Speciation of arsenic metabolites in chicken meat and in human cells

by

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Abstract

A phenylarsenical, 3-nitro-4-hydroxyphenylarsonic acid (Roxarsone®, Rox), has been used extensively as a poultry feed additive for over 60 years. However, little is known about the concentrations of different arsenic species present in chicken meat as a consequence of feeding Rox to chickens. The first objective of my thesis research is to study the speciation of Rox in chicken meat, providing relevant information on human exposure to arsenic through the ingestion of chicken meat. The second objective is to study accumulation, transport, and metabolism of Rox and the related arsenic species using human cells, improving a mechanistic understanding.

To quantify arsenic species in chicken meat, I first developed an enzyme-assisted extraction method which enhanced the extraction efficiency of arsenic from 28% using water/methanol extraction to 55% using papain digestion. The efficiency was further enhanced to 88% by using ultrasonication combined with the papain digestion. Arsenic species were separated using high performance liquid chromatography (HPLC). Inductively coupled plasma mass spectrometry (ICPMS) and electrospray ionization tandem mass spectrometry (ESI-MS/MS) were used in combination to quantify and identify arsenic species. The method of papain-assisted extraction and HPLC-ICPMS determination achieved detection limits of 1.0-1.8 μg arsenic per kg chicken breast meat for arsenobetaine (AsB), inorganic arsenite (As^{III}), dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), inorganic arsenate (As^{V}), Rox, and N-acetyl-4-hydroxy-m-arsanilic acid (NAHAA).

To determine whether the feeding of Rox to chicken leads to the increased concentrations of other arsenic species in chicken breast meat, I studied the arsenic species

in the breast meat from chickens raised in a 35-day feeding study. Eight hundred chickens were fed a diet supplemented with Rox and another 800 chickens were fed a control diet. The results from the analyses of 229 chicken samples showed that Rox, AsB, As^{III}, MMA, DMA and an unidentified arsenic metabolite (Un) were detectable in breast meat from the Rox-fed chickens. The concentrations of arsenic species, except AsB, were significantly higher in the Rox-fed than the control chickens. The concentrations of As^{III}, DMA, MMA, and Un decreased after Rox feeding was stopped. Seven days after termination of Rox feeding, the concentrations of As^{III} (3.1 μg/kg), Rox (0.4 μg/kg), and Un (0.8 μg/kg) in the Rox-fed chickens were still significantly higher than the control.

To examine whether the bioavailability of Rox is lower than As^{III} and AsB, I studied the accumulation and transepithelial transport of arsenic species using the Caco-2 cells. After Caco-2 cells were exposed for 24 h to Rox, AsB, or As^{III}, the accumulated Rox in Caco-2 cells was 6-20 times less than AsB and As^{III}. The permeability of Rox from the apical to basolateral side of the Caco-2 monolayers was much less than As^{III} and AsB.

To study the metabolism of Rox in human cells, I treated HepG2 cells and human primary hepatocytes with Rox for 24 h and measured the arsenic metabolites. The results showed that both HepG2 and human hepatocytes could metabolize Rox to 3-amino-4-hydroxylphenlarsonic acid (3AHPAA) and As^{V}. Human primary hepatocytes also had the ability to metabolize Rox to MMA and NAHAA. A new and previously unknown metabolite was identified as a thiolated Rox. The characterization of this new metabolite was achieved using a newly developed HPLC-ESI-Qudrupole Time-of-Flight (QToF) high-resolution mass spectrometry method.

To understand how a detected metabolite NAHAA was formed, I assessed whether N-acetyltransferases were responsible for the acetylation of a precursor, 3AHPAA, to NAHAA. Preliminary results showed that the pigeon liver N-acetyltransferases and chicken liver cytosol could acetyl 3AHPAA to NAHAA. The use of human liver cytosol and human N-acetyltransferase 1 did not produce detectable NAHAA.

This dissertation shows speciation of arsenic in chicken meat and in human liver and colon cells, with an emphasis on Rox and its related arsenic species. Analytical methods enabled the sensitive detection of arsenic species and the identification of new arsenic metabolites. Feeding of Rox to chickens increased the concentrations of five arsenic species in breast meat. The quantitative determination of arsenic species in chicken meat provided useful information for assessing human exposure to arsenic from consumption of chicken meat. Studies using human colon and liver cells improved understanding of arsenic transport and metabolism.

Preface

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Accumulation and transport of roxarsone, arsenobetaine, and inorganic arsenic using the human immortalized Caco-2 cell line. J. Agric. Food. Chem. Published online; doi: 10.1021/acs.jafc.6b03341. Copyright [2016] American Chemical Society. I was responsible for the cell experiment, HPLC-MS analyses, data analyses, and manuscript writing. Dr. Leslie assisted with the revision of the manuscript. My supervisory Dr. Le involved in concept formation and manuscript revision.

The research projects in Chapter 5 and 7 involved the use of human primary hepatocytes. The use of hepatocytes, as a part of the study titled "The role of transport proteins in toxicology", received research ethics approval from the Health Research Ethics Board (HREB) - Biomedical Panel, Study ID: Pro00001646_REN1, Approved on Nov 29, 2015.

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List of Abbreviations

As	Arsenic
AsB	Arsenobetaine
As^{III}	Inorganic arsenite
As^{V}	Inorganic arsenate
DMA	Dimethylarsinic acid
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
HBSS	Hank's balanced salt solution
HPLC	High performance liquid chromatography
ICPMS	Inductively coupled plasma mass spectrometry
LY	Lucifer Yellow
MMA	Monomethylarsonic acid
NAHAA	N-acetyl-4-hydroxy-m-arsanilic acid
NAT	N-acetyltransferases
PABA	p-aminobenzoic acid
QToF	Qudrupole Time-of-Flight mass spectrometry
Rox	Roxarsone
SMZ	Sulfamethoxazole
TER	Transepithelial resistance
Un	Unidentified arsenic species
XIC	Extracted ion chromatogram
3AHPAA	3-amino-4-hydroxylphenlarsonic acid

Chapter 1. Introduction

1.1. The global concern of arsenic in drinking water

On the periodic table, arsenic (As), with the atomic number 33 and an atomic mass of 74.92 g/mol, belongs to Group 5 along with nitrogen, phosphorus, antimony, and bismuth. It is a metalloid possessing properties of both metals and non-metals. The only stable and naturally occurring isotope of arsenic is ^{75}As. The most common valence states of arsenic are -3, 0, +3, and +5 (ATSDR 2007). Under oxidizing conditions, As usually exist as H_3AsO_4, which is in a class of compound called arsenate (As^V, +5 valence state). Alternatively, in mildly reducing conditions, As is generally present as H_3AsO_3, which is in a class of compound called arsenite (As^{III}, +3 valence state) (Duker et al. 2005). The name, structure, and chemical properties of arsenic compounds relevant to the study of the retention, metabolism, and excretion of a phenyl-arsenic compound, Roxarsone, in chickens and human cells, are shown in Table 1.

The concentration of arsenic is around 1.7 μg/L in seawater and <1 μg/L in uncontaminated lakes or streams (Matschullat 2000). The majority of arsenic in water systems (ocean, streams, lakes, and estuaries) originates from submarine volcanism and atmospheric deposition (Matschullat 2000). The primary water-soluble forms of arsenic are As^{III} and As^V (Duker et al. 2005). In southwestern Taiwan, well water contaminated with high concentrations of arsenic (> 50 μg/L) had been used by residents for more than 50 years. In this area, the blackfoot disease was identified (Chen et al. 1988), along with the observation of a dose-response relationship between chronic exposure to arsenic and incidences of lung and bladder cancers (Chiou et al. 1995) and non-insulin-dependent diabetes (Tseng et al. 2000). In northern Chile, the average concentration of arsenic in

drinking water reached 570 μg/L between 1955 and 1969 (Smith et al. 1998). Bladder cancer mortality and lung cancer mortality in this area was markedly elevated for the people aged over 30 in the year 1989-1993, suggesting that the ingestion of drinking water containing high levels of arsenic is a highly-possible cause of lung and bladder cancers (Smith et al. 1998). The epidemiological relationship between arsenic and diseases found in Chile and Taiwan was consistent with the studies in Japan (Tsuda et al. 1995) and Argentina (Hopenhayn-Rich et al. 1996). In 2000, Smith et al. (2000) reported that millions of people in Bangladesh had been exposed to high concentration of arsenic from their tube wells; this remains the largest arsenic poisoning of a population reported in history. Because of these findings, the World Health Organization (WHO) set the limit for the maximum tolerable arsenic concentration in drinking water as 10 μg/L (WHO 2008). However, to date, arsenic concentrations in the ground water above this guideline limit have still been detected in many countries and areas, including Bangladesh, India, Vietnam, Thailand, China, Brazil, Mexico, Germany, United Kingdom, USA, and Canada. (Nordstrom 2002).

1.2. Human exposure to arsenic

Drinking water is not the only source of human exposure to arsenic. The application of arsenic as herbicides in golf courses, livestock feed additives, medicine, pesticides, pigments, and wood preservatives can introduce arsenic into the environment, causing human exposure to arsenic (USEPA 2010). Using arsenic to harden alloys, combust fossil fuels, and to manufacture glass, semiconductors, electrical devices, and textiles contribute to occupational arsenic exposure (USEPA 2010). In the general

population, the main source of arsenic exposure is via ingestion of food containing arsenic (ATSDR 2007). Intake of arsenic from air and soil is usually at much lower levels. It has been estimated that the average daily dietary intake of arsenic by adults in the United States is 53 μg/day (Yost et al. 1998). Meat, fish, and poultry account for 80% of dietary arsenic intake (ATSDR 2007). With the exception of these, most foods contain very low arsenic concentrations (< 20 μg As/kg wet weight) (Dabeka et al. 1993). Seafood contains much higher arsenic concentrations, which possibly is the result of the ingestion of plankton by marine organisms (Caumette et al. 2012). However, the predominant species of arsenic in seafood, which accounts for the high arsenic concentrations, is arsenobetaine (AsB), which is regarded as non-toxic to humans (Tseng 2009).

1.3. Biotransformation of arsenic

All living organisms are exposed to arsenic in one form or another and have evolved strategies to cope with this exposure. One of the most commonly encountered strategies is methylation (Challenger et al. 1933; Le et al. 2000). In this strategy, As^{V} is first reduced to As^{III} and then methylated to monomethylarsonic acid (MMA^{V}). Next, MMA^{V} is reduced to MMA^{III} and then methylated to dimethylarsinic acid (DMA^{V}). Finally, DMA^{V} is reduced to DMA^{III} and then methylated to trimethylarsine oxide (TMAO). TMAO has been found in both humans (Styblo et al. 2002) and rats (Cohen et al. 2002). This methylation pathway was first confirmed by Frederick Challenger and his co-workers (Challenger et al. 1945). It was once regarded as a detoxification mechanism because the methylated arsenic(V) species (MMA^{V} and DMA^{V}) are much less toxic than their inorganic precursors. However, the finding that methylated arsenic(III) species

(MMA^{III} and DMA^{III}) are more toxic than their precursors suggests that methylation is not strictly a detoxification process, but also an activation process (Stýblo et al. 2002).

Another metabolic pathway occurring in organisms is thiolation. Thio-arsenicals are structural homologs of oxo-arsenicals with the replacement of one or more arsenic-bound oxygen atoms with sulfur atoms (Wang et al. 2015). *In vitro* studies showed that DMA^{III} taken up by human red blood cells was converted to dimethylmonothioarsinic acid ($DMMTA^{V}$) (Suzuki et al. 2004). Additionally, rat liver cytosol was found to convert DMA^{III} to $DMMTA^{V}$ or dimethyldithioarsinic acid ($DMDTA^{V}$) (Naranmandura and Suzuki 2008).

1.4. The toxicity of arsenic

As^{III} is a well-established human toxicant and carcinogen. Symptoms of acute arsenic poisoning may include nausea, vomiting, diarrhea, blood in the urine, stomach pain, hair loss, and convulsions (USEPA 2010). Epidemiological studies have shown that chronic exposure to inorganic arsenic is strongly associated with the prevalence of lung, bladder, and skin cancers (Chen et al. 1992; Levine et al. 1988), and increased risk of cerebrovascular disease (Chen et al. 1995), hypertension (Chiou et al. 1997), and many other adverse health effects (WHO 2012; Yoshida et al. 2004).

The toxicity of arsenic is highly dependent on the chemical species of arsenic (ATSDR 2007). The median lethal doses of arsenic species vary by ten thousand folds from the most toxic to the least toxic arsenic species. The order of arsenic toxicity is $DMA^{III} > MMA^{III} > As^{III} > As^{V} > DMA^{V} > MMA^{V} > AsB \approx$ non-toxic. Hence, although it is necessary to determine the concentration of the total arsenic, it is even more important to

determine the individual concentrations of each arsenic species present, in order to more accurately assess the level of toxicity exerted by arsenic.

1.5. Phenylarsenicals

1.5.1. The occurrence of phenylarsenicals

Phenylarsinicals play a significant role in environmental contamination. There are no naturally-occurring phenylarsenicals in the environment. All phenylarsinicals are synthesized compounds that are introduced into environmental systems by anthropogenic activities. Phenylarsenicals have been used as warfare compounds, drugs, and feed additives. Arsenic-based warfare compounds are diphenylarsinic acids (Daus et al. 2008; Kinoshita et al. 2005), which were produced during the First and Second World Wars, in large scales of several thousand tons. At the beginning of the 20th century, two phenylarsenicals, salvarsan and melarsoprol, had been discovered as active agents against syphilis and sleeping sickness and were used as medicine for these diseases despite their high toxicities (Betz and Klufers 2009). Recently, a series of new phenylarsenicals, e.g. S-dimethylarsino-glutathione (ZI0-101) (Dilda and Hogg 2007; Evaluation 2009; Garnier et al. 2014) and phenylarsine oxide (PAO) (Ralph 2008), have been synthesized and evaluated as antitumor drugs (Dilda and Hogg 2007; Liu et al. 2003). Nowadays, the most abundant phenylarsenicals are monophenylarsonic acids which are used as feed additives.

1.5.1.1.The use of phenylarsenicals as feed additives

The use of phenylarsenicals as feed additive has been approved since the 1940s (Jones 2007a). The approved phenylarsenicals include 4-hydroxy-3-nitrophenylarsonic

acid (Roxarsone, Rox), p-arsanilic acid (4-aminophenylarsonic acid, ASA), 4-nitrophenylarsonic acid (4NPAA, Nitarsone), p-ureidophenylarsonic acid (pUPAA), and phenylarsonic acid (PAA) (structures in Table 1). When used as feed additives, these compounds are effective in reducing hemorrhage and mortality from different kinds of coccidian, such as *Eimeria tenella* and *Eimeria acervulina* (Izquierdo et al. 1987; Kowalski and Reid 1975). Rox also help birds develop a considerable immunity toward subsequent infection with *Eimeria tenella* (Goble 1949).

Variation in the substituents on the aromatic ring results in differences in the growth-promoting and disease-controlling effects of these compounds. For example, the addition of a nitro group in the 3-position of the 4-hydroxy compound was indicated to have increased toxicity in chickens (Morehouse and Mayfield 1946). Therefore, Rox and ASA are approved as animal feed additives for both poultry and swine, whereas 4NPAA and pUPAA are only approved for controlling blackhead disease in turkeys.

1.5.1.2.One of the most-used phenylarsenic feed additives - Roxarsone

In the United States, Rox was estimated to be used in 69.8% and 73.9% of the broiler starter feeds and broiler grower feeds, respectively, from 1995 to 2000 (Chapman and Johnson 2002). Rox is not allowed in layer feeds but is approved at 22.7 to 45.4 g/ton (25 to 50 ppm) in broiler feeds (Jones 2007b). The rates of depletion of Rox from chickens were established using chicken muscle, skin, liver, and kidney (Morrison 1969). Highest residues at 150-790 µg/kg were found in the liver (Morrison 1969). Rapid depletion of residues occurs during the first five days off medication. The residue levels of arsenic in poultry meat considered safe are 0.5 ppm in muscle, 1 ppm in skin/fat, and 3 ppm in liver

(US FDA 2004). Withdrawal of the drug 5 days before slaughter results in arsenic levels well below these tolerance established by the FDA (Garbarino et al. 2003; Kerr et al. 1969). A summary of the past studies on the total arsenic in poultry can be found in Nachman et al. (2013).

The use of arsenical feed additives was prohibited in Europe in 1999 (Nachman and Baron 2013). In 2011, US FDA scientists found that the concentrations of inorganic arsenic in the livers of chickens treated with Rox were increased compared to the concentrations in the livers of the untreated control chickens (Kawalek et al. 2011). On the basis of this observation, the FDA withdrew the approval of Rox in 2013 (US FDA 2013). Pfizer Inc. and Zoetis Inc., who had ownership of Rox (trade name: 3-Nitro®) in sequence, voluntarily suspended the sale and withdrew the product from the US in 2014-2015. While Pfizer Inc. has suspended the sale of Rox in the US, it continues to manufacture and export Rox overseas. Other countries make their own decisions on the use of arsenic-based chicken feed additives within the context of their own communities and regulatory systems (US FDA 2015).

Phenylarsenical-contaminated poultry litter deposited or applied as fertilizer in crop fields leads to elevated arsenic concentrations in soil and leaching water, resulting in arsenic contamination of the environment. In sludge, Rox is rapidly transformed to its amination counterpart, 3-amino-4-hydroxyphenylarsonic acid (3AHPAA) in the absence of oxygen (Cortinas et al. 2006; Nakajima et al. 2005). In the presence of oxygen, Rox is primarily degraded to As^{V} in about 30 days, mostly by biotic degradation (Garbarino et al. 2003).

In the chicken body, Rox is mostly excreted unchanged in the chicken litter (Moody and Williams 1964). The recent work of Nachman et al. (2013), together with the 2011 US FDA study (Kawalek 2011), suggested that Rox may be partially biotransformed to inorganic arsenicals in the chicken body. Information on the metabolism of Rox in chicken is very limited (Conklin et al. 2012; Kawalek 2011; Overby and Straube 1965; Peng et al. 2014). Furthermore, it is unclear whether other arsenic metabolites are produced in Rox-fed chickens. Therefore, it is crucial to determine the magnitude that the concentrations of the more toxic arsenic species, e.g. As^{III}, increase due to the feeding of Rox to chicken.

1.5.2. Speciation of monophenylarsenicals in biological and environmental samples

1.5.2.1. Extracting and enriching technologies

Extracting and enriching methods for monophenylarsenicals in biological and environmental samples includes accelerated solvent extraction (ASE), enzyme-assisted extraction, solid phase micro-extraction (SPME), liquid-liquid microextraction (LLME), and capillary electrophoresis (CE).

ASA, 4NPAA, and Rox were extracted from the porcine and chicken liver samples using ASE (Cui et al. 2012). Enhanced extraction efficiency was achieved by using elevated temperature and pressure. Using an extraction temperature of 80°C and extraction pressure of 1,500 psi, this method achieved detection limits of 0.24, 0.74, and 0.41 ng/mL for ASA, 4-NPAA, and Rox, respectively.

Enzymatic hydrolysis has been employed to enhance the extraction efficiency of the traditional water-methanol extraction system for arsenic species in solid biological

samples. The advantage of using enzymes, especially protease, is that enzymes can break down specific bonds of the matrix under mild conditions (temperature and pH), without causing the destruction and conversion of different arsenic species during extraction. Therefore, enzymatic hydrolysis becomes an appealing method to extract phenyl arsenic compounds from solid biological samples. Liu et al. (2015) used papain hydrolysis to extract arsenic species from chicken muscle. This method achieved better extraction efficiency (55%) than that of traditional 1:1 water/methanol extraction (28%), and more arsenic species were extracted. Pepsin hydrolysis of seafood for extracting other arsenic species (As^{III}, As^{V}, DMA^{V}, and AsB) has also been reported (Moreda-Piñeiro et al. 2011).

Roerdink and Aldstadt (2004) used SPME to extract ASA and Rox from complex matrices (surface water from a hog farm, a turkey farm, and the Milwaukee River). The sample was acidified and then derivatized with 1, 3-propanedithiol (PDT). A 65-μm polydimethylsiloxane-divinylbenzene (PDMS-DVB) SPME fiber was used to extract the derived phenylarsenicals. A detection limit of 2.69 μg/L and a recovery of 103±10.9% for Rox were achieved. In terms of chemical derivatization, the researchers introduced another reaction between ASA and 4-dimetylaminobenzaldehyde (DMAB) (Roerdink and Aldstadt 2005), which can form new compounds named p-(4-dimethylaminophenyl)phenylarsonic acid (p-DAPPA), which can then, in turn, be detected by long pathlength absorbance spectrophotometry (LPAS). This method is selective for ASA from other organic arsenic compounds, such as DMA^{V}, MMA^{V}, and Rox. The limit of detection of this method was 21.2 μg/L and recovery was 100.5 ± 13.7 % for ASA.

LLME has been applied for the enrichment of phenyl arsenic compounds. Guo et al. (Guo et al. 2013) developed an ionic liquid based carrier-mediated hollow fiber liquid-liquid-liquid microextraction (IL carrier-mediated HF-LLLME) to extract and enrich 3AHPAA, PAA, 4NPAA, and ASA from chicken and feed samples. The arsenic species were first extracted from chicken meat or feed by water using ultrasonication. Then, the pH of the extractant was adjusted to 10.2 to ensure all analytes were in their ionic forms. The arsenic species in the aqueous phase interacted with the ionic liquid $[MTOA]^+[Cl]^-$ in toluene (20% (v/v)), which was impregnated in the pores of a hollow fiber, then back extracted into a tiny volume (μL level) of 0.3 mol/L NaBr as the acceptor phase in the lumen of the hollow fiber. The gradient concentration of Br^- and anionic arsenic species is the driving force for the migration of analytes from outside to the inside of the hallow fiber. This method achieved enrichment factors in the range of 86 to 372-fold. The recovery was 79.2–105.4% for the spiked chicken and feed samples. Another HF-LLLME system was developed by Li and Hu (2011). This method was applied on PAA, ASA, 4HPAA, 4NPAA, and 3NHPAA. The target analytes were extracted from 5 mL aqueous samples (donor solution, pH 2.15) through a thin phase of tributyl phosphate (TBP) inside the pores of a polypropylene hollow fiber to an 18 μL 0.8 mmol/L Tris acceptor solution inside the lumen of the hollow fiber. Phenylarsenic compounds complex with TBP probably through hydrogen bonding and hydrophobic interactions. The driving force is the pH gradient between the donor phase and the acceptor phase. The enrichment factor was around 155-fold.

The use of ionic liquid based LLME could be traced back to the work of Monasterio and Wuilloud (2010). They used tetradecyl(trihexyl)phosphonium chloride

(CYPHOS®IL 101) to enrich pentavalent arsenic species including As^V, DMA^V, and MMA^V. These arsenic compounds form As-molybdate heteropoly acid complex with molybdenum, which can further form ion pairs with CYPHOS®IL 101, and then be extracted into chloroform. Though phenylarsenicals have benzene ring, they are still quite polar. The idea of pairing phenylarsenicals with ionic liquid is a good way to improve the hydrophobicity of the phenylarsenicals to achieve the goal of extracting them from the aqueous to the organic phase.

For the phenylarsenicals initially present in the organic phase, Wang et al. (Wang et al. 2011) successfully developed an ultrasound-assisted dispersive LLME to extract trace amount of Rox from different kinds of edible oil. Hexane (1.25 mL, disperser solvent) with 50 μL of pH 7 ammonium formate buffer solution (50μL, extraction solvent) was injected rapidly into 3 g of edible oil containing Rox. The solution was then centrifuged at 10,000 rpm and the extraction solvent was deposited at the bottom of a conical test tube. The recovery obtained was above 90% and the limit of detection was 5.8 ng/g.

CE can serve to perform preconcentration. In the work of Li and Hu (Li and Hu 2011) mentioned above, besides HF-LLLME, the researchers also used sample stacking technique on CE for the online preconcentration of PAA, ASA, 4HPAA, 4NPAA, and 3NHPAA. After filling a capillary with 25 mM carbonate buffer (pH 9.75) as the background electrolyte (BGE), a long water plug (91% length of separation capillary) was injected into the capillary. Then, electronic field (+- 10 kV) was applied at the capillary outlets, and the acceptor solution obtained by HF-LLLME was injected into the capillary at −10 kV for 180 s. Under the electric field, the target anions moved rapidly into the capillary and stacked at the boundary between the water zone and BGE zone. Once the

anions reached the boundary, their velocities slowed down because the strength of the electric field decreased in the BGE zones. Finally, the water was expelled from the inlet of the separation capillary. On-line CE preconcentration improved the enriching factor of HF-LLLME for the target analytes from 155-fold to 1780-fold.

1.5.2.2.Separation technologies using high performance liquid chromatography (HPLC)

The separation of phenylarsenicals is usually done by HPLC. In HPLC, a mobile phase driven by mechanical pumps carries the sample into a separation column. The sample mixture interacts with the stationary phase packed inside the column. Select compounds in the sample mixture retain strongly on the stationary phase and thus travel down the column slowly, while others interact with the stationary phase less strongly and travel faster with the mobile phase. The differential partitioning between the stationary and mobile phases results in the separation of analytes. As shown in Table 1, the first pKa's of the typical monophenylarsenicals are in the range of 2 to 4. The low $\log K_{ow}$'s indicate that monophenylarsenicals are quite polar. On the basis of their chemical properties, the ideal separation mechanisms for these compounds are ionic exchange and reverse phase.

By manipulating the ionic properties of phenylarsenicals, their retention times in HPLC can be varied. At a pH higher than 6, the majority of phenylarsenicals are anions. They can bind to the cationic packing materials in the anion exchange columns. High concentration (mM level) of salts is added to the aqueous mobile phase to cause a competition between the anions from the salt and the anionic arsenic species. The result is the elution of the arsenic species in the order of increasing anionic strength, which dictates their ability to compete with the salts and be retained on the column longer. Several studies

used anion exchange chromatography with methanol/60 mM NH_4HCO_3 (5:95 v/v, pH 8.75) as the mobile phase (Liu et al. 2015; Peng et al. 2014; Yang et al. 2016). By running a gradient elution, 3AHPAA, N-acetyl-4-hydroxyl-phenylarsonic acid (NAHAA), and Rox were baseline-resolved. Ion pair chromatography shares a similar separation mechanism with anionic exchange chromatography. It uses the ion pair reagent to coat the packing materials of the reverse phase column (e.g. C_{18}), which forms a cationic layer on the packing materials for the anionic compounds to bind. In the work of Guo et al. (2013), the researchers used C_{18} column with 10.0 mM malonic acid, 2.5 mM ion-pairing reagent sodium butanesulfonate, and methanol/water (5:95 v/v) as the mobile phase to successfully separate ASA, 4-hydroxyphenylarsonic acid (4HPAA), Rox, PAA, and 4NPAA.

In strongly acidic solution (e.g. pH < 2), most phenylarsenicals are in their non-ionic forms. In this case, reversed phase columns can be used to separate them on the basis of their polarity. Cui et al. (2012) used C_{18} column and methanol/50 mM KH_2PO_4 (5:95 v/v, 0.1 % v/v trifluoroacetic acid, pH 1.67) as the mobile phase, allowing for a separation of 4NPAA, ASA, and Rox.

1.5.2.3.Identification technologies using mass spectrometry (MS)

To date, the most popular method for detecting arsenic species is inductively coupled plasma mass spectrometry (ICPMS). In ICPMS, an aqueous sample is delivered by pneumatic nebulization into a high-temperature plasma (~8,000K), where the analytes undergo atomization and then ionization. ICP can break down any compound into its constructing elements, enabling very high sensitivity element-specific detection. ICP works well with both simple and complex matrices (Arroyo-Abad et al. 2011b, 2011a).

The elemental ions created by the ICP are extracted into ion optic by a series of differentially pumped vacuums and the ions are then separated based on their mass-to-charge ratio by a mass spectrometer, which is most commonly a quadrupole or triple quadrupoles, although double-focusing magnetic sector and time-of-flight mass spectrometers have also been used.

The major type of interference occurring in ICPMS for arsenic detection is isobaric interference. This occurs when arsenic is measured in a sample containing a chloride (Cl) matrix, such as tissue or cell samples. Cl binds to the argon (Ar) plasma gas and $ArCl^+$ polyatomic ions are formed, which have the same m/z as ^{75}As. As a result, quantitative analysis can have an error due to $ArCl^+$. One way to reduce the isobaric interference caused by $ArCl^+$ is by introducing oxygen (O_2) gas into the reaction cell to collide with and remove $ArCl^+$. In this case, As is converted into AsO^+ which is monitored at m/z 91. Another way of avoiding isobaric interference when detecting As by ICPMS is to introduce helium (He) gas into the reaction cell to suppress the kinetics of the ions derived from the sample matrix, including $NaAr^+$, ClO^+, $ArCl^+$, etc. Isobaric interferences can also be corrected using mathematical interference correction. In this case, the knowledge that $ArCl^+$ is present at m/z 75 and m/z 77 in the proportion to the isotope ratio of ^{35}Cl: ^{37}Cl, 3:1, can be used to correct for the interference at m/z 75. The counts at m/z 75 resulting from $Ar^{35}Cl^+$ are calculated based on the intensity of counts at m/z 77, which are the result of $Ar^{37}Cl^+$. By subtracting $ArCl^+$ from the count at m/z 75, a corrected arsenic signal can be obtained. However, selenium (Se) has an isotope at m/z 77 and any Se in the sample will increase the counts at m/z 77 accordingly. Fortunately, Se also has an isotope at m/z 82. By measuring the intensity of Se at m/z 82, the Se counts at m/z 77 can be calculated

and subtracted allowing for the determination of the counts at m/z 77 due to $Ar^{37}Cl^{+}$. As a result, the counts of arsenic can be obtained using Equation 1 (USEPA 1994). In my studies, I employ helium gas to reduce the interference of $ArCl^{+}$.

$$Count_{As} = Count_{(m/z\ 75)} - 3.127 \times Count_{(m/z\ 77)} - 0.815 \times Count_{(m/z\ 82)} \quad [1]$$

Electrospray ionization mass spectrometry (ESIMS) is able to provide molecular information on a chemical compound. Electrospray ionization is a so-called "soft ionization" technique, which maintains the integrity of the molecule by ionizing it while keeping it intact. A solution containing phenylarsenicals flowed through a capillary tube to which a high-voltage is applied; the result is a fine spray of charged droplets containing phenylarsenical ions. The inert gas, nitrogen, can help high-flow electrosprays with additional nebulization. The charged droplets undergo further solvent evaporation due to the flow of nitrogen gas, until the point when the Rayleigh limit is reached, which results in Coulomb fission, ejecting the ions into the gas phase. The ions then travel through the mass analyzer to the detector. Many types of mass analyzer, including quadrupole, tandem quadrupole, time-of-flight, and ion trap, can be used for the mass selection process. The major technique for sensitively detecting phenylarsenicals is tandem mass spectrometry (MS/MS), including selected ion monitoring (SIM) and multiple reactions monitoring (MRM). In SIM, the first quadrupole transmits an ion with a certain m/z. This ion collides with the nitrogen gas in the collision cell and a chosen fragment ion (with a specific m/z) was transmitted through the third quadrupole to the detector. This process can efficiently decrease the background of detection and increase the detection sensitivity for the ion of interest. The instrument rational of MRM is same with SIM but MRM is used for multiple ion pairs.

MRM has been used to identify Rox, ASA, 4HPAA, 4NPAA, and pUPAA (Jia et al. 2014). In our lab, we developed an MRM method on ESI-MS/MS for the detection of 8 arsenic species (AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3AHPAA, NAHAA, and Rox), including three phenylarsenicals. ESI-MS/MS is often used complementarily with ICPMS to identify and quantify arsenic species at the same time. The operating conditions for complementary ICPMS and ESI-MS/MS method can be viewed in several previous papers (Peng et al. 2014; Yang et al. 2016).

1.6. Study hypotheses and objectives

The practice of feeding Rox to chickens has lasted for more than 60 years. Although the European Union ceased the use of arsenicals as feed additives in 1999 (European Commission 1999) and the US FDA withdrew the approval of Rox in 2013 (US FDA 2013), Rox continues to be legally used in many other countries (Huang et al. 2014; Yao et al. 2013).

Chicken is the number one meat consumed in North America on a per capita basis, with a supply of 17.7 billion kg per year (AAFC 2013; ERS 2014). It is paramount to assess the concentrations of individual arsenic species in this highly-consumed food. This information will enable a proper assessment of human exposure to arsenic species and determination of the relative contributions of arsenic species from the various sources. Although several studies have reported on the concentrations of total arsenic in Rox-fed chickens and in chicken meat purchased from food markets (Batista et al. 2012; Doyle and Spaulding 1978; Jelinek and Corneliussen 1977; Lasky et al. 2004), information provided on the specific arsenic species is limited (Mao et al. 2011; Pizarro et al. 2003; Polatajko

and Szpunar 2004; Sanchez-Rodas et al. 2006; Sanz et al. 2005). It is still unknown whether the use of Rox as a chicken feed additive increases the concentration of other arsenic species in chicken meat. Moreover, how effects of the ingestion of chicken fed Rox on human health remains a question.

To fill the knowledge gap, my thesis focuses on understanding the retention, metabolism, and excretion of Rox in chickens, and the relevance of human exposure to arsenic from the ingestion of chicken meat. My overall hypothesis is that: the feeding of Rox to chickens increases the concentration of various arsenic species in chicken breast meat and that Rox can be taken up and metabolized by the human body. To test my hypothesis, I propose the specific objectives as follows:

The primary objective of Chapter 2 is to develop an analytical method that is necessary for the determination of individual arsenic species that may be present at trace concentrations in chicken meat. Accurately identifying and quantifying arsenic species in chicken meat is challenging due to low concentrations of arsenic species. Therefore, previous work has often focused on chicken liver and feces, both of which contain higher concentrations of arsenic species (Conklin et al. 2012; Falnoga et al. 2000; Kawalek 2011; Peng et al. 2014; Rosal et al. 2005; Salisbury et al. 1991). Determining the concentration of individual arsenic species is important because the toxicity of arsenic is highly dependent on its chemical species. (Charoensuk et al. 2009; Naranmandura et al. 2011; Shen et al. 2013; Styblo et al. 2000). Currently, there is no analytical method available for determining individual arsenic species present at trace levels in chicken meat. The analytical challenges include: (1) the development of an efficient separation technique, which is required to differentiate individual arsenic species; (2) concentrations of each

arsenic species are likely very low, and thus the detection technique used needs to be highly sensitive; and (3) trace amounts of arsenic species may be difficult to extract from chicken tissue, and thus an efficient method of sample preparation is required. The topic of the second chapter of my thesis is to develop an analytical method that addresses the aforementioned challenges enabling the selective separation and sensitive detection of different arsenic species in chicken meat. Separation of the arsenic species in chicken breast meat will be performed using anion exchange chromatography. ICPMS and ESIMS will be employed simultaneously for the determination of individual arsenic compounds. Parameters such as the sensitivity, of the method will be assessed.

The emphasis of my third chapter is on determining and comparing the concentrations of arsenic species in the breast meat of chickens fed a Rox-supplemented diet and a control (Rox-free) diet. The study design is a controlled feeding study that involves 1600 chickens from two common commercial strains. In the first four weeks, half of the chickens (800) were fed a diet supplemented with Rox and the other 800 chickens were fed a control diet. This design allows for the study of the uptake and metabolism of Rox. In the final week, all chickens were fed a Rox-free diet, allowing for the study of the Rox elimination kinetics over a 7-day period. Samples were collected on 16 days. On each day, 16 chickens were sacrificed and breast tissue samples were obtained. Following the development the methods for arsenic extraction, chromatography separation, and mass spectrometry detection discussed in chapter 2, these methods were applied to the analyses of arsenic species in chicken breast tissues collected from the 35-day feeding study. The main questions addressed by this study include: What arsenic species are present in chicken meat? What are their concentrations? What are the temporal patterns of arsenic

species in chickens over the feeding period? How fast do the concentrations of arsenic species in chicken breast decrease after the feeding of Rox stops? What are residual concentrations of arsenic species in chicken breast meat 5-7 days after the feeding of Rox stops? Our collaborator Dr. Martin Zuidhof from the Department of Agriculture at the University of Alberta provided the chicken samples. The kinetics of the uptake and elimination of arsenic species in the chicken breast meats during the 35 days were also evaluated.

The objective of Chapter 4 is to improve our understanding of the cellular accumulation and transepithelial transport of Rox by human cells. Chapter 4 was built on the basis of the results discussed in Chapter 3. In the feeding study, the concentration of Rox in the chickens' diet was 500 times higher than the background concentration of AsB. However, the concentrations of Rox and AsB in the chicken breast tissues were on the same order of magnitude. What could be the reason for this intriguing result? It was hypothesized that the bioavailability of Rox is much lower than of AsB. To test this hypothesis, I used one of the most common models, the Caco-2 cell line, to analyze the intestinal absorption of Rox. Intestinal epithelial cells are regarded as the first barrier for contaminants penetrating the body via the oral route. The Caco-2 cell line was established from human colon adenocarcinoma cells and has been widely used to examine absorption mechanisms and to estimate the permeability of drugs, nutrients, and minerals. In the case of arsenic, the Caco-2 cell line has been used to study the accumulation and transport of As^{V}, As^{III}, AsB, DMA, MMA, trimethylarsine oxide, tetramethylarsonium ion, and arsenosugars in standard solutions and in food products. To date, there is little information regarding the intestinal absorption of Rox in humans.

The study described in Chapter 5 also represents an extension of Chapter 3, with an emphasis on the metabolism of Rox. The primary objective of this study described is to investigate the metabolism of Rox using human immortalized cell lines and primary hepatocytes. Results from the analyses of chicken breast meat in Chapter 3 showed the presence of multiple arsenic species in chickens fed Rox. These arsenic species are possible metabolites of Rox. In addition, although the arsenic species were excreted rapidly from the chickens during the Rox withdrawal period, the residual arsenic concentrations in the chicken breast meat seven days after terminating Rox feeding remained significantly higher in the Rox-fed chickens than in the control chickens. Therefore, human consumption of chicken meat could result in exposure to residual Rox. There is no information on the human metabolism of Rox. The information provided in Chapter 5 will fill an important knowledge gap - understanding the metabolism of Rox and determining the metabolites of Rox is important because the arsenic metabolites may have higher toxicity than Rox.

Chapter 6 focuses on identifying a new metabolite of Rox in human primary hepatocytes. In Chapter 5, I found that, with the incubation with Rox, several unidentified arsenic species were present in both the cell lysate and cell culture medium of the hepatocytes. I propose a major undertaking to identify and characterize the new arsenic species that were observed in the preliminary analyses. These unidentified arsenic species were not seen in the hepatocytes incubated with As^{III}, suggesting that the unidentified arsenic species could be new metabolites of Rox. Characterizing the structure of these previously unidentified Rox metabolites could help us to better understand the metabolism of Rox in humans.

The aim of Chapter 7 is to examine the possible acetylation of 3-AHPAA to form NAHAA. This study is also motivated by the results of Chapter 3, in particular, the observation of 3-AHPAA and NAHAA in chicken meat. Previous studies reported the presence of 3-AHPAA and NAHAA in the chicken litter, but it could not be concluded whether these arsenic species were formed in the chicken body or by microorganisms in the litter. The determination of 3-AHPAA and NAHAA in chicken meat suggests that these compounds are formed in the chicken body. I hypothesize that 3-AHPAA can be enzymatically transformed to NAHAA. To test this hypothesis, I conducted *in vitro* experiments to demonstrate the involvement of arylamine acetyltransferases in the acetylation of a 3-AHPAA to NAHAA.

The anticipated outcome of my research will fill the knowledge gap on the accumulation, transport, and metabolism of Rox in chickens and humans. It will provide useful information for assessing arsenic exposure through the ingestion of chickens fed Rox, and provide new analytical methods for the study of arsenic species in complicated biological samples. The knowledge gained from these studies will help improve our molecular understanding of arsenic health effects.

1.7. References

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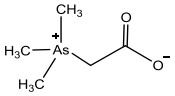
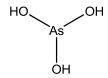
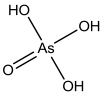
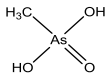
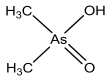
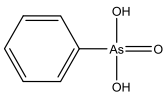
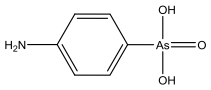
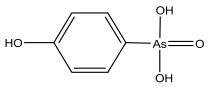
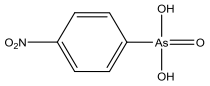
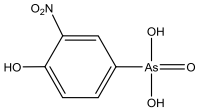
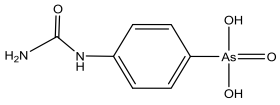
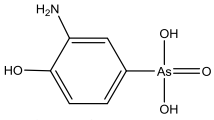
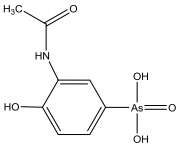
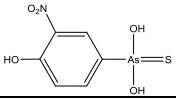
Table 1.1 Names, structures and chemical properties of the arsenic compounds mentioned in my thesis. Values were cited from Mao et al. 2011.

