ORIGIN AND FATE OF ARSENOBETAINE IN MUSHROOMS

ORIGINE ET DESTIN D'ARSENOBETAÏNE DANS LES CHAMPIGNONS

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Abstract

Arsenic (As) occurs ubiquitously in the environment in many different forms (referred to as species) that differ in their physical, chemical and biological properties as well as toxicities. Only one of these species, arsenobetaine (AB), is considered to be non-toxic. AB has been identified in high proportions and concentrations in many marine organisms, but it is found to a much lesser extent in the terrestrial environment. The fruiting bodies of some terrestrial fungi (i.e., mushrooms) are one of the few terrestrial organisms that contain AB in high proportions, thereby offering a unique opportunity to investigate the formation of AB in a non-marine system. This thesis examines the total arsenic concentration and arsenic species present in mushrooms, as well as potential formation pathways for AB.

To investigate the impact of the growth substrate and the role of microbial communities on arsenic speciation in mushrooms, the arsenic speciation was compared in log- and soil-growing mushrooms. The total arsenic concentrations, measured with inductively coupled plasma mass spectrometry (ICP-MS), and arsenic speciation, measured with high performance liquid chromatography (HPLC) – ICP-MS, were determined for 10 soil-growing and nine log-growing mushrooms collected from an area with naturally elevated arsenic concentrations. The predominant arsenic species were similar among phylogenetic groups and morphologies, and corresponded to previous trends observed in these categories. AB was detected in several of the log-growing species, indicating that the growth substrate and/or microbial community are not solely responsible for the presence/absence of AB. Measurements using X-ray absorption spectroscopy (XAS), specifically X-ray absorption near edge structure (XANES), of arsenic speciation in mycelia of mushrooms containing AB, revealed no AB in mycelia either from logs or the ground, verifying the specificity of AB to the reproductive (fruiting body) stage of the mushroom. The role of AB in mushrooms was hypothesized to be similar to that of glycine betaine, a known osmolyte. Betaine (measured with HPLC-mass spectrometry) and previously measured AB concentrations were compared for 46 mushrooms. Low betaine concentrations were typically accompanied by an absence of AB and AB was generally detected in mushrooms with a high betaine concentration. This suggests a similar, but not competitive role for these two compounds, indicating that mushrooms capable of utilizing betaine may also be able to use AB.

Currently, there has been no research conducted on the possible speciation changes in mushrooms as a result of common preparation methods for consumption, that is, cooking. As mushrooms are one of the only commonly consumed terrestrial organisms that contain high proportions AB, it is crucial to identify if speciation changes to more toxic species are taking place. A total of 12 edible mushrooms

were subjected to a variety of different high temperature preparation methods (frying, baking, and barbequing). For most mushrooms, cooking, specifically frying, resulted in a decrease in the overall total arsenic concentration. However, this was accompanied by changes in the proportions of the arsenic species. For some mushrooms, the thermal treatments resulted in the methylation of some arsenic species to form trimethylarsine oxide (TMAO). Degradation of AB to the more toxic tetramethylarsonium ion (TETRA) was also observed.

Synthetic experiments were conducted to investigate the feasibility of two different hypothesized AB formation pathways in the terrestrial environment. The first synthetic experiment confirmed that TMAO could be transformed to AB using glutathione (GSH) and iodoacetic acid (IoAA). Iodoacetic acid was identified in two of three soils examined in the present study, verifying that all of these reactants can be found in soils and making this a very plausible formation pathway for AB in the terrestrial environment. In the second synthetic experiment dimethylarsinous acid (DMA(III)) was investigated as a potential precursor to AB, but no AB was identified in the final reaction products. However, this may have been due to experimental conditions and DMA(III) should continue to be investigated as a potential precursor to AB.

The findings of this thesis have increased the knowledge about factors influencing the origin, role, and fate of AB in mushrooms and a synthetic pathway for the formation of AB under naturally occurring environmental conditions has been identified. However, future study is warranted to determine the exact formation pathway for this compound in mushrooms.

Résumé

Arsenic (As) est omniprésent dans l'environnement et existe sous différentes formes (nommées espèces) qui diffèrent par leurs propriétés physiques, chimiques et biologiques ainsi que par leurs toxicités. Une seule de ces espèces, arsénobetaïne (AB), est considérée comme non toxique. L'AB a été identifiée en proportion et concentration élevées dans de nombreux organismes marins, mais est peu présent dans l'environnement terrestre. Les organes de fructification de certains champignons terrestres (i.e. champignons) sont l'un des rares organismes terrestres qui contient de l'AB en proportion élevée; offrant ainsi une occasion unique d'étudier leur formation dans un système non-marin. Cette thèse examine la concentration total et les diverses espèces d'arsenic présent dans les champignons, ainsi que les voies de formation potentielle de l'AB.

Pour étudier l'impact du substrat de croissance et le rôle des communautés microbiennes sur la spéciation de l'arsenic dans les champignons, ceux-ci ont été cultivés en sol et sur bûche. La concentration d'arsenic total, mesurée par spectroscopie de masse à plasma à couplage inductif (ICP-MS), ainsi que la spéciation, mesurée par la chromatographie en phase liquide à haute performance (HPLC) – ICP-MS, ont été déterminée pour 10 sols et 9 bûches provenant de zone historiquement contaminée par l'arsenic. Les espèces d'arsenic prédominant étaient similaires parmi les groupes phylogénétiques et morphologies, et correspondent aux tendances antérieures observées dans ces catégories. L'AB a été détectée dans plusieurs espèces de champignon poussant sur bûche, ce qui indique que le substrat de croissance et/ou de les communautés microbiennes ne sont pas les seuls responsable de la présence absence d'AB.

Les mesures par spectroscopie d'absorption des rayons X (XAS), plus précisément spectroscopie de structure près du front d'absorption des rayons X (XANES), n'a révélé aucune AB dans les mycéliums du sol ou des bûches, montrant la spécificité de l'AB pour l'organe de fructification lors du stade reproduction. L'hypothèse a été émis que le rôle de l'AB dans les champignons est semblable à celle de la glycine bétaïne, un osmolyte connu. La bétaïne (mesurés par HPLC et spectrométrie de masse) et les concentrations AB précédemment mesurées ont été comparées pour 46 champignons. Les faibles concentrations de bétaïne sont généralement accompagnées d'une absence d'AB et l'AB a généralement été détectée dans les champignons avec une concentration élevé de bétaïne. Ceci suggère un rôle similaire, mais pas compétitif pour ces deux composés, ce qui indique que les champignons capables d'utiliser bétaïne peuvent également être en mesure d'utiliser AB.

Présentement, aucune recherche n'a été menée sur les possibles changements de spéciation de l'arsenic dans les champignons lors de leur préparation pour la

consommation humaine. Puisque les champignons sont l'un des seuls organismes terrestres couramment consommés contenant des proportions élevées d'AB, il est crucial d'identifier si la spéciation de l'arsenic change pour des espèces plus toxiques. Un total de 12 champignons comestibles ont été soumis à une variété de différentes cuisons à haute température (friture, cuisson, et barbecue). Pour la plupart des champignons le mode de cuisson, particulièrement la friture, a entraîné une diminution de la concentration de l'arsenic total. Cependant, ceci était accompagné par des changements dans les proportions des espèces d'arsenic. Pour certains champignons, les traitements thermiques ont donné lieu à la méthylation de certaines espèces d'arsenic pour former du triméthylarsine oxyde (TMAO). La dégradation de l'AB en un ion plus toxique, le tétraméthylammonium (TETRA), a également été observée.

Des expériences de synthèse ont été menées afin étudier la faisabilité des deux hypothétiques voies de formation de l'AB dans l'environnement terrestre. La première expérience a confirmé que le TMAO synthétique peut être transformé en AB en utilisant le glutathion (GSH) et l'acide iodoacétique (IoAA). L'acide iodoacétique a été identifiée dans deux des trois sols examinés lors de cette étude, montrant que ce réactif peut être présent dans les sols, rendant cette voie de formation de l'AB très plausible dans l'environnement terrestre. Dans la seconde expérience, l'acide diméthylarsenic (DMA(III)) a été étudiée comme précurseur potentiel de l'AB, mais aucune AB n'a été identifié dans les produits finaux de la réaction. Toutefois, cela peut être dû aux conditions expérimentales et DMA (III) devrait continuer à être étudiée comme un précurseur potentiel de l'AB.

Les résultats de cette thèse ont permis d'accroitre les connaissances sur les facteurs qui influencent l'origine, le rôle et le destin de l'AB dans les champignons, ainsi que l'identification d'une voie de synthèse en conditions naturels. Cependant, de futures études sont nécessaires afin de déterminer les voies exactes de formation dans les champignons.

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List of Abbreviations

Abbreviation	Definition
µXANES	micro X-ray absoprtion near edge structure
AB	Arsenobetaine
AB-Br^-	Arsenobetaine bromide
AC	Arsenocholine
AC-Br^-	Arsenocholine bromide
Acetyl-CoA	Acetyl-coenzyme A
AC-I^-	Arsenocholine iodide
APL	Acute promyelocytic leukemia
APS	Advanced photon source
As	Arsenic
As(III)	Arsenite
As(-III)	Arsine
As(V)	Arsenate
AsS	Arsenosugar
ATP	Adenosine tri-phosphate
ATRA	all-*trans*-retinoic acid
ATSDR	Agency for Toxic Substances and Disease Registry
BBQ	Barbequed
BC	British Columbia
BCR	Community Bureau of Reference
BM	Bending magnet
CRM	Certified reference material
CCME	Canadian Council of Ministers of the Environment
DDW	Double-deionized water
DMA(III)	Dimethylarsinous acid
DMA(III)I	Dimethylarsinous iodide
DMA(V)	Dimethylarsinic acid
DMAA	Dimethylarsinoyl acetic acid
DMAE	Dimethylarsinoyl ethanol
DMAsSs	Dimethylated arsenosugars
DNA	Deoxyribonucleic acid
dw	Dry Weight
EE	Extraction efficiency

Abbreviation	Definition
ESI-MS	Electrospray mass spectrometry
$FADH_2$	Flavin adenine dinucleotide
FAO	Food and Agriculture Organization
FB	Fruiting Body
GSH	Glutathione
GST	Glutathione S-transferase
HA	Hard acid
HB	Hard base
HPLC	High performance liquid chromatography
HSAB	Hard/soft acid/base
HSP27	Heat shock protein 27
iAs	Inorganic arsenic
ICP-MS	Inductively coupled plasma mass spectrometry
ID	Insertion device
IM	Intramuscular
IoAA	Iodoacetic acid
IP	Intraperitoneal
IRIS	Integrated risk information system
KGDH	Ketoglutarate dehydrogenase
KI	Potassium iodide
LD_{50}	Lethal Dose 50%
LOD	Limit of detection
LOQ	Limit of quantification
$MetCB_{12}$	Methylcobalamin
MGPB	Mushroom growth promoting bacteria
MMA(III)	Monomethylarsonous acid
MMA(V)	Monomethylarsonic acid
MMTA	monomethylthioarsonic acid
MOA	Mode of action
MRM	Multiple reaction monitoring
MS/MS	mass spectrometry/mass spectrometry
NADH	nicotinamide adenine dinucleotide
NS	Not specified
NSERC	Natural Sciences and Engineering Research Council
ON	Ontario

Abbreviation	Definition
PARP-1	Poly(ADP-ribose) polymerase-1
PDC	Pyruvate dehydrogenase complex
PHD	Pyruvate dehydrogenase
PML-RARa	promyelocytic leukemia protein retinoic acid receptor alpha
PNC/XSD	Pacific Northwest Consortium/X-ray Science Division
PNP	Purine nucleoside phosphorylase
QA/QC	Quality assurance/quality control
RNA	Ribonucleic acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPD	Relative percent difference
SA	Soft acid
SAM	S-adenosyl methionine
SB	Soft base
TDS	Total dietary study
TETRA	Tetramethylarsonium ion
TMA(III)	Trimethylarsine
TMAO	Trimethylarsine oxide
TMAsSs	Trimethylated arsenosugars
UK	Unknown
USA	United States
WHO	World Health Organization
ww	Wet weight
XANES	X-Ray absorption near-edge structure
XAS	X-Ray absorption spectroscopy
XPA	Xeroderma pigmentosum group A protein
YK	Yellowknife

Chapter 1 – Introduction

Arsenic is an element found in the environment in many different chemical forms (referred to as species), each having unique chemical, physical and biological properties. The different species vary in their toxicities, with the more toxic arsenic species including inorganic forms, such as arsenite (As(III)) and arsenate (As(V)). Generally, pentavalent organoarsenic compounds (i.e. containing a carbon-arsenic bond) are less toxic than their trivalent analogues and arsenobetaine (AB) is the only species considered to be non-toxic. Marine organisms tend to contain higher concentrations of arsenic (from less than 1 to more than 50 mg/kg, wet weight (ww) (Kohlmeyer et al., 2003)), whereas freshwater and terrestrial biota usually contain less than 1 mg/kg, ww (Aitio and Becking, 2001). While marine organisms contain more arsenic, it is generally in the form of less or non-toxic species (i.e. AB, arsenocholine (AC) and arsenosugars (AsSs)) (Edmonds and Francesconi, 1993), with AB being prevalent mostly in higher trophic level organisms like animals. The terrestrial environment (i.e. soil, groundwater, and plants) is typically dominated by inorganic arsenic species (Ma et al., 2014). One of the few exceptions to this generalization is the fruiting body of terrestrial fungi (i.e. mushrooms), which can contain high proportions of AB. This provides a unique system that is simpler than higher level marine organisms in which to analyze the formation pathway, role, and fate of AB.

Chapter 2 of this thesis is a literature review that discusses the intricacies of arsenic toxicity, its mechanisms of action, and use as a potent chemotherapeutic agent against acute promyelocytic leukemia (APL). The review also examines the toxicology of AB, how it behaves in the human body, and abiotic/chemical and biologically mediated potential synthetic pathways for AB formation. The chapter considers the main route by which humans are exposed to arsenic – through food – and discusses potential transformations that may take place during food preparation that could result in more toxic arsenic species. This chapter concludes with identifying the current research gaps in the literature. In short, the main areas requiring further investigation are the following:

1) *The exact organism(s)/pathways of formation for AB in terrestrial fungi.* It has been suggested that the varying microbial communities in the growth substrate (i.e. if the mushroom is growing in soil vs. on a log) may impact the composition of arsenic within the mushroom. It has been suggested that microbes may play an important role in AB formation. In this thesis, it was therefore hypothesized that log-growing mushrooms would not contain AB as a result of a microbial community in the growth substrate that is different (possibly smaller or less diverse) from that in soil.

2) *AB is thought to play an osmolytic role in mushrooms*, but this has not been proven. AB is structurally similar to glycine betaine, which is known for its osmolytic function in various organisms. AB may act osmolytically in the fruiting body of mushrooms to help maintain the structural integrity, ensuring the cap remains turgid and elevated for effective spore dispersal. It is hypothesized that AB concentrations will correlate with betaine concentrations, demonstrating their relationship and endorsing the role of AB as an osmolyte.

3) *Changes to the total arsenic and arsenic speciation as a result of cooking.* This has never been examined in mushrooms. Studies have been conducted for seafood (which typically contains high proportions of AB), and it was demonstrated that an increase in more toxic species may occur. It is considered that similar transformations will occur in mushrooms as a result of cooking.

4) *The exact pathway in which AB is formed in the fruiting body of mushrooms.* There is very little literature describing feasible formation pathways for AB in the terrestrial environment; pathways may be different from those in the marine environment (where AsSs are thought to be involved), since AsSs are generally not present in the terrestrial environment. Pathways using naturally occuring relevant arsenic compounds as precursors to AB are investigated in this thesis. It is hypothesized that both dimethylarsinous acid (DMA(III)) and trimethylarsine oxide (TMAO) may be possible precursors to AB.

In Chapter 3, sample collection, materials, and experimental methods are discussed. The quality assurance/quality control (QA/QC) measures applied during analytical measurements are outlined and the QA/QC results are discussed in order to provide the context for the use of measured data throughout the thesis.

Chapter 4 of this thesis describes the results associated with the mushroom-focused research. Total arsenic and arsenic speciation results for soil- and log-growing mushrooms are examined to determine the influence of growing environment and substrate on the arsenic speciation in mushrooms. Additionally, AB's role in water retention in mushrooms is investigated, specifically whether it is similar to that of osmolytes like betaine, a compound with a structure similar to that of AB.

In Chapter 4, the fate of the arsenic species after cooking (frying, baking, and/or barbequing) wild and store-bought mushrooms is also discussed. The results from this section are put in the context of relative toxicity to humans when cooked mushrooms are eaten.

The more synthetic-focused experiments, exploring the origins and formation of AB via pathways that have the potential to be exclusive of biological organisms, are presented in Chapter 5. The results from two potential synthetic pathways are

presented, and the validity of one of them is tested by examining the presence of one of the starting reactants (iodoacetic acid) in environmental samples.

Chapter 2 – Literature Review

2.1. Introduction to Arsenic

2.1.1. Arsenic in the Environment

Arsenic is a metalloid found in water, soil, and air as a result of both natural and anthropogenic sources. Research suggests that about one quarter of the atmospheric arsenic is of natural origin, mainly from volcanic eruptions of gas and ash but also from the weathering/erosion of rocks and minerals and biological processes (Wang and Mulligan, 2006, Aitio and Becking, 2001). Natural global emissions of arsenic and arsenic compounds are estimated to be approximately 8000 tonnes per year (Rossman, 2003).

The human contribution to arsenic contamination of the environment is mainly a result of processes such as high-temperature combustion (e.g. coal and oil combustion), metal treatments, ammunition factories, the use of pesticides and fertilizers, mining and smelting, and wood preservation (Oremland and Stolz, 2003, Matschullat, 2000, Pacyna, 1987). Copper smelting and the combustion of fossil fuels, specifically, are the two predominant sources of arsenic, together accounting for about sixty percent of anthropogenic arsenic emission (Martin et al., 2014, Chilvers and Peterson, 1987). Anthropogenic arsenic emissions are three times greater than natural emissions, at approximately 24,000 tonnes per year (Rossman, 2003).

In the pedosphere, arsenic concentrations are highly variable and depend on the location and environmental conditions (Matschullat, 2000). For example, arsenic concentrations are higher near man-made sources such as pesticide manufacturing plants or mines (Aitio and Becking, 2001). Arsenic cannot be destroyed in the environment; it can only transform into different chemical forms or change the way it is bound (Agency for Toxic Substances and Disease Registry (ATSDR), 2007). Arsenic and its compounds are included in Group 1 of the Priority Substances List on the basis of the potential harm that these compounds can pose to the environment and human health (Environment Canada, 2013).

2.1.2. Chemical Forms of Arsenic

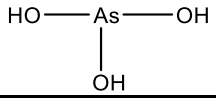
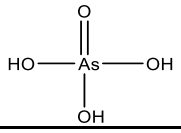
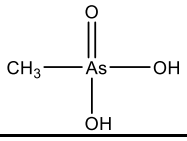
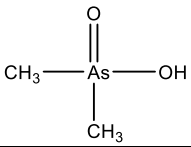
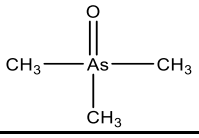
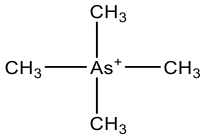
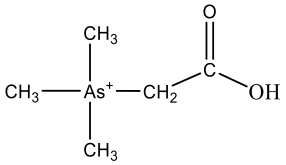
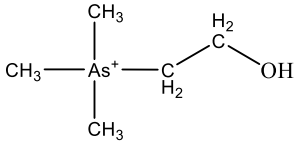
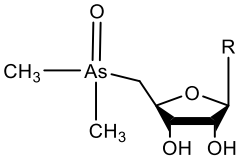
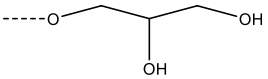
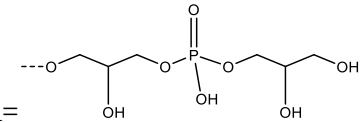
Arsenic (As), element number 33 on the periodic table, occurs in nature in many different forms (referred to as species) that differ in their physical, chemical, biological, and toxicological properties and have very diverse toxicities (Sharma and Sohn, 2009). Arsenic can exist in four oxidation states (+5, +3, 0, and -3). The +3 and +5 forms are the most common in the environment. The presence of the -3 state in the environment is debatable because the oxidation state of As in As-H

bonds is unclear due to the relatively similar electronegativities of As and H (Cullen and Reimer, 1989).

Organoarsenic compounds are those that are formed when arsenic and carbon share a covalent bond (Table 2-1) (Agency for Toxic Substances and Disease Registry (ATSDR), 2007). Arsenic in combination with other elements (such as oxygen, chlorine, or sulfur) for a category of inorganic arsenic (iAs) compounds.

In aqueous, oxygenated environments (under oxidizing conditions), the most prevalent form of iAs is arsenate (As(V) as $H_2AsO_4^-$ (at pH<6.9) and $HAsO_4^{2-}$ (at pH>6.9). In anoxic environments (under reducing conditions), arsenite is more common (As(III) as H_3AsO_3 (at pH< 9.2) and $H_2AsO_3^-$ (at pH>9.2)) (Rakhunde et al., 2012, Bos et al., 2011). Both of these forms of iAs are toxic and can interconvert when there are changes to the redox potential and pH (Cullen and Reimer, 1989). Monomethylarsonic acid (MMA(V)) and dimethylarsinic acid (DMA(V)) are stable pentavalent methylated versions of these two iAs species. The trivalent versions of these compounds also exist, as monomethylarsonous acid (MMA(III)) and dimethylarsinous acid (DMA(III)). In addition to iAs compounds and their methylated derivatives, other arsenic compounds exist, such as AB (sometimes called “fish arsenic”), AC and arsenosugars (AsS) (Sadee et al., 2015).

Table 2-1: Organoarsenicals and inorganic arsenic species found in the environment, which are of toxicological relevance, along with their associated abbreviations, chemical formula and structure.

Name	Acronym	Formula	Structure
Inorganic Arsenic (iAs) Compounds			
Arsenite (arsenous acid)	As(III)	$As(OH)_3$	
Arsenate (arsenic acid)	As(V)	$AsO(OH)_3$	
Organoarsenic Compounds			
Monomethylarsonic acid	MMA(V)	$(CH_3)AsO(OH)_2$	
Dimethylarsinic acid	DMA(V)	$(CH_3)_2AsO(OH)$	
Trimethylarsine oxide	TMAO	$(CH_3)_3AsO$	
Tetramethylarsonium ion	TETRA	$(CH_3)_4As^+$	
Arsenobetaine	AB	$(CH_3)_3As^+CH_2COO^-$	
Arsenocholine	AC	$(CH_3)_3As^+CH_2CH_2OH$	
Arsenosugars	AsS		Example: AsS-OH  R= AsS-PO_4  R=

2.1.3. Toxicity of Arsenic Compounds

Arsenic's ubiquitous presence in the environment means that humans can be exposed through many different pathways. These include inhalation, dermal contact, and ingestion of both arsenic–containing food and water.

The main source of arsenic exposure for humans is through food, except in areas where the drinking water is naturally contaminated with arsenic (in its inorganic form) (Aitio and Becking, 2001). The severity of arsenic exposure depends on two factors, the dose (the amount of arsenic and the frequency of the exposure) and the chemical species. The toxicity and mobility of each arsenic species depends on its structure, as well as the valence/oxidation state of the arsenic atom, as mentioned in the previous section (National Research Council, 1999, Edmonds and Francesconi, 1993).

The toxicity of arsenic is divided into two categories, acute (short term) and chronic (long-term) exposure. Acute arsenic poisoning can result in nausea, vomiting, abdominal pain, severe diarrhoea, bloody urine, shock, convulsions, coma, and even death (Ratnaike, 2003). The acute toxicity of different arsenic species in terms of their LD_{50} values (the milligrams (mg) of arsenic per kilogram (kg) of body weight that results in the death of 50 percent of a population of test animals) is provided in Table 2-2. Discrepancies between these values and other literature values can be attributed to the varying experimental conditions (i.e. different modes of administration and different species of animals). Generally, for organoarsenic species, the trivalent analog of a compound is more toxic than its pentavalent form and inorganic compounds can be more toxic than organoarsenicals (usually in the +5 oxidation state) (Reimer et al., 2010). An example of increased toxicity of an organoarsenical (in the +3 oxidation state) compared with iAs is the lower LD_{50} of MMA(III) compared with inorganic As(III) in hamsters. The lethal dose is known for adult humans for some of the toxic inorganic species because of documented poisoning cases. For arsenic trioxide, a fatal dose is between 100 to 200 mg (approximately the amount of one or two standard-size pills) (Benramdane et al., 1999a).

Chronic exposure to iAs can lead to various cancers (including lung, skin, liver, kidney, and bladder), skin lesions, respiratory diseases, bone marrow depression, diabetes, and cardiovascular diseases (Hughes, 2002, Singh et al., 2015). The most common neurological effect of long-term arsenic exposure is peripheral neuropathy and the gastrointestinal effects are usually expressed as toxic hepatitis accompanied by increased levels of liver enzymes (Jomova et al., 2011).

AB is considered the only non-toxic arsenical, even at high doses, and does not undergo biotransformation during the human digestion process (López-Gonzálvez et al., 1995). In a study by Kaise et al. (1985), 10 g of AB/kg body weight

(equivalent to about 6 g of arsenic) was administered to mice with no adverse effects. No LD_{50} could be established, since that was considered to be the maximum dose of AB that could be administered. The toxicology and metabolism of AB will be reviewed in more detail in Section 2.5.

Table 2-2: Acute toxicity of arsenic species in laboratory animals in order of most toxic (dark red) to least/non-toxic (dark green). The different routes of administration are intraperitoneal (IP), intramuscular (IM), or were not specified (NS).

Chemical	Animal	Route of Administration	LD_{50} (mg As/kg)	
MMA(III)	Hamster	IP	2	[a]
Arsine	Mouse	IP	2.9	[b]
	Rabbit	IP	2.4	[b]
	Rat	NS	2.9	[c]
Arsenite (As(III))	Rat/Mouse	Oral	5.8	[d]
	Rat	NS	8	[c]
	Mouse	IM	8	[e]
	Hamster	IP	8	[a]
Arsenate (As(V))	Rat/Mouse	Oral	54	[d]
	Rat	NS	20	[c]
	Mouse	IM	22	[e]
Arsenic trioxide	Mouse	Oral	26	[f]
	Rat	Oral	15	[g]
TETRA	Rat/Mouse	Oral	890	[h]
MMA(V)	Mouse	Oral	1800	[i]
	Rat	Oral	1101	[j]
	Rat	NS	700-1800	[c]
DMA(V)	Mouse	Oral	648	[i]
	Rat/Mouse	Oral	1200-2600	[h]
	Rat	Oral	1315	[j]
TMA(III)	Mouse	Oral	5000	[k]
TMAO	Mouse	Oral	5500	[i]
Arsenocholine (AC)	Rat	NS	6500	[c]
Arsenobetaine (AB)	Rat/Mouse	Oral	>6 000	[c, h]
	Mouse	Oral	>4260	[f]

[a](Petrick et al., 2001), [b](Levvy, 1946), [c](Sadee et al., 2015), [d] (Schroeder and Balassa, 1966), [e](Bencko et al., 1978), [f](Kaise et al., 1985), [g](Harrisson et al., 1958), [h] (Hedegaard and Sloth, 2011), [i](Kaise et al., 1989), [j](Aitio and Becking, 2001), [k](Raab and Feldmann, 2003)

2.2. Arsenic in the Biotic Environment

The amount of arsenic in living animals, plants, and microorganisms depends on the amount of arsenic that is present and on the type of organism (Aitio and Becking, 2001). As arsenic is naturally occurring, all living creatures will contain some arsenic. Arsenic is generally present in marine (sea-living) organisms at higher levels than in terrestrial organisms (including those in fresh water). Marine organisms tend to contain higher concentrations of arsenic (from less than1 to more than 50 mg/kg, wet weight (ww) (Kohlmeyer et al., 2003)), whereas freshwater and terrestrial biota usually contain less than 1 mg/kg, ww (Aitio and Becking, 2001). While marine organisms contain more arsenic, it is generally present in less or non-toxic species (e.g. AB, AC, and AsS) (Edmonds and Francesconi, 1993). The terrestrial environment (i.e. soil, groundwater, and plants) is typically dominated by iAs species (Ma et al., 2014). The formation, presence, and toxicity of these different arsenic species will be explained in the following sections.

2.2.1. Chemical Pathway for Methylation

In humans, and in most mammalian species, ingested iAs undergoes a series of reduction and oxidative methylation steps before the majority is excreted in the urine (Bumpus and Aust, 1987, Rossman, 2003). The first report of the formation of methylated species from iAs in fungi was published by Frederick Challenger and his coworkers in 1933 (Challenger et al., 1933). The pathway proposed that the methylation process involves a successive enzymatic transfer of a methyl group from a donor atom to an accepter atom. This process is referred to as biomethylation when it takes place within a living organism (Edmonds and Francesconi, 1987). As seen in Figure 2-1, this pathway is mediated by arsenic methyltransferase enzymes and S-adenosylmethionine (SAM) (the methyl donor) (Rehman and Naranmandura, 2012). In this process, As(V) is reduced to As(III) and then undergoes oxidative methylation to generate monomethylarsonic acid(V) (MMA(V)) (Reimer et al., 2010). MMA(V) is then reduced to the trivalent methylated metabolite monomethylarsonous acid (MMA(III)). This process repeats, forming dimethylated species: dimethylarsinic acid (DMA(V) and dimethylarsinous acid (DMA(III)) (Bergquist et al., 2009). However, this pathway does not always go to completion and metabolites can be released at different stages and in varying proportions depending on the organism and the composition and concentration of the arsenical substrate (Cullen, 2014). Other pathways for biomethylation have been suggested (i.e. Hayakawa et al. (2005)), but the Challenger Pathway remains the most rational and commonly accepted.

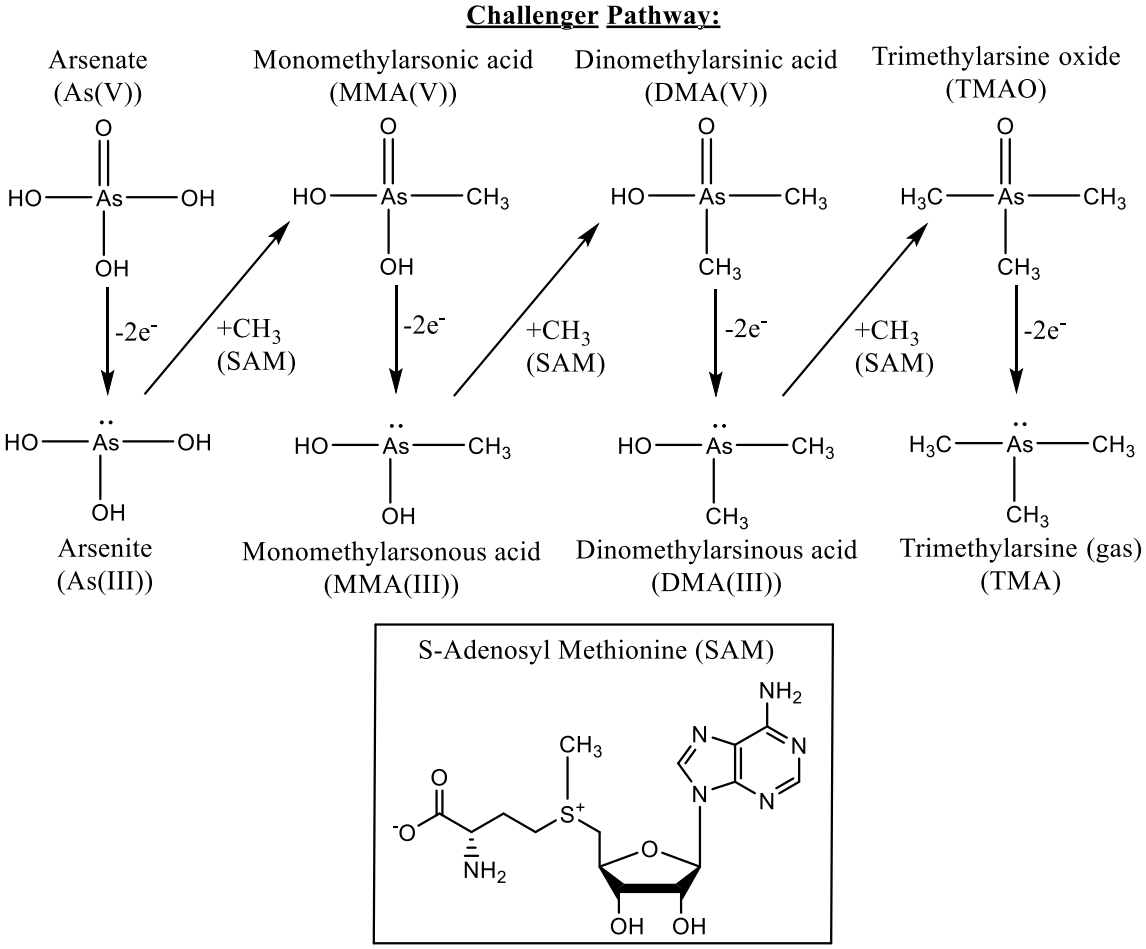


Figure 2-1: A representation of the biomethylation process for arsenic, referred to as the Challenger pathway. Modified from Zhao et al. (2010) and Rehman and Naranmandura (2012).

2.3. Arsenic Toxicology

When consumed, arsenic is taken up into the bloodstream after absorption from the gastrointestinal tract. From there, it is distributed between plasma and red blood cells, taken up into cells, metabolized, and/or begins to interact with proteins and enzymes (Molin et al., 2015b). In humans, the main route of arsenic excretion is in the urine and, on average, human urine contains 10–30% iAs, 10–20% MMA (III and V), and 60–80% DMA (III and V) (Vahter and Concha, 2001). The following sections describe specific toxicological aspects of arsenic and some of the fundamental properties (such as binding affinities for sulfur, ability to bind to proteins, induction of DNA damage, and ROS generation) of arsenic compounds as a way of explaining their most likely mechanisms of toxicity. Some areas of complexity will also be reviewed (such as the intra/interspecies variation, the use of arsenic as a medicine, and the resemblance of arsenate and phosphate). Arsenobetaine as a non-toxic arsenical, and a major focus of this thesis, will be discussed in the next section, Section 2.4.

2.3.1. Toxicity of Methylated Species

Biomethylation of arsenic was considered to be a detoxification pathway for many years because the predominant end products – namely MMA and DMA – are less toxic than iAs. However, during this process, trivalent methylated arsenic intermediates, MMA(III) and DMA(III), are formed and these have been found to be more toxic than iAs. Individuals who excrete a higher proportion of ingested arsenic as urinary MMA(III) have shown to have an increased risk of arsenic-associated cancers (Dangleben et al., 2013, Petrick et al., 2001). Petrick et al. (2001) demonstrated that MMA(III) is more toxic than arsenite during *in vitro* exposure of arsenic to Chang human hepatocytes and in hamsters (Hughes, 2002). Furthermore, in a study investigating several types of human cells (from the liver, skin, urinary bladder, and lung) and primary rat hepatocytes, MMA(III) proved to be more toxic than arsenite in all cell types, and DMA(III) was found to be at least as cytotoxic as arsenite for most cell types (Styblo et al., 2000).

2.3.2. Toxicity of Inorganic Species

It has been established that arsenite is more acutely toxic than arsenate (approximately 10 times) based on LD_{50} values (Schroeder and Balassa, 1966, Ma et al., 2014, Chatterjee et al., 1995). However, the exact reasons behind this are still being investigated. Some of the differences in toxicity may be a result of the different rates of cellular uptake. At equimolar concentrations, arsenite accumulation has been shown to be faster than that of arsenate (Lerman et al., 1983, Vega et al., 2001). In a study by Bertolero et al. (1987), cellular uptake of arsenite was four times greater than that of arsenate. It appears that both arsenite

and arsenate are actively transported into cells via transporters whose substrates they resemble (Rossman, 2003). Specifically, arsenate bears a strong resemblance to phosphate, and is therefore taken up into cells through phosphate transporters (Huang and Lee, 1996, Cullen, 2014). Arsenite, on the other hand, can be transported by aquaglycoporins (which typically transport water and glycerol) and is uncharged at physiological pH, allowing it to pass through cell membranes more quickly than arsenate (Liu et al., 2002). In the human body, and in many other mammalian species, arsenate is reduced to arsenite as part of a biotransformation process. This step may be considered a type of bioactivation, as arsenite is much more reactive with tissue components (likely due to its strong affinity for sulfhydryl groups, detailed further in Section 2.1) (Vahter and Concha, 2001). The reduction of arsenate to arsenite results in similar toxicities of the two species in chronic exposure scenarios.

The transport mechanisms of organic arsenicals are unknown but may involve organic ion transporters (Rossman, 2003). It has also been suggested that organic methylated trivalent species are highly reactive and more potent inhibitors of glutathione (GSH) reductase and thioredoxin reductase, in comparison with arsenite or pentavalent metabolites (Styblo et al., 1997, Lin et al., 1999). The implications of GSH reductase inhibition is discussed further in Section 2.1.4.3.

2.3.3. Affinity for Sulfur

Many of the toxic effects of arsenic have been linked to its strong affinity for sulfur. The fundamental attraction between arsenic and sulfur can be explained by Pearson's hard/soft (Lewis) acid/base (HSAB) principle. This principle is described below, followed by a discussion of the interactions of the different species with sulfur.

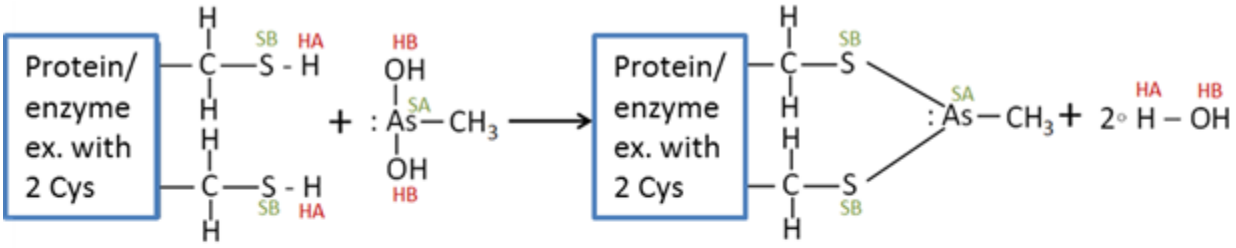
2.3.3.1. Hard/soft acid/base principle

In the Lewis theory of acid-base reactions, the Lewis base donates a lone pair of electrons, whereas a Lewis acid accepts electrons. There are four main properties used to classify chemical compounds as hard or soft acids or bases: electronegativity, molecular size, charge, and polarizability. 'Soft' acids or bases are large elements with low charge densities and are strongly polarizable. 'Hard' acids and bases are those elements which are smaller, have higher charge densities, and are weakly polarizable. The HSAB theory states that soft acids (like Cu^{+}, Hg^{2+}, and Pb^{2+}) react and form stronger bonds with soft bases (like I^{-}, Br^{-}, and S^{2-}), whereas hard acids (like H^{+}, Ca^{2+}, and Fe^{3+}) react and form stronger bonds with hard bases (like F^{-}, O^{2-}, and H_2O) (Chappell et al., 2001). Due to the polarizability of soft acids and bases, their interaction is more covalent in nature. Hard acids and bases, however, have a higher charge-to-radius ratio resulting in interactions that are more electrostatic in nature.

2.3.3.2. Affinity of As(III) for sulfur and implications for toxicity

In terms of As(III) compounds with OH groups (such as arsenite, MMA(III), and DMA(III)) binding to sulfur-containing proteins or enzymes, the interaction is complex (Figure 2-2). Simply put, each bond can be considered to be shared between an acid and a base. In As(III) compounds with hydroxyl groups, the arsenic is the acid and the hydroxides are the bases. For the cysteine groups in the protein or enzyme, sulfur is the base and the protons are the acids. As(III) and H^+ (proton) are both Lewis acids, with H^+ being the harder acid (due to its charge density) and As(III) being considered the softer acid. Similarly, sulfide and hydroxide groups are both Lewis bases, but sulfide is the softer base between the two. Therefore, they will switch partners and a metathesis reaction (double displacement between Lewis acidic and basic portions of two species) takes place. It is important to note that the classification of hardness or softness of an acid or base occurs as a scale of hardness or softness. A specific group should not always be classified as a 'hard/soft' acid or base, but rather 'harder' or 'softer' than that of which it is being compared to.

The bond dissociation energies seen in Figure 2-2 are based on thermochemical (Darwent, 1970) or mass spectroscopic (Luo, 2007) measurements at a standard temperature of 298 K and are given in kilojoules per mole (kJ/mol). Overall, the reaction has a negative enthalpy and is therefore considered an exothermic reaction. The values are not estimates for the bonds in the given compounds, but rather values for those bonds in similar compounds.



Bonds Broken

Bond	Number	ΔH (kJ/mol)
S – H	2	361[c]
As – O	2	484[b]
SUM:		1690

Bonds Formed

Bond	Number	ΔH (kJ/mol)
O – H	2	498[a]
As – S	2	380[b]
SUM:		1756

$$Enthalpy\ (\Delta H) = \sum \Delta H(Bonds\ Broken) - \sum \Delta H(Bonds\ Formed)$$

$$\Delta H = 1690\ {}^{kJ}/_{mol} - 1756\ {}^{kJ}/_{mol}$$

$$\Delta H = -66\ {}^{kJ}/_{mol}$$

Figure 2-2: A representation of the interaction between MMA(III) and two cysteine groups in a protein. The harder acids/bases (HA/HB) are labeled in red and the softer acids/bases (SA/SB) are labeled in blue. The equation below the representation indicates the bond dissociation energies in kJ/mol. *Bond dissociation values obtained from [a](Darwent, 1970), [b](Luo, 2007), and [c](Luo, 2002). The bond energies are based on arsenic monoxide for As-O and arsenic monosulfide for As-S. The bond dissociation energy for the O-H bond was based on the value for water (H_2O), as water is formed in the latter part of the reaction. The S-H bond dissociation energy value is based on the energy of the hydrogen dissociating from sulfur bonded to a methyl group (methylthiol).

As(III) compounds with one or more hydroxyl groups have a high affinity for and readily react with the sulfhydryl groups, allowing them to bind to cysteines in peptides and proteins and cause misfolding and impairment or inhibition of the protein's function (Shen et al., 2013). Furthermore, these changes to the conformation and/or function of a protein can alter its interactions with other important proteins. As a result, it has been suggested that arsenic-protein binding plays a key role in negatively affecting the health of those exposed (Johnson et al., 2016). The mechanism of arsenic toxicity is therefore considered to be a result of As(III) attacking proteins (through sulfhydryl groups) rather than attacking other biomolecules like DNA. Many of the possible protein, peptide, and enzyme targets of trivalent arsenic species include DNA repair enzymes, transcription factors, steroid hormone receptors, zinc finger proteins, and tubulin (Muenyi and Ljungman, 2015). As a result of trivalent arsenicals binding to these targets, DNA repair and growth factors can be altered, there can be changes to cell proliferation, and altered DNA methylation patterns can all result in toxic or carcinogenic effects (Chappell et al., 2001).

The HSAB principle can also be used to explain the toxicity differences between MMA(III), DMA(III) and trimethylarsine (TMA(III)), which vary as MMA(III) toxicity > DMA(III) > TMA(III). This ranking is based on the LD_{50} values presented in Table 2. No LD_{50} value is available for DMA(III), but it is assumed to be less toxic than MMA(III) and more toxic than its pentavalent counterpart. The number of binding sites for sulfur is reduced as more methyl groups are attached to arsenic (Chappell et al., 2001), since methyl groups are softer bases and therefore are not as easily exchanged as hard bases (like hydroxide groups). For example, MMA(III) with two hydroxide groups can react with two sulfur-containing ligands, making it more toxic than DMA(III) which can only react with one sulfhydryl group. TMA(III) has three methyl groups and thus no hydroxides available for displacement by sulfhydryls, which may explain its lower toxicity.

2.3.3.3. Affinity of As(V) for sulfur and implications for toxicity

Arsenite is considered a softer acid because it has a low oxidation state (+3) and is easily polarizable, whereas arsenate (+5) is considered a harder Lewis acid due to its higher charge-to-radius ratio (Pearson, 1968). Therefore, arsenate is often found bound to oxygen in compounds and is consequently less toxic and less likely to interfere with protein and enzyme function (Chappell et al., 2001). However, in biological systems, arsenate is quickly reduced to arsenite and other methylated species via the Challenger pathway. In a protein binding experiment using rabbit erythrocytes, arsenite was shown to bind the fastest, followed by arsenate, and MMA(V) and DMA(V) were much slower (Delnomdedieu et al., 1995).

2.3.4. Arsenic Binding to Proteins

Arsenic has been shown to bind to and inactivate over 200 enzymes (Ratnaike, 2003). In most arsenic-protein binding experiments, trivalent arsenic (as arsenite) or phenylarsine oxide (PAO(III)) have been used (Yan et al., 2009, Snow et al., 1999, Styblo et al., 1997, Le Cabec and Maridonneau-Parini, 1995, Estrov et al., 1999, Kapahi et al., 2000). PAO(III), although not a biologically relevant metabolite, is frequently used because it is relatively stable, whereas other trivalent organoarsenicals (i.e. DMA(III) and (MMA(III)) are not stable in solution, being prone to rapid oxidation to pentavalent species (Yan et al., 2009, Gong et al., 2001).

Proteins susceptible to arsenic binding (with As(III) and PAO(III)) have been identified in several types of human cells: AG06 keratinocytes (Snow et al., 1999), human lymphoblastoid cells (Menzel et al., 1999), human MCF-7 breast cancer cells (Zhang et al., 2007), both the membrane and nuclear fraction of A549 human lung carcinoma cells (Yan et al., 2009), and human HepG2 hepatocarcinoma cells (Mizumura et al., 2010). The types of proteins thus identified were examined for trends in their function in this review and seven functional categories could be identified (Appendix A, Table A-1): structural proteins, metabolic enzymes, regulatory proteins, proteins related to protein synthesis/regulation, stress response proteins, transport proteins, and DNA/RNA related proteins. To date, most of these have not been studied further except for those which are discussed in more detail in the following sections.

Some of the most common reoccurring proteins identified in all of the cell line studies described above include actin, tubulin, keratin, peroxiredoxin-1, heat shock protein 27 (HSP27), thioredoxin reductase, pyruvate dehydrogenase, thioredoxin reductase, glucose transporters (e.g., GLUT4), and several DNA ligases. The inhibition of many of these proteins makes them more susceptible to other damage induced by arsenic. For example, the peroxiredoxin-1 gene encodes a protein that is a member of the peroxiredoxin family of antioxidant enzymes that help combat oxidative stress. Inhibition of this protein makes cells more susceptible to oxidative damage (discussed further in Section 2.3.4.2). Furthermore, when several DNA ligases are inhibited, the cell experiences a reduced capacity to repair irregularities and breaks in DNA (detailed in Section 2.3.4.4).

2.3.4.1. Pyruvate Dehydrogenase

Pyruvate dehydrogenase (PDH), a metabolic enzyme, is a component of the pyruvate dehydrogenase complex (PDC) that is involved in transforming pyruvate into acetyl coenzyme A (acetyl-CoA), which is then used in the citric acid cycle to produce cellular energy in the form of adenosine triphosphate (ATP) (Wieland, 1983). PDH is responsible for the first two reactions in the PDC, decarboxylation

of pyruvate and reductive acetylation of lipoic acid (Reed, 1969). Arsenite can inhibit the PDC by binding to the lipoic acid moiety (Figure 2-3) (Shen et al., 2013). Inhibition of the PDC results in a lack of acetyl-CoA production which limits the citric acid cycle and ultimately reduces the production of ATP. This was demonstrated in human erythrocytes by Carter (1998), who observed ATP depletion after incubation with 10 mM arsenate and arsenite for 5 hours. A lack of ATP can lead to cell damage and death.

A study by Bergquist et al. (2009) demonstrated that three trivalent arsenicals, arsenite, MMA(III), and DMA(III), are capable of inhibiting both the PDC complex and the ketoglutarate dehydrogenase (KGDH) complex via direct binding. MMA(III) was shown to be the strongest inhibitor of both the PDC and the KGDH complex, followed by arsenite. This is because MMA(III) formed a more stable binary complex in the active site of the enzyme. DMA(III) only showed inhibition after an increased incubation time. No pentavalent arsenicals inhibited either the PDC or the KGDH. This is likely attributable to the HSAB explained in Section 2.1.3.1. and the availability of binding sites to sulfur.

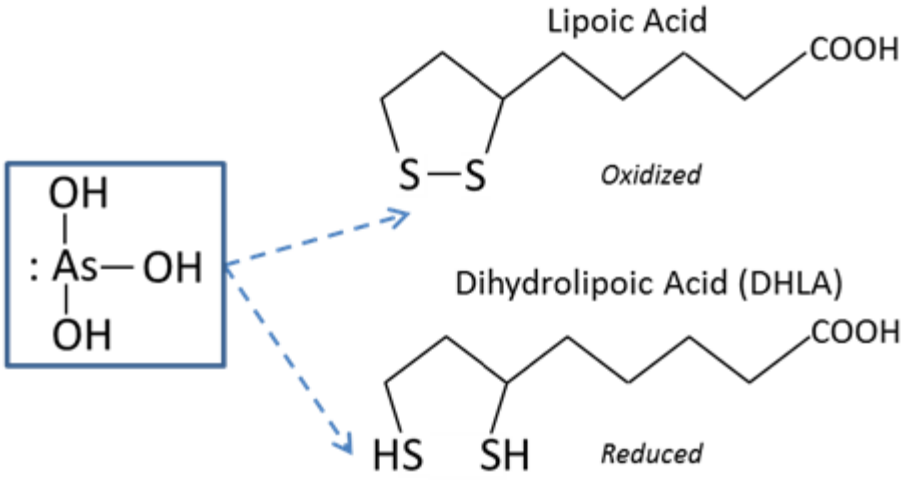


Figure 2-3: A representation of the interaction between trivalent arsenic species (shown in this example as arsenite) and lipoic acid in the reduced and oxidized forms. Modified from (Biewenga et al., 1997)

The PDC and KGDH complex both play a key role in the citric acid cycle, which takes place in the mitochondria. Products of the citric acid cycle include carbon dioxide, ATP, reduced nicotinamide adenine dinucleotide (NADH), and reduced flavin adenine dinucleotide ($FADH_2$) (Krebs and Johnson, 1937). Arsenic binding to the PDC and KGDH complexes inhibits their activity, therefore inhibiting the

citric acid cycle. This can result in depleted mitochondrial NADH resulting in oxidative stress and the production of reactive oxygen species (ROS) that, in the absence of arsenic, would have been managed (Shen et al., 2013).

It has also been suggested that the generation of reactive oxygen species by arsenite may be responsible for PDC inhibition, in addition to direct arsenic-thiol binding. A study by Samikkannu et al. (2003) found that arsenite-induced ROS production (i.e., ROS that are not a result of direct binding in the PDC or KGDH, but a result of arsenite binding to critical thiols) can also lead to PDC inhibition. Therefore, the binding of trivalent arsenic can inhibit the activity of the complexes, which can then be perpetuated by the production of ROS and have further inhibitory effects.

