EVALUATION OF ARSENIC CONCENTRATIONS IN APPLE PRODUCTS AND ITS POTENTIAL HEALTH EFFECTS

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ABSTRACT

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The presence of heavy metals such as arsenic in fruit juices has been raised as a concern because of their potential toxicity to humans. Prior surveys of arsenic concentrations in apple juice have not considered the source of the fruit and/or juice concentrate used in the juice manufacturing. Therefore, it is unclear if differences exist between juices produced from apple juice concentrate (which, in the US, would largely be produced using imported apple juice concentrate) and fresh juices produced from domestic apples. The overall goal of this research is to establish a risk profile for apple juice and cider with respect to potential contamination with arsenic and other metals. The specific objectives of this research are to evaluate the *in vitro* toxicity of inorganic arsenic on a human intestinal cell line (Caco-2 cells) as well as to assess the concentrations of metals in samples of shelf-stable apple juice and fresh apple cider obtained at retail operations and cider mills throughout Michigan, and to determine the relationship of metal concentrations in fresh apples with that in orchard soil samples (from Michigan orchards). In this project, we found that the uptake of arsenite (As III) from the apical to basolateral side of Caco-2 cell monolayers was favored over arsenate (As V) uptake. This indicates that the toxicity of arsenic was dependent on its species. In addition, this project demonstrated that apple ciders produced from 100% local Michigan apples contain significantly less arsenic than samples of retail shelf stable juices that are predominantly produced using imported apple juice concentrate. The overall results of this project demonstrated that apple ciders and juices manufactured using apples grown in Michigan are safer with lower arsenic concentrations and the results of this

research can be useful in establishing the basis for action levels for heavy metals in juice products.

For all their selfless sacrifices, I was given everything that they did not have. Without them, I would not be who I am today. I can never thank you enough. This accomplishment is dedicated to you, Bố Mẹ.

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KEY TO ABBREVIATIONS

Al	Aluminum
As	Arsenic
AsB	Arsenobetaine
ATSDR	Agency for Toxic Substances and Disease Registry
BAF	Bioaccumulation Factor
Cd	Cadmium
CDI	Chronic Daily Intake
CFSAN	Center for Food Safety and Applied Nutrition
Cr	Chromium
CSF	Cancer Slope Factor
Cu	Copper
DALY	Disability-adjusted life year
DMA	Dimethylarsonous Acid
DMEM	Dulbecco's Modified Eagle Medium
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
GSH/ GSSG	Glutathione/ oxidized glutathione
HI	Hazard Index
HQ	Hazard Quotient
IARC	International Agency for Research on Cancer

ICP-MS	Inductively Coupled Plasma- Mass Spectrometer
IL	Interleukin
ILCR	Incremental Lifetime Cancer Risk
JECFA	Joint Expert Committee on Food Additives
LADD	Lifetime Average Daily Dose
LOQ	Limit of Quantification
MCL	Maximum Contaminant Level
MCP-1	Monocyte Chemoattractant Protein
MMA	Monomethyl Arsinous Acid
Mn	Manganese
MRL	Maximum Residue Limit
MRL	Minimal Risk Level
NO	Nitric Oxide
OM	Organic Matter
Pb	Lead
RfD	Reference Dose
ROS	Reactive Oxygen Species
TDS	Total Diet Study
TEER	Transepithelial Electrical Resistance
TMAO	Trimethylarsine oxide

CHAPTER 1: INTRODUCTION, CHAPTER SUMMARIES, SIGNIFICANCE AND LITERATURE REVIEW

INTRODUCTION

Arsenic is a natural element on earth which can be easily found in soil. Arsenic can cause contamination in soil, water and food via natural process or human activities. Many countries have issues with elevated arsenic concentration in groundwater such as Vietnam, Chile, China, Bangladesh, Argentina, etc. whereas in the United States most arsenic exposure is through food sources because of strict federal regulation of arsenic levels in drinking water (Flora, 2015).

There are many species of arsenic compounds and they can be divided into two groups: inorganic and organic. However, the most concern is with inorganic arsenic because of its higher toxicity to humans as compared to organic forms. Trivalent and pentavalent arsenicals and their metabolites like dimethylarsonous acid (DMA) and monomethyl arsinous acid (MMA) are a big concern because they are associated with diseases such as skin, lung, and bladder cancers (Melak et al., 2014). Therefore, it is important to understand their absorption kinetics in the gastrointestinal tract and their bioavailability to the circulation system to more fully understand their potential toxicity.

Besides drinking water, humans can also be exposed to arsenic via food like rice and beverages like juices. There are many studies on arsenic in rice, but relatively few on juices and other beverages (Abedin et al., 2002; Duxbury et al., 2011). Therefore, more comprehensive evaluation of arsenic levels in beverages is essential for assessing the potential risk of arsenic to human health.

Fruit juices and some fruit-based beverages have been found to contain total arsenic and arsenic species higher than the U.S. Environmental Protection Agency (EPA) limit for inorganic arsenic in drinking water (10 µg/L) (Conklin and Chen, 2012; Roberge et al., 2009; Wilson et al., 2012). In a study conducted by Consumers Union (2012), 10% of 88 apple and grape juices contained total arsenic exceeding 10 µg/L. The authors also found that 25% of juice samples exceeded the federal limit for lead in bottled drinking water (5 µg/L) (Consumer Reports, 2012). Surveillance of 247 apple juices conducted by the U.S. Food and Drug Administration (FDA) showed that 8% of samples had total arsenic concentration higher than 10 µg/L. Other studies also found that fruit juices contained heavy metals like arsenic and lead (Conklin and Chen, 2012; Consumer Reports, 2012; Roberge et al., 2009; Wilson et al., 2012). However, information related to the source of arsenic in food is limited and examining random samples from the market may not be sufficient to identify beverages with concentrations of heavy metals exceeding federal regulatory limits.

Human exposure to arsenic from drinking water has been reduced in the U.S because of stricter regulation from the government. This has resulted in food and beverages, rather than drinking water, becoming a major contributor to exposure to these metals in the U.S. A recent assessment by the World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA) concluded that food is a major source of inorganic arsenic exposure. The European Food Safety Authority (EFSA) also concluded that it is important to reduce the arsenic exposure through diet (EFSA, 2009). Unfortunately, there currently are no regulatory limits for arsenic concentrations in fruit juice in the U.S. The FDA did propose an action level of 10 µg/L for inorganic arsenic in apple juice in 2013, but this action level has never been formally adopted by the agency.

The objectives of this research were to evaluate the toxicity of different inorganic arsenic species *in vitro* using the intestinal model Caco-2 cell line, to assess the concentrations of heavy metals in samples of apple juice and in apple cider available for sale in Michigan, and to investigate the relationship of the heavy metal content in orchard soils to those levels in apple tissues and juices produced by these orchards.

CHAPTER SUMMARIES

Chapter 1 presents a concise review of the literature beginning with some potential sources of arsenic, their basic chemistry and toxicity. Arsenic present in the orchard soils can either be naturally occurring or could have been added via historical application of lead arsenate pesticides. This chapter is comprised of two major sections: 1) arsenic characterization (what arsenic compounds are in foods, where these arsenic compounds come from, and how they can contaminate food products); and 2) their potential effects on human health. Chapter 1 also provides background information for the motivation guiding my research in the Bourquin laboratory.

Chapter 2 focuses on understanding the intestinal absorption of various forms of arsenic using the intestinal model Caco-2 cell line *in vitro*. There were two inorganic arsenic species tested in this experiment: arsenate (As (V)) and arsenite (As (III)). First, the effect of different arsenic forms on Caco-2 cellular monolayer integrity which served as a model of the small intestinal epithelial layer was determined and secondly, the uptake of various forms of arsenic in Caco-2 cells was evaluated. The working hypothesis of this chapter is that a proportion of administered arsenic will be absorbed by the cells, and some will be transported through the basolateral membrane. To test this hypothesis, Caco-2 cells were seeded on Transwell inserts and

the cells and media were collected for arsenic analysis after exposure. This research presents evidence that the uptake and transport of As (III), the most toxic form, is more efficient than that of As (V).

The focus of **chapter 3** was to determine the concentrations of total arsenic in apple juice produced from Michigan apples and compare these concentrations to those in commercial apple juices prepared from imported or domestic juice concentrates and purchased from retail grocery stores in Michigan. In this study, we expanded our interest to examine the concentration of other metals such as aluminum, chromium, copper, cadmium, manganese and lead in juice products, since little research has been conducted on these metals in juices. The working hypothesis of this research is that the concentrations of arsenic and other metals are higher in juices produced using imported concentrate than in juices produced using Michigan apples. To test this hypothesis, juice samples were collected at retail grocery stores and Michigan cider mills and the metals were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). We present evidence that the concentration of these metals observed in apple juices are unlikely to cause harmful effects in humans.

Chapter 4 further explores the relationship of arsenic and lead concentrations in soil and those in fruit and leaves harvested at selected apple orchards in Michigan. Arsenic and lead concentration in apple tree tissues and orchard soils were the main focus in this study because these were the active ingredients of lead arsenate pesticide, which was widely used in fruit orchards in the 20^{th} century. The working hypothesis of the research presented on chapter 4 is that orchard plots established prior to 1988 – the year in which this pesticide was banned in the U.S. – will have higher soil contents of arsenic and lead compared to orchards established after

1988. To test this hypothesis, soil and tree tissue samples were collected at different areas in several Michigan farms and then were analyzed using microwave extraction and ICP-MS. Fruit samples also were processed into juice and pomace fractions to assess the partitioning of arsenic and lead during juice processing. This research presents evidence that the concentration of lead is higher in orchard plots which were established prior to 1988 as compared to the plots established after 1988. The data from this chapter also proves that apple trees are not accumulators of lead and arsenic.

In **chapter 5**, the collective findings in chapter 2-4 are summarized as conclusions for this research. I briefly propose future research directions based on the findings of chapters 2-4. The future directions will enable us to better understand the source of metal contamination to apple juices and how this information can help us to control and eliminate the contamination of toxic metal in juices.

SIGNIFICANCE

Apple juice is one potential food that can contribute exposure to inorganic arsenic. Apple juice or apple juice concentrate is a primary ingredient not only in apple juice, ciders, and cocktails, but also in other fruit blends such as grape and berry juice. Therefore, apple juice is concerning as a potential source of dietary inorganic arsenic exposure. Recently, arsenic exposure via fruit juice consumption has captured the attention of the public because children consume more apple juice relative to their body weight than do adults (Carrington et al., 2013). Children in the U.S. (6-11 years old) drink approximately 1.6 L of fluid per day, which includes 0.46 L of plain water and 0.23 L of fruit juices (Wilson et al., 2012).

Some studies evaluating the concentration of arsenic in juice have demonstrated that arsenic in juice may be a significant health concern. Roberge et al (2009) found arsenic concentrations ranging between 5.4 to 29.5 μg/L in five apple juices and ciders. In January 2012, the magazine Consumer Reports published an article in which 28 apple juices and three grape juices were tested for total arsenic level, with results indicating that approximately 10% of juice samples exceeded 10 μg/L total arsenic (Consumer Reports, 2012). More recently, researchers from Health Canada reported that, out of 96 fruit juices and fruit drink products tested, 10% contained inorganic arsenic concentrations above 10 μg/L. Inorganic arsenic concentrations in these samples ranged from 0.5 to 23.9 μg/L. However, these studies all analyzed samples that were randomly collected from retail grocery stores without knowing about the source of the juice ingredients. Therefore, the source of contamination with these heavy metals in juices was unknown. Similarly, to arsenic, there are limited studies on lead concentrations in juices. Currently in the U.S., there is no legal standard for arsenic or lead concentration in fruit juices except for an action level of arsenic in apple juice of 10 μg/L proposed by the U.S. FDA in 2013 (*11*) and a maximum level of lead in juices adopted by the Codex Alimentarius Commission in 2015 (Codex, 2015). In this study, we will conduct systematic surveillance on national and local brands of apple juices available in Michigan.

Considerable data on arsenic and lead contamination are available for ground water and other foods, but few studies have evaluated arsenic concentrations in fruit drinks and, to the best of our knowledge, no study has examined the relationship between the concentration of these heavy metals in soils from fruit orchards and the fruit produced from these orchards in Michigan. Therefore, this will be the first study assessing total arsenic and other metal contents in apple

juices, orchard soils and tree tissues to characterize its effect on the potential safety of the produce as well as of juices in Michigan.

This research is expected to provide more data to support the establishment of arsenic and lead maximum levels in juices as well as improve the knowledge about sources of arsenic and lead contamination in apple juices for consumers.

LITERATURE REVIEW

Arsenic and human health effects

Arsenic is widely distributed naturally in the earth's soil and it is classified as a known human carcinogen (Roberge et al., 2009). Arsenic is found in both inorganic and organic forms, and the inorganic form (inAs) has greater toxicity to humans and other animals compared to organic arsenic (Carrington et al., 2013; Weber and Hendrickson, 2006). Inorganic arsenic exposure has been associated with cancers such as lung, bladder and skin cancer (Bates et al., 1995; Smith et al., 1992).

Humans can be exposed to arsenic by different sources via consumption of contaminated food, water, air, and occupational exposure, which can cause harmful effects on human health. It was estimated that about 100 million persons globally are exposed to arsenic at levels higher than 50 μg/L. Exposure to arsenic at ranges from 10 to 300 μg/L can lead to adverse effects such as skin lesions, circulatory disorders, neurological complications, diabetes, respiratory complications, hepatic and renal dysfunction including mortality due to chronic diseases (Abdul et al., 2015). Occurrence of skin lesions including melanosis, keratosis, and pigmentation can develop 5-10 years after exposure to arsenic. This can happen even when humans are exposed to arsenic via drinking water containing relatively low concentrations (≤ 10 μg/L) of arsenic

(Ahsan, 2000). Arsenic can also deposit in other keratin rich areas such as nails, and can lead to formation of distinct white lines (Mees' lines) in the nails of fingers and toes (Abdul et al., 2015).

Immediately after exposure, arsenic enters the systemic circulation and can accumulate in enterocytes and bind to hemoglobin because trivalent arsenicals strongly bind to sulfhydryl groups of α-chains and two β-chains in hemoglobin (Lu et al., 2004). This can reduce uptake of oxygen by cells and reduce the lifespan of erythrocytes leading to anemia. Arsenic can cross the blood brain barrier, so the brain is a key target organ of arsenic. Arsenic exposure can affect concentration, memory and verbal learning skills due to disorganization of the cytoskeletal framework leading to axonal degradation (Abdul et al., 2015). Besides exposure to arsenic via food and drinking water, humans can be exposed to arsenic via inhalation during mining or milling of ores. Arsenic fume or dust can lead to respiratory complications (chronic cough, bronchitis). High levels of arsenic exposure through inhalation can increase morbidity and mortality rate (Saha et al., 1999).

Long-term exposure to arsenic can cause harmful effects to the cardiovascular system including the heart and blood vessels (arterial occlusion). Arsenic can lead to the development of atherosclerosis in which artery walls thicken as a result of inflammation and proliferation of smooth muscle cells (Simeonova and Luster, 2004a). Arsenic exposure can induce different pathophysiological events that lead to the atherogenesis and other cardiovascular complications such as hypertension, ischemic heart disease, etc. Arsenic can react directly with sulfhydryl groups which are a component of many macromolecules involved in cell signaling, such as receptors, integrins, or protein phosphatases. This interaction can obstruct cellular metabolism and lead to endothelial cell damage (dysfunction). Endothelial dysfunction can be indirectly

induced by alteration of cellular redox state (Abdul et al., 2015). Exposure to arsenic can increase oxidative stress and increase reactive oxidative species (ROS) activity. These ROS can act as signaling molecules that can induce the activities of nuclear transcription factors such as NF-kB and AP-1(Simeonova and Luster, 2004a). These transcription factors can regulate inflammatory gene expression and stimulate the release of chemokines (monocyte chemoattractant protein (MCP-1) and interleukin (IL)-8 in the endothelium. ROS such as the superoxide anion can interact with nitric oxide (NO) and cause NO inactivation. Attenuation of NO bioavailability is strongly associated with endothelial dysfunction (Simeonova and Luster, 2004b) . All of these activities can lead to the development of endothelial insufficiency and increase expression of genes associated with inflammation and atherosclerosis cardiovascular disease.

Arsenic can exist in different organic and inorganic forms and each has different toxicokinetic effects in humans and animal species. In the environment and in food, inorganic arsenic can exist in the trivalent (As (III)) or pentavalent state (As (V)); As (III) tends to be more toxic than As (V), particularly *in vitro* (Cohen et al., 2002; Vega et al., 2001). After being taken up by cells, As (V) is rapidly reduced to As (III) and can generate methylated arsenic metabolites such as dimethylarsonous acid (DMA) and monomethyl arsinous acid (MMA) in both the trivalent and pentavalent forms. While the pentavalent form of methylated arsenic metabolites are considered detoxification products of inorganic arsenic, the trivalent forms have been connected in the toxicity of inorganic arsenic (Cohen et al., 2013).

MMA and DMA are stable in the environment in the pentavalent state (i.e., MMA^V and DMA^V). When these compounds are ingested in humans and most animals, they are excreted unmetabolized in the urine. In a long-term bioassays, when rats were administrated MMA for

two years, no tumors or any pre-neoplastic lesions formed (Arnold et al., 2003). However, administration of high doses of DMA in drinking water or the diet resulted in urinary bladder tumors in rats in two-year bioassays (Arnold et al., 2006; Wei et al., 1999). Both MMA and DMA are metabolites of inorganic arsenic. Trivalent and pentavalent forms of MMA and DMA are generated from the ingestion of inorganic arsenic when arsenic is taken into the hepatocytes and undergoes oxidative methylation. The toxicokinetic effects of these methylated arsenicals are different when MMA and DMA are ingested directly from food or the environment. When MMA or DMA is ingested directly, they are excreted mostly unchanged (except in the rat). In humans, exposure to MMA and DMA does not generate the toxic trivalent arsenical. In contrast, the trivalent forms of MMA and DMA are generated from inorganic arsenic, and these forms have been implicated in the toxicity of inorganic arsenic (Cohen et al., 2006).

Other organic arsenic forms include trimethylarsine oxide (TMAO), arsenobetaine (AsB), arsenocholine and arsenosugars. TMAO is a metabolite of arsenic but it is a major metabolite in rat urine and has not been detected in human urine(Cohen et al., 2006) . Arsenobetaine is an arsenical form found in marine animals. Studies show that arsenobetaine is excreted unchanged after ingestion, so it is not a concern. Asenocholine is chemically similar to arsenobetaine but it is not commonly found in food except shrimp (Borak and Hosgood, 2007). The Agency of Toxic Substances and Disease Registry (ATSDR) has reported that arsenocholine is “essentially nontoxic”. Arsenosugars are present in seaweed and mollusks. These are compounds of arsenic (DMA^{V}, DMA^{III}, thiolated DMA^{V} or trimethylarsine) with a sugar and a glycerol, phosphate, sulfonate or sulfate moiety (Feldmann and Krupp, 2011). Arsenosugars can be metabolized to different compounds including DMA and thiolated DMA. Currently, no human studies have

assessed the toxicity of arsenosugars. However, recent research suggested that thiolated DMA ^{V} was more toxic than arsenite in human bladder cells (Leffers et al., 2013a).

