Arsenic Speciation in Poultry Kidney

by

Xuan Sun

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Chemistry
University of Alberta

ABSTRACT

An organoarsenic compound, 3-nitro-4-hydroxyphenyl arsenic acid (also known as Roxarsone), has been used as a feed additive. Roxarsone was approved by the Food and Drug Administration (FDA) to control diseases in poultry, and to improve weight gain, feed efficiency, and meat pigmentation. Contrary to the previous belief that most of the Roxarsone is excreted unchanged in the manure, researchers from the FDA recently reported increases in inorganic arsenic concentration in the liver of a small number of chickens fed with Roxarsone. This thesis focuses on a much larger scale feeding study, involving a subset of 142 chickens from a total of 1600 chickens over a 35-day period. Within this subset, 71 chickens were fed a Roxarsone-supplemented diet, and the other 71 chickens were fed a control diet not supplemented with Roxarsone. The objectives of this research are to develop a method for arsenic speciation analysis and to quantify arsenic species in chicken kidney. Kidney samples were treated with pepsin, and the extracts were analyzed for arsenic species using high performance liquid chromatography (HPLC) separation with simultaneous detection by inductively coupled plasma mass spectrometry (ICP-MS) and electrospray ionization tandem mass spectrometry (ESI MS/MS). The temporal profile of each arsenic species was acquired and the analyses show the presence of eleven arsenic compounds in the extracts of the chicken kidney samples. HPLC-ICP-MS allowed for the quantification of the arsenic species, and ESI MS/MS provided complementary information for the identification of the arsenic species. Results from the analyses of both the control and the Roxarsone-

fed chickens are important to our understanding of arsenic metabolism, distribution, and retention in chicken.

PREFACE

Some of the research conducted for this thesis forms part of an interdisciplinary collaboration, led by Professor X. Chris Le at the University of Alberta, with Professor Martin Zuidhof and Professor Gary Kachanoski being the lead collaborators at the University of Alberta. The method referred to in chapter 2 was established by myself, with the assistance of Zongling Yang, Hanyong Peng and Professor X. Chris Le. All of the temporal profiles and all of the data analyses in chapter 3, and concluding analysis in chapter 4 are my original work. No part of this thesis has been previously published.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Dr. Chris Le, for the acceptance into his research group. His guidance, advice and support provided me with the help needed to accomplish my research project and thesis. His knowledge and enthusiasm for research give me a great motivation to think and learn. I have learned a lot from his group meetings, discussions, and suggestions.

Secondly, I would like to express my gratitude to Dr. Leluo Guan and Dr. Michael Serpe, who serve as my committee members, for their critical review and comments on my thesis.

I also want to thank the present and past group members of Dr. Le's group. I am very fortunate to have worked and studied in this collaborative environment. Special thanks to Ms. Xiufen Lu, Mr. Hanyong Peng, Ms. Qingqing Liu and Ms. Aleks Popowich for their technical assistance and discussion of my research work. Thanks Ms. Katerina Carastathis, and all other members of the Division of Analytical and Environmental Toxicology for their friendship and support during my study.

Thanks to Dr. Gary Kachanoski, and Dr. Martin Zuidhof for their help with my research project. And finally, thanks to Mr. Aaron Wipf and Ms. Janae Preteau for their help with sample collection.

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

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

As^{III}	arsenite
As^{V}	arsenate
AsB	arsenobetaine
DMA^{III}	dimethylhydroxyarsine
DMA^{V}	dimethylarsinic acid
ESI-MS	electrospray ionization mass spectrometry
HNO_3	nitric acid
HPLC	high performance liquid chromatography
H_2SO_4	sulfuric acid
ICP-MS	inductively coupled plasma mass spectrometry
LOD	limit of detection
MMA^{III}	monomethyl-dihydroxyarsine
MMA^{V}	monomethylarsonic acid
MRM	multiple reaction monitoring
3-AHPAA	3-amino-4-hydroxy-phenylarsonic acid
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 exposure is one of the most important environmental health risks in the world. It contributes significantly to the burden of a series of preventable diseases worldwide, and it is specifically associated with increased risk of cancer, diabetes, and cardiovascular diseases. The presence of arsenic in the environment is a result of both natural and anthropogenic activities. Arsenic exposure from groundwater is the most serious threat to mankind around the world. However, the expansion of arsenical drug use in poultry production brings new issues to the researchers: whether or not the abuse of arsenical drugs increases the risks of global human arsenic exposure.

1.1 ARSENIC BACKGROUND

As a ubiquitous semi-metallic element in the environment, arsenic naturally occurs in over 200 different mineral forms in the Earth's crust.[1] Arsenic sulfide was converted into arsenate which was introduced into the environment through mineral rock weathering.[2] Before human activities had any effect on the environment, the distributions of arsenic in soils, water, air and living organisms were regulated by natural cycling balance. With the development of industry and agriculture, the rate of mobilization of arsenic caused by anthropogenic activity (mining and refineries, smelting, glass making, pesticide manufacture) is three folds higher than natural arsenic cycling. Arsenic can be found in a large variety of media (ground water, soils, plants, marine organisms, sediments, body tissue and fluids, etc.), in a variety of different forms and concentrations. Figure 1.1 depicts simplified relations among arsenic in water, atmosphere, and soils and rocks.

There are more than 50 arsenic compounds present in the environment systems, ranging

from highly toxic arsenite (As^{III}), to nontoxic arsenobetaine. Arsenic can exist in 5 valence states (-3, 0, +2, +3, and +5), depending on environmental conditions. The oxidation state of arsenic can be -3 (as existing in AsH_3), 0 (as element arsenic), +2 (as existing in As_4S_4), +3 [as existing in $As(OH)_3$], and +5 [as existing in $AsO(OH)_3$]. Element arsenic rarely occurs in nature, and the oxidation state (-3 valence states) is found only in extremely reduced environment. Inorganic arsenite (As^{III}, +3) can be oxidized into arsenate (As^{V}, +5). Thus, inorganic arsenite are usually found in anaerobic conditions, and inorganic arsenate are most prevalent in oxic conditions. [3] Inorganic arsenic compound can also be methylated into monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}), and trimethylarsine oxide (TMAO) by microorganisms under oxidizing conditions.[4,5]

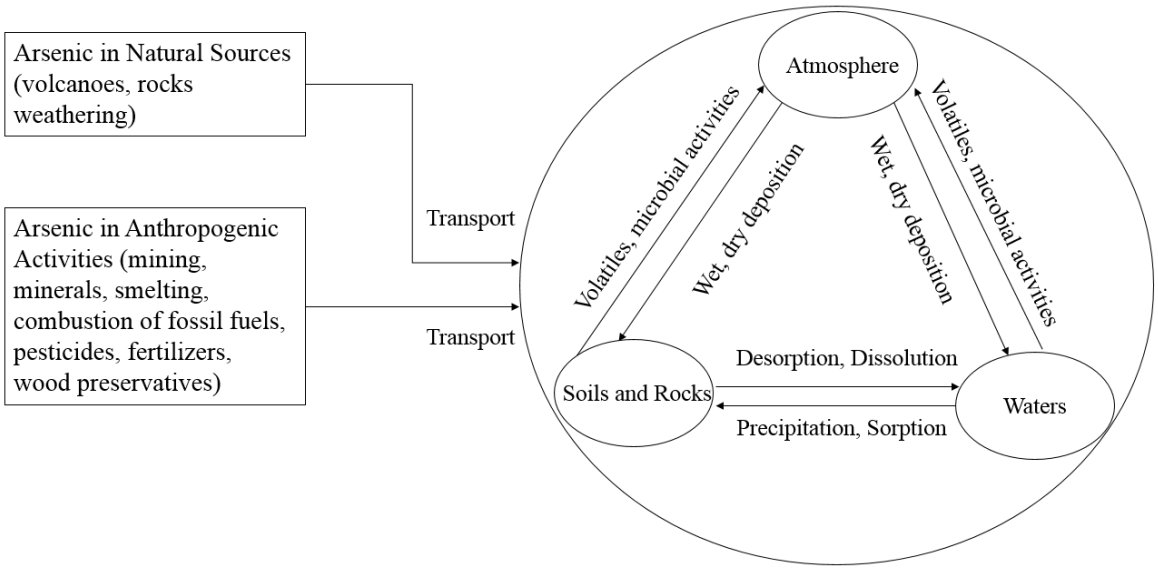


Figure 1.1 A simplified diagram of arsenic in the environment. For simplicity, biological organisms, such as animals and plants, are not included in this diagram. .

1.2 DIETARY EXPOSURE OF ARSENIC

1.2.1 Arsenic in Drinking Water

Arsenic can enter the food chain through a variety of routes, including water and soils as major sources. Groundwater contamination by arsenic is the most widespread way, from which arsenic can be ingested by humans directly, or distributed to plants and animals.[6] The major arsenic exposure pathway is believed to be from drinking contaminated groundwater. About 100 million people are suffering from the toxic effects of arsenic due to natural groundwater contamination.[7] The current World Health Organization (WHO) tolerance level of arsenic in drinking water is 10 μg/L.[8] Levels of arsenic present in water typically depend on bed-rock type.[9] In the U.S., most areas contain arsenic at concentrations lower than 10 μg/L, except for the western mountainous regions in New Mexico, Utah, Arizona, and Nevada, where sedimentary deposits from rocks contribute to higher levels of arsenic.[10] In Canada, there are some "hot spots" (>10 μg/L) in parts of Alberta, British Columbia, Manitoba, New Brunswick, Newfoundland and Labrador, Nova Scotia, Québec, and Saskatchewan.[11] Arsenic ground water problems are also found in India (West-Bengal), Vietnam, Taiwan, Mexico, Argentina, Chile, Hungary, Romania.[12-16]

1.2.2 Arsenic in Food

Plant-based foods, particularly rice, are a significant source of arsenic.[17,18] The anaerobic growing condition and the specific plant physiological characteristics of rice may be relevant to arsenic in rice.[19] Half of the world's population is supported by rice and its products. Rice contains approximately tenfold elevated arsenic concentrations, compared to all other staple dietary grains.[20] In China, inorganic arsenic is the dominant arsenic species in rice, while high percentages of DMA are found in rice from the Unites States and European Union.[21] The total arsenic concentration in some rice samples from the U.S. can reach levels as high as 220 ng/g, which is over an order of magnitude greater than the typical concentrations found in rice from China. The most detailed

global assessment of total arsenic and inorganic arsenic concentrations in rice grain, to date, was published by Meharg et al in 2009.[21] This study shows an elevated risk of bladder and lung cancers from rice intake. Risks are highest in countries such as Bangladesh that have high rice consumption and highly arsenic contaminated rice from anthropogenic activity.

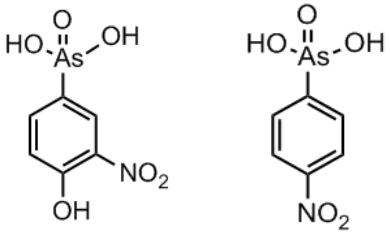
Arsenic was also found in apples,[22] grapes,[23] lettuce,[24] lima beans,[25] and orange juice[26] because of the naturally occurring arsenic in the soil and the use of arsenical-containing pesticides. While the broad use of arsenical feed additives leads to elevated arsenic concentrations in beef, [27]chicken, milk, [28] and eggs [29,30]. Most of these foods contain very low arsenic concentrations compared to that in seafood.[31] However, the higher concentrations of arsenic in seafood are primarily due to arsenobetaine or arsenochlorine, which have very low toxicity, or are essentially non-toxic and low-toxic forms respectively.

There were also some frightening cases of accidental arsenic poisoning through arsenic contaminated food and beverages reported in Japan,[32] Germany,[33] and China.[34] In 1972, 417 patients in Japan were poisoned by soya-sauce, in which an arsenic containing amino acid was used in the making. The arsenic levels in the soya-sauce ranged from 5.6 to 71.6 mg/L. Similarly, powdered milk contaminated with arsenic containing sodium phosphate introduced 13.5-21.0 mg/kg of arsenic to consumers in 1955 in Japan. 180 vinedressers and cellarmn were exposed to arsenic containing pesticides used on wine grapes; those exposed people were found to have symptoms of chronic arsenic poisoning, and about 23 % had evidence of vascular disorders of the extremities.[35] In China, some coals contain high levels of arsenic and caused arsenic poisoning among residents in one city and four prefectures in Guizhou Province.[34]

1.2.3 Arsenical Use in Poultry Feed

Arsenicals have been widely used in the production of poultry, pigs, and cattle in the

U.S. and other countries. In the United States, several organoarsenic compounds were approved by the U.S. Food and Drug Administration (FDA) as feed additives for use in poultry and swine. The use of arsenical in poultry promotes poultry growth, increase feed efficiency and pigmentation, as well as control poultry disease. Roxarsone (3-nitro-4-hydroxyphenylarsonic acid) was approved by the FDA in 1944 for use for both poultry growth promotion and disease control. In Latin America and Asia, arsenical feed additives are increasingly used in poultry and swine farming, due to the explosive development of industrial-model animal production and high demand of meat production



Roxarsone Nitarsone

FDA approval of the use of roxarsone imposes a subsequent restriction, which mandates a 5 day roxarsone withdrawal period, before slaughtered. However, little is known about how much arsenic remains in the poultry after the 5 withdrawal days, as a result of the longer period of ingesting the arsenical drug. The regulation of arsenic remain in poultry products is complex in the United States. The Environmental Protection Agency (EPA), the U.S. Department of Agriculture (USDA), and the FDA share the responsibility for control of controlling pesticides, veterinary drugs, and environmental contaminants in meat and egg products. The FDA sets the tolerance level of arsenic in poultry residues (0.5 μg/g total As in chicken muscle and 2.0 μg/g total As in chicken liver), whereas the monitoring of these levels of arsenic in residue was performed by the USDA under the National Residue Program (NRP). The current arsenic standard for meats was set before 1963, and it did not consider the different toxicity of each arsenic species. The regulation only imposes a restriction on the total arsenic concentration in poultry, which can be misleading for the estimation of health risks, and toxic and non-toxic arsenic

species uptake. In June 2011, the main manufacturer of Roxarsone, Pfizer subsidiary, Alpharma, voluntarily discontinued selling Roxarsone in Poultry feed in the U.S. Currently, Roxarsone and Nitarsone (a chemically similar arsenical drug) products are still being marketed in some areas of the U.S and Canada. There is a need for researchers to investigate the potential health risks for poultry consumers.

1.2.4 Arsenic Remain in Poultry

The safe tolerance for total arsenic in poultry residue is 0.5 μg/g in poultry meat residues; and 2 μg/g to liver and kidney residues (US food and Drug Administration, 1997). In 1969, Morrison[36] tested the total arsenic distribution in the liver, muscle, skin, and kidneys of broiler chickens fed Roxarsone after 5-days withdrawal period. The result indicates that less than 0.1 ppm arsenic was found in poultry muscle and skin, and that arsenic remained in poultry kidney and liver at levels of 0.39 ppm, and 0.13 ppm, respectively. Lasky calculated the total and inorganic arsenic concentrations in chicken liver, and estimated the quantity of arsenic remaining in chicken muscle, based on the data from United States Department of Agriculture Food Safety and Inspection Service from 1989 to 2000.[37] The author expressed concern about arsenic residues in meat, which may lead to people ingestion of a mean of 1.3 to 5.2 μg per day of inorganic arsenic from chicken. For those who eat much more chicken than average, the tolerable daily intake is greater than World Health Organization (WHO) recommendation. The tolerable arsenic intake level recommended by WHO in drinking water is 10 μg/L per day.[8]

The bioaccumulation of arsenic in different tissues through ROX feeding can be significantly different from tissue to liver. The observed concentration in different chicken tissues in the decreasing order are as: liver > heart > leg and breast muscles. The concentration of arsenic was higher in liver and heart, compared to breast muscles and leg.[38-40]

1.3 ARSENIC METABOLISM

In humans, 40 to 70% of inorganic arsenic is metabolized, and excreted within 48 h. Arsenic is eliminated from the body primarily through the liver and kidney. Other less important routes of arsenic clearance include feces, sweat, skin desquamation and incorporation into hair and nail.[40] The metabolism of arsenic can potentially convert the most toxic forms of arsenic into less toxic form, followed by their excretion.

Various bio-species, such as yeast, fungi, algae, plants and animals were found to transform inorganic arsenic into the methyl-derivatives.[41-44] In mammals, inorganic arsenate is reduced into arsenite with the aid of reduced glutathione (GSH) in blood. [45] Then, inorganic arsenite can undergo methylation in liver to form monomethylarsonic acid (MMA^V), and dimethylarsinic acid (DMA^V) catalyzed by methyltransferases. [46] The whole methylation pathway depends on the reduction by GSH, and oxidative addition of methyl groups. [47] MMA^V and DMA^V are less toxic than inorganic arsenic, and are easily excreted in the urine. By monitoring levels of DMA^V and MMA^V in urine, the concentration of overall arsenic ingestion can be estimated. Methylation of DMA to tri-methylarsine oxide has also been reported in mice and hamsters.[48]

At the cellular level, inorganic arsenic can bind to thiol groups of proteins. The tissues rich in thiol group may effectively trap arsenic, i.e., keratin-rich tissue, intestinal walls, epididymis, thyroid glands and lens of the eye. [49] In mice, [74] arsenic was found to retain longest in the lung, intestinal walls, thyroid gland and the lens of the eye.[50] Radiolabeled arsenic in mice also showed the highest arsenic concentrations in the bile and kidneys. Arsenate was cleared more rapidly than arsenite from all soft tissues except for the kidneys.[51]

Methylated derivatives of arsenosugars, arsenobetaine, arsenocholine and arsenolipids were also observed in aquatic organisms, including seaweeds, shrimps, lobsters, and fish.[52,53] However, arsenobetaine is unlikely to be metabolized further in the human

body, and it has been demonstrated that arsenobetaine can be excreted rapidly after ingestion.[54,55]

1.4 HEALTH EFFECTS OF ARSENIC

1.4.1 Chronic Exposure

Inorganic arsenicals have been classified as Group I carcinogens in 1987 by the International Agency for Research on Cancer (IARC). [56] Long-term exposure to arsenic can result in chronic arsenic poisoning (arsenicosis). The clinical features of arsenicosis in humans include non-cancerous effects of hyper- and hypo-pigmentation, hypertension, keratosis, cardiovascular diseases and diabetes. The cancerous effects include cancer of the skin, lungs and bladder. [57,58] In Taiwan, residents suffered from severe Black Foot Disease (BFD) have been found to be associated with chronic exposure to high levels of arsenic in drinking water. [59,60] About 60-100 million people in India and Bangladesh are currently subject to skin lesions as a result of drinking arsenic-contaminated water. [61,62] Chronic arsenic poisoning may also lead to damage of the internal organs of the respiratory, [63] digestive, circulatory, neural, renal and immune systems, [57,58,64] as well as the impairment of cognitive abilities,[65] and reduced thymic function in infants.[66]

1.4.2 Acute Exposure

Currently, acute arsenic poisonings still occur, but are uncommon. [67] In most cases, acute arsenic poisoning occurs from accidental ingestion of insecticides or pesticides, and less commonly from attempted suicide. The median lethal dose of inorganic arsenic in acute exposure to human is about 0.6 mg/kg per day. [68] Depending on the quantity consumed, death usually occurs within 24 hours to four days. The clinical manifestations of acute arsenic poisoning are nausea, vomiting, colicky abdominal pain, profuse water diarrhea, and excessive salivation.[69] Other symptoms include acute psychosis, a diffuse skin rash, toxic cardiomyopathy, and seizures.[70] Urinary arsenic

concentration is the best indicator for recent arsenic poisoning in 1-2 days.

1.5 ARSENIC SPECIATION

1.5.1 Sampling and Sample Preservation

In the past, sampling problems like loss of analytes and contamination always plagued researchers in the trace element analysis of arsenic. Nowadays, those problems can be partially understood and controlled.[71] There are three major elements for quality assurance in sampling: planning, documentation and control. By clearly subdividing the sampling steps, standardized sampling methods can be written as a standard operating procedures (SOP).[72] Strictly following the standard operating procedures, trained personnel can achieve reliable sampling results during the whole process. If no portable sampling technique can be established, then it may be necessary to have an on-site analysis method. For example, samples contain volatile arsenic components should be extracted immediately after collection.

Stabilization and preservation of arsenic species in different matrix is another major concern for arsenic speciation. The interconversion of As^{III} and As^{V}, and the methylation of inorganic arsenic may happen during the sample storage period.[73-75] The stability of different arsenic species is dependent on temperature, pH, and the sample matrix. Furthermore, changes in weather conditions, humidity, and the surrounding environment may also lead to alterations of arsenic species in the original sample. Temperatures below -20 ℃ will minimize the microbial activities, and then avoid transformation of the analytes.[76] Dark storage of sample is also a practical solution to prevent other ions from catalyzing the arsenic conversion.[76,77] Moreover, additives like methanol, [78] mineral acid (HCl), [79] and phosphoric acid [78] added into the sample solution may also be helpful for the stabilization of arsenic species.

1.5.2 Extraction of Arsenic Species

Most arsenic compounds in environmental samples are present at very low levels, which may require extraction steps to release and enrich the analytes from the original matrix. [80] In most cases, traditional solvent extractions were based on the principal of liquid-liquid extraction (LLE) and liquid-solid extraction (LSE) depending on the sample phase. With an appropriate affinity towards one or more of the analytes to the extraction solvent, the target analytes can be transferred into the extract. In order to increase the extraction efficiency, some techniques such as shaking, heating, sonication, or microwave-assisted extraction are used to promote extraction distribution and shorten the extraction time.

1.5.2.1 Solvent Extraction

Considering the possibility of evaporation from analytes, methanol is ideal for solvent extraction. Although some research shows that methanol is weak for inorganic arsenicals extraction, [81] water will strengthen the solubility of inorganic arsenical and increase the extraction efficiency. Water-methanol mixture was highly recommended for the extraction of arsenic due to its good affinity to both polar and non-polar arsenic compounds. Besides, various pH and redox reagents additives may be applied to the sample, in order to aid in the release of arsenic bound to the primary matrix.[71] Chloroform and methanol were also frequently used, although handling chloroform is difficult in actual operation.[82] Other solvent like ethanol, dioxane, [83] acetone, [84] hexane, [85,86] or dichloromethane [87] were occasionally used for organoarsenic extraction in water and sediment containing arsenic warfare agents.

1.5.2.2 Enzyme Extraction

Enzyme extraction is known for its high specificity and efficiency for protein destruction, which is adequate for the release of arsenic from a protein-rich matrix.[88] The enzymes used in arsenic speciation are hydrolytic enzymes, which includes Lipase, Amylases, and Proteases.[89] Enzyme activity is dependent on the temperature and the

pH of the buffer. When reaching the maximum temperature, the enzyme will not be stable. Similarly, the enzyme will be denaturized and inactive outside the pH range. At the optimum pH and temperature, the activity of the enzyme will reach a plateau. Enzyme extraction is always performed at the optimum or medium condition of the particular enzymes. Long incubation time of 24 hours are the most widely chosen by investigators.[22,90,91]

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

High performance liquid chromatography (HPLC) is an efficient conventional separation method for arsenic speciation. There are many chromatographic modes for HPLC separation, such as ion exchange, ion pairing, reverse phase and size exclusion chromatography. Most toxic arsenic species, such as As^{III}, As^{V}, MMA^{V}, DMA^{V} are ionic compounds, so ion chromatography (IC) is the most frequently used for the separation of arsenic species. The principal of ion exchange chromatography is based on the diverse affinity of analyte ions towards an ion exchanger, which can be controlled by a proton association-dissociation equilibrium (pKa). [92] Many As-species have a pKa < 8.0.[93] Hence, neutral and negatively charged As-species under high pH can be differentiated by anion exchange chromatography.[94] Furthermore, anion-exchange columns are more tolerant to changes in matrix.[95] More and more chromatographic commercial columns now are available for the anion exchange separation of arsenic species, like Hamilton PRP-X100, Dionex DX100 and Dionex AS 7. Various methods have been developed, and can achieved good separation for AsB, As^{III}, As^{V}, MMA^{V} and DMA^{V}.[96-98]

Cation-exchange may not retain the two most toxic arsenic species (As^{III} and As^{V}). However, cation-exchange chromatography is useful for the separation of AsB, AsC, TMAO and Me_4As^{+} species.[99-102] In most cases, anion exchange are explored to be the primary separation and if required, cation-exchange may secondarily separate the less

toxic organic cationic arsenicals.

1.5.4 Arsenic Species Detection Using Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

The technology of inductively coupled plasma (ICP) was first brought up in the early 1960's.[103] The high temperature (up to 10,000K) of the plasma atomizes and ionizes all forms of arsenic so that the response of arsenic does not vary with different species. By coupling with mass spectrometry (MS), ions are focused and selected by mass to charge ratio (*m/z*) through mass analyzed, usually a quadrupole. For arsenic analysis, *m/z* 75 is the only stable isotope in nature for arsenic. The detector only received ion signals for ions *m/z* 75, proportional to the ion concentration, while all other ions are filtered out. The combination of inductively coupled plasma (ICP) with MS can achieve a limit of detection as low as μg/L for arsenic determination. The drawback of ICP-MS is the possible interference of chloride due to the formation of argon chloride ($^{40}Ar^{35}Cl$) in the plasma, which has the same mass as arsenic (^{75}As).

HPLC combined with ICP-MS provides highly efficient and reliable separations, along with adequate detection and quantification of non-volatile elements, such as arsenic. However, identification of arsenic compounds by HPLC-ICP-MS requires the availability of arsenic standards, which seems to be difficult for unknown arsenic species. And there is a possibility of misinterpreting two species, if the retention time shifts, or two species have the same retention time.

1.5.5 Arsenic Species Identification Using Electrospray Ionization Mass Spectrometry (ESI-MS)

Electrospray ionization mass spectrometry (ESI-MS) can provide molecular information for the verification of arsenic compounds. In recent years, ESI-MS has been widely used for the determination of arsenic compounds, and even for the identification of new arsenic species.[104-106] The liquid analytes can be dispersed into a fine aerosol by

electrospray.[107] An inert gas (nitrogen or carbon dioxide) can facilitate nebulization of the solvent. Then the aerosol is introduced into the vacuum stage through a capillary carrying a potential difference of approximately 3000~5000V. As the solvent evaporates from the charged droplet, the droplet become unstable and subsequently undergoes fission. These gas-phase ions are then focused into the mass analyzer, and finally pass to the detector.

Different types of information for the target molecules can be obtained, depending on how the generated ion is further ionized. For simple verification, the molecular ion can provide information of the molecular mass of the target analyte. Only one single quadrupole device performs mass analysis for the purpose of monitoring particular ion masses (single ion monitoring (SIM)), or to scan the full mass range. Further information can be obtained through fragment ions generated by collisions of the molecular ion with molecules of a neutral gas in the process of collision-induced dissociation (CID). A second mass analyzer (tandem MS or MS/MS) can be used to screen specific fragment ions (single or multiple reaction monitoring, SRM or MRM), or to scan the full mass range, which provides structure information of the molecules.

By matching the optimum ionization conditions with optimum separation conditions, ESI-MS can be coupled with HPLC for the characterization of arsenic species eluted from column.[106] The compounds in the samples of interest can be identified by both matching the retention time with standards in chromatography, or based on the fraction ions present in ESI-MS. However, HPLC-ESI-MS is more susceptible to matrix effects, and the detection limit is higher, compared to ICP-MS. The strict requirements for high purity sample and ion interference prevent ESI-MS for the fully successful application in biological samples. The quantitation and identification of arsenic species in complicated matrices may need to combine HPLC-ICP-MS with ESI-MS.

1.5.6 Arsenic Speciation by Using HPLC-ICP-MS Combine with ESI-MS

HPLC-ICP-MS coupled with ESI-MS for arsenic speciation has been extensively developed in recent years.[106,108,109] As shown in Figure 1.2, HPLC-ICP-MS was combined with ESI-MS for the purpose of simultaneous quantitation and verification of the arsenic species. Treated samples were introduced into HPLC and separated at low flow rate. Then, the separated arsenic compounds were split by a tee splitter in specific ratio between the ICP-MS and ESI-MS. HPLC-ICP-MS allowed for quantification of arsenic species, and ESI MS/MS provided complementary information for the identification of arsenic species.

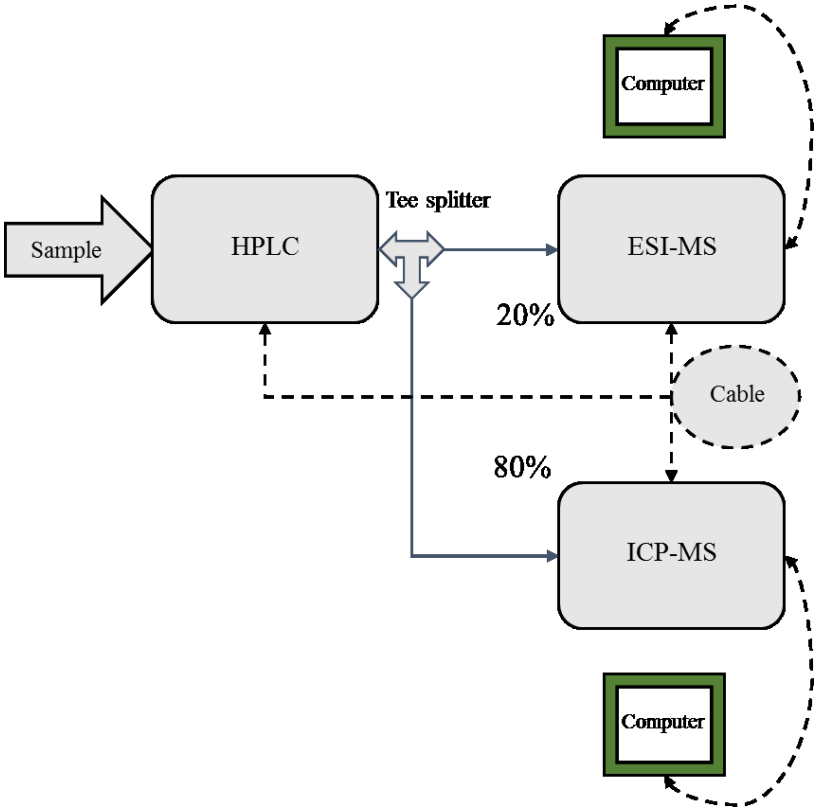


Figure 1.2 Schematic showing HPLC-ICP-MS coupled with ESI-MS systems

1.6 STUDY HYPOTHESIS AND OBJECTIVES

I hypothesize that ROX feeding will increase the concentration of arsenic in poultry kidney, and that ROX can be transformed and metabolized in poultry, which leads to elevated inorganic and organic arsenic residues in poultry. In order to test these hypothesizes, I will focus on the following two major objectives:

Firstly, it is necessary to develop a highly efficient and specific method for the arsenic speciation in poultry kidney. Enzyme assisted extraction of the arsenic from kidney samples will be tested and optimized. Separation of arsenic species will be achieved using anion exchange chromatography. ICP-MS will be applied to determine the concentration of arsenic. Then, ESI-MS will be used to further confirm the identity of each suspected arsenic species. The method of enzyme-assisted extraction combined with HPLC-ICP-MS and ESI-MS will be fully developed and assessed.

Secondly, a large-scale feeding experiment will be performed to estimate the effect of ROX feeding on the levels of arsenic in poultry. The chromatography and mass spectrometry method developed above will be used to monitor arsenic species in poultry kidneys. Kidney samples will be obtained from chickens in both the Control and the ROX feeding groups, over a 35-day feeding period. Temporal profiles of arsenic species will provide information on the uptake, elimination, and metabolism of arsenic species. The concentrations of inorganic and organic arsenic in poultry 7 days after the feeding of ROX stops will provide information about the residual concentration of arsenic species and whether ROX feeding increases risks of arsenic exposure for chicken consumers.

1.7 REFERENCE

(1) H. Onishi, K. H. W. *Handbook of Geochemistry, Springer-Verlag*, **1969**. II-1, *33-B5*

(2) Shacklette, H. T.; Boerngen, J. G. *Element Concentrations in Soils and Other Surficial Materials of the Conterminous United States* **1984**. *103*.1270

(3) Cox, D. P.; Alexande.M *Applied Microbiology* **1973**, *25*, 408.

(4) Reichert, F.; Trelles, R. *An. Asoc. Quim. Argent* **1921**, *9*, 89.

(5) McBride, B.; Wolfe, R. *Biochemistry-Us* **1971**, *10*, 4312.

(6) Ferguson, J. F.; Gavis, J. *Water Research* **1972**, *6*, 1259.

(7) Ravenscroft, P.; Brammer, H.; Richards, K. *Arsenic pollution: a global synthesis*; John Wiley & Sons, **2011**; *94*.103

(8) World Health Organization (WHO). *Guidelines for drinking-water quality: recommendations*; World Health Organization, **2004**; *Vol 1*, 1

(9) Komorowicz, I.; Barałkiewicz, D. *Talanta* **2011**, *84*, 247.

(10) Frost, F. J.; Muller, T.; Petersen, H. V.; Thomson, B.; Tollestrup, K. *Journal of Exposure Science and Environmental Epidemiology* **2003**, *13*, 231.

(11) McGuigan, C. F.; Hamula, C. L.; Huang, S.; Gabos, S.; Le, X. C. *Environmental Reviews* **2010**, *18*, 291.

(12) Nickson, R.; McArthur, J.; Burgess, W.; Ahmed, K. M.; Ravenscroft, P.; Rahman, M. *Nature* **1998**, *395*, 338.

(13) Chowdhury, U. K.; Biswas, B. K.; Chowdhury, T. R.; Samanta, G.; Mandal, B. K.; Basu, G. C.; Chanda, C. R.; Lodh, D.; Saha, K. C.; Mukherjee, S. K. *Environmental Health Perspectives* **2000**, *108*, 393.

(14) Nickson, R.; McArthur, J.; Ravenscroft, P.; Burgess, W.; Ahmed, K. *Applied Geochemistry* **2000**, *15*, 403.

(15) Smedley, P.; Kinniburgh, D. *Applied Geochemistry* **2002**, *17*, 517.

(16) Pal, T.; Mukherjee, P. K.; Sengupta, S. *Current Science* **2002**, *82*, 554.

(17) Zhang, W.; Cai, Y.; Tu, C.; Ma, L. Q. *Science of the Total Environment* **2002**, *300*, 167.

(18) Meharg, A. A.; Rahman, M. M. *Environmental Science & Technology* **2003**, *37*, 229.

(19) Zhao, F.-J.; McGrath, S. P.; Meharg, A. A. *Annual Review of Plant Biology, Vol 61* **2010**, *61*, 535.

(20) Williams, P. N.; Villada, A.; Deacon, C.; Raab, A.; Figuerola, J.; Green, A. J.; Feldmann, J.; Meharg, A. A. *Environmental Science & Technology* **2007**, *41*, 6854.

(21) Meharg, A. A.; Williams, P. N.; Adomako, E.; Lawgali, Y. Y.; Deacon, C.; Villada, A.; Cambell, R. C.; Sun, G.; Zhu, Y.-G.; Feldmann, J. *Environmental Science & Technology* **2009**, *43*, 1612.

(22) Caruso, J. A.; Heitkemper, D. T.; B'Hymer, C. *Analyst* **2001**, *126*, 136.

(23) Herce-Pagliai, C.; Moreno, I.; Gonzalez, G.; Repetto, M.; Cameán, A. *Food Additives & Contaminants* **2002**, *19*, 542.

(24) Xu, J.; Thornton, I. *Environmental Geochemistry and Health* **1985**, *7*, 131.

(25) Ventura-Lima, J.; Bogo, M. R.; Monserrat, J. M. *Ecotox Environ Safe* **2011**, *74*, 211.

(26) Ghimire, K. N.; Inoue, K.; Makino, K.; Miyajima, T. *Separation Science and Technology* **2002**, *37*, 2785.

(27) Jorhem, L.; Slorach, S.; Sundström, B.; Ohlin, B. *Food Additives & Contaminants* **1991**, *8*, 201.

(28) Ulman, C.; Gezer, S.; Anal, Ö.; Töre, I. R.; Kirca, Ü. *Water, Air, and Soil Pollution* **1998**, *101*, 411.

(29) Evans, R. J.; Bandemer, S. L. *Analytical Chemistry* **1954**, *26*, 595.

(30) Donoghue, D. J.; Hairston, H.; Cope, C. V.; Bartholomew, M. J.; Wagner, D. D. *Journal of Food Protection®* **1994**, *57*, 218.

(31) Tam, G.; Charbonneau, S.; Bryce, F.; Pomroy, C.; Sandi, E. *Toxicology and Applied Pharmacology* **1979**, *50*, 319.

(32) Tsuchiya, K. *Environmental Health Perspectives* **1977**, *19*, 35.

(33) Lüchtrath, H. *Journal of Cancer Research and Clinical Oncology* **1983**, *105*, 173.

(34) Rahman, M. M.; Sengupta, M. K.; Chowdhury, U. K.; Lodh, D.; Das, B.; Ahamed, S.; Mandal, D.; Hossain, M. A.; Mukherjee, S. C.; Pati, S., CSIRO Publishing, **2006**, *38*, 29.

(35) Lüchtrath, H. *Deutsche Medizinische Wochenschrift (1946)* **1972**, *97*, 21.

(36) Morrison, J. L. *Journal of Agricultural and Food Chemistry* **1969**, *17*, 1288.

(37) Lasky, T.; Sun, W. Y.; Kadry, A.; Hoffman, M. K. *Environmental Health Perspectives* **2004**, *112*, 18.

(38) Chan, P. C.; Huff, J. *Journal of Environmental Science & Health Part C* **1997**, *15*, 83.

(39) Chung, J. S.; Kalman, D. A.; Moore, L. E.; Kosnett, M. J.; Arroyo, A. P.; Beeris, M.; Mazumder, D. G.; Hernandez, A. L.; Smith, A. H. *Environmental Health Perspectives* **2002**, *110*, 729.

(40) Gul Kazi, T.; Qadir Shah, A.; Imran Afridi, H.; Ali Shah, N.; Balal Arain, M. *Ecotox Environ Safe* **2013**, *87*, 120.

(41) Maeda, S. *Arsenic in Environment. Part I: Cycling and Characterization*; John Wiley & Sons Inc, **1994**. 155

(42) Cullen, W. R.; Reimer, K. J. *Chemical Reviews* **1989**, *89*, 713.

(43) Aposhian, H. V. *Annual Review of Pharmacology and Toxicology* **1997**, *37*, 397.

(44) Thomas, D. J.; Styblo, M.; Lin, S. *Toxicology and Applied Pharmacology* **2001**, *176*, 127.

(45) Marafante, E.; Vahter, M.; Envall, J. *Chemico-biological Interactions* **1985**, *56*, 225.

(46) Stýblo, M.; Drobná, Z.; Jaspers, I.; Lin, S.; Thomas, D. J. *Environmental Health Perspectives* **2002**, *110*, 767.

(47) Thompson, D. J. *Chemico-biological Interactions* **1993**, *88*, 89.

(48) Marafante, E.; Vahter, M. *Environ. Res.* **1987**, *42*, 72.

(49) Vahter, M. *Applied Organometallic Chemistry* **1994**, *8*, 175.

(50) Vahter, M.; Marafante, E.; Dencker, L. *Science of the Total Environment* **1983**, *30*, 197.

(51) Lindgren, A.; Vahter, M.; Dencker, L. *Acta Pharmacologica et Toxicologica*

1982, *51*, 253.

(52) Edmonds, J.; Francesconi, K. *Nature* **1981**, *289*, 602.

(53) Edmonds, J.; Francesconi, K. *Experientia* **1987**, *43*, 553.

(54) Freeman, H.; Uthe, J.; Fleming, R.; Odense, P.; Ackman, R.; Landry, G.; Musial, C. *Bulletin of Environmental Contamination and Toxicology* **1979**, *22*, 224.

(55) Le, X. C.; Cullen, W. R.; Reimer, K. J. *Clinical Chemistry* **1994**, *40*, 617.

(56) Arsenic, I. A. R. C. "arsenic compounds (Group 1)." *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans"* **1987**.*7*, 1

(57) ATSDR *US department of Health & Human Service, Public Health Sevice*, *Agency for Toxic Substances and Disease Registry*. **2000**, 428

(58) IPCS *Environmental Health Criteria Series* **2001**, 224.

(59) Chen, C.; Hsu, L.; Tseng, C.; Hsueh, Y.; Chiou, H. *Arsenic Exposure and Health Effects (Chappell WR, Abemathy CO, Calderon RL, eds). New York: Elsevier Science* **1999**, *12*, 113.

(60) Tseng, W.-P. *Environmental Health Perspectives* **1977**, *19*, 109.

(61) Ahmad, S. A.; Sayed, S. U.; Barua, S.; Khan, M. H.; Jalil, A.; Hadi, S. A.; Talukder, H. K. *Environmental Health Perspectives* **2001**, *109*, 629.

(62) Chakraborti, D.; Basu, G. K.; Biswas, L. K.; Chowdhury, U. K.; Rahman, M. M.; Paul, K.; Chowdhury, T. R.; Chanda, C. R.; Lodh, D.; Ray, S. L. *Characterization of Arsenic-bearing Sediments in the Gangetic Delta of West Bengal, India*, *Arsenic exposure and health effects* **2001**, *4*, 27

(63) Dauphiné, D. C.; Ferreccio, C.; Guntur, S.; Yuan, Y.; Hammond, S. K.; Balmes, J.; Smith, A. H.; Steinmaus, C. *International Archives of Occupational and Environmental Health* **2011**, *84*, 591.

(64) Andrew, A. S.; Jewell, D. A.; Mason, R. A.; Whitfield, M. L.; Moore, J. H.; Karagas, M. R. *Environmental Health Perspectives* **2008**, *116*, 524.

(65) Chen, Y.; Parvez, F.; Gamble, M.; Islam, T.; Ahmed, A.; Argos, M.; Graziano, J. H.; Ahsan, H. *Toxicology and Applied Pharmacology* **2009**, *239*, 184.

(66) Ahmed, S.; Ahsan, K. B.; Kippler, M.; Mily, A.; Wagatsuma, Y.; Hoque, A. W.; Ngom, P. T.; El Arifeen, S.; Raqib, R.; Vahter, M. *Toxicol Sci* **2012**, *129*, 305.

(67) Bronstein, A. C.; Spyker, D. A.; Cantilena, L. R.; Green, J. L.; Rumack, B. H.; Dart, R. C. *Clinical Toxicology* **2011**, *49*, 910.

(68) SCHOOLMEESTER, W. L.; WHITE, D. R. *Southern Medical Journal* **1980**, *73*, 198.

(69) Mueller, P.; Benowitz, N. *Emergency Medicine Clinics of North America* **1989**, *7*, 667.

(70) Campbell, J. P.; Alvarez, J. *American Family Physician* **1989**, *40*, 93.

(71) Francesconi, K. A.; Kuehnelt, D. *Analyst* **2004**, *129*, 373.

(72) Wagner, G. *Science of the Total Environment* **1995**, *176*, 63.

(73) Le.X. C.; Cullen, W. R.; Reimer, K. J. *Environmental Science & Technology* **1994**, *28*, 1598.

(74) Segura, M.; Muñoz, J.; Madrid, Y.; Cámara, C. *Analytical and Bioanalytical Chemistry* **2002**, *374*, 513.

(75) Martınez-Bravo, Y.; Roig-Navarro, A.; Lopez, F.; Hernandez, F. *Journal of Chromatography A* **2001**, *926*, 265.

(76) Hall, G. M. *Journal of Analytical Atomic Spectrometry* **1999**, *14*, 205.

