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Detection and confirmation of veterinary drug residues in commercially available frozen shrimp

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DETECTION AND CONFIRMATION OF VETERINARY DRUG RESIDUES IN COMMERCIALLY AVAILABLE FROZEN SHRIMP

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Dedicated to my parents, Lisa and Jason Johnson.

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ABSTRACT

Aquaculture has grown rapidly as the world's wild-caught fisheries approach their sustainable limits. Feed conversion in aquaculture is more efficient than in terrestrial animals. Thus with a growing world population, seafood produced through aquaculture can provide a high quality source of protein. Aquaculture systems rely on high stocking densities and commercial feeds to increase production and profitability, which increase animal stress and susceptibility to disease. Veterinary drugs are commonly used to prevent and treat disease outbreaks. Several of these drugs are banned for use in shrimp farming in the United States. These drugs can be toxic to humans, with side effects that can be fatal. There is also an increased risk of developing antibiotic resistant strains of human pathogens, including *Bacillus* and *Vibrio* species. The Food and Drug Administration is responsible for the safety of all fish and fishery products entering the United States, but funding for testing is limited. Examples of drugs with high enforcement priority include chloramphenicol, nitrofurans, fluoroquinolones and quinolones, malachite green, and steroid hormones. State testing has repeatedly resulted in the detection of banned drugs. The objective of this study was to quantify veterinary drug residues in commercially available frozen shrimp.

Imported, farm-raised shrimp samples were purchased from local supermarkets and include shrimp from seven brands and six different countries. A preliminary screening was done using rapid ELISA kits to test for chloramphenicol, malachite green, nitrofurans, and fluoroquinolones. Samples tested positive for malachite green and fluoroquinolones; all samples tested negative for chloramphenicol and nitrofurans. ELISA results were confirmed using liquid chromatography with tandem mass

spectrometry. Drug residues in shrimp samples were confirmed for chloramphenicol at concentrations ranging from 0.30 to 0.49 ppb, and enrofloxacin from 1.22 to 5.95 ppb. Results suggest that current testing by the FDA may not be adequately addressing imported seafood safety. Concurrently analyzed wild-caught shrimp from the US tested negative for all veterinary drugs considered.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Aquaculture has grown rapidly as the world's fisheries have reached their sustainable limits. Aquaculture systems rely on high stocking densities and commercial feeds to increase production and profitability, which increases animal stress and susceptibility to disease. Veterinary drugs, including those that are known to cause adverse human health effects, are commonly used to prevent and treat disease outbreaks, making routine testing essential.

Several veterinary drugs are illegal for use in food-producing animals in the United States because of their toxicity to humans, their linkage to fatal diseases, and antibiotic resistance in human pathogens including Bacillus and Vibrio species. The Food and Drug Administration is responsible for the safety of all fish and fishery products entering the United States, but funding for testing is limited. State testing has repeatedly resulted in the detection of banned veterinary drugs. Current testing and enforcement may be insufficient. Contaminated product is still entering the country because exporting countries often don't have sufficient resources for alternative means of combating disease.

Previous studies have focused on the use of veterinary drugs in aquaculture, including the history of their use, their impacts on the environment and human health, the toxicity of historically used antimicrobials, and alternatives to unsafe veterinary drugs. Methods have been developed for the rapid screening of animal and food samples using enzyme-linked immunosorbent assays (ELISA), and several methods have been developed for the detection of veterinary drug residues. The best and most

sensitive method is high-pressure liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS). The instrument significantly reduces background signal and allows measurement at very low levels.

The purpose of this study was to screen for and confirm the presence of illegal veterinary drug residues in shrimp. Commercially available frozen shrimp samples were tested for chloramphenicol, fluoroquinolones, malachite green, and nitrofurans, which are drugs that have high enforcement priority in the United States due to their adverse health affects. The methods used to confirm the presence of residues were procedures preferred by FDA laboratories for the detection of drug and chemical residues in food.

In this thesis, we describe a series of experiments to screen for the four aforementioned veterinary drugs using ELISA and confirm positive results using LC-MS/MS. A review of related literature, description of methods used, results, and a discussion of the results follow.

1.2 Shrimp Aquaculture

Aquaculture is the farming of aquatic organisms, including both plants and animals, with the implication of some form of intervention in the rearing process, such as regular stocking, feeding, or protection from predators. The primary purpose of aquaculture is for food production, but it is also used for recreation, stock restoration, and biofuel production. Information about the early history of aquaculture is unclear; however, there is evidence of commercial fish farming in Egypt as early as 2500 BC and detailed records of aquaculture in China from 1100 BC [1]. Early aquaculture production was characterized by low stocking densities and utilized minimal inputs in the form of land, water, feed, fertilizers, and energy.

Interest in the culture of shrimp was prompted by an increased market demand and the inability of capture fisheries to meet that demand [2]. The shrimp farming industry experienced rapid growth and diversification in the 1980s [1]with market expansion occurring in economically advanced countries [2]. The export market and opportunity to earn foreign exchange attracted support from individual governments and international assistance agencies and investment by private industry [2]. Developing countries were provided financial assistance from the World Bank system, beginning as capital investment and expanding to include extension, research, training, and technology development, with the primary recipients being China and India [1].

The rapid growth in population during the 20th century contributed to an increased demand for seafood. As capture fisheries reached their maximum sustainable limits at 90 million metric tons per year, the aquaculture industry has grown at an accelerated rate to become a major contributor to the world fish supply [3]. Over the last five decades, the world fish food supply has grown dramatically due to steady growth in fish production and improved distribution channels. Between 1980 and 2010, world food fish production by aquaculture grew by nearly 12 times. In 2011, annual global aquaculture production accounted for 41% of total world fisheries production by weight[4]. In 2012, more than 91% of the total supply of edible fishery products in the United States was from imports, and shrimp imports, valued at $4.5 billion, accounted for 27 percent of the value of total edible imports [5].

Protein-energy malnutrition is a leading contributor to the global burden of disease [6]. Almost 20% of the world population's consumption of animal protein intake is from finfish and shellfish [4]. Seafood production from aquaculture provides an essential

source of protein for the growing human population[3], thus making aquaculture an important animal food-producing sector and important cash crop in both developed and developing countries[7]. In 2012, the major exporters of shrimp to the US (by volume) were Thailand, Ecuador, Indonesia, India, Viet Nam, and China [5]. The contribution of aquaculture to the world's production of seafood is expected to increase. Aquaculture is a viable option in developing nations because it offers opportunities to alleviate poverty by increasing employment and community development and reduces the overexploitation of natural resources [8]. Seafood is a more efficient protein source compared to other major commercial species; the feed conversion ratio (FCR) for fish is lower, meaning that fish requires less feed mass input to produce the same amount of body mass output. Fish do not expend energy to maintain body temperature, they use less energy to maintain their position, and lose less energy in protein catabolism and excretion of nitrogen.

