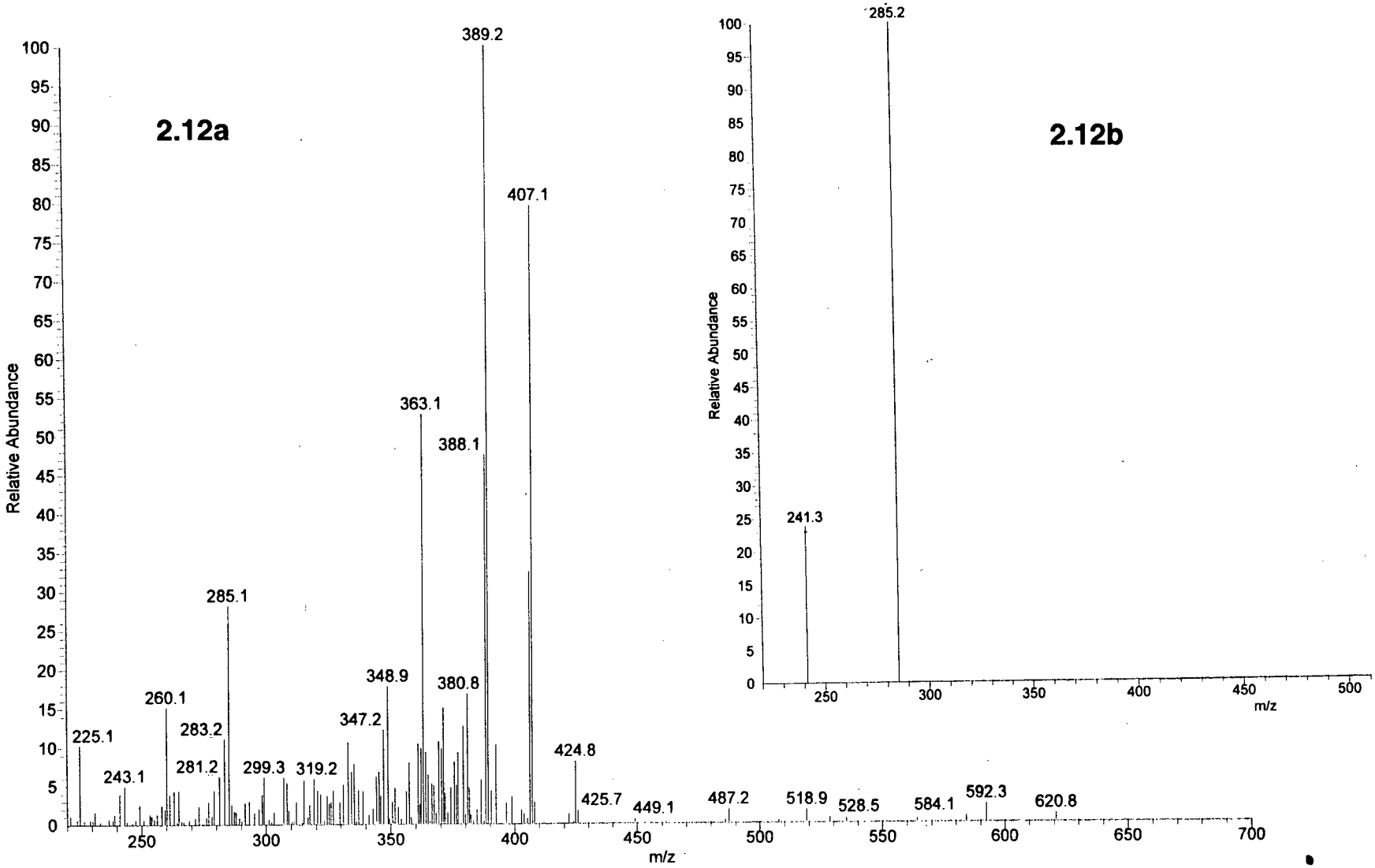

Figure 2.12a. Mass spectra for precursor ion m/z 407 in kelp extract subjected to CID, negative mode. **Figure 2.12b.** Precursor ion m/z 407 selected, then m/z 285. Suggested structures for m/z 285 and 241 in Figure 2.9 and text.

The strongest argument for the presence of arsenosugars in this extract is the evidence for arsenosugar XIII because the MS-MS, as well as MS-MS-MS experiments, show characteristic fragments. The identification of the other compounds, based on this data alone, is not as clear. The major reason for the problems with compound identification in the kelp extract is the low levels of the compounds, especially in the presence of large amounts of matrix components. Chromatographic separation followed by these MS experiments would improve the identification of the arsenosugars.

These results are very useful in characterizing the arsenosugar standards, verifying the cationic behaviour of arsenosugar X, the similar anionic behaviours of arsenosugars XI and XII, and the strongly anionic character of arsenosugar XIII, which was also observed in the HPLC behaviour of these compounds. Moreover, this mass spectral information adds to that currently available in the literature.

2.2. Methods for the Analysis of Antimony

2.2.1. Introduction

Methods for the analysis of total antimony in samples are similar to those used for arsenic, which are described briefly in section 2.1.1.

Speciation techniques for antimony are fewer in number. A recent review lists the known methods for speciation, including extraction techniques; electrochemical techniques; coupled techniques such as hydride generation-gas chromatography (HG-GC) with flame-in-tube AAS or ICP-MS detection; and high-performance liquid chromatography (HPLC) with ICP-OES, ICP-MS or HG-AAS detection[62]. Of these, only HG-GC-AAS, HG-GC-MS and HPLC-ICP-MS methods were used for this work.

HPLC techniques are well established for the speciation of arsenic compounds (section 2.1.1), but only recently have HPLC methods been developed to speciate antimony. In the first report of such methods, ICP-OES detection and a cation exchange column were used to separate Sb (III) and Sb (V) in electrolyte solutions[63]. Anion exchange techniques coupled to ICP-MS or ICP-OES were explored by other authors, where mobile phases such as tartrate at pH 5.5[64] and phthalate at pH 5[65,66,67], 2 mM KOH and EDTA at pH 4.5[68] were used successfully to separate Sb (III) and Sb (V), and Me_3SbCl_2 or Me_3SbO[67,68]. These methods have been used with some success to analyze real environmental samples[65,67,68]. Some of these methods were also attempted for the separation of dimethylantimony compounds of unknown structure but with no success[69].

The application of HPLC to antimony speciation is limited by the lack of standard compounds available; for example, no standard dimethyl and monomethylantimony (V) compounds are commercially available, or easily synthesized. At the pHs studied for most of the

HPLC method development, Sb (V) exists as the anion $Sb(OH)_6^-$ whereas Sb (III) exists as $Sb(OH)_3$ when the oxide is dissolved, or as a complexed form such as the antimonyl tartrate, $[Sb_2(C_4O_6H_2)_2]^{2-}$. The Sb (V) anion is not strongly retained on an anion exchange column and is hence easily eluted from an anion exchange HPLC column. Uncomplexed Sb (III), however, precipitates easily as the oxide, and in the complexed anionic form is strongly retained on an anion exchange column. Me_3SbCl_2 most likely exists at neutral pH as $Me_3Sb(OH)_2$ and is not retained or is retained to a minor extent on an anion exchange column[67,68].

As described in section 2.1.1 for arsenic, HG-GC speciation techniques can be used to detect antimony species in samples as inorganic antimony compounds in the +3 and +5 oxidation states, which are derivatized to form SbH_3[70,71,72]. It is also be used for compounds that can be derivatized to form methyl-, dimethyl- and trimethylstibines ($MeSbH_2$, Me_2SbH and Me_3Sb)[70,73].

A problem has been noted by various researchers attempting to apply the hydride generation reaction to the generation of methylated stibines: when trimethylantimony dichloride (Me_3SbCl_2), trimethylantimony dihydroxide ($Me_3Sb(OH)_2$) and dimethylantimony dihyroperoxychloride ($Me_2SbCl(O_2H)_2$) were reacted with borohydride and acid to form their corresponding stibine, four peaks corresponding to SbH_3, $MeSbH_2$, Me_2SbH, and Me_3Sb, appeared for each compound, rather than the anticipated ones of Me_3Sb or Me_2SbH[73,74]. Dodd *et al.*[73] postulated that this "rearrangement" occurred when the reaction apparatus had not been conditioned properly and found that the problem disappeared when the apparatus was rinsed with the reagents ($NaBH_4$ solution and acid solution) for three minutes or more.

In the following section, some problems with the hydride generation derivatization technique will be described for antimony, including examples of rearrangement/ demethylation which can lead to misinterpretation of analytical results.

2.2.2. Experimental

2.2.2.1. Chemicals and reagents

Antimony (V) and (III) standards were obtained as potassium hexahydroxyantimonate, $KSb(OH)_6$ (Aldrich), and potassium antimonyl tartrate, $K_2Sb_2(C_4O_6H_2)_2$ (Aldrich). Me_3SbCl_2 was synthesized as described elsewhere[75]. Stock solutions were made by dissolving these compounds in deionized water and diluting the resulting solutions to 1000 or 100 mg L^{-1} as Sb. Standard working solutions were made by diluting the stock solution with deionized water as necessary. $NaBH_4$ (reagent grade, Aldrich) was dissolved in deionized water fresh daily to provide a concentration of 2% w/v. Glacial acetic acid, citric acid, sodium hydroxide (for pH adjustment), maleic acid and concentrated sulfuric acid were all reagent grade and obtained from common distributors.

2.2.2.2. Method of analysis for HG-GC-AAS

Three methods were used: Methods 1, 2 and 3. The apparatus for Methods 1 and 2 was composed of a semi-continuous flow, hydride generation system developed for arsenic analysis[76], coupled to an atomic absorption spectrometer (Varian AA1275) fitted with an Sb lamp (Varian) operating at a wavelength of 217.6 nm. One modification was made to the basic apparatus in the form of using a gas-liquid separator[77] that resulted in less analyte carryover. The apparatus consisted of Tygon tubing for the peristaltic pump, and PTFE tubing (1/8" OD) for the remainder. The glass gas-liquid separator was silanized with $(CH_3)_2SiCl_2$ before use.

For Method 3 the semi-continuous flow and batch modes of analysis were combined. Figure 2.13 shows a schematic diagram of the apparatus used. Unsilanized glass batch reactors

of 60 ml volume were incorporated into the apparatus from Method 1. A gas-liquid separator was not used for Method 3 since the gases were separated from the liquids in the batch reactors. The AAS, peristaltic pump and tubing were identical to those used in Method 1.

For all methods, data were collected from the AAS and processed directly by using an HP 3390A integrator, or were analyzed with the aid of Shimadzu EZChrom software.

For Method 1, a peristaltic pump was used to deliver standard or sample solution (ranging from 5 μL to 200 μL for standards, and from 1 mL to 5 mL for samples) to mix with the acid or buffer and then to mix with a solution of $NaBH_4$ (2% w/v) in a reaction coil. For Method 2, standard solution was mixed with a solution of $NaBH_4$ (2% w/v) in the mixing coil, and the gas-liquid mixture was mixed with 1M H_2SO_4. For both Methods 1 and 2, the gases evolved were separated in the gas-liquid separator and then swept by a flow of helium through a PTFE U-tube at -78 °C (dry ice/acetone) to remove water and into a PTFE U-tube, where they were trapped at -196 °C (liquid N_2). Continuous hydride generation and trapping were carried out for 3 minutes. The peristaltic pump was then stopped (making the system semi-continuous) and the second U-tube was heated to 60 °C, allowing the gases to be swept with He at a flow rate of 40 mL/min onto a Poropak PS column, which was then heated from 70 °C to 150 °C at a rate of 30 °C/min, whereby the gases were separated. They were then detected by AAS.

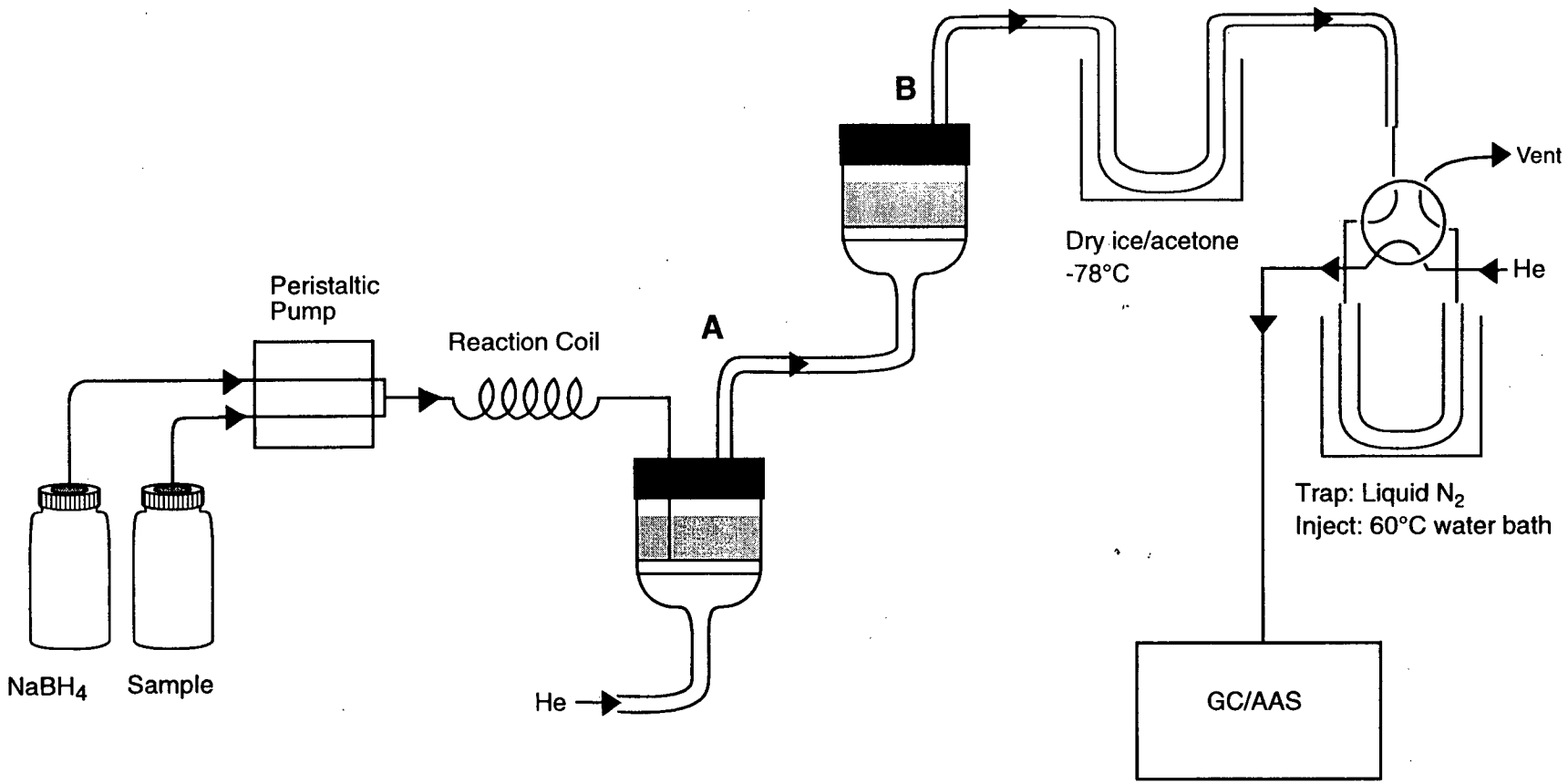

Figure 2.13. Schematic diagram for hydride generation of stibines, Method 3.

For Method 3, the peristaltic pump delivered standard solution and 2% (w/v) $NaBH_4$ solution to the first batch reaction vessel (A). A volume of 30 mL was delivered to reactor A while helium gas was bubbled through the glass frit to strip the solution of gases. The gas stream was bubbled through the contents of reactor B (see Figure 2.13). Throughout the process, the gas stream was dried and trapped at -196 °C and the remaining procedure was identical to the one previously described for Methods 1 and 2. Reactants were added to reactor B in Method 3 from a 1 mL syringe inserted between the rubber stopper and the side of the reactor.

Measurement of pH was carried out with an Accumet Model 15 pH meter (Fisher Scientific) after the sample and acid had been mixed, but before the $NaBH_4$ was added.

2.2.2.3. Sample preparation

Mycelia of the pink oyster mushroom *Pleurotus flabellatus* (Western Biologicals, Aldergrove, BC) were grown with shaking in 400 mL potato dextrose broth (Difco) in a 1 L Erlenmeyer flask. Me_3SbCl_2 solution was added to the broth to give a concentration of 1 ppm in antimony. After a 14 day growing period, the mycelia, as spherical pellets, were harvested by centrifugation and rinsed with distilled water. They were then homogenized with an Ultraturrax T25 homogenizer (Jak & Kunkel) to give a solution of lysed fungal cells, and analyzed by using HG-GC-AAS. A control experiment was carried out in which the fungus was grown in the same manner, only without the addition of antimony to the potato dextrose broth. See Chapter 3 for more details about this experiment.

2.2.3. Results and Discussion

2.2.3.1. Demethylation of trimethylantimony species in aqueous solution during analysis by using GC-HG-AAS

Dodd *et al.*[73] observed that peaks corresponding to SbH_3, $MeSbH_2$, and Me_2SbH, as well as the expected Me_3Sb, appeared when the hydride generation apparatus had not been preconditioned with the reagents used for analysis (2% $NaBH_4$ and 4 M acetic acid). In an attempt to replicate this observation, experiments were carried out by using the same method, but with an AAS instead of an MS as the detector. Me_3SbCl_2 was analyzed after a preconditioning step which consisted of rinsing all tubing with water, as well as after rinsing with 2% $NaBH_4$ and 4 M acetic acid. In all replicates (five for each), the results were the same when Me_3SbCl_2 was reduced and analyzed: Me_3Sb and a minor amount of Me_2SbH (corresponding to <2% demethylation) were detected. The large amount of demethylation that was observed by Dodd *et al.*[73] could not be replicated.

2.2.3.1.1. The effect of acid

When HCl was used as the acid in the reaction to adjust the pH of the sample, increased demethylation was observed. Different concentrations of HCl led to the appearance of SbH_3, $MeSbH_2$ and Me_2SbH in differing amounts, indicating a pH dependence for the demethylation process. Hence, demethylation of Me_3SbCl_2 at different acidities was studied, and the results obtained are illustrated in Figure 2.14. It was assumed that the reaction efficiency and detector response for each species is the same (which was observed to be the case for Me_3Sb and SbH_3), allowing the amount of each stibine to be determined. A minimum of three replicates were carried out at each pH, but reproducibility of the normalized amounts was poor, with relative

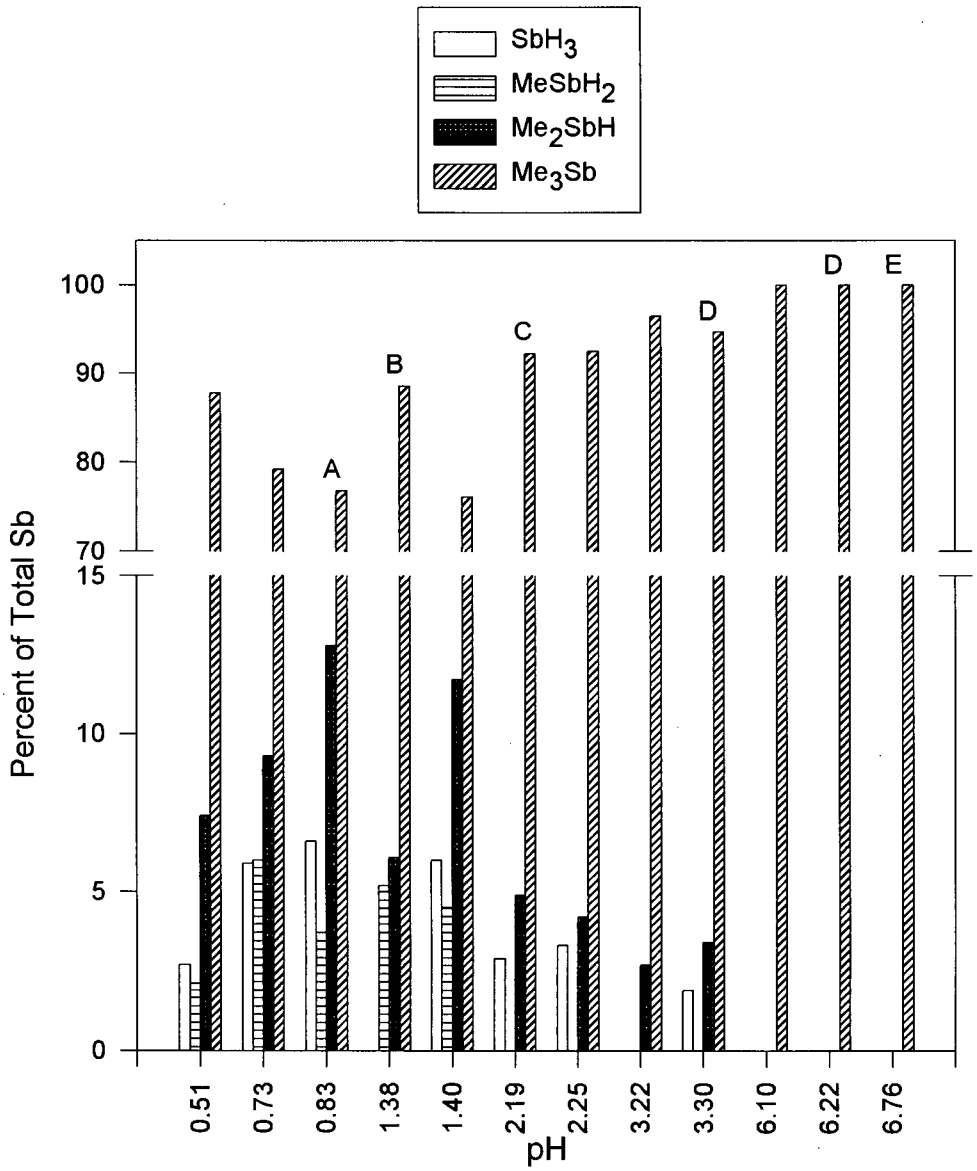

Figure 2.14. Percent amounts of stibines generated from Me_3SbCl_2 at varying pH, when using HG-GC-AAS. Amounts of stibines may not add up to 100% because of imprecision in blank correction. Except where indicated, unbuffered HCl was used to adjust the pH. A = sulfuric acid, B = maleic acid, C = citric acid, D = citrate buffer, E = water.

standard deviations averaging 22% (0.1% up to 50% for amounts approaching the detection limit). The acid used in most of the experiments was unbuffered HCl, but at some pHs different buffers and acids were tested to determine if the demethylation was specific to HCl. These buffers and acids are specified in Figure 2.14. Small amounts of Me_2SbH appear at pH 3.22 and pH 3.30, but at pH 6.10 no demethylation is seen for the concentration of Me_3SbCl_2 being studied.

Although a statistically significant dependence of demethylation on the acid used was not observed for the acid concentrations studied (see Figure 2.14), it must be noted that certain acid systems give less demethylation than would be expected considering the pH. For example, reactions carried out by using 4 M acetic acid (pH 2.2) resulted in statistically significantly less demethylation than experiments carried out at similar pH, by using other acids (see Table 2.5 and compare to results for 0.01 M HCl and 0.1 M citric acid). However, when 0.6 M acetic acid was tested (Table 2.5), the amount of demethylation was not statistically significantly different from other acid systems at similar pH, but the amount of demethylation was calculated to be statistically significantly different from that observed when 4 M acetic acid was used. More concentrated solutions of citric acid (0.5 M and 1 M) also showed amounts of demethylation that were calculated to be statically significantly less than that observed when 0.1 M citric acid was used (Table 2.5). More studies must be carried out to elucidate the mechanism of the demethylation phenomenon and hence to explain this observed behaviour.

Table 2.5. Comparison of amounts of demethylation when using different concentrations of acids. Lower values of percent Me_3Sb of total Sb indicate higher amounts of demethylation.

Acid used	pH (calculated)[a]	% Me_3Sb of total Sb (SD[c])
0.01 M HCl	2.25[b]	92.5 (0.6)
0.6 M acetic acid	2.6	94 (2)
4 M acetic acid	2.2	98 (2)
0.1 M citric acid	2.19[b], 2.2	92.2 (0.7)
0.5 M citric acid	1.9	96.6 (0.6)
1 M citric acid	1.7	95.9 (0.9)

[a] Unless otherwise stated, pH is calculated by using $pH=-\log(\sqrt{K_a} \times [HA]/2)$; where $K_a = 1.75 \times 10^{-5}$ for acetic acid; $K_a = 7.44 \times 10^{-4}$ for citric acid; [HA] = concentration of acid. [HA] is divided by 2 because of dilution during mixing of the acid in the HG apparatus.
[b] pH was measured after mixing the acid solution with water, as described in section 2.2.2.2.
[c] SD = standard deviation, calculated from 3 replicate analyses.

2.2.3.1.2. The effect of concentration

When high levels of Me_3SbCl_2 (500 - 1000 ng) were analyzed, demethylation was observed at neutral pH as well. Demethylation can only be seen when high levels of Me_3SbCl_2 are analyzed, even though the amount of demethylation is low at neutral pH (<1%), because the levels of SbH_3, $MeSbH_2$ and Me_2SbH are sufficiently high for detection. The detection limits are estimated to be between 1 ng and 5 ng for all the stibines, although standards are not available for methyl- and dimethylantimony species.

The demethylation pattern is not dependent on the concentration of antimony being analyzed. Small ranges of concentration (2 orders of magnitude) were tested, however, so the possibility that the demethylation pattern changes at higher concentrations cannot be discounted.

2.2.3.1.3. The effect of sample matrix

To illustrate the importance of establishing the presence of demethylation during the analysis of trimethylantimony species, a sample known to contain trimethylantimony species was analyzed. We found that the sample matrix in this example has an effect on the hydride generation of methylstibines. The term "sample matrix" refers to any chemical components other than the analytes (antimony species) that make up the sample.

Figure 2.15a shows a chromatogram of an aqueous extract of the fungus *Pleurotus flabellatus* that had been grown in liquid culture and amended with Me_3SbCl_2. The stibines were generated with the aid of aqueous borohydride with no acid or buffer, conditions under which the Me_3SbCl_2 standard in water described in section 2.2.3.1.1 showed minimum demethylation (pH 5.6). Figure 2.15a shows peaks in addition to the one corresponding to the expected trimethylstibine for this sample.

Figure 2.15b shows the same fungus extract analyzed while using a 0.05M citrate buffer at pH 6.2. Most of the demethylation appearing in Figure 2.15a was eliminated by using a buffer. To determine whether the demethylation observed in Figure 2.15a was caused by the sample matrix, a control sample from the mushroom culture that had not been amended with antimony, and hence with the identical matrix but found previously to contain a concentration of antimony less than 0.05 ppm (Chapter 3), was analyzed after the addition of 200 ng of Me_3SbCl_2. The sample was analyzed in the same manner as the sample in Figure 2.15a and the result is shown in Figure 2.15c. The same demethylation pattern can be seen, indicating that this specific sample matrix causes the demethylation.

The efficiency of the hydride generation reaction is decreased by the sample matrix, as shown by Figure 2.15d, which is the chromatogram that resulted when 200 ng of Me_3SbCl_2

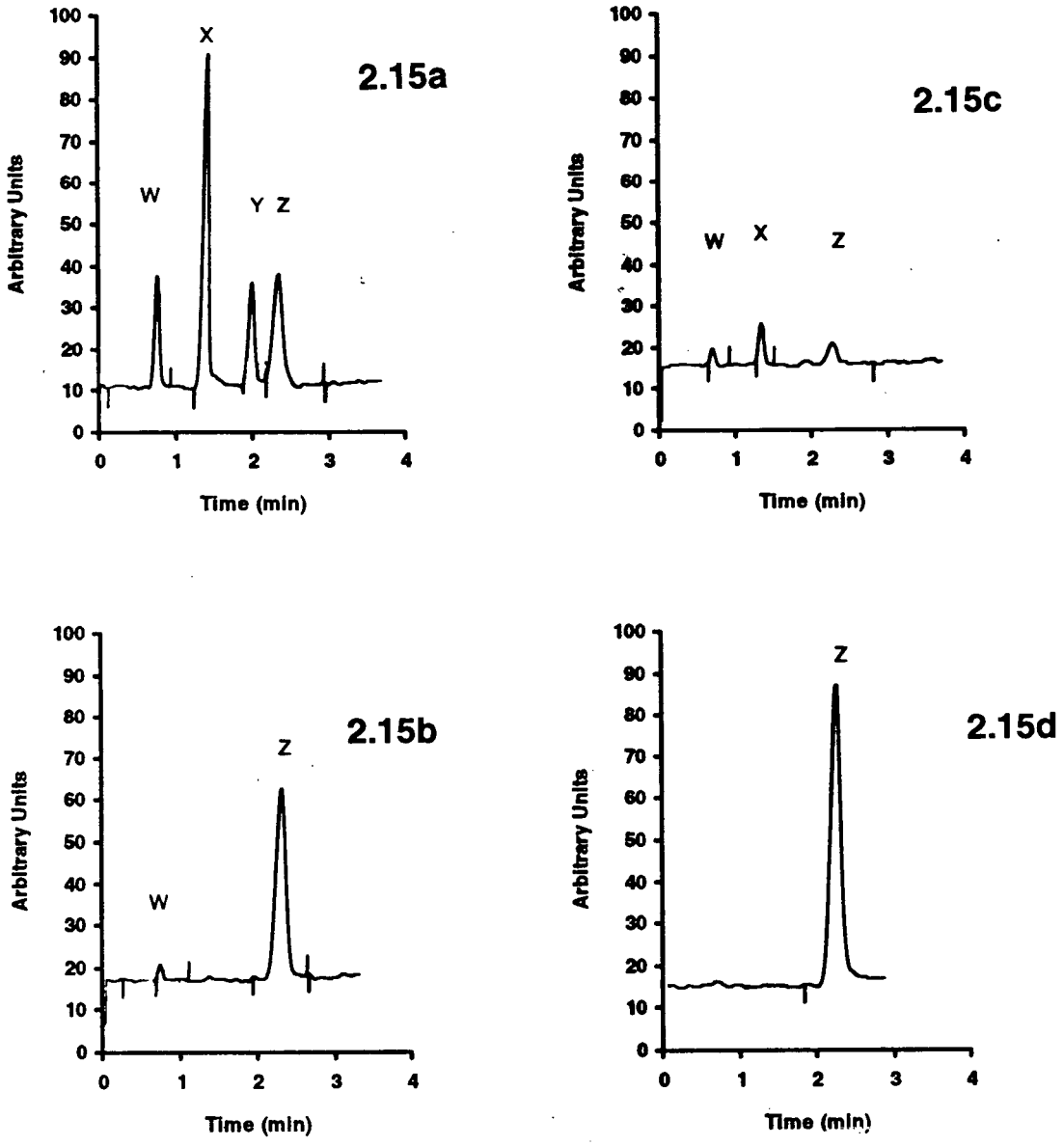

Figure 2.15a. Chromatogram of an aqueous fungus extract analyzed at neutral pH (water only, unbuffered). **Figure 2.15b.** Chromatogram of the same aqueous fungus extract analyzed at pH 6.2, citrate buffer. **Figure 2.15c.** Chromatogram of antimony-free aqueous fungus extract spiked with 200 ng Me_3SbCl_2, analyzed as in Figure 2.15a. **Figure 2.15d.** Chromatogram of 200 ng Me_3SbCl_2 in water analyzed as in Figure 2.15a. W = SbH_3, X = $MeSbH_2$, Y = Me_2SbH, Z = Me_3Sb.

dissolved in water was analyzed in the same manner as the sample in Figure 2.15a and Figure 2.15c. The amount of total hydrides in Figure 2.15d is much greater than that in Figure 2.15c. Figures 2.15(a-d) show the importance of matrix effects in this fungal extract on the hydride generation of Me_3Sb.

2.2.3.1.4. Other studies to determine causes of demethylation

Additional studies were carried out to qualitatively determine causes of demethylation, and are summarized in Table 2.6.

Table 2.6. Qualitative tudies to determine causes of demethylation. See Figure 2.12 for locations of reactors A and B in Method 3. Reactor B contained 20 ml of 1M H_2SO_4 and an amount of 100 ng (as Sb) of Me_3SbCl_2 was reacted in each experiment. **I, II** and **III** are sequential in time.

Expt. No.	Method	Reactions	Demethylation?
1	3	**I.** $Me_3SbCl_2 + NaBH_4$ (reactor A) **II.** Gaseous products from **I** bubbled through H_2SO_4 (reactor B)	no
2	2	**I.** $Me_3SbCl_2 + NaBH_4$ **II.** (Products from **I**) + H_2SO_4	yes
3	3	**I.** 2 ml $NaBH_4$ (2% w/v) + H_2SO_4 (reactor B) **II.** $Me_3SbCl_2 + NaBH_4$ (reactor A) **III.** Gaseous products from **II** bubbled through liquid products from **I** (reactor B)	yes
4	3	**I.** $Me_3SbCl_2 + NaBH_4$ (reactor A) **II.** Gaseous products from **I** bubbled through H_2SO_4 (reactor B) **III.** (Liquid products from **II** in reactor B) + 2 ml $NaBH_4$ (2% w/v)	yes

Me_3SbCl_2 is stable in 1M DCl in D_2O by using NMR analysis[78], and therefore demethylation is not taking place prior to the hydride generation reaction. Me_3Sb was generated at neutral conditions and then bubbled through H_2SO_4, and it was found to be stable to acid (Table 2.6, Experiment 1). However, when both borohydride and acid are present after trimethylstibine is generated, demethylation is observed (Table 2.6, Experiments 2 and 3). Trimethylstibine appears to dissolve in acid (or possibly become reoxidized to trimethylantimony oxide) and demethylates during the reaction with sodium borohydride (Table 2.6, Experiment 4). Therefore demethylation is taking place during the reaction, as a result of a product of the acid/borohydride reaction. As well, trimethylstibine, once formed, is unstable to a product of the acid/borohydride reaction.

2.2.3.1.5. Suggested reasons for demethylation

"Molecular rearrangement" of methylarsines has been observed in the past[79,80,81], mostly in the form of demethylation, but also resulting in the formation of higher methylated compounds from lesser methylated ones. The previous studies do not give an explanation for the rearrangement, but rather link it to certain factors. For example, when 0.5 M H_2SO_4 was used in the derivatization reaction, demethylation of monomethyl- and dimethylarsines resulted[81]. The present study (section 2.2.3.1.1) shows a similar trend, in that the use of acid during the hydride generation of Me_3SbCl_2 results in demethylation products. However in another study the use of acid pH during the hydride generation reaction reduced the amount of demethylation[79]. In the same study[79] the use of a sodium borohydride pellet rather than aqueous solution and elimination of dissolved oxygen also reduced or eliminated the amount of demethylation, and this was attributed to an increase in the rate of the reduction reaction. In other words, these authors postulated that a faster rate of reaction results in less demethylation.

Mechanistic pathways for demethylation or rearrangement have not been postulated in the literature. The mechanism of the hydride generation reaction itself is not well known but studies with deuterium labeled sodium borohydride and arsenic compounds indicate that the hydrides result from direct H^- transfer from the borohydride[82].

The present study indicates that demethylation takes place in solution during the hydride generation reaction. It may then be postulated to take place at one or both of the following stages: during the actual reduction, or after Me_3Sb is formed.

To address the possibility of demethylation taking place during the reaction, the following reactions may be postulated to take place for the reduction of Me_3SbCl_2:

$$Me_3SbCl_2 + 2H^- \rightarrow Me_3SbH_2 + 2Cl^- \qquad (1)$$

$$Me_3SbH_2 \rightarrow Me_3Sb + H_2 \qquad (2)$$

Because the Sb-C bond is of similar strength to Sb-H but less than that of Sb-Cl[83], [84], methyl groups may be lost during these reactions. The intermediate compound Me_3SbH_2 may be unstable to H^+ or to products of the reaction between acid and borohydride.

Me_3Sb, once formed, appears to be unstable to the products of the acid/borohydride reaction (section 2.2.3.1.4). The presence of borane, BH_3 or diborane, B_2H_6, known to be products from the reaction of BH_4^- with HCl[85] may be a key factor affecting the stability of Me_3Sb. Adducts with BH_3 may form, such as Me_3SbBH_3 which decomposes at room temperature[86]. The decomposition of Me_3SbBH_3 may follow a similar pathway as that proposed for the reaction of Me_4Pb and B_2H_6 to Me_3B and metallic Pb, which involves stepwise loss of methyl groups from the lead compound[87]. Me_2SbHBH_3 has been isolated and reacts further to form the stable compound Me_2SbBH_2[88]. The relative instability of the Me-Sb bond in Me_3Sb is

also seen by using mass spectrometry[89] (see also Chapter 6) where the first methyl group is easily lost from Me_3Sb. More experiments are necessary to deduce the mechanism of both the reduction reaction and the demethylation and rearrangement phenomena.

Therefore, when analyzing a sample for methylated antimony compounds by the method of hydride generation, the reaction conditions should be carefully tested with standard compounds such as Me_3SbCl_2. The issue of "rearrangement" associated with methyl- or dimethylantimony(V) compounds has not been addressed, since no standard compounds are available, but similar problems may exist for these species, making the synthesis and study of these compounds imperative. As well, the behaviour of methylated antimony species in the +3 oxidation state is completely unknown, although their presence in the environment is not unlikely. Earlier hydride generation studies that report the presence of methylated antimony compounds should be regarded with caution, especially any quantitative aspects, since the presence and amounts of these compounds may be an artifact of the method.

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Chapter 3

ARSENIC AND ANTIMONY IN MUSHROOMS

3.1. Introduction

Mushrooms are fruiting bodies which participate in the reproductive cycle of a fungal organism. The fungus consists of filamentous cells (hyphae) that grow through a substrate to form a mass called the mycelium. Fungi that produce mushrooms belong to one of two divisions, the Basidiomycota and Ascomycota, based on the type of reproductive cell inside the mushroom used for spore production (basidium or asci). Spores are the reproductive cells that trigger the birth of new fungal organisms. Most of the commonly known fungi that produce mushrooms belong to the division Basidiomycota.

Interest in the arsenic content in mushrooms has increased recently. Some species of mushrooms appear to accumulate arsenic and other metals from soil[1], and their potential as biological pollution indicators has been discussed[2]. For mushrooms that accumulate arsenic, and are edible, such as *Laccaria amethystina*, toxicological consequences (if any) to consumers have been of concern[3]. For these reasons, the uptake and speciation of arsenic in mushrooms has been studied. Determining the bioavailability and species of arsenic in mushrooms, especially those containing elevated levels, helps in toxicological risk assessment.

Chemical processes taking place in the terrestrial environment have not been studied to the extent to which those in the marine environment have been. The recent findings of arsenobetaine and arsenocholine in mushrooms have led researchers to draw similarities between marine and terrestrial pathways for the formation of arsenic compounds[4,5,6]. The presence of

arsenobetaine in mushrooms in higher taxonomic positions (i.e., being more highly evolved)[7] is similar to the presence of arsenobetaine in higher marine organisms, such as marine animals[8].

Although some authors believe that the fungi producing the mushrooms are responsible for the bio-synthesis of more complex arsenic forms, such as arsenobetaine[7], no proof exists for this hypothesis. This theory is favoured for two reasons. One is that arsenobetaine has not been found in soil[4]. Arsenobetaine was, however, found in ant-hill material[9], and its presence in estuarine waters was recently confirmed[10]. The second reason is that similar chemical forms of arsenic have been seen in mushroom species collected from different locations[3]. In support of the fungus biosynthesis theory, the fungi *Agaricus placomyces* and *Pleurotus* sp. (producing edible mushrooms) methylate arsenic to a small extent[11].

Very little is known about the interaction of antimony with fungi, or with terrestrial organisms in general. The chemical similarities of antimony and arsenic has led to the hypothesis that fungi may interact with antimony in the way that they do with arsenic. For example, antimony was postulated to be methylated by the fungus *Scopulariopsis brevicaulis*, and this has, in fact, been shown to take place to a very small degree[12,13]. The identity of antimony species in fungi which produce mushrooms is completely unknown.

The following chapter describes work exploring three objectives. The first is to determine the arsenic species in edible mushrooms that are readily available in supermarkets. The second is to determine if mycelia in axenic culture (i.e., containing only one organism) are responsible for the formation of complex arsenicals by growing pure cultures of fungi that are capable of producing mushrooms in arsenic amended media. The third is to learn more about biological interactions with antimony, by growing fruiting bodies on antimony-containing substrate, and by culturing fungi that can produce mushrooms in antimony amended media.

3.2. Experimental

3.2.1. Chemicals and reagents

Sodium arsenate, $Na_2HAsO_4.7H_2O$ (Aldrich), arsenic trioxide, As_2O_3 (Alfa), methanearsonic acid, $CH_3AsO(OH)_2$ (Vineland Chemical), and cacodylic acid, $(CH_3)_2AsO(OH)$ (Vineland Chemical) were dissolved in deionized water to make standard solutions. Extracts of kelp powder (Galloway's, Vancouver, BC) and Nori (*Porphyra tenera*) of known arsenosugar content were obtained as described elsewhere[14] and were used as laboratory standards in order to establish the retention times of arsenosugars. The identity and retention times of the arsenosugars were verified by comparison to pure arsenosugars generously donated by K. Francesconi and T. Kaise. Arsenobetaine[15], arsenocholine[16], trimethylarsine oxide[17], and tetramethylarsonium iodide[18] were had been synthesized previously according to standard methods. Identification of arsenicals in samples was made by comparison of retention times to those in standards.

Antimony (V) and (III) standards were obtained as potassium hexahydroxyantimonate, $KSb(OH)_6$ (Aldrich), antimony trichloride, $SbCl_3$ (Aldrich), and potassium antimonyl tartrate, $K_2Sb_2(C_4O_6H_2)_2$ (Aldrich). Me_3SbCl_2 was synthesized as described elsewhere[19]. Stock solutions were made by dissolving these compounds (except $SbCl_3$) in deionized water to 1000 or 100 ppm as Sb. $SbCl_3$ was dissolved in 6 M HCl to 1000 ppm as Sb. Standard working solutions were made by diluting the stock solutions with deionized water as necessary.

For hydride generation analysis, $NaBH_4$ (reagent grade, Aldrich) was dissolved in deionized water fresh daily to give a concentration of 2% w/v. Ammonium citrate buffer at a concentration of 0.05 M and pH 6 (1 M ammonium hydroxide, MicroSelect, Fluka, and

analytical reagent grade citric acid, BDH) and 1 M HCl (Environmental grade, Alfa Aesar) were used.

Other chemicals for the preparation of mobile phases were analytical-reagent grade or higher in purity. They included methanol (HPLC grade, Fisher), concentrated phosphoric acid (Aldrich), ammonium hydroxide (1M, Fluka), pyridine (Fisher), formic acid (BDH), potassium hydroxide (KOH, Aldrich), tetraethylammonium hydroxide, (TEAH, 20% in water, Aldrich), and malonic acid (BDH). Mobile phases were filtered through 0.45 μm cellulose nitrate filters (Millipore) prior to use.

The arsenosugar stock solution used in the *Scleroderma citrinum* experiments was obtained by extracting commercially available macroalgae species (available for human consumption), which are known to contain arsenosugar X and XI, and DMA[14]. The sample consisting of 28 g Nova Scotian dulse (*Palmaria palmata*), 6.5 g Taiwanese Nori (*Porphyra tenera*) and 6.7 g Japanese Yaki Nori (*Porphyra tenera*, all purchased at Vancouver supermarkets) was extracted with approximately 1.5 L of MeOH/water (1:1) three times by sonication (2 hours), and the liquid was separated from the solid between sonications by filtration through Whatman #1 filter paper. The extracts were combined and concentrated to a final volume of approximately 400 mL by evaporation with a constant stream of air overnight at room temperature.

3.2.2. Apparatus and method of analysis

3.2.2.1. HG-GC-AAS analysis for antimony speciation

The apparatus was composed of a semi-continuous flow, hydride generation system developed for arsenic analysis,[20] coupled to an atomic absorption spectrometer (Varian AA1275) fitted with an Sb lamp (Varian) operating at a wavelength of 217.6 nm. One modification was made to the basic apparatus in the form of using a gas-liquid separator[21] that resulted in less analyte carryover. The apparatus consisted of Tygon tubing for the peristaltic pump, and PTFE tubing (1/8" OD) for the remainder. The glass gas-liquid separator was silanized with $(CH_3)_2SiCl_2$ before use. Data were collected from the AAS and process directly by using an HP 3390A integrator, or were analyzed with the aid of Shimadzu EZChrom software.

A peristaltic pump was used to deliver standard or sample solution (usually 0.1 mL to 3 mL) to mix with the acid or buffer and then to mix with a solution of $NaBH_4$ (2% w/v) in a reaction coil. The gases evolved were separated in the gas-liquid separator and then swept by a flow of helium into a PTFE U-tube, where they were trapped at -196 °C. Continuous hydride generation and trapping were carried out for 3 minutes. The peristaltic pump was then stopped (making the system semi-continuous) and the U-tube was heated to 70 °C, allowing the gases to be swept with He at a flow rate of 40 mL/min onto a Poropak PS column, which was then heated from 70 °C to 150 °C at a rate of 30 °C/min, whereby the gases were separated. They were then detected by AAS.