(77) Bednar, A.; Garbarino, J.; Ranville, J.; Wildeman, T. *Environmental Science & Technology* **2002**, *36*, 2213.

(78) Pizarro, I.; Gomez, M.; Camara, C.; Palacios, M. A. *Analytica Chimica Acta* **2003**, *495*, 85.

(79) Oliveira, V.; Sarmiento, A.; Gómez-Ariza, J.; Nieto, J.; Sánchez-Rodas, D. *Talanta* **2006**, *69*, 1182.

(80) Namieśnik, J.; Górecki, T. *Pol. J. Environ. Stud* **2001**, *10*, 77.

(81) Edmonds, J.; Shibata, Y.; Prince, R.; Francesconi, K.; Morita, M. *Journal of the Marine Biological Association of the United Kingdom* **1994**, *74*, 463.

(82) Leermakers, M.; Baeyens, W.; De Gieter, M.; Smedts, B.; Meert, C.; De Bisschop, H.; Morabito, R.; Quevauviller, P. *TrAC Trends in Analytical Chemistry* **2006**, *25*, 1.

(83) Stan'kov, I.; Sergeeva, A.; Tarasov, S. *Journal of Analytical Chemistry* **2000**, *55*, 66.

(84) Haas, R.; Krippendorf, A. *Environmental Science and Pollution Research* **1997**, *4*, 123.

(85) Schoene, K.; Steinhanses, J.; Bruckert, H.-J.; König, A. *Journal of Chromatography A* **1992**, *605*, 257.

(86) Epure, G.; Grigoriu, N.; Filipescu, L. *University Politehnica of Bucharest, Scientific Bulletin Series B: Chemistry and Materials Science* **2010**, *3*, 72.

(87) Tørnes, J. A.; Opstad, A. M.; Johnsen, B. A. *Science of the Total Environment* **2006**, *356*, 235.

(88) Carpenter, R. C. *Analytica Chimica Acta* **1981**, *125*, 209.

(89) Bermejo, P.; Capelo, J.; Mota, A.; Madrid, Y.; Cámara, C. *TrAC Trends in Analytical Chemistry* **2004**, *23*, 654.

(90) Forsyth, D. S.; Marshall, W. D. *Environmental Science & Technology* **1986**, *20*, 1033.

(91) Gilon, N.; PotinGautier, M.; Astruc, M. *Journal of Chromatography A* **1996**, *750*, 327.

(92) Fritz, J. S.; Gjerde, D. T. Wiley, *Ion chromatography*. John Wiley & Sons, **2009**. *31*, 1

(93) Zimmermann, M. D.; Tossell, J. A. *The Journal of Physical Chemistry A* **2009**, *113*, 5105.

(94) Larsen, E. H. *Spectrochimica Acta Part B: Atomic Spectroscopy* **1998**, *53*, 253.

(95) Beauchemin, D.; Siu, K.; McLaren, J. W.; Berman, S. S. *J. Anal. At. Spectrom.* **1989**, *4*, 285.

(96) Zheng, J.; Goessler, W.; Kosmus, W. *Microchimica Acta* **1998**, *130*, 71.

(97) Lintschinger, J.; Schramel, P.; Hatalak-Rauscher, A.; Wendler, I.; Michalke, B. *Fresenius' Journal of Analytical Chemistry* **1998**, *362*, 313.

(98) Ackley, K. L.; B'Hymer, C.; Sutton, K. L.; Caruso, J. A. *Journal of Analytical Atomic Spectrometry* **1999**, *14*, 845.

(99) Larsen, E. H.; Pritzl, G.; Hansen, S. H. *Journal of Analytical Atomic Spectrometry* **1993**, *8*, 1075.

(100) Campbell, N.; Reece, J. Biology, 7th edition, San Francisco, USA: Pearson,

Benjamin Cummings. **2005**, *7*, 956

(101) Shibata, Y.; Morita, M. *Applied Organometallic Chemistry* **1992**, *6*, 343.

(102) Vacchina,V.; Połeć, K.; Szpunar, J. *Journal of Analytical Atomic Spectrometry* **1999**, *14*, 1557.

(103) Wendt, R. H.; Fassel, V. A. *Analytical Chemistry* **1965**, *37*, 920.

(104) Inoue, Y.; Sakai, T.; Shimizu, N.; Yoshida, K.; Chen, H.; Kuroda, K.; Endo, G. *Applied Organometallic Chemistry* **1999**, *13*, 81.

(105) Moreda-Pineiro, J.; Alonso-Rodriguez, E.; Romaris-Hortas, V.; Moreda-Pineiro, A.; Lopez-Mahia, P.; Muniategui-Lorenzo, S.; Prada-Rodriguez, D.; Bermejo-Barrera, P. *Food Chemistry* **2012**, *130*, 552.

(106) Hansen, H. R. *J. Anal. At. Spectrom* **2003**, *18*,474.

(107) Pozniak, B.; Cole, R. *J Am Soc Mass Spectrom* **2007**, *18*, 737.

(108) McSheehy, S.; Pohl, P. L.; Lobinski, R.; Szpunar, J. *Analyst* **2001**, *126*, 1055.

(109) Mattusch, J.; Möller, D.; González, M. P. E.; Wennrich, R. *Analytical and Bioanalytical Chemistry* **2008**, *390*, 1707.

CHAPTER 2

DEVELOPMENT OF A METHOD FOR ARSENIC SPECIATION ANALYSIS OF CHICKEN KIDNEY

2.1 INTRODUCTION

The identification and quantification of different arsenic (As) species in complicated matrices (including biological tissues, sediment and soil) has been a major challenge of analytical chemistry for many years. Investigation of arsenicals in biological samples involves multiple steps in the analytical procedure[1] (such as extraction, pre-concentration, purification, chromatographic separation, and specific detection). Each of these steps can increase the risk of analyte loss and the contamination of samples. To better study the distribution of different arsenic species in poultry kidney, it is necessary to have highly sensitive and specific method, which includes high efficiency of extraction, optimal performance in separation and sensitive detection.

Enzymatic hydrolysis is a chemically mild but efficient method that is able to liberate species from the matrices, and thus satisfies the strict requirements of arsenic extraction from biological samples.[2] Hydrolytic enzymes are used in sample digestion by introducing water at specific bonds of the substrate during catalysis. The initial use of enzyme digestion was to extract Cd, Cu, Pb and Ti from human liver and kidney tissue,[3] with subsequent detection by flame atomic absorption spectrometry (F-AAS). In 2001, trypsin and pancreatin extraction coupled with HPLC-ICP-MS successfully achieved detection of AsB, DMA^V, MMA^V in baby food.[4] There are three different types of hydrolytic enzymes (lipases, amylases, and proteases), classified as fat-hydrolyzed, starch and glycogen decomposed, proteinases and peptidases in the analytical literature. The function of those enzymes and how they perform in sample treatments is shown in Table 2.1.

Table 2.1 Enzymes for sample treatment.

Name	Function
Pancreatin	Protein degrading, break down fats, proteins and starch.
Trypsin	Lysine or arginine proteases, which leaves behind the protein as peptide chains having none or one lysine or arginine residue
Subtilisin	Serine proteases, which initiate the nucleophilic attack on the peptide (amide) bond through a serine residue at the active site
Pepsin	Efficient in cleaving peptide bonds between hydrophobic and preferably aromatic amino acids such as phenylalanine, tryptophan, and tyrosine
Pronase E	Hydrolysis of protein or polypeptides, or isolation of amino acid-glycoside from proteins.
Papain	Breaks peptide bonds and involves deprotonating of Cysteine or Histidine

Currently, the most widely used laboratory technique for arsenic characterization is high-performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS). Compared to traditional detectors, ICP-MS has high sensitivity, multi-element capability and wide linear dynamic range of detection. For trace element speciation analysis, the majority of published literature used ICP-MS as a detector for arsenic speciation[5-7]. In 2001, Jackson and Bertsch [6] reported determination of p-ASA and ROX in poultry wastes using HPLC coupling with ICP-MS. To elute ROX from Dionex AS7 column, strong acid, HNO_3, was used as the eluent. Good separation for six As-species in two single isocratic eluent concentration (2.5mM, 50mM) was achieved. Disadvantage of their method is oxidation of As^{III} during separation. To avoid this, two single separations using different eluents were performed. In the most recently published paper, Grant et al. [8] used HPLC-ICP-MS to determine arsenic species in chicken liver that acquired from a local market and ROX was identified as one of major arsenic with minor amounts of As^{V}.

The rapid monitoring of organoarsenic compounds using HPLC-ICP-MS requires the availability of standards for the identification of new species. The complexity of arsenic speciation and matrix effects may lead to coelution of two species or shift in retention time for uncontrolled ion-pairs. [9] The technique of electrospray ionization mass spectrometry (ESI-MS) can partially solve this problem by providing a large number of molecular information for identification of arsenic compounds[10]. In 1996, Corr and Larsen [11] reported tandem mass spectra of four arsenosugars and provided the possibility of arsenic identification by ESI-MS/MS. Since then, the application of ESI-MS/MS for arsenic identification has increased dramatically[9,12-15]. The limitation of ESI-MS for the identification of arsenic compounds is the requirements for high concentration of analytes and low sample matrix. For biological sample, it is very difficult to overcome matrix effect and ion interferences during ESI-MS analysis. The combination of HPLC-ICP MS with ESI-MS/MS has demonstrated the possibility for identification and quantification of arsenical species simultaneously.

The objective of this chapter was to develop a method for arsenic speciation analysis in poultry kidney. The method involves enzyme digestion for extraction of arsenic species, HPLC separation, and both ICP-MS and ESI-MS/MS detections. The method was used to extract, separate and identify arsenicals in the kidney of poultry fed with both Roxarsone® containing diet (ROX Group) and Roxarsone-free diet (Control Group).

2.2 EXPERIMENT

2.2.1 Reagents and Standards

All reagents used in the study were of analytical grade. Ammonium bicarbonate (Fluka), ammonium hydroxide (Fisher) and HPLC grade methanol (Fisher) were prepared for HPLC mobile phase.

Pepsin from *procine gastric mucosa* (powder, Sigma-Aldrich), pancreatin form *porcine pancreas* (powder, Sigma-Aldrich), subtilisin protease from *Bacillus licheniformis* (Type VIII, lyophilized powder, Sigma-Aldrich, pronase E from *Streptomyces griseus* (Type XIW, powder, Sigma-Aldrich), papain from *papaya latex* (lyophilized powder, Sigma-Aldrich) were stored at 4 ℃ before enzyme solutions of these enzymes are made.

All arsenic standard stock solutions were kept at 4℃ before use. Arsenobetaine (98%, Tri Chemical Laboratories Inc., Japan), sodium m-arsenite (97.0%, Sigma, US), cacodylic acid (98%, Sigma, US), monosodium acid methane arsonate (99.0%, Chem Service, West Chester, PA), sodium arsenate (99.4% Sigma, US), 3-amino-4-hyroxyphenylarsonic acid (Pfalz and Bauer, Inc., Waterbury, CT), N-acetyl-4-hydroxy-m-arsanilic acid (N-AHAA, Pfalz and Bauer, Inc.), and 3-nitro-4-hydroxyphenylarsonica acid (Roxarsone, 98.1% purity, Sigma-Aldrich, St. Louis, MO) were used for preparation of AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and ROX standard stock solution. All stock solutions (100 mg As/L) were made by dissolving corresponding purified solids in deionized water. Environmental calibration

standard 5850 (Agilent Technologies, U.S.) served as primary calibration standard. Concentrations of arsenic species were standardized against this primary standard using direct injected ICP-MS analysis. Standard reference material (SRM) 1640 trace elements in natural water from National Institute of Standards and Technology (Gaithersburg, MD) was used as a quality control measure for total arsenic analysis. Speciation analysis standard solutions of eight arsenic species were diluted daily from arsenic standard stock solution.

2.2.2 Sample Collection

Kidney samples from Lake Poultry Processor® (St. Paul, AB) and Warburg Poultry Processor® (Warburg, AB) were collected by the owners of the facilities and were shipped on ice to University of Alberta, Canada. The poultry in these two organic poultry processors were claimed to be fed with a ROX free diet. Individual Lake Poultry and Warburg Poultry kidney samples were separately homogenized in a blender (Kitchen Aid). Blended samples were stored in sealable bags (Ziploc®) at -80 ℃ prior to analysis.

20 μg/L of eight standard arsenic species mixtures were added to 0.5 g kidney samples, and then incubated for 1 hour. This "spiked" sample was used to determine arsenic recoveries for different enzymes during method development.

The feeding experiment was conducted in the Poultry Research Centre, University of Alberta. The detailed sample collection procedures are shown in Section 3.2.1. The kidney samples acquired from the feeding experiment on Day 28 in the ROX-fed group were used for mass balance calculation and extraction efficiency evaluation.

2.2.3 Sample Treatment

2.2.3.1 Extraction of Arsenic Species from Poultry Kidney

(1) Traditional Extraction of Arsenic Species from Poultry Kidney

Poultry kidney sample were stored at -80 ℃. They were defrosted and then homogenized using disposable spatula (210mm, Sigma-Aldrich) on the day of analysis. 0.5 g of the homogenate was then weighed into 15 mL tube. To this, 10.00 mL of methanol-water solution (volume 1:1) was added, and then centrifuged at 3500 g for 10 min. Repeated extractions were conducted, and the supernatant portions were combined into a 50 mL beaker. Then the beaker was placed on a hot plate at 40 ℃ to evaporate methanol. The solution then transferred into a 15 mL tube and dilute with water to 5 mL, the spiraled into 1 mL syringe-filter (0.45 μm, Sigma-Aldrich). The filtered solution was used for arsenic speciation in HPLC-ICP-MS/ ESI MS/MS.

(2)Enzyme-assisted Extraction of Arsenic Species from Poultry Kidney

Enzyme Solution Preparation

The buffer solutions were prepared at the optimum pH for each different enzyme (Table 2.2). To adjust the pH of the buffer solution, 0.5% HCl (99.8%, Optima) and ammonium bicarbonate (Fluka) were used. Then, 1.00 g enzyme powder was weighed and dissolved into 50 mL buffer solutions. Trace arsenic species in each pure enzyme solution was determined by HPLC-ICP-MS. Every time before each sample treatment, the fresh enzyme solution will be made and added into homogenized kidney sample.

Enzyme-assisted Extraction of Arsenic Species from Poultry Kidney

Poultry kidney sample were stored at -80 ℃. They were defrosted and then homogenized using disposable spatula (210mm, Sigma-Aldrich) on the day of analysis. One aliquot of sample (~0.5 g) was left for acid digestion in total analysis. Another aliquot of homogenate (~0.5 g) was weighed into a 10mL tube. Of the enzyme solution, 5.00 mL enzyme solution (mass ratio = 1:5) (pepsin, papain, pancreatin parameter

optimization) was separately added into the corresponding tube. Ultrasonic homogenization (KitchenAid) was used to further homogenize the mixture. The mixture was incubated at optimum temperature (shown in Table 2.2) for overnight to allow further digestion of the kidney sample. The following day, the mixture was centrifuged for 15 min. One aliquot of the extract on the top layer was spiraled into a 1 mL syringe-filtered (0.45 μm, Sigma-Aldrich). The filtered solution was analyzed for arsenic speciation using HPLC-ICP-MS/ESI MS/MS.

Table 2.2 Temperature and pH Conditions for Enzymes Extraction

Pancreatin	Trypsin	Subtilisin	Pepsin	Pronase E	Papain
pH=8.0	pH=8.0	pH=8.0	pH=2.0	pH=7.5	pH=7.5
37 ℃	37 ℃	55 ℃	37 ℃	37 ℃	45 ℃

2.2.3.2 Acid Digestion of Poultry Kidney Samples

Of the homogenized kidney sample, 0.5 g was weighed into a 100 mL beaker. To the beaker, 30 mL concentrated sulfuric acid (H_2SO_4) was poured followed by 10 mL concentrated nitric acid. The beaker was covered by a watch glass and the mixture was digested overnight. The next day, the beaker was heated to 150 ℃ for further digestion until the solution became transparent. The temperature then was raised to 300℃ to evaporate the nitric acid. After the reddish mist above the beaker disappeared, the temperature was raised to 450 ℃ to evaporate all acids until the final volume was less than 1 mL. Deionized water was added to dilute the acids to 3 mL and the solution was subsequently syringe-filtered (0.45 μm). Reference material (DOLT-4 fish litter tissue) was digested and determined in the same manner. Poultry kidney sample before extraction, sample extracts after extraction, residue on Day 28 and reference material were processed in triplicate. The extraction efficiency was also evaluated based on the mass balance calculation from acid digested kidney samples on Day 28.

2.2.4 Sample Analysis

2.2.4.1 Arsenic Speciation Analysis

Arsenic species in the filtered digested sample extracts were separated by PRP-X110 anion exchange column (7μm particle size, 150×4.1mm; Hamilton, Reno, NV) with a guard column (PRP-X110S; Hamilton). Agilent 1100 series (Agilent Technologies, Germany) HPLC system was used. Mobile phases prepared for gradient elution were as follows: A) 5% methanol; B) 120 mM ammonium bicarbonate (NH_4HCO_3) and 5% methanol, pH adjusted to 8.75. Mobile phase solutions were filtered through a 0.45 μm membrane and sonicated for 15 minutes before HPLC separation. Gradient elution program used for separation is shown in Table 2.3 and Figure 2.1. The HPLC setup was identical for both ICP-MS and ESI-MS detections.

Table 2.3 Gradient Elution Conditions for HPLC Separation. The flow rate was maintained constant at 2.0 mL/min. Mobile phase B started from 0% to 30% during the first 2 min, and stayed at 30% for 3 min. From 5 min to 8 min, the mobile phase B kept increasing to 100% and held for 9 min. After 17min, the mobile phase B returned to 0 % and maintained at 0 % for another four minute (18-22 min).

Time (min)	A	B	Flow rate (mL/min)
0.0	100%	0	2.0
2.0	70%	30%	2.0
5.0	70%	30%	2.0
8.0	0	100%	2.0
17.0	0	100%	2.0
18.0	100%	0	2.0
22.0	100%	0	2.0

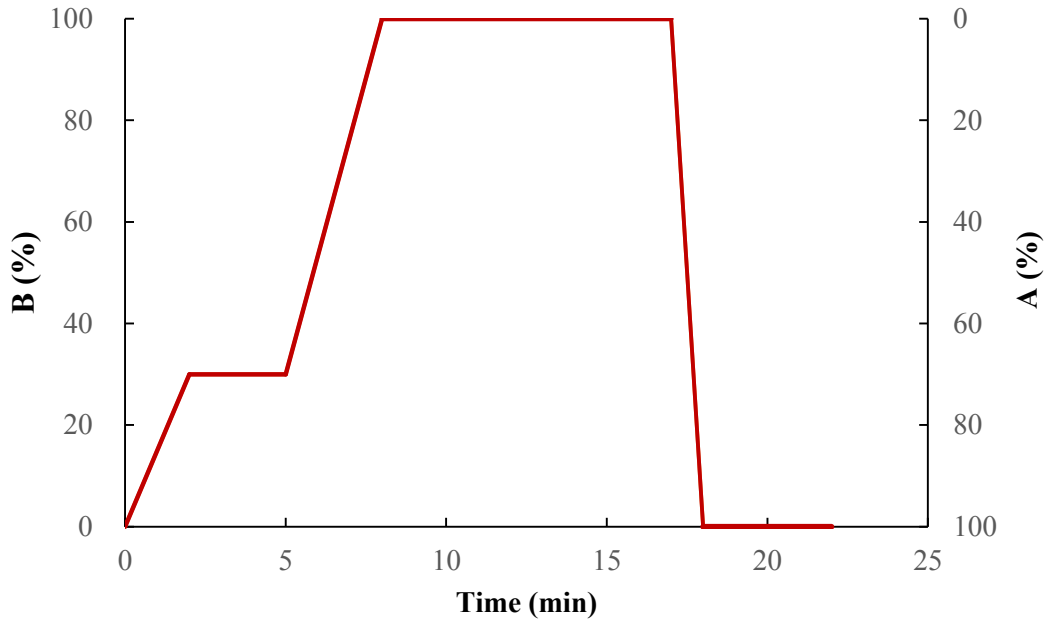


Figure 2.1 Gradient elution program for HPLC separation

2.2.4.2 Total Arsenic Analysis

Acid digested sample was directly introduced by ASX-5110 autosampler (CETAC, Omaha, NE) into the ICP-MS (Agilent 7500cs; Agilent Technologies, Japan). The mass to charge ratio *m/z* 75 was monitored for arsenic. The operating parameters of ICP-MS are shown in Table 2.4. Arsenic standards with a variety of concentrations are prepared in 1% HNO_3 and the concentration of arsenic standards are 0.1 µg/L, 0.5 µg/L, 1.0 µg/L, 5.0 µg/L, 10 µg/L, 20 µg/L. Standard Reference Material (SRM) 1640a trace elements in natural water was used to verify for the daily accuracy of instrument prior to sample analyzed.

Table 2.4 ICP-MS Operating Parameters for Arsenic Speciation in poultry kidney

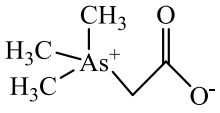
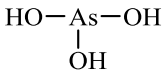
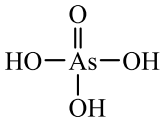
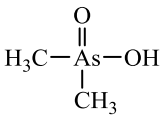
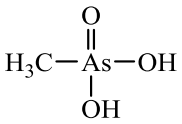
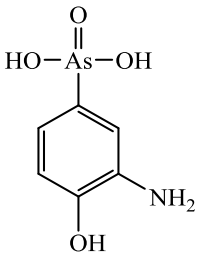
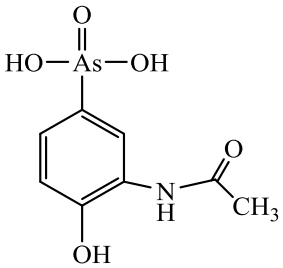
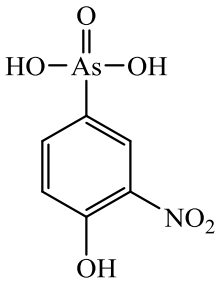
ICP-MS Parameters	
RF Power	1500 W
Ar flow rate	Carrier gas: 0.95 L/min
	Makeup gas: 0.15 L/min
Collision gas	He
	Gas flow: 3.5-4.5 mL/min
Isotope monitored	^{75}As
Points per peak	3

2.3 RESULTS AND DISCUSSION

2.3.1 Determination of Arsenic Species in Poultry Kidney by Using HPLC-ICP-MS

The arsenic species of interest in this study are listed in Table 2.5. These include the chicken feed additive Roxarsone (3-nitro-4-hydroxyphenylarsonic acid) and its potential metabolites, 3-amino-4-hydroxyphenylarsonic acid (3-AHPAA) and N-acetyl-4-hydroxyl-m-acrsanilic acid (N-AHAA). They also include inorganic arsenite (As^{III}), arsenate (As^{V}), and the methylarsenicals, monomethylarsonic acid (MMA^{V}), and dimethylarsinic acid (DMA^{V}). These are included because background levels of inorganic arsenicals are present in the chicken feed (as shown in Figure A.1), and the inorganic arsenicals can be metabolized to MMA^{V} and DMA^{V}. Arsenobetaine (AsB) is also included because arsenobetaine is a major arsenic species present in seafood and because the chicken feed contains “fish meal” as a protein source.

Table 2.5 Arsenic species included in this study

Abbrev.	Names	Structure
AsB	Trimethylarsonioacetate	
As^{III}	Arsenite	
As^{V}	Arsenate	
DMA^{V}	Dimethylarsinic acid	
MMA^{V}	Monomethylarsonic acid	
3- AHPAA	3-amino-4-hydroxyphenylarsonic acid	
N-AHAA	N-acetyl-4-hydroxyphenylarsonic acid	
Rox	3-nitro-4-hydroxyphenylarsonic acid	

Eight arsenic species in standard mixture 5 μg/L each were separated on a PRP-X110S anion exchange column and detected with ICP-MS. All arsenic species can be baseline-resolved within 15 minutes. The elution order was as follows: AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and Roxarsone (Figure 2.2.a).

A kidney sample collected on the Day 28 of the feeding experiment was also analyzed using the same method. The chromatogram is shown in Figure 2.2.b. The retention time match suggests the presence of AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and Roxarsone in poultry kidney on Day 28.

The matrix effect of kidney sample makes the peak of 3-AHPAA, N-AHAA and ROX shift slightly compared with the standard. In order to further identify the above eight arsenic species, standards of each of the expected arsenic species were spiked into the sample and analyzed by HPLC-ICP-MS. If the peak of the suspected arsenic species in kidney increased with the corresponding addition of the arsenic standard, the suspected species is tentatively identified. Chromatograms from the analyses of the kidney sample (red traces) and the sample supplemented with individual arsenic species (black traces) are shown in Figure 2.3 (a-h). These include the addition of arsenobetaine (AsB), inorganic arsenite (As^{III}), dimethylarsinic acid (DMA^{V}), monomethylarsonic acid (MMA^{V}), inorganic arsenate (As^{V}), 3-amino-4-hydroxyphenylarsonic acid (3-AHPAA), N-acetyl-4-hydroxy-m-arsanilic acid (N-AHAA), and Roxarsone, respectively.

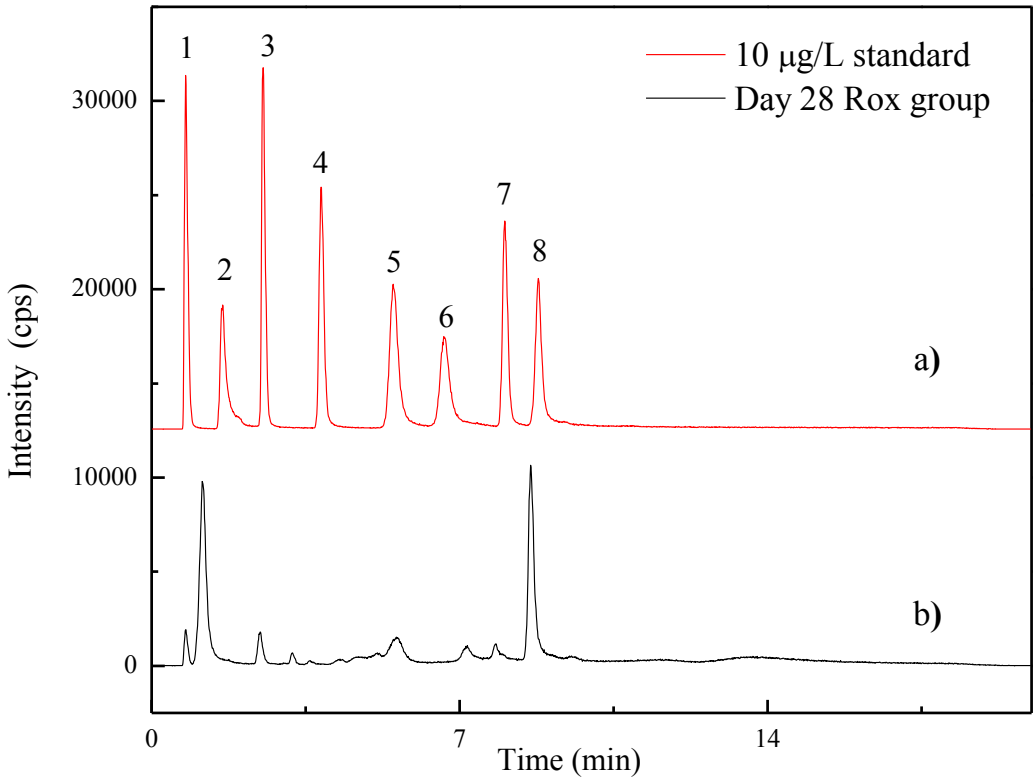


Figure 2.2 Chromatograms obtained from HPLC-ICP-MS analyses of 10.0 µg/L arsenic standards (a) and a kidney sample (b). The peaks correspond to (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) N-AHAA and (8) Roxarsone. The kidney sample was collected on Day 28 from ROX-fed group in Pen # 13, strain Ross 308.

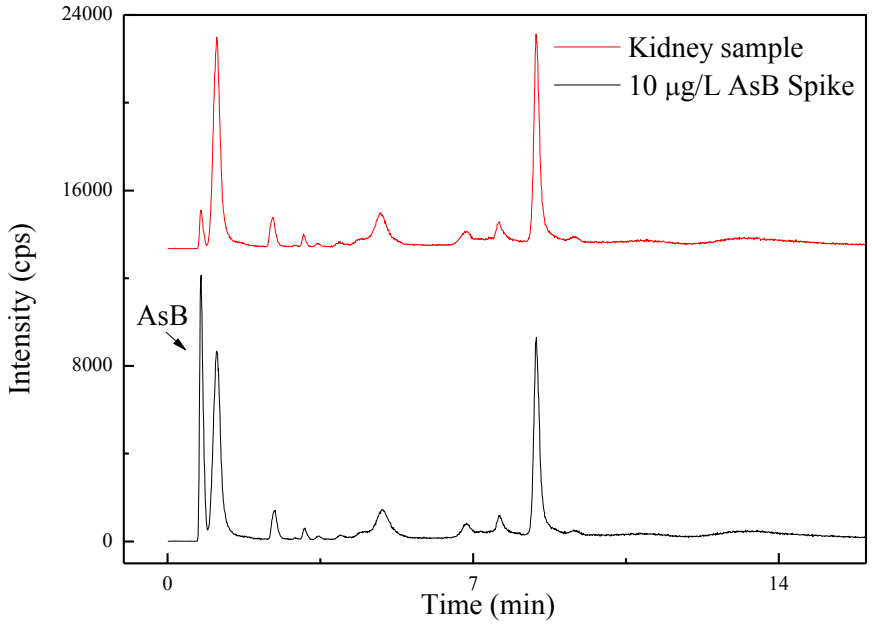


Figure 2.3 (a) Chromatograms obtained from HPLC-ICP-MS analyses of a kidney sample (top trace) and the same sample spiked with 10 µg/L of arsenobetaine (AsB) (bottom trace).

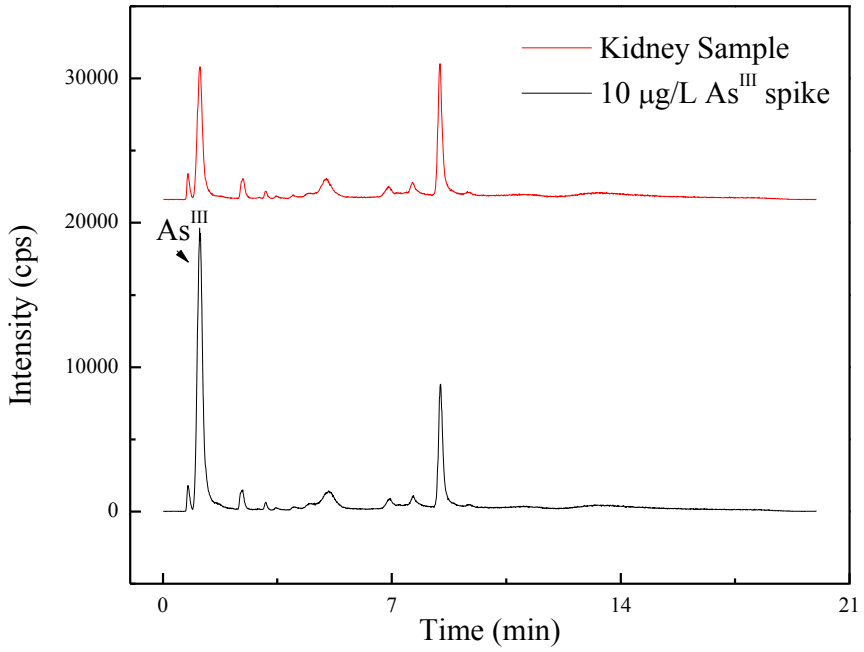


Figure 2.3 (b) Chromatograms obtained from HPLC-ICP-MS analyses of a kidney sample (top trace) and the same sample spiked with 10 µg/L of inorganic arsenite (As^{III}) (bottom trace).

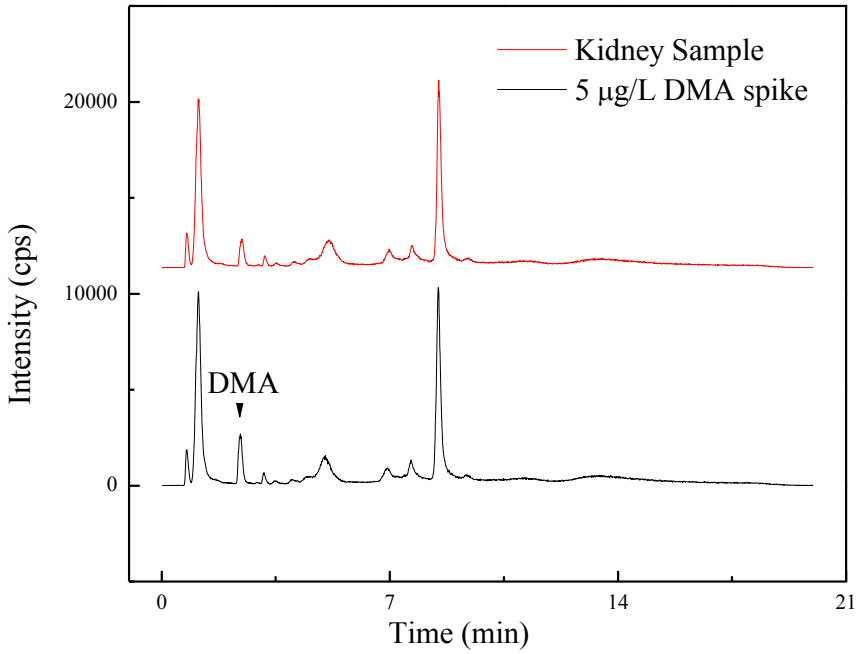


Figure 2.3 (c) Chromatograms obtained from HPLC-ICP-MS analyses of a kidney sample (top trace) and the same sample spiked with 5 µg/L of dimethylarsinic acid (DMA) (bottom trace).

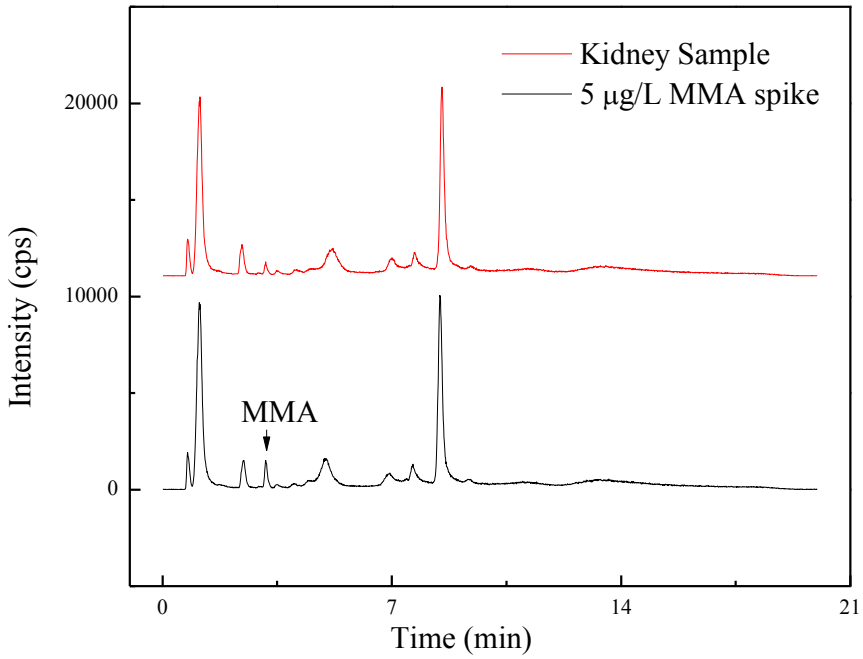


Figure 2.3 (d) Chromatograms obtained from HPLC-ICP-MS analyses of a kidney sample (top trace) and the same sample spiked with 5 µg/L of monomethylarsonic acid (MMA^V) (bottom trace)

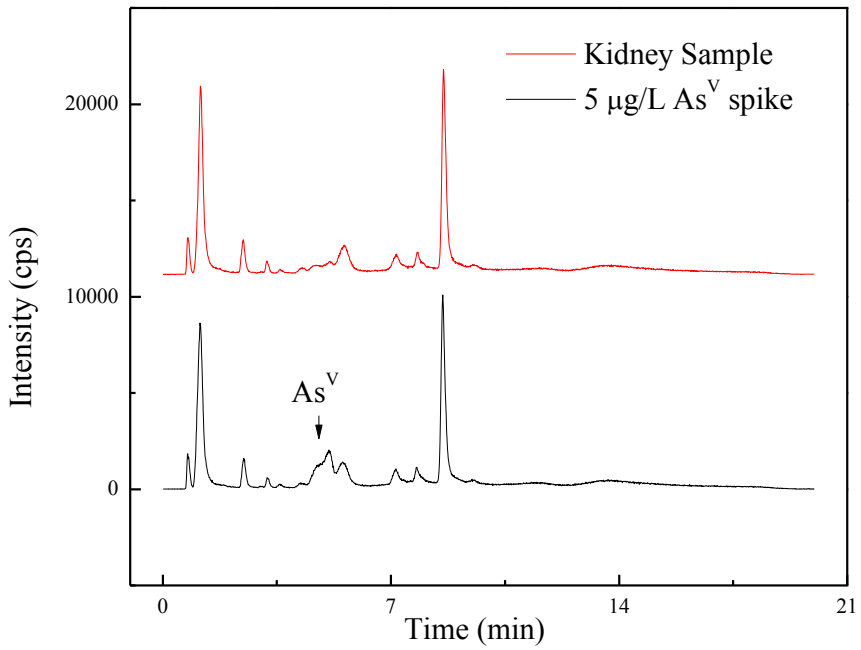


Figure 2.3 (e) Chromatograms obtained from HPLC-ICP-MS analyses of a kidney sample (top trace) and the same sample spiked with 5 µg/L of inorganic arsenate (As^{V}) (bottom trace)

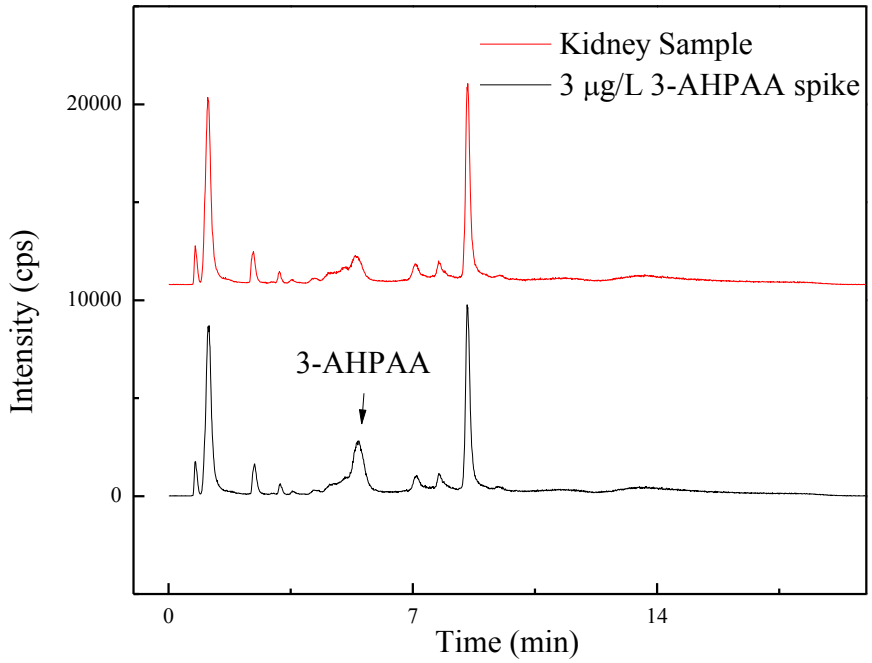


Figure 2.3 (f) Chromatograms obtained from HPLC-ICP-MS analyses of a kidney sample (top trace) and the same sample spiked with 3 µg/L of 3-amino-4-hydroxy-phenylarsonic acid (3-AHPAA) (bottom trace)

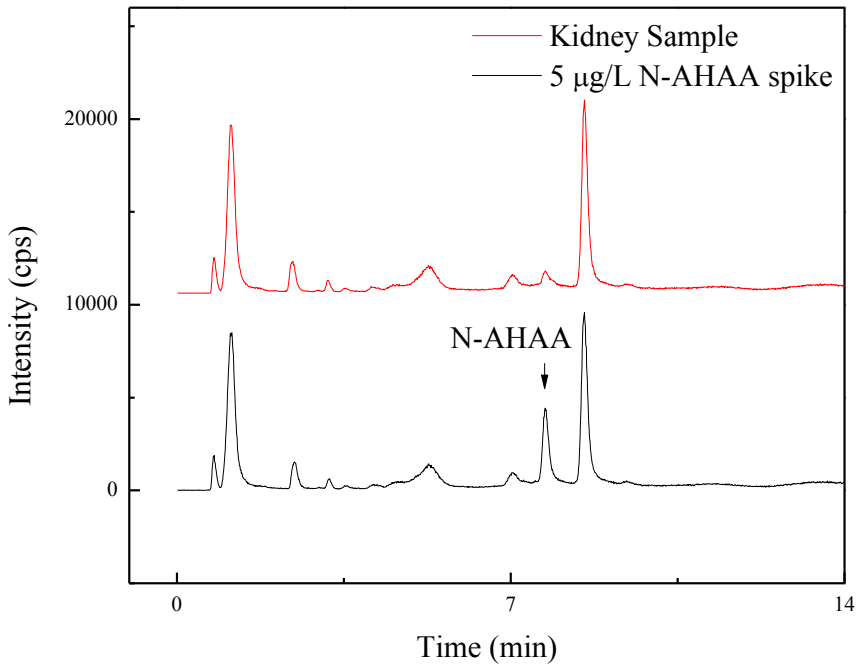


Figure 2.3 (g) Chromatograms obtained from HPLC-ICP-MS analyses of a kidney sample (top trace) and the same sample spiked with 5 µg/L of N-acetyl-4-hydroxy-m-arsanilic acid (N-AHAA) (bottom trace)

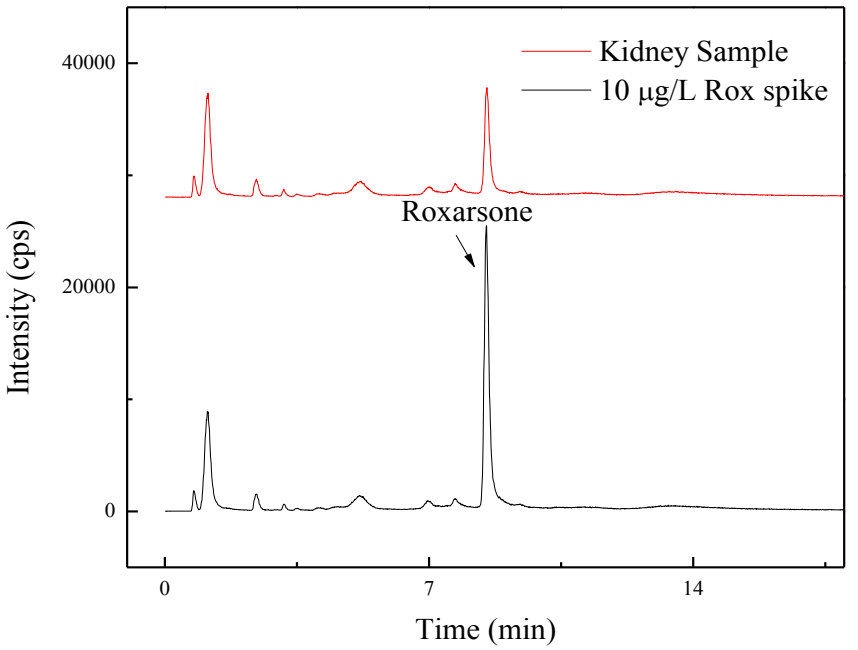


Figure 2.3 (h) Chromatograms obtained from HPLC-ICP-MS analyses of a kidney sample (top trace) and the same sample spiked with 10 µg/L of 3-nitro-4-hydroxy-phenylarsonic acid (ROX) (bottom trace)

2.3.2 Identification of Arsenic Species in Poultry Kidney by Using HPLC-ESI-MS

The identification of arsenic species was further supported by results from HPLC-ESI-MS. The optimized operating parameters and MRM transitions condition are shown in Table 2.6 and Table 2.7.

