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ANALYSIS OF VETERINARY DRUG RESIDUES IN IMPORTED AND DOMESTIC CRAWFISH USING LIQUID CHROMATOGRAPHY—MASS SPECTROMETRY

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Environmental Sciences

by
Emily Kate Wall
B.S., St. Lawrence University, 2013
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ABSTRACT

Aquaculture production has greatly increased over the past few decades, and will continue to grow as the world fisheries become overfished and demand for seafood increases. With increased production comes more intense cultivation methods and heavy use of formulated feeds that may contain veterinary drug residues. Currently no antibiotics are allowed in the U.S. for crawfish aquaculture; yet, detectable levels of various antibiotics have been found in imported seafood samples. The FDA is responsible for testing aquaculture products entering the United States, but only has the capabilities to test a minimal amount of those imports. Additionally, for crawfish there is only one published FDA method to test for chloramphenicol, and they have yet to publish a method to test for multiple veterinary drug residues. Therefore, the objective of this study was to develop a method that could test various antibiotics in commercially available frozen crawfish, and use that method to test imported and domestic crawfish.

Crawfish were obtained from the Aquaculture Research Station at Louisiana State University, and were used as blank crawfish to validate a method to test for chloramphenicol, florfenicol, enrofloxacin, ciprofloxacin, and sarafloxacin using liquid chromatography—mass spectrometry. In short, the tissue was extracted with dilute acetic acid and acetonitrile with added sodium chloride. After centrifugation, the extract was evaporated to dryness with nitrogen and reconstituted in mobile phase. The extract was passed through a syringe and 0.2µm PVDF membrane filter into an auto-sampler vial. A Waters Acquity TQD LC/MS/MS operated in the positive and negative ion mode; ciprofloxacin, enrofloxacin, and sarafloxacin in positive ion mode, and florfenicol and chloramphenicol in negative ion mode. Results indicated acceptable method performance characteristics for selectivity, linearity, accuracy (recovery), precision

(RSD), and MDL and LOQ. Though ciprofloxacin did show some of the lowest recoveries, and chloramphenicol did have quite high RSD values. Retail samples tested negative for most of the veterinary drug residues with the exception of chloramphenicol in one Louisiana brand at an average concentration of 0.91 ng/g, and in a Chinese brand at an average concentration of 0.52 ng/g.

1 CHAPTER ONE: INTRODUCTION

Annual global aquaculture production has more than tripled within the past 15 years[1], similarly, farm-raised crawfish production and value has increased—with a 25 percent increase in 2014[2]. In the United States, Louisiana accounts for the majority of U.S crawfish sales and consumption, with demand being met from imported crawfish primarily from China. As demand for aquaculture products rises, cultivation methods will intensify leading to non-hygienic and stressful conditions that will rely on heavy amounts of antibiotics administered in fish feed in order to combat disease[1]. Many times farmers turn to unapproved drugs, especially since relatively few new veterinary drugs are approved for aquaculture. Furthermore, countries that export crawfish to the U.S., specifically developing countries, are subject to fewer regulations and lower production standards.

In the United States there are no approved antibiotic drugs for the production of crawfish or shrimp[3], yet in the past chloramphenicol has been detected in crawfish imported from China, and other veterinary drug residues, such as the fluoroquinolones, have been detected in other seafood aquaculture products. The USFDA has identified a number of aquaculture drugs that are of high enforcement priority and not approved for use in the U.S.; these drugs include chloramphenicol, nitrofurans, fluoroquinolones and quinolones, malachite green, and steroid hormones[4]. Florfenicol is one of the few antibiotics approved for use in aquaculture in the U.S., though only for salmon and catfish.

Aquaculture is expected to increase to 40% of total global seafood production[5], and with this potential increase it is imperative that there is a method to monitor antibiotic residues in order to ensure the safety of the food supply and protect public health. Chloramphenicol can cause adverse side effects such as irreversible aplastic anemia, and the widespread use of these

antibiotics can trigger antibiotic resistant pathogens that can be transmitted to humans and hinder the use of important antibiotics used to treat human infections. In order to lessen these adverse effects, aquaculture products need to be tested to ensure they are following USFDA guidelines and do not contain detectable antibiotic residues.

Therefore, it is essential to have a rugged method that is efficient of both time and materials to analyze crawfish for a variety of antibiotics. Currently the FDA has only published a method for chloramphenicol in crawfish, and lacks a method that looks at multiple antibiotic residues. Consequently, the purpose of this investigation was to develop a single liquid chromatography-mass spectrometry method for chloramphenicol, florfenicol, enrofloxacin, ciprofloxacin, and sarafloxacin residues in retail frozen crawfish based upon previous methods.

2 CHAPTER TWO: LITERATURE REVIEW

2.1 Crawfish

2.1.1 Production and Consumption

In Louisiana scientists have scientifically described specimens of what appear to be 39 distinctive species of crawfish[6]. The Louisiana crawfish harvests however are only composed of two species—the red swamp crawfish *Procambarus clarkii* and to a lesser extent the white river crawfish *Procambarus zonangulus*[7]. The red swamp crawfish is the most desired species in the marketplace; they live two years or less, have high juvenile survival, and are capable of spawning year-round in the southern United States with some reproducing more than once per year[7]. Most crawfish in Louisiana is farm raised in shallow earthen ponds[8], and is the state's most valuable aquacultural commodity[2]. Wild crawfish make up about 12 percent of the harvest[9] and are mostly caught in the Atchafalaya Basin[2].

Crawfish production differs from most other aquacultural enterprises in three main ways. First, its primary feed is forage rather than formulated feeds; second, crawfish are often double-cropped or rotated with other field crops; and third, because production is highly seasonal the fresh product cannot be marketed year-round[9]. Another difference is that crawfish ponds are not stocked with hatchery-reared young[7]. Instead, farmers rely on reproduction by unharvested crawfish from the previous year, or on mature crawfish (from natural habitats or culture ponds) that are stocked to produce young naturally[7-8]. This stocking is usually only needed in new ponds or when a pond has been idle for a year or more[8].

The primary forage crop for crawfish aquaculture is rice, though sorghum-sudangass or other natural vegetation also serves as a food source[7]. Supplemental feeding has not yet been shown to predictably increase yields or size of crawfish at harvest[7]. Over 50 percent of

Louisiana's crawfish aquaculture is practiced in conjunction with rice production[7], but other strategies of growing crawfish also exist such as monocropping. In monocropping systems crawfish is the sole crop harvested and production typically occurs in the same physical location for several production cycles or longer[8]. For aquaculture done in conjunction with rice, the production strategy is a crop rotation system. Two basic crop rotation systems exist: rice-crawfish-rice and rice-crawfish-fallow (or rice-crawfish-soybean)[8]. Crawfish can either be produced in permanent rotation with a rice crop year after year in the same location, or in a field rotation with rice, with restocking of crawfish each rotational cycle[7]. Crawfish production follows the rice harvest, and the forage crop used for growing crawfish is the crop residue and re-growth of the rice stubble after grain harvesting[8].

Crawfish aquaculture works well with rice production partly because the economics of rice production have weakened and many producers turned to crawfish as an accessory crop[7]. In addition, crawfish aquaculture fits well into existing rice farm operations through the use of marginal agricultural lands, crop rotations, and permanent farm labor and equipment during off-peak farming periods[7]. Rice is also semi-aquatic and tends to survive well in flooded crawfish ponds, while also delivering plant fragments to the detrital pool[7]. Rice is typically grown and harvested during the summer, and crawfish are reared during autumn, winter, and early spring[8].

Current farming practices flood and drain ponds to mimic the natural flooding and drying cycle in the Atchafalaya River basin[7]. This control allows farmers to positively influence water quality, food resources, and other factors within their ponds[7]. Crawfish survive the dry intervals by digging or retreating to burrows where they can avoid predators, acquire the moisture necessary for survival, and reproduce in safety[8]. The pond flooding, as well as rainfall, allows the emergence of crawfish that have been living inside the burrows[8].

Harvesting crawfish relies upon the passive technique of baited traps[8]. Harvesting occurs generally from March through June, when densities of marketable crawfish are highest and crawfish are most active[7]. Since marketable crawfish are continual over much of the season, regular and frequent harvests are necessary, the duration of which is influenced by cost of harvesting, marketing price, and catch[8]. Harvesting frequently is also necessary due to the aggressive and territorial nature of crawfish; harvesting removes the larger individuals from the population, reducing aggression and leaving space and food resources for undersized crawfish to thrive[7].

2.1.2 Market and Trade

The first record of a commercial crawfish harvest in the United States was in 1880 where 23,400 pounds were harvested with a value of $2,140[7]. In 2014 there was a 25 percent increase in farm-raised crawfish production and value, with a production of 127 million pounds of crawfish valued at $172 million[2]. According to the 2005 U.S. Census of Aquaculture, Louisiana accounted for 96.4 percent of U.S. crawfish sales[9] with small harvests of farmed crawfish occurring in other states, such as Texas, Arkansas, Mississippi, Alabama, and the Carolinas[7]. The U.S International Trade Commission found that U.S. consumption of crawfish tail meat fluctuated between 1997 and 2002, but was 178.7 percent higher in 2002 than in 1997[10]. Most of the U.S. consumption occurs in Louisiana; in 2002 per capita consumption was 10.4 pounds, whereas the rest of the United States was only at 0.25 pounds[11].

Louisiana is the largest producer of crawfish in part because the red swamp crawfish lifecycle is well-suited to the annual sequence of spring flooding and summer dry period common to large river systems and floodplains in the southern U.S.[8]. Additionally, regional demand is high and fuels the growth of the aquaculture industry. Global expansion of the red

swamp crawfish in pond culture is unlikely however, due to concerns associated with non-native introduction and this species ability to wreak havoc in ecological sensitive habitats[8]. The red swamp crawfish is quite hardy and adaptive, which allows it to out-compete some aquatic species and cause devastation to key plants[8]. Moreover, it is a suspected vector of the crayfish plague fungus, which was notorious for eliminating many populations of native European crayfish[8].

Most crawfish is sold live as whole crawfish or fresh tail meat, with some being sold as frozen tail meat[11]. Live crawfish are sold primarily for crawfish boils in the spring, and peeled tail meat is used in various Cajun dishes that are consumed year-round[11]. When crawfish are abundant or when live markets become saturated, a portion of the annual crop is processed and sold as fresh or frozen abdominal or "tail" meat[7]. The smaller crawfish are usually processed for the tail meat market, leaving the larger ones for the more profitable live market[7]. Most fresh crawfish tail meat is sold during the season in which domestic crawfish are harvested, as well as to the region in and around Louisiana due to its short shelf life[10]. It is for these reasons that about 70 percent of Louisiana's production of crawfish is consumed in Louisiana and neighboring states[11]. Most frozen crawfish tail meat is sold during the off-season, and has the ability to be sold outside of the Louisiana region[10].

In order to balance the U.S. demand for crawfish tail meat, frozen meat is imported, primarily from China, but also from Spain and Trinidad and Tobago[10]. Domestic crawfish tail meat and imports are direct competitors in the U.S. market; market participants note that quality and consistency is the most important factor in their purchasing decision, followed by pricing differences[10]. Subject import volume increased 279.3 percent between 1997 and 2002, well in excess of the 178.7 percent increase in U.S. consumption over that period[10]. Increases in subject

import volume and market share coincided with suppressed domestic prices and declines in domestic production, sales volumes, capacity utilization, employment, and financial performance[12]. Another result of the imports from China is that now less than ten percent of the annual domestic crop is processed for tail meat[7]. Prior to Chinese imports there were over 100 licensed crawfish processors in Louisiana, but as of recently that number was in the low 30's[7]. This has lead to a dramatic reduction in processing (peeling) capacity in Louisiana, and as a result each year thousands of tons of smaller crawfish are not harvested[7].