History of lead arsenate pesticide

Arsenical pesticides have been used in agriculture for centuries. The earliest arsenical insecticide use was arsenic sulfide in China as early as 900 A.D. The copper acetoarsenite known as Paris green was first used in 1867 to control Colorado potato beetle in the USA. Fruit growers soon adopted this insecticide to control codling moth (*Cydia pomonella*) in apple. Among arsenical insecticides, lead arsenate was the most extensively used. It was used widely and rapidly throughout the world because of its long lasting pesticidal effect as it can adhere well to the surface of plants (F. Peryea, 1998). Lead arsenate insecticides were preferred to control codling moth on apples due to its high efficacy and low phytotoxicity. It also was used on other fruit species, garden crops and turf grasses. However, in 1919, fear and concern about the risk of excessive residue of lead and arsenic on fruits and vegetables arose. It was found that existing practices failed to adequately remove arsenic residues while washing produce (Codling, 2011a; F. Peryea, 1998). During subsequent years, steps were taken to limit the use of arsenical pesticides.

Lead arsenate use was terminated in Washington state in 1948 when the organochlorine dichorodiphenyltrichloroethane (DDT) was sold commercially. Continuing use of lead arsenate pesticide in the USA was documented in Michigan, Pennsylvania, and Georgia during the mid-1960s. All lead arsenate-based insecticides were officially banned on 1 August 1988 in the USA (F. Peryea, 1998). Concentrations of lead and arsenic in orchard soils increased significantly because of repeated pesticide application. Alloway (1995) discovered that lead level ranged from 500-1500 mg/kg and arsenic level ranged from 200-500 mg/kg in soils of orchards having a

history of lead arsenate application, whereas the concentration was 2 to 300 mg/kg and 0.1 to 20 mg/kg for lead and arsenic, respectively in uncontaminated orchards (Alloway, 1995; Codling, 2011b). Lead and arsenic concentrations were variable depending on soil properties, type of orchards (peach, apple, plum), organic matter content and history of pesticide use. For example, the level of heavy metals in peach orchards was lower than that in apple orchards due to the infrequency and lower application rate of lead arsenate spraying in the former (Codling, 2011a).

Because lead and arsenic do not dissolve, biodegrade or decay, and generally are not rapidly absorbed by plants, they persist in the soil long after application. When lead arsenate reaches the soil, it undergoes hydrolysis and the lead and arsenic are no longer chemically associated in soil. Consequently, both heavy metals are usually distributed in the top soil in area where lead arsenate was used. Some studies have shown that the level of arsenic in the top 15 to 20 cm of soils is higher than in the 15-40 cm depth. Arsenic is more soluble than lead, so it can move through the soil profile regardless of the soil type. The ratio of lead/arsenic (Pb/As) decreases with increasing soil depth, indicating greater downward movement of arsenic relative to lead (Codling, 2011a; F. Peryea, 1998). The addition of soluble phosphorous compounds can reduce lead solubility but enhance arsenic mobility (Peryea, 1991; Peryea and Kammereck, 1997). In one soil survey from 31 apple orchards, lead concentration mean was 821 mg/kg, close to the original amount applied (817 mg/kg) while average concentration of arsenic was 188 mg/kg which is lower than the 245 mg/kg that was used (Codling, 2011b).

Arsenic may enter food by different pathways. For example, arsenic is naturally present in soil and groundwater and can be taken up by growing plants. There is concern that prior use of lead arsenate as an insecticide in fruit orchards may have increased soil arsenic and lead

concentrations, with the possibility that these metals can be taken up by plants and be present in the edible fruits. It is well established that plants can absorb arsenic, but the extent of soil to plant transfer is dependent upon the plant species and the bioavailability of the metals in the soil (Carrington et al., 2013; Weber and Hendrickson, 2006).

Arsenic and Lead Level in Fruit Juices

While arsenic can enter food in many ways through natural sources or anthropological uses, lead contamination in the environment occurs primarily through human activities. Arsenic is a natural element, so it can be present in soil and groundwater, which can be absorbed by plants. In addition, the previous use of lead arsenate-based pesticides in agriculture can leave the residue in the soil and lead to contamination in food and beverages (Mandal and Suzuki, 2002).

In the U.S., there is a federal standard guideline for allowable arsenic and lead levels in drinking water, but no regulation regarding the presence of these heavy metals in juices currently exists. Roberge et al (2009) analyzed total arsenic in 12 juice samples (two apple ciders, five apple juices and five grape juices). The authors have found that 10 of these juices had total arsenic concentrations higher than 10 µg/L and the highest arsenic level was found in grape juice (47.59 µg/L). Among these samples, eight had inorganic arsenic levels exceeding 10 µg/L and the highest inorganic arsenic level (35.65 µg/L) was found in grape juice (Roberge et al., 2009). This has increased the attention of the public on safety of juice because inorganic arsenic is more toxic than organic arsenic species.

In response to the concern of the public on juice safety, Consumers Union tested different fruit juices in 2011 and found that 10% of tested samples contained arsenic exceeding the EPA drinking water standard of 10 µg/L (Consumer Reports, 2012). In the Consumers Union study,

28 apple juices and 3 grape juices were purchased from retail stores in Connecticut, New Jersey, and New York. Each of the juices was bought in different lot numbers either in ready to drink bottles, juice boxes or cans of concentrate. This made up to total of 88 samples. The authors also found that 25% of tested samples exceeded the federal limit for lead in bottled drinking water (5 µg/L) (CDC, 2017). The highest lead level detected in apple juice was 13.6 µg/L and for grape juice was 15.9 µg/L. The arsenic level was also found to be higher in grape juice (24.7 µg/L) than in apple juice (13.9 µg/L). In this study, they also concluded that inorganic arsenic was the predominant form of arsenic in these samples. For example, the highest concentration of arsenic was 24.7 µg/L found in grape juice, which contained 20.48 µg/L inorganic arsenic (17). In the same year, the U.S. FDA released data on As concentrations in 247 apple juice samples the agency tested from 2005 to 2011 (FDA, 2011). The results showed that 8% of these juices had total arsenic levels higher than the current federal standard for arsenic in drinking water, with the highest concentration being 45 µg/L. Among these samples, the highest concentration of inorganic arsenic was found in concentrated apple juice (43 µg/L).

In another study on levels of arsenic and lead in apple juices and other fruit juices, Wilson et al. (2012) reported that 12 of 37 juices bought at local grocery stores (Seattle, WA) contained total arsenic nearly at or higher than 10 µg/L, with the highest level found in grape juice (24.8 µg/L). Lead was detected in more than 94% of samples analyzed, with the highest level being 13.4 µg/L in apple-based juice. None of these samples exceeded the federal limit for lead in drinking water (15 µg/L) (Wilson et al., 2012). Interestingly, the authors found that arsenic was detected in more samples and in greater quantities than lead for all types of juices in this study. Even though both lead and arsenic would be expected from apples grown in orchards with previous lead arsenate pesticide application, the difference of these two heavy metals in

juice can be explained by relatively high arsenic conversion rates by tree fruits, the lower mobility of lead, or the relative lead/arsenic ratio in lead arsenate pesticides (Wilson et al., 2012).

CHAPTER 2. EVALUATE THE EFFECT OF ARSENIC IN *IN VITRO* INTESTINAL MODEL CACO- 2 CELL LINE.

ABSTRACT

There are many different sources of human exposure to inorganic arsenic (arsenite and arsenate) but food and drinking water are two main sources. After ingestion, these arsenic compounds will reach the intestinal epithelium - the first barrier for arsenic absorption. The purpose of this study is to characterize the capability of arsenite (As (III)) and arsenate (As (V)) across the human intestinal epithelium. A human intestinal cell line (Caco-2) was used to determine the total uptake of As (III) (exposed in the form of As_2O_3) and As (V) at different concentrations (10, 20, 30, 40, 50, 60 mg/kg). The concentration of arsenic in the cells and the culture medium after exposure was analyzed using Inductively Coupled Plasma- Mass Spectrometry (ICP_MS) and the integrity of the cell monolayers was monitored by Transepithelial Electrical Resistance (TEER) during the assays. Across all the concentrations, both As (III) and As (V) significantly reduced the TEER value of Caco-2 cells after one-hour of exposure, but As (III) caused a greater reduction in TEER. In addition, greater concentration of As (III) in the medium resulted in greater arsenic transport to basolateral media and greater arsenic retention in the cells. After 4 hours of exposure, As (V) had lower uptake than that of As (III); the uptake percentages ranged between 5.1-8.4% and 20.6-30.8 % for As (V) and As (III), respectively. The results indicated that arsenic absorption in human gastrointestinal tract is dependent on its chemical species.

INTRODUCTION

Arsenic (As) is a natural chemical on Earth and can be found in two forms: organic (when linked with carbon and hydrogen) and inorganic (when combined with oxygen, chlorine and sulfur, etc.) (ATSDR, 2007). Inorganic arsenic is known to have higher toxicity than organic arsenic forms, and is classified as a carcinogen for humans (group 1) by the International Agency for Research on Cancer (IARC) (WHO, 2010). There are two common states of the inorganic form of arsenic in the environment, which are known as arsenite (As (III)) and arsenate (As (V)) (Vahter, 2002). Humans can be exposed to arsenic by different routes from food, water, soil ingestion and from air via inhalation (Meacher et al., 2002). Food, however, is the greatest contributor to inorganic arsenic intake and drinking water is the second leading source while the contribution of air inhalation and soil ingestion is negligible.

There is a strong evidence that ingestion of inorganic arsenic can lead to variety of health problems from acute toxicity to chronic disease. Arsenic can induce skin lesions, hypertension, black foot disease and many types of cancers (Chen et al., 1995; Engel et al., 1994; Kadono et al., 2002; Tseng, 1977). Long term exposure to arsenic can cause skin cancer and other types of cancer such as bladder, kidney, lung cancer, etc (Martinez et al., 2011).

Arsenic can cause harmful effects to the organism after crossing the first physiological barrier - the intestinal epithelium - and distribution to the tissues via the bloodstream. Consequently, evaluating the potential of different forms of inorganic arsenic to cross the intestinal barrier is an interesting aspect. However, given the cost and technical challenge of using animal models to estimate intestinal absorption, an *in vitro* system was considered to be a more flexible and lower cost alternative. One *in vitro* model that has been used widely to study drug absorption and evaluate uptake of minerals is the Caco-2 cell system (*3, 10, 17, 18*). These

cells can develop and act similarly to small intestinal epithelial cells after differentiating into polarized enterocyte-like monolayers. Its application is a great alternative to animal studies for studying uptake and/or transport of toxic agents. Caco-2 cells grown using the Transwell system were chosen for this study because the cells are grown in a system having a two-chamber model: the upper apical side being analogous to the intestinal lumen, and the lower basolateral side being analogous to the blood side.

Since arsenic toxicity depends on its bioavailability or the dose that reaches the systemic circulation, it is important to know the uptake of arsenic in the cell. Therefore, the aim of this study was to evaluate the intestinal absorption and the uptake of inorganic arsenic (As III and As V) in human cells using an *in vitro* model of human intestinal absorption.

MATERIALS AND METHODS

Arsenic standards

A commercial standard solution of As (V) prepared from H_3AsO_4 (1,000 mg/ L, Merck, Spain) was used for As (V) experiments. For As (III), the stock standard solution (1,000 mg/L) was prepared as described by Laparra et al. (Laparra et al., 2005). Briefly, 1.32 g of arsenic trioxide (Sigma-Aldrich, MO) was dissolved in 25 mL of 20% (w/v) KOH solution, neutralized with 20% (v/v) H_2SO_4 and diluted to 1 L with 1% (v/v) H_2SO_4.

Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). The complete culture medium contained high glucose Dulbecco's Modified Eagle Medium (DMEM from Sigma-Aldrich; St. Louis, MO), 10% Fetal bovine serum (FBS from Sigma-Aldrich; St. Louis, MO), and 1% (v/v) antibiotic – penicillin and streptomycin solution (Gibco,

Sigma-Aldrich; St. Louis, MO). The cells were maintained at 37 ^{o}C in an incubator with an atmosphere of 5% CO_2 and 95% air at constant humidity, the culture medium was changed every other day.

For arsenic uptake experiments, Caco-2 cells were seeded onto polycarbonate Transwell inserts (Corning; Corning, NY) of 24 mm diameter and 0.4 µm pore size at a density of $5x10^4$ cells/cm^2. The Transwell inserts were placed into six well plates dividing the apical from the basal compartments. A total of 1.5 mL medium was added to the apical compartment and 2 mL medium to the basolateral chamber. Media was changed every 48 h. The cells were used for uptake experiments at 15-21 day post-seeding when Caco-2 cells were fully confluent and exhibited maximum differentiation (Ellwood et al., 1993).

Monolayer integrity

To maintain the integrity of the cellular monolayer and full development of functional tight junctions in the Caco-2 cells before the transport and uptake experiment, transepithelial electrical resistant (TEER) values were monitored every 48 h. The TEER values were checked using a Millicell ERS meter (EMD Millipore Co.; Billerica, MA, USA). Only those filters that had TEER value >250 Ωcm^2 at the beginning were used. During the experiment, TEER was monitored every 1 hour.

Arsenic uptake experiment

Retention and transport experiments were conducted with cells grown on Transwell filters for 15-21 days after seeding. Before the arsenic uptake experiment, the medium was aspirated from the apical and basolateral chambers and the cell monolayers were washed three times with phosphate buffered saline (PBS from Sigma Aldrich; St. Louis, MO) at a temperature of 37 ^{o}C and pH of 7.4 to remove any unattached cells. Afterward, 1.5 mL of DMEM media with

different concentrations of As (III) and As (V) was added to each apical chamber and 2 mL of fresh medium containing no arsenic was added to each basolateral chamber (Figure 2.1). The concentrations of As_2O_3 and As (V) used for this experiment were 0, 10, 20, 30, 40, 50, and 60 mg/L. The transepithelial electrical resistance was monitored every 60 min during assays (0, 1, 2, 3, and 4 h).

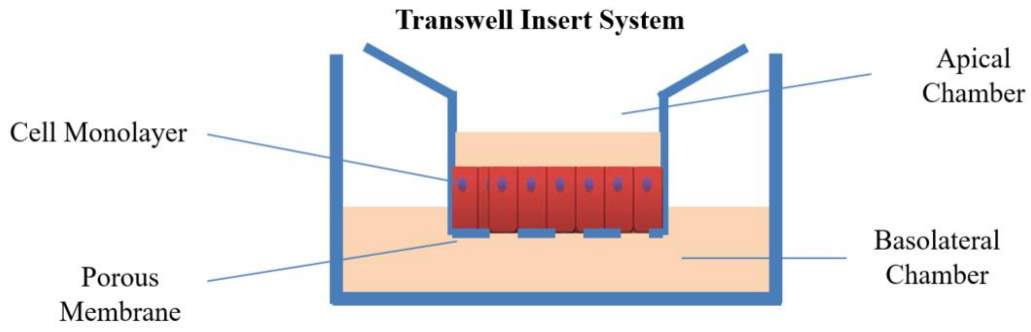

Figure 2.1. Transwell system with Caco-2 cells

Cell cultures were incubated for 4 h at 37 ºC, 5% CO_2 and 95% relative humidity. At the end of incubation, both apical and basolateral media of the inserts were recovered by aspiration, and total arsenic was analyzed to evaluate transepithelial transport. The cell surface of the monolayers was washed three times with PBS, detached with trypsin-EDTA (from Sigma-Aldrich), and recovered with 0.5 mL of DMEM media. The cell solutions were collected and then centrifuged to collect cell pellets using microcentrifuge (Sorvall Legend Micro 17, Thermal Scientific, MA) and lysed with 0.1% Triton 100X (from Sigma-Aldrich), and the total arsenic was analyzed to evaluate arsenic retention.

Total Arsenic Determination

Triplicate samples of cell monolayers, and apical and basolateral media were analyzed for total arsenic content by inductively coupled plasma – mass spectrometry (ICP-MS) (Model 7500ce; Agilent Technologies Inc., IL. USA). These data were used to calculate retention, transport and total uptake of each arsenic species.

Statistical analysis

All treatments consisted of 3 replicates and data are presented as means ± SEM. Least square means were compared using the Fisher Least Significant Difference method using SAS 9.4 software (SAS Institute, Inc., Cary, NC) and were considered significant when P-values were less than 0.05.

RESULTS

Effect of As species on TEER value of the cell monolayer

The TEER of the monolayers was higher than 600 $\Omega.cm^2$ before the experiment started, indicating that the cell monolayers were intact in the Transwell inserts, and no holes presented after 15-21 days of culture (Srinivasan et al., 2015). When Caco-2 cells were treated with As (III) (in the form of As_2O_3) and As (V) (in the form of H_3AsO_4), the TEER values were dependent on the presence of each species. Across all the concentrations, both As_2O_3 and As V reduced the TEER value of Caco-2 cells after 1-hour exposure. TEER values were lower in cells exposed to As_2O_3 than As V and these differences were statistically different starting at 2 hours after treatment (Figure 2.2).

As can be seen in Figure 2.3, exposure of Caco-2 cells to As (V) did not influence TEER values between 0 and 4 h after exposure to all tested concentrations except 30 mg/kg. In contrast, As (III) exposure caused a significant reduction of TEER value at the lowest concentration tested

(10 mg/kg); the TEER value reduced 30% after 2 h exposure. At the highest concentration of As_2O_3 (60 mg/kg), the TEER value was reduction 50% after 4 hours of incubation.

When As (III) and As (V) was added into the apical media, there was a greater transport of As (III) than of As (V) during 4 h of incubation. At the highest concentrations (60 mg/kg) of the two species, the absolute transport of As (III) was five-fold higher than that of As (V) (Figure 2.4). At low concentrations (10 to 30 mg/kg), there was no change in the transport of As (V) to basolateral chamber in 4 h. However, when the concentration increased to 40 mg/kg, we observed that the transport was increased after 3 hours of exposure.

