ARSENIC SPECIATION OF SWINE URINE FOR POSSIBLE USE IN HUMAN EXPOSURE ASSESSMENTS

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By

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The undersigned, appointed by the Dean of the Graduate School,

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ABSTRACT

Millions of people are exposed to arsenic in the United States and worldwide. Commonly found arsenic species in human urine are AsIII (arsenite), AsV (arsenate), MMA (monomethyl arsenic acid), DMA (dimethylarsinic acid) and AB (arsenobetaine). Evidence has shown that these species vary in toxicity, and since each of these metabolites can be detected through analysis, they have the potential to be used as biomarkers for human exposure. For human exposure assessments in areas that have naturally occurring arsenic contaminated sources, or those who live or work near contaminated environmental sites where arsenic has been used, it is important to fully understand what species of arsenic residents are being exposed to in order to grasp the risk of arsenic exposure specifically and in its entirety.

Since it is difficult to determine direct human exposures, a swine model was used as a surrogate. Swine urine was collected from two different swine studies where animals were given non-toxic doses of arsenic contaminated soil and another group receiving a soluble reference dose using sodium arsenate for comparison. The urine samples from these studies were used to modify an arsenic speciation method using high-performance liquid chromatography and inductively coupled plasma mass spectrometry (LCICPMS). It is evident that when comparing the percent of arsenic species found in swine urine samples with what is found in humans a correlation can be made. There was a range of 64-74% DMA in swine samples for all test soils where a range of 60-75% DMA has been reported in human urine samples. This further

illustrates the importance of arsenic speciation in swine urine since it does appear that it could correlate to human exposure. If proper measurement systems are utilized to quantify As species of health concern, dosed swine can be used to assess and predict human toxicological effects of arsenic exposure.

CHAPTER 1 INTRODUCTION

Overview

Millions of people are exposed to arsenic in the United States and worldwide. Arsenic is a naturally occurring metalloid; meaning that is has both metal and water properties and that is widely distributed in the Earth's crust (ATSDR, 2007). Depending on the form of arsenic, whether it is in an inorganic or organic state, can affect outcomes of exposure to populations due the fact that inorganic arsenic is significantly more toxic then organic arsenic. Inorganic arsenic is well absorbed from the gastrointestinal tract and then distributed through the body while being metabolized by methylation and then excreted primarily by urine (Klaassen, 2008). The methylation process provides a unique look at the toxicity of arsenic because it is metabolized quite differently in the body depending on chemical species (ATSDR, 2007). Not only does arsenic have the potential to cause significant toxicity, but the U. S. Department of Health and Human Services, the International Agency for Research on Cancer (IARC) and the U. S. Environmental Protection Agency (EPA) have all recognized inorganic arsenic as a known human carcinogen (ATSDR, 2007).

Inorganic arsenic occurs naturally in the soil and in many kinds of rock, especially in minerals and ores that contain copper or lead (ATSDR, 2007). When these ores are heated in smelters, most of the arsenic goes to the stack and enters the air as fine dust (ATSDR, 2007). Arsenic cannot be destroyed in the environment; it can only change its form or become attached to or separated from particles (ATSDR, 2007). Many arsenic

compounds can dissolve in water thus enters into lakes, rivers or groundwater by dissolving in rain or snow or through the discharge of industrial wastes (ATSDR, 2007). Arsenic ultimately ends up in the soil or sediment since arsenic will stick to particles in the water or sediment on the bottom of lakes or rivers (ATSDR, 2007).

Arsenic exposure can cause a wide variety of ailments. Chronic exposure to inorganic arsenic targets the skin and can induce a series of characteristic epithelium striations and commonly results in skin cancer. (ATSDR, 2007). The single-most characteristic effect of long term oral exposure to inorganic arsenic is pattern of skin changes including patches of darkened skin and the appearance of small warts or corns on the palms, soles and torso (ATSDR, 2007). Ingestion of large doses of inorganic arsenic can be fatal, but is not common in an exposure case (Klaassen, 2008). Symptoms of acute intoxication include fever, anorexia, hepatomegaly, melanosis, cardiac arrhythmia, and in fatal cases, cardiac failure (Klaassen, 2008). Arsenic exposure, especially during an exposure in a work environment, can cause nausea, vomiting, shortness of breath, and headaches (ATSDR, 2007).

Arsenic is also classified as a known human carcinogen and is associated with tumors of the skin, lung and urinary bladder and possibly the kidney, liver and prostrate (Klaassen, 2008). There are several federal agencies that advocate the reduction of arsenic exposure to at risk populations. The EPA, the Occupational Safety and Health Administration (OSHA) and the Food and Drug Administration (FDA) have developed regulations for arsenic. The EPA has set limits of the amount of arsenic that industrial sources can release into the environment and in the case of inorganic arsenic they have

banned the use in pesticides. The EPA has also lowered the limit for arsenic in drinking water to 10 ppb (ATSDR, 2007). The OSHA has established a permissible exposure limit for airborne arsenic in various workplaces that use inorganic arsenic (ATSDR, 2007). Even though there are many regulations on arsenic, exposure still poses a significant risk to certain populations.

Everyone has more than likely been exposed to arsenic at some point in their lives from air, water or food; however, there are those populations that are at increased risk. Children are much more susceptible to the side effects of arsenic than adults who were exposed. There are very few studies that look at prevalence or risk in children. Calderon et al. (2001) has reported that there is an association with lower verbal IQ and poorer long-term memory in children. Studies have also indicated that there is increased risk of childhood cancers due to exposure *in utero* and in early childhood (Infant-Rivard, 2001). However, adults who are working in an environment where arsenic is used are at increased risk of exposure as well as have increased risks of spontaneous abortions, stillbirths and cancer (Ahmed 2001, Kwok 2006, Infant-Rivard 2001). Adults will also be affected later in life due to the latency of health effects such as cancer caused by exposure *in utero* or as a child (Smith, 1998).

Not only are children and adults vulnerable, but there are increased risks associated with where a person lives and works too. This includes some areas of the United States such as California and Nevada where unusually high natural levels of arsenic in soil or water are found (Smith, 2009). There are also populations that have lived in areas such as Bangladesh, Argentina and Northern Chile who were once exposed

and are living with the consequence of that exposure. In addition to natural sources of arsenic contamination, hazardous waste sites may contain large quantities of arsenic. If the material is not properly disposed, it can enter surrounding water or soil. If you live near such a site, you could be exposed to elevated levels of arsenic. One fortunate aspect is that arsenic is no longer produced in the United States due to the fact that processes such as manufacturing of wood preservatives and inorganic arsenic compounds can no longer be used in agriculture (ATSDR, 2007). However, arsenic is ranked first on the CERCLA (Comprehensive Environmental Response, Compensation, and Liability Act) priority list of hazardous substances indicating its impact on both the environment and populations at risk for exposure are of a high level of concern (CERCLA, 2009). People whose work involves arsenic production such as copper or lead smelting, wood treating or pesticide application are also at risk of exposure. Sawing, sanding, or burning arsenic-treated wood could cause inhalational exposure. Populations are also vulnerable who live near agricultural areas where arsenic is used on crops and the soil can become contaminated (ATSDR, 2007).

There have been extensive studies performed on arsenic exposure and its prevalence and risk associated with different diseases. Most of these studies have been conducted in areas where high concentrations of arsenic are in drinking water. In the past areas such as Bangladesh, Northern Chile and India have reported high arsenic levels in their water. Just recently studies have been focused more in the United States to look at lower levels of arsenic exposure and risk. The US population has a significant risk of being exposed to arsenic.

Figure 1-1: Arsenic Distribution in Groundwater in the U.S. shows locations in the United States where there has been arsenic detected in drinking water. Arsenic found in drinking water can be caused by mining and industry in the area or naturally occurring contamination due to the geology of certain areas. With such few studies (Smith 2009, Zierold 2004, Navas-Acien 2008) addressing low dose long term exposure to populations in the US, many unknowns remain.

Arsenic in the Environment

Arsenic cannot be destroyed in the environment; it can only change its form, or become attached to or separated from particles (ATSDR, 2007). It may change its form by reacting with oxygen or other molecules present in air, water, or soil, or by the action of bacteria that live in soil or sediment (ATSDR, 2007). Many common arsenic compounds can dissolve in water; thus, arsenic can get into lakes, rivers, or groundwater by dissolving in rain or snow or through the discharge of industrial wastes (ATSDR, 2007). Some arsenic will stick to particles in the water or sediment on the bottom of lakes or rivers and some will be carried along by water. Ultimately, most arsenic ends up in the soil or sediment (ATSDR, 2007). The mechanisms by which arsenic can enter into groundwater is described below.

Arsenic is released into groundwater from natural weathering of soil and rocks and in areas of volcanic activity (ATSDR, 2007). Anthropogenic sources of arsenic released into water include mining, nonferrous metals, smelting, waste water, dumping of sewage sludge, coal burning power plants, manufacturing processes, urban runoff, atmospheric deposition and poultry farms (Garbarino 2003, Nriagu 1998, Pacyna 1995).

A contributory part of mining and coal burning power plants is arsenic leaching from abandoned mine tailings and fly ash waste piles (ATSDR, 2007) as well as significant amounts of arsenic is released in liquid effluents from gold-milling operations (Environmental Canada, 1993). Leaching of arsenic from soil, landfills or slag deposits is another source of arsenic in groundwater (Francis 1987, Wadge 1987). Soil receives arsenic from a variety of anthropogenic sources including ash residue from power plants, smelting operations, mining wastes, and municipal, commercial and industrial waste (ATSDR, 2007).

Arsenic occurs naturally in the environment in two valence states, arsenic (III) and arsenic (V) (Ruby, 1999). Typically arsenic (III) is present in anoxic condition while arsenic (IV) is the dominant form of arsenic in oxic soils. The presence of arsenic as anionic species causes it to be quite mobile in soils when it occurs in a soluble form (Ruby, 1999). Arsenic may occur as sulfide minerals (arsenopyrite) at mining and milling sites (Ruby, 1999). In soils arsenic may be present as the anthropogenic form in which it was deposited such as; lead and calcium arsenates from pesticides, arsenic pentoxide from herbicides and fungicides, copper-chrome arsenate from wood treating, or arsenic disulfide from tanning operations or as various soil alteration phases of variable composition such as arsenic in iron and manganese oxides and in phosphate materials (Ruby 1999). Arsenic displays a propensity to coprecipitate with iron to from iron-arsenic oxides the most common soil alteration phase for arsenic (Davis, 1996).

Arsenic is commonly concentrated in sulfide-bearing mineral deposits; especially those associated with gold mineralization and have a strong affinity for pyrite (Kokler,

2001). Arsenic can be easily solubilized in groundwaters depending on pH, redox conditions, temperature and solution composition (Smedley, 2002). A small number of source materials are now recognized as significant contributors to arsenic in water supplies including organic-rich or black shales, Holocene alluvial sediments with slow flushing rates, mineralized and mined areas (including gold deposits), volcanogenic sources and thermal springs (Nordstrom, 2002). In sediment high dissolved arsenic can occur through increased pH above 8.5 or the onset of reductive iron dissolution (Smedley, 2002). Additional facts that promote arsenic solubility are high concentrations of phosphate, bicarbonate, silicate and organic matter in the groundwater (Nordstrom, 2002).

In a number of areas around the world oxidation and dissolution of arsenian pyrite and arsenopyrite are additional processes that lead to high concentrations of arsenic in groundwater (Welch, 2000). The oxidation can be promoted naturally through infiltrating oxygenated groundwater (Mueller, 2001) or through lowering of the groundwater table into a stratigraphic zone containing arsenic-rich sulfides (Schreiber, 2000). The highest natural arsenic concentrations in the U.S. are in Fairbanks, Alaska at 1 to 10 mg/L where arsenopyrite-rich rocks are being oxidized and also possibility some iron reduction (Mueller, 2001). Present data suggest that arsenic is released to groundwater through reduction of arseniferous iron-oxyhydroxidates when anoxic condition develops during sediment burial (Nickson 1998). This process is driven by the microbial oxidation of organic C, concentrations of which reach 6% C in aquifer sediment (Sahu, 2012). Arsenic released from iron oxide appears to be the most common cause

of widespread groundwater contamination. This can occur in response to different types of geochemical conditions including release of arsenic into the groundwater through reaction of iron oxide with organic carbon (USGS, 1998).

Millions of people worldwide are exposed to arsenic-contaminated drinking water. Arsenic can easily be solubilized into groundwater from various geological sources. Three of the most studied areas where arsenic-contaminated water poses a significant public risk are Bangladesh, West Bengal, India and Taiwan. Bangladesh and West Bengal, India populations are exposed to up to 3,200 ug/L arsenic with a potential exposed population of 30 and 6 million respectively (Nordstrom, 2002). There is a smaller population of around 200,000 that are potentially exposed in Taiwan with up to 1820 ug/L of arsenic in contaminated groundwater (Nordstrom, 2002). While in Taiwan it seems fewer people are affected and maybe less of a concern, it is one of the most documented areas for arsenic related studies. There are several environmental conditions that make these parts of the world more susceptible to arsenic leaching. Both Bangladesh and West Bengal, India have very similar geological attributes such as alluvial and deltaic sediments with high phosphate.

Bangladesh and West Bengal, India are reducing environments for arsenic sorption into groundwater (Smedley, 2002). Arsenic leaching into the area groundwater is caused by alluvial and deltaic aquifers (Smedley, 2002)). The affected aquifers are generally shallow of Holocene age and comprise of micaceous sands, silts and clays deposited by the Ganges, Brahmaputra and Hegha River systems and their precursors (Smedley, 2002). Sediments are derived from upland Himalayan catchments and from

basement complexes of the northern and western parts of West Bengal. In the most affected areas the aquifer sediments are capped by a layer of silt, which effectively restricts entry of air to the aquifers. This with the presence of recent organic matter deposited with the sediments results in the development of highly reducing conditions that favor the mobilization of arsenic (Smedley, 2002).

Taiwan has significantly different environmental conditions including coastal zones and black shales (Nordstrom, 2002). Aquifers with carbonaceous shales can lead to high dissolved arsenic concentrations (Nordstrom, 2002). Two other environmental conditions can lead to high arsenic and they include closed basins in arid-to-semiarid climates and strongly reducing aquifers, often composed of alluvial sediments but with low sulfate concentrations much like those conditions found in the United States. For the United States there are some very similar geologic conditions as compared to Bangladesh and West Bengal, India. However, with its size and varying geological regions there are several different environmental conditions that contribute to arsenic leaching into groundwater. Those include thermal springs (oxidation of arsenic can be promoted naturally through infiltrating oxygenated groundwaters), closed basin lakes and various rocks (Nordstrom, 2002). Unfortunately, these generalities do not allow prediction of high or low dissolved arsenic concentrations. Arsenic released from iron oxide appears to be the most common cause of widespread arsenic concentrations (USGS, 1998). Arsenic is released into groundwater through reaction of iron oxide with organic carbon. Iron oxide can also be released to alkaline groundwater found particularly in the western states. Geothermal arsenic leaching does not affect a large

part of the US except for the Yellowstone geothermal system that causes high amounts of arsenic in the Madison and Missouri Rivers (USGS, 1998).

The United States has a vast array of environmental conditions that allows for similar, yet also different conditions that promote arsenic solubilization in its groundwater. The worst affected area of the United States is the southwestern region including the states of Nevada, California and Arizona. Additionally within the last decade aquifers in Maine, Michigan, Minnesota, South Dakota, Oklahoma and Wisconsin have been found with high arsenic concentrations (Smedley, 2002). In Nevada it appears that groundwater is primarily present under reducing conditions, having low dissolved-O_2 concentrations and high concentrations of dissolved organic C, Mn and Fe with the groundwaters associated with high pH (Welch, 1998). There is a little bit different story in California where there is a large range of groundwater arsenic concentrations found in the Tulare Basin of the San Joaquin Valley (Fuji, 1995). Redox conditions in the aquifers appear to be highly variable and the high arsenic concentrations are found in both oxidizing and reduction conditions. Robertson (1988) also noted the presence of high arsenic concentrations in groundwaters under oxidizing condition in alluvial aquifers in the basin and Range Province in Arizona.

A major difference between the environmental conditions of West Bengal, India, Bangladesh and Taiwan as compared to the United States is arsenic contamination from mining activities. Several mining areas in the United States have significant problems with acid mine drainage resulting from extensive oxidation of iron sulfide mineral (Smedley, 2002). In these areas pH values can be extremely low and iron oxides,

produced from the oxidation reaction, dissolves and releases bound arsenic (Smedley, 2002). Some areas also have some extremely acidic mine-drainage waters with negative pH values and arsenic concentrations in the mg 1^{-1} range (Nordstrom, 2002).

Of all the various sources of arsenic in the environment, drinking water poses the greatest threat to human health (Smedley, 2002). It has been reported that groundwater provides drinking water for 1.5 billion people daily and to many more in times of surface water scarcity (DFID, 2001). Drinking water is derived from a variety of sources including surface water, groundwater and rainwater. These sources vary in terms of arsenic risk with most high concentrations of arsenic found in groundwater (Smedley, 2002). Arsenic is a ubiquitous element found in the atmosphere, soils and rocks, natural waters and organisms (Smedley, 2002). Arsenic is mobilized through a combination of natural processes such as weathering reactions, biological activity and volcanic emissions as well as through a wide range of anthropogenic activities (Smedley, 2002).

Transport and partitioning of arsenic in water depends on the chemical form of the arsenic and on interactions with other materials present (ATSDR, 2007). Under oxidizing and mildly reducing conditions groundwater arsenic concentrations are usually controlled by adsorption rather than by mineral precipitation (ATSDR, 2007). Redox potential and pH are the most important factors controlling arsenic speciation into the environment. Under oxidizing conditions $H_2AsO_4^-$ (As V) is dominant at low pH while at higher pH $HAsO_4^{2-}$ becomes dominant (Smedley, 2002). Under reducing conditions at pH less the 9.2, the uncharged arsenite species $H_3AsO_3^0$ (As III) will predominate

(Brookins 1998, Yan 2000). Smedley and Kinniburgh (2002) describe two triggers that can lead to the release of arsenic into groundwater on a large scale. The first is pH. At the higher pH conditions found in semi-arid or arid environments, usually a result of mineral weathering and high evaporation rates. Those conditions can lead to increased leaching of arsenic (Smedley, 2002). The second trigger that Smedley and Kinniburgh (2002) describe is the development of strongly reducing conditions at near-neutral pH values leading to the desorption of arsenic from mineral oxides. The reductive dissolution of Fe and Mn oxides can also lead to arsenic release (Smedley, 2002). Another characteristic feature of high arsenic groundwater areas is the large degree of spatial variability in arsenic concentrations in the groundwaters. It is difficult or almost impossible to predict the likely concentration of arsenic in a particular well from those of neighboring groundwater sources (Smedley, 2002).

Manipulation of these natural mechanisms to prevent the leaching of arsenic into groundwater would be extremely difficult. One would have to be able to control the pH and oxidation state of the environment as well as control the spatial variability. Not only would one need to control those three key conditions, but seasonal patterns of arsenic speciation has also been observed (ATSDR, 2007) and would therefore also have to be controlled as well. It is not feasible to reduce arsenic contaminated groundwater in the natural environment. The basic treatment is to supply populations with arsenic free drinking water (Smith, 2000). This means the most predominate solution is the removal of that source. Since there is little to no literature regarding the natural prevention of arsenic in groundwater, there are several methods that can be used for

the treatment of arsenic contaminated drinking water. These methods include adsorption-co precipitation using iron and aluminum salts, adsorption on active alumina, activated carbon and activated bauxite, reverse osmosis, ion exchange and oxidation followed by filtration (Viraraghaven, 1999).

Arsenic Speciation

Arsenic can undergo both chemical and biological transformations into various species in the environment and in numerous organisms. Arsenic in water can undergo a complex series of transformations including oxidation-reduction reactions, ligand exchange, precipitation and biotransformation (EPA 1979, 1984, Sanders 1994 and Welch 1988). Factors that most strongly influence fate process in water are pH, metal sulfide and sulfide ion concentrations, iron concentrations, temperature, salinity, distribution and composition of biota, season and the nature and concentration of organic matter (EPA 1979, Farago 1997, Redman 2002, Wakao 1988). Inorganic species of arsenic are predominant in the environment. Arsenic (V) generally dominates in oxidizing environment such as surface water and arsenic (III) dominates under reducing conditions such as may occur in groundwater containing high levels of arsenic. However, the reduction of arsenate to arsenite is slow (ATSDR, 2007). Some organoarsenicals are also present in water and usually in the form of MMA and DMA but in low concentrations (Eisler, 2004).

Arsenic in soil is found as a complex mixture of mineral phases such as co-precipitated and sorbed species as well as dissolved species (Roberts, 2007). The

degree of arsenic solubility in soil will depend on the amount of arsenic distributed between these different mineral phases (ATDSR, 2007). The dissolution of arsenic is also affected by particle size. The arsenic cycle in soil is complex with many biotic and abiotic processes controlling its overall fate and environmental impact. Arsenic in soil exists in various oxidation states and chemical species depending upon the pH and oxidation-reduction potential. Transformations between the various oxidation states and species of arsenic occur as a result of biotic or abiotic processes (Bhumbla, 1994). While degradation of an organic compound is typically considered complete mineralization, in the case of organic compounds, the element arsenic itself cannot be degraded however the organic portion of the molecule can be metabolized (Woolson, 1976). Arsenicals applied to soils may be methylated by microorganisms to arsines and organic forms may be mineralized to inorganic forms (ATSDR, 2007). However, organic arsenical pesticides do not degrade by hydrolysis or by soil photolysis.

Humans are exposed to arsenic either through the ingestion of contaminated water, soil, dermal contact and through the air. Arsenic is metabolized quite differently in the body depending on the chemical species administered (Suzuki, 2002). Natural organic arsenics such as arsenobetaine, arsenocholine and arsenosugars of marine products are taken up and absorbed efficiently and then excreted rapidly in their intact forms into urine without showing appreciable effects in the body (Cullen 1989, Francesoni 1993, Shibata 1992, Edmonds 1977, Irgolic 1992). Inorganic arsenic is metabolized efficiently by humans and then excreted into the urine mostly in the form

of DMA after being metabolized by consecutive reduction and methylation reactions (Vahter 1999, Vahter 1994, Mandal 2001, Shiobara 2001).

Three different processes occur in human cells for the biotransformation of arsenic. First, the reduction of pentavalent to trivalent arsenic species. Second is methylation, and third is the replacement of hydroxyl by thiol groups (Dopp, 2010). The reduction and methylation of arsenic occurs is several steps. Arsenate and monomethylarsenic acid (MMA^{V}) reductases in mammalian cells catalyze the reduction of arsenate to arsenite and of MMA^{V} to monomethylarsonous acid (MMA^{III}) (Laparra 2003). The methylation of arsenite is catalyzed by arsenite methyltransferase with S-adenosylmethionine (SAM) as a methyl group donor (Laparra 2003). The first methylation step forms MMA^{V} where MMA^{V} is then reduced to MMA^{III} and further methylated to yield DMA^{V} in the presence of MMA^{III} methyltransferase (Cullen, 1989). Similar reduction of DMA^{V} to DMA^{III} is the third methylation step forming trimethylarsenic oxide ($TMA^{V}O$) (Calabrese 1997). **Figure 1-2: Arsenic Methylation Pathway** describes the methylation pathway of arsenic. Arsenic in the body can be present in six different species but can also be present bound to biological constituents (Suzuki, 2002).

While the methyl transfer system is a well established the pathway for the biomethylation of arsenic a new pathway has recently been proposed (Dopp, 2010). The long accepted pathway as described above consists of a series of reductions of pentavalent to trivalent species (Dopp, 2010). The new proposed metabolic pathway suggests that trivalent arsenic species bound to glutathione are methylated without

being oxidized (Hayakawa 2005). Hayakawa et al. suggests that arsenic glutathione complexes are the preferred substrate for methylation. However Thomas et al. (2004) has demonstrated that glutathione is not essential but can be replaced by other reducing systems yielding much higher conversion rates. This suggests that glutathione has an indirect role in the methylation of arsenic (Thomas, 2004).

Arsenic may be found in food and total arsenic may be substantially higher in certain seafood but the general consensus is that about 85-90% of the arsenic in the edible parts of marine food and shellfish is organic arsenic and approximately 10% is inorganic (EPA, 2003). The creation of organoarsenics in a marine environment is unique. They are created from the uptake of inorganic arsenic via the phosphate transport mechanism (Meunier, 2010). The normal cell response is reduction and elimination of arsenic as arsenite however some of the arsenic is methylated in a random process and through subsequent transformations creating organoarsenics such as arsenobetaine, arsenocholine and arsenosugars (Meunier, 2010).

Arsenic speciation is the key to arsenic toxicity. Inorganic arsenic is metabolized by consecutive reduction and methylation reactions to dimethylated arsenic (DMA) and then excreted into the urine (Suzuki, 2002). In humans this process is not complete and some arsenic remains as inorganic or as MMA (monomethylarsenic acid) (Smith, 2009). This process is no longer considered a detoxification process since some of the metabolites are also considered toxic (Smith, 2009). **Figure 1-2** shows the methylation process of arsenic. Since each of these metabolites can be detected through analysis, they have to potential to be used as biomarkers. Roychowdhury et al. (2003) conducted

a study to examine 19 families that had been exposed to arsenic-contaminated groundwater in West Bengal, India. After collecting urine samples it was found that the average of arsenite, arsenate, MMA and DMA in urine were 23.1, 59.0, 24.6 and 127.4 ng/mL when the families were exposed to water concentrations from 0.64 to 75.5 ppb (Roychowdhury, 2003). The average proportion of MMA and DMA was also reported as 75.7% and had mixed results when correlating relations between species and total arsenic when looking at the average as a whole and several families using one source (Roychowdhury, 2003). Huang et al. (2008) examined the association between urinary arsenic species and the incidence of urothelial carcinoma (UC). They found significantly higher percentages of MMA and lower percentages of DMA existed among the patients with UC than among healthy residents. Urinary DMA was shown to have an inverse associate with risk of UC (Huang, 2008). Smith and Steinmaus (2009) evaluated several studies that looked at the relationship between arsenic metabolism and risks of arsenic-related disease and concluded that elevated proportions of MMA have higher risks of arsenic-caused cancer and other arsenic-related health effects than those who excrete lower proportions (Smith, 2009). These studies show the potential of using urinary arsenic species as biomarkers to help determine risk of, not only cancer, but potentially other chronic diseases as well.

Health Effects of Arsenic

Arsenic poisoning can affect several target systems of the body; therefore, there are numerous clinical signs of exposure. Clinical signs can vary depending on if exposure is acute or chronic and by the route of exposure such as oral, ingestion or by dermal

contact. Acute high dose exposure can produce encephalopathy, with signs and symptoms of headache, lethargy, mental confusion, hallucinations, seizures and coma (ATSDR, 2007). Fever, anorexia, melanosis, cardiac arrhythmia and eventually cardiac failure can also present as signs of acute exposure (Klaassen, 2008). Symptoms from an acute exposure through ingestion usually occurs within 30 minutes of exposure and include constriction of the throat, gastric pain, vomiting, diarrhea, muscle cramps, hypertension and tachycardia followed by multi-organ failure (Komaromy-Hiller, 2001). For acute exposure to arsine gas through inhalation clinical signs are nonspecific but within 1-2 days after exposure abdominal pain, hematuria and jaundice are fairly common (Komaromy-Hiller, 2001). Arsine gas exposure can also be accompanied by hemoglobinuria, renal failure and anemia and can be fatal in some cases (ATSDR, 2007).