2.2 Aquaculture

2.2.1 Veterinary Drug Use in Aquaculture

Aquaculture is the farming of aquatic organisms, including finfish and shellfish, by individuals, groups, or corporations using interventions such as feed, medications, controlled breeding, or containment that act to enhance production[1]. Evidence shows aquaculture existed in Egypt and China as early as 2500 B.C. and 1100 B.C, respectively[1]. Currently annual global aquaculture production has more than tripled within the past 15 years, and by 2015 was predicted to account for 39% of total global seafood production by weight[1]. The primary production facilities are located in a few Asian countries, and China alone accounts for about 71 percent of total global aquaculture production[1].

Global aquaculture will only continue to grow as the world fisheries become overfished and demand for seafood continues to increase. As demand for aquaculture products rises, cultivation methods will intensify to include high stock density and volume, and heavy use of formulated feeds containing antibiotics, antifungals, and other pharmaceuticals[1]. As a result of the non-hygienic and stressful conditions present in aquaculture facilities, the risk for bacterial infection is high[1]. In order to combat disease, heavy amounts of antibiotics are administered in

fish feed prophylactically at sub-therapeutic concentrations (disease prevention) and therapeutically (disease treatment), which can then lead to residual concentrations left in seafood[1]. Antibiotics are typically applied in three ways at sub-therapeutic concentrations: a single antibiotic for an extended period; a rotating class of multiple antibiotics; and a gradient regimen where antibiotic concentrations are gradually increased[13].

There are no uniform global standards for aquaculture and antibiotic use, and in the case of developing countries there are limited or no regulations in place. When regulations do exist, there is little enforcement by local authorities. New animals drugs in the United States that are added to aquaculture feed are subject to FDA approval, but the same cannot be said for other countries—particularly the developing countries where most aquaculture takes place[13]. Furthermore, relatively few new animal drugs have been approved for aquaculture in the US, and as a result many aquaculture growers may use unapproved new animal drugs or general-purpose chemicals not permitted for drug use[14].

2.2.2 Environmental Impacts

The main environmental impacts of marine aquaculture include biological pollution, organic pollution and eutrophication, habitat modification, and chemical pollution[15]. In terms of biological pollution, aquaculture facilities can unintentionally release farmed fish and their parasites and pathogens into the environment[15]. Escapes of these organisms could impact the wild aquatic species in the area by out-competing native species, and through the introduction and spread of disease. Aquaculture operations can negatively impact water quality by contributing to nutrient loading and eutrophication through wastes entering the aquatic environment[15]. These factors can damage natural ecosystems and the species living in them, as well as facilities obstructing wild animals' use of their natural surroundings[15]. Lastly,

aquaculture results in chemical pollution through the use of antibiotics, parasiticides, pesticides, hormones, pigments, minerals, and vitamins[15]. Research indicates that 70-80% of the drug used in aquaculture ends up in the environment[16] with the potential to spread into and persist in the environment. One study found the 90% dissipation time (DT_{90}) for enrofloxacin and florfenicol to be greater than 150 days in terrestrial soil and marine sediments[17]. These environmental residues can then promote the development of multiple antimicrobial-resistant bacteria[16-17].

2.2.3 Human Health Impacts

The aquaculture process and use of antibiotics can contribute to adverse human health impacts through the accumulation of residual antibiotic in edible tissue, and the development of antibiotic resistant bacteria. Between the 1990's and today, the number of publications linking bacterial resistance to seafood has increased by 800%[5]. Bacterial antibiotic resistance arises through mutations in bacterial DNA or through horizontal gene transfer mechanisms[1]. The resistance genes in fish pathogens are often the same as those found in human pathogens, and most of these genes are transferable[16]. Additionally, some antibiotics used in aquaculture are also used in human medicine; therefore, if antibiotic resistant bacteria occur in aquaculture this could impede the use of some important antibiotics used to treat human infections[15].

The use of unapproved drugs or misuse of approved drugs in aquacultured products poses a potential human health hazard[4]. When a veterinary drug does not have or exceeds the maximum residue limit (MRL) or has not been approved for use in aquaculture, the risk to the public health is not known and at certain concentrations may represent a hazard to consumers[16]. Generally, low level exposures to antibiotic residues are not likely to cause immediate toxic effects among the general public; however, the chronic effects are largely unknown[1]. The potential immediate and long range human health consequences may include hypersensitivity

reactions, toxicity-related reactions, potential carcinogenic and mutagenic effects, and increasing prevalence of antibiotic-resistant microorganisms[14].

The greatest risk to public health is assumed to be the development of a reservoir of transferable resistance genes in bacteria in aquatic environments, which can further disseminate and eventually become antibiotic resistant human pathogens[16]. Even low-level exposures well below regulatory limits can still promote the emergence of multi-drug resistant microorganisms[5]. Antimicrobial resistant bacteria may have a range of adverse effects on human health including increased frequency and duration of illness, treatment failure, and loss of therapeutic options[14]. Direct spread of resistance from aquatic environments to humans may occur from consumption of aquaculture food products or through drinking water, and direct contact with water or aquaculture food products[16]. Specific populations at risk include individuals working in aquaculture, populations living around these facilities, and consumers who regularly prepare and eat aquacultured products[1].

2.3 Antibiotic Residues Impacting Food Safety

In the United States, the FDA's Center for Veterinary Medicine (CVM) has identified high enforcement priority aquaculture drugs that historically have been used without FDA approval[4]. High priority drugs are known or suspected carcinogens; known serious toxicological hazards; suspected serious toxicological hazards believed to have substantial use in aquaculture; and antimicrobials likely to confer bacterial resistance to drugs used in human medicine[18]. These drugs should not be used in fish that is to be consumed and include chloramphenicol; nitrofurans; fluoroquinolones and quinolones; malachite green; and steroid hormones[4]. No aquacultured species may contain a residue of an unapproved drug, and may not contain a residue level of an approved drug that is above FDA tolerance limits[4]. Based on which drugs are often used in

aquaculture, the compounds chosen as target analytes were chloramphenicol, florfenicol, enrofloxacin, ciprofloxacin, and sarafloxacin.

2.3.1 Chloramphenicol

Chloramphenicol (CAP) (Figure 2.1) is produced for commercial use by chemical synthesis, and is biosynthesized by the soil organism *Streptomyces venezuelae* and several other *Actinomycetes*[19]. It was the first mass produced antibiotic and was widely used globally as a human antibiotic due to its effectiveness against typhoid[20]. Chloramphenicol is a broad-spectrum antibiotic that is highly potent and active against gram-positive and gram-negative bacteria[21]. It is not adsorbed to clay or soil and is thus highly mobile; the half life in soil at 25°C is 4.5 days, in pond water at 25°C and pH 8 the half life is 10.3 days, and the half life at 37°C and pH 6 is 20.8 days[22]. Bioconcentration in aquatic organisms is not likely as the log K_{ow} is 1.14[22].

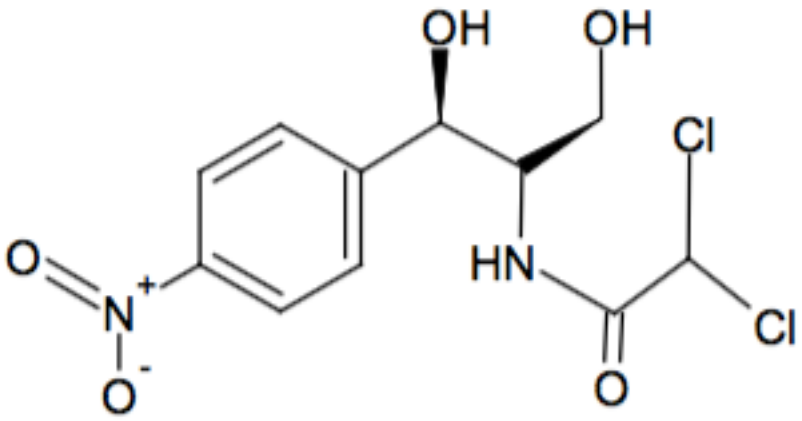

Figure 2.1. Chemical structure of chloramphenicol.

Presently it is scarcely used in human medication[20], and is not approved for use in food-producing animals[14]. The restrictions are due to its risk of severe human disease, particularly an idiosyncratic dose-independent aplastic anemia in humans, as well as other concerns about the genotoxicity of the drug and its metabolites, and its embryo and fetotoxicity[16]. In addition, it is a

suspected carcinogen and may affect the reproductive system in humans[14]. Even low concentrations of chloramphenicol can cause aplastic anemia, a form of anemia when the bone marrow ceases to produce sufficient red and white blood cells[20]. It is an irreversible condition with a 70% case fatality rate; those that do recover experience a high incidence of acute leukemia[16]. A syndrome of cyanosis and cardiovascular collapse known as Grey syndrome may also occur, especially in newborn babies[21]. Due to these risks, a safe level of exposure has not been determined and as such no ADI (acceptable daily intake) or MRLs (maximum residue levels) are established[14, 16]. However, there are no reported cases of aplastic anemia due to consumption of chloramphenicol residues in foods[16].

Chloramphenicol is banned in food-producing animals in the United States, the European Union, Canada, Australia, Japan, and China[20]. However despite these restrictions, it is one of the most commonly used antibiotics by aquaculture producers, and has been detected in national monitoring programs in products such as shrimp and crawfish[5, 16]. In 2001 shrimp imported into Europe from Asian countries were found to have between 1 and 10 ppb of chloramphenicol[20]. In 2003 the European Union banned the import of shrimp and crawfish from China because chloramphenicol had been found[23]. Additionally, as recently as 2014 Denmark had detected chloramphenicol in frozen red swamp crawfish from China at 1.2, 1.95, and 2.49 ppb[24]. Furthermore, since chloramphenicol has a zero-tolerance limit, it is important that these monitoring programs are able to detect it at low levels in biological samples. Therefore, a minimum required performance limit (MRPL) for chloramphenicol was set at 0.3 μg/kg (ppb) in food of animal origin in the EU, United States, and China[25].

2.3.2 Florfenicol

Florfenicol (FF) (Figure 2.2) is a synthetically produced broad-spectrum antibacterial agent specifically developed for veterinary use[17]. Florfenicol was first evaluated as a therapeutic agent in fish in the early 1980s and is still used today[16]. It has a wide range of activity similar to that of chloramphenicol, though it does not carry the risk of inducing human aplastic anemia[26]. Florfenicol lacks the nitro group located on the chloramphenicol aromatic ring that has been associated with the chloramphenicol induced non-dose related irreversible aplastic anemia in humans[26]. However, it is theoretically possible that florfenicol could cause some dose-dependent reversible bone marrow suppression, as chloramphenicol and thiamphenicol are known to do[26]. Florfenicol also has similar characteristics to that of chloramphenicol, it is highly mobile in soil with half lives in three sediment-water systems ranging from 8 to 19 days, and is not likely to bioaccumulate (log K_{ow} of 0.37)[27].

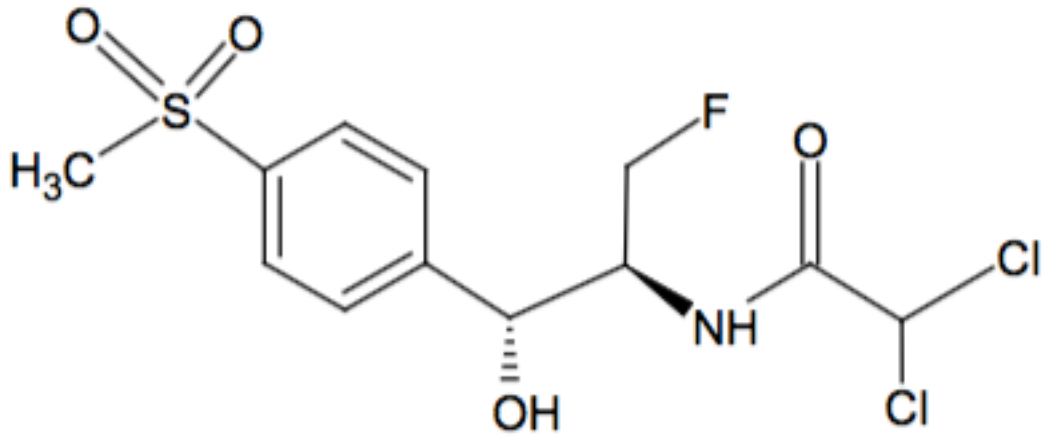

Figure 2.2. Chemical structure of florfenicol.