In shrimp culture there are differences among various species with respect to environmental requirements, feeding, behavior, and compatibility with other species [2]. Considerations to take into account include water salinity (10-40 ppt), temperature tolerance (18-33 °C), the character of soil in the culture facilities, feed quality, and response to high-density culture [2]. The compatibility of different penaeid species in polyculture is highly dependent upon these factors, but rotational production of different species can be done according to seasonal changes of salinity and temperature [2]. The most common species of aquacultured shrimp are the white leg (*Penaeus vannamei*) and black tiger (*Peneaus monodon*) species. *P. vannamei* originate from the eastern Pacific Ocean, from Sonora, Mexico to Peru, and are ideal for farming because of their

ability to grow in very shallow water [9]. These shrimp grow up to 230 mm in length and have a maximum carapace length of 90 mm. *P. vannamei* is a highly euryhaline species that can tolerate salinities ranging from 0-50 ppt and temperatures from 22-32 °C [2]. *P. monodon* are native to the western Indo-Pacific, from southeast Africa to Pakistan and Japan [9]. The maximum length of these shrimp is 336 mm and they weigh from 60 to 130 grams [9]. *P. monodon* is euryhaline and can withstand almost fresh-water conditions, although 10-25 ppt is considered optimum, and their temperature tolerance ranges from 12-37.5 °C [2].

Shrimp culture is mainly carried out using traditional pond systems. While traditional systems utilized natural stocking through the intake of tidal water carrying large numbers of shrimp larvae, hatchery units and nursery ponds are now used to grow larvae to an advanced juvenile stage before transfer to production ponds. Although earthen ponds are the predominate system in shrimp aquaculture, farms with semi-intensive culture systems often have nurseries and rearing ponds with concrete dikes [2]. Recirculating aquaculture tank production systems are generally used for intensive shrimp and prawn culture, where water is continually exchanged and recycled to maintain dissolved oxygen levels and remove metabolic waste products. Biological filtration using nitrifying bacteria and solids removal are important components of recirculating systems. Certain species can be produced using raceway systems, in which water is exchanged multiple times daily [2].

1.3 Veterinary Drug use in Shrimp Aquaculture

Worldwide, aquaculture systems continue to increase in number and intensity in response to the rising demand for aquaculture products. [3]. The tremendous increase in

aquaculture production has been accompanied by potentially detrimental health effects in human and animals associated with the dissemination of considerable quantities of veterinary drugs into the environment [10]. As has occurred in other types of animal husbandry, the expansion and intensification of commercial aquaculture has increased stressors under which fish are being raised, resulted in the prevalence of pathogens in both culture systems and the natural aquatic environment, and made imperative the use of veterinary medicines to maintain healthy stocks, prevent and treat disease outbreaks, and maximize yield [8,11]. The intensification of culture methods is accomplished through high stocking densities, the use of medicated feeds, and the heavy application of pesticides. [3]. The types of medication used to treat aquatic species include vaccines, antibiotics, antiparasitics, antifungal agents, and immunostimulants [12]. The use of these products, with the intent to improve health management and biosecurity within aquaculture, has made it possible to achieve great advances in aquaculture production capacity [8].

In developing countries, the use of a veterinary drugs is prevalent in intensive marine shrimp farming to achieve sustainable production. Important issues that effect drug use in the aquaculture industry include the integrity of the environment, the safety of target animals and humans who consume them, and the safety of persons who administer the compounds. There are three primary ways in which antibiotics are used in aquaculture: 1) therapeutically, to treat existing disease, 2) prophylactically, at subtherapeutic concentrations, and 3) subtherapeutically, for production enhancement[13]. Antibiotics are typically administered in the water, often as components of fish feed, and are occasionally injected [13].

1.4 Impact on the Environment and Human Health

Using large amounts of a variety of antibiotics, including non-biodegradable antibiotics and those that are important for use in human medicine, ensures that they remain in the aquatic environment and exert selective pressure for long periods of time[10]. Veterinary drugs are deposited in the environment in the form of uneaten food and fish waste. Thus, they can penetrate into the sediment, be carried by currents to be dispersed over a wide area, and be ingested by wild fish and shellfish [10]. Veterinary drug use in aquaculture can result in a reduction in mortality during disease events and an overall better survival rate [14]; however, it is important to consider the potential negative impacts, including environmental degradation, the development of antimicrobial resistance among bacterial pathogens, and toxicological effects on non-target organisms.

1.4.1 Environmental Impacts

The benefits of shrimp aquaculture are numerous, but adequate environmental safeguards must be in place to prevent environmental degradation. The main environmental effects of marine aquaculture are caused by the introduction of invasive species that threaten biodiversity, organic pollution and eutrophication, chemical pollution, and habitat modification [15]. The presence of unconsumed fish feed and metabolic waste increases the input of nitrogen, carbon, and phosphorous into the aquaculture environment and results in eutrophication [10]. Furthermore, aquaculture environments and the fish and shellfish harvested from them can have elevated levels of antibiotic residues, antibiotic-resistant bacteria, and organic pollutants compared to their wild counterparts [3].

The existence of large amounts of antibiotics in the water and sediment can affect the flora and plankton in culture systems, causing shifts in the diversity of the microbial communities and affecting the structure and activity of microbiota [16]. Several groups of veterinary drugs are known to be of environmental concern because of their historical, measurable impacts on the environment [17]. The heavy use of antibiotics inhibits the microbiota at the base trophic level in the water and sediment from performing important metabolic functions, promoting algal blooms and anoxic conditions that could potentially lead to impacts on fish and human health [10].

1.4.2 Antimicrobial Resistance

Antibiotics are important for human therapy as well as disease management in aquaculture, but their prudent and responsible use is essential because of their ability to pollute the environment and challenge microbial populations. The widespread use of antimicrobial agents in shrimp culture has led to accumulation of residues in the water and sediment and the emergence of antimicrobial resistance in in environmental bacteria [18]. It has also resulted in an increase of antimicrobial resistance in shrimp pathogens and the transfer of resistance determinants to terrestrial bacteria and human pathogens [19,20]. Antimicrobial resistance is a major public health concern and is widely recognized as a priority issue for the aquaculture industry. Antibiotic residues and resistant bacteria, including resistant strains of Vibrio and Bacillus species, have been detected in Vietnamese shrimp ponds [19]. The extent of antimicrobial resistance resulting from antimicrobial use in aquaculture is yet to be determined [21].