3.2.2.2. HPLC-ICP-MS analysis for antimony and arsenic speciation

The HPLC apparatus consisted of a Waters 510 double piston pump, a Rheodyne six-port injection valve with a 20 μL loop, in line filters, a guard column for each analytical column packed with the same stationary phase, and the analytical column. Columns and mobile phases are listed in Table 3.2. In some cases of plasma instability and detector drift, 10 ppb Rh was added to the mobile phase to provide a constant background signal. A VG Plasmaquad PQ2 Turbo ICP-MS (VG Elemental) was used as a detector. Parameters for the ICP-MS are given in Table 3.3. The m/z monitored were 121 and 123 (Sb), 75 (As), 77 82 (Se and Ar-^{37}Cl) and 103 (Rh) where applicable. The HPLC was coupled to the spray chamber of the ICP-MS by using a minimum of PTFE tubing (10 cm x 0.5 mm i.d.) with the appropriate PTFE fittings.

Extracts and media were diluted as necessary, filtered through 0.45 μm syringe filters (Millipore) and analyzed by HPLC-ICP-MS using the conditions given in Table 3.1 (arsenic speciation), Table 3.2 (antimony speciation) and Table 3.3 (ICP-MS detection). Data were processed and analyzed by using chomatographic software[22], and when semi-quantitative concentrations of arsenic and antimony compounds were determined, external calibration curves were used.

3.2.2.3. ICP-MS analysis for total arsenic and antimony concentrations

The ICP-MS described above, outfitted with a peristaltic pump and injection loop for flow injection introduction, was used for the determination of total arsenic and antimony in samples. The parameters listed in Table 3.3 were used, except that time resolved analysis was not used for these analyses. Solutions and standards were diluted with 1% (v/v) nitric acid (doubly distilled in quartz, Seastar) and Rh (10 ppb) was added as an internal standard.

Table 3.1. HPLC conditions for arsenic speciation

Chromatography	Column	Mobile phase	Flowrate (mL/min)
Anion exchange	Hamilton PRP-X100, 150 × 4.6 or 250 × 4.6 mm	20 mM ammonium phosphate, pH 6.0	1.0 or 1.5
Cation exchange	Supelcosil LC-SCX or Whatman SCX Partisil 5, 250 × 4.6 mm	20 mM pyridinium formate, pH 2.7	1.0
Ion-pairing	GL Sciences ODS, 250 × 4.6 mm	10 mM TEAH, 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	0.8

Table 3.2. HPLC conditions for antimony speciation

Chromatography	Column	Mobile phase	Flowrate (mL/min)
Anion exchange[23]	Hamilton PRP-X100, 150 × 4.6 mm	2 mM KOH	1.0
Ion-pairing	Hamilton PRP-1, 150 × 4.6 mm	10 mM TEAH, 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	0.8

Table 3.3. Operation parameters for ICP-MS

Feature	Specific Conditions
Forward radio-frequency power	1350 W
Reflected power	<10 W
Cooling gas flow rate (Ar)	13.8 L/min
Intermediate (auxiliary) gas flow rate (Ar)	0.65 L/min
Nebulizer gas flow rate (Ar)	1.002 L/min
Nebulizer type	de Galan
Analysis mode	Time Resolved Analysis (TRA) for HPLC
Quadrupole pressure	9×10^{-7} mbar
Expansion pressure	2.5 mbar

3.2.3. Cultivation of *Pleurotus flabellatus* fruiting bodies on a solid substrate

To qualitatively determine antimony uptake by mushrooms, the fungus *P. flabellatus* (commonly known as the pink oyster mushroom) was grown on a solid soil medium (wood chips, sawdust and bran, about 25% moisture) contained in polyethylene bags. The bags containing 4 kg substrate were sterilized and inoculated with *P. flabellatus* mycelium, at Western Biologicals Ltd., Aldergrove BC. The bags were opened in a biological safety cabinet at UBC and antimony solutions (10 mL of 500 ppm as Sb) were added through a 0.2 μm syringe filter (Nalgene), dropwise, and the top 15 cm of soil was mixed with a flame-sterilized spatula. The mixing was inadequate and hence concentrations were not homogeneous throughout the bag. Four bags were prepared, containing: (A) potassium antimonyl (III) tartrate; (B), potassium antimonate (V); (C) Me_3SbCl_2; and (D) no antimony. The bags were kept in a dark incubation chamber at 15 °C for 1 week, and then the temperature was increased to 20 °C for another week with illumination for 12 hours/day. The humidity was kept high by placing open dishes of water

around the bags, and by spraying the bags daily with deionized water. After 2 weeks fruiting bodies appeared and they were harvested and frozen until analysis.

3.2.4. Preparation of pure submerged cultures of fungi

P. flabellatus was grown from a culture obtained from Western Biologicals Ltd., Aldergrove, BC, in potato dextrose broth (Difco), a general liquid medium for fungus. The following procedures were used for all culture experiments. Solutions used to amend the media were sterilized by using syringe filters (0.22 μm, Nalgene or Millipore, cellulose acetate), and they were added to give the appropriate starting concentration in each treatment (see Table 3.5). Seed culture containing mycelia that were free of antimony and arsenic was added in a volume that was approximately 10% of the volume of the broth (see Table 3.5 for volumes). The fungi were incubated on a shaker (forming spherical mycelia for *P. flabellatus*, *S. citrinum* and *S. crispa*, and broken filaments of mycelia for *M. procera*) for the duration of the experiment (Table 3.5) at 26 °C. For *P. flabellatus*, the spherical mycelia were harvested by centrifugation of the biomass/liquid mixture and washing of the mycelia with deionized water. For the other cultures, the biomass was harvested by vacuum filtration (Whatman #1 filter paper) and rinsed a minimum of three times with deionized water.

Scleroderma citrinum, commonly known as the earthball, and resembling puffballs in shape and reproduction, was obtained from an uncontaminated wood chip substrate in Vancouver. A number of fruiting body specimens were collected, cleaned manually by abrasion and with water to remove dirt and other debris, and sterilized on the outside surfaces with 30% hydrogen peroxide.

Table 3.4. Summary of pure culture experiments. AB = arsenobetaine; AsS = arsenosugars from algae extract; control experiments contain non-living cells.

Sb or As species	No. of replicates	Duration of experiment (days)	Approximate concentration (ppm)	Culture volume (mL)
P. flabellatus				
Sb (III)	2	14	1	400
Sb (V)	2	14	1	400
Me_3SbCl_2	2	14	1	400
No Sb or As	2	14	0	400
Sb (III) control	2	14	1	400
Sb (V) control	1	14	1	400
Me_3SbCl_2 control	1	14	1	400
S. citrinum				
Sb (III)	2	43	10	400
Sb (V)	2	43	10	400
Me_3SbCl_2	2	43	10	400
As (V)	2	43	1	400
AB	2	43	1	400
AsS	3	27	0.3	100
No Sb or As	2	43	0	400
Sb (III) control	1	63	10	50
Sb (V) control	1	63	10	50
As (V) control	1	63	1	50
AB control	1	63	1	50
AsS control	1	27	0.3	50
M. procera				
As (V)	2	35	1	400
No Sb or As	1	35	0	400
As (V) control	1	35	1	200
S. crispa				
As (V)	2	35	1	400
No Sb or As	1	35	0	400
As (V) control	1	35	1	200

All other culturing steps were carried out aseptically in a biological safety cabinet. The specimens were cut open and a small piece of the fungus inside (containing spores) was used to inoculate sterile potato dextrose agar plates and potato dextrose broth. Within 2 weeks, growth was seen both on the plates (as a filamentous mycelium) and in the broth (as small white spherical mycelia, as for *P. flabellatus*). These cultures were then used to seed other stock cultures and the experimental cultures.

M. procera and *S. crispa* were obtained from the American Culture Collection as axenic cultures, which were then used to inoculate YM broth (see Table 3.4 for YM broth ingredients, following the recipe recommended by Difco[24]). The submerged cultures obtained were used to seed the experimental cultures.

Table 3.5. YM Broth ingredients and composition

Ingredients per liter	Weight in grams
Yeast extract, Difco	3
Malt extract, Sigma	3
Peptone, Difco	5
Dextrose, Fisher Scientific	10
pH ± 0.2 at 25 °C	6.2

Controls were prepared, containing Sb and As species, potato dextrose broth or YM broth and mushroom mycelia that had been autoclaved. The controls were incubated for a minimum of the same time period as the live cell experiments, and the liquid was collected by filtration (Whatman #1 filter paper). No organisms appeared to be growing in any flasks containing autoclaved cells, determined by visual inspection of the flasks, and by visual inspection of potato dextrose agar and nutrient agar that had been streaked with the autoclaved cells.

No contamination by other organisms was observed, either macroscopically (i.e. no cloudy solutions, which indicate bacterial infection) or microscopically, except in an experiment containing *Sparassis crispa* and amended with As (V). Hence the experiments (except for the one containing live cells of *S. crispa*) were assumed to axenic, meaning that only one organism was present in each experiment.

A portion (1-5 mL) of the medium for each experiment was collected after the addition of arsenic or antimony (referred to as "medium before") and reserved for analysis. The medium for each experiment was collected after harvesting of the biomass (referred to as "medium after") as well. All biomass and medium samples were frozen (-20° C) immediately until sample preparation and analysis.

3.2.5. Sample preparation and analysis

Edible mushrooms were obtained canned or dried from Vancouver supermarkets. Canned mushrooms (oyster mushroom and straw mushroom) were homogenized in a blender with the liquid in the can, and freeze-dried. The freeze-dried material, and dried mushrooms were pulverized by using a mortar and pestle. The powders were then extracted. Extractions were carried out by weighing 2 g (± 0.5 mg) of the dried powders into 50 mL or 15 mL centrifuge tubes, adding 10-15 mL MeOH/Water (1:1), sonicating for 20 minutes, centrifuging for 20 minutes and decanting the liquid layer into a RBF. Each sample was sonicated and centrifuged a total of 5 times. The decanted extracts for each sample were pooled and rotovapped to near dryness (1-2 mL) and then dissolved in 5 or 10 mL of deionized water.

P. flabellatus fruiting bodies were freeze-dried and pulverized by using a mortar and pestle. A soil sample consisting of equal amounts of soil from each bag was oven dried and weighed to calculate R (R = fresh weight/dry weight). Extractions were carried out as described

above, except that for mushrooms, 0.5 g (± 0.5 mg) was weighed and extracted, and final extract volumes were 10 mL; and for soils, 3 g (fresh weight) was extracted (± 0.01 g) and final volumes were 10 mL.

Samples of the fruiting bodies for *P. flabellatus* were digested with acid for determination of total antimony content. The freeze-dried mushroom powders were weighed (0.5 g ± 0.5 mg) into a 500 mL round bottomed flask (RBF). Concentrated nitric acid (3 mL, doubly distilled in quartz, Seastar, Sidney, BC), hydrogen peroxide (3 mL, 30% in water, reagent grade, Fisher) and concentrated sulfuric acid (1 mL, reagent grade, Fisher) were added to each sample. The samples in the RBFs were boiled for 3 hours by using a heating mantle and a reflux apparatus[25]. After all the samples had cooled, the clear solutions remaining were diluted to 10 mL with deionized water and stored at 4 °C until analysis.

Medium samples were diluted with deionized water and filtered as necessary, and analyzed with no further preparation. Fresh weight biomass samples (*P. flabellatus* and *S. citrinum*) were prepared by weighing 10 g (± 0.01 g) of filtered and washed mycelia spheres and homogenizing the spheres with an Ultraturrax T25 homogenizer (Jak & Kunkel). The samples were then centrifuged, and the resulting supernatant fraction was filtered. Dry weight samples (all species of fungi) were obtained by freeze-drying the mycelia and pulverizing them with a mortar and pestle. The samples were then prepared by weighing 0.25 g (± 0.5 mg) of the dry powder, extracting with 5 g (± 0.5 mg) deionized water by sonication for 2 hours, centrifuging the slurry, and collecting and filtering the resulting supernatant for analysis.

3.2.6. Isolation of an unknown compound containing antimony

An unknown antimony-containing compound was observed in all medium samples amended with inorganic antimony species when they were analyzed by using ion-pairing chromatography HPLC-ICP-MS. Due to the large proportions of this compound in most samples, an attempt was made to isolate and partially characterize it.

A sample of potato dextrose broth medium containing Sb (V) in which *P. flabellatus* had been cultured was used. Undiluted medium (1 mL) was injected onto a PRP-1 column (4.6 cm × 15 cm) and the fraction eluting between 5 and 15 minutes was collected (a mobile phase of 10 mM TEAH/4.5 mM malonic acid at pH 6.8 was used; see Table 3.2 for chromatographic details). This was repeated 9 times so that a total of 10 mL was injected onto the column. The fractions were pooled and rotovapped to dryness and then dissolved in about 2 mL with deionized water. Ethanol at -20 °C (4 mL) was added and the mixture was kept at -20 °C for 24 hours. The mixture was centrifuged, and the supernatant was then applied to a 30 mL Sephedex LH20 (Pharmacia) in methanol column (3 × 10 cm). Methanol (90 mL) was applied to the column and collected, apart from the first 15 mL, and then evaporated to dryness. The solids remaining were dissolved in 1 mL of deionized water.

3.3. Results and Discussion

3.3.1 Arsenic species in edible mushrooms

Much of the recent interest in arsenic in mushrooms stems from their potential as dietary sources of arsenic, especially in specimens collected from areas high in arsenic. For example, because of the presence of DMA in the choice edible *Laccaria amethystina*, it has been recommended that the ingestion of this mushroom grown on arsenic-contaminated soil be avoided[3]. Therefore we were interested in analyzing some edible mushrooms commonly available in Vancouver supermarkets, to determine the identity of arsenic species present in the MeOH/water extractable portion. In past studies, the arsenic profile for the same species of mushroom collected from different locations and containing different levels of arsenic was found to be similar[3]. Thus we hypothesized that knowledge of the speciation of arsenic in mushrooms meant for human consumption might allow us to predict the speciation in the same mushrooms containing higher levels of arsenic.

The mushrooms analyzed in this study were bought both in the dried form and in cans. Dried mushrooms included wooden ears (probably *Auricula auricularia*), which is a fungus used in many Chinese dishes; shiitake mushrooms (*Lentinus edodes*); two samples of porcini mushrooms, Porcini 1 (most likely *Boletus* sp.) and Porcini 2 (unidentified mushrooms that were most likely *Agaricus* sp., or portobello mushrooms); chanterelle mushrooms (*Cantherellus cibarius*); and a dried mushroom powder of unknown composition. Two species of mushrooms were obtained in cans; they were oyster mushrooms (most likely *Pleurotus ostreatus*) and straw mushrooms (probably *Volvariella volvacea*). The arsenic species extracted from these mushrooms by using MeOH/water (1:1) are summarized in Table 3.6.

Table 3.6. Arsenic species in edible mushrooms, in ppb dry weight (SD[a]). "Trace" amounts are greater than the limit of detection (LOD) but less than 2 × LOD.

Mushroom	As (III)	As (V)	MMA	DMA	AB	Me_4As^+	Sum of species[b]
Wooden ears	trace	17 (4)	23 (5)	trace	<3	<3	46
Shiitake	210 (30)	130 (10)	trace	18 (4)	<5	<5	360
Porcini 1	56 (3)	30 (10)	trace	46 (1))	<5	10 (4)	150
Porcini 2	<5	30 (10)	trace	70 (20)	54 (6)	<5	160
Chanterelle	trace	22	<5	trace	<5	<5	32
Mushroom powder	<9	210 (50)	trace	230 (60)	910 (50)	<9	1360
Oyster mushrooms	30 (10)	28 (1)	30	140 (10)	<5	<5	230
Straw mushrooms	<5	36	<5	trace	<5	<5	41

[a] SD = standard deviation, obtained from analysis of extracts by using two different chromatographic systems (anion and cation exchange, see Table 3.1) with ICP-MS detection.
[b] Calculation of sum of arsenic species included trace amounts estimated at the detection limit, which was 5 ppb for all mushrooms, except for *Auricula* sp. (3 ppb) and mushroom powder (9 ppb).

The dried mushroom mentioned earlier, referred to as Porcini 2 and tentatively identified as *Agaricus* sp., was packaged under the common name "porcini" mushroom, the common name for *Boletus* sp. However, the appearance of the mushrooms revealed the fact that they had been identified incorrectly, since mushroom gills were observed, and *Boletus* sp. have pores rather than gills. Gills, pores or spines are found on the underside of many mushrooms with caps. The observation of a different profile of arsenic species in the unidentified mushrooms, compared with that found for a (presumed) correctly identified sample of porcini mushrooms (Porcini 1, *Boletus* sp.) supports the suggestion that the Porcini 2 mushrooms were identified incorrectly as porcini mushrooms. The most important difference in the arsenic profiles is the presence of arsenobetaine (34% of arsenic extracted) in the unidentified mushrooms. A number of mushrooms belonging to the genus *Agaricus* were analyzed in a previous study[7] and all contained arsenobetaine, ranging in proportion from 55 to 96% of the arsenic extracted. Therefore it is not unreasonable to suggest that the unidentified mushroom may belong to the genus *Agaricus*.

The only other mushroom sample that contains arsenobetaine is the mushroom powder of unknown composition. The arsenic species found in the powder are similar to those found in *Agaricus bisporus*, both in identity and proportions[7] (*Agaricus bisporus*: As (V) 12%, MMA 6%, DMA 27%, AB 55%; see Table 3.7). *Agaricus bisporus* is a mushroom commonly cultivated and eaten, and is also known as the button or white mushroom. Most likely the mushroom powder is composed of this species of mushroom.

Table 3.7. Comparison of proportions of arsenic species in mushrooms (%) in the current study with those found in published studies (indicated by a footnote).

Mushroom	As (III)	As (V)	MMA	DMA	AB	Me_4As^+
Porcini 2	0	19	3	44	34	0
Mushroom powder	0	15	1	17	67	0
Agaricus bisporus[a]	0	12	6	27	55	0
Oyster mushroom	13	12	13	61	0	0
Pleurotus ostreatus[b]	60	39	1	0	0	0
Straw mushroom	0	88	0	12	0	0
Volvariella volvacea 1[a]	trace	4	8	78	10	0
Volvariella volvacea 2[a]	trace	6	trace	94	trace	0
Porcini 1	37	20	3	31	0	7
Amanita caesara[a]	32	38	0	13	0	17
Agaricus campester[a]	0	0	trace	trace	96	4

[a] Results from Slejkovec *et al.* (1997)[7].
[b] Results from Slejkovec *et al.* (1996)[11]

In general, appreciable proportions of inorganic arsenic were extracted from most of the mushrooms. If these mushrooms were collected from areas contaminated with arsenic and

similar arsenic species were present, the presence of higher levels of inorganic arsenic could be toxicologically important.

Extractable arsenic species have been identified for the first time in wooden ears fungus (*Auricula auricularia*), shiitake mushrooms (*Lentinus edodes*), porcini mushrooms (*Boletus* sp.) and chanterelle mushrooms (*Cantharellus cibarius*). The low levels of arsenic in the sample of chanterelle mushrooms made the speciation of arsenic difficult. Me_4As^+, a compound not observed in any other mushrooms in this study, is observed in Porcini 1 (*Boletus* sp.). This compound has been observed in other mushroom species, including *Amanita* sp. and *Agaricus* sp. in previous studies (see Table 3.7)[7].

The low levels of arsenic in straw mushrooms made identification of arsenic species difficult. Arsenate appears to be the dominant species in this study, but DMA was found to be the major species in the presumed same mushroom in a previous study[7]. The species of arsenic in this mushroom differed between two different specimens in the previous study (see Table 3.7); one specimen contained inorganic arsenic, arsenobetaine and MMA, as well as DMA, and the other contained mostly DMA and a small amount of arsenate. Therefore the speciation of arsenic appears to be inconsistent for this mushroom species. Different arsenic profiles may arise from different microbial environments for the different specimens, if uptake of the arsenic compounds is taking place. Different metabolic pathways may be followed as well.

When *Pleurotus* sp. were cultivated in soil amended with inorganic arsenic, only inorganic arsenic was observed in the mushroom, with 1% conversion to MMA (see Table 3.7)[11]. In this study, DMA is the major species of arsenic extracted from oyster mushrooms (*Pleurotus* sp.), with inorganic arsenic and MMA present as well. This may indicate differences in the growing environment of the fungus, and that the arsenic species present in this mushroom are due to uptake from the environment rather than biosynthesis by the mushroom. Slejkovec *et*

al.[11], however, suggested that the time scale for the study involving cultivation of *Pleurotus* sp. was not long enough for the mycelium or fruiting body to biosynthesize other arsenic species. This hypothesis may also account for the differences in arsenic species observed.

3.3.2. The interaction of arsenic species with pure submerged cultures of fungi

3.3.2.1. Culture experiments with Scleroderma citrinum

As mentioned earlier, *Scleroderma citrinum* is commonly known as the earthball, and is similar to a puffball in appearance. It forms spherical fruiting bodies that can range up to 12 cm in diameter, and the spore mass inside is violet-black, becoming powdery and pale greenish or lilac gray when mature. Spores are propagated when the fruiting body is mature and opens. This mushroom was chosen because of its availability and also because of its resemblance to puffballs. Puffballs were found to contain arsenobetaine in previous studies[7].

Experiments were conducted in which the growth medium was amended with arsenate (As (V)), arsenobetaine (AB) and a mixture of arsenosugars (AsS). Arsenate is the most likely form of arsenic available in soil[26] and probably to fungi in the environment. Arsenobetaine is a possible end product for some mushroom species and so it was of interest to determine if any chemical changes take place as a result of mushroom metabolism. Arsenosugars are postulated to be intermediates in the biosynthesis of arsenobetaine[27] and therefore their fate was of interest.

Concentrations of total arsenic in media and biomass for these experiments, as well as a bioconcentration factor (BCF) for the fresh weight biomass are summarized in Table 3.8. A BCF is defined as the quotient of the concentration of a material (in this case, arsenic) in an organism divided by the concentration of the material in the solution in which the organism has been living[28]. It can be calculated by using the following relation:

$$BCF = ([As]_{biomass}/[As]_{ma}); \text{ ma} = \text{medium after.}$$

Total concentrations were obtained by using flow injection ICP-MS analysis as described in section 3.2.2.3, except for the samples from the arsenosugar amended experiment. These concentrations were obtained by summing the concentrations of arsenic species determined by HPLC-ICP-MS (as described in section 3.2.2.2).

If a BCF is greater than 1, concentration of arsenic by the organism from the medium is taking place[29]. Concentration by *S. citrinum* was taking place only for arsenobetaine because a BCF of 23 (Table 3.8) is observed. In fact, only 7% of the arsenic remains in solution, indicating that most of the arsenic is taken up by the organism. *S. citrinum* does not appear to accumulate arsenate or arsenosugars. The low levels of arsenic in the biomass for the arsenate-amended experiment may indicate that exclusion or fast excretion of inorganic arsenic is taking place by *S. citrinum*.

Table 3.8. Total concentrations of arsenic obtained by ICP-MS analysis (except where indicated) for experiments conducted with *S. citrinum*. AB = arsenobetaine, AsS = arsenosugar mixture

Experiment/sample	Concentration of As (ppm) (SD[a])	BCF ($[As]_{biomass}/[As]_{ma}$)[b]
As (V) amended		
Medium before	1.23 (0.03)	
Medium after	1.34 (0.05)	
Biomass	0.20 (0.03)	0.15
AB amended		
Medium before	1.30 (0.03)	
Medium after	0.09 (0.03)	
Biomass	2.04 (0.04)	23
AsS amended[c]		
Medium before	0.37[c]	
Medium after	0.30[c]	
Biomass	0.24[c]	0.80

[a] SD = standard deviation, calculated from duplicate biological experiments.
[b] BCF = Bioconcentration factor, calculated as $[As]_{biomass}/[As]_{ma}$; where ma = medium after.
[c] These total arsenic concentrations were not obtained by flow injection ICP-MS analysis, but rather from the sum of arsenic species determined by HPLC-ICP-MS. See text for more detail.

The proportions of arsenic species in samples from arsenic-amended *S. citrinum* experiments are summarized in Table 3.9. The speciation of arsenic is expressed in proportions rather than absolute amounts because the analyses for most experiments were semi-quantitative.

Table 3.9. Proportions of arsenic species (%) in experiments conducted with *S. citrinum*; control experiments contain non-living cells and arsenic; AB = arsenobetaine, AsS = arsenosugar mixture

Experiment/sample	As(III)	As(V)	DMA	Sugar X	Sugar XI	AB
As (V) amended						
Medium before	2.8	97.2	0	0	0	0
Medium after	80.3	19.7	0	0	0	0
Biomass	87.5	12.5	0	0	0	0
Control medium after	0.7	99.3	0	0	0	0
AB amended						
Medium before	0	1.0	0	0	0	99.0
Medium after	0	0	0	0	0	100
Biomass	0.6	0	0	0	0	99.4
Control medium before	0	0	0	0	0	100
Control medium after	0	0	0	0	0	100
AsS amended						
Medium before	0	0	10.2	14.1	75.7	0
Medium after	0	0	9.9	34.1	56.0	0
Biomass	0	0	10.0	34.2	55.9	0
Control medium before	0	4.6	9.3	12.8	73.3	0
Control medium after	0	0	8.9	14.5	76.6	0

For the experiments in which the growth medium was amended with arsenate, reduction to arsenite by the mycelia appears to be taking place (Table 3.9). No reduction took place in the control containing arsenate and non-living cells, indicating that the reduction was due to the biological activity of the mycelia, rather than chemical transformations in the medium. The speciation of arsenic in the biomass was similar to that in the medium. Most likely the fungus takes up arsenate, reduces it to arsenite and excretes it as arsenite.

As shown by the concentrations of total arsenic in media and biomass for the experiments in which arsenobetaine was added (Table 3.8), accumulation of arsenobetaine from the medium

takes place, and Table 3.9 shows that arsenobetaine remains unchanged. This result may indicate that should arsenobetaine be present in the growing environment of a mushroom-producing fungus in the wild, it will be taken up efficiently by the fungus and not be detectable in the soil.

The proportions of arsenic species in the medium at the beginning of the experiment ("medium before") in Table 3.9 represents the composition of the arsenosugar mix used for the experiments (extract of dulse and Nori, see section 3.2.1). The proportions of arsenic species are similar for the control media at the beginning and at the end of the experiment. This indicates that changes in proportions are caused by the biological action of the fungus. The proportion of DMA remains constant, but the amount of arsenosugar X increases, while that of arsenosugar XI decreases. The proportions in the biomass and in the medium at the end of the experiment are similar. However, no arsenobetaine or other arsenic species are observed from the interaction of the fungus with arsenosugars.

In summary, these experiments show that accumulation of arsenobetaine by *S. citrinum* takes place. As well, the mycelium form of this fungus in pure culture appears to be unable to biosynthesize methylarsenic species or other organoarsenic species from arsenate and arsenosugars. The fungus is responsible for the reduction of arsenate to arsenite, and the probable transformation of arsenosugar XI to arsenosugar X.

3.3.2.2. Culture experiments with Macrolepiota procera and Sparassis crispa

Macrolepiota procera is commonly known as the parasol mushroom and it is a choice edible, growing up to 25 cm in diameter. It possesses a stem and cap with gills underneath, and the surface of the cap is scaly. *Sparassis crispa* is also a choice edible and its appearance is described as resembling that of a cauliflower, ranging from 10 to 60 cm[30]. Both these mushrooms were chosen for biological experiments because of the interesting arsenic speciation

results found for specimens collected from the wild. These published results from Slejkovec *et al.*[7] are summarized in Table 3.10. The major compound found in a wild specimen of *M. procera* was arsenobetaine and it seems reasonable that this fungus might be capable of biosynthesizing arsenobetaine. Arsenocholine, an arsenic compound found in minor amounts in the marine environment[8], was found in large amounts in wild specimens of *S. crispa*, in addition to an appreciable amount of unknown arsenic species[7].

Table 3.10. Arsenic species (% of arsenic extracted) found in wild specimens of *M. procera* and *S. crispa* (from Slejkovec *et al.*[7]); AB = arsenobetaine, AC = arsenocholine

Mushroom	AB	As(III), MMA, DMA, As (V)	AC	Unknowns[a]
M. procera	100	trace	0	0
S. crispa 1	31	3 (As(V))	66	0
S. crispa 2	trace	trace	45	55

[a] Unknown compound on cation exchange HPLC system.

The present arsenic speciation results for experiments conducted with the two species of fungus, using arsenate to amend the growth medium, are summarized in Table 3.11.

Table 3.11. Concentrations of arsenic species in experiments conducted with *M. procera* and *S. crispa* in ppm (biomass is dry weight except where indicated); control experiments contain non-living cells and arsenic.

Experiment/Sample	As (III)	As (V)	DMA	TMAO	Sum of species	BCF[c]
M. procera						
Medium before	<0.004	1.01	<0.004	<0.004	1.01	
Medium after	0.062	0.67	<0.004	<0.004	0.73	
Biomass (dry weight)	1.33	0.67	<0.02	<0.02	2.00	
Biomass (fresh weight)[a]	0.11	0.06	<0.002	<0.002	0.17	0.23
Control medium after	<0.004	0.75	<0.004	<0.004	0.75	
S. crispa						
Medium before	<0.004	1.08	<0.004	<0.004	1.08	
Medium after	<0.004	0.85	0.043	0.26	1.15	
Biomass (dry weight)	2.56	2.62	0.29	1.8	7.27	
Biomass (fresh weight)[b]	0.14	0.14	0.02	0.1	0.40	0.34
Control medium before	<0.004	1.05	<0.004	<0.004	1.05	
Control medium after	<0.004	0.95	<0.004	<0.004	0.95	

[a] Fresh weight concentration is calculated as [biomass concentration (dry weight)]/R, where R = fresh weight of biomass/dry weight of biomass; R for *M. procera* is 11.9.
[b] Fresh weight concentration is determined as for *M. procera*, R for *S. crispa* is 18.2.
[c] BCF = bioconcentration factor; see Table 3.8 for calculation.

Because the BCF values are less than 1 (Table 3.11), no accumulation of arsenate is taking place by these fungi. The cultures of *S. crispa* were not axenic and therefore the conclusion cannot be drawn that the methylated compounds (DMA and TMAO) are present due to metabolism by the fungus, rather than by other organisms (e.g., bacteria) present. *M. procera* biomass contains arsenic mostly as arsenite but excretes very little of it, which may indicate that arsenite is sequestered by the organism after it is formed, or that the excretion process is slower

than the time scale of these experiments. Neither arsenobetaine nor arsenocholine are present in the biomass or in the media, which may reflect the inability of these species to biosynthesize these compounds.

3.3.2.3. Summary of the interaction of arsenic with fungi that can produce mushrooms

Mycelia of mushroom-producing fungi grown in pure culture appear to be unable to synthesize arsenobetaine or arsenocholine, and reduction of arsenate to arsenite appears to be the only chemical process taking place. Arsenobetaine is efficiently taken up by *S. citrinum* which suggests that if any arsenobetaine is present in the growing environment of wild fungi, it may be accumulated by the mushroom. Similar results were observed by Slejkovec *et al.*[11]. That is, only a small amount of methylation by *Pleurotus* sp. fruiting bodies and *Agaricus placomyces* mycelia in pure culture was observed. Additionally, when the growth medium for pure cultures of *Agaricus placomyces* was amended with arsenobetaine and tetramethylarsonium ion, these two compounds were taken up to a high extent[11]. The authors suggested that the biosynthesis of arsenobetaine may not be observed because of the short time scale of the laboratory experiments[11], which is probably the case for *S. citrinum*, *M. procera* and *S. crispa* as well.

3.3.3. The interaction of antimony species with fungi

3.3.3.1. Cultivation of Pleurotus flabellatus fruiting bodies

Although antimony has been listed as a US-EPA priority pollutant, very little is known about its interaction with living organisms. Mushrooms were grown in soil that had been amended with antimony in experiments designed to contribute information about the interaction of antimony with fungi. It was of interest to determine, qualitatively, if antimony is taken up under these conditions and to obtain speciation information. The strawberry oyster mushroom *Pleurotus flabellatus* was chosen because of its availability as a pure culture (Western Biologicals Ltd., Aldergrove, BC) as well as its fast growth rate. As mentioned in section 3.2.3, the fruiting bodies were cultivated on a solid substrate contained in polyethylene bags, a common method used by home mushroom growers. When the conditions are ideal, the fruiting bodies push through holes in the polyethylene to appear on the outside of the bag. Oyster mushrooms have short lateral stems and they are gilled on the underside of the cap. *Pleurotus flabellatus* is pink in colour and produces fruit in 2 weeks from the time of substrate inoculation.

Four bags were prepared, containing: (A) potassium antimonyl (III) tartrate; (B), potassium antimonate (V); (C) Me_3SbCl_2; and (D) no antimony. The concentrations of total antimony in mushrooms from bags A, B and C were elevated compared to D, the mushroom that had been exposed to substrate not amended with antimony (see Table 3.12). Prior to acid digestion, $Sb(OH)_6^-$, $SbCl_4^-$, $K_2Sb_2(C_4O_6H_2)_2$ and Me_3SbCl_2 (0.125 μg each, to give a total of 0.5 μg of Sb) were spiked into the round bottom flask (RBF) containing the mushroom D, and an average recovery of 95% was obtained (see Table 3.12). These results indicate that the acid digestion procedure was adequate to dissolve antimony in these forms and probably that bound to the mushroom matrix.

Table 3.12. Antimony in mushrooms after acid digestion, analyzed by hydride generation-GC-AAS.

Experiment	Sb species[a]	[Sb] (ppm dry weight) in mushrooms
A	Sb (III)	6.5
A (following extraction[b])	Sb (III)	5.1
B	Sb (V)	1.6
C	Me3SbCl2	1.0
C (following extraction)	Me3SbCl2	0.86
D	no Sb	0.04
D (spiked, replicate 1)	no Sb	0.42[c]
D (spiked, replicate 2)	no Sb	0.53[c]

[a] Sb species in this column are those added to amend the mushroom growing soil.
[b] Extraction was carried out by using MeOH/water (1:1), as detailed in section 3.2.5.
[c] Concentration of spike = 0.50 ppm

Very little antimony in the mushrooms is in a form extractable by MeOH/H_2O (1:1) for mushrooms A and C, leaving the remainder bound up with the residue (Table 3.12 and Table 3.13). Semi-quantitative amounts of antimony species found in mushroom and soil extracts are summarized in Table 3.13. Sb (III) is oxidized to Sb (V), but the possibility of oxidation by the mushrooms is unverified since no biomass-free control experiment (soil, bag and Sb (III) only) was carried out; thus, the oxidation of Sb (III) by the soil or by the air cannot ruled out. The oxidation state of antimony remains the same (i.e., Sb (V)) in both the soil and mushroom for experiment B. In experiment C, Me_3SbCl_2 is taken up by the mushroom unchanged (i.e., no methyl groups were lost) and it also remains unchanged in the soil. Recoveries of soil spiked with all three antimony species, followed by extraction, are very low (<20%), indicating that the antimony species are strongly absorbed or adsorbed to the soil.

Table 3.13. Antimony extracted from mushrooms and soils[a] (ppm dry weight), HG-GC-AAS analysis. "Trace" amounts are greater than the limit of detection (LOD) but less than 3 × LOD. When summing species, trace amounts were given a value of the LOD.

Sample	Sb (III)	Sb (V)[b]	Me_3SbCl_2	Sum of Sb species
Mushroom A	<0.02	0.087	<0.02	0.087
Soil A	<0.003	1.0	<0.003	1.0
Mushroom B	<0.02	0.062	<0.02	0.062
Soil B	<0.003	0.46	<0.003	0.046
Mushroom C	<0.0002	0.001	0.0033	0.0043
Soil C	<0.001	0.016	2.3	2.32
Mushroom D	<0.02	trace	<0.02	0.01
Soil D	<0.003	trace	<0.003	0.003
Spiked soil D	0.016	0.29	0.25	0.556
% recovery[c]	0.1%	16%	14%	

[a] Dry weight concentrations for soils were obtained by multiplying fresh weight concentrations by R = 1.85.
[b] Limit of detection (LOD) for Sb (V) was 0.01 ppm for mushrooms, except for mushroom C, for which a LOD of 0.0002 ppm was estimated, and 0.003 ppm for soils.
[c] Concentration of spike = 1.84 ppm each

3.3.3.2. Culture experiments with Pleurotus flabellatus

3.3.3.2.1. ICP-MS and HG-GC-AAS analysis of biomass extracts and media

More experiments with *P. flabellatus* were carried out, with the following points in mind. The problems of antimony absorption or adsorption to soil was avoided by growing the fungus in submerged culture to form mycelial spheres. We wished to confirm the oxidation of Sb (III) to Sb (V) by comparison with controls containing antimony and non-living cells. Finally, higher concentrations of antimony were used to allow the possible observation of new antimony species by HPLC-ICP-MS.

A summary of concentrations in media at the beginning and the end of the experiments is given in Table 3.14. Two analysis techniques were used: hydride generation-GC-AAS, to obtain

speciation information; and ICP-MS, to determine the total amount of Sb. The concentrations of antimony acquired by using HG-GC-AAS differed from those obtained by using ICP-MS for the experiments in which inorganic antimony was used (Sb (III) and Sb (V)). This may indicate the presence of an antimony complex or compound that cannot be derivatized to a hydride under the analysis conditions. The concentrations of inorganic antimony species were determined either by using the method of standard additions, or by using an external calibration curve based on standards in a matching matrix. Both these methods of quantification minimize the likelihood of inhibition of hydride formation from inorganic antimony because of a matrix effect. No such difference is seen for the experiment in which Me_3SbCl_2 is used, except for the media at the end of the experiment, which show a decrease in the amount of hydride formed from Me_3SbCl_2. No appreciable differences are seen in the concentrations of total Sb (determined by using ICP-MS) in the media at the beginning and the end of the experiment.

Table 3.14. Antimony in media and biomass extracts of *Pleurotus flabellatus* grown in submerged culture (ppm in solution, ppm fresh weight for biomass) (SD[a]). HG-GC-AAS analysis was used for speciation, ICP-MS was used for total Sb; control experiments contain non-living cells; na = not analyzed.

Experiment/sample	Sb (III)	Sb (V)	Me_3Sb-[b]	Total (ICP-MS)
Sb (III) amended				
Medium before	0.40 (0.01)	na	< 0.05	1.2 (0.2)
Medium after	0.001	0.35 (0.04)	< 0.001	1.09 (0.04)
Biomass extract	0.004	0.47	< 0.002	na
Control medium before	0.39 (0.08)	na	< 0.05	1.14 (0.01)
Control medium after	0.37 (0.06)	na	< 0.05	1.108 (0.003)
Sb (V) amended				
Medium before	na	0.4 (0.1)	< 0.03	1.2 (0.2)
Medium after	na	0.4 (0.3)	< 0.05	1.2 (0.2)
Biomass extract	< 0.002	0.57	< 0.002	na
Control medium before	na	na	na	1.27
Control medium after	na	na	na	1.36
Me_3SbCl_2 amended				
Medium before	< 0.05	na	1.11 (0.05)	1.2 (0.1)
Medium after	< 0.01	na	0.45 (0.03)	1.1 (0.1)
Biomass extract	< 0.0008	< 0.0008	0.42	na
Control medium before	< 0.05	na	1.29	1.1
Control medium after	< 0.05	na	1.22	1.0

[a] SD = standard deviation, calculated for replicate experiments.
[b] Me_3Sb- is a compound containing Me_3Sb, determined by HG-GC-AAS, meaning that the exact structure of the species prior to derivatization is unknown.

The concentrations of antimony in the fresh biomass after homogenization were determined to be about 0.5 ppm fresh weight, by HG-GC-AAS, for all three treatments (Table

3.14). This concentration is similar to the concentrations obtained by HG-GC-AAS for the media samples at the end of the experiment (0.35 to 0.45, see Table 3.14), which may indicate that no bioconcentration of antimony is taking place by the mushroom mycelia. The antimony species found by using the method of HG-GC-AAS in the mushrooms treated with Sb (III) and Sb (V) is Sb (V). The species of antimony found in the mushrooms treated with Me_3SbCl_2 is a compound containing Me_3Sb-.

3.3.3.2.2. HPLC-ICP-MS analysis of biomass extracts and media

Two HPLC systems were used with ICP-MS as a detector: anion exchange chromatography with a mobile phase of 2 mM KOH[23] (Method A), and ion-pairing chromatography, with a mobile phase of 10 mM TEAH/4.5 mM malonic acid at pH 6.8 and a polymeric reversed phase column (Method B); see Table 3.3 for details. The separation of Me_3SbCl_2 and Sb (V) (as $Sb(OH)_6^-$) by using these two chromatographic systems with ICP-MS detection is presented in Figure 3.1. Sb (III) standards are not eluted regardless of the method used. In Figure 3.1a, Me_3SbCl_2 elutes unretained on the column in the anion exchange system (Method A) and $Sb(OH)_6^-$ is retained, affording a separation between the two compounds; this was observed previously as well[23]. When using the ion-pairing chromatography system (Method B), both $Sb(OH)_6^-$ and Me_3SbCl_2 are retained and separated (Figure 3.1b).

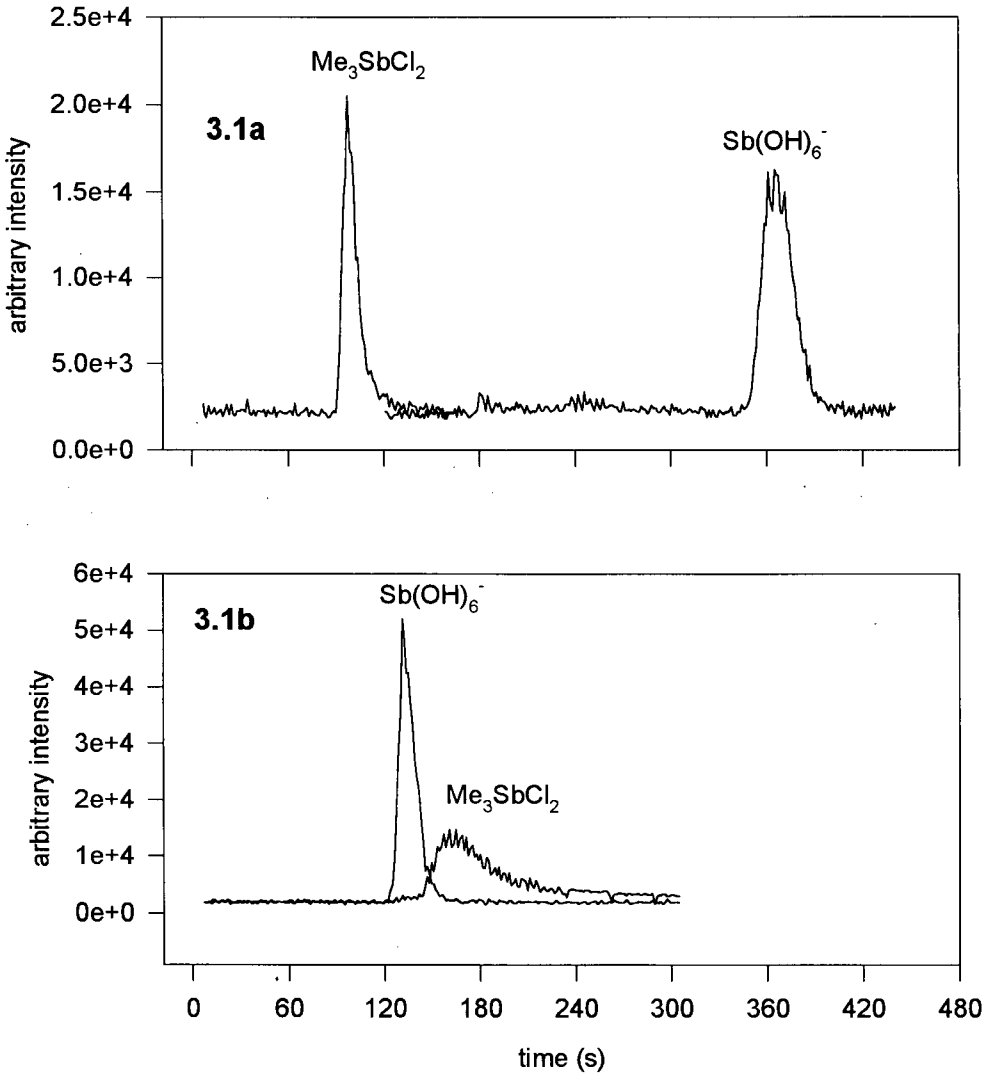

Figure 3.1. Chromatograms of standard antimony compounds (100 ppb each) on two HPLC-ICP-MS systems. **3.1a.** 2 mM KOH, PRP-X100 antion exchange column. **3.1b.** 10mM TEAH, 4.5 mM malonic acid, pH 6.8, 0.1% MeOH, PRP-1 reversed phase column.

When Method A was used for HPLC analysis, several unknown antimony-containing compounds, labeled A1, A2 and A3, were observed. A chromatogram is shown in Figure 3.2 for a sample (medium after for the experiment amended with Sb (V)) in which the unknown compounds A1, A2 and A3 are present. The relative amounts of antimony compounds when some of the samples were analyzed are summarized in Table 3.15. The HPLC analysis was qualitative only, hence absolute amounts are not given, and the assumption was made that the ionization in the plasma and response was similar for all antimony compounds.