2.3.4.2. Production of Other Reactive Species

Due to the biomethylation and speciation changes of arsenic in the body, it is expected that arsenic will cycle through different oxidation states. This creates a redox cycle where the simultaneous reduction and oxidation of arsenic compounds could generate reactive oxygen species (ROS) or other free radical species (Chappell et al., 2001). Reactive oxygen and nitrogen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and nitric oxide (NO) are known to be important in mutagenesis and carcinogenesis (Wiseman and Halliwell, 1996). In their review, Shi et al. (2004) outline several studies in which arsenic exposure resulted in the generation of ROS in various cellular systems. However, they state that the exact mechanism behind this remains unclear. Arsenic-induced ROS generation in cells can lead to DNA damage, lipid peroxidation, redox enzyme activity, and decreased antioxidant levels (Lee-Chen et al., 1993, Nakamuro and Sayato, 1981). At low levels, ROS have been found to influence gene expression and act as secondary messengers (Esteve-Nunez et al., 2001). At higher levels, ROS not only promote cell apoptosis, but they can also cause DNA damage and oxidative stress-induced damage (Alarifi et al., 2013). Mutagenesis and DNA damage caused by ROS likely contributes to the initiation of cancer (Valentín et al., 2006). Furthermore, the inhibition of enzymes by trivalent arsenic weakens the cell's ability to protect against oxidants and leaves them hypersensitive to arsenic toxicity (Hughes, 2002).

2.3.4.3. Glutathione and Managing Reactive Species

Glutathione (GSH) (a tripeptide of cysteine, glutamic acid, and glycine) is suggested to be important in maintaining a proper cellular redox state, and depletion of GSH is considered to be an indicator of oxidative stress (Hall et al., 2013). There are several ways in which arsenic exposure has been associated with GSH depletion: 1) GSH is consumed when it acts as an electron donor for the conversion of pentavalent to trivalent arsenic species, 2) trivalent arsenicals have a

high affinity for GSH (due to the sulfhydryl group), and can bind the GSH, and 3) free radicals produced during arsenic metabolism can oxidize GSH (Chrysostomou et al., 2015, Shi et al., 2004). GSH depletion as a result of chronic arsenic exposure has been observed in several epidemiological studies including Peters et al. (2015), Hall et al. (2013), Niedzwiecki et al. (2013) and Wang et al. (2007). In arsenic-resistant Chinese hamster ovary cells, when cellular GSH levels were depleted using buthionine and the cells were re-exposed to arsenic, their resistance to As(III) was lowered to that of the non-resistant cell line (Lee et al., 1989). Lowered GSH levels decrease the ability of cells to cope with ROS and other reactive species, which further enhances the damage induced by arsenic exposure.

2.3.4.4. DNA Damage

Trivalent arsenic species have been shown to bind to and inhibit several proteins responsible for DNA repair and regulation. These proteins include ATP-dependent DNA helicase 2 subunit 1 and DNA-dependent protein kinase catalytic subunit, which were identified in the nuclear fraction of human lung carcinoma cells (Yan et al., 2009), and DNA replication licensing factor MCM6, which was identified in a human breast cancer cell line (Zhang et al., 2007). Other DNA repair proteins inhibited by arsenite include poly(ADP-ribose) polymerase-1 (PARP-1), formamidopyrimidine-DNA glycosylase (Fpg), and Xeroderma pigmentosum group A protein (XPA) (Shen et al., 2013). All of these proteins contain a zinc finger DNA binding domain, which is associated with recognizing damaged DNA. A particular zinc finger DNA repair protein of interest is PARP-1, as it is highly sensitive to arsenic inhibition and is crucial in the DNA damage repair networks (Zhou et al., 2011). The inhibition of PARP-1 may be caused by As(III) replacing zinc at one of the proteins' binding sites (Ding et al., 2009) thereby interfering with the structural and chemical integrity of the protein. In particular, PARP-1 is a direct molecular target for arsenite, with arsenite preferentially binding to zinc fingers with more than three cysteine residues (Zhou et al., 2011). Arsenite interaction with zinc finger proteins is an important molecular mechanism for arsenic toxicity and carcinogenesis as it limits the cell's ability to repair damaged DNA (Zhou et al., 2014).

In addition to causing DNA damage, Yan et al. (2009) showed that arsenic can bind to free cysteine groups on DNA-dependent protein kinase, ATP-dependent helicase II, and topoisomerase 2 alpha, which are all linked to DNA repair and maintenance of genome stability. Therefore, arsenic exposure not only damages DNA by increasing reactive species concentrations in cells, but it propagates the problem by inhibiting the cellular mechanisms that cope with and correct compromised DNA.

2.3.5. Inter/Intra Species Variation in Arsenic Toxicity

One of the problems with respect to determining the exact mechanism of toxicity for arsenic exposure is the variation, both intraspecies (e.g., between humans) and interspecies (e.g., between humans and other animals). There is no accepted model for studying arsenic toxicity, and more specifically arsenic carcinogenesis. Previous animals tested have shown to differ in the way they handle arsenic exposure. For example, rat hemoglobin has a higher affinity for trivalent arsenic species than human hemoglobin. This leads to accumulation of arsenicals in the red blood cells of rats, but not other species (Lu et al., 2004). This then results in different half-lives for arsenic in blood between different animal species. In humans, most arsenic is typically cleared from the body rather quickly, with a half-time of approximately 40-60 hours (Agency for Toxic Substances and Disease Registry (ATSDR), 2007). However, clearance is even faster in mice and hampsters, with 90% removal observed within 48 hours (ATSDR, 2007).

Different animal species also vary in the extent to which they methylate iAs and which species are predominantly excreted (Cohen et al., 2006, Obinaju, 2009). The lack of animal models has hindered mechanistic studies of arsenic toxicity and carcinogenesis in the past (Rossman, 2003).

In some animal species, arsenic exposure on its own is not enough to induce carcinogenesis, but will act as a co-carcinogen (Rossman et al., 2002). In these cases, arsenic exposure will only lead to carcinogenesis if another genotoxic agent is present. In a study by Burns et al. (2004), hairless mice were exposed to different combinations of sodium arsenite and/or ultraviolet radiation (UVR). Untreated mice and mice only exposed to sodium arsenite developed no tumours. Mice treated with only UVR did exhibit relatively low levels of carcinoma, but dietary arsenite markedly enhanced this UVR-induced cancer yield. Rossman (2003) suggests that arsenite (and potentially some of its metabolites) act as co-carcinogens by activating signal transduction pathways that increase cell proliferation (Germolec et al., 1996), decrease anti-proliferative signaling (Trouba et al., 2000), and override the checkpoints controlling cell division after DNA damage (Kaltreider et al., 1999). Although this behaviour of arsenic co-carcinogenesis has typically been observed in mice, several epidemiological studies also implicate arsenic as a co-carcinogen in humans (Hertz-Piccioto, 2000). In two studies, populations exposed to arsenic in drinking water showed an increase in lung cancer in smokers in comparison with non-smokers, suggesting a synergy between the carcinogens (Chiou et al., 1995, Tsuda et al., 1995).

Although humans typically accumulate and methylate arsenic in similar ways, differences have been detected between gender and ethnicity. In a study by Loffredo et al. (2003), total arsenic and distribution of arsenic species in the urine of men and women from Mexico, China, and Chile were analyzed. As expected,

the predominant urinary arsenic species was DMA(V). However, differences between the ratios of arsenic species were detected between men and women for all three populations (the Chinese and Mexican women excreted higher proportions of DMA than their male counterparts, while Chilean females excreted lower proportions of DMA) and these differences varied by population (the Chinese population had a higher proportion of MMA in their urine than either of the other populations). Therefore, the exact composition of arsenic species excreted by humans is unpredictable and may depend on factors such as the geographical environment or gender.

It has also been suggested that some people are more sensitive to arsenic exposure than others even if their ethnic background is the same. Specifically, chronically-exposed individuals from West Bengal who had arsenic-induced skin lesions also had a 10-fold higher prevalence of bronchiectasis (a disease in which there is damage to the bronchial tubes in the lungs) in comparison to those who did not have lesions, despite the fact that both groups had the same arsenic exposure (Mazumder et al., 2005). This can perhaps be attributed to a genetic predisposition to arsenic sensitivity. For example, several studies attribute arsenic sensitivities to variations in genes such as; purine nucleoside phosphorylase (PNP) (De Chaudhuri et al., 2008), glutathione S-transferase (GST) M1 (Chiou et al., 1997), and other genetic polymorphisms (Wu et al., 2014). In several other studies, men were found to be more susceptible to arsenic toxicity than women (Mazumder et al., 2005, De et al., 2004, Gonsebatt et al., 1997). The exact reasons for this have not yet been determined, but it has been hypothesized that it is due to a sex hormone-related increased methylation capacity in women (Watanabe et al., 2001).

Overall, the variance associated with the degree of arsenic methylation in different animals, its role as a co-carcinogen, and the discrepancies of arsenic methylation between humans make it very difficult to pinpoint the exact mechanism of toxicity. As a result of these variances, there is no accepted animal model for studying arsenic toxicity or carcinogenesis. Furthermore, because humans have demonstrated different sensitivities to arsenic, even epidemiological studies may not be applicable to the general population, especially in different geographical areas or if there is a large gender difference.

2.3.6. Paradox of Arsenic: Use as Medicine

The properties of arsenic that make it a dangerous toxicant and carcinogen are also responsible for its unique chemotherapeutic abilities. Research has demonstrated that arsenic is a potent chemotherapeutic agent against acute promyelocytic leukemia (APL), a cancer of white blood cells, and when used in conjunction can enhance the effectiveness of existing chemotherapeutics (Muenyi and Ljungman, 2015).

In APL, there is an abnormal accumulation of developing granulocytes (a class of white blood cells that have granules of enzymes present in their cytoplasm) called promyelocytes (Roldan et al., 2013). Typical treatment for newly diagnosed APL consists of all-*trans*-retinoic acid (ATRA) and anthracycline-based chemotherapy, typically resulting in cure rates now surpassing 80% (Lo-Coco et al., 2013). Arsenic trioxide has also been shown to be effective in the treatment of APL (Zhang et al., 1996).

Arsenic trioxide treatment of APL acts through binding to the cysteine residues of zinc finger proteins located within the disease-specific promyelocytic leukemia protein retinoic acid receptor alpha (PML-RARα) (Zhang et al., 2010). The presence of PML- RARα is a crucial receptor involved in the malignancy and proliferation of the leukemic promyelocytes that cause APL. Arsenic trioxide binding to the PML-RARα leads to its degradation, which results in cell differentiation and induction of apoptosis of the leukemic promyelocytes (Zhang et al., 2010). In addition to PML-RARα binding, arsenic trioxide can also act to prompt apoptosis, differentiation, and growth arrest (Jeanne et al., 2010). Arsenic trioxide is able to generate ROS, deplete GSH levels, and interrupt mitochondrial cycles, which all result in induced apoptosis of APL cells (as they are more sensitive to arsenic trioxide than healthy cells, having reduced GSH levels) (Davison et al., 2002). In patients that are resistant to ATRA treatment on its own, utilization of arsenic trioxide may decrease the ATRA resistance mainly by potentially allowing for arsenic trioxide to bind to the PML-RARα (which can be blocked in ATRA-resistant patients due to a mutation in the ligand binding domain), and also by further promoting conditions that promote apoptosis (Tomita et al., 2013, Davison et al., 2002, Jeanne et al., 2010).

A combination of arsenic trioxide and ATRA treatments has demonstrated to be synergistic at both the biological and clinical levels (Shao et al., 1998, Bachleitner-Hofmann et al., 2002, de Thé and Chen, 2010, Hu et al., 2009). After treating 85 patients with ATRA and arsenic trioxide, Hu et al. (2009) reported that 80 patients (94.1%) had entered complete remission, and of those patients the five-year relapse-free survival and overall survival were 94.8% and 97.4%, respectively. The toxicity profile of the treatment plan was referred to as “mild and reversible” and urine arsenic concentrations returned to well below the safe limit 24 months after treatment. ATRA and arsenic trioxide treatment of APL by Lo-Coco et al. (2013) resulted in two-year event-free survival (defined as achievement of remission without relapses or death) in 97% of patients, which was overall greater than the ATRA-chemotherapy group (only 86%). ATRA-arsenic trioxide treatment, in comparison with ATRA-chemotherapy, was associated with fewer infections, less hematologic toxicity, but increased hepatic toxicity. This indicates that the combination of ATRA and arsenic trioxide is a very effective and minimally toxic treatment for newly diagnosed APL.

Arsenic trioxide has been reported as one of the most toxic arsenic species and is highly carcinogenic. However, in patients with APL, in particular those who are resistant to ATRA treatment, arsenic trioxide can be an extremely effective chemotherapeutic agent.

2.3.7. Arsenate

Arsenate shares many physiochemical properties with phosphate (nearly identical pKa values, similarly charged oxygen atoms, and very similar size) and can thus be taken up inadvertently by cells through phosphorus pathways (Elias et al., 2012). It has been suggested that due to their similarities, arsenate can compete with and replace phosphate in many biochemical reactions (Dani, 2011). Biomolecules formed in this way are thought to rapidly decompose, and the term used to describe this decomposition is 'arsenolysis' (Thomas, 2010). Biologically, arsenolysis is capable of disrupting glycolysis and oxidative phosphorylation, which can result in reduced ATP formation. Human red blood cells incubated with arsenate *in vitro* for 5 hours have shown depleted ATP levels (Carter, 1998). However, there is very little literature supporting this as a viable mechanism of toxicity. Because arsenate is readily transformed to arsenite in almost all organisms, it is unlikely that arsenate contributes to the overall toxicity of arsenic exposure. The arsenate substitution for phosphate is likely just coincidental (Dani, 2011).

2.4. Arsenobetaine (AB)

Humans are mainly exposed to arsenic through food, except in areas where the drinking water is naturally contaminated with arsenic (in its inorganic form) (Aitio and Becking, 2001). Arsenic is found in a wide range of food categories (notably, seafood, rice, cereals, mushrooms, and poultry) with a high variation of concentrations and species (Cascio, 2011, Jomova et al., 2011). The daily intake of total arsenic from food and beverages is highly dependent on the population of interest. Seafood is known to be the most significant source of arsenic in the diet and it generally contains very little iAs (lower than 0.1 mg/kg) (Sloth, 2013). The dominant species in marine fish and most other seafood is AB, generally constituting more than 70% of the total arsenic content (Rose and Fernandes, 2013). The fruiting body of terrestrial fungi, commonly known as mushrooms, can also contain AB as the predominant arsenical depending on the species of fungi (Nearing et al., 2014a). Unlike the arsenic species discussed in the previous section, AB is the only species with no known toxicity. The reasons behind this lack of toxicity and studies investigating the effects of exposure are discussed in the following sections. What happens within the human body once AB is ingested will also be reviewed.

2.4.1. Toxicology of Arsenobetaine

AB appears to be metabolically unavailable to both humans and animal species. It is rapidly excreted (typically within 1-2 days) in the urine with no significant metabolites (Francesconi et al., 2013, Le et al., 1994). In a study by Kaise et al. (1985), 10 g of AB/kg body weight (equivalent to about 6 g of arsenic) was administered to male mice without any adverse effects. No LD_{50} could be established, since that was considered to be the maximum dose of AB that could be administered. AB was detected in the urine of these mice in an unaltered form, suggesting that it is excreted without being metabolized.

Other studies have shown the same result with lower doses of AB. Irvin and Irgolic (1988) examined the effect of AB on rat embryos. They found that AB, even at concentrations of several hundred grams of arsenic per kilogram bodyweight, did not exert any toxic effects. Sakurai et al. (2004) orally administered AB to mice once a day for four days, with a total administered amount of 6.5 g AB/kg bodyweight (equivalent to approximately 2.7 g As/kg bodyweight) (designed to mimic the amounts of AB likely contained in marine animals that are commonly consumed). They found that AB was distributed to the immune organs (such as the spleen and thymus), but no toxic effects were observed.

Sabbioni et al. (1991) compared the toxicity of iAs and AB in the mouse embryo cell line BALB/3T3 clone A 31-1-1. They found that unlike iAs (in this case, sodium arsenite), which completely inhibited cell growth and colony formation at a concentration of 13 mg/L (equivalent to 7.5 mg As/L), AB was non-toxic and non-transforming, even at concentrations of 89 mg AB/L (equivalent to 37 mg As/L). They attribute the absence of cytotoxic effects or transformations of AB to the different degree of cellular retention and intracellular behaviour. AB showed low retention efficiency, it was unable to interact with components inside the cell and no cellular biotransformations were observed. In a more recent study by Rangkadilok et al. (2015), the *in vitro* cytotoxicity of several elements (including cadmium, manganese, and arsenic (both iAs and AB)) using human cancer cells (T47D, A549, and Jurkat cells) was investigated. Overall, a decrease in cell viability was observed in T47D and Jurkat cells as a result of exposure to iAs, but not to AB.

Therefore, there is currently no literature available that reports toxic effects of AB, which is likely attributable to its structural properties. It appears to not interact with cells or cell components, which may be due to the fact that AB is fully saturated with bonds that are not easily broken (As-C). Moreover, AB has a strong structural resemblance to glycine betaine (hereafter referred to as ‘betaine’) (Figure 2-4), which is naturally present in human blood plasma between 2.3 and 7.0 mg/L (Lever et al., 1994). In many mammalian and aquatic organisms, betaine acts as an osmolyte to maintain cell volume and osmotic balance under changing salinity

(Adair et al., 2005). In mammals, betaine is also capable of acting as a methyl donor and can provide protection against protein denaturation (Schwahn et al., 2003). Therefore, based on the functions of betaine, it is expected to remain available in the blood (i.e. not bind to plasma proteins or transform) and the structurally analogous AB is expected to do the same (Pei and Gailer, 2009). This was confirmed in a study by Pei and Gailer (2009), in which AB was unable to bind to either human or rabbit blood plasma proteins.

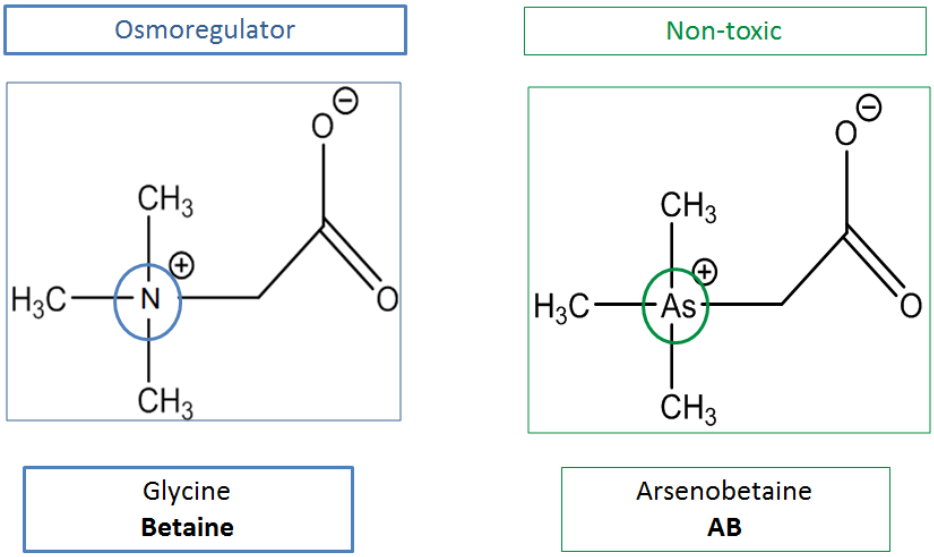


Figure 2-4: Structural comparison of glycine betaine and arsenobetaine (AB).

2.4.2. Arsenobetaine Behaviour in the Human Body

It is generally understood that AB is efficiently absorbed and excreted (Molin et al., 2015b). In humans, the typical half-life of AB is less than 20 hours (Molin et al., 2012). However, this may not hold true for other mammalian species. Vahter et al. (1983) investigated the excretion rate of radiolabeled AB in mice, rabbits, and rats. For mice and rats, they found that over 98% of the administered dose was excreted within 3 days of intravenous injection, whereas only 75% of the dose was excreted for the rabbit. In other words, 25% of the administered AB remained after three days. To determine the retention and distribution of AB in humans, Brown et al. (1990) used isotopically labeled AB. They found that AB was rapidly dispersed in soft tissues, with no localization of AB concentrations in any region or organ. This measurement was taken after 24 hours, after which most of the AB had already been excreted, which means that the dispersion of AB was applicable to long-term storage of AB. In all of the subjects analyzed (a total of six volunteers) less than 1% of ingested radioactivity remained in the body after 24 days.

The absorption of AB depends on many factors, such as the presence of other food in the gastrointestinal tract, and the food matrix (Molin et al., 2015b). It is unlikely that AB is transformed or degraded during normal digestion, although Harrington et al. (2008) demonstrated that microorganisms in the human gastrointestinal tract are capable of AB degradation after an incubation period of 30 days.

In a study by Molin et al. (2012), individuals were fed a single test meal of cod, farmed salmon, blue mussel, or potato (the control), and their urine was analyzed for total arsenic content as well as iAs, AB, DMA(V), and MMA(V). The amount of total arsenic excreted in the urine within 0 – 72 hours varied among the groups, ranging from an average of 49% for the blue mussel group to 74% for the cod group. The total urinary excretion of AB, estimated with a mathematical model, for the blue mussel and cod groups was higher than the amount of ingested AB. Originally, the amount of excreted AB as a % of the ingested amount was 90% for the cod group and 99% for the blue mussel group. However, the corresponding average absorption percentages (AB absorption percent was assumed to be equal to total absorption percent (total arsenic excreted divided by total arsenic ingested multiplied by 100%)) was 81% for the cod group and 56% for the blue mussel group. Correcting the AB excretion based on the absorption percent put the cod and blue mussel groups over 100%.

Molin et al. (2012) suggest the apparent increase in AB could be due to possible endogenous formation of AB from other organic arsenicals present in the test meals or in the body. The increase in AB excreted could also be a result of arsenosugar transformation. Arsenosugars (arsenic-containing carbohydrates) are common constituents of marine organisms and algae, including species consumed as human food (like blue mussels) (Figure 2-5) (Francesconi et al., 2002). However, it is also possible that in the Molin et al. (2012) study, previously ingested AB was released.

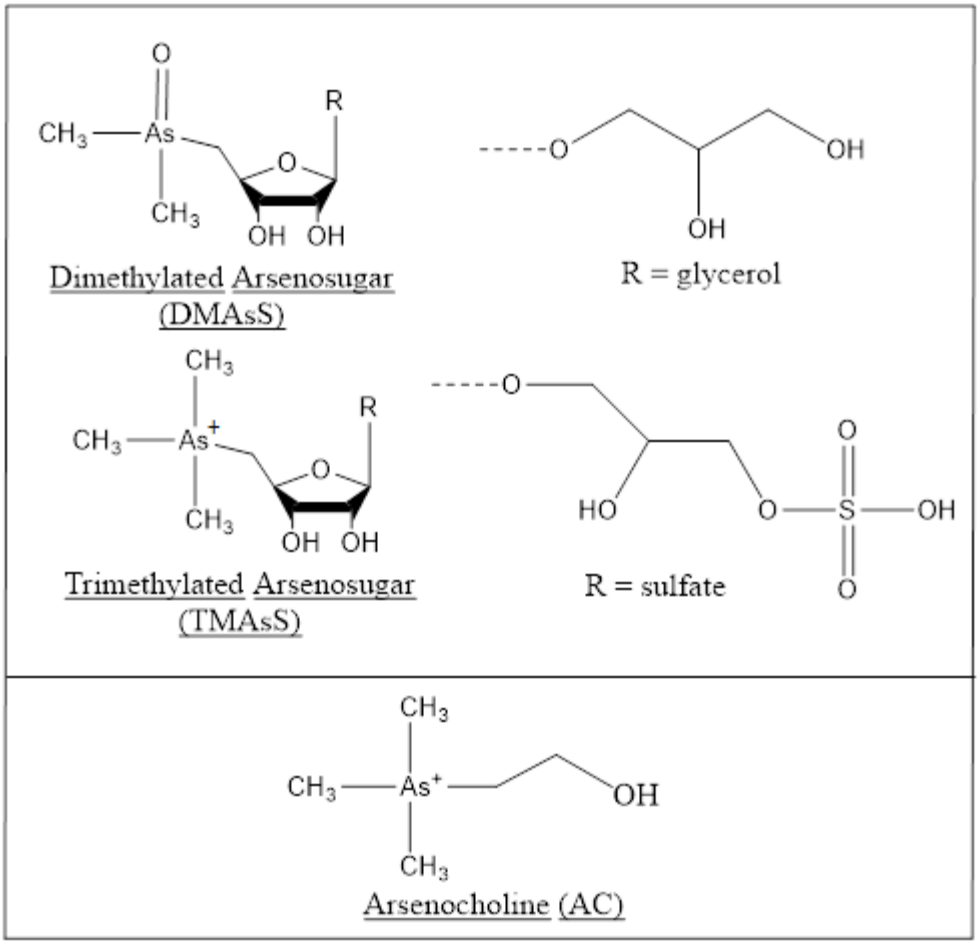


Figure 2-5: Top: Structural representation of the chemical building blocks used to make arsenosugars with two examples shown. Bottom: A representation of the chemical structure of arsenocholine (AC). Modified from Fukuda et al. (2011) and Feldmann and Krupp (2011).

In a study by Newcombe et al. (2010), AB levels in the urine of five volunteers on an AB-free diet for twelve days were analyzed. They found that the AB was still present in the urine five days after commencing the AB-free diet. The urine concentrations varied between 0.2 and 12.2 µg AB/L for three of the volunteers and the other two volunteers had AB concentrations below the limit of detection. Similarly, Molin et al. (2012) found 46% of ingested AB in the urine of their control group (fed potatoes instead of seafood) after two days. These results indicate that it is possible that one or more of the following may be true: a) AB accumulated in the tissues is slowly released over time, b) AB is a human

metabolite formed from other arsenicals, or c) there is a possible steady state of AB in the human body/urine.

2.5. Synthesis of Arsenobetaine

2.5.1. Review of Arsenobetaine Formation Pathways

2.5.1.1. Abiotic Pathways

Several experiments have demonstrated that AB can be formed abiotically (in this case, a reaction that takes place in the absence of biological mediation (i.e. does not require living cells or enzymes derived from living cells for the reaction itself to take place)). For the purpose of this research, a reaction is still classified as abiotic even if it requires certain conditions (such as reducing conditions) that are produced by biological organisms or if it requires reactants that come from biological means. In this review, the use of the term 'abiotic' implies that the reaction can be carried out experimentally, without the intervention of any organisms.

One of the first syntheses of AB (Pathway 1, Figure 2-6) was done by Cannon et al. (1981) who were investigating the structure and synthesis of AB in the western rock lobster.

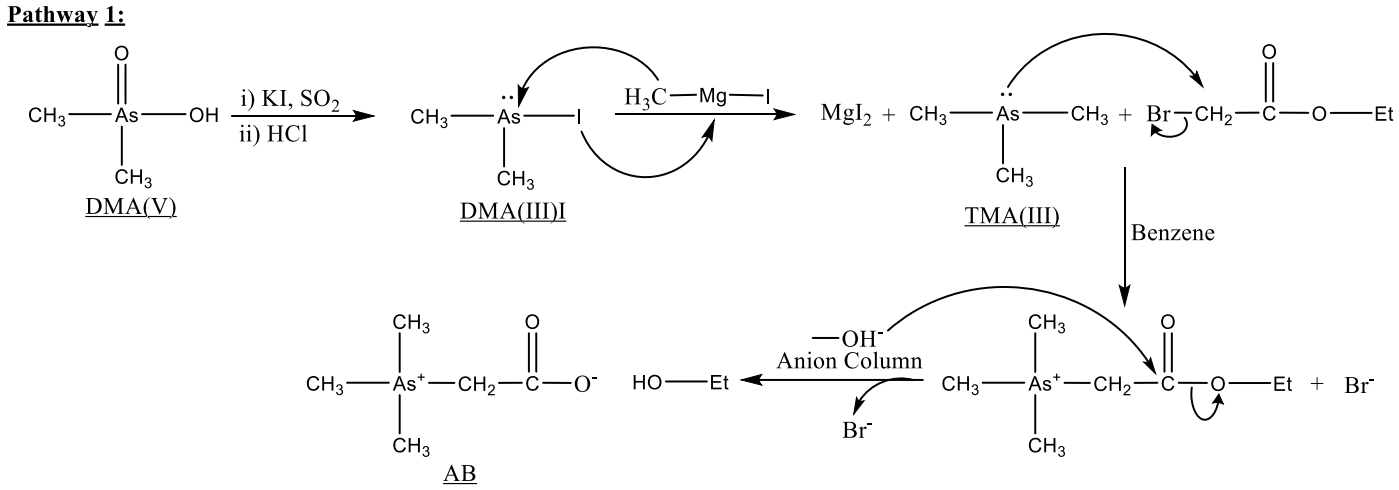


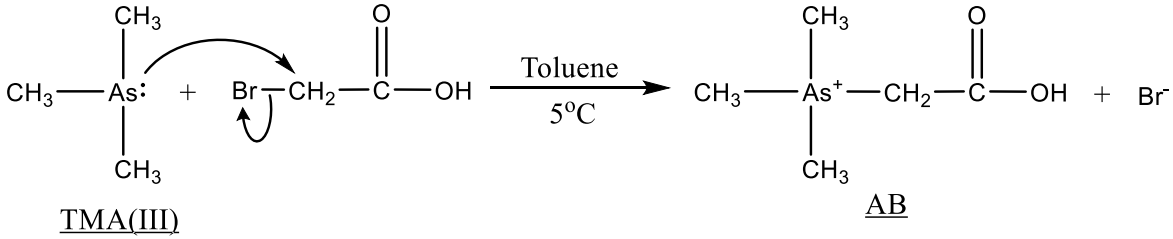
Figure 2-6: A schematic representation and hypothesized reaction mechanism for the synthesis of arsenobetaine (AB) from dimethylarsenic acid (DMA(V)), as proposed by Cannon et al. (1981).

For this synthesis, DMA(V) was converted into dimethyliodoarsine (DMA(III)I), following methods previously outlined by (Burrows and Turner, 1920). In this case, DMA(V) is reduced by sulfur dioxide (SO_2) in the presence of KI and diluted hydrochloric acid. DMA(III)I forms as a yellow oil at the end of this process and can be separated and dried. DMA(III)I was then treated with methylmagnesium iodide to obtain trimethylarsine (TMA(III)) via a metathesis reaction with the Grignard reagent (originally suggested by Challenger and Ellis (1935)). The TMA(III) was then oxidized via electrophilic attack of the alpha carbon of ethyl bromoacetate, creating a new As-C bond and releasing Br^- (similar to a single displacement of a Lewis base). This substance was then passed through a strongly basic anion exchange column (Dowex 2(OH^-)), where nucleophilic attack of the hydroxide to the ester resulted in formation of AB. It is possible that this process could take place in the environment if all of the required conditions are met.

In a complex procedure, Minhas et al. (1998) were able to synthesize arsenobetaine bromide and arsenocholine bromide/iodide from trimethylarsine at 96% yield and 89% yield, respectively (Pathway 2 a) and b), Figure 2-7).

Pathway 2:

2 a)



2 b)

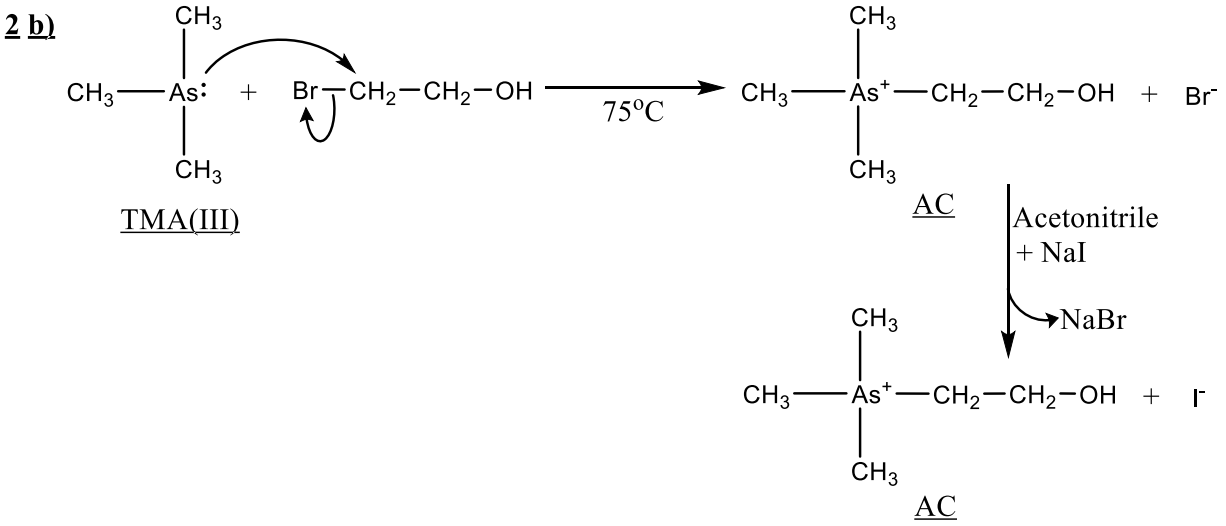


Figure 2-7: Schematic representations and hypothesized reaction mechanisms for the synthesis of arsenobetaine bromide (AB-Br^-) and arsenocholine bromide/iodide (AC-Br^-/I^-) from trimethylarsine (TMA(III)) as discussed in Minhas et al. (1998).

For pathway 2a), trimethylarsine (TMA(III) was added to toluene and cooled to 5°C. Bromoacetic acid was added to this solution, resulting in the immediate precipitation of arsenobetaine bromide (AB-Br^-). For pathway 2b), TMA(III) was heated at 75°C with $BrCH_2CH_2OH$ in a sealed vial for 72 hours. The product, arsenocholine bromide (AC-Br^-), separated after cooling the vial to room temperature and was collected and dried. Arsenocholine iodide (AC-I^-) was formed by combining AC-Br^- in a tinfoil-covered flask containing acetonitrile. Sodium iodide was added and the solution was covered and stirred for two hours. This resulted in the exchange of bromide and iodide (i.e. a metathesis reaction). Sodium bromide then was filtered off and AC-I^- precipitated after cooling to 5°C. In both cases, the mechanism proceeds via a nucleophilic attack of arsenic on the carbon adjacent to bromine, oxidizing arsenic and generating AB or AC.

These abiotic experiments demonstrate that AB can be synthesized through a variety of chemical processes and pathways. However, it is unlikely that these pathways are feasible in nature (as published) due to the compounds and environmental manipulation required.

Nakamura et al. (2008) published the formation of AB by exposing trimethylarsine oxide (TMAO) to iodoacetic acid (IoAA) in the presence of glutathione (GSH) (Pathway 3, Figure 2-8).

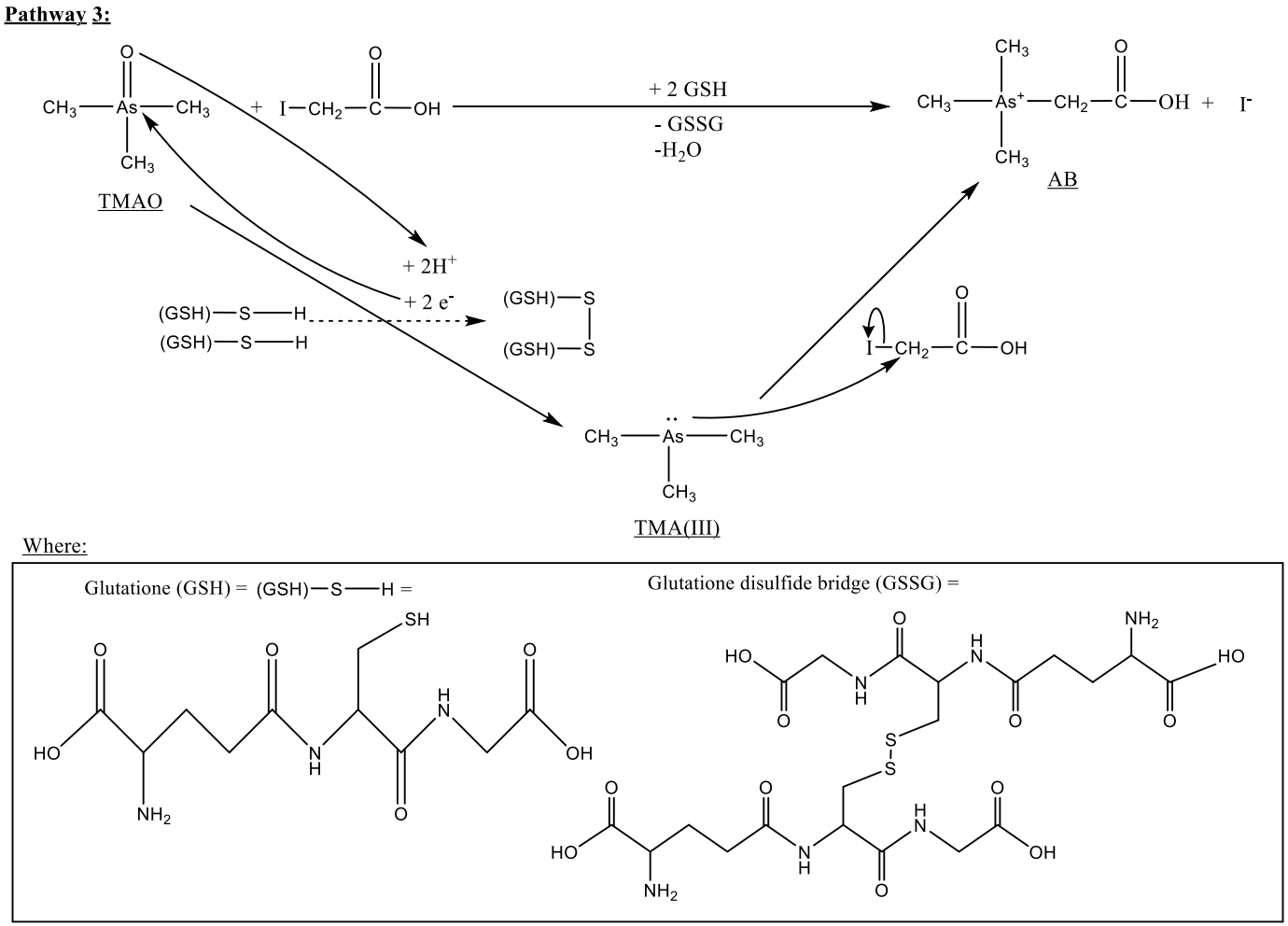


Figure 2-8: A schematic representation and hypothesized reaction mechanism for the synthesis of arsenobetaine (AB) from trimethylarsine oxide (TMAO) using glutathione (GSH) and iodoacetic acid (IoAA) as discussed in Nakamura et al. (2008).

For this reaction, TMAO was combined in solution with GSH and IoAA in a 100 mM phosphoric acid-citric acid buffer at a pH of 5 at 37°C for two hours. The exact mechanism is not described. It is hypothesized (as shown in Figure 2-8) that arsenic in TMAO is reduced to As(III) via the formation of a disulfide bridge between two GSH compounds resulting in TMA(III). From there, TMA(III) can be oxidized via electrophilic attack of the methylene carbon of iodoacetic acid, creating a new As-C bond and releasing I^- (similar to what is observed in the previously described pathways (1 and 2)).

It is possible that this pathway could occur in the environment, if IoAA and GSH are present in the surroundings. GSH is likely present in the environment as it is present in nearly all living organisms and is involved in several metabolic and physiological processes (Grill, 2001). IoAA has been detected in disinfected drinking water (as it is often a by-product of disinfection) (Shi and Adams, 2009) and in rivers and streams (as a result of wastewater treatment) (Duirk et al., 2011). Iodoacetic acid has also been detected in rain and snow (Gilfedder et al., 2008). It is hypothesized that IoAA is also present in the soil. This will be further investigated in Chapter 5, Section 5.2.

Abiotic experiments by de Bettencourt et al. (2011) indicated that GSH was an important compound in forming a number of complexes that could be key intermediates in the potential AB formation pathway. First of all, GSH was shown to react with DMA(V) in water to produce a GS-DMA(III) complex (Pathway 4a, Figure 2-9), highlighting the reducing capabilities of GSH. This was also demonstrated with monomethylarsonic acid (MMA(V)) as the starting compound combined with GSH in water, with the addition of S-adenosyl methionine (SAM) after 2 hours (Pathway 4b, Figure 2-10). This GS-DMA(III) complex was then shown to react with SAM to produce a GS-TMA(V) complex, which reacted with water to form TMAO (Pathway 4c, Figure 2-11). The GS-TMA(V) complex was demonstrated to react with methylcobalamin ($MetCB_{12}$) to form tetramethylarsonium ion (TETRA) (Pathway 4d, Figure 2-12).

Pathway 4:

4 a)

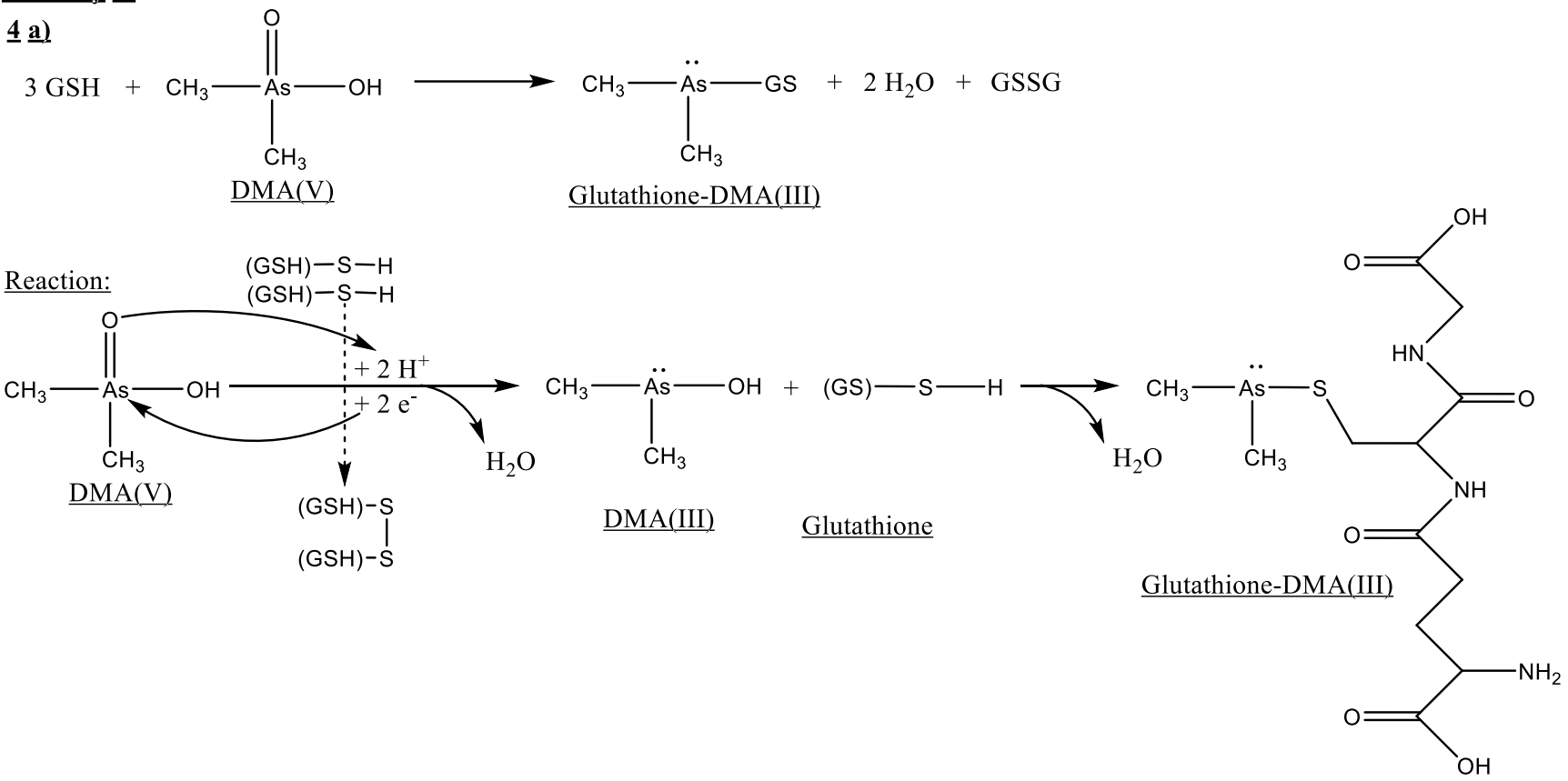


Figure 2-9: Schematic representations of the synthesis and hypothesized reaction mechanism for the synthesis of glutathione-dimethylarsinous acid complex (GS-DMA(III)) outlined by de Bettencourt et al. (2011). For this synthesis, dimethylarsinic acid (DMA(V)) (5 mg) and glutathione (GSH) (20 mg) were combined in 2.5 mL of degassed deionized water and left to react for two hours.

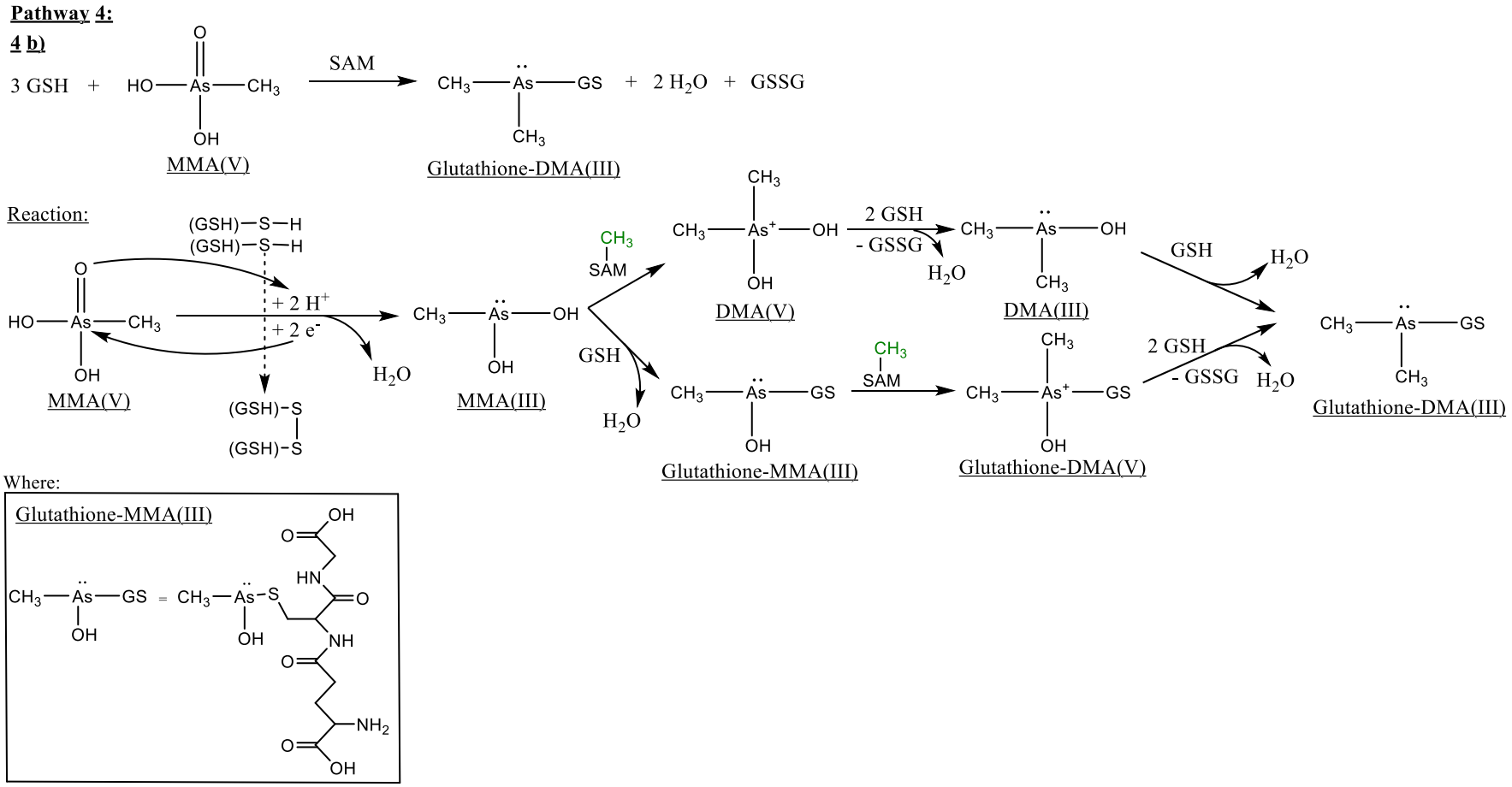
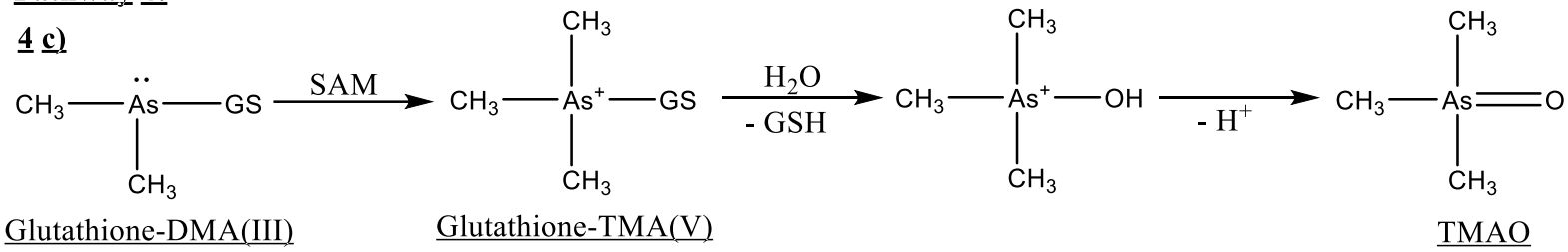


Figure 2-10 : Schematic representations of the synthesis and hypothesized reaction mechanism for the synthesis of glutathione-dimethylarsinous acid complex (GS-DMA(III)) from monomethylarsonic acid (MMA(V)) outlined by de Bettencourt et al. (2011). For this synthesis, MMA(V) and glutathione (GSH) were combined in degassed deionized water and left to react for two hours. After this reaction, S-adenosyl methionine was added and the reaction was left for another 4 hours. The reaction is adapted from de Bettencourt et al. (2011) and a possible mechanism is suggested. Several intermediates (DMA(V), DMA(III), GS-MMA(III), and GS-DMA(V)) are shown between MMA(III) and GS-DMA(III), as the order of the reaction is unknown and both pathways are possible.