There are two types of hazards associated with antimicrobial resistance, as identified by the 1996 Joint FAO/OIE/WHO Expert Consultation on Antimicrobial Use in Aquaculture and Antimicrobial Resistance: the development of acquired resistance in aquatic bacteria that can infect humans, and the development of acquired resistance in bacteria in aquatic environments whereby such resistant bacteria can act as a reservoir of resistant genes that can be further disseminated and ultimately end up in human pathogens [22]. The human health consequences of antimicrobial resistance in bacteria include an increased frequency of treatment failures and an increased severity of infection, which can lead to longer illness duration, increased frequency of bloodstream infections, and higher mortality [22]. High-risk populations include individuals working in aquaculture facilities, populations living around aquaculture facilities, and consumers who regularly eat aquaculture products [3]. Although there are no documented cases of human infections from antimicrobial resistant bacteria from aquaculture products [14], there is a need for better information about the potential for human exposure to contaminants and human health risks [3].

1.4.3 Residues of Food Safety Concern

In addition to posing environmental problems and creating antimicrobial resistance, the use of veterinary drugs or their residues in commercialized shrimp products can cause serious toxicity. Acute and chronic toxicities have been evaluated and are well documented in literature [13]. In most cases, the amount of drug residues ingested by an individual who consumes contaminated animal tissues will be considerably less than that consumed as a primary drug [23]. The lack of documented cases of direct toxicity from antibiotics and their metabolites in animal tissue indicates

that the probability of occurrence is extremely low [24,25]. There is exception in chloramphenicol, a drug that causes dose-independent aplastic anemia [13].

The United States Food and Drug Administration's Center for Veterinary Medicine (CVM) is responsible for setting enforcement priorities for drug use in shellfish for human consumption. Enforcement priorities are based on the safety status of the compound, user safety, environmental safety, and the extent of data available for enforcement priority determination. Known or suspected carcinogens and known serious toxicological hazards are high priority compounds. Examples of drugs with high enforcement priority include chloramphenicol, nitrofurans, fluoroquinolones and quinolones, malachite green, and steroid hormones [26].

1.4.4 Chloramphenicol

Chloramphenicol

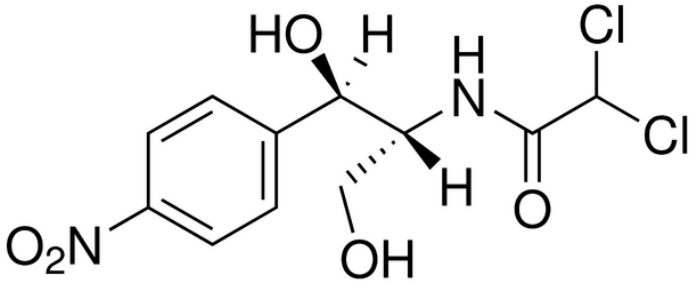


Figure 1.1: Chemical structure of chloramphenicol

Introduced in 1949, chloramphenicol was the first broad-spectrum antibiotic [27]. It was isolated from *Streptomyces venezuelae* in soil from Venezuela and was widely used because of its high efficacy against a wide range of organisms, low cost, and ease of synthesis and administration [28]. In the early 1950s, serious toxicities related to chloramphenicol administration were reported in adults and children, and its use began to decline. Two types of chloramphenicol toxicity are potentially fatal: idiosyncratic

aplastic anemia and dose-dependent gray baby syndrome. The most common toxicity is reversible, dose-dependent bone marrow suppression which occurs due to inhibition of mitochondrial membranous protein synthesis and results in immune system impairment[29]. Grey baby syndrome is a potentially fatal disease that can occur in children as well as adults and is characterized by abdominal distension, vomiting, metabolic acidosis, progressive pallid cyanosis, irregular respiration, hypothermia, hypotension, and vasomotor collapse [28]. Using the recommended reduced dosage of chloramphenicol for infants and neonates can prevent gray baby syndrome [27]. The development of aplastic anemia after oral administration of chloramphenicol occurs in genetically predisposed individuals and is well-established, but must be taken into perspective; while fatal aplastic anemia is estimated to occur in one of 24,500 – 40,800 cases [30], fatal anaphylaxis occurs in one of 67,000 patients treated with penicillin.

Chloramphenicol is widely used in veterinary medicine for both food and companion animals because of its activity against the main veterinary pathogens. While it has never been approved for use in food-producing animals in the United States, it is used extensively in other countries to treat bacterial infections [28].

1.4.5 Fluoroquinolones

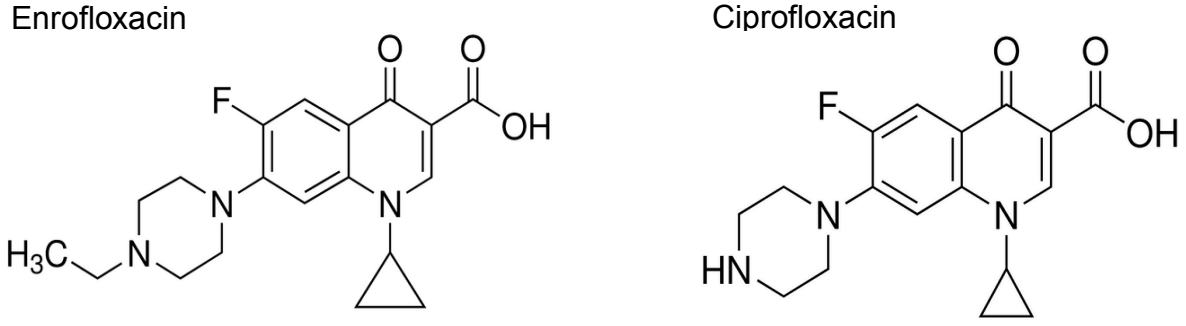


Figure 1.2: Chemical structures of enrofloxacin and primary metabolite ciprofloxacin

Fluoroquinolones are broad-spectrum antibiotics that are used to treat bacterial diseases in aquaculture and have been associated with multiple, severe toxicities, including hemolysis, renal failure, thrombocytopenia, and cardiac arrhythmia [31]. The most commonly observed adverse affects during therapy with fluoroquinolones are reactions of the gastrointestinal tract and central nervous system. The development of quinolone drugs began with the non-fluorinated drug nalidixic acid in the early 1960s and continued in the 1980s with the first 6-fluorinated derivatives, which have enhanced activity against Gram-negative bacteria[31].

