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Further Assessment of PPCPs in Feed Grade Chicken Feather Meal Including Potential Sources

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FURTHER ASSESSMENT OF PPCPs IN FEED GRADE CHICKEN FEATHER MEAL INCLUDING POTENTIAL SOURCES

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by
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ABSTRACT

The poultry and rendering industries have played important roles in both the diets of humans as well as environmentally sustainable development. However, a recent article published by Dr. D. C. Love and co-workers at Johns Hopkins Center for a Livable Future revealed concerns that pharmaceutical and personal care products (PPCPs) might be able to re-enter the human food supply by being present in feed grade chicken feather meal, i.e., be consumed by and accumulate in the tissue of chickens or other animals. Their study showed twenty-four PPCPs that were detected above the reporting limits in a total of twelve feather meal samples (five feed grade and seven fertilizer grade) bought in the United States and China.[1]

Since this publication may have flaws in terms of the specific origin of the samples of chicken feather meal, and insufficient evidence existed to support the source of the contamination found in the feather meal samples, it was thought that addition studies were warranted. Samples of raw chicken feathers and fresh chicken feather meal were either collected or received from three (3) rendering plants from different geographical regions of the country. To analyze the samples, EPA method 1694 was followed, and the conditions of an HPLC-MS/MS (high-performance liquid chromatography tandem mass spectrometer) were adjusted to test the performance of low-concentration (ppb level) drug detection of sixteen analytes: acetaminophen, erythromycin, norgestimate, sulfachloropyridazine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxazole, thiabendazole, enrofloxacin, norfloxacin, 1,7-dimethylxanthine, caffeine, ofloxacin, and diphenhydramine. The results

indicated that the mass spectrometer used, which contains a quadrupole ion-trap analyzer, has a lower sensitivity and thus a higher detection limit for the aforementioned sixteen analytes than a triple quadrupole analyzer, which is the standard instrument recommended by the EPA method and AXYS Analytical Services Ltd. In order to continue the project, the samples were then sent to AXYS Analytical Services Ltd., the same firm that carried out the analyses for the Johns Hopkins study. The results showed that these samples of chicken feather meal were not nearly as contaminated as those studied in the aforementioned publication, and that the contaminants may come in part from the raw chicken feathers and accumulates in the dissolved air filtration (DAF) system. The poultry industry should take care of the source of diphenhydramine, anhydrochlortetracycline (ATCT), and sulfadimethoxine, which were in high concentrations in our samples.

DEDICATION

This is dedicated to:

Dr. Joseph S. Thrasher, who gave me the opportunity to join this Rendering project, and helped me revise my thesis; Dr. Qiaoli Liang, who guided me in using the HPLC-MS/MS instrument at the University of Alabama and discussed the problems I met; the Animal Co-Products Research & Education Center, who funded the project; and my coworker graduate student Chen Liu and other group members, who gave me strong support.

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CHAPTER ONE

INTRODUCTION

I. The Poultry and Rendering Industries

The United States poultry industry is the world's largest producer.[2] In 2013, the U.S. manufactured 8.52 billion broilers and sold 185 million chickens.[3] Meanwhile, the poultry industry produces an estimate of 11 billion pounds of waste every year.[4] In this case, the rendering industry has over 100 years of history in making continuous efforts in converting the secondary animal by-products into beneficial commodities,[5] and to help avoid the serious potential hazards brought by unprocessed animal by-products to animal health, human health, and the environment.[6] Annually, the rendering industry recycles around 59 billion pounds of perishable materials from poultry and livestock, meat and food processing. The supermarket and restaurant industry contribute the majority of its by-products to the feed industry as high-energy fats and high-quality protein ingredients for diet supplement and other efficient productions.[7] Among these, about 2 billion kilograms (5 billion pounds) of feathers, one of the poultry industry's by-products, is produced annually. Feather meal is the most common final product produced from the feathers.[8] Through hydrolyzing under great pressure and heat, grinding and drying, feathers are converted to feather meal and are used to formulate animal feed and organic fertilizer.[9]

In the poultry industry, companies provide the chicken feed and FDA (food & drug administration) approved pharmaceutical compounds and vaccines to maintain flock health.[10] A total of forty-four feed additives are currently allowed in the market

according to the Feed Additive Compendium in 2015.[11] For example, the additives that are allowed for chicken nutritional needs (egg production/feed efficiency/growth promotion) and medicinal uses (air saculitis/chronic respiratory disease (CRD)/blackhead/histomaniasis/bluecomb/mud fever/non-specific enteritis etc.) are listed in Table 1.1.

Table 1.1. Use level of nutritional addivtives and medines from the Feed Additive Compendium 2015 by chicken species in 2015.[11] (The use levels may vary in terms of the level needed to prevent/cure different diseases).

Feed Additives	Nutritional Uses level (g/ton)	Medicinal Uses level (g/ton)
Amprolium		~113.5-227
Bacitracin Methylene Disalicylate	~4-50	~100-200
Bacitracin Zinc	~4-50	~100-200
Bambermycins	1-2	
Chlortetracycline	10-50	~100-500
Clopidol		113.5
Cyromazine		5.0 (ppm)
Decoquinate		27.2
Diclazuril		0.91
Hygromycin B		8-12
Lasalocid		68-113
Lincomycin	~2-4	2
Monensin		90-110
Narasin		~54-90
Narasin/Nicarbazin		54-90
Neomycin/Oxytetracycline	10-50	~100-500
Nicarbazin		~90.8-181.6
Nitarsone		170.1
Oxytetracycline	10-50	~100-500
Penicillin	~2.4-50	
Robenidine Hydrochloride		30
Salinomycin		40-60
Semduramicin		22.7
Sulfadimethoxine and Ormetoprim 5:3		113.5 and 68.1
Tylosin	~4-50	800-1000
Virginiamycin	~5-15	20
Zoalene		~36.3-113.5

In the rendering industry, raw materials are handled and treated by heat to remove the moisture content and micro-organisms. Through draining and pressing, melted oil is separated from the solids, and rest of the solids fraction is then grounded into a

powder.[12] Four rendering process methods exist: sterilization of animal by-products, which kills all forms of microbials; hydrolyzing of feather by-products, which add water to feather by-products by chemical process to cause the feathers fall apart; coagulating of blood by-products and drying of animal by-products.[13]

II. Project Objectives

Dr. D. C. Love and his co-workers at the Johns Hopkins Center for a Livable Future were concerned that the pharmaceuticals induced by feed additive or other sources of antimicrobials in the poultry industry might stay in secondary production, even after the rendering process, and re-enter the human food supply through chicken feather meal.[1] In this case, these pharmaceuticals become pollutants or PPCPs (Pharmaceuticals and Personal Care Products), defined by the EPA (Environmental Protection Agency) as "any product used by individuals for personal health or cosmetic reasons or used by agribusiness to enhance growth or health of livestock."[14] In Dr. D. C. Love's paper, published in 2012 in *Environmental Science & Technology*, he consigned AXYS Analytical Services Ltd. to test a total of twelve feather meal samples from the U.S. and China and stated that 19 out of the 59 tested antimicrobials were detected in the samples, including six drug classes: sulfonamides, macrolides, fluoroquinolones, tetracyclines, folic acid antagonists, and streptogramins. He alerted that the antimicrobials detected are approved for use in industrial poultry production,[1] suggesting these PPCPs were introduced by the poultry industry.

However, as pointed out by C. L. Hofacre of the University of Georgia's College of Veterinary Medicine, this study seems to be flawed as both insufficient information

about the source, age, etc. of the chicken feather meal samples was given and no attempt was made to eliminate possible sources of contamination.[15] Mark Bland, a member of the American Association of Avian Pathologist, also mentioned that seven out of twelve samples studied in the paper were described as "fertilizers", which should not be considered as "reentry route," and the analytes were found near the detection limit.[16] Besides, Barry Kelly suggested the sampling technique is questionable due to the fact that samples from China could not be considered as a representative of the sample conditions for U.S.[17] In addition, no information was given in the original publication suggesting how much the measured levels of PPCPs lie above either background levels or minimum detection limits. Furthermore, Dr. Love and his coworkers later published two corrections to their article in which the sample that they originally claimed to have been from North Carolina, was actually from Pennsylvania, and the number of turkeys raised in the U.S. should be 247 million instead of 80 million per year.[18]

The primary objective of this project was to either refute or substantiate the conclusions in this publication that raised concerns about pharmaceuticals and personal care products (PPCPs) reentering the human food supply from chicken feather meal. Chicken feather meal samples (feed grade) of defined origin, as well as the corresponding samples of water (both plant and recycled flush), municipal water, and chicken feathers were collected and prepared for analysis. In the Johns Hopkins paper, the PPCPs detected above the reporting limits were listed and divided into the groups below: acetaminophen, erythromycin, norgestimate, sulfachloropyridazine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxazole, thiabendazole, enrofloxacin,

norfloxacin, 1,7-dimethylxanthine, caffeine, ofloxacin, diphenhydramine, and ormetoprim from the Group 1 PPCPs; and 4-epioxytetracycline (EOTC), isochlortetracycline (ICTC), and oxytetracycline (OTC) from the Group 2 PPCPs. PPCPs are partitioned into four groups based on their analysis conditions. Group 1 and Group 2 PPCPs are analyzed under acidic extraction and positive electrospray ionization (ESI+) conditions, but the conditions of the LC gradient programs (flow mixture and flow rate) are different; Group 3 PPCPs are analyzed under acidic extraction and negative electrospray ionization (ESI-), while Group 4 PPCPs are analyzed under basic extraction and ESI+.[18] More detail information can be found in Appendix A, Table A.6-A.9. Depending on the results, the project aimed to provide more refutable data and/or determine the source or cause of contamination.

III. LC/MS/MS in Analysis of Contaminants

Among all the newly developed analytical tools and methods, various spectroscopies (atomic absorption spectroscopy, atomic emission spectroscopy, Raman spectroscopy, X-ray fluorescence spectroscopy, etc.), mass spectrometry, thermal analysis, etc., LC/MS/MS has shown the greatest success in the analysis of contaminants due to the limitation of gas chromatography in separations of non-volatile and thermally unstable chemicals and to improvement in mass spectrometry in terms of the atmospheric pressure ionization and electrospray techniques. [19] HPLC is a separation technique that is involved in the adsorption, partition, and ion exchange between the mobile and stationary phases, which is widely used in the separation of various pharmaceuticals, pesticides, biological samples, etc. [20] In chromatography, the interval between the instant

of injection and the detection of the component is called the retention time, which usually varies with the identity of each component.[21] The structural information of each composition is analyzed by the MS/MS. The mass of precursor ion and fragmentation ion generated in the collision cell are distinct and unique. Figure 1.1 shows the chemical structure of norgestimate and the MS/MS spectrum of precursor ion and fragmentation ion, which is also called parent-daughter ions. In the case of norgestimate, the precursor (parent) is charged by ESI and formed $[C_{23}H_{31}NO_3+H]^+$ ion, the mass/charge ratio (m/z) is 370. During the collision, norgestimate ion goes through the McLafferty rearrangement and lose the newly formed acetyl, the main fragmentation (daughter) ion is $[C_{21}H_{27}NO+H]^+$ and its m/z is 310.

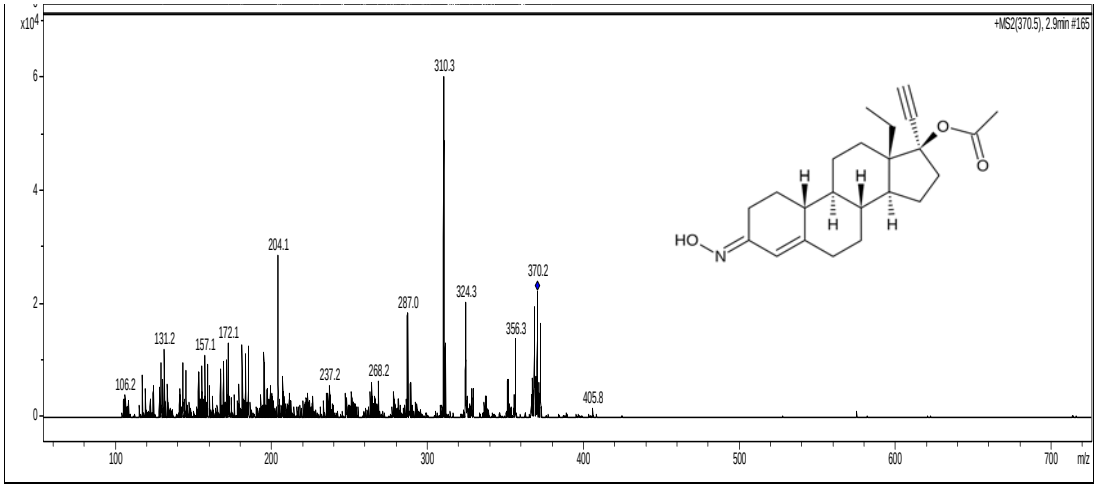

Figure 1.1: The chemical structure and MS/MS spectrum of norgestimate, measured with electrospray ionization-quadrupole ion trap MS/MS.

Quadrupole time-of-flight (Q-TOF), triple quadrupole (QqQ), and quadrupole ion-trap (QIT) MS/MS are the three most often used analysis methods for structural information. The Q-TOF MS/MS can give full-scan data of the precursor ion and accurate mass of fragmentation ions, so it is more sensitive in scan mode compared to the third quadrupole of the QqQ. But the cost is high, and Q-TOF can only do quasi-selected reaction monitoring (quasi-SRM), which means it has less sensitivity for quantitation of analytes. The QqQ MS/MS consists of two single quadrupoles with a collision cell in between, combining the simplicity of a quadrupole MS with the high efficiency of a TOF MS analyzer.[20] The QqQ is sensitive for quantitation with multiple reaction monitoring (MRM) [sometimes called selected reaction monitoring (SRM)], and the cost is moderate, so it has often been used in quantitative analysis. But the QqQ is the least sensitive in scan mode. The QIT contains two hyperbolic electrodes and a ring electrode. A QIT can create stable trajectories for ions of either a certain m/z or a m/z range; the unwanted ions will collide with the wall or eject from the trap.[23-25] Ion selection, ion activation, and acquisition of fragment ion spectra happen in the same place, which makes it possible to do higher order MS^n detection.[26,27] Also, recently developed QIT MS/MS can also do SRM experiments. However, a low-mass cutoff (LMCO) is the major disadvantage of QIT, which means it cannot simultaneously store ions over the full m/z range.

Therefore, a secondary objective of this project is to investigate the possibility to use the least expensive cost QIT analyzer instead of the QqQ analyzer in mass spectrometer of HPLC-MS/MS instrument, which is suggested in EPA Method 1694 and

also used by the authors of Reference 1 at AXYS Analytical Services Ltd. If the HPLC-QIT-MS^n can also do a good job in the quantitative detection of PPCPs, then one could also extend the application of QIT-MS to the trace level of drugs in the environment.

CHAPTER TWO

EXPERIMENTAL

I. Sample Collection

Three pre-tests chicken feather meal samples in bags from Plant A, Plant B and Plant C were provided by Dr. Annel Greene, Director of ACREC.

A field trip was made to Plant 1, located in the southeastern United States, to collect test samples for the study. A tour of the rendering plant was given to help understand how the samples were processed in order to better trace the possible sources of contaminants. Samples of both fresh chicken feather meal and chicken feathers were collected in wide-mouth bottles (Thermo Scientific, heavy-duty bottle, HDPE, 2-L/Thermo Scientific amber rectangular bottle, HDPE, 2-L). Composite samples of the plant's poultry feather meal product over a recent three-month period were also received. Later local water samples [plant water (two sources), dissolved-air filtration or DAF water-in, dissolved-air filtration or DAF water-out, municipal water, etc.] were collected in clean bottles (Thermo Scientific, heavy-duty bottle, HDPE, 4-L/Wheaton 4-L square bottle with vinylite–lined cap) for trace analysis. (The results for the liquid samples will be discussed in my co-worker Ms. Chen Liu's Thesis as well as in any joint publications that will also include the results being described herein for the solid samples.) Samples collected from the plant were maintained in a cooler around 0 °C with either blue ice or ice. Samples were stored in a freezer (< -10 °C) in the laboratory within three (3) hours after the sample collection. A bag of peat moss was also purchased from the local store, which was treated to study the matrix effect and used for the quality control group.

Later permission was received from two other plants: Plant 2 and 3 in U.S. to obtain their raw samples. Instead of collecting the chicken feathers and chicken feather meal samples from a single poultry processing plant, gathering samples from a number of plants in different areas of the U.S. will represent a broader spectrum of product originating from many different poultry processing plants around the country. Necessary supplies were shipped to these Plants: four new bottles (Thermo Scientific amber rectangular bottle, HDPE, 2-L) for storing the chicken feather and chicken feather meal; enough large bottles (1-L) for DAF water-in, DAF water-out, plant water, and municipal water samples; and blue ice with other packing material in a cooler, so that the plants could collect and ship fresh samples at low temperature to our laboratory at the Advanced Materials Research Laboratory in Anderson, SC. At the same time, the chain of custody paper work was sent with the cooler to guarantee that personnel at the plant would record the required information on each sample. Sample names, collecting location, and collection date and time could be found on the chain of custody form, which became an important factor to determine the authenticity and conditions of samples when collected.

After the bottles filled with target samples were received, information on the chain of custody paperwork was recorded and organized in files. For each solid samples, 10-20 g of well mixed samples was grounded into particles smaller than 1mm, and the powders were then put into 50-mL new centrifuge tubes with screw caps (CORNING, plug seal cap, polypropylene). For each liquid samples with visible particles inside, 2-L of well-shaken samples were filtered through a hazardous waste filtration system (Millipore YT30 142HW) with microfiber filters of 47-mm circles to remove the visible

particles. Each clear aqueous solution was stored in 1-L heavy-duty bottles; and the filtered particles were collected in 50-mL centrifuge tubes and treated as solid samples for future tests. Each type of sample was prepared in more than two aliquots, one aliquot was used for the PPCPs test, one was saved for a back-up, and the reminder of raw materials were stored in the original sample bottles. Then every centrifuge tubes and bottles was numbered such that the order would match the information offered by the chain of custody paperwork. Later, each sample bottle or tube was sealed in one or two zip-lock bags to avoid possible cross contamination. All the samples were kept in a freezer in the dark at < -10 °C until proceeding to the sample preparation step.

II. Sample Processing

Collected samples and peat moss went through the following steps, and each sample batch was extracted during the same 12-hour shift.

i. Preparation of Chemicals and Materials

Reagents and commercial standards were obtained from commercial vendors. Reagents: sodium phosphate monobasic monohydrate (ACS reagent, CAS no. 10049-21-5, Ricca), ethylenediaminetetraacetic acid tetrasodium salt dehydrate (98%-102%, CAS no. 10378-23-1, Sigma), methanol (ACS reagent, CAS no. 67-56-1, Fisher Scientific), dichloromethane (CAS no. 75-09-2, Macron), hydrochloric acid solution 6 N (certified 5.96-6.05N, CAS no. 7732-18-5, Fisher Scientific), formic acid (LC-MS ultra, CAS no. 64-18-6, Fluka); sixteen commercial standards: acetaminophen (≥98%, Cambridge Isotope Lab), sulfamethazine (≥98%, Cambridge Isotope Lab), sulfamethoxazole (≥98%, Cambridge Isotope Lab), diphenhydramine•HCl (Cerilliant), thiabendazole (99.8%, CAS

no. 148-79-8, Fluka), 1,7-dimethylxanthine (CAS no. 611-59-66, Sigma), caffeine (CAS no. 58-08-2, Sigma), norgestimate (CAS no. 35189-28-7, Sigma), sulfachloropyridazine (99.4%, CAS no. 80-32-0, Fluka), sulfamerazine (99.9%, CAS no. 127-79-7, Fluka), norfloxacin (CAS no. 70458-96-7, Fluka), sulfadimethoxine (99.9%, CAS no. 122-11-2, Fluka), enrofloxacin (99.0%, CAS no. 193106-60-6, Fluka), ofloxacin (99.8%, CAS no. 82419-36-1, Fluka) , sulfamethizole (98.9%, CAS no. 144-82-1, Fluka), erythromycin (≥98%, Cambridge Isotope Lab); five labeled compounds: atrazine (Ring-$^{13}C_3$, 99%, Cambridge Isotope Lab), erythromycin (*N,N*-dimethyl-$^{13}C_2$, 90%, Cambridge Isotope Lab), sulfamethazine (phenyl-$^{13}C_6$, 90%, Cambridge Isotope Lab), sulfamethoxazole (ring-$^{13}C_6$, 99%, Cambridge Isotope Lab), caffeine (trimethyl-$^{13}C_3$, 99%, Cambridge Isotope Lab). The concentrations of each solution was prepared as shown in Appendix A, Table A.1. In Table A.1, the original concentrations of each commercial standards and labeled internal standards was determined and calculated based on the target concentrations needed in the EPA method 1694.[19]

Solid commercial standards were weighted by using a precision analytical balance of an elemental analysis instrument (PerkinElmer AD 6 autobalance controller). Liquid commercial standards, including five internal standards were also measured by pipette. After the chemicals were individually placed in micro-centrifuge tubes with screw caps (Neptune™ 2.0-mL pre-sterile conical-bottom), a certain amount of methanol was added to each tube, and the solutions were diluted to their targeted concentrations (the volumes of methanol added to the chemicals were calculated and are shown in Appendix A, Table A.1). For some solid chemicals, a secondary solvent (acetonitrile or DMSO, etc. referred

to in Appendix A, Table A.1 in the solvent column) was applied due the solubility properties of different pharmaceuticals.