Similar to chloramphenicol, florfenicol is also one of the top antibiotics used by heavy aquaculture producers[5]. Due to the ban of chloramphenicol in food-producing animals,

florfenicol has been widely used in veterinary medicine to prevent and treat infection with sensitive bacteria[17]. In a survey in China looking at shrimp and carp aquaculture, florfenicol was found to be the second most widely used antibiotic[28]. Today it is approved for use in the US for medicated feed to control mortality in catfish and salmonids[4].

2.3.3 Fluoroquinolones: Enrofloxacin, Ciprofloxacin, and Sarafloxacin

Enrofloxacin (ENR), ciprofloxacin (CIP), and sarafloxacin (SARA) (Figure 2.3) are later-generation fluoroquinolones (FQs)[29], a class of broad-spectrum synthetic bacterial medicines effective against gram-negative bacteria[17]. They are effective against these bacteria through the inhibition of bacterial DNA gyrase, thus preventing DNA synthesis[30]. Unlike CAP and FF, fluoroquinolones bind tightly to soil and are immobile, though similarly they have little chance to bioaccumulate. Fluoroquinolones are a critically important class of synthetic antibiotics as they are used to treat serious infections in humans[14]. Both the FDA and WHO ranked them as essential antimicrobial agents for human health and animal health and welfare[14]. Enrofloxacin is used for non-food producing animals, ciprofloxacin is approved to treat human diseases and is not used in veterinary applications, and sarafloxacin was developed only for use in animals[31]. In mammalian and nonmammalian species, enrofloxacin is N-dealkylated to its metabolite ciprofloxacin, which contributes to enrofloxacin's activity and is itself a potent antimicrobial[32].

The most commonly used fluoroquinolones are enrofloxacin and ciprofloxacin in human medicine and animal husbandry[17]. Sarafloxacin was the first fluoroquinolone approved in the United States for use in food-producing animals, but has been withdrawn from the market owing to concerns about microbial resistance[33]. In China, sarafloxacin is licensed for use in aquaculture as it has been shown to effectively control common bacterial infections of cultured fishes[31]. Enrofloxacin is also licensed for use in aquaculture in China[32], and is commonly administered as

in-feed antibacterials[17]. In the survey in China looking at shrimp and carp aquaculture, enrofloxacin was the fourth most widely used antibiotic[28]. Ciprofloxacin on the other hand is banned for use in aquaculture in China[28].

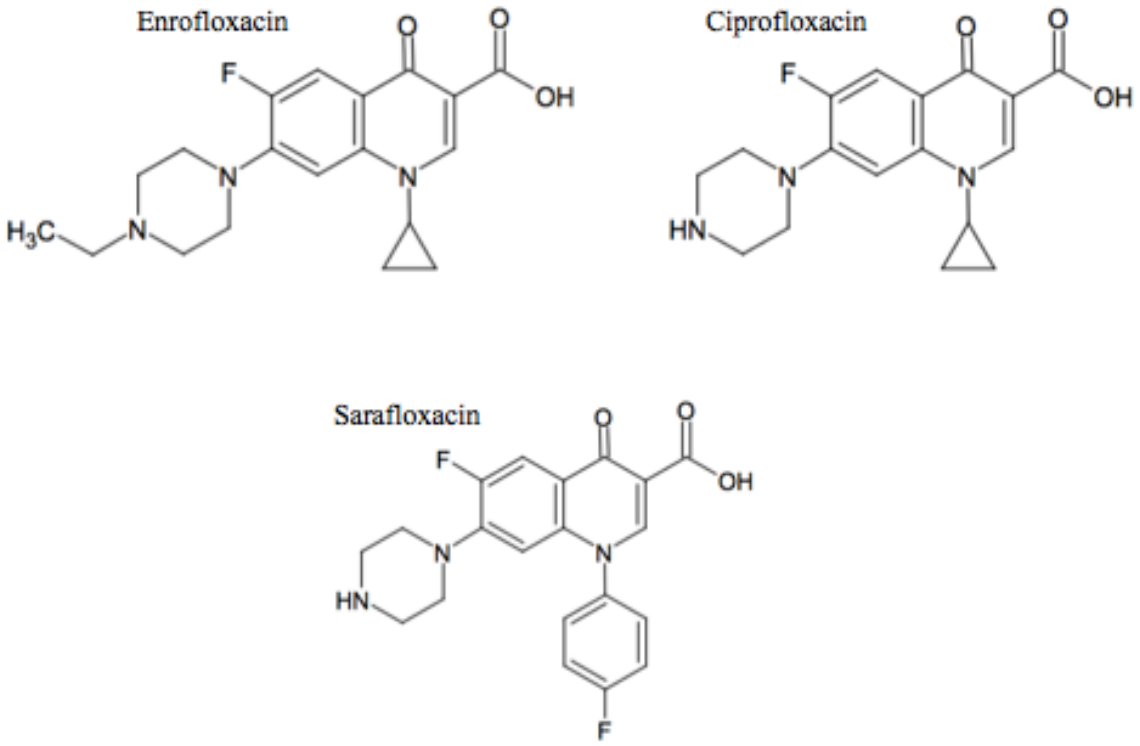

Figure 2.3. Chemical structure of the fluoroquinolones: enrofloxacin, ciprofloxacin, and sarafloxacin.

In the US, the FDA prohibited the extra-label use of fluoroquinolones in food producing animals in 1997 based on evidence that widespread use in food animals would promote the evolution of drug-resistant pathogens that could be transmitted to humans through the food chain[14]. As a result, the ability to treat human diseases with these antimicrobial drugs may have been compromised[14]. Furthermore, none of the fluoroquinolones are approved in the United State for use as aquaculture therapeutic agents, and such use is considered to be unsafe[14, 30].

2.4 Regulations for Antibiotic Use in Aquaculture

2.4.1 International Regulations

The World Trade Organization's Sanitary and Phytosanitary Agreement (SPS agreement) states that countries have the right to establish measures and the appropriate level of protection to safeguard the life and health of their population. Scientific evidence should be used to establish these control measures, and these measures should not be taken to only favor the domestic industry[34]. Many countries work together to establish measures of protection; for example, the U.S. and Canada, under the United States and Canada Free Trade Agreement, have harmonized human food safety requirements for approval of drugs used in food animals, and have agreed on identical tolerances for 37 animal drugs[13]. The U.S., the European Economic Community (EEC), and Japan are involved in a trilateral program, the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH)[35], and as a result also have similar requirements for animal drug approval and tolerance levels[13].

An international effort for risk assessment is with the World Health Organization (WHO) and Food and Agriculture Organization (FAO) Joint Expert Committee on Food Additives (JECFA), which evaluates human food safety data on selected animal drugs for the Codex Committee on Residues of Veterinary Drugs in Foods[13]. JECFA is a committee of animal drug experts from the codex committee countries, and works to evaluate toxicological and residue data on priority animal drugs, as well as establishing tolerance levels[13]. Priority animal drugs are established by the Codex Committee on Residues of Veterinary Drugs in Foods based on veterinary drugs that may pose a consumer safety problem and/or that may have potential adverse impacts on international trade[34].

In China, there are several laws and regulations that manage the aquaculture industry, these include the Fisheries Law of the People's Republic of China, the Marine Environment Protection Law of the People's Republic of China, the Law of the People's Republic of China on Agricultural Product Quality Safety, and the Food Safety Law of the People's Republic of China[36]. The Fisheries Law states that the use of antimicrobials in Chinese aquaculture should be appropriate and that the wastewater effluent discharges should not pollute the surrounding aquatic area, but lacks a clear statement on how this can be evaluated and avoided[37]. In terms of surveillance of veterinary medicine use in Chinese aquaculture, the Fish Drug Surveillance Department was established in 2005, and the Ministry of Agriculture has organized a national inspection team to conduct inspections in major aquaculture producing areas[28]. However, despite the appearance of regulation by multiple government departments, aquaculture facilities in China operate largely without permits[36].

2.4.2 U.S. Regulations

The Federal Food, Drug and Cosmetic Act (FFDCA) and its amendments determine the legal marketing and use of veterinary drugs in the U.S.[35]. The USFDA's Center for Veterinary Medicine (CVM) has responsibility for approval and monitoring activities that govern animal drug use in the U.S. They work to ensure that new drugs pass a rigorous approval process, and regulate the manufacture and distribution of drugs intended for food and companion animals[13]. Drugs are approved by the FDA for specific species, dose, route of administration, frequency of administration and conditions of use[35]. Labeled withdrawal times must be followed to ensure that no harmful drug residues are present in the edible tissue of the animal when it is harvested for human consumption[14]. If there is no tolerance established, any amount of drug residue found is considered a violation and renders the product adulterated[14]. The FDA may also prohibit extra-

label use of certain drugs that pose a public health concern, which includes chloramphenicol and fluoroquinolones[35].

The Seafood Hazard Analysis and Critical Control Point (HACCP) Regulation was promulgated under the FFDCA, and requires both domestic and foreign processors to prevent the introduction of contaminants into the seafood they process or that they transport through commerce[14]. FDA-CVM cooperates with USDA's Food Safety Inspection Service and FDA's Center for Food Safety and Applied Nutrition to monitor for unsafe residues in food and to take enforcement action when there are violations[35]. The regulatory sanctions that FDA has available to apply to domestic processors that are non-compliant are warnings letters, seizure of products, injunction against further non-compliant practices, or prosecution. The options available with respect to foreign processors that are non-compliant include placing the affected products on import alert for detention without physical examination[14].

Routine testing of aquaculture drug residues was initiated in 2002, under the Chemotherapeutics in Seafood Compliance Program, to collect and test samples of selected imported and domestic aquacultured seafood products for the presence of unapproved chemical compounds[14]. Any seafood products containing unapproved animal drug residues are considered to be adulterated under the FFDCA and are not permitted to be sold in, or imported into the U.S.[14]. However, testing on imported aquaculture products is limited to a few chemicals and the frequency of testing is rather low compared to the amount of imported aquacultured products that are potentially contaminated[1]. For example, in 2007 approximately 868,000 fish and fishery products were imported into the U.S., and FDA examined or obtained samples from only approximately 1.2 percent of those[14].

2.5 Method Validation

In order to develop a method that can be used by other agencies to identify animal drug residues, it is important that it follow the proper validation protocol. Some general guidelines to assess method performance are to prepare and analyze method blanks, matrix blanks, and matrix spikes of known concentration[38]. Use of various types of blanks allows the analyst to insure measured concentrations are not influenced by background contamination or matrix interference. Matrix blanks are used to establish background level (presence or absence) of analytes and to verify that sample matrix and equipment used does not interfere with or affect the analytical signal[38]. Matrix spikes, or laboratory-fortified matrix, includes fortifying blank matrix with a known concentration of analyte, and can be used to calculate spike recoveries, accuracy, and precision[38]. The laboratory-fortified matrix should be made up of known concentration of at least three different concentration levels: low, middle, and high, and carried through the complete sample preparation procedure[39].

