Determination of Arsenicals in Chicken Skin

by

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ABSTRACT

Roxarsone® (ROX, 3-nitro-4-hydroxyphenylarsonic acid) has been used in the poultry industry to prevent coccidiosis, enhance feed efficiency, and to promote growth of the broiler chickens. However, the metabolism, distribution, and elimination of ROX from poultry are not well understood. The focus of this research is the determination of the total arsenic concentration and arsenic species present in chicken skin. Skin samples were collected from chickens in a 35-day feeding experiment. One portion of the skin samples was digested with acids, and the concentration of total arsenic was determined using inductively coupled plasma mass spectrometry (ICPMS). For the determination of the arsenic species, chicken skin samples were digested using enzymes, and the arsenic species were extracted. The arsenic species were determined by using high performance liquid chromatography (HPLC) separation paired with ICPMS and electrospray ionization mass spectrometry (ESIMS) detection methods. Arsenic concentrations in the skin of the ROX-fed chickens are significantly higher than those in the skin of the control chickens. In the ROX-fed chickens, ROX and its major arsenic metabolites are detectable. Temporal profiles of arsenic species, from all 35 days of the feeding experiment, indicate that ROX is partially metabolized in the chicken and that the metabolites are distributed to various other organs.

PREFACE

This thesis is an original work by Chenming Cao. No part of this thesis has been previously published.

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LIST OF ABBREVIATIONS

3-AHPAA	3-amino-4-hydroxy-phenylarsonic acid
As^{III}	arsenite
As^{V}	arsenate
AsB	arsenobetaine
DMA^{III}	dimethylarsinous acid
DMA^{V}/DMA	dimethylarsinic acid
ESIMS	electrospray ionization mass spectrometry
HNO_3	nitric acid
HPLC	high performance liquid chromatography
H_2SO_4	sulfuric acid
ICPMS	inductively coupled plasma mass spectrometry
LD_{50}	median lethal dose
LOD	limit of detection
LOQ	limit of quantitation
MMA^{III}	monomethylarsonous acid
MMA^{V}/MMA	monomethylarsonic acid

MRM	multiple reaction monitoring
N-AHAA	N-acetyl-4-hydroxy-m-arsanilic acid
ROX	Roxarsone (3-nitro-4-hydroxy-phenylarsonic acid)
STD	standard
TMA^{III}	trimethylarsine
TMAO	trimethylarsine oxide

CHAPTER 1

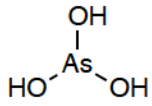
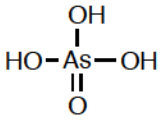
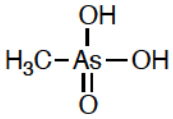
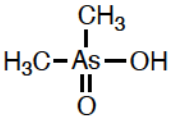
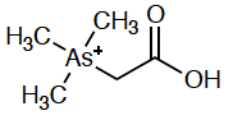
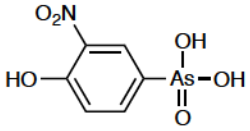
INTRODUCTION

1.1 ARSENIC AND ITS PROPERTIES

1.1.1 Arsenic Chemistry

Arsenic is an element in Group 15 of the Periodic Table. It has the atomic number 33 and an atomic mass of 74.92 g/mol [1]. It is chemically categorized as a semimetal or a metalloid. Arsenic is present in various chemical forms (Table 1.1), which can be classified as organic or inorganic arsenicals. There are four common oxidation states for arsenic, -3, 0, +2, +3, and +5 [1]. Many factors, such as oxidation, reduction, and pH can affect the speciation of arsenic. Trivalent arsenic (As^{III}) is prevalent under anaerobic or reducing conditions; under oxidizing conditions and aerobic environments, the pentavalent species becomes more stable and predominates [1, 2]. The effect of pH on the speciation of arsenic is based on the relationship between the pH value of the environment and the pKa values of the arsenicals. pH can influence the conversion between neutral arsenicals and their corresponding ionic arsenicals [3].

Table 1.1 Arsenic species described in the thesis

Species	Abbreviation	Chemical Structure	pKa values
Arsenite	As^{III}		9.2, 12.1, 13.4 [4]
Arsenate	As^{V}		2.3, 6.7, 11.6 [4]
Monomethylarsonic acid	MMA		3.6, 8.2 [5]
Dimethylarsinic acid	DMA		6.2 [4]
Arsenobetaine	AsB		4.7 [6]
3-nitro-4-hydroxy-phenylarsonic acid	ROX		3.5, 6.5, 9.6 [7]

1.1.2 Arsenic Toxicity

Arsenic is known to be both a poison and a carcinogen. However, not all the arsenicals are highly toxic. The toxicities of arsenic compounds are species-dependent. Table 1.2 lists some commonly studied arsenic compounds and their acute toxicities (LD_{50} values). Usually inorganic arsenic (As^{III}, As^{V}) is more toxic than organic species (most commonly MMA^{V} and DMA^{V}). However, recent studies indicate that methylated trivalent metabolites (MMA^{III} and DMA^{III}) have higher toxicities than their pentavalent counterparts and even inorganic arsenic [8-11]. Some other arsenicals, arsenobetaine, arsenocholine, and arsenosugars, that are abundant in marine organisms, are considered much less toxic or even nontoxic [12-14].

The toxicity of arsenic can occur by a series of mechanisms. It has been found that arsenate (As^{V}) can be disruptive by competing with phosphate. The similarity in charge and size between arsenic and phosphorus allows the replacement of phosphate by arsenate to occur [15, 16]. One example is the replacement of phosphate in ATP by a so-called arsenolysis process [17]. This can cause rapid hydrolysis of high-energy bonds and effectively uncoupling oxidative phosphorylation. Arsenate may also replace the phosphorus in DNA, causing DNA damage [16, 18]. But experimental evidence for arsenic replacement of phosphorus in DNA is lacking.

The other most common toxic mode of arsenic is the inactivation of enzyme systems [15]. It is known that arsenite (As^{III}) can inhibit more than 200 different enzymes [19]. As^{III} readily forms strong bonds with thiol group, disrupting sulfur

bearing enzymes and amino acids, such as cysteine and methionine. Besides, As^{III} inhibits pyruvate and succinate oxidation pathways, as well as the tricarboxylic cycle, and can greatly impair gluconeogenesis that will result in cell damage [15, 20].

The toxic effects of arsenic are dose-dependent. Exposure to the minimal lethal dose of arsenic would cause an acute arsenic toxic effect. In an adult human, the lethal range of inorganic arsenic is estimated at a dose of 1-3 mg As/kg [21]. Symptoms from acute arsenic poisoning may include nausea, diarrhea, vomiting, bloody urine, hair loss, stomach pain, and convulsions, potentially ending in coma or death [15, 22]. Exposure to an elevated level of arsenic for a long term, results in a variety of chronic toxic effects. Epidemiological studies performed in many regions including Chile and Taiwan show that people are suffering from cancers in the skin, liver, lungs, urinary bladder and kidneys as a result of chronic exposure to arsenic [23-25]. Noncancerous health implications, such as skin lesions in Bangladesh and India [26, 27], and blackfoot disease in Taiwan [28], are also reported. Chronic exposure to arsenic has been found to have a strong correlation with increased risk of heart disease [29], chronic lower respiratory disease [30], diabetes [31], possible harm to fetuses [32], and children's intellectual function [33].

Table 1.2 Acute toxicities of the commonly studied arsenic species

Species	Abbreviation	Animal	LD_{50} (24h)
Arsenite	As^{III}	Mice	34.5 mg/kg [12]
Arsenate	As^{V}	Mice	100 mg/kg [34]
Monomethylarsonic acid	MMA	Mice	1800 mg/kg [35]
Dimethylarsinic acid	DMA	Mice	1200 mg/kg [35]
Arsenobetaine	AsB	Mice	>10,000 mg/kg [12]
3-nitro-4-hydroxy-phenylarsonic acid	ROX	Mice	244 mg/kg [36]
		Chicken	110 mg/kg [36]

1.2 ARSENIC IN THE NATURAL ENVIRONMENT

Arsenic is a ubiquitous element. It exists in the earth's crust, rock, soil, water, air, and in the biosphere. It has relatively high abundance; ranking 20th among the trace elements [37]. Arsenic can be found in rocks with concentration ranging from 0.5 to 2.5 mg/kg [38]. It is a major component in more than 200 minerals, and the most common arsenic minerals are arsenopyrite, galena, iron pyrite, chalcopyrite, realgar, orpiment, and sphalerite [39, 40]. Due to natural sources, such as weathering of As-containing rocks, 45,000 tons of As are released from the earth's crust per year [41]. Due to the parent rock breaking down process, arsenic can be distributed to sediments and soils. Arsenic concentrations in soils are higher than those in rocks [42]. Uncontaminated soils usually contain 1-40 mg/kg of arsenic. The arsenic concentrations in soil vary among geographic regions and they are influenced by the climate, soil components, and redox potential status [38, 40]. Arsenic can be released into surface water and surrounding groundwater through weathering and leaching of arsenic-containing rocks and soils. Its concentrations in water are as high as 1 mg/L with a mean of 3 μg/L in sea water, 1.7 μg/L in river water, 1 μg/L in precipitation, and 280 μg/L in saline lakes [43]. Arsenic can also be deposited into the atmosphere by volcanic and geyser activities [1], as well as volatilization from microorganisms [44]. In air, arsenic is predominantly absorbed onto particulate matters [45].

1.3 ARSENIC FROM ANTHROPOGENIC SOURCES

Compared to natural sources, anthropogenic activities release more arsenic into the environment. The anthropogenic sources, including industrial effluents from mining and ore smelting, fertilizer and livestock feed additives, arsenic-containing pesticides, and wood preservatives, exceed natural sources by 3:1 [38]. Metal smelting may emit arsenic trioxide (As_2O_3) as a by-product. Nearly 90% of the total anthropogenic arsenic was used for the production of a popular antifungal wood preservative, chromated copper arsenate (CCA) [1, 45]. Arsenic had been widely used in agricultural as well. Arsenic was made into cotton desiccants, herbicides, insecticides, and pesticides in early years [46]. Some organic arsenicals are added into animal feed for disease prevention and growth promotion [47].

Phenylarsonic acids are one category of feed additives in the poultry industry and they are one of the major anthropogenic arsenic sources. 3-nitro-4-hydroxyphenylarsonic acid (Roxarsone ®, ROX) (Structure available in Table 1.1) is an example of an arsenic-containing feed additives. ROX was used for the past 60 years, to promote weight gain, enhance feed efficiency, improve pigmentation, and to control the intestinal parasites that cause coccidiosis [47, 48]. The ROX ingested by chicken is believed to be mainly excreted unchanged into the waste [49]. However, heightened levels of inorganic arsenic, converted from ROX, have been detected in the livers of ROX-fed chickens [50]. The use of ROX would introduce arsenic into the environment, such as agricultural land and water, through poultry litter [51]. It can also contaminate the food chain directly by

ingestion of ROX-fed chickens, or indirectly by other animals or plants through feed or fertilizer made from chicken byproduct (feathers, skin, heads, bones and viscera) [52, 53].

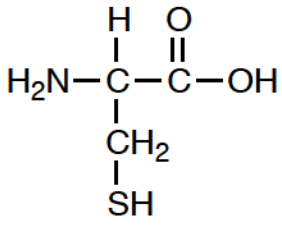
1.4 ARSENIC IN BIOLOGICAL SYSTEMS

1.4.1 Arsenic Binding to Proteins

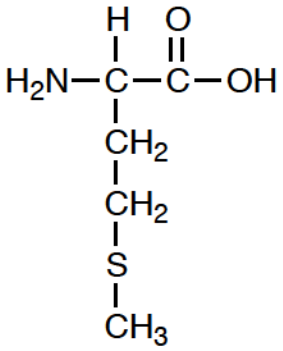
Arsenic can be accumulated in living organism. One possible mechanism of bioaccumulation is through arsenic binding to proteins and amino acids. This binding process takes place between trivalent arsenic and sulfur bearing amino acids, such as cysteine and methionine (Figure 1.1). Cysteine contains a thiol group (-SH), which displays nucleophilicity. Thiol groups are able to replace the hydroxyl group (-OH) in trivalent arsenic, forming a stable arsenic-sulfur bond. This interaction shows different stoichiometry depending on the speciation of arsenic (Figure 1.2). As^{III}, MMA^{III} and DMA^{III} are capable of binding to 3, 2 and 1 cysteines, respectively [37, 54, 55]. Methionine contains single sulfur that is incorporated into the chain structure, making it less accessible for binding. It is possible for methionine to convert into cysteine through metabolic conversions [56], making it able to bind trivalent arsenic. The arsenic and amino acid binding process has been reported in many cysteine-rich proteins, such as metallothionein [54, 55], hemoglobin [57], and keratin [58, 59], which are related to either the arsenic detoxification or the arsenic accumulation process.

Studies note that pH has an effect on how readily arsenic will bind with cysteine. Toyama et al. [60] explored the interaction between As^{III} and metallothionein at pH 2 and 7.4. The study found that the arsenic to cysteine ratio

1:3 was maintained at 7.4, but at 2, the reaction was inhibited. The pH dependence indicates that at a pH of 5.7-5.9, the As^{III}-cysteine binding reaction should be favorable.



(a) Cysteine



(b) Methionine

Figure 1.1 Structures of sulfur bearing amino acids (a) Cysteine, (b) Methionine.

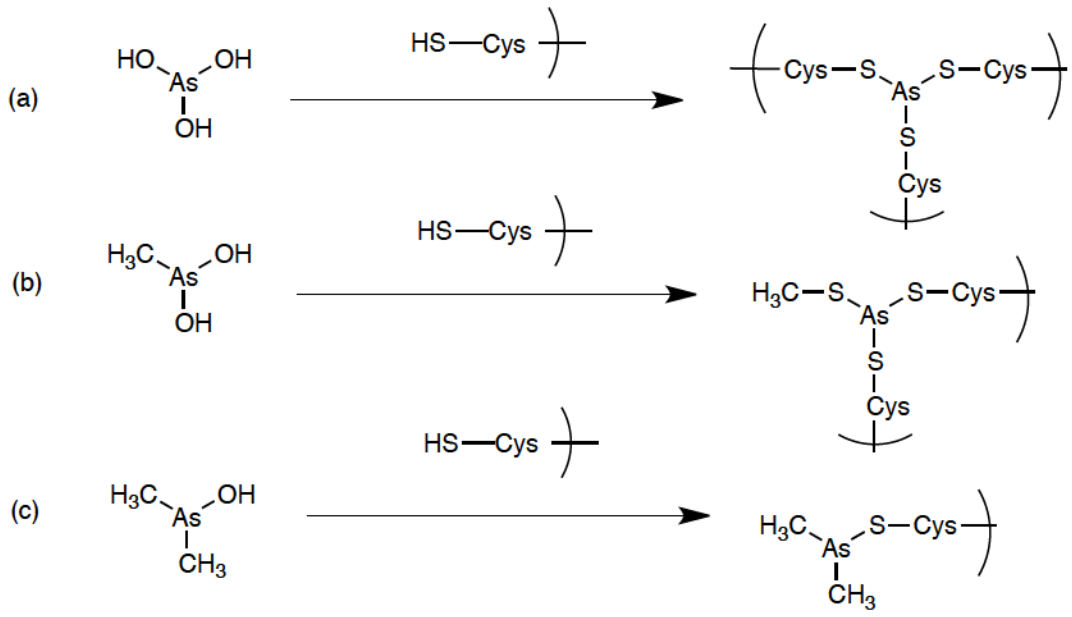


Figure 1.2 Mechanism of the binding between cysteine-containing protein and (a) As^{III}, (b) MMA^{III} and (c) DMA^{III} [54].

1.4.2 Arsenic in Plants and Animals

Most living organisms do not generate arsenic in their inner systems, and any arsenic found in living tissue is more likely due to the arsenic accumulation process. Arsenic is transported very slowly throughout all living organisms, if at all, therefore, the amount of arsenic accumulated depends almost solely on the amount of arsenic the organism is exposed to.

Some plants have the ability to remove arsenic from soil by their roots and transport it to their above ground shoots and leaves [61]. Plants accumulate 0.01-5 μg/g of arsenic (dry weight basis). At these levels, animals have very little risk of being poisoned due to the consumption of plants. Usually plants will die or suffer greatly before actually accumulating toxic concentrations of arsenic [38].

Arsenic is also cumulative in animal tissue. Among marine animals, arsenic can accumulate to levels of 0.005 to 0.3 mg/kg in some mollusks, crustaceans and coelenterates [62]. The average arsenic concentration in freshwater fish is about 0.14 μg/g on the basis of total wet weight [63]. The concentrations of arsenic in marine animals are higher than those in animals living on land. In mammals, arsenic accumulates in certain areas of ectodermic tissues, primarily the hair and nails. The domestic animals generally contain less than 0.3 μg/g on a wet weight basis [38].

1.4.3 Arsenic in Human

Humans are exposed to many different forms of arsenic species via food, water, and other environmental media. Arsenic can be taken in by humans

through respiratory, oral and dermal routes. The arsenic levels in humans is similar to that in domestic animals, which is lower than 0.3 μg/g (wet weight basis) [38]. Total human body arsenic content varies between 3 and 4 mg and tends to increase with age [64].

Arsenic accumulation has been found in skin, hair and nails because trivalent arsenicals have a high affinity for these keratin-rich tissues [65]. The normal amount of arsenic in hair is about 0.08-0.25 μg/g. An arsenic concentration of 1.0 μg/g or higher in human hair has been set as the limit that signifies the presence of excess arsenic and poisoning [66]. The normal arsenic concentration in nail tissue is 0.34 ± 0.25 μg/g [67], whereas the concentration of arsenic in nail clippings from a patient with arsenic poisoning can be in the range of 20-130 μg/g [68]. Arsenic levels in hair and nails are usually used as indicators of past arsenic exposure [69].

Analyses reveal that most bodily tissues contain arsenic with a concentration about 0.3-147 μg/g (dry weight), except hair, nails and teeth [1]. Arsenic distribution in tissues is dependent on several factors, including blood perfusion, tissue volumes, diffusion coefficients, membrane characteristics, and tissue affinities [38]. Arsenic exists in human tissues in different chemical forms. The inorganic arsenic species can be methylated by consecutive methylation reactions (Figure 1.3) and oxidation-reduction reactions between the trivalent and pentavalent arsenicals [70].

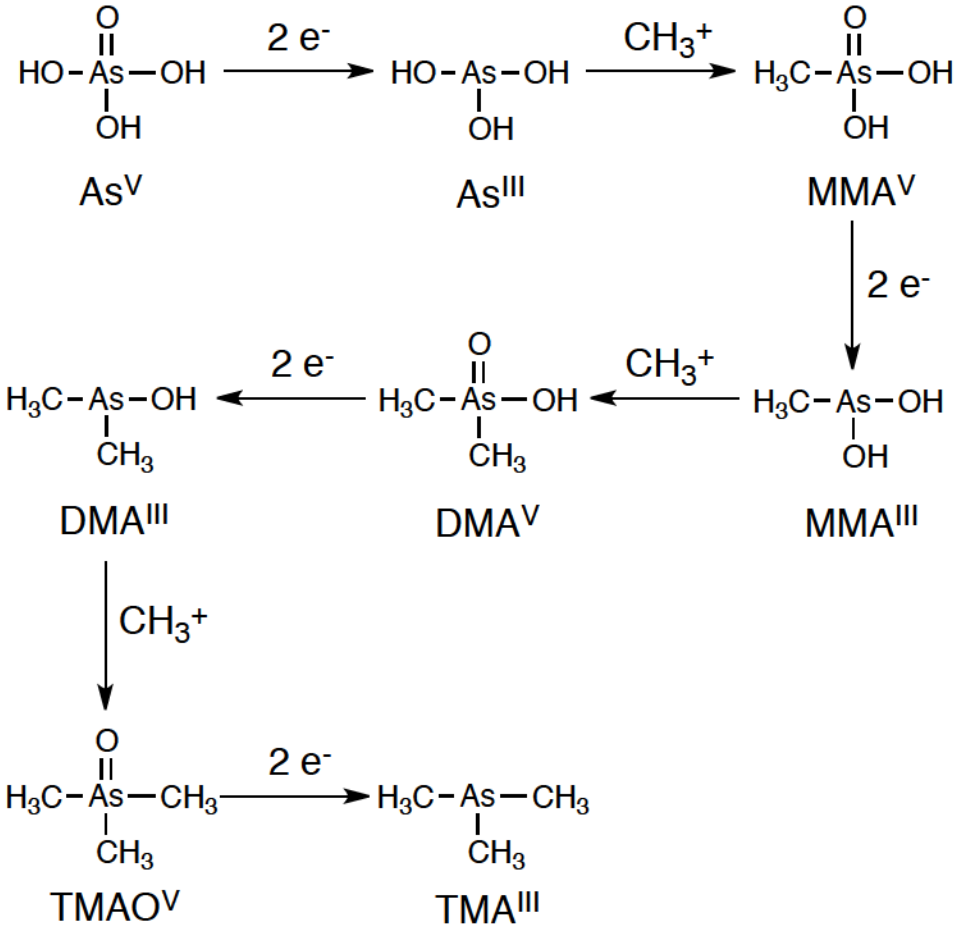


Figure 1.3 Pathway of biomethylation of arsenicals [70].

1.4.4 Arsenic in Poultry Tissues

The arsenic-containing feed additive, ROX, was previous widely used in the poultry industry. It was estimated that 1.7 to 2.2 million pounds of ROX were fed to poultry each year, and each chicken was fed about 3.5 mg of arsenic a day throughout their six-week life [71].

The distribution of arsenic in chickens has been investigated. One study used ^{76}As isotope to track arsenic in the body of chickens, and found an accumulation in feathers 12 hours after the oral dose [72]. Lasky et al. [73] found an average 0.39 μg/g arsenic in chicken livers and some muscle tissues collected from the data sources of United States Department of Agriculture (USDA). The data showed that the arsenic accumulation in livers could be 2 to 11 times higher in the liver than in muscle tissue, depending on how long the arsenic was removed from the feed before slaughter. There has also been a comparison of arsenic concentrations in young chickens to mature chickens; consistently higher arsenic concentrations were found in younger chickens.

Dean et al. [74] analyzed samples of chicken tissue from chickens fed a diet containing ROX as well as fish meal. They reported no traces of ROX in the chicken meat. Another study reported the presence of AsB, As^{V}, and DMA in a candidate reference material containing chicken meat [75]. Recent research performed by the US FDA (United States Food and Drug Administration) showed the discovery of new phenylarsonic compounds from the liver of ROX-fed chickens. These new arsenic species are possible metabolites of ROX [50].

1.5 TECHNOLOGIES FOR ARSENIC ANALYSIS

Analytical techniques capable of determining arsenic concentrations are important prerequisites in the study of the distribution of arsenic. Since the chemical form of arsenic greatly influences its toxicity and bioavailability, as well as its behaviour in the environment, there is a need to develop analytical methods that are able to determine the relative concentration of the individual arsenic species in samples [76, 77].

1.5.1 Sampling and Pretreatment of Samples

Two of the most important considerations during sample collection and storage are the prevention of contamination and the minimization of changes to arsenic speciation. The sample container, for example, high-density polyethylene bottles, and glassware were thoroughly prewashed with acid, then with water before use, in order to remove any arsenic residue and traces of oxidizing or reducing agent that may alter the original form of the arsenic species. It is essential to store samples at low temperature, such as -20 °C, to preserve the chemical integrity of the samples by preventing the transformation of the analytes due to microbial activities [78]. Sample homogenization is necessary prior to further sample processing, in order to evenly distribute all the substance in the sample, and to minimize errors attributable to poor representation.

1.5.1.1 Digestion of Solid Sample

Most trace element analysis techniques can only analyze samples in liquid form. Arsenic in solid samples needs to be solubilized prior to speciation analysis. For total arsenic analysis, oxidative digestion is widely accepted as a common

sample pretreatment approach, although acid digestion [79] and dry ashing [80] are the two main methods. These methods require very harsh reaction conditions, such as strong oxidizing acids, high temperature and even high pressure to decompose the solid matrices [81]. They can generally provide very high digestion efficiency, which can even reach 100%. Microwave-assisted digestion is a newly developed technique that has also been used for a wide variety of samples for total element analysis, such as soil, sediments, and marine organisms [82, 83].

1.5.1.2 Extraction of Arsenic Species

The pretreatment of samples for arsenic speciation is a requirement. It should release arsenic species with their original form, while simultaneously providing high extraction efficiency. Commonly used extraction methods involve solvent extraction and enzymatic extraction.

Solvent extraction provides a simple and robust extraction, and it is ideal for samples containing complex matrices. Methanol/water, methanol, or water are the most commonly used extraction solvents for arsenic speciation [84-86]. Other chemical solutions, such as trifluoroacetic acid [87] and nitric acid [88] have also been shown to quantitatively remove arsenic from solid samples for speciation. Solvent extraction is usually assisted with microwave [89, 90], ultrasound [88, 91], or pressurized [92] techniques.

Enzyme-assisted extraction utilizes enzymes to help break down biological materials and to release arsenic within specific samples by mimicking the physical and biochemical processes in the living systems, such as the human digestive tract

[93]. This approach is widely used for a variety of food samples including rice [93, 94]. Typical enzyme materials contain proteases (pepsin and trypsin, for instance) [94, 95], amylases [96] and even enzyme mixtures [97, 98]. This type of extraction is fast and very efficient in preserving arsenic species [97, 99], and has also been reported to be combined with microwave energy for satisfactory extraction recoveries [98, 100].

1.5.2 Analytical Methods for Total Arsenic Analysis

There are a great number of analytical techniques available for the determination of total arsenic. These techniques may include colorimetry/spectrophotometry [101, 102], atomic absorption spectrometry (AAS) [82, 103], atomic fluorescence spectrometry (AFS) [104], inductively coupled plasma atomic emission spectrometry (ICP-AES) [105], and inductively coupled plasma mass spectrometry (ICPMS) [87, 106]. The most popular technique today is ICPMS. Its advantages include excellent sensitivity, low limits of detection (LOD), multi-element capabilities, wide linear dynamic range, and high sample throughput [107].

In ICPMS, the analyte is first introduced by a peristaltic pump to the nebulizer, where the liquid solution is converted into an aerosol by argon (Ar) gas. After passing through a spray chamber to remove the larger droplets, the sample aerosol moves into the torch body, and it is vaporized and ionized by the inductively coupled plasma, at extremely high temperature (up to 10,000 K). The ions produced then pass through an interface into the mass spectrometer, which operates under room temperature and high vacuum conditions, where the ions are

focused and separated based on their mass-to-charge ratios (*m/z*). A quadrupole is the most widely used mass analyzer and is set to select the stable ^{75}As ions. At specific voltages, only the ions with selected *m/z* 75.0 can reach the detector. Finally, the ions are measured using an electron multiplier, and are collected by a counter for the selected ^{75}As ions. A mass spectrum is generated and the peak intensity of ^{75}As is directly proportional to the initial concentration of arsenic in the sample solution. With ICPMS, arsenic in concentrations from parts per trillion (ppt) to parts per million (ppm) can be measured in a single analysis within 1-3 min.

1.5.3 Analytical Methods for Arsenic Speciation Analysis

ICPMS provides high sensitivity detection for element analysis. But ICPMS can only differentiate the isotope masses rather than the chemical forms. ICPMS cannot achieve speciation analysis on its own. Hyphenated techniques that combine chromatographic separation methods such as high performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE) with ICPMS are required for the determination of arsenic species at trace levels in environmental and biological samples [76, 108, 109].

HPLC is a widely used analytical technique for the separation of a variety of components in a mixture solution. It separates analytes according to their differential partitioning between the stationary and mobile phases. Various types of chromatographic columns are commercially available, differing in the size and type of the stationary phase made up of packed particles inside the columns. Many chromatographic modes have been developed, including ion exchange, ion

pairing, reverse phase, and size exclusion chromatography, all with different column properties. HPLC is extensively used in different modes for separating a wide range of arsenic species in environmental and biological samples [70, 76, 110].

The combination of HPLC with ICPMS for arsenic speciation offers excellent sensitivity and specificity. However, no molecular information of arsenic species is obtainable, as the atomization of all arsenic compounds happens during the harsh ionization process in ICP. Identification of the arsenic species relies solely on the comparison between the HPLC retention time of the standards and that of the sample. It is susceptible to misidentifications if different species have identical chromatographic retention times [111]. Identification of unknown or new arsenicals that have no standards can be difficult. Electrospray ionization mass spectrometry (ESIMS) has become the most common method of obtaining complementary information in order to identify unknown peaks. ESIMS is a soft ionization technique and it can reserve the molecular information. In ESIMS, a sample solution containing the analytes of interest is dispersed into a fine spray of charged droplets by a high-voltage power applied to a capillary tube where the liquid flows through. An inert gas, such as nitrogen and carbon dioxide, can help high-flow electrosprays with additional nebulization. The charged droplets undergo further solvent evaporation until the Rayleigh limit is reached, which results in Coulomb fission and produces gas phase ions [112]. These sample ions then travel through the mass analyzer to the detector. Many types of mass analyzer, including quadrupole, tandem quadrupole, time-of-flight, and ion trap,

are used for the mass selection process. The major techniques for detection are selected ion monitoring (SIM) and multiple reaction monitoring (MRM).

Recently, ESIMS has been employed in conjunction with HPLC-ICPMS for speciation studies [113-117]. The HPLC-ICPMS/ESIMS system provides simultaneous detections for identification and quantitation of arsenic species.

1.6 THESIS OBJECTIVES

This research focuses on the study of arsenic in the skin of ROX-fed chickens. Chicken skin is selected, as it is keratin-rich and has great potential to bind arsenic. The study of arsenic accumulation and arsenic metabolism can be achieved by conducting total arsenic analysis and arsenic speciation analysis. Three major objectives of the research are shown as follows:

First, a proper quantitation method for the determination of arsenic concentrations in chicken skin needs to be developed. Because of trace concentrations of arsenic present in chicken skin, ICPMS capable of high sensitivity will be employed. Conditions of both sample digestion and sample detection will be optimized. Parameters such as sensitivity, linearity and accuracy of the method will be assessed.

Secondly, the ICPMS method will be applied to the determination of arsenic concentrations in skin samples from chickens used in a ROX feeding experiment. Changes in the concentrations of arsenic in chicken skin will be monitored over the period of five-week feeding time. The temporal profiles will be evaluated in order to better understand the arsenic uptake and elimination kinetics.

Finally, major arsenic species present in chicken skin will be examined. The HPLC-ICPMS/ESIMS system will be developed and employed to study the arsenic species in skin samples on specific days. This will contribute to an overall objective of understanding the ROX metabolism.

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CHAPTER 2

DEVELOPMENT OF A METHOD FOR DETERMINING TOTAL ARSENIC CONCENTRATION IN CHICKEN SKIN

2.1 INTRODUCTION

Arsenic is a toxic element that is widely spread in water, food, soil, dust, wood and other materials [1]. Toxicology studies have found that chronic exposure to arsenic could cause cancers, cardiovascular diseases, type II diabetes and other adverse health effects [2-4]. In order to maintain health, it is of great importance to monitor the arsenic content in high-consumption food, such as chicken, and in environmental samples.

Current arsenic detection and quantitation techniques involve spectrophotometry [5], hydride generation atomic fluorescence spectrometry (HGAFS) [6], and inductively coupled plasma mass spectrometry (ICPMS) [7]. ICPMS provides extreme sensitivity to achieve lower detection limits (μg/kg to ng/kg range), wide linear dynamic range (up to 5 or 6 orders of magnitude), high precision (0.5% to 5%) and high sample throughput, it is suitable for trace level of arsenic analysis [8].

However, ICPMS has difficulty in analyzing solid samples. Solid samples must be converted to solutions prior to detection. One commonly used conversion method is wet digestion using oxidizing acids (such as nitric acid, HNO_3). This acid digestion has several advantages: it is effective on both inorganic and organic materials, and it reduces or eliminates interference by destroying sample matrix.

But acid digestion is also limited by several factors, such as the low maximum digestion temperature, the risk of contamination through laboratory air, and the danger of losses of trace elements. These can be solved by adding sulfuric acid (H_2SO_4), setting up reflux condenser and using an excess of acid (mainly HNO_3) [9].

In this chapter, the objective was to develop an ICPMS method for the determination of arsenic in chicken skin. This method is necessary for the work to be described later in Chapter 3, namely determining the total arsenic concentration in skin of chickens treated with Roxarsone® (ROX).

2.2 MATERIALS AND METHODS

2.2.1 Reagents and Standards

Deionized water obtained from a Milli-Q water purification system (18.2 MΩ·cm, Millipore, Molsheim, France) was used throughout the experiment. Certified ACS plus concentrated nitric acid (HNO_3) and concentrated sulfuric acid (H_2SO_4) were purchased from Fisher Scientific, and were used for digestion of chicken skin samples. Environmental calibration standard (Agilent Technologies, U.S.) was used as a working standard solution, freshly prepared for each analytical session. Standard reference material (SRM) 1640a (trace elements in natural water) was obtained from National Institute of Standards and Technology (Gaithersburg, MD), and was used as a quality control measure of total arsenic determination. DORM-4 (fish muscle certified reference material for trace metals) was obtained form National Research Council Canada, and was used for method validation.

All glassware and plastic bottles used in the experiment were thoroughly washed then soaked overnight in 10% HNO_3 and were thoroughly rinsed with deionized water before use.

2.2.2 Sample Pretreatment

Chicken skin samples were stored at -80 °C. They were thawed on the day of analysis. After removal of feathers and visible fat, these samples were then cut into small pieces by dissecting scissors. The small pieces were placed in a kitchen blender for homogenization. The homogenized skin sample was then weighed into a 50 mL beaker. In the fume hood, concentrated HNO_3 and H_2SO_4 were slowly and sequentially added into the beaker with a 1:3 volume ratio. Then, the beaker was covered with a watch glass, and the mixture was left overnight for digestion. On the following day, the beaker was placed on a hotplate and heated to 120 °C for further digestion until the solution turned transparent. The temperature was increased to 200 °C to remove the excess HNO_3. Then the watch glass was removed, and the temperature was raised to 500 °C to further evaporate the acids to almost dryness (volume was less than 0.5 mL). The entire digested content was transferred into a 15 mL centrifuge tube (Fisher Scientific) and diluted by deionized water afterwards. This solution was then vortexed, and aspirated into a 10-mL syringe (BD Biosciences, Franklin Lakes, NJ), filtered through a 0.45 μm membrane (Mandel, Guelph, ON, Canada) before total arsenic analysis. In this wet digestion procedure, several parameters (mass of sample, total acid amount, final volume of diluted solution) were optimized and they were listed in Table 2.1. Certified reference material of DORM-4 fish protein (0.3 g) was digested and

analyzed in the same manner as skin samples. It was used to ensure the accuracy of the method. Each of the skin samples and reference materials was processed in triplicate. Blanks containing only the acids were prepared in triplicate and were included with each set of samples as well.

Table 2.1 Optimized parameters for digestion and analysis of chicken skin

Parameter	Value	
Mass of Skin Sample	0.2 g	0.5g
Total Acid Amount	20 mL	40 mL
Final Volume of Diluted Solution	4 mL	10 mL

2.2.3 Sample Analysis

The arsenic concentration in the diluted solutions was determined by using ICPMS (Agilent 7500cs; Agilent Technologies, Japan). 2 mL of sample solution was introduced by an ASX-510 autosampler (CETAC, Omaha, NE) and then transferred into ICPMS nebulizing system by a peristaltic pump at a speed of 0.4 r/s. The instrumental conditions were optimized before each analytical session. These conditions are shown in Table 2.2. Arsenic was monitored at *m/z* 75.0 under the helium mode to prevent the interference of polyatomic ion argon chloride $ArCl^+$. Because of the collision between $ArCl^+$ and helium, $ArCl^+$ would be dissociated before reaching the detector [10, 11].

At the beginning of each analytical session, external calibration was conducted by serial concentrations of arsenic standards (0.1, 0.5, 1, 5, 10 and 50 µg/L). These arsenic standards were prepared in 1% HNO_3 from the primary arsenic standard (10mg As/L). The daily results accuracy was checked by standard reference material 1640a prior to samples. Afterwards, the limit of detection and limit of quantitation were determined for evaluation of the quantitation method. It was achieved by analyzing 20 procedure blanks which containing only acids [12].

Table 2.2 Optimum instrumental conditions for ICPMS operation

Plasma parameters—	
RF power	1500 W
Plasma gas flow rate	15 L/min
Carrier gas flow rate	0.99 L/min
Makeup gas flow rate	0.1 L/min
Sampler and skimmer	Nickel, 1 and 0.4 mm id[a]
Spray chamber temperature	2 °C
Ion lenses setting	Optimized for best sensitivity of 1 µg/L Li, Y and Tl in 2 wt%[b] HNO_3 tuning solution (Agilent Technologies, U.S.)
Reaction/collision cell parameters—	
He gas flow rate	3.2 mL/min
Octpole bias	-18 V
Quadrupole bias	-15 V
Data acquisition parameters—	
Peak pattern	FullQuant (3 points)
Integration time (per point)	0.1 s
Replicates	3

[a] id: inner diameter;

[b] wt%: weight percent.

2.3 RESULTS

2.3.1 Optimization of Acid Digestion

Initial experiments using 0.2 g or 0.5 g skin samples showed that 0.2 g skin sample was sufficient for digestion and analysis (Figure 2.1). Figure 2.2 illustrated the total arsenic concentrations in 0.2 g skin samples after digested with 20 mL acids (5 mL HNO_3 and 15 mL H_2SO_4) and 40 mL acids (10 mL HNO_3 and 30 mL H_2SO_4). The total arsenic concentrations in 0.2 g skin samples, when diluted with 4 mL and 10 mL deionized water, were shown in Figure 2.3.

2.3.2 Total Arsenic Analysis Using ICPMS

Total arsenic analysis of chicken skin is achieved by using ICPMS due to higher sensitivity. One example of the calibration curve was shown in Figure 2.4. By analyzing 20 procedural blank samples, we obtained standard deviation. On the basis of 10 times the standard deviation, the limit of quantitation of our method was 0.06 µg (As)/L (solution). In order to ensure the accuracy of our method, we determined arsenic concentrations in standard reference materials. Our measured values and the certified values were shown in Table 2.3.

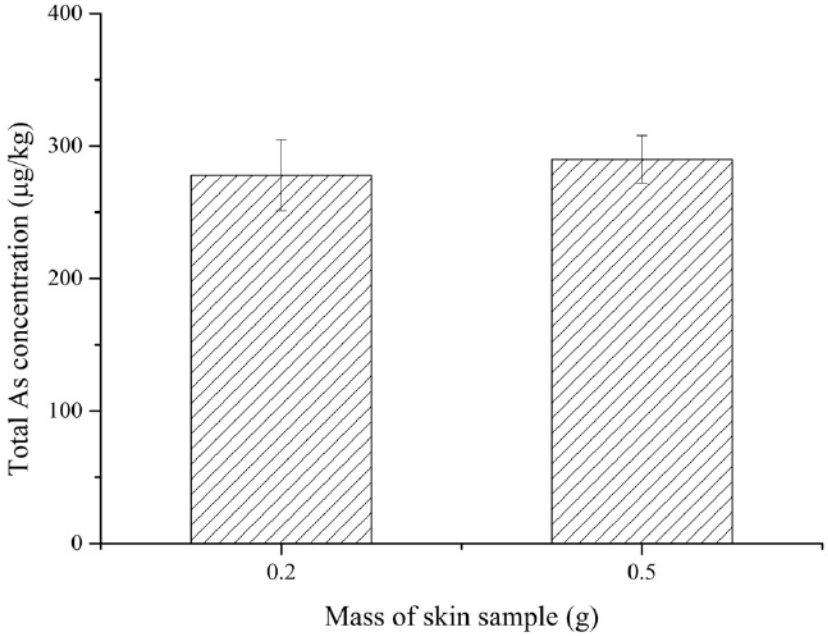

Figure 2.1 Total arsenic concentrations in two different amounts (0.2 g and 0.5 g) of chicken skin samples. The skin sample was collected on day 28 from pen 1, Cobb 500. The concentrations were calculated to the unit of µg (As)/kg (skin, wet weight) for comparison.

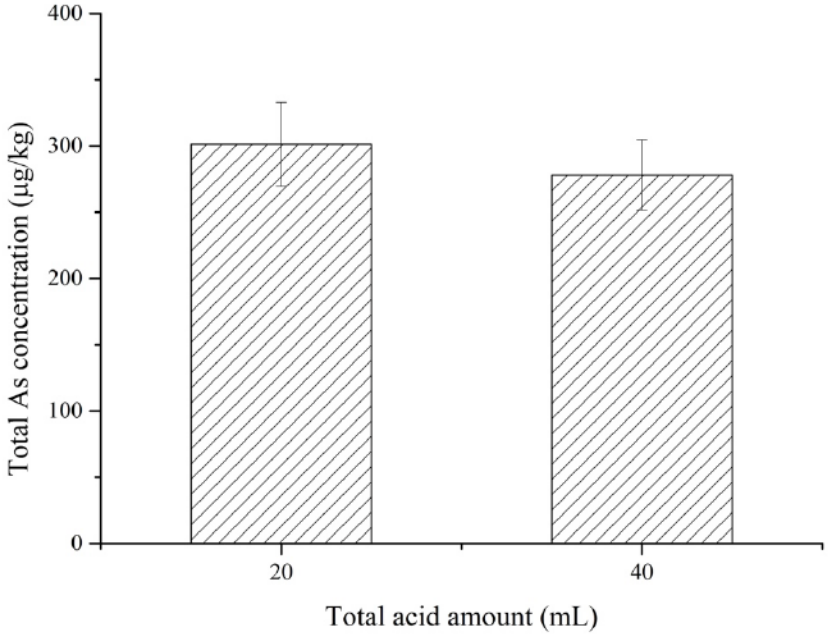

Figure 2.2 Total arsenic concentrations in 0.2 g chicken skin samples after digestion using 20 mL acids (5 mL HNO_3 and 15 mL H_2SO_4) and 40 mL acids (10 mL HNO_3 and 30 mL H_2SO_4). The skin sample was collected on day 28 from pen 1, Cobb 500. The concentrations were calculated to the unit of µg (As)/kg (skin, wet weight) for comparison. There is no significant difference ($p>0.05$) between the two.