Arsenobetaine was monitored using the positive ionization mode. All other seven arsenic species were monitored using the negative ionization mode. Figure 2.4 shows chromatograms from the HPLC-ESI-MS and HPLC-ICP-MS analyses of a chicken kidney extract spiked with 10 μg/L of arsenic standards. The separation was achieved on a single column. The effluent flow was split 80% to the ICP-MS and 20% to the ESI-MS. The simultaneous detection by ICP-MS and ESI-MS was complementary. While the ICP-MS provided arsenic-specific detection at m/z 75.0, the ESI-MS detection used specific MRM transitions (Table 2.7) that represented the specific fragment ions and the parent molecular ions of the arsenic species. The retention time of each arsenic species between the two chromatograms are consistent. This is expected because both chromatograms were from the same HPLC separation. HPLC separation with simultaneous ICP-MS and ESI-MS detections was used for identification and quantitation of arsenic species.

Table 2.6 Selected operating parameters of ESI-MS (5500 QTRAP) using MRM Mode

ESI-MS Parameters		
	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)	500 ℃	500 ℃
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 2.7 Condition used for the fragmentation of arsenic species and the MRM transitions chosen for detection.

Arsenic Species	Polarity	Molecular ion (m/z)	Characteristic fragments	DP (V)	CE (V)	CXP (V)
AsB	Pos	179	105	71	37	9
			120	71	28	11
As^{III}	Neg	125	107	-10	-18	-15
DMA^{V}	Neg	137	107	-70	-30	-11
			122	-70	-18	-13
MMA^{V}	Neg	139	107	-40	-40	-43
			124	-40	-24	-7
As^{V}	Neg	141	107	-15	-58	-13
			123	-15	-20	-7
3-AHPAA	Neg	232	107	-20	-64	-11
			123	-20	-28	-25

N-AHAA	Neg	274	165	-45	-26	-9
			123	-45	-36	-11
ROX	Neg	262	107	-30	-94	-15
			123	-30	-38	-11

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

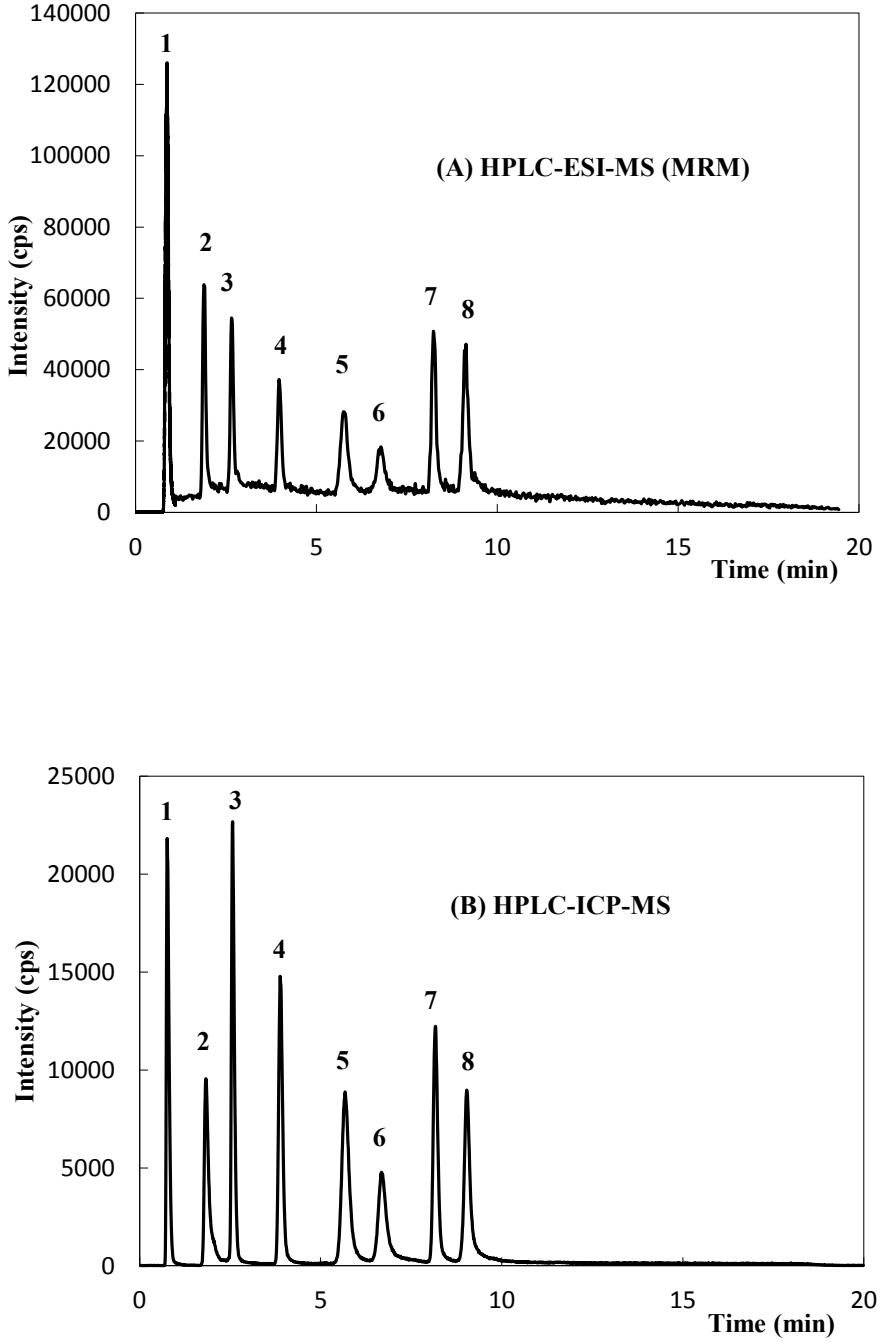


Figure 2.4 Chromatograms obtained from the same HPLC separation with both ESI-MS (top trace) and ICP-MS (bottom trace) detections. The sample was a chicken kidney extract spiked with 10.0 µg/L of (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) N-AHAA and (8) Roxarsone.

2.3.3 Enzyme-assisted Extraction of Arsenic Species from Poultry Kidney

2.3.3.1 Total arsenic in poultry kidney from Lake Poultry and Warburg

The total concentrations of arsenic in kidney samples obtained from Lake Poultry Processor and Warburg Poultry Processor were 2.7 μg/kg (RSD=5.9%). These concentrations are similar to background value, reported from a market survey of chicken meat. [16]

2.3.3.2 Arsenic species in six enzyme solutions

Figure 2.5 shows the HPLC-ICP-MS results from the analyses of six enzyme solutions in their respective optimum buffers. Only pepsin (Figure 2.5e) and papain (Figure 2.5f) have relatively low background of arsenic (<0.5 μg/L). The analyses of pancreatin, subtilisin, and trypsin show an unknown arsenic peak between 3-AHPAA and N-AHAA. The concentration of this unknown species is more than 0.5 μg/L. In order to achieve low detection limit for arsenic speciation in poultry kidney, I chose the enzymes (pepsin, papain, and pancreatin) that have relatively low arsenic background for enzyme extraction.

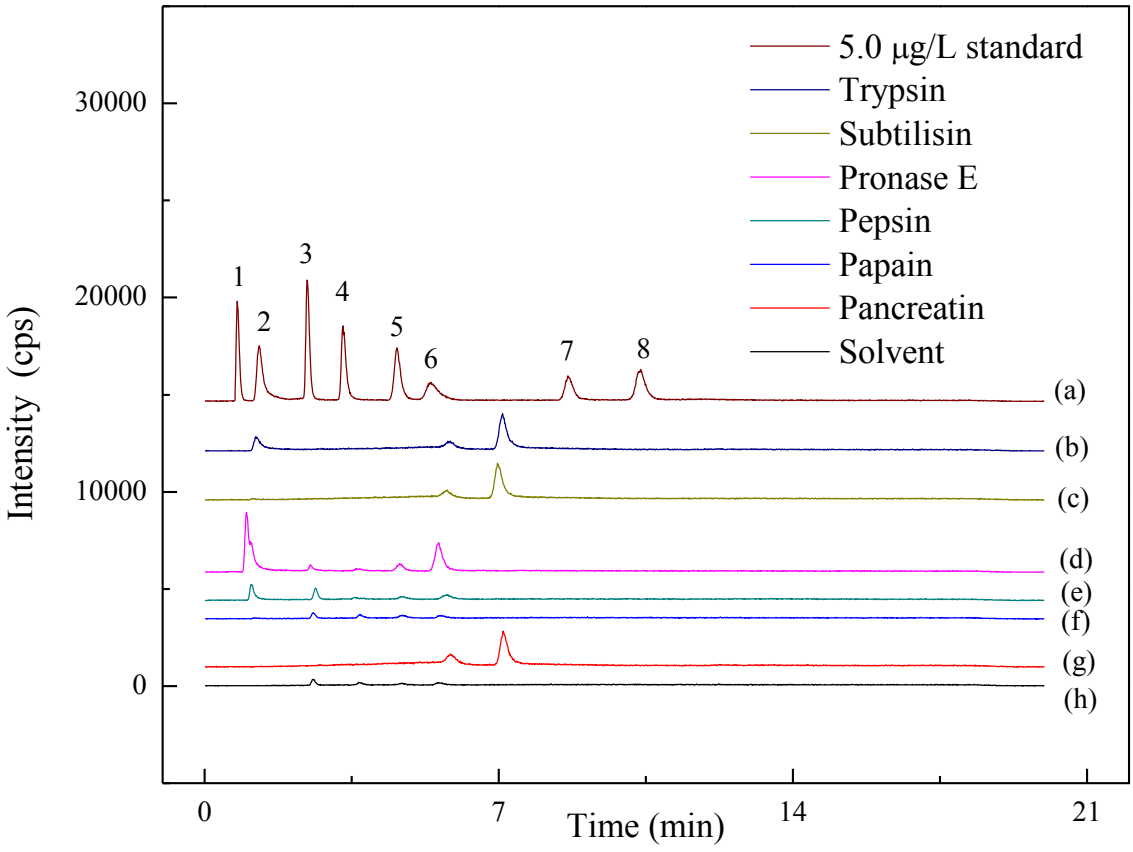


Figure 2.5 Chromatograms obtained from HPLC-ICP-MS analyses of arsenic standards (a) and seven enzyme solutions (b - h). (a) A standard solution containing 5 μg/L of (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) N-AHAA and (8) Roxarsone. (b) Trypsin in 5% $NH_3 \cdot H_2O$ buffer solution, pH=8.0; (c) Subtilisin in 5% $NH_3 \cdot H_2O$ buffer solution, pH=8.0; (d) Pronase E in 20 mM NH_3HCO_3 solution, pH=7.5; (e) Pepsin in 0.5% HCl buffer solution, pH=2.0; (f) Papain in 20 mM NH_3HCO_3 solution, pH=7.5; (g) Pancreatin in 5% $NH_3 \cdot H_2O$ buffer solution (h) CH_3OH:H_2O (1:1) solution in, pH=8.0

2.3.3.3 Comparison of Extraction Recovery of Arsenic Species using Three Different Enzymes

Eight arsenic mixture standards (20 μg/L each) were spiked into replicate aliquots of a kidney sample. These replicate samples were then separately treated with pepsin, papain, and pancreatin. The recoveries of each arsenic species were compared. Representative chromatograms are shown in Figure 2.6. The pepsin treatment can achieve the best recovery for all eight arsenic species in poultry kidney. Papain treatment results in lower recovery of Roxarsone. The pancreatin treatment cannot efficiently recover most of the arsenic species, such as DMA, 3-AHPAA and Roxarsone. Therefore, pepsin was chosen to be used in subsequent experiments.

2.3.3.4 Comparison of Pepsin Treatment with Solvent Extraction

A mixture of water-methanol is commonly used to extract arsenic species from biological samples. The treatment of kidney samples with pepsin was compared to the extraction with water-methanol. Figure 2.7 shows the chromatograms obtained from the HPLC-ICP-MS analyses of the pepsin-treated extract and the water-methanol extract of the kidney sample. The methanol-water extraction cannot achieve as good recovery as pepsin for As^{III}, DMA, and Roxarsone. The results demonstrate that the pepsin treatment is able to improve extraction of arsenic species from poultry kidney.

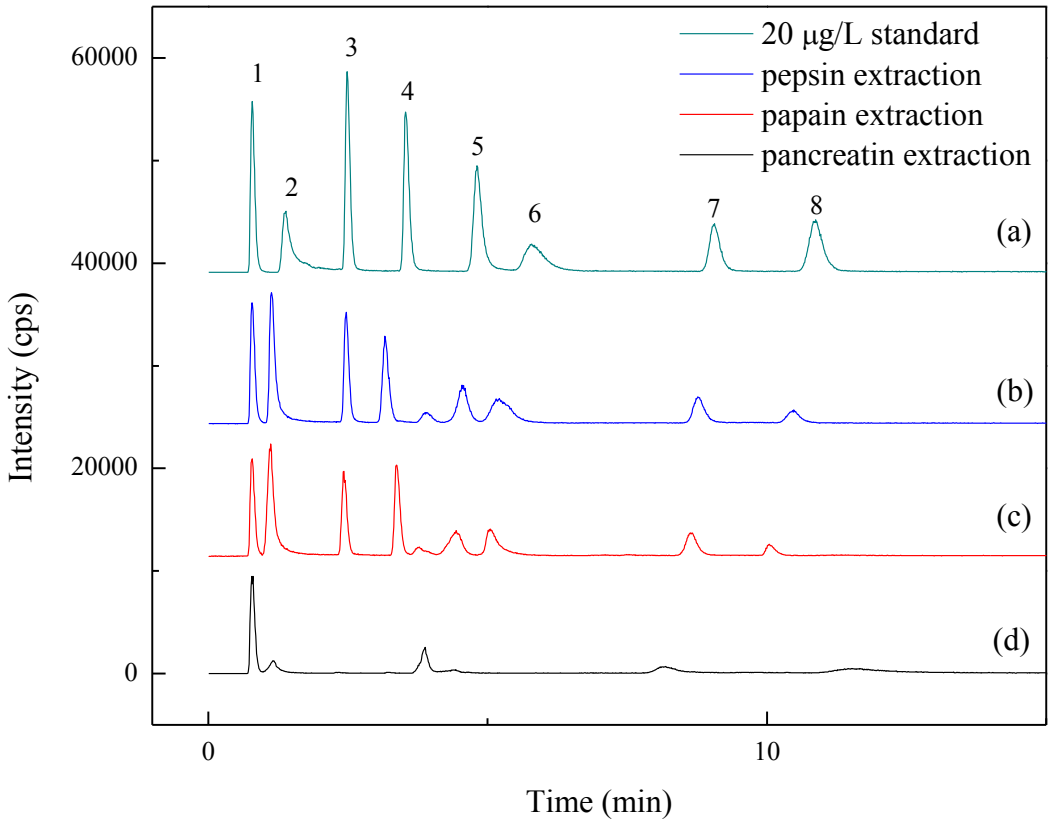


Figure 2.6 Chromatograms obtained from HPLC-ICP-MS analyses of replicate kidney samples treated with pepsin (b), papain (c) and pancreatin (d). Prior to the enzyme treatment, the kidney sample was spiked with 20 µg/L each of eight arsenic species: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) N-AHAA and (8) Roxarsone. (a) 5 µg/L mixture standard; (b) Kidney sample treated with pepsin; (c) Kidney sample treated with papain; (d) Kidney sample treated with pancreatin.

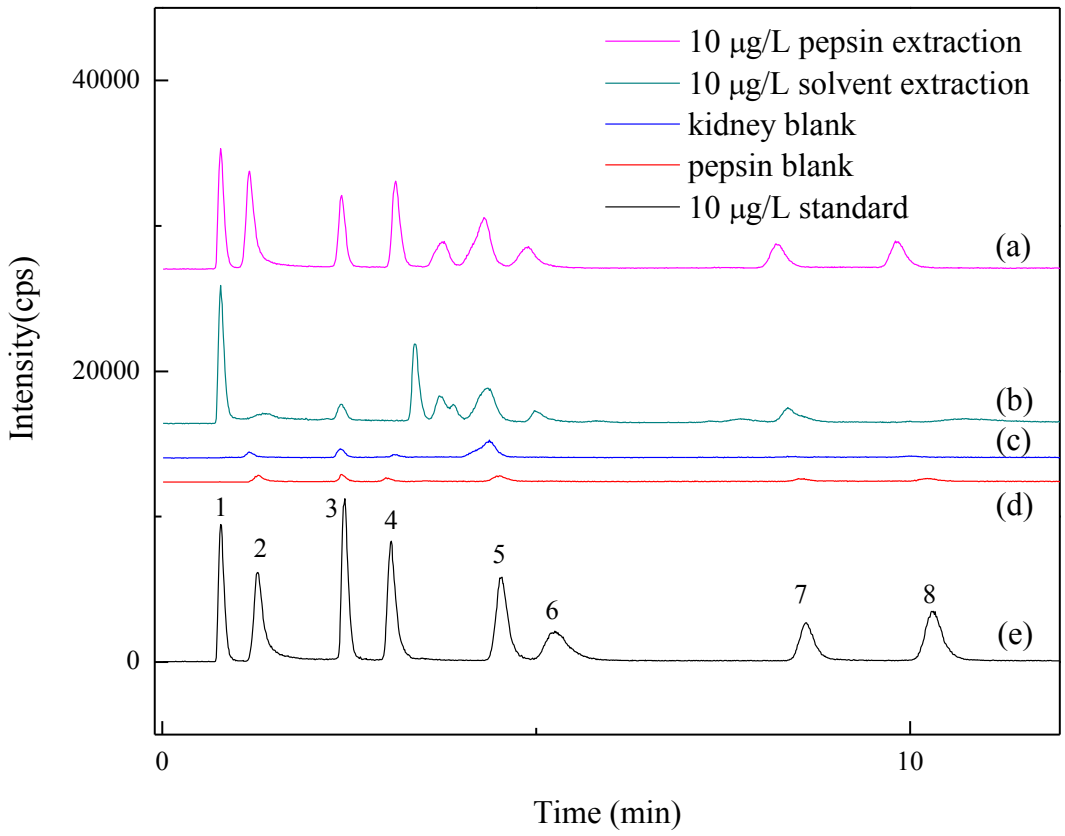


Figure 2.7 Chromatograms obtained from HPLC-ICP-MS analyses of kidney samples treated with pepsin or extracted with water-methanol. (a) Kidney samples spiked with 10 µg/L arsenic standards and treated with pepsin; (b) kidney sample spiked with 10 µg/L arsenic standards and extracted with a mixture of water-methanol (50% each); (c) kidney sample treated with pepsin; (d) pepsin in buffer; (e) standard solution containing 10 µg/L each of (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) N-AHAA and (8) Roxarsone.

2.3.3.5 Recovery of Eight Arsenic Species Extracted with Pepsin Treatment

The overall recoveries of eight arsenic species, involving the pepsin treatment, sample dilution, and HPLC-ICP-MS analysis, are shown in Figure 2.8. The recoveries ranged from 49% for DMA to 95% for AsB. These recovery values are in general better than using the method of water-methanol extraction (Figure 2.7). Although the overall recoveries (49% - 95%) are not ideal, the results show that the combination of the pepsin treatment of kidney sample with HPLC-ICP-MS analysis of the extract remains the best option available. The recovery of Roxarsone, the expected major arsenic species in Roxarsone-treated chickens, is 52%.

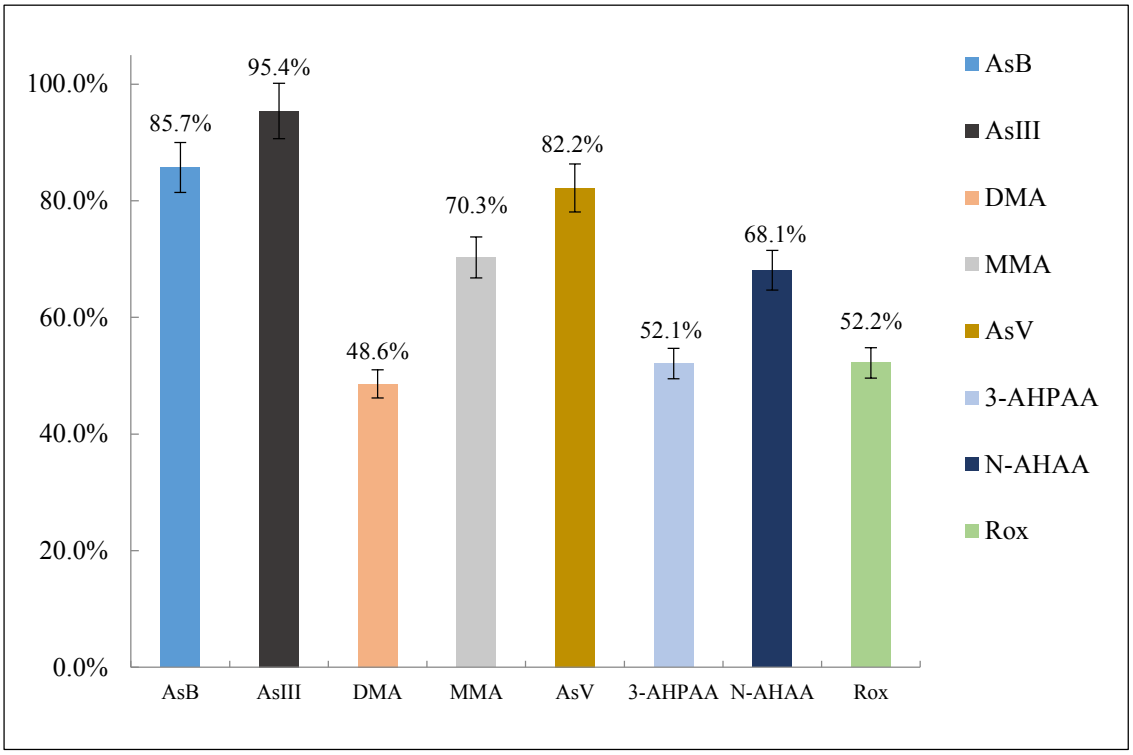


Figure 2.8 Recoveries of eight arsenic species (AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, ROX) extracted by pepsin treatment and analyzed using HPLC-ICP-MS. Recoveries were based on the determination of arsenic species spiked to chicken kidney samples. The error bars represent one standard deviation from triplicate analyses of spiked kidney homogenate. The kidney sample was from Warburg Poultry Processor.

2.3.4 Extraction Efficiency of Arsenic Species for Pepsin-treated Kidney Samples

For evaluation of the extraction efficiency of arsenic species from poultry kidney, aliquots of a kidney sample from a Roxarsone-fed chicken on Day 28 were treated as following. One aliquot of the sample was treated with pepsin, and the extracts and the residue were separately digested with acids and analyzed with ICP-MS for total arsenic concentrations. Another aliquot of the kidney sample was directly digested with acid and analyzed with ICP-MS for total arsenic. The mass balance of arsenic between the two sets of analyses was obtained. The percent of arsenic extracted with the pepsin treatment over the total concentration of arsenic in the kidney sample represent the extraction efficiency.

Similarly, methanol-water extraction of the replicate aliquots of the kidney sample was performed. The mass balance of arsenic and extraction efficiency were obtained similarly.

Figure 2.9 shows the total arsenic concentration in the kidney sample, the concentrations of arsenic in the extracts and in the residues, and the sum of arsenic concentrations in the extract and residue. The extraction efficiency with the pepsin treatment is 64.3%. The extraction efficiency using methanol-water is 27.4%. With the procedure of the pepsin treatment, the sum of arsenic in the extract and in the residue equals to the total arsenic concentration in the kidney sample, suggesting that there is no loss of arsenic during the treatment processes. The lack of mass balance in the methanol-water procedures indicates that there is a loss of arsenic during the treatment process. The methanol-water extraction requires evaporating methanol and transferring the solution for several times that may cause sample loss. For the pepsin extraction, the loss of sample is minimum as the sample processing avoids evaporation and dilution of enzyme solution.

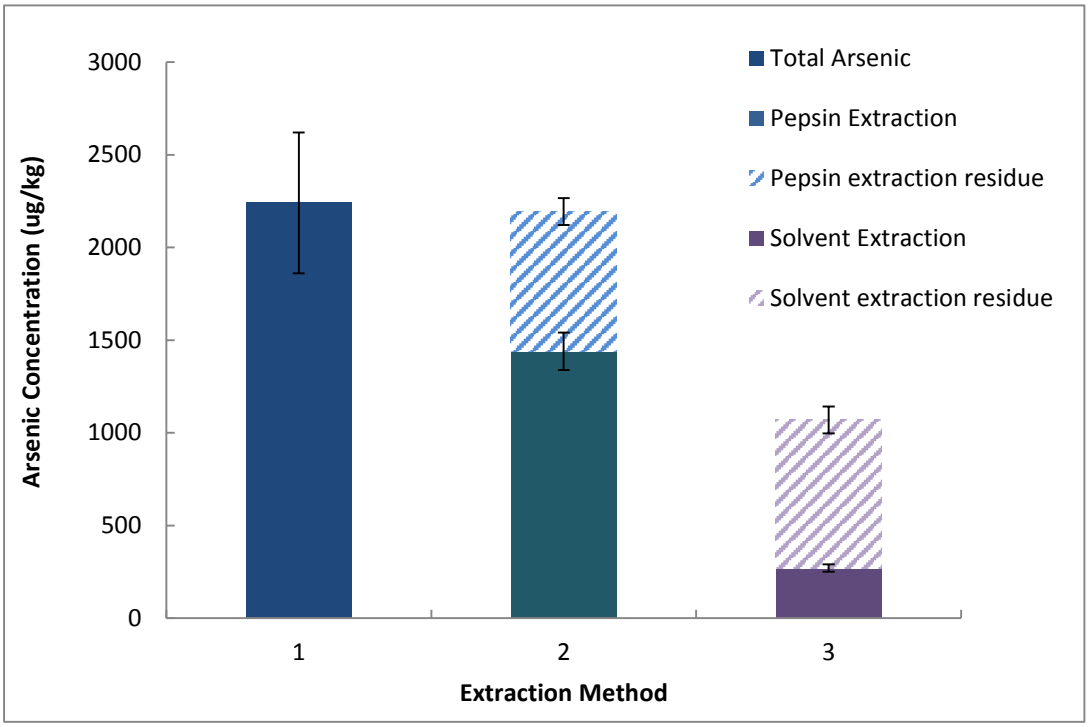


Figure 2.9 Extraction efficiency for pepsin extraction and solvent extraction for arsenic species in poultry kidney sample. Residues after pepsin and solvent extraction were also digested and analyzed. Kidney samples were acquired from ROX-Group on Day 28 (Pen #11, Ross 308). The first aliquot of the kidney sample was directly digested with acid and analyzed with ICP-MS for total arsenic. The second aliquot of the sample was treated with pepsin, and the extracts and the residue were separately digested with acids and analyzed with ICP-MS for total arsenic concentrations. The third aliquot of the sample was treated with methanol/water, and the extracts and the residue were separately digested with acids and analyzed with ICP-MS for total arsenic concentrations. The error bars represent one standard deviation from triplicate analyses from total arsenic concentration.

2.3.5 Quantitation of Arsenic Species Using HPLC-ICP-MS

After the arsenic species were identified using simultaneous HPLC-ICP-MS and HPLC-ESI-MS/MS, HPLC-ICP-MS was further used for the quantitation of individual arsenic species. Chromatograms from the HPLC-ICP-MS analyses of 8 arsenic standard species, at concentrations of 0.1, 0.5, 1.0, 5.0, 10.0, and 20.0 µg/L, are shown in Figure 2.10. The calibration equation and correlation coefficient are listed in Table 2.8.

Method limits of detection (LOD) for arsenic species in chicken kidney (µg/kg), taking into account of sample extraction, dilution, and HPLC-ICP-MS analysis, were between 1.0 µg/kg and 2.0 µg/kg as shown in Table 2.9. The method LOD was 2.0 µg/kg as As for 3-AHPAA and 1.0 µg/kg as As or better for other arsenic species. The low LOD is suitable for quantitation of arsenic species in poultry kidney.

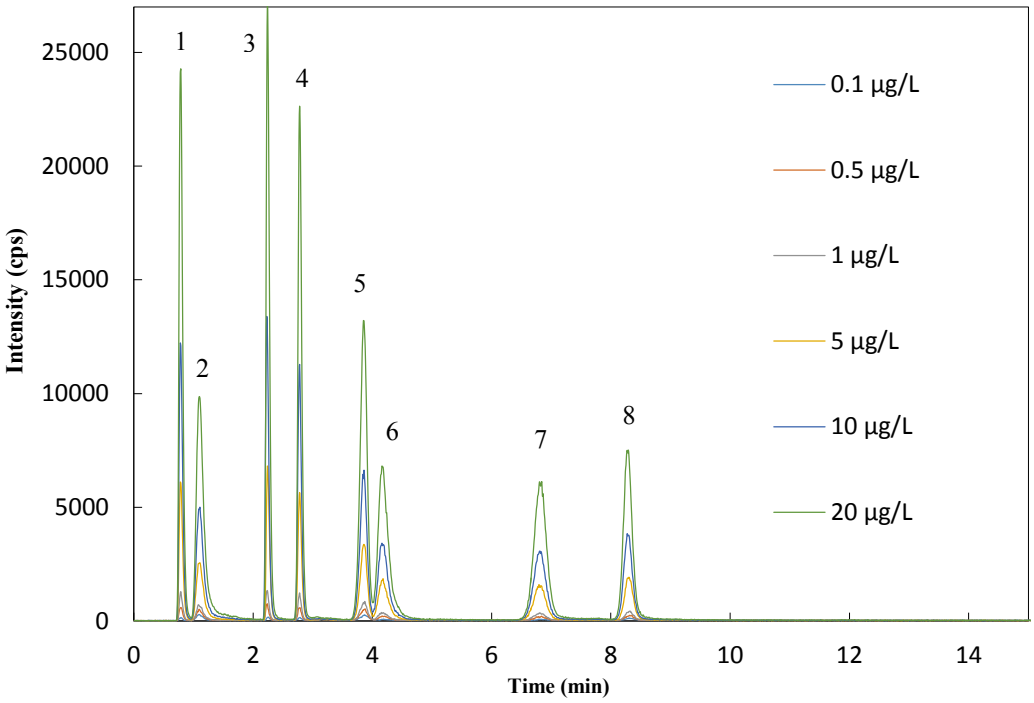


Figure 2.10 Representative chromatograms obtained from the HPLC-ICP-MS analyses of eight arsenic species at concentration ranging from 0.1 µg/L to 20 µg/L. The arsenic species were (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) N-AHAA and (8) Roxarsone. Gradient elution condition were shown in Table 2.3. Their concentration were 0.1, 0.5, 1.0, 5.0, 10, and 20 µg/L.

Table 2.8 The calibration equation and correlation coefficient for arsenic species

Arsenic Species	Calibration equation	R^2
AsB	y = 54270x - 8369.2	0.9999
As^{III}	y = 44019x - 5439.7	0.9992
DMA^{V}	y = 54657x - 7478.3	0.9999
MMA^{V}	y = 50436x - 4519.2	1.0000
As^{V}	y = 51042x + 9282.4	1.0000
3-AHPAA	y = 35239x – 12854	0.9998
N-AHAA	y = 44916x – 11028	0.9999
Roxarsone	y = 39096x - 4839.4	0.9999

Table 2.9 Limits of detection (LOD) for arsenic species in poultry kidney. Concentration units are micrograms of As per kilogram of wet weight kidney.

Arsenic Species	LOD(μg/kg)
AsB	1.0
As^{III}	1.0
DMA^{V}	1.0
MMA^{V}	1.0
As^{V}	1.0
3-AHPAA	2.0
N-AHAA	1.0
ROX	1.0

2.4 CONCLUSIONS

The extraction method was optimized and pepsin was chosen for arsenic extraction from poultry kidney. The overall recoveries for eight arsenic species ranged from 49~95%. The overall extraction efficiency was 64.3%. Compared with reported arsenic extraction method, such as traditional solvent extraction, the enzyme-assisted extraction method did not require the evaporation of extracts. Thus, the enzyme-assisted extraction method can avoid the loss and contamination of the analytes. The established method is easy to operate, and can significantly increase the overall extraction efficiency of arsenic species.

HPLC separation was coupled to simultaneous ICP-MS and ESI-MS detections. Following the same HPLC separation, ICP-MS provided element-specific detection at m/z 75.0 and the multiple reaction monitoring (MRM) using ESI-MS/MS provided molecular information of the arsenic species. Target arsenic species including AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and ROX, were baseline resolved using anion exchange chromatography. The LOD for arsenic quantitation using HPLC-ICP-MS was as low as 1.0 μg/kg, which is suitable for quantifying existing arsenic-containing compounds in poultry kidney samples. Previous study only relied on single technique for arsenic quantification or identification. However, either ICPMS or ESIMS has its own merits and defects if used separately. High sensitivity and wide dynamic range of ICP-MS is ideal for quantification of arsenic species, but no structural information of arsenic species can be obtained from ICP-MS analyses. While ESI-MS is able to provide a wealth of structural information of analytes of interest, the detection limit of ESI-MS is poorer, and ESI-MS is more susceptible to matrix effects and ion interferences. The combination of HPLC-ICP-MS and ESI-MS can overcome the shortcomings of each technique, and provide complementary information for arsenic speciation analysis.

2.5 REFERENCES

(1) Rosenberg, E.; Ariese, F. *Trace Element Speciation for Environment, Food and Health*. *The Royal Society of Chemistry, Cambridge*, UK, **2001**, *17*, 164

(2) Bermejo, P.; Capelo, J.; Mota, A.; Madrid, Y.; Cámara, C. TrAC *Trends in Analytical Chemistry* **2004**, *23*, 654.

(3) Carpenter, R. C. *Analytica Chimica Acta* **1981**, *125*, 209.

(4) Pardo-Martinez, M.; Vinas, P.; Fisher, A.; Hill, S. J. *Analytica Chimica Acta* **2001**, *441*, 29.

(5) Harrington, C. F.; Clough, R.; Hansen, H. R.; Hill, S. J.; Pergantis, S. A.; Tyson, J. F. *Journal of Analytical Atomic Spectrometry* **2009**, *24*, 999.

(6) Jackson, B. P.; Bertsch, P. M. *Environmental Science & Technology* **2001**, *35*, 4868.

(7) Wang, P.; Zhao, G.; Tian, J.; Su, X. *Journal of Agricultural and Food Chemistry* **2010**, *58*, 5263.

(8) Grant, T. D, Jones, J. F. *Journal American Water Works Association*, **1977**, *69*,12

(9) McSheehy, S.; Marcinek, M.; Chassaigne, H.; Szpunar, *J. Analytica Chimica Acta* **2000**, *410*, 71.

(10) Hansen, H. R.; Raab, A.; Feldmann, J. *Journal of Analytical Atomic Spectrometry* **2003**, *18*, 474.

(11) Corr, J. J.; Larsen, E. H. *Journal of Analytical Atomic Spectrometry* **1996**, *11*, 1215.

(12) Gallagher, P. A.; Wei, X. Y.; Shoemaker, J. A.; Brockhoff, C. A.; Creed, J. T. *Journal of Analytical Atomic Spectrometry* **1999**, *14*, 1829.

(13) Pergantis, S. A.; Wangkarn, S.; Francesconi, K. A.; Thomas-Oates, J. E. *Analytical Chemistry* **2000**, *72*, 357.

(14) Madsen, A. D.; Goessler, W.; Pedersen, S. N.; Francesconi, K. A. *Journal of Analytical Atomic Spectrometry* **2000**, *15*, 657.

(15) Pedersen, S. N.; Francesconi, K. A. *Rapid Commun Mass Sp* **2000**, *14*, 641.

(16) Nachman, K. E.; Baron, P. A.; Raber, G.; Francesconi, K. A.; Navas-Acien, A.;

D. C. *Environ Health Perspect* **2013**, *121*, 818.

CHAPTER 3

DETERMINATION OF ARSENIC SPECIES IN THE KIDNEY OF CHICKENS FED THE ROXARSONE-SUPPLEMENETD FOOD OR THE CONTROL FOOD

3.1 INTRODUCTION

3-nitro-4-hydroxyphenyl arsenic acid (also known as Roxarsone) has been approved for use as a feed additive in chicken, turkeys and swine [1-4]. The use of Roxarsone in chicken feed was intended to control disease of chicken, and to improve the weight gain, feed efficiency, and meat pigmentation.[3-5] In the past, it was believed that most of the ingested Roxarsone was excreted unchanged. However, a report by FDA in 2012 showed increases in the concentration of inorganic arsenic in chicken liver.[4] We are conducting of feeding study of a much larger scale, involving a total of 1600 chickens. We fed 800 chickens with a Roxarsone-supplemented diet and another 800 chickens with a Control diet for up to 35 days. This thesis focuses on the arsenic speciation analysis in kidney of a subset of 142 chickens from a total of 1600 chickens. Of this subset, 71 chickens were fed with a Roxarsone-supplemented diet, and the other 71 chickens were fed with a control diet not supplemented with Roxarsone. This study mainly focuses on the distribution of individual arsenic species in chicken kidney over the 35-day feeding period.

In North America, chicken kidney is usually discarded, or processed for use as fertilizer and pet food. However, people in Asia and Europe consume kidney as food. To our knowledge, arsenic species in kidney of Roxarsone-fed poultry has never been characterized before. It is important to investigate if metabolites from Roxarsone are present in the kidney of poultry and how much of each arsenic species remains in kidney after the stop of Roxarsone feeding. Furthermore, determination of metabolites of

Roxarsone in kidney may help us better understand the transformation of different arsenic species in chicken body.[5,6]

3.2 EXPERIEMENT

3.2.1 Sample Collection

A 35-day chicken feeding experiment was carried out at the Poultry Research Centre, University of Alberta, Canada. The study protocol was approved by the Animal Care and Use Committee for Livestock, Faculty of Agricultural, Environment, and Life Sciences, University of Alberta (Protocol # 094/05/10). In total, 1600 chickens, representing two commercial strains (Cobb 500 and Ross 308) of broiler chickens, were used. This thesis involved the analysis of a subset of 192 chicken kidney samples.

Both strains had 8 pens each, which were divided into two groups (Figure 3.1), Rox Group (chicken fed with a Roxarsone diet) and Control Group (chicken fed with a Roxarsone free diet). Samples were collected on Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35. On each day, one chicken from each pen was euthanized and the kidney sample was gathered. Therefore, 16 chicken kidney samples were obtained on each collection day. In total, 192 kidney samples were collected throughout the whole experiment. The sample were all stored at -80 ℃ until analysis.

Poultry food used was prepared by the Poultry Research Centre, University of Alberta. There were three periods (uptake period, growth period, and ROX withdrawal period) as shown in Table A.1 (Appendix) for poultry feeding. During the uptake period, from Day 0 to Day 14, the chickens in the ROX group were fed with ROX-supplemented diet. The growth period lasted from Day 14 to Day 28, and the chickens in the ROX group were supplied with ROX-supplemented diet for growth period. After Day 28, there were 7 days of the withdrawal period for chickens in the ROX group, where ROX-

free diets were provided to the chickens in this period. Chickens in the control group were fed with ROX-free diet for the entire feeding period.

Typical chromatograms of chicken food samples are shown in Figure A.1 (Appendix) and the quantitative results of feed samples are summarized in Table A.2 (Appendix). ROX-supplemented diets (ROX starter and ROX grower) had total arsenic exceeding 18000 µg As/kg, mainly in the form of ROX itself. Metabolites, 3-AHPAA or N-AHAA were not observed in the feed. The analysis of six diets also showed the presence of small amounts of AsB, DMA^V and As^V.

Chicken body weights over the entire feeding period were monitored as well. They were summarized in Appendix Table A.3. There is no significant difference ($P > 0.05$) in the body weights of two strains of chickens in the control and the ROX-treated groups.

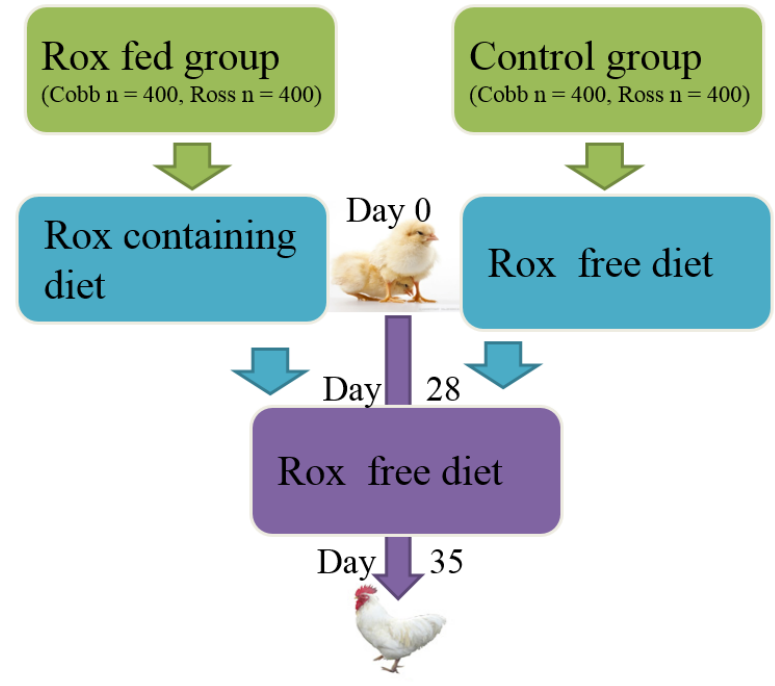


Figure 3.1 Schedules of feeding 1600 chickens a Roxarsone-containing diet or a control diet (Roxarsone free diet)

3.2.2 Sample Treatment

0.5 g of homogenized chicken kidney sample was weighed into a 10 mL polypropylene centrifuge tube (Thermo-Fisher Scientific). Accurate sample weight was recorded. 5.00 mL of pepsin solution was added to the tube by pipet. Then an ultrasonic homogenize probe (Kitchen Aid) was used to further homogenize the mixture.

After vortexing, the mixture was incubated at 37 ℃ overnight to allow pepsin to thoroughly digest the kidney sample. The next day, the mixture was centrifuged at 3000 g for 15 minutes. Solution on the top layer was removed and syringe-filtered (0.45 μm membrane). A glass auto sampler vial with the 500 μL inlet was served to keep the solution prior to HPLC-ICP-MS/ESI-MS analysis. Pure pepsin solution was prepared as a process control. Kidney samples on all sampling days from both groups were prepared and analyzed as the same way in triplicate. The homogenized probe was cleaned between samples with hot water, soaked for 10 min in water methanol (1:1) bath, and rinsed with deionized water. Other laboratory equipment in direct contact with samples were all one-time used.