1.4.6 Malachite Green

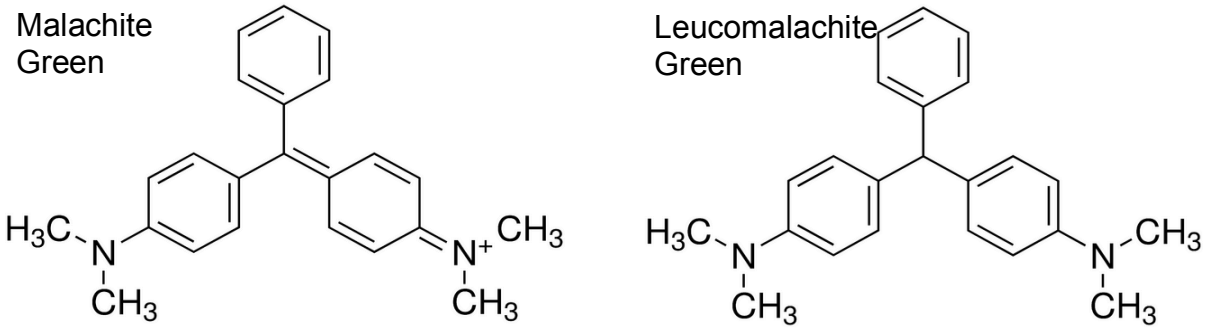


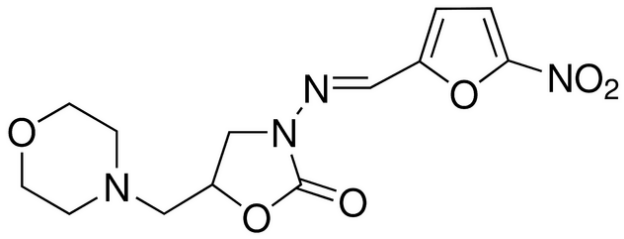
Figure 1.3: Chemical structures of malachite green and primary metabolite leucomalachite green

Malachite Green is most commonly known for its use in the dye industry and as a therapeutic agent for fish [32]. It has been widely used all over the world in the fish farming industry as a fungicide, ectoparasiticide, and disinfectant. However, it is highly cytotoxic to mammalian cells, with the ability to induce cell transformation and lipid peroxidation, thereby acting as a liver tumor enhancing agent [33]. Human exposure to malachite green occurs most notably through its use as an antifungal agent in aquaculture systems. Malachite green is metabolized to leucomalachite green upon

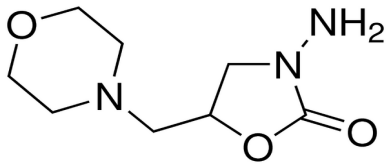
absorption into the tissue, thus the method of analysis must be capable of determining both compounds to detect its presence in seafood products [32].

The discovery of toxic health effects of malachite green led to the prohibition of its use in food production by the US FDA, but because it is effective, readily available, and relatively inexpensive, it has been used in aquaculture since the early 1930s and is considered the most effective antifungal agent by many in the fish industry [34]. Therefore, consumers of farmed fish and workers in the aquaculture industry are at risk for exposure to malachite green [32]. Malachite green is structurally similar to other triphenylmethane dyes, such as gentian violet, which are known to be carcinogenic [35]. Studies indicate that malachite green causes reproductive abnormalities in rabbits and fish [36] and enhances the formation of hepatic tumors in rats [8]. Concern over exposure to malachite green is attributable to studies suggesting it may cause adverse effects, but there is inadequate evidence to evaluate carcinogenicity or determine the risk of exposure to the dye [32].

1.4.7 Nitrofurans



Furaltadone



AMOZ

Figure 1.4: Chemical structures of furaltadone and primary metabolite 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ)

Nitrofurans are a group of synthetic, broad-spectrum antibiotics characterized by the 5-nitrofuran ring in their structure and by their effectiveness against bacteria. The

most widely used nitrofurans are furazolidone, furaltadone, nitrofurazone, and nitrofurantoin. In the tissue, these compounds metabolize to 3-amino-2-oxazolidinone (AOZ), 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ), semicarbazide (SEM), and 1-aminohydantoin (AHD). Detection of nitrofurans is based on the determination of the metabolites. Due to concerns about potential carcinogenicity of the drug residues and their potential to cause harmful effects on human health, the FDA banned their use in food-producing animals in 2002, but residues continue to be found in imported shrimp because of their ready availability for veterinary therapy. Animal studies have resulted in tumor production from dietary exposure to nitrofurans [37].

In the United States and Europe, any confirmed concentration of nitrofuran residues in edible animal tissues is prohibited, although the European Commission has established a maximum residue performance limit (MRPL) of 1μg/kg for nitrofuran metabolites [37]. Nitrofuran antibiotics are cheap and effective for the promotion of growth and prevention of disease and are therefore still used in some countries. This necessitates sampling and monitoring procedures in order to ensure consumer safety[37]. Studies on the stability of nitrofuran metabolites have demonstrated that 67-100% of residues remain after storage and cooking by a variety of methods [38].

1.5 Alternatives to Antibiotics in Aquaculture Disease Management

The need to minimize the use of antibiotics in aquaculture disease management is widely recognized by the aquaculture industry and academia, and research has focused on methods to reduce the impact of disease in aquaculture [39]. The aquaculture industry has experienced major losses caused by disease outbreaks, relying on non-selective chemotherapeutic agents or antimicrobials that not only target the pathogen,

but also affect normal flora. Alternatives to antimicrobial agents for the management of disease in aquaculture systems include vaccination, immunostimulants, probiotics, bioremediators, bacteriophage therapy, and holistic approaches [14].

Good aquaculture practices (or best aquaculture practices) are essential for the reduction of stress and promotion of animal health. The establishment of these procedures can substantially reduce issues associated with disease and the need for chemotherapeutic intervention [40]. Vaccination has proven successful for bacterial disease prevention in salmonid aquaculture, but there are few vaccines available for shrimp because of the poorly developed immune systems of invertebrates [39]. Immunostimulants have been used to modulate immune responses in finfish and shellfish aquaculture and have the potential to improve resistance against a wide range of pathogens [39]. Probiotics such as *Bacillus*, *Vibrio*, and *Pseudomonas* spp. are widely used in shrimp aquaculture in order to enhance the populations of beneficial microorganisms, improve water and sediment quality, suppress pathogenic bacteria, stimulate the immune system, and improve digestion [41]. The use of bioremediators such as nitrifying and photosynthetic bacteria to manage the environment are promising alternatives for disease prevention and outbreak management [39]. Bacteriophage therapy is now being explored in the medical field for treatment of antibacterial-resistant pathogens and has potential application in aquaculture. Bacteriophages only lyse target bacteria, unlike antibiotics, and would not suppress the beneficial flora [39].