Pathway 4:

4 c)



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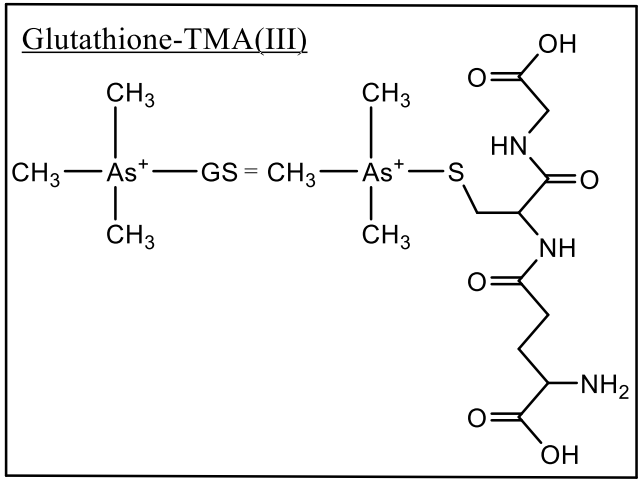
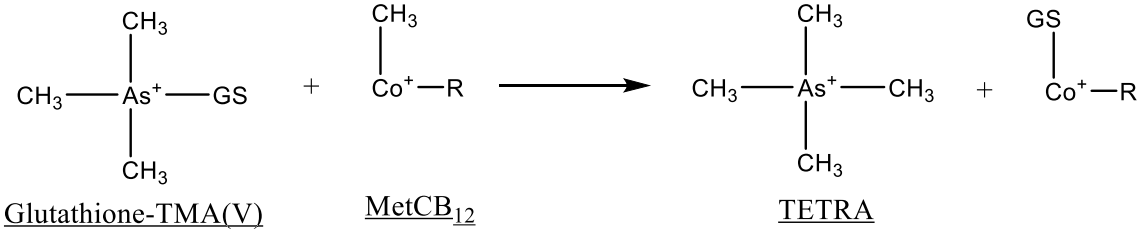


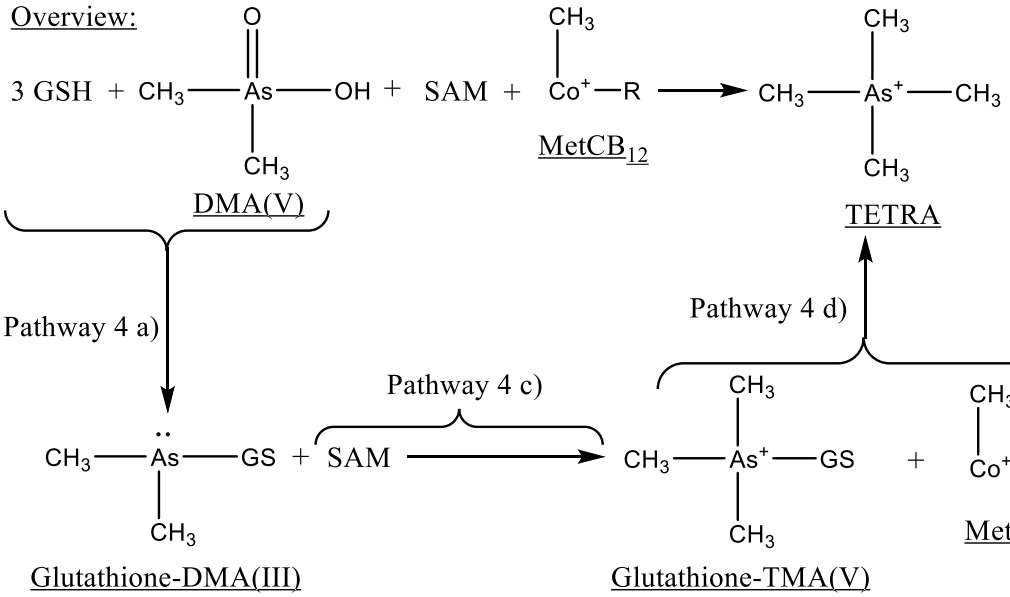
Figure 2-11: A schematic representation of the synthesis of glutathione-trimethylarsine complex (GS-TMA(III)) and trimethylarsenic oxide (TMAO) outlined by de Bettencourt et al. (2011). For this synthesis, S-adenosyl methionine (SAM) was added to the reaction of DMA(V) and GSH (Pathway 4a) and left to react for three hours, resulting in the formation of the GS-TMA(III) complex. This complex was demonstrated to be easily hydrolyzed in the presence of water, resulting in the formation of TMAO.

Pathway 4:

4 d)



Overview:



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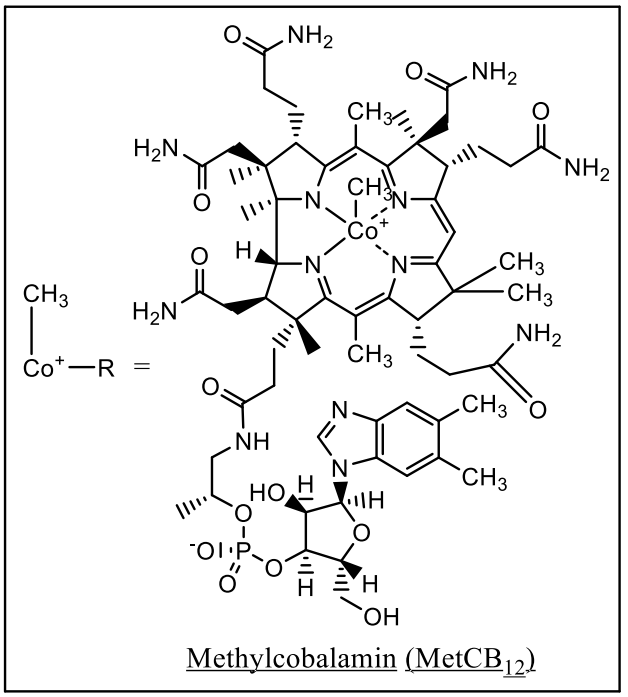

Figure 2-12: A schematic representation of the synthesis of tetramethylarsonium ion (TETRA) outlined by de Bettencourt et al. (2011). For this synthesis, methylcobalamin ($MetCB_{12}$) was added to the reaction of DMA(V), GSH, and SAM (Pathway 4c) and left to react for an additional four hours, resulting in the formation of TETRA.

In all these reactions, the reagents typically play similar roles. GSH has two functions: it can reduce As(V) to As(III) or it can act as a Lewis base and exchange with a hydroxyl group (or potentially another anion) (i.e., it can be exchanged with other arsenic substituents via a metathesis reaction). SAM acts as a positive (carbonium) methyl donor in all of these reactions and therefore cannot exchange with a negative group on arsenic. The positive methyl group on SAM is typically attacked by As(III) (Lewis acid attacked by Lewis base), creating a new arsenic-carbon bond and oxidizing As(III) to As(V). In these situations, the addition of the methyl group does not result in the discharge of any other substituents (demonstrated in pathway 4c). On the other hand, $MetCB_{12}$ may act as a negative (carbanion) methyl donor. Therefore, it could potentially exchange with other negative groups attached to arsenic (such as the hydroxyl and GS groups mentioned previously). In this case, there is no change to the oxidation state of the arsenic compound and a 'switch' of the groups (i.e. metathesis reaction) takes place.

All of the reactants in these pathways are commonly found in the environment, suggesting that they could be viable in the natural environment. For these reactions, no alterations were made to the environment (i.e. the reactants were simply added at different intervals and left to react for a total of approximately 10 hours) (de Bettencourt et al., 2011). In some cases, the initial GS-arsenic complexes may not be as prevalent in nature, but can be synthesized by reacting DMA(V) or MMA(V) with GSH and/or SAM (de Bettencourt et al., 2011). Therefore, these reactions seem highly plausible and show how the addition of the fourth methyl group on TETRA can be added. This offers a glimpse into how the fourth substituent could be added to arsenic to give AB, which, like TETRA, has a positive charge on the arsenic atom. The pathways proposed by de Bettencourt et al. (2011) offer a hypothetical precursory formation for AB, if an acetate group (-CH_2COO^-) (such as glyoxylate) can be added instead of another methyl group. However, further investigation is needed to determine if this would actually take place.

The abiotic experiments by de Bettencourt et al. (2011) also demonstrate possible formations of arsenosugars from glutathione-arsenic complexes (Pathway 4 e), Figure 2-13).

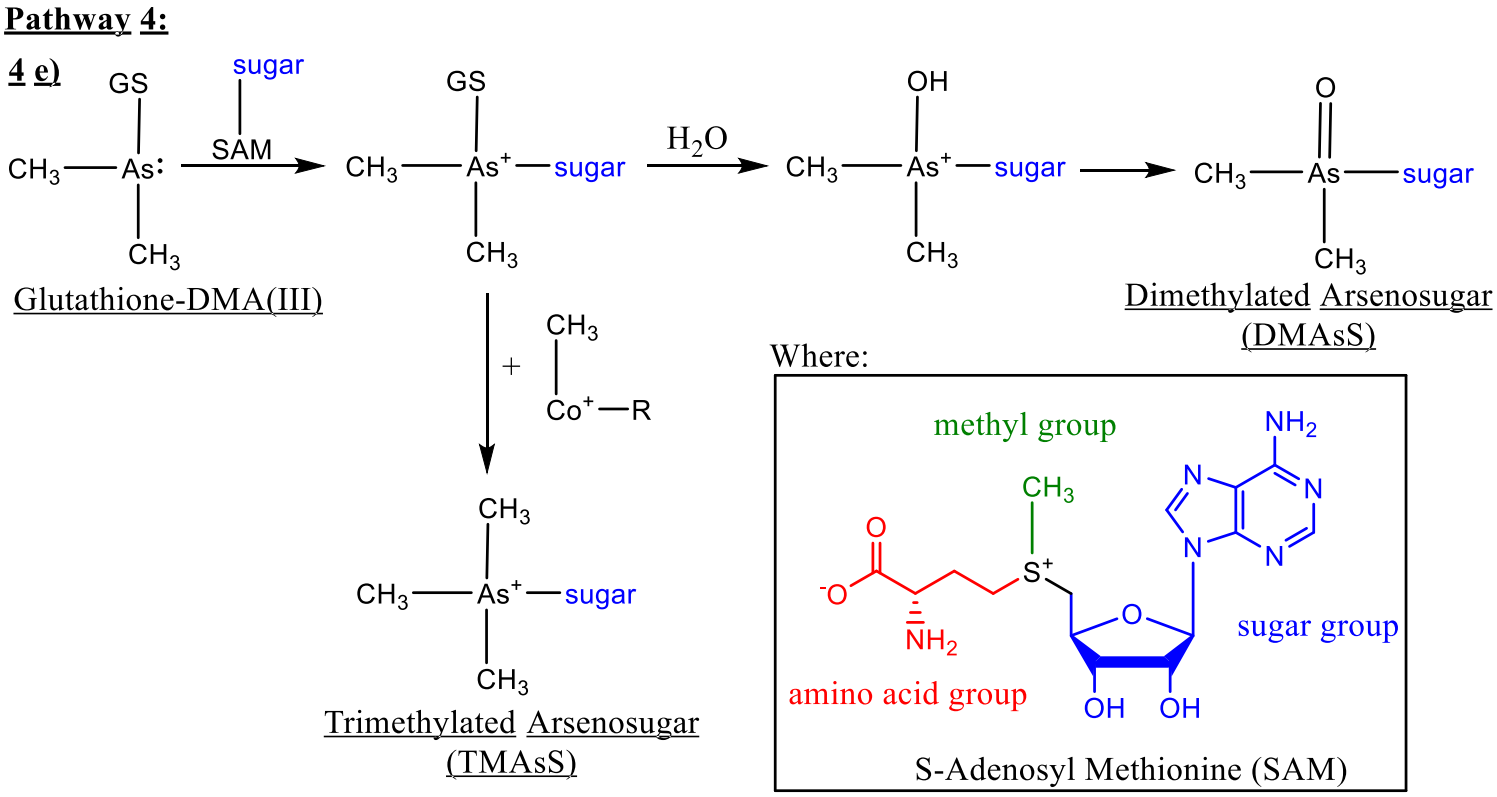

Figure 2-13: Schematic representations for the synthesis of dimethylated arsenosugars (DMAsSs) and trimethylated arsenosugars (TMAsSs) using the glutathione-dimethylarsinous acid complex (GS-DMA(III)) as the initial reactant (as suggested by de Bettencourt et al. (2011)).

The synthesis of these arsenosugars from the trivalent arsenic-glutathione complex offers a possible formation pathway for arsenosugars in the environment. The involvement of arsenosugars in the formation of AB will be discussed in the following section.

2.5.1.2. Biologically Mediated Pathways

The biosynthesis of non-toxic AB remains unclear, although it has been shown to specifically accumulate in marine organisms and some terrestrial fungi. It is possible that the accumulation of AB is the result of a detoxification process in marine organisms; however, it is more likely linked to the salinity of the environment (Caumette et al., 2012). The higher concentrations of AB in marine organisms are more commonly attributed to the osmolytic properties of the molecule because of its structural resemblance to betaine [$(CH_3)_3N^+CH_2COO^-$] (Molin et al., 2015a). Betaine is a nitrogen betaine used as an osmolyte by aquatic organisms to maintain osmotic balance under conditions of changing salinity (Adair et al., 2005). In terrestrial fungi, AB is thought to play a similar role. High AB concentrations have been found in some (but not all) mushrooms, specifically in the cap and outer stalk, where it may accompany other osmolytes to maintain a rigid cap suitable for holding and dispersing spores (Nearing et al., 2014a, Smith et al., 2007).

Currently, the main hypothesis for the mechanism of AB formation is through the degradation of dimethylated arsenosugars (DMAsSs) or trimethylated arsenosugars (TMAsSs), or alternatively from DMA(III). These arsenosugars are likely formed through a similar reaction to what was suggested by de Bettencourt et al. (2011) (Pathway 4 e, Figure 2-13), however, without GSH involved in the reaction. The formation of arsenosugars without an arsenic-glutathione initial complex is demonstrated in Figure 2-14 for DMAsSs (pathway 5) and TMAsSs (pathway 6). The formation of AB from arsenosugars could explain the higher levels of AB in urine seen in research by Molin et al. (2015b) and Fukuda et al. (2011).

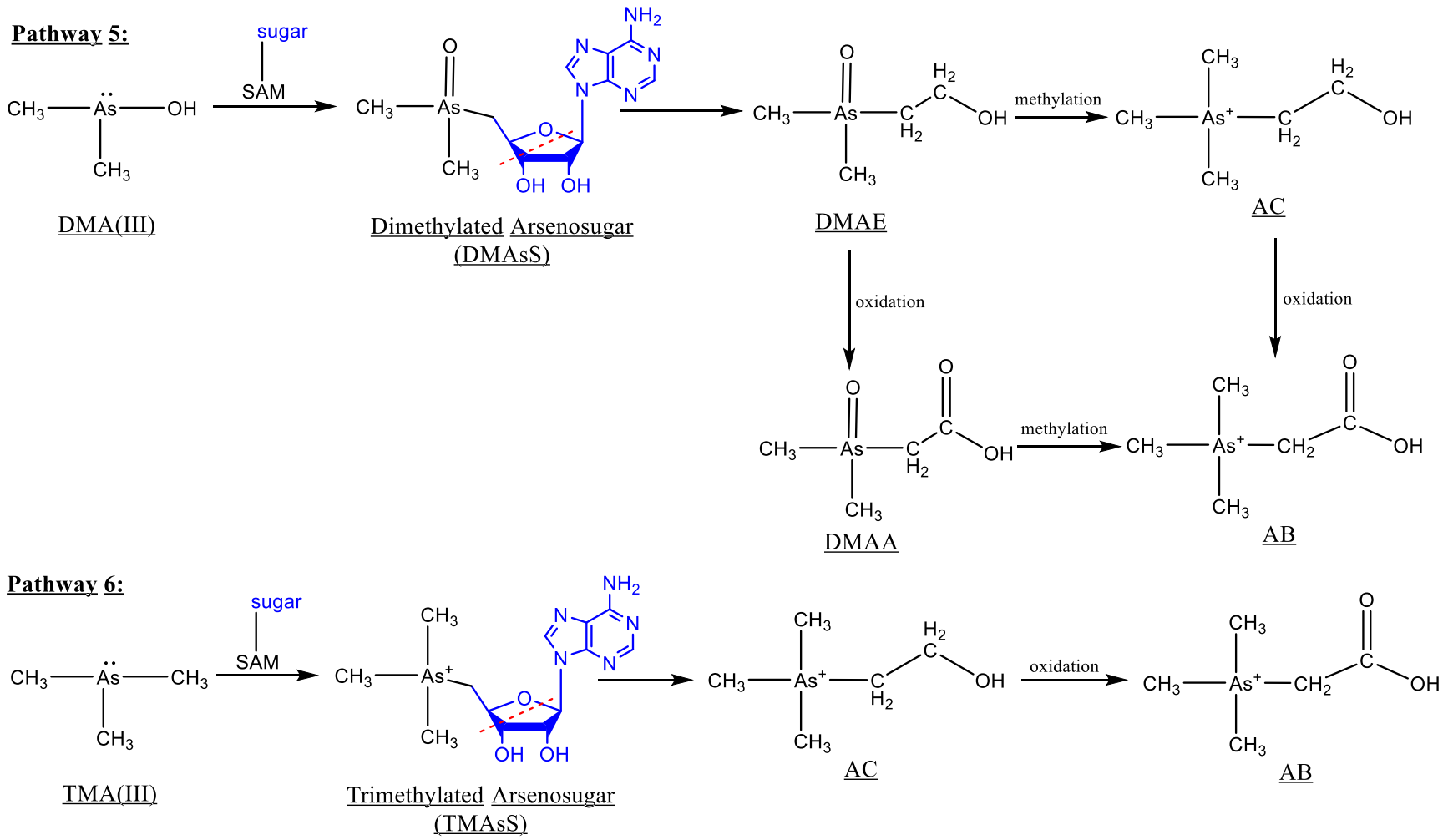

Figure 2-14: A schematic representation of the proposed mechanism of formation of arsenobetaine (AB) from arsenosugars. The formation of dimethylated arsenosugars (DMAsSs) and trimethylated arsenosugars (TMAsSs) is suggested to be similar to what was outlined by de Bettencourt et al. (2011). The suggested subsequent degradation of these sugars is indicated by a dashed red line. The formation of AB from these sugars is due to a combination of oxidation and methylation reactions.

In the Challenger pathway (Figure 2-1), SAM acts as a methyl-group donor. However, it can also act as a source of adenosyl groups for the production of arsenosugars (Reimer et al., 2010). In this case, shown as pathway 5 in Figure 2-14, dimethylated arsenosugars generated from SAM can be degraded to form dimethylarsinoyl ethanol (DMAE), which can then be methylated (either via the reduction and oxidative methylation pathway described earlier with GSH and SAM or potentially via an anion exchange with a negative methyl group, such as the one on $MetCB_{12}$) to form AC or oxidized to form dimethylarsinoyl acetic acid (DMAA). AB is then formed when AC is oxidized, or when DMAA undergoes methylation (again, by a negative methyl group). This pathway is the most widely accepted theory, since high levels of dimethylated arsenosugars are present at the base of aquatic food chains (Duncan et al., 2010). Support for this pathway is provided by Edmonds and Francesconi (1987), who identified arsenic-containing ribofuranosides (arsenosugars) in algae, and DMAE as a product of their degradation. DMAA, DMAE, and AC have also been detected in low concentrations in several marine animals and algae (Reimer et al., 2010). The mechanism by which these sugars are converted to DMAA, DMAE, or AC remains unclear. This is an area that lacks mechanistic studies and more research is needed to determine exactly how the arsenosugars degrade.

The conversion of AC to AB has been demonstrated in several studies. In a laboratory study using mice, rats, and rabbits, the conversion of AC to AB was demonstrated by Marafante et al. (1984). Microorganisms from Japanese sediment were also found capable of converting AC to AB (Hanaoka et al., 1992). In research by Francesconi et al. (1989), fish (yelloweye mullet) that were fed AC readily converted it to AB which was retained in the tissues. However, the exact mechanism of transformation remains unclear.

In pathway 6, trimethylated arsenosugars (whose exact formation pathway is unknown, but may be via the methylation of dimethylated arsenosugars or generated from SAM) undergo degradation to form AC and then oxidization to form AB. Francesconi et al. (1992) demonstrated the degradation of trimethylated sugars to AC. A separate study by Francesconi et al. (1999) found that shrimp accumulated very little (0.9%) dimethylated arsenosugar and the arsenosugar remained unchanged. However, the shrimp accumulated more of the trimethylated arsenosugars and almost half of the accumulated amount was converted to AB. Although this mechanism accounts for some AB formation, the small amount of trimethylated species generally found in the environment suggests that other pathways may be more likely (Caumette et al., 2012).

In a separate pathway, DMA(III) has been hypothesized as a precursor to DMAA (possibly from glyoxylate (or pyruvate)), which is then methylated to form AB (Edmonds, 2000) (glyoxylate: Pathway 7, Figure 2-16, pyruvate: Pathway 8,

Figure 2-17). The differences in the structure between glyoxylate and pyruvate are shown in Figure 2-15.

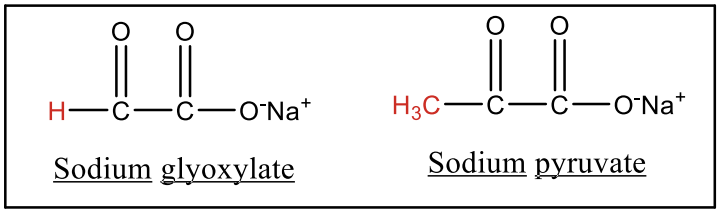

Figure 2-15: A comparison of the chemical structure of sodium glyoxylate and sodium pyruvate. The difference in the chemical structures is highlighted in red.

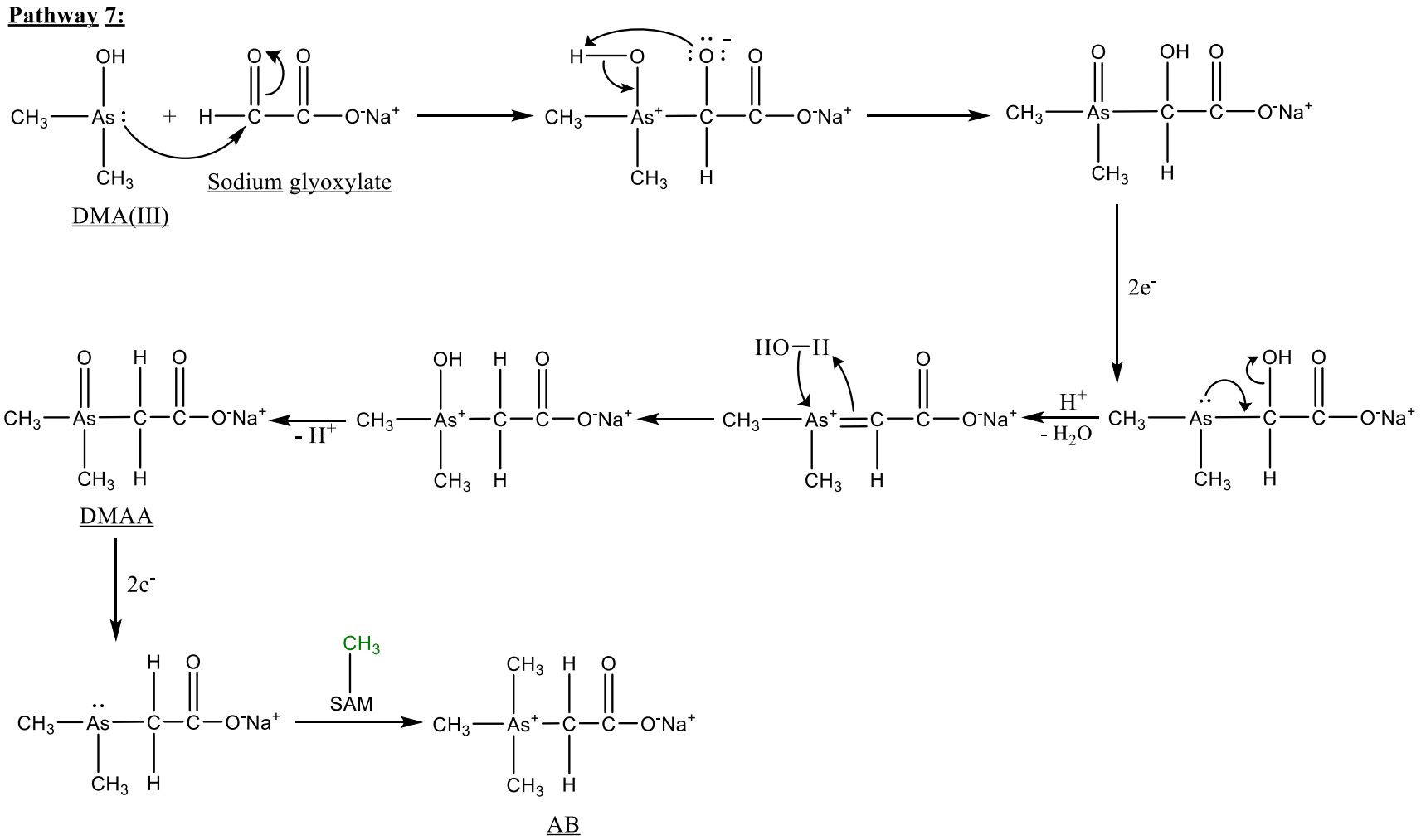

Figure 2-16: A schematic representation of the a possible formation pathway for arsenobetaine (AB) from dimethylarsinous acid (DMA(III)) using sodium glyoxylate. In this reaction, dimethylarsinoyl acetic acid (DMAA) exists as an intermediate compound. This pathway was originally proposed by Edmonds (2000).

Pathway 8:

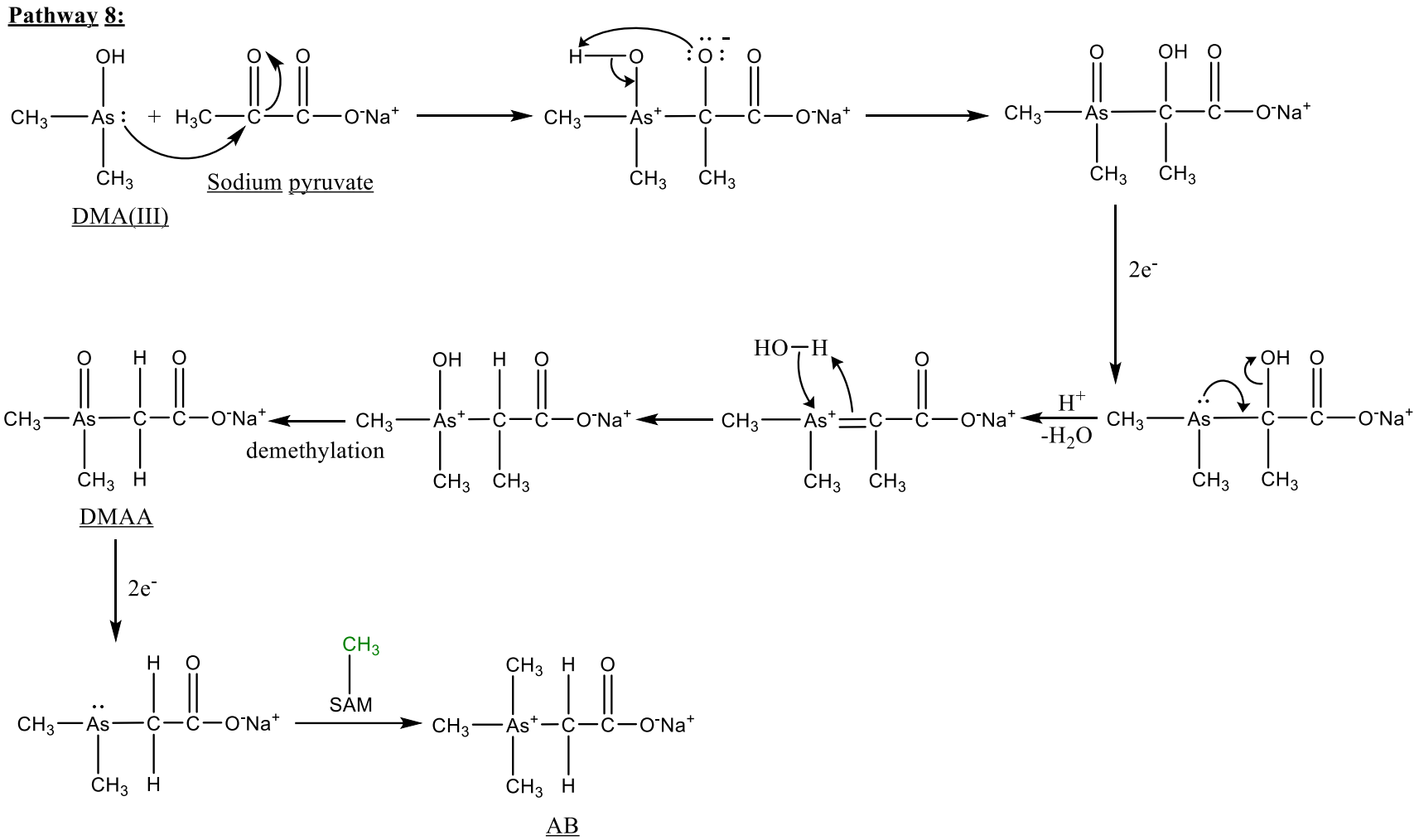

Figure 2-17: A schematic representation of the a possible formation pathway for arsenobetaine (AB) from dimethylarsinous acid (DMA(III)) using sodium pyruvate. In this case, a demethylation of the 2-carbon is necessary in order to form AB. In this reaction, dimethylarsinoyl acetic acid (DMAA) may exist as an intermediate compound, depending when the demethylation occurs. This pathway was originally proposed by Edmonds (2000).

In these reactions, DMA(III) would be oxidized via nucleophilic attack, creating a new As-C bond (as was observed for several of the reactions in the previous section). After some electron shuffling, it is possible that this compound is reduced by either pyruvate or glyoxylate. However, this depends on the reduction potential of these compounds in the reaction and another reducing agent (such as GSH) may be required.

At this point, for the reaction using glyoxylate (pathway 7), it is hypothesized that DMAA may form after the introduction of water. After further reduction (potentially by glyoxylate or another reducing agent) and the addition of SAM (capable of methylcarbonium attack), AB may be produced. A similar reaction may take place when pyruvate is used (pathway 8). However, a demethylation of the 2-carbon, or potentially, a shift in the methyl group from carbon to arsenic would also be required.

If neither glyoxylate nor pyruvate are able to reduce arsenic in this reaction, then future experiments should be done using glutathione (GSH) to reduce arsenic in combination with SAM as a methyl donor, or methylcobalamin ($MetCB_{12}$) as a methylcarbanion transfer agent.

The 'arsenylation' of glyoxylate parallels its amination, producing arsenic compounds that are analogous to α-amino acids. This speculated pathway offers some support as to why AB and betaine are so similar (Edmonds, 2000). Furthermore, this pathway offers a route to AB in environments where arsenosugars are not found in high proportions or concentrations (such as in the terrestrial environment, particularly in the fruiting body of mushrooms).

In summary, out of the three pathways listed, none have been demonstrated to fruition. In pathway 6, the formation of AB from TMAsSs and AC was demonstrated by Francesconi et al. (1999), however the exact formation of TMAsSs has yet to be determined. The last step of pathway 5 and 7 was demonstrated by Ritchie et al. (2004), who showed that by using lysed-cell extract of a *Pseudomonas* species, DMAA could be converted to AB and DMA(V). Supplementing the reaction with SAM promoted both the rate and extent of AB formation (Ritchie et al., 2004). The early steps of all pathways still require more experimentation and have yet to be demonstrated.

2.5.2. Arsenobetaine Formation in Terrestrial Fungi

AB has been identified as a common arsenic species in the fruiting bodies, which are commonly referred to as mushrooms, of a wide range of fungal species. Specifically, the Agaricaceae family (including the white button mushroom, *Agaricus bisporus*) contains AB as its primary arsenic compound in all the species analyzed to date (Nearing et al., 2014a). As mentioned previously, it is proposed

that AB plays an osmolytic role in terrestrial fungi, similar to its role in marine organisms. The high proportion of AB in puffball or gilled mushrooms supports this theory, as these morphologies require osmolytes to maintain their fruiting body structure (Nearing et al., 2014a). However, the exact formation pathway of AB in fungi is unclear. In the following sections, the role of the mushroom species itself, growth conditions, and microorganisms in the formation of AB will be discussed.

It has been proposed that the fungi producing the mushrooms and/or associated organisms are responsible for AB formation, as AB is not generally found in soil and it has been identified in mushroom species collected from different locations (Koch et al., 2000). Therefore, it is possible that the fungi produce AB at some point in their life cycle (during fruiting body formation) or, alternatively, that it is accumulated from the surrounding environment where AB or its precursors are produced by microorganisms. In a study of 46 different fungal species, Nearing et al. (2014a) found that major arsenic species were consistent within phylogenetic groups, and that AB was the most prevalent species within both the Lycoperdaceae and Agaricaceae families. However, in log-growing mushrooms, no AB was detected, reiterating the theory of microbial influence on AB formation, since log and soil microbial communities are likely different. In a subsequent study, Nearing et al. (2015b) found that the vegetative life stage (the mycelium) of fungi is not responsible for AB formation, indicated by the lack of arsenic transformations and absence of AB.

AB accumulation in *A. bisporus* was shown to be significant when exposed to environmentally relevant concentrations of arsenic (190 to 360 mg/kg in substrate) (Smith et al., 2007). This supports the hypothesis that AB is a product of fungal arsenic metabolism, possibly in conjunction with the metabolism of microbes associated specifically with the fungus. Methylated arsenic species, including TMAO (but not AB), were detected in the uninoculated compost, indicating that some microorganisms were able to survive the heat sterilization process (similar to pasteurization, used to prepare the compost in these experiments) and methylate the added iAs. In a study by Soeroes et al. (2005), exposure of *A. bisporus* to high iAs concentrations (1000 mg/kg in mushroom substrate) led to AB concentrations half of that seen in the culture not exposed to arsenic, although both concentrations were less than 0.5 mg/kg. They suggest that the high iAs concentrations in some way inhibits the formation of AB but the differences may also be attributable to variability between cultivation experiments (control and treated substrates were not produced in replicates).

2.5.2.1. Role of Microorganisms in Arsenobetaine Formation

Microorganisms are capable of affecting both fungal growth and arsenic speciation. When cultivating mushrooms, the transition from the vegetative phase to the reproductive (fruiting body) stage depends on the presence of a casing layer with

specific physical, chemical, and microbiological properties (Ebadi et al., 2012). In a study by Zarenejad et al. (2012), 247 bacterial isolates were identified in the casing layer from 14 different edible mushroom farms. Of these, the researchers identified 23 strains as organisms that they termed mushroom growth promoting bacteria (MGPB). This classification was used for bacteria that they found to stimulate and promote primordia and fruiting body formation via undefined pathways, including several *Pseudomonas* isolates and *E. coli* strains. *Pseuodomonas putida* promoted the highest increase in *A. bisporus* production (Zarenejad et al., 2012). The symbiotic relationship between the presence of MGPB and fungal growth has been shown to cause a significant increase in mushroom production, reduction in the time until harvest, and an increase in the total polysaccharide-protein complex content (Young et al., 2013). However, the interaction is not always a good thing and although *Pseuodomonas putida* stimulates fruiting body formation, other *Pseuodomonas* can cause mushroom diseases (Scherlach et al., 2013).

There is limited research on the methylation of arsenic by microbes associated with mushrooms or even plants. As previously mentioned, Smith et al. (2007) found methylated organoarsenicals in mushroom compost, with TMAO as the primary product of microbial arsenate transformation. In plant research, Lomax et al. (2012) conducted an experiment where they exposed plants (rice, tomato, and red clover) to iAs under axenic conditions, and determined that no methylated arsenic species were produced. The plants were able to take up MMA(V) and DMA(V), but not produce it on their own. They also grew rice seedlings and rice plants in non-sterile, arsenite- or DMA(V)- containing conditions, where they identified the formation of methylated arsenic. These results indicate that the studied plants are unable to methylate iAs, and instead, take up methylated arsenic produced by microorganisms (Lomax et al., 2012). Zhao et al. (2013) suggest that methylated arsenic species in the plants studied so far (including rice) originate from the soil and that methylation in the soil depends on soil conditions. In contrast, Cullen et al. (1989) showed that pure suspension cultures of plants (specifically the Madagascar periwinkle, *Catharanthus roseus*) methylated MMA(V) to DMA(V) in small amounts, after uptake of the MMA(V). They suggest that *C. roseus* cells have a greater cytosolic capacity to accumulate methylarsonate than to transform it.

If microorganisms associated with fungal production also occur in the marine environment, it is possible that they may be, at least partially, responsible for AB formation.

2.6. Human Exposure to Arsenic

Humans are mainly exposed to arsenic through food, except in areas where the drinking water is naturally contaminated with arsenic (Aitio and Becking, 2001).

These areas include Bangladesh and West Bengal, India, where some of the most contaminated sources of drinking water have been identified with concentrations ranging from under 50 μg/L up to and above 300 μg/L (Chakraborti et al., 2015). The WHO guideline value for drinking water is 10 μg/L (based on the consumption of 2 L of water per day) (Bos et al., 2011).

Humans can also be exposed to arsenic via inhalation. However, the amount of arsenic inhaled is generally very small, except in areas that are industrially polluted (near mining, mineral processing, and metallurgical extraction industries), or if people smoke (arsenic compounds are among the several hundred chemicals present in cigarette smoke) (Cascio, 2011, Martin et al., 2014). Pulmonary exposure to arsenic can contribute to approximately 1 μg/day in non-smokers and approximately 10 μg/day in smokers (Aitio and Becking, 2001).

2.6.1. Arsenic in food

Arsenic is found in a wide range of food categories (notably, seafood, rice, cereals, mushrooms, and poultry) with a high variation of concentrations and species (Table 2-3) (Cascio, 2011, Jomova et al., 2011). The daily intake of total arsenic from food and beverages is highly dependent on the population of interest. This data can be obtained from total dietary studies (TDSs), in Canada (Health Canada, 2009) and the United Kingdom (European Food Safety Association, 2009), as well as from large-scale studies like the French study "CALIPSO" (Leblanc, 2008). However, the total daily arsenic intake does not specify the ingested species, which is important because toxicological effects and safe limits are generally established for iAs. iAs (largely considered to be the most toxic, as discussed in Section 2.1.3.) has been specifically reported by Xue et al. (2010) for North America, in the CALIPSO study, and in the European Union (European Food Safety Association, 2009), Japanese (Oguri et al., 2014) and Chinese (Li et al., 2011) TDSs, but not by Health Canada.

In North America, the mean total arsenic exposure from food is 0.38 μg/kg per day, of which 0.05 μg/kg per day is iAs (approximately 13%) (Xue et al., 2010). For a 150 lb individual (68.2 kg), this would be equal to 26 μg/day, of which 3.41 μg/day is predicted to be iAs. However, in countries where populations consume larger volumes of high-iAs foods (such as rice or cereal), the daily intake may be much higher (Bos et al., 2011). For example, the average iAs consumption in Japan is estimated to be between 19 and 59 μg/day due to the consumption of rice and hijiki (Oguri and Yoshinaga, 2013). In a study of residents in Shizuoka city, Japan, the greatest iAs intake was from cereals (13 μg/day per person), followed by algae (5.7 μg/day per person), constituting 90% of the total daily adult iAs consumption (21-24 μg/day per person) (Oguri et al., 2014). In China, the daily iAs is estimated to be around 42 μg/day, with rice being the largest contributor to total iAs consumption (about 60%) for all of the different regions (Li et al., 2011).

The Agency for Toxic Substances and Disease Registry (ATSDR) suggests a minimal risk level (MRL) (the daily human exposure that is likely to be without appreciable risk of adverse health effects) for iAs of 5 μg/kg per day (equates to 300 μg/day for a 60 kg person) for acute exposure (exposure taking place during a period of 14 days or less) and 0.3 μg/kg per day (equates to 18 μg/day for a 60 kg person) for chronic exposure (one year or longer) (ATSDR, 2014). The latter value is also the oral reference dose for arsenic currently published by the U.S. EPA Integrated Risk Information System (IRIS, 1991). The Joint Food and Agriculture Organization of the United Nations (FAO)/WHO Expert Committee on Food Additives (JECFA) determined that the lower limit dose for a 0.5% increased incidence of lung cancer (1 in 200 incremental cancer risk) from epidemiological data is 3.0 μg/kg bodyweight per day (equates to 180 μg/day for a 60 kg person) (WHO, 2010). Therefore, the cancer risk posed by the dietary intake of iAs in locations like Japanese and China may not be negligible.

In Canada, the only limits currently in existence for arsenic in food are based on total arsenic in fish protein, bone meal, and juice (3.5 ppm, 1.0 ppm, and 0.1 ppm, respectively) (Canadian Food Inspection Agency, 2016). Australia and New Zealand Food Standards Code specifies a maximum level of total arsenic in cereals (1 mg/kg) and maximum iAs levels for crustaceans (2 mg/kg), fish (2 mg/kg), molluscs (1 mg/kg), and seaweed (1 mg/kg) (Food Standards Australia & New Zealand, 2015). China has more extensive legislation on the maximum levels of total arsenic in juice, edible fats, coco products (e.g. coco butter and chocolate), and sugar (all between 0.1 and 1 mg/kg) and iAs in grains (0.1-0.2 mg/kg) and in fruit, vegetables, meat, eggs, fresh milk, and liquor (all 0.05 mg/kg) (USDA, 2006). Furthermore, fish, algae, shells, shrimp, crabs, and other aquatic foods all have restricted iAs levels (with the highest permissible concentration being 1.5 mg/kg dry weight for algae) ((USDA, 2006) translated from (China Food Safety Regulations, 2005)).

The species of arsenic present in commonly consumed foods are reviewed in Table 2-3. These foods are organized on the basis of their origin (marine or terrestrial). As stated in the introduction, the predominant arsenic species in almost all of the marine-origin samples is AB.

Table 2-3: A representation of the typical arsenic species identified in marine (blue) and terrestrial (green) organisms commonly eaten in many cultures. AB is present in almost all of the marine organisms.

Food		**Arsenic Species Generally Found**	**Reference**
Shrimp		AB, AC	(Branch, 1990)
		AB, DMA(V), As(III), As(V)	(Sadee et al., 2015)
Haddock		AB, As(V)	(Branch, 1990)
Crab		AB, AC	(Branch, 1990)
Cod		AB, As(V)	(Branch, 1990)
Mackerel		AB, DMA(V), As(V)	(Branch, 1990)
Seaweed	Yakinori	As(III), As(V), MMA(V), DMA(V), AB	(Morita et al., 1981)
	Hijiki	Mainly iAs (As(III), As(V)), and arsenosugars	(Rose et al., 2007), (Yokoi and Konomi, 2012)
	Other Species	Arsenosugars, AB, As(III), MMA(V), DMA(V), As(V),	(Benramdane et al., 1999b, Van Hulle et al., 2002)
Shellfish		As(III), As(V), MMA(V), DMA(V), AB and AC	(Branch, 1990)
Herring		AB, DMA(V), organoarsenicals	(Lischka et al., 2013)
Tuna		AB, DMA(V), MMA(V), As(III), As(V)	(Raber et al., 2012)
Bivalves (Mussel)		AC, AB, As(III), DMA(V), MMA(V), As(V)	(Benramdane et al., 1999b)
		AB, AC, DMA(V), MMA(V), As(III) and As(V)	(Sadee et al., 2015)
		AB, arsenosugars, As(III), As(V)	(Li et al., 2003)
		AB, DMAA, As(V), arsenosugars	(Lai et al., 2004)
Bivalves (Oyster)		AB, DMA(V), MMA(V), As(V), arsenosugars	(Sadee et al., 2015) (Li et al., 2003)
Bivalves (Clam)		AB, arsenosugars	(Li et al., 2003)

Food	Arsenic Species Generally Found	Reference
Fruit and Vegetables	As(III), As(V), MMAA, DMA(V), TMAO	(Branch, 1990)
Fruit and Vegetables	As(III), As(V), DMA(V) and MMA(V)	(Sadee et al., 2015)
Potato	DMA(V), As(III), As(V)	(Branch, 1990)
Carrot	DMA(V), As(III), MMAA	(Branch, 1990)
	MMA(III), MMA(V), DMA(V), monomethylthioarsonic acid (MMTA), As(III), As(V)	(Amaral et al., 2013)
	As(III), MMA(V), As(V)	(Vela et al., 2001)
Leek	DMA(V), As(III), As(V)	(Branch, 1990)
Rhubarb	DMA(V), As(III), MMA(V)	(Branch, 1990)
Berries	As(III), As(V), DMA(V)	(Koch et al., 2013)
Apple	As(III), DMA(V), MMA(V), As(V)	(Sadee et al., 2015) (Amaral et al., 2013)
Rice (including white long, brown long, basmati, risotto, jasmine)	As(III), As(V), DMA(V), MMA(V)	(Jackson and Punshon, 2015)
	As(III), DMA(V), As(V)	(Williams et al., 2005)

Although seafood is known to be the most significant source of arsenic in the diet, it generally contains very little iAs (lower than 0.1 mg/kg) (Sloth, 2013). The dominant species in marine fish and most other seafood is AB, generally constituting more than 70% of the total arsenic content (Rose and Fernandes, 2013). However, arsenosugars have been found in high concentrations in algae and algae-feeders (e.g. mussels and oysters) and arsenolipids have been identified in fish oils and fish species with higher fat content (e.g. capelin and tuna) (Guéguen et al., 2011, Molin et al., 2015a).

In a British TDS, total and iAs were identified in the main food types (Food Standards Agency, 2009). In this study, fish had the highest total arsenic (3.99 mg/kg, dry weight (dw)), followed by poultry (0.022 mg/kg, dw), but cereals had the highest proportion of iAs (0.012 mg/kg, dw or 67%) in comparison with fish (0.015 mg/kg, dw or 0.003%). These values show that although a lower overall proportion may be seen in fish, a higher or comparable concentration may still be the result. However, some marine species have been found to have uniquely high iAs. These include marine algae, like hijiki (*Hizikia fusiforme*) (arsenate > 60 mg/kg) and bivalves, like blue mussels (*Mytilus edulis*) (iAs between 0.001 and 4.5 mg As/kg) (Molin et al., 2015a).

Rice and rice-based products have also been identified as significant dietary sources of iAs (Raber et al., 2012). Rice contains both As(III) and As(V) in proportions that are generally higher than organic arsenic (DMA and MMA), but the organic/inorganic ratios vary depending on the source location of the rice (Cascio, 2011). The proportion of iAs to total arsenic in rice ranges between 10-93% , and brown rice typically has more arsenic and a higher percent of iAs than white rice (Molin et al., 2015a). China is presently the only country that has implemented national legislation for maximum inorganic concentration in rice (at 0.15 mg/kg) (USDA, 2006).

2.6.2. Arsenic Species in Mushrooms

Speciation analysis of arsenic compounds in terrestrial fungi (i.e. mushrooms) has identified the presence of a number of different arsenic compounds, including AB, AC, arsenosugars, TETRA, TMAO, DMA(V), MMA(V), and iAs (Table 2-4). Health Canada has not set a limit on the amount of arsenic in commercially produced mushrooms (Canadian Food Inspection Agency, 2016). China is the only country to date that has established maximum total arsenic concentrations for fresh (0.5 mg/kg) and dried (1.0 mg/kg) mushrooms (Llorente-Mirandes et al., 2014, Ministry of Health of China, 2003).

Of the different species included in Table 2-4, the edible mushrooms containing AB as their most prevalent form of arsenic include all of the puffball mushrooms and almost all of the *Agaricus* species. The other mushrooms that contain AB as

their main species were gilled (fried chicken mushroom and shaggy mane mushroom). One pored mushroom (Hollow Bolete mushroom) had predominantly AB, but this was an exception and most pored mushrooms contained predominantly DMA. A partial association of predominantly AB with mushroom morphology (puffball and gills) has been explained by the theory that AB acts as an osmolyte, helping to maintain the structure (and reproductive processes such as spore dispersal) (Nearing et al., 2014a).

Table 2-4: The main arsenic compounds found in some edible mushroom species grown in a commercial setting and from both contaminated and uncontaminated locations. DMA and MMA are in the (V) oxidation state.

Mushroom (*Species*)	iAs	AB	DMA	MMA	TMAO	AC	TETRA	Origin
Bolete (with pored caps)								Contaminated Soil, [a]
Slippery Jack (*Suillus luteus*)								
White Button (*Agaricus bisporus*)								
Deceiver (*Laccaria laccata*)								
White Button (*Agaricus bisporus*)								Store-bought, [b]
Portobello (*Agaricus brunnescens*)								
Cremini (*Agaricus brunnescens*)								
Straw (*Volvariella volvacea*)								
Enoki (*Flammulina velutipes*)								
Shiitake (*Lentinula edodes*)								
Oyster (P*leurotus ostreatus*)								
Black chanterelle (*Craterellus cornucopioides*)								
Golden chanterelle (*Cantharellus cibarius*)								
King Bolete, pored (*Boletus edulis*)								
Lobster (*Hypomyces lactifluorum*)								
White Tree/Wood Ear (*Tremella fuciformis*)								
Cloud/Wood Ear (*Auricularia polytricha*)								
Giant Puffball (*Calvatia gigantea*)								Uncontaminated Soil, [b]
Common/Warted Puffball (*Lycoperdon perlatum*)								
Gomphidius oregonensis (no common name)								
Hollow Bolete (*Suillus cavipes*)								
Giant Puffball (*Calvatia gigantea*)								Contaminated Soil, [b]
Common/Warted Puffball (*Lycoperdon perlatum*)								
Meadow (*Agaricus campestris*)								
Shaggy Mane (*Coprinus comatus*)								
Fried Chicken (*Lyophyllum decastes*)								
Weeping Milkcap (*Lactarius volemus*)								
Weeping/Granulated Bolete (*Suillus granulatus*)								
Larch Bolete (*Suillus grevillei*)								
Blue-staining/Poor Man's Slippery Jack (*Suillus tomentosus*)								
Dryad's Saddle (*Polyporus squamosus*)								
White Button (*Agaricus bisporus*)								Mushroom Facility, [c]

*Grey boxes indicate that the species was present, and pink boxes indicate that specific species had the highest percent of total arsenic.

[a] (Koch et al., 2013), [b] (Nearing et al., 2014a), [c](Smith et al., 2007), [d](Llorente-Mirandes et al., 2014)

2.7. The Effect of Cooking Treatments

The risk associated with consuming arsenic-containing foods has generally been based on the raw products. However, food is generally subject to a variety of processes (such as washing, peeling, freezing, storage, canning, sterilization, and cooking) prior to consumption. During these procedures it is possible for the chemical forms of arsenic to undergo alterations, potentially making them more (or less) toxic. The effect of processing on total arsenic and arsenic species should be incorporated into the risk assessment in order to accurately portray the potential risk associated with food intake.

Freezing has not been shown to cause any significant changes to the total arsenic concentration or species and does not need to be considered as a processing treatment (Dahl et al., 2010).

The toxicity of arsenic exposure depends on two main factors: the total arsenic concentration and the species of arsenic present. In the following sections, the changes in these two factors as a result of food processing will be examined. This review investigates the changes in arsenic concentration/species in marine fish and seafood, canned products, and foods of terrestrial origin (with a subsection specific to mushrooms).

