ARSENIC SPECIATION WITHIN THE MAMMALIAN METABOLISM

by
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ABSTRACT

Inorganic arsenic (iAs) is a ubiquitous element in the environment- it innately exists within the earth's crust and mobilizes into the atmosphere, soil, water, and within organisms [1]. Information on arsenic speciation within tissues is limited, although speciation is vital given that each arsenic species has different qualitative and quantitative differences in toxicity [2]. The goal of this thesis is to (1) provide a non-systematic literature review to compare the current state of literature and define the gaps in mammalian arsenic speciation and note arsenic metabolism within the lungs, liver, and spleen and to (2) develop arsenic extraction and analysis methods to assess arsenic in lung, spleen, and liver tissues of male and female C57BL6 mice exposed to 0, and 1000 ppb of sodium (meta) arsenite in drinking water. Arsenic species iAsIII, iAsV, MMA, and DMA were examined through use of high-performance liquid chromatography joined with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) within spiked mammalian tissues. Arsenic species were detected during a preliminary assessment of 1000 ppb dosed spleen and lung**.** This research evaluates a method for arsenic speciation in different tissues. The use of these method in follow-up studies will provide insight for the potential biochemical pathways, toxicological mechanisms, and allow for future inferences about arsenic species in mammalian tissue.

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DEDICATION

May this thesis serve to honor the life and memory of Dr. Edward J. Bouwer.

I dedicate this thesis to my profoundly loving parents, Nagwa El Berry Moustafa and Ahmed Massoud Moustafa.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

As	Arsenic
AS3MT	Arsenite methyltransferase
AQP9	Aquaporin 9
BCR	Bovine liver certified reference material
CRM	Certified reference material
Conc.	Concentration
CPS	Counts per second
DDI	Double distilled water
DB	Digest blank
DMA	DMA total or DMAIII + DMAV
DMAIII	Dimethylarsinous acid, dimethylarsenite
DMAV	Dimethylarsenic acid, dimethylarsinate
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
g	Grams
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GSSG	Glutathione disulfide
HCl	Hydrochloric acid
HNO_3	Nitric acid
HPLC	High-performance liquid chromatography
iAsIII	Inorganic arsenic 3, Trivalent inorganic arsenic, arsenite
iAsV	Inorganic arsenic 5, Pentavalent inorganic arsenic, arsenate
ICP-MS	Inductively coupled plasma mass spectrometry
ISTD	Internal Standard
L	Liter

LOD	Limit of detection
MeOH	Methanol
μg	Microgram
μm	Micrometer
mL	Milliliter
mm^2	Millimeters squared
MMA	MMA total or MMAIII + MMAV
MMAIII	Monomethylarsonous acid, methylarsonous acid, methylarsonite
MMAV	Monomethylarsonic acid, methylarsonic acid
MS	mass spectrometry
m/z	mass-to-charge ratio
$NaAsO_2$	Sodium arsenite, sodium (meta) arsenite, sodium iAsIII
$(NH4)_3PO_4$	Ammonium phosphate
OTC	Ornithine carbamoyl transferase
pH	Potential of hydrogen
PNP	Purine nucleotide phosphorylase
ppb	Parts per billion (μg/L)
ppm	parts per million
RNS	Reactive oxygenated species
ROS	Reactive Nitrogen Species
RPS	Revolutions per second
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SRM	Standard reference material
TMAH	Tetramethylammonium hydroxide
TMAO	Trimethylarsine oxide
V	Volts
Vol	Volume

CHAPTER I

The Metabolism of Arsenic Species within the Mammalian Hepatic, Pulmonary, and Splenic Systems: A Literature Review

1.1 Introduction

Arsenic is a ubiquitous element in the environment- it exists within the earth's crust and mobilizes into the atmosphere, soil, water, and within organisms [1]. Due to its pervasiveness, individuals are readily exposed to arsenic by inhalation, ingestion, and dermally [2]. Arsenic is especially a concern in drinking water. The World Health Organization (WHO) has adopted the standard for arsenic in drinking water as 0.01 mg/l or 10 parts per billion (ppb); however, it is reported that at least 140 million individuals ingest water above this guideline [3].

The U.S. Environmental Protection Agency (EPA), International Agency for Research on Cancer (IARC), and Department of Health and Human Services all recognize inorganic arsenic as a human carcinogen [2]. Arsenic exposure via drinking water has been associated with various adverse health effects such as diabetes mellitus [4, 5], cirrhotic portal hypertension [6, 7], cardiovascular diseases [8, 9], bronchiectasis [10], and impaired neurological function [6, 7]. Dark patches of hyperpigmentation on extremities are considered the "pathologic hallmark of chronic arsenic exposure" [2]. Furthermore, the toxic effects of arsenic are dependent on its species [11]; given that each arsenic species has distinct qualitative and quantitative differences in toxicity [10]; however, the mechanism of chemical action of arsenic toxicity is not entirely known [12]. Thus, collecting data and current literature on arsenic speciation should be approached with urgency as arsenic exposure is a defined threat to public health [13].

Currently, information on arsenic speciation within tissues is limited. Cumulative internal dose of arsenic may be dependent on the species present and accumulated within the body [14]. Therefore, analysis of arsenic species within tissue, especially when exposed chronically, can indicate patterns of arsenic metabolism [15]. Determination of arsenic species in specific organ tissues can lead to a better understanding of the mechanisms of toxicity and offer insight for hazard identification and exposure assessment [10]. The goal of this review is to summarize the scope of the current state of literature regarding arsenic species within mammalian lung, liver, and spleen.

1.2 The Chemical Actions and Characteristics of Arsenic

1.2.1 The Environmental Relevance of Arsenic

Arsenic is a metalloid as it displays physical and chemical properties of both metals and nonmetals [16, 17]. It is a part of the nitrogen family, exists as a free element, covalently bonds with hydrogen, carbon, and oxygen, and is present in the environment as sulfide ores [18]. Unlike metals which are only mobile within acidic conditions, arsenic is readily soluble in acidic (pH 6), neutral, and alkaline (pH 6.6–7.8) settings [1, 19-21]. Arsenic is a major constituent of more than 245 mineral species [20] and subsists into two major groups: inorganic arsenic and organic arsenic (12) (Figure 1). Arsenic also exists in two different oxidation states: pentavalent (V) and trivalent (III and –III) [22, 23]. Each arsenic species has different chemical properties and can reside within different tissues in the human body. Arsenic retention in specific tissues varies based on the amount of exposure, the species, and the methylation potential [16].

Humans are exposed to arsenic through multiple routes, including: intake of contaminated water, ingestion of food (fish is a common source of arsenic for example), and inhalation of ambient air (Figure 1) [24]. Weathering reactions such as volcanic emissions or high winds can displace arsenic in the environment [1]. Arsenic exposure

can also be brought on by anthropogenic activities such as coal industries and the manufacturing of pesticides, wood treatment sprays, and nonferrous alloys [2]. Arsenate, or pentavalent inorganic arsenic (iAsV), is the prevalent and stable arsenic species in drinking water [16] except within reducing conditions (e.g. when ammonia is released by microbial processes in water), where arsenite, also referred as trivalent inorganic arsenic (iAsIII), will dominate water's arsenic composition [17]. Paradoxically, as far back as 1786, arsenic compounds were designed for therapeutics [25]. Arsenic has been indicated to treat dermatological disease, syphilis, hematological disorders, respiratory diseases, and marked the creation of chemotherapy [25, 26]. Arsenic trioxide is still used today to alleviate the symptoms of leukemia [25].

1.2.2 Arsenic Metabolism Pathways

Arsenic's chemistry makes distinguishing its species challenging [20, 21]. Organic is defined by a carbon bond in the chemical framework [27]. Organic species are monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) [13] (Table 1, Figure 1). Arsenic can undergo eight electron reductions and exists in four valence or oxidation states: V, III, 0, and –III [7, 17]. Arsenic metabolism is characterized by reduction reactions of pentavalent arsenic into trivalent form and oxidation reactions where trivalent arsenic is methylated and converted to pentavalent form [17]. The transformation of arsenic within the body is further explained by first pass metabolism [22].

Table 1. Arsenic Species of Interest regarding the Mammalian Metabolism. (Table 1 was created referencing Sattar et al. (2016) [26] and Chen et al. (2014) [11].

Table 1. Arsenic Species of Interest regarding the Mammalian Metabolism			
	Common Names	Common Abbreviations	Chemical Structure
Inorganic	Arsenic Acid, Arsenate, Inorganic AsV	AsV, iAsV	AsO(OH)3
	Arsenous Acid, Arsenious Acid, Arsenite, Inorganic AsIII	AsIII, iAsIII	As(OH)3
Organic	Monomethylarsonic Acid, Methylarsonic Acid	MMAV, MAsV	CH3AsO(OH)2
	Monomethylarsonous Acid, Methylarsonous Acid, Methylarsonite	MMAIII, MAsIII	(CH3)3As(OH)2
	Dimethylarsenic Acid, Dimethylarsinate	DMAV, DMAsV	(CH3)2AsO(OH)
	Dimethylarsinous Acid, Dimethylarsenite	DMAIII, DMAsIII	(CH3)2As(OH)
	Trimethylarsine Oxide	TMAO, TMAsVO, TMAOV	(CH3)3AsO
	Arsenobetaine	AsB	(CH3)3 As+ CH2 COOH
	Arsenocholine	AsC	(CH3)3 As+ CH2 -CH2 OH

Inorganic AsV is typically ingested via water. In the body, iAsV is reduced to iAsIII, by the enzyme AsV reductase (Figure 2A). Glutathione (GSH) can permit arsenic reduction as an essential co-factor to create an oxidized glutathione disulfide product (GSSG) [28] iAsV can further be reduced to iAsIII without enzymes by interaction with thiols [14, 21] or by other endogenous reductants such as purine nucleotide phosphorylase (PNP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ornithine carbamoyl

transferase (OTC) [28-30].

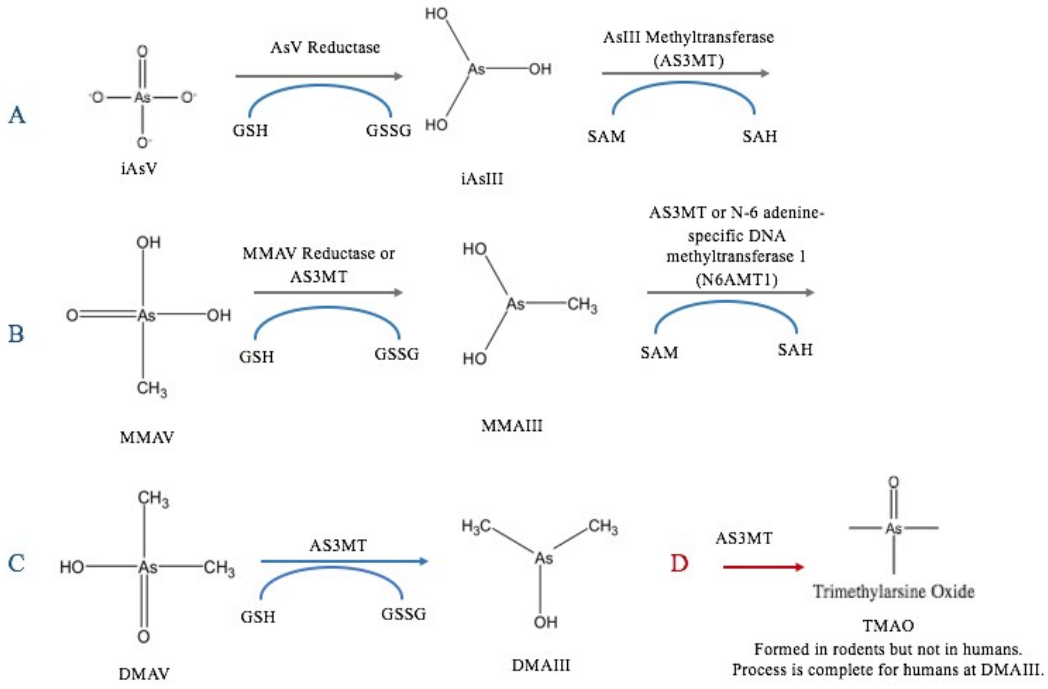


Figure 1. Arsenic Metabolic Chemical Pathway. The arsenic metabolism is a series of oxidation and reduction reactions. **A)** Inorganic AsV is reduced to iAsIII, by the enzyme AsV reductase using GSH, which is oxidized to glutathione disulfide in the process. AS3MT permits the oxidation of iAsIII to MMAV. AS3MT removes SAM from DNA

methylation. **B)** MMAV is then reduced to MMAIII using MMAV reductase or AS3MT as an enzyme. MMAIII is oxidized into the first dimethylated form (DMAV) via AS3MT or N6AMT1. **C)** DMAV is then converted to DMAIII, by AS3MT, which is the final form in the human metabolism. **D)** Rodents however trimethylate DMAIII into TMAO. (Figure 2 was created through Pubchem [31] and ChemDraw [32] with reference to Hall et al., 2012 [29], Reichard and et al., 2010 [30], and Chen et al., 2014 [13], Khairul et al. (2017)[14], Tamaki (1992) [21], Healy et al. (1998) [21].)