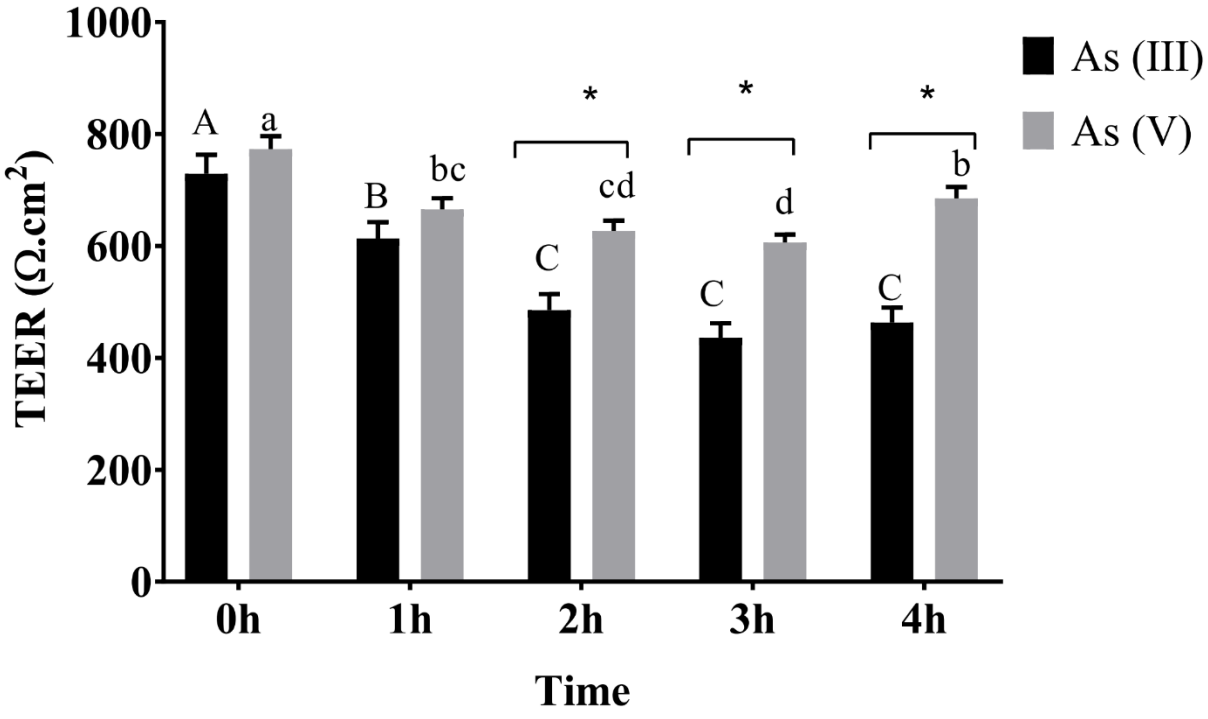

Figure 2.2. Effect of As III (As_2O_3) and As V (H_3AsO_4) on TEER value after 4 hours exposure. Data are mean ± SEM (n = 3). Symbols: * Indicates significant difference from As (III) and As (V) ($P < 0.05$); letters indicate significant difference from 0 h and 1, 2, 3, and 4 h in each As species ($P < 0.05$).

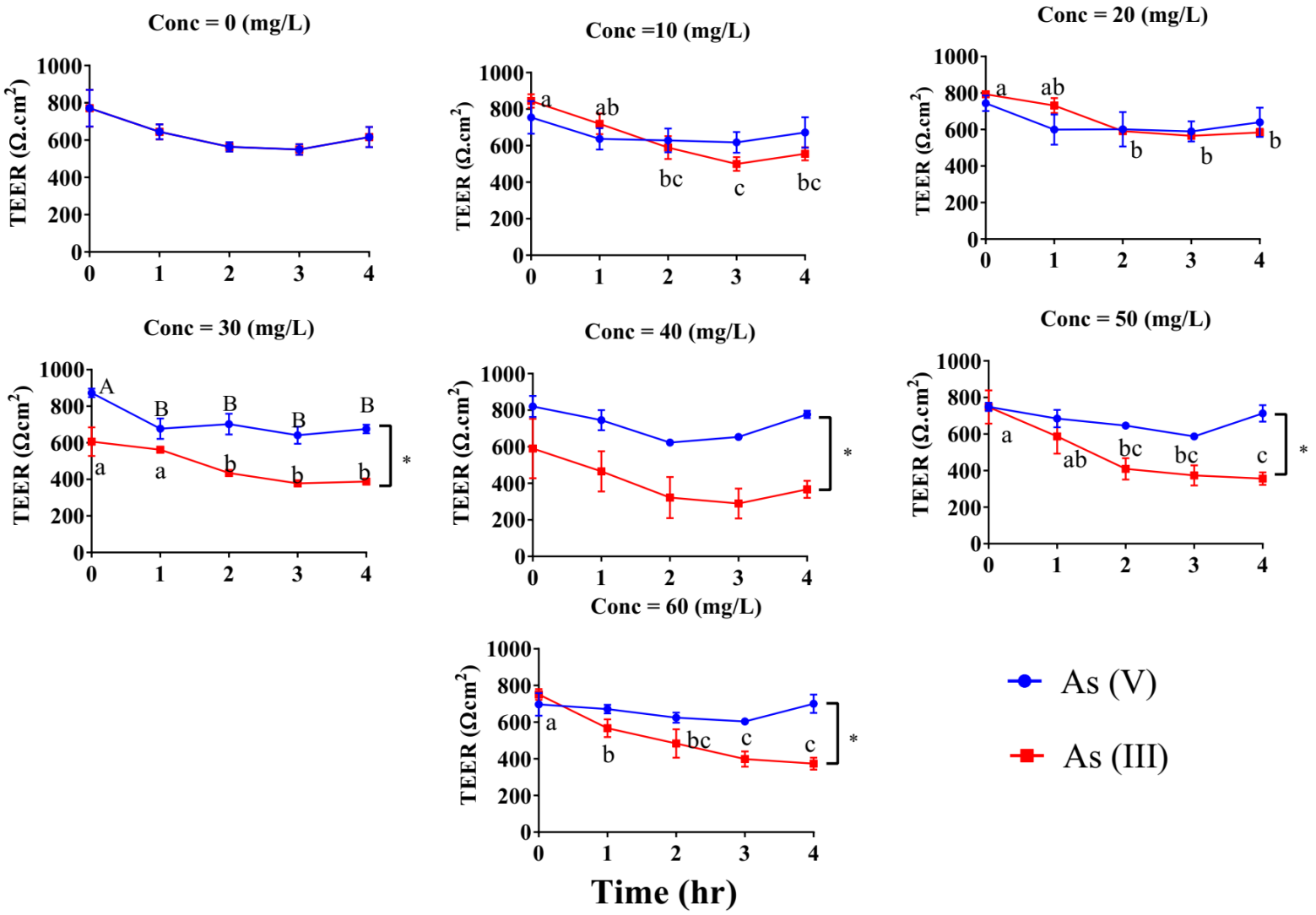

Figure 2.3. Effect of As III (As_2O_3) and As V (H_3AsO_4) on TEER value at different concentration after 4 hours exposure. Data are mean ± SEM (n = 3). * ($P < 0.05$): As (III) vs. As (V); letters ($P<0.05$): 0 hr vs. 1,2,3,4 hr in each As species ($P < 0.05$).

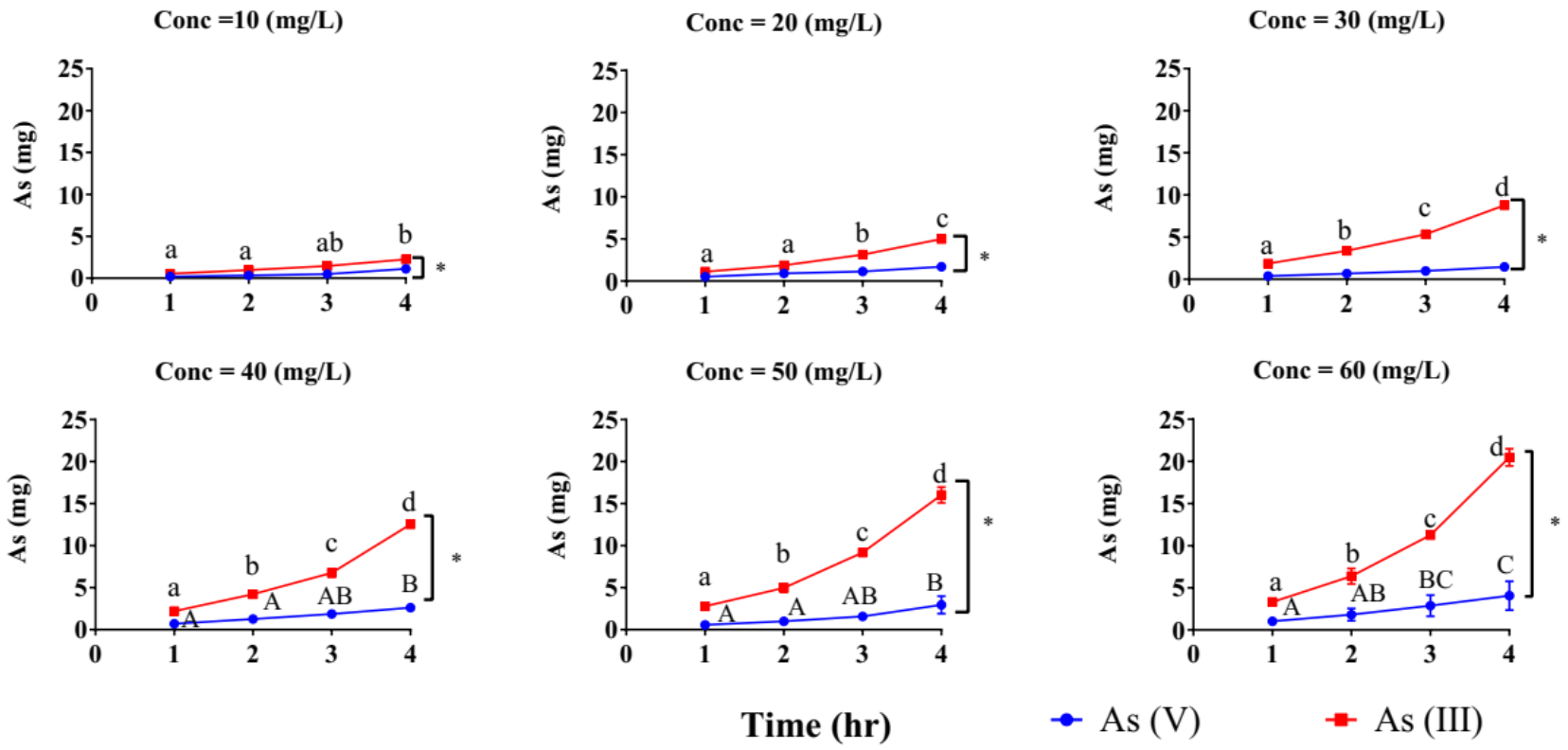

Figure 2.4. Transport of As III (As_2O_3) and As V (H_3AsO_4) to basolateral media during incubation of 4 h. Data are mean ± SEM (n = 3). * (P < 0.05): As (III) vs. As (V); letters (P<0.05): 0 hr vs. 1,2,3,4 hr in each As species (P < 0.05**).** For some points, the error bars did not show if they would be shorter than the height of the symbol.

As can be seen in Figure 2.4, As (III) exposure significantly increased basolateral transport at the lowest concentration (10 mg/kg), the amount of As (III) transported to basolateral media at 4 h was fourfold that at 1 h. In addition, the greater the concentration of As (III), the greater arsenic transport to basolateral media and the greater the arsenic retention in cells. In contrast, the As (V) cell retention and transport did not change when the concentration of As (V) increased. When expressed as percentage of retention with respect to the amount added, the highest retention was obtained at the lowest concentration of As (III) (Table 2.1).

Table 2.1. Cell retention and transport of As (III) (As_2O_3) and As (V) by Caco-2 cells after 4h incubation

Species added (mg/kg)		Cell monolayer (retention)		Basal medium (Transport)	
As (III)	As (V)	(mg)[1]	(%)[2]	(mg)[3]	(%)[2]
10		0.08 ± 0.01^{a}	0.66 ± 0.03^{a}	2.2 ± 0.6^{a}	20.6 ± 1.7^{a}
20		0.08 ± 0.01^{a}	0.36 ± 0.03^{b}	5.0 ± 0.6^{b}	22.9 ± 1.7^{ab}
30		0.09 ± 0.02^{a}	0.27 ± 0.03^{c}	8.8 ± 0.7^{c}	27.0 ± 2.1^{bc}
40		0.15 ± 0.01^{b}	0.32 ± 0.03^{bc}	12.5 ± 0.7^{d}	28.7 ± 2.1^{c}
50		0.17 ± 0.01^{bc}	0.29 ± 0.03^{bc}	16.0 ± 0.6^{e}	29.2 ± 1.7^{c}
60		0.18 ± 0.01^{c}	0.25 ± 0.03^{c}	20.5 ± 0.7^{f}	30.8 ± 1.9^{c}
	10	0.005 ± 0.01^{d}	0.04 ± 0.03^{d}	1.1 ± 0.6^{a}	8.4 ± 1.7^{d}
	20	0.008 ± 0.01^{d}	0.03 ± 0.03^{d}	1.7 ± 0.6^{a}	6.6 ± 1.7^{d}
	30	0.01 ± 0.01^{d}	0.03 ± 0.03^{d}	1.5 ± 0.7^{a}	4.0 ± 1.9^{d}
	40	0.02 ± 0.01^{d}	0.03 ± 0.03^{d}	2.6 ± 0.6^{a}	5.3 ± 1.7^{d}
	50	0.02 ± 0.01^{d}	0.02 ± 0.03^{d}	2.9 ± 0.7^{ag}	5.1 ± 2.1^{d}
	60	0.02 ± 0.01^{d}	0.02 ± 0.03^{d}	4.1 ± 0.7^{bg}	5.7 ± 2.1^{d}

Values are expressed as means ± SEM of three independent replicates (n = 3). Different superscript letters within a same column indicate significant differences ($P < 0.05$) between arsenic contents or percentages. [1] Retention of arsenic in the cells. [2] Percentage of retention or transport calculated with respect to the amount added. [3] Transport of arsenic into basolateral chamber after 4 hours.

The total arsenic uptake including retention and transport is presented in Figure 2.5; smaller uptake was observed for As (V) as compared to As (III). There was no difference in total uptake of As (V) when the arsenic concentration increased. In contrast, as the concentration of As (III) increased, there was greater total uptake. There was a statistically significant positive

relationship between total uptake and As (III) concentration. The correlation between As (III) concentration and total As uptake was strong as the r^2 for the regression analysis was 0.93 (P = 0.002) (Data not shown).

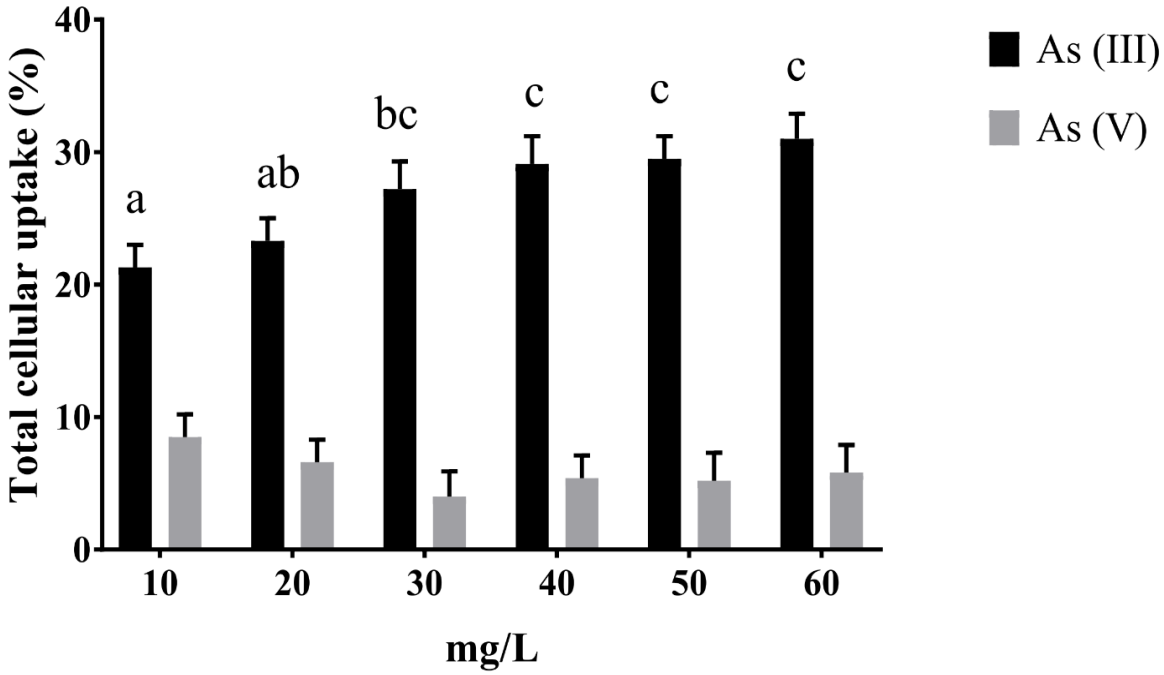

Figure 2.5. Total arsenic cell uptake after 4 h exposure. Data are mean ± SEM (n = 3). Letters indicate significant difference from among concentration tested (P < 0.05).

DISCUSSION

Arsenic has been well-established as a hazardous chemical, causing a variety of adverse effects and cancer in humans (Chen et al., 1995; Engel et al., 1994; Kadono et al., 2002; Tseng, 1977). However, the specific effects of arsenic on metabolic and immune functions are poorly understood (Calatayud et al., 2014). Arsenic needs to cross the first and largest physiological barrier - the intestinal epithelium - into the lamina propria and ultimately the circulatory system, to effect its toxicity. Therefore, in the present work, the absorption and transport of inorganic As species were evaluated in a Caco-2 model of the human intestinal epithelium. This cell model

has been used previously to evaluate the effects of many different compounds in toxicology assays (Boveri et al., 2004; Loh et al., 2012; Piret et al., 2012). Even though Caco-2 cells alone do not fully represent the whole gastrointestinal (GI) tract because it is missing the function of mucus production, it can differentiate into a polarized enterocyte-like monolayer and act similarly to small intestinal epithelial cells (Laparra et al., 2005; Srinivasan et al., 2015). In this study, the assays of retention and transport of inorganic As were conducted in 4 hours since chyme requires at least this amount of time to pass from the pylorus to the ileocecal valve in humans (Laparra et al., 2005).

The results obtained with Caco-2 cell culture show that both the trivalent and pentavalent forms of As reduced the TEER value measured across Caco-2 cell monolayers after 1 h exposure. However, As (III) had more effect on TEER reduction than the pentavalent species of As. As (III) reduced TEER value in the cells up to 37% after 4 h, while only 11% reduction happened in Caco-2 cell monolayers exposed to As (V) (Figure 2.2). TEER is the measurement of electrical resistance across a cellular monolayer and is an indicator of the integrity of the tight junctions and of the cell monolayer. Even though the TEER values in this experiment were higher than 100 Ω.cm2 (below which is considered as a "leaky" value), the reduction in TEER value showed that both inorganic arsenics may change the pore size of the tight junctions and modify permeability of the monolayers (Srinivasan et al., 2015).

Across all the As concentrations tested for both inorganic species, As (III) modified the TEER value to a greater extent than As (V). The reduction of TEER value was higher in cells exposed to As (III) compared to As (V) during incubation at the concentrations of 30 to 60 mg/kg. These results were similar to the previous study of Laparra et. al (2005). The authors found that As (V) did not modify the TEER value, but As (III) can reduce the TEER value up to

37% after 4 h exposure. The modification of the cell monolayer indicated that the tight junctions were the early sites of As (III)-induced injury (Laparra et al., 2005). In other research, Rao et. al. (2000) showed that this reduction can be associated with the formation of reactive oxygen species and lead to the reduction of glutathione (GSH)/ oxidized glutathione (GSSG) ratio (Rao et al., 2000). In the present study, we did not test the content of GSH or GSSH, but it is well-known that GSH is the primary target of trivalent arsenic because of its high affinity for sulfhydryl groups; and the exposure to trivalent arsenicals can change the balance of GSH-GSSG (Abdul et al., 2015; Bode and Dong, 2002; Shen et al., 2013).