2.7.1. Marine Fish and Seafood

2.7.1.1. Concentration Changes

Throughout the various food preparation stages, changes to the arsenic concentration and speciation are most likely to occur during thermal treatment (Devesa et al., 2008). Heat has been shown to cause both increases and decreases in the total arsenic levels in foods. Increases in concentration, probably related to decreases in percent moisture content (although this was not reported) were seen in studies of salted cod and bivalves after cooking (Devesa et al., 2001a), sea bass fillets after frying and microwave cooking (Ersoy et al. (2006), and in fish (cultivated in arsenic contaminated ponds in Taiwan) after frying, grilling, and baking (Ling et al). Moreover, significant increases in the concentration of iAs (measured in wet weight) were observed after cooking bivalves and squid, but again this is likely due to the decrease in weight that takes place during cooking via partial dehydration (Devesa et al., 2001a). The results of these studies suggest that the concentration and speciation of arsenic after cooking should be taken into consideration when assessing the risk to human health.

In addition to the decrease in wet weight concentration resulting from moisture loss, decreases in arsenic concentrations have been attributed to its volatilization or solubilisation (into liquids that are not consumed) during the cooking process

(Devesa et al., 2001a). Lai et al. (2004) found that, after cooking, the total amount of arsenic in cooked mussel was less than that of the raw mussel, but that the distribution of water-soluble arsenic species remained the same. In this case, some of the arsenic compounds were lost into the broth during the steaming process (confirmed by the detection of significant amounts of arsenic species in the broth in similar proportions to those which were observed in the raw mussel). This was seen again in a study by García Sartal et al. (2012) where four types of edible seaweed (Kombu, Wakame, Nori, and Sea Lettuce) were cooked (boiled, water poured off after cooking) resulting in an overall decrease in arsenic concentration. Both the raw and cooked samples were freeze-dried before analysis, and results were expressed as dry weights. The increase in temperature in the aqueous media was enough to cause a release of arsenic species, but not enough to cause any transformations.

2.7.1.2. Speciation Changes

To determine the exact effect of temperatures traditionally used in cooking or sterilization on the stability of the various arsenic species found in food, Devesa et al. (2001b) conducted a study of AB, DMA(V), MMA(V), TMAO, TETRA, and AC heated in buffered aqueous solution at different temperatures (85-190 °C) and for different lengths of time. Various pH levels (4.5, 5.5, 6.5, and 8.0) were applied during the heating process. The results indicate that no transformations of arsenic species take place below 120 °C. However, between 150 and 190 °C, a partial decomposition of AB occurs, producing TMAO at 150 °C and TMAO and TETRA at temperatures of 160 °C or above (Devesa et al., 2001b). The proportions of these transformations depend on both temperature and duration of the heat exposure. This study on arsenic standards led to several subsequent studies of the transformations that take place in cooked seafood (Table 5). Devesa et al. (2005) analyzed 64 seafood products (fish, bivalves, squid, and crustaceans) subjected to different cooking treatments (grilling, roasting, baking, stewing, boiling, steaming, and microwaving). In the cooked products, AB remained the major arsenic species, followed by DMA(V) and TETRA. Specifically, cooked sardines and bivalves showed an increase in DMA(V), and an increase or appearance of TETRA (accompanied by a loss in AB) for cooked megrim, anchovy, Atlantic horse mackerel, and sardines (Súñer et al., 2002). This demonstrates that the observed degradation during thermal treatment of the standards also takes place during cooking processes for food. In a study by Lai et al. (2004), steaming the mussels caused an overall decrease in the proportion of AB (likely solubilized into the broth), as well as an increase in the proportions of DMA(V) and As(V). Hanaoka et al. (2001a) roasted muscles of marine animals (star-spotted shark (*Mustelus manazo*) and red crayfish (*Panulirus longipes femoristriga*) and observed the conversion of AB to TETRA and, in smaller concentrations, As(V). In this experiment, more transformation occurred when the muscle tissues were cooked

(and burned) on a grill (56% transformation of AB) compared to when they were grilled in a pan (47%). These studies indicate that AB is likely to undergo transformation to TETRA during cooking processes. Dahl et al. (2010) suggest that this transformation takes place via the decarboxylation of AB to form TETRA.

Higher concentrations of TETRA in food after cooking increases the risk associated with its consumption. TETRA has a relatively high acute toxicity (oral LD_{50} of 890 mg As/kg for the iodide and 580 mg As/kg for the chloride), making it more toxic than several other arsenic species (including DMA(V) and MMA(V)) (Table 2) (Kaise et al., 1989, Hanaoka et al., 2001a). TETRA can also inhibit cell growth at concentrations of 8 mg/cm^3 and is more cytotoxic than both AB and AC (Kaise et al., 1998). However, the transformation of AB to TETRA in cooked seafood is generally associated with a decrease in the overall arsenic concentration and resulting TETRA concentrations are typically not high enough to pose a serious risk (not reaching concentrations higher than 0.571 μg/g, ww for grilled anchovy in the study by Devesa et al. (2005)). Nonetheless, these transformations confirm that arsenic species from food should be measured for the product in the form in which it is ingested, in order to determine the actual arsenic exposure and risk.

Table 2-5: A review of arsenic speciation transformations observed after cooking treatments.

Food	Cooking Treatment	Primary Species in Raw Sample	Speciation Changes After Cooking	Reference
Sole, dory, hake, and sardine	Baked, fried or grilled	AB	Appearance of TETRA	(Devesa et al., 2001c)
Sardines and bivalves	Grilled, roasted, baked, stewed, boiled, steamed or microwaved	AB	Increase in DMA(V)	(Devesa et al., 2005)
Megrim, anchovy, Atlantic horse mackerel, and sardine		AB	Increase/appearance of TETRA	
Star-spotted shark and red crayfish	Burned on grill or pan	AB	Appearance of TETRA	(Hanaoka et al., 2001a)
Anemone (*Anemonia sulcate)*	Fried	DMA(V)	An overall decrease in all species (AB, DMA(V), MMA(V), As(V), TETRA, TMAO, AC) was observed	(Contreras-Acuña et al., 2013)
Shark and Lobster Muscle	Flame-cooked	AB	Appearance of TETRA	(Hanaoka, 2001)
Blue mussels (*Mytilus edulis*)	Steamed	AB	Increase in DMA(V) and As(V)	(Lai et al., 2004)
Gilthead sea bream, meagre and salmon	Boiled, grilled or roasted	AB	No significant transformations reported	(Coelho et al., 2013)
Edible seaweed (Kombu, Wakame, Nori and Sea Lettuce)	Boiled	AsS	No significant transformations reported	(García Sartal et al., 2012)

2.7.1.3. Canned Products

Foods are often prepared as canned products for personal use and commercial sale because the canning process is a preservation method that increases shelf life. During the canning process, sterilization by heat is usually carried out to protect the food against bacteria. The high temperatures used during this process have been shown to cause considerable solubilisation of arsenic (demonstrated for commercially purchased canned seafood products) (Vélez et al., 1997). This causes a decrease in the total arsenic of the preserved seafood, but since the brine can be consumed with the product, the total ingested arsenic was not significantly affected.

When fish are subjected to the canning process, the associated liquids may give the fish product a neutral or mildly acidic pH (in the range of 5.8-7), or an even more acidic pH when stored in oils or natural juices (pH between 4.5-5.8) (Devesa et al., 2001b). However, as mentioned previously, arsenic standards remained unaltered at pH levels 4.5, 5.5, 6.5, and 8.0, at a temperature of 120 °C (Devesa et al., 2001b). On the other hand, in a TDS by Sirot et al. (2009) reporting arsenic species in a variety of seafood products, some speciation changes can be observed when the fresh/frozen samples are compared to canned samples of the same seafood type (Table 2-1). There is a decrease in all arsenic species in the canned samples except for the canned anchovy (where there is an increase in DMA(V) and a slight increase in iAs) and canned mackerel (where there is a slight increase in MMA(V) and DMA(V)). Further research is needed to determine the stability of arsenic species (specifically AB) during the canning process and storage in both fish and other AB-containing food (i.e. mushrooms).

Table 2-6: Arsenic concentrations (in µg/g fresh weight) for fresh/frozen seafood products in comparison with the canned version of the same seafood type (but not the exact same sample).

Species	Preparation Stage	Number of Samples	AB	MMA(V)	DMA(V)	iAs	Sum of Species
Tuna (*Thunnus alalunga*, *Euthynnus pelamis*)	Fresh/ Frozen	4	1.79	0.029	0.016	0.009	2.45
	Canned	5	0.94	0.004	0.011	0.004	0.81
Crab (*Cancer pagurus*)	Fresh/ Frozen	3	13.1	0.004	0.051	0.253	16.8
	Canned	1	2.05	0.004	0.003	0.068	2.23
Anchovy (*Engraulis encrasicolus*)	Fresh/ Frozen	1	0.72	0.025	0.003	0.015	0.94
	Canned	2	0.57	0.019	0.036	0.019	0.8
Sardine (*Sardina pilchardus*)	Fresh/ Frozen	4	5.81	0.025	0.1	0.048	6.02
	Canned	1	2.14	0.004	0.018	0.031	3.54
Mackerel (*Scomber scombrus*)	Fresh/ Frozen	4	1.7	0.078	0.138	0.029	2.41
	Canned	1	0.18	0.082	0.186	0.007	0.7

(Sirot et al., 2009)

2.7.2. Foods of Terrestrial Origin

Similar to the observations seen for seafood, cooking – namely, broiling, roasting, baking, and frying – foods, such as ground beef, poultry, and freshwater fish, has been shown to cause an increase in the total arsenic concentration in the final wet weight product from water loss (Dabeka et al., 1992, Perelló et al., 2008).

A common way of cooking terrestrial foods of plant origin, like rice and vegetables, is boiling in water. In these situations, the initial arsenic concentration in the water itself can contribute to concentration changes in the food being boiled. In a study by Bae et al. (2002), arsenic in rice boiled in arsenic-containing water in Bangladesh was found to be higher than in the raw rice. In Bangladesh, rice is boiled with an excessive amount of water, and the water remaining after cooking is discarded. This suggests an absorption effect of rice grains, or alternatively, concentration of arsenic in the water as a result of water evaporation during cooking. The increase in iAs after boiling can be significant and 5- to 17-fold increases were observed in a similar study by Laparra et al. (2005) (rice was added to arsenic-containing water and boiled to dryness). On the other hand, when uncontaminated water is used for boiling, the total amount of arsenic is typically reduced (observed for vegetables (She and Kheng, 1992) and pasta (Cubadda et al., 2003) where water was discarded after cooking). Other techniques shown to decrease arsenic in food, such as rice and seaweed, include pre-rinsing them before consumption and cooking them in large amounts of water (Lynch et al., 2014, Raab et al., 2008, Hanaoka et al., 2001b).

In a study to assess arsenic intake in Region Lagunera, Mexico, pinto beans, potatoes, sauce, pork, and beef (all boiled) as well as fried eggs and corn-based flat-bread tortillas were all compared to determine their contribution to daily arsenic intake (Del Razo et al., 2002). They found that in the Lagos de Moreno region, (where the total arsenic in tap water is over 400 μg/L) foods that absorbed the most water during preparation and cooking had the highest arsenic levels (e.g. pinto beans and pasta soup), in comparison with foods with low water content (e.g. tortillas) (Del Razo et al., 2002). The pinto beans were boiled in water for the longest duration (before the water was poured off) and had the highest mean arsenic concentration.

2.7.3. Effect of Cooking on Terrestrial Fungi

Mushrooms can be consumed both raw and cooked. The technique used to cook the mushroom often depends on its size and characteristics. Smaller, sliced, chopped, or halved mushrooms are generally sautéed or fried in oil or butter. Larger-capped mushrooms (like portobello and shiitake) are normally grilled, roasted, or broiled (and brushed with oil). Mushrooms can also be microwaved, although this is less common. Frying has been found to cause the greatest moisture

loss (compared to grilling or boiling) and should therefore be suspected for causing the greatest increase in total wet weight arsenic concentration (Bassey et al., 2014). Mushrooms are rarely boiled as a form of preparation and when heated in soups, the temperature is unlikely to surpass 120°C. Since AB transformations only occur after temperatures surpass 150°C (Devesa et al., 2001b), cooking techniques that reach the highest temperature will likely have the most impact on arsenic species transformation in mushrooms. As seen in the study by Hanaoka et al. (2001a), completely cooking (and burning) the marine animal muscle tissues on a grill caused more transformation than when it was grilled in a pan. Since grilling mushrooms, although not to the extent of charring them, is a common cooking technique, it is likely transformations occur during cooking and AB is transformed into TETRA. The most cultivated mushroom worldwide is *A. bisporus* (button mushroom) (Reis et al., 2012) (already noted for its high AB content) (Nearing et al., 2014a). Since it is highly consumed, it may be necessary to consider the effect of thermal treatments on arsenical species (specifically AB) when determining risk associated with mushroom intake.

2.8. Summary of knowledge gaps and relevance to thesis

Arsenic is found ubiquitously throughout the environment in many different species, which each have unique chemical, physical, and biological properties. Inorganic arsenic species (arsenite and arsenate) are among the most toxic, while AB is the only species considered non-toxic. Upon ingestion, iAs undergoes a series of reduction and oxidative methylation steps before the majority is excreted in urine as DMA(V) (for humans). The toxicity of arsenite and other trivalent arsenicals is attributed to several different properties: they inhibit proteins and enzymes, promote the generation of reactive species, and decrease the capacity of cells to cope with reactive species or DNA damage. However, these properties also make arsenic a potent chemotherapeutic agent against APL.

Where there are no natural or anthropogenic sources of arsenic contamination, food is the main source of intake for humans. Arsenic has been identified in many foods (rice, cereals, and mushrooms), but is most significantly observed in seafood. AB is the principal arsenic form in both seafood and many mushrooms species. AB does not exhibit any of the toxic effects related to the other arsenic species as it appears to be metabolically unavailable to both humans and animals. It is efficiently absorbed and excreted, although some studies suggest possible steady-state retention of AB. Common preparations of food, such as cooking treatments, have been found to alter the arsenic speciation in seafood such as the degradation of AB to dimethylarsinic acid (DMA(V)) and tetramethylarsonium ion (TETRA). Changes in the arsenic species indicate that it may be necessary to consider the effect of preparation methods, such as thermal treatments, on arsenic speciation when determining risk associated with mushroom intake.

Currently, there has been no research conducted on the possible speciation changes in mushrooms as a result of common preparations of mushrooms, such as cooking treatments. As mushrooms are one of the only commonly consumed terrestrial organisms that contain high proportions AB, it is crucial to identify if speciation changes to more toxic species are taking place. In Chapter 4, the changes in total arsenic concentrations and arsenic speciation will be examined in a variety of edible mushroom species before and after common preparation procedures (frying, baking, and barbequing). The total arsenic and arsenic speciation will also be investigated in canned mushrooms subject to cooking treatments (frying and baking).

There are several hypothesized pathways for the formation of AB, but none have been confirmed or identified in biological organisms. In the marine environment, it is likely that AB is formed through pathways originating from arsenosugars. In the terrestrial environment and in the human gastrointestinal tract, where no arsenosugars are present, other pathways, involving DMA(III), GSH, SAM, or other compounds, are more probable. In terms of its formation in terrestrial fungi, it is possible that the fruiting body of the mushrooms produces AB, that they accumulate it from the surrounding environment (where microorganisms are responsible for generating AB or its precursors), or that there is a symbiotic relationship between the fungi and the microbial community that results in AB formation. By determining the organism responsible for AB formation or elucidating the biosynthetic pathway for AB formation, remediation approaches may be developed for arsenic contaminated areas.

In order to investigate the role of microorganisms in AB formation, the total arsenic and arsenic speciation in mushrooms grown on logs is compared to that of those grown in soil (Chapter 4). The microbial community in these two substrates is different, and may therefore yield different arsenic compositions even in the same mushroom species. This thesis aims to determine the extent to which the growth environment (log vs. soil) can alter the arsenic composition of a mushroom, specifically the presence/absence of AB.

Furthermore, no research has been conducted to determine why mushrooms can contain such high proportions of AB. It has been hypothesized to play a similar, osmolytic role like betaine. This thesis will examine any possible correlations of AB and betaine in mushrooms to examine this hypothesis.

Chapter 5 of the thesis aims to investigate possible formation pathways of AB in terrestrial fungi by examining some of the synthesis reactions described in Section 2.5. The AB synthesis presented by Nakamura et al. (2008) involving TMAO, iodoacetic acid, and GSH will be investigated and the presence of iodoacetic acid in soil is examined. Pathway (c) (Figure 2-8) will also be examined to determine if DMA(III) is a possible precursor for AB. This pathway, proposed by Edmonds

(2000), suggests that the arsenylation of glyoxylate should yield AB. Both of these pathways offer plausible formation routes for AB in the terrestrial environment, as they do not rely on the presence of arsenosugars.

Chapter 3 – Methods

3.1. Chemicals and Reagents

The chemicals and reagents used for the arsenic speciation analysis and total arsenic determinations are similar to those outlined by Nearing et al. (2014a). Double-deionized water (DDW) was prepared in-house to a minimum resistance of 18 MΩ·cm (E-pure Barnstead). For arsenic extractions, DDW was used with HPLC grade methanol (Fisher Scientific) and 2% nitric acid (v/v) (Fisher Scientific). For total arsenic analysis, matrix spikes and calibration curves were prepared from a stock solution of 1000 µg/mL total arsenic (arsenic pentoxide, Inorganic Ventures). Different sources of standards were used for the calibration curves than for quality control calibration check solutions (arsenic pentoxide, SPC Science).

For HPLC-ICP-MS analysis, different sources of standards were used for the calibration curves than for quality control calibration check solutions and matrix spikes, when available. This was done for As(III) (in hydrochloric acid, 90% from Fluka and 99.999% purity from Aldrich), As(V) (1000 ppm from Aldrich and 1000 ppm from Inorganic Ventures), DMA(V) (cacodylic acid in water, >99% from Fluka and 99% purity from City Chemical), AB (Wako and Argus Chemicals), and TMAO (trimethylarsine oxide, Wako and Argus Chemicals). Only one source was available for AC (arsenocholine bromide, Argus Chemicals), MMA(V) (monosodium acid methane arsonate sesquihydrate, 99.0% purity Chemservice), and TETRA (Wako and Argus Chemicals). Arsenosugars used as standards were extracted from brown algae (*Fucus vesiculosus*) collected in Nova Scotia, Canada. DMAA, dimethylarsinoylacetic acid, and DMAE, dimethylarsinolethanol, were kindly donated by Kevin Francesconi (University of Graz, Austria). For arsenic extractions, the certified reference material (CRM) BCR®- 627, Tuna Fish, was obtained from European Commission Institute for Reference Materials and Measurements. For total digests, CRM BCR®-482, Lichen, was obtained from the same source and CRM Bushes, Twigs, and Leaves GWB®-07603 was obtained from the Institute of Geophysical and Geochemical Exploration (Langfang, China).

For the betaine extraction from soil, a standard solution was prepared using betaine hydrochloride (>99%, Sigma). For the iodoacetic acid extractions from soil, extractant solutions were prepared using calcium chloride dihydrate (certified A.C.S, Fisher Scientific) and oxalic acid (98%, anhydrous, Acros Organics). Iodoacetic acid (>98.0%, Sigma-Aldrich) and potassium iodide (15% W/V, LabChem Inc., Pittsburgh, PA) were used to make standard solutions.

For the synthetic experiments, dimethylarsinous iodide, DMA(III)I, was obtained from Dr. William Cullen's research group at the University of British Columbia

and was stored in the freezer to avoid any degradation or oxidation of the species. The potassium phosphate-citric acid buffer was prepared using potassium phosphate monobasic (>99%, Sigma-Aldrich) and citric acid monohydrate (certified A.C.S., Caledon Laboratories). Other reagents in the synthetic experiments included calcium hydroxide (>95.0%, Caledon Laboratories), sodium pyruvate (>99%, Sigma-Aldrich), glyoxylic acid (50% wt. % in water, Aldrich Chemistry), and S-adenosyl methionine (SAM) (S-(5'-Adenosyl)-L-methionine chloride dihydrochloride, >75%, Sigma Life Science).

3.2. Collection and Preparation of Mushroom Samples

Mushrooms were collected from local grocery stores (in Kingston, Ontario (ON)) and from background areas at Odessa, ON, Canada. Background areas are those without any known anthropogenic sources of arsenic. Mushrooms were also collected from areas impacted by historical gold mining activities, referred to as contaminated locations. For this research, mushrooms were collected from Deloro, ON, an area with higher than normal arsenic concentrations in Canada. This site has naturally elevated arsenic concentrations ranging from 20 – 120mg/kg in the soil (Nearing et al., (2014a). The location of this site is shown in Figure 3-1. This location is adjacent to an old gold mine that was operational from the late 1800s to the early 1900s. Mushrooms collected from this site are reffered to as originating from an elevated location. The locations from which samples were collected for the glycine betaine (referred to as just betaine from this point on) analysis are described in Nearing et al. (2014a). Briefly, these mushrooms were obtained from: grocery stores, British Columbia (BC) (only background areas), ON background and contaminated/elevated areas, and Yellowknife (YK) background and contaminated areas.

Figure 3-1: Map and expanded portion of Deloro, Ontario. Mushrooms were collected from a park area in the town.

Both the wild mushrooms picked by hand (wearing protective latex gloves) and fresh mushrooms purchased from the grocery store were stored in paper bags and kept cool until the time of processing in the laboratory (maximum 2 days). Processing included identifying the species of the wild mushrooms (using spore prints, microscopic examination in some cases, and matching of mushroom features to those in Lincoff (1981)), washing both the wild and store bought mushrooms with tap water to remove soil, bugs, and other particles, freezing them whole or in cubed samples, and dehydrating them using a FreeZone 6 freeze-dryer (Labconco, Kansas City, MO, USA). After the mushrooms were dried, they were pulverized using a Master Chef Automatic Coffee Grinder or with a ceramic mortar and pestle, and kept at room temperature in 250 mL wide-mouth Fisherbrand® disposable polypropylene jars or 50 mL Fisherbrand® disposable polypropylene centrifuge tubes (depending on the amount) until extraction. Between samples, the grinder or mortar and pestle were rinsed and cleaned with methanol and Kimtech Science® Kimwipes® delicate task wipers. A small amount of the next sample was ground and discarded prior to pulverizing the remainder of the sample.

The store-bought/edible (ON background) mushrooms underwent a variety of thermal treatments (frying, barbequing, and baking) and were processed similarly. After they were collected or purchased from the grocery store, they were stored in their original packaging until processing (no more than 48 hours). Processing included washing the mushrooms and slicing them into thin (approximately 0.5-1.5

cm thick) strips or bite-sized cubes (2-3 cm^2). Canned mushrooms were drained and rinsed in tap water in order to mimic a typical preparation/cooking process. The liquid from the canned mushrooms was collected in 50 mL Fisherbrand® disposable polypropylene centrifuge tubes. After washing, all mushrooms were blotted dry with paper towel. Wild mushrooms that were barbequed were left in larger pieces.

The three different cooking techniques (frying, barbequing, and baking) differed in the way the thermal treatment was applied. For the fried mushrooms, the mushrooms were fried in store-bought canola oil that showed no detectable arsenic, either before or after the frying process. The same digestion method outlined in Section 3.3.2 was used to digest the cooking oil samples. Barbequing was done on a propane barbeque until the mushrooms had prominent grill-lines and appeared fully cooked (Figure 3-2). Frying took place on medium-high heat for approximately 20 minutes, until the mushrooms were fully cooked (turned brown, decreased in moisture, and appeared crispy on some edges) (Figure 3-3). Baking took place on aluminium foil sheets at 450°F (approximately 232°C) for approximately 15-20 minutes, until fully cooked (Figure 3-4).

In total, 12 mushroom samples were subjected to cooking treatments. Four different mushroom species (saffron milk cap (*Lactarius deliciosus*), rough-stemmed bolete (*Leccinum scabrum*), penny bun (*Boletus edulis*), and giant puffball (*Calvatia gigantea*) were collected from Odessa, ON and were subjected to frying and barbequing; analysis of fresh mushrooms brings the number of samples for this set to 12. Four different species of fresh mushrooms (portobello and crimini (brown strain of *Agaricus bisporus*), white button (white strain of *Agaricus bisporus*), oyster (*Pleurotus ostreatus*), shiitake (*Lentinula edodes*), and enoki (*Flammulina velutipes*) were purchased from a local grocery store in Kingston (Loblaws, 1100 Princess St, Kingston, ON K7L 5G8), and were fried and baked (18 samples). Two varieties of canned mushroom (whole and pre-sliced white button mushrooms (white strain of *A. bisporus*), products of Canada) were purchased from the same location and were also fried and baked (6 samples). Therefore, a total of 36 samples were analyzed.

Figure 3-2: Pictures of the progressive stages of mushrooms that were subjected to barbequing. The left hand side shows the raw mushrooms, the center shows the barbequing process, and the right hand side shows the final product that was used for analysis.

Figure 3-3: Pictures of the progressive stages of mushrooms that were subjected to frying. The left hand side shows the raw mushrooms, the center shows the frying process (on medium-high heat for approximately 20 minutes) for three of the six mushrooms, and the right hand side shows the final product that was used for analysis.

Figure 3-4: Pictures of the progressive stages of mushrooms that were subjected to baking. The left hand side shows the raw mushrooms and the right hand side shows the final product that was used for analysis after baking (450°F (approximately 232°C) for approximately 15-20 minutes on aluminum foil sheets).

3.3. Total arsenic and arsenic species analysis in mushrooms

The total arsenic content and arsenic species composition was determined for wild mushrooms, raw mushrooms, and each type of cooked mushroom following an extraction procedure. The total arsenic concentration within the mushrooms was determined by adding the total arsenic in the extracts and the total arsenic in the final residues from the extraction. Some mushrooms were digested without prior extraction (i.e. the original mushroom material) for mass balance checks. This was done when there was sufficient sample available in order to compare totals from the extract plus the residue (henceforth referred to as 'extract + residue') with the totals from the completely digested sample. Residue digests and digests of the original samples were analyzed for total arsenic. The methods for these steps are described in the following sections.

3.3.1. Arsenic species extraction

All mushroom samples were extracted in duplicate following methods described by Whaley-Martin et al. (2012b). A total of 0.25 g of dried sample was extracted using 5 mL of 50% (by volume) aqueous methanol in a 50 mL Fisherbrand® disposable polypropylene centrifuge tube. The mixtures were vortexed and shaken end-over-end for approximately 12 hours (overnight) at room temperature in a Rugged Rotator (Model: 099A RD4512, Glas-Col, USA), then placed in an ultrasonic bath for 20 min before being centrifuged at 3500 rpm for 30 min at 15 °C in a Sorvall Legend RT Centrifuge (Thermo Electron Coorporation, 2007). The supernatant was decanted into a 15 mL Fisherbrand® disposable polypropylene centrifuge tubes and the ultrasonication and centrifugation steps were repeated 2 more times (i.e. a total of 15 mL MeOH/H_2O was added), collecting all the supernatants together. Methanol was removed from the supernatant by evaporation at 60 °C using a Rapidvap (Labconco, Kansas City, MO, USA) to a final volume of approximately 7.5 mL. The methanol/water extraction procedure does not cause any transformation of the arsenic species (Mir et al., 2007). Some As(III) may oxidize to As(V) during the drying portion of the sample preparation. However, iAs values are reported as a sum (of As(III) and As(V)), negating the issue of As(III) transformation. Extracts were syringe filtered using disposable 0.45 µm filters (Millipore® polypropylene 25 mm diameter hydrophilic polyvinylidene fluoride (PVDF) durapore membrane) into 15 mL Fisherbrand® disposable polypropylene centrifuge tubes and kept frozen until analysis. Tuna fish (CRM BCR®- 627), Lichen (CRM BCR®-482), and/or Bushes, Twigs, and Leaves (GWB®-07603) were included with the extract samples.

3.3.2. Digestion for Total Arsenic

Extraction residues were digested with 10 mL trace metal grade nitric acid (HNO_3) (~ 70%, Fisher Scientific) at 120°C until the sample was reduced to 1–2 mL in 50 mL glass test tubes. Samples were then diluted with 10 mL of double deionised water (DDW) (prepared in-house to a minimum resistance of 18 MΩ·cm (E-pure Barnstead)) and syringe filtered as described for the extracts.

Digestion of original mushroom samples was the same as that of the residues, except 0.5 g of mushroom sample was used instead of the residual material from the extract. Tuna fish (CRM BCR®- 627), Lichen (CRM BCR®-482), and/or Bushes, Twigs, and Leaves (GWB®-07603) were included with the digests.

3.3.3. Total arsenic analysis of extracts and digests:

All samples (extracts, residue digests, and total digests) were analyzed for total arsenic using inductively coupled plasma mass spectrometry (ICP-MS). The ICP-MS operating conditions followed those described by Caumette et al. (2011). The instrument used was an ICP-MS DRC II from PerkinElmer (PerkinElmer, MA). Measurements were made with arsenic oxide (AsO^{91}) in dynamic reaction cell (DRC) mode using oxygen as the reaction gas at a flow rate of 0.9 mL/min. This allows for chemical modification of the ion beam to eliminate interferences. Arsenic signals were measured based on m/z AsO^{91} recovery. The internal standard monitored during total analysis was rhodium (10 µg/L) introduced via a T-tube. Other instrumental tests included instrumental blanks and calibration checks, which were run every 10 samples. Instrument blanks were also run every 10 samples and were always below the limit of detection (LOD) (0.1 µg As/L). The LOD of 0.1 µg As/L for the instrument blanks was based on the lowest measurable calibration concentration (0.1 µg As/L solution). For the method blanks, the LOD was based on the lowest measurable calibration concentration multiplied by the highest dilution factor (approximately 40 for the extract and 53 for the residue) resulting in LODs of 4 and 5 µg As/kg mushroom (based on the instrument LOD of 0.1 µg As/L). The LOD for the extract + residue total concentration was established to be the lower of the two values (4 µg As/kg mushroom) because the sum was considered detectable if the lowest detectable portion could be detected. Instrument quality control checks that included a 5 µg/L and 50 µg/L solution for total arsenic were run every 10 samples and generally found to be within an acceptable range (80-120 % recovery) (Canadian Council of Ministers of the Environment (CCME), 2016) with exceptions explained in Section 3.4.

3.3.4. Arsenic speciation analysis of extracts:

All of the extracts from the methanol/water extraction procedure were analyzed for arsenic speciation using high performance liquid chromatography (HPLC) (anion

and cation exchange) coupled to ICP-MS. Again, the HPLC-ICP-MS instrumental operating conditions followed those outlined in Caumette et al. (2011). The anion exchange chromatography was performed using a Hamilton PRPX100 anion exchange column with a gradient mobile phase (A: 4 mM of ammonium nitrate (NH_4NO_3) (99.999% purity, Aldrich) and B: 60 mM NH_4NO_3, pH = 8.65). The gradient was 100% A 0-2 min, 100% B 3-6.5 min, 100% A 7.5-10.75 min, 100% B 11-13 min, and 100% A 13.25-15 min with a flow rate of 1 mL/min. Anion exchange was used to identify: As(III), As(V), MMA(V), and DMA(V). Cation exchange chromatography was performed with a Chrompack Ionosphere C cation exchange column with a mobile phase of 20 mM pyridine (99% purity, Sigma), pH = 2.7. Cation exchange was used to identify: DMA(V), AB, TMAO, AC, TETRA, and arsenosugars (for DMA(III) and TMAO experiments). All chromatographic speciation data were analyzed with Peak Fit Version 4.12. All mushrooms were analyzed for arsenic speciation and all compounds that could be matched with a known standard were quantified using the calibration curve for that arsenic compound.

Instrument blanks were also run every 10 samples and always demonstrated an absence of peaks. For speciation analysis, a limit of quantification, LOQ (1 µg As/L) was established, which was based on the lowest calibration concentration. This quantity was considered an LOQ instead of an LOD because the standard concentrations did not range lower than 1 µg As/L but some peaks could be seen in some samples that were lower than 1 µg As/L. For the method blanks, the LOQ was determined to be the lowest calibration concentration multiplied by the highest dilution factor for the extracts. As stated previously, the highest dilution factor for the extracts had an average of approximately 40, resulting in an LOQ of 40 µg As/kg mushroom. As mentioned, in some cases arsenic species below the LOQ could be quantified but they contain a high amount of uncertainty. In other cases, peaks were visible, but could not be quantified (the area under the peak could not be measured using the software available), and these peaks were labeled as ‘trace’.

3.4. Quality Assurance and Quality Control

Certified reference material (CRM) Tuna Fish Tissue (Reference Material Number 627) was run for total arsenic (extract + residue method) and for arsenic speciation (certified values: 4.8 ± 0.3 mg/kg total As, 3.9 ± 0.23 mg/kg AB and 0.15 ± 0.022 mg/kg DMA(V)), and was included with every 10 samples. Method blanks were also included with every 10 samples and showed no detectable arsenic. Matrix spikes (adding a known amount of arsenic to a sample prior to analysis) were also prepared immediately before analysis (10% frequency) for total As and As speciation analysis. As mentioned previously, all samples were extracted (and residues were available to obtain total arsenic from extract + residue) in duplicate.

3.4.1. Experiment A – Mushrooms collected from Deloro, ON

Mushrooms were collected from Deloro, ON at two time points, in September and October, 2014. Therefore, analysis was done for the two sample sets at different periods of time, each with their own respective QA/QC. The results of the QA/QC are discussed in the following two sections.

3.4.1.1. Experiment A1 –Collected in September, 2014

The QA/QC results for the analysis of total arsenic and arsenic species in mushrooms collected from Deloro, ON in September, 2014 are outlined in Table 3-1. For this experiment, there were a total of 10 samples, but the extracts were only analyzed for eight samples. This was because two mushroom samples (*Trametes versicolor* and *Gleophyllum sepiarium*) were too difficult to pulverise into a relatively homogenous powder (required for extraction), and thus they could not be extracted. Nine of the 10 samples underwent total digestion (of the original samples), but there was not enough sample remaining to digest the *Russula fragilis* mushroom.

Table 3-1: Quality assurance and quality control (QA/QC) results for the analysis of total arsenic and arsenic speciation in Experiment A1 – Total arsenic and arsenic speciation in mushrooms collected from Deloro in September, 2014.

QC test	Assessment metric	N	Range	Average
Total arsenic by ICP-MS				
Extract Totals				
Replicates	Relative percent difference (%)	8	4-31	19
Matrix spike	Percent recovery (%)	2	40-73	55
Residue totals				
Replicates	Relative percent difference (%)	8	4-43	22
Matrix spike	Percent recovery (%)	2	3-11	7
Total digest totals				
Replicates	Relative percent difference (%)	9	1-42	13
Matrix spike	Percent recovery (%)	2	70-84	77
Certified reference materials	Percent recovery tuna fish (%)	1	80	80
	Percent recovery lichen (%)	1	43	43
Overall totals				
Certified reference materials	Percent recovery tuna fish (%) (extract + residue)	1	92	92
Instrument blanks	Concentration (µg/L)	18	<0.1	<0.1
Instrument quality checks	Percent recovery (%)	22	83-100	91
Method blanks	Concentration (µg/kg mushroom)	3	<4.0	<4.0
Extraction efficiency	Percent of extracted arsenic/extract + residue total (%)[b]	16	1-27	7
	Percent of extracted arsenic/total digest total (%)[b]	14	1-53	19
Arsenic species by HPLC-ICP-MS				
Replicates	Relative percent difference (%)	36[a]	0-46	15
Matrix spike	Percent recovery (%)	20	101-269	148
Instrument blanks	Concentration (µg/L)	4	<1.0	<1.0
Instrument quality checks	Percent recovery (%)	24	96-118	105
Certified reference materials (tuna fish)	Percent recovery AB (%)	2	105-115	110
	Percent recovery DMA (anion) (%)	2	109,109	109
Method blanks	Concentration (µg/kg mushroom)	2	<40	<40
Column recovery	Percent recovery (%)[b]	8	60-111	85

[a] The number of replicates for arsenic species by HPLC-ICP-MS is very high because the relative percent difference was calculated for all of the samples for each species (iAs, DMA (anion), MMA, AB, TMAO, and AC.
[b] The extraction efficiencies are based on the corrected extract, residue, and total digest values and the column recoveries are based on the corrected extract values.

For total arsenic analysis, all samples were analyzed in duplicate with an average relative percent difference (RPD)[1] of 19% for the extracts, 22% for the residues, and 13% for the total digest.

Two matrix spikes were analyzed for the extracted solution, the digestion of the residue, and the total digest. The average matrix spike recoveries were 55% for the extracts, 7% for the residues, and 77% for the total digest. This indicates that there is a strong matrix effect in the analysis, causing the signals to be lowered. Therefore, it was assumed that the matrix effects taking place in the matrix spikes (which were prepared in samples) was likely also taking place in the remainder of the samples. In order to correct for this, the extract, residue, and total digest values were corrected for their respective matrix spike recovery (sample value/(% matrix spike recovery/100)). When comparing the total arsenic from summing the extract + residue with the total arsenic when the entire mushroom sample was digested and analysed, the extract + residue results are equivalent to the total digest results without corrections, but 5 times higher than the total digest results, after the corrections had been applied. This will be discussed further in the following section (Section 3.4.1.2).

The average percent recovery of total arsenic for the CRM (tuna fish, obtained from the extract + residue) was 92%, which was considered to be within an acceptable range. The average percent recovery of total arsenic for the CRM (tuna fish, obtained from the total digest) was 80%, which was considered to be within an acceptable range. The average percent recovery for lichen from the total digest was only 43%, which is below the acceptable range. Results from previous digestions of this CRM (68 to 102%, average of 78%) (Nearing et al., 2014a) suggest that this material may be difficult to digest using the applied methods but the recovery in this digestion batch is inexplicably low and the issue remains unresolved. If matrix correction is applied, the tuna fish recovery is 103% and the lichen recovery is 56%; acceptance of the total arsenic results for this batch is based on the tuna fish recovery (both uncorrected and corrected). It was not necessary to correct the CRM values on the basis of the matrix spike recoveries for the extract + residue total arsenic and speciation results, probably because the matrix of the dried tuna fish did not interfere with the extraction process as significantly as the mushroom matrix.

[1] $Relative\ percent\ difference\ (RPD) = \frac{|Value\ 1 - Value\ 2|}{\left(\frac{Value\ 1 + Value\ 2}{2}\right)} \times 100\%$

Extraction efficiencies (EEs) were calculated by dividing the total arsenic in the extract by the sum of arsenic in the extract plus the residue, using the values corrected by the matrix spike recoveries. For this sample set, they ranged from 1-27% and had an average of approximately 7%. When the total arsenic in the extract was divided by the total digest arsenic value, the EEs ranged from 1-53%, with an average of 19% (again, the corrected values were used). When uncorrected values were used, the EEs for extract/extract + residue were 7-75% (average of 32%) and 4-38% (average of 17%) for the extract/total digests. The correction for the total digest is small, and therefore doesn't appear to have a large impact. However, correcting the values for the extract and residue considerably decreased the extraction efficiency. Using the uncorrected versions of the EEs speaks to the degree in which arsenic was extracted from the mushrooms without taking any matrix effects into consideration (which is why the corrected values are used here). Mushrooms tend to have a wide range of extraction efficiencies; however, extraction efficiency has not been found to correlate with the total arsenic (Nearing et al., 2014a). It is likely that the arsenic that was not extracted is insoluble in the methanol-water extraction solution or bound to lipids/other cellular components (Koch et al., 2000).

The analysis of arsenic species was also done in duplicate because all of the mushrooms were extracted in duplicate. The RPD was calculated for each arsenic species present in the sample (inorganic arsenic (As(III) and As(V)), DMA(V), MMA(V), AB, TMAO and AC).

The percent recovery of the matrix spikes during speciation analysis showed matrix effects in the anion exchange chromatography analysis with average percent recoveries for the two spiked samples as: As(III): 176%, DMA(V): 144%, MMA(V): 171%, and As(V): 216%. For the cation exchange, the average percent recoveries for the three spiked samples were DMA(V) 114%, AB 123%, TMAO 121%, and AC 117%, which indicate minimal matrix effects (the ranges are within 70-130%, the normally acceptable range (CCME, 2016)). Therefore, based on the matrix spike recoveries the concentrations were corrected for the anion species but not the cation species.

Instrument quality checks were run every 10 samples. A total of three quality checks were done for both the anion and cation exchange and the percent recovery of each species was determined. Overall, the average percent recovery of the quality checks for all species was 105%. Instrument blanks were also run every 10 samples and always demonstrated an absence of peaks.

The percent recovery for the CRM was determined for two species, AB and DMA(V) (anion value) and had an average of 110% and 109%, respectively. The anion value was used for DMA(V) in order to remain consistent with the DMA(V) values that were used for the samples. These recoveries were not corrected for the

matrix spike recovery because 1) AB is a cation species (for which no correction was applied) and 2) the recovery of DMA(V) from the anion column was 109% without any adjustment. This is well within the acceptable range and indicates that perhaps there is less matrix effect from the dried tuna than from the mushroom. If the matrix spike correction is applied for the DMA(V) CRM recovery, the average would be approximately 76%, which is still within the accepted range (±30% of the certified value (CCME, 2016)).

The percent column recovery was determined by dividing the measured sum of species by the total arsenic determined in the extract, and then multiplying the result by 100 to determine the percentage (Equation 1). After correcting the extract totals and anion species with the matrix spike recoveries, the column recoveries ranged between 60%-111% with an average of 85%. This means that most, but not all, of the arsenic extracted was quantified through the HPLC analysis. Some of the arsenic species that were not captured in this analysis may have been below the LOQ or retained by the column. Indeed, the lower column recoveries were associated with samples that contained trace amounts of species.

$Equation$ 1:

$$\%\ Column\ Recovery = \frac{Sum\ of\ detected\ arsenic\ species}{Total\ arsenic\ detected\ in\ extract} \times 100\%$$

3.4.1.2. Experiment A2 –Collected in October, 2014

The QA/QC results for the analysis of total arsenic and arsenic species in mushrooms collected from Deloro, ON in October, 2014 are outlined in Table 3-2. For this experiment, there were a total of 11 samples. All samples were analyzed in duplicate except for one (*Hypoxylon fragiforme* mushroom), as there was not enough sample to complete the extraction twice.

Table 3-2: Quality assurance and quality control (QA/QC) results for the analysis of total arsenic and arsenic speciation in Experiment A2 – Total arsenic and arsenic speciation in soil-growing and log-growing mushrooms collected from Deloro in October, 2014.

QC test	Assessment metric	N	Range	Average
Total arsenic by ICP-MS				
Extract Totals				
Replicates	Relative percent difference (%)	10	1-76	26
Matrix spike	Percent recovery (%)	2	112-114	113
Residue totals				
Replicates	Relative percent difference (%)	10	3-56	22
Matrix spike	Percent recovery (%)	2	91-96	94
Total digest totals				
Replicates	Relative percent difference (%)	4	3-33	18
Matrix spike	Percent recovery (%)	2	72-112	92
Certified reference materials	Percent recovery tuna fish (%)	1	78	78
	Percent recovery bushes, twigs, and leaves (%)[a]	1	71	71
Overall totals				
Certified reference materials (extract + residue)	Percent recovery tuna fish (%)	1	102	102
	Percent Recovery bushes, twigs, and leaves (%)	1	74	74
Instrument blanks	Concentration (µg/L)	17	<0.1	<0.1
Instrument quality checks	Percent recovery (%)	22	80-200[b]	115
Method blanks	Concentration (µg/kg mushroom)	3	<4.0	<4.0
Extraction efficiency	Percent of extracted arsenic/extract residue total (%)	22	1-87	45
	Percent of extracted arsenic /total digest total (%)		1-109	43
Arsenic species by HPLC-ICP-MS				
Replicates	Relative percent difference (%)	41[c]	1-74	21
Matrix spike	Percent recovery (%)	8	92-121	103
Instrument blanks	Concentration (µg/L)	4	<1.0	<1.0
Instrument quality checks	Percent recovery (%)	36	92-109	101
Certified reference materials (tuna fish)	Percent recovery AB (%)	2	120-121	120
	Percent recovery DMA (anion) (%)	2	89-95	92
Method blanks	Concentration (µg/kg mushroom)	2	<40	<40
Column recovery	Percent recovery (%)	12	12-22	17

[a] The bushes, twigs, and leaves CRM was used because there was no more Lichen available.

[b] Of the 22 instrument quality checks that were run, the majority were well within the accepted range (100±20%). However, the last six checks (that were run concurrent to the extract samples) appeared to be higher than the rest, and as a result increased the range of recoveries up to 200%. Without these six samples, the range of instrument QC checks would be 80-119%, with an average of 96%.

[c] The number of replicates for Arsenic species by HPLC-ICP-MS is very high because the relative percent difference was calculated for all of the samples for each species (iAs, DMA (anion), MMA, AB, TMAO, and AC.

For total arsenic analysis, the samples that were analyzed in duplicate had an average RPD of 24% (average of RPDs for total analysis of extracts, residue digests, and total digests).

Two matrix spikes were analyzed for each of the extracted solution and the digestion of the residue. The average matrix spike recoveries were 113% for the extract, 94% for the residues, and 92% for the total digest. This indicates that there was not a strong matrix effect at the time of the analysis. Therefore, no correction was applied to the data (contrary to what was done with the previous data set). All of the wild mushrooms vary drastically in their texture, composition, and structure. This sample set contained more log-growing mushrooms than the previous sample set, and it is possible that log-growing mushrooms have a matrix that interferes less than that of soil-growing mushrooms. Alternatively or additionally, instrumental conditions during the time of the first run may have led to differences in the effect of matrix components on the analysis.

Instrument quality checks were analyzed using 5 µg As/L and 50 µg As/L solutions every 10 samples and had an average percent recovery of 115%. Of the 22 instrument quality checks that were run, the first 16 were within the accepted range (100±20%, (CCME, 2016)) with a range of 80-119% and an average of 96%. However, the last 6 checks appeared to be higher than the rest (range of 133-200%, average of 165%), and as a result increased the range of recoveries up to 200%. These QC checks were recorded throughout the analysis of the extract samples and show that the instrument conditions may have changed during the analysis of these samples. The impact of the recoveries is further discussed at the end of this section.

The percent recovery for the tuna fish CRM was 102% for the extract + residue method and 78% for the total digest method. The bushes, twigs, and leaves sample was also run as a CRM because lichen was not available. The percent recovery for the bushes, twigs, and leaves was 74% for the extract + residue method and 71% for the total digest method. These values were all considered to be within an acceptable range (100±30%) (CCME, 2016).

EEs (from the extract total divided by the extract + residue) ranged from 1-87% of total arsenic and had an average of 45%. When the EEs were calculated based on the total arsenic in the extract divided by the arsenic in the total digest the values ranged from 1% -109%, with an average of 43%. These EEs are slightly higher than the first data set (which had EEs of 32% for extract/extract + residue and 19% for extract/total digest), even though the method was the same. This confirms the unpredictability of EEs regardless of method and/or total arsenic content.

As previously mentioned, for the analysis of arsenic species, each sample was also run in duplicate except for the *Hypoxylon fragiforme* mushroom. The RPD was calculated for each arsenic species present in the sample (inorganic arsenic (As(III)

and As(V), DMA(V), MMA(V), AB, TMAO and AC). The RPDs ranged from 1%-74%, with an average of 22%.

The percent recovery of the matrix spikes during speciation analysis showed few matrix effects for this data set. Matrix spike recoveries ranged from 92%-103% with an average of 103%.

Instrument quality checks were run every 10 samples during speciation analysis. A total of three quality checks were done for both the anion and cation exchange and the percent recovery of each species was determined. Overall, the average percent recovery of the instrument quality checks for all species was 101%.

The percent recovery for the tuna fish CRM was determined for two species, AB (which had an average of 101%) and DMA(V) (anion) (which had an average of 120%). The DMA(V) value obtained on the anion exchange column was used for both the CRM and the samples.

The percent column recovery was determined using the same method outlined in the previous section. The column recoveries ranged from 12%-22%, with an average of 17%. However, the CRM had a column recovery of 97%. This suggests that there is an issue with the values for the total arsenic analysis in the extract for the mushroom samples (but not for the CRM). When comparing the total arsenic obtained from the extract + residue method to the total arsenic when the entire mushroom sample was digested and analysed (carried out where there was enough sample, as a mass balance check), the extract + residue results are approximately 5 times higher than that of the total digest results. This, along with the increase in the instrument QC check recoveries during the analysis of the extracts, suggests that total arsenic in extract values for mushroom samples may be inexplicably high. This issue remains unresolved because the samples could not be re-run: insufficient sample extract volumes remained for reanalysis, and the instrument became unavailable for use. The acceptability of the CRM data suggests that the issue resides in the specific analysis at the time of these specific extracts. The results of the speciation analysis can still be used qualitatively, as the proportions of arsenic species in the samples are still believed to be accurate.

The 5 times greater values in the extract + residue measurements compared with the total digest values were observed in both the Deloro, ON September (Experiment A1, using corrected values) and October (Experiment A2) sample sets. Therefore, when comparing these sample sets the total arsenic value from the total digest will be used when available. This allows comparability with other results for mushrooms from Deloro, in Nearing et al. (2014a). When there was no total digest done, a scaled down estimate ($1/5^{th}$) of the extract + residue measurement will be used. In conclusion, the inconsistencies with the mass balance check (total digest values vs extract + residue values) remain unresolved

between the two sample sets. Therefore, the results of the analysis will be compared qualitatively and semi-quantitatively (by using proportions). Exact concentrations of arsenic species in the mushrooms will not be compared between the two analyses.

3.4.2. Experiment B – Raw/Cooked wild & store-bought fresh/canned mushrooms

The QA/QC results for the analysis of total arsenic and arsenic species in raw and cooked fresh mushrooms collected from Odessa, ON and fresh/canned mushrooms from a local Loblaw's in Kingston, ON are outlined in Table 3-3. For this experiment, 36 samples were analyzed, all of which were run in duplicate.

Table 3-3: Quality assurance and quality control (QA/QC) results for the analysis of total arsenic and arsenic speciation in Experiment B – Total arsenic and arsenic speciation in wild and store-bought fresh/canned mushrooms.

QC test	Assessment metric	N	Range	Average
Total arsenic by ICP-MS				
Extract Totals				
Replicates	Relative percent difference (%)	36	0-158	27
Matrix Spike	Percent recovery (%)	6	81-114	100
Residue Totals				
Replicates	Relative percent difference (%)	36	0-116	33
Matrix Spike	Percent recovery (%)	6	84-124	102
Total Digest Totals				
Replicates	Relative percent difference (%)	35	0-40	12
Matrix Spike	Percent recovery (%)	6	74-114	93
Certified Reference Materials	Percent recovery tuna fish (%)	3	84-100	91
	Percent recovery lichen (%)	2	75-105	90
	Percent Recovery bushes, twigs, and leaves (%)	1	84	84
Overall Totals				
Certified Reference Materials	Percent Recovery Tuna Fish (%) (Extract + residue)	3	85-102	95
Instrument Blanks	Concentration (µg/L)	62	<0.1	<0.1
Instrument Quality Checks	Percent recovery (%)	90	75-128	97
Method Blanks	Concentration (µg/kg mushroom)	9	<4.0	<4.0
Extraction Efficiency	Percent of Extracted Arsenic/Extract residue total (%)	72	6-95	66
	Percent of Extracted Arsenic/total digest total (%)	70	1-195	50
Arsenic species by HPLC-ICP-MS				
Replicates	Relative percent difference (%)	82 [a]	0-64	16
Matrix Spike	Percent recovery (%)	48	80-241	117
Instrument Blanks	Concentration (µg/L)	10	<1.0	<1.0
Instrument Quality Checks	Percent recovery (%)	80	78-125	100
Certified Reference Materials (Tuna Fish)	Percent recovery AB (%)	3	81-92	85
	Percent recovery DMA (anion) (%)	3	85-106	94
Method Blanks	Concentration (µg/kg mushroom)	4	<40	<40
Column Recovery	Percent recovery (%)	36	67-138	108

[a] The number of replicates for arsenic species by HPLC-ICP-MS is very high because the relative percent difference was calculated for all of the samples for each species (iAs, DMA(V) (anion), MMA(V), AB, TMAO, and AC.

