University of Alberta

Arsenic Speciation Towards Understanding the Environmental Fate of 3-Nitro-4-hydroxyphenylarsonic Acid

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

Department of Chemistry

Spring 2013
Edmonton, Alberta

ABSTRACT

A common practice in the poultry industry has been the addition of phenylarsenicals to the feed for the animals. However, the fate of these arsenicals is not clear. This thesis focuses on the identification and quantitation of arsenic species in litter of chickens that were fed either a basal diet or the basal diet supplemented with 3-nitro-4-hydroxyphenylarsonic acid (Roxarsone®, ROX) over a 35-day period. An analytical technique using high performance liquid chromatography (HPLC) separation with simultaneous detection by both inductively coupled plasma mass spectrometry (ICPMS) and electrospray ionization tandem mass spectrometry (ESI-MS/MS) was developed. This hyphenated technique enabled the determination of eight arsenic species, including the feed additive ROX and its potential biotransformation products. 3-amino-4-hydroxyphenylarsonic acid (3-AHPAA) and N-acetyl-4-hydroxy-m-arsanilic acid (N-AHAA) were identified, and they accounted for 5-27% of total arsenic in the litter of chickens fed the ROX-supplemented diet. The unchanged ROX remained as the major arsenic species, accounting for > 60% of the total arsenic. The concentrations of 3-AHPAA, N-AHAA, arsenite, arsenate, monomethylarsonic acid, dimethylarsinic acid, were consistently higher in the litter samples from the ROX-fed chickens than from the control chickens. These results suggest that ROX can be converted to several arsenic species. This research contributes to a better understanding of the fate of the common arsenic feed additive used in poultry.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Dr. X. Chris Le, for his support and confidence in my abilities. Under his guidance, I was able to apply pure analytical chemistry knowledge to environmental and toxicological studies. His sharing of expertise greatly broadened my horizons, and his enthusiasm and dedication for research motivated me to devote myself into learning. It's a great pleasure working with him.

I would also like to thank my committee members, Dr. Liang Li and Dr. Hongquan Zhang, for their valuable comments and critical review of my thesis.

Thanks to Dr. Gary Kachanoski and Dr. Martin Zuidhof for their insightful thoughts and for making this project possible.

Special thanks to Ms. Xiufen Lu, Mr. Hanyong Peng, Dr. Rongfu Huang, Dr. Shengwen Shen, and Ms. Jinhua Li for their expertise and technical assistance for this work.

I would like to thank Mr. Sanjay Gupta and Dr. Yichao Qian for their help with sample collection.

Thanks to Dr. Xing-Fang Li, Dr. Lydia Chen, Dr. Baowei Chen, Mr. Feng Li, Ms. Wei Wang, Ms. Brittany Dever, Mr. Justin Geisel, and Ms. Madeleine Jensen-Fontaine for their suggestions and discussions in the lab.

I would like to thank Ms. Katerina Carastathis and all other members of the Division of Analytical and Environmental Toxicology for your friendship and support during my study.

I would also like to acknowledge the Graduate Teaching Assistantship and Graduate Research Assistantship from the Department of Chemistry.

Thanks to my friends I met here in Edmonton for their encouragement and support throughout my program. My life has been enriched by their smiles.

Lastly, many thanks to my family who are always behind me. It is their constant love, understanding, and support that help me through tough times.

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LIST OF ABBREVIATIONS

3-AHPAA	3-amino-4-hydroxy-phenylarsonic acid
As^{III}	arsenite
As^{V}	arsenate
AsB	arsenobetaine
DMA^{III}	dimethylarsinous acid
DMA^{V}	dimethylarsinic acid
ESIMS	electrospray ionization mass spectrometry
HNO_3	nitric acid
HPLC	high performance liquid chromatography
H_2SO_4	sulfuric acid
ICPMS	inductively coupled plasma mass spectrometry
LOD	limit of detection
MMA^{III}	monomethylarsonous acid
MMA^{V}	monomethylarsonic acid
MRM	multiple reaction monitoring
N-AHAA	N-acetyl-4-hydroxy-m-arsanilic acid

ROX	3-nitro-4-hydroxy-phenylarsonic acid
SIM	selected ion monitoring
TMA^{III}	trimethylarsine
$TMAO^{V}$	trimethylarsine oxide

CHAPTER 1

INTRODUCTION

Arsenic, ubiquitous in nature, is best known as a notorious poison to multicellular life. It is widely distributed in the earth's crust, with an average level of 1.8 mg/kg. Its cycling in the environment and biological systems is regulated by natural and anthropogenic activities. Humans are exposed to arsenic through a variety of sources. Applications of arsenic, such as poultry feed additives, have drawn significant attention from the public and the scientific community.

1.1 ARSENIC CHEMISTRY

Arsenic is a Group 15 element with an atomic mass of 74.92 g/mol. It is chemically categorized as a metalloid, possessing properties of both metals and non-metals. One stable isotope, ^{75}As, constitutes naturally occurring arsenic [1]. Elemental arsenic is rarely found in nature. There are a number of forms of arsenic that can be generally classified as organic or inorganic arsenicals, ranging from highly toxic arsenite (As^{III}) to nontoxic arsenobetaine [2-4]. -3, 0, +3, and +5 are the four common oxidation states for arsenic [3]. Arsenite (+3) may predominate in anaerobic or reducing conditions, while arsenic in the oxidation state of +5 (such as arsenate, dimethylarsinic acid, arsenobetaine) becomes the most stable and readily detectable form under normal environmental conditions [3, 4].

1.2 OCCURRENCE OF ARSENIC

1.2.1 Arsenic in Natural Sources

Arsenic widely exists in the earth's crust, rock, soil, water, air, and biosphere. It can be potentially mobilized from one medium to another, during which its chemical form may change due to microbial activities or reactions with other compounds. Crustal arsenic is distributed in more than 200 minerals. The most common ores of arsenic are sulfides, including arsenopyrite (FeAsS), orpiment (As_2S_3), and realgar (AsS) [5]. Formation of smaller rocks, sediments and soils from the bedrock can result in the distribution of arsenic that is extensively present in the earth's crust. Arsenic can be released into surface water and surrounding groundwater through weathering and leaching, respectively, of arsenic-bearing rocks and soils. Coal-fired power generation, volcanic action, smelting of copper, lead, cobalt, and gold ores, and vegetation burning at high temperatures lead to the emission of arsenic into the air. Arsenic is predominantly absorbed onto particulate matters [6], while microorganisms can generate volatile methylated arsenic compounds under both aerobic and anaerobic conditions to release arsenic to the atmosphere [7]. Arsenic is also rich in living organisms. The major form of arsenic found in fish and other seafood is nontoxic arsenobetaine [8-10].

1.2.2 Arsenic in Occupational Applications

Anthropogenic activities, including combustion of fossil fuels, metal and glass production, leaching from mining wastes, and application of fertilizers, livestock feed additives, wood preservatives and pesticides also add arsenic to the

environment. Approximately 90% of the arsenic produced around the world is used to prepare inorganic arsenic trioxide (As_2O_3), which can be used for the production of the wood preservative copper chromated arsenate (CCA) [6]. Applications of arsenic compounds as pesticides, herbicides, and insecticides are now declining [11]. Some organic arsenicals, such as Roxarsone®, are added purposefully into animal feed due to their antibiotic and growth-promoting properties[12]. Roxarsone ingested by chickens can be excreted into waste, and then spread into the environment by land application of poultry litter as a fertilizer [13, 14]. Hindmarsh and McCurdy's study [15] reveals that anthropogenic input of arsenic contributes more than natural sources to the environmental arsenic.

1.2.2.1 Use of Roxarsone in Poultry Feed

Phenylarsonic acids are widely used as feed additives in poultry industry. The first approved and also the most commonly used drug is 3-nitro-4-hydroxyphenylarsonic acid (Roxarsone®, ROX) (Figure 1.1). It is added into poultry feed to promote weight gain, improve feed efficiency, and control intestinal parasites that cause coccidiosis [12]. The ROX ingested by chickens is believed to be mainly excreted unchanged into the waste [16]. However, higher levels of inorganic arsenic (arsenate and arsenite) converted from ROX have been recently detected in the livers of chickens fed ROX than in the untreated chickens [17]. Because of the carcinogenic risks associated with inorganic arsenic, the manufacturer of ROX, Pfizer subsidiary Alpharma, has voluntarily discontinued selling ROX in poultry feed in the U.S. since June 2011 [18].

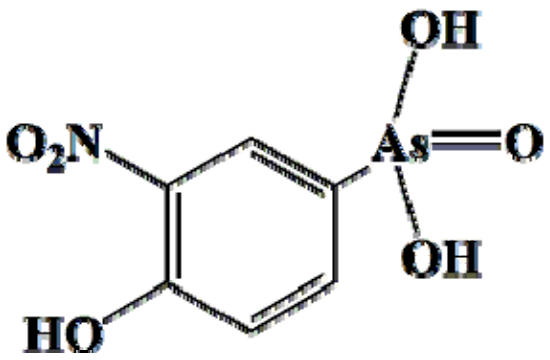


Figure 1.1 Chemical structure of 3-nitro-4-hydroxyphenylarsonic acid, commonly known under the commercial name of Roxarsone® (ROX).

1.2.2.2 Arsenic in Poultry Litter

Each year an estimated 12 to 23 billion kilograms (kg) of poultry litter is produced in the U.S. [19]. Garbarino et al. [20] estimated that the annual chicken litter could contain 250,000 kg of arsenic, if 70% of the 8.3 billion chickens raised in the U.S. [21] were fed a diet containing 45.4 g of ROX per ton of the diet [22, 23], since each bird excretes approximately 150 mg of ROX over the 42-day growth period. Up to 90% of the poultry litter in the U.S. is subsequently disposed of to agricultural lands as a fertilizer [14]. 70–90% of total arsenic in the chicken waste is water-soluble [20, 24, 25], and arsenic may be taken up by crops grown in arsenic-amended fields [26, 27]. Continuous and intense uses of poultry litter for land application can introduce arsenic into the environment [25, 28-30]. Poultry litter is also fed to beef cattle as an economical source of protein, minerals, and energy [31, 32]. 20 to 25% of the litter is used for feeding in Virginia [32]. Some industrial companies in the U.K. and the U.S., such as Fibrowatt [33], are also utilizing poultry litter as electrical and heating fuels [34].

ROX is believed to be the dominant arsenic species in poultry litter [20, 24, 35, 36]. Meanwhile, several arsenic compounds, such as arsenate (As^{V}) [20, 35] and 3-amino-4-hydroxyphenylarsonic acid (3-AHPAA) [35, 36], have been indicated as metabolites of ROX. Unknown arsenic species were also found in poultry litter [20, 35], which need further investigation.

1.2.3 Human Exposure to Arsenic

Arsenic contamination in groundwater can cause serious public health issues in many regions where people rely on groundwater as their source for drinking water. The World Health Organization's guideline for the maximum arsenic level in drinking water is 10 μg/L. Groundwater in most areas within the U.S. contains arsenic at concentrations lower than 10 μg/L, except for the western mountainous regions in New Mexico, Utah, Arizona, and Nevada due to sedimentary deposits from rocks having high levels of arsenic [37]. In Canada, there have been some arsenic "hot spots" (> 10 μg/L) reported in parts of Alberta, British Columbia, Manitoba, New Brunswick, Newfoundland and Labrador, Nova Scotia, Québec, and Saskatchewan [38]. Up to 22 million people in most regions of Bangladesh (50 out of 64 districts) are exposed to arsenic contamination in drinking water at a level of greater than 50 μg/L [39]. Instead of being restricted to West Bengal, a larger population in more areas have been recognized to be severely affected by arsenic-contaminated drinking water in India [40]. Similar situations appeared in the Blackfoot-disease area of Taiwan [41], and many other regions in China [42]. Elevated concentrations of arsenic (> 10 μg/L) in drinking water were also found in Chile, Argentina, Mexico, Peru, Thailand and Australia [41, 43].

Attention on human exposure to arsenic is now extending beyond water to rice, owing to a recent study published in the Proceedings of the National Academy of Sciences (PNAS) [44]. Rice plants were found to readily extract arsenic from the environment, which results in possible arsenic poisoning from

rice [44]. For the Chinese population, arsenic intake from rice overweighs that through drinking water due to the great consumption of rice [45]. The major form of arsenic in rice from China is inorganic arsenic [45], which is a known human carcinogen. China has already developed a statutory limit for arsenic in food (0.15 μg inorganic arsenic/g) [44]. Rice grown in U.S. has an average concentration of 0.26 μg As/g, five times higher than found in rice from Europe, India and Bangladesh [46]. Fish and other seafood are also significant sources of dietary arsenic for human [47, 48]. Arsenic was found to be present in seafood at high concentrations of several hundred micrograms per gram [48], but mainly in the form of arsenobetaine which is generally considered to be of no toxicological concern [8-10]. It is believed that less harmful organic arsenic is the major form of arsenic in food [49], where inorganic arsenic only accounts for 1-3 % of the total arsenic [50].

1.3 ARSENIC METABOLISM

Inorganic arsenic (arsenite and arsenate) is the dominant arsenic species present in nature. It can be metabolized in humans and other mammals through a stepwise biomethylation process after ingestion [51]. The sequential methylation pathway involves alternate reductions of arsenic (pentavalent to trivalent) followed by oxidative additions of a methyl group. As shown in Figure 1.2, a series of arsenic metabolites are revealed, including monomethylarsonic acid (MMA^V), monomethylarsonous acid (MMA^{III}), dimethylarsinic acid (DMA^V), dimethylarsinous acid (DMA^{III}), trimethylarsine oxide ($TMAO^V$), and trimethylarsine (TMA^{III}). In most mammal species, dimethyl arsenic is usually the

final form of metabolites [52]. Some bacteria and also rats can produce $TMAO^V$ and even TMA^{III} through further methylations of arsenic [52].

It has been found that the primary metabolites in human urine are DMA^V and MMA^V, which are less toxic than parental inorganic arsenic [41]. As a result, arsenic methylation above was previously considered to be a detoxification process until the finding regarding MMA^{III} and DMA^{III}. Although these two intermediate trivalent metabolites are chemically unstable, recently developed analytical techniques are able to observe and confirm their presence in the urine samples from people exposed to high concentrations of arsenic in drinking water [53-55]. Numerous studies have illustrated that MMA^{III} and DMA^{III} may have higher toxicity than inorganic arsenic [56-59]. Therefore, the arsenic biomethylation is no longer deemed as a detoxification process [60, 61]. Much attention has been paid to the toxicity of individual arsenic metabolites.

Arsenobetaine, the major arsenic species in seafood, is believed to excrete rapidly from the human body without any metabolic process after ingestion [8, 62, 63]. However, not all organoarsenicals from marine organisms are excreted unchanged. Mussels, seaweed, clams, and oysters contain a large amount of arsenosugars [64-66]. Le et al [66] conducted arsenic speciation analyses of mussels and human urine samples obtained before and after the ingestion of seaweed. They demonstrated that arsenosugars can be metabolized into other arsenic compounds, among which DMA^V is the dominant product. Similar studies have confirmed the metabolism of arsenosugars [65, 67, 68].

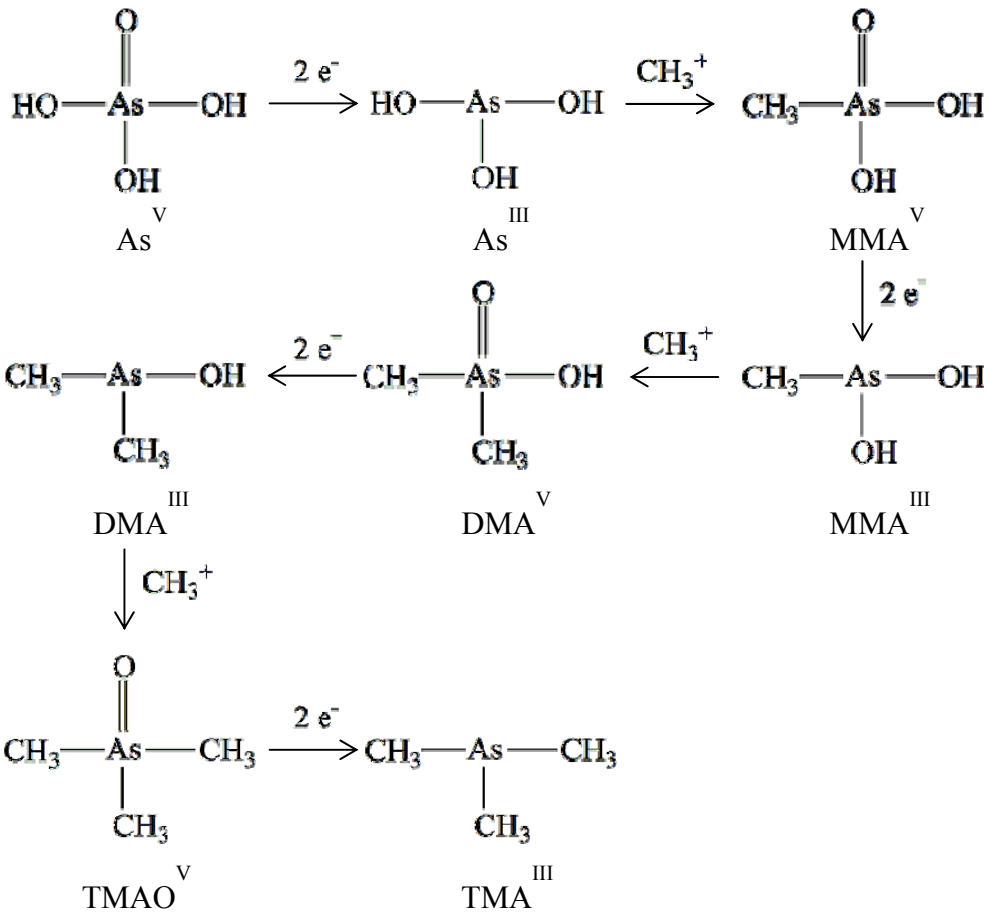


Figure 1.2 Pathway of arsenic biomethylation, showing a sequence of a two-electron reduction followed by the oxidative addition of a methyl group [69].

1.4 HEALTH EFFECTS OF ARSENIC

1.4.1 Chronic Exposure

Chronic ingestion of arsenic-contaminated drinking water and food is the major source of arsenic exposure for humans [70]. A variety of adverse health effects can follow after chronic exposure to arsenic, including cancerous and noncancerous diseases. Epidemiological studies in many regions including Chile and Taiwan show that people are suffering from cancers in the bladder, skin, liver, lungs and kidneys [71, 72]. Skin lesions identified in Bangladesh, India [40, 42], and Blackfoot disease found in Taiwan [41] are the most noticeable noncancerous effects. Arsenic has also been associated with increased risk of heart disease [73], chronic lower respiratory disease [74], diabetes [75], possible harm to fetuses [44], and children's intellectual function [76].

1.4.2 Acute Exposure

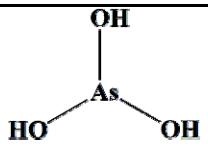
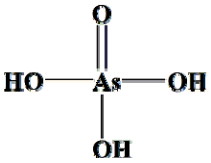
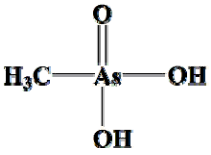
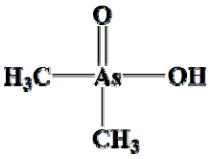
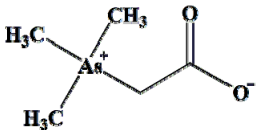
As_2O_3 was a popular poison dating back to the Middle Ages. Arsenic-drugged wine was allegedly the cause of Napoleon's death. The estimated minimal lethal dose of arsenic in adults is 1 mg/kg per day [6, 61]. Symptoms from acute arsenic poisoning may include nausea, diarrhea, vomiting, blood in the urine, hair loss, stomach pain, and more convulsions. It also potentially ends with coma to death [77].

1.5 ARSENIC SPECIATION

There are more than 50 arsenic species present in nature. The toxicities of arsenic compounds strongly depend on their chemical forms. Table 1.1 lists the

chemical structures and acute toxicities (LD_{50} values) of the most commonly studied arsenic species. The distinction between inorganic arsenic and organic arsenic is essential because inorganic arsenic (As^{III}, As^{V}) is generally more toxic than organic species (most commonly MMA^{V} and DMA^{V}), posing tremendous epidemic poisoning risk. However, recent studies consistently indicate that methylated trivalent metabolites (MMA^{III} and DMA^{III}) have higher toxicities than their pentavalent counterparts and even inorganic arsenic [56-59]. Some other arsenicals, arsenobetaine, arsenocholine, and arsenosugars that are abundant in marine organisms, are considered much less toxic or even nontoxic [8-10]. Therefore, assessments of risks associated with arsenic exposure to the environment and human health cannot be based solely on total arsenic, because it masks species-dependent toxicity. Arsenic speciation provides a way to quantify different arsenic forms and helps more accurately assess the toxicological effects of arsenic.

Table 1.1 Chemical structures and toxicities of the commonly studied arsenic species.

Name	Abbreviation	Chemical Structure	LD_{50} in Mice
Arsenite	As^{III}		34.5 mg/kg [8]
Arsenate	As^{V}		100 mg/kg [78]
Monomethylarsonic acid	MMA^{V}		1800 mg/kg [79]
Dimethylarsinic acid	DMA^{V}		1200 mg/kg [79]
Arsenobetaine	AsB		> 10,000 mg/kg [8]

1.5.1 Sampling and Pre-treatment of Samples

To ensure the quality of a specific sample, sampling needs to be designed ahead of time and then documented [80]. There are many factors that should be considered during the sample collection process, including weather conditions, equipment and transport selections, and even personnel training. It is essential to store environmental and biological samples at low temperatures, such as -20 °C, to avoid transformation of the analytes due to microbial activities. Freeze-drying is an alternate way for sample preservation. The moisture in the sample can be calculated by measuring the sample before and after the freeze-drying process. Sample homogenization is necessary before further processing to evenly distribute all substances in the sample, and to minimize errors attributable to poor representation.

1.5.2 Extraction of Arsenic Species

Arsenic in solid samples needs to be solubilized prior to speciation analysis, while arsenic-containing water samples can be analyzed without any extraction procedure. It is crucial to evaluate the extraction efficiency of arsenic species in the specific sample as 100% efficiency by a single chemical extraction in solid matrices is not always attainable. Therefore, optimization of the extraction method for each matrix investigated is necessary. A combination of different extractants can also be used to achieve desirable extraction efficiency [81].

1.5.2.1 Solvent Extraction

Solvent extraction is simple, robust, and ideal for samples containing complex matrices. Methanol/water, methanol, or water is the most commonly used extraction solvent for arsenic speciation [82-85]. Methanol is a good solvent for extracting organic arsenic because of its high solubility in polar organic solvents. However, it has been reported that inorganic arsenic is poorly extracted by methanol [86]. Water, instead, is probably a better solvent for extracting inorganic arsenic since it can easily penetrate the sample matrix. Moreover, it is polar and can extract the organic arsenic species to some extent as well. Other chemical solutions, such as trifluoroacetic acid [87] and nitric acid [88, 89], have also been shown to quantitatively remove arsenic from solid matrices for speciation. Solvent extraction is usually assisted with microwave [88, 90, 91], ultrasound [89, 92], or pressurized [93] techniques.

1.5.2.2 Enzymatic Extraction

Enzymatic extraction utilizes enzymes to reflect the bioaccessible fraction of arsenic within specific samples by mimicking the physical and biochemical processes in the living systems, such as the human digestive tract [94, 95]. This approach is widely used for a variety of food samples including rice [94-96]. Typical enzyme materials contain proteases (pepsin and trypsin, for instance) [96, 97], amylases [98] and even enzyme mixtures [99, 100]. This type of extraction is fast and very efficient in preserving arsenic species [99, 101], and has also been reported to be combined with microwave energy for satisfactory extraction recoveries [100, 102].

1.5.3 Separation of Arsenic Species Using High Performance Liquid Chromatography (HPLC)

HPLC is an analytical technique for the separation of a variety of constituents in a mixture. Small volumes of the mixture (sample) are normally introduced into the HPLC by either a manual syringe injection or an autosampler injection. The mobile phase driven by mechanical pumps carries the mixture into a separation column. The stationary phase packed inside the column interacts with the sample mixture. Some compounds in the sample mixture retain strongly on the stationary phase and thus travel slowly down the column, while others interact with the stationary phase less strongly and travel faster with the mobile phase. Therefore, differential partitioning between the stationary and mobile phases results in the final separation of analytes.

Various types of chromatographic columns are commercially available, differing in the size and type of the stationary phase on packed particles inside the columns. Many chromatographic modes have been developed, including ion exchange, ion pairing, reverse phase and size exclusion chromatography with different column properties. HPLC in different modes is extensively used for separating a wide range of arsenic species in environmental and biological samples [69, 103-108].

1.5.4 Detection of Arsenic Species Using Inductively Coupled Plasma Mass Spectrometry (ICPMS)

In recent years, ICPMS is most commonly used to combine with HPLC for arsenic speciation [69, 103-108]. ICPMS is an element-specific, sensitive, high resolution detection technique that can handle both simple and complex matrices for trace elements analysis [109].

The analyte of interest is first introduced into the ICPMS via a nebulizer where the liquid solution is converted into an aerosol by argon (Ar) gas. Inductively coupled plasma at extremely high temperatures (up to 10,000 K) ionizes the small droplets of sample transported to the torch. The ions then pass through an interface to the mass spectrometer operating under room temperature and high vacuum conditions, where the ions are focused and separated according to their mass-to-charge ratios (*m/z*). Quadrupole is the most popular mass analyzer and is set to select ^{75}As, the sole stable isotope present in nature. Only the ions with selected *m/z* 75 can reach the detector, while others are filtered out. Chloride needs to be avoided introducing into the instrument as the isobaric ion $^{35}Cl+^{40}Ar$ may interfere with the analysis of ^{75}As. High sensitivity can be achieved since an electron multiplier as the detector is used to obtain counts per second for the selected ^{75}As. It is important to keep in mind that ICPMS is designed to measure analytes at very low concentrations. The instrument can readily be overwhelmed by large quantities of ions, derived from samples at part per million (ppm) concentration levels. Cross contamination of samples may also result. Therefore,

it is good practice to estimate the concentrations of compounds in a sample before introducing them into the ICPMS.

Although ICPMS provides many advantages for arsenic detection, no structural information of arsenic species can be obtained. High temperature ICP breaks all arsenic compounds apart. HPLC-ICPMS alone is susceptible to misidentifications if different species have identical chromatographic retention times [110]. Identification of unknown or new arsenicals that have no standards can be difficult.

1.5.5 Detection of Arsenic Species Using Electrospray Ionization Mass Spectrometry (ESIMS)

ESIMS is able to provide a wealth of structural information of analytes of interest. Chromatography is needed for the separation of diverse forms of arsenic, and the removal of complex matrix interferences [111] that challenge the ESI detection [112]. ESI offers a convenient interface available for converting ions from liquid phase to gas phase, which leads to a simple connection with HPLC separations [113, 114] . Online coupling of the ESIMS with the HPLC further enhances the capability for determination of individual arsenic compounds [115, 116], especially the unknown species [117, 118]. Anion exchange chromatography is most commonly used in separating a variety of inorganic or organic arsenicals [115], as most arsenic compounds can be negatively charged.

A sample solution containing the analytes of interest is dispersed into a fine spray of charged droplets by a high-voltage power applied to a capillary tube

where the liquid flows through. An inert gas, such as nitrogen and carbon dioxide, can help high-flow electrosprays with additional nebulization [119]. The charged droplets undergo further solvent evaporation until the Rayleigh limit is reached, which results in Coulomb fission and produces gas phase ions. These sample 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, are used for the mass selection process. The major techniques for detection are selected ion monitoring (SIM) and multiple reaction monitoring (MRM).

Before connecting the HPLC to the ESIMS, infusion studies of arsenic standards corresponding to the suspected arsenic species of interest in the sample should be conducted on the ESIMS. This standard procedure is to optimize detection conditions, and to verify the detectability of the expected compounds. SIM detection was very popular prior to the development of triple quadrupoles. But it can be problematic when multiple compounds in the sample have the same *m/z* and coelute. On the contrary, two species are less likely to have identical transitions in the MRM mode unless both molecular ions and fragment ions for these two compounds are the same. This situation is even rarer when at least two MRM transitions are monitored for each individual species [120].

However, ESIMS suffers from higher detection limits than ICPMS, and is more susceptible to matrix effects and ion interferences [112, 121, 122].

1.5.6 Application of HPLC-ICPMS/ESIMS in Speciation Studies

Either ICPMS or ESIMS has its own merits and defects if used separately. By combining the ICPMS with the ESIMS after a single HPLC separation, the complementarity can overcome the shortcomings of each technique. Application of the HPLC-ICPMS/ESIMS system has gained popularity in recent years [118, 123-126].

As shown in Figure 1.3, the eluent from the HPLC separation is split between the ICPMS and the ESIMS. A low flow rate in the ESIMS is necessary to allow efficient ionization of the liquid injected [127]. Simultaneous detections by the two mass spectrometers provide a superior tool for identification and quantification of arsenic species.

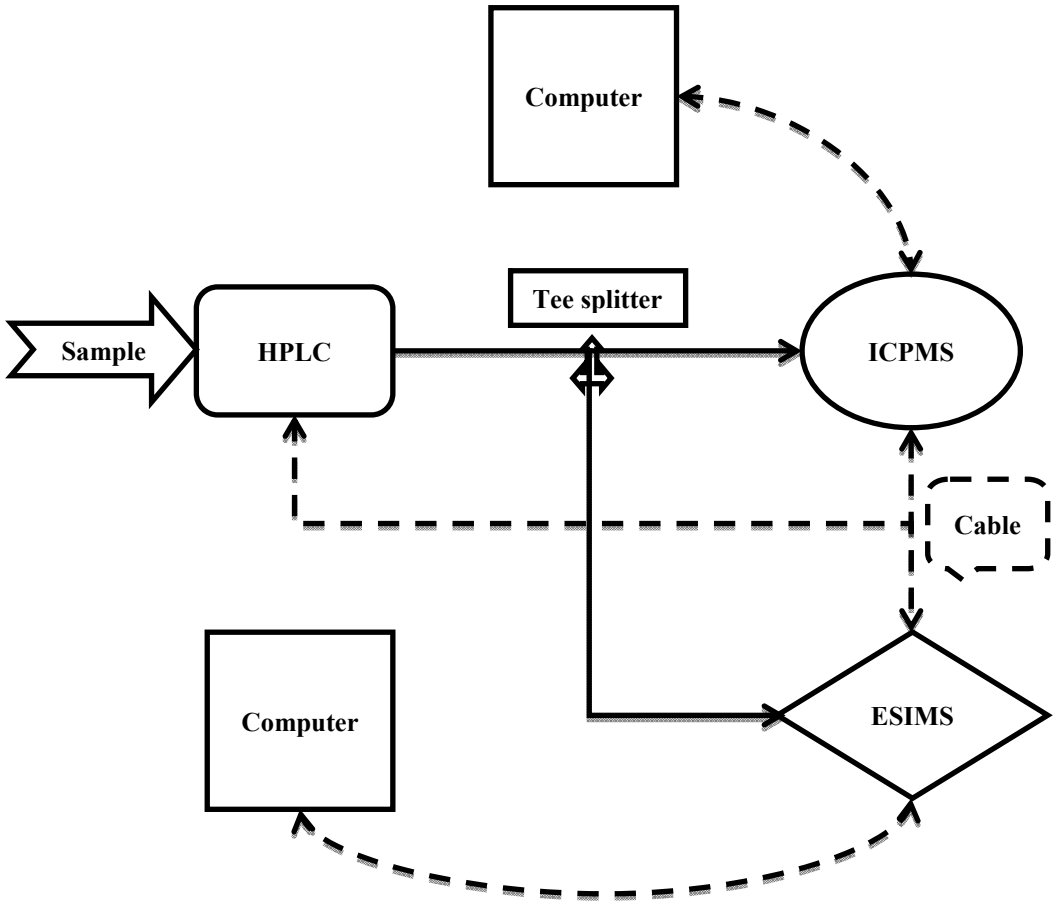


Figure 1.3 Schematic diagram of the HPLC-ICPMS/ESIMS system.

1.6 STUDY HYPOTHESIS AND OBJECTIVES

This thesis hypothesizes that ROX can be converted to other arsenic species. This hypothesis can be tested by conducting arsenic speciation analyses. There are two major objectives in order to investigate the hypothesis, as follows.

First of all, a suitable and efficient method needs to be developed for arsenic speciation in environmental biomass samples. Separation of arsenic species in poultry litter will be performed based on anion exchange chromatography. ICPMS will be employed for determination of individual arsenic compounds by spiking suspected arsenic standards into the sample for chromatographic comparison. Further confirmation of the identities of each suspected arsenic species in litter will be conducted in MRM mode using ESIMS. Simultaneous ICPMS and ESIMS detections of analytes from a single HPLC separation will then be designed for environmental arsenic exposure monitoring. Parameters such as sensitivity, linearity and accuracy of the method will be assessed.

Secondly, the HPLC-ICPMS/ESIMS system will be used to investigate arsenic speciation and quantification in poultry litter following a controlled ROX feeding experiment. Most of the ROX ingested is excreted unchanged [16], while a small amount of ROX may be converted to other forms of arsenic and found in waste [20, 35, 36]. The chemical form of arsenic plays an important role in its toxicity and impact on the environment and human health [69, 128]. The distribution changes of each arsenic compound in poultry litter, with a focus on ROX metabolites, will be evaluated over the period of feeding.

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CHAPTER 2

DEVELOPMENT OF AN HPLC-ICPMS/ESIMS METHOD FOR ARSENIC SPECIATION ANALYSIS

2.1 INTRODUCTION

More than 50 arsenic species are present in natural environment and biological systems. As with most elements, the chemical form of arsenic determines its toxicity and its impact on the environment. Inorganic arsenic (As^{III}, As^{V}) is a known human carcinogen, and is generally more toxic than organic arsenic (typically MMA^{V}, DMA^{V}) [1-6]. Arsenic trioxide, a trivalent form of arsenic, is by all means one of the most efficient poisons with a LD_{50} of 34.5 mg/kg in mice [7]. However, arsenobetaine (AsB) that commonly exists in marine creatures [8-10] is completely non-toxic with a LD_{50} of higher than 10,000 mg/kg in mice [7]. Different arsenic species can possibly co-exist in environmental samples. In these cases, arsenic speciation, rather than total arsenic determination, is necessary to evaluate arsenic exposure of the environment and humans.