After the preparations of the sample of each PPCP with a known concentration, a mixture of sixteen commercial standards and $^{13}C_3$-atrazine was prepared with a series of concentrations for producing calibration curves. The concentrations of each analyte in CS-1, CS-2, CS-3, CS-4 and CS-5 are listed in Appendix A, Table A.2, which strictly followed EPA method 1694.[19] Later the concentrations were adjusted based on the instrumental performance, and the new concentrations are shown in Appendix A, Table A.3. More information will be discussed in Chapter Three.

The buffer solution was prepared by weighing 1.93 g $NaH_2PO_4 \bullet H_2O$ in 99 mL of reagent water, and then adding 1 mL 85% H_3PO_4.[19]

ii. Sample Preparation

Based on the EPA protocol, the particle size of the samples should be smaller than 1 mm. Since chicken feathers and some chicken feather meal particles were larger than 1 mm, samples had to be ground, homogenized, or blended first. Hard particles were reduced by grinding with a mortar and pestle, and then the softer particles were reduced by grinding in a cryogenic mill (6870 Freezer/Mill: SPEX Sample Prep). The sample was frozen with liquid nitrogen to improve the grinding efficiency. Also, two peat moss (brand: Miracle-Gro®, Sphagnum peat moss) samples were ground and used as blank and ongoing precision and recovery standard (OPR) for reference matrix aliquots. Filtered solids from DAF water samples (only DFA water contained visible particles, other water samples were clear) were treated differently from the above-mentioned solid samples,

instead of grinding, buffer solution was added, and the solids were homogenized in centrifuge tubes by stirring with a glass rod. The pH of the final solution was adjusted to 2 ± 0.5 (Fisher scientific™ accumet™ AB15 Basic pH meter). All operations were carried out in a fume hood in order to prevent particles from contaminating the work environment.

A quantity of 0.25-0.10 g of well-mixed chicken feather and chicken feather meal samples were weighed (Sartorius balance) separately, and each sample was placed into a centrifuge tube (CORNING, plug seal cap, polypropylene). The exact weights of net samples were recorded. The same weighing procedure was followed for two samples of 0.25 g each of peat moss, which were used for the method blank and the OPR sample for the acid fraction. After weighing, all solid samples were stored in closed container to prevent further drying.

iii. Acid Fraction

An amount of 0.25 g of each sample or peat moss was placed in a 50-mL centrifuge tube, then 15-mL of pH = 2 phosphate buffer was added, followed by 5 min vortexing (BV 1000 vortex mixer, 115 VAC, 60 Hz, 1.5 amps). The sixteen commercial standards mentioned in the *Preparation of Chemicals and Materials* section were spiked only into the peat moss aliquot that served as the acid fraction OPR, the injection volume followed Appendix A, Table A.1. (The volumes were adjusted based on the method performance, since the injection volumes needed to be increased when the recovery was zero.) The five labeled compounds were spiked into the acid fractions of the samples and

QC (blank and OPR) samples. The pH of each solution was kept to 2.0 ± 0.5 by adding a buffer solution. After each addition, the centrifuge tube was again vortexed.

iv. Concentration and Extraction

After the spiking step, 20 mL of acetonitrile was added to each of the solid samples and the QC samples, followed by 30 minutes of sonication (Aquasonic™ ultrasonic cleaner, model 150HT, 50/60 Hz) and 5-10 minutes of centrifugation at 3000 rpm (VWR clinical 200 centrifuge). Extract (supernatants) of each entire sample and the QC samples were decanted into separate, clean 250-mL round-bottomed flasks. Then 15-mL of phosphate buffer was added to each sample and the QC samples and the pH was adjusted to 2.0 ± 0.5 with HCl. A second extraction was performed by repeating the steps above, and the extract was added to their respective flasks. For the third extraction, only 15 mL of acetonitrile was added to each of the tubes. The tube was sonicated and centrifuged, and the supernatants were decanted into their respective round-bottomed flasks. A filtration through a hazardous waste filtration system (Millipore YT30 142HW, filter paper: Whatman™ GF/A, 47 mm circles) was carried out if particles were visible in any extract. In this case, squeeze bottles were used to rinse the filter three times with reagent water and three times with acetonitrile.

The collected extracts from the acid fraction of the solid samples and QC samples were concentrated separately to a final volume of 20 - 30 mL by rotary evaporation (Büchi water bath B-481, Büchi rotavapor R-124, Welch DuoSeal vacuum pump) at 50 ºC. Immediately after concentration, 200 mL of reagent water and 500 mg of $Na_4EDTA \bullet 2H_2O$ were added to the acid fraction extract.

v. Cleanup

The SPE apparatus (Oasis HLB 20-cc Vac Cartridge, 1 g sorbent per cartridge, 60-µm particle size) was assembled and the SPE HLB cartridges were attached to it. An SPE HLB cartridge was conditioned by eluting it first with 20 mL of methanol followed by 6-mL of reagent water at pH 2.0 ± 0.5. These eluents were discarded after use. After conditioning of the cartridges, the prepared samples were each loaded onto a separate cartridge at a flow rate of 5-10 mL min^{-1}. The flow rate was controlled by a multi-position extraction manifold (Agilent Vac Elut SPS 24 Manifold with PYREX borosilicate glass tube, culture, disposable rimless, 10 × 75 mm). Once the entire sample passed through the cartridge, the acid fraction cartridge was washed with 10-mL of reagent water to remove the EDTA. Then the cartridges were then dried under vacuum for approximately 5 min, followed by treatment with 12-mL of methanol, which was used to elute the analytes under vacuum, and later the elution was completed by gravity. Each eluent was collected in a respective clean centrifuge tube (Corning 15-mL centrifuge tube, plug seal cap, polypropylene), and concentrated to near dryness under a gentle stream of nitrogen in a water bath held at 50 ± 5 °C.

At last, 3-mL of methanol was added to the concentrated acid extracts, including the blank and OPR samples. The final volume was adjusted to 4.0 ± 0.1 mL with 0.1% formic acid solution. If visible particles were presented in the extract, or if the extract was cloudy, the extract was filtered through a 0.2-µm filter (pressure filter). A quantify of 1-mL of each clear extract was transferred to an HPLC/MS/MS autosampler vial for

analysis. The remaining 3-mL of extract was treated as backup and stored in a refrigerator.

The whole process is summarized in the Figure 2.1 below, the aqueous sample processing steps can be found in my co-worker Ms. Chen Liu's Thesis.

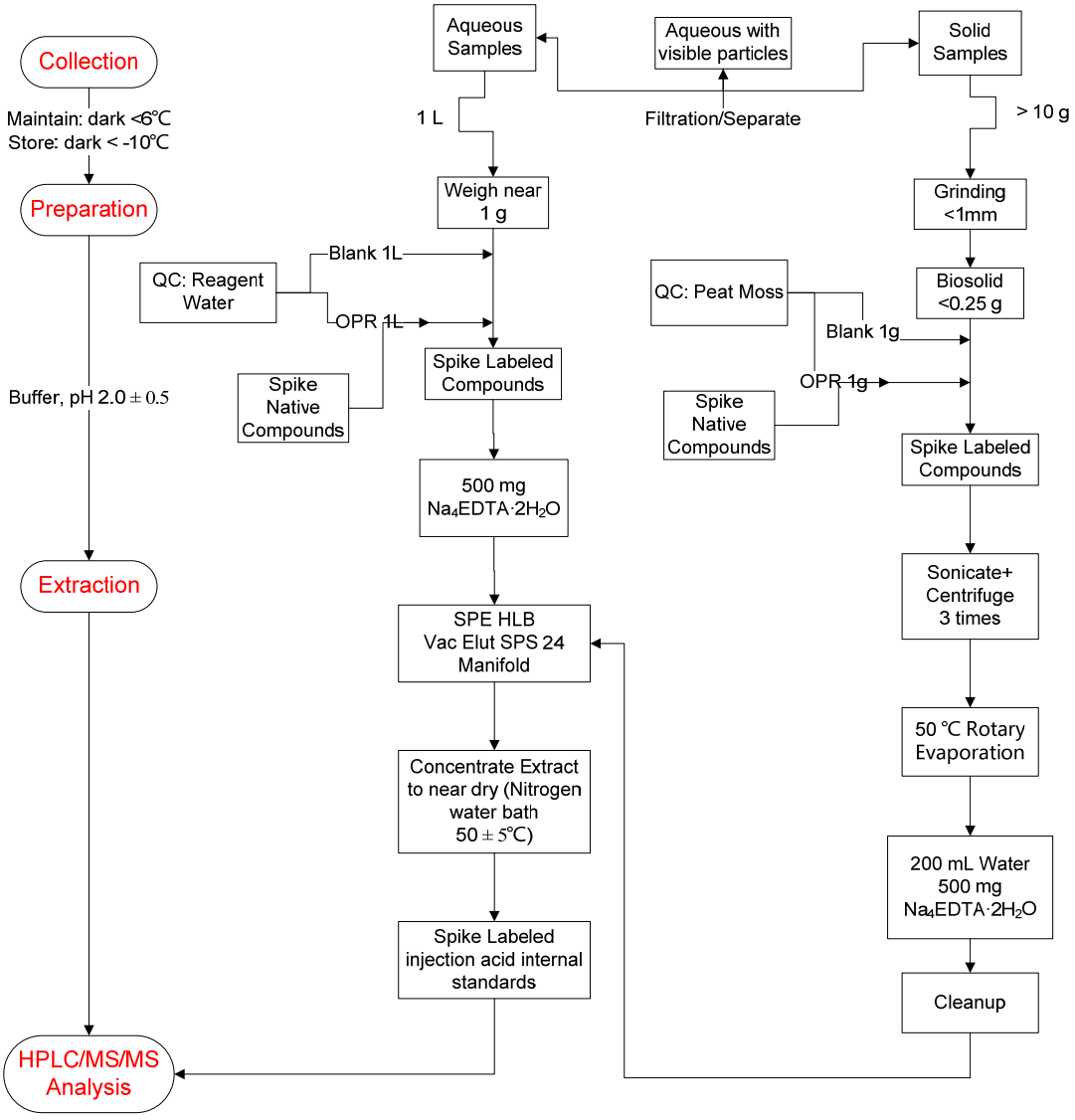

Figure 2.1: Flow chart of determination of Group 1 pharmaceuticals and personal care products by LC/MS/MS.

III. Instrumentation

After the samples were extracted and concentrated, they were ready for analysis by HPLC-MS/MS. An Agilent Technologies 1200 series HPLC tandem Bruker ESI/QIT MS^n system (column: XTerra MS C_{18} 125Å, particle size 3.5 μm, 1 mm × 50 mm) was used as the HPLC/MS/MS system. The LC conditions are shown in Appendix A, Table A.10. The MS control software used was the *Esquire 6.2.581.3 version*, and the HPLC/MS/MS control software station was *HyStar 3.2.44 SR2* version.

First, 1 mL of a mixture of the total twenty-one analytes (sixteen native analytes and five labeled compounds) under CS-3 concentration was injected into the HPLC-MS/MS to verify the instrumental performance. The parameters: parent-daughter ions m/z's, amplifier (adjusted to obtain a high voltage without waveform distortion) and low mass cutoff values (quadrupole rejects low m/z ion)[28] were typed into the HyStar software control panel to identify the chemical compositions. Initial values strictly followed the EPA method 1694,[19] and the adjusted parent-daughter ions m/z values, amplifier values and cutoff values were determined by MS-MS infusion (controlled by Esquire software) of each pure commercial analyte. The final parameters are summarized in Appendix A, Table A.4.

For example, acetaminophen, the parent-daughter ions m/z are 152.2-110.1; the original cutoff value was 100, and the amplifier value was 1. The above data was entered into the Esquire 6.2.581.3 control panel. After infusion of an aliquot of the mixture of standards or pure acetaminophen at CS-3 concentration, the MS spectrum of acetaminophen (152.2-110.1) would be shown on the screen (see Figure 1.1). The cutoff

values and amplifier values could be manually changed and optimized to obtain the best peak (highest signal-to-noise ratio, or S/N) in the spectrum in situ. These steps were repeated with all of the other PPCPs and labeled standards, and the spectra and settings were recorded for future set up in the MRM/SRM mode in HPLC-MS/MS, which, instead of doing one analyte at a time allows for as many as ten (10) channels to be used simultaneously. Thus, the structural information of ten (10) analytes can be analyzed by MS/MS at once. Additional information will be discussed in Chapter Three.

After the parameters of total twenty-one target analytes were determined, HPLC-MS/MS programs were set up for calibration curves and samples analysis. Since at most 10 analytes channels can be monitored in the MRM mode, the total of 21 analytes had to be divided into three (3) subgroups, which basically means in order to collect the data on all 21 analytes, each sample need to be run three times. Also, in order to show reproducibility, two sample trails were done for each subgroup. In sum, in order to collect the data on all of the analytes in one sample, the sample extract had to be run a total of six times. More information will be discussed in Chapter Three.

Since commercial standards are pure, while the composition of each sample extract was complicated, verification tests were needed to make sure that the HPLC column and the MS electrospray ionization components were not contaminated. Mixtures of commercial standards at the concentration level CS-3 were treated as verification group to validate the performance of the HPLC/MS/MS. If the retention time (RT) of any analyte fell beyond 0.4 min of the predicted RTs or the calculated concentration of any analytes as invalid, and the column would be replaced and/or the MS instrument would

be cleaned. If the former, then calibration curves would be reproduced for the new column. More discussion will be given in Chapter Three, Section IV.

Since one objective of the current study is to extend the application of QIT MS^n in PPCPs detection, it is necessary to compare the Instrumental Detection Limit (IDL, defines as "the lowest concentration that an analytical instrument can measure. "[29]) of the ESI/QIT MS^n system and QqQ MS/MS system. Since the limit of detection (LOD) is defined as "the lowest concentration level that can be determined to be statistically different from a blank", sometimes, the IDL and LOD are operationally the same.[30] Thus, the IDLs of each analyte was measured and calculated in the way described below.

The mixture of standards containing analyte X (symbol X represents analyte, e.g.: acetaminophen) with the concentration but near the expected limit of detection (S/N of the peak is within 5-10, which can be read from the window of the software) with the internal standard $^{13}C_3$-Atrazine was prepared in triplicate. After the mixture was injected into the HPLC/MS/MS instrument via an auto-sampler, the standard deviation (S_X) of the set of peak areas of this analyte can be calculated, and then it can be applied to the following equation to calculate the IDL:[31,32]

$$IDL = t_\alpha S_X$$

t_α: one-sided student t-distribution with n-1 degree of freedom and the level of significance level is α; S_X: estimate of the true standard deviation of the distribution of sample means.

The same protocol was applied to calculate the IDLs for all the analytes. Sample calculation can be found in Chapter Three.

CHAPTER THREE

RESULTS AND DISCUSSION

I. Settings for HPLC/MS/MS

After the mixture of commercial standards was injected into the HPLC/MS/MS with the parameters of analytes set up in the way that was mentioned in Chapter Two, Section II, the data was saved as a yep file (BDal Compass Analysis file) and read by the *Bruker Compass DataAnalysis* software. A total ion current (CIT) chromatogram of a mixture of nine commercial standards (1,7-dimethylxanthine, caffeine, $^{13}C_3$-caffeine, thiabendazole, $^{13}C_3$-atrazine, sulfamethoxazole, diphenhydramine, $^{13}C_6$-sulfamethoxazole, $^{13}C_6$-sulfamethazine) at the concentration CS-3 is shown as an example below in Figure 3.1.

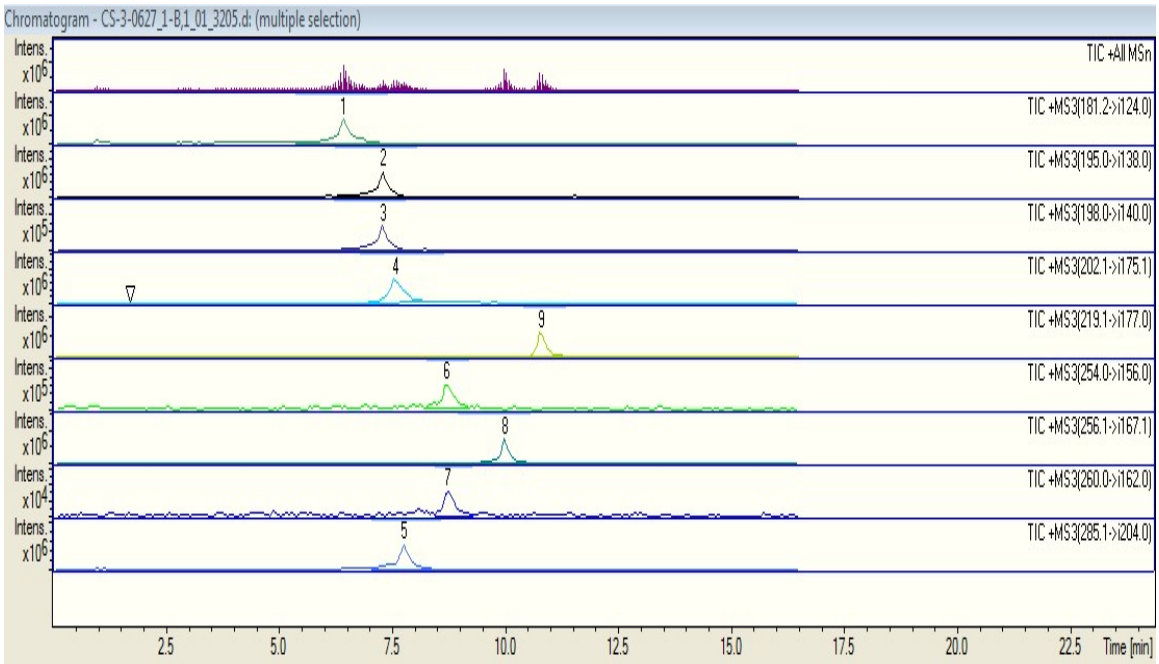

Figure 3.1: Chromatogram of a mixture of commercial standards (1,7-dimethylxanthine, caffeine, $^{13}C_3$-caffeine, thiabendazole, $^{13}C_3$-atrazine, sulfamethoxazole, diphenhydramine, $^{13}C_6$-sulfamethoxazole, and $^{13}C_6$-sulfamethazine), the concentration of each analytes are given in Appendix A, Table A.3, column CS-3, respectively.

In Figure 3.1, parent-daughter ions m/z are marked at the top right of each row, and the retention times (RTs) of each analytes can be read from the x-axis of this chromatogram. For example, the parent-daughter ions m/z of first analyte (the second row) was 181.1-124.1, plus the retention time of 6.5 minutes can be read from the chromatogram, matching the information of 1,7-dimethylxanthine shown in Appendix A, Table A.4. (or the appendix of EPA method 1694 appendix[19]). In this way, all twenty-one analytes were determined by the m/z ratios of the parent-daughter ions and the RTs in order to help double-check the authenticity of each analyte, respectively. Otherwise, if there was no peak appeared at the m/z ratios of the target parent-daughter ions with the correct RTs, it means that this analyte was either nonexistent, or below the detection limit.