For total arsenic analysis, the average RPD for the duplicate samples ranged from 0%-52% with an average of 18%. The largest range (0%-51%) of RPDs was in the fresh mushrooms purchased from the grocery store, with an average RPD of 19%. Only two of the 18 duplicates for this sample set had values that were well above the acceptable range. Both of these samples were cooked via the frying method. Fried mushrooms tended to be harder to homogenize than raw or baked samples due to the oil. Therefore, the larger differences in the values could be due to heterogeneity in the samples. The other sample sets had ranges of 1%-38% and 2%-19% for the canned and wild mushrooms, respectively. Therefore, the reproducibility in the experiment was determined to be acceptable.

Two matrix spikes were analyzed for both the extracted solution and the digestion of the residue for each sample set. The matrix spike recoveries for the extract and residues ranged from 81%-124% recovery with an average of 101%. All of these recoveries were considered to be within an appropriate range of recovery (CCME, 2016). This indicates that there was not a strong matrix effect in the analysis of any of the sample sets. Therefore, no correction was applied to the data.

Instrument quality checks were analyzed using 5 µg As/L and 50 µg As/L solutions every 10 samples and had an average percent recovery of 96%. Of the 90 instrument quality checks that were run, the majority were well within the accepted range (100 ± 20%, (CCME, 2016)) and only seven were outside of this range (but were not greater than 100 ± 30%).

The percent recoveries for tuna fish CRM were an average of 95% for the extract + residue total and 91% for the total digest total. The lichen CRM had an average percent recovery of 90% for the total digest total and the bushes, twigs, and leaves CRM had 84% recovery (also for the total digest total). All of these values were considered to be within an acceptable range.

The EEs had a wide range for all of the sample sets. For the extract/extract + residue EEs, the mushrooms from Odessa, ON ranged from 29%-95%, fresh grocery store mushrooms ranged from 6%-88%, and canned mushrooms ranged from 63%-90%. Overall, the extract/extract + residue EEs ranged from 6%-95% and had an average of approximately 66%. For the extract/total digest EEs, the mushrooms from Odessa, ON ranged from 10%-51%, fresh grocery store mushrooms ranged from 1%-195%, and canned mushrooms ranged from 27%-54%. Overall, the extract/total digest EEs ranged from 1%-195% and had an average of approximately 50%.

During speciation analysis, the RPD between duplicate samples was calculated for each arsenic species present in each sample (inorganic arsenic (As(III) and As(V)), DMA(V), MMA(V), AB, TMAO, and AC). The RPDs ranged from 0%-78% with an average of 17%.

The percent recoveries of the matrix spikes during speciation analysis varied for each sample set. For the canned and fresh grocery store mushrooms, matrix spike recoveries during speciation analysis ranged from 80%-129%, with an average of 102%. Therefore, no correction was applied to either of these data sets. However, matrix spike recoveries for the wild mushrooms collected from Odessa ranged from 86%-241% with an average of 146%. For this sample set, the average percent recoveries for the two spiked samples in the anion exchange were: As(III): 171%, DMA(V): 138%, MMA(V): 147%, and As(V): 157%. For the cation exchange, the average percent recoveries for the three spiked samples were: DMA(V): 187%, AB: 91%, TMAO: 144%, and AC: 134%. Therefore, based on the matrix spike recoveries the concentrations were corrected for all of the anion species and for TMAO. The DMA cation values were not corrected, because they weren't used for reporting; instead the DMA anion values were used. AC values were not corrected as no AC was detected in any of the samples. AB values were not corrected as the matrix spike recovery was determined to be within an acceptable range (CCME, 2016).

Instrument quality checks were run every 10 samples during speciation analysis. Overall, the average percent recovery of the quality checks for all species ranged from 78%-125% with an average of 100%.

The percent recovery for the CRM was determined for two species, and had an average of 85% for AB and 94% for DMA(V) (based on the anion value). As in the other experiments, the anion value was used for DMA(V) in order to remain consistent with the DMA(V) values that were used for the samples.

The percent column recovery was determined using the same method outlined in Section 3.4.1 using the corrected species values. The column recoveries for all of the sample sets ranged from 67%-138%, with an average of 108%. The lower column recoveries did not correlate with trace recoveries or low arsenic concentration.

A mass balance check was carried out for this set of data, like the other data discussed in Section 3.6.1, by comparing the total arsenic from extract + residue with the total arsenic when the entire mushroom sample was digested and analysed. The extract + residue results are an average of 2.6 times lower for the Odessa samples, equivalent for the fresh grocery store mushrooms, and 2 times lower for the canned samples. This is opposite to what was observed in the Deloro mushrooms, where the extract + residue totals were five times greater. Although these sample sets vary to a much smaller degree or, in the case of the fresh grocery store mushrooms, not at all, the total arsenic value from the total digest will be used when available in order to remain consistent. The inconsistencies with the mass balance check (total digest values vs extract + residue values) remain unresolved between all of the sample sets. There is no clear reason to explain why

the extract + residues values consistently fluctuated in varying degrees in relation to the total digest values. Therefore, the results of the analysis will be compared qualitatively and semi-quantitatively (by using proportions). Exact concentrations of arsenic species in the mushrooms will not be compared between the sample sets.

3.5. Arsenic Speciation Analysis using X-Ray Absorption Spectroscopy (XAS)

X-ray absorption spectroscopy (XAS), specifically X-ray absorption near edge structure (XANES) analysis, was performed at the Advanced Photon Source (APS) Pacific Northwest Consortium/X-ray Science Division (PNC/XSD) on the bending magnet (BM) beamline, Sector 20. XANES spectra of the arsenic Kα-edge (11868 eV) were recorded in fluorescence mode using a solid state Ge detector (Canberra model GL0055PS) while monitoring incident and transmitted intensities in N_2-filled transmission ionization chambers. Dried and homogenized mushroom samples were placed in a sample holder, held between two layers of Kapton™ tape and kept at 100 K using a liquid N_2 cryostat (Model 22 CTI Cryodyne Refrigerator System, Janis). A total of 3 to 5 scans were collected with a 0.5 eV step size over the edge region and averaged prior to background removal and normalization to edge jump. The Si(111) double crystal monochromator was calibrated using the first inflection point of the gold LIII absorption edge (11919.7 eV). A reference gold foil was measured simultaneously with all samples. Additional experimental setup information can be found in Smith et al. (2005) and Nearing et al. (2014a). XANES spectra of the arsenic Kα-edge (11868 eV) were fit within -20 to +30 eV from the arsenic E_O using Athena software. Frozen As(III) and As(V) standards (Koch et al., 2011), and liquid AB, DMA(V), and TMAO (Smith et al., 2005) previously measured by our group were used for fittings.

Three-dimensional analysis was also performed at the APS PNC/XSD on the insertion device (ID) beamline, Sector 20. Samples of a freeze-dried puffball were sealed in Kapton tape for measurement at room temperature. The X-ray undulator beam used for the ID beamline passed through a LN2-cooled Si(111) monochromator and was focused to a cross-section of 2×2 μm^2 using Kirkpatrick-Baez mirrors, with the beam incident on the sample stage at a 38° angle. Samples were affixed with tape to a plastic holder mounted on an xy-stage (Melles-Griot, Rochester, NY) capable of submicron stepping. The xy-stage was mounted on an xyz stage (Advanced Design Consulting (ADC), USA) with approximately 0.5 μm resolution which is the typical operating set-up at the 20ID beamline (Heald et al., 2007). For finer resolution depth-scanning, a high-resolution stage (Newport Corporation) was added. Incident beam intensity was monitored using a He-filled transmission ion chamber (incident intensity ~ 9×10^{10} ph/s at 12 keV). A germanium microchannel optic (Woll et al., 2014), lithographically fabricated at Cornell University, was mounted in front of a single-element Vortex® silicon drift

detector. The tissue sample was scanned either in horizontal position and depth or in horizontal and vertical positions with the full fluorescence energy spectrum collected at each voxel at an excitation energy of 12 keV. The elements analyzed were potassium, calcium, copper, zinc, manganese, iron, and arsenic.

At points of interest μXANES were collected to determine the arsenic speciation. For the μXANES collection a total of 3 to 5 scans were collected with a 0.5 eV step size over the edge region and averaged prior to background removal and normalization to edge jump. XANES spectra of the arsenic Kα-edge (11868 eV) were fit within -20 to +30 eV from the arsenic E_O using Athena software. No change in white line energy was observed from first to last scan, indicating the beam was not changing the arsenic speciation during the XANES measurements. Frozen As(III), As(V) and As(V) glycerol standards (Koch et al., 2011), and liquid AB, DMA(V), TMAO, and arsenic glutathione ($As(Glu)_3$) (Smith et al., 2005) previously measured by our group were used for fittings.

3.6. Betaine Extraction

Betaine extraction was done in accordance with the optimized method outlined by Bessieres et al. (1999). Previously dried and analyzed mushroom samples from Nearing et al. (2014a) were used. For each mushroom, 0.2 g of dried sample and 6 mL of cold DDW (approximately 4 °C) were weighed into 15 mL Fisherbrand® disposable polypropylene centrifuge tubes. The mixtures were vortexed and shaken end-over-end using a Rugged Rotator (Model: 099A RD4512, Glas-Col, USA), for approximately 20 minutes at 4 °C before being centrifuged at 3000 rpm for 15 minutes at 4 °C. The supernatant was decanted and syringe filtered using disposable 0.45 μm filters (Millipore® polypropylene 25 mm diameter hydrophilic PVDF durapore membrane) into 15 mL Fisherbrand® disposable polypropylene centrifuge tubes. The samples were then diluted 100x before undergoing analysis.

Betaine was analyzed using an Agilent 1260 HPLC (outfitted with an Agilent ZORBAX Eclipse Plus C-18 column, 2.1 mm X 100 mm, 1.8 μm column) coupled with an Agilent 5460 Triple-quadruple MS/MS Mass Spectrometer under positive electrospray ionization (ESI+) mode. Multiple reaction monitoring (MRM) using the transition 118 *m/z* > 59 *m/z* was selected for quantitative analysis (Preedy, 2015). Parameters optimized for the MS/MS analysis are: fragmentor voltage, 135 V; collision energy, 20 V; cell accelerator voltage, 7 V.

The QA/QC results for the analysis of betaine in the dried mushrooms collected (total number of samples was 48) are outlined in Table 3-4. For every 10 samples a solution with a known concentration of betaine (in water), a matrix spike, a duplicate, and a blank were included. The HPLC-MS instrument LOD for betaine in the extract is 0.00012 mg/L. This blank was based on the lowest calibration concentration. The method blank LOD was determined to be 0.014 mg/L. This was

determined by multiplying the highest dilution factor (120) by the lowest calibration concentration. All blanks were below the LOD and all samples had values well above the LOD.

As no certified reference material was available for glycine betaine, a surrogate was created by soaking 0.2 g of mushroom in DDW to saturation (approximately 2 mL) with a spike of approximately 50 µL of 1000 mg/L betaine solution overnight (approximately 12 hours). The surrogate was brought up to 6 mL and underwent the same procedure as the other samples. The surrogate was run in quintuplicate and the average percent recovery was determined to be 29%. This value was used to correct the extraction concentrations of the other mushroom samples, as is typical for the analysis of organic chemicals (CCME, 2016). Instrument quality checks were also completed using a betaine solution and the average percent recovery was determined to be 81%.

Table 3-4: Quality assurance and quality control (QA/QC) results for the analysis of total betaine concentration in mushroom extracts through HPLC-MS.

Instrument	Measurement	Medium	N	Range	Average
HPLC-MS	Total Betaine Concentration (from mushroom extracts)	Replicates (relative percent difference, %)	5	0-6	3
		Surrogate (spiked mushroom) (percent recovery, %)	5	26-32	29
		Betaine solution (percent recovery, %)	5	101-105	103
		Method blanks (mg/kg mushroom)	8	<0.014	<0.014
		Instrument blanks (mg/L)	1	<0.00012	<0.00012
		Instrument quality checks (percent recovery, %)	4	75-96	81

3.7. Iodoacetic Acid Extraction from Soil

Iodoacetic acid was extracted from three different soils; two were collected from Odessa, ON in 2012 and 2015, and the other was collected from Deloro Woods in Deloro, ON in 2012. The soils were dried in a Ecotherm Laboratory Oven (Precision, Winchester, VA, USA) until the weight remained consistent, for a total of approximately 12 hours. Two different extraction methods were used; 0.1 M calcium chloride ($CaCl_2$) and 0.5 M oxalic acid. Calcium chloride was used as an extract based on its identification as a "universal extractant" for soils (Houba et al., 2000). van Erp et al. (1998) used 0.1 M calcium chloride to extract a multitude of nutrients from soil and Marchetti et al. (1994) used calcium chloride to extract iodine from ashed soil. Oxalic acid was selected as an extractant based on its use in Whitehead (1973). They demonstrated that 0.5 M oxalic acid solutions had a higher iodine solubility than 0.1 M calcium chloride solutions. Therefore, oxalic acid could potentially extract more iodoacetic acid from the soil than the calcium chloride. The two extractant solutions were prepared in 125 mL narrow-mouth Fisherbrand® Nalgene® bottles.

For each soil, 1 g of soil and 10 mL of the extractant solution were weighed into 15 mL Fisherbrand® disposable polypropylene centrifuge tubes. The mixtures were vortexed and shaken end-over-end using a Rugged Rotator (Model: 099A RD4512, Glas-Col, USA) for 4 hours. The mixtures were then stored in the fridge for approximately 72 hours (over the weekend). The day before analysis, the mixtures were centrifuged at 3500 rpm for 20 minutes at 20 °C. The supernatant was decanted and syringe filtered using disposable 0.45 µm filters (Millipore® polypropylene 25 mm diameter hydrophilic PVDF durapore membrane) into 15 mL Fisherbrand® disposable polypropylene centrifuge tubes and kept in the fridge.

The samples were analyzed using HPLC anion exchange chromatography coupled to ICP-MS. For the analysis of iodine (specifically iodoacetic acid), the method was adapted from the work of Shi and Adams (2009). A Hamilton PRPX100 10µm 250x4.6MM column was used along with an autosampler (Perkin Elmer Flexar LC) cooled to 7°C with 50 µL sample injection. An isocratic elution with mobile phase consisting of 200 mM ammonium nitrate (analytical grade, 99.999% Aldrich) was used at pH 8, adjusted with ammonium hydroxide. The flow rate was set to 1.5 mL/min and each run lasted 20 min. The instrument used was an ICP-MS DRC II from PerkinElmer (PerkinElmer, MA). The internal standard monitored during total iodine analysis was rhodium (10 µg/L) and no internal standard was used for speciation. All measurements were made with m/z for iodine (m/z 127) and bromine (m/z 79) in standard mode. Iodoacetic acid and potassium iodide solutions were prepared in water (1 mg/L) and then diluted 100 times before analysis (which correlates to the sample preparation). These solutions were run as

calibration/QC checks and used to determine the location of the iodoacetic acid and potassium iodide peaks.

The QA/QC results for the analysis of iodine in the dried soils are outlined in Table 3-5. As no certified reference material was available for iodoacetic acid in soil, a surrogate was created by spiking 1 g of soil with 1 mL of a 1 mg/L iodoacetic acid solution and then carrying out the extraction with the two different solutions. Standard solutions of iodoacetic acid and potassium iodide were prepared in DDW and analyzed alongside the soil extract samples to compare the retention times of possible peaks. Iodoacetic acid was also spiked into the extractant solutions without any soil (in duplicate), to determine the effect of the solution on the iodoacetic acid peak, and the percent recovery was an average of 100%. The duplicates were also prepared for each of the extractant solutions for one of the soils (n=4), the surrogates (n=4), matrix spikes (n=4), and iodine spikes (n=4), resulting in a total of 12 duplicates with an average RPD of 5% for the iodine peak. The observed peak was iodoacetic acid for all samples except for Soil A extracted with calcium chloride, in which case it was the KI.

Instrumental tests included instrument blanks and calibration checks. Instrument blanks were run throughout the analysis and were under the instrument LOD (1.0 µg/L). This LOD is based on the lowest calibration concentration for iodoacetic acid. The two extractant solutions were run on their own as method blanks in duplicate and were below the method LOD (11 µg/kg soil). This LOD was determined to be lowest calibration concentration multiplied by the highest dilution factor for the soil extracts (approximately 11).

Table 3-5: Quality assurance and quality control (QA/QC) results for the analysis of iodine speciation in soil extracts through HPLS-ICP-MS.

Instrument	Measurement	Medium	N	Range	Average
HPLC-ICP-MS	Iodine Species (from soil extracts)	Replicates (relative percent difference, %)	12	1-8	5
		Surrogate (spiked soil) (percent recovery, %)	4	93-106	100
		Matrix spike (spiked extract) (percent recovery, %)	4	91-105	99
		Iodoacetic acid in blank extractant solutions (percent recovery, %)	4	93-104	100
		Method blanks (µg/kg soil)	8	<11	<11
		Instrument blanks (µg/L)	1	<1.0	<1.0
		Instrument quality checks (percent recovery iodoacetic acid, %)	2	102-103	102
		Instrument quality checks (percent recovery potassium iodide, %)	2	97-103	100

3.8. Synthetic methods

3.8.1. Attempted synthesis of AB from DMA(III)I

Diluted solutions of calcium hydroxide ($Ca(OH)_2$) (0.2 g in 100 mL DDW), sodium pyruvate (1.0 g in 100 mL DDW), and glyoxylic acid (0.2 g in 100 mL) were prepared. An equimolar solution of calcium hydroxide ($Ca(OH)_2$), sodium pyruvate, and glyoxylic acid was prepared by weighing 1.0 g of the diluted solutions into 100 mL of DDW in a 125 mL narrow-mouth Fisherbrand® Nalgene® bottle. The solution was degassed by bubbling nitrogen gas through the solution for approximately one hour in a glove bag (Pyramid Glove Bag, PVC with Butyl Gloves, Erlab Captair) and then was stored in the sealed glove bag. DMA(III)I was only opened and used under deoxygenated conditions in a glove bag with a steady feed of nitrogen gas. A solution of 10 ppm DMA(III)I was prepared in the glove bag in degassed DDW (by bubbling through nitrogen gas for approximately 30 minutes) and 1 mL of this was added to the calcium hydroxide, sodium pyruvate, and glyoxylic acid solution to obtain a final concentration of approximately 1 ppm DMA(III)I. This solution was left in the glove bag and allowed to react for two hours. In order to promote mixing and prevent oxidation, nitrogen gas was continuously bubbled through the solution. 1.5 mL samples were collected into HPLC vials at this stage in order to look for arsenic species, including dimethylarsinoyl acetate (DMAA). An equimolar concentration of S-adenosyl methionine (SAM) (0.01 g) was added to 17 mL of this solution and allowed to react for another 2 hours in the glove bag with nitrogen gas bubbled through the solution. Samples were collected at this point and stored in the fridge until analysis (approximately 12 hours).

3.8.2. Attempted synthesis of AB from TMAO, Iodoacetic acid, and Glutathione (GSH)

The conditions of this experiment were designed to mimic those outlined in Nakamura et al. (2008), although very few details were given in that publication. A 100 mM phosphoric acid-citric acid buffer was prepared with DDW to maintain a pH of 5 in a 240 mL narrow-mouth Fisherbrand® Nalgene® bottle. Glutathione (0.5 g), iodoacetic acid (0.3 g) and TMAO (50 µL of 100 mg/L solution) were added to 50 mL of the buffer and heated at 37°C for 2 hours. The final concentrations of these species were 0.033 M for glutathione and iodoacetic acid and 1.3×10^{-3} mM for TMAO. The sample was divided in two, with one half stored in the freezer until analysis and the other half left to continue reacting at room temperature (20°C).

3.8.3. Analysis of arsenic species in synthetic reactions

The samples were analyzed using HPLC cation exchange chromatography as described in section 3.3.4. Instrument quality control checks were included every 10 samples in this analysis using 10 and 50 µg As/L solutions. A calibration curve was also analyzed using 5, 10, 50, and 100 µg As/L solutions before and after all of the samples were run. Specifically, standards of AB, DMA(V), TMAO, AC, TETRA, DMAA, DMAE, and arsenosugars were run to determine retention times and peak characteristics.

The QA/QC results for the analysis of arsenic species are outlined in Table 3-6. Instrumental blanks were included before and after analysis and no visible peaks were detected. No method blanks were included in this analysis.

Matrix spikes were included for each reaction using a spike solution containing 10 mg/L solution of DMA(V), AB, TMAO, and AC. After one round of analysis, the samples were stored in the freezer for approximately two weeks and then diluted 100x and analyzed again. Dilutions were carried out by adding 1.5 mL of water to 0.15 g of sample. Matrix spikes are reported in Table 3-6 for the 100x diluted samples. The diluted samples were also spiked individually with known standards of compounds that were the desired product or likely present in order to compare retention time and peak characteristics. The analysis of the product solutions from synthetic experiments focused on the identification of peaks rather than the concentrations of potential products. Only matrix spikes were quantified by peak fitting.

Samples (before the dilution) were prepared in a minimum of triplicate, and it was determined qualitatively (by graphing the chromatograms on the same chart, Figure 3-5) that there were no differences between any replicates. PeakFit was not used to qualify the differences because only the identity of the arsenic compounds in the solutions (i.e., position of peaks) was of interest, and the shape of the detected peak indicated that other peaks were hidden and could not be quantified. No replicates were run of diluted samples but each diluted sample was individually spiked with different, known arsenic compounds for comparison (as discussed previously), and results are shown in Table 3-6.

Table 3-6: Quality assurance and quality control (QA/QC) results for the analysis of arsenic speciation in synthetic experiments through HPLS-ICP-MS.

Instrument	Measurement	Medium	N	Range	Average
HPLC-ICP-MS	Arsenic species from synthetic experiments	Instrument quality checks (percent recovery, %)	32	92-110	101
		Instrument blanks (µg/L)	4	<1.0	<1.0
		Matrix spike (percent recovery DMA(V), %)	4	95-118	107
		Matrix spike (percent recovery AB, %)	4	82-114	105
		Matrix spike (percent recovery TMAO, %)	4	106-118	113
		Matrix spike (percent recovery AC, %)	4	101-103	102

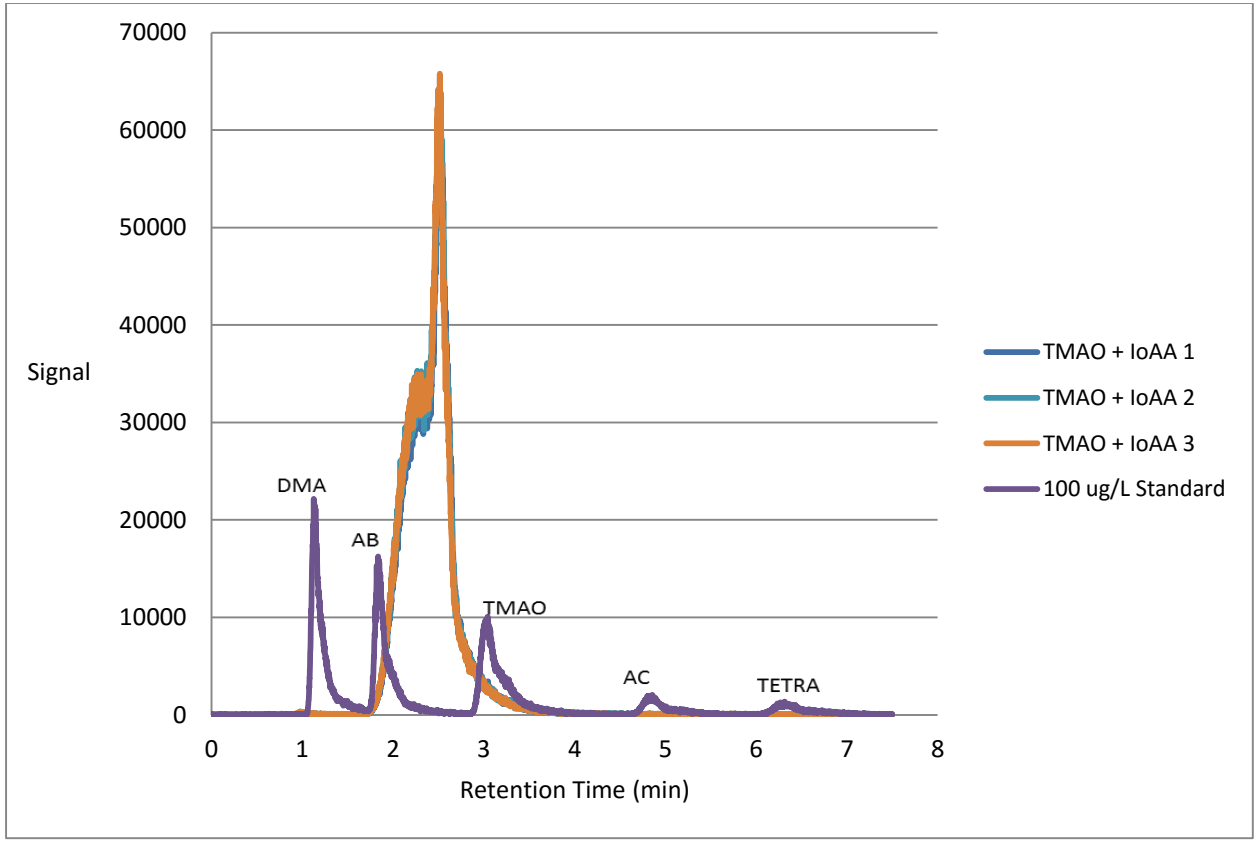

Figure 3-5: Cation exchange chromatograms for the TMAO + IoAA + GSH experiment for the first sample that was prepared in triplicate. It is clear that there is very little difference between the three analyses. A 100 µg/L calibration solution is also shown to demonstrate peak locations of known compounds.

Chapter 4 – Mushroom Results/Discussion

4.1. Deloro Mushrooms

Mushrooms were collected from Deloro, ON, which is considered a location with elevated arsenic concentrations, although they are probably naturally high. These mushrooms were analyzed using HPLC-ICP-MS to determine the total arsenic concentration and arsenic species present. Soil-growing (10 samples) and log-growing (nine samples) were collected in order to investigate the impact of the growth substrate on total arsenic and arsenic speciation, specifically the presence/absence of AB. The results from this analysis are presented and discussed in the following sections.

4.1.1. Total Arsenic

As discussed in the previous chapter (Chapter 3 - Section 3.4.1), the total arsenic values used for comparison for the samples collected in Deloro, ON are the total arsenic values from a total digest of the sample (when available). When there was no total digest completed (due to lack of sample) an amended sum of the extract and residue total arsenic concentration is reported. During the two sample collections (September and October, 2014) a variety of soil-growing (n = 10) and log-growing (n = 11) mushrooms were collected. Overall, the soil-growing mushrooms typically had higher total arsenic concentrations (ranging from 0.36-93 mg/kg dry weight (dw), with an average of 12 mg/kg, dw) than the log-growing species (ranging from 0.33-2.5 mg/kg dw, with an average of 1.1 mg/kg dw). In samples collected by Nearing et al. (2014a), the soil-growing mushrooms also had higher total arsenic concentrations (n = 65, ranging from 0.02-59 mg/kg, dw, with an average of 5.7 mg/kg, dw) than the log-growing mushrooms (n = 8, ranging from 0.04-7.8 mg/kg, dw, with an average of 1.1 mg/kg, dw).

The total arsenic concentrations in mushrooms collected from Deloro, ON by Nearing et al. (2014a) were compared to the total arsenic concentration in mushrooms collected for this research. As only one log-growing mushroom was collected by Nearing et al. (2014a) from this specific location, only soil-growing mushrooms were compared. A non-parametric comparison of two samples (Mann-Whitney test) was conducted using XLStat software (XLSTAT Version 2016.04.32310 with Microsoft Excel 2010). It was determined that there was no significant difference ($p<0.05$) between total arsenic concentrations in soil-growing mushrooms in Nearing et al. (2014a) and the present arsenic concentrations in soil-growing mushrooms. Another Mann-Whitney test was used to compare the combined arsenic concentrations in soil-growing mushrooms (from present samples and from Nearing et al. (2014a)) to those in log-growing mushrooms. It was determined that the soil-growing mushrooms contained significantly higher

arsenic concentrations ($p<0.05$). This is likely because soils have higher arsenic concentrations for uptake than trees or decaying logs (Cheng et al., 2005, Reimer and Cullen, 2009).

4.1.2. Arsenic speciation

The arsenic speciation in the samples was examined to determine the species present and to see if there were differences and similarities between mushroom samples, and trends related to the mushroom growing environment (soil or log substrate). Results (as proportions) are summarized in Table 4-1 and Figure 4-1.

Table 4-1: Major arsenic compounds in soil-and log-growing mushrooms from Deloro, ON that were collected in September and October, 2014.

Species	Source	Total As* (mg/kg,dw)	Proportion of Sum of Species (%)							Predominant Compound
			iAs	DMA	MMA	AB	TMAO	AC	UK1	
Hygrophorus bakerensis	Soil, ON	0.68 [a] ± 0.28	32	52	4.9		7.3	3.1		DMA
Hypomyces lactifluorum		0.543 [a] ± 0.008	64	20	7.8		8.3			iAs
Boletus bicolor		1.26 [a] ± 0.02	22	76	2.5	trace				DMA
Cerrena unicolor		0.36 [a] ± 0.04	20	14	66					MMA
Stereum ostrea		0.43 [a] ± 0.09	39	31	13		11	6.9		iAs
Russula fragilis		6.1 [a, b] ± 1.4	74	19	7					iAs
Calvatia gigantea		92.6 [a] ± 0.6				100				AB
Tricholoma Aurantium		7.9 [b] ± 0.6	trace	5.7		84	8.2	2.6	trace	AB
Galerina Autumnalis		7.2	1	50	0.47	48				DMA
Coprinus atramentarius		2.959 [b] ± 0.005	51	45		4.1				iAs
Pseudofistulina radicata	Log, ON	0.520 [a] ± 0.003	7.1	5.6	70	7.6	5.4	4.7		MMA
Exidia glandulosa		0.33	12	61	4.0	11			12	DMA
Plicaturopsis crispa		1.6		85		15				DMA
Phlebia tremellosa		2.0	trace	79	trace	15			6.5	DMA
Trametes versicolor		0.52 [a] ± 0.17	16	54	6.4				23	DMA
Ischnoderma resinosum		0.41 ± 0.05	39	46	3.6				11	DMA
Hypoxylon fragiforme		0.39 [b]	18	33	7.8	23	trace		19	DMA
Pholiota malicola		2.5 ± 0.6	3.1	14		65		11	7.0	AB
Lycoperdon pyriforme		1.78 ± 0.05	9.4	18		51			22	AB

*As discussed in Section 3.4.1, the total arsenic concentration correlates to the value obtained from the total digest of the sample. The uncertainty is the difference between the total arsenic concentrations for the duplicate samples. In some cases there was not enough sample remaining to conduct duplicate analysis. In these cases, no uncertainty is presented.

[a] These total arsenic values are corrected based on the matrix spike recoveries discussed in Section 3.4.1.1.

[b] These total arsenic values are corrected values from the extract + residue analysis (not enough sample remained to conduct a separate total digest of the mushroom).

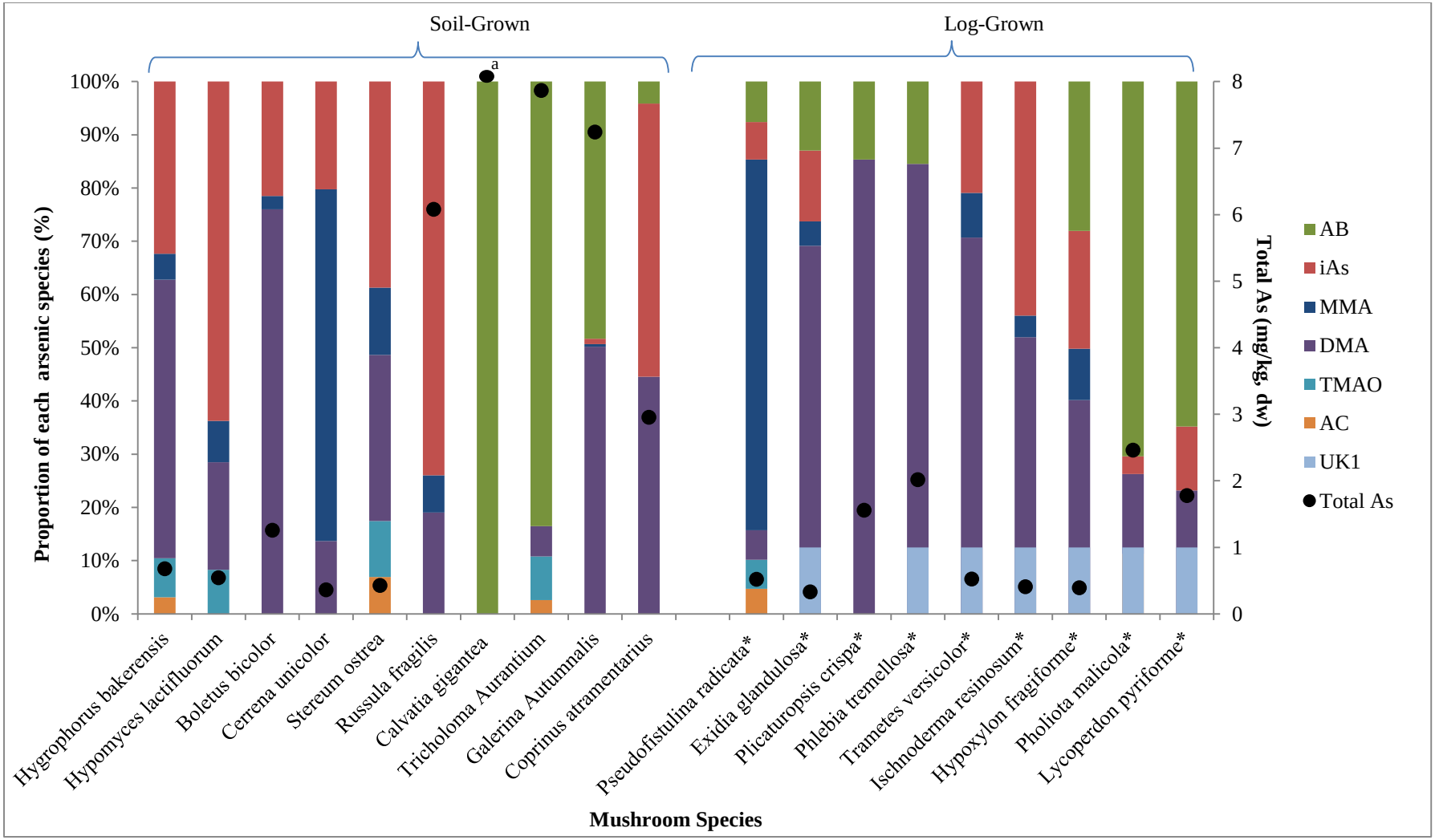

Figure 4-1: Average proportion of arsenic compounds (left axis) and total arsenic (right axis and black circles) in Deloro mushrooms. The 10 samples on the left-hand side are soil-growing and the 9 samples on the right-hand side are log-growing, indicated further by a '*" symbol after the species name. [a] The *Calvatia gigantea* mushroom had a total arsenic concentration of 93 mg/kg, dw, which is much higher than the other mushrooms, and therefore indicated above the axis.

4.1.2.1. General speciation trends

The predominant (i.e. highest proportion) arsenic compound identified in each mushroom species is outlined in Table 4-1. The major compounds in this sample set were compared with those identified in Nearing et al. (2014a) when the same or similar mushroom species were analyzed (Table 4-2). All of the mushrooms were compared with the same species except for the *Boletus bicolor* mushroom (which was compared to the *Boletus edulis* mushroom) and the *Russula fragilis* mushroom (which was compared to the *Russula emetica* mushroom), as those were the closest species available.

Table 4-2: Predominant arsenic species in same-species mushrooms from samples collected for this research in comparison to those collected by Nearing et al. (2014a). All of these mushrooms were collected from Deloro, ON and the *Lycoperdon pyriforme* mushroom was log-growing in both sample sets.

Species	Predominant compounds*, this study	Total As (mg/kg, dw), this study	Predominant compounds*, Nearing et al. (2014a)	Total As (mg/kg, dw), Nearing et al. (2014a)
Boletus bicolor [a]	DMA (76%), iAs (22%)	1.3	iAs (71%), DMA (12%)	0.12
Calvatia gigantea	AB (100%)	93	AB (100%)	59
Coprinus atramentarius	iAs (51%), DMA(45%)	3.0	TMAO (40%), DMA (38%), iAs (14%)	11
Hypomyces lactifluorum	iAs (64%), DMA (20%)	0.39	iAs (80%), DMA (20%)	0.06
Russula fragilis [b]	iAs (74%), DMA (19%)	6.1	iAs (23%), DMA (13%)	0.068
Lycoperdon pyriforme	AB (51%), Unknown compound (22%), DMA (18%)	1.8	DMA (76%), iAs (24%)	0.057

**Predominant compounds are those that were greater than 10% of the sum of species.*

[a] No literature value was available for a *Boletus bicolor* mushroom with elevated arsenic concentrations; the predominant arsenic species from the store bought *Boletus edulis* was used, as it was the closest species available.

[b] No literature value was available for *Russula fragilis* mushroom with elevated arsenic concentrations; predominant arsenic species from the *Russula emetica* collected in BC (from an area with low arsenic concentrations) was used, as it was the closest species available.

Of the six mushrooms that were compared, three were found to have the same predominant arsenic compound. Specifically, only the puffball mushroom (*Calvatia gigantea*) had AB as the predominant compound for both samples sets with the exact same proportions (100% AB for both sample sets). The puffball mushroom from Deloro (Ontario, elevated arsenic) also had the highest total arsenic concentration in each sample set (93 mg/kg, dw for sample collected in September and 59 mg/kg, dw for sample collected by Nearing et al. (2014a)). The other two mushrooms that had the same predominant compounds were the *Hypomyces lactifluorum* mushroom and the *Russula fragilis* mushroom. The *H. lactifluorum* mushroom had very similar proportions of iAs (65% for this sample set and 80% for the sample collected by Nearing et al. (2014a)) and the DMA(V) proportions were identical (20% for both). The *R. fragilis* mushroom had the same predominant compound (iAs), but with very different proportions (74% for this sample set and 23% for the sample collected by Nearing et al. (2014a)). However, the proportions of DMA(V) were very similar (19% and 13%). The variance in the proportion of iAs is likely due to the comparison of similar, but not identical, species.

The three mushrooms that did not have the same predominant compound were the *Boletus bicolor*, *Coprinus atramentarius*, and *Lycoperdon pyriforme*. The *B. bicolor* mushroom had inverse proportions of the same compounds in the two sample sets, 76% DMA(V) and 22% iAs for this sample set and 71% iAs and 12% DMA(V) for the sample *Boletus edulis* collected by Nearing et al. (2014a). Again, the differences in the iAs proportions of these two mushroom species could be due to the comparsion of similar, but not identical mushroom species. The *C. atramentarius* and *L. pyriforme* mushrooms varied both in the proportions and presence of predominant species, even though identical mushroom species were compared. The discrepancies in the major arsenic compounds could be a result of the different sampling times (Nearing et al. (2014a) samples were collected in 2012, mushrooms from this study were collected in 2014), or varying total arsenic concentrations and extraction efficiencies. Additionally, differences in substrate conditions could have affected the results. For example, both of the *L. pyriforme* mushrooms were collected from a log, but the environmental conditions were different. The log collected from Deloro for this research (which had AB as the predominant species) was quite small and significantly decayed. The log from which the *L. pyriforme* mushroom was collected for Nearing et al. (2014a) (with DMA as the predominant species) was large and in the very early stages of decay. An increase in the amount of microbes in the decaying log might account for the high proportion of AB in that sample. The issues with respect to the mass balance check discussed in Section 3.4.1 could have also impacted the results.

An unknown compound (referred to as unknown 1 (UK1)) was identified in several of the mushroom samples during anion exchange chromatography. This peak had a

retention time of approximately 3.2 minutes and was emitted after the cation compound peak but before the peak for As(III). It is hypothesized that this compound could be a peak with cationic character. The unknown is present in most samples that contain AB (and in some cases, in similar proportions). The AB retention time during anion exchange chromatography is approximately 3.1 minutes (Appendix B, Figure B-1), which makes it a likely candidate for the unknown compound in samples that contain AB. However, there are some samples for which this does not hold true: the unknown was detected but no AB was seen in the cation exchange chromatogram. An unknown compound was detected in a small number of samples in Nearing et al. (2014a) with a retention time of 2.3 minutes, before the cation peak. It seems unlikely that these could be the same arsenic compound, but more research is needed to determine exactly what this compound is in the samples that do not contain AB.

4.1.2.2. Morphologic and phylogenetic trends in arsenic speciation

Initially, it was hypothesized by Šlejkovec et al. (1997) that the taxonomic or evolutionary status of fungus could be associated with the arsenic compound patterns within a mushroom and that more evolved fungal species (such as puffballs) contain more complex organoarsenicals. However, more currently it has been suggested by Nearing et al. (2014a) that it is not the advancement of the fungal species, but rather the physiological similarities that are indicative of arsenic metabolite patterns. In order to compare the distribution of arsenic species, the mushrooms were grouped based on their phylogenetic placement (Appendix B, Table B-1) and fruiting body morphology (Appendix B, Table B-2).

When looking at phylogenetic groupings of mushrooms from this study, the Agaricales order (which includes puffballs) mainly contained AB as the predominant arsenic compound (four out of the eight mushrooms) and the Polyporales mainly contained DMA(V) (three of the four mushrooms). For the Boletales order, the predominant arsenic species was DMA(V) (one of one mushroom). The order of Russulales contained iAs as the predominant species for both of the mushrooms. All of these observations are consistent with findings by Nearing et al. (2014a), although a higher proportion of Agaricales order mushrooms (30 of 47) contained AB as the predominant species in previous observations.

In the Auriculariales order, the *Exidia glandulosa* (black ear/witches butter) jelly mushroom was found to have DMA (61%) as the predominant species, with smaller proportions of other species (iAs: 12%, unknown: 12%, AB: 11%, and MMA(V): 4%). In the Auriculariales order mushroom (*Auricularia polytricha*) from Nearing et al. (2014a), the predominant species was iAs (60%), with smaller proportions of DMA(V) (39%) and trace amounts of MMA(V). Several other

orders of mushrooms were analyzed in this mushroom study that had not been previously analyzed. In the Amylocorticiales order, the *Plicaturopsis crispa* (crimped gill) mushroom was found to have DMA as the predominant species. In the Xylariales order, the pored *Hypoxylon fragiforme* (red cushion) mushroom was also found to have DMA(V) as the primary species.

In the Hypocreales order, the *Hypomyces lactifluorum* (lobster) mushroom was found to have iAs (64%) as the predominant species, with a smaller proportion of DMA(V) (20%). This is very similar to the *H. lactifluorum* mushroom from Nearing et al. (2014a), in which the predominant arsenic species was iAs (80%) with a small proportion of DMA(V) (20%). The lobster mushroom is unique in that it is technically a parasitic fungus that completely engulfs another mushroom, typically of the *Russula* species (Arora, 2001). If this is the case, it is likely the *Russula* mushroom that is storing iAs. However, the *H. lactifluorum* has also been reported to attack various other *Boletus* and *Polyporus* species of mushrooms (Bessette and Bessette, 2006). Both of these mushroom species contain DMA as the predominant arsenic compound. Therefore, if the *H. lactifluorum* the mushroom is infecting these types of mushrooms, it may be breaking down the DMA into iAs. However, without a sample of host mushroom it is impossible to confirm this.

When organizing the mushrooms based on the fruiting body morphology, AB was identified in puffball (two out of two mushrooms) and gilled (two of nine mushrooms) morphologies. AB was not observed as the primary species in any of the pored or jelly morphologies. DMA(V) was the most common predominant species for the gilled (four of nine mushrooms) and pored (four of seven mushrooms) morphologies. These findings support the theory that AB may act as an osmolyte (due to its structural similarity to betaine), as it is found in morphologies that require more osmolytic pressure to maintain the structural integrity of the fungus. In puffball and gilled mushrooms, AB may help with osmolytic regulation to ensure the cap remains turgid and elevated for effective spore dispersal. It is possible that pored mushrooms may not require as much osmolytic regulation, and therefore accumulate less AB. Further investigation into the correlation between betaine and AB concentrations is discussed in Section 4.1.3.

4.1.2.3. AB in Soil-Growing vs. Log-Growing Mushrooms

The average proportion of arsenic compounds and total arsenic in soil- and log-growing mushrooms from Deloro, ON are shown in Figure 4-1. It has been suggested that AB (or its precursors) may be produced by the surrounding microbial community, rather than by the fungus itself. Smith et al. (2007) demonstrated that microbial communities found in the growth substrate material for the commercial cultivation of *A. bisporus* were capable of transforming As(V)

to TMAO when the fungus was absent, but AB was only seen when the fungus was present. Nearing et al. (2014a) found that the major arsenic compound present in the fruiting body of a given mushroom was similar (regardless of geographical location and soil arsenic concentration), except when comparing log-growing and soil-growing mushrooms. In the analysis of the tree-growing birch polyphore (*Piptoporus betulinus*) mushroom, AB was only detected in trace amounts in the pores of the mushroom and no AB was detected in the cap or center portion of the fruiting body (Environmental Sciences Group (ESG), unpublished data). The difference between log and soil substrates was hypothesized to be a result of differences in microbial communities. This suggests that the presence of AB may be dependent upon the growth environment and/or microbial community surrounding the mushroom during development and reproduction.

As discussed previously, only nine of the 11 log-growing samples underwent the extraction process. Of these nine samples, seven contained AB (7.6% to 65%). Two of these log-growing species (*L. pyriforme* and *Pholiota malicola*) even contained AB as the predominant compound. The *L. pyriforme* mushroom had a total arsenic concentration of 1.8 mg/kg, dw and an EE of 82%, with 51% of arsenic species as AB. The *P. malicola* mushroom had a total arsenic concentration of 2.5 mg/kg dw and an EE of 95%, with 65% of arsenic species as AB. In contrast, the *L. pyriforme* mushroom growing on a log from Nearing et al. (2014a) had a total arsenic concentration of 0.057 mg/kg dw, with 0% of arsenic species as AB. Therefore, although the two *L. pyriforme* mushrooms were both log-growing from ON backround sites, they have completely different compositions of arsenic. As stated previously, the log substrates that they were growing on had very different compositions (both in size and state of decomposition), which could account for some of the variation. This, along with the high proportion of AB-containing log-growing species in this sample set, suggests that it may be more than just the variation in the microbial community in the soil vs. log that is responsible for the absence/presence of AB.

4.1.2.4. AB Correlations with Total Arsenic

The correlation of arsenic species with the total arsenic concentration has been studied in several experiments. Whaley-Martin et al. (2012a) found a strong positive correlation between the concentration of iAs and total arsenic in blue mussels (*Mytilus edulis*) collected from an arsenic-contaminated area. This area had a strong arsenic concentration gradient from background concentrations to very high levels of contamination, which was the result of historical mining activities. Along this gradient, they observed that the concentration of organoarsenic compounds (mainly AB) remained relatively constant despite the increase in total arsenic. In experiments by Soeroes et al. (2005), *Agaricus bisporus* mushroom grown on substrate amended with As(V) (1000 mg As/kg) contained

higher proportions of iAs (22.8 µg As/g, dw) than those cultivated on the control/un-amended substrates (substrate: 3.8 µg As/g, mushroom: 0.5 µg As/g, dw). The control mushrooms had approximately two times the concentration of AB as the mushrooms grown on the amended substrate, even though they had a lower total arsenic concentration. Conversely, Nearing et al. (2014a) found no correlation between iAs and total arsenic concentration in mushrooms. Furthermore, they did observe a positive correlation between AB concentration and total arsenic for mushrooms that contained AB.

For the mushrooms collected in this study, when comparing the total arsenic with either the concentration or proportion of AB in the sample, samples with high total arsenic concentrations tend to have high proportions of AB (100% AB, 84% AB and 48% AB for the three highest total arsenic concentrations). Total arsenic concentrations ranged from 0.36-6.1 mg/kg, dw for mushrooms that did not contain AB and from 0.33-93 mg/kg, dw for mushrooms that contained AB. For all mushrooms, the linear trend line for the total arsenic concentration as a function of the concentration of AB has an R^2 value of 0.7, which indicates a slight positive correlation. This trend line is likely skewed by the low arsenic concentrations of the log growing mushrooms and the low extraction efficiency values for the samples collected in September. Proportions are based on the sum of species measured (not on the total arsenic value) and therefore, it is possible that a large portion of the unextracted arsenic could have been AB. A non-parametric Spearman correlation test was conducted using XL stat software, and a significant positive trend was identified between the concentration of AB and the total arsenic concentration at the 95% confidence level ($p<0.05$). The same test was done for the proportion of AB to the total arsenic concentration and again, a significant correlation was detected at the 95% confidence level ($p<0.05$). No significant correlation was observed between total arsenic concentration and the concentration or proportion of iAs species using the Spearman correlation test.

The correlation of AB and total arsenic concentration/proportion results from this mushroom study correspond to the correlations observed by Nearing et al. (2014). Therefore, it is likely that when mushrooms contain high concentrations of arsenic, a high proportion of that will be in the form of AB. AB, when present, has been shown to be preferentially accumulated over other compounds by mushrooms during the vegetative life cycle (mycelium growth) (demonstrated in *Agaricus bisporus* mushrooms, which typically contain AB as the predominant species, but not to the same degree in *Suillus luteus* (predominant species typically DMA) or *Sparassis crispa* (predominant species typically AC) (Nearing et al., 2015b)). Preferential accumulation of AB has also been associated with fruiting body formation (the life stage at which these mushrooms were collected). This indicates that the mycelium may be selectively accumulating AB and transporting it to the fruiting body or transforming iAs to AB precursors for uptake by the fruiting body.

Upon examination of the mushrooms in the present study with the lowest concentrations of arsenic, a higher proportion of iAs tends to be seen (although as mentioned this was not statistically significant). iAs could be toxic to mushrooms, and has no use within the mushroom. Therefore, if a mushroom stores its arsenic primarily as iAs, it is likely to resist accumulating a high concentration of arsenic.