The greater rate of transport of As (III) compared to As (V) related to the dependence of transport on the culture media. In the present study, DMEM with 109 mg/L NaH_2PO_4 was used, and phosphate may inhibit the transport and uptake of As (V) (Sigma-Aldrich, 2017). Pentavalent arsenic has a similar structure to phosphate and it has been demonstrated that As (V) transport is mediated via the phosphate transporter (Calatayud et al., 2010; Gonzalez et al., 1995; Huang and Lee, 1996). When similar experiments were conducted in KB cells cultured in phosphate-free medium, the authors found that the toxicity of As (III) did not change as compared to cells cultured in regular media, but toxicity and intracellular accumulation of As (V) was increased 40 times and reached the same cytotoxicity as that of As (III) (Huang and Lee, 1996). They also found that at a dose of 10 mg/L sodium phosphate could reduce As (V) uptake up to 50%. Phosphate is a major inorganic salt in all culture media and, because of its similar structure to As (V), phosphate ions can compete with As (V) for binding sites. Thus, it has limited the transport and uptake of As (V). In contrast, As (III) uptake is not affected by the presence of phosphate. Calatayud et al. (2010) reported that transport of As (V) was reduced as the phosphate concentration in the medium increased. Second, pH of the cultured medium was

7.2-7.4, which is close to physiological pH, and pH can affect the uptake of these arsenic species by changing their charges. The pKa values for As (V) are 2.3, 6.7, and 11.6 whereas the pKa for As (III) is 9.2 (Calatayud et al., 2011, 2010). In this case, As (V) is predominantly found in anionic form, while As (III) is nonionized; and this uncharged form will be preferably transported through the membranes compared to ionized forms (Calatayud et al., 2010). The observation in the present study may change in the human intestinal tract because pH values vary between 5 and 8 along the intestinal tract, potentially resulting in changes in arsenic transport (Fallingborg, 1999; Neuhoff et al., 2005).

Similar to the increase of transport by time and concentration of As (III) shown in Figure 2.4 and Table 2.1; we observed that there was an increase of cell As retention with increasing concentrations of trivalent arsenic but not pentavalent arsenic (Table 2.1). This indicates that, for all concentrations tested for As (III) in this experiment, there was no saturable component in the cellular uptake. This demonstrates that As (III) transport may involve facilitated diffusion (Laparra et al., 2005).

When total cellular uptake (%) was calculated as retention plus transport (Figure 2.5), we observed results consistent with previous studies (Calatayud et al., 2011, 2010; Laparra et al., 2005). The total uptake of As (V) through the monolayer after 4 h was of lesser magnitude than the total uptake of As (III). As mentioned before, the culture conditions may affect the uptake of each As species. In the present study, the greater the exposure to As (III), the greater total cellular uptake of As (III) and this relationship was relatively linear. This supports the idea that the transport is by facilitated diffusion mechanism, but it is possible that other transport systems may assist the transport of As (III) through the monolayer (Calatayud et al., 2012, 2011).

In summary, this experiment has provided useful information about the uptake and/or transport of arsenic through Caco-2 cells. The *in vitro* system mimics the absorption of arsenic by the human intestinal tract *in vivo*. We have demonstrated that arsenic (III) uptake from the apical to basolateral side of Caco-2 cell monolayers was favored over As (V) uptake. This result helps us understand the relative bioavailability of two arsenical species when humans ingest arsenic-contained food or water. The Caco-2 model is a useful model of human intestinal epithelia cells to evaluate the transport and uptake of inorganic species. However, the *in vivo* intestinal tract not only contains enterocytes, but also mucus secreting cells, macrophages, dendritic cells, and other lymphocytes. Therefore, to evaluate the interaction between intestinal epithelial cells and immune system cells on the uptake and/or transport of arsenic, a model of co-culture of intestinal cells and different immune cells could be used.

CHAPTER 3. ASSESSMENT OF SELECTED METAL CONCENTRATIONS IN SHELF-STABLE COMMERCIAL APPLE JUICES AND FRESH APPLE CIDERS IN MICHIGAN

ABSTRACT

Consumer concern about the presence of arsenic and other metals in fruit juices has been heightened due to potential toxicity. However, it is unclear if differences in metal concentrations exist between shelf stable apple juices and fresh juices produced from U.S. grown apples. This research was conducted to assess the concentrations of different metals, particularly arsenic (As), aluminum (Al), cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb) and manganese (Mn), in shelf-stable apple juices and fresh apple ciders obtained at retail establishments and cider mills throughout Michigan. Samples of shelf-stable apple juice and fresh apple cider, obtained in the fall of 2015 and 2016, were analyzed for metal concentrations by inductively coupled plasma mass spectrometry (ICP-MS). A risk assessment was also conducted to access the potential non-carcinogenic toxicity (Hazard Index - HI) via chronic apple juice daily intake for all metal exposures and to estimate incremental lifetime cancer risk (ILCR) by using the U.S. EPA cancer slope factor for inorganic As. Averaged across both sampling years, As was detected in quantifiable concentrations (> 1 µg/L) in 77% of juice samples, but only 16% of cider samples. Among those samples containing quantifiable As and Pb, the total As and Pb concentrations in apple juice and cider were similar. Cu (120-157 µg/L) and Mn (197-325 µg/L) were detected in all samples. Al and Cr levels in quantifiable samples were not significantly different among juice categories, but the percentage of samples containing these metals was higher in apple juices. The results indicate that these metals do not increase non-carcinogenic toxicity risk (HI <1) and the ILCR resulting from inorganic arsenic exposure via drinking apple juice is acceptable.

INTRODUCTION

Heavy metal contamination in food is not a new issue. In the U.S., it has been evaluated by the federal government for many years. For example, the Center for Food Safety and Applied Nutrition (CFSAN) of the U.S. FDA conducts the Total Diet Study (TDS) annually, which also includes the evaluation of total arsenic in the diet (FDA, 2017a). These evaluations can help to determine the level of the contaminants and estimate the dietary intake of those contaminants. While arsenic can enter food in many ways through natural sources or anthropology, lead contamination in the environment occurs primarily through human activities. Arsenic is a natural element, so it can be present in soil and groundwater and can be absorbed by plants. In addition, the previous use of lead arsenate-based pesticides in agriculture can leave residues of these metals in the soil and lead to contamination in food and beverages (Mandal and Suzuki, 2002).

Apple juice has been found to occasionally contain arsenic and lead concentrations higher than the U.S. EPA limit for drinking water (Consumer Reports, 2012; Roberge et al., 2009; USFDA, 2011; Wilson et al., 2012). Young children and elderly persons have greater juice consumption compared to the general population, and children in particular are susceptible to adverse health effects associated with lead and arsenic exposure. Currently, the U.S. FDA has proposed an action level for arsenic (10 µg/L) but not for lead in apple juice. There are no standard guidelines regarding daily consumption of juice to reduce the risk of heavy metal exposure. The Agency for Toxic Substances and Disease Registry (ATSDR) listed the reference dose (RfD) of chronic arsenic exposure as 0.0003 mg/kg/day, whereas an RfD was not set for lead (ATSDR, 2007). For people who drink large quantities of fruit juice, their As consumption can exceed the RfD. Thus, to more fully characterize the levels of heavy metals in specific fruit juices, this research examined the arsenic and lead levels in different fruit juices manufactured

by large scale commercial processors and small-scale cider mills. The results from this research may be useful to update FDA about health risk assessment for arsenic and lead in fruit juice and help to evaluate the actual health risk associated with consumption of arsenic and lead-containing juices.

Considerable data on arsenic and lead contamination are available for groundwater and other foods and some fruit drinks. The current study expanded the analysis to include these and other metals such as aluminum (Al), cadmium (Cd), copper (Cu), chromium (Cr) and manganese (Mn). By screening a larger array of metals present in apple juice, this research can be used to evaluate the potential risks that may result from metal exposure via apple juice consumption.

The objectives of this study were: (a) to assess the concentrations of different metals in shelf-stable apple juices and fresh apple ciders obtained at retail establishments and cider mills throughout Michigan in 2015 and 2016; and (b) to evaluate the potential human health risks associate with these metals in apple juices and apple cider by Hazard Index (HI) and Incremental Lifetime Cancer Risk (ILCR).

MATERIALS AND METHODS

Item collection

Fruit juice products were purchased from different retail grocery stores and cider mills in Michigan from June to December of 2015 and 2016. Samples of shelf-stable apple juice (n=39) and fresh apple cider (n=160) were assigned a unique identification code in order to: 1) maintain anonymity for companies supplying the samples, and 2) blind the analyst to the sample source. Aliquots of each juice were transferred to polypropylene tubes and stored at 4 ^{o}C for analysis the next day. The rest of samples were stored at -20 ^{o}C to prevent degradation.

Sample preparation for analysis

Fruit samples were analyzed for metals by the Michigan State University Diagnostic Center for Population and Animal Health (DCPAH) using inductively coupled plasma – mass spectrometry (ICP-MS) (Model 7500ce; Agilent Technologies Inc., IL. USA). The method has been described by Wahlen et al. (2005). Each element was calibrated using a 4-point linear curve of the analyte: internal standard response ratio. Metals were analyzed in helium mode, which removed spectral interference at specific mass for different metals (Wahlen, R., L. Evans, J. Turner, 2005). The reported limit of quantification (LOQ) for all metals in this analysis was 1 µg/L except for Cr, which had an LOQ of 5 µg/L. To estimate the mean value for all samples, we assumed that the samples having metal concentration less than LOQ contained 0.5 µg/L for all metals except Cr, for which we assumed samples containing detectable Cr less than the LOQ contained 2.5 µg/L (EPA, 2000). The mean value of quantifiable samples was calculated as the average of samples containing metal concentrations higher than the LOQ.

Exposure and risk estimation

Exposure estimation

The mean values of each metal measured in the apple juice were used to calculate the chronic daily intake (CDI). The exposure to these metals from juice consumption were characterized based on CDI values. The following equation was used to determine the CDI of the seven metals analyzed in this study (EPA, 1992):

$$CDI = C \times DI/BW$$

Where CDI is the chronic daily intake (mg/kg-day^{-1}), C is the concentration of each metal found in juice samples (mg/L). DI is the average daily intake rate of apple juice (L/day) and BW is the body weight (kg) of an individual.

Data from US EPA's What We Eat in America (WWEIA) - Food Commodity Intake Database (FCID), 2005-2010 was used to estimate consumption of juice for different age groups (0-70 years). The average apple juice consumption presented in Table 3.4 was extracted from FoodRisk for two food categories: “apple, juice” and “apple, juice-baby food” (FoodRisk, 2016). Juice consumption was counted for apple juice drinkers only. Currently, there are no available estimates for apple cider consumption in the U.S. We assumed that 1% and 5% of total apple juice consumption was from apple cider for the US population aged 0-6 years and the population aged 7 years and older, respectively (Table 3.4). These assumptions were based on approximate numbers of cider mills in the U.S. and average annual volumes of apple cider production in Michigan cider mills (Bobe et al., 2007; FDA, 2001).

Non-cancer risk assessment

A hazard quotient (HQ) was used to evaluate the noncancer risk associated with metal intake resulting from apple juice consumption. The Hazard quotient (HQ) was calculated using the reference dose (RfD) data as published by Tvermoes et al (2014). Briefly, the EPA reference doses for As, Cd, Cr, and Mn were used to calculate the HQs for these metals while the EPA screening level RfD were used to calculate the HQ for Al and Cu because no RfD has been set for these two metals (EPA, 2018) (Appendix Table A5.1). HQs were calculated using the following formula:

HQ = CDI / (RfD or screening level RfD)

The sum of all of these six metals were expressed as hazard index (HI) risks (EPA, 2005). This index showed the cumulative non-carcinogenic risks that these metals can pose to apple juice drinkers in the worst-case scenario assessment:

$HI = HQ_{Al} + HQ_{As} + HQ_{Cd} + HQ_{Cr} + HQ_{Cu} + HQ_{Mn}$

Lead was not included in this HQ or HI calculation because no oral RfD value or any related guidance has been established.

Cancer risk assessment

Cancer risk was only calculated for inorganic As exposure in this study because there are no cancer slope factors associated with oral exposures to other metals (except cadmium, which was not detected in this study). In this study, we only analyzed total arsenic. The concentration of inorganic arsenic in apple juices was estimated based on a surveillance study on apple juice conducted by FDA, wherein 74.8% of arsenic in apple juice was determined to be inorganic arsenic (FDA, 2011). The incremental lifetime cancer risk (ILCR) was used to express the carcinogenic risk for inorganic As exposure resulting from apple juice consumption, which is the cancer slope factor (CSF) multiplied by the life time average daily dose (LADD). LADD (mg/kg-day) was calculated by the following equation (Tvermoes et al., 2014):

$$LADD = C_{metal} \times \left(0.001\frac{mg}{\mu g}\right) \times \left(1\ \frac{L}{kg}\right) \times \left(0.001\frac{kg}{g}\right) \times \sum \left(\frac{IRi \times EDi}{AT}\right)$$

where C_{metal} is concentration of inorganic As in apple juice (μg/L); C_{mean} = 1.79 μg/L; $C_{mean\ of\ quantifiable\ samples}$ = 2.24 μg/L; C_{FDA} = 10 μg/L; IRi is body weight normalized juice consumption rate for the i^{th} age group (g/kg-day); EDi is exposure duration for the i^{th} age group (years); and AT is a lifetime of 70 years (Appendix Table A5.2).

The cancer slope factor for U.S. EPA was used to estimate the cancer risk of inorganic arsenic (1.5 per mg/kg-day).

Statistical analysis of juice samples

Data were analyzed using the FREQ and MIXED procedures of SAS, version 9.4 (SAS Institute, Inc., Cary, NC). Least square means were compared using the Fisher Least Significant Difference method and were considered significant when P-values were less than 0.05.

RESULTS

Metal concentrations

Table 3.1 shows the results for analysis of metals in apple juices collected in 2015 and 2016. Aluminum concentration ranged from less than 1 to 907 μg/L, and samples collected in 2015 had higher Al levels as compared to samples collected in 2016. However, there was no difference in Al concentration among those samples containing quantifiable Al concentrations (> 1 μg/L). Arsenic concentration ranged from <1 to 9.5 μg/L and there was no difference among samples collected in different years. Cadmium concentrations were less than 1 μg/L for all apple juices tested. The average Cr concentration (range <5 to 65 μg/L) in 2016 samples was higher than in 2015 samples, but there was no difference in Cr concentration among quantifiable samples (samples contained Cr higher than 5 μg/L). Cu and Mn were detected in all apple juice samples with average concentrations of 120.1 μg/L and 324.7 μg/L, respectively. The concentration of Cu in apple juice in 2016 was significantly higher than that in 2015; however, there was no difference in Mn and Pb concentration among the two years (Table 3.1).

There was no difference in concentration of metals in apple cider samples between the two years of sampling except that the concentration of Al was higher in 2016 for quantifiable samples (>1 μg/L) (Table 3.2). Only one of 160 cider samples contained cadmium at a concentration above the LOQ; the Cd concentration in this sample was 1.9 μg/L.

Table 3.1. Metal concentrations in apple juices (n=39) purchased from Michigan retail grocery stores in 2015 and 2016.

Metal	Metal frequency (above LOQ)		Range (µg/L)		Average (µg/L)		Average of quantifiable samples (µg/L)	
	2015	2016	2015	2016	2015	2016	2015	2016
Al	94.1[a]	36.4[b]	< 1 - 806	< 1 - 907	321 ± 55.9[a]	144 ± 54.7[b]	341 ± 55.6	396 ± 103
As	88.2	68.1	< 1 - 8.5	< 1 - 9.5	2.6 ± 0.5	2.3 ± 0.4	2.9 ± 0.5	3.1 ± 0.5
Cd	0	0	< 1	< 1	< 1	< 1	< 1	< 1
Cr	17.7[a]	81.8[b]	< 5 - 14	<5 - 65	3.9 ± 0.8[a]	17 ± 3.5[b]	10.4 ± 2.4	20.4 ± 3.8
Cu	100	100	12.3 - 132	25.6 - 276	55 ± 8.5[a]	170 ± 15.1[b]	55.3 ± 8.5[a]	170 ± 15.1[b]
Mn	100	100	38.2 - 1689	21.2 - 2408	379 ± 86.6	283 ± 107	379 ± 86.6	283 ± 107
Pb	52.9	31.8	< 1 - 6.8	< 1 - 5.6	1.7 ± 0.4	1.2 ± 0.3	2.8 ± 0.6	2.6 ± 0.7

Values are means ± SEMs. Within each row for each parameter, means followed by different letters are significantly different (P<0.05). Metal frequency (% of samples) above limit of quantification (5 µg/L for Cr and 1 µg/L for other metals).

Table 3.2. Metal concentrations in fresh apple ciders (n=160) purchased from Michigan cider mills in 2015 and 2016.

Metal	Metal frequency (above LOQ)		Range (µg/L)		Average (µg/L)		Average of quantifiable samples (µg/L)	
	2015	2015	2015	2016	2015	2016	2015	2016
Al	38.5[a]	1.2[b]	< 1 - 934	< 1 - 514	30.1 ± 12.5	6.8 ± 6.3	77.5 ± 31.0[a]	514 ± 202[b]
As	11.5	19.5	< 0 - 8.7	< 0 - 11.5	0.8 ± 0.1	0.9 ± 0.2	3.2 ± 0.9	2.6 ± 0.7
Cd	0	1.2	< 1	< 1 - 1.9	< 1	0.5 ± 0.02	< 1	1.9
Cr	10.3[a]	28.1[b]	< 5 - 27.3	< 5 - 196	3.3 ± 0.4	6.8 ± 2.4	10.5 ± 2.5	17.7 ± 8.1
Cu	100	100	26.5 - 999	23.53 - 282	161 ± 16.5	153 ± 8.0	161 ± 16.5	153 ± 8.0
Mn	100	100	35.8 - 567	41.7 - 1983	159 ± 10.6	233 ± 27.8	159 ± 10.6	233 ± 27.8
Pb	43.6	50	< 1 - 28.4	< 1 - 26.2	2.1 ± 0.4	3.1 ± 0.5	4.1 ± 0.9	5.7 ± 0.9

Values are means ± SEMs. Within each row for each parameter, means followed by different letters are significantly different (P<0.05). Metal frequency (% of samples) above limit of quantification (5 µg/L for Cr and 1 µg/L for other metals).