II. Calibration Curves

After the series of mixture of standards of concentrations from CS-1 to CS-5 (Appendix A, Table A.2) were prepared individually as described in Chapter Two, Section III, the HPLC-MS/MS was set up for running each mixture. However, only diphenhydramine (0.5 ng mL^{-1}), thiabendazole (1.25 ng mL^{-1}) and 1,7-dimethylxanthine (0.125 μg mL^{-1}) were detected in the mixture of CS-1 concentration (the lowest concentration in the series), and more than half of the target analytes in the mixtures could not be detected under the concentration of CS-2. This means that the ESI/QIT MS^n

is not as sensitive as an QqQ MS/MS in quantitation of acetaminophen (< 0.15 μg mL^{-1}), erythromycin (< 0.75 ng mL^{-1}), norgestimate (< 7.5 ng mL^{-1}), sulfachloropyridazine (< 3.75 ng mL^{-1}), sulfadimethoxine (< 0.75 ng mL^{-1}), sulfamerazine (< 1.5 ng mL^{-1}), sulfamethazine (< 1.5 ng mL^{-1}), sulfamethizole (< 1.5 ng mL^{-1}), sulfamethoxazole (< 1.5 ng mL^{-1}), enrofloxacin (< 7.5 μg mL^{-1}), norfloxacin (< 37.5 ng mL^{-1}), caffeine (< 37.5 ng mL^{-1}) and ofloxacin (< 3.75 ng mL^{-1}). In order to draw reliable calibration curves for the native analytes, each analyte from concentrations CS-1 to CS-5 must be detected by the HPLC-MS/MS with peaks with a S/N threshold of 5. Thus, the concentrations of sixteen analytes (CS-1 to CS-5) were increased to higher values, and the new concentrations used for the calibration curves are shown in Appendix A, Table A.3. The relationship of these new concentrations values are expressed by following equations (*CS-n* represents the concentration of the PPCP in the CS-n; the concentration of CS-5 were not changed):

$$\text{CS-1} = \text{CS-5} \times 0.075$$

$$\text{CS-2} = \text{CS-5} \times 0.1$$

$$\text{CS-3} = \text{CS-5} \times 0.25$$

$$\text{CS-4} = \text{CS-5} \times 0.4$$

A series of sample calculations for producing the calibration curve of norgestimate is discussed below.

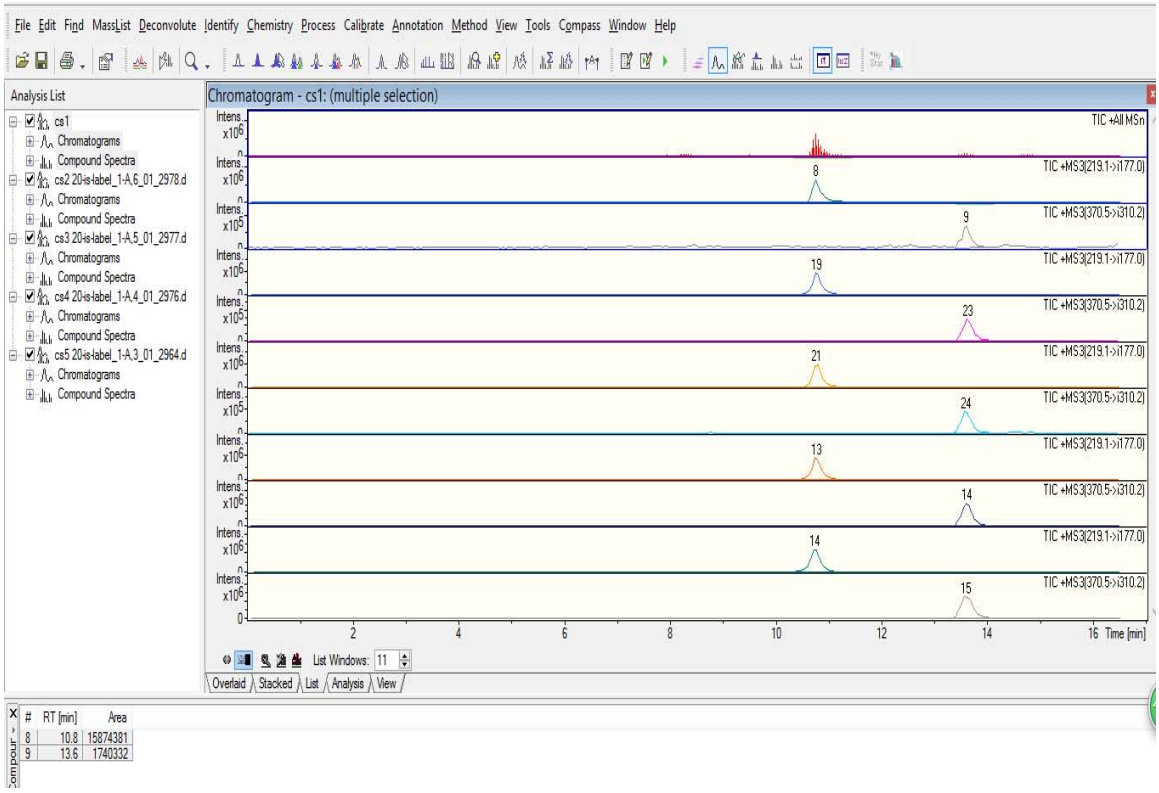

Figure 3.2: Chromatograms of norgestimate (parent-daughter ions m/z: 370.5-310.2, RT 13.7 min) with the concentration of 0.0375 μg mL^{-1}, 0.05 μg mL^{-1}, 0.0125 μg mL^{-1}, 0.2 μg mL^{-1}, and 0.5 μg mL^{-1} and internal standard $^{13}C_3$-atrazine (parent-daughter ions m/z was 219.1-177.0, RT 10.8 min, the concentration was 0.05 μg mL^{-1}).

In Figure 3.2, when the concentration of norgestimate was 0.0375 μg mL^{-1} (in CS-1), the peak area of norgestimate with a RT 13.6 min could be read from the chromatogram as 1740332, the peak area of the internal standard $^{13}C_3$-atrazine with RT 10.8 min was 15874381, the area ratio was $\frac{1740332}{15874381} = 0.1096315$. The mixture of commercial standards with the same concentration was run in triplicate, and the calculation of the peak area ratio was carried out in the same way mentioned above. Since

the three ratios show acceptable reproducibility, the average was calculated as the peak area ratio of norgestimate with a concentration of 0.0375 μg mL^{-1} (CS-1). Then, the peak area ratios of norgestimate with a concentration of 0.05 μg mL^{-1}, 0.0125 μg mL^{-1}, 0.2 μg mL^{-1}, and 0.5 μg mL^{-1} were calculated in the same way, and all of the values are summarized in Table 3.1. Then the calibration curve of norgestimate was produced, and it is shown with its regression equation in Figure 3.3.

Table 3.1. Data for creating the calibration curve of norgestimate with the new series of concentrations.

Concentrations μg mL^{-1}	RT (min)	Peak Area of Norgestimate	Peak Area of $^{13}C_3$-Atrazine	Ratio	Ratio Average
0.0375 (CS-1)	13.6	1740332	15874381	0.1096315	0.144199
	13.7	1966207	16361792	0.120171	
	13.6	3305427	16299327	0.202795	
0.05 (CS-2)	13.6	2273561	14133545	0.160863	0.176120
	13.6	2648194	14287191	0.185354	
	13.7	3466668	17315109	0.182143	
0.125 (CS-3)	13.7	6646075	15501192	0.428746	0.557033
	13.7	7161678	11394837	0.628502	
	13.6	11655314	18987231	0.61385	
0.2 (CS-4)	13.7	10916585	11490142	0.950083	0.956082
	13.7	11279025	11751612	0.959785	
	13.6	16047291	16744217	0.958378	
0.5 (CS-5)	13.7	25476431	10195048	2.498903	2.446172
	13.6	24509373	10563435	2.320209	
	13.6	35344971	14029092	2.519405	

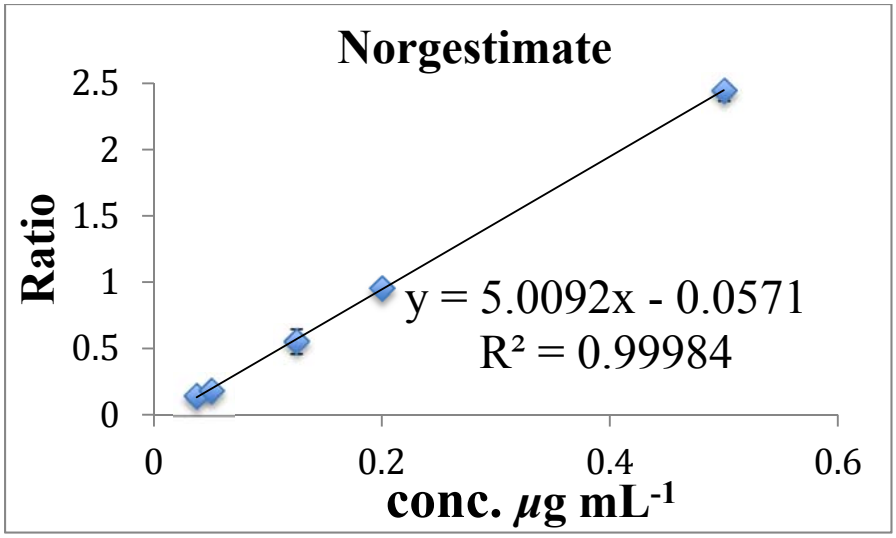

Figure 3.3: The calibration curve of norgestimate.

The calibration curves with corresponding regression equations for each of the other fifteen native analytes are shown in Appendix A, Figure A.1-Figure A.15. These data were used in the verification of the existence and the quantitation of the concentration of the targeted analytes.

III. Instrumental Detection Limit

Most of the target analytes with the original CS-1 concentration in EPA method 1694 could not be detected with a S/N above 5. IDLs of each analytes in the ESI/QIT instrument should be calculated and compared with that of the QqQ instrument. The IDL calculation of ofloxacin is shown as an example below.

First, the IDL of ofloxacin was estimated based on the former detection performance with low concentration. Solutions of ofloxacin with concentration of 1.25 ng mL^{-1} and 3.75 ng mL^{-1} could not be detected by the ESI/QIT MS/MS, and a signal for this analyte was not detected until the concentration was raised to 18.75 ng mL^{-1} with a S/N

around 10. Therefore, the estimated IDL for ofloxacin was 20 ng mL^{-1}. Later, as explained in Chapter, Two Section III, a solution of 20 ng mL^{-1} of ofloxacin was prepared in triplicate and run by the HPLC-MS/MS, the results are listed below.

Table 3.2: Data with which to calculate the IDL for ofloxacin.

Trails	Ratio	Concentration (ng mL)
1	0.462196	18.89
2	0.265477	16.53
3	0.294667	16.88
Average	--	17.44
Standard Deviation	--	1.27
Detection Limit	--	8.87

From above section, the calibration curve of ofloxacin is (See Appendix A. Figure A.15):

$$\text{Ratio} = 83.32 \times \text{conc.}\ (\mu g\ mL^{-1}) - 1.112$$

Ratio = 0.462196 was plugged in the equation above, and the concentration was calculated as 18.89338 μg mL^{-1}. Besides, t-distribution with n-1 (= 2) degree of freedom at 0.01 level of significance is 6.96,[33] so the IDL for ofloxacin is 6.96 × 1.27 ng mL^{-1} = 8.87 ng mL^{-1}. All the IDLs of each analytes are shown in the Appendix A, Table A.5.

From the results, the IDLs of diphenhydramine, thiabendazole, caffeine, sulfadimethoxine, enrofloxacin, ofloxacin, norgestimate, sulfachloropyridazine, sulfamethizole, sulfamethoxazole and sulfamerazine are in ng mL^{-1} level or higher, which

explains the situation why QIT MS/MS instrument could not detect the lower concentrations of these analytes in the original CS-1 and CS-2 concentration levels in EPA method 1694.

Symbol "NA" (in Appendix A, Table A.5) means that the IDL is not available due to either out of stock of the analytes or more experiments are required to calculate the IDLs. For example, 1,7-dimethylxanthine was prepared in 125 ng mL^{-1}, but no signal was found in the mass chromatograms, which means the concentration needs to be increased until the S/N would be around 5-10 (i.e.: 187.5 ng mL^{-1} with S/N is 5.5); Meanwhile, norgestimate was prepared in a concentration of 37.5 ng mL^{-1}, and the S/N was 22, which is larger than 10 and means that the estimated concentration should be lower.

In summary, since QIT-MS/MS cannot measure the concentrations as low as the instrument (QqQ-MS/MS) used in the EPA method for acetaminophen (0.050 μg mL^{-1}), erythromycin (0.25 ng mL^{-1}), norgestimate (2.5 ng mL^{-1}), sulfachloropyridazine (1.25 ng mL^{-1}), sulfadimethoxine (0.25 ng mL^{-1}), sulfamerazine (0.5 ng mL^{-1}), sulfamethazine (0.5 ng mL^{-1}), sulfamethizole (0.5 ng mL^{-1}), sulfamethoxazole (0.5 ng mL^{-1}), enrofloxacin (2.5 μg mL^{-1}), norfloxacin (12.5 ng mL^{-1}), caffeine (12.5 ng mL^{-1}) and ofloxacin (1.25 ng mL^{-1}), the IDLs of these analytes detected by QqQ-MS/MS would be smaller than that of QIT-MS/MS, respectively.

IV. Analyses of Solid Samples From Rendering Plants

i. Results from pre-test chicken feather meal samples

Once the extract samples and QC samples were run by HPLC-MS/MS, mass chromatogram was analyzed in the same way as described in Sections II and III in this

Chapter. Each sample was run two times to show reproducibility. Figure 3.4 is the chromatogram from the pre-test chicken feather meal sample produced by Plant A.

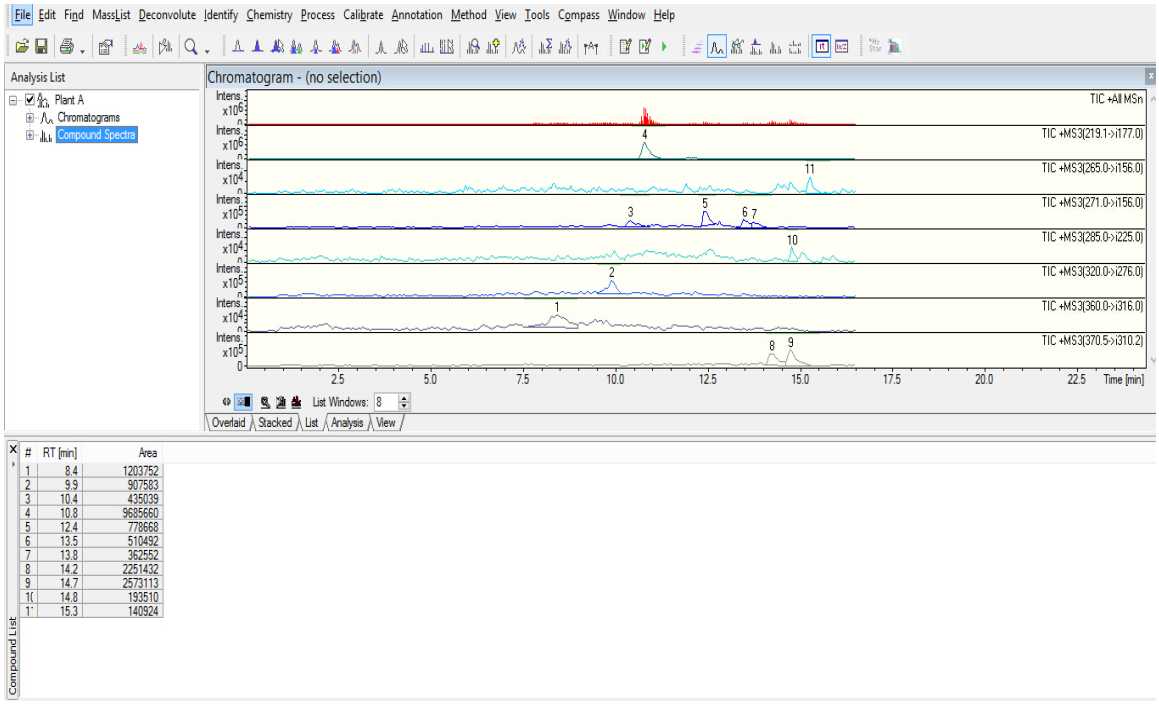

Figure 3.4: The chromatogram of the pre-test chicken feather meal sample produced by Plant A.

In order to verify the existence of target analytes, both parent-daughter ions m/z and RT were strictly compared with Appendix A, Table A.4. For example, in Figure 3.4, parent-daughter ions m/z in the second row was 219.1-177.0 and the RT was 10.7 min, matching $^{13}C_3$-atrazine; while in the last row, the parent-daughter ions m/z was 370.5-310.2, but the RT (14.2 min and 14.7 min) does not match the RT of norgestimate (13.7 min). To verify the performance of HPLC and MS/MS, the verification groups (mixture of standards with the concentrations of CS-3) were inserted into the run order of samples.

The parent-daughter ions m/z and the RTs of each analytes should both match the data shown in Appendix A, Table A.4. If the column of HPLC was contaminated, the RTs would change; if the parent-daughter ions m/z changes, the calibration curves would shift.

The only valid signal was from enrofloxacin, of which parent-daughter ions m/z was 360.0-316.0 and RT was 8.4. The area ratio was $\frac{1203752}{9685660} = 0.124282$ in the first trail, and 0.066408 in the second trail, so the average was 0.0953. However, enrofloxacin peak was also found in the total ion chromatogram of QC blank group, which means there might existed matrix effect or contamination induced by the background, and the background area ratio 0.0765 should be removed, so the final ratio was 0.0953-0.0765 = 0.0188, then plugged in the calibration curve of enrofloxacin: Ratio = 33.604 conc. (μg mL^{-1}) – 0.4784. So the concentration of enrofloxacin was 14.80 ng mL^{-1}. Converted back to the original concentration, where the final extract was 4 mL, the total enrofloxacin collected in this extract was 14.80 ng mL^{-1} × 4 mL =59.20 ng from 0.2487 g of chicken feather meal (raw material). The concentration in ppb was 59.20 ng / 0.2487 g = 238.04 ng g^{-1} = 238.04 ppb.

All the results are shown in Appendix B, Table B.1. Only enrofloxacin (238.04 ng g^{-1}) was detected in the Plant A sample; norfloxacin (2324.51 ng g-1) was detected in Plant B sample; while sulfamethazine (1412.33 ng g^{-1}) and sulfadimethoxine (75.02 ng g^{-1}) were detected in the Plant C sample. Other analytes were not detected from these samples. Attention should be directed to the fact that the concentration of the found

analytes were very low. For example, enrofloxacin was found at 14.80 ng mL^{-1} in the extract, which was below the lowest concentration (37.5 ng mL^{-1}) in the calibration curve of enrofloxacin. The error brought by the detection sensitivity might not be ignored.

The recovery was calculated for the OPR samples, in the same way as with the chicken feather meal samples. The results are shown in the last column in Appendix B, Table B.1. However, the zero recoveries of ten analytes (sulfamethazine, sulfamethoxazole, 1,7-dimethylxanthine, caffeine, norgestimate, sulfachloropyridazine, sulfamerazine, sulfadimethoxine, sulfamethizole and erythromycin) and poor recovery of thiabendazole (only 2.8%) from peat moss were seen in the tests, which lead to an unreliability in our results of these analytes. Therefore, the original spiking volumes of analytes into the OPR sample were increased in order to improve the percent recovery as well as the method performance.

ii. Results of PPCPs in samples from Plant 1

The spiking volume was increased from 30 μL to 90 μL for diphenhydramine, thiabendazole, caffeine, norfloxacin, sulfadimethoxine and enrofloxacin in the OPR samples. Norgestimate, sulfachloropyridazine, sulfamethizole, and erythromycin were spiked at a four-fold volume increased when compared to the original volumes in EPA Method 1694, respectively. While sulfamethoxazole, 1,7-dimethylxanthine, and sulfamerazine were spiked at a five-fold volume (See Appendix A. Table A.5).