Name	Abbreviation	Structure	pKa	LogP [a]
Arsenobetaine	AsB			
Arsenite	As^{III}		9.2, 12	
Arsenate	As^{V}		2.3, 6.7, 12	-1.9
Monomethylarsonic acid	MMA^{V}		4.6, 7.8	-1.8
Dimethylarsinic acid	DMA^{V}		6.2	-1.6
Phenylarsonic acid	PAA		3.6, 8.8	-0.0010
p-Arsanilic acid	ASA		2.0, 4.0, 8.9	-1.3
4-hydroxyphenylarsonic acid	4HPAA		3.9	-0.70
4-Nitrophenylarsonic aicd (Nitarsone)	4NPAA		3.0	-0.30
4-Hydroxy-3-nitrophenylarsonic acid (Roxarsone)	Rox		3.5	-0.51
p-Ureidophenylarsonic acid	pUPAA			-1.4 [a]
3-amino-4-hydroxyphenylarsonic acid	3AHPAA			-1.8 [a]
N-acetyl-4-hydroxymarsanilic acid	NAHAA			-1.5 [a]
4-hydroxy-3-nitro-mononthiol-phenylarsonic acid	Thiolated Rox			0.090 [a]

[a] Values were calculated by ACD/ChemSketch 10.0 (Advanced Chemistry Devalopment, Inc., Ontario, Canada).

Chapter 2. A method of papain extraction coupled to liquid chromatography-mass spectrometry for the determination of arsenic species in chicken breasts [1]

2.1. Introduction

Humans are mainly exposed to arsenic (As) through ingestion of food and water. Chronic exposure to high concentrations of arsenic is associated with a variety of adverse health effects. As a consequence, regulatory agencies around the world have tightened guidelines on arsenic in water. For example, the World Health Organization (WHO 2011), the United States Environmental Protection Agency (EPA 2001), and Health Canada (Health Canada 2006) have guidelines for arsenic (10 μg/L) in drinking water. However, many arsenic species can be present in food (Cullen and Reimer 1989; Le et al. 1994; Schoof et al.1993; Ruttens et al. 2012; Bundschuh et al. 2012; Raber et al. 2012; Carbonell-Barrachina et al. 2012; Hu et al. 2015; Nachman and Baron 2013). Arsenic in food can range from highly toxic inorganic arsenite to the virtually non-toxic arsenobetaine. The relative toxicity of different arsenic species, e.g., in terms of medium lethal concentration (LC_{50}), varies by 3-4 orders of magnitude (Charoensuk et al. 2009; Hughes et al. 2011; Naranmandura et al. 2011; Shen et al. 2013; Styblo et al. 2000). Therefore the determination of total arsenic in food is not sufficient for human health risk assessment; it is necessary to determine the speciation of arsenic in food.

Extensive research has been carried out on the speciation of arsenic in seafood (McSheehy et al. 2002, 2003; Beauchemin et al. 1998; Pergantis et al. 2000; Whaley-Martin et al. 2012; Pétursdóttir et al. 2014; Niegel and Matysik 2010) for the need of

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differentiating toxic arsenic species (e.g., inorganic arsenicals) from those of high concentration but little toxicity (e.g., arsenobetaine). Recently, there has been much attention to the determination of arsenic in rice and chicken because of the relatively high concentrations of arsenic in these food items (Chapman and Johnson 2002; Conklin et al. 2012; de la Calle et al. 2011; Koch et al. 2013; Zhu et al. 2008). The source of arsenic in chickens is mainly due to the use of Roxarsone (Rox), an organoarsenical, as a feed additive to control infection and promote growth of chickens (Chapman and Johnson 2002). Chicken is one of the most consumed meats, and it may contribute a considerable amount of arsenic to the total dietary exposure. However, little is known about the arsenic speciation in chicken meat (Nachman and Baron 2013; Conklin et al. 2012; Lasky et al. 2004). The primary objective of this research was to develop a method that enable the determination of arsenic species present in chicken meat at trace concentration.

Determination of trace concentrations of various arsenic species in solid samples, such as chicken meat, requires appropriate extraction (Amaral et al. 2013; Feldmann et al. 1999; Leermakers et al. 2006; Rubio et al. 2010; Santos et al. 2013) followed by efficient separation and sensitive detection (B'Hymer and Caruso 2004; Chen et al. 2014; Chen and Belzile 2010; Dietz et al. 2007; Feldmann et al. 1999; Ma et al. 2014; Maher et al. 2012; Santos et al. 2013; Yehiayan et al. 2009). Most of the highly sensitive methods for arsenic speciation have used high performance liquid chromatography (HPLC) separation with detection of atomic fluorescence, inductively coupled plasma mass spectrometry (ICPMS), and electrospray ionization mass spectrometry (ESIMS) (McSheehy et al. 2002, Whaley-Martin 2012; B'Hymer and Caruso 2004; Chen et al. 2014; Chen and Belzile 2010; Dietz et al. 2007; Feldmann et al. 1999; Ma et al. 2014; Maher et al. 2012; Santos et al. 2013;

Yehiayan et al. 2009) . For solid samples, such as chicken meat, arsenic species must be extracted into solution amenable for HPLC analysis. The method of extraction must be efficient and must not change the pertinent property of the original arsenic species. Methods such as ultrasound water-bath assisted extraction (Moreda-Piñeiro et al. 2011b) and microwave-enhanced extraction (Wolle et al. 2014) have been employed to enhance the extraction of arsenic from seafood. But extraction of arsenic species from chicken meat has not been fully tested.

The quantitative and reproducible extraction of arsenic species was considered as the most challenging aspect in the speciation analysis of organoarsenicals relevant to chicken feed (Liu et al. 2013). Both organic and inorganic arsenic species are expected to be present in chicken breasts. Harsh digestion conditions could alter arsenic species, whereas mild extraction conditions might not efficiently release the arsenic species that could potentially be bound to proteins. To maintain the integrity of arsenic species, I chose to use mild pH and temperature conditions. To enhance extraction efficiency, I incorporated proteolytic enzymes to digest proteins. Previous research has demonstrated several applications of enzymatic extraction for chemical speciation studies (Bermejo et al. 2004; Bermejo-Barrera 2002; Lu et al. 2009; Caruso et al. 2001; Vale et al. 2008; Sanz et al. 2007). Enzymes such as pronase E (Bermejo-Barrera 2002), amylase (Caruso et al. 2001), and lipase (Sanz et al. 2007) have been used for extraction of arsenic species from seafood, freeze-dried apple, and hair samples, respectively. Enzymatic extraction has not been demonstrated for arsenic speciation in chicken meat.

I chose to test a number of proteolytic enzymes, such as bromelain, papain, pepsin, proteinease k, and trypsin, for extraction of arsenic species from chicken breast meat.

Because of their proteolytic activities, some of these proteases have been widely used for meat tenderization. For example, papain can degrade both myofibrillar and collagen proteins. From plant sources, papain is relatively inexpensive among the various proteolytic enzymes.

The concentration of arsenic in chicken meat has been reported to be on the order of sub-mg kg^{-1} (Conklin et al. 2012; Lasky et al. 2004). The concentrations of individual arsenic species in chicken meat are expected to be on the order of μg kg^{-1}. Therefore, highly sensitive detection and efficient separation approaches are required to enable determination of trace amounts of individual arsenic species in chicken breast meat. I chose HPLC separation because of its demonstrated capability for resolving various arsenic species. To achieve highly-sensitive quantitation and identification of arsenic species, I incorporated HPLC with simultaneous detection by both ICPMS and ESIMS. I report here speciation of arsenic in chicken breast meat using the method of enzyme-assisted extraction, HPLC separation, and mass spectrometry detection. The analytical method should contribute to improving human exposure assessment associated with arsenic intake from chicken meat.

2.2. Experimental methods

2.2.1. Instrumentation

A PRP-X110S anion exchange column (7 μm particle size, 150×4.1 mm; Hamilton, Reno, NV), installed with an Agilent 1100 series HPLC system (Agilent Technologies, Germany), was used for separation of arsenic species. An Agilent 7500cs ICPMS system (Agilent Technologies, Japan) and an AB SCIEX 5500 QTRAP ESIMS system (Concord,

ON, Canada) were used for detection. The operating conditions for these two mass spectrometers are summarized in Table 2.1. The eluent from the HPLC column was split so that 80% of the flow (1.6 mL/min) was introduced to ICPMS and 20% of the flow (0.4 mL/min) was introduced to ESIMS. This split was achieved by using a 300 series stainless steel tee (Valco Canada, Brockville, ON, Canada). A schematic of the HPLC coupled with ICMPS and ESIMS is shown in Figure 2.1.

2.2.2. Reagents and arsenic standards.

Stock solution (10 mg/L) of arsenobetaine (AsB), arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}), N-acetyl-4-hydroxy-m-arsanilic acid (NAHAA), and 3-nitro-4-hydroxyphenylarsonic acid (Rox) were prepared from arsenobetaine (98% purity, Tri Chemical Laboratories Inc., Japan), sodium m-arsenite (97.0%, Sigma, St. Louis, MO), sodium arsenate (99.4%, Sigma), monosodium acid methane arsonate (99.0%, Chem Service, West Chester, PA), cacodylic acid (98%, Sigma), N-acetyl-4-hydroxy-m-arsanilic acid (Pfaltz and Bauer Inc.), and 3-nitro-4-hydroxyphenylarsonic acid (98.1%, Sigma-Aldrich, St. Louis, MO), respectively. The concentrations of these arsenic species were calibrated against a primary arsenic standard (Agilent Technologies, U.S.) and were determined using ICPMS. Standard solutions of arsenic species (0.1, 0.5, 1, 5, and 10 µg/L) were freshly prepared daily by serial dilution from the stock solutions. Milli-Q18.2 MΩ·cm deionized water (Millipore Corporation, Billerica. MA) and HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ) were used as solvents. Proteinase K was purchased from Qiagen. All other proteases and trifluoroacetic acid (TFA) (99%) were purchased from Sigma.

2.2.3. Standard reference materials

Three standard reference materials (SRM) were used in this study. SRM1640a (trace elements in natural water) was obtained from National Institute of Standards and Technology (Gaithersburg, MD). It contains inorganic arsenate and arsenite, and the certified value is 8.075 ± 0.070 μg/L for total arsenic. In the present study, SRM1640a was used for quality control in calibration and in the determination of total arsenic. DROM-4 (dogfish muscle) was obtained from National Research Council of Canada (Ottawa, ON, Canada). The certified value (6.80 ± 0.64 mg/kg) is for total arsenic concentration. DROM-4 was used in this study for the quality control of the acid digestion of the sample followed by the determination of total arsenic concentration. BCR627 (tuna fish meat) was obtained from Institute for Reference Materials and Measurements (IRMM), Belgium. It has certified concentrations of arsenobetaine (52 ± 3 μmol/kg) and dimethylarsinic acid (2.0 ± 0.3 μmol/kg). BCR627 was used for assessing the extraction efficiency followed by speciation analyses of AsB and DMA.

2.2.4. Chicken meat samples

One set of chicken breast meat samples were collected from a 35-day poultry feeding study that was conducted at the Poultry Research Centre, University of Alberta. An exposed group of 800 chickens, randomly divided and housed in 8 pens (100 chickens per pen), were fed a Roxarsone-supplemented diet during the first 4 weeks (28 days), and then fed a basal diet during the last week (day 29-35) of the feeding experiment. A control group of anther 800 chickens, housed in another 8 pens, were fed a basal diet that was not supplemented with Roxarsone throughout the entire 5-week feeding period. On each of the

pre-designed sampling days, one chicken from each of the 16 pens was euthanized, and breast meat samples from each of the 16 chickens were collected. In the present study of developing an analytical method, breast meat samples from chickens housed in six pens were tested. Each breast meat sample was homogenized separately in a blender. The homogenized samples were freeze-dried in a freeze dryer (FTS Systems, Stone Ridge, NY, USA). The freeze-dried samples were stored as crumbled powder in a -20 ^{o}C freezer. Several chicken breast meat samples from day 21 and day 28 of the feeding experiment were tested in the present study. A composite sample was used to examine the extraction efficiency of various arsenic species. This composite sample was prepared by combining a fraction of freeze-dried samples from 6 different chickens collected on Day 28 of the feeding experiment.

Another set of chicken breast meat samples were obtained from a local grocery store in Edmonton, Alberta, Canada. These samples were used to test the effect of sample matrix in the determination of arsenic species using the method described in this paper.

2.2.5. Extraction of arsenic species

Several methods of extraction were compared. These methods involved the use of methanol/water, trifluoroacetic acid (TFA), protease enzymes (papain, pepsin, trypsin, proteinase k, and bromelain), and ultrasonication. Ultrasonication followed by enzyme-assisted extraction using papain was the final method of choice for extraction of arsenic species from chicken breast meat samples.

Procedures for ultrasonication were modified from the method of Sanz et al. (Sanz et al. 2005). A Misonix sonicator 4000 (Qsonica, LLC. CT, USA) was used.

Approximately 0.3 g of freeze-dried powdered chicken breast sample was weighted with a precision of 0.1 mg, and was added into a 15-mL centrifuge tube. Papain (30 mg) and deionized water (5 mL) were added to the tube, and the tube was then placed in a 60-^{o}C water bath. The sample solution was then sonicated at 30% amplitude and 20 KHz for 2 min, followed by a stop for 1 min, and further sonication for another 2 min. The mixture of the sample and papain in deionized water was incubated in a 60-^{o}C water bath for 6 hours. After the incubation, the temperature of the water bath was increased to 95 ^{o}C to stop the enzyme activity. The tube containing the sample extracts was centrifuged at 4000 g for 10 min. The supernatant was removed from the tube and filtered through a 0.45 μm membrane prior to HPLC analysis.

Extraction with the assistance of only enzymes (papain, pepsin, trypsin, proteinase k, and bromelain), and not with sonication, was performed similarly, except that the amount of the enzymes and the incubation temperature varied with the enzymes. A freeze-dried sample (approximately 0.3 g weighed with a precision of 0.1 mg) was incubated with 30 mg trypsin in 5 mL deionized water at 37 ^{o}C for 6 hours, or incubated with 30 mg papain in 5 mL deionized water at 60 ^{o}C for 6 hours, or incubated with 30 mg bromelain in 5 mL deionized water at 55 ^{o}C for 6 hours. Extraction with proteinase k involved 0.3 g freeze-dried sample, 480 μL proteinase k, and 4.52 mL deionized water, and incubation at 60 ^{o}C for 6 hours. For pepsin-assisted extraction, 0.3 g freeze-dried sample and 30 mg pepsin were added to 5 mL deionized water containing 0.5% HCl, and the solution was incubated at 37 ^{o}C for 6 hours.

Extraction with trifluoroacetic acid (TFA) was tested on several samples. A freeze-dried chicken breast sample (0.3 g) was weighed into a 15-mL centrifuge tube, to which 5

mL of 2 M TFA was added. The tube was then placed in a 95-^{o}C water bath for 6 hours (Ackerman et al. 2005).

For comparison, samples were also extracted with a mixture of 50% water and 50% methanol. A freeze-dried sample (0.3 g) was added to a 15-mL centrifuge tube, to which 10 mL of water and methanol solution (at 1:1 volume ratio) was added. This mixture was placed into an ultrasonication water-bath and was sonicated for 40 min. After centrifugation, the supernatant was dispensed into a 50-mL beaker. Another 10 mL of water and methanol solution was added to the centrifuge tube, and the content was sonicated for 40 min. The supernatant was combined into the 50-mL beaker. The process of extraction was repeated for a third time, and the extract was combined in the beaker. The beaker containing the combined extracts was placed on a hot plate that was heated to 80 ^{o}C to allow for evaporation until about 0.5 mL solution remaining. The condensed extract was quantitatively transferred to a 15-mL tube and was diluted to 5 mL. The solution was filtered through a 0.45 μm membrane prior to HPLC analysis. The residue was digested with nitric acid and the total arsenic concentration in the residue was determined using ICPMS.

2.2.6. Acid digestion and determination of total arsenic

The method of acid digestion was modified from the EPA method 3050B (EPA 1996). Briefly, a freeze-dried powder sample (0.3 g) was weighed into a 50-mL beaker, to which 25 mL concentrated nitric acid (HNO_3) was slowly added. The beaker was covered with a watch glass and left in a fume hood overnight. In the following morning, the beaker was placed on a hot plate that was heated to 200 ^{o}C. Digestion was complete when the

solution became transparent and it was yellowish in color. The watch glass was then removed to allow for evaporation of the acid from the beaker until about 0.5 mL solution remaining. The residual solution was quantitatively transferred to a 15-mL tube and diluted to 5 mL with deionized water. The solution was either diluted with deionized water by another 10 times or directly analyzed for total arsenic using ICPMS. For quality assurance, standard reference material DROM-4 (dogfish muscle) was digested in the same manner and analyzed using ICPMS. Standard reference material SRM1640a was also used for quality assurance.

For determination of total arsenic in extracts, each extract was diluted by 10 times and the diluted solution was divided into 3 aliquots. SRM 1640a was added to two aliquots, making these aliquots to contain additional 5 μg/L and 10 μg/L arsenic, respectively. Total arsenic concentration in the extract was determined using ICPMS and the standard addition method to minimize any matrix effect.

The concentrations of arsenic in the extract and in the digested residue of the same sample were compared with the total arsenic concentration in the acid-digested sample. The sum of arsenic in the extract and in the residue was consistent with the total arsenic. A comparison between the concentration of arsenic in the extract and the total arsenic concentration provided information on the extraction efficiency (Table 2.2).

2.2.7. Speciation analysis using HPLC separation and both ICPMS and ESIMS detection

HPLC separation of arsenic species was modified from the method of Peng et al.(Peng et al. 2014). In brief, an anion exchange column (Hamilton PRP-X110S) was used

along with two mobile phases and a gradient elution program. Mobile phase A contained 5% methanol and 95% deionized water. Mobile phase B contained 5% methanol and 60 mM NH_4HCO_3 in deionized water, pH 8.75. The gradient program started with 100% mobile phase A and 0% mobile phase B. Mobile phase B was linearly increased to 40% during the first 10 min, with corresponding decrease of mobile phase A to 60%. From 10 min to 17 min, mobile phase B continued to increase linearly to 100%. From 17 min to 18 min, mobile phase B returned to 0% and mobile phase A increased to 100%. 100% mobile phase A remained to the end of the chromatographic run (22 min). The flow rate was 2 mL/min.

The HPLC effluent was split to ICPMS (1.6 mL/min) and ESIMS (0.4 mL/min) for simultaneous ICPMS and ESIMS detection (Figure 2.1). The operating parameters for these two mass spectrometers are shown in Table 2.1. ICPMS provided element specific detection of arsenic at m/z 75. ESIMS detection was based on multiple reaction monitoring (MRM) of the parent and fragment ions. The selected ion transitions for the MRM detection of seven arsenic species are summarized in Table 2.3. The detection limit (LOD) and limit of quantification (LOQ) are summarized in Table 2.4.

2.3. Results and discussion

2.3.1. HPLC separation with simultaneous ICPMS and ESIMS detection of arsenic species

To enable speciation of trace levels of arsenic in chicken meat, I first developed a method using HPLC separation followed by simultaneous detection with both ICPMS and ESIMS. An 80% fraction of the HPLC effluent was split to ICPMS and the remaining 20%

of the HPLC effluent flow was introduced to ESIMS (Figure 2.1). Therefore, a single HPLC analysis gives rise to two chromatograms, because of the simultaneous on-line detection by ICPMS (Figure 2.2a) and by ESIMS (Figure 2.2b). Each of the seven arsenic species between the two chromatograms has an identical retention time (Figure 2.2). The ICPMS provides element-specific detection of arsenic (m/z 75), while the ESIMS allows for detection of molecular and fragment ions of the arsenic compounds through multiple reaction monitoring (MRM).

ESIMS detection of the characteristic MRM transitions (Table 2.3) for each of the arsenic species enables the specific detection of individual arsenic species present in a sample containing seven arsenic species (Figure 2.3b-h). Except for inorganic arsenite that has only one strong MRM transition (125/107, Figure 2.3c), other six arsenic species can be detected with two characteristic MRM transitions, including arsenobetaine (179/105 and 179/120, Figure 2.3b), DMA (137/107 and 137/122, Figure 2.3d), MMA (139/107 and 139/124, Figure 2.3e), inorganic arsenate (141/107 and 141/123, Figure 2.3f), N-acetyl-hydroxy-m-arsanillic acid (274/123 and 274/165, Figure 2.3g), and Roxarsone (262/107 and 262/123, Figure 2.3h). Therefore, the simultaneous ICPMS (Figure 2.3a) and ESIMS (Figure 2.3b-h) MRM detections provide complementary information, enhancing determination of trace arsenic species. The seven arsenic species shown in Figure 2.3 was spiked to deionized water containing papain that was subsequently used for enzyme-assisted extraction of chicken samples.

2.3.2. Extraction of arsenic species from a standard reference material

Arsenic species in solid samples must be extracted into solutions, making the samples amenable to HPLC analysis. To develop an extraction method, I initially examined extraction of arsenic species present in a standard reference material, BCR627 (tuna fish meat). I choose this standard reference material because it has certified values for AsB (52 ± 3 μmol/kg or 3.90 ± 0.23 mg As/kg) and DMA (2.0 ± 0.3 μmol/kg or 0.15 ± 0.02 mg As/kg), the two predominant arsenic species in this tuna fish meat sample. There is no chicken standard reference material with certified arsenic speciation information.

Using a mixture of water and methanol at a 1:1 volume ratio, I was able to extract 3.15 ± 0.71 mg/kg of total arsenic into the methanol/water solution. This represents an extraction efficiency of 66%. Acid digestion and analysis of the residue using ICPMS showed the presence of 1.34 ± 0.15 mg/kg of total arsenic remaining. The sum of arsenic (4.49 mg/kg) in the extract and in the residue is consistent with the total certified value of 4.80 ± 0.30 mg/kg. Using this mass balance approach, I demonstrate that there is no loss or contamination of the sample during the extraction and handling processes.

HPLC-ICPMS analysis of the methanol/water extract showed that the concentration of AsB was 2.62 ± 0.60 mg/kg and the concentration of DMA was 0.12 ± 0.02 mg/kg. The certified concentrations of AsB and DMA are 3.90 ± 0.23 mg/kg and 0.15 ± 0.02 mg/kg, respectively. Comparisons between the certified values and the measured concentrations of AsB and DMA in the extract suggest that the extraction efficiencies were 67% for AsB and 80% for DMA. This degree of extraction efficiency is commonly experienced by others, reporting extraction efficacy ranging from 30% to 80% (Rubio et al. 2010; Amaral et al. 2013; Sanz et al. 2005).

2.3.3. Extraction of arsenic species from chicken meat

The preliminary analysis of total arsenic in chicken meat showed that arsenic concentrations were an order of magnitude lower than that in the tuna fish standard reference material BCR627. Initially, I tested extraction using a mixture of water and methanol at 1:1 volume ratio. From a chicken breast meat sample that contained 0.75 ± 0.01 mg/kg (748 ± 10 μg/kg) total arsenic, I could only extract 0.21 ± 0.02 mg/kg (208 ± 25 μg/kg) total arsenic, representing an extraction efficiency of 28%. The concentration of total arsenic remaining in the residue was 0.47 ± 0.01 mg/kg (or 465 ± 12 μg/kg). For the determination of arsenic speciation present in chicken meat at trace concentrations, extraction of arsenic species from chicken meat must be improved.

I explored the use of proteases to improve the extraction efficiency. Enzymes from plant extracts, such as papain, bromelain and ficin, have been widely used for meat tenderization because of their proteolytic activities (Ashie et al. 2002; Bekhit et al. 2014; Wang et al. 1958). Papain, for example, has low substrate specificities and can catalyze the hydrolysis of a wide range of bonds including peptide, amide, ester, and thiol ester (Bekhit 2014). Papain can significantly degrade both myofibrillar and collagen proteins, yielding protein fragments of several sizes (Ashie et al. 2002). I reasoned that the use of proteolytic enzymes could enhance the extraction of arsenic species from chicken meat.

Figure 2.4 shows chromatograms from the HPLC-ICPMS analyses of arsenic species extracted from chicken breast meat. With the use of either papain (Figure 2.4a) or pepsin (Figure 2.4b), a number of arsenic species were extracted from the meat sample. HPLC-ICPMS analyses of the extract sample spiked with seven arsenic standards show the presence of these arsenic species as well as five new arsenic species (U1-U5).

I then compared the use of papain, bromelain, trypsin, pepsin, and protease K with water/methanol extraction and trifluoroacetic acid (TFA) extraction (Table 2.2). With the use of papain or proteinase K, the extraction efficiency was increased to 55-58% from 28% when the methanol/water mixture was used for extraction. The use of trypsin, bromelain, and pepsin also improved the extraction efficiency as compared to the methanol/water extraction.

Figure 2.5 shows chromatograms from the HPLC-ICPMS analyses of chicken meat extracts when methanol/water, papain, trypsin, bromelain, pepsin, proteinase K, and TFA were used for extraction. As compared to water/methanol extraction (Figure 2.5a), the use of enzyme-assisted extraction resulted in extracting larger number of arsenic species and higher concentrations. For example, with the use of papain-assisted extraction (Figure 2.5b), 10 arsenic species were extracted into the solution, as compared to 8 arsenic species extracted into water/methanol mixture (Figure 2.5a). The concentrations of arsenic species in the papain-assisted extract were 2 times of those in the water/methanol extract, evident from the doubled intensities of peaks in Figure 2.5b as compared to those in Figure 2.5a. These results are consistent with the preliminary results of the overall extraction efficacy increase from 28% to 55% (Table 2.2).

A comparison of the use of the five enzymes indicates that papain and proteinase K achieved higher extraction efficiency (Table 2.2 and Figure 2.5). This is consistent with previous reports showing that papain performed better than other proteases, such as trypsin, pepsin, bromelain, ficin, and calpain, in digesting meat (Sullivan and Calkins 2010; Iizuka and Aishima 2000; Miyada and Tappel 1956).

Trifluoroacetic acid (TFA) has been used previously to treat samples prior to the determination of total arsenic and/or arsenic species (Ackerman et al. 2005). I also tested the use of TFA for the extraction of arsenic species from chicken meat. However, the use of TFA for extraction resulted in the conversion of As^{V} to As^{III} (Figure 2.5g). This finding is consistent with previous reports showing that an increase in acidity of urine samples with the addition of HCl led to conversion of As^{V} to As^{III} (Feldmann et al. 1999). Therefore, in the subsequent speciation analysis, I did not use TFA for sample treatment.

2.3.4. Optimization of the papain-assisted extraction of arsenic species from chicken meat

Having shown that papain and proteinase K improved the efficiency of extracting arsenic species from chicken meat, I further optimized the extraction conditions. I chose to focus on papain-assisted extraction because papain is much less expensive than proteinase K. The chicken breast meat samples were composites from six chickens receiving roxarsone-containing feed for 21 or 28 days.

I found that the amount of papain necessary for the enzyme-assisted extraction was one tenth of the chicken meat (dry weight). I tested the extraction efficiency after incubation of the chicken meat with papain for 1, 2, 4, 6, and 12 hours. I found that the extraction efficiency reached plateau after four hours of incubation with papain. I further introduced the treatment of the chicken sample using an ultrasonication probe prior to the papain-assisted extraction. Other studies have suggested that ultrasonication could enhance the extraction efficiency, because ultrasonication could help to break the cell membrane (Sanz et al. 2005; Vale et al. 2008). With the combination of ultrasonication and papain-

assisted extraction, the concentrations of AsB, DMA, MMA, Rox, Unknown 1, and Unknown 5 in the extract were increased by 1.7, 1.6, 1.9, 1.1, 1.7, and 2.1 times as compared to those in the papain-assisted extraction only (Table 2.2). Therefore, the overall extraction efficiency was improved from 55% to 90%.

2.3.5. Determination of arsenic species in a composite chicken meat sample

I have further demonstrated an application of the method of ultrasonication, papain-assisted extraction, HPLC separation, and ICPMS/ESIMS detection. Figure 2.6 shows typical chromatograms from the speciation analysis of a composite chicken breast meat sample collected from six chickens on day 28 of the feeding experiment. Detection with ICPMS (Figure 2.6a) shows the presence of 9 peaks, corresponding to 9 arsenic species. ESIMS detection with total ion monitoring (Figure 2.6b) shows predominantly one peak that corresponds to arsenobetaine (Figure 2.6c). Multiple reaction monitoring (MRM) (Figures 2.6c-6f) enables detection of As^{III} (Figure 2.6d), DMA (Figure 2.6e), and Roxarsone (Figure 2.6f).

On the basis of the ICPMS measurements, I obtained the concentrations of arsenic species in the chicken breast meat sample as following: AsB (58 ± 1 μg/kg), As^{III} (55 ± 2 μg/kg), As^{V} (12 ± 1 μg/kg), MMA (2.5 ± 0.3 μg/kg), DMA (26 ± 1 μg/kg), Roxarsone (9.2 ± 0.7 μg/kg), and four unidentified arsenic species (U1: 18 ± 1 μg/kg, U2: 1.2 ± 0.2 μg/kg, U3: 5.3 ± 0.2 μg/kg, and U5: 9.3 ± 1.3 μg/kg, respectively). This chicken breast meat sample was collected from six chickens on day 28 of the feeding experiment. These results show that the method is useful for the arsenic speciation analysis in chicken meat. These concentrations suggest that chicken could be a main source of food arsenic

considering that overall daily intake of arsenic has been reported to be ~ 40 μg per day (Schoof et al. 1993). The method is useful for assessing human exposure to arsenic species and for studying arsenic metabolism.

2.4. Conclusion

I have demonstrated the development and application of an arsenic speciation method that combines ultrasonication with enzyme-assisted extraction, HPLC separation, and simultaneous ICPMS and ESIMS detection. This method enabled the determination of AsB, As^{III}, As^{V}, MMA, DMA, and Rox. Five arsenic species in the chicken breast meat samples remained unidentified. The concentration of the unidentified arsenic species was on the order of μg/kg. Some method of pre-concentration of these arsenic species would be necessary for the characterization and identification of these new arsenic species.

Chicken is the most consumed meat in North America, with a consumption rate of one billion kilograms per year (AAFC 2011; US ERS 2012). The information on the concentration of individual arsenic species in chicken meat is required for understanding human exposure to arsenic species and the potential health risk. Results on the concentrations of specific arsenic species in chicken will be necessary for improving regulatory policy that protects public health. In addition, the mechanisms of Roxarsone metabolism in chicken (or by humans following ingestion of chicken meat) are poorly understood. Further development of analytical methods that enable identification of new metabolites and determination of arsenic species distribution will contribute to an improved understanding of the metabolism of Roxarsone. The knowledge gained will help

improve molecular understanding of arsenic health effects and arsenic speciation in other systems.

2.5. References

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Table 2.1 The optimized operating conditions for ICPMS and ESIMS

ICPMS	ESIMS
Radio frequency power: 1500 W	Ion spray voltage (IS): 4500 V (for
Sample depth: 7.0 mm	positive ionization) or -4500 V (negative
Carrier gas: 1 L min^{-1}	ionization)
Makeup gas: 0.13 L min^{-1}	Temperature (TEM): 500 ^{o}C
Spray chamber temperature (TEM): 2 ^{o}C	Curtain gas (CUR): 30 psi
Reaction cell He gas: 3.0 mL min^{-1}	GS1: 50 psi
Monitored m/z: 75	GS2: 50 psi
	CAD: high
	EP: 10 V(positive mode)
	or -10 V (negative)

Table 2.2 Arsenic content in the extract and residue after a chicken meat sample was extracted using different methods. The chicken breast meat sample was a composite from six chickens on Day28 of the feeding experiment.

Extraction Method	As in extract ($\mu g\ kg^{-1}$)	As in residue ($\mu g\ kg^{-1}$)	Extraction efficiency (%)
Total arsenic	748 ± 10		
Water-methanol (1:1)	208 ± 25	465 ± 12	28
Trypsin	307 ± 3	480 ± 68	41
Pepsin	328 ± 8	442 ± 62	44
Bromelain	315 ± 10	383 ± 15	42
Proteinase K	432 ± 57	308 ± 13	58
Papain	415 ± 23	303 ± 7	55 *

*Further improvement by the treatment of chicken samples using an ultrasonication probe with papain-assisted extraction resulted in an overall extraction efficiency of 90%.

Table 2.3 Selected ion transitions for multiple reaction monitoring (MRM) of seven arsenic species and the optimal operating conditions of ESI-MS/MS.

Arsenic species	Polarity	Parent ion (m/z)	Product ion (m/z)	Product ion formula	Decluster potential (DP)	Collision energy (CE)	Cell exit potential (CXP)
AsB	positive	179	105	$(CH_3)_2As^+$	71	37	9
			120	$(CH_3)_3As^+$	71	28	11
As^{III}	negative	125	107	AsO_2^-	-10	-18	-15
DMA	negative	137	107	AsO_2^-	-70	-30	-11
			122	$CH_3AsO_2^-$	-70	-18	-13
MMA	negative	139	107	AsO_2^-	-40	-40	-43
			124	AsO_3H^-	-40	-24	-7
As^V	negative	141	107	AsO_2^-	-15	-53	-10
			123	AsO_3^-	-15	-20	-7
NAHAA	negative	274	165	$C_8H_8NO_3^-$	-45	-36	-11
			123	AsO_3^-	-45	-26	-9
Rox	negative	262	123	AsO_3^-	-30	-38	-11
			107	AsO_2^-	-30	-94	-15

Table 2.4 LODs and LOQs of HPLC-ICPMS for the determination of seven arsenic species.

As species	LODs ($\mu g\ kg^{-1}$, n=7)	LOQs ($\mu g\ kg^{-1}$, n=7)
AsB	1.0	3.1
As^{III}	1.8	5.8
DMA	1.5	4.8
MMA	1.7	5.2
As^{V}	1.7	5.2
NAHAA	1.3	4.2
Rox	1.2	3.2

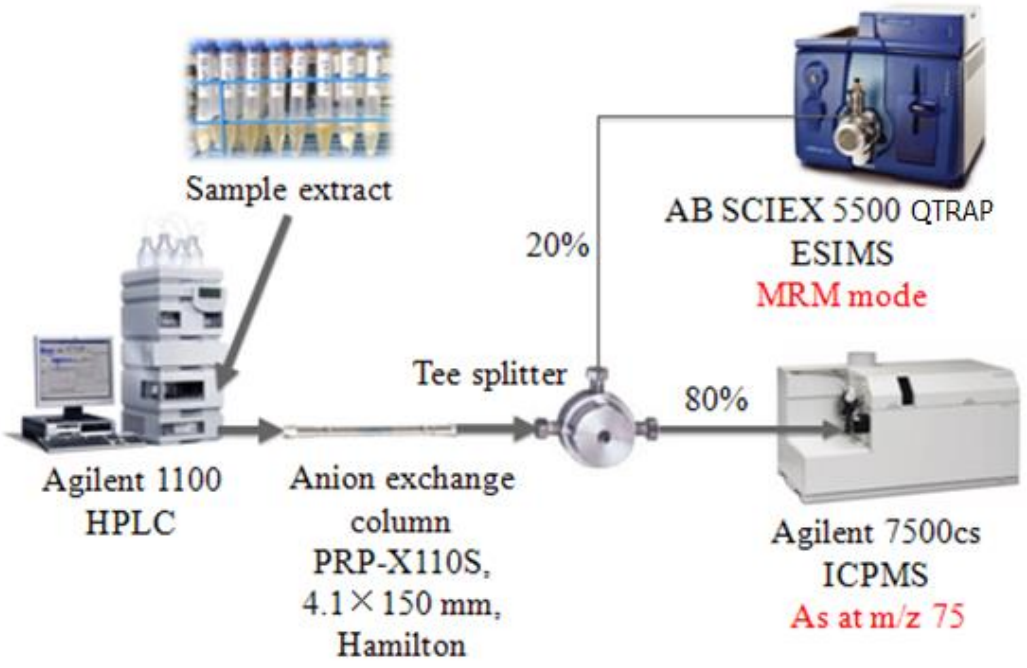

Figure 2.1 Schematic showing split of HPLC effluent to ICPMS and ESIMS for simultaneous on-line detection.

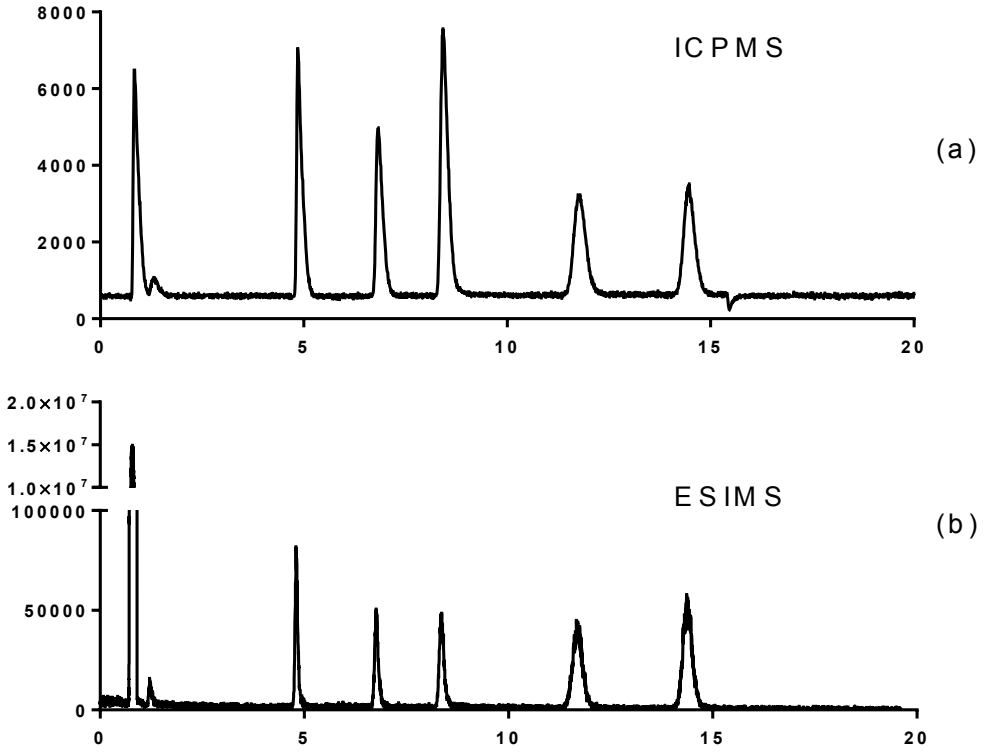

Figure 2.2 Chromatograms obtained from HPLC separation and simultaneous detection of seven arsenic species by ICPMS (a) and ESIMS (b). The concentrations of arsenic species were 10 μg L^{-1}. The arsenic species were added to 5 mL of deionized water containing 30 mg papain. The peaks from left to right correspond to AsB, As^{III}, DMA, MMA, As^{V}, NAHAA, and Rox.

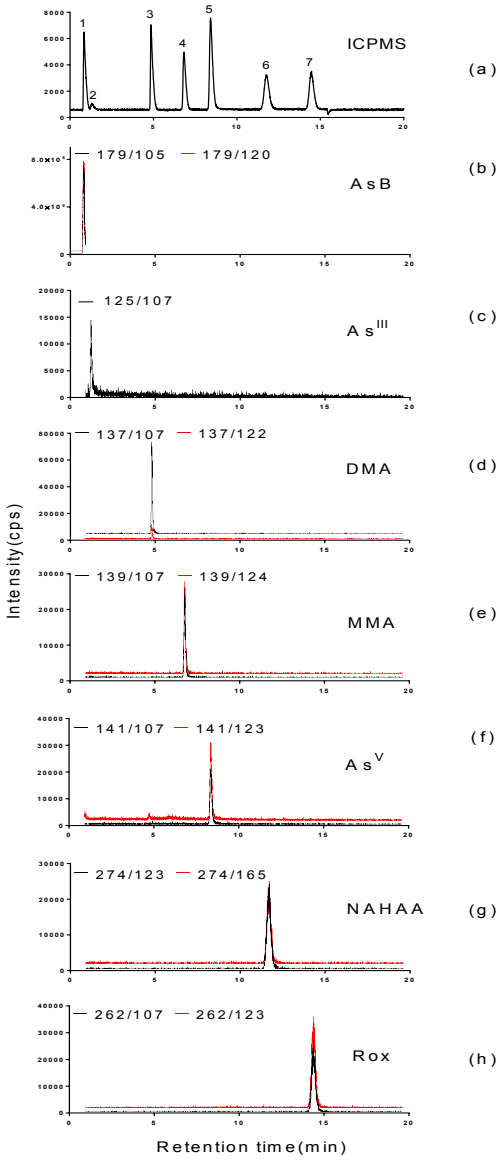

Figure 2.3 Chromatograms from a HPLC-ICPMS/ESIMS analysis of seven arsenic species in 5 mL deionized water containing 30 mg papain. A strong anion exchange column (PRP-X110S) was used for separation. The mobile phase was 60 mM ammonium bicarbonate and 5% methanol at pH 8.75. Arsenic at m/z 75 was monitored with ICPMS (a). ESIMS detection with multiple reaction monitoring (MRM) mode was operated under positive ionization for AsB (b) and negative ionization for the other six arsenic species (c-h). The seven peaks correspond to AsB (peak 1), inorganic As^{III} (peak 2), DMA (peak 3), MMA (peak 4), inorganic As^{V} (peak 5), NAHAA (peak 6), and Roxarsone (peak 7).