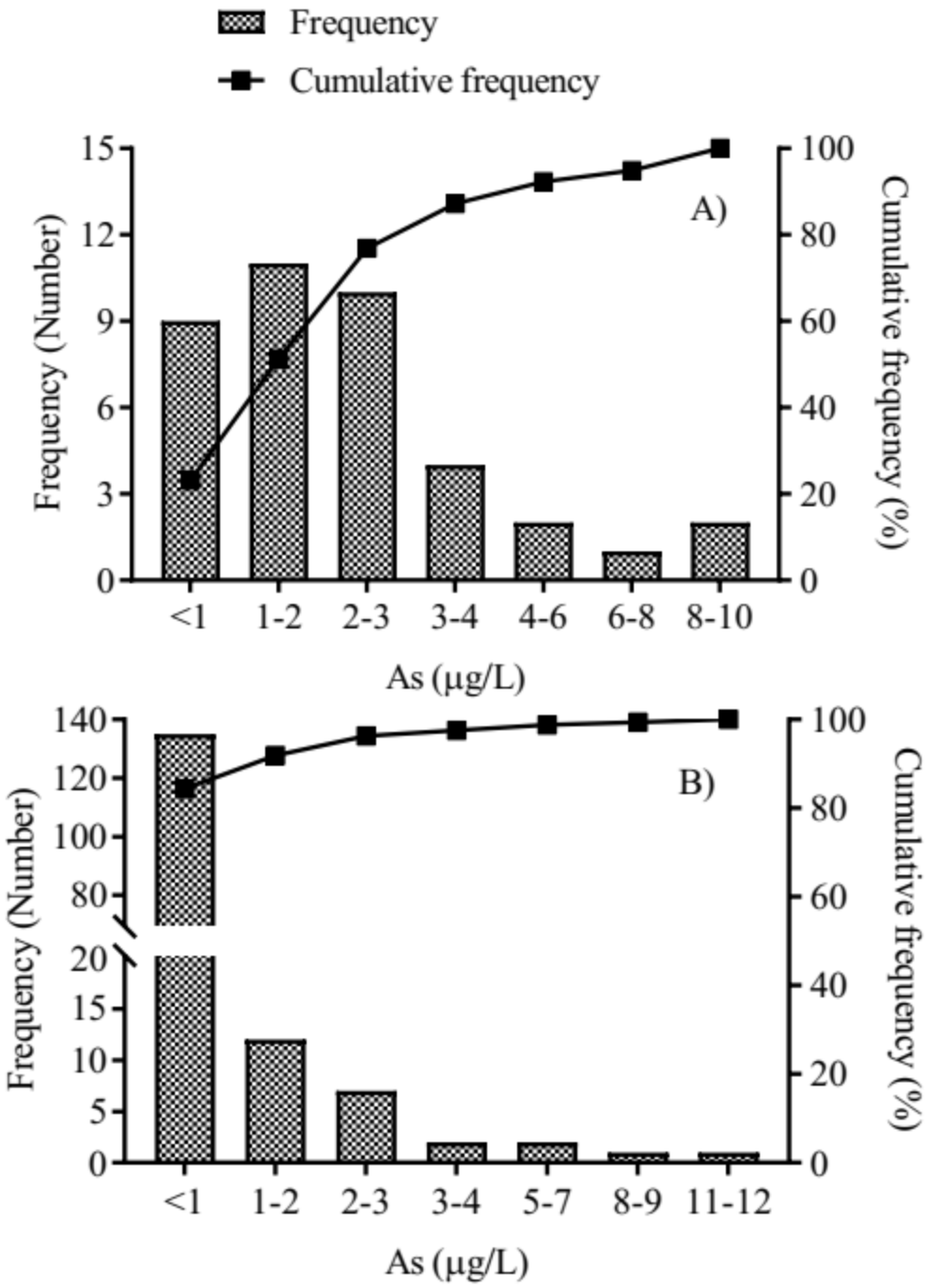

Figure 3.1. Cumulative distribution of total arsenic in apple juices (A) and fresh apple ciders (B) in Michigan.

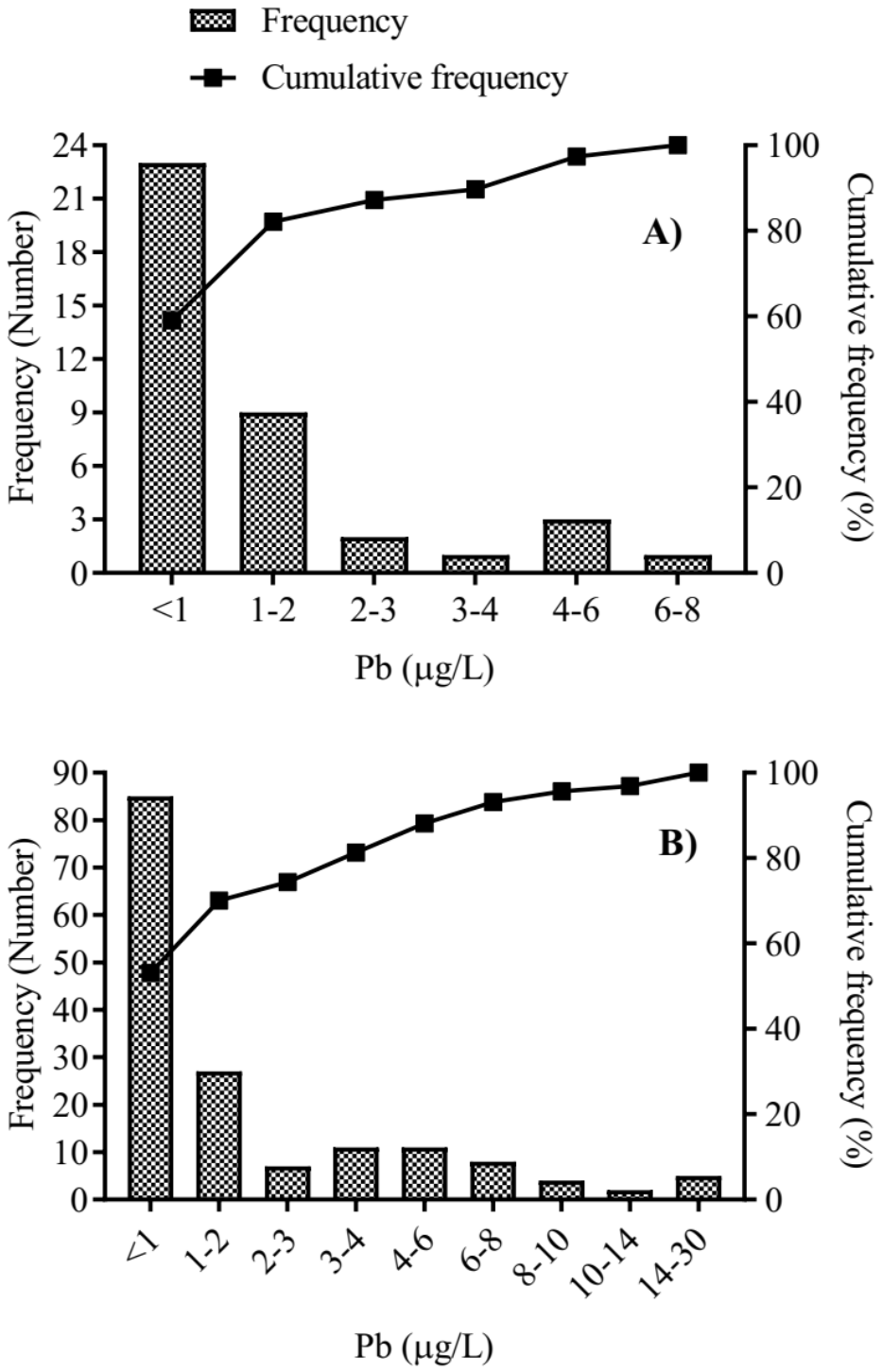

Figure 3.2. Cumulative distribution of lead in apple juices (A) and fresh apple ciders (B) in Michigan.

Of the nearly 200 samples of juice and cider we gathered over the two-year period, tests showed that only one cider sample contained total arsenic in excess of 10 µg/L, which is the proposed action level for inorganic arsenic in apple juice established by FDA in 2013. In addition, while the assay method could quantify arsenic concentrations as small as 1 part per billion (ppb), only 16 percent of the cider samples contained any detectable arsenic above this LOQ (Figure 3.1).

Table 3.3 shows the results for analysis of metals in apple cider and juice samples collected over two years. Averaged across both years, lead was detected (> 1 µg/L) in 41% of shelf stable apple juice samples and 47% of apple cider samples. Among those samples containing quantifiable lead, the lead concentrations detected in apple juice (2.7 µg/L) and in apple cider (4.9 µg/L) were not significantly different. Among all samples analyzed, seven apple cider samples contained lead concentrations exceeding 10 µg/l, with the maximum lead level detected being 28.3 µg/L. No samples of shelf stable apple juice contained lead concentrations that exceeded 10 µg/L (Figure 3.2). Averaged across both years, As was detected (> 1 µg/L) in 77% of juice samples, but only in 16% of cider samples (Table 3.3). Among those samples containing quantifiable As and Pb, the total As and Pb concentrations in apple juice and cider were similar. Cu and Mn was detected in all samples, and their mean concentrations differed in apple juice and cider tested. Al and Cr levels in quantifiable samples were not significantly different among juice categories, but the percentage of samples containing these metals was about three times higher in apple juices than in cider samples.

Table 3.3. Metal concentrations in apple cider vs apple juices in Michigan (2015 – 2016).

Metal	Metal frequency (above LOQ)		Average (µg/L)		Average of quantifiable samples (µg/L)	
	Apple juice (n=39)	Apple cider (n=160)	Apple juice	Apple cider	Apple juice	Apple cider
Al	61.5[a]	19.4[b]	221 ± 41.4[a]	18.1 ± 7[b]	368 ± 44	296 ± 103
As	76.9[a]	15.6[b]	2.4 ± 0.3[a]	0.86 ± 0.1[b]	3.0 ± 0.4	2.9 ± 0.4
Cd	0	0.6	<1	0.51 ± 0.01	<1	0.01 ± 0.01
Cr	53.9[a]	19.4[b]	11.4 ± 2.2[a]	5.1 ± 1.2[b]	15.4 ± 8.6	14.1 ± 5.7
Cu	100	100	120 ± 13[a]	157 ± 9[b]	120 ± 13.0[a]	157 ± 9[b]
Mn	100	100	325 ± 71[a]	197 ± 15[b]	325 ± 71[a]	197 ± 15.4[b]
Pb	41	46.9	1.4 ± 0.3	2.6 ± 0.4	2.7 ± 1.2	4.9 ± 0.6

Values are means ± SEMs. Within each row for each parameter, means followed by different letters are significantly different (P<0.05). Metal frequency (% of samples) above limit of quantification (5 µg/L for Cr and 1 µg/L for other metals).

Evaluation of non-cancer risk

Apple juice and apple cider consumption by different age groups is presented in Table 3.4. Among all of age groups and among two types of juice, children from one to two years of age consumed the highest amount of apple juice on a gram per kilogram body weight basis (13.4 g/kg/day), so this group was used to calculate CDI as well as the HQ values for each metal and the HI value to estimate the potential health impact of metals in apple juice. The CDI value was evaluated using both the mean concentration of all samples and the average concentration of quantifiable samples (Table 3.5).

Table 3.4. Average apple juice consumption by age group derived from Food Commodity Intake Database, 2005-2010.

Age Range	Consumption per weight (g/kg/day)	Apple juice consumption * (g/kg/day)	Apple cider consumption* (g/kg/day)
3 to < 6 months	11.21	11.1	0.11
6 to < 12 months	10.19	10.09	0.10
1 to < 2 years	13.38	13.25	0.13
2 to < 3 years	10.86	10.75	0.11
3 to < 6 years	6.48	6.42	0.07
6 to < 11 years	2.82	2.68	0.14
11 to < 16 years	1.65	1.57	0.08
16 to < 21 years	1.31	1.24	0.07
21 to < 50 years	1.22	1.16	0.06
50 to < 70 years	0.8	0.76	0.04

* For the US population aged 0-6 years, we assumed that 1% of total apple juice consumption was apple cider. For the US population aged 7 years and older, we assumed that apple cider accounted for 5% of total apple juice consumption.

Hazard quotients for each metal (except Pb) were calculated using the mean of all samples and the mean of quantifiable samples (Table 3.3) and then compared to the reference dose. The HQ for inorganic As was calculated using the concentration of total arsenic in this study with assumption that 74.8% of total arsenic was inorganic species (FDA, 2011). Among the six metals, inorganic As and Cr yielded the highest HQs of 0.08 and 0.05, respectively. Similar results were found when estimating the non-carcinogenic risk using the mean value of quantifiable samples with the highest HQ of inorganic As (0.1); however, this value was four-times lower than HQ of inorganic As using the FDA maximum contaminant level for bottled water (0.44) (Table 3.5).

The sum of the individual HQs was calculated as a hazard index to estimate the cumulative non-cancer risk. HI calculated from the mean of all samples and mean of quantifiable samples were both less than one whereas HI using FDA maximum contaminant level for bottled water exceeded one (Table 3.5).

Table 3. 5. Chronic daily intake (CDI) of metals from apple juice consumption for a child from 1 to 2 years of age.

Metal	Mean CDI (µg/kg/day)	Mean CDI (quantifiable samples) (µg/kg/day)	FDA CDI[c] (bottle water) (µg/kg/day)	RfD (µg/kg/day)	Mean HQ	Mean HQ (quantifiable samples)	FDA HQ
Al[a]	2.93	4.88	2.64	1000	0.003	0.005	0.003
InAs*	0.02	0.03	0.13	0.3	0.08	0.1	0.44
Cd	NA	NA	0.07	0.5	NA	NA	0.13
Cr	0.15	0.20	1.32	3	0.05	0.07	0.44
Cu	1.59	1.59	13	40	0.04	0.04	0.33
Pb[b]	0.02	0.04	0.07	NA	NA	NA	NA
Mn	4.30	4.30	0.66	140	0.03	0.03	0.005
Sum HI					**0.2**	**0.245**	**1.35**

* Inorganic arsenic level was estimated based on FDA surveillance (74.8% of arsenic is inorganic species).
[a] No RfD value is established, so the EPA screening level RfD was used.
[b] No RfD or screening level RfD was available for Pb, so Pb was excluded for HQ and HI calculation.
[c] This value was calculated by using FDA limit for each metal (can be found in supplement Table S1) multiplied with daily intake of apple juice for 1-2-year-old group per kilogram body weight.

Evaluation of cancer risk

The incremental lifetime cancer risk (ILCR) was used to express the carcinogenic risk for inorganic As exposure resulting from apple juice consumption. Values used to estimate ILCR for each age group can be found in appendix Table A5.2. The cancer risk for adults (ages 0-70) was 5×10^{-6} and 6×10^{-6} when using mean values of all samples and mean values of quantifiable samples, respectively. These ILCR estimates were about 5 times less than ILCR calculated using

the current FDA proposed action level for inorganic As concentration in apple juice (10 µg/L) (Table 3.6).

Table 3.6. Estimated lifetime average daily dose (LADD mg/kg/day) and cancer risk.

Mean chronic daily intake (all samples)		Mean chronic daily intake (quantifiable samples)		FDA action level in apple juice	
LADD	**Risk**	**LADD**	**Risk**	**LADD**	**Risk**
3.3^{E-06}	4.9^{E-06}	4.1^{E-06}	6.1^{E-06}	18^{E-06}	27^{E-06}

ILCR was calculated using the EPA cancer slope factor for arsenic: 1.5 mg/kg/day.
LADD estimation can be found in appendix Table A5.3

DISCUSSION

Apple juice is one of the most favorite drinks and is the second most consumed juice in the United States after orange juice (USDA Economic Research Service, 2015). However, there is a rising concern of potential health impact when consuming metal-contaminated juices. This study assessed concentrations of different metals in apple juices and apple cider manufactured by large scale commercial processors and small-scale cider mills.

The levels of arsenic in apple juice and apple cider in this study were lower than that reported in studies by other researchers (Tvermoes et al., 2014; Wang et al., 2017; Wilson et al., 2012). There are many factors that potentially influence metal concentrations in apple juice such as geographical location of soil, differences in agricultural practices, apple juice types as well as the origin of the apple juice (Tvermoes et al., 2014). In this study, shelf stable apple juices were more likely to contain quantifiable arsenic (77% of samples) compared to apple cider. First, it is possible that the juice processing increases the arsenic concentration in juice samples as compared to non- processed apple cider. Wang et al. (2017) has shown that heavy metals such as As can be transferred to juice during filtration. The authors found that food-grade filter aids containing arsenic levels higher than 3 ppm raised the level of arsenic in apple juice from 4 to 7

times due to the filtration (Wang et al., 2017). Second, it is possible that both the juice concentrate and water used to reconstitute the juice concentrate to produce single strength apple juice can be significant sources of arsenic. The majority (95%) of shelf stable apple juice samples tested in this study were apple juice concentrate diluted with water. Further work is needed to evaluate the metal concentrations in juice concentrate and in water used for dilution to ascertain which source is more responsible for arsenic in the finished apple juice products.

In contrast with arsenic, lead concentrations were not different among apple juice and apple cider samples, and the mean values were similar to previous studies (Tvermoes et al., 2014; Wilson et al., 2012). Similar results have been reported by Wang et al. (2017), who found no differences in lead concentration in apple juice as compared to unfiltered juice (Wang et al., 2017). This indicates that the filtration process affects arsenic level but not lead in apple juice.

Concentrations of Cu and Cr in apple juice, but not in apple cider, were different among the two years of sampling. The levels of Cu and Cr detected in apple juice in this study were higher than that observed by Tvermoes et. al (2014). The concentration of these metals in apple juice might be influenced by location of apple orchard and the origin of the juice. In contrast, apple cider had consistent concentration of Cr and Cu among the two seasons, when samples were collected at the same locations in Michigan each year.

All samples contained less than 1 µg/L cadmium except one cider sample in 2016 that contained 1.9 µg/L. Mn concentration in apple juice and apple cider ranged from 21.2 to 2408 µg/L, with 95% of samples exceeding the current FDA bottled water standard of 50 µg/L in all samples tested (Table 3.3) (FDA, 2017b). Likewise, average Al concentration in all apple juices exceeded the maximum contaminant level (MCL) for Al in drinking water (50-200 µg/L). It is worth noting that the FDA bottled water standards for Mn and Al are based on the EPA National

Drinking Water Regulations, which were not based on health risk but rather for aesthetic purposes for public drinking water such as taste, color and odor (EPA, 2017). However, there is also a concern about the toxicity of Al and Mn, as consuming high concentrations of these metals can cause adverse neurological disorders. For example, high levels of Mn in drinking water are associated with hyperactive behaviors and impaired cognitive development (O'Neal and Zheng, 2015). Similarly, Al exposure has been linked to different neurological diseases such as Alzheimer's disease and autism spectrum disorders (Shaw and Tomljenovic, 2013). However, when using the concentration of Al and Mn in this study to calculate the non-cancer risk for the highest exposed group resulting from apple juice consumption, the hazard quotient for both Al and Mn were less than 1 (Table 3.5). This means that Al and Mn in apple juice cause no significant risk for non-cancer effects or do not likely harm human health.