The results of the quantitative detection of PPCPs in the chicken feather and chicken feather meal samples from Plant 1 are presented in Appendix B, Table B.2. The samples turned out to be very clean, that is to say, none of the analytes was detected in

the samples. The verification groups discussed in the earlier chapter were examined after every several runs of samples, all the results were valid due to the consistency of the RT and peak area ratio of each analyte. However, sulfamethazine, sulfamethoxazole, 1,7-dimethylxanthine, norgestimate, sulfachloropyridazine, sulfamerazine, sulfamethizole and erythromycin still showed zero percent recovery, which brings of the uncertainty of the existence of the above PPCPs. Therefore, it is necessary to test the chicken feather and chicken feather meal samples from the Plant 1, the Plant 2 and the Plant 3, respectively, as well as the quality control samples of peat moss via HPLC-QqQ MS/MS, and meanwhile detect a broader range of PPCPs.

CHAPTER FOUR

AXYS ANALYTICAL SERVICES LTD.

Since some of the analytes have zero recoveries, which might due to the matrices issues or our samples of peat moss, (AXYS Analytical Services Ltd. also reported that troubles were met in finding a peat moss sample that give excellent recovery.) and the low sensitivity of the HPLC-QIT-MS^n system, samples from Plant 1, Plant 2, and Plant 3 in the U.S. were sent to AXYS Analytical Services Ltd., the same firm that the scientists from Johns Hopkins used.

The earlier ground chicken feathers, chicken feather meals, and filtered DAF-in solid samples from Plant 1, 2 and 3, as well as the ground peat moss were prepared in different centrifuge tubes and sealed in double zip-lock bags. All of the samples and their backups were then packed in a cooler with blue ice, and the cooler was shipped to AXYS Analytical Services Ltd. in Canada.

AXYS Analytical Services Ltd. tested a broader range of PPCPs: 46 Group 1 analytes and 14 Group 2 analytes for each sample. All of the analytes and their respective concentrations that were found in the solid samples from the three rendering plants are shown in Appendix C, Tables C.1-Table C.5. The analytes for which concentrations were above the reporting limits (considered positive detection) are summarized in Table 4.1 and Table 4.2.

Table 4.1. Summary of Group 1 PPCPs that were detected above the reporting limit in all solid samples.

[Drug Class] Analytes Group 1 (Out of 46 Total Analytes)	DAF-in solid (Plant 1)	Chicken feather meal (Plant 1)	Chicken feathers (Plant 1)	Chicken feather meal (Plant 2)	Chicken feathers (Plant 2)	DAF-in solid (Plant 2)	Chicken feather meal (Plant 3)	Chicken feathers (Plant 3)	Peat moss	Number of Samples with This Analyte
UNITS	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	
Azithromycin	17									**1**
Caffeine	29.4				37.7					**2**
Carbamazepine	16.2									**1**
Ciprofloxacin	80.4									**1**
Diphenhydramine	5.11									**1**
Enrofloxacin						5.11				**1**
Erythromycin-H_2O			5.06						5.82	**2**
Lomefloxacin	7.12									**1**
Ofloxacin	9.74									**1**
Ormetoprim				3.02	2.37	1.36				**3**
Sulfadimethoxine		0.71		3.74	2.92	2.41	13.5	0.823		**6**
Number of Analytes Detected in Sample with concentration above the reporting limit	**7**	**1**	**1**	**2**	**3**	**3**	**1**	**1**	**1**	

Table 4.2. Summary of Group 2 PPCPs that were detected above the reporting limit in all solid samples.

[Drug Class] Analytes Group 2 (Out of 14 Total Analytes)	DAF-in solid (A)	Chicken feather meal (Plant 1)	Chicken feathers (Plant 1)	Chicken feather meal (Plant 2)	Chicken feathers (Plant 2)	DAF-in solid (Plant 2)	Chicken feather meal (Plant 3)	Chicken feathers (Plant 3)	Peat moss	Number of Samples with This Analyte
UNITS	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	ng g^{-1} (dry weight basis)	
Anhydrochlortetracycline [ACTC]	29	32.9	30.2		33.3	24.2		32.2		**6**
Doxycycline									21.6	**1**
4-Epioxytetracycline [EOTC]	22.8		29							**2**
Isochlortetracycline [ICTC]							41.7			**1**
Oxytetracycline [OTC]	66.5		154							**2**
Number of Analytes Detected in Sample with concentration above the reporting limit	**3**	**1**	**3**	**0**	**1**	**1**	**1**	**1**	**1**	
Total Number of Analytes in Samples (Group 1 and 2) with concentration above the reporting limit	**10**	**2**	**4**	**2**	**4**	**4**	**2**	**2**	**2**	

A total eleven out of forty-six Group 1 analytes were detected above the reporting limits: they are azithromycin, caffeine, carbamazepine, ciprofloxacin, diphenhydramine, enrofloxacin, erythromycin-H_2O, lomefloxacin, ofloxacin, ormetoprim, and sulfadimethoxine. A total of five out of fourteen Group 2 analytes were detected above the reporting limits: they are ACTC, doxycycline, EOTC, ICTC, and OTC.

Among the aforementioned sixteen positive analytes, only four analytes out of sixty PPCPs, namely ormetoprim (3.02 ppb, Plant 2), sulfadimethoxine (0.71 ppb, Plant 1; 3.74 ppb, Plant 2; 13.5 ppb, Plant 3), ACTC (32.9 ppb, Plant1), and ICTC (41.7 ppb, Plant 3), were detected above the reporting limits in the feed grade chicken feather meal

samples. The analytes acetaminophen, erythromycin, norgestimate, sulfachloropyridazine, enrofloxacin, norfloxacin, 1,7-dimethylxanthine, caffeine and ofloxacin, which were claimed to have positive detections in the samples of U.S. feed grade chicken feather meal in the Johns Hopkins paper, turned out to be negative detections (concentrations below the reporting limits). However, it should be pointed out that some of the concentrations of PPCPs detected in the current solid samples were higher than the concentrations reported in the Johns Hopkins study: ormetoprim (0.6 ppb, Idaho; 1.7 ppb, Tennessee), sulfadimethoxine (0.9 ppb, Idaho; 3.4 ppb, Tennessee), ICTC (16.6 ppb, California; 5.8 ppb, Idaho).[1] ACTC was not reported to be positive detection in Johns Hopkins paper, while this PPCP was detected in the feather meal sample from Plant 1.

The DAF water-in solids from rendering Plant 1 contained the highest number of analytes: 10 positive detections for PPCPs. And the DAF water-in solid from Plant 2 contained the second highest number of analytes: 4. A total of thirteen (13) different analytes were detected in the DAF water-in solids, namely azithromycin, caffeine, carbamazepine, ciprofloxacin, diphenhydramine, enrofloxacin, lomefloxacin, ofloxiacin, ormetoprim, sulfadimethoxine, OTC, EOTC and ACTC.

In the samples of chicken feathers, seven (7) analytes, namely caffeine (37.7 ppb, Plant 2), erythromycin-H_2O (5.06 ppb, Plant 1), ormetoprim (2.37 ppb, Plant 2), sulfadimethoxine (2.92 ppb, Plant 2; 0.823 ppb, Plant 3), ACTC (30.2 ppb, Plant 1; 33.3 ppb, Plant 2; 32.2 ppb, Plant 3), EOTC (29 ppb, Plant 1), and OTC (154 ppb, Plant 1) were detected above the reporting limits.

Peat moss is the matrix used as a quality control group and to evaluate the method performance (blank samples and OPR samples). Even two PPCPs, erythromycin-H_2O (5.82 ppb) and doxycycline (21.6 ppb) were found in a sample of the peat moss used in our laboratory.

From the above discussion, one can see that sulfadimethoxine and ACTC were found in six (6) out of nine (9) solid samples; followed by ormetoprim, which was found in three (3) solid samples, and caffeine and oxytetracycline were found in two (2) solid samples.

From Appendix C, Table C.1 to Table C.5, cloxacillin, oxacilline, penicillin G, and roxithromycin were either not quantifiable or the concentration were estimated. One can see that real samples also have matrix effects, and unknown compounds from the extraction of the sample may interfere with the analytical results.

It is also possible that a correlation exists between the analytes found in the chicken feathers, which are the raw material for chicken feather meal, and the analytes found in either the chicken feather meal and/or the solid from DAF water-in samples. In order to check the null hypothesis of the mean concentration of each analyte in the chicken feather meal, chicken feathers and solid from the DAF water-in system, a "randomized complete block design" was created in the SAS studio academic program. In each statistical test, the observation is the concentration of each analytes reported by AXYS, the treatment is the three different sample types, and the block is the target sixty analytes.

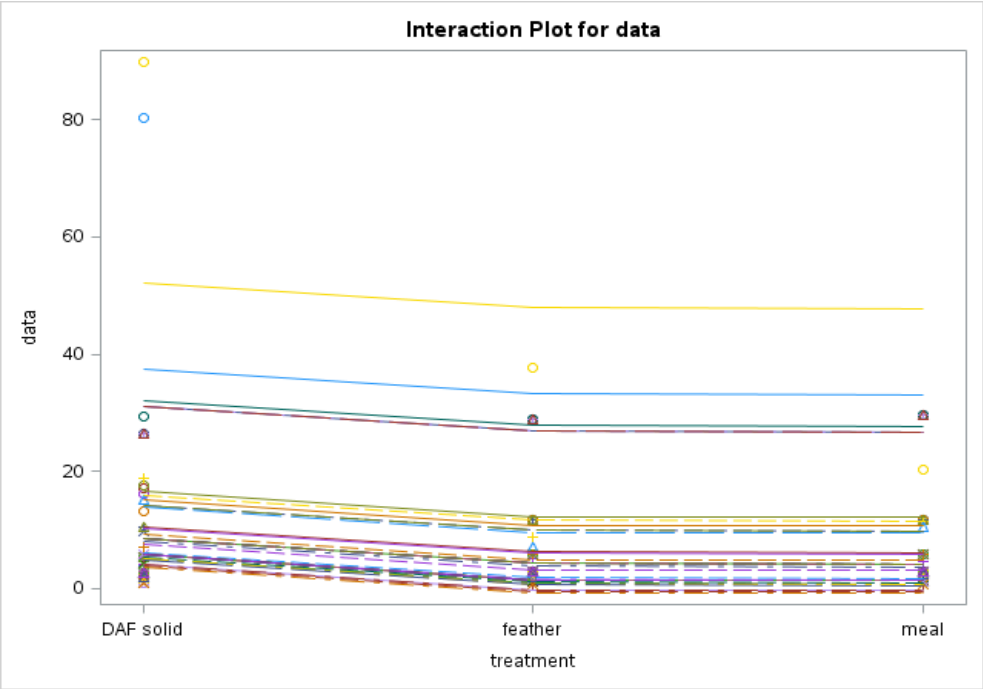

Figure 4.1. The concentrations of 60 analytes (in ppb) in samples of DAF water-in solid, chicken feathers, and chicken feather meal from Plant 1. A colorful line represents a single analyte, while the colorful symbols dots represent the outliers.

Table 4.3. Plant 1: T-test (LSD) for concentration (above), this test controls the Type I error rate; and Tukey's studentized range (HSD) test for concentration (below), this test controls the Type I experimental error rate. The level of significance is 0.05.

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**treatment**
A	11.775	43	DAF solid
B	7.518	43	Feather
B			
B	7.370	43	Meal

Means with the same letter are not significantly different.			
Tukey Grouping	**Mean**	**N**	**treatment**
A	11.775	43	DAF solid
B	7.518	43	Feather
B			
B	7.370	43	Meal

From Table 4.3, one can see in Plant 1 that no significant difference exists between the mean concentrations of analytes in the samples of chicken feather meal and chicken feathers. While, there is a significant difference between the solid from DAF water-in system and the other two samples.

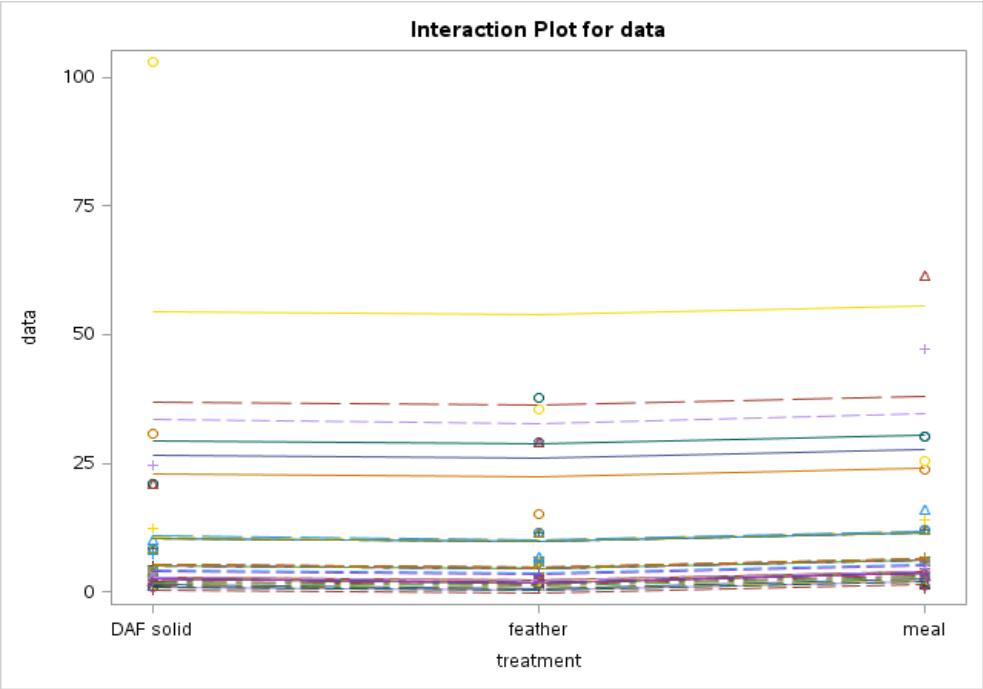

Figure 4.2. The concentrations of 60 analytes (in ppb) in samples of DAF water-in solid, chicken feathers, and chicken feather meal from Plant 2. A colorful line represents a single analyte, while the colorful symbol dots represent the outliers.

Table 4.4. Plant 2: T-test (LSD) for concentration (above), this test controls the Type I error rate; and Tukey's studentized range (HSD) test for concentration (below), this test controls the Type I experimental error rate. The level of significance is 0.05.

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**treatment**
A	9.441	43	Meal
A			
A	8.347	43	DAF solid
A			
A	7.713	43	Feather

Means with the same letter are not significantly different.			
Tukey Grouping	**Mean**	**N**	**treatment**
A	9.441	43	Meal
A			
A	8.347	43	DAF solid
A			
A	7.713	43	Feather

From Table 4.4, one can see in Plant 2 that no significantly difference exists between the mean concentrations of each analyte in the sample of feather meal, chicken feathers and DAF water-in solid.

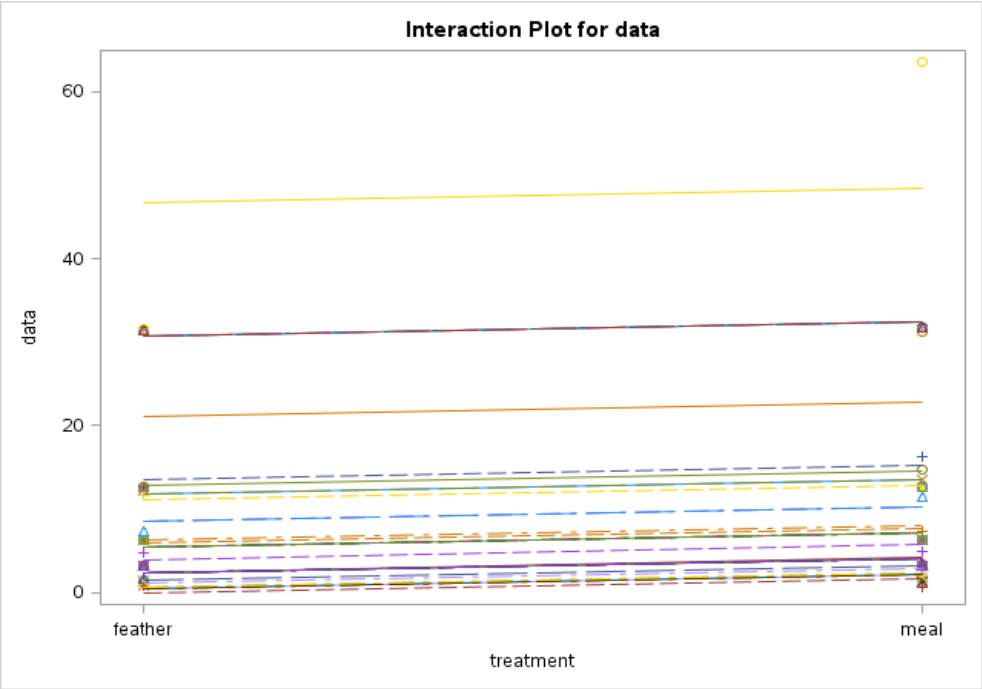

Figure 4.3. The concentrations of 60 analytes (in ppb) in samples of chicken feathers and chicken feather meal from Plant 3. A colorful line represents a single analyte, while the colorful symbols dots represent the outliers.

Table 4.5. Plant 3: T-test (LSD) for concentration (above), this test controls the Type I error rate; and Tukey's studentized range (HSD) test for concentration (below), this test controls the Type I experimental error rate. The level of significance is 0.05.

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**treatment**
A	9.8348	43	Meal
A			
A	8.0907	43	Feather

Means with the same letter are not significantly different.			
Tukey Grouping	**Mean**	**N**	**treatment**
A	9.8348	43	Meal
A			
A	8.0907	43	Feather

From Table 4.5, one can see in Plant 3 that no significant difference exists between the mean concentrations of each analyte in the sample of chicken feather meal and chicken feathers.

In conclusion, the statistical tests show that the concentrations of each analyte in the sample of chicken feather and chicken feather meal are correlated with each other.

Since the samples of chicken feather and chicken feather meal from Plant 1 and the samples of peat moss were analyzed on both HPLC-ESI/QIT MS^n system (see Appendix B, Table B.2) and an HPLC-QqQ MS/MS system [see Appendix C, Table C.1, Table C.4 (column: DAF-in solid from Plant 1) and Table C.5 (column: peat moss)], a comparison of the performances of these two instruments is possible. The same analytes were detected in the same samples by the QqQ analyzer MS (AYXS): erythromycin (5.06

ppb) in chicken feather meal; sulfadimethoxine (0.71 ppb) in chicken feather meal; and thiabendazole (5.11 ppb), caffeine (37.7 ppb), and ofloxacin (9.74 ppb) in DAF-out solids have were not detected by the ESI/QIT MS/MS due to the relatively higher IDLs. For example, 37.7 ppb of caffeine was detected in 0.25 g of DAF-in solids with the QqQ analyzer MS (AXYS). Had all of this caffeine been extracted into a final volume 4-mL for injection into an LC-MS system, the concentration of caffeine would have been 2.35 ng mL^{-1}, which is much lower than the IDL of caffeine (13.07 ng mL^{-1}) determined on the HPLC-ESI/QIT MS^n system. This does not even take into account that the percent recovery for caffeine was 46% on our instrument (see Appendix A, Table A.5), while AXYS had a better percent recovery (85%).

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTION

1. The fresh chicken feather meal (feed grade) analyzed in this study does not contain as many PPCPs as was found in the samples reported by Johns Hopkins Center for a Livable Future. But one should note that higher concentrations were found for four detected analytes: ormetoprim, sulfadimethoxine, ICTC and ACTC.

2. The poultry industry should consider the use of ACTC and sulfadimethoxine versus the levels of these analytes found in the samples of chicken feather meal analyzed in this study.

3. A QIT-MS/MS instrument is not as sensitive as a QqQ-MS/MS instrument, and the detection limits were found to be higher than on a QqQ-MS/MS instrument for all of the sixteen (16) target analytes studied: acetaminophen, erythromycin, norgestimate, sulfachloropyridazine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxazole, thiabendazole, enrofloxacin, norfloxacin, 1,7-dimethylxanthine, caffeine, ofloxacin and diphenhydramine.