4.1.3. Betaine analysis

AB is structurally similar to the osmolyte glycine betaine (henceforth referred to as betaine): the chemical structures of the two compounds are identical except for the central atom, which is arsenic in AB, and nitrogen in betaine. AB has been found to adventitiously accumulate in marine organisms and is proposed to have an osmolytic function (Clowes and Francesconi, 2004). AB has been seen to be negatively correlated with betaine in marine organisms (Fujihara et al., 2003) suggesting that AB is competitively taken up or used alongside betaine. AB has also been hypothesized to correspondingly accumulate in mushrooms as an osmolyte to help maintain fruiting body structure for effective spore dispersal and tugor pressure maintenance (Nearing et al., 2014a, Smith et al., 2007). To ascertain if any relationships exists between AB and betaine concentrations in mushrooms, the betaine concentrations were measured in mushrooms previously studied by Nearing et al. (2014a).

The betaine and AB concentrations in these mushrooms are shown in Figure 4-2.

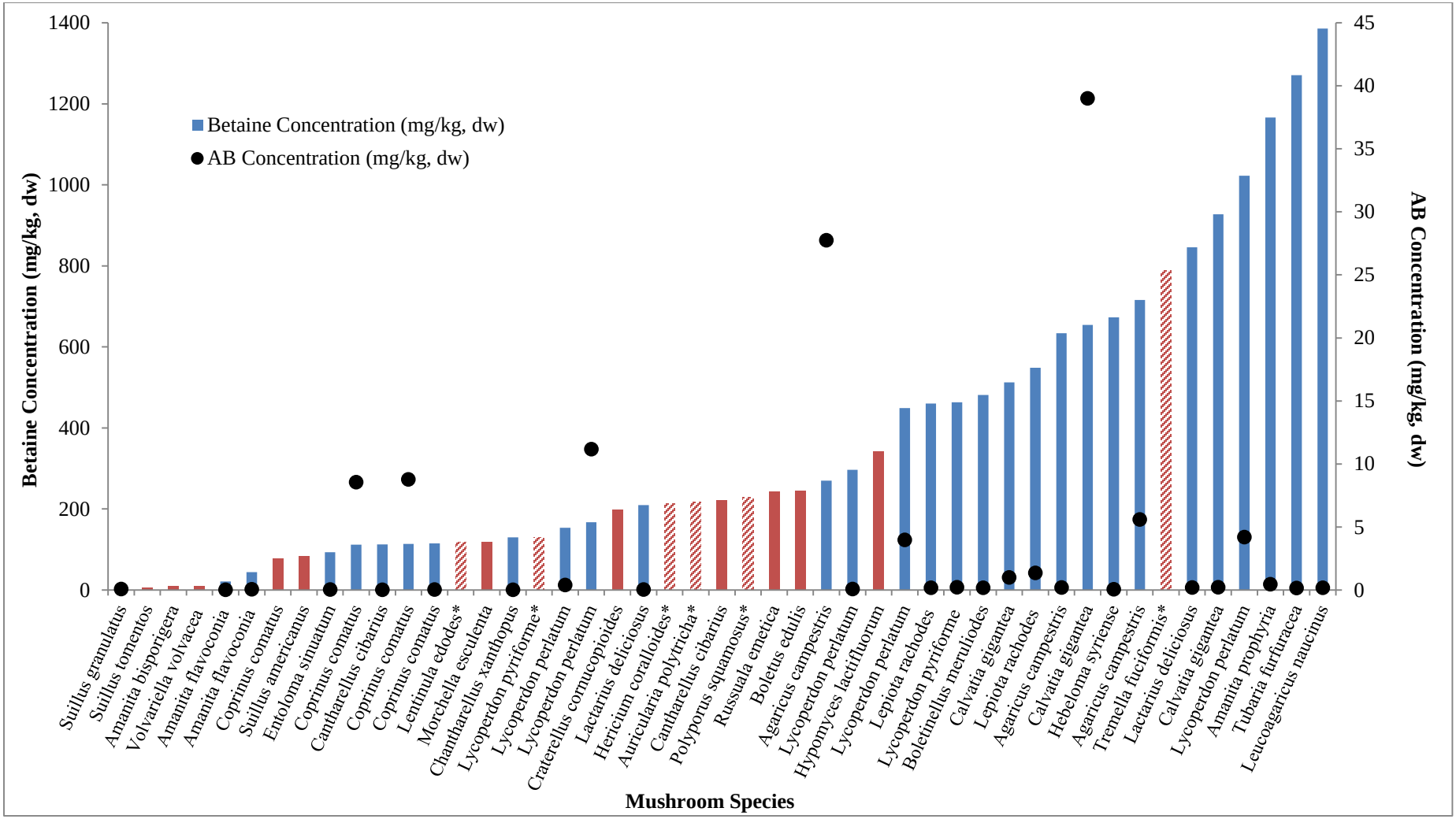

Figure 4-2: Betaine concentration (left axis and bars) and arsenobetaine (AB) concentration (right axis and black circles) in mushrooms collected by Nearing et al. (2014a). The data are organized from lowest to highest betaine concentration. Log-growing mushrooms are indicated by a '*' symbol after the species name and their betaine bars have diagonal hatching. Red bars (solid and hatched) indicate the presence of betaine but no AB.

The betaine concentration results indicate a fairly constant increase; that is, no obvious threshold from low to high betaine concentrations is seen. This also suggests a fairly even or normal distribution of betaine concentrations in the studied mushrooms.

For the majority of the samples containing higher concentrations of betaine (right-hand side of Figure 4-2), AB was detected in the sample. The only exception to this was the log-growing *Tremella fuciformis* mushroom, which did not contain AB. In the majority of the mushrooms with lower betaine concentrations (left-hand side to center of Figure 4-2), AB was not detected. This confirms the hypothesis that when there is betaine present, it is also likely that AB is present. It is possible that if mushrooms require betaine, AB may be of use to them as well and is more likely to be taken up along with the betaine. If mushrooms are able to make use of betaine, they are more likely to be able to use AB.

Generally, there is no clear correlation between the betaine and AB concentration. A Spearman correlation test was conducted using XLStat software, and no significant correlation was identified. However, a significant correlation was identified between the betaine concentration and proportion of AB (Spearman correlation test, $p<0.05$). This suggests that with increasing betaine concentrations, the predominance of AB increases; in other words, AB-predominant mushrooms are also those that have more betaine.

Betaine concentrations have been measured in several edible mushrooms; crimini (*Agaricus bisporus* (brown)) (1467 mg/kg, dw), white button (*A. bisporus*) (1245 mg/kg, dw), oyster (*Pleurotus ostreatus*) (1053 mg/kg, dw), Portobello (*Agaricus bisporus* (brown)) (684 mg/kg, dw), enoki (*Flammulina velutipes*) (122 mg/kg, dw), and shiitake (*Lentinula edodes*) (25 mg/kg, dw) (Self Nutrition Data, 2014). None of these species were analyzed for AB and betaine in the present study, although a species related to *A. bisporus*, *A. campestris*, had 270 to 715 mg/kg betaine in the present study. The *Agaricus* species in the list of the edible mushrooms for which betaine concentrations have been reported have the highest betaine concentrations and are also known to contain AB as their predominant arsenic compound. Overall, these results indicate that AB is taken up alongside betaine, not competitively as proposed by Fujihara et al. (2003) (for pinnipeds, seabirds, and sea tutles).

4.1.4. Arsenic speciation analysis using X-Ray absorption spectroscopy (XAS)

HPLC-ICP-MS analysis can be complemented with XAS, when total arsenic concentrations are high enough, to overcome the dependence on EEs by examining the unaltered sample (Nearing et al., 2014b). Three mushroom species (*Calvatia gigantia*, *Ischnoderma resinosum*, and *Pholiota malicola*) were analyzed at the

Advanced Photon Source (APS) (Figure 4-3). These mushrooms were analyzed using the bending magnet (BM) line to determine the major arsenic compounds present. The arsenic distribution in one of those species (*C. gigantea*) was mapped using the insertion device (ID) line and μXANES were collected at two points. The BM line was also used to analyze the speciation in the bulk (not spatially resolved) soil and mycelium (both from the soil and growing on a log) adjacent to a puffball mushroom growing in the soil and on a log (Figure 4-8).

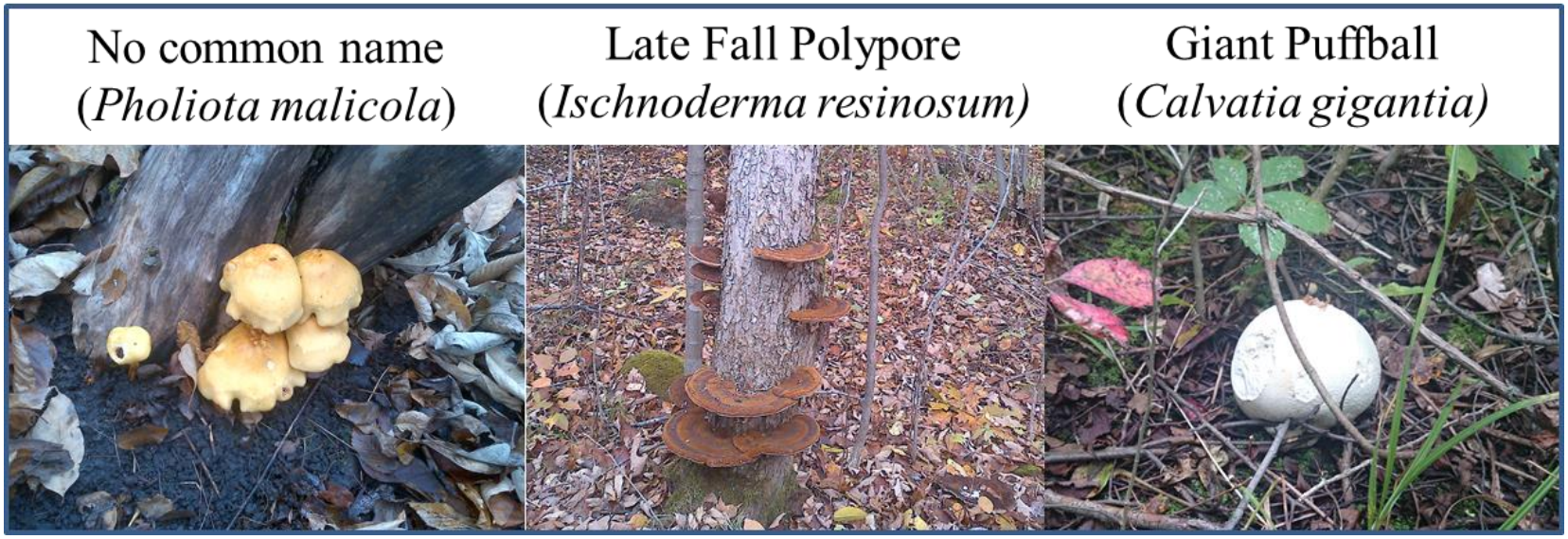

Figure 4-3: Three mushroom species (*Calvatia gigantia*, *Ischnoderma resinosum*, and *Pholiota malicola*) that were collected from Deloro, ON and analyzed at the Advanced Photon Source (APS).

The XANES results from the BM line are compared with those from the HPLC-ICP-MS results (discussed in Section 4.1.2.) in Table 4-3. The XANES predominant species corresponds with the HPLC-ICP-MS predominant arsenic species for all of the samples. Both of the log-growing mushrooms had very low total arsenic concentrations (0.41 mg/kg dw for the *I. resinosum* and 2.5 mg/kg dw for the *P.malicola* mushroom). The *P.malicola* mushroom had AB/AC as the predominant compound. XANES cannot distinguish between these two compounds because they have the same white line energies. Complementing XANES analysis with HPLC allows for separation of AC and AB. In the *I. resinosum* mushroom, the predominant compound was DMA. For XANES analysis, total arsenic concentrations ideally need to be above 1 mg/kg (Nearing et al., 2014b). The mushrooms were selected as representatives of log-growing mushrooms for XANES analysis before the total concentrations were known, in hopes that they would have had detectable total arsenic concentrations. The concentrations were in fact lower than 1 mg/kg (measured by ICP-MS after the XANES measurements were collected), making the arsenic difficult to see and the spectra difficult to interpret.

Table 4-3: Comparison of the total arsenic and arsenic speciation proportions from HPLC-ICP-MS with those from X-ray absorption near-edge structure (XANES) fitting results for the same mushroom. All of these mushrooms were collected from Deloro, ON and mushrooms with a `*`symbol were log-growing.

Species	Total As (mg/kg, dw)	Proportion of sum of species (%) from HPLC-ICP-MS							Extraction efficiency (%)	Predominant compound (HPLC-ICP-MS)	Predominant compound and proportion (XAS)
		iAs	DMA	MMA	AB	TMAO	AC	UK1			
Calvatia gigantia	92.6				100				25	AB	AB (100%)
*Ischnoderma resinosum**	0.41	39	46	3.6				11	95	DMA	DMA
*Pholiota malicola**	2.5	3.1	14		65		11	7	95	AB	AB/AC*

*XANES cannot distinguish between AB and AC due to the fact that it identifies compounds based on the oxidation state and immediately adjacent atoms. In both of the compounds, arsenic is immediately bonded to 4 carbon atoms, giving AB and AC the same whiteline energy.

C. gigantea was further examined using the ID line, to map the distribution of the arsenic and other elements in the mushroom tissue. For this analysis, three scans were completed. A conceptual model for the location of these scans is shown in Figure 4-4. Scan 1 was carried out into the depth of the sample (along the x-z axis), from the surface of the sample to 0.011 cm into the sample. Scan 2 was taken along the x-y axis at the edge of the puffball (so that the skin of the puffball was included) at a depth of 0.004 cm into the sample. Scan 3 was also taken along the x-y axis, but in the interior of the puffball away from the skin, at a depth of 0.003 cm. Maps of the elemental distribution of iron, potassium, calcium, manganese, zinc, copper, and arsenic for each of the scans are shown in Figure 4-5 (scan 1), Figure 4-6 (scan 2), and Figure 4-7 (scan 3).

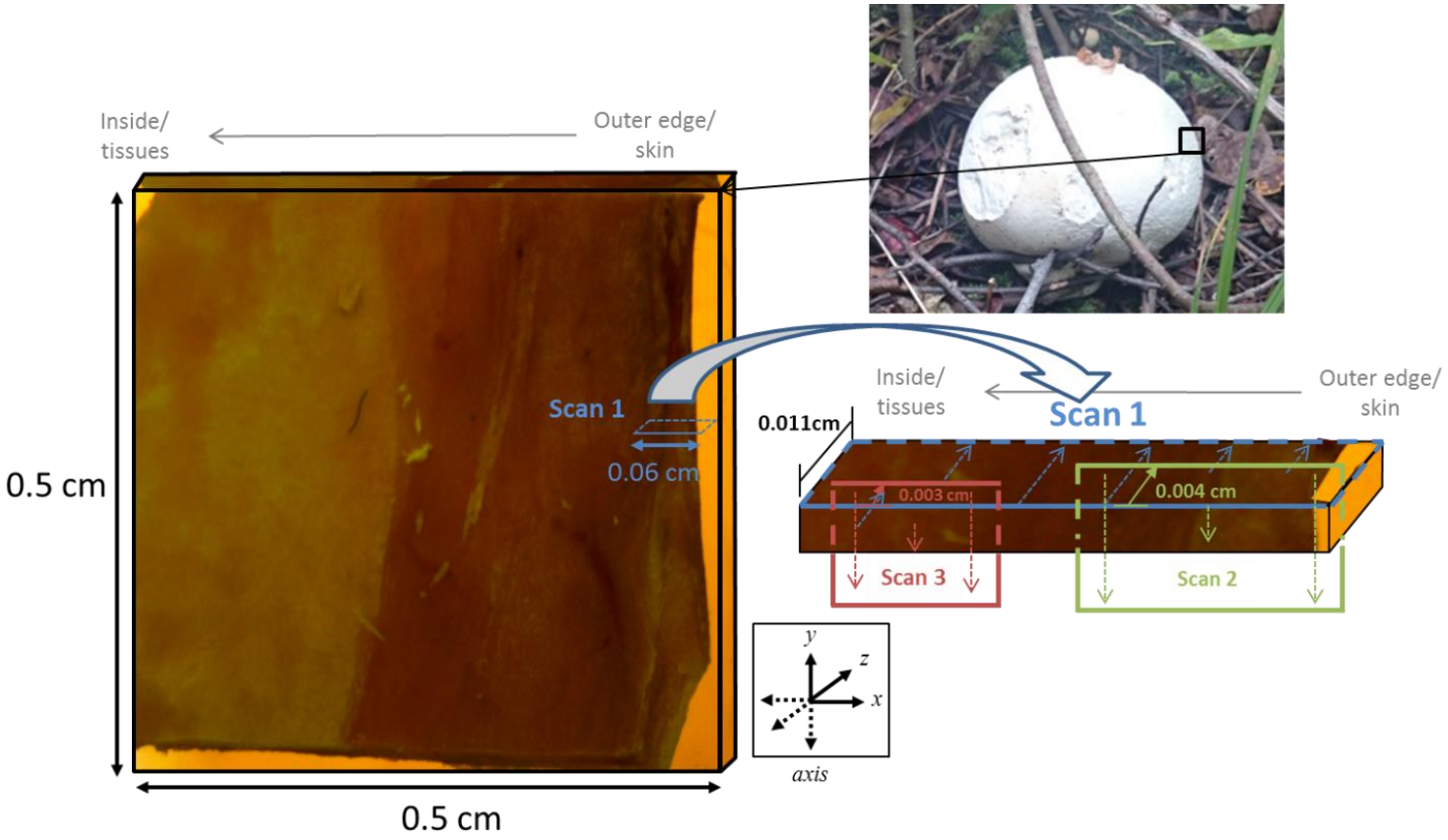

Figure 4-4: Conceptual model of the three-dimensional (3D) XAS imaging scans that were done on the giant puffball (*Calvatia gigantea*) mushroom collected from Deloro, ON using the insertion device (ID) line. Scan 1 was done from the surface of the sample to 0.011 cm into the sample. Scan 2 was taken at a depth of 0.004 cm into the sample and scan 3 was taken at a depth of 0.003 cm.

Scan 1 (Figure 4-5) shows the elemental dispersion from the outer edge or ‘skin’ of the mushroom (right hand side) into the tissue of the mushroom (left hand side). The hotspots of iron, potassium, and manganese are likely the result of soil particles that were not removed during the washing process and may have entered the sample when they were cut. Apart from these hotspots, a gradient of high to low signal appears from the outside (skin) to the inner tissues of the mushroom. The high concentration near the skin is likely a dense area that resulted from cutting the mushroom (and consequently compressing the mushroom tissue) as opposed to an indication of contamination. The best attempt was made to cut the mushroom as straight and uniformly as possible when it was fresh, and the sectioned tissue was then pressed between two glass slides before drying. The tissue may be different thicknesses and the cut edge may appear more uneven because it did not dry evenly or the moisture was not uniformly distributed throughout the mushroom. The hole that appears to the right side of the center of the image was likely present in the fresh mushroom tissue, as Puffballs are typically very porous.

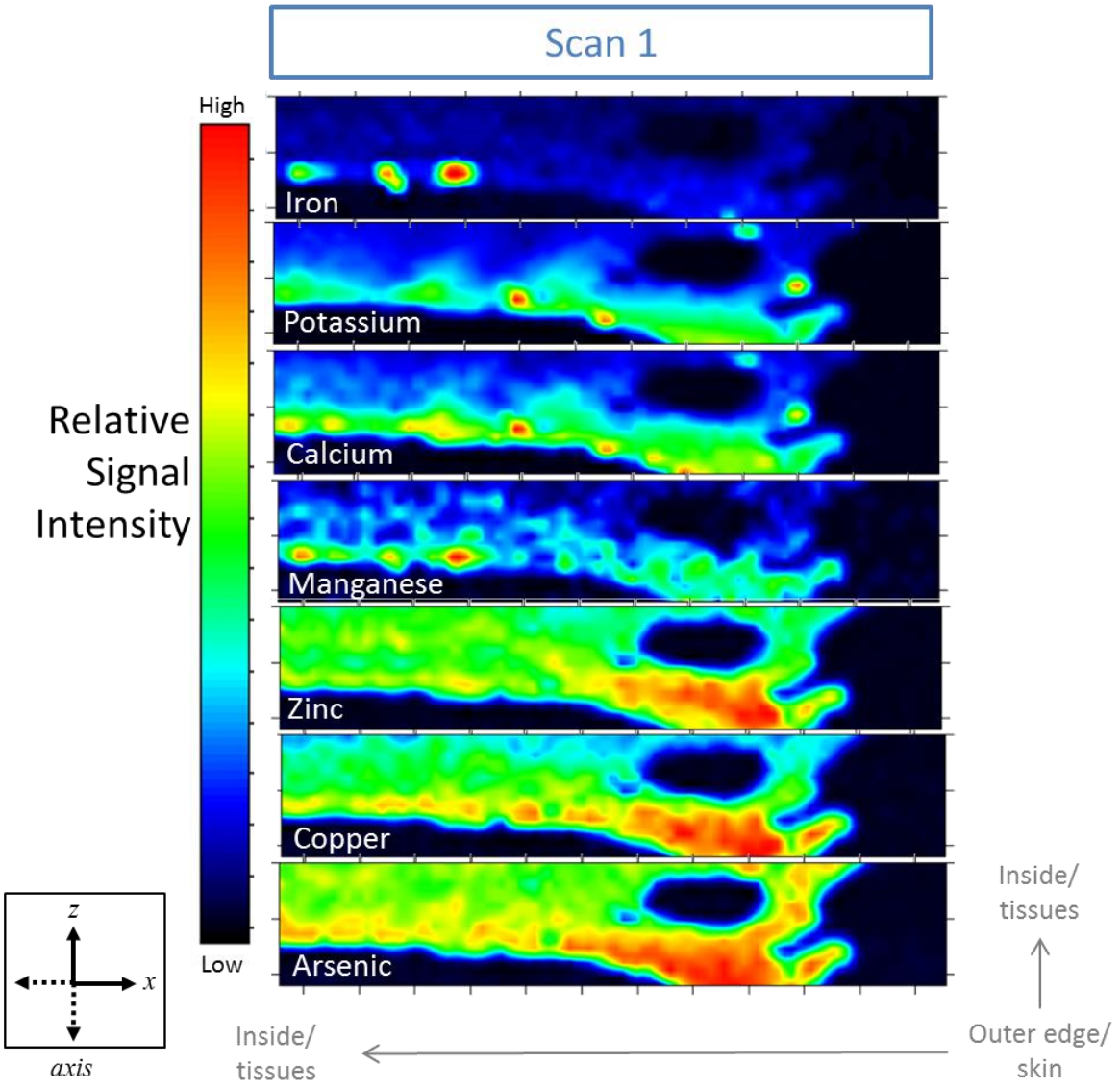

Figure 4-5: Map of elemental distribution for cross section of giant puffball (*Calvatia gigantea*) mushroom collected from Deloro, ON – Scan 1. The right-hand side is the outside or 'skin' of the mushroom and the left-hand side is further into the mushroom tissue.

Scan 2 (Figure 4-6) also shows the elemental dispersion from the outer edge of the sample (right hand side) into the inner tissues of the mushroom. However, instead of scanning from the surface cut edge of the sample, scan 2 looks at an x-y plane within the sample tissue. Scanning within the tissue (beyond the cut edge in scan 1) in the x-y plane produces a scan that is not as greatly influenced by the sample thickness and/or sample preparation, as the inner tissues are the least altered (not cut or exposed to air). Therefore, scans 2 and 3 should provide a more accurate representation of the distribution of elements in the Puffball tissue. It appears that there is very little iron within the sample, which is consistent with Scan 1 (where iron hot spots were attributed to soil particles). Potassium and calcium appear to have co-located hotspots, which may be crystals. Potassium has been identified as one of the major elements in many mushroom species and is particularly concentrated in the fruiting body of the mushroom (Kalač, 2009). Calcium has also been identified in several mushroom tissues, although at a lower concentrations (Falandysz et al., 2001). Calcium (particularly calcium chloride) in mushrooms is suggested to be responsible for the strong white colour in some mushrooms (i.e. the extremely white *C. gigantea* mushroom) and increased calcium concentrations have been shown to reduce browning in mushrooms after they have been harvested (Kukura et al., 1998). More research is needed to examine the specific roles of potassium and calcium in mushrooms in order to determine why high concentration zones are correspondingly located. Copper, zinc and manganese are observed to be similarly distributed. Most of the elements appear to have a higher signal intensity in the lower left corner, which may be attributed to a thicker/denser portion of tissue.

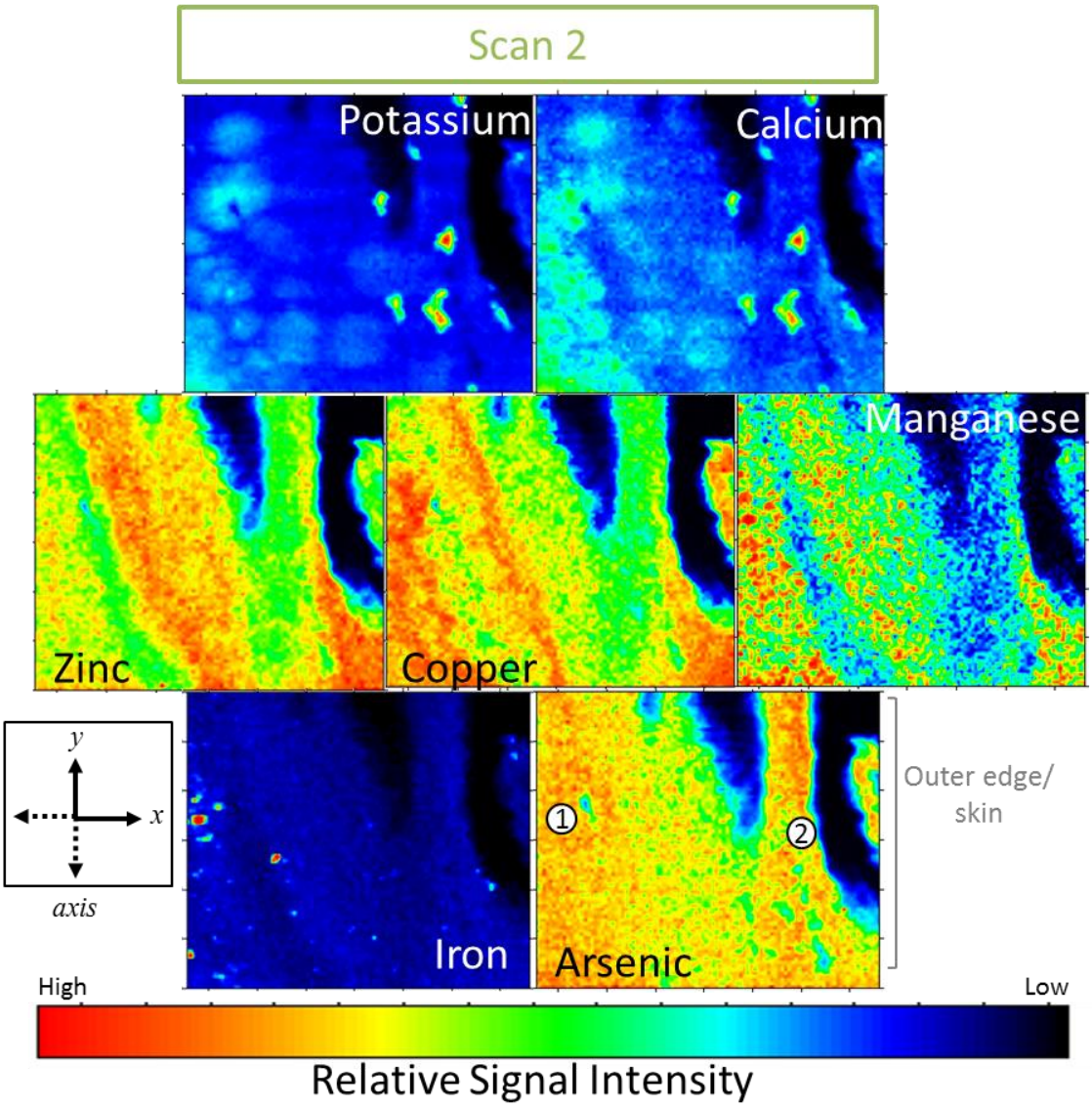

Figure 4-6: Map of elemental distribution for cross section of giant puffball (*Calvatia gigantea*) mushroom collected from Deloro, ON– Scan 2. The right-hand side is the outer side of the mushroom and the left-hand side is further into the mushroom tissue. The numbers indicate the locations where µXANES were collected.

Arsenic appears to be uniformly distributed throughout the plane. The microbeam scanning (μXANES analysis) (i.e. the collection of XANES spectra at specific locations during two or three-dimensional XRF imaging) was done at two locations on the sample to determine arsenic speciation at the point. The numbers on the arsenic map indicate the locations where μXANES were collected. Point number one was identified to be 100% As(V) and point number two was identified as 100% As(III). Therefore, although arsenic appears to be uniformly distributed, As(III) was present at the outer edge of the mushroom, but further into the mushroom tissue (or at least at point one), the predominant species was As(V). AB was not detected at either of the μXANES points, even though it was identified as the predominant arsenic species in this mushroom sample. This is because the information from μXANES is specific to particular locations, rather than the speciation throughout the whole sample.

Scan 3 (Figure 4-7) shows the elemental dispersion within a centre plane of the mushroom sample.

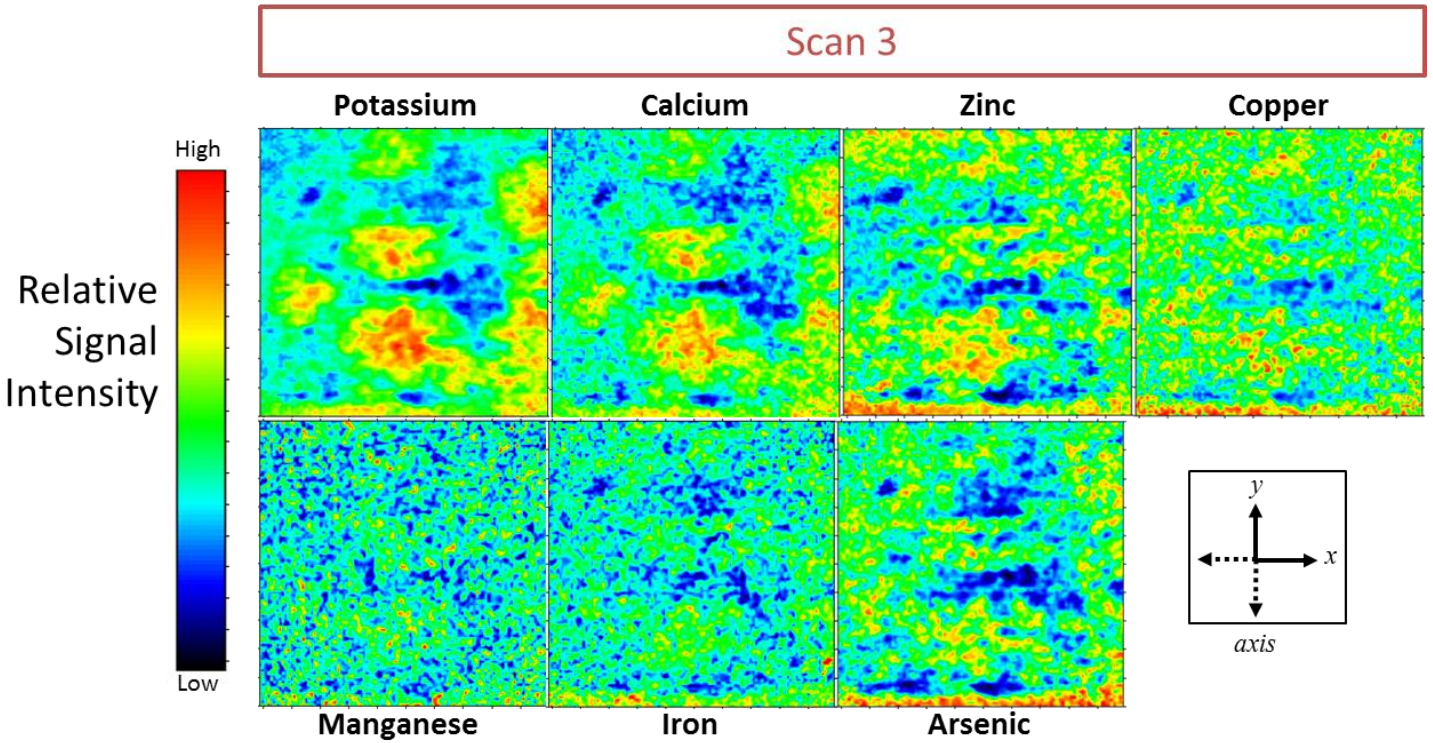

Figure 4-7: Map of elemental distribution for cross section of giant puffball (*Calvatia gigantea*) mushroom collected from Deloro, ON– Scan 3. This cross section is from the middle of the mushroom tissue, and therefore, no outside edge of the sample is observed.

As seen in scan 2, potassium and calcium have similar areas of increased signal with an appearance of possible globules, which may be increased tissue associated with some granular distribution of the mushroom tissue. However, the less pronounced localization of the other elements (zinc, copper, manganese, iron, and arsenic), which all appear to be uniformly distributed, suggests that these globules or grains may be specific to calcium and potassium. An increase in signal for all elements, but especially arsenic, zinc and copper, is observed at the bottom of each map. This could be a result of an increase in tissue thickness/density towards the bottom of the sample that may have occurred during the drying process.

The BM line was used to identify the predominant arsenic species in the soil and mycelium (growing in the soil and on a log) adjacent to a puffball mushroom (Figure 4-8). In both the soil and the mycelium growing in the soil, As(V) was the only arsenical seen. In the log-growing mycelium, As(III) was the only arsenical. AB was not detected in any of the samples. This correlates with results from Nearing et al. (2015b), who found that AB is not formed during the vegetative life stage of the mushroom, but rather during fruiting body formation. The As(V) in the soil mycelium may indicate that As(V) is directly taken up by the mycelium, whereas the As(III) in the log-growing mycelium indicates that maybe As(III) was present in the log and absorbed by the mycelium in that form. As(III) is the form of arsenic commonly found in plants that have not been processed (Smith et al., 2008). However, the difference between oxidation states in the log vs. soil growing mycelium should be interpreted cautiously because As(III) is easily oxidized to As(V) and it is possible that arsenic in the soil or wood from the log could fluctuate between these two states.

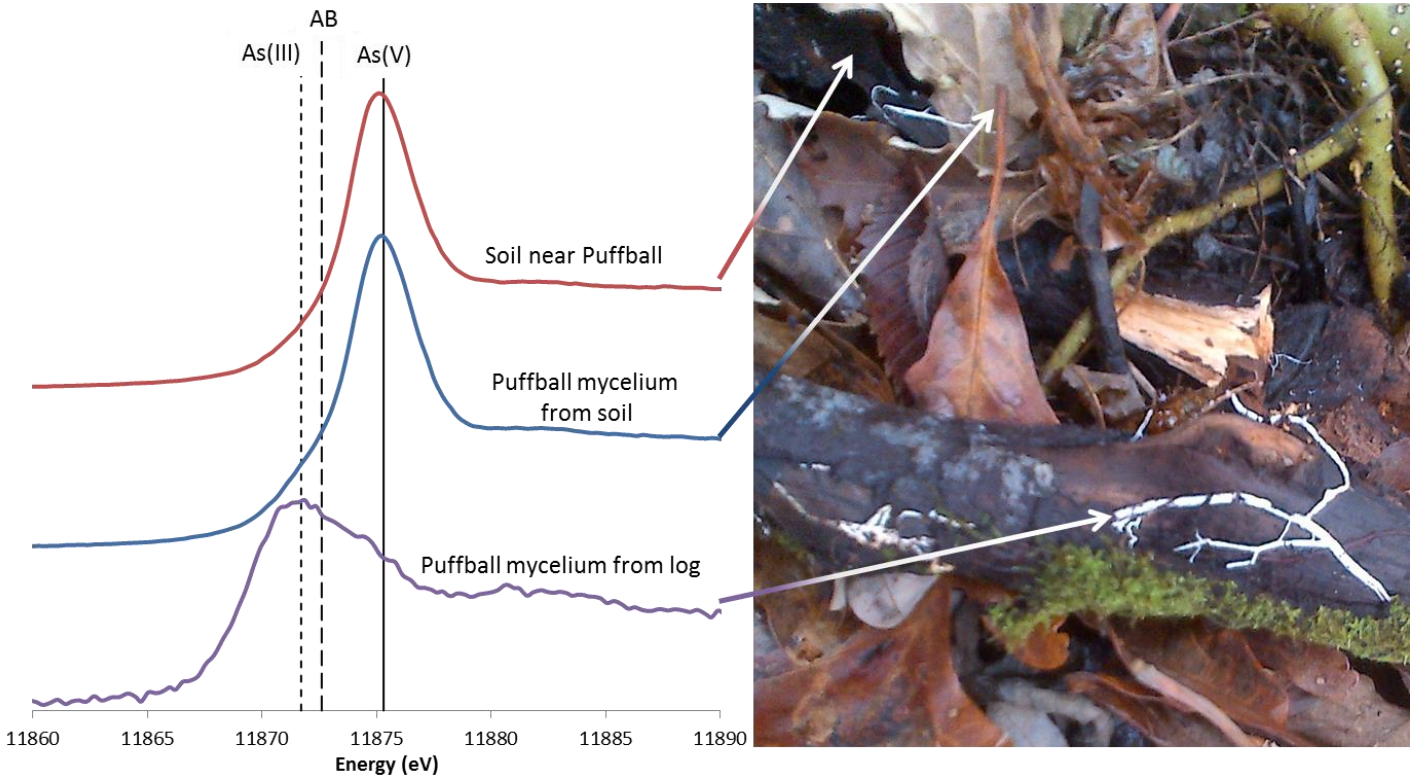

Figure 4-8: Samples from the soil surrounding a puffball mushroom (red), the mycelium growing in the soil (blue), and the mycelium growing in a log (purple) were analyzed using the bending magnet (BM) beam line. The X-ray absorption near edge structure (XANES) spectra are shown on the left hand side of this figure, and standard energies for As(III), AB, and As(V) are indicated along the x-axis. The concentration of arsenic was extremely low in the mycelium from the log, resulting in an unclear signal. Only the major peak (As(III)) in this sample was considered identifiable.

4.2. Cooked Mushrooms

In total, 12 mushroom samples were subjected to cooking treatments. Four different mushroom species (saffron milk cap (*Lactarius deliciosus*), rough-stemmed bolete (*Leccinum scabrum*), penny bun (*Boletus edulis*), and giant puffball (*Calvatia gigantea*) were collected from Odessa, ON and were subject to frying and barbequing. Four different species of fresh mushrooms (portobello and crimini (brown strain of *Agaricus bisporus*), white button (white strain of *Agaricus bisporus*), oyster (*Pleurotus ostreatus*), shiitake (*Lentinula edodes*), and enoki (*Flammulina velutipes*) were purchased from a local grocery store, and fried and baked. Finally, two canned mushroom varieties (white button mushrooms (white strain of *A. bisporus*), sliced and whole) were also fried and baked. The total arsenic concentration and arsenic speciation in these 12 mushrooms were examined pre- and post-cooking to determine the species present and to see if any transformations occurred during the varying thermal treatments. Results (as proportions) are summarized in Table 4-4 and Figure 4-9 (for mushrooms collected from Odessa, ON) and Table 4-5, Figure 4-10, and Figure 4-11 (for mushrooms purchased from the grocery store in Kingston, ON).

An unknown arsenic species was identified in several of the mushrooms from the local grocery store during the anion exchange. This compound (UK (anion)) had an average retention time of approximately 4.3 minutes. This peak was emitted after the cation peak, but before the As(III) peak. As none of the mushrooms containing the unknown compound had any cation species, it is unlikely that this compound is a second cation peak. Therefore, it is likely an unidentified anionic species, such as DMA(III) or a thiolated arsenic compound. The retention time for DMA(III) is before that of As(III) (Appendix B, Figure B-1). As with the unknown compound from the Section 4, more research is needed to determine the identity of the compound. This could be done by analyzing the sample in conjunction with other standards or using another complementary analysis method (such as electrospray mass spectrometry ESI-MS as suggested in Nearing et al. (2014b)) to obtain more information on the compound structure).

Table 4-4: Major arsenic compounds (as proportions) in raw, fried, and barbequed (BBQ) mushroom samples from Odessa, ON.

Sample origin	Mushroom (common name)	Sample description	Proportion of sum of species (%)							Major compound	Total As (mg/kg, dw)*	Column recovery (%)
			iAs	DMA	MMA	AB	TMAO	AC	TETRA			
Odessa, ON	Saffron Milk Cap	Raw	18	11	21	51	0	0	0	AB	0.22 ± 0.03	37
		Fried	57	trace	19	24	0	0	trace	iAs	0.081 ± 0.002	66
		BBQ	44	trace	13	28	0	0	16	iAs	0.12 ± 0.02	70
	Rough-Stemmed Bolete	Raw	28	72	0	0	0	0	0	DMA	0.18 ± 0.01	74
		Fried	36	64	0	0	0	0	0	DMA	0.069 ± 0.005	73
		BBQ	34	54	12	0	0	0	0	DMA	0.14 ± 0.02	72
	Penny Bun	Raw	18	73	4.4	0	5.2	0	0	DMA	0.34 ± 0.04	91
		Fried	27	73	trace	0	0	0	0	DMA	0.15 ± 0.03	82
		BBQ	26	66	8.3	0	0	0	0	DMA	0.21 ± 0.02	85
	Puffball	Raw	22	6.9	0	71	0	0	0	AB	0.83 ± 0.08	95
		Fried	23	5.5	0	63	0	0	7.6	AB	0.48 ± 0.03	92
		BBQ	19	5.9	0	58	0	0	17	AB	0.84 ± 0.09	87

*As discussed in Section 3.4.2., the total arsenic concentration correlates to the value obtained from the total digest of the sample. The uncertainty is the difference between the total arsenic concentrations for the duplicate samples.

Table 4-5: Major arsenic compounds (as proportions) in raw, fried, and baked fresh and canned mushroom samples from a local grocery store in Kingston, ON.

Sample origin	Mushroom (common name)	Sample description	Proportion of sum of species (%)							Major compound	Total As (mg/kg, dw)*	Column recovery (%)
			iAs	DMA	MMA	AB	TMAO	AC	UK (anion)			
Grocery store, fresh mushrooms	Portobello	Raw	69	15	15	0	0	0	0	iAs	0.053 ± 0.001	138
		Fried	48	trace	trace	0	52	0	0	TMAO	0.020 ± 0.004	122
		Baked	100	0	trace	0	0	0	0	iAs	0.026 ± 0.003	115
	White Button	Raw	68	32	trace	0	0	0	0	iAs	0.064 ± 0.013	124
		Fried	73	27	0.0	0	0	0	0	iAs	0.030 ± 0.005	97
		Baked	67	33	0.0	0	0	0	0	iAs	0.036 ± 0.005	84
	Oyster	Raw	50	trace	13	0	0	0	37	iAs	0.099 ± 0.021	137
		Fried	48	0	29	0	0	0	23	iAs	0.062 ± 0.016	116
		Baked	36	trace	trace	0	0	0	64	iAs	0.095 ± 0.001	104
	Shiitake	Raw	100	trace	trace	0	0	0	0	iAs	0.039 ± 0.001	130
		Fried	100	0	0	0	0	0	trace	iAs	0.028 ± 0.006	103
		Baked	49	trace	trace	0	0	0	51	iAs	0.041 ± 0.016	122
	Enoki	Raw	36	8.2	9.6	47	0	0	0	AB	0.12 ± 0.02	131
		Fried	77	10	13	0	0	0	0	iAs	0.056 ± 0.011	118
		Baked	67	14	19	0	0	0	0	iAs	0.054 ± 0.002	118
	Crimini	Raw	59	41	0	0	0	0	0	iAs	0.026 ± 0.001	100
		Fried	58	31	11	0	0	0	0	iAs	0.041 ± 0.008	106
		Baked	trace	36	0	0	64	0	0	TMAO	0.034 ± 0.005	101
Grocery store, canned	Canned White Button (Sliced)	Raw	trace	28	0	72	0	0	0	AB	0.804 ± 0.004	97
		Fried	trace	26	0	74	0	0	0	AB	0.47 ± 0.06	113
		Baked	trace	31	0	69	0	0	0	AB	0.60 ± 0.02	98
	Canned White Button (Whole)	Raw	20	39	0	41	0	0	0	AB	1.1 ± 0.1	118
		Fried	17	33	0	50	0	0	0	AB	0.82 ± 0.01	135
		Baked	15	46	0	39	0	0	0	DMA	1.05 ± 0.02	106

*As discussed in Section 3.4.2., the total arsenic concentration correlates to the value obtained from the total digest of the sample. The uncertainty is the difference between the total arsenic concentrations for the duplicate samples.

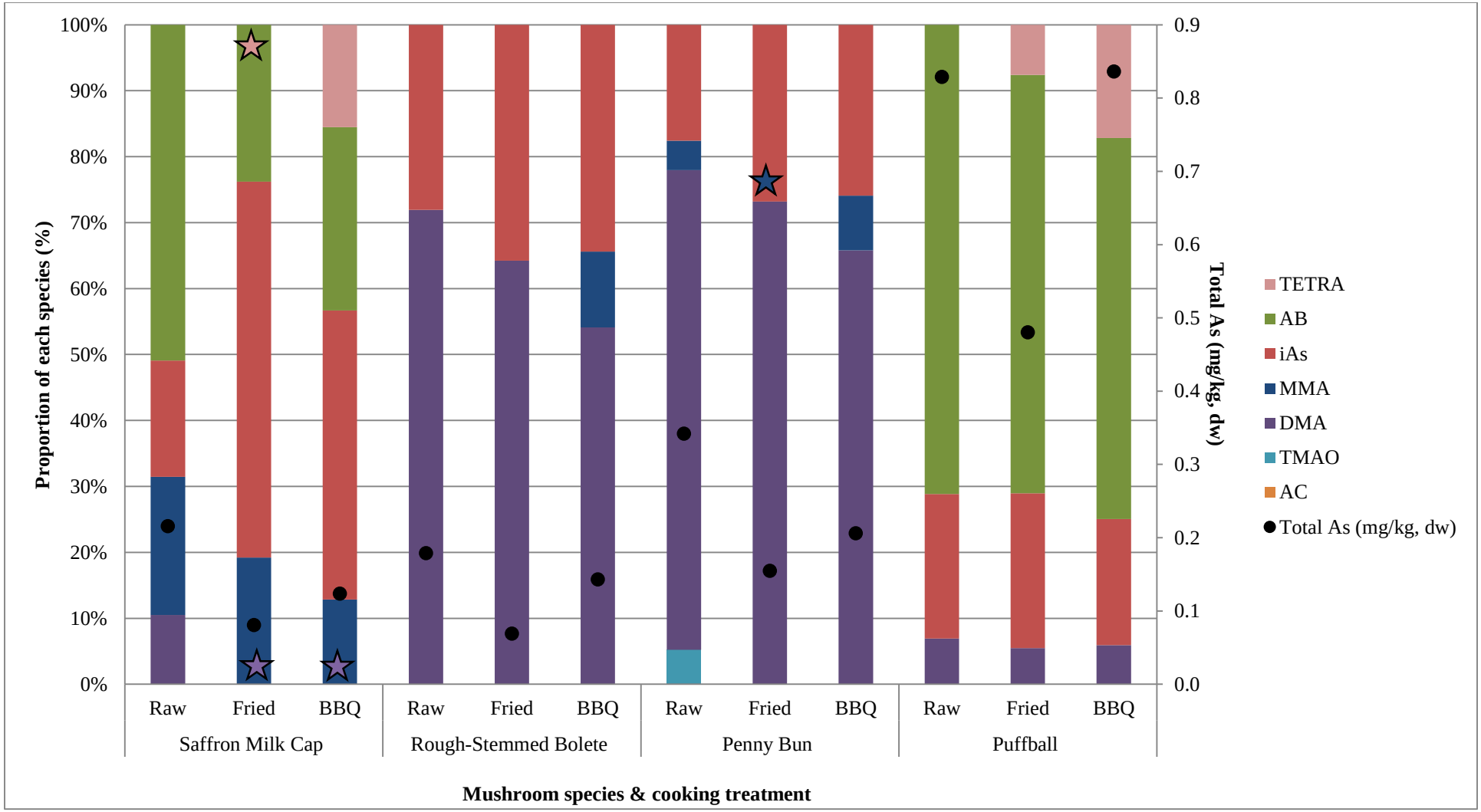

Figure 4-9: Average proportion of arsenic compounds (left axis) and total arsenic (right axis and black circles) in raw, fried, and barbequed (BBQ) mushrooms from Odessa, ON. Stars indicate that trace amounts of the corresponding species (by colour) were identified in the sample.

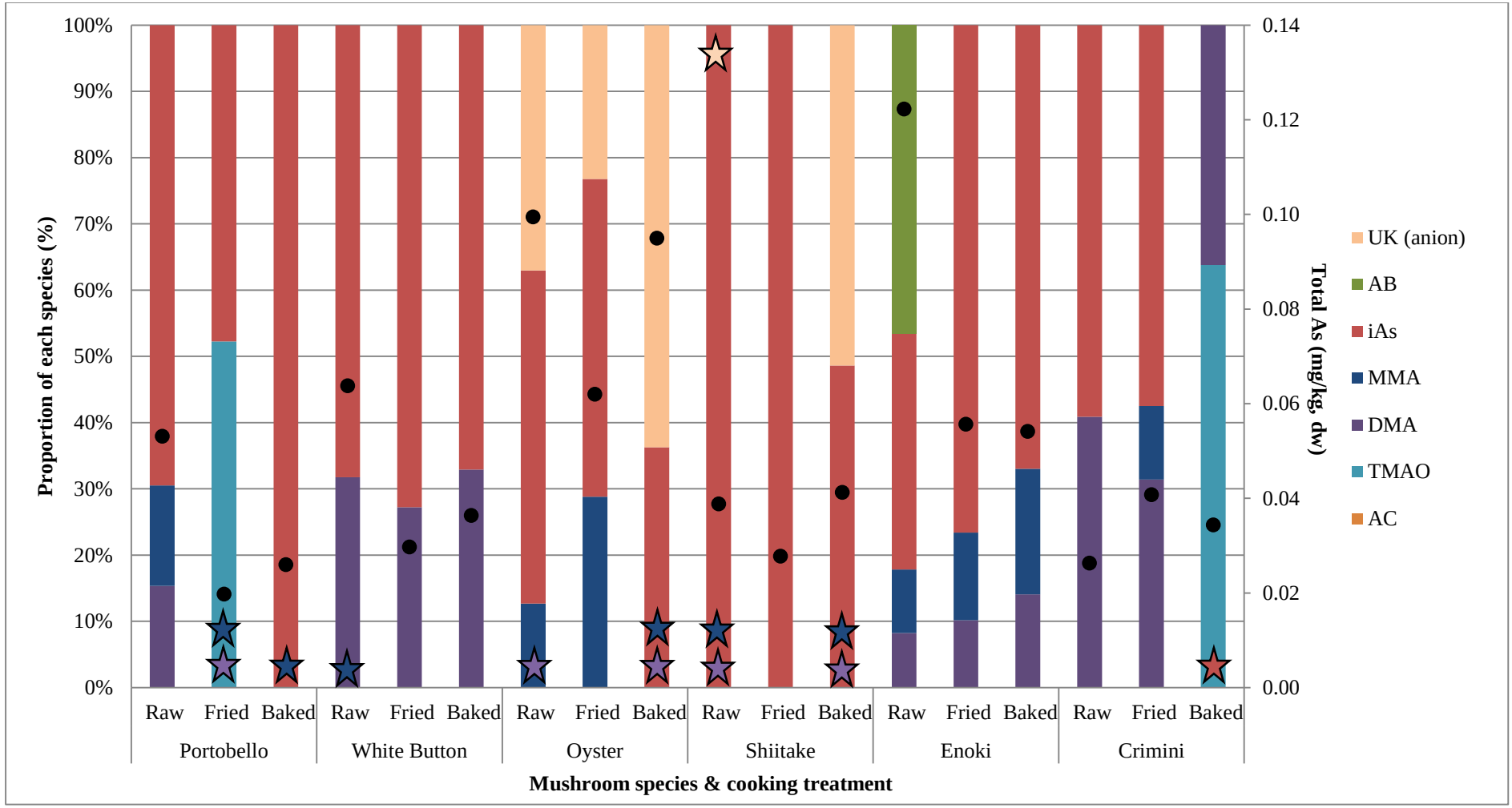

Figure 4-10: Average proportion of arsenic compounds (left axis) and total arsenic (right axis and black circles) in raw, fried, and baked fresh mushrooms from Loblaw's grocery store in Kingston, ON. Stars indicate that trace amounts of the corresponding species (by colour) were identified in the sample.