Table 3.15. Relative amounts of antimony compounds (%) in some *P. flabellatus* samples analyzed by using Method A. Control experiments contain non-living cells and antimony; A1-A3= unknown compounds.

Experiment/sample	Me_3Sb-	$Sb(OH)_6^-$	A1	A2	A3
Sb(V) amended					
Biomass extract	0	57	43	0	0
Medium before	0	66	18	14	0
Medium after	0	46	15	16	23
Control medium after	0	44	25	31	0
Sb(III) amended					
Biomass extract	0	56	44	0	0
Medium after	0	73	0	11	15
Me_3SbCl_2 amended					
Biomass extract	95	5	0	0	0
Medium after	99	1	0	0	0

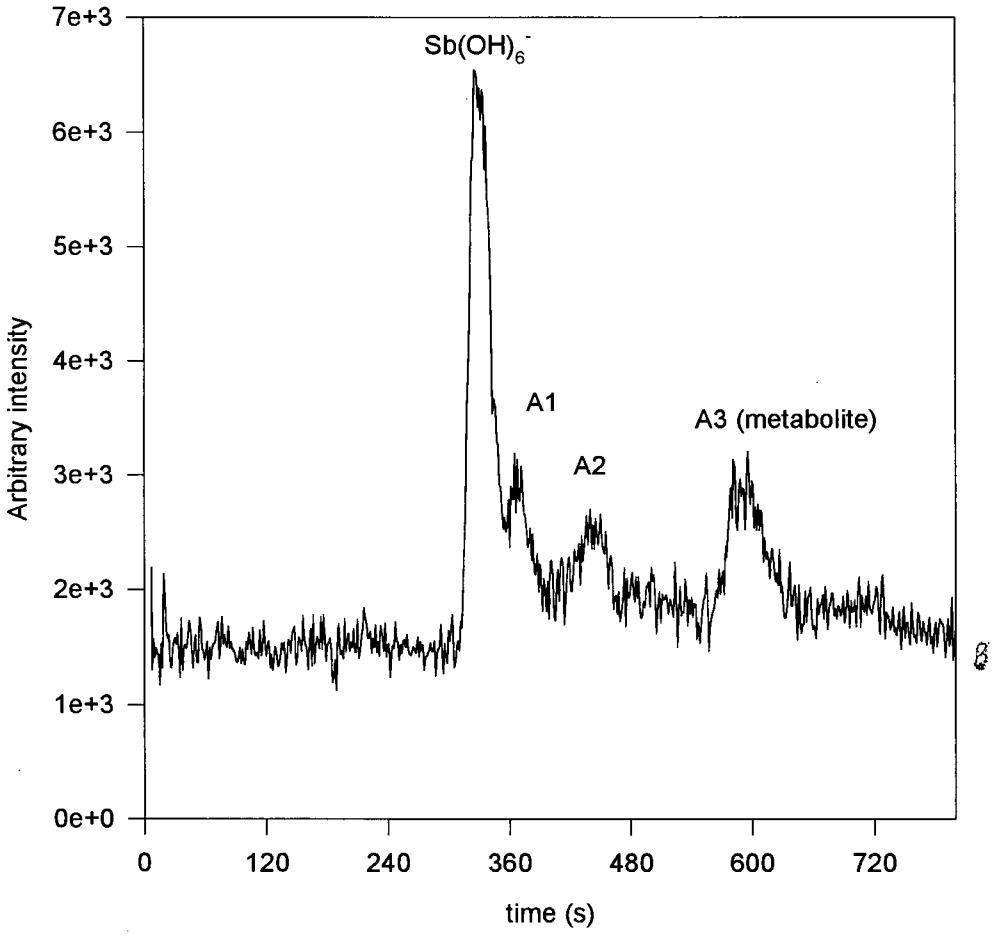

Figure 3.2. Chromatogram (Method A) of medium after 14 days of growth *for P. flabellatus* amended with Sb (V).

Upon examining Table 3.15, it can be observed that a large amount of A1 (43 and 44%) is present in biomass extracts for the experiments treated with inorganic antimony. A3 is seen only in media sampled at the end of the experiments amended with Sb (III) and Sb (V). For the experiment amended with Sb (V), A3 is not present in the medium at the beginning of the experiment (medium before), nor in the control medium at the end of the growing period (control medium after). Thus these results suggest that a metabolite is formed from inorganic antimony starting compounds by *P. flabellatus*, and excreted into the medium. This metabolite might contain antimony, or it may bind to antimony already present in the medium. No new species are formed when Me_3SbCl_2 is used as the starting compound.

When Method B was used for HPLC analysis, unknown antimony-containing compounds, labeled B1, B2, B3 and B4, were observed as well. Three chromatograms obtained by using Method B are shown in Figure 3.3. The first chromatogram (Figure 3.3a) is for the medium at the beginning of the experiment amended with Sb (V) (medium before), the second chromatogram (Figure 3.3b) was obtained from the medium at the end of the same experiment (medium after) and the third chromatogram (Figure 3.3c) represents a fresh weight extract of the biomass collected from this experiment. Unknown B4 is a major antimony-containing compound in the medium samples, but it is not present in the biomass extract. Chromatograms for samples from the experiment in which Sb (III) was used to amend the medium are similar to those in Figure 3.3 and are hence not shown. Due to its presence in all medium samples, including all controls, B4 cannot be considered a metabolite. B3 is found in small amounts in biomass extracts for experiments amended with inorganic antimony but not in medium samples; however, its presence as a metabolite was not determined.

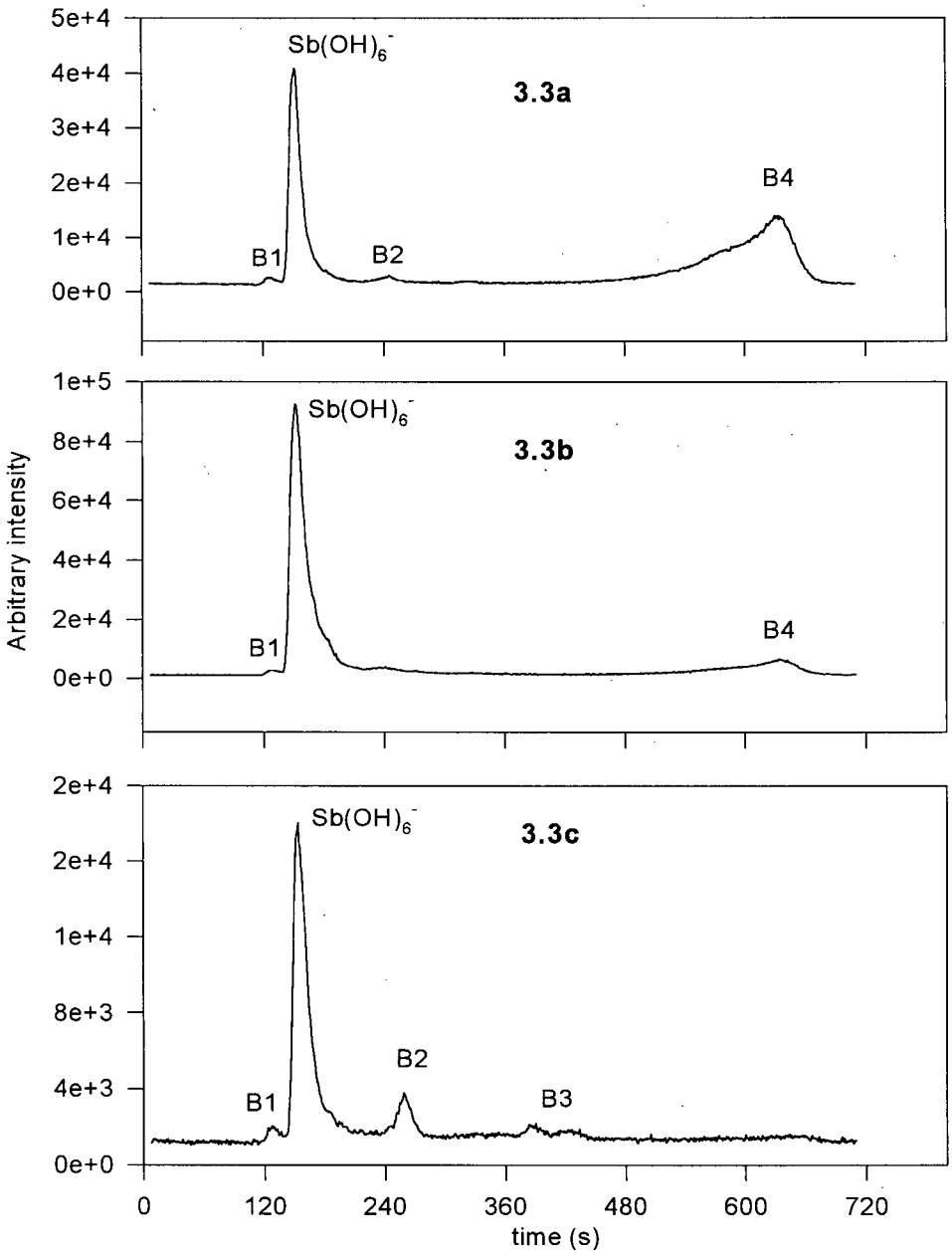

Figure 3.3. Chromatograms of *P. flabellatus* media and biomass extracts (Method B) for experiments amended with Sb (V). **3.3a.** Medium before. **3.3b.** Medium after. **3.3c.** biomass extract (dry weight). B1, B2, B3 and B4 are unknown Sb-containing compounds.

Because of the ubiquitous presence of B4, an attempt was made to characterize it. As detailed in section 3.2.6, B4 was isolated from a medium sample ("medium after", amended with Sb (V)) by using HPLC. Ethanol (-20° C) was added to the isolation product to precipitate proteins according to the method published by Ma *et al.*[31] The sample was then applied to a Sephedex LH20 column to remove excessive salts. The final isolated compound was analyzed qualitatively. HG-GC-AAS analysis when using 1M HCl (acid pH) to adjust the pH of the reaction afforded only SbH_3. When HG-GC-AAS analysis was carried out at neutral pH, stibines were not produced. Hence the final isolated compound possesses the oxidation state of +5 and does not contain methyl groups bound to the antimony. However, the presence of antimony-containing compounds of unknown oxidation state that are not derivatized to hydrides cannot be discounted. This type of compound was suggested to be present in media from these experiments in section 3.3.3.2.1 and Table 3.14.

The chromatogram obtained when the isolated unknown compound was qualitatively analyzed by using Method B is shown in Figure 3.4. The first chromatogram (Figure 3.4a) was obtained when the sample used for the isolation procedure was analyzed; this was the medium at the end of the experiment amended with Sb (V) (medium after), and prior to the isolation procedure. The second chromatogram (Figure 3.4b) represents the final isolated product, after ethanol precipitation and Sephedex LH20 clean-up. Each of the chromatograms also shows the relative amounts of the compound (assuming similar detector response), and it can be seen that $Sb(OH)_6^-$ and B4 were present in each sample. Only B4 was the starting compound for the chromatogram in Figure 3.4b, indicating that B4 may in fact be an Sb (V) compound in equilibrium with $Sb(OH)_6^-$.

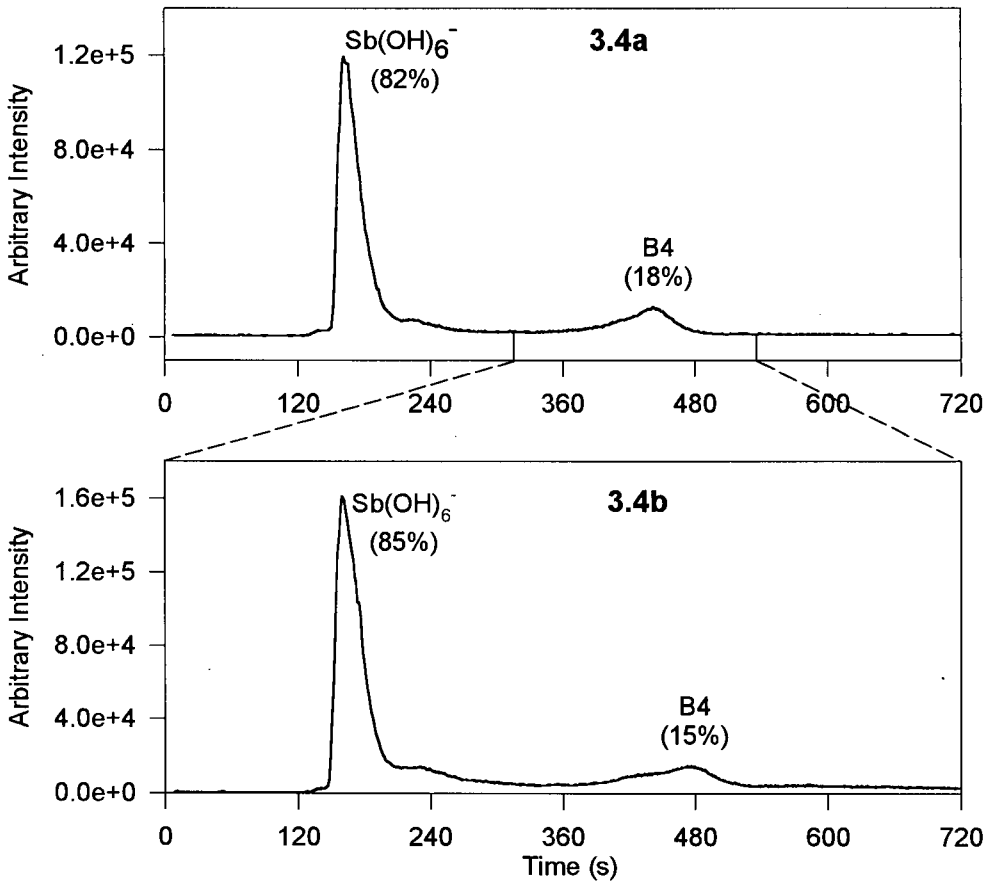

Figure 3.4. Chromatograms of unknown B4 (Method B) showing proportions of each compound. **3.4a.** Medium after for *P. flabellatus* amended with Sb (V). **3.4b.** B4 after HPLC fraction collection, ethanol precipitation and Sephedex LH20 clean-up.

The equilibrium may be represented by the following equation:

$$B4 \rightleftharpoons Sb(OH)_6^-$$

When B4 is isolated, the equilibrium is re-established so that $Sb(OH)_6^-$ is present. Other attempts to characterize this compound were unsuccessful.

Enhanced oxidation of Sb (III) to Sb (V) appears to be taking place due to the presence of *P. flabellatus*, as suggested by the results in Table 3.14. Equal amounts of Sb (III) were found in the medium at the start of the experiment (amended with Sb (III)), and in the controls treated with Sb (III) at the beginning and the end of the experiment. Raw area counts (quantification was not carried out) are shown for $Sb(OH)_6^-$ present in media (Figure 3.5). Although some oxidation is taking place in the medium in the absence of living cells, enhanced oxidation is taking place by *P. flabellatus* (Figure 3.5).

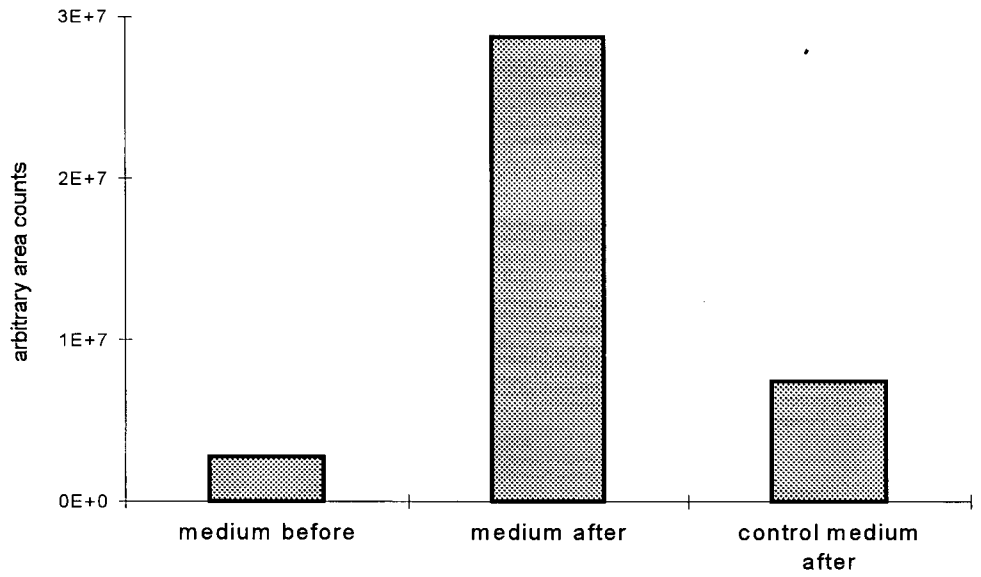

Figure 3.5. Raw area counts for $Sb(OH)_6^-$ in media for *P. flabellatus* grown in Sb (III)-amended culture.

3.3.3.3. Culture experiments with Scleroderma citrinum

The earthball, *Scleroderma citrinum,* was grown in pure submerged culture amended with antimony compounds to determine the interaction of antimony with this organism. The concentrations of total antimony in medium and biomass samples for cultures amended with antimony compounds are summarized in Table 3.16. The concentrations in media do not differ appreciably from the beginning (medium before) to the end (medium after) of the experiments. Bioconcentration factors are similar for the three antimony compounds studied and are approximately 0.2, indicating that no accumulation of antimony takes place by the fungus.

Table 3.16. Concentration of total antimony (ppm) (SD[a]) in media and biomass extracts of *Scleroderma citrinum* grown in submerged culture (ppm for solutions, ppm fresh weight for biomass). ICP-MS analysis was used for the analysis.

Experiment/sample	[Sb]	BCF $([Sb]_{biomass}/[Sb]_{ma})^b$
Sb (III) amended		
Medium before	12.0 (0.5)	
Medium after	10.9 (0.3)	
Biomass extract	2.0 (0.7)	0.18
Sb(V) amended		
Medium before	12.7 (0.7)	
Medium after	12.2 (0.3)	
Biomass extract	2.5 (0.3)	0.20
Me_3SbCl_2 amended		
Medium before	10.9 (0.7)	
Medium after	10.7 (0.4)	
Biomass extract	1.8 (0.4)	0.17

[a] SD = standard deviation, calculated for replicate experiments.
[b] BCF = Bioconcentration factor, calculated as $[Sb]_{biomass}/[Sb]_{ma}$; where ma = medium after.

The chromatograms resulting from the analysis of biomass extracts for the three experiments by using Method A (anion exchange HPLC-ICP-MS with 2 mM KOH) are shown in Figure 3.6. The first two chromatograms were obtained from the analysis of the biomass extracts for *S. citrinum* grown with Sb (III) (Figure 3.6a) and Sb (V) (Figure 3.6b). A second antimony-containing peak of unknown identity and with a retention time similar to that of A1 in Table 3.15, is observed in both these chromatograms. The third chromatogram (obtained by analyzing the biomass extract from the Me_3SbCl_2 amended culture) shows the presence of Me_3SbCl_2 (or the hydrolyzed form, $Me_3Sb(OH)_2$), most likely unchanged, in the biomass (Figure 3.6c). The medium samples analyzed by using Method A (not shown) contain only Sb (V) for the experiments amended with inorganic antimony, and only Me_3SbCl_2 for the experiment amended with Me_3SbCl_2. No new antimony compounds were detected by using this method of analysis, contrasting with the finding of a metabolite formed by *P. flabellatus*.

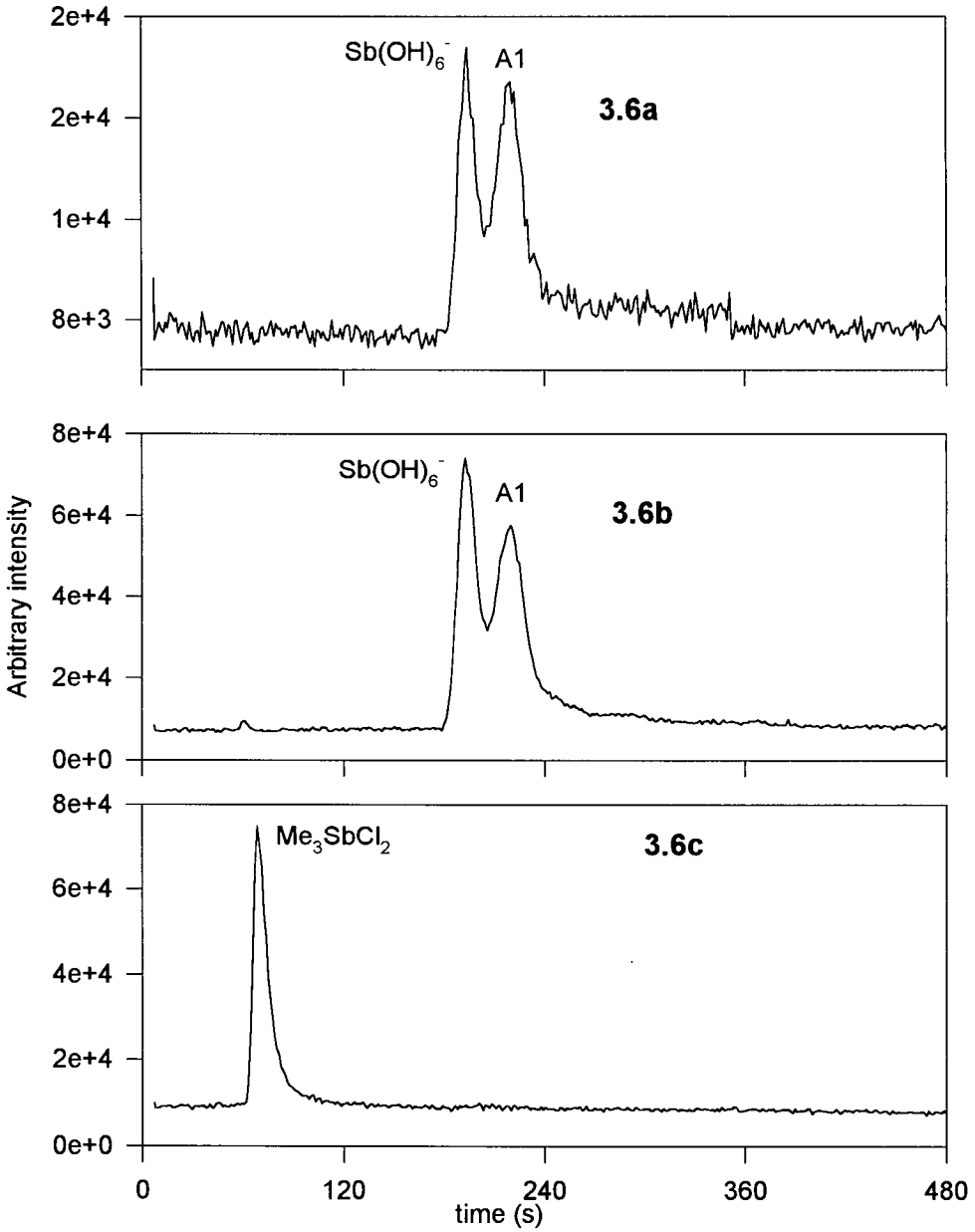

Figure 3.6. Chromatograms for biomass extracts (fresh weight) for *S. citrinum* experiments (Method A). **3.6a.** Experiment amended with Sb (III). **3.6b.** Experiment amended with Sb (V). **3.6c.** Experiment amended with Me_3SbCl_2. A1 is an unknown Sb-containing compound.

When the samples were analyzed by using Method B (ion-pairing HPLC-ICP-MS with 10 mM TEAH/4.5 mM malonic acid, pH 6.8), the unknown described in the previous section, B4, was present in all samples, including biomass extracts. The identification of B4 in these samples was based on the similar retention time of the peak compared with that observed in the samples for *P. flabellatus*. Considering only the area counts for $Sb(OH)_6^-$, increased oxidation of Sb (III) to Sb (V) in the presence of the live fungus does not take place in these experiments.

3.3.3.4. Summary of the interaction of antimony with fungi that can produce mushrooms

Elevated levels of antimony were observed in fruiting bodies of *P. flabellatus* grown on substrate amended with antimony compounds, compared with levels found in the mushrooms grown on non-Sb containing substrate. Sb (V) was the only antimony species extracted from the mushrooms grown with both Sb (III) and Sb (V), and a Me_3Sb-containing compound was extracted from mushrooms grown with Me_3SbCl_2.

No appreciable differences were observed in total antimony concentrations in culture media for both *P. flabellatus* and *S. citrinum*, when the media were amended with antimony compounds. Thus the mycelia do not accumulate these antimony compounds. Previous studies with other fungi have shown different results. *Scopulariopsis brevicaulis* appears to take up Sb (III) from solution (losses of 30% were observed)[13], and *Saccharomyces cerevisiae* completely took up Sb (III) but not Sb (V)[32].

When Method A was used for the analysis of biomass extracts and media, a metabolite was detected, presumably produced from the interaction of *P. flabellatus* with inorganic antimony compounds. No metabolites were detected for *S. citrinum* by using Method A. The antimony species in biomass extracts were similar for *P. flabellatus* and *S. citrinum* experiments,

showing a second antimony-containing compound eluting closely with $Sb(OH)_6^-$. Further studies to identify unknowns A1 and A3 are recommended.

When Method B was used for analysis, the unknown compound B4 appeared in nearly all biomass extracts and media. Thus an attempt was made to characterize it. B4 may be in the +5 oxidation state and it appears to be in equilibrium with $Sb(OH)_6^-$. We suggest that further studies address either this compound's identification or elimination (e.g., by using a growth medium with minimum carbon sources and salts).

Enhanced oxidation of Sb (III) to Sb (V) by *P. flabellatus* takes place, although oxidation by other factors is appreciable.

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Chapter 4

ARSENIC IN THE MEAGER CREEK HOT SPRINGS ENVIRONMENT

4.1. Introduction

The Meager Creek hot springs are located north of Pemberton, in British Columbia, Canada. Hot springs are formed when water percolates through permeable rock or fractures, is heated by the earth's crust at depth, and is then driven to the earth's surface by a combination of artesian flow and thermal convection[1]. Hot water is able to dissolve minerals over time, so that elevated levels of metals and metalloids are often associated with hot springs. For example, arsenic concentrations in water from hot springs in Yellowstone National Park have been documented to reach 1.6 ppm[2]. Levels of total arsenic reported for a number of Japanese hot springs range from non-detectable to 25 ppm[3]. The presence of arsenic in geothermal waters is due to the dissolution of arsenic containing minerals such as arsenopyrite, niccolite, enargite, orpiment, realgar, proustite and other pyrites[4], and the most probable form of arsenic in hydrothermal solutions is thought to be arsenite[5,6]. Pyrite is the most likely host for arsenic at Meager Creek[7].

The hot springs at Meager Creek provide an opportunity for studying the environmental chemistry of metal(loid)s such as arsenic. Arsenic is a known poison and carcinogen, and its toxicity is dependent on the chemical form, or species, that it takes. For example arsenobetaine ($(CH_3)_3As^+CH_2COO^-$) can be found in marine animals and mushrooms, and is much less toxic than arsenous acid ($As(OH)_3$). Some arsenic compounds found in the environment are listed in Table 1.1 (Chapter 1).

Very little is known about arsenic speciation in a hot springs environment specifically, although limited knowledge is available about non-marine ecosystems. For example, arsenic speciation was determined in a river in Japan receiving drainage from hot springs[8], but samples were not collected from the immediate hot springs environment. Samples analyzed included a green alga, a diatom, freshwater fish, a freshwater prawn, a marsh snail and fly larvae, and all samples contained DMA and TMA when they were analyzed by HG-GC-AAS following alkaline digestion of extracts. These results are inconclusive because the methodology used involves a digestion procedure, which may change the species of arsenic, and an analytical tool that utilizes derivatization and hence does not permit identification of the arsenic species in solution. A series of studies on freshwater and terrestrial plant uptake of radioactive arsenic has been published[9,10,11] and arsenolipids were found to be formed in aquatic plants, although no conclusive structural elucidation was carried out. In another study five species of halophytes from an estuarine environment were analyzed for arsenic species and content[12]. The authors claim that compounds such as arsenobetaine, arsenocholine, tetramethylarsonium, TMAO, DMA and an arsenosugar were found in plant extracts and that metabolic synthesis of these compounds is taking place within the plants.

The present study was undertaken to determine, semi-quantitatively, arsenic levels and speciation in water and biota at Meager Creek hot springs to extend knowledge about the chemistry of arsenic in freshwater/terrestrial environments[7-10].

4.2. Experimental

4.2.1. Chemicals and reagents

Arsenic standards were obtained as sodium arsenate, $Na_2HAsO_4.7H_2O$ (Aldrich), arsenic trioxide, As_2O_3 (Alfa), methanearsonic acid, $CH_3AsO(OH)_2$ (Vineland Chemical), and cacodylic acid, $(CH_3)_2AsO(OH)$ (BDH), and were dissolved in deionized water to make standard solutions. Extracts of kelp powder (Galloway's, Vancouver, BC) and Nori (*Porphyra tenera*) of known arsenosugar content[13] were used to identify the retention times of arsenosugars; these were verified by comparison to pure arsenosugars generously donated by K. Francesconi and T. Kaise. Arsenobetaine[14], arsenocholine[15], trimethylarsine oxide[16], and tetramethylarsonium iodide[17] had been synthesized previously according to standard methods. Methanol (HPLC grade, Fisher), tetraethylammonium hydroxide (TEAH, 20% in water, Aldrich), malonic acid (BDH), concentrated phosphoric acid (Aldrich), ammonium hydroxide (1M, Fluka), pyridine (Fisher), and formic acid (BDH) were used as reagents for mobile phases and extractions.

4.2.2. Sampling

Sampling was carried out in November, 1996 and July, 1997. Sample locations are shown in Figure 4.1. Water was sampled by hand into polypropylene bottles that had been acid washed previously. Biota were sampled by hand, stored in Ziploc® bags and kept cool until processing in the lab. There, they were washed thoroughly with tap water to remove soil and other particles, rinsed with deionized (1 Mohm) water, and frozen. Microbial mats were rinsed with only a minimum of deionized water before freezing, to prevent loss of mat composition. The samples were then freeze-dried and pulverized to a fine powder for analysis.

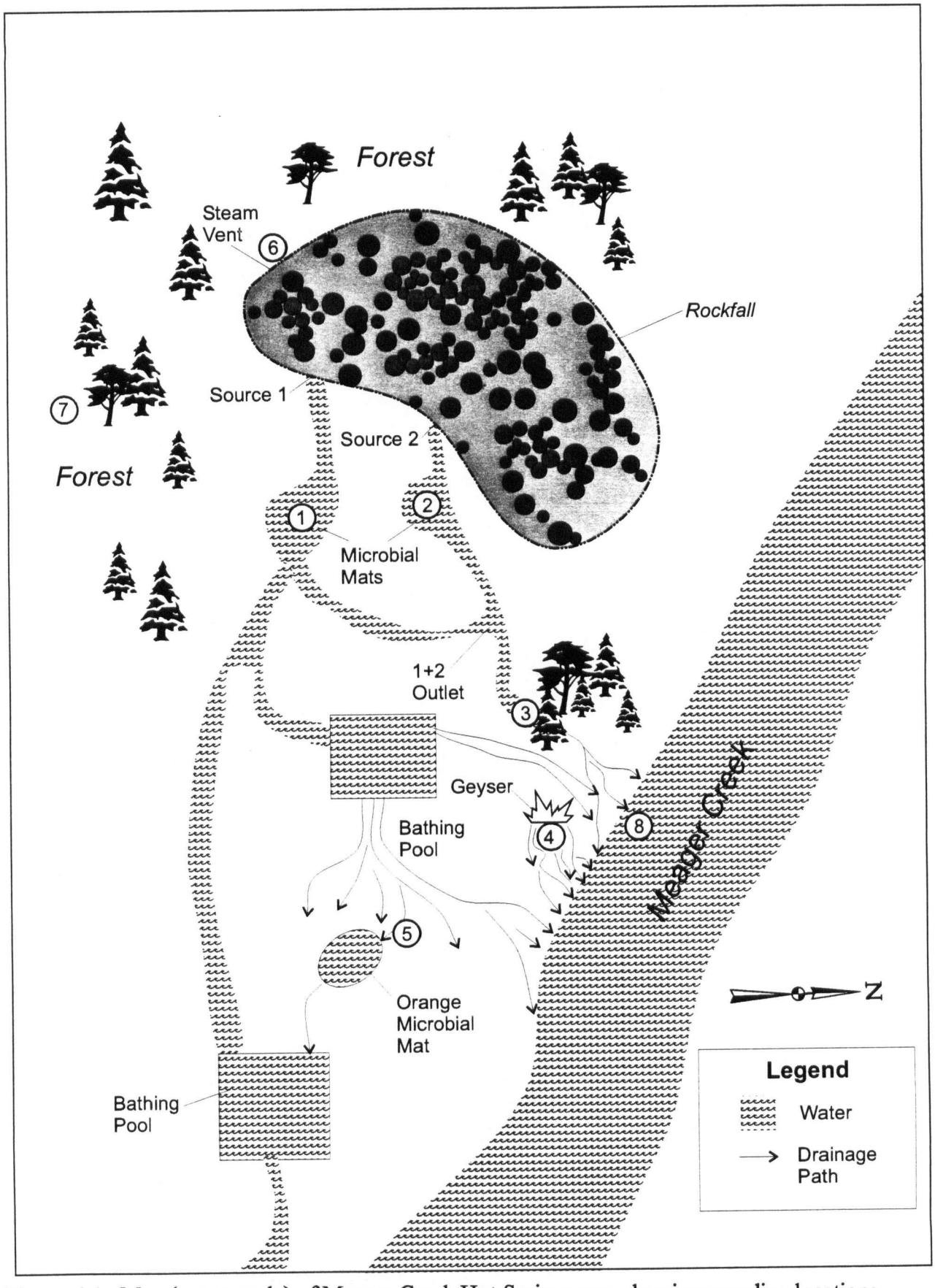

Figure 4.1. Map (not to scale) of Meager Creek Hot Springs area showing sampling locations

Identification of plants and mushrooms (including bracket fungus) was carried out by using field guide books[18,19,20]. Assistance from Ms. O. Lee and Dr. W. B. Schofield (Botany Department, UBC), and from Mr. James Black (Vancouver Mycological Society) is greatly appreciated in the identification of moss, lichens and mushrooms.

4.2.3. Sample preparation and analysis

For the determination of total arsenic content by using ICP-MS, all samples were analyzed in duplicate resulting in <2% standard deviation. Water samples were analyzed directly by ICP-MS (VG PlasmaQuad, VG Elemental) using Rh (10 ppb) as an internal standard. Acid digestions of biota samples were carried out after weighing (0.3 g ± 0.5 mg) the freeze-dried powders into either a 40 mL glass vial or a 500 mL round bottomed flask (RBF). Concentrated nitric acid (3 mL, doubly distilled in quartz, Seastar, Sidney, BC) was added to each sample, and additionally, hydrogen peroxide (3 mL, 30% in water, reagent grade, Fisher) was added to each sample in glass vials. The samples in glass vials were heated directly on a hot plate and boiled for 3 hours. The contents of the RBFs were boiled for 2 hours by using a heating mantle and a reflux apparatus[21] and then cooled. Hydrogen peroxide (3 mL) was added to the RBFs and the solutions were heated for another hour. After all the samples had cooled, the clear solutions remaining were diluted to 25 mL with deionized water and stored until analysis. The acid digests were analyzed by using ICP-MS, with Rh (10 ppb) as an internal standard, and by monitoring m/z 75 and 103 for arsenic and rhodium, respectively. ICP-MS parameters are given in Table 4.1.

Extractions were carried out by weighing 0.5 to 1 g (± 0.5 mg) of the freeze-dried powders into 50 mL or 15 mL centrifuge tubes, adding 10-15 mL MeOH/Water (1:1), sonicating for 20 minutes, centrifuging for 20 minutes and decanting the liquid layer into a RBF. Each

sample was sonicated and centrifuged a total of 5 times. The decanted extracts for each sample were pooled and rotovapped to near dryness (1-2 mL) and then dissolved in 5 or 10 mL of deionized water. The extracts were filtered through 0.45 μm syringe filters (Millipore) and analyzed by HPLC-ICP-MS using the conditions given in Tables 4.1 and 4.2. Data from the ICP-MS were processed by using chromatographic software[22], and identification of arsenicals in samples was made by comparison of retention times with those of standards by using at least two chromatographic systems. Semi-quantitative concentrations of arsenic compounds were determined by using external calibration curves for each compound corresponding to a matching standard, or to DMA for arsenosugars.

Table 4.1. Operation parameters for ICP-MS

Feature	Specific Conditions
Forward radio-frequency power	1350 W
Reflected power	<10 W
Cooling gas flow rate (Ar)	13.8 L/min
Intermediate (auxiliary) gas flow rate (Ar)	0.65 L/min
Nebulizer gas flow rate (Ar)	1.002 L/min
Nebulizer type	de Galan
Analysis mode	Time Resolved Analysis (TRA) for HPLC
Quadrupole pressure	9×10^{-7} mbar
Expansion pressure	2.5 mbar

Table 4.2. HPLC conditions for arsenic speciation

Chromatography	Column	Mobile phase	Flowrate (mL/min)
Anion exchange	Hamilton PRP-X100, 150 × 4.6 or 250 × 4.6 mm	20 mM ammonium phosphate, pH 6.0	1.0 or 1.5
Cation exchange	Supelcosil LC-SCX or Whatman SCX Partisil 5, 250 × 4.6 mm	20 mM pyridinium formate, pH 2.7	1.0
Ion-pairing	GL Sciences ODS, 250 × 4.6 mm	10 mM TEAH, 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	0.8

4.3. Results and Discussion

4.3.1. Total concentrations of arsenic in water samples

The concentrations of arsenic and sampling locations (Figure 4.1) for water samples taken in November 1996 and July 1997 are shown in Table 4.3. These results show no seasonal variations from midsummer to autumn sampling, with the arsenic levels being very similar with respect to both the locations sampled and the sampling date (range of 237-303 ppb). The concentration of arsenic in the hot springs water (average concentration of 280 ppb) is two orders of magnitude higher than in the cold Meager Creek water (5.4 ppb), reflecting the action of hot water on arsenic containing minerals. The cooler temperature observed at location 1 in November was probably a result of cooler air temperatures (-5 to 0 °C) and precipitation in the form of snow. The oxygen concentration ($[O_2]$) results show that the water was well oxygenated at the source, at outlet 1+2, and at the top of the microbial mats, but more reducing conditions exist under the microbial mats and in the sediments. HPLC-ICP-MS analysis of waters[23] showed only the presence of arsenate, except for trace levels of arsenite at location 2. This indicates that if the arsenic was dissolved initially from minerals as arsenite, oxidation to arsenate took place before expulsion of the water from the source. Arsenate is the species of arsenic expected in most waters[24], although others have found up to 80% of total arsenic as arsenite in Hot Creek, California[25].

Table 4.3. Some physical and chemical characteristics of Meager Creek waters (see Figure 4.1 for sample locations), na = not analyzed.

Location	Date	[As] (ppb) (SD)[a]	[O_2] (ppm) %	T (°C)	pH
Source 2	Nov. 1996	237 (8)	na	na	na
1	Nov. 1996	303 (2)[b]	3.04, 56.7%[c] 1.08, 20.2%[d]	44	6.7
4 (geyser)	Nov. 1996	288	na	na	na
8 (Meager Creek)	Nov. 1996	5.4	na	3	8.3
Source 2	July 1997	na	2.70, 38.4%	56	6.4
1	July 1997	286[b]	0.60, 8.5%[d]	52	6.3
2	July 1997	277[b]	0.75, 10.0%[e]	41	6.8
1 + 2 outlet	July 1997	289[b]	4.20, 59.7%	49	6.3

[a] SD = standard deviation from analysis of duplicate samples; other concentrations are for single samples only.
[b] Also in Feldmann *et al.*[23]
[c] Measurement taken at surface of microbial mat.
[d] Measurement taken below microbial mat.
[e] Measurement taken in sediment below microbial mat.

4.3.2. Total concentrations of arsenic in biota

The concentrations of arsenic in acid digested samples of biota sampled at two different times, in November 1996 and in July 1997 are shown in Table 4.4. Sampling locations correspond to numbers on the map of the sampling area in Figure 4.1.

Table 4.4. Samples, sampling location (see Figure 4.1), sampling times and arsenic levels (ppm dry weight) (SD for duplicate digestions) in biota samples, B = background

Sample	Arsenic (ppm)	Sampling time	Sampling location
Top layer, microbial mat	290 (20)	Nov 1996	1
Brown microbial mat,	82 (8)	Nov 1996	5
Algae 1 (deep green algae)	249	Nov 1996	2
Sedge, *Scirpus* sp.	7.1 (0.4)	Nov 1996	1
Cedar, *Thuja plicata*	0.96	Nov 1996	3
Moss at geyser, *Fumaria hygrometrica*	237 (2)	Nov 1996	4
Fleabane, *Erigeron* sp.	14 (2)	Nov 1996	1, 2
Brown lichen, *Bryoria* sp.	0.30	Nov 1996	6
Yellow lichen, *Alectoria* sp.	0.12	Nov 1996	6
Orange microbial mat, bottom	59	July 1997	5
Orange microbial mat, top	108	July 1997	5
Algae 2 (green algae)	56	July 1997	5
Sedge, *Scirpus* sp.	4.5	July 1997	1
Moss at geyser, *Fumaria hygrometrica*	350	July 1997	4
Moss at stream, *Fumaria hygrometrica*	91	July 1997	3
Fleabane, *Erigeron* sp.	3.9	July 1997	1, 2
Monkey flower, *Mimulus* sp.	8.7	July 1997	1, 2
Brown lichen, *Bryoria* sp.	0.30	July 1997	6
Brown lichen, *Bryoria* sp. (B)	4.8	July 1997	7
Yellow lichen, *Alectoria* sp.	0.16	July 1997	6
Yellow lichen, *Alectoria* sp. (B)	0.55	July 1997	7
Cup mushroom, *Tarzetta cupularis*	0.09	July 1997	7
Fawn mushroom, *Pluteus cervinus*	0.10	July 1997	7
Bracket fungus, *Fomitopsis pinicola*	< 0.07	July 1997	7

Microbial mats are found covering the rocks over which the hot water flows from the source into the nearby river. These mats are 1-6 thick and consist of a variety of organisms, usually predominantly bacteria and cyanobacteria but also including fungi and algae (their microbiological makeup is discussed in a later section)[26]. Their appearance can be described as alternating layers of green, brown and orange with pockets of purple. Strings of deep green material can be found on rocks in areas of faster water flow and these were labeled Algae 1. "Brown microbial mat" refers to brown slime covering the bottom of a cooler stream (about 30° C), and Algae 2 refers to a sample of stringy, absorbent algae collected near location 4. Finally, a thick mat of bright orange was found in July in a stagnant pool of cooler water, and this mat was also composed of layers, with orange on top, brown and green layers, and a white crust on the bottom.

All algae and microbial mat samples contain high levels of arsenic, ranging from 56-290 ppm (dry weight), and the microbial mat from location 1 contains the highest amount. The lowest amounts of arsenic in these samples are observed in the orange microbial mat (59 and 108 ppm), the brown microbial mat (82 ppm) and Algae 2 (56 ppm), all taken from locations furthest away from the hot springs source, with the exception of Algae 2. Dilution (i.e., snow in November), precipitation (as, for example, $Ca_3(AsO_4)_2$), absorption by biota, or adsorption of the dissolved arsenic may result in lower levels of arsenic in water farther away from the source, and these lower arsenic levels in the water would result in lower levels in the microbial mat samples. No seasonal changes were studied for these samples.

Moss (*Fumaria hygrometrica*) was sampled at location 4, a geyser source, the result of drilling by BC Hydro in 1974, and also at location 3, a cooler stream about 200 m away from sources 1 and 2 (See Figure 4.1). A greater arsenic content is observed for the moss from location 4 in July (350 ppm) with respect to that in November (237 ppm), and both these

amounts are higher compared with the arsenic content in moss from location 3 (91 ppm). Although the differences have not been statistically validated by taking replicate samples from each location and time, they may be due to the following possibilities. The arsenic content in samples taken from the same location in midsummer and late autumn may reflect seasonal differences in arsenic uptake rates and accumulation. Lower levels of arsenic may have been present in the water at the stream location for the reasons mentioned above, leading to lower levels in the moss taken from the stream. Finally, different levels of submergence of the mosses in the arsenic containing water might cause differences in arsenic concentration.

Sedge (*Scirpus* sp., most likely Olney's bulrush, a species known to grow at Meager Creek Hot springs[1]) and fleabane (*Erigeron* sp.) samples contain higher levels of arsenic in the late autumn (7.1 ppm for *Scirpus* sp. and 14 ppm for *Erigeron* sp.) compared with the samples taken in summer (4.5 ppm for *Scirpus* sp. and 3.9 ppm for *Erigeron* sp.), but again these differences have not been statistically validated. Nevertheless, because the levels of arsenic in the water are the same at both these times, the higher arsenic levels observed in the plant samples from late autumn may indicate that the rate of arsenic accumulation exceeds the rate of arsenic depuration through the growing season. Studies on accumulation of arsenic in plants growing on mine dumps showed a similar trend, where accumulation rates were associated with physiological growth of the plants[27].