AsIII methyltransferase (AS3MT) then oxidizes iAsIII and catalyzes the addition of a methyl group to make MMAV [14] (Figure 2A and 2B). AS3MT utilizes s-adenosylmethionine (SAM) as the methyl donor and forms s-adenosylhomocysteine (SAH) [33]. MMAV is then reduced to MMAIII via MMAV reductase or AS3MT (Figure 2B) [30, 34]. MMAIII is oxidized and methylated to become DMAV by AS3MT or N-6 adenine-specific DNA methyltransferase 1 (N6AMT1) (Figure 2B and 2C) [34, 35]. DMAV is then reduced to DMAIII in humans (Figure 2C). Finally, MMAV and DMAV are excreted in the urine, DMAV typically being at a higher concentration (Figure 1) [14, 36]. For rodents, oxidative methylation continues to form trimethylarsine oxide (TMAO) from DMAIII (Figure 2D) [10, 22].

Once arsenic is ingested, it can be taken up by tissues via aquaporin 9 (AQP9) [37-39]. Aquaglyceroporins are a subfamily of aquaporins that facilitate the permeation of small molecules and water molecules between plasma and tissue [40, 41]. AQP9 is particularly vital for the transport of iAsIII, but has been theorized to transport multiple species of arsenic [37-39]. AQP9 is not explicit to arsenic transport since mammals also permeate AQP9 from glycerol and other small neutral solutes [38].

1.2.3 Arsenic Methylation and Toxicity

Methylation of arsenic within the body was formerly theorized to be for detoxification and bioactivation [14, 20]. Methylation is now recognized to be dependent on exposure conditions and dose [14]. Furthermore, products or intermediates yielded through arsenic methylation are potentially more reactive [42]. The ability to methylate does not protect cells against the acute toxicity of trivalent arsenicals [43]. The trivalent forms of both inorganic and organic arsenic are more toxic and carcinogenic than the pentavalent forms [11, 44, 45]. Multiple studies have shown that MMAIII is more toxic than any other species of arsenic [45-47] (Table 2). In fact, in 2005 it was discovered MMAIII is over 100 times more potent than iAsIII as an *in vitro* inhibitor of thioredoxin reductase [20]. Organic arsenic species which have been found to be non-toxic include arsenobetaine (AsB) and arsenocholine (AsC) [13], which require extreme oxidation conditions to decompose [21]. Petrick and colleagues (2000) used human hepatocytes to rank arsenic species by toxicity: MMA(III) > iAsIII > iAsV > MMA(V) = or > DMA(V) [47]. Although DMAIII was not included in Petrick et al.'s study, it should be acknowledged that DMAIII has greater toxicity than MMAV and less than iAsV [11, 44, 45].

Table 2. Mammalian Arsenic Species- Toxicity Rankings and Endpoints. Arsenic species vary in toxicity. Arsenic species have been reported to target various regions of the mammalian body. (Table 2 was created in reference to Devesa et al. (2004) [48], Drobná et al. (2004) [55], Hughes et al. (2006) [8], Jamova et al. (2011) [42], Li et al. (2017) [47], Le et al. (2000) [52], Mandal et al. (2007) [49], Mass (2001) [43], Reichard et al. (2010) [29], Styblo (2000) [56], Vahter et al. (1984) [50], and Yoshino et al. (2009) [51].)

Table 2. Mammalian Arsenic Species - Toxicity Rankings & Endpoints			
Toxicity Ranking	As Species	Tissue and Areas of Concentration in Body	Organic and Inorganic Total

1	MMAIII	Urine [19] Spleen, Bone marrow, Thymus, Blood Plasma [48]	MMA combined: Blood [49] Urine [19] Hippocampus, Cerebral Cortex, Cerebellum [49] Liver [50]
5	MMAV	Urine [30] Fingernails, Blood plasma [51] Liver [50] Spleen [49] Bladder [44]	
2	iAsIII	Hair, Fingernails [11] Blood Plasma, Liver, Lung, Kidneys [52]	Inorganic As: Lungs, Liver, Kidneys [52]
3	iAsV	Urine, Fingernails, Blood Plasma [53] Liver, lung, kidneys [52]	
4	DMAIII	Urine [54] Fingernails [51] Liver [50]	DMA combined: Bladder, Cerebral Cortex, Cerebellum, Kidney, Bladder [49] Lungs [55] Red Blood cells, Gastrointestinal Tract [52]
6	DMAV	Urine [30] Fingernail, blood plasma, liver [56] Bladder [44] Kidney [44]	

As3MT promoter methylation is triggered by even moderate exposure to arsenic, and aberrant As3MT expression is correlated to a weakened arsenic metabolism [57]. Arsenic methylation is confirmed to be correlated to As3MT gene variation [58]. As3MT genetic polymorphisms may influence arsenic methylation in children, potentially to a lesser extent than in adults [58]. A 2016 study which tested arsenic species within urine of children in Taiwan revealed that the *AS3MT* high-risk haplotype is related to developmental delay affecting motor skills and cognition [59]. The Atacama Desert has the highest arsenic levels in the Americas (>1,000 μg/L) and its residents have been exposed to arsenic for over 7,000 years without epidemiological emergencies- it is

theorized to be due to adaptations of the methyltransferase *AS3MT* gene [60, 61].

In humans, low excretion of DMAV or MMAV in the urine represents poor methylation and can indicate poor arsenic metabolism [62]. In addition, the methylation of inorganic metabolites is thought to influence where arsenic species distribute within the human body [63]. Inorganic AsIII is methylated more rapidly than iAsV, perhaps due to iAsIII having greater uptake into tissues which methylate arsenic [19]. Nevertheless, questions persist regarding how and where MMAIII (as well as other species of arsenic) target within tissue [45-47]. Multiple studies have shown that females are more efficient at metabolizing arsenic [64-66]. In fact, pregnant females have been shown to metabolize inorganic arsenic into DMA more rapidly than both non-pregnant female and male counterparts, which is suggested to reduce health risks to both the fetus and the mother [67].

1.2.4 Chemical and Molecular Mechanisms of Arsenic Toxicity

Arsenic's toxic action is thought to depend on its structure and chemical reactions. To date several mechanisms for arsenic toxicity have been described (as reviewed in Hughes 2002 [68]). Oxidative stress is one of the predominant mechanisms of arsenic toxicity [69]. Unbound arsenic produces reactive oxygen intermediates (e.g. MMA) during reduction-oxidation reaction cycling; in addition, such metabolic activation can elicit lipid peroxidation, DNA damage, and impair proteins [69, 70]. Arsenic mediated generation of reactive oxygen (ROS) and nitrogen species (RNS) within systems results in cellular damage, apoptosis, and activation of oxidative sensitive signaling pathways [69].

Trivalent methylated arsenic species inhibit thioredoxin reductase as well as GSH reductase (and therefore, the reduction of GSSG) [43]. Gene expression regulating the binding of transcription factors to DNA is influenced by thioredoxin levels (as reviewed in

Hughes [68]). Enzyme inhibition can result in altered reduction-oxidation status of cells and decreased protection against cell oxidants [43, 68].

Arsenic and phosphorus have similar electron orbitals and oxidation states [18]. Arsenic's chemical resemblance to phosphorus and affinity to form covalent bonds with sulfur are two additional causes for its toxicity [18]. Pentavalent inorganic arsenic, iAsV, specifically resembles phosphate and replaces it in critical biochemical processes [39]. The replacement is initiated by iAsV's reaction with glucose to form glucose-6-iAsV, which resembles 6-phosphogluconate and glucose-6-phosphate, and therefore acts as a substrate toglucose-6-phosphate dehydrogenase during glycolysis [68].

Oxidative phosphorylation uncoupling occurs by pentavalent arsenic when adenosine diphosphate (ADP) creates ADP-iAsV instead of ATP in the absence of the high-energy ATP phosphate bonds [68]. Trivalent arsenicals, iAsIII, react directly with sulfhydryl groups, which are a key constituent of proteins [44]. It is possible that many arsenic pathways are mediated by the binding of proteins [22].

Another mechanism of arsenic toxicity is due to the fact that both DNA methylation and iAs methylation steps require SAM as a methyl donor [33]. In the presence of arsenic, SAM is depleted by AS3MT to methylate arsenic rather than carry out important methylation functions such as methylate DNA [30]. DNA methyltransferase (DNMT) catalyzes the transfer of a methyl of from SAM to the C5′ position of cytosine at CpG dinucleotides, creating 5-methylcytosine (as reviewed by Reichard et al. [30]). Mass and Wang (1997) findings suggest the CpG within the entire genome can become hypermethylated upon iAsIII exposure of adenocarcinoma cells, due to diminished ability of *Sss*I methylase to remove methyl groups from SAM for DNA [71]. Zhong and Mass (2001) alternatively found hypomethylation and hypermethylation in DNA of human respiratory cells after several weeks of iAsIII exposure [45].

Trivalent arsenicals, such as iAsIII, react directly with sulfhydryl groups, which are a key constituent of proteins and therefore can hinder protein activity [68]. It is possible that many arsenic pathways are mediated by the binding of proteins [70]. MMAIII specifically can hinder the mitochondrial electron transport chain (ETC) [14, 72]. Moreover, MMAIII restrains the activity of ETC complexes II and IV, which results in electron leakage from complex I and III which creates reactive oxygen species in mitochondria, therefore an induction of mitochondrial dysfunction takes place and can trigger apoptosis) [14, 72]. Despite extensive research the molecular and cellular mechanisms involved in arsenic toxicity in mammals are still not thoroughly defined given arsenic's complicated metabolism [65].

1.2.5 Differences in Arsenic Mammalian Metabolisms

A myriad of studies discuss arsenic species composition within blood as it serves to understand arsenic circulation in the metabolism. After only four hours of being intravenously exposed to iAsIII and iAsV, rats showed 95% of arsenic levels in blood to be in the form of DMA [73]. Interestingly, arsenic amounts in blood are low compared to liver, kidney, lungs, or bladder [19]. For humans, the half-life of inorganic arsenic is roughly 10 hours and 70% of arsenic is excreted through urine [74]. Unlike humans and other mammals, following exposure to inorganic arsenic, rats metabolize arsenic readily within red blood cells [52] and have an increased binding affinity towards trivalent forms of arsenic (iAsIII, DMAIII, and MMAIII) [75]. Twaddle et. al (2019) compared rhesus monkeys and mice dosed with sodium iAsIII and to find both animals had predominant formation of DMAV within plasma while erythrocyte composition suggested covalent binding of arsenic in the magnitude of DMAIII > MMAIII > inorganic arsenic species [76]. Therefore, DMA is reported to be the predominant species of arsenic in the blood of rats, mice, and monkeys; MMA (MMAIII and MMAV), though not the predominant species, is also recorded in murine blood [19, 73].

Mice and rats are better at methylation than humans [14, 22]. Some mammalian species exhibit minimal methylation capacity or lack methylation altogether. For example, chimpanzees cannot methylate arsenic while marmoset, tamarin monkeys, and guinea pigs appear to deficiently methylate iAsIII [22, 77-79]. Another example is dogs, which have a greater capacity to methylate iAs species in hepatocytes than human, mice, or rabbits [80]. It is possible that without the As3MT enzyme, which only exists in some mammals, methylation of arsenic is hindered [20, 81]. Table 1 summarizes the differences in arsenic metabolism between mammalian species.

Table 3. Differences in Arsenic Metabolism by Mammal

Table 3. Differences in Arsenic Metabolism by Mammal		
Species	Findings	Source
Chimpanzees	Cannot methylate inorganic arsenic	Vahter et al., 2005
Dogs	Dogs have a greater capacity to methylate iAs in hepatocytes than human, mice, or rabbits.	Drobna et al., 2010
Guinea Pigs	Deficiently methylate iAsIII	Healy et al., 1997
Hamsters	High MMAV reductase activity in bladder	Sampayo-Reyes et al., 2000
Humans	Six metabolites: iAsV, iAsIII, MMAV, MMAIII, DMAV, and DMAIII	2012 (27), Reichard and Puga, 2010 (29), Khairul et al. (2017), Tamaki (1992), Healy et al. (1998)
Marmoset Monkeys	Cannot methylate inorganic arsenic	Vahter, 1999
Mice	Mice metabolize arsenic more efficiently than humans; methylate arsenic further than humans into TMAO.	Hughes et al., 2003 Vahter, 1999
Rats	Rat hemoglobin has an increased binding affinity towards trivalent forms of arsenic (iAsIII, DMAIII, and MMAIII). Longer DMA half-life in rats compared to other mammals, including humans. DMA is more extensively methylated in rats.	Lu et al., 2004 ASTDR, 2007
Tamarin Monkeys	Cannot methylate arsenic	Vahter, 1999

1.3 Arsenic Presence in Different Tissues- The Liver, Spleen, and Lungs

The liver, lungs, and spleen are all important organs which can indicate the nuances of the arsenic metabolism and are pathologic targets of arsenic (Figure 2). The first organ to process arsenic in the metabolic pathway is the liver, therefore the liver has been suggested to have the highest rates of total arsenic, and inorganic arsenic, as well as enzymes or aquaporins to facilitate arsenic transport [37-39]. Arsenic species target the spleen more so than various other tissues such as the liver [42]. Post-mortem studies have confirmed that arsenic species can be found in the lungs upon chronic exposure (from either ingestion or inhalation) [52, 82-86].