There have been several analytical techniques used for arsenic speciation, ranging from inexpensive spectrophotometry, to costly hydride generation atomic fluorescence spectrometry, to more complicated coupled systems such as high performance liquid chromatography combined with inductively coupled plasma mass spectrometry (HPLC-ICPMS) and capillary electrophoresis interfaced with inductively coupled plasma mass spectrometry (CE-ICPMS) [11-15]. So far, HPLC-ICPMS is the most commonly used method for arsenic speciation analysis

because of its separation capability, detection sensitivity, and compatibility with various environmental samples. But being only element-specific, ICPMS is susceptible to erroneous identifications if different species have identical chromatographic retention times. Using different separation methods in tandem for confirmative identification is possible but time-consuming. Electrospray ionization mass spectrometry (ESIMS) can provide a wealth of structural information for determination of arsenic compounds [16, 17]. However, ESIMS is more susceptible to matrix effects and ion interferences. By combining HPLC separation with both ICPMS for quantification and ESIMS for identification, we are able to determine a variety of arsenic species.

The objective of this chapter was to develop an HPLC-ICPMS/ESIMS method for arsenic speciation analysis. This analytical technique was used to separate and identify arsenicals in litter of poultry fed Roxarsone® (ROX).

2.2 MATERIALS AND METHODS

2.2.1 Reagents and Standards

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), 3-nitro-4-hydroxyphenylarsonic acid (98.1% purity, Sigma-Aldrich, St. Louis, MO), and arsenobetaine (98%, Tri Chemical Laboratories Inc., Japan) were used to prepare, As^{III}, As^{V}, MMA^{V}, DMA^{V}, ROX and AsB stock solutions containing 1000 mg As/L in 18.2 MΩ·cm deionized water, respectively. 3-amino-4-hydroxyphenylarsonic acid (3-AHPAA) and N-acetyl-4-hydroxy-m-arsanilic acid (N-AHAA) were purchased from Pfaltz and

Bauer, Inc. (Waterbury, CT). Both stock solutions at 100 mg As/L were prepared by dissolving their purified solids in deionized water with 5% HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ). Environmental calibration standard (Agilent Technologies, U.S.) served as the primary arsenic standard for calibration of concentrations of all arsenic stock solutions using direct injection ICPMS. Standard reference material (SRM) 1640a Trace Elements in Natural Water was obtained from the National Institute of Standards and Technology (Gaithersburg, MD), and was used as a quality control measure of above total arsenic determination. Stock solutions were kept at 4 °C prior to the time of use. Arsenic standard solutions for speciation analysis were prepared daily from stock solutions using deionized water as the diluent.

2.2.2 Sample Processing

2.2.2.1 Extraction of Arsenic Species from Poultry Litter

Poultry litter samples were frozen at -20 °C. They were removed from the freezer and homogenized using a blade coffee grinder (KitchenAid, Mississauga, ON, Canada) on the day of analysis. The homogenate was weighed, vortexed, ultrasonicated using 10 mL of methanol-water solution (volume 1:1), and then centrifuged at 3500 g for 10 min. Repeated extractions were conducted, and the supernatant portions were combined. Table 2.1 shows the extraction parameters (mass of sample, time of sonication, number of extraction times) that were optimized. An aliquot of the extract was then aspirated into a 1-mL syringe (BD Biosciences, Franklin Lakes, NJ), filtered through a 0.45 μm membrane (Mandel, Guelph, ON, Canada), and diluted prior to arsenic speciation or total arsenic

analysis. The extraction of the litter samples was performed in triplicate. The litter residues were kept at -20 °C until acid digestion.

Table 2.1 Optimized parameters for the extraction of arsenic species in poultry litter.

Parameter	Setting			
Mass of Sample	0.5 g		1.0 g	
Time of Sonication	10 min	15 min	20 min	30 min
Number of Extraction Times	3 times	4 times		5 times

2.2.2.2 Acid Digestion of Poultry Litter Samples

Extraction of arsenic species from litter samples using a methanol-water mixture could possibly result in low extraction efficiency [18]. The sum concentration of arsenic species extracted may be lower than the total arsenic concentration determined from acid digestion of the litter samples, because a portion of arsenic could remain in the residues after extraction. Therefore, in order to evaluate extraction efficiency as well as to obtain a mass balance of total arsenic in the litter samples, acid digestion of the litter residues from methanol-water extraction and the original poultry litter samples were conducted.

A 0.5 g portion of the homogenized sample was weighed into a 100 mL beaker. In a fume hood, 30 mL concentrated (95.0–98.0%) sulfuric acid (H_2SO_4) was poured into the beaker, followed by slowly adding 10 mL concentrated (68.0–70.0%) nitric acid (HNO_3). Then, the beaker was covered with a watch glass for overnight to allow digestion to occur. The next day, the beaker was placed on a hotplate and heated to 150 °C for further digestion until the solution became transparent. The watch glass was then removed, and the temperature was raised to 450 °C to evaporate the acids until a volume of less than 2 mL solution remained. The entire content in the beaker was quantitatively transferred into a 15 mL centrifuge tube. Deionized water was added to reconstitute the sample to 10 mL. This solution was subsequently syringe-filtered (0.45 μm), and diluted in deionized water with 1% HNO_3 before total arsenic analysis. The litter residues and the certified reference material of DOLT-4 fish litter tissue (National Research Council Canada) were digested and analysed in the same manner as

original litter samples. DOLT-4 was used to ensure the accuracy of the method. Each of the litter samples, residues and reference materials was processed in triplicate.

2.2.3 Sample Analysis

2.2.3.1 Total Arsenic Analysis

Diluted digested samples were injected into the ICPMS (Agilent 7500cs; Agilent Technologies, Japan) using an ASX-510 autosampler (CETAC, Omaha, NE). Arsenic was monitored at *m/z* 75. ICPMS was operated in a helium mode to prevent interference from argon chloride ($^{40}Ar^{35}Cl^{+}$). Collisions between helium gas and $^{40}Ar^{35}Cl^{+}$ decrease the energy of $^{40}Ar^{35}Cl^{+}$ so that it cannot reach the mass detector [19].

Serial concentrations of arsenic standards (0.5, 1, 2, 5, 10, and 20 μg/L) were prepared in 1% HNO_3 from the primary arsenic standard (10 mg As/L) for the calibration of total arsenic analysis. The standard reference material was 1640a Trace Elements in Natural Water s, and it was analyzed prior to samples to check the daily accuracy of the results.

2.2.3.2 Speciation Analysis

HPLC Separation

HPLC (Agilent 1100 series; Agilent Technologies, Germany) separation of arsenic species in samples was performed on a PRP-X110S anion exchange column (7 μm particle size, 150 × 4.1 mm; Hamilton, Reno, NV) with an appropriate guard column (PRP-X110S; Hamilton) by a gradient elution using

ammonium bicarbonate (NH_4HCO_3) (Sigma-Aldrich) in deionized water with 5% methanol as the mobile phase. NH_4HCO_3 is an ideal buffer for interfacing with both ICPMS and ESIMS. Addition of methanol to the eluent improves ionization efficiency in the plasma so that the signal intensity of arsenic can be enhanced, and also maintains detection limits unchanged [13]. Two mobile phases prepared for the separation were as follows: (A) 60 mM ammonium bicarbonate, 5% methanol, pH adjusted to 8.7; (B) 5% methanol. They were filtered through a 0.45 μm membrane and sonicated for 15 min prior to HPLC use. The gradient elution program used for the separation is shown in Table 2.2 and Figure 2.1. The injection volume was 30 μL for all samples and standards. The same HPLC separation was used with detection by both ICPMS and ESIMS.

Table 2.2 Gradient elution conditions for HPLC separation. The program started with a linear gradient from 100% mobile phase B to 100% mobile phase A during the first 3 min. The composition stayed at 100% A from 3 min to 11 min. From 11 min to 14 min, the mobile phase returned from 100% A to 100% B in a linear gradient, and maintained at 100% B for another minute (14–15 min). Flow rate was kept at 2.4 mL/min for the entire 15 min.

Time	A	B	Flow rate (mL/min)
0.00	0	100%	2.4
3.00	100%	0	2.4
11.00	100%	0	2.4
14.00	0	100%	2.4
15.00	0	100%	2.4

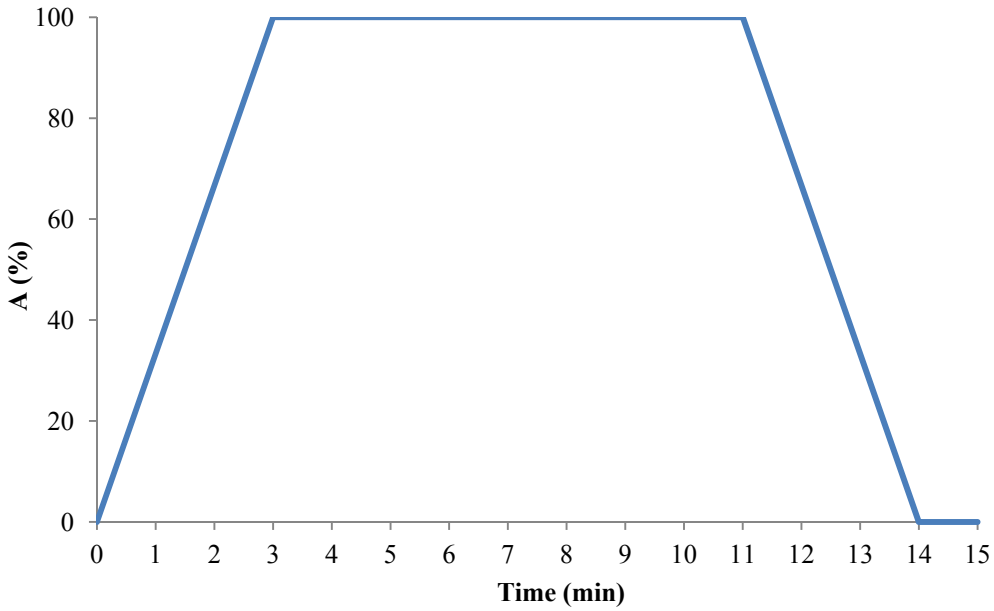


Figure 2.1 Gradient elution program for HPLC separation.

HPLC-ICPMS Analysis

The outlet of the HPLC column was directly connected to the nebulizer of the Agilent 7500cs Octopole ICPMS. Arsenic species were monitored as As^{+} (*m/z*=75) in helium reaction mode. Poultry litter extracts were initially analyzed along with a standard mixture containing seven arsenicals: AsB, As^{III}, As^{V}, MMA^{V}, DMA^{V}, 3-AHPAA, and ROX. A combination approach of retention time matching and sample spiking with arsenic standards was conducted to identify the suspected arsenic species present in the poultry litter. Calibration curves across all species were obtained prior to litter samples on a daily basis by running standard mixtures in the range of 0.05–20 μg As/L for each arsenic compound. Limits of detection (LOD) were estimated based on 3σ of the baseline noise.

HPLC-ESIMS Analysis

To further confirm the identities of arsenic species, litter extracts were also analyzed using the HPLC system coupled to an AB SCIEX 5500 QTRAP ESIMS (Concord, ON, Canada). Initially, each arsenic standard with a concentration range of 1 to 10 μg As/L was introduced directly by a syringe pump into the ESIMS system, in order to optimize ESIMS operating parameters. Precursor and product ion matches between suspected species in the litter extract and expected standards were performed in the multiple reaction monitoring (MRM) mode. The ionization mode was switched from positive (for AsB) to negative (for other arsenic species) at 1.3 min. AsB is a cationic arsenic compound and can only be detected in positive mode.

HPLC-ICPMS/ESIMS Analysis

In order to obtain comprehensive quantification results from ICPMS and confirmative identification information from ESIMS simultaneously, the litter extracts were analyzed using HPLC-ICPMS/ESIMS for arsenic speciation. As shown in Figure 1.3, the system contains ICPMS (Agilent 7500cs) and ESIMS (AB SCIEX 5500 QTRAP) detection in parallel after HPLC (Agilent 1100 series) separation as described above. The eluent generated from LC system was split between the ICPMS and the ESIMS with a 4:1 ratio by a 300 series stainless steel tee (Valco Canada, Brockville, ON). The results detected by the ICPMS at *m/z* 75 (As^+) were compared with those obtained simultaneously by the ESIMS in MRM mode to confirm the identities of individual arsenicals.

2.3 RESULTS

2.3.1 Total Arsenic in Poultry Litter Extracts

Figure 2.2 illustrates the total arsenic concentrations in the litter extracts after 0.5 g of the litter sample was sonicated for four different times: 10 min, 15 min, 20 min, and 30 min. The samples were extracted three times, and diluted 100-fold for total arsenic analysis. As shown in Figure 2.3, two different masses (0.5 g and 1.0 g) of samples were both extracted five times prior to total arsenic analysis (sonicating for 20 min). The supernatant of each of the five extractions was also collected and analyzed separately in Figure 2.4 (a) and 2.4 (b).

2.3.2 Total Arsenic in Poultry Litter and Extract Residues after Acid Digestion

Total arsenic concentrations in both the original poultry litter samples and the extraction residues were determined separately using ICPMS after HNO_3/H_2SO_4 (volume 1:3) digestion of triplicate samples (Table 2.3). Extraction efficiency was calculated by the total arsenic in the litter extract divided by the total arsenic in the digested litter, in order to evaluate the methanol-water extraction method. Based on the total arsenic results, digested poultry litter samples were best diluted 100-fold so that the arsenic concentrations of samples could fall within the range of the calibration curve (0.5-20 μg/L).

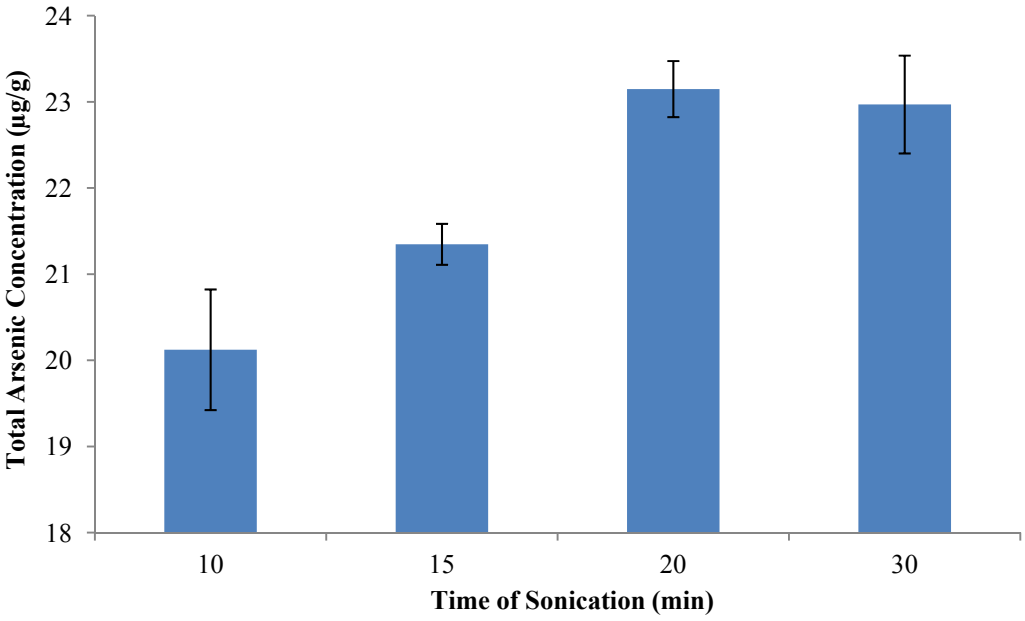


Figure 2.2 Total arsenic concentrations in poultry litter extracts after sonication for 10 min, 15 min, 20 min, and 30 min. The studied litter sample was collected on day 28 from pen #15, Ross 308. A slight increase in the total arsenic occurs by extending sonication from 10 min to 20 min. Then the total concentrations start to reach the plateau.

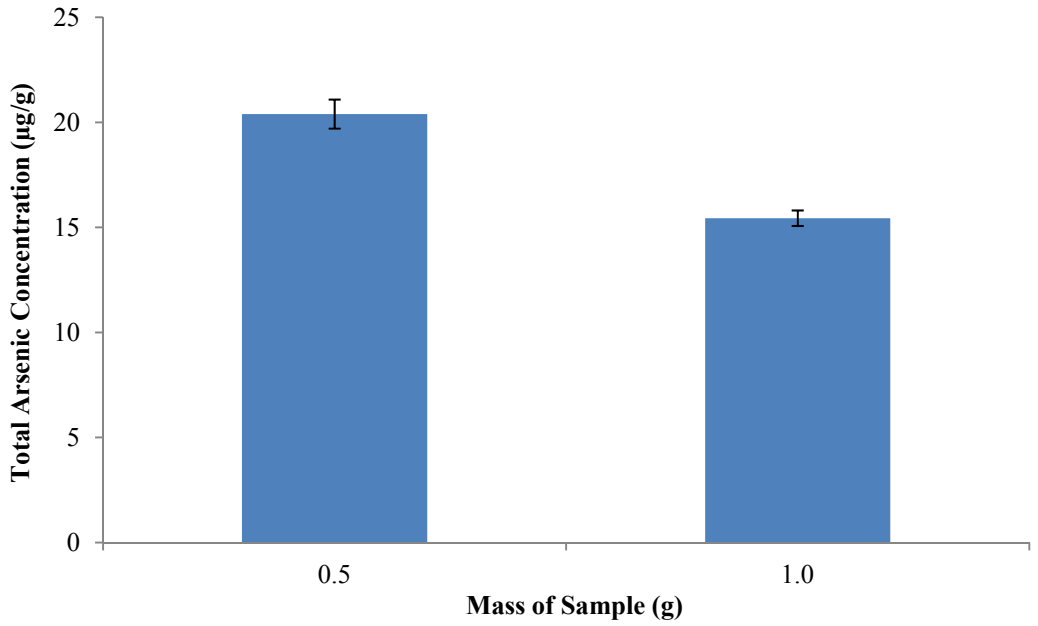


Figure 2.3 Total arsenic concentrations in poultry litter extracts from two different amounts (0.5 g and 1.0 g) of litter samples. The studied litter sample was collected on day 28 from pen #24, Cobb 500. The concentrations were calculated to the unit of µg/g for comparison. There is a decrease in the total arsenic if 0.5 g more samples was extracted.

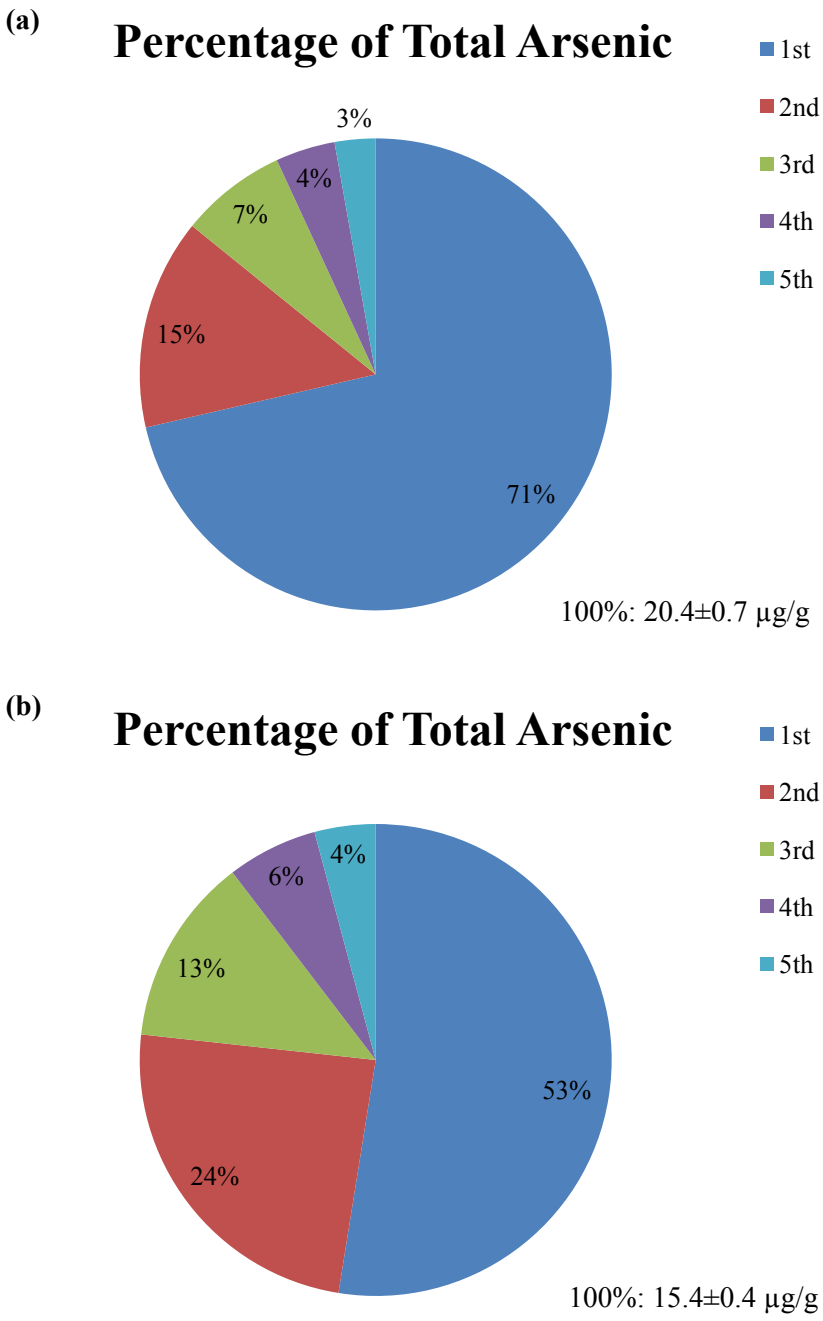


Figure 2.4 Relative concentrations of total arsenic from each of the five sequential extractions. The studied litter sample was collected on day 28 from pen #24, Cobb 500. (a) 0.5 g and (b) 1.0 g of the homogenized sample were used for extraction.

Table 2.3 Arsenic content in poultry litter obtained from the analyses of extracts and acid digestion solutions. The studied litter sample was collected on day 28 from pen #15, Ross 308.

Parameter	Value
Total Arsenic in Litter Extract (μg/g)	23.1 ± 0.3
Total Arsenic in the Digested Residue (μg/g)	9.4 ± 0.3
Sum of Arsenic in the Extract and Residue (μg/g)	32.5 ± 0.5
Total Arsenic in the Digested Litter (μg/g)	32.0 ± 0.9
Extraction Efficiency (%)	72

2.3.3 Determination of Arsenic Species in Poultry Litter Using HPLC-ICPMS

Figure 2.5 (a) shows a chromatogram for the separation of seven arsenic species in a standard mixture using a PRP-X110S anion exchange column. All arsenic species can be baseline-resolved within 10 min using HPLC-ICPMS. The elution order was as follows: AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, and ROX. ROX has an average retention time of 8.8 min, which is not as strongly retained as previous observations on the PRP-X100 anion exchange column [20, 21]. Figure 2.5 (b) shows the method was also able to separate all arsenic-containing compounds in litter extract. The retention time match suggests the presence of AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, ROX, and an unknown species of interest in poultry litter.

To further demonstrate the true identities of the above seven arsenic species, we first spiked the litter extract with each of the expected arsenic standards and analyzed the spiked samples using HPLC-ICPMS. If the peak of the suspected arsenic species in litter elutes with that of the standard arsenic spike, the identity of the suspected arsenic species is assumed to be the same as that of the expected arsenic standard [Figure 2.6 (a-g)].

As illustrated in Figure 2.5, the unknown peak eluted between 3-AHPAA and ROX. Plausible arsenic standards with related structures to 3-AHPAA and ROX were therefore spiked into the litter extract, and we found that the unknown had a retention time match with N-acetyl-4-hydroxy-m-arsanilic acid (N-AHAA) [Figure 2.6 (h)].

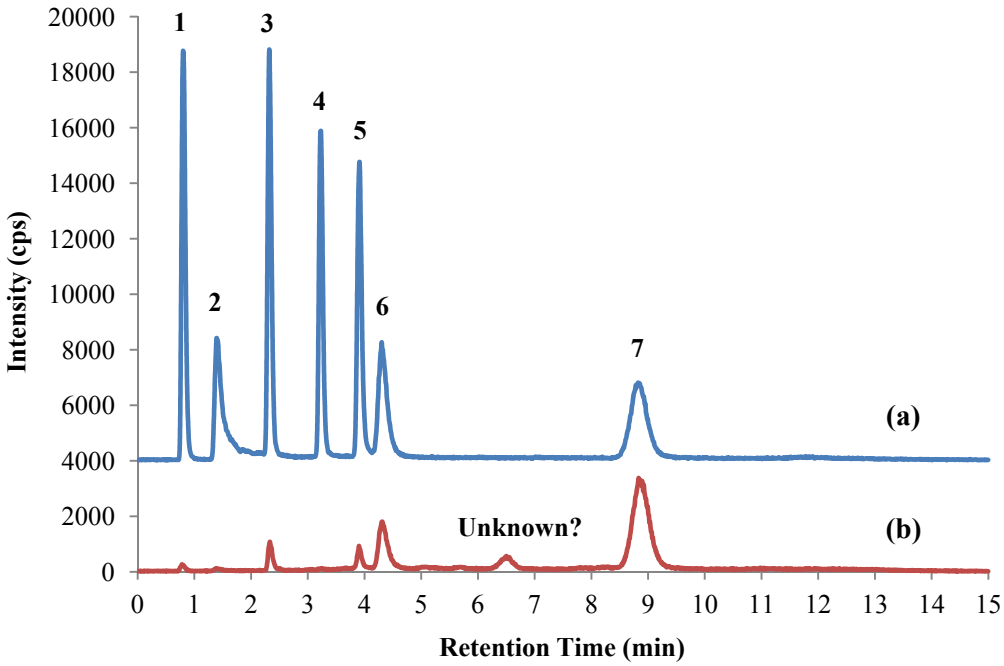


Figure 2.5 Chromatograms from HPLC-ICPMS analyses of (a) a standard mixture of seven arsenic species at 10 µg As/L each and (b) a litter sample of ROX-fed chickens collected on day 28 from pen #11, Ross 308. An unknown peak was observed. Peaks: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) ROX.

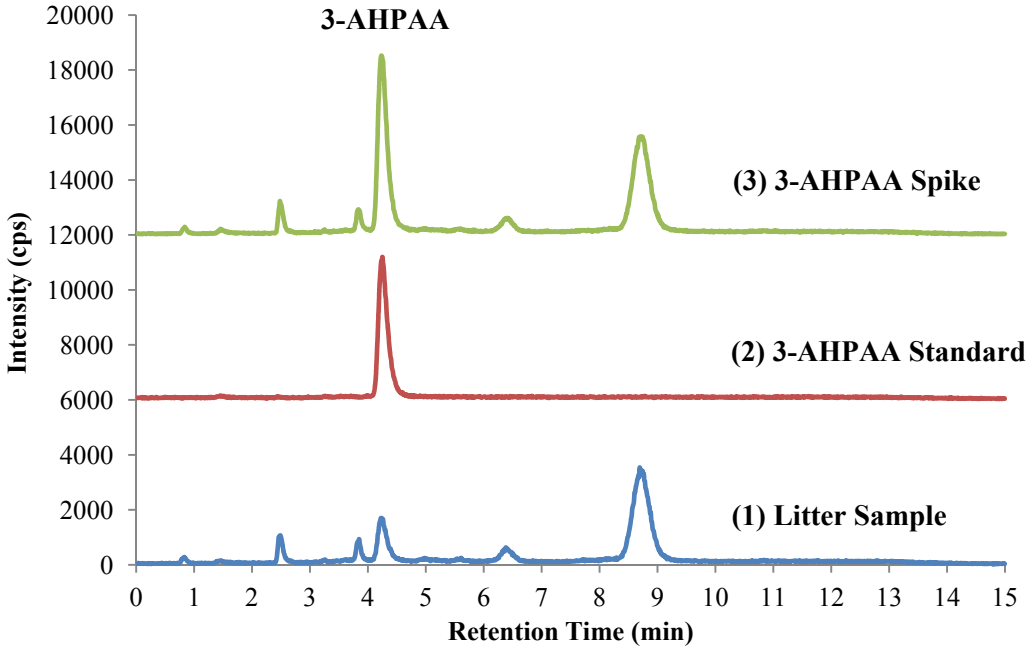


Figure 2.6 (a) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a litter sample with co-injections of 3-AHPAA standard for identity confirmation. (1) A litter sample collected on day 28 from ROX-fed chickens in pen #11, Ross 308. (2) 10 µg/L 3-AHPAA standard. (3) Litter sample with a 10 µg/L 3-AHPAA standard spike.

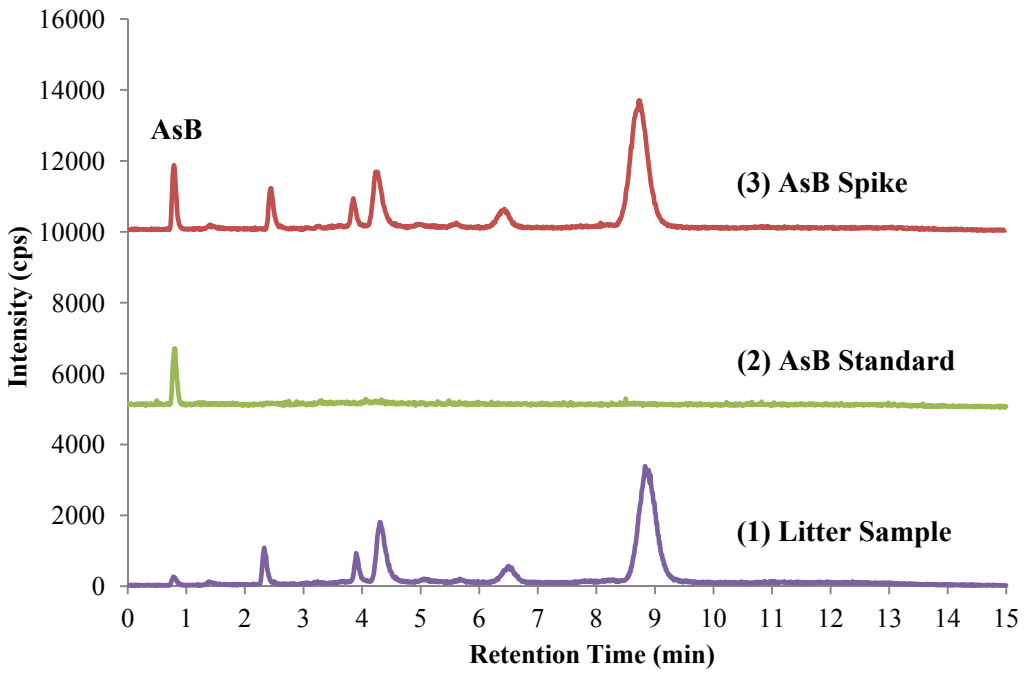


Figure 2.6 (b) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a litter sample with co-injections of AsB standard for identity confirmation. (1) A litter sample collected on day 28 from ROX-fed chickens in pen #11, Ross 308. (2) 1 µg/L AsB standard. (3) Litter sample with a 1 µg/L AsB standard spike.

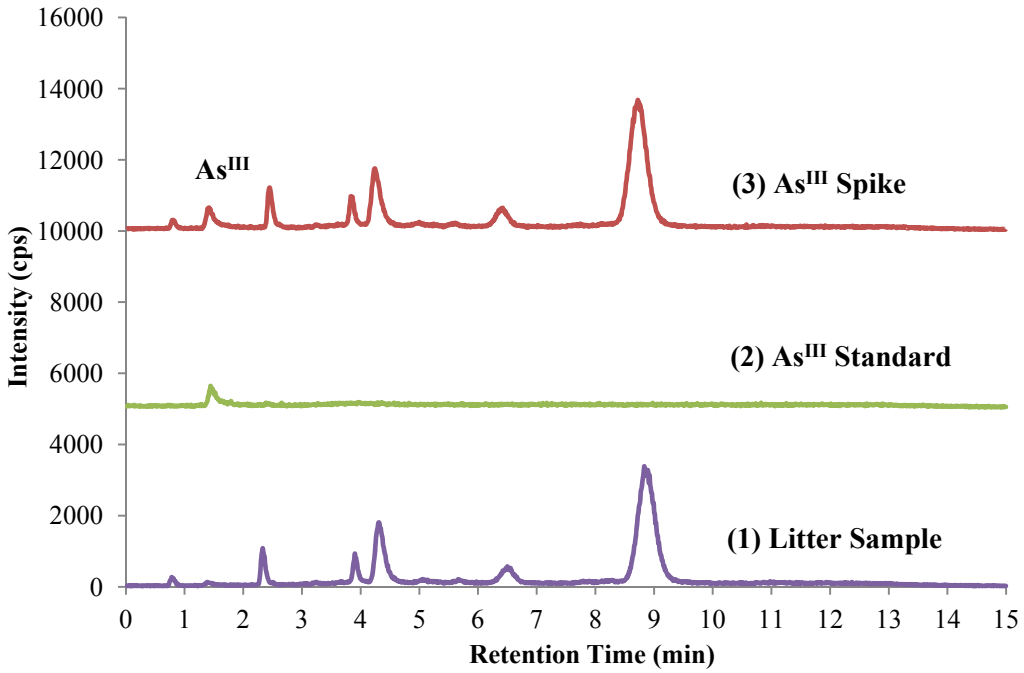


Figure 2.6 (c) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a litter sample with co-injections of As^{III} standard for identity confirmation. (1) A litter sample collected on day 28 from ROX-fed chickens in pen #11, Ross 308. (2) 1 µg/L As^{III} standard. (3) Litter sample with a 1 µg/L As^{III} standard spike.

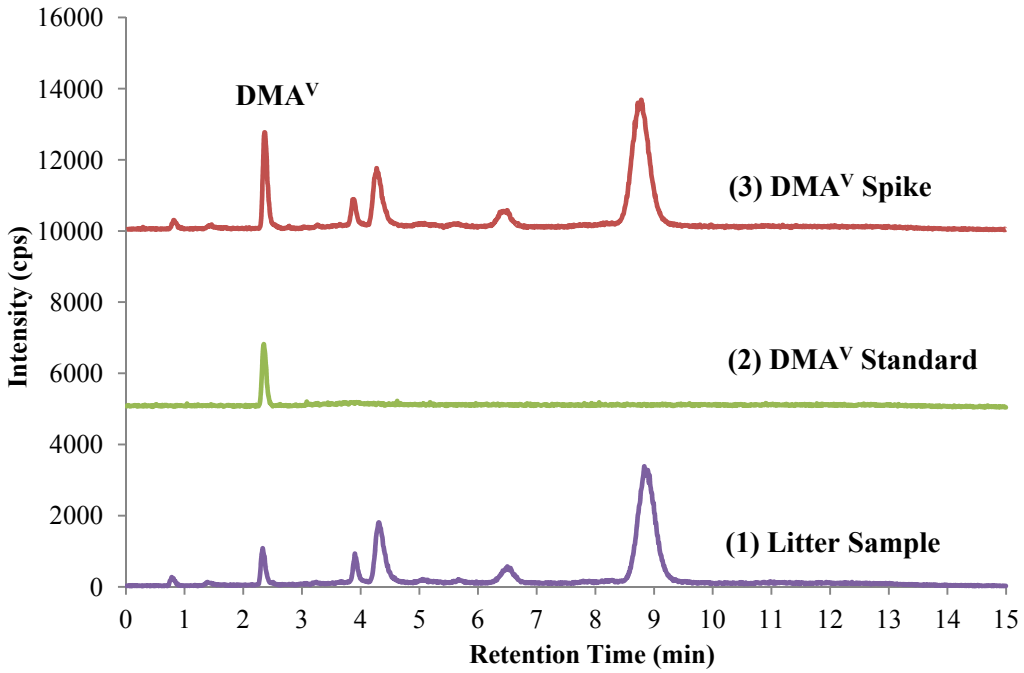


Figure 2.6 (d) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a litter sample with co-injections of DMA^V standard for identity confirmation. (1) A litter sample collected on day 28 from ROX-fed chickens in pen #11, Ross 308. (2) 1 µg/L DMA^V standard. (3) Litter sample with a 1 µg/L DMA^V standard spike.