4. A correlation exists between the concentrations of sixty analytes in the samples of chicken feathers and chicken feather meal based on the large quantities of data reported by AXYS Analytical Services Ltd.

5. The DAF water systems in the rendering plants appear to be a place of accumulation of PPCPs during the rendering process of chicken feather meal, e.g., especially in Plant 1. Thus consideration should be given with respect to whether an economical way could be found for removal of PPCPs at this point in the process so that

the risk of further contamination of chicken feather meal product is reduced. More study and discussions can be find in the Thesis of my co-worker Mrs. Chen Liu.[33]

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DEFINITIONS

Flag:

H = concentration is estimated;

N = authentic recovery is not within method/contract control limits;

NQ = data not quantifiable;

U = not detected at the reporting limit;

V = surrogate recovery is not within method/contract limits.

MAX = concentration is an estimate maximum value.

APPENDICES

Appendix A

Parameters for Sample Preparation and Instrumentation Set-up

Table A.1. Concentrations of each PPCPs commercial standards and spiking volumes (acid extraction).

Compounds	Weight (mg)	V of solvent (μL)	Original conc. (mg mL^{-1})	Target conc. (μg mL^{-1})	Solvent	Inject V (μL)
Acetaminophen	NA	NA	100	100	Acetonitrile	30
Erythromycin	NA	NA	0.1	100	Acetonitrile	30
Norgestimate	0.656	656	1	1000	Methanol	30
Sulfachloropyridazine	2.281	1000	2.281	2.5	Acetonitrile	30
Sulfadimethoxine	1.4	1400	1	0.5	Methanol	30
Sulfamerazine	1.257	1000	1.257	1	Methanol	30
Sulfamethazine	1.984	992	2	1	Methanol	30
Sulfamethizole	0.663	663	1	1	Methanol	30
Sulfamethoxazole	NA	NA	0.1	1	Acetonitrile	30
Thiabendazole	1.065	1065	1	1000	Methanol	30
Enrofloxacin	0.968	968	1	1000	Methanol: Acetonitrile =1:1	30
Norfloxacin	1.226	613	2	2000	Glacial acetic acid/water	30
Diphenhydramine	NA	NA	1	1	Methanol	30
Oxytetracycline Dihydrate	0.674	625	1	0.5	DMSO	200
1,7-Dimethylxanthine	1.6	1600	1	1000		
Caffeine	NA	NA	1	1000		
Ofloxacin	1	1000	1	1000		
Labelled compound			μg mL^{-1}			
$^{13}C_6$-Sulfamethoxazole	NA	NA	100	1	Acetonitrile	100
$^{13}C_3$-Caffeine	NA	NA	100	3	Methanol	100
$^{13}C_2$-Erythromycin	NA	NA	100	1	Acetonitrile	100
$^{13}C_6$-Sulfamethazine	NA	NA	100	1	Acetonitrile	100
Internal standard						
$^{13}C_3$-Atrazine	NA	NA	100	2.5	Nonane	80

Table A.2. Concentration of calibration standards for Group 1 compounds (μg mL^{-1}) in EPA method.[19]

Compound	CS-1	CS-2	CS-3	CS-4	CS-5
Acetaminophen	0.050	0.15	0.75	2.5	10
Erythromycin	0.00025	0.00075	0.00375	0.0125	0.05
Norgestimate	0.0025	0.0075	0.0375	0.125	0.5
Sulfachloropyridazine	0.00125	0.00375	0.01875	0.0625	0.25
Sulfadimethoxine	0.00025	0.00075	0.00375	0.0125	0.05
Sulfamerazine	0.0005	0.0015	0.0075	0.025	0.1
Sulfamethazine	0.0005	0.0015	0.0075	0.025	0.1
Sulfamethizole	0.0005	0.0015	0.0075	0.025	0.1
Sulfamethoxazole	0.0005	0.0015	0.0075	0.025	0.1
Thiabendazole	0.00125	0.00375	0.0187	0.0625	0.25
Enrofloxacin	0.0025	0.0075	0.0375	0.125	0.5
Norfloxacin	0.0125	0.0375	0.187	0.625	2.5
Diphenhydramine	0.0005	0.0015	0.0075	0.025	0.1
1,7-Dimethylxanthine	0.125	0.375	1.870	6.250	25
Caffeine	0.0125	0.0375	0.187	0.625	2.5
Ofloxacin	0.00125	0.00375	0.01875	0.0625	0.25
Internal standard					
$^{13}C_3$-Atrazine	0.05	0.05	0.05	0.05	0.05
$^{13}C_3$-Caffeine	0.075	0.075	0.075	0.075	0.075
$^{13}C_6$-Sulfamethoxazole	0.025	0.025	0.025	0.025	0.025
$^{13}C_2$-Erythromycin	0.025	0.025	0.025	0.025	0.025
$^{13}C_6$-Sulfamethazine	0.025	0.025	0.025	0.025	0.025

Table A.3. New concentration of calibration standards for Group 1 compounds (μg mL^{-1}).

Compound	CS-1	CS-2	CS-3	CS-4	CS-5
Acetaminophen	0.75	1	2.5	4	10
Erythromycin	0.00375	0.005	0.0125	0.02	0.05
Norgestimate	0.0375	0.05	0.125	0.2	0.5
Sulfachloropyridazine	0.01875	0.025	0.0625	0.1	0.25
Sulfadimethoxine	0.00375	0.005	0.0125	0.02	0.05
Sulfamerazine	0.0075	0.01	0.025	0.04	0.1
Sulfamethazine	0.0075	0.01	0.025	0.04	0.1
Sulfamethizole	0.0075	0.01	0.025	0.04	0.1
Sulfamethoxazole	0.0075	0.01	0.025	0.04	0.1
Thiabendazole	0.01875	0.025	0.0625	0.1	0.25
Enrofloxacin	0.0375	0.05	0.125	0.2	0.5
Norfloxacin	0.1875	0.25	0.625	1	2.5
Diphenhydramine	0.0075	0.01	0.025	0.04	0.1
1,7-Dimethylxanthine	1.875	2.5	6.25	10	25
Caffeine	0.1875	0.25	0.65	1	2.5
Ofloxacin	0.01875	0.025	0.0625	0.1	0.25
Internal standard					
$^{13}C_3$-Atrazine	0.05	0.05	0.05	0.05	0.05
$^{13}C_3$-Caffeine	0.075	0.075	0.075	0.075	0.075
$^{13}C_6$-Sulfamethoxazole	0.025	0.025	0.025	0.025	0.025
$^{13}C_2$-Erythromycin	0.025	0.025	0.025	0.025	0.025
$^{13}C_6$-Sulfamethazine	0.025	0.025	0.025	0.025	0.025

Calibration Curve for Different Analytes

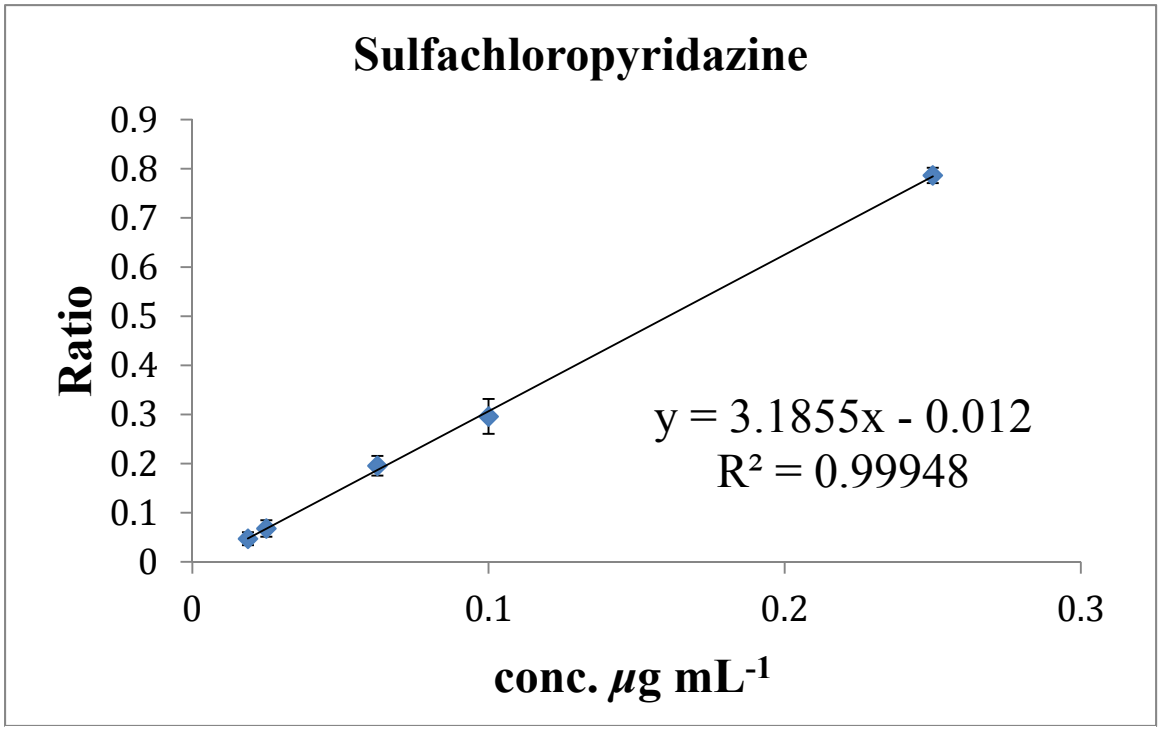

Figure A.1: The calibration curve of sulfachloropyridazine.

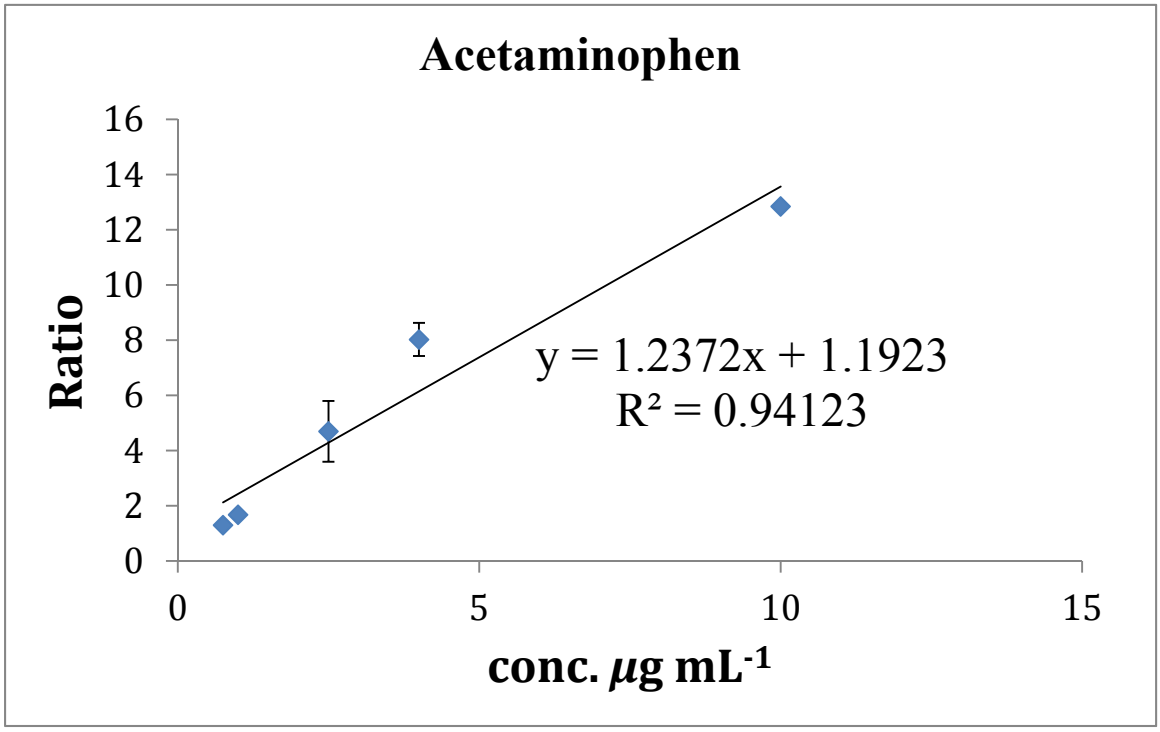

Figure A.2: The calibration curve of acetaminophen.

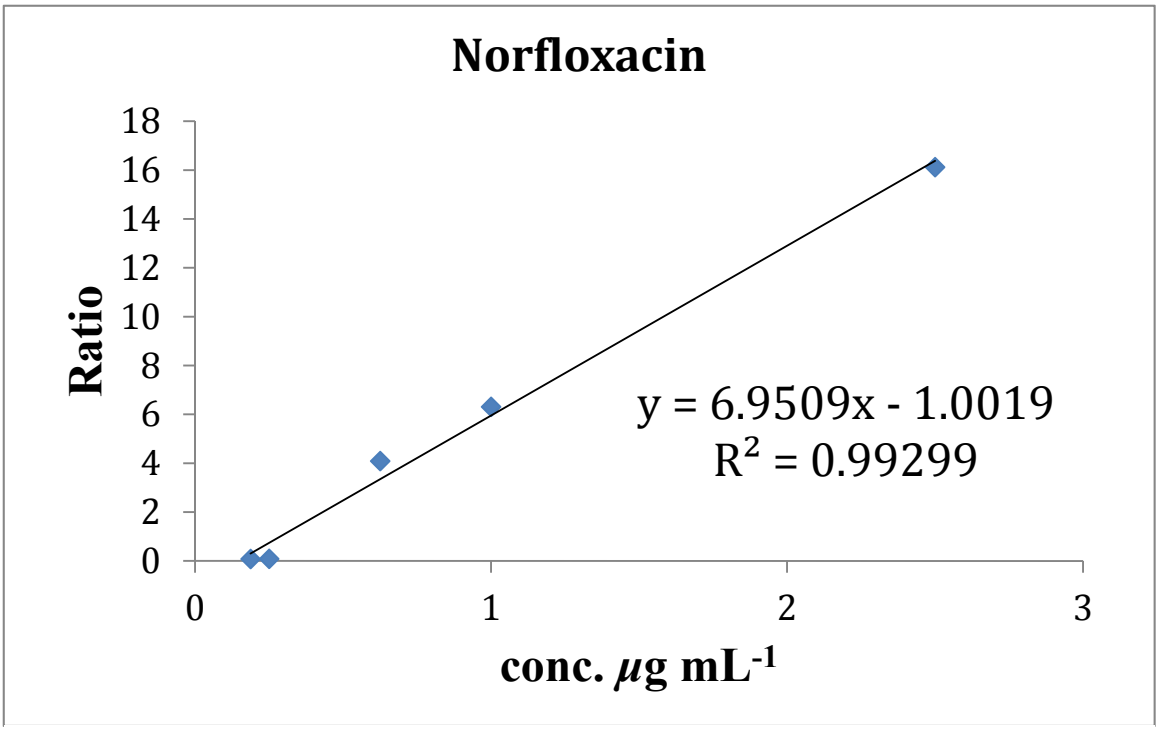

Figure A.3: The calibration curve of norfloxacin.

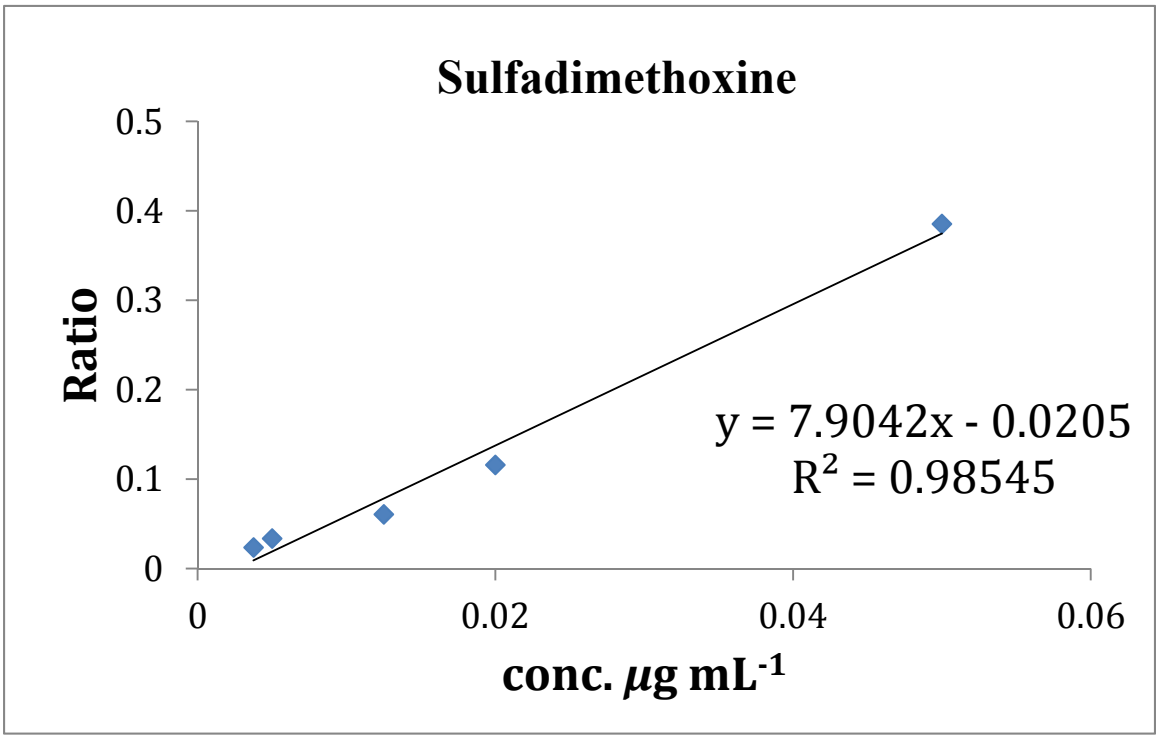

Figure A.4: The calibration curve of norfloxacin.

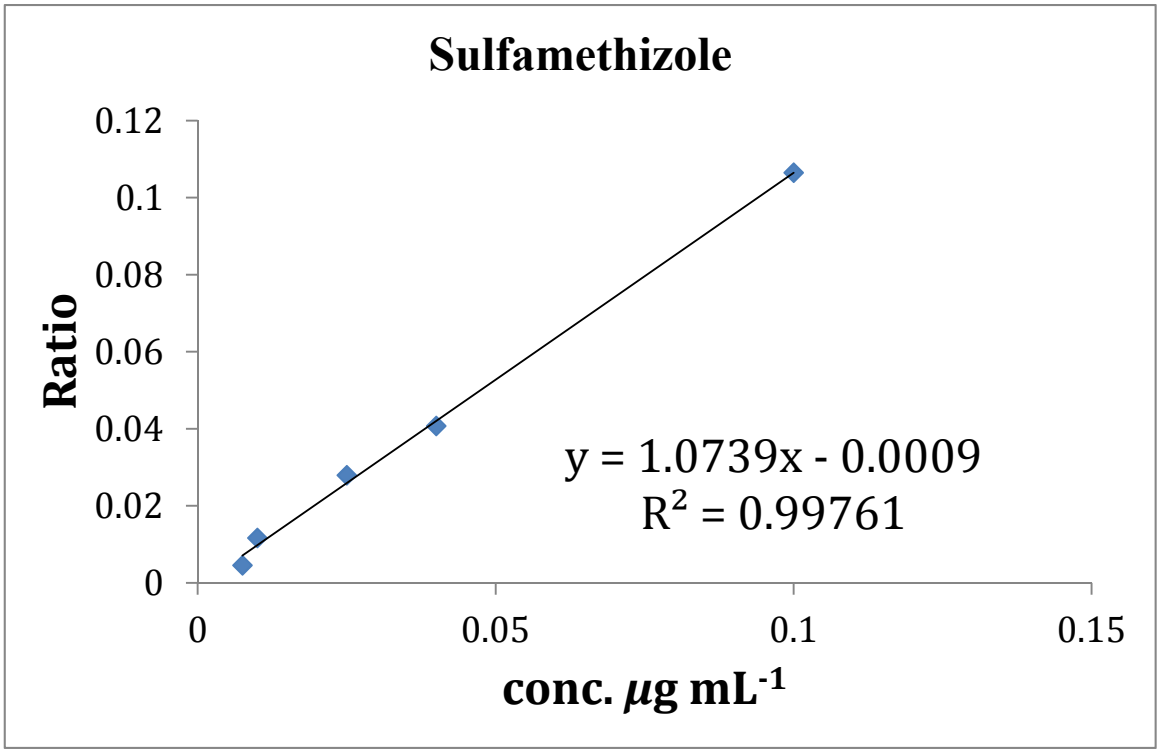

Figure A.5: The calibration curve of sulfamethizole.