Gender, exposure dose, enzymatic availability (e.g. AS3MT, N6AMT1, MMAV reductase, or AsV reductase), presence of AQP9, and genetics all have been reported to affect methylation capacity and arsenic metabolism [14, 64, 65, 74, 79]. Studies with increased dose models report increased total arsenic and the presence of arsenic species [14, 48]. It is known that arsenic species (iAsIII, iAsV, MMAV, MMAIII, DMAIII, and DMAV) are present in spleens, lungs, and liver after exposure of inorganic arsenic [48, 87]. However, different ratios of species exist in each organ. This is theorized to be due to the manner in which arsenic is metabolized within tissues [16]. For example, if one organ has a lower ability to methylate arsenic, it is associated with higher total arsenic tissue concentrations [16]. In the next sections, arsenic species are explored in the lungs, liver, and spleen to help to define the knowledge gaps in arsenic metabolism.

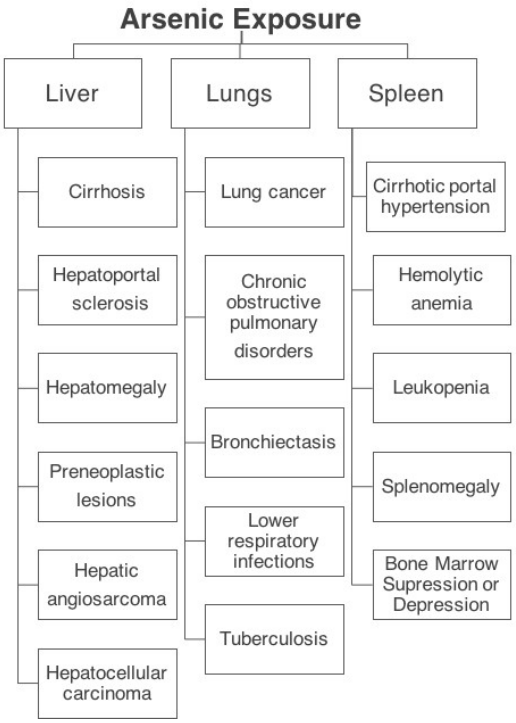


Figure 2: Arsenic Pathology as it relates to the Liver, Lungs, and Spleen. (Figure 2 was developed with reference to the following publications: Abdul et al. (2015) [88], Chen et al. 2011 [9], Datta et al. (1979) [89], Hall et al. (2006) [8], IARC Monographs (2004) [90], Jomova et al. (2011) [44],Parvez et al. (2013) [91], Rahman et al. (2011) [92], U.S. Department of Health and Human Services (2007) [2]).

1.3.1 Arsenic Speciation and Metabolism within the Liver

The liver is a site of arsenic carcinogenesis- as reviewed by Liu and Waalkes (2008) arsenic exposure can result in hepatocellular carcinoma or angiosarcoma, preneoplastic lesions, hepatomegaly, hepatoportal sclerosis, fibrosis, and even cirrhosis [93]. Because of its position in the portal circulation, liver is a major site for metabolism of ingested inorganic arsenic. Upon ingestion, arsenic is readily absorbed by the gastrointestinal tract and directed to the liver, the primary site of arsenic methylation as it is rich with GSH [22, 94].

In the liver, AQP9 is distinct in the basolateral membrane of hepatocytes (50). Consequently, Shinkai and colleagues (2009) suggest AQP9 is a transportation channel that contributes to iAsIII cytotoxicity in mouse hepatocytes [41]. Carbrey et al. (2009) agrees and additionally proposes that AQP9's transportation mechanisms are a route for metabolism by the liver which elicits partial protection for mammals [38] from arsenic toxicity [38]. *AS3MT induced inhibition of DNMT action is nearly distinct to the liver and potentially, other AS3MT-expressing cell types [30]*. Within human hepatocytes, Inorganic arsenic is metabolized by the expression of membrane transporters such as MRP2, GLUT2 [95]. It is still uncertain how arsenic species travel from the liver into other tissues and how other cells uptake arsenic from hepatocytes [96].

Various studies have verified inorganic arsenic presence in the liver and its transport out of the liver to determine the arsenic pathway and how it relates to methylation in tissues [14, 30, 49, 52]. iAsV is reduced to its trivalent form primarily in the liver and blood [12, 26]. After iAsV is reduced to iAsIII, it is readily taken up by the hepatocytes [22, 73]. Cell culture studies have shown that human primary hepatocytes produce all methylated metabolites of iAs that are excreted in human urine: MMAIII, MMAV, DMAIII, and DMAV [50]. It is estimated that 1 kg of human liver can methylate up to 14.8 µmoles of iAsIII (i.e., 1,108 µg As) during 1 hour [95]. It is still unclear whether iAsIII or iAsV is the predominant arsenic species in the liver [22, 73]. Using rat studies, Lerman et al. (1983) found that iAsIII is readily taken up by hepatocytes than iAsV [73]. Vahter et al., (1984) discovered that when iAsIII was injected into mice and rabbit models, it caused elevated total arsenic concentration in the liver followed by the lungs [52]. Similarly, Li and colleagues (2017) orally dosed mice to 2500, 5000,10000, and 20000 ppb of NaAsO2 and after a mere 6 to 9 hours, the liver: had the most abundant inorganic arsenic, the lowest values of MMA, and the highest levels of total arsenic [49].

1.3.2 Arsenic Speciation and Metabolism within the Spleen

Arsenic has been linked to splenic hypertrophy and is known to cause other diseases correlated to the spleen [88]. Arsenic exposure via ingestion has been confirmed to yield arsenic within the spleen [37]. For example, after eight weeks of exposing mice to $NaAsO_2$, the spleen contained 2- to 5- fold higher levels of total arsenic compared to the liver, kidney, and heart and furthermore; 37- fold higher than the lungs [97]. Consequently, dermal can result in arsenic uptake by the spleen and blood five days following exposure at a rate of 1–33 µg/cm2/hour (as reviewed in the 2007 *Toxicological Profile for Arsenic)* [2].

Mice exposed to low concentrations of iAsIII water in vivo have shown suppressed spleen cell function [98]. Also, MMAIII has been shown to be more efficient than iAsIII at inhibiting spleen cell function [99]. Despite iAsIII targeting the spleen, iAsIII exposure has been reported to have no effect on splenic weight and cell recovery within mouse models [100]. In 2016, C57BL/6J mice were dosed with 100 and 500 ppb of iAsIII to analyze arsenic within spleens) [48]. iAsV, followed by DMAIII, were found to be the most dominant species at both doses, and there was no effect on spleen weight or cell recovery [48]. In 100ppb dosed mice, the amount of arsenic per species was: iAsV (11.62 ± 2.89pg) >> DMAV (0.97 ±0.71pg) > MMAV (0.29 ± 0.08pg) > MMAIII (0.12 ± 0.05pg) > iAsIII (0.11 ± 0.08pg). Rankings slightly varied in 500ppb dosed mice ((iAsV (11.33 ± 2.86) > DMAIII (1.83 ± 0.42) > MMAV (1.61 ± 2.13) > DMAV (1.15 ± 0.27) > MMAIII (0.22 ± 0.17) > iAsIII (0.51 ± 0.26)). Species concentrations thus increased as expected with the higher doses of exposure [48]. Furthermore, a dose-dependent increase of intracellular MMAIII was found within bone marrow and thymus cells of the same mice while it was nearly undetectable in the spleen at even 500 ppb of iAsIII exposure [48].

The spleen has immune function as it is a source of T cell storage, specifically for systemic immune cell responses. It appears even low exposure of trivalent arsenic can influence T-cells within the spleen [99, 101]. Upon studying peripheral blood

mononuclear cells in humans, Burchiel et al. (2014) found T-cell proliferation at increasingly low levels of iAsIII (0.1–10 nM) and T-cell suppression by 100 nM MMAIII [99]. Consequently, Soto-Peña and partners observed suppression of phytohemmagglutinin (PHA)-induced T cell proliferation in children aged 6-10 years of age within the Zimapán community in the Mexican State of Hidalgo who were ingesting less than 100 ppb of iAsIII in drinking water [101].

1.3.3 Arsenic Speciation and Metabolism within the Lungs

Arsenic is one of the toxicants linked to both malignant and non-malignant respiratory disease [102]. Chronic and acute arsenic exposure is linked to a multitude of respiratory diseases such as lung cancer, nonmalignant lung disease, bronchiectasis, and respiratory infections [92]. Moreover, arsenic exposure during pregnancy increases the risk of lower respiratory infection and morbidity at infancy [63]. In 2011, Putila and Guo matched cancer registries, arsenic stream sediment, and soil concentrations (from the United States Geological Survey), smoking status, age, and socioeconomic status [103]. Findings concluded that arsenic is significantly associated with lung cancer incidence, even with the previously stated variables controlled [65]. Parvez et al. (2013) examined water sources for 950 individuals and confirmed that low- to moderate-doses of arsenic from water are associated with impaired lung function [104].

Arsenic can influence lung pathology if ingested or inhaled, which although atypical with chemical exposure, is confirmed by multiple studies in Taiwan [83], Chile [84, 85], and Argentina [86]. Rationale regarding why arsenic species present in the lungs when arsenic is ingested is enigmatic. Some theorize it is because the lung is perfused with blood which may carry arsenic species [102]; The lung may play a critical role as a location for arsenic methylation as it has iAsIII methyltransferase activity equivalent to that of the liver [23]. Furthermore, several studies have indicated that lungs can have prolonged contact with arsenic compared to other organs [17, 38, 67]. Lung epithelial

cells have been documented to poorly convert inorganic arsenic to organic arsenic [68] which is considered a cause for pulmonary damage [104]. Organs of Swedish smelter workers were posthumously studied and confirmed that liver samples had diminished levels of arsenic, while the content in the lungs remained high [82]. Interestingly, between 1989-2000, residents of Antofagasta, Chile were observed to have arsenic induced lung disorders after experiencing early-life arsenic exposure, which was significantly linked to mortality [105].

In 2008, Kenyon et al. exposed mice to iAsV-treated water for 12 weeks and discovered that lungs had higher concentrations of arsenic than the liver [17]. As verified by hydride generation atomic absorption spectrometry (HG-AAS), DMAIII and DMAV (DMA total) were the most dominant arsenic metabolites in murine lungs when exposed to iAsV treated water [19]. DMA has frequently been recorded in mammalian lungs [19, 49, 52, 106]. DMA total can concentrate in the lungs in as little as 2-4 hours following iAsV exposure [19, 39]. Consequently, Li and colleagues (2017) observed DMA in lungs at consistent time points throughout their animal studies as well and to be the most dominant species when compared to the liver, kidney, and brain following $NaAsO_2$ exposure [49]. Li and partners also discovered MMA levels were found to be the lowest in the liver but ranked the second greatest in the lungs [49].

Experts debate where and how DMA species are methylated during the arsenic metabolic pathway [49, 107, 108]. Studies suggest that DMA forms in the liver and is then transported to the lungs where it accrues over a short period of time [19, 62]. Yet, inorganic and organic arsenic circulates in the blood and travels to the lung to be metabolized and eventually methylated into DMA [19, 62]. In addition, upon exposure to methylated arsenic (DMA and MMA species), DMA still reveals prominence within the lungs [52, 107, 109]. For example, studies which exposed rats and mice to DMAV intravenously found concentrated DMA in the lungs [52, 109]. This suggests that DMA is

sequestered to the lung regardless of the capacity of the lung or other organs ability to methylate arsenic [109].

1.4 Conclusion

Arsenic undergoes a series of reduction and oxidation reactions which impact the mammalian metabolism resulting in disease and morbidity [2, 110]. Arsenic mechanisms of toxic action include (but are not limited to): ROS generation [69], inhibition of enzymatic actions [43, 68], alteration of the ETC [14, 72], elicitation of lipid peroxidation, DNA damage, and the impairment proteins [69, 70]. The actions of arsenic species with greater toxicity (i.e. MMAIII) can be linked to chemical mechanisms to help understand the roles of speciation within molecular mechanisms [43, 68, 111]. Albeit MMAIII is determined to be over 100 times more toxic than iAsIII [20], its action in the mammalian metabolism still requires more exploration.

Examining arsenic metabolism in the spleen, lungs, and liver offers insight to arsenic speciation's role in different tissues. The liver is claimed to metabolize and transport arsenic effectively [14, 64, 65, 74, 79]. Remarking on arsenic speciation in the liver, the first organ in the arsenic metabolic pathway [22], can promote further theories and study of the arsenic metabolic pathway. By noting elevated DMA in the lungs [52, 107, 109], one can infer that the lungs are one of the last organs to be targeted by arsenic or that DMA directly sequesters to the lungs upon exposure [109]. The spleen still remains ambiguous in the study of arsenic speciation and further examination can bring understanding to arsenic speciation's effect on immunity and blood circulation.