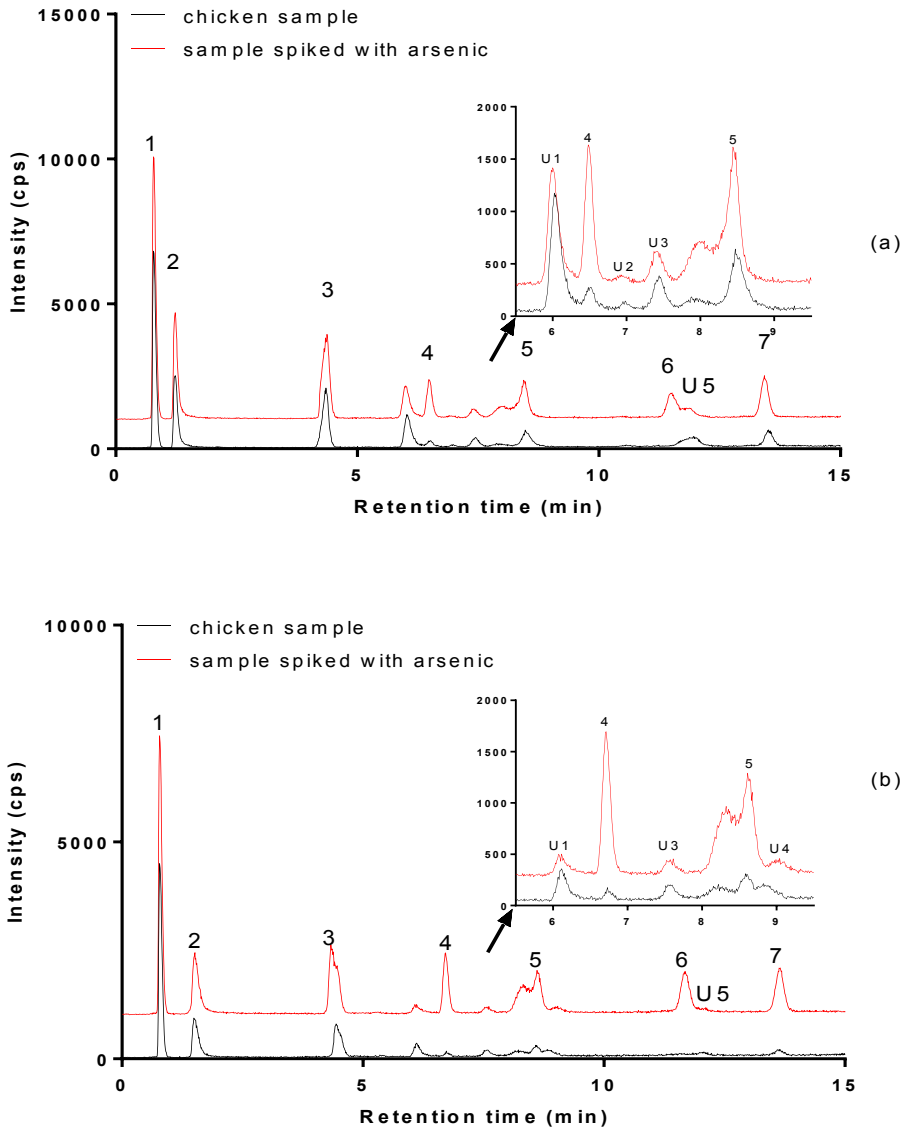

Figure 2.4 HPLC-ICPMS analyses of Day28 chicken breast samples. Chromatograms from the analyses of chicken samples are compared with those of seven arsenic species standards spiked into these chicken samples. The peaks labeled with numbers 1 through to 7 correspond to AsB, As^{III}, DMA , MMA, As^{V}, NAHAA and Rox, respectively. The five peaks labeled with U1 through to U5 correspond to new arsenic species with unidentified chemical structures. The chicken breast meat sample was either extracted with either papain papain digestion (a) or pepsin digestion (b).

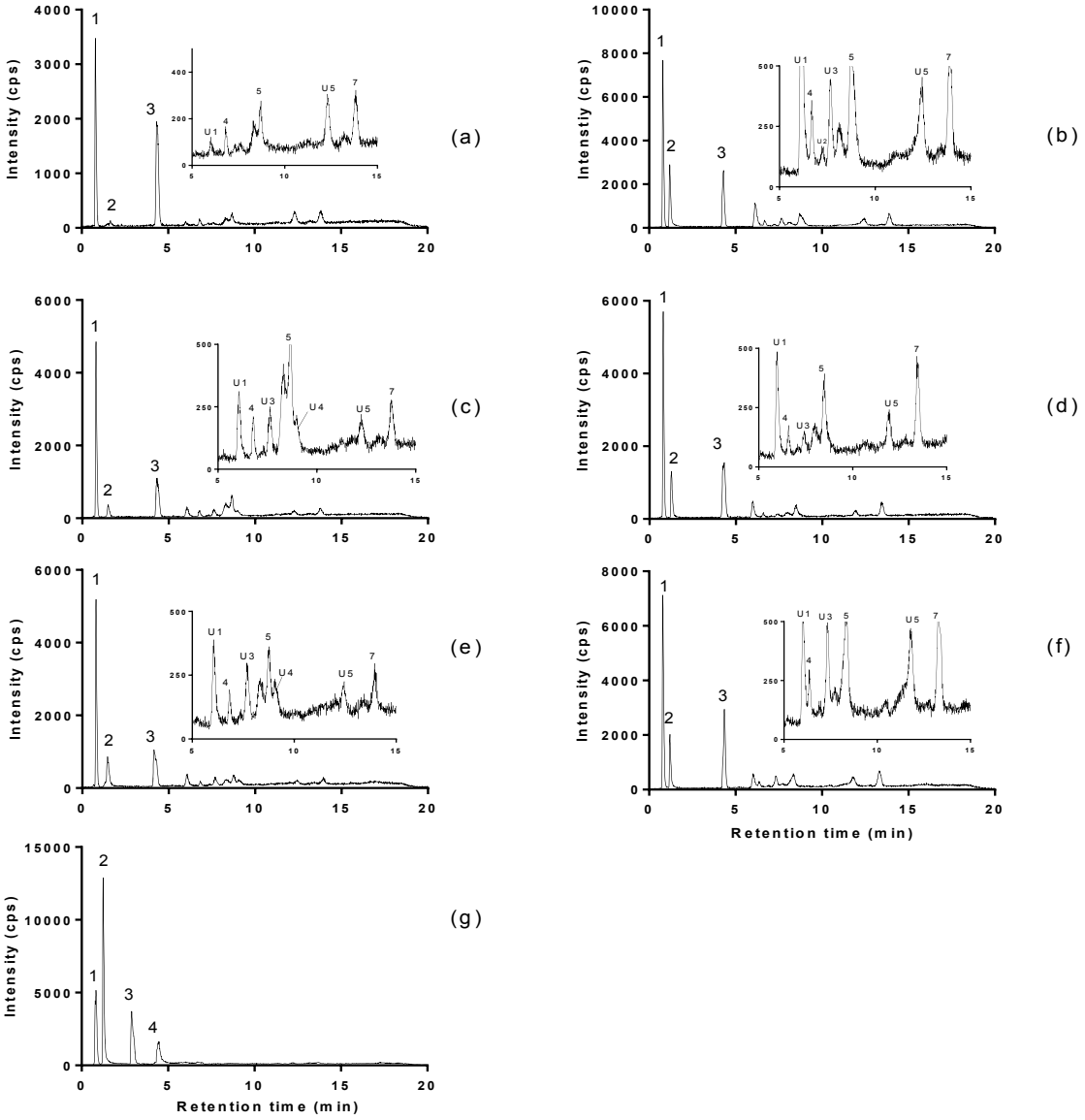

Figure 2.5 Chromatograms obtained from HPLC-ICPMS analyses of a chicken meat sample after different extraction methods. The peaks labeled with numbers 1 through to 7 correspond to AsB, As^{III}, DMA , MMA, As^{V}, NAHAA and Rox, respectively. The unidentified arsenic species are labelled with U1 through to U5 in the inset. The extraction methods were (a) water-methanol, (b) papain, (c) trypsin, (d) bromelain, (e) pepsin, (f) proteinase K, and (g) TFA.

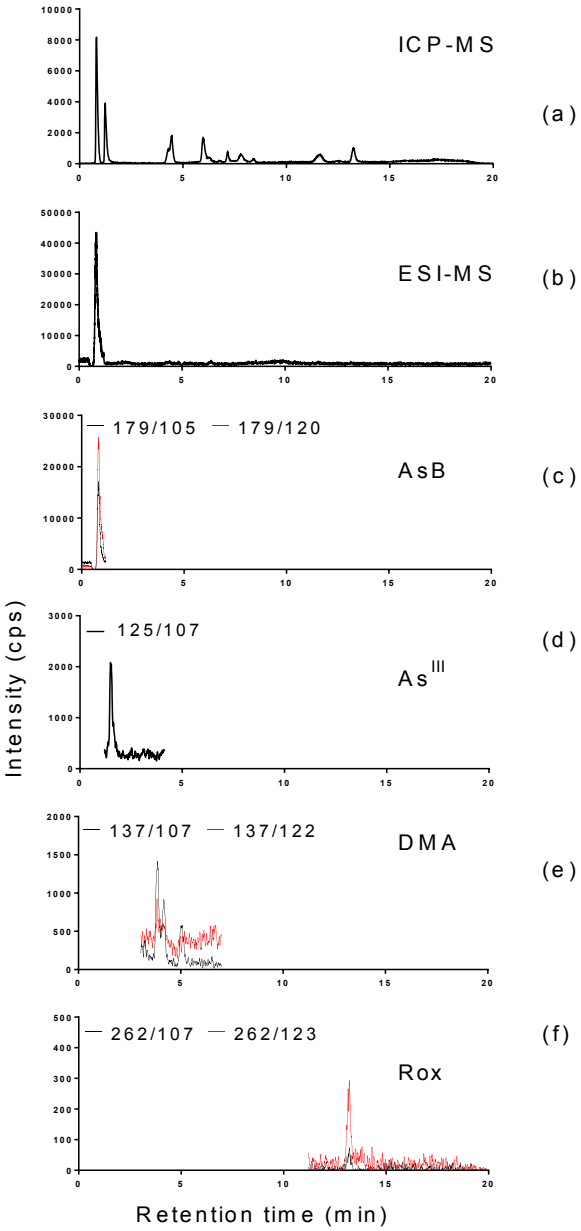

Figure 2.6 Chromatograms obtained from HPLC separation and simultaneous ICPMS (a) and ESIMS (b-f) detection for the determination of arsenic species in a chicken breast meat sample. The sample was obtained on Day 28 of the feeding experiment. AsB, As^{III}, DMA and Rox are detectable using the MRM mode of ESI-MS/MS.

Chapter 3. Arsenic species in chicken breast: temporal variations of metabolites, elimination kinetics, and residual concentrations [2]

3.1. Introduction

Since 1944 when the United States Food and Drug Administration (FDA) first approved the use of 3-nitro-4- hydroxyphenylarsonic acid (Roxarsone, Rox) as an animal feed additive, this organoarsenic compound has been extensively used in the poultry industry for more than 60 years to alleviate coccidiosis, promote growth and weight gain, and improve pigmentation of chickens (Chapman and Johnson 2002; Kowalski and Reid 1975; US FDA 2015a). However, there have been considerable concerns over the use of Rox because of potential human exposure to arsenic species through the consumption of chicken (Conklin et al. 2012; Kawalek et al. 2011; Lasky et al. 2004; Lasky 2013; Nachman et al. 2013). From 1999, European Union ceased the use of arsenicals as feed additives (European Commission 1999). In 2011, an US FDA study (Kawalek et al. 2011) reported that feeding of broiler chickens with Rox attributed to the increased concentrations of inorganic arsenicals in chicken livers. In response to the FDA study, the manufacturer of Rox in the US has voluntarily suspended its supplies (US FDA 2015a). In 2013, US FDA withdrew the approval of Rox (US FDA 2013). However, Rox continues to be legally used in many other countries (Huang et al. 2014; Yao et al. 2013).

Although several studies have reported on the concentration of arsenic in Rox-fed chickens or in chicken meat purchased from food markets (Batista et al. 2012; Doyle and

[2] A version of this chapter has been published in *Environ. Health Perspect.* 2016, 124:1174–1181. Dr. Martin J. Zuidhof's group maintained and cared for the chickens and Dr. Zuidhof was involved with the statistical analyses and revision of the manuscript. Xiufen Lu, Dr. Hanyong Peng, and Dr. Xing-Fang Li assisted with the study design and data analysis. Dr. X. Chris Le contributed to the concept formation and manuscript revision.

Spaulding 1978; Jelinek and Corneliussen 1977; Lasky et al. 2004), the information on the specific arsenic species is limited (Mao et al. 2011; Pizarro et al. 2003; Polatajko and Szpunar 2004; Sanchez-Rodas et al. 2006; Sanz et al. 2005). Determining the concentrations of individual arsenic species is important because the toxicity of arsenic is highly dependent on its chemical species. The median lethal concentrations of arsenic species vary by several orders of magnitude from the most toxic to the least toxic arsenic species (Charoensuk et al. 2009; Naranmandura et al. 2011; Shen et al. 2013; Styblo et al. 2000). Though Rox itself is of low toxicity to the test animals (Sullivan and Al-Timimi 1972), its toxicity to human is not well understood. Furthermore, it is not clear how much other arsenic metabolites may be produced in Rox-fed chicken. It is crucial to determine the magnitude of increases in the concentrations of the more toxic arsenic species, e.g. arsenite (As^{III}).

Chicken is the No.1 meat consumed in North America on a per capita basis, with a supply of 17.7 billion kg per year (AAFC 2013; ERS 2014). It is paramount to assess the concentrations of individual arsenic species in this highly-consumed food. The information will enable assessment of human exposure to arsenic species and determination of the relative contributions of arsenic species from the various sources.

Information on the metabolism of Rox in chicken is very limited (Conklin et al. 2012; Kawalek 2011; Overby and Straube 1965; Peng et al. 2014). Accurately identifying and quantifying arsenic species in chicken meat is challenging due to low concentrations of arsenic species. Therefore previous work has often focused on chicken livers and feces that contained higher concentrations of arsenic species (Conklin et al. 2012; Falnoga et al. 2000; Kawalek 2011; Peng et al. 2014; Rosal et al. 2005; Salisbury et al. 1991). Recent

work of Nachman et al. (2013) determined arsenic species in chicken samples collected in a US-based market basket survey. This study found the concentrations of inorganic arsenicals were higher in conventional chickens (geometric mean (GM) = 1.8 μg/kg; 95% confidential interval (CI): 1.4, 2.3) than in antibiotic-free (GM = 0.7 μg/kg; 95% CI: 0.5, 1.0) or organic (GM = 0.6 μg/kg; 95% CI: 0.5, 0.8) chickens. The study also found a correlation between the higher concentrations of inorganic arsenicals (GM = 2.3 μg/kg; 95% CI: 1.7, 3.1) in the presence of Rox (GM = 1.3 μg/kg; 95% CI: 1.0, 1.7) in the chicken samples compared to the concentrations of inorganic arsenicals (GM = 0.8 μg/kg; 95% CI: 0.7, 1.0) in Rox-negative samples. This correlation suggests that feeding of Rox may increase concentrations of As^{III} in chicken meat. This finding, together with the 2011 US FDA study (Kawalek 2011), suggests that Rox may be partially biotransformed to inorganic arsenicals in the chicken body. However, it is still unknown whether feeding of Rox increases concentrations of other arsenic species in chicken meat. Moreover, how these arsenic species change with the growth of chicken fed Rox remains a question.

To fill the knowledge gap, a controlled feeding study that involved 1600 chickens of two common commercial strains was initiated. In the first four weeks, half of the chickens (800) were fed a diet supplemented with Rox and the other 800 chickens were fed a control diet. This design allows us to study the uptake and metabolism of Rox. In the final week, all chickens were fed Rox-free diet. This allows us to study the elimination kinetics over the 7-day period. I determined whether the feeding of Rox increased arsenic metabolites, e.g., arsenite and dimethylarsinic acid (DMA^V), in chicken breasts and the degree to which arsenic metabolites were eliminated from chicken breast meat after the feeding of Rox stopped.

3.2. Experimental methods

3.2.1. Chicken breast meat samples

Chicken breast meat samples were collected from a 35-day poultry feeding study that was conducted at the Poultry Research Centre, University of Alberta. The chickens were fed by Dr. Martin Zuidhof's group. The feeding experiment started when the chickens were hatched (day 0). A total of 1600 chickens, of two commercial broiler strains (Ross 308 and Cobb 500) were used. These 1600 chickens were equally divided into Rox-fed group and control group. The control group of 800 chickens, randomly divided and housed in 8 pens (100 chickens per pen), was fed a basal diet that was not supplemented with Roxarsone throughout the entire 5-week feeding period. The basal (control) diet had trace concentrations of arsenobetaine (AsB) (average 0.03-0.1μg/g), arsenate (As^{V}) (0.04-0.1 μg/g), and DMA^{V} (0.03-0.04 μg/g), and no detectable As^{III} or monomethylarsonic acid (MMA^{V}). The presence of AsB was due to the inclusion of a fish supplement as a protein source in the feed. The trace concentrations of arsenic in the basal diet are consistent with background levels commonly found in animal feeds. The Rox-fed group of another 800 chickens, randomly divided and housed in another 8 pens (100 chickens per pen), were fed a Roxarsone-supplemented diet during the first 28 days (4 weeks), and then fed the basal diet during the last week (day 29-35). The Roxarsone-supplemented diet was prepared from the basal diet with the addition of Roxarsone (18 ± 1 μg/g measured as arsenic), a standard supplementation dose in common poultry practice (USFDA 2015b). The last week of feeding without Roxarsone supplementation exceeds FDA regulations of withdrawal of Roxarsone for 5 days prior to processing in order to allow elimination of arsenic from the chicken bodies. Drinking water from the same source (<1 μg/L arsenic)

was available to all the chickens throughout the entire 35-day period. On days 0, 1, 2, 3, 4, 7, 14, 21, 28, 29, 30, 31, 32, 33, 34, and 35, one bird per pen was euthanized, weighed, and the breast meat was collected. Raw samples were stored at -80^{o}C. In all cases, 16 chickens, one each from the 8 control pens and one each from the 8 Rox-fed pens, were euthanized and sampled on each day. Unfortunately, a few labels came off the sampling bag after freezing. To maintain integrity of the samples, I discarded any samples with questionable labeling. As a consequence, I analyzed 11-16 samples from each of the 16 sampling days, for a total of 229 samples.

All procedures involving animals were reviewed and approved by the University of Alberta Animal Care and Use Committee: Livestock (protocol #094). The feeding design and the age of chickens at breast sample collection are summarized in Table 3.1.

3.2.2. **Determination of arsenic species**.

I analyzed all 229 chicken breast samples (114 from the control chickens and 115 from the Rox-fed chickens) for arsenic speciation using a previously developed method (Liu et al. 2015). Briefly, arsenic species in 0.5 g of freeze-dried samples were extracted using an enzyme-assisted extraction method, and each extract was analyzed in duplicate for arsenic speciation using high performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICPMS). Identities of arsenic species were confirmed using HPLC separation with simultaneous detection by ICPMS and electrospray ionization mass spectrometry. Detailed analytical procedures are included in Supplementary Materials and the method evaluation has been described previously (Liu et al. 2015; Peng et al. 2014a).

The detection limits (LOD), obtained according to the method of US EPA (2015) by seven replicate analyses of chicken breast meat samples spiked with 0.2 µg/L arsenic standards, were 1.0 µg/kg for AsB, 1.8 µg/kg for As^{III}, 1.5 µg/kg for DMA^{V}, 1.7 µg/kg for MMA^{V}, and 1.2 µg/kg for Rox. I used three standard reference materials, SRM1640a (trace elements in natural water), DORM-4 (fish muscle), and BCR627 (tuna), for method development. Because there was currently no chicken meat standard reference material certified for arsenic species, I prepared an in-house reference sample by adding 10 µg/L As standard mixture to a low-arsenic chicken breast meat sample purchased from supermarket. This reference sample was analyzed in triplicates along with each of the seven batches of chicken breast samples analyzed. The measured concentrations were AsB (mean ± SD, 11.1 ± 0.6 µg /L; coefficient of variation (CV)=6%; n=21), As^{III} (12 ± 1 µg /L; CV= 8%; n=21), DMA^{V} (10 ± 1 µg /L; CV= 10%; n=21), MMA^{V} (11 ± 1 µg /L; CV= 10%, n=21), As^{V} (10 ± 1 µg /L; CV= 12%; n=21), and Rox (11 ± 1 µg /L; CV= 11%; n=21). During each batch of analysis, I also analyzed a solution containing 4.5 µg/L AsB, a stable arsenic species. The results (mean ± SD, 4.3 ± 0.2 µg/L; CV=5.7%) indicated good consistency between batches.

3.2.3. Statistical analysis

Statistical analyses were performed by using SPSS version 20.0 (IBM Corp, Armonk, NY). Arithmetic mean, standard deviation, and coefficient of variation of arsenic concentrations were calculated based on the results from replicate analyses of multiple chicken samples in each test group. Sample size (n) in the tables and figures referred to the

number of different chickens. They were each from one of the 16 pens that initially contained 100 chickens per pen.

I used repeated measures two-way analysis of variance (ANOVA) to analyze the effect of Roxarsone treatment and age on the concentration of arsenic species over 35 days. The two-way ANOVA took account of the treatment difference over time in the concentration of arsenic species. I also tested sex (male and female) and strains (Ross and Cobb) on the concentrations of arsenic species; however, their effects were not significant for any arsenic species. Therefore, I did not report the effects of sex and strains in comparison.

Mann-Whitney U-test was used to analyze the significance of difference between Rox-fed and control chickens on day 35. Spearman correlation test was performed to investigate the relationship between different arsenic species. Recognizing that most of the data for As^{III}, Unknown, and Rox in the control group were below detection limit, I conducted Sign test for these three species (Table 3.2) by comparing the range of their concentrations in the Rox-fed chickens to the detection limit. I used repeated measures two-way ANOVA to compare the concentrations of arsenic species on different days (Table 3.3). This allowed us to assess starting on which day after the termination of Rox feeding the concentrations of arsenic species were no longer significantly different.

3.2.4. Pharmacokinetic analysis

The concentrations of arsenic species in chicken breast tissues were determined at each time point (day 28 to 35). The pharmacokinetic parameters, including elimination rate constant (K) and elimination half-life ($t_{1/2}$), were determined by the compartmental method

using Graphpad Prism 6 (GraphPad Software, San Diego, CA, USA). The formula for one-phase decay model is expressed as: $Y= (Y_0 - Yt)*\exp(-K*X) + Yt$, where Y_0 is the Y value when X (time) is zero; Yt is the Y value at infinite time or when Y value does not change significantly with time; K is the rate constant. Half-life is computed as ln(2)/K.

3.3. Results and discussion

3.3.1. Arsenic species found in chicken breasts

Figure 3.1 shows typical chromatograms obtained from the analyses of a pair of chicken breast samples, one from the control group and the other from the Rox-fed group, both collected on day 28 of the feeding experiment. The chicken sample from the control group showed the presence of AsB as the major arsenic species (Figure 3.1, top trace). The chicken sample from the Rox-fed group showed the presence of detectable AsB, As^{III}, DMA^{V}, MMA^{V}, Rox, and an unidentified arsenical (Unknown) (Figure 3.1, bottom trace).

Rox was not detectable in any of the samples from the 114 control chickens, but it was detected in all samples from the 115 Rox-fed chickens. Inorganic arsenite (As^{III}) and methylated arsenicals (DMA^{V} and MMA^{V}) were detected more frequently in the Rox-fed chicken samples than in the control chicken samples. As^{III}, DMA^{V} and MMA^{V} were detected in 98% (113 samples), 93% (107), and 100% (115), respectively, of the Rox-fed chicken samples; they were detectable in 26% (22), 92% (106), and 92% (106) of the control chicken samples. The concentration of AsV in both the control and Rox-fed chickens was below detection limit of 1.7 µg/kg. A possible explanation for the low concentration of As^{V} in the chicken berast could be that a substantial fraction of absorbed As^{V} was reduced to As^{III} (Vahter and Envall 1983; Vahter and Marafante 1985; Radabaugh

and Aposhan 2000) before it was distributed in chicken breasts. A new arsenic species, whose chemical structure has yet to be identified, was detectable in 114 samples (99%) from the Rox-fed chickens. This new arsenic species was not detectable in any of the samples from the control chickens. Arsenobetaine (AsB) was detectable in all samples from both the control and Rox-fed chickens. Each of these arsenic species was quantified and the results from the analyses of 114 control chicken samples and 115 Rox-fed samples were summarized in Table 3.4.

3.3.2. Comparison between the control and Rox-fed chickens

Table 3.5 shows the results from the repeated measures two-way ANOVA of each arsenic species present in more than 100 control chickens and more than 100 Rox-fed chickens. The comparison between the Rox-fed chickens and the control chickens in the concentrations of five arsenic species, including As^{III} ($P \leq 0.001$), DMA^{V} ($P \leq 0.001$), MMA^{V} ($P = 0.01$), Unknown ($P \leq 0.001$), and Rox ($P \leq 0.001$), showed significantly higher arsenic in the Rox-fed chickens than in the control chickens. The effect of age of chickens was significant for the concentrations of all six arsenic species ($P \leq 0.001$). The effect of Roxarsone treatment changed significantly with age for the concentrations of all arsenic species ($P \leq 0.001$) except AsB ($P = 0.63$).

AsB was the only species that had no significant difference ($P = 0.76$) in the concentration between the control chickens and the Rox-fed chickens. This result was understandable because the basal diet for all chickens contained approximately 0.3-0.9 μg/g AsB. The source of AsB was from fish that is commonly used as a protein source in chicken diets. In this study, AsB was present at similar concentrations in the food to both

the control group and Rox-fed group of chickens. Therefore, AsB was an appropriate internal standard.

3.3.3. Temporal profiles of each arsenic species

From the speciation analyses of 229 chicken samples collected on different days over the 35-day feeding experiment, I was able to obtain temporal profiles for individual arsenic species. Because each group of chickens was exposed to the same feed and because AsB was not metabolized, I normalized the concentrations of individual arsenic species in each chicken against the concentration of AsB in the respective chicken. With AsB as an internal standard, this normalization minimizes potential analytical fluctuations. Data without normalization against AsB was shown in Supplementary Materials Figure 3.2.

Figure 3.3 shows that the concentrations of As^{III}, DMA^{V}, MMA^{V} and Unknown in the Rox-fed chickens increased in a similar trend to that of Rox during the first 28 days when these chickens were fed Rox-containing diet. Their concentrations all reached maximum on day 28, the last day that Rox was fed. The rapid decreases in arsenic concentrations from day 28 to day 35 reflected elimination of arsenic from the chickens during the Rox withdrawal period. The elimination kinetics will be discussed later. The apparent lower concentrations of arsenic species between day 7 and day 21 could be due to rapid growth of chickens, resulting in distribution of arsenic species in larger masses of chicken breasts. Indeed, Figure 3.3f shows rapid body weight gains of both groups of chickens in this period. Taking into account of the chicken growth (and body weight), I multiplied the concentration of each arsenic species by the sample-specific body weight. Figure 3.4 shows continual increases of As^{III}, DMA^{V}, MMA^{V}, Rox, and the Unknown

arsenic species in the Rox-fed chickens in the first 28 days. The average amount of arsenic species in the chickens fed 28 days of Rox were 38 ± 19 μg As^{III}, 20 ± 16 μg DMA^{V}, 13 ± 5 μg MMA^{V}, 8 ± 3 μg Rox, and 8 ± 3 μg Unknown arsenic species.

3.3.4. Elimination of arsenic species

Figure 3.5 summarizes elimination of individual arsenic species from the Rox-fed chicken breasts after the feeding of Rox stopped on day 28. These results show patterns of decreasing arsenic concentrations in the chicken breast from day 28 to day 35. Fitting the concentrations of arsenic species on each day after the termination of Rox feeding with a one-phase exponential decay model enabled us to estimate the elimination kinetics and half-life of individual arsenic species. As shown in Table 3.6, the half-lives for all arsenic species are less than 1 day. As^{III} has the longest retention in chicken breast ($t_{1/2}$ = 1 day) and DMA^{V} has the shortest retention ($t_{1/2}$ = 0.4 day). The other three arsenic species, Rox, MMA^{V} and the new metabolite had a similar half-life ($t_{1/2}$ = 0.7 day).

Figure 3.5 also shows that after several days of elimination, the concentrations of arsenic species appears to have no significant further decrease. I conducted repeated measures two-way ANOVA on the arsenic concentration data from day 28 through to day 35. I found that for the faster eliminating species DMA^{V} and MMA^{V}, starting on day 30 their concentrations did not significantly differ from the final concentrations on day 35. The P-value for comparison between day 29 (or day 28) and day 35 were <0.01, while the P-value for comparison between day 30 (or age older than day 30) and day 35 were >0.76 for DMA^{V} and MMA^{V}. For As^{III}, Unknown, and Rox , starting on day 31 their concentrations did not significantly differ from their concentrations on day 35. The P-value

for comparison between day 30 (or age younger than day 30) and day 35 were <0.02, while the P-value for comparison between day 31 (or age older than day 31) and day 35 were >0.14 for As^{III}, Unknown, and Rox.

Starting on Day 29, all chickens were fed the diet containing no Rox. By day 35, the Rox-fed chickens had seven days to excrete arsenic from the body. The poultry industry standard regulated by US FDA (2015b) is to have a 5-day clearance period. My results show that majority of arsenic species was excreted rapidly, with half-lives ranging from 0.4 day for DMA^{V} to 0.7 day for MMA^{V}, Rox and Unknown arsenic species, and 1 day for As^{III}. Trivalent arsenicals readily interact with cysteine groups in proteins (Shen et al. 2013), such as tubulin and myosin (Menzel et al. 1999); these interactions could contribute to the longer retention of As^{III} in chicken breasts. Adding papain enhanced the extraction of As^{III} from chicken breasts (Liu et al. 2015) also suggested As^{III} could be present in bound form. After five days following the withdrawal of Rox from the feed, there was no further significant decrease of arsenic concentrations in chicken breast meat. Thus, a five-day clearance period seems reasonable.

3.3.5. Residual arsenic species after termination of Rox feeding

Although Figure 3.6 shows rapid clearance of arsenic species, it was not clear whether the residual arsenic remaining in chicken breast was significantly different comparing the control and the Rox-fed chickens. Therefore, I compared arsenic concentrations in 8 control chickens and 8 Rox-fed chickens on the last day. Figure 3.6 shows the concentrations of arsenic species in the control and Rox-fed chickens on day 35. The results of Mann Whitney U tests are shown in Table 3.7. Except for AsB (P=0.88) and

MMA^V (P=0.13), As^{III} (P=0.01), DMA^V (P=0.02), Unknown (P<0.001), and Rox (P<0.001) in the Rox-fed group were significantly higher than those in the control group. Therefore, even after a five-day or seven-day clearance period, the concentrations of four arsenic species, As^{III}, DMA^V, Rox and the Unknown, are significantly higher in the Rox-fed chickens than in the control chickens. The arsenic species in the chicken breasts were not completely cleared to the background level in the control.

The concentrations of residual As^{III} in Rox-fed chicken were from 0.41 to 3.1 µg/kg in chicken breasts (Figure 3.6 and Table 3.7). The concentrations of As^{III}, Rox, DMA^V, MMA^V, and Unknown were an order of magnitude lower than the concentrations of AsB (31 ± 11 µg/kg in the control chickens and 34 ± 14 µg/kg in the Rox-fed chickens).

In previous studies, Morrison (1969) and Brugman *et al.* (1967) pointed out that feeding chicken or lamb on chicken litter containing Roxarsone did not cause arsenic residues to accumulate in the edible tissues. However, the authors also mentioned that the amount of litter consumed was not large enough to lead to any detectable increase of arsenic. Nachman *et al.* (2013) detected the concentrations of inorganic arsenicals (arsenite and arsenate together) in conventional supermarket chicken meat samples and found the concentrations in Rox-positive samples had geometric mean (GM) of 2.3 µg/kg (95% CI: 1.7, 3.1). The concentration of Rox in Rox-positive samples had GM of 1.3 µg/kg (95% CI: 1.0, 1.7). In my study, the overall concentrations of arsenic species in the chicken breast meat after 7-day withdrawal period were similar to those reported by Nachman *et al.* (2013). The concentration of Rox (0.41±0.04 µg/kg) on day 35 was slightly lower than the results of Nachman *et al.* (2013) and the concentration of As^{III} (3.1±1.6 µg/kg) was slightly higher. In addition to the determination of As^{III} and Rox in the chicken

breast meat, I also detected MMA^V (1.4±0.4 μg/kg), DMA^V (1.8±0.5 μg/kg), and a new arsenic metabolite (0.8±0.3 μg/kg) whose chemical structure is yet to be identified.

Using the concentrations of arsenic species I determined in the chicken breast meat after the 7-day withdrawal period, I could estimate the human daily intake of arsenic from the consumption of these Rox-fed chicken. The residual concentration of As^{III} in Rox-fed chicken was 3.1±1.6 μg/kg. For an average consumption of 98 g chicken per day (ERS 2014), the average daily intake of As^{III} from eating this chicken would be 0.3±0.2 μg/day. The summed concentrations of all arsenic metabolites (excluding the non-toxic arsenobetaine) in Rox-fed chicken samples after 7-day withdrawal was 7.6 μg/kg. From an average consumption of 98 g chicken meat per day, the average daily intake of all arsenic metabolites from chicken breast meat would be 0.7 μg/day or 0.01 μg/(day kg body weight) for a 70-kg adult. This is much lower than the WHO (2011) provisional tolerable daily intake value of 3 μg/(day kg body weight) for inorganic arsenic. As a comparison, the upper limit of arsenic in drinking water is 10 μg/L (WHO 2008). The daily intake of arsenic from 2 liters of water containing 10 μg/L arsenic would be 20 μg/day, or 0.3 μg/(day kg) for 70-kg adults. Water and food are the primary sources of human exposure to arsenic (Hughes et al. 2011; Kile et al. 2007; Schoof et al. 1999; Tao and Bolger 1999; Williams et al. 2005; WHO 2011). Trace concentrations of arsenic are present in all food items as arsenic is naturally occurring in the environment. Although the contribution of arsenic from chicken breast meat is low, it is important to minimize exposure to arsenic from all possible sources.

3.3.6. Correlation between arsenic species

Rox showed significant correlation with As^{III} ($r = 0.74$, $P<0.001$), DMA^{V} ($r = 0.80$, $P<0.001$), MMA^{V} ($r = 0.71$, $P<0.001$), and Unknown ($r = 0.87$, $P<0.001$). Especially for the Unknown arsenic species, such a strong correlation with Rox suggests it might be a direct metabolite of Rox.

3.4. Conclusions

The present study provides information on the concentrations of individual arsenic species in chicken breast throughout the 35-day feeding period. The results show that the feeding of Roxarsone to chicken increases the concentrations of As^{III}, Rox, and a new arsenic metabolite in chicken breast meat. Although arsenic species are excreted rapidly from the chickens after feeding of Rox stops, the residual arsenic concentrations in chicken breast meat seven days after terminating Rox feeding remain significantly higher in the Rox-fed chickens than in the control chickens. However, consumption of moderate amount of chicken breast meat does not exceed the WHO provisional tolerable daily arsenic intake level.

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Table 3.1 Summary of the feeding experiment design and time of sample collection.

Feeding design						Age (days) at breast sample collection
Broiler strain	Group	Starter period (Day 0-14)	Grower period (Day 15-28)	Withdrawal period (Day 29-35)	n (chickens/pens)	
Ross 308	Rox-fed	Rox-supplemented diet	Rox-supplemented diet	Rox-free diet	400/4	0, 1, 2, 3, 4, 7, 14, 21, 28, 29, 30, 31, 32, 33, 34, 35
	Control	Rox-free diet	Rox-free diet	Rox-free diet	400/4	
Cobb 500	Rox-fed	Rox-supplemented diet	Rox-supplemented diet	Rox-free diet	400/4	
	Control	Rox-free diet	Rox-free diet	Rox-free diet	400/4	

Table 3.2 Sign test comparing As^{III}, Unknown arsenic species, and Rox between the control and Rox-treated groups over the 35-day feeding period.

As species	Control (μg/kg)		Rox-fed (μg/kg)		P value
	Range [a]	Median [a]	Range [b]	Median	
As^{III}	0.36-0.36	0.36	N.D.[a] -70.6	6.54	<0.001*
Unknown	0.26-0.26	0.26	N.D-9.91	1.45	<0.001*
Rox	0.24-0.24	0.24	N.D.-18.6	1.90	<0.001*

* P-value is significant at the 0.05 level.

[a] Non-detectable concentrations of As^{III}, Unknown, and Rox were denoted by 2 times of their detection limits of 1.8 μg/kg for As^{III}, 1.3 μg/kg for Unknown, and 1.2 μg/kg for Rox in the chicken breast meat samples in dry weight.

[b] N.D.: below detection limit of As^{III}, Unknown, and Rox.

Table 3.3 P values from the repeated measures two-way ANOVA comparing the concentration of each arsenic species in the last 7 days of the 35-day feeding study. The concentration of each arsenic species on day 35 was used as the reference for comparison with the other days (age). For example, the concentration of As^{III} on day 28 was compared to the concentration of As^{III} on day 35. After comparison, the P value was generated for day 28 in the As^{III} column.

Age	As^{III}	DMA^{V}	MMA^{V}	Unknown	Rox
28	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
29	<0.001*	<0.001*	0.007*	<0.001*	<0.001*
30	0.002*	0.94	0.76	0.02*	0.01*
31	0.27	0.88	0.91	0.14	0.33
32	0.93	0.91	0.80	0.86	0.29
33	0.81	0.90	0.93	0.56	0.66
34	0.92	0.87	0.97	0.72	0.61

* The difference of mean is significant at 0.05 level.

Table 3.4 Concentrations (μg/kg) of individual arsenic species in the breast meat samples of 114 control chickens and 115 Rox-fed chickens over the 35-day feeding period.

Age	As(III) in Control			As(III) in Rox-fed			Unknown[a] in Control			Unknown in Rox-fed			Rox in Control			Rox in Rox-fed			n[c] of Control	n of Rox-fed
	mean	SD	CV	mean	SD	CV	mean	SD	CV	mean	SD	CV	mean	SD	CV	mean	SD	CV		
Day 0	N.D[b]	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	8	8
Day 1	3.54	1.10	31%	4.60	2.27	49%	N.D	N.D	N.D	1.72	0.61	35%	N.D	N.D	N.D	5.92	1.92	32%	8	8
Day 2	1.27	1.14	90%	11.54	5.43	47%	N.D	N.D	N.D	4.68	2.54	54%	N.D	N.D	N.D	9.44	5.18	55%	6	6
Day 3	N.D	N.D	N.D	11.63	2.95	25%	N.D	N.D	N.D	4.99	1.51	30%	N.D	N.D	N.D	11.27	1.93	17%	8	7
Day4	N.D	N.D	N.D	21.59	8.00	37%	N.D	N.D	N.D	6.04	2.51	42%	N.D	N.D	N.D	12.11	3.97	33%	8	8
Day 7	N.D	N.D	N.D	27.78	7.39	27%	N.D	N.D	N.D	3.83	1.06	28%	N.D	N.D	N.D	5.06	1.06	21%	8	8
Day 14	N.D	N.D	N.D	10.67	4.30	40%	N.D	N.D	N.D	2.33	1.21	52%	N.D	N.D	N.D	2.77	0.65	23%	7	8
Day 21	0.57	0.22	39%	3.93	0.93	24%	N.D	N.D	N.D	0.61	0.25	41%	N.D	N.D	N.D	1.51	0.32	21%	8	7
Day 28	N.D	N.D	N.D	30.11	18.33	61%	N.D	N.D	N.D	5.03	1.44	29%	N.D	N.D	N.D	5.14	2.11	41%	8	8
Day 29	N.D	N.D	N.D	19.4	3.46	18%	N.D	N.D	N.D	3.20	0.33	10%	N.D	N.D	N.D	3.69	0.7	19%	6	5
Day 30	N.D	N.D	N.D	14.95	5.89	39%	N.D	N.D	N.D	2.16	0.68	31%	N.D	N.D	N.D	1.62	0.16	10%	6	7
Day 31	N.D	N.D	N.D	4.24	0.38	9%	N.D	N.D	N.D	0.98	0.28	29%	N.D	N.D	N.D	0.66	0.22	33%	7	8
Day 32	N.D	N.D	N.D	2.89	0.63	22%	N.D	N.D	N.D	0.63	0.21	33%	N.D	N.D	N.D	0.69	0.14	20%	5	7
Day 33	N.D	N.D	N.D	2.57	1.25	49%	N.D	N.D	N.D	0.45	0.13	29%	N.D	N.D	N.D	0.54	0.21	39%	7	7
Day 34	N.D	N.D	N.D	2.47	0.55	22%	N.D	N.D	N.D	0.73	0.16	22%	N.D	N.D	N.D	0.48	0.11	23%	6	5
Day 35	N.D	N.D	N.D	3.10	1.61	52%	N.D	N.D	N.D	0.82	0.29	35%	N.D	N.D	N.D	0.41	0.04	10%	8	8

Table 3.4(con't). Concentrations (μg/kg) of individual arsenic species in the breast meat samples of 114 control chickens and 115 Rox-fed chickens over the 35-day feeding period.