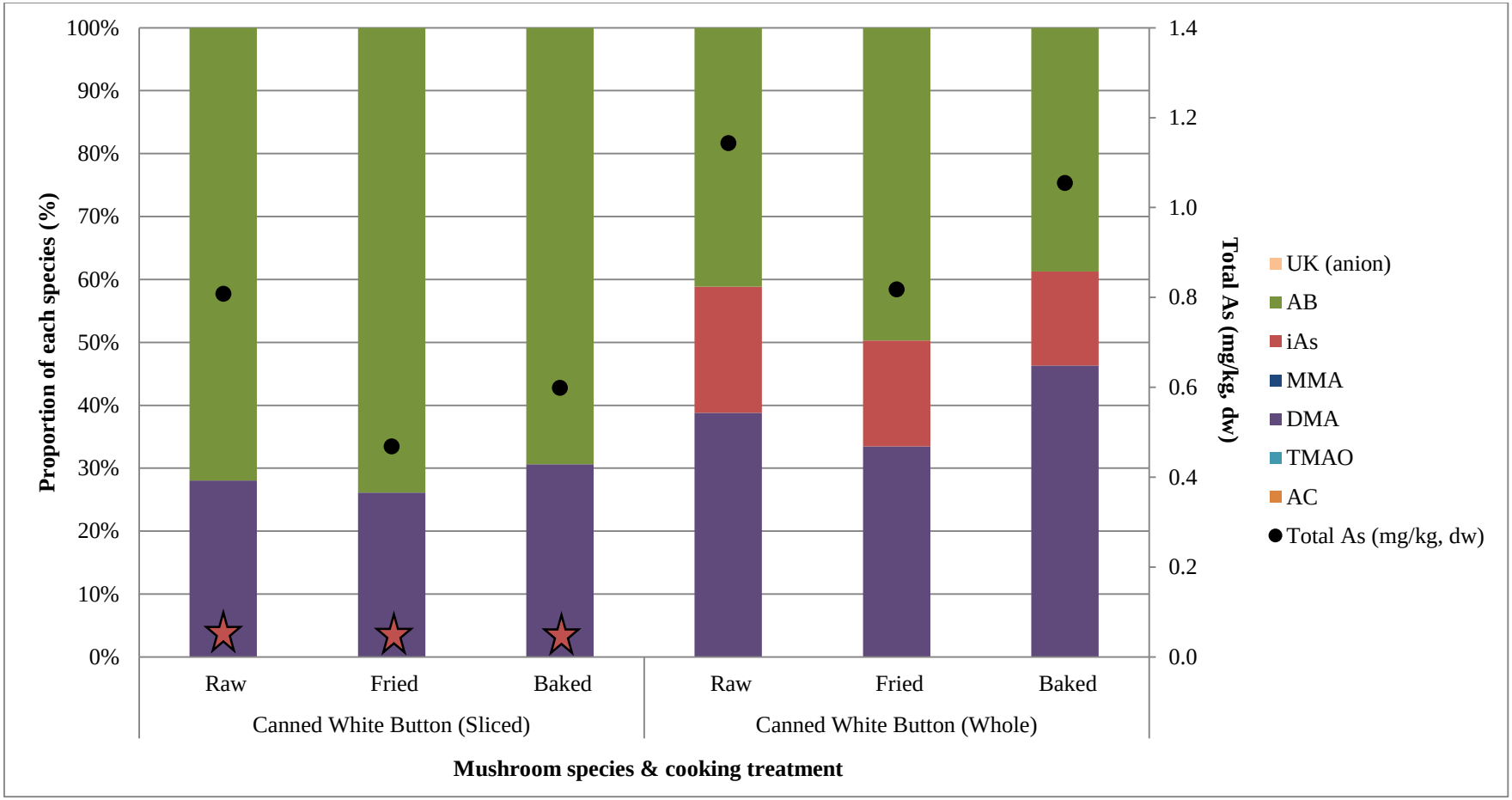

Figure 4-11: Average proportion of arsenic compounds (left axis) and total arsenic (right axis and black circles) in raw, fried, and baked canned mushrooms from Loblaw's grocery store in Kingston, ON. Stars indicate that trace amounts of the corresponding species (by colour) were identified in the sample.

4.2.1. Total Arsenic

The total arsenic concentrations in the raw mushroom samples are summarized and compared to literature values in Table 4-6. The total arsenic concentrations in the fresh, raw mushroom samples range from 0.026 mg/kg, dw (for the crimini mushroom (brown strain of *A. bisporus*)) to 0.83 mg/kg, dw (for the giant puffball (*C. gigantea*)).

Table 4-6: A comparison of the total concentration from raw mushroom samples collected from Odessa, ON and purchased from a local grocery store in Kingston, ON to literature values for the same mushroom species (unless otherwise identified).

Sample Origin	Common Name	Species	Total As (mg/kg, dw) Raw Sample	Total As (mg/kg, dw) Literature Value	Source, notes
Odessa, ON	Saffron Milk Cap	*Lactarius deliciosus*	0.22	0.253	[a], ON Background
	Rough-Stemmed Bolete	*Leccinum scabrum**	0.18	0.12*	[a], Store bought, *Boletus sp*.*
	Penny Bun	*Boletus edulis*	0.34	0.12	[a], Store bought
	Puffball	*Calvatia gigantea*	0.83	0.27-1.3, (average: 0.82)	[a], range and average of 4 ON Background samples
Grocery store, fresh mushrooms	Portobello	*Agaricus bisporus* (brown)	0.053	0.090	[a], Store bought
	White Button	*Agaricus bisporus*	0.064	0.102	[a], Store bought
	Oyster	*Pleurotus ostreatus*	0.10	0.048	[a], Store bought
	Shiitake	*Lentinula edodes*	0.039	0.23	[a], Store bought
	Enoki	*Flammulina velutipes*	0.12	0.04	[a], Store bought
	Crimini	*Agaricus bisporus* (brown)	0.026	0.020	[a], Store bought
Grocery store, canned	White Button (Sliced)	*Agaricus bisporus*	0.81	0.043	[b], canned button mushrooms
	White Button (Whole)	*Agaricus bisporus*	1.1	0.043	[b], canned button mushrooms

[a](Nearing et al., 2014a)
[b](Zbinden et al., 2000)
*Comparison of the rough-stemmed bolete mushroom (*Leccinum scabrum*), which was formerly classed as *Boletus scaber*, is made to *Boletus edulis*. This was done in the absence of a literature value for an ON background or store bought *L. scabrum* mushroom, as *Boletus edulis* was the closest species with available data.

Overall, the total arsenic values in the fresh samples collected for this experiment compare well to values reported by Nearing et al. (2014a), especially considering that they were not from the same location and were collected several years apart. The canned mushrooms however, have the greatest difference between reported values from this experiment and the literature values reported by Zbinden et al. (2000). The total arsenic concentrations from the canned white button mushrooms from this analysis (0.81 mg/kg, dw for pre-sliced and 1.1 mg/kg, dw for whole mushrooms) are almost twenty times higher than the literature value for the same species of canned mushroom (0.043 mg/kg, dw).

Cooking treatments consistently lowered the total arsenic concentration. Frying, barbequing and baking resulted in a decrease in total arsenic for almost all samples. The only exception was the crimini mushroom, for which a very slight increase in the total arsenic concentration was observed for both the fried and baked sample. Overall, the largest changes in the total arsenic concentrations were seen after frying the mushroom. Only two of the 12 samples did not follow this trend. The enoki mushroom had relatively similar arsenic concentration for the fried and baked sample (both much lower than the total arsenic in the raw sample), and the crimini mushroom, as mentioned previously, showed an increase in total arsenic after frying. This general trend does not coincide with results from Ling et al. (2014), who found that frying resulted in the greatest increase in the total arsenic concentration of cooked seafood (in comparison to grilling and baking). The loss in the total arsenic that occurred in the majority of the samples was most likely attributable to dissolution of arsenic into the water phase of the mushroom sample and removal of this water (and the arsenic in it) through aerosol formation during the high energy frying process, through dripping during barbequing and drying onto the foil during baking. It is possible that the total arsenic concentrations in the barbequed samples were less affected than the fried samples because there is less water loss through dripping compared with the aerosol formation during frying. In future experiments, the drippings or aerosol produced during the cooking process should be collected to investigate this and establish mass balance.

4.2.2. Arsenic speciation

The predominant arsenic species in the raw mushroom samples are summarized and compared to literature values in Table 4-7. For six of the 12 mushrooms, the predominant arsenic species from this analysis corresponds to the literature values.

Table 4-7: A comparison of the predominant arsenic species from raw mushroom samples collected from Odessa, ON and purchased from Loblaw's in Kingston, ON to literature values for the same mushroom species (unless otherwise identified).

Sample Origin	Common Name	Species	Predominant Species*, Raw Sample	Predominant Species*, Literature Value	Source, Notes
Odessa, ON	Saffron Milk Cap	*Lactarius deliciosus*	AB (51%), MMA (21%), iAs (18%), DMA (11%)	AB (100%)	[a], ON Background
	Rough-Stemmed Bolete	*Leccinum scabrum***	DMA (72%), iAs (28%)	iAs (71%), DMA (12%)	[a], Store bought, *Boletus sp*.**
	Penny Bun	*Boletus edulis*	DMA (73%), iAs (18%)	iAs (71%), DMA (12%)	[a], Store bought
	Puffball	*Calvatia gigantea*	AB (71%), iAs (22%)	AB (range from 84-100%, average 93%)	[a], average of 4 ON background samples
Grocery store, fresh mushrooms	Portobello	*Agaricus bisporus* (brown)	iAs (69%), DMA (15%), MMA (15%)	AB (42%), iAs (30%), DMA (27%)	[a], Store bought
	White Button	*Agaricus bisporus*	iAs (68%), DMA (32%)	AB (40%), DMA (31%), iAs (24%)	[a], Store bought
	Oyster	*Pleurotus ostreatus*	iAs (50%), MMA (13%)	DMA (79%), AB (11%)	[a], Store bought
	Shiitake	*Lentinula edodes*	iAs (100%)	iAs (67%), DMA (33%)	[a], Store bought
	Enoki	*Flammulina velutipes*	AB (47%), iAs (36%)	iAs (86%), DMA (33%)	[a], Store bought
	Crimini	*Agaricus bisporus* (brown)	iAs (59%), DMA (41%)	iAs (35%), DMA(33%), AB(33%)	[a], Store bought
Grocery store, canned	White Button (Sliced)	*Agaricus bisporus*	AB (72%), DMA (28%)	AB (40%), DMA (31%), iAs (24%)	[a], Store bought, fresh
	White Button (Whole)	*Agaricus bisporus*	AB (41%), DMA (39%), iAs (20%)	AB (40%), DMA (31%), iAs (24%)	[a], Store bought, fresh

[a](Nearing et al., 2014a)

*Predominant compounds are those that were greater than 10% of the sum of species.

**Comparison of the rough-stemmed bolete mushroom (*Leccinum scabrum*), which was formerly classed as *Boletus scaber*, is made to *Boletus edulis*. This was done in the absence of a literature value for an ON background or store bought *L. scabrum* mushroom, as *Boletus edulis* was the closest species with available data.

The rough-stemmed bolete mushroom (*L. scabrum*) was formerly classed as *Boletus scaber*. As no literature value was available for an ON background or store bought *L. scabrum* mushroom, the ON background predominant arsenic species from the *Boletus edulis* was used, as it was the closest species available. The two mushrooms have the same predominant species, however they are in inverse proportions (DMA (72%) and iAs (28%) for this sample set and iAs (71%), DMA (12%) for sample from Nearing et al. (2014a)). The mushrooms with the same predominant species were the: saffron milk cap (*Lactarius deliciosus*), puffball (*Calvatia gigantea*), shiitake (*Lentinula edodes*), crimini (*Agaricus bisporus* (brown)) and canned (white button (sliced and whole) *Agaricus bisporus*). For all of the *Agaricus bisporus* mushrooms (both the white and brown strains), AB was only detected in the literature value. However, for the enoki (*Flammulina velutipes*) mushroom, AB was only detected in the mushroom collected for this research whereas DMA (and no AB) was detected in the sample from Nearing et al. (2014a). The mushroom which varied most drastically from the literature proportions was the oyster (*Pleurotus ostreatus*) mushroom, for which the predominant species were iAs (50%) and MMA (13%) for this sample set and DMA (79%) and AB (11%) for the sample form Nearing et al. (2014a). The reasons for differences in grocery store mushrooms are unknown – they may be similar to those in wild mushrooms (factors like different growing environments, substrate preparation, etc) – and warrant further study. For example, a comprehensive survey of mushrooms from different suppliers and at different times, may be useful.

4.2.2.1. Arsenic Speciation Changes in Raw Vs, Cooked Fresh Mushrooms

For the all of the raw mushrooms containing AB (saffron milk cap, giant puffball, and enoki mushroom), there was a decrease in the AB content after both cooking treatments (Table 4-4, Table 4-5, Figure 4-9, Figure 4-10, and Figure 4-11). For the saffron milk cap and giant puffball, there was also a decrease in the amount of DMA and the formation of TETRA was observed. For both mushrooms, TETRA was generated to a greater extent for the barbequed sample. The degradation of AB to TETRA is similar to results from Hanaoka et al. (2001a) who observed the transformation of AB to TETRA in the roasted muscle of marine animals. Devesa et al. (2005) also reported an increase or appearance of TETRA in cooked megrim, anchovy, Atlantic horse mackerel, and sardines, and an increase in DMA in cooked sardines and bivalves. In the enoki mushroom, although AB was observed in the raw sample and not in the fried or baked sample, the formation of TETRA was not observed. Instead, the proportion of iAs increased and the proportions of DMA and MMA remained relatively the same. This suggests that in this case, AB was transformed to iAs and demethylation occurred.

For mushrooms that did not contain AB in the raw sample, changes in arsenic speciation as a result of cooking treatments varied greatly and trends were difficult to identify. In the rough-stemmed bolete mushroom, iAs and DMA were present in the raw and fried sample, but MMA was additionally detected in the barbequed sample. For the white button mushroom, the trace amount of MMA in the raw sample was not detected in the fried or baked sample. For the penny bun mushroom, the small proportion of TMAO found in the raw sample was not observed in the fried or barbequed samples; instead, the proportions of iAs increased.

For the crimini mushroom, only iAs and DMA were present in the raw sample. In addition to these compounds, MMA was found in the fried sample and TMAO was found in the baked sample. Similarly, for the portobello mushroom, iAs, DMA and MMA were detected in the raw sample, but the formation of TMAO was observed in the fried sample. In the baked sample, all compounds had been broken down into iAs. The unknown compound was detected in the oyster and shiitake mushroom. For the oyster mushroom, the only change in speciation was an increase in the proportion of the unknown compound in the baked sample. For the shiitake mushroom, the raw sample contained predominantly iAs, with trace amounts of DMA and MMA. The unknown compound was found in both the fried and baked sample.

The formation of TMAO in the fried Portobello mushroom and the baked crimini mushroom, along with the formation of the unknown compound (hypothesized to be DMA(III) or a thiolated compound) in the baked shiitake mushroom and an increase in the proportion of the unknown in the baked oyster mushroom, indicate that methylation may be taking place during the cooking process. In research by McSheehy et al. (2005), As(III) (5 mg/L) was transformed into AC, TMA, and TMAO using UV irradiation in a solution containing 1.4 M (20% v/v) acetic acid. This suggests that in high energy situations, such as cooking or under UV light, the methylation of arsenic species may occur. This is contrary to the demethylation that was observed for the enoki mushroom. Therefore, the speciation changes that occur during cooking may depend on several factors (such as the mushroom species matrix, arsenic species present in the raw sample, pH, temperature, and water content).

In order to get a semi-quantitative idea of the changes in toxicity, each of the species was given a different toxicity weighting: iAs = 1, methylated species and AC = 0.1, and AB = 0. These toxicity weights are assigned on the basis of the order of magnitude for the LD_{50} values (Chapter 2, Table 2-2). Organoarsenicals have toxicities that are an order of magnitude lower than those for iAs compounds, and AB is the only non-toxic compound. The unknown anion compound was given a toxicity weight of 1 in order remain protective of human health. It is possible that

the unknown compound is DMA(III) (which is nearly as toxic as iAs) and until this can be rejected, the toxicity weight should remain consistent with that of iAs. The proportion of each arsenic species was multiplied by its toxicity weighting, and the sum all of these values was used to compare the changes in toxicity based on the changes in speciation (Equation 2, Figure 4-12). To get a better idea of the actual change in toxicity, these values were then multiplied by the total arsenic concentration in that sample (Equation 3, Figure 4-13). Compounds that had trace amounts of an arsenic species were not included (i.e. their concentration was reduced to zero) as their proportions could not be accurately quantified.

Equation 2:

$$Speciation\ toxicity\ score, sample\ i = \sum Proportion\ of\ species_x \times toxicity\ weight_x$$

Where *i* = mushroom sample (raw, baked, BBQed or fried)
x = arsenic species (iAs, DMA, MMA, AC, AB, or UK)

Equation 3:

$$Toxicity\ score = Speciation\ toxicity\ score \times total\ arsenic\ concentration\ (\frac{mg}{kg}, dw)$$

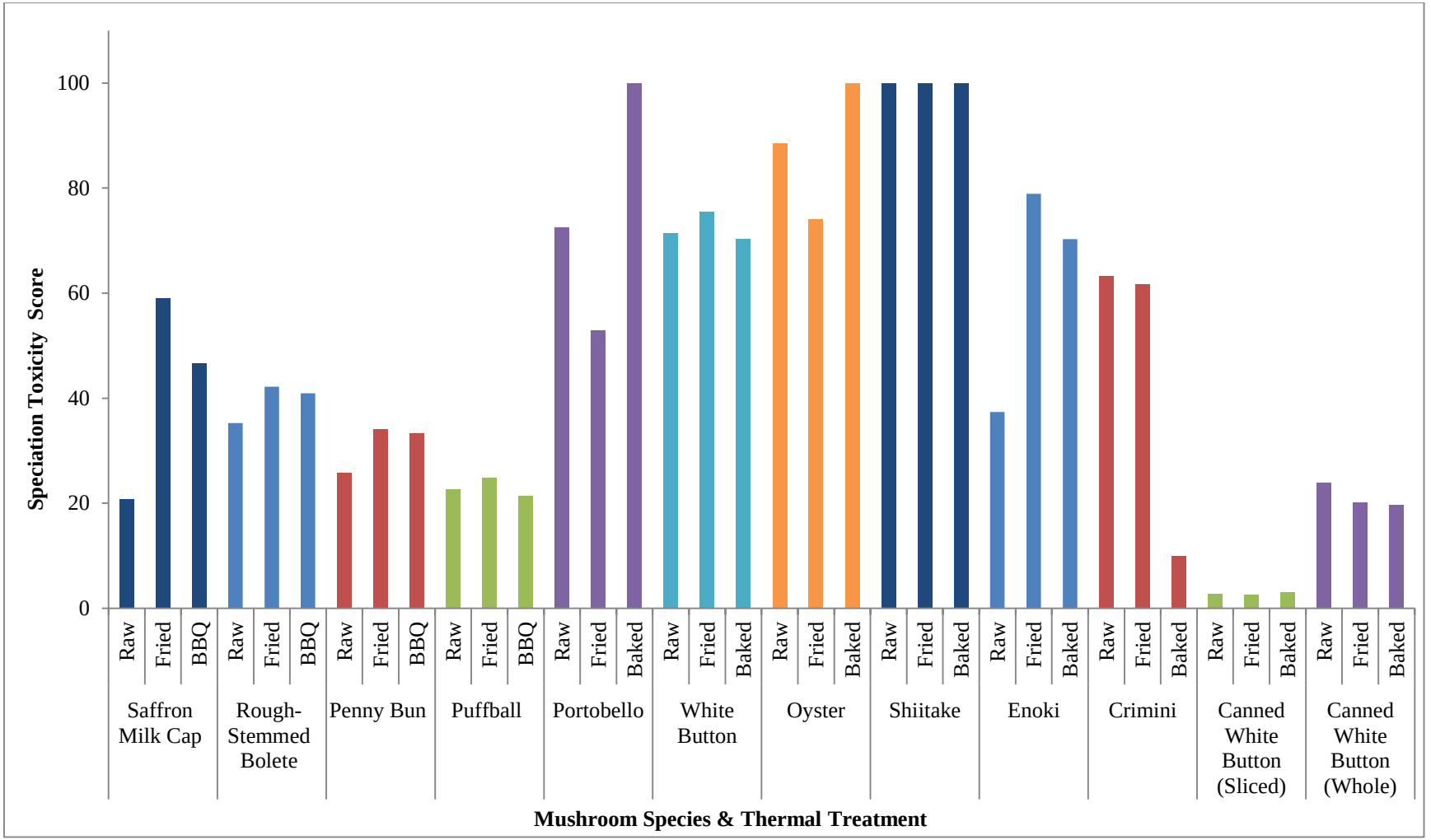

Figure 4-12: A semi-quantitative comparison of the toxicity of each mushroom based on the sum of all of the species present in the mushroom multiplied by their respective toxicity weights. This value is classified as the speciation toxicity score.

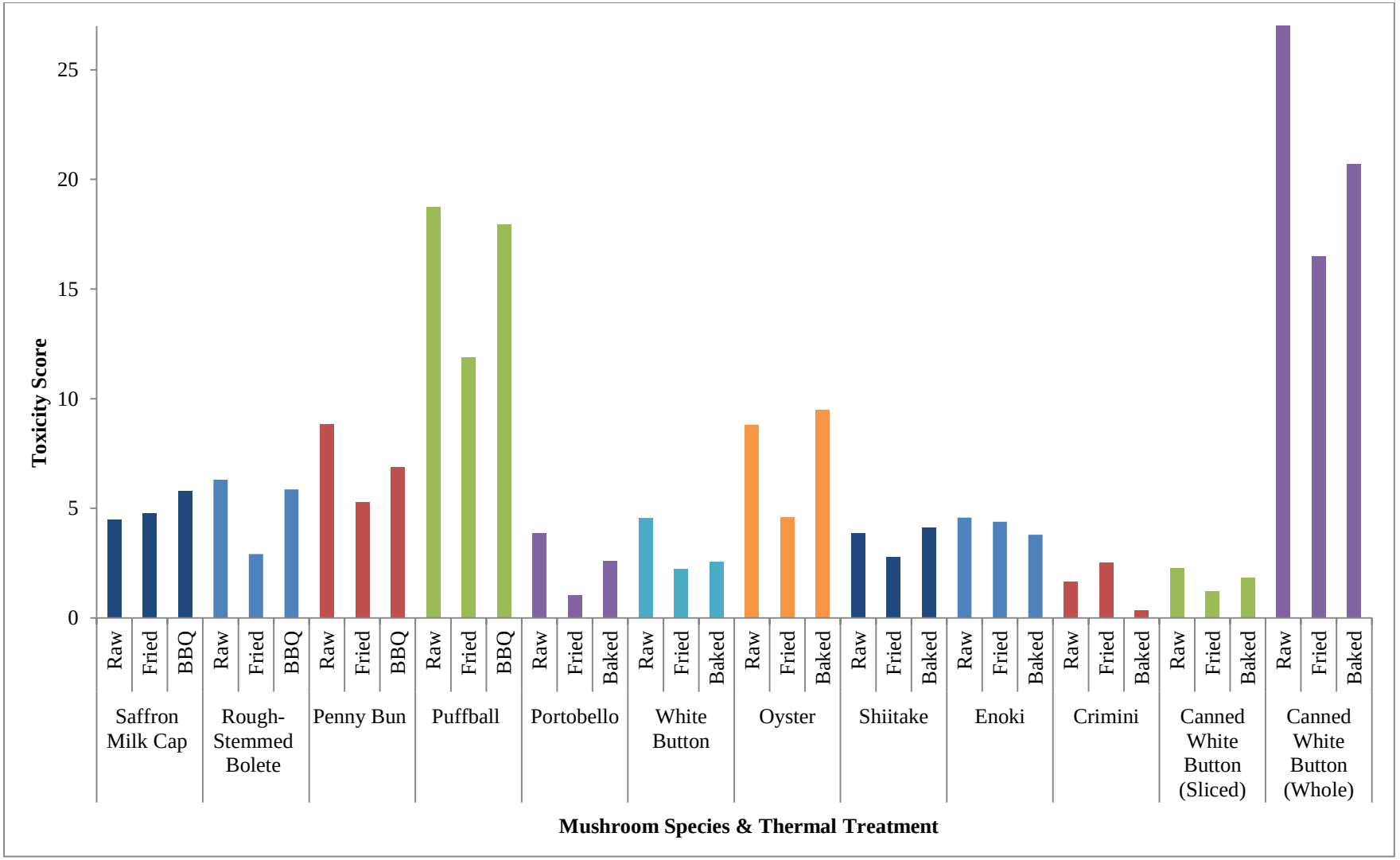

Figure 4-13: A semi-quantitative comparison of the overall toxicity of each mushroom based on the speciation toxicity score multiplied by the total arsenic concentration.

The speciation toxicity scores (Equation 1, Figure 4-12) indicate that for five of the 10 fresh mushroom species (saffron milk cap, rough-stemmed bolete, penny bun, portobello, and enoki), both of the cooked samples have a higher toxicity than the raw sample. The only exception to this is the portobello mushroom, for which only the baked sample was more toxic than the raw sample (the fried sample was less toxic). For the other mushroom species, the puffball, white button, shiitake, and canned mushroom samples showed very little changes in the toxicity between raw and cooked samples. For the crimini mushroom, the raw and fried samples had very similar toxicities and the baked sample was less toxic.

The overall toxicity scores (Equation 2, Figure 4-13) indicate a different set of trends. For almost all of the mushrooms (rough-stemmed bolete, penny bun, puffball, portobello, white button, and both canned mushrooms) the raw mushroom had the highest toxicity. This is attributable to the fact that the raw samples consistently had higher total arsenic concentrations than the cooked samples. The exceptions to this trend were the saffron milk cap, enoki, oyster, shiitake, and crimini mushrooms. For the saffron milk cap and enoki mushrooms, the toxicities were relatively similar for all of the samples. For the oyster and shiitake mushrooms, the baked sample was slightly higher than the raw sample. For the crimini mushroom, the fried sample had the highest toxicity.

Overall, these results indicate two main trends, 1) when AB is present in the raw sample, it is likely to decrease in proportion after cooking, and 2) thermal treatments can affect the arsenic speciation in many ways, usually resulting in an increase in the proportion of more toxic species, but lower total arsenic concentration (i.e. a lower overall toxicity score). In both trends, the species of arsenic in the cooked form are more toxic than the raw form. Considering that food is the main source of arsenic intake for humans (when there are no natural or anthropogenic sources of contamination), preparation methods are important when determining the risk associated with consuming arsenic-containing foods. However, the increase in toxic species is accompanied by a decrease in the total arsenic content, therefore reducing the risk associated with consuming these foods.

4.2.2.2. Arsenic speciation changes in raw vs. cooked canned mushrooms

Although the canned mushrooms had the highest total arsenic concentrations, they also had large proportions of AB. However, unlike the fresh mushrooms, there were no changes in the arsenic speciation after either cooking treatment. When the overall toxicity scores were compared, the canned white button mushroom (whole) had the highest toxicity. This is attributable to the larger proportion of iAs and high total arsenic concentration found in this sample. In terms of speciation, it was hypothesized that during the canning process it is possible for speciation changes to occur from the high temperatures experienced during sterilization. It is clear that

this does not happen, and that the arsenic species in canned mushrooms are much less susceptible to changes in arsenic speciation from cooking treatments than fresh mushrooms. It is possible that the processing of the mushrooms prior to the canning process may remove the less stable forms of arsenic. Therefore, it is important to look at both the speciation and total arsenic when consuming canned mushrooms. Both of the canned mushrooms had relatively similar total arsenic concentrations, yet only the canned variety with proportions of iAs (20% in raw, 17% in fried, and 15% in baked) had sizeable toxicity scores.

Chapter 5 – Synthetic Results/Discussion

As discussed in Chapter 2, the toxicity of arsenic is highly dependent on its chemical form, with only one species (AB) being non-toxic. Although several studies have investigated the presence of AB in a variety of mushroom species, the exact formation pathway has yet to be determined. Nearing et al. (2015b) found that the mycelium (the vegetative life stage) of mushrooms did not appear to biosynthesize AB, but adventitiously accumulate it over other arsenic species and we hypothesize that the AB can then be transported to the fruiting body of the mushroom. When investigating the growth and reproductive life stages of mushrooms, Nearing et al. (2015a) found that AB was exclusively detected in the fruiting body stage of mushroom growth. An increase in microbial diversity was detected at the time of fruiting body formation, but when the bacteria were cultivated and exposed to a variety of arsenic species, no transformations were observed. Therefore, although several different potential biosynthetic pathways for AB have been hypothesized, it still remains unclear which organism(s) or chemical pathways are responsible for AB formation. The following synthetic experiments were conducted to test possible chemical, rather than biological, formation pathways for AB.

5.1. Trimethylarsine oxide (TMAO) and Iodoacetic Acid (IoAA) Reaction

As stated in section 3.8.2, the conditions of this experiment were designed to mimic those outlined in Nakamura et al. (2008), in order to determine the reproducibility of their experiment (Figure 5-1). The experiments were carried out using TMAO with iodoacetic acid to attempt to produce AB. Chromatograms of the undiluted samples (after the reaction) are shown in Figure 5-2 and 100x dilution of the sample (spiked with standard solutions) is shown in Figure 5-3.

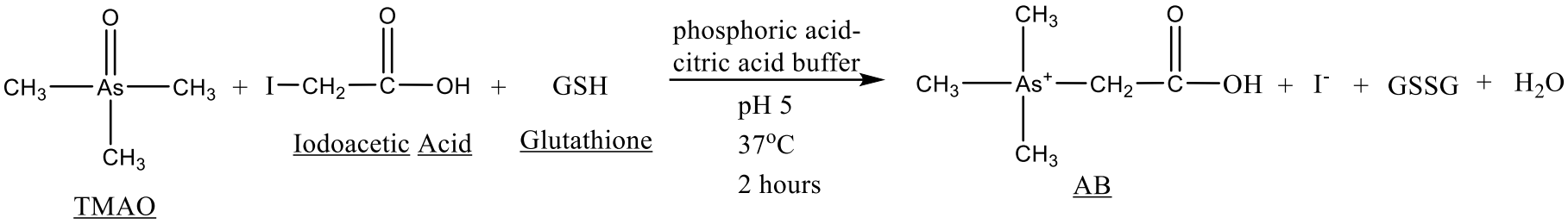

Figure 5-1: A schematic representation of the reaction of trimethylarsine oxide (TMAO), glutathione (GSH), and iodoacetic acid (IoAA) to form arsenobetaine (AB) as presented in Nakamura et al. (2008). The reaction was carried out in a 100 mM phosphoric acid-citric acid buffer that was prepared with DDW to maintain a pH of 5. The solution was heated at 37°C for 2 hours.

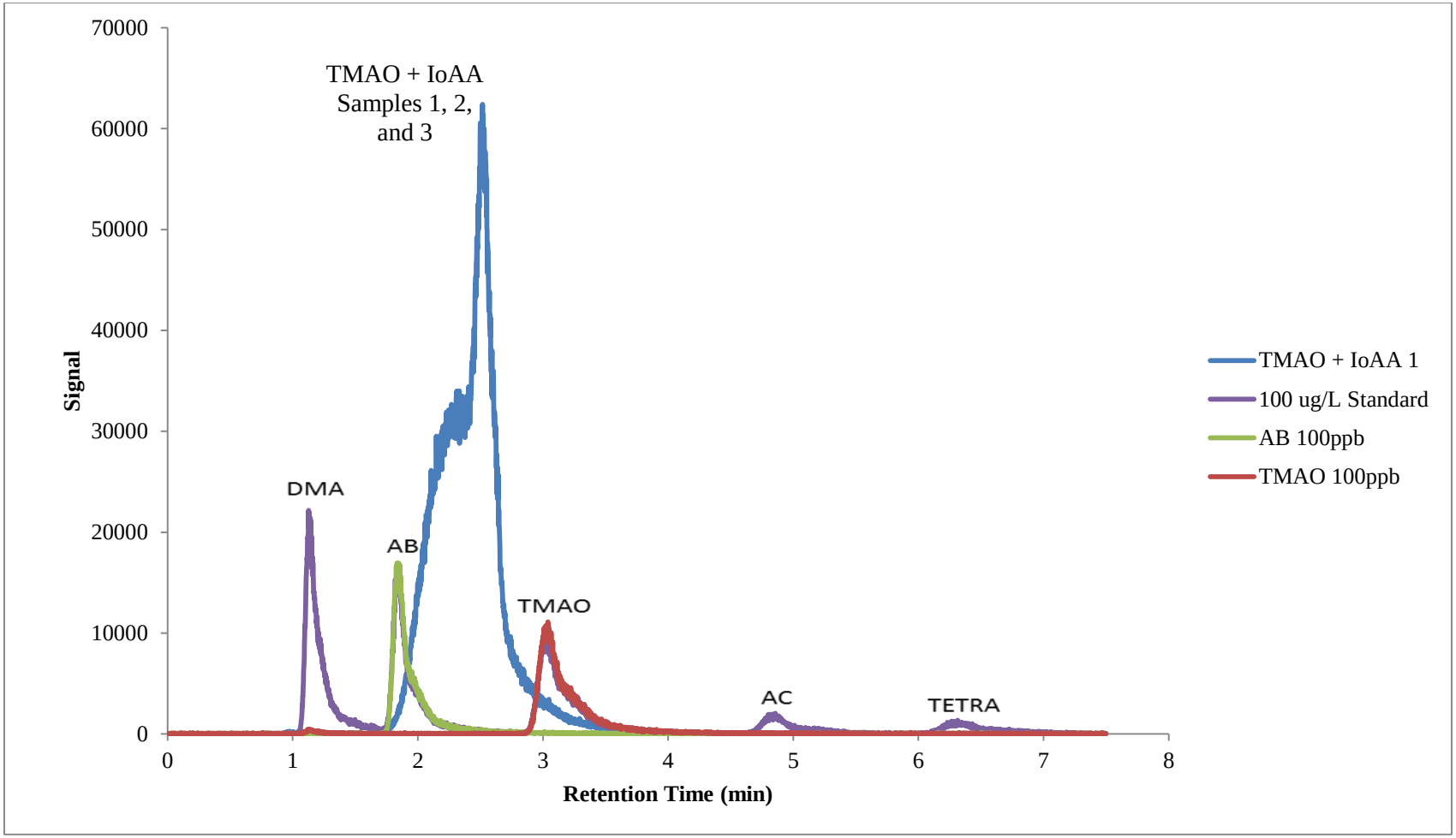

Figure 5-2: HPLC chromatograms of one of the undiluted TMAO + IoAA samples in comparison with 100 µg/L standard solution (purple), a 100 µg/L solution of AB (green), and a 100 µg/L solution of TMAO (red). The peak for the TMAO + IoAA sample was detected between the AB and TMAO peaks, and was therefore diluted 100x in order to try to differentiate the peaks.

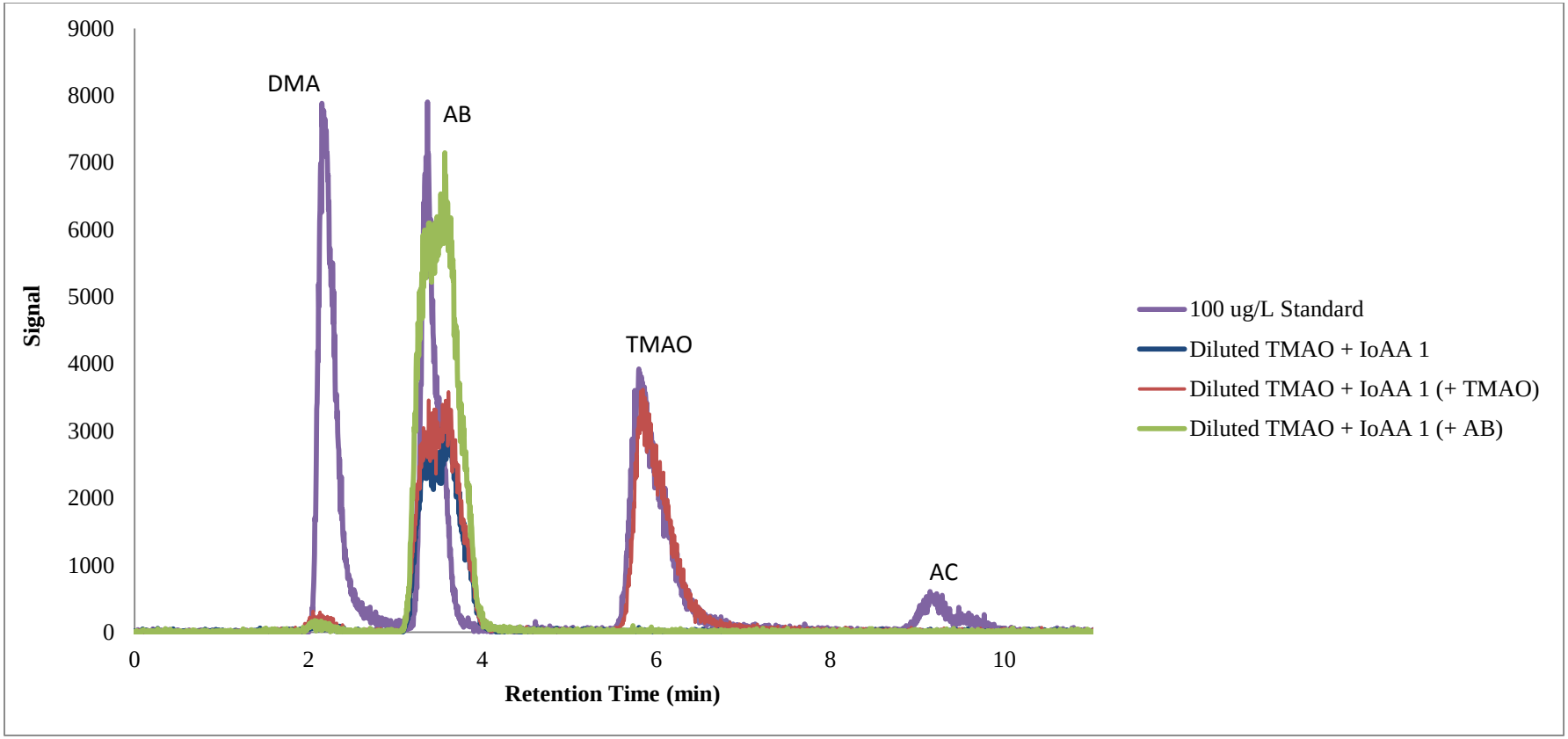

Figure 5-3: The HPLC chromatograms of the 100x diluted TMAO to AB sample (dark blue) spiked to a concentration of 100 µg/L with AB (green) and TMAO (red). A 100 µg/L standard solution (purple) is shown in the background for a comparison of standard peaks.

Figure 5-2 shows the chromatogram of the products from the reaction of TMAO, GSH, and iodoacetic acid (IoAA) mixed into the pH 5 phosphoric acid-citric acid buffer and heated at 37°C for 2 hours (and then stored in the freezer until analysis). A large peak can be seen between the standard peaks of AB and TMAO, and therefore, it was unclear if AB had actually been formed. To allow for better chromatographic separation of the peaks, one of the triplicate samples was diluted 100x. This diluted sample was analyzed independently and was also analyzed after being spiked with 1 mg/L AB and 1 mg/L TMAO solutions (so that the sample being analyzed had a final concentration of 100 µg/L). The chromatograms for these samples are shown in Figure 5-3. The un-spiked sample, shown in dark blue, was eluted at the same point (with an almost identical retention time) as the AB standard peak from the 100 µg/L standard solution. This suggests that AB was formed during the reaction. This was further confirmed by spiking the sample with AB (shown in green) and detecting only one peak. The spiking experiment showed that the peak shape was changed slightly in the matrix of the reaction mixture. The addition of TMAO to the sample showed only the TMAO at the same retention time of TMAO in a standard solution, indicating that the product peak was not TMAO.

In research by Smith et al. (2007), TMAO was detected as the major arsenic compound in As(V) amended compost when no fungus was present. This indicates that although microorganisms may not be capable of forming AB, they are capable of forming TMAO. The results from this experiment confirm the results of Nakamura et al. (2008), that TMAO is a possible precursor to AB and that if GSH and IoAA are present in soils with a pH of approximately 5, AB can be formed. As discussed in Chapter 2 (Section 2.3.1.1.), GSH is most likely the reducing agent, reducing As(V) to As(III), which is followed by an electrophilic attack of the methylene carbon of iodoacetic acid to arsenic (creating a new As-C bond and releasing I^-). GSH is likely present in the environment as it is present in nearly all living organisms and is involved in several metabolic and physiological processes (Grill, 2001). Therefore, experiments needed to be conducted to determine the presence of IoAA in soil.

5.2. Identification of iodoacetic acid (IoAA) in Soil

Three soils were tested in this pilot experiment to ascertain if iodoacetic acid is present in soils associated with mushrooms. Soil 1 was collected from Odessa, ON in 2015 near a spent puffball mushroom, Soil 2 was also collected from Odessa, ON in 2012 near a shaggy mane mushroom, and Soil 3 was collected from Deloro Woods in Deloro, ON in 2012 near a spent puffball mushroom. The chromatograms for the extraction of iodine from Soil 1 for both extractant solutions are shown in Figure 5-4 and the concentration of extracted potassium iodide and IoAA are shown in Figure 5-5. It can be seen that KI was extracted with

calcium chloride but IoAA was not, whereas the reverse was true for oxalic acid. As hypothesized in Section 3.7, oxalic acid was therefore demonstrated to be a better extraction solution for IoAA from the soil than the calcium chloride, due to the fact that IoAA is more soluble in oxalic acid.

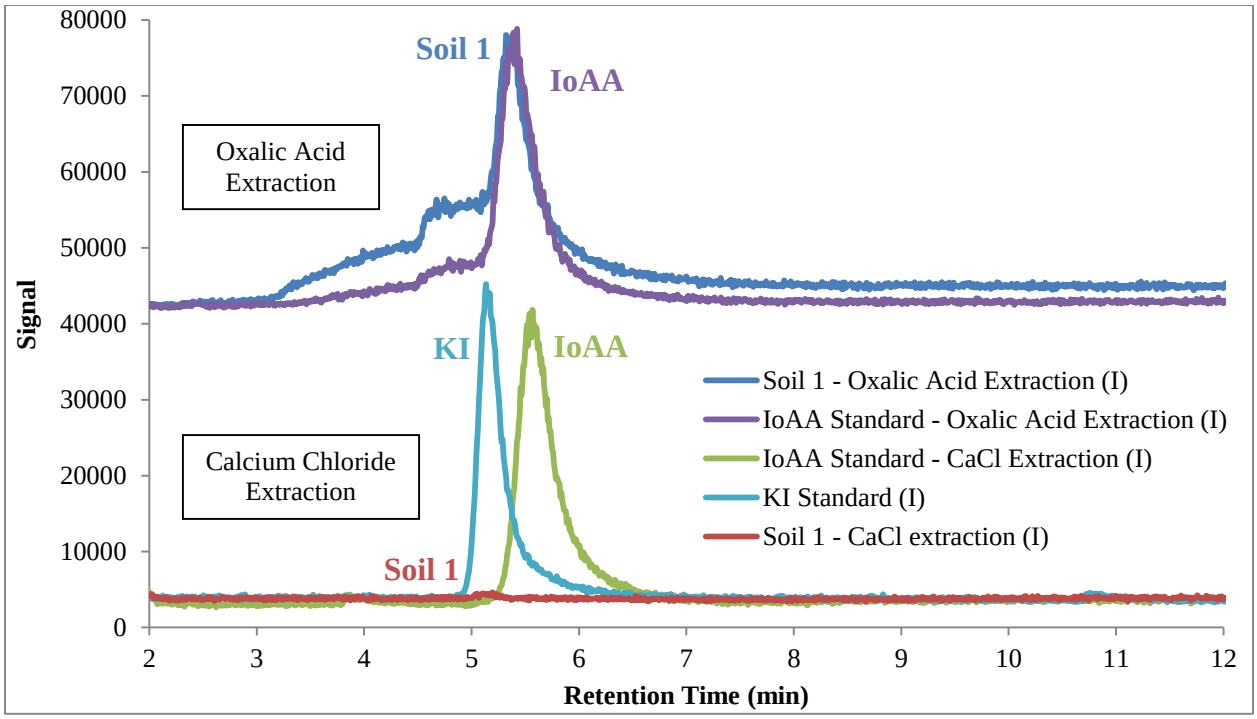

Figure 5-4: Chromatograms for Soil 1 extraction using oxalic acid (upper graph) and calcium chloride ($CaCl_2$) (lower graph). Both graphs include the iodoacetic acid standard that was incorporated into the extractant solution for analysis. The lower graph includes the potassium iodide (KI) standard, as the retention time for this peak aligns with the peak detected in the sample.

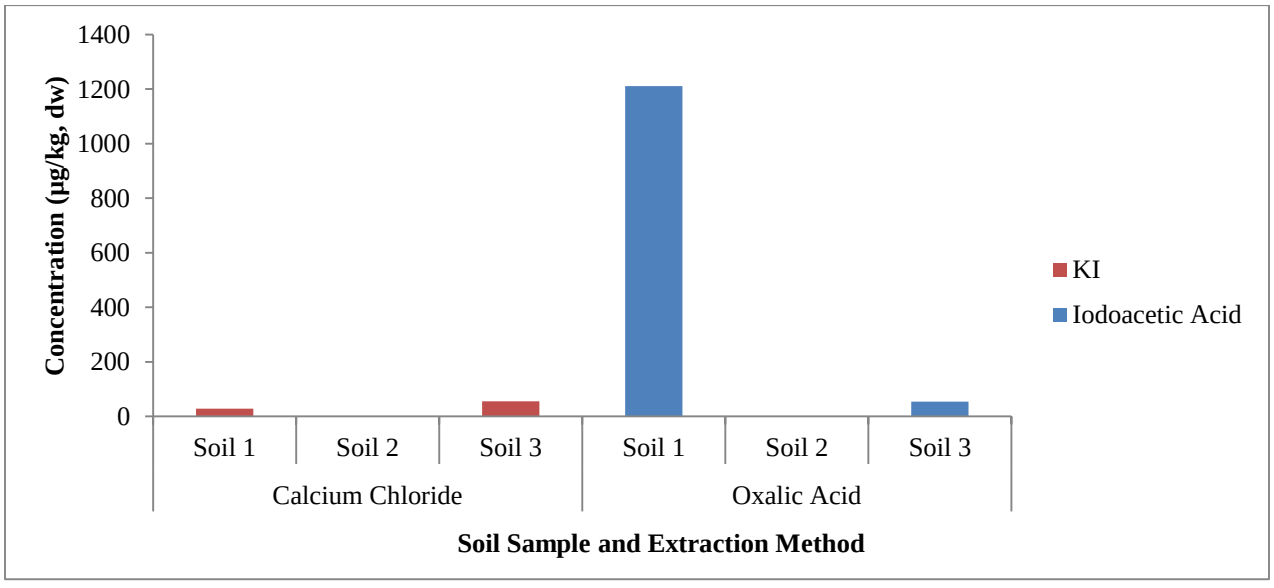

Figure 5-5: Concentrations of potassium iodide (KI, red) and iodoacetic acid (blue) in three soil samples using two extraction methods, 0.1 M calcium chloride (shown on the left) and 0.5 M oxalic acid (shown on the right).

IoAA was identified in the two soils that were collected adjacent to locations where puffball mushrooms had been growing (at the time of collection the mushrooms were considered to be 'spent'). No IoAA was found in Soil 2, collected adjacent to a shaggy mane (*Coprinus comatus*) mushroom. As stated previously in this research and in analysis done by Nearing et al. (2014a), the predominant arsenic species in puffball mushrooms is AB, whereas the predominant species in shaggy mane can vary (out of the two shaggy mane mushrooms collected from ON background sites by Nearing et al. (2014a) only one had AB as the predominant species, while the other had DMA). Soil 2 was collected adjacent to the shaggy maine that had DMA as the predominant species (100%) and only trace amounts of AB. Therefore, the lack of IoAA in the soil could be associated with the lack of AB in the shaggy mane mushroom.

Therefore, it is highly plausable that AB is being formed abiotically in the soil as a result of a transformation of TMAO with GSH and the IoAA present in the soil. Furthermore, after its formation the AB may be quickly accumulated within the fruiting body, which explains why AB is not detected in the soil. Future research is needed to determine if this reaction will continue to take place with other reducing agents (other than GSH) and at soil temperatures.

5.3. Transformation of DMA(III) to AB

It was hypothesized by Edmonds (2000) that AB could be formed via the transformation of DMA(III) to DMAA using glyoxylate and pyruvate and followed by SAM to obtain AB as the final product (Chapter 2, Section 2.3.1.2., Figure 2-16 and 2-17). This hypothesis was tested using calcium hydroxide, DMA(III)I, sodium pyruvate and glyoxylic acid as described in Section 3.8.1 to first attempt to form DMAA. SAM was then added to a portion of this solution in order to test if AB would be formed. The scheme for this reaction is shown in Figure 5-6.