Performance characteristics that should be evaluated in order to validate a new quantitative method should include selectivity, linearity, accuracy (recovery), precision (repeatability), limit of detection, and limit of quantitation[38]. It is understood that with a large multi-residue method not all analytes will meet recommended acceptability ranges, but the performance for each compound should be tested and reported so that the accuracy and precision are known and are sufficient for the intended purpose of the method[38].

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample[40]. For negative control samples, there should be no interference peaks near the retention times for all analytes. Linearity is shown with a calibration curve displaying the relationship between instrument response and known

concentrations of the analyte, and is generated for each analyte by spiking the matrix with the analyte at known concentrations[40]. It should consist of five to eight concentrations prepared from a common stock and performed in duplicate[41]. The standard curve linear regression coefficient of determination, R^2, should be greater than or equal to 0.995[39].

Accuracy describes the closeness of mean test results obtained by the method to the true value of the analyte, and is determined by replicate analysis of samples containing known concentrations of the analyte[40]. Accuracy should be measured as recovery for a minimum of three replicates at each of three concentrations in the range of expected concentrations[39]. Recovery values should range from 40%-120% for method levels of 1 ppb, and 60-115% for method levels of 10 ppb[38]. The precision of an analytical method is the agreement between a set of replicate measurements without assumption of knowledge of the true value[39]. Precision can be further classified as repeatability and intermediate precision. Repeatability is the precision obtained under conditions where independent test results are obtained with the same method on identical test items in the same test facility by the same operator using the same equipment within short intervals of time, and intermediate precision is within-laboratory precision obtained under variable conditions such as different days[38]. Repeatability precision for method levels of 1 and 10 ppb should be between 11-44% (acceptable values of RSD_r are between ½ and 2 times the RSD of 22%)[38].

The limit of detection (LOD) is the lowest concentration of analyte in a sample that can be determined to be statistically different from a blank[39], and is often referred to as the method detection limit (MDL)[38]. MDL is the minimum concentration of a substance than can be measured and reported with 99% confidence that the analyte concentration is greater than zero, and is determined from analysis of a sample in a given matrix containing the analyte[39]. The limit

of quantitation (LOQ) is the level above which quantitative results may be determined with acceptable accuracy and precision[39]. It is defined as equal to 10 times the standard deviation of the results for a series of replicates used to determine a justifiable limit of detection[42].

3 CHAPTER THREE: MATERIALS AND METHODS

3.1 Chemical Reagents and Materials

LC-MS grade acetonitrile from EMD Millipore Corp (Billerica, MA), reagent grade glacial acetic acid from Ricca Chemical Company (Arlington, TX), and sodium chloride from Fisher Scientific (Fair Lawn, NJ) were used for sample preparation**.** LC-MS grade acetonitrile (Fisher Chemical, Fair Lawn, NJ), Milli-Q purified water, and formic acid (Acros, New Jersey) were used for the mobile phase. Acetic acid solution (1% v/v) was prepared with water purified to 18.2 megaohms by a Millipore Sigma (Billerica, MA) filtration system. Ceramic homogenizer pellets were obtained from Agilent Technologies (Santa Clara, CA). Polypropylene syringes and 17 mm PVDF 0.2 μm syringe filters were purchased from Thermo Scientific (Waltham, MA). The LC column used was an Acquity UPLC BEH C18, 1.7 x 50 μm purchased from Waters (Milford, MA).

3.2 Standard Solutions

Neat standards of enrofloxacin (ENR) (≥98.0%), ciprofloxacin (CIP) (≥98.0%), sarafloxacin (SARA) (≥97.2%), florfenicol (FF) (analytical standard, for drug analysis), and chloramphenicol (CAP) (≥98.0%) were purchased from Sigma Aldrich (St. Louis, MO). Selected standard solution concentrations were made based on a method developed for the analysis of veterinary drug residues in frog legs and other aquacultured species[43]. Separate stock solutions for each analyte were prepared in acetonitrile at concentrations of 500 μg/mL (ppm) for ENR, CIP, SARA, and FF, and 200 μg/mL (ppm) for CAP, and were reported to be stable for one year[43]. An intermediate mixed standard solution was prepared in acetonitrile at concentrations of 500 ng/mL (ppb) for ENR, CIP, SARA, and FF, and 100 ng/mL for CAP, and

was reported to be stable for one month[43]. Additional details of daily standards prepared in blank matrix (containing ≥ 95% matrix), as well as fortified standards in tissue are given in Table 3.1.

Table 3.1. Nominal standard solution concentrations.

	Approx concn of stds (ng/mL) in matrix (fortified stds (ng/g) in tissue)	
Level	ENR, CIP, SARA, FF	CAP
2x	25 (10)	5 (2)
1x	12.5 (5)	2.5 (1)
0.5x	6.25 (2.5)	1.25 (0.5)

[a]The extraction procedure concentrates samples by a factor of 2.5.

3.3 Sample Procurement

Domestic and imported frozen retail crawfish were obtained from local grocery stores, and transported to the Department of Environmental Science at Louisiana State University Agricultural and Mechanical College in Baton Rouge, LA. The samples were stored at -80°C until further processing. For method validation, about 1-2 pounds of live crawfish were obtained from Dr. C. Greg Lutz at the Aquaculture Research Station at Louisiana State University in Baton Rouge, LA. These crawfish were put into the -80°C freezer overnight, thawed and peeled the next day, and put into the freezer until further processing.

3.4 Extraction Procedure

Crawfish were extracted based on a method developed for the analysis of veterinary drug residues in frog legs and other aquacultured species[43]. A portion of the frozen crawfish tails (2.5 ± 0.03 g) was weighed into a 50 mL polypropylene centrifuge tube. Validation samples were fortified by adding an appropriate amount of the intermediate mixed standard to the semi-frozen tissue. After sitting for approximately 15 minutes, 2.0 g of sodium chloride, 10.0 mL of acetonitrile, and 5.0 mL of 1% acetic acid in water were added to each tube. The samples were

homogenized using a Waverly H100 handheld homogenizer (Waverly, IA) for less than a minute. A ceramic homogenizer pellet was then added to each tube and placed on a vortex genie 2 (Scientific Industries, Bohemia, NY) at maximum speed (1700-2300 rpm) for 8 minutes. Later on in method development a Robot Coupe Blixer 2 food processor (Ridgeland, MS) became available, and was used to grind the crawfish tails prior to extraction. The Robot Coupe extraction method was used for analysis of the seven fortified blanks used to calculate MDL and LOQ, and for retail sample analysis. Validation samples were fortified by adding an appropriate amount of the intermediate mixed matrix to the already ground tissue, and allowed to sit for 15 minutes. Next 2.0 g of sodium chloride, 10.0 mL of acetonitrile, 5.0 mL of 1% acetic acid in water, and a ceramic homogenizer was added to each tube. Since the tissue was already ground, the sample was mixed using the vortex genie 2 and the ceramic homogenizer pellet, and did not need to be homogenized by hand. From this point everything was treated similarly.

After the tubes were vortexed, they were placed in a centrifuge at 10°C for approximately 8 minutes at about 3500 rpm. The top organic layer was removed with a Pasteur pipet and placed into another clean 50 mL centrifuge tube. An additional 10 mL of acetonitrile was added to the original tissue mix, and put back on the vortex genie at maximum speed and into the centrifuge at 3500 rpm for 8 minutes each. The top organic layer was again removed and combined with the other acetonitrile layer, and then evaporated on an N-EVAP from Organomation Associates, Inc. (Berlin, MA) heated to 55°C for approximately 60 minutes. The residue was reconstituted in 1.0 mL of 10% acetonitrile and 0.1% formic acid (overall) in water, and then sonicated for about 5 minutes. After sonication the samples were centrifuged at 3500 rpm for 8 minutes at ambient temperature. The extracts were each transferred to a 1 mL plastic syringe and passed through a 0.2 μm PVDF syringe filter into an LC vial for analysis.

3.5 Liquid Chromatography-Mass Spectrometry Instrumentation

Compounds were separated by liquid chromatography and quantified using a triple quad mass spectrometer in multiple reaction monitoring (MRM) mode. MRM is a targeted mass spectrometry technique that allows the detection and quantification of specific molecules in complex mixtures[44]. Separate methods were used for positive and negative MS/MS analysis (Table 3.2). Samples were analyzed with a Waters Acquity TQD LC/MS/MS initially in either positive or negative ion mode and then re-injected for analysis using methods for the opposite polarity; CIP, ENR, and SARA were measured in positive ion mode, and FF and CAP in negative ion mode. All compounds were separated on an Acquity UPLC BEH C18, 1.7 x 50 μm column using a gradient mobile phase consisting of water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B) run in gradient mode (Table 3.2).

3.6 Data Analysis

For the estimation of accuracy (recovery) and precision, blank crawfish from the Aquaculture Research Center were fortified at three different concentration levels: 10.0, 5.0, and 2.5 ng/g for ENR, CIP, SARA, and FF, and 2, 1, and 0.5 ng/g for CAP, corresponding to 0.5, 1, and 2 times the level of interest (Table 3.1). Concentrations of the analytes were interpolated from a one-point matrix matched standard. Accuracy was calculated from the spike recoveries, using Equation 1[41].

$$\text{Recovery } \% = (C_f - C_u) \times 100 / C_a \quad (1)$$

Where C_f is the concentration of the fortified sample, C_u is the concentration of the unfortified or "blank" sample, and C_a is the calculated (not analyzed) concentration of analyte added to the test sample[41].

Table 3.2. Instrumental Parameters of the Waters Acquity TQD LC/MS/MS.

Mass Spectrometry	ES Pos	ES Neg
Capillary (kV)	3.84	2.29
Cone (V)	40	30
Extractor (V)	3	2
Source Temperature (°C)	120	110
Desolvation Temperature (°C)	400	450
Desolvation Gas Flow (L/Hr)	500	600
Argon Collision Gas Flow (mL/min)	0.18	0.19
LC		
Column	Acquity UPLC BEH C18, 1.7 x 50 µm	
Mobile Phase A	Water with 0.1% Formic Acid	
Mobile Phase B	Acetonitrile with 0.1% Formic Acid	
Flow Rate	0.3 mL/min	
Column Temperature (°C)	30°C	
Injection Volume	15 µL	
LC Gradient	A	B
Initial Time	95%	5.0%
1 min	95%	5.0%
3 min	40%	60%
5 min	5.0%	95%
7 min	5.0%	95%
7.1 min	95%	5.0%
10 min	95%	5.0%

Precision, RSD, was calculated from the three different days of recovery data using Equation 2[41].

$$RSD\ \% = s_r \times 100 / \bar{x} \quad (2)$$

Where s_r is repeatability standard deviation, and $\bar{x}$ is the average spike recovery for the three days.

MDL was calculated according to the U.S. Environmental Protection Agency's MDL procedure published in 40 CFR (Code of Federal Regulations) Part 136, Appendix B, rev. 1.11[45]. A minimum of seven aliquots of the sample are to be used to calculate MDL[45], so in order to calculate MDL, LOQ, and within day RSD, seven blanks were fortified at the 0.5x level. The MDL was calculated by multiplying the standard deviation of those values by the t test value at the 99% confidence interval (MDL=standard deviation x 3.143 for one-tailed Student's t test, n=7) per the EPA method for determination of the MDL[45]. LOQ is mathematically defined as 10 times the standard deviation of the results for a series of replicates used to determine a justifiable limit of detection[42], and therefore was calculated by multiplying the standard deviation of the seven fortified blanks by 10.

4 CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Tuning the LC/MS/MS Method

Prior to analysis of the samples, the mass spectrometer was tuned to each particular analyte to maximize signal intensity and establish product (daughter) ions. Multiple reaction monitoring (MRM) utilizes the three quadrupoles to select for specified transitions from one or more precursor ions to multiple product ions[44]. The fragmentation of target compounds to form product ions has been reported in the literature; the *m/z* values and molecular formulas for the most important ions are shown in (Table 4.1). MRM-MS sensitivity is dependent upon proper tuning of instrument parameters such as cone voltage and collision energy in order to generate optimal fragmentation and maximal signal transmission of the product ions[44]. Two MRM transitions were monitored for each analyte; the ion that showed the most intensity was used as the quantifier ion and the ion with less intensity was used as the qualifier ion (Table 4.2).