Apple juice is a primary ingredient not only in apple juice, ciders, and cocktails but also in other fruit blends such as grape and berry juice. Therefore, apple juice is a potential source of dietary toxic chemical exposure. Recently, arsenic exposure via fruit juice consumption has captured the attention of the public because children consume more apple juice relative to their body weight than adults do (Carrington et al., 2013). Children in the U.S. (6 -11 years old) drink approximately 1.6 L of fluid per day, which includes 0.46 L of plain water and 0.23 L of fruit juices (Wilson et al., 2012). In the current study, children from one to two years of age consumed the highest amount of apple juice per day per kg body weight basis (13.4 g/kg/day) among all age groups, so apple juice consumption by this age group was used to estimate risk of non-cancer effects. All of the estimated HQ values for Al, As, Cr, Cu, Mn were less than one, meaning that there was not an increased risk for non-carcinogenic health effects by these metals via drinking apple juice for mean of all samples and mean of quantifiable samples (Table 3.5). Similarly, total

risk of non-cancer effects (HI) was less than one for both mean values. Among these HQs, As and Cr were the highest contributors to overall risk which ranged from 30-41% for As and 25-29% for Cr. However, the overall HI estimated based on mean value of all samples and quantifiable samples were still lower than that calculated using the FDA maximum contaminant level for bottled water (HI = 1.3). Therefore, metal concentrations in the national apple juice brands assessed in this study had relatively low non-cancer risk.

Pb was not included in the risk assessment for non-cancer effects in this study because currently there is no reference dose value established for Pb. Even though frequency of detecting Pb and the mean Pb concentrations in apple juice and apple cider was not statistically different (Table 3.3), some apple cider samples contained Pb concentrations that were higher than the EPA standard for drinking water (15 µg/L) (Figure 3.2). However, there was no illness predicted due to lead in apple juice using the FDA-iRisk model to estimate disability adjusted life years (DALYs) with health endpoints of reduced intelligence quotient in children (0-6 ages) and hypertensive heart disease in adults (Almutairi, 2016). Disability-adjusted life year (DALY) estimates include both mortality and morbidity associated with illnesses and one DALY is equal with one lost year of healthy life across the population (WHO, 2014). Almutairi (2016) found the mean exposure of lead in these apple juice and apple cider samples was relatively low with no illnesses predicted. However, potential adverse effects associated with lead exposure cannot be ignored because its adverse effect depends on many factors such as duration and amount of exposure as well as age of the exposed individual (Almutairi, 2016).

The incremental lifetime cancer risks of As in this study was lower than the risk evaluation by Tvermoes et al. (2014) (2×10^{-5}) even though the same EPA cancer slope factor

was used. There are some differences between these studies. First, the database of apple juice consumption used for exposure estimates was different in both studies. In the current study, we used results from the U.S. EPA's What We Eat in America - Food Commodity Intake Database, 2005-2010 whereas the data from 2009-2010 National Health and Nutrition Examination Survey (NHANES) was used in Tvermoes study. Second, the average total As concentration (2.4-3.0 µg/L) of apple juices measured in the present study was lower than the average concentration of those in previous studies (5.3 µg/L) (Tvermoes et al., 2014). In addition to geographical factors and apple juice origin in these studies also affected the variation of As level in juices. These factors led to differences in the estimated chronic daily intake for the two studies, and consequently the estimated cancer risks were different. Similarly, the As level in this study was lower than the average total As concentration in the FDA report (5.2 µg/L) so the ILCR was lower than the risk evaluated by FDA (Carrington et al., 2013). In the cancer risk assessment for arsenic in apple juice report by FDA, Carrington et al. (2013) estimated eight cases per million people would develop urinary tract and lung cancer from exposure to inorganic arsenic. Carrington et al. (2013) used the slope factors for urinary tract and lung cancer, whereas we used the EPA oral slope factor developed from the prevalence of skin cancer (EPA, 1991). Those factors may lead to different ILCR of the current study and the FDA report. In general, according to EPA, the acceptable risk level ranges from 10^{-4} to 10^{-6} (WHO, 2001), so the ILCR estimated in this study for the group consuming the highest amount of apple juice would fall within the acceptable range as deemed by EPA.

The non-cancer risk and ILCR were not calculated for arsenic and other metals in apple cider because no specific data on apple cider consumption were available. However, we assumed that only a small amount of apple cider was consumed (we assumed 1% of total apple juice

consumption for children and 5% of total apple juice consumption for adult). Based on the risk evaluation of apple juice consumption in this study, we predict that arsenic exposure via apple cider is not likely to be harmful to human health.

There are limitations to the current analysis. We did not compare inorganic and organic juices, so the results may not be completely representative for retail juice products available nationally. The varieties of apples used for making apple cider were unknown and apple varieties used for cider production likely differ considerably among these cider mills. Therefore, it is difficult to identify which factors led to the differences in arsenic and lead levels among these samples. Furthermore, this study estimated the risk from inorganic arsenic exposure from apple juice consumption and did not account risk associated with arsenic exposures via other food, water and soil.

CONCLUSIONS

The current study found that arsenic was detected less frequently in apple cider samples than in commercial shelf stable apple juices. Most of the As concentrations detected in this study were less than the proposed action level indicated by FDA for inorganic As in apple juice (10 μg/L). In addition, all of juice samples in the present study contained lead at concentration less than 30 μg/L. It is important to note that the U.S. FDA has not established a maximum level for lead in fruit juices. However, the Codex Alimentarius Commission adopted a maximum level of lead in fruit juices of 0.03 mg/kg (roughly equal to 30 μg/L) during the 38th session of the Codex Alimentarius Commission in 2015 (Codex, 2015). Although Codex standards are not necessarily binding, they do represent the best current advice regarding maximum lead levels in juice products in international commerce. Additionally, the metals in apple juice and apple cider do not pose a significant risk to human health based on the levels of the metals detected and the

anticipated average daily intakes. Moreover, the evaluation of arsenic levels in fruit juices may support guidelines published by FDA on the proposed action level for arsenic in apple juice.

CHAPTER 4. RELATIONSHIP OF METAL CONCENTRATIONS IN SOIL AND THAT IN FRUIT AND LEAVES OF APPLE TREES AT SELECTED ORCHARDS IN MICHIGAN

ABSTRACT

Historically, lead-arsenate pesticides were commonly used in fruit orchards. Residues of metals from this historical use can persist in soils for decades, which can result in potential risk for humans if they consume fruits grown on the contaminated soil. This research was conducted to assess lead and arsenic levels in apples, leaves and orchard soils where the apples were grown to determine the relationship between metal levels in fruits and fruit products vs. orchard soils. Soil and tree tissue samples were collected from several Michigan farms and then were analyzed using microwave extraction and inductively coupled plasma mass spectrometry (ICP-MS). Soil samples were taken at depths of 0-20 cm and 20-40 cm at a distance of one meter from the tree trunk. Fruit samples were also processed into juice and pomace fractions to assess the partitioning of arsenic and lead during juice processing. Lead concentration was significantly higher in topsoil (9.4 µg/kg) as compared to that in the subsoil (6.9 µg/kg), but arsenic content did not differ between the two soil layers ($P>0.05$). Lead concentrations in apple leaves were correlated with lead in topsoil (0-20 cm) ($P=0.03$). Concentrations of total arsenic in all juice samples were less than 1 µg/L and showed less potential than lead for uptake and translocation to fruits. There was no significant relationship between soil arsenic content and the content in juice, pomace and leaf samples ($P>0.05$). Results of this research indicate that lead and arsenic concentrations in apples and apple products from Michigan orchards are unlikely to be impacted by the contamination of these metals in orchard soils.

INTRODUCTION

Arsenical pesticides have been used in agriculture for centuries. The earliest arsenical insecticide used was arsenic sulfide in China as early as 900 A.D. The copper acetoarsenite known as Paris green was first used in 1867 to control Colorado potato beetle in the USA. Fruit growers soon adopted this insecticide to control codling moth (*Cydia pomonella*) in apple. Among arsenical insecticides, lead arsenate was the most extensively used during the 20th century. Lead arsenate was used globally because of its long lasting pesticidal effect, as it can adhere well to the surface of plants (F. Peryea, 1998). Lead arsenate was preferred for control codling moth on apples due to its high efficacy and low phytotoxicity. It also was used on other fruit species, garden crops and turf grasses. However, in 1919, fear and concern about the risk of excessive residues of lead and arsenic on fruits and vegetables arose. It was found that existing practices failed to adequately remove arsenic (As) residues while washing produce (Codling, 2011a; F. Peryea, 1998).

All lead arsenate-based insecticides were officially banned on 1 August 1988 in the USA (F. Peryea, 1998). However, residues from lead arsenate use persist in orchard soils for decades and can contaminate produce grown in these soils. The levels of arsenic and lead in orchard soils vary depending on the duration, the amount and the frequency of pesticide applied (Schooley et al., 2008). The concentrations of arsenic and lead in lead-arsenate contaminated soil is a concern because these metals could pose a potential risk to humans if high concentrations are incorporated into produce from these orchards.

A few studies have evaluated the concentrations of arsenic in fruit juices and fruit beverages in the U.S. (Roberge et al., 2009; Tvermoes et al., 2014; Wilson et al., 2012).

However, none of these studies have identified the source of the fruits used to manufacture the juices and/or assessed the metal levels in orchard soils where the apples were grown. This is an important knowledge gap as soil As and Pb concentrations vary significantly depending on location due to natural variation in soil concentrations of these metals as well as anthropogenic activity such as the intentional application of lead arsenate. We are unaware of any prior research that has determined the relationship of lead and arsenic concentrations in orchard soils, fruit, and the resulting juice produced from that fruit. Therefore, it is important to determine if the concentrations of these two heavy metals in fruits and tree tissues are correlated with their concentrations in orchard soils.

The objectives of this study were: (a) to assess the distribution of Pb and As in the soil at different depths; (b) to evaluate the distribution of these metals in the juice and pomace fractions of apple fruits; and (c) to determine the relationship between the concentrations of these metals in orchard soils and tree tissues.

MATERIALS AND METHODS

Soil sampling

Soil samples were obtained from orchard plots that differed in date of establishment: (1) "old" orchard plots established prior to 1988, and (2) "new" orchard blocks established after 1988. All samples were collected during the 2016 season. The selection of 1988 as a break point for characterizing the relative age of the orchard plots was based on the year in which lead arsenate was banned as a pesticide in the U.S.

Four orchards in Michigan were selected for sampling. Since orchard plots were of different sizes, each plot was sub-divided into three-acre sampling areas. In each sampling area of the orchard plots, two trees were selected randomly for sampling. Soil samples were taken to a

depth of 40 cm at a distance one meter from the tree trunk using a core sampler (Oakfield Apparatus, Fond du Lac, WI). Each core sample was divided into two fractions based on soil depth; 0-20 cm and 20-40 cm. Four core samples of soil were taken under each tree. These four core samples were combined to produce composite samples. The core samples were separated by depth, mixed thoroughly in a clean bucket, and soil sample bags were filled with subsamples (200 g). The longitude and latitude coordinates corresponding to each sample were collected as records to uniquely identity each plot. Soil samples were air-dried at 37 ^{o}C in 24 hours, then rolled and sieved through a 2-mm sieve prior to analysis.

Tree tissues

Samples of leaves and fruits were collected from the same trees used for soil sampling. Forty leaves and six to eight mature apple fruits were collected from each tree at the same time that soil sampling was completed. The variety of each apple tree sampled was recorded. Fruits and leaves were rinsed under distilled water to minimize metal contamination from dust. The leaf samples were dried at 70 ^{o}C (24 hours) in a forced-air oven, ground in a coffee mill (Hamilton Beach; Glen Allen, VA, USA), and then stored in a container for analysis. Apples, including peels and cores, were sliced and sub-samples (2-4 g triplicate samples) were used to determine moisture content by drying for 24 h at 100 ^{o}C. The remaining apple material was juiced by using a juice extractor (Acme 6001, Waring Commercial, USA) and separated into two fractions: juice and pomace. These samples were stored at 4 ^{o}C until analysis for metal concentrations; samples were typically prepared for analysis on the same day.

Determination of total arsenic and lead in soils and tree tissues

The juice samples were analyzed for metals directly by inductively coupled plasma – mass spectrometry (ICP-MS) (Model 7500ce; Agilent Technologies Inc., IL. USA) while the

soils, apple leaves, and pomace samples (after being dried at 70 ^{o}C in a forced air oven) were extracted by microwave digestion (Anton Parr Multiwave 3000, Ashland, VA, USA) and analyzed for metal concentrations by ICP-MS. The dried, finely powdered soil or sediment sample (0.2-1.0 g) was weighed into a dry, clean Teflon digestion vessel and 6 mL of 1:3 nitric acid: hydrochloric acid (Sigma – Aldrich, MO) was added. The vessel was closed, placed into the rotor, and tightened. The loaded rotor was then placed into the microwave oven. The microwave conditions for digestion were: stage 1: microwave power 1200 W, 300 PSI, and ramp for 2 min; stage 2: microwave power 1200 W, 300 PSI, and ramp for 3 min followed by a 5-min hold. After cooling for 30 min, the vessels were opened carefully. The cooled digests were diluted to 20 g with deionized water (Rahman et al., 2009). The samples were filtered using Whatman Grade 42 filter paper before analysis by ICP-MS. The ICP-MS analyses were conducted at the Michigan State University (MSU) Diagnostic Center for Population and Animal Health. The limit of quantification (LOQ) for lead and arsenic was 1 µg/kg. Data for metal concentrations in soil and leaves were reported as mg/kg dry weight basis.

Organic matter and pH measurement

Organic matter and pH in the soil samples were analyzed by the Soil and Plant Nutrient Laboratory at MSU. Total organic matter was determined based on loss on ignition by heating dried and ground soil (5 ± 0.001g) in a muffle furnace at 360 ^{o}C for 2 hrs. The samples were allowed to cool to room temperature prior to weighing. pH in soil was determined by adding 5 grams of soil to 10 mL of distilled water. pH was then measured using glass and reference pH electrodes, or a combination electrode connected to a pH meter. The pH meter was calibrated with pH 7.0 and pH 4.0 buffers before analyzing samples.

Bioaccumulation factor

Bioaccumulation factor (BAF) is a ratio of metal concentration in the edible parts of plant ($C_{plant\ tissue}$) to the concentration of metal in soil (C_{soil}) in mg/kg (Boim et al., 2016; Oti, 2015). It was calculated as follows:

$BAF = C_{plant\ tissue} / C_{soil}$

Where $C_{plant\ tissue}$ is the concentration of metals in apple fruit which was estimated using concentration of lead and arsenic quantified in apple juice and apple pomace; and C_{soil} was calculated by taking the mean of the metal concentration of topsoil and subsoil in this study.

Statistical Analysis

Three replicates per sample were analyzed for all of the experiments. Data were statistically analyzed using the REG and MIXED procedures of SAS, version 9.4 (SAS Institute Inc., Cary NC, USA). Least square means were compared using the Fisher Least Significant Difference method and were considered significant when P-values were less than 0.05. In instances where As concentrations determined by ICP-MS were detectable but less than the LOQ (1 µg/kg)**,** 0.5 µg/kg was used as a default value (EPA, 2000).

RESULTS

Total arsenic and lead in soil and apple tissues.

Lead (Pb) concentration was higher than arsenic (As) concentration in the soils and there was a significant difference in Pb concentration depending on soil depth (Table 4.1). Lead concentrations in topsoil (9.4 mg/kg) was higher than that in subsoil (6.9 mg/kg) ($P < 0.05$); whereas arsenic concentration was similar in topsoil and subsoil (Table 4.1). Lead concentration was higher than arsenic concentration in leaves, juice and residue. Most juice samples pressed from fresh apples contained less than one µg/kg of As.

Table 4.1. Concentration of lead and arsenic (mg/kg) in soil samples and apple tissues obtained from selected orchards in Michigan (N=20).

	Topsoil[1] (0-20 cm)	Subsoil[1] (20-40 cm)	Leaves[1]	Juice[2]	Residue[2]
Lead	9.4 ± 0.67 [a]	6.9 ± 0.67 [b]	0.23 ± 0.01 [*]	0.0061 ± 0.001[*]	0.013 ± 0.001[*]
Arsenic	2.9 ± 0.48	3.2 ± 0.48	0.067 ± 0.01[**]	<0.001 [**]	0.007 ± 0.0004[**]

Result are reported as mean ± SEM (n=20). LOQs for Pb and As were 0.001 mg/kg.
[*,**] Means in the same column with different superscripts are significantly different (P<0.05).
[a,b] Means in the same row with different superscripts are significantly different (P<0.05).
[1] Data were reported as mg/kg dry weight basis.
[2] Data were reported as mg/kg wet weight basis.

The concentrations of As and Pb detected in samples of topsoil and subsoil at new and old orchard plots are presented in Figure 4.1. The lead level in all soil samples was higher than that of arsenic in both new and old orchard plots. Lead concentrations in both topsoil and subsoil were significantly higher in old plots as compared to the new plots. Conversely, there was no significant difference in arsenic concentration between old and new blocks for either soil depth (Figure 4.1).

Apple fruits were processed into juice and pomace fractions. The pomace was dried before analysis for arsenic and lead concentration. The distribution of lead and arsenic in the juice and pomace fractions is presented in Figure 4.2. Lead partitioned more in the juice portion (64.7%) as compared to the pomace (35.3%); whereas arsenic partitioned more in pomace 60.7%) than in juice (39.3%).

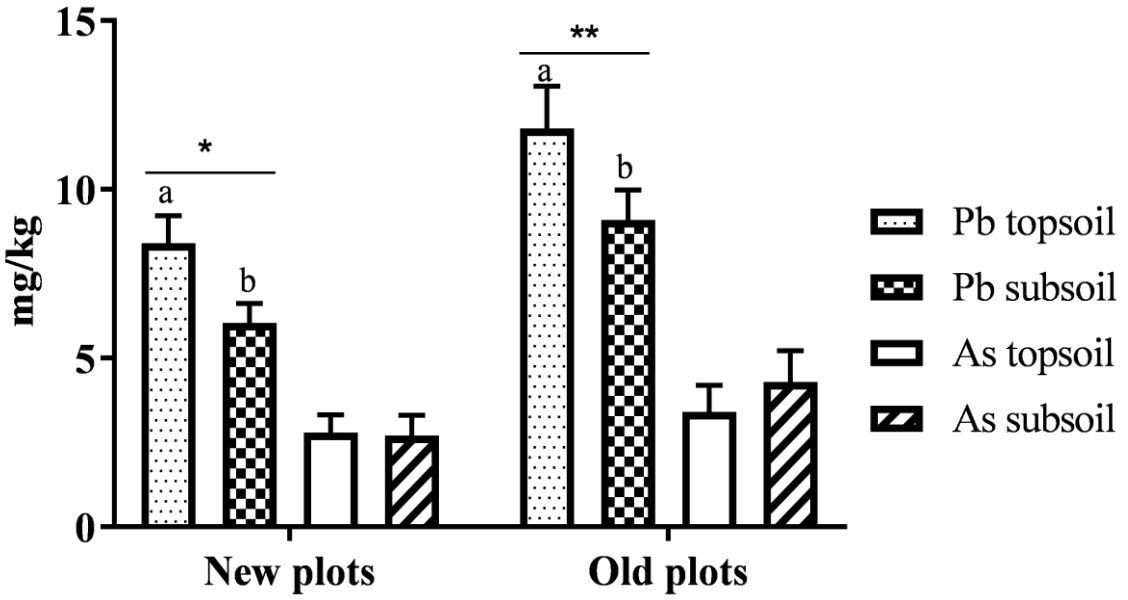

Figure 4.1. Concentration of lead and arsenic in soil in old and new orchard sites in Michigan. (n=20). Data were reported as mg/kg dry weight basis. The topsoil is soil from 0-20 cm depth and subsoil is from 20-40 cm depth. New plots were established after 1988, and old plots were established prior to 1988. ^{a,b} ($p < 0.05$) vs topsoil + subsoil. $^{*, **}$ ($p<0.05$) vs new + old plots.