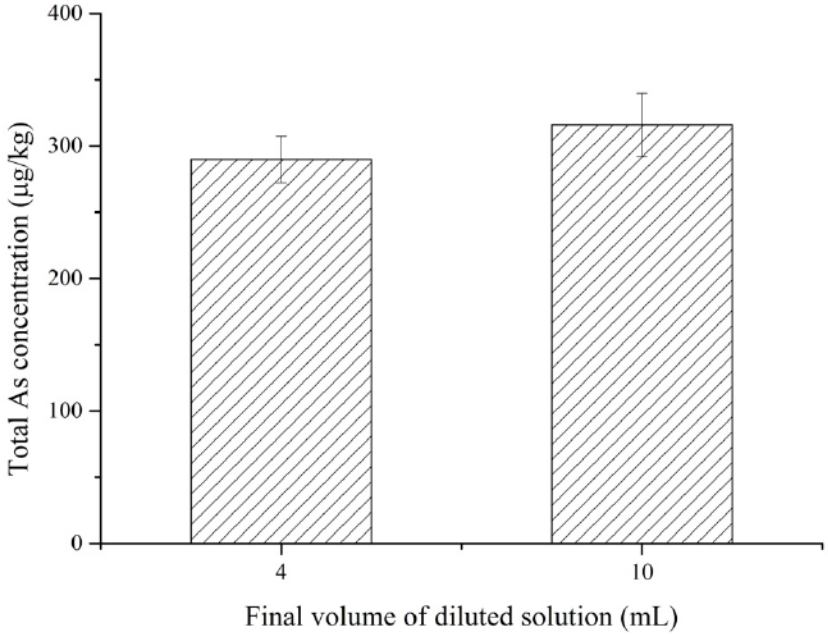

Figure 2.3 Total arsenic concentrations in 0.2 g chicken skin samples after digestion and dilution with 4 mL and 10 mL deionized water. The skin sample was collected on day 28 from pen 11, Ross 308. The concentrations were calculated to the unit of µg (As)/kg (skin, wet weight) for comparison. There is no significant difference ($p>0.05$) between the two.

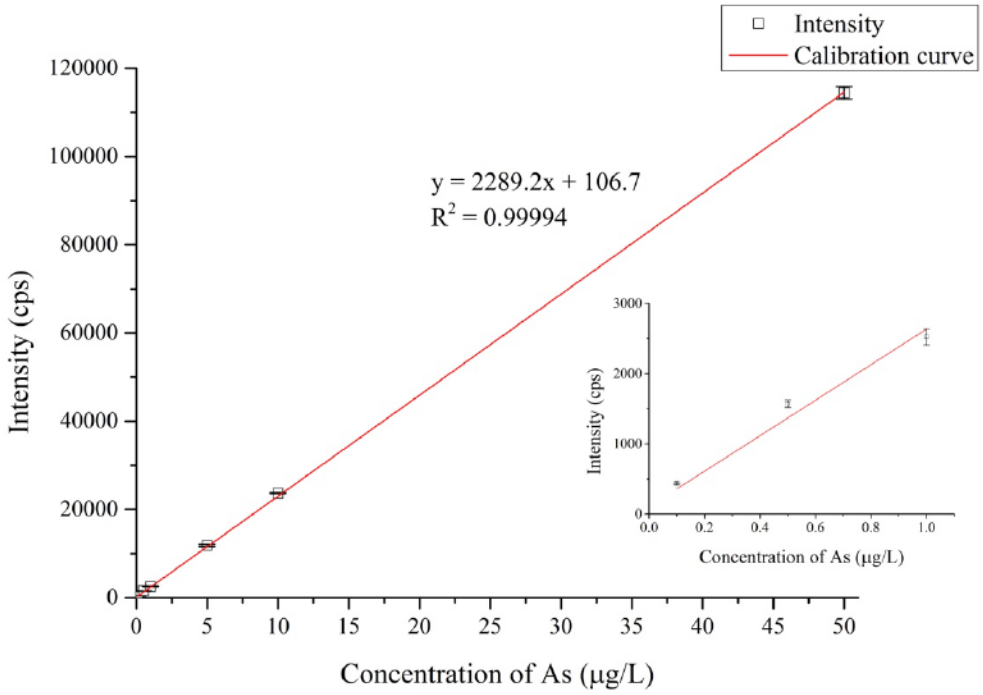

Figure 2.4 A calibration curve generated from the total arsenic analyses of 0.1, 0.5, 1, 5, 10 and 50 µg/L arsenic standards using ICPMS. The inset shows the concentration range from 0.1 to 1 µg/L.

Table 2.3 Comparison of measured and certified arsenic concentration in reference materials

Reference Material	Certified As Concentration	Measured As Concentration	Repeat Times	Unit	Accuracy (%)	Purpose
1640a	8.075 ± 0.070	8.076	1	µg/L	100	Quality control of calibration curve
DORM-4	6.80 ± 0.64	7.20 ± 0.46	3	mg/kg	106 ± 7	Acid digestion method validation

2.4 DISSCUSIONS

2.4.1 Optimization of Acid Digestion

The mass of skin samples for digestion was first optimized. The measured values showed the total arsenic concentrations in the diluted solution with the unit µg/L and they were converted to the total arsenic concentrations in chicken skin with the unit µg/kg for comparison. Due to the limitation of the skin sample (the masses of some samples were less than 0.5 g), the mass of homogenized skin sample was determined as 0.2 g for the acid digestion process in subsequent experiments.

Another important parameter for the digestion process is the amount of acid needed. Large amounts of acid would increase the extent of digestion, but it would require a longer time to evaporate, bring higher background and increase the risk when handling it. When decreasing the total acid amount from 40 mL to 20 mL, we did not observe any residue left in the acid solution. By comparing the results in Figure 2.2, it can be seen that the total arsenic concentration detected in the same amount of skin was similar between the use of 20 mL acids and 40 mL acids. The arsenic concentration in the blank solution when using 20 mL acids for digestion was below the limit of quantitation. Therefore, in order to maintain high extent of acid digestion, 20 mL acids including 5 mL HNO_3 and 15 mL H_2SO_4 were used.

Similarly, the effect of final diluted solution volume in the sample preparation process was explored. The comparison of total arsenic in 0.2 g homogenized skin, when diluted to 4 mL and 10 mL for ICPMS analysis, was

illustrated in Figure 2.3. We found that the calculated value was similar between the two dilutions. However, after evaporation, the final acid content was mainly H_2SO_4 whose volume was less than 0.5 mL. When diluted to 4 mL, the concentration of H_2SO_4 can be as high as 12.5% (v/v), while the concentration of H_2SO_4 would be 5% (v/v) when diluted to 10 mL. On one hand, high concentration of acid would affect sample introduction system and nebulization system of ICPMS and reduce the instrument sensitivity [13, 14]. On the other hand, high concentration of H_2SO_4 (higher than 5%) would also contaminate even damage the sampler and skimmer, and influence the performance of ICPMS [15]. Thus, the final volume of diluted solution was set as 10 mL, and during the acid evaporation process, the amount of acid left in the beaker was controlled as less as possible.

2.4.2 Total Arsenic Analysis Using ICPMS

ICP-MS offers extremely low limit of detection and limit of quantitation ranging from sub part per billion (ppb) to parts per trillion (ppt) for most elements [8]. In our case, we calculated the limit of quantitation (LOQ) using the following equation.

$$LOQ = (b+10\ \sigma)/s$$

where s is the slope of the calibration curve, σ is the standard deviation of the response obtained from 20 replicate procedural blanks, and b is the average response of 20 replicate procedural blanks [12].

The LOQ of our ICPMS method for total arsenic analysis is 0.082 ppb (μg/L) in the diluted solution, and it is sufficient for the quantitation of the digested skin sample.

The accuracy and the validity of acid digestion method were checked by parallel analyses of reference materials. The measured arsenic concentration showed good agreements with the certified values (Table 2.3). Therefore, our total arsenic analysis method is validated and suitable for further study of the chicken skin samples in ROX feeding experiment.

2.5 CONCLUSIONS

The acid digestion method was developed, and the optimized conditions were as follows: 0.2 g as the mass of homogenized chicken skin, 20 mL (5 mL HNO_3 and 15 mL H_2SO_4) as the amount of acids for digestion, 10 mL as the final volume of the diluted solution for ICPMS analysis. This technique required small amount of skin sample. It also generated low background because of the relatively pure reagent. It will be used for the subsequent preparation of the chicken skin samples in the following feeding experiment.

An ICPMS quantitation method for arsenic was developed as well, with high sensitivity (2289.2 cps/(μg/L)), low LOQ (0.082 ppb), good linear response (R^2=0.9999) and good accuracy (close to certified values in Table 2.3). It is suitable for total arsenic analysis of the chicken skin samples.

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CHAPTER 3

APPLICATION OF THE ICPMS METHOD FOR TOTAL ARSENIC CONCENTRATION ANALYSIS IN CHICKEN SKIN[1]

3.1 INTRODUCTION

Roxarsone® (ROX, 3-nitro-4-hydroxyphenylarsonic acid) has been previously used in the poultry industry to prevent coccidiosis, enhance feed efficiency, and promote growth [1, 2]. However, recent research has reported that ROX could be transformed into toxic inorganic arsenic species in chicken [3]. Roxarsone, inorganic arsenic and a few other arsenic species were detected in chicken meat samples from a US-based market survey [4]. Skin consists of keratin, a cysteine-rich protein. On the basis of arsenic-sulfur interaction, skin has great potential to bind arsenic. But few studies focused on arsenic in chicken skin. It is still less known about the arsenic concentration in skin of ROX-treated chicken. As chicken skin could be consumed as food in our daily life, systematic and comprehensive study of arsenic amount in chicken skin is necessary for ensuring our human health.

Here we applied our acid digestion and ICPMS quantitation method to the study of total arsenic concentration in skin of either ROX-treated chickens or control chickens. We explored the arsenic uptake and elimination process in

[1] Note: In this chapter, Table 3.1 was constructed in collaboration with Mr. Zonglin Yang.

chicken skin by monitoring the arsenic concentration in skin over the whole feeding period.

3.2 MATERIALS AND METHODS

A 35-day chicken feeding experiment was carried out at the Poultry Research Centre, University of Alberta. This study was approved by animal welfare committee in the Department of Agricultural, Food and Nutritional Science. In total, 1600 chickens, representing two strains (Cobb 500 and Ross 308), were used. For each strain, there were 800 chickens initially placed and raised in 8 pens (100 chickens in each pen). These 800 chickens were then divided into two groups: 400 chickens in 4 pens would be fed with ROX-containing diet and served as the ROX-treatment group; the remaining 400 chickens in the other 4 pens would be fed with ROX-free diet and served as the control group. During the entire 35-day feeding period, drinking water from the same source (< 1 μg/L arsenic) was supplied to the chickens. Diets, including starter feed, grower feed and finisher feed, were provided to the chickens sequentially for 2 weeks, 2 weeks and 1 week. The composition information for the diets was shown in Appendix Table A.1. Some arsenicals were detected in the diets and their concentrations were summarized in Table 3.1. For the ROX-treated group, ROX was withdrawn from the diet at the last week (day 29 to day 35) in compliance with United States Food and Drug Administration (US FDA) regulations in order to allow elimination of arsenic from chickens' bodies. The information of the feeding experiment is summarized in Table 3.2.

3.2.1 Sample Collection

Chicken skin samples were obtained from the Poultry Research Centre, University of Alberta. They were collected on days 0-4, day 7, day 14, day 21, and days 28-35 (16 days in total), representing the samples from uptake, growth and ROX withdrawal period. At each sampling day, one chicken from each pen was slaughtered, and a portion of skin was removed from the body for our arsenic concentration study. In total, 16 chicken skin samples were collected each day: one from each of the eight control pens and one from each of the eight ROX-treated pens. These skin samples from different days varied in mass, and their masses ranged from 0.2 g (sample in day 0) to 3.9 g (sample in day 35).

All samples were properly labeled and sealed in plastic bags. They were frozen and stored at -80 °C until the time of analysis.

Table 3.1 Concentrations of arsenic species detected in ROX-treated and untreated feed samples.[a]

	Concentration of Arsenic Species (mean ± SD) (µg/g)				
Feed Type	AsB	DMA	As^V	ROX	Sum of As
ROX Starter	0.054 ± 0.004	0.031 ± 0.006	0.059 ± 0.009	18.3 ± 0.9	18.4 ± 0.9
ROX Grower	0.034 ± 0.005	0.036 ± 0.003	0.072 ± 0.002	18 ± 1	18 ± 1
ROX Finisher	0.026 ± 0.002	0.034 ± 0.009	0.044 ± 0.008	0.11 ± 0.02	0.21 ± 0.02
Control Starter	0.097 ± 0.003	0.033 ± 0.004	0.12 ± 0.01	N.D.	0.25 ± 0.01
Control Grower	0.035 ± 0.003	0.042 ± 0.006	0.05 ± 0.01	0.31 ± 0.03	0.43 ± 0.03
Control Finisher	0.030 ± 0.002	0.035 ± 0.004	0.07 ± 0.01	0.17 ± 0.03	0.31 ± 0.03

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

Table 3.2 Information of the feeding experiment

Chicken Strain	Group	Feeding Plan			Pen Number
		Uptake period (day 0 – day 14)	Growth period (day 15 – day 28)	ROX elimination period (day 29 – day 35)	
Cobb 500	ROX-treated	ROX Starter (ROX-containing diet)	ROX Grower (ROX-containing diet)	ROX Finisher (ROX-free diet)	1, 16, 19, 24
	Control	Control Starter (ROX-free diet)	Control Grower (ROX-free diet)	Control Finisher (ROX-free diet)	5, 6, 18, 25
Ross 308	ROX-treated	ROX Starter (ROX-containing diet)	ROX Grower (ROX-containing diet)	ROX Finisher (ROX-free diet)	11, 13, 15, 29
	Control	Control Starter (ROX-free diet)	Control Grower (ROX-free diet)	Control Finisher (ROX-free diet)	3, 12, 17, 22

3.2.2 Sample Pretreatment

Skin samples from the same collection day were processed during the same day. They were first thawed at the room temperature. After feathers and visible fat were removed, the skin sample was then homogenized by using dissecting scissors. Afterwards, 0.2 g homogenized skin sample was weighed into a clean 50 mL beaker, and 5 mL HNO_3 and 15 mL H_2SO_4 were sequentially and slowly added into the beaker. The beaker was then covered with a watch glass and left in the fume hood overnight for sample digestion. On the next day, the beaker was placed on a hotplate and heated at 120 ℃ until the content became transparent. Then the watch glass was removed, and the beaker was heated at 500 ℃ for evaporation of the acids. When there was only a drop of acid content left in the beaker, the heating procedure was stopped. The acid content was then transferred into a 15 mL polypropylene centrifuge tube and diluted to 10 mL with deionized water. This sample solution was finally syringe-filtered through a 0.45 μm membrane into another centrifuge tube prior to total arsenic concentration analysis. Sample blanks containing only acids were prepared using the same way. Skin sample was digested and analyzed in triplicate. For those samples with lower masses, they were prepared duplicate (for samples from day 7 and day 14) or only once (for samples from day 0-4).

In this process, certified reference material DORM-4 was prepared in a similar way for quality control. 0.3 g DORM-4 was digested and then diluted to 100 mL each time. They were prepared and analyzed in triplicate.

3.2.3 Total Arsenic Analysis

Skin samples from the same collection day were analyzed using ICPMS during the same day. Before sample analysis, external calibration was first conducted for the quantitation of As in skin samples. It was achieved by analyzing arsenic standards with concentrations ranging from 0.1 to 50 µg As/L. Arsenic signal was monitored at *m/z* 75.0 under the helium mode for both standards and samples. In order to check the instrument drift, 10 µg As/L standard solution was analyzed once every twenty samples.

3.3 RESULTS

3.3.1 Arsenic Concentration in Chicken Skin Over The Entire Feeding Period

The total arsenic concentration data in different sampling days were summarized in Table 3.3. According to these data, the temporal changes of total arsenic concentrations in the skin samples of chickens from Control Group Strain Cobb 500 (Control Cobb), Control Group Strain Ross 308 (Control Ross), ROX-fed Group Strain Cobb 500 (ROX Cobb) and ROX-fed Group Strain Ross 308 (ROX Ross) were obtained and illustrated in Figure 3.1. Paired t-tests were carried out for skin samples on the same sampling day between ROX-treated group and control group from the same strain, and between Cobb strain and Ross strain from the same group. The ROX-fed groups between the two strains of chicken showed similar trends ($p>0.05$ comparing samples from the same day but different strains) during the 35-day feeding period. Likewise, the control groups between the two strains of chicken showed similar results on arsenic

concentrations ($p>0.05$) over the 35-day period. For chickens from the same strain but different groups, significant difference was observed. The concentrations of arsenic in the ROX-fed chicken skin were significantly higher than the concentration in the skin of control chickens.

3.3.2 Body Weights of Chickens

Chicken body weights over the entire feeding period were monitored as well. They were summarized in Appendix Table A.2. The temporal change of chicken body weight was illustrated in Figure 3.2. Paired t-tests were conducted for the chickens sacrificed on the same day between ROX-fed group and control group for both strains. 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.

Table 3.3 Total arsenic concentrations detected in the skin samples.[a]

Total Arsenic Concentration (mean ± SD) (µg/kg)				
Day	Control Cobb	Control Ross	ROX Cobb	ROX Ross
0	11.9 ± 0.6	14 ± 2	4 ± 3	4 ± 2
1	14 ± 7	17 ± 7	52 ± 16	52 ± 34
2	12.2 ± 0.2	16 ± 5	117 ± 4	114 ± 30
3	20 ± 4	24 ± 5	147 ± 40	155 ± 26
4	31 ± 7	39 ± 4	277 ± 13	238 ± 43
7	58 ± 12	53 ± 17	344 ± 23	327 ± 69
14	42 ± 6	49 ± 4	297 ± 46	272 ± 10
21	60 ± 10	66.5 ± 0.3	201 ± 66	236 ± 29
28	76 ± 10	77 ± 2	334 ± 41	370 ± 80
29	88 ± 10	78 ± 11	246 ± 22	232 ± 17
30	66 ± 2	62 ± 5	159 ± 12	177 ± 76
31	83 ± 17	84 ± 4	183 ± 13	197 ± 11
32	62 ± 7	56 ± 6	113 ± 24	120 ± 28
33	64 ± 12	66 ± 8	130 ± 15	124 ± 5
34	56 ± 17	65 ± 7	94 ±10	98 ± 8
35	66 ± 4	60 ± 2	104 ± 4	91 ± 5

[a] SD: standard deviation.

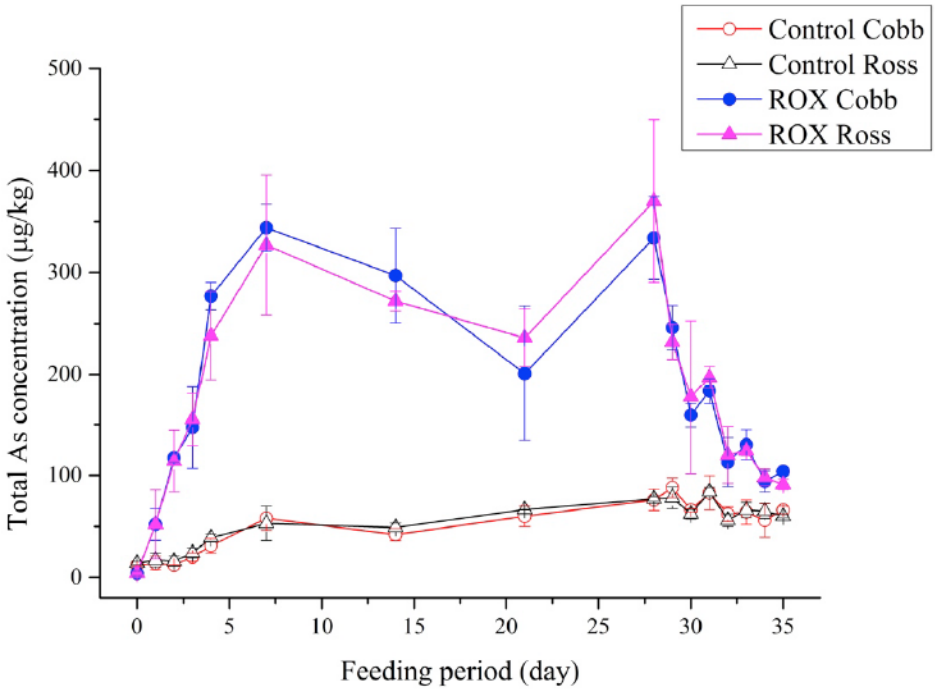

Figure 3.1 Total arsenic concentrations in the skin samples of chickens from Control Cobb, Control Ross, ROX Cobb and ROX Ross groups. The skin samples were collected on 16 different days (day 0-4, day 7, day 14, day 21, day 28-35). Four chickens were sampled from each group on each day. Each data point represents average arsenic concentration and standard deviation from the analyses of four chicken samples (n=4).

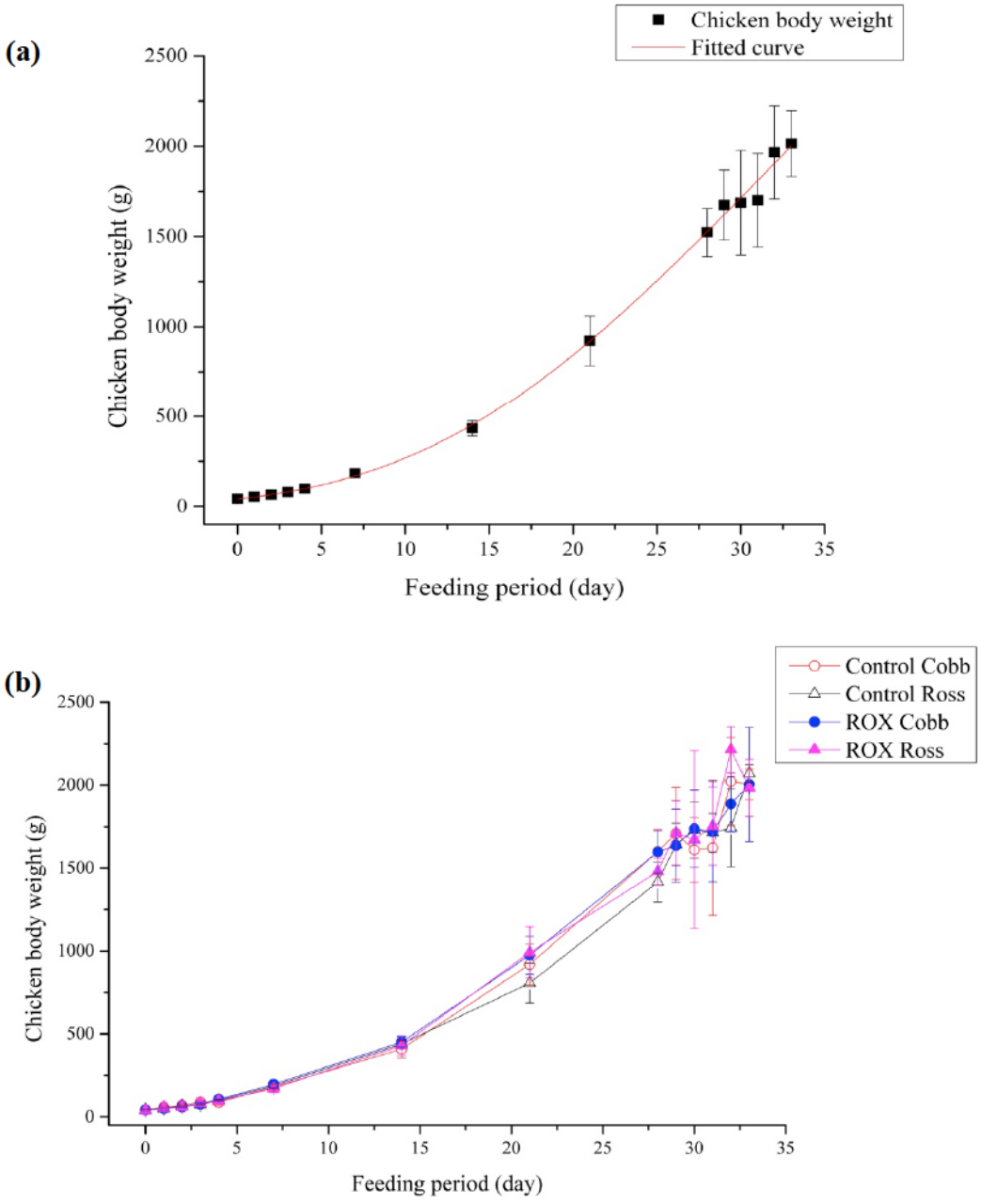

Figure 3.2 (a) The body weights for all chickens during the feeding period. The fitted curve function was $y=5401.8\times e^{-e^{-0.048\times(x-32.9)}}$, $R^2=0.99762$.

(b) Body weights of chickens from four separate groups over the 35-day feeding period. Each data point represents average and standard deviation from four chickens (n=4).

3.3.3 Arsenic Concentration in Chicken Skin During The First 28 Days

In the feeding experiment, ROX-containing diet (containing 0.025% ROX) was fed to the ROX-treatment group in the first four weeks. Figure 3.3 showed the total arsenic concentration changes in the skin samples from different groups and different strains during this period. Significant differences ($p<0.05$) were observed between ROX-fed group and control group for both strains on each day from day 1 to day 28 on the basis of paired t-test.

In order to explore the kinetics of arsenic uptake during day 0 to day 28, curve fitting was conducted, and arsenic uptake rates were estimated for comparison. The total arsenic concentrations for ROX Cobb group during day 0 to day 28 and the fit curve were shown in Figure 3.4. Here I used the Gompertz function in the software "origin", which is usually used for modeling the animal growth. The total arsenic concentration in skin rapidly increased during day 0 to day 7, and the arsenic uptake rates were ranging from 11.3 μg/(kg·day) (day 0) to 91.6 μg/(kg·day) (day 2). Then, the total arsenic concentration reached a plateau, and the total arsenic concentration remained relatively steady during day 7 to day 28. The total arsenic concentrations in skin samples from ROX Ross group demonstrated a similar trend, and the arsenic uptake rates during day 0 to day 7 were ranging from 12.6 μg/(kg·day) (day 0) to 74.8 μg/(kg·day) (day 2). The total arsenic concentration trend curve for ROX Ross group was shown in Figure 3.5.

Unlike the ROX-treatment groups, the control groups did not show very dramatic change during this arsenic uptake period. The total arsenic concentration trend curve for Control Cobb group was shown in Figure 3.6. Here I used the

same function for the curve fitting for the control groups. But large deviations were observed for the arsenic concentrations on day 4 and day 7. Generally, the concentration of arsenic in skin of Control Cobb chickens increased slowly during day 0 to day 28. The As uptake rates on each day were below 2.9 μg/(kg·day). The Control Ross group showed similar total arsenic concentration trend and its trend was illustrated in Figure 3.7.

The comparison of total arsenic concentration in skin samples from ROX-treatment group and control group on day 28 was shown in Figure 3.8. For both strains, significant differences ($p<0.05$, $p=0$ for Cobb strain, $p=0.02$ for Ross strain) were observed between ROX-fed group and control group. ROX-fed group showed higher arsenic concentrations in skin. For the skin samples in the same group (either ROX-fed group or control group), there was no statistically significant difference ($p>0.05$, $p=0.53$ for ROX-fed group, $p=0.81$ for control group) in arsenic concentration between two strains of chickens.

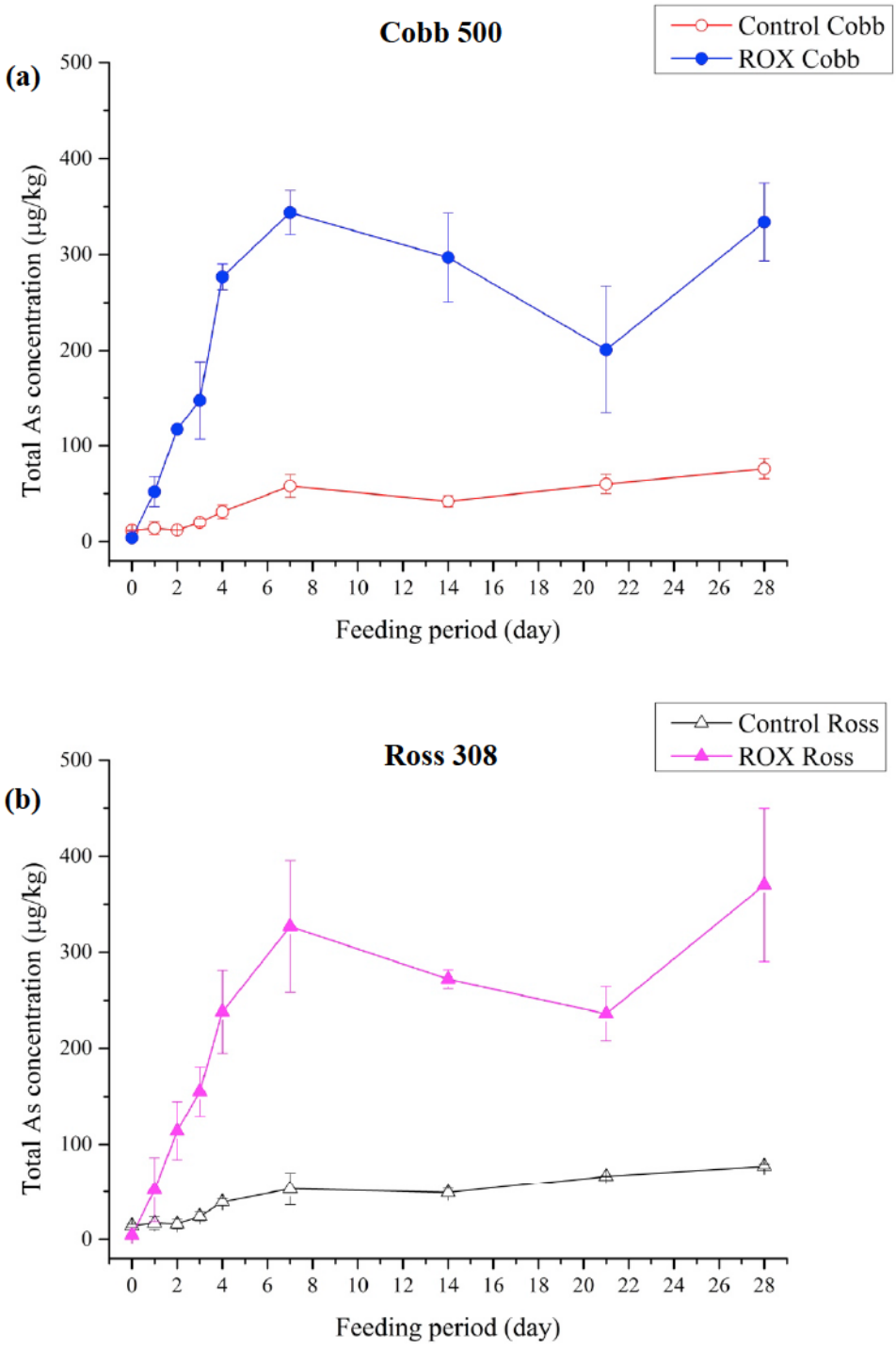

Figure 3.3 Total arsenic concentrations in the skin samples of chickens from strain (a) Cobb 500 and (b) Ross 308 during As uptake period (day 0-28).

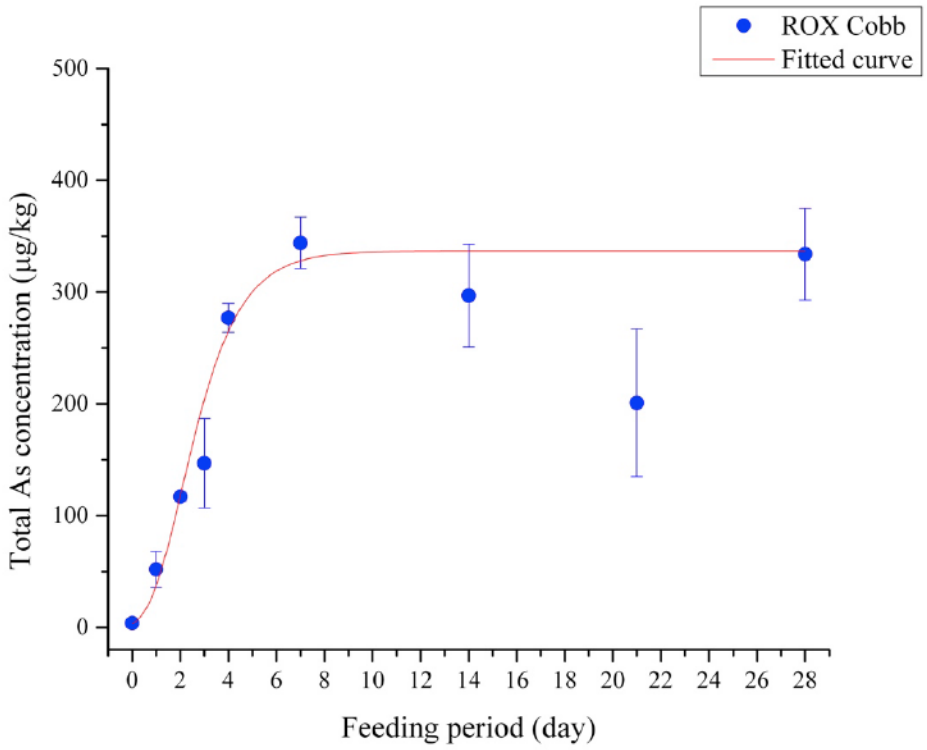

Figure 3.4 Total arsenic concentrations in skin samples of chickens from ROX Cobb group during day 0 to day 28. The fitted curve function is $y=336.8\times e^{-e^{-0.74\times(x-2.07)}}$, $R^2=0.98838$. Y is the total As concentration, while x is the feeding day. The As uptake rate is $y'=336.8\times0.74\times e^{-e^{-0.74\times(x-2.07)}-0.74\times(x-2.07)}$. Y' is the As uptake rate, while x is the feeding day.

Feeding period (day)	0	1	2	3	4	7	14	21	28
As uptake rate (µg/(kg·day))	11.3	60.5	91.6	75.8	47.0	6.3	0.0	0.0	0.0

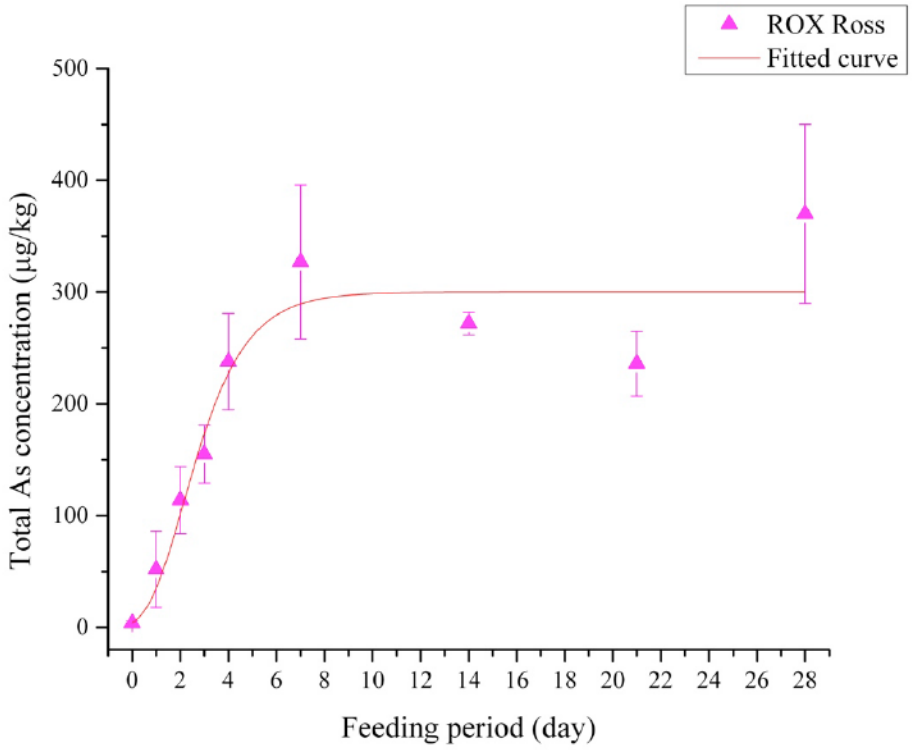

Figure 3.5 Total arsenic concentrations in skin samples of chickens from ROX Ross group during day 0 to day 28. The fitted curve function is $y=300.0\times e^{-e^{-0.68\times(x-2.12)}}$, $R^2=0.98277$. Y is the total As concentration, while x is the feeding day. The As uptake rate is $y'=300.0\times0.68\times e^{-e^{-0.68\times(x-2.12)}-0.68\times(x-2.12)}$. Y' is the As uptake rate, while x is the feeding day.

Feeding period (day)	0	1	2	3	4	7	14	21	28
As uptake rate (µg/(kg·day))	12.6	51.3	74.8	64.7	43.0	7.1	0.0	0.0	0.0

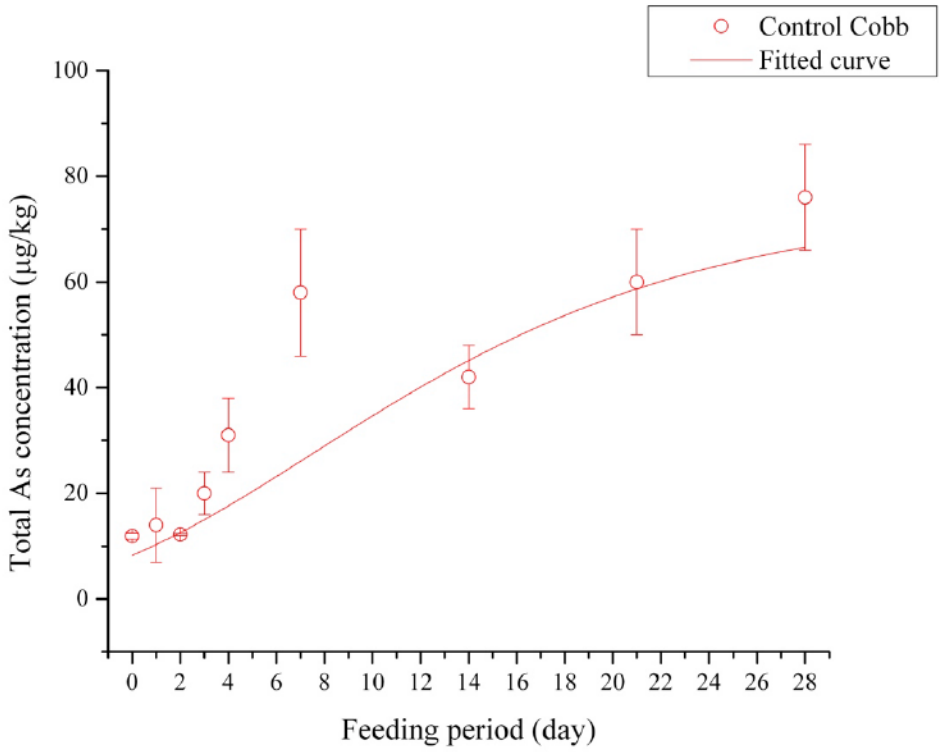

Figure 3.6 Total arsenic concentrations in skin samples of chickens from Control Cobb group during day 0 to day 28. The fitted curve function is $y=74.7\times e^{-e^{-0.105\times(x-7.49)}}$ ($0<x<28$), $R^2=0.52950$. Y is the total As concentration, while x is the feeding day. The As uptake rate is $y'=74.7\times0.105\times e^{-e^{-0.105\times(x-7.49)}-0.105\times(x-7.49)}$. Y' is the As uptake rate, while x is the feeding day.