Table 4.1. Product ions used to identify residues.

Compound	Precursor Ion *(m/z)*	Product Ion *(m/z)*	Product Ion Formula	Refs
CIP	332.22	288.1	$C_{16}H_{19}FN_3O^+$	[43]
		245.11	$C_{14}H_{14}FN_2O^+$	
ENR	360.25	342.13	$C_{19}H_{21}FN_3O_2^+$	[43]
		316.17	$C_{18}H_{23}FN_3O^+$	
SARA	386.28	342.1	$C_{19}H_{18}N_3OF_2^+$	[46]
		299.07	$C_{17}H_{13}N_2OF_2^+$	
CAP	321.04	256.89	$C_{10}H_{10}N_2O_4Cl^-$	[43]
		151.91	$C_7H_6NO_3^-$	
FF	356.05	335.82	$C_{12}H_{12}Cl_2NO_4S^-$	[47]
		184.91	$C_8H_9O_3S^-$	

Standards in mobile phase were injected at decreasing concentrations to establish if these drug residues could reliably be detected at the level of interest. The levels of interest (1x levels) were set using current testing target levels, also considered regulatory action target levels; these values are supported by CVM as sufficient for detecting the presence of each residue[48]. Target levels for the fluoroquinolones are 5 ng/g[48], which was also chosen as the level of interest for florfenicol (Table 4.2). For chloramphenicol, regulatory laboratories look at the maximum required performance limit (MRPL), which is no more and no less than the concentration level that laboratories in the European Community should at least be able to detect and confirm[49]. Chloramphenicol currently has a MRPL of 0.3 ng/g in food of animal origin[25], rather than the previous level of 1 ng/g. However, in this investigation the signal intensity was not sufficient for low level standards so the level of interest was set to 1 ng/g, which is consistent with the study for frog legs this method is based off of (Table 4.2)[43]. Additionally, there was some background contamination of chloramphenicol in the blank samples used for validation, likely due to its natural occurrence, so it was not possible to get an accurate reading below the 0.5x level, even though the signal was high.

4.2 Method Validation and Quantitation of Target Residues in Crawfish

4.2.1 Selectivity

No interference peaks were found near the retention times for the selected transitions for the five analytes under the chromatographic conditions used for the negative control samples of solvent blank and reagent blank, with the exception for enrofloxacin's qualifier ion (360.25>342.13). For the matrix blank samples used for validation, chloramphenicol was detected at low levels in all samples tested, therefore the blank areas were subtracted from fortified sample areas when calculating recovery and detection and quantitation limits.

Table 4.2. MRM transitions used for detection and quantitation.

Analyte	Ion Mode	MRM Transitions	Cone, V	CE, eV[a]	RT	Level of interest[b] (ng/g)
Ciprofloxacin	Positive	332.22>288.1[c]; 332.22>245.11	40	20	2.34	5
Enrofloxacin	Positive	360.25>316.17[c]; 360.25>342.13	40	20	2.41	5
Sarafloxacin	Positive	386.28>299.07[c]; 386.28>342.10	40	30; 20	2.51	5
Florfenicol	Negative	356.05> 335.82[c]; 356.05> 184.91	30	10; 20	2.79	5
Chloramphenicol	Negative	321.04>151.91[c]; 321.04> 256.89	30	20; 10	2.88	1

[a] CE=Collision energy

[b] The level of interest corresponds to 1x level

[c] Primary MRM for quantitation

Others have also found chloramphenicol levels when there is no known use of the antibiotic being used. Products of animal origin originating from Asian countries entering the European market were found to be noncompliant (containing CAP) on a regular basis, even when there was no history of chloramphenicol use in those countries[50]. The background levels of chloramphenicol are potentially due to chloramphenicol occurring naturally in soil and crops, which can then be taken up by the animal resulting in residues in animal products not due to illegal use of the drug[51]. In 2010 the detection of CAP in different families of Mongolian plants as well as in soil was reported, suggesting that CAP is produced in the soil and the plants absorb CAP through their root systems[50]. More recent studies suggest that CAP can be naturally produced in sterile and natural soils by soil bacteria and transferred to crops[19], and another study

found 35% of cereal straw samples from the Netherlands, France, UK, Germany, and Denmark to contain CAP—with the highest level at 6.3 μg/kg[51].

4.2.2 Linearity

The working range of the method was determined by analyzing 5 different calibration standards. For matrix-matched calibration standards, concentrations were 76.0, 50.0, 25.0, 12.5, and 6.25 ng/mL for the FQs and FF, and 15.2, 10.0, 5.0, 2.5, and 1.25 ng/mL for CAP. Linear regression analysis was used to calculate the calibration curve, which was linear between the lowest and highest concentration, with correlation coefficients ≥ 0.972 (Appendix Figure 7.1). Solvent standard curves were also generated using concentrations of 50.0, 25.0, 12.5, and 6.25 ng/mL for the FQs and FF, and 10.0, 5.0, 2.5, and 1.25 ng/mL for CAP. Linear regression curves were linear with correlation coefficients ≥0.963 (Appendix Figure 7.2).

4.2.3 Accuracy and Precision

To determine recoveries (measure of accuracy), blank samples were fortified with a mixed standard solution at three different levels of concentration on three different occasions (October 12, 2017; October 25, 2017; and November 1, 2017). After quantifying the fortified samples using a one-point matrix matched standard, recovery was calculated according to Equation 1, and results are given in Table 4.3. Overall, the mean recoveries ranged from 36.7 to 97.6%, and generally fell within the acceptance criteria of 40-120% for method levels of 1 ppb, and 60-115% for method levels of 10 ppb[38].

Precision (RSD) for ciprofloxacin, enrofloxacin, sarafloxacin, and florfenicol was acceptable with values of 2.68-32.46% across the three days (Table 4.3). However, precision (RSD) for chloramphenicol was quite high across the three days (57.52-72.75%) (Table 4.3). The

high RSD values could possibly be due to the background levels of chloramphenicol. Repeatability precision for 7 samples fortified at the 0.5x level had RSDs ranging from 8-14%, with the exception of chloramphenicol which was 35% (Appendix Table 7.1).

Table 4.3. Accuracy studied at 2x, 1x, and 0.5x for FQs, FF, and CAP in crawfish.

Compounds	Level	Concentration (ng/g)	Accuracy Recovery, % (RSD)[a] (n=3)	MDL[b] (ng/g)	LOQ[c] (ng/g)
Ciprofloxacin	2x	10	53.03 (32.46)	0.44	1.38
	1x	5	68.74 (30.53)		
	0.5x	2.5	36.65 (25.06)		
Enrofloxacin	2x	10	71.86 (4.29)	0.53	1.69
	1x	5	52.80 (19.53)		
	0.5x	2.5	71.57 (31.12)		
Sarafloxacin	2x	10	76.92 (2.68)	0.50	1.59
	1x	5	76.11 (6.34)		
	0.5x	2.5	88.60 (12.39)		
Florfenicol	2x	10	92.25 (14.02)	0.71	2.25
	1x	5	97.64 (25.20)		
	0.5x	2.5	89.90 (10.52)		
Chloramphenicol	2x	2	63.01 (72.75)	0.27	0.85
	1x	1	53.54 (59.56)		
	0.5x	0.5	40.98 (57.52)		

[a] Recoveries based on comparison to a single point matrix matched standard.

[b] Method detection limit (MDL) = SD × 3.143, where SD is standard deviation from quantitative results of 0.5x spikes (n = 7)

[c] LOQ= 10 x SD, where SD is standard deviation from quantitative results of 0.5x spikes (n=7)

4.2.4 MDL and LOQ

MDL and LOQ were calculated from the standard deviation of residue concentrations in blank samples fortified at the 0.5x level. Detection and quantitation limits of the five veterinary drug residues are given in Table 4.3. The detection limits were between 0.27 and 0.71 ng/g, and the quantitation limits varied between 0.85 and 2.25 ng/g. The blank sample run with this data set had background levels of chloramphenicol near the 0.5x fortified levels, but was still less than each fortified sample. There was not enough blank crawfish left to run another 7 sample blanks to look at MDL of the blanks to determine if it was higher than the MDL for the fortification samples. The previous blanks that had been run were examined to calculate a MDL, which resulted in a similar value to what was previously obtained for the spiked samples. Therefore it was determined to use the MDL of the 0.5x fortified samples, since those were assumed to be more accurate as they had all been analyzed under the same conditions.

4.3 Matrix Effects

Matrix effects can impact quantitative analysis of compounds at trace levels. In order to determine whether the matrix was enhancing or suppressing the signal, standards at 50 and 10 ng/mL prepared in solvent were compared to standards prepared with blank matrix, and showed general matrix enhancement. Other papers have reported matrix enhancement for ENR and CAP in catfish, and slight enhancement in frog legs[43]. Furthermore, another investigation found matrix enhancement for CIP in salmon, but suppressed in shrimp and tilapia[52]. Matrix enhancement in this investigation was further demonstrated by calculating recoveries of fortified samples using solvent standard curves and one-point matrix matched standards (Table 4.4). The high recoveries calculated when using solvent standard curves indicate matrix enhancement, with the exception of chloramphenicol which had similar recovery values. To minimize the matrix effect, recoveries

were calculated using matrix standards; it was also established that using a one-point matrix matched standard as opposed to a matrix matched standard curve provided slightly better results and was more efficient of resources and time.

Table 4.4. Recoveries of 1x fortified samples using solvent standard curves and one-point matrix matched standards.

Analyte	Level of interest (1x) (ng/g)	Solvent standard curve recovery, % (n=1)	Matrix std-1 point recovery, % (n=3)
Enrofloxacin	5	116.9	52.8
Ciprofloxacin	5	228.5	68.7
Sarafloxacin	5	280.9	76.1
Florfenicol	5	163.5	97.6
Chloramphenicol	1	50.2	53.5

4.4 Targeted residues in retail samples

This method was used to test two brands of Louisiana crawfish and two brands of Chinese crawfish, with three samples per brand tested. One Louisiana brand showed levels of chloramphenicol above the LOQ at an average concentration of 0.91 ng/g (Figure 4.1).

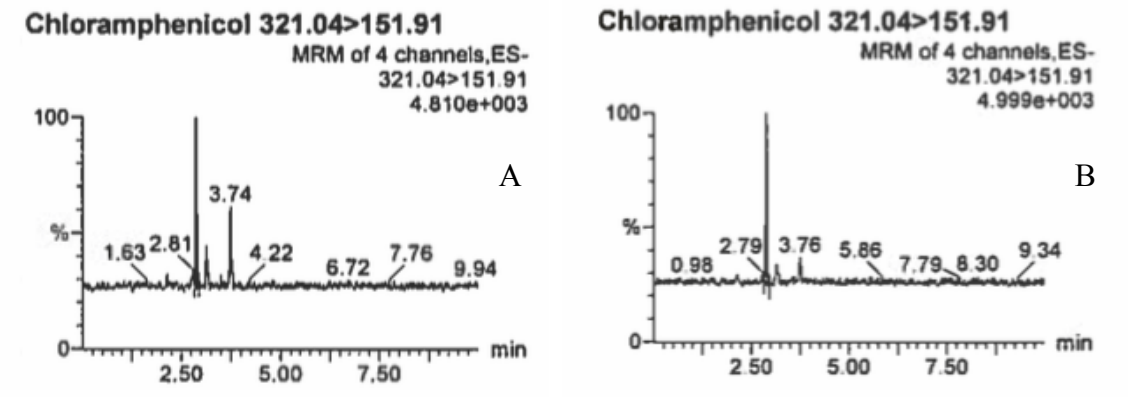

Figure 4.1. Matrix matched standard (2.5 ng/mL) of chloramphenicol for Louisiana #1 (A), and positive sample result from Louisiana #1 sample 3 (B).