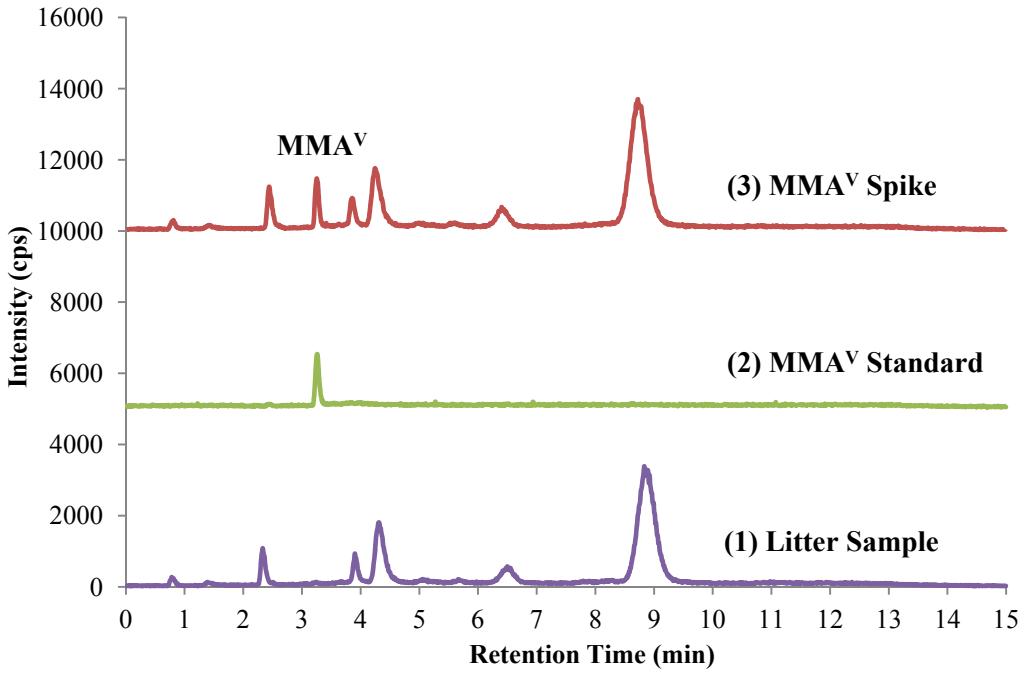


Figure 2.6 (e) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a litter sample with co-injections of MMA^V standard for identity confirmation. (1) A litter sample collected on day 28 from ROX-fed chickens in pen #11, Ross 308. (2) 1 µg/L MMA^V standard. (3) Litter sample with a 1 µg/L MMA^V standard spike.

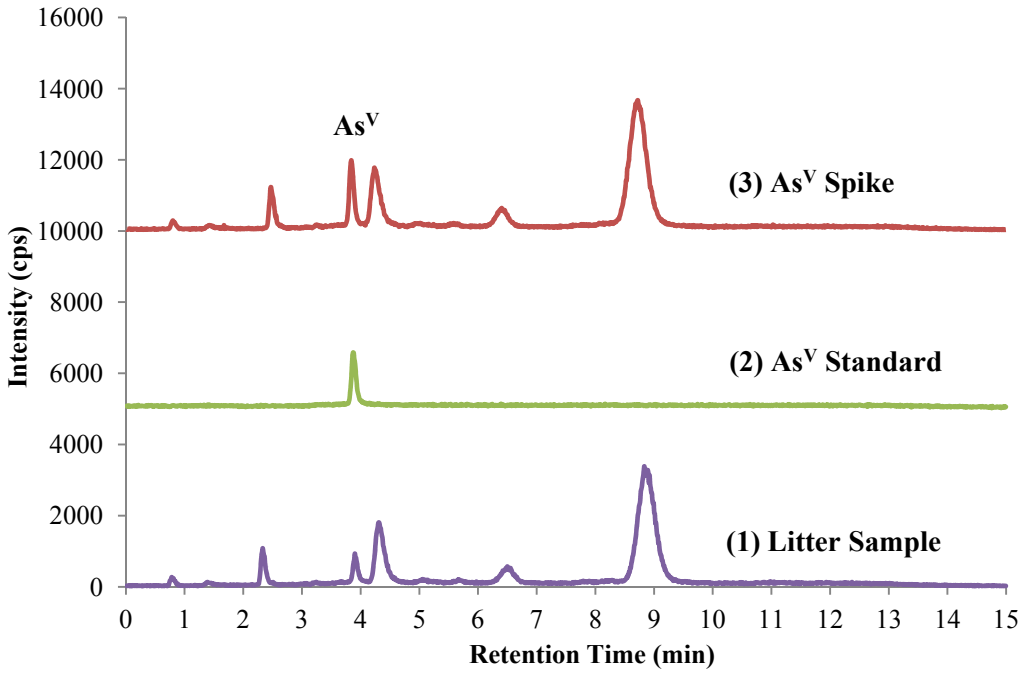


Figure 2.6 (f) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a litter sample with co-injections of As^V standard for identity confirmation. (1) A litter sample collected on day 28 from ROX-fed chickens in pen #11, Ross 308. (2) 1 µg/L As^V standard. (3) Litter sample with a 1 µg/L As^V standard spike.

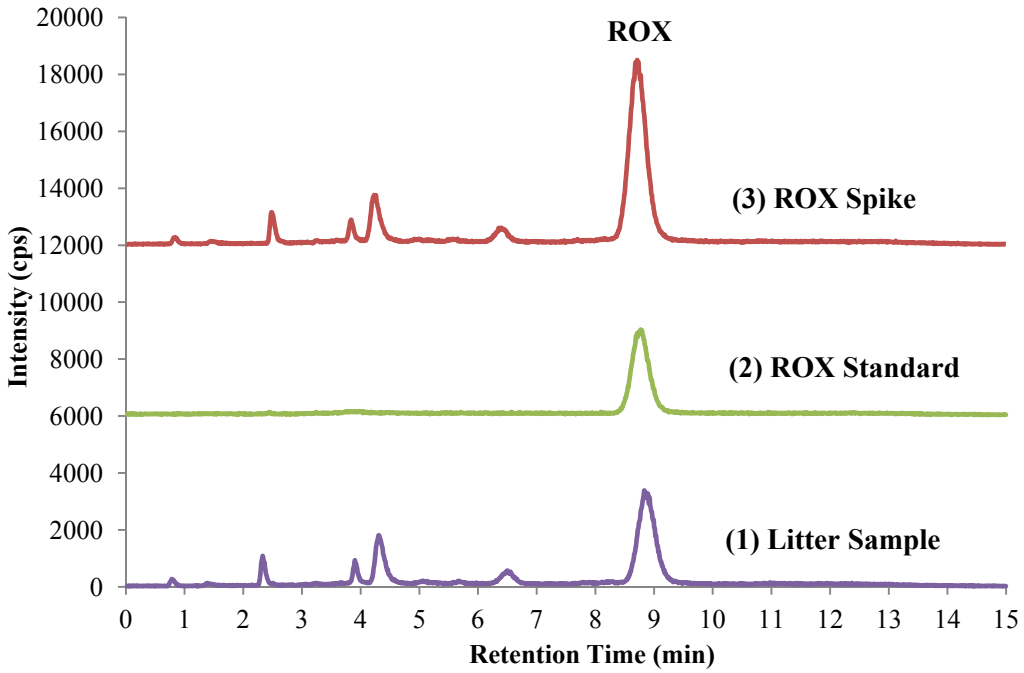


Figure 2.6 (g) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a litter sample with co-injections of ROX standard for identity confirmation. (1) A litter sample collected on day 28 from ROX-fed chickens in pen #11, Ross 308. (2) 10 µg/L ROX standard. (3) Litter sample with a 10 µg/L ROX standard spike.

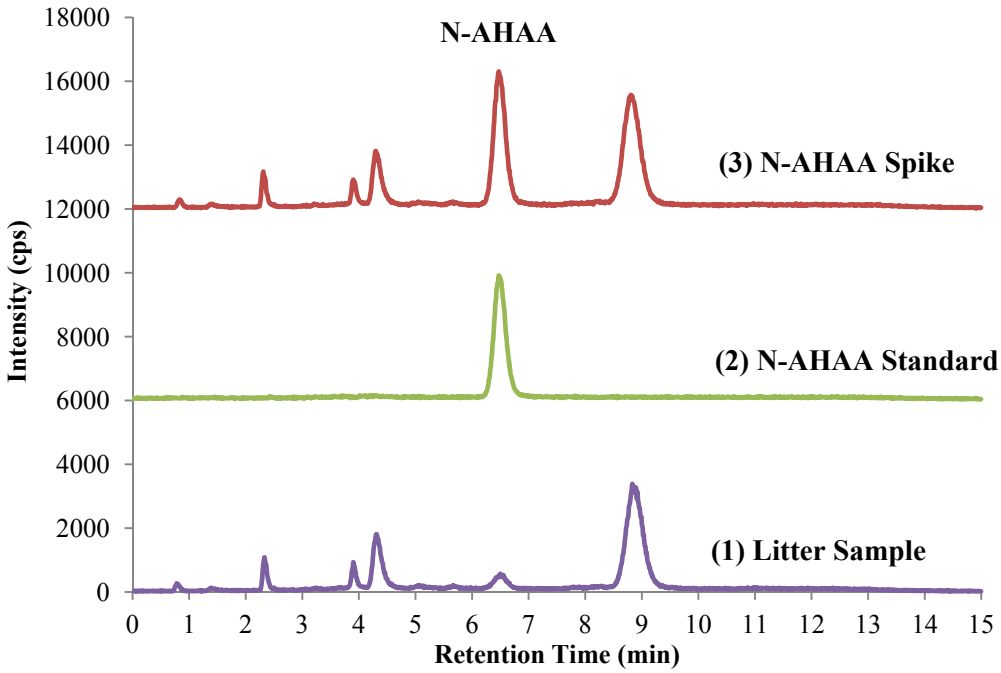


Figure 2.6 (h) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a litter sample with co-injections of N-AHAA standard for identification of the unknown. (1) A litter sample collected on day 28 from ROX-fed chickens in pen #11, Ross 308. (2) 10 µg/L N-AHAA standard. (3) Litter sample with a 10 µg/L N-AHAA standard spike.

2.3.4 Identification of Arsenic Species in Poultry Litter Using HPLC-ESIMS

The identities of all detected arsenic species including the putative N-AHAA in the litter extract were further confirmed by HPLC-ESIMS. Figures 2.7, 2.8, and 2.9 show typical MS/MS spectra from the ESIMS analyses of standard solutions of 10 μg/L ROX (Figure 2.7), 3-AHPAA (Figure 2.8), and N-AHAA (Figure 2.9). These spectra were obtained from direct infusion of the arsenic standards and the MS/MS analyses under the conditions shown in Table 2.4. After optimizing operating parameters (Table 2.4) and MRM transition conditions (Table 2.5) of individual arsenicals, HPLC-ESIMS analyses were performed on litter extracts. As shown in Figure 2.10 (a), all arsenic compounds except As^{III} were separated and detected in MRM mode. Their retention times agree well with those generated by HPLC-ICPMS [Figure 2.10 (b)]. Typical MRM chromatograms of N-AHAA in litter extracts are given in Figure 2.11. Figure 2.12 shows typical chromatograms form MRM detection of arsenic species in a little extract, suggesting the presence of 3-AHPAA. Likewise, Figure 2.13 shows the presence of ROX in a litter extract from the analysis by HPLC separation and MRM detection. Thus, the presence of AsB, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, ROX, as well as N-AHAA in poultry litter was verified.

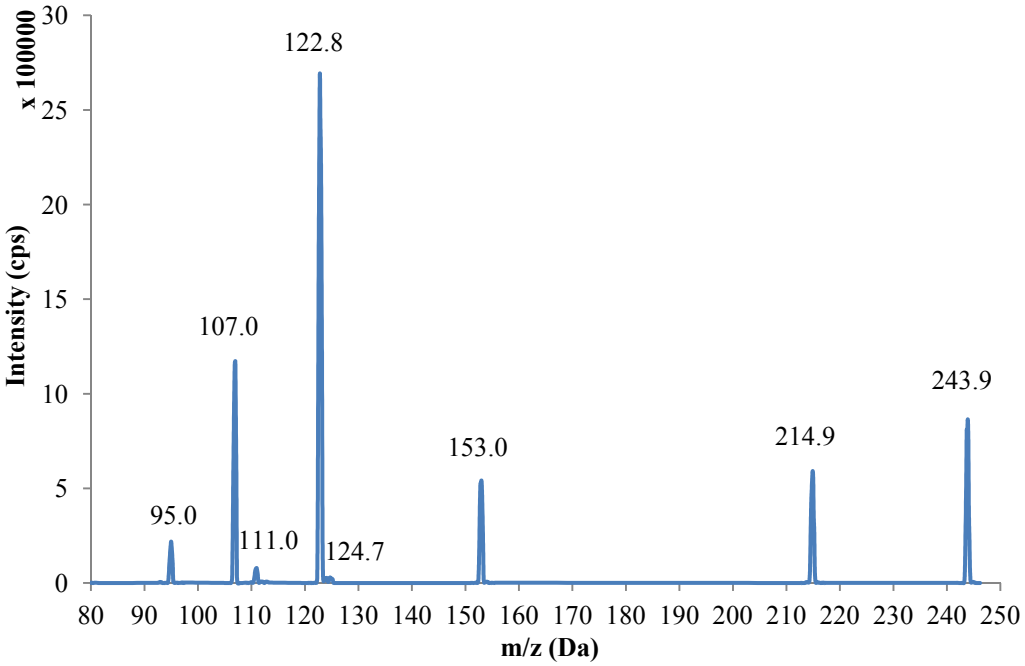


Figure 2.7 Typical MS/MS spectrum from the ESIMS analysis of the ROX standard solution at 10 µg As/L.

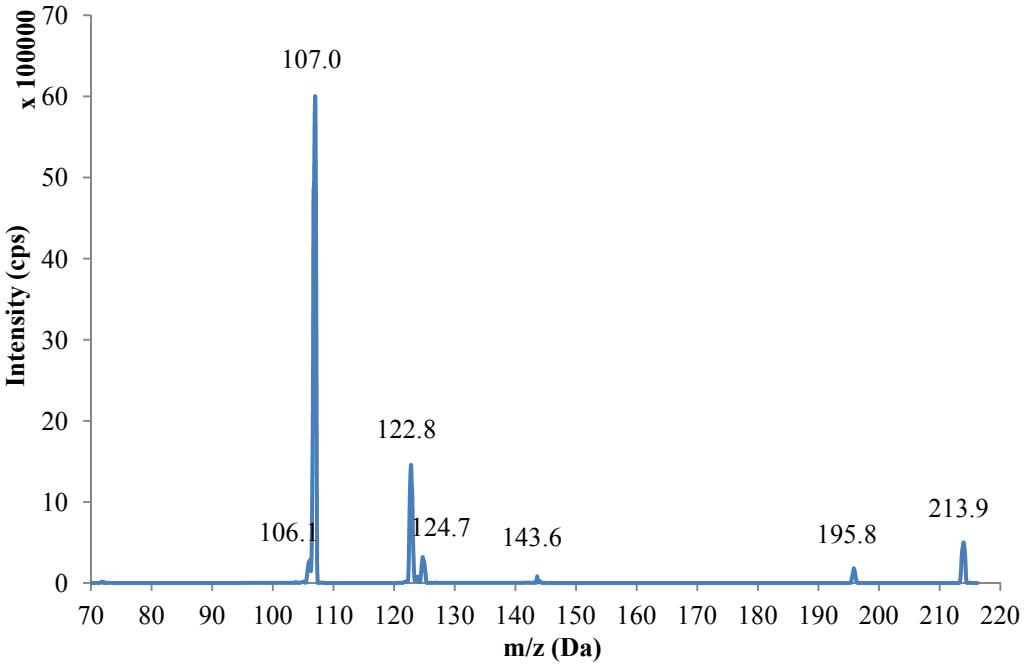


Figure 2.8 Typical MS/MS spectrum from the ESIMS analysis of the 3-AHPAA standard solution at 10 µg As/L.

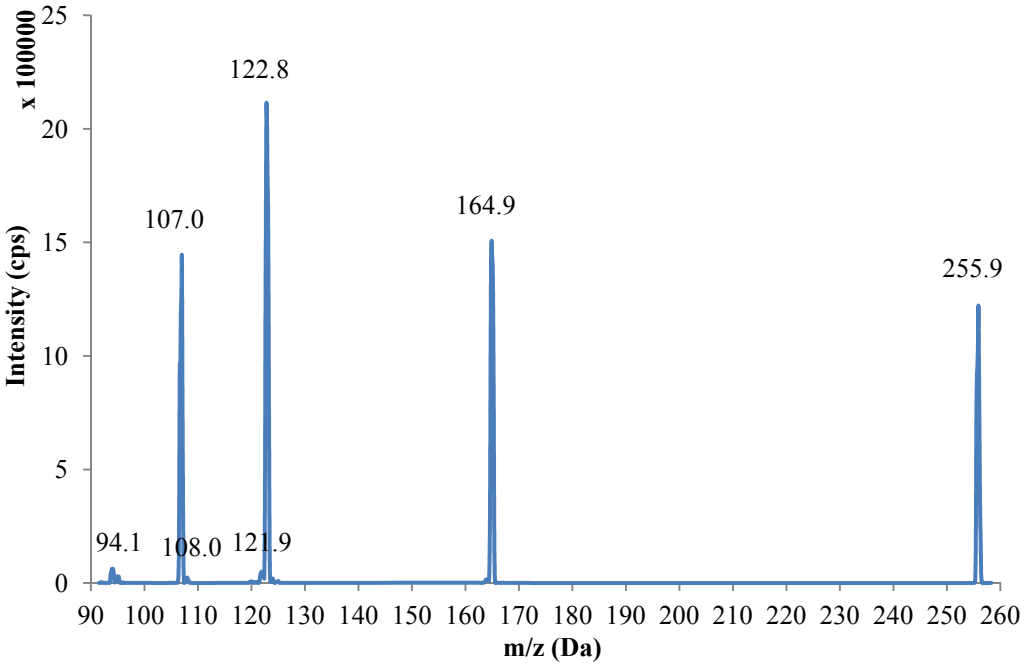
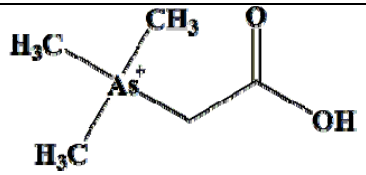
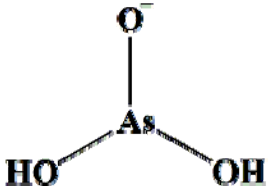
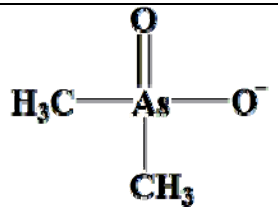


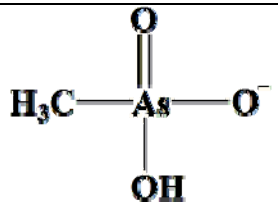
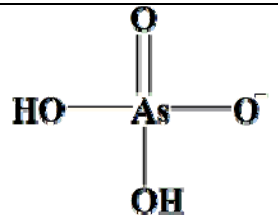
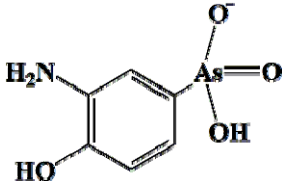
Figure 2.9 Typical MS/MS spectrum from the ESIMS analysis of the N-AHAA standard solution at 10 µg As/L.

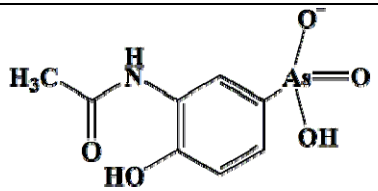
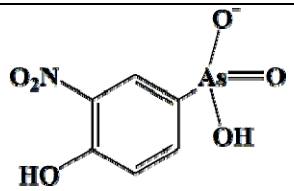
Table 2.4 Selected operating parameters of the 5500 QTRAP ESIMS.

Parameter	Value in Positive Ionization Mode	Value in Negative Ionization Mode
Curtain Gas (CUR)	30 psi	30 psi
Collision Gas (CAD)	High	High
Ionspray Voltage (IS)	4500 V	-4500 V
Temperature (TEM)	600 °C	600 °C
Ion Source Gas 1 (GS1)	50 psi	50 psi
Ion Source Gas 2 (GS2)	50 psi	50 psi
Entrance Potential (EP)	10 V	-10 V
Dwell Time for Each Transition	150 ms	150 ms

Table 2.5 MRM parameters for arsenic speciation using HPLC-ESIMS.

Arsenic Species	Polarity	Molecular ion structure	Molecular ion (m/z)	Characteristic fragments	Fragment structure	DP (V)	CE (V)	CXP (V)
AsB	Pos		179	105	$(CH_3)_2As^+$	71	37	9
				120	$(CH_3)_3As^+$	71	28	11
As^{III}	Neg		125	107	AsO_2^-	-10	-18	-15
DMA^V	Neg		137	107	AsO_2^-	-70	-30	-11
				122	$CH_3AsO_2^-$	-70	-18	-13

Arsenic Species	Polarity	Molecular ion structure	Molecular ion (m/z)	Characteristic fragments	Fragment structure	DP (V)	CE (V)	CXP (V)
MMA^V	Neg		139	107	AsO_2^-	-40	-40	-43
				124	AsO_3H^-	-40	-24	-7
As^V	Neg		141	107	AsO_2^-	-15	-58	-13
				123	AsO_3^-	-15	-20	-7
3-AHPAA	Neg		232	107	AsO_2^-	-20	-64	-11
				123	AsO_3^-	-20	-28	-25

Arsenic Species	Polarity	Molecular ion structure	Molecular ion (m/z)	Characteristic fragments	Fragment structure	DP (V)	CE (V)	CXP (V)
N-AHAA	Neg		274	107	AsO_2^-	-45	-72	-13
				123	AsO_3^-	-45	-36	-11
ROX	Neg		262	107	AsO_2^-	-30	-94	-15
				123	AsO_3^-	-30	-38	-11

Note: DP: Declustering Potential; CE: Collision Energy; CXP: Cell Exit Potential.

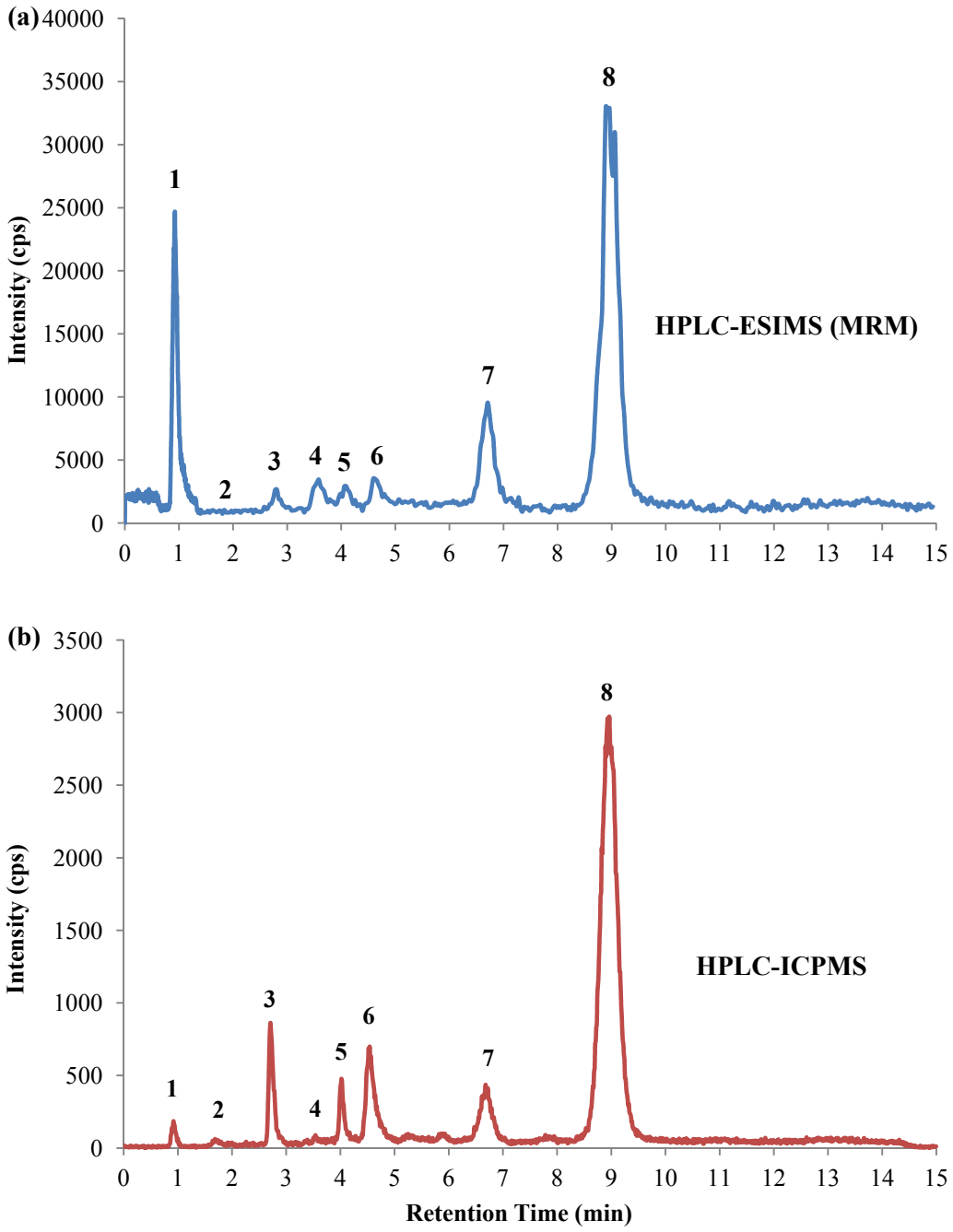


Figure 2.10 Chromatograms showing analyses of a litter sample collected on day 28 from ROX-fed chickens (pen #24, Cobb 500) using (a) HPLC-ESIMS and (b) HPLC-ICPMS. Peaks: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) N-AHAA, (8) ROX.

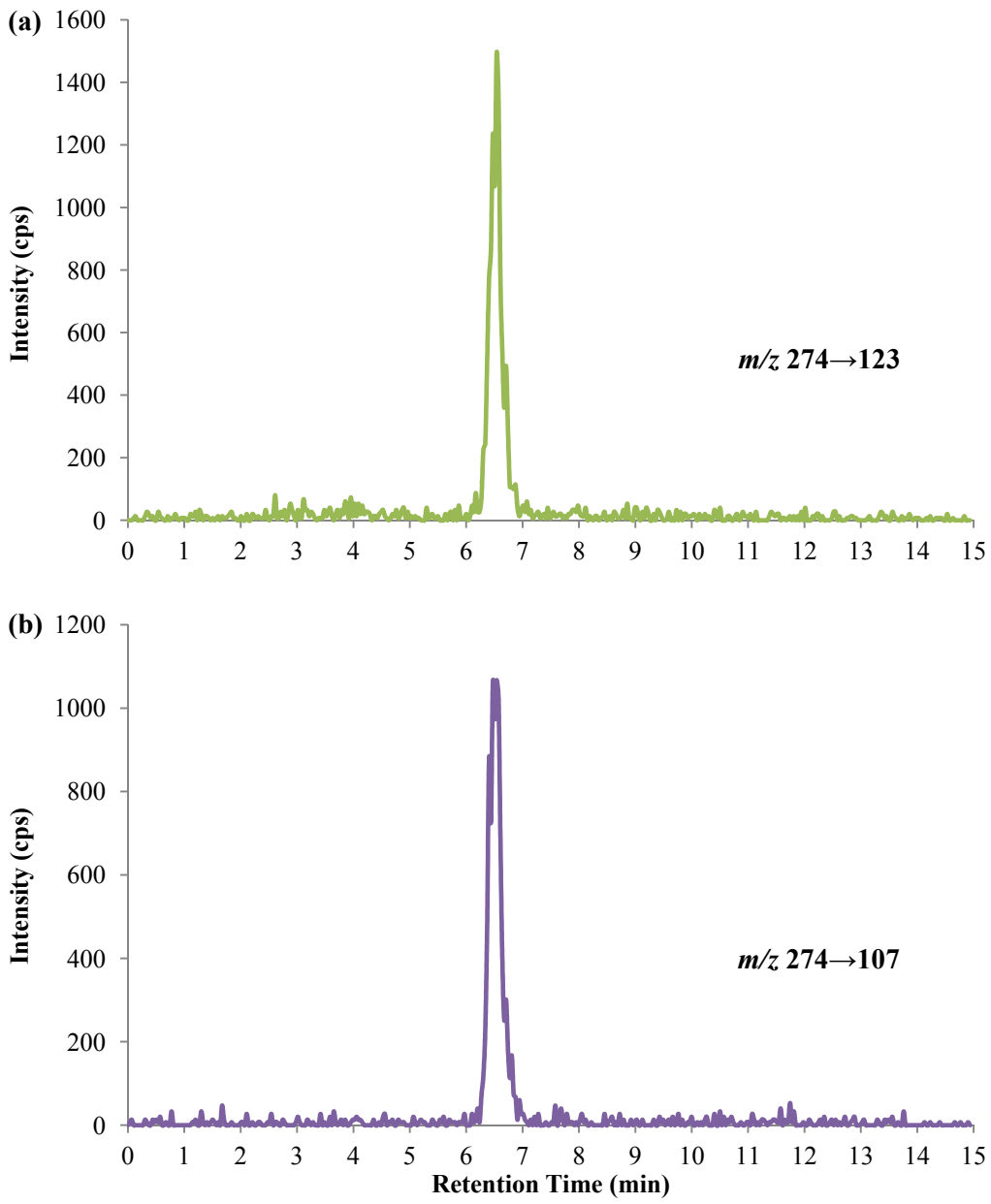


Figure 2.11 Chromatograms generated from MRM analysis of a litter sample collected on day 28 from ROX-fed chickens (pen #11, Ross 308) selecting the transitions (a) *m/z* 274 to 123 and (b) *m/z* 274 to 107, both for N-AHAA.

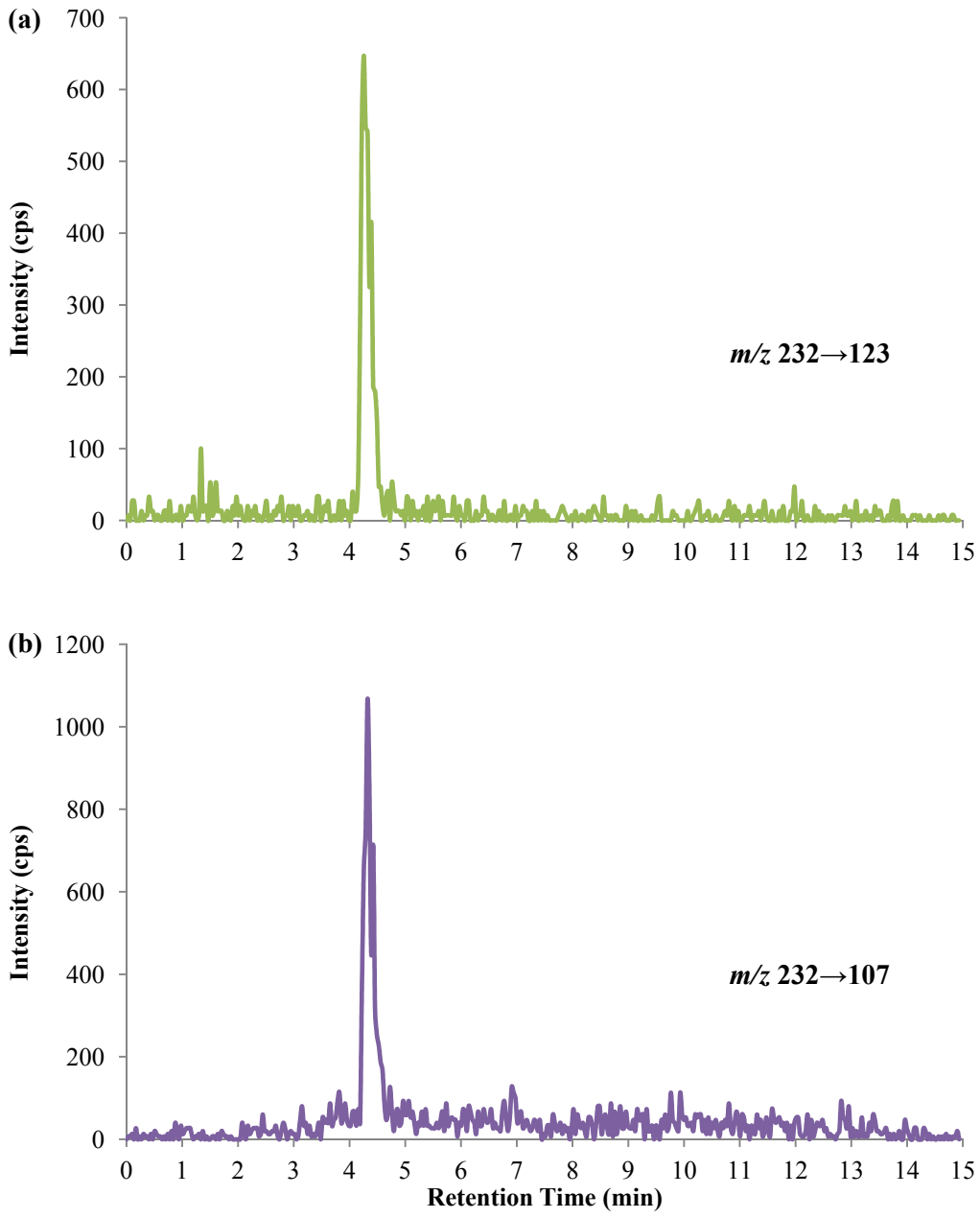


Figure 2.12 Chromatograms obtained from the analysis of a litter sample using HPLC separation and MS/MS detection based on MRM transitions of (a) m/z 232 to 123 and (b) m/z 232 to 107. These MRM transitions were selected for monitoring 3-AHPAA. The litter sample was collected on day 28 from ROX-fed chickens (pen #11, Ross 308).