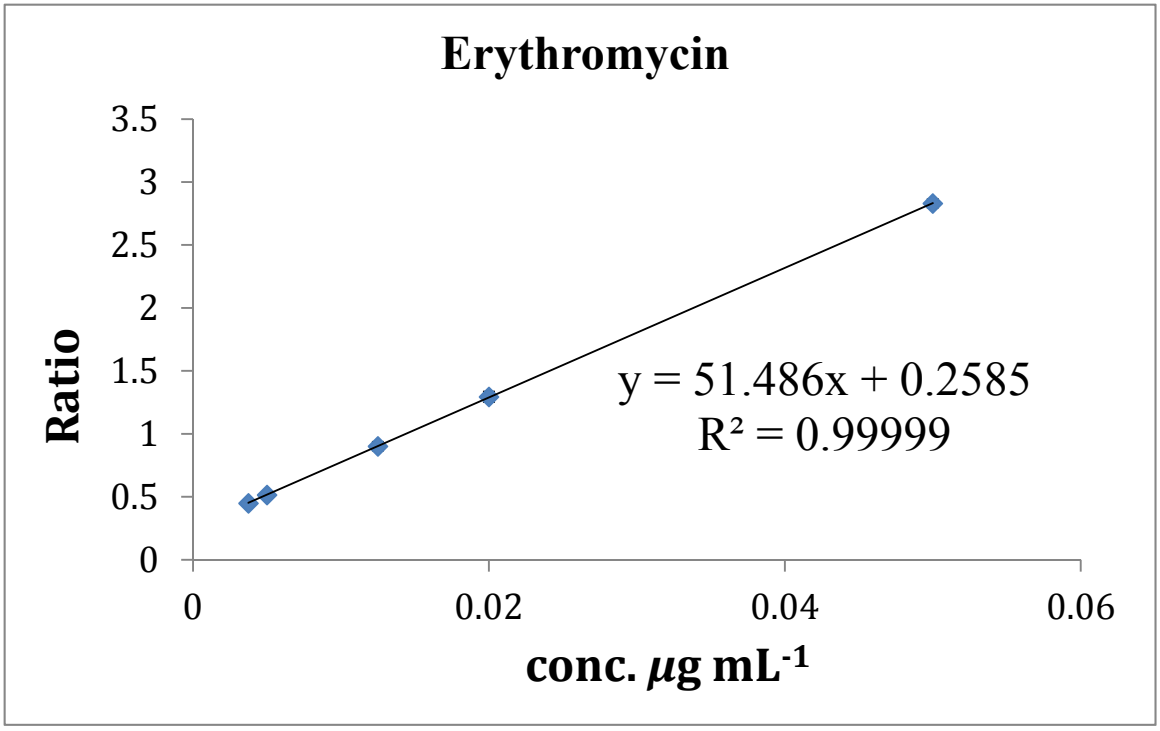

Figure A.6: The calibration curve of erythromycin.

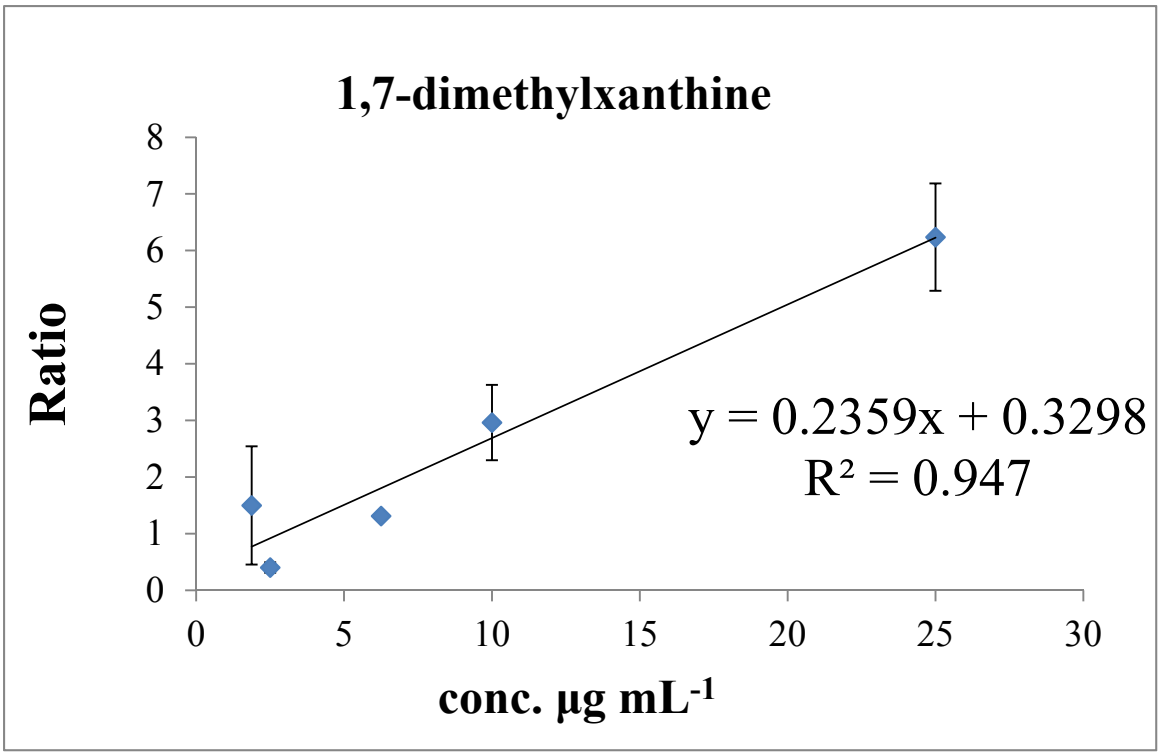

Figure A.7: The calibration curve of 1,7-dimethylxanthine.

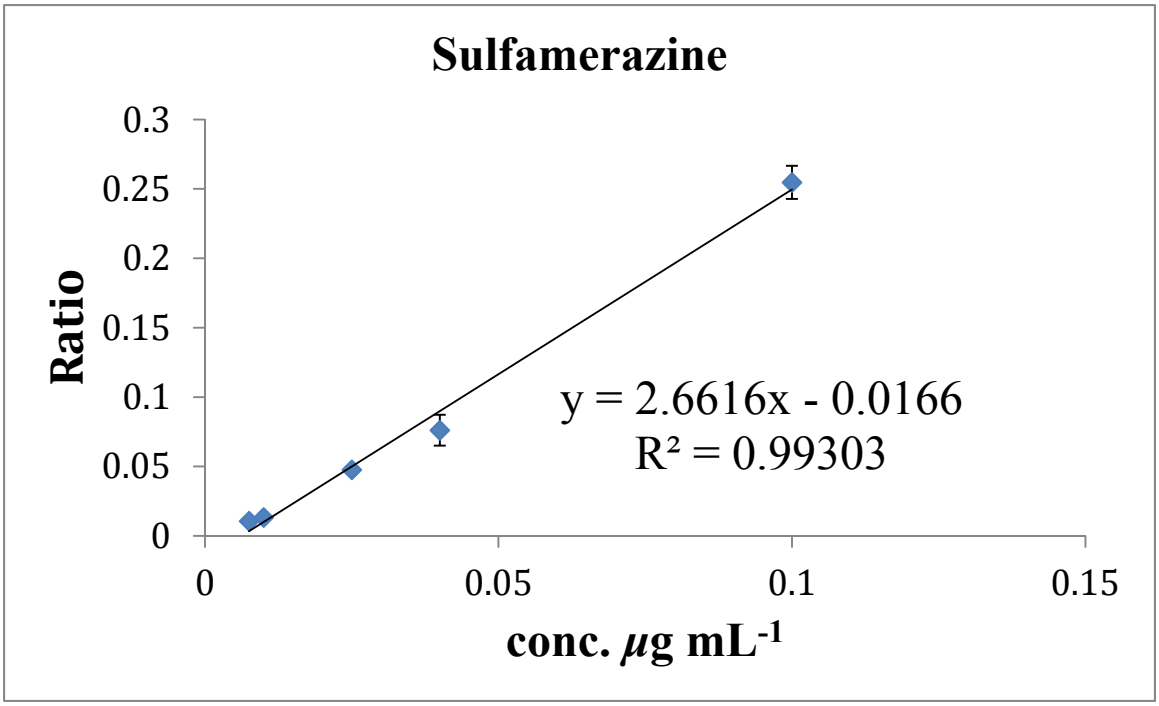

Figure A.8: The calibration curve of sulfamerazine.

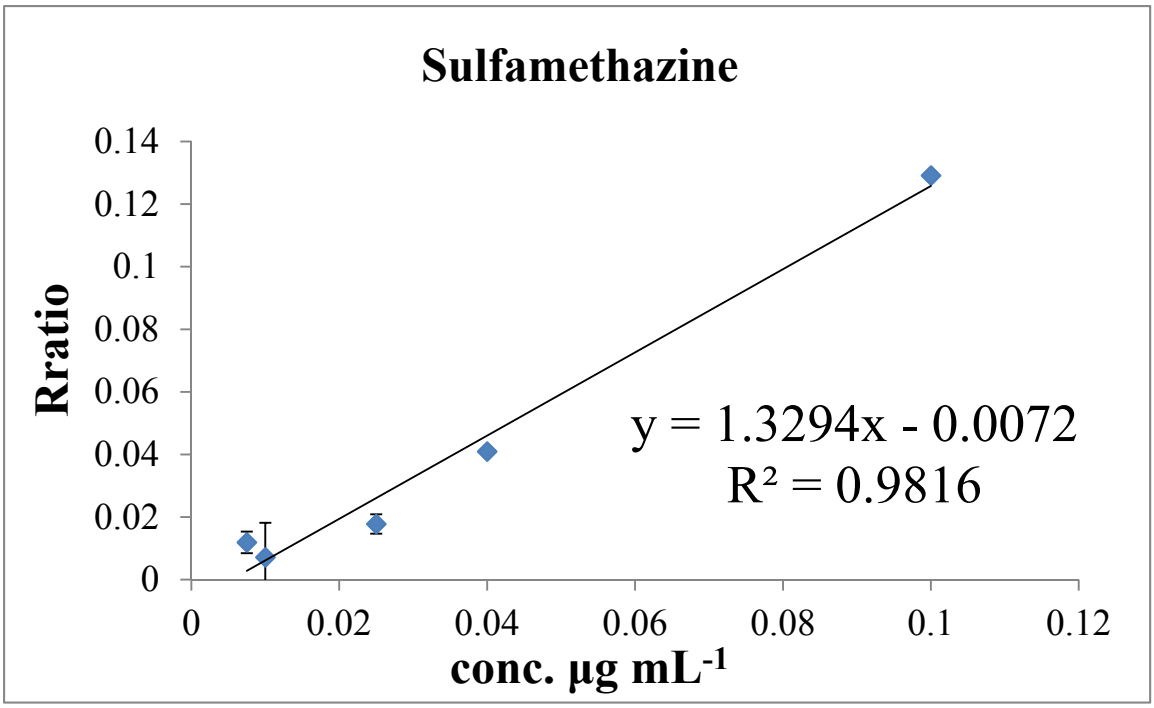

Figure A.9: The calibration curve of sulfamethazine.

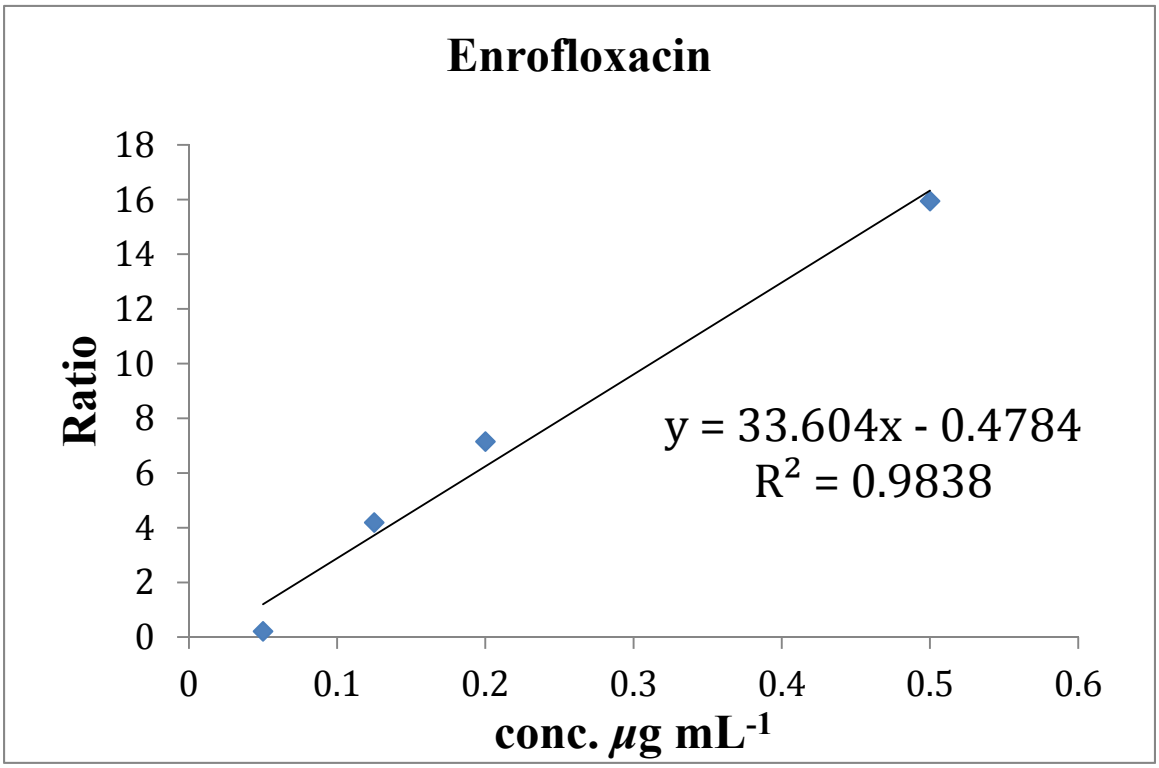

Figure A.10: The calibration curve of enrofloxacin.

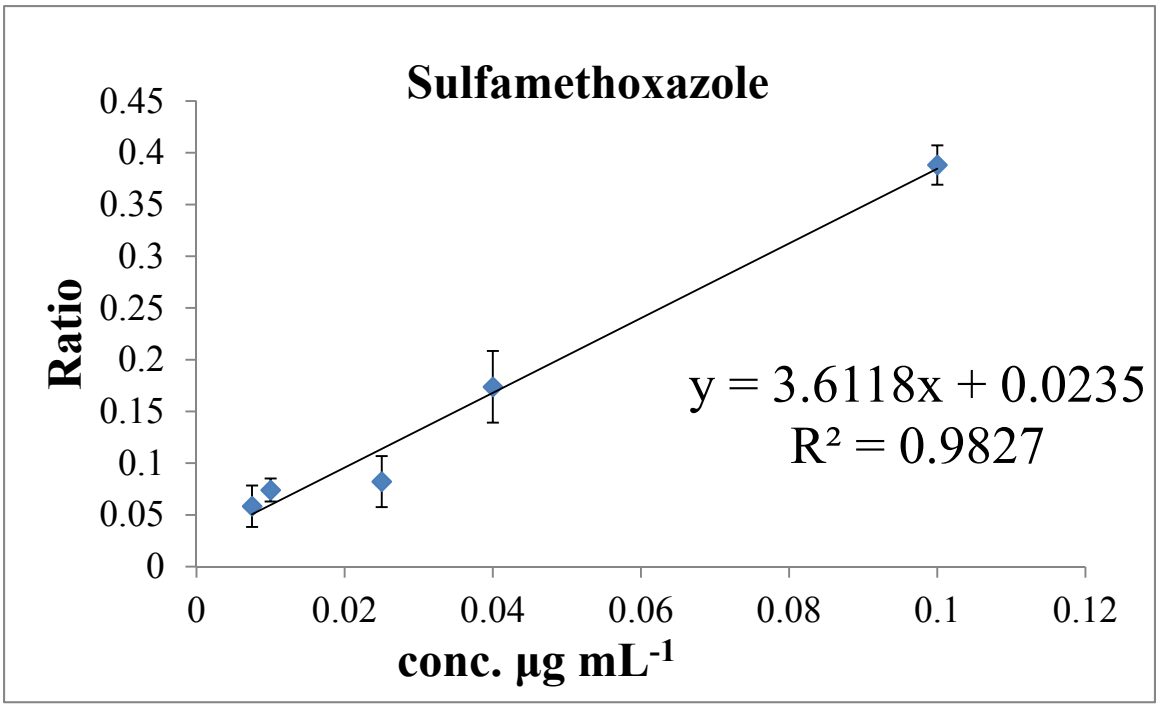

Figure A.11: The calibration curve of sulfamethoxazole.

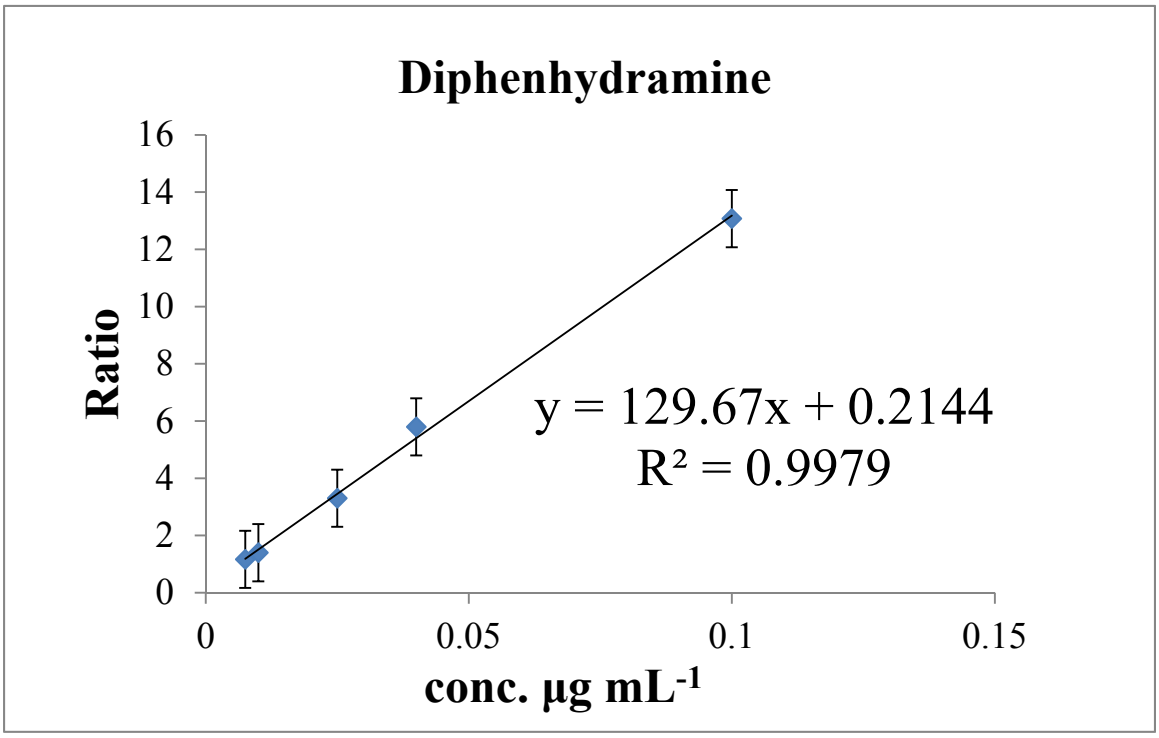

Figure A.12: The calibration curve of diphenhydramine.