Common signs observed from a chronic exposure to arsenic usually presents with dermatological, neurological, hematological, and gastrointestinal symptoms including hyperkeratosis and numbness and tingling in the extremities (Komaromy-Hiller, 2001). Chronic arsenic exposure presents as recurring abdominal symptoms such as nausea, vomiting and unexpected weight loss (Klaassen, 2008). Liver injury is common and can result in jaundice, abdominal pain and hepatomegaly (NRC, 2001). The skin is a major target organ in chronic exposure and results in unique changes in skin epithelium (Klaassen, 2008) and is thought to be the single most characteristic effect of long-term inorganic arsenic exposure (ATSDR, 2007). Diffuse or spotted hyperpigmentation and hypopigmentation can occur between six months and three years after chronic exposure with Palmar-Planter hyperkeratosis (NRC, 2001) as well as

the development of warts or corns (ATSDR, 2007). Distinct transverse white bands called Mee's lines across the fingernails of an exposed person are also an indication of long-term exposure (Klaassen, 2008). Peripheral vascular disease has been observed in persons that have ingested inorganic arsenic contaminated drinking water (Klaassen, 2008). Blackfoot disease can occur in extreme cases where acrocyanosis and Raynaud's phenomenon can progress to endarteritis and gangrene in the lower extremities (Yu, 2002). Arsenic induced vascular effects have been reported in Chile, Mexico, India and China though the severity that has been shown in the Taiwanese population suggesting other unidentified environmental factors are also involved (Yu, 2002).

While arsenic poisoning, both acute and chronic, cause a wide range of clinical symptoms that affects numerous target systems, arsenic is also a carcinogen. Arsenic-induced skin cancers include basal cell carcinomas and squamous cell carcinomas both arising from areas of arsenic-induced hyperkeratosis (Klaassen, 2008). There is also an association of internal tumors in humans and arsenic exposure including tumors of the bladder and lung and potentially the liver, kidney and prostate (NRC, 2001).

Pathological findings for arsenic exposure in humans and in animal models are limited and there is little data on acute exposures. Neuropathy is a common symptom for arsenic exposure. Dying-back axonopathy with demyelination appears in histological examinations under repeated exposure (ATSDR, 2005). For oral arsenic exposure evidence of pulmonary edema, dermoepidermic separation, hepatomegaly, glomerular congestion, gastrointestinal bleeding (Quatrehomme, 1992), and increased serum levels of AST, LDH, BUN, bilirubin and creatinine (Levin-Scherz, 1987) can be found along with,

stomach adhesions and eroded epithelium (ATSDR, 2005). Santra et al. (2002) demonstrated that in an oral chronic exposure mouse study there were increased liver weight, altered liver histopathology and decreased hepatic enzymes were observed. Yeh and Su (1963) observed generalized atherosclerosis involving large, medium-size and small arteries as well as systemic arterial intimal thickening in small and medium-size arteries involving heart, gastrointestinal tract, liver, skin and pancreas with patients who had Blackfoot disease resulting from chronic arsenic exposure.

There is evidence from human studies involving inhalation exposure that gross pigmentation with hyperkeratinization (Perry, 1948) and lung cancer including epidermoid carcinoma; small cell carcinoma and adenocarcinoma were observed (Axelson 1978, Newman 1976, Pershagen 1987, Qiao 1997, Wicks 1981). There are no studies on dermal exposure in humans on respiratory, cardiovascular, hematological, hepatic, renal and endocrine systems describing pathological characteristics (ATSDR, 2007). Rabbits that were exposed to arsenic dermally showed no significant changes in urinalysis, blood chemistry or other observed changes for respiratory, cardiovascular, hematological, hepatic, renal or endocrine target systems (ATSDR, 2007).

There are several known mechanisms of action associated with arsenic toxicity and carcinogenicity. The trivalent compounds of arsenic are thio-reactive. This inhibits enzymes or alters proteins by reacting with proteinaceous thiol groups (Klaassen, 2008). Pentavalent arsenate is an uncoupler of mitochondrial oxidative phosphorylation and arsine gas is a potent hemolytic agent (NRC, 2001). Arsenic and its metabolites have been shown to produce oxidants and oxidative DNA damage, alteration in DNA

methylation status and genomic instability, impaired DNA damage repair, and enhanced cell proliferation (NRC, 2001; Rossman, 2003).

Unlike many carcinogens, arsenic is not a mutagen in bacteria and acts weakly in mammalian cells but can induce chromosomal abnormalities, aneuploidy, and micronuclei formation (Klaassen, 2008). Arsenic can also act as a co-mutagen and/or co-carcinogen (Rossman, 2003). This is evident by a study described by Germolec et al (1998) where animal models have shown that arsenic is a rodent skin tumor co-promoter with 12-*O*-teradeconoyl phorbol-13-acetate in v-Ha-*ras* mutant Tg.AC mice. Rossman et al (2001) describes arsenic as a co-carcinogen with UV irradiation in hairless mice. In rats it has also been shown that the methylated arsenic species DMA^{5+} is a urinary bladder tumor initiator and promoter (Wei, 2002) and produces urothelial cytotoxicity and proliferation regeneration with continuous exposure (Cohen, 2001). Since there is a corollary in humans with increased mortality from lung cancer in young adults following *in utero* exposure to arsenic, there has been an attempt to determine the mechanism of toxicity (Smith, 2006). It has been hypothesized that arsenic may also act as an estrogen-signaling agent to produce hepatocarcinogenic effects (Waalkes, 2004).

Inorganic arsenics cause substantial acute and chronic toxicity and arsenic itself is a known carcinogen. Not only is arsenic one of the first chemicals to be recognized to cause cancer (Smith, 2002), there has been evidence since the 1930's that arsenic in drinking water could cause skin cancer (Arguello, 1938). During the 1960's evidence emerged that arsenic in drinking water might cause internal cancers such as lung and

urinary tract (Bergoglio 1964, Biagini 1968). But in 1985 there was increased mortality shown from several cancers including lung, bladder and kidney cancers in Taiwan (Chen, 1985) where populations were exposed to high level of arsenic from contaminated water sources. Chen et al. (1988) found that bladder cancer mortality rates for those with more than 600 ug/liter of arsenic in their water were more than 30 to 60 times the rate of unexposed populations.

While one may argue that arsenic in drinking water is an international problem since there are well-documented areas around the world where populations have been affected by arsenic contamination in water sources including Chile, Argentina, Taiwan and Bangladesh (Nordstrom, 2002), the United States should be an area of concern as well. Two reports from the NRC (1999, 2001) affirmed that cancer risks might be on the order of 1 in 100 for 50 μg/liter of arsenic in drinking water. This estimated cancer risk is more than 100 times greater than that for any other drinking water contaminant with a MCL (Smith, 2002). While cancer risk is a concern with arsenic exposure there have also been recent links to arsenic and other chronic diseases.

A study conducted by Zierold et al. (2004) evaluated the prevalence of 9 different chronic diseases in adults who drank well water for 20 or more years in an at risk area in Wisconsin. It was reported that individuals with well water arsenic concentrations between 2ug/L and 10ug/L were significantly more likely to report having depression than individuals exposed to <2ug/L (Zierold, 2004). Also, individuals who were exposed to contaminated water >10ug/L were significantly more likely to report having had cardiac bypass surgery, high blood pressure, and circulatory problems

than those exposed to well water at <2ug/L arsenic (Zierold, 2004). Mazumder, Et al. (2000) found a prevalence of cough, shortness of breath and chest sounds in the lungs of those with increasing concentrations of arsenic in drinking water and these symptoms were most pronounced in those subjects that had skin lesions, a symptom of arsenic poisoning. There is also an indication that arsenic exposure is associated with the prevalence of type 2 diabetes in the United States. A study conducted by Navas-Acien Et al. (2008) evaluated the percent of arsenic in urine from participants in a previous National Health and Nutritional Examination Survey (NHANES). This study shows individuals with type 2 diabetes have 26% higher level of total arsenic in their urine indicating an association with arsenic urine concentrations and type 2 diabetes (Navas-Acien, 2008).

Everyone has more than likely been exposed to arsenic at some point in their lives from air, water or food; however, there are those populations that are at increased risk. Children are much more susceptible to the side effects of arsenic than adults who were exposed. There are very few studies that look at prevalence or risk in children. Calderon et al. (2001) has reported that there is an association with lower verbal IQ and poorer long-term memory in children. Studies have also indicated that there is increased risk of childhood cancers due to exposure *in utero* and in early childhood (Infant-Rivard, 2001). However, adults have increased risks of spontaneous abortions, stillbirths and cancer (Ahmed 2001, Kwok 2006, Infant-Rivard 2001), and adults will also be affected later in life due to the latency of health effects such as cancer caused by exposure *in utero* or as a child (Smith, 1998).

Summary

Not only are children and adults vulnerable, but there are increased risks of where a person lives and works too. This includes some areas of the United States where unusually high natural levels of arsenic in soil or water are found in California and Nevada (Smith, 2009). Other than natural sources of contamination, hazardous waste sites can contain large quantities of arsenic where the waste can easily contaminate groundwater sources. One fortunate aspect is that arsenic is no longer produced in the United States for processes such as manufacturing of wood preservatives and inorganic arsenic compounds can no longer be used in agriculture (ATSDR, 2007); however, arsenic is ranked first on the CERCLA (Comprehensive Environmental Response, Compensation, and Liability Act) priority list of hazardous substances indicating its impact on both the environment and populations at risk for exposure are of a high level of concern (CERCLA, 2009).

While there are numerous studies related to inorganic arsenic, very little data is available on low-dose exposures in the United States let alone studies examining individual arsenic species. Since there is emerging evidence that arsenic at low-doses is linked to numerous chronic disorders, one thing is certain, there is profound evidence that arsenic causes cancer. When there is significant direct human epidemiological evidence that a substance causes cancer, the focus should be on margins of safety, avoiding extensive statistical manipulations of data and excessive debate about potential uncertainties (Smith, 2002).

References

1. Agency for Toxic Substances and Disease Registry (ATSDR): Toxicology Profile for Arsenic, 2007.
2. Ahmad SA, et al.: Arsenic in drinking water and pregnancy outcomes. *Environ. Health Perspect* 109:629-31, 2001.
3. Arguello RA, Cenget DD, Tello EE: Cancer and endemic arsenism in the Cordoba Region. *Argent Dermat* 22*:* 461**,** 1938.
4. Axelson O, Dahlgren E, Jansson CD, et al.: Arsenic exposure and mortality: A case referent study from a Swedish copper smelter. *Br J Ind Med* 35:8-15, 1978.
5. Bergoglio, RM*:* Mortalidad por cáncer en zonas de aguas arsenicales de la Provincia de Córdoba, República Argentina. *Presa Med Arg* 51:994, 1964*.*
6. Biagini RE, Bertola VG, Martin A, Mosquera J: Histochemical localization of arsenic in the skin of patients with chronic arsenic poisoning*. Arch Argent Dermatol* 16*:*172, 1966*.*
7. Bhumbla DK, Keefer RF: Arsenic mobilization and bioavailability in soils. In: Nriagu JO (Ed) arsenic in the environment, part I: Cycling and characterization. New York, NY; John Wiley & Sons, Inc 51-82, 1994.
8. Brookins D.G. Eh-pH Diagrams of Geochemistry. Springer-Verlag, Berlin, 1998.

9. Calabrese, EJ, Stanek EJ, James RC, Robers SM: Soil ingestion: a concern for acute toxicity in children. *Environ. Health Persp* 105:1354-1358, 1997.
10. Calderon, J. Et al.: Exposure to arsenic and lead and neuropsychological development in Mexican children. *Environ Res* 85:69-76, 2001.
11. CERCLA Priority List of Hazardous Substances (CERCLA), 2009. ATDSR accessed at http://www.atsdr.cdc.gov/cercla/07list.html on 09/01/2011.
12. Chen CJ, Chaung YC, Ling TM, Wu HY: Malignant neoplasms among residents of a Blackfoot Disease-endemic area in Taiwan: High-arsenic artesian well water and cancers. *Cancer Res* 45:5895, 1985.
13. Cohen SM, Yamamoto S, Cano M, Arnold L: Urothelial cytotoxicity and regeneration induced by dimethylarsinic acid in rats. *Toxicol Sci* 59:68-74, 2001.
14. Cullen WR, Reimer KJ: Arsenic speciation in the environment. *Chem Rev* 89:713, 1989.
15. Davis A, Ruby MV, Bloom M, et al.: Mineralogic constraints on the bioavailability of arsenic in smelter-impacted soils. *Environ Sci technol* 30:392-399, 1996.
16. DFID: Addressing the Water Crisis: Healthier and more productive lives for people. UK Department of international development, London, 2001.
17. Dopp E, Kligerman AD, Diaz-Bone RA: Organoarsenicals. Uptake, Metabolism, and Toxicity Chapter 7 In Metal Ions in Life Sciences. (Eds) A. Sigel, H. Sigel and R. Sigel: *Royal Society of Chemistry*. 231-265, 2010.

18. Edmonds JS, Francesoni KA, Cannon RJ, Raston CL, et al.: Isolation, crystal structure and synthesis of arsenobetaine, the arsenical constituent of the western rock lobster. *Tetrahedron Lett* 18:1543, 1977.
19. Eisler R: A review of arsenic hazards to plants and animals with emphasis of fishery and wildlife resources, in: W.R. happell, C.O. Abernathy, C.R. Cothern (Eds), Arsenic Exposure and Health, *Sci Technol let* Northwood, UK, 185-259, 2004.
20. Environmental Canada: Canadian environmental protection act priority substances list assessment report, arsenic and its compounds. Ottawa, Canada: Canada Communication Group, 1993.
21. Environmental Protection Agency (EPA): Health Assessment Document for Arsenic. Research Triangle Park: US EPA, EPA600823021F, 1984.
22. Environmental Protection Agency (EPA): Water-related environmental fate of 129 priority pollutants: Vol I. Introduction and technical background, metals and inorganics, pesticides and PCB's. Washington, DC: US EPA office of water planning and standard, EPA440479028a, 1979.
23. Farago ME: Arsenic in the marine environment. In: Gianguzza A, Pelizetti E, Sammartano S (Eds). Marine Chemistry. Netherlands: Kluwer Academic Publishers 275-291, 1997.
24. Fuji R, Swain WC: Areal distribution of selected trace elements, salinity and major ions in shallow ground water, Tulare Basin, Southern San

Joaquin Valley, California. *US Geol Surv Water-Resour Investig* Rep. 95: 4048, 1995.

25. Huang Yung-Kai, et al: Arsenic Exposure, Urinary Arsenic Speciation, and the Incidence of Urothelial Carcinoma: a Twelve Year Follow-up Study. *Cancer Causes Control* 19:829-839, 2008.
26. Infant-Rivard C, et al.: Drinking water contaminants and childhood leukemia. E*pidemiology* 12:13-19, 2001.
27. Irgolic KJ: Arsenic, in: M. Stoeppler (Ed), Hazardous metals in the environment, Elsevier, Amsterdam, 287-350, 1992.
28. Klaassen CD: *Casaraett & Doull's Toxicology the basic science of poisons*. New York: McGraw Hill Medical, 2008.
29. Kokler A, et al.: The US Geological Survey Workshop on Arsenic in the Environment, February 21-22, 2001.
30. Komaromy-Hiller G.: Arsenic, Mercury, and Cadmium In The Clinical Toxicology Laboratory, Contemporary Practice of Poisoning Evaluation. (Ed) L. M. Shaw Washington, DC: AACC Press 383-399, 2001.
31. Kwok RK, et al.: Arsenic in drinking water and reproductive health outcomes: a study of participants in the Bangladesh Integrated Nutritional Programme. *J Health Popul Nutr* 24:190-205, 2006.
32. LaParra JM, Velez D, Barbera R, Farre R, Montoro R: Estimation of arsenic bioaccessibility in edible seaweed by an *in vitro* digestion method. *J Agric Food Chem* 53:8829-8833, 2003.

33. Levin-Scherz JK, Patrick JD, Weber FH, et al.: Acute arsenic ingestion. *Ann Emerg Med* 16:702-704, 1987.

34. Mandal BK, Orgra Y, Suzuki KT: Identification of dimethylarsenous and monomethylarsonous acids in human urine of the arsenic-affected areas in west Bengal, India. *Chem Res Toxicol* 14:371, 2001.

35. Mueller S, Verplanck P, Goldforb R: Ground-water studies in Fairbanks, Alaska-a better understanding of some of the United States' highest natural arsenic concentrations: USGS Fact Sheet FS-111-01, November 2001.

36. Mazumder D, Guha N, et al.: Arsenic in drinking water and the prevalence of respiratory effects in West Bengal, India. *Intl J of Epidem* 29:1047-1052, 2000.

37. Meunier L, Wragg J, Koch I, Reimer K: Method variables affecting the bioaccessibility of arsenic in soil. *J Env Sci and Hlth, Part A: Toxic/Hazardous Substances and Environmental Engineering* 45(5):517-526, 2010.

38. National Research Council (NRC): Arsenic in Drinking Water (update). Washington, DC: National Research Council, National Academy, 2001.

39. Navas-Acien A, Silbergeld EK, Pastor-Barrisuo R, Guallar E: Arsenic exposure and prevalence of type 2 diabetes in US adults. *JAMA* 300: 814-822, 2008.

40. Newman JA, Archer VE, Saccomanno G, et al.: Histologic types of bronchogenic carcinoma among members of copper-mining and smelting communities. *Ann N Y Acad Sci* 271:260-268, 1976.

41. Nickson R: Arsenic in the air. *Nature* 295:338, 1998.

42. Nordstrom DK: Worldwide Occurrences of arsenic in ground water. *Science* 296: 2143, 2002.

43. Perry K, Bowler RG, Buckell HM, et al.: Studies in the incidence of cancer in a factory handling inorganic compounds of arsenic--II: Clinical and environmental investigations. *Br J Ind Med* 5:6-15, 1948.

44. Pershagen G, Bergman F, Klominek J, et al.: Histological types of lung cancer among smelter workers exposed to arsenic. *Br J Ind Med* 44:454-458, 1987.

45. Qiao Y, Taylor PR, Yao S, et al.: Risk factors and early detection of lung cancer in a cohort of Chinese tin miners. *Ann Epidemiol* 7:533-541, 1997.

46. Quatrehomme G, Ricq O, Lapalus P, et al.: Acute arsenic intoxication: Forensic and toxicological aspects (an observation). *J Forensic Sci* 37:1163-1171, 1992.

47. Redman AD, Macalady DL, Ahmann D: Natural organic matter affects arsenic speciation and sorption onto hematite. *Environ Sci Technol* 36:2889-2896, 2002.

48. Robertson FN. Arsenic in groundwater under oxidizing conditions, southwest United States. *Environ Geochem Health* 11:171-185, 1989.

49. Rossman TG, Uddin AN, Burns FJ, et al.: Arsenite is a cocarcinogen with solar ultraviolet radiation for mouse skin: An animal model for arsenic carcinogenesis. *Toxicol Appl Pharmacol* 176:64-71, 2001.

50. Rossman TG: Mechanism of arsenic carcinogenesis: An integrated approach. *Mutat Res* 533:37-65, 2003.

51. Roychowdhury T, Tokunaga H, Ando M: Survey of arsenic and other heavy metals in food composites and drinking water and estimation of dietary intake by the villagers from an arsenic-affected area of west Bengal, India. *Sci of Total Env* 308(1-3):15-35, 2003.

52. Ruby MV, Schoof R, Brattin W, et al.: Advances in evaluating the oral bioavailability of inorganics in soil for use in human health risk assessment. *Environ Schi Technol* 33:3697-3705, 1999.

53. Sahu SJ, Nath B, Roy S, Mandal B, Chatterjee D: Bioavailability of arsenic in the soil horizon: a laboratory column study. *Environ Earth Sci* 65:813-821, 2012.

54. Sanders JG, Riedel GF, Osmann RW: Arsenic cycling and its impact in estuarine and coastal marine ecosystems. In: Briagu JO (Ed). Arsenic in the environment, part I: cycling and characterization. New York, NY: John Wiley & Sons Inc, 289-308, 1994.

55. Santra A, Maiti A, Das S, et al.: Hepatic damage caused by chronic arsenic toxicity in experimental animals. *J Toxicol Clin Toxicol* 38(4):395-405, 2000.

56. Schreiber ME, Simo JA, Freiberg PG: Stratigraphic and geochemical controls on naturally occurring arsenic in groundwater, eastern Wisconsin, USA. *Hydrogeology J* 8:161-176, 2000.

57. Shibata Y, Morita M, Fuwa K: Selenium and arsenic in biology: their chemical forms and biological functions. *Adv Biophys* 28:31, 1992.

58. Shiobara Y, Ogra Y, Suzuki KT: Animal species difference in the uptake of dimethylarsenous acid (DMAIII) by red blood cells. *Chem Res Toxicol* 14:1446, 2001.

59. Smedley PL, Kinniburgh DG: A review of the source, behavior and distribution of arsenic in natural waters. *Appl Geochem* 17:517, 2002.

60. Smith A, Lingas E, Rahman M: Contamination of drinking water by arsenic in Bangladesh: a public health emergency. *Bulletin of the World Health organization* 79:1093-1103, 2000.

61. Smith A, Steinmnaus CM: Health effects of arsenic and chromium in drinking water: recent human findings. *Annu Rev Public Health* 30:107-22, 2009.

62. Smith A, et. al: Marked increase in Bladder and lung cancer mortality in a region of Northern Chile due to arsenic in drinking water. *American Journal of Epidemiology* 147:660-669, 1998.

63. Smith AH, Lopipero PA, Bates MN, Steinmaus CM: Arsenic epidemiology and drinking water standards. *Science* 296:2143, 2002.

64. Smith AH, Marshall G, Yuan Y, et al.: Increased mortality from lung cancer and bronchiectasis in young adults after exposure to arsenic in utero and in early childhood. *Environ health Perspect* 114:1293-1296, 2006.

65. Suzuki KT, Mandal BK, Orgra Y: Speciation of arsenic in body fluids.

66. Thomas DJ, Waters SB, Styblo M: Elucidating the pathway for arsenic methylation. *Tox of appl pharm* 198(3):319-326, 2004.

67. United States Geological Survey (USGS): Trace elements national synthesis project 1998 Conference paper: Arsenic in ground water supplies of the United States, 1998.

68. Vahter M: Methylation of inorganic arsenic in different mammalian species and population groups. *Sci Prog* 82:69, 1999.

69. Vahter M: Species difference in the metabolism of arsenic compounds. *Appl Organomet Chem* 8:175, 1994.

70. Viraraghaven T, Subramanian KS, Aruldoss JA: Arsenic in drinking water-problems and solution. *Water Science and Technology* 40:69-76, 1999.

71. Waalkes MP, Lui J, Chen H, et al: Estrogen signaling in livers of male mice with hepatocellular carcinoma induced by exposure to arsenic in utero. *J Natl Cancer Inst* 96:466-474, 2004.

72. Wadge A, Hutton M: The leachability and chemical speciation of selected trace elements in fly ash from coal combustion and refuse incineration. *Environ Pollut* 48:85-99, 1987.

73. Wakao N, Koyatsu H, Komai Y, et al: Microbial oxidation of arsenite and occurrence of arsenite-oxidizing bacteria in acid mine water fro a sulfur-pyrite mine. *Geomicrobiol J 6:11-24, 1988.*

74. Wei M, Wanibuchi H, Morimura K, et al.: Carcinogenicity of dimethylarsinic acid in male F344 rats and genetic alterations in induced urinary bladder tumors. *Carcinogenesis* 23:1387-1397, 2002.

75. Welch AH, Lico MS: Factors controlling As and U in shallow ground water, southern Carson Desert, Nevada. *Appl. Geochem*. 13:521-539, 1998.

76. Welch AH, Westjohn DB, Helsel DR, Wanty RB: Arsenic in groundwater of the United States: occurrence and geochemistry. *Groundwater* 38:589, 2000.

77. Wicks MJ, Archer VE, Auerbach O, et al.: Arsenic exposure in a copper smelter as related to histological type of lung cancer. *Am J Ind Med* 2:25-31, 1981.

78. Woolson EA: Generation of dimethyl arsine from soil. 1976 Meeting of the weed science society of America. February 3-5 No. 218, 1976.

79. Yan X, Yan P, Kerrich R, Hendry MH: Distribution of arsenic (III), arsenic (V) and total arsenic in pore waters from thick till and clay-rich aquifer sequence, Saskatchewan, Canada. *Geochim Cosmochim Acta* 64:2637-2648, 2002.

80. Yeh S, Su H: A pathological study on the Blackfoot disease in Taiwan. *Rep Inst Pathol Natl Taiwan Univ* 14:25-73, 1963.

81. Yu HS, Lee CH, Chen GS: Peripheral vascular diseases resulting from chronic arsenical poisoning. *J dermatol* 29:123-130, 2002.

82. Zierold KM, et al.: Prevalence of Chronic Diseases in Adults Exposed to Arsenic-Contaminated Drinking Water. *American Journal of Public Health* 94:No. 11, 2004.

CHAPTER 2 SWINE RELATIVE BIOAVAILABILITY STUDIES

Background

Millions of people are exposed to arsenic in the United States and worldwide. Arsenic is ubiquitous in the Earth's crust and people encounter many chemical forms that vary in toxicity. Most toxic naturally occurring arsenic compounds are inorganic forms and their monomethylated metabolites (Styblo, 2002). It is used to manufacture glass, feed additives, pigments, medicinals, wood preservatives, pesticides and semiconductor products and food (ATSDR, 2007). It is estimated that 25% of the North American dietary intake of arsenic is in the inorganic form (Yost, 1998). Exposure to inorganic arsenic can result in a variety of adverse health effects such as skin disorders, nerve impairment, cancer of the liver, bladder, kidneys, prostate, and lungs, and even death from large doses (ATSDR 2007, Hall 2002). Humans are exposed to inorganic arsenic primarily through ingestion of water or inhalation and work place exposure (ATSDR 2007, Suzuki 2002, Dopp 2010). Once ingested arsenic is well absorbed and then distributed throughout the body after circulating through the liver where it is mostly metabolized by methylation, and then excreted primarily in the urine (Tam 1979, Pomroy 1980, Buchet 1981). Five arsenic species are commonly found in human urine and they are AsIII (arsenite), AsV (arsenate), MMA (monomethylarsenic acid), DMA (dimethylarsinic acid) and AB (arsenobetaine). Far less toxic are the organic arsenic compounds (Sakurai 2002, Francesconi 1994, Kojina 2002). Usually exposure to organic

arsenic occurs through the ingestion of fish, shellfish or seaweed (Yost 1998, Dopp 2010).

Arsenic speciation is the key to arsenic toxicity. Inorganic arsenic is metabolized by consecutive reduction and methylation reactions to dimethylated arsenic (DMA) and then excreted into the urine (Suzuki, 2002). In humans, this process is not complete and some arsenic remains as inorganic or as MMA (monomethylarsenic acid) (Smith, 2009). This process is no longer considered a detoxification process since some of the metabolites are also considered quite toxic (Smith, 2009). Since each of these metabolites can be detected through analysis, they have the potential to be used as biomarkers.