Feeding period (day)	0	1	2	3	4	7	14	21	28
As uptake rate (µg/(kg·day))	1.9	2.1	2.4	2.5	2.7	2.9	2.4	1.5	0.8

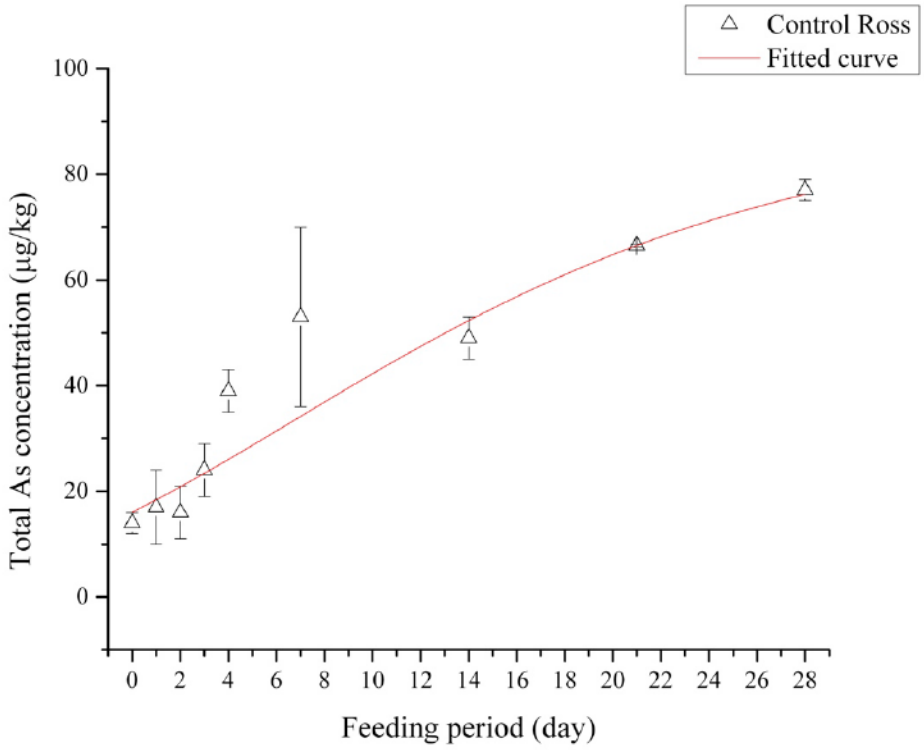

Figure 3.7 Total arsenic concentrations in skin samples of chickens from Control Ross group during day 0 to day 28. The fitted curve function was $y=90.8\times e^{-e^{-0.082\times(x-6.72)}}$ ($0<x<28$), $R^2=0.98008$. Y is the total As concentration, while x is the feeding day. The As uptake rate is $y'=90.8\times0.082\times e^{-e^{-0.082\times(x-6.72)}-0.082\times(x-6.72)}$. Y' is the As uptake rate, while x is the feeding day.

Feeding period (day)	0	1	2	3	4	7	14	21	28
As uptake rate (µg/(kg·day))	2.3	2.4	2.5	2.6	2.7	2.7	2.4	1.7	1.1

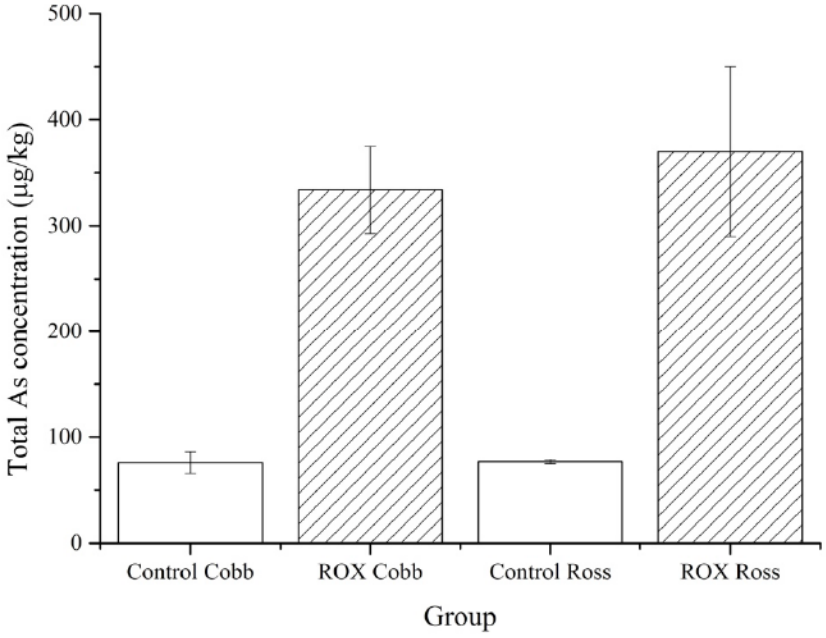

Figure 3.8 Total arsenic concentrations in skin samples from ROX-treatment groups and control groups (n=4 for each group) at day 28.

3.3.4 Arsenic Concentration in Chicken Skin Following Withdraw of ROX from The Feed

In the last week of the feeding experiment, the ROX component was withdrawn from the ROX group diet. Both ROX group and control group were fed with ROX-free diet allowing the chickens to eliminate the ROX from their bodies. The total arsenic concentrations in skin samples of chickens from all of the four groups were monitored, and they were illustrated in Figure 3.9. Significant differences ($p<0.05$) were observed between ROX-fed group and control group for both strains during this ROX withdrawal period (from day 28 to day 35). For both control groups, the total arsenic concentrations fluctuated slightly during day 28 to day 35. However, the total arsenic concentration trends of ROX-fed groups were quite different from those of control groups. The total arsenic concentrations for both ROX-treated groups decayed exponentially during this period.

In order to explore arsenic elimination kinetics, I calculated the logarithm of the total arsenic concentrations of ROX Cobb chickens. The fitted curve were shown in Figure 3.10 (a) and (b). Here I fitted the logarithm As concentration with both linear function and piecewise linear function. These two functions corresponded to the single-phase elimination model and biphasic elimination model. When using the piecewise linear function for fitting, I obtained lower residual sum. Biphasic elimination model is suitable for the arsenic elimination study. According to the slopes (k), the half-lives ($t_{1/2}$) of As in skin could be calculated ($t_{1/2}=\ln 2/k$). The half-life of arsenic in skin was 4.3 day (day 28-30)

and 14.1 day (day 30-35). The logarithm of the total arsenic concentrations of ROX Ross chickens and the fitted curves were shown in Figure 3.11 (a) and (b). The half-life of arsenic in skin for ROX Ross group was 4.3 day (day 28-30) and 10.3 day (day 30-35).

The total arsenic concentrations in skin samples of chickens from both ROX-treatment groups and control groups at day 35 were compared in Figure 3.12. Significant differences in total arsenic concentrations ($p<0.05$, $p=0.007$ for Cobb strain, $p=0.005$ for Ross strain) were observed between ROX-fed group and control group for both strains. The chickens from ROX-fed groups retained higher concentrations of arsenic in their skins than the chickens from control groups.

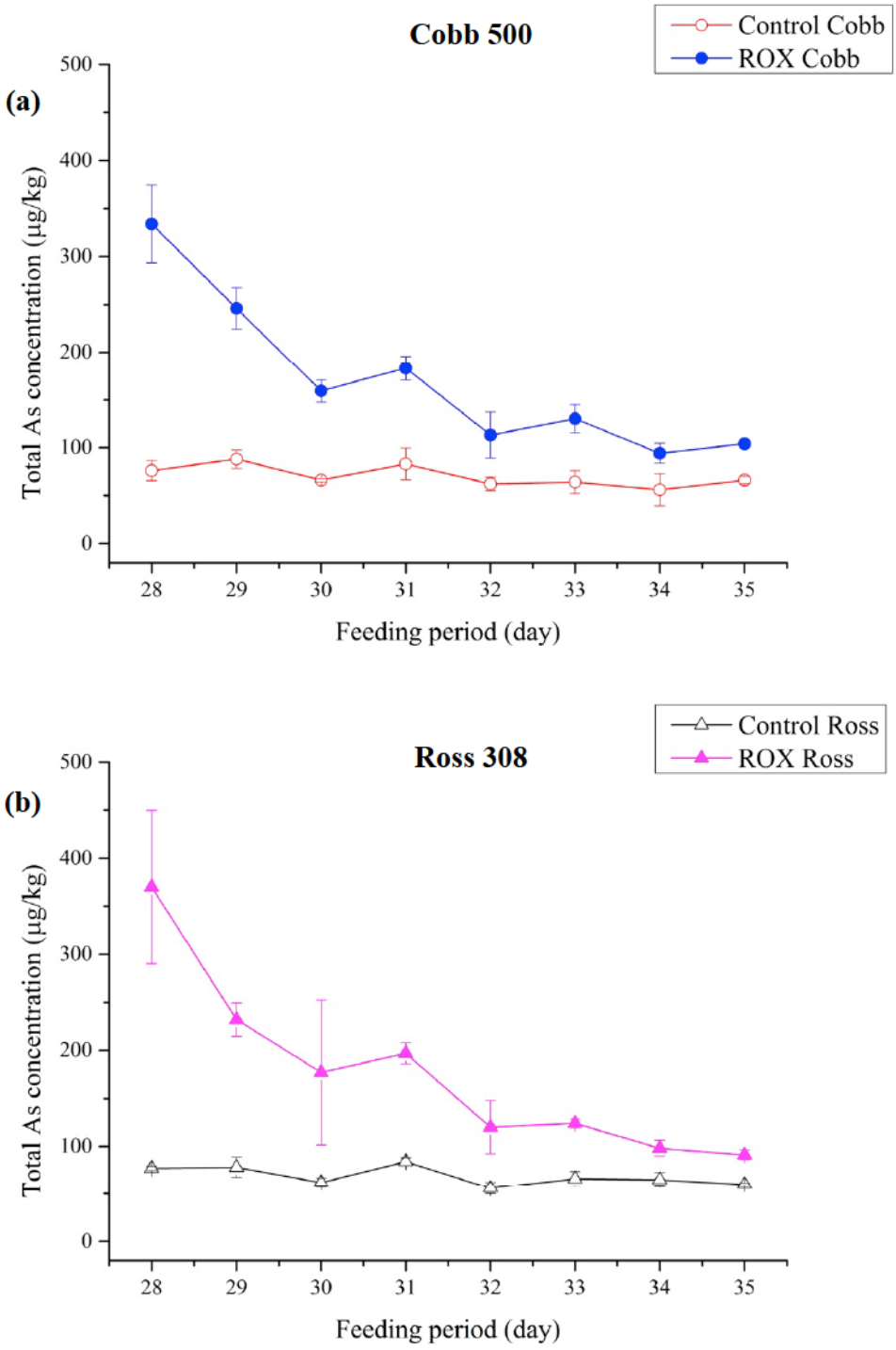

Figure 3.9 Total arsenic concentrations in the skin samples of chickens from strain (a) Cobb 500 and (b) Ross 308 during As elimination period (day 28-35).

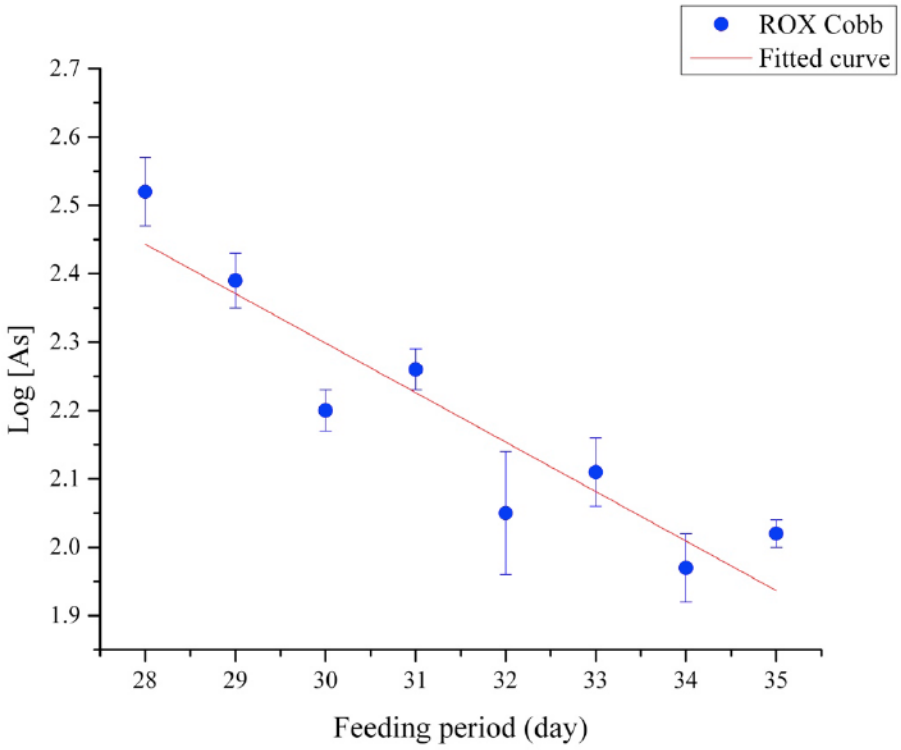

Figure 3.10 (i) The logarithm of total arsenic concentrations in skin samples of chickens from ROX Cobb group during day 28 to day 35 with linear fit. The fitted curve function was $y=4.47-0.072x$ ($28<x<35$), $R^2=0.83143$. Y is the total As concentration, while x is the feeding day. The residual sum of squares is 0.0371. This is used for single-phase elimination kinetic.

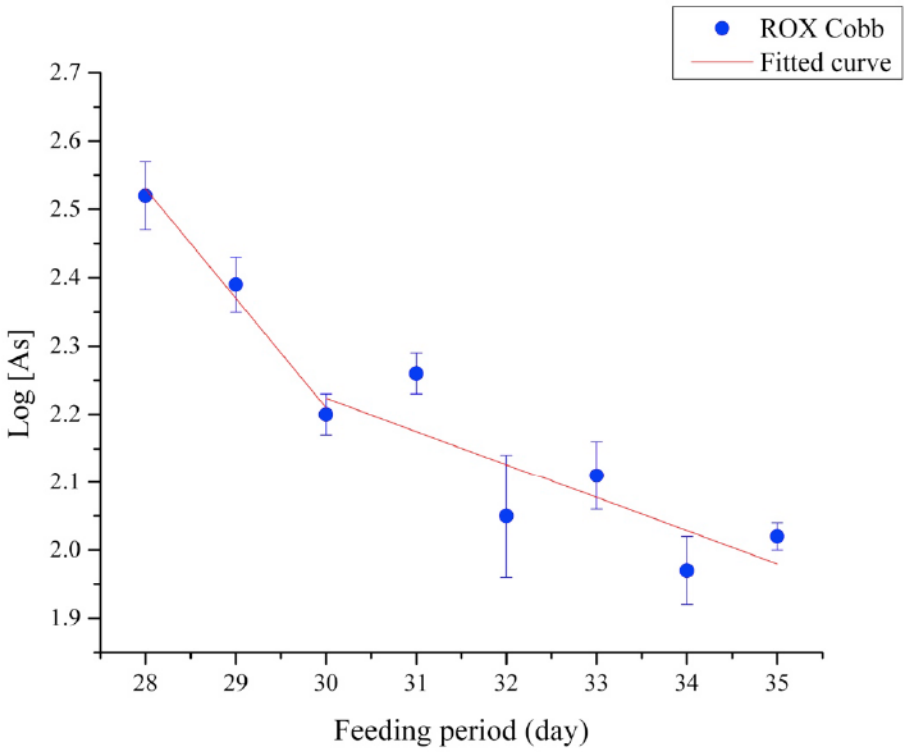

Figure 3.10 (ii) The logarithm of total arsenic concentrations in skin samples of chickens from ROX Cobb group during day 28 to day 35 with piecewise linear fit. The fitted curve function was $y=7.01-0.16x$ ($28<x<30$), $R^2=0.97683$; $y=3.69-0.049x$ ($30<x<35$), $R^2=0.59927$. Y is the total As concentration, while x is the feeding day. The residual sum of squares is 0.0006+0.0197=0.0203. This is used for biphasic elimination kinetic.

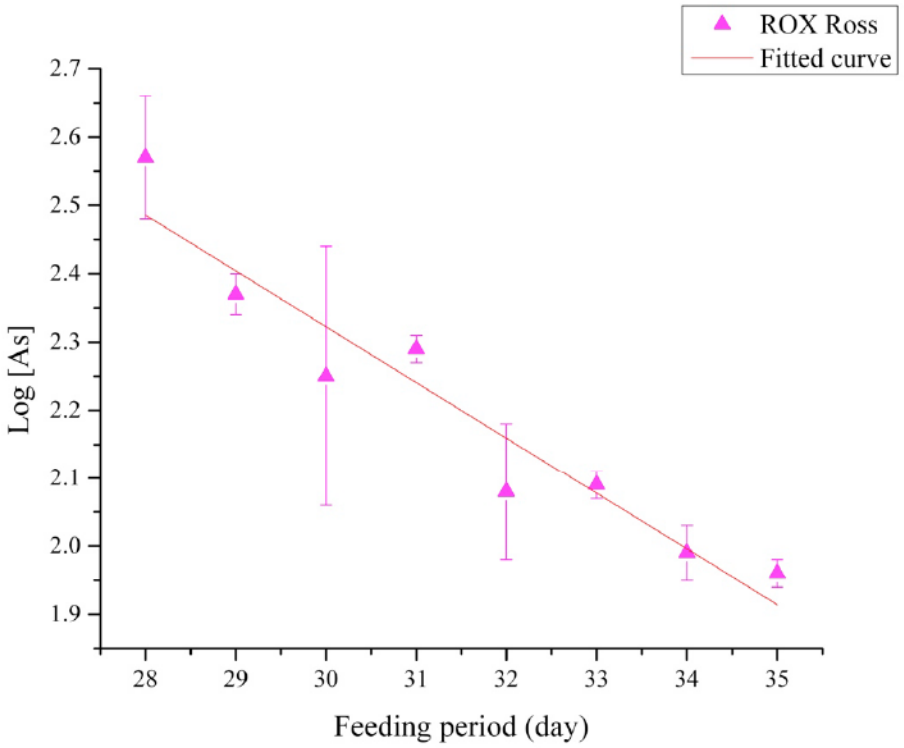

Figure 3.11 (i) The logarithm of total arsenic concentrations in skin samples of chickens from ROX Ross group during day 28 to day 35 with linear fit. The fitted curve function was $y=4.77-0.082x$ ($28<x<35$), $R^2=0.90622$. Y is the total As concentration, while x is the feeding day. The residual sum of squares is 0.0245. This is used for single-phase elimination kinetic.

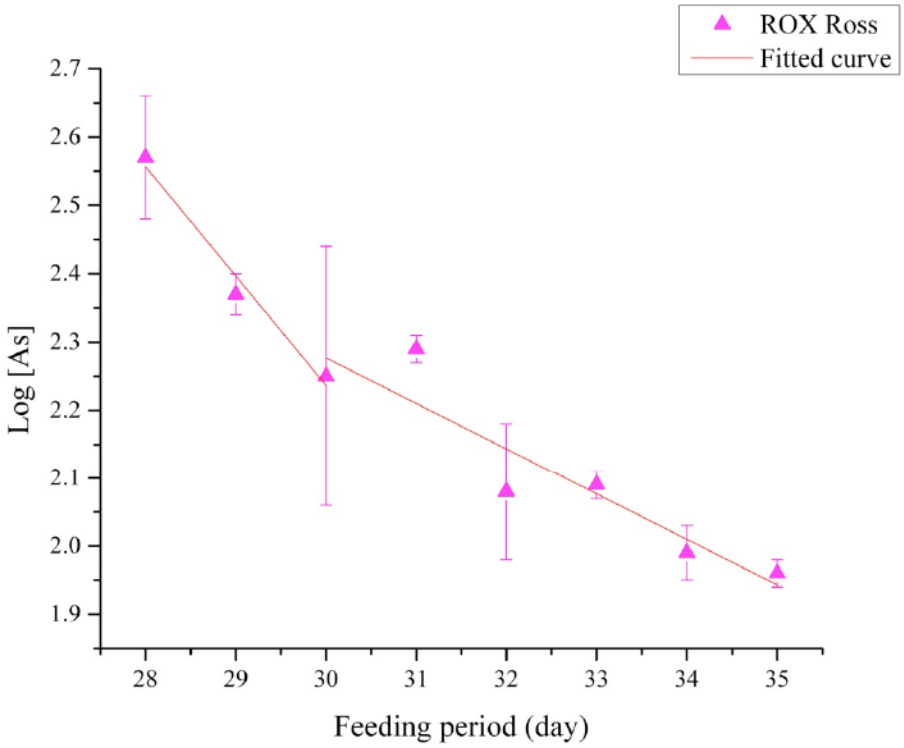

Figure 3.11 (ii) The logarithm of total arsenic concentrations in skin samples of chickens from ROX Ross group during day 28 to day 35 with piecewise linear fit. The fitted curve function was $y=7.03-0.16x$ ($28<x<30$), $R^2=0.95918$; $y=4.28-0.067x$ ($30<x<35$), $R^2=0.83402$. Y is the total As concentration, while x is the feeding day. The residual sum of squares is 0.0011+0.0120=0.0131. This is used for biphasic elimination kinetic.

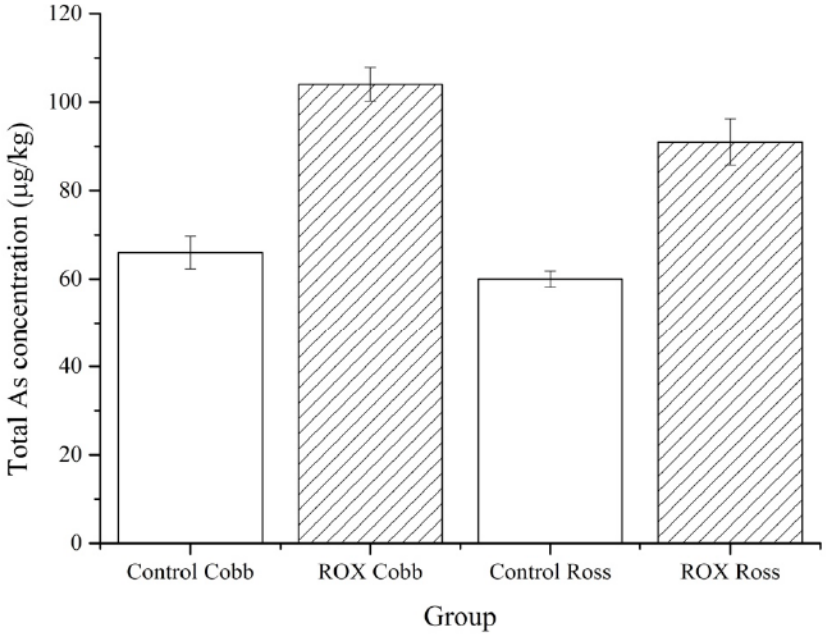

Figure 3.12 Total arsenic concentrations in skin samples from ROX-treatment groups and control groups (n=4 for each group) at day 35.

3.4 DISCUSSION

3.4.1 Total Arsenic Concentration Trend

When chickens of the same age and the same strain were compared, ROX-fed chickens had more arsenic residue in their skin than control chickens (Table 3.3 and Figure 3.1). For both ROX-fed groups, similar total arsenic concentration trends ($p>0.05$) in chicken skin were observed (Figure 3.1). For both control groups, similar trends ($p>0.05$) were found as well (Figure 3.1). This suggests that both strains of chickens behave similarly with respect to Roxarsone treatment. Chicken strain did not influence the uptake and elimination of arsenic in chicken skin.

3.4.2 Arsenic Uptake Kinetics in Chicken Skin

The difference in diets for the chickens from ROX-fed group and control group led to the difference of the total arsenic concentrations in chicken skin from day 0 to day 28. As illustrated in Figure 3.3 (a) and (b), chickens from ROX-fed groups accumulated more arsenic in their skin than chickens from control groups because of the ROX component in the feed. The difference in feed also led to the difference in arsenic uptake rate. Generally, the arsenic uptake rates in skin for ROX-treated chickens were higher than that for control chickens.

After four weeks' feeding period, great difference was shown between control group and ROX-treated group for both strains. At day 28, the total arsenic concentrations in skin for chickens in control groups were only 76 ± 10 μg/kg for Cobb strain and 77 ± 2 μg/kg for Ross strain (Table 3.3). However, the total arsenic concentrations in skin for chickens in ROX-treated groups were 334 ± 41

μg/kg for Cobb strain and 370 ± 80 μg/kg for Ross strain (Table 3.3), which were 4 times higher.

Although the arsenic concentrations in the skin of the ROX-treated chickens were elevated, the average concentrations were still below the tolerance levels for human consumption (500 μg/kg for uncooked tissues of chickens) set by the United States Food and Drug Administration (US FDA) [5]. The concentrations of total arsenic in the skin of ROX-treated chickens on day 28 ranged from 296 μg/kg to 372 μg/kg for Cobb strain and 316 μg/kg to 672 μg/kg for Ross strain. One of these eight chickens at the peak of the arsenic concentration in the skin exceeding the FDA tolerance level.

3.4.3 Arsenic Elimination Kinetics in Chicken Skin

During the last week, the chickens were all fed with ROX-free basal diet. Because there was little change in the feed for control groups, the total arsenic concentrations in skin for control groups did not change very much.

The arsenic concentrations in skin of ROX-fed chickens decreased dramatically as ROX was withdrawn from the chicken feed. Both ROX-fed groups showed biphasic elimination. At the first stage (day 28 to day 30), the arsenic concentrations decreased rapidly and the half-life of As is 4.3 day. After that, the arsenic concentration elimination slowed down and the half-life of As is about 14.1 day. The biphasic elimination would be due to the influence of arsenic in the diet and environment. Arsenic exists in the ROX finisher diet (Table 3.1)

and water. During the last week, arsenic from these sources could affect the elimination of As in chicken skin.

At day 35, the end of feeding period, 69% (for Cobb strain) and 75% (for Ross strain) arsenic were eliminated from chicken skin. As a result, 104 ± 4 μg/kg and 91 ± 5 μg/kg arsenic were left in the skin of chickens from ROX Cobb group and ROX Ross group (Table 3.3). But compared to the total arsenic concentrations in control groups (66 ± 4 μg/kg for Control Cobb group and 60 ± 2 μg/kg for Control Ross group), these concentrations were still higher. Even with one-week elimination period, there was still significant difference ($p<0.05$) in total arsenic concentrations between ROX-treatment groups and control groups.

3.4.4 Perspectives on Estimated Daily Intake of Arsenic from Chicken Skin

According to the arsenic concentrations in ROX-fed chicken skin on the last feeding day, we can estimate the daily intake of arsenic through eating chicken skin. The average daily consumption of chicken is 0.10 kg based on the data from the National Chicken Council [6]. The mean percentage of skin in the whole chicken is 15% [7]. The estimated daily intake of arsenic from chicken skin would be 0.10 kg × 15% × 104 μg/kg = 1.6 μg per day. For a 70-kg person, this would be 1.6 μg/70 kg = 0.023 μg/kg body weight per day. The current tolerable arsenic daily intake level set by World Health Organization (WHO) is 3.0 μg/kg body weight per day [8]. The daily intake of arsenic from chicken skin is lower than the WHO value. There are other sources of arsenic intake, for example from water. The maximum contaminant level for arsenic in drinking water is 10 μg/L. For a daily consumption of 2 L water, the estimated maximum daily intake of arsenic

from 2 L drinking water would be 2 L × 10 μg/L =20 μg. The daily intake of arsenic from chicken skin is 1.6 μg per day, which is lower than from water.

3.5 CONCLUSIONS

Arsenic uptake and elimination kinetics in chicken skin were studied by applying our acid digestion and ICPMS detection method. During the whole feeding experiment, significant differences of the arsenic concentrations in skin were found between ROX-treated groups and control groups. Because of the addition of ROX in the feed, the chickens in ROX-treatment group showed large arsenic uptake and elimination rate. The arsenic concentrations detected in skin from the control chickens and from the ROX-fed chickens, after a 7-day withdrawal period, were within US FDA regulatory limits. The daily intake of arsenic through eating chicken skin was estimated, which is lower than the tolerable arsenic daily intake level set by WHO.

3.6 REFERENCES

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CHAPTER 4

DETERMINATION OF ARSENIC SPECIES IN CHICKEN SKIN BY USING AN HPLC-ICPMS/ESIMS METHOD[1]

4.1 INTRODUCTION

Arsenic has many forms present in the natural environment and biological systems. The toxicity of arsenic is highly species-dependent, ranging from highly toxic to nontoxic. For example, arsenic trioxide, is one of the most efficient poisons with an LD_{50} of 34.5 mg/kg in mice [1]. However, arsenobetaine (AsB), which is usually found in seafood [2, 3], has an LD_{50} value higher than 10,000 mg/kg in mice [1] and can be consider as nontoxic compound. In skin samples, many arsenic species can exist. Speciation analysis is necessary in our case for the study of ROX metabolism and concentration in chicken.

Several speciation studies were conducted in the liver and muscle of ROX-fed chickens for investigation of the metabolism of ROX [4-8]. However, it is little known about the arsenic species in chicken skin, and there is also a lack of a method for speciation of arsenic for chicken skin. Inductively coupled plasma mass spectrometry (ICPMS) is good for element analysis with high sensitivity.

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[1] Note: 1. Figure 4.25 was constructed in collaboration with Ms. Qingqing Liu and Mr. Hanyong Peng;

2. Table 4.12 and 4.13 were constructed was constructed in collaboration with Ms. Qingqing Liu, Mr. Hanyong Peng and Ms. Xuan Sun;

3. Figure 4.26 and 4.27 were constructed in collaboration with Mr. Zonglin Yang.

When combine with separation technique, such as high performance liquid chromatography (HPLC), it can be used for speciation study and it is compatible with various environmental samples. But ICPMS is only element-specific, and problems about identifications could happen when species cannot be distinguished by chromatographic retention time. Electrospray ionization mass spectrometry (ESIMS) can provide structural information for determination of arsenic compounds [9, 10]. By combining HPLC with ICPMS and ESIMS, we can achieve simultaneously quantitation and identification of arsenic species in samples. Here by using the HPLC-ICPMS/ESIMS system, we conducted the speciation of arsenic in the skin of selected ROX-treatment chickens and control chickens.

4.2 MATERIALS AND METHODS

4.2.1 Reagents and Standards

100 mg As/L arsenic standard stock solutions were prepared. Among these standards, AsB, As^{III}, As^{V}, DMA and MMA stock solutions were obtained by dissolving arsenobetaine (98%, Tri Chemical Laboratories Inc., Japan), sodium m-arsenite (97.0%, Sigma, St. Louis, MO), sodium arsenate (99.4%, Sigma), cacodylic acid (98%, Sigma) and monosodium acid methane arsonate (99.0%, Chem Service, West Chester, PA) in 18.2 MΩ·cm deionized water. ROX stock solutions were prepared by dissolving 3-nitro-4-hydroxyphenylarsonic acid (98.1% purity, Sigma-Aldrich, St. Louis, MO) in HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ). 3-amino-4-hydroxyphenylarsonic acid (3-AHPAA) and N-acetyl-4-hydroxy-m-arsanilic acid (N-AHAA) were purchased from Pfaltz

and Bauer, Inc. (Waterbury, CT). Methylated ROX, methylated 3-AHPAA and methylated N-AHAA were synthesized from ROX, 3-AHPAA and N-AHAA through a methylation reaction in our group. These standard stock solutions were prepared by dissolving their purified solids in deionized water with 50% HPLC grade methanol. All the stock solutions were kept at 4 °C prior to the time of use. Arsenic standard solutions used for speciation analysis were prepared daily from stock solutions using deionized water as diluent.

Pancreatin, trypsin, pepsin and papain were purchased from Sigma-Aldrich, and were used to release protein-bound arsenic compounds in chicken skin. Chicken skin samples from day 28 and day 35 were used for the arsenic speciation study.

4.2.2 Sample Processing

Skin samples were first thawed at room temperature. After thawing, feathers and visible fat were removed, and the skin sample was then cut into small pieces using dissecting scissors. 0.5 g of the chicken skin pieces was accurately weighed into a 15 mL polypropylene centrifuge tube (Fisher Scientific) and 2 mL deionized water was added to the tube. The sample solution was then vortex-mixed thoroughly and further homogenized by Powergen 125 homogenizer (Fisher Scientific) for 3 min. Then, 0.5 mL of 0.1 g/mL enzyme solution and 0.5 mL buffer solution were added to the sample solution. Conditions were adjusted for the enzyme digestion reaction (Table 4.1) and the reaction was carried out overnight. The following day, the sample solution was sonicated for 5 min followed by centrifugation at 4000 rpm for 30 min. The supernatant was collected

by a 3-mL syringe (BD Biosciences, Franklin Lakes, NJ) and filtered by an Amicon Ultra-0.5 centrifugal filter (10,000 MWCO, EMD Millipore Corporation, Billerica, MA). Finally, 100 µL of the filtered solution was transferred to a glass autosampler vial prior to arsenic speciation analysis. Sample blanks containing no chicken sample were also prepared in a similar fashion for comparison.

Table 4.1 Reaction conditions for enzyme extraction

Enzyme	Buffer Solution	Optimized pH	Optimized Temperature	Instrument for Reaction
Pancreatin	60 mM NH_4HCO_3	7.8	37 ℃	Incubator
Trypsin	60 mM NH_4HCO_3	7.8	37 ℃	Incubator
Papain	Water	6-7	60 ℃	Water bath
Pepsin	3% HCl	2-4	37 ℃	Incubator

4.2.3 Arsenic Speciation Analysis

Agilent 1100 series HPLC system (Agilent Technologies, Germany) was used for the separation of arsenic species in sample solutions. The separation was performed on a PRP-X110S anion exchange column (7 µm particle size, 150 × 4.1 mm; Hamilton, Reno, NV) with an appropriate guard column (PRP-X110S; Hamilton). Two mobile phases were prepared as follows: (A) 5% methanol; (B) 60 mM ammonium bicarbonate (NH_4HCO_3) (Sigma-Aldrich) with the pH adjusted to 8.75 with 10% ammonium hydroxide. NH_4HCO_3 is an ideal buffer for use with both ICPMS and ESIMS. Having methanol in the eluent helps improve ionization efficiency in the plasma, it enhances the signal intensity of arsenic and maintains consistent detection limits [11]. Both mobile phases were filtered through a 0.45 µm membrane and sonicated for 15 min prior to use. The gradient elution program used for the separation is shown in Table 4.2. The injection volume was 50 µL for all samples and standards.

After sample injection and HPLC separation, the eluent was split at a ratio of 4 to 1 by a 300 series stainless steel tee (Valco Canada, Brockville, ON) between an Agilent 7500cs Octopole ICPMS (Japan) and an AB SCIEX 5500 QTRAP ESIMS (Concord, ON, Canada). In HPLC-ICPMS, arsenic species were monitored at m/z=75.0 (As^+) in helium reaction mode. Quantitation of individual arsenic species was achieved with external calibrations of the corresponding arsenic standard solution. Identification of suspected arsenic species present in the chicken skin was achieved by a combination approach of retention time matching and sample spiking with arsenic standards. In HPLC-ESIMS, multiple reaction

monitoring (MRM) mode was used. Arsenic species were detected in positive ionization mode for the first 1.1 min (for AsB) and negative ionization mode for the remaining 18.9 min (for other arsenic species). Precursor and product ion matches between species in skin samples and corresponding standards were performed here to further confirm the identities of individual arsenicals. By using this HPLC-ICPMS/ESIMS technique, we were able to obtain comprehensive quantitative results from ICPMS and confirmative identification information from ESIMS simultaneously.

During each analytical session, mixtures of eight arsenic standards (including AsB, As^{III}, DMA, MMA, As^{V}, 3-AHPAA, N-AHAA and ROX) were prepared with concentrations ranging from 0.05 to 20 μg As/L to span the expected range of arsenic concentrations in chicken skin. Arsenic standards in enzyme solutions were also prepared in order to correct possible enzyme matrix effects. The calibration standards were analyzed prior to chicken samples. Accuracy of the calibrations was checked by analyzing certified reference material No. 18 (human urine, National Institute for Environmental Studies, Ibaraki, Japan). Limits of detection (LOD) were estimated based on 3σ of the baseline noise in the chromatograms. During sample analysis, instrument drift was checked by analyzing a standard mixture of 5 μg As/L once every ten samples.

Table 4.2 Gradient elution conditions for HPLC separation. The program started with a linear gradient from 100% mobile phase A to 40% mobile phase B during the first 2 min. The composition stayed constant at 40% B from 2 min to 5 min. From 5 min to 8 min, the mobile phase changed from 40% B to 100% B in a linear gradient, and stayed at 100% B from 8 min to 15 min. From 15 min to 16 min, the mobile phase returned from 100% B to 100% A in a linear gradient, and maintained at 100% A for another 4 minute (16-20 min). The flow rate was kept at 2.0 mL/min for the entire 20 min.

Time	A	B
0.00	100%	0
2.00	60%	40%
5.00	60%	40%
8.00	0	100%
15.00	0	100%
16.00	100%	0
20.00	100%	0

4.3 RESULTS

4.3.1 Comparison of Enzymatic Extraction

Figure 4.1 illustrates the arsenic species present in the enzyme solutions used for extraction. This gives us information about the background arsenic speciation generated from the enzymes. In these enzymes, inorganic arsenicals (As^{III} and As^{V}) and methylated arsenicals (DMA and MMA) had been detected, among these species, As^{V} was most prevalent. When comparing the concentration of arsenic species in the enzymes, we found that pancreatin and trypsin have more arsenic than papain and pepsin.

In order to explore the extraction effectiveness of each enzyme, a spike experiment was conducted. Figure 4.2 shows the arsenic species present in the enzyme extracts of the mixture of control chicken skin samples and 10 ppb arsenic standard. The recoveries of the spike experiment using each of the four enzymes were calculated and summarized in Table 4.3. All of these enzymes showed good recoveries when used for extracting AsB, inorganic arsenicals and methylated arsenicals. However, when extracting phenylarsonic compounds (ROX, 3-AHPAA, N-AHAA), different extraction behaviors were observed in the four enzymes. Pancreatin and trypsin were not good to extract ROX, and pepsin showed less than 10% recovery for 3-AHPAA. Only papain had good recoveries for all of the phenylarsonic compounds.

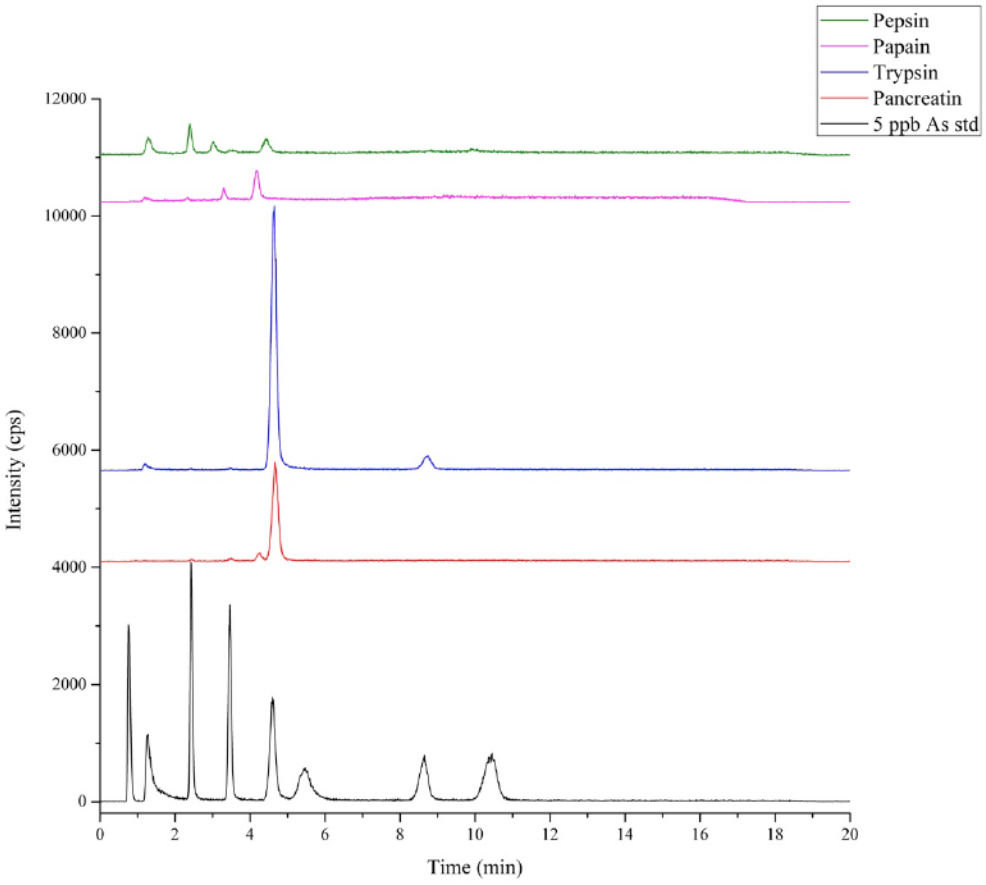


Figure 4.1 Chromatograms of arsenic species present in the enzyme solution. Peaks in As standard from left to right are AsB, As^{III}, DMA, MMA, As^{V}, 3-AHPAA, N-AHAA, and ROX.