There are a number of alternative options available for health and disease management in aquaculture to reduce reliance upon antimicrobial agents [39]. Healthy seed supply, probiotic use, optimum water quality maintenance, and lower stocking

densities are suggested for the control of disease in shrimp farming [42]. The use of alternatives to antimicrobial agents in aquaculture is critical for the protection of human and animal health and experience in other sectors of animal husbandry has demonstrated that these changes can be made without detrimental financial effects [10].

1.6 Laws and Regulations for Veterinary Drug Use in Aquaculture

1.6.1 International Regulations

The issue of antimicrobial resistance among bacterial pathogens caused by the use of antimicrobial drugs in aquaculture has been deliberated for a considerable length of time, but improved laboratory methods for detecting drug residues around 2001 led to heightened concern associated with the use of drugs in aquaculture and disruptions of trade in aquaculture products [14]. The World Trade Organization's Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) sets the basic rules for food safety and gives countries the right to set their own standards to protect the health of their citizens, using whatever measures and inspection methods they determine to be appropriate for their consumers. The regulations must be based on available scientific evidence and should not be used to favor domestic industry [14].

At the international level, the Codex Alimentarius Commission (CAC) is responsible for providing advice on risk management concerning veterinary drug residues. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) provides independent scientific advice for risk assessment through the evaluation of available data [14]. The risk assessment process is used to establish acceptable daily intake (ADI) and maximum residue limits (MRLs) to protect the health of consumers and ensure fair trade of foods [7]. Veterinary drugs with toxic or carcinogenic potential, such as

chloramphenicol and nitrofurans, are not evaluated and, therefore, no ADI or MRL is established. These drugs are banned for use in food-producing animals in most countries [14]. Regulatory authorities generally adopt a zero tolerance approach for veterinary drugs without an established ADI/MRL, and the major veterinary drugs involved are chloramphenicol, nitrofuran metabolites and malachite green [14].

1.6.2 United States Regulations

The United States of America is a member of the Codex Alimentarius Commission. The United States Food and Drug Administration (FDA) has jurisdiction over veterinary drugs and farmed shrimp in the United States. The legal marketing and use of veterinary drugs in the US is determined by the Federal Food, Drug, and Cosmetic Act (FFDCA) and its amendments [7]. Approvals are for specific products and include evaluation of human food safety, target animal safety and effectiveness, environmental safety, and user safety [43]. The agency works with individual states to ensure the safety of seafood products, approve drugs and feed additives, monitor manufacturing, distribution, and use of fish drugs, provide technical assistance and training, and provide the necessary oversight required to ensure fish food products are safe, wholesome, and properly labeled [53].

The US FDA's Center for Veterinary Medicine (FDA-CVM) animal drugs and animal feed and therefore has a critical role in protecting human and animal health in the United States. The agency ensures the safety and effectiveness of animal drugs and is responsible for the safety of treated seafood products in the US. The CVM recognizes that antimicrobial resistance is an important public health issue and addresses potential risks associated with the use of antimicrobials in animals through

the new animal drug approval process, post-approval monitoring, and surveillance [7]. A new animal drug is considered to be unsafe and in violation of the law if its use does not conform to its FDA approved, conditionally approved, or indexed indications. The agency may deny approval if the proposed use of a drug fails to meet FDA's reasonable certainty of no harm standard [7].

The FDA prohibits the extra-label use of certain drugs or classes of drugs in food-producing animals that pose a risk to public health. The following drugs (both animal and human formulations), families of drugs, and substances are prohibited for extra-label uses in food-producing animals:

- Chloramphenicol
- Clenbuterol
- Diethylstillbestrol
- Ipronidazole
- Other nitroimidazoles
- Furazolidone, Nitrofurazone, other nitrofurans
- Sulfonamide drugs in lactating dairy cattle
- Fluoroquinolones
- Glycopeptides[7].

Typically, the FDA pulls samples randomly from various importers and tests them in their own laboratories. If positives are found, an import alert is issued against the foreign supplier and all products coming into the country from that supplier must have testing done by a private laboratory until multiple shipments pass the specifications for antibiotics.

CHAPTER 2. MATERIALS AND METHODS

2.1 Sample Procurement

Twenty-seven frozen samples of imported, farm-raised shrimp were obtained from five retail grocery stores in Baton Rouge, Louisiana. Samples included shrimp from Thailand, India, Indonesia, Vietnam, China, Ecuador, Vietnam-Thailand, and India-Indonesia. Fourteen frozen samples of domestic, wild-caught shrimp were obtained from six retail grocery stores in Baton Rouge, LA. Frozen samples were transported to the Department of Food Science at Louisiana State University Agricultural and Mechanical College in Baton Rouge, LA and were stored at -80°C until further processing.

2.2 ELISA Screening

ELISA test kits were purchased from Bioo Scientific Corporation (Austin, Texas) to screen for four aquaculture drug residues: MaxSignal Chloramphenicol ELISA Test Kit (1020-03A), MaxSignal Fluoroquinolone ELISA Test Kit (1024-01), MaxSignal Malachite Green/Leucomalachite Green Test Kit (1019-06A), and MaxSignal Furaltadone (AMOZ) ELISA Test Kit (1020-03A). The kit components were stored at 8°C, according to the manufacturer's instructions. Frozen shrimp samples were prepared and analyzed according to the protocols provided with the test kits. Shrimp samples were homogenized using a food processor (Kitchen Aid Contour Silver 7 Cup – Model #KFP0711CU). Optical densities of samples were measured using a Model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, CA). Standard curves were constructed by plotting the mean relative absorbance (%) obtained from each reference standard against its concentration in ng/mL on a logarithmic curve. The mean relative

absorbance values for each sample were used to determine the corresponding concentration of the tested drug in ppb.