Not only are humans exposed to arsenic by ingestion of water and food, but also by inhalation and through occupational means and exposure from arsenic-contaminated soil. This is an important exposure route for environmental contaminants, especially for children (Calabrese 1997, Vanwignen 1990). Human exposure to contaminated soil occurs near mining sites and other industrial areas that use arsenic. Inorganic arsenic occurs naturally in the soil and in many kinds of rock, especially in minerals and ores that contain copper or lead (ATSDR, 2007). Arsenic cannot be destroyed in the environment; it can only change its form or become attached to or separated from particles (ATSDR, 2007). Many arsenic compounds can dissolve in water and enter lakes, rivers or under groundwater by dissolving in rain or snow or through the discharge of industrial wastes (ATSDR, 2007). Arsenic ultimately ends up in the soil or sediment since arsenic will stick to particles in the water or sediment on the bottom of

lakes and rivers (ATSDR, 2007). For human exposure assessments in areas that have naturally occurring arsenic contaminated water sources or those who live and work near contaminated environmental sites where arsenic has been used, it is important to fully understand what species of arsenic residents are being exposed to, in order to grasp the risk of arsenic exposure in its entirety. Understanding the exposure pathway of arsenic by soil contamination is important when assessing public health risks associated with arsenic containing soils (Rodriquez, 2003).

Since human exposure to arsenic is a substantial public health concern, animal models can be used to help determine risk of exposure and health effects. The determination of bioavailability using swine is a common practice used during human health risk assessments.

Materials and Methods

Subjects used in Bioavailability studies

Toxicological studies have long been performed in animal models to determine mechanisms of action, effective and safe dosing and human risk of toxicity from human exposure to toxic substances (Brent 2004, Weis 1991, Ruby 1999, Bannon 2009). Juvenile swine are primarily selected for use because they are a good physiological model for gastrointestinal absorption in children (Sutton 2000), and the current model used to determine risk of arsenic exposure to those that have been exposed. Reliable analysis of the potential hazard to humans from ingestion of a chemical depends upon accurate information on a number of key parameters, including the concentration of the

chemical in environmental media (soil, dust, water, food, air, paint), intake rates of each medium, and the rate and extent of absorption ("bioavailability") of the chemical into the blood from each ingested medium (Casteel, 1996). The amount of a chemical that actually enters the blood from an ingested medium depends on the physical-chemical properties of the chemical and the medium (Casteel, 1996). Some metals in soil may exist, at least in part, as poorly water-soluble minerals, and may also exist inside particles of inert matrix such as rock or slag of variable size, shape, and association. These chemical and physical properties may influence the absorption or bioavailability of the metals when ingested (Casteel, 1996). Thus, equal ingested doses of different forms of a chemical in different media may not be of equal health concern.

Study Design

Three different bioavailability studies have been conducted and will be described in this a subsequent chapters. These studies will be named EPA, this study was conducted for the EPA, and California Department of Toxic Substances (CaDTS) study #1 and study #2. The test materials and a reference material (sodium arsenate, NaAs) were administered to groups of four or five juvenile swine for 14 days. The studies included a non-treated group of three animals to serve as a control for determining background arsenic levels. All doses were administered orally. The studies were performed as nearly as possible within the spirit and guidelines of Good Laboratory Practices (GLP: 40 CFR 792). **Table 2-1: Study Design and Dosing Information Conduct for the EPA**, **Table 2-2: Study Design and Dosing Information Conducted for the California Department of Toxic Substances Study #1** and **Table 2-3: Study Design and Dosing Information for the**

California Department of Toxic Substances Study #2 describe the design of each test materials used, concentrations of test materials and dosing information.

Juvenile swine used for this study were intact males from a health monitored herd and were purchased from Chinn Farms, Clarence, Missouri. The number of animals purchased for the study was several more than required by the protocol to permit culling of unhealthy and over- or under-sized pigs. These animals were purchased at an age of about 5-6 weeks (weaning occurs at age 3 weeks) and housed in individual stainless steel cages. The animals were then held under quarantine for one week to allow acclimation prior to dosing. A certified veterinary clinician examined each animal and any animals that appeared to be in poor health during this quarantine period were excluded from the study. To minimize weight variations among animals and groups, extra animals most different in body weight (either heavier or lighter) five days prior to exposure (day -5) were also excluded from the study. The remaining animals were assigned to dose groups at random.

When exposure began (day zero), the animals were about 6-7 weeks old. The animals were weighed at the beginning of the study and every three days during the course of the study. All animals were examined daily by an attending veterinarian while on study and were subjected to examination at necropsy by a veterinarian in order to assess overall animal health.

Diet

Animals were weaned onto standard pig chow (made at the University of Missouri Animal Science Feed Mill). The feed was nutritionally complete.

Prior to the start of dosing and throughout the dosing period, each day every animal was given an amount of feed equal to 4.0% of the mean body weight of all animals on study. Feed amounts were adjusted every three days, when animals were weighed. Feed was administered in two equal portions, at 11:00 AM and 5:00 PM daily.

Drinking water was provided *ad libitum* via self-activated watering nozzles within each cage.

Dosing

Animals were exposed to dosing materials (sodium arsenate or sieved test material) for 14 days, with the dose for each day being administered in two equal portions beginning at 9:00 AM and 3:00 PM (two hours before feeding). Pigs were dosed two hours before feeding to ensure that they were in a semi-fasted state. To facilitate dose administration dosing materials were placed in a small depression in a ball of dough consisting of moistened feed (typically about 5g), and the dough was pinched shut to contain the soil. The dough was then placed in the feeder at dosing time.

Target arsenic doses (expressed as µg of arsenic per kg of body weight per day) for animals in each group were determined in the study design. The daily mass of arsenic administered (either as sodium arsenate or as sieved test material) to animals in each group was calculated by multiplying the target dose (µg/kg-day) for that group by the anticipated average weight of the animals (kg) over the course of the study:

$$Mass\ (\mu g / day) = Dose\ (\mu g / kg - day) \cdot Average\ Body\ Weight\ (kg)$$

The average body weight expected during the course of the study was estimated by measuring the average body weight of all animals and throughout the study from 0-5, 6-9 and 10-13 days to calculate dose. After completion of the study, the true mean body weight was calculated using the actual body weights (measured every three days during the study), and the resulting true mean body weight was used to calculate the actual doses achieved. Any missed or late doses were recorded and the actual doses adjusted accordingly.

Relative Bioavailability

The relative oral bioavailability of arsenic was assessed by comparing the absorption of arsenic from the soil samples ("test materials") to that of sodium arsenate. Groups of four or five swine were given oral doses of sodium arsenate or a test material twice a day for 14 days. Groups of three non-treated swine served as a control.

The amount of arsenic absorbed by each animal was evaluated by measuring the amount of arsenic excreted in the urine (collected over 48-hour periods beginning on days 6, 9, and 12). The urinary excretion fraction (UEF) is the ratio of the amount excreted per 48 hours divided by the dose given per 48 hours. UEF was calculated for the test materials and the sodium arsenate using simultaneous weighted linear regression. The relative bioavailability (RBA) of arsenic in each test material compared to sodium arsenate was calculated as follows:

RBA=UEF (test soil) / UEF (sodium Arsenate)

Sample Collection and preparation for analysis

Samples of urine were collected from each animal for 48-hour periods on days 6 to 7, 9 to 10 and 12 to 13 of the study. Collection began at 8:00 AM and ended 48 hours later. The urine was collected in a plastic bucket placed beneath each metabolism cage, which was emptied into a plastic storage bottle. Aluminum screens were placed under the cages to minimize contamination with feces or spilled food. Due to the length of the collection period, collection containers were emptied periodically (typically twice daily) into separate plastic bottles to ensure that there was no loss of sample due to overflow.

At the end of each collection period, the total urine volume for each animal was measured and a 60-mL portion was removed and acidified with 0.6 mL concentrated trace metal nitric acid. All samples were refrigerated until arsenic analysis.

Samples were collected for arsenic speciation analysis by two different means. For one 48 hour urine sample collection a 15 mL aliquot of urine was immediately frozen. Another set of the same urine was collected then acidified with trace metal nitric acid to 1%. These samples were then refrigerated.

For The EPA and CaDTS Study #1 total arsenic data, swine urine was prepped by digesting 25-mL samples of urine by refluxing and then heating to dryness in the presence of magnesium nitrate and concentrated nitric acid. Following magnesium nitrate digestion, samples were transferred to a muffle furnace and ashed at 500°C. The digested and ashed residue was dissolved in hydrochloric acid and analyzed by the

hydride generation technique. Analysis was conducted by L. E. T., Inc., (Columbia, Missouri)

For CaDTS Study #2 Urine samples were analyzed for arsenic by Ce^2l environmental laboratories (Lee's Summit, Missouri) by ICP-MS. In brief, all calibration standards, QC controls and samples were prepared for analysis at 1/10 dilutions. The dilutions were prepared with 2% HNO_3 and de-ionized water solution with Gallium as the internal standard at a concentration of 50 ug/L.

A number of quality control (QC) steps were taken during all three study analyses to evaluate the accuracy of the analytical procedures. Quality Controls are used to monitor instrument performance, drift and human error. These quality control steps include:

- Duplicates (Sample Preparation Replicates)

  Known duplicate samples were selected at random for each urine collection. Duplicates are used to assure samples were prepared properly, observe any matrix interferes and also to check instrument performance over time. Also a random selection of about 10% of all urine samples generated during the study were prepared for laboratory analysis in duplicate (i.e., two separate subsamples of urine were digested) and submitted to the laboratory in a blind fashion. During analysis, every tenth sample is also analyzed in duplicate. Duplicate results for urine samples typically agreed within 10% relative percent difference (RPD).

- Laboratory Quality Control and Control Standards

  Laboratory low, medium and high controls as well as a laboratory control standard were tested periodically during sample analysis. These controls are used to assure that the instrument was calibrated properly and to assure that the calibration is maintained throughout the analysis.

- Blanks

  Laboratory blank samples were run along with each batch of samples at a rate of about 10%. Blanks never yielded a measurable level of arsenic (all results <1 µg/L).

- Spike Recovery

  During analysis, one feed and water sample and every tenth urine sample was spiked with known amounts of arsenic (sodium arsenate) and the recovery of the added arsenic was measured. These recoveries are used to assure that there are no matrix interference and to also monitor overall performance over time during analysis.

  Summary of QC Results

  Based on the results of all of the QC samples and steps described above, it is concluded that the analytical results are of sufficient quality for derivation of reliable estimates of arsenic absorption from the test materials.

References

1. Agency for Toxic Substances and Disease Registry (ATSDR): Toxicological profile for arsenic. Atlanta, GA: Public Health service: 2007.
2. Bannon DI, Drexler JW, Fent GM, Casteel SW, Hunter PJ, Brattin WJ, Major MA: Evaluation of small arms range soils for metal contamination and lead bioavailability. *Envrion Sci Technol* 43:9071-9076, 2009.
3. Brent RL: Utilization of animal studies to determine the effects and human risks of Environmental toxicants (drugs, chemicals, and physical agents). *Pediatrics* 113:984-995, 2004.
4. Buchet JP, Lauwerys R, Roels H: Urinary exretion of inorganic arsenic and its metabolites after repeated ingestion of sodium metaarsenite by volunteers. *Int Arch Occup Envrion Health* 48:111-118, 1981.
5. Calabrese EJ, Stanek, EJ, James RC, Roberts SM: Soil ingestion: a concern for acute toxicity in children. *Envrion Health Persp* 05:1354-1358, 1997.
6. Casteel SW, Cowart RP, Weis CP, Henningsen GW, Hoffman E, Brattin WJ, Starost MF, Payne JT, Stockham SL, Becker SV, Turk JR: A swine model for determining the bioavailability of lead from contaminated media. In: Advances in swine in biomedical research. Tumbleson and Schook eds. Plenum Press, New York. 637-46, 1996.
7. Dopp E, Kilgerman AD, Diaz-Bone RA: Organoarsenicals. Uptake, Metabolism, and Toxicity. *Met. Ions Life Sci* 7:231-265, 2010.

8. Francesconi KA, Edmonds JS: Arsenic and marine organisms. *Advances in Inorganic Chemistry* 41:147-189, 1994.
9. Kojina C, Sakurai T, Ochiai M, Kumata H, Qu W, Waalkes MP, et al.: Cytotoxicological aspects of the organic arsenic compound arsenobetaine in marine animals. *Applied Organometallic Chemistry* 16:421-426, 2002.
10. Pomroy C, Charbonneau SM, McCullough RS, Tam GK: Human retention studies with 74As. *Toxicol Appl Pharmacol* 53:550-556, 1980.
11. Rodriguez RR, Basta NT, Casteel SW, Armstrong FP, Ward DC: Chemical extraction methods to assess bioavailable arsenic in soil and solid media. *J Environ Quality* 32:876-884, 2003.
12. Ruby MV, Schoof R, Brattin W, Glodade M, Post G, Harnois M, Mosby DE, Casteel SW, Berti W, et al.: Advances in evaluating the oral bioavailability of inorganics in soil for use in human health risk assessment. *Environmental Science and Technology* 33:3697-3705, 1998.
13. Sakurai T: Biological effects of organic arsenic compounds in seafood. *Applied Organometallic Chemistry* 16:401-405, 2002.
14. Smith AH, Steinmaus CM: Health effects of arsenic and chromium in drinking water: recent human findings. *Annu rev Public Health* 30:107-122, 2009.
15. Styblo M, Drobna Z, Jaspers I, Lin S, Thomas DJ: The role of biomethylation in toxicity and carcinogenicity of arsenic: a research update. *Environmental Health Perspectives* 110:767-71, 2002.

16. Sutton KL, Heitkemper DT, in: Caruso JA, Sutton KL, Ackley (Eds), Elemental speciation: New approaches for trace element analysis. Elsevier, Amsterdam 501-530, 2000.

17. Suzuki KT, Mandal BK, Orgra Y: Speciation of arsenic in body fluids. *Talanta* 58:111-119, 2002.

18. Tam GK, Charbonneau SM, Bryce F, Pomroy C, Sandi E: Metabolism of inorganic arsenic (74As) in humans following oral ingestion. *Toxicol Appl Pharmacol* 50:319-22, 1979.

19. Vanwignen JH, Clausing P, Brunekreef B: Estimated soil ingestion by children. *Environ Res* 51:147-162, 1990.

20. Weis CP, LaVelle JM: Characteristics to consider when choosing bioavailability In: The proceedings of the international symposium on bioavailability and the dietary update of lead. *Science and Technology Letters* 3:113-119, 1993.

21. Yost LJ, Schoof RA, Aucoin R: Intake of inorganic arsenic in the North American Diet/ *Hum Ecol Risk Assess* 4:137-152, 1998.

CHAPTER 3 ARSENIC SPECIATION METHOD DEVELOPMENT

Introduction

Since bioavailability studies in animal models are used to help assess risk to the human population, being able to decipher which arsenic species a human population is being exposed to using the swine model is valuable information for future risk assessments. Separating arsenic species by High-performance Liquid Chromatography (HPLC) followed by detection using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is well established (Suzuki 2002, Hata 2007, B'Hymer 2004, Sutton, 2000, Verdon 2009). However, separation of arsenic species in swine urine where swine have been exposed to arsenic through soil and standard bioavailability studies has not been published before. This chapter describes the methods for the speciation of arsenic in swine urine from two bioavailability studies of arsenic-contaminated soils and proposes some future applications for this type of analysis.

The method development and final method for Arsenic Speciation by LC-ICP-MS is describes in Chapter 3. All method development was conducted at the Missouri Department of Health and Senior Services, Missouri State Public Health Laboratory (MSPHL) within the Chemistry testing Unit in Jefferson City, Missouri.

Clinical Relevance and Summary of Test Principle

Arsenic is a toxic and carcinogenic metalloid that is naturally occurring in the Earth's crust, in soil, rocks and minerals (Klaassen, 2008). It cannot be destroyed by the environment, it can only change form or become separated from other particles (ATSDR,

2007). Arsenicals have been used since ancient times as drugs and even today are very effective against acute promyelocytic leukemia (Klaassen, 2008). Even though elemental arsenic exists, it is usually found in the environment with other elements such as oxygen, chlorine, and sulfur (ATSDR, 2007). Arsenic is of interest due to the possible accumulation in food, water (Montes-Bayon, 2003) and soil. Not only can people be exposed to normal levels of arsenic through the environment by air, water, soil and food, but also through occupational exposure through human activity. In 2003, the United States was the world's largest consumer of arsenic (ATSDR, 2007).

Environmental exposure mainly occurs in arsenic-contaminated water (Klaassen, 2008) and through soil at mining sites. Arsenic in drinking water is often from natural sources. Most of the U.S. drinking water contains arsenic levels lower than 5 ppb; however, it has been estimated that 25 million people in Bangladesh alone drink water with arsenic levels above 50ppb (Klaassen, 2008). The enforceable standard for arsenic is a maximum contaminant level (MCL) and is set at 10 parts per billion (ppb) by the U.S. Environmental Protection Agency and the World Health Organization (Montes-Bayon 2003, EPA 2010). There are some areas of the United States that contain unusually high levels of arsenic, and by living in these areas, the drinking water could be contaminated (Klaassen, 2008). Children may also take in higher amounts of arsenic in these elevated areas due to contaminated soil (B'Hymer, 1999). Food, especially seafood, may contribute significantly to daily arsenic intake (Klaassen, 2008). Arsenic in seafood is largely in an organic form called arsenobetaine that is much less toxic than the inorganic forms (ATSDR 2007, Hata 2007).

Inorganic arsenic is well absorbed 80-90% from the gastrointestinal track. It is then distributed throughout the body while being metabolized by methylation as described in **Figure 1-2: Arsenic Methylation Pathway**, and then excreted primarily by urine (Klaassen, 2008). Both inorganic and organic forms of arsenic leave the body by urine and will be eliminated within several days with some retained within the body for several months (ATSDR, 2007). Arsenic exposure can happen through ingestion, dermal contact or through the air. Skin is a potential route of exposure to arsenic, and systemic toxicity has been reported in persons having dermal contact with solutions of inorganic arsenic (Klaassen, 2008). Airborne arsenic is largely trivalent arsenic oxide and deposition in the airways and absorption of arsenicals from lungs is dependent on particle size and chemical form (Klaassen, 2008). Arsenic has a predilection for skin and is excreted by desquamation of skin and in sweat (Klaassen, 2008). It also concentrates in forming fingernails and hair (Klaassen, 2008). Arsenic exposure produces characteristic transverse white bands across the fingernails, which appears about six weeks after the onset of symptoms of arsenic toxicity and can be used as a biomarker for exposure (Klaassen, 2008).

The toxicity and carcinogenicity of arsenic depend on its species (Baranov, 1999) (See **Figure 3-1: Arsenic Species**). Arsenic species are a good index to predict the risk of arsenic related disease (Stanelle, 2010). The toxicity of the various arsenic species varies a great deal. Arsenite (AsIII) and arsenate (AsV) have a DL50 of 14 and 20 mg/kg while monomethylarsenic acid (MMA) and dimethylarsinic acid (DMA) have a DL50 of 700-1800 and 700-2600 mg/kg respectively (Speciation, 2009). The toxicity of arsenic is

sometimes referred to as synonyms for poison; however, those arsenic compounds in fish and other seafood are actually as harmless as table salt (Speciation, 2009). Arsenocholine and arsenobetaine (AB) have a DL50 of greater than 10000 (Speciation, 2009).

Test Principle

Elemental speciation is defined as the identification and quantitation of the chemical form of an element, including metals and nonmetals (Vela, 1993). The chemical form can be the oxidation state of an inorganic form; it can also depend on the type and number of substituents of organometallic compounds (Vela, 1993). From a risk assessment perspective it is no longer sufficient to quantitate the total elemental content of samples to define toxicity; therefore, elemental speciation offers a continuing challenge for the analytical chemist (Vela, 1993). Liquid chromatography (LC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) is one of the most sensitive and versatile methods used for detecting various arsenic species (B'Hymer, 2004). Several separation techniques are available, but the ones most often used for elemental speciation are chromatographic techniques such as gas chromatography (GC), liquid chromatography (LC), super critical fluid chromatography (SFC), hydride generation and electrothermal vaporization (Ritsema 1998; Vela 1993). Element-selective detection can be achieved with many techniques, including graphite furnace atomic absorption spectroscopy (GFAAS), flame/laser-excited atomic fluorescence spectroscopy, plasma atomic emission spectroscopy (AES), and plasma MS (Vela, 1993). GFAAS and flame/laser-excited atomic fluorescence spectroscopy do not allow the on-

line detection desirable for chromatography (Vela, 1993). Plasma spectroscopy is preferred since it offers low limits of detection with wide linear dynamic ranges and increased sensitivity of 2 - 3 orders of magnitude (subnanogram to subpicogram levels) with the additional capability of isotopic analysis (Vela, 1993).

Two complementary techniques are necessary for trace element speciation. One provides an efficient and reliable separation procedure, and the other provides adequate detection and quantitation. The coupling of these techniques requires sample introduction compatibility and minor instrumentation modifications with maximum efficiency and response for each technique (Vela, 1993).

The ICP is most commonly used for spectrometric analyses, and its basic operation has been described extensively. The ICP is formed in a quartz torch that consists of an assembly of three concentric tubes (Vela, 1993). A plasma is a gas or a mixture of gases in which a fraction of the atoms or molecules is ionized. Plasma gas, typically argon, is passed through these tubes at different flow rates and each tube has a specific function (Vela, 1993). The flow carried between the intermediate and the outer tubes is known as the support flow or cooling gas. The plasma gas is introduced tangentially and forms a vortex flow to the center, providing a toroidal shaped plasma (Vela, 1993). Another function of this flow is to cool the inside walls of the torch and prevent it from melting (Vela, 1993). The nebulizer gas, which flows through the inner tube, transports the sample in the form of an aerosol or gas to the plasma (Vela, 1993). This flow, which comes between the inner and the intermediate tubes, also is termed the auxiliary flow and its function is to position the plasma and keep the plasma

removed from the torch (Vela, 1993). Power is coupled to the torch via a water-cooled load coil by use of a radio frequency generator (Vela, 1993). The plasma is produced when electrons from an external source are imbedded into the region of the induction coil and ionize the neutral plasma gas (Vela, 1993). Once the argon conducts, the plasma forms spontaneously and maintains temperatures of 6000-8000 K (Vela, 1993).

Several methods can be used for introducing liquid or gaseous samples to the plasma. The most common method is solution nebulization from an aqueous sample (Vela, 1993). The main purpose of a nebulizer is to produce an aerosol that can be introduced to the plasma through the inner tube of the torch. A spray chamber is also necessary for separating larger droplets produced by the nebulizer and reducing the solvent load to the plasma. Both LC and flow injection involve interfacing with the spray chamber-nebulizer system.

The most important characteristics of the ICP include high temperatures, long analyte residence times, high electron number densities, and a relatively inert environment (Montaser, 1992). The combination of these properties leads to total desolvation (if solutions are introduced), nearly complete solute vaporization, and a high atomization/ionization efficiency (Vela, 1993). The potential of simultaneous, multi-element analysis with the ICP is realized by using a suitable detection system. The efficiency of the ICP in producing singly-charged positive ions for most elements makes it an effective ionization source for mass spectrometry (MS). The presence of doubly-charged ions is expected only from the elements with low second ionization potential (Hauk, 1986). A two- or three-stage differentially pumped interface is used to extract

ions from the atmospheric-pressure plasma into the low-pressure mass spectrometer (Vela, 1993). Ions pass through a cooled sampling cone with an orifice - 1 mm in diameter and the gas expands behind this first orifice, and a portion passes through a second orifice in the skimmer cone (Vela, 1993). A series of ion lenses, maintained at appropriate voltages, are used to focus the ions into the quadrupole mass analyzer (Vela, 1993). The ions are transmitted through the quadrupole on the basis of their mass-to-charge (m/z) values and are then detected by using an electron multiplier (Vela, 1993). The use of a quadrupole mass analyzer gives better than unit mass resolution over a mass range up to m/z = 300 and the system is considered a sequential multi-element analyzer that has scan times < 20 ms for one sweep (Vela, 1993). The signal intensity is a function of the number of analyte ions (both excited and neutral) in the plasma and the mass dependent transport through the mass spectrometer (Vela, 1993).

LC is commonly used for separating ionic, polar, and non-polar compounds as well as complex ions and neutral species (Vela, 1993). It is the most popular technique for elemental speciation with ICP-MS detection using either reverse phase or ion-exchange chromatography (Montes-Bayon, 2003). Ion-exchange chromatography can be applied to the separation of both ionic and non-ionic compounds. The formation of ionic complexes or the use of ligand-exchange reactions is necessary to resolve non-polar compounds (Vela 1993, B'Hymer 2004). Buffered aqueous salt solutions containing moderate amounts of methanol or acetonitrile are used as mobile phases, whereas the extent of ionization, sample retention, and selectivity is controlled by variations in pH (Vela, 1993).

One limitation of using ICP-MS detection for arsenic speciation is the presence of an isobaric interference at m/z = 75 (B'Hymer, 2004). This interference results from the formation of the polyatomic ion $ArCl^+$ when chlorine is present in a sample reactions with the plasma gas. A reaction cell can be used to minimize interference (B'Hymer, 2004). The reaction cell is pressurized with an appropriate reaction gas and contains a quadrupole that allows for the elimination or reduction of argon-based polyatomic interferences by interaction of the reaction gas with the interfering polyatomic species in the incoming ion beam (B'Hymer, 2004). The quadrupole in the reaction cell allows elimination of unwanted reaction by-products that would otherwise react to form new interferences (Baranov 1999, Tanner 2000).

LC-ICP-MS is the preferred choice of detection for arsenic speciation. ICP-MS offers several advantages for general speciation analysis detection over several other detection methods. Elements such as arsenic normally present in nature at trace or ultra-trace levels are divided among several species of which their contribution to the total elemental weight is minimal (Montes-Bayon, 2003). The introduction of ICP-MS allowed for a new capability of speciation detection. Using LC coupled with ICP-MS allows for the separation of arsenic species before detection with the ICP-MS. Ion exchange chromatography in based on the interactions of charged analytes with the charged functional groups of the stationary phase (Montes-Bayon, 2003). Arsenic species can bind to charged sites on an anion-exchange column (Montes-Bayon, 2003) and can separate species such as arsenite, arsenate, monomethylarsenic acid (MMA), dimethylarsinic acid (DMA), arsenobetaine (AsB) and arsenocholine (AsC) (B'Hymer,

2004). Using a gradient then allows for the separation of these species that can then be detected using the ICP-MS (Montes-Bayon, 2003).

Other advantages to using LC-ICP-MS for arsenic speciation include having high sensitivity, a wide linear detection system and having multi-element and multi-isotope detection (Suzuki, 2002). While LC techniques, primarily ion-exchange chromatography, allows for low detection limit separation. The ease of coupling a LC instrument to an ICP-MS for detection is another important advantage (B'Hymer, 2004). Sample matrixes can be complex when testing for blood, urine or other tissues and given the diversity of the arsenic species that are detectable and low levels of detection required only solidifies that the LC-ICP-MS is an excellent tool for arsenic speciation detection (Keng-Chang, 2011). With the increase in knowledge related to arsenic species and their effects it will only become all that more important to be able to efficiently detect arsenic species using the most sensitive and reliable detection techniques available.