CHAPTER II

Development of Methods for Arsenic Speciation via High Performance Liquid Chromatography and Inductively Coupled Plasma Mass Spectrometry

Chapter II Abstract

Introduction: There is a paucity of published literature on arsenic speciation in mammalian tissues with relation to gender and dose. Part of the reason could be explained by the lack of consensus of methods for the determination of species.
Goal: The long-term goal of this project is to assess arsenic species within mammalian tissue to understand the arsenic metabolism. The short-term goal is to develop a method to determine the relevant arsenic species.
Methods: A method for detecting arsenic species was tested using high-performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (HPLC-ICP-MS). The Hamilton PRPX-100 Anion Exchange Column was chosen, and several mobile phases were tested using laboratory standards to optimize the separation of each species. Lung and spleen samples were spiked with 1000 ppb of iAsIII, iAsV, DMA total, and MMA total to test recovery. One male C57/BL6 mouse lung and spleen dosed at 1000 ppb were analyzed to test feasibility. Linearity, sensitivity, precision, accuracy and resolution were tested.
Results: Four arsenic species (iAsIII, iAsV, MMA, and DMA) were observed via HPLC-ICP-MS analysis after tissue spiked with 1000 ppb were extracted using EDTA. DMA (110 ng DMA/ g tissue) was recovered from the lungs of mice dosed at 1000 ppb during pilot method development tests. EDTA showed no interaction with arsenic for extraction and furthermore, EDTA permitted the ability to extract tissue through a 0.2μm filter for HPLC injection.
Discussion / Conclusion: The findings gathered from this research will help inform metabolic pathways of arsenic, explain variability of species of arsenic within tissues, and further assist to explain how dose can impact disease.

2.1 Introduction

2.*1.1 The Relevance of Arsenic and the Importance of Speciation*

Arsenic is responsible for adverse health outcomes of hundreds of millions of people [3], Individuals are readily exposed to arsenic via, ingestion, dermal routes, and inhalation [2, 70]. Arsenic is a ubiquitous metalloid that exists as multiple species, each of which yield different toxicities and chemical actions [10, 12, 112]. Within the mammalian metabolism, arsenic undergoes a series of reduction-oxidation cycles which result in six arsenic species: pentavalent inorganic arsenic (iAsV), trivalent inorganic arsenic (iAsIII), monomethylarsonic acid (MMAV), monomethylarsonous acid (MMAIII), dimethylarsinic acid (DMAV), and dimethylarsonous acid (DMAIII) (Table 1) [13, 30, 33]. Among these species, MMAIII is the most toxic; however, it is not certain [5] how and where each species of arsenic metabolizes within tissue [45, 46]. Though it is known that the severity of arsenic's effects differ based on the species an individual is exposed to, how much one is exposed to, and even one's gender, there is a lack of research which defines such variables.

Arsenic is metabolized through first pass effect and therefore, the liver has been shown to have high total arsenic when compared with various organs (lungs, kidney, etc.) [37-39]. Arsenic species presenting in the lungs can result from both ingestion or inhalation [83-86] and its clearance from the lungs is gradual (which can even be observed posthumously [52, 82]. The spleen's immune and hematopoietic roles are critical to human health- which makes it important to understand how arsenic targets the spleen and why arsenic found at higher levels compared to several other tissues, including the liver [42].

Table 1. Arsenic Species of Interest within the Human Metabolism. Six arsenic species exist within the human metabolism; MMAIII being the most toxic species followed by iAsIII, iAsV, DMAIII, MMAV, and the least toxic is DMAV [5]. Names of species are determined by their methylation and oxidation state being pentavalent or trivalent [13, 30, 33].

Table 1. Arsenic Species of Interest within the Human Metabolism				
	Toxicity Rank	Common Names	Common Abbreviations	Chemical Structure
Inorganic	3	Arsenic Acid, Arsenate, Inorganic AsV	AsV, iAsV	$AsO(OH)_3$
	2	Arsenous Acid, Arsenious Acid, Arsenite, Inorganic AsIII	AsIII, iAsIII	$As(OH)_3$
Organic	5	Monomethylarsonic Acid, Methylarsonic Acid	MMAV, MAsV	$CH_3AsO(OH)_2$
	1	Monomethylarsonous Acid, Methylarsonous Acid, Methylarsonite	MMAIII, MAsIII	$(CH_3)_3As(OH)_2$
	6	Dimethylarsenic Acid, Dimethylarsinate	DMAV, DMAsV	$(CH_3)_2AsO(OH)$
	4	Dimethylarsinous Acid, Dimethylarsenite	DMAIII, DMAsIII	$(CH_3)_2As(OH)$

2.1.2 Importance of Method Development for Arsenic Detection

No methods have been standardized to measure arsenic within tissue by the EPA or any federal agencies, this includes the measurement of total arsenic as well as arsenic speciation [2].

Total arsenic is typically measured using inductively coupled plasma mass spectrometry (ICP-MS) or graphite furnace atomic absorption (GFAA). ICP-MS has the advantage of analyzing many elements simultaneously and having a very low limit of detection [37] whereas GFAA can only measure one element at a time and is not as sensitive.

There are multiple ways to determine the species of arsenic [113]. High Performance Liquid Chromatography coupled with ICP-MS (HPLC-ICP-MS) can show a vast range of arsenic species as it can separate chemicals by polarity and pH [27]. Another method is X-Ray Absorption Fine Structure Spectroscopy (XAFS) [1]. utilizing a synchrotron light source, which is a powerful way to differentiate between species. However, access to the synchrotron is limited and time consuming. Gas phase chromatography is typically well suited for separation; however, many arsenic species are not volatile or not stable at the temperature required in gas phase [27].

Determining an appropriate mobile phase to separate arsenic species with the HPLC-ICP-MS is critical to attain ideal peak retention and ionization of the analyte molecules. Mobile phase parameters were discussed with professionals of Johns Hopkins University (Baltimore, MD) and Agilent Technologies (Santa Clara, CA).

Arsenic speciation studies utilizing HPLC have varying mobile phases, some of which require ammonium phosphate despite phosphate within mobile phases causing build-up on the interface of cones. Ammonium phosphate however, contains surface ions as well as oppositely charged ions within the mobile phase and therefore, exchanges equilibria well during the stationary phase [114]. Ethylenediaminetetraacetic acid (EDTA) and hydrochloric acid are often used in mobile phases as stabilizers. Mobile phases also often contain methanol, which can help with enhancing the signal due to its carbon bonds.

The most important parameters to consider in the selection of the column that is used to separate arsenic species within the HPLC are packing and column length. Packing options include polystyrene divinylbenzene (PS-DVB) and trimethylammonium which are polymeric packing materials stable across pH ranges from 1 to 13 and therefore, one single column can analyze varying ions [115]. Available column lengths range from 50 mm to 250 mm. A long column allows ideal separation of peaks (Agilent).

Helium is a large halogen which can assist to remove argon chloride interferences from HPLC-ICP-MS results [37]. Chlorides interfere with arsenic speciation as it forms large and intense peaks. This specifically occurs from the polyatomic ion argon (mass of 40) chloride (mass of 35) because it shares the same mass-to-charge ratio (m/z) as the arsenic isotope [37].

2.1.3 The Importance of Sample Preparation

Sample preparation is a critical step in order to avoid aerobic conditions that oxidize trivalent arsenic to the pentavalent form (iAsV, MMAV, DMAV) [116]. DMAIII is highly unstable under aerobic conditions [113]. and arsenic species undergo species change in aqueous solutions [117, 118]. Thus, in order to capture the arsenic species as they exist in each tissue at the time of sampling, contact with oxidizing or reducing conditions need to be minimized or avoided during sample preparation.

The use of C57BL/6 mice to model uptake and metabolism of arsenic is particularly advantageous as they have proven to be sensitive to arsenic exposure [119] are genetically and physiologically near identical and have elucidated metabolic pathways and biological mechanisms [120]. Mouse organs are typically “snap frozen” by introducing liquid nitrogen immediately to reduce morphological distortion or lyse of cells within tissue [121]. as well as minimize aerobic conditions that might oxidize arsenic species. Tissues should not be fixed or buffered with any solutions to avoid reaction of arsenic species; e.g. trivalent arsenicals have an affinity for sulfhydryl groups of proteins and therefore protein or nutrient-rich solutions should be avoided since they may alter speciation [25].

1.3.4 Arsenic Extraction from Tissues

Trivalent species of arsenic are the most difficult to preserve in a sample as they oxidize readily in the environment; however, methylated forms of arsenic (MMAIII and DMAIII) have been detected as metabolites of urine with the treatment of chelating agents [32]. Ethylenediamine tetraacetic acid or EDTA is an anticoagulant defined by a polyprotic acid with two amine groups that have lone-pair electrons which permit the chelation of metal ions [122]. Chelating agents have long been indicated for therapy to alleviate arsenicosis [123]. AsIII oxidation to AsV during sample preparation and storage procedures has been prevented in water through the use of EDTA and other chelation agents [124].

The main goals of this study were: 1) to develop and validate a method for the efficient extraction of arsenic from mammalian tissues that preserves the species present at time of sampling; 2) to optimize the hardware needed for the identification of arsenic species which would be present in the lungs, liver, and spleen of mammalian species.

2.2.0 Materials and Methods

An optimized analytical method for analysis of arsenic species from mammalian tissues using high performance liquid chromatography (HPLC) coupled with inductively coupled–mass spectrometry (ICP-MS) was adapted with modifications from methods reported by Yathavakilla et al., (2008) [125], Hanen et al., (2004) [126], Guimarães et al., (2018) [127], Kawalek et al. (2011) [128], Nam et al., (2006) [129], Jackson et al., (2001) [130]. Organ samples from mice dosed with 0, 100 ppb, and 1000 ppb arsenic were collected for method optimization experiments. Multiple samples of lungs, livers and spleens were extracted and weighed before analysis.

Arsenic species separation was performed using high performance liquid chromatography (HPLC) (model 1260, Agilent Technologies, Santa Clara, CA) in tandem with an inductively coupled plasma mass spectrometry (ICP-MS) (7500 ce

series, Agilent). The two instruments in unison can determine the presence of arsenic species and the ICP-MS alone can measure total arsenic [21, 22].

2.2.1 Animal Exposure to Arsenic

One hundred and twenty C57BL/6 mice (Charles River, Wilmington, Massachusetts), were exposed ad libitum to sodium (meta) arsenite (sodium iAsIII or $NaAsO_2$) (Sigma Aldrich, St. Louis, MO, USA) mixed with water (Crystal Geyser Natural Spring Water, Weed, CA) for eight weeks starting at four to five weeks of age (Table 2). Groups of mice were exposed to 0, 100, and 1000 parts per billion (ppb = µg/L) of inorganic arsenic to model no exposure, moderate exposure, and elevated exposure respectively (Table 2). When the mice were about 12 weeks of age, they were sacrificed. Lung, liver, and spleen samples were harvested, snap-frozen in liquid nitrogen (N_2) immediately after collection and stored in a freezer at -80° Celsius. Not all organs were analyzed for this thesis.

Animal use and care was approved by Johns Hopkins Bloomberg School of Public Health and follows all protocols enforced by the Institutional Animal Care and Use Committee (IACUC), which complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Table 2. C57BL/6 Mice Dosing and Organ Inventory. Mice were dosed with 100ppb and 1000ppb of $NaAsO_2$ (or sodium iAsIII) for eight weeks. One hundred and twenty total organs were collected.

Table 2. C57BL/6 Mice Dosing and Organ Inventory							
	Control- No Arsenic		100 ppb iAsIII		1000 ppb iAsIII		Totals
C57BL6 Mouse Organ	male	female	male	female	male	female	
Spleen	10	10	5	5	5	5	40

Liver	10	10	5	5	5	5	40
Lung	10	10	5	5	5	5	40
Totals	30	30	15	15	15	15	120
	60		30		30		

2.2.2 Arsenic Extraction from Tissues

After organs were thawed on ice for 2-3 hours in a dark space (Figure 1A), the weight of each organ was determined. Several extraction methods were tested to evaluate yield and efficiency of extraction, and to select the one that best preserved arsenic species. In this paper we report extraction using EDTA [131, 132], heat [125, 127, 129, 133] and TMAH [128]. All extraction methods were conducted as indicated in Figure 1. (EDTA was replaced with TMAH for that extraction method).

Figure 1.A

Figure 1.B

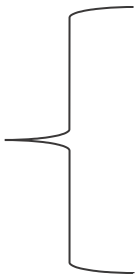


Figure 1.C

Figure 1. Lung, Liver, and Spleen Extraction Methods for HPLC-ICP-MS. (Figure 1 was created through BioRender [134]).

2.2.2.1 Extraction with EDTA

After the organ mass was determined, 0.5 mL of double distilled (DDI) water was added to the cryovial along with 1.6mg EDTA per 1g of tissue, based on guidelines from Sigma Aldrich [135]. The cryovial was then inverted and vortexed until the organ peeled off the sides of the cryovial (less than one minute). Then, the organ was placed in a 1510 Branson ultrasonic bath at 25°C for 30 minutes.

Following the first sonication, the organ was homogenized with sterile hemostat scissors and tweezers (Figure 1B) taking care not to scratch the plastic within the cryovial to avoid introducing unwanted chemicals into the organ sample. The homogenized sample was then placed on a cell strainer (Falcon (A Corning Brand) Cell Strainer, 70μm nylon). The cell strainer was placed on the 50-mL tube utilizing sterile procedure. Using a pestle (CellTreat Scientific Products Cell Strainer Pestle), the organ was strained into the 50-mL tube. The remaining amount of DDI water (1 to 1.5mL depending on organ weight) was used to: 1) rinse off the hemostat scissors, pestle, and tweezers, 2) added to the cryovial and vortexed to remove any remaining parts of the organ sample and, 3) rinse any remaining tissue sample out of the strainer. The 50-mL tube with the homogenized

and strained organ sample was then vortexed and put in an ultrasonic bath at 25°C for 30 minutes.