Bracket fungus and mushrooms (see Table 4.4 for Latin names), sampled from the forested area nearby (i.e., not directly adjacent to the hot springs) contain low levels of arsenic (0.10 ppm and lower, Table 4.3). This may indicate that the amount of arsenic being transported via the atmosphere from the hot springs is negligible or very low. Lichens (*Bryoria* sp. and *Alectoria* sp.) collected from a tree directly adjacent to a steam vent (location 6) do not contain levels of arsenic that are elevated (0.12-0.30 ppm) with respect to samples from the forest,

location 7 (0.55-4.8 ppm), indicating that the lichens are not being impacted by the steam vent.

Cedar leaves (*Thuja plicata*) were sampled directly above a warm stream that drained from the hot springs source into the river. The source of arsenic in the cedar was probably the soil in which the tree was growing, rather than the steam generated by the stream.

4.3.3. Arsenic speciation in biota samples

Semi-quantitative amounts of arsenic species in samples from Meager Creek have been estimated and are listed in Table 4.5. The structures, names and abbreviations for arsenic compounds can be found in Table 1.1, Chapter 1.

Table 4.5. Estimated concentrations of arsenic species in biota samples in ppm dry weight (SD[a] in brackets); "trace" amounts are greater than the limit of detection (LOD) but less than 2 × LOD..

Sample, time (location)	As(III)	As(V)	MMA	DMA	Sugar X	Sugar XI
Top layer, microbial mat, Nov 1996 (1)	53 (7)	23.4 (0.8)	1.8	<0.03	10	2.4
Brown microbial mat, Nov 1996 (5)	0.5 (0.4)	1.4 (0.4)	<0.04	<0.04	<0.03	0.09
Algae 1 (deep green algae), Nov 1996 (2)	<0.04	3.8 (0.8)	<0.04	<0.04	0.8 (0.2)	0.17
Scirpus sp., Nov 1996 (1)	0.25 (0.08)	0.63 (0.06)	0.027	<0.01	<0.07	<0.01
Thuja plicata, Nov 1996 (3)	0.13 (0.04)	0.04 (0.01)	0.013	0.015 (0.006)	<0.006	<0.008
Fumaria hygrometrica, Nov 1996 (4)	<0.01	1.2 (0.2)	<0.01	<0.01	0.18 (0.13)	<0.01
Erigeron sp., Nov 1996 (1,2)	3.5 (0.7)	2.2 (0.2)	<0.01	0.93 (0.66)	<0.007	<0.01
Bryoria sp., Nov 1996 (6)	trace	0.05 (0.01)	<0.02	<0.02	<0.01	trace
Alectoria sp., Nov 1996 (6)	trace	trace	<0.02	<0.02	<0.01	trace
Orange microbial mat, bottom, July 1997 (5)	0.23	1.2	0.09	0.28	1.3 (0.7)	0.12
Orange microbial mat, top, July 1997 (5)	0.09	1.2	0	0.09	0.27	0.19
Algae 2 (green algae), July 1997 (4)	<0.02	2.1 (0.7)	0.23	0.07	1.0 (0.5)	0.34
Scirpus sp., July 1997 (1)	0.35	1.0	<0.01	0.04	<0.007	<0.01
Fumaria hygrometrica, July 1997 (4)	<0.01	3 (1)	<0.01	0.7	0.28 (0.18)	0.03
Fumaria hygrometrica, July 1997 (3)	0.6	0.7	0.05	0.07	0.31	<0.01
Erigeron sp., July 1997 (1,2)	0.6	1.0	<0.02	0.22	<0.01	<0.02
Mimulus sp., July 1997[b] (1,2)	3 (1)	2.8 (0.7)	<0.02	<0.02	<0.01	<0.02
Bryoria sp., July 1997 (6)	<0.01	0.03	<0.01	<0.01	<0.01	<0.01
Alectoria sp., July 1997 (6)	<0.01	0.03	<0.01	<0.01	<0.01	<0.01
Alectoria sp. (background), July 1997 (7)	0.032 (0.005)	0.045 (0.009)	<0.01	<0.01	<0.007	0.03
Cladonia sp., July 1997 (6)	0.08 (0.02)	0.11 (0.01)	<0.02	trace	trace	<0.02
Fomitopsis pinicola July 1997 (7)	0.02	0.04	<0.01	<0.01	<0.01	<0.01
Tarzetta cupularis, July 1997 (7)	trace	0.028	<0.01	0.020	trace	<0.01
Pluteus cervinus, July 1997 (7)	0.023	0.027	<0.01	trace	<0.01	<0.01

[a] SD = standard deviation, calculated from duplicate analyses (by using anion and cation exchange HPLC-ICP-MS).

[b] This sample also contains tetramethylarsonium ion (Me_4As^+) in an amount of 0.014 ppm dry weight.

4.3.3.1. Algae and microbial mat samples

All algae and microbial mat samples contain arsenosugars (Table 5), with the orange microbial mat containing the highest amount proportionally (44% of sum of extracted arsenic species). Only arsenosugars X and XI are observed in samples and in most cases the major arsenosugar is arsenosugar X.

Sample chromatograms for Algae 1 and extracts used as laboratory standards (from kelp and Nori) are shown in Figure 4.2, and they show the selectivity of the different chromatographic techniques. Figure 4.2a (ion-pairing chromatography) shows the presence of arsenosugar XI and the absence of arsenite. The absence of arsenite allows arsenosugar X to be seen in Figure 4.2c (anion exchange chromatography), and the presence of arsenosugar XI is confirmed in this chromatogram as well. Figure 4.2b (cation exchange chromatography) corroborates the presence of arsenosugar X, well separated from the other arsenic compounds. Another sample chromatogram is illustrated in Figure 4.3, showing the matching retention times for arsenosugar XI when a sample (top layer, microbial mat) is spiked with an extract containing arsenosugar XI (Nori).

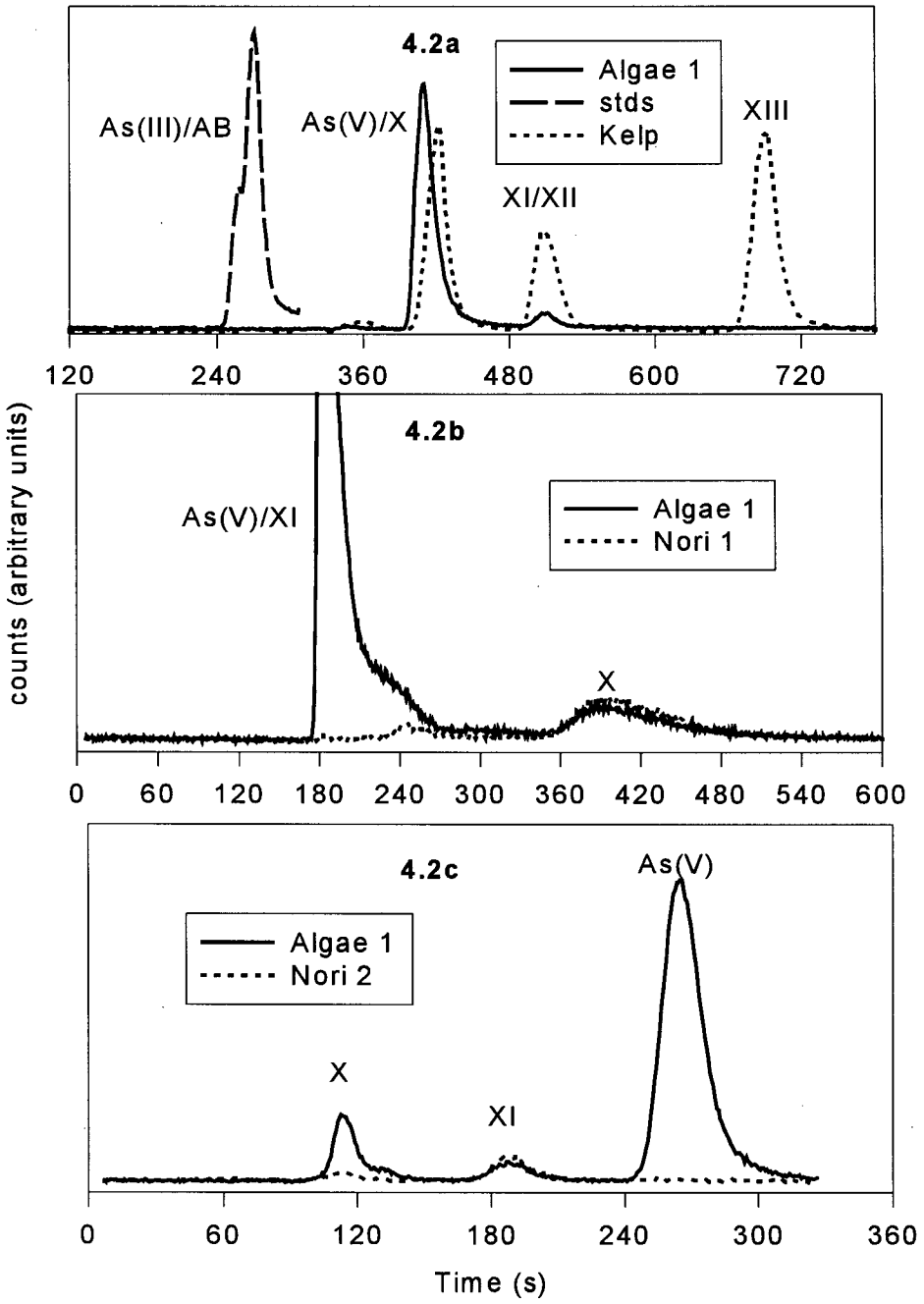

Figure 4.2. Chromatograms for Algae 1 and laboratory standards showing the presence of arsenosugars X and XI. Extracts of Nori labeled Nori 1 and Nori 2 contain predominantly arsenosugar X and XI, respectively. **4.2a.** C18 column, 10 mM TEAH/4.5 mM malonic acid/pH 6.8 mobile phase. **4.2b.** Cation exchange column, 20 mM pyridinium formate/pH 2.7 mobile phase. **4.2c.** Anion exchange column, 20 mM ammonium phosphate/pH 6.0 mobile phase.

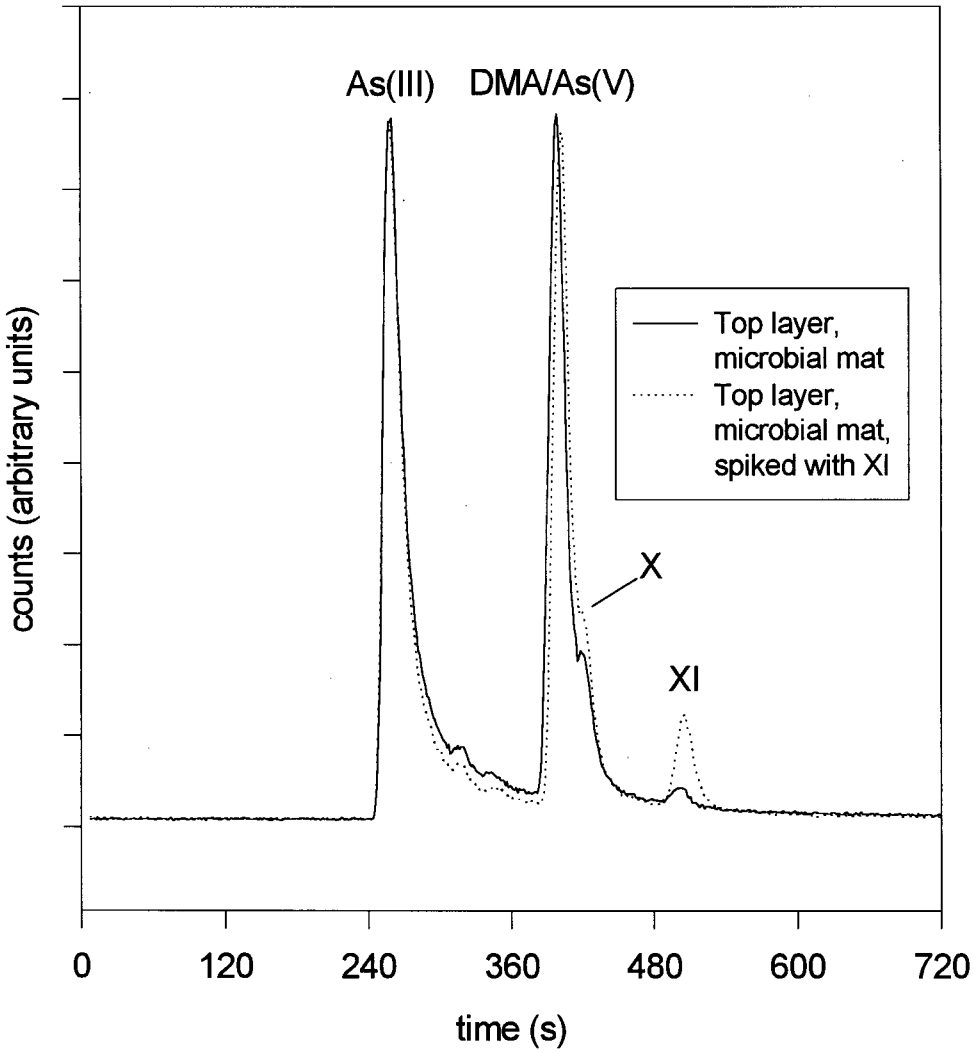

Figure 4.3. Chromatograms of a microbial mat extract (top layer, microbial mat) and extract spiked with arsenosugar XI, showing co-elution of arsenosugar XI by using ion-pairing HPLC-ICP-MS (C18 column, 10 mM TEAH/4.5 mM malonic acid/pH 6.8 mobile phase).

Arsenosugars have been found in the environment previously in marine algae, which contain, among others, arsenosugars X through XIII, often as the only species of water soluble arsenic[13,28,29,30]. In marine phytoplankton *Heterosigma akashiwo* and *Skelotonema costatum* (a diatom), arsenosugars XI and XIII were found to be the major compounds in each organism, respectively[31]. Arsenosugar XIII was the major metabolite produced when another marine diatom, *Chaetoceros concavicornis*, was exposed to arsenate[32]. This previous work may indicate that arsenosugars are formed *de novo* by these lower trophic level marine organisms. The *de novo* synthesis of arsenosugars by the marine macroalga *Fucus spiralis* has been postulated to take place, based on the transformation of radiolabeled arsenate in seawater to water soluble organoarsenic compounds within the alga, and subsequent speculation that the compounds were arsenosugars X and XI[30,33]. Other marine green algae, *Dunaliella* sp. and *Polyphysa peniculus* do not form arsenosugars in pure culture; the former reduces arsenate to arsenite[34] and the latter methylates arsenic species[35].

Arsenosugars appear to be not as common in terrestrial and freshwater environments. The freshwater algae, *Chlorella* sp. have been studied extensively and the extractable arsenic species after exposure to arsenate is predominantly arsenate, or inorganic arsenic[36,37,38]. Goessler *et al.*[38], however, found that in addition to arsenate, *Chlorella Böhm* forms small amounts of an unidentified arsenical, arsenite, MMA and DMA. Comparing the HPLC retention time of the unknown compound to the retention time for arsenosugar XI in our studies, and noting that a similar chromatographic system to ours was used, may allow the unknown compound to be tentatively identified as arsenosugar XI. A commercial sample of the terrestrial soil/freshwater cyanobacterium, *Nostoc* sp. contains arsenosugar X[13], making up 32% of the total arsenic, but in pure culture *Nostoc* sp. takes up arsenate as inorganic arsenic, with less than 1% DMA formed[39]. Likewise, another freshwater cyanobacterium *Phormidium* sp. contains mostly inorganic arsenic

that is not free, but NaOH digestable[40]. A freshwater diatom was found to contain DMA (81%) by using an inconclusive method of speciation[8], allowing for the possibility that the DMA found is actually derived from arsenosugars in the diatom. Hence arsenosugars are possibly formed *de novo* by certain species of marine algae (micro and macro), freshwater cyanobacteria and freshwater algae.

Microbial mats have been described as "a heterotrophic and autotrophic community, dominated by cyanobacteria...annealed tightly together by slimy secretions from various microbial components"[41]. The microbial compositions of mats from Yellowstone National Park and elsewhere have been found to depend on the pH, the temperature, and the H_2S concentration in the water[26]. Based on those factors, microbial mats at locations 1 and 2 at Meager Creek possibly contain cyanobacteria species such as *Synechococcus lividus*, the photosynthetic green nonsulfur bacteria *Chlorflexus* sp. and the purple bacterium *Chromatium tepidum*[26]. However, the mats are almost certainly not homogeneous with respect to time or area and many other bacteria are present, as mentioned earlier. For example, 22 species of bacteria and 1 fungus have been cultured from the microbial mat at location 1[42].

Therefore, the arsenosugars found in the mats are probably synthesized by cyanobacteria or other bacteria. The high proportion of inorganic species, especially arsenate, in most samples, may imply that most of the arsenate in the water is not metabolized by the microbes making up the mat. Similar behaviour has been observed with bacteria cultured from a marsh area in Yellowknife, NWT[43]. Arsenite was extracted from the microbial mat sample taken from location 1, which is not surprising considering the reducing conditions imposed by the mat (Table 4.3). This suggests that arsenate-reducing bacteria are present in this mat. The microbial mat from location 1 contains MMA, and the orange microbial mat from location 5 contains DMA (Table 4.5), indicating that methylation is taking place by organisms in these mats.

Microbial mats were present in the algae sampling locations, and hence the arsenosugars and other arsenic compounds extracted from algae may be due to uptake from their environment. Therefore although the possibility exists that these algae form arsenosugars, no conclusions can be made about their capability to do so.

A difference to note between freshwater and marine arsenosugar formation is the finding of only arsenosugars X and XI in Meager Creek and other terrestrial samples[13,38], whereas arsenosugars XII and XIII, as well as arsenosugars X and XI, are common in marine algae. Among marine algae, differences have also been noted; arsenosugars X and XI predominate in Rhodophyta and Chlorophyta, and arsenosugars XII and XIII are the major compounds in Phaeophyta[30]. The phylum Phaeophyta is almost exclusively marine and hence the lack of sugars XII and XIII in the freshwater environment is not surprising, if these sugars are restricted to Phaeophyta. Microbial mats appear to follow the same chemotaxonomic trend as Rhodophyta and Chlorophyta. However, the reasons for the trends are not clear, since the aglycones (R^+ groups in the pathway shown in Figure 1.2., Chapter 1) are found as common metabolites in all organisms[30].

4.3.3.2. Vascular plants (sedge, cedar, fleabane, monkey flower)

The identities and amounts of arsenic species associated with an organism probably reflect at least four things: (a) the presence of arsenicals outside the organism, in its food source; (b) the ability of these compounds to enter the organism; (c) the ability, if any, of the organism to synthesize arsenic compounds and (d) the presence of arsenicals adsorbed onto the outside surface. At this point it is unclear how much these factors influence the speciation of arsenic found in plants. In previous studies, Nissen *et al.*[9] and de Bettencourt *et al.*[12] have implied that arsenicals other than arsenate found in plants were metabolites and hence synthesized by plants,

but de Bettencourt *et al.* also allowed for the possibility that arsenic speciation in halophytes reflected the arsenic species found in the estuarine waters from which they were sampled[12,44]. In other studies, *Catharanthus roseus* (periwinkle) was conclusively shown to methylate MMA to DMA in 4% yield in pure plant tissue culture[45]. Other authors believe that trace amounts of methylated species in plants are due to uptake from the soil after the compounds are formed by microbial activity in the soil[46]. This theory is supported by studies in which beans and vascular aquatic plants absorbed MMA from soil and water[47,48,49].

The major extractable arsenic species in vascular plants from Meager Creek hot springs are arsenite and arsenate (Table 4.5). All the plants, with the exception of cedar, were growing in wet soil that consisted of microbial mats, at the edges of the streams. Although the soil in which the plants were growing was not sampled, it is reasonable to suggest that there may be arsenosugars in the root environments of the plants. Therefore it is interesting to note the lack of arsenosugars in the plants. These results are consistent with some of the studies mentioned above, where inorganic arsenic species are the major water soluble species of arsenic found in plants.

Differences are seen between the July and November samples for sedge and fleabane (Figure 4.4). In sedge, higher levels of arsenite and MMA are seen in November, and in fleabane, higher levels of arsenite are seen but slightly lower levels of DMA are seen in November. These observations may suggest that more biological activity is taking place outside the plant, and accumulation of these compounds is taking place, leading to higher levels of arsenite and methyl species inside the plant. However, no seasonal reduction or methylation was evident in the water sampled at any locations, although water analyzed from microbial mats incubated anaerobically over time acquired arsenite and methylarsenic species[23]. Arsenic biomethylation and reduction by plants is a possibility as well, and the metabolism taking place

may parallel the increased growth of the plant during the summer.

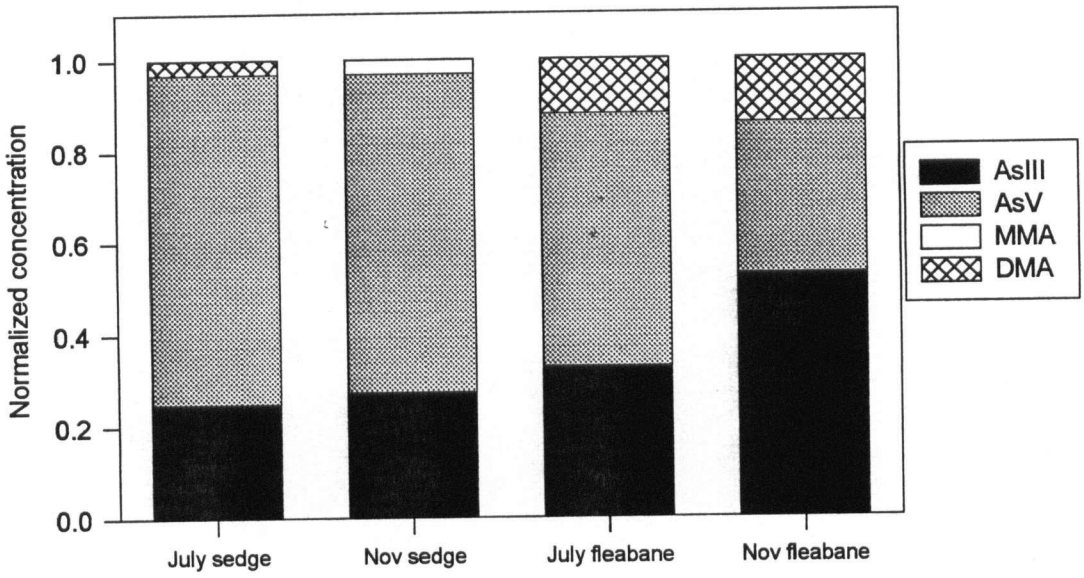

Figure 4.4. Seasonal arsenic speciation in higher plants, sedge (*Scirpus* sp.) and fleabane (*Erigeron* sp.), shown as proportions of total arsenic extracted.

Interestingly, the major extractable arsenic species in cedar is arsenite (Table 4.5). When pine seedlings, *Pinus* sp., were allowed to take up radiolabeled arsenate through their roots hydroponically, arsenite was found as the major species in roots and shoots[10], which may indicate reduction by the plant. Arsenate was found to be more toxic than arsenite to *Catharanthus roseus*[45]. Hence reduction to arsenite by cedar, and by the other vascular plants collected from Meager Creek, may in fact be a detoxification mechanism.

The monkey flower *Mimulus* sp., in addition to containing nearly equal amounts of extractable arsenite and arsenate (2.8-3 ppm dry weight), contains a small amount (0.014 ppm) of tetramethylarsonium ion (Table 4.5, including footnotes to the table). This is a compound that was not found in the microbial mats, in water, or in water after fermentation of microbial mat[23].

The occurrence of Me_4As^+ may indicate the presence of a specific microenvironment in which this compound is available to the plant, or an ability of the plant to synthesize Me_4As^+. Marine sediments cultured aerobically with MMA and DMA released Me_4As^+ into the culture medium in small amounts[50] and tetraalkylated arsenicals (but not Me_4As^+) were reported to have been found in estuarine waters[44,51]. Nevertheless, Me_4As^+ has otherwise not been found in pure or mixed culture experiments, in sediments, or in waters; this information together with the lack of the compound in Meager Creek waters may suggest that methylation is indeed taking place in this plant. The formation of Me_4As^+ is thought to be the final product of the methylation pathway proposed by Challenger, with methylation of trimethylarsine by methyl donors such as S-adenosylmethionine (SAM) or methyl halides[24]. However, normally the reaction sequence stops at or before the formation of Me_3AsO (or Me_3As). An alternative pathway has been suggested, which involves the exchange of the ribosyl group on a precursor trimethylarsonioriboside (Chapter 1, Figure 1.3, intermediate **3.1**) for a methyl group[52]. The complete absence of the intermediate trimethylarsonioriboside, as well as the absence of its precursor molecule, arsenosugar XIII, in the terrestrial environment, makes this pathway unlikely in terrestrial plants.

In summary, the speciation of water soluble arsenic in vascular plants can be generalized as follows: plants may be selective for inorganic arsenic, they may be able to biomethylate arsenic in small amounts and they may detoxify arsenate by reducing it to arsenite, which may be less toxic to them.

4.3.3.3. Moss

Arsenosugar X was detected in all moss samples (Table 4.5). Microbial colonies were observed in and around the moss samples, and despite the washing procedure, most likely contaminated the samples. The sampling methods used in the present study are not capable of

differentiating between the contribution from microbial mat components interspersed throughout the moss sample and the contribution from arsenosugar uptake by the moss from its environment, or of metabolism within the moss.

The normalized concentrations of arsenic species in moss are shown in Figure 4.5. Seasonal variations (from July to November) include a greater proportion of arsenosugar X in November, and the presence of methyl species in July. The most likely reason for these differences is that the microbial population in the moss environment changes throughout the growing season. The same reasoning can be used to explain the differences in the moss samples taken from different locations. For example, the moss sampled at the geyser location (location 4) appears to contain less arsenosugar, proportionally, than the moss sampled at the cooler stream location (location 3). This larger proportion of arsenosugar X as well as the presence of arsenite is also seen in the orange microbial mat taken from a cooler location (Table 4.5). Therefore the microbial community in the vicinity of the moss sample at the cooler stream location may resemble a microbial mat at a cooler location, which may consist of more arsenic-metabolizing organisms.

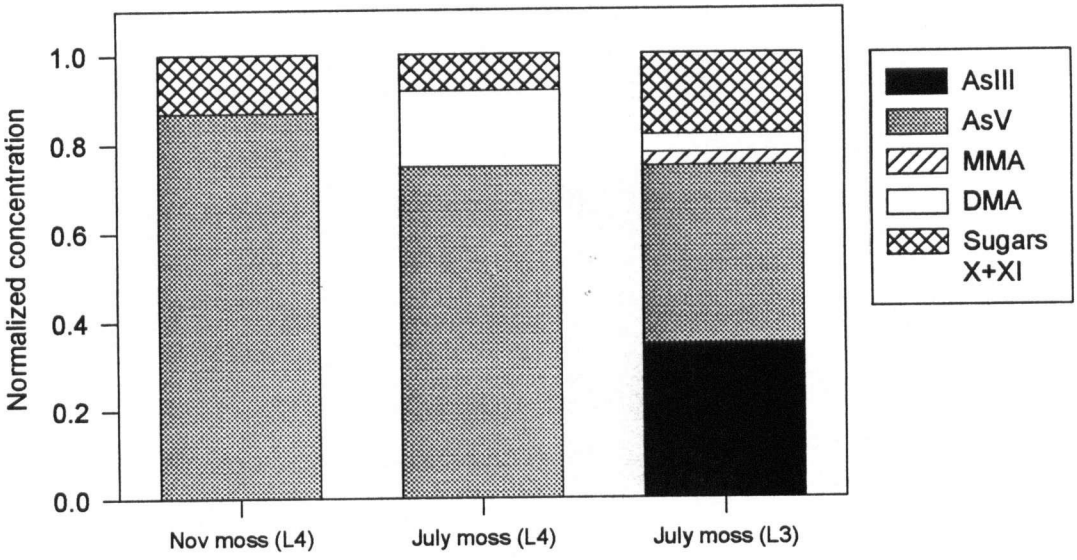

Figure 4.5. Seasonal and spatial arsenic speciation in moss (*Fumaria hygrometrica*) shown as proportions of total arsenic extracted, L = location. July and November moss are from the geyser location (L4) and the second July moss is from the stream location (L3).

4.3.3.4. Fungi including lichens

The levels of arsenic compounds in all fungi samples (Table 4.5) are at or close to the limit of detection for the HPLC-ICP-MS methods used. Brown and yellow lichens both contain arsenosugar XI, as does the yellow lichen sampled from a location not expected to be impacted by the hot springs (location 7). Pixie cups contain trace levels of arsenosugar X. The presence of arsenosugars in these samples is very likely a result of synthesis by the indigenous algae or cyanobacteria living symbiotically with the fungus, making up the lichen, since the organisms were relatively isolated, and not exposed to the microbial mats with their sugar-synthesizing organisms. The organisms associated with both pixie cups (*Cladonia* sp.) and yellow lichen (*Alectoria* sp.) are green algae of the genus *Trebouxia*[53]. Of course, the possibility cannot be

discounted of sugars being present due to uptake or adsorption from other organisms living in the immediate surroundings of the lichens, or as a result of metabolism by the fungus.

The results for the two mushrooms sampled add to the current knowledge of arsenic speciation in mushrooms. Inorganic species are the major species found, as well as DMA in minor amounts (Table 4.5). Arsenosugar X was detected in *Tarzetta cupularis*, but in trace amounts; verification of this result, especially in specimens containing higher levels of arsenic, would be very interesting. A small amount of an arsenosugar was reported in another mushroom, *Laccaria amethystina*, but the authors considered additional chromatographic confirmation to be necessary[54]. Very low levels of water soluble arsenic are present in these fungi.

4.3.3.5. Extraction efficiency for arsenic species

The sums of arsenic species extracted are low for all samples analyzed. The % amounts of extracted arsenic with respect to the total, obtained by dividing the sum of species in Table 4.5 by the total arsenic in Table 4.4, are shown in Table 4.6. This calculation does not take into account the amount of arsenic that may have been extracted but was not observable by using these chromatographic methods. In past studies, this amount has been observed to be significant in some samples, even when using the method of standard additions for quantification[55]. Analytical problems have been reported when significant levels of sodium and potassium were present in samples; that is, a suppressed chromatographic signal for arsenobetaine resulted from the co-elution of sodium and potassium ions during cation exchange HPLC-ICP-MS[56]. The suppression of the signal for earlier eluting arsenic species (e.g., anionic species and arsenosugar X) is possible as well, although such an effect was not reported[56].

Table 4.6. Percent amounts of arsenic extracted.

Sample	% Arsenic extracted
Top layer, microbial mat, Nov 1996	31
Brown microbial mat, Nov 1996	2.4
Algae 1 (deep green algae), Nov 1996	1.9
Sedge, *Scirpus* sp., Nov 1996	13
Cedar, *Thuja plicata*, Nov 1996	21
Moss at geyser, *Fumaria hygrometrica*, Nov 1996	0.6
Fleabane, *Erigeron* sp., Nov 1996	47
Brown lichen, *Bryoria* sp., Nov 1996	28
Yellow lichen, *Alectoria* sp., Nov 1996	41
Orange microbial mat, bottom, July 1997	5.4
Orange microbial mat, top, July 1997	1.7
Algae 2 (green algae), July 1997	6.7
Sedge, *Scirpus* sp., July 1997	31
Moss at geyser, *Fumaria hygrometrica*, July 1997	1.1
Moss at stream, *Fumaria hygrometrica*, July 1997	1.9
Fleabane, *Erigeron* sp., July 1997	47
Monkey flower, *Mimulus* sp., July 1997	67
Brown lichen, *Bryoria* sp., July 1997	10
Yellow lichen, *Alectoria* sp., July 1997	19
Yellow lichen, *Alectoria* sp., July 1997 (B)	19
Cup mushroom, *Tarzetta cupularis*, July 1997	70
Fawn mushroom, *Pluteus cervinus*, July 1997	63

Extractable amounts of arsenic range from 0.6 % for moss to 70 % for the mushroom *Tarzetta cupularis*. From vascular plants, 13-67% of total arsenic was extracted (mean of 38%), from moss, 0.6-1.9% of total arsenic was extracted (mean of 1.2 %), and from fungi, 10-70% of total arsenic was extracted (mean of 36%). The extraction method used has been successful for

marine plants and animals[57,58] and is assumed to extract water-soluble species, yet it appears to be insufficient for the extraction of these samples. The levels of inorganic arsenic may be underestimated, because in other studies the highest amounts of inorganic arsenic species from terrestrial samples were extracted with water alone[59]. Insufficient extraction may also result from the presence of arsenic compounds that are nonpolar and not soluble in methanol/water (1:1). In other studies where the presence of arsenolipids was postulated, an extraction technique with hot ethanol was used[9,10,11]. Other researchers have used a sequential extraction technique consisting of Soxhlet extraction with 80% methanol, cold 5% chloroacetic acid, warm 75% ethanol and hot 5% chloroacetic acid to extract all but 20% of arsenic from wheat seeds[60]. These extraction techniques are likely to extract compounds that are more nonpolar in nature. Additionally, prior freeze-drying of samples has been recently found to result in lower extraction efficiencies, although the reasons for this are unclear[55]. Arsenic that is not extracted by methanol/water (1:1) might be tightly bound to lipids; to cell wall components of plants, including insoluble cellulose, calcium or magnesium pectates, or lignin; and to chitin or other cell components of fungal samples.

Low extraction efficiencies are associated with microbial mats and algae (1.7-31%, mean of 18%), a result also seen with *Nostoc* sp. (34%)[13] and possibly with *Phormidium*, where the arsenic in the cyanobacterium was thought to be not free and possibly bound up in a non-water soluble form[40]. Metals (possibly including arsenic) can bind to bioflocculants secreted by microbial mats[41], and this may limit extraction efficiencies for arsenic. More specifically, arsenate may coprecipitate with insoluble minerals as a result of microbial mat biomineralization. Evidence exists for the formation of hydrated iron and manganese oxides, and iron and aluminum silicates on bacterial cells in freshwater microbial mats[61,62], which are minerals known to co-precipitate with arsenate. There is also evidence of ferric arsenate precipitation as a result of

bacterial action[63].

4.3.4. Summary

Arsenosugars are apparently formed by cyanobacteria/bacteria, possibly by algae in the lichens *Cladonia* sp., *Bryoria* sp. and *Alectoria* sp., and possibly by the fungus *Tarzetta cupularis*. The arsenic speciation in vascular plants is mostly inorganic arsenic in an amount of at least 13-67% of the total arsenic and significant amounts of arsenite are present in almost all the samples. This may be a concern for higher trophic level organisms, such as mammals, but past studies have shown that even very high concentrations in higher plants have not been harmful to grazers, probably due to fast excretion rates of any toxic forms of arsenic[64]. The identity of some water-soluble species in these biota was determined, but the speciation of the majority of arsenic is unknown. The significance of water-soluble arsenic is not clear, although in some circumstances it reflects the species that are most likely bioavailable for other organisms.

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Chapter 5

ARSENIC IN THE YELLOWKNIFE ENVIRONMENT

5.1. Introduction

Yellowknife is located on Great Slave Lake, in the Northwest Territories, Canada. A major industry in the city is gold mining, and two gold mines, the Royal Oak Giant Mine and the Miramar Con Mine, are presently in operation. The gold in the mined ore is associated with arsenopyrite (FeAsS), and hence arsenic waste is generated during the smelting operation. For example, when milling began at Giant Mine in 1948, aerial emissions of 7.3 tonnes as arsenic trioxide per day were released[1]. Currently, aerial emissions of only 1.7 tonnes as arsenic trioxide per year are released from Giant Mine, and 0.5 tonnes of arsenic per year are discharged with the effluent into Baker Creek.

The proximity of the two gold mines to the city of Yellowknife has allowed researchers to study the effects of mining and arsenic on the surrounding environment. Arsenic chemistry has been studied to some extent in Pud, Kam, Meg, Keg and Peg watershed areas, which receive the drainage from Con Mine waste. Arsenic mobility was found to be controlled by remobilization of historically contaminated sediments rather than by present day mining practices[2]. Waters, sediments and porewaters were analyzed for inorganic and methyl arsenic species[3] and methylarsenic (III) compounds were discovered although they were not identified. Macrophytes (aquatic plants) were analyzed for total arsenic[4] and concentrations were found to be elevated compared with specimens collected from uncontaminated areas. Toxic effects of reduced plant health and lack of biodiversity were observed in areas of high arsenic content.

Anaerobic microbes isolated from a sediment core from Kam Lake were found to methylate arsenic to monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine oxide (TMAO), methylarsenic (III) compounds, arsine and trimethylarsine[5].

As discussed in the previous chapter, little is known about arsenic speciation in biota in the freshwater and terrestrial environments. The high levels of arsenic in the Yellowknife area allows us to study the forms that arsenic takes in the available biota. As well, these results can be compared with those obtained from Meager Creek.

5.2. Experimental

5.2.1 Chemicals and reagents

Arsenic standards were obtained as sodium arsenate, $Na_2HAsO_4.7H_2O$ (Aldrich), arsenic trioxide, As_2O_3 (Alfa), methanearsonic acid, $CH_3AsO(OH)_2$ (Vineland Chemical), and cacodylic acid, $(CH_3)_2AsO(OH)$ (BDH) were dissolved in deionized water to make standard solutions. Extracts of kelp powder (Galloway's, Vancouver, BC) and Nori (*Porphyra tenera*) of known arsenosugar content[6] were used to identify the retention times of arsenosugars; the retention times were then verified by comparison to those obtained from pure arsenosugars generously donated by K. Francesconi and T. Kaise. Arsenobetaine[7], arsenocholine[8], trimethylarsine oxide[9], and tetramethylarsonium iodide[10] had been synthesized previously according to standard methods. Methanol (HPLC grade, Fisher), tetraethylammonium hydroxide (TEAH, 20% in water, Aldrich), malonic acid (BDH), concentrated phosphoric acid (Aldrich), ammonium hydroxide (1M, Fluka), pyridine (Fisher), and formic acid (BDH) were used as reagents for mobile phases and extractions.

5.2.2. Sampling

Sampling was carried out in June and August of 1997. Sample locations are shown in Figure 5.1a and 5.1b. Water was sampled by hand into polypropylene bottles that had been previously acid washed. Some samples were split and one of the aliquots was filtered through 1 μm cellulose nitrate filters (Sartorius) before freezing.

Sediment samples were collected by using a trowel and they were frozen in Ziploc® bags. They were air dried over a period of two days, then ground in a mortar and pestle. Particles that passed through a 60 mesh sieve were subsequently extracted.

Most biota were sampled by hand and all were stored in Ziploc® bags and kept cool until processing in the lab. There, they were washed thoroughly with tap water to remove soil and other particles, rinsed with deionized (1 Mohm) water, and frozen.

Mussels were sampled by hand as well as by using an Eckman grab, and a different freshwater species was collected from Campbell River for comparison. They were shelled before freezing, as were large specimens of snails. Smaller specimens of snails(< 0.5 cm in length) were frozen and processed whole.

Fish were caught by using a gillnet, and they were gutted before freezing. Roe was removed from one fish and this was kept and frozen for analysis. The fish were thawed at a later date and filleted and skinned to obtain muscle samples, which were refrozen.

Pond scum, or algal/microbial mats of unknown composition, sampled from a puddle beside Baker Creek (location 4) were washed with a minimum of deionized water to minimize sample loss before freezing. All frozen biota samples were then freeze-dried and pulverized to a fine powder for analysis.

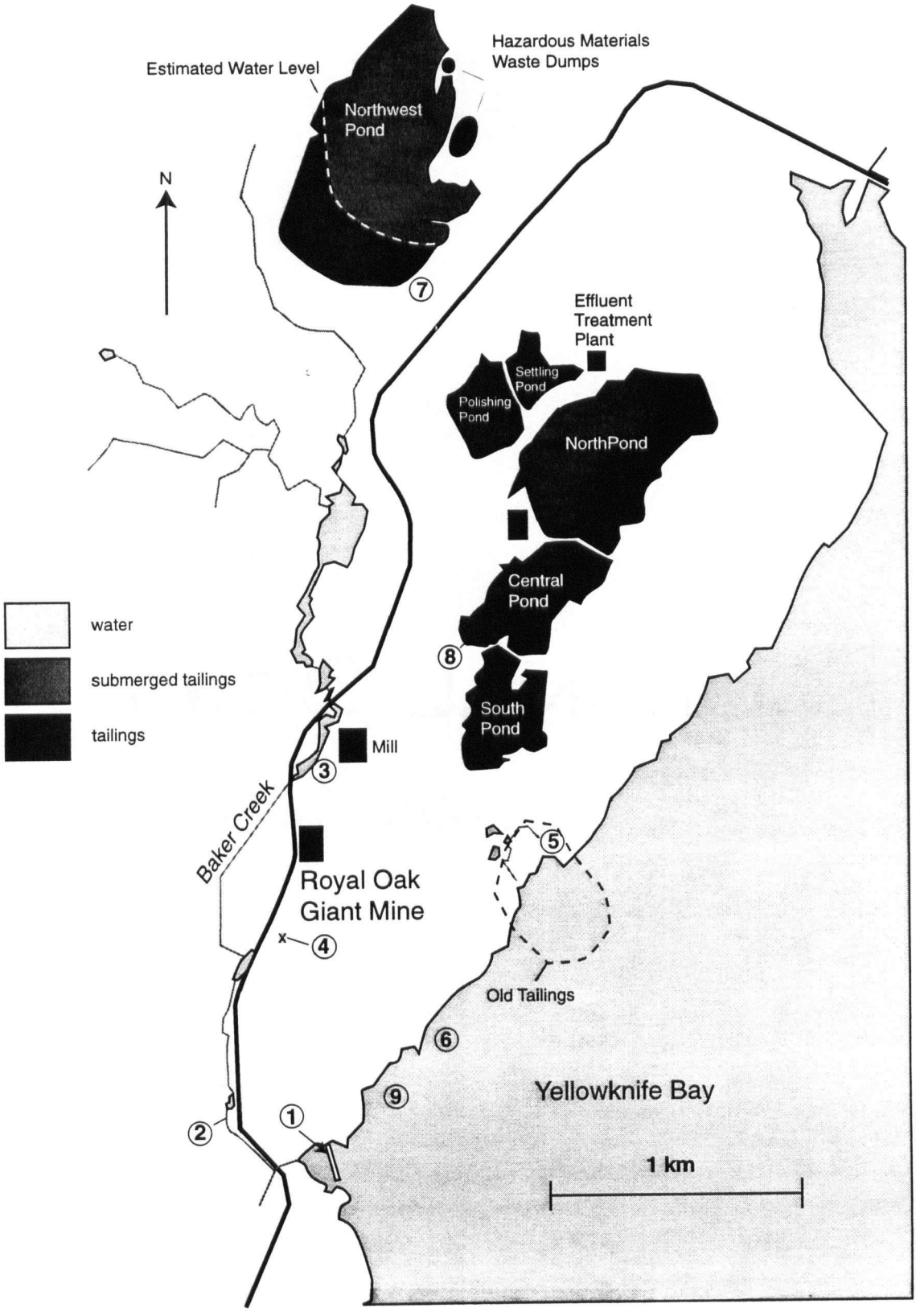

Figure 5.1a. Map of Royal Oak Giant mine property and surrounding area, with sample locations.