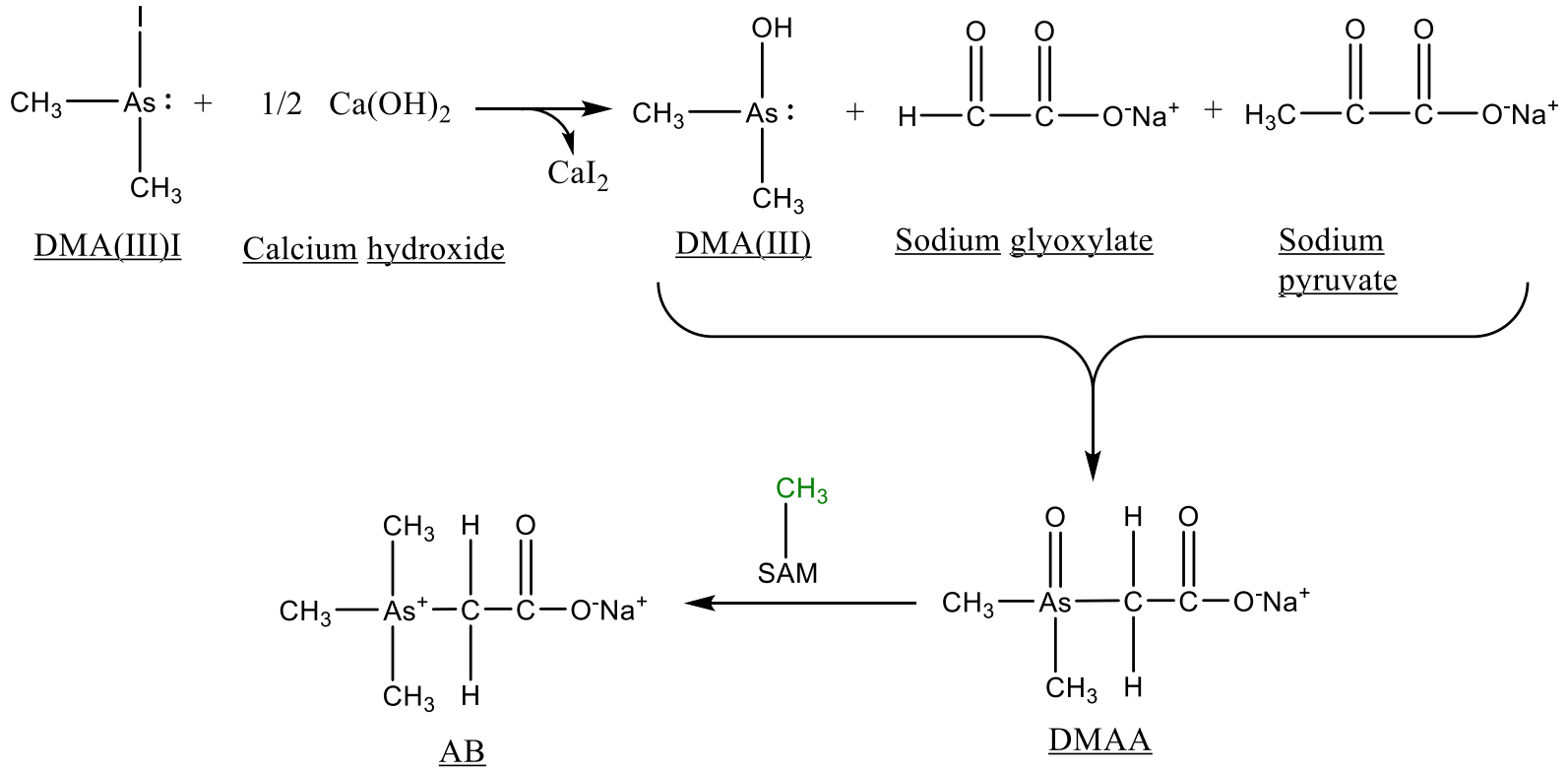

Figure 5-6: A schematic representation for the synthesis of arsenobetaine (AB) from dimethylarsinous iodide (DMA(III)I). For this reaction, DMA(III)I was combined in degassed DDW with calcium hydroxide ($Ca(OH)_2$), sodium pyruvate, and sodium glyoxylate and allowed to react for 2 hours. It was hypothesized that this would result in the formation of dimethylarsinoyl acetate (DMAA). To add an additional methyl group to DMAA, S-adenosyl methionine (SAM) was added to this solution and allowed to react for another 2 hours, ideally resulting in the formation of AB.

For this reaction, the calcium hydroxide was used to replace the I in DMA(III)I with a hydroxide group (via a metathesis reaction). From this point, it is hypothesized to follow the same mechanism of reaction previously outlined in Chapter 2 (Section 2.3.1.2., Pathway 7, Figure 2-15).

The solutions were first analyzed without any dilution and were analyzed approximately two weeks later with a 100x dilution. Although some of the arsenic species may not be stable over a two week storage time, the two compounds of greatest interest, DMAA and AB, are thought to be stable under most conditions ((Devesa et al., 2008)). Additionally, the DMA(III) appears to elute with AB and/or TMAO and oxidation of this peak (through storage for two weeks) allows a clearer visualization of the AB peak. Chromatograms of the above reactions are shown in Figure 5-7 and Figure 5-8. A comparison of the chromatograms for the solutions before and after SAM was added is shown in Figure 5-9.

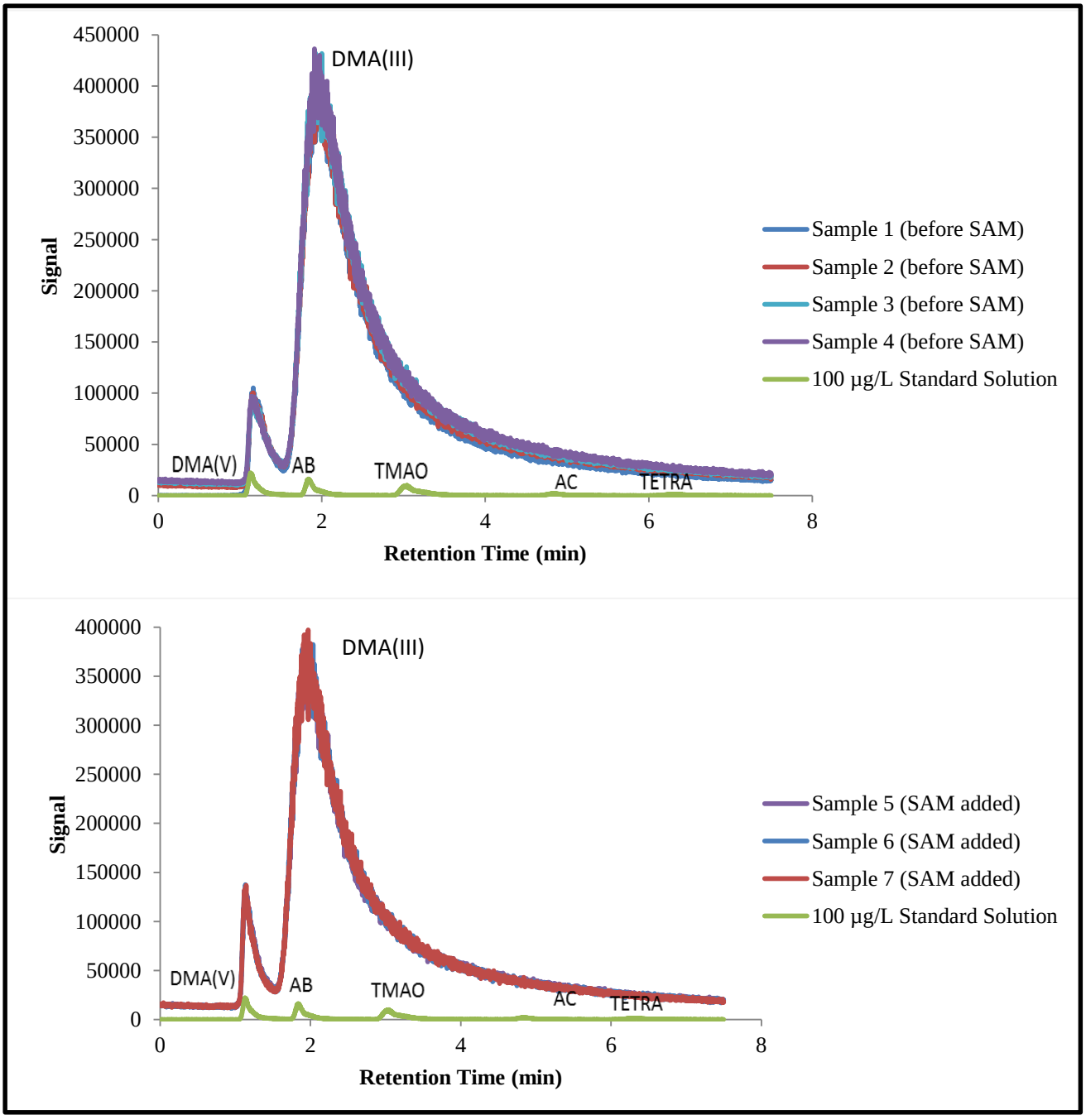

Figure 5-7: Chromatograms of the DMA(III) solution before SAM was added (in quadruplicate, samples 1-4, upper graph) and after (in triplicate, samples 5-7, lower graph). A 100 µg/L standard solution is shown (in green) for retention time comparison).

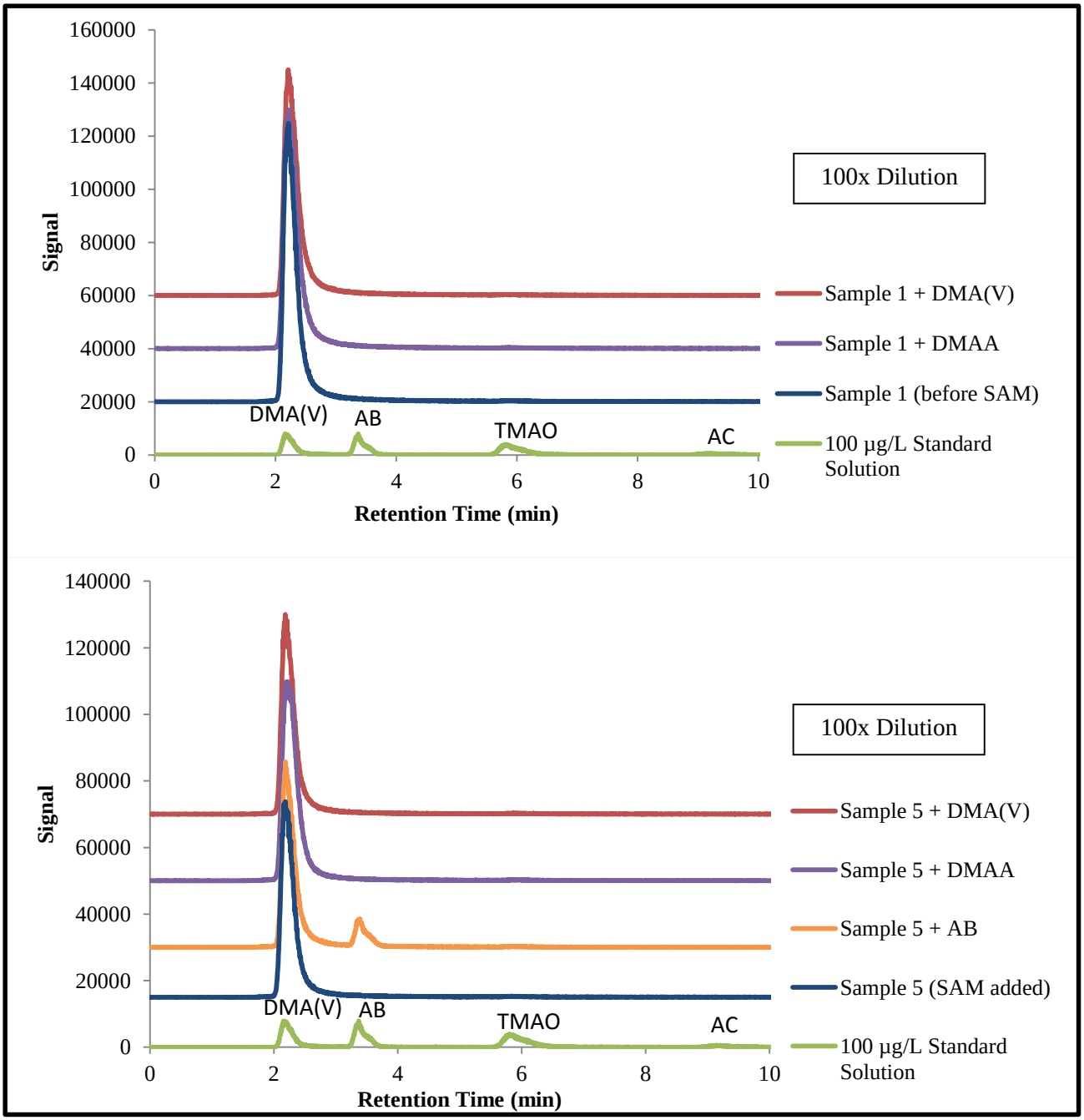

Figure 5-8: Upper graph: Chromatogram of the 100x diluted DMA(III) solution before SAM was added (Sample 1, dark blue). Sample 1 was also spiked with DMAA (purple) and DMA(V) (red). Lower graph: Chromatogram of the 100x diluted DMA(III) solution after SAM was added (Sample 5, dark blue). Sample 5 was also spiked with DMAA (purple), AB (orange) and DMA(V) (red). A 100 µg/L standard solution is shown in both graphs (in green) for retention time comparison.

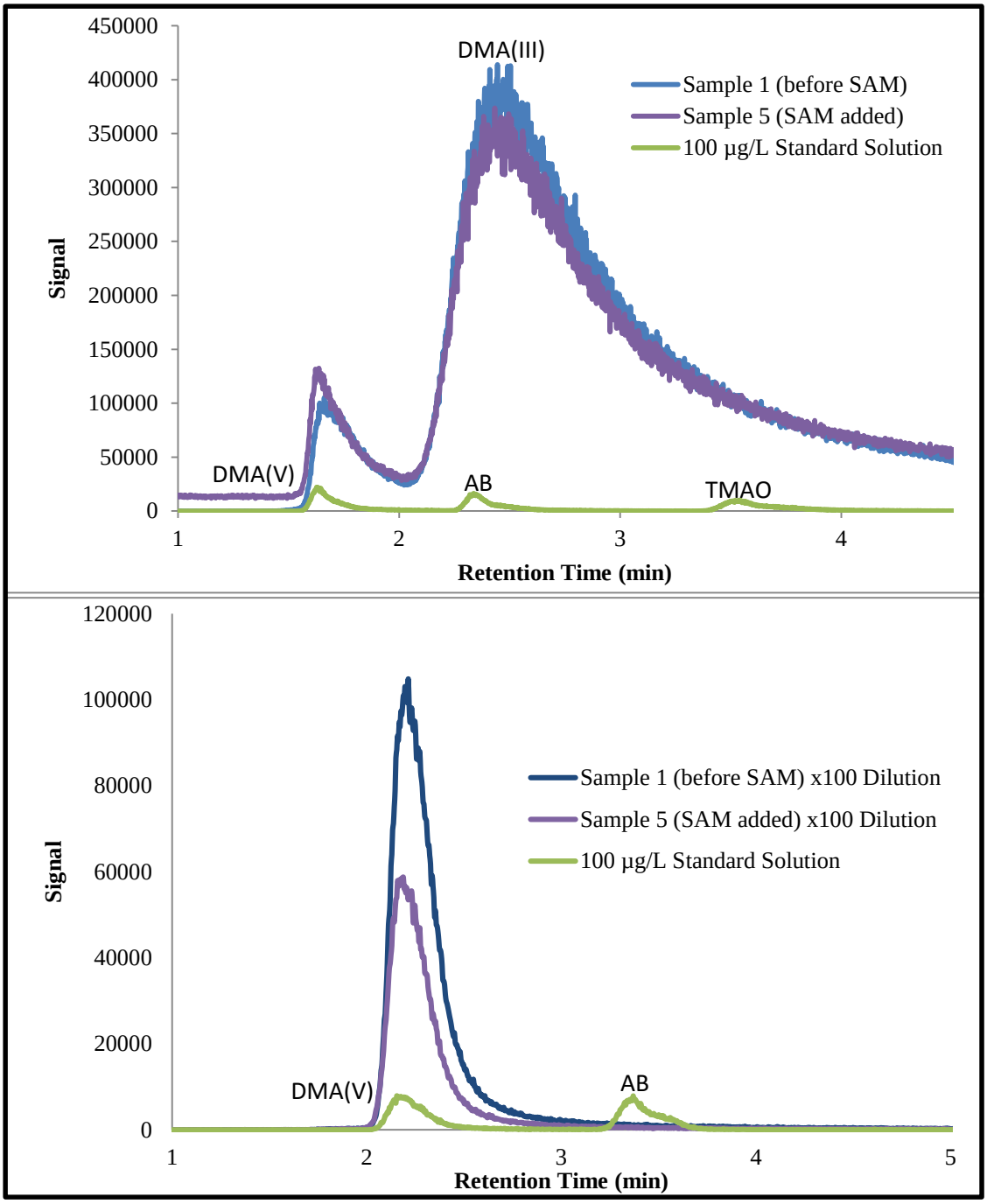

Figure 5-9: Upper graph: Chromatograms of the DMA(III) solution before SAM was added (Sample 1, dark blue) and after (Sample 5, purple). Lower graph: Chromatogram of the 100x diluted DMA(III) solution before SAM was added (Sample 1, dark blue) and after (Sample 5, purple). A 100 µg/L standard solution is shown in both graphs (in green) for retention time comparison.

5.3.1. Formation of DMAA and AB

It is unclear in the analysis of both the undiluted (Figure 5-7) and 100x diluted (Figure 5-8) samples whether DMAA was formed. Spiking the 100x diluted sample (before SAM was added) with DMAA did not cause an additional peak to be formed, indicating that the DMA(V) peak may be co-eluting with the DMAA peak. When arsenic standards were run on their own, the retention times for DMA(V) (1.1 minutes) and DMAA (1.2 minutes) were very similar (Appendix C, Figure C-1). However, it is clear that AB was not formed after the addition of SAM in either the undiluted (Figure 5-7) or 100x diluted (Figure 5-8) samples. When the 100x diluted sample was spiked with AB, a unique peak was formed which indicated that any potential AB is not being masked within the DMA(V) peak. Furthermore, as shown in Figure 5-9, the addition of SAM had no impact on the arsenic species. The decrease in concentration seen in the solution where SAM was added is a result of adding the SAM solution to the reaction and therefore lowering the concentration.

These results do not indicate that this pathway of AB formation is impossible, but rather that it did not work under the conditions used for this experiment. It is possible that the addition of SAM could cause DMAA to form AB, but if DMAA was not formed during the primary phase of this experiment, then the addition of SAM would have no impact. In research by *Ritchie et al. (2004),* DMAA was shown to form AB when exposed to the lysed bacterial extract of *Pseudomonas fluorescens*.

Further research is needed to examine the possible transformation of DMA(III) to DMAA and to AB under different experimental conditions. Firstly, the solution of DMA(III)I should be analyzed before and after the reaction with $Ca(OH)_2$ to determine if DMA(III) (i.e., $(CH_3)_2AsOH$) is synthesized. It is possible that DMA(III) may oxidize to DMA(V) and prevent the reaction from proceeding (therefore resulting in the DMA(V) peak observed after reaction). An As(III) species is required to attack the pyruvate or glyoxylate, as seen in Figure 2-17 and Figure 2-18. Adding a reducing agent, like GSH, may afford reduction to DMA(III) (if indeed it was oxidized to DMA(V)) (demonstrated in Figure 2-9). Secondly, the addition of pyruvate and glyoxylate to synthezise DMAA should be further invesitigated. Furthermore, in the case of adding pyruvate (which has an additional methyl group on the alpha carbon) a demethylating agent may be required to remove the methyl group and produce DMAA. The presence of DMAA at this point in the reaction should be determined. At this point, the addition of SAM should yield AB. However, SAM has been demonstrated to react with As(III) species. Therefore, DMAA may need to be reduced prior to the electrophilic attack of SAM. The addition of GSH (as a reducing agent) in conjunction with SAM should be investigated, as well as the addition of $MetCB_{12}$ to the solution

containing DMAA (as this may result in a metathesis reaction). This attempt gave some insight into the reactions and the stability of the solutions and can be used to aid in the design of future experiments.

Chapter 6 – Summary and Conclusions

Overall, the objective of this research was to determine the conditions under which AB could be formed in the terrestrial environment and to narrow down potential formation pathways. Furthermore, the research aimed to identify the fate of arsenic species in food (specifically AB) in order to more accurately determine the risk associated with consumption.

In summary, it was determined that the predominant arsenic species in mushrooms is not always consistent. This suggests that external factors may play a key role in determining the composition of arsenic in the fruiting body. However, most of the predominant arsenic species in mushrooms aligned with trends previously observed in Nearing et al. (2014a). This suggests that the factors previously identified as important, morphology and phylogenetics, may still have some impact on arsenic speciation. Furthermore, the detection of AB in log-growing mushrooms indicates that growth substrate and microbial community alone do not account for the presence/absence of AB. In the future, more research should be done to investigate the degree to which all of the factors influence arsenic speciation, specifically AB formation and retention. Similarly, more research is needed to determine the exact correlation between total arsenic concentration and the concentration and/or proportion of AB and iAs.

AB is hypothesized to play an osmolytic role in mushrooms, thought to aidi in the maintenance of turgor pressure and keep the stalk and cap rigid. It was determined that lower betaine (a structurally similar osmolyte) concentrations are typically accompanied by an absence of AB. Correspondingly, a higher presence of AB was detected in mushrooms with higher betaine concentrations. This suggests that there is some correlation between the two compounds, but that the presence of AB is not controlled by any one element.

Results for XAS analysis confirm the absence of AB in the vegetative growth stage of mushrooms (the mycelium). Mapping a small section of a puffball mushroom demonstrated that arsenic was uniformely distributed throught the mushroom tissue, but appears to occur as iAs near the skin. Different distributions were observerd for other elements, but more research is needed to determine the significance of the

In edible mushrooms, thermal treatments (frying, barbequing, and baking) generally decreased the total arsenic concentration and caused some alterations in the arsenic species proportions. In some cases, the thermal treatments resulted in methylation of arsenic compounds to more toxic species. AB, when present in the raw sample, was also observed to degrade to form the more toxic TETRA. However, the speciation changes are also typically accompanied by a decrease in

the total arsenic concentration, resulting in an overall decrease in the toxicity of the mushrooms after cooking. Therefore, the risk associated with mushroom consumption should be based on the total arsenic concentration, arsenic speciation, and preparation methods.

For the more synthetic aspects of this research, the transformation of TMAO to AB was confirmed using GSH as a reducing agent and IoAA. This is a possible pathway for AB in the terrestrial environment, as IoAA is found in the soil and GSH is probably readily available. DMA(III) was not a precursor to AB as hypothesized, but this could be a result of experimental conditions. More research is needed to investigate the capability of DMA(III) to be transformed to AB. Experiments should be conducted using carbanion methyl donors (such as $MetCB_{12}$) as a methylating agent or a combination of a reducing agent (like GSH) with a carbonium methylating agent (like SAM).

In conclusion, more research is still needed to determine the exact mechanism of formation of AB in mushrooms. The extent of influencing factors such as growth substrate, soil composition (e.g., presence of iodoacetic acid and GSH), morphology, phylogenetics, and betaine concentration on the presence and proportion of AB needs to be further investigated. The concentration of betaine in fresh mushrooms should also be compared to the betaine concentration in spent mushrooms (i.e. after the spores have been dispersed). This may give more information on the function of AB in mushrooms. The risk associated with mushroom consumption should be based on the total arsenic concentration and arsenic speciation in the form in which the mushroom will be consumed (i.e. after it is cooked). The presence of TMAO, GSH and IoAA in soils should be quantified to confirm this as a viable AB formation pathway. Additional experiments could include growing mushrooms in soil amended with TMAO, GSH, and IoAA to determine if AB is produced and accumulated by the mushrooms. Additionally, it may be valuable to amend soils with different species of inorganic arsenic or mono/dimethylated species to determine if TMAO is formed. Finally, more experiments should be conducted using DMA(III) as a precursor. It is hypothesized that adding a reducing agent, such as GSH, may allow this reaction to take place.

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Appendices

A. Additional information for Chapter 2

Table A-1: Proteins identified as being susceptible to arsenic binding from human hepatocarcinoma cells (shown in green), human breast cancer cells (shown in purple), and the nuclear fraction (shown in red) and membrane fraction (shown in blue) of A549 human lung carcinoma cells. Proteins that are written twice under the same function in different colours come from different cell lines. Proteins from the same cell line that fall under more than one functional category were written twice and separated into their respective categories (ex. Fructose-bisphosphate aldolase A is both a structural protein and a metabolic enzyme).

Proteins related to protein synthesis/regulation	**Stress response related proteins**
Elongation factor 1-alpha 2	Heat shock cognate 71 kDa protein
Elongation factor1-alpha1	Stress-70 protein
Heterogeneous nuclear ribonucleoprotein A/B	heat shock protein
Heterogeneous nuclear ribonucleoprotein A3	Hsp 90βc
Heterogeneous nuclear ribonucleoprotein C-like 1	Hsp27c
Heterogeneous nuclear ribonucleoprotein D0	Hsp70-4c
Heterogeneous nuclear ribonucleoprotein K	Poly(rC)-binding protein 1
Heterogeneous nuclear ribonucleoprotein L	Protein disulfide isomerase precursorb
Heterogeneous nuclear ribonucleoprotein Q	T-complex protein 1, delta subunit
Heterogeneous nuclear ribonucleoprotein R	T-complex protein 1, theta subunit
Heterogeneous nuclear ribonucleoproteins A2/B1	Tumor rejection antigen 1 (GRP94)c
Heterogeneous nuclear ribonucleoproteins C1/C2	Protein disulfide-isomerase A6 precursor
Elongation factor 1-delta	Protein disulfide isomerase related protein (PDSIRP5)
Elongation factor 2	
Eukaryotic translation initiation factor 4A1/2	
HnRNP A2/B1	
HnRNP C1/2	
HnRNP K	

Transport Proteins	DNA/RNA Related Proteins
4F2 cell-surface antigen heavy chain	Interleukin enhancer-binding factor 2
4F2 cell-surface antigen heavy chain	ATP-dependent DNA helicase 2 subunit 1
ADP/ATP translocase 1	DNA-dependent protein kinase catalytic subunit
ADP/ATP translocase 3	DNA replication licensing factor MCM6
Interleukin enhancer-binding factor 2	DNA topoisomerase 2-alpha
Leucine-rich repeat-containing protein 59	ATP-dependent RNA helicase A
Phosphate carrier protein	Non-POU domain-containing octamer-binding protein
Voltage-dependent anion-selective channel protein 2	40S ribosomal protein S14
Voltage-dependent anion-selective channel protein 3	Nucleolar RNA helicase 2
Clathrin heavy chain 1	Polypyrimidine tract-binding protein 1
Dynein cytoplasmic 1 heavy chain 1	Small nuclear ribonucleoprotein Sm D2
GTP-binding nuclear protein RAN	Splicing factor, arginine/serine-rich 3
Importin 90	U2 small nuclear ribonucleoprotein A′

Structural Proteins	Metabolic Enzymes	Regulatory Proteins
Actin	3-hydroxyacyl-CoA dehydrogenase type-2	78 kDa glucose-regulated protein precursor
Actin, aortic smooth muscle	Fructose-bisphosphate aldolase A	Anterior gradient protein 2 homologue precursor
Annexin A2	Glucose-6-phosphate 1-dehydrogenase	Nucleophosmin
Annexin A2	Peroxiredoxin-1	Probable ATP-dependent RNA helicase DDX17
Filamin-A	Peroxiredoxin-1	Probable ATP-dependent RNA helicase DDX5
Fructose-bisphosphate aldolase A	Peroxiredoxin-1	Ras-related protein Rab-10
Keratin, type I cytoskeletal 10	Peroxiredoxin-2	Ras-related protein Rab-15
Keratin, type I cytoskeletal 10	Protein disulfide-isomerase A6 precursor	Ras-related protein Rab-1A
Keratin, type I cytoskeletal 9	Pyruvate kinase isozymes	Ras-related protein Rab-1B
Keratin, type I cytoskeletal 9	6-Phosphogluconate dehydrogenase	Ras-related protein Rab-35
Keratin, type II cytoskeletal 1	ATP synthase alpha chain	Ras-related protein Rab-7
Keratin, type II cytoskeletal 1	ATP-AMP transphosphorylase 2	Reticulon-4
Keratin, type II cytoskeletal 2 epidermal	d-3-Phosphoglycerate dehydrogenase	Stomatin-like protein 2
Keratin, type II cytoskeletal 2 epidermal	Glyceraldehyde-3-phosphate dehydrogenase	Thioredoxin domain-containing protein 1 precursor
Lamin-A/C	Lactate dehydrogenase B chain	
Lamin-B1	Malate dehydrogenase	14-3-3 protein tau
Matrin-3	Methylcrotonyl-CoA carboxylase alpha chain	Nucleolar protein p120
Spectrin alpha chain	Neural enolase	Poly(rC)-binding protein 1
Spectrin beta chain, brain 1	Palmitoyl-protein thioesterase	Proteasome subunit alpha type 6
Tubulin alpha-ubiquitous chain	Peroxiredoxin 4b	Puromycin-sensitive aminopeptidase
Vimentin	Phosphoglycerate kinase 1	Rho GDP-dissociation inhibitor 1
Actin, cytoplasmic 1	pPhosohoglycerate kinase-1	Ribophorin I (glycosylation)
Alpha-actinin 4	Protein disulfide isomerase precursorb	Thioredoxin reductase
Annexin II	Protein disulfide isomerase related protein (PDSIRP5)	
Cytokeratin 2-1	Protein disulfide isomerase related protein (PDSIRP5)	
Cytokeratin e2	pyrophosphatase	
Filamin A	Pyruvate carboxylase	
Lamina-associated polypeptide 2	Pyruvate kinase M2 (PKM2)	
Tubulin β2b	Rho GDP-dissociation inhibitor 1	
Tubulin β5b	Transketolase	
β-Actinb	Triosephosphate isomerase	
β-Tubulin	Ubiquitin-activating enzyme E1	

B. Additional information for Chapter 4

Table B-1: Phylogenetic classifications of species collected from Deloro, ON. The mushrooms are organized based on class, order, family, and then species. The predominant arsenic compound and fruiting body morphologies of each species are shown to the right of the taxonomy.

Class	**Order**	**Family**	**Species**	**Common Name**	**Predominant Compound**	**Fruiting body Morphology**
Agaricomycetes	Agaricales	Agaricaceae	*Calvatia gigantea*	Giant puffball	AB	Puffball
			*Lycoperdon pyriforme**	Pear-shaped puffball	AB	Puffball
		Fistulinaceae	*Pseudofistulina radicata**	n/a	MMA	Pored
		Hygrophoraceae	*Hygrophorus bakerensis*	Brown waxy cap	DMA	Gilled
		Hymenogastraceae	*Galerina Autumnalis*	Deadly galerina	DMA	Gilled
		Psathyrellaceae	*Coprinus atramentarius*	Inky cap	iAs	Gilled
		Strophariaceae	*Pholiota malicola**	n/a	AB	Gilled
		Tricholomataceae	*Tricholoma Aurantium*	Golden orange	AB	Gilled
	Amylocorticiales	Amylocorticiaceae	*Plicaturopsis crispa**	Crimped gill	DMA	Gilled
	Auriculariales	Auriculariaceae	*Exidia glandulosa**	Black ear/Witches butter	DMA	Jelly
	Boletales	Boletaceae	*Boletus bicolor*	Two-colour bolete	DMA	Pored
	Russulales	Russulaceae	*Russula fragilis*	Fragile brittlegill	iAs	Gilled
		Stereaceae	*Stereum ostrea*	False turkey-tail or Golden curtain crust	iAs	Gilled
	Polyporales	Fomitopsidaceae	*Ischnoderma resinosum**	late fall polypore	DMA	Pored
		Polyporaceae	*Trametes versicolor**	Turkey tail	DMA	Pored
Basidiomycetes		Meruliaceae	*Phlebia tremellosa**	n/a	DMA	Gilled
		Polyporaceae	*Cerrena unicolor*	Mossy maze polypore	MMA	Pored
Euascomycotina	Xylariales	Xylariaceae	*Hypoxylon fragiforme**	Red cushion	DMA	Pored
Sordariomycetes	Hypocreales	Hypocreaceae	*Hypomyces lactifluorum*	Lobster mushroom	iAs	Pored

'*' indicates that the mushroom was log growing.

Table B-2: Phylogenetic classifications of species collected from Deloro, ON. The mushrooms are organized based on the fruiting body morphologies, followed by the taxonomy (class, order, family, and then species).

Fruiting body Morphology	Class	Order	Family	Species	Common Name	Predominant Compound
Puffball	Agaricomycetes	Agaricales	Agaricaceae	*Calvatia gigantea*	Giant puffball	AB
	Agaricomycetes	Agaricales	Agaricaceae	*Lycoperdon pyriforme**	Pear-shaped puffball	AB
Gilled	Agaricomycetes	Agaricales	Strophariaceae	*Pholiota malicola**	n/a	AB
	Agaricomycetes	Agaricales	Tricholomataceae	*Tricholoma Aurantium*	Golden orange	AB
	Agaricomycetes	Agaricales	Hygrophoraceae	*Hygrophorus bakerensis*	Brown waxy cap	DMA
	Agaricomycetes	Agaricales	Hymenogastraceae	*Galerina Autumnalis*	Deadly galerina	DMA
	Agaricomycetes	Amylocorticiales	Amylocorticiaceae	*Plicaturopsis crispa**	Crimped gill	DMA
	Basidiomycetes	Polyporales	Meruliaceae	*Phlebia tremellosa**	n/a	DMA
	Agaricomycetes	Agaricales	Psathyrellaceae	*Coprinus atramentarius*	Inky cap	iAs
	Agaricomycetes	Russulales	Russulaceae	*Russula fragilis*	Fragile brittlegill	iAs
	Agaricomycetes	Russulales	Stereaceae	*Stereum ostrea*	False turkey-tail or Golden curtain crust	iAs
Pored	Agaricomycetes	Boletales	Boletaceae	*Boletus bicolor*	Two-colour bolete	DMA
	Agaricomycetes	Polyporales	Fomitopsidaceae	*Ischnoderma resinosum**	late fall polypore	DMA
	Agaricomycetes	Polyporales	Polyporaceae	*Trametes versicolor**	Turkey tail	DMA
	Euascomycotina	Xylariales	Xylariaceae	*Hypoxylon fragiforme**	Red cushion	DMA
	Sordariomycetes	Hypocreales	Hypocreaceae	*Hypomyces lactifluorum*	Lobster mushroom	iAs
	Agaricomycetes	Agaricales	Fistulinaceae	*Pseudofistulina radicata**	n/a	MMA
	Basidiomycetes	Polyporales	Polyporaceae	*Cerrena unicolor*	Mossy maze polypore	MMA
Jelly	Agaricomycetes	Auriculariales	Auriculariaceae	*Exidia glandulosa**	Black ear/Witches butter	DMA

'*' indicates that the mushroom was log growing.

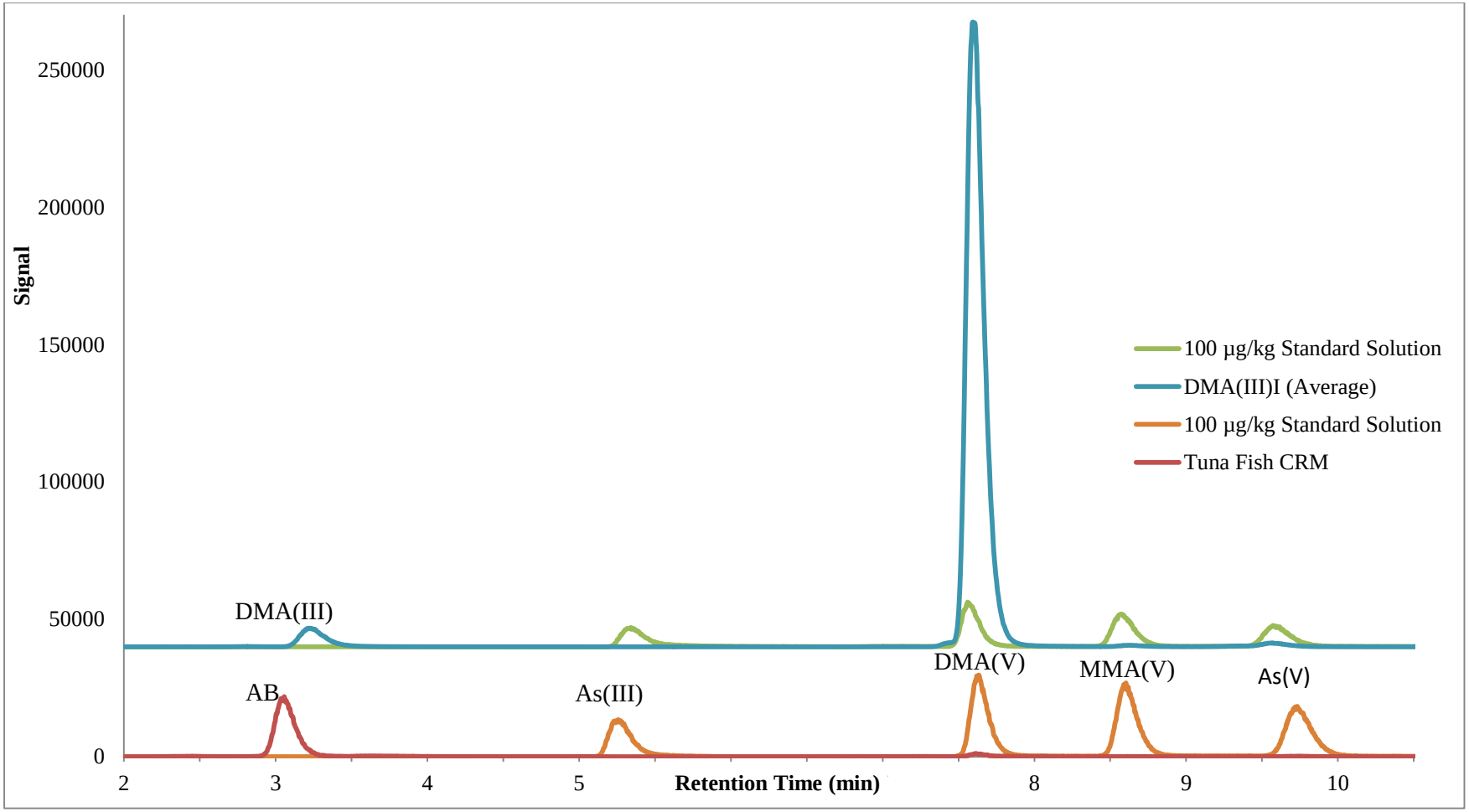

Figure B- 1: The HPLC anion chromatogram of tuna fish CRM (BCR®- 627) (red) which has certified speciation values of 3.9 ± 0.23 mg/kg AB and 0.15 ± 0.022 mg/kg DMA(V). The HPLC anion chromatogram for DMA(III)I is shown in blue and is the average of the triplicate samples that were analyzed. The 100 µg/L standard solutions for As(III), DMA(V), MMA(V), and As(V) were analyzed concurrently (under the same instrumental conditions) as their corresponding sample (i.e. the orange standard corresponds to the tuna fish analysis, and the green standard corresponds to the DMA(III)I analysis.

C. Additional information for Chapter 5

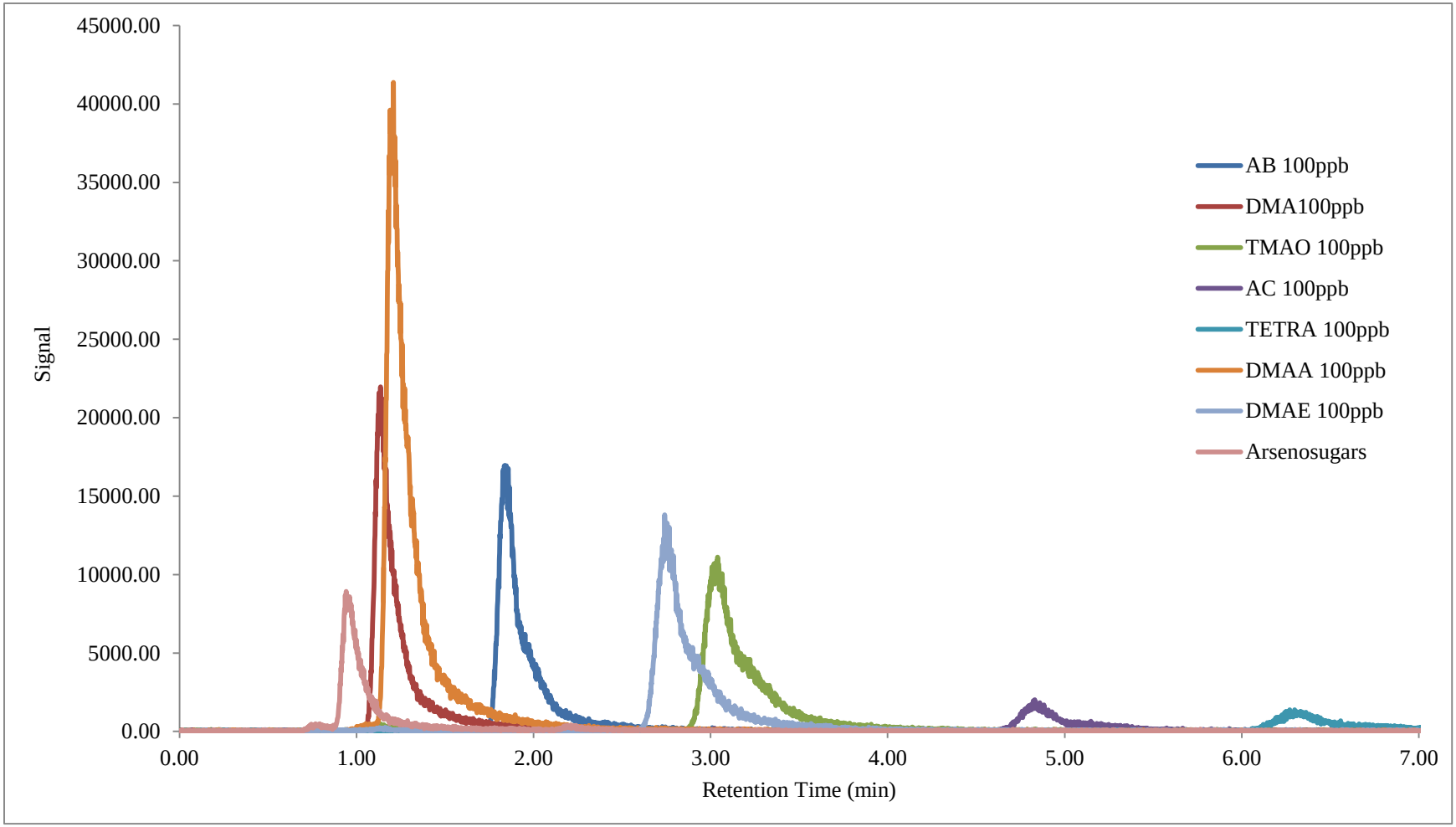

Figure C-1: The HPLC cation chromatograms of the 100 µg/L standard solutions for AB, DMA, TMAO, AC, TETRA, DMAA, DMAE, and a mix of arsenosugars.

D. Supplemental Information

Table D-1: Concentrations (dry weight) in soil- and log-growing mushrooms from Deloro, ON that were collected in September and October, 2014.

Sample	Source	Total As (mg/kg)	Concentration (ug/kg)							Total As in Extract (mg/kg)	Column Recovery (%)	Extraction Efficiency (%)
			iAs	DMA	MMA	AB	TMAO	AC	UK1			
Hygrophorus bakerensis	Soil, ON Elevated	0.68 [a]	125	203	19	0.0	28	12	0.0	0.063	60	5.0
Hypomyces lactifluorum	Soil, ON Elevated	0.543 [a]	203	64	25	0.0	26	0.0	0.0	0.039	79	4.2
Boletus bicolor	Soil, ON Elevated	1.26 [a]	151	534	17	trace	0.0	0.0	0.0	0.091	76	6.0
Cerrena unicolor	Soil, ON Elevated	0.36 [a]	21	14	69	0.0	0.0	0.0	0.0	0.0092	83	1.2
Stereum ostrea	Soil, ON Elevated	0.43 [a]	76	61	25	0.0	21	14	0.0	0.021	87	2.5
Russula fragilis	Soil, ON Elevated	6.1 [a, b]	839	216	79	0.0	0.0	0.0	0.0	1.1	103	7.5
Calvatia gigantea	Soil, ON Elevated	92.6 [a]	0.0	0.0	0.0	7553	0.0	0.0	0.0	6.8	111	25
Tricholoma Aurantium	Soil, ON Elevated	7.9 [b]	trace	306	0	4467	437	139	trace	39	14	98
Galerina Autumnalis	Soil, ON Elevated	7.2	64	3198	30	3079	0.0	0.0	0.0	48	13	96
Coprinus atramentarius	Soil, ON Elevated	2.959 [b]	870	755	0	70	0.0	0.0	0.0	14	12	92
Pseudofistulina radicata*	Log, ON Elevated	0.520 [a]	18	14	177	19	14	12	0.0	0.022	83	4.9
Exidia glandulosa*	Log, ON Elevated	0.33	62	321	21	60	0.0	0.0	62	3.3	22	84
Plicaturopsis crispa*	Log, ON Elevated	1.6	0.0	2206	0.0	379	0.0	0.0	0.0	16	19	98
Phlebia tremellosa*	Log, ON Elevated	2.0	trace	931	trace	171	0.0	0.0	77	8.7	14	96
Trametes versicolor*	Log, ON Elevated	0.52 [a]	59	199	24	0.0	0.0	0.0	86	1.9	20	91
Ischnoderma resinosum*	Log, ON Elevated	0.41	201	238	19	0.0	0.0	0.0	55	3.0	17	95
Hypoxylon fragiforme*	Log, ON Elevated	0.39 [b]	59	107	26	75	trace	0.0	61	1.7	19	86
Pholiota malicola*	Log, ON Elevated	2.5	47	212	0.0	991	0.0	158	106	10	15	95
Lycoperdon pyriforme*	Log, ON Elevated	1.78	65	126	0.0	353	0.0	0.0	151	4.4	16	82

[a] These total arsenic values are corrected based on the matrix spike recoveries discussed in Section 3.4.1.1.

[b] These total arsenic values are corrected values from the extract + residue analysis (not enough sample remained to conduct a separate total digest of the mushroom).

Table D-2: Major arsenic compounds (as concentrations) in raw, fried, and barbequed (BBQ) mushroom samples from Odessa, ON.

Sample Origin	Species	Sample Description	Total As (mg/kg)	Concentration (ug/kg)							Total As in Extract (mg/kg)	Column Recovery (%)	Extraction Efficiency (%)
				iAs	DMA	MMA	AB	TMAO	AC	TETRA			
Odessa, ON	Saffron Milk Cap	Raw	0.22	46.9	28.0	55.6	135.3	0.0	0.0	0.0	0.22	123	37
		Fried	0.081	115.3	trace	38.8	48.1	0.0	0.0	trace	0.15	135	66
		BBQ	0.12	93.3	trace	27.3	59.1	0.0	0.0	33.1	0.22	98	70
	Rough-Stemmed Bolete	Raw	0.18	40.8	104.7	0.0	0.0	0.0	0.0	0.0	0.18	81	74
		Fried	0.069	33.6	60.3	0.0	0.0	0.0	0.0	0.0	0.069	136	73
		BBQ	0.14	61.7	97.1	20.6	0.0	0.0	0.0	0.0	0.14	125	72
	Penny Bun	Raw	0.34	54.3	224.7	13.7	0.0	16.1	0.0	0.0	0.34	90	91
		Fried	0.15	36.4	99.5	trace	0.0	0.0	0.0	0.0	0.15	88	82
		BBQ	0.21	55.0	139.6	17.6	0.0	0.0	0.0	0.0	0.21	103	85
	Puffball	Raw	0.83	121.3	38.4	0.0	394.2	0.0	0.0	0.0	0.83	67	95
		Fried	0.48	117.4	27.4	0.0	317.4	0.0	0.0	38.0	0.48	104	92
		BBQ	0.84	125.8	39.0	0.0	379.8	0.0	0.0	112.8	0.84	79	87

Table D-3: Major arsenic compounds (as concentrations) in raw, fried, and baked fresh and canned mushroom samples from a local grocery store in Kingston, ON.

Sample Origin	Species	Sample Description	Total As (mg/kg)	Concentration (ug/kg)							Total As in Extract (mg/kg)	Column Recovery (%)	Extraction Efficiency (%)
				iAs	DMA	MMA	AB	TMAO	AC	UK (anion)			
Grocery store, fresh mushrooms	Portobello	Raw	0.053	141	31	31	0.0	0.0	0.0	0.0	0.15	138	68
		Fried	0.020	72	trace	trace	0.0	79	0.0	0.0	0.12	122	70
		Baked	0.026	65	0.0	trace	0.0	0.0	0.0	0.0	0.056	115	9
	White Button	Raw	0.064	63	29	trace	0.0	0.0	0.0	0.0	0.074	124	45
		Fried	0.030	50	19	0.0	0.0	0.0	0.0	0.0	0.072	97	43
		Baked	0.036	44	22	0.0	0.0	0.0	0.0	0.0	0.079	84	40
	Oyster	Raw	0.10	94	trace	24	0.0	0.0	0.0	69	0.14	137	64
		Fried	0.062	60	0.0	36	0.0	0.0	0.0	29	0.11	116	39
		Baked	0.095	54	trace	trace	0.0	0.0	0.0	95	0.14	104	87
	Shiitake	Raw	0.039	78	trace	trace	0.0	0.0	0.0	0.0	0.060	130	71
		Fried	0.028	43	0.0	0.0	0.0	0.0	0.0	trace	0.042	103	28
		Baked	0.041	48	trace	trace	0.0	0.0	0.0	51	0.081	122	80
	Enoki	Raw	0.12	97	22	26	127	0.0	0.0	0.0	0.21	131	60
		Fried	0.056	107	14	18	0.0	0.0	0.0	0.0	0.12	118	45
		Baked	0.054	65	14	18	0.0	0.0	0.0	0.0	0.08	118	49
	Crimini	Raw	0.026	42	29	0.0	0.0	0.0	0.0	0.0	0.070	100	54
		Fried	0.041	37	20	7.2	0.0	0.0	0.0	0.0	0.061	106	53
		Baked	0.034	trace	27	0.0	0.0	48	0.0	0.0	0.075	101	60
Grocery store, canned mushrooms	Canned White Button (Sliced)	Raw	0.81	trace	51	0.0	130	0.0	0.0	0.0	0.29	97	85
		Fried	0.47	trace	43	0.0	122	0.0	0.0	0.0	0.23	113	87
		Baked	0.60	trace	48	0.0	110	0.0	0.0	0.0	0.25	98	88
	Canned White Button (Whole)	Raw	1.1	62	120	0.0	127	0.0	0.0	0.0	0.40	118	69
		Fried	0.82	40	80	0.0	119	0.0	0.0	0.0	0.28	135	73
		Baked	1.1	46	142	0.0	119	0.0	0.0	0.0	0.41	106	69