Age	AsB in Control			AsB in Rox-fed			DMA^V in Control			DMA^V in Rox-fed			MMA^V in Control			MMA^V In Rox-fed			n of Control	n of Rox-fed
	mean	SD	CV	mean	SD	CV	mean	SD	CV	mean	SD	CV	mean	SD	CV	mean	SD	CV		
Day 0	N.D	N.D	N.D	N.D.	N.D.	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	8	8
Day 1	5.58	1.34	24%	5.37	1.65	31%	1.43	0.74	52%	1.92	0.58	30%	0.52	0.22	42%	1.25	0.31	25%	8	8
Day 2	14.95	7.41	50%	23.94	10.24	43%	2.42	0.53	22%	4.52	1.12	25%	1.39	0.16	12%	3.13	0.48	15%	6	6
Day 3	27.68	5.66	20%	33.18	9.18	28%	2.99	0.95	32%	4.62	1.56	34%	1.44	0.5	35%	2.4	0.91	38%	8	7
Day4	37.9	12.67	33%	36.01	7.28	20%	2.53	0.41	16%	5.37	1.59	30%	1.73	0.53	31%	4.49	1.58	35%	8	8
Day 7	22.8	2.76	12%	27.22	5.67	21%	2.26	0.63	28%	3.69	1.03	28%	3.50	1.07	31%	5.99	1.45	24%	8	8
Day 14	31.58	6.08	19%	30.72	4.4	14%	1.93	0.26	13%	2.82	1.16	41%	1.38	0.39	28%	2.14	0.19	9%	7	8
Day 21	17.57	7.76	44%	14.34	3.61	25%	1.89	0.69	37%	2.37	0.49	21%	1.17	0.61	52%	1.93	0.79	41%	8	7
Day 28	25.94	8.07	31%	24.77	5.42	22%	3.43	1.97	57%	13.48	11.47	85%	4.30	1.97	46%	8.67	3.77	43%	8	8
Day 29	37.99	11.59	31%	30.93	10.26	33%	2.69	0.67	25%	11.96	4.04	34%	2.43	0.4	16%	6.07	2.18	36%	6	5
Day 30	40.66	11.42	28%	37.09	16.88	46%	1.68	0.65	39%	1.81	0.35	19%	1.65	0.44	27%	2.04	0.45	22%	6	7
Day 31	21.68	6.40	30%	18.61	3.64	20%	1.29	0.4	31%	0.9	0.12	13%	0.79	0.23	29%	0.85	0.16	19%	7	8
Day 32	27.46	9.17	33%	25.59	9.11	36%	1.55	0.21	14%	1.55	0.5	32%	1.32	0.23	17%	1.33	0.44	33%	5	7
Day 33	25.55	6.91	27%	24.48	5.95	24%	0.75	0.17	23%	1.18	0.26	22%	0.69	0.14	20%	1.01	0.27	27%	7	7
Day 34	29.40	12.49	42%	22.13	6.3	28%	1.00	1.06	106%	1.00	0.74	74%	1.22	0.49	40%	1.04	0.29	28%	6	5
Day 35	30.99	11.3	36%	33.5	13.93	42%	1.32	0.18	14%	1.8	0.48	27%	1.14	0.27	24%	1.42	0.41	29%	8	8

[a] Unknown: an arsenic species whose chemical structure is not yet identified.

[b] N.D.: below detection limit of 1.0 μg/kg for AsB, 1.8 μg/kg for As^{III}, 1.5 μg/kg for DMA^V, 1.7 μg/kg for MMA^V, 1.3 μg/kg for Unknown, and 1.2 μg/kg for Rox in the chicken breast meat samples in dry weight.

[c] n is the number of chickens

Table 3.5 P values from repeated measures 2-way ANOVA comparing the concentrations of each arsenic species between the control and Rox-fed groups over the 35-day feeding period.

	AsB	As^{III}	DMA^{V}	MMA^{V}	Unknown	Rox
Treatment	0.76	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Age	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Treatment x Age	0.63	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*

* Statistically significant.

Table 3.6 The elimination rate constant (K), elimination half-life ($t_{1/2}$), Y_0 and Y_t for individual arsenic species in the one-phase decay elimination model.

	As^{III}	DMA^{V}	MMA^{V}	Unknown	Rox
K (day^{-1})	0.69	1.90	0.90	0.93	0.99
$t_{1/2}$ (day)	1.00	0.37	0.73	0.74	0.70
(95% CI)	(0.70, 1.80)	(0.28, 0.58)	(0.50, 1.35)	(0.54, 1.20)	(0.52, 1.11)
Y_0	2.38	4.86	0.82	0.51	0.56
Y_t	0.06	0.02	0.04	0.02	0.02

Table 3.7 Mann Whitney U tests comparing the concentrations of individual arsenic species in the breast samples between the 8 control chickens and 8 Rox-fed chickens on Day 35.

	Control (μg/kg) (mean ± SD)	Rox-fed (μg/kg) (mean ± SD)	P value
AsB	31 ± 11	34 ± 14	0.88
As^{III}	N.D[b]	3.1 ± 1.6	0.01*
DMA^{V}	1.3 ± 0.2	1.8 ± 0.5	0.02*
MMA^{V}	1.1 ± 0.3	1.4 ± 0.4	0.13
Unknown	N.D	0.82 ± 0.29	<0.001*
Rox	N.D	0.41 ± 0.04	<0.001*

[a] Comparison was done for each pair containing one sample from control group and one sample from Rox-fed group of the same strain of chickens. Breasts samples were collected on Day 35, seven days after termination of Roxarsone feeding.

[b] N.D.: below detection limit of 1.0 μg/kg for AsB, 1.8 μg/kg for As^{III}, 1.5 μg/kg for DMA^{V}, 1.7 μg/kg for MMA^{V}, 1.3 μg/kg for Unknown, and 1.2 μg/kg for Rox in the chicken breast meat samples in dry weight.

* Statistically significant.

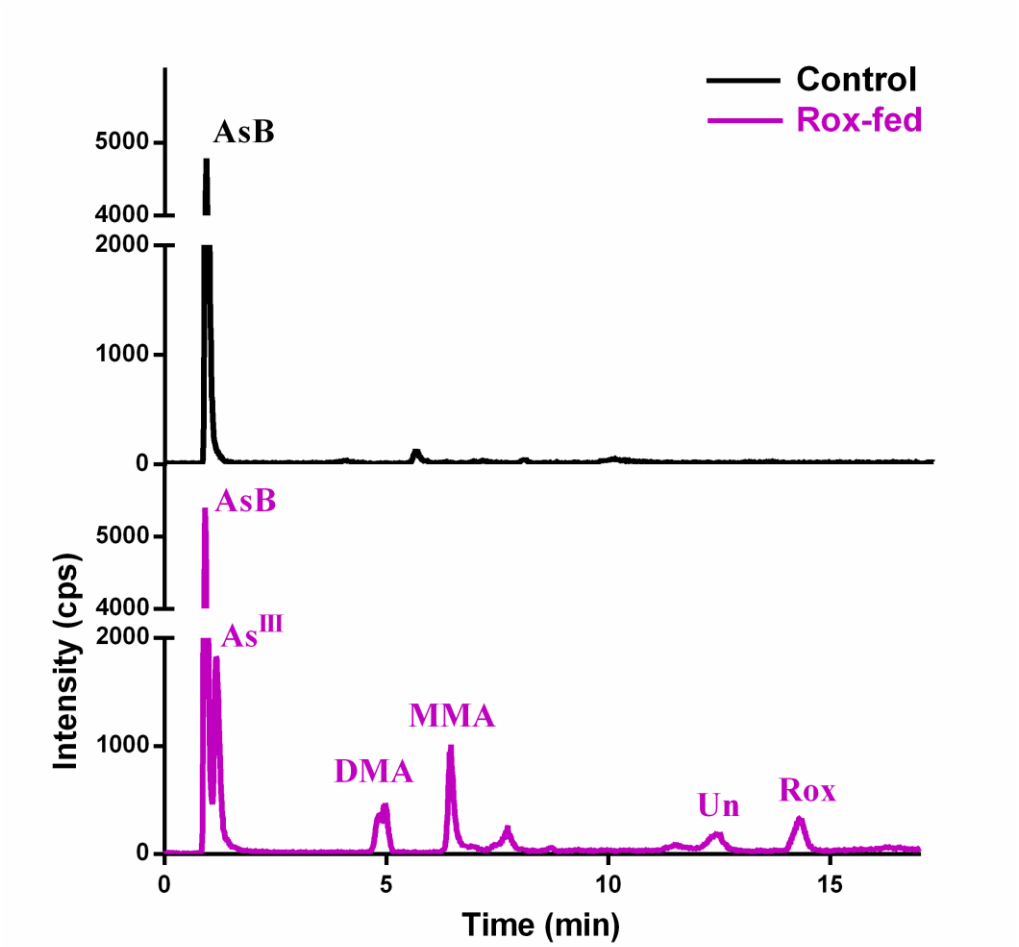

Figure 3.1 Chromatograms obtained from HPLC-ICPMS analyses of breast samples from a control chicken (top trace) and a Rox-fed chicken (bottom trace) collected on day 28 of the feeding experiment. The control chicken was given a basal diet not containing Roxarsone. The Rox-fed chicken was given a diet containing approximately 18 mg/kg Roxarsone during the first 28 days. Only arsenobetaine (AsB) was consistently present in the control chicken breast samples. AsB, arsenite (As^{III}), dimethylarsinic acid (DMA^{V}), monomethylarsonic acid (MMA^{V}), Roxarsone, and an Unknown arsenic species (Un) are detected in the Rox-fed chicken breast samples.

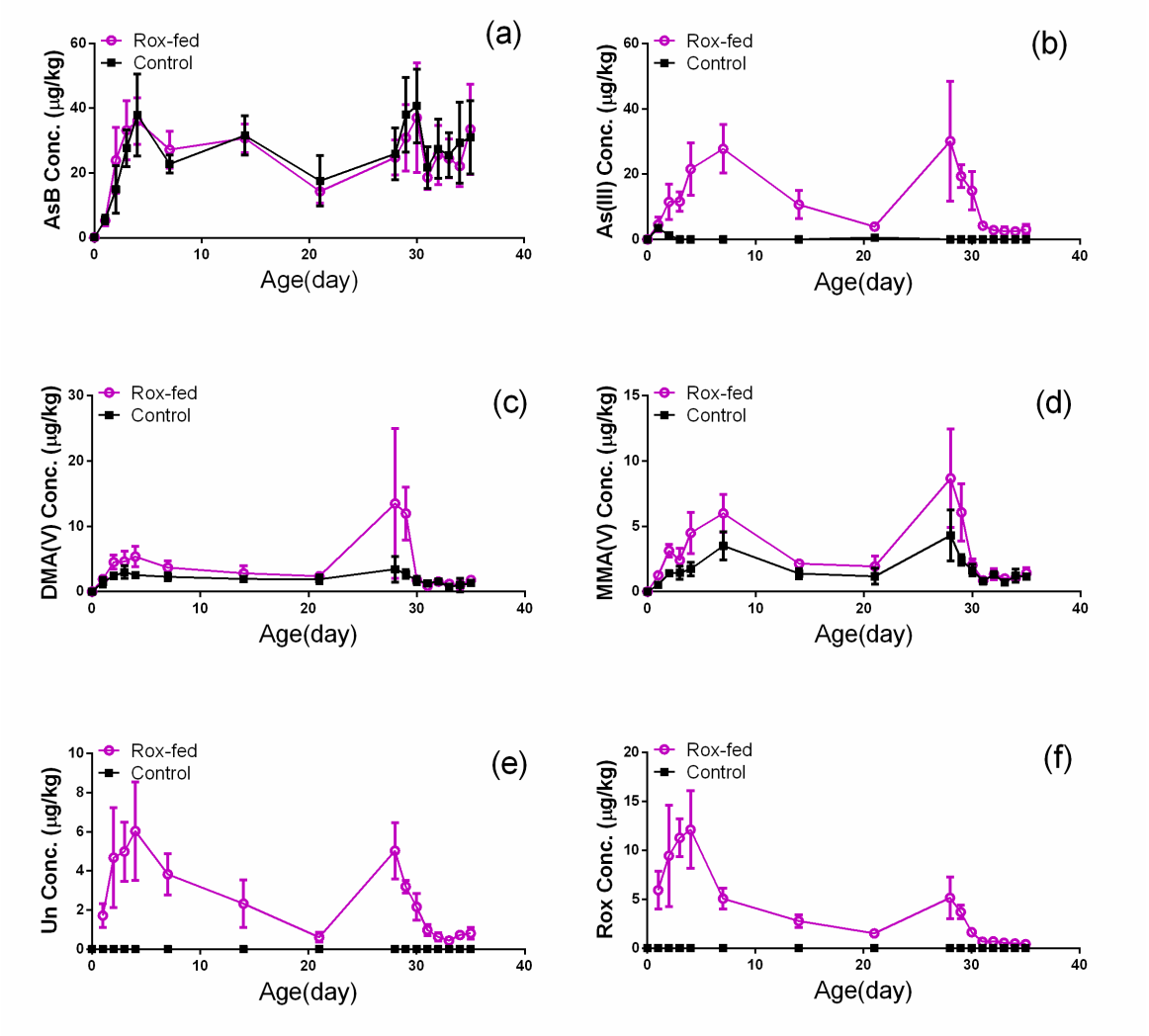

Figure 3.2 Concentration of each arsenic species, without normalization against AsB, in the breast samples of control and Rox-fed chickens over the 35-day feeding period. Error bars represent standard deviation from replicate analyses of each of 5-8 chicken samples.

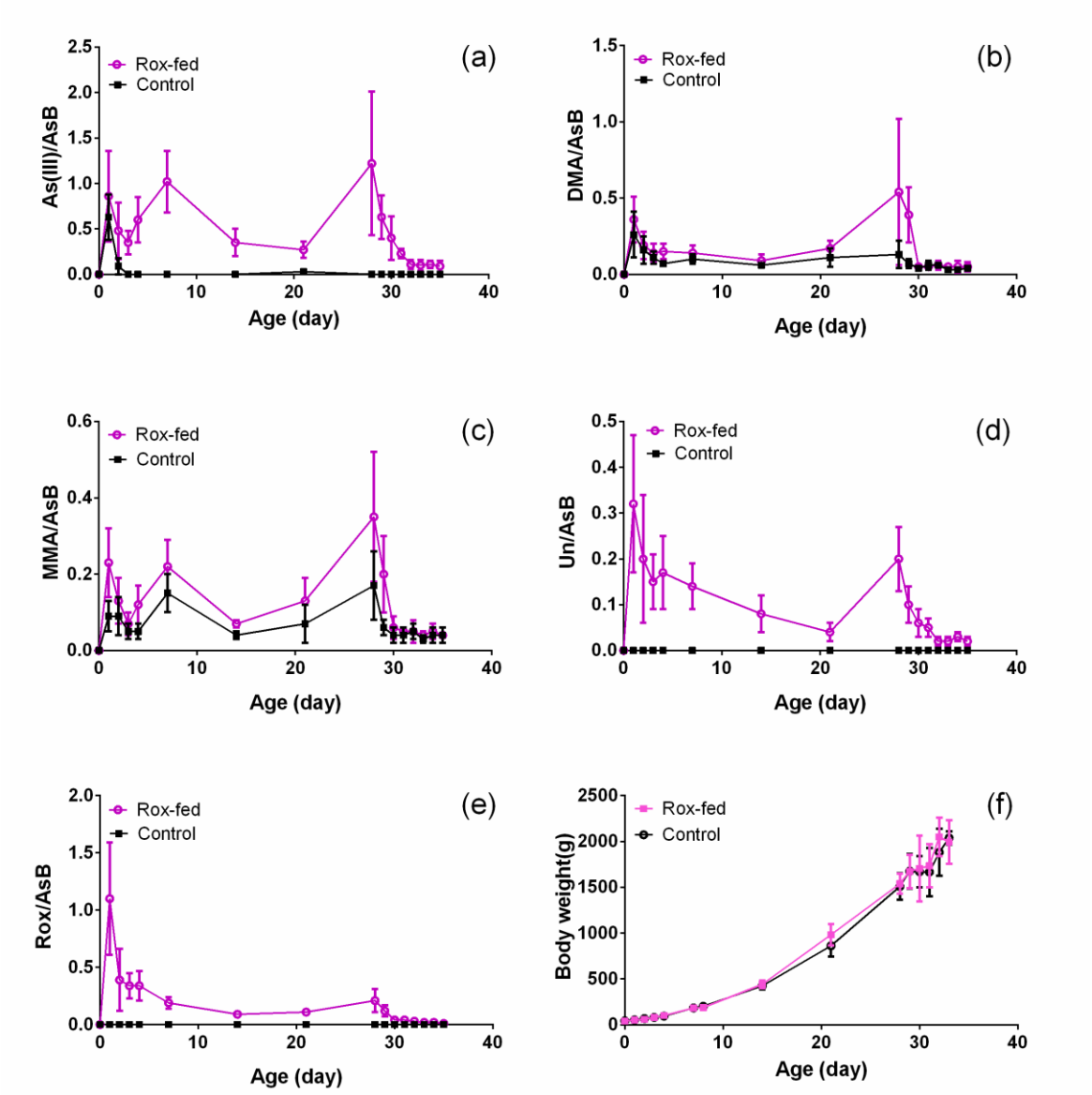

Figure 3.3(a-e) Concentration of each arsenic species, normalized against AsB, in the breast samples of control chickens and Rox-fed chickens over the entire 35-day feeding period. (f) Body weight of chickens over the 35-day feeding experiment. Error bars represent standard deviation from replicate analyses of each of 5-8 chicken samples.

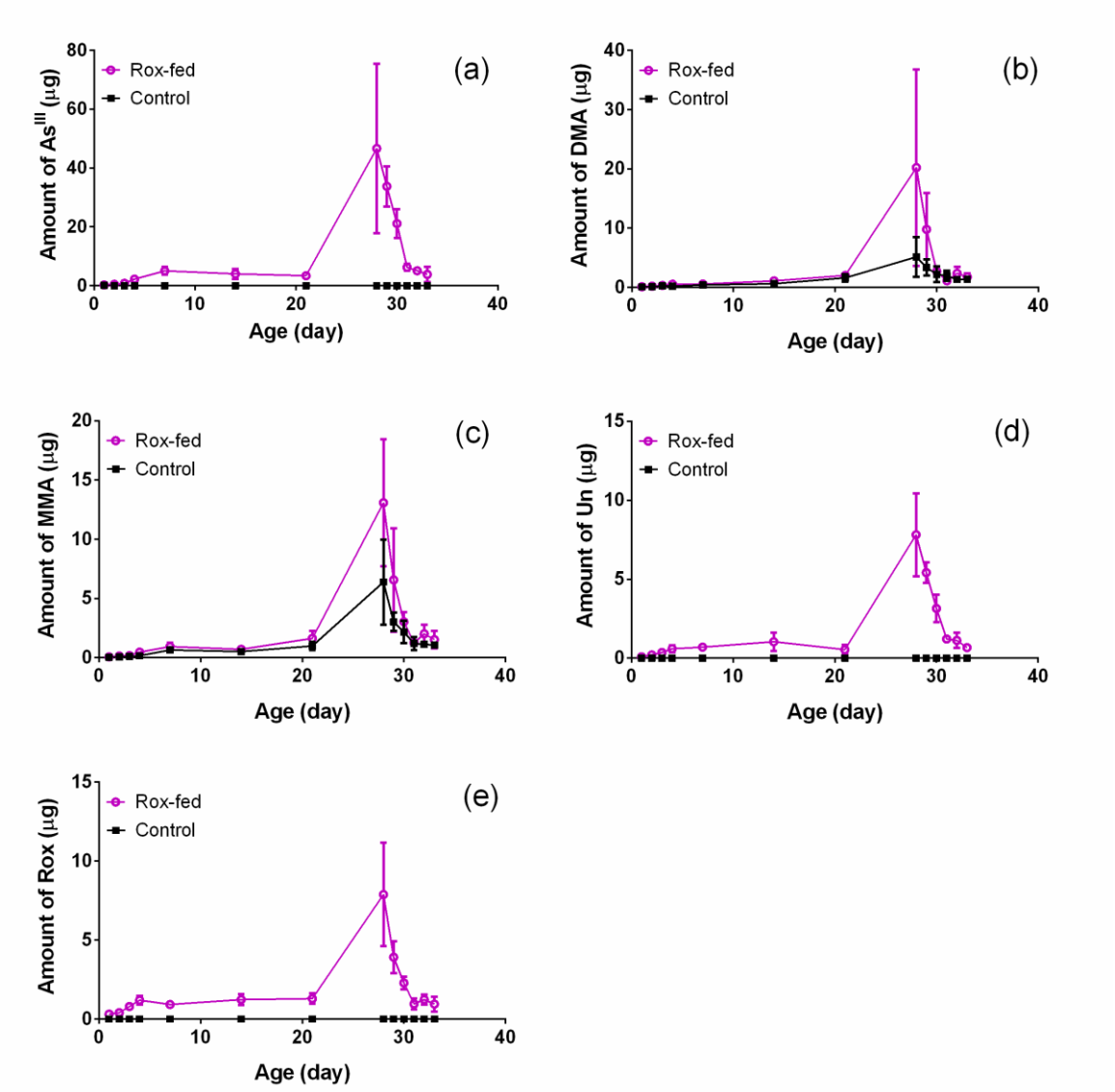

Figure 3.4 Content of each arsenic species in the breast samples of control and Rox-fed chickens. The amount of arsenic species (μg) was obtained by multiplying the concentrations of arsenic species in each sample by its sample-specific body weight. Each error bar represents a standard deviation from replicate analyses of 5-8 chicken samples.

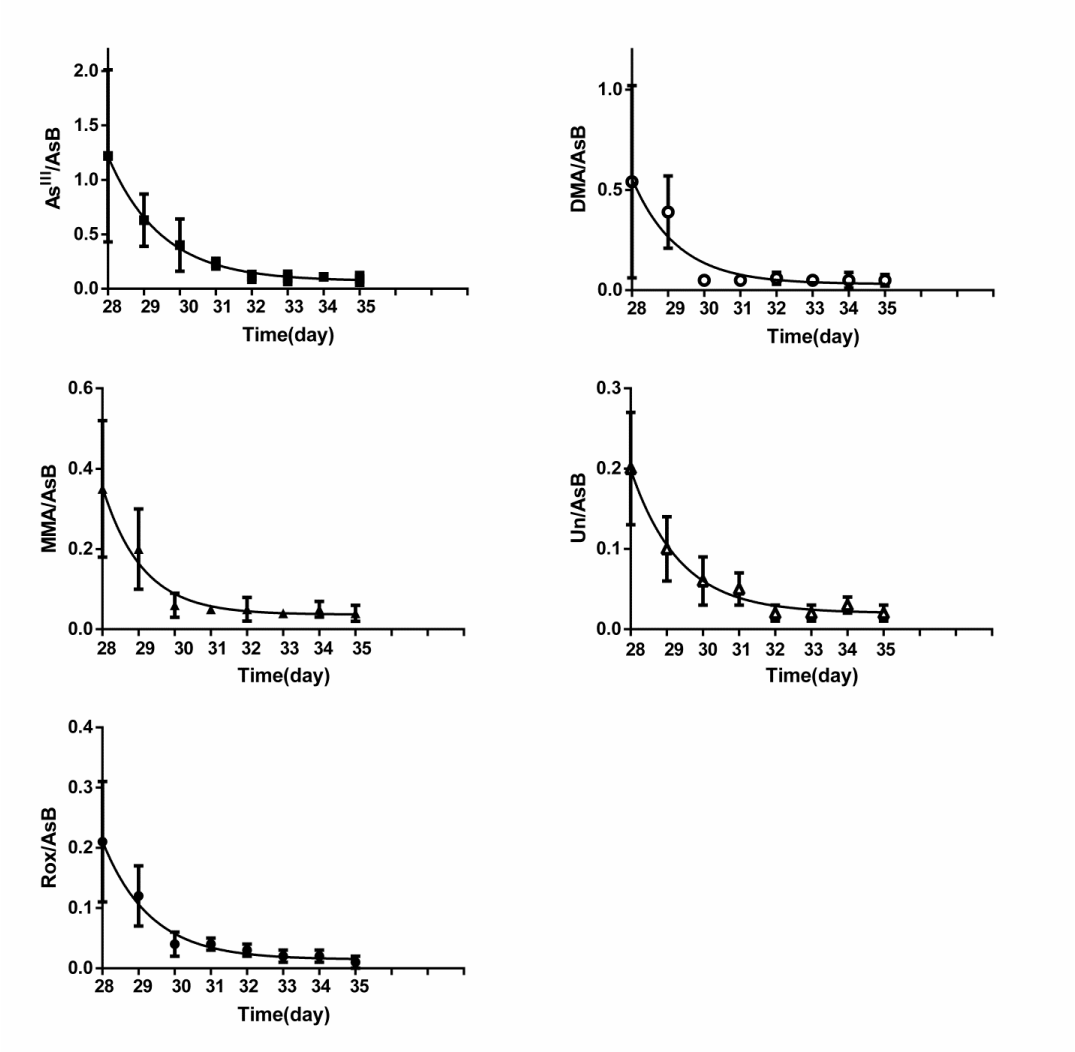

Figure 3.5 Concentration of each arsenic species, normalized against AsB, in the breast samples of Rox-fed chicken. Eight Rox-fed samples were collected each day from day 28 to day 35. Day 28 was the last day when these chickens were fed Roxarsone. From day 29 to day 35, all chickens were fed the control food that did not contain Roxarsone. Data points were presented as mean and one standard deviation from replicate analyses of each of the 5-8 breast samples. The curve represents the best fit of the data using one-phase exponential decay function.

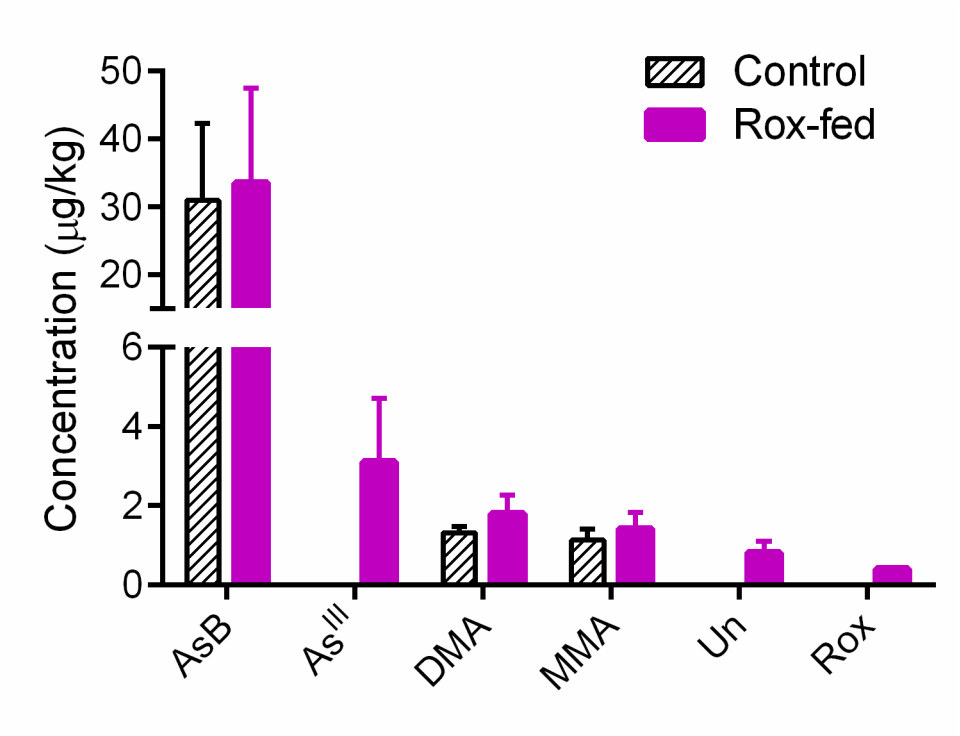

Figure 3.6 The mean concentration of individual arsenic species in eight control chickens and eight Rox-fed chickens on Day 35 (final day) of the feeding experiment. This was seven days after the final feeding of Roxarsone on day 28. Error bars represent standard deviation from four replicate measurements of each of the eight chicken samples. The concentrations of As^{III}, Rox, and Unknown are significantly higher ($P < 0.01$) in the Rox-fed chickens than in the control chickens. The concentrations of arsenobetaine (AsB) are not significantly different ($P > 0.01$) between the control and the Rox-fed chickens.

Chapter 4. Accumulation and transport of Roxarsone, arsenobetaine, and inorganic arsenic using the human immortalized Caco-2 cell line [3]

4.1. Introduction

Roxarsone (Rox, 3-nitro-4-hydroxyphenylarsonic acid) is an organoarsenic compound that was widely used for more than 60 years by the poultry industry as a feed additive to promote chicken growth and prevent infection (Nachman et al. 2013). Results from chicken feeding studies showed that most of the ingested Rox was excreted into the chicken feces (Jones 2007). However, even after a seven-day clearance period when the feeding of Rox stopped, a trace concentration of arsenic species were detectable in the chicken breast meat (Liu et al. 2016) and liver (Peng et al. 2014). The arsenic species detected in chicken meat included Rox, arsenobetaine (AsB), arsenite (As^{III}), and dimethylarsinic acid (DMA^{V}) (Nachman et al. 2013; Liu et al. 2015, 2016). Chicken is the number one meat consumed in North America, with an average intake of 80 grams per day (AAFC 2016; USERS 2014). Consumption of Rox-fed chicken could result in the ingestion of Rox and these other arsenic species (Liu et al. 2016). Chronic exposure of humans to high concentrations of inorganic arsenicals can lead to increased risk of several forms of cancers and various adverse health effects (National Research Council 2001; Smith and Steinmaus 2009; Hughes et al. 2011; Maull et al. 2012; Naujokas et al. 2013). Little is known about the toxicity and health risk from human ingestion of Rox.

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Human exposure to arsenic is generally estimated from the arsenic intake, using the data obtained from measurements of arsenic concentrations in food and drinking water. Such estimations neglect the bioavailability of the arsenic species. Bioavailability is the fraction of an administered compound that reaches the systemic circulation. In a study of feeding chickens with Rox, I added 18 ± 1 μg/g arsenic in the form of Rox to the Rox-containing chicken feed that also contained 0.03 ± 0.005 μg/g AsB (Yang et al. 2016). After chicken had been fed for a continuous 28-day period, I found 25 ± 5 ng/g AsB and 5 ± 2 ng/g Rox in the breast meat (n=229) (Liu et al 2016). From a continuous 7-day feeding period, I detected 63 ± 23 ng/g AsB and 151 ± 30 ng/g Rox in the liver of 250 chickens (Peng et al. 2014). Although the concentration of Rox was 500 times higher than AsB in the chicken feed, the concentrations of Rox and AsB in the chicken tissues were on the same order of magnitude. What could be the reason for this apparent intriguing result? I hypothesize that the bioavailability of Rox is much lower than AsB, which could contribute to the observation from the chicken feeding study.

To test my hypothesis, I use one of the most common models, Caco-2 cell line which is established from human colon adenocarcinoma cells, to analyze intestinal absorption. Intestinal epithelial cells are regarded as the first barrier for contaminants penetrating the body via the oral route, and Caco-2 cells have been widely used to examine absorption mechanisms and to estimate permeability of drugs, nutrients, and minerals. In the case of arsenic, the Caco-2 cell line has been used to study the accumulation and transport of As^{V}, As^{III}, AsB, DMA, monomethylarsonic acid (MMA), trimethylarsine oxide, tetramethylarsonium ion, and arsenosugars in standard solutions and in seafood (Calatayud et al. 2010, 2012a, 2012b; Laparra et al. 2007; Leffers et al. 2013). To date,

there is little information about the intestinal absorption of Rox in humans. The objective of this study is to provide important information for understanding the cellular accumulation and transepithelial transport of Rox by human cells.

4.2. Experimental methods

4.2.1. Chemicals

Arsenobetaine (98% purity) was purchased from Tri Chemical Laboratories Inc., (Japan). Procedures for its synthesis have been described elsewhere (Cullen et al. 2016). Sodium m-arsenite (97% purity), sodium arsenate (99.4% purity), 3-nitro-4-hydroxyphenylarsonic acid (Rox, 98% purity), and 3-amino-4-hydroxyphenylarsonic acid (3AHPAA) were purchased from Sigma-Aldrich (St. Louis, MO). The concentrations of the arsenic species were calibrated against a primary arsenic standard (Agilent Technologies) and were determined using inductively coupled plasma mass spectrometry (ICPMS) (Chen et al. 2013; Liu et al. 2015). Milli-Q18.2 MΩ·cm deionized water (Millipore Corporation, Billerica, MA) were used to prepare arsenic solutions. Fetal bovine serum (FBS), ammonium bicarbonate (NH_4HCO_3), Hank's balanced salt solution (HBSS), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich. Dulbecco's modified Eagle's medium (DMEM) was purchased from Corning (Corning, NY). Trypsin-EDTA, phosphate buffered saline (PBS), nonessential amino acids (NEAA), penicillin-streptomycin solution (Pen-Strep), trypan blue, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Gibco (Burlington, ON). RIPA buffer (pH 8.0) containing 50 mM Tris-HCl, 150 mM sodium chloride, 1% Igepal

CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) was also from Gibco. Lucifer yellow (LY) was from Thermo Fisher Scientific (Waltham, MA).

4.2.2. Immortalized cell cultures

Caco-2 cells (American Type Culture Collection, Manassa, VA) were grown in DMEM medium supplemented with 10% FBS, 1% NEAA, and 1% Pen-Strep. For transepithelial transport studies, Caco-2 cells were grown and differentiated on Transwell® polyester permeable inserts (24 mm diameter, 0.4 μM pore size; Costar Corp., NY) in 6-well plates (Corning, NY). The cells were seeded at a density of 2.7×10^4 cells/cm^2. The medium (1.5 mL) was added to the apical compartment (the donor solution) and 2 mL was added to the basolateral compartment (the receptor solution). The culture medium was changed every second day. The transport experiments were performed using the Caco-2 monolayers that had been grown on the inserts for 21-29 days. By day 21, the monolayer became fully differentiated (Hubatsch et al. 2007). Transepithelial resistance (TER) across the cell monolayer was measured every 3 days using the Millicell-ERS-voltmeter (Millipore). Caco-2 cells between passages 5 and 15 were used and incubated at 37^{o}C in a humidified incubator with 95% air and 5% CO_2.

Both polarized and non-polarized Caco-2 cells were used in this study. Polarized Caco-2 cells are different from non-polarized Caco-2 cells in that polarized Caco-2 cells have an apical and basolateral surface defined by tight junctions. Polarized cells are useful for studying the transepithelial passage of compounds such as Roxarsone because polarized cells have transcellular (across the cell) and paracellular (between the cells) transport pathways. Therefore, I used polarized Caco-2 monolayers for the transport study

(Figure 4.1). Because transport of compounds across the polarized Caco-2 monolayer can be very rapid, accumulation would be difficult to measure. Therefore, I conducted the accumulation studies using non-polarized Caco-2 cells. Accumulation within the non-polarized cells can occur only through transcellular processes and is likely to be prolonged compared to polarized cells.

4.2.3. Intracellular accumulation of arsenic species

To 45 wells were each seeded the same number of Caco-2 cells (at a density of 2.7 $\times 10^4$ cells/cm^2). The DMEM medium supplemented with 3 μM Rox was added to the first 15 wells. The DMEM medium supplemented with 3 μM AsB was added to the second 15 wells. And the DMEM medium supplemented with 3 μM As^{III} was added to the third 15 wells. These wells were placed in a humidified incubator maintained at 37^{o}C and 95% air / 5% CO_2. At the given incubation times (2, 4, 8, 12, and 24 h), three wells from the incubation of each arsenic species were taken out for analysis. Caco-2 cells from each of these wells were separately harvested. Cells were washed with PBS twice and lysed with 500 μL RIPA buffer. HNO_3 (40 μL, 60%) was added to 200 μL cell lysate and the mixture was incubated at 70 ^{o}C overnight to digest the organic contents in the cell lysate. After acid digestion, the solution was diluted 10-fold and filtered over a 0.45-μm membrane. The concentration of total arsenic in the digested solution was determined using inductively coupled plasma mass spectrometry (ICPMS).

4.2.4. Apical to basolateral transport of arsenic species across Caco-2 monolayers

The integrity of the Caco-2 monolayers was assessed by determining the transepithelial resistance (TER) value before and after each experiment. In addition, the hydrophilic Lucifer Yellow (LY), which permeates monolayers exclusively through the paracellular route, was used to test the monolayer integrity. LY (100 μM) was added to the apical compartment of the inserts of the transport experiments. After a 30-minute incubation, the fluorescence of the LY in the basolateral compartment was measured at excitation/emission wavelengths of 483/535 nm using a fluorescence microplate reader (Beckman Coulter, CA).

The transport experiments (Figure 4.1) were conducted in HBSS medium with 0.35 g/L $NaHCO_3$ and 25 mM HEPES, pH 7.4. Before each experiment, the cell monolayers were washed and equilibrated in HBSS for 15 min. Arsenic-free HBSS (2 mL) was added to the basolateral compartment (the acceptor solution). As^{III}, As^{V}, AsB and Rox (3 μM each) were separately prepared in HBSS and 1.5 mL of each solution was separately added to the apical compartment (the donor solution). Each arsenic species was tested in triplicate. At each sampling time (0.5, 1, 2, 4, 6, 8, and 24 h), half of the solution (1 mL) from the basolateral compartment (the acceptor solution) was removed and replaced with the same volume (1 mL) of arsenic-free HBSS medium. Removing solutions (and the transported test compound) from the acceptor compartment at the different testing period is a common practice in studying cellular transport (Hubatsch et al. 2007). This practice maintains appropriate conditions for the cellular transport, by ensuring that the concentration of the test compound in the acceptor compartment does not exceed 10% of that in the donor compartment.

The apparent permeability coefficient (P_{app}, cm/s) was determined from the amount of arsenic species transported per unit time following Equation 1:

$$P_{app} = (dQ/dt)\ (V/AC_0) \quad [1]$$

Where dQ/dt is the steady-state flux (μmol/s), V is the volume of the acceptor solution (mL), A is the surface area of the filter (cm^2), and C_0 is the initial concentration of the test compound in the donor solution (μM).

To assess the effect of temperature on the transport of Rox, I determined the P_{app} at 37°C and 4°C. HBSS (1.5 mL) containing 50 μM Rox was added to the apical compartment. During the experiment, the cells were either kept on a 37°C cell culture warming plate (Bel-Art™ Scienceware™) or on ice for the 4°C experiment. The P_{app} at 1 h was determined using the method described above and Equation 1.

To evaluate the paracellular transport of Rox, cell monolayers were incubated with 2.5 mM EDTA in PBS without Ca^{2+} and Mg^{2+} for 5 min. Depletion of Ca^{2+} was to open up the intercellular tight junctions (Ménez et al. 2007), allowing for paracellular transport. Rox (50 μM) was prepared in HBSS without Ca^{2+} and 1.5 mL of this Rox solution was added to the apical compartment. Samples from the basolateral compartment were analyzed at 15 min, 30 min, 45 min and 1 h. The P_{app} values were determined using Equation 1.

4.2.5. Cell viability

The number of viable cells at the end of each experiment was quantified using the trypan blue exclusion assay. The experiment was excluded if the cell viability was less than 95%.

4.2.6. Determination of arsenic concentration using ICPMS

An Agilent 7500cs Octopole reaction system ICPMS (Agilent Technologies, Japan) was used for the detection of arsenic at m/z 75. The inductively coupled plasma was operated at a radio frequency of 1500 W. The flow rates of argon carrier gas and make up gas were 1 L/min and 0.13 L/min for the sample nebulization. The flow rates of argon plasma gas and auxiliary gas were 15 L/min and 0.2 L/min, respectively, for maintaining stable plasma. Helium gas at a flow rate of 3.5 mL/min was introduced to the octopole reaction/collision cell of the ICPMS system, to reduce possible isobaric interference of ArCl on the determination of As.

Quantitation was performed by external calibration with the arsenic standard solution (Agilent Technologies). The accuracy of the calibration curve was validated by standard reference material 1640a (National Institute of Standards and Technology, Gaithersburg, MD.) which has a certified value of 8.0 ± 0.07 μg/kg for arsenic. My analysis of this reference material gave an arsenic concentration of 8.1 ± 0.04 μg/kg, in agreement with the certified value. I have also determined the concentration of total arsenic in fish standard reference material DORM-4 (National Research Council of Canada). My result 6.3 ± 0.1 μg/kg is consistent with the certified value of 6.8 ± 0.6 μg/kg.

In all experiments, parallel control experiments were carried out, and the arsenic concentrations measured. In all experiments, the background concentrations of arsenic (blank values) were very low and were subtracted from the concentration of arsenic in the test cells or cell medium. For example, the concentrations of total arsenic in the non-polarized Caco-2 cells incubated with 3 μM arsenic for 24 h were 2-46 ng/mg proteins.

The concentrations of the arsenic in control wells were below 0.2 ng/mg proteins. For transport study, the concentrations of total arsenic incubated with 3 μM arsenic for 24 h in the basolateral compartment was 50-300 ng/10^6 cells. The concentrations of total arsenic in 8 sets of control wells were below 2 ng/10^6 cells.