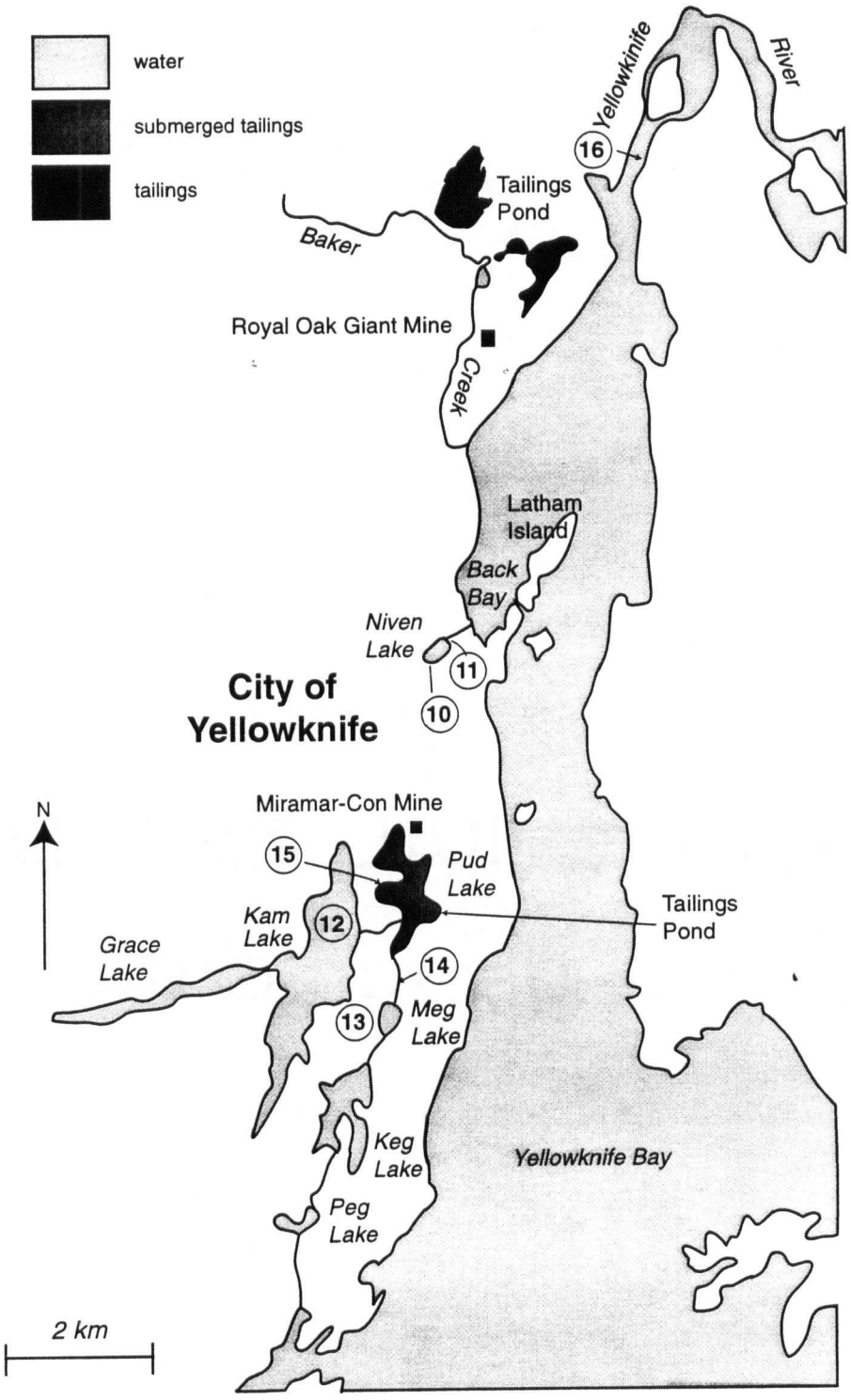

Figure 5.1b. Map of Yellowknife, showing Royal Oak Giant Mine and Miramar Con Mine, and sampling locations.

Mussels and snails[11], plants[12] and mushrooms[13,14] were identified by using field guide books. Assistance from O. Lee, Dr. W. B. Schofield and Julie Oliveira (Botany Department, UBC) is greatly appreciated in the identification of moss, lichens and algae. We are very grateful also to Chris Ollson (Royal Military College) for fish identification, Mike Fournier (Yellowknife, NWT) for assistance with plant identification, and James Black (Vancouver Mycological Society) for help with mushroom identification.

5.2.3. Sample preparation and analysis

For the determination of total arsenic content by using ICP-MS, all samples except water samples were analyzed in duplicate resulting in <2% standard deviation. Water samples were analyzed only once directly by ICP-MS (VG PlasmaQuad, VG Elemental) using Rh (10 ppb) as an internal standard. Acid digestions of biota samples were carried out after weighing (0.3 g ± 0.5 mg) the freeze-dried powders into either a 500 mL round bottomed flask (RBF). Concentrated nitric acid in a volume of 3 mL (doubly distilled in quartz, Seastar, Sidney, BC) was added to each sample. The samples were boiled for 2 hours by using a heating mantle and a reflux apparatus[15] and then cooled. Hydrogen peroxide (3 mL, 30% in water, reagent grade, Fisher) was added to the RBFs and the solutions were heated for another hour. After all the samples had cooled, the clear solutions remaining were diluted to 25 mL with deionized water and stored until analysis. The acid digests were analyzed by using ICP-MS, with Rh (10 ppb) as an internal standard, and by monitoring m/z 75 and 103 for arsenic and rhodium, respectively. ICP-MS parameters are given in Table 5.1.

Extractions were carried out by weighing 0.5 to 1 g (± 0.5 mg) of the freeze-dried powders into 50 mL or 15 mL centrifuge tubes, adding 10-15 mL MeOH/Water (1:1), sonicating for 20 minutes, centrifuging for 20 minutes and decanting the liquid layer into a RBF. Each

sample was sonicated and centrifuged a total of 5 times. The decanted extracts for each sample were pooled and rotovapped to near dryness (1-2 mL) and then diluted to 5 or 10 mL with deionized water.

Fish, fish roe, mussels, snails, and oyster tissue certified reference material (CRM) 1566, obtained from NIST, were digested with protease, based on methods for enzymatic digestion[16,17,18]. An accurately weighed 1 g (±0.5 mg) sample was combined with 0.02-0.05 g of protease (Type VIII, No. P-5380, Sigma) in a plastic 50 mL centrifuge tube. Ammonium carbonate (BDH) buffer at a concentration of 0.1 M and pH 7.2 (adjusted with nitric acid) was added and the tube was sealed and vortexed. The samples were shaken for 4 hours at 37 °C, then centrifuged, and the supernatant was diluted to 25 mL with deionized water. To determine the extent of arsenic solubilization by the protease, oyster tissue samples were extracted with buffer alone (no protease) and using the same procedure as that described for protease digestions.

Water samples, extracts and protease digestions (PDs) were filtered through 0.45 μm syringe filters (Millipore) and analyzed by HPLC-ICP-MS using the conditions given in Tables 5.1 and 5.2. Data from the ICP-MS were processed by using chromatographic software[19], and identification of arsenicals in samples was made by comparison of retention times with those of standards by using at least two chromatographic systems. Semi-quantitative concentrations of arsenic compounds were determined by using external calibration curves for each compound corresponding to a matching standard, or to DMA for arsenosugars.

Table 5.1. Operation parameters for ICP-MS

Feature	Specific Conditions
Forward radio-frequency power	1350 W
Reflected power	<10 W
Cooling gas flow rate (Ar)	13.8 L/min
Intermediate (auxiliary) gas flow rate (Ar)	0.65 L/min
Nebulizer gas flow rate (Ar)	1.002 L/min
Nebulizer type	de Galan
Analysis mode	Time Resolved Analysis (TRA) for HPLC
Quadrupole pressure	9×10^{-7} mbar
Expansion pressure	2.5 mbar

Table 5.2. HPLC conditions for arsenic speciation

Chromatography	Column	Mobile phase	Flowrate (mL/min)
Anion exchange	Hamilton PRP-X100, 150 × 4.6 or 250 × 4.6 mm	20 mM ammonium phosphate, pH 6.0	1.0 or 1.5
Cation exchange	Supelcosil LC-SCX or Whatman SCX Partisil 5, 250 × 4.6 mm	20 mM pyridinium formate, pH 2.7	1.0
Ion-pairing	GL Sciences ODS, 250 × 4.6 mm	10 mM TEAH, 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	0.8

5.3. Results and Discussion

Arsenic compounds were identified by comparing retention times of arsenic compounds in samples with those of standard compounds. If the retention time for an arsenic compound in a sample was the same as that for a standard compound, the arsenic compound was concluded to have the same identity as the standard compound. If the presence of arsenobetaine or cationic species (such as TMAO, arsenocholine, and Me_4As^+) was indicated after analysis with anion exchange HPLC-ICP-MS, the identities of such species were confirmed by analysis with cation exchange HPLC-ICP-MS method. Cation exchange chromatography was also used to confirm the presence of arsenosugar X. This analytical method separates the aforementioned peaks from other peaks that co-elute with them on other chromatographic systems. The identities of arsenosugars were confirmed by using ion pairing chromatography. Some samples were spiked with standards to confirm that retention times of arsenic compounds did not depend on matrix.

The primary purpose of the present study was to determine the identity of detectable arsenic species that could be extracted by using MeOH/water (1:1) extraction. The amounts of the arsenic species found were calculated semi-quantitatively, to determine approximate levels of the compounds (i.e., major or minor components). Standard deviations shown in Table 5.6 and for subsequent results were calculated with quantities obtained from analysis on the different chromatographic systems, where applicable. In some cases, only one number was obtained if the better sensitivity and selectivity of a chromatographic system allowed the species to be seen on one system but not another. The relative standard deviation, on average, is estimated to be about 30%.

5.3.1. Water and sediment samples

Water and soil were sampled in a few locations from which biota were sampled to ascertain the arsenic speciation and concentrations in the surrounding aqueous environment of these biota. Total arsenic concentrations and speciation results for waters, and estimated water soluble species (extracted with MeOH/water 1:1) for soils are summarized in Table 5.3.

Table 5.3. Concentrations of total arsenic and arsenic species in water samples (ppb) and soil extracts (ppm) (SD)[a]. Total concentrations were determined by ICP-MS as described in section 5.2.3. "Trace" amount is 1 ppb (i.e., at detection limit); nd = not determined.

Sample (location)	As (III)	As (V)	MMA	DMA	Sum of species	Total (ICP-MS)
Pore water (1)	296	6.2	<1	29	331	980
Surface water (1)	2.5	98	<1	<1	101	140 (30)†
Surface water (2)	<1	126	<1	<1	126	nd
Surface water (3)	<1	184	<1	<1	184	195 (3)‡
Standing water (4)	1.4	350 (40)†	<1	trace	352	480
Con effluent (14)	<1	5.3	<1	<1	5.3	32 (26)†
Surface water (10)	<1	20.3	2.5	<1	23	68
Surface water (11)	trace	26 (5)‡	2.5 (0.8)‡	<1	30	54 (4)‡
Sediment extract (1)	19.1	0.65	<0.05	<0.05	19.8	nd
Sediment extract (2)	14.4	2.0	<0.05	<0.05	16.4	nd
Sediment extract (3)	10.8	2.8	<0.05	<0.05	13.6	nd

[a] SD = Standard deviation, based on duplicate analysis at two different dilutions† or analysis of duplicate samples‡.

In all surface waters the major arsenic compound is arsenate. Minor amounts of methyl species are observed in water samples from Niven Lake (locations 10 and 11) and from location

4, which were areas of prolific plant and algal growth. Only small amounts of arsenite are present in some samples, except for the pore water squeezed from a sediment in the Baker Creek marsh, which contains arsenite as the major compound. This indicates strongly reducing conditions in the sediment. Not all of the arsenic was accounted for in some samples by using HPLC-ICP-MS analysis; the largest discrepancies are observed for the pore water sample, Con effluent, and Niven Lake waters (locations 10 and 11). Species such as methylated arsenic(III)thiols, of the form $(CH_3)_nAs^{III}(SR)_{3-n}$ (n=1,2,3), as well as those involving binding of arsenic to colloidal organic matter such as humic and fulvic substances, were postulated to be present in waters from Yellowknife in a previous study[3]. The HPLC behaviour for such arsenic species is unknown and they might not be detectable by using the HPLC-ICP-MS methods in this study.

The concentrations of arsenic species in Table 5.3 are in ppm dry weight of sediment. The major extractable species from sediments is arsenite, which probably reflects the insolubility of arsenate in the sediments, and it may reflect reducing conditions as well. If the MeOH/water technique can be used to approximate the species that are water soluble, then it appears that arsenite is the major species available to organisms growing in the sediment.

5.3.2. Freshwater fish

Although arsenic speciation has been studied in some detail for marine fish, very little information is available about the forms of arsenic in freshwater fish. Because of this lack of information, a study was conducted to examine arsenic in fish from Yellowknife. It was also of interest to see if higher levels of arsenic (compared with published background levels) would be present in the fish as a result of their exposure to higher levels of arsenic in the water and sediments. The sampling area chosen was the Baker Creek outlet in Yellowknife Bay (location

9, Figure 5.1a). No fish are observed in Baker Creek itself after the mine discharge period begins each summer, although the lack of fish in Baker Creek is thought to be due to elevated levels of ammonia rather than metals[20]. Four different fish species were caught: whitefish (*Coregonus clupeaformis*), sucker (*Catostomus commersoni*), walleye (*Stizostedion vitreum*) and pike (*Esox lucius*). Fish muscle was chosen as the part of the fish to be analyzed because of its significance to consumers.

5.3.2.1. Total arsenic in fish

Some physical characteristics, the moisture content, and the arsenic content in each fish sampled are shown in Table 5.4. For most of these and other samples, acid digestions were carried out once, and analyzed in duplicate by using ICP-MS. Relative standard deviations obtained from duplicate analyses were less than 2% in all cases. When sucker 1, pike 2 and whitefish 3 were digested in duplicate, the average relative standard deviation between digestions was less than 5%. Standard deviations for other samples may be estimated to be 5% of the total arsenic concentration.

Total arsenic concentrations have previously been found to be much higher in marine fish than in freshwater fish, and the present results confirm this observation. The highest concentration of arsenic in this study is 3.1 ppm dry weight in a whitefish, whereas arsenic concentrations in marine fish range from 3.5 ppm dry weight for mackerel to 196 ppm dry weight for plaice collected from uncontaminated areas[16]. Converting these arsenic concentrations to fresh weight concentrations (using [As]/R where R is fresh weight/dry weight in Table 5.4) gives concentrations ranging from 0.07 ppm for whitefish 3 to 0.72 for whitefish 1.

Table 5.4. Total arsenic concentrations in fish from location 9.

Fish	Sex	Weight (g)	Moisture content in % (R)[a]	Arsenic concentration (ppm dry weight) (SD)[b]
Whitefish 1	Male	846	77 (4.33)	3.1
Whitefish 2	Female	586	76 (4.24)	0.84
Whitefish 3	Female	1453	74 (3.88)	0.28 (0.02)
Whitefish 3 roe	---	137		0.25
Sucker 1	Male	656	78 (4.65)	1.24 (0.01)
Sucker 2	Male	457	81 (5.35)	0.98
Walleye 1	Male	386	79 (4.75)	0.46
Walleye 2	Male	507	77 (4.37)	0.85
Pike 1	Male	628	79 (4.72)	1.30
Pike 2	Male	988	80 (5.08)	1.40 (0.09)

[a] Moisture content was calculated by using (R-1)/R*100%; R = fresh weight/ dry weight.
[b] SD = Standard deviation, based on duplicate acid digestions.

These numbers can be compared with reported arsenic concentrations in freshwater fish sampled from pristine locations, ranging from 0.025 ppm fresh weight for pike to 0.132 ppm fresh weight for white sucker[21,22]. The concentrations of the fish sampled in this study are lower than those found in rainbow trout and freshwater smelt purchased from a Japanese market (1.46 and 1.08 ppm fresh weight)[23]. Most likely the levels of arsenic in Yellowknife fish are not elevated compared with fish analyzed in previous studies[21,22,23], but more samples should be analyzed to confirm this. The fish analyzed in this study all contain levels below a maximum permissible concentration of arsenic in fish for human consumption, set by the Australian National Health and Medical Research Council, of 1.14 mg/kg (ppm) fresh weight[21].

The sample size from this study is too small to draw any correlations between physical characteristics and arsenic concentration. However, for sucker, walleye and pike, the arsenic

concentration appears to increase with increasing fish size, a trend that has been documented previously[24]. This does not appear to be the case for whitefish. This trend may not be observed for every species of fish, and there may be differences based on sex.

The concentration of arsenic in the roe is approximately the same as the fish from which it was taken. The roe was very fatty and difficult to digest, and care had to be taken to keep the rate of heating slow to prevent charring. In yellowtail flounder *Pleuronectes ferruginea*, the arsenic concentrations in spawned eggs were significantly lower than those found in developing gonads and fish muscle[25], an observation that contrasts with the results found in this study (i.e., that the concentrations are the same). Clearly, more studies are necessary to determine if any trends are observable for arsenic concentrations in gonads and muscle for freshwater fish.

5.3.2.2. Arsenic speciation in fish

Enzymatic digestion has been used previously for fish and shellfish samples. Branch *et al.*[16] used trypsin, a protease, to disrupt the lipid-protein membrane in fish samples and release the cell contents. They found that trypsin digestion gave greater recoveries for arsenic from a certified reference material, DORM-1 (dogfish muscle) and whiting; lower recoveries for plaice, mackerel and a lemon sole specimen; and similar results for cod, when compared with methanol/chloroform (2:1) extraction. Forsyth *et al.*[17,18] used crude protease and lipase to digest samples for tin and lead speciation and observed reduced matrix effects during analysis, effective release of organotin analytes from marine food matrices, and adequate recovery of alkyllead standards. Oyster tissue and dogfish muscle CRMs were digested with a combination of protease and lipase to yield quantitative recovery of selenium[26].

It was predicted in this work that if recoveries were similar for enzymatic digestion, using a non-specific protease, compared with acid digestion to determine total arsenic in a CRM, then

protease digestion might be a suitable method for releasing arsenic from a protein-rich sample matrix, as well as other elements such as antimony. The results obtained when total arsenic was determined in oyster tissue CRM (NIST 1566) in protease digestions, acid digestions, and aqueous (buffer) extracts are shown in Table 5.5. About 70% of arsenic is recovered from aqueous (buffer) extraction alone, whereas 100% of arsenic is released from the matrix using protease digestion. Higher levels of antimony are present after using the protease digestion, although all values obtained are significantly higher than the non-certified value (0.01 ppm). The reasons for this are unclear, and more studies should be carried out to obtain a more reliable value for the acid digestion procedure to use for comparison. Protease digestion was subsequently used for fish, shellfish and snail samples in an attempt to maximize extraction of other elements, as well as arsenic.

Table 5.5. Comparison of arsenic concentrations (SD)[a] by using protease and acid digestion methods for oyster tissue (NIST 1566). The certified concentration of arsenic is 14.0 ± 1.2 ppm, and the non-certified value for Sb is 0.01 ppm.

Digestion procedure	Concentration of As (ppm)	Concentration of Sb (ppm)
Acid digestion	14.0 (0.2)	0.3 (0.2)[b]
Protease digestion, pH 7.2	13.8 (0.2)	0.22 (0.08)
Buffer extraction, pH 7.2	11.4 (0.3)	0.17 (0.07)

[a] SD = standard deviation, obtained from 3 replicate extractions.
[b] SD for this sample was obtained from 2 replicate digestions.

Table 5.6. Concentrations of arsenic species in fish (ppm dry weight) (SD)[a] from location 9. "Trace" amounts are greater than or at the limit of detection (LOD) but less than 3 × LOD. Sum of As species include trace amounts (given a value of LOD).

Fish	Method[b]	As(V)	MMA	DMA	AB	Sugar XI	Unknown	Sum of As species	% EE/DE[c]
Whitefish 1	PD	<0.01	trace	<0.01	0.28 (0.03)	<0.01	0.4 X[d]	0.69	22
Whitefish 2	PD	<0.01	<0.01	0.04 (0.03)	0.28 (0.13)	<0.01	0.11 X	0.43	52
Whitefish 3	PD	<0.01	<0.01	<0.01	0.052 (0.008)	<0.01	<0.01	0.052	19
Sucker 1	PD	0.05 (0.02)	0.03	0.023 (0.005)	0.25 (0.06)	0.15 (0.03)	0.19 Y[e]	0.70	56
Sucker 1	M/W	trace	<0.01	0.09 (0.06)	0.3 (0.1)	0.13 (0.03)	0.19 Y	0.72	60
Sucker 2	PD	<0.01	<0.01	0.029 (0.004)	0.24 (0.15)	0.11(0.04)	0.19 Y	0.57	58
Sucker 2	M/W	0.03	<0.01	0.19 (0.06)	0.26 (0.06)	trace	0.26 Y	0.75	77
Walleye 1	PD	<0.01	<0.01	trace	0.04 (0.02)	<0.01	0.04 X	0.09	20
Walleye 2	PD	<0.01	0.03	trace	0.18 (0.06)	<0.01	0.05 X	0.27	32
Pike 1	PD	<0.01	<0.01	0.8 (0.1)	0.17 (0.08)	<0.01	0.6 Y	1.57	120
Pike 1	M/W	<0.01	<0.01	0.33 (0.02)	0.19 (0.07)	<0.01	0.23 Y	0.75	58
Pike 2	PD	<0.01	<0.01	0.5 (0.1)	0.22 (0.13)	<0.01	0.17 Y	0.89	64
Pike 2	M/W	<0.01	<0.01	0.38 (0.04)	0.26 (0.07)	<0.01	<0.01	0.64	46

[a] SD = standard deviation, based on analyses of samples on 2 or 3 different chromatographic systems; see text for more detail.
[b] Method of sample preparation: PD = protease digestion; M/W = MeOH/Water (1:1) extraction.
[c] % EE/DE = % Extraction/digestion efficiency, calculated as (Sum of As species)/(Total arsenic from Table 5.4) * 100%.
[d] X = Unknown arsenic-containing compound that corresponds to an early-eluting peak on the cation exchange chromatographic system. [e] Y = Unknown arsenic-containing compounds that correspond to late eluting peaks on the ion-pairing chromatographic system.

The semi-quantitative amounts of arsenic species found in fish are summarized in Table 5.6. The most notable feature in these speciation results is the presence of arsenobetaine in all fish species and individuals. Arsenobetaine is the major arsenic compound found in most marine fish[16,27]. However, none (i.e., less than 5 ppb arsenobetaine) was found in a previous study of freshwater fish including bass, pike, carp, yellow perch, striped perch, pickerel (walleye) and whitefish, even though methods that had been developed specifically to identify arsenobetaine were employed[22].

Another remarkable result is the presence of arsenosugar XI in suckers. Arsenosugars have been found in only one other fish, a marine fish (silver drummer) that feeds on algae[28]. Most of the arsenic in the silver drummer was as TMAO (rather than arsenobetaine) with arsenosugars XI and XIII in the muscle, as well as arsenosugars X, XI, XII and XIII (with the structures shown in Table 1.1, Chapter 1) in the digestive tract. The source of the arsenosugars in the silver drummer fish was postulated to be its food source, brown and red algae. Suckers are bottom feeders and may also acquire arsenosugar XI through their food, which implies that some benthic organisms are able to synthesize arsenosugars. The results in the previous chapter imply that cyanobacteria or other bacteria are able to synthesize arsenosugars X and XI and hence it is not unreasonable to suggest that organisms in the sediment may be capable of doing so.

The major arsenic compound in pike is DMA. Pike is a pelagic carnivorous feeder, meaning that it derives its food from the water and not from the sediment (e.g., other fish). The stomach contents of pike sampled for this study consisted of invertebrates and small fish. Invertebrates may contain DMA, or arsenosugars. Arsenosugars may be broken down to DMA (observed in the human body[29] and in mice[30]). For example, as described in the next section, mussels and snails contain arsenosugars X and XI, and DMA, among other arsenic species,

although DMA is not the major species. It might be expected that pike would accumulate more arsenobetaine, because its diet includes small fish, which probably contain arsenobetaine. However, pike appears to preferentially accumulate DMA. High levels of DMA have been observed in other fish; mackerel contained up to 25% of its total arsenic as DMA[16].

Two types of unknown compounds are present in these samples. Unknown X in Table 5.6 was observed in very low levels in whitefish and walleye, and had an early retention time on the cation exchange system. It co-eluted with arsenate and arsenosugar XI, but attempts to identify the unknown by using other chromatographic systems were unsuccessful. Unknown Y compounds (in Table 5.6) appeared as two late eluting peaks on the ion-pairing chromatographic system as shown in Figure 5.2 (retention times approximately 700 s and 1000 s). These compounds did not co-chromatograph with arsenosugars X, XI, XII or XIII in kelp extract, as illustrated in Figure 5.2. The chromatograms in Figure 5.2 are scaled individually to facilitate comparison of peak retention times. It was surmised that the compounds should be anionic if they are late eluting on the ion-pairing system used, but they did not appear on the anion exchange system. Possibly they were irreversibly bound to the anion exchange column. Unknown compounds eluting at similar retention times (with respect to DMA) on the same chromatographic system have been observed as arsenosugar metabolites in human urine[29].

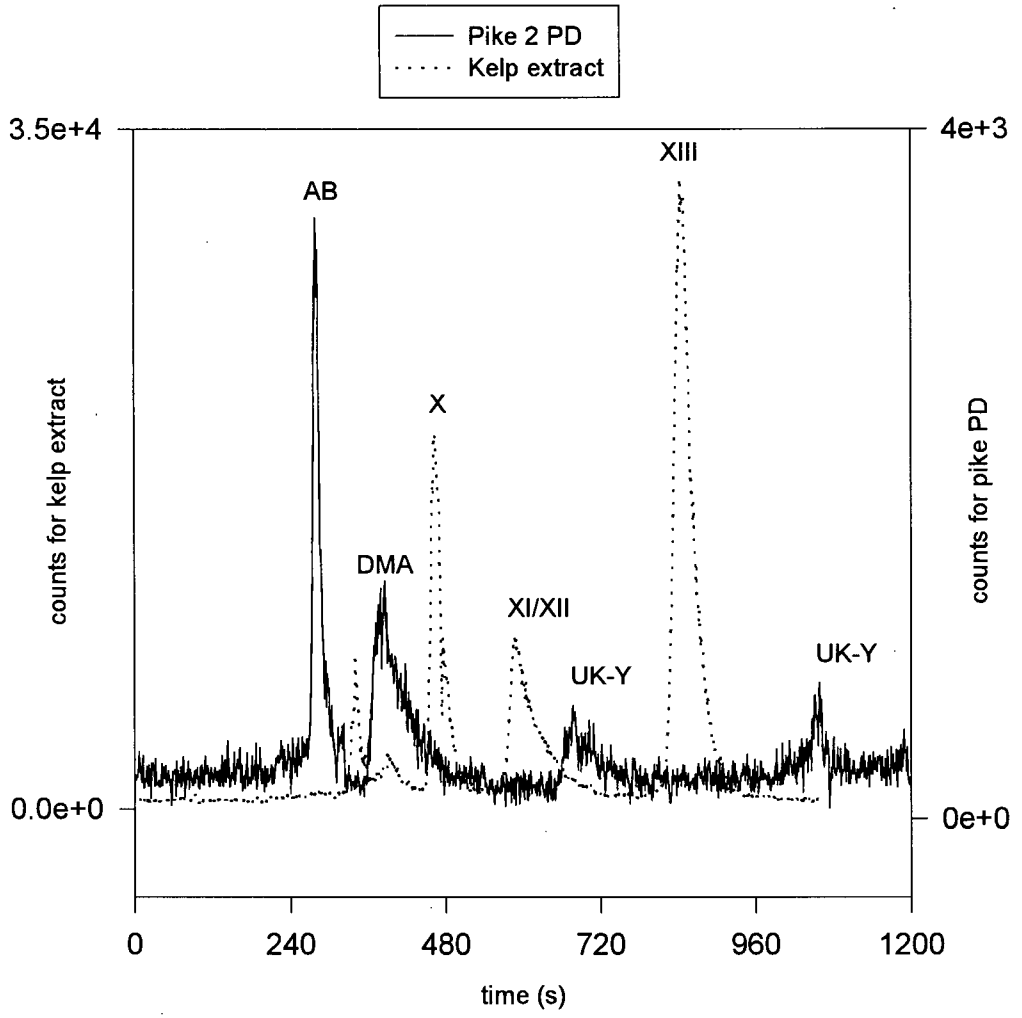

Figure 5.2. Chromatogram of protease digest (PD) of a Yellowknife fish (Pike 2), showing small amounts of two unknown compounds (UK-Y) mentioned in Table 5.6. Ion-pairing chromatography (C18 column, 10mM TEAH/4.5 mM malonic acid, pH 6.8, 0.1% MeOH; see Table 5.2) with ICP-MS detection was used. Abbreviations for arsenic species are in Table 1.1.

Branch *et al.*[16] observed that the trypsin digest of a single specimen of plaice contained more DMA than the organic extract, and they postulated that partial degradation of arsenobetaine to DMA by trypsin was taking place. To determine if the presence of DMA in pike, and the unusual presence of arsenosugar in sucker was a result of the protease digestion, rather than representative of the arsenic content in the fish, MeOH/water (1:1) extractions were performed for comparison.

No notable differences in arsenobetaine content in sucker are seen between the protease digestion results and the MeOH/water extraction (Table 5.6), although a more significant difference is observed in the amount of DMA extracted. The greatest difference appears for sucker 2, where not only is more DMA present, but less arsenosugar XI is observed when MeOH/water was used. The reasons for this are not clear. Possibly the arsenosugar in this matrix is bound to protein and the protease digestion is required to release it. Likewise, some DMA may be bound to a product of the protease digestion that precipitates or binds irreversibly to the chromatographic columns, whereas it may be effectively extracted as a free ion by using MeOH/water and can then be detected. Another possibility is that arsenosugar XI breaks down to form DMA during the MeOH/water extraction process.

For pike, no significant differences are seen in arsenobetaine amounts by using the two methods (Table 5.6). Less DMA is extracted by using MeOH/water for both fish, which is the opposite observation as that for the sucker. Less unknown compound appears after MeOH/water extraction compared with protease digestion as well. Some of these compounds were probably released by the action of the protease on the matrix, and were probably not available for extraction by MeOH/water. When protease digestion was used, the sum of arsenic species observed (Table 5.6) is greater than 100% of the total arsenic (Table 5.5) for pike 1; however, considering that standard deviations are at least 30%, the amount detected after

protease digestion was within this amount and hence probably not significantly different from 100%.

Extraction or digestion efficiencies were determined by using the relation:

$$\text{Extraction/digestion efficiency (\%)} = (\text{sum of As species})/(\text{total As}) \times 100\%.$$

These values are somewhat higher for MeOH/water extraction compared to protease digestion for sucker, and lower for pike. Reasons for the non-extraction of certain species (i.e., greater amounts of DMA extracted from sucker, and smaller amounts of DMA extracted from pike, when using MeOH/water) are suggested above and these probably account for these differences in the sum of arsenic species detected. However, this comparison study is too small to draw any conclusions about the effectiveness of the different extraction techniques, except that the extraction of arsenic species appears to be matrix dependent and varies between different species of fish.

Extraction/digestion efficiencies for most of the fish are lower than 100%, ranging from 19 to 78% (except pike 1 by PD). Protease digestion was expected to have solubilized most of the arsenic, although time constraints did not allow determinations of the total arsenic in the protease digestions to be made. However, if most of the arsenic was solubilized, a large amount of arsenic is unaccounted for in these results. This arsenic may be bound to molecules such as peptides or incompletely hydrolyzed proteins, that may precipitate or remain irreversibly bound on the chromatographic systems used here. Arsenic is known to bind to cytosolic proteins from rabbit and rat liver[31,32].

In a previous study, 13.6 to 68% of arsenic remained in the residue from the trypsin digest for marine fish[16]. Some of the arsenic in the present study may have remained in the

residue after protease digestion, especially if it was lipid bound. Lipid bound arsenic was postulated to be significant in mackerel, which is a fatty fish[16]. The lowest digestion efficiency observed in the present results was for whitefish 3, which is a fatty fish as well, as evidenced by an apparent lipid layer being present after the protease digestion procedure. Hence lipid bound arsenic may be significant for these samples, and experiments using lipase, to hydrolyze lipids, would be useful.

To summarize, this study has shown that arsenobetaine is a major extractable arsenic species in freshwater fish. Arsenosugar XI was observed for the first time in freshwater fish, and DMA was observed to be the major arsenic species in pike. Although DMA has been linked to increased tumor growth in mice exposed to carcinogens[33], the levels of DMA in these fish are too low to be of toxicological concern. Protease digestion and MeOH/water extractions gave comparable results in terms of arsenic species observed, although quantities varied, and more experiments are necessary to verify the differences. Extraction/digestion efficiencies were generally low, and again, more experiments are necessary to determine the reasons for this.

5.3.3. Freshwater shellfish

5.3.3.1. Total arsenic in shellfish

The arsenic concentrations in freshwater mussels and snails collected from Yellowknife and from Campbell River are shown in Table 5.7. The freshwater mussel collected from Yellowknife was identified as *Anadonta grandis simpsoniana* and the snails are *Stagnicola* sp. The mussel from Campbell River is *Margaritifera falcata*. The mussels were collected from areas containing low levels of arsenic. The snails were collected from the marsh area at the Baker Creek outlet (location 1) and also from Baker Creek outside the mill area of Giant Mine

(location 3). The mussel concentrations should thus reflect typical background concentrations, but the snails are most likely to have been impacted by high levels of arsenic.

Table 5.7. Moisture content and arsenic concentration in freshwater shellfish. All shellfish are from Yellowknife, except where specified; CR = Campbell River; nd = not determined.

Sample, sampling time (location)	Moisture content (%)[a] (R=fresh weight/ dry weight)	Arsenic concentration (ppm dry weight)
Anadonta sp., June (16)	nd	6.0
Anadonta sp., August (16)	90 (9.8)	7.4
Margaritifera sp. (CR)	nd	3.1
Stagnicola sp., shelled[b] (1+3)	81 (5.2)	82
Stagnicola sp., whole[b] (1+3)	69 (3.2)	83

[a] Moisture content was calculated by using (R-1)/R*100%; R=fresh weight/ dry weight.[c] nd = not determined.
[b] The same species of snails was analyzed, but processing differed; see section 5.2.2.

These concentrations obtained for arsenic in freshwater mussels (3.1-7.4 ppm dry weight) are similar to those for marine mussels, where values ranging from 8.47 to 15 ppm dry weight and 2.1 to 3.7 ppm fresh weight have been found[34,35,36,37]. The levels of arsenic in these freshwater snails (*Stagnicola* sp.) are similar to levels found in marine gastropods, which can range from 4.2 to 233 ppm dry weight[38,39,40]. A freshwater snail contained 0.186 ppm (fresh weight) arsenic[41], which is an order of magnitude lower than the comparable concentrations in *Stagnicola* sp. in the present study, of 1.6 to 2.6 ppm arsenic (fresh weight).

5.3.3.2. Speciation of arsenic in shellfish

The arsenic species that were determined following protease digestion (PD) and MeOH/water methods are shown in Table 5.8.

Table 5.8. Concentrations of arsenic species in freshwater mussels and snails (ppm dry weight) (SD)[a] from Yellowknife, except where indicated. CR = Campbell River; NP = not performed, concentration not determined because sample was not analyzed by using cation exchange chromatography. S = shelled; W = whole. "Trace" amounts are greater than or at the limit of detection (LOD) but less than 3 × LOD. Sum of As species include trace amounts (given a value of LOD).

Sample, method[b]	As (III)	As (V)	MMA	DMA	AB	Sugar X	Sugar XI	TMAO	Me_4As^+	Unknown	Sum of As species	% EE/DE[c]
Anadonta sp., June, PD	<0.05	0.3 (0.1)	<0.05	0.19	<0.04	1.1 (0.4)	0.3 (0.1)	<0.05	<0.03	<0.03	1.9	31
Anadonta sp., Aug, PD	<0.04	0.13 (0.05)	trace	0.12	<0.03	1.2 (0.3)	0.38 (0.05)	<0.04	<0.02	0.3 (0.2)	2.2	30
Anadonta sp., Aug, M/W	0.10	0.07 (0.02)	trace	1.0 (0.3)	<0.01	0.6 (0.2)	0.9 (0.2)	NP	NP	<0.01	2.7	36
Margaritifera sp. (CR), PD[d]	0.005 (0.002)	<0.001	trace	0.03 (0.02)	<0.001	0.05 (0.01)	0.15 (0.03)	NP	NP	<0.002	0.24	---
Margaritifera sp. (CR), M/W	0.12	0.04 (0.01)	<0.01	<0.01	<0.01	0.4 (0.2)	0.6 (0.1)	NP	NP	0.11 (0.03)	1.3	42
Stagnicola sp. (S), PD	6	4.8 (0.8)	0.20	0.6	0.19	1.1	<0.04	1.1	18 (10)	<0.02	32	39
Stagnicola sp. (S), M/W	4	1.8	0.5	0.4	<0.01	NP	<0.01	NP	16	<0.01	23	28
Stagnicola sp. (W), PD	2 (1)	4 (1)	<0.04	0.6	<0.04	0.5	<0.04	0.5	6.3 (3.7)	<0.02	14	17

[a] SD = standard deviation, see Table 5.6 for details

[b] Method of sample preparation; PD = protease digestion; M/W = MeOH/water (1:1) extraction

[c] % EE/DE = % Extraction/digestion efficiency, calculated as (Sum of As species)/(Total arsenic from Table 5.7) * 100%.

[d] Concentrations for this sample are on a fresh weight basis.

The most striking result in Table 5.8 is the small amount, if any, of arsenobetaine in freshwater mussels and snails. A small amount was extracted from the Campbell River mussel (3% of total arsenic extracted, fresh weight) but this was not reproducible when the extraction was repeated on a dry weight basis. A small amount (0.6%) is present in shelled snails.

The absence of arsenobetaine has been observed for an estuarine mussel *Corbicula japonica*[34] and was suggested to be related to the low salinity of its environment. Glycinebetaine is used by marine animals for osmo-regulation[42], and it has been suggested that arsenobetaine is accumulated in marine animals because of its similarity to glycinebetaine. Therefore since estuarine and freshwater animals live in lower salinity regions, their need for osmo-regulation is reduced, resulting in less arsenobetaine entering the body of a freshwater animal and/or excretion of more arsenobetaine. These processes would result in less arsenobetaine being accumulated in the body of the animal. The absence of arsenobetaine in the animals analyzed in the present study supports the view that arsenobetaine is accumulated from the environment, i.e. by marine animals. Previous studies have also indicated that the presence of arsenobetaine in marine mussels is a result of bioaccumulation (i.e., from food)[43].

The major arsenic compounds in the mussels are arsenosugars X and XI (Table 5.8). The arsenic speciation in Yellowknife mussels collected in June and August differs only in the smaller amount of As (V) and a significant amount of an unknown species detected using the ion pairing chromatographic method, in the mussel collected in August (following PD). The unknown arsenic species had a retention time on the ion-pairing system similar to that observed for one of the unknown compounds in pike protease digests (780 s, cf. 700 s in pike).

As (V) is present in all mussel samples. MeOH/water (1:1) extracted As (III) from the Yellowknife mussel collected in August and the Campbell River mussel. In these specimens, the levels of inorganic arsenic found are not of toxicological concern to humans (should the mussels

be ingested). However, inorganic arsenic levels may be significant in situations where exposure of mussels to arsenic is much higher, and if accumulation of arsenic by mussels takes place.

Some MeOH/water extractions were performed to validate the arsenic species detected after using the PD technique, as for the fish samples. The sum of arsenic species is slightly higher by using MeOH/water extraction for the Yellowknife mussel collected in August (2.7 ppm cf. 2.2 ppm for PD), but a lower sum results from MeOH/water extraction of shelled snails (23 ppm cf. 32 ppm for PD).

Like the results for suckers, MeOH/water apparently extracts (or renders detectable) more DMA from Yellowknife mussels (1 ppm) than does PD (0.12 ppm). The reasons suggested for the differences in the sucker results probably apply here as well.

More arsenosugar X is observed in protease digests, which may suggest that arsenosugar XI is being enzymatically degraded to arsenosugar X. The decomposition of arsenosugar XI to arsenosugar X in aqueous extracts during storage has been documented previously[34]. However, this observation was not made for arsenosugar XI in sucker and hence it appears that individual matrices affect these processes.

The results for the Campbell River mussel suggest that differences between fresh weight and dry weight extraction may be significant, in terms of extraction efficiency and species extracted or detected. Better extraction efficiencies have been observed for fresh weight compared to dry weight extractions for marine mussels[44]. For the two analyses of Campbell River mussels, differences in arsenic species are seen. Arsenosugars were observed in all samples, but small amounts of arsenite, MMA and DMA are observed following fresh weight PD of *Margaritifera* sp., whereas arsenite, arsenate and an unknown species are present following dry weight extraction (Table 5.8). The differences in arsenic species may be due to differences in the preparation technique (fresh weight or dry weight, and PD or MeOH/water extraction), or to

sample inhomogeneity. Sample inhomogeneity was observed by Shibata *et al.*[34] for a freeze-dried reference material and was considered to be quite significant. In this study, arsenosugar XIII was observed for a single fresh weight PD of the Campbell River mussel (results not shown), but it was not found in a duplicate fresh weight PD, nor in the dry weight MeOH/water extraction. The presence of arsenosugar XIII in the freshwater environment, had it been reproducible, would have been very significant, since up to now, only arsenosugars X and XI have been observed in the terrestrial environment as discussed in section 4.3.3.1 (Chapter 4).

In snails (*Stagnicola* sp.), the major extractable arsenic compound, in a proportion of 40-60% of arsenic extracted, is tetramethylarsonium ion. Inorganic species (As (III) and As (V)) amount to 25-40% of the total arsenic extracted, which is a major difference from marine snails. In a marine snail, *Tectus pyramis,* the major compounds were found to be arsenobetaine (35-67% of arsenic extracted) and tetramethylarsonium ion (6-26% of arsenic extracted)[38]. In other marine gastropods, arsenobetaine was also found to be the major arsenic compound, with inorganic arsenic, DMA, arsenocholine, the tetramethylarsonium ion or arsenosugar X as minor constituents[27,40,45]. Uncharacterized trimethylarsenic (62% of total arsenic) and dimethylarsenic (27% of total arsenic) species were postulated to be present in a freshwater snail[41].

The role of tetramethylarsonium ion is not clear. It has been suggested that it may serve as a precursor to or a decomposition product of arsenobetaine. However, its presence in snails, where the uptake or synthesis of arsenobetaine appears not to be significant, indicates that this is probably not the case. The tetramethylarsonium ion may arise from a metabolic pathway independent of one that would produce arsenobetaine, and is probably prevalent in freshwater gastropods. Methylation, possibly following the Challenger mechanism, might be taking place. The presence of MMA, DMA and TMAO in the snails, which would be intermediate compounds in the mechanism, supports this hypothesis.

Arsenobetaine is present in shelled snails in a small amount (0.6%), so the processes that occur in marine systems may also be taking place here, but to a smaller extent. Arsenosugar X was also detected in snails as a minor component (3.5%). The different sample preparation techniques used for the shelled snails may indicate that protease digestion releases more arsenic than MeOH/water extraction, although more studies should be done to verify this. The MeOH/water extract of snails was incompletely analyzed (by anion exchange and ion-pairing chromatography only), but the total amount of arsenic given in Table 5.8 reflects that which would be seen if cation exchange chromatography was used, within the estimated uncertainty of 30%.

Extraction/digestion efficiencies are lower than 50% for all samples analyzed. The same reasons as those given for the fish extraction efficiencies apply here: protease digestion and MeOH/water extraction may not solubilize all the arsenic, or the solubilized arsenic may not be detectable by using these chromatographic systems. When MeOH/water (1:1) was used to extract arsenic from marine mussels (*Mytulis edulis* in all cases) in other studies, only 52 to 60% of arsenic was extracted[34,36]. In another study, 47% of arsenic in *Mytulis edulis* was accounted for by HPLC-ICP-MS analysis as water soluble species, with 17% extracted in chloroform, and 32% remaining in the solid residue[35]. Arsenic bound to lipids or other types of molecules such as proteins appears to be significant in mussel matrix. Another extraction method (MeOH/water, 9:1, with rehydration of dry weight samples before extraction) has proven to yield higher extraction efficiencies (95-100%) for marine mussels, although a significant amount remained unaccounted for after chromatographic analysis[44].

Chitin is a component of snails, since the radula (similar to a tongue) and parts of the digestive system are composed of this material[46]. Protease digestion is inadequate for the solubilization of chitin and more specific enzymes such as chitinase and β-(1-3)-glucanase are

necessary[47,48]. In other studies, extraction of the marine snail *T. pyramis* with methanol was unable to dissolve 12 to 25% of total arsenic[38] which are significant amounts, but not as great as those unaccounted for in the present study.

Mollusk shells, made of calcium carbonate crystals within a protein framework known as conchiolin, were found to require rigorous extraction with 2M HCl[49] to dissolve arsenic. Hence it is expected that the mild sample preparation used in this study would not be capable of extracting arsenic from shells. This reasoning explains the observation that the extraction efficiency of whole snails is about half that of shelled snails, since the sample was probably diluted by the presence of shells from which arsenic could not be extracted. Arsenic is most likely present in snail shells, because it has been found in other mollusk shells[49].

To summarize, the speciation of extractable arsenic in freshwater shellfish, containing very little or undetectable amounts of arsenobetaine, supports the suggestion that arsenobetaine accumulation in marine animals is related to osmo-regulation. Arsenosugars X and XI are the major compounds in freshwater mussels, similar to findings in marine mussels. Tetramethylarsonium ion is the major compound in freshwater snails, although inorganic arsenic is present in significant levels as well. The high accumulation of inorganic compounds in snails could present toxicological problems for higher trophic organisms. Large amounts of arsenic remain unidentified and this could be lipid or chitin bound. More work is necessary to elucidate the nature of this arsenic.

5.3.4. Plants

Higher plants were collected from the Yellowknife area to determine the speciation of arsenic in plants. Sampling areas (refer to Figures 5.1a and 5.1b) included Kam Lake which

received accidental mining effluent discharges in the early 1970s from the Con Mine, and has also been influenced by raw sewage from Yellowknife[50] (location 12). Baker Creek, currently receiving mine effluent from the Giant Mine, was sampled in different areas: the marsh (location 1) and mill (location 3) areas, as well as an intermediate location in the creek (location 2). Niven Lake was also chosen as a sampling location, because it was a former sewage pond and was expected to contain residual elevated levels of arsenic and other metals, and because of prolific plant and algal growth. Two locations are specified, one on the east side of the lake, near the site of a former dump (location 10) and the other at the north end of the lake where the lake drains into Back Bay (location 11). Cattail, *Typha latifolia,* was common to all the sampling locations and horsetail, *Equisetum fluviatile,* was found in Kam Lake and Baker Creek. These two plants were sampled (shoots only) from different sites to determine if any location specific differences in arsenic speciation could be seen. The aquatic plants, burweed, *Sparganium augustifolium,* and pondweed, *Potomogetan richardsonii,* were sampled from the water in Yellowknife Bay near a former Giant Mine tailings pond (location 6).