LC-ICP-MS, liquid chromatography inductively coupled plasma mass spectrometry is the method used to determine various arsenic species. The concentration of arsenate (As V), arsenite (As III), MMA, DMA and AB are determined by using high performance liquid chromatography (HPLC) to separate the species coupled to an ICP-DRC-MS to detect the arsenic species. This analytical technique is based on separation by anion-exchange chromatography (IC) followed by detection using quadrupole ICP-MS technology and includes DRC™ technology (Baranou, 1999), which minimizes or eliminates many argon-based polyatomic interferences (Tanner, 2000). Column separation is largely achieved due to differences in charge-charge interactions

of each negatively charged arsenic component in the mobile phase with the positively-charged quaternary ammonium groups bound at the column's solid-liquid interface. Upon exit from the column, the chromatographic eluent goes through a nebulizer where it is converted into an aerosol upon entering the spray chamber. Carried by a stream of argon gas, a portion of the aerosol is transported through the spray chamber and then through the central channel of the plasma where it is heated to temperatures of 6000-8000° K. This thermal energy atomizes and ionizes the sample. The ions and the argon enter the mass spectrometer through an interface that separates the ICP, which is operating at atmospheric pressure (approximately 760 torr), from the mass spectrometer, which is operating at approximately 10^{-6} torr. The mass spectrometer permits detection of ions at each mass-to-charge ratio in rapid sequence which allows the determination of individual isotopes of an element. Once inside the mass spectrometer the ions pass through the ion optics, then through the DRC™, and finally through the mass-analyzing quadrupole before being detected as they strike the surface of the detector. The ion optics uses an electrical field to focus the ion beam into the DRC™. The DRC™ component is pressurized with an appropriate reaction gas and contains a quadrupole. In the DRC™, elimination or reduction of argon-based polyatomic interferences takes place through the interaction of the reaction gas with the interfering polyatomic species in the incoming ion beam. The quadrupole in the DRC™ allows elimination of unwanted reaction by-products that would otherwise react to form new interferences (Jones, 2008).

Three different swine studies were conducted where swine urine was collected for arsenic speciation experiments. These studies were bioavailability studies where study A examined urine collected in one 48 hour period where multiple doses of one test material were used and study B and C had one dose concentration with several test materials using the sodium arsenate control group to estimate relative bioavailability.

Total arsenic was determined in the feed and water used during both Studies A, B and C to ensure that there was minimal exposure to background sources of arsenic. The feed samples that were analyzed were found to contain ≤0.2ug/g of arsenic and the water samples collected contained <1.0ug/L total arsenic. It can be concluded that the feed and water arsenic concentrations would not interfere with total arsenic or arsenic speciated data described below.

Separations of Species in Swine Urine

Figure 3-2: Chromatograph of Arsenic Species by LC IC-MS shows a chromatograph of the five main species found in human urine. Calibration standards were established for each species and calibration concentrations were determined by what species were seen during swine urine analysis. The calibration standards for As III, AB and MMA were 2, 5, 10, 25, 50 and 100 ug/L while the calibration standards for DMA and As V were 5, 25, 50, 100, 250 and 500 ug/L. Quality control concentrations for the check standard and spike for As III, AB, MMA and DMA and As V were 25, 100, 50 and 150 ug/L respectively as describes in **Table 3-1: Standard and Quality Control Concentrations used for Arsenic Speciation**. Only a correlation coefficient >0.99 was accepted for analysis.

pH study

During the development of the arsenic speciation method with swine urine there were some observations of some of the species showing inconsistent retention times and split peaks. Since all standards and controls are analyzed with a diluent consisting of 25% control urine and 75% 0.5M ammonium acetate, the control urine was adjusted to several different pH concentrations and spiked with standards in order to see if there was an optimum pH to stabilize the inconsistent peaks.

Table 3-2: Average Concentration of Arsenic Species at Various pHs of Spiked Control Swine Urine shows the average concentration of the five arsenic species analyzed using control urine that was adjusted to varying pH concentrations using trace metal nitric acid. These samples were analyzed in triplicate at the same species concentration. MMA was observed having a split peak at pH 3, 5 and 7 and As V having a double peak at pH 3, 5 and 7. MMA and DMA show the most instability with varying pH concentrations. **Table 3-2** describes the expected values for MMA occur at pH 0 and 1. For DMA, the expected values do not vary as MMA does, but as indicated by **Figure 3-3: Retention Time Shifts of Arsenic Species at varying pH Concentrations of Spiked Control Urine**, the peak retention time clearly shifts left from pH 7 to pH 0 as well as separation from an unknown arsenic species peak. **Figure 3-3** also depicts the single peak for MMA at pH 0 and split peaks at pH 7. It was decided it was necessary to use 1% trace metal nitric acid to acidify the calibration standard intermediates as well as the control urine diluent to maintain a pH of zero to assure the stability of the arsenic species peaks.

Method Modifications

Along with the acidification of the standards and control urine to stabilize the separation of arsenic species, several other small modifications were made to the method provided by CDC (Verdon, 2009). The analysis software was different than that in the CDC method. Perkin Elmer Chromera software was used instead of TotalChrom since that is what was available on the ICP-MS used for arsenic speciation. Also the two six-port switching valves were set up slightly different. The CDC method had one of the switching valves hard wired to the computer. The set up used for swine urine speciation connected the second switching valve through the HPLC pump. The two switching valves also had slightly different timing. Timed event 1 (TE1) was set with a step at 0.1 and 0.2 as compared to 0.5 and 1.0. The only other noteworthy change between the two methods was the internal standard. Due to cost the swine urine speciation method used a multi-element standard containing arsenic instead of using trimethylarsine oxide (TMAO).

Sample Preparation Comparison

Most published studies regarding sample collection for arsenic speciation in urine state to freeze the samples instead of acidifying them (B'Hymer 2004). Urine samples are usually treated with nitric acid for analysis of total arsenic (Sutton, 2000); however, when evaluating urine for arsenic species it is theorized that oxidation could occur when using nitric acid resulting in inaccurate species detection (B'Hymer, 2004). During the EPA Study, one 48 hour urine collection was collected and frozen immediately, as well as one that was acidified with 1% nitric acid according to the study

design for total arsenic determination. Since both sample collection types were available, both were analyzed for arsenic species.

Figure 3-4: Comparison of Sample Storage and Analysis Methods-Sodium Arsenate and **Figure 3-5: Comparison of Sample Storage and Analysis Meth-Test Material** shows the total arsenic concentrations from study A using the sodium arsenate control group and one test material group. The blue data (◆) is the total arsenic that was determined and reported for the bioavailability study that was conducted. This data was analyzed by hydride generation technique using a PerkinElmer 3100 atomic absorption spectrometer. The red data (■) is the total arsenic from the frozen samples determined by summing each individual species concentration that was detected. The green data (▲) is the total arsenic/48 hours in swine urine from the acidified samples and was determined by summing each individual species concentration detected. Analysis of variance revealed significant differences in average total arsenic between doses examining both the sodium arsenate and test material data as shown in **Table 3-3: Type III Test of Fixed Effects for EPA Study Sodium Arsenate Sample Storage Study** (F=52.98, DF=2, p value=<.0001). This is to be expected since as the dose is increased the average total arsenic will increase. The Type III fixed-effects model of analysis of variance applies to situations in which one or more treatments is applied to the subjects of the experiment to see if the response variable values change. This allows the estimation of the ranges of response variable values that the treatment would generate in the population as a whole.

A least squares means was also conducted to examine differences in dose within each sample preparation group as described above. **Table 3-4: Least Squares Mean for EPA Study Sodium Arsenate Sample Storage Study** shows that within each sample preparation group there is a difference between the average total arsenic and increasing dose as was expected. **Figure 3-4: Comparison of Sample Storage and Analysis Methods-Sodium Arsenate** also reveals that there is considerable difference in response for the total arsenic from the frozen versus the acidified samples.

Table 3-5: Differences of Least Squares Means-Repeated Measures for EPA Study Sodium Arsenate Sample Storage Study shows the repeated measures least square means with adjustment using Tukey-Kramer that demonstrates a difference between the total arsenic data from acidified samples and determined by hydride generation (Preparation 1) and arsenic samples that were frozen (Preparation 2) (t=9.48, df=60, adj p-value=<.0001). There was also a significant difference between frozen samples (Preparation 2) and acidified total arsenic samples determined by LC-ICP-MS (t=-8.22, df=60, adj p value=<.0001). However, there was not a difference between total arsenic determined by hydride generation (Preparation 1) and total arsenic determined by LC-ICP-MS (Preparation 3) (t=1.26, df=60, adj p-value=0.4218). The data shows that freezing swine urine is not the optimum for arsenic recovery since there is a significant difference between arsenic recovered with acidified urine versus frozen urine. It is theorized that since there was a large amount of **Figure 3-4: Comparison of Sample Storage and Analysis Methods-Sodium Arsenate** particulate matter that settled out of the frozen samples that some arsenic may have had a

reduction in solubility accounting for a lower overall total arsenic. The frozen samples were then acidified with nitric acid to see if additional arsenic could be recovered. This is depicted in **Figure 3-4: Comparison of Sample Storage and Analysis Methods-Sodium Arsenate** with the data denoted with a purple (X). There were not enough samples to do an extensive study or statistical analysis to show that no recovery occurred; however, it does indicate that further recovery was not observed.

Figure 3-6: Chromatograph Depicting a Comparison of a Sample that has been Frozen and Acidified shows a chromatograph of the frozen urine sample compared to the acidified swine urine sample to reiterate the fact that recovery of arsenic was significantly different between the two samples preservation methods. **Figure 3-7: Urinary Arsenic Species Comparison of Sample Storage Techniques** show the percent of species per sample for the frozen and the acidified samples. **Table 3-6: Comparison of Sample Storage and the Percent Species Detected for EPA Study** depicts the numeric percentages in comparing the percent species of frozen vs. acidified swine urine sample preparations. In the acidified samples (**Figure 3-7**) as compared to the frozen samples (**Figure 3-7**), the acidified samples do show an increase in percent DMA and As III, and a decreased percent in As V. This could be an indication that some breakdown has occurred since As V is converted to As III and on to DMA during methylation. Most studies suggest avoiding acidification, but it was found that compared with total arsenic determined from another source, that acidifying had better total arsenic results when summed compared to freezing alone. According to these data, the frozen samples provide a better picture of what species exist in the urine, while the acidified urine

shows that some oxidation may be occurring with the increase of DMA. This would be expected with the breakdown of other species, but surprisingly shows an increase in As III. Since these frozen samples as well as the acidified urine samples were stored several months before analysis and the fact that the frozen samples could not compare to total arsenic that was detected in study A by acidification, it was decided to evaluate the acidified collection method more closely during study B and C.

Statistical analysis

Statistical analysis was performed using Microsoft Excel and SAS.

Safety Precautions

Several safety precautions are taken when conducting this method. The arsenic species are known to be toxic and carcinogenic. Care was taken to avoid inhalation or dermal exposure to Arsenous acid (As III), Arsenic acid (As V), Monomethylarsenic acid (MMA), Dimethylarsinic acid (DMA), Arsenobetaine (AB) and any other reagents used in this method. Gloves, lab coats, and safety glasses were worn at all times while handling these reagents.

Because of the possibility of being exposed to various microbiological hazards, take appropriate measures to avoid any direct contact with urine specimens. Wear proper personal protective equipment, including gloves, a lab coat, and safety glasses while handling all urine products.

There are minimal instrument hazards when performing this procedure using standard safety practices. Possible hazards include ultraviolet radiation, high voltages,

radio-frequency radiation, and high temperatures. Avoid direct contact with the mechanical and electronic components, unless all power to the instrument is off. Generally, mechanical and electronic maintenance and repair should be performed only by qualified technicians. Before operating the instrument, read the information in the *PerkinElmer ELAN® ICP-MS System Safety Manual*.

Follow standard safety precautions when performing this procedure, including the use of a lab coat, safety glasses, gloves, and a chemical fume hood and/or biological safety cabinet.

Procedures for Collecting, Storing, and Handling Specimens

A. Special instructions

No special instructions such as fasting, special diets, etc. are required.

B. Sample collection

Juvenile Swine Urine

Samples of urine are collected from each animal for 48-hour periods. Urine is collected in a plastic bucket that has been placed beneath each cage, which is emptied into a plastic storage bottle. Aluminum screens are placed under the cages to minimize contaminations with feces or spilled food. Due to length of collection period collection containers are emptied periodically into a separate plastic bottle to ensure that there was no loss of sample due to overflow. After the 48 hour urine collection an aliquot of urine is placed in a 250 mL bottle with 1% nitric acid. An aliquot of the acidified urine samples are transferred to a 15 mL Falcon tube and refrigerated until use.

C. Sample handling

Samples can be transported at room temperature but are then stored at 5°C +/- 3°C until needed.

D. Sample quantity

The optimal amount of specimen is at least 5 mL, minimum is approximately 1 mL.

E. Unacceptable specimens

The criteria for unacceptable specimens are a low volume (< 1 mL) and suspected contamination such as a leaking or damaged sample container. Specimen contact with dust or dirt may compromise test results. Maintenance of temperature during shipment should be verified by examining the shipment for the presence of unmelted ice in the frozen ice packs. A description of reasons for each rejected sample should be recorded.

Equipment

- Perkin Elmer ELAN II DRC equipped with Series 200 liquid chromatography system with autosampler with ELAN and Chromera software
- Vortexer
- Centrifuge
- Ultracold freezer

Supplies

- 18 MΩ water
- Base urine, pooled from control groups of juvenile swine
- *PRP-X100* anion exchange column (4.6 x 150 mm, 5 µm particle size), Hamilton part number 79174
- Pipettes with tips capable of dispensing 40, 400 and 1000 µL volumes.
- Polypropylene crimp top autosampler vials, *Fisher Scientific* 03-397-61
- Autosampler vial pre-slit crimp cap, *Fisher Scientific*, part number 03-396AA
- Sample racks
- Acetonitrile HPLC grade
- Nitric Acid-Trace Metal
- Methanol HPLC grade
- Ammonium carbonate
- Tris (hydroxymethyl) aminomethane
- Ammonium Sulfate
- Ammonium Acetate
- Acetic acid, Glacial
- Ammonium hydroxide
- Multi-element metals standard that includes As (SPEX)
- Liquid argon

- Twister spray chamber (50 mL volume) *Glass Expansion Inc*., part number 20-809-0296HE
- SeaSpray nebulizer with HPLC sample port, *Glass Expansion Inc*, part number AR30-1-USS2E
- HPLC interface kit, *Glass Expansion Inc*, part number FT-16-8
- 10% hydrogen in argon gas mixture, ≥ 99.999% purity

Standards

- Arsenic (III) SPEX CertiPrep CAS# As+3[7440-38-2]
- Arsenic (V) SPEX CertiPrep CAS# As +5[7440-38-2]
- Arsenobetaine (AB) Fluka CAS # 64436-13-1
- Dimthylarsinic acid Chem Service
- Disodium methyl arsenate (monomethyl arsenic acid) Chem Service

Quality Control Materials

A. NIST Controls Standard Reference Material 2669 As Species in Frozen Human urine. Two Levels 1 and 2.

B. Internal Standard Peak Performance P/N 4400-SPIKE1-100 multi-element including As.

C. Pooled Urine Control see Reagents Preparation section for preparations.

Reagents Preparation

A. HPLC Buffers

0.1 M Ammonium Acetate, pH 5. Dissolve 5.44 g of ammonium acetate and 1.68 mL of concentrated glacial acetic acid into approximately 900 mL of 18 MΩ·cm water. Adjust pH to 5.0 using drop wise additions of either 10% ammonium hydroxide or glacial acetic acid. Complete volume to 1000 mL with 18 MΩ·cm water. Mix thoroughly. Expires in one year. Prepare ahead of time or as needed. Note that this solution is not prepared as a dilution of 0.5 ammonium acecate, pH 5. *Use for sample prep*

0.5 M Ammonium Carbonate. Dissolve 48.05 g of ammonium carbonate into approximately 900 mL of 18 MΩ·cm water. Complete volume to 1000 mL with 18 MΩ·cm water. Mix thoroughly. Expires in one year. Prepare ahead of time or as needed.

0.5 M TRIS Buffer. Dissolve 60.57 g of tris(hydroxymethyl)aminomethane in approximately 900 mL of 18 MΩ·cm water. Complete to 1000 mL with 18 MΩ·cm water. Expires in one year. Prepare ahead of time or as needed.

0.5 M Ammonium Sulfate. Dissolve 66.07 g of ammonium sulfate in approximately 900 mL of 18 MΩ·cm water. Complete to 1000 mL with 18 MΩ·cm water. Expires in one year. Prepare ahead of time or as needed.

Buffer A (Make fresh for every analysis)

HPLC Buffer A Preparation. May be prepared ahead of time before the day of analysis. To a clean 1 liter or greater capacity beaker containing a clean magnetic stir bar add approximately 477.5 m L of 18 MΩ·cm water (≥ 18 MΩ·cm). Add the following:

(a) 10.0 mL of 0.50 M ammonium carbonate
(b) 10.0 mL of 0.50 M TRIS buffer
(c) 2.5 mL of methanol.

Buffer B (Make fresh for every analysis)

HPLC Buffer B Preparation. May be prepared ahead of time before the day of analysis. To a clean 1 liter or greater capacity beaker containing a clean magnetic stir bar, add approximately 462.5 mL of 18 MΩ·cm water (≥ 18 MΩ·cm). Add the following:

(a) 10.0 mL of 0.50 M ammonium carbonate
(b) 10.0 mL of 0.50 M TRIS buffer
(c) 2.5 mL of methanol.
(d) 15.0 mL of 0.50 M ammonium sulfate

Buffer C (Make when needed)

50% Acetonitrile. To make autosampler rinse solution add 200 mL of acetonitrile and 200 mL of 18 MΩ·cm water. Mix. Expires in 1 year. Prepare ahead of time if needed.

Buffer D Column storage (Make when needed)

5% Acetonitrile. To make column storage solution, add 50 mL of acetonitrile (HPLC or Spectrophotometer grade) to 950 mL of 18 MΩ·cm water. Mix. Expires in 1 year. Prepare ahead of time or as needed.

B. Acidified Base Urine (Make when needed)

1. Pipette 10 mL of control base to a 15 mL Falcon tube
2. Add 1% HNO_3, sometime urine may foam or bubble so make sure to this is under a hood.
3. Centrifuge for 10 minutes at 4000 rpm

C. Diluent (make daily)

1. Pipette 2.50 mLs of pooled control base urine that has been acidified
2. Add 7.50 mL of 0.5M Ammonium carbonate

D. Standards

1. Stock solutions

Table 3-7: Standard Stock Solution Preparation describes the preparation of the standard stock solutions. Stock Solutions expire in 1 year with the exception of MMA which expires in 3 months.

NOTE: Once solutions are made Total As is determined by the MSPHL Metals in Water method to determine exact concentration.

2. Intermediate preparation

Two different concentrations of intermediates are prepared and are listed below. Determine the amount of each intermediate or stock solution that needs to be added to each of the intermediates A and B and dilute with 18 MΩ·cm water in 50 mL falcon tubes and acidify with 1% HNO_3. Expiration is 1 month. **Table 3-8: Intermediate Concentrations** describes the intermediate concentrations needed to make the calibration standards.

E. Internal Standard

The internal standard solution is 5ug/L made in DI water.

1. Make a 1000 ug/L intermediate
2. Dilute to 5ug/L in DI water

F. Controls

The Control Urine is prepared by pooling several different and acidified urine samples and determined the concentrations of each species. 1mL aliquots will be made and stored at 25°C +/- 5°C. This is will be used during each day of analysis.

G. ICPMS Rinse Solution

To prepare the ICPMS rinse solution, add 4000 mL DI add 40 mL Trace Metal HNO3 and 800 uL of 1000 mg/L gold. This solution expires in six months.

Instrument Method

A. Optimization file CVAA.dac

Lens voltage optimized using arsenic and ICP RF power set at 1450. All other parameters optimized for best performance.

B. Chromera method parameters

Method is named As Speciation method under the Group As Speciation method group. **Figure 3-8: Arsenic Speciation Method in Chromera** describes the setup of the speciation method in Chromera.

Procedure

Day before Analysis

A. Column Equilibration

1. If time allows, make sure the proper spray chamber is connected, make Buffer A and B and equilibrate the LC column for 30 minutes to one hour the day before an analysis.

To equilibrate column, select STRT or F8 on the pump. Make sure flow is set to 1.0 and Buffer A is at 1%.

Day of Analysis

B. Prepare the instrument and warm solutions to room temperature.

1. Remove Urine samples from Ultracold freezer if samples have been previously frozen. Remove Control Urine from refrigerator.
2. Log on to the instrument computer.
3. Select **Start Menu > Programs > PerkinElmer > Chromera > ChromeraConfigurationApp** to enable Chromera to the current user. Scroll to the **chemistry** line of the current user and check the **use this configuration** checkbox. Save the configuration.
4. Open ELAN software.
5. Check the waste container.
6. Check liquid argon.
7. Set LC autosampler to 4 degrees C. **F1** PELT, set temp to 4, return.
8. Install column.

9. Make LC Buffer A and B and check needle rinse solution for quantity. See section **9.** for procedure to make these reagents.
10. Put line A in mobile phase. Keep the mobile phase bottle lid loose for breathing.
11. Check switching valve to make sure it is set to 1.
12. Using the **pump** key on the LC panel, assure that the pump flow is set to 1.00 and 100%A (alternatively, check if METHOD 02 is loaded in HPLC pump module. If not, press **F6 DIR**, select Method 2, press **F4 RCL**, and then press the blue return button.
13. Press **F8 STRT**.
14. Install spray chamber for CVAA/As Speciation. This method uses three ELAN peristaltic pump lines; a small one from the 6-port valve to the spray chamber, a large one from the spray chamber to the waste line and a small one for internal standard. The ELAN peristaltic pump runs CCW.
15. Make sure switching valve is properly connected-see **Figure 3-9: Switching Valve Setup** for how switching valve is set up.
16. Turn on recirculator.
17. Attach CETAC autosampler pump tubing clamp.
18. Record base vacuum pressure.
19. Start plasma.
20. Put CETAC autosampler rinse line in DI.

21. Open the MIW method in order to identify correct autosampler, or alternatively select method **Sampling** tab, then **Select** button, and choose Cetac ASX-500 autosampler and tray AS500b.try.
22. Send the probe to rinse by selecting **Sampling** tab, then **Probe** button, then **Go to Rinse button**.
23. Wait 30 minutes for instrument and standards to warm up.

C. Prepare standards and samples

1. For Standards see **Table 3-9: Calibration and QC Preparation Sheet**.
2. For NIST Controls
    a. Pipette 300uL of NIST Control Level 1 and 2 into an autosampler vial
    b. Add 100uL of 0.5M ammonium Acetate buffer acidified with 1_HNO_3.
3. For Samples and Urine Control
    a. Centrifuge samples at 4000 for 10 minutes
    b. Pipette 600 uL of urine sample and add 200 uL of 0.5M ammonium acetate buffer into 800 mL autosampler vial.
    c. Gently mix samples.

D. Optimize the lens voltage and do a daily performance check

1. Put CETAC autosampler rinse line in MIW rinse solution.
2. Create a new dataset.
3. Optimize the lens voltage. Use the MIW standard 3, usually in position 5, for the optimization.

4. Open the Optimizing Lens Voltage CVAA worklist, which should open the Lens Calibration CVAA method and CVAA.dac.
5. Make optimizing solution. **Table 3-10: Optimization Solution Stock Solutions** describes what stock solutions are needed for the optimization solution. **Table 3-11: Intermediate Optimization Solution Preparation** describes how to prepare the intermediates to make the optimization solution and **Table 3-12: Working Optimization Solution** describes how to make the optimization solution.
6. In the **Optimization** window, select **get analyte list**, and choose **As**. Under **parameter description**, select **lens voltage**. Record the current lens voltage. Optimize. To view the lens voltage curve, open **Real Time** and choose **numeric**. Go to **Smarttune**. Select the CVAA.dac optimization file and the current dataset. Under Optimization, open the Daily Performance CDC CVAA.mth.
7. Perform the daily performance check. **Table 3-13: Preparation of the Daily Performance Check Solution** describes how to make the daily performance check solution. If the check doesn't pass, adjust the nebulizer gas. If the check still doesn't pass, perform additional optimization. Assure that CVAA.dac file is selected. Perform a pulse stage optimization, a CRO optimization, and then a Cell Path Voltage optimization. Record the base vacuum pressure.
8. Send the probe to standby.

9. If necessary, restart mobile phase flow.

E. Analyze samples

1. Open Chromera. Don't use the Chromera button from the Start menu. Use the button in the ELAN software.
2. Select **To Sequence** icon.
3. Open the **As Speciation sequence** by selecting **File > Open Sequence** and expanding (click + sign) the **As Speciation sequence template group**. Highlight **As Speciation sequence** and press **OK**. See **Table 3-14: A Typical Arsenic Speciation Run Sequence** for the sequence format. Add samples if needed. Run a Check Standard every ten samples and at the end.
4. Select **File > Save Sequence As** to save both the **Group:** "folder" name and the "file" **Name** as the date plus letter of analysis; e.g., the first analysis of January 31, 2009 is named "20090131a".
5. Put samples in autosampler.
6. Start sequence by clicking **Start Sequence** icon.
7. If the Run Time Graphics window is not displayed, select the **To Run Time Graphics** icon.
8. Record column pressure.

F. Process data

1. After data collection (don't try data processing while Chromera is collecting data), select **File > Reprocess Data**. In the **Batch** tab, locate and click the +

sign to expand the current **Batch Name**. Highlight it and press **Next** or select **Select Samples** tab. **Figure 3-10: Processing Method for Chromera** shows the processing method for data analysis using Chromera.

2. The sequence will be displayed in the small **Reprocess** window. Use the window edges or corner to drag the window to nearly full screen. Click the + sign to expand all standards and sample rows. Leave all standards and samples checked. For all standards and samples, click on the down arrow at **Decision Point Type** to select **Peak Detection Review**. Additionally to Standard "S6" **Decision Point Type** add selections 2) **Calibration Review**, and 3) **Report**, **Calibration.rpt**, and **Printer**.
3. Click on the **Reprocess** button at the bottom of the **Reprocess** window. One at a time, a **Peak Detection** window of the chromatograms will be displayed. If the peak is not properly integrated, change in the integration parameters and press **Save PD Method & Update Results**. Once the integration is correct, **Close** the integration window. **Be patient because Chromera data processing is slow here.**
4. After Standard "S6" the calibration will be displayed. In the Species Calibration window, select the Species tab and expand **As 75**, then expand **Species**. The correlation coefficient (R-squared) should be at least 0.999, or QC may not be acceptable. If removing one calibration point (unchecking) doesn't produce at least 0.999, the column may require maintenance or

replacement. **Be even more patient after Standard "S6" because the Chromera data processing is even slower at this point.**

5. Review the results by selecting **To Results Preview**. Select **Print Preview** and print it. Write the Group sequence on the report, along with analyst initials.
6. The hardcopy instrument report includes the printed **Calibration.rpt,** the printed **Results Preview,** and the printed **Daily Performance Check**.
7. QC values will need to be entered in the Microsoft Excel book As Speciation.xls and the QC limits need to be checked in NWAQA under As Speciation. Once all QC has been checked the report will need to be submitted for peer review.

G. Shut down the instrument

1. Exit Chromera. Flush the LC and column with storage buffer (5% acetonitrile) for approximately 15 minutes. Store the column in storage buffer. Shut off peltier on the autosampler.
2. Move the CETAC autosampler probe to rinse. Rinse the spray chamber with MIW acid rinse, then water, and then allow it to dry. Move the CETAC autosampler probe to standby, then release autosampler pump tubing clamp.
3. Stop the plasma. Assure that argon gas flow to the instrument is stopped. Shut off the recirculator. Release the Elan peristaltic pump tubing.