3.2.3 Arsenic Speciation Analysis

Mixtures of eight arsenic standards with a series of concentration ranging from 0.1 to 20 μg /L were prepared from arsenic standard stock solution, and finally diluted into pure enzyme solutions. An external calibration curve was constructed for each individual arsenical, and was drawn to quantitate arsenic concentrations in kidney sample extracts prior to the kidney sample analysis. Fish certified standard reference material (SRM) DORM-4 for AsB and DMA was analyzed to check instrument drift for every ten sample.

Samples were injected into Agilent 1100 series HPLC (Germany) by autosampler with an injection volume of 40 μL. The separation and detection method was as described in Chapter 2. A PRP-X110S anion exchange column with a guard column from

Hamilton was used to separate arsenic species. The gradient elution program with two mobile phases (A: 5% methanol, B: 60mM ammonium bicarbonate (NH_4HCO_3) in 5% methanol, PH=8.75) was used to separate all eight different arsenic species in 15 minutes. The signal at m/z 75 (As^+) was monitored and used to quantitate arsenic concentration by ICP-MS (Agilent 700cs Octopole, Japan). ESI-MS (AB SCIEX 5500 Q trap, ON, Canada) in MRM mode was used to identify arsenic species. Each sample was analyzed in triplicates. The combination of HPLC-ICP-MS and ESI-MS allowed the simultaneously identification and qualification of arsenic species in poultry kidney samples.

3.3 RESULTS AND DISCUSSION

3.3.1 Arsenic Speciation in Poultry Kidney Sample fed with ROX and Control diet

Figure 3.2 shows one example of typical HPLC-ICP-MS chromatograms obtained from analyses of kidney samples of both control and ROX-fed chickens on Day 28. The dominant arsenic species in poultry kidney are AsB, As^{III}, DMA^V, and Roxarsone. Other trace amount of arsenic compounds (MMA^V. As^V, 3-AHPAA, N-AHAA) were also detected. The retention time of each species is consistent with that of the respective arsenic standard. Typical HPLC-ICP-MS chromatograms obtained from analyses of kidney samples of both control and ROX-fed chickens on Day 0, Day 4, Day 7, Day 14, Day 28, and Day 35 are also shown in Appendix Figure A.3.

The kidney samples collected on 13 different days from the Control chickens and the Rox-fed chickens were analyzed in same manner. There were two unknown arsenic species in poultry kidney. One elutes is between MMA^V and As^V. The other unknown peak is between 3-AHPAA and N-AHAA. By comparing with the nearest identified peaks, the concentrations of the two unknown arsenic species were estimated against calibrations of the nearest arsenic species on the same chromatogram. The arsenic concentrations for each species vary with the feeding period. Table 3.1 and Table 3.2

demonstrate the average of arsenic concentrations of each species on each feeding days from both the Control Group and the ROX Group. Detailed concentrations of arsenic species (µg/kg) in each kidney sample of the Control Group and the ROX-fed group on Day 35, Day 34, Day 32, Day 30, Day 28, Day 21, Day 14, Day 7, Day 4, Day 3, Day 2, Day 1, Day 0 are shown in Appendix Table A.5 and Table A.6.

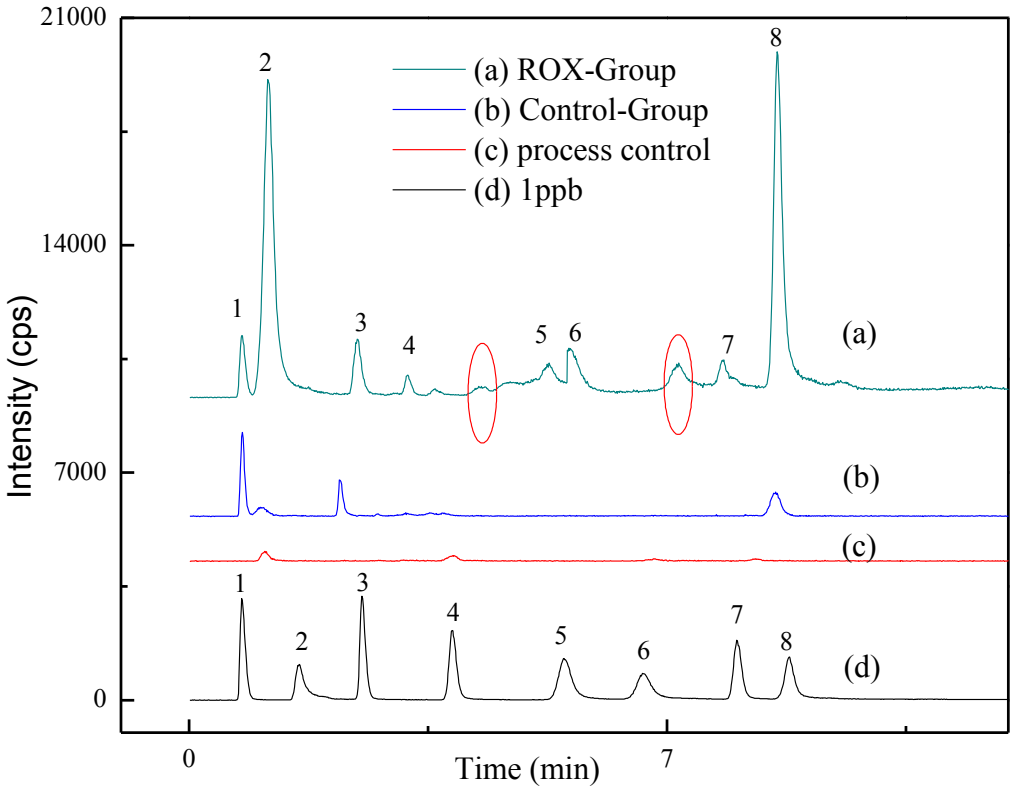


Figure 3.2 Typical chromatograms obtained from HPLC-ICP-MS analyses of kidney samples collected on Day 28 from (a) Roxarsone chicken (Cobb strain #500, pen #24) and (b) Control chicken (Cobb strain #500, pen #6). (c) Control kidney sample from Warburg (d) Stock arsenic standards including (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) As^{V}, (6) 3-AHPAA, (7) N-AHAA and (8) Roxarsone.

Table 3.1 Mean arsenic concentrations (µg/kg) in poultry kidney from the control Group on each sampling day

	AsB (µg /kg)	As^{III} (µg /kg)	DMA (µg /kg)	MMA^{V} (µg /kg)	Unknown1 (µg /kg)	As^{V} (µg /kg)	3-AHPAA (µg /kg)	Unknown2 (µg /kg)	N-AHAA (µg /kg)	Roxarsone (µg /kg)
Day0	N.D.	1.92	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Day1	4.76	5.57	5.06	2.04	N.D.	N.D.	N.D	N.D.	N.D.	N.D.
Day2	6.74	4.57	3.70	1.88	1.97	N.D.	N.D	N.D.	<LOD	<LOD
Day3	10.03	1.18	4.16	1.31	1.80	N.D.	N.D	N.D.	<LOD	1.25
Day4	10.51	3.66	4.23	N.D.	<LOD	<LOD	N.D.	N.D.	2.15	N.D.
Day7	32.27	4.65	6.16	2.29	<LOD	<LOD	N.D.	N.D	N.D.	N.D.
Day14	28.85	<LOD	6.70	<LOD	<LOD	<LOD	N.D.	N.D.	N.D.	7.16
Day21	18.84	2.21	11.80	3.46	3.58	<LOD	N.D.	N.D	N.D	25.91
Day28	15.76	6.64	11.48	1.44	1.56	<LOD	N.D.	N.D.	N.D.	24.60
Day30	16.49	5.99	6.54	3.76	2.14	1.52	N.D.	N.D.	N.D.	22.03
Day32	15.74	1.06	7.25	2.00	3.37	4.74	N.D.	N.D.	N.D.	25.11
Day34	16.97	5.20	8.84	2.29	N.D.	4.36	N.D.	N.D.	N.D.	26.70
Day35	12.24	1.89	5.39	2.92	N.D.	1.92	2.50	N.D.	1.33	23.18

*N.D. Not Detectable

Table 3.2 Mean arsenic concentration in (µg/kg) in poultry kidney from the ROX Group on each sampling day

	AsB (µg /kg)	As^{III} (µg /kg)	DMA (µg /kg)	MMA^{V} (µg /kg)	Unknown1 (µg /kg)	As^{V} (µg /kg)	3-AHPAA (µg /kg)	Unknown2 (µg /kg)	N-AHAA (µg /kg)	Roxarsone (µg /kg)
Day0	N.D.	N.D	1.98	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Day1	5.07	7.18	6.82	3.39	N.D.	N.D.	N.D.	N.D.	N.D.	29.71
Day2	6.90	11.96	6.40	2.51	3.18	10.19	N.D.	N.D.	1.68	55.72
Day3	12.38	13.60	6.71	2.44	2.55	14.51	6.64	N.D.	1.28	75.80
Day4	15.70	18.20	5.45	2.01	1.51	13.07	17.49	7.01	3.52	51.86
Day7	38.29	33.8	9.55	12.34	1.65	18.39	13.80	12.79	9.50	72.46
Day14	23.50	16.15	9.27	1.55	N.D.	6.64	11.35	3.88	9.03	66.69
Day21	13.42	51.15	15.96	4.84	4.15	6.90	24.12	14.11	7.39	64.17
Day28	13.99	94.36	25.11	3.60	2.69	2.82	18.47	2.67	2.75	105.81
Day30	15.46	10.96	7.60	4.28	4.34	1.89	2.12	N.D.	N.D.	32.33
Day32	13.42	2.00	6.25	2.22	3.33	2.52	2.00	N.D.	N.D.	27.38
Day34	11.70	9.47	9.30	3.04	1.78	2.60	1.89	N.D.	N.D.	40.48
Day35	15.14	5.10	5.06	2.96	4.04	2.09	2.96	N.D.	N.D.	33.10

*N.D. Not Detectable

3.3.2 Temporal Profiles of Arsenic Speciation in Poultry Kidney

3.3.2.1 Temporal profiles of Total Arsenic Concentration in Poultry Kidney

Figure 3.3 shows the temporal profile of total arsenic concentration from both the control group and the ROX-fed group. Figure A.8 in Appendix shows the temporal profile of the amount of total arsenic in chicken kidney from both the control group and ROX-fed group. The amount of arsenic was estimated by multiplying the concentrations of total arsenic species in each kidney sample by the specific kidney sample weight. The total arsenic concentrations in poultry kidney are the sum of individual arsenic species including AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, Roxarsone, and two unknown arsenic species. The concentration of the unknown arsenic species was estimated by calibrating against the nearest arsenic peaks. The total arsenic concentration gradually increases until the end of feeding day in control Group, whereas the total arsenic concentration increases with the ROX feeding from Day 0 to Day 28, and reaches the highest value on Day 28 in ROX-fed group. After Day 28, since ROX-fed groups were supplied with Roxarsone-free diet, the total arsenic concentration decreases dramatically.

The paired t-test is conducted between two strains, Cobb and Ross, for both ROX-fed group and the control group. The P value demonstrate that there is no significant difference between two strains ($P > 0.05$). Based on this result, we do not differentiate two strains of the ROX-fed group and the control group in the following temporal profiles of each arsenic species in Section 3.3.2.2 and Section 3.3.2.3

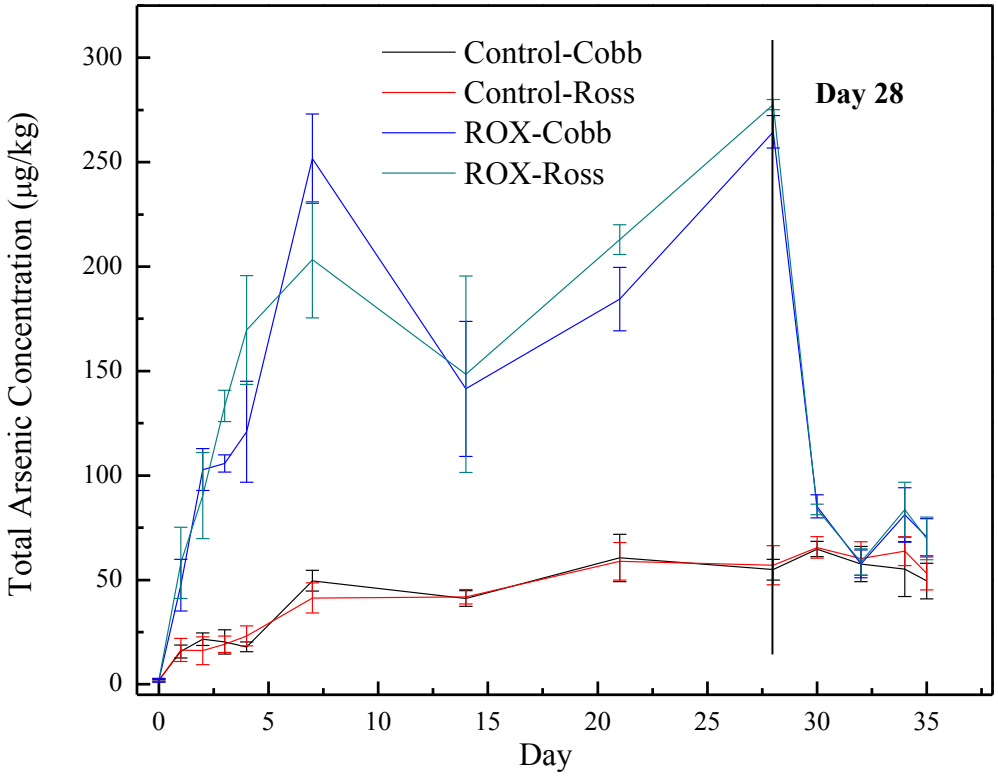


Figure 3.3 Temporal profiles of total arsenic in chicken kidney during a 35-day feeding period from both Control group (black square) and ROX-group (red circle) in poultry kidney. The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). The concentration of total arsenic is the sum of all individual arsenic species. On each day, 4 poultry kidney samples were collected from each strain (Cobb or Ross) on each group (the control group or the ROX-fed group).The error bars represent one standard deviations from triplicate analyses of 4 kidney samples on each sampling day.

3.3.2.2 Temporal profiles of Different Arsenic Species Concentration in Poultry Kidney

Figure 3.4 to Figure 3.8 display the temporal profiles of each arsenic species including AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, Rox, Unknown1, and Unknown2, from the control groups and the ROX groups during a 35-day feeding period. Previous determination in Section 3.3.1 showed no significant difference between two strains, Cobb and Ross, so two trains were combined to study the temporal profiles of both the control group and the ROX-fed group.

As a dominant arsenic species in poultry kidney, the concentration of Roxarsone in kidney sample is much higher than any other arsenic species on each sampling day (shown in Figure 3.4 (b)). The uptake of ROX in poultry significantly elevated the concentration of Roxarsone in kidney sample, compared with poultry kidney samples from the control groups. On Day 28, the concentration of Roxarsone reached the peak value (108 ± 17 μg/kg).

AsB in poultry kidney sample was introduced by fish meal in poultry food as a protein source. So the concentration of AsB for both control and ROX groups was similar as expected on each given day (shown in Figure 3.4 (a)). The change of AsB concentration with feeding period was probably related to the poultry grown weight and diet uptake. After chicken weight becomes stable, the uptake of diet gradually stays stable. The concentration of AsB then has no large variation after Day 28.

The temporal profiles of inorganic arsenite are different from inorganic arsenate (shown in Figure 3.5). The concentration of inorganic arsenite is much higher than concentration of inorganic arsenate. From Day 0 to Day 28, the inorganic arsenite in poultry kidney keep accumulating and reach as high as 90 ± 8 μg/kg on Day 28. After withdrawal of Roxarsone on Day 28, the elimination of As^{III} is fast. Seven days after the withdrawal, the As^{III} remain in poultry kidney is only approximately 5 μg/kg.

Some reports [7,8] suggest that arsenate tends to be reduced to arsenite in kidney and there is a longer lag period for arsenate to be methylated into DMA. Inorganic arsenate concentration increases initially (Fig 3.5 (b)), and the concentration of inorganic arsenate reach the highest value only after four days of ROX feeding. The concentration of Roxarsone also has a small peak value on the Day 4. The coincidence of the peak value on the same day for both As^{V} and Roxarsone indicates that Roxarsone may be responsible for the increase of As^{V} in the kidney.

The difference between ROX-fed group and control group for DMA^{V} and MMA^{V} (shown in Figure 3.6) is not as large compared with other arsenic species between two groups. Also, the concentration of these two species is much lower than other arsenic species. Most of DMA^{V} and MMA^{V} in poultry kidney are suspected to be metabolites of inorganic As^{III} and As^{V}. Although the variation patterns of DMA^{V} and MMA^{V} are less understandable as compared with other arsenic species in chicken kidney, significant difference between ROX-group and control group still indicates that: the potential increases of DMA^{V} and MMA^{V} concentrations in poultry kidney could be related to the ROX feeding.

The presence of 3-AHPAA, N-AHAA in poultry kidney sample are considered as the direct metabolites of Roxarsone. The concentration of these two arsenic species are very low (maximum 10-20 μg/kg), and the temporal profiles of these two species (Figure 3.7) are very similar to that of Roxarsone (Shown in Figure 3.4.b). In addition, these two species were detected at least two days after ROX feeding on ROX group in poultry kidney. The elimination of these two species are fast after Roxarsone supplemented food stopped after Day 28.

Two unknown arsenic species were also found in poultry kidney (shown in Figure 3.8). The identification of unknown arsenic species in poultry kidney is difficult, due to low concentration of these two species and potential matrix effect. Since the temporal profiles of these two unknown arsenic species are similar with the temporal profile of

Roxarsone, these two unknown arsenic species were also suspected as another two direct metabolites of Roxarsone.

The uptake and elimination of arsenic species are also shown as typical chromatograms in Appendix Figure A.4 and Figure A.5 respectively. Figure A.4 shows typical chromatograms obtained from HPLC-ICP-MS analyses of kidney samples on Day 0, Day 4, Day 7, Day 14, and Day 28 from ROX-fed chickens. Figure A.5 shows typical chromatograms obtained from HPLC-ICP-MS analyses of kidney samples on Day 0, Day 28, and Day 35 from ROX-fed chickens.

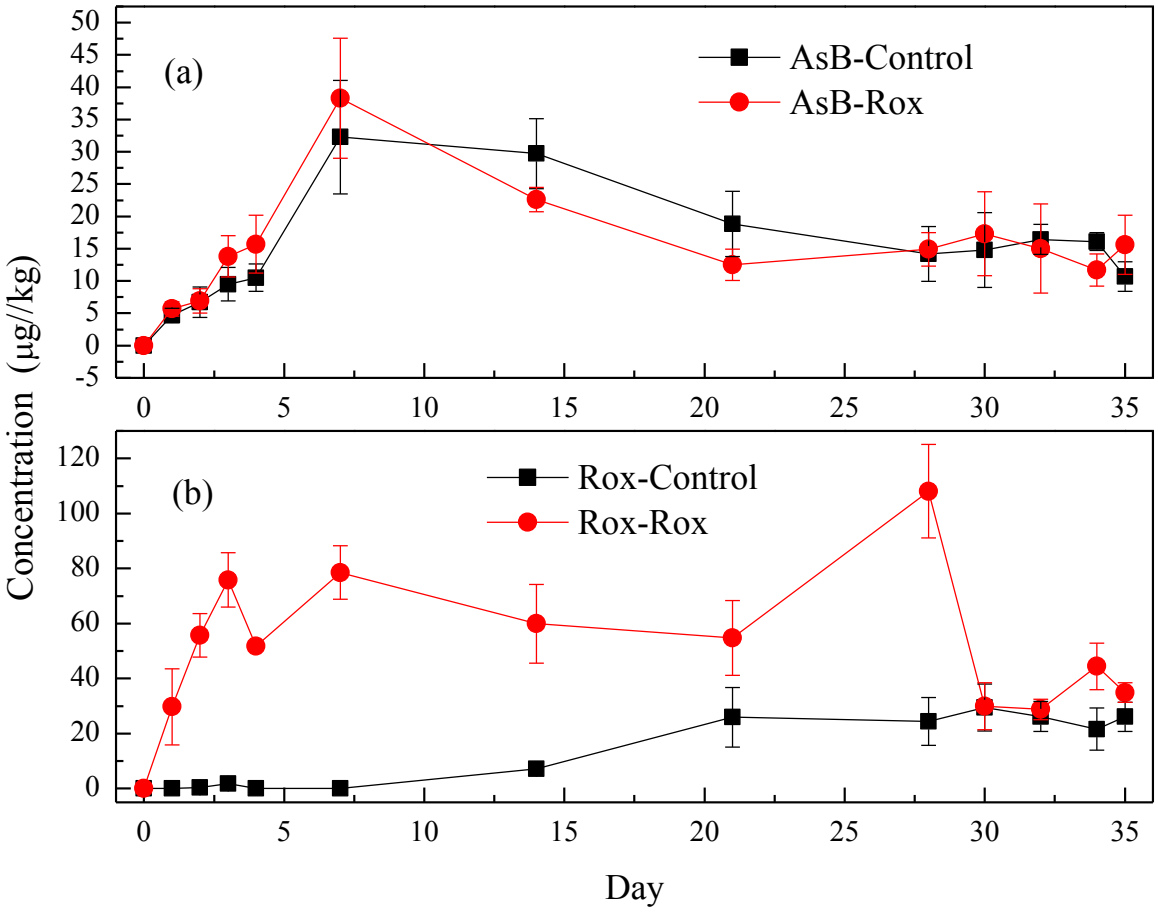


Figure 3.4 Temporal profiles of AsB and ROX in poultry kidney during a 35-day feeding period for both the control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 chicken kidney samples. (a) Temporal profile of AsB. (b) Temporal profile of inorganic ROX

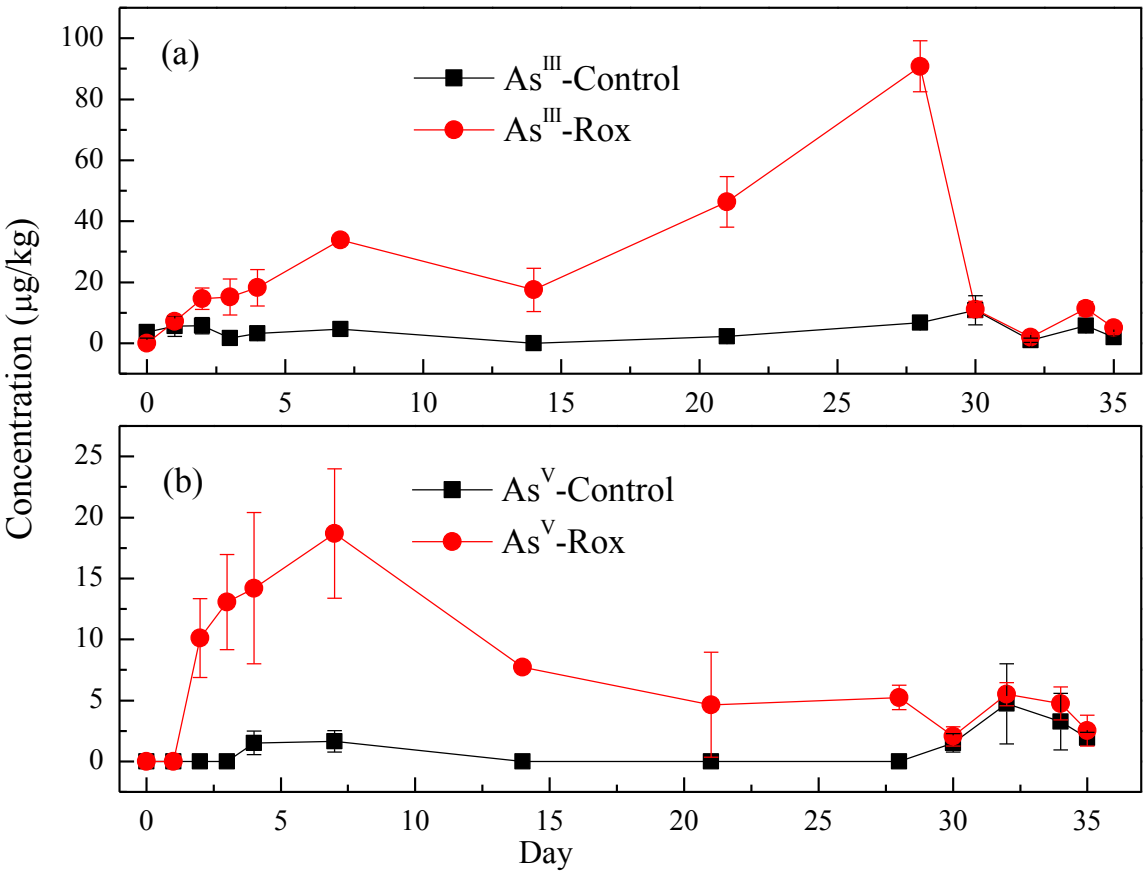


Figure 3.5 Temporal profiles of inorganic arsenicals during a 35-day feeding period for both the control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 chicken kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples. (a) Temporal profile of inorganic arsenite. (b) Temporal profile of inorganic arsenate.

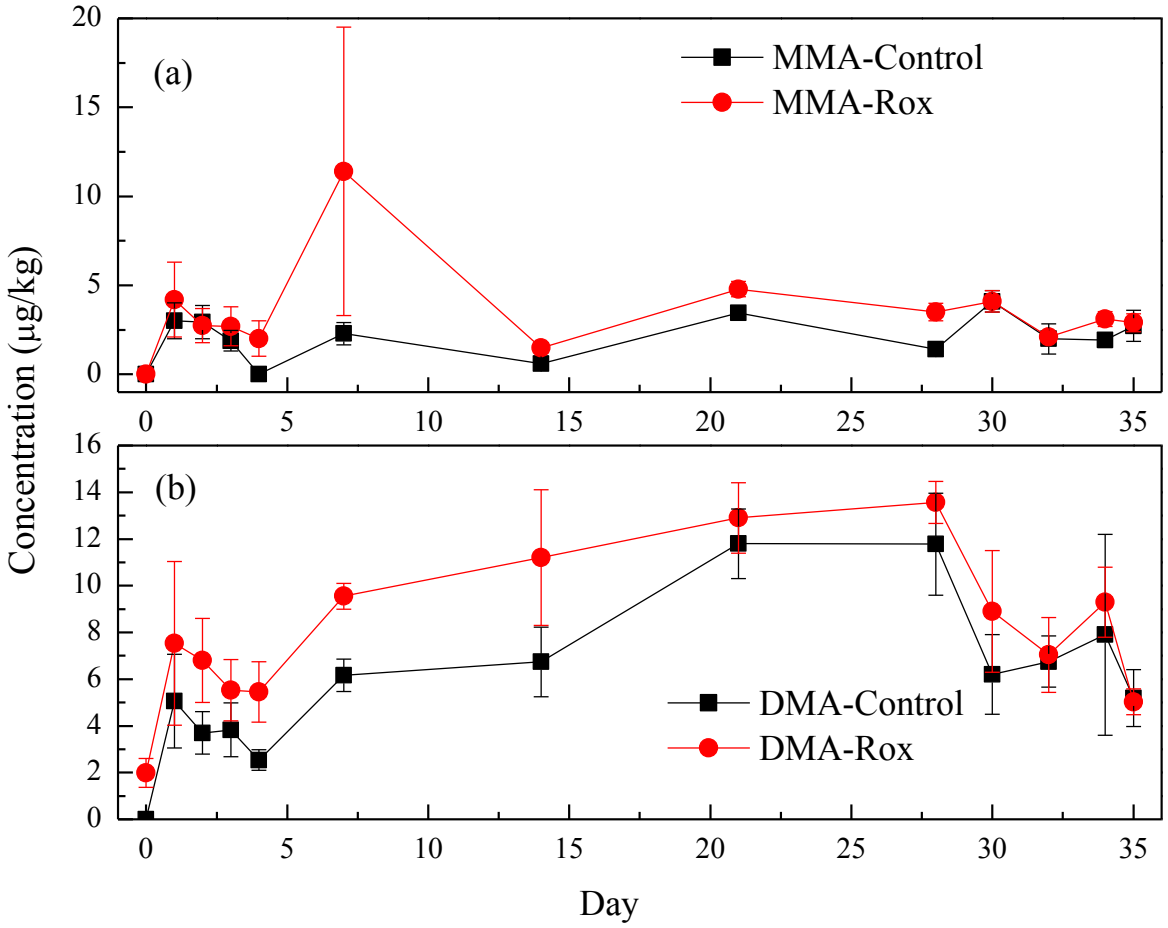


Figure 3.6 Temporal profiles of methylated arsenic species during a 35-day feeding period for both the control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples. (a) Temporal profile of monomethylarsonic acid. (b) Temporal profile of dimethylarsinic acid.

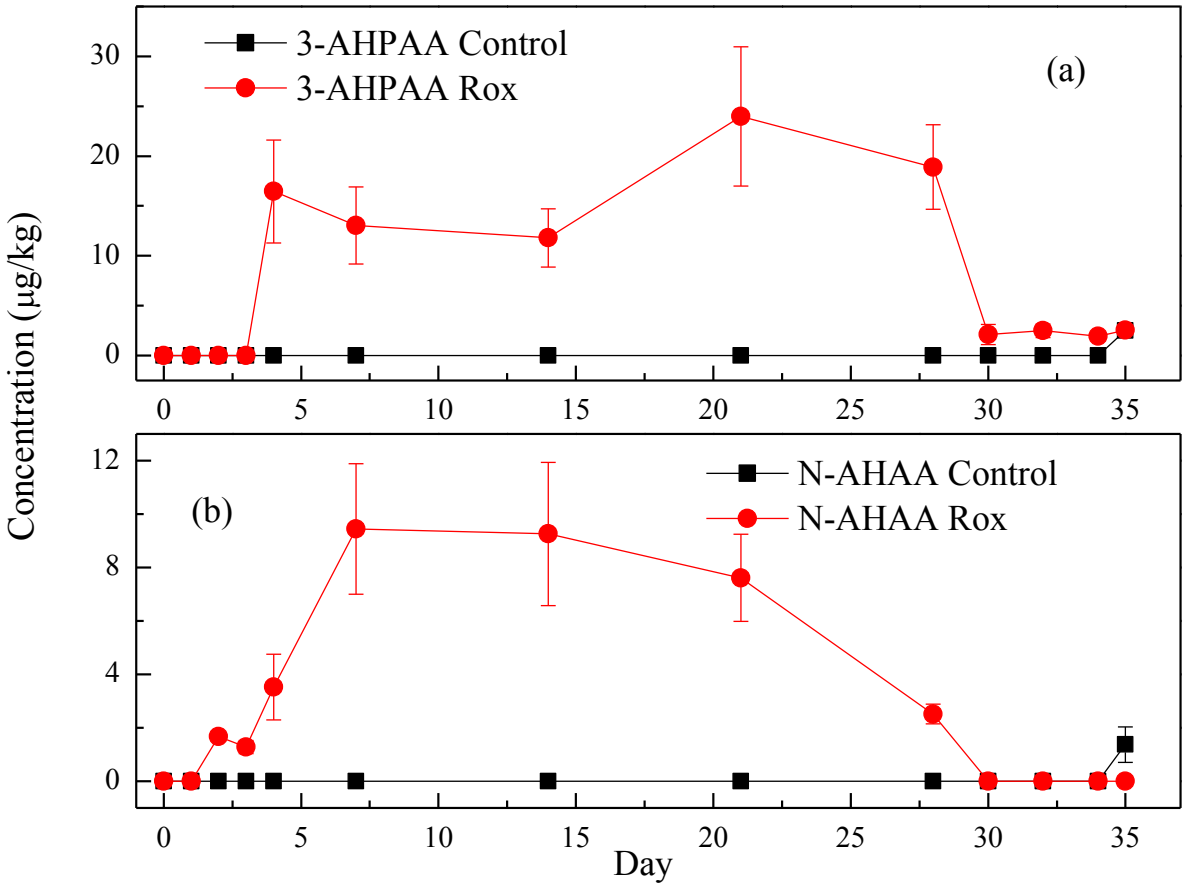


Figure 3.7 Temporal profiles of phenylarsonic species during a 35-day feeding period for both the control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples. (a) Temporal profile of 3-amino-4-hydroxy-phenylarsonic acid. (b) Temporal profile of N-acetyl-4-hydroxy-m-arsanilic acid.

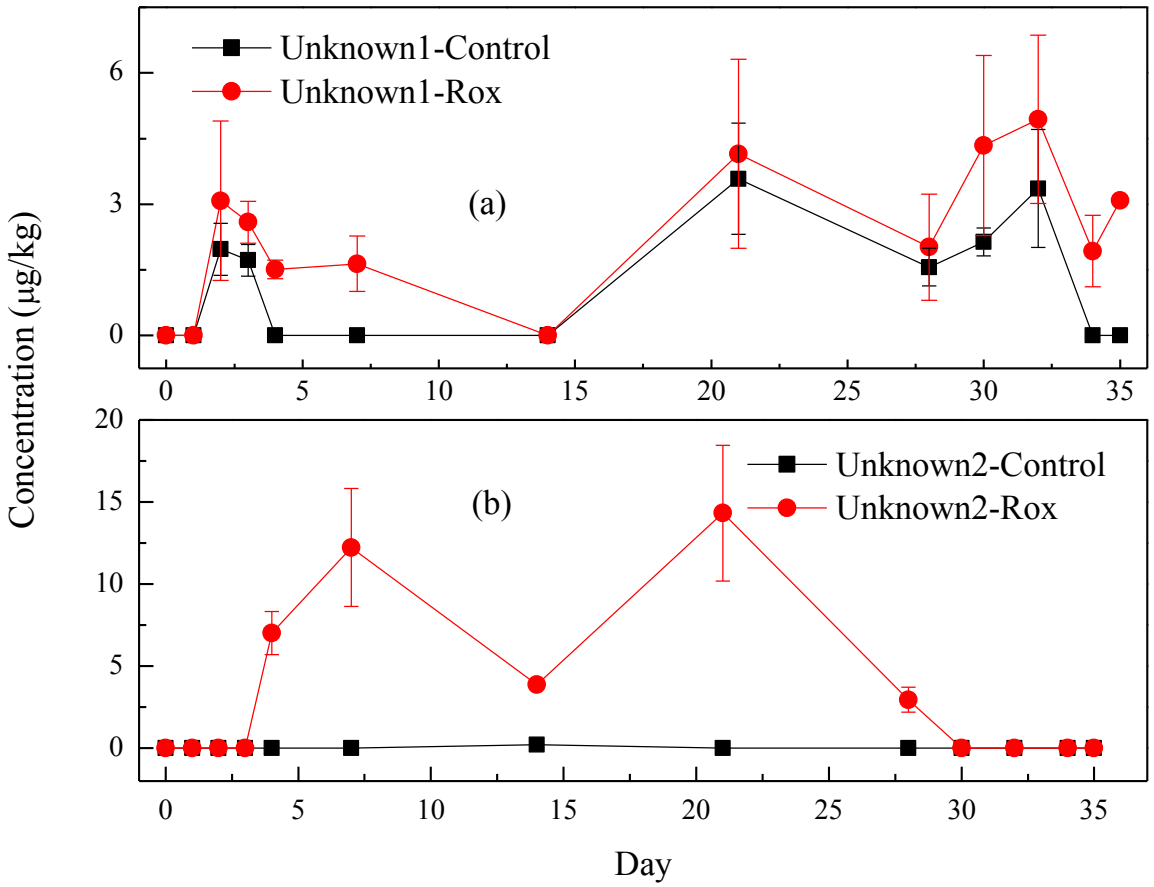


Figure 3.8 Temporal profiles of two unknown arsenic species during a 35-day feeding period for both the control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples. (a) Temporal profile of Unknown 1. (b) Temporal profile of Unknown 2.

3.3.2.3 Temporal profiles of Different Arsenic Species Concentration normalized against AsB

AsB is commonly present in marine organisms, e.g. crustaceans, bivalves, and fish. Fish meals and bones are often made into "fish meal", as a protein source for other animals. In the present feeding experiment, "fish meal" was added to the chicken feed. Both the control chickens and the ROX-fed chickens were exposed to similar concentrations of AsB in their feeds (Appendix Table A.2). AsB is not metabolized in organisms. Therefore, AsB can serve as a good internal standard for the normalized of the concentration of other arsenic species. Considering mass and fat discrepancy between each poultry kidney sample, the concentrations of seven arsenic species were normalized against the concentration of AsB. The results are displayed in Figure 3.9 and Figure 3.10. Figure 3.9 reflects the relative concentrations of each arsenic species normalized against AsB from the ROX-treated group. Figure 3.10 (a)-Figure 3.10 (f) demonstrate differences between Control Group and ROX-fed group for each arsenic species normalized by AsB. The observed difference between the ROX-fed group and the control group in normalized As^{III}, As^{V}, 3-AHPAA and N-AHAA are consistent with the results of their absolute concentrations (Figure 3.3-3.4). These results suggest that Roxarsone can be transformed to these arsenic that are detectable in poultry kidney.

For inorganic arsenic, the normalized concentration of As^{V} (in Figure 3.10 (c)) increase sharply after four days of Roxarsone feeding. The normalized concentration of As^{III} (in Figure 3.10 (b)) fluctuate slightly until Day 14, and then reach a peak value on Day 28. The normalized concentrations of Roxarsone (in Figure 3.10 (a)) also reach the highest value on Day 4 and Day 28, corresponding with the changes of inorganic As^{V} and As^{III}. It is probably because Roxarsone could be degraded into inorganic arsenate, and then reduced to inorganic arsenite in the presence of reductase in poultry kidney.

The variations of the normalized concentrations of 3-AHPAA (in Figure 3.10 (f)) and

N-AHAA (in Figure 3.10 (g)) are consistent with the changes of the concentration of Roxarsone in poultry kidney. Among seven arsenic species in poultry kidney, the differences in normalized concentrations of 3-AHPAA and N-AHAA between the control-Group and the Rox-Group are the most significant. 3-AHPAA and N-AHAA are not detectable in the kidney samples of control chickens.

Similar variation patterns for normalized DMA^V and MMA^V in both the control group and the Roxarsone-fed group are shown in Figure 3.10 (d) and (e).

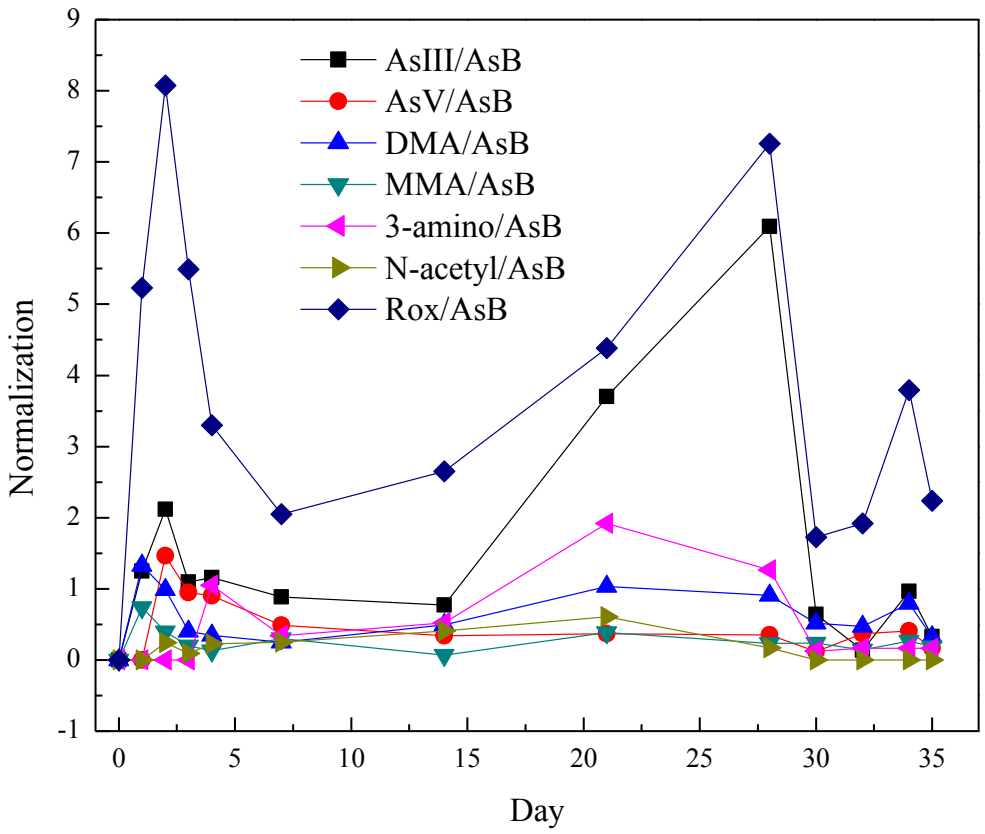


Figure 3.9 Temporal profiles of individual arsenic species normalized against arsenobetaine concentration in kidney samples from ROX-fed group. Each trace line represents temporal profile of one individual arsenic species in ROX-fed group, and the trace line is also shown as red trace with error bars in Figure 3.10 (a)-(g) corresponding to each individual arsenic species. The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35) from the ROX group. On each day, 8 kidney samples were collected from ROX-fed group.

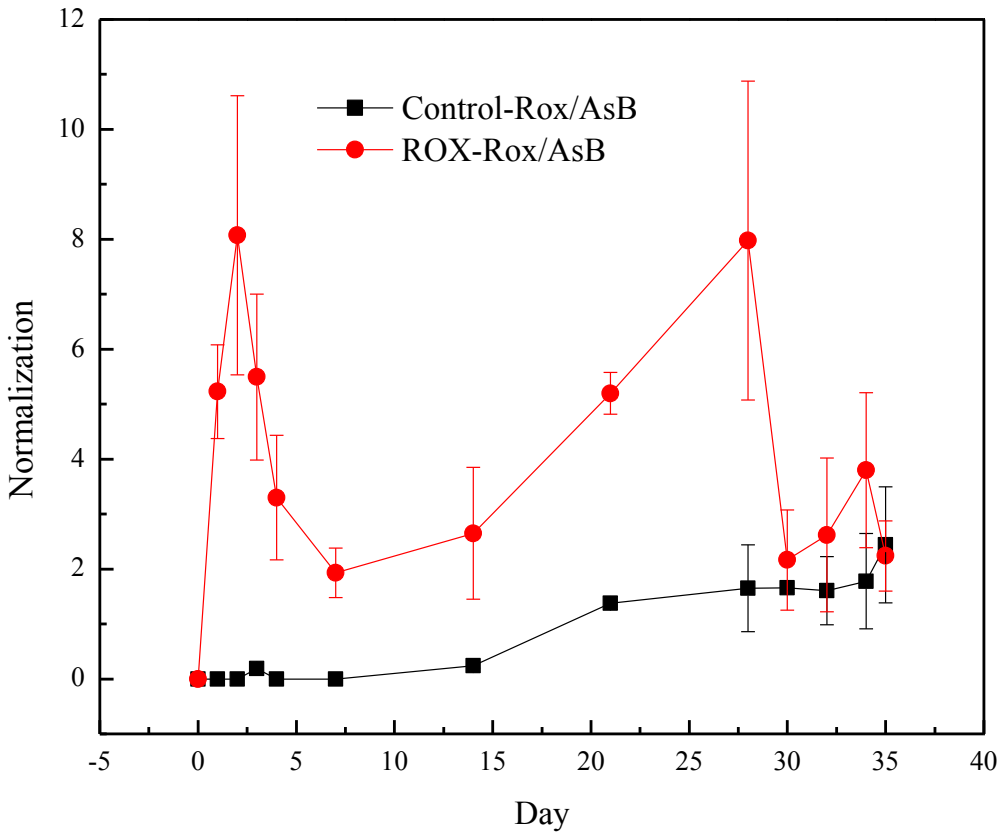


Figure 3.10 (a) Temporal profiles of Roxarsone normalized against arsenobetaine for both the Control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples.