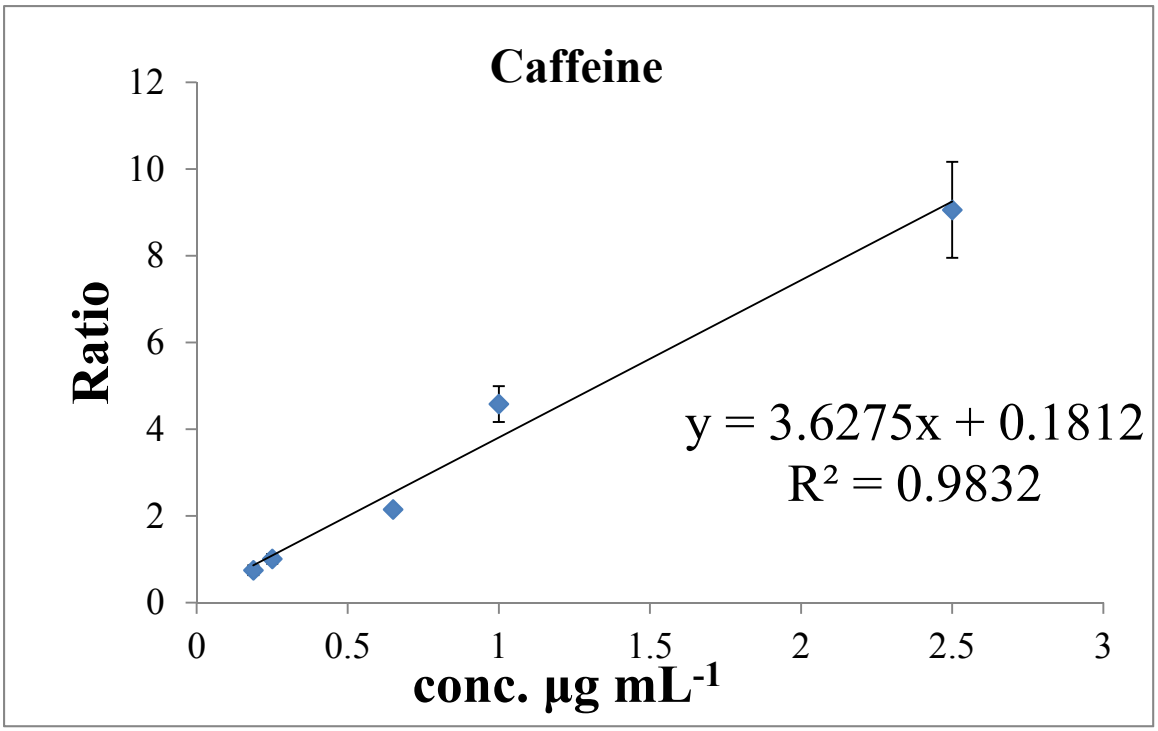

Figure A.13: The calibration curve of caffeine.

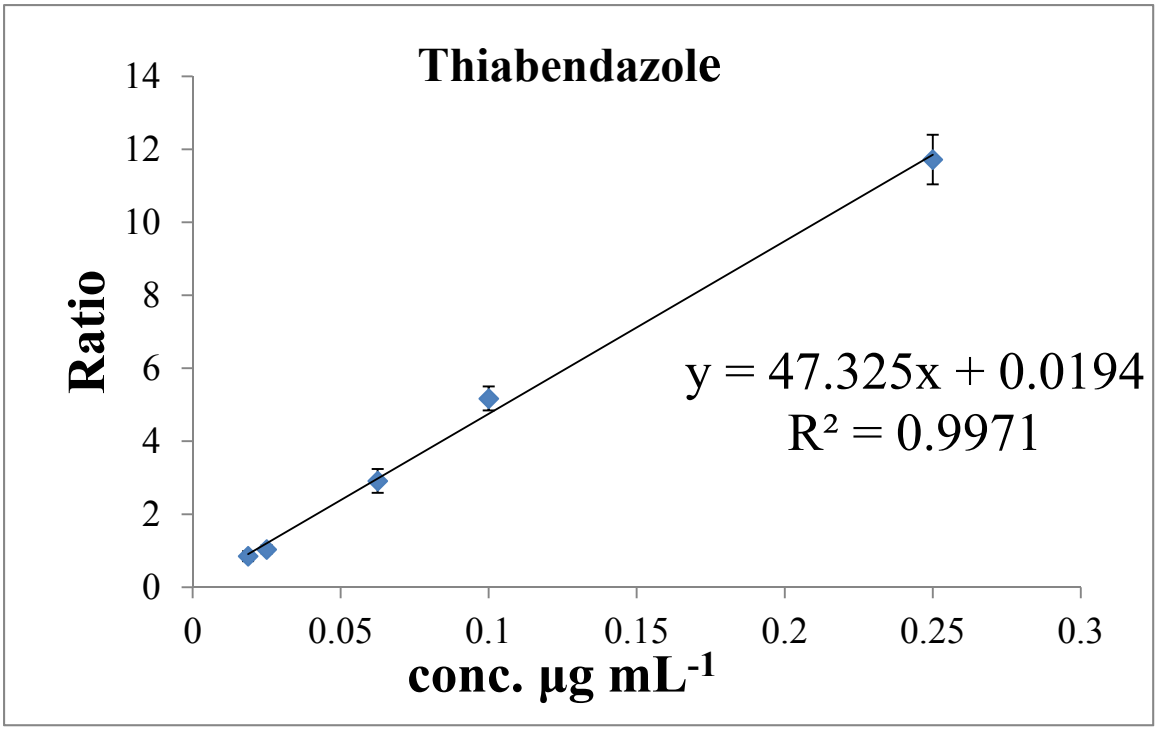

Figure A.14: The calibration curve of thiabendazole.

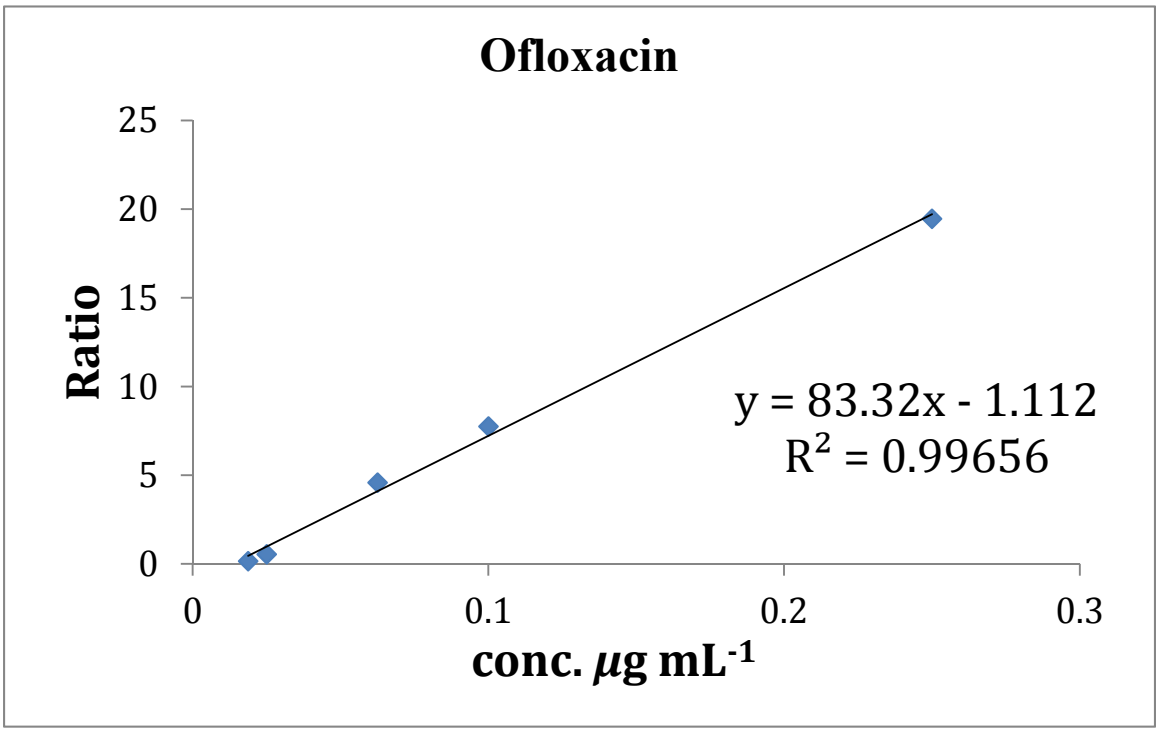

Figure A.15: The calibration curve of ofloxacin.

Table A.4. Parent-daughter ions settings in HPLC/MS/MS program.

Analytes	Parent-daughter ions	Cutoff	Amplify	Retention time
Acetaminophen	152.2-110.0	100	1	5.2
Sulfamethazine	279.0-196.0	90	0.7	7.8
Sulfamethizole	271.0-187.0	100	1	8
Sulfamethoxazole	254.0-156.0	85	0.5	8.8
Diphenhydramine	256.1-167.1	80	0.9	10
Thiabendazole	202.1-175.1	100	0.8	7.6
1,7-Dimethylxanthine	181.2-124.0	80	1	6.5
Caffeine	195.0-138.0	90	0.9	7.3
Norgestimate	370.5-310.2	100	0.5	13.7
Sulfachloropyridazine	285.0-156.0	77	1	8.5
Sulfamerazine	265.0-156.0	72	0.5	7.1
Norfloxacin	320.0-276.0	85	0.6	8.1
Sulfadimethoxine	311.0-156.0	90	0.65	9.6
Enrofloxacin	360.0-316.0	97	0.7	8.3
Ofloxacin	362.2-318.0	98	0.5	8.3
Erythromycin	734.4-576.4	198	0.7	10
$^{13}C_3$-Caffeine	198.0-140.0	90	0.7	7.3
$^{13}C_6$-Sulfamethazine	285.1-204.0	100	1.2	7.8
$^{13}C_6$-Sulfamethoxazole	260.0-162.0	85	0.7	8.7
$^{13}C_2$-Erythromycin	736.4-578.4	260	0.5	10.1
$^{13}C_3$-Atrazine	219.1-177.0	80	0.75	10.9

Table A.5. Percent recoveries from peat moss and IDLs for pure commercial standards.

[Drug Class] Analytes Group 1	Spiking volume (μL)	Recovery (%)	IDL (ng mL^{-1})
Diphenhydramine	90	55.6	1.04
Thiabendazole	90	239.3	4.87
Caffeine	90	46	13.07
Norfloxacin	90	70	305.10
Sulfadimethoxine	90	128.7	9.24
Enrofloxacin	90	67.1	20.87
Ofloxacin	90	62	8.87
Norgestimate	120	18.5	NA
Sulfachloropyridazine	120	0	45.16
Sulfamethizole	120	0	26.94
Erythromycin	120	0	NA
Sulfamethoxazole	150	8.31	3.10
1,7-Dimethylxanthine	150	106.16	NA
Sulfamerazine	150	0	13.07

Table A.6. Group 1 – Acidic extraction, ESI (+) instrument conditions in EPA method 1694.[19]

LC Gradient Program		LC Flow Rate (mL/min)	Gradient	General LC Conditions	
Time (min)	Flow Mixture[1]			Column Temp	40 °C
0.0	95% Solvent A 5% Solvent B	0.15	1	Flow Rate	0.15-0.30 mL/min
4.0	95% Solvent A 5% Solvent B	0.25	6	Max Pressure	345 bar
22.5	12% Solvent A 88% Solvent B	0.30	6	Autosampler tray temperature	4 °C
23.0	100% Solvent B	0.30	6	MS Conditions	
26.0	100% Solvent B	0.30	6	Source Temp	140 °C
26.5	95% Solvent A 5% Solvent B	0.15	6	Desolvation Temp	350°C
33	95% Solvent A 5% Solvent B	0.15	6	Cone / Desolvation Gas Rate	80 L/hr / 400 L/hr

1. Solvent A = 0.3% Formic Acid and 0.1% Ammonium Formate in HPLC water
   Solvent B = 1:1 Acetonitrile:Methanol

Table A.7. Group 2 – Acidic extraction, ESI (+) instrument conditions in EPA method 1694.[19]

LC Gradient Program		LC Flow Rate (mL/min)	Gradient	General LC Conditions	
Time (min)	Flow Mixture[1]			Column Temp	40 °C
0.0	10% Solvent A 90% Solvent B	0.20	1	Flow Rate	0.20-0.23 mL/min
1.0	10% Solvent A 90% Solvent B	0.20	6	Max Pressure	345 bar
18.0	40% Solvent A 60% Solvent B	0.23	6	Autosampler tray temperature	4 °C
20.0	90% Solvent A 10% Solvent B	0.23	6	MS Conditions	
24.0	90% Solvent A 10% Solvent B	0.23	6	Source Temp	120 °C
24.3	10% Solvent A 90% Solvent B	0.20	6	Desolvation Temp	400°C
28	10% Solvent A 90% Solvent B	0.20	6	Cone / Desolvation Gas Rate	70 L/hr / 450 L/hr

1. Solvent A = 1:1 acetonitrile:methanol, with 5 mM Oxalic Acid
   Solvent B = HPLC H_2O, with 5 mM Oxalic Acid

Table A.8. Group 3 – Acidic extraction, ESI (-) instrument conditions in EPA method 1694.[19]

LC Gradient Program		LC Flow Rate (mL/min)	Gradient	General LC Conditions	
Time (min)	Flow Mixture[1]			Column Temp	40 °C
0.0	60% Solvent A 40% Solvent B	0.2	1	Flow Rate	0.200 mL/min
0.5	60% Solvent A 40% Solvent B	0.2	6	Max Pressure	345 bar
7.0	100% Solvent B	0.2	6	Autosampler tray temperature	4 °C
12.5	100% Solvent B	0.2	6	MS Conditions	
12.7	60% Solvent A 40% Solvent B	0.2	6	Source Temp.	100 °C
16	60% Solvent A 40% Solvent B	0.2	1	Desolvation Temp.	350°C
				Cone / Desolvation Gas Rate	50 L/hr / 300 L/hr

2. Solvent A = 0.1% Ammonium Acetate and 0.1% Acetic Acid in HPLC water
   Solvent B = 1:1 Methanol:Acetonitrile

Table A.9. Group 4 – Basic extraction, ESI (+) instrument conditions in EPA method 1694.[19]

LC Gradient Program		LC Flow Rate (mL/min)	Gradient	General LC Conditions	
Time (min)	Flow Mixture[1]			Column Temp	40 °C
0.0	2% Solvent A 98% Solvent B	0.25	1	Flow Rate	0.25 mL/min
5.0	30% Solvent A 70% Solvent B	0.25	6	Max Pressure	345 bar
12.0	30% Solvent A 70% Solvent B	0.25	6	Autosampler tray temperature	4 °C
12.5	2% Solvent A 98% Solvent B	0.25	6	MS Conditions	
16.0	2% Solvent A 98% Solvent B	0.25	6	Source Temp	120 °C
				Desolvation Temp	350°C
				Cone / Desolvation Gas Rate	70 L/hr / 400 L/hr

1. Solvent A = 0.1% Acetic Acid/Ammonium Acetate Buffer
   Solvent B = Acetonitrile

Table A.10. Group 1 – Acidic extraction, ESI (+) instrument conditions in HPLC-MS/MS at the University of Alabama.

LC Gradient Program		LC Flow Rate (mL/min)	Gradient	General LC Conditions	
Time (min)	Flow Mixture[1]			Column Temp	40 °C
0.0	95% Solvent A 5% Solvent B	0.15	1	Flow Rate	0.15-0.30 mL/min
2.0	95% Solvent A 5% Solvent B	0.25	6	Max Pressure	345 bar
10.5	12% Solvent A 88% Solvent B	0.30	6	Autosampler tray temperature	4 °C
11.5	100% Solvent B	0.30	6	MS Conditions	
13.0	100% Solvent B	0.30	6	Source Temp	140 °C
13.5	95% Solvent A 5% Solvent B	0.15	6	Desolvation Temp	350°C
16.5	95% Solvent A 5% Solvent B	0.15	6	Cone / Desolvation Gas Rate	80 L/hr / 400 L/hr

1. Solvent A = 0.3% Formic Acid and 0.1% Ammonium Formate in HPLC water
   Solvent B = 1:1 Acetonitrile:Methanol

Appendix B

The Concentration of PPCPs in Solid Samples Detected at Alabama

Table B.1. The concentration of each PPCPs in three pre-test chicken feather samples from Dr. Greene.

[Drug Class] Analytes	Plant A ppb ($ng\ g^{-1}$)	Plant B ppb ($ng\ g^{-1}$)	Plant C ppb ($ng\ g^{-1}$)	Recovery from peat moss (%)
Sulfamethazine	U	U	1412.33	0
Sulfamethoxazole	U	U	U	0
Diphenhydramine	U	U	U	67.0
Thiabendazole	U	U	U	2.8
1,7-Dimethylxanthine	U	U	U	0
Caffeine	U	U	U	0
Norgestimate	U	U	U	0
Sulfachloropyridazine	U	U	U	0
Sulfamerazine	U	U	U	0
Norfloxacin	U	2324.51	U	84.6
Sulfadimethoxine	U	U	75.02	0
Enrofloxacin	238.04	U	U	47.1
Ofloxacin	U	U	U	77.0
Sulfamethizole	U	U	U	0
Erythromycin	U	U	U	0

Table B.2. The concentration of each PPCPs in the chicken feather, chicken feather meal, DAF water-in and DAF water-out samples collected from Plant 1 in the U.S.

[Drug Class] Analytes	Chicken feather ppb (ng/g)	Chicken feather meal	DAF in solid	DAF out solid	Recovery from peat moss (%)
Sulfamethazine	U	U	U	U	0
Sulfamethoxazole	U	U	U	U	0
Diphenhydramine	U	U	U	U	55.6
Thiabendazole	U	U	U	U	239.3
1,7-Dimethylxanthine	U	U	U	U	0
Caffeine	U	U	U	U	46.0
Norgestimate	U	U	U	U	0
Sulfachloropyridazine	U	U	U	U	0
Sulfamerazine	U	U	U	U	0
Norfloxacin	U	U	U	U	70.0
Sulfadimethoxine	U	U	U	U	128.7
Enrofloxacin	U	U	U	U	46.1
Ofloxacin	U	U	U	U	62.0
Sulfamethizole	U	U	U	U	0
Acetaminophen	U	U	U	U	133.5
Erythromycin	U	U	U	U	0

Appendix C

The Concentration of PPCPs in Solid Samples Reported by AXYS Analytical Services Ltd.

Table C.1. The concentration of each PPCPs detected in the chicken feather and chicken feather meal samples collected from Plant 1 in the U.S.