Reportable Range of Results

a. Linearity limits

The reportable range of results using this method is reported in **Table 3-15: Reportable Range of Results for Arsenic Speciation**. The lowest reportable limit is the lowest standard. The highest reportable limit is the highest standard.

b. Limit of Detection

Limit of Detection is the concentration of the lowest Standard.

c. Accuracy

See the As Speciation Validation for accuracy calculations in Chapter 3. The accuracy is established by determining the recovery of As Species from spiked samples during the initial validation. In order to examine the consistency of the recovery over the range of concentrations encountered, the measurements are taken using the two QC standards, covering the range of expected concentrations.

d. Precision

As Speciation Validation for accuracy calculations can be found in Chapter 3.

e. Analytical specificity

The LC separation provides analytical specificity. The As Species peaks are located in an area of the chromatogram with no visible interferences. The internal standard peak does not co-elute with any of the As Species peaks.

Quality Assessment

A. Quality Assessment

Quality Assessment procedures follow standard practices. Daily experimental checks are made on the stability of the analytical system. The instrument performance is checked before each use. Blanks and standards, as well as QC materials, are added to each day's run sequence. The blank is used to detect any contamination in the system or in the spiking solutions and/or reagents. Calibration standard retention times are examined to ensure the correct chromatographic peak. A linear calibration is developed for the batch using a complete set of calibration standards. The results from the analysis of a QC standard obtained using this calibration are compared with acceptance criteria to assure the proper operation of the analysis.

B. Establishing QC limits

Quality Control limits are established for the QC materials through validation with at least seven (7) analyses. These characterization runs include previously characterized QC materials for comparison purposes and are used to establish control during the analytical runs.

Statistical information on the mean, standard deviation, and confidence limits are calculated for the validation data set. Individual quality control charts for the characterization runs are created, examined, and quality control limits are used to ensure analytical stability. See the CVAA-Lewisite metabolite Excel QC book for precision and accuracy calculations.

The following criteria are the minimum requirements for evaluation of the QC validation results. An analytical validation run is considered "out-of-control" if:

(1) A Warning Limit of ± 2s (2* the standard deviation) confidence intervals is acceptable but the quality control needs to be monitored and adjustments made when deemed appropriate.

(2) A Control Limit of ± 3s (3 * the standard deviation) confidence intervalsis considered "out of control". If any control is outside of the 3s range immediate corrective action needs to be taken.

(3) If two consecutive QC concentrations are outside of the same ± 2s confidence interval, the second of those runs is considered "out-of-control".

If the QC result is "out-of-control," the cause of the failure must be determined and corrected. Additional QC criteria may be added if consistent with laboratory policy.

Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

a. Low analyte response

The standard 1 peak must be visible. If it is not visible, rerun standard 1. If the peak is still not visible, perform maintenance on the system.

b. Analyte in standards or QC materials

If an inordinately large amount of analyte is measured in one of the calibration standards or QC materials, but this is not seen in the remainder of the samples, this indicates a contamination of this particular sample. The source of this incident should be investigated to prevent repeat occurrences, but no further action is required.

If the As III peak is the same height at the DMA peak during analysis, this is an indication that the intermediate standard stock isfailing. This will also be indicated during QC analysis with lower concentrations than expected. As V can also show lower concentration values that usually occur simultaneously with As III degradation.

c. Analyte in all samples

If an inordinately large amount of analyte is present in all measurements for a particularsequence, it is likely that one or more of the spiking solutions are contaminated. If necessary, prepare new solutions.

d. QC sample outside of 3σ confidence limits

If the concentration of one or more of the control materials fall outside the 3σ confidence limits, one of the above causes is likely. Follow the steps outlined above to isolate and correct the problem. No analytical results may be reported for runs not in statistical control. Before beginning another analytical run, several QC materials (in the case of QC failure) or calibration verification samples (in the case of calibration failure) are analyzed. Once calibration and/or quality control have been reestablished, analytical runs may be resumed.

Limitations of Method, Interfering Substances and Conditions

Care is required in order to prevent contamination of QC materials, standards, and samples. If chromatographic resolution begins to deteriorate, corrective action must be taken to restore resolution.

The argon chloride (ArCl) interferences on arsenic (^{75}As) are eliminated by the operation of the DRC™ under the parameters noted in the sections above during the speciated arsenic analysis.

The chromatogram may show other urinary arsenic species and polyatomic isobaric interferences (Ar+Cl-) other than the analyte of interest. Due to the nature of the matrix analyzed in this procedure, occasional arsenic species from unknown substances might be encountered but should not elute with the same retention time as current As Species and the internal standard. Interference with the reference standards results in rejection of that analysis. If repeating the analysis does not remove the interference with the reference standard, the results for that analyte are not reportable.

Reference Ranges (Normal Values)

The reference range for human exposure for each arsenic species (see **Table 3-16: Reference Range for Arsenic Species in Humans**) is based on literature reports and from periodic review of accumulated data collected during the analysis of urine samples representing a normal, healthy population believed to be free of unusual exposure to

arsenic. Where data is absent or scant, references ranges are based on the scientific literature, if available (Jones, 2008).

Critical Call Results ("Panic Values")

Due to this method being used for swine studies, there are no critical call results at this time.

Specimen Storage and Handling During Testing

Specimens should be stored at 5±3°C until ready to be extracted for analysis. If the measurement of the extracted samples is delayed until the next day, samples should be refrigerated at 5±3°C.

Alternate Methods for Performing Test or Storing Specimens if Test System Fails

Alternate procedures do not exist for the measurement of urinary As Species, if the analytical system fails, storage of unprepared specimens at -70±5°C is recommended until the analytical system is again operational.

a. Length of time samples may be stored. Repeat measurements of QC samples stored at 5±3°C show no degradation of the analytes.
b. Proper storage procedures

Urine samples should be stored at 5±3°C. Reference materials should be stored according to package insert.

Test Result Reporting System; Protocol for Reporting Critical Calls (if Applicable)

Results are generally reported to integer values of ug/L.

Proficiency Testing

a. Scope of PT

In accordance to MSPHL policies, proficiency test must be preformed twice per year on all methods. Since this method was created in-house, there is no known PT program that supports As Speciation however the CDC will provide human arsenic samples that can be used for proficiency testing purposes. If a problem should arise with this arrangement with CDC to provide human arsenic speciation samples then a Chemistry Unit supervisor will prepare five blind samples for the analyst twice per year.

b. Requirements of PT

Samples are analyzed in accordance with the documented method. Analytical results are reviewed by a Chemistry Unit supervisor.

c. Documentation of PT

A successful PT Challenge requires a score of 80% correct.

Chromera maintenance: Backing up and Restoring the Methods, Sequences, and the Database

A Chromera method or sequence may be exported (saved) to an XML format file. This is recommended, so that methods and sequences can be imported (opened) back into a new database, as discussed below. To export, open Chromera, and activate the method window or the sequence window, and then choose >File>Save as XML. Name the file appropriately so that it can be identified for importing.

The current version of Chromera (v. 1.2) stores all data (methods, sequences, & chromatograms) in a cost-free-use database created through Microsoft Open SQL Server. Since the software was provided in kind, there are limitations to the database: 1) the maximum allowable size of the database is 2 gigabytes; 2) if the database exceeds the 2 gigabyte size, Chromera software will either cease working, or will perform erratically, and; 3) there is no way to ascertain how big the database currently is. Based upon MSPHL experience running only one method (CVAA), the database size should still be < 2 gigabytes after one year.

To backup, restore, or create a new database, close Chromera. Then choose >Start>Programs>Perkin Elmer>DataManager. A dropdown menu in the DataManager allows the user to **Backup**, **Restore**, or **Create New** a database.

Before creating a database using **Create New**, **Backup** the current database (unless the current database is already corrupted). **Create New** will delete all saved methods and sequences, so the parameters will need to be rekeyed, or imported through Chromera as directed above.

Restore is used to recover backed up databases. Remember to **Backup** the current database before restoring an archived database, or current data will be lost.

References

1. ATSDR Toxicology Profile of Arsenic, US Department of Health and Human Services, August 2007.
2. B'Hymer C, Caruso JA: Arsenic and its speciation analysis using high-performance liquid chromatography and inductively coupled plasma mass spectrometry. *J Chromatogr A* 1045:1-13, 2004.
3. Baranov VI, Tanner SD: A dynamic reaction cell for inductively coupled plasma mass spectroscopy (ICP-DRC-MS. Part 1. The rf-field energy contribution in thermodynamics of ion-molecule reaction. *J Anal At Spectrom* 14:1133-1142, 1999.
4. Hata A, et al.: "HPLC-ICP-MS Speciation Analysis of Arsenic in Urine of Japanese Subjects without Occupational Exposure." *Journal of Occupational Health* 49:217-223, 2007.
5. Houk RS: Mass spectrometry of inductively couple plasma. *Anal Chem* 58:97A-105A, 1986.
6. Keng-Chang H, Chien-Che S, Yeou-Lih H: Arsenic speciation in biomedical sciences: Recent advances and applications. *Kaohsiung J Medical Sciences* 27:382-389, 2011.
7. Klaassen CD: Casaraett and Doull's Toxicology the basic science of poisons. New York: McGraw Hill Medical, 2008.
8. Montaser A, Golightly DW: Inductively Coupled Plasmas in Analytical Spectrometry; VCH Publishers: New York, 1992.

9. Montes-Bayon M, DeNicola K, Caruso JA: Liquid chromatography-inductively coupled plasma mass spectrometry. *J Chromatrogr A* 1000:457-476, 2003.
10. Ritsema, R, Dukan L, Navarro, R, Van Leeuwne, W, Oliveria N, Wolfs P, Lebret E: Speciation of arsenic compounds in Urine by LC-ICP MS. *Appl Organometal Chem* 12:591-599, 1998.
11. Stanelle RD, McShane WJ, Dodova EN, Pappas RS, Kobelski RJ: Rapid analysis of Lewisite metabolites in urine by high-performance liquid chromatography-inductively coupled plasma-mass spectrometry. *J Anal Toxicol* 34:122-8, 2010.
12. Sutton KL, Heitkemper DT, in: Caruso JA, Sutton KL, Ackley (Eds), Elemental speciation: New approaches for trace element analysis. Elsevier, Amsterdam 501-530, 2000.
13. Suzuki KT, Mandal BK, Orgra Y: Speciation of arsenic in body fluids. *Talanta* 58:111-119, 2002.
14. Tanner S, Baranov VI, Vollkopf U: A dynamic reaction cell for inductively coupled plasma mass spectroscopy (ICP-DRC-MS. Part III. Optimization and analytical performance. *J Anal At Spectrom* 15:1261-1269, 2000.
15. Vela N, Olson LK, Caruso JA: Elemental Speciation with Plasma Mass Spectrometry. *Analytical Chemistry* 13:585A-597A, 1993.
16. Verdon CP, Caldwell KL, Fresquez MR, Jones RL: Determination of seven arsenic compounds in urine by HPLC-ICP-DRC-MS: a CDC population biomonitoring method. *Anal Bioanal Chem* 393:939-47, 2009.

17. www.EPA.gov accessed on 2/16/2010.

18. www.speciation.net/Public/Document/2008/03/16/3525.html (speciation) accessed on 12/30/09.

CHAPTER 4 ARSENIC SPECIATION METHOD VALIDATION

Introduction

The Validation of Swine Urine Arsenic Speciation Method on LCICPMS was conducted at the Missouri State Public Health Laboratory (MSPHL), Jefferson City, Missouri. The validation used the arsenic speciation method describes in Chapter 2.

This study has been conducted in order to validate the method of Determination of As Speciation in Swine Urine by LCICPMS. This method is capable of separating As Species; arsenite (As III), arsenate (AsV), dimethylarsinic acid (DMA), Monomethylarsenic acid (MMA) and arsenobetaine (AB) in swine urine for research purposes.

Study Discussion

The study validation was completed for As III, As V, DMA and AB between 03/28/2011 and 05/12/2011. The study validation for the MMA components was conducted from 03/28/2011 through 06/16/2011. Additional validation of MMA and the NIST controls was required due to a pH problem with the species that was causing the LC peaks to split. This was corrected by acidifying all standards with nitric acid and using an acidified buffer when making up the NIST controls.

A calibration, check standard, spike, duplicate spike, urine control and two NIST controls for all five species were used to aid in the validation and their determined limits are below.

Analysts

Laura Naught 03/28/11-06/11/11

Validation of the As Speciation Control Limits

Description of Study

A minimum of seven runs were completed for each control parameter. Limits for each control were determined by using the data analysis package on Microsoft Excel. Once the descriptive statistics were determined then limits were calculated by using +/- 2 standard deviations for warning limits and +/-3 standard deviations for the control limits. Some limits have been forced to a smaller range than what has been calculated due to the variability of some species already found in control urine and also the variability in species as the sample is withdrawn from the sample tube. Some limits were wider than what would normally be acceptable. For example, the standard acceptable limits for sample spikes in the Chemistry Unit are usually set to either 80-120% or 70-130%.

Calibration range-The acceptable correlation coefficient calibration range has been set to 0.990-1.00.

NIST controls- NIST controls are in a human urine matrix so the values are not within the provided limits. Due to this fact and that this is a method for research

purposes the NIST controls were analyzed a minimum of seven times to determine quality control limits.

Urine Control- The urine control is created by pooling several known positive urine samples into one sample. This sample has been aliquoted and will be analyzed during each analysis.

Spike- Spiked samples were also analyzed as a duplicate to determine a relative percent difference. There has been some variability with some species but these limits has been set to help account for some of this variability but also maintain a high standard for quality control.

Duplicate Spike- All duplicate samples are reported as relative percent difference and the lower limit has been set to 0 since there are no negative differences.

Study Results

Tables 4-1: AB Quality Control Limits, 4-2: As III Quality Control Limits, 4-3: DMA Quality Control Limits, 4-4: MMA Quality Limits and **4-5: As V Quality Control Limits** show the quality control limits for each control and are listed by each As species in separate tables. Each table includes calculated quality control limits for the correlation coefficient, check standard, NIST control levels, urine control spike and duplicate spike values for AB, As III, DMA, MMA and As V arsenic species.

Precision of the method was also determined and results can be seen in **Table 4-6: Swine Urine Sample Precision**.

Reporting Limits

Table 3-15. Reportable range of results for As Speciation describes the reportable range for AB, As III, DMA, MMA and As V. The lowest reportable limit is the lowest standard. The highest reportable limit is the highest standard.

Calibration Range

The calibration range for AB As III and MMA are 2, 5, 10, 25, 50 and 100 ug/L. The calibration range for DMA and As V are 5, 10, 25, 50, 100, 500 ug/L

Linearity limits

Linearity has been shown up to 2000 ug/L by showing the calculated value is within +/- 10% of the target value. If a sample does run above the highest reportable limit it can be reanalyzed by dilution or by running a higher calibration value and showing linearity within +/- 10%.

Limit of Detection

Limit of Detection is the concentration of the lowest Standard.

Proficiency Testing

The CDC provided four unknown samples to be used as proficiency testing samples. The results were only known by the unit manager and were graded

upon completion of the analysis. These samples were in a human urine matrix and were treated the same as the NIST controls during sample preparation.

Proficiency Testing Results

Four samples were analyzed for five As species. It was decided to use a range of positive and negative for grading purposed since there are significant variables that could affect the results and because we were only provided target values and not a range for each species. Of the 20 analyses there was one, As V PAsS 1007, where it was reported as a non-detected peak and there was a known value for the PT sample. Even though the value is significantly lower than standard one this will be counted as a miss. The grade for this PT event is 19/20 or 95%.

The PT samples were also divided into ranges of concentrations; low, medium and high and the values were compared for each species.

Low range- 15/16 94% due to variability with the sample target values and the fact that some are below our reporting range there were 15 out of 16 samples that were a positive match. As V PAsS 1007 was incorrectly identified as a non-detect when there was in fact a positive value given. Medium range- 3/3 100% all were within 25% of the target value given by CDC. High-1/1 100% the result was within +/- 20% of the target value given by CDC.

This method is considered proficient due to the overall grade of 95% for proficiency testing. This is within the standard acceptable range of 80% being acceptable.

RBA Comparison Using Different Analysis Methods

During CaDTS the studies total arsenic was also determined separately through hydride generation (CaDTS Study #1) and ICP-MS (CaDTS Study #2) as part of the normal study design for determining the Relative Bioavailability (RBA). **Figure 4-1: Graphic Depiction of the Average Percent (%) Relative Bioavailability (RBA) Comparing Two Different Analysis Techniques for California Department of Toxic Substances Study #1 and Figure 4-2: Graphic Depiction of the Average Percent (%) Relative Bioavailability (RBA) Comparing Two Different Analysis Techniques for California Department of Toxic Substances Study #2** shows the comparison of the average relative bioavailability (RBA) of the total arsenic concentration determined by hydride generation and ICP-MS to the LC-ICP-MS arsenic speciation method. As shown in **Table 4-4: Method Analysis of Average Percent Relative Bioavailability (RBA) for California Department of Toxic Substances Studies**, **Figure 4-1** and **4-2** there was little difference between analytical methods used to determine the average percent RBA. Analysis of variance was conducted to determine significance between the calculated percent RBA using the two different analysis techniques. Data indicates that there was no significant difference in **Table 4-8: Type III Test of Fixed Effects California Department of Toxic Substances Study #1** (F=0.02, df=12, p-value=0.8801) and (F=3.49, df=12, p=value=0.0865) as shown

in **Table 4-9: Type III Test of Fixed Effects California Department of Toxic Substances Study #2.**

Conclusion

The Validation study and statistical results presented in Chapter 4 show that the Arsenic Speciation of Swine Urine by LC-ICP-MS is a valid method. This is due to acceptable quality control limits, successful proficiency testing results and statistically similar RBA data comparing two different analysis methods. Due to these factors it has been determined that the arsenic speciation of swine urine by LC-ICP-MS is valid. This method is also capable of quantitating five different arsenic species in swine urine.

CHAPTER 5 ARSENIC SPECIATION RESULTS

CaDTS studies were performed similar to the EPA Study except for six different soils were used for this study at only one dosing level. This was done to maximize the number of soils used and was more budget efficient. The two CaDTS studies had samples collected for all three 48 hour urine collections so this data is the average of all three 48 hour collections. **Figure 5-1: Urinary Arsenic Species in Percent for the Two Studies Conducted for the California Department of Toxic Substances** depicts graphically average percentages while **Table 5-1: Average Percent (%) Arsenic for Study #1 and #2 for the California Department of Toxic Substances** show the numerical values. One major difference between the EPA and the two CaDTS study analyses is that the two CaDTS studies speciation occurred within one month of study conclusion therefore seems a better depiction of the species present. The EPA Study samples were not analyzed for several months after sample collection. Another interesting factor with the CaDTS studies, data is that some small amounts of AB were detected; whereas, during the EPA Study it was not. This could be due to two factors. One is that the samples were analyzed closer to the end the study than with the EPA study and there less potential for the biotransformation of arsenic to occur during sample storage. The second explanation is that there was Chromera software upgrade during this time. While there were no changes made to sample processing method used during analysis and results processing the software did allow for better integration.

Even though general practice is to freeze urine samples for arsenic speciation analysis, it was discovered that frozen urine samples could not recover the total arsenic concentrations determined by acidification indicating that frozen samples give a less accurate depiction of arsenic quantitation in swine urine. Also, the acidified speciated urine was not significantly different from the total arsenic urine concentration indicating that the arsenic speciation method is producing quantitatively accurate results and that the acidified urine is still capable of detecting accurate arsenic species and able to match calculated RBA when comparing the two methods.

It is evident that when comparing the percent of species found in the swine urine samples from the two CaDTS studies with what is found in humans a correlation can be made. For these studies there was a range of 64-74% and 64-77% DMA respectively for all six soils; whereas, a range of 60-75% DMA has been reported in human urine samples (Hopenhayn-Rich, 1993). This further illustrates the importance of the speciation of arsenic in swine urine since it does appear that it could correlate to human exposure to arsenic. If proper measurement systems are utilized to quantify arsenic species of health concern, dosed swine can be used to assess and predict human toxicological effects of arsenic exposure.

When relative bioavailability (RBA) of a chemical in a site medium (e.g., soil) is available the information can be used to improve the accuracy of exposure and risk calculations. RBA data can be used to adjust default oral toxicity values (reference dose and slope factor) to account for differences in absorption between the chemical ingested in water and the chemical ingested in site media, assuming the toxicity factors

are based on a readily soluble form of the chemical. With the development of an arsenic speciation method for swine urine, even more information could be used to conduct human risk assessments.

Several epidemiological studies have indicated that not only occupational exposure, but also long-term exposure to inorganic arsenic in drinking water can increase risk of cancer of the skin, bladder and kidney (Rahman 2011, Chen 1992, IARC 2004). The toxicity and carcinogenicity depends on the arsenic species (Eguchi 1997, Vahter 2002, Hirano 2004). DMA or its derivatives are believed to be the ultimate carcinogens (Shen 2006, Wanibuchi 2004). It has been reported that human urine shows a percentage range of 60-75% DMA excretion (Hopenhayn-Rich, 1993). The two CaDTS studies gave a range between 64-77% DMA indicating that using a swine model correlates nicely to humans for exposure assessments. MMA has also been linked to toxicity. Huang et al. (2008) examined the association between urinary arsenic species and the incidence of urothelial carcinoma (UC). They found significantly higher percentages of MMA and lower percentages of DMA existed among the patients with UC than among healthy residents. Urinary DMA was shown to have an inverse association with risk of UC (Huang, 2008). Smith and Steinmaus (2009) evaluated several studies that looked at the relationship between arsenic metabolism and risks of arsenic-related disease and concluded that elevated proportions of MMA have higher risks of arsenic-caused cancer and other arsenic-related health effects than those who excrete lower proportions (Smith, 2009). One disadvantage of using an animal model is that there was no MMA detected due to the fact that most animals can fully metabolize

arsenic to DMA. MMA is found in human urine due to a metabolic deficiency thereby showing the value in using speciation as a biomarker for determining risk (Smith, 2009).

Arsenic exposure in the United States has been overlooked until recently. All of the U.S. population has been exposed to arsenic. Unlike other parts of the world arsenic in the U.S. is found at lower concentrations in drinking water and in arsenic-contaminated soil; however, that does not make the problem any less relevant. Recent studies have linked arsenic exposure with prevalence of type II diabetes (Navas-Acien, 2005), depression, high blood pressure (Zierold, 2004) and increased risk of cancer (Smith, 2009). Only the Naves-Acien et al (2005) study evaluated speciated arsenic in urine relative to disease. Much of the data that exists looked at the prevalence and risk of arsenic exposure from other countries where known exposures are extremely high. However, exposure can be difficult to determine due to the fact that it is hard to know how much was actually consumed; and that is especially complicated with humans. This is where the use of an animal model could be important especially with soil exposure. It is difficult to know how much soil or dust has been ingested by a human population, but the amount of exposure can be precisely controlled in an animal study.

Clearly arsenic exposure has a negative impact on human health by its association with chronic illnesses and cancer. Most studies regarding risk of disease from arsenic exposure have been conducted outside the United States and most are associated with water exposure. There are currently no available data regarding the speciation of arsenic in populations in the United States exposed to arsenic-contaminated soil living near mining or manufacturing sites. There is also limited

information on health risks of populations in those areas. There is no doubt that arsenic is a good biomarker for exposure, but evidence has suggested that speciation can provide even more information regarding exposure and risk that could help the understanding of low-dose, long-term exposure risks in the United States. This study shows the potential for using the swine model for urinary arsenic speciation as biomarkers to help determine risk of not only cancer, but potentially other chronic diseases as well.

References

1. Chen CJ, Chen CW, Wu MM, Kuo TL: Cancer potential in liver, lung, bladder and kidney due to ingested inorganic arsenic in drinking water. *Br J Cancer* 66:888-892, 1992.
2. Eguchi N, Kuroda K, Endo G: Metabolites of arsenic induced tetraploids and mitotic arrest in cultured cells. *Arch Enivon Contam Toxicol* 32:141-145, 1997.
3. Hirano S, Kobayashi Y, Cui X, Kanno s, Hayakawa T, Shraim A.: The accumulation and toxicity of methylated arsenicals in endothelial cells: important roles of thiol compounds. *Toxicol Appl Pharmacol* 198:458-467, 2004.
4. Hopenhayn-Rich C, Smith AH, Goeden HM: Human studies do not support the methylation threshold hypothesis for the toxicity of inorganic arsenic. *Environ Res* 60:161-77, 1993.
5. International Agency for Research on Cancer (IARC). Some drinking water disinfectants and contaminants, including arsenic. IARC monographs on the evaluations of carcinogenic risk to humans 84. Lyon: IARC 37-267, 2004.
6. Navas-Acien A, Sharrett AR, Silbergeld EK, Schwartz BS, Nachman KE, et al.: Arsenic exposure and cardiovascular disease: a systematic review of the epidemiologic evidence. *Am J Epidemiol* 162:1037-49, 2005.

7. Rahman MM, Chowdhury UK, Mukherjee SC, Mondal BK, Paul K, Lodh D, Biswas BK, Chanda CR, Basu GK, Saha KC, Roy S, Das R, Palit SK, Quamruzzaman Q, Chakraborti D: Chronic arsenic toxicity in Bangladesh and west Bengal, India-a review and commentary. *J Toxicol Clin Toxicol* 39:683-700, 2011.
8. Smith AH, Steinmaus CM: Health effects of arsenic and chromium in drinking water: Recent human findings. *Annu Rev Public Health* 30:107-22, 2009.
9. Vahter M: Mechanisms of arsenic biotransformation. *Toxicology* 181-182:211-217, 2002.
10. Zierold KM, Knobeloch L, Anderson H: Prevalence of chronic diseases in adults exposed to arsenic-contaminated drinking water. *American Journal of Public Health* 94:1936-1937, 2004.

CHAPTER 6 IMPLICATIONS FOR HUMAN EXPOSURE

Prevalence of Disease Associated with Arsenic Exposure

Recent studies have suggested that chronic exposure to arsenic in drinking water is associated with chronic diseases. A study conducted by Zierold, et al. (2004) evaluated the prevalence of nine (9) different chronic diseases in adults who drank well water for twenty or more years in an at risk area in Wisconsin. It was reported that individuals with well water arsenic concentrations between 2ug/L and 10ug/L were significantly more likely to report having depression than individuals exposed to <2ug/L (Zierold, 2004). Individuals who were exposed to contaminated water >10ug/L were significantly more likely to report having had cardiac bypass surgery, high blood pressure, and circulatory problems than those exposed to well water at <2ug/L arsenic (Zierold, 2004). Mazumder, Et al. (2000) found a prevalence of cough, shortness of breath and chest sounds in the lungs of those who had increased concentrations of arsenic in their drinking water. These symptoms were most pronounced in those subjects that had skin lesions, a symptom of arsenic poisoning. Prevalence odds ratio estimates were significantly increased for those who were exposed to high levels of arsenic in drinking water and were reported at 7.8 (95% CI: 3.1-19.5) for females and 5.0 (95% CI: 2.6-9.9) for males for cough; as well as, 9.6 (95% CI: 4.0-22.9) and 6.9 (95% CI: 3.1-15.0) for chest sound for females and males respectively (Muzaumder, 2000). There is also an indication that arsenic exposure is associated with the prevalence of type 2 diabetes in the United States. A study conducted by Navas-Acien Et al. (2008)

evaluated the percent of arsenic in urine from participants in a previous National Health and Nutritional Examination Survey (NHANES). This study shows individuals with type 2 diabetes have 26% higher level of total arsenic in their urine indicating an association with arsenic urine concentrations and type 2 diabetes (Navas-Acien, 2008).