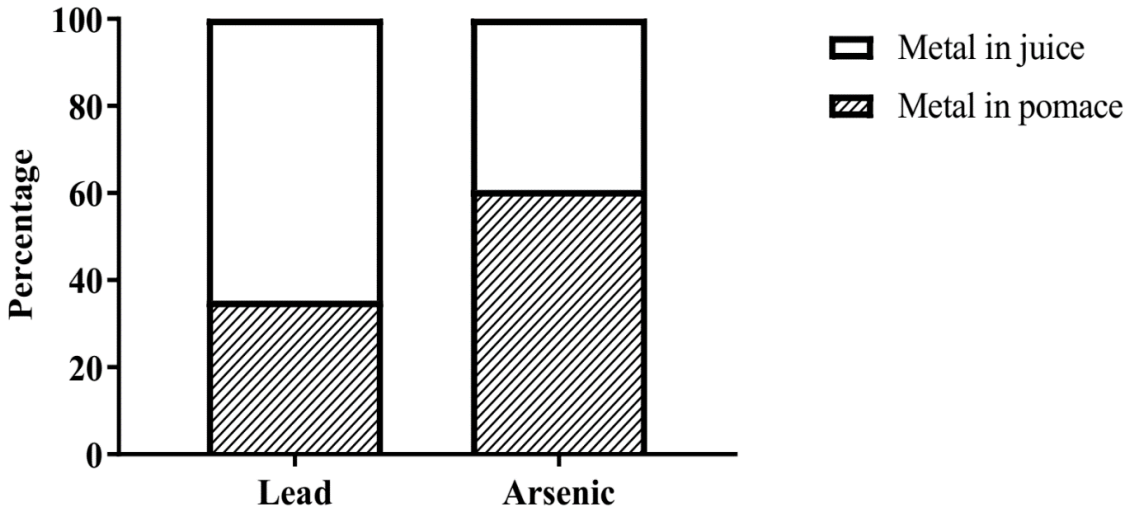

Figure 4.2. Lead and arsenic distribution in apple juice and pomace fractions.

The concentrations of lead and arsenic in apple juice and pomace analyzed by ICP-MS were used to estimate the concentration of these metals in the apple fruits based on the weight of juice and pomace portions as compared to original weight of apples (Figure 4.3). Similar to the distribution of lead and arsenic in soil, the lead level (7.6 µg/kg) was higher than that of arsenic (2.1 µg/kg) in whole apple fruits. However, there were no significant differences in lead or arsenic concentrations in apples sampled from old or new orchard plots (Figure 4.3). The concentrations of lead and arsenic in apples were much less than that allowed by the U.S. EPA for these metals in drinking water, which is 10 µg/kg for arsenic and 15 µg/kg for lead.

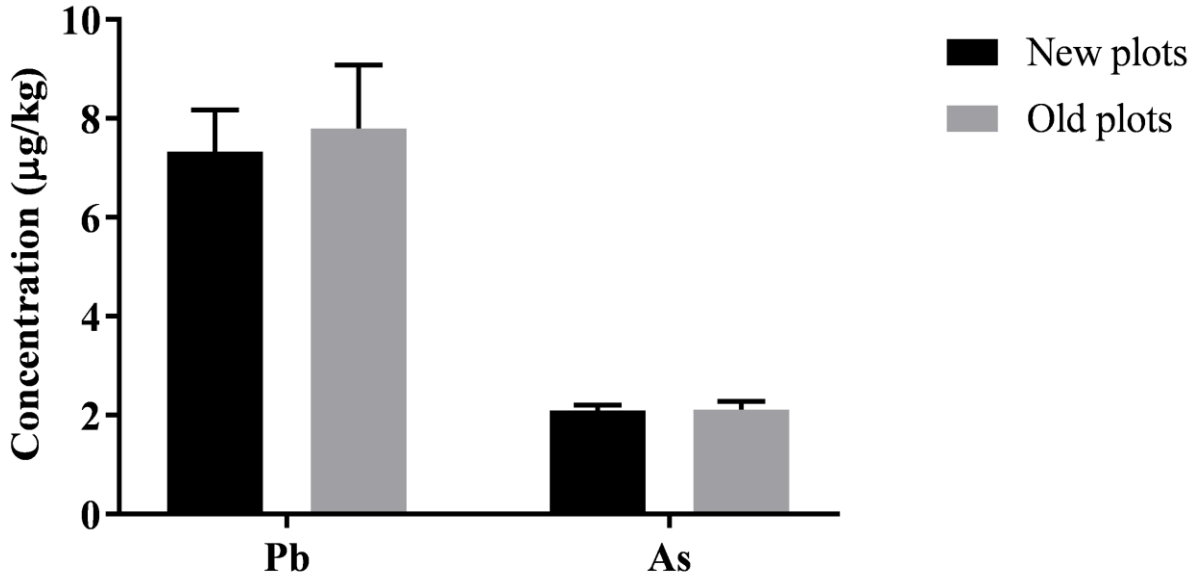

Figure 4.3. Concentration of lead and arsenic in apples obtained from old and new orchard plots. Data were reported as mg/kg wet weight basis.

Organic matter and pH measurement

Average organic matter and pH values in the topsoil and subsoil samples are presented in Figure 4.4. There was a significant difference in organic matter concentration in soil samples obtained from the two depths. Organic matter was 2.6% and 1.6% in topsoil and subsoil,

respectively. Mean pH of topsoil samples was significantly higher than that observed in subsoil. The pH of topsoil ranged from pH 7.0-7.6 whereas the pH of subsoil ranged from 5.5-7.4.

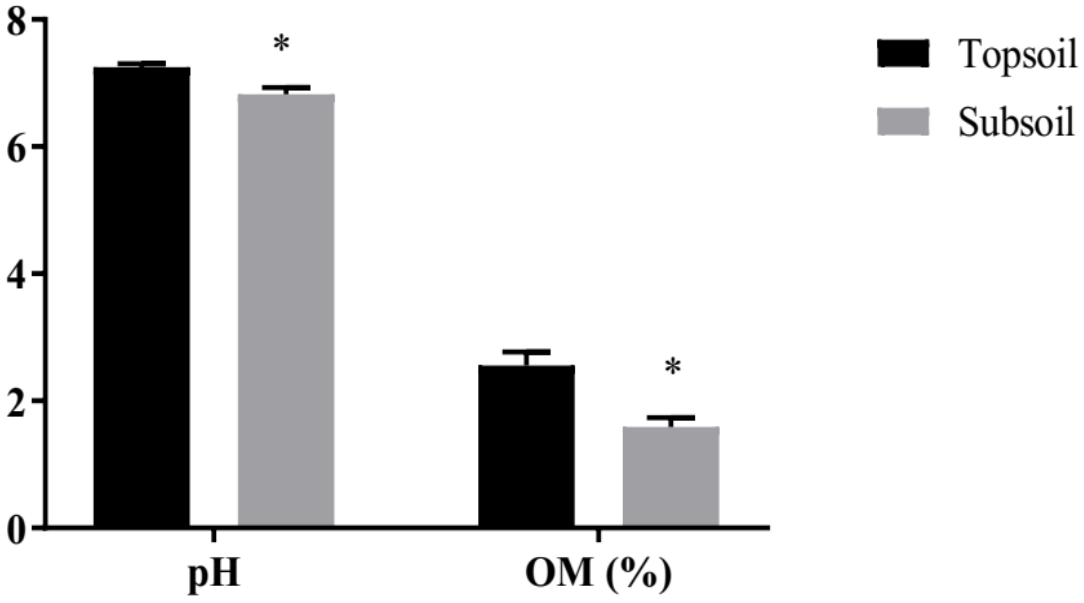

Figure 4.4. Organic matter (OM) concentration (% of dry matter) and pH levels in apple orchard soils. * Means in each category are statistically different ($P < 0.05$).

Bioaccumulation factor

BAFs of As and Pb were equally low (0.01). BAF less than 1 indicates that a plant is characterized as an excluder, whereas plants are categorized as accumulators and hyper-accumulators if the BAF value is higher than 1 and 10, respectively (Bu-Olayan and Thomas, 2009).

Regression Analysis

Linear regression analysis was conducted to assess potential relationships among lead and arsenic concentrations in soil, leaves, juice and pomace. No significant relationship was

detected between soil As concentrations and that found in leaves, juice or pomace (Table 4.2). There was a statistically significant relationship between Pb level in soil and in leaves, as well as for Pb level in juice and pomace. However, these relationships were relatively weak as the r^2 for the regression analysis was approximately 0.2 for both (Table 4.2).

Table 4.2. Linear regression equations describing relationships between arsenic and lead concentrations in soil, leaves, juice, and pomace (residue).

	Regression equation	**R^2**	**P**
Arsenic	$As_{leaf} = 0.07 - 0.0002\ As_{soil1}$	0	0.97
	$As_{leaf} = 0.08 - 0.003\ As_{soil2}$	0.02	0.54
	$As_{residue} = 0.006 + 0.02\ As_{leave}$	0.16	0.08
	$As_{residue} = 0.006 + 0.00008\ As_{soil1} + 0.0001\ As_{soil2}$	0.07	0.6
Lead	**$Pb_{leaf} = 0.16 + 0.007\ Pb_{soil1}$**	**0.24**	**0.03**
	$Pb_{leaf} = 0.19 + 0.007\ Pb_{soil2}$	0.1	0.16
	$Pb_{juice} = 0.007 - 5E\text{-}05\ Pb_{soil1}$	0	0.81
	$Pb_{juice} = 0.007 - 7E\text{-}05\ Pb_{soil2}$	0	0.82
	$Pb_{juice} = 0.003 - 0.0002\ Pb_{soil1} - E\text{-}05\ Pb_{soil2} + 0.006\ Pb_{leaf} + 0.27\ Pb_{residue}$	0.24	0.35
	$Pb_{juice} = 0.005 + 0.005\ Pb_{leave}$	0.01	0.75
	$Pb_{juice} = 0.003 + 0.27\ Pb_{residue}$	**0.22**	**0.04**
	$Pb_{residue} = 0.01 + 0.0002\ Pb_{soil1} + 0.00006\ Pb_{soil2}$	0.03	0.79

Bold: Significant effect ($P < 0.05$)

DISCUSSION

Lead arsenate was first used in apple orchards to prevent codling moth infestation in the 1890s. It was used for approximately 100 years and even though it was banned in the United

States in 1988, the persistence of residual lead and arsenic resulting from the use of this pesticide in soil still raises questions about its potential risk to human and environmental health across the United States (Hood, 2006; Schooley et al., 2008). Michigan consistently is among the top four largest apple producing states in the U.S and lead arsenate was used extensively in Michigan orchards in prior decades. This long history of lead arsenate pesticide use in apple orchards has the potential to contribute to the buildup of arsenic and lead concentrations in orchard soils. This study assessed the distribution of these heavy metals in a subset of apple orchards in Michigan and also assessed the relationship between the concentrations of these metals in orchard soils and tree tissues.

The higher concentration of Pb in the topsoil relative to the subsoil is similar to that in previous studies of soil Pb concentrations in soils having a history of lead arsenate use. Jones and Hatch (1937) reported that total Pb and As concentration in the top 20 cm of soil was higher than the subsoil (20-40 cm) in 21 commercial orchards in Oregon (Jones, J. S.;Hatch, 1937). Later, Peryea and Creger (1994) found similar trends in soil lead concentrations. However, the concentrations of lead and arsenic observed by Peryea and Creger (1994) were much higher than those in our current study. Peryea and Creger (1994) reported Pb concentrations ranging from 445 to 2,213 mg/kg and As concentrations ranging from 57.8 to 363.8 mg/kg (Peryea and Creger, 1994). It is possible that the concentrations of Pb and As found by Peryea and Greger (1994) were much higher because their research was completed shortly after lead arsenate use as a pesticide was banned in the U.S. in 1988. Peryea and Greger (1994) assessed six contaminated orchards in the state of Washington, which had different spraying application rate per season and geographical location. By contrast, a study conducted in New York state reported that Pb concentration ranged from 1.48 to 720 mg/kg dry weight and As concentration ranged from 1.6

to 141 mg/kg (Merwin et al., 1994); concentrations that were intermediate between those of Peryea and Creger and our observations.

The lack of a difference in As concentration between two soil depths may be due to a low solubility and lower mobility of Pb in soils as compared to arsenic. It has been demonstrated that As is more soluble than Pb so it can move through the soil profile regardless of the soil type (Codling, 2011b; F. Peryea, 1998; Peryea and Creger, 1994). In summary, the Pb and As concentrations observed in the orchard sites in Michigan were considerably lower than those reported in earlier studies in other states. The metal concentrations observed in the present study fall into "background level" of Michigan soils. In Michigan, background levels for arsenic found in soil range from <0.3 to 22.8 mg/kg and <0.4 to 38.9 mg/kg for lead (MDEQ, 2005).

After 1914, lead arsenate pesticides were commercially manufactured in two formulations: acid lead arsenate and basic lead arsenate. The Pb/As concentration ratio is 1 for acid lead arsenate ($PbHAsO_4$) and 1.67 for basic lead arsenate [$Pb_4(PbOH)(AsO_4)_3$] (Peryea and Creger, 1994). However, in this study, we found that the average Pb/As ratios in soil ranged from 2 to 7 (data not shown) which is higher than the original ratio of the commercial pesticide. We speculate that as arsenic leaches through the soil profile over time (as compared to lead, which is relatively static in soil), the Pb/As ratio increases compared to the original ratio of the pesticide. Peryea et. al. (1994) found that the Pb/As ratio was reduced when the soil depth increased because of reduction of lead level and increase of arsenic level by leaching when the soil depth increased (Peryea and Creger, 1994). However, this ratio also can be affected by the natural presence of these metals in the soils.

The hypothesis of this study was that concentrations of lead and arsenic would be higher in orchard plots established prior to 1988. We observed no difference in As levels when

comparing old and new orchard plots, but the Pb level was higher in both the topsoil and subsoil layers in old plots compared to new plots (Figure 4.1). This suggests that Pb is a better indicator for tracing lead arsenate pesticide accumulation because As is more mobile than lead in soils (Elfving et al., 1994).

The highest concentrations of lead were detected in topsoil (0-20 cm), which also has higher organic matter and higher pH levels compared to subsoil. There are many factors that can affect metal mobility, including soil composition, pH, soil redox state, metal speciation and application of phosphate fertilizers, etc. (Weber and Hendrickson, 2006). The low mobility of Pb may be related to its stable Pb^{2+} form in the soil, which can bind with organic matter and thereby contribute to Pb accumulation in the topsoil. In addition, the subsoil has lower pH than the topsoil, which can reduce the sorption of most trace metal forms (Rieuwerts, 2007). However, this explanation may not apply for As since there was no difference in arsenic concentration for the two soil depths. Arsenic may form soluble complexes with organic matter, enhancing As solubility and increasing the leaching of As (Wang and Mulligan, 2006).

As and Pb concentrations in tree tissues were consistently low compared to the concentration of these metals in soil (Table 4.1), suggesting that soil As and Pb had low phytoavailability. As and Pb concentrations in apple fruits were lower than in leaves, consistent with previous studies observed in fruit trees grown on lead arsenate-contaminated soil (Creger and Peryea, 1992; F. J. Peryea, 1998; Peryea, 2002). Lead and arsenic concentrations in apple fruits did not differ among apple trees planted in old and new plots (Figure 4.3). This observation is consistent with other studies showing that fruit trees grown in lead- or arsenic-contaminated soil without serious phytotoxicity and did not contain hazardous residues (Creger and Peryea, 1992; Merwin et al., 1994).

After processing apples into juice and pomace fractions, we found that the majority of lead was present in the juice fraction whereas the majority of arsenic was present in the pomace (Figure 4.2). We are unaware of any other research on the fractionation of lead and arsenic in juice and pomace fractions of apples. This observation is relevant to processors of commercial apple juice and apple juice concentrate. This result also was consistent with the results of a market basket survey of nearly 200 apple juices and apple ciders around Michigan, wherein we observed that lead was present in more samples with higher concentration than arsenic (45% vs. 27%; Cao and Bourquin, 2017). However, Wilson et al. (2012) found that arsenic was present in more samples as compared to lead across all types of juices. There are some differences among these two studies. First, in the later study, most of the samples were national brand labels and diluted from fruit concentrate procured from several countries, predominantly the U.S., China, and Argentina but also from Austria, Brazil, Chile, Germany, New Zealand, and Turkey. Since soil metal concentrations can vary considerably from region to region, and several factors can influence the extent to which metals in soil are taken up by plants, it is expected that juice products originating from different countries would have considerable variation in metal concentrations. Second, Wilson et al. (2012) tested metal concentrations in juice samples, but could not determine the relationship of lead and arsenic concentrations in these products with that in soils from their points of origin (level of heavy metal in fruits and fruit products vs. these in orchard soils) (Wilson et al., 2012).

To evaluate the metal translocation from soil to apple tree tissues, regression analyses were conducted to assess the relationships between the concentrations of lead and arsenic in soil, leaves, juice and pomace. This analysis could not consider As in juice because all of the juice samples prepared in this study had arsenic concentrations lower than the LOQ (1 µg/L). We

observed no significant relationship between As concentration in soil and all tree tissues (Table 4.2). There were statistically significant and positive correlations between the leaf lead content and the lead content in topsoil samples and those in juice and pomace. The concentration of lead in leaves increased when concentration of lead in the topsoil increased. Collectively, these results demonstrated that there was no correlation of metal concentrations in fruit and in soil in the present study. In contrast, previous studies showed that arsenic concentrations in apricot and Gala apple fruits were positively related to soil arsenic levels (Creger and Peryea, 1992). These differing results might be partially due to different methods used for soil extraction prior to metal analysis. In the present study we extracted metals in soil samples using HNO_3 whereas Creger and Peryea (1992) used HCl for soil extractions. In a study of five soil testing methods, Peryea (2002) found that different extraction methods can affect the measured concentration of metals in soil. Secondly, fruit cultivars can vary in their potential extent of metal translocation, leading to differing concentrations of metals in the edible fruit tissues. Finally, and perhaps most importantly, the concentrations of lead and arsenic in the fruit and soil reported by Creger and Peryea (1992) were much higher than that in our current study. The larger range of metal concentrations reported by Creger and Peryea (1992) would result in a greater likelihood of detecting a significant relationship between soil and fruit metal concentrations using regression analysis.