2.2.1 Chloramphenicol

Three grams of the homogenized sample was weighed into 15 mL centrifuge tubes, mixed with 6 mL of ethyl acetate, then vortexed for 3 minutes at maximum speed. The sample was centrifuged for 5 minutes at 4,000 x g at room temperature and 4 mL of the ethyl acetate supernatant was transferred into a new 15 mL centrifuge tube. The sample was dried at 60°C using a centrifugal solvent evaporator. The dried residue was dissolved in 2 mL of n-hexane and 1 mL of Sample Extraction Buffer was added and mixed by vortexing at maximum speed for 2 minutes. The sample was centrifuged for 10 minutes at 4,000 x g at room temperature and the upper hexane layer was discarded. One hundred microliters of the lower aqueous layer was used per well for the assay (dilution factor = 5).

One hundred microliters of each CAP standard and each sample was added in duplicate into different wells. 50 µL of CAP-HRP conjugate was added and the plate was incubated for 1 hour at room temperature in the dark. The plate was washed 3 times with 250 µL of Wash Solution. One hundred microliters of TMB substrate was added and the plate was incubated for 20 minutes at room temperature. After incubation, 100 µL of Stop Buffer was added to stop the enzyme reaction and the plate was read immediately at 450 nm wavelength.

2.2.2 Fluoroquinolones

Fat was not removed from the sample, as per the manufacturer's instruction. One gram of the homogenized sample was weighed into 15 mL centrifuge. Four milliliters of

70% methanol was added and vortexed for 10 minutes at maximum speed. The sample was centrifuged for 5 minutes at 4,000 x g at room temperature and 0.5 mL of the supernatant was transferred to a new 15 mL centrifuge tube. 0.5 mL of Sample Extraction Buffer was added and mixed well. Fifty microliters was used for the assay (dilution factor = 10).

Fifty microliters of each enrofloxacin standard and each sample was added in duplicate into different wells. One hundred microliters of Antibody #1 was added and the plate was incubated for 30 minutes at room temperature. The plate was washed 3 times with 250 µL of Wash Solution. One hundred microliters of TMB substrate was added and the plate was incubated for 15 minutes at room temperature. After incubation, 100 µL of Stop Buffer was added to stop the enzyme reaction and the plate was read immediately following the addition of Stop Buffer on a microplate reader at 450 nm wavelength.

2.2.3 Malachite Green

Two grams of the homogenized sample was weighed into 15 mL centrifuge tubes. One milliliter of Sample Extraction Buffer A, 0.4 mL of Sample Extraction Buffer B and 6.0 mL of acetonitrile were added and vortexed for 4 minutes at maximum speed. The sample was centrifuged for 10 minutes at 4,000 x g and 2 mL of the upper organic layer was transferred to a new 15 mL centrifuge tube containing 300 mg of MG Clean Up Mix. The sample was vortexed for 1 minute at maximum speed and left at room temperature for 10 minutes then centrifuged for 10 minutes at 4,000 x g. One milliliter of the supernatant was transferred to a 2 mL microcentrifuge tube and dried. One hundred microliters of Oxidant Solution was added to the dried sample and vortexed vigorously

for 1 minute, centrifuged for 10 seconds, and left at room temperature for 15 minutes. Four hundred microliters of Sample Extraction Buffer C and 650 µL of n-hexane were added to the sample and vortexed vigorously for 1 minute. The sample was centrifuged at 4,000 x g for 5 minutes and the upper organic layer was discarded. Ninety microliters of the lower aqueous layer was used for the assay (dilution factor = 1.5).

Ninety microliters of each MG standard and each sample was added in duplicate into different wells. Thirty microliters of MG-Biotin Conjugate was added and the plate was incubated for 30 minutes at room temperature in the dark. The plate was washed 3 times with 250 µL of Wash Solution. One hundred microliters of TMB substrate was added and the plate was incubated for 15 minutes at room temperature. After incubation, 100 µL of Stop Buffer was added to stop the enzyme reaction and the plate was read immediately at 450 nm wavelength.

2.2.4 Nitrofurans

One gram of the homogenized sample was weighed into 15 mL centrifuge tubes. 0.5 mL of Sample Extraction Buffer, 3.5 mL of distilled water, 0.5 mL of 1 M hydrochloric acid (HCl), and 20 µL of 50 mM 2-Nitrobenzaldehyde were added and vortexed for 30 seconds at maximum speed. The sample were incubated for 3 hours at 50°C and vortexed for 5 seconds every hour during the incubation. Five milliliters of 0.1 M dipotassium phosphate (K_2HPO_4), 0.4 mL of sodium hydroxide (NaOH), and 6 mL of ethyl acetate were added and vortexed for 30 seconds. The sample was centrifuged at 4,000 x g for 10 minutes at room temperature and 3.0 mL of the ethyl acetate supernatant (corresponding to 0.5 g of the original sample) was transferred into a new 15 mL centrifuge tube. A centrifugal solvent evaporator was used to dry the sample at

60°C under reduced pressure. The dried residue was dissolved in 1 mL of n-hexane and 1 mL of Sample Extraction Buffer was added and vortexed for 2 minutes. The sample was centrifuged at 4,000 x g for 10 minutes at room temperature and 100 µL of the lower aqueous layer was used per well for the assay (dilution factor = 2).

One hundred microliters of each AMOZ standard and each sample was added in duplicate into different wells. Fifty microliters of AMOZ-HRP Conjugate was added and the plate was incubated for 30 minutes at room temperature. The plate was washed 3 times with 250 µL of Wash Solution. One hundred microliters of TMB substrate was added and the plate was incubated for 20 minutes at room temperature. After incubation, 100 µL of Stop Buffer was added to stop the enzyme reaction and the plate was read immediately at 450 nm wavelength.

2.3 Liquid Chromatographic–Mass Spectrometric Confirmation of Residues

Quantitation of chloramphenicol and fluoroquinolone residues was accomplished using a modification of AOAC Official Method 995.09 [44]. Malachite green was quantified using a modified method for the quantitative determination of triphenylmethane dyes in aquaculture products [45]. Standards, internal standards, and all other chemicals were of analytical or HPLC grade and purchased from Sigma Aldrich Co. (St. Louis, MO).

2.3.1 Chloramphenicol and Enrofloxacin Quantitation

Chloramphenicol and enrofloxacin were extracted from tissue with pH 4 buffer. Filtered extract was cleaned up on C18 solid-phase extraction column. Compounds were separated by liquid chromatography using a C8 column and measured using a triple quadrupole mass spectrometer. Results were corrected for recovery of each analyte for each analytical run.