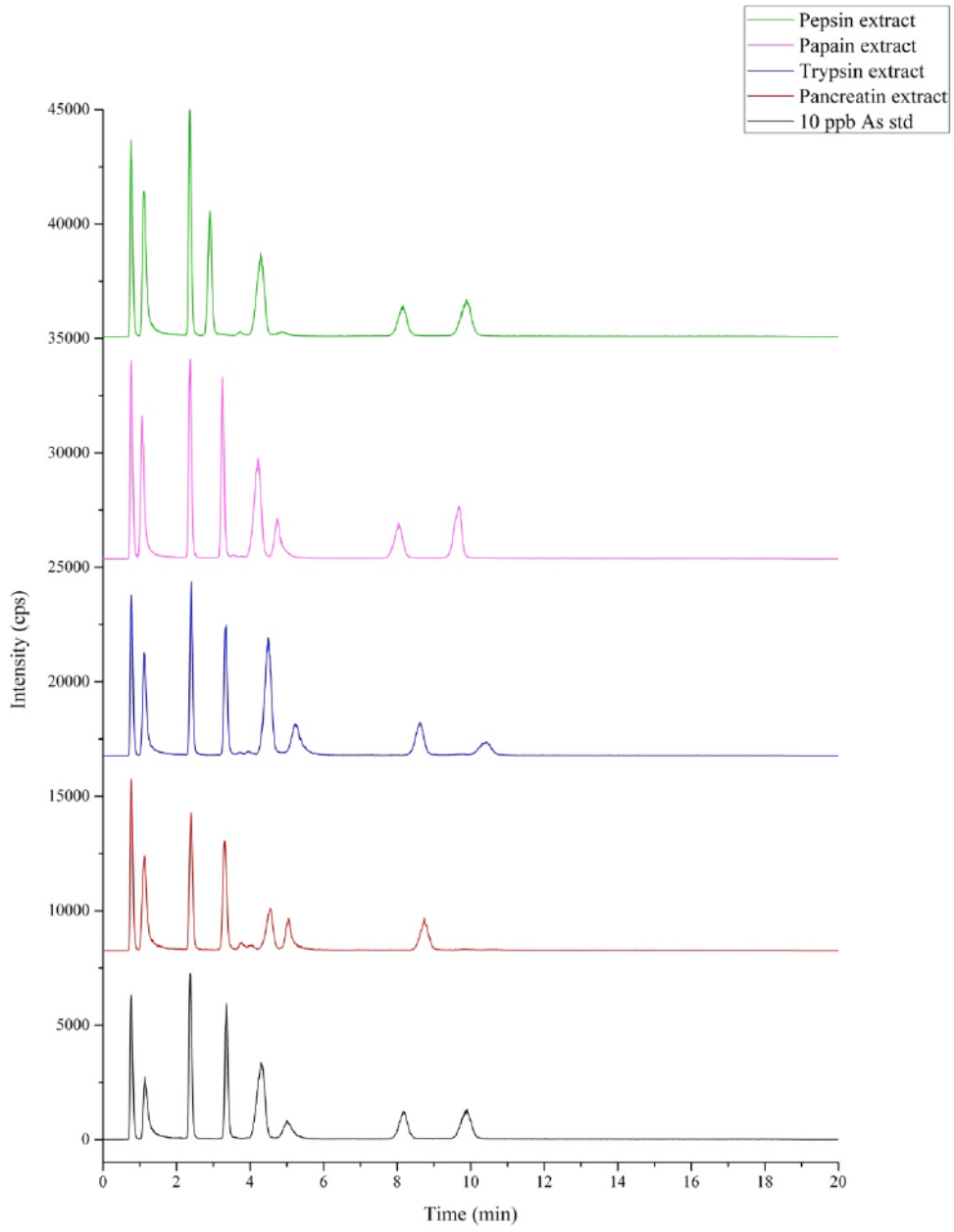


Figure 4.2 Chromatograms of arsenic species present in enzyme extracts from the spike experiment. Peaks in As standard from left to right are AsB, As^{III}, DMA, MMA, As^{V}, 3-AHPAA, N-AHAA, and ROX.

Table 4.3 Recovery of spiked arsenic species by using enzyme extraction.[a]

Recovery of Arsenic Species (mean ± SD) (%)								
Enzyme	AsB	As^{III}	DMA	MMA	As^{V}	3-AHPAA	N-AHAA	ROX
Pancreatin	95 ± 4	128 ± 23	76 ± 4	72 ± 3	27 ± 40	51 ± 4	71 ± 1	12 ± 20
Trypsin	88 ± 5	104 ± 10	74 ± 4	66 ± 6	19 ± 19	82 ± 21	61 ± 4	16 ± 14
Papain	113 ± 20	130 ± 22	98 ± 15	87 ± 13	75 ± 16	72 ± 10	78 ± 12	84 ± 13
Pepsin	135 ± 4	142 ± 6	122 ± 4	100 ± 3	113 ± 5	3 ± 6	96 ± 2	86 ± 1

[a] SD: standard deviation.

4.3.2 Arsenic Species in Chicken Skin

Figure 4.3 shows the chromatograms from the HPLC-ICPMS/ESIMS analyses of arsenic species in the mixed arsenic standard solution. All eight arsenic compounds (AsB, As^{III}, DMA, MMA, As^{V}, 3-AHPAA, N-AHAA, and ROX) were well separated by current gradient elution program. They were also well detected by ICPMS [Figure 4.3 (a)] and ESIMS [Figure 4.3 (b)] with consistent retention times.

A typical chromatogram of the arsenic speciation analysis of skin sample was demonstrated in Figure 4.4. Eight major arsenic-containing compounds were detected in the skin sample. These species were further confirmed by individual arsenic standard spiking experiment using HPLC-ICPMS [Figure 4.5 (i)-(viii)] as well as the MRM detection using HPLC-ESIMS [Figure 4.6 (i)-(viii)].

Apart from the eight major arsenic compounds, there were five more unknown species present in the skin sample (Figure 4.4). Unknown peak 3, 4 and 5 had retention times similar to that of phenylarsonic compounds. Plausible arsenic compounds with related structures to 3-AHPAA, N-AHAA and ROX, were synthesized in our group. Through the spike experiment, we found that unknown compounds 3, 4 and 5 had retention times matched with the methylated products of 3-AHPAA, N-AHAA and ROX [Figure 4.5 (ix)-(xi)], respectively. Their identities were then further confirmed by MRM detection [Figure 4.6 (ix)-(xi)]. For HPLC-ESIMS analyses, the optimized operating conditions and MRM transition conditions of individual arsenicals are summarized in Table 4.4 and Table 4.5.

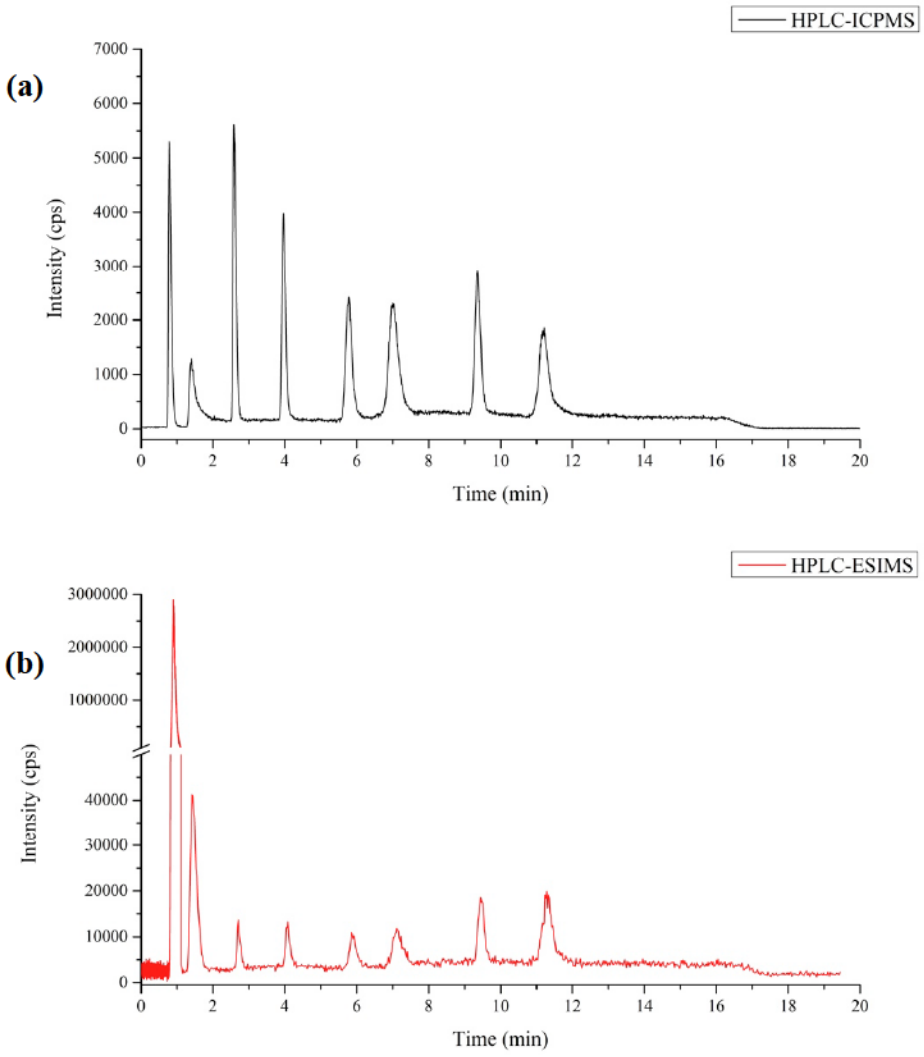


Figure 4.3 Chromatograms of 5 µg/L mixed arsenic standard obtained from (a) HPLC-ICPMS, (b) HPLC-ESIMS. Peaks from left to right are AsB, As^{III}, DMA, MMA, As^{V}, 3-AHPAA, N-AHAA, and ROX.

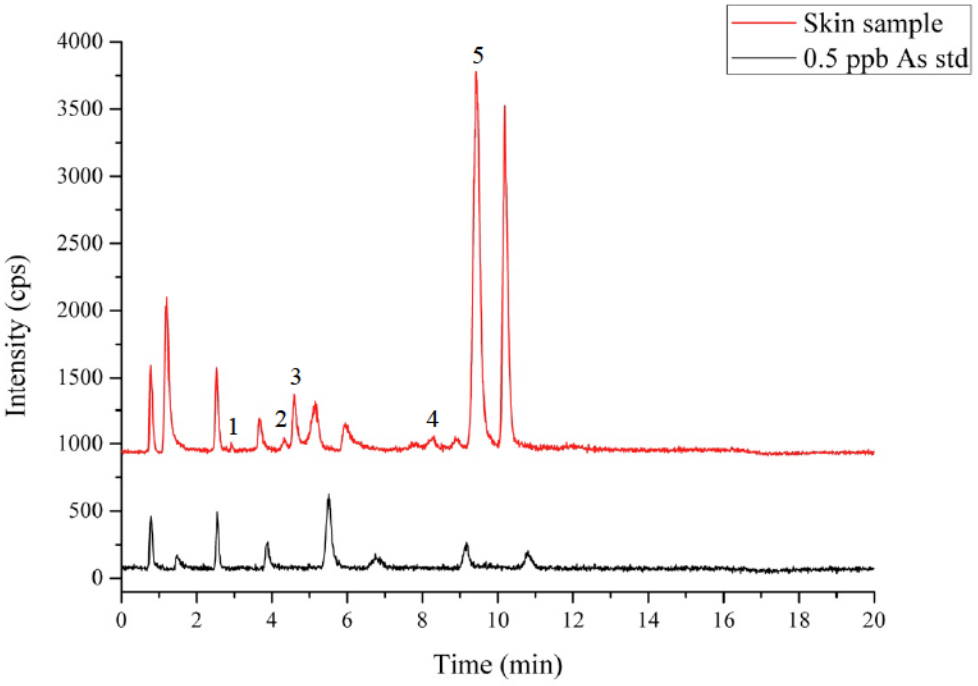
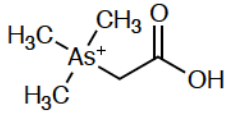
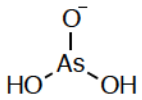
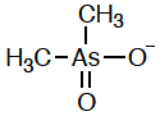


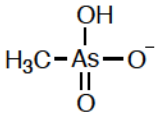
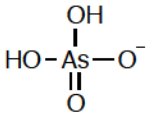
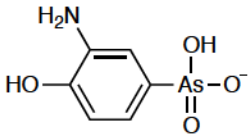
Figure 4.4 Chromatogram of skin sample of pen 15 Ross 308 ROX-treated chicken collected from day 28. Peaks in As standard from left to right are AsB, As^{III}, DMA, MMA, As^{V}, 3-AHPAA, N-AHAA, and ROX. Five unknown arsenic compounds were detected, which were labeled 1 to 5.

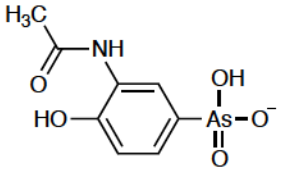
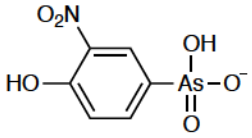
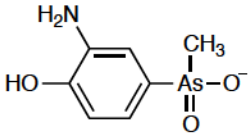
Table 4.4 Optimized operating conditions of the 5500 QTRAP ESIMS

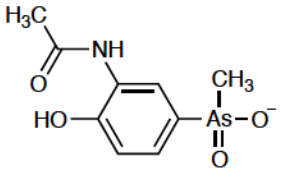
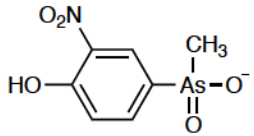
Parameter	Value in Positive Ionization Mode	Value in Negative Ionization Mode
Curtain Gas (CUR)	30 psi	30 psi
Collision Gas (CAD)	High	High
Ionspray Voltage (IS)	4500 V	-4500 V
Temperature (TEM)	600 °C	600 °C
Ion Source Gas 1 (GS1)	50 psi	50 psi
Ion Source Gas 2 (GS2)	50 psi	50 psi
Entrance Potential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

Table 4.5 MRM information and parameters for arsenic speciation using HPLC-ESIMS

Arsenic Species	Polarity	Molecular Ion Structure	Molecular Ion (*m/z*)	Characteristic Fragments	Fragment Structure	Compound Parameters		
						DP (V)	CE (V)	CXP (V)
AsB	Pos		179.0	105.0	$(CH_3)_2As^+$	71	37	9
				120.0	$(CH_3)_3As^+$	71	28	11
As^{III}	Neg		125.0	107.0	AsO_2^-	-10	-18	-15
DMA	Neg		137.0	107.0	AsO_2^-	-70	-30	-11
				122.0	$CH_3AsO_2^-$	-70	-18	-13

Arsenic Species	Polarity	Molecular Ion Structure	Molecular Ion (*m/z*)	Characteristic Fragments	Fragment Structure	Compound Parameters		
						DP (V)	CE (V)	CXP (V)
MMA	Neg		139.0	107.0	AsO_2^-	-40	-40	-43
				124.0	AsO_3H^-	-40	-24	-7
As^V	Neg		141.0	107.0	AsO_2^-	-15	-58	-13
				123.0	AsO_3^-	-15	-20	-7
3-AHPAA	Neg		232.0	107.0	AsO_2^-	-20	-64	-11
				123.0	AsO_3^-	-20	-28	-25

Arsenic Species	Polarity	Molecular Ion Structure	Molecular Ion (*m/z*)	Characteristic Fragments	Fragment Structure	Compound Parameters		
						DP (V)	CE (V)	CXP (V)
N-AHAA	Neg		274.0	123.0	AsO_3^-	-45	-26	-9
				165.0	$C_8H_8NO_3^-$	-45	-36	-11
ROX	Neg		262.0	107.0	AsO_2^-	-30	-94	-15
				123.0	AsO_3^-	-30	-38	-11
Methylated 3-AHPAA	Neg		230.0	107.0	AsO_2^-	-45	-70	-12
				123.0	AsO_3^-	-45	-30	-9

Arsenic Species	Polarity	Molecular Ion Structure	Molecular Ion (*m/z*)	Characteristic Fragments	Fragment Structure	Compound Parameters		
						DP (V)	CE (V)	CXP (V)
Methylated N-AHAA	Neg		272.0	108.0	$C_6H_6NO^-$	-65	-34	-1
				230.0	$C_7H_9AsNO_3^-$	-20	-24	-9
Methylated ROX	Neg		260.0	107.0	AsO_2^-	-45	-66	-15
				138.0	$C_6H_5NO_3^-$	-45	-26	-13

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

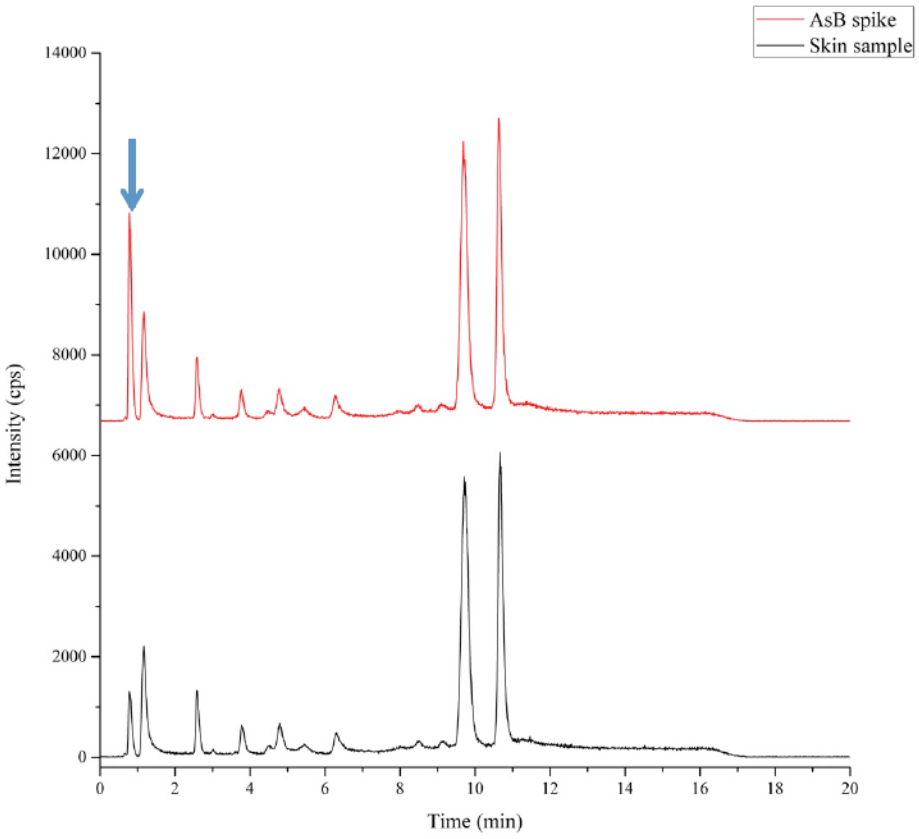


Figure 4.5 (i) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a skin sample with AsB standard spike for identity confirmation. Skin sample was collected on day 28 from ROX-fed chicken in pen 15, Ross 308. 2 µg/L AsB standard was spiked in skin sample.

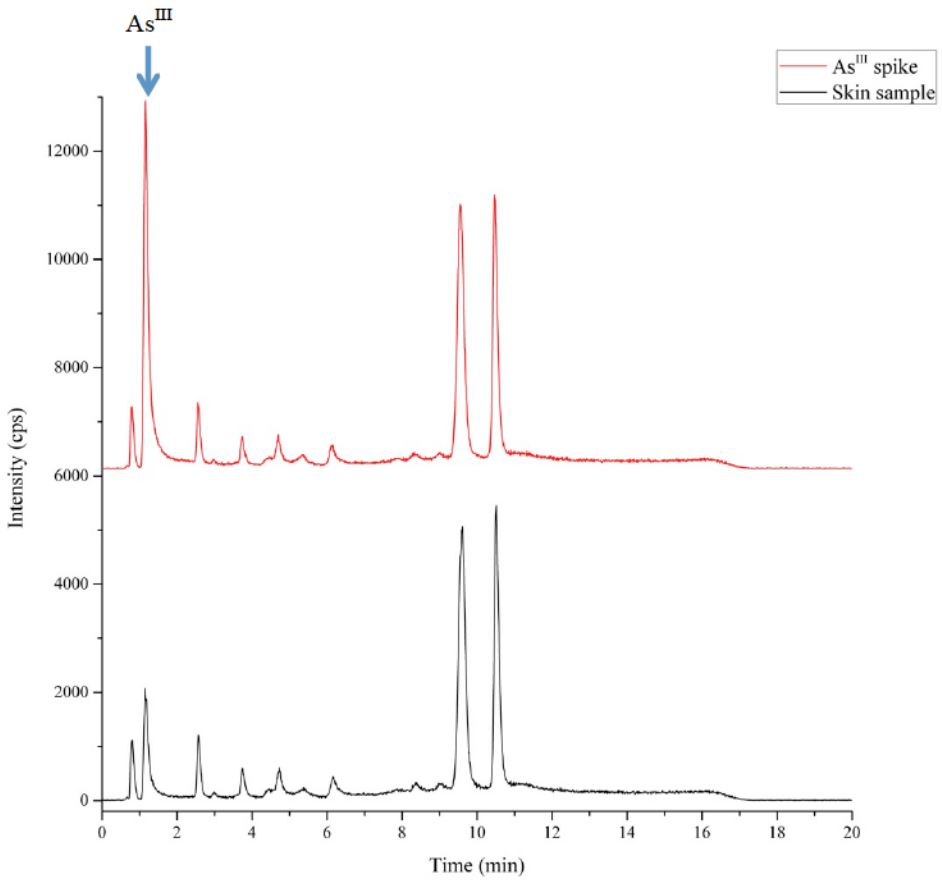


Figure 4.5 (ii) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a skin sample with As^{III} standard spike for identity confirmation. Skin sample was collected on day 28 from ROX-fed chicken in pen 15, Ross 308. 10 µg/L As^{III} standard was spiked in skin sample.

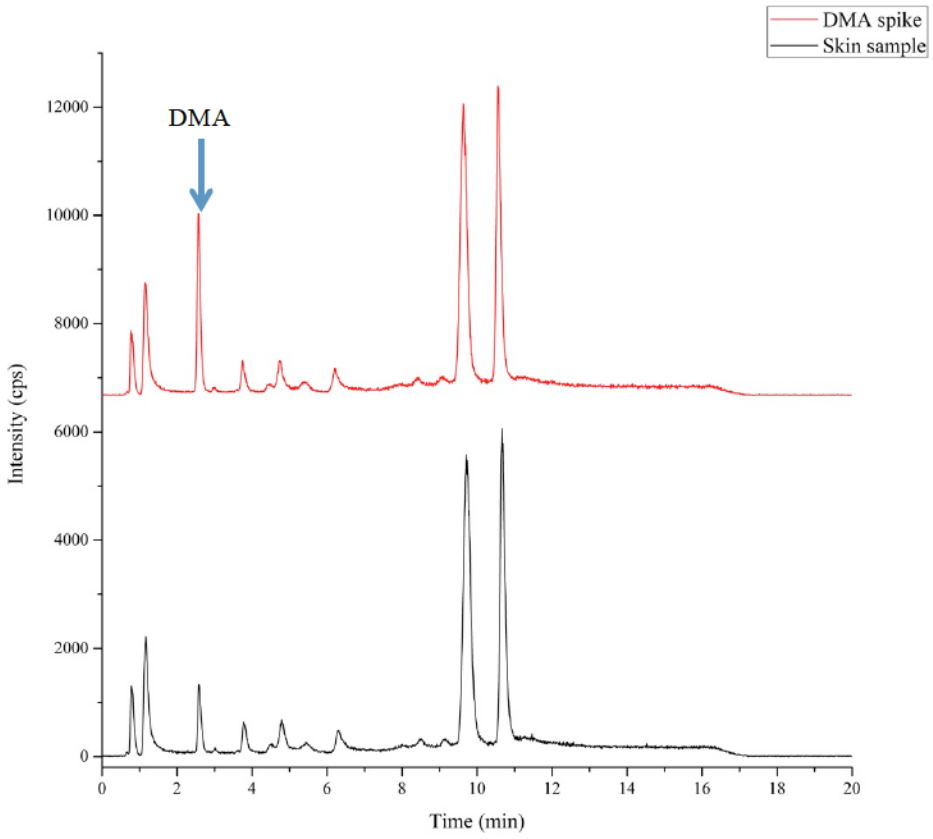


Figure 4.5 (iii) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a skin sample with DMA standard spike for identity confirmation. Skin sample was collected on day 28 from ROX-fed chicken in pen 15, Ross 308. 2 µg/L DMA standard was spiked in skin sample.

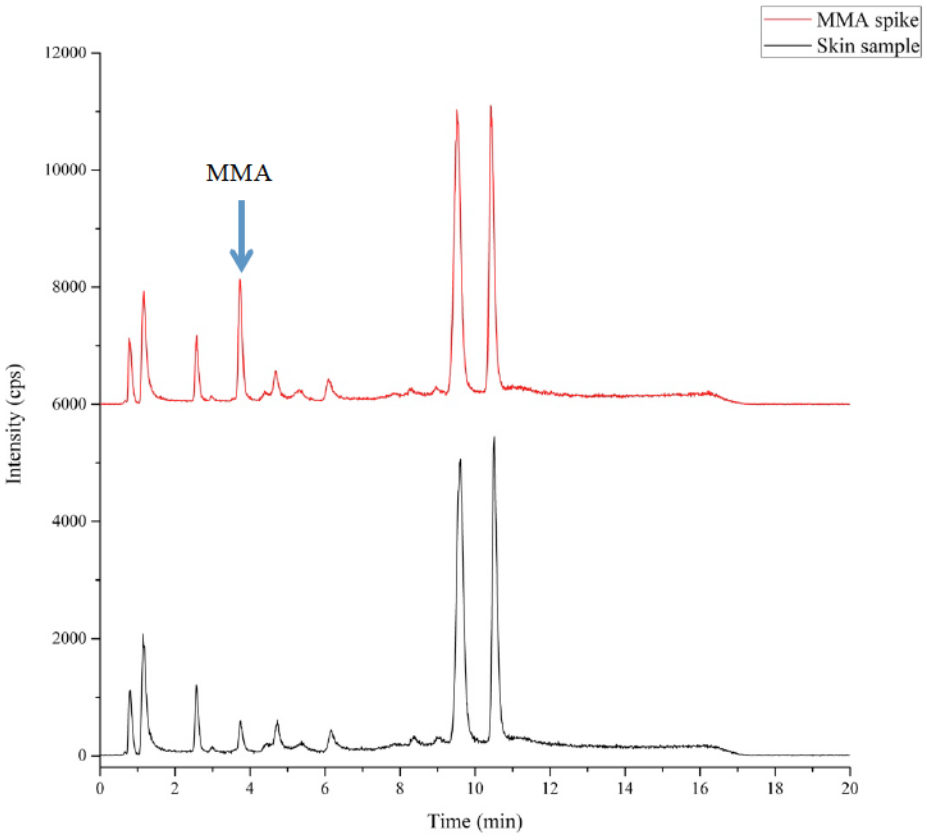


Figure 4.5 (iv) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a skin sample with MMA standard spike for identity confirmation. Skin sample was collected on day 28 from ROX-fed chicken in pen 15, Ross 308. 2 µg/L MMA standard was spiked in skin sample.

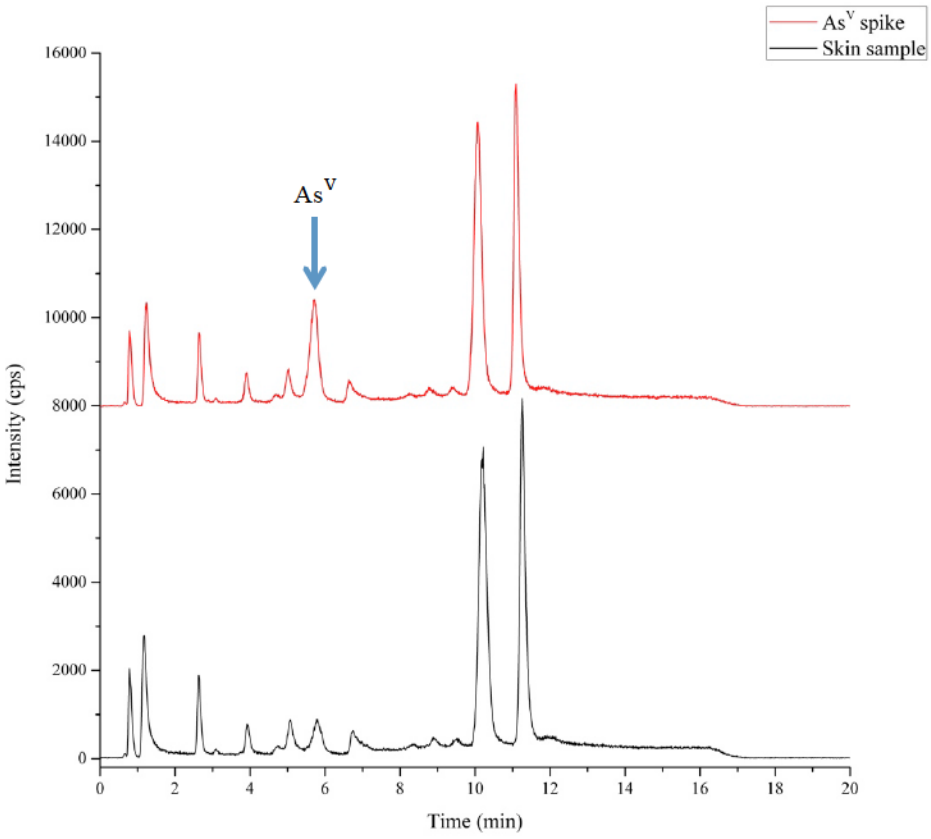


Figure 4.5 (v) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a skin sample with As^V standard spike for identity confirmation. Skin sample was collected on day 28 from ROX-fed chicken in pen 15, Ross 308. 3 µg/L As^V standard was spiked in skin sample.

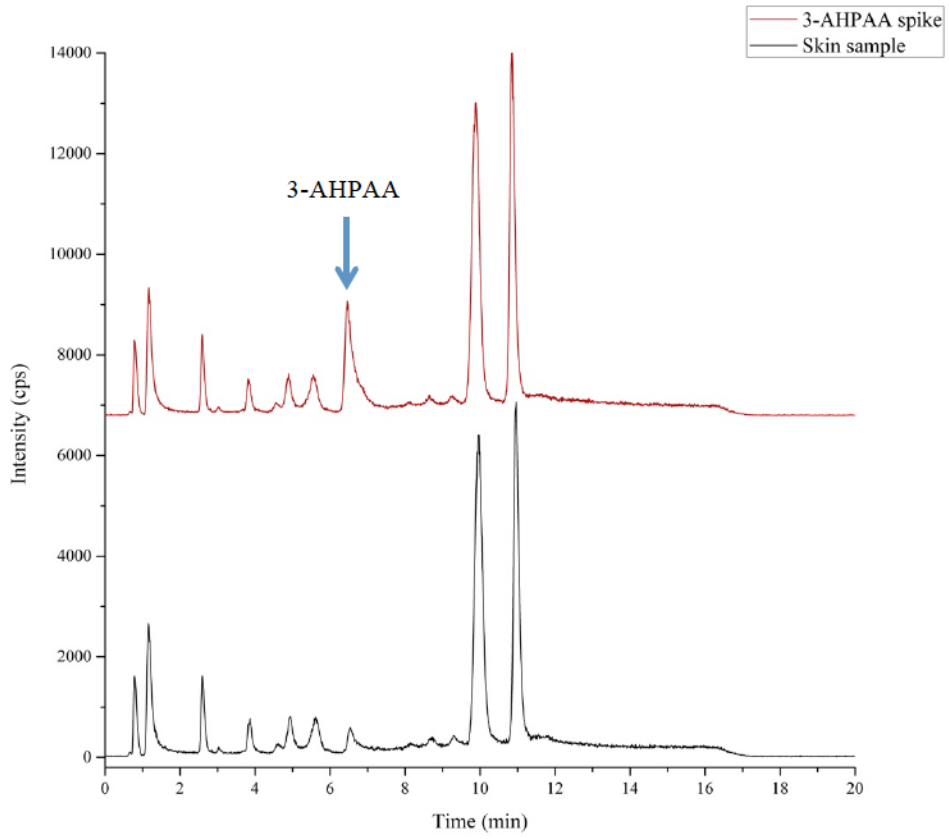


Figure 4.5 (vi) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a skin sample with 3-AHPAA standard spike for identity confirmation. Skin sample was collected on day 28 from ROX-fed chicken in pen 15, Ross 308. 5 µg/L 3-AHPAA standard was spiked in skin sample.

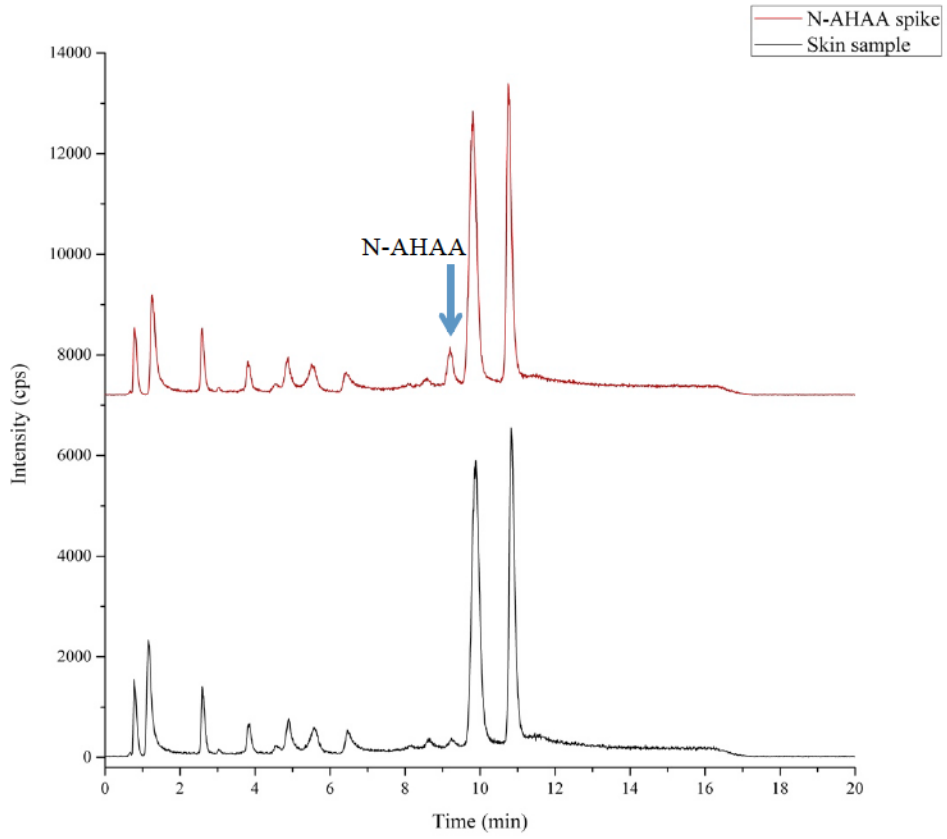


Figure 4.5 (vii) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a skin sample with N-AHAA standard spike for identity confirmation. Skin sample was collected on day 28 from ROX-fed chicken in pen 15, Ross 308. 1 µg/L N-AHAA standard was spiked in skin sample.

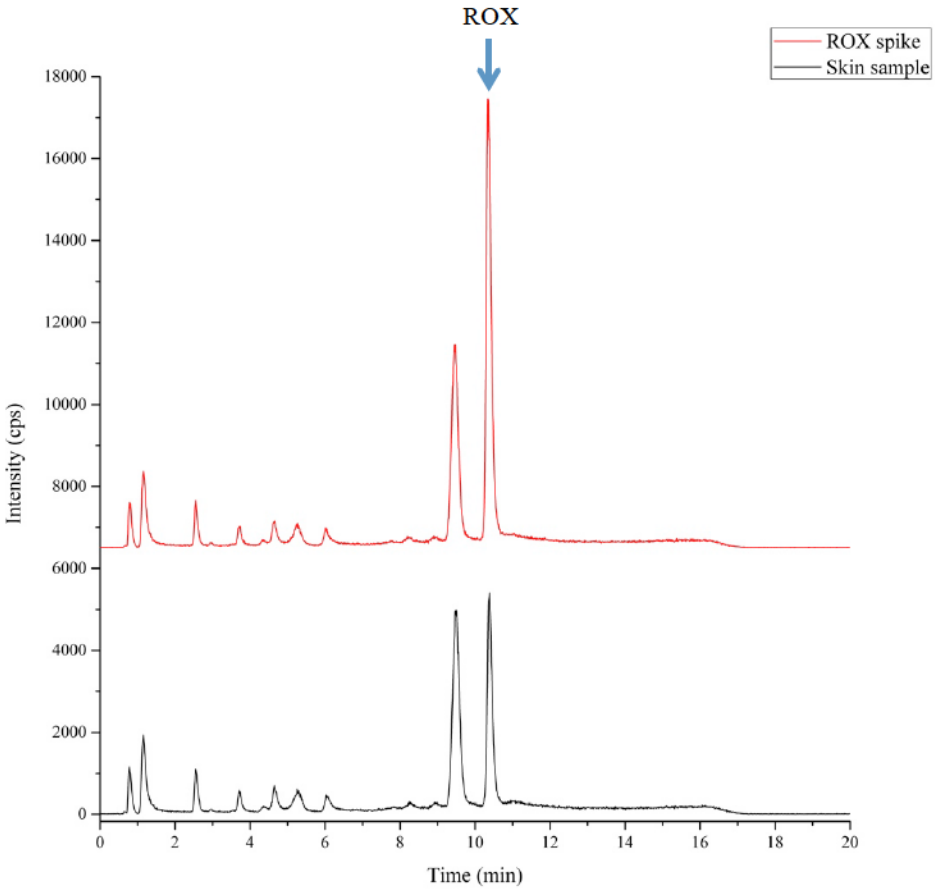


Figure 4.5 (viii) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a skin sample with ROX standard spike for identity confirmation. Skin sample was collected on day 28 from ROX-fed chicken in pen 15, Ross 308. 14 µg/L ROX standard was spiked in skin sample.

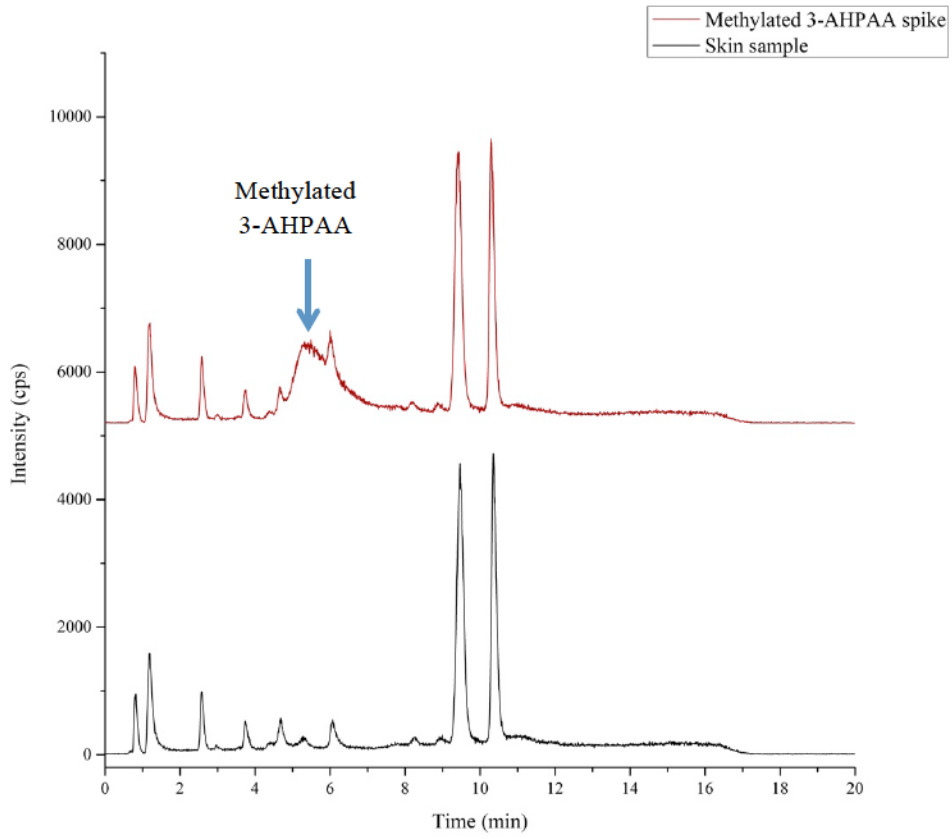


Figure 4.5 (ix) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a skin sample with methylated 3-AHPAA standard spike for identity confirmation. Skin sample was collected on day 28 from ROX-fed chicken in pen 15, Ross 308. 5 µg/L methylated 3-AHPAA standard was spiked in skin sample.

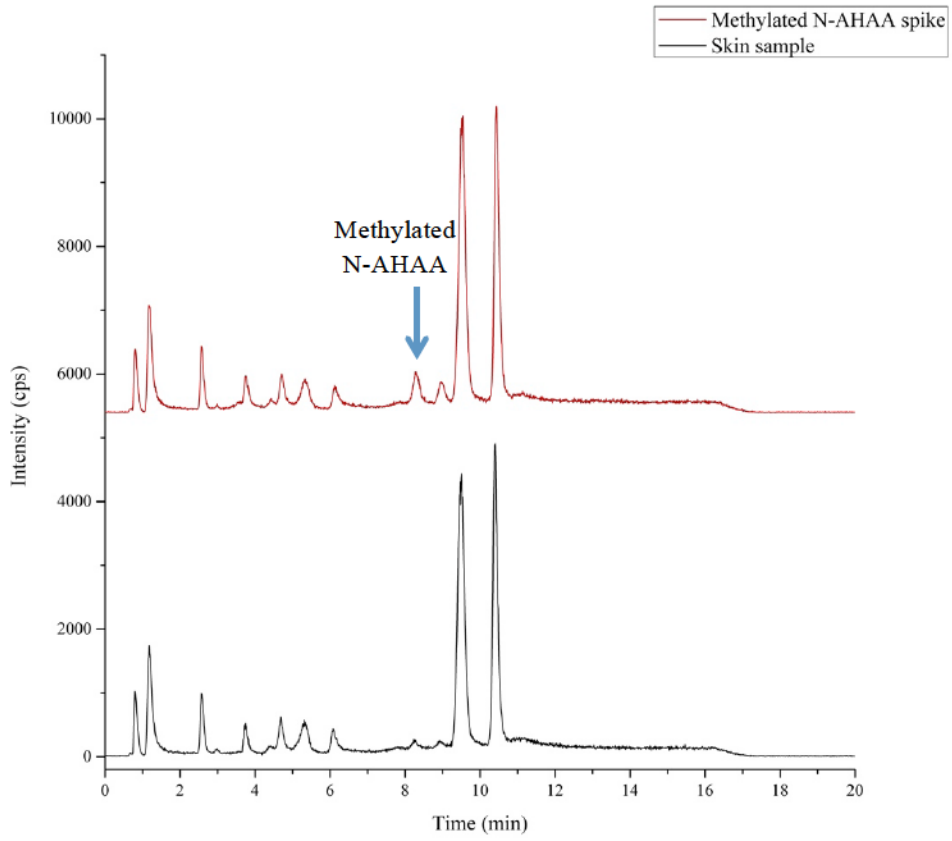


Figure 4.5 (x) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a skin sample with methylated N-AHAA standard spike for identity confirmation. Skin sample was collected on day 28 from ROX-fed chicken in pen 15, Ross 308. 5 µg/L methylated N-AHAA standard was spiked in skin sample.