4.2.7. Determination of arsenic species using HPLC-ICPMS

The HPLC-ICPMS method for the determination of arsenic species was modified from that of Liu et al.[5] Briefly, an anion exchange column (Hamilton PRP-X110S) was used to separate arsenic species. Mobile phase A contained 5% methanol and 95% deionized water. Mobile phase B contained 5% methanol and 60 mM NH_4HCO_3 in deionized water, pH 8.75. The gradient elution program (40 min) started with 100% mobile phase A and 0% mobile phase B. Mobile phase B was linearly increased to 100% for the first 35 minutes and then decreased to 0% in the following one minute and kept at 0% for 4 min. The HPLC effluent was directly introduced to the ICPMS using a short PEEK tubing (0.5 mm internal diameter and 30 cm in length). Arsenic was monitored at m/z 75. My analysis of arsenic species in fish standard reference material BCR627 (Institute for Reference Materials and Measurements, Belgium) showed 3.8 ± 0.6 mg/kg of AsB and 0.14 ± 0.10 mg/kg dimethylarsinic acid (DMA). These are in agreement with the certified value of 3.90 ± 0.23 mg/kg for AsB and 0.15 ± 0.02 mg/kg for DMA.

After Caco-2 cells were incubated with arsenic species at multiple concentrations for different time periods (as described in Section 2.3), cells were washed with PBS twice, lysed with 200 μL RIPA buffer, and diluted to a final volume of 500 μL with deionized water. The cell lysates were centrifuged at 8000×g for 5 min to remove the cell debris. An

aliquot of 10 μL supernatant was used to determine the protein concentrations by Bradford assay. The remaining supernatant was filtered with 3000 Da cut-off at 14000×g for 30 min using an ultracentrifugation unit (Millipore, Canada). The filtrate was analyzed for arsenic species using HPLC-ICPMS.

4.2.8. Statistical analysis

Statistical analyses were conducted on SPSS version 20.0 (IBM Corp, Armonk, NY). Independent t-test was used to compare the results between the P_{app} of As^{V} and Rox, and between the P_{app} of Rox at different temperatures.

4.3. Results and discussion

4.3.1. Metabolism of Rox by Caco-2 cells

After Caco-2 cells were incubated with 20 μM Rox for 24 h, the majority of Rox remained unchanged, as demonstrated from the HPLC-ICPMS analysis of arsenic species in the cell lysate (Figure 4.2). Only very small amounts of As^{V} and 3-amino-4-hydroxyphenylarsonic acid (3AHPAA) were detected in the cells. The concentration of As^{V} and 3AHPAA accounted for only 0.4% and 1.2% of the concentration of total arsenic, respectively.

4.3.2. Cellular accumulation of arsenic species in non-polarized Caco-2 cells

To test my overall hypothesis that the bioavailability of Rox is lower than that of AsB and As^{III}, I first measured cellular accumulation of these arsenic species in non-polarized plated Caco-2 cells incubated with Rox (3 or 30 μM), AsB (3 μM), or As^{III} (3

μM) for the times indicated (Figure 4.3). After a 12-h incubation with 3 μM Rox, the amount of total arsenic in the Caco-2 cells was 2.4 ± 0.5 ng/mg intracellular proteins. In contrast, after a 12-h incubation with 3 μM AsB or 3 μM As^{III}, the amount of arsenic in the Caco-2 cells reached 17 ± 2 and 43 ± 3 ng/mg intracellular proteins, respectively. After 24 h of incubation with the same concentration (3 μM) of Rox, AsB, and As^{III}, the accumulated amount of Rox was about one-sixth and one-twentieth of that of AsB and As^{III}, respectively.

After the Caco-2 cells were exposed to 30 μM Rox for 24 h, the accumulated amount of arsenic was 34 ± 4 ng/mg intracellular proteins. This amount was about 10-times of the arsenic amount in the cells exposed to 3 μM Rox, indicating that the accumulation of Rox was dose-dependent.

My results showed that Rox, As^{III}, and AsB were able to go into the cells but only a portion can be accumulated in the non-polarized Caco-2 cells. The percentage of arsenic accumulated in the cells was determined by 100% × (concentration of arsenic in cells × cell lysate volume) / (arsenic dosage in culture medium). A maximum of 12.7% of As^{III} was retained in the Caco-2 cells and the accumulations of AsB and Rox were even lower, amounting to 3% and 1% of the initial treatment concentration, respectively. My results are consistent with previous observation of the low cellular accumulation of As^{III} (0.87–2.28%) and As^{V} (0.14–0.39%) in Caco-2 cells (Laparra et al. 2005).

4.3.3. Cellular transport of arsenic species across polarized Caco-2 monolayers

4.3.3.1. Transport of arsenic species from the apical to basolateral compartment of Caco-2 monolayers.

Polarized Caco-2 cells are useful for studying the trans-epithelial transport because these cells have transcellular (across the cell) and paracellular (between the cells) transport pathways. The transport of As^{III}, As^{V}, AsB, and Rox from the apical to basolateral compartments of the Caco-2 monolayer is shown in Figure 4.4. The transported amount of arsenic increased linearly with time over the first 4 h for the four arsenic species tested. After that there was a reduction in the transport rate, likely due to the decrease in the concentration of arsenic species in the apical compartment (Calatayud et al. 2011). The apparent permeability coefficients at 4 h were $(0.27 \pm 0.02) \times 10^{-6}$ cm/s for Rox, $(0.35 \pm 0.02) \times 10^{-6}$ cm/s for As^{V}, $(0.76 \pm 0.02) \times 10^{-6}$ cm/s for AsB, and $(1.75 \pm 0.08) \times 10^{-6}$ cm/s for As^{III}. The permeability coefficients at 24 h were $(0.24 \pm 0.02) \times 10^{-6}$ cm/s for Rox, $(0.30 \pm 0.04) \times 10^{-6}$ cm/s for As^{V}, $(0.6 \pm 0.1) \times 10^{-6}$ cm/s for AsB, and $(1.02 \pm 0.05) \times 10^{-6}$ cm/s for As^{III}.

In all transport studies, the TER values measured at the end of the experiment were within the range of ±5% of the TER values measured at the beginning of the experiment. At the end of the arsenic transport experiments, < 0.3% of Lucifer Yellow (LY) in the apical compartment was detected in the basolateral compartment. The results of both the TER and LY measurements indicate that the polarized Caco-2 monolayer remained intact, in the presence of the different arsenic species, for the duration of the experiment.

P_{app} of Rox was 2 times lower than that of AsB and 4 times lower than that of As^{III}. Leffers et al. (2013) determined the P_{app} of As^{III} after 24-h exposure as $(1.6 \pm 0.5) \times 10^{-6}$

cm/s, which was consistent with my results after 24 h for As^{III} ($(1.0 \pm 0.1) \times 10^{-6}$ cm/s). In the present study, the transported amount of AsB at 4 h was 3.4% of the initial amount of AsB added to the cell culture. This result was also consistent with that of Laparra et al. (2007), who reported that 1.7% of AsB was transported through Caco-2 monolayer in 4 h.

Calatayud et al. (2011) measured the transport of As^{III}, DMA^{III}, and MMA^{III} by Caco-2 cells. The P_{app} value for As^{III} at 2 h was $(4.6 \pm 0.3) \times 10^{-6}$ cm/s. In an earlier paper, Calatayud et al. tested the transport of As^{V} in Caco-2 cells (Calatayud et al. 2012). The P_{app} for As^{V} at 2 h was $(1.00 \pm 0.05) \times 10^{-6}$ cm/s. I found that the P_{app} at 2 h for As^{III} and As^{V} were $(1.6 \pm 0.1) \times 10^{-6}$ cm/s and $(4.6 \pm 0.2) \times 10^{-7}$ cm/s, respectively. My results were 2-3 times lower than the results in Calatayud et al. (2011, 2012). Differences in transport medium and cell conditions (e.g., culture conditions, passage) should be taken into consideration when comparing the results obtained in different studies (Calatayud et al. 2012; Laparra et al. 2005; Roggenbeck et al. 2016). For example, HBSS was more efficient than DMEM as the transport medium according to the results of Mathieu et al. (1999). According to the results of Yee (1997), compounds with P_{app} less than 1×10^{-6} cm/s were regarded to have low permeability; compounds with P_{app} between 1×10^{-6} cm/s and 10×10^{-6} cm/s were regarded to have moderate permeability. In my study, Rox had low permeability with P_{app} values on the order of 10^{-7} cm/s.

My results indicate that the bioavailability of Rox is much lower than AsB and As^{III}. These results are consistent with the finding from previous feeding studies (Liu et al. 2016; Peng et al. 2014). After chickens ate a feed containing 18 μg/g Rox and 0.034 μg/g AsB for 28 days, the average concentrations of Rox and AsB in the breast meat of chickens (n=229) were 5 ± 2 μg/kg and 25 ± 5 μg/kg, respectively. The average total concentration

of Rox and its possible metabolites in the chicken breast meat was 5-30 µg/kg. Thus, despite a 529-fold higher concentration of Rox (18 µg/g) than AsB (0.034 µg/g), their concentrations in the chicken tissues were on the same order of magnitude. My results in this study suggest that the low bioavailability of Rox could contribute to the inproportionally lower concentration of Rox in the chicken tissues than in the chicken feed. Other important contributors to the observed overall differences between Rox and AsB include differences in their disposition and elimination rates. Previous studies have also shown that AsB is rapidly excreted into human urine (Edmonds and Francesconi 1977; Crecelius 1977; Freeman et al. 1979; Vahter et al. 1983; Le et al. 1993, 1994). There is no information on how fast Rox and its metabolites are eliminated from the human body.

4.3.3.2.Transport of Rox across polarized Caco-2 monolayers decreased with temperature

The transport of Rox across polarized Caco-2 monolayers was reduced by 63% at 4^{o}C compared with 37^{o}C (Figure 4.5). This decrease was significant ($P < 0.001$) in the transport of Rox from the apical to the basolateral compartment when the temperature was decreased.

4.3.3.3.Paracellular transport of arsenic species across polarized Caco-2 monolayers

Molecules can go across the intestinal epithelium cells into the blood by one or more of three main pathways: passive diffusion through the paracellular (between the cells) pathway, passive diffusion through the transcellular (across the cell) pathway, and carrier-mediated transcellular transport (passive or active) (Sugano et al. 2010; Chávez-Capilla et al. 2016). To investigate the pathway of Rox transport, I evaluated the involvement of

paracellular transport in the overall transport of Rox. Compounds can pass across polarized epithelia, such as Caco-2 monolayers, through paracellular and/or transcellular routes. Paracellular transport occurs between adjacent cells and is dependent upon the permeability of tight junctions. Under steady-state conditions, the tight junctions allow the diffusion of small hydrophilic ions and inert molecules of small size. Small hydrophilic compounds, such as Lucifer yellow, mannitol, and lactulose, cross epithelial layers exclusively through paracellular transport, and therefore, are often used as paracellular transport markers. The configuration of tight junctions is altered by EDTA through its chelation of Ca^{2+}, resulting in the opening of tight junctions (Ward et al. 2000; Rothen-Rutishauser et al. 2002). Opening up the tight junctions does not affect the passage of molecules that traverse the epithelial layer exclusively through transcellular routes, but increases the passage of molecules that have a paracellular transport route (Calatayud et al. 2011).

In my experiments, the TER values were decreased significantly (to 74% of initial TER) after treating the cells with 2.5 mM EDTA for 5 min. The P_{app} of Rox increased from $(1.85 \pm 0.09) \times 10^{-7}$ cm/s to $(6.2 \pm 0.5) \times 10^{-6}$ cm/s after the addition of EDTA to open up the tight junctions (Figure 4.6). This 33-fold increase in P_{app} of Rox indicated that paracellular transport contributes to the overall transepithelial transport of Rox. However, this ratio between P_{app} after and before opening up tight junctions was 2.5-fold lower than the ratio of the positive control Lucifer yellow (83-fold increase, from $(0.900 \pm 0.003) \times 10^{-7}$ cm/s to $(6.7 \pm 0.3) \times 10^{-6}$ cm/s). Therefore, my results suggest the coexistence of transcellular transport and the paracellular route for the transport of Rox (Calatayud et al. 2010).

My results indicate that paracellular transport is a substantial route for moving Rox across the Caco-2 monolayers. From the experiments of altering the structure of tight junctions by using EDTA to chelate Ca^{2+}, I found that the transport of Rox was increased by 33 times, suggesting the involvement of paracellular transport. The same treatment of the Caco-2 cells resulted in an 84 fold increase in the transport of Lucifer Yellow, which is known to be highly dependent on paracellular transport, suggesting that paracellular transport was not the only route for translocating Rox from the apical to basolateral side of Caco-2 monolayers. The cell-membrane-permeability of Rox also supported the existence of transcellular transport.

4.4. Conclusion

Because of the existence of Rox in the meat of chickens and the large human consumption of chicken meat every year, the study of the intestinal transport and accumulation of Rox using human cell model systems is important, for the health effects of Rox to be understood. The bioavailability of some other arsenic species, including inorganic arsenicals, DMA, MMA, and AsB, have been investigated (Calatayud et al. 2010, 2011, 2012a, 2012b; Laparra et al. 2005a, 2005b, 2007; Leffers et al. 2013). But this paper is the first report on these aspects of Rox transport. In this study, I have shown that Rox can be accumulated by non-polarized Caco-2 cells and in a dose-dependent manner. Compared to As^{III}, As^{V}, and AsB, both the accumulation in non-polarized Caco-2 cells and transport across polarized Caco-2 cells for Rox are much lower. The transport of Rox is temperature-dependent and paracellular transport is a contributing component. The present study contributes new information for evaluating the bioavailability of Rox in humans,

which is important for arsenic exposure assessment, particularly from the consumption of poultry products that may contain Rox

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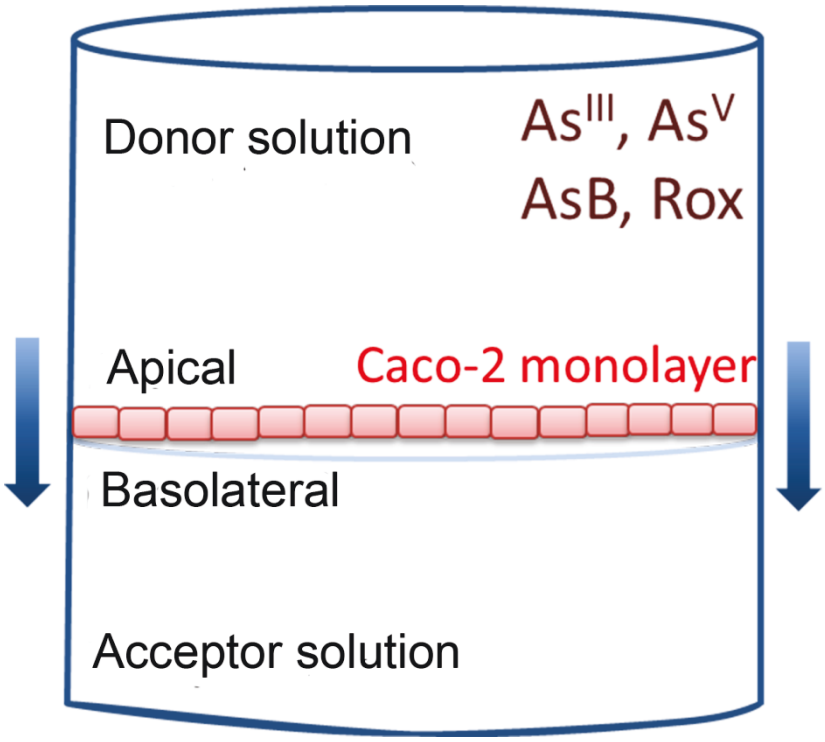

Figure 4.1 Schematic showing a study of the transport of arsenic species using polarized Caco-2 cells. A monolayer of Caco-2 cells was formed on a membrane. A donor solution containing the test arsenic species was placed in the top chamber on the apical side of the Caco-2 monolayer. An acceptor solution (initially arsenic-free buffer) was placed in the bottom chamber on the basolateral side of the Caco-2 monolayer. The acceptor solution was repeatedly sampled for analysis. The integrity of the Caco-2 monolayers was assessed by determining the transepithelial resistance (TER) before and after each experiment.

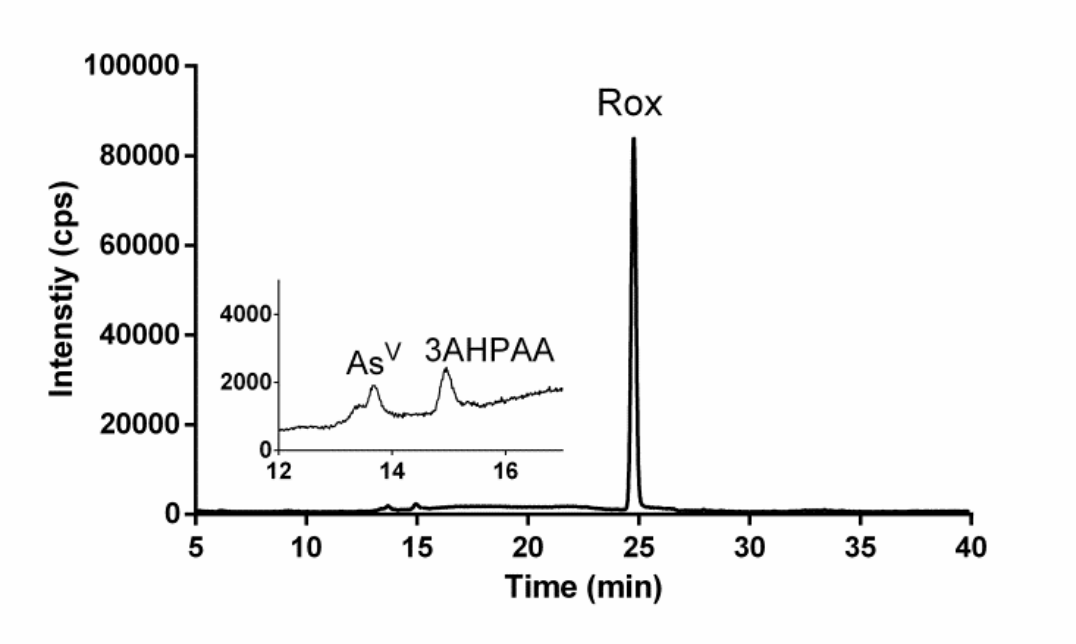

Figure 4.2. Chromatogram obtained from the HPLC-ICPMS analysis of arsenic species present in Caco-2 cells. Non-polarized Caco-2 cells were incubated with 20 μM Rox for 24 h. After the cells were washed and lysed, the cell lysate was centrifuged and filtered. An aliquot of the filtrate was analyzed for arsenic species using HPLC-ICPMS. The inset shows a portion of the chromatogram in expanded scales.

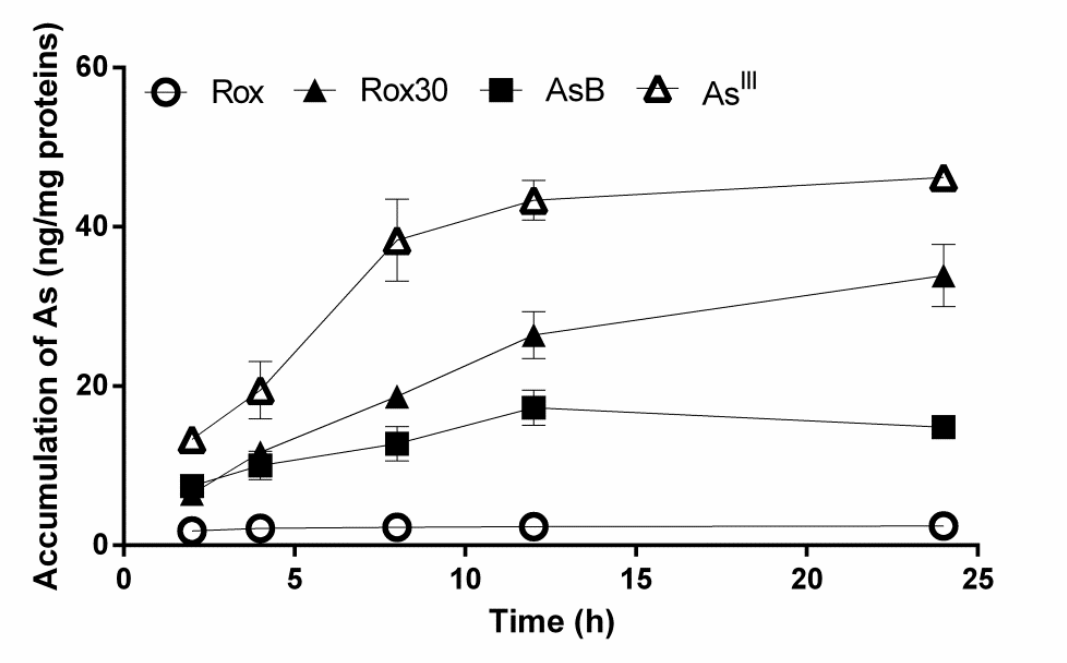

Figure 4.3. Arsenic accumulation by non-polarized Caco-2 cells. The non-polarized Caco-2 cells were exposed to 3 μM of Roxarsone (Rox), arsenite (As^{III}), arsenobetaine (AsB) or 30 μM of Roxarsone (Rox30). After exposure for the indicated time, the total arsenic concentration in the cell lysate was measured using ICPMS. Points represent mean ± standard deviation of three independent experiments.

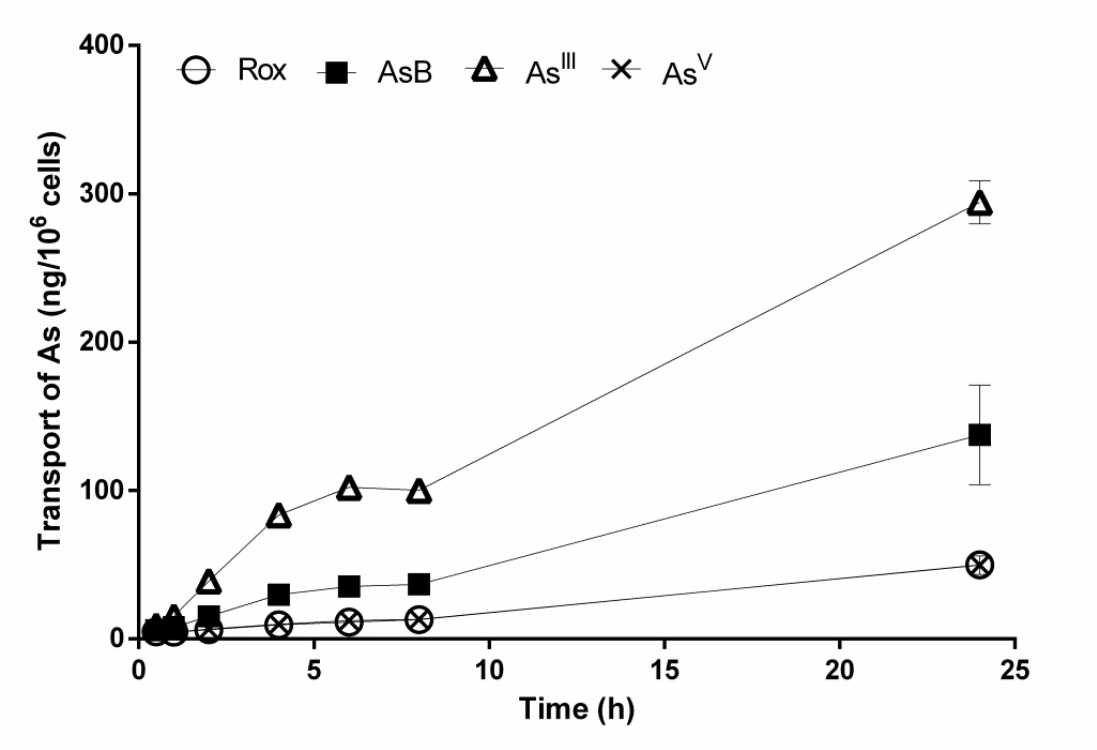

Figure 4.4. Transport of arsenicals from the apical (donor) to basolateral (receptor) sides of polarized Caco-2 cell monolayers. The medium in the apical compartment of the cell monolayers contained 3 μM of Rox, As^{V}, As^{III}, or AsB. At the indicated time points, the total arsenic concentration in the medium from the basolateral side of the cell monolayers was measured using ICPMS. Points represent mean ± standard deviation of three independent experiments.

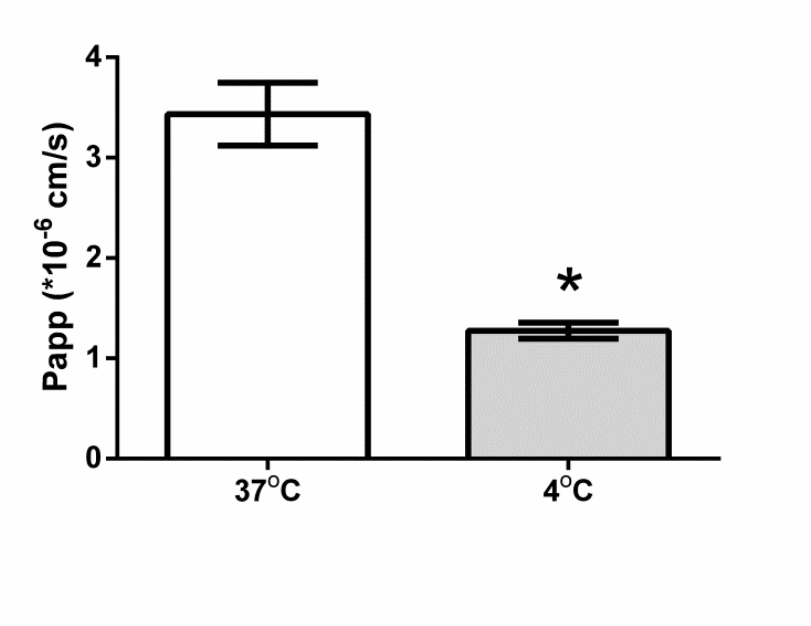

Figure 4.5. Temperature dependence of the apparent permeability coefficient (P_{app}) of Rox across the polarized Caco-2 cell monolayers. The medium in the apical compartment of the cell monolayer contained 50 μM Rox and the cell monolayers were kept at 37°C (open bar) or on ice (solid bar) for 1 h. The total arsenic concentration in the medium from the basolateral side of the cell monolayers was then measured using ICPMS. Bars represent mean ± standard deviation of three independent experiments. The P_{app} at 4°C is significantly ($P < 0.001$) lower than P_{app} at 37°C.

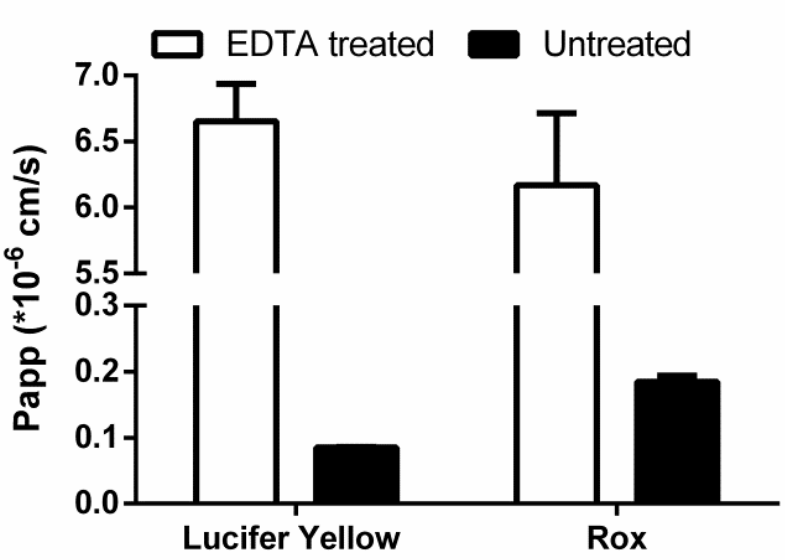

Figure 4.6. Effect of paracellular transport on the permeability (P_{app}) of Rox across polarized Caco-2 cell monolayers. The monolayer was treated with 2.5 mM EDTA (EDTA-treated, open bar) to open up the tight junctions or without EDTA (Untreated, solid bar) for 5 min. The total arsenic concentration in the medium from the basolateral side of the cell monolayers was then measured using ICPMS. Lucifer Yellow was used as the positive control. Bars represent mean ± standard deviation of three independent experiments.

Chapter 5. Characterization of Roxarsone metabolism by human immortalized cell lines and primary hepatocytes [4]

5.1. Introduction

Roxarsone (Rox, 3-nitro-4-hydroxyphenylarsonic acid) is an arsenic-containing compound that has been widely used as a chicken feed additive since the 1940s. Rox prevents the coccidiosis caused by the intestinal parasite *Eimeria tenella* and thus promotes chicken growth and improves feed efficiency (Nachman et al. 2013). The toxicity of As is known to be dependent upon the chemical species with inorganic arsenicals, arsenite (As^{III}) and arsenate (As^{V}) considered to be highly toxic. The outcome of chronic exposure to inorganic arsenicals can lead to various forms of cancers and several other adverse health effects (Hughes et al. 2011; Kapaj et al. 2006). Some organic arsenicals found in food, such as arsenobetaine (AsB), arsenosugars, arsenolipids and Rox, are regarded to be essentially nontoxic (Moreda–Piñeiro et al., 2012; US FDA, 2016).

Some evidence suggests that chickens can metabolize Rox to other arsenic (As) species, such as arsenite (As^{III}) and dimethylarsinic acid (DMA^{V}) (Stolz et al. 2007; Wershaw et al. 2003; Conklin et al. 2012; Rosal et al. 2005). To understand the potential health risks from eating meat from chicken fed Rox, it is important to investigate the cellular metabolism of Rox using human models. After ingestion of chicken meat containing Rox, gastrointestinal digestion constitutes a first step in which Rox can be metabolized, thus influencing its absorption. Intestine and liver are the sites for first-pass

[4] A version of this chapter is in preparation to submit for publication. Dr. Elaine Leslie provided the human primary hepatocytes and was involved in concept formation and manuscript revision. Dr. X.Chris Le assisted with manuscript revision.

metabolism, i.e. metabolism that occurs before a drug reaches the systemic circulation. Orally administered Rox must pass across the intestinal epithelium to enter the portal circulation for passage to the liver, the primary site of arsenic metabolism (Thomas et al., 2001; US EPA, 2010). The US FDA (2016) found As^{III}, As^{V}, DMA, MMA, 3-amino-4-hydroxylphenlarsonic acid (3AHPAA), N-acetyl-4-hydroxyphenylarsonic acid (NAHAA) and 7 unknown arsenic species present in the livers of chickens fed Rox.

In the current manuscript, the metabolism of Rox was investigated using three different human cell models. Rox biotransformation in the human colon adenocarcinoma Caco-2 epithelial cell line was studied as a model of intestinal metabolism. The biotransformation of Rox was also studied using the human hepatocellular carcinoma HepG2 epithelial cell line and primary human hepatocytes as models for hepatic metabolism. Results of this study provide important information for understanding the cellular metabolism of Rox by human cells.

5.2. Experimental methods

5.2.1. Chemicals

Arsenobetaine (98% purity) was purchased from Tri Chemical Laboratories Inc., (Japan). Sodium m-arsenite (97% purity), sodium arsenate (99.4% purity), 3-nitro-4-hydroxyphenylarsonic acid (Rox, 98.1% purity), 3AHPAA, fetal bovine serum (FBS), ammonium bicarbonate ($NaHCO_3$), Hank's balanced salt solution (HBSS) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO). 3-amino-4-hydroxyphenylarsonic acid (3AHPAA) and N-acetyl-4-hydroxy-m-arsanilic acid (NAHAA) were from Pfaltz and Bauer Inc. (Waterbury, CT).

The concentrations of the arsenic species were calibrated against a primary arsenic standard (Agilent Technologies, U.S.) and were determined using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500cs, Agilent Technologies, Germany). Milli-Q18.2 MΩ·cm deionized water (Millipore Corporation, Billerica, MA) were used to prepare arsenic solutions. Dulbecco's modified Eagle's medium (DMEM) were purchased from Corning (Corning, NY). Eagle's Minimum Essential Medium was purchased from ATCC (Manassas, U.S.). Trypsin-EDTA, phosphate buffered saline (PBS), nonessential amino acids (NEAA), penicillin-streptomycin solution (Penstrep), trypan blue, L-glutamine, ITS^+ (6.25 μg/ml insulin, 6.25 μg/ml transferrin and 6.25 ng/ml selenium), Dextran, RIPA buffer [50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate] were purchased from Gibco (Burlington, ON).

5.2.2. Immortalized cell cultures

Caco-2 cells (ATCC, Manassas, U.S.) were grown in DMEM with 10% FBS, 1% NEAA and 1% Penstrep. HepG2 cells (ATCC, Manassas, U.S.) were grown in EMEM containing 10% FBS and 1% Penstrep. Caco-2 and HepG2 cells were used between passage 5 to 15 and 7 to 17, respectively. Both cell lines were incubated at 37^{o}C with 5% CO_2.

5.2.3. The culture of primary human hepatocytes

Human hepatocytes were from two adults (>18 years old) patients undergoing hepatic resection at the University of Alberta Hospital (Edmonton, Alberta) by qualified

medical staff with the approval of the University of Alberta human ethics review board. Patients were free of infectious disease (Hepatitis B/C and HIV) and significant liver disease. Hepatocytes from livers were isolated using a modified 2-step collagenase perfusion method (Mercer et al. 2001) and cultured in suspension or plated on collagen coated plates. The suspension culture followed the procedure of Martin et al.(2005) with slight modifications. In brief, isolated hepatocytes were incubated in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in 15-ml tubes (10^6 cells/mL, 1.5 mL/tube) at 37°C and 5% CO_2 for 15 min. Hepatocytes were also seeded on type I rat tail collagen Biocoat 6-well culture plates following the procedure of Roggenbeck et al. (2015) , except without collagen overlay.

5.2.4. The treatment of primary human hepatocytes

Isolated hepatocytes in suspension culture were incubated for 4 h with either 1 μM As^{III}, 20 μM Rox, or 100 μM Rox. For hepatocytes cultured on collagen coated plates, ~18 h after seeding, arsenicals (1 μM As^{III}, 20 μM Rox, or 100 μM Rox) were added into the culture media and then incubated for 24 h.

5.2.5. Analysis of intracellular concentration of arsenic species

After incubating Caco-2, HepG2 and primary human hepatocytes with arsenic species at multiple concentrations for different time periods, cells were washed with PBS twice, lysed with 200 μL RIPA buffer, and made to a final volume of 500 μL with deionized water. The cell lysates were centrifuged at 8000×g for 5 min to remove the cell debris. In the supernatant, 10 μL was used to determine the protein concentrations by

Bradford assay and 300 μL was filtered using an ultracentrifugation unit (Millipore, Canada) with a 3000 Dalton cut-off at 14000×g for 30 minutes. The filtrates were used for speciation analyses by HPLC-ICP-MS and HPLC-ESI-MS.

HPLC separation of arsenic species was modified from the method of Liu et al. (2015). In brief, an anion exchange column (Hamilton PRP-X110S) was used to separate arsenic species. Mobile phase A contained 5% methanol and 95% deionized water. Mobile phase B contained 5% methanol and 60 mM NH_4HCO_3 in deionized water, pH 8.75. For the analysis of Caco-2 and HepG2 cells, the gradient eluting program (40 min) started with 100% mobile phase A and 0% mobile phase B. Mobile phase B was linearly increased to 100% for the first 35 minutes and then decreased to 0% in the following one minute and kept at 0% for 4 min. For the analysis of human primary hepatocytes, the gradient eluting program (29 min) starts with 100% mobile phase A and 0% mobile phase B. Mobile phase B was linearlyer increased to 100% for the first 24 minutes and then decreased to 0% in the following one minute and kept at 0% for 4 min.

The identification of arsenic species was modified from Liu et al. (2015). Briefly, the HPLC effluent was split to ICP-MS (1.6 mL/min) and electrospray ionization mass spectrometry (ESI-MS, 0.4 mL/min) for simultaneous detection**.** The confirmation of the structures of Rox and 3AHPAA in HepG2 cell lysate from the multiple reactions monitoring (MRM) mode in ESI-tandem MS (ESI-MS/MS) is shown in Figure 5.1**.**

5.3. Results and discussion

5.3.1. Metabolism of Rox by Caco-2 and HepG2 cells.

After Caco-2 cells were exposed to 20 μM Rox for 24 h, a very small amount of As^V [$1 \pm 0.1) \times 10^{-3}$ ng/10^5 cells] and 3AHPAA [$(3 \pm 0.1) \times 10^{-3}$ ng/10^5 cells] was detected in cell lysates (Figure 5.2a). The majority of Rox remained unchanged. Since the liver is the major site of biotransformation, I investigated Rox metabolism in HepG2 cells (Figure 5.2b). After HepG2 cells were exposed to 20 μM Rox for 24 h, Rox was biotransformed to (0.1 ± 0.01) ng/10^5 cells of 3AHPAA. NAHAA and three unknown arsenic species were also present in the HepG2 cell lysate (Figure 5.2b). Compared to the metabolites generated in Caco-2 cells after 24 h exposure, HepG2 cells metabolized Rox more extensively and resulted in the formation of more arsenic species (Figure 5.2).

The liver plays a major role in metabolism and the body's defense mechanisms. One of the most important functions of the liver is the detoxification of toxic substances that enter the body through ingestion, inhalation, or dermal absorption. So it is reasonable to see that higher percentage of Rox was metabolized to less toxic 3AHPAA in HepG2 cells (7.2%) than Caco-2 cells (1.2%). And HepG2 cells also metabolized Rox to less toxic NAHAA while Caco-2 did not.

HepG2 was more efficient in biotransforming Rox to other arsenic species than Caco-2 cells. The sum concentration of metabolites accounted for 1.6% and 2-7% of the concentration of total arsenic detected for Caco-2 and HepG2, respectively. The principal product of Rox biotransformation in HepG2 cells was 3AHPAA. This finding agreed with that of Moody and Williams (1964) who found that hens metabolized 18% Rox to 3AHPAA as the major metabolite.

5.3.2. The kinetics of Rox metabolism in HepG2 cells

I studied the metabolism of Rox in HepG2 cells over a 72 h exposure period to 50 μM Rox. The concentrations of three major metabolites, DMA, As^{V} and 3AHPAA, over time are shown in Figure 5.3. The concentrations of DMA, As^{V} and 3AHPAA increased over time. The concentration of Rox reached its peak value at 24 h.

Figure 5.4 shows the concentrations of Rox metabolites when HepG2 cells were exposed to different concentrations (20, 50, and 100 μM) of Rox for 24 h. The concentration of DMA did not change significantly, whereas 3AHPAA and As^{V} cellular accumulation increased with increased exposure concentration. MMA and NAHAA became detectable when the HepG2 cells were exposed to 100 μM Rox.

The elimination of Rox and its metabolites from HepG2 cells was studied by incubating the cells with 50 μM Rox for 24 h and then changing to fresh arsenic-free media (Figure 5.5). The levels of arsenic species were monitored in the cells over the 8 h post-media change. In Figure 5.5, comparing the concentrations of arsenic species detected at the starting point of elimination, 4 h after changing the medium and 8 h after changing the medium, DMA, As^{V}, 3AHPAA and Rox had reduced intracellular concentrations. The arsenic species might be effluxed out from the cells.

5.3.3. Metabolism of Rox by plated and suspended primary human hepatocytes

The metabolism of Rox by primary human hepatocytes isolated from two different donors was investigated. Methylation of arsenic after treatment with As^{III} has been previously characterized using plated primary human hepatocytes and therefore As^{III} was

used as the positive control. According to Styblo et al. (1999), when human hepatocytes were exposed to 1 μM As^{III}, about 20 pmol MMA and 5 pmol DMA were produced in every 2×10^5 cells over a 24-h exposure period. The apparent methylation rate (AMR, calculated as the amount of As^{III} converted to MMA and DMA per h per 10^6 cells) was between 3.1-35.7 pmol/h/10^6 cells and the ratio between DMA and MMA was within 0.03-2.9 over 4 hepatocyte preparations, indicating considerable inter-individual variability in methylation (1999). In my study, the plated hepatocytes exposed to 1 μM As^{III} produced 13.3 pmol MMA and 10.7 pmol DMA for every 2×10^5 cells for human 1 and 11.5 pmol MMA and 19.7 pmol DMA for human 2. The AMR was 5 pmol/h/10^6 cells and 6.5 pmol/h/10^6 cells for human 1 and 2, respectively. The ratios of DMA/MMA were 0.8 and 1.7, for human 1 and 2, respectively. The results were at the same magnitude or within the range of the results shown in the work of Styblo et al. (1999), which showed that the hepatocytes in my study were metabolically active. As expected from previous metabolic studies, the freshly isolated hepatocytes in suspension had higher metabolic activity than the plated culture over 48 h (Kocarek et al. 1993; LeCluyse et al. 1996; Richert et al. 2002). The AMR for suspended cells was 22.9 pmol/h/10^6 cells for human 1 and 34.3 pmol/h/10^6 cells for human 2.

The chromatograms of arsenic species in hepatocytes from human 1 cultured on plates and in suspension are shown in Figure 5.6a and 5.6b, respectively. The concentrations of intracellular arsenic species in primary human hepatocytes in suspension and seeded on culture plates are shown in Figure 5.7. Hepatocytes were exposed to 20 or 100 μM Rox. In plated cells (Figure 5.7a, left panel), the concentration of MMA, As^V, 3AHPAA and NAHAA increased with the higher concentration of Rox. MMA and

3AHPAA were the major metabolites when cells were exposed to 100 μM Rox. In suspended cells (Figure 5.7b, right panel), the concentration of MMA and As^V did not vary significantly with the increased treatment concentration. The concentrations of NAHAA in several samples were below the limit of detection.

The species of Rox metabolites present in the HepG2 cells and hepatocytes are similar, including MMA, As^V, 3AHPAA and NAHAA (Figure 5.7 and Table 5.1). When cells were exposed to 20 μM Rox, HepG2 cells produced more DMA (not detected in hepatocytes) and 3AHPAA (not detected in hepatocytes) but less MMA (not detected in HepG2 cells) and As^V (>14-fold lower for HepG2 compared to hepatocytes from human 1). At this Rox concentration NAHAA was not detected in HepG2 or hepatocytes. When cells were exposed to 100 μM Rox, the comparison between human 1 hepatocytes and HepG2 cells suggests that the primary hepatocytes have the potential to produce more As^V (7-fold), MMA (53-fold), 3AHPAA (5-fold) and NAHAA (3-fold).

Consistent with previous studies using As^{III} (Styblo et al. 1999), there was inter-individual variability in the metabolic activity of my hepatocyte preparations for Rox. Hepatocytes from human 2 produced more As^V but less DMA, MMA, 3AHPAA and NAHAA than HepG2 cells (Figure 5.7 and Table 5.1). The As^V concentration generated in the hepatocytes from human 2 was 94- and 16-times more than the ones from HepG2 cells when the cells were exposed to 20 μM and 100 μM Rox, respectively. DMA, MMA, 3AHPAA and NAHAA were non-detectable in hepatocytes from human 2 at both concentration levels.

Rox has relatively low bioavailability compared to other arsenic species such as inorganic arsenite and arsenobetaine (unpublished data). The low percentage of the

administrated Rox taken up by human cells can undergo metabolism. However, according to my results, the metabolites only account for 7% or less of the total Rox taken up by the cells.

5.4. Conclusion

My study has demonstrated that the Caco-2, HepG2 cells and primary human hepatocytes are capable of converting Rox to other arsenic species. These results suggest that Rox could be metabolized before entering the systematic circulation. HepG2 and human hepatocytes metabolized Rox to 3-amino-4-hydroxylphenlarsonic acid (3AHPAA), As^{V} and several unknown arsenic species. 3AHPAA is the principal metabolite of Rox in HepG2 cells. HepG2 cells and primary human hepatocytes produced similar Rox metabolites. The primary hepatocytes had the ability to generate more individual metabolites (e.g., MMA, 3AHPAA and NAHAA) than HepG2 cells, however, the metabolic profile for Rox varied between the two humans hepatocyte preparations studied. The present study contributes new information for evaluating the metabolism of Rox in humans, which is important for arsenic exposure assessment after ingestion of chicken meat containing Rox. There were also several unknown arsenic species present in the HepG2 cells and hepatocytes. Identifying these arsenic species will provide more information about human Rox metabolism.

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Table 5.1 The intracellular concentrations of arsenic species present in plated primary human hepatocytes from two individuals, designated Human 1 and 2 and HepG2 cells (n=3). Cells were treated with 20 or 100 μM Rox for 24 h.

	20 μM Rox					100 μM Rox				
	HepG2 cells (mean ± SD, μg/g protein)	Human 1 (μg/g protein)		Human 2 (μg/g protein)		HepG2 cells (mean ± SD, μg/g protein)	Human 1 (μg/g protein)		Human 2 (μg/g protein)	
DMA	0.04 ± 0.03	N.D.	N.D.	N.D.	N.D.	0.04 ± 0.01	N.D.	N.D.	N.D.	N.D.
MMA	N.D.	0.8	0.8	N.D.	N.D.	0.03 ± 0.01	1.7	1.5	N.D.	N.D.
As^V	0.02 ± 0.002	0.3	0.3	1.9	1.9	0.09 ± 0.01	0.7	0.6	1.4	1.9
3AHPAA	0.07 ± 0.02	N.D.[a]	N.D.	N.D.	N.D.	0.27 ± 0.05	1.8	1	N.D.	N.D.
NAHAA	N.D.	N.D.	N.D.	N.D.	N.D.	0.1 ± 0.04	0.4	0.2	N.D.	N.D.

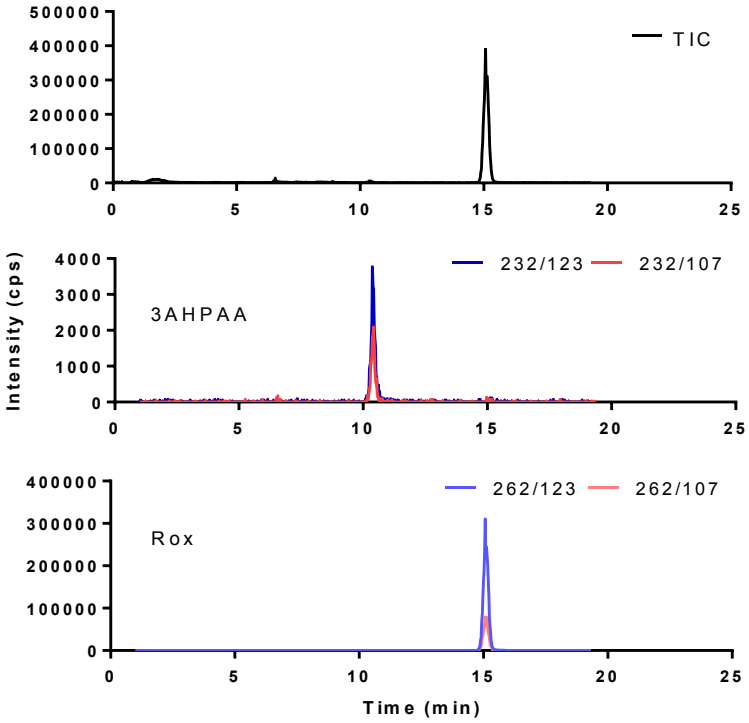

Figure 5.1 Chromatograms obtained from HPLC-ESI-MS/MS analysis of arsenic species in HepG2 cell lysate sample. ESI-MS/MS was used to confirm the structures of the detected arsenic compounds in HepG2 cell exposed to 20μM Rox. In MRM mode, because Rox and 3AHPAA had relatively higher concentrations than other arsenic species and their signal intensities on MS were less suppressed by the matrix, they could be more easily detected than other arsenic species. Here I only show the chromatograms of these two arsenic species in MRM mode.

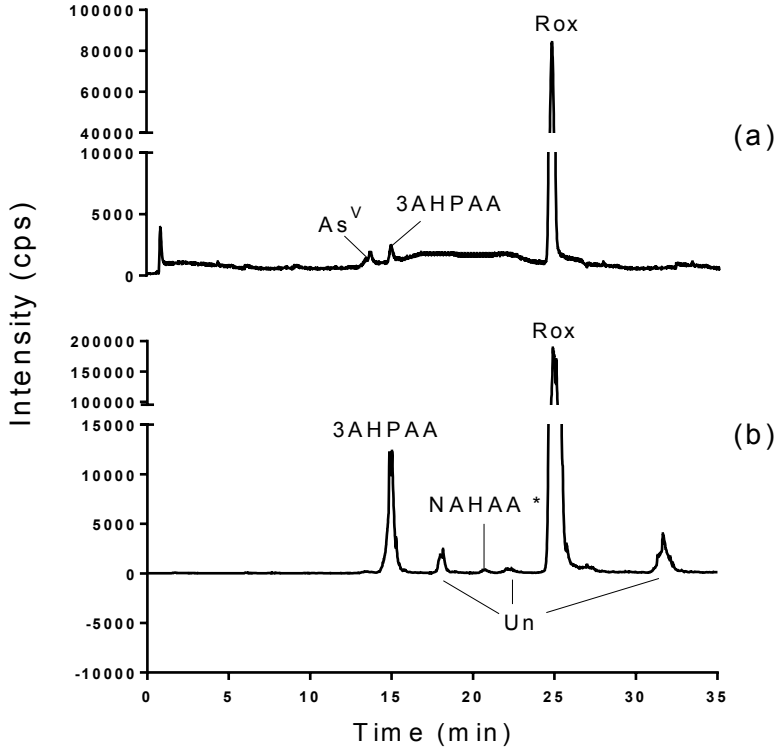

Figure 5.2 Different arsenic species present in immortalized cell lines after Rox treatment. (a) Non-polarized Caco-2 cells and (b) HepG2 cells were exposed to 20 μM Rox. Unknown arsenic species were labelled as Un.

*NAHAA in HepG2 cells exposed to 20 μM Rox was below detection limit.

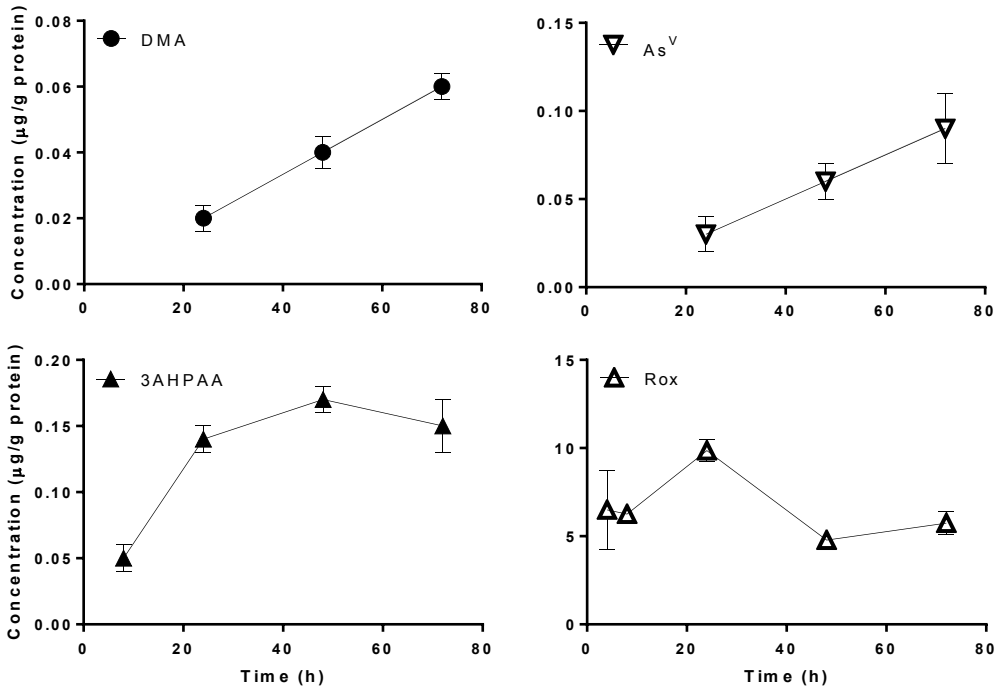

Figure 5.3 Time profile of the arsenic species in HepG2 cells exposed to Rox. HepG2 cells were exposed to 50 µM Rox. At the indicated time points, the concentrations of the indicated arsenic species in the cell lysate were measured by HPLC-ICPMS. Points represent mean ± standard deviation of three independent experiments.

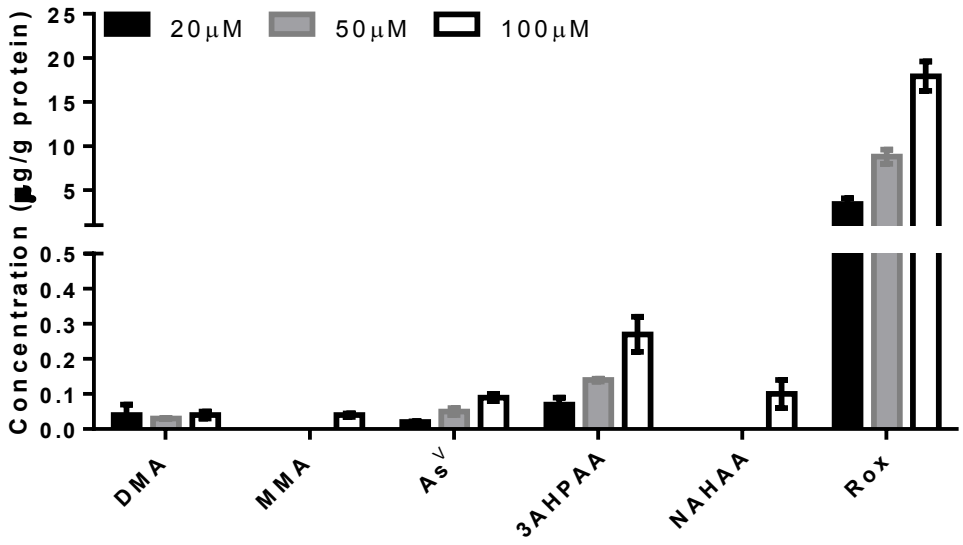

Figure 5.4 The effect of Rox concentration on the intracellular level of different arsenic species in HepG2 cells. HepG2 cells were exposed to 20 μM (blackbar), 50 μM (grey bar) and 100 μM (open bar) Rox. After 24 h, the concentrations of different arsenic species in the cell lysate were measured by HPLC-ICPMS. Bars represent mean ± standard deviation of three independent experiments.

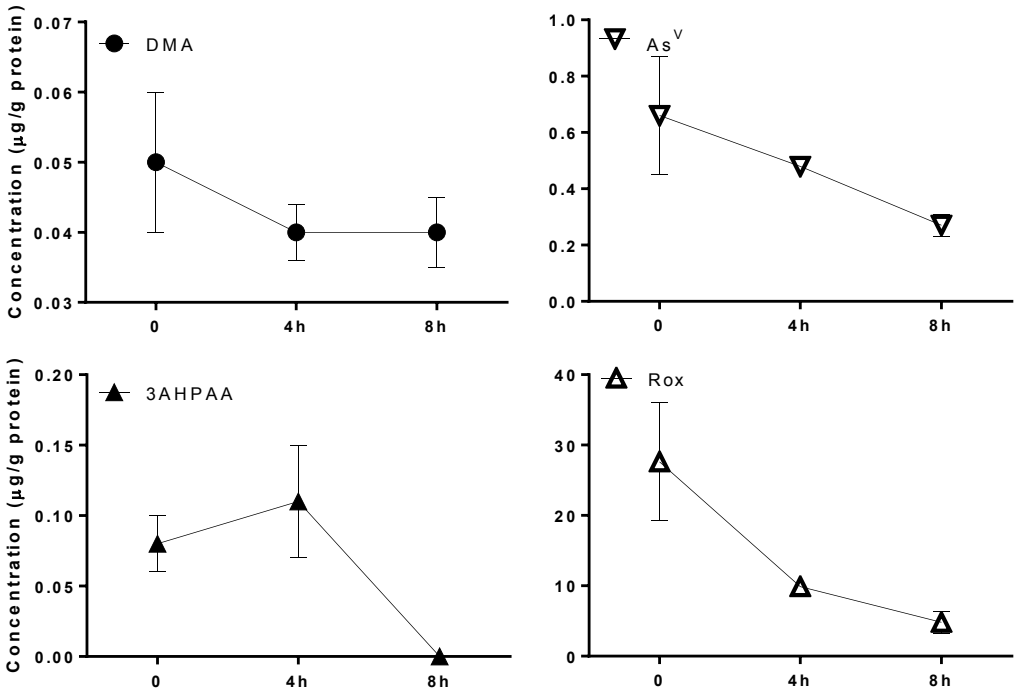

Figure 5.5 The intracellular concentrations of different arsenic species remaining in HepG2 cells over time after removal of Rox from the medium. HepG2 cells were exposed to 50 µM Rox for 24 h. The culture medium was then replaced with Rox-free medium and the levels of the remaining intracellular arsenic species measured. T=0 was the starting point of the Rox removal. Points represent mean ± standard deviation of three independent experiments.

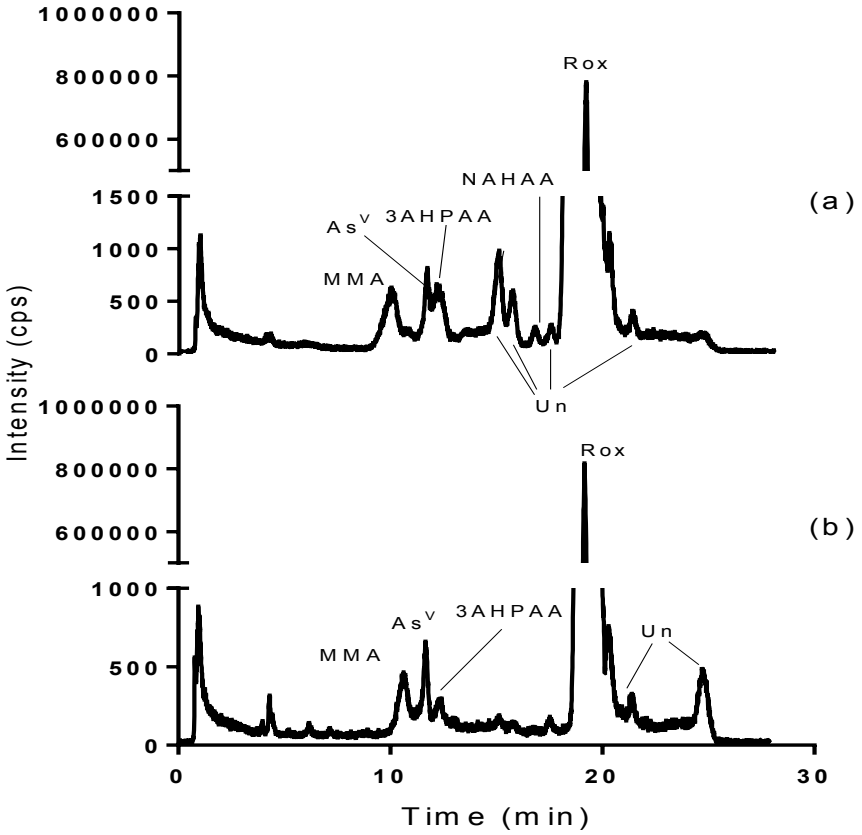

Figure 5.6 Primary hepatocytes from human 1 in (a) plate culture and (b) suspension culture were exposed to 100 μM Rox. After 24 h, the arsenic species in the cell lysates were detected by HPLC-ICPMS. Unknown arsenic species were labelled as Un.

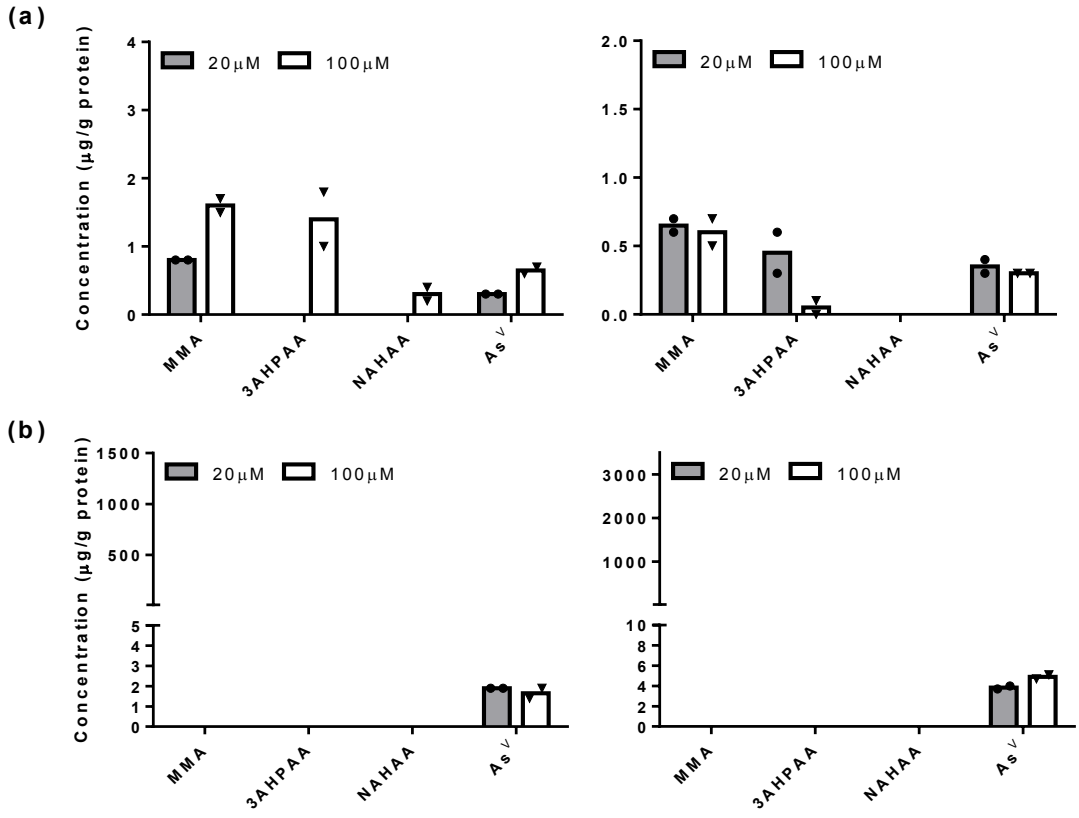

Figure 5.7 The intracellular concentrations of arsenic species present in suspended or plated primary human hepatocytes exposed to Rox. (a) Hepatocytes from human 1 were plated on Biocoat 6-well plate (left panel) or in suspension (right panel). (b) Hepatocytes from human 2 plated on Biocoat 6-well plate (left panel) or in suspension (right panel). These cells were exposed to 20 µM Rox (closed bar) or 100 µM Rox (open bar). The arsenic species were detected in the cell lysates from suspension culture after 4-h exposure and from plate culture after 24-h exposure by HPLC-ICPMS. The mean of duplicate values are plotted for all conditions.

Chapter 6. The identification of new Roxarsone metabolite in primary human hepatocytes [5]

6.1. Introduction

An organoarsenic compound, Roxarsone (Rox, 3-nitro-4-hydroxyphenylarsonic acid), has been widely used as a chicken feed additive since the 1940s. It prevents the coccidiosis, promotes chicken growth, and improves feed efficiency (Nachman et al. 2013). The toxicity of arsenic (As) is known to be dependent upon its chemical species. The outcome of chronic exposure to inorganic arsenicals, arsenite (As^{III}) and arsenate (As^{V}), can lead to lung and bladder cancers and several other adverse health effects (Hughes et al. 2011; Kapaj et al. 2006). Different from inorganic arsenicals, Rox is regarded to be essentially nontoxic (Moreda–Piñeiro et al., 2012; US FDA, 2016). However, previous studies suggest that chickens can metabolize Rox to other more toxic arsenic species, such as As^{III} and dimethylarsinic acid (DMA^{V}) (Stolz et al. 2007; Wershaw et al. 2003; Conklin et al. 2012; Rosal et al. 2005). Eating meat from chicken fed Rox causes potential risk of increased arsenic exposure. In my previous study (Chapter 5), Rox was found to be metabolized to several unidentified arsenic species in human primary hepatocytes treated with Rox. Identifying those arsenic species is necessary because the knowledge of discovering arsenic species contributes to the illustration of arsenic metabolic pathways.

ESI-tandem mass spectrometry (MS/MS) is good for identification of unknown compounds but its sensitivity for arsenic species is often very low. Arsenic species at trace

[5] A version of this chapter is in preparation to submit for publication. Dr. Elaine Leslie provided the human primary hepatocytes. Dr. Hongquan Zhang and Dr. X.Chris Le assisted with manuscript revision.

level in biological samples is very difficult to be detected. Compared to simple MS scan, precursor ion scanning (PIS) has improved sensitivity for the molecules that belong to a particular class and can be recognized from a common diagnostic fragment ion. It is MS/MS scan in which the third quadrupole (Q3) is set to a fixed mass and the first quadrupole (Q1) sweeps a mass range. However, PIS is limited by two drawbacks for the identification of unknown compounds with known fragments. First, Q3 usually allows transmitting ions in a wide window (1 Da) to maximize the yield of fragment ions from the collision cell. Fragments originating from other species and from background might be selected, which can result in false positive identification. Secondly, only a single precursor ion scan can be acquired at a time. If precursor ions of multiple product ions have to be profiled, multiple PISs have to be sequenced together in a cycle, making it time-consuming and prone to lose sensitivity. For example, I have tried to acquire 9 PISs together at a scan rate of 2000 Da/s and in the m/z range of 100-1000 in Q1, the total acquiring time was 4 sec per cycle which was relatively slow for a sharp chromatographic peak (e.g., peak width of 30 sec). Increasing the scan rate to the highest value 12000 Da/s decreased the total acquiring time to 0.72 sec, however, the sensitivity was largely decreased at such a high scan rate.

To improve the sensitivity of detection of arsenic species on ESI-MS/MS, in this work, I used a strategy known as MS^{ALL}. This technique works on hybrid quadrupole time-of-flight (QToF) mass spectrometer. In general, two ToF scans are looped in one cycle. These two scans have the same source and mass analyzer parameters, except that the first scan has high collision energy (CE) and the second has low CE. The first scan maintains the intact structure of ions in samples, while the second scan sends all ions formed in the

ion source to the collision cell for fragmentation. Each scan takes 0.25 sec and thus one cycle takes 0.5 sec. The time required for one cycle is even shorter than the PIS with the highest scan rate. Moreover, this strategy, in contrast to PSI, records data for all product ions. In this way, the full MS scan and MS/MS (without specifying precursor ions) scan data are collected from a single injection. In addition, the inherent features of the TOF analyzer, such as high mass accuracy and resolution, can contribute to overcoming the limitations, such as the false positive identification, of PSI on triple quadrupole mass spectrometers (QqQ MS).

The main objective of this study is to identify one of those unknown arsenic species present in human primary hepatocytes by using the MS^{ALL} strategy. I chose to focus on the unidentified arsenic species that had the highest concentration in the samples from the metabolism study.

6.2. Experimental methods

6.2.1. Materials and reagents

Stock solution (1 mg/L) of arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}), 3-nitro-4-hydroxyphenylarsonic acid (3AHPAA), N-acetyl-4-hydroxy-m-arsanilic acid (NAHAA), and 3-nitro-4-hydroxyphenylarsonic acid (Rox) were prepared from sodium m-arsenite (97.0%, Sigma-Aldrich, St. Louis, MO), sodium arsenate (99.4%, Sigma-Aldrich), monosodium acid methane arsonate (99.0%, Chem Service, West Chester, PA), cacodylic acid (98%, Sigma-Aldrich), 3-amino-4-hydroxyphenylarsonic acid (Pfaltz and Bauer Inc., Waterbury, CT), N-acetyl-4-hydroxy-m-arsanilic acid (Pfaltz and Bauer Inc., Waterbury, CT), and 3-nitro-4-

hydroxyphenylarsonic acid (98.1%, Sigma-Aldrich), respectively. The concentrations of the arsenic species were calibrated against a primary arsenic standard (Agilent Technologies, U.S.) and were determined using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500cs, Agilent Technologies, Germany). Milli-Q18.2 MΩ·cm deionized water (Millipore Corporation, Billerica, MA) were used to prepare arsenic solutions. Phenol red-free Dulbecco's modified Eagle's medium (DMEM) were purchased from Corning (Corning, NY). Human recombinant insulin was purchased from Life Technologies (Carlsbad, CA). Trypsin-EDTA, phosphate buffered saline (PBS), nonessential amino acids (NEAA), penicillin-streptomycin solution (Penstrep), trypan blue, L-glutamine, Dextran, RIPA buffer [50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate] were purchased from Gibco (Burlington, ON). ITS^+ (6.25 μg/ml insulin, 6.25 μg/ml transferrin, and 6.25 ng/ml selenium) and Biocoat culture plates were purchased from BD Biosciences (Franklin Lakes, NJ). Ammonium bicarbonate was from Sigma-Aldrich and methanol was from Thermo Fisher Scientific (Waltham, MA).

6.2.2. The culture and Rox treatment of primary human hepatocytes

Human hepatocytes were collected from two adults (>18 years old) patients undergoing hepatic resection at the University of Alberta Hospital (Edmonton, Alberta) by qualified medical staff with the approval of the University of Alberta human ethics review board. Patients were free of infectious diseases (Hepatitis B/C and HIV) and significant liver diseases. Hepatocytes were isolated using a modified 2-step collagenase perfusion method (Mercer et al. 2001) and seeded on type I rat tail collagen Biocoat 6-well culture

plates following the procedure of Roggenbeck et al. (2015) , except without collagen overlay. After isolated hepatocytes were seeded on collagen coated plates for about 18 h, 20 μM or 100 μM Rox was added into the culture media and hepatocytes were then incubated for another 24 h.

6.2.3. Sample preparation

After incubation of primary human hepatocytes with Rox for 24 h, cells were washed with 1×PBS twice, lysed with 200 μL RIPA buffer, and made to a final volume of 500 μL by using deionized water. The cell lysates were centrifuged at 8000×g for 5 min to remove the cell debris. Supernatant (300 μL) was filtered using an ultracentrifugation unit (Millipore, Canada) with a 3000 Dalton cut-off at 14000×g for 30 minutes. The filtrates were used for speciation analyses.

6.2.4. Synthesis of thiolated Rox standard

Thiolated Rox standard was synthesized according to the previously reported method (Hua Naranmandura et al. 2006). In brief, Rox (10 mM) was incubated with Na_2S (16 mM) in 10 mL deionized water. H_2SO_4 (16 mM) was added dropwise to the incubation mixture. The reaction mixture was stood for 1 h and the solution was diluted 10^4 times and filtered through 0.45 μm filter for use. The purity of the synthesized mtRox is 90% which is calculated based on the ratio of chromatographic peak area.

6.2.5. Arsenic speciation analysis using high-performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICPMS)

An Agilent 1290 series HPLC system, an anion exchange column (PRP X110s, 150 mm × 4.6 mm, 7-µm particle size; Hamilton,) and a reverse phase column (ODS-3, 100 mm × 2 mm, 3-µm particle size; Phenomenex, Torrance, CA) were used for the separation of arsenicals. In the anion exchange separation, the mobile phase consisted of 5% methanol in water as mobile phase A and 60mM NH_4HCO_3, 5% methanol in water (pH 8.75) as mobile phase B. The flow rate was 2 mL/min. A 10 µL aliquot of the cell medium sample was injected and the separation was performed in a gradient. The mobile phase B was increased from 0% to 100% in the first 10 min, then kept at 100% from 10 to 20 min and decreased to 0% in the next 1 min. The column was kept at 0% mobile phase B for 3 min for equilibration.

In the reverse phase separation, the mobile phase consisted of 0.2% formic acid in water (pH 3) as mobile phase A and methanol as mobile phase B. The flow rate was 0.15 mL/min. A 10 µL aliquot of the cell medium sample was injected and the separation was performed in a gradient. The mobile phase B was linearly increased from 5% to 30% in the first 10 min, then increased from 30% to 50% from 10 to 30 min and decreased to 5% in the next 1 min. The column was kept at 5% mobile phase B for 9 min for equilibration.

The effluent from HPLC system was directly introduced into the nebulizer of a 7500cs ICPMS. Arsenic was monitored at m/z 75. When separation on HPLC was run in anion exchange mode, pure argon was used as the carrier gas. Other instrument parameters were shown in Supplementary table 1, left column. When separation was run in reverse phase mode, 20% oxygen in argon gas was used in combination of the pure argon gas and

it made up for 20% of the total carrier gas. The flow rate of pure argon in carrier gas was decreased to 0.7-0.8 L/min. Spray chamber temperature was decreased to -5^{o}C. Other parameters were kept the same.

6.2.6. The MS^{ALL} technique using HPLC coupled with electrospray ionization (ESI) hybrid quadrupole time-of-flight mass spectrometry (QToF MS)

Reverse phase HPLC was hyphenated with ESI quadrupole Time of Flight mass spectrometry (QToF MS) TripleToF 5600 (AB Sciex, Concord, Ontario, Canada) to identify the unknown arsenic species. The separation condition was described above. The instrument parameters for QToF were shown in Table 2, middle column. An MS^{ALL} method was used (Bateman et al. 2007; Tiller et al. 2008; Wrona et al. 2005; Zhu et al. 2014). In this method, two ToF scans were looped in one cycle. These two ToF scans had the same source parameter, except that the first ToF scan had collision energy (CE) of -5 eV and the second one had CE of -45 eV. The first ToF scan maintained the structure of the intact ions, while the second ToF scan fragmented ions present in the first scan. Each scan took 0.25 second, therefore each cycle took 0.5 seconds. The retention time of specific arsenic-containing fragments detected in the second scan help to locate the retention time of the arsenic species in the first scan.

For the detection of arsenic species, the retention time of the precursor ion in the first ToF scan should be the same as the retention time of the arsenic fragments in the second scan. The workflow of the data analysis is that, after data was collected, I first searched for the specific arsenic fragments in the second scan. If there were arsenic fragment ions, their retention time was used to find the candidate precursors in the first

scan. The "Non-Targeted Peak Finding" function in the XIC Manager which is an add-in for the PeakView^{TM} 2.0 software (AB Sciex, Ontario, Canada) was used to find all peaks in the first scan. This function shows found peaks with ion mass and retention time in a table. From this table, I chose the ions with peak found in the time range in which the arsenic fragments have the highest intensity. After candidate precursor ions were selected, product ion mode on QToF was used to ascertain whether these ions generate the arsenic fragments.

6.2.7. Arsenic speciation analysis using HPLC coupled with ESI triple-quadrupole mass spectrometry (QqQ MS)

Reverse phase HPLC was hyphenated with ESI QqQ MS QTRAP 5500 (AB Sciex, Concord, Ontario, Canada) and ICPMS at the same time to confirm the existence of monothiol-Rox in the cell medium. After HPLC separation, the sample was split into the two mass spectrometers. The instrument parameters were shown inTable 2, right column.

6.3. Results and discussion

6.3.1. Identification of the presence of an unknown Rox metabolite by using HPLC-ICPMS

I first used HPLC-ICPMS to examine the metabolism of Rox in human primary hepatocytes. Figure 1a shows typical anion exchange chromatograms of arsenic species from the analyses of the cell culture medium (black trace) and cell lysate (red trace) of the plated human primary hepatocytes that were exposed to 20 μM Rox for 24 hours. Both chromatograms show the presence of an arsenic species (Un) having a retention time of

25.7 min. To identify whether this arsenic species was a metabolite of Rox, I then collected the chromatograms of 20 μM Rox incubated with cell culture medium for 24 hours (purple trace) and a standard solution of Rox (blue trace). No arsenic species is present at around 25-26 min in the both blue and purple traces, which indicates that Un does not come from any conversion of Rox in the cell culture medium or the original Rox solution. Thus, Un is a Rox metabolite generated in human primary hepatocytes.

To examine whether Un was an unknown arsenic species, I compared the retention time of Un with those of six possible metabolites of Rox (Figure 1b). Previous studies (Liu et al. 2016; Yang et al. 2016) found the feeding of Rox to chicken caused the increased concentrations of arsenite (As^{III}), arsenate (As^{V}), dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), 3-amino-4-hydroxyl-phenylarsonic acid (3AHPAA) and N-acetyl-4-hydroxyl-phenylarsonic acid (NAHAA) in chicken breast meat and litter. Therefore, I mixed these six arsenic species and Rox in a standard solution and determined their retention times using same chromatographic conditions as Un analysis (Figure 1b). Compared to these six arsenic species, Un has a different retention time, suggesting that Un has not been identified previously.

To obtain more information on Un, I further studied the retention behavior of Un on the reverse phase column. Because the concentration of Un in the culture medium was higher than in the cell lysate and I had much more volume of culture medium than cell lysate, I used the cell medium for subsequent Un identification. Figure 2a is the reverse phase chromatogram of arsenic species from the analysis of the cell culture medium. Un had a retention time of 19.3 min that is longer than that of Rox. Therefore, Un showed a longer retention timer than Rox on both anion exchange and reverse phase columns. To

verify if the unknown peaks after Rox in anion exchange and reverse phase chromatography were derived from the same arsenic species, I collected the first fraction (18-20 min) containing unknown arsenic species and the second fraction (22-24 min) serving as background in reserve phase chromatography. The two fractions were injected into the anion-exchange column for separation. The first fraction led to a peak that had the same retention time as Un, whereas the second fraction did not generate any peak at the same retention time (Figure 2b). Thus, although separation principles are largely different for anion exchange and reverse phase chromatography, Un shows a stronger retention than Rox on both columns. This allows us to suspect that Un might be a sulfur-containing arsenic species, because previous studies showed that thiolated arsenic species had longer retention than their oxygenated counterparts on anion exchange (Hansen et al. 2004; Raml et al. 2007; Yathavakilla et al. 2008) and reverse phase columns (Fricke et al. 2005).

6.3.2. Identification of the structure of the unknown Rox metabolite by using HPLC-ESI-QToF-MS

In order to elucidate the structure of the Un, I initially used ESI-MS/MS to obtain molecular weight and fragment information of Un. However, the concentration of Un in the cell medium is less than 10 ppb (estimated by using calibration curve of Rox), which is lower than the sensitivity of conventional ESI-MS/MS for analysis of arsenic species. Additionally, the complicated matrix further increases the difficulty of identifying the structure of Un. To confront these challenges, I combined an MS technique known as MS^{ALL} with a fragment list prepared to include possible fragments of Un.

MS^{ALL} includes two looped ToF scans in each cycle. The two scans use same other source parameters, except that the collision energy (CE) of the second scan is higher than that of the first scan. The low CE allows the first scan to preferentially generate spectra of molecular ions, whereas the high CE enhances the in-source fragmentation, generating more fragment ions in spectra of the second scan. Therefore, the MS^{ALL} technique enables simultaneous generation of spectra of molecular ions and fragment ions.

In order to extract useful information from spectra generated by MS^{ALL}, I created a fragment list that included possible fragment ions of Un. Based on the retention behavior of Un on anion exchange and reverse phase columns, I suspected that Un might be a sulphur-containing arsenic species. Therefore, I searched literature for determined fragment ions of the sulphur-containing arsenic species. In the negative mode, Wallschläger and Stadey (2007) determined mass signals of the reaction solutions of arsenite and sulfide by using ESI-QToF and they detected fragments AsO_3^-, $AsSO^-$, $AsSO_2^-$, AsS_2^- and AsS_2O^- from the reaction solution. Peng et al. (2014) detected AsO_2^- and AsO_3^- as the fragment ions of Rox standard by using ESI-QqQ MS . I also searched for methyl-arsenic fragments and methyl-thiol-arsenic fragments, because methylated arsenicals have been found to be converted to thiol-methylated arsenicals (Suzuki et al. 2004). In the positive mode, AsO^+, $As(CH_2)_2^+$, $As(CH_3)_2OH_2^+$ and $As(CH_3)_2^+$ was detected in the works of identification of arsenolipid which have methyl groups bind to arsenic atom, (Amayo et al. 2011, 2013; Arroyo-Abad et al. 2013). $As(CH_3)_2S^+$ was detected as the fragment ion of dimethylthioarsinic acid and trimethylarsine sulfide (Adair et al. 2007). AsO^+ and AsS^+ are general fragments for detecting arsenic species contains oxygen and sulfur atom in their

structures. Table 1 lists these fragment ions that are sorted in ESI positive and negative modes.

I then used the list of fragment ions to extract chromatogram of Un that is obscured within XIC of the second scan of MS^{ALL} by matrix ions. I first analyzed the cell medium sample by using reverse phase HPLC-ESI-QToF in the negative mode. Chromatograms extracted from XIC by using ions AsO_2^- (m/z 106.912 ± 0.005), $AsSO^-$ (m/z 122.889 ± 0.005), and $AsSO_2^-$ (m/z 138.884 ± 0.005) showed a peak within17-19 min (Figure 3). The peak retention time of these ions was 18.2 min. I then used the Non-Targeted Peak Finding function in the XIC Manager to find all peaks in the first ToF scan. The finding criteria were set as: 'Approximate LC peak width' was 1 min; 'Minimum intensity in counts' was 5 counts, and 'Chemical noise intensity multiplier' was 1.5. I chose the ions with a peak having the retention time in the time range of 18.0 to 18.4 min in which this arsenic fragments had the highest intensity. The chosen ions were listed in Table 3. I further excluded those precursor ions (575.153, 507.159, 575.158 and 296.993) based on one fact that the chromatogram shape of a precursor ion is similar to those of the fragment ions (Figure 4). The rest ions were then tested by product ion mode on QToF. The ion with m/z 277.9 was the only one that produced the arsenic-containing fragment ions (Figure 5).

Similarly, I also applied the list of fragment ions to XIC of the second scan generated by reverse phase HPLC-ESI-QToF in the positive mode. Chromatograms extracted from XIC by using ions AsO^+ (m/z 90.916 ± 0.005) and AsS^+ (m/z 106.894 ± 0.005) showed a peak at around 18 min (Figure 6). The peak finding having same finding criteria as the negative mode was used to search for the precursor ions in the first ToF scan. The possible precursor ions were shown in Table 3. All candidate ions were checked by

product ion mode because that they all showed similar chromatograms to those of AsO^+ and AsS^+ (Figure 7). Only the ion with m/z of 279.9 produced the product ion of m/z 90.916 and 106.894 (Figure 8).

Therefore, both negative and positive modes demonstrated that Un has a theoretical molecular mass of 278. 9. In addition, Un is able to generate fragment ions at m/z 106.912, 122.889 and 138.884 in negative mode and at m/z 90.916 and 106.894 in positive mode. I can collectively deduce its formula could be $AsS(OH)_2C_6H_3(OH)(NO_2)$ (4-Hydroxy-3-nitro-monothiobenzenearsonic acid) (see structures in Figure 9).

6.3.3. Confirming the structure of an unknown Rox metabolite by using a synthesized standard and HPCL-ESI-MS/MS

To further confirm the structure of Un, I synthesized thiolated Rox having the identified structure by using the reported approach (cite reference). The purity of the synthesized thiolated Rox is 90%. I used this synthesized thiolated Rox as a standard to compare with Un in cell culture medium for their chromatography and MS properties. I first compared the thiolated Rox standard with Un in the cell medium for their retention behavior on anion exchange column by using HPLC-ICPMS. The unknown arsenic species had the same retention time as thiolated Rox standard (Figure 10). I further spiked the thiolated Rox standard into the cell medium sample and analyzed this spiked sample. Only a single peak at the retention time of Un was observed and the peak intensity was increased compared to the cell medium sample without spiking thiolated Rox standard (Figure 10). Therefore, the thiolated Rox standard and Un have same chromatographic retention property.

I then used the multiple reaction monitoring (MRM) detections to compare thiolated Rox standard with Un for their fragment ions. To establish the transition ion pairs, I collected MS/MS spectra of the thiolated Rox standard. Figure 11a shows that MS spectrum of thiolated Rox standard that has peaks with m/z of 277.9120, 278.9148, and 279.9093. These measured values were in good agreement with its theoretical values 277.9110, 278.9137, and 279.9094 (mass error < 4, 4, and 0.3 ppm, respectively). Under collision energy of -45 eV (Figure 11b), thiolated Rox could be fragmented to $AsSO_2^-$ (m/z 138.888), $AsSO^-$ (m/z 122.892), $C_4HNO_3^-$ (m/z 110.994), AsO_2^- (m/z 106.915) and $C_4HNO_2^-$ (m/z 94.999). These fragment ions were in agreement with their theoretical values of m/z 138.884, 122.889, 110.996, 106.912 and 95.001(mass error < 28, 24, 18, 28 and 21 ppm, respectively). Rox was characterized under the same condition (supplementary materials). Rox was fragmented to AsO_3^- (m/z 122.910) and AsO_2^- (m/z 106.916) under CE of -45 eV (Figure 12).

Having obtained the information on the parent and fragment ions of the thiolated Rox standard, I selected five major fragment ions to establish MRM transition ion pairs. These five transition ion pairs 278/95, 278/107, 278/111, 278/123, and 278/139 were then used to detect Un in the cell medium. If thiolated Rox standard and Un have the same molecular structure, I expect all five transition ion pairs lead to a peak that has the same retention time as Un that is analyzed by HPLC-ICPMS. I also used transition ion pairs 262/107 and 262/123 to monitor Rox. To test this, I incorporated simultaneous detection of both ICPMS and ESIMS with reverse phase HPLC separation. The eluent from reverse phase column was split into two portions for simultaneous detection by ICPMS and ESI-triple Quadrupole. Figure 13 shows the chromatograms detected by the two mass

spectrometers. All 5 MRM transitions resulted in a peak that had the same retention time (18.2 min) of Un detected by ICPMS, which supports that this Un has the same structure as the thiolated Rox standard.

With the addition of H_2O_2, thiolated Rox was immediately converted into Rox and As^V. Figure 14 showed that with the addition of H_2O_2, the peak of thiolated Rox disappeared and the height of the peaks of Rox and As^V increased. The standard solution of 7 arsenic species was spiked into the cell medium incubated with H_2O_2. The peaks of 7 arsenic standards confirmed the retention time of Rox and As^V.

6.4. Conclusion

The unidentified arsenic species in the primary human hepatocytes incubated with Rox was confirmed as thiolated Rox. Using the MS^{ALL}, thiolated Rox was successfully identified at a very low concertation (10 ppb). This strategy is not only helpful for identifying the thiol-containing arsenic species but is also good for some other arsenic species which could generate specific fragment ion. Further studies on how this arsenic compound is formed might be interesting to look at. This could further our understanding on how human liver cells handle different arsenic compounds.

According to the results in Figure 5.6 (Chapter 5), there were still another 3 Rox metabolites presenting in human primary hepatocytes. Future work can be taken out to elucidate the structures of those unknowns using high resolution, accurate MS/MS. Other methods include chemical/enzyme reactivity, retention time predictions, in silico MS/MS predictions etc. can also be used to elucidate the structures of the unknowns.

6.5. References

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Table 6.1 The list of arsenic-containing fragment ions expected in positive and negative mode.

Positive mode		Negative mode	
Formula	Theoretical m/z	Formula	Theoretical m/z
AsO^+	90.9159	AsO_2^-	106.9120
AsS^+	106.8936	AsO_3^-	122.9069
$As(CH_2)_2^+$	104.9680	$AsSO^-$	122.8891
$As(CH_3)_2^+$	102.9521	$AsSO_2^-$	138.8835
$As(CH_3)_2S^+$	136.9406	AsS_2^-	138.8663
$As(CH_3)_2OH_2^+$	122.9786	AsS_2O^-	154.8612

Table 6.2. The optimized operating conditions for ICPMS and ESIMS

ICPMS	ESI QToFMS	ESI QqQMS
Radio frequency power: 1500 W	Ion spray voltage (IS): + 5500 V (pos)	Ion spray voltage (IS): -4500 V
Sample depth: 7.0 mm	- 4500 V (neg)	
Carrier gas: 0.9-1.0 L/min	Temperature (TEM): 400 ^{o}C (pos)	Temperature (TEM): 500 ^{o}C
Makeup gas: 0 L/min	500 ^{o}C (neg)	
Spray chamber temperature (TEM): 2 ^{o}C	Curtain gas (CUR): 20 psi	Curtain gas (CUR): 30 psi
Reaction cell He gas: 3.0 mL/min	GS1: 40 psi	GS1: 40 psi
Monitored m/z: 75	GS2: 50 psi	GS2: 50 psi
	CAD: high	CAD: high
	EP: ± 10	EP: -10
	DP: +80 eV (pos); -40 eV (neg)	DP: -40 eV
		CE: -45 eV
		CXP: -15

Table 6.3. The list of candidate precursor ions in the positive and negative mode.

Negative mode		Positive mode	
m/z	Retention time (min)	m/z	Retention time (min)
575.153	18.09	243.287	18.12
465.185	18.12	286.109	18.16
507.159	18.18	473.661	18.16
277.914	18.18	279.926	18.22
575.158	18.18	477.311	18.23
296.993	18.24	225.113	18.26
479.202	18.36	256.264	18.28
		476.307	18.34
		308.161	18.41
		349.155	18.41
		489.179	18.44
		259.166	18.44

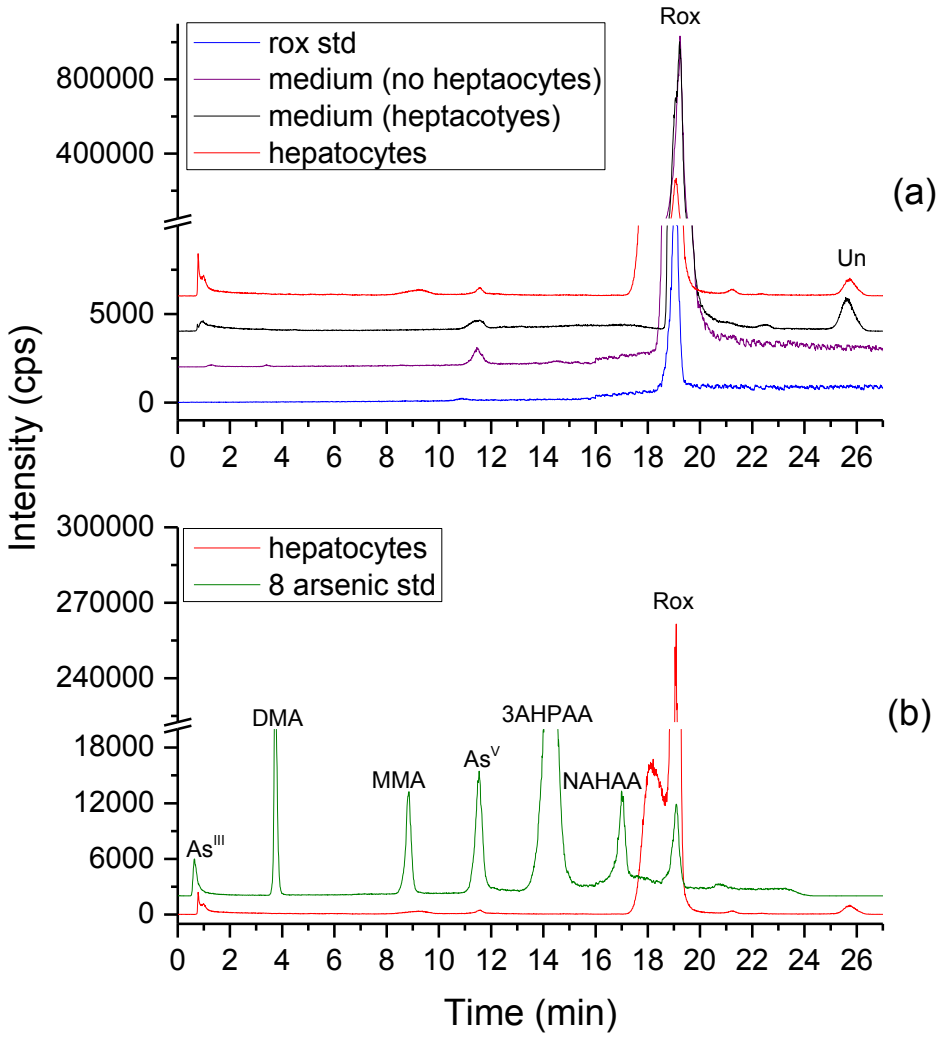

Figure 6.1. Chromatograms from the anion exchange separation and ICPMS detection of arsenic species in in the (a) cell lysate of plated human primary hepatocytes exposed to 20 µM Rox for 24 hours (black trace) and cell culture medium of plated human primary exposed to 100 µM Rox for 24 hours (red trace); (b) cell lysate (black trace) and cell medium (red trace) of plated human primary hepatocytes exposed to 1 µM As(III) for 24 hours.

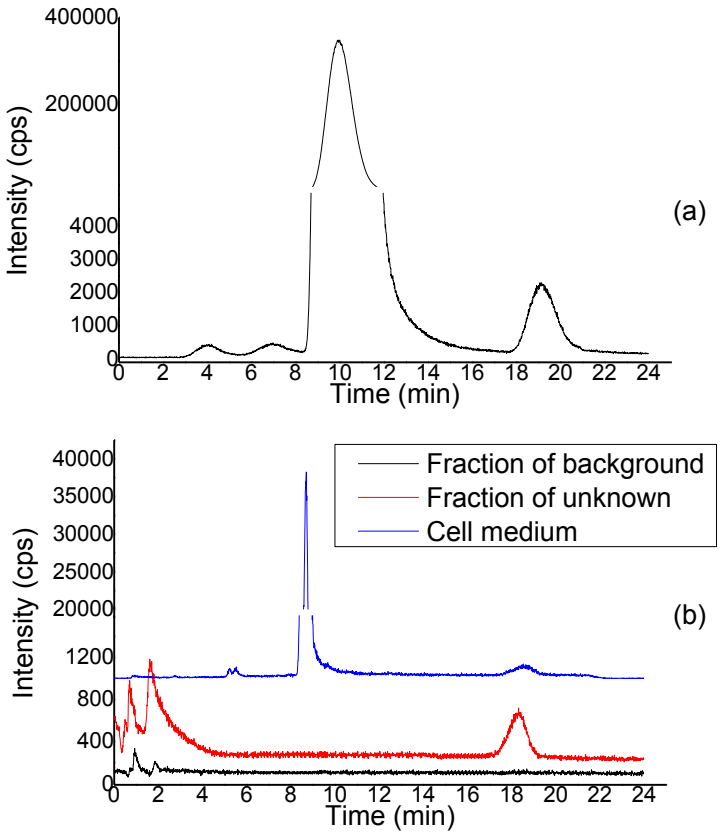

Figure 6.2. Chromatogram from (a) the reverse phase separation and ICPMS detection of arsenic species in cell medium; (b) anion exchange separation and ICPMS detection of cell medium (blue trace), the fraction of an unknown arsenic species (18-20 min) from reverse phase chromatographic run (red trace) and the fraction of background (22-24 min) from reverse phase chromatographic run (black trace).

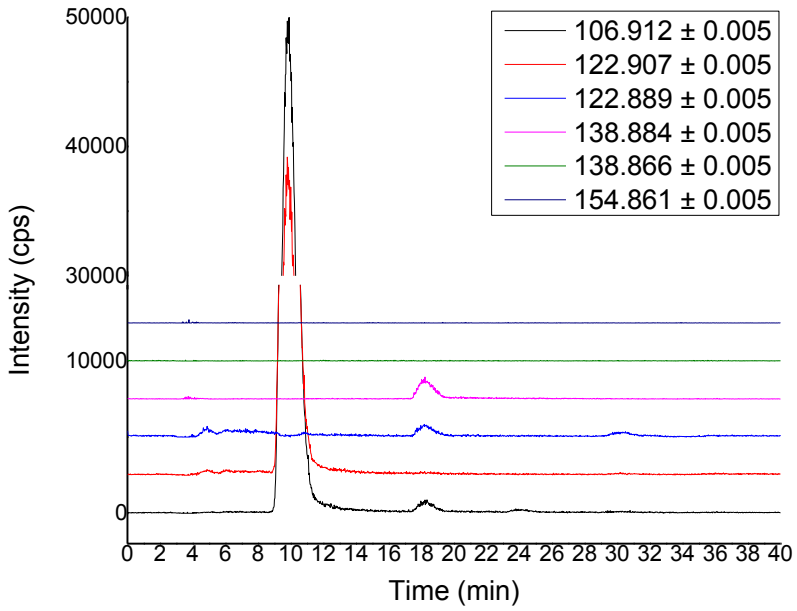

Figure 6.3. Chromatograms from reverse phase separation and ESI-QToF detection of cell medium monitored at m/z of the arsenic fragments (shown in Table 6.1) in the negative mode.

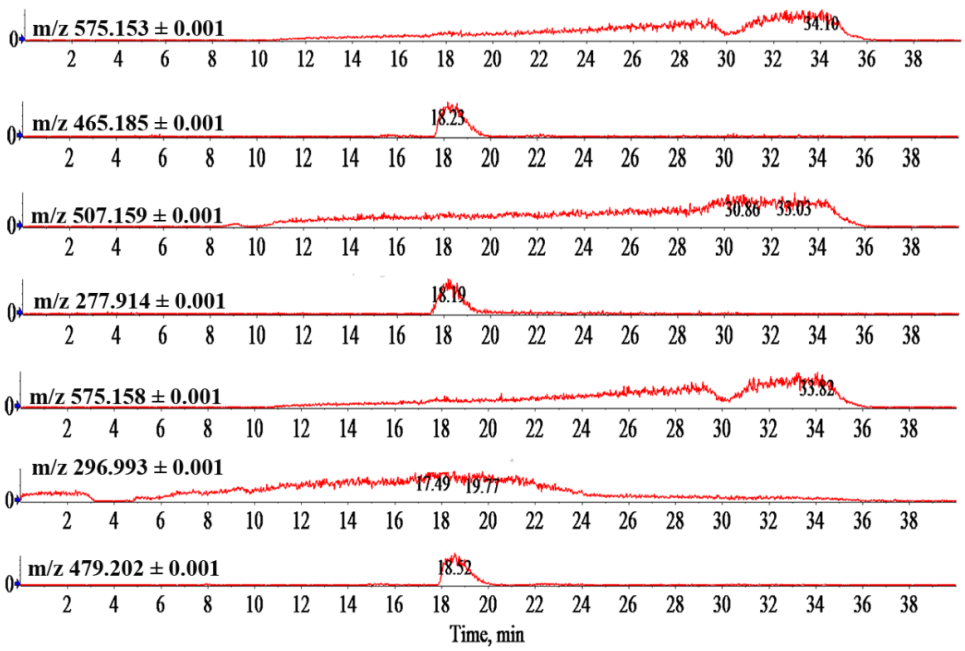

Figure 6.4 Chromatograms from reverse phase separation and ESI-QToF detection of cell medium monitored at m/z of the candidate precursor ions (shown in Table 6.3) in the negative mode.

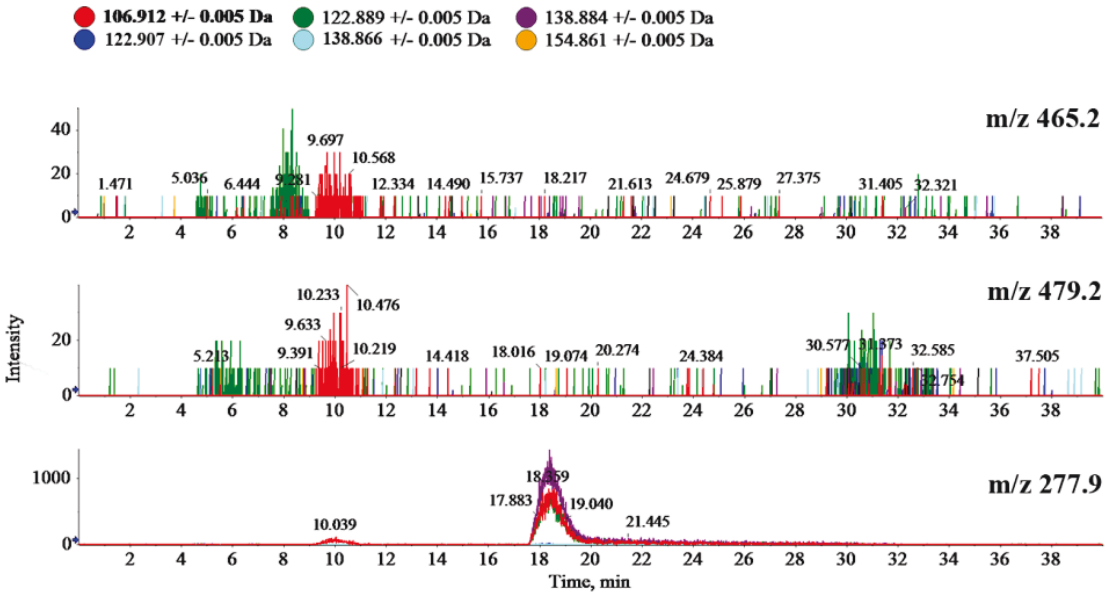

Figure 6.5. Chromatograms from reverse phase separation and ESI-QToF detection of cell medium monitored in product ion mode in negative mode. The parent ions were monitored at m/z 465.2, 479.2 and 277.9. The product ions were monitored at m/z 106.912, 122.907, 122.889, 138.866, 138.884 and 154.861.

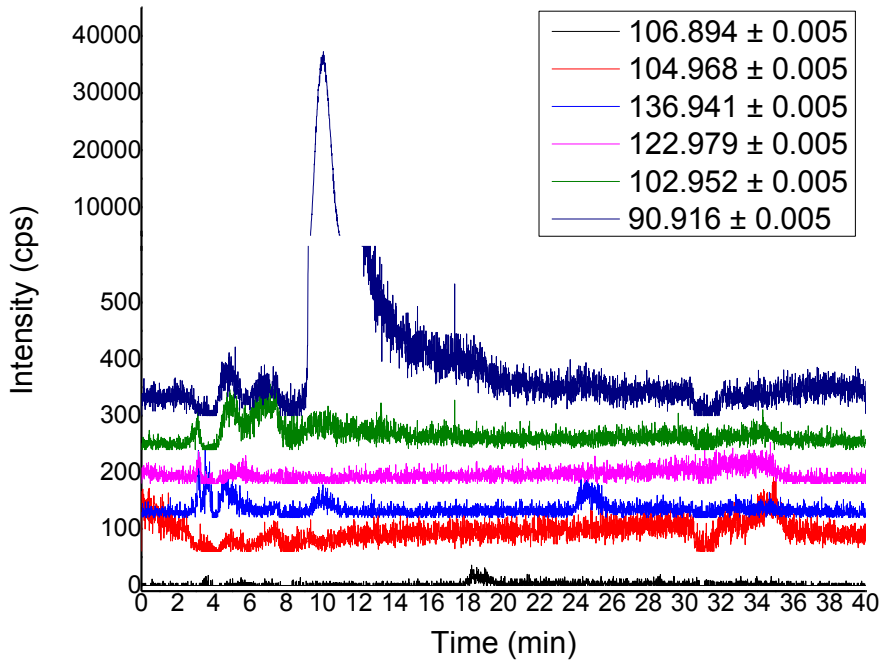

Figure 6.6. Chromatograms from reverse phase separation and ESI-QToF detection of cell medium monitored at m/z of the arsenic fragments (shown in Table 6.1) in the positive mode.

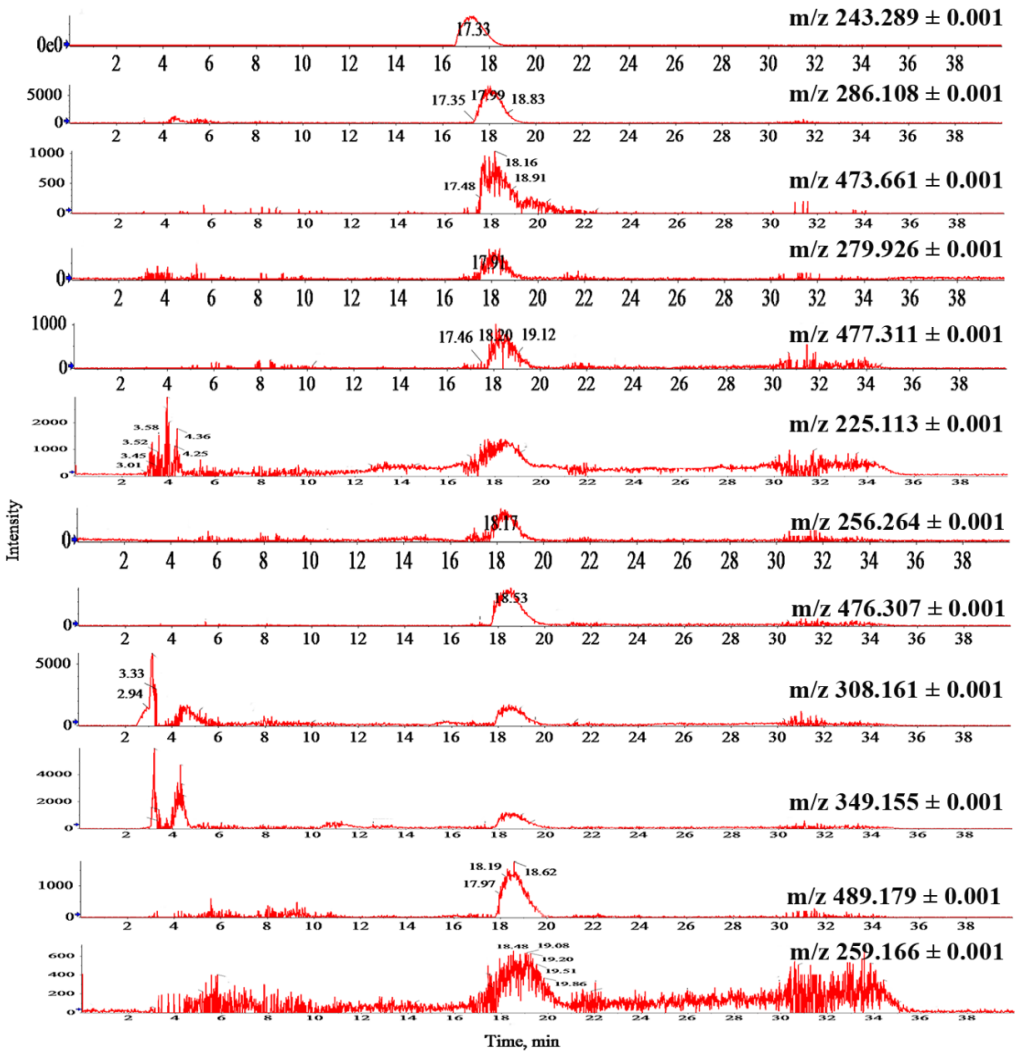

Figure 6.7 Chromatograms from reverse phase separation and ESI-QToF detection of cell medium monitored at m/z of the candidate precursor ions (Shown in Table 6.3) in the positive mode.

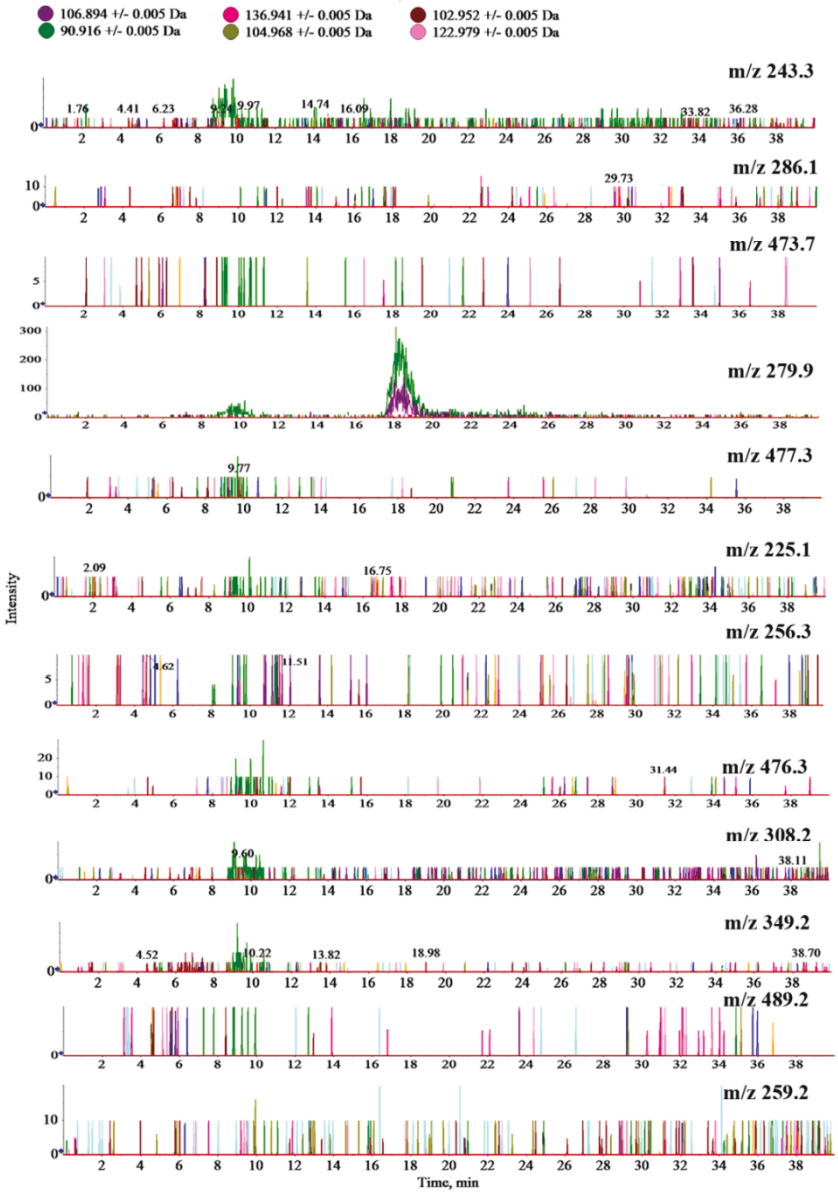

Figure 6.8 Chromatograms from reverse phase separation and ESI-QToF detection of cell medium monitored in product ion mode in positive mode. The parent ions were monitored at m/z of the candidate ions shown in Table 2 in positive mode. The product ions were monitored at m/z 90.916, 106.894, 136.944, 104.968, 102.952 and 122.979.

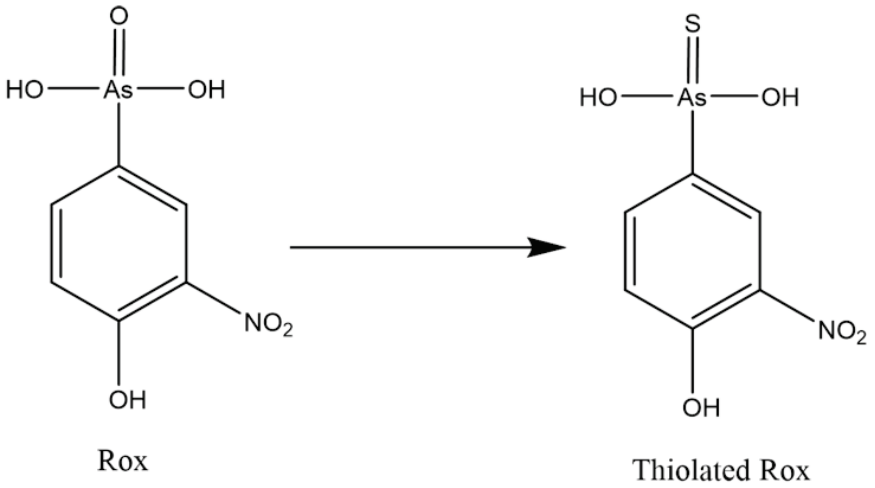

Figure 6.9 The structures of Rox and thiolated Rox.

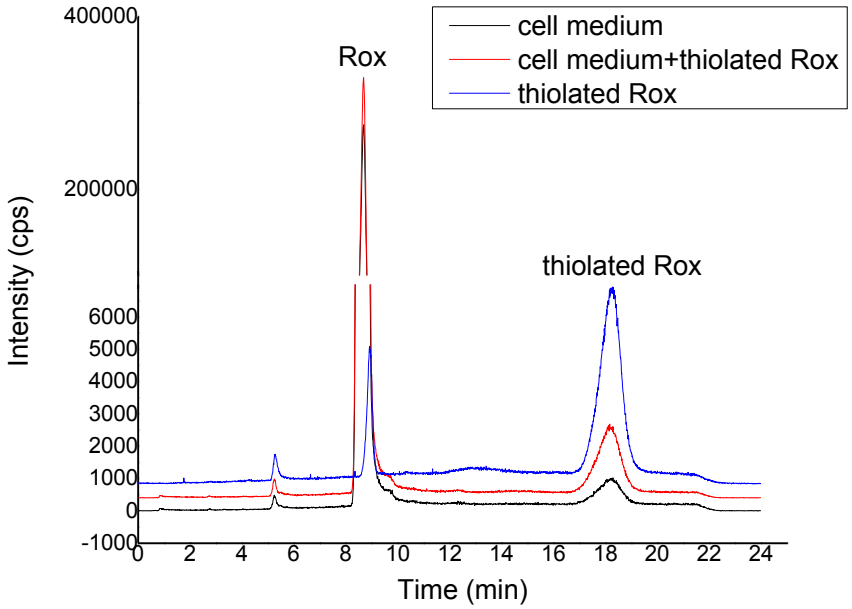

Figure 6.10 Chromatograms from anion exchange separation and ICPMS detection of the cell medium (black trace), synthesized thiolated Rox (with Rox and arsenate as impurities inside, blue trace), and cell medium spiked with thiolated Rox standard solution (red trace).

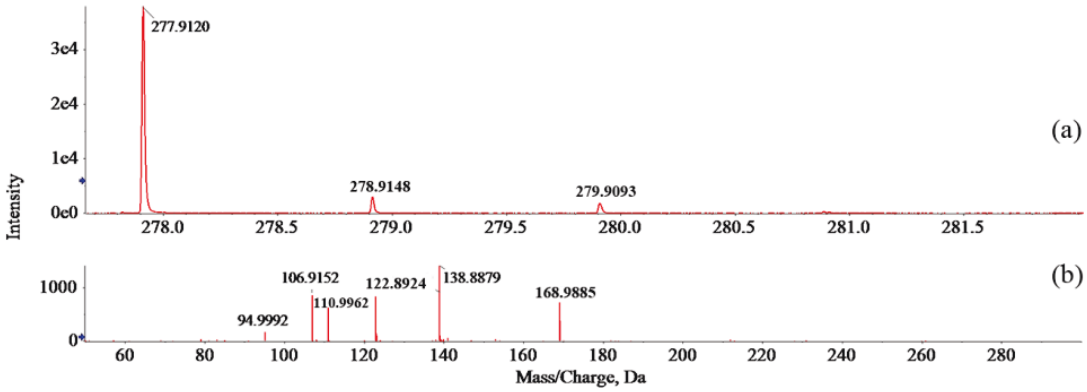

Figure 6.11. Mass spectral obtained from QToF MS analyses of a compound eluting from the reverse phase HPLC column at min, corresponding to Rox: (a) accurate mass of Rox; (b) product ion scan of Rox under 45eV collision energy.

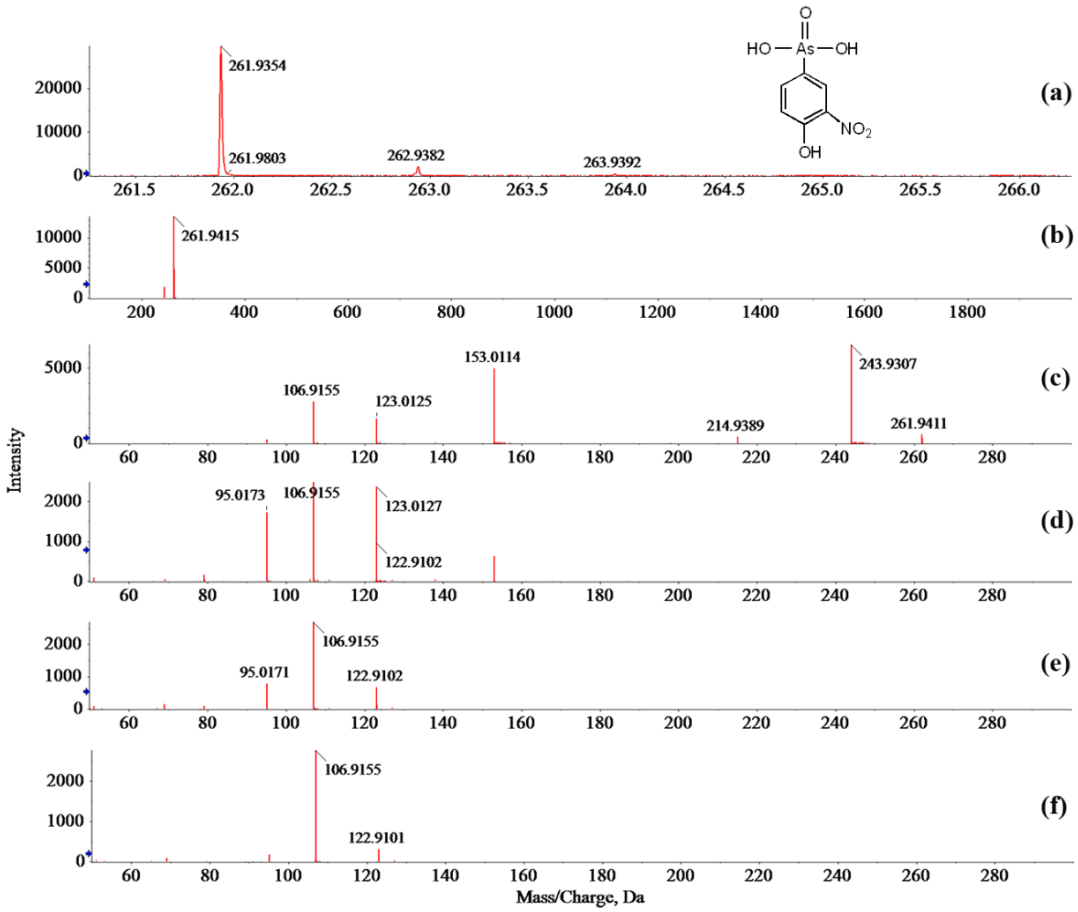

Figure 6.12 Mass spectral obtained from QToF MS analyses of a compound eluting from the reverse phase HPLC column at min, corresponding to Rox: (a) accurate mass of Rox; (b) product ion scan of Rox under 5eV collision energy; (c) product ion scan of Rox under 25eV collision energy; (d) product ion scan of Rox under 45eV collision energy; (e) product ion scan of Rox under 65eV collision energy; (f) product ion scan of Rox under 85eV collision energy. The structure of Rox is shown in (a).

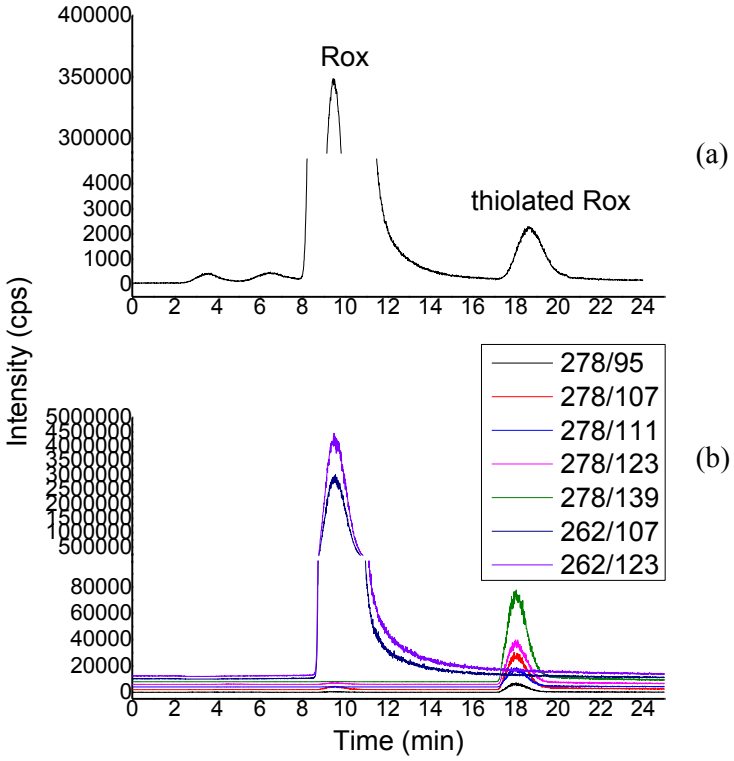

Figure 6.13. Chromatograms from reverse phase separation of cell medium. The cell medium was detected by (a) ICPMS and (b) ESI-triple Quadrupole at the same time. In ESI-triple Quadrupole, thiolated Rox was monitored by 5 transition ion pairs (278/95, 278/107, 278/111, 278/123, and 278/139) and Rox was monitored by 2 transition ion pairs (262/107 and 262/123).

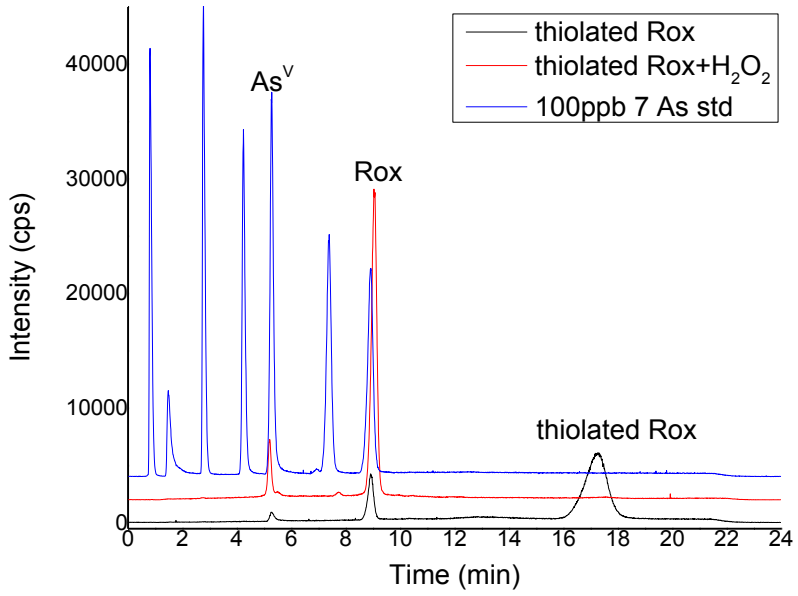

Figure 6.14. Chromatograms from anion exchange separation and ICPMS detection of synthesized thiolated Rox solution (with Rox and arsenate as impurities inside) (black trace), synthesized thiolated Rox solution treated with 6% H_2O_2 (red trace), and the standard solution of 100ppb 7 arsenic species (blue trace).

Chapter 7. Biotransformation of 3-amino-4-hydroxyphenylarsonic acid to N-acetyl-4-hydroxyphenylarsonic acid by poultry and human N-acetyltransferases [6]

7.1. Introduction

Phenylarsonic acid derivatives are extensively used as feed additives in the poultry industry. The most commonly-used one is Roxarsone (Rox) which is used to control coccidial intestinal parasites and promote chicken growth. After feeding chicken Roxarsone, Moody and Williams (1964) found that about 25% of the excreted Arsenic (As) in chicken feces was in form of the Roxarsone degradation product 3-amino-4-hydroxyphenylarsonic acid (3-AHPAA). In the chicken feeding study conducted by our group, I detected 3-AHPAA and N-acetyl-4-hydroxyphenylarsonic acid (NAHAA) in the manure and livers of chickens fed Rox (Peng et al. 2014; Yang et al. 2016). Figure 7.1 shows the chromatogram of arsenic species in the liver of chicken fed with Rox.