Following sonication, the sample was centrifuged at 1400 rotations per minute (RPM) for 40 minutes with a ten-minute deceleration (Figure 2C). When completed, the supernatant was aliquoted into a new 50 mL tube while the pellet was stored for later analysis. Following a method by Li et al. (2017) [49], the supernatant was analyzed. The supernatant was centrifuged again at 1400 RPM for 40 minutes with a ten-minute deceleration. The supernatant was removed and filtered sequentially using 13mm syringe filters, first with a 0.8 μm pore followed by a 0.2 μm filter (Pall Corporation Acrodisc 13mm Syringe Filter with 0.8 μm Supor Membrane, Pall Gelman Laboratory Acrodisc 13mm Syringe Filters with 0.2 μm Supor (PES) Membrane for HPLC Sample Preparation and Solvent or Aqueous Filtration). If the syringe yielded any resistance upon attempting to push the supernatant through the filter, the filter was changed. One to four filters (of either 0.2 or 0.8 μm) were used for each organ- numbers of filters varied. The final filtered supernatant products were transferred into 2 mL amber vials compatible with the HPLC and stored in an inert atmosphere in 4°C up to 12 hours before analysis.

2.2.2.2 Extraction with Heat

After thawing and weighing, lung, liver, and spleen samples were sonicated at 25°C for 30 minutes twice and homogenized using cell strainers in 50 mL tubes as described above. The 50 mL tubes with organ samples were put in a beaker with water and placed on a hot plate at 90°C for three hours to replicate the methods of Guimaraes et al. (2018) [127] as well as other arsenic extraction methods which utilize heat [125, 129, 133]. The water was monitored with a thermometer every 20 minutes over the three-hour span to assure the temperature was stable. The heated samples were then centrifuged twice at 2000 RPM for 40 minutes with a ten-minute deceleration. The supernatant was

separated from the sample following each centrifugation and the pellet was stored for later analysis. Both the supernatant and pellet were stored under argon as described below, and placed in 4°C for up to 12 hours.

2.2.2.3 Extraction with TMAH

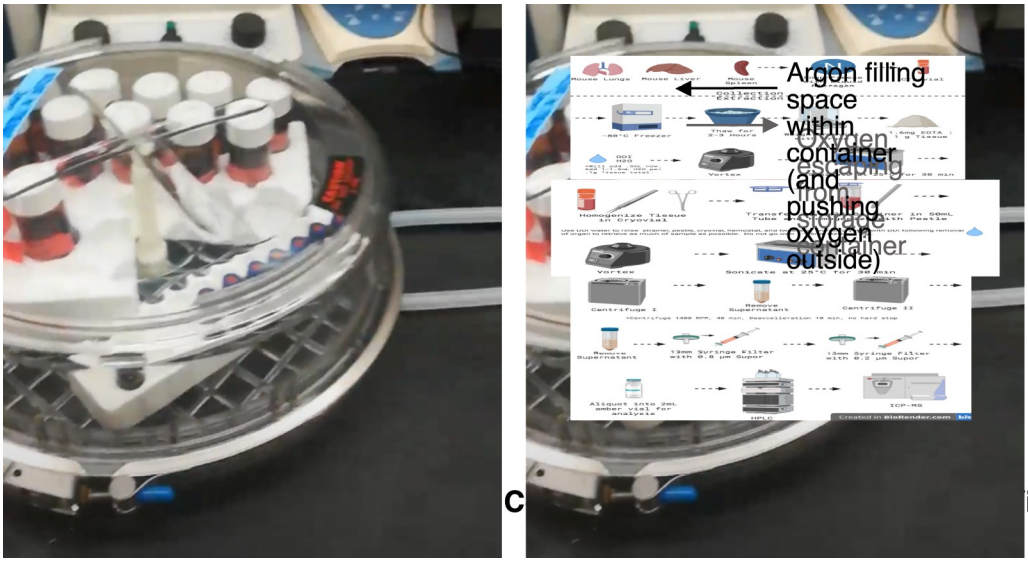
The same extraction procedure described in Figure 1 was followed using 25% Tetramethylammonium hydroxide (TMAH) instead of EDTA (Alfa Aeser, Haverhill, MA) [131, 132]. TMAH was used in a concentration of 3 mL per 0.5 grams of organ tissue along with 6.5 mL DDI water [128].

2.2.3 Storage Under Inert Atmosphere

The extracted organ samples were stored in 2mL amber vials with twist-on septum seal lids to decrease light exposure, which may cause speciation changes [25]. Additionally, 30 mL amber vials were used for all reference materials or standards. The septum seal permitted the ability to safely add argon gas into the vial to provide an inert atmosphere and therefore reduce changes in arsenic species. Two 18-gauge needles were inserted into the septum just high enough to avoid touching the solution within the vial; this allows for the release of oxygen within the vial to go into the atmosphere through one needle while argon flows in through the second needle. Argon flows through a rotameter set to 35-45 mL/ min. Argon was injected for 30 minutes into each 30 mL vial , and 2 minutes into each 2 mL vials to allow approximately 10 air changes within each vial and ensure no air is left inside.

All vials were placed in a re-sealable 5600 mL chamber, which was also filled with argon to create an inert atmosphere (Figure 2) and stored at 4° C. Samples were stored in this chamber for a maximum of 12 hours before analysis to minimize oxidation or reduction

of the samples.



C ials

2.2.4 HPLC and ICP-MS Settings

A mobile phase was created using 10 milliliters (mL) of 1% methanol (ACS Certified, Thermo Fisher, Waltham, Massachusetts), 2 mL of 1 M (moles/liter) of dibasic ammonium hydrogen phosphate (BB-2627, Boston BioProducts, Ashland, MA), and 58.4 mg of .0002 M of ethylenediamine tetraacetic acid (EDTA) salt. All chemicals were added to a 1L Teflon bottle and then DDI water was added to the remaining ~988 mL to make 1L mobile phase.

Three pH solutions were tested: 2.36, 6.15, and 9.14. The pH of the mobile phase is important as each arsenic species each carry multiple different pKa values [20]. The pH of the mobile phase was considered the most successful if it allowed us to detect all species simultaneously.

Female and male mouse organs were analyzed for arsenic species using Agilent 1260 high performance liquid chromatography (HPLC) in tandem with Agilent 7500ce series inductively coupled plasma mass spectrometry (ICP-MS) with Masshunter and Chemstation software. For the HPLC, a PRPX-100 Anion Exchange Column (Hamilton, Reno, NV) with polyether ether ketone (PEEK) lining, a diameter of 4.6mm and 250 mm

in length. The size of the bead packing within the column is 5 μm for a volume of 4,155 mm3. The corresponding PEEK guard column (PRPX-100 Anion Exchange Analytical Guard, Hamilton, Reno, NV) was placed before the PRPX-100 column. The guard column retains impurities from the sample to avoid damage to the analytical column. The column was conditioned prior to conducting any analysis, calibration, or runs. Conditions of the HPLC and ICP-MS were set as seen in Tables 2 and 3.

All bottles and tubes used were rinsed overnight with 10% Optima grade nitric acid (HNO_3) (Fisher Scientific, Waltham, MA) to remove any potential residual metals. Glass materials were avoided - arsenic is often used in glass making and arsenic may potentially leach out of glass and interfere with results [136].

An internal standard (ISTD) of 5 ppb iAsV (SPEX, NIST, Gaithersburg, MD) was used to normalize the raw arsenic signal, or the counts, to the internal standard counts in order to correct for drifts or fluctuations. The ISTD was always injected at the start of each HPLC-ICP-MS sample injection to bypass the column. Inorganic arsenic five was chosen as it is the most stable in an aerobic environment and iAsV was measured in every run.

Table 3. Settings utilized with the *Agilent* 1260 High Performance Liquid Chromatography (HPLC). Settings were determined through literature review.

Table 3. Settings utilized with the *Agilent* 1260 High Performance Liquid Chromatography (HPLC).	
Mobile phase composition	10 mL 1% methanol, 2 mL of 1 M dibasic ammonium hydrogen phosphate, 58.4 mg of .0002 M EDTA
Mobile phase pH	pH 6 (±0.05)
Mobile phase flow rate	1 mL/min
Injection volume	50 μL
Column temperature	Ambient
Acquisition time	900 s (15 min)
Column	PRPX-100 Anion Exchange HPLC Column, PEEK lining Diameter 4.6mm, Length is 250 mm

Table 4. Settings utilized with the Agilent 7500ce Series Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Settings were determined through literature review.

Table 4. Settings utilized with the Agilent 7500ce Series Inductively Coupled Plasma Mass Spectrometry (ICP-MS)			
RF Power	1530 W	Spray Chamber Temp.	2°C
RF Matching	1.80 V	Collision cell	He Gas , 5.0 mL/ min
Auxilliary (makeup) gas flow	0.10 L/min	Data acquisition Mode & Ions Monitored	Time-resolved, m/z 75 for 75As+ , and m/z 77 for 40Ar37Cl+
Nebulizer (carrier) gas flow	0.81 L/min	Octopole Bias	Negative 15 V
Nebulizer type	Micromist	Quadrupole Bias	Negative 18 V
Sampling Depth	7.6 mm	Cell Entrance Voltage	negative 30 V
Peristaltic Pump Speed	0.30 rps	Cell Exit Voltage	Negative 40 V

2.2.5 Total Arsenic Analysis

Twelve total organs from female mice were analyzed to evaluate if EDTA impacts the presence of arsenic within tissue and to determine the concentration of total arsenic in tissues (Table 4). Total arsenic was measured in each organ via ICP-MS. The organs were split into four groups: 1) 1000 ppb dosed mouse organs with no EDTA, 2) 1000 ppb dosed mouse organs with EDTA, 3) 0 dosed mouse organs with no EDTA 4) 0 ppb dosed mouse organs with EDTA. Each group had one female lung, a female liver, and a female spleen.

EDTA was dissolved in double distilled water using a 1.6 mg of EDTA to 1 mL of water ratio. EDTA solution was added directly to the six organs within their cryovials. Cryovials were inverted and let stand for 30 minutes to permit coagulation. Samples were then transferred to 50 mL conical tubes. All samples were digested using an atmospheric pressure and open vessel digestion method inside a microwave (MARS-5, CEM, Charlotte, NC, US) using Sarstedt 50 mL conical tubes which had holes drilled into the caps in-house and were used. To each tube, 5 mL of HNO_3 was added.

For quality control, bovine liver certified reference material was used (BCR- 185R, 0.0330 +/- 0.0029 mg/kg arsenic, Institute for Reference Materials and Measurements (Geel, Belgium)). From the BCR-185R powder, 0.56 g was weighed into a 50-mL polypropylene tube and 5 mL of concentrated Optima nitric acid (Fischer Scientific, Waltham, MA) and 5 mL of double distilled water was added at the start of digestion. The bovine liver reference material was then microwaved with the samples at 110 °C with 10-minute ramp to temperature followed by a 5-minute hold time for one round for samples; the BCR powder was not completely digested after the first round of microwaving and subjected to a second round of microwaving with an additional 5 mL of nitric acid added. Following microwaving, 5 mL of DDI water was added to all samples, bovine reference materials, and digest blanks.

Three digest blanks were tested, which were composed of 5 mL of concentrated Optima nitric acid and digested by the exact same method as the samples. All run samples were prepared by adding 4.2 mL of diluent and 75 μL of MeOH (for carbon enhancement of the As signal) to 400 μL of acid digests (including samples, RMs, and digest blanks) or calibration standard stocks. Six point calibration standards were prepared from a stock standard of As 100 mg/mL (High Purity Standards, North Charleston, SC) at 0.1, 0.5, 2, 10, 50, and 500 μg/L. Standards were prepared fresh before the run from intermediate solutions containing 2% Optima HNO_3.

The diluent used in all samples contained: 0.5% (v/v) concentrated Optima grade hydrochloric acid, 5 μg/L Peak Performance Multi-elemental internal standard (CPI International, Santa Rosa, CA), 0.005% (v/v) Triton-X detergent solution (Sigma Aldrich, St. Louis, MO), and DDI water. Triton-X is a detergent used in the diluent to help proteins become better solubilized in the solution [137]. Yttrium (Y) was used as the internal standard element for normalization of the arsenic signal.

Table 5. Total Arsenic EDTA Comparison Experiment Set-Up. Evaluation of arsenic extraction efficiency using EDTA. Total arsenic was measured in 12 organs via ICP-MS. The organs were split up into four groups: 1) 1000 PPB dosed mouse organs with no EDTA, 2) 1000 PPB dosed mouse organs with EDTA, 3) 0 PPB dosed mouse organs with no EDTA 4) 0 PPB dosed mouse organs with EDTA

Table 5. Total Arsenic EDTA Comparison Experiment Set-Up	
No EDTA	EDTA
Female 1000 PPB Dosed Mice	
1 Lung 1 Liver 1 Spleen	1 Lung 1 Liver 1 Spleen
Female Control Mice (0 PPB)	
1 Lung 1 Liver 1 Spleen	1 Lung 1 Liver 1 Spleen

2.2.6 MMA Separation

Because MMAIII is the most toxic arsenic species, MMAIII and MMAV were separated from total MMA (MMAIII + MMAV) by following the exact methods established by Reay and Asher in 1977 [138]. Sulfuric acid (Fisher Scientific, Waltham, MA), *Optima* grade nitric acid, sodium meta-bisulfite (EM Chemicals, Burlington, MA) and sodium thiosulfate (Sigma-Aldrich, St. Louis, MO) were combined to make the Reay and Asher reagent. National Institute of Standards and Technology (NIST) (Gaithersburg, MD) standard reference material for MMA total (1ppm, 17.64 mg/kg (+-) of MMA) was left in a biosafety hood overnight to permit oxidation of the arsenic species. The 1 ppm reference material was then added to reagents in a one to one ratio and incubated at room temperature (25°C) for eighty-one minutes.