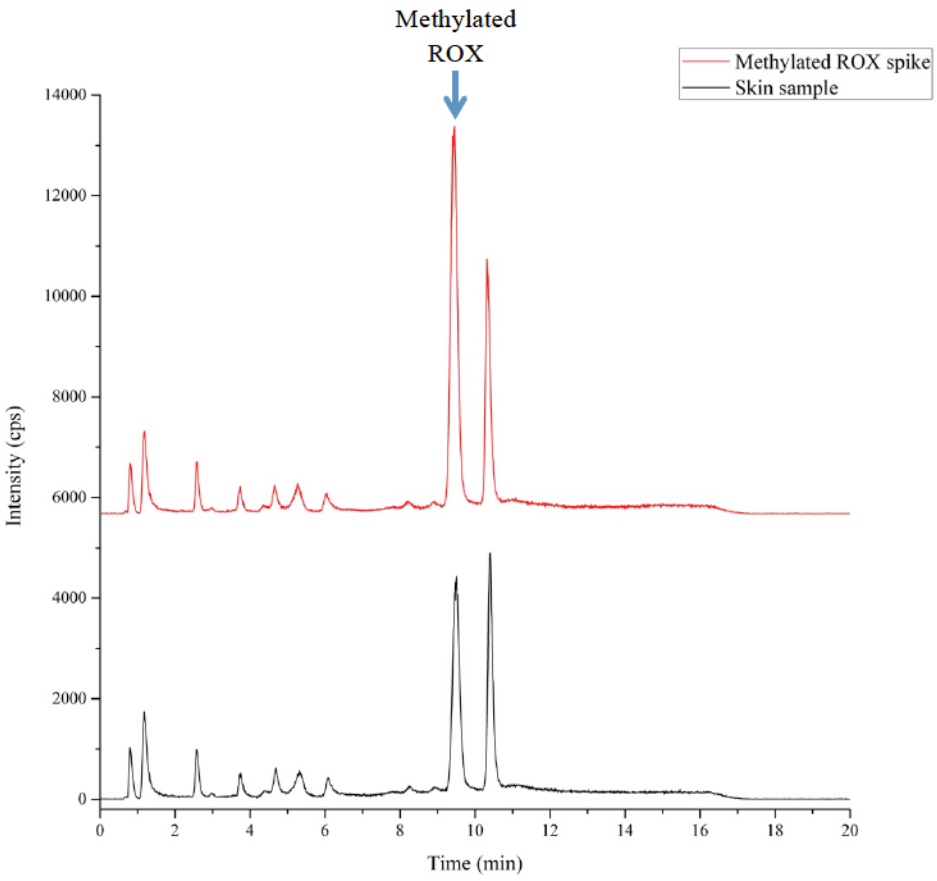


Figure 4.5 (xi) Chromatograms obtained from HPLC-ICPMS analyses of arsenic in a skin sample with methylated ROX standard spike for identity confirmation. Skin sample was collected on day 28 from ROX-fed chicken in pen 15, Ross 308. 10 µg/L methylated ROX standard was spiked in skin sample.

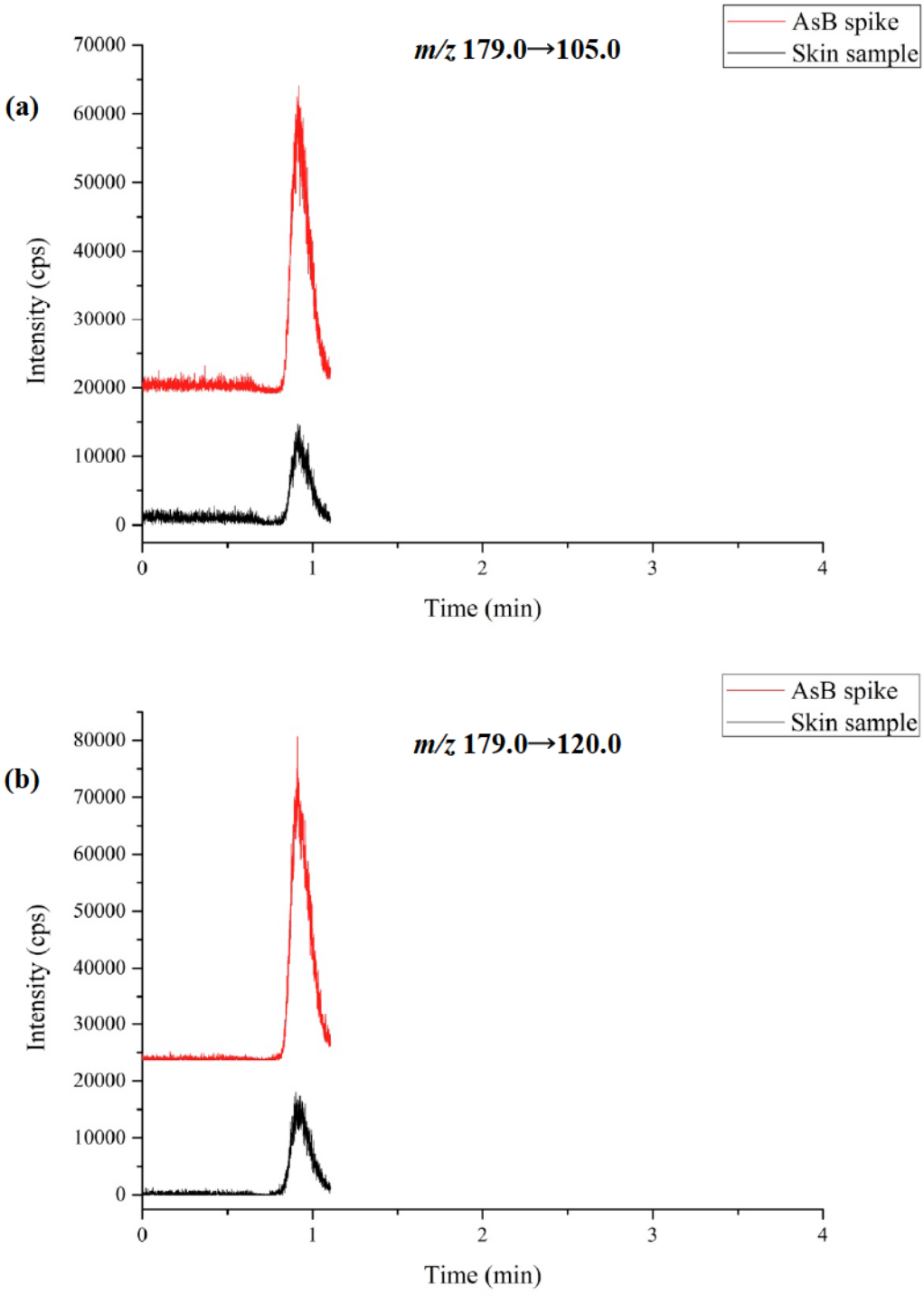


Figure 4.6 (i) Chromatograms from MRM analyses of a skin sample of ROX-fed chicken (pen 15, Ross 308) with AsB spike using the transitions (a) *m/z* 179.0 to 105.0 and (b) *m/z* 179.0 to 120.0.

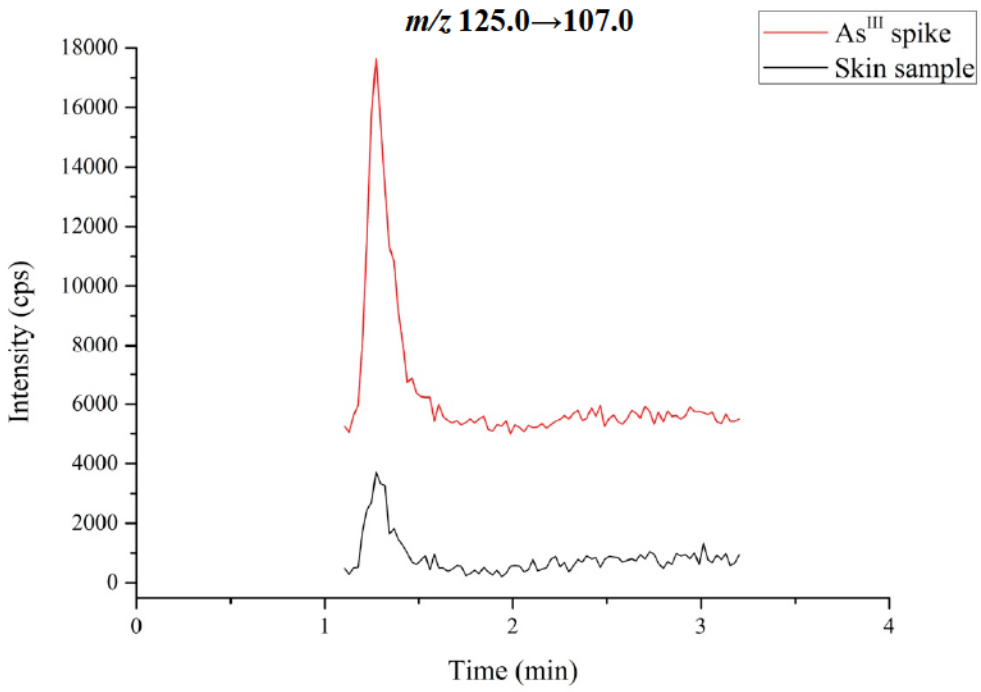


Figure 4.6 (ii) Chromatograms from MRM analyses of a skin sample of ROX-fed chicken (pen 15, Ross 308) with As^{III} spike using the transition *m/z* 125.0 to 107.0.

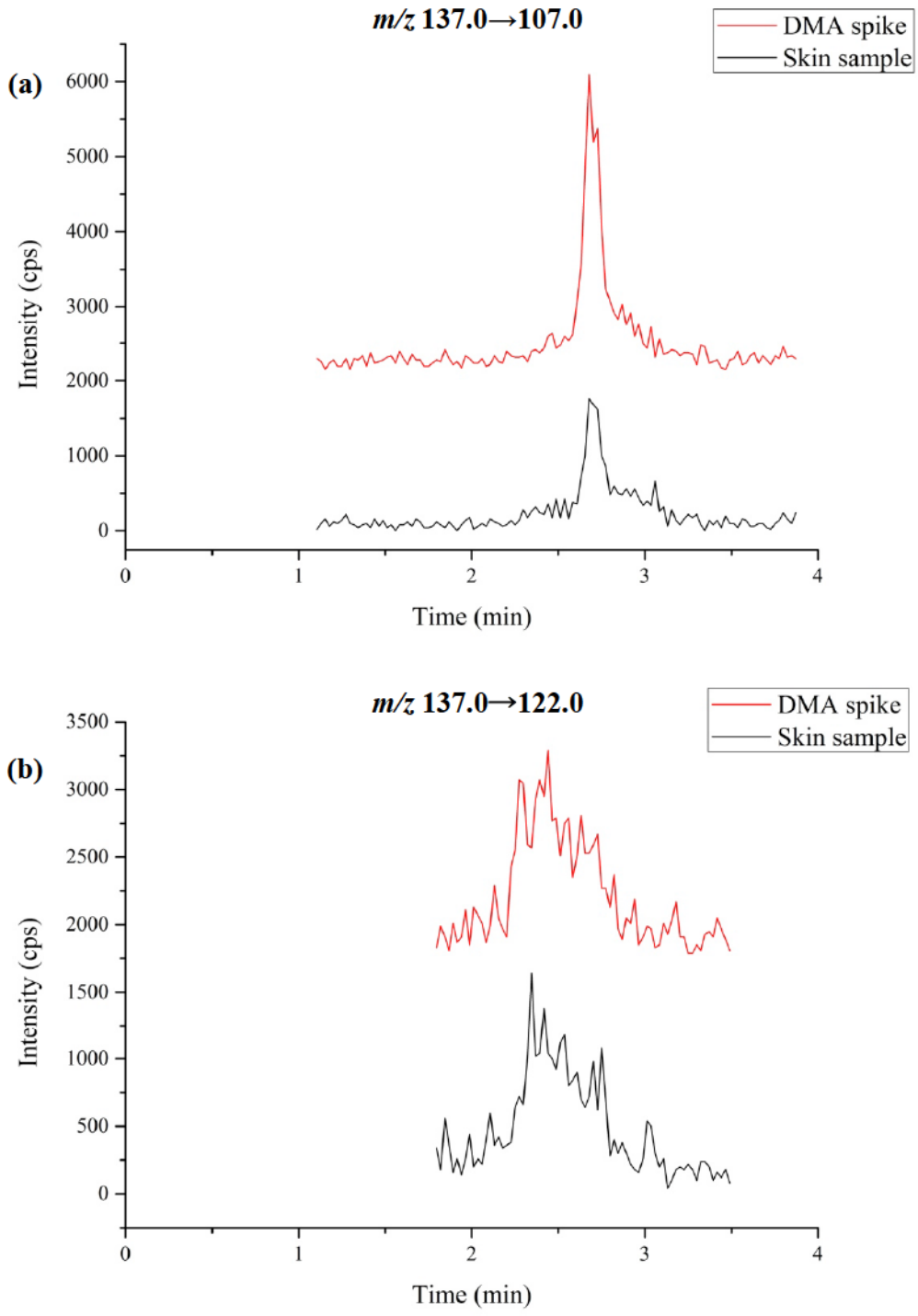


Figure 4.6 (iii) Chromatograms from MRM analyses of a skin sample of ROX-fed chicken (pen 15, Ross 308) with DMA spike using the transitions (a) *m/z* 137.0 to 107.0 and (b) *m/z* 137.0 to 122.0.

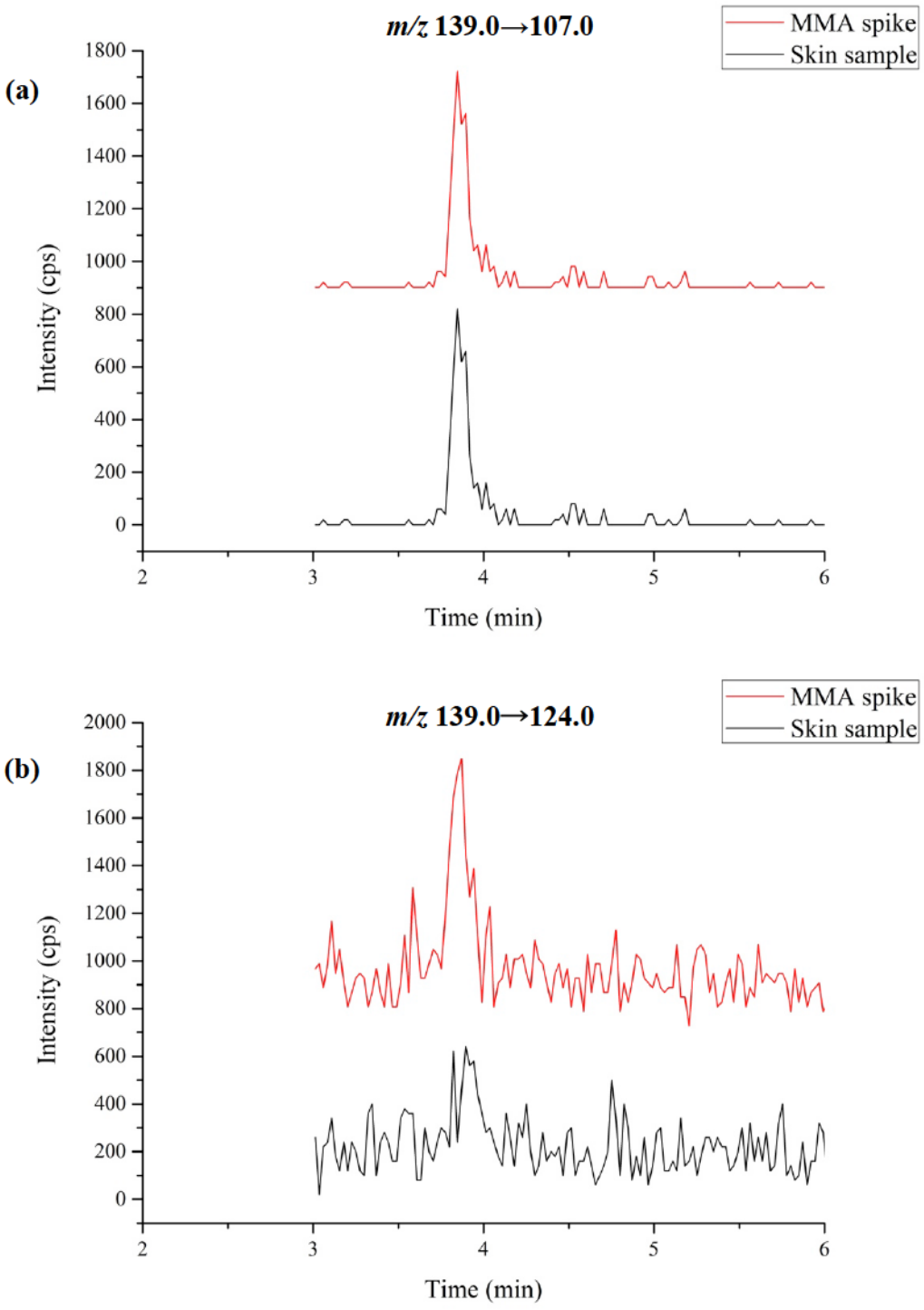


Figure 4.6 (iv) Chromatograms from MRM analyses of a skin sample of ROX-fed chicken (pen 15, Ross 308) with MMA spike using the transitions (a) *m/z* 139.0 to 107.0 and (b) *m/z* 139.0 to 124.0.

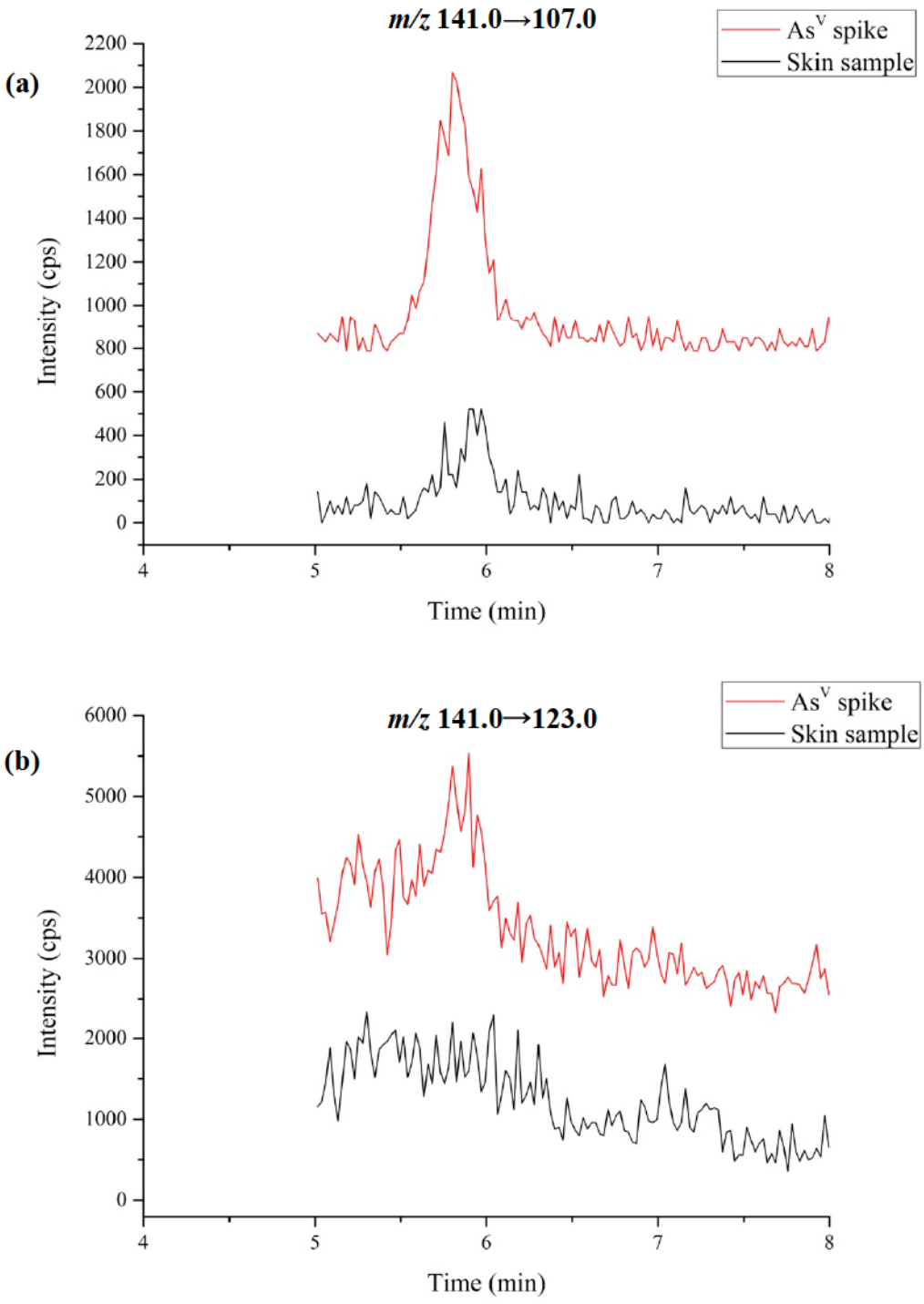


Figure 4.6 (v) Chromatograms from MRM analyses of a skin sample of ROX-fed chicken (pen 15, Ross 308) with As^V spike using the transitions (a) *m/z* 141.0 to 107.0 and (b) *m/z* 141.0 to 123.0.

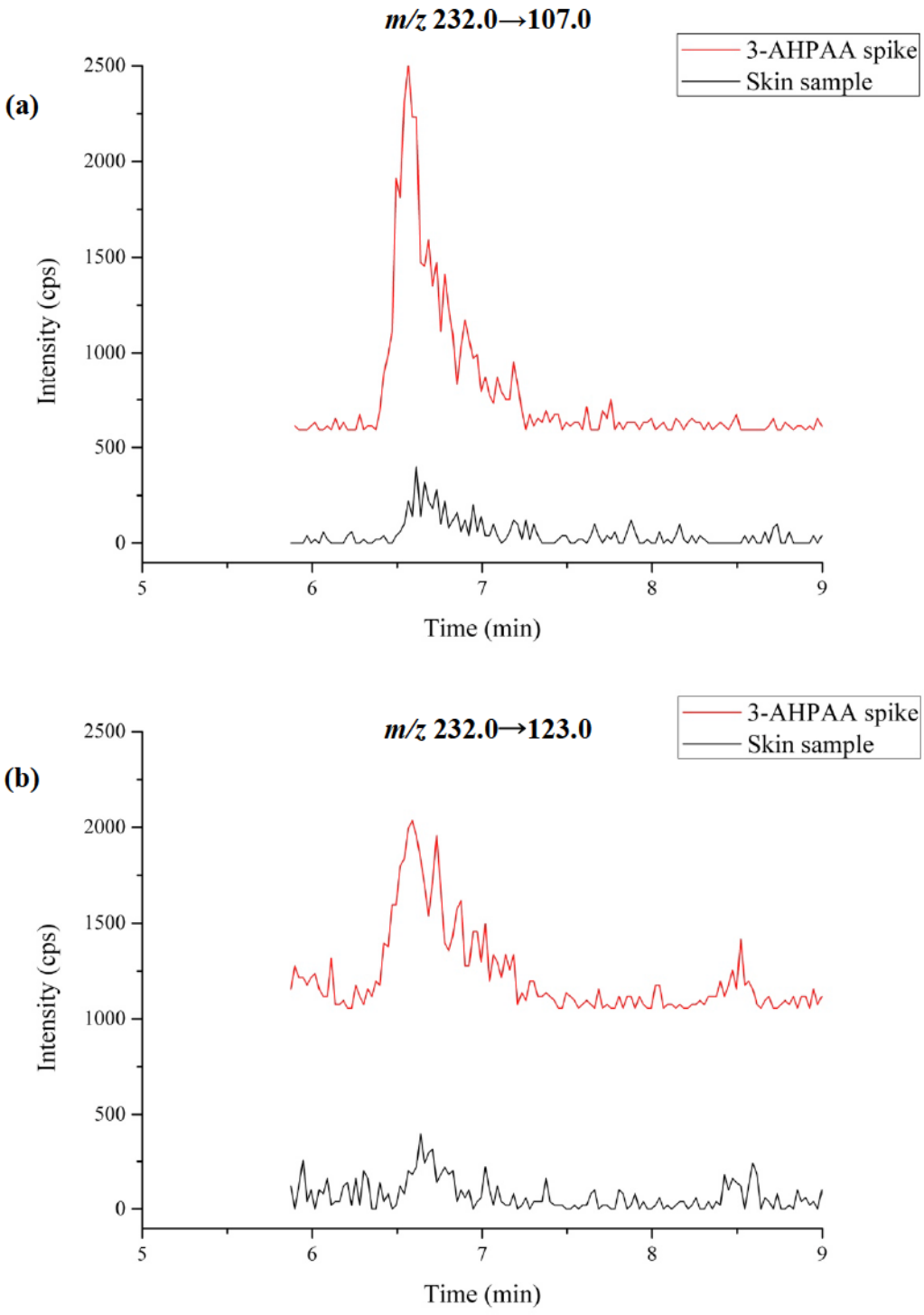


Figure 4.6 (vi) Chromatograms from MRM analyses of a skin sample of ROX-fed chicken (pen 15, Ross 308) with 3-AHPAA spike using the transitions (a) *m/z* 232.0 to 107.0 and (b) *m/z* 232.0 to 123.0.

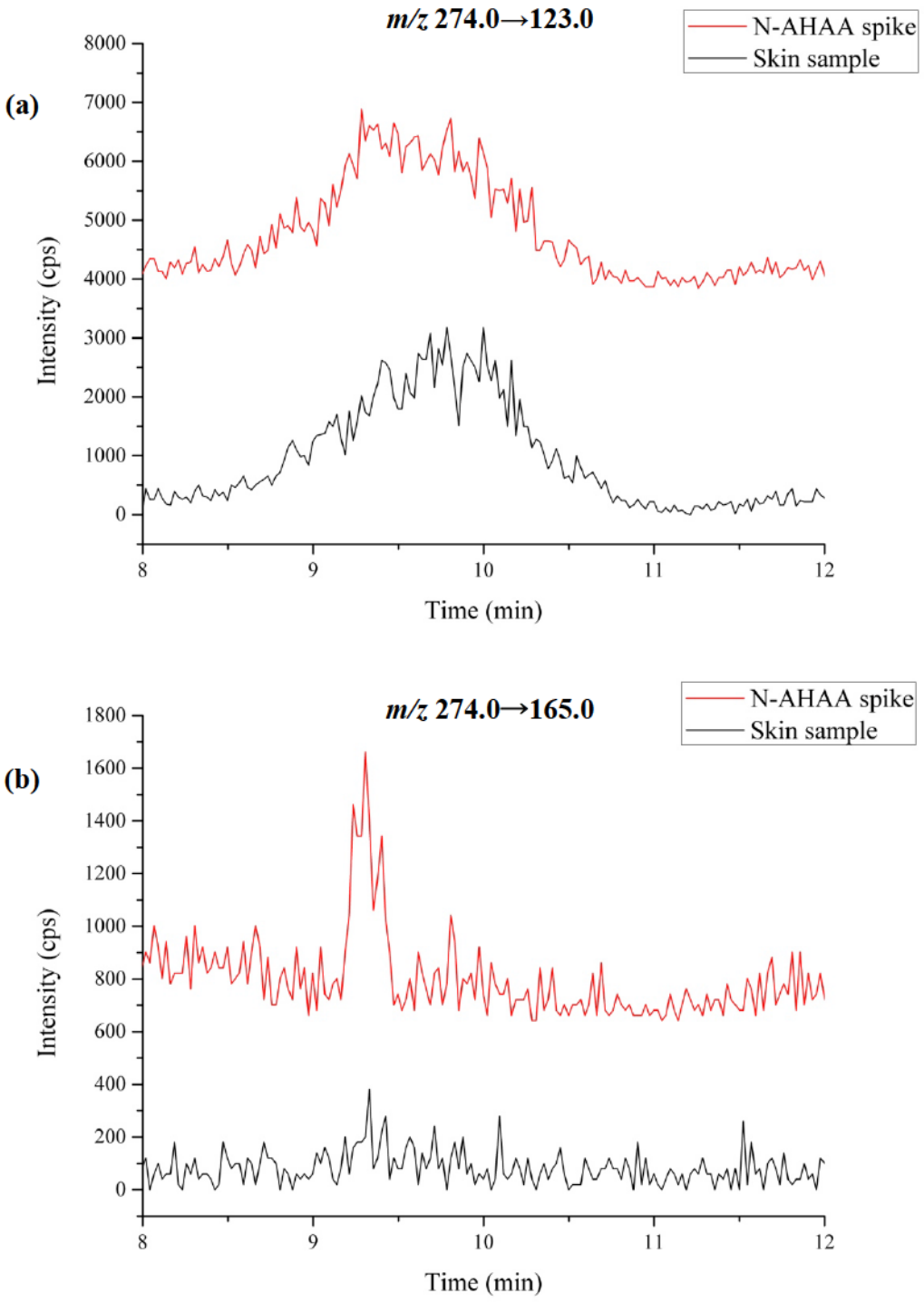


Figure 4.6 (vii) Chromatograms from MRM analyses of a skin sample of ROX-fed chicken (pen 15, Ross 308) with N-AHAA spike using the transitions (a) *m/z* 274.0 to 123.0 and (b) *m/z* 274.0 to 165.0.

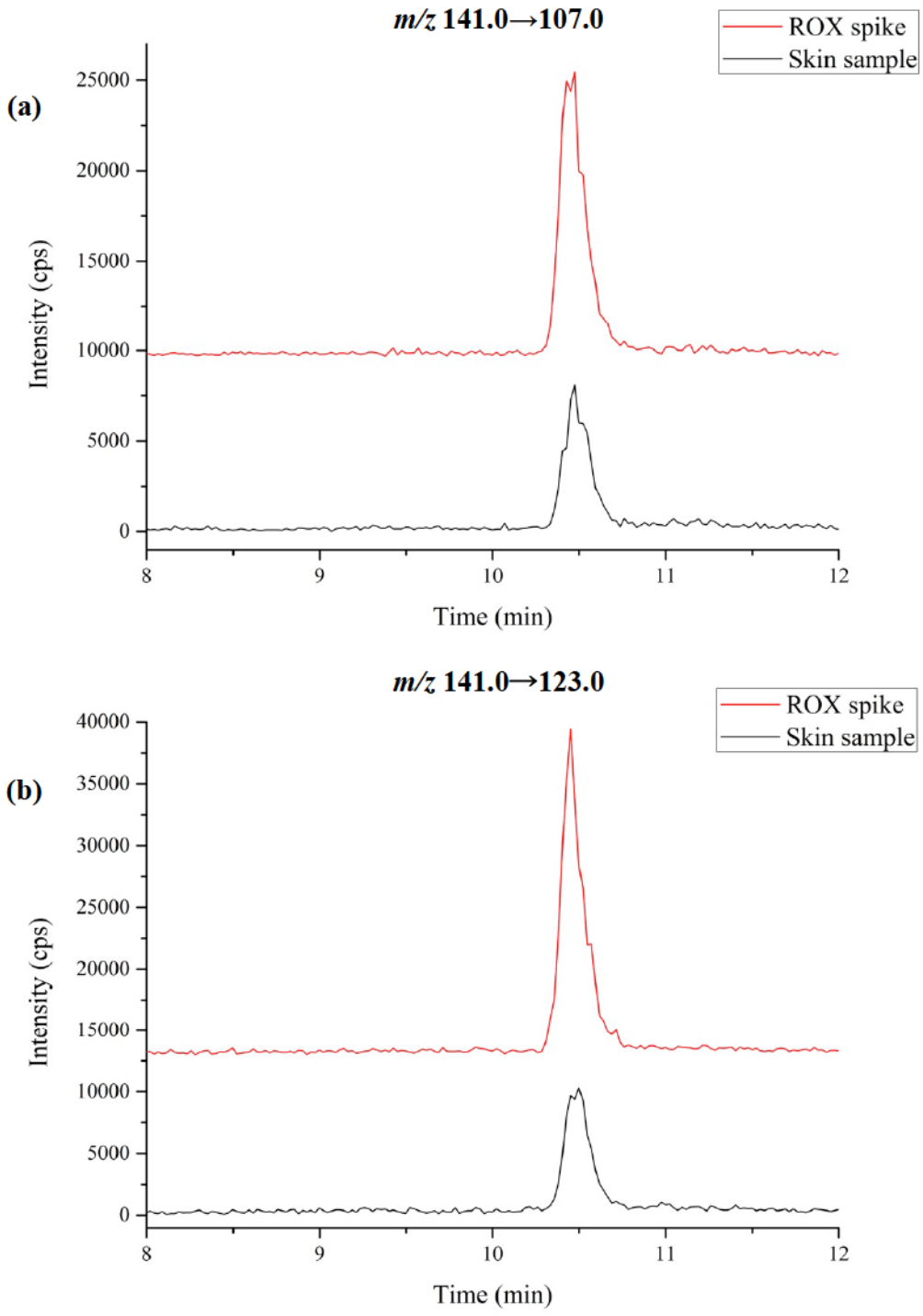


Figure 4.6 (viii) Chromatograms from MRM analyses of a skin sample of ROX-fed chicken (pen 15, Ross 308) with ROX spike using the transitions (a) *m/z* 262.0 to 107.0 and (b) *m/z* 262.0 to 123.0.

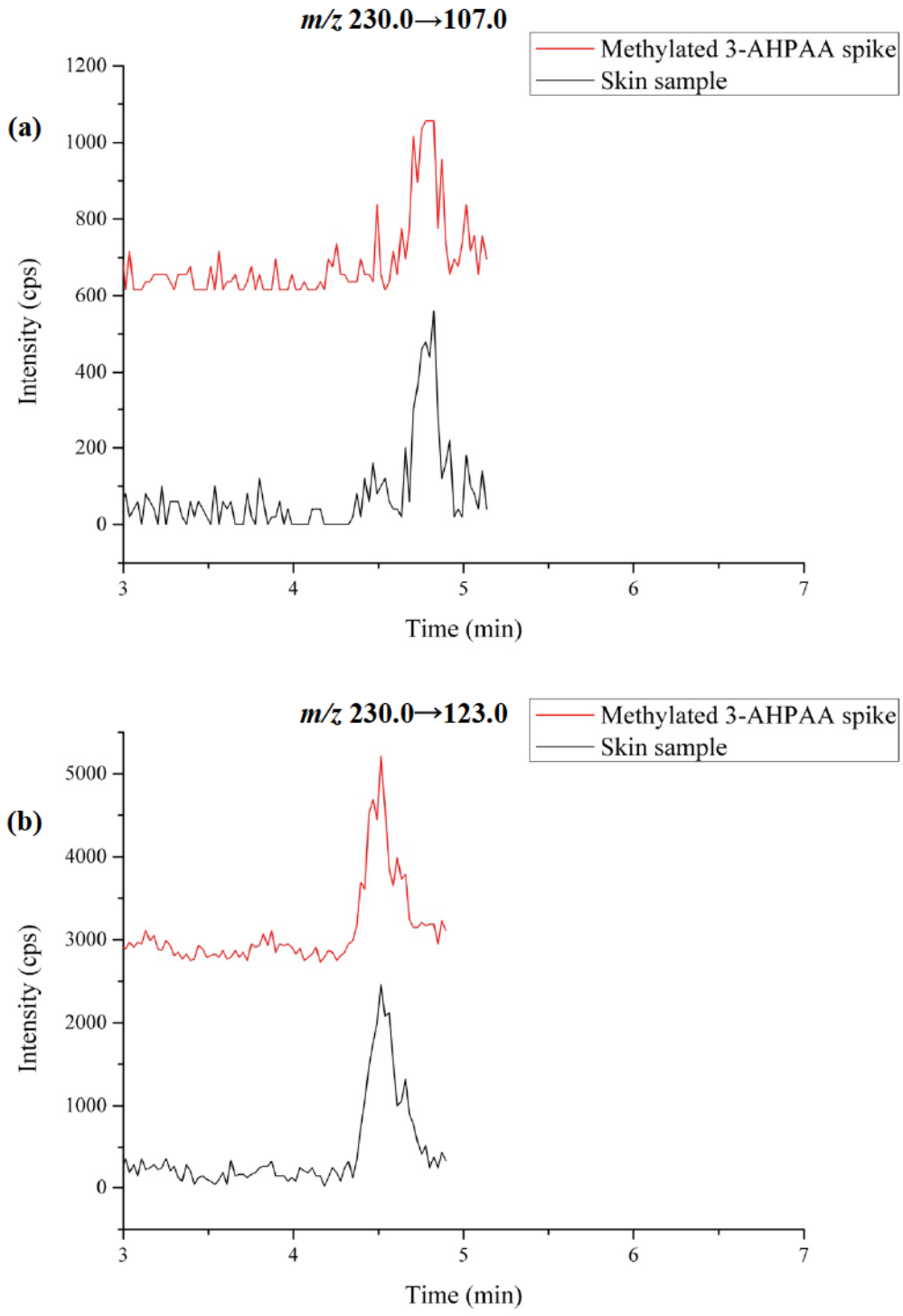


Figure 4.6 (ix) Chromatograms from MRM analyses of a skin sample of ROX-fed chicken (pen 15, Ross 308) with methylated 3-AHPAA spike using the transitions (a) *m/z* 230.0 to 107.0 and (b) *m/z* 230.0 to 123.0.

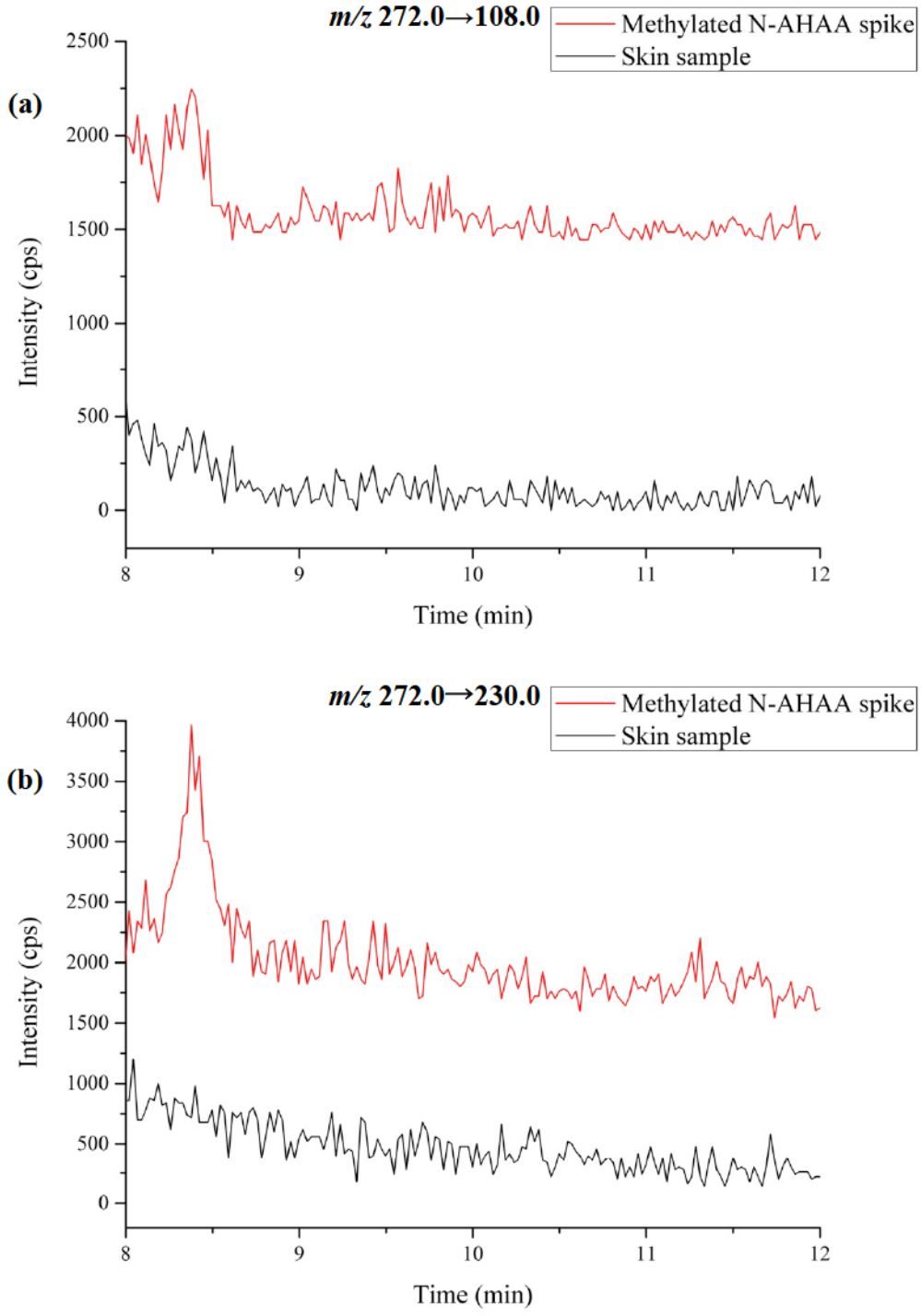


Figure 4.6 (x) Chromatograms from MRM analyses of a skin sample of ROX-fed chicken (pen 15, Ross 308) with methylated N-AHAA spike using the transitions (a) *m/z* 272.0 to 108.0 and (b) *m/z* 272.0 to 230.0.