One Chinese sample showed levels of chloramphenicol averaging 0.52 ng/g, which is above the MDL of 0.27 ng/g, but below the LOQ at 0.85 ng/g. This means it is statistically different from zero, but does not carry acceptable accuracy and precision and is not reliably quantifiable. No other veterinary drug residues were detected in any samples, as seen below in Table 4.5.

Table 4.5. Concentration of veterinary drugs (ppb) in crawfish using LC/MS/MS.

Origin	Analyte	MDL (LOQ) (ng/g)	Concentration[a] (ng/g)
China			
1	Chloramphenicol	0.27 (0.85)	0.52±0.20
	Florfenicol	0.71 (2.25)	ND
	Enrofloxacin	0.53 (1.69)	ND
	Ciprofloxacin	0.44 (1.38)	ND
	Sarafloxacin	0.5 (1.59)	ND
2	Chloramphenicol		ND
	Florfenicol		ND
	Enrofloxacin		ND
	Ciprofloxacin		ND
	Sarafloxacin		ND
Louisiana			
1	Chloramphenicol	0.27 (0.85)	0.91±0.33
	Florfenicol	0.71 (2.25)	ND
	Enrofloxacin	0.53 (1.69)	ND
	Ciprofloxacin	0.44 (1.38)	ND
	Sarafloxacin	0.5 (1.59)	ND
2	Chloramphenicol		NA[b]
	Florfenicol		ND
	Enrofloxacin		ND
	Ciprofloxacin		ND
	Sarafloxacin		ND

[a] Reported as mean ± SD (n=3)

[b] Adequate MS/MS spectra not obtained

5 CHAPTER FIVE: SUMMARY AND CONCLUSIONS

The objective of this research was to expand upon past methods to validate a single method that could look at multiple veterinary drug residues in retail frozen crawfish. The use of unapproved veterinary drugs can lead to accumulation in crawfish tissue and also lead to antibiotic resistant bacteria that can then be passed onto the consumer. These can have deleterious effects on human health, especially with antibiotics such as chloramphenicol which can cause severe health consequences at dose-independent levels. Therefore, routine testing of multiple veterinary drug residues in food is important in order to protect public health.

The results of this investigation showed acceptable method performance characteristics when looking at selectivity, linearity, accuracy (recovery), precision (repeatability), limit of detection, and limit of quantitation. CIP demonstrated some of the lowest recoveries, though this was also found by others for frog legs using Q-TOF and LC-fluorescence[43]. One of the difficulties throughout method development was always having a background level of chloramphenicol and the inability to get a true blank. This made it impossible to obtain the level of interest at the MRPL of 0.3 ng/g. The lowest level standard tested was at 0.5 ng/g and this was just above the background levels for chloramphenicol. This background contamination could also have affected the precision, which had quite high RSD values for spike recovery at the three concentrations.

The background contamination could be due to chloramphenicol occurring naturally in plant material, which is used as forage material and could be transferred to animal tissues[50]. It is known that the soil organism *S. venezuelae* and related organisms can biosynthesize CAP, and a few studies have quantified CAP in grasses and herbs[50], cereal straw[51], and soil—with this particular study showing that in a single day over 100 ug/kg of chloramphenicol can be produced

by *S. venezuelae* in nonsterile topsoil, a level that significantly exceeds the concentration calculated that could result in the detection of residues in crops[19]. The biology of the formation of CAP in arable soil is largely not understood[51], though one study showed that CAP production and *S. venezuelae* growth rate are strongly related and depend on factors such as temperature and soil organic matter—with increased production after addition of carbon and nitrogen sources[19].

Analysis of retail samples showed two samples with elevated levels of chloramphenicol, and no other veterinary drug residues being detected. One sample from China showed levels of chloramphenicol averaging 0.52 ng/g, which is above the MDL of 0.27 ng/g, but below the LOQ of 0.85 ng/g. This means it is statistically different from zero, but does not carry acceptable accuracy and precision and is not reliably quantifiable. Another sample, this one being from Louisiana, showed a higher average level of chloramphenicol at 0.91 ng/g, which was above the LOQ and can thus be considered reliably quantifiable. However, it should be remembered that there were background levels of chloramphenicol in supposedly blank tissue, and with chloramphenicol potentially occurring naturally it is possible that products of animal origin can contain residues of CAP that are not due to illegal use of the drug[50].

Further testing is needed to investigate the safety of other imported crawfish that may be purchased by restaurants and not found in local grocery stores, including that from countries other than China. Other aquaculture species should also be examined to determine the extent of imported seafood contamination. Additionally, due to recent studies finding CAP occurring naturally in soil and crops, the occurrence in crops is probably more prevalent than we think and therefore soil and rice straw, which is the main forage material for crawfish, should be tested for chloramphenicol. A survey looking at different areas where crawfish are grown should be undertaken, including natural areas such as the Atchafalaya Basin.

6 REFERENCES

1. Sapkota, A.; Sapkota, A. R.; Kucharski, M.; Burke, J.; McKenzie, S.; Walker, P.; Lawrence, R., Aquaculture practices and potential human health risks: Current knowledge and future priorities. *Environment International* **2008,** *34* (8), 1215-1226.

2. Louisiana State University Agricultural Center, Louisiana Summary Agriculture & Natural Resources 2014 Data. 2382 ed.; 2014; pp. 1-342. http://www.lsuagcenter.com/agsummary/Data.html (accessed September 9, 2016).

3. Benbrook, C. M., Antibiotic Drug Use in U.S. Aquaculture. The Northwest Science and Environmental Policy Center, Ed. Institute for Agriculture and Trade Policy Report 2002; pp 1-18.

4. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Chapter 11: Aquaculture Drugs In *Fish and Fishery Products Hazards and Controls Guidance*, 4 ed.; 2011.

5. Done, H. Y.; Halden, R. U., Reconnaissance of 47 antibiotics and associated microbial risks in seafood sold in the United States. *Journal Of Hazardous Materials* **2015,** *282*, 10-17.

6. Walls, J. G., *Crawfishes of Louisiana*. Baton Rouge, La.: Louisiana State University Press: 2009.

7. McClain, W. R.; Romaire, R. P.; Lutz, C. G.; Shirley, M. G., *Louisiana crawfish production manual*. [Baton Rouge, La.] : LSU Agricultural Center: 2007.

8. McClain, W. R.; Romaire, R. P., Cultured Aquatic Species Information Programme, Procambarus clarkii. FAO Fisheries and Aquaculture Department, Ed. Rome, 2007.

9. Gillespie, J.; Guidry, K.; Boucher, R., Crawfish Production: Financial Characteristics and Record-Keeping Options. Southern Regional Aquaculture Center,: 2012; Vol. SRAC Publication No. 2405.

10. U.S International Trade Commission, *Crawfish Tail Meat from China: Investigation No. 731-TA-752 (Second Review)* Washington, District of Columbia : United States International Trade Commission: 2008.

11. Nyaupane, N. P.; Gillespie, J. M., Factors Influencing Producers' Marketing Decisions in the Louisiana Crawfish Industry. *Journal of Food Distribution Research* **2011,** *42* (2).

12. U.S International Trade Commission, *Crawfish tail meat from China: Investigation no. 731-TA-752 (third review)*. Washington, District of Columbia : United States International Trade Commission: 2014.

13. Committee on Drug Use in Food Animals, Panel on Animal Health, Food Safety, and Public Health, Board on Agriculture, National Research Council, Food and Nutrition Board, Institute of Medicine, *The use of drugs in food animals. [electronic resource] : benefits and risks*. Washington, D.C. : National Academy Press: 1999.

14. Eschenbach, A. C. v. *Enhanced Aquaculture and Seafood Inspection - Report to Congress, Food and Drug Administration Amendments Act of 2007, Public Law 110-85, Section 1006 – Enhanced Aquaculture and Seafood Inspection*; 2008.

15. Goldburg, R.; Elliott, M. S.; Naylor, R., Marine aquaculture in the United States: environmental impacts and policy options. *Pew Oceans Commission* **2001**, 1-33

16. FAO/OIE/WHO, Antimicrobial Use in Aquaculture and Antimicrobial Resistance. World Health Organization: Switzerland: 2006; pp 1-107.

17. Wei, R.; Ge, F.; Chen, M.; Wang, R., Occurrence of Ciprofloxacin, Enrofloxacin, and Florfenicol in Animal Wastewater and Water Resources. *Journal of Environmental Quality* **2012,** *41* (5), 1481-1486.

18. HFV-200, Center for Veterinary Medicine, Enforcement Priorities for Drug Use in Aquaculture In *Center for Veterinary Medicine Program Policy and Procedures Manual* 2011; pp 1-15.

19. Romkens, P.; Berendsen, B.; Pikkemaat, M.; Wegh, R.; van Sisseren, M.; Stolker, L.; Nielen, M.; Romkens, P., Occurrence of Chloramphenicol in Crops through Natural Production by Bacteria in Soil. *J. Agric. Food Chem.* **2013,** *61*, 4004-4010.

20. Hanekamp, J. C.; Bast, A., Antibiotics exposure and health risks: Chloramphenicol. *Environmental Toxicology and Pharmacology* **2015,** *39* (1), 213-220.

21. Ali, I.; Aboul-Enein, H. Y.; Gupta, V. K.; Singh, P.; Negi, U., Analyses of Chloramphenicol in Biological Samples by HPLC. *Analytical Letters* **2009,** *42*, 1368–1381.

22. J. Wongtavatchai, J. G., McLean, F. Ramos, and D. Arnold, WHO Food Additives Series: 53, Chloramphenicol World Health Organization, Ed. 2004.

23. Department of Agriculture and Forestry Office of the Commissioner, Emergency Rules: Chloramphenicol in Shrimp and Crawfish Testing and Sale *Louisiana Register* **2003,** *29* (6), 831-834.

24. European Commission, RASFF Portal, 2014.ACD. 15/01/2014 ed.; https://webgate.ec.europa.eu/rasff-window/portal/?event=SearchByKeyword&NewSearch=1&Keywords=chloramphenicol.

25. Zhang, Q.-J.; Peng, T.; Chen, D.-D.; Xie, J.; Wang, X.; Wang, G.-M.; Nie, C.-M., Determination of chloramphenicol residues in aquatic products using immunoaffinity

column cleanup and high performance liquid chromatography with ultraviolet detection. *Journal Of AOAC International* **2013,** *96* (4), 897-901.

26. American Academy of Veterinary Pharmacology and Therapeutics, Florfenicol (Veterinary—Systemic) http://c.ymcdn.com/sites/www.aavpt.org/resource/resmgr/imported/florfenicol.pdf **2007**.

27. Intervet Inc, Environmental Assessment for Aquaflor (Florfenicol) for Freshwater-reared Finfish in Recirculating Aquaculture Systems. **2013**, 1-87.

28. Xinhua, Y.; Wen, C., Use of veterinary medicines in Chinese aquaculture: current status. In *Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production*, Bondad-Reantaso, M. G.; Arthur, J. R.; Subasinghe, R. P., Eds. FAO Fisheries and Aquaculture Technical Paper 2012; Vol. 547, pp 51-67.

29. Johnston, L.; Mackay, L.; Croft, M., Determination of quinolones and fluoroquinolones in fish tissue and seafood by high-performance liquid chromatography with electrospray ionisation tandem mass spectrometric detection. *Journal Of Chromatography.* **2002,** *982* (1), 97-109.