2.2.7 Quality Control

2.2.7.1 Standard Reference Materials

Standard reference materials (SRMs) help to evaluate efficiency, percent recovery, and detection. Certiprep inorganic AsIII and iAsV standards were ordered from Spex (Metuchen, NJ). The iAsV standard has 1000 mg/L of iAsV in H_2O while the iAsIII standard has 1000 mg/L iAsIII in 2% HCl. DMA and MMA standards were ordered from National Institute of Standards and Technology (NIST) (Gaithersburg, MD). A total DMA SRM (combined DMAIII and DMAV, SRM# 3031) was ordered from NIST which has 20.47 mg/kg of DMA (k= 2.73). Lastly, a total MMA standard (combined MMAIII and MMAV, SRM# 3030) was ordered from NIST with a concentration of 17.64 mg/kg (+-) of MMA (k= 2.12).

2.2.7.2 Calibration Standards for Speciation Preparation

For each arsenic species, a five-point calibration curve was constructed. Calibration concentrations of: 0, 25, 50, 100, 500, and 1000 ppb of combined arsenic species (MMA total, DMA total, iAsIII, and iAsV) were used. Standards were prepared by serial dilution using a 1000 ppb mixed species solution, diluting with the mobile phase.

Initial Concentration (Ci) x Initial Volume (Vi) **Equation 1**
= Final Concentration (Cf) x Final Volume (Vf)

2.2.7.3 Total Arsenic Mass Fraction Calculations

The mass fractions, or μg of arsenic per gram of tissue, was calculated using the mass of arsenic obtained from the ICP-MS divided by the organ mass (equation 2). The total Volume of Digest (V_T, in mL) was obtained by adding the volume of sample during extraction (V_S, in mL) plus the 10 mL of digestion acid (nitric acid).The mass of As (in μg)

per g of tissue was calculated by multiplying the concentration from the ICP-MS (μg per L) by the total volume of digest (L).

Mass Fraction = μg of As / Organ Weight (g) **Equation 2**

2.2.7.4 Limit Of Detection

Three digest blanks (composed of nitric acid) were used to calculate the limit of detection (LOD) and the average was used for blank subtraction. The LOD was calculated to be 0.161 ug of arsenic/L and was determined by equation 3.

LOD (ug/L) = 3 x (standard deviation of the blanks) **Equation 3**

Blank subtraction was done to account for noise on the ICP-MS, potential contamination of the reagents, and to measure interferences or suppression of the signal which would be caused by the chemical or the digestion methods. The three digest blanks were averaged to be 1.67 μg/L for blank subtraction.

2.2.7.5 Spiked Sample Methods

In order to evaluate the sensitivity of our method to detect arsenic species in the presence of the complex tissue matrix, 6 tissue samples were spiked with 1000 ppb of standard reference materials (SRM) described in section 2.6.1 for iAsIII, iAsV, MMA, and DMA in mobile phase (2 lungs, 2 livers, and 2 spleens). Equation 1 was used to determine the amount of each SRM added to each organ. After the tissues were spiked, they were processed following our EDTA extraction method described above (Figure 1). After extraction, each sample was analyzed using HPLC-ICP-MS.

2.3.0 Results

Arsenic extraction and analysis optimization studies were conducted using the HPLC-ICP-MS operating conditions described above. Total arsenic, as well as several arsenic species were detected in spiked samples. The mobile phase was determined to be the most advantageous at a pH of 6.

2.3.1 Arsenic Extraction for Speciation Results

2.3.1.1 Extraction Method Results

Extraction with Heat. Out of all the heated organ samples (one lung, one liver, and one spleen), the final supernatant of the spleen was the only organ that could go through a 0.2 μm syringe filter. The spleen supernatant could filter through the 0.2 μm filter only if the supernatant went through the 0.8 μm filter first.

Extraction with TMAH. None of the organs treated with TMAH during extraction could be filtered with a 0.8 μm syringe filter. For the liver and spleen samples, following the first and second centrifuge steps, the resulting supernatants presented with precipitates that tore the 0.8 μm filter.

2.3.1.2 Extraction with EDTA Results

Supernatant from all organs treated with EDTA passed through both 0.8 and 0.2 μm filter sieves and therefore were the only ones that were analyzed using the HPLC-ICP-MS. Figure 2 shows tissue treated with and without EDTA.

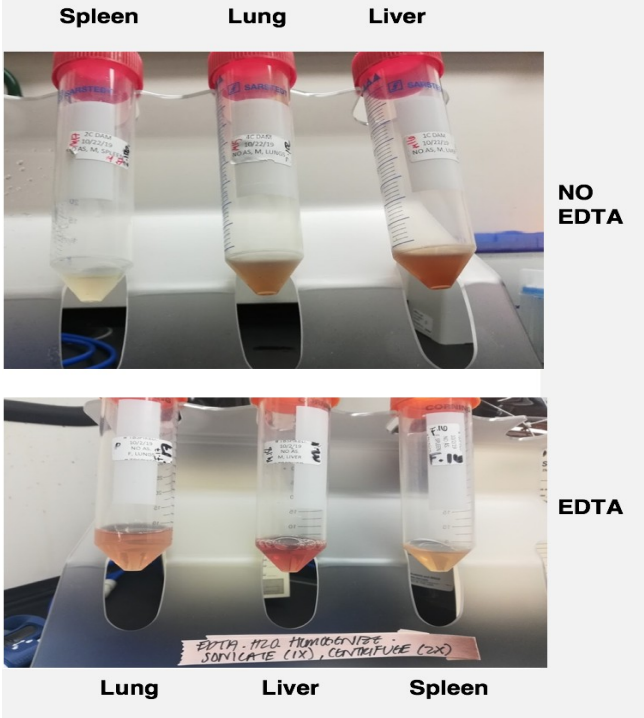


Figure 2. EDTA Treated Tissue for Arsenic Species Extraction. Mouse lungs, livers, and spleens were extracted for HPLC-ICP-MS analysis using EDTA to prevent coagulation of tissue. In the non-EDTA treated tissue, supernatant is cloudy, opaque, and could not pass through a 0.2 μm filter. The EDTA treated organs, successfully passed through a 0.2 μm filter for HPLC-ICP-MS analysis.

2.3.1.3 Extraction of Total Arsenic from EDTA Results- Treated Tissue of Dosed Mice

Tissue treated with EDTA (compared to tissue not treated with EDTA) does not influence the recovery of arsenic within organs (Table 6 and Table 7). Recoveries of total arsenic were similar with EDTA treated tissue and non-EDTA tissue, for the 1000 ppb dosed mice and 0 ppb dosed mice.

Table 6. Evaluation of EDTA for Extraction of Arsenic from Dosed Tissues. Recoveries of total arsenic were similar with EDTA treated tissue, non-EDTA tissue, 1000 ppb dosed mice, and 0 ppb dosed mice.

Table 6. Evaluation of EDTA for Extraction of Arsenic from Dosed Tissues									
Organ Sample	Concentration (PPB)	CPS	Organ Wt (g)	Extract Volume (mL)	Total Volume of Digest (L)	Volume Digest (µg of As) x Blank Subtractio	Mass Fraction with Blank Subtraction (µg As / g tissue)	Certified Value	Percent Recovery
Lung Samples									
1000PPB, Lung with EDTA	6.87	589	0.878	1.5	0.012	0.079	0.090		
1000PPB, Lung without EDTA	6.43	542	0.101	1.5	0.012	0.074	0.732		
0PPB, Lung with EDTA	9.94	919	0.685	1.5	0.012	0.114	0.167		
0PPB, Lung, without EDTA	7.98	709	0.104	1.5	0.012	0.092	0.883		
Liver Samples									
1000PPB, Liver with EDTA	6.19	517	0.687	1.8	0.012	0.073	0.106		
1000PPB, Liver without EDTA	6.94	597	0.869	1.8	0.012	0.082	0.094		
0PPB, Liver with EDTA	6.97	600	0.724	1.8	0.012	0.082	0.114		
0PPB, Liver without EDTA	4.43	328	0.849	1.8	0.012	0.052	0.062		
Spleen Samples									
1000PPB, Spleen with EDTA	5.58	451	0.083	1.25	0.011	0.063	0.756		
1000PPB, Spleen without EDTA	5.88	483	0.091	1.25	0.011	0.066	0.727		
0PPB, Spleen with EDTA	4.92	380	0.095	1.25	0.011	0.055	0.582		
0PPB, Spleen without EDTA	5.82	477	0.085	1.25	0.011	0.065	0.770		
BCR1	3.33	210	0.56	0	0.01	0.017	0.030	0.03	99%
BCR2	3.31	208	0.56	0	0.01	0.016	0.029	0.03	98%
DB1 (Digest blank)	1.59	23.3							
DB2 (Digest blank)	1.71	35.6							
DB3 (Digest blank)	1.71	35.6							
Water CRM	100	10629							

*BCR – Bovine liver certified reference material *DB – Digest blank *CPS- Counts per second *CRM- Certified Reference Material

2.3.1.4 Calibration Results

The calibration curve confirmed it is possible to see all four species within one chromatogram (Figure 3). The calibration standard counts at 1 ppb are higher than those at 0 ppb (the mobile phase) which confirms that we can detect arsenic species at concentrations as low as 1 ppb (Table 7). The R^2 values reveal the calibration curve is linear for all arsenic species (see Figure 5A and 5B for reference). R^2 values are as follows: iAsIII = 0.9758, DMA = 0.9999, MMA= 0.9998, iAsV = 0.9929. Calibration was completed through MassHunter which produced counts per second (cps).

Table 7. Arsenic Species Calibration Curve Data. Arsenic species were calculated at 1, 5, 25, 50, 100, 500, and 100 ppb of arsenic species. HPLC-ICP-MS data revealed chromatographic peak areas and counts per second.

Table 7. Calibration Counts and Peak Areas from HPLC-ICP-MS								
Standard Conc (ppb)	iAsIII		DMA		MMA		AsV	
	Area	Count	Area	Count	Area	Count	Area	Count
0 (mobile phase)	2.20E1	2.20E1	1.30E1	1.30E1	2.90E1	2.90E1	2.90E1	2.90E1
1	6.10E1	6.10E1	3.68E3	3.68E3	5.82E3	5.82E3	1.59E3	1.59E3
5	1.06E2	1.06E2	2.21E4	2.21E4	2.14E4	2.14E4	3.62E3	3.62E3
25	1.00E3	1.00E3	1.07E5	1.07E5	1.13E5	1.13E5	1.68E4	1.68E4
50	1.56E3	1.56E3	2.28E5	2.28E5	2.28E5	2.28E5	3.80E4	3.80E4
100	7.89E3	7.89E3	4.65E5	4.65E5	4.80E5	4.80E5	8.93E4	8.93E4
500	1.07E5	1.07E5	2.29E6	2.29E6	2.29E6	2.29E6	3.62E5	3.62E5
1000	2.95E5	2.95E5	4.47E6	4.47E6	4.47E6	4.47E6	6.24E5	6.24E5

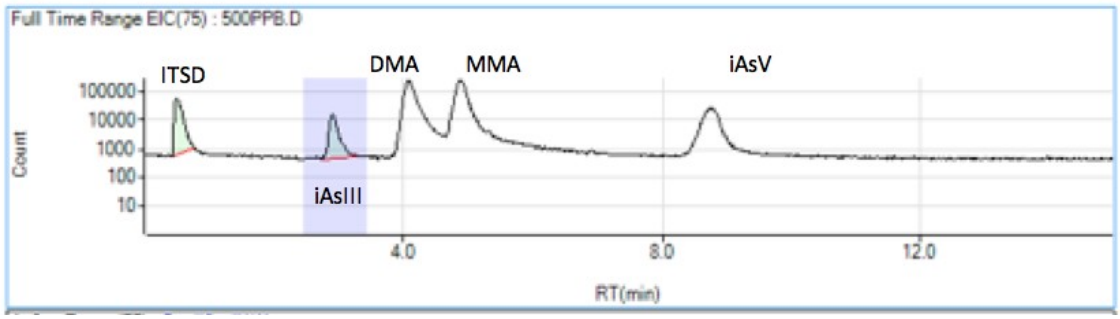


Figure 3. Anion Exchange Chromatogram Showing 500 ppb Mixed Species Standards (iAsIII, DMA, MMA, iAsV). ISTD of 5ppb iAsV was injected before the start of each run. PRP X-100 (250 X 4.6MM) column with 1mL/min flow rate. MP= MeOH $(NH_4)_3PO_4$ and EDTA, pH of 6. As species detected by ICP-MS in He mode at m/z of 75.