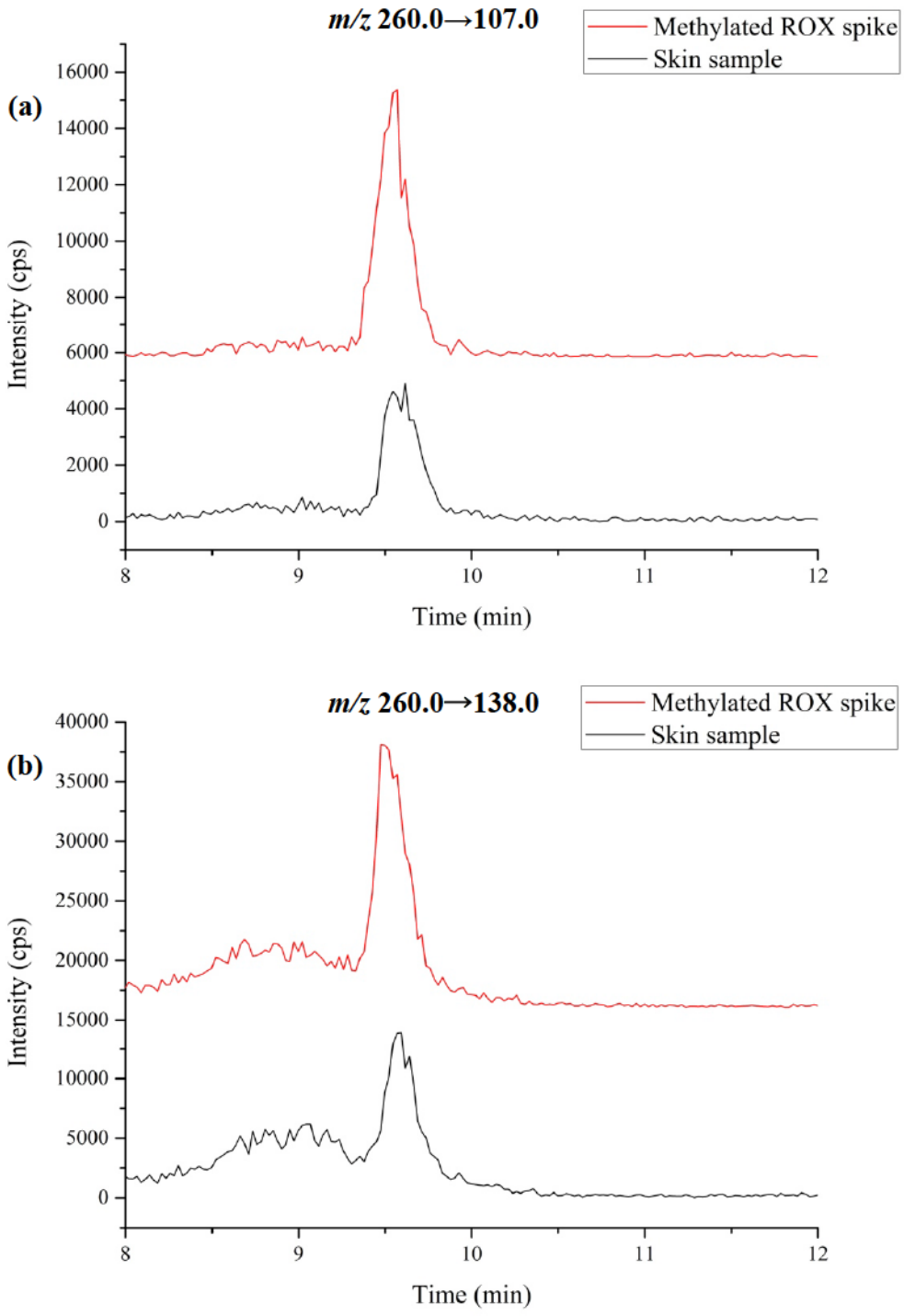


Figure 4.6 (xi) Chromatograms from MRM analyses of a skin sample of ROX-fed chicken (pen 15, Ross 308) with methylated ROX spike using the transitions (a) *m/z* 260.0 to 107.0 and (b) *m/z* 260.0 to 138.0.

We further quantified the concentrations of arsenic species using the HPLC-ICPMS/ESIMS system. As shown in Table 4.6, our system showed low LOD values that are capable of determining trace amounts of arsenicals. By applying the HPLC-ICPMS/ESIMS technique, the arsenic species in chicken skin samples from two different days (day 28 and day 35) were studied. For skin samples from the ROX-fed chickens, similar arsenic species were detected in these two days although the concentrations differ (Figure 4.7). Concentrations of arsenicals in the skin of ROX-fed chickens from Cobb and 500 Ross 308 strains were summarized in Table 4.7 and 4.8. ROX and methylated ROX had higher concentrations than any other arsenic species on each given day for both strains. When comparing the sum of arsenic compounds on day 28 and day 35 (Figure 4.8), we observed a significant decrease in the sum of arsenic compounds in ROX-fed chickens after one week's ROX withdraw period, which agreed with our finding in the total arsenic analysis (Figure 3.9).

Table 4.6 Limits of detection (LOD) for selected arsenic compounds.

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

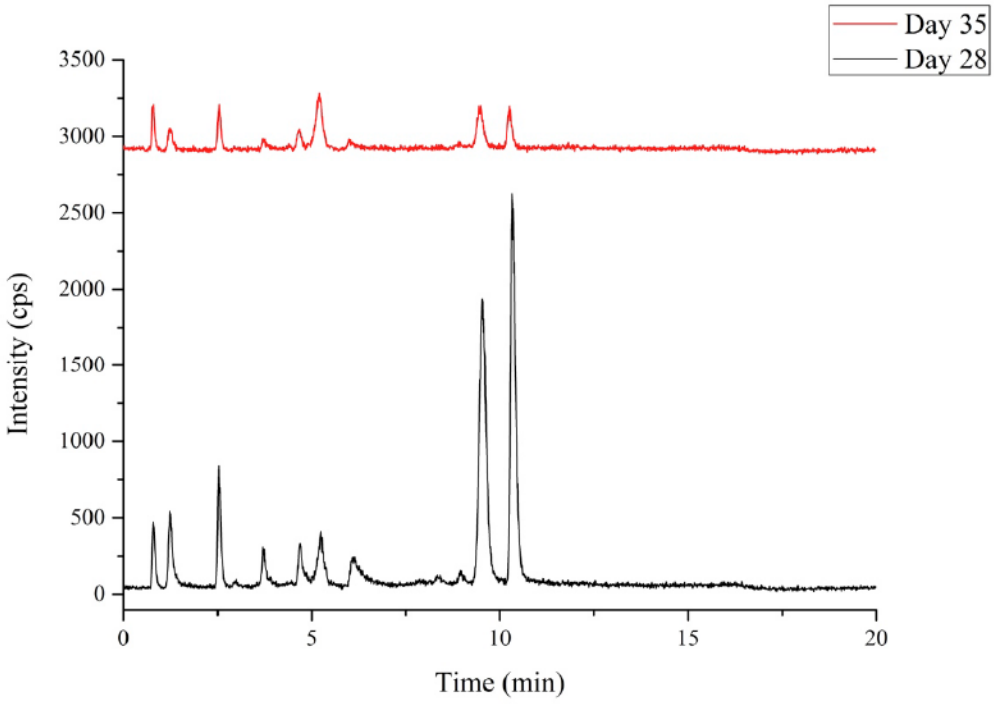


Figure 4.7 Chromatograms obtained from the HPLC-ICPMS/ESIMS analyses of skin samples collected on day 28 and day 35 from ROX-fed chickens (pen 11, Ross 308).

Table 4.7 Concentrations of arsenicals detected in the skin samples from ROX-fed chickens (strain Cobb 500) (n=4 for each day).[a]

Concentration of Arsenicals (mean ± SD) (µg/kg)		
	Day 28	Day 35
AsB	4 ± 2	5 ± 2
As^{III}	18 ± 3	3.5 ± 0.4
DMA	5 ± 1	2.60 ± 0.05
Unknown 1[b]	0.1 ± 0.2	N.D.
MMA	3.2 ± 0.2	2.0 ± 0.3
Unknown 2[c]	N.D.	N.D.
Methylated 3-AHPAA[d]	2 ± 1	4 ± 4
As^{V}	1.8 ± 0.7	1.6 ± 0.8
3-AHPAA	6 ± 1	0.30 ± 0.02
Methylated N-AHAA[e]	0.3 ± 0.4	N.D.
N-AHAA	0.9 ± 0.4	N.D.
Methylated ROX	53 ± 18	7 ± 3
ROX	40 ± 11	3 ± 1
Sum of Arsenicals	136 ± 22	29 ± 5

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

[b] Concentration estimated according to the calibration curve of DMA

[c] Concentration estimated according to the calibration curve of MMA

[d] Concentration estimated according to the calibration curve of 3-AHPAA

[e] Concentration estimated according to the calibration curve of N-AHAA

Table 4.8 Concentrations of arsenicals detected in the skin samples from ROX-fed chickens (strain Ross 308) (n=4 for each day).[a]

Concentration of Arsenicals (mean ± SD) (µg/kg)		
	Day 28	Day 35
AsB	13 ± 8	4.2 ± 0.9
As^{III}	18 ± 3	3.5 ± 0.4
DMA	6 ± 1	4 ± 2
Unknown 1[b]	0.42 ± 0.02	N.D.
MMA	5 ± 1	2.3 ± 0.6
Unknown 2[c]	0.08 ± 0.15	1 ± 1
Methylated 3-AHPAA[d]	3 ± 2	1.5 ± 0.7
As^{V}	3 ± 1	1.3 ± 0.5
3-AHPAA	6.8 ± 0.5	0.6 ± 0.5
Methylated N-AHAA[e]	0.6 ± 0.6	N.D.
N-AHAA	1.0 ± 0.1	0.2 ± 0.3
Methylated ROX	62 ± 17	9 ± 2
ROX	52 ± 8	3.2 ± 0.6
Sum of Arsenicals	157 ± 21	30 ± 4

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

[b] Concentration estimated according to the calibration curve of DMA

[c] Concentration estimated according to the calibration curve of MMA

[d] Concentration estimated according to the calibration curve of 3-AHPAA

[e] Concentration estimated according to the calibration curve of N-AHAA

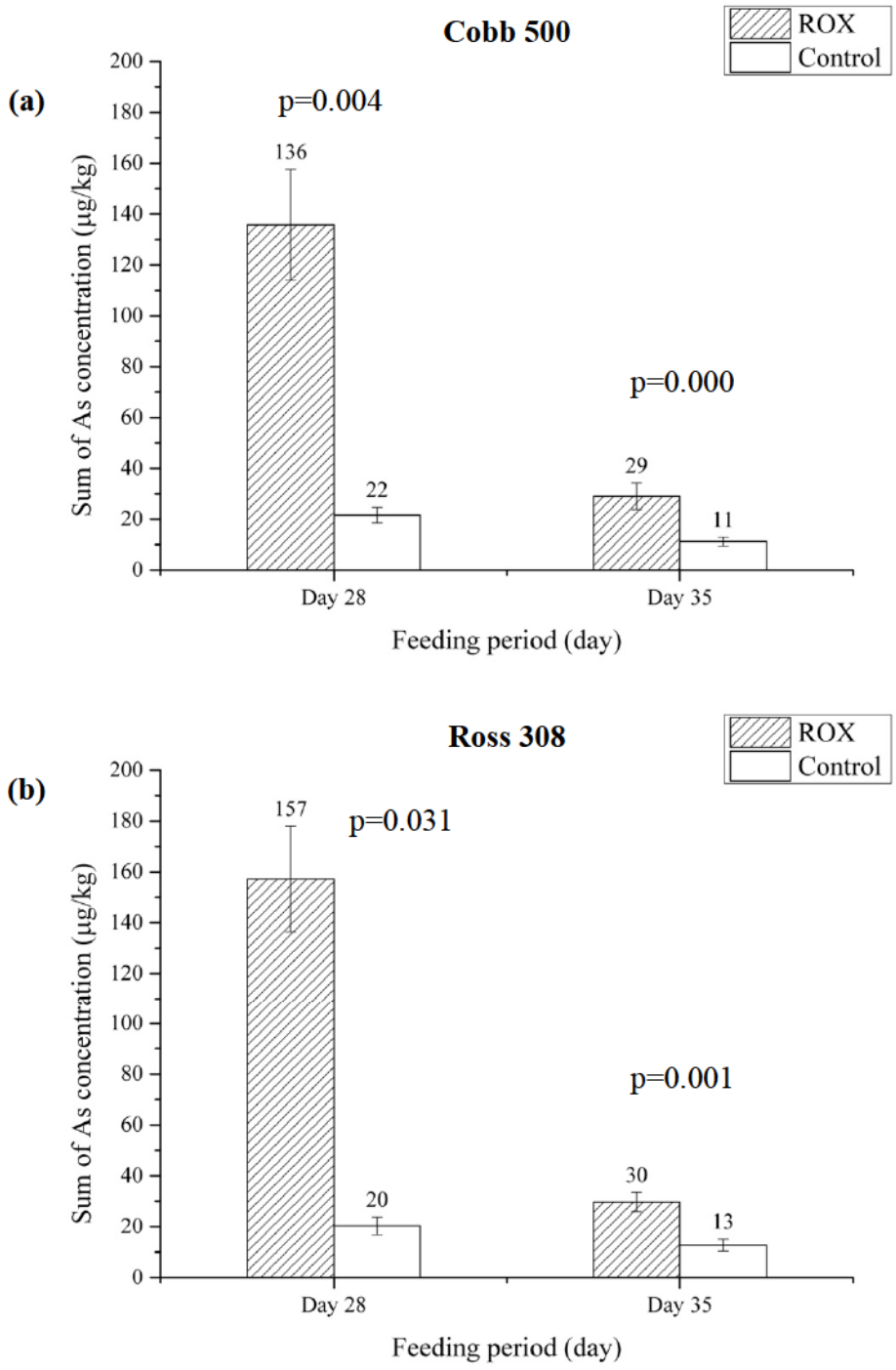


Figure 4.8 Sum of arsenicals in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308. Day 28 was the last day of feeding ROX. Day 35 was seven days after stopping feeding ROX to the chickens.

We further looked at relative concentrations of each arsenic compound as percentages of the sum of arsenicals. Figure 4.9 indicates that ROX and methylated ROX were the most dominant arsenic species in the skin of ROX-fed chickens on both day 28 and day 35. The total percentage of ROX and its methylated product was greater than 70% on day 28, and then it dropped to about 40% on day 35. As^{III} accounted for approximately 10% of the total concentration of arsenicals on both days. Other phenylarsonic compounds, such as 3-AHPAA, N-AHAA and their methylated products were also detectable in the skin of ROX-fed chickens on both day 28 and day 35. 3-AHPAA and methylated 3-AHPAA displayed higher percentages on day 28. But on day 35, their concentrations were lower than AsB and inorganic arsenic. Unlike the 3-AHPAA, N-AHAA and methylated N-AHAA had lower concentrations on both days. The result of N-AHAA in skin was different from that in chicken litter and chicken liver [8, 11].

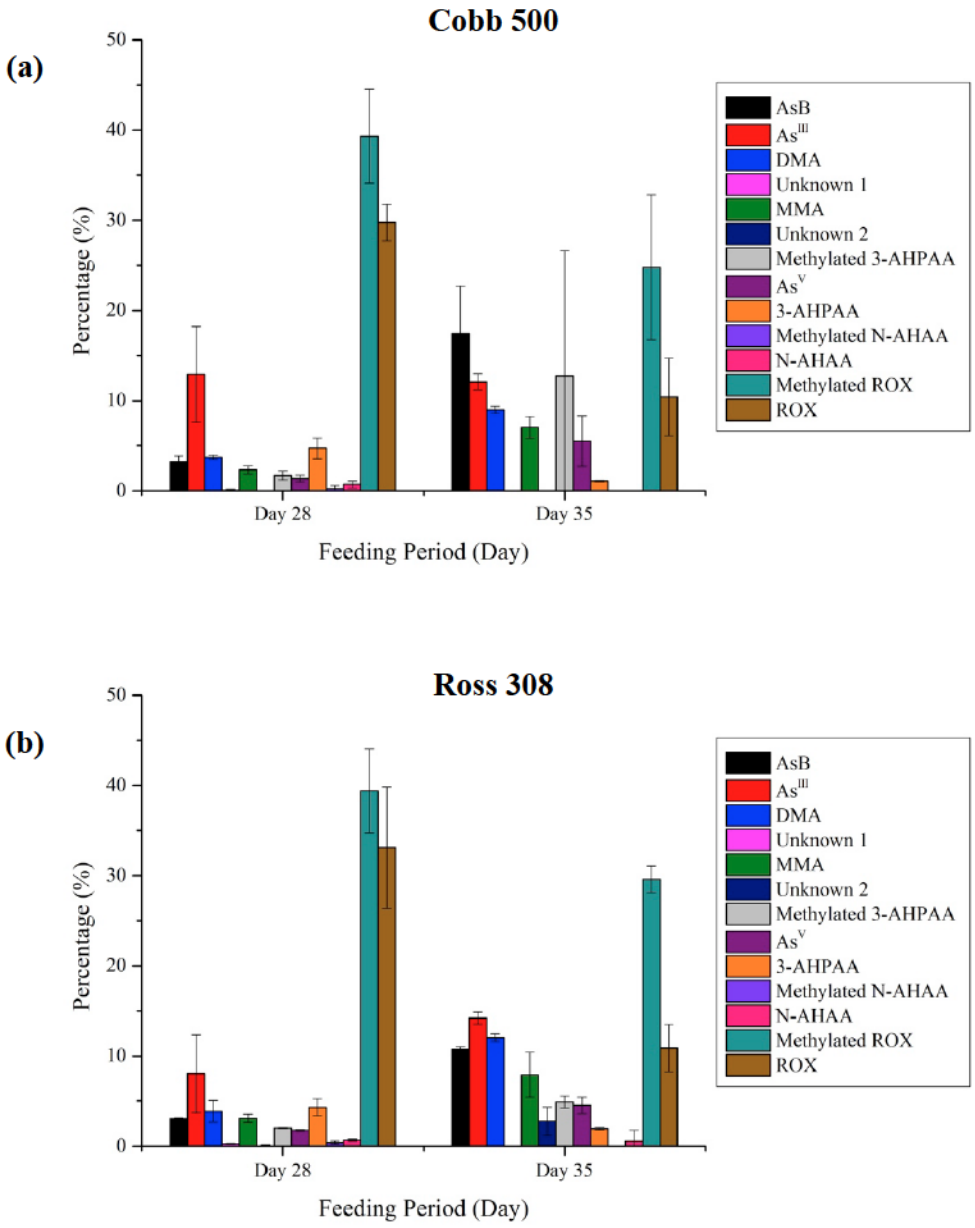


Figure 4.9 Percentages of different arsenicals in the skin samples of ROX-fed chickens from strain (a) Cobb 500 and (b) Ross 308. The skin samples were collected on day 28 and day 35.

Skin samples from the control group showed the presence of smaller number of arsenic species (Figure 4.10). As illustrated in Table 4.9 and 4.10, concentrations of all arsenic species were very low in the skin of control chickens from both Cobb 500 and Ross 308 strains. Trace amounts of phenylarsonic compounds were detected in the samples on day 28 and day 35. Unlike that in the skin of ROX-fed chickens, the sum of arsenic did not have a dramatic change in the skin of control chickens between day 28 and day 35 (Figure 4.8). On the last day (day 35), the sum of arsenic in the skin of ROX-fed chickens and that in the skin of control chickens was very close, but the control chickens did have less arsenic in their skin. The percentages of arsenic species in the skin of control chickens are displayed in Figure 4.11. In the skin of control chickens, AsB and DMA were the major arsenic species instead of ROX and methylated ROX, and each of them had a percentage higher than 20% during these two days.

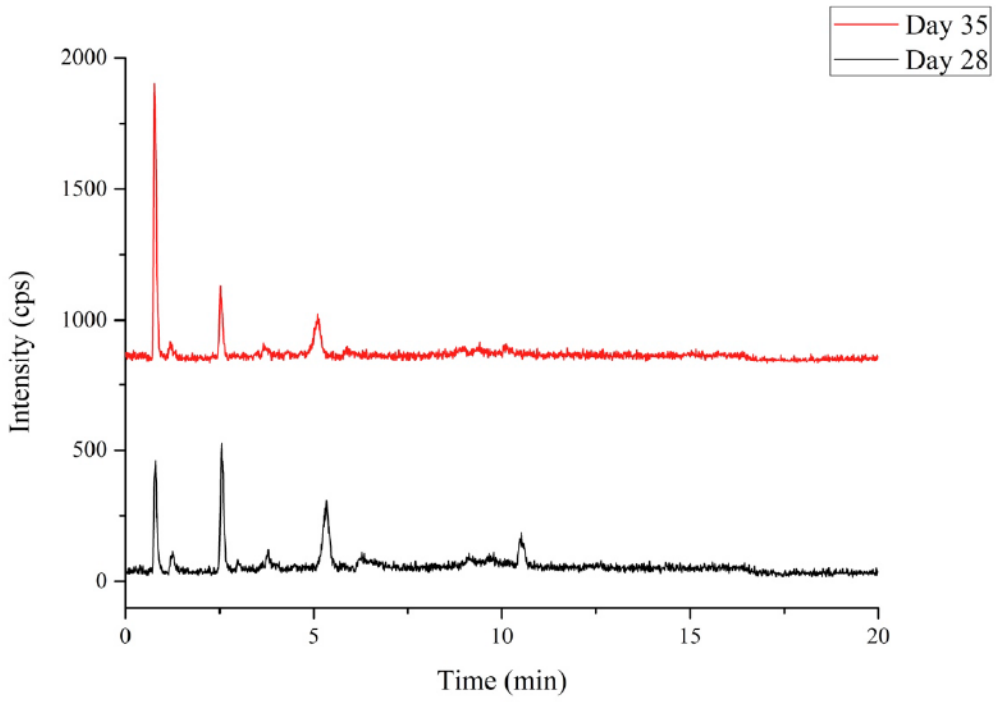


Figure 4.10 Chromatograms obtained from the HPLC-ICPMS/ESIMS analyses of skin samples collected on day 28 and day 35 from control chickens (pen 12, Ross 308).

Table 4.9 Concentrations of arsenicals detected in the skin samples from control chickens (strain Cobb 500) (n=4 for each day).[a]

Concentration of Arsenicals (mean ± SD) (µg/kg)		
	Day 28	Day 35
AsB	6 ± 1	3 ± 2
As^{III}	1.4 ± 0.3	1.8 ± 0.2
DMA	6 ± 1	2.9 ± 0.3
Unknown 1[b]	0.52 ± 0.05	N.D.
MMA	1.7 ± 0.2	1.7 ± 0.1
Unknown 2[c]	0.4 ± 0.9	N.D.
Methylated 3-AHPAA[d]	N.D.	N.D.
As^{V}	0.9 ± 0.4	1.1 ± 0.4
3-AHPAA	2.1 ± 0.6	0.1 ± 0.2
Methylated N-AHAA[e]	N.D.	N.D.
N-AHAA	0.1 ± 0.2	N.D.
Methylated ROX	1 ± 1	0.3 ± 0.5
ROX	2 ± 1	N.D.
Sum of Arsenicals	22 ± 3	11 ± 2

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

[b] Concentration estimated according to the calibration curve of DMA

[c] Concentration estimated according to the calibration curve of MMA

[d] Concentration estimated according to the calibration curve of 3-AHPAA

[e] Concentration estimated according to the calibration curve of N-AHAA

Table 4.10 Concentrations of arsenicals detected in the skin samples from control chickens (strain Ross 308) (n=4 for each day).[a]

Concentration of Arsenicals (mean ± SD) (µg/kg)		
	Day 28	Day 35
AsB	5 ± 1	5 ± 2
As^{III}	1.7 ± 0.3	2.2 ± 0.9
DMA	4 ± 1	3.0 ± 0.7
Unknown 1[b]	0.5 ± 0.3	N.D.
MMA	1.9 ± 0.5	2.0 ± 0.5
Unknown 2[c]	2 ± 2	N.D.
Methylated 3-AHPAA[d]	N.D.	N.D.
As^{V}	1.1 ± 0.7	0.3 ± 0.4
3-AHPAA	1.5 ± 0.2	0.1 ± 0.2
Methylated N-AHAA[e]	N.D.	N.D.
N-AHAA	0.33 ± 0.03	N.D.
Methylated ROX	0.5 ± 0.7	N.D.
ROX	2 ± 2	N.D.
Sum of Arsenicals	20 ± 3	13 ± 2

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

[b] Concentration estimated according to the calibration curve of DMA

[c] Concentration estimated according to the calibration curve of MMA

[d] Concentration estimated according to the calibration curve of 3-AHPAA

[e] Concentration estimated according to the calibration curve of N-AHAA

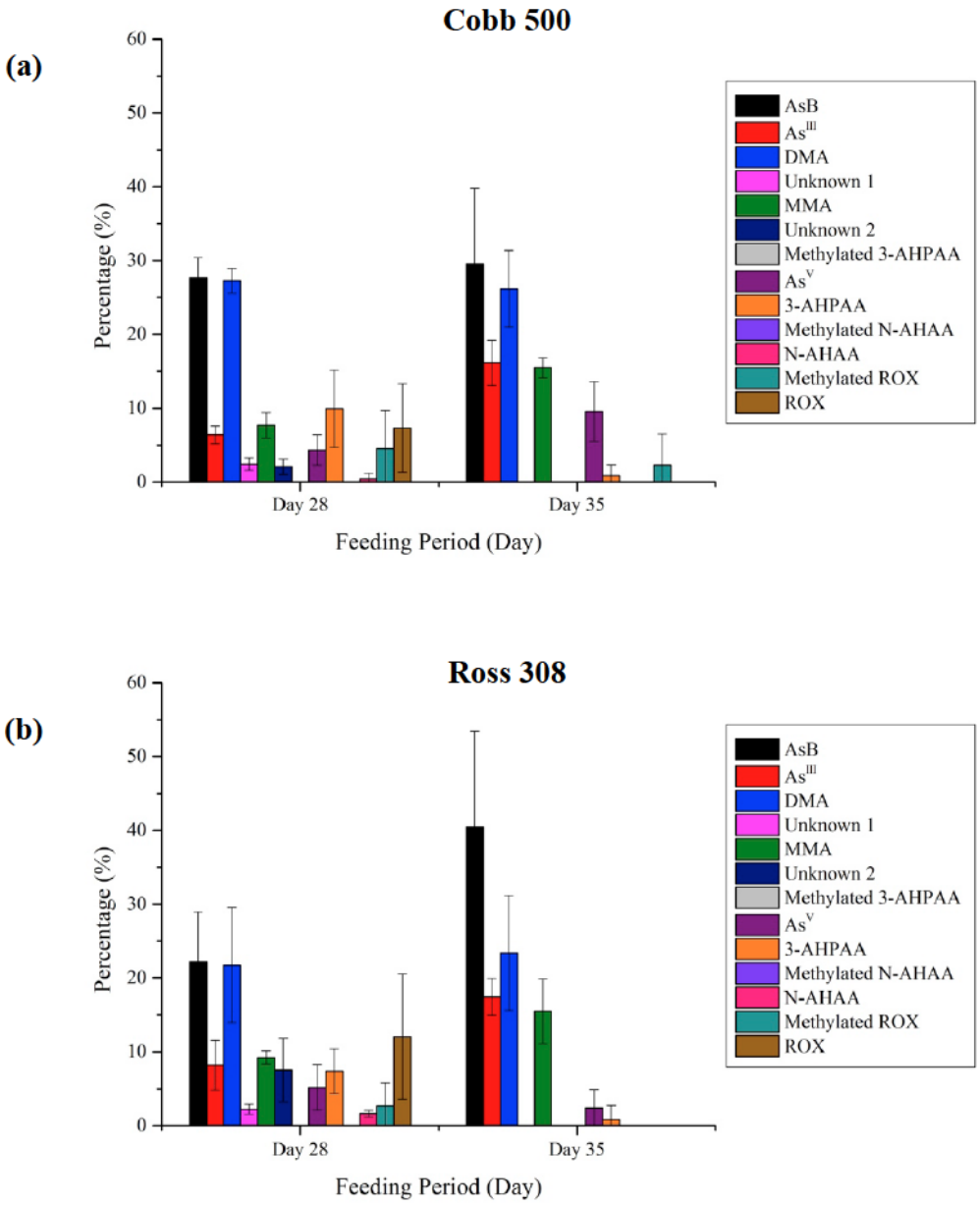


Figure 4.11 Percentages of different arsenicals in the skin samples of control chickens from strain (a) Cobb 500 and (b) Ross 308. The skin samples were collected on day 28 and day 35.

By comparing above speciation results in the skin, we can find that ROX was one of the major factors causing the difference in the sum of arsenic between the ROX-fed group and control group. This can also be seen from the ROX concentration comparison within the same day in Figure 4.12. More ROX had been detected in skin of ROX-fed chickens than in skin of control chickens. However, when the amount of ROX was subtracted from the sum of arsenic compounds, there was still a significant difference existing between ROX-fed group and control group (Figure 4.13). In order to explore the other compounds leading to the difference in the sum of arsenic, the temporal changes of other identified individual arsenicals were further studied. The results are illustrated in Figure 4.14-4.24. Paired t-tests were conducted for each species on each day (day 28 and day 35) between ROX-treated group and control group. P values for comparing ROX-treated group and control group in the concentrations of different arsenicals were included in Table 4.11.

The presence of AsB in the skin was most probably from the fish meal used in the chicken feed. As shown in Figure 4.14, the concentrations of AsB in the skin samples from ROX-fed and control chickens were very close to each other as expected on both day 28 and day 35, indicating it was not related to ROX.

But for phenylarsonic compounds (Figure 4.15-4.19), greater concentrations were observed in the ROX-fed group than in the control group for both strains. This kind of relationships was really similar to that of ROX (Figure 4.12), suggesting that transformation from ROX to phenylarsonic compounds could happen.

The results on inorganic arsenic (As^{III} and As^{V}) and the methylated arsenicals (MMA and DMA) appear to be more complicated. While the concentrations of As^{III} and total inorganic arsenic (As^{III} and As^{V}) are significantly greater in the ROX-treated chickens than in the control chickens (Figure 4.20 and Figure 4.22), the difference in the concentrations of MMA (Figure 4.23) and DMA (Figure 4.24) are not significant between the ROX-treated and the control chickens. In principle, Roxarsone could be degraded to inorganic arsenic which in turn could be methylated to MMA and DMA. The relatively low concentrations of MMA and DMA in the chicken skin did not allow for conclusive interpretation.

The comparison of concentration of individual arsenical in the same groups of chickens but on different days was conducted as well. For control chickens, all the arsenicals detected in chicken skin displayed similar concentrations on both day 28 and day 35 (Figure 4.12, 4.14-4.24). However, for ROX-fed chickens, the concentration relationships were quite different on these two days. As shown in Figure 4.12, more ROX had been detected in skin of ROX-fed chickens on day 28. The concentration of ROX in chicken skin on day 28 was 15-20 times greater than that on day 35. That was because of the removal of ROX from the feed on day 28. ROX was not administered to the chickens during the last week (day 28-35). ROX was eliminated from chicken skin during that period. Similar relationships were also found in the comparison results of As^{III} and other phenylarsonic compounds (Figure 4.15-4.20). But for AsB, DMA, MMA and As^{V}, the concentrations on both day 28 and day 35 were quite close to each other (Figure 4.14, 4.21, 4.23, 4.24).

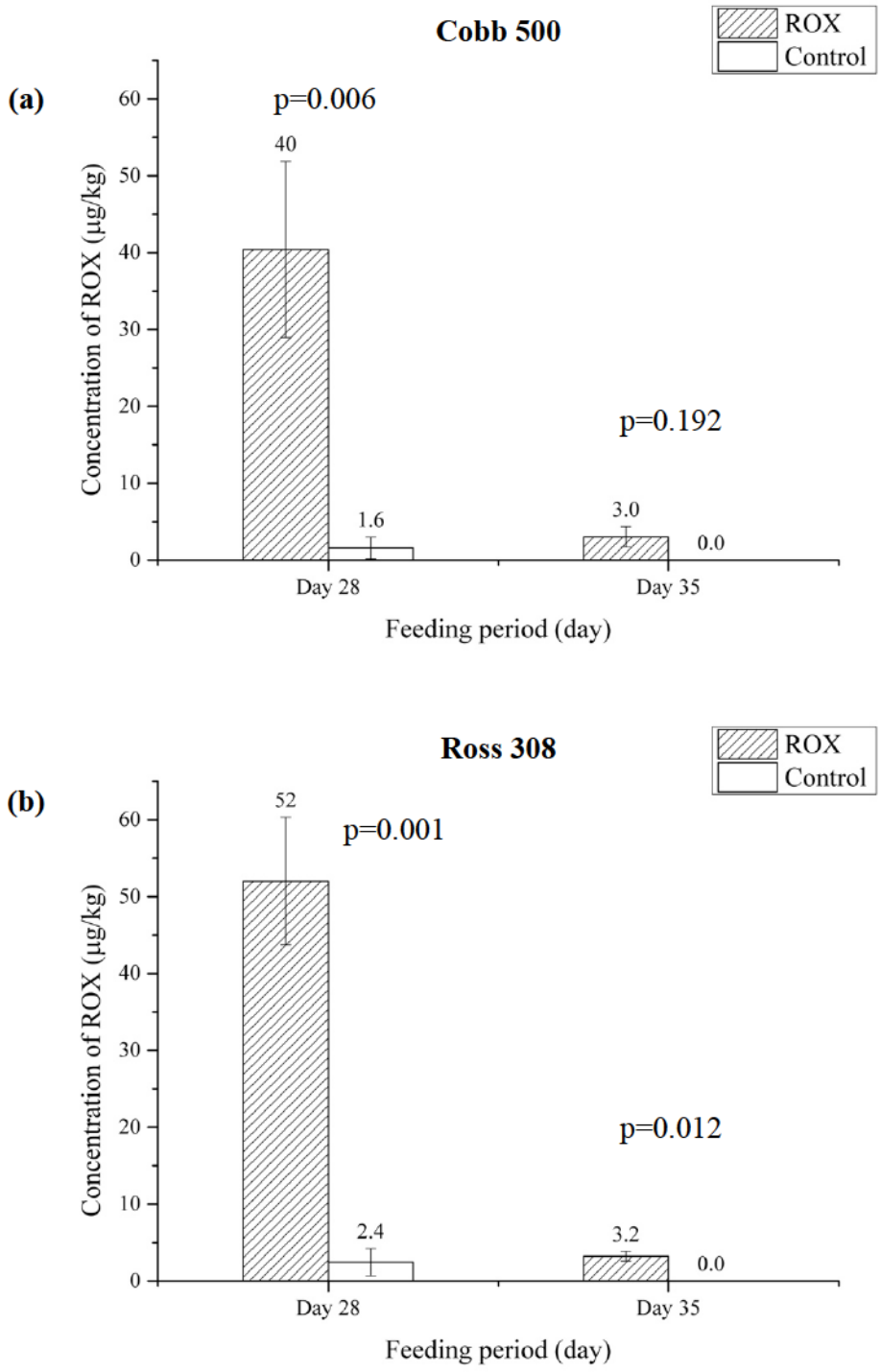


Figure 4.12 Concentration of ROX in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

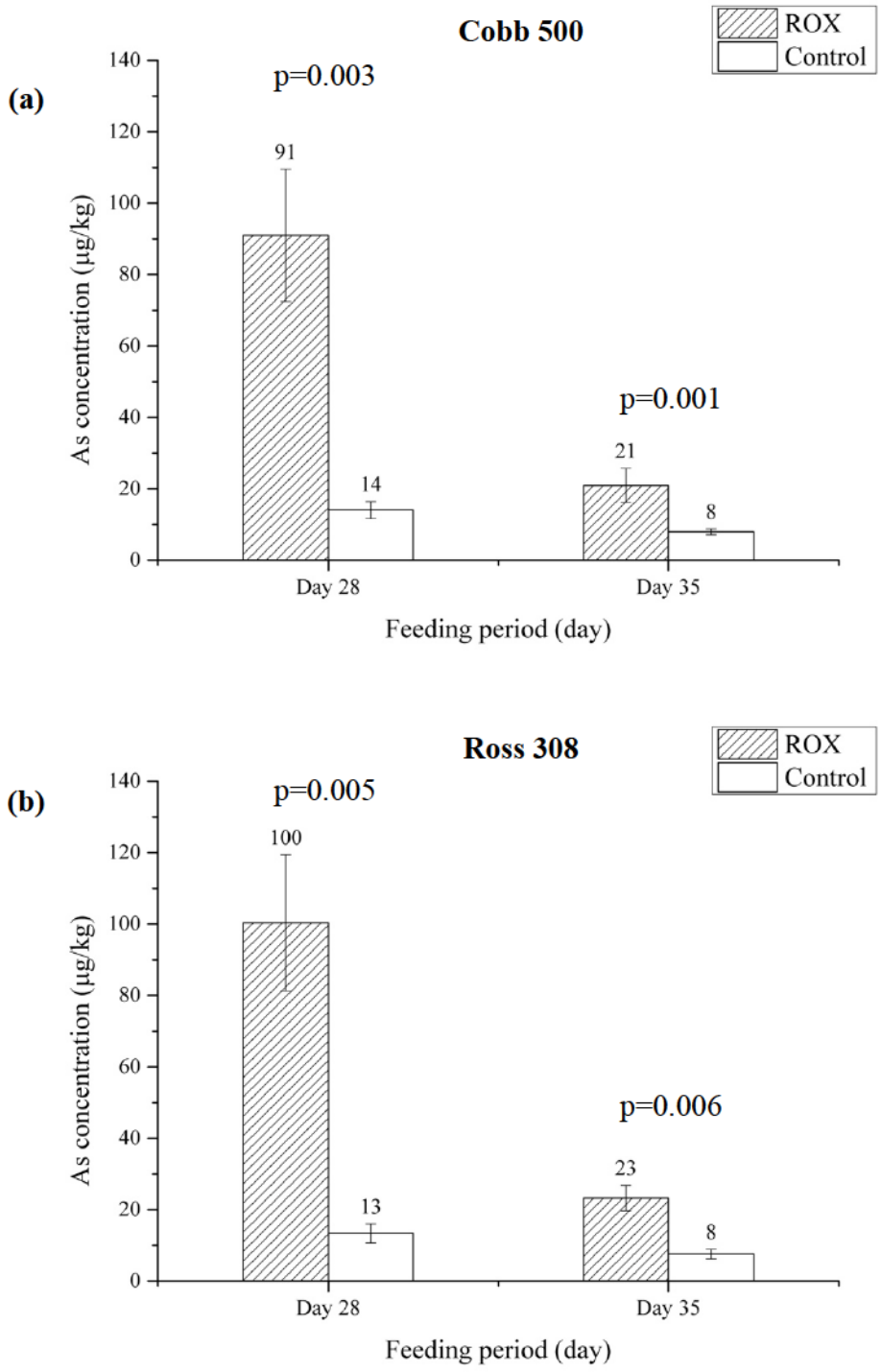


Figure 4.13 Concentration of all arsenicals except ROX in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