Five grams ± 0.05 g of shrimp tissue homogenate was weighed into 50 mL polypropylene centrifuge tubes and fortified with 0.5 µg/mL analyte. Twenty milliliters McIlvaine buffer-EDTA solution was added to each sample and blended for 30 seconds with a homogenizer, rinsing the probe twice with 2 mL McIlvaine buffer-EDTA solution into each tube. Tubes were shaken for 10 minutes using a flat-bed shaker at high speed then centrifuged for 10 minutes at 2500 x g. The supernatant was transferred to a new centrifuge tube and the extraction was repeated twice using 10 mL McIlvaine buffer-EDTA solution. The combined supernatants were centrifuged for 20 minutes at 2500 x g. The extract was filtered through glass microfiber filter paper, grade GF/B, 5.5 cm using a Büchner funnel into 125 mL sidearm flask by applying gentle vacuum to sidearm. The centrifuge tube was rinsed twice with 2 mL McIlvaine buffer-EDTA solution and filtered into the sidearm flask. SPE cartridges (6 mL, 500 mg C18) were conditioned with 20 mL methanol followed by 20 mL H2O. The extract was applied to SPE cartridge with 75 mL reservoir attached; the sidearm flask was rinsed twice with 2 mL McIlvaine buffer-EDTA and washings were added to the reservoir. When test extract was loaded, the SPE cartridge was washed using 20 mL H2O from rinsing the sidearm flask. The cartridge was allowed to run dry following H2O wash and air was drawn through for at least 2 more minutes. Compounds were eluted from the cartridge into 10 mL volumetric flasks using 6 mL methanolic oxalic acid. Vacuum was increased to maximum at the end of elution to remove residual solvent from the cartridge. Eluate was diluted to 10 mL with H2O. The test solution was filtered through 13 mm, 0.45 µm filtration cartridges into LC vials for analysis.

Chloramphenicol and enrofloxacin concentrations were determined using a Waters Acquity ultra performance liquid chromatography (UPLC) system (Milford, MA) coupled to a XEVO triple quadrupole mass spectrometer. The columns used were a Phenomenex Kinetex C-18 column (Torrance, CA) with 2.6 µm particle size and dimensions of 2.1 millimeter (mm) X 50 mm and a Phenomenex C18 guard column (2.1 mm X 5 mm, µm particle size). The mobile phase consisted of water with 0.1% formic acid (mobile phase A for enrofloxacin, positive ion mode), water with 0.1% acetic acid (mobile phase A for chloramphenicol, negative ion mode), and methanol (mobile phase B). The initial percentage of A was 88%, which was reduced to 40% at 5 minutes, and ramped back up to 88% at 6.5 minutes, with 0.7 mL/min constant flow rate.

2.3.2 Malachite Green Quantitation

Leucomalachite green was isolated from the matrix by liquid-liquid extraction with acetonitrile. Determination was performed using LC-MS/MS with positive electrospray ionization. Deuterated internal standards were used to improve quantitation. The method has been validated according to the EU criteria of Commission Decision 2002/657/EC in accordance with the minimum required performance limit (MRPL) set at 2 $\mu g/kg^{-1}$ for the sum of MG and LMG.

Shrimp tissue homogenate was fortified with 2 µg/mL internal standard (D5-leucomalachite green). Five hundred microliters of hydroxylamine solution was added, the sample was mixed, then allowed to stand for 10 minutes in the dark. Eight milliliters of acetonitrile and 1 g (±0.1 g) of anhydrous magnesium sulfate were added and the tube was vortexed vigorously for 1 minute at maximum speed, then shaken for 10 minutes with a rotative stirrer at 100 x g. The tube was centrifuged at 2000 x g for 5

minutes at 4 °C. The supernatant was transferred to a new clean tube and evaporated to dryness at 50 °C under nitrogen. The dried residue was reconstituted in 800 µL of acetonitrile/ascorbic acid (100/1; v/v). The extract was transferred to an Eppendorf tube and centrifuged at 20,000 x g for 5 minutes then filtered through a 0.45 µm PVDF filter into an LC vial prior to LC-MS/MS analysis.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Detection and Confirmation of Drug Residues

ELISA kits were used to rapidly screen shrimp samples to determine which drug residues were present. Chloramphenicol, fluoroquinolones, malachite green, and nitrofurans were the drugs chosen for screening because of their ability to cause severe adverse effects in humans and their high enforcement priority status. The four veterinary drugs analyzed, the detection limits for all methods, and current FDA detection levels in parts per billion are listed in Table 3.1. ELISA (Enzyme Linked Immnosorbent Assay) has become a favorable option for portable and high throughput screening for drug residues in animal products because of its advantages over conventional methods, which can be extremely expensive and time-consuming. Commercial ELISA kits are simple, rapid, sensitive, cost-effective, and have shorter processing times. These advantages enable government agencies, seafood processors, and quality assurance organizations to detect drug residues in fish and shrimp matrices at the required sensitivities without complicated clean up steps.

Table 3.1. Veterinary drugs analyzed, detection limits of methods used (ppb), and current FDA detection levels (ppb)

Analyte	Detection Limit		Current FDA
	ELISA	LC-MS/MS	Detection Levels
Chloramphenicol	0.025	0.3	0.3
Fluoroquinolones	0.2	1	5
Malachite green	0.1	0.4	1
Nitrofurans	0.05	NT	1

LC-MS/MS = liquid chromatography coupled with tandem mass spectrometry detection; NT = not tested

Rapid assay kits from Bioo Scientific Corporation (Austin, Texas) were used to determine if drug residues were present in shrimp samples. The methods are based on competitive colorimetric ELISA assays. The drug antibody has been coated in the plate wells. During the analysis, sample is added along with an enzyme-conjugated antibody, tagged with a peroxidase enzyme, that targets the drug antibody coated on the plate wells. If the target is present in the sample, it will compete for the drug antibody, thereby preventing the enzyme-conjugated antibody from binding. The resulting color intensity, after addition of substrate, has an inverse relationship with the target concentration in the sample.

Of the 27 samples analyzed in this study, 25 were found to contain detectable levels of veterinary drug residues and 20 contained more than one detectable residue. Two of the four analytes monitored in this study were detected using ELISA. Fluoroquinolones and malachite green were detected and the results are presented in Table 3.2. Nitrofurans were not detected in any of the samples tested. The most frequently observed residue was malachite green, followed by fluoroquinolones. To confirm ELISA results, samples that tested positive for both of the drugs using ELISA (fluoroquinolones and malachite green) were sent to an external laboratory for analysis. Although no samples were positive for chloramphenicol, it was also analyzed using confirmatory testing because of its severe adverse health effects, notably its ability to cause fatal aplastic anemia, and because even the spiked samples analyzed using ELISA were negative, suggesting that the extraction procedure was unsuccessful.