Risks Associated with Exposure to Arsenic

There have been several studies conducted with exposure to high concentrations of arsenic in drinking water looking at both children and adult exposures. These studies are usually reported as relative risk and standardized mortality rates. Studies have indicated that when comparing exposed women versus unexposed women a relative risk of spontaneous abortion was reported as 2.5 (95% CI 1.5-4.3) and a relative risk for stillbirths as 2.5 (95% CI 1.3-4.9) (Ahmad, 2001). Women with arsenic skin lesions, indicating direct and long-term exposure had an increased risk of stillbirths with a relative risk of 13.1(CI: 3.2-54) (Kwok, 2006). Infant mortality relative risk is estimated at 1.17 (CI: 1.02-1.32) from a study involving over 29,000 pregnancies (Rahman, 2007). Childhood cancer has also been reported that a relative risk is estimated at 1.39 (CI: 0.7-2.76) for lymphoblastic leukemia (Infant-Rivard, 2001). There have been studies that also examine adults who have been exposed *in utero* or in early childhood. It was reported that the standardized mortality ratio for lung cancer in adults who were born just before the height of exposure and had been exposed in early childhood was 7.0 (CI 5.4-8.9) and 12.4 (CI 3.3-31.7) for bronchitis (Smith, 1998). For those adults who were born during high exposure, a standardized mortality ratio of 6.1 (CI 3.5-9.9) for lung cancer and 46.2 (CI 21.1-87.7) for bronchitis (Smith, 1998) was evident.

There is also evidence that myocardial infarction in northern Chile showed that mortality increased soon after high exposure began (Yuan, 2007), and reports indicate that long-term arsenic exposure from drinking water is associated with urinary bladder cancer in Chile and Argentina (Huang, 2008). Mortality was also studied in northern Chile where significant arsenic exposure has occurred and this area was compared to the rest of Chile. It was found that rates for bladder, skin, lung and kidney cancer were increased. Bladder cancer showed an increased standard mortality rate of 6.0 (95% CI 4.8-7.4) in men and 8.2 (95% CI 6.3-10.5) in women (Smith, 2010). Lung cancer mortality in northern Chile was reported in men as the standard mortality rate of 3.8 (95% CI 3.5-4.1) and in women 3.1 (95% CI 2.7-3.7) (Smith, 2010).

Arsenic cancer rates in various regions around the world

Epidemiological studies in areas such as Bangladesh, Taiwan and West Bengal, India have demonstrated a significant increase in the risk of lung, skin, liver, bladder and other cancers associated with the high levels of arsenic in drinking water (Tchounwou, 2004). Since areas such as Bangladesh, West Bengal, India and Taiwan are exposed to significantly higher arsenic in certain groundwater sources their cancer risk is higher. The U.S. EPA estimates the lifetime risk for developing skin cancer from the ingestion of arsenic in water ranges from 1 per 1000 to 2 per 1000 (EPA, 1998). Tseng (1968) conducted a study of a large population in Taiwan and found a clear dose-response between drinking water and the prevalence of skin cancer. His study estimated that by the age of 60 more than 1 in 10 would develop skin cancer (Tseng, 1968). This is a significantly increased risk of exposure as compared to those in the United States.

Though epidemiological studies are limited on cancer risks from around the world, Smith (2002) is uncertain what the cancer risk will be in Bangladesh due to latency of cancer. However, it is sure to be significant.

In many areas where arsenic-contaminated drinking water is prevalent, the main causes of death with chronic ingestion are internal cancers. Dramatic increases in mortality from internal cancers have been reported in Taiwan (Chen, 1992) and Chile (Smith, 1998). In Taiwan populations exposed to high concentrations of arsenic in their drinking water with an average of 800 ug/L of arsenic have an estimated relative risk of bladder cancer between 30 and 60 (Chen 1992, Smith 1998). In northern Chile between 5-10% of all deaths occurring over the age of 30 were attributed to arsenic-caused internal cancers particularly bladder and lung (Smith, 1998). In Chile the average arsenic exposure was 500 ug/L over 10-20 years (Smith, 1998). Numerous studies have been conducted showing an increase of skin and internal cancers all over the world, and they clearly show a dose-response relationship. The more arsenic one is exposed to for longer periods of time, the greater risk there is to having certain cancers.

However, in the United States the cancer risk is a little bit more difficult to estimate. There is no accurate data on the average arsenic levels in drinking water for the US but the estimated range is from 2.0 to 2.5 ug/L (EPA 1998, ATSDR 2007). Using extrapolated data from cancer risk studies from Taiwan and other high risk areas, an average arsenic drinking water level of 2.5 ug/L would yield an estimated lifetime risk of dying from liver, lung, bladder or kidney cancer due to arsenic in drinking water at 1 in a 1000 (Smith, 1998). Using the previous EPA standard of 50 ug/L it has been estimated

that the life risk could be as high as 13 per 1000 persons exposed (Smith, 1992). Using the same estimation methods it is also suggested that person exposed to 500 ug/L of arsenic could have a lifetime risk of 13 per 100 people (Smith, 1998). In the latest U.S. National Research Council report concluded that exposure to 50 ug/L could easily result in a combined cancer risk of 1 in 100 (NRC, 1999).

While there are clearly different regions around the world that have been or are currently being exposed to arsenic-contaminated groundwater, several factors determine if there are increased risks for cancer. While there can be significantly different geological environments that cause arsenic to enter the groundwater there seems to be numerous conditions that control that and while some conditions are prone to more solubilization than others, it is still hard to predict where high arsenic will occur. While it appears there may be some correlation to location and an increased cancer risk, it is clear that it all comes down to the dose and length of exposure. The higher dose of exposure for longer periods of time shows that there is a definite increase in cancer risk. Cancer latency can also cause difficulties in determine risk and also the exact amount of dose and the period of exposure all play a part and those factors are difficult to determine. One thing is certain; arsenic-contaminated groundwater does show a clear link to skin and other internal cancers. Although the evidence of carcinogenicity of arsenic in humans seems strong the mechanism by which it produces tumors in humans is not completely understood (Tchounwou, 2004).

Determining Risk

Assessing risk factors to environmental contaminates such as arsenic can be difficult. Traditional approaches, especially with cancer studies, can provide insufficient data in regards to sample size and inadequate exposure assessments (Bencko, 2011). It can be difficult to quantitatively determine exposure and often has to be done retrospectively because it is difficult to know how much water or soil an individual has ingested or has been exposed to on their skin (Bencko, 2011). There are two ways that this problem could be remedied. One is the use of biomarkers to assess exposure. Total arsenic and its metabolites can all be detected in urine. By sampling those exposed one could assess the amount exposure from what was determined as exposure in the exposure assessment. Not all species of arsenic are toxic and if a population is able to readily digest that arsenic then their risk could be very different from another exposure. The other option could be to use an animal model to assess risk to a human population. Both assessing biomarkers and using an animal model are proposed in this method hypothesis and will be discussed later in the paper.

Animal Models

Toxicological studies have long been performed on an animal model to determine mechanisms of action, effective and safe dosing and human risk of toxicity from human exposure to toxic substances (Brent, 2004). Juvenile swine are primarily selected for use because they are considered to be a good physiological model for gastrointestinal absorption in children (Casteel, 2010), and the current model used to determine risk of arsenic exposure to those that have been exposed. Reliable analysis of

the potential hazard to humans from ingestion of a chemical depends upon accurate information on a number of key parameters. These parameters include the concentration of the chemical in environmental media (soil, dust, water, food, air, paint), intake rates of each medium, and the rate and extent of absorption ("bioavailability") of the chemical by the body from each ingested medium (Casteel, 2010). The amount of a chemical that actually enters the body from an ingested medium depends on the physical-chemical properties of the chemical and the medium (Casteel, 2010). Some metals in soil may exist, at least in part, as poorly water-soluble minerals, and may also exist inside particles of inert matrix such as rock or slag of variable size, shape, and association. These chemical and physical properties may influence the absorption or bioavailability of the metals when ingested (Casteel, 2010). Thus, equal ingested doses of different forms of a chemical in different media may not be of equal health concern.

For example, the Environmental Protection Agency (EPA) will perform a risk assessment on a population that lives near or has been exposed to arsenic-contaminated soil; these sites have usually been declared Superfund sites. One way to determine risk is by measuring the bioavailability of arsenic, meaning, how much of this particular arsenic-contaminated soil is readily absorbed by a human. The relative bioavailability (RBA) of a soil is determined by exposing swine to contaminated soil by measuring the level of arsenic in urine. The RBA that is calculated then aids the EPA and other regulatory agencies in determining risk of exposure for those who live near the exposed area. Animal models are a good model to use to determine risk of human

exposure. It is difficult to seek volunteers to expose themselves to toxic substances and monitor these effects and also monitoring takes time and long-term studies are expensive and difficult to keep track of study participants over this long period of time. If a link can be correlated between an animal model and human risk this could provide invaluable information needed to assess past, ongoing and future exposures.

References

1. Agency for Toxic Substances and Disease Registry (ATSDR), Toxicology Profile for Arsenic, 2007.
2. Ahmad SA, et al.: Arsenic in drinking water and pregnancy outcomes. *Environ Health Perspect* 109:629-31, 2001.
3. Bencko V: Use of Epidemiological Data for Cancer Risk Assessment: approaches, concepts, and issues. *The Open Epidemiology Journal* 4:94-98, 2011.
4. Brent RL: Utilization of Animal Studies to Determine Effects and Human Risks of Environmental Toxicants (Drugs, Chemicals and Physical Agents). *Pediatrics* 113:4, 2004.
5. Calderon J, et al.: Exposure to arsenic and lead and neuropsychological development in Mexican children. *Environ Res* 85:69-76, 2001.
6. Castell SW, et al.: Relative Bioavailability of Arsenic in Two Soils for the Iron King Mine. Prepared for: US Environmental Protection Agency Office of Superfund remediation Technology Innovation. February 25, 2010.
7. Chen CJ, Chen CW, Wu MM, Kuo TL: Cancer potential in liver, lung, bladder and kidney due to ingested inorganic arsenic in drinking water. *Br J Cancer* 66:888-892, 1992.

8. Huang Yung-Kai, et al.: Arsenic Exposure, Urinary Arsenic Speciation, and the Incidence of Urothelial Carcinoma: a Twelve Year Follow-up Study. *Cancer Causes Control* 19:829-839, 2008.
9. Infant-Rivard C., et al.: Drinking water contaminants and childhood leukemia. E*pidemiology* 12:13-19, 2001.
10. Kwok RK, et al: Arsenic in drinking water and reproductive health outcomes: a study of participants in the Bangladesh Integrated Nutritional Programme *J Health Popul Nutr* 24:190-205, 2006.
11. Mazumder D, Guha N, et al.: Arsenic in drinking water and the prevalence of respiratory effects in West Bengal, India. *International Journal of Epidemiology* 29:1047-1052, 2000.
12. Navas-Acien A, Sharrett AR, Silbergeld EK, Schwartz BS, Nachman KE, et al.: Arsenic exposure and cardiovascular disease: a systematic review of the epidemiologic evidence. *Am J Epidemiol* 162:1037-49, 2005.
13. National Research Council (NRC): Arsenic in Drinking Water. Washington, DC: National Research Council, National Academy, 1999.
14. Rahman M, et al.: Association of arsenic exposure during pregnancy with fetal loss and infant death: a cohort study in Bangladesh. *Am J Epidemiol* 165:1389-96, 2007.
15. Smith AH, et al.: Marked increase in bladder and lung cancer mortality in a region of Northern Chile due to arsenic in drinking water. *Am J Epidemiol* 147:660-69, 1998.

16. Smith AH, Steinmaus CM: Health Effects of Arsenic and Chromium in Drinking Water: Recent Human Findings. *Annu Rev public Health* 30:107-22, 2009.

17. Smith AH: University of California, Berkeley School of Public Health. Arsenic Health Effects Research Program access as http://asrg.berkeley.edu on 05/26/11, 2010.

18. Tchounwou TB, Centeno JA, Patlolla AK: Arsenic toxicity, mutagenesis, and carcinogenesis-a health risk assessment and management approach. *Molecular and Cellular Biochemistry* 255: 47-55, 2004.

19. Tseng WP, et. al: Prevalence of skin cancer in an endemic area of chronic arsenicism in Taiwan. *Journal of National Cancer Institute* 40: 453-463, 1968.

20. US EPA (EPA): Special report on ingested inorganic arsenic skin cancer; nutritional essentiality Risk assessment forum. Publication No. EAP 625/3-87/013, Washington DC, EPA, 1998.

21. Yuan Y, et al.: Acute myocardial infarction mortality in comparison with lung and bladder cancer mortality in arsenic-exposed region II of Chile from 1950-2000. *Am J Epdiemiol* 166:1381-91, 2007.

22. Zierold KM, et al.: Prevalence of Chronic Diseases in Adults Exposed to Arsenic-Contaminated Drinking Water. *American Journal of Public Health* 94:11, 2004.

CHAPTER 7 SUMMARY AND CONCLUSIONS

Clearly arsenic exposure has a negative impact on human health by being associated with chronic illnesses and cancer as described throughout this paper. Most studies regarding risk of disease from arsenic exposure has been conducted outside the United States and mostly associated with water exposure. There is currently no available data regarding the speciation of arsenic in populations in the United States exposed to arsenic-contaminated soil living near mining or manufacturing sites and very few regarding exposure to contaminated water sources. There is also limited information on health risks of populations in those areas. Presented in this study (or paper) is strong evidence that using arsenic species in urine as a biomarker and the success of using animal models to help determine human risks. If a link can be correlated between an animal model and human risk this could provide invaluable information needed to assess past, ongoing and future exposures.

APPENDIX A

Chapter 1 Figures

Figure 1-1: Arsenic Distribution of Groundwater in the United States

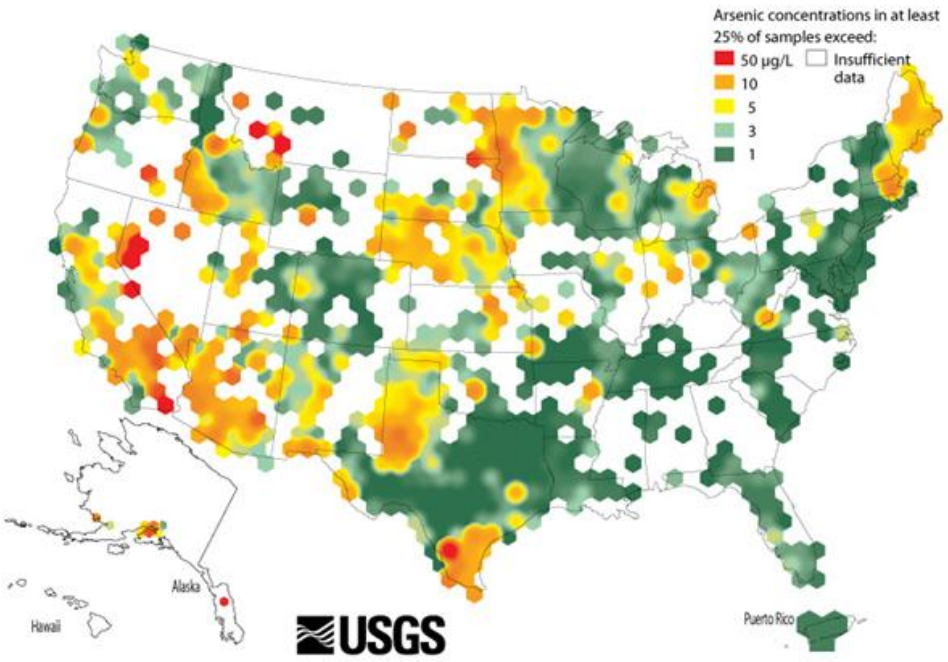


Figure 1-1 shows the distribution of arsenic contaminated groundwater in the United States. Concentrations vary with orange and red areas indicating the highest concentrations. Map from www.water.usgs.gov.

Figure 1-2: Arsenic Methylation Pathway

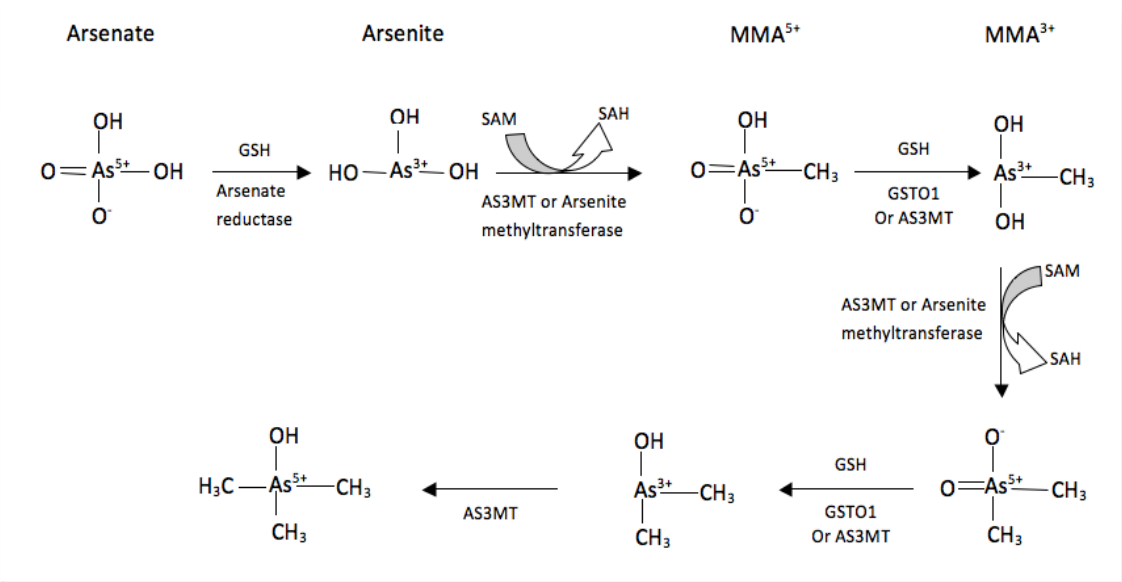


Figure 1-2 describes the methylation pathway of arsenic. Arsenate and monomethylarsonic acid (MMA^{V}) reductases in mammalian cells catalyze the reduction of arsenate to arsenite and of MMA^{V} to monomethylarsonous acid (MMA^{III}). The methylation of arsenite is catalyzed by arsenite methyltransferase with S-adenosylmethionine (SAM) as a methyl group donor. The first methylation step forms MMA^{V} where MMA^{V} is then reduced to MMA^{III} and further methylated to yield DMA^{V} in the presence of MMA^{III} methyltransferase . Similar reduction of DMA^{V} to DMA^{III} is the third methylation step forming trimethylarsenic oxide ($TMA^{V}O$).

APPENDIX B

Chapter 2 Tables

Table 2-1 Study Design and Dosing Information for Study Conducted for the EPA

Group	Group Name Abbreviation	Dose Material Administered	As Conc of the material (ug/g or ug/ul)	No. Pigs in Group	Arsenic Dose		
					Target (ug/kg BW-day)	Actual[a] (ug/kg BW-day)	Actual[b] (ug-day)
1	NaAs	Sodium Arsenate	2	4	25	25	339
2	NaAs	Sodium Arsenate	10	4	50	50	678
3	NaAs	Sodium Arsenate	10	4	100	100	1354
4	TM1	ASARCO	181.9	4	40	40	542
5	TM1	ASARCO	181.9	4	60	60	813
6	TM1	ASARCO	181.9	4	120	120	1625
7	TM2	Hawaii	500	4	40	40	542
8	TM2	Hawaii	500	4	60	60	813
9	TM2	Hawaii	500	4	120	120	1625
10	Control	Negative control	0	3	0	0	0

[a]Calculated as the administered daily dose divided by the measured or extrapolated daily body weight, averaged over days 0-14 for each animal and each group

[b]Calculated as the mass of soil or sodium arsenate solution administered times the concentration of the soil or sodium arsenate solution. Doses were administered in two equal portions given at 9:00 AM and 3:00 PM each day. Doses were held constant based on the expected mean during the exposure interval (14 days).

Table 2-2: Study Design and Dosing Information for a Study Conducted for the California Department of Toxic Substances, Study #1

Group	Group Name Abbreviation	Dose Material Administered	As Conc of the material (ug/g or ug/uL)	No. Pigs in Group	Target (ug/kg BW-day)	Actual[a] (ug/kg BW-day)	Actual[b] (ug-day) Dose Prep 1 (day 0-4)	Actual[b] (ug-day) Dose Prep 2 (day 5-9)	Actual[b] (ug-day) Dose Prep 3 (day 10-13)	Average Actual[b] dose over 0-13 days
1	TM1	EM01-1-1.3	302	5	60	60	634.2	676.8	760.2	690.4
2	TM2	EM03-0-1.3	2541	5	60	60	634.2	676.8	760.2	690.4
3	TM3	EM08-0-0.2	633	5	60	60	634.2	676.8	760.2	690.4
4	TM4	EM18-0-2	10482	5	60	60	634.2	676.8	760.2	690.4
5	TM5	EM19-0-1	370	5	60	60	634.2	676.8	760.2	690.4
6	TM6	EM21-1-3	12041	5	60	60	634.2	676.8	760.2	690.4
7	NaAs	Sodium Arsenate	10	5	50	50	528.5	564	633.5	575.3
8	Control	Negative control	0	3	0	0	0	0	0	0

[a]Calculated as the administered daily dose divided by the measured or extrapolated daily body weight, averaged over days 0-5, 6-10 and 11-14 for each animal and each group.

[b]Calculated as the mass of soil or sodium arsenate solution administered times the concentration of the soil or sodium arsenate solution.

Dose were administered in two equal portions given at 8:00 AM and 3:00 PM each day. Doses were held constant based on the expected mean weight during each dosing period (day 0-4, 5-9 and 10-13).

Table 2-3: Study Design and Dosing Information for a Study Conducted for the California Department of Toxic Substances, Study #2

Group	Group Name Abbreviation	Dose Material Administered	As Conc of the material (ug/g or ug/uL)	No. Pigs in Group	Target (ug/kg BW-day)	Actual[a] (ug/kg BW-day)	Actual[b] (ug-day) Dose Prep (day 0-13)
1	TM1	EM05	1906	5	60	60	913.2
2	TM2	EM13	1237	5	60	60	913.2
3	TM3	EM15	12095	5	60	60	913.2
4	TM4	EM20	5647	5	60	60	913.2
5	TM5	RG01	200	5	60	60	913.2
6	TM6	RG03	610	5	60	60	913.2
7	NaAs	Sodium Arsenate	10	5	50	50	761
8	Control	Negative control	0	3	0	0	0

[a]Calculated as the administered daily dose divided by the measured or extrapolated daily body weight, averaged over days 0-14 for each animal and each group.

[b]Calculated as the mass of soil or sodium arsenate solution administered times the concentration of the soil or sodium arsenate solution.

Dose was administered in two equal portions given at 8:00 AM and 3:00 PM each day. Doses were held constant based on the expected mean weight during each doing period (14 days)

APPENDIX C

Chapter 3 Tables

Table 3-1: Standard and Quality Control Concentration Used for Arsenic Speciation

	AsIII (ug/L)	AB (ug/L)	DMA (ug/L)	MMA (ug/L)	As V (ug/L)
Standard 1	2	2	5	2	5
Standard 2	5	5	25	5	25
Standard 3	10	10	50	10	50
Standard 4	25	25	100	25	100
Standard 5	50	50	250	50	250
Standard 6	100	100	500	100	500
Check Standard	25	25	100	25	100
Spike Value	50	50	150	50	150

Table 5-1 describes the standard and quality control concentrations used for arsenic speciation by LC-ICP-MS analysis.

Table 3-2: Average Concentration of Arsenic Species at Various pHs of Spiked Control Swine Urine

pH	AB (ug/L)	As III (ug/L)	DMA (ug/L)	MMA (ug/L)	As V (ug/L)
0	27.30	24.70	110.91	24.05	94.19
1	25.36	26.87	110.22	23.84	106.68
3	25.04	26.14	107.40	23.61[1]	106.64[2]
5	25.93	26.00	139.48	21.97[1]	92.74[2]
7	25.27	24.80	107.09	13.54[1]	118.46[2]

The data in Table 4-4 shows the average concentration of spiked control swine urine for each arsenic species determined by LC-ICP-MS analyzed in triplicate. The spiked concentrations were expected to be 25.00 ug/L for AB, As III and MMA and 100.00 ug/L for DMA and As V. It was observed that MMA showed a [1]split peak as the pH of the spiked control urine was increased. As V also showed what would be described as a [2]double peak at higher pH concentrations making it difficult to integrate. As the pH was decreased the double peak separated and there was a clear As V peak and an addition unidentified arsenic peak.

Table 3-3: Type III Test of Fixed Effects for EPA Study Sodium Arsenate Sample Storage Study

Effect	Num DF	Den DF	F Value	Pr>F
Dose	2	60	52.98	<.0001
ug As/48 hours	5	60	20.82	<.0001
Dose*ug As/48 hours	10	60	0.87	0.5634

Table 5-2 describes the type III test of fixed effects for a study conducted for the U.S. Environmental Protection Agency for both the sodium arsenate and test material 1 study groups. The data indicate there was a significant difference between dosing groups which is to be expected. Statistical data analysis determined using SAS.

Table 3-4: Least Squares Means for EPA Study Sodium Arsenate Sample Storage Study

Effect	Estimate	Standard Error	DF	t Value	Pr>|t|
Preparation 1	6.6745	0.07634	60	87.43	<.0001
Preparation 2	5.7440	0.07634	60	75.24	<.0001
Preparation 3	6.5506	0.07634	60	85.81	<.0001

Table 5-3 describes the least square means for a study conducted for the U.S. Environmental Protection Agency sodium arsenate study group. The analysis indicates there was a significant difference between dosing groups which is to be expected. Preparation 1=Total As ug/48 hour acidified and analyzed by hydride generation, preparation 2=As species summed for total As ug/48 hour frozen samples analyzed by LCICPMS and preparation 3=As species summed for total As ug/48 hour acidified samples analyzed by LCICPMS. Statistical data analysis determined using SAS.

Table 3-5: Differences of Least Squares Means-Repeated Measures for EPA Study Sodium Arsenate Sample Storage Study

Effect	Preparation	Preparation	Estimate	Standard Error	DF	t-Value	Adj P
Preparation	1	2	0.9304	0.09816	60	9.48	<.0001
Preparation	1	3	0.1239	0.09816	60	1.26	0.4218
Preparation	2	3	-0.8065	0.09816	60	-8.22	<.0001

Table 5-4 shows the repeated measures least square means with adjustment using Tukey-Kramer that demonstrates a difference between the total arsenic data from acidified samples and determined by hydride generation (Preparation 1) and arsenic samples that were frozen (Preparation 2). There was also a significant difference between frozen samples (Preparation 2) and acidified total arsenic samples determined by LC-ICP-MS (Preparation 3). There was no difference between total arsenic determination by hydride generation (Preparation 1) and total arsenic determined by LC-ICP-MS (Preparation 3). Statistical data analysis determined using SAS.

Table 3-6: Comparions of Sample Storage and the Percent Species Detected for a Study Conducted for the EPA Study

Dose Group	AsIII % Frozen	AsIII % Acidified	DMA % Frozen	DMA % Acidified	AsV % Frozen	AsV% Acidified
Group 1	0.95	6.80	80.33	89.27	18.72	3.74
Group 2	1.98	4.15	70.46	93.48	27.56	1.95
Group 3	1.62	5.51	83.66	90.88	14.65	3.16
Group 4	0.00	4.10	60.42	92.76	39.58	3.14
Group 5	0.40	5.99	52.40	89.65	47.20	4.37
Group 6	1.28	6.61	76.77	89.96	21.95	2.59
Group 7	0.00	5.08	72.56	88.03	27.44	6.63
Group 8	1.36	4.39	71.84	92.96	26.79	1.89
Group 9	1.84	3.37	86.63	92.30	11.53	3.11

Table 5-5 describes the percentage of each species determined by LC-ICP-MS comparing the sample storage process of freezing vs. acidification.