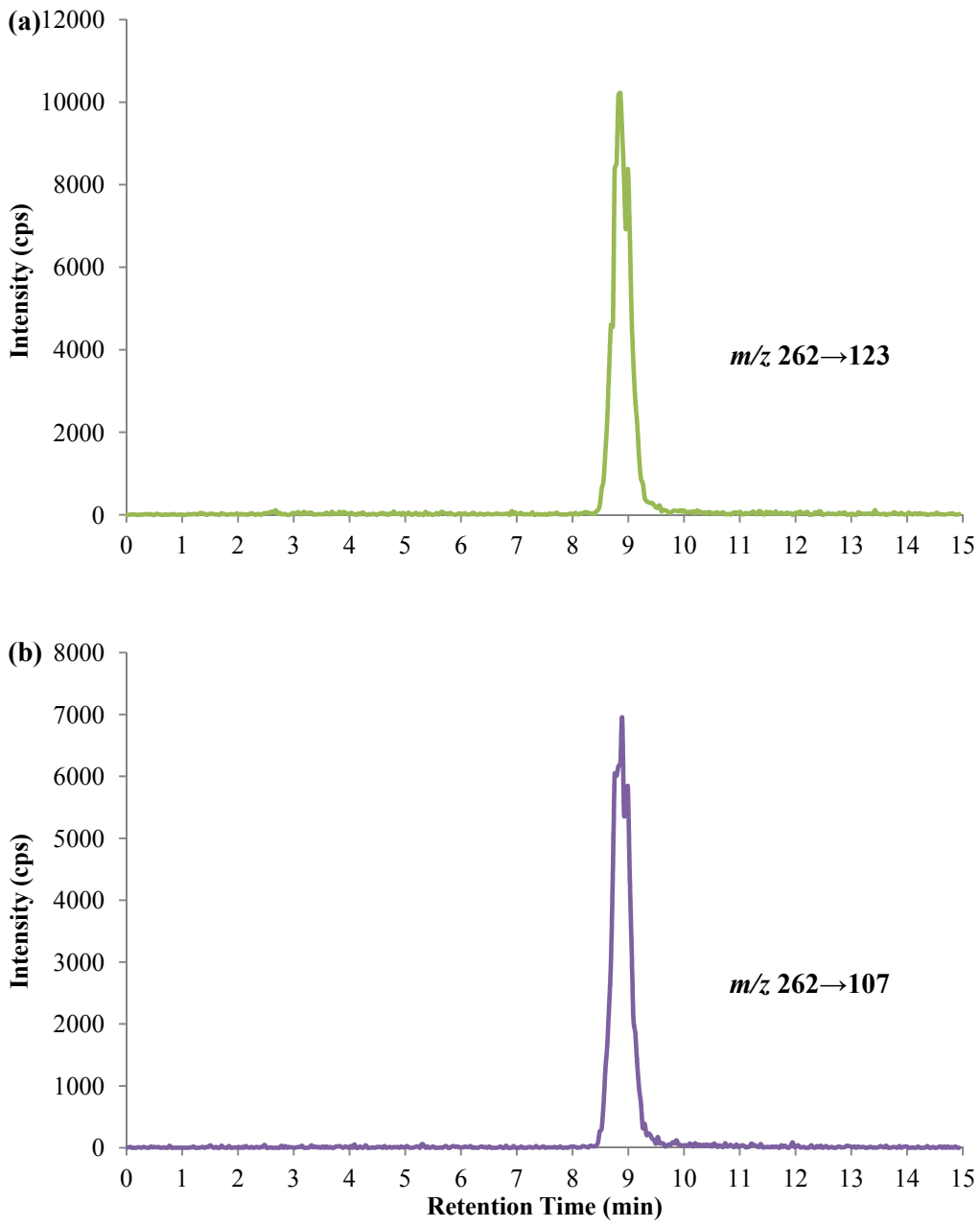


Figure 2.13 Chromatograms obtained from the analysis of a litter sample using HPLC separation and MS/MS detection based on MRM transitions of (a) m/z 262 to 123 and (b) m/z 262 to 107. These MRM transitions were chosen for the detection of ROX. The litter sample was collected on day 28 from ROX-fed chickens (pen #11, Ross 308).

2.3.5 Development of HPLC-ICPMS/ESIMS Method for Arsenic Speciation in Poultry Litter

Finally, HPLC separation combined with ICPMS and ESIMS detections described above was chosen to analyze poultry litter samples. ESIMS is molecular-specific. Overlapping the ICPMS chromatograms with those of the ESIMS accelerates the identification for arsenic-containing compounds. Besides, the quantification of arsenic species in litter relies on the ICPMS because of its lower detection limits (higher sensitivity). Our system demonstrated LOD of 0.1 μg/L as As for three phenylarsonic compounds and 0.05 μg/L as As or better for the other five arsenic species using ICPMS (Table 2.6). Typical chromatograms from an HPLC-ICPMS/ESIMS analysis were the combination of Figure 2.10 (a) and 2.10 (b). Examples of the calibration curves of all eight confirmed arsenic species analyzed by ICPMS are illustrated in Figure 2.14 (a-h).

Table 2.6 Limits of detection (LOD) for arsenic species using HPLC-ICPMS for quantification.

Arsenic Species	LOD (μg/L)
AsB	0.02
As^{III}	0.05
DMA^{V}	0.02
MMA^{V}	0.02
As^{V}	0.05
3-AHPAA	0.1
N-AHAA	0.1
ROX	0.1

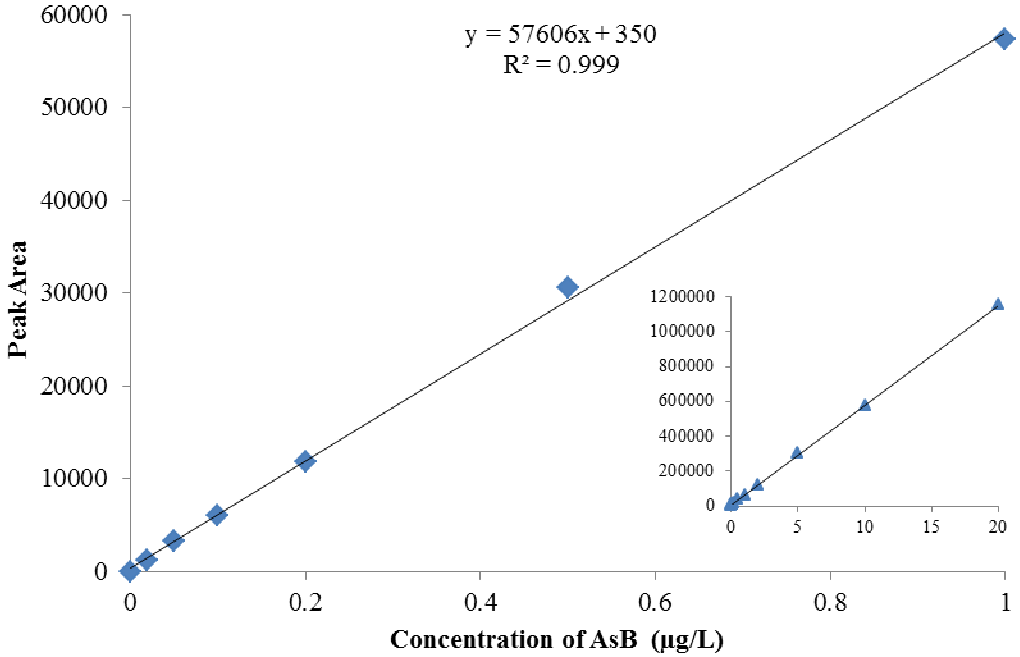


Figure 2.14 (a) A calibration curve generated from the HPLC-ICPMS analyses of 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 µg/L AsB standards (working range). The inset shows the concentration range from 0 to 20 µg/L.

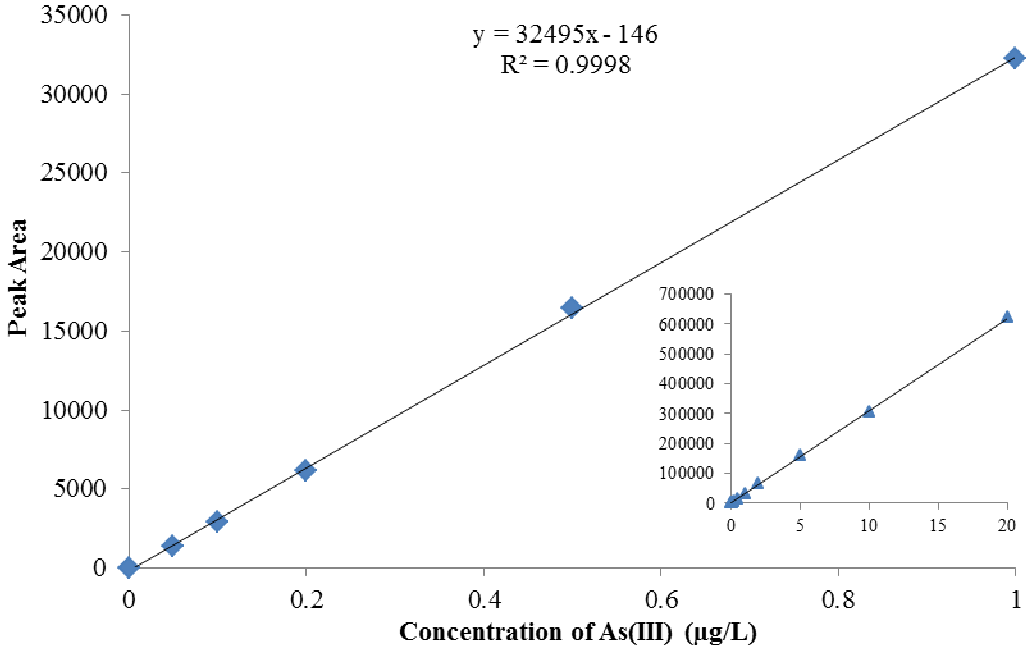


Figure 2.14 (b) A calibration curve generated from the HPLC-ICPMS analyses of 0.05, 0.1, 0.2, 0.5, and 1.0 µg/L As^{III} standards (working range). The inset shows the concentration range from 0 to 20 µg/L.

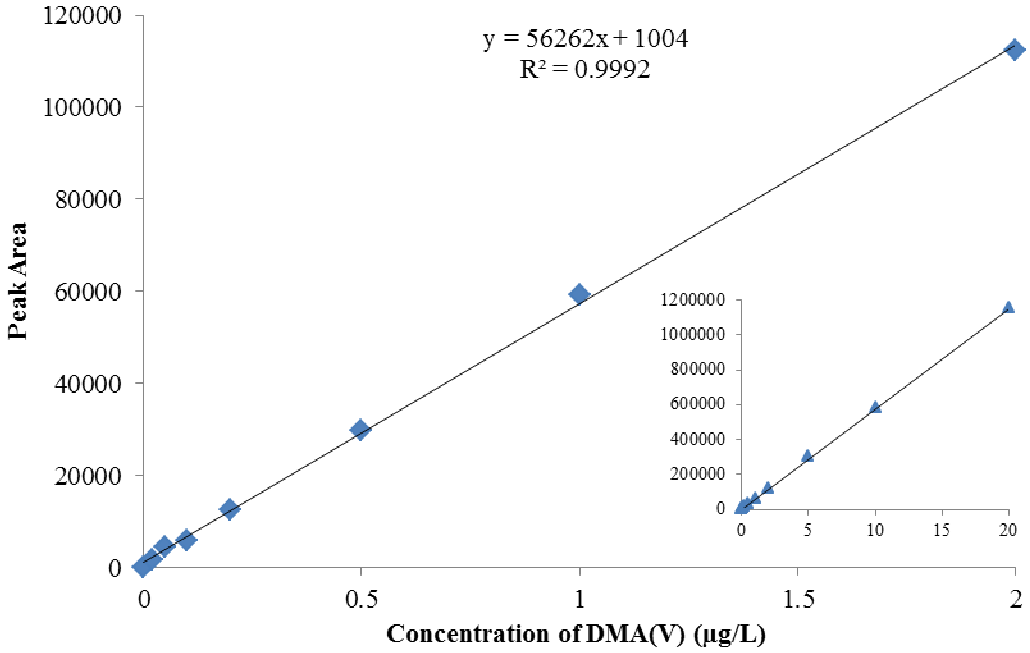


Figure 2.14 (c) A calibration curve generated from the HPLC-ICPMS analyses of 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 µg/L DMA^V standards (working range). The inset shows the concentration range from 0 to 20 µg/L.

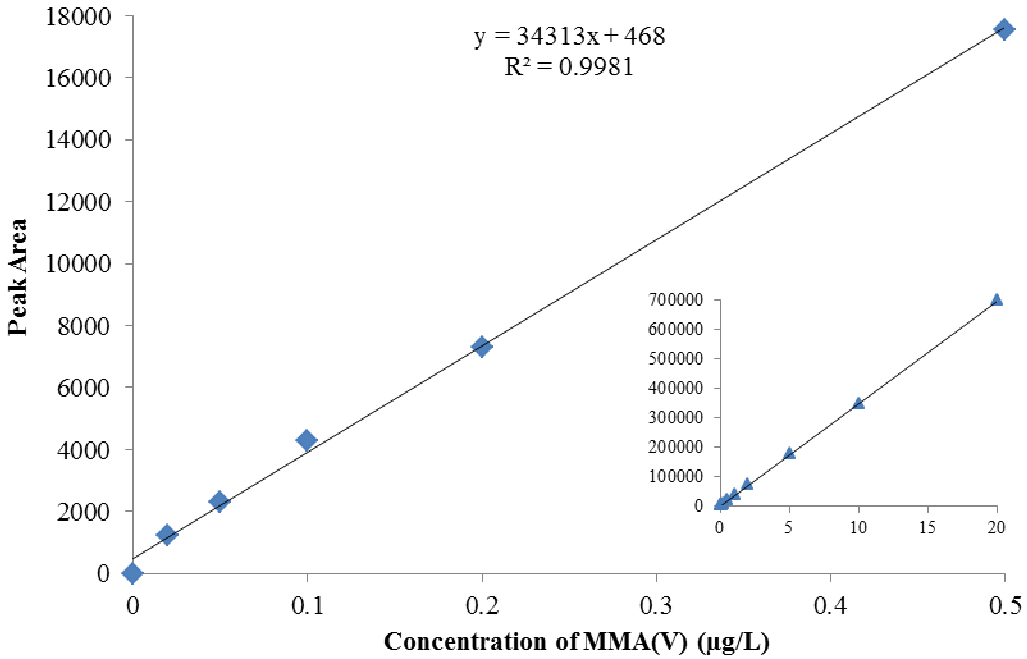


Figure 2.14 (d) A calibration curve generated from the HPLC-ICPMS analyses of 0.02, 0.05, 0.1, 0.2, and 0.5 µg/L MMA^V standards (working range). The inset shows the concentration range from 0 to 20 µg/L.

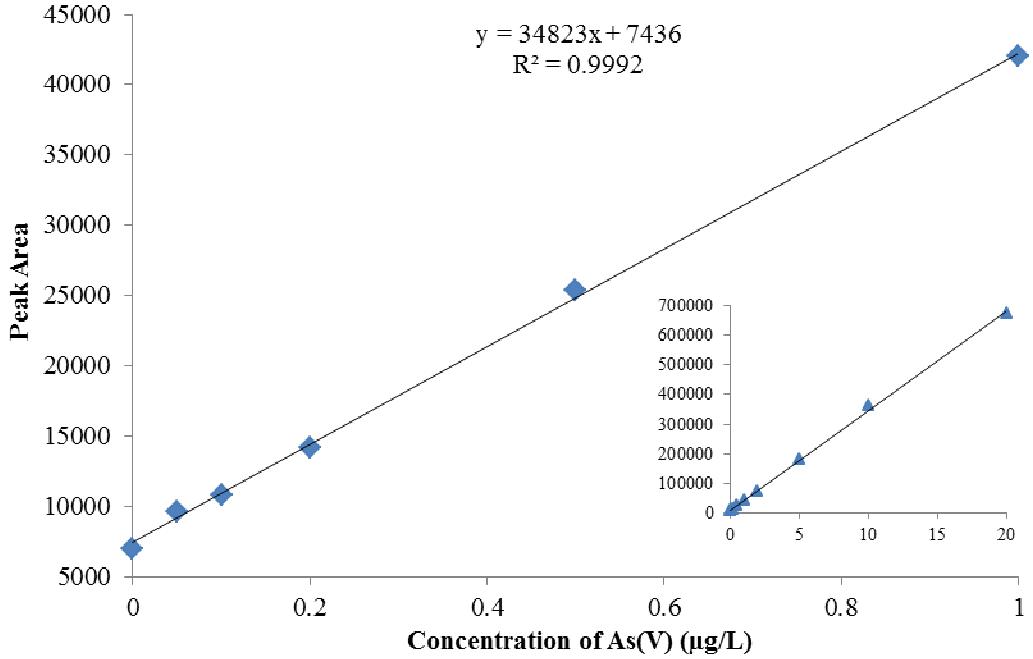


Figure 2.14 (e) A calibration curve generated from the HPLC-ICPMS analyses of 0.05, 0.1, 0.2, 0.5, and 1.0 µg/L As^{V} standards (working range). The inset shows the concentration range from 0 to 20 µg/L.

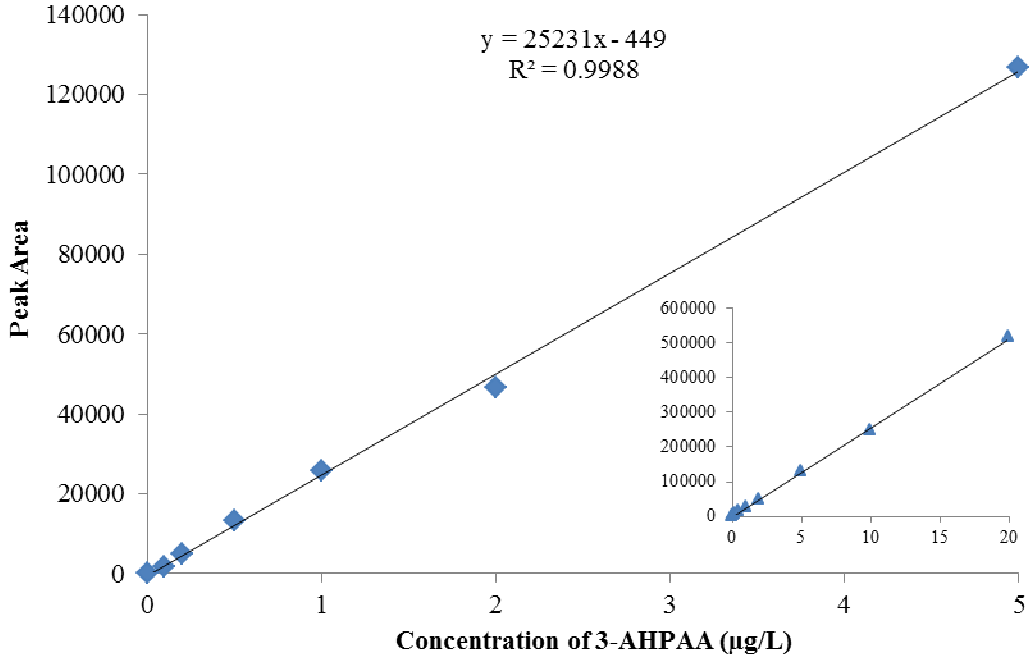


Figure 2.14 (f) A calibration curve generated from the HPLC-ICPMS analyses of 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 µg/L 3-AHPAA standards (working range). The inset shows the concentration range from 0 to 20 µg/L.

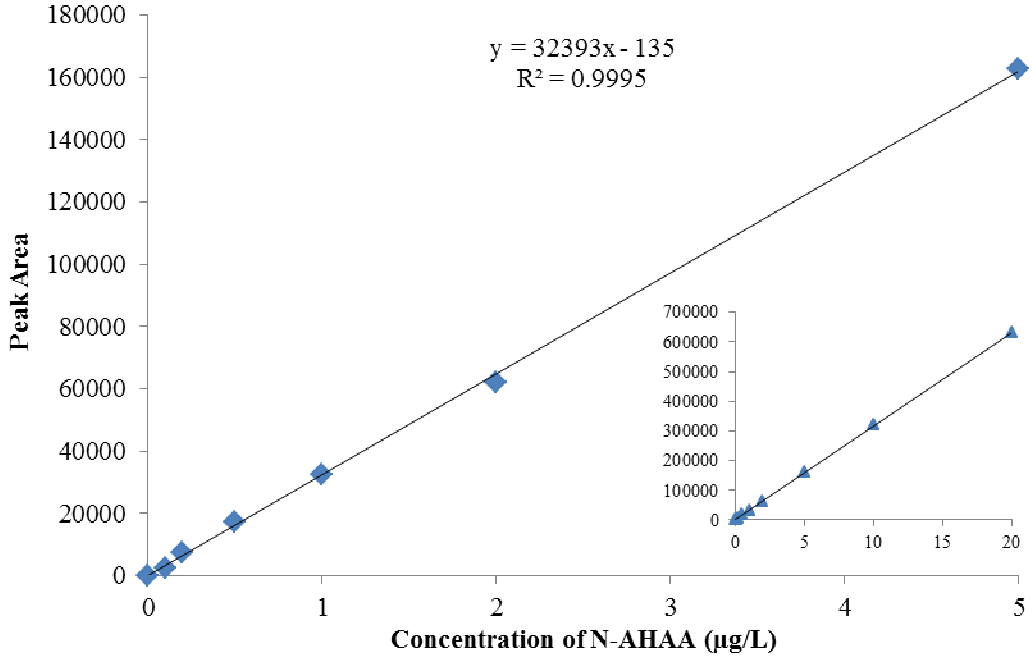


Figure 2.14 (g) A calibration curve generated from the HPLC-ICPMS analyses of 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 µg/L N-AHAA standards (working range). The inset shows the concentration range from 0 to 20 µg/L.

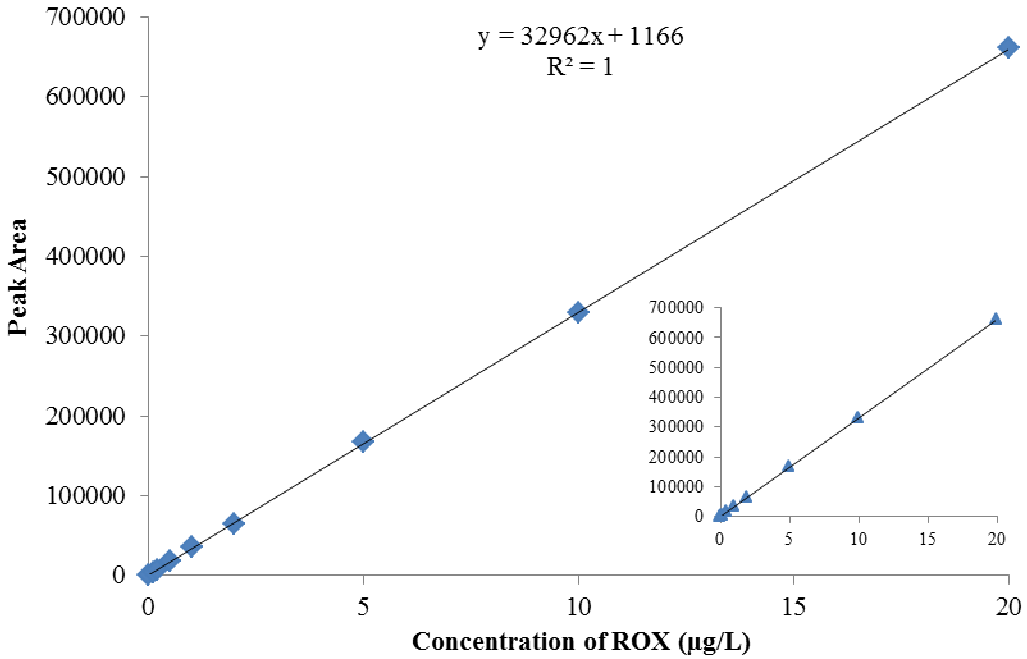


Figure 2.14 (h) A calibration curve generated from the HPLC-ICPMS analyses of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, and 20 µg/L ROX standards (working range). The inset shows the concentration range from 0 to 20 µg/L.

2.4 DISCUSSION

2.4.1 Optimization of Sample Extraction

For the same amount of samples and under the same extraction conditions, by increasing the time of sonication from 10 min to 20 min, there was an average 3.0 μg/g increase of the total arsenic concentrations in the litter extract. However, the total arsenic values remained constant if the time of sonication was extended to 30 min. Thus, 20 min was used for each time of extraction in subsequent experiments.

Similarly, the mass of samples for extraction was optimized. The measure values of total arsenic concentrations were in the unit of μg/L, which needed to be converted to the unit of μg/g for comparison. A sharp decrease in the total arsenic occurred by adding 0.5 g more samples (Figure 2.3). Therefore, 0.5 g of the homogenized samples was weighed for the extraction process in subsequent experiments. By comparing Figure 2.4 (a) with 2.4 (b), it can be seen that if using 0.5 g, instead of 1.0 g, as the mass of sample, the first time of extraction was more efficient (71% > 53%). The arsenic species could be extracted more fully after three times of extraction because 93% of 20.4±0.7 μg/g is higher than 90% of 15.4±0.4 μg/g. The last two extractions only accounted for less than 7% of total arsenic in Figure 2.4 (a). Therefore, no more than three times of extraction was tried.

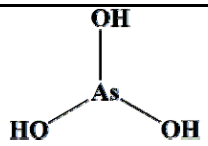
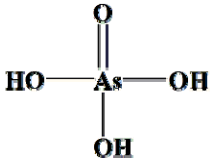
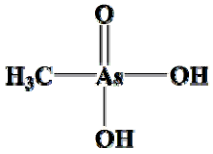
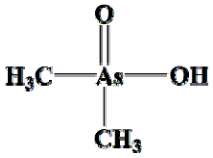
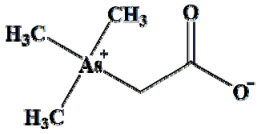
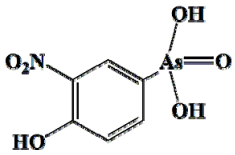
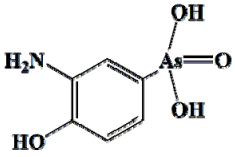
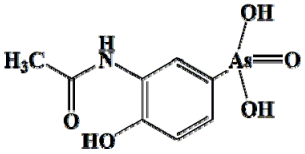
As shown in Table 2.3, the mean total arsenic concentration in the poultry litter we collected was 32.0 μg/g (±0.9 μg/g) from chickens fed ROX, which is similar with the results reported elsewhere [22-24]. The optimized extraction

method provided a fairly good extraction efficiency of 72% using a methanol-water mixture. The mass balance of litter samples was also achieved by including the portion of arsenic left in the residues.

2.4.2 Identification of Arsenic Species in Poultry Litter Using HPLC-ICPMS/ESIMS

Chemical structures of all arsenic species involved in this study are listed in Table 2.7. ROX and six other arsenic standards (As^{III}, As^{V}, MMA^{V}, DMA^{V}, 3-AHPAA, and AsB) were selected for initial chromatography, due to their potential as biotransformation products of ROX [13-15, 23]. AsB is a non-toxic substance that commonly exists in fish meal and seafood [7, 8, 10] which are significant sources of dietary arsenic for farmed poultry [25, 26]. Baseline resolution of these seven arsenic species in a standard mixture was achieved on the PRP-X110S anion exchange column within 10 min [Figure 2.5 (a)]. By matching the retention times of arsenic species in poultry litter [Figure 2.5 (b)] with those in the above standard mixture using HPLC-ICPMS, the presence of AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, ROX, and an unknown species in poultry litter was indicated.

Table 2.7 Chemical structures and pK_a values of arsenic species studied.

Name	Abbreviation	Chemical Structure	pK_a Values
Arsenite	As^{III}		9.2, 12.1, 13.4
Arsenate	As^{V}		2.3; 6.8; 11.6
Monomethylarsonic acid	MMA^{V}		3.6; 8.2
Dimethylarsinic acid	DMA^{V}		6.2
Arsenobetaine	AsB		4.7
3-nitro-4-hydroxy-phenylarsonic acid	ROX		3.5, 5.7, 9.1
3-amino-4-hydroxy-phenylarsonic acid	3-AHPAA		-
N-acetyl-4-hydroxy-m-arsanilic acid	N-AHAA		-

To confirm the identity of each arsenic peak and to investigate the unknown peak, different concentrations of arsenic standards were added to the litter extract separately and analyzed under the same conditions. Typical chromatograms from the 3-AHPAA spiking experiment are presented in Figure 2.6 (a). The resulting peak of suspected 3-AHPAA in the spiked litter extract was symmetrical, with height increased as expected. Similar results were obtained for AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, and ROX spikings [Figure 2.6 (b-g)]. Another phenylarsonic acid, N-AHAA, was found to co-elute with the unknown species in the litter sample [Figure 2.6 (h)]. The presence of ROX, As^{III}, As^{V}, MMA^{V}, DMA^{V}, and 3-AHPAA in litter has been reported in the literature [14, 15]. However, the finding of N-AHAA in poultry litter in this study is novel.

Although HPLC-ICPMS provides identification information by matching the retention times of expected arsenic standards with suspected arsenic species in the litter, there are still limitations if different species have identical chromatographic retention times. ESIMS provides useful structural information of arsenic species of interest, especially of arsenic compounds never reported in poultry litter. In Figure 2.10 (a), all arsenic-containing compounds except As^{III} were detectable in MRM mode, thereby verifying their identities in the litter sample. The undetectability of As^{III} in litter samples by the QTRAP mass spectrometer could be due to its inherently low concentration and poor ionization efficiency.

Figure 2.11 (a) and 2.11 (b) show example chromatograms of the identification of N-AHAA in poultry litter in MRM mode. MS/MS fragmentations

of the suspected arsenic species revealed two MRM transitions, *m/z* 274 to 123 and *m/z* 274 to 107, which are characteristic of N-AHAA. Both fragments are very common to arsenic-containing compounds, corresponding to AsO_3^- and AsO_2^-, respectively. Likewise, the identities of the other arsenic species were confirmed (Figure 2.12 and 2.13) by the simultaneous monitoring of their respective MRM transitions (Table 2.5).

However, ESIMS suffers from higher LOD, matrix effects and ion interferences. ICPMS is more sensitive, reproducible, and robust for arsenic quantification in poultry litter. By combining ESIMS with ICPMS after a single HPLC separation, we were able to achieve both confirmative identification and reliable quantification simultaneously.

It has been reported [27-29] that the LOD values for arsenicals using the HPLC-ICPMS method is 0.2–2.0 μg/L. As shown in Table 2.6, better results for eight arsenic species were achieved based on ICPMS data in our study. All standard calibration curves are typically linear with R^2 values higher than 0.998 [Figure 2.14 (a-h)].

2.5 CONCLUSIONS

The methanol-water extraction method was optimized as follows: 0.5 g as the mass of sample; 20 min as the time of sonication; 3 times as the number of extraction times. This technique will be used for subsequent extraction of arsenic species in environmental waste samples.

An HPLC-ICPMS/ESIMS method was developed for the purpose of arsenic speciation in environmental waste samples. The successful separation of AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and ROX was performed on anion exchange chromatography. The ESIMS detection was reliable enough to provide their structural information and confirm their presences. N-AHAA, a previously unreported species in the litter of poultry fed ROX, was identified. The high sensitivity of ICPMS detection offered LOD values as low as 0.02 μg As/L, which is preferred for quantifying the existing arsenic-containing compounds in the litter samples.

2.6 REFERENCES

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CHAPTER 3

APPLICATION OF THE HPLC-ICPMS/ESIMS METHOD FOR ARSENIC SPECIATION IN POULTRY LITTER

3.1 INTRODUCTION

3-nitro-4-hydroxyphenylarsonic acid (Roxarsone®, ROX) has been well known as the first approved arsenic-containing drug commonly used in poultry industry. It is fed to chickens to control coccidial intestinal parasites, promote weight gain, and improve feed efficiency [1]. Most ROX ingested by chickens is believed to be excreted unchanged into the waste [2]. Approximately 12 to 23 billion kilograms of poultry litter is estimated to be produced annually in the U.S. [3], of which 90% is subsequently applied to agricultural lands as a fertilizer [4]. Poultry litter is also used as an economical source of protein, minerals, and energy to feed beef cattle [5, 6], or as electrical and heating fuels to support industrial companies [7, 8]. Numerous concerns have been expressed associated with arsenic exposure in poultry litter when poultry litter arsenic is released into the environment [9-15].

Several studies [12, 13, 16, 17] have shown that ROX is the dominant arsenic species in poultry litter, with minor amounts of arsenate (As^{V}), arsenite (As^{III}), monomethylarsonic acid (MMA^{V}), and dimethylarsinic acid (DMA^{V}) also detected. However, the distribution of individual arsenic compounds in chicken waste may vary over the feeding period. No quantitative information is available regarding temporal changes of these arsenicals. There is also evidence that ROX

is partly converted to inorganic As^V [12, 13] and 3-amino-4-hydroxyphenylarsonic acid (3-AHPAA) [13, 17]. Whether ROX could be metabolized to other arsenic species has not been extensively studied. Considering the widespread use of poultry litter, systematic arsenic speciation of poultry litter is required and will be critical to fully assess the risks of ROX and its metabolites to the environment and human health.

Using the hyphenated HPLC-ICPMS/ESIMS system developed, we report here the speciation of arsenic in the litter of chickens fed either ROX-supplemented diet or control diet. We show distribution changes of each arsenic species in poultry litter, with a focus on ROX metabolites over the period of feeding.

3.2 MATERIALS AND METHODS

A 35-day poultry feeding study was conducted at the Poultry Research Centre, University of Alberta. A total of 1200 chickens, representing two strains Ross 308 and Cobb 500, were used. For each strain, 300 chickens were placed in 3 pens (100 chickens in each pen) to serve as the control group, and another 300 chickens were raised in 3 additional pens (also 100 chickens per pen) to serve as the ROX-treatment group. Drinking water from the same source (< 1 μg/L arsenic) was available to the chickens throughout the entire 35-day period. The chickens were provided with starter feed during the first two weeks. After an additional two-week supply of grower feed, finisher diets were fed to the chickens for the last week before the end of the trial. For the ROX-treated group, ROX was withdrawn from the finisher diet one week prior to slaughter in compliance with

U.S. FDA regulations in order to allow elimination of arsenic from chickens' bodies. The strain of chickens, pen identification numbers, number of chickens, composition of diets, duration of feeding, time of litter collection are summarized in Table 3.1.

3.2.1 Sample Collection

Poultry litter samples were obtained from the Poultry Research Centre, University of Alberta. They were collected on day 14, day 24, day 28, day 30 and day 35 respectively. Fresh wood chips served as the pen bedding material. On each of the first four collection days, five identical spots of accumulated litter samples from each pen were gathered and combined, representing the whole litter from each pen. Only at the end (day 35) was a full litter collected from each pen.