5.3.4.1. Total arsenic in plants

The concentrations of total arsenic in plants are summarized in Table 5.9a. Uptake of arsenic by macrophytes was studied previously[4] for plants collected near Con Mine and some of these results are also shown for comparison in Table 5.9b.

Table 5.9a. Arsenic concentrations in plants from Yellowknife.

Sample, Time	Location	Arsenic concentration (ppm dry weight)
Emergent plants (shoots)		
Horsetail *Equisetum fluviatile*, June	12	30
Horsetail *Equisetum fluviatile*, June	1	48
Horsetail *Equisetum fluviatile*, June	3	260
Cattail *Typha latifolia*, June	2	5.0
Cattail *Typha latifolia*, June	11	0.52
Cattail *Typha latifolia*, June	12	3.8
Bidens cernua, August	10	100
Submergent plants		
Milfoil, *Myriophyllum* sp., June (whole plant)	11	78
Milfoil, *Myriophyllum* sp., June (whole plants)	10	39
Milfoil, *Myriophyllum* sp., August (whole plant)	10	17.4
Duckweed, *Lemna minor*, August (whole plants)	10	28
Burweed *Sparganium augustifolium*, August (shoots)	6	2.5
Pondweed, *Potomogetan richardsonii*, August (shoots)	6	20

Table 5.9b. Arsenic concentrations in plants from Yellowknife, previous study[4].

Sample, Time	Location	Arsenic concentration (ppm dry weight)
Emergent plants (shoots)		
Typha latifolia	near Con Mine	17.2; range <1.0-38
Equisetum fluviatile	near Con Mine	34; range 5.5-91
Triglochin palustre, arrow grass	near Con Mine	40
Submergent plants		
Potomogetan pectinatus (whole plants)	near Con Mine	1219; range 190-4990
Myriophyllum exalbescens (whole plants)	near Con Mine	143; range 30-255
Sparganium sp. (shoots)	near Con Mine	28

Typha sp. contains the lowest levels of total arsenic, and this is especially obvious when the levels are compared with levels in other plants collected from the same sample location. For example, compare *Typha* sp. (3.8 ppm) with *Equisetum* sp. (30 ppm) at location 12 (Kam Lake); and *Typha* sp. (0.52 ppm) with *Myriophyllum* sp. (78 ppm) at location 11 (Niven Lake). In a previous study[4], the average concentration of arsenic in *Typha* sp. shoots was found to be 17.2 ppm dry weight with a range of <1.0 to 38 ppm dry weight, which is higher than the levels found in this study (0.52 to 5.0 ppm dry weight). However, the small sample size in this study precludes any direct comparison, except that *Typha* sp. shoots contained the lowest levels of total arsenic in the previous study as well as in the present one. Dushenko *et al.* postulated that the lower levels of arsenic in *Typha* sp., as well as its abundance at all sampling sites (observed in

the present study as well) indicates a greater tolerance to arsenic contamination by using mechanisms for the exclusion of arsenic[4].

Equisetum sp. contains high levels of arsenic at all the locations from which it was sampled (Table 5.9). Most likely the levels in these samples of *Equisetum* sp. are elevated (30 to 260 ppm dry weight) compared with background levels (5.5 ppm dry weight) in *Equisetum* sp. (sampled from Grace Lake, a location relatively unaffected by mine waste discharge) reported in the previous study[4]. The other emergent plant in the present study is *Bidens cernua*, a flowering plant that grew prolifically at the edges of Niven Lake in August, and the arsenic concentration in the shoots is 100 ppm dry weight. This concentration is higher than the average concentrations of arsenic found for all of the emergent plants sampled in the previous study (Table 5.9)[4].

The submergent plants *Sparganium* sp., *Potomogetan richardsonii*, *Myriophyllum* sp. and *Lemna minor* contain arsenic in concentrations ranging from 2.5 to 78 ppm dry weight. The previous study showed that submergent plants contained more arsenic than emergent plants[4] (Table 5.9) but the sample size and number of sampling locations in the present study was too small to make a direct comparison.

The concentration of arsenic in *Myriophyllum* sp. appears to decrease throughout the summer at the same location (39 ppm dry weight in June compared to 17.4 ppm dry weight in August), although this observation needs to be statistically verified. Grasses accumulate arsenic from soil at a faster rate when physiological growth of the plant is significant and accumulation of arsenic slows down when plant growth slows[51]. As described in Chapter 4, higher levels of arsenic were observed for fleabane and sedge at the end of the growing season. The results for *Myriophyllum* sp. indicate that an opposite trend is taking place, (i.e., arsenic concentration is lower at the end of the growing season) although the reasons for this are unclear. Possibly the rate of arsenic depuration exceeds that of uptake while plant growth is taking place.

5.3.4.2. Arsenic speciation in plants

The arsenic species found in the plants collected in Yellowknife are listed in Table 5.10. The most noteworthy feature is the predominance of inorganic arsenic (As (III) and As (V)) as the extractable species, amounting to greater than 90% for all plants except for *Lemna minor*, which contains 80% of extractable arsenic as inorganic arsenic. For *Equisetum* sp. and *Sparganium* sp. inorganic arsenic forms were the only arsenic species extracted.

Table 5.10. Concentrations of arsenic species in Yellowknife plants (ppm dry weight) (SD)[a]. "Trace" amounts are greater than or at the limit of detection (LOD) but less than 3 × LOD. Sum of As species include trace amounts (given a value of LOD).

Sample, time (location)	As (III)	As (V)	MMA	DMA	Sugar X	Sugar XI	Me_4As^+	Sum of As species	% EE[b]
Typha latifolia, June (12)	0.6 (0.2)	2 (1)	trace	trace	<0.02	<0.02	<0.01	2.6	70
Equisetum fluviatile, June (12)	7	7	<0.02	<0.02	<0.02	<0.02	<0.01	14	47
Equisetum sp., June (1)	25	36	<0.02	<0.02	<0.02	<0.02	<0.01	61	130
Equisetum sp., June (3)	65 (4)	40 (20)	<0.1	<0.1	<0.1	<0.1	<0.05	105	40
Typha sp., June (2)	0.8	2.8	trace	trace	<0.02	<0.02	<0.01	3.6	72
Sparganium augustifolium, Aug. (6)	0.28 (0.04)	0.64 (0.08)	<0.01	<0.01	<0.01	<0.01	<0.007	0.92	37
Potomogetan richardsonii, Aug. (6)	0.3 (0.1)	2.0 (0.5)	<0.01	0.04	0.15	<0.01	0.05 (0.02)	2.5	13
Typha sp., June (11)	0.18 (0.05)	0.4 (0.1)	trace	0.04	<0.01	<0.01	trace	0.64	120
Myriophyllum sp., June (11)	25 (2)	14 (1)	0.40	0.30	<0.02	<0.02	trace	40	51
Myriophyllum sp., June (10)	13 (2)	2.5 (0.3)	0.8	<0.01	<0.01	<0.01	0.033 (0.007)	16	42
Myriophyllum sp., Aug. (10)	2.00 (0.06)	5.11 (0.06)	0.22	0.20	trace	0.07	trace	7.6	44
Lemna minor, Aug. (10)	1.1 (0.2)	4 (1)	0.26 (0.06)	0.2 (0.1)	0.5 (0.2)	0.18 (0.04)	0.11 (0.03)	6.4	23
Bidens cernua, Aug. (10)	1.3 (0.6)	14.3 (0.5)	0.4	0.15	<0.01	<0.01	0.08 (0.02)	16	16

[a] SD = standard deviation, obtained from analyses of 2 chromatographic systems (anion and cation exchange).
[b] % EE = % Extraction efficiency, calculated as (Sum of As species)/(Total arsenic from Table 5.9) * 100%.

The proportions of arsenic species, especially As (III) and As (V), are consistent between plants of the same species, as seen for *Typha* sp., *Equisetum* sp. and *Myriophyllum* sp. collected in June (see Table 5.11). The major arsenic species extracted from *Typha* sp. is As (V), for *Myriophyllum* sp. (June) it is As (III), and equal amounts of As (V) and As (III) were extracted from *Equisetum* sp. As mentioned in Chapter 4, As (III) is less toxic to periwinkle than As (V)[52], and may be less toxic to higher plants from Meager Creek. Its presence in plants from Yellowknife may indicate that reduction of arsenate is taking place by the plant as a detoxification mechanism. The major species of arsenic in all water samples was arsenate (Table 5.3). However, the major extractable species from Baker Creek sediments was arsenite (see Table 5.3) which may be taken up unchanged by plants.

Table 5.11. Percent arsenic species of total arsenic extracted from plants (SD)[a].

Plant	As (III)	As (V)	Methyl (DMA + MMA)	Sugars (X + XI)	Me_4As^+
Typha latifolia[b]	24 (3)	71 (8)	4.8 (4.4)	0	0
Equisetum fluviatile[b]	50 (10)	50 (10)	0	0	0
Sparganium augustifolium	30	70	0	0	0
Potomogetan richardsonii	11.8	78.7	1.6	5.9	2.0
Myriophyllum sp., June[d]	71 (12)	25 (14)	3.4 (2.2)	0	0.1 (0.1)
Myriophyllum sp., August	26	67.3	5.5	0.9	0
Lemna minor	17.3	63	7.2	10.7	1.7
Bidens cernua	8	88	3.4	0	0.5
Average for submergents collected in August[c]	21 (8)	70 (7)	3.6 (3.3)	4.4 (5.0)	0.9 (1.1)

[a] SD = standard deviation, calculated for plants of the same species sampled from different areas and with different absolute concentrations of arsenic. Where no SD is indicated, only one sample was collected, so n=1.
[b] For SD, n = 3. [c] For SD, n = 2..
[d] Submergents collected in August include: *Sparganium* sp., *Potomogetan* sp., *Myriophyllum* sp. and *Lemna minor*; for SD, n = 4.

The difference between arsenate and arsenite distributions for *Myriophyllum* sp. between June and August is interesting to note. The sample collected in August contains the same proportion of arsenate as the June sample contains of arsenite (approximately 70%). This may indicate that oxidation is taking place throughout the summer, or it may indicate a slowing of the microbial activity (in the plant's environment) or plant activity to produce As (III), which may be related to decreased plant growth in August.

In general, submergent plants collected in August contain predominantly As (V) (see Table 5.11) and therefore the trend observed for *Myriophyllum* sp. may extend to other submergent plants. Dushenko *et al.*[4], found that submergent plants accumulated more arsenic than emergent plants (Table 5.9). They postulated that leaf uptake from the surrounding water column was significant, and that no arsenic exclusion mechanism exists for submergents. The presence of arsenate as the major extractable arsenic compound in submergents after a summer of exposure may mirror the surrounding environment, and indeed, the major arsenic species in Yellowknife waters is arsenate.

Minor amounts (less than 5%) of methylated arsenic (MMA and DMA) were extracted from all samples of *Typha* sp. (Table 5.10). The absence of methylated arsenic from extracts of other plants collected from the same location as *Typha* sp. (such as *Equisetum* sp. at Kam Lake) may indicate some specificity on the part of *Typha* sp. to accumulate these species or to methylate arsenic from its surroundings. Minor amounts of methyl arsenic are also observed in *Potomogetan* sp., *Myriophyllum* sp. collected in both June and August, and in *Bidens cernua*. A larger amount (7.2%) of methyl arsenic is present in *Lemna minor*. In all these species except *Typha* sp. and *Equisetum* sp., Me_4As^+ was observed at some point (e.g., in a *Myriophyllum* sp. specimen in June, but not in August) Arsenosugars are also present in *Potomogetan* sp.,

Myriophyllum sp. and *Lemna minor*. Evidence exists for both the synthesis and uptake of methyl arsenic (as MMA and DMA) from its environment, as discussed in Chapter 4[52,53,54]. Me_4As^+ was found in a vascular plant from Meager Creek and its presence in these samples, albeit at very low levels, indicates a wider distribution than might otherwise be expected, considering the rarity of its presence in sediments or waters, as discussed in Chapter 4[55,56]. Halophytes apparently contained TMAO, Me_4As^+, arsenocholine and arsenobetaine[57] and the presence of these compounds was attributed to the presence of similar compounds in the surrounding water.

The discovery of arsenosugars in some of these plants (Table 5.10), amounting to as much as 11% of extracted arsenic (absolute concentration 0.68 ppm dry weight) in *Lemna minor*, represents the first finding of arsenosugars in higher terrestrial plants. Halophytes (salt marsh plants) were proposed to contain an arsenosugar but no identification (i.e., comparison with standard arsenosugar compounds) was carried out[57]. Interestingly, only arsenosugars X and XI are observed in the present study, which has also been the case so far for Meager Creek samples, and Yellowknife fish and shellfish. The arsenosugars are found only in submergent plants. *Myriophyllum* sp. and *Lemna minor* were growing in physical contact with algae, which may be a possible source of arsenosugars (mentioned in the next section).

Submergent plants, in general, contain a larger proportion of organoarsenic species (on average, 9% of extracted arsenic, and up to 20% of extracted arsenic for *Lemna minor*; see Table 5.11) compared with emergent plants (up to 4.8 %). The reasons for this trend are unclear. Due to their propensity for uptake from their environment, as discussed by Dushenko *et al.*[4], submergent plants may be acquiring organoarsenic from their environment, but the possibility of synthesis by the plants cannot be discounted.

Extraction efficiencies are less than 100% in most cases, although two cases of efficiencies greater than 100% were obtained. In both these cases (*Equisetum* sp. from Baker

Creek marsh and *Typha* sp. from Niven Lake) the standard deviations in the speciation results are about 30% and the error in these numbers may account for these unusual results. The lowest extraction efficiencies are observed for *Potomogetan richardsonii*, *Bidens cernua* and *Lemna minor*. Unextracted arsenic could be bound to lipids, or to cell wall components, including insoluble cellulose, calcium or magnesium pectates, or lignin.

5.3.5. Algae and microbial mats

Algae and microbial mats were sampled from a few locations in Yellowknife. Microbial mats were sampled from standing water next to, but not a part of, Baker Creek (location 4, Figure 5.1a), in both June and August. In June only the microbial mat was present in the standing water, whereas in August other plants (*Typha* sp. and *Equisetum* sp.) were present, but very little of the microbial mat was present. A microbial mat was also sampled from a small pond near a former Giant Mine tailings pond (location 5). Location 10 in Niven Lake was sampled for two different types of algae, which were painstakingly separated from each other and other organisms, and washed thoroughly. The exact composition of the microbial mats from locations 4 and 5 was unknown and most likely included a combination of bacteria (including cyanobacteria) and freshwater algae (including diatoms). The organisms possibly belong to genera such as *Microcystis*, *Oscillatoria*, *Spirulina* or *Aphanizomenon*, which have been found in other freshwater microbial mats[58]. Two species of green algae were sampled from Niven Lake: *Enteromorpha intestinalis*, and Algae 1, of unknown identification, but possibly *Cladorpha* sp.

5.3.5.1. Total arsenic in algae

Arsenic concentrations in the microbial mats are high, ranging from 390 to 2500 ppm dry weight (Table 5.12). These levels are higher than those found in Meager Creek microbial mats (maximum of 278 ppm dry weight).

Table 5.12. Arsenic concentrations in algae from Yellowknife.

Sample, Time	Location	Arsenic concentration (ppm dry weight)
Microbial mat, June	4	2500
Microbial mat, August	4	1100
Microbial mat, June	5	390
Enteromorpha intestinalis, August	10	6.6
Algae 1, August	10	30

A bioconcentration factor (BCF)[59] can be calculated for these algae, by using fresh weight concentrations and the following relations, where fw is fresh weight and dw is dry weight:

$[As_{fw}] = [As_{dw}]/R$; where

R = fresh weight mass of sample/dry weight mass of sample

$BCF = [As_{fw}]/[As_{water}]$

Factors of 0.86 and 0.38 are calculated for the microbial mats sampled from location 4 in June and August, respectively (R = 6). If the factors are less than 1, then no bioconcentration is taking place[4] and this is likely the case for these samples.

Concentration factors can be calculated for the samples from location 10 as well; they are 9 (R = 10) for *Enteromorpha intestinalis*, and 68 (R = 6.5) for Algae 1. The water concentrations used for BCF calculations for the August samples are those determined in the June samples in Table 5.3 because concentrations of arsenic are not expected to change by more than about 50% in a location, from June to August[60]. The BCFs obtained indicate that *Enteromorpha intestinalis* and Algae 1 bioconcentrate and bioaccumulate arsenic. BCFs for marine and freshwater algae have been reported to be as high as 200 to 3000[61].

5.3.5.2. Arsenic speciation in algae

The arsenic species in the microbial mats sampled from location 4 in June and August show very few differences (See Table 5.13). The major species in these samples are inorganic arsenic, and the proportion of arsenite appears to increase slightly from June to August, where [arsenite]/[arsenate] was 0.47 in June and 0.70 in August. On the other hand, the proportions of DMA and arsenosugar X, although small in June, decrease to trace levels in August. The reasons for this are unclear and the differences are probably too small to draw conclusions.

Table 5.13. Concentrations of arsenic species in Yellowknife algae (ppm) (SD)[a], organized by location (location numbers from Figure 5.1). "Trace" amounts are greater than or at the limit of detection (LOD) but less than 3 × LOD. Sum of As species include trace amounts (given a value of LOD).

Sample, time	As (III)	As (V)	MMA	DMA	Sugar X	Sugar XI	Sum of As species	% EE[b]
Location 4								
Microbial mat, June	8 (3)	17 (10)	<0.02	0.30	0.9	<0.02	26	1.0
Microbial mat, Aug.	16.0 (0.6)	23 (3)	<0.02	trace	trace	<0.02	39	3.5
Location 5								
Microbial mat, June	<0.02	30 (10)	0.22	0.61 (0.04)	3.1 (0.9)	0.4	34	8.7
Location 10								
Enteromorpha intestinalis, Aug.	<0.01	2.4 (0.9)	<0.01	<0.01	0.20 (0.06)	0.07	2.7	41
Algae 1, Aug.	<0.01	2.1 (0.3)	0.3 (0.2)	0.21 (0.07)	2.5 (0.5)	1.3 (0.3)	6.4	21

[a] SD = standard deviation, obtained from analyses of 2 chromatographic systems (anion and cation exchange).
[b] % EE = % Extraction efficiency was calculated as (Sum of As species)/(Total arsenic from Table 5.12) * 100%.

The microbial mat sampled from the pond at location 5 contains arsenate as its major species, and 12.6% of the arsenic extracted occurs as methylated arsenic and arsenosugars (Table 5.13). The absence of arsenite in this sample compared with samples from location 4 probably indicates a lack of organisms that reduce arsenate to arsenite. Arsenosugars X and XI (10% of arsenic extracted) are present in this sample, whereas only arsenosugar X (3.4% of arsenic extracted for the June sample) is present in samples from location 4. The finding of arsenosugars in these samples and in microbial mats from Meager Creek in levels ranging from 0.8 to 44% of water soluble arsenic (Chapter 4) indicates that their synthesis is not restricted to thermophilic organisms. That is, among the non-thermophilic organisms that make up the microbial mats from Yellowknife, some appear to be capable of synthesizing arsenosugars X and XI.

The algae sampled from location 10 belong to the phyllum of Chlorophyta. Because they are macroscopic it was possible to thoroughly wash them without disrupting or losing the organisms. From Table 5.13, it can be seen that arsenate is the major water soluble arsenic species (90% for *Enteromorpha intestinalis* and 33% for algae 1) in these algae. Arsenate is the only inorganic species present, indicating that no reduction to arsenite takes place by, or in the environment of these algae. Appreciable amounts of arsenosugars are present, especially in Algae 1 (sum of arsenosugars X and XI was 59% of extracted arsenic). This represents the highest proportion of arsenosugars found in freshwater organisms from both the Yellowknife and the Meager Creek environment. The terrestrial cyanobacterium *Nostoc* sp. contains the highest proportion of arsenosugars in any terrestrial organism (arsenosugar X was the major arsenic compound extracted)[6]. The presence of only arsenosugars X and XI (and not arsenosugars XII and XIII) in the present study are in agreement with the findings from Meager Creek.

These freshwater algae are probably synthesizing arsenosugars, although the possibility of uptake from microscopic organisms living in close physical contact with the algae and possibly

excreting arsenosugars cannot be discounted based on these studies. A unicellular marine diatom is apparently capable of synthesizing arsenosugars[62], and freshwater analogues of these microscopic organisms may exist.

Low extraction efficiencies are observed for algae and especially for microbial mats (Table 5.13). Low extraction efficiencies were also found for Meager Creek microbial mats (3-33%) and algae (1.1-7%). Only 1% of total arsenic was extracted from the microbial mat from location 4 that contained the highest levels of arsenic of all algae samples (2500 ppm dry weight). The low extraction efficiencies from microbial mats might be a result of arsenate coprecipitatation with iron and manganese oxides onto or in the microbial mats, making the arsenic insoluble in MeOH/water (1:1). As discussed in Chapter 4, evidence exists to suggest that biomineralization of arsenic in microbial mats could take place[63,64,65].

The highest extraction efficiencies are observed for *Enteromorpha intestinalis* (49%) and Algae 1 (20%). Lipid soluble arsenic, which would have been extracted to only a very small extent by using MeOH/water (1:1) may make up some of the unextracted arsenic in these algae samples. Arsenic may also be bound to cell wall components such as those mentioned in 5.3.4.2.

5.3.6. Mosses

5.3.6.1 Total arsenic in mosses

The total arsenic concentrations found in mosses collected from Yellowknife are summarized in Table 5.14. Two samples of *Drepanocladus* sp. were found growing underwater (locations 1 and 4) and another sample of *Drepanocladus* sp. as well as the other mosses were terrestrial (see Table 5.14 for location numbers). The identity of mosses 1 and 2 are not certain

but they are most likely the same species, *Fumaria hygrometrica*. Although *Drepanocladus* sp. was identified by genus only, all specimens of this moss are of the same unknown species[66].

Table 5.14. Arsenic concentrations in mosses from Yellowknife.

Sample, Time	Location	Arsenic concentration (ppm dry weight)
Drepanocladus sp., June	1	1220
Drepanocladus sp., August	1	490
Drepanocladus sp., August	4	880
Moss 1[a], June	5	1130
Moss 2[a], August	7	1900
Pohlia sp., August	15	1310
Drepanocladus sp., August	15	770

[a] Moss 1 and Moss 2 are suggested to be the same species, namely *Fumaria hygrometrica*.

The terrestrial mosses contain, in general, the highest levels of arsenic, and the sample from location 7 (Giant Mine tailings pond) contains the highest amount (1900 ppm dry weight, see Table 5.14). For terrestrial mosses, washing was probably not adequate to remove the soil in which they were growing, which may explain the very high levels of arsenic found in them. It was possible to sample aquatic *Drepanocladus* sp. so that sediment was not included and thus the levels in these moss samples more accurately represent the actual concentrations of arsenic in the moss. *Drepanocladus* sp. from location 1 appears to decrease in arsenic concentration from June to August, although the sampling locations were not exactly the same and replicate samples were not taken to verify this observation. Meager Creek samples of *Fumaria hygrometrica* also showed seasonal differences, where the concentration of arsenic in a November sample was

lower than that of a July sample. As with the Meager Creek samples, the seasonal differences in these samples may reflect differences in arsenic uptake rates and accumulation.

5.3.6.2. Arsenic speciation in mosses

The arsenic species found in mosses are summarized in Table 5.15. Inorganic species of arsenic are the major and only species extracted from all moss samples, except from *Drepanocladus* sp. From all three locations, *Drepanocladus* sp. (1, 4 and 15) contain more extractable As (V) than As (III), whereas mosses 1 and 2, presumed to be the same species, show no trend.

The most remarkable feature of these results is the occurrence of Me_4As^+ in *Drepanocladus* sp. sampled from location 1 in June and August, but the lack of it in the same species of moss sampled from the two different locations 4 and 15. The major arsenic species present in snails, which were living in the moss, is Me_4As^+. In a similar way, arsenosugars are present in *Fumaria* sp. from Meager Creek (Chapter 4), but not in the same or similar moss species from Yellowknife locations. These results may indicate that the arsenic species extracted from moss reflect the surrounding environment. This supports the hypothesis that for mosses, the source of arsenic species is uptake from the environment, rather than *de novo* synthesis. The arsenic species found in mosses from Meager Creek and Yellowknife, compared with the arsenic species found in some organisms (snails in Yellowknife, microbial mats in Meager Creek) that live in close physical contact with the mosses, are shown in Figure 5.3.

Table 5.15. Concentrations of arsenic species in Yellowknife mosses (ppm) (SD)[a]. "Trace" amounts are greater than or at the limit of detection (LOD) but less than 3 × LOD. Sum of As species include trace amounts (given a value of LOD).

Sample, time	Location	As (III)	As (V)	MMA	DMA	Me_4As^+	Sum of As species	% EE[b]
Drepanocladus sp., June	1	50 (10)	100 (50)	<0.2	<0.2	4.2 (0.1)	154	13
Drepanocladus sp., Aug.	1	21 (1)	49 (7)	<0.02	<0.02	2 (1)	72	15
Drepanocladus sp., Aug	2	10 (3)	60 (10)	trace	<0.06	<0.01	70	8.0
Moss 1, June	5	24.5 (0.6)	4 (1)	<0.02	<0.02	<0.01	29	2.6
Moss 2, Aug.	7	4.86 (0.08)	27 (5)	<0.06	<0.06	<0.03	32	1.7
Pohlia sp., Aug	15	55 (6)	17 (5)	<0.06	<0.06	<0.03	72	5.5
Drepanocladus sp., Aug.	15	10.8 (0.3)	11.4 (0.3)	<0.06	0.96 (0.09)	<0.03	23	3.0

[a] SD = standard deviation, obtained from analyses on 2 chromatographic systems (anion and cation exchange).
[b] % EE = % Extraction efficiency was calculated as (Sum of As species)/(Total arsenic from Table 5.14) * 100%.

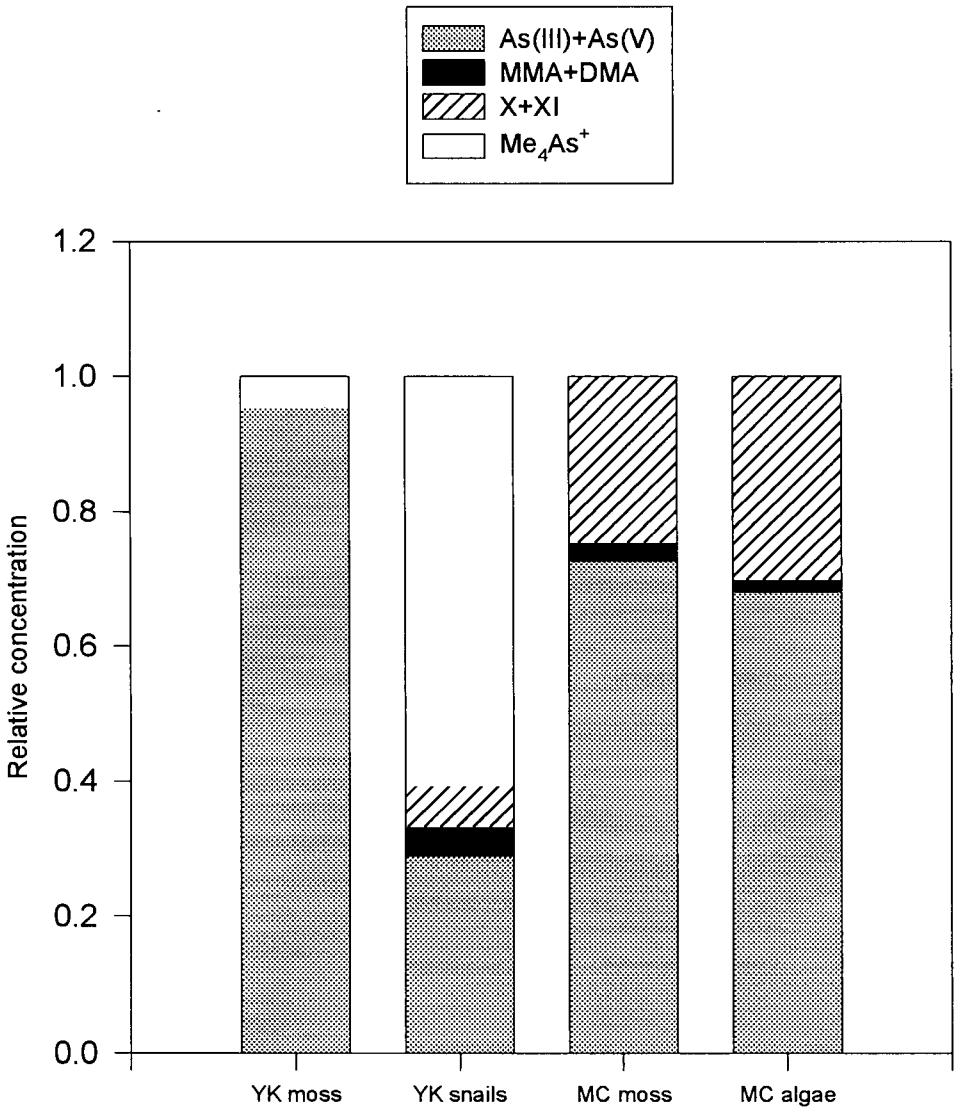

Figure 5.3. Relative amounts of arsenic species in moss and associated organisms from Yellowknife (YK) and Meager Creek (MC), showing similarity in speciation between the moss and the organism (snails or algae) living in close physical contact with it. Abbreviations for arsenic species are found in Table 1.1.

Extraction efficiencies for moss samples are low, ranging from 1.7% for moss 2 from location 7 to 15% for *Drepanocladus* sp. sampled in August from location 1. Low extraction efficiencies were observed for mosses from Meager Creek as well. Limited extraction might be expected from the terrestrial mosses from which soil could not be completely removed, since arsenic in soil occurs in mostly non-water soluble forms, such as minerals, or adsorbed onto iron and manganese oxides. Arsenic appears to be bound to other plant components of the mosses for the non-terrestrial species, which may include lipids and cell wall components (see section 5.3.4.2.)

5.3.7. Lichens and mushrooms

Pixie cup (PC) lichens (*Cladonia* sp., not positively identified as the same species) were found at different locations: PC1 near Kam Lake (location 12), PC2 near Meg Lake (location 13), and PC3 in the Con tailings pond area (location 15). Other lichens were collected as well near Meg Lake (location 13) and one species was found on rocks close to the water line of the Giant Mine tailings pond (lichen 4, from location 7). Identifications are *Cladina* sp. for lichen 1 and *Cladonia* sp. for lichens 2 and 3 (lichens 2 and 3 were not the same species). The identity of lichen 4 remains unknown but it was not *Cladonia* sp. Lichens 1, 2 and 3 were growing together.

Mushrooms *Paxillus involutus*, *Psathyrella candolleana* and *Leccinum scabrum* were found near Meg Lake (location 13). Meg Lake is the first lake in the Meg-Keg-Peg drainage system, into which Con Mine effluent flows, and therefore it is the first lake to receive Con Mine effluent. The shaggy mane mushroom, *Coprinus comatus*, was found growing in the dry and sandy former Giant Mine tailings pond (location 8). Puffballs *Lycoperdon pyriforme* were sampled at two different locations. Mature specimens were found at the edges of the currently

used Giant Mine tailings pond (location 7), and younger specimens were found at the edges of the Con Mine tailings pond (location 15).

5.3.7.1. Total arsenic in lichens and mushrooms

Total arsenic concentrations in lichens and mushrooms from Yellowknife are summarized in Table 5.16. Levels of arsenic in lichens are higher than the levels in Meager Creek samples, which contained a maximum of 4.79 ppm dry weight of arsenic (Chapter 4). The highest concentration of arsenic in *Cladonia* sp. is observed in the sample from the Con Mine tailings pond, location 15 (520 ppm); it was likely to have been submerged at times of slow drainage from the tailings pond. The highest level of arsenic in all lichens occurs in Lichen 4 from the Giant Mine tailings pond (location 7). This was also the sampling location for the puffball mushroom, *Lycoperdon* sp., which contains the highest level of arsenic among mushrooms. The lichens that were sampled together, Lichens 1, 2 and 3, contain similar levels of arsenic, with an average of 47 (SD of 9) ppm dry weight. This may indicate that these different species of lichens accumulate arsenic to a similar extent.

Table 5.16. Arsenic concentrations in lichens and fungi from Yellowknife.

Sample, time	Location	Arsenic concentration (ppm dry weight)
PC1, June	12	14.3
PC1, residue[a], June	12	6.4
PC2, August	13	29
PC2, residue[b], Aug.	13	15.9 (0.7)[c]
PC3, Aug.	15	520
Lichen 1, Aug.	15	38
Lichen 2, Aug.	15	49
Lichen 3, Aug.	15	55
Lichen 4, Aug.	7	2300
Paxillus involutus, Aug.	13	36
Psathyrella candolleana, Aug.	13	13.6
Leccinum scabrum, Aug.	13	8.3
Coprinus comatus, Aug.	8	410
Lycoperdon pyriforme, Aug.	7	1010

[a] Sample obtained when the residue remaining after MeOH/water extraction was acid digested. [b] Residues following duplicate extractions, as for PC1 (location 12). [c] Standard deviation obtained from total arsenic content in residues of duplicate extractions.

Some of the mushrooms contain higher levels of arsenic than mushrooms collected from uncontaminated areas. For example, the specimen of *Lycoperdon* sp. analyzed in the present study contains 1010 ppm dry weight of arsenic, whereas the published background values are 0.46 to 2.81 ppm dry weight for the same family of mushrooms[67]. *Lycoperdon* sp. from the Con Mine tailings pond (location 15) was not analyzed for total arsenic because the sample size was sufficient only for speciation analysis. Total arsenic levels for *Psyathyrella* sp. and *Leccinum* sp. collected from uncontaminated areas were less than 0.2 ppm dry weight[68,69] and for *Paxillus involutus*, they were 5.7-5.9 ppm[68]. Arsenic levels in *Paxillus involutus*, *Psathyrella*

candolleana and *Leccinum scabrum* in the present study are apparently elevated compared with the levels published for similar species collected from uncontaminated areas. The levels in the present study, however, are still close to the range of background concentrations (non-detectable to 15 ppm dry weight of arsenic) for most mushrooms[68,67]. The *Coprinus comatus* mushrooms from location 8 are appreciably elevated in arsenic concentration with respect to a published literature amount of < 0.1 ppm for *Coprinus micaceus*[67]. Its arsenic concentration is also elevated even when compared with the exceptionally high background levels (up to 130 ppm dry weight) found in *Laccaria* sp.[70]

5.3.7.2. Arsenic speciation in lichens and fungi

The arsenic species extracted from lichens and mushrooms are summarized in Table 5.17. The major arsenic species in lichens are As (III) and As (V), with the sum of these two species making up 62 to 93 % of the total extracted arsenic for the lichen specimens analyzed.

Arsenobetaine has been found for the first time in lichens, and it is present in all the lichens sampled. Chromatograms of PC1 and PC2 extracts, obtained from cation exchange HPLC-ICP-MS analysis, are shown in Figure 5.4. Arsenobetaine was identified in PC1 by spiking the sample extract with standard arsenobetaine and demonstrating co-chromatography of the suspected component with the authentic material (Figure 5.4a). It was identified in PC2 (and other lichen samples) by matching the retention time of the presumed arsenobetaine peak in the sample with that of the standard (Figure 5.4b).

Table 5.17a. Concentrations of arsenic species in Yellowknife lichens (ppm) (SD)[a]. PC = pixie cup lichen. "Trace" amounts are greater than or at the limit of detection (LOD) but less than 3 × LOD. Sum of As species include trace amounts (given a value of LOD).

Sample (location)	As (III)	As (V)	MMA	DMA	Sugar X	AB	TMAO	Me_4As^+	Unknown	Sum of As species	% EE[b]
PC1 (12)	0.8 (0.2)	0.8 (0.3)	<0.02	trace	<0.01	0.2 (0.1)	<0.02	<0.01	<0.01	1.8	13
PC2 (13)	6 (1)	1.4 (0.4)	0.26	0.4 (0.1)	0.6 (0.3)	1.31 (0.06)	1.8 (0.5)	0.039 (0.002)	0.3 X[c] (0.1)	12	42
PC3 (15)	29 (6)	13	1.9	1.6	<0.03	1.9	<0.06	<0.03	<0.03	47	9.1
Lichen 1 (13)	6.4 (0.4)	3.0	<0.04	1.0	<0.02	0.6	0.5	<0.02	0.19 X	12	31
Lichen 2 (13)	4.6 (0.7)	1.4	0.39	<0.04	<0.02	0.79 (0.02)	0.5 (0.1)	<0.02	<0.02	7.7	16
Lichen 3 (13)	4.2 (0.8)	3.5 (0.3)	0.5	0.38	0.4 (0.2)	0.4	0.6	<0.02	<0.02	10	18
Lichen 4 (7)	0.5	24 (7)	1.0	0.7	<0.05	trace	<0.10	<0.05	<0.05	26	1.1

[a] SD = standard deviation, obtained from analyses with 2 chromatographic systems (anion and cation exchange) and replicate analyses.
[b] % EE = % Extraction efficiency, calculated as (Sum of As species)/(Total arsenic from Table 5.16) * 100%.
[c] Unknown X = peaks eluting between arsenobetaine and tetramethylarsonium ion on the cation exchange system.

Table 5.17b. Concentrations of arsenic species in Yellowknife fungi (ppm) (SD)[a]. "Trace" amounts are greater than or at the limit of detection (LOD) but less than 3 × LOD. Sum of As species include trace amounts (given a value of LOD).

Sample (location)	As (III)	As (V)	MMA	DMA	Sugar XI	AB	AC	Me_4As^+	Unknown	Sum of As species	% EE[b]
Paxillus involutus (13)	1.1 (0.1)	0.7 (0.2)	<0.05	16 (3)	0.8	<0.01	<0.01	0.11 (0.04)	0.3X (0.1), 11Y[d] (3)	30	83
Psathyrella candolleana (13)	1.4 (0.2)	4.4 (0.3)	0.14 (0.01)	0.6 (0.1)	<0.01	0.30	<0.07	0.17 (0.06)	<0.07	7.0	51
Leccinum scabrum (13)	0.17 (0.02)	0.08 (0.02)	<0.01	6.5 (0.6)	<0.01	<0.07	<0.07	<0.07	<0.07	6.8	85
Coprinus comatus (8)	2.0	4 (1)	<0.02	1.7 (0.2)	<0.02	60 (20)	0.3	<0.1	0.4X	68	17
Lycoperdon pyriforme (7)	3.3	21 (1)	6	10 (3)	<0.2	30 (10)	0.6	0.5	<0.1	71	7.1
Lycoperdon pyriforme (15)	12	1.2	<0.2	2.5 (0.6)	<0.2	60 (10)	0.6	0.30	0.5X (0.1)	77	nd[e]

[a] SD = standard deviation, obtained from analyses with 2 chromatographic systems (anion and cation exchange) and replicate analyses.
[b] % EE = % Extraction efficiency, calculated as (Sum of As species)/(Total arsenic from Table 5.16) * 100%.
[c] Unknown X = peaks eluting between arsenobetaine and tetramethylarsonium ion on the cation exchange system.
[d] Unknown Y = a broad peak that eluted slightly before arsenobetaine on the cation exchange system.
[e] nd = not determined, because the total arsenic concentration was not determined.

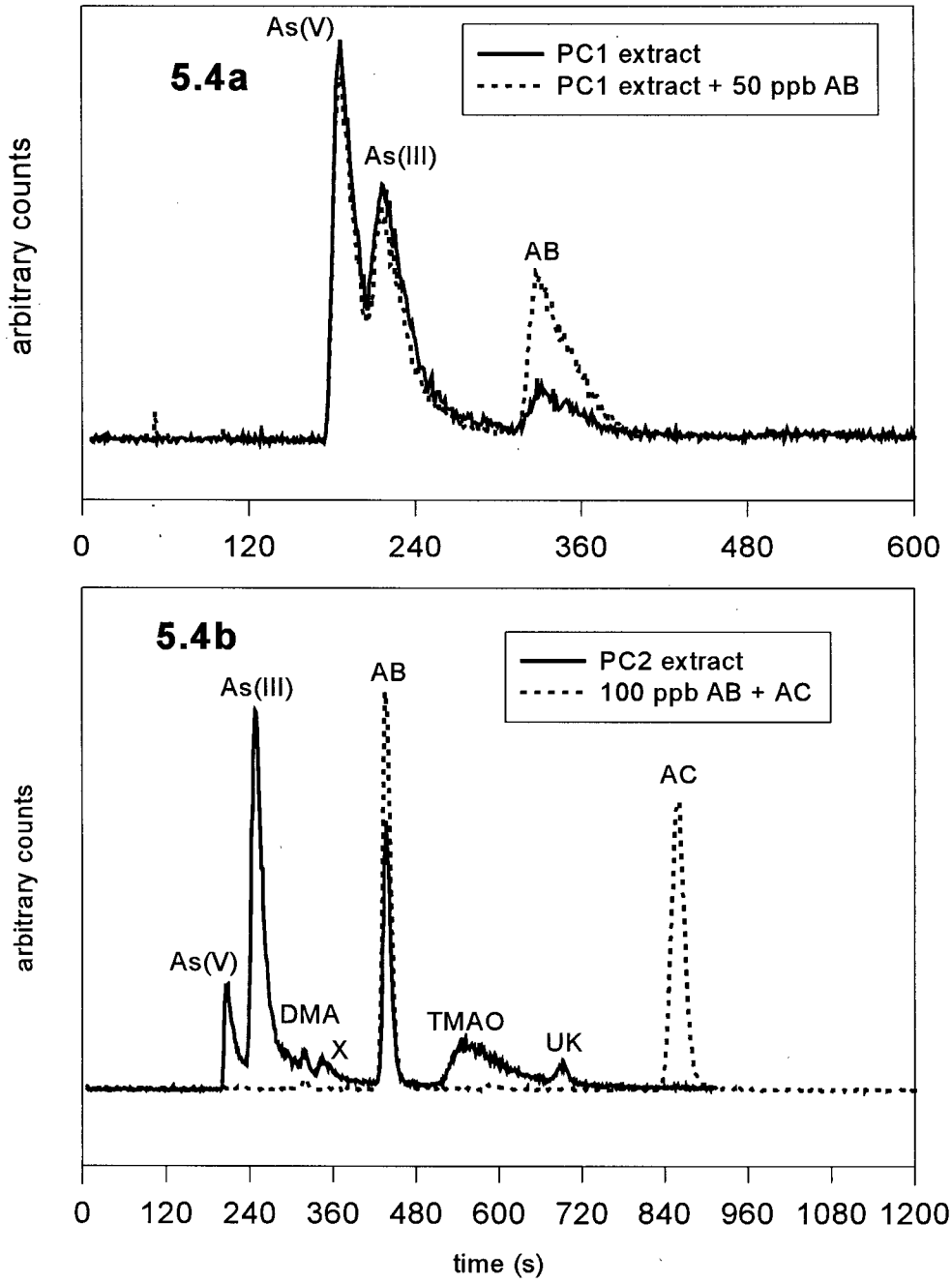

Figure 5.4. Arsenobetaine (AB) in lichens by using cation exchange HPLC-ICP-MS (see Table 5.2 for details). **5.4a.** Chromatograms of Yellowknife lichen (PC1) extract and extract spiked with 50 ppb AB standard. **5.4b.** Chromatograms of Yellowknife lichen (PC2) extract and 100 ppb AB and AC standards. See Table 1.1 for abbreviations, UK= unknown compound.

Arsenosugar X is present in minor amounts in two lichen samples, PC2 (5% of extracted arsenic) and Lichen 3 (4% of extracted arsenic) from location 13. Arsenosugars were not detected in Lichens 1 and 2, which were growing together with Lichen 3 at location 13 (Table 5.17a).

Some similarities exist for all lichen species that were sampled from the Con Mine Meg-Kam Lakes drainage system (locations 12, 13 and 15), summarized in Table 5.18. The proportion of As (III) is similar for all samples (42-61% of extracted arsenic, see Table 5.18), but a broader range is observed for the relative amounts of As (V) in the lichens (12-44% of extracted arsenic). Amounts of arsenobetaine range from 4 to 11%, and similar amounts of TMAO were observed for the three lichen species (lichens 1, 2 and 3) that were growing together (4-6.5 % of extracted arsenic). Similarities in arsenic speciation between these three lichens might be expected, due to their common environment.