The bioaccumulation factors of As and Pb calculated from this research were very low. This indicates that the mobilization of trace metals from soil through the roots to the edible fruits was negligible and apple trees were not an accumulator for lead and arsenic. Previous research has shown that tree fruits can tolerate Pb and As when grown on soils containing relatively high concentrations of these heavy metals (Merwin et al., 1994). However, this factor may vary

depending on the concentration of these metals in soil and with different cultivars. Therefore, it would be interesting to carry out more research to assess metal uptake by different fruit varieties and in different geographies.

There are some limitations to this study. The results of this research might not reflect the impact of prior pesticide use if the orchard soils have been disturbed by plowing or otherwise mixing the soil using tillage implements prior to planting. Historical records of pesticide sprays were not available for the plots used in this research, so it is impossible to know exactly the amount of arsenic or lead applied in the past.

In conclusion, this is the first study characterizing the relationship between metal concentrations in apple tree tissues and orchard soils in Michigan. The results showed that apple trees were not accumulators of lead and arsenic. Moreover, the distribution of arsenic and lead in orchard soils was different depending on soil depth increments. However, arsenic and lead were present at relatively low concentrations both in the soil and tree tissues. Results of this research indicate that lead and arsenic concentrations in apples from Michigan orchards are unlikely to cause harm to human health.

CHAPTER 5. SUMMARY AND FUTURE STUDIES

SUMMARY

Human can be exposed to arsenic by different sources via consumption of contaminated food, water, air and occupational exposure, which can cause harmful effects on human health. However, there are several potential factors can affect arsenic toxicity in human such as its chemical properties, arsenic concentration, its bioavailability in human. The results from this research support the idea that human absorption of arsenic is dependent on the species of As, in which As (III) had the greater absorption and cellular accumulation than As (V). In addition, we demonstrated that it is important to understand any potential health risks posed to human via arsenic-contain food. This is especially critical in children because children consume more apple juice relative to their body weight than do adults. The risk assessment carried out in this project demonstrate that there are relatively low risks for children from arsenic and other metal exposure via apple juice consumption. The results from this project suggest that apples grown on arsenic-contained soil from Michigan orchards are unlikely cause harm to human health.

FUTURE DIRECTIONS

Studies to explore the effect of co-culture of Caco-2/HT29-MTX cells and food matrix on metabolism of inorganic arsenic

In chapter 2 of this dissertation, we found that As (III) uptake by Caco-2 cells grown on Transwell inserts and transfer of As (III) from the apical to basolateral side of these inserts was favored over that of As (V) uptake and transfer in this model. Using this model, we found that As (III) is more available for absorption. However, the *in vivo* intestinal tract not only contains

enterocytes, but also mucus secreting cells, macrophages, dendritic cells, and other lymphocytes. These cells can affect bioaccumulation and transformation of inorganic arsenic which can either promote or reduce As toxicity. Therefore, it would be interesting to conduct a similar experiment using a model of co-culture of Caco-2/HT29-MTX cells because the HT29 cells produce mucus which can create another barrier that would potentially hinder As uptake (Kleiveland, 2015). During co-cultivation of these two cells lines, HT29-MTX will secret a mucin layer on the top of the epithelial cells which would more closely represent the normal intestinal epithelium, enterocytes and goblet cells (Behrens et al., 2001; Kleiveland, 2015).

Second, in the present experiment we used aqueous standards of As, so we did not account for the food matrix interaction. It would be interesting to conduct similar experiments using apple juice to evaluate the effect of arsenic binding with other components in apple juice on the transport of As into the monolayer. It has been shown that bioaccessibility values of pure As standards were significantly higher than that in rice-bound As, because inorganic As bound tightly to thiol groups of rice protein, limiting its release from the matrix (Alava et al., 2013). Therefore, it would be useful to investigate the bioavailability of inorganic arsenic in apple juice vs. apple cider because they differ in fiber content.

Studies to understand if the reconstitution procedure of apple juice from juice concentrate increases the arsenic level

The results of the study presented in chapter 3 demonstrated that the metals in apple juice and apple cider do not pose a significant risk to human health based on the levels of the metals detected and the anticipated average daily intakes of apple juice. Furthermore, As, Al, and Cr were detected less frequently in apple cider samples than in commercial shelf stable apple juice. However, there was no explanation for this difference between these juice types. In 2011, FDA

published data on arsenic concentrations in apple juice concentrate from 2005-2011 as a part of the Toxic Elements Food and Foodware Program, in which total arsenic concentration ranged from 1-236 µg/kg (FDA, 2011). These apple juice concentrate samples were imported from different countries such as Argentina, Brazil, China, Chile, Mexico, and Turkey, so it is possible that juice concentrate is the predominant source for these metals in shelf stable juices. It would be useful to collect additional samples of juice concentrate and analyze for metal levels. Besides collecting these samples of apple juice and juice concentrate from the retail stores, we also propose to collect shelf-stable juice samples from manufacturers in Michigan during processing as well as the juice concentrates and water used to reconstitute the juices. This approach would enable us to assess levels of metals contributed by concentrate versus water to the finished products.

Studies to evaluate the effect of apple cultivars on the accumulation of arsenic and other metals in apples.

The study in chapter 4 demonstrated that arsenic and lead were present at relatively low concentrations both in the soil and tree tissues in Michigan orchards. Moreover, the distribution of arsenic and lead in orchard soils was different depending on soil depth. However, the numbers of the orchards examined in the present study were limited and, therefore, may not be fully representative of all apple orchards in Michigan. It would be useful to expand this study to include several additional orchards geographically distributed throughout Michigan in order to improve our confidence that the results are consistent across Michigan orchards. Secondly, in this study, the varieties of apples were ignored when comparing the accumulation of arsenic and lead in the fruits. Therefore, we propose to expand this research by also systematically examining the impact of apple varieties on metal uptake and distribution. In particular, the apple

cultivars most commonly used for producing apple cider and apple juice concentrate should be assessed. In previous studies, Creger and Peryea (1992) demonstrated that arsenic phytotoxicity symptoms of apple and apricot trees were species and cultivar-specific (Creger and Peryea, 1992). Consequently, this may affect to the concentration of the metals in the tree fruits because some cultivars may be tolerant to As exposure and have no phytotoxicity symptoms even though they may take up high amounts of As.

APPENDICES

APPENDIX A. CELL PROLIFERATION AND CYTOTOXICITY ASSAY

The number of viable cells was determined by using Cell Counting Kit-8 (CCK-8) assay (Dojindo, Maryland, USA). This assay measured the amount of the formazan dye generated by dehydrogenase in cells which is directly proportional to the number of living cells.

Cytotoxicity of inorganic arsenic was determined by cytotoxicity LDH assay (Dojindo, Maryland, USA). The cytotoxicity (%) was determined by measuring lactate dehydrogenase (LDH) activity release from damaged cells in apical medium. The oxidation of the coenzyme nicotinamide-adenine dinucleotide (NADH) by the LDH released in the presence of an electron mediator will produce an orange formazan dye, which is proportional to that of released LDH into the medium as an indicator of cytotoxicity.

The Caco-2 cells was were seeded onto polycarbonate Transwell inserts (Corning; Corning, NY) of 24 mm diameter and 0.4 μm pore size at a density of $5x10^4$ cells/cm^2. The Transwell inserts were placed into six well plates dividing the apical from the basal compartments. A total of 1.5 mL medium was added to the apical compartment and 2 mL medium to the basolateral chamber. Media was changed every 48 h.

The experiments were conducted with cells grown on filters for 15-21 days after seeding. Before the arsenic exposure experiment, the medium was aspirated from the apical and basolateral chambers and the cell monolayers were washed three times with phosphate buffered saline (PBS from Sigma Aldrich; St. Louis, MO) at a temperature of 37 ^oC and pH of 7.4 to remove any unattached cells. Afterward, 1.5 mL of DMEM media with different concentrations of As (III) (added in the form of As_2O_3) and As (V) (added in the form of H_3AsO_4) was added to each apical chamber and 2 mL of fresh medium containing no arsenic was added to each

basolateral chamber. The concentrations of As_2O_3 and H_3AsO_4 used for this experiment were 0, 0.01, 0.1, 1, 10, 20, 50 mg/L.

After 4-hour exposure with arsenic, 100 µL of the apical media from each well was added to clear 96-well plate, following by adding 100 µL of Working Solution and incubation for 30 minutes at room temperature. After incubation, 50 µL of the Stop Solution was added to each well. The plates were measured for absorbance at 490 nm using a microplate reader (PerkinElmer, MA, USA). 100 µL of the cells without exposure to arsenic were lysed with Lysis Buffer, and the supernatant after centrifugation was used as positive control for the cytotoxicity assay. The results of this assay are presented in Figure A5.1.

The cell surface of the monolayers in the Transwell system was washed three times with PBS, detached with trypsin-EDTA (from Sigma-Aldrich), and recovered with 0.5 mL of DMEM media. 100 µL of cell suspension was added in each well of 96-well plate, following by 10 µL of the CCk-8 solution. The plate was incubated for 2 hours in the incubator (at 37 ^oC, 5% CO_2) and the absorbance was measured at 450 nm using a microplate reader. The results of this assay are presented in Figure A5.2.

After 4 hours exposure to As (III) and (V), we observed no LDH release to the media, indicating that the two inorganic As species did not cause cell damage or cell death in this experiment. However, these arsenic compounds significantly affected cell proliferation. When Caco-2 cells were exposed to As (V) at the concentration of 20 (mg/L), the cell viability was significantly reduced as compared to the control. In contrast, As (III) affected the cell viability at lower concentration (1 mg/L). However, there was no differential effect of As (III) and (V) on the cell viability at tested concentrations.

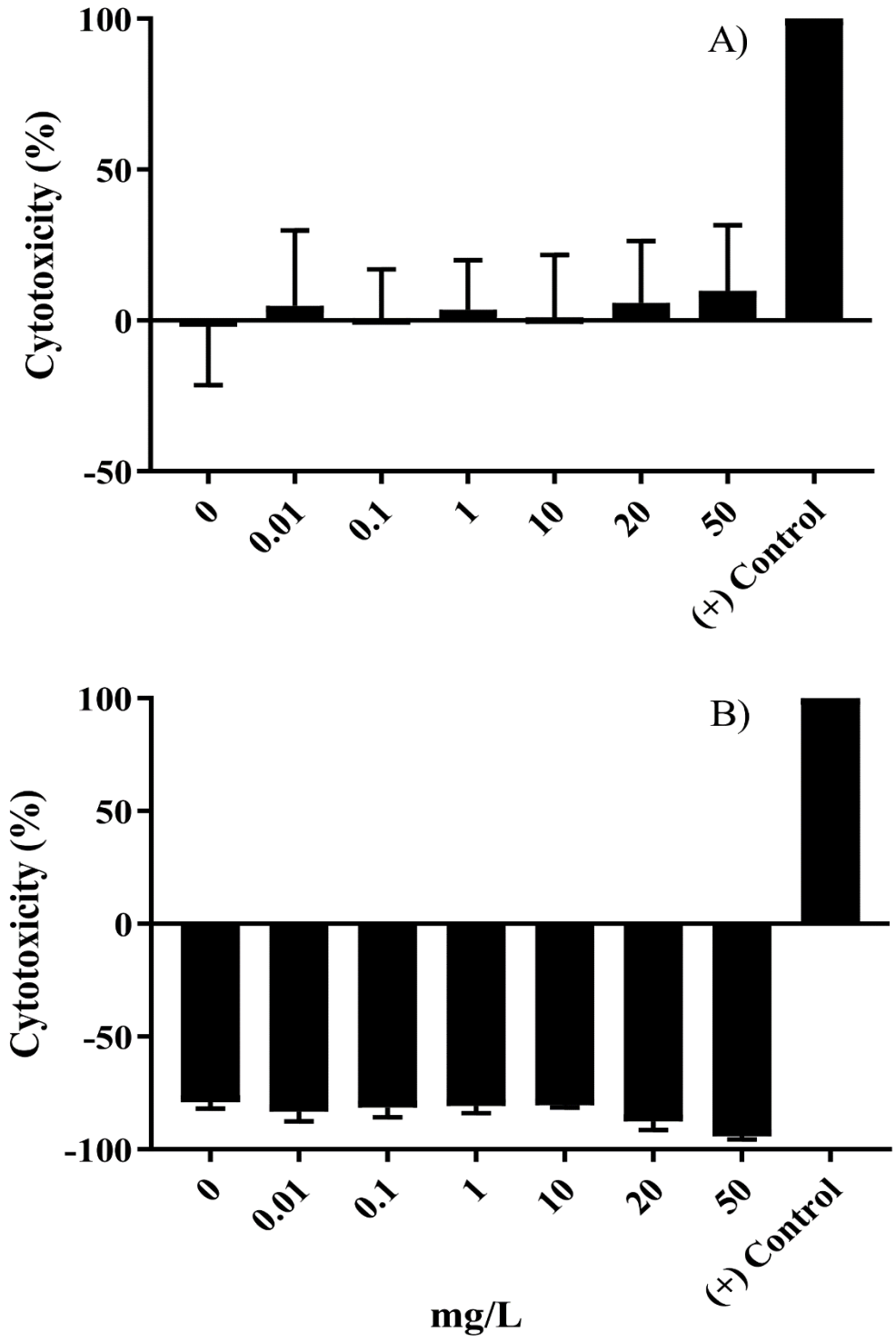

Figure A5.1. Cytotoxicity of As (III) (Fig. A) and As (V) (Fig. B) on Caco-2 cells after 4h exposure.

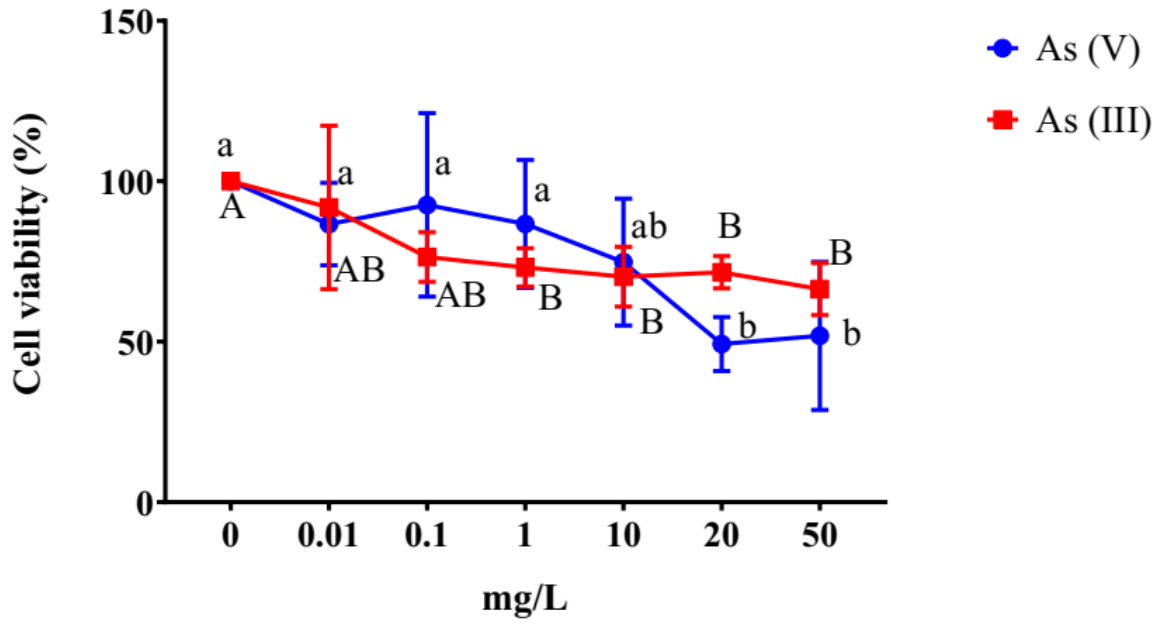

Figure A5.2. Cell viability (%) of Caco-2 cells after exposure to As (III) and As (V) for 4h.

Base on the result of these preliminary studies, we concluded that, at low concentration (0.01 to 1 mg/L) two inorganic arsenic species did not damage Caco-2 cells. In the next experiment, we tested the effect of these arsenic compounds on Caco-2 cells at higher concentrations from 10 to 60 mg/L. The starting concentration was decided because we did not observe the negative effects of As on Caco-2 cells at low concentration and 10 mg/L was 1000-fold greater as compared to the federal government regulation for inorganic arsenic in drinking water as well as an action level for inorganic arsenic in apple juice (Carrington et al., 2013).

APPENDIX B. LIFETIME AVERAGE DAILY DOSE ESTIMATION

Table A5.1. Various guidelines for metal content in drinking water (EPA) and bottled water (FDA) (adapted from Tvermoes et. al. (2014)).

Metal	Chronic MRL (mg/kg-day)	FDA limit (mg/L)	EPA MCL (mg/L)	RfD (mg/kg-day)
Aluminum (Al) [a,d]	1.0	0.20	0.05–0.20	1.0
Inorganic Arsenic (As)	0.0003	0.01	0.01	0.0003
Cadmium (Cd)	0.0001	0.005	0.005	0.0005
Chromium (Cr) [b]	0.0009	0.10	0.10	0.003
Copper (Cu) [a,c]	NA	1.0	1.3	0.04
Lead (Pb) [c]	NA	0.005	0.015	NA
Manganese (Mn) [d]	NA	0.05	0.05	0.14

MRL: minimal risk level; MCL: maximum contaminant level; RfD: chronic oral reference dose; NA: not applicable.
[a] No RfD value is established for this metal; therefore, the EPA screening level RfD is listed.
[b]The RfD for Cr is based on the RfD for Chromium (VI).
[c]The MCL is based on the action level; the maximum contaminant level goal (MCLG) for Pb is 0 mg/L and for Cu is 1.3 mg/L.
[d] The MCL is a secondary non-enforceable standard. Exceeding the secondary MCL for Al may be associated with aesthetic effects such as discolored water. For Mn, exceeding the secondary MCL may be associated with discolored water as well as corrosion and staining.

Table A5.2. Values of apple juice intake used to estimate incremental lifetime cancer risk (ILCR)

Age Group	Body weight normalized CDI of juice (IR) (g/kg-day)	Exposure duration (ED) in years for that age group (year)	(IR X ED)/AT (g/kg-day)
3 to <6 months	11.10	0.25	0.04
6 to <12 months	10.09	0.5	0.07
1 to <2 years	13.25	1	0.19
2 to <3 years	10.75	1	0.15
3 to <6 years	6.42	3	0.27
6 to <11 years	2.68	5	0.19
11 to <16 years	1.57	5	0.11
16 to <21 years	1.24	5	0.09
21 to <50 years	1.16	29	0.48
50 to <70 years	0.76	20	0.22
Sum			1.82

CDI: chronic daily intake; IR: body weight normalized CDI of juice; ED: exposure duration; AT: lifetime exposure duration (70 yrs.).

Table A5.3. Values of inorganic arsenic used to estimate lifetime average daily dose (LADD) (mg/kg-day)

(µg/L)	Average metal concentration	Average metal concentration above limit of quantification	FDA action level in apple juices
Total Arsenic	2.4	3	
Inorganic Arsenic	1.79	2.24	10
LADD	3.9^{E-06}	4.1^{E-06}	18^{E--06}

*: inorganic arsenic level is estimated based on FDA surveillance of arsenic in apple juice (74.8% of arsenic is inorganic species) (FDA, 2011).

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