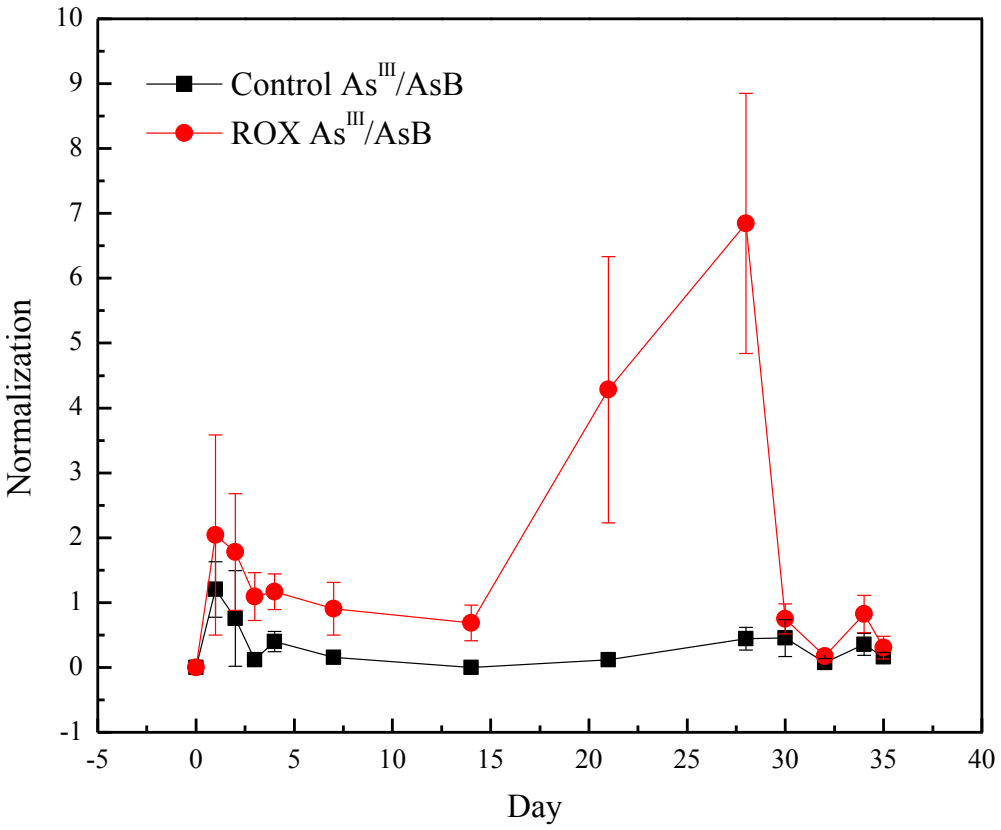


Figure 3.10 (b) Temporal profiles of inorganic arsenite normalized against arsenobetaine for both the Control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples.

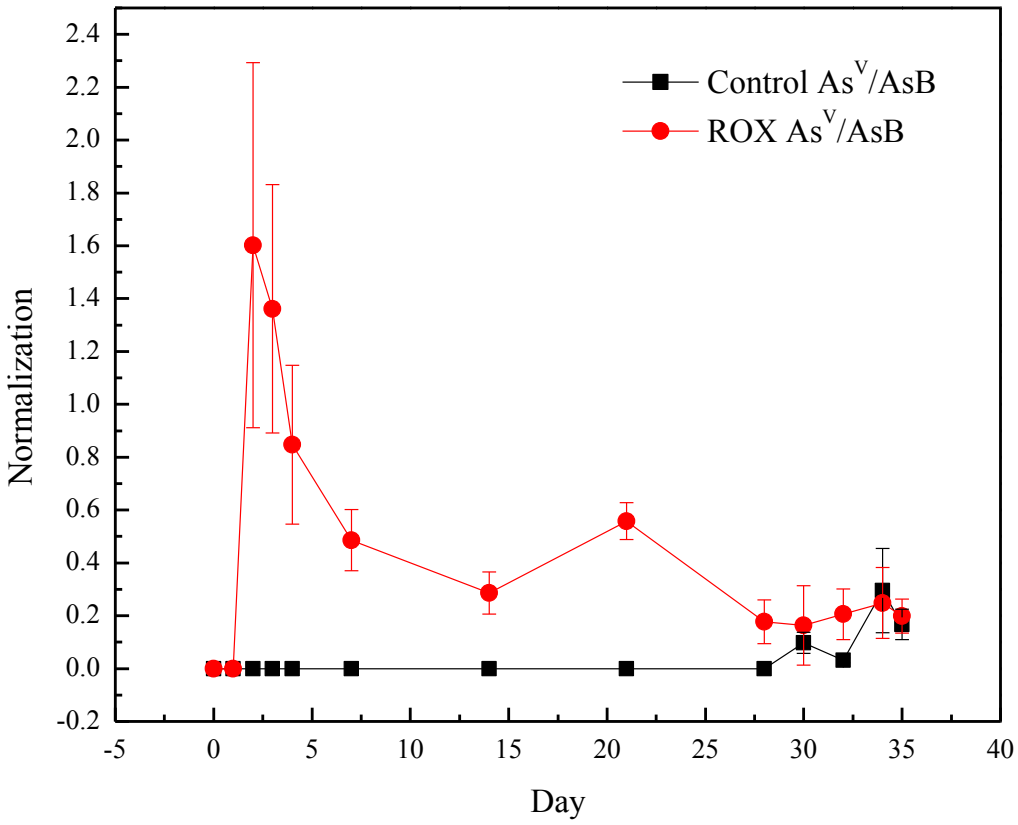


Figure 3.10 (c) Temporal profiles of inorganic arsenate normalized against arsenobetaine for both the Control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples.

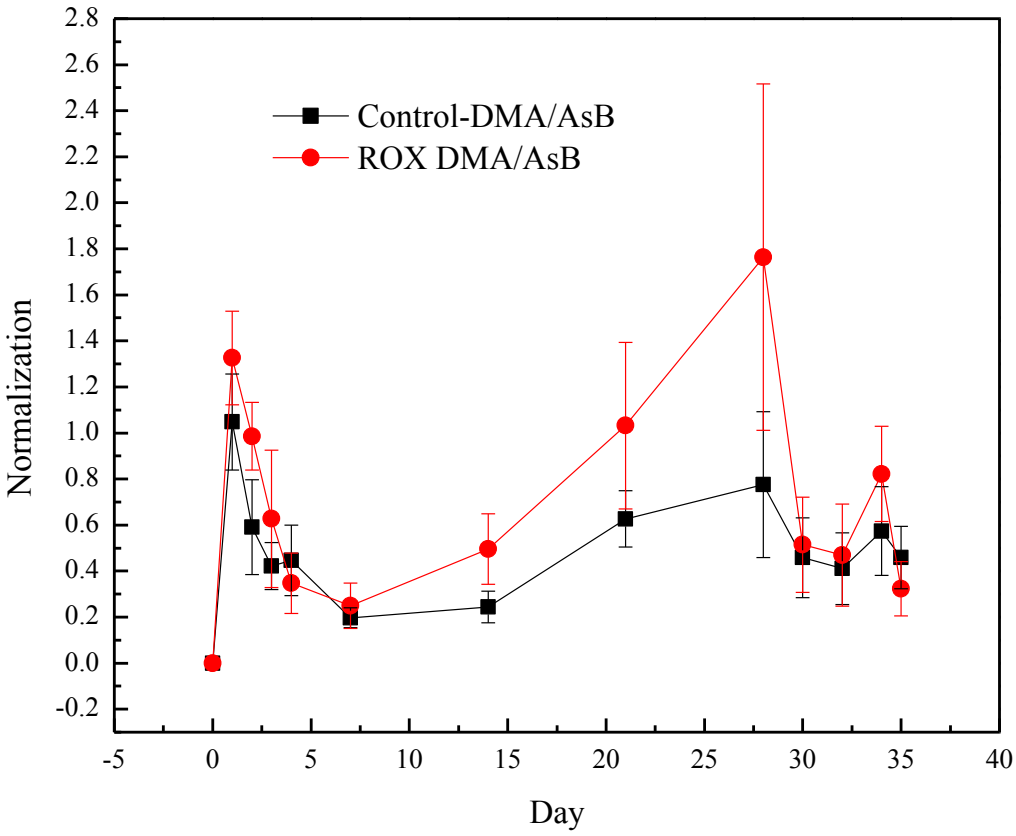


Figure 3.10 (d) Temporal profiles of dimethylarsinic acid normalized against arsenobetaine for both the Control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples.

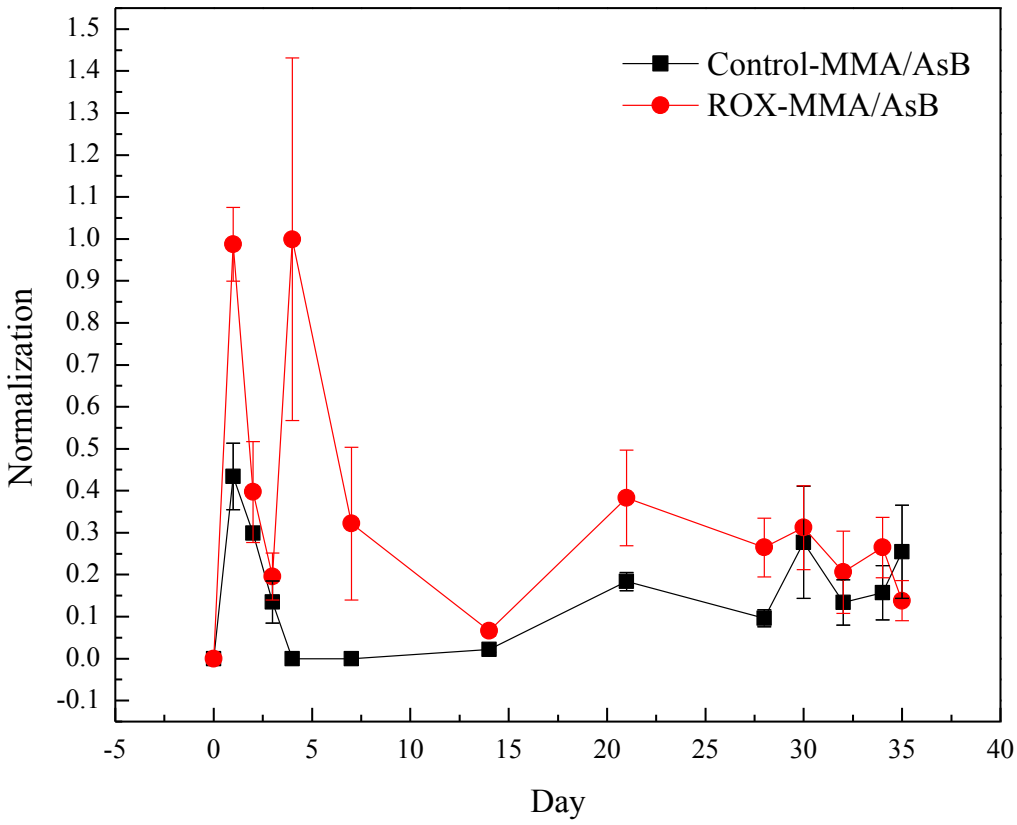


Figure 3.10 (e) Temporal profiles of monomethylarsonic acid normalized against arsenobetaine for both the Control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples.

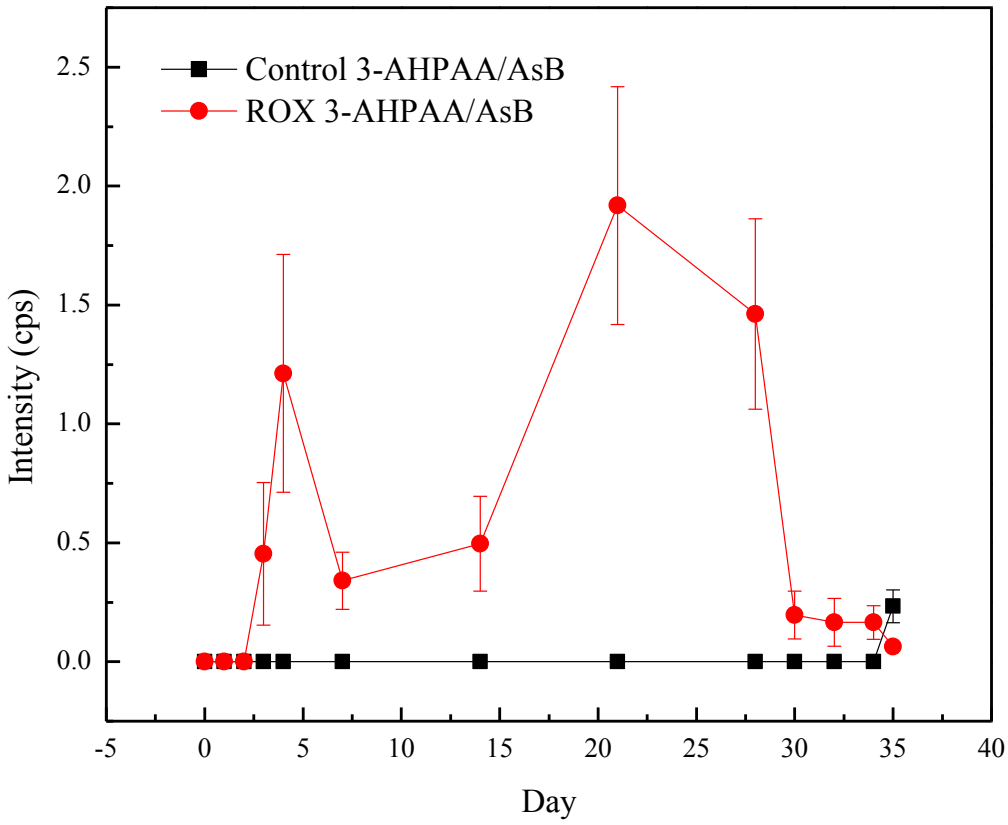


Figure 3.10 (f) Temporal profiles of 3-amino-4-hydroxy-phenylarsonic acid normalized against arsenobetaine for both the Control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples.

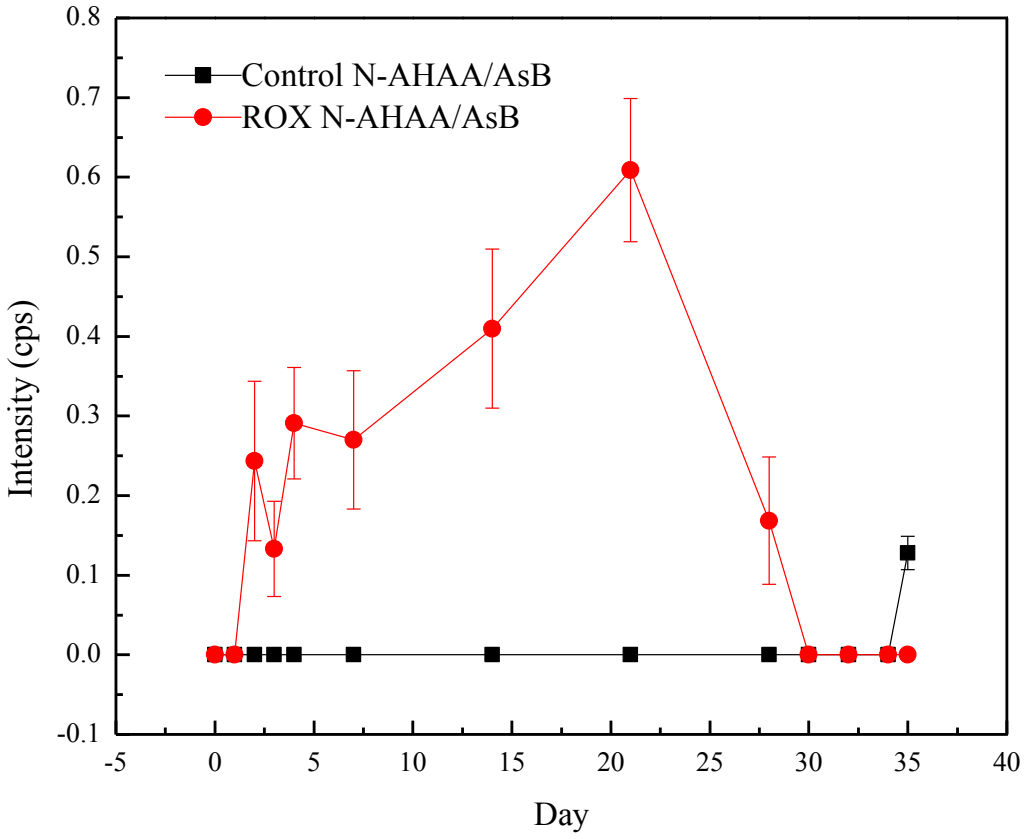


Figure 3.10 (g) Temporal profiles of N-acetyl-4-hydroxy-m-arsanilic acid normalized against arsenobetaine for both the Control group (black square) and the ROX group (red circle). The kidney samples were collected on 13 different days (Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30, Day 32, Day 34, and Day 35). On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples.

3.3.3 Correlation of Different Arsenic Species with Roxarsone in Poultry Kidney

Statistical relationship between any two arsenic species in ROX-fed group from Day 7 to Day 35 was analyzed to better evaluate the correlation between arsenic species. Figure 3.11 demonstrates the linear relationship between Roxarsone and other six arsenic species including As^{III}, As^{V}, DMA^{V}, MMA^{V}, 3-AHPAA, and N-AHAA. Table 3.3 shows the correlation coefficients and P values of two arsenic species. The correlation coefficient was obtained by dividing the covariance of the two arsenic species by the product of their standard deviations. The correlation coefficient reflects the strength of linear relationship between two arsenic species. Values between 0.3 and 0.7 indicate a moderate positive linear relationship, and values between 0.7 and 1.0 indicate a strong positive linear relationship. The P-value is the probability when the correlation coefficient is in fact zero (null hypothesis). If this probability is lower than the conventional 1% ($P < 0.01$), the correlation coefficient is called statistically significant.

In kidney samples from the ROX-Group, moderate to strong correlations were observed among inorganic arsenite, Roxarsone, 3-AHPAA and N-AHAA (shown in Table 3.3) Strong positive correlations between Rox-As^{III}, Rox-DMA, Rox-3-AHPAA, and ROX-N-AHAA (shown in Figure 3.11.1-3.11.3), indicate that the elevated concentrations of N-AHAA, 3-AHPAA, As^{III}, and DMA in the ROX-fed chickens are due to the feeding of Rox. Nevertheless, correlations of these arsenic species with As^{V} and MMA^{V} were relatively weak (coefficient ranging from 0.02-0.39) (Table 3.3). The weak correlation among Roxarsone, As^{V} and MMA^{V} does not suggest that there is no metabolic conversion from Roxarsone to As^{V} and MMA^{V} within poultry kidney. In fact, Roxarsone is able to hydrolyze into arsenate, and then be methylated into MMA^{V} and DMA^{V}.[9] However, the concentrations of As^{V} and MMA^{V} are too low to allow for observation of a clear correlation with Rox concentration.

The biotransformation among different arsenic species is complicated. We suspected

that the Roxarsone may degraded into As^{V}, then As^{V} can be reduced to As^{III}, which in turn be further methylated into MMA^{V} and DMA^{V}. For 3-AHPAA and N-AHAA, the same temporal profiles with that of Rox and high correlation coefficients both suggest that these two phenylarsonic species could be direct metabolites of Roxarsone.

Possible pathways for the metabolism of Roxarsone are shown in Appendix Figure A.2. Roxarsone may be reduced to 3-AHPAA, which may further be acetylated to N-AHAA. Both 3-AHPAA and N-AHAA have been detected in the kidney sample and previously reported in chicken liver by FDA[4] (U.S. Food and Drug Agency, 2011) and Hanyong Peng in our group (Peng et al. unpublished). Roxarsone, 3-AHPAA, and N-AHAA may each be methylated to the respective methyl arsenicals. Roxarsone may also be degraded to As^{V}, which in turn can be reduced to form As^{III} and methylated to form MMA^{V} and DMA^{V}. In this study, we have detected Rox, 3-AHPAA, N-AHAA, As^{V}, As^{III}, MMA^{V}, and DMA^{V}. These are consistent with the half of the proposed pathways for Roxarsone metabolism (Appendix Figure A.2) We did not identify methylated ROX, methylated 3-AHPAA or methylated N-AHAA, although some of the methylated arsenic species could be the suspected unknown arsenic species in chicken kidney. Further study is needed to identify these arsenic species in chicken kidney.

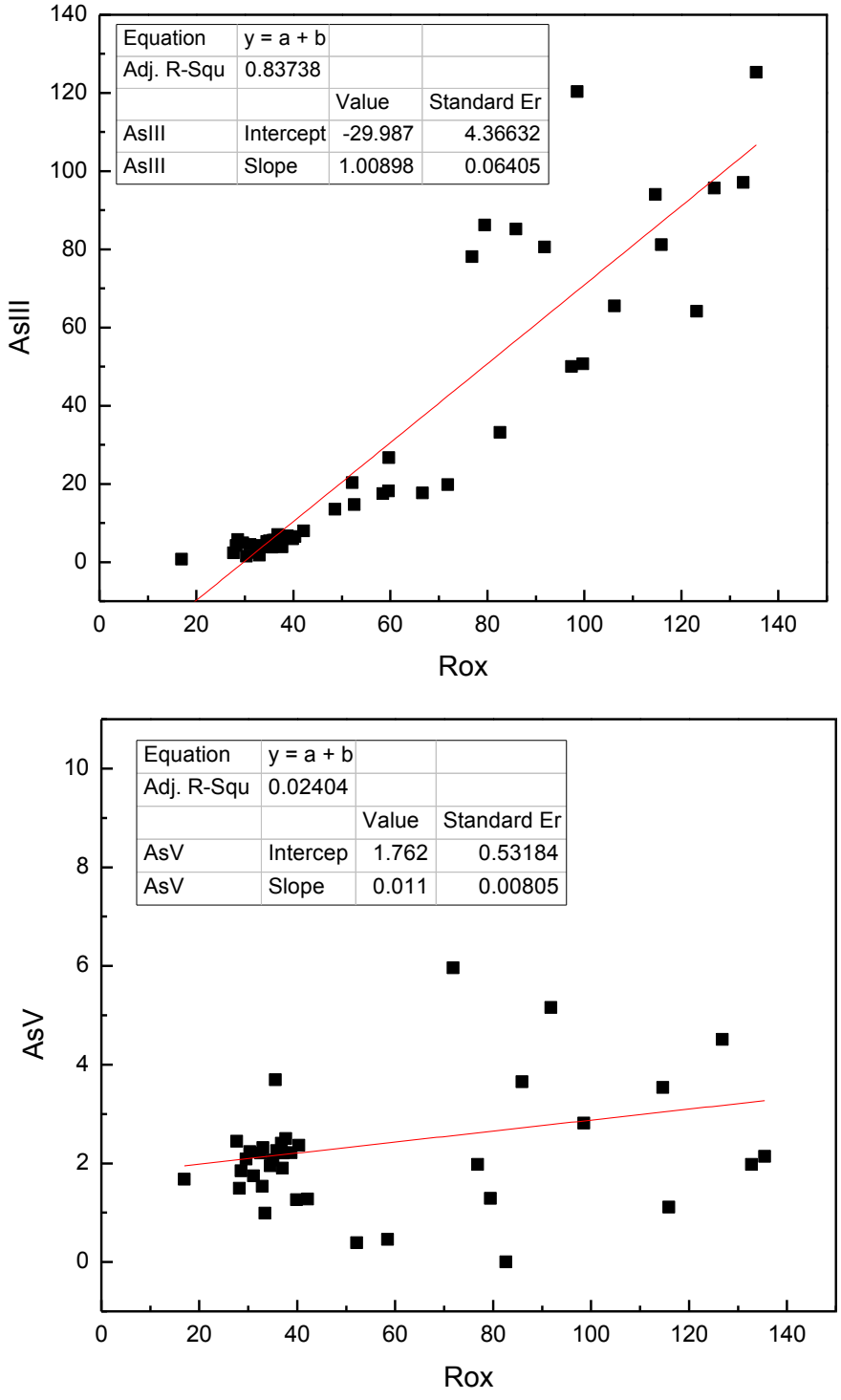


Figure 3.11.1 Correlation of ROX with inorganic arsenic species. Correlation of ROX with As^{III} was shown on top, R^2=0.8374, N=64. Correlation of ROX with As^{V} was shown on bottom, R^2=0.0240, N=64.

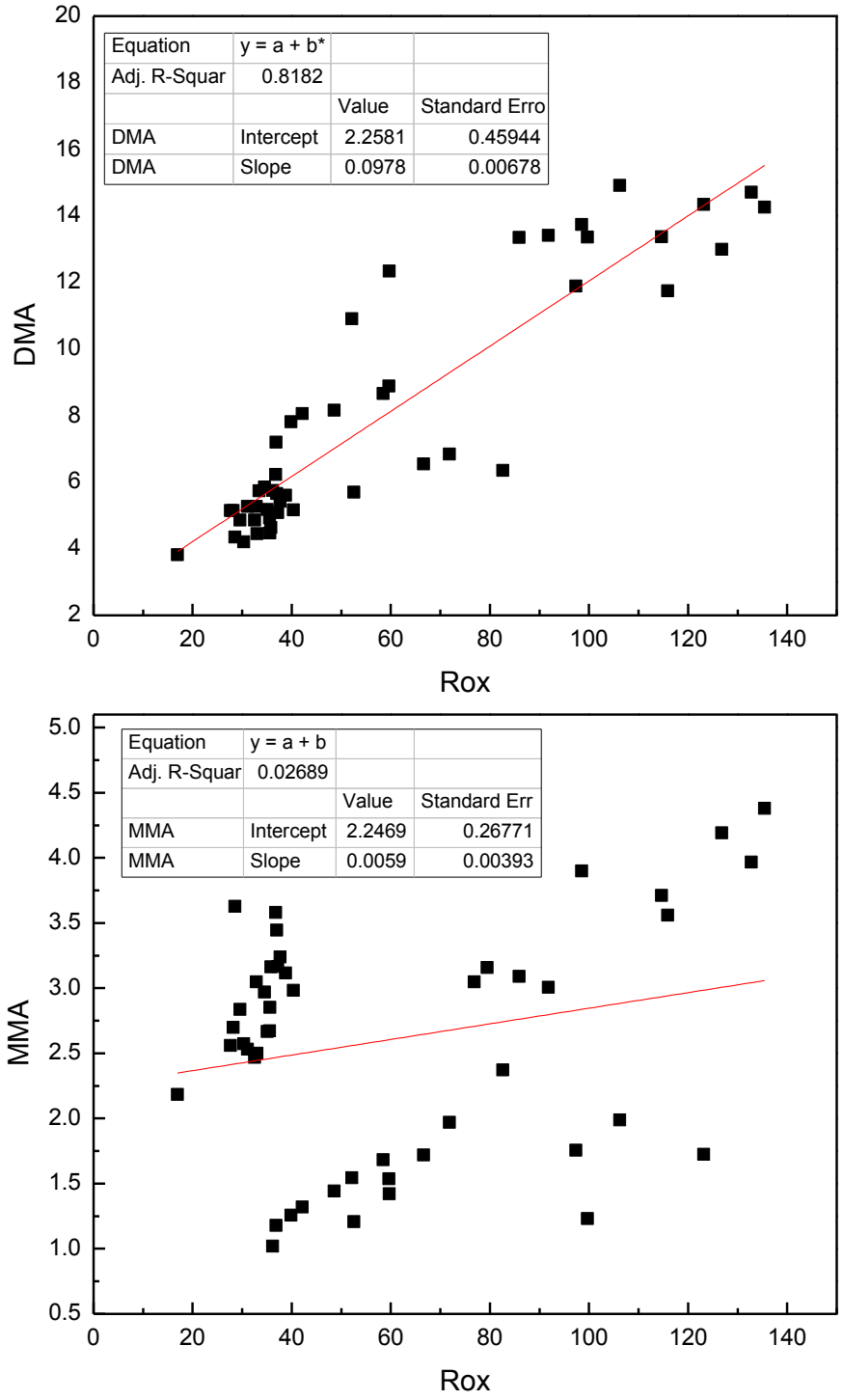


Figure 3.11.2 Correlation of ROX with methylated arsenic species. Correlation of ROX with DMA^V was shown on top, R^2=0.8182, N=64. Correlation of ROX with MMA^V was shown on bottom, R^2=0.0267, N=64.

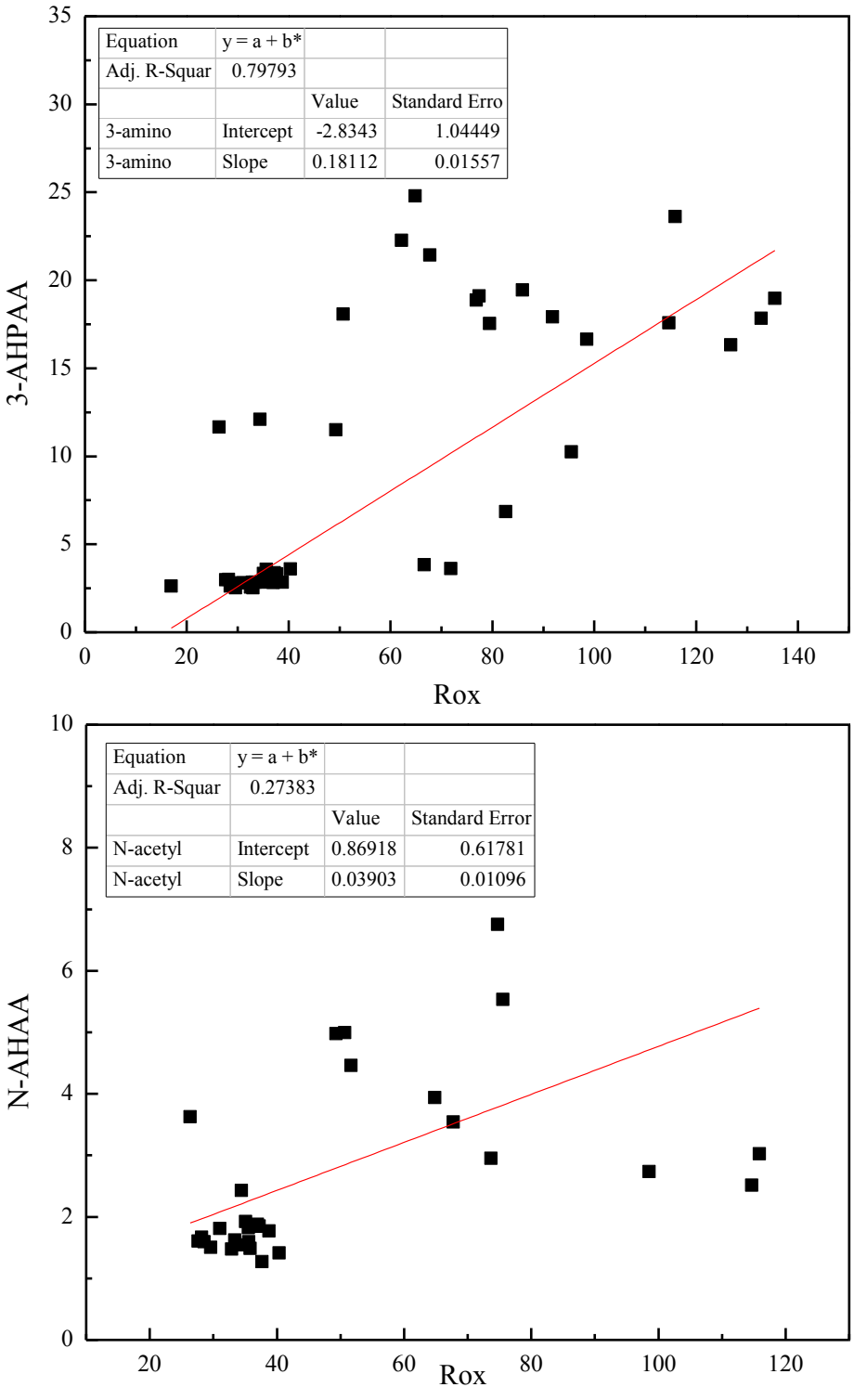


Figure 3.11.3 Correlation of ROX with phenylarsonic species. Correlation of ROX with 3-AHPAA was shown on top, R^2=0.7979, N=32. Correlation of ROX with N-AHAA was shown on bottom, R^2=0.2738, N=26

Table 3.3 Correlation coefficients and P values for arsenic species in poultry kidney samples from the ROX-fed group

		As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	Rox
AsIII	R	1.00	0.89**	0.39	0.27	0.96**	0.88**	0.91**
	P	-	<0.001	0.005	0.109	<0.001	<0.001	<0.001
DMA^{V}	R	0.89**	1.00	0.06	0.26	0.96**	0.88**	0.66**
	P	<0.001	-	0.676	0.129	<0.001	<0.001	<0.001
MMA^{V}	R	0.39**	0.06	1.00	0.02	0.49**	0.39	0.21
	P	0.005	0.676	-	0.195	0.002	0.073	0.133
As^{V}	R	0.26	0.26	0.21	1.0	0.18	0.10	0.27
	P	0.109	0.129	0.195	-	0.297	0.655	0.109
3-AHPAA	R	0.96**	0.96**	0.49	0.187	1.00	0.78**	0.73**
	P	<0.001	<0.001	0.002	0.297	-	<0.001	<0.001
N-AHAA	R	0.47	0.88**	0.40	0.10	0.78**	1.00	0.46**
	P	<0.001	<0.001	0.073	0.655	<0.001	-	0.007
ROX	R	0.92**	0.91**	0.22	0.27	0.73**	0.47**	1.00
	P	<0.001	<0.001	0.134	0.1089	<0.001	0.008	-

N=64 for As^{III}, DMA^{V}, MMA^{V}, As^{V}, ROX, N=32 for 3-AHPAA, N=26 for N-AHAA.

** Correlation is significant at $P < 0.01$.

3.3.4 Elimination of Different Arsenic Species in Poultry Kidney after ROX Feeding Withdrawal

The temporal profiles of each arsenic species (shown in Figure 3.10) demonstrate that the concentrations of all arsenic species except AsB, decreases rapidly after the stop of ROX feeding on Day 28. In order to evaluate the level of elimination of eight arsenic species seven days after the ROX-feeding stopped, the concentrations of eight arsenic species in poultry kidney for both groups on Day 28 and Day 35 are compared in Figure 3.12.

On Day 28, the differences between the control group and the Rox-treated group are significant for As^{III}, As^{V}, 3-AHPAA, N-AHAA and Rox. The withdrawal of ROX for seven days remarkably reduced the differences between Control Group and Rox Group for these arsenic species. The elimination rate of ROX is as high as 13 μg/day. For most arsenic species except AsB, the concentration decrease at least 3 times after 7 days of ROX feeding withdrawal in Rox-Group.

The regulation of FDA mandated a ROX withdrawal period of 5 days before poultry being slaughtered. From our result, the concentration of arsenic in poultry kidney decreased quickly after ROX feeding stopped. I did not have kidney samples from Day 33, i.e. 5 days after the feeding of ROX stopped. However, I have samples from Day 32; which represents 4 days following the ROX withdrawal. The results from Day 32 are shown in Figure 3.12 (middle graph). The patterns and concentrations of each arsenic species on Day 32 (Figure 3.12 middle graph) are similar to those on Day 35 (Figure 3.12 (bottom graph)).

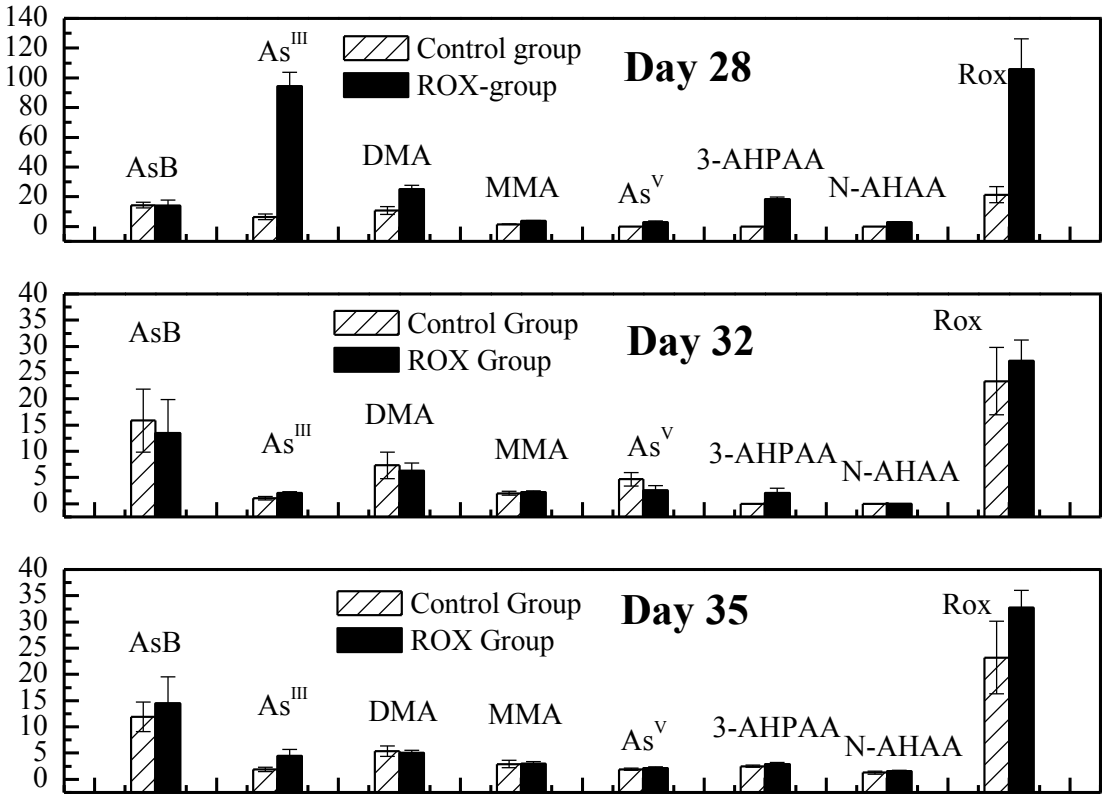


Figure 3.12 Comparison of the concentrations of individual arsenic species in kidney samples between the ROX-Group and the Control-Group on three sampling days; Day 28 (shown on top graph), Day 32 (shown on middle graph) and Day 35 (shown on bottom graph). Day 28 was the day when the feeding of ROX stopped. Arsenic species include AsB, As^{III}, As^{V}, DMA^{V}, MMA^{V}, 3-AHPAA, N-AHAA and Roxarsone. On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples.

3.3.5 Comparison of Arsenic Species by Types

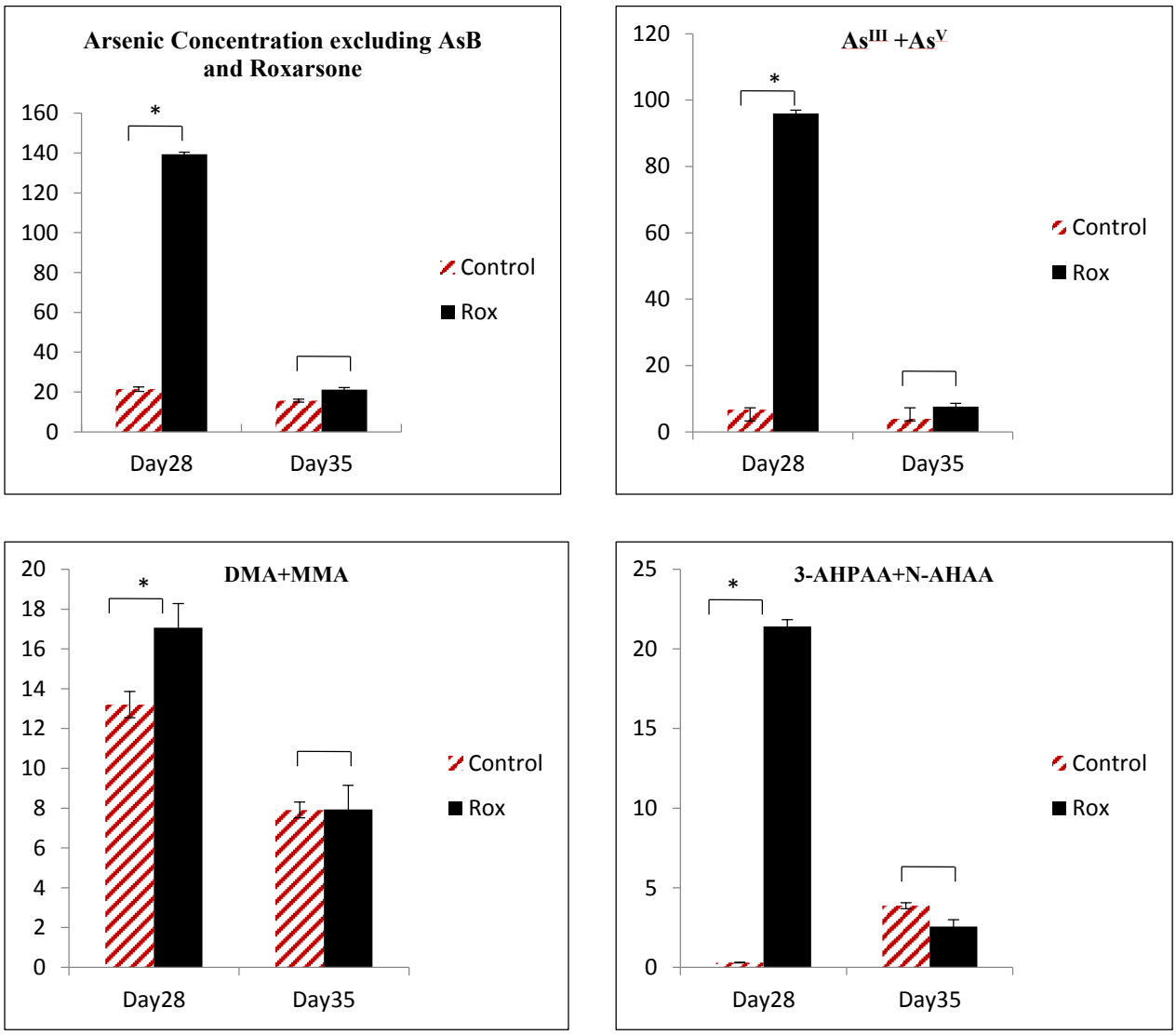
Figure 3.13 shows concentrations of four types of arsenic species in chicken kidney from the control group and the ROX-fed group on Day 28 and Day 35.

For the summed concentrations of arsenic species excluded AsB and Roxarsone, the difference between ROX-treated Group and Control Group is significant on Day 28. After the seven-day period of ROX withdrawal, the difference is not significant between the control and the ROX-fed group.

Concentrations of the inorganic arsenic ($As^{III} + As^{V}$) are significantly different between Rox-treated Group and Control Group on Day 28. This difference is not significant on Day 35. Similarly, the 3-AHPAA and N-AHAA are significantly increased in the kidney of the ROX-fed chickens on Day 28. There is no significant difference on Day 35.

The concentrations of the methylated arsenicals (MMA^{V}+DMA^{V}) are also significantly increased in the kidney of the ROX-fed chickens on Day 28, although the increase is not as substantial as for inorganic arsenicals and phenylarsenicals. There is no difference in the concentrations of methylarsenicals between the control and the ROX-fed chickens on Day 35.