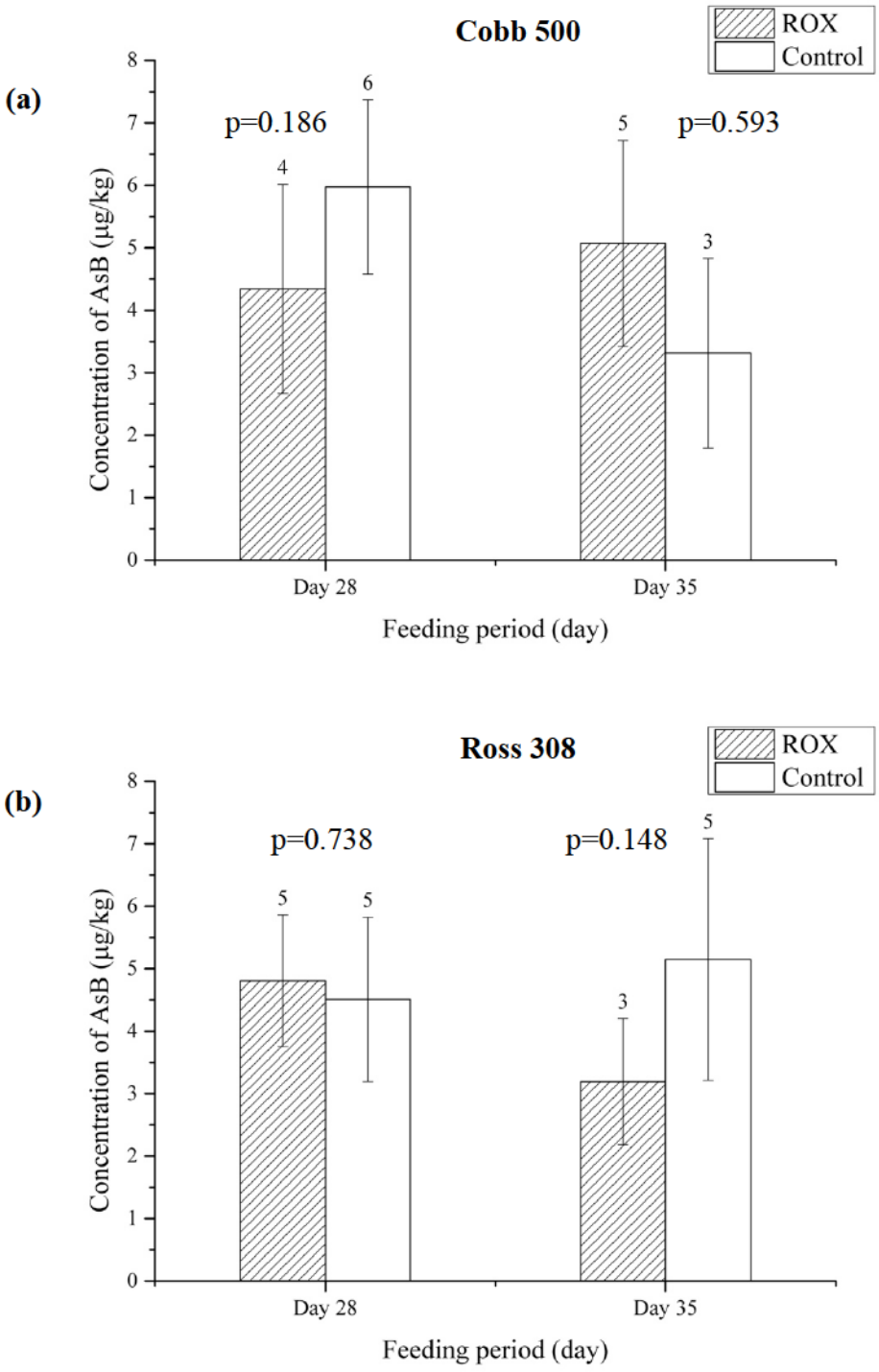


Figure 4.14 Concentration of AsB in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

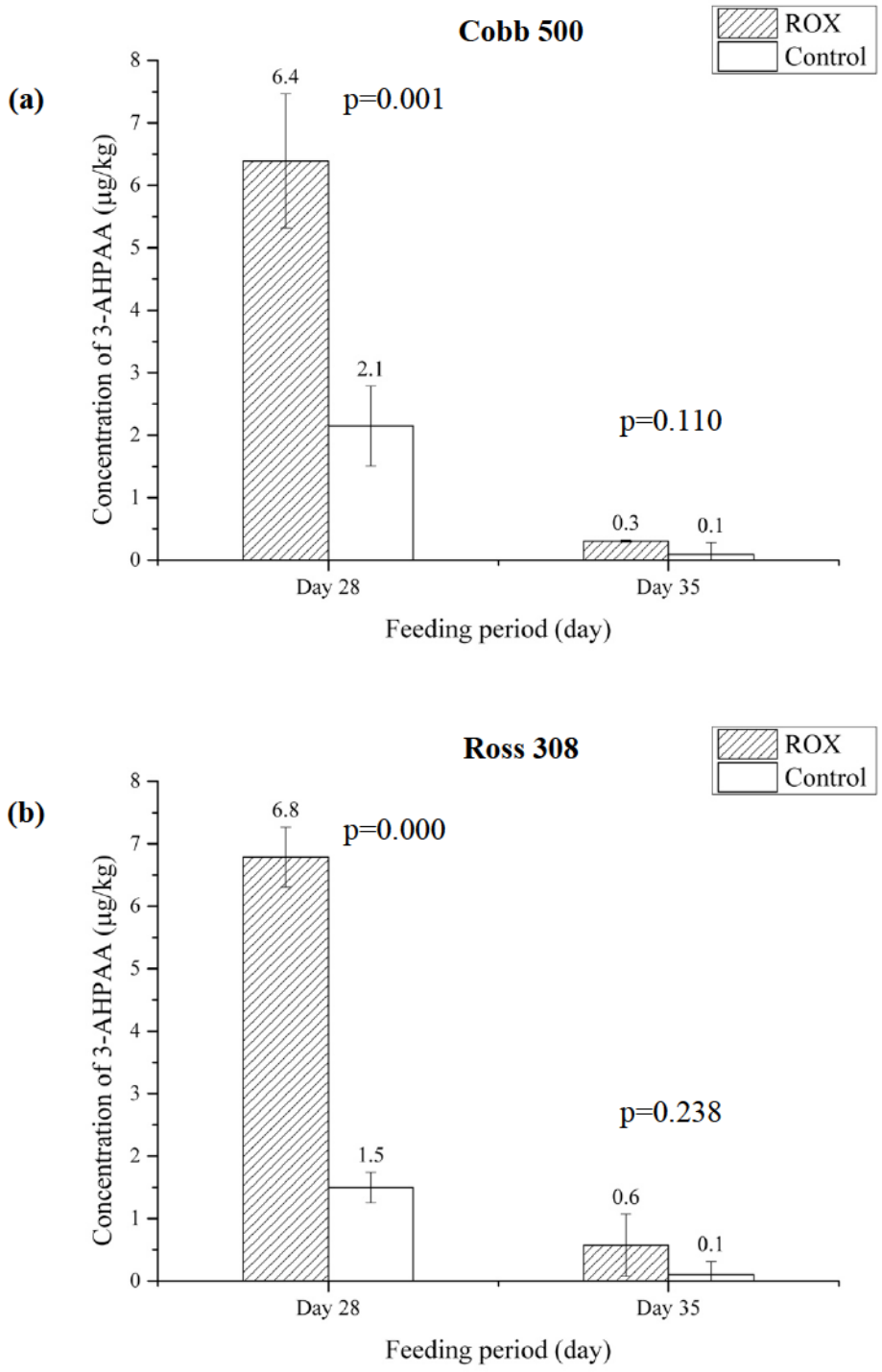


Figure 4.15 Concentration of 3-AHPAA in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

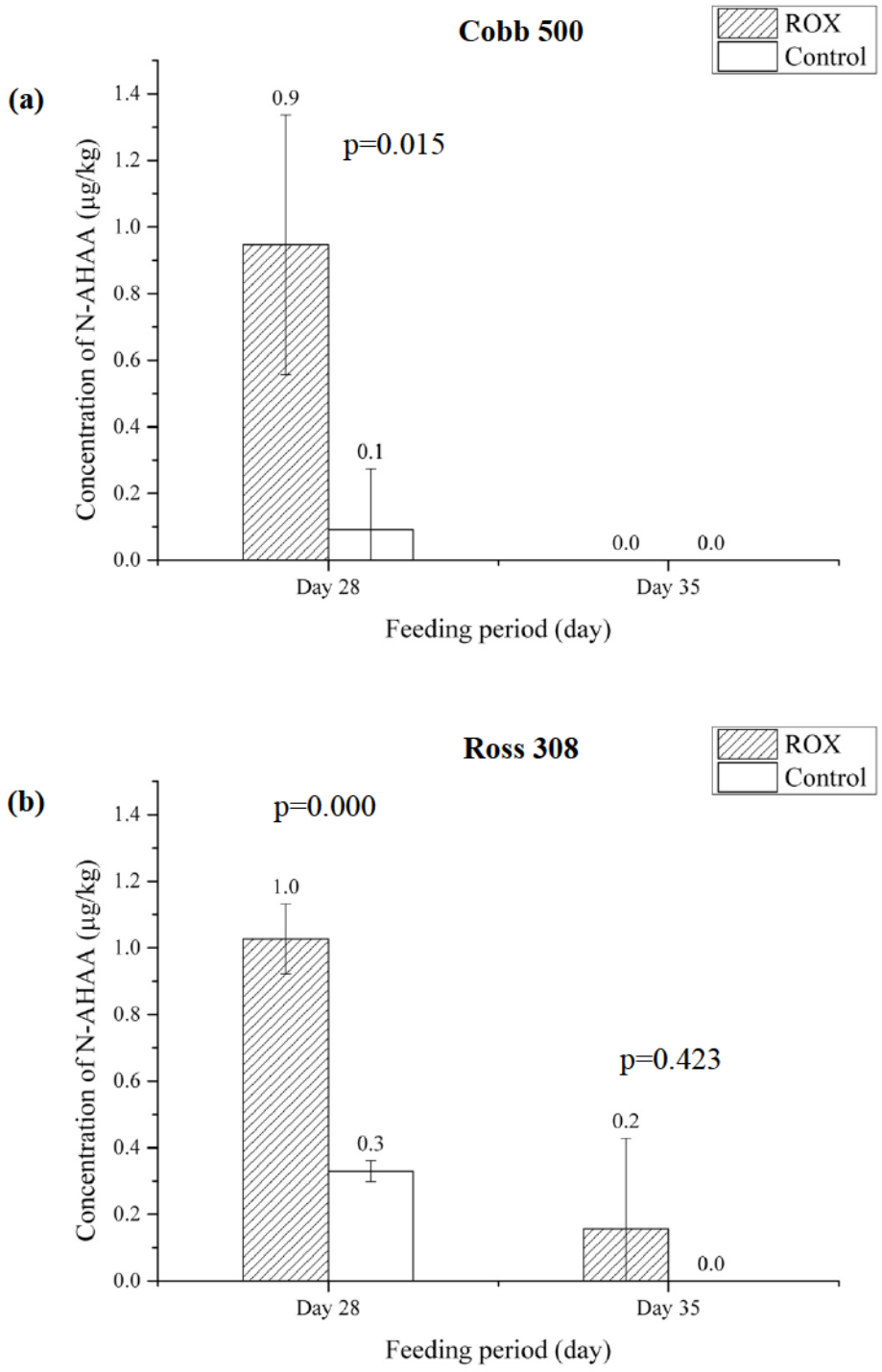


Figure 4.16 Concentration of N-AHAA in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

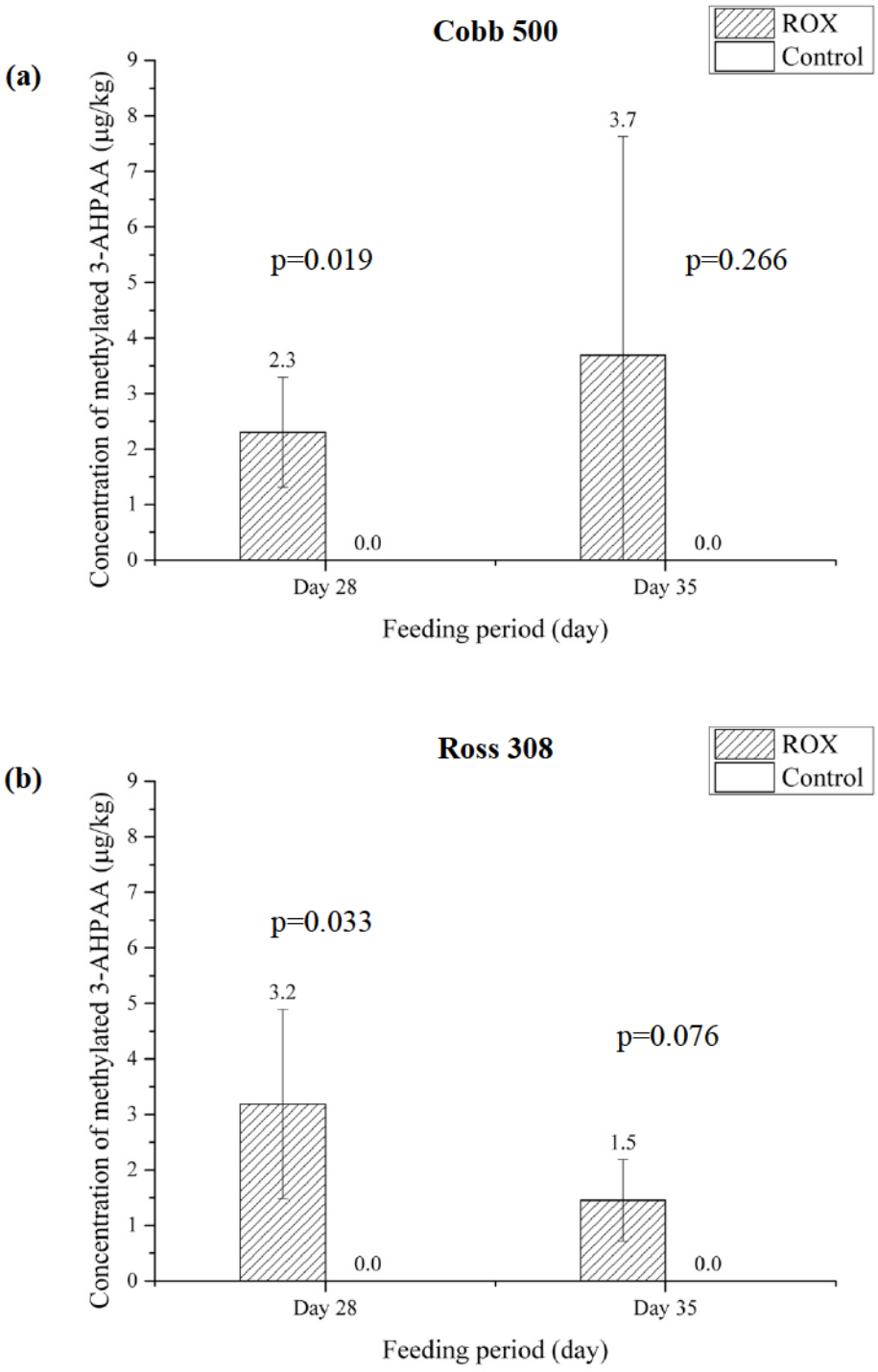


Figure 4.17 Concentration of methylated 3-AHPAA in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

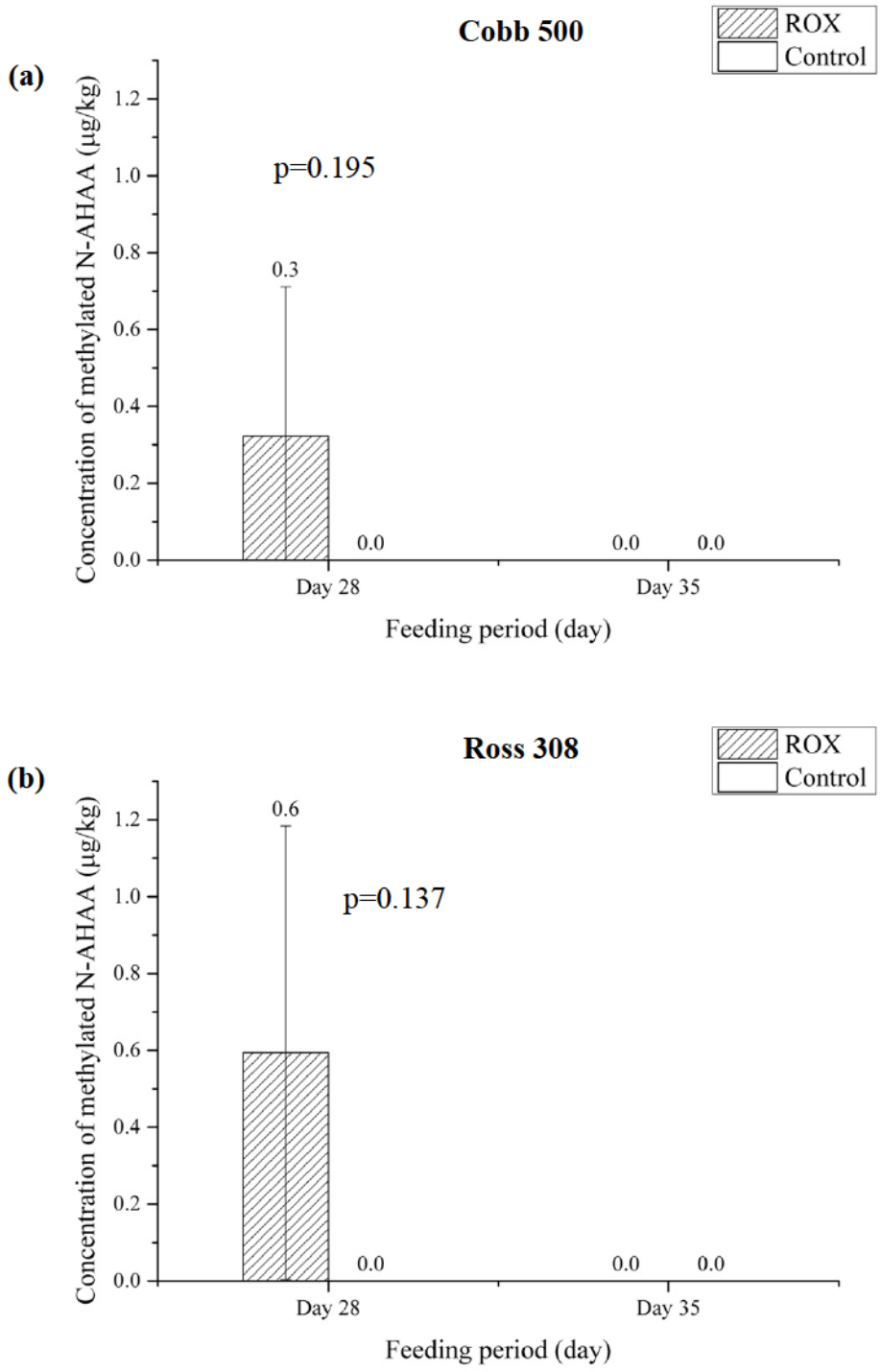


Figure 4.18 Concentration of methylated N-AHAA in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

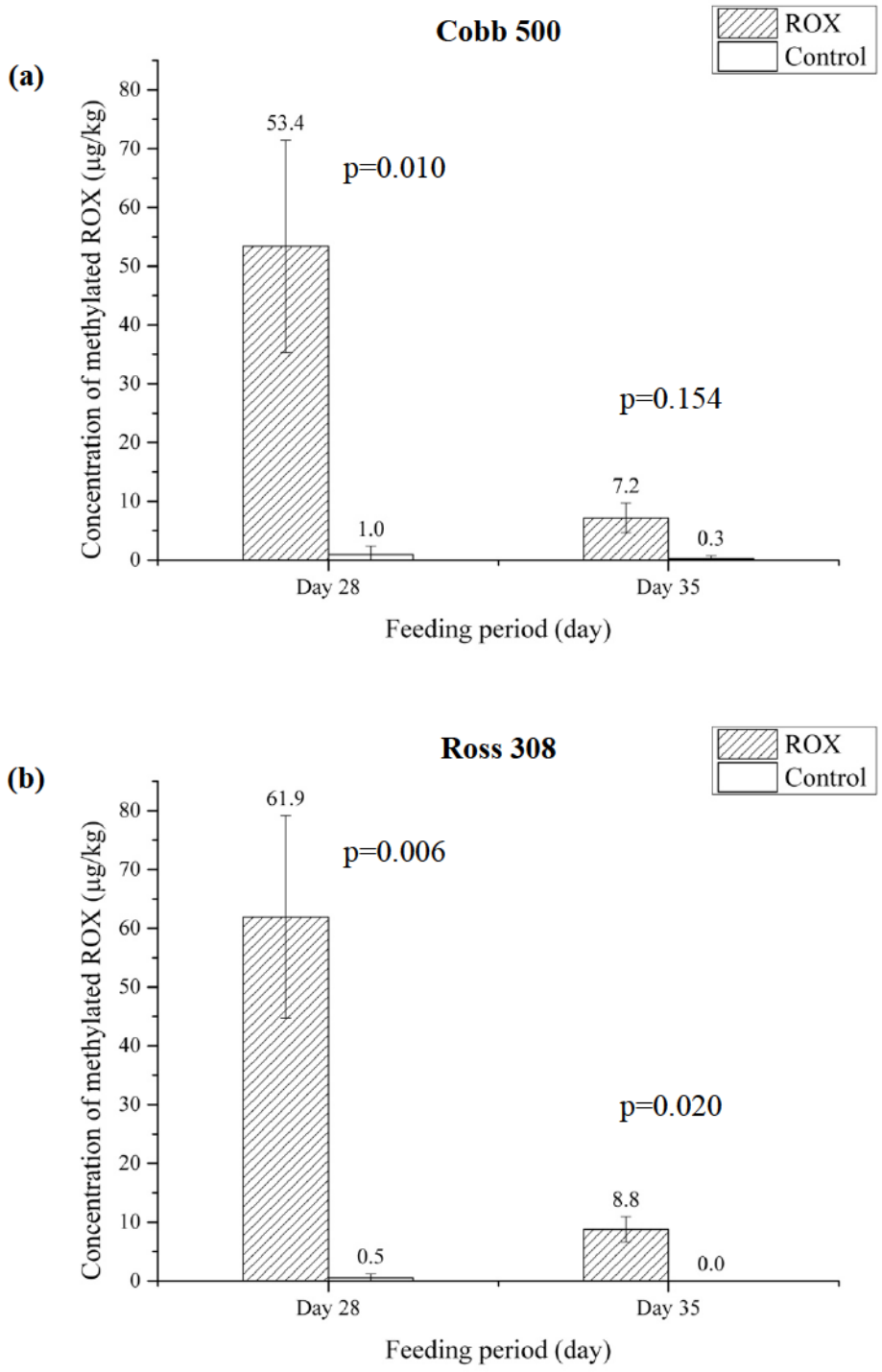


Figure 4.19 Concentration of methylated ROX in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

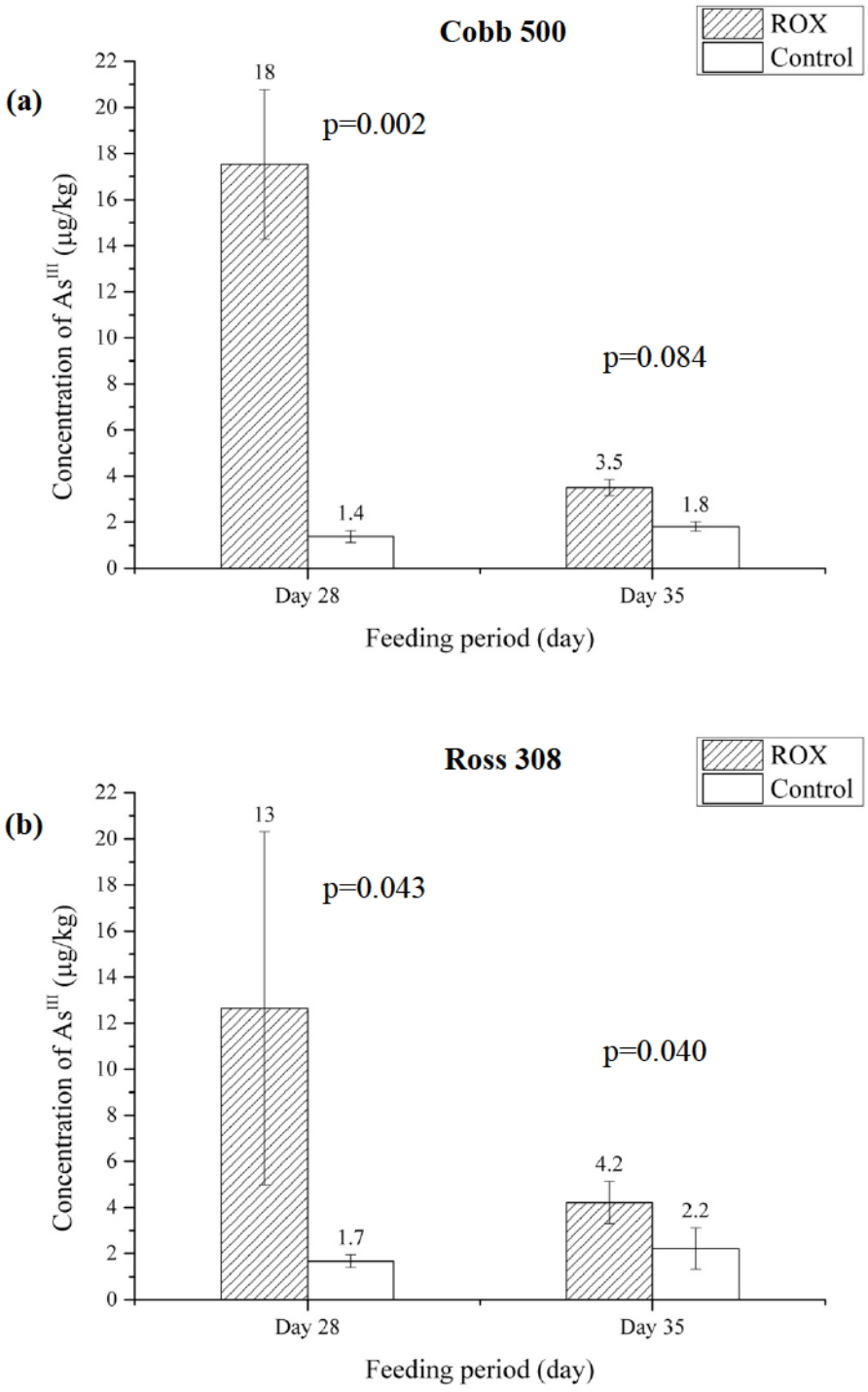


Figure 4.20 Concentration of As^{III} in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

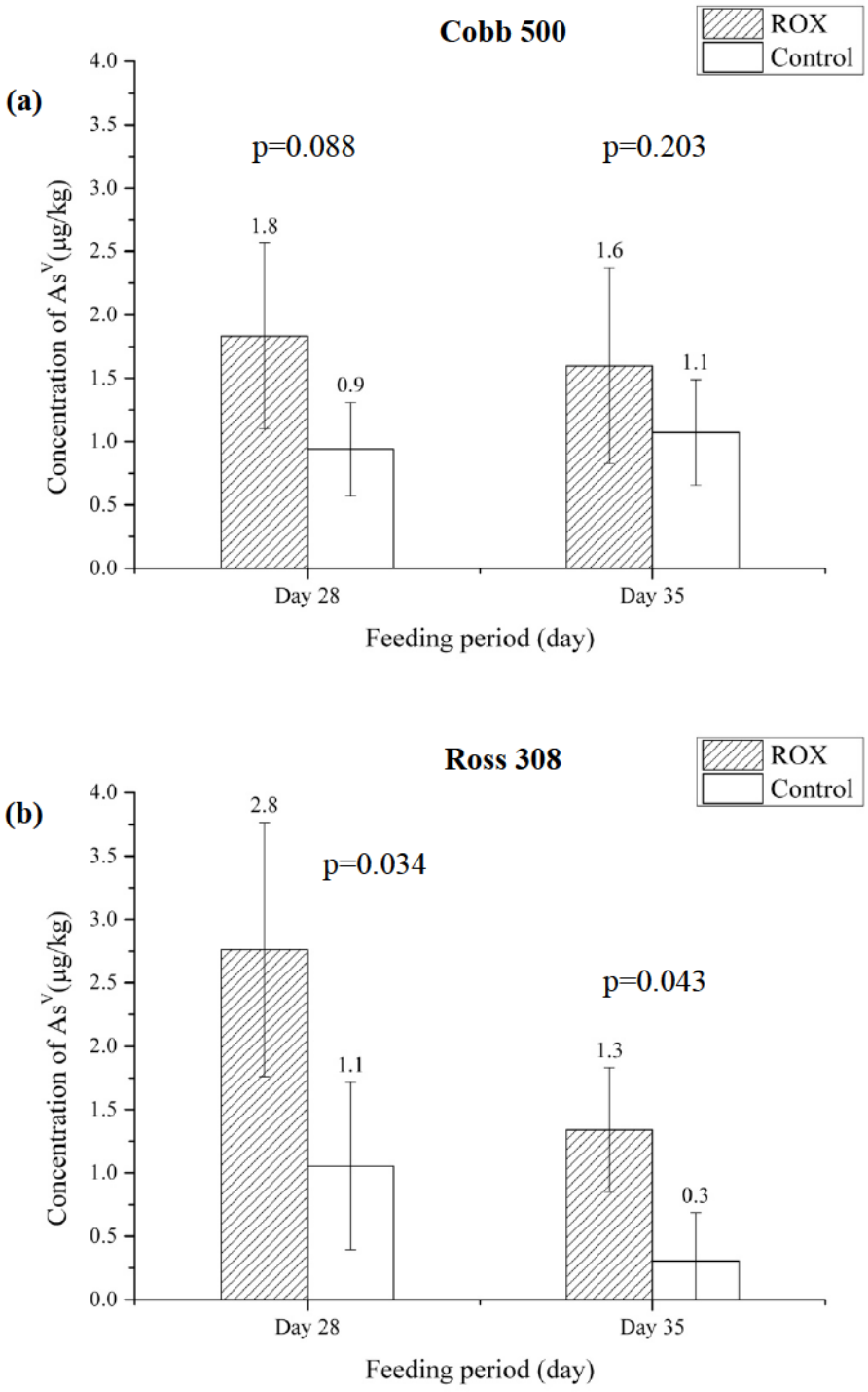


Figure 4.21 Concentration of As^{V} in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

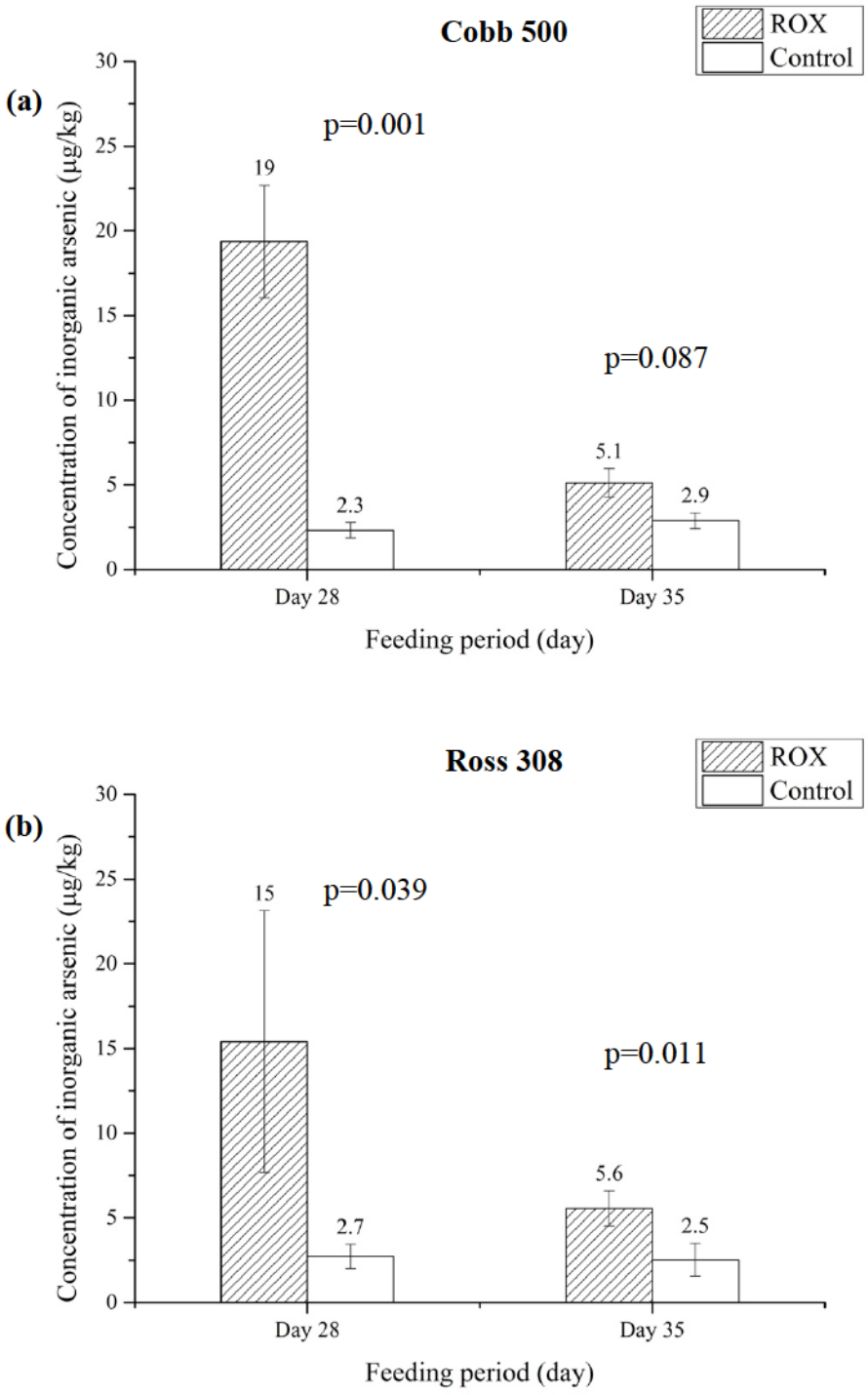


Figure 4.22 Concentration of inorganic arsenic (As^{III} and As^{V}) in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

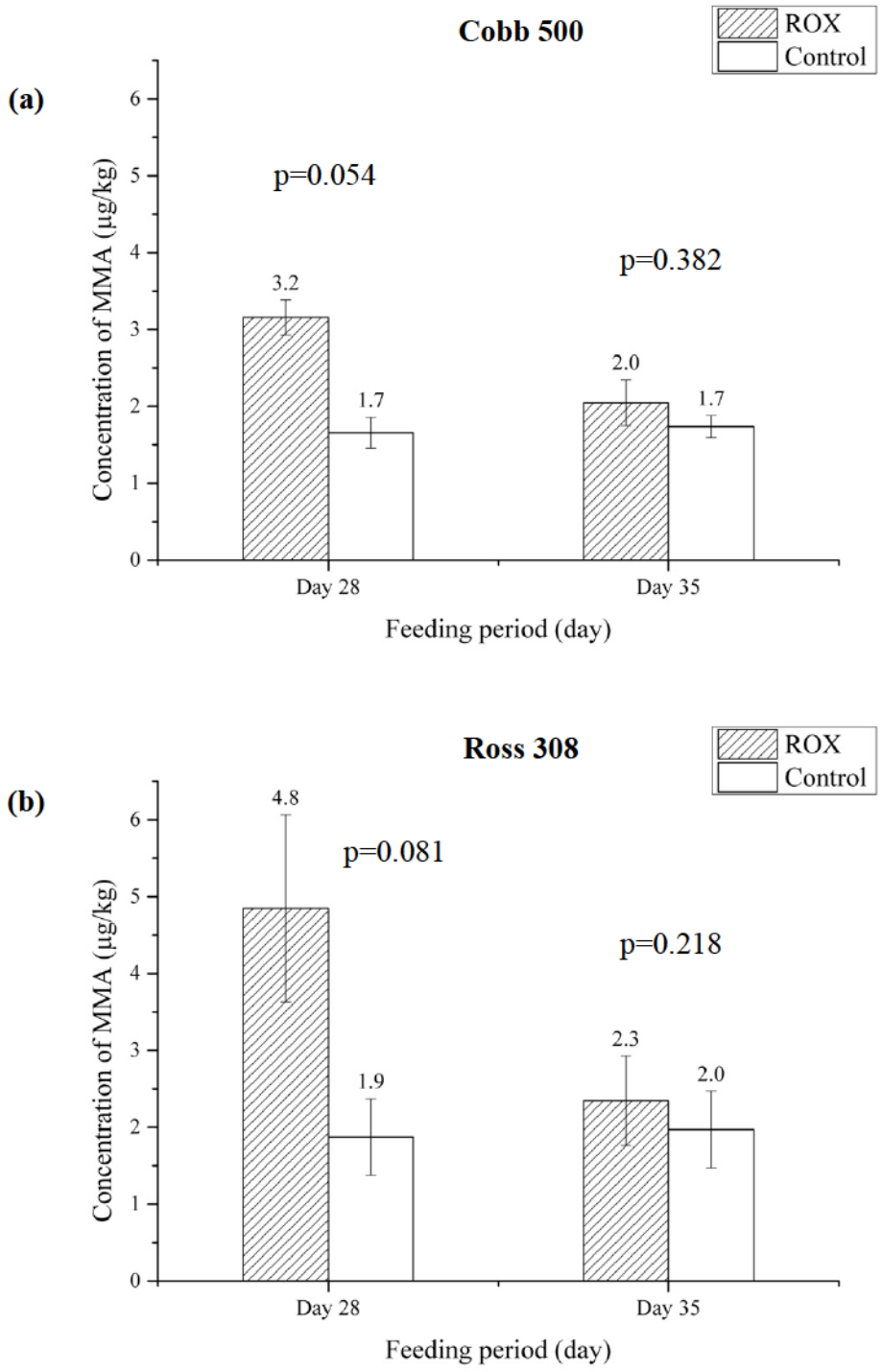


Figure 4.23 Concentration of MMA in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

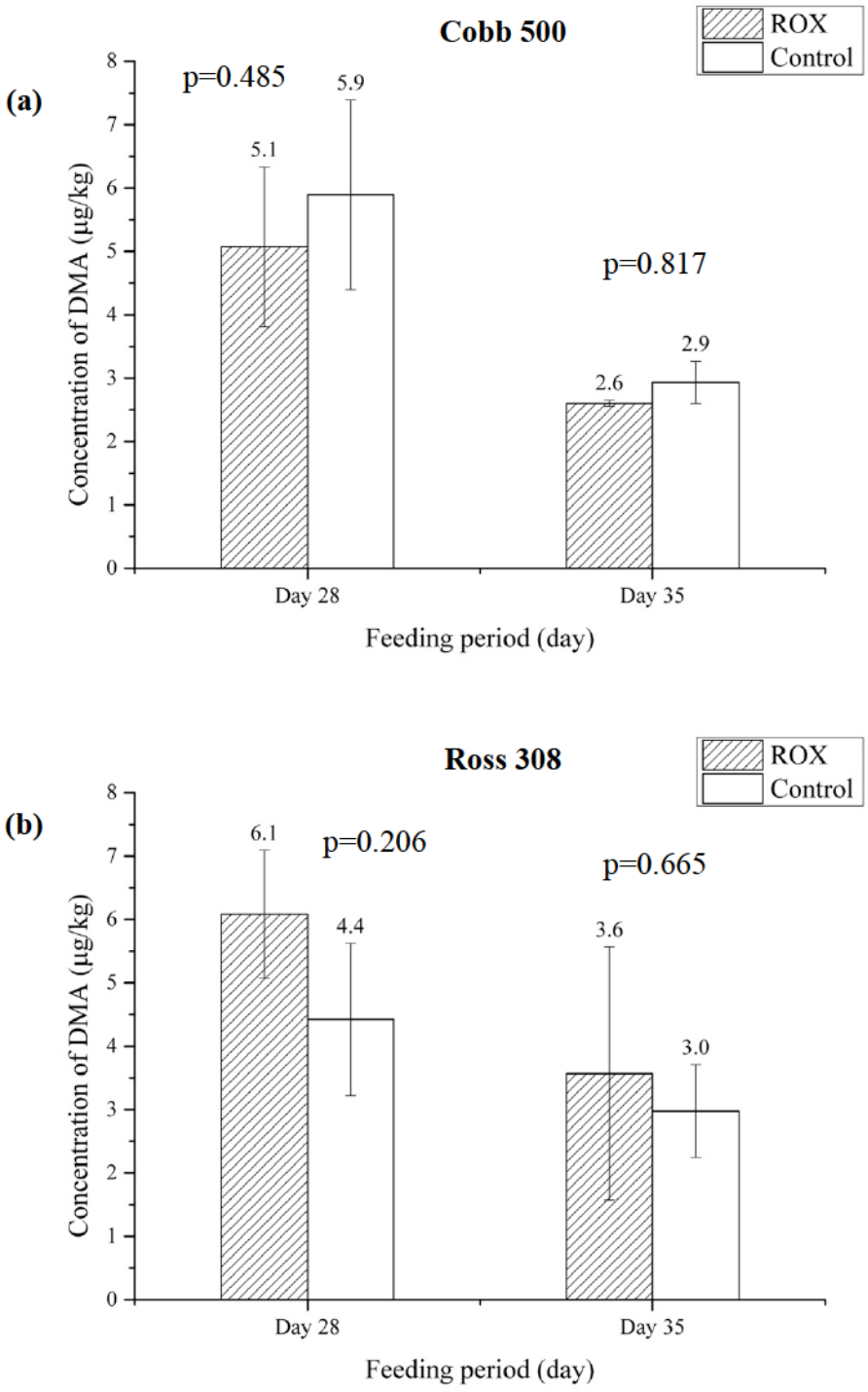


Figure 4.24 Concentration of DMA in chicken skin samples from strains (a) Cobb 500 and (b) Ross 308.

Table 4.11 P values for comparison between ROX-treated group and control group in the concentrations of arsenicals (t-test).