Figure 4.A

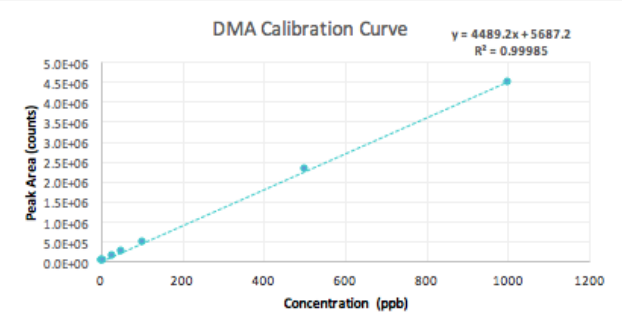


Figure 4.B

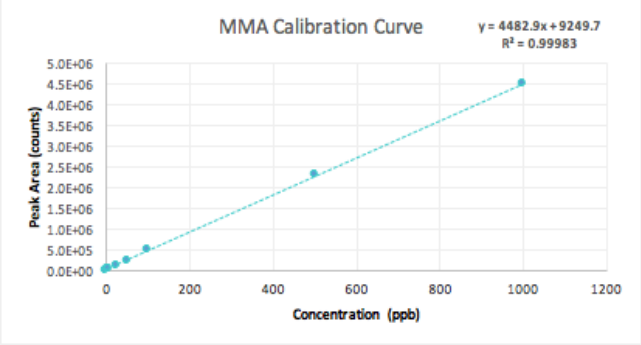


Figure 4. DMA and MMA Species Calibration Curve. A) DMA Calibration Curve from 0 to 1000ppb standards, DMA R^2 = 0.9999 **B)** MMA Calibration Curve from 0 to 1000ppb standards, MMA R^2= 0.9998.

2.3.1.5 Spiked Sample Results

1000 ppb spiked lung, liver, and spleen organs were analyzed for the recovery of iAsIII, DMA, MMA, and iAsV (Table 8). Recovery of each arsenic species was seen in each organ. MMA is noted to be too sensitive and therefore, percent recovery does not appear to be accurate. The greatest percent recovery was from iAsV species for all three organs. All organs yielded chromatograms with all four arsenic species (Figure 5).

Table 8. Spiked Organ Arsenic Species Concentrations and Percent Recoveries.

Lung, liver, and spleens were spiked with 1000 ppb of mixed arsenic species standards and observed for percent recoveries. Expected concentration of spike is 167.5 ppb.

Table 8. Spiked Organ Arsenic Species Concentrations and Percent Recoveries									
Mouse Organ		iAsIII		DMA		MMA		iAsV	
1000 ppb Spiked Organs	Organ Wt (g)	Conc. (ppb)	Percent Recovery	Conc. (ppb)	Percent Recovery	Conc. (ppb)	Percent Recovery	Conc. (ppb)	Percent Recovery
Spiked Lung	0.18	161	96	112	67	394	235	179	107
Spiked Liver	1.35	37	22	80	48	4.62E6	2.76E6	155	93
Spiked Spleen	0.25	23	13	13	8	4.30E6	2.57E6	24	14

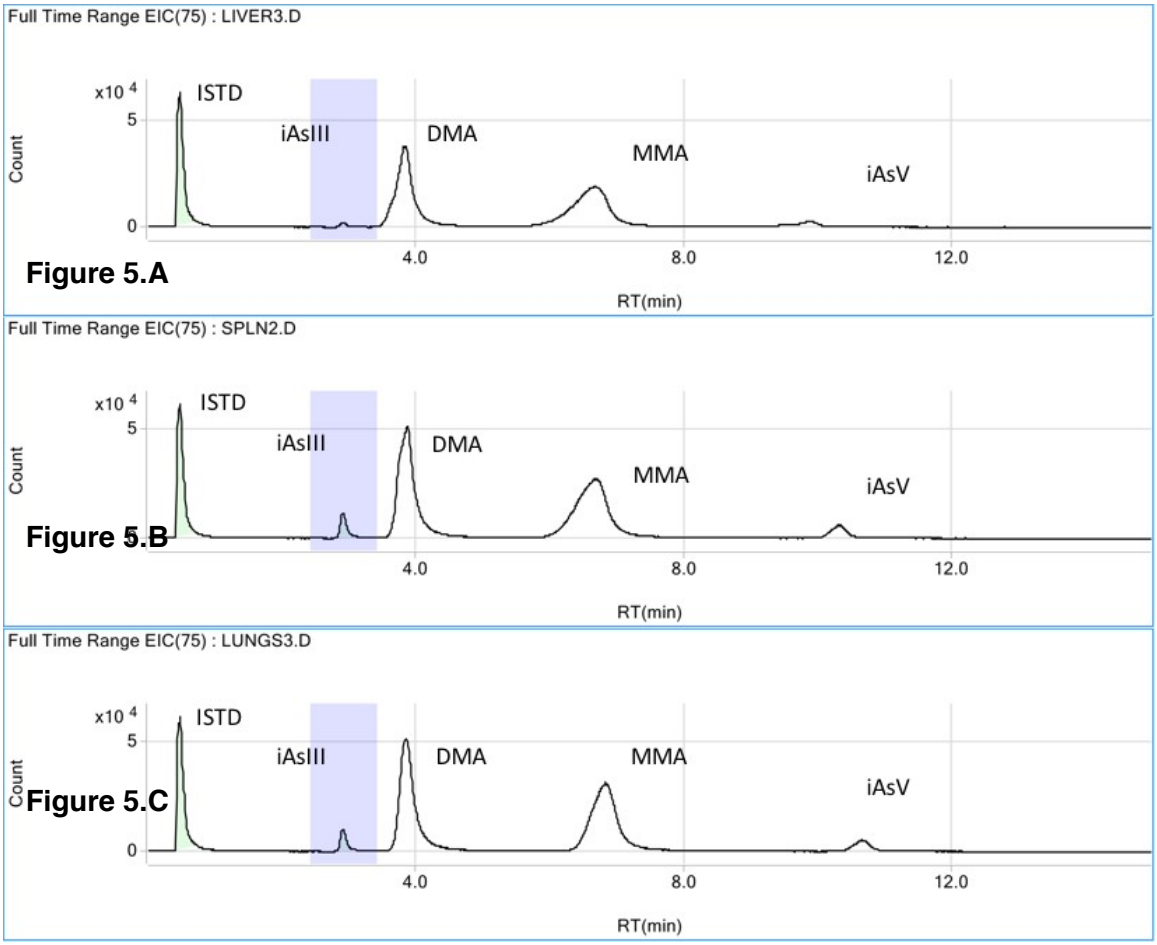


Figure 5. Spiked 1000 ppb Lung, Liver and Spleen Chromatogram. Chromatograms showing 1000 ppb multi-species Spike-In **A)** Liver **B)** Spleen and **C)** Lung. PRPX-100 (250 X 4.6MM) column, 1 mL/min flow rate. MP= MeOH $(NH_4)_3PO_4$ and EDTA, pH of 6. As species detected by ICP-MS in He mode at m/z of 75. The first peak seen in each chromatogram is the internal standard (ISTD) of 5 ppb iAsV. Peaks were identified as follows peak at around 120 seconds or 3 minutes) is iAsIII. Peak at around 4 minutes is DMA. Peak seen before eight minutes is MMA. The last peak is iAsV, which is the most polar of the species and therefore comes off the column last.

2.3.1.6 Arsenic Speciation Results

Pilot data was obtained from HPLC-ICP-MS to ascertain the effectiveness of arsenic speciation extraction (Figure 1). DMA was found in the 1000 ppb dosed male lung at a concentration of 110 ng of DMA per gram of tissue (Table 9).

Table 9. Arsenic Speciation in 0 ppb and 1000 ppb Dosed Tissue via HPLC-ICP-MS

Table 9. Arsenic Speciation in 0 ppb and 1000 ppb Dosed Tissue via HPLC-ICP-MS						
1000 ppb Samples			Mass Fractions (ng As Species/ g Organ)			
Organ Type		Organ Wt (g)	iAsIII	DMA	MMA	iAsV
0 ppb (Control)	**0 ppb Lung**	**0.177**	1	1	8163	0
	0 ppb Liver	**1.344**	0	0	386	0
	0 ppb Spleen	**0.128**	0	1	2499	0
1000 ppb Dosed	**1000 ppb Lung**	**0.265**	0	110	5452	0
	1000 ppb Spleen	**0.187**	0	1	5689	0

2.3.1.7 MMA Separation Results

MMAIII and MMAV were successfully separated (Figure 6) using methods from Reay and Asher (1977) [138]. Peaks could not be assigned to the respective MMA species.

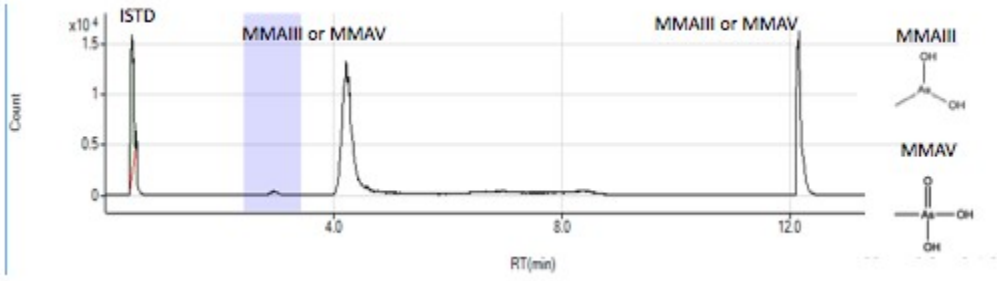


Figure 6. MMA Species Separation. Anion exchange chromatography showing 1000 ppm NIST MMA standard being separated to MMAIII and MMAV. The separation of MMAIII and MMAV was successful. A PRPX-100 (250 X 4.6MM) column used a 1mL/min flow rate. MP= MeOH $(NH_4)_3PO_4$ and EDTA, pH of 9.

2.4.0 Discussion and Future Directions

2.4.1 Arsenic Extraction Discussion and Future Directions

2.4.1.1 Extraction with Heat Discussion and Future Directions

Temperature changes during extraction proves to challenge the identification of arsenic species [24, 25]. Some studies allow samples to sit as long as 12 hours at room temperature [35, 36] however, this was decided against to prevent interconversion of arsenic species [37]. Organs were heated in this experiment to replicate the methods of Guimaraes et al. (2018) [38] as well as other arsenic extraction methods which utilize heat [39-41]. Heating was discovered to be ineffective at breaking down tissue to 0.2μm and furthermore, heating can break down the tissue to the point of hypopigmentation of the tissues.

2.4.1.2 Extraction with TMAH Discussion and Future Directions

TMAH not only was unsuccessful in breaking down tissue, but it is a strong base and therefore may cause speciation changes, such as trivalent species converting to pentavalent species [42]. Furthermore, TMAH can influence ion chromatography products [42]. Although many studies suggest TMAH use with arsenic speciation [131, 132] it was deemed unfit for this project after method development.

2.4.2 Arsenic Speciation Discussion and Future Directions

Arsenic speciation analysis with HPLC-ICP-MS has been successful with urine samples [25, 44, 45]. However, mammalian tissues are a more complicated matrix as one has to break down the organic samples without ruining the integrity of the chemicals, for example MMAIII and DMAIII are unstable in oxidative conditions. HPLC-ICP-MS may commonly be used for the analysis of arsenic species in aqueous matrixes. However, its success is limited by the extraction methods to convert a solid to a liquid (which can result in the loss of integrity of the species) and it relies on matching peaks to standards [46].

It is challenging to observe both trivalent and pentavalent species in one complex as trivalent species will oxidize while pentavalent reduce- especially the methylated species

[113, 117, 118]. It may serve as advantageous to extract and test for the trivalent or pentavalent species separately. However- this would not be representative of the species present in the tissue at the time of sacrifice or exposure. One would be reducing all of the pentavalent species (and vice versa) and therefore, the recorded concentrations would be an over representation of the presence of a species.

Although isocratic elution has been successful in separating arsenic species [139] a step gradient analysis may have proved effective as gradient separations can yield a better resolution, and faster reduction of retention times [33]. For example, B'Hymer and Caruso (2002) successfully separated AsC, AsB, AsIII, DMAV, MMAV and AsV from apple samples with the same column (PRPX-100) following a gradient elution [114]. Nonetheless, isocratic elution keeps the chromatographic system in a permanent equilibrium, which is particularly convenient for routine analysis (as reviewed by Benramdane et al. (1999) [37]).

2.4.3 MMA Separation Discussion and Future Directions

MMAIII and MMAV were successfully separated (Figure 6), however; peaks cannot be assigned to the correct MMA species as the m/z ratio of the ICP-MS was set to only read a mass of 75 and; therefore, cannot determine constituents with molecular weight. Since Reay and Asher's methods were designed in 1977 and have seldom been repeated, it is important to note that the methods can successfully separate MMA species and should be used in the future [138]. Because of sulfhydryl groups in the reagent mixture, the extraction methods cannot be repeated with DMA as other metabolites will be present [126].

2.4.4 Extraction of Total Arsenic from EDTA- Treated Tissue of Dosed Mice

There was no significant difference in the mass fractions of arsenic recovered from tissues from mice that had been dosed with 1000 ppb and those who had not been dosed. EDTA did not improve the recovery (Table 5). Total arsenic analysis with more samples needs to be completed to confirm results. Furthermore, total arsenic in whole organs was analyzed in this thesis. For the arsenic speciation analysis, final extracted samples which were injected by the HPLC-ICP-MS were in supernatant form and the pellet was not used for analysis (Figure 1). To further understand how and where arsenic species congregate, it would be beneficial to attain total arsenic concentrations of both the supernatant and the pellet of the extracted tissue separately. The pellet, however, cannot be analyzed for speciation by HPLC as it cannot break down to 0.2 μm, as the instrument requires.