The significant increases in the concentrations of the inorganic arsenicals ($As^{III} + As^{V}$), phenylarsenicals (3-AHPAA + N-AHAA), and methylarsenicals ($MMA^{V} + DMA^{V}$) on the ROX-fed chickens as compared to the control chickens on Day 28 suggest that the increases are due to ROX-feeding.



* At the 0.05 level, the two groups have the significant difference

Figure 3.13 Comparison of the summed concentrations of four types of arsenic species in chicken kidney from both the ROX-Group and the control-Group on Day 28 and Day 35. On each day, 8 kidney samples were collected from the ROX-fed group, and another 8 kidney samples were collected from the control group. The error bars represent one standard deviation from triplicate analyses of 8 kidney samples.

3.3.6 Residual Concentrations of Arsenic Species in Poultry Kidney after the 7 day Withdrawal

The feeding of ROX stopped on Day 28. The concentrations of arsenic species in kidney samples collected on Day 35 represent the residual arsenic remaining after a 7-day clearance period. The concentrations of each arsenic species after the 7-day clearance period are shown in Figures 3.14-3.20, Appendix Table A.4.3 and Table A.4.4. The paired t-test was conducted between two groups, the control group and the ROX-fed group. Since each strain contains four chicken kidney samples from the control group, and another four kidney samples from the ROX-fed group, the chickens in the same strains were paired between the control group and the ROX-fed group. The paired t-test results are shown as two asterisks on the Figure 3.14-3.20.

On Day 35, the concentrations of the residual ROX in the ROX-fed chickens (32.7 ± 3.3 μg/kg, or 32.7 ± 3.5 μg/kg from the Cobb strain and 33.4 ± 6.4 μg/kg from the Ross strain) were significantly higher than those in the control chickens (23.2 ± 6.9 μg/kg) (Figure 3.14 a). There is no significant difference in the AsB concentrations between the control chickens (11.9 ± 2.8 μg/kg) and the ROX-fed chickens (14.5 ± 5.0 μg/kg) (Figure 3.14 b).

The concentrations of residual As^{III} and As^{V} in chicken kidney on Day 35 are shown in Figure 3.15. The concentrations of As^{III} in the ROX-fed chickens (4.5 ± 1.2 μg/kg), seven days after the ROX feeding stopped, are significantly higher than that of residual concentrations in control chicken (1.9 ± 0.4 μg/kg) (Figure 3.15 a). Differences in the As^{V} concentrations between the control and the ROX-fed chickens are not significant (Figure 3.15b).

Figure 3.16 shows the sum of inorganic arsenic concentrations (As^{III} +As^{V}), in kidney of the control chickens and the ROX-fed chickens on Day 35, i.e. seven days after termination of ROX feeding. These results show overall the total concentrations of the

inorganic arsenicals ($As^{III} + As^{V}$) in the ROX-fed chickens (6.3 ± 1.2 μg/kg or 6.3 ± 1.5 μg/kg for the Cobb strain and 6.8 ± 0.5 μg/kg for the Ross strain) are significantly higher ($P < 0.05$) than these in the control chickens (3.8 ± 0.5 μg/kg) after the 7-day clearance period.

Figure 3.17 shows the concentrations of MMA^{V} and DMA^{V} remaining in the kidney of the control chickens and the ROX-fed chickens seven days after the feeding of ROX stopped. There is no significant difference in the MMA^{V} + DMA^{V} concentrations between the control and the ROX-fed chickens. Similarly, there is no significant difference in the total concentrations of the methylarsenical ($MMA^{V} + DMA^{V}$) between the control (8.3 ± 0.6 μg/kg) and the ROX-fed (8.0 ± 0.6 μg/kg) chickens (Figure 3.18).

Figure 3.19 shows the residual concentrations of 3-AHPAA and N-AHAA in kidney of the control chickens and the ROX-fed chickens seven days after the end of ROX feeding. 3-AHPAA and N-AHAA are not detectable in the kidney of most control chickens. They are detectable in the kidney of many ROX-fed chickens. The difference is significant between the control and the ROX-fed groups.

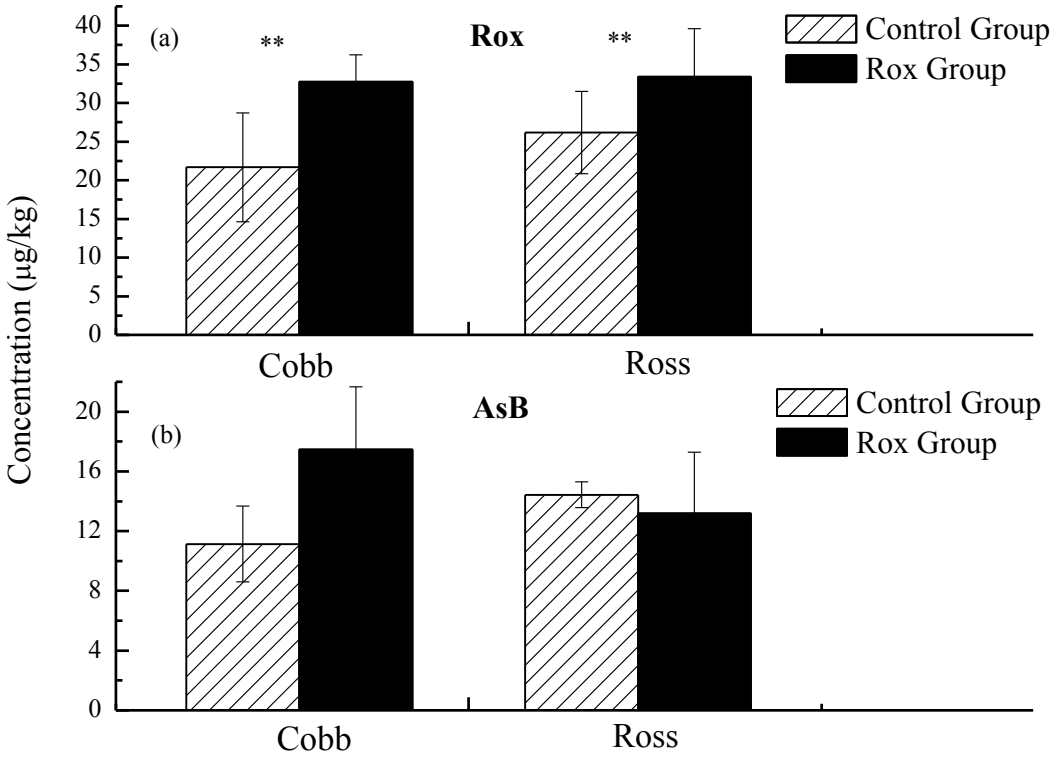
The total concentrations of the phenylarsenicals (3-AHPAA + N-AHAA) in the ROX-fed chickens (8.3 ± 0.9 μg/kg) are significantly higher than those in the control chickens (3.8 ± 0.3 μg/kg) (Figure 3.20).

The sum of all arsenic species, excluding the non-toxic arsenobetaine, is compared in Figure 3.21. After the 7-day clearance period, the concentrations of all arsenic species, except AsB, in the ROX-fed chickens are (55.7 ± 4.8 μg/kg). This is compare to 39.1 ± 7.9 μg/kg in the control chickens. A pared t-test shows that the two values are significantly different ($P < 0.05$).

Figure 3.22 shows the total concentrations of all detectable arsenic species, including AsB, inorganic As^{III}+ As^{V}, methylarsenicals MMA^{V}+DMA^{V}, phenylarsenicals

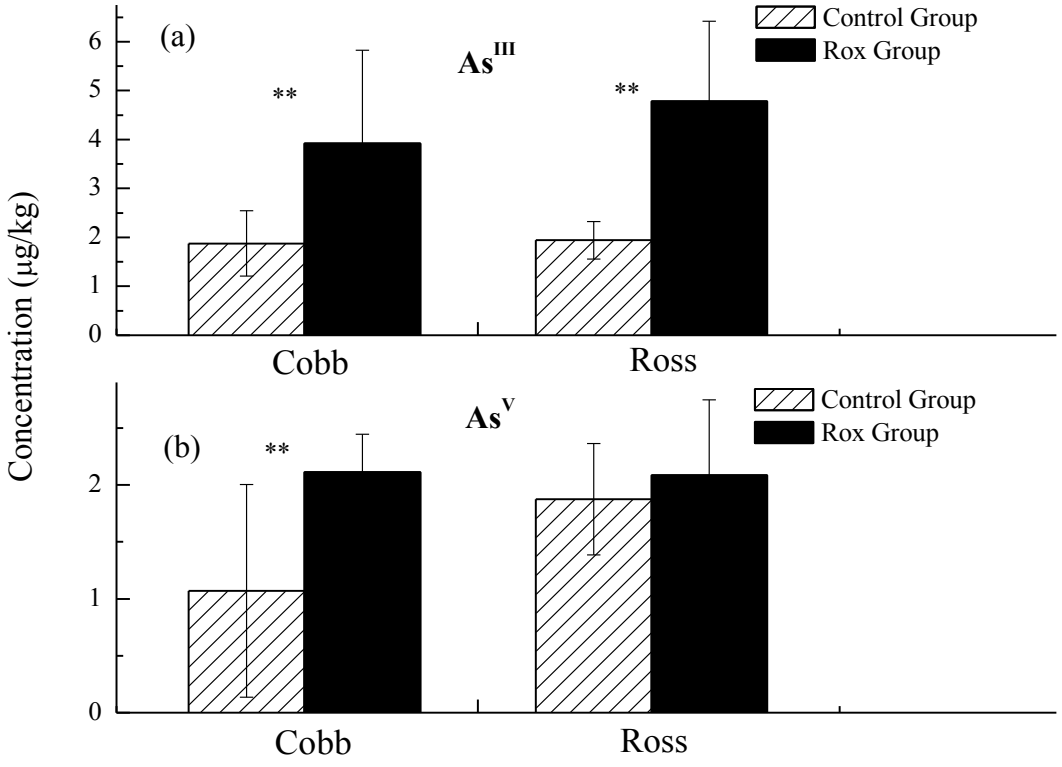
ROX+3-AHPAA + N-AHAA, and unknown arsenicals, in the kidney samples collected from the control chickens and the ROX-fed chickens seven days after the termination of feeding ROX. The total As concentrations in the control chickens are 51.0 ± 7.8 μg/kg. The total residual As concentrations in the ROX-fed chickens are 70.1 ± 8.9 μg/kg. The difference between the two groups is statistically significant ($P < 0.05$).

The tolerance of FDA for total arsenic in poultry muscle and live tissues are 0.5 and 2 mg/kg, respectively. However, the safety standards for inorganic arsenic species in foods have not been established by FDA. The concentrations of total arsenic in the kidney of the control chickens (51.0 ± 7.8 μg/kg) and the ROX-fed chicken (70.1 ± 8.9 μg/kg) on Day 35 are below the FDA tolerance values for chicken muscles (500 μg/kg) and liver (2000 μg/kg).



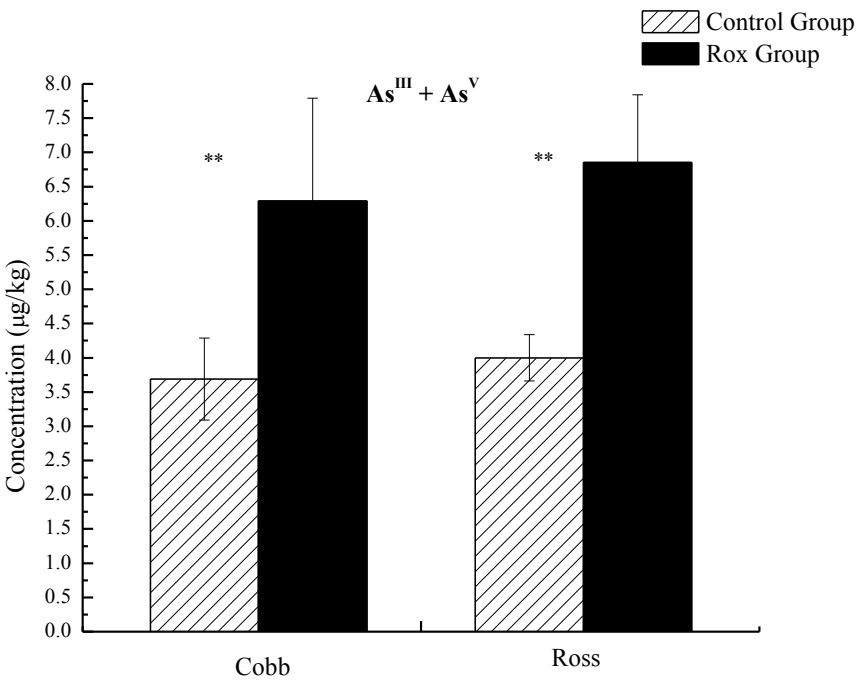
** At the 0.05 level, the two groups have the significant difference

Figure 3.14 Comparison of arsenobetaine and Roxarsone concentrations in chicken kidney between the ROX-Group and the Control-Group on Day 35. (a) Roxarsone concentration in both strains (Cobb 500, Ross 308) from the ROX-Group and the Control-Group. (b) AsB concentration in both strains (Cobb 500, Ross 308) from the ROX-Group and the Control-Group. The error bars represent one standard deviation from triplicate analyses of 4 kidney samples.



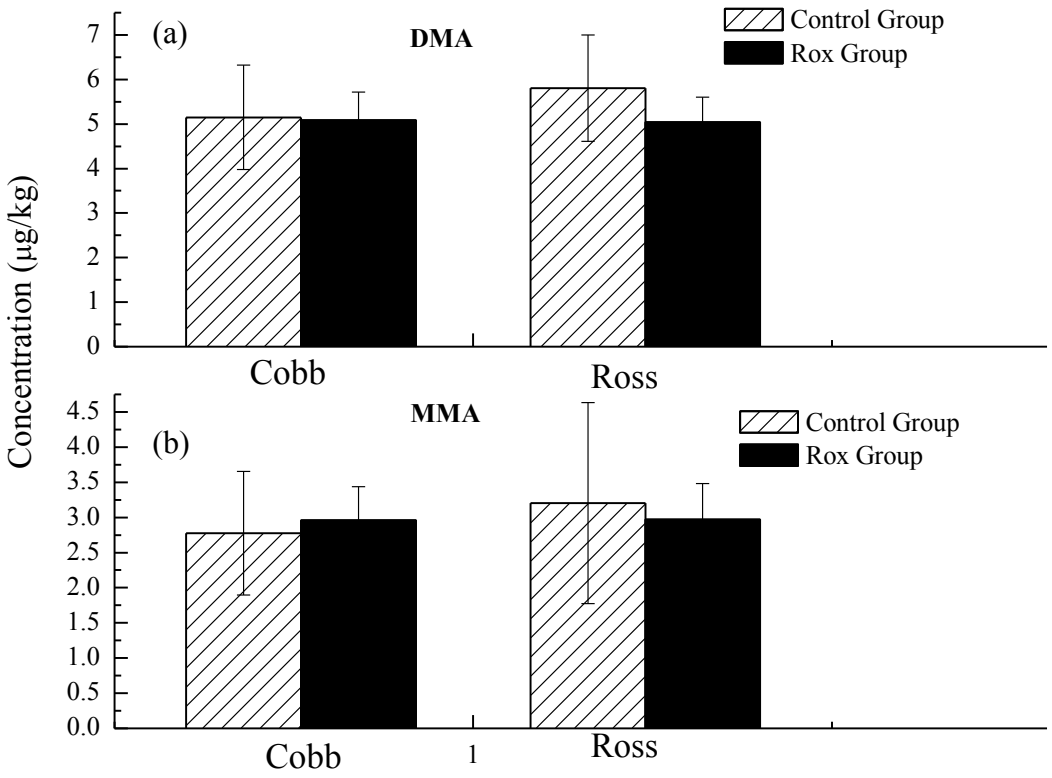
** At the 0.05 level, the two groups have the significant difference

Figure 3.15 Comparison of inorganic arsenic concentrations in chicken kidney between the ROX-Group and the Control-Group on Day 35. (a) As^{III} concentration in both strains (Cobb 500, Ross 308) from the ROX-Group and the Control-Group. (b) As^{V} concentration in both strains (Cobb 500, Ross 308) from the ROX-Group and the Control-Group. The error bars represent one standard deviation of triplicate analyses from 4 kidney samples.



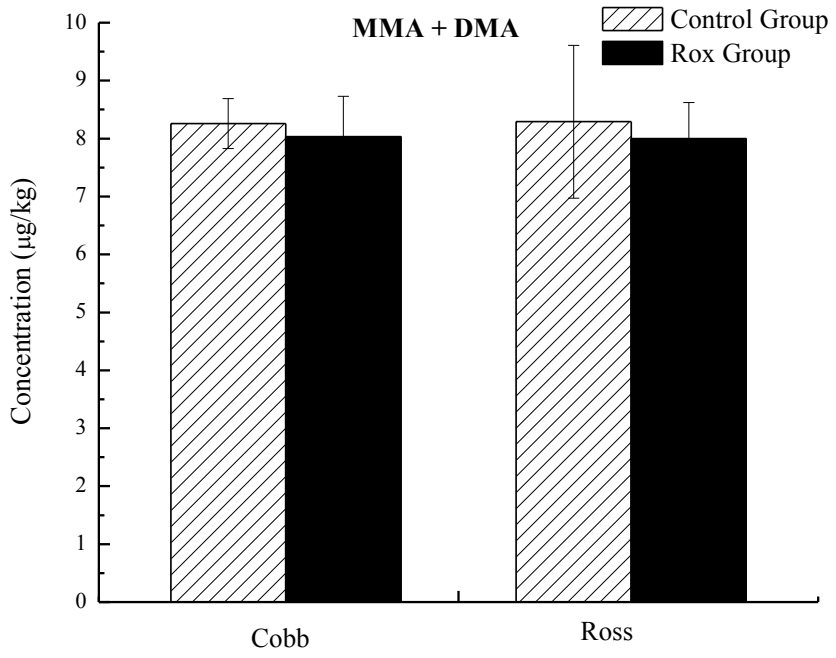
** At the 0.05 level, the two groups have the significant difference

Figure 3.16 Comparison of total inorganic arsenic concentrations (As^{III} + As^{V}) in chicken kidney between the ROX-Group and the Control-Group in both strains (Cobb 500, Ross 308) on Day 35. The error bars represent one standard deviation of triplicate analyses from 4 kidney samples.



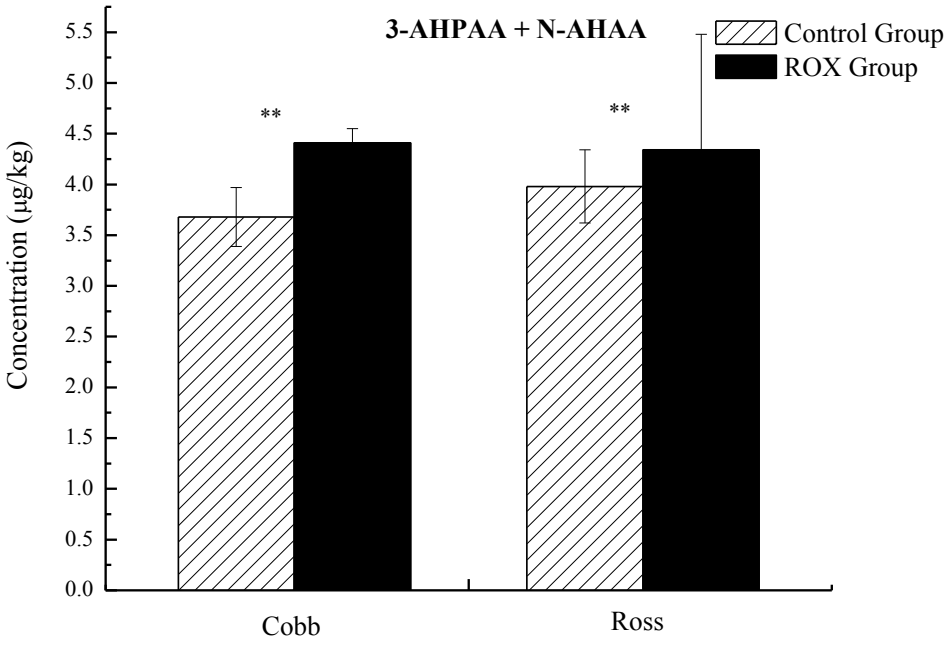
** At the 0.05 level, the two groups have the significant difference

Figure 3.17 Comparison of the concentration of the methylated arsenic in chicken kidney between the ROX-Group and the Control-Group in both strains (Cobb 500, Ross 308) on Day 35. (a) DMA^V concentration in both strains (Cobb 500, Ross 308) from the ROX-Group and the Control-Group. (b) MMA^V concentration in both strains (Cobb 500, Ross 308) from the ROX-Group and the Control-Group. The error bars represent one standard deviation from triplicate analyses of 4 kidney samples.



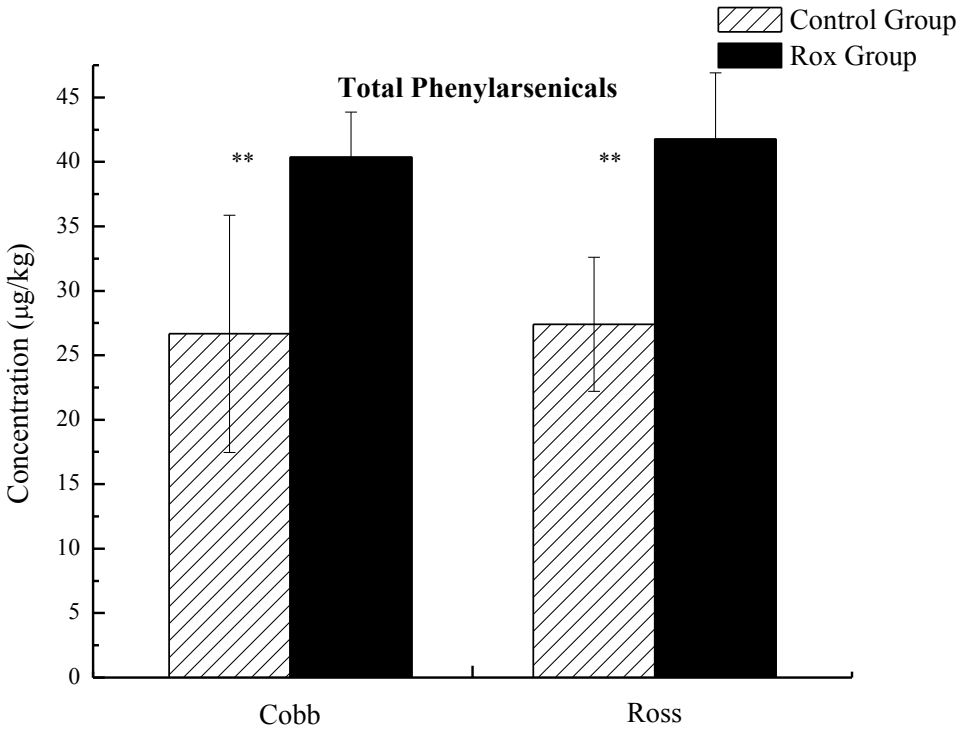
** At the 0.05 level, the two groups have the significant difference

Figure 3.18 Comparison of the total concentration of methyl arsenicals (MMA^V+ DMA^V) in chicken kidney between the ROX-Group and the Control-Group in both strains (Cobb 500, Ross 308) on Day 35. The error bars represent one standard deviation from triplicate analyses of 4 kidney samples.



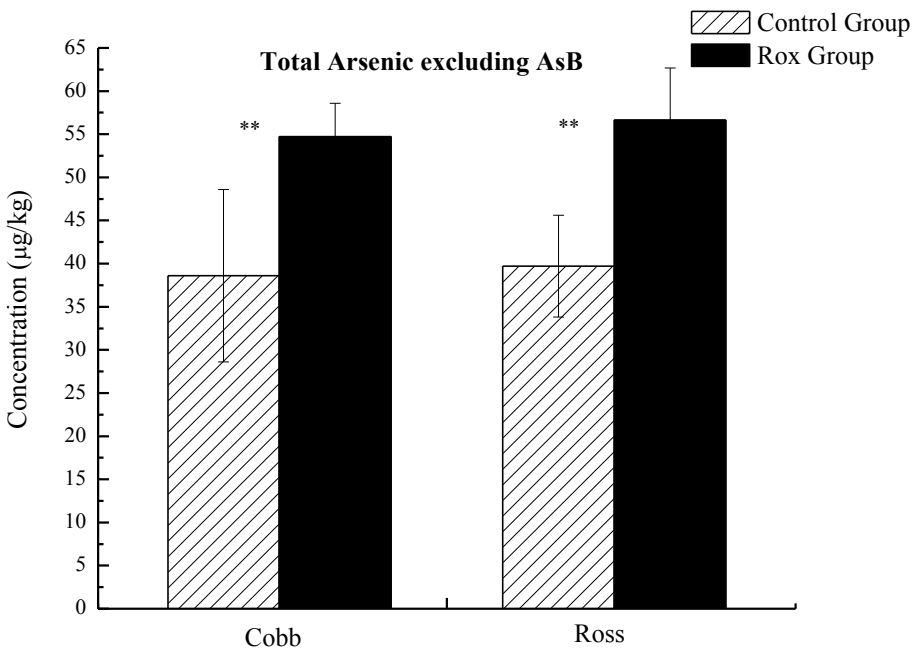
** At the 0.05 level, the two groups have the significant difference

Figure 3.19 Comparison of the total concentration of 3-AHPAA and N-AHAA in chicken kidney between the ROX-Group and the Control-Group in both strains (Cobb 500, Ross 308) on Day 35. The error bars represent one standard deviation from triplicate analyses of 4 kidney samples.



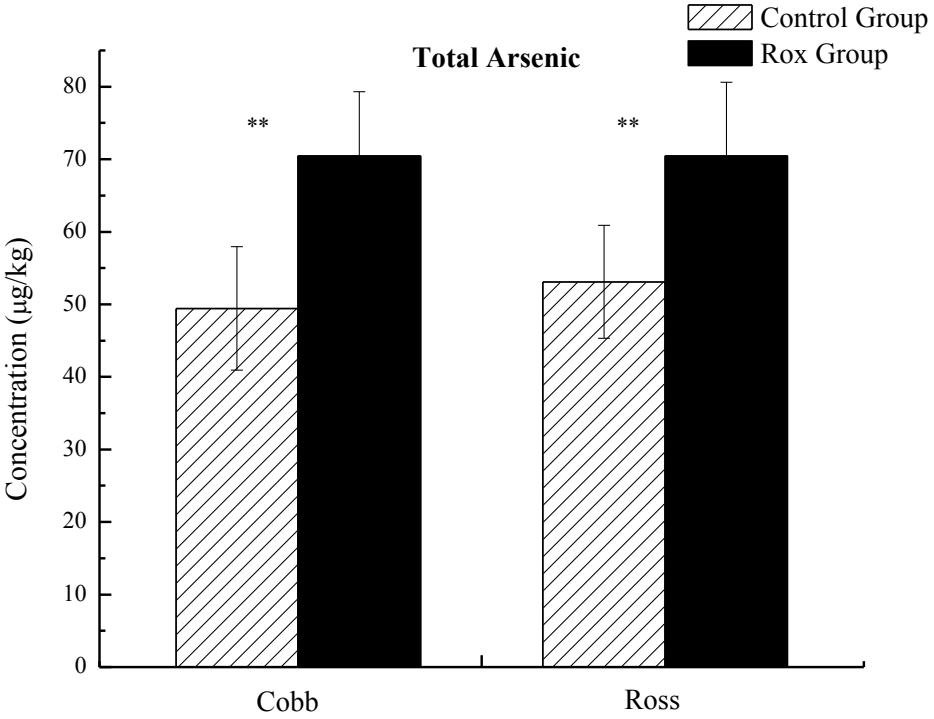
** At the 0.05 level, the two groups have the significant difference

Figure 3.20 Comparison of the total concentration of phenylarsenicals (ROX, 3-AHPAA, N-AHAA, and unknown arsenic) in chicken kidney between the ROX-Group and the Control-Group in both strains (Cobb 500, Ross 308) on Day 35. The error bars represent one standard deviation from triplicate analyses of 4 kidney samples.



** At the 0.05 level, the two groups have the significant difference.

Figure 3.21 Comparison of the sum of all arsenic species excluding the non-toxic AsB in chicken kidney between the ROX-Group and the Control-Group in both strains (Cobb 500, Ross 308) on Day 35. The error bars represent one standard deviation from triplicate analyses of 4 kidney samples.



** At the 0.05 level, the two groups have the significant difference

Figure 3.22 Comparison of the sum of all arsenic species in chicken kidney between the ROX-Group and the Control-Group in both strains (Cobb 500, Ross 308) on Day 35. The error bars represent one standard deviation from triplicate analyses of 4 kidney samples.

3.4 CONCLUSIONS

The homogenized kidney samples were treated with pepsin. The extracts were analyzed for arsenic species using high performance liquid chromatography (HPLC) separation with simultaneous detection by ICP-MS and electrospray ionization tandem mass spectrometry (ESI-MS/MS). HPLC-ICP-MS allowed for quantification of arsenic species, and ESI-MS/MS provided complementary information for the identification of arsenic species. Our analyses shows the presence of ten arsenic species in the extracts of chicken kidney samples. The results from the analysis of both the control and the ROX-fed chickens are useful for a better understanding of arsenic metabolism, distribution, and retention in chicken.

The data presented in our feeding experiment suggest that arsenic concentrations in poultry without withdrawal of Roxarsone feeding can be approximately 10-times greater than those in poultry fed with non-ROX diet. The higher concentrations of inorganic arsenicals ($As^{III} + As^{V}$), phenylarseniclas (Rox, and 3-AHPAA and N-AHAA), and methylarsenicals (MMA^{V}+DMA^{V}) observed in poultry kidney is consistent with consumption of Roxarsone feed additives.

After 7 days of withdrawal, the concentrations of arsenic in poultry kidney decrease dramatically. However, the concentrations of inorganic arsenic in Rox-fed group (6.6 ± 1.2 µg/kg) slightly higher than those in the control group (3.8 ± 0.5 µg/kg) of poultry kidney. The residual concentration of total arsenic in the control chickens and in the ROX-fed chickens are below the FDA tolerance levels for chicken muscles (500 µg/kg) and liver (2000 µg/kg)

3.5 REFERENCES

(1) Braude, R.; Wallace, H.; Cunha, T. *Antibiotics & chemotherapy* **1953**, *3*, 271.

(2) National Research Council, Arsenic in Drinking Water, *National Academy Press* **1999**, *4*, 31

(3) Wegener, H. C.; Aarestrup, F. M.; Jensen, L. B.; Hammerum, A. M.; Bager, F. *Emerging infectious diseases* **1999**, *5*, 329.

(4) Joseph Kawalek, Mary C. Carson, Sean D. Conklin, Karyn Howard, *U.S. Food and Drug Agency*, **2011**, Analysts' Report for Study *275*, 30

(5) Alp, O.; Zhang, Y. F.; Merino, E. J.; Caruso, J. A. *Metallomics* **2011**, *3*, 482.

(6) Smith, A. H.; Hopenhaynrich, C.; Bates, M. N.; Goeden, H. M.; Hertzpicciotto, I.; Duggan, H. M.; Wood, R.; Kosnett, M. J.; Smith, M. T. *Environmental Health Perspectives* **1992**, *97*, 259.

(7) Lerman, S.; Clarkson, T. W. *Fundamental and Applied Toxicology* **1983**, *3*, 309.

(8) George Cherian, M.; Goyer, R. A. *Life sciences* **1978**, *23*, 1.

(9) Pizarro, I.; Gomez, M. M.; Fodor, P.; Palacios, M. A.; Camara, C. *Biological Trace Element Research* **2004**, *99*, 129.

(10) Conklin, S. D.; Shockey, N.; Kubachka, K.; Howard, K. D.; Carson, M. C. *Journal of Agricultural and Food Chemistry* **2012**, *60*, 9394.

CHAPTER4

GENERAL DISCUSSION AND CONCLUSIONS

4.1 REVIEW OF THESIS OBJECTIVES

An organoarsenic compound, 3-nitro-4-hydroxyphenyl arsenic acid (also known as Roxarsone), was added to poultry food to control intestinal parasites and improve meat pigmentation.[1] Most of ingested ROX was excreted in chicken manure. However, the remaining ROX in the chicken body may be metabolized and transformed into other more toxic arsenic species, which will then increase the levels of arsenic exposure by chicken consumers.[2] In order to study the distribution of arsenic species in the chicken fed with Roxarsone food, we conducted a large scale feeding experiment, involving 1600 chickens, over a 35-day feeding period. The objective of this thesis was to investigate arsenic species in kidney samples from a subset of 192 chickens, fed with either the ROX-supplemented food or the Control food without ROX. An analytical method has been described in Chapter 2, which hyphenated high-performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICP-MS), and electrospray ionization mass spectrometry (ESI-MS). The method was intended for the arsenic speciation in poultry kidney. In Chapter 3, the established method was applied to determine the concentrations of arsenic species in poultry kidney.

4.2 SUMMARY OF RESULTS

The hyphenated HPLC-ICP-MS/ESI-MS method could simultaneously quantify and confirm ten arsenic species in chicken kidney. The anion exchange chromatography can separate all eight target arsenic species, including arsenobetaine (AsB), inorganic 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), within 15 min. The detection limit for HPLC-ICP-MS is 5.0 μg/kg for 3-AHPAA, and 1.0 μg/kg for other arsenic species. The pepsin extraction can achieve ~64% extraction efficiency of As from chicken kidney.

Since chickens were fed with ROX supplemented food in the ROX treated group, the dominant arsenic species determined in kidney samples is Roxarsone as expected. However, elevated concentrations of inorganic arsenic were also detected in the ROX treated group compared with the Control group. The very strong positive correlation between ROX and inorganic arsenite was observed in poultry kidney. Inorganic arsenic accounts for about 36% of total arsenic on Day 28, and 10% of total arsenic after 7 days of withdrawal of ROX.

The concentrations of methylated arsenic species (MMA^V and DMA^V) are much lower than inorganic arsenic species in chicken kidney. The differences between ROX-treated group and Control group for methylated arsenic are not as apparent as other arsenic species.

Two significant biotransformation products, 3-AHPAA and N-AHAA were also detected in poultry kidney. There are no 3-AHPAA and N-AHAA contained in chicken food as shown in chromatograms obtained from HPLC-ICP analyses of chicken food (Figure A.1). These two species are direct metabolites of ROX in poultry kidney. The temporal profiles of 3-AHPAA and N-AHAA share the same trends with ROX during a 35-days feeding. Another two unknown arsenic species were also detected in chicken kidney. The concentration of unknown arsenic species are very low in the extracts. The identification of these two unknown arsenic species is very difficult.

The total arsenic concentration after 7 days of ROX withdrawal is ~70 μg/kg (0.07 ppm), which is below FDA regulation (2 ppm) for total arsenic concentration in kidney. Assuming that one adult (body weight ~70 kg) would consume 50 g of chicken kidney per day. The total arsenic intake from chicken kidney would be 3.5 μg per day. The

World Health Organization (WHO) guideline for arsenic in drinking water is 10 μg/L. One adult who drink 2 L water per day could ingest a maximum of 20 μg arsenic daily. For comparison, the arsenic intake from 50 g chicken kidney would be 6 times lower than that of maximum arsenic intake from drinking water.

Arsenic levels in different organs were also compared in Tables A.4.1-A.4.4. Arsenic concentrations in liver, muscle, and skin were obtained from works of Hanyong Peng, Qingqing Liu, and Chenming Cao, respectively. The sum of concentrations of all arsenic species excluding AsB in chicken kidney is lower than that in chicken liver, but higher than that in chicken muscle and skin. That may be because chicken kidney and liver contain more sulfur that could bind to arsenic. Also, liver and kidney are main metabolic organs. Since chicken were fed with the same amount of arsenobetaine, arsenobetaine remained in kidney are close to that in liver for both groups on both Day 28 and Day 35. Arsenic levels in kidney, liver, muscle, and skin are all related to the intake of ROX-containing diet. After 7 days' withdrawal of Roxarsone, arsenic levels in kidney, liver, muscle, and skin all sharply decreased compared with the concentrations of arsenic on Day 28.

Overall, this thesis has established a method for arsenic speciation in poultry kidney, including extraction, separation, detection and verification. The arsenic speciation analysis in the ROX-fed chickens as compared to the control chickens provided useful information: (1) the temporal profiles of each arsenic species in poultry kidney; (2) concentration patterns and correlation among the various arsenic species; (3) rapid elimination of arsenic species after stop of ROX feeding; and (4) the residual concentration of arsenic species following a withdrawal (clearance) period.

4.3 FUTURE RESEARCH

The complicated matrix of poultry kidney sample makes the identification of unknown arsenic species very difficult. Future research is needed to establish an efficient

enrichment and extraction method, to further purify the kidney extracts. Besides, the ionization efficiency of As^V, 3-AHPAA is poor during the ESI-MS analysis.[3] It would useful to enhance the ionization of As^V and 3-AHPAA.

Whether the transformation of ROX takes place in poultry kidney, or elsewhere in poultry is unknown. In order to determine that the metabolism of ROX truly take place in the poultry kidney, analyses of fresh blank poultry kidney incubated with ROX will be useful. Further research could also identify enzymes that are required for the transformation of remained arsenic.

The temporal profile of each arsenic species provided detailed information for distribution of arsenic in poultry kidney. However, it is also important to combine this research with other results, such as arsenic speciation in poultry muscle and liver. The correlation between arsenic in kidney and in other tissues and organs of chicken may need to be further investigated.

In 2013, Maryland became the first state to ban Rox in poultry production. It is intended to reduce human exposure to arsenic from chicken. However, Nitarsone (which has chemically similar structure with that of Roxarsone) was exempted from the Maryland law. Although Nitarsone may behave similarly to Roxarsone, there is no study on the metabolism of Nitarsone or the distribution and elimination of Nitarsone and its metabolites. In order to protect public health and eliminate the potential risk of dietary arsenic exposure, similar determination method can be applied to investigate the Nitarsone feeding to the poultry.

4.4 REFERENCES

(1) Silbergeld, E. K.; Nachman, K. In *Environmental Challenges in the Pacific Basin*; Carpenter, D. O., Ed. **2008**; *1140,* 346.

(2) Nachman, K. E.; Baron, P. A.; Raber, G.; Francesconi, K. A.; Navas-Acien, A.; D. C. *Environ Health Perspect* **2013**, *121*, 818.

(3) Lobinski, R.; Schaumloffel, D.; Szpunar, J. *Mass Spectrometry Reviews* **2006**, *25*, 255.

BIBLIOGRAPHY

(1) H. Onishi, K. H. W. *Handbook of Geochemistry, Springer-Verlag*, **1969**. II-1, *33-B5*

(2) Shacklette, H. T.; Boerngen, J. G. *Element Concentrations in Soils and Other Surficial Materials of the Conterminous United States* **1984**. *103.*1270

(3) Cox, D. P.; Alexande.M *Applied Microbiology* **1973**, *25*, 408.

(4) Reichert, F.; Trelles, R. *An. Asoc. Quim. Argent* **1921**, *9*, 89.

(5) McBride, B.; Wolfe, R. *Biochemistry-Us* **1971**, *10*, 4312.

(6) Ferguson, J. F.; Gavis, J. *Water Research* **1972**, *6*, 1259.

(7) Ravenscroft, P.; Brammer, H.; Richards, K. *Arsenic pollution: a global synthesis*; John Wiley & Sons, **2011**; *94*.103

(8) World Health Organization (WHO). *Guidelines for drinking-water quality: recommendations*; World Health Organization, **2004**; *Vol 1*, 1

(9) Komorowicz, I.; Barałkiewicz, D. *Talanta* **2011**, *84*, 247.

(10) Frost, F. J.; Muller, T.; Petersen, H. V.; Thomson, B.; Tollestrup, K. *Journal of Exposure Science and Environmental Epidemiology* **2003**, *13*, 231.

(11) McGuigan, C. F.; Hamula, C. L.; Huang, S.; Gabos, S.; Le, X. C. *Environmental Reviews* **2010**, *18*, 291.

(12) Nickson, R.; McArthur, J.; Burgess, W.; Ahmed, K. M.; Ravenscroft, P.; Rahman, M. *Nature* **1998**, *395*, 338.

(13) Chowdhury, U. K.; Biswas, B. K.; Chowdhury, T. R.; Samanta, G.; Mandal, B. K.; Basu, G. C.; Chanda, C. R.; Lodh, D.; Saha, K. C.; Mukherjee, S. K. *Environmental Health Perspectives* **2000**, *108*, 393.

(14) Nickson, R.; McArthur, J.; Ravenscroft, P.; Burgess, W.; Ahmed, K. *Applied Geochemistry* **2000**, *15*, 403.

(15) Smedley, P.; Kinniburgh, D. *Applied Geochemistry* **2002**, *17*, 517.

(16) Pal, T.; Mukherjee, P. K.; Sengupta, S. *Current Science* **2002**, *82*, 554.

(17) Zhang, W.; Cai, Y.; Tu, C.; Ma, L. Q. *Science of the Total Environment* **2002**, *300*, 167.

(18) Meharg, A. A.; Rahman, M. M. *Environmental Science & Technology* **2003**, *37*, 229.

(19) Zhao, F.-J.; McGrath, S. P.; Meharg, A. A. *Annual Review of Plant Biology, Vol 61* **2010**, *61*, 535.

(20) Williams, P. N.; Villada, A.; Deacon, C.; Raab, A.; Figuerola, J.; Green, A. J.; Feldmann, J.; Meharg, A. A. *Environmental Science & Technology* **2007**, *41*, 6854.

(21) Meharg, A. A.; Williams, P. N.; Adomako, E.; Lawgali, Y. Y.; Deacon, C.; Villada, A.; Cambell, R. C.; Sun, G.; Zhu, Y.-G.; Feldmann, J. *Environmental Science & Technology* **2009**, *43*, 1612.

(22) Caruso, J. A.; Heitkemper, D. T.; B'Hymer, C. *Analyst* **2001**, *126*, 136.

(23) Herce-Pagliai, C.; Moreno, I.; Gonzalez, G.; Repetto, M.; Cameán, A. *Food Additives & Contaminants* **2002**, *19*, 542.

(24) Xu, J.; Thornton, I. *Environmental Geochemistry and Health* **1985**, *7*, 131.

(25) Ventura-Lima, J.; Bogo, M. R.; Monserrat, J. M. *Ecotox Environ Safe* **2011**, *74*, 211.

(26) Ghimire, K. N.; Inoue, K.; Makino, K.; Miyajima, T. *Separation Science and Technology* **2002**, *37*, 2785.

(27) Jorhem, L.; Slorach, S.; Sundström, B.; Ohlin, B. *Food Additives & Contaminants* **1991**, *8*, 201.

(28) Ulman, C.; Gezer, S.; Anal, Ö.; Töre, I. R.; Kirca, Ü. *Water, Air, and Soil Pollution* **1998**, *101*, 411.

(29) Evans, R. J.; Bandemer, S. L. *Analytical Chemistry* **1954**, *26*, 595.

(30) Donoghue, D. J.; Hairston, H.; Cope, C. V.; Bartholomew, M. J.; Wagner, D. D. *Journal of Food Protection®* **1994**, *57*, 218.

(31) Tam, G.; Charbonneau, S.; Bryce, F.; Pomroy, C.; Sandi, E. *Toxicology and Applied Pharmacology* **1979**, *50*, 319.

(32) Tsuchiya, K. *Environmental Health Perspectives* **1977**, *19*, 35.

(33) Lüchtrath, H. *Journal of Cancer Research and Clinical Oncology* **1983**, *105*, 173.