Feeding Period	**Day 28**		**Day 35**	
Strain	**Cobb 500**	**Ross 308**	**Cobb 500**	**Ross 308**
Species	**P value**	**P value**	**P value**	**P value**
AsB	0.186	0.738	0.593	0.148
As^{III}	0.002	0.043	0.084	0.040
DMA	0.485	0.206	0.817	0.665
Unknown 1	0.019	0.515	/	/
MMA	0.054	0.081	0.382	0.218
Unknown 2	0.391	0.254	/	0.423
Methylated 3-AHPAA	0.019	0.033	0.266	0.076
As^{V}	0.088	0.034	0.203	0.043
3-AHPAA	0.001	0.000	0.110	0.238
Methylated N-AHAA	0.195	0.137	/	/
N-AHAA	0.015	0.000	/	0.423
Methylated ROX	0.010	0.006	0.154	0.020
ROX	0.006	0.001	0.192	0.012
Sum of Arsenicals	0.004	0.031	0.000	0.001

4.4 DISCUSSION

4.4.1 Extraction of Arsenic

Enzymes were used for extracting arsenic from the chicken skin. In these enzyme solutions, some arsenic compounds had been detected, which were mainly inorganic arsenic compounds (As^{III} and As^{V}) and methylated arsenic compounds (DMA and MMA) (Figure 4.2). Among these arsenic species, As^{V} was the dominant species in all the four enzymes. The mechanism of arsenicals retained in enzymes was not clear. One possible way could be through the interaction between arsenic compounds and thiol groups in the enzyme. Concentration of arsenicals varied in different enzymes. In pancreatin and trypsin solutions, As^{V} had a concentration about 5 ppb that is 10 times greater than that in our skin sample extract. The high concentrations of arsenicals in enzymes would bring the interference for trace arsenic species detection.

Apart from the arsenic background of the enzyme, the recovery is another key point for enzyme extraction. The results of spike experiment (Table 4.3 and Figure 4.3) showed that pancreatin and trypsin had less than 30% recoveries for ROX, and they were not good for extracting ROX from chicken skin. It had also been found that pepsin had only 5% recovery for 3-AHPAA. The low recovery for arsenic compounds would affect the accuracy of speciation analysis. Papain had good recoveries for all eight major arsenic compounds ranging from 70% to 130%, and it showed low arsenic background. Therefore, as the most suitable one of these four enzymes, papain was selected for extracting arsenicals from chicken skin in our arsenic speciation study. The unique catalytic mechanism of papain

would be one possible reason that papain works well comparing to other enzymes. Papain is a cysteine protease, and it will digest most protein substrates more extensively than the pancreatic proteases (pancreatin and trypsin). Papain exhibits broad specificity, cleaving peptide bonds of basic amino acids, leucine, or glycine. It also hydrolyzes esters and amides. Papain could digest the chicken skin more completely and as a result, more arsenicals release from chicken skin.

4.4.2 Arsenic in Chicken Skin

By using our HPLC-ICPMS/ESIMS method, we achieved baseline resolution in the separation of eight major arsenic species (AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and ROX) within 15 min. By matching the retention times of arsenic species in chicken skin with those in the arsenic standard mixture using HPLC-ICPMS, the presence of AsB, As^{III}, DMA^{V}, MMA^{V}, As^{V}, 3-AHPAA, N-AHAA, and ROX in chicken skin was indicated. In order to confirm the identity of each arsenic peak, different concentrations of arsenic standards were added to the skin extract separately and analyzed under the same conditions. By observing the peak height change in the chromatograms, we could conclude that the peak with height change belonged to the arsenic species we added. The identification of arsenic species was also performed by using HPLC-ESIMS as ESIMS provided useful molecular information. The identities of arsenic species were further confirmed by simultaneous monitoring of the MRM transitions from the molecular ion to characteristic MS/MS fragments (Table 4.5). In chicken skin, five more unknown arsenic species had been discovered, and three of them had been tentatively identified as the methylated products of 3-

AHPAA, N-AHAA and ROX using the above identification method. As many methylation enzymes exist in chicken body, these methylated phenylarsonic compounds are possible to be converted through the methylation pathway. The finding of these methylated phenylarsonic products is novel, and it is first reported in this study.

In the HPLC-ICPMS/ESIMS system, the concentration of each arsenical was quantified against the calibration curve of the corresponding arsenic standard solution. According to the calibration curves and chromatograms, the LOD values were estimated as well. It has been reported that the LOD values for arsenicals using the HPLC-ICPMS method is 0.2-2.0 μg/L [12, 13]. As shown in Table 2.6, better results for eight arsenic species were achieved based on ICPMS data in our study.

Skin samples from day 28 and day 35 were studied by using this HPLC-ICPMS method. Samples in these two days reflected the final arsenic level and arsenic species in skin before and after ROX elimination. As illustrated in Figure 4.7, thirteen arsenic species with variable concentrations were present in the skin samples collected on these two days from ROX-fed chickens. Figure 4.9 indicates ROX and methylated ROX predominated in the skin samples of the ROX-treated group. These two compounds contributed to 68%-72% of the sum of arsenic on day 28 and 35%-41% of the sum of arsenic on day 35.

Five major ROX metabolites in total were identified in chicken skin. These ROX metabolites are methylated ROX, 3-AHPAA, methylated 3-AHPAA, N-

AHAA and methylated N-AHAA. These compounds showed similar concentration trends to that of ROX from day 28 to day 35. Because they were neither added in diets nor observed in control skin, conversion from ROX as the parent molecule is the most reasonable explanation of their presence in the skin of ROX-fed chickens considering their structural similarity. Inorganic arsenicals (As^{III} and As^{V}) consisted of 10-20% of the total arsenic. The sum of As^{III} and As^{V} demonstrated similar trends to that of ROX, suggesting that ROX may be partially degraded to inorganic arsenicals. Background levels of AsB, DMA, MMA were also detected in chicken skin samples. The presence of AsB was possibly a result of ingestion of the basal diet which contained fish meal. It does not change in the chicken body [14-16]. The source of DMA and MMA was not clear. These two arsenicals were probably the metabolites of inorganic arsenicals.

Possible pathways for the metabolism of Roxarsone are shown in Figure 4.25. Roxarsone can be reduced to 3-AHPAA which can further be acetylated to N-AHAA. Both 3-AHPAA and N-AHAA have been detected in the skin sample and previously reported in chicken liver [8, 17]. Roxarsone, 3-AHPAA and N-AHAA could be methylated to the respective methylated phenylarsenicals. Roxarsone may also be degraded to As^{V}, which in turn can be reduced to form As^{III} and methylated to form MMA and DMA.

The sum of arsenic on day 28 was 6 times greater than that on day 35 as ROX was withdrawn from the chicken feed during the final week. This result agrees with our finding of total arsenic concentrations.

In control skin samples, trace amount of ROX, methylated ROX and 3-AHPAA were detected. A possible reason would be the contamination of the chicken feed. AsB and DMA predominated in the control skin samples for both Cobb 500 and Ross 308 strains, which contributed to over 40% in total of all species present. The sum of arsenic in the skin of the control chickens did not change much from day 28 to day 35.

The concentrations of major arsenicals in chicken skin were also compared with that in other chicken organs (muscle, liver and kidney). Table 4.12 and Table 4.12 summarized the arsenic concentrations in different organs of ROX-fed chickens and control chickens. Generally, on both day 28 and day 35, the concentrations of arsenic in different organs demonstrated the same order in both ROX-fed chickens and control chickens. Liver had the highest sum of arsenic concentrations, followed by kidney, skin and muscle. Liver and kidney are the main metabolic organs and have high sulfur content, and skin has high content of cysteine-rich keratin. The differences in these organs' composition and function could lead to the difference of arsenic concentrations.

The concentrations of major arsenicals in chicken feed were also studied in our group for evaluating biotransformation of ROX. Typical chromatograms of feed samples are shown in Figure 4.26 and 4.27, and the quantitative results are summarized in Table 3.1.There was ROX with concentrations greater than 18 μg/g in the ROX-supplemented diets (ROX starter and ROX grower), which are major dietary sources of ROX and other arsenic metabolites of ROX-fed chickens. Consistently small amounts of AsB were detected in all diets, which

explained the presence of AsB in all skin samples. DMA and As^{V} were also present in diets. Their concentrations were about the same level as AsB. The technique was able to separate and determine As^{III}, MMA, 3-AHPAA, and N-AHAA and methylated phenylarsenicals; but they were not detectable in any of the six feed samples. Biotransformation from the existing arsenic species in diets was the only contributor to their presence in chicken skin. Probably due to feed cross-contamination, the existence of ROX in three feed samples (ROX finisher, control grower and control finisher) was observed. This could explain the existence of trace amounts of ROX, and other phenylarsenicals in the control skin samples (Figure 4.10, Table 4.9 and 4.10).

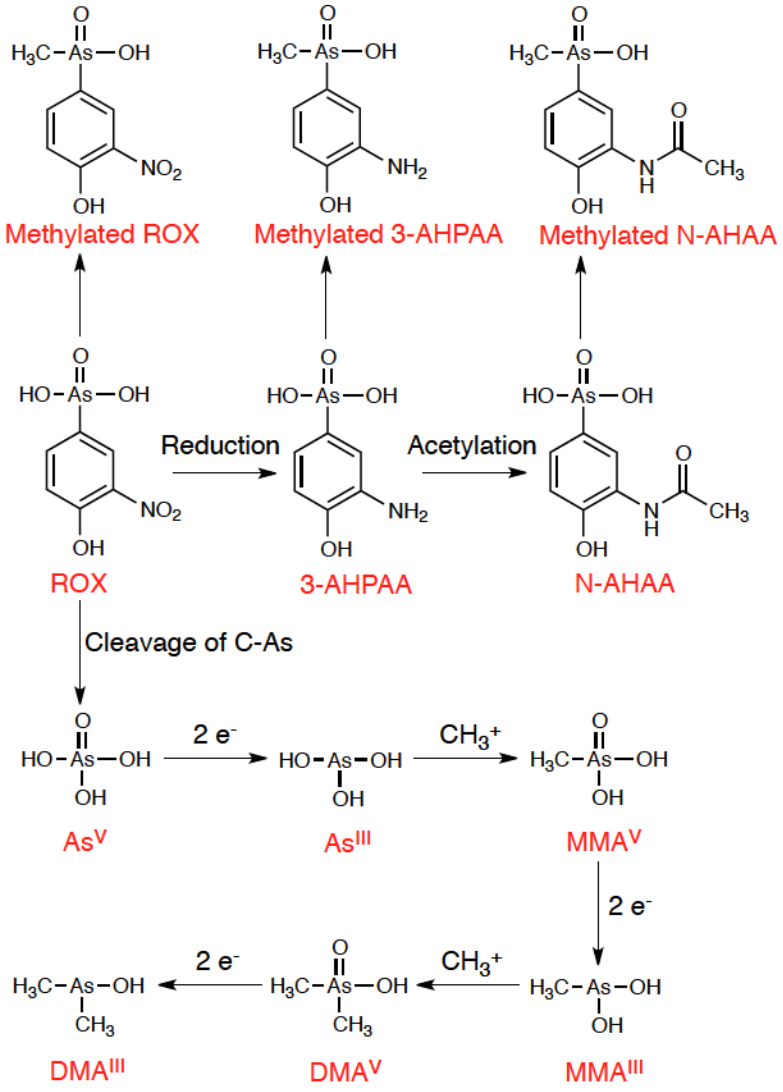


Figure 4.25 Possible pathways for the metabolism of Roxarsone in chicken. This figure was obtained from Ms. Qingqing Liu of the Dr. Chris Le group at the University of Alberta.

Table 4.12 Arsenic concentrations in different organs of ROX-fed chicken on day 28 and day 35.[a]

	Concentration of Arsenicals (mean ± SD) (μg/kg)							
	Day 28				Day 35			
	Skin	Muscle	Liver	Kidney	Skin	Muscle	Liver	Kidney
ROX	46 ± 11	5 ± 2	297 ± 71	106 ± 21	3.1 ± 0.8	0.41 ± 0.04	11 ± 2	33 ± 3
As^{III}+As^{V}	17 ± 6	30 ± 20	141 ± 65	97 ± 10	5.4 ± 0.9	3 ± 2	20 ± 4	7 ± 1
MMA+DMA	9 ± 2	22 ± 13	77 ± 86	29 ± 26	5 ± 2	3.2 ± 0.7	6 ± 2	8.0 ± 0.6
AsB	5 ± 1	25 ± 6	16 ± 4	14 ± 4	4 ± 1	33 ± 15	17 ± 6	15 ± 5
Sum of phenylarsenicals	68 ± 17	5 ± 2	636 ± 116	23 ± 2	11 ± 3	0.8 ± 0.3	143 ± 81	4.6 ± 0.4
Sum of As excluding AsB	142 ± 21	62 ± 24	1151 ± 174	256 ± 12	25 ± 4	8 ± 2	179 ± 81	56 ± 5

[a] SD: standard deviation.

Note: This table was constructed in collaboration with Ms. Qingqing Liu, Mr. Hanyong Peng and Ms. Xuan Sun. Data for chicken muscle was obtained from Ms. Qingqing Liu. Data for chicken liver was obtained from Mr. Hanyong Peng. Data for chicken kidney was obtained from Ms. Xuan Sun.

Table 4.13 Arsenic concentrations in different organs of control chicken on day 28 and day 35.[a]

	Concentration of Arsenicals (mean ± SD) (μg/kg)							
	Day 28				Day 35			
	Skin	Muscle	Liver	Kidney	Skin	Muscle	Liver	Kidney
ROX	2 ± 2	N.D.	9 ± 5	21 ± 5	N.D.	N.D.	3.1 ± 0.9	23 ± 7
$As^{III}+As^{V}$	2.5 ± 0.6	N.D.	6 ± 3	7 ± 2	2.7 ± 0.8	N.D.	15 ± 5	3.8 ± 0.5
MMA+DMA	7 ± 2	8 ± 7	5 ± 10	12 ± 3	4.8 ± 0.6	2.5 ± 0.3	3 ± 2	8.3 ± 0.8
AsB	5 ± 2	26 ± 9	18 ± 6	14 ± 2	4 ± 2	31 ± 12	23 ± 10	12 ± 3
Sum of phenylarsenicals	3 ± 1	N.D.	15 ± 8	1.4 ± 0.2	0.3 ± 0.4	N.D.	10 ± 3	3.8 ± 0.3
Sum of As excluding AsB	16 ± 3	8 ± 7	35 ± 14	42 ± 9	8 ± 1	2.5 ± 0.3	31 ± 6	39 ± 8

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

Note: This table was constructed in collaboration with Ms. Qingqing Liu, Mr. Hanyong Peng and Ms. Xuan Sun. Data for chicken muscle was obtained from Ms. Qingqing Liu. Data for chicken liver was obtained from Mr. Hanyong Peng. Data for chicken kidney was obtained from Ms. Xuan Sun.

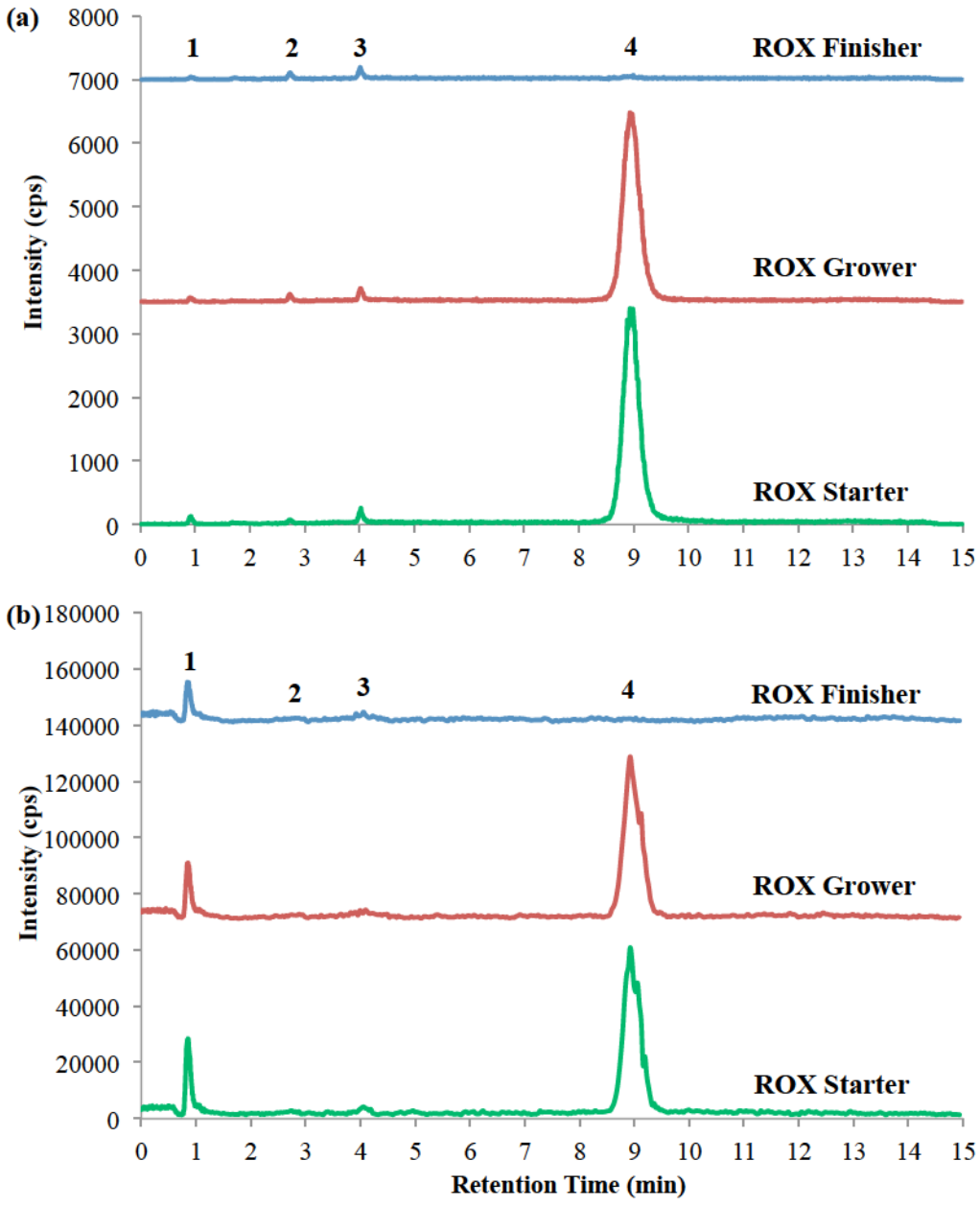


Figure 4.26 Chromatograms obtained from the analyses of ROX-treated feed samples using (a) HPLC-ICPMS and (b) HPLC-ESIMS. Peaks: (1) AsB, (2) DMA, (3) As^{V}, (4) ROX. This figure was obtained from Mr. Zonglin Yang of the Dr. Chris Le group at the University of Alberta.

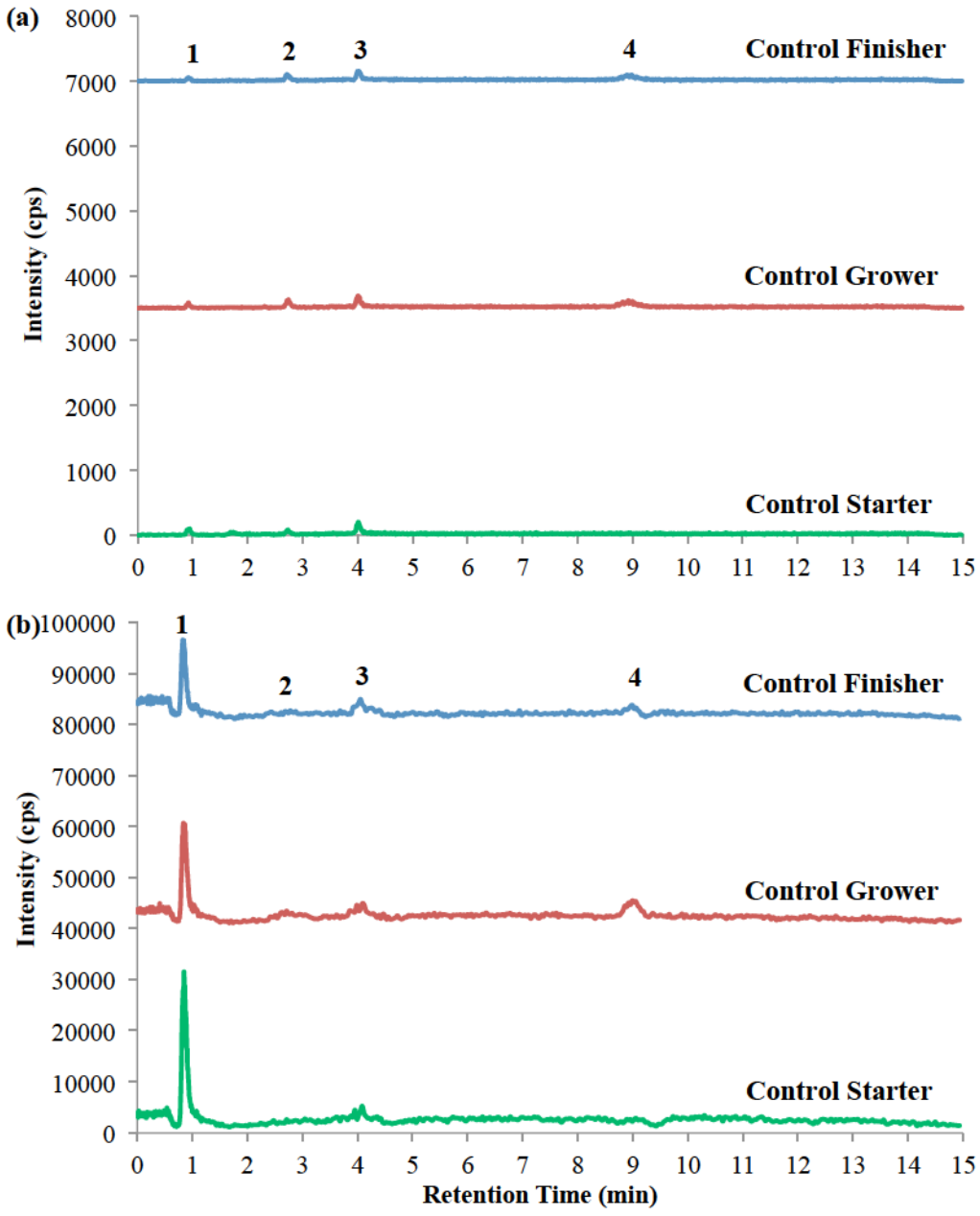


Figure 4.27 Chromatograms obtained from the analyses of untreated feed samples using (a) HPLC-ICPMS and (b) HPLC-ESIMS. Peaks: (1) AsB, (2) DMA, (3) As^{V}, (4) ROX. This figure was obtained from Mr. Zonglin Yang of the Dr. Chris Le group at the University of Alberta.

4.5 CONCLUSIONS

Arsenic species present in chicken skin were extracted with the assistance of papain digestion. These arsenicals in the extract were then determined by using HPLC-ICPMS/ESIMS. This method provided complementary identification and quantitation of arsenic species in chicken skin. Methylated products of 3-AHPAA, N-AHAA and ROX, were observed in chicken skin for the first time. These compounds and 3-AHPAA, N-AHAA are likely metabolites of ROX. Investigations of the mechanism of formation of methylated 3-AHPAA, methylated N-AHAA and methylated ROX would be very useful.

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CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 REVIEW OF THESIS OBJECTIVES

Roxarsone® (ROX, 3-nitro-4-hydroxyphenylarsonic acid) has been added to chicken feed in order to prevent coccidiosis, improve feed efficiency, and promote growth. This phenylarsenical may be metabolized in chicken body and generate some more toxic arsenic compounds. The toxic inorganic arsenic compounds have already been found in liver and muscle of ROX-fed chickens, but little information is known about arsenic in chicken skin. Chicken skin is consumed directly as food, and it is also processed into animal feed as protein source. The objective of this thesis was to study the total arsenic concentration and arsenic speciation in skin of chickens fed either ROX-supplemented feed or the control feed. In Chapter 2, I have described a method using inductively coupled plasma mass spectrometry (ICPMS). I then applied the method to the determination of total arsenic concentration in chicken skin from a 35-day feed experiment (Chapter 3). In Chapter 4, I further determined the arsenic species in selected chicken skin samples.

5.2 SUMMARY OF RESULTS

ICPMS quantitation was developed with high sensitivity, low limit of quantitation (LOQ), good linear response and good accuracy. A H_2SO_4/HNO_3 digestion method was also optimized for the treatment of the solid skin samples.

During the 35-day feeding period, arsenic concentration in chicken skin increased rapidly during the first week and decreased quickly in the last week after the feeding of ROX stopped. When comparing the arsenic concentrations in skin samples on the same day, I detected higher concentrations of arsenic in the skin of ROX-treated chickens than in the skin of control chickens. ROX played an important role in the higher concentration of arsenic in chicken skin. The ROX-fed chickens had 5 times high arsenic in the skin than the control chickens at the end of the ROX supplement period on day 28. During the last week, ROX was removed from the chicken feed, and this led to the decrease of arsenic concentration in skin of ROX-fed chickens. Most of the arsenic was eliminated by the end of the ROX withdraw period. However, on the last day of the feeding period, the arsenic concentration was still significant higher in skin of ROX-fed chickens compared to that of control chickens. The concentrations of total arsenic in skin of both ROX-fed chickens and control chickens were all below the tolerance level (500 μg/kg) of US Food and Drug Administration.

Arsenic species was extracted with the assistance of papain digestion and determined by using a hyphenated HPLC-ICPMS/ESIMS method. This method enabled the simultaneous identification (from ESIMS) and quantitation (from ICPMS) of arsenic species after a single HPLC separation. Successful separation of arsenobetaine (AsB), arsenite (As^{III}), dimethylarsinic acid (DMA^{V}), monomethylarsonic acid (MMA^{V}), arsenate (As^{V}), 3-amino-4-hydroxy-phenylarsonic acid (3-AHPAA), N-acetyl-4-hydroxy-m-arsanilic acid (N-AHAA), and 3-nitro-4-hydroxy-phenylarsonic acid (ROX) was achieved within 15 min

using anion exchange chromatography. Apart from these eight major arsenic compounds, five more arsenic species were newly discovered, and three of them had been tentatively identified as the methylated products of 3-AHPAA, N-AHAA and ROX. Speciation analyses of skin samples collected on day 28 and day 35 showed that the major arsenic species in the ROX-treated groups were ROX and methylated ROX, accounting for 70% of the total arsenic on day 28 and 40% of the total arsenic on day 35. Several significant biotransformation products, 3-AHPAA, N-AHAA, methylated 3-AHPAA and methylated N-AHAA were identified. They consisted of 7% of the total arsenic. Inorganic arsenicals (As^{III} and As^{V}) have a total percentage about 15%-20%, and most of them are in the trivalent form. In control chicken skin samples, the dominant species were AsB and DMA, accounting for greater than 40% of total arsenic. The concentration of total arsenic in the control skin was 6 times lower than that in the skin of ROX-fed chickens on day 28, and it was half of the value of the concentration of total arsenic in the skin of ROX-fed chickens on day 35. This relationship agrees with our finding on the total arsenic concentrations obtained from direct ICPMS analyses.

5.3 FUTURE RESEARCH

The digestion of solid samples is one of the most important parts for the total element study. Current acid digestion method provided good accuracy (99%-113% recovery for certified reference material), but it was time-consuming (2 days for sample digestion). Other techniques, such as microwave digestion, could be investigated to further improve the digestion process [1].

For arsenic speciation, extracting all the arsenic species efficiently from solid samples is challenging. I only examined the use of different types of enzyme. More parameters, such as the enzyme-substrate ratio and solvent volume could be explored to improve the extraction efficiency. The concentrations of most arsenic species in chicken skin samples were very low. Preconcentration techniques may be useful. Freeze-drying technique could be applied for concentration [2]. Because of the use of enzyme in the extraction process, the matrix in the extract was complicated. Other techniques, such as solid phase extraction (SPE) [3], solid phase microextraction (SPME) [4], ionic liquid combined with hollow fiber liquid-liquid microextraction [5] could be investigated to remove the matrix and concentrate the analytes.

In the skin of ROX-fed chickens, 3-AHPAA, N-AHAA, methylated 3-AHPAA, methylated N-AHAA and methylated ROX were detectable. The formation of 3-AHPAA from ROX has been reported through the reduction of the nitro group in ROX to an amino group [6, 7]. Further research is required to understand the formation of N-AHAA and the methylated phenylarsonic compounds. Besides, there were also two other unknown arsenic species present in chicken skin, and their structures and formation have not been explored.

This thesis has shown that a small amount of ROX was converted to several other arsenic compounds. It is still unknown whether this conversion process occurred in chicken skin or in other organs. A comprehensive study can be carried out about the distribution of arsenic compounds in different chicken tissues to further investigate the metabolism of ROX in chicken body.

5.4 REFERENCES

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APPENDIX

SUPPORTING INFORMATION

Table A.1 Compositions of chicken feed. This information was provided by our collaborator Dr. Martin Zuidhof at the University of Alberta.

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

Ingredient Name	Percent	Percent	Percent	Percent	Percent	Percent
Dicalcium Phosphate	1.546	1.005	1.081	1.546	1.005	1.081
Salt, Plain (NaCl)	0.426	0.337	0.358	0.426	0.337	0.358
L-Lysine	0.232	0.151	0.154	0.232	0.151	0.154
DL-Methionine	0.229	0.096	0.089	0.229	0.096	0.089
L-Threonine	0.048	0.101	0.025	0.048	0.101	0.025
Broiler Vitamin Premix (0.5% inclusion)	0.50	0.50	0.50	0.50	0.50	0.50
Choline Chloride Premix (0.5% inclusion)	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin E 5000 IU/kg	0.30	0.30	0.30	0.30	0.30	0.30
Generic Enzyme (0.05% inclusion)	0.05	0.05	0.05	0.05	0.05	0.05
Coccidiostat (Amprol)	0.05	0.05	0.05	0.05	0.05	0.05
Antibiotic Growth Promoter (ROX)	0	0	0	0.025	0.025	0.025
Total	100	100	100	100	100	100

Table A.2 Chicken body weight during the feeding period.[a] This information was provided by our collaborator Dr. Martin Zuidhof at the University of Alberta.

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
29	1709 ± 277	1636 ± 221	1643 ± 127	1711 ± 194
30	1610 ± 195	1737 ± 233	1729 ± 169	1672 ± 536
31	1623 ± 407	1719 ± 301	1713 ± 117	1752 ± 235
32	2022 ± 263	1887 ± 168	1742 ± 234	2213 ± 139
33	2004 ± 92	2004 ± 345	2070 ± 54	1984 ± 172

[a] SD: standard deviation.

Table A.3 Detailed concentrations of total arsenic in each chicken skin sample.[a]

Sample Group: ROX-fed Chickens (Strain Cobb 500)				
Total Arsenic Concentration (mean ± SD) (µg/kg)				
Day	Pen 1	Pen 16	Pen 19	Pen 24
0	1.5	5.9	6.7	3.7
1	41.2	24.1	63.4	/
2	/	119.7	365.9	230.3
3	154.8	103.2	182.3	234.1
4	267.3	500.7	286.2	351.5
7	528 ± 3	345 ± 22	596 ± 41	557 ± 49
14	403.6	285 ± 24	348 ± 17	258 ± 4
21	148 ± 18	163 ± 8	161 ± 15	277 ± 8
28	365 ± 66	301 ± 32	296 ± 43	372 ± 33
29	243 ± 51	256 ± 49	268 ± 10	217 ± 53
30	160 ± 21	162 ± 9	143 ± 9	170 ± 8
31	191 ± 7	168 ± 23	178 ± 11	195 ± 44
32	/	125 ± 10	85 ± 11	128 ± 6
33	111 ± 5	127 ± 5	144 ± 27	137 ± 8
34	81 ± 8	101 ± 3	93 ± 12	104 ± 6
35	101 ± 8	/	154 ± 20	107 ± 13

[a] SD: standard deviation.

Sample Group: ROX-fed Chickens (Strain Ross 308)				
Total Arsenic Concentration (mean ± SD) (µg/kg)				
Day	Pen 11	Pen 13	Pen 15	Pen 29
0	3.3	5.3	19.8	2.3
1	405.4	14.7	62.6	79.9
2	63.4	202.0	16.0	85.5
3	183.4	147.0	77.7	133.7
4	272.5	200.9	277.7	199.6
7	341 ± 14	306 ± 31	412 ± 5	248 ± 58
14	273 ± 29	261 ± 8	205 ± 7	281 ± 34
21	225 ± 15	290 ± 17	214 ± 4	268 ± 25
28	316 ± 24	462 ± 34	332 ± 13	672 ± 99
29	243 ± 8	241 ± 22	206 ± 29	239 ± 9
30	117 ± 7	262 ± 12	151 ± 4	46 ± 5
31	661 ± 129	207 ± 20	199 ± 23	186 ± 13
32	99 ± 10	138 ± 21	134 ± 9	88 ± 2
33	119 ± 18	124 ± 15	130 ± 8	124 ± 8
34	93 ± 2	91 ± 10	100 ± 29	109 ± 10
35	86 ± 22	173 ± 11	92 ± 14	95 ± 9

[a] SD: standard deviation.

Sample Group: Control Chickens (Strain Cobb 500)				
Total Arsenic Concentration (mean ± SD) (µg/kg)				
Day	Pen 5	Pen 6	Pen 18	Pen 25
0	33.2	12.4	11.4	147.0
1	10.3	8.2	15.5	23.0
2	12.4	12.1	96.3	55.3
3	18.6	15.3	23.3	23.3
4	32.7	21.4	32.8	38.2
7	48.2	69.3 ± 0.9	47.1 ± 0.8	66.9
14	38 ± 5	49 ± 6	36 ± 7	44 ± 6
21	59 ± 3	59 ± 1	73 ± 6	48.3 ± 0.4
28	65 ± 14	77 ± 4	85 ± 2	614 ± 10
29	98 ± 21	83 ± 7	78 ± 21	95 ± 3
30	68 ± 14	66 ± 10	65 ± 2	197 ± 18
31	59 ± 10	93 ± 9	96 ± 2	85 ± 8
32	60 ± 10	55 ± 9	72 ± 6	60 ± 4
33	56 ± 10	58 ± 8	78 ± 6	109 ± 14
34	47 ± 6	42 ± 2	56 ± 7	79 ± 30
35	67.4 ± 0.9	62 ± 6	65 ± 5	71 ± 3

[a] SD: standard deviation.

Sample Group: Control Chickens (Strain Ross 308)				
Total Arsenic Concentration (mean ± SD) (µg/kg)				
Day	Pen 3	Pen 12	Pen 17	Pen 22
0	15.3	12.4	13.3	49.2
1	10.1	18.7	14.9	25.7
2	12.7	96.7	19.5	37.5
3	27.9	25.2	26.5	17.0
4	36.3	38.3	44.4	38.6
7	42.2	50 ± 4	77 ± 2	41.6
14	49 ± 6	44 ± 3	52 ± 4	52 ± 3
21	66 ± 5	67 ± 3	101 ± 12	92 ± 9
28	392 ± 22	77 ± 3	80 ± 6	75 ± 5
29	70 ± 11	88 ± 10	86 ± 11	68 ± 18
30	63 ± 6	56 ± 6	61 ± 11	68 ± 4
31	81 ± 6	83 ± 18	90 ± 13	83 ± 3
32	57 ± 14	54 ± 5	49 ± 2	63 ± 1
33	55 ± 4	71 ± 11	67 ± 7	69 ± 5
34	58.6 ± 0.5	75 ± 5	60 ± 7	66 ± 2
35	58 ± 10	59 ± 8	106 ± 37	62 ± 10

[a] SD: standard deviation.

Table A.4 Detailed concentrations of arsenic species in each selected chicken skin sample.[a]

Sample Group	**ROX-fed Chickens (Strain Cobb 500)**							
	Concentration of Arsenic Species (μg/kg)							
Feeding Period	**Day 28**				**Day 35**			
Species	**Pen 1**	**Pen 16**	**Pen 19**	**Pen 24**	**Pen 1**	**Pen 16**	**Pen 19**	**Pen 24**
AsB	3.438	3.877	3.240	6.818	6.238	/	2.231	3.905
As^{III}	20.55	13.20	19.35	17.05	3.762	/	5.804	3.262
DMA	4.000	5.385	4.184	6.722	2.568	/	3.372	2.638
Unknown 1	N.D.	0.3874	N.D.	N.D.	N.D.	/	N.D.	N.D.
MMA	2.841	3.383	3.197	3.205	1.833	/	10.25	2.258
Unknown 2	N.D.	N.D.	N.D.	N.D.	N.D.	/	N.D.	N.D.
Methylated 3-AHPAA	1.015	3.362	2.166	2.679	0.9061	/	1.028	6.475
As^{V}	1.312	2.916	1.490	1.612	1.052	/	2.812	2.145
3-AHPAA	4.851	6.661	7.361	6.691	0.2934	/	13.51	0.3152
Methylated N-AHAA	N.D.	0.5100	0.7808	N.D.	N.D.	/	0.7244	N.D.
N-AHAA	0.5579	1.141	1.395	0.6924	N.D.	/	3.989	N.D.
Methylated ROX	39.23	68.99	36.26	69.02	8.983	/	16.96	5.409
ROX	28.46	46.64	33.42	53.19	3.961	/	21.78	2.083

[a] N.D.: below detection limit; Pen 16 sample on day 35 is not available.

Sample Group	ROX-fed Chickens (Strain Ross 308)							
	Concentration of Arsenic Species (µg/kg)							
Feeding Period	**Day 28**				**Day 35**			
Species	**Pen 11**	**Pen 13**	**Pen 15**	**Pen 29**	**Pen 11**	**Pen 13**	**Pen 15**	**Pen 29**
AsB	3.794	4.246	4.978	6.206	2.721	4.015	4.352	2.505
As^{III}	10.05	10.68	23.73	6.125	3.646	11.72	5.269	3.728
DMA	6.797	110.5	5.372	231.6	2.550	10.05	5.868	2.287
Unknown 1	0.4378	N.D.	0.3992	0.4165	N.D.	N.D.	N.D.	N.D.
MMA	3.938	5.539	3.707	6.204	1.933	3.121	34.83	2.758
Unknown 2	N.D.	N.D.	0.3013	N.D.	N.D.	N.D.	2.451	N.D.
Methylated 3-AHPAA	4.025	1.676	5.185	1.866	1.796	8.035	0.6102	1.957
As^{V}	1.648	3.263	2.259	3.887	1.804	1.966	0.8280	1.391
3-AHPAA	6.868	7.163	6.091	7.031	0.8549	22.71	N.D.	0.8678
Methylated N-AHAA	0.7203	N.D.	1.360	0.2961	N.D.	N.D.	N.D.	N.D.
N-AHAA	1.106	0.8726	1.073	1.058	0.4707	4.242	N.D.	N.D.
Methylated ROX	59.87	51.81	86.89	49.21	7.428	6.581	11.27	7.632
ROX	48.04	60.54	42.42	57.11	3.932	7.974	2.954	2.796

[a] N.D.: below detection limit.

Sample Group	Control Chickens (Strain Cobb 500)							
	Concentration of Arsenic Species (µg/kg)							
Feeding Period	**Day 28**				**Day 35**			
Species	**Pen 5**	**Pen 6**	**Pen 18**	**Pen 25**	**Pen 5**	**Pen 6**	**Pen 18**	**Pen 25**
AsB	4.475	6.833	7.446	5.143	1.782	3.030	3.031	5.410
As^{III}	1.294	1.289	1.768	1.173	1.750	2.089	1.806	1.606
DMA	4.606	5.544	7.532	338.0	3.118	2.574	3.303	2.746
Unknown 1	0.5580	0.5444	0.5311	0.4541	N.D.	N.D.	N.D.	N.D.
MMA	1.440	1.917	1.685	1.588	1.552	1.825	1.699	1.874
Unknown 2	N.D.	N.D.	N.D.	1.788	N.D.	N.D.	N.D.	N.D.
Methylated 3-AHPAA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
As^{V}	1.148	0.6628	1.353	0.5908	1.064	1.434	1.299	0.4889
3-AHPAA	2.552	2.829	1.657	1.551	N.D.	N.D.	N.D.	0.3756
Methylated N-AHAA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
N-AHAA	N.D.	N.D.	0.3655	N.D.	N.D.	N.D.	N.D.	N.D.
Methylated ROX	N.D.	0.9653	2.962	N.D.	N.D.	N.D.	1.027	N.D.
ROX	N.D.	1.229	3.454	1.640	N.D.	N.D.	N.D.	N.D.

[a] N.D.: below detection limit.

Sample Group	Control Chickens (Strain Ross 308)							
	Concentration of Arsenic Species (µg/kg)							
Feeding Period	**Day 28**				**Day 35**			
Species	**Pen 3**	**Pen 12**	**Pen 17**	**Pen 22**	**Pen 3**	**Pen 12**	**Pen 17**	**Pen 22**
AsB	3.662	3.623	6.424	4.330	5.424	7.740	4.122	3.310
As^{III}	1.838	1.965	1.405	1.459	1.830	1.670	3.562	1.808
DMA	254.1	4.277	5.688	3.302	2.348	2.397	3.860	3.298
Unknown 1	N.D.	0.3886	0.7544	0.6693	N.D.	N.D.	N.D.	N.D.
MMA	2.573	1.397	1.812	1.714	2.596	1.613	2.145	1.527
Unknown 2	4.368	N.D.	N.D.	1.745	N.D.	N.D.	N.D.	N.D.
Methylated 3-AHPAA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
As^{V}	1.260	0.2968	0.8093	1.852	N.D.	N.D.	0.7781	0.4485
3-AHPAA	1.438	1.822	1.238	1.496	0.4183	N.D.	N.D.	N.D.
Methylated N-AHAA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
N-AHAA	0.3078	0.3496	0.3629	0.2980	N.D.	N.D.	N.D.	N.D.
Methylated ROX	N.D.	N.D.	0.7297	1.442	N.D.	N.D.	21.32	N.D.
ROX	2.198	1.700	0.9195	4.978	N.D.	N.D.	3.908	N.D.

[a] N.D.: below detection limit.