Table 3.2. Concentration of veterinary drugs (ppb) in shrimp as detected by ELISA

Country of Origin	Fluoroquinolone Concentration	Malachite Green Concentration
Thailand	ND	2.776 ± 0.00
	ND	1.432 ± 0.02
	ND	1.438 ± 0.03
	1.312 ± 0.05	1.311 ± 0.00
	ND	3.031 ± 0.02
	1.798 ± 0.07	3.757 ± 0.00
	1.262 ± 0.07	1.567 ± 0.02
	1.735 ± 0.11	1.306 ± 0.01
	1.979 ± 0.10	1.800 ± 0.01
	1.316 ± 0.01	0.936 ± 0.01
	ND	3.151 ± 0.02
	4.174 ± 0.09	1.883 ± 0.02
	1.647 ± 0.10	1.144 ± 0.04
	2.361 ± 0.03	1.910 ± 0.00
	1.701 ± 0.12	1.986 ± 0.01
Indonesia	1.468 ± 0.09	2.077 ± 0.03
	1.636 ± 0.07	0.723 ± 0.04
	1.442 ± 0.06	0.676 ± 0.02
	ND	ND
	ND	ND
India	2.220 ± 0.02	1.037 ± 0.03
	1.536 ± 0.00	0.354 ± 0.63
Vietnam	1.792 ± 0.03	2.181 ± 0.01
China	1.594 ± 0.07	1.902 ± 0.00
Ecuador	2.174 ± 0.01	1.041 ± 0.02
Vietnam-Thailand	2.837 ± 0.11	1.111 ± 0.03
India-Indonesia	1.378 ± 0.08	1.151 ± 0.02
United States	ND	ND

Values are expressed as mean±SD

3.2.1 Chloramphenicol

The detection limit for the chloramphenicol ELISA was 0.025 ppb in fish and shrimp. The antibody is 100% cross-reactive with chloramphenicol and exhibits 76.8% cross-reactivity with the main metabolite chloramphenicol glucuronide (Table 3.3). No samples tested positive for chloramphenicol residues. The average variability in

absorbance between duplicate samples was 4.5%, where 25% was considered acceptable. This was determined as follows:

$$\frac{2(x-y)}{x+y} * 100$$

Table 3.3: Cross-reactivity profile for chloramphenicol ELISA

Analytes	Cross-reactivity (%)
Chloramphenicol	100
Chloramphenicol Glucuronide	100
Chloramphenicol base	90
Thiamphenicol	45
Tetracyclines	36
Gentamicin	31
Ampicillin	21
Florfenicol	10

Using LC-MS/MS, chloramphenicol residues were detected in three of the five samples that were tested. The concentrations ranged from 0.30 to 0.49 ppb. Samples 1, 2, and 4 had detectable levels of chloramphenicol, despite the negative ELISA results for those samples. The multiple reaction monitoring (MRM) extracted ion chromatogram (XIC) for chloramphenicol in samples 1, 2, and 4 are presented in figures 3.1, 3.2, and 3.3, respectively. Published reports have demonstrated that fish and shrimp purchased at the retail level may contain veterinary drug residues that are banned for use in food-producing animals in the United States [46]. An analysis of veterinary drug residues in fish in shrimp samples collected in Canada between 1993 and 2004 resulted in the detection of drug residues in nine of the 30 samples analyzed, with four containing more than one detectable residue [46]. A survey of animal products in China detected chloramphenicol residues in 23 out of 28 aquacultured fish samples in 2013 [47].

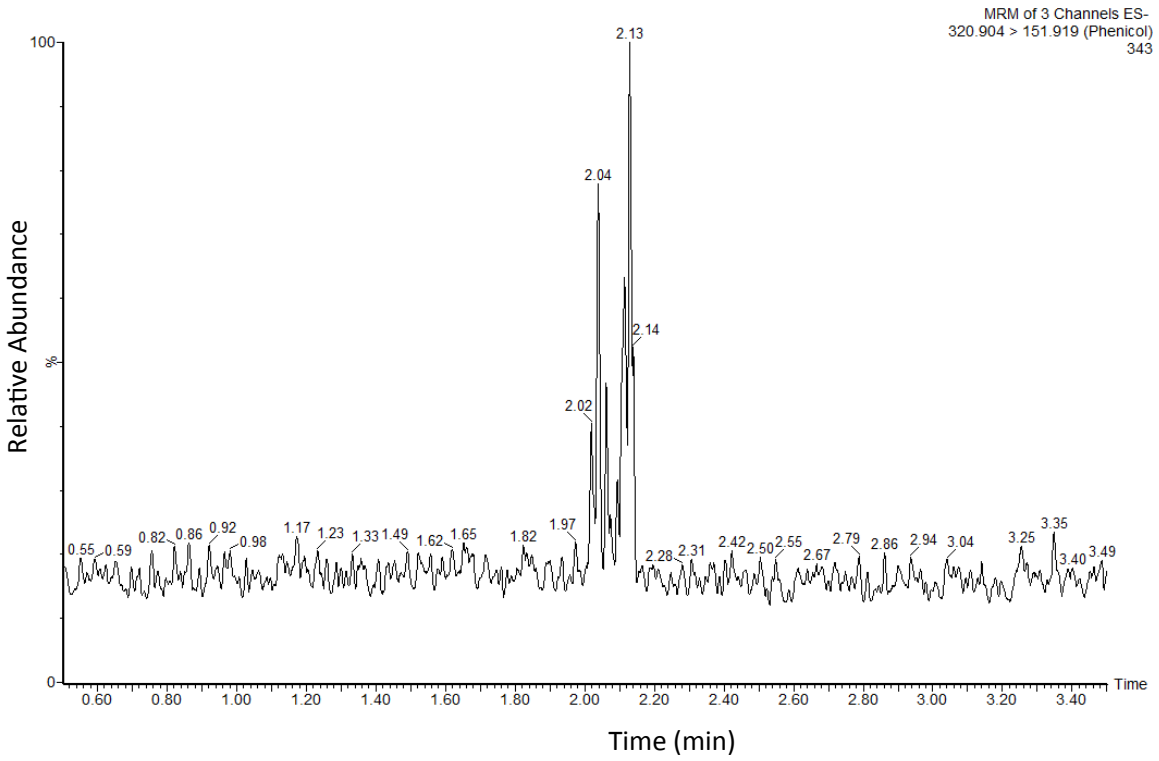


Figure 3.1: Extracted ion chromatogram for chloramphenicol in sample 1