	Chicken feather meal from Plant 1		Chicken feather from Plant 1	
Sample Size	0.508g (dry)		0.519g (dry)	
UNITS	Flag	ng g^{-1} (dry weight basis)	Flag	ng g^{-1} (dry weight basis)
Acetaminophen	U	29.5	U	28.9
Azithromycin	U	2.95	U	2.89
Caffeine	U	29.5	U	28.9
Carbadox	U	2.95	U	2.89
Carbamazepine	U	2.95	U	2.89
Cefotaxime	U	11.8	U	11.6
Ciprofloxacin	U	11.8	U	11.6
Clarithromycin	U	2.95	U	2.89
Clinafloxacin	U	11.8	U	11.6
Cloxacillin	U H	5.91	U H	5.78
Dehydronifedipine	U	1.18	U	1.16
Diphenhydramine	U	1.18	U	1.16
Diltiazem	U	0.591	U	0.729
Digoxin	U	11.8	U	11.6
Digoxigenin	U	20.2	U	37.6
Enrofloxacin	U	5.91	U	5.78
Erythromycin-H2O	U	4.53		5.06
Flumequine	U	2.95	U	2.89
Fluoxetine	U	2.95	U	2.89
Lincomycin	U	5.91	U	5.78
Lomefloxacin	U	5.91	U	5.78
Miconazole	U	2.95	U	2.89
Norfloxacin	U	29.5	U	28.9
Norgestimate	U	11.5	U	8.66
Ofloxacin	U	2.95	U	2.89
Ormetoprim	U	1.18	U	1.16
Oxacillin	U H	5.91	U H	5.78
Oxolinic Acid	U	1.18	U	1.16
Penicillin G	U H	5.91	U H	5.78

Penicillin V	U	5.91	U	5.78
Roxithromycin	NQ		NQ	
Sarafloxacin	U	29.5	U	28.9
Sulfachloropyridazine	U	2.95	U	2.89
Sulfadiazine	U	2.95	U	2.89
Sulfadimethoxine		0.71	U	0.578
Sulfamerazine	U	1.29	U	1.33
Sulfamethazine	U	1.18	U	2.04
Sulfamethizole	U	2.45	U	2.15
Sulfamethoxazole	U	2.42	U	1.9
Sulfanilamide	U	29.5	U	28.9
Sulfathiazole	U	2.95	U	2.89
Thiabendazole	U	2.95	U	2.89
Trimethoprim	U	2.95	U	2.89
Tylosin	U	11.8	U	11.6
Virginiamycin M1	U	10.6	U	6.95
1,7-Dimethylxanthine	U	118	U	116
% Moisture		4.21		62.7
$^{13}C_2$-^{15}N-Acetaminophen (% Recovery)	V	227		149
$^{13}C_3$-Caffeine (% Recovery)	V	177		113
d10-Carbamazepine (% Recovery)		82.3		86.8
$^{13}C_3$-^{15}N-Ciprofloxacin (% Recovery)		150		111
$^{13}C_2$-Erythromycin-H2O (% Recovery)		96.8		91.9
D_5-Fluoxetine (% Recovery)		62		80.7
$^{13}C_6$-Sulfamethazine (% Recovery)		92		71.5
$^{13}C_6$-Sulfamethoxazole (% Recovery)		91.4		80.4
D_6-Thiabendazole (% Recovery)		48.5		65.5
$^{13}C_3$-Trimethoprim (% Recovery)		120		108
Anhydrochlortetracycline [ACTC]		32.9		30.2
Anhydrotetracycline [ATC]	U	29.5	U	28.9
Chlortetracycline [CTC]	U	11.8	U	11.6
Demeclocycline	U	29.5	U	28.9
Doxycycline	U	11.8	U	11.6
4-Epianhydrochlortetracycline [EACTC]	U	118	U	116

4-Epianhydrotetracycline [EATC]	U	29.5	U	28.9
4-Epichlortetracycline [ECTC]	U	29.5	U	28.9
4-Epioxytetracycline [EOTC]	U	11.8		29
4-Epitetracycline [ETC]	U	11.8	U	11.6
Isochlortetracycline [ICTC]	U	11.8	U	11.6
Minocycline	U	118	U	116
Oxytetracycline [OTC]	U	11.8		154
Tetracycline [TC]	U	11.8	U	11.6
% Moisture		4.21		62.7
D6-Thiabendazole (% Recovery)		96.9		95.4

Table C.2. The concentration of each PPCPs detected in the chicken feather and chicken feather meal samples collected from Plant 2 in the U.S.

	Chicken feather meal from Plant 2		Chicken feather from Plant 2	
Sample Size	0.498g (dry)		0.517g (dry)	
UNITS	Flag	ng g^{-1} (dry weight basis)	Flag	ng g^{-1} (dry weight basis)
Acetaminophen	U	30.1	U	29
Azithromycin	U	3.48	U	2.9
Caffeine	U	30.1		37.7
Carbadox	U	3.01	U	2.9
Carbamazepine	U	3.01	U	2.9
Cefotaxime	U	12.1	U	11.6
Ciprofloxacin	U	12.1	U	11.6
Clarithromycin	U	3.01	U	2.9
Clinafloxacin	U	23.7	U	15.1
Cloxacillin	U H	6.03	U H	5.81
Dehydronifedipine	U	1.21	U	1.16
Diphenhydramine	U	1.21	U	1.16
Diltiazem	U	0.603	U	0.581
Digoxin	U	12.1	U	11.6
Digoxigenin	U	25.5	U	35.4
Enrofloxacin	U	6.03	U	5.81
Erythromycin-H2O	U	4.62	U	4.45
Flumequine	U	3.01	U	2.9
Fluoxetine	U	3.29	U	2.9
Lincomycin	U	6.03	U	5.81
Lomefloxacin	U	6.71	U	5.81
Miconazole	U	3.01	U	2.9
Norfloxacin	U	47.2	U	29
Norgestimate	U	14.1	U	5.81
Ofloxacin	U	3.01	U	2.9
Ormetoprim		3.02		2.37
Oxacillin	U H	6.03	U H	5.81
Oxolinic Acid	U	1.58	U	1.16
Penicillin G	U H	6.03	U H	5.81
Penicillin V	U	6.03	U	5.81
Roxithromycin	NQ		NQ	
Sarafloxacin	U	30.1	U	29
Sulfachloropyridazine	U	3.01	U	2.9
Sulfadiazine	U	3.01	U	2.9
Sulfadimethoxine		3.74		2.92

Sulfamerazine	U	2	U	1.16
Sulfamethazine	U	4.82	U	1.16
Sulfamethizole	U	2.04	U	1.16
Sulfamethoxazole	U	1.73	U	1.73
Sulfanilamide	U	61.5	U	29
Sulfathiazole	U	3.01	U	2.9
Thiabendazole	U	3.01	U	2.9
Trimethoprim	U	3.01	U	2.9
Tylosin	U	12.1	U	11.6
Virginiamycin M1	U	16	U	6.85
1,7-Dimethylxanthine	U	121	U	116
% Moisture		0.46		67.3
$^{13}C_2$-^{15}N-Acetaminophen (% Recovery)	V	262		147
$^{13}C_3$-Caffeine (% Recovery)	V	157		113
d_{10}-Carbamazepine (% Recovery)		77.3		100
$13C_3$-^{15}N-Ciprofloxacin (% Recovery)		105		139
$13C_2$-Erythromycin-H2O (% Recovery)		105		100
D_5-Fluoxetine (% Recovery)		55		89.6
$^{13}C_6$-Sulfamethazine (% Recovery)		74.4		94
$^{13}C_6$-Sulfamethoxazole (% Recovery)		103		99.2
D_6-Thiabendazole (% Recovery)		46		89.2
$^{13}C_3$-Trimethoprim (% Recovery)		117		118
Anhydrochlortetracycline [ACTC]	U	30.1		33.3
Anhydrotetracycline [ATC]	U	30.1	U	29
Chlortetracycline [CTC]	U	13	U	11.6
Demeclocycline	U	30.1	U	29
Doxycycline	U	12.1	U	11.6
4-Epianhydrochlortetracycline [EACTC]	U	121	U	116
4-Epianhydrotetracycline [EATC]	U	30.1	U	29

4-Epichlortetracycline [ECTC]	U	30.1	U	29
4-Epioxytetracycline [EOTC]	U	12.1	U	11.6
4-Epitetracycline [ETC]	U	12.1	U	11.6
Isochlortetracycline [ICTC]	U	12.1	U	11.6
Minocycline	U	121	U	116
Oxytetracycline [OTC]	U	12.1	U	11.6
Tetracycline [TC]	U	12.1	U	11.6
% Moisture		0.46		67.3
D6-Thiabendazole (% Recovery)		90.2		96.7

Table C.3. The concentration of each PPCPs detected in the chicken feather and chicken feather meal samples collected from Plant 3 in the U.S.

	Chicken feather from Plant 3		Chicken feather meal from Plant 3	
Sample Size	0.476g (dry)		0.472g (dry)	
UNITS	Flag	ng g^{-1} (dry weight basis)	Flag	ng g^{-1} (dry weight basis)
Acetaminophen	U	31.5	U	31.8
Azithromycin	U	3.15	U	3.46
Caffeine	U	31.5	U	31.8
Carbadox	U	3.15	U	3.18
Carbamazepine	U	3.15	U	3.18
Cefotaxime	U	12.6	U	14.7
Ciprofloxacin	U	12.6	U	12.7
Clarithromycin	U	3.15	U	3.18
Clinafloxacin	U	12.6	U	31.2
Cloxacillin	U H	6.3	U H	6.36
Dehydronifedipine	U	1.26	U	1.27
Diphenhydramine	U	1.26	U	1.27
Diltiazem	U	0.879	U	0.705
Digoxin	U	12.6	U	16.2
Digoxigenin	U	31.6	U	63.6
Enrofloxacin	U	6.3	U	6.36
Erythromycin-H2O	U	4.83	U	4.87
Flumequine	U	3.15	U	3.18
Fluoxetine	U	3.15	U	3.21
Lincomycin	U	6.3	U	6.36
Lomefloxacin	U	6.3	U	7.38
Miconazole	U	3.15	U	3.18
Norfloxacin	U	31.5	U	31.8
Norgestimate	U	11.6	U	12.4
Ofloxacin	U	3.15	U	3.18
Ormetoprim	U	1.26	U	1.27
Oxacillin	U H	6.3	U H	6.36
Oxolinic Acid	U	1.26	U	1.27
Penicillin G	H	12.4	U H	6.36
Penicillin V	U	6.3	U	6.36
Roxithromycin	NQ		NQ	
Sarafloxacin	U	31.5	U	31.8
Sulfachloropyridazine	U	3.15	U	3.18
Sulfadiazine	U	3.15	U	3.18

Sulfadimethoxine		0.823		13.5
Sulfamerazine	U	1.26	U	1.85
Sulfamethazine	U	1.54	U	2.59
Sulfamethizole	U	1.26	U	1.76
Sulfamethoxazole	U	1.61	U	3.15
Sulfanilamide	U	31.5	U	31.8
Sulfathiazole	U	3.15	U	3.18
Thiabendazole	U	3.15	U	3.18
Trimethoprim	U	3.15	U	3.18
Tylosin	U	12.6	U	12.7
Virginiamycin M1	U	7.31	U	11.5
1,7-Dimethylxanthine	U	126	U	127
% Moisture		56.3		3.67
$^{13}C_2$-^{15}N-Acetaminophen (% Recovery)	V	162	V	293
$^{13}C_3$-Caffeine (% Recovery)		137	V	147
d_{10}-Carbamazepine (% Recovery)		96.1		93
$^{13}C_3$-^{15}N-Ciprofloxacin (% Recovery)		114		115
$^{13}C_2$-Erythromycin-H2O (% Recovery)		94		116
D_5-Fluoxetine (% Recovery)		86.3		66
$^{13}C_6$-Sulfamethazine (% Recovery)		89.7		89.4
$^{13}C_6$-Sulfamethoxazole (% Recovery)		112		108
D_6-Thiabendazole (% Recovery)		79		46.9
$^{13}C_3$-Trimethoprim (% Recovery)		113		133
Anhydrochlortetracycline [ACTC]		32.2	U	31.8
Anhydrotetracycline [ATC]	U	31.5	U	31.8
Chlortetracycline [CTC]	U	12.6	U	15
Demeclocycline	U	31.5	U	31.8
Doxycycline	U	12.6	U	12.7
4-Epianhydrochlortetracycline [EACTC]	U	126	U	127
4-Epianhydrotetracycline [EATC]	U	31.5	U	31.8
4-Epichlortetracycline [ECTC]	U	31.5	U	35.7

4-Epioxytetracycline [EOTC]	U	12.6	U	12.7
4-Epitetracycline [ETC]	U	12.6	U	12.7
Isochlortetracycline [ICTC]	U	12.6		41.7
Minocycline	U	126	U	127
Oxytetracycline [OTC]	U	12.6	U	12.7
Tetracycline [TC]	U	12.6	U	12.7
% Moisture		56.3		3.67
D6-Thiabendazole (% Recovery)		107		103

Table C.4. The concentration of each PPCPs detected in the DAF water-in samples collected from Plant 1 and Plant 2 in the U.S.

	DAF-in solid from Plant 1		DAF-in solid from Plant 2	
Sample Size	0.566g (dry)		0.718g (dry)	
UNITS	Flag	ng g^{-1} (dry weight basis)	Flag	ng g^{-1} (dry weight basis)
Acetaminophen	U	26.5	U	20.9
Azithromycin		17	U	2.66
Caffeine		29.4	U	20.9
Carbadox	U	2.65	U	2.09
Carbamazepine		16.2	U	2.09
Cefotaxime	U	17.6	U	8.36
Ciprofloxacin		80.4	U	8.36
Clarithromycin	U	2.65	U	2.09
Clinafloxacin	U	13.1	U	30.8
Cloxacillin	U H	5.3	U H	4.18
Dehydronifedipine	U	1.06	U	0.836
Diphenhydramine		5.11	U	0.836
Diltiazem	U	1.56	U	0.476
Digoxin	U	10.6	U	8.36
Digoxigenin	U	89.9	U	103
Enrofloxacin	U	5.3		5.11
Erythromycin-H2O	U	4.06	U	3.21
Flumequine	U	2.65	U	2.09
Fluoxetine	U	3.76	U	7.42
Lincomycin	U	5.3	U	4.18
Lomefloxacin		7.12	U	4.18
Miconazole	U	2.65	U	2.09
Norfloxacin	U	26.5	U	24.7
Norgestimate	U	18.9	U	12.4
Ofloxacin		9.74	U	2.09
Ormetoprim	U	1.06		1.36
Oxacillin	U H	5.3	U H	4.18
Oxolinic Acid	U	4.39	U	1.17
Penicillin G	U H	5.3	U H	4.18
Penicillin V	U	5.3	U	4.18
Roxithromycin	NQ		NQ	
Sarafloxacin	U	26.5	U	20.9
Sulfachloropyridazine	U	2.65	U	2.09
Sulfadiazine	U	2.65	U	2.09

Sulfadimethoxine	U	0.838		2.41
Sulfamerazine	U	4.01	U	2.38
Sulfamethazine	U	5.87	U	3.31
Sulfamethizole	U	1.76	U	1.32
Sulfamethoxazole	U	1.92	U	1.23
Sulfanilamide	U	26.5	U	20.9
Sulfathiazole	U	2.65	U	2.09
Thiabendazole	U	2.65	U	2.09
Trimethoprim	U	2.65	U	2.09
Tylosin	U	10.6	U	8.36
Virginiamycin M1	U	15.2	U	10.1
1,7-Dimethylxanthine	U	106	U	83.6
% Moisture		65.7		71.2
^{13}C2-15N-Acetaminophen (% Recovery)	V	187	V	24.8
$^{13}C_3$-Caffeine (% Recovery)	V	186	V	203
d_{10}-Carbamazepine (% Recovery)		69.5		102
$^{13}C_3$-^{15}N-Ciprofloxacin (% Recovery)		136	V	162
$^{13}C_2$-Erythromycin-H2O (% Recovery)		82.6		112
D_5-Fluoxetine (% Recovery)		36.9		58.8
$^{13}C_6$-Sulfamethazine (% Recovery)		49.5		61.7
$^{13}C_6$-Sulfamethoxazole (% Recovery)		76.7		68.5
D_6-Thiabendazole (% Recovery)		47.7		75.2
$^{13}C_3$-Trimethoprim (% Recovery)		92.4	V	160
Anhydrochlortetracycline [ACTC]		29		24.2
Anhydrotetracycline [ATC]	U	26.5	U	20.9
Chlortetracycline [CTC]	U	11.2	U	8.6
Demeclocycline	U	26.5	U	20.9
Doxycycline	U	10.6	U	8.36

4-Epianhydrochlortetracycline [EACTC]	U	106	U	83.6
4-Epianhydrotetracycline [EATC]	U	26.5	U	20.9
4-Epichlortetracycline [ECTC]	U	26.5	U	20.9
4-Epioxytetracycline [EOTC]		22.8	U	8.36
4-Epitetracycline [ETC]	U	10.6	U	8.36
Isochlortetracycline [ICTC]	U	10.6	U	8.36
Minocycline	U	106	U	83.6
Oxytetracycline [OTC]		66.5	U	8.36
Tetracycline [TC]	U	10.6	U	8.36
% Moisture		65.7		71.2
D6-Thiabendazole (% Recovery)		97.8		98.8

Table C.5. The concentration of each PPCPs detected in the peat moss samples, lab blank and OPR quality control group.

	Peat moss		Lab Blank		Spiked Matrix	
Sample Size	0.398g (dry)		0.500g			
UNITS	Flag	ng g^{-1} (dry weight basis)	Flag	ng g^{-1}	Flag	% Recovery
Acetaminophen	U	37.7	U	30		96.5
Azithromycin	U	3.77	U	3		24
Caffeine	U	37.7	U	30		85
Carbadox	U	3.77	U	3		73.9
Carbamazepine	U	3.77	U	3		118
Cefotaxime	U	15.1	U	12		113
Ciprofloxacin	U	363	U	63.6		88.9
Clarithromycin	U	3.77	U	3		98.8
Clinafloxacin	U	408	U	189		185
Cloxacillin	U H	7.55	U H	6	H N	201
Dehydronifedipine	U	1.51	U	1.2		98.5
Diphenhydramine	U	1.51	U	1.2		92.4
Diltiazem	U	0.755	U	0.6		107
Digoxin	U	15.1	U	12		101
Digoxigenin	U	156	U	12		135
Enrofloxacin	U	32.4	U	12.3		133
Erythromycin-H2O		5.82	U	4.6		126
Flumequine	U	3.77	U	3		90.9
Fluoxetine	U	3.77	U	3		92.8
Lincomycin	U	7.55	U	6		163
Lomefloxacin	U	56.9	U	32.8		229
Miconazole	U	3.77	U	3		70.9
Norfloxacin	U	852	U	205		111
Norgestimate	U	11	U	6		93
Ofloxacin	U	3.77	U	3		183
Ormetoprim	U	1.51	U	1.2		83.5
Oxacillin	U H	7.55	U H	6	H N	152
Oxolinic Acid	U	1.51	U	1.2		105
Penicillin G	U H	7.55	U H	6	H N	146
Penicillin V	U	7.55	U	6	N	183
Roxithromycin	NQ		NQ		NQ	
Sarafloxacin	U	37.7	U	30		151
Sulfachloropyridazine	U	3.77	U	3		127
Sulfadiazine	U	3.77	U	3		110
Sulfadimethoxine	U	0.755	U	0.6		95.7
Sulfamerazine	U	2.42	U	1.2		94
Sulfamethazine	U	2.06	U	1.2		101
Sulfamethizole	U	1.51	U	1.2		70.9

Sulfamethoxazole	U	1.85	U	1.2		98.6
Sulfanilamide	U	55.4	U	30		57.9
Sulfathiazole	U	3.77	U	3		75.9
Thiabendazole	U	3.77	U	3		107
Trimethoprim	U	3.77	U	3		92.6
Tylosin	U	15.1	U	12		93.6
Virginiamycin M1	U	13.1	U	6.61		134
1,7-Dimethylxanthine	U	151	U	120		175
% Moisture		26.4				
$^{13}C_2$-^{15}N-Acetaminophen (% Recovery)	V	183		119		119
$^{13}C_3$-Caffeine (% Recovery)		129		87.6		109
d_{10}-Carbamazepine (% Recovery)		113		110		95.1
$^{13}C_3$-^{15}N-Ciprofloxacin (% Recovery)	V	4.48	V	6.24		16.6
$^{13}C_2$-Erythromycin-H2O (% Recovery)		67.1		84.8		85.4
D_5-Fluoxetine (% Recovery)		54.5		77.5		79.1
$^{13}C_6$-Sulfamethazine (% Recovery)		70.5		100		86.7
$^{13}C_6$-Sulfamethoxazole (% Recovery)		108		111		101
D_6-Thiabendazole (% Recovery)		58.2		69.3		75.1
$^{13}C_3$-Trimethoprim (% Recovery)		77.4		78.4		84.9
Anhydrochlortetracycline [ACTC]	U	44.9		40.2		38
Anhydrotetracycline [ATC]	U	37.7	U	30		34.9
Chlortetracycline [CTC]	U	16.7	U	12		105
Demeclocycline	U	37.7	U	30		56.9
Doxycycline		21.6	U	12		80.6
4-Epianhydrochlortetracycline [EACTC]	U	151	U	120		13.8

4-Epianhydrotetracycline [EATC]	U	37.7	U	30		38.8
4-Epichlortetracycline [ECTC]	U	38.5	U	30		107
4-Epioxytetracycline [EOTC]	U	15.1	U	12		74.5
4-Epitetracycline [ETC]	U	15.1	U	12		111
Isochlortetracycline [ICTC]	U	15.1	U	12	MAX	70.2
Minocycline	U	151	U	120		14.8
Oxytetracycline [OTC]	U	15.1	U	12		93.8
Tetracycline [TC]	U	15.1	U	12		92.3
% Moisture		26.4				
D6-Thiabendazole (% Recovery)		56.3		74.7		75.5