Poultry feed was prepared by the Poultry Research Centre. The supplement of ROX to the feed was also prepared by the Poultry Research Centre. A total of six ROX-supplemented and control feed samples (starter diet × 2, grower diet × 2, finisher diet × 2) were obtained in parallel with the litter samples from the Poultry Research Centre, and were analyzed for arsenic speciation. The compositions of the poultry feed are summarized in Appendix Table A.1.

All samples were frozen and stored at -20 °C until the time of analysis.

Table 3.1 Information of feeding experiments.

Poultry Strain	Group	Feeding plan			Pen Number	Number of Chickens	Time of Litter Collection
		Uptake period (day 0 – day 14)	**Growth period (day 14 – day 28)**	**ROX withdrawal period (day 28 – day 35)**			
Ross 308	ROX-fed	ROX Starter (ROX-supplemented diet)	ROX Grower (ROX-supplemented diet)	ROX Finisher (ROX-free diet)	11	100	day 14, day 24, day 28, day 30, day 35
					13	100	
					15	100	
	control	Control Starter (ROX-free diet)	Control Grower (ROX-free diet)	Control Finisher (ROX-free diet)	12	100	
					17	100	
					22	100	
Cobb 500	ROX-fed	ROX Starter (ROX-supplemented diet)	ROX Grower (ROX-supplemented diet)	ROX Finisher (ROX-free diet)	1	100	
					19	100	
					24	100	
	control	Control Starter (ROX-free diet)	Control Grower (ROX-free diet)	Control Finisher (ROX-free diet)	5	100	
					6	100	
					25	100	

3.2.2 Sample Processing

On the day of analysis, poultry litter or feed samples were homogenized using a blade grinder. 0.5 g of the homogenate was accurately weighed into a 50 mL polypropylene centrifuge tube (Fisher Scientific), and 10 mL of methanol-water solution (volume 1:1) was added. The sample solution was vortex-mixed thoroughly and sonicated for 20 min, and then centrifuged at 3500 g for additional 10 min. The supernatant was removed and kept in a new centrifuge tube. Extraction of the same sample solution was repeated twice. An aliquot of the combined supernatant was then syringe-filtered through a 0.45 μm membrane, and diluted 10-fold with deionized water into a glass autosampler vial prior to speciation analysis of arsenic using HPLC-ICPMS/ESIMS. Sample blanks containing only the methanol-water mixture were also prepared. Each sample was extracted and analyzed in triplicate.

Additionally, control litter homogenate was incubated with the ROX standard for 1 h or 2 months, respectively. 0.5 g of the fortified mixture was immediately processed in the same manner as described above for subsequent arsenic speciation analysis to evaluate the extraction procedure and storage condition.

3.2.3 Arsenic Speciation Analysis

A PRP-X110S anion exchange column (7 μm particle size, 150 × 4.1 mm) from Hamilton (Reno, NV) with a guard column was used for HPLC separation of arsenic species. The mobile phases were 5% methanol and 60 mM ammonium bicarbonate in 5% methanol (pH adjusted to 8.7 with 10% ammonium hydroxide).

The gradient elution program developed in Chapter 2 (Table 2.2) was used. Samples were injected using an Agilent 1100 series HPLC (Germany) for separation, and the eluent was split at a ratio of 4 to 1 between an Agilent 7500cs Octopole ICPMS (Japan) and an AB SCIEX 5500 QTRAP ESIMS (Concord, ON, Canada), respectively. The injection volume was 30 µL for all samples and standards. Each sample was analyzed in triplicate.

Mixtures of eight arsenic standards were prepared with concentrations ranging from 0.05 to 20 µg As/L to incorporate expected arsenic concentrations in poultry litter and feed extracts. The calibration standards were prepared daily from each of the stock solutions, and analyzed prior to the samples. Instrument drift was checked by analyzing a standard mixture at 5 µg As/L once every ten samples.

The signals detected by the ICPMS at *m/z* 75 (As^+) were compared with those obtained simultaneously by the ESIMS in MRM mode to confirm the identities of each arsenic species in samples. The concentration of individual arsenical present was determined based on external calibration of the corresponding arsenic standard solution. The application of the HPLC-ICPMS/ESIMS system to the analyses of poultry litter and feed samples allowed us to obtain comprehensive quantification results from the ICPMS and also confirmative identification information from the ESIMS.

3.3 RESULTS

3.3.1 Arsenic Species in Poultry Litter from Each Time of Extraction

In Chapter 2, total arsenic was studied when we optimized the extraction procedure. But it remained unclear how much of each arsenic species was present during each time of extractions. After the HPLC-ICPMS/ESIMS method was developed, we conducted arsenic speciation in each of the five supernatant extracts (Figure 3.1). A large amount of arsenic, especially in the form of ROX, was extracted during the first two extractions, whereas nearly no arsenic was detected in the litter extract after three extractions.

3.3.2 Arsenic Species in ROX-incubated Control Litter

After a control litter sample was incubated with the ROX standard for 1 h, no additional peaks except ROX appeared (Figure 3.2). Arsenic species other than ROX present in the fortified litter sample remained the same in quantity, suggesting that ROX was stable and did not convert to other arsenic compounds during the sample extraction. Similar result was observed in the same control litter incubated with the ROX standard for 2 months (Figure 3.3). This also indicates that the storage condition at -20 °C could prevent degradation of ROX in the litter.

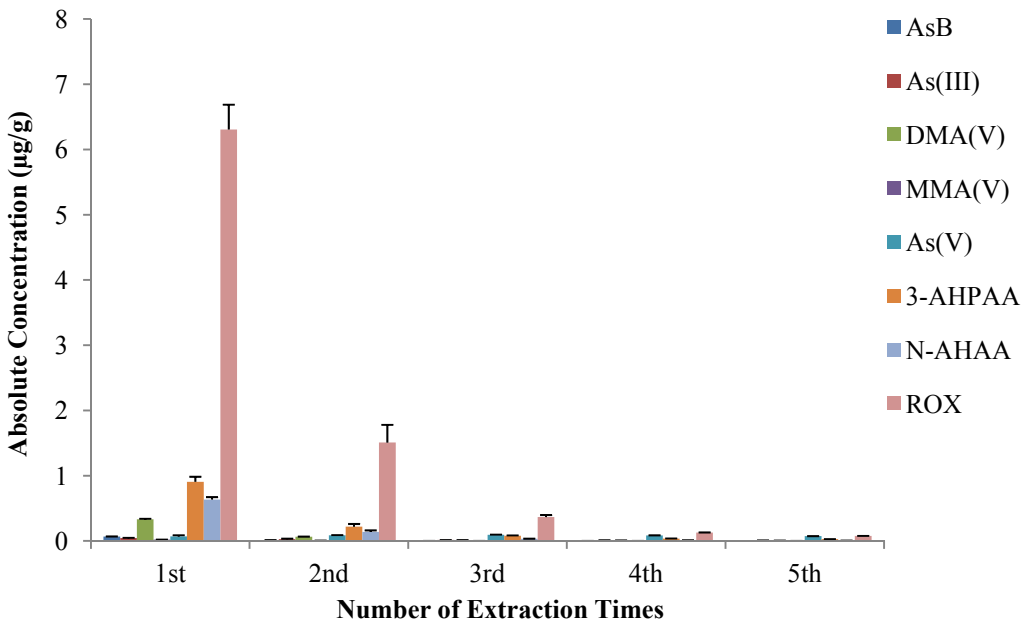

Figure 3.1 Arsenic speciation patterns from each of the five sequential extractions. The studied litter sample was collected on day 28 from pen #24, Cobb 500. The supernatant from each extraction was collected and analyzed separately. Most arsenic was extracted during the first two extractions. ROX is the predominant species in the litter extracts.

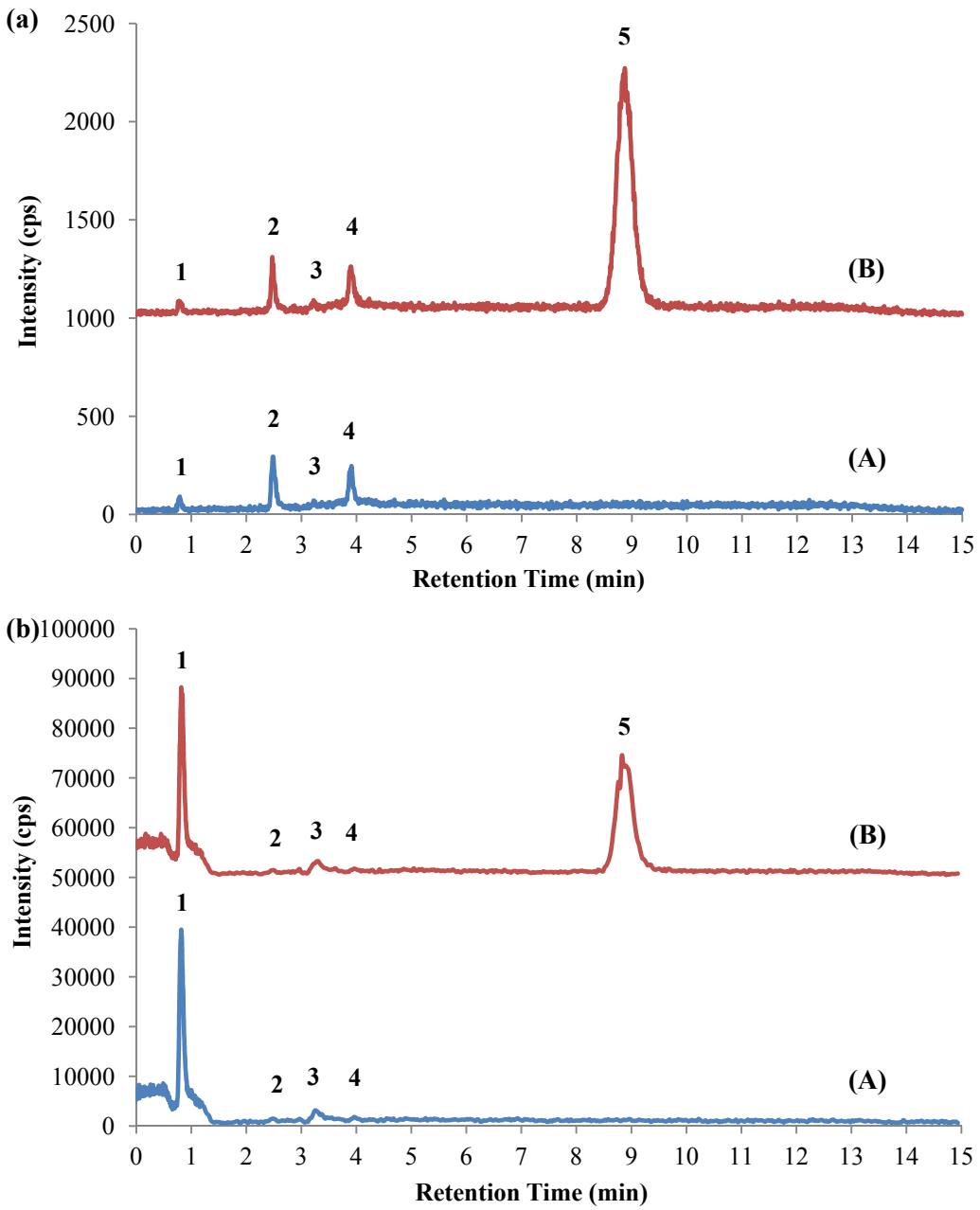

Figure 3.2 (a) HPLC-ICPMS and (b) HPLC-ESIMS chromatograms from the analyses of (A) a litter sample collected on day 14 from control chickens (pen #17, Ross 308) and (B) the same litter sample after incubation with the ROX standard for 1 h. Peaks: (1) AsB, (2) DMA^V, (3) MMA^V, (4) As^V, (5) ROX.

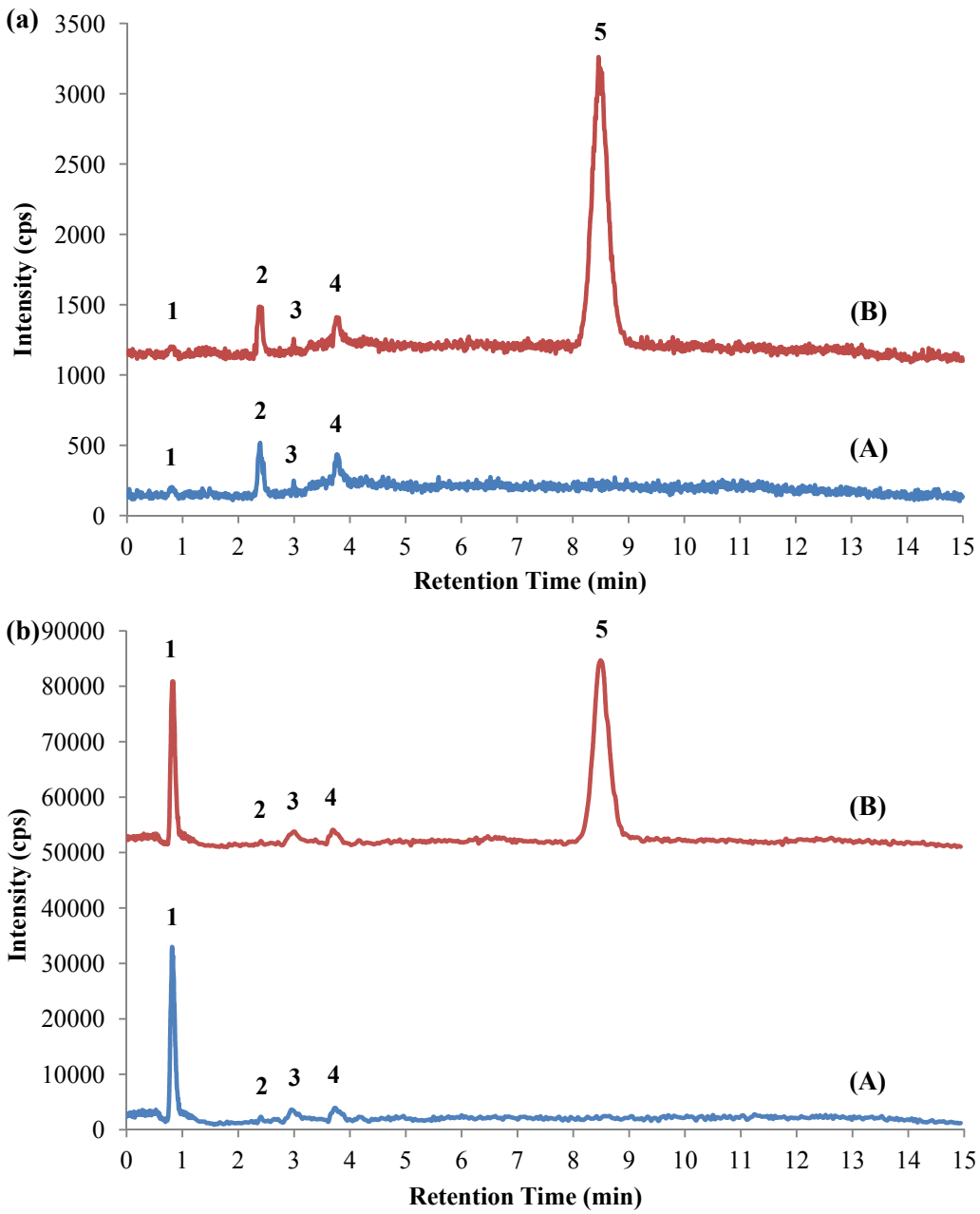

Figure 3.3 (a) HPLC-ICPMS and (b) HPLC-ESIMS chromatograms from the analyses of (A) a litter sample collected on day 14 from control chickens (pen #17, Ross 308) and (B) the same litter sample after incubation with the ROX standard for 2 months. Peaks: (1) AsB, (2) DMA^V, (3) MMA^V, (4) As^V, (5) ROX.

3.3.3 Arsenic Species in Poultry Litter

Figure 3.4 shows typical chromatograms from the HPLC-ICPMS/ESIMS analyses of arsenic species in a litter sample. All eight arsenic-containing compounds (AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and ROX) were well-detected by ICPMS [Figure 3.4 (a)], whose retention times are consistent with those generated in MRM mode [Figure 3.4 (b)]. This particular sample was diluted 10-fold due to the elevated concentration of ROX.

The litter samples collected on five different days from the ROX-fed chickens were analyzed by arsenic speciation. Similar speciation patterns were observed (Figure 3.5). Concentrations of eight arsenicals in the litter of ROX-fed chickens from both Ross 308 and Cobb 500 strains were calculated and summarized in Table 3.2 and 3.3. ROX had a much higher concentration than any other arsenic species on each given day for both strains. As shown in Figure 3.6, there was a slight increase in the sum of arsenic before day 28. At the last collection time point (day 35), the sum of arsenic value returned to a value statistically even much lower than that on day 14.

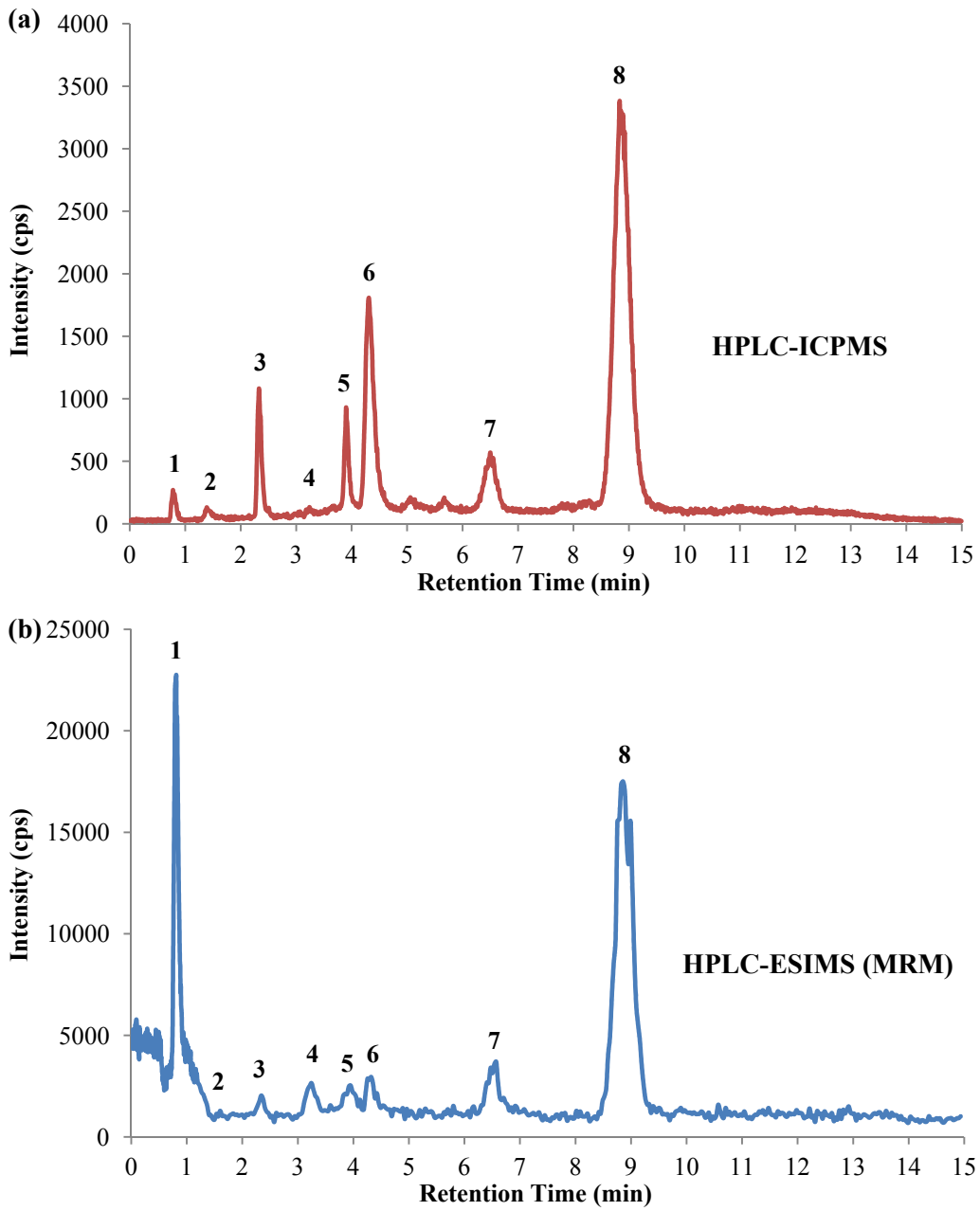

Figure 3.4 Chromatograms showing analyses of a litter sample collected on day 28 from ROX-fed chickens (pen #11, Ross 308) using (a) HPLC-ICPMS and (b) HPLC-ESIMS. Peaks: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) N-AHAA, (8) ROX.

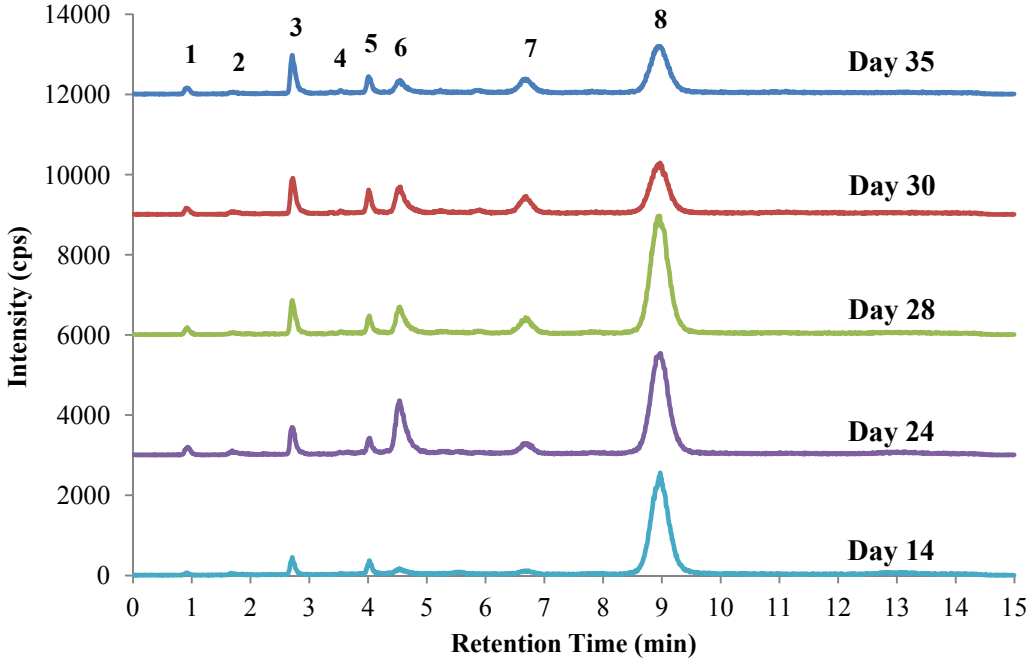

Figure 3.5 Chromatograms obtained from the analyses of litter samples collected on five different days (day 14, 24, 28, 30, and 35) from ROX-fed chickens (pen #24, Cobb 500) using HPLC-ICPMS/ESIMS. Only chromatograms from ICPMS detections are shown. Peaks: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) N-AHAA, (8) ROX.

Table 3.2 Concentrations of arsenic species detected in the litter samples from ROX-fed chickens (strain Ross 308). [a]

	Concentration of Arsenic Species (mean ± SD) (µg/g)							
Day	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	ROX
14	0.035 ± 0.007	0.09 ± 0.03	0.20 ± 0.08	0.022 ± 0.007	0.11 ± 0.07	0.6 ± 0.3	0.22 ± 0.06	7 ± 1
24	0.115 ± 0.009	0.16 ± 0.07	0.34 ± 0.08	0.024 ± 0.004	0.22 ± 0.09	1.7 ± 0.4	0.5 ± 0.1	7.1 ± 0.9
28	0.090 ± 0.004	0.16 ± 0.06	0.43 ± 0.03	0.025 ± 0.004	0.18 ± 0.06	2.0 ± 0.4	0.68 ± 0.04	7.1 ± 0.8
30	0.079 ± 0.005	0.11 ± 0.07	0.39 ± 0.03	0.024 ± 0.009	0.11 ± 0.07	1.06 ± 0.09	0.61 ± 0.07	7 ± 1
35	0.08 ± 0.01	0.10 ± 0.06	0.41 ± 0.07	0.024 ± 0.007	0.10 ± 0.07	0.79 ± 0.03	0.53 ± 0.08	4.8 ± 0.8

[a] SD: standard deviation.

Table 3.3 Concentrations of arsenic species detected in the litter samples from ROX-fed chickens (strain Cobb 500). [a]

	Concentration of Arsenic Species (mean ± SD) (µg/g)							
Day	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	ROX
14	0.037 ± 0.008	0.11 ± 0.07	0.18 ± 0.03	0.011 ± 0.006	0.09 ± 0.04	0.24 ± 0.04	0.20 ± 0.02	7.6 ± 0.7
24	0.113 ± 0.01	0.17 ± 0.06	0.29 ± 0.06	0.014 ± 0.005	0.15 ± 0.06	1.2 ± 0.4	0.44 ± 0.05	6 ± 1
28	0.095 ± 0.02	0.202 ± 0.005	0.42 ± 0.05	0.020 ± 0.005	0.15 ± 0.02	1.4 ± 0.4	0.72 ± 0.04	7 ± 2
30	0.093 ± 0.01	0.23 ± 0.05	0.45 ± 0.03	0.028 ± 0.005	0.18 ± 0.02	1.08 ± 0.05	0.69 ± 0.08	4.0 ± 0.6
35	0.087 ± 0.007	0.16 ± 0.05	0.5 ± 0.1	0.023 ± 0.003	0.12 ± 0.04	0.6 ± 0.1	0.64 ± 0.07	3.7 ± 0.4

[a] SD: standard deviation.

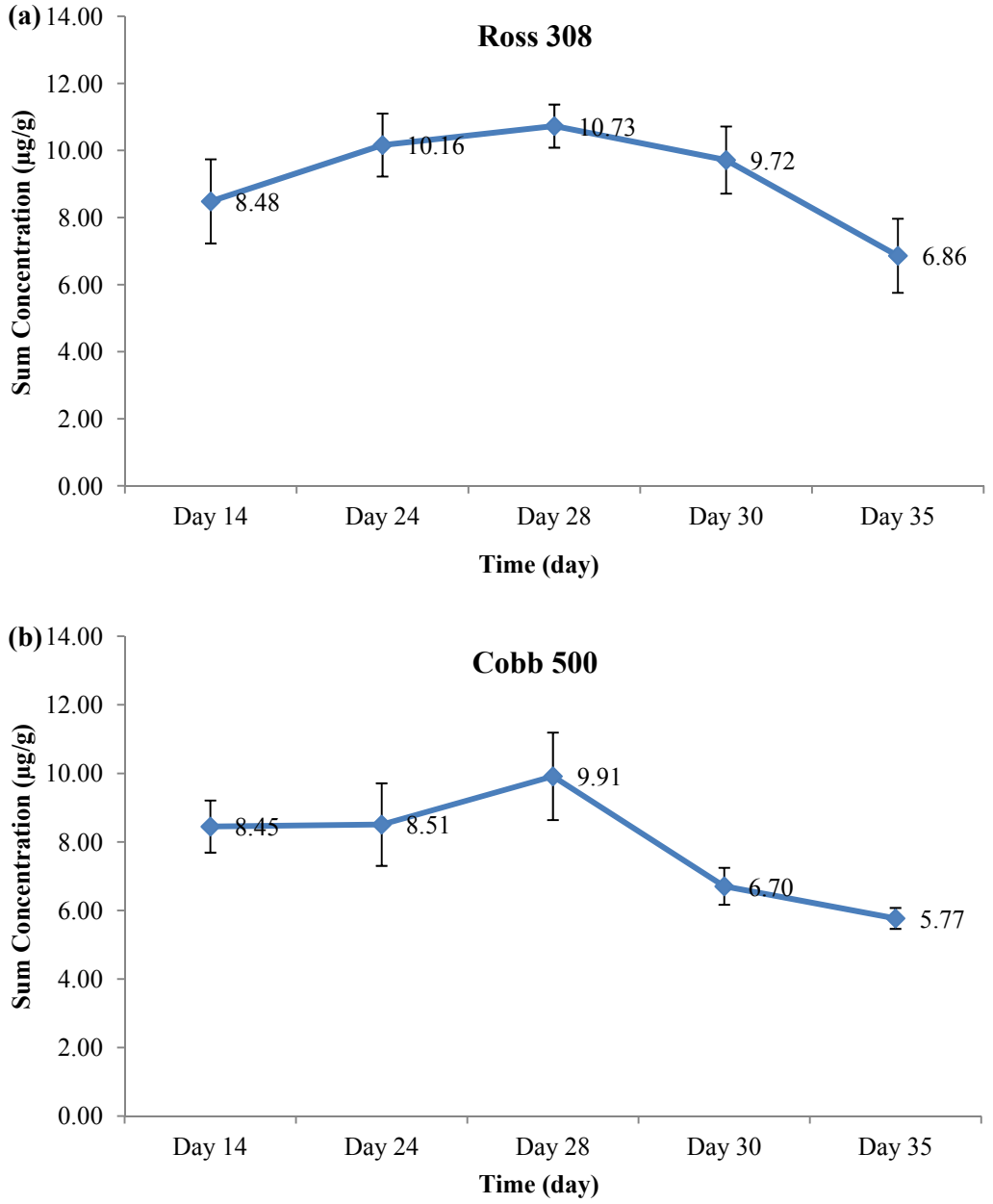

Figure 3.6 The sum of arsenic species in the litter samples of ROX-fed chickens from strain (a) Ross 308 and (b) Cobb 500. The litter samples were collected on five different days (day 14, 24, 28, 30, and 35). The sum of arsenic peaked on day 28 when ROX was first withdrawn from the feed.

However, considering the amounts of wood chips and moisture varied in the litter samples collected from five different days, we further looked at relative concentrations of each arsenic compound as percentages of the sum during the period of feeding, in order to better evaluate the temporal changes of arsenic species. Figure 3.7 (a) indicates that ROX was the most dominant arsenic species with percentages greater than 60% in the litter of Ross 308 chickens fed ROX. The concentrations of all arsenicals varied over the period of feeding. The 3-AHPAA and N-AHAA displayed higher concentrations among other arsenic metabolites on each given day. Similar results were observed in the litter of Cobb 500 chickens fed ROX [Figure 3.7 (b)].

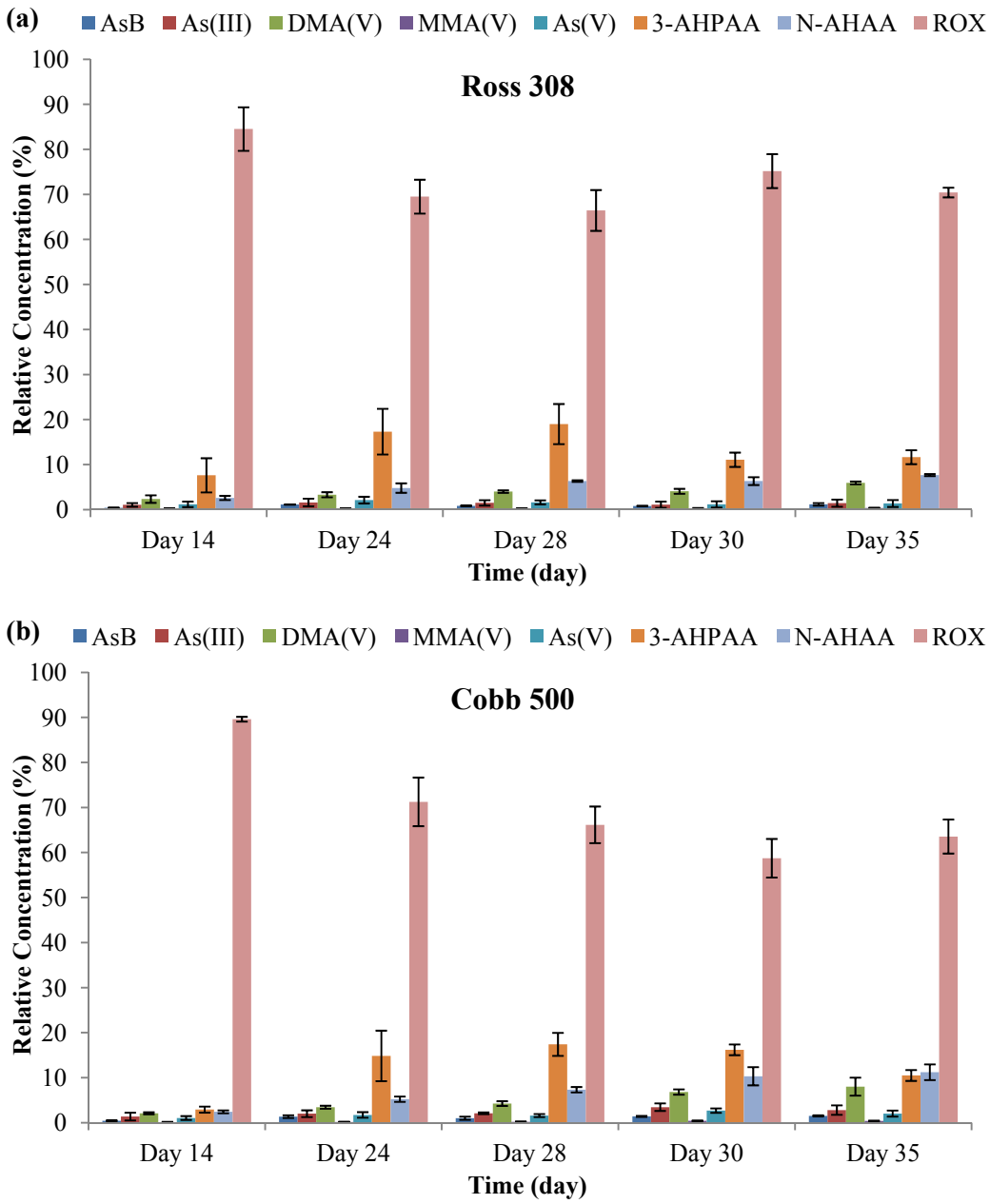

Figure 3.7 Relative concentrations of AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and ROX in the litter samples of ROX-fed chickens from strain (a) Ross 308 and (b) Cobb 500. The litter samples were collected on five different days (day 14, 24, 28, 30, and 35). ROX was the predominant arsenic species in all samples.

For the control group, similar speciation patterns were also obtained (Figure 3.8). As illustrated in Table 3.4 and 3.5, concentrations of all arsenic species were very low in the control litter of chickens from both Ross 308 and Cobb 500 strains. Traces of ROX, 3-AHPAA and N-AHAA could be detected in the litter collected after day 24. Figure 3.9 shows the sum of arsenic reached the maximum on the last day (day 35). But the peak values (0.6-0.7 μg/g) were much lower than those (around 10 μg/g) in the litter of ROX-fed chickens. Instead of ROX, DMA^V was the major arsenic species in the control litter with percentages higher than 45% (Figure 3.10).