The distribution of arsenic species found in the lichens collected near Con Mine (locations 12, 13, and 15) differs from that in the Giant Mine lichen (location 7) as summarized in Table 5.18. The major differences observed are the predominance of As (V) extracted from Lichen 4, and the small proportion of arsenobetaine found in Lichen 4 (0.5%) compared with the amounts found in the other lichens (4 to 11%). These differences may be caused by differences in lichen species (and hence metabolism of arsenic), and/or differences in their environments. For example, the major form of arsenic is probably as arsenate adsorbed onto ferric hydroxide in the Giant Mine tailings and the major form of arsenic in the tailings pond water is arsenate[60]. Unlike lichen 4, the Con Mine lichen samples were less likely to be submerged in tailings pond water, except for one sample (PC3). The arsenic speciation might reflect the extent to which the lichen is directly impacted by the tailings water.

Table 5.18. Proportions of total arsenic extracted, in % of arsenic species for lichens and mushrooms.

Sample (location)	As (III)	As (V)	Methyl + Sugars	AB	TMAO + cations
PC1 (12)	44	44	1.9	11	0
PC2 (13)	50	12	10	11	18
PC3 (15)	61	27	7.4	4.0	0
Lichen 1 (13)	55	26	8.6	5.1	5.9
Lichen 2 (13)	60	18	5.1	10.3	6.5
Lichen 3 (13)	42	35	13	4.0	6.0
Average for Con Mine lichens (12, 13, 15)	52 (8)[a]	27 (11)	7.7 (3.9)	7.5 (3.5)	6.1 (6.5)
Lichen 4 (7)	1.9	91	6.5	0.5	0
Lycoperdon sp. (7)	4.6	29	22	42	7.8
Lycoperdon sp. (15)	16	1.6	3.2	78	1.8

[a] Standard deviation of proportions of each compound.

The mushroom *Lycoperdon* sp. from the Giant Mine tailings pond (location 7) also contains a proportionally higher amount of arsenate, although the major arsenic species in both specimens of *Lycoperdon* sp. is arsenobetaine. Arsenobetaine was also observed to be the major arsenic species extracted from *Lycoperdon echinatum* (78%), *Lycoperdon perlatum* (88%) and *Lycoperdon pyriforme* (62%) in a previous study; minor components included As (V), As (III), MMA and DMA[67]. An greater variety of arsenic species is observed in the *Lycoperdon* sp. from Giant Mine tailings pond (location 7) compared with that in *Lycoperdon* sp. from the Con Mine tailings pond (location 15); see Figure 5.4 and Table 5.18. The specimen from location 7 was observed to be in a more mature form, and this may account for the differences in speciation

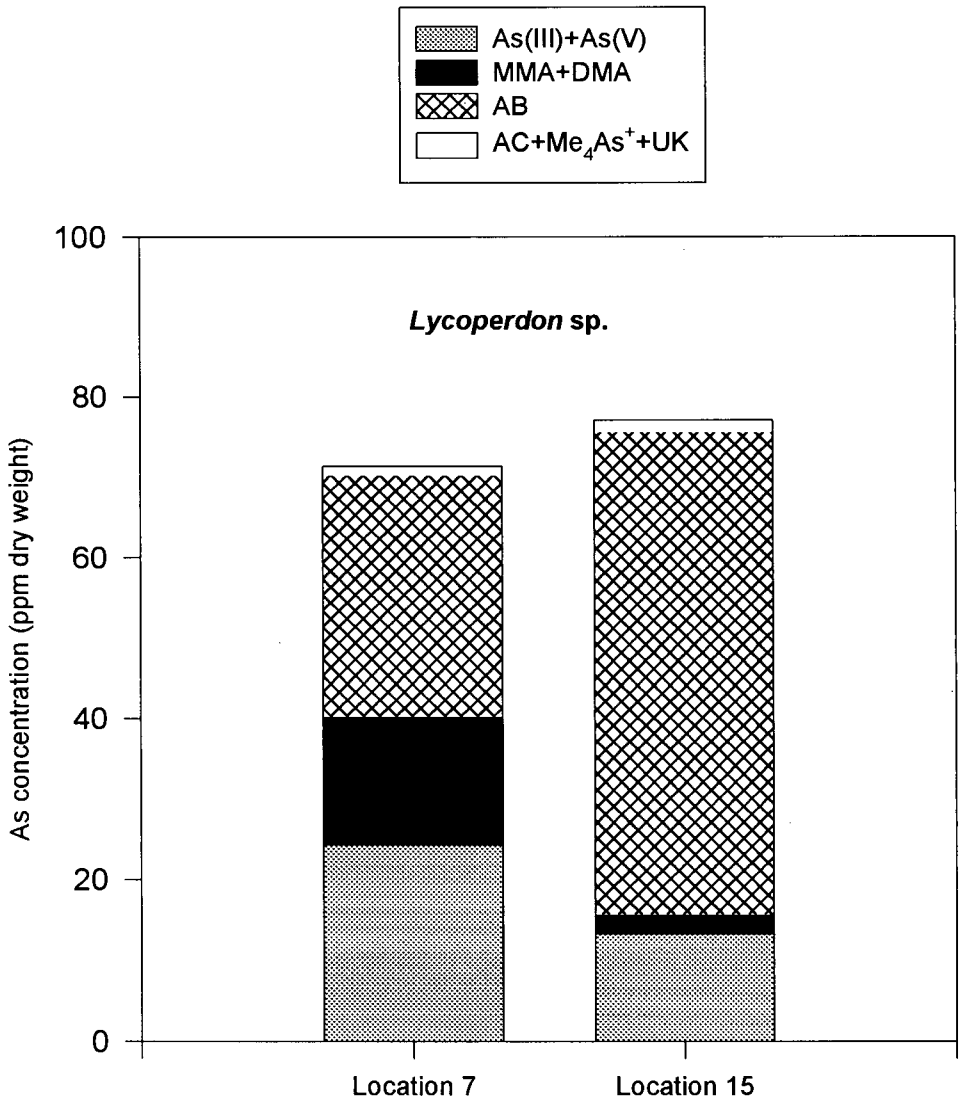

Figure 5.5. Relative amounts of arsenic species in the puffball mushroom *Lycoperdon* sp. from location 7 (Giant Mine tailings pond) and location 15 (Con Mine tailings pond). Differences in speciation are seen for *Lycoperdon* sp. from different locations. Abbreviations for arsenic compounds are in Table 1.1; UK = unknown compound.

observed. Again, the microbial environment influencing the fungus may also cause differences in arsenic speciation.

The arsenic speciation has been determined for water-soluble species in the shaggy mane mushroom *Coprinus comatus* for the first time, and the major arsenic compound is arsenobetaine (88% of extracted arsenic). Minor components include inorganic arsenic, DMA, arsenocholine and an unknown arsenic compound.

Paxillus involutus grew in abundance next to the Con Mine effluent stream. Its arsenic content is unusual because a major proportion of arsenic extracted (36%) is in an unidentified form (unknown compound Y). The peak for unknown Y was broad and it eluted near the retention time of arsenobetaine on the cation exchange system (see Figure 5.6 for chromatogram.) Neither unknown Y, nor a minor component, unknown X, could be identified as any of the arsenic compounds available to us as standards; some of the cationic arsenic standards are chromatographed in Figure 5.6. An unknown peak possessing a retention time similar to that for unknown Y, on almost the identical chromatographic system, was observed in mussels by Larsen *et al.*[35] The authors established that the compound was neither 2-dimethylarsinylethanol ($Me_2As(O)CH_2CH_2OH$) nor glycerylphosphoryl-arsenocholine ($Me_3As^+CH_2CH_2O\text{-}PO_2^-\text{-}OCH_2CHOHCH_2OH$).

The other major arsenic compound extracted from *Paxillus involutus* is DMA (53% of extracted arsenic). As (III) and (V), Me_4As^+, another unknown species (unknown X), and a small amount of arsenosugar XI (2.6% of arsenic extracted) occur as minor components.

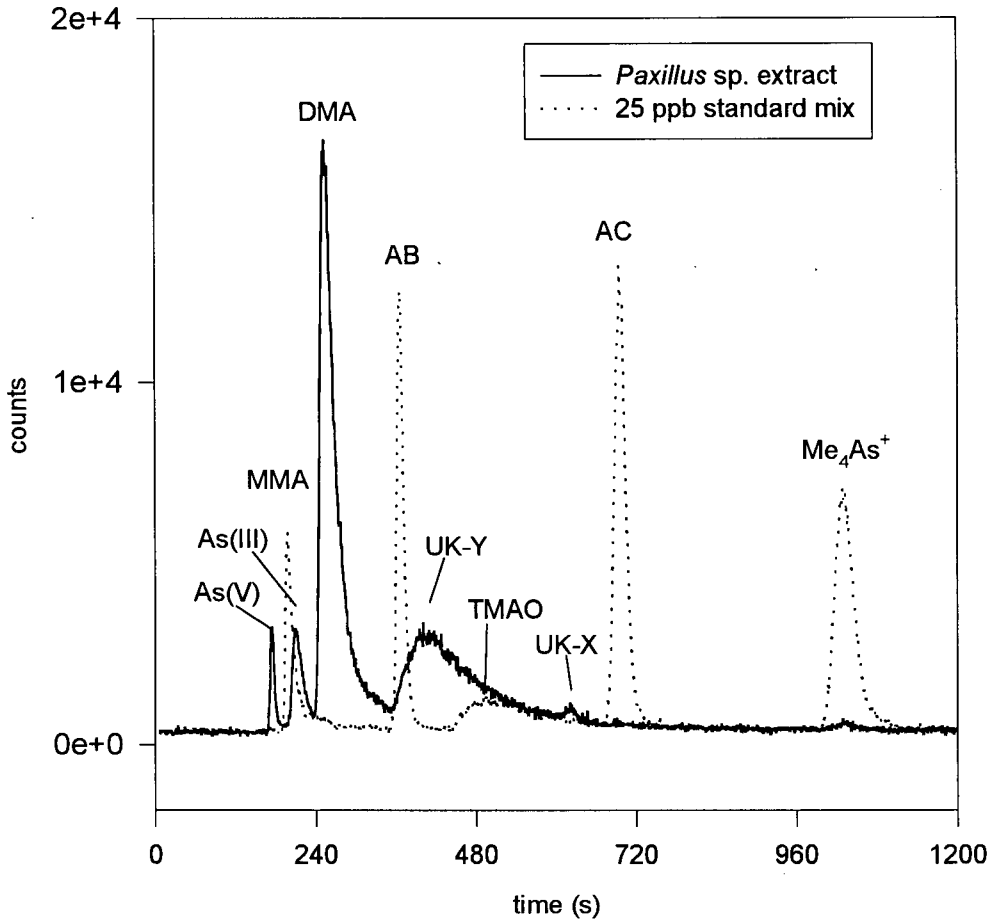

Figure 5.6. Chromatogram of *Paxillus involutus* extract (diluted 10x), showing the presence of unknown compounds X and Y (UK-X and UK-Y, respectively), as well as DMA, inorganic arsenic, and a small amount of Me_4As^+. Standards (including 25 ppb of MMA, AB, TMAO and AC) do not co-chromatograph with UK-X and UK-Y. Abbreviations for arsenic compounds are found in Table 1.1.

The major arsenic compound found in *Psathyrella candolleana* is arsenate, making up 63% of the total arsenic extracted. The next most abundant compound is arsenite, and minor components are MMA, DMA, arsenobetaine and Me_4As^+. This mushroom species is classified in the same family as *Coprinus* sp. (Coprinaceae)[69]. Similarities have been observed in arsenic contents of different mushroom species within the same family[67], and thus it is not surprising to find some of the same compounds (e.g., arsenobetaine) in *Psathyrella candolleana* and *Coprinus comatus*. Both mushrooms are edible but the major water-soluble arsenic species in *Psathyrella candolleana*, As (V) and As (III), are toxic to humans, whereas in *Coprinus comatus* the major species, AB, is not.

Leccinum scabrum contains mostly DMA with minor amounts of inorganic arsenic. In comparison, the three major arsenic compounds extracted from *Boletus edulis* (Chapter 3) were arsenite, arsenate and DMA. These two mushrooms belong to the same family (Boletaceae)[69], and again, similarities in arsenic species can be seen.

It has been suggested that in more "primitive" genera of mushrooms, arsenobetaine occurs much less frequently than in genera that are more highly evolved, such as puffballs[67]. On this basis, *Paxillus involutus,* and *Leccinum scabrum* may be more primitive mushrooms.

The presence of arsenosugar XI in *Paxillus involutus* represents one of the first reports of arsenosugars in mushrooms. Arsenosugar XI was tentatively identified in an extract of *Laccaria amethystina*, but further chromatographic confirmation was considered to be necessary[70].

Small amounts (less than 1% of extracted arsenic) of arsenocholine were found in *Coprinus comatus* and *Lycoperdon* sp. Arsenocholine has been observed only in small amounts in marine samples, even though studies have demonstrated that this compound can be readily bio-transformed into arsenobetaine by sediments[71]. However, arsenocholine has been observed

previously in mushrooms, being one of the major extracted arsenic species in *Amanita muscaria*[72] as well as in *Sparassis crispa*[67].

Other researchers[67,73] have suggested that the fungus itself is responsible for synthesizing arsenicals, such as arsenobetaine, that had not previously been found in the terrestrial environment. The absence of these compounds in soil (although arsenobetaine was recently found in ant hill material[74]) provides strong support for their hypothesis. However, experiments summarized in Chapter 3 indicate that synthesis of arsenobetaine or arsenocholine does not take place in pure culture of fungi. In the present study, the arsenic compounds found in a mushroom species (*Lycoperdon* sp.) appear to depend on the location, indicating that the surrounding environment has a strong influence on the extractable arsenic species from a mushroom. As well, it was shown in Chapter 3 that arsenobetaine in the culture medium was readily taken up by fungus. If arsenobetaine was being synthesized by soil organisms, or organisms associated with the mycelia, it would be taken up efficiently by the fungus and not be detectable in the soil. Experiments with organisms cultured from the environment of cultivated or wild mushrooms might help to elucidate the source of complex arsenicals such as arsenobetaine, arsenocholine and arsenosugars.

Extraction efficiencies for lichens and fungi range from 1.1% for Lichen 4 from location 7 to >80% for *Paxillus involutus* and *Leccinum scabrum* from location 13. The average extraction efficiency for lichens collected from the Con Mine area (locations 12, 13 and 15) is 21% with a standard deviation of 12. The lowest extraction efficiencies are observed for PC3 from location 15, and Lichen 4 from location 7, which are the lichens containing the highest levels of total arsenic.

The mushrooms *Coprinus comatus* and *Lycoperdon* sp. contain the highest levels of total arsenic and their extraction efficiencies are the lowest. Extraction efficiencies for *Lycoperdon* sp.

in a previously published report ranged from 24% to 128%[67], whereas 68% of arsenic was extracted from *Laccaria amthystina*[70] containing levels of arsenic comparable to those in *Coprinus* sp. and *Lycoperdon* sp. The present results suggest a negative correlation between extraction efficiency and arsenic concentration in lichens and mushrooms. However, the reasons for this apparent correlation remain unclear. A possibility is that some of the arsenic contributing to high total levels of arsenic is in a non-extractable form, such as in a mineralized form on the outside of the specimen, or bound to chitin or other cell components of the fungus. The distribution and metabolism of arsenic may not be uniform for different levels of arsenic uptake for fungi, which may explain differences in extraction efficiencies.

Residues of two extracted lichens were analyzed for total arsenic (see Table 5.16) and extraction efficiencies from these results were calculated as:

$$\% \text{ Extraction efficiency} = [(\text{total arsenic})-(\text{arsenic in residue})]/(\text{total arsenic}) \times 100\%.$$

Based on this calculation, 55% of total arsenic was extracted from PC1, which differs from the extraction efficiency of 13% in Table 5.17 (calculated by summing the arsenic species detected). An extraction efficiency of 45% was calculated by using the arsenic concentration in the PC2 residue, which agrees quite well with the 42% reported in Table 5.17. The difference in extraction efficiencies for PC1 indicates that some arsenic that was extracted was not observed by using the chromatographic systems available. This was suggested previously for other samples as well.

5.4. Summary

A large amount of information about arsenic in the terrestrial environment was obtained from the study of Yellowknife biota. An important observation was, as for the Meager Creek study, the low proportions of water soluble arsenic species (evidenced by low extraction efficiencies) in the majority of the biota studied. Arsenic that is not extracted is probably bound to lipids, cell components, proteins, or exists as minerals. Although the extractable portion can be approximated to represent the arsenic species that are bioavailable, the availability of the non-extractable arsenic cannot be assumed to be negligible, since the metabolism of such arsenic by higher trophic level organisms has not been reported.

Freshwater fish were found to contain arsenobetaine, in contrast to previous reports. Sucker was found to contain arsenosugar XI, and probably obtains the arsenosugar from its food source, implying that benthic organisms are capable of synthesizing these arsenic compounds. The major extractable arsenic compound in pike was found to be DMA, but the source is unknown. Amounts of arsenobetaine in freshwater mussels were negligible or non-existent, supporting the idea that the uptake of arsenobetaine in the marine environment is related to osmo-regulatory processes.

Mostly inorganic arsenic species were extracted from plants, mosses, algae and lichens. Plant species appear to be consistent in their uptake and metabolism of arsenic, which may indicate some transport mechanisms specific to the plant. Arsenosugars were found for the first time in higher plants. Freshwater green algae, as well as microbial mats, contain arsenosugars, probably indicating synthesis by the organisms.

Arsenobetaine was found for the first time in lichens. Differences in arsenic speciation are seen for the mushroom *Lycoperdon* sp. from different locations, which may indicate that

complex arsenicals, such as arsenobetaine, are formed as a part of the fungus microbial community and taken up by the fungus, rather than synthesized by the fungus itself.

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Chapter 6

ANTIMONY IN ENVIRONMENTAL SAMPLES

6.1. Introduction

Antimony can enter the environment as a result of rock weathering, soil runoff and through effluents from mining and smelting. Its compounds are used as flame retardants in plastics and textiles, as additives in metal alloys, as doping agents in semiconductors, and as antiparasitic drugs[1]. Because of the toxicity of some of its compounds, antimony is listed by the United States Environmental Protection Agency (US-EPA) as a priority pollutant[2]. The determination of antimony species in the environment is necessary in order to assess the toxicity and mobility of antimony.

Usually concentrations of naturally occurring antimony are about 5-10% those of arsenic[3] and the two elements are often found together in mineral deposits[4]. Because elevated levels of arsenic are found in the Meager Creek and Yellowknife environments (Chapters 4 and 5), and because elevated antimony levels were found previously in biota from the Yellowknife environment[5], we were interested in increasing the range of samples previously analyzed for antimony content. Although methods for antimony speciation by using HPLC with element specific detection have been developed[6,7,8], determination of methylantimony species other than Me_3SbCl_2 by using these methods have not been successful[9]. Therefore, we chose to use the method of hydride generation-gas chromatography (HG-GC) with AAS and MS detection for the analysis of antimony in environmental samples, in spite of the limitations and problems associated with this speciation method (see Chapter 2 and references at the end of this chapter[5,10,11]).

6.2. Experimental

6.2.1. Chemicals and reagents

Antimony (V) and (III) standards were obtained as potassium hexahydroxyantimonate, $KSb(OH)_6$ (Aldrich), and potassium antimonyl tartrate, $K_2Sb_2(C_4O_6H_2)_2$ (Aldrich). Me_3SbCl_2 was synthesized as described elsewhere[12]. Stock solutions were made by dissolving these compounds in deionized water and diluting the resulting solutions to 1000 or 100 mg L^{-1} as Sb. Standard working solutions were made by diluting the stock solution with deionized water as necessary.

For hydride generation analysis, $NaBH_4$ (reagent grade, Aldrich) was dissolved in deionized water fresh daily to provide a concentration of 2% w/v. Ammonium citrate buffer at a concentration of 0.05 M and pH 6 (1 M ammonium hydroxide, MicroSelect, Fluka, and analytical reagent grade citric acid, BDH) and 1 M HCl (Environmental grade, Alfa Aesar) were used for pH adjustment during derivatization.

6.2.2. Sampling and sample preparation

Sampling was carried out at Meager Creek (MC) and in Yellowknife (YK), as described in Chapters 4 and 5, with sample locations shown in Figure 6.1a (MC) and Figure 6.1b (YK). Water was sampled by hand into polypropylene bottles that had been acid washed previously. Biota were sampled by hand, stored in Ziploc® bags and kept cool until processing. They were washed thoroughly with tap water to remove soil and other particles, rinsed with deionized (1 Mohm) water, and frozen. The samples were freeze-dried and pulverized to a fine powder for analysis. Snails from Yellowknife were dissected to remove the soft tissue prior to freezing.

Samples were digested with acid for the determination of total antimony content. The freeze-dried powders were accurately weighed (0.5 g ± 0.5 mg) into a 500 mL round bottomed flask (RBF). Concentrated nitric acid (3 mL, doubly distilled in quartz, Seastar, Sidney, BC) and hydrogen peroxide (3 mL, 30% in water, reagent grade, Fisher) were added to each sample. The samples in the RBFs were boiled for 3 hours by using a heating mantle and a reflux apparatus[13]. After all the samples had cooled, the clear solutions remaining were diluted to 25 mL with deionized water and stored at 4°C until analysis.

Extractions were carried out by weighing 0.5 g (± 0.5 mg) of the dried powders into 50 mL or 15 mL centrifuge tubes, adding 10-15 mL MeOH/water (1:1), sonicating for 20 minutes, centrifuging for 20 minutes, and decanting the liquid layer into a RBF. Each sample was sonicated and centrifuged a total of 5 times. The decanted extracts for each sample were pooled and rotovapped to near dryness (1-2 mL) and then diluted to 5 or 10 mL with deionized water. Moss (*Drepanocladus* sp.) from Yellowknife locations 1 and 4, and snails (*Stagnicola* sp.) from Yellowknife locations 1+3, were extracted in a larger quantity to permit analysis by using HG-GC-AAS and HG-GC-MS. Masses of 1 or 2 g were weighed out and extracts were made up to a final volume of 20 mL or 40 mL, respectively.

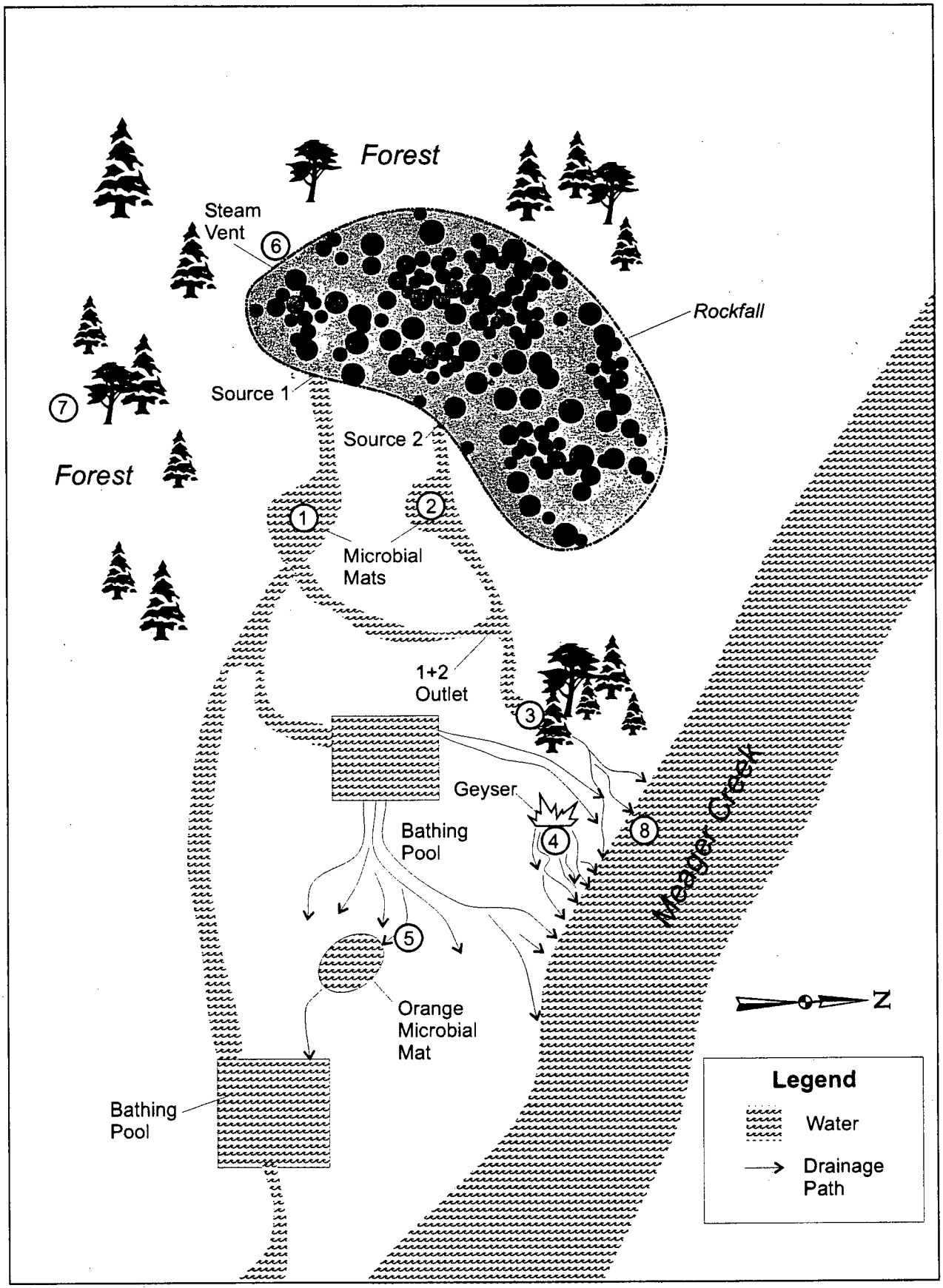


Figure 6.1a. Map (not to scale) of Meager Creek Hot Springs area showing sampling locations.

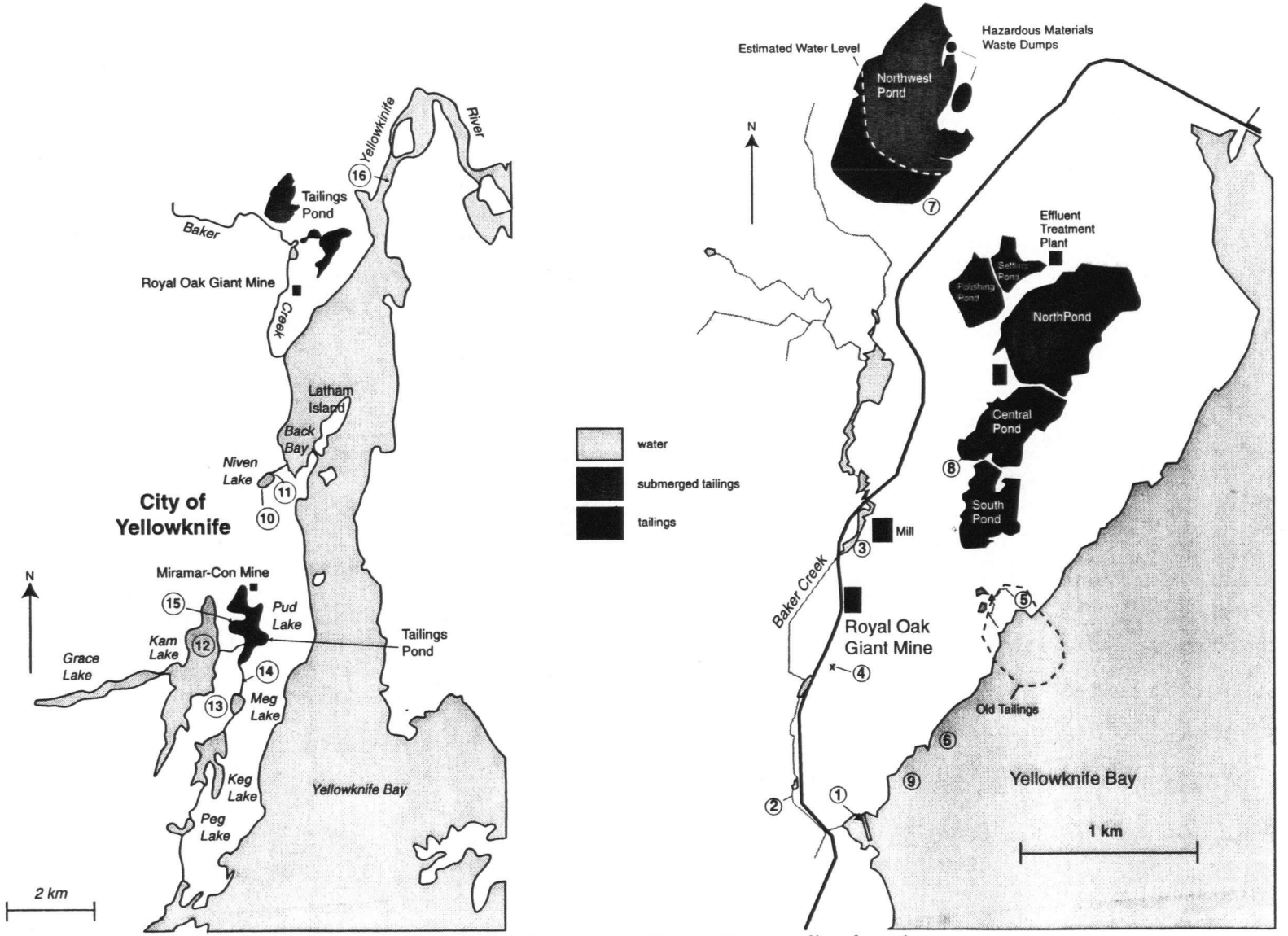


Figure 6.1b. Maps of Yellowknife sampling locations.

6.2.3. ICP-MS analysis for total arsenic and antimony concentrations

Acid digested samples and water samples were analyzed by ICP-MS for total antimony content. A VG Plasmaquad PQ2 Turbo ICP-MS (VG Elemental) outfitted with a peristaltic pump and injection loop for flow injection introduction was used. Parameters for the ICP-MS are given in Table 6.1. The m/z monitored were 121 (Sb), 123 (Sb) and 103 (Rh). Acid digested samples were diluted with 1% nitric acid (doubly distilled in quartz, Seastar) and Rh (10 ppb) was added as an internal standard to diluted samples and water samples. Quantification was carried out by using an external calibration curve derived from Sb standards made in 1% nitric acid and containing 10 ppb Rh.

Table 6.1. Operation parameters for ICP-MS

Feature	Parameter
Forward radio-frequency power	1350 W
Reflected power	<10 W
Cooling gas (Ar) flow rate	13.8 L/min
Intermediate (auxiliary) gas (Ar) flow rate	0.65 L/min
Nebulizer gas (Ar) flow rate	1.002 L/min
Nebulizer type	de Galan
Quadrupole pressure	9×10^{-7} mbar
Expansion pressure	2.5 mbar

6.2.4. HG-GC-AAS analysis for antimony speciation

The apparatus was composed of a semi-continuous flow, hydride generation system developed for arsenic analysis,[14] coupled to an atomic absorption spectrometer (Varian AA1275) fitted with an Sb lamp (Varian) operating at a wavelength of 217.6 nm, or at 231.4 nm for a few

samples (section 6.3.2). One modification was made to the basic apparatus in the form of using a gas-liquid separator[15] that resulted in less analyte carryover. The apparatus consisted of Tygon tubing for the peristaltic pump, and PTFE tubing (1/8" OD) for the remainder. Data were collected from the AAS and processed directly by using an HP 3390A integrator, or were analyzed with the aid of Shimadzu EZChrom software run on a PC.

A peristaltic pump was used to deliver standard or sample solution (ranging from 5 μL to 200 μL for standards, and from 1 mL to 100 mL for samples) to mix with the acid or buffer and then to mix with a solution of $NaBH_4$ (2% w/v) in a reaction coil. The gases evolved were separated in the gas-liquid separator and then swept by a flow of helium into a PTFE U-tube, where they were trapped at -196 °C. Continuous hydride generation and trapping were carried out for 3 minutes. The peristaltic pump was then stopped (making the system semi-continuous) and the U-tube was heated to 70 °C, allowing the gases to be swept with He at a flow rate of 40 mL/min onto a Poropak PS column, which was then heated from 70 °C to 150 °C at a rate of 30 °C/min, whereby the gases were separated. They were then detected by AAS. Semi-quantitative amounts were calculated by using external calibration curves.

6.2.5. HG-GC-MS analysis for confirmation of methylantimony species

Extracts of samples and standard solutions of Me_3SbCl_2 were reacted with $NaBH_4$ to form methylantimony hydrides. The reactions were performed in a 15 mL vial (sealed with a PTFE-faced silicone or neoprene septum, 16mm, Supelco) by using the appropriate volume of sample (5 mL for moss extracts and 10 mL for snail extract) or Me_3SbCl_2 standard solution, and 1 mL deionized water (for standards) and then injecting 0.5 mL of 6% $NaBH_4$ solution through the septum. All reactions were carried out without the addition of acid or buffer, with the exception of one reaction in which Me_3SbCl_2 standard solution was first made acidic by adding

an equal volume of 1 M HCl, in order to generate $MeSbH_2$ and Me_2SbH in addition to Me_3Sb. These methods were qualitative only.

For the analysis, a GC-MS system consisting of a Star 3400Cx gas chromatograph (Varian), equipped with a 1078 temperature programmable injector (Varian) and interfaced to a Saturn 4D ion-trap mass spectrometer (Varian) was employed. A gas tight syringe (1.0 mL, Gastight #1001, Hamilton) was rinsed with 5 mL of lab air and then used to inject 1 mL of headspace generated from the samples onto a capillary column (PTE™-5, 30m x 0.32mm, 0.25 μm, Supelco 2-4143, poly (5% diphenyl / 95% dimethylsiloxane)). The injector was kept at 100 °C. The temperature program started at 40 °C, and stopped at 150 °C with a heating ramp of 15 °C/min. The parameters used are shown in Table 6.2.

Table 6.2. GC-MS parameters.

GC method	
Injector temperature	100 °C
Column temperature program	40 °C, 15 °C/min to 150 °C
Transfer line temperature	200 °C
Column	PTE™-5, 30 m × 0.32 mm, 0.25 μm, Supelco
MS method	
Mass range	115-180 m/z
Scan time	0.4 s
Segment length	4.5 min
Ion Mode	Electron Impact
Multiplier	2150 V
Target	15 400
Ionization current	20 μA
Manifold temperature	260 °C

6.3. Results and Discussion

6.3.1. Antimony species and total antimony in environmental samples

The semi-quantitative amounts of antimony species in environmental samples detected by using the method of HG-GC-AAS, as well as total antimony, determined by using ICP-MS, are shown in Table 6.3. The antimony contents in biota and water from Yellowknife and Meager Creek are summarized in Table 6.3a (biota) and Table 6.3b (water). Absolute detection limits of 1 ng for Sb (III) and methyl antimony species were estimated and relative standard deviations between replicate analyses can be estimated to be 20%. The biota samples were chosen for analysis by HG-GC-AAS based on their ability to meet one of two criteria: (a) the presence of antimony in sample extracts was observed during HPLC-ICP-MS analysis or (b) total antimony content greater than 10 ppm dry weight was measured.

6.3.1.1. Inorganic antimony species

In all biota sample extracts and water samples, inorganic Sb (V) is the major antimony species (Tables 6.3a and 6.3b). Very few biota samples have been speciated for antimony previously[5,16], but other researchers have also shown that Sb (V) is the major antimony compound in water samples[3,8,17,18,19]. Thermodynamically, Sb (V) is predicted to be the most stable oxidation state under most environmental, oxygenated conditions (pH 5 to 8)[20] and therefore it is not surprising to find that Sb (V) is the most abundant extractable species of antimony in these samples.

Table 6.3a. Total antimony, extracted[a] antimony species and estimated extraction efficiency in extracts of environmental biota samples (ppm dry weight). YK = Yellowknife; MC = Meager Creek; na = not analyzed. Extraction efficiency estimated by calculating (Sum of Sb species)/(Total Sb)× 100%.

Sample (location #)	Sb (V)	Sb (III)	Me_2Sb-	Me_3Sb-	Sum of Sb species	Total Sb (acid digested)	Extraction Efficiency (%)
YK Biota samples							
Moss 1, June (5)	1.4	<0.007	<0.007	<0.007	1.4	190	0.7
Drepanocladus sp., June (1)	2.5 (0.6)[b]	0.009	0.046	<0.004	2.56	12	21
Drepanocladus sp., Aug. (1)	2.82 (0.05)[b]	0.011	0.044	<0.004	2.88	60	4.8
Drepanocladus sp., Aug. (4)	2.0	0.012	0.17 (0.01)[b]	<0.004	2.18	28	7.8
Stagnicola sp., Aug. (1+3)	0.7	<0.002	0.005	0.024	0.73	6	12
Typha sp. (12)	0.19	<0.004	<0.004	<0.004	0.19	0.20	95
Bidens cernua (10)	0.031	<0.01	<0.01	<0.01	0.031	0.7	4.4
Lemna minor (10)	0.05	<0.01	<0.01	<0.01	0.05	0.39	13
Myriophyllum sp., Aug. (10)	0.032	<0.02	<0.02	<0.02	0.032	0.28	11
Potomogetan sp. (6)	0.028	<0.01	<0.01	<0.01	0.028	0.8	3.5
Sparganium sp. (6)	0.096	<0.01	<0.01	<0.01	0.096	0.26	37
Lichen 4 (7)	5	<0.01	<0.01	<0.01	5	120	4.2
Cladonia sp. (13)	0.046	<0.01	<0.01	<0.01	0.046	1.4	3.3
Lycoperdon sp. (7)	0.46	<0.005	<0.005	<0.005	0.46	60	0.8
Coprinus sp. (8)	5	<0.005	<0.005	<0.005	5	34	15
MC Biota samples							
Mimulus sp. (1+2)	0.07	<0.007	<0.007	<0.007	0.07	0.5	14

[a] Samples were extracted by using MeOH/water (1:1) as described in section 6.2.2, and antimony species were detected by using HG-GC-AAS as described in section 6.3.4.

[b] Value in parentheses is standard deviation, calculated from duplicate analyses.

Table 6.3b. Total antimony, antimony species[a] and percent Sb species of total Sb in environmental samples of water (ppb). See Table 6.3a for abbreviations. Percent Sb species of total represents the amount of antimony detectable by HG-GC-AAS, calculated by using (Sum of Sb species)/(Total Sb)× 100%.

Sample and location	Sb (V)	Sb (III)	Me_2Sb-	Me_3Sb-	Sum of species	Total Sb	Percent Sb species of total (%)
YK Water Samples (ppb)							
Location 1	240	<0.03	<0.03	<0.03	240	260	92
Location 2	220	<0.03	<0.03	<0.03	220	na	na
Location 3	300	<0.03	<0.03	<0.03	300	380 (50)[c]	79
Location 4	30	0.10 (0.02)[b]	0.335 (0.007)[b]	0.13 (0.05)[b]	30.6	50	60
Location 10	1.3	<0.03	<0.03	<0.03	1.3	3.7	35
Location 11	0.38	0.08	<0.03	<0.03	0.46	1.8 (0.2)[c]	26
Con effluent, location 14	18	<0.03	<0.03	<0.03	18	31	58
MC Water Samples (ppb)							
Location 1, Sept. 1996	6 (4)[b]	0.08	<0.03	<0.03	6	na	na
Location 2, Sept. 1996	8.2 (0.9)[b]	0.34	<0.03	<0.03	8.5	5	170
Location S2, Nov. 1996	2.4	0.06	<0.03	<0.03	2.5	5	50
Location S1, July 1997	6	0.09	<0.03	<0.03	6	na	na
Location S2, July 1997	4.3	0.05 (0.02)[b]	<0.01	0.04	4.4	na	na
Location 4, Sept. 1996	1.2	0.14	<0.03	<0.03	1.3	12	11
Location 1+2, July 1997	3.0	0.07	<0.03	<0.03	3.1	na	na

[a] Antimony species detectable by using HG-GC-AAS as described in section 6.3.4.
[b] Value in parantheses is standard deviation, calculated from duplicate analyses.
[c] Value in parantheses is standard deviation, calculated from analysis of duplicate samples.

Inorganic Sb (III) is present in *Drepanocladus* sp. (moss) samples from Yellowknife (YK) location 1 and Yellowknife (YK) location 4, as shown in Table 6.3a. Sb (III) is also present in all Meager Creek (MC) waters and water from YK location 4 and location 11 (Table 6.3b). Other studies have shown that Sb (III) is produced in the photic zone of an estuarine inlet, which may indicate that biological activity is responsible for the presence of Sb (III)[21]. In the same study, reducing conditions, including the presence of H_2S, did not result in significant reduction of Sb (V) to Sb (III) in water, although the formation of Sb(III)-S compounds was postulated[21]. In the present study, water from Meager Creek was sampled from locations near microbial mats, which have been shown to exist under reducing conditions (Chapter 4) and to produce Me_3Sb[22]. Reducing conditions and microbial metabolism may lead to the presence of Sb (III) in these waters.

6.3.1.2. Methylated antimony species

Methylated antimony species were detected in a few samples by using HG-GC-AAS. Biota samples containing methylantimony species include *Drepanocladus* sp. from YK locations 1 and 4 and snails (*Stagnicola* sp.) from YK locations 1+3 (Table 6.3a). A chromatogram showing stibine, dimethylstibine and trimethylstibine that were generated from a standing water sample (YK location 4) is shown in Figure 6.2.

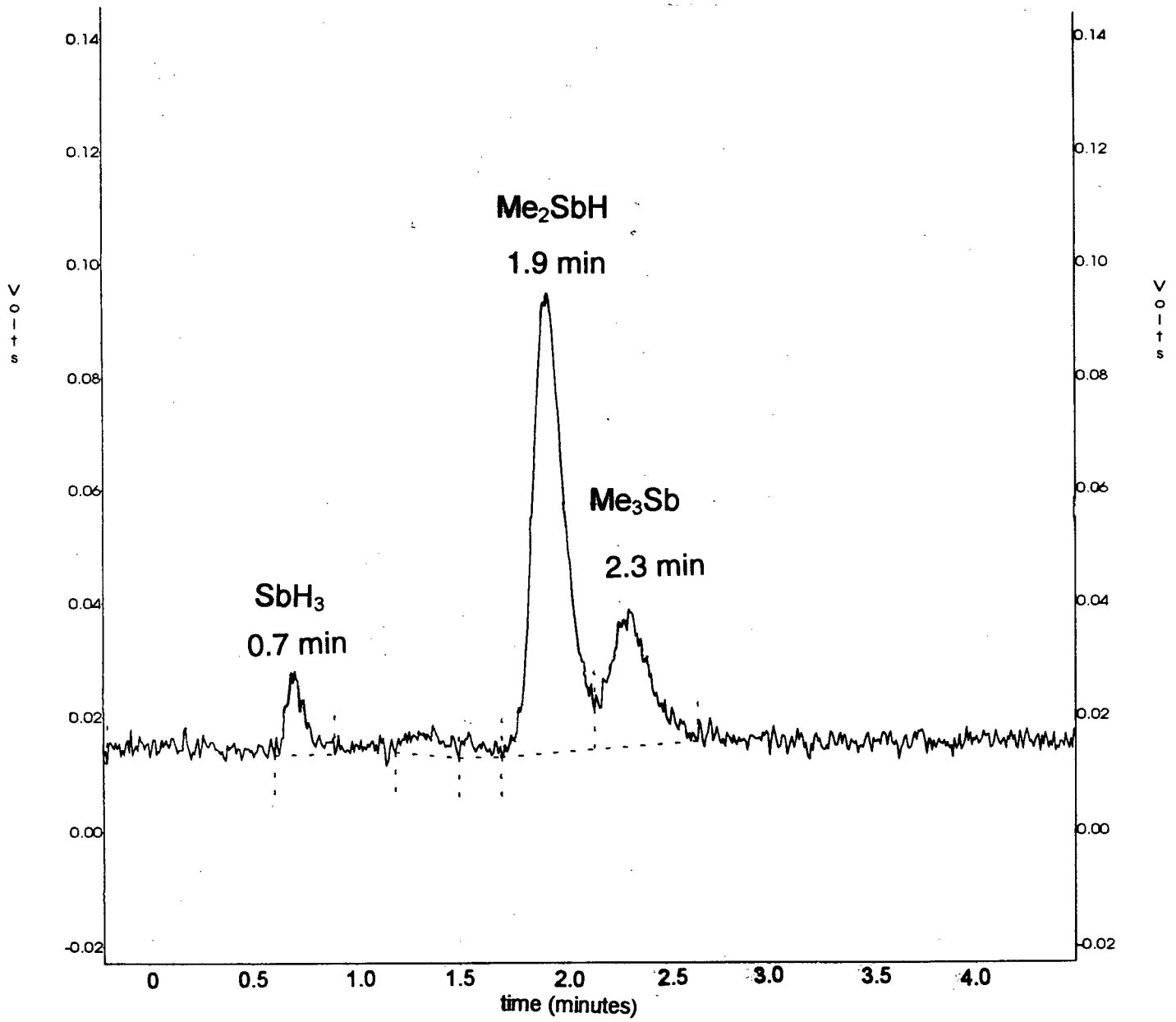


Figure 6.2. Chromatogram obtained by using HG-GC-AAS (217.6 nm) showing stibines generated at neutral pH from 100 mL of a sample of standing water from location 4 in Yellowknife.