Table 3-7 Standard Stock Solution Preparation

Arsenic Species	Range of Weight	Solvent Used to Dissolve	Final Volume After Dissolution
Arsenobetaine (AB)	0.02–0.03 g	Water (18 MΩ·cm)	10.00 mL
Disodium methyl arsenate (MMA)	0.20–0.25 g	Water (18 MΩ·cm)	50.00 mL
Dimethylarsinic acid (DMA)	0.20–0.25 g	Water (18 MΩ·cm)	100.0 mL

Table 2-1 describes the standard stick solutions that are required to conduct the arsenic speciation method by LC-ICP-MS. Stock Solutions expire in 1 year with the exception of MMA which expires in 3 months.

Table 3-8 Intermediate Concentrations

intermediate standards	Intermediate identified	Concentration, ug/L
V, DMA	A	1000 ug/L
III, MMA, AB	B	250 ug/L

Table 2-2 describes the intermediate concentrations made from the standard stock solutions shown in Table 2-1. Species with the same concentrations are combined into two different intermediate standards as describes above. Intermediate stock solutions expire after three months.

Table 3-9 Working Calibration and QC Preparation Sheet

	ug/L	DMA, As V 1,000ug/L INT uL	ug/L	As III, AB, MMA 250ug/L INT uL	Diluent uL 25% Control Urine 75% Buffer
Standard 1	5	**4**	2	**6.4**	789.6
Standard 2	25	**20**	5	**16**	764
Standard 3	50	**40**	10	**32**	728
Standard 4	100	**80**	25	**80**	640
Standard 5	250	**200**	50	**160**	440
Standard 6	500	**400**	100	**320**	80
Chk Std	100	**80**	25	**80**	640
Spk/Dup Spk	150	**160**	50	**160**	280 urine, 200 buffer

Table 2-3 describes how to prepare the calibration standards and quality control samples for use in the arsenic speciation method by LC-ICP-MS.

Table 3-10 Optimization Solution Stock Solutions

Elements	Source	Concentration
Ag Al As Ba Be Cd Cr Co Mo Pb Se Sb Sn Sr Ti Tl V Ni	Perkin Elmer kit	100 mg/L
Th U	Perkin Elmer kit	100 mg/L
Hg	Perkin Elmer kit	10 mg/L
Li Cu Fe Mn Zn	Spex Single element	1000 mg/L

An Optimization solution is used each day of use for the ICP-MS. Table 24 describes the stock solutions needed to prepare the optimization solution. Preparation of solutions containing Hg should be prepared every two weeks or as needed. The intermediates that do not contain Hg should be prepared every three months or as needed.

Table 3-11 Intermediate Optimization Solution Preparation

Calibration Standard Intermediates	Concentration	Standard Source	Preparation Instructions
Ag Al As Ba Be Cd Cr Co Mo Pb Se Sb Sn Sr Ti Tl V Ni	1 mg\L	Perkin Elmer kit 100 mg/L	In a 100 mL volumetric add DI, 2 mL of Nitric acid 1+1 with 10 mg/L Au, and 1 mL of stock from standard source.
Th U	1 mg\L	Perkin Elmer kit 100 mg/L	In a 100 mL volumetric add DI, 2 mL of Nitric acid 1+1 with 10 mg/L Au, and 1 mL of stock from standard source.
Hg	0.1 mg/L	Perkin Elmer kit 10 mg/L	In a 100 mL volumetric add DI, 2 mL of Nitric acid 1+1 with 10 mg/L Au, and 1 mL of stock from standard source.
Li	1 mg\L	Single element 1000 mg/L	In a 100 mL volumetric add DI, 2 mL of Nitric acid 1+1 with 10 mg/L Au, and 100 uL of stock from standard source.
Fe Mn Cu Zn	10 mg\L	Single element 1000 mg/L	In a 100 mL volumetric add DI, 2 mL of Nitric acid 1+1 with 10 mg/L Au, and 1 mL of stock from each standard source.

Table 2-5 describes the intermediate stock solutions required to make the optimization solution. This optimization solution will be used daily before any analysis on the ICP-MS. Preparation of solutions containing Hg should be prepared every two weeks or as needed. The intermediates that do not contain Hg should be prepared every three months or as needed. Stock solutions may be purchased from a reputable commercial source or prepared from ultra high-purity grade chemicals or metals (99.99-99.999% pure).

Table 3-12 Working Optimization Solution

	Elements and Concentration	Preparation Instructions
Working Optimization Solution	Ag Al As Ba Be Cd Cr Co Mo Pb Se Sb Sn Sr Ti Tl V Ni Th U and Li = 100 ug/L Hg = 10 ug/L Fe Mn Cu and Zn = 1.10 mg/L	In a 100 mL volumetric add DI, 2 mL of Nitric acid 1+1 with 10 mg/L Au, 10 mL of 10 mg/L Fe Mn Cu and Zn Int., and 10 mL of the other four calibration standard intermediates.

Table 2-6 describes the preparation of the working optimization solution. Optimization of the ICP-MS is required daily before use.

Table 3-13 Preparation of Daily Performance Check Solution

Element	SRM Conc. mg/L	SRM Aliquot Volume (μL) per 100 mL for Intermediate (1 mg/L)	Intermediate Aliquot Volume (μL) per 200 mL for Performance Check Solution (1 ug/L)
Be	1,000	100	200
Co	1,000	100	200
Pb	1,000	100	200
U	1,000	100	200
In	1000	100	200
Ce	1000	100	200
Ba	1000	100	200

Table 2-7 describes the preparation of the Daily Performance Check Solution. The Daily Performance Check solution is an aqueous mixture of the elements Be, U, In, Ce, Co, Pb and Ba at 1 μg/L in 0.5% Optima conc. HNO_3. This solution is used to check the sensitivity, oxides, doubly-charged ions and background of the instrument. To prepare the intermediate and working solution add 0.5 ml of Optima conc. HNO_3 to each 100 ml volumetric used. This solution expires in three months.

Table 3-14 A Typical Arsenic Speciation Run Sequence

Sample Type	Sample Name	Vial	Inst. Method	Standard Level	Injections	Dil. Factor	Peak Det. Meth
Sample	Blank	1	As Speciation		1	1	As Speciation
Standard	S1	2	As Speciation	1	1		As Speciation
Standard	S2	3	As Speciation	2	1		As Speciation
Standard	S3	4	As Speciation	3	1		As Speciation
Standard	S4	5	As Speciation	4	1		As Speciation
Standard	S5	6	As Speciation	5	1		As Speciation
Standard	S6	7	As Speciation	6	1		As Speciation
Sample	*Chk STD	8	As Speciation		1	1	As Speciation
Sample	NIST 1	9	As Speciation		1	1	As Speciation
Sample	NIST 2	10	As Speciation		1	1	As Speciation
Sample	Urine Control	11	As Speciation		1	1	As Speciation
Sample	Sample	12	As Speciation		1	1	As Speciation
Sample	*Sample SPK	13	As Speciation		1	1	As Speciation
Sample	*Sample DupSPK	14	As Speciation		1	1	As Speciation

Table 2-8 shows a typical arsenic speciation by LC-ICP-MS analysis run sequence that is prepared using the instruments Chromera software. *A Check Standard and Spike and Duplicate Spike are analyzed after every 10 samples.

Table 3-15 Reportable Range of Results for Arsenic Speciation

Compound	Lowest reportable limit ug/L)	Highest reportable limit (ug/L)
AB	2	250
As III	2	250
DMA	5	500
MMA	2	250
As V	5	500

Table 2-9 shows the reportage range of results that was determined through a validation study on the arsenic speciation method by LC-ICP-MS. Linearity has been shown up to 2000 ug/L by showing that the measured value is within +/- 10% of the target value. If a sample does run above the highest reportable limit it can be reanalyzed by dilution or by running a higher calibration value and showing linearity within +/- 10%.

Table 3-16 Reference Range for Arsenic Species for Humans

REFERENCE RANGES FOR ARSENIC SPECIES	
Species Chemical Name	**Reference Range[1], µg/L**
Arsenobetaine	<LOD – 7.9
Arsenocholine	<LOD
Trimethylarsine oxide	<LOD
Monomethylarsenic acid	<LOD – 7.1
Dimethylarsinic acid	1.8 – 12.2
Arsenous (III) acid	<LOD – 2.5
Arsenic (V) acid	<LOD – 3.2
[1] There are no established reference ranges for arsenic species. Above ranges are estimates based on CDC unpublished data. 5-95 percentile of randomly selected NHANES 2002 samples n=48.	

Table 2-10 describes the reference range for arsenic species for humans. There have not been studies completed on reference ranges for swine urine.

Chapter 3 Figures

Figure 3-1 Arsenic Species

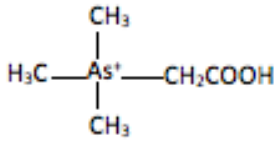
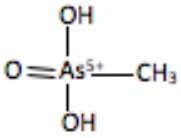
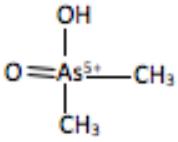
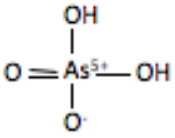
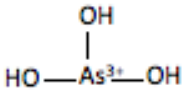
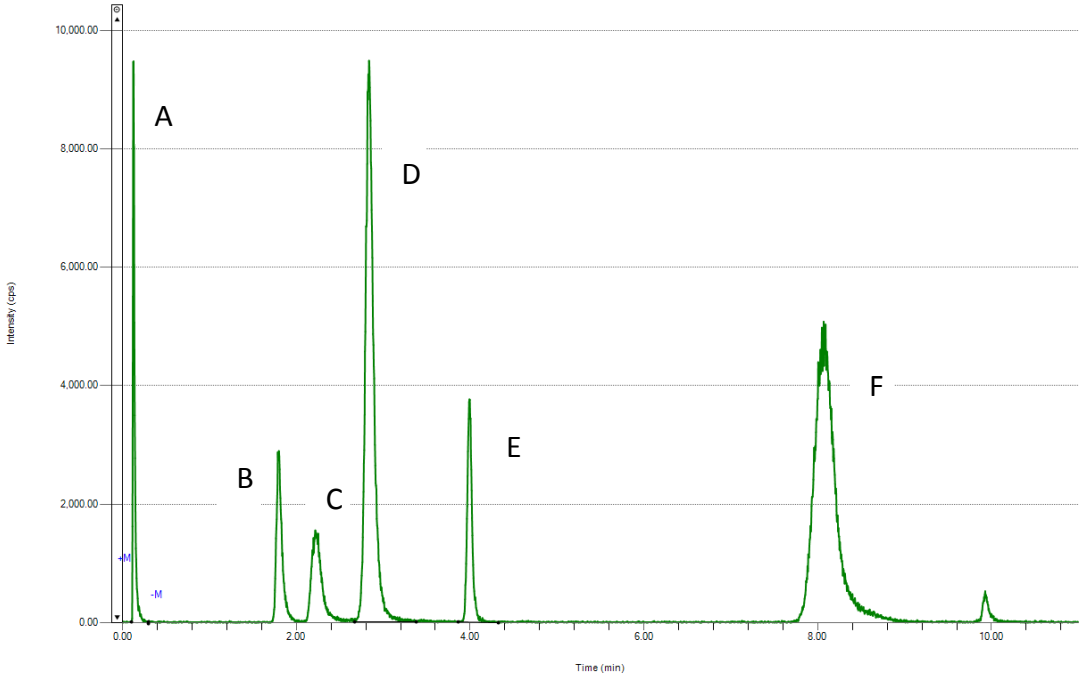
Name	Abbreviation	Structure
Arsenobetaine	AB	
Monomethyl arsenic Acid	MMA	
Dimethylarsinic Acid	DMA	
Arsenic (V) acid (arsenate)	As (V)	
Arsenous (III) acid (arsenite)	As (III)	

Figure 3-1 shows the five typical arsenic species found in human urine and their chemical structure.

Figure 3-2: Chromatograph of arsenic species by LC ICP-MS



The chromatograph in Figure 5-1 depicts the seperation of five arsenic species and the internal standard; A) multi-element internal standard, B) Sodium arsenite (As III), C) Arsenobetaine (AB), D) Dimethylarsinic Acid (DMA), E) Monomethylarsenic Acid (MMA) and F) sodium arsenate (As V).

Figure 3-3: Retention Time Shifts of Arsenic Species at Varying pH Concentrations of Spiked Control Swine Urine

pH 7=Black Line
pH 0=Red line

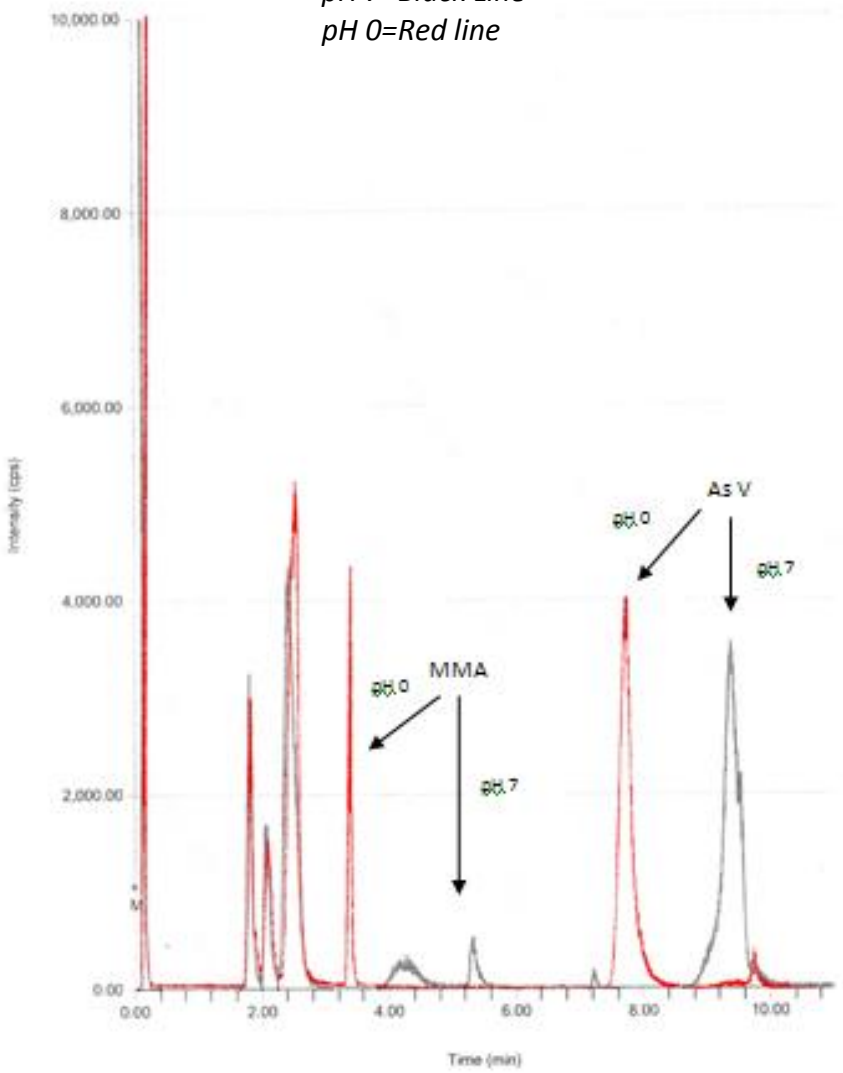


Figure 3-3 demonstrates a standard arsenic species chromatograph and two different pH concentrations of stock solution using control swine urine. At pH 7, black line, MMA is a split peak and As V has an extra peak that made it difficult to integrate. Both MMA and As V peaks were also shifted to the right. Upon acidifying the control swine urine to pH 0, red line, MMA shifted to the left and form one peak sharp peak while As V also shifted to left and separated from the unknown arsenic peak to ease integration. Table 4-4 describes the concentrations of each species at several varying pHs.

Figure 3-4: Comparison of Sample Storage and Analysis Methods Sodium Arsenate

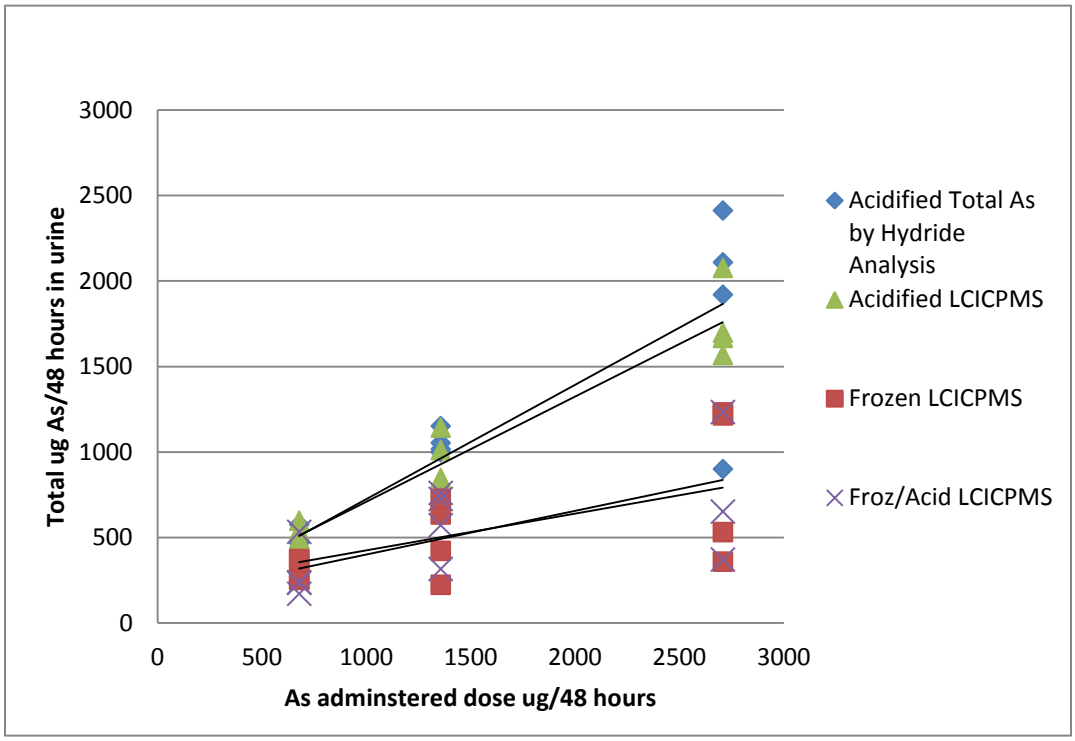


Figure 5-2 shows the dose response of arsenic excreted in swine urine where groups of swine were exposed to varying doses of sodium arsenate (reference group). Acidified total arsenic concentrations were determined by hydride analysis and depicted in blue (◆). LC-ICP-MS analysis was used to speciate the urine arsenic. The species were then summed to determine total arsenic concentrations. Those data are depicted in green (▲) for samples that were stored acidified, red (■) for urine samples that were frozen and purple (X) for samples that were originally frozen then acidified.

Figure 3-5: Comparison of Sample Storage and Analysis Methods Test Material 1

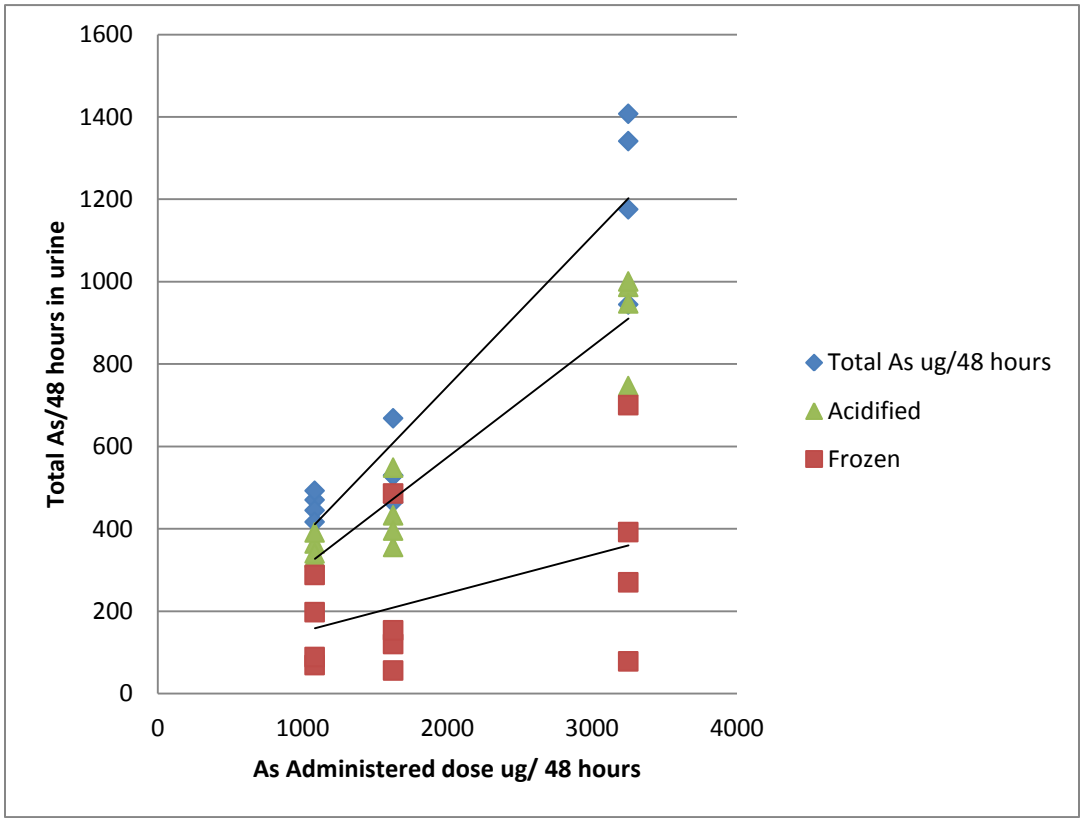


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Figure 3-6: Chromatograph Depicting a Comparison of a Swine Urine Sample that has been Frozen and Acidified.

Grey=Frozen Chromatograph
Green=Acidified Chromatograph

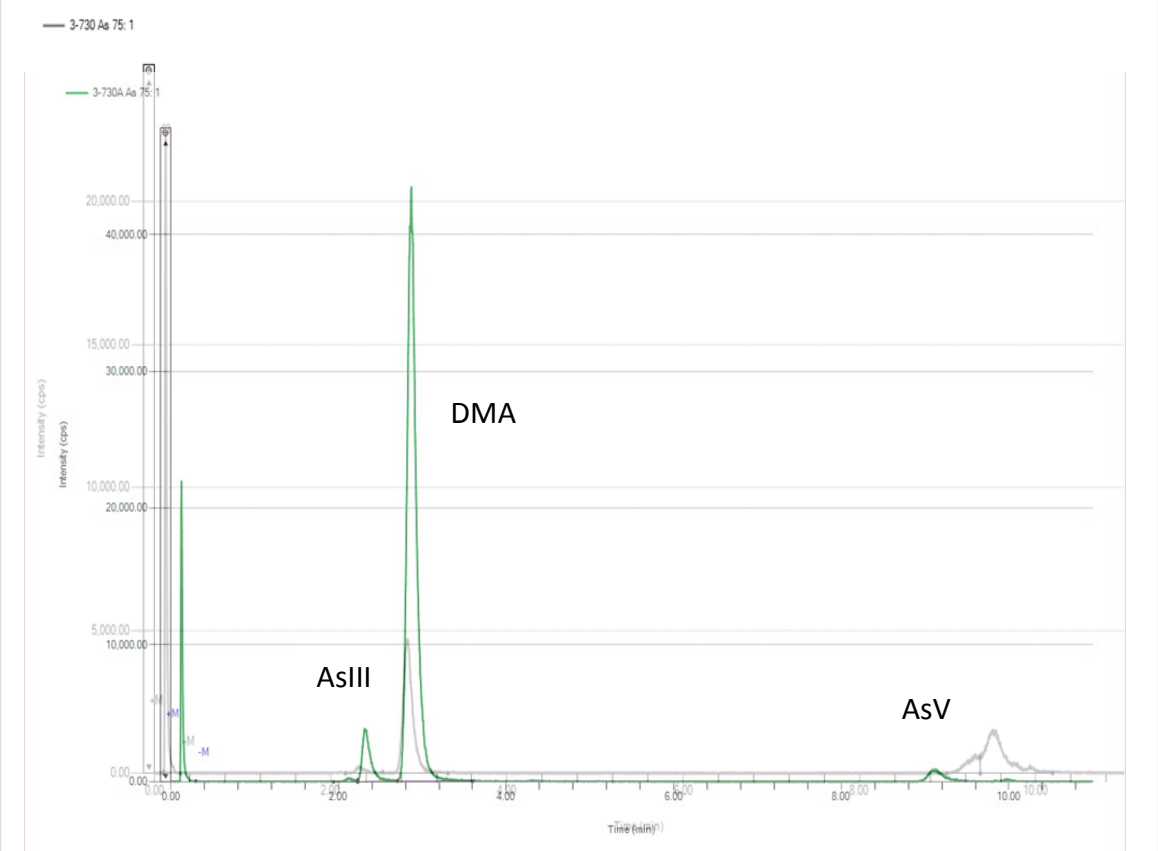


Figure 5-4 shows a graphic depiction of the difference intensities detected after storing swine urine samples frozen and acidified. The frozen sample is shown in the grey line and has a very low intensity peak for both AB and DMA but a large wide peak for as V. The green line shows the sample acidified. The acidified chromatograph shows two three very distinct peaks for easy integration and also shows an increased intensity for AB and DMA.

Figure 3-7: Urinary Arsenic Species Comparison of Sample Storage Techniques

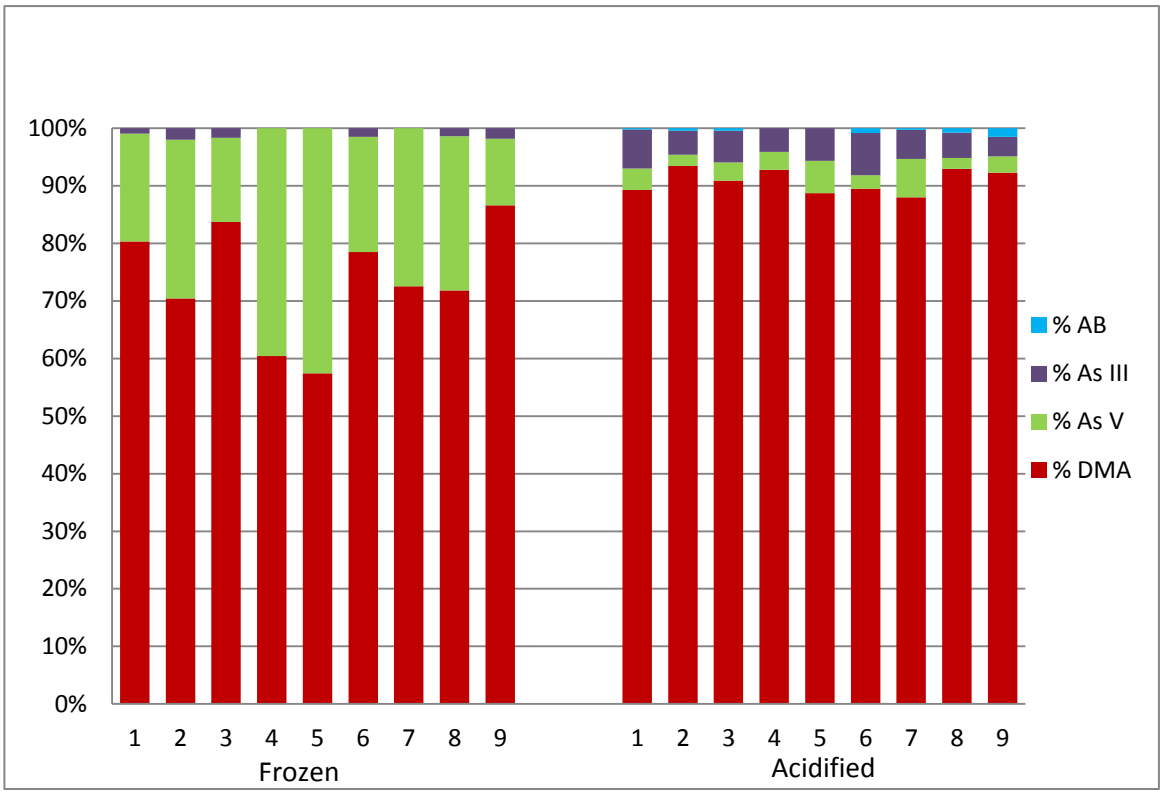


Figure 5-5 shows the average percent of arsenic species analyzed by LCICPMS comparing the sample storage techniques of freezing and acidificed urine samples. The numbers associated with each column are associated with dosing groups. Columns 1,2 and 3 are swine dose groups for soil Test Material 1; 4,5 and 6 are swine dose groups for soil Test Material 2; 7, 8 and 9 are dose groups for sodium arsenate, the reference material for study A.