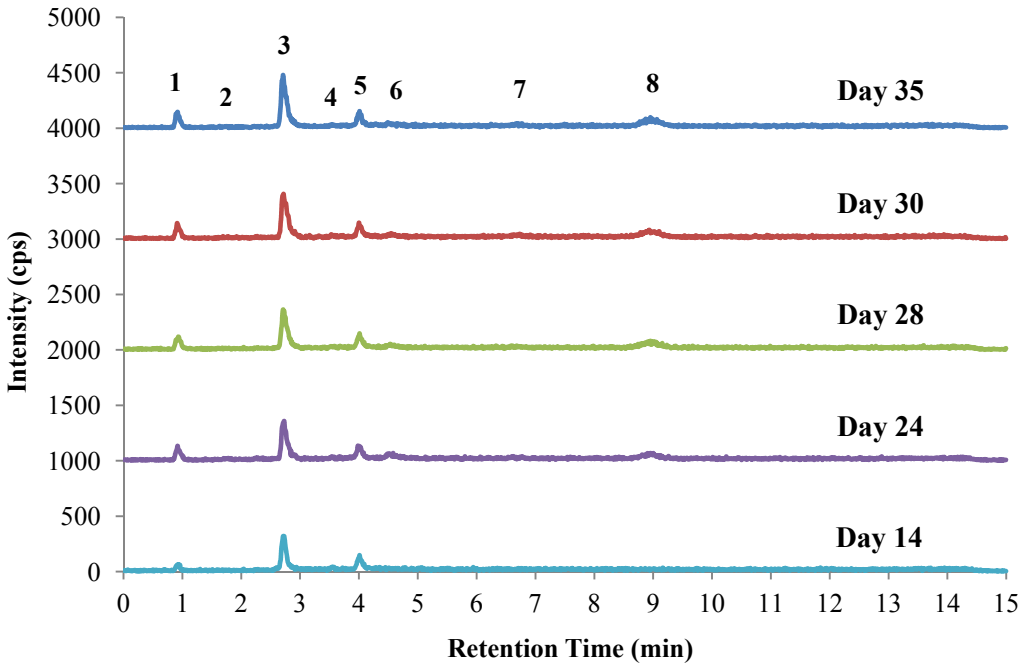

Figure 3.8 Chromatograms obtained from the HPLC-ICPMS/ESIMS analyses of litter samples collected on five different days (day 14, 24, 28, 30, and 35) from control chickens (pen #25, Cobb 500). Only chromatograms from ICPMS detections are shown. Peaks: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) N-AHAA, (8) ROX.

Table 3.4 Concentrations of arsenic species detected in the litter samples from control chickens (strain Ross 308). [a]

	Concentration of Arsenic Species (mean ± SD) (µg/g)							
Day	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	ROX
14	0.025 ± 0.008	0.006 ± 0.001	0.11 ± 0.02	0.004 ± 0.001	0.04 ± 0.02	N.D.	N.D.	N.D.
24	0.080 ± 0.006	0.018 ± 0.002	0.23 ± 0.01	0.0039 ± 0.0001	0.047 ± 0.006	0.04 ± 0.01	0.02 ± 0.01	0.09 ± 0.04
28	0.069 ± 0.002	0.014 ± 0.004	0.26 ± 0.01	0.0036 ± 0.0001	0.046 ± 0.008	0.030 ± 0.003	0.020 ± 0.008	0.11 ± 0.05
30	0.072 ± 0.007	0.015 ± 0.003	0.29 ± 0.02	0.0037 ± 0.0001	0.05 ± 0.01	0.031 ± 0.004	0.03 ± 0.01	0.11 ± 0.06
35	0.084 ± 0.008	0.017 ± 0.004	0.34 ± 0.01	0.0049 ± 0.0003	0.05 ± 0.01	0.0306 ± 0.0004	0.03 ± 0.02	0.13 ± 0.04

[a] N.D.: below detection limit. SD: standard deviation.

Table 3.5 Concentrations of arsenic species detected in the litter samples from control chickens (strain Cobb 500). [a]

	Concentration of Arsenic Species (mean ± SD) (µg/g)							
Day	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	ROX
14	0.022 ± 0.005	0.006 ± 0.002	0.12 ± 0.02	0.0032 ± 0.0006	0.05 ± 0.01	N.D.	N.D.	N.D.
24	0.067 ± 0.001	0.02 ± 0.01	0.248 ± 0.009	0.006 ± 0.003	0.06 ± 0.02	0.03 ± 0.02	0.010 ± 0.009	0.08 ± 0.01
28	0.066 ± 0.004	0.012 ± 0.007	0.29 ± 0.02	0.006 ± 0.003	0.05 ± 0.01	0.03 ± 0.01	0.016 ± 0.009	0.09 ± 0.01
30	0.064 ± 0.003	0.014 ± 0.007	0.30 ± 0.01	0.005 ± 0.002	0.06 ± 0.02	0.03 ± 0.01	0.016 ± 0.009	0.11 ± 0.01
35	0.074 ± 0.003	0.014 ± 0.006	0.34 ± 0.03	0.006 ± 0.002	0.05 ± 0.02	0.026 ± 0.009	0.017 ± 0.006	0.092 ± 0.003

[a] N.D.: below detection limit. SD: standard deviation.

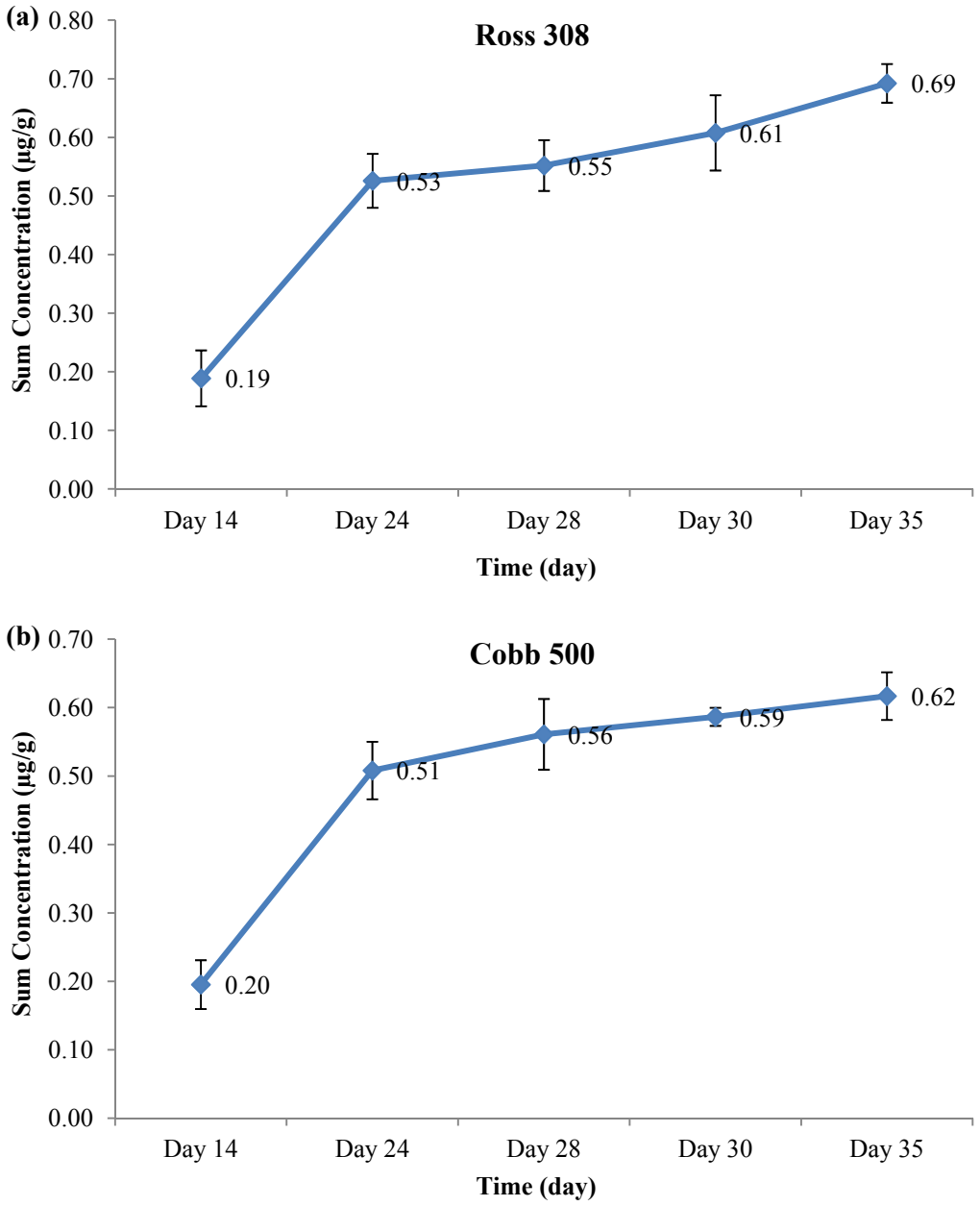

Figure 3.9 The sum of arsenic species in the litter samples of control chickens of strain (a) Ross 308 and (b) Cobb 500. The litter samples were collected on five different days (day 14, 24, 28, 30, and 35). The sum of arsenic reached the peak on day 35.

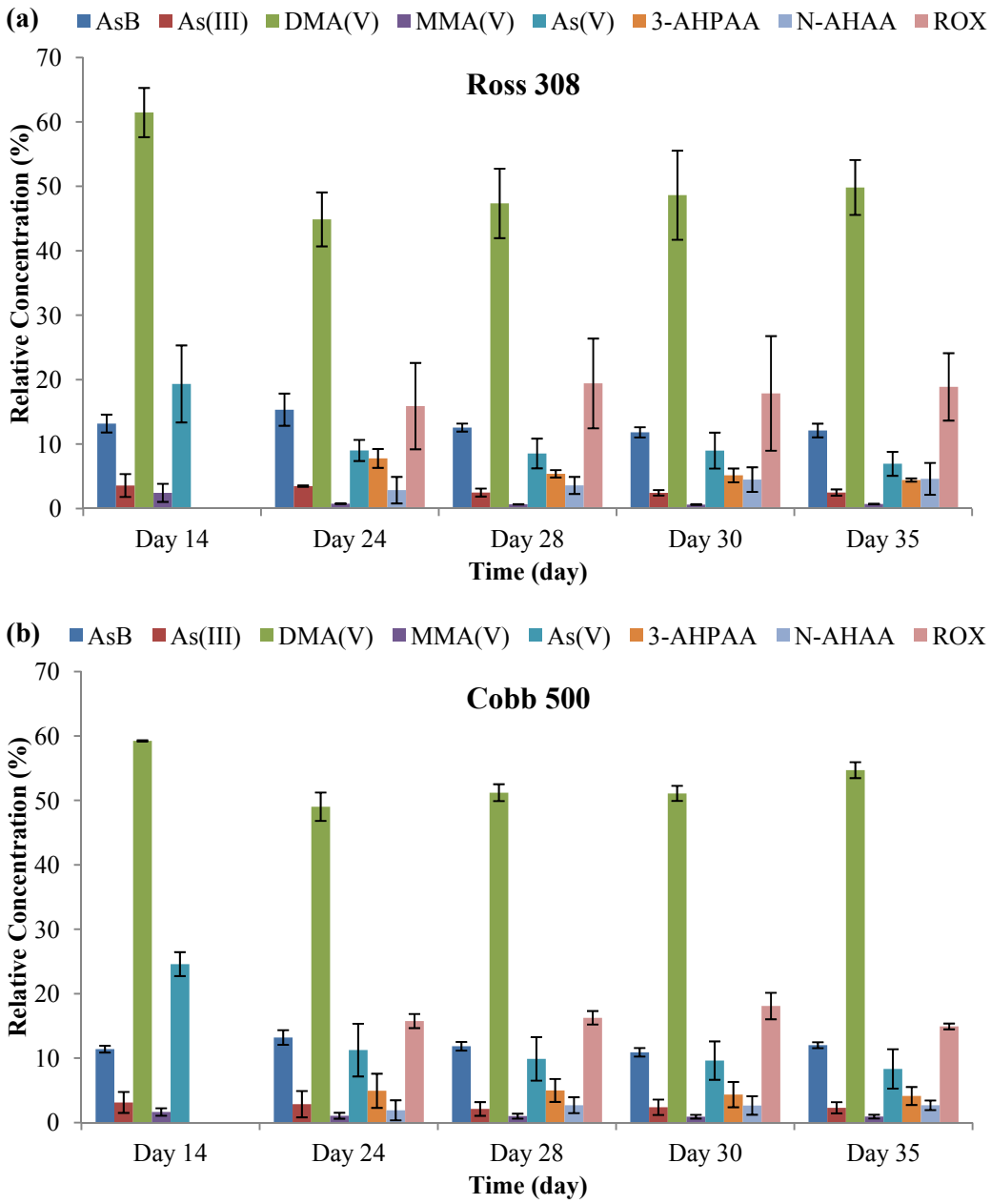

Figure 3.10 Relative concentrations of AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and ROX in the litter samples of control chickens of strain (a) Ross 308 and (b) Cobb 500. The litter samples were collected on five different days (day 14, 24, 28, 30, and 35). DMA^{V} was the major arsenic species in all samples.

By comparing above speciation results in the litter, it can be found that the difference in the sum of arsenic between the ROX-fed group and control group was mainly due to the quantitative disparities of three phenylarsonic acids (ROX, 3-AHPAA, and N-AHAA). However, the distribution changes of the other five arsenic species in the litter were hardly noticeable during the period of feeding. The presence of AsB in the litter was most probably because of the fish meal used in the poultry feed. As shown in Figure 3.11, the concentrations of AsB in the litter samples from ROX-fed and control chickens were very similar as expected on each given day, indicating it was not from conversion of ROX. We also studied the sum of inorganic and methyl arsenicals (As^{III}, As^{V}, MMA^{V}, and DMA^{V}), and compared the results between the ROX-fed group and control group to see whether degradation of ROX into these arsenic species occurred because they are more hazardous due to higher toxicities. Only slight differences in the sum of arsenic between the two groups were observed in Figure 3.12, which were much lower than the differences obtained when all arsenic species were summed as shown in Figure 3.6 and 3.9.

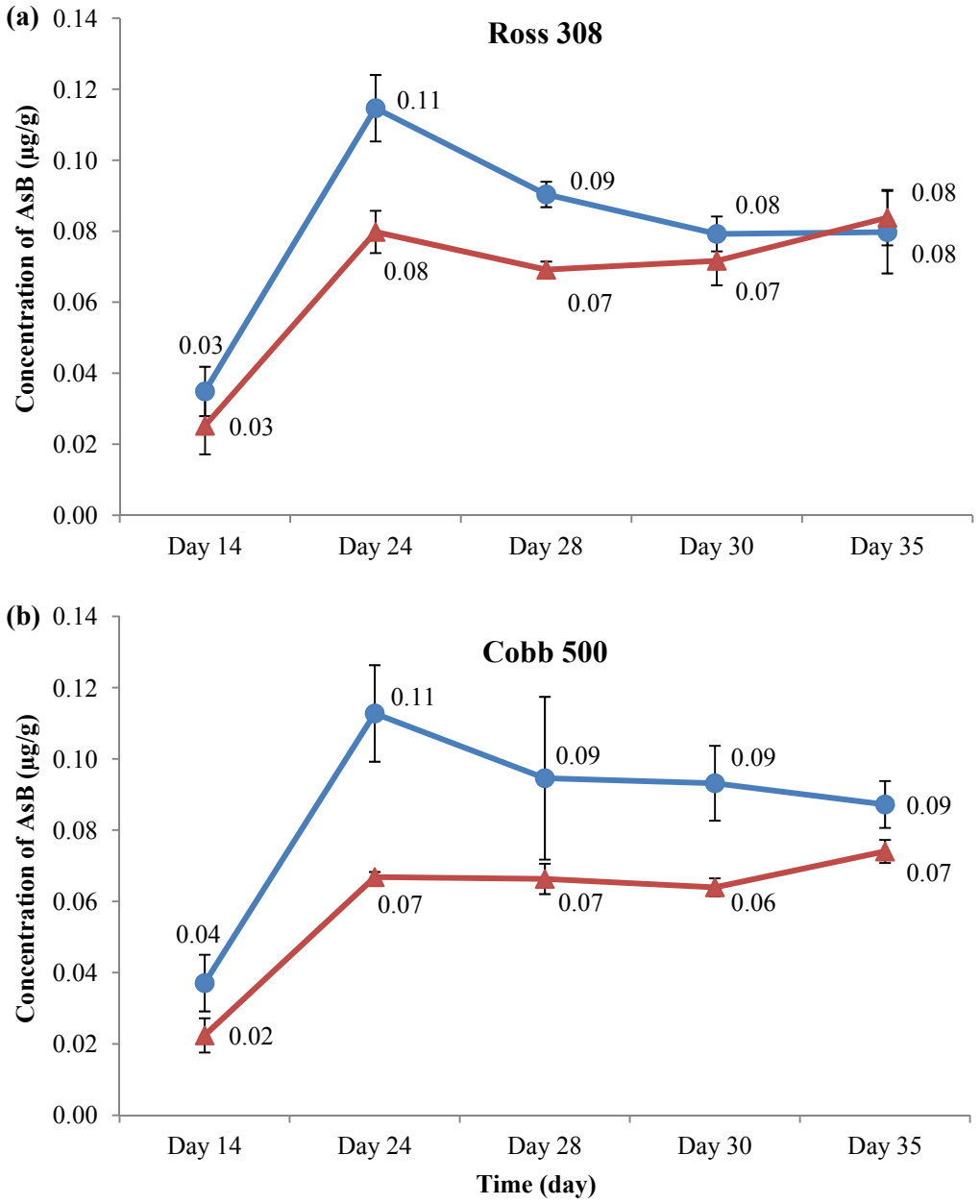

Figure 3.11 The concentrations of AsB in each litter sample of the ROX-fed (circles) and control (triangles) chickens from strain (a) Ross 308 and (b) Cobb 500.

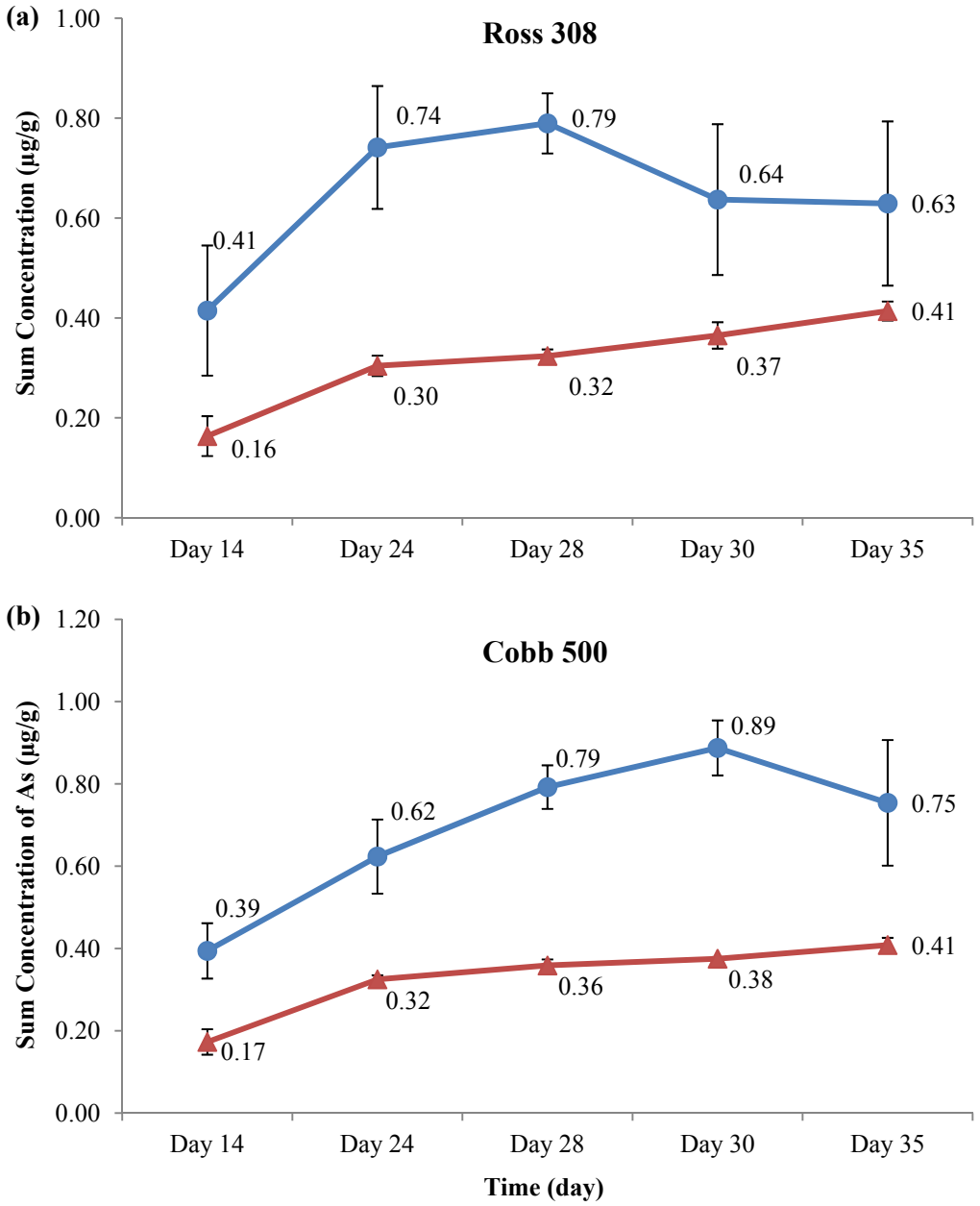

Figure 3.12 The sum of inorganic and methyl arsenicals (As^{III}, As^{V}, MMA^{V}, and DMA^{V}) in each litter sample from the ROX-fed (circles) and control (triangles) chickens from strain (a) Ross 308 and (b) Cobb 500.

By normalizing the concentrations of seven arsenic species against the concentrations of AsB in each litter sample, we were able to control for variables caused by wood chips and moisture in the litter. The results are summarized in Tables 3.6–3.9. Figures 3.13–3.18 illustrate that greater ratios of six arsenic species over AsB were obtained in the ROX-fed group than in the control group for both strains, suggesting that ROX can be converted to these arsenic species, especially N-AHAA and 3-AHPAA. Similar ratios were observed between two groups in Figure 3.19, indicating transformation to DMA^V rarely happened. P values for comparing ROX-treated group and control group in the concentrations of N-AHAA or 3-AHPAA against the concentrations of AsB are included in Table 3.10, showing significant differences of N-AHAA and 3-AHPAA between litter samples from these two groups.

We also normalized the sum of inorganic and methyl arsenicals against the concentration of AsB on each given day. Figure 3.20 shows higher ratios for the ROX-fed group than the control group over the period of feeding. However, similar variation trends of the ratios were obtained between the two groups.

Table 3.6 Concentrations of arsenic species normalized against the concentrations of AsB in each litter sample from ROX-fed chickens (strain Ross 308) [a]

	Normalized Ratio (mean ± SD)						
Day	As^{III}/AsB	DMA^{V}/AsB	MMA^{V}/AsB	As^{V}/AsB	3-AHPAA /AsB	N-AHAA /AsB	ROX /AsB
14	3 ± 1	6 ± 2	0.6 ± 0.1	3 ± 1	18 ± 8	6.3 ± 0.7	210 ± 30
24	1.4 ± 0.7	2.9 ± 0.5	0.21 ± 0.02	1.9 ± 0.7	15 ± 4	4 ± 1	62 ± 4
28	1.8 ± 0.7	4.7 ± 0.4	0.27 ± 0.05	2.0 ± 0.7	22 ± 4	7.6 ± 0.7	80 ± 10
30	1.4 ± 0.8	4.9 ± 0.4	0.3 ± 0.1	1.4 ± 0.8	13 ± 1	7.7 ± 0.7	92 ± 9
35	1.2 ± 0.6	5 ± 1	0.3 ± 0.1	1.3 ± 0.9	10 ± 1	7 ± 1	60 ± 10

[a] SD: standard deviation.

Table 3.7 Concentrations of arsenic species normalized against the concentrations of AsB in each litter sample from ROX-fed chickens (strain Cobb 500) [a]

	Normalized Ratio (mean ± SD)						
Day	As^{III}/AsB	DMA^{V}/AsB	MMA^{V}/AsB	As^{V}/AsB	3-AHPAA /AsB	N-AHAA /AsB	ROX /AsB
14	3 ± 3	5 ± 1	0.3 ± 0.1	2 ± 1	6.5 ± 0.2	5.6 ± 0.6	210 ± 50
24	1.5 ± 0.8	2.6 ± 0.6	0.12 ± 0.03	1.3 ± 0.5	11 ± 5	3.9 ± 0.4	50 ± 10
28	2.2 ± 0.5	5 ± 1	0.213 ± 0.008	1.7 ± 0.3	15 ± 3	8 ± 2	80 ± 30
30	2.5 ± 0.7	4.9 ± 0.7	0.30 ± 0.04	2.0 ± 0.4	11.6 ± 0.8	7 ± 2	43 ± 5
35	1.8 ± 0.6	5 ± 2	0.26 ± 0.04	1.4 ± 0.5	6.9 ± 0.9	7 ± 1	42 ± 3

[a] SD: standard deviation.

Table 3.8 Concentrations of arsenic species normalized against the concentrations of AsB in each litter sample from control chickens (strain Ross 308) [a]

	Normalized Ratio (mean ± SD)						
Day	As^{III}/AsB	DMA^{V}/AsB	MMA^{V}/AsB	As^{V}/AsB	3-AHPAA /AsB	N-AHAA /AsB	ROX /AsB
14	0.3 ± 0.2	4.7 ± 0.7	0.2 ± 0.1	1.5 ± 0.4	N/A	N/A	N/A
24	0.23 ± 0.04	3.0 ± 0.3	0.049 ± 0.004	0.59 ± 0.08	0.5 ± 0.2	0.2 ± 0.1	1.1 ± 0.6
28	0.20 ± 0.06	3.8 ± 0.3	0.052 ± 0.001	0.7 ± 0.1	0.43 ± 0.04	0.3 ± 0.1	1.6 ± 0.6
30	0.21 ± 0.02	4.1 ± 0.6	0.052 ± 0.006	0.8 ± 0.2	0.4 ± 0.1	0.4 ± 0.2	1.5 ± 0.8
35	0.20 ± 0.03	4.1 ± 0.5	0.059 ± 0.002	0.6 ± 0.2	0.37 ± 0.04	0.4 ± 0.2	1.6 ± 0.5

[a] N/A: not applicable. SD: standard deviation.

Table 3.9 Concentrations of arsenic species normalized against the concentrations of AsB in each litter sample from control chickens (strain Cobb 500) [a]

	Normalized Ratio (mean ± SD)						
Day	As^{III}/AsB	DMA^{V}/AsB	MMA^{V}/AsB	As^{V}/AsB	3-AHPAA /AsB	N-AHAA /AsB	ROX /AsB
14	0.3 ± 0.2	5.2 ± 0.2	0.15 ± 0.06	2.1 ± 0.2	N/A	N/A	N/A
24	0.2 ± 0.2	3.7 ± 0.2	0.08 ± 0.04	0.8 ± 0.2	0.4 ± 0.2	0.2 ± 0.1	1.2 ± 0.2
28	0.2 ± 0.1	4.3 ± 0.2	0.08 ± 0.03	0.8 ± 0.2	0.4 ± 0.2	0.2 ± 0.1	1.4 ± 0.2
30	0.2 ± 0.1	4.7 ± 0.4	0.08 ± 0.03	0.9 ± 0.2	0.4 ± 0.2	0.2 ± 0.2	1.7 ± 0.1
35	0.19 ± 0.08	4.6 ± 0.2	0.08 ± 0.02	0.7 ± 0.2	0.3 ± 0.1	0.22 ± 0.07	1.24 ± 0.03

[a] N/A: not applicable. SD: standard deviation.

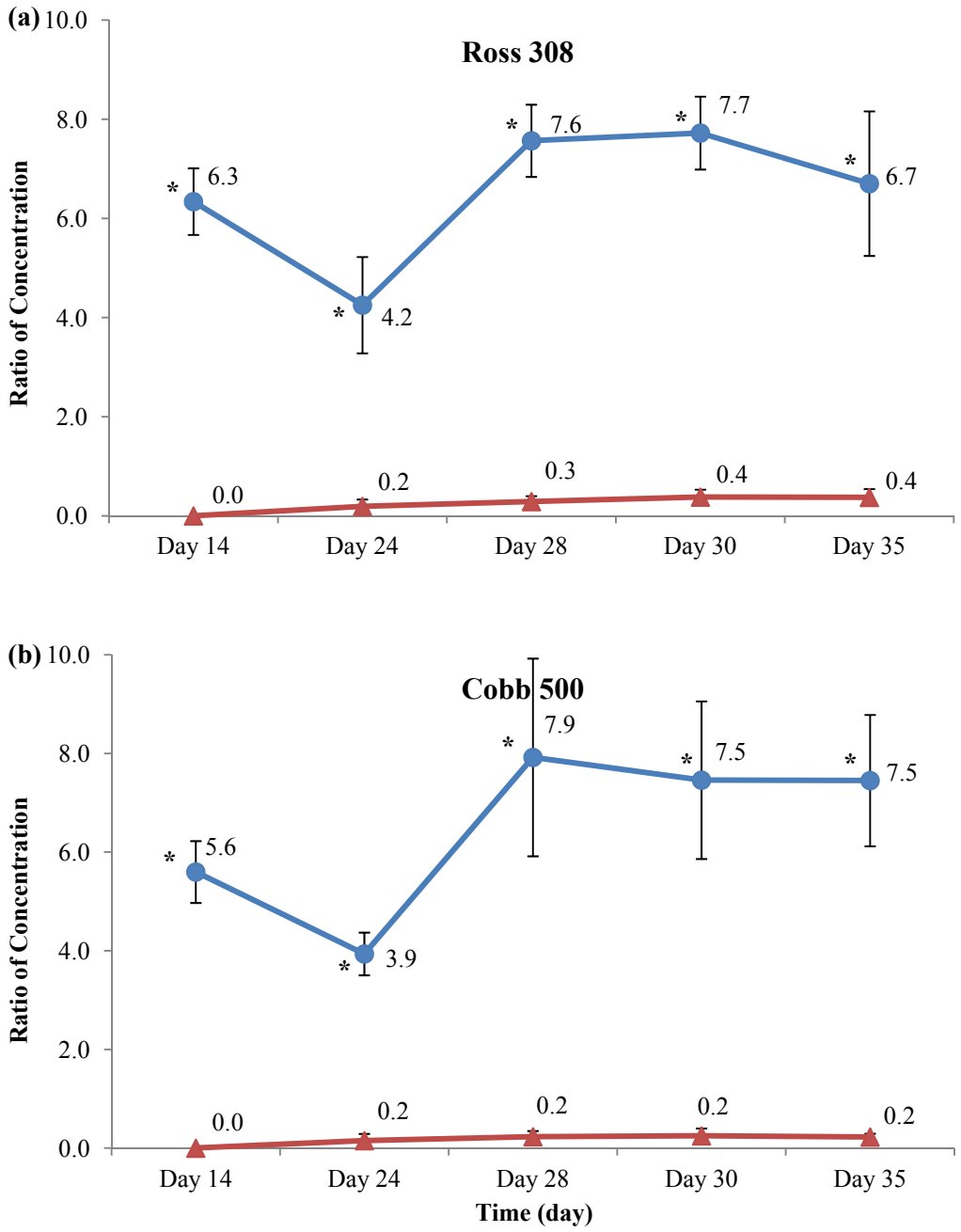

Figure 3.13 Concentrations of N-AHAA normalized against the concentrations of AsB in each litter sample from the ROX-fed (circles) and control (triangles) chickens from strain (a) Ross 308 and (b) Cobb 500. * $P < 0.05$ when comparing the ratios between ROX-fed group and control group on that given day.

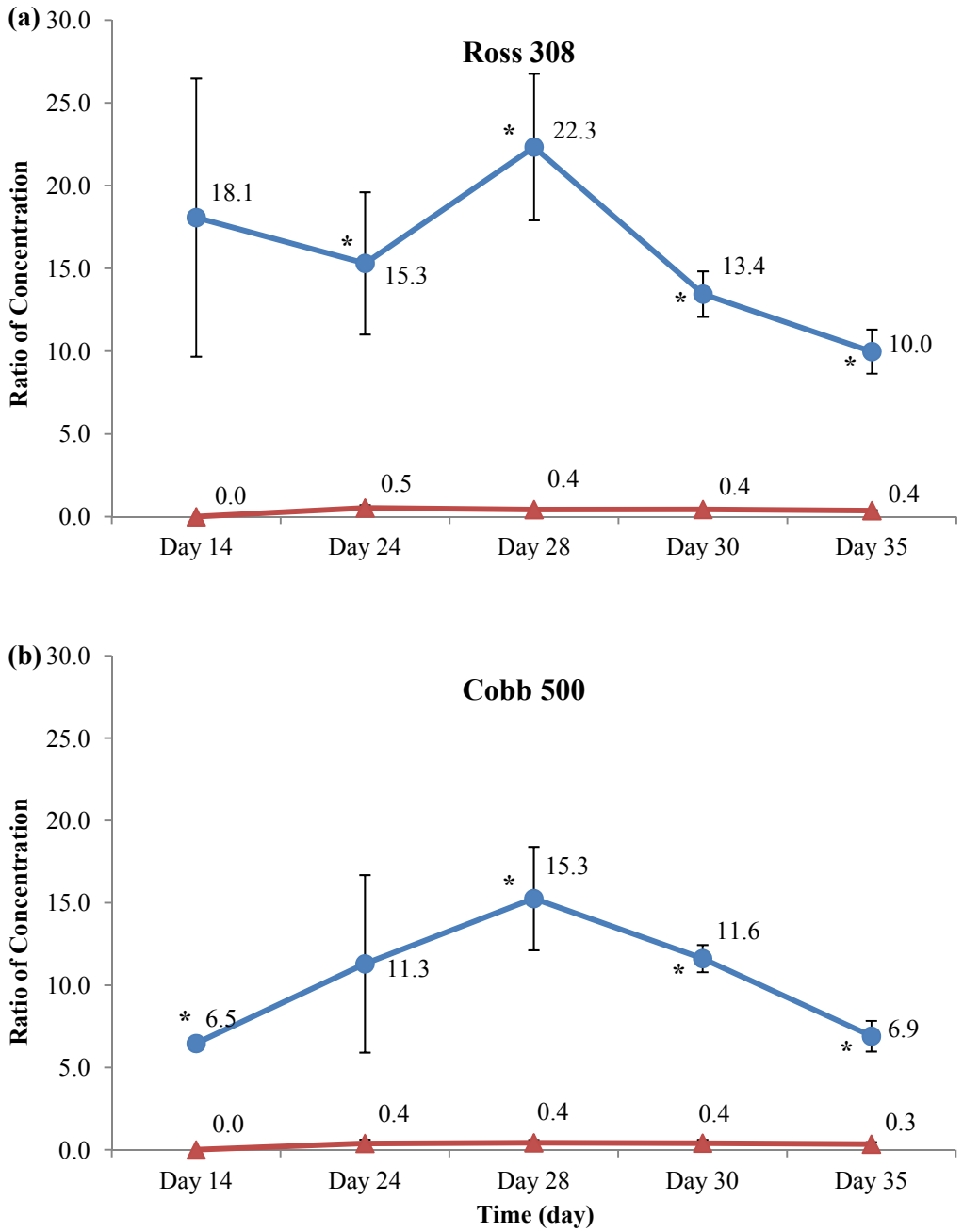

Figure 3.14 Concentrations of 3-AHPAA normalized against the concentrations of AsB in each litter sample from the ROX-fed (circles) and control (triangles) chickens from strain (a) Ross 308 and (b) Cobb 500. * $P < 0.05$ when comparing the ratios between ROX-fed group and control group on that given day.