(34) Rahman, M. M.; Sengupta, M. K.; Chowdhury, U. K.; Lodh, D.; Das, B.; Ahamed, S.; Mandal, D.; Hossain, M. A.; Mukherjee, S. C.; Pati, S., CSIRO Publishing, **2006**, *38*, 29.

(35) Lüchtrath, H. *Deutsche Medizinische Wochenschrift (1946)* **1972**, *97*, 21.

(36) Morrison, J. L. *Journal of Agricultural and Food Chemistry* **1969**, *17*, 1288.

(37) Lasky, T.; Sun, W. Y.; Kadry, A.; Hoffman, M. K. *Environmental Health Perspectives* **2004**, *112*, 18.

(38) Chan, P. C.; Huff, J. *Journal of Environmental Science & Health Part C* **1997**, *15*, 83.

(39) Chung, J. S.; Kalman, D. A.; Moore, L. E.; Kosnett, M. J.; Arroyo, A. P.; Beeris, M.; Mazumder, D. G.; Hernandez, A. L.; Smith, A. H. *Environmental Health Perspectives* **2002**, *110*, 729.

(40) Gul Kazi, T.; Qadir Shah, A.; Imran Afridi, H.; Ali Shah, N.; Balal Arain, M. *Ecotox Environ Safe* **2013**, *87*, 120.

(41) Maeda, S. *Arsenic in Environment. Part I: Cycling and Characterization*; John Wiley & Sons Inc, **1994**. 155

(42) Cullen, W. R.; Reimer, K. J. *Chemical Reviews* **1989**, *89*, 713.

(43) Aposhian, H. V. *Annual Review of Pharmacology and Toxicology* **1997**, *37*, 397.

(44) Thomas, D. J.; Styblo, M.; Lin, S. *Toxicology and Applied Pharmacology* **2001**, *176*, 127.

(45) Marafante, E.; Vahter, M.; Envall, J. *Chemico-biological Interactions* **1985**, *56*, 225.

(46) Stýblo, M.; Drobná, Z.; Jaspers, I.; Lin, S.; Thomas, D. J. *Environmental Health Perspectives* **2002**, *110*, 767.

(47) Thompson, D. J. *Chemico-biological Interactions* **1993**, *88*, 89.

(48) Marafante, E.; Vahter, M. *Environ. Res.* **1987**, *42*, 72.

(49) Vahter, M. *Applied Organometallic Chemistry* **1994**, *8*, 175.

(50) Vahter, M.; Marafante, E.; Dencker, L. *Science of the Total Environment* **1983**, *30*, 197.

(51) Lindgren, A.; Vahter, M.; Dencker, L. *Acta Pharmacologica et Toxicologica*

1982, *51*, 253.

(52) Edmonds, J.; Francesconi, K. *Nature* **1981**, *289*, 602.

(53) Edmonds, J.; Francesconi, K. *Experientia* **1987**, *43*, 553.

(54) Freeman, H.; Uthe, J.; Fleming, R.; Odense, P.; Ackman, R.; Landry, G.; Musial, C. *Bulletin of Environmental Contamination and Toxicology* **1979**, *22*, 224.

(55) Le, X. C.; Cullen, W. R.; Reimer, K. J. *Clinical Chemistry* **1994**, *40*, 617.

(56) Arsenic, I. A. R. C. "arsenic compounds (Group 1)." *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans"* **1987**.*7*, 1

(57) ATSDR *US department of Health & Human Service, Public Health Sevice*, *Agency for Toxic Substances and Disease Registry*. **2000**, 428

(58) IPCS *Environmental Health Criteria Series* **2001**, 224.

(59) Chen, C.; Hsu, L.; Tseng, C.; Hsueh, Y.; Chiou, H. *Arsenic Exposure and Health Effects (Chappell WR, Abemathy CO, Calderon RL, eds). New York: Elsevier Science* **1999**, *12*, 113.

(60) Tseng, W.-P. *Environmental Health Perspectives* **1977**, *19*, 109.

(61) Ahmad, S. A.; Sayed, S. U.; Barua, S.; Khan, M. H.; Jalil, A.; Hadi, S. A.; Talukder, H. K. *Environmental Health Perspectives* **2001**, *109*, 629.

(62) Chakraborti, D.; Basu, G. K.; Biswas, L. K.; Chowdhury, U. K.; Rahman, M. M.; Paul, K.; Chowdhury, T. R.; Chanda, C. R.; Lodh, D.; Ray, S. L. *Characterization of Arsenic-bearing Sediments in the Gangetic Delta of West Bengal, India*, *Arsenic exposure and health effects* **2001**, *4*, 27

(63) Dauphiné, D. C.; Ferreccio, C.; Guntur, S.; Yuan, Y.; Hammond, S. K.; Balmes, J.; Smith, A. H.; Steinmaus, C. *International Archives of Occupational and Environmental Health* **2011**, *84*, 591.

(64) Andrew, A. S.; Jewell, D. A.; Mason, R. A.; Whitfield, M. L.; Moore, J. H.; Karagas, M. R. *Environmental Health Perspectives* **2008**, *116*, 524.

(65) Chen, Y.; Parvez, F.; Gamble, M.; Islam, T.; Ahmed, A.; Argos, M.; Graziano, J. H.; Ahsan, H. *Toxicology and Applied Pharmacology* **2009**, *239*, 184.

(66) Ahmed, S.; Ahsan, K. B.; Kippler, M.; Mily, A.; Wagatsuma, Y.; Hoque, A. W.; Ngom, P. T.; El Arifeen, S.; Raqib, R.; Vahter, M. *Toxicol Sci* **2012**, *129*, 305.

(67) Bronstein, A. C.; Spyker, D. A.; Cantilena, L. R.; Green, J. L.; Rumack, B. H.; Dart, R. C. *Clinical Toxicology* **2011**, *49*, 910.

(68) SCHOOLMEESTER, W. L.; WHITE, D. R. *Southern Medical Journal* **1980**, *73*, 198.

(69) Mueller, P.; Benowitz, N. *Emergency Medicine Clinics of North America* **1989**, *7*, 667.

(70) Campbell, J. P.; Alvarez, J. *American Family Physician* **1989**, *40*, 93.

(71) Francesconi, K. A.; Kuehnelt, D. *Analyst* **2004**, *129*, 373.

(72) Wagner, G. *Science of the Total Environment* **1995**, *176*, 63.

(73) Le.X. C.; Cullen, W. R.; Reimer, K. J. *Environmental Science & Technology* **1994**, *28*, 1598.

(74) Segura, M.; Muñoz, J.; Madrid, Y.; Cámara, C. *Analytical and Bioanalytical Chemistry* **2002**, *374*, 513.

(75) Martınez-Bravo, Y.; Roig-Navarro, A.; Lopez, F.; Hernandez, F. *Journal of Chromatography A* **2001**, *926*, 265.

(76) Hall, G. M. *Journal of Analytical Atomic Spectrometry* **1999**, *14*, 205.

(77) Bednar, A.; Garbarino, J.; Ranville, J.; Wildeman, T. *Environmental Science & Technology* **2002**, *36*, 2213.

(78) Pizarro, I.; Gomez, M.; Camara, C.; Palacios, M. A. *Analytica Chimica Acta* **2003**, *495*, 85.

(79) Oliveira, V.; Sarmiento, A.; Gómez-Ariza, J.; Nieto, J.; Sánchez-Rodas, D. *Talanta* **2006**, *69*, 1182.

(80) Namieśnik, J.; Górecki, T. *Pol. J. Environ. Stud* **2001**, *10*, 77.

(81) Edmonds, J.; Shibata, Y.; Prince, R.; Francesconi, K.; Morita, M. *Journal of the Marine Biological Association of the United Kingdom* **1994**, *74*, 463.

(82) Leermakers, M.; Baeyens, W.; De Gieter, M.; Smedts, B.; Meert, C.; De Bisschop, H.; Morabito, R.; Quevauviller, P. *TrAC Trends in Analytical Chemistry* **2006**, *25*, 1.

(83) Stan'kov, I.; Sergeeva, A.; Tarasov, S. *Journal of Analytical Chemistry* **2000**, *55*, 66.

(84) Haas, R.; Krippendorf, A. *Environmental Science and Pollution Research* **1997**, *4*, 123.

(85) Schoene, K.; Steinhanses, J.; Bruckert, H.-J.; König, A. *Journal of Chromatography A* **1992**, *605*, 257.

(86) Epure, G.; Grigoriu, N.; Filipescu, L. *University Politehnica of Bucharest, Scientific Bulletin Series B: Chemistry and Materials Science* **2010**, *3*, 72.

(87) Tørnes, J. A.; Opstad, A. M.; Johnsen, B. A. *Science of the Total Environment* **2006**, *356*, 235.

(88) Carpenter, R. C. *Analytica Chimica Acta* **1981**, *125*, 209.

(89) Bermejo, P.; Capelo, J.; Mota, A.; Madrid, Y.; Cámara, C. *TrAC Trends in Analytical Chemistry* **2004**, *23*, 654.

(90) Forsyth, D. S.; Marshall, W. D. *Environmental Science & Technology* **1986**, *20*, 1033.

(91) Gilon, N.; PotinGautier, M.; Astruc, M. *Journal of Chromatography A* **1996**, *750*, 327.

(92) Fritz, J. S.; Gjerde, D. T. Wiley, *Ion chromatography*. John Wiley & Sons, **2009**. *31*, 1

(93) Zimmermann, M. D.; Tossell, J. A. *The Journal of Physical Chemistry A* **2009**, *113*, 5105.

(94) Larsen, E. H. *Spectrochimica Acta Part B: Atomic Spectroscopy* **1998**, *53*, 253.

(95) Beauchemin, D.; Siu, K.; McLaren, J. W.; Berman, S. S. *J. Anal. At. Spectrom.* **1989**, *4*, 285.

(96) Zheng, J.; Goessler, W.; Kosmus, W. *Microchimica Acta* **1998**, *130*, 71.

(97) Lintschinger, J.; Schramel, P.; Hatalak-Rauscher, A.; Wendler, I.; Michalke, B. *Fresenius' Journal of Analytical Chemistry* **1998**, *362*, 313.

(98) Ackley, K. L.; B'Hymer, C.; Sutton, K. L.; Caruso, J. A. *Journal of Analytical Atomic Spectrometry* **1999**, *14*, 845.

(99) Larsen, E. H.; Pritzl, G.; Hansen, S. H. *Journal of Analytical Atomic Spectrometry* **1993**, *8*, 1075.

(100) Campbell, N.; Reece, J. Biology, 7th edition, San Francisco, USA: Pearson,

Benjamin Cummings. **2005**, *7*, 956

(101) Shibata, Y.; Morita, M. *Applied Organometallic Chemistry* **1992**, *6*, 343.

(102) Vacchina,V.; Połeć, K.; Szpunar, J. *Journal of Analytical Atomic Spectrometry* **1999**, *14*, 1557.

(103) Wendt, R. H.; Fassel, V. A. *Analytical Chemistry* **1965**, *37*, 920.

(104) Inoue, Y.; Sakai, T.; Shimizu, N.; Yoshida, K.; Chen, H.; Kuroda, K.; Endo, G. *Applied Organometallic Chemistry* **1999**, *13*, 81.

(105) Moreda-Pineiro, J.; Alonso-Rodriguez, E.; Romaris-Hortas, V.; Moreda-Pineiro, A.; Lopez-Mahia, P.; Muniategui-Lorenzo, S.; Prada-Rodriguez, D.; Bermejo-Barrera, P. *Food Chemistry* **2012**, *130*, 552.

(106) Hansen, H. R. *J. Anal. At. Spectrom* **2003**, *18*,474.

(107) Pozniak, B.; Cole, R. *J Am Soc Mass Spectrom* **2007**, *18*, 737.

(108) McSheehy, S.; Pohl, P. L.; Lobinski, R.; Szpunar, J. *Analyst* **2001**, *126*, 1055.

(109) Mattusch, J.; Möller, D.; González, M. P. E.; Wennrich, R. *Analytical and Bioanalytical Chemistry* **2008**, *390*, 1707.

(110) Rosenberg, E.; Ariese, F. *Trace Element Speciation for Environment, Food and Health*. *The Royal Society of Chemistry, Cambridge*, UK, **2001**, *17*, 164

(111) Bermejo, P.; Capelo, J.; Mota, A.; Madrid, Y.; Cámara, C. TrAC *Trends in Analytical Chemistry* **2004**, *23*, 654.

(112) Carpenter, R. C. *Analytica Chimica Acta* **1981**, *125*, 209.

(113) Pardo-Martinez, M.; Vinas, P.; Fisher, A.; Hill, S. J. *Analytica Chimica Acta* **2001**, *441*, 29.

(114) Harrington, C. F.; Clough, R.; Hansen, H. R.; Hill, S. J.; Pergantis, S. A.; Tyson, J. F. *Journal of Analytical Atomic Spectrometry* **2009**, *24*, 999.

(115) Jackson, B. P.; Bertsch, P. M. *Environmental Science & Technology* **2001**, *35*, 4868.

(116) Wang, P.; Zhao, G.; Tian, J.; Su, X. *Journal of Agricultural and Food Chemistry* **2010**, *58*, 5263.

(117) Grant, T. D, Jones, J. F. *Journal American Water Works Association*, **1977**, *69*,12

(118) McSheehy, S.; Marcinek, M.; Chassaigne, H.; Szpunar, *J. Analytica Chimica*

Acta **2000**, *410*, 71.

(119) Hansen, H. R.; Raab, A.; Feldmann, J. *Journal of Analytical Atomic Spectrometry* **2003**, *18*, 474.

(120) Corr, J. J.; Larsen, E. H. *Journal of Analytical Atomic Spectrometry* **1996**, *11*, 1215.

(121) Gallagher, P. A.; Wei, X. Y.; Shoemaker, J. A.; Brockhoff, C. A.; Creed, J. T. *Journal of Analytical Atomic Spectrometry* **1999**, *14*, 1829.

(122) Pergantis, S. A.; Wangkarn, S.; Francesconi, K. A.; Thomas-Oates, J. E. *Analytical Chemistry* **2000**, *72*, 357.

(123) Madsen, A. D.; Goessler, W.; Pedersen, S. N.; Francesconi, K. A. *Journal of Analytical Atomic Spectrometry* **2000**, *15*, 657.

(124) Pedersen, S. N.; Francesconi, K. A. *Rapid Commun Mass Sp* **2000**, *14*, 641.

(125) Nachman, K. E.; Baron, P. A.; Raber, G.; Francesconi, K. A.; Navas-Acien, A.; D. C. *Environ Health Perspect* **2013**, *121*, 818.

(126) Braude, R.; Wallace, H.; Cunha, T. *Antibiotics & chemotherapy* **1953**, *3*, 271.

(127) National Research Council, Arsenic in Drinking Water, *National Academy Press* **1999**, *4*, 31

(128) Wegener, H. C.; Aarestrup, F. M.; Jensen, L. B.; Hammerum, A. M.; Bager, F. *Emerging infectious diseases* **1999**, *5*, 329.

(129) Joseph Kawalek, Mary C. Carson, Sean D. Conklin, Karyn Howard, *U.S. Food and Drug Agency*, **2011**, Analysts' Report for Study *275*, 30

(130) Alp, O.; Zhang, Y. F.; Merino, E. J.; Caruso, J. A. *Metallomics* **2011**, *3*, 482.

(131) Smith, A. H.; Hopenhaynrich, C.; Bates, M. N.; Goeden, H. M.; Hertzpicciotto, I.; Duggan, H. M.; Wood, R.; Kosnett, M. J.; Smith, M. T. *Environmental Health Perspectives* **1992**, *97*, 259.

(132) Lerman, S.; Clarkson, T. W. *Fundamental and Applied Toxicology* **1983**, *3*, 309.

(133) George Cherian, M.; Goyer, R. A. *Life sciences* **1978**, *23*, 1.

(134) Pizarro, I.; Gomez, M. M.; Fodor, P.; Palacios, M. A.; Camara, C. *Biological Trace Element Research* **2004**, *99*, 129.

(135) Conklin, S. D.; Shockey, N.; Kubachka, K.; Howard, K. D.; Carson, M. C.

Journal of Agricultural and Food Chemistry **2012**, *60*, 9394.

(136) Lobinski, R.; Schaumloffel, D.; Szpunar, J. *Mass Spectrometry Reviews* **2006**, *25*, 255.

APPENDIX

SUPPORTING INFORMATION

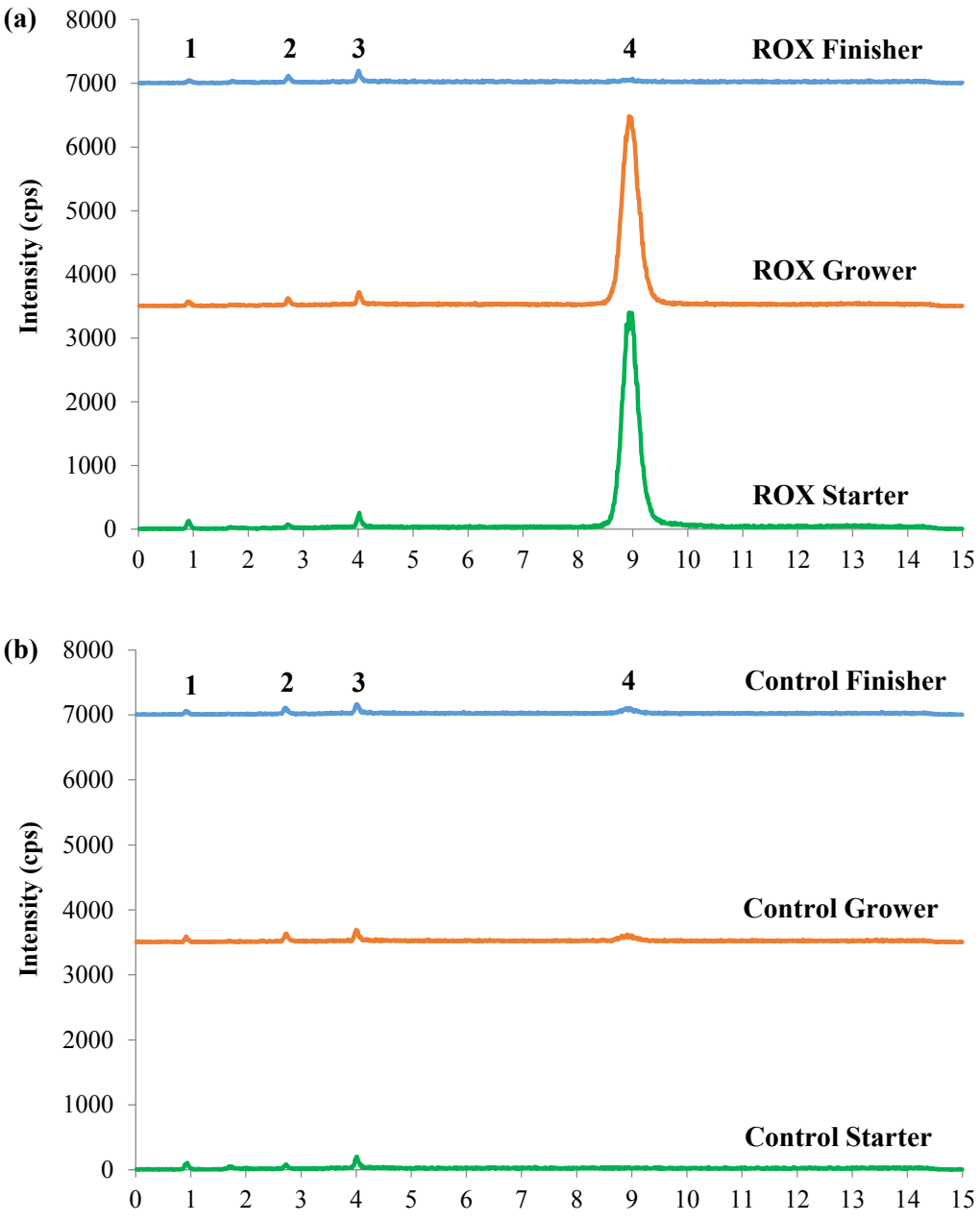


Figure A.1 Chromatograms obtained from the HPLC-ICP-MS analyses of chicken food supplemented with Roxarsone (a) or the control food not supplemented with Roxarsone (b) Peaks: (1) AsB, (2) DMA^{V}, (3) As^{V}, (4) ROX. (This figure was obtained by Zongling Yang in his MSc thesis)

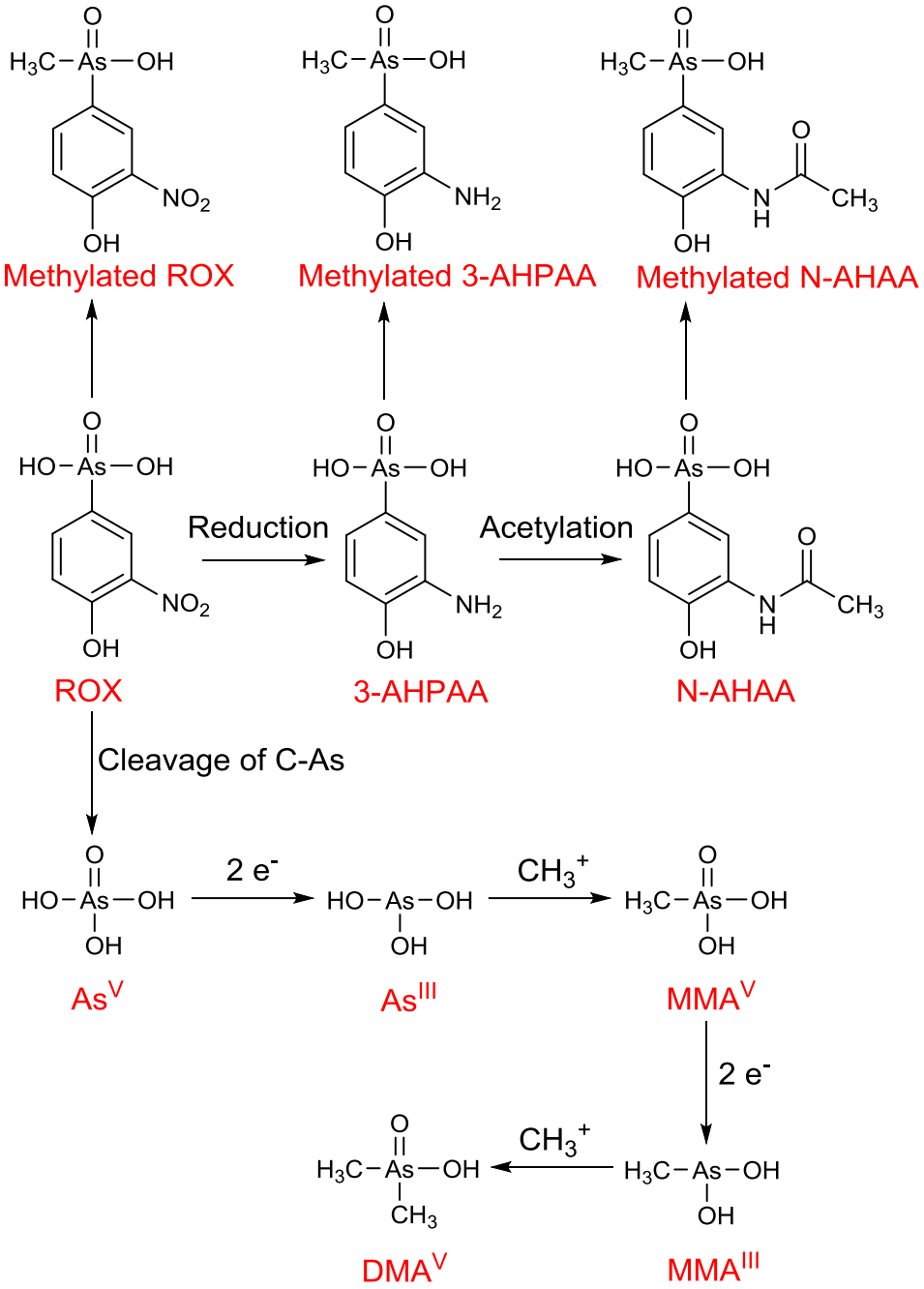
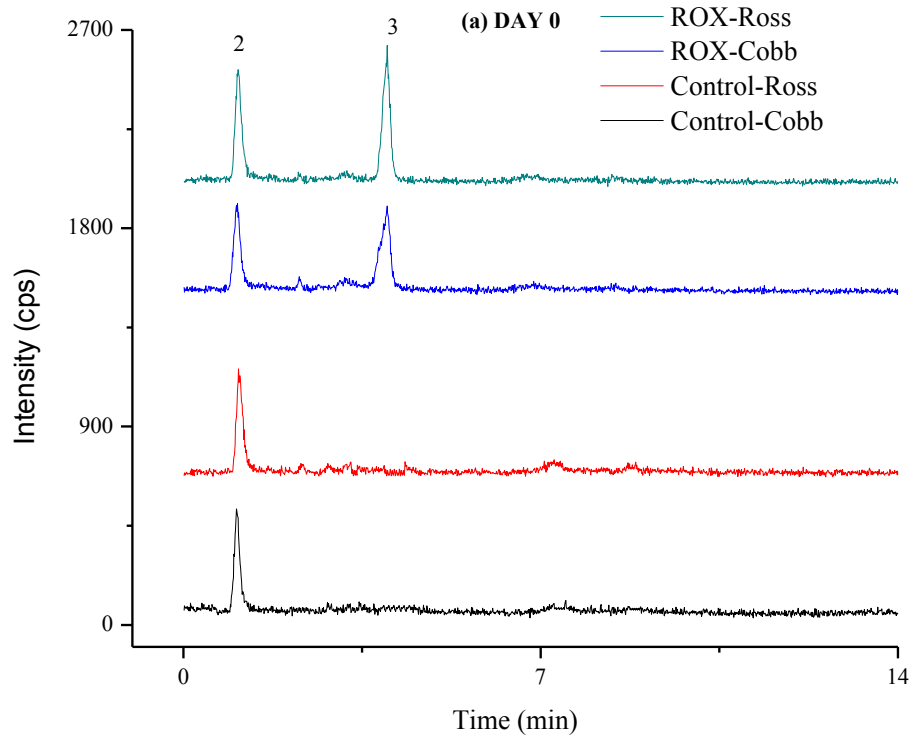
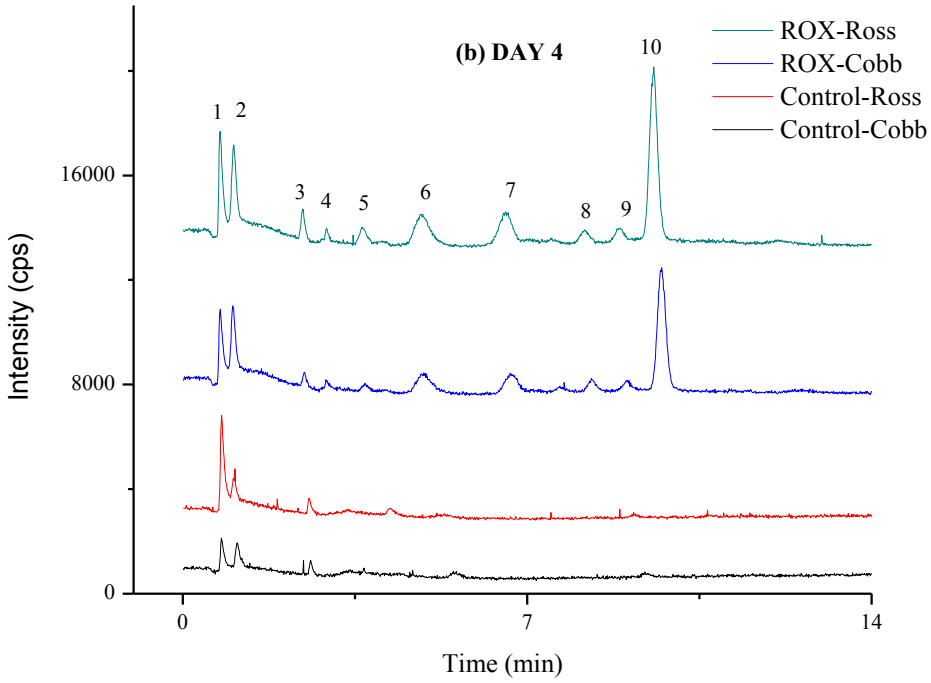
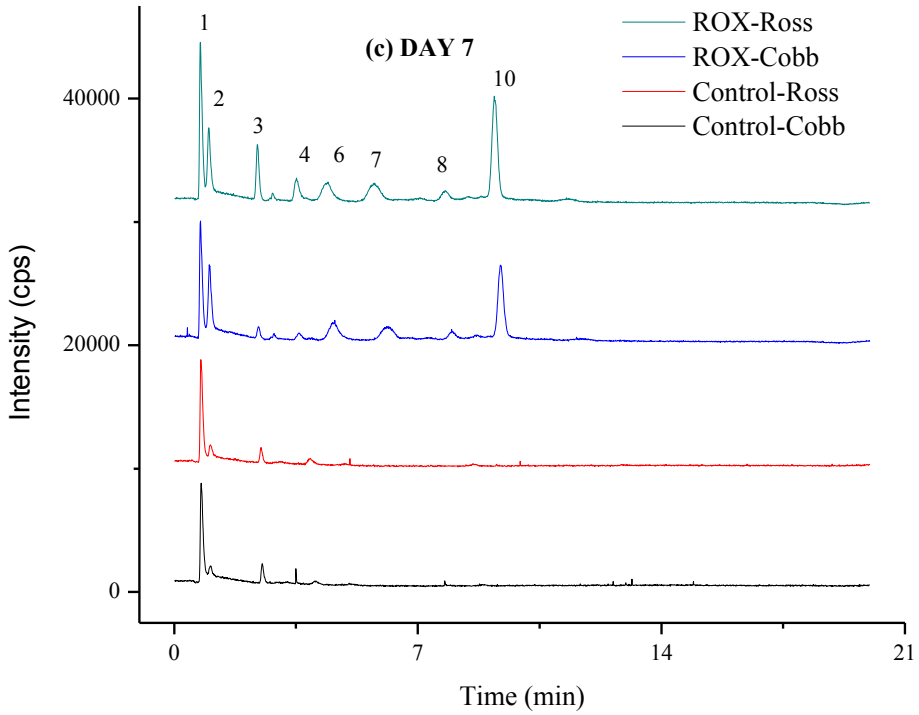
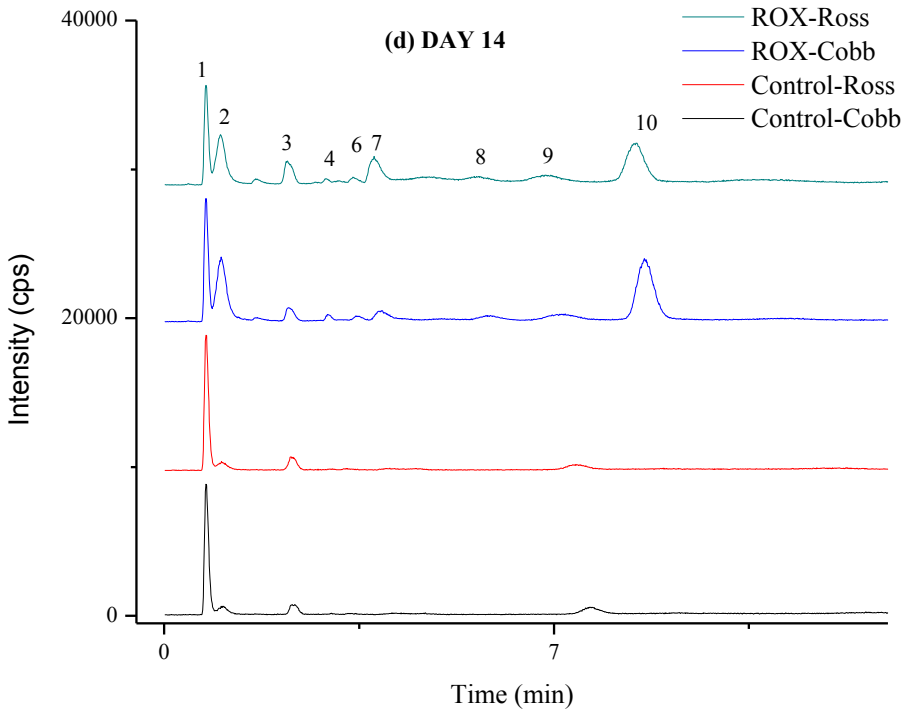


Figure A.2 Possible pathways for the metabolism of Roxarsone in chicken. This figure was obtained with the assistance of Hanyong Peng, Qingqing Liu, and Chenming Cao of the University of Alberta.


(a) DAY 0
ROX-Ross
ROX-Cobb
Control-Ross
Control-Cobb
2
3
2700
1800
900
0
Intensity (cps)
0
7
14
Time (min)


(b) DAY 4
ROX-Ross
ROX-Cobb
Control-Ross
Control-Cobb
1
2
3
4
5
6
7
8
9
10
16000
8000
0
Intensity (cps)
0
7
14
Time (min)


(c) DAY 7
ROX-Ross
ROX-Cobb
Control-Ross
Control-Cobb
1
2
3
4
6
7
8
10
40000
20000
0
Intensity (cps)
0
7
14
21
Time (min)


(d) DAY 14
ROX-Ross
ROX-Cobb
Control-Ross
Control-Cobb
1
2
3
4
6
7
8
9
10
40000
20000
0
Intensity (cps)
0
7
Time (min)

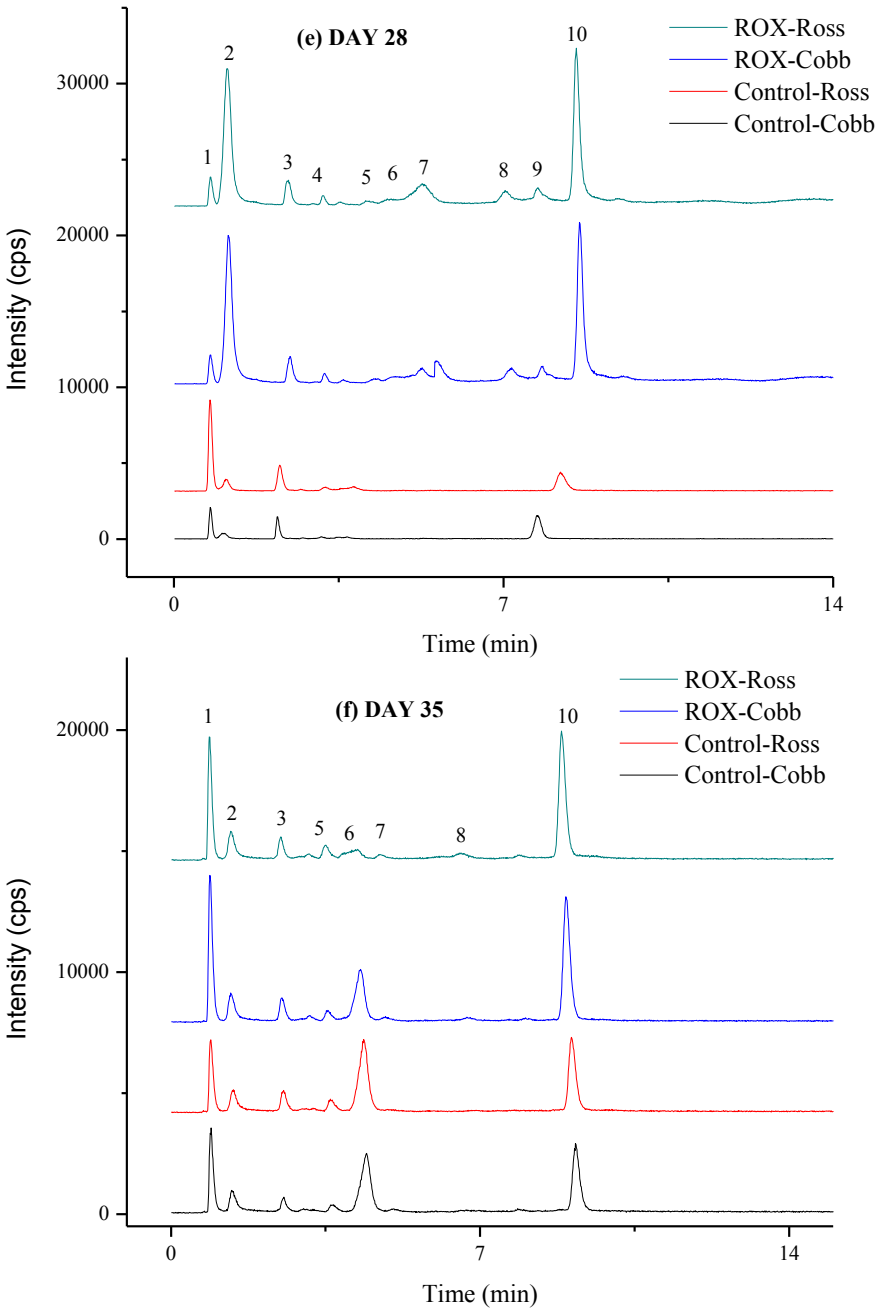


Figure A.3 Typical chromatograms obtained from HPLC-ICP-MS analyses of kidney samples collected on (a) Day 0, (b) Day 4, (c) Day 7, (d) Day 14, (e) Day 28 and (f) Day 35 from the Rox-fed chicken (including Cobb strain: blue trace and Ross strain: green trace) and the control chicken (including Cobb strain: black trace, and Ross strain: red trace). Peaks: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) Unknown 1, (6) As^{V}, (7) 3-AHPAA, (8) Unknown 2, (9) N-AHAA, and (10) Roxarsone.

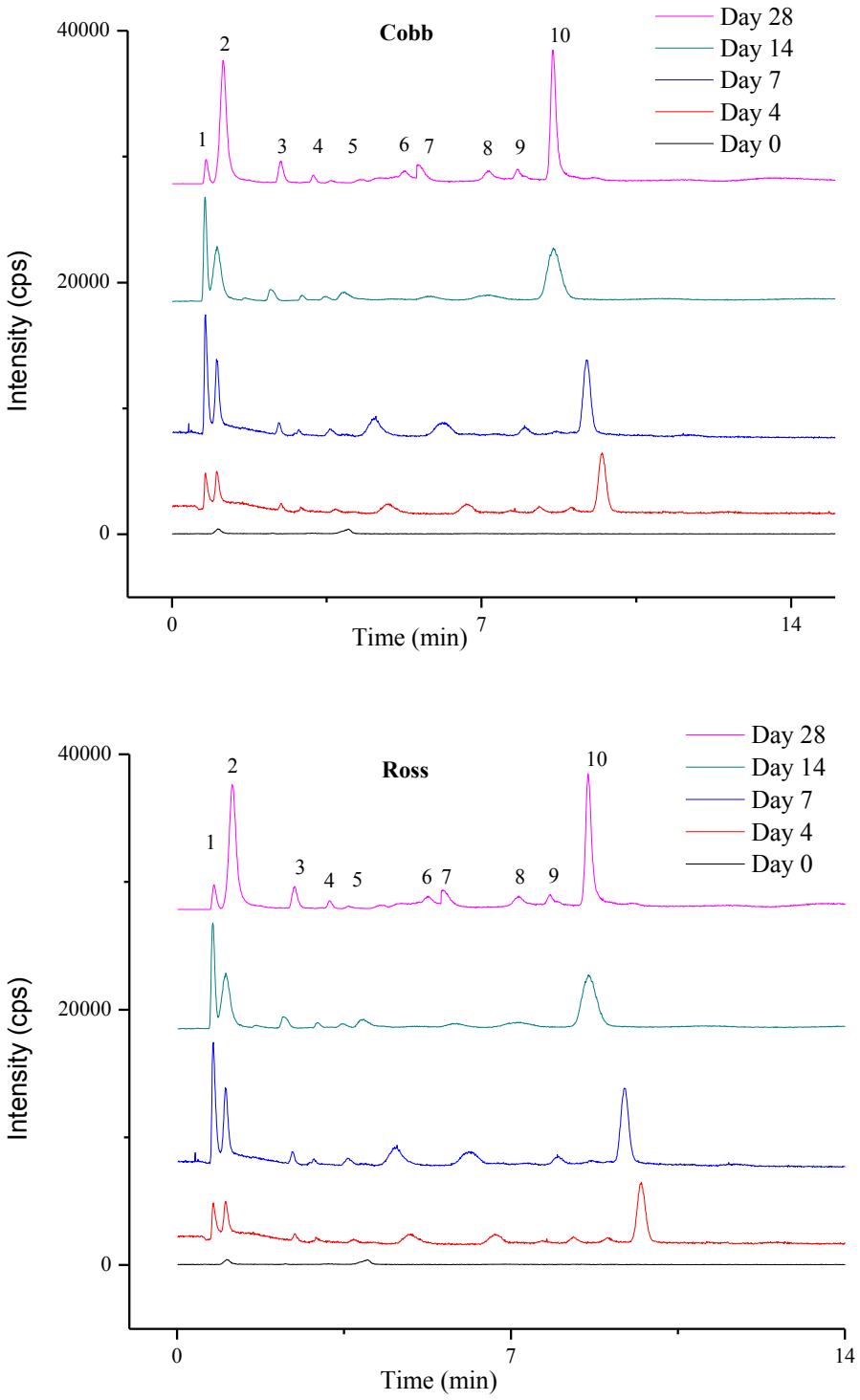


Figure A.4 Typical chromatograms obtained from HPLC-ICP-MS analyses of kidney samples on Day 0, Day 4, Day 7, Day 14, and Day 28 from ROX-fed chickens in both Cobb and Ross strains. Peaks: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) Unknown 1, (6) As^{V}, (7) 3-AHPAA, (8) Unknown 2, (9) N-AHAA, and (10) Roxarsone.