Figure 3-8 Arsenic Speciation Method in Chromera

Name	Description	Group
As Speciation 032811		As Speciation

ICP-MS Settings	Run Time(min)	DRC Mode	RPq
	11	DRC-B	0.5

Tuning File	Optimization File
C:\Elandata\T…\Defult.tune	*C:\Elandata\O…\CVAA.doc*

Analyte	Mass	Use MSIS Constant
As 75	74.9	None

Species	Units	S1	S2	S3	S4	S5	S6
As III	ug/L	2	5	10	25	50	100
As V	ug/L	5	25	50	100	250	500
AB	ug/L	2	5	10	25	50	100
DMA	ug/L	5	25	50	100	250	500
MMA	ug/L	2	5	10	25	50	100
INSTD	ug/L						

LC Pump Settings	Transition
	Gradient

Step	Step type	step time(min)	Flow(ml/min)	%A	%B
0	Equil	0.1	1.0		100
1	Run	5.5	0.8		100
2	Run	5.5	1.4		100

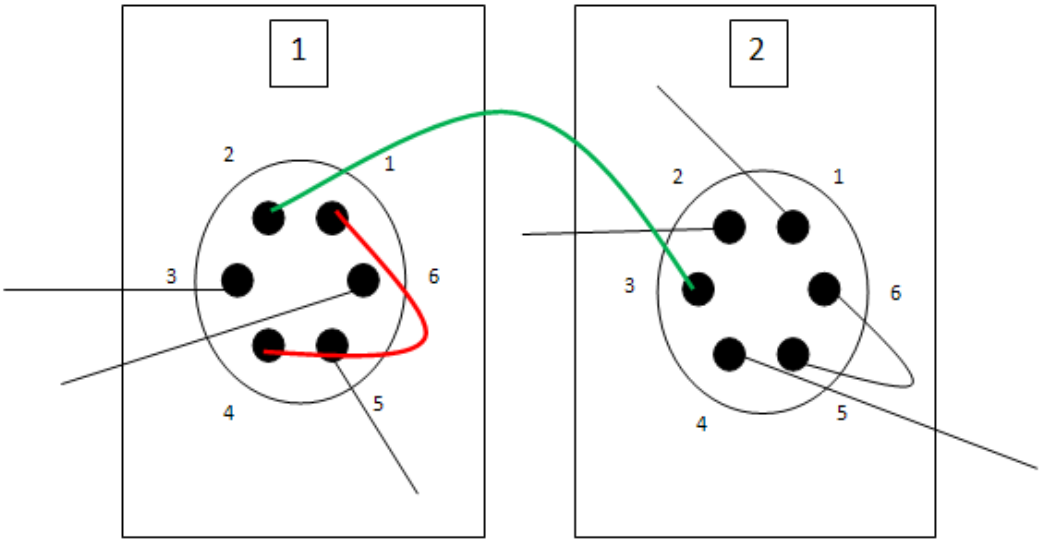
Step Type	TE time (min)	TE
Run	0.1	TE1
Run	0.2	TE1

Autosampler	Injection Vol (ul)	Tray Temp C	Tolerance (+/- C)
	50.0	4	2.0

LC Oven Settings	Temp C
	25.0

Figure 2-2 shows the arsenic speciation by LC-ICP-MS method setup using Chromera software. The method setup describes what tuning and optimization files need to be use as well as the LC pump, instrument and switching valve setup.

Figure 3-9 Switching Valve Setup



	1	2	3	4	5	6
Switching Valve 1	INST loop that connects with V1/4	Sample transfer loop from V2/3	Nebulizer spray chamber	INST loop that connects with V1/1	INSTD Waste	INSTD entry
Switching Valve 2	Waste	Rinse solution	Sample transfer loop from V1/2	Column sample line	Not used loop to V2/6	Not used loop to V2/5

Figure 2-3 depicts the duel switching value setup required for the arsenic speciation by LC-ICP-MS analysis. The switching values are use to inject an external internal standard and control the amount of sample injected to the ICP-MS from the LC.

Figure 3-10 Processing Method for Chromera

Name

As Speciation

Analyte	Mass	Bunching factor	Area Threshold	Noise Threshold
As	74.9216	10	10	20

Species	Retention Time	Peak Start	Peak En	Use Peak area	Use tallest
INSTD	0.114	0.7	0.3	YES	NO

	Type		Reference Standard		
	Uses a Retention Time Reference				
DMA	2.705	2.6	3.2	YES	NO
	Type		Reference Standard		
	Uses a CIS		INSTD (this setting for all species)		
As III	2.321	2.2	2.65	YES	NO
AB	1.917	1.8	2.031	YES	NO
MMA	4.955	4.4	6.0	YES	NO
As V	9.534	9.3	9.9	YES	NO

EVENT

Start Manual Integration- this is sometimes needed to aid in the identification of peaks

***Save PD Method & Update Results** saves changes only for the chromatogram that is open and will also update the chromatogram when attempting to integrate peaks.

***Save New PD Method & Update Results** saves changes to the entire reprocessing method. Use caution if going to use this option, since the reprocessing method will then need to be updated during your next sequence set up and data reprocessing.

Figure 3-10 describes the setup for the processing method using the Chromera Software. After instrument analysis of each sample the processing method allows for further analysis of each sample chromatograph. Integration of peaks and concentrations of species are determined through this processing method.

APPENDIX D

Chapter 4 Tables

Table 4-1: AB Quality Control Limits

Control	Calibration (correlation coefficient)	Check Standard ug/L	NIST level 1 ug/L	NIST level 2* ug/L	Urine Control ug/L	Spike Percent (%)	Duplicate Spike RPD
True Value	1.00	25	12.4	1.43		100	
Mean Value		25.48	11.21		1.23	98.05	5.94
Sample #		18	9		9	10	9
Range of results for Study (ug/L)		21.99-31.55	9.03-15.5		0.65-1.48	91.1-103.2	0.3-15.5
Acceptable range	0.99-1.00	17.94-33.03	5.49-16.92		0.66-1.8	85.72-110.38 **80-120****	0-21.67
Precision 1 Std Deviation Value		2.51	1.9		0.19	4.11	5.24

Table 3-1 describes the quality control limits for arsenic species AB that were determined during the method validation process. *NIST Level 2 for AB is incomplete and will not be used in regular QC. AB and As III will sometimes be a double peak and AB is hard to integrate therefore will not be used.
**Spike value limits have been set to 80-120%.

Table 4-2: As III Quality Control Limits

Control	Calibration (correlation coefficient)	Check Standard ug/L	NIST level 1 ug/L	NIST level 2* ug/L	Urine Control ug/L	Spike %	Duplicate Spike RPD
True Value	1.00	25	1.47	5.03		100	
Mean Value		24.22	0.60		19.08	93.18	8
Sample #		18	5		9	10	9
Range of results for Study (ug/L)		21.75-28.5	0.38-1.15		18.31-20.07	78.1-102.6	0.3-16.1
Acceptable range	0.99-1.00	18.93-29.50	0-1.54		17.42-20.74	66.51-119.85 **70-130****	0-25.55
Precision 1 Std Deviation Value		1.76	0.32		0.55	8.89	5.85

Table 3-2 describes the quality control limits for arsenic species As III that were determined during the method validation process. * NIST level 2 for As III is incomplete and will not be used for regular QC. AB and As III will sometimes form a double peak that is difficult to integrate.

** Spike value limits have been set to 70-130%. These limits are a little wider due to variability in samples.

Table 4-3: DMA Quality Control Limits

Control	Calibration (correlation coefficient)	Check Standard ug/L	NIST level 1 ug/L	NIST level 2 ug/L	Urine Control ug/L	Spike %	Duplicate Spike RPD
True Value	1.00	100	3.47	25.3		100	
Mean Value		98.54	4.61	21.12	149.05	106.4	5.30
Sample #		18	8	9	9	10	9
Range of results for Study (ug/L)		83.35-121.52	3.84-5.38	19.01-25.94	145.5-151.62	61.5-135.6	0.56-10.8
Acceptable range	0.99-1.00	72.01-125.06	2.86-6.36	15.18-27.07	142.4-155.7	34.75-178.09 **70-130***	0-15.34
Precision 1 Std Deviation Value		8.84	0.58	1.98	2.22	23.9	3.35

Table 3-3 describes the quality control limits for arsenic species DMA that were determined during the method validation process. * Spike value limits have been set to 70-130%.

Table 4-4: MMA Quality Control Limits

Control	Calibration (correlation coefficient)	Check Standard ug/L	NIST level 1 ug/L	NIST level 2 ug/L	Urine Control ug/L	Spike %	Duplicate Spike RPD
True Value	1.00	25	1.87	7.18		100	
Mean Value		23.65	1.72	5.37		93.66	6.44
Sample #		15	8	9		14	14
Range of results for Study (ug/L)		16.33-27.84	1.21-2.25	4.15-8.7		72.7-113.4	0.1-26.2
Acceptable range	0.99-1.00	14.93-29.47	0.72-2.72	1.07-9.67		58.08-117.39 **70-130****	0-27.09
Precision 1 Std Deviation Value		2.91	0.33	1.43		11.86	6.88

Table 3-4 describes the quality control limits for arsenic species MMA that were determined during the method validation process. *MMA is not present in Swine Urine. **Spike value limits have been set to 70-130%.

Table 4-5: As V Quality Control Limits

Control	Calibration (correlation coefficient)	Check Standard ug/L	NIST level 1 ug/L	NIST level 2 ug/L	Urine Control ug/L	Spike %	Duplicate Spike RPD
True Value	1.00	100	2.41	6.16		100	
Mean Value		97.79	3.91	10.15	16.75	110.54	5.15
Sample #		18	8	9	9	9	9
Range of results for Study (ug/L)		85.72-126.00	2.42-4.93	7.76-13.29	14.87-19.35	80.9-130.3	0.1-15.2
Acceptable range	0.99-1.00	68.64-126.93	1.10-6.70	5.03-14.16	12.96-20.54	60.34-144.01 **70-130***	0-21.34
Precision 1 Std Deviation Value		9.71	0.93	3.33	1.26	16.7	5.39

Table 3-5 describes the quality control limits for arsenic species As V that were determined during the method validation process. *Spike value limits have been set to 70-130%.

Table 4-6: Swine Urine Sample Precision

Sample ID #	Date of Analysis	AB ug/L*	As III ug/L	DMA ug/L	As V ug/L
5-720	03/29/11		1.38	35.94	52.77
5-720	03/29/11		1.30	36.12	44.87
5-720	03/30/11		1.67	38.15	54.91
5-720	04/14/11		2.78	52.68	51.94
5-720	04/19/11		2.45	54.15	32.95
5-720	04/26/11		1.77	56.57	62.78
1 Std deviation precision			0.596	9.821	10.158
8-723	03/29/11		10.42	394.81	69.60
8-723	03/29/11		10.05	401.48	70.02
8-723	03/30/11		12.89	400.37	70.79
8-723	04/14/11		13.68	390.47	58.98
8-723	04/19/11		15.81	454.55	70.99
8-723	05/05/11		5.89	446.96	85.74
1 Std deviation precision			3.464	28.25	8.54

Table 3-6 shows the analysis of two swine urine samples used to assess the precision of the arsenic speciation method by LC-ICP-MS. Swine Urine Samples 5-720 and 8-723 were analyzed six times over several different days to look at the reproduceability of method. The standard deviation was determined and each species showed acceptable precision. *AB was not consistently present in any of the samples that were repeated.

Table 4-7: Method Analysis Comparison of Average Percent Bioavailability for Two Studies Conducted for the California Department of Toxic Substances.

Test Material	Test Material	Average %RBA over three Urine Collections (95% CI)
EM01-1-1.3	Total As Hydride Analysis	23.7 (10.9-36.5)
EM01-1-1.3	Speciated Total	26.0 (1.4-50.5)
EM03-0-1.3	Total As Hydride Analysis	15.3 (11.7-18.8)
EM03-0-1.3	Speciated Total	13.9 (3.2-24.7)
EM08-0-0.2	Total As Hydride Analysis	19.2 (16.9-21.4)
EM08-0-0.2	Speciated Total	19.7 (12.4-27.0)
EM18-0-2	Total As Hydride Analysis	4.0 (3.3-4.6)
EM18-0-2	Speciated Total	3.7 (-2.5-9.4)
EM19-0-1	Total As Hydride Analysis	11.7 (8.3-15.2)
EM19-0-1	Speciated Total	11.2 (7.8-14.7)
EM21-1-3	Total As Hydride Analysis	23.0 (17.6-28.5)
EM21-1-3	Speciated Total	22.7 (18.8-26.6)
EM05	Total As ICP-MS	15.3 (15.22-15.5)
EM05	Speciated Total	18.3 (26.3-10.2)
EM13	Total As ICP-MS	12.5 (5.1-19.9)
EM13	Speciated Total	11.5 (13.1-9.9)
EM15	Total As ICP-MS	19.7 (13.1-26.2)
EM15	Speciated Total	15.8 (21.1-10.6)
EM20	Total As ICP-MS	22.7 (21.1-24.3)
EM20	Speciated Total	20.1 (23.1-17.0)
RG01	Total As ICP-MS	12.4 (7.6-17.2)
RG01	Speciated Total	10.2 (14.0-6.4)
RG03	Total As ICP-MS	11.8 (6.9-16.6)
RG03	Speciated Total	11.4 (17.4-5.5)

Table 5-7 compares the calculated RBA determined by arsenic analysis methods of hydride generation and LC-ICP-MS and ICP-MS to LC-ICP-MS for two studies conducted for the California Department of Toxic Substances.

Table 4-8: Type III Test of Fixed Effects for a RBA Study #1 conducted for the California Department of Toxic Substances

Effect	Num DF	Den DF	F Value	Pr>F
Preparation	1	12	0.02	0.8801
Soil	5	12	6.87	0.0030
Prep*soil	5	12	0.29	0.9120

Table 5-8 demonstrates that there is no significant difference between the calculated RBA using the arsenic analysis of hydride generation and LC-ICP-MS (Preparation). Statistical analysis was determined using SAS.

Table 4-9: Type III Test of Fixed Effects for a RBA Study #2 conducted for the California Department of Toxic Substances

Effect	Num DF	Den DF	F Value	Pr>F
Preparation	1	12	3.49	0.0865
Soil	5	12	22.83	<.0001
Prep*soil	5	12	2.21	0.1211

Table 5-9 demonstrates that there is no significant difference between the calculated RBA using the arsenic analysis of ICP-MS and LC-ICP-MS (Preparation). Statistical analysis was determined using SAS.

Chapter 4 Figures

Figure 4-1: Graphic Depiction of the Average Percent (%) Relative Bioavailability (RBA) for Study #1 for the California Department of Toxic Substances comparing two Different Analysis Techniques

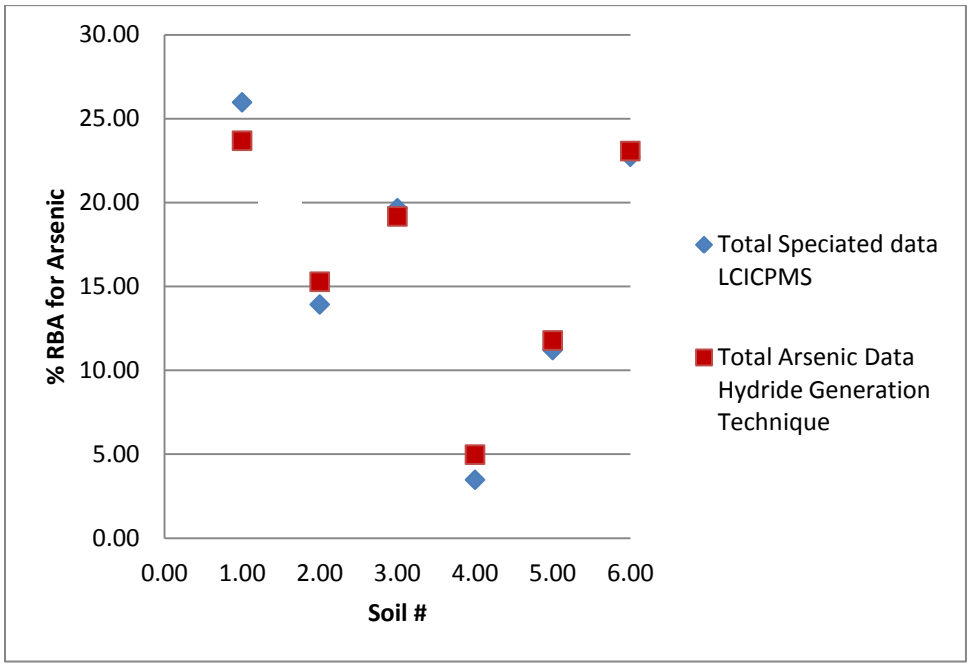


Figure 5-7 shows a graphic depiction comparison of the calculated % RBA from two different types of analyses, hydride generation for total arsenic and LC-ICP-MS speciation that totals the species for the determination of total arsenic concentration. Soli # represents the six test material soils used in study B.

Figure 4-2: Graphic Depiction of the Average Percent (%) Relative Bioavailability (RBA) for Study #2 for the California Department of Toxic Substances comparing two Different Analysis Techniques

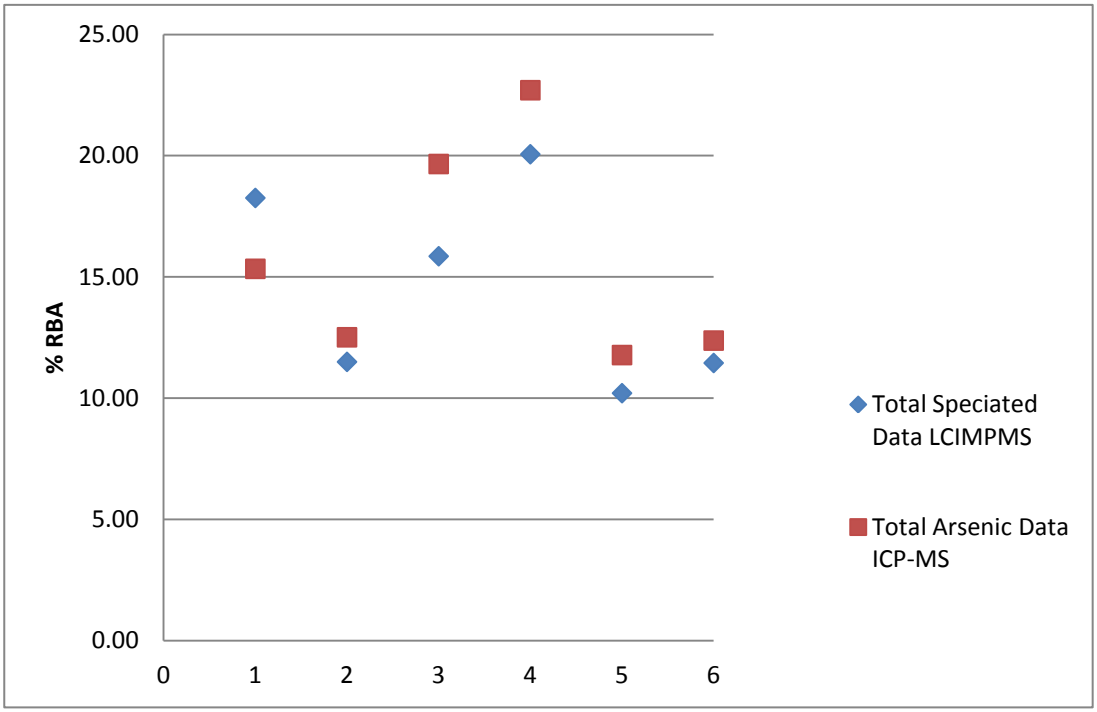


Figure 5-9 shows a graphic depiction comparison of the calculated % RBA from two different types of analyses, ICP-MS for total arsenic and LC-ICP-MS speciation that totals the species for the determination of total arsenic concentration. Soli # represents the six test material soils used in study C.

APPENDIX E

Chapter 5 Tables

Table 5-1: Average Percent (%) Arsenic Species ug/L for Two Studies Conducted for the California Department of Toxic Substances

	Avg total As ug/48 hr	Avg % DMA	Avg % As V	Avg % As III	Avg % AB
EM01-1-1.3	198.33	77.91	18.36	2.76	0.98
EM03-0-1.3	142.50	74.68	21.62	2.77	0.93
EM08-0-0.2	181.66	64.05	31.44	3.34	1.16
EM18-0-2	79.26	68.75	28.39	2.32	0.54
EM19-0-1	123.18	72.64	22.86	3.20	1.29
EM21-1-3	201.49	72.14	25.10	2.35	0.42
Reference	586.05	75.82	20.76	3.02	0.39
Control	47.44	76.47	20.74	2.45	0.35
EM05	198.33	77.91	18.36	2.76	0.98
EM13	142.50	74.68	21.62	2.77	0.93
EM15	181.66	64.05	31.44	3.34	1.16
EM20	79.26	68.75	28.39	2.32	0.54
RG01	123.18	72.64	22.86	3.20	1.29
RG03	201.49	72.14	25.10	2.35	0.42
Reference	586.05	75.82	20.76	3.02	0.39
Control	47.44	76.47	20.74	2.45	0.35

Table 5-6 describes the average percent detected for each species for all test materials, reference and control groups for two studies conducted for the California Department of Toxic Substances.

Chapter 5 Figures

Figure 5-1: Graphic Depiction of Percent Arsenic Species for Study #1 and #2 for the California Department of Toxic Substances

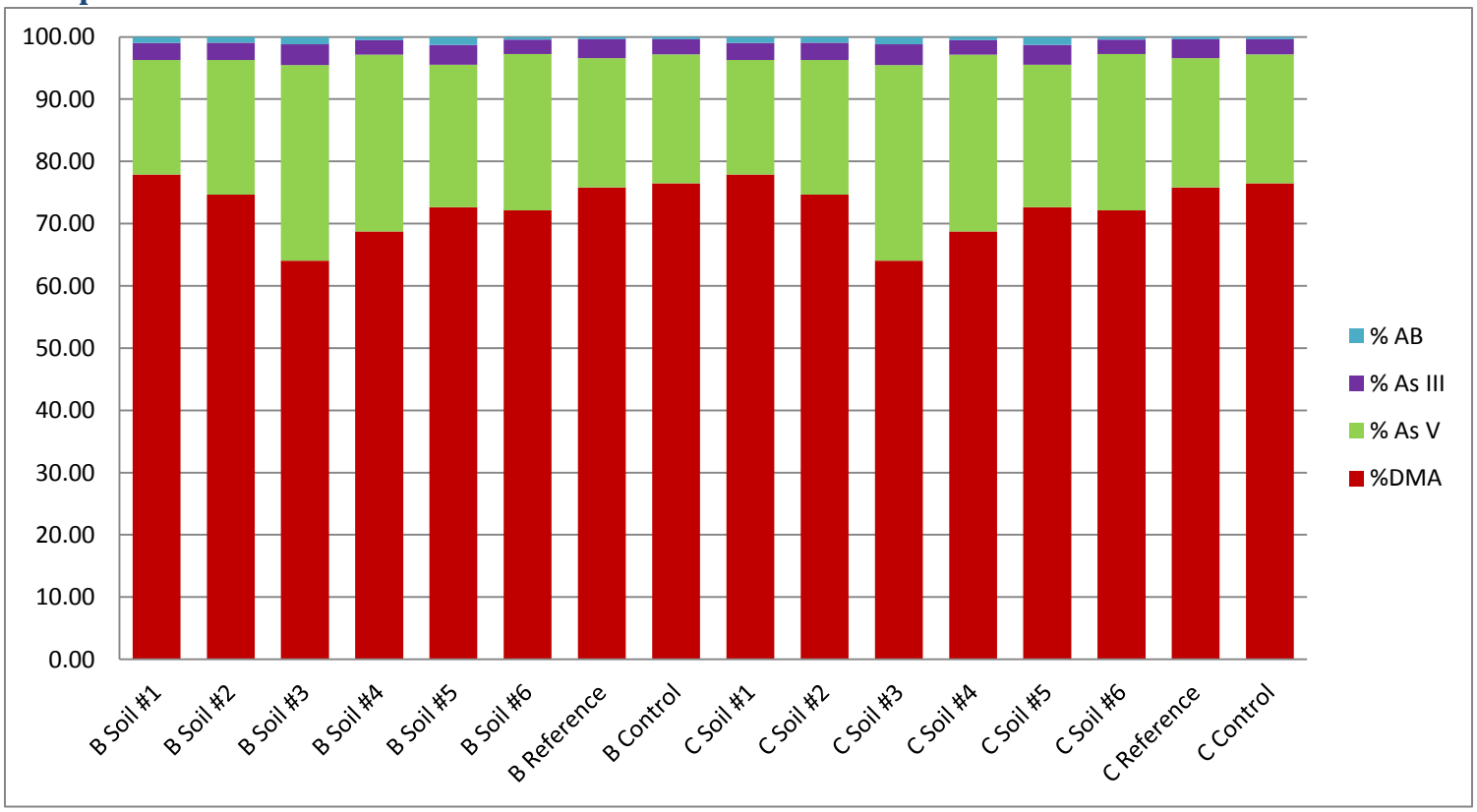


Figure 5-6 shows the percent average species for study B and C for all soil test materials and the reference and control groups for each.

VITA

Laura Eisinger Naught was born June 15th, 1978 in Jefferson City, MO. Laura was raised in Jefferson City, MO where her parents, Don and Linda Eisinger, still reside.

Laura attended Drury University in Springfield, MO where she received her Bachelor of Arts degree in 1996 (Major Biology and Chemistry, Minor Global Studies). She then worked as a Research Specialist at the University of Missouri-Columbia in the Biochemistry Department. During that time she received her Masters' in Biochemistry in 2005. She then attended the University of Missouri-Columbia to pursue her doctoral degree in toxicology.

Laura Is currently employed as the Quality Systems Officer for the Missouri State Public Health Laboratory, Department of Health and Senior Services, State of Missouri. She resides in Jefferson City, MO with her husband William Naught.