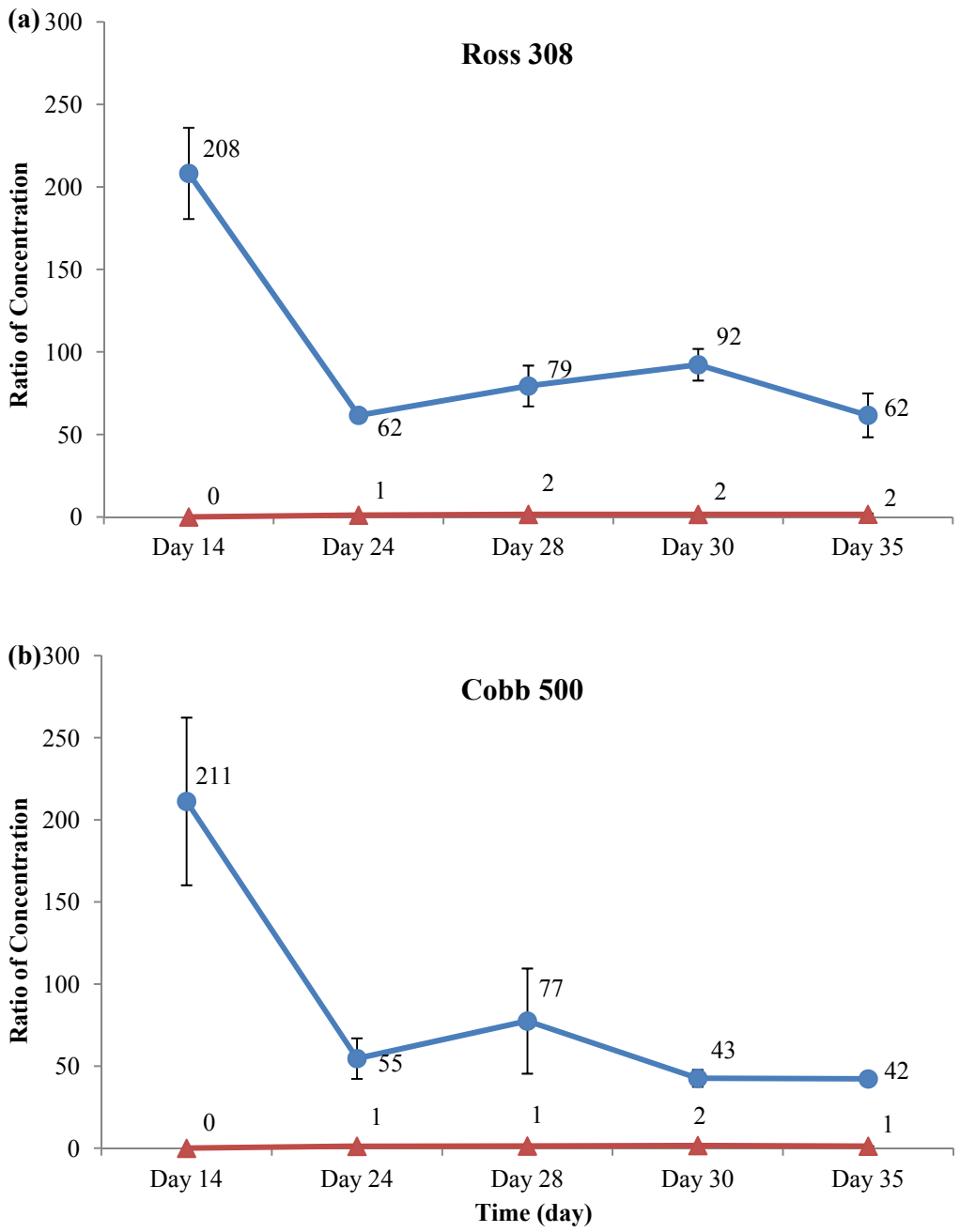

Figure 3.15 Concentrations of ROX normalized against the concentrations of AsB in each litter sample from the ROX-fed (circles) and control (triangles) chickens from strain (a) Ross 308 and (b) Cobb 500.

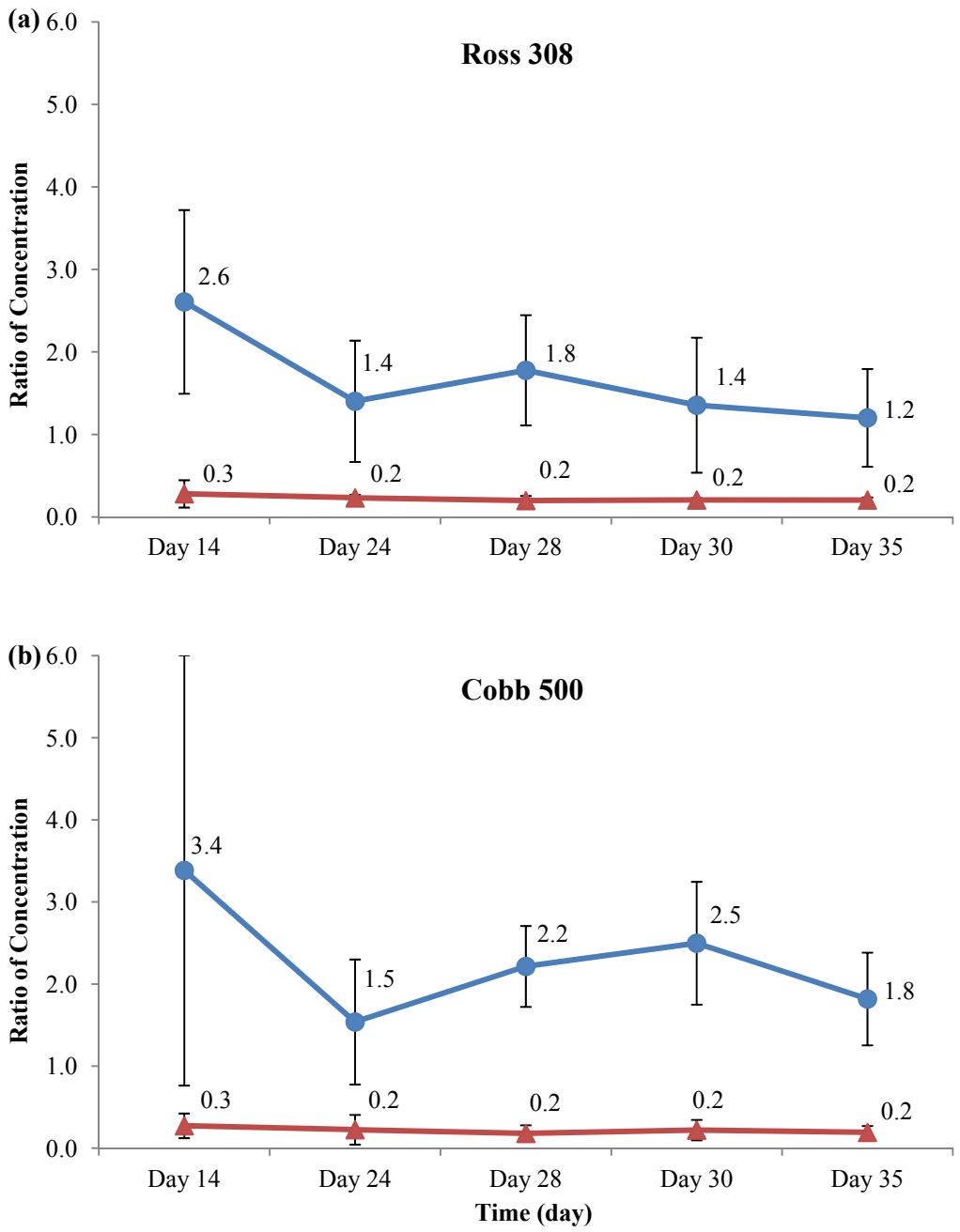

Figure 3.16 Concentrations of As^{III} normalized against the concentrations of AsB in each litter sample from the ROX-fed (circles) and control (triangles) chickens from strain (a) Ross 308 and (b) Cobb 500.

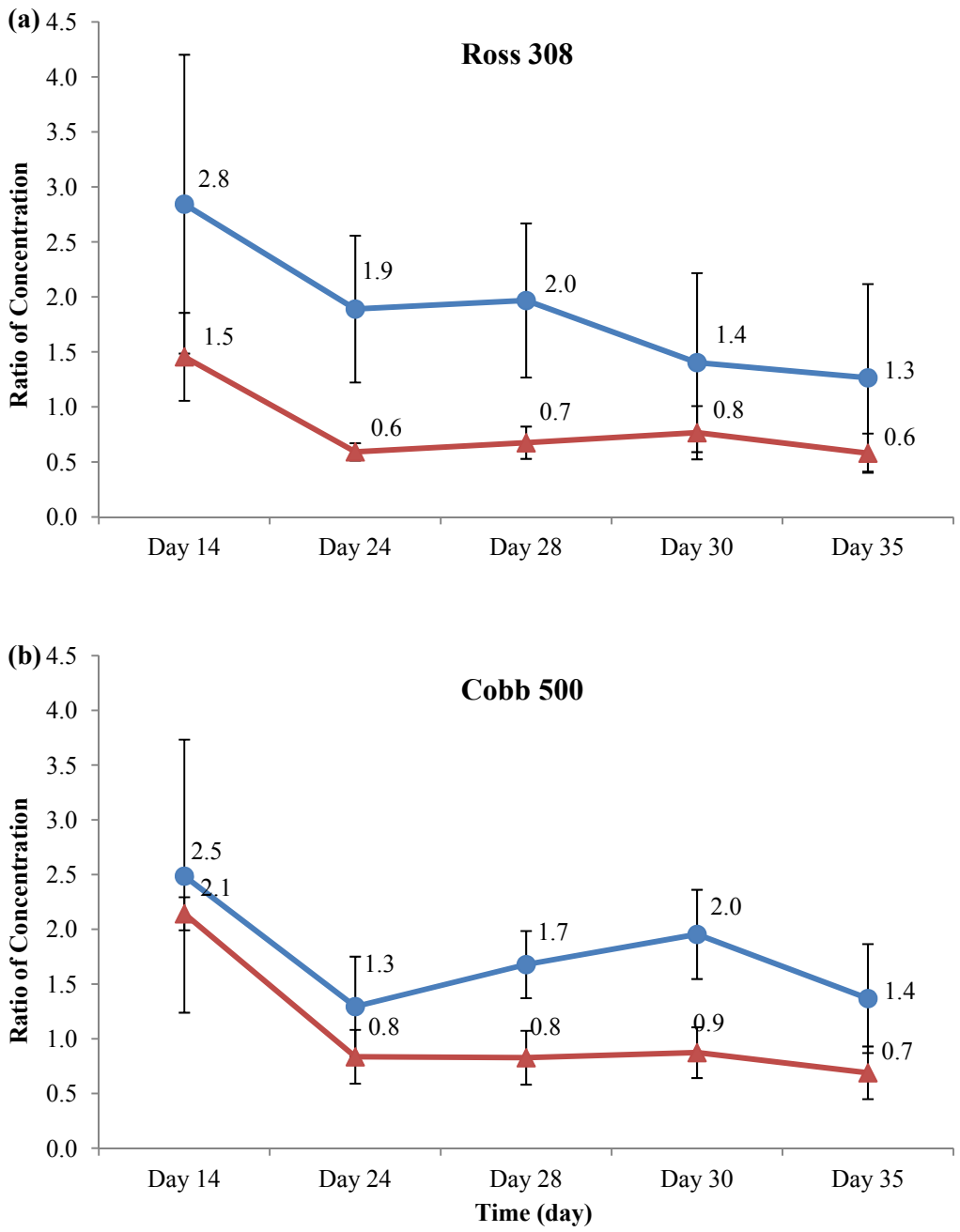

Figure 3.17 Concentrations of As^V normalized against the concentrations of AsB in each litter sample from the ROX-fed (circles) and control (triangles) chickens from strain (a) Ross 308 and (b) Cobb 500.

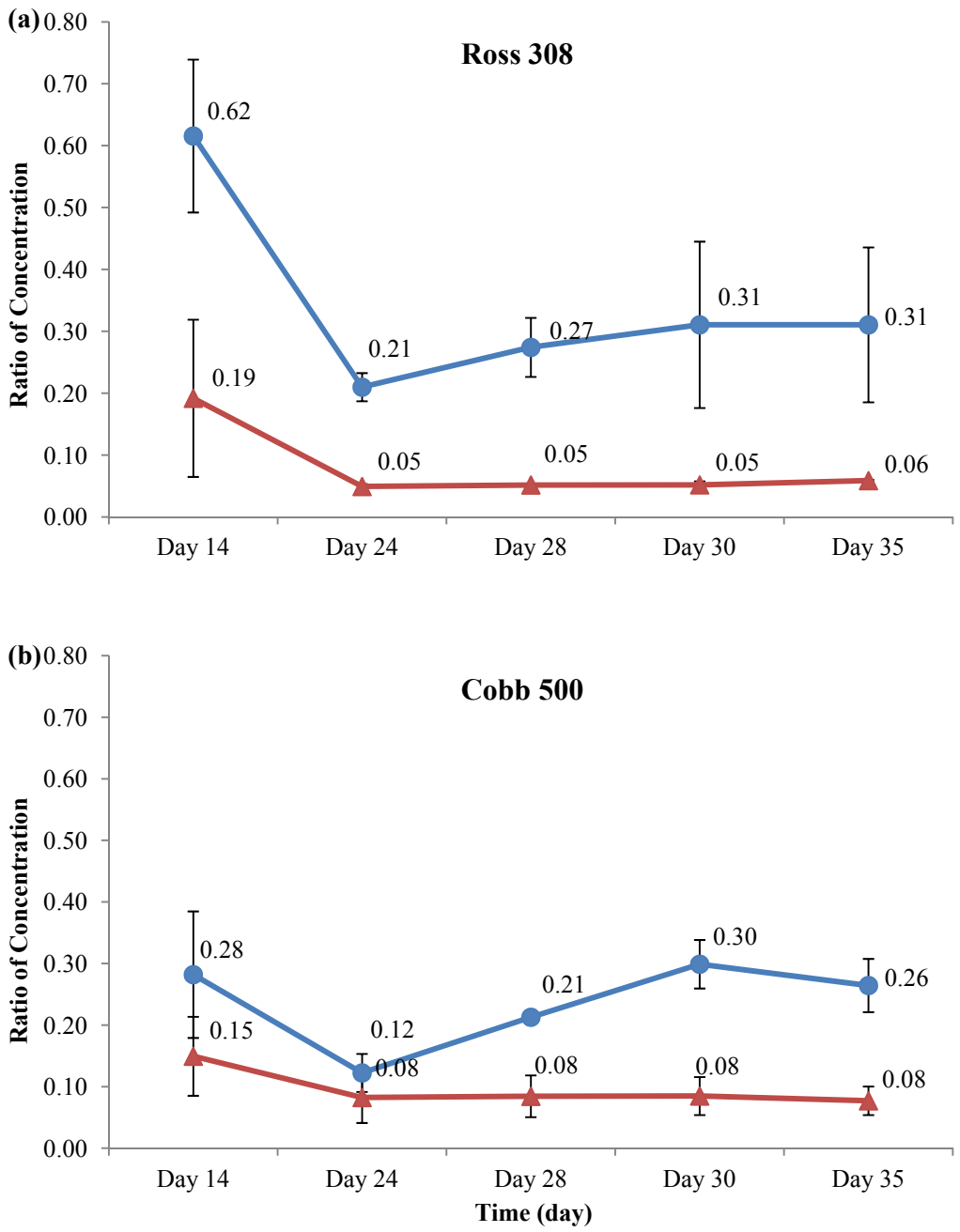

Figure 3.18 Concentrations of MMA^V normalized against the concentrations of AsB in each litter sample from the ROX-fed (circles) and control (triangles) chickens from strain (a) Ross 308 and (b) Cobb 500.

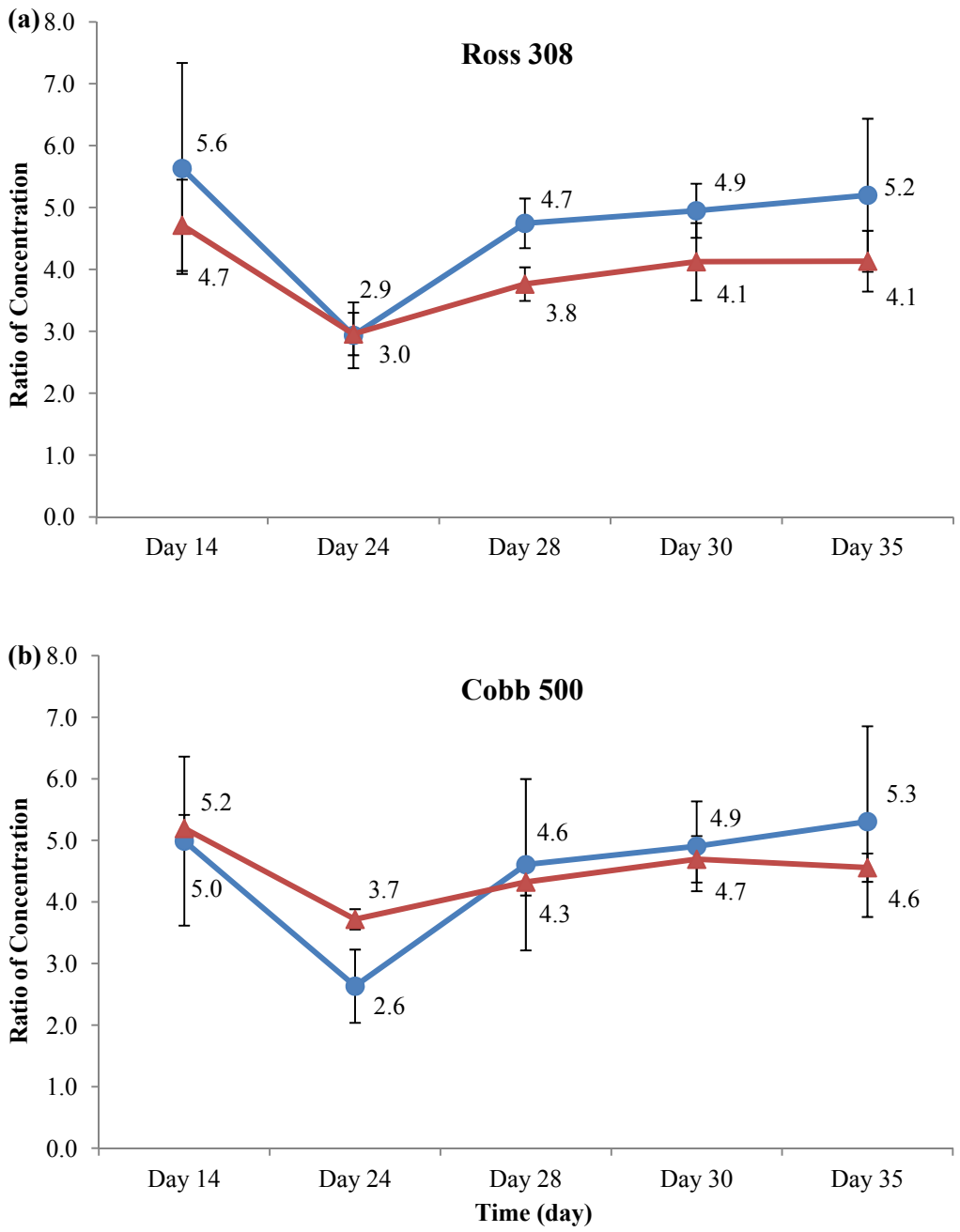

Figure 3.19 Concentrations of DMA^V normalized against the concentrations of AsB in each litter sample from the ROX-fed (circles) and control (triangles) chickens from strain (a) Ross 308 and (b) Cobb 500.

Table 3.10 P values for comparing ROX-treated group and control group in the concentrations of N-AHAA or 3-AHPAA against the concentrations of AsB.

Ratio	Strain	Day 14	Day 24	Day 28	Day 30	Day 35
N-AHAA/AsB	Ross 308	0.000	0.002	0.000	0.000	0.016
	Cobb 500	0.000	0.000	0.003	0.001	0.011
3-AHPAA/AsB	Ross 308	0.065	0.027	0.013	0.004	0.000
	Cobb 500	0.000	0.072	0.001	0.000	0.006

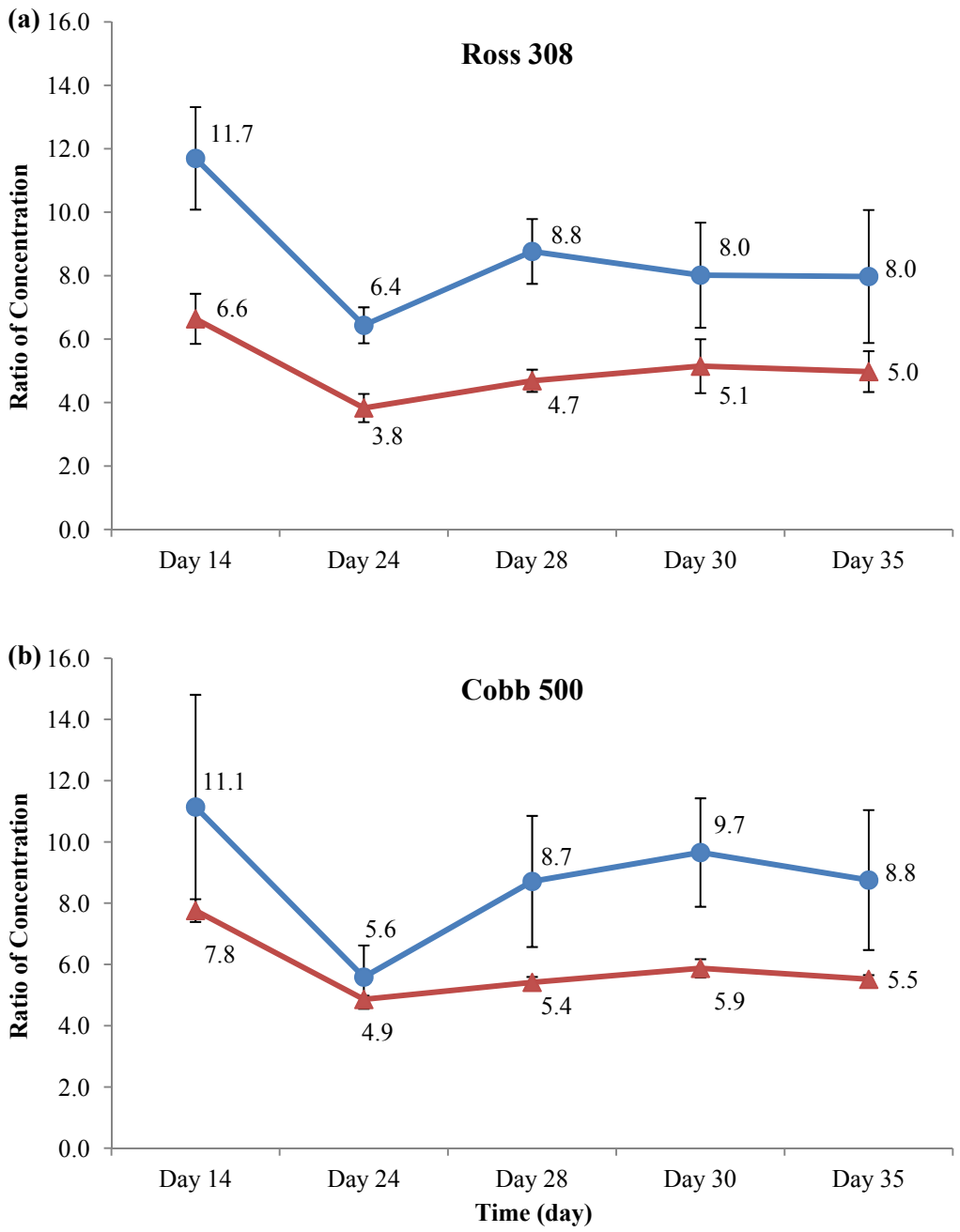

Figure 3.20 The ratio of the sum concentration of inorganic and methyl arsenicals (As^{III}, As^{V}, MMA^{V}, and DMA^{V}) against the concentration of AsB in each litter sample from the ROX-fed (circles) and control (triangles) chickens from strain (a) Ross 308 and (b) Cobb 500.

3.3.4 Arsenic Species in Feed

Chemical integrity of ROX in the feed is a prerequisite for evaluating biotransformation of ROX in poultry litter. Typical chromatograms of feed samples are shown in Figure 3.21 and 3.22, and the quantitative results are summarized in Table 3.10. ROX-supplemented diets (ROX starter and ROX grower) had total arsenic exceeding 18 μg As/g, mainly in the form of ROX itself. No 3-AHPAA or N-AHAA was observed in the feed. The analysis of six diets also showed the presence of small amounts of AsB, DMA^V and As^V.

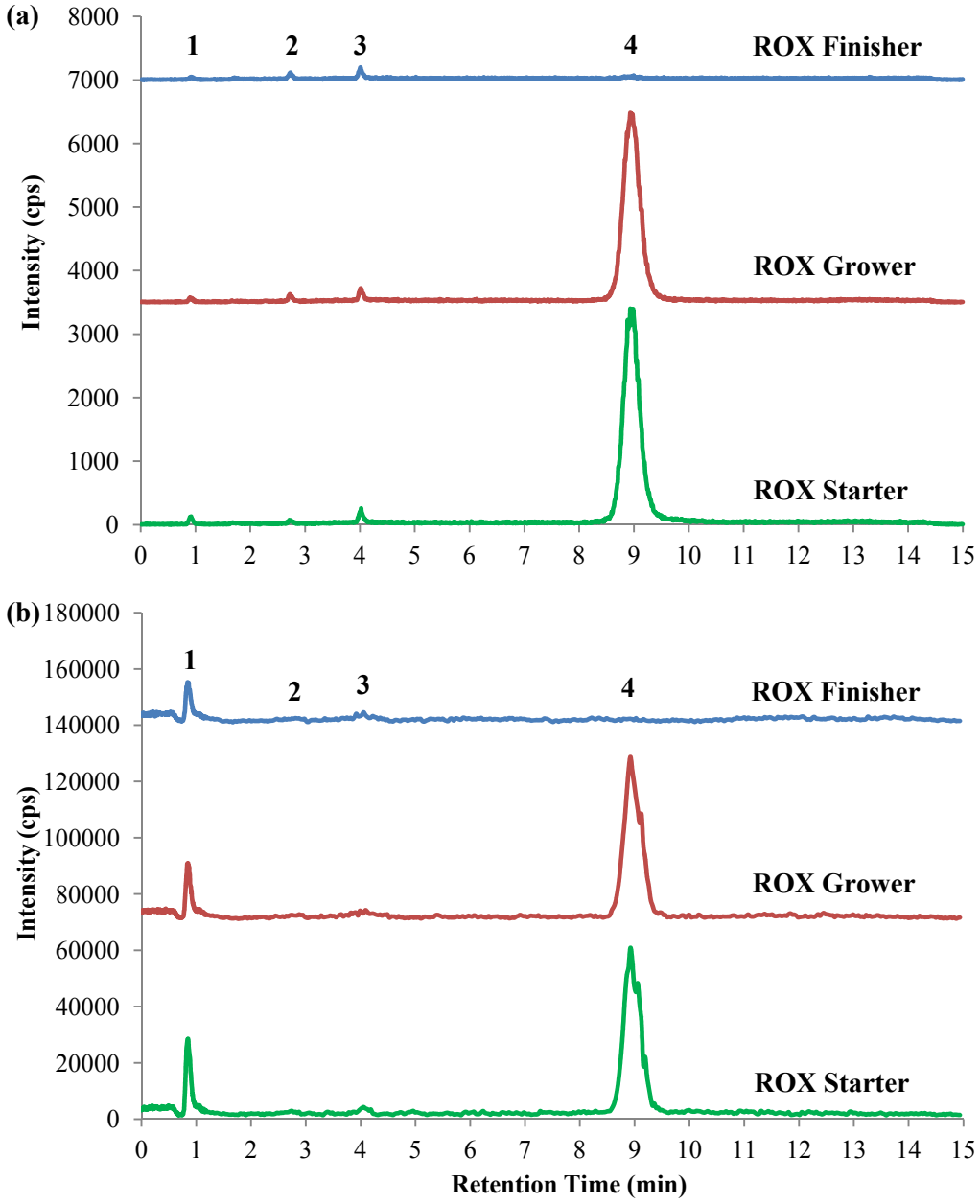

Figure 3.21 Chromatograms obtained from the analyses of ROX-treated feed samples using (a) HPLC-ICPMS and (b) HPLC-ESIMS. Peaks: (1) AsB, (2) DMA^{V}, (3) As^{V}, (4) ROX.

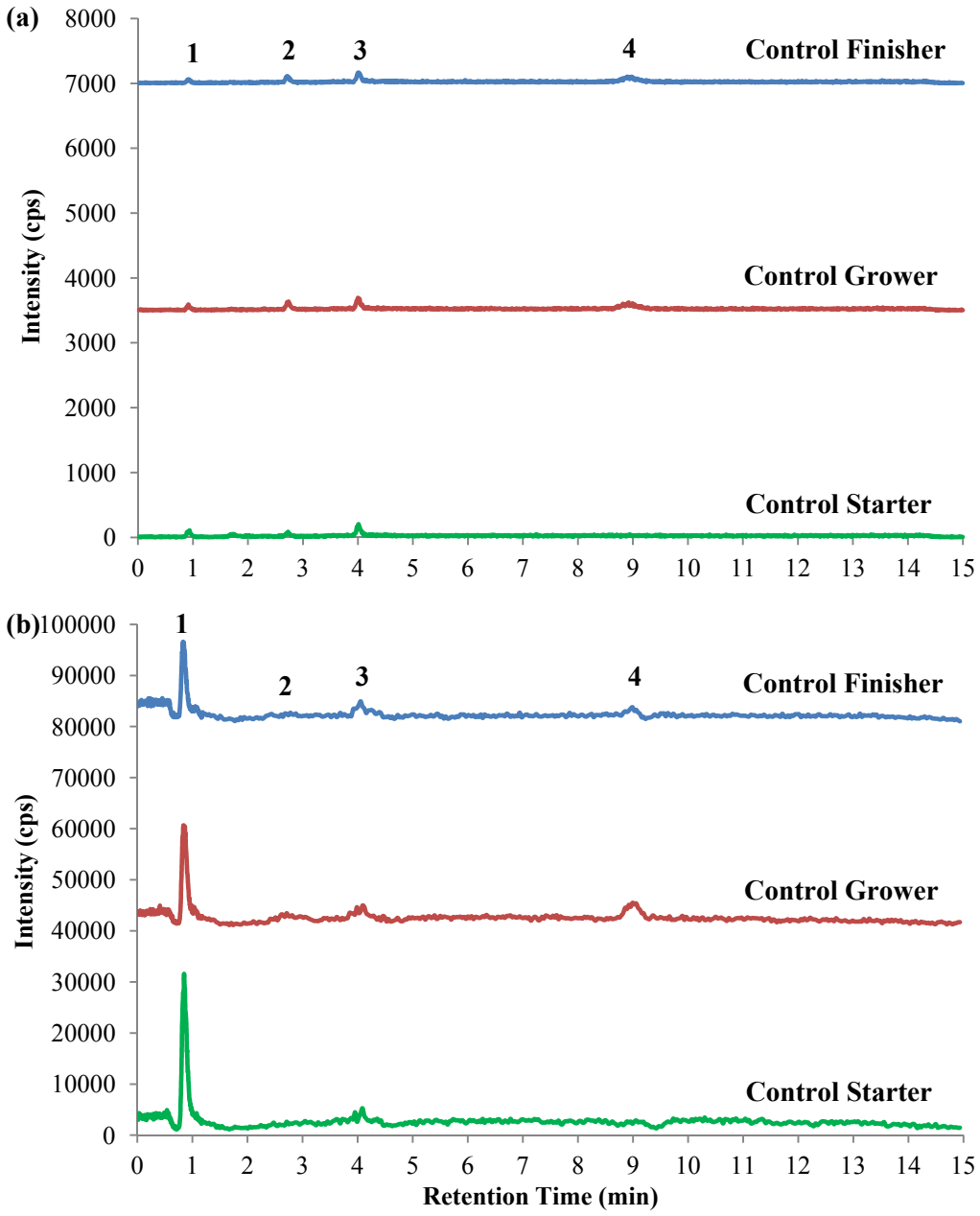

Figure 3.22 Chromatograms obtained from the analyses of untreated feed samples using (a) HPLC-ICPMS and (b) HPLC-ESIMS. Peaks: (1) AsB, (2) DMA^{V}, (3) As^{V}, (4) ROX.

Table 3.11 Concentrations of arsenic species detected in ROX-treated and untreated feed samples.[a]

Concentration of Arsenic Species (mean ± SD) (µg/g)						
Feed Type	**AsB**	**As^{III}**	**DMA^{V}**	**MMA^{V}**	**As^{V}**	**ROX**
ROX Starter	0.054 ± 0.004	N.D.	0.031 ± 0.006	N.D.	0.059 ± 0.009	18.3 ± 0.9
ROX Grower	0.034 ± 0.005	N.D.	0.036 ± 0.003	N.D.	0.072 ± 0.002	18 ± 1
ROX Finisher	0.026 ± 0.002	N.D.	0.034 ± 0.009	N.D.	0.044 ± 0.008	0.11 ± 0.02
Control Starter	0.097 ± 0.003	N.D.	0.033 ± 0.004	N.D.	0.12 ± 0.01	N.D.
Control Grower	0.035 ± 0.003	N.D.	0.042 ± 0.006	N.D.	0.05 ± 0.01	0.31 ± 0.03
Control Finisher	0.030 ± 0.002	N.D.	0.035 ± 0.004	N.D.	0.07 ± 0.01	0.17 ± 0.03

[a] N.D.: below detection limit. SD: standard deviation.