30. Flores-Miranda, B. M.; Espinosa-Plascencia, A.; Gómez-Jiménez, S.; López-Zavala, A. A.; González-Carrillo, H.; Bermúdez-Almada, M. d. C., Accumulation and Elimination of Enrofloxacin and Ciprofloxacin in Tissues of Shrimp Litopenaeus vannamei under Laboratory and Farm Conditions. *ISRN Pharmaceutics* **2012**, 1-7.

31. Fang, X.; Zhou, J.; Liu, X., Pharmacokinetics of sarafloxacin in allogynogenetic silver crucian carp, Carassius auratus gibelio. *Fish physiology and biochemistry* **2016,** *42* (1), 335-341.

32. Liang, J. P.; Li, J.; Li, J. T.; Liu, P.; Chang, Z. Q.; Nie, G. X., Accumulation and elimination of enrofloxacin and its metabolite ciprofloxacin in the ridgetail white prawn Exopalaemon carinicauda following medicated feed and bath administration. *Journal of Veterinary Pharmacology and Therapeutics* **2014,** *37* (5), 508-514.

33. Choi, J.-H.; Na, T. W.; Mamun, M. I. R.; Abd El-Aty, A. M.; Shin, E.-H.; Park, J.-H.; Shim, J.-H., Bufferized solvent extraction and HPLC fluorometric detection method for sarafloxacin in pig and chicken muscles. *Biomedical Chromatography: BMC* **2011,** *25* (3), 405-411.

34. Karunasagar, I., Public health and trade impact of antimicrobial use in aquaculture. In *Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production*, Bondad-Reantaso, M. G.; Arthur, J. R.; Subasinghe, R. P., Eds. FAO Fisheries and Aquaculture Technical Paper: 2012; Vol. 547, pp 1-9.

35. Matysczak, J.; Prater, D. A., Legislation and regulatory efforts in the United States of America relevant to the use of antimicrobials in aquaculture. In *Improving biosecurity*

through prudent and responsible use of veterinary medicines in aquatic food production, Bondad-Reantaso, M. G.; Arthur, J. R.; Subasinghe, R. P., Eds. FAO Fisheries and Aquaculture Technical Paper 2012; Vol. 547, pp 119-126.

36. Homan, A.; Tingting, D., Making aquaculture accountable through third-party certification and consumer protection law in the United States and China. *Vermont Law Review* **2014,** *39* (1), 135-159.

37. Sun, M.; Chang, Z.; Van den Brink, P. J.; Li, J.; Zhao, F.; Rico, A., Environmental and human health risks of antimicrobials used in Fenneropenaeus chinensis aquaculture production in China. *Environmental Science And Pollution Research International* **2016,** *23* (15), 15689-15702.

38. US Food & Drug Administration: Office of Foods and Veterinary Medicine, Guidelines for the Validation of Chemical Methods for the FDA FVM Program. 2 ed.; 2015.

39. US Food & Drug Administration: Office of Regulatory Affairs, Methods, Method Verification and Validation 2014; Vol. 1.7, pp 1-19.

40. U.S. Department of Health and Human Services, Food & Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine,, Guidance for Industry--Bioanalytical Method Validation Rockville, MD, 2001.

41. AOAC International, AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals 2002.

42. Wisconsin Department of Natural Resources: Laboratory Certification Program, ANALYTICAL DETECTION LIMIT GUIDANCE & Laboratory Guide for Determining Method Detection Limits. PUBL-TS-056-96: 1996.

43. Turnipseed, S. B.; Clark, S. B.; Storey, J. M.; Carr, J. R., Analysis of Veterinary Drug Residues in Frog Legs and Other Aquacultured Species Using Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry. *Journal of agricultural and food chemistry* **2012,** (18), 4430-4439.

44. Sherwood, C. A.; Eastham, A.; Lee, L. W.; Risler, J.; Mirzaei, H.; Falkner, J. A.; Martin, D. B., Rapid optimization of MRM-MS instrument parameters by subtle alteration of precursor and product m/z targets. *Journal Of Proteome Research* **2009,** *8* (7), 3746-3751.

45. Environmental Protection Agency, 40 CFR Appendix B to Part 136 - Definition and Procedure for the Determination of the Method Detection Limit-Revision 1.11. U.S. Government Publishing Office 2011; Vol. 23, pp 343-346.

46. Schilling, J. B.; Cepa, S. P.; Menacherry, S. D.; Bavda, L. T.; Heard, B. M.; Stockwell, B. L., Liquid chromatography combined with tandem mass spectrometry for the confirmation of sarafloxacin in catfish tissue. *Analytical Chemistry* **1996,** *68* (11), 1905-1909.

47. Stravs M; Schymanski E; Singer H; Department of Environmental Chemistry; Eawag and Thomaidis N; University of Athens, Spectrum EQ364151 for Florfenicol. Eawag, Duebendorf, Switzerland, 2015.

48. U.S. Food and Drug Administration, Chemotherapeutics in Aquaculture Seafood Compliance Program 2017.

49. J.C. Hanekamp, G. F., K. Olieman, Chloramphenicol, food safety and precautionary thinking in Europe *Env. Liability* **2003,** *11*, 209-221.

50. Berendsen, B.; Stolker, L.; de Jong, J.; Nielen, M.; Tserendorj, E.; Sodnomdarjaa, R.; Cannavan, A.; Elliott, C., Evidence of natural occurrence of the banned antibiotic chloramphenicol in herbs and grass. *Analytical & Bioanalytical Chemistry* **2010,** *397* (5), 1955-1963.

51. Nordkvist, E.; Zuidema, T.; Herbes, R. G.; Berendsen, B. J. A., Occurrence of chloramphenicol in cereal straw in north-western Europe. *Food Additives & Contaminant: Part A* **2016,** *33* (5), 798-803.

52. J.N. Pearce, B. G. B., J.M. van de Riet, M.D. Casey, and R.A. Potter, Determination of fluoroquinolones in aquaculture products by ultra-performance liquid chromatography-tandem mass spectrometry. *Food Additives & Contaminants* **2009,** *26* (1), 39-46.

7 APPENDIX—SUPPLEMENTAL DATA

Table 7.1. Intraday precision (RSD) and average recovery for 0.5x fortified samples (n=7).

Analyte	0.5x #1	0.5x #2	0.5x #3	0.5x #4	0.5x #5	0.5x #6	0.5x #7	Avg Recovery, %	RSD, %
CAP	0.133	0.198	0.148	0.300	0.251	0.303	0.360	48.35	35.33
FF	1.366	1.729	1.931	1.658	1.843	1.789	2.077	70.824	12.697
CIP	0.928	0.928	1.265	0.864	1.088	0.948	0.922	39.681	13.957
ENR	1.423	1.750	1.207	1.434	1.347	1.318	1.467	56.830	11.914
SARA	1.734	1.812	2.072	2.110	1.858	2.078	2.098	78.643	8.074

Table 7.2. Matrix effects of standards at 50 and 10 ng/mL prepared in solvent vs. standards prepared with blank matrix.

Analyte	Concentration (ng/mL)	Solvent Standard Area	Matrix Standard Area
Enrofloxacin	50	4807.79	16263.80
	10	450.74	3433.30
Ciprofloxacin	50	1376.704	4453.15
	10	264.302	754.37
Sarafloxacin	50	677.58	6166.19
	10	66.65	1240.90
Florfenicol	50	1617.67	2214.07
	10	266.70	357.36
Chloramphenicol	50	3084.95	2120.45
	10	292.89	460.56

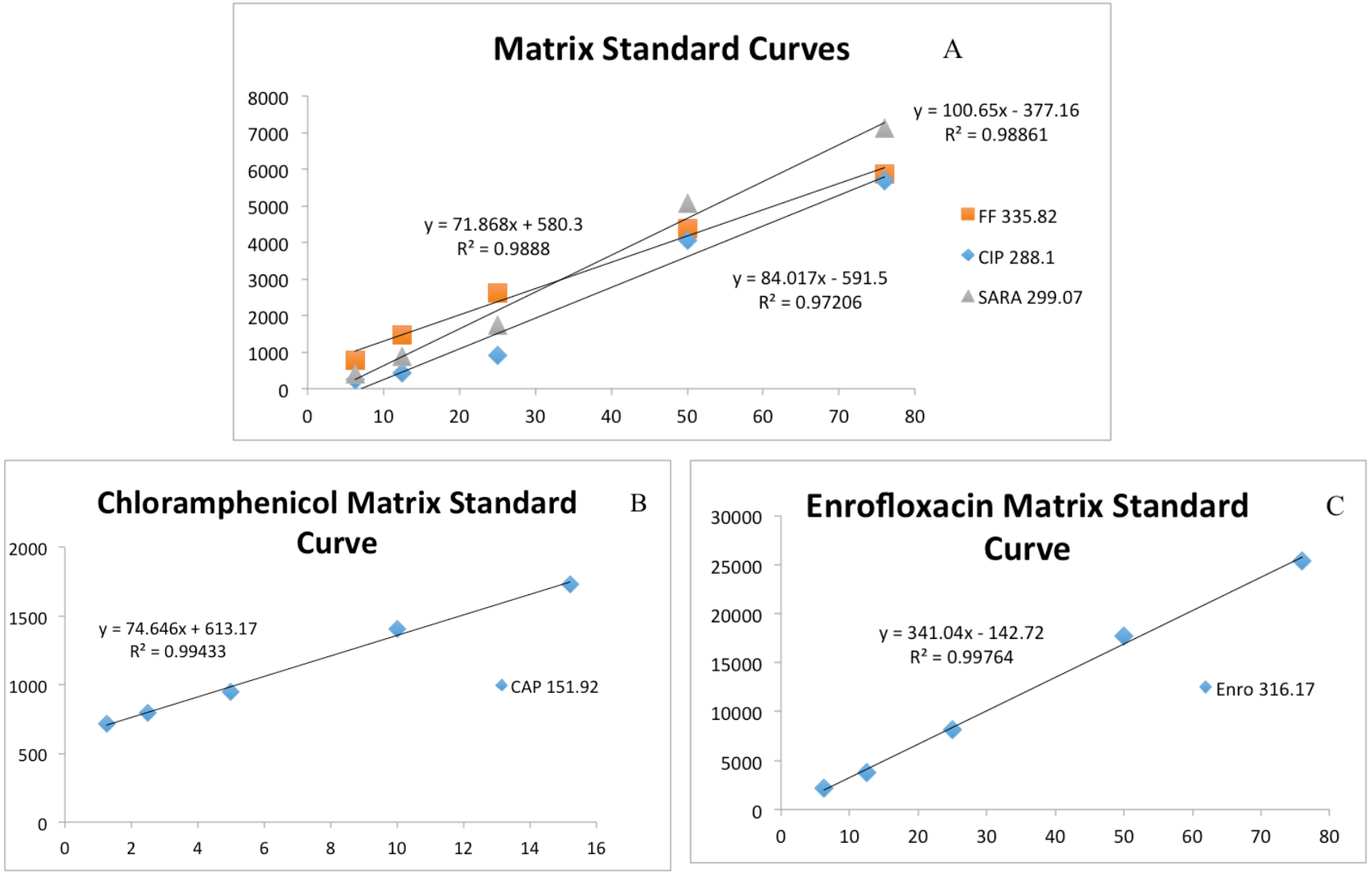

Figure 7.1. Matrix standard curves for florfenicol, ciprofloxacin, sarafloxacin (A); chloramphenicol (B); and enrofloxacin (C).