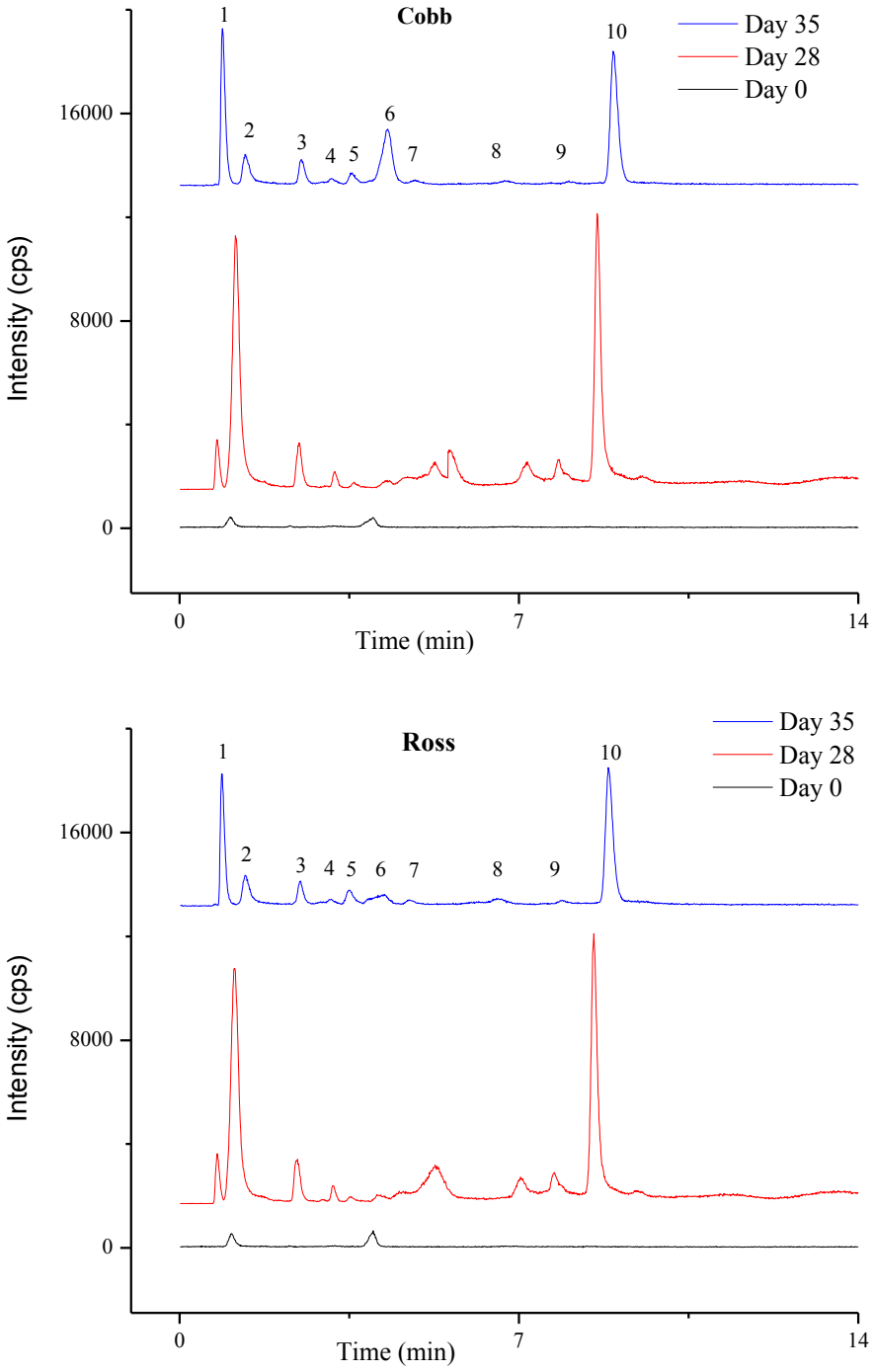


Figure A.5 Typical chromatograms obtained from HPLC-ICP-MS analyses of kidney samples on Day 0, Day 28, and Day 35 from ROX-fed chickens in both Cobb and Ross strains. Peaks: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) Unknown 1, (6) As^{V}, (7) 3-AHPAA, (8) Unknown 2, (9) N-AHAA, and (10) Roxarsone.

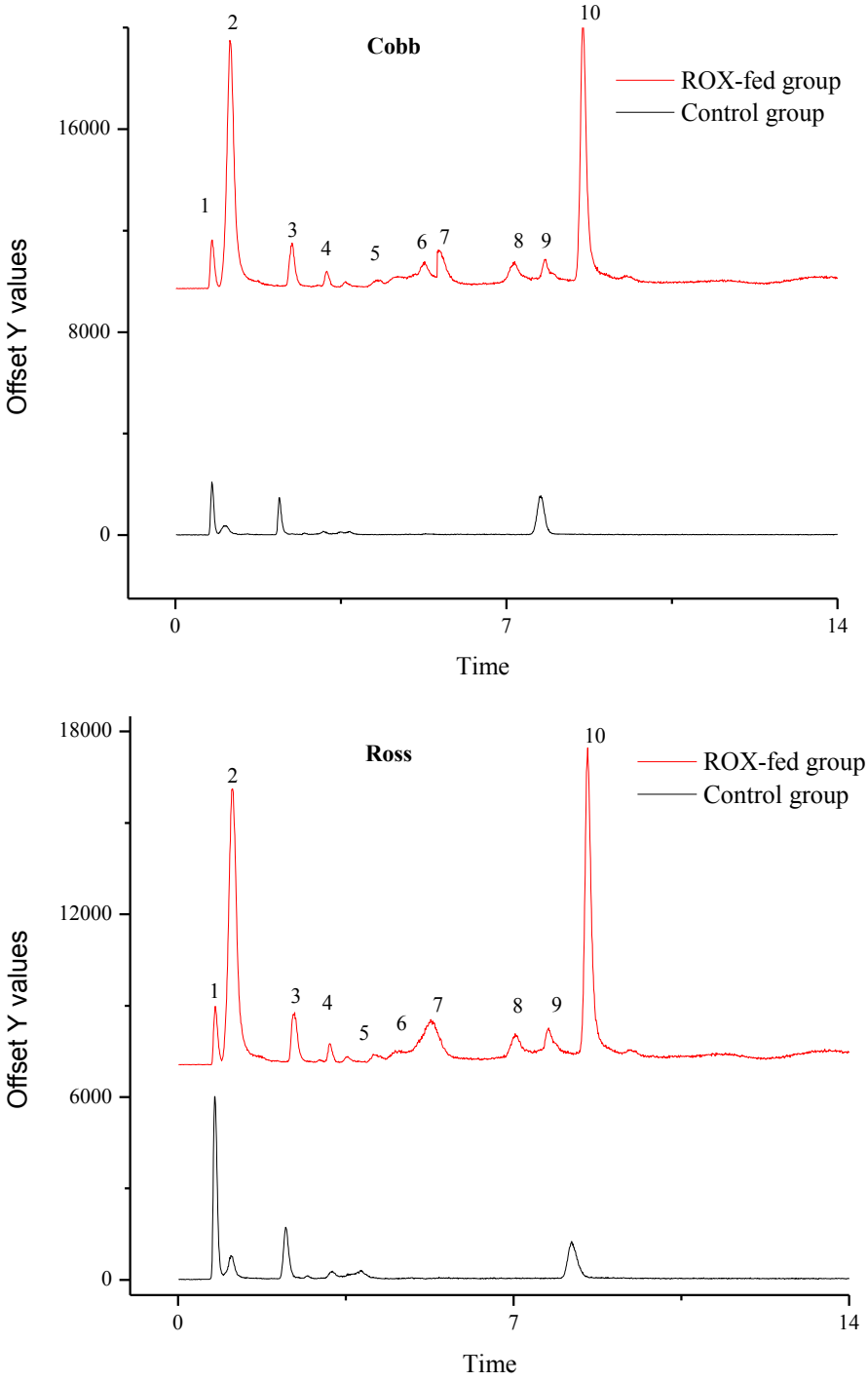


Figure A.6 Typical chromatograms obtained from HPLC-ICP-MS analyses of kidney samples on Day 28 from both the control chickens and the ROX-fed chickens in both Cobb and Ross strains. Peaks: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) Unknown 1, (6) As^{V}, (7) 3-AHPAA, (8) Unknown 2, (9) N-AHAA, and (10) Roxarsone.

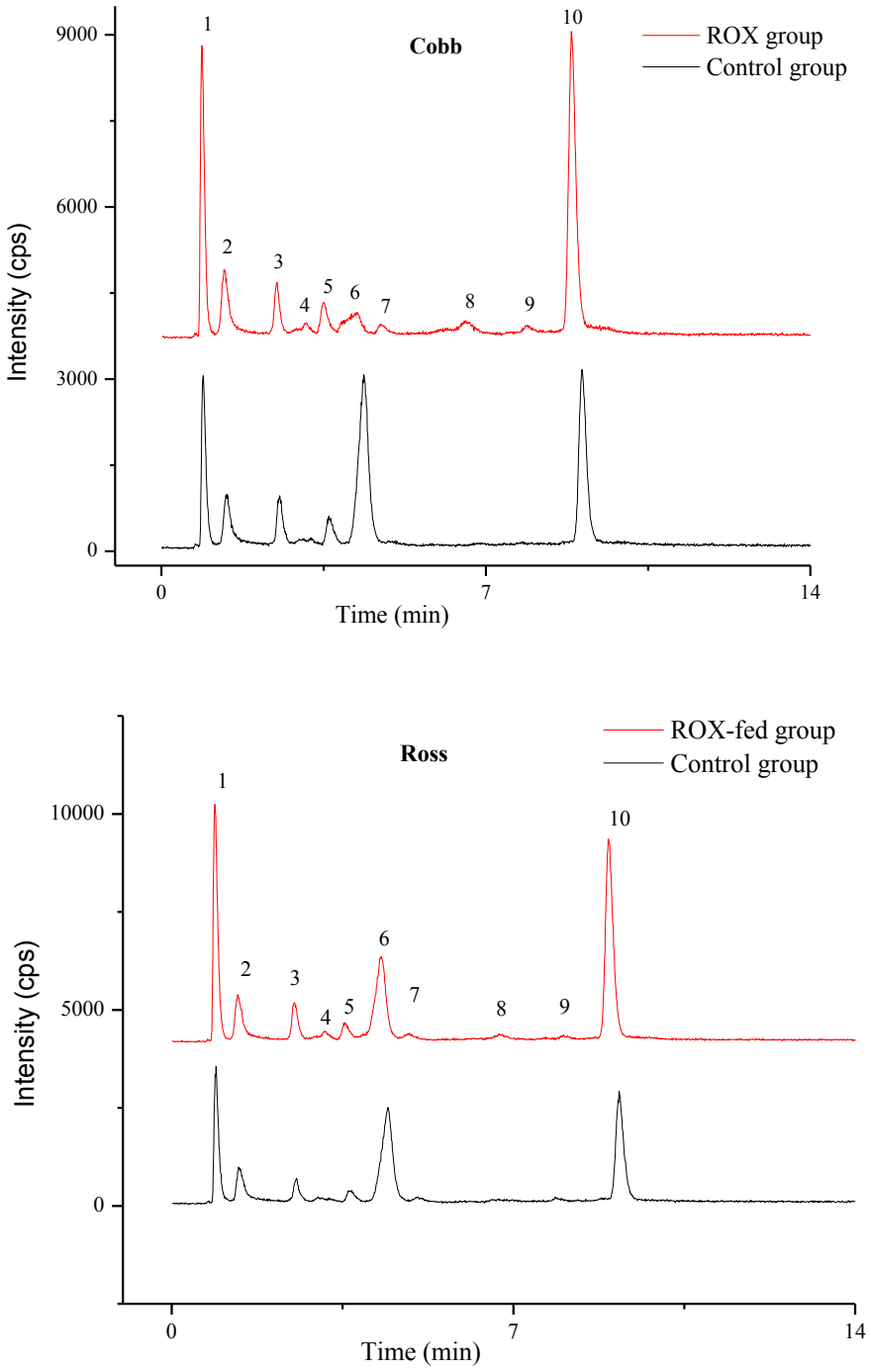


Figure A.7 Typical chromatograms obtained from HPLC-ICP-MS analyses of kidney samples on Day 35 from both the control chickens and the ROX-fed chickens in both Cobb and Ross strains. Peaks: (1) AsB, (2) As^{III}, (3) DMA^{V}, (4) MMA^{V}, (5) Unknown 1, (6) As^{V}, (7) 3-AHPAA, (8) Unknown 2, (9) N-AHAA, and (10) Roxarsone.

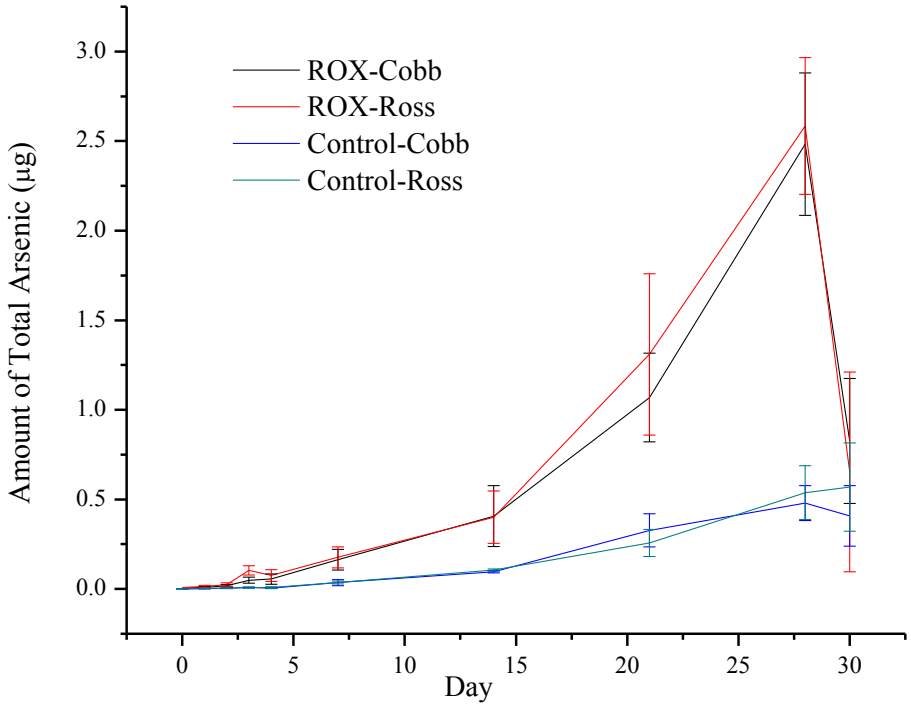


Figure A.8 Temporal profiles showing the amount of total arsenic in the kidney of chickens over the feeding period. The amount of total arsenic was estimated by multiplying the concentrations of total arsenic species in each kidney sample by specific kidney sample weight. The error bars represent one standard deviation from triplicate analyses of 4 chicken kidney samples of each treatment group. In total, 16 samples were included for each sampling day.

Table A.1 Information of feeding experiments.

Poultry Strain	**Group**	**Number of Pen**	**Uptake Period (Day 0-Day 14)**	**Growth period (Day 14-Day 28)**	**ROX withdrawal period (Day 28-Day 35)**
Ross 308 (n=800)	ROX-fed (n=400)	11,13,15,29	ROX starter (ROX-supplemented diet)	ROX Grower (ROX-supplemented diet)	ROX Finisher (ROX-free diet)
	Control (n=400)	3,12,17,22	Control Starter (ROX-free diet)	Control Grower (ROX-free diet)	Control Finisher (ROX-free diet)
Cobb 500 (n=800)	ROX-fed (n=400)	1,16, 19, 24	ROX starter (ROX-supplemented diet)	ROX Grover (ROX-supplemented diet)	ROX finisher (ROX-free diet)
	Control (n=400)	5, 6, 18, 25	Control starter (ROX-free diet)	Control Grower (ROX-free diet)	Control Finisher (ROX-free diet)

Table A.2 Concentrations of arsenic species detected in ROX-treated and untreated feed samples.[a]

Concentration of Arsenic Species (mean ± SD) (µg/kg)						
Feed Type	**AsB**	**As^{III}**	**DMA^{V}**	**MMA^{V}**	**As^{V}**	**ROX**
ROX Starter	54 ± 4	N.D.	31 ± 6	N.D.	59 ± 9	18300±900
ROX Grower	34 ± 5	N.D.	36 ± 3	N.D.	72 ± 2	18000±1000
ROX Finisher	26 ± 2	N.D.	34 ± 9	N.D.	44 ± 8	110 ± 20
Control Starter	97 ± 3	N.D.	33 ± 4	N.D.	120 ± 10	N.D.
Control Grower	35 ± 3	N.D.	42 ± 6	N.D.	50 ± 10	310 ± 30
Control Finisher	30 ± 2	N.D.	35 ± 4	N.D.	70 ± 10	170 ± 30

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

Table A.3 Chicken body weight during the feeding period [a].

Body Weight (mean ± SD) (g)				
Day	Control Cobb	ROX Cobb	Control Ross	ROX Ross
0	44 ± 1	42 ± 2	41 ± 5	40 ± 2
1	57 ± 8	50 ± 7	50 ± 7	56 ± 4
2	68 ± 5	60 ± 7	67 ± 6	61 ± 5
3	89 ± 5	77 ± 8	73 ± 5	82 ± 10
4	88 ± 15	106 ± 6	100 ± 6	96 ± 14
7	180 ± 11	196 ± 17	187 ± 12	171 ± 24
14	409 ± 53	451 ± 32	440 ± 19	430 ± 59
21	919 ± 123	975 ± 115	806 ± 119	991 ± 154
28	1599 ± 134	1597 ± 127	1416 ± 121	1479 ± 81
30	1610 ± 195	1737 ± 233	1729 ± 169	1672 ± 536
32	2022 ± 263	1887 ± 168	1742 ± 234	2213 ± 139

Table A.4.1 Comparison of arsenic concentration in different organs in chicken on Day 28 from control group. Liver, muscle, and skin concentration was obtained by Hanyong Peng, Qingqing Liu, and Chenming Cao, respectively

Arsenic Species	Kidney	Liver	Muscle	Skin
AsB	14.4±2.0	18.2±5.3	25.9±8.6	5.2±1.5
ROX	21.4±5.4	8.6±5.2	N.D.	2.0±1.6
As^{III}+As^{V}	6.5±1.9	5.7±3.2	N.D.	2.5±0.5
DMA^{V}+MMA^{V}	12.2± 2.8	5.3±9.4	7.7±9.2	6.9±1.5
Sum of phenyl-arsonic acid	1.4±0.2	15.5±8.8	N.D.	2.8±1.2
Sum of As excluding AsB	41.6 ±8.5	34.6±1.5	7.7±9.2	15.7±3.0
Sum of all As	56.0 ±6.9	53.2±17.4	33.7±9.4	18.3±6.1

N.D. not detectable

Table A.4.2 Comparison of arsenic concentration in different organs in chicken on Day 28 from ROX treated group. Liver, muscle, and skin concentration was obtained by Hanyong Peng, Qingqing Liu, and Chenming Cao, respectively

Arsenic Species	Kidney	Liver	Muscle	Skin
AsB	14.0±3.7	16.1±4.1	24.8±5.8	4.6±1.3
ROX	105.8±20.5	297.4±93.1	5.1±2.3	46.2±11.1
As^{III}+As^{V}	97.2±10.1	140.9±70.1	30.1±19.6	17.4±6.1
DMA^{V}+MMA^{V}	28.7±25.8	76.5±8.9	22.2±15.3	9.4±1.7
Sum of phenyl-arsonic acid	24.0±3.0	492.9±78.6	5.0±1.5	68.4±17.1
Sum of As excluding AsB	255.8±11.5	869.4±194.6	62.4±15.0	141.7±21.4
Sum of all As	269.8±9.1	1166.7±249.4	87.2±19.5	151.2±26.1

N.D. not detectable

Table A.4.3 Comparison of arsenic concentration in different organs in chicken on Day 35 from control group. Liver, muscle, and skin concentration was obtained by Hanyong Peng, Qingqing Liu, and Chenming Cao, respectively

Arsenic species	Kidney	Liver	Muscle	Skin
AsB	11.9±2.8	22.1±9.3	31.0±11.2	4.2±1.9
ROX	23.2±6.9	3.2±0.9	N.D.	N.D.
As^{III}+As^{V}	3.8±0.5	14.8±5.6	N.D.	2.7±0.8
DMA^{V}+MMA^{V}	8.3±0.8	3.4±1.3	2.5±0.5	4.8±0.6
Sum of phenyl-arsonic acid	3.8±0.3	9.4±4.3	N.D.	0.25±0.43
Sum of As excluding AsB	39.1±7.9	31.3±4.7	2.5±0.5	7.8±1.1
Sum of all As	51.0±7.8	50.5±16.6	33.5±0.5	11.5±2.1

N.D. not detectable

Table A.4.4 Comparison of arsenic concentration in different organs in chicken on Day 35 from ROX treated group. Liver, muscle, and skin concentration was obtained by Hanyong Peng, Qingqing Liu, and Chenming Cao, respectively

Arsenic species	Kidney	Liver	Muscle	Skin
AsB	14.5±5.0	17.1±5.7	33.5±14.9	4.0±1.3
ROX	32.7±3.3	11.0±2.2	0.4±0.0	3.1±0.8
As^{III}+As^{V}	6.6 ± 1.2	19.9±6.6	3.1±1.7	5.4±0.9
DMA^{V}+MMA^{V}	8.0±0.6	5.5±2.1	3.2±0.9	5.4±1.6
Sum of phenyl-arsonic acid	8.3±0.9	141.6±94.9	0.8 ± 0.3	11.1±3.2
Sum of As excluding AsB	55.7±4.8	179.4 ± 95.8	7.6 ± 2.0	25.4±4.0
Sum of all As	70.1±8.9	196.5 ± 97.5	44.1±15.1	29.6±3.5

N.D. not detectable

Table A.5 Detailed concentration of arsenic species (μg/kg) in each kidney sample in the control group on Day 35, Day 34, Day 32, Day 30, Day 28, Day 21, Day 14, Day 7, Day 4, Day 3, Day 2, Day 1, Day 0

DAY35									
		AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	Roxarsone
pen5-con-cobb		7.4399	2.1974	5.5050	3.2610	2.1288	2.8784	1.1041	36.0470
pen6-con-cobb		10.0392	1.5848	4.9320	2.8730	2.0872	2.4239	1.0869	19.1613
pen18-on-cobb		11.087	1.1279	6.2530	2.2088	1.7477	2.2935	1.0681	15.7503
pen25-on-cobb		14.7249	2.1202	5.7286	2.2859	1.7782	2.5496	1.3233	20.9545
pen3-con-ros		11.1644	2.3600	3.4195	3.4392	2.0295	2.3969	1.2093	18.1268
pen17-con-ross		14.3433	2.0166	6.2238	2.3348	1.8875	2.5385	1.7974	27.5536
pen22-con-ross		14.5296	1.8646	5.3908	4.0759	1.8507	2.4780	1.5231	24.8014
DAY34									
		AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	Roxarsone		
pen5-con-cobb		18.3346	6.2717	8.3161	2.2214	2.3751	30.0339		
pen6-con-cobb		11.5470	3.7294	6.9718	2.1652	2.5967	14.7013		
pen18-on-cobb		9.8849	3.9812	5.7005	2.0231	3.9757	20.4904		
pen25-on-cobb		25.5626	5.0779	6.7272	2.4666	4.1038	21.2157		
pen3-con-ross		12.3904	6.3075	10.4693	2.4934	4.9797	34.6141		
pen12-con-ross		18.2496	5.4866	12.9170	2.2323	5.1938	23.5900		
pen17-con-ross		28.0819	4.3994	10.7425	2.3280	6.2429	32.0363		
pen22-con-ross		11.7501	7.0026	8.9235	2.4085	5.4055	36.9017		

DAY32									
		AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	Roxarsone	Unknown1	
pen5-con-cobb		18.8821	1.2919	10.8791	2.6229	6.5303	N.D.	3.2649	
pen6-con-cobb		14.9899	1.1740	6.8163	1.5519	2.5556	14.9307	N.D.	
pen18-on-cobb		16.9654	<LOD	6.1995	1.6874	4.9525	26.0153	3.4271	
pen25-on-cobb		11.6723	<LOD	2.9472	2.0867	4.9236	18.6078	3.4852	
pen3-con-ross		14.3490	<LOD	8.6852	2.4437	4.3662	24.8495	3.4018	
pen17-con-ross		18.8592	1.7607	8.9980	1.9575	3.8417	33.4121	N.D.	
pen22-con-ross		15.4434	<LOD	6.9166	1.7091	5.6955	22.4309	N.D.	
DAY30									
		AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	Roxarsone	Unknown1	
pen5-con-cobb		18.0541	5.4727	5.5680	5.0387	1.5318	19.5474	3.6689	
pen6-con-cobb		14.0404	5.4744	5.9441	4.3214	1.5321	20.9047	2.9819	
pen18-on-cobb		10.9215	5.7418	6.3240	4.4503	1.2267	23.5098	2.8474	
pen25-on-cobb		11.5425	5.4999	5.9078	3.6163	1.0291	20.7808	1.5607	
pen3-con-ross		27.1534	5.5239	8.4616	3.0752	2.0349	23.1026	1.8551	
pen12-con-ross		25.7255	5.6103	7.3615	3.0065	2.1025	21.4195	2.1515	
pen17-con-ross		24.1146	5.5979	6.3525	3.7065	1.9265	20.1748	2.7998	
pen22-con-ross		21.6800	5.3509	5.2125	4.2288	1.9168	17.7205	2.7952	

DAY28							
		AsB	As^{III}	DMA^{V}	MMA^{V}	Roxarsone	Unknown1
pen5-con-cobb		15.7195	8.0756	9.4105	1.5122	19.8151	1.5106
pen6-con-cobb		14.6105	9.3802	9.0679	1.4565	23.1240	1.5733
pen18-on-cobb		13.7209	5.8102	8.8668	1.2755	18.1238	1.5832
pen3-con-ross		11.0755	6.0421	15.5734	1.5568	30.5757	1.6386
pen17-con-ross		14.1322	5.4776	12.5252	1.5182	22.1942	1.2515
pen22-con-ross		16.9670	4.3391	9.0858	1.4132	14.7143	1.1210

DAY14					
		AsB	DMA^{V}	MMA^{V}	Roxarsone
pen5-con-cobb		23.5801	6.1070	<LOD	7.2903
pen6-con-cobb		26.0401	6.8968	<LOD	7.0948
pen25-on-cobb		29.2638	7.9425	<LOD	7.1643
pen3-con-ross		32.8347	4.7801	<LOD	7.0004
pen12-con-ross		29.6000	5.1942	<LOD	7.1660
pen17-con-ross		25.0762	5.2766	<LOD	7.2904

DAY7					
		AsB	As^{III}	DMA^{V}	MMA^{V}
pen5-con-cobb		34.1309	3.4731	8.5456	2.2962
pen6-con-cobb		38.0840	4.7781	5.0466	2.2718
pen18-on-cobb		31.2883	4.6467	4.9729	2.9447

pen25-on-cobb	41.0119	4.8971	8.6026	1.4935	
pen3-con-ross	23.2373	4.9242	5.1890	2.7533	
pen12-con-ross	29.4738	4.5720	4.9963	1.9553	
pen17-con-ross	37.2196	4.8390	7.0561	2.5461	
pen22-con-ross	24.1702	4.4941	5.4730	2.3799	

DAY4					
		AsB	As^{III}	DMA^{V}	N-AHAA
pen5-con-cobb		6.5721	3.8522	3.7255	2.0095
pen6-con-cobb		10.2925	3.8143	3.7076	2.7884
pen18-on-cobb		9.6249	3.2040	4.0987	2.1844
pen25-on-cobb		7.5612	3.4435	3.6822	1.2478
pen3-con-ross		16.0885	3.5778	5.8801	2.2831
pen12-con-ross		5.9587	3.8299	4.3901	3.0069
pen17-con-ross		16.6751	3.5791	3.8297	2.0423
pen22-con-ross		11.3500	3.9950	4.5316	1.6924

DAY3							
		AsB	As^{III}	DMA^{V}	MMA^{V}	Roxarsone	Unknown1
pen5-con-cobb		9.8528	1.3401	4.3142	<LOD	0.8935	1.9653
pen6-con-cobb		5.4298	0.2410	2.7706	<LOD	1.7802	1.3169
pen18-on-cobb		12.6024	0.8143	5.2212	1.4218	1.0480	2.6394
pen25-on-cobb		13.6536	1.0748	5.3485	1.5498	1.8201	2.0889

pen3-con-ross	7.5068	1.1027	2.4242	<LOD	0.5788	1.2799
pen12-con-ross	11.6637	1.2444	3.1891	<LOD	0.8847	1.9102
pen17-con-ross	10.0496	1.8991	6.0305	2.5398	1.3998	1.3486
pen22-con-ross	9.4808	1.7282	4.0069	1.5174	1.6111	1.8692

DAY2						
		AsB	As^{III}	DMA^{V}	MMA^{V}	Unknown1
pen5-con-cobb		7.1318	5.8402	3.9084	1.8854	1.7442
pen6-con-cobb		10.6612	6.7772	3.5360	2.4842	2.2499
pen18-on-cobb		3.4052	8.4647	3.5385	1.8798	1.2584
pen25-on-cobb		7.4979	4.2700	4.0109	2.0060	4.0053
pen3-con-ross		4.3081	<LOD	1.9301	1.1210	1.0219
pen12-con-ross		8.4389	6.2903	4.8864	2.0541	2.6064
pen17-con-ross		5.1417	1.0411	3.1409	1.4325	1.8595
pen22-con-ross		7.3901	3.1670	4.6657	2.2116	1.0344

DAY1						
		AsB	As^{III}	DMA^{V}	MMA^{V}	
pen5-con-cobb		2.9526	5.8174	2.3401	1.0582	
pen6-con-cobb		5.2560	6.2377	5.9297	2.0312	
pen18-on-cobb		5.9523	5.5922	5.0612	N.D.	
pen25-on-cobb		4.0148	5.7661	5.0025	N.D.	
pen3-con-ross		4.0040	2.3508	3.7322	2.2015	
pen12-con-ross		5.4594	6.4050	7.4355	2.1542	

pen17-con-ross		5.7314	6.4746	5.8671	2.7489	
pen22-con-ross		N.D.	5.8922	5.1120	N.D.	
DAY0						
		As^{III}				
pen5-con-cobb		1.8737				
pen6-con-cobb		1.3227				
pen18-con-cobb		2.4110				
pen25-con-cobb		2.1230				
pen3-con-ross		2.3526				
pen12-con-ross		1.3227				
pen17-con-ross		1.2678				
pen22-con-ross		2.6632				

Note: Under the HPLC conditions shown in Figure 2.2, unknown 1 elutes at 4.8 min, between MMA^{V} and 3-amino, unknown 2 elutes at 7.2 min, between 3-amino and N-acetyl.

Table A.6 Detailed concentration of arsenic species (μg/kg) in each kidney sample in the ROX feeding group on Day 35, Day 34, Day 32, Day 30, Day 28, Day 21, Day 14, Day 7, Day 4, Day 3, Day 2, Day 1, Day 0

DAY35									
	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	Roxarsone	Unknown1	N-AHAA
pen1-rox-cobb	20.3784	4.6673	5.4499	3.2443	1.9676	2.9913	35.8353	4.0901	1.5429
pen16-rox-cobb	18.5610	1.8735	4.5087	2.5137	2.2517	2.6304	31.9459	4.2006	1.7489
pen19-rox-cobb	7.0023	5.7753	4.3471	3.6267	1.8443	2.6128	28.5396	3.1611	1.6033
pen24-rox-cobb	16.9305	4.6057	5.5485	2.8911	2.2013	2.9028	31.8079	4.2911	1.6122
pen11-rox-ross	10.6770	4.4887	5.5767	3.3339	1.4768	2.8923	32.0295	3.6009	1.8644
pen13-rox-ross	16.2082	5.2244	5.0621	2.8354	2.6536	3.3170	35.9339	4.1367	1.4977
pen15-rox-ross	17.3101	5.3014	4.7594	2.9984	2.2349	3.5105	37.2494	4.2567	1.6374
pen29-rox-ross	8.6124	4.0986	4.7580	2.7123	1.9446	2.6593	28.4247	4.0372	N.D.
DAY 34									
	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	Roxarsone	Unknown1	
pen1-rox-cobb	13.3077	5.3104	7.4192	2.6753	3.0356	<LOD	27.2320	1.6404	
pen16-rox-cobb	12.1928	8.6744	12.2854	3.3292	2.2198	2.1648	37.5066	N.D.	
pen19-rox-cobb	14.2825	9.2925	11.3801	3.3331	2.5233	1.9325	39.0208	2.0331	
pen24-rox-cobb	10.3687	8.6693	8.2644	3.1316	2.4017	<LOD	33.0767	1.8773	
pen11-rox-ross	9.6842	8.5422	7.3040	3.1371	2.2565	N.D.	36.6085	N.D.	
pen13-rox-ross	13.2567	14.5749	9.1731	3.0683	2.5586	N.D.	56.5286	N.D.	

pen15-rox-ross	12.6058	12.3281	10.8954	3.1331	3.1560	N.D.	45.4307	N.D.	
pen29-rox-ross	9.4807	8.3384	9.3266	2.7097	2.6186	N.D.	47.4646	N.D.	
DAY 32									
	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	Roxarsone	Unknown1	
pen1-rox-cobb	24.8219	1.9185	8.6744	2.2089	4.5279	<LOD	21.8160	N.D.	
pen16-rox-cobb	6.6123	N.D.	6.5328	2.2087	1.7080	<LOD	29.0838	2.9369	
pen19-rox-cobb	13.0899	2.0184	4.9697	1.9566	2.0388	<LOD	31.2054	3.7710	
pen24-rox-cobb	15.5286	2.0847	8.0096	2.2617	2.2152	N.D.	22.1796	3.0219	
pen11-rox-ross	18.6134	2.5834	5.5513	2.1695	2.4038	2.5063	30.8824	2.8540	
pen13-rox-ross	14.7653	N.D.	7.0039	2.8127	2.1103	2.7367	24.2012	4.3271	
pen15-rox-ross	6.3673	1.8021	4.4184	2.2360	2.3413	2.8622	30.7717	3.9968	
pen29-rox-ross	8.0690	2.0083	5.0732	2.1269	3.0116	2.9635	27.8198	2.7520	
DAY 30									
	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	Roxarsone		
pen1-rox-cobb	12.7087	9.5895	11.3412	4.2950	2.0924	<LOD	37.2545		
pen16-rox-cobb	22.6355	11.3876	9.4269	5.0469	1.6098	2.9502	32.6573		
pen19-rox-cobb	22.6878	12.9303	6.0129	4.7030	1.8006	N.D.	43.8871		
pen24-rox-cobb	12.9066	10.5141	9.2075	4.0559	1.6599	3.1694	42.3098		
pen11-rox-ross	13.5245	10.8225	7.6650	4.8471	1.4546	<LOD	23.6129		
pen13-rox-ross	22.7754	15.7243	6.6850	5.2308	2.5088	N.D.	34.4676		
pen15-rox-ross	10.3637	13.1254	6.8246	3.8052	1.5369	2.6463	35.7563		
pen29-rox-ross	6.0943	3.5741	3.6604	2.3313	2.4761	2.2327	9.4444		

DAY 28										
	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	Roxarsone	Unknown1	Unknown2
pen1-rox-cobb	11.1610	87.5805	12.5573	3.6351	2.3255	20.6070	3.0260	115.2708	1.7884	3.7306
pen16-rox-cobb	13.8276	102.9143	13.8342	3.6918	3.6468	18.4464	N.D.	113.6132	1.9305	1.4243
pen24-rox-cobb	19.7852	102.7824	13.5419	3.4940	3.2328	18.0519	2.5138	92.2253	N.D.	2.9186
pen11-rox-ross	10.3503	96.3872	13.8464	4.0791	3.2416	17.0768	2.7379	129.7971	1.8314	N.D.
pen29-rox-ross	14.8551	82.1628	71.7988	3.1026	1.6338	18.2027	N.D.	78.1642	4.4294	1.5207
DAY 21										
	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	Roxarsone	Unknown1	Unknown2
pen1-rox-cobb	18.2702	39.2276	14.0288	4.5534	3.8526	24.7026	6.8871	63.2365	3.9746	16.8261
pen16-rox-cobb	9.4761	54.4251	12.1627	5.1013	10.6442	17.5979	N.D.	37.9118	4.0041	15.8262
pen19-rox-cobb	14.8440	45.3125	12.7010	4.7317	5.4346	36.2293	7.9056	63.3107	N.D.	N.D.
pen11-rox-ross	12.0238	38.8382	17.4931	4.7336	3.9598	25.1383	N.D.	53.5318	N.D.	N.D.
pen13-rox-ross	9.6152	103.8763	16.7422	5.1600	3.8251	17.7358	N.D.	141.0609	N.D.	N.D.
pen15-rox-ross	15.0500	42.7960	22.3441	4.8673	12.6977	21.1958	N.D.	51.6310	4.6600	9.6790
DAY 14										
	AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	Roxarsone	Unknown2	
pen1-rox-cobb	32.2643	23.5358	6.5774	2.0206	7.7350	14.7626	10.5801	73.7033	4.4245	
pen16-rox-cobb	19.8913	10.1378	5.7250	1.1131	6.7519	13.1744	10.3468	44.3525	N.D.	
pen19-rox-cobb	19.9034	16.4009	8.5675	1.5537	5.7232	15.1924	10.4892	55.5432	3.8768	
pen13-rox-ross	23.0791	22.3352	11.7090	1.5742	7.1604	5.9266	7.3479	69.7484	3.3934	
pen15-rox-ross	22.5658	6.3856	7.6910	1.2525	6.4117	5.1229	6.7970	39.6124	3.8012	
pen29-rox-ross	22.1835	16.1285	14.2038	1.6478	6.1103	14.5384	9.0583	109.7097	N.D.	

DAY 7											
		AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	Roxarsone	Unknown1	Unknown2
pen1-rox-cobb		36.1821	68.8291	9.0513	6.2526	24.4028	10.2519	9.4491	95.4924	1.6423	14.1643
pen16-rox-cobb		51.4866	28.5059	21.1875	20.3898	19.0185	13.0412	9.4305	82.2570	N.D.	N.D.
pen19-rox-cobb		38.0634	49.7547	16.3207	3.2783	22.0114	13.0100	9.4624	88.9357	1.6843	17.6842
pen24-rox-cobb		33.5210	40.3753	8.6558	3.3170	22.1264	N.D.	9.8923	93.5810	1.6400	12.4112
pen11-rox-ross		34.8372	24.9244	6.2396	7.5457	13.4152	12.0905	9.5716	61.5563	N.D.	10.7446
pen13-rox-ross		44.2230	32.3513	6.5176	22.7623	21.2409	20.6880	9.5031	67.9271	N.D.	N.D.
pen15-rox-ross		32.4927	22.9153	7.4162	17.2146	16.0443	10.3433	9.2724	58.1538	N.D.	N.D.
DAY 4											
		AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	Roxarsone	Unknown1	Unknown2
pen1-rox-cobb		11.1705	12.1465	6.7564	15.0201	6.5139	11.5095	4.9746	49.2778	N.D.	N.D.
pen16-rox-cobb		19.6528	20.4070	3.9317	9.9081	9.2000	11.6539	4.5672	38.5354	1.7404	N.D.
pen19-rox-cobb		14.7061	13.7089	6.2630	9.7639	9.0650	N.D.	3.6288	34.8765	1.8845	N.D.
pen24-rox-cobb		18.2819	18.4477	6.6964	18.1673	16.9367	N.D.	5.1383	69.1400		N.D.
pen11-rox-ross		15.0441	20.6340	6.7524	20.1692	18.8120	24.7881	3.9393	64.8127	1.5823	7.0069
pen13-rox-ross		12.3983	13.8421	4.2102	12.1610	11.3105	21.4362	4.4622	51.6685	1.0342	7.0310
pen15-rox-ross		15.0251	26.5426	5.1265	25.1924	23.5174	18.0743	3.5409	67.7456	1.0645	N.D.
DAY 3											
		AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	3-AHPAA	N-AHAA	Roxarsone	Unknown1	
pen1-rox-cobb		10.4089	7.9620	5.7536	1.3881	15.5109	4.7456	<LOD	72.4866	2.8900	
pen16-rox-cobb		17.8464	17.6740	5.9163	4.6512	11.6023	8.5311	<LOD	86.1094	2.7213	

pen19-rox-cobb	7.5168	10.8109	5.4163	2.0221	10.6226	<LOD	3.0950	42.5814	N.D.	
pen24-rox-cobb	7.6183	4.6875	4.6678	0.9767	15.2668	N.D.	N.D.	28.6509	N.D.	
pen11-rox-ross	17.7654	16.0073	6.8045	3.2126	17.9004	<LOD	<LOD	61.6171	N.D.	
pen13-rox-ross	15.4702	24.7566	N.D.	2.6906	N.D.	N.D.	N.D.	88.3490	2.0301	
pen15-rox-ross	10.0996	13.5315	11.7361	2.1548	16.1427	10.3340	1.3017	75.5707	N.D.	
DAY 2										
		AsB	As^{III}	DMA^{V}	MMA^{V}	As^{V}	N-AHAA	Roxarsone	Unknown1	
pen1-rox-cobb		10.9616	14.0761	10.1905	4.5455	6.9565	<LOD	60.6885	7.0307	
pen16-rox-cobb		5.0882	10.0422	3.2872	2.8251	14.6150	1.9230	58.3422	1.6846	
pen19-rox-cobb		7.0240	19.8779	7.4543	2.3882	12.5970	<LOD	53.6074	3.2410	
pen24-rox-cobb		5.8256	16.8674	4.9712	2.3429	6.5648	1.8123	51.1273	2.3305	
pen11-rox-ross		7.8440	6.5235	6.8478	2.1486	10.7659	2.0672	33.2709	3.0764	
pen13-rox-ross		7.3673	12.1418	7.1448	2.2406	8.6183	<LOD	66.7641	N.D.	
pen15-rox-ross		5.3289	1.2664	4.6818	<LOD	11.3691	2.4353	43.9003	2.9424	
pen29-rox-ross		5.8675	14.9188	6.6412	2.7256	10.0816	1.9123	67.1342	2.0079	
DAY 1										
		AsB	As^{III}	DMA^{V}	MMA^{V}	Roxarsone				
pen1-rox-cobb		2.4635	5.2480	2.5945	2.4463	14.1588				
pen16-rox-cobb		1.7417	9.3422	2.5668	4.8929	33.0554				
pen19-rox-cobb		3.9441	6.0276	6.5089	4.9452	18.1731				
pen11-rox-ross		4.6821	9.9795	6.0876	2.2838	48.5816				
pen13-rox-ross		5.9868	7.0968	7.4310	1.5761	21.8168				
pen15-rox-ross		10.9204	11.7076	13.7083	2.6301	35.0628				

pen29-rox-ross		5.7786	5.1098	8.8726	4.9781	18.0460				
Day 0										
		As^{III}	DMA^{V}							
pen1-rox-cobb		1.7802	1.3018							
pen16-rox-cobb		2.9294	2.5263							
pen13-rox-ross		1.5698	2.1359							

Note: Under the HPLC conditions shown in Figure 2.2, unknown 1 elutes at 4.8 min, between MMA^{V} and 3-amino, unknown 2 elutes at 7.2 min, between 3-amino and N-acetyl.

Table A.7 Detailed weight (g) of each kidney sample in both the control group and the ROX feeding group on Day 0, Day 1, Day 2, Day 3, Day 4, Day 7, Day 14, Day 21, Day 28, Day 30

	Chicken kidney sample weight /g									
Pen	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 30**
1	0.530	0.344	0.115	0.484	0.30	0.50	3.0	4.6	9.9	8.7
3	0.310	0.256	0.444	0.637	0.36	0.86	2.4	3.8	10.4	8.7
5	0.231	0.288	0.121	0.609	0.13	0.51	2.4	4.3	10.5	7.3
6	0.163	0.080	0.151	0.525	0.26	0.81	2.5	4.2	7.5	5.8
11	0.324	0.219	0.116	0.652	0.35	1.34	2.1	5.5	10.6	9.1
12	0.445	0.173	0.231	0.480	0.16	0.95	2.6	5.2	6.1	10.4
13	0.417	0.100	0.274	0.559	0.41	0.71	2.7	5.9	10.5	10.4
15	0.110	0.103	0.316	0.516	0.61	0.75	2.5	7.1	8.1	10.6
16	0.064	0.282	0.285	0.268	0.74	0.57	1.9	5.7	10.3	9.1
17	0.534	0.077	0.355	0.178	0.51	0.91	2.6	3.6	9.3	8.4
18	0.246	0.095	0.345	0.326	0.19	0.44	2.2	6.2	8.2	7.3
19	0.114	0.271	0.145	0.383	0.30	0.49	3.5	7.1	3.4	8.6
22	0.183	0.181	0.136	0.367	0.20	0.82	1.9	4.6	8.2	9.3
24	0.136	0.138	0.172	0.620	0.46	1.11	3.3	8.1	7.9	7.6
25	0.403	0.068	0.235	0.252	0.20	1.01	2.1	7.2	7.9	11.8
29	0.209	0.123	0.305	0.501	0.43	0.54	2.8	5.6	8.0	10.2