2.5.0 Conclusion

Methods to extract arsenic species from tissue were developed (Figure 1), but additional experiments to improve their efficacy must be conducted. EDTA proved to be beneficial to attain a tissue particle size less than or equal to .2 μm - which is necessary to avoid impurities from obstructing the HPLC column. Calibration curves confirmed that separation and determination of four arsenic species (iAsIII, DMA, MMA, and iAsV) is possible. When 1000 ppb spiked lung, liver, and spleen tissue were extracted, all four stock standards (iAsIII, DMA, MMA, and iAsV) were detected by the HPLC-ICP-MS. Total arsenic analysis via ICP-MS did not reveal much of a difference between 0 ppb and 1000 ppb dosed tissue (Table 6). Additional trials must be completed to explore the results. A pilot trial using tissues from mice exposed to 1000 ppb in-vivo determined 110 ng of DMA per gram of tissue in a murine lung. The pilot study was not able to determine other species in the lung or in a spleen tissue (Table 9). This thesis promotes further exploration to compare arsenic speciation within tissues. Arsenic speciation within organs complemented by total arsenic analysis is particularly valuable as it gives a ratio of arsenic within the organ. The findings from this research intend to help inform

the current state of arsenic speciation to define the variability of toxic species of arsenic within the metabolism.

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CURRICULUM VITAE

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EDUCATION

MASTER OF SCIENCE IN ENVIRONMENTAL HEALTH ENGINEERING — Expected May 2020
JOHNS HOPKINS BLOOMBERG SCHOOL OF PUBLIC HEALTH
THESIS: ARSENIC SPECULATION WITHIN THE MAMMALIAN METABOLISM

BACHELOR OF SCIENCE IN COMMUNITY HEALTH
UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN — May 2014
Concentration in Health Education

CERTIFICATIONS

JOHNS HOPKINS BLOOMBERG SCHOOL OF PUBLIC HEALTH — Baltimore, MD
Certificate of Risk Sciences and Public Policy — May 2019
Certificate of Humane Sciences and Toxicology

AMERICAN ACADEMY OF PHLEBOTOMY TECHNICIANS — Baltimore, MD
Phlebotomy Technician Certification — Jan. 2019

AMERICAN HEART ASSOCIATION — Downers Grove, IL
Cardiopulmonary Resuscitation, AED & First Aid Certification — Aug. 2017

TECHNOLOGY CENTER OF DUPAGE — Addison, IL
Nursing Assistant Certification — May 2009

RESEARCH EXPERIENCE

GRADUATE RESEARCH ASSISTANT — Baltimore, MD
EXPOSURE SCIENCES & ENVIRONMENTAL EPIDEMIOLOGY LABORATORY — Sept. 2018 - Present
Principal Investigator: Ana Maria Rule, PhD
Johns Hopkins Bloomberg School of Public Health

- Independently conduct analysis with high performance liquid chromatography (HPLC) and inductively coupled plasma mass spectroscopy (ICP-MS)
- Examine the presence of metals and harmful compounds within electronic cigarette devices
- Collect and analyze biospecimens from cigarette smokers, non-smokers, and electronic device smokers
- Set up air monitors to measure pollution from anthropogenic sources (incinerators, uncovered coal ash, waste sites, densely trafficked truck routes)
- Recruit participants, conduct surveys, and establish community relationships

GRADUATE RESEARCH ASSISTANT — Baltimore, MD
TOXICOLOGY, PHYSIOLOGY, & MOLECULAR MECHANISMS LABORATORY — Sept. 2018 - Present
Principal Investigator: Fenna Sillé, PhD

Johns Hopkins Bloomberg School of Public Health

- Investigate the impact of arsenic exposure in murine models which includes handling, sacrificing, and collecting specimens per IACUC regulation
- Develop extraction techniques of mammalian lungs, livers, and spleens for further analysis via HPLC
- Learn toxicology laboratory techniques such as enzyme-linked immunosorbent assays (ELISAs), real- time polymerase chain reactions (RT-PCR), tissue culture, & peripheral blood mononuclear cell (PBMC) extraction from whole blood

RESEARCH AND MEDICAL ASSISTANT — Naperville, IL
HOBSON MEADOWS FAMILY MEDICINE — Oct. 2014 - Nov. 2016

- Optimized research with the Agency for Healthcare Research and Quality to assess the delivery of primary care and elaborate on the needs of its patients
- Prepared spreadsheets through SPSS and Excel to study patient demographics and clinic habits
- Served as a liaison between insurance agencies and pharmacies to ensure appropriate charges, coverage, and benefits
- Independently performed radiological and specimen testing as well as assisted with minor surgical procedures

TEACHING EXPERIENCE

TEACHING ASSISTANT: RISK POLICY, MANAGEMENT, & COMMUNICATION — Baltimore, MD
DEPARTMENT OF HEALTH POLICY AND MANAGEMENT — Oct. 2019 - Present
Johns Hopkins Bloomberg School of Public Health

- Help graduate students understand decision making and communication within public policy
- Promote students to examine societal context for scientific, economic, and legal reasons
- Grade assignments, aid the professor with technology, and keep track of student performance

TEACHING ASSISTANT: QUANTITATIVE TOOLS FOR MANAGEMENT — Baltimore, MD
DEPARTMENT OF HEALTH POLICY AND MANAGEMENT — June 2019 - July 2019
Johns Hopkins Bloomberg School of Public Health

- Taught graduate students how to use analytical tools such as Microsoft Access, Microsoft Excel (with Analysis Toolpak), and Tableau Public for healthcare facility management

BIOLOGY AND SPANISH TUTOR — Glen Ellyn, IL
COLLEGE OF DUPAGE — Aug. 2013 - Jan. 2014

- Approached by professors and individual students to tutor one-on-one and lead group study
- Taught beginner to intermediate levels of biology and Spanish levels III
- Created study guides and worksheets to cater to each student

PROFESSIONAL ASSOCIATIONS

VICE PRESIDENT OF PUBLIC HEALTH PROMOTION & COMMUNITY AFFAIRS — Baltimore, MD
April 2019 -

JOHNS HOPKINS SCHOOL OF PUBLIC HEALTH STUDENT ASSEMBLY Present

- Elected by student body to establish relationships with community representatives of Baltimore city for the Bloomberg School of Public Health
- Lead events on campus to cater to underserved populations (food drives, school supply donations, and health advocacy seminars, etc.)

HEALTH EDUCATOR AND ADVOCATE Downers Grove, IL
COMPTIA WELLNESS COMMITTEE June 2017 - Aug. 2018

- Implemented and designed programs for staff wellness and preventative medicine
- Collected data regarding demographics and health determinants to determine the best forms of health promotion and preventative medicine for international employees of CompTIA
- Set-up and personally give presentations regarding a wide array of health topics

COMMUNITY MEMBER Downers Grove, IL
TECHNOLOGY ASSOCIATIONS June 2017 - Aug. 2018

Advancing Diversity in Technology Community
Advancing Women in Technology Community
Information Technology Future Leaders Community

TREASURER Urbana, IL
ILLINI FORESTERS Jan. 2012 - May 2014

- Volunteered to maintain the ecological health of forest preserves, parks, prairies, and plantations
- Assisted with cleanup of local parks, controlled burning of tall grass prairies, planting trees, guiding trail walks, and assisting local landowners with conservation protocols
- Competed in university forestry conclaves

HONORS AND AWARDS

MCKINLEY HEALTH CENTER'S SPECIAL POPULATIONS
Distinguished Alumni Award
Awarded December 2017
Urbana-Champaign, IL

UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN
Institutional Honors: Spring 2014
Deans List: Spring 2013
Urbana-Champaign, IL

EDWARD HOSPITAL
Academic Employee Scholarship
Awarded for the following terms:
Fall 2009 & 2010, Spring 2010 & 2011
Naperville, IL

COMMUNITY INVOLVEMENT

FUNDRAISING VOLUNTEER COORDINATOR Elgin, IL
HOULIHAN CLAN FOUNDATION May 2016 - Present

- Monitor and help plan events which raise money for families who face financial burden or adversity due to medical issues
- Count donations, find vendors to donate items, and assure success of annual events

FUSE STUDIO VOLUNTEER Glen Ellyn, IL
HADLEY JUNIOR HIGH SCHOOL Aug. 2017 - Aug. 2018

- Supported middle-school students complete challenges regarding science, technology, engineering, art, and mathematics
- Promoted hands on learning by helping students create bionic arms, three-dimensional printed structures, architectural designs, and other independent learning modules

COMPOSTING AND GENERAL HORTICULTURE VOLUNTEER Chicago, IL
GARFIELD PARK CONSERVATORY ALLIANCE Sept. 2017 - Aug. 2018

- Completed different methods of composting and gave demonstrations to the public, as well
  school groups, to advocate its importance to the environment
- Gained knowledge of tree and plant identification
- Maintained propagation for greenhouses, community plots, and gardens

TECH VOLUNTEER Chicago, IL
TECHGIRLZ Nov. 2017 - Aug. 2018

- Empowered women and young girls to build and understand technology
- Endorsed free workshops which teach IT concepts such as programming, application design, computer components, circuit board set-up, virtual/ augmented reality, etc.

GIRL SCOUTS AND CREATING IT FUTURES VOLUNTEER Vernon Hills, IL
GIRL SCOUTS OF GREATER CHICAGO AND NORTHWEST INDIANA Oct. 2017

- Provided mentorship to pre-teenage girls by fostering independence and problem solving
- Encouraged critical thought to formulate solutions to barriers in assignments

CHILDCARE VOLUNTEER Urbana, IL
CRISIS NURSERY Aug. 2011 - Mar. 2012

- Delivered a nondiscriminatory environment which assured safety to children and families of
  abuse or neglect
- Provided emergency childcare in addition to working weekly

BIG SISTER VOLUNTEER Joliet, IL
BIG BROTHERS BIG SISTERS OF WILL AND GRUNDY COUNTIES Aug. 2010 - Aug. 2011

- Established relationships with children who face adversity to improve their quality of life, encourage success, and offer guidance
- Authorized medical contact in case of emergency

ADDITIONAL EXPERIENCE

MEMBER OPERATIONS COORDINATOR Downers Grove, IL

COMPTIA: INFORMATION TECHNOLOGY INDUSTRY & ASSOCIATION — April 2017 - Aug. 2018

- Regularly presented at technology education seminars to acknowledge trends and incoming concerns within technology
- Promoted technology certifications, research, and education through a non-profit
- Managed international member accounts of technology vendors, distributors, professors, and students
- Assisted in finance and organization of the charitable program "Tech Leaders Giving Circle"

SPECIAL POPULATIONS' OUTREACH COORDINATOR & EDUCATOR — Oswego, IL — Jan. 2012 - May 2014
MCKINLEY HEALTH CENTER

- Presented research findings at university classrooms, cultural centers, medical institutions, and community facilities
- Planned and led events for socioeconomically underserved subgroups to ensure the delivery of health education, medicine, and clinic resources
- Created, promoted, and lead health prevention programs for the public
- Fundraised for each event and was responsible for the management of their budget

SURGICAL INTERN — Urbana, IL
PRESENCE COVENANT MEDICAL CENTER — Aug. 2013 - Aug. 2014

- Audited surgical operations and introduced programming to raise efficiency of five departments
- Instilled protocols of quality assurance and good clinical practice for staff to follow
- Shadowed surgeries and made patient rounds following procedures

PATIENT SUPPORT MANAGER, DISPATCHER, & PATIENT TRANSPORTER — Glen Ellyn, IL — Dec. 2010 - Aug. 2012
EDWARD HOSPITAL

- Volunteered for two years then hired to manage the scheduling of over 300 patients
- Responded to all hospital codes or assigned personnel to aid with trauma

PRESENTATIONS & POSTER SESSIONS

Metallotherapeutics Research Center's first Mid-Atlantic Symposium — Baltimore, MD — November 2019
"Frontiers in Metals in Medicine" University of Maryland School of Pharmacy
Presented "Determination of Arsenic Speciation in Mammalian Tissue through HPLC-ICP-MS to Explore Gender, Dose, and Toxicological Influences"

CompTIA Deep Dive Presentation Series — Downers Grove, IL
Technology Innovations and Research — October 2017
Presented "Deep Dive: A Breakdown of CompTIA's Content & Technology Standards"

ADDITIONAL COMPETENCIES

LABORATORY SKILLS	RT-PCR, tissue culture, animal handling, HPLC, ICP-MS, ELISAs, as well as air monitoring via impactors, small samplers, and total dust cassettes
LABORATORY SOFTWARE	MassHunter and IRIS
ELECTRONIC MEDICAL RECORDS	E-Clinical Works, Epic, and Meditech

STATISTICAL SOFTWARE	MS Excel, Minitab, Smartsheet, Crystal Ball, SPSS, and Tableau
GRAPHICS & DESIGN	Adobe Illustrator and Adobe Photoshop
LANGUAGES	Arabic and Spanish

CONTACTS FOR REFERENCE

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