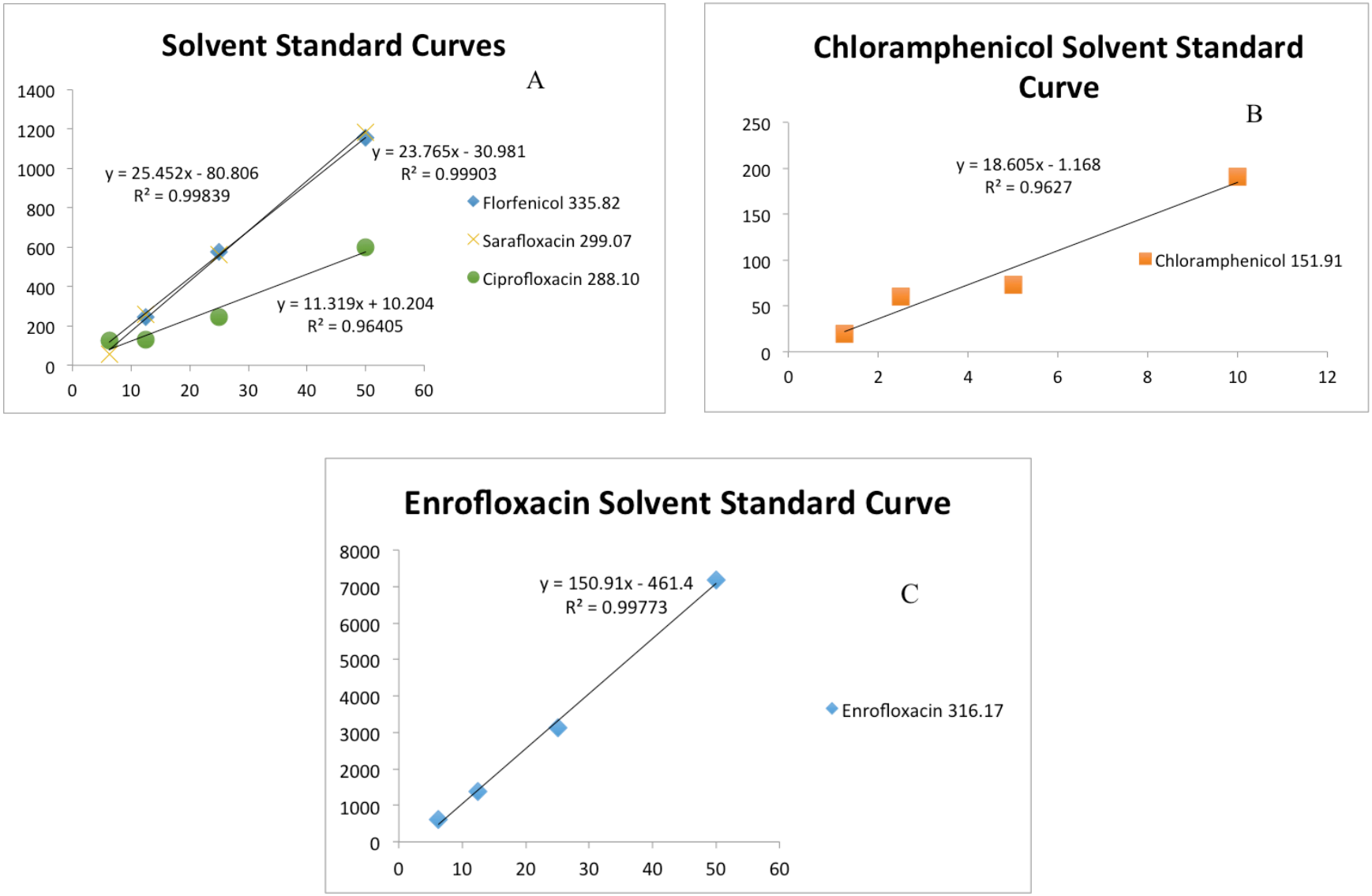

Figure 7.2. Solvent standard curves for florfenicol, sarafloxacin, and ciprofloxacin (A); chloramphenicol (B); and enrofloxacin (C).

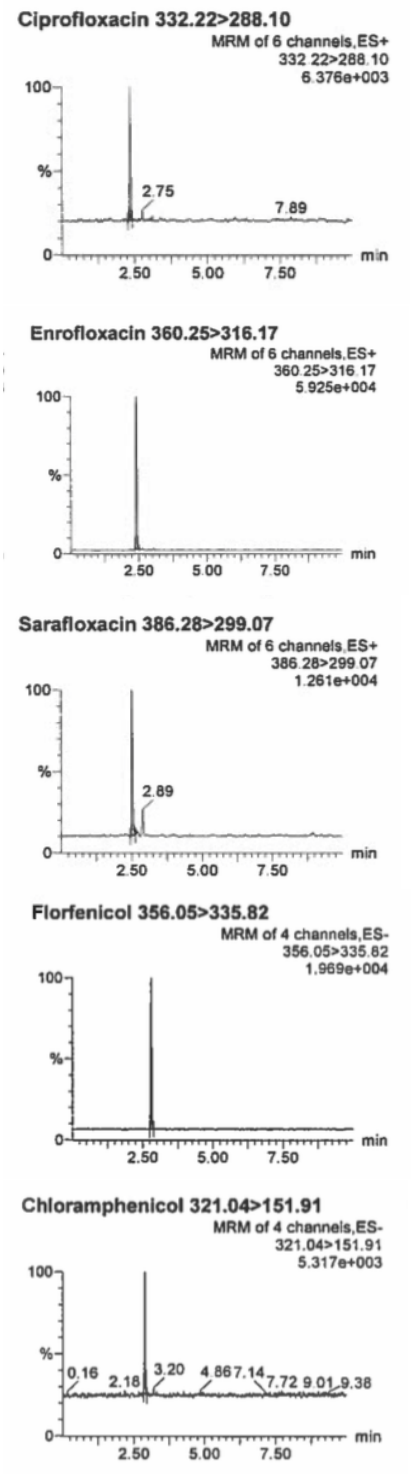

Figure 7.3. Chromatograms for matrix calibration standards of the three FQs and FF at 6.25 ng/mL and CAP at 1.25 ng/mL.

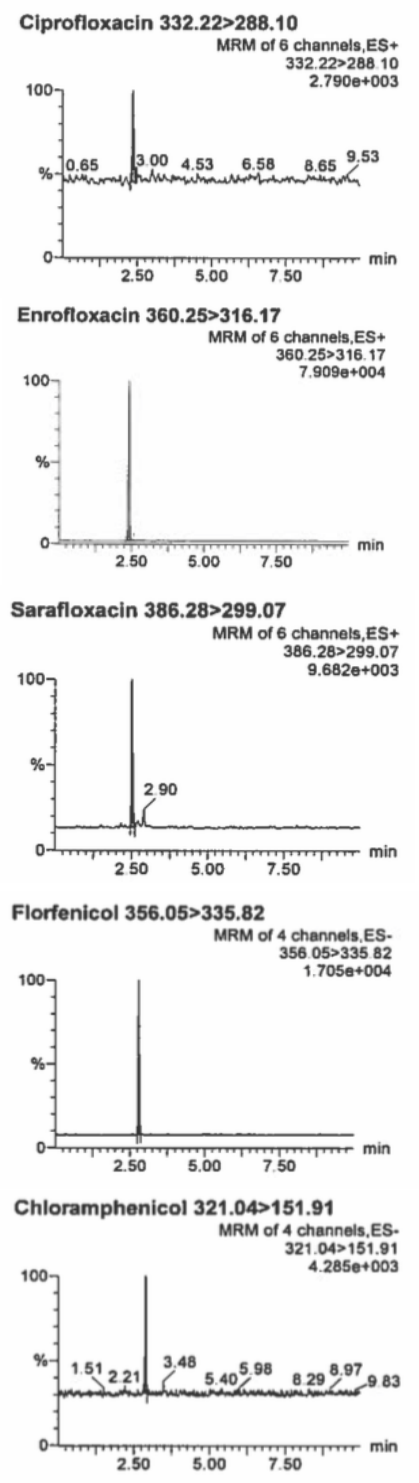

Figure 7.4. Chromatograms for fortification standards of the three FQs, FF, and CAP at the 0.5x level.

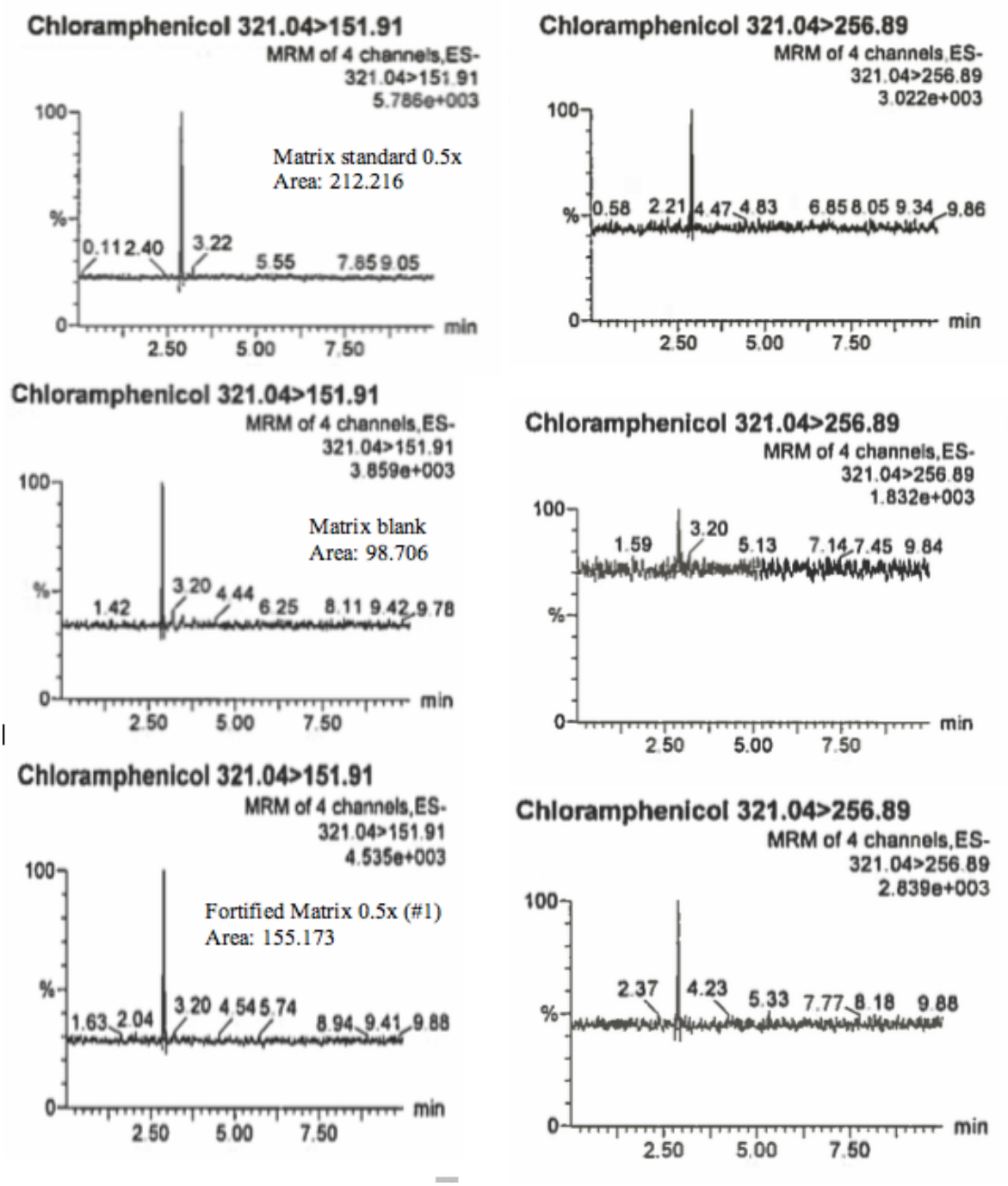

Figure 7.5. Chromatograms of quantifier ion (151.91) and qualifier ion (256.89) of chloramphenicol showing the 0.5x matrix standard, matrix blank, and 0.5x fortified matrix (rep #1) from run to calculate MDL.

8 VITA

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