The moss samples from Yellowknife that contained methylantimony compounds were all identified as *Drepanocladus* sp. Interestingly, no detectable antimony species could be extracted from another sample of *Drepanocladus* sp., at YK location 15 (see Figure 6.1b). The inability to extract antimony in *Drepanocladus* sp. from YK location 15 is probably because of lower levels of total antimony in this sample. The discovery of methylantimony compounds in snails represents, to our knowledge, the first finding of methylated antimony in an animal.

Methylantimony compounds in biota have been observed rarely in past studies. Dodd *et al.* identified predominantly a trimethylantimony compound in one sample and a dimethylantimony compound in another sample of the same species of macrophyte from Yellowknife[5]. The results from the present study differ from that by Dodd *et al.*[5] because the dimethylantimony compound is possibly the same in all three moss samples. Analysis of a soil extract by using HPLC-ICP-MS indicated the presence of a $Me_3Sb(V)$ species[7].

A dimethylantimony compound is present in a water sample from YK location 4 and a trimethylantimony compound is present in a water sample from MC location S2 (Table 6.3b). Monomethylantimony and dimethylantimony compounds, assumed to be methylstibonic acid and dimethylstibinic acid, have been observed in river, estuary and sea water samples by other researchers and their presence was attributed to biological activity[21,23].

The dimethylantimony compound present in the Yellowknife water sample (YK location 4) may be similar to the dimethyl compound in the moss sampled from the same location (YK location 4), and its presence in both samples may indicate uptake and/or excretion. From these results it is impossible to differentiate between the possibility of the moss forming a dimethylantimony species as a metabolite and excreting it into the water, and the possibility of microorganisms in the water or sediment forming the compound and its being taken up by the moss. Both scenarios are possible.

The presence of trimethylantimony species in Meager Creek water may be a result of the production of Me_3Sb by the microbial mats nearby. Recent studies have indicated that Me_3Sb would be oxidized rapidly to Me_3SbO[24,25], but other studies suggest that if the Challenger mechanism is followed for the methylation of antimony (oxidative addition of methyl groups, followed by reduction to the stibine, see Chapter 1, Figure 1.1) the final reduction step to Me_3Sb takes place only to a very small extent[9]. Therefore the trimethylantimony compound in the Meager Creek water sample may be present as a result of oxidation following Me_3Sb production in the mats, or of biological antimony methylation only as far as Me_3SbO.

6.3.1.3. Extraction efficiencies for biota and percent Sb species of total Sb in waters

Extraction efficiencies of antimony from biota range from 0.7 to 95%. However, extraction efficiencies for all samples except one are below 37% (Table 6.3a). Clearly the extraction method using MeOH/water (1:1) inadequately extracts antimony from these samples. The reasons for this are likely similar to those given for low extraction efficiencies of arsenic from biota: antimony may be strongly bound to cellular components such as lipids, cellulose, lignin or carbohydrates. For example, metals are known to bind strongly to fungal cell walls[26], and antimony may thus be strongly bound to cellular components. Accordingly, extraction efficiencies from fungus samples (lichen and mushrooms) ranged from 0.8 to 15% (Table 6.3a). *Typhus* sp. (cattail) from YK location 12 was extracted nearly quantitatively (Table 6.3a).

Some of the antimony extracted from these samples may not have been detected by using HG-GC-AAS, which may account for the differences in amounts detected in waters by HG-GC-AAS compared with the levels of total antimony in waters (Table 6.3b, last column). Arsenic species that are "hidden" to HG-GC-AAS detection, without strong digestion techniques (e.g., UV photolysis or microwave digestion), have been found in sediment pore water samples from

Yellowknife[27]. This arsenic was suggested to be bound to colloidal organic matter, or in the form of organoarsenic compounds (such as arsenocholine or arsenosugars)[27]. In the same way, antimony that is complexed strongly to organic groups may not form hydrides under the conditions used, or Sb-S compounds may exist as well. Sb(III)-S compounds have been proposed to be present in estuarine and interstitial waters, detected by acidifying and degassing samples before HG-GC-AAS analysis at pH 6[21]. This method was not carried out in the present study and hence the possibility has not been ruled out that such compounds are present.

6.3.1.4. Total concentrations of antimony compared with arsenic

The total concentrations of arsenic (from Chapters 4 and 5) and antimony (from Table 6.3) in some Yellowknife and Meager Creek biota and water samples are summarized in Table 6.4. An average [Sb]/[As] ratio of 6.4% can be calculated for the biota samples. For water samples other than those taken from Baker Creek (locations 1 and 3) and the effluent from Con Mine (location 13), the average ratio is 4.0%.

Table 6.4. Comparison of total concentrations of antimony and arsenic (ppm unless otherwise stated) for selected samples

Sample	[Sb]	[As]	[Sb]/[As] × 100%
YK Biota samples (location #)			
Moss 1, June (5)	190	1130	17
Drepanocladus sp., June (1)	12	1220	9.8
Drepanocladus sp., August (1)	60	490	12
Drepanocladus sp., August (4)	28	880	3.2
Stagnicola sp., August (1+3)	6	82	7.3
Typha sp. (12)	0.20	3.8	5.3
Bidens cernua (10)	0.7	100	0.7
Lemna minor (10)	0.39	28	1.4
Myriophyllum sp., August (10)	0.28	17.4	1.6
Potomogetan richardsonii (6)	0.8	20	4.0
Sparganium augustifolium (6)	0.26	2.5	10
Lichen 4 (7)	120	2300	5.2
Cladonia sp. (13)	1.4	29	4.8
Lycoperdon sp. (7)	60	1010	5.9
Coprinus sp. (8)	34	410	8.3
MC Biota samples (location #)			
Mimulus sp. (1+2)	0.5	8.7	5.7
YK Water Samples in ppb			
Location 10	3.7	68	5.4
Location 11	1.8	53	3.4
Location 4	50	740	6.8
Location 1	260	120	220
Location 3	380	220	170
Con effluent, location 14	31	51	61
MC Water Samples in ppb			
Location 2,	5	277	1.8
Location S2	5	237	2.1
Location 4	12	288	4.2

Ratios of [Sb]/[As] in soil and rocks average about 10%[1,4] and therefore it may appear that uptake of antimony by biota is slightly less than that of arsenic, a phenomenon that has been observed before[4]. A slight deviation of the average ratio obtained (6.4%) from the typical crustal ratio (10%) might also be observed if soil ratios were lower than 10% in the Yellowknife environment. The ratio in waters is slightly lower (4%) than the ratio in biota. Although these slight differences are noted, the results are still within an order of magnitude of the typical relative levels of antimony and arsenic in the natural environment.

It is interesting to note that the concentrations of antimony in waters from Baker Creek (YK locations 1,2 and 3) and in Con Mine effluent (YK location 14) are similar to those of arsenic. The probable reason for this is that arsenic is effectively removed by alkaline precipitation with ferric sulfate from the effluents, before the effluents are discharged into the environment[28], whereas antimony is not. Co-precipitation of arsenic with ferric hydroxides is postulated to take place during effluent treatment, and it has been observed that antimony is removed 10 times less efficiently than arsenic from solution by co-precipitation with ferric and manganese hydroxides[17].

6.3.2. The confirmation of antimony in samples containing methylantimony compounds by using HG-GC-AAS

The presence of methylantimony species in samples was confirmed by two methods: (a) by using HG-GC-AAS at a different wavelength to corroborate that peaks are due to antimony compounds and not the result of spectral interferences, and (b) by using HG-GC-MS to confirm the structure and presence of hydrides derived from samples (next section, 6.3.3). The three moss samples, from YK locations 1 and 4, as well as the water sample from YK location 4, were analyzed by using HG-GC-AAS with the AAS operating at a wavelength of 231.4 nm, which is a

secondary absorption line specific to antimony. Peaks appeared at the same retention times as those found at a wavelength of 217.6 nm, and in similar abundances, as summarized in Table 6.5. The snail extract was not analyzed because of limited sample size.

Table 6.5. Relative amounts (% of sum of methyl species, estimated by normalizing area counts) for methyantimony peaks in moss and water samples.

Sample (YK location #)	Sb (III) (RT=0.7)	Me_2Sb- (RT=1.9)	Me_3Sb- (RT=2.3)
AAS at 231.4 nm			
Drepanocladus sp. June (1)	12	88	0
Drepanocladus sp., Aug. (1)	10	90	0
Drepanocladus sp., Aug. (4)	5	95	0
Water, Aug. (4)	17.5	59	23.5
AAS at 217.6 nm			
Drepanocladus sp., June (1)	17	83	0
Drepanocladus sp., Aug. (1)	23	77	0
Drepanocladus sp., Aug. (4)	6	94	0
Water, Aug. (4)	17.5	59	23.5

6.3.3. The use of headspace HG-GC-MS for the speciation of antimony compounds

Me_3Sb was generated in sealed vials from standard solutions of Me_3SbCl_2 by using hydride generation methodology. A sample of the headspace was injected into the GC-MS. A detection limit of 0.08 ng Sb for Me_3Sb was obtained, corresponding to 1 ng Sb in solution before derivatization. However, the analysis of headspace following hydride generation suffers from imprecision, since RSD values no better than 20% were observed for 5 replicate analyses.

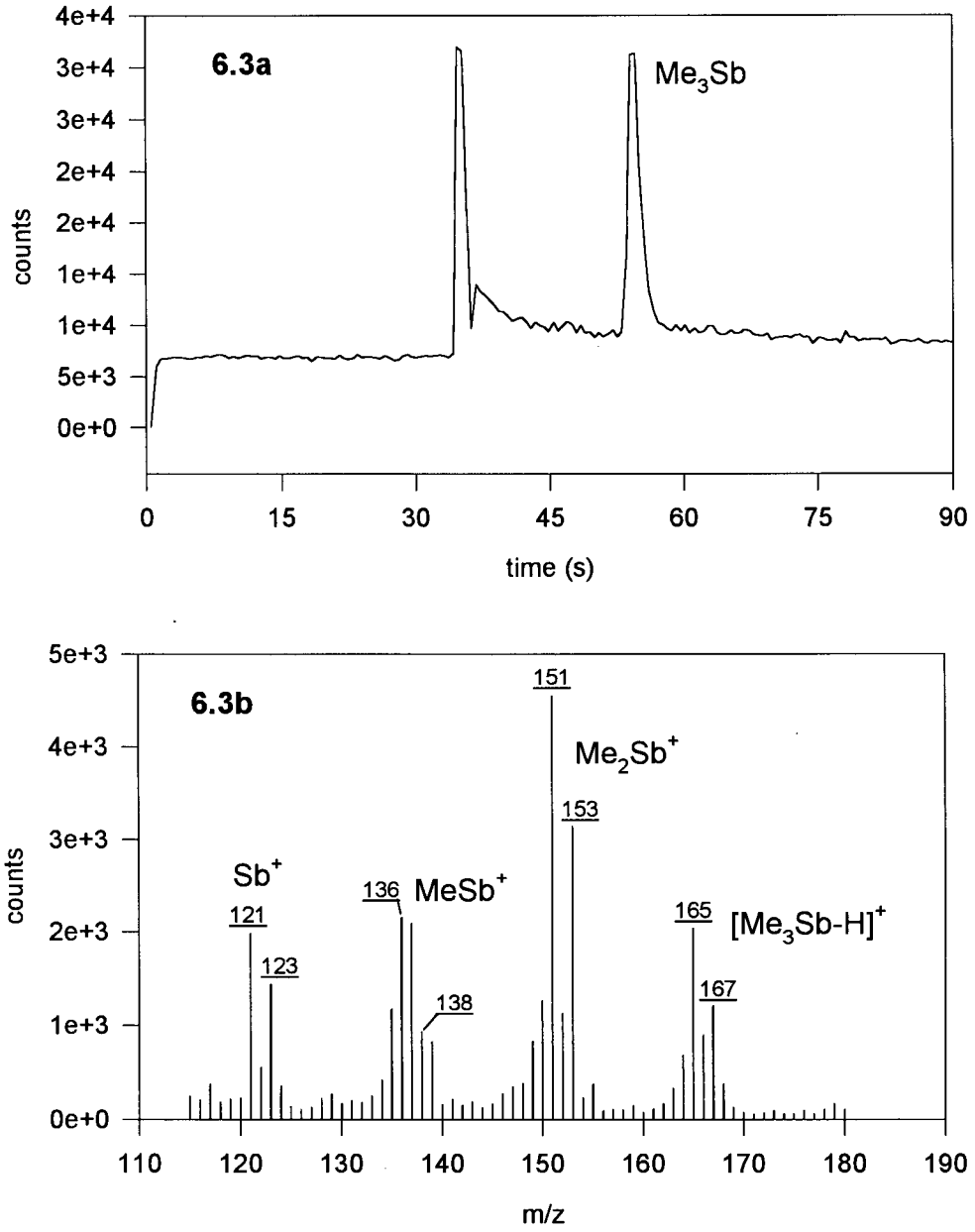


Figure 6.3a. Total ion chromatogram resulting from headspace-HG-GC-MS analysis showing Me_3Sb generated from 30 ng Me_3SbCl_2 (neutral). **Figure 6.3b.** Mass spectrum at 54.60 s corresponding to Me_3Sb.

In Figure 6.3, a chromatogram and mass spectrum are shown, corresponding to standard trimethylstibine (30 ng Sb as Me_3Sb) generated by using 30 μL of 1000 ppb Me_3SbCl_2 solution with 1 mL of deionized water and 0.5 mL of 6% (w/v) $NaBH_4$ solution. The dominant characteristic of all mass spectra involving antimony compounds is the appearance of the isotopic pattern due to masses of 121 and 123 (naturally occurring at about 52:48) in all Sb-containing fragments. This isotopic pattern is observed in Figure 6.3 at m/z 165/167, corresponding to $[Me_3{}^{121}Sb\text{-}H]^+/[Me_3{}^{123}Sb\text{-}H]^+$; at m/z 151/153 corresponding to $Me_2{}^{121}Sb^+/Me_2{}^{123}Sb^+$; at m/z 136/138, corresponding to $Me^{121}Sb^+/Me^{123}Sb^+$; and at m/z 121/123 corresponding to $^{121}Sb^+/^{123}Sb^+$. This mass spectrum is similar to one obtained previously by using the same GC-MS[29] and also to spectra obtained by using a quadrupole mass spectrometer[5]. The loss of a methyl group from methylstibines is the prevalent fragmentation pattern and is considered to be typical for methylated organometallic compounds[30].

As discussed in Chapter 2, enhanced demethylation of trimethylstibine is observed when the hydride generation reaction is performed at low pH. Therefore, acidic HG conditions resulting in demethylation can be used to generate mass spectra for methylstibine and dimethylstibine. In Figure 6.4, the chromatogram obtained from hydride generation of Me_3SbCl_2 (100 ng Sb) at low pH (Figure 6.4a) and the mass spectra obtained for the peaks assumed to be methylstibine (Figure 6.4b) and dimethylstibine (Figure 6.4c) are shown. The fragmentation pattern for dimethylstibine is similar to that observed for trimethylstibine, because the most abundant m/z appears as a result of methyl loss ($MeSb^+$ from Me_2SbH). The mass spectrum for the peak at a retention time of 39.27s most likely corresponds to the one expected for $MeSbH_2$, even though the high background levels obscures a clear, characteristic fragmentation pattern. However, fragments at m/z 121/123 and 136/138 were observed, corresponding to Sb^+ and $MeSb^+$, respectively.

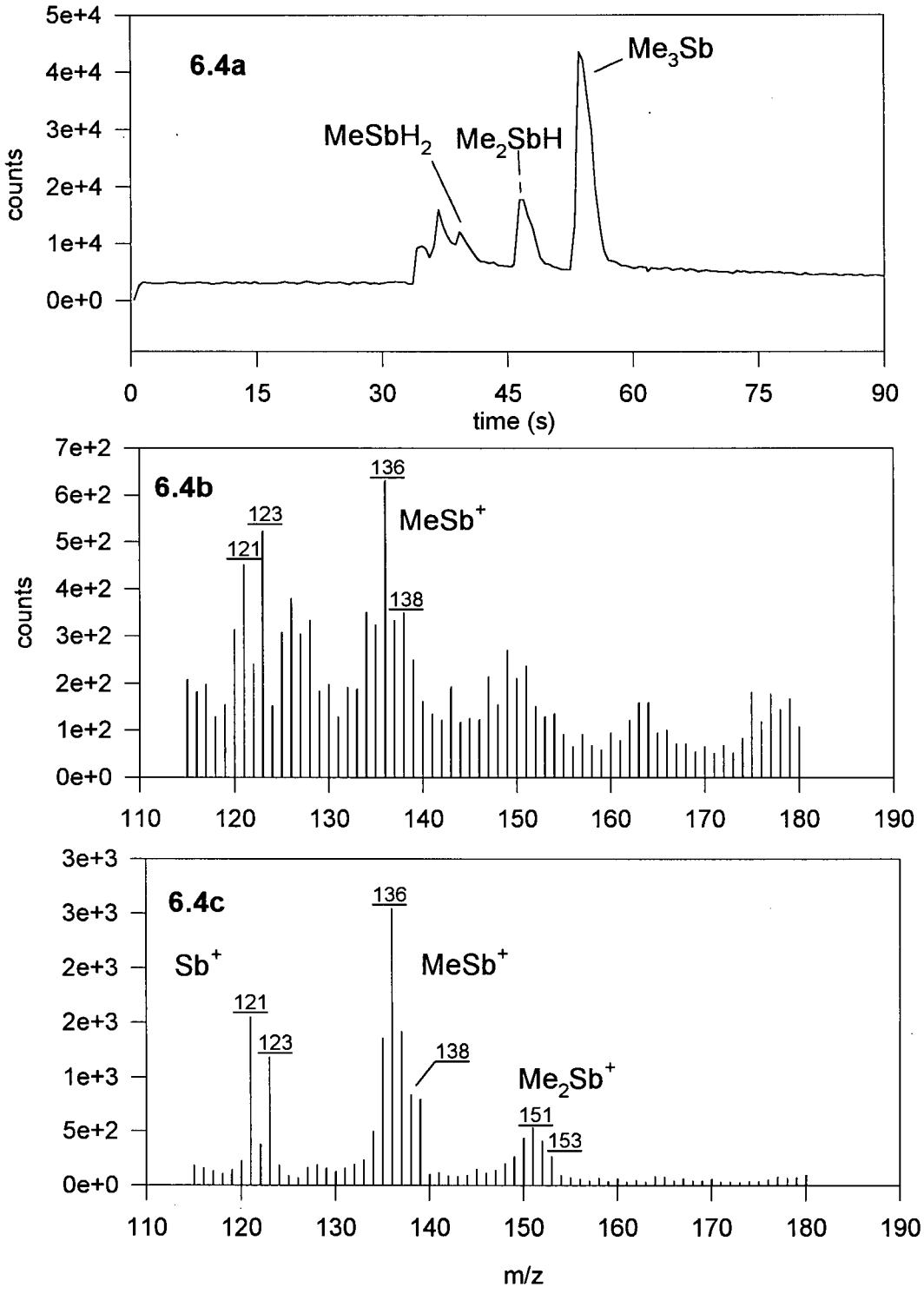
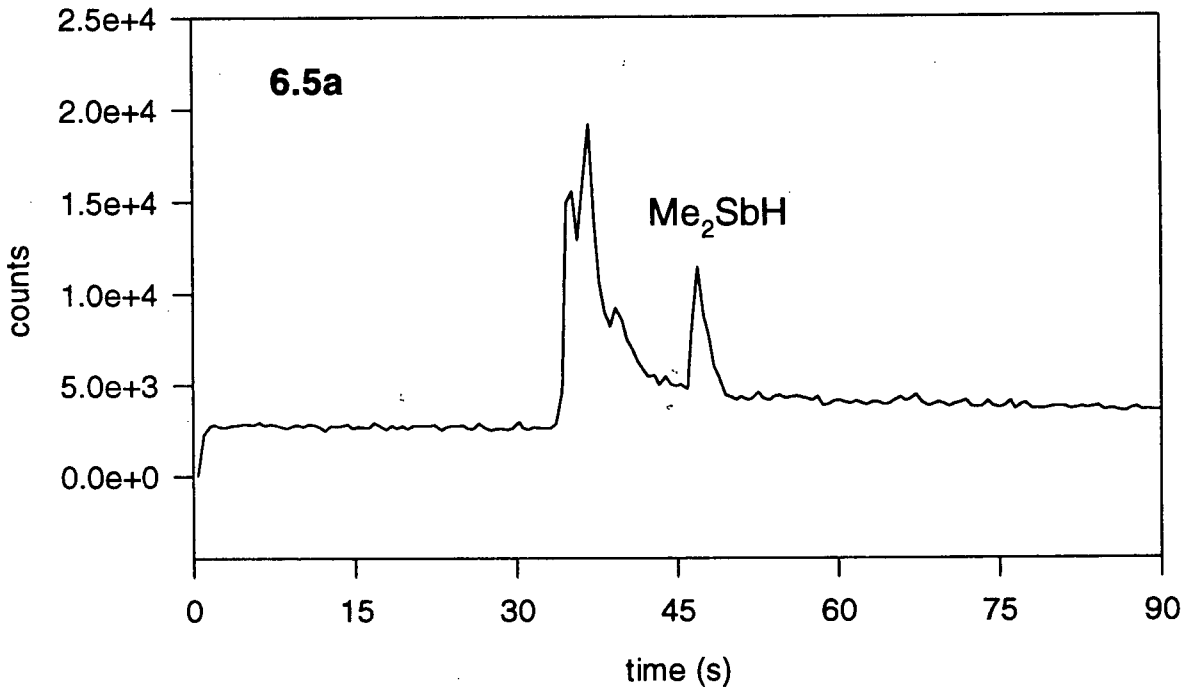


Figure 6.4a. Total ion chromatogram resulting from headspace-HG-GC-MS analysis showing stibines generated from 100 ng Me_3SbCl_2 (1 M HCl). **Figure 6.4b.** Mass spectrum at 39.27 s corresponding to $MeSbH_2$. **Figure 6.4c.** Mass spectrum at 49.61 s corresponding to Me_2SbH.

The use of HG-GC-MS was useful in the past for the identification and confirmation of methylated antimony species in plant samples from Yellowknife[5]. The direct injection of fractions of gas samples into an ion trap GC-MS resulted in the conclusive identification of trimethylstibine, trimethylbismuthine and methyltin compounds in landfill and fermentation gases[29]. The headspace HG-GC-MS method developed in this work was anticipated to provide information about the presence of methylstibines following HG of Yellowknife samples.

Chromatograms and mass spectra for peaks corresponding in retention time to dimethylstibine for moss samples from Yellowknife are shown in Figures 6.5, 6.6 and 6.7. The mass spectra indicate that the compound found in the headspace following hydride generation of the sample extracts is indeed dimethylstibine, by comparison with the mass spectrum shown in Figure 6.4c.

The chromatogram and mass spectra for the peaks corresponding in retention time to dimethylstibine and trimethylstibine for the snail extract are shown in Figure 6.8. Again, comparison of the mass spectra with those for standards (Figures 6.3b and 6.4c) indicates the presence of dimethyl- and trimethylstibine following HG of the extract. Differences in m/z abundances are probably due to interfering ions causing slightly different fragmentation patterns. For example, in Figure 6.8c (the mass spectrum for the peak corresponding to trimethylstibine) m/z 166 and 168 are observed (corresponding to Me_3Sb^+) rather than 165 and 167 (corresponding to $[Me_3Sb\text{-}H]^+$), which were the m/z observed for the standard compound (Figure 6.3b). For this sample, background subtraction was necessary to isolate the major m/z of interest, because of low levels of antimony in the extract and large amounts of other matrix components.



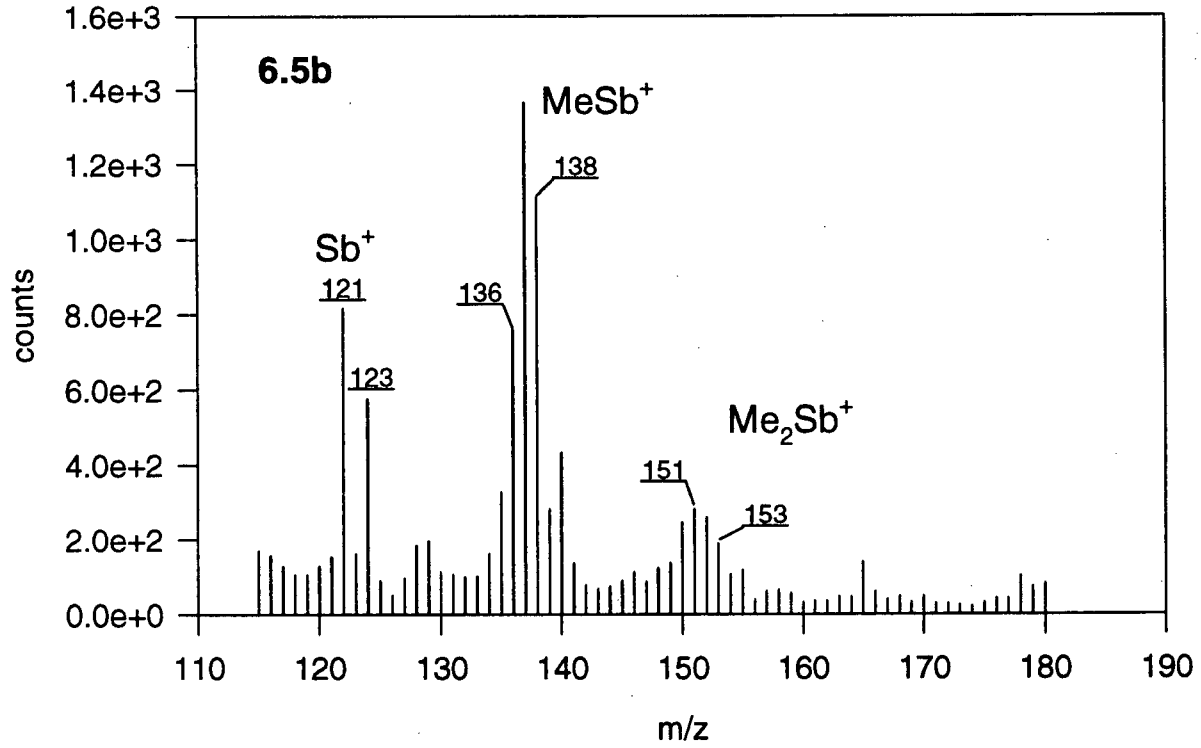


Figure 6.5a. Total ion chromatogram resulting from headspace-HG-GC-MS analysis of 5 mL of moss extract (June, YK Location 1) showing Me_2SbH. **Figure 6.5b.** Mass spectrum at 46.90 s corresponding to Me_2SbH.

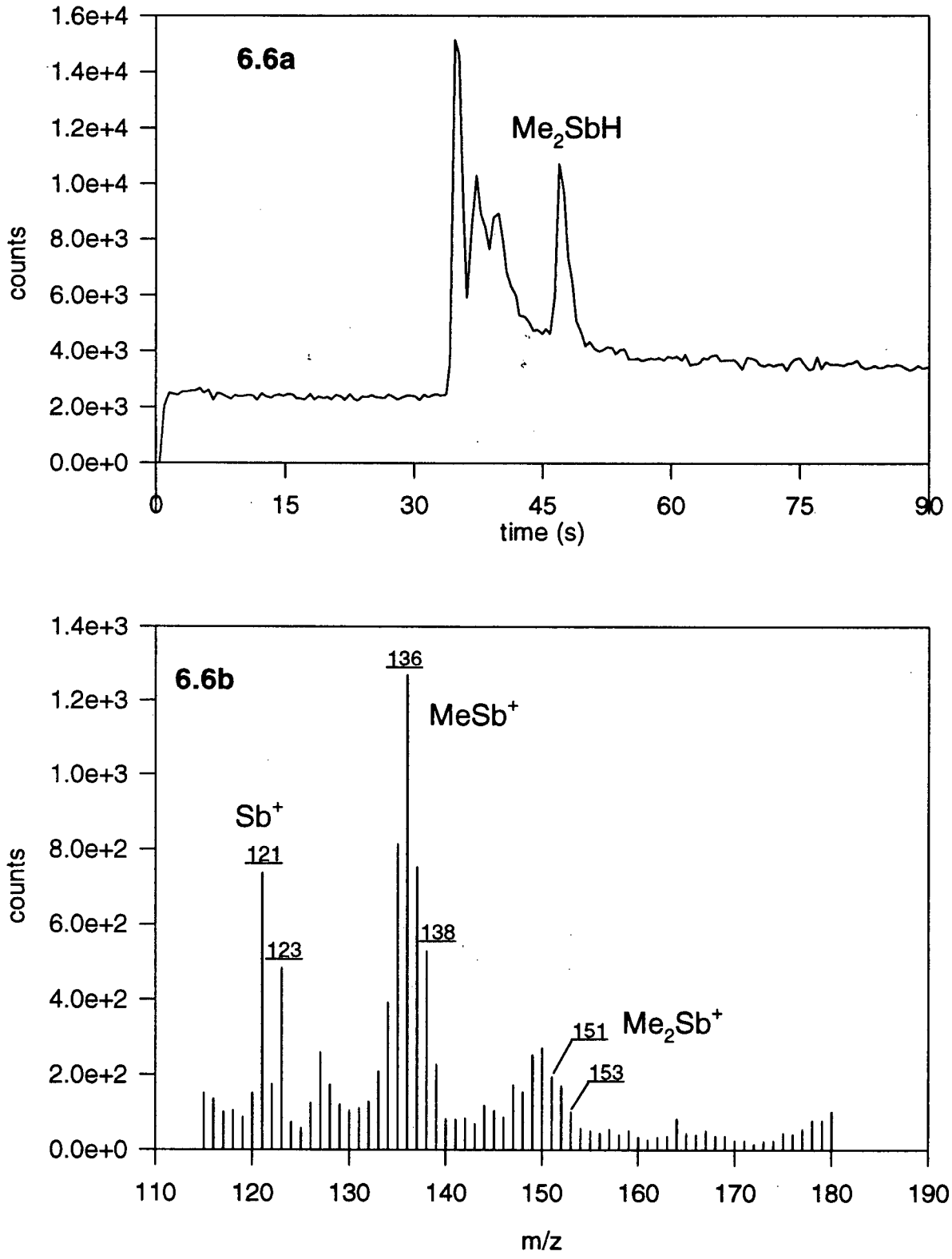


Figure 6.6a. Total ion chromatogram resulting from headspace-HG-GC-MS analysis of 5 mL of moss extract (August, YK Location 1) showing Me_2SbH. **Figure 6.6b.** Mass spectrum at 46.96 s corresponding to Me_2SbH.

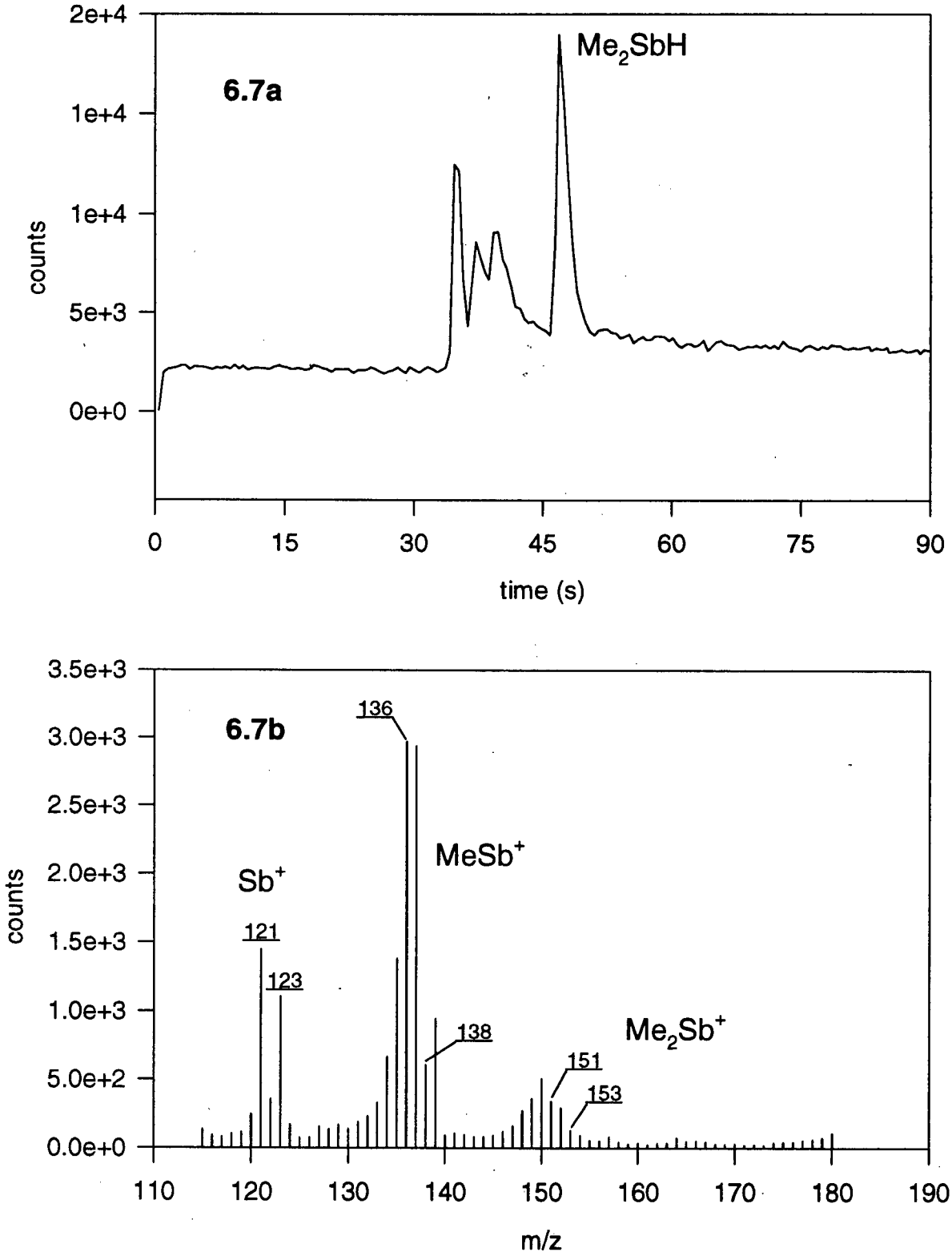


Figure 6.7a. Total ion chromatogram resulting from headspace-HG-GC-MS analysis of 5 mL of moss extract (August, YK Location 4) showing Me_2SbH. **Figure 6.7b.** Mass spectrum at 46.91 s corresponding to Me_2SbH.

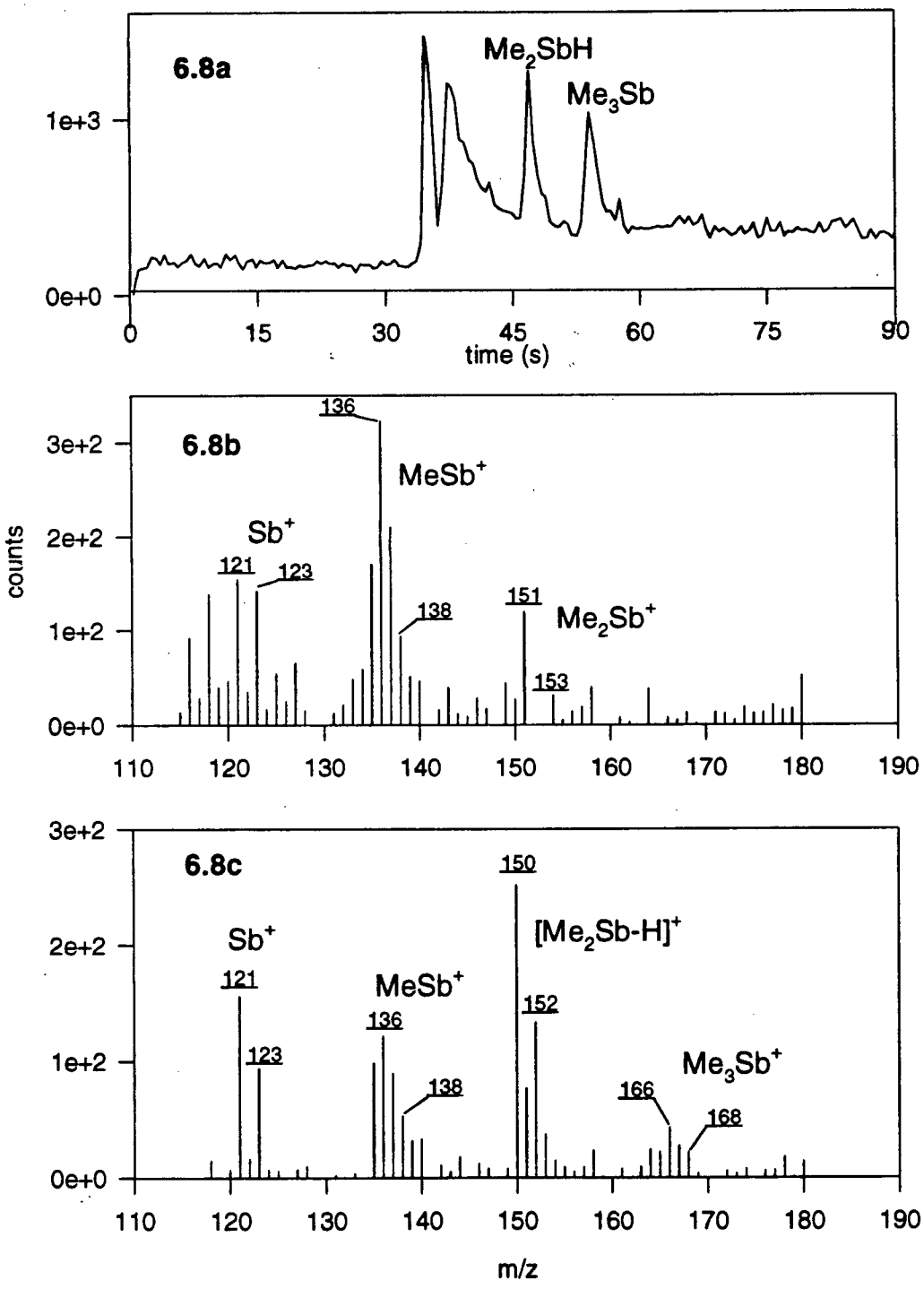


Figure 6.8a. Chromatogram (sum of Sb containing ions) resulting from headspace-HG-GC-MS analysis of 10 mL of snail extract (YK Location 1+3) showing Me_2SbH and Me_3Sb.
Figure 6.8b. Background corrected mass spectrum at 46.96 s corresponding to Me_2SbH.
Figure 6.8c. Background corrected mass spectrum at 54.05 s corresponding to Me_3Sb.

6.4. Summary

Antimony was extracted from environmental biota samples from Yellowknife and Meager Creek, with extraction efficiencies ranging from 0.7 to 37% for all samples except for *Typha* sp., from which 95% of antimony was extracted. Total antimony levels in most samples, when compared with arsenic levels, reflected the relative abundance of naturally occurring antimony (about 5-10% of arsenic). The exceptions were water samples from Baker Creek, receiving mine effluent from Giant Mine in Yellowknife, which contained antimony at concentrations similar to those for arsenic. This probably indicates that treatment of effluent to remove arsenic is successful, but that antimony is inefficiently removed during the treatment process.

Speciation analysis was carried out by using HG-GC-AAS. The major antimony species in all samples, including biota extracts and water, was Sb (V). Sb (III) and methylated antimony species were detected in some samples as well. The presence of methylated antimony species in moss from Yellowknife and a water sample from Yellowknife was confirmed by using HG-GC-AAS at a second absorption wavelength, increasing the likelihood that the peaks obtained are due to the presence of antimony compounds. A headspace HG-GC-MS method was developed for the speciation of antimony compounds and this was used to confirm methylantimony species in the headspace following HG of extracts of moss and snail samples from Yellowknife.

Because of the abundance of *Drepanocladus* sp. at location 1 in Yellowknife, and the seasonal consistency in its methylantimony content, this species of moss can be used as a laboratory standard for dimethylantimony. Future work could involve the use of moss extracts to study HPLC behaviour of the dimethylantimony compound present in the moss by using ICP-MS detection or MS detection for structural information.

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Chapter 7

CONCLUSIONS AND FUTURE WORK

Considerable knowledge was gained about arsenic and antimony species in the terrestrial environment.

Existing methods for speciation analysis were adapted for this work. Three HPLC methods with ICP-MS detection allowed separation and identification of at least 11 arsenic species. The agreement of retention times between chromatographic peaks in samples and standards, by using more than one method, corroborates identifications based on retention times. Arsenosugar standards were analyzed by using tandem ESI-IT-MS and fragmentation patterns specific to this mass analyzer were obtained. Only partial identification of arsenosugars in a crude kelp extract was achieved by using MS-MS and MS-MS-MS techniques (only arsenosugar XIII could be identified with any certainty). Future work should address clean-up of the kelp extract (e.g., the use of HPLC methods, before introduction to ESI-IT-MS).

The demethylation of trimethylantimony species during analysis by using HG-GC-AAS was characterized as being dependent on pH; lower pH resulted in more demethylation. Mechanistic reasons for demethylation were sought and it was established that methyl groups were lost during the HG reaction, rather than as a result of instability of the starting compound (Me_3SbCl_2) or the product (Me_3Sb) to acid. Care is recommended (and was taken) in the analysis of environmental or other samples for

antimony by using HG-GC methods to ensure that the methylantimony species identified are not an artifact of the method.

Non-toxic and very low levels of toxic water soluble arsenic species were identified in some edible mushrooms, and consequently these do not present a health concern to consumers. It would be interesting to determine if arsenic speciation changes as the arsenic concentration increases for the mushrooms analyzed. In some cases the extractable arsenic was inorganic and hence higher levels might become of greater toxicological concern. Pure culture experiments with fungi that can produce mushrooms indicated that the synthesis of arsenobetaine, arsenocholine or arsenosugars does not take place by the mycelia during the short experiment times. Arsenobetaine is accumulated by *Scleroderma citrinum* which may suggest that if any arsenobetaine is present in the growing environment of wild mushrooms, it may be accumulated by the fungus.

The interaction of antimony with fungus was confounded by the ubiquitous presence of an unknown antimony compound for which only partial characterization was accomplished. *Pleurotus flabellatus* formed an antimony-containing metabolite (of unknown identity, detected by using HPLC-ICP-MS) from inorganic antimony. This fungus also oxidized Sb (III) to $Sb(OH)_6^-$. Future experiments should include growth media that contain a minimum amount of salts and/or carbon sources, to simplify the matrix and hopefully eliminate the presence of unidentified antimony-binding compounds.

Novel results were obtained from a study of the arsenic species in samples from two terrestrial environments: a hot springs environment (Meager Creek, BC) and from an area impacted by smelting and mining (Yellowknife, NWT). Arsenosugars are apparently synthesized by cyanobacteria and other bacteria in microbial mats from both locations.

Thus thermophilic as well as non-thermophilic organisms have this capability. Small amounts of arsenosugars were also found in lichens, in higher plants, in some mushrooms, in green algae (belonging to the phylum Chlorophyta), in freshwater mussels and in suckers. Because they are bottom feeders, suckers probably accumulate arsenosugars from benthic organisms. Suckers also contain arsenobetaine which was the major arsenic compound found in all other fish analyzed, except for pike. In pike, the major detectable arsenic compound was DMA.

On the other hand, neither freshwater mussels nor snails contain appreciable quantities of arsenobetaine. This is in direct contrast to findings in the marine environment where marine mussels and gastropods usually contain arsenobetaine as a major or as the only arsenic compound. Freshwater mussels contain mostly arsenosugars and snails contained mostly tetramethylarsonium ion, as well as inorganic arsenic.

Arsenobetaine was found for the first time in lichens and it is present in all specimens from Yellowknife. It was also found in some mushrooms, including *Coprinus comatus* and *Lycoperdon pyriforme*. The identity of arsenic species was determined for the first time in *Paxillus involutus, Psathyrella candolleana* and *Leccinum scabrum*.

The major arsenic species extracted from higher plants, as well as lichens, mosses, algae and microbial mats were inorganic (arsenite and arsenate). A large amount of arsenic remained unextracted or undetected in all types of samples. The nature of this arsenic is unknown and hence more studies are imperative to determine its chemical and toxicological significance. Arsenic that is not extracted may be bound to lipids, cell components, proteins, or it may exist in a mineral form.

Antimony species were determined in some samples from Meager Creek and Yellowknife, and mostly Sb (V) was found (although, as for arsenic, a large fraction remained unextracted). In moss samples, however, a dimethylantimony species in the form of a compound that resulted in dimethylstibine being formed from hydride generation of the extract, is present. Derivatization of extracts of the same moss species from two different locations, and from the same location at different times (June and August) yielded dimethylstibine in all cases. Future work to identify this compound would be very interesting.

Arsenic speciation in the terrestrial environment, in general, does not appear to be as complex as in the marine environment. For example, arsenosugars and arsenobetaine, which are the major arsenic compounds in marine plants and animals, respectively, occur either rarely or in very small amounts in terrestrial samples. This may indicate that different metabolic pathways are followed in the terrestrial environment, or that the processes in the marine environment are not important in the terrestrial environment.

Little is known about the speciation of antimony in the environment, and this study helps to establish a knowledge base for this element. Inorganic antimony appears to be the predominant species and there is no evidence yet for antimony analogues of arsenosugars or arsenobetaine. However the apparent absence of these compounds may be due to the present lack of appropriate analytical methodology for antimony speciation.