Toxicities of individual arsenic species range from virtually nontoxic, e.g., arsenobetaine (AsB), to known human carcinogen arsenite (Conklin et al. 2012; Feldmann and Krupp 2011). It is important to identify the metabolism pathways and biological fates of arsenic compounds because the metabolites sometimes might be more toxic or more biologically available than their parent compounds. It was illustrated by the results that arsenite was released from the Roxarsone metabolism (Nachman et al. 2013) . And a new and water-soluble azobenzene derivative of unknown toxicity and biological availability was produced by the conversion of 3-AHPAA (Wershaw et al. 2003).

[6] A version of this chapter is in preparation to submit for publication. Dr. Martin Zuidhof provided the fresh chicken livers. Dr. Elaine Leslie provided the frozen human liver samples. Dr. X.Chris Le assisted with manuscript revision.

3-AHPAA is a phenylarsonic acid with an amino group on it. N-acetylation is a major route of biotransformation for xenobiotics containing an aromatic amine (R-NH_2), such as 3-AHPAA, or a hydrazine group (R-NH-$COCH_3$) and hydrazide (R-NH-NH-$COCH_3$). Like methylation, N-acetylation masks an amine with a nonionizable group, so that many N-acetylated metabolites are less water-soluble than the parent compound (Klaassen 2013).

The N-acetylation of xenobiotics is catalyzed by N-acetyltransferases (NATs) and requires the cofactor acetyl coenzyme An (acetyl-CoA). The reaction occurs in two sequential steps according to a ping-pong Bi-Bi mechanism (Hein 1988). In the first step, the acetyl group from acetyl-CoA is transferred to a cysteine residue in the NATs active site with the release of CoA. In the second step, the acetyl group is transferred from the acetylated NATs to the amino group of the substrate. NATs are cytosolic enzymes found in liver and many other tissues of most mammalian species (Vatsis et al. 1995). The arylamine compounds can either be detoxified by NATs or bioactivated to metabolites that have the potential to cause toxicity such as cancer (Butcher et al. 2008). Rox is reduced to 3-AHPAA, therefore subsequent acetylation is possible (Figure 7.2). In the present work, I investigated that whether NATs were responsible for the acetylation of 3-AHPAA and the production of NAHAA. Through doing this research, I could have a better understanding of the biotransformation mechanism of Roxarsone *in vitro*.

7.2. Experimental methods

7.2.1. Chemicals

Sucrose, dihydrogen potassium phosphate, ethylenediaminetetraacetic acid (EDTA), leupeptin, dithiothreitol (DTT), Tris-HCl, Acetyl coenzyme A, pigeon liver N-acetyltransferases (one isoform with Enzyme Commission Number 2.3.1.5), acetyl-DL-carnitine, carnitine acetyltransferase, p-aminobenzoic acid (PABA), 4-acetamidobenzoic acid, sulfamethoxazole (SMZ) were purchased from Sigma-Aldrich (St. Louis, MO). Human N-acetyltransferases 1 recombinant protein was from Abnova (Walnut, CA). 3-amino-4-hydroxyphenylarsonic acid (3AHPAA) and N-acetyl-4-hydroxy-m-arsanilic acid (NAHAA) were from Pfaltz and Bauer Inc. (Waterbury, CT).

7.2.2. Preparation of chicken and human liver cytosol

Fresh chicken livers were provided by Dr. Martin Zuidhof from Department of Agriculture, University of Alberta. The livers were cut into small cubes and stored at -80^{o}C before use. Frozen human liver samples were provided by Dr. Elaine Leslie from Department of Physiology, University of Alberta. Cytosol was prepared as previously described (Andres, Klem, Szabo, & Weber, 1985; Andres, Vogel, Tarr, Johnson, & Weber, 1987; Grant, Mörike, Eichelbaum, & Meyer, 1990; Winter & Unadkat, 2005). In brief, homogenizing buffer contained 250 mM sucrose, 100 mM dihydrogen potassium phosphate, 1 mM EDTA, 1 μg/ml leupeptin, 1 mM DTT, and 10 mM Tris-HCl (pH 7.4). Chicken cytosol was prepared by homogenizing 10 g of chicken liver on ice in 30 mL buffer. Human cytosol was prepared by homogenizing 0.5 g of human liver in 1.5 mL buffer. The liver homogenate was centrifuged at 15,000 ×g at 4 ^{o}C for 20 minutes. The

supernatant was centrifuged again at 100,000 ×g at 4 ^{o}C for 1 hour. The supernatant was used immediately or fresh-frozen in liquid nitrogen and then stored at - 80^{o}C.

7.2.3. Acetylation assay

Assays were conducted by the method reported by Grant et al. (Grant et al. 1991). The concentration of Acetyl coenzyme A was fixed at 100 μM. Total reaction solution volume was 90 μl. Incubations contained: 40 μl of chicken liver cytosol, pigeon liver NAT, human liver cytosol NAT1or human liver cytosol (the concentration of proteins in four enzyme sources was at 0.1 μg/μL) ; 20 μl of acetyl CoA regenerating system (containing 4.5 mM acetyl-DL-carnitine and 0.02 unit of carnitine acetyltransferase) dissolved in 225 mM triethanolamine-HCl, 4.5 mM DL-dithiothreitol, 4.5 mM EDTA at pH 7.4 (Andres et al. 1985); 20 μl of acetyl coenzyme A (450 μM in water) and 10 μl of 540 μM of 3-AHPAA, PABA or SMZ. The initial concentrations of proteins in pigeon liver NAT and human liver cytosol were 27.5 μg/μL and 0.13 μg/μL, respectively. The concentration of proteins was diluted to 0.1 μg/μL before use. The incubation was kept in a 37 ^{o}C water bath for 1 hour and reactions were terminated by the addition of 90 μl methanol. The solution was ultracentrifuged through filters with 3000 Dalton cut-off at 14,000 ×g for 30 minutes. The filtrate was loaded onto the HPLC column for separation.

7.2.4. Separation and quantification of target compounds

HPLC separation of arsenic species was performed on a PRP-X110s anion exchange column (Hamilton). Two mobile phases were prepared as follows: (A) 5% methanol; (B) 60 mM ammonium bicarbonate, 5% methanol, pH adjusted to 8.75. The flow rate was kept

at 400 µL/min for a 15-minute HPLC gradient elution. Phase B was increased to 100% within 10 minutes, decreased to 0% in the following 1 minute and kept at 0% for another 4 minutes. The sample injection volume was 20 µL. Electrospray ionization – triple quadrupole mass spectrometry (ESI-MS) in MRM mode in negative mode was used to detect 3-AHPAA and NAHAA. The parameters for the mass spectrometry were shown in Table 7.1. The transition ion pairs were 232/107, 232/123 for 3-AHPAA and 274/165, 274/123 for NAHAA.

PABA and its acetylated products 4-acetamidobenzoic acid (acetyl-PABA), and SMZ and its acetylated products acetyl-SMZ were separated by reverse phase column (Phenomenex Luna® C18 (2): 100 x 2 mm; 3-µm particle size; 100 Å), eluted at a flow rate of 150 µL/min. Elution was carried with 0.1% trifluoroacetic acid in water as phase A and 0.1% trifluoroacetic acid in acetonitrile as phase B. Phase B increased from 20% to 95% in the first 20 minutes, went back to 20% in the following 1 minute and kept at 20% for another 10 minutes. PABA, acetyl-PABA, SMZ, and acetyl-SMZ were detected in MRM mode in positive mode. The transition ion pairs were 138/120 and 138/77 for PABA, 180/138, 180/94, and 180/77 for acetyl-PABA, 279/124 and 279/186 for SMZ, and 321/124 and 321/134 for acetyl-SMZ.

7.3. Results and discussion

Pigeon liver NAT, human NAT1 recombinant proteins, chicken liver cytosol and human liver cytosol were used as the sources of N-acetylation transferases. They were incubated with 3-AHPAA individually to acetylate 3-AHPAA to NAHAA. In Figure 7.3a,

pigeon liver NAT acetylated 3-AHPAA to larger amount of NAHAA (7.6 µg) than chicken liver cytosol (0.23 µg NAHAA, Figure 7.3b).

Human NAT1 did not produce any NAHAA. I used PABA as the positive control to test the acetylation ability of human NAT1. Figure 7.4 showed that PABA was acetylated to acetyl-PABA while there was no NAHAA produced when there was the same amount of human NAT1 used to acetylate 3-AHPAA. Human liver cytosol could not acetylate 3-AHPAA to NAHAA. Both ESI-MS and ICP-MS results in Figure 7.5 suggested that no NAHAA was formed in the reaction mixture. PABA and SMZ were used as the positive control to test the acetylation ability of human liver cytosol. In Figure 7.6, human liver cytosol could acetylate PABA to acetyl-PABA and SMZ to acetyl-SMZ.

I compared the protein sequences (FASTA data) among pigeon NAT, chicken NAT, and human NAT1 by doing sequence alignments using the Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).The results are shown in Figure 7.7 and Table 7.2. Chicken NAT shared 100% identity with pigeon NAT and only 47% identity with human NAT.

I noticed the human hepatocytes and human liver cancer cells produced NAHAA when these two kinds of cells were incubated with Roxarsone. Even though human NAT1 could not acetylate 3-AHPAA, other human NAT might serve to acetylate 3-AHPAA to NAHAA. There are always species differences in the substrate specificity of NATs, for example, PABA is preferentially N-acetylated by NAT1 in human but NAT2 in mice (Klaassen 2013). Even though the human liver cytosol used here cannot acetylate 3-AHPAA. This could be because the concentration or activity of the enzyme in the prepared cytosol was too low to see the results.

7.4. Conclusion

The evidence that chicken and pigeon NATs could acetylate 3-AHPAA to NAHAA is provided. The acetylation pathway of forming NAHAA from 3-AHPAA is possible.

7.5. References

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Table 7.1 The parameters of ESI-MS for the detection of 3-AHPAA, NAHAA, PABA, and acetyl-PABA.

Compound	Transition ion pairs	Declustering potential	Entrance potential	Collison energy	Collision cell exit potential	Curtain gas	IonSpray Voltage	Temperature	Ion source gas 1	Ion source gas 2
3-AHPAA	232/107	50	-20	-64	-11	30	-4500	500	50	50
	232/123	50	-20	-28	-25					
NAHAA	274/123	50	-45	-36	-11					
	274/165	50	-45	-26	-9					
SMZ	279/124	26	10	30	10					
	279/186									
Acetyl-SMZ	321/124	86		30	4					
	321/134			35						
PABA	138/120	80	10			30	5000	450	30	30
	138/77									
Acetyl-PABA	180/138			30	15					
	180/94									
	180/77									

Table 7.2. Percent Identity matrix of sequence alignment among human NAT1(P18440), chicken NAT (NP_990857.2), and pigeon NAT (AAA48590.1). The identity matrix was created by Clustal Omega 1.2.3.

	Human NAT1	Chicken NAT	Pigeon NAT
Human NAT1	100.00%	46.90%	47.55%
Chicken NAT		100.00%	100.00%
Pigeon NAT			100.00%

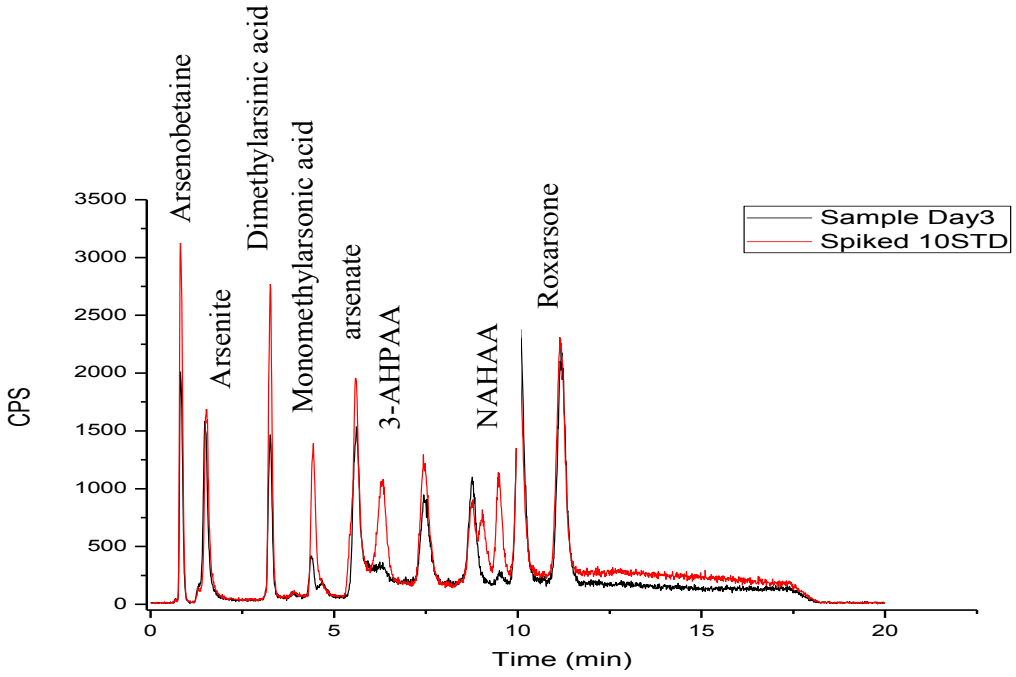

Figure 7.1 Arsenic speciation in the livers of chicken fed Roxarsone. 3-AHPAA and NAHPAA present in the chicken liver sample.

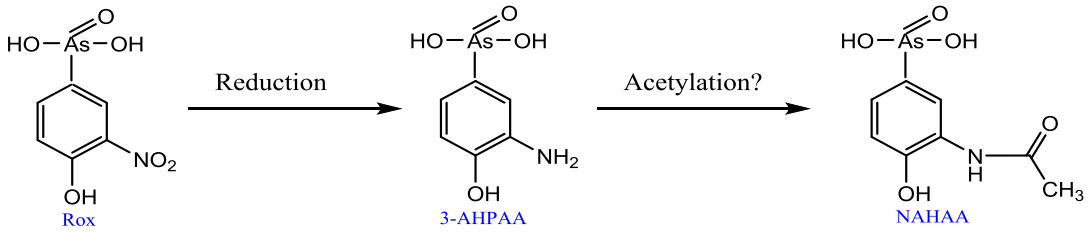

Figure 7.2 Structures of arsenic compounds of interest

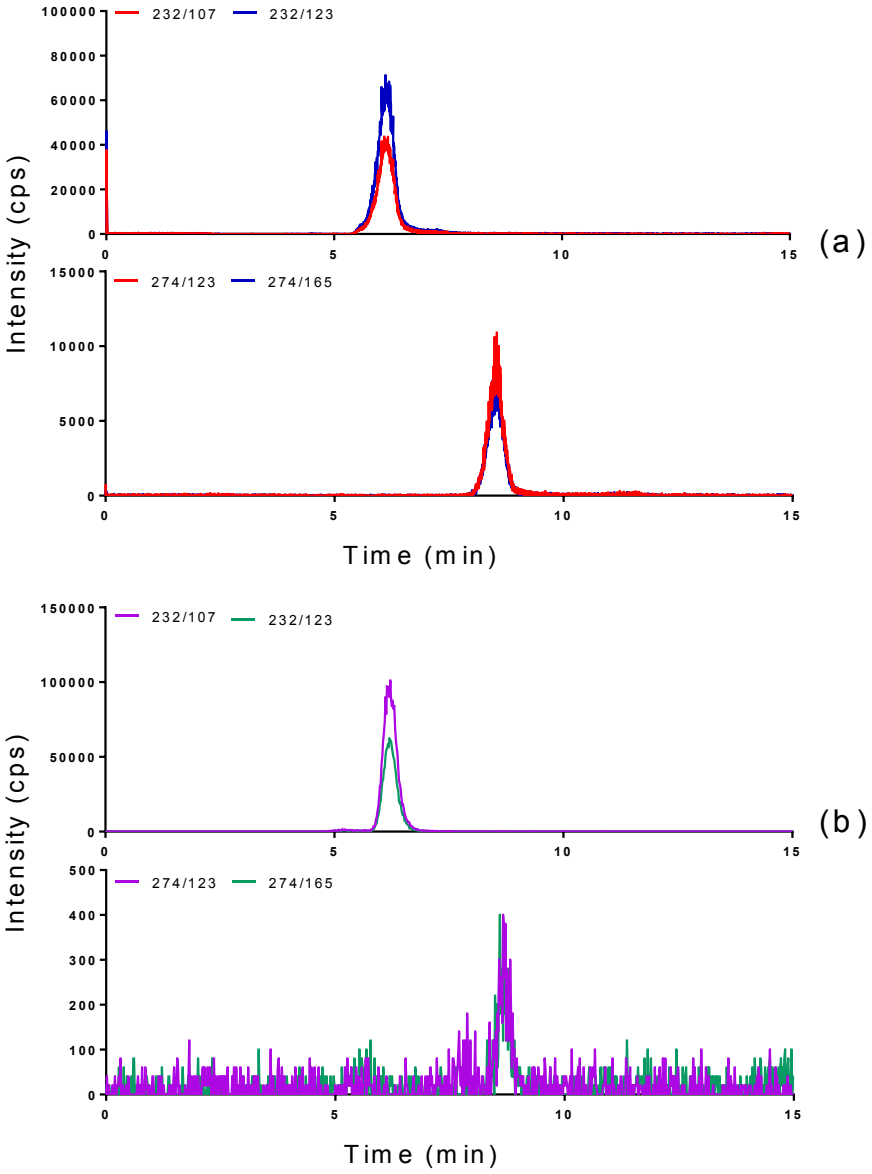

Figure 7.3 The chromatograms of 3-AHPAA monitored at 232/107 and 232/123 and NAHAA monitored at 274/123 and 274/165: (a) pigeon liver NAT was incubated with 3-AHPAA to produce NAHAA; (b) chicken liver cytosol was incubated with 3-AHPAA to produce NAHAA.

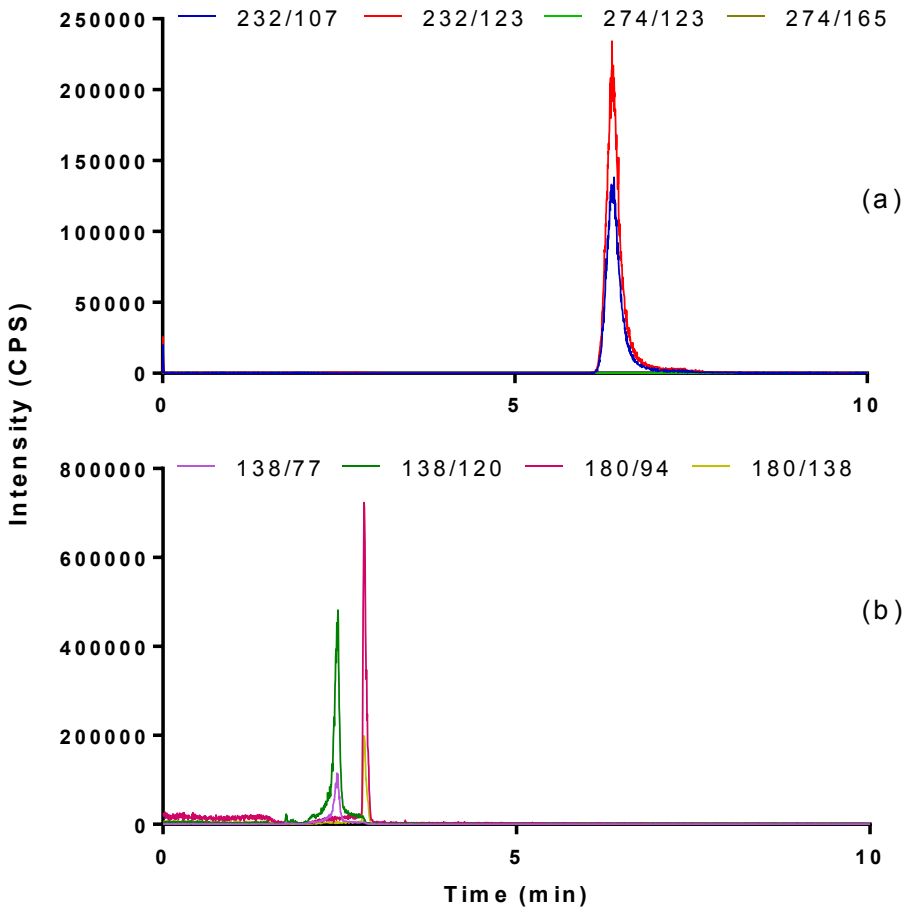

Figure 7.4 The chromatograms of 3-AHPAA monitored at 232/107 and 232/123 and NAHAA monitored at 274/123 and 274/165: (a) Human NAT1 was incubated with 3-AHPAA; (b) PABA was used as the positive control to test the acetylation activity of human NAT1. PABA was monitored at 138/77 and 138/120 while acetyl-PABA was monitored at 180/94 and 180/138.

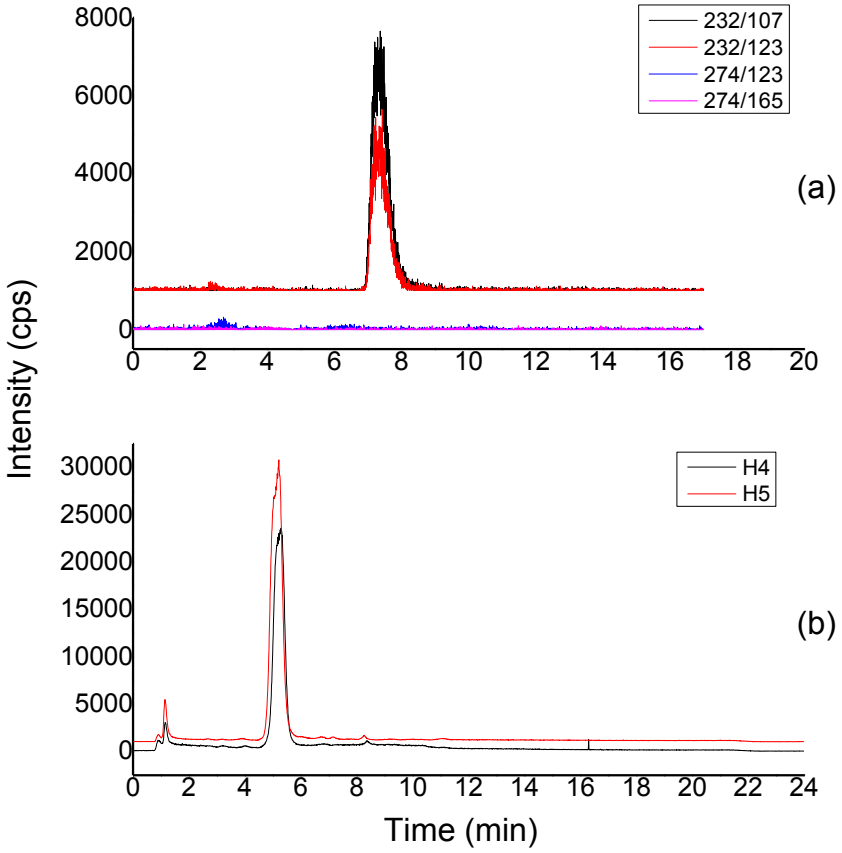

Figure 7.5 The chromatograms of (a) HPLC-ESIMS analyses of human liver cytosol (H4) incubated with 3-AHPAA. The transition ion pairs monitored were 232/107 and 232/123 for 3-AHPAA, and 274/123 and 274/165 for NAHAA; (b) HPLC-ICPMS analyses of human liver cytosol H4 (black trace) and H5 (red trace) incubated with 3-AHPAA.

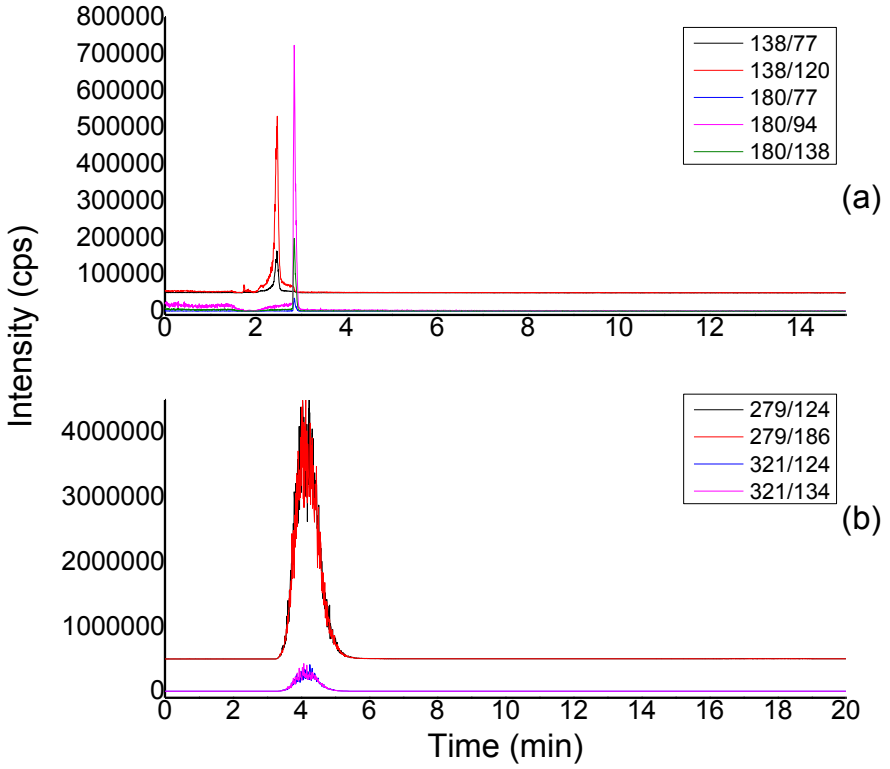

Figure 7.6 The chromatograms of HPLC-ESIMS analyses of human liver cytosol H5 incubated with PABA and SMZ. PABA and SMZ were used as positive control to test the acetylation ability of human liver cytosol. (a) PABA was monitored at 138/77 and 138/120 and acetyl-PABA monitored at 180/94, 180/77 and 180/138; (b) SMZ was monitored at 279/124 and 279/186 and acetyl-SMZ was monitored at 321/124 and 321/134.

Figure 7.7 The alignment among pigeon NAT (AAA48590.1), chicken NAT (NP_990857.2), and human NAT1(P18440).

Chapter 8. Conclusions

8.1. Introduction

Arsenic (As) is a metalloid that occurs naturally in the earth's crust at 0.5-2.5 mg/kg (ATSDR 2007). It is found as more than 50 chemical species in environmental and biological systems (Cullen and Reimer 1989; Gong et al. 2002). Epidemiological studies have shown that chronic exposure to inorganic arsenic is strongly associated with the prevalence of lung, bladder and skin cancers (Chen et al. 1992; Levine et al. 1988) and the increased risk of cardiovascular disease, type 2 diabetes, and adverse pregnancy outcomes (WHO 2012; Yoshida et al. 2004). The toxicity of arsenic is highly dependent on the chemical species of arsenic, and the median lethal doses of arsenic species vary by ten thousand folds from the most toxic to the least toxic arsenic species (Tseng 2009). WHO has set the maximum tolerable concentration of arsenic in drinking water as 10 μg/L (WHO 2008).

Inorganic arsenic can undergo methylation to become mono-, di-, and tri-methyl arsenicals. While the pentavalent methyl arsenicals (MMA^V and DMA^V) are less toxic that inorganic arsenic, the trivalent methyl arsenicals (MMA^{III} and DMA^{III}) are more toxic inorganic arsenic species (Styblo et al. 2000). Therefore, it is critical to characterize different arsenic species and determine their concentrations in order to provide meaningful human exposure and health risk assessment.

3-nitro-4-hydroxyphenylarsonic acid (Roxarsone®, Rox) has been used extensively as a poultry feed additive to control intestinal parasites that cause coccidiosis and to promote the poultry growth (Chapman and Johnson 2002; Jones 2007; Lasky et al. 2004). Previous studies measuring total arsenic concentrations in chickens have shown that

arsenic was distributed to the whole chicken body and that a 5-day withdrawal period resulted in substantial decrease in arsenic concentration in chickens (Garbarino 2003; Kazi 2012). However, little is known about the concentrations of different arsenic species present in chickens as a consequence of feeding Roxarsone to chickens. Human exposure to As might be increased due to the ingestion of chicken fed Roxarsone. The uptake and metabolism of Rox in human body remains unclear.

My thesis research is aimed to fill the knowledge gap on Roxarsone. To accomplish the goal, I first developed an analytical method to sensitively determine arsenic species in the breast meat of chickens (Chapter 2). Then this method was applied on the chicken breast samples that were collected from a 35-day poultry feeding study (Chapter 3). The accumulation and transport of Rox through the human colon cancer cells (Caco-2) were investigated (Chapter 4). And the metabolism of Roxarsone in human liver cancer cells (hepG2) and human primary hepatocytes was studied (Chapter 5). The unidentified Roxarsone metabolite present in human primary hepatocytes was characterized (Chapter 6). And finally, the acetylation of one Roxarsone metabolite, 3AHPAA, to another metabolite, NAHAA, was assessed (Chapter 7). The final chapter present the summaries of the major findings from my research (Chapter 2-7), and the overall conclusions on my thesis work and perspectives on future works.

8.2. Major findings

8.2.1. Development of extraction, separation, and detection methods for the determination of arsenic species

Currently, there is no analytical method available for determining individual

arsenic species present at trace levels in chicken meat. Analytical challenges include: (1) nearly ten arsenic species are likely present, and thus an efficient separation technique is required to differentiate individual arsenic species; (2) concentrations of each arsenic species are likely very low, and thus a detection technique needs to be highly sensitive; and (3) trace amounts of arsenic species may be difficult to extract from chicken tissue, and thus an efficient method of sample preparation is required. Ideally, all arsenic species should be completely extracted into the solution. The integrity of arsenic species should be maintained during the extraction process. This is a major analytical challenge.

I have developed a high performance liquid chromatography (HPLC) technique that is able to separate seven relevant arsenic species. To quantify the amount of individual arsenic species, I linked HPLC with inductively coupled plasma mass spectrometry (ICPMS) and electrospray ionization (ESI) tandem mass spectrometry (MS/MS). This combination is unique and advantageous because ICPMS enables highly sensitive detection of arsenic and ESI-MS/MS provides molecular information for confirmative identification. Using this method I have now achieved detection limits of 0.06-0.11 μg/L for seven arsenic species.

I have examined several extraction reagents, including water-methanol, trifluoroacetic acid (TFA), and enzymes (trypsin, pepsin, papain, bromelain, proteinase K), combined with ultrasonication. With the use of papain-assisted extraction, 10 arsenic species were extracted and detected, as compared to 8 detectable arsenic species in the water/methanol extract. The overall extraction efficiency was also improved using a combination of ultrasonication and papain digestion, as compared to the conventional water/methanol extraction.

Finally, the method is an enzyme-enhanced extraction of arsenic species from chicken meat, separation using anion exchange chromatography (HPLC), and simultaneous detection with both inductively coupled plasma mass spectrometry (ICPMS) and electrospray ionization tandem mass spectrometry (ESIMS). Detection limits were in the range of 1.0-1.8 μg arsenic per kg chicken breast meat (dry weight) for seven arsenic species: arsenobetaine (AsB), inorganic arsenite (As^{III}), dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), inorganic arsenate (As^{V}), 3-nitro-4-hydroxyphenylarsonic acid (Roxarsone), and N-acetyl-4-hydroxy-m-arsanilic acid (NAHAA). This detection capability will allow me to detect trace concentrations of arsenic in chicken samples.

8.2.2. Feeding of Roxarsone to chicken induced the increased concentrations of other arsenic species in chicken breast tissue

Chicken is the No.1 consumed meat in North America, with a consumption rate of one billion kilograms per year (Statistics Canada 2012). The practice of feeding 3-nitro-4-hydroxyphenylarsonic acid (Roxarsone, Rox) to chickens lasted for over 60 years. Concentrations of arsenic in chicken range from μg/kg to mg/kg. However, the fate of Rox and arsenic metabolites remaining in chicken are poorly understood. Having developed the methods for arsenic extraction, chromatography separation, and mass spectrometry detection, I have applied these methods to the analyses of arsenic species in chicken breast tissues. The tissue samples were obtained from 256 chickens, representing both strains (Ross308 and Cobb500). Half of them were fed a diet supplemented with Roxarsone and the other half were fed a control diet not supplemented with Roxarsone.

Rox, arsenobetaine, arsenite, monomethylarsonic acid, dimethylarsinic acid, and a new arsenic metabolite, were detected in breast meat from chickens fed Rox. The concentrations of arsenic species, except arsenobetaine, were significantly higher in the Rox-fed than in the control chickens. As^{III}, DMA^{V}, MMA^{V}, and Un in the Rox-treated chickens, increased in a similar trend during the Rox-feeding period (from day 1 to day 28). Their concentrations decrease after Rox feeding was stopped (day 29 to day 35). The half-lives of elimination of these arsenic species were 0.4-1 day. Seven days after termination of Rox feeding, the concentrations of arsenite (3.1 μg/kg), Rox (0.4 μg/kg), and a new arsenic metabolite (0.8 μg/kg) were significantly higher in the Rox-fed chickens than the control. Feeding of Rox to chickens increased the concentrations of five arsenic species in the breast meat. Although most arsenic species were excreted rapidly when the feeding of Rox stopped, arsenic species remaining in the Rox-fed chickens were higher than the background levels.

8.2.3. Cellular accumulation and transport of Roxarsone in the human colon cancer cells Caco-2.

Residual amounts of Rox can be present in chicken meat, which gives rise to potential human exposure to Rox. But studies on the bioavailability of Rox in humans are scarce. I reported in the fourth chapter that the accumulation and transepithelial transport of Rox using the human colon-derived adenocarcinoma cell line (Caco-2) model. The cellular accumulation and transepithelial passage of Rox in Caco-2 cells were evaluated and compared to that of AsB, As^{III}, and As^{V}. After a 24-h exposure, the accumulated Rox was 6-20 times less than AsB and As^{III}. The permeability of Rox from the apical to

basolateral side of the Caco-2 monolayers was similar to As^{V} but less than As^{III} and AsB. The result of lower bioavailability of Rox was consistent with the previous observations that relatively lower amounts of Rox retained in the breast meat of Rox-fed chickens. These data provide useful information on the intestinal bioavailability of Rox and assessing the impact of human exposure to Rox.

8.2.4. The metabolism of Roxarsone in human liver cancer cells (HepG2) and human primary hepatocytes.

Following the study in Chapter 4, the studies on the metabolism of Rox in humans are also scarce. The study in Chapter 5 used the human hepatoma cell line (HepG2) and primary human hepatocytes as models for the intestinal and hepatic metabolism of Rox. HepG2 and human hepatocytes metabolized Rox to 3-amino-4-hydroxylphenlarsonic acid (3AHPAA), As^{V} and several unknown arsenic species. 3AHPAA is the principal metabolite of Rox in HepG2 cells. Human hepatocytes had the ability to metabolize Rox to MMA and N-acetyl-4-hydroxy-m-arsanilic acid (NAHAA). There was variation in the metabolism ability in different individuals. These results indicated that Rox could be metabolized by human cells, especially of hepatic origin (primary hepatocytes and HepG2 cells).

8.2.5. Identification and characterization of new arsenic metabolites of Rox in human primary hepatocytes

There were several unidentified arsenic species present in the human primary hepatocytes exposed to Rox. Because the toxicity of arsenic is highly dependent on the

species of arsenic, it is essential to identify these new arsenic species. I developed a method using HPLC-ESI-QToF to identify one of the unidentified arsenic species. The structure of this new compound was also be characterized. The results showed that it was monothiol-Roxarsone. Identification of this new thiolated arsenical is novel.

8.2.6. Acetylation of 3AHPAA to NAHAA

In this study, I incubated a pigeon liver N-acetyltransferases, chicken liver cytosol, human NAT1 or human liver cytosol with 3AHPAA to see whether these enzyme sources containing N-acetyltransferase could acetyl 3AHPAA to NAHAA. Results showed that the pigeon liver N-acetyltransferases and chicken liver cytosol had the ability to acetyl 3AHPAA to NAHAA. However, human liver cytosol and human NAT1 showed no acetylation ability for 3AHPAA. The results confirmed that 3AHPAA could be acetylated to NAHAA in the chicken body.

8.3. Conclusion

In this work, the knowledge on the uptake, metabolism and excretion of Rox from chicken bodies to human cells is expanded. The results of the feeding study showed that the feeding of Roxarsone could increase the concentrations of arsenite, DMA, and an unknown arsenic species in chicken breast tissues, even after 7-day withdrawal period. Caco-2 studies showed that the accumulation and transport of Roxarsone were lower than arsenite and arsenobetaine. This low uptake of Rox attenuates the human exposure to arsenic from the ingestion of Rox-containing chicken meat. However, the metabolism study in HepG2 and human primary hepatocytes demonstrated that Roxarsone could be

metabolized to other arsenic species. Some of the metabolites have higher toxicity than Rox itself. Therefore we need to pay attention to Rox-fed chickens.

The residual arsenic species will result in potential increase in human exposure to arsenic through the ingestion of chicken fed Roxarsone. However, the daily exposure from the ingestion of Rox-containing chicken meat is likely 30-fold lower than the assumed exposure from drinking water with arsenic at maximum allowance level. Even though the exposure amount is low, reducing arsenic in the chicken meat is necessary to minimize the risk of human exposure. Therefore I recommend the government agencies to take action on banning the use of Roxarsone. When Roxarsone is not available, farmers may rely more heavily on existing anticoccidial drugs or seek alternatives for controlling the disease through vaccines or better management practices.

8.4. Significance of the work

This research fills the knowledge gap on the occurrence, accumulation, transport, and metabolism of Roxarsone in chicken bodies and human cells. Results on the concentrations of specific arsenic species in chicken meat are helpful for improving the regulatory policy that protects public health. The analytical methods for studying arsenic species in chicken breast meat and the methods for identifying new arsenic metabolites are applicable to study arsenic species in other systems. Examining the bioavailability and metabolism of Roxarsone in human cells can help assess the impact of human exposure to Rox. Characterizing new arsenic metabolites in human cells contributes to the understanding the metabolism of arsenic in humans. The outcomes are important to both fundamental and applied aspect of arsenic research.

8.5. Future research

The companies producing Roxarsone suspended their sale under the supervision of FDA in the United States, but it continues to manufacture and export Roxarsone overseas (US FDA 2015). United States imports averagely 113 million pounds of chickens from 2010 to 2014 (ERS 2014), so there is still a high chance of getting Rox-fed chicken in the market. Whether Canadians are still likely exposed to arsenic despite recent change in FDA can be investigated through randomly analyze chicken at regional grocery stores with known origin to their meat products.

The concentration of arsenic species in chicken breast samples at day 28 is the most significant variable in my thesis. The variability of arsenic concentration at day 28 which is the last day of feeding Roxarsone to chickens is large. Differences in feed intake and increase in chicken body weight can contribute to this large variation. Future studies could address the effect of these confounding variables on the concentrations of arsenic species on day-to-day basis.

Absorption of arsenic species could happen at any side along the GI tract. Why Rox has lower biovailability than other arsenic species might be due to the stomach acid hydrolysis of Rox. Therefore the stability (e.g. pH stability) of Rox before the entering the small interstine needs to be studied to confirm that the lower bioavailblity of Rox results from lower uptake of Rox by enterocytes. Why Rox has lower uptake by Caco-2 cells than arsenite and arsenobetaine can also be studied. The mechanism of Rox uptake by cells remains unclear. The transcellular transport of arsenite, arsenate and MMA^{III} all requires transporters on the apical side of enterocytes (Roggenbeck et al. 2016). Investigation of the transporters facilitating the cellular uptake of Rox would be interesting.

I found the thiolated Rox as a new arsenic species in the hepatocytes. The cytotoxicity of this arsenic species needs to be examined. And how it formed, the enzymes associated with the thiolation, and their role in the bioactivation of arsenic species with unknown toxicity can be investigated. Other than thiolated Rox, there are still some other unknown Rox metabolites presenting in the hepatocytes or HepG2 cells. It is good to put a light on the structures of these unidentified arsenic species, which is useful to understand the pathway and mechanism of arsenic metabolism in human body. Investigation on whether these compounds are thiolated arsenic species, thiol-methylated arsenic species or arsenic-GSH conjugation can be conducted. The mass information of these unknown arsenic species cannot be provided in this thesis because these arsenic species cannot be separated from Rox in the reverse phase chromatography. Separation conditions can be optimized. Strategies that go beyond MS or MS/MS method, for example, in silico MS/MS and retention modelling, can be used to identify the unknown arsenic species.

MS^{ALL} method is very senstitive for the detection and identification of unknown arsenic species in a complex biological matrix. In Chapter 6, I can detecte thiolated Rox at about 10 μg/L in the cell culture medium. This method is not only good for identifying the thiol-containing arsenic species but also can also be applied to other arsenic species which can generate specific fragment ions.

In my acetylation study, that no NAHAA formed from acetylation of 3AHPAA by human NATs might attribute to two reasons. The first one is that other NATs instead of human NAT1 is responsible for the acetylation of 3AHPAA. And in my experiment, there might be loss of activity of NATs during the preparation of liver cytosol. So in future study, pure human NATs with higher activity can used to investigate its acetylation ability on

3AHPAA. Another possible reason is that NAHAA is not formed by the acetylation of 3AHPAA. If this is true, studies on the how Rox is converted to NAHAA is worth doing.

8.6. References

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