3.4 DISCUSSION

3.4.1 Extraction of Arsenic

Most arsenic was extracted during the first two extractions. The major arsenic species in the litter extracts was ROX, accounting for over 73% of all species. Small amounts of 3-AHPAA, N-AHAA, and other arsenic-containing compounds were also present. After three extractions, there was a sharp decrease in the concentration of arsenic in the fourth and fifth extracts, which agrees with the results of total arsenic analysis given in Figure 2.4 (a). Therefore, all samples were extracted three times prior to arsenic speciation analysis. It should be pointed out that the presence of ROX metabolites was not an artifact from the extraction and storage procedures. After incubating a control litter sample with the ROX standard for either 1 h (Figure 3.2) or 2 months (Figure 3.3), the concentrations of all arsenic species other than ROX remained the same.

3.4.2 Arsenic in Poultry Litter

The HPLC-ICPMS/ESIMS method developed was able to separate eight arsenic species of interest (AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and ROX) within 10 min, and also confirm their presence in the poultry litter. The concentration of each arsenical can be quantified against the calibration curve of the corresponding arsenic standard solution. The relative intensity of each arsenic species was a little different between the two detections (ICPMS and ESIMS), which reflects the nature of the two different ionization methods.

As illustrated in Figure 3.5, eight arsenic species of interest with variable concentrations were present in the litter samples collected on five different days

from ROX-fed chickens. Figure 3.7 indicates ROX predominated in the litter samples of the ROX-treated group, which is similar to what was reported in the literature [1-4]. Most ROX was excreted unchanged by Ross 308 chickens and remained its chemical form in the litter. ROX contributed to between 66% and 85% of the sum arsenic.

Two major ROX metabolites, 3-AHPAA and N-AHAA, were identified. They represented 8–20% (3-AHPAA) and 3-8% (N-AHAA) of the total arsenic. Because they were neither added in diets nor observed in the control litter (day 14), conversion from ROX as the parent molecule is the most reasonable explanation of their presence in the litter of chickens fed ROX considering their structural similarity. Inorganic arsenicals (the sum of As^{III} and As^{V}) only consisted of 2–4% (mean 2.8%) of total arsenic. Background levels of MMA^{V}, DMA^{V}, and AsB were also detected in the chicken litter samples. The presence of AsB was possibly a result of ingestion of the basal diet which contained fish meal. AsB has been known to be rapidly excreted from animal bodies unchanged [18-20]. The sum of arsenic reached the maximum on day 28 when ROX was withdrawn from the chicken diet [Figure 3.6 (a)]. A same amount of litter was collected at a certain area on each day. Thereby the accumulation of litter on the fixed area of woodchips during ROX treatment increased the percentage of actual litter in the samples obtained, which leads to the modest rise before day 28. During the final week, the sum of arsenic in litter decreased as expected because no ROX was fed to the chickens. Arsenic in the litter of chickens fed ROX from strain Cobb 500 followed a similar pattern [Figure 3.6 (b) and 3.7 (b)].

Different from control litter samples collected on day 24, 28, 30, and 35, there was no ROX, 3-AHPAA, or N-AHAA being detected in the sample obtained on day 14. Two feed samples (control grower and control finisher), to which no ROX was intentionally added, might have been contaminated with traces of ROX (Table 3.11), and this could be the reason why trace amounts of the three phenylarsonic compounds were detected in the control litter collected after day 24 (Figure 3.10). Instead of ROX, DMA^V predominated in the control litter for both strains (Ross 308 and Cobb 500), which contributed to over 45% of all species present. The sum of arsenic was below 0.7 μg/g on each given day (Figure 3.9), approximately 10-fold lower than those in the litter of ROX-fed chickens (5.8–10.7 μg/g). The slight increase in the sum of arsenic over the feeding period is likely because of continuous intake of background arsenic in the feed.

3.4.3 Arsenic in Poultry Feed

There was ROX with concentrations greater than 18 μg As/g in the ROX-supplemented diets (ROX starter and ROX grower), which are major dietary sources of ROX and other arsenic metabolites in the litter of ROX-fed chickens. Consistently small amounts of AsB were detected in all diets, which explained the presence of AsB in all litter samples. DMA^V and As^V were also present in diets. Their concentrations were about the same level as AsB. The technique was able to separate and determine As^{III}, MMA^V, 3-AHPAA, and N-AHAA; but they were not detectable in any of the six feed samples. Biotransformation from the existing arsenic species in diets was the only contributor to their presence in poultry litter. 3-AHPAA and N-AHAA appeared to be the major conversion products of ROX

because high concentrations of ROX were always accompanied by large amounts of 3-AHPAA and N-AHAA in the litter of ROX-fed chickens (Figure 3.7). While in the control litter samples, only trace amounts of ROX, 3-AHPAA, and N-AHAA were detected (Figure 3.10). The existence of ROX in three feed samples (ROX finisher, control grower and control finisher) was probably a result of feed cross-contamination.

3.5 CONCLUSIONS

The HPLC-ICPMS/ESIMS method developed in this study provided comprehensive identification and quantitation of arsenic species in poultry litter and feed. N-AHAA, a previously unreported compound in chicken litter, was observed. The dominant arsenic species in the litter of ROX-treated chickens was unchanged ROX (> 60%). ROX can be converted to several arsenic species, among which 3-AHPAA and N-AHAA were the major metabolites. No transformation of ROX occurred during sample extraction and storage. Mechanistic investigations of the formation of N-AHAA are under way. Since reductive conversion of the nitro group of ROX to an amino group in forming 3-AHPAA is possible [21, 22], a portion of the 3-AHPAA could be further acetylated to produce N-AHAA [23].

3.6 REFERENCES

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

4.1 REVIEW OF THESIS OBJECTIVES

Roxarsone® (ROX), 3-nitro-4-hydroxy-phenylarsonic acid, has been added to poultry feed to promote weight gain, improve feed efficiency, and control coccidial intestinal parasites. Much of the ingested ROX ends up in chicken litter. 90% of chicken litter is applied as a fertilizer to agricultural land. Assessing environmental impact requires identification and quantitation of arsenic species in chicken litter samples. The objective of this thesis was to study arsenic speciation in litter of chickens fed either ROX-supplemented food or the control feed. In Chapter 2, I have described an arsenic speciation method using high performance liquid chromatography (HPLC), inductively coupled plasma mass spectrometry (ICPMS), and electrospray ionization mass spectrometry (ESIMS). I then applied the method to the determination of arsenic species in chicken litter from a 35-day feeding study (Chapter 3).

4.2 SUMMARY OF RESULTS

The hyphenated HPLC-ICPMS/ESIMS method enabled the simultaneous identification (from ESIMS) and quantitation (from ICPMS) of arsenic species after a single HPLC separation. Successful separation of arsenobetaine (AsB), arsenite (As^{III}), dimethylarsinic acid (DMA^{V}), monomethylarsonic acid (MMA^{V}), arsenate (As^{V}), 3-amino-4-hydroxy-phenylarsonic acid (3-AHPAA), N-acetyl-4-hydroxy-m-arsanilic acid (N-AHAA), and 3-nitro-4-hydroxy-phenylarsonic acid

(ROX) was achieved within 10 min using anion exchange chromatography. A methanol-water extraction method was also optimized for the extraction of arsenic species from poultry litter samples. No conversion of ROX occurred during sample extraction and storage.

N-AHAA, a previously unreported species in the litter of ROX-fed chickens, was identified. Following a 35-day feeding regimen, speciation analyses of litter samples collected on days 14, 24, 28, 30, and 35 showed that the major arsenic species in the ROX-treated groups was ROX, accounting for 60-90% of the total arsenic. Two significant biotransformation products, 3-AHPAA and N-AHAA, were identified. They represented 3-19% (3-AHPAA) and 3-12% (N-AHAA) of the total arsenic. Inorganic arsenicals (the sum of As^{III} and As^{V}) consisted of 2-6% (mean 3.5%) of total arsenic. In the control litter samples, DMA^{V} predominated, accounting for greater than 45% of total arsenic. The concentration of total arsenic in the control was 10-fold lower than that in the litter of treated chickens. Also, the concentrations of total inorganic and methyl arsenicals (As^{III}, As^{V}, MMA^{V}, and DMA^{V}) were consistently higher in the litter of the ROX-fed chickens than those in the control chickens. These results suggest conversion of ROX to several arsenic species. AsB was detected in the feed that contained fish supplement, which was responsible for the observed background level of AsB in the chicken litter.

This thesis research demonstrates the development of a chromatography-mass spectrometry method and its application to study of environmental, agricultural, and health relevance. The findings of the new metabolite and the

concentrations of the various arsenic species will contribute to better understanding of the biotransformation and the fate of arsenic species.

4.3 FUTURE RESEARCH

Extracting all arsenic species efficiently from solid samples is challenging. While the optimized methanol-water extraction method provided a good extraction efficiency of 72%, other techniques, such as microwave assisted extraction, could be investigated to further improve the extraction of arsenic species from litter samples.

Analysis of 10-fold diluted poultry litter sample by HPLC-ICPMS showed the presence of a small amount of As^{III}. But it was not detectable using HPLC-ESIMS in the MRM mode, because As^{III} had poor ionization efficiency. Post-HPLC column derivatization of As^{III} with dimercaptosuccinic acid (DMSA) could enhance ionization (of this complex), thereby improving detection of As^{III} by ESIMS [1].

In the litter of ROX-fed chickens, 3-AHPAA and N-AHAA displayed higher concentrations than other arsenic metabolites. The formation of 3-AHPAA from ROX has been indicated as a result of a reduction of the nitro group in ROX to an amino group [2, 3]. Further research is needed to understand the formation of N-AHAA.

Investigating ROX metabolites in poultry litter plays an important role in assessing potential impact of applying poultry litter to agricultural land. This thesis research has shown that a small amount of ROX was converted to several

arsenic species. It is not known where the conversion takes place. In order to find out whether the transformation of ROX occurs within the chicken's digestive tract or during the litter composting, sample collections both before and after the litter composting are necessary. Analyses of freshly collected litter samples will be useful to clarify the question of ROX transformation.

Arsenic speciation analyses of feed and litter provide an overall view of the distribution of each arsenic species in these samples. Any correlation between the ingestion of arsenicals from the feed and the excretion of arsenic species in the litter may need further investigations.

4.4 REFERENCES

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APPENDIX A

SUPPORTING INFORMATION

Table A.1 Compositions of chicken feed.

Feed Group	**Control**			**ROX-treated**		
Feed Name	**Starter (0-14 d)**	**Grower (15-28 d)**	**Finisher (29-35 d)**	**Starter (0-14 d)**	**Grower (15-28 d)**	**Finisher (29-35 d)**
Ingredient Name	**Percent**	**Percent**	**Percent**	**Percent**	**Percent**	**Percent**
Corn, Yellow, Grain	18.009	18.009	15.008	18.005	18.005	15.004
Fat, Vegetable	3.775	3.365	4.131	3.774	3.364	4.130
Fish Meal Menhaden	3.002	5.003	3.509	3.001	5.001	3.508
Soybean Meal Deh - Plant 1	26.880	16.221	15.105	26.873	16.217	15.102
Wheat, Hard, Grain	42.952	53.263	58.074	42.941	53.250	58.059
Calcium Carbonate	1.501	1.048	1.066	1.500	1.048	1.066
Dicalcium Phosphate	1.546	1.005	1.081	1.546	1.005	1.081
Salt, Plain (NaCl)	0.426	0.337	0.358	0.426	0.337	0.358

Ingredient Name	Percent	Percent	Percent	Percent	Percent	Percent
L-Lysine	0.232	0.151	0.154	0.232	0.151	0.154
DL-Methionine	0.229	0.096	0.089	0.229	0.096	0.089
L-Threonine	0.048	0.101	0.025	0.048	0.101	0.025
Broiler Vitamin Premix (0.5% inclusion)	0.50	0.50	0.50	0.50	0.50	0.50
Choline Chloride Premix (0.5% inclusion)	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin E 5000 IU/kg	0.30	0.30	0.30	0.30	0.30	0.30
Generic Enzyme (0.05% inclusion)	0.05	0.05	0.05	0.05	0.05	0.05
Coccidiostat (Amprol)	0.05	0.05	0.05	0.05	0.05	0.05
Antibiotic Growth Promoter	0	0	0	0.025	0.025	0.025
Total	100	100	100	100	100	100

Table A.2 Detailed concentrations of arsenic species in each litter sample.

Litter Sample Group:		ROX-fed Chickens (Strain Ross 308)															
		Arsenic Species															
Day		AsB		As^{III}		DMA^{V}		MMA^{V}		As^{V}		3-AHPAA		N-AHAA		ROX	
	Pen #	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Day 14	11	0.035	0.003	0.051	0.007	0.26	0.02	0.026	0.003	0.10	0.04	0.98	0.08	0.25	0.02	6.4	0.5
	13	0.028	0.003	0.102	0.005	0.115	0.006	0.014	0.006	0.04	0.02	0.4	0.1	0.16	0.03	7	2
	15	0.042	0.002	0.111	0.008	0.22	0.01	0.025	0.002	0.18	0.03	0.54	0.04	0.26	0.02	8.5	0.5
	mean	0.035		0.09		0.20		0.022		0.11		0.6		0.22		7	
	SD	0.007		0.03		0.08		0.007		0.07		0.3		0.06		1	
Day 24	11	0.114	0.005	0.080	0.008	0.380	0.004	0.025	0.001	0.27	0.02	1.367	0.006	0.51	0.03	7.40	0.01
	13	0.106	0.008	0.229	0.009	0.25	0.01	0.019	0.006	0.12	0.05	2.1	0.2	0.34	0.01	6.1	0.4
	15	0.124	0.004	0.17	0.01	0.390	0.004	0.028	0.003	0.27	0.02	1.71	0.01	0.63	0.03	7.8	0.2
	mean	0.115		0.16		0.34		0.024		0.22		1.7		0.5		7.1	
	SD	0.009		0.07		0.08		0.004		0.09		0.4		0.1		0.9	
Day 28	11	0.09	0.01	0.09	0.02	0.46	0.03	0.028	0.005	0.20	0.02	2.47	0.07	0.71	0.07	6.7	0.3
	13	0.094	0.003	0.202	0.006	0.40	0.02	0.021	0.008	0.11	0.01	2.0	0.1	0.6	0.1	6.6	1.0
	15	0.087	0.004	0.19	0.01	0.42	0.03	0.025	0.003	0.22	0.05	1.61	0.06	0.71	0.06	8.1	0.2
	mean	0.090		0.16		0.43		0.025		0.18		2.0		0.68		7.1	
	SD	0.004		0.06		0.03		0.004		0.06		0.4		0.04		0.8	
Day 30	11	0.07	0.01	0.04	0.02	0.39	0.02	0.033	0.001	0.08	0.02	1.06	0.08	0.56	0.03	6.5	0.2
	13	0.082	0.006	0.12	0.01	0.37	0.02	0.015	0.003	0.069	0.007	1.0	0.2	0.58	0.06	9	1
	15	0.082	0.001	0.18	0.01	0.42	0.01	0.024	0.002	0.191	0.008	1.15	0.07	0.69	0.03	7.0	0.2
	mean	0.079		0.11		0.39		0.024		0.11		1.06		0.61		7	
	SD	0.005		0.07		0.03		0.009		0.07		0.09		0.07		1	
Day 35	11	0.067	0.009	0.04	0.02	0.41	0.02	0.028	0.002	0.08	0.02	0.76	0.05	0.51	0.03	4.6	0.5
	13	0.090	0.005	0.122	0.008	0.34	0.02	0.016	0.005	0.04	0.02	0.8	0.1	0.45	0.04	4.2	0.5
	15	0.082	0.003	0.14	0.02	0.47	0.03	0.028	0.004	0.18	0.03	0.81	0.06	0.61	0.02	5.8	0.3
	mean	0.08		0.10		0.41		0.024		0.10		0.79		0.53		4.8	
	SD	0.01		0.06		0.07		0.007		0.07		0.03		0.08		0.8	

Litter Sample Group:		ROX-fed Chickens (Strain Cobb 500)															
		Arsenic Species															
Day		AsB		As^{III}		DMA^{V}		MMA^{V}		As^{V}		3-AHPAA		N-AHAA		ROX	
	Pen #	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Day 14	1	0.045	0.006	0.07	0.01	0.16	0.04	0.02	0.01	0.074	0.009	0.29	0.05	0.22	0.04	7	1
	19	0.036	0.002	0.08	0.01	0.217	0.006	0.0112	0.0001	0.141	0.004	0.23	0.01	0.206	0.006	8.4	0.2
	24	0.030	0.005	0.190	0.008	0.16	0.01	0.005	0.003	0.06	0.01	0.2	0.1	0.18	0.03	7	1
	mean	0.037		0.11		0.18		0.011		0.09		0.24		0.20		7.6	
	SD	0.008		0.07		0.03		0.006		0.04		0.04		0.02		0.7	
Day 24	1	0.12	0.02	0.14	0.02	0.24	0.05	0.015	0.005	0.141	0.002	1.16	0.08	0.42	0.06	5.0	0.6
	19	0.118	0.002	0.122	0.003	0.37	0.01	0.018	0.002	0.21	0.02	0.83	0.05	0.50	0.02	7.5	0.3
	24	0.10	0.01	0.24	0.02	0.27	0.06	0.009	0.005	0.09	0.03	1.7	0.3	0.40	0.09	6	1
	mean	0.11		0.17		0.29		0.014		0.15		1.2		0.44		6	
	SD	0.01		0.06		0.06		0.005		0.06		0.4		0.05		1	
Day 28	1	0.12	0.01	0.20	0.02	0.37	0.06	0.026	0.008	0.16	0.02	1.75	0.06	0.69	0.08	5.3	0.7
	19	0.083	0.003	0.20	0.01	0.473	0.005	0.018	0.003	0.16	0.01	1.56	0.03	0.695	0.004	6.7	0.2
	24	0.080	0.006	0.207	0.008	0.41	0.04	0.016	0.005	0.14	0.04	1.0	0.2	0.8	0.2	9	3
	mean	0.09		0.202		0.42		0.020		0.15		1.4		0.72		7	
	SD	0.02		0.005		0.05		0.005		0.02		0.4		0.04		2	
Day 30	1	0.10	0.02	0.17	0.02	0.42	0.06	0.033	0.009	0.18	0.05	1.12	0.03	0.7	0.1	3.8	0.3
	19	0.096	0.003	0.25	0.02	0.48	0.01	0.024	0.003	0.158	0.004	1.08	0.03	0.59	0.01	4.6	0.1
	24	0.081	0.003	0.259	0.005	0.46	0.04	0.03	0.01	0.20	0.05	1.0	0.1	0.8	0.2	3	1
	mean	0.09		0.23		0.45		0.028		0.18		1.08		0.69		4.0	
	SD	0.01		0.05		0.03		0.005		0.02		0.05		0.08		0.6	
Day 35	1	0.094	0.009	0.14	0.01	0.34	0.03	0.020	0.004	0.08	0.02	0.71	0.05	0.56	0.06	4.1	0.3
	19	0.082	0.002	0.12	0.01	0.54	0.01	0.023	0.002	0.143	0.006	0.60	0.02	0.681	0.009	3.69	0.01
	24	0.09	0.01	0.21	0.02	0.48	0.04	0.03	0.01	0.13	0.05	0.50	0.05	0.7	0.2	3.3	1.0
	mean	0.087		0.16		0.5		0.023		0.12		0.6		0.64		3.7	
	SD	0.007		0.05		0.1		0.003		0.04		0.1		0.07		0.4	

Litter Sample Group:		Control Chickens (Strain Ross 308)															
		Arsenic Species															
Day		AsB		As^{III}		DMA^{V}		MMA^{V}		As^{V}		3-AHPAA		N-AHAA		ROX	
	Pen #	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Day 14	12	0.029	0.002	0.0052	0.0004	0.13	0.01	0.0039	0.0002	0.055	0.005	N.D.		N.D.		N.D.	
	17	0.030	0.002	0.0058	0.0004	0.13	0.01	0.0032	0.0008	0.043	0.008	N.D.		N.D.		N.D.	
	22	0.016	0.003	0.0075	0.0007	0.09	0.01	0.0054	0.0009	0.017	0.002	N.D.		N.D.		N.D.	
	mean	0.025		0.006		0.11		0.004		0.04							
	SD	0.008		0.001		0.02		0.001		0.02							
Day 24	12	0.086	0.004	0.017	0.002	0.24	0.01	0.0039	0.0006	0.052	0.006	0.036	0.003	0.008	0.002	0.039	0.002
	17	0.074	0.002	0.020	0.005	0.25	0.01	0.0039	0.0003	0.049	0.006	0.05	0.01	0.010	0.001	0.111	0.008
	22	0.080	0.002	0.018	0.002	0.219	0.005	0.004	0.001	0.040	0.006	0.034	0.003	0.028	0.009	0.106	0.002
	mean	0.080		0.018		0.23		0.0039		0.047		0.04		0.02		0.09	
	SD	0.006		0.002		0.01		0.0001		0.006		0.01		0.01		0.04	
Day 28	12	0.067	0.003	0.0092	0.0004	0.27	0.01	0.0034	0.0006	0.056	0.002	0.028	0.002	0.013	0.001	0.058	0.003
	17	0.0700	0.0007	0.015	0.004	0.26	0.01	0.004	0.001	0.041	0.004	0.028	0.002	0.018	0.001	0.14	0.01
	22	0.0709	0.0003	0.018	0.002	0.248	0.006	0.004	0.001	0.042	0.003	0.034	0.003	0.029	0.003	0.129	0.009
	mean	0.069		0.014		0.26		0.0036		0.046		0.030		0.020		0.11	
	SD	0.002		0.004		0.01		0.0001		0.008		0.003		0.008		0.05	
Day 30	12	0.064	0.003	0.012	0.001	0.31	0.01	0.0037	0.0007	0.065	0.006	0.034	0.003	0.014	0.001	0.041	0.004
	17	0.073	0.001	0.014	0.004	0.301	0.007	0.0038	0.0009	0.05	0.01	0.032	0.009	0.031	0.002	0.157	0.006
	22	0.078	0.001	0.018	0.002	0.272	0.004	0.004	0.001	0.041	0.002	0.026	0.002	0.039	0.003	0.139	0.003
	mean	0.072		0.015		0.29		0.0037		0.05		0.031		0.03		0.11	
	SD	0.007		0.003		0.02		0.0001		0.01		0.004		0.01		0.06	
Day 35	12	0.078	0.003	0.013	0.001	0.36	0.01	0.0047	0.0006	0.06	0.01	0.0309	0.0004	0.0176	0.0004	0.095	0.005
	17	0.081	0.007	0.018	0.004	0.34	0.02	0.0048	0.0006	0.0465	0.0005	0.031	0.002	0.027	0.004	0.18	0.01
	22	0.093	0.002	0.021	0.001	0.337	0.007	0.005	0.002	0.038	0.003	0.030	0.002	0.052	0.004	0.122	0.002
	mean	0.084		0.017		0.34		0.0049		0.05		0.0305		0.03		0.13	
	SD	0.008		0.004		0.01		0.0003		0.01		0.0004		0.02		0.04	

Litter Sample Group:		Control Chickens (Strain Cobb 500)															
		Arsenic Species															
Day		AsB		As^{III}		DMA^{V}		MMA^{V}		As^{V}		3-AHPAA		N-AHAA		ROX	
	Pen #	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Day 14	5	0.024	0.001	0.0045	0.0006	0.127	0.005	0.002	0.002	0.056	0.003	N.D.		N.D.		N.D.	
	6	0.017	0.004	0.0076	0.0004	0.09	0.02	0.004	0.003	0.034	0.003	N.D.		N.D.		N.D.	
	25	0.026	0.001	0.0049	0.0004	0.129	0.008	0.0032	0.0006	0.054	0.003	N.D.		N.D.		N.D.	
	mean	0.022		0.006		0.12		0.0032		0.05							
	SD	0.005		0.002		0.02		0.0006		0.01							
Day 24	5	0.068	0.002	0.0074	0.0009	0.242	0.009	0.005	0.001	0.072	0.004	0.011	0.002	0.006	0.001	0.070	0.001
	6	0.067	0.007	0.029	0.001	0.258	0.004	0.008	0.003	0.038	0.004	0.042	0.003	0.021	0.004	0.09	0.02
	25	0.065	0.002	0.0088	0.0007	0.24	0.01	0.0028	0.0003	0.06	0.01	0.024	0.001	0.004	0.002	0.078	0.006
	mean	0.067		0.02		0.248		0.006		0.06		0.03		0.010		0.08	
	SD	0.001		0.01		0.009		0.003		0.02		0.02		0.009		0.01	
Day 28	5	0.067	0.004	0.0071	0.0006	0.272	0.007	0.0057	0.0003	0.068	0.006	0.0161	0.0002	0.012	0.002	0.081	0.004
	6	0.070	0.007	0.021	0.003	0.31	0.01	0.008	0.001	0.039	0.007	0.041	0.008	0.026	0.006	0.11	0.02
	25	0.062	0.001	0.0089	0.0008	0.280	0.007	0.0031	0.0005	0.06	0.01	0.029	0.002	0.010	0.001	0.086	0.004
	mean	0.066		0.012		0.29		0.006		0.05		0.03		0.016		0.09	
	SD	0.004		0.007		0.02		0.003		0.01		0.01		0.009		0.01	
Day 30	5	0.066	0.005	0.010	0.002	0.29	0.02	0.0054	0.0009	0.066	0.008	0.013	0.001	0.011	0.001	0.110	0.006
	6	0.061	0.004	0.022	0.003	0.313	0.007	0.007	0.001	0.04	0.01	0.037	0.005	0.026	0.006	0.09	0.01
	25	0.064	0.003	0.0098	0.0007	0.30	0.02	0.0036	0.0005	0.07	0.01	0.027	0.001	0.010	0.002	0.114	0.007
	mean	0.064		0.014		0.30		0.005		0.06		0.03		0.016		0.11	
	SD	0.003		0.007		0.01		0.002		0.02		0.01		0.009		0.01	
Day 35	5	0.075	0.004	0.009	0.002	0.323	0.007	0.0065	0.0003	0.062	0.006	0.015	0.003	0.014	0.003	0.091	0.002
	6	0.077	0.004	0.021	0.002	0.37	0.02	0.007	0.002	0.032	0.003	0.0333	0.0002	0.023	0.004	0.09	0.01
	25	0.071	0.002	0.0126	0.0006	0.32	0.01	0.0036	0.0002	0.06	0.01	0.029	0.001	0.0128	0.0002	0.090	0.002
	mean	0.074		0.014		0.34		0.006		0.05		0.026		0.017		0.092	
	SD	0.003		0.006		0.03		0.002		0.02		0.009		0.006		0.002	

APPENDIX B

CYTOTOXICITY OF PHENYLARSONIC ACIDS

B.1 INTRODUCTION

Three phenylarsonic acids, ROX, 3-AHPAA and N-AHAA, were found as major arsenic species present in the litter of poultry fed ROX. Considering the extensive use of poultry litter as an agricultural fertilizer, it is meaningful to profile the toxicity of these three arsenicals with similar structures.

Real-time cell electronic sensing (RT-CES) has been demonstrated to provide sensitive and continuous real-time monitoring of cellular responses to a variety of chemicals [1-4]. In vitro cytotoxicity values (IC_{50}) can be generated from cell-based assays to evaluate toxicity ranking of chemicals. Our objective is to study the effects of ROX, 3-AHPAA and N-AHAA on T24 (human bladder carcinoma) cells using the RT-CES technique.

B.2 MATERIALS AND METHODS

B.2.1 Reagents and Cell Culture Conditions

1 M standards of ROX, 3-AHPAA, and N-AHAA were prepared separately in dimethyl sulphoxide (DMSO) (Sigma, St. Louis, Mo). Hydrochloric acid (Alfa Aesar, Ward Hill, MA) was added into 3-AHPAA and N-AHAA standard solutions with 10% volume of DMSO to aid in dissolution. Concentrations of standard solutions were calibrated against the environmental calibration standard (Agilent Technologies, U.S.) using direct injection ICPMS. T24 (human bladder carcinoma) cells, McCoy's 5A modified medium with L-glutamine, and minimum

essential medium eagle were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). 1×DPBS, 0.05% Trypsin-EDTA and penicillin/streptomycin (P/S) were purchased from Gibco (Burlington, ON, Canada). Fetal bovine serum (FBS) was obtained from Sigma-Aldrich (Oakville, ON, Canada). T24 cells were cultured in McCoy's 5A modified medium with 10% FBS and 1% P/S at 37 °C with 5.0% CO_2.

B.2.2 RT-CES Cytotoxicity Testing

The cytotoxic effects of three phenylarsonic acids on T24 cells were measured using the RT-CES 96× system (ACEA Biosciences, San Diego, CA), following the procedures described previously [1]. The number of seeding cells was 5000 cells/100 µL. Cells were allowed to grow for approximately 20 h until cell index (CI) reached a value of 1. Then 3-AHPAA (0.5-2 mM), N-AHAA (5-10 mM), and ROX (5.5-10 mM) with serial concentrations were introduced to the culture media (200 µL/well) for testing. Negative control (untreated) and solvent control (treated cells with only 1.275% v/v DMSO) were also included. Each analyte was tested in triplicate, and monitored for 80 h. The data collected was used to generate dose-response curves as well as IC_{50} (a concentration inhibiting 50% cell growth) for individual arsenicals.

B.3 RESULTS AND DISCUSSION

The T24 cellular responses to phenylarsonic acids exposure were monitored continuously for 80 h. CI for each microwell was recorded automatically by the RT-CES system on a one-hour basis. As shown in Figure B.1, controls without arsenic treatment exhibited higher CI values, providing real-time control of normal cell growth in the microwells. In response to arsenic exposure, however, concentration-dependent cellular responses to all three arsenicals were demonstrated. The measured CI decreased with increasing concentrations of arsenic compounds. With sufficient concentrations of 3-AHPAA (> 0.8 mM), N-AHAA (> 7 mM), or ROX (> 6 mM), CI can be lowered to zero within 80 h, indicating no viable cells were attached to the microelectrodes after a period of time. Because a lower concentration can cause CI to decrease, 3-AHPAA treatment showed more severe toxic effect on T24 cells than the other two arsenicals.

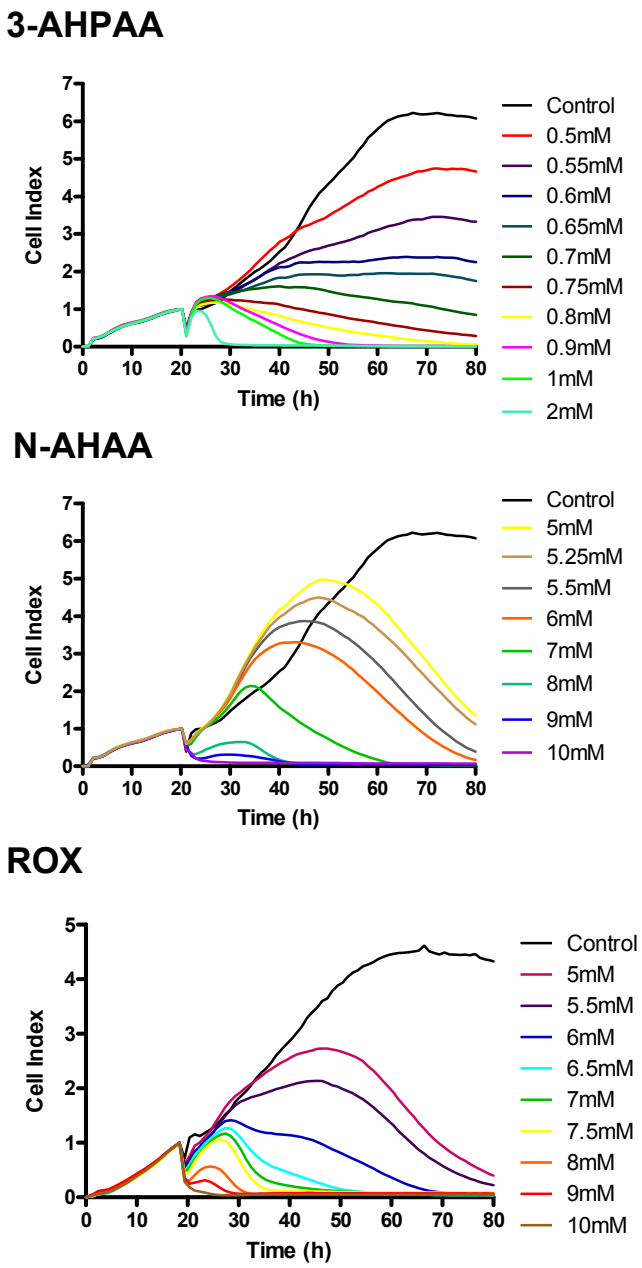

Figure B.1 Typical RT-CES curves showing toxic effects of three phenylarsonic acids (3-AHPAA, N-AHAA, and ROX) on T24 cells. Arsenic compounds at various concentrations were introduced to cell culture media after 20 h. CI was recorded once every hour.

Figure B.2 illustrates typical IC_{50} curves of T24 cells responding to 3-AHPAA, N-AHAA, and ROX treatments over 70 h after the introduction of the arsenic compounds to the culture. IC50 values for T24 cells after 24 h or 48 h post-treatment of individual arsenicals were generated, and summarized in Table B.1. Toxic effects of the three phenylarsonic acids on T24 cells were generally in the order of 3-AHPAA > ROX > N-AHAA.

B.4 CONCLUSIONS

The cytotoxicities of all three phenylarsonic acids were very low, among which 3-AHPAA displayed a relatively higher toxic effect on T24 cells than N-AHAA and ROX.

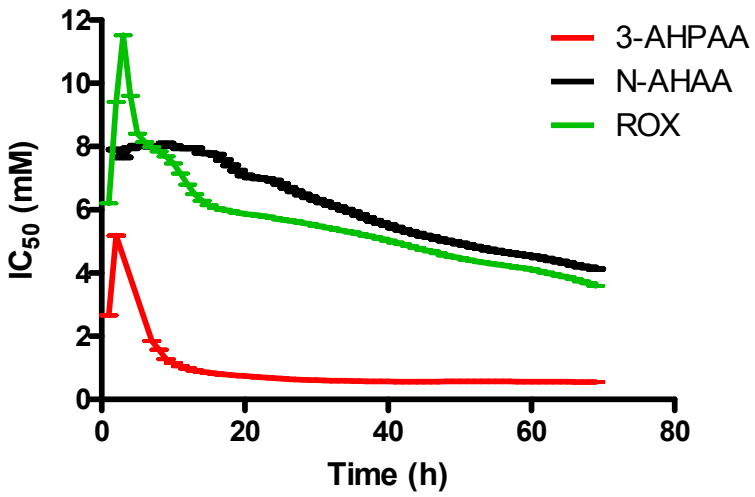

Figure B.2 IC_{50} curves for three phenylarsonic acids (3-AHPAA, N-AHAA, and ROX) over 70 h post-treatment.

Table B.1 IC_{50} values for T24 cells responding to 3-AHPAA, N-AHAA, and ROX treatments.

IC_{50} values (mM) for three phenylarsonic acids on T24		
Arsenic Species	After 24 h	After 48 h
3-AHPAA	0.68	0.57
N-AHAA	6.9	5.0
ROX	5.7	4.6

B.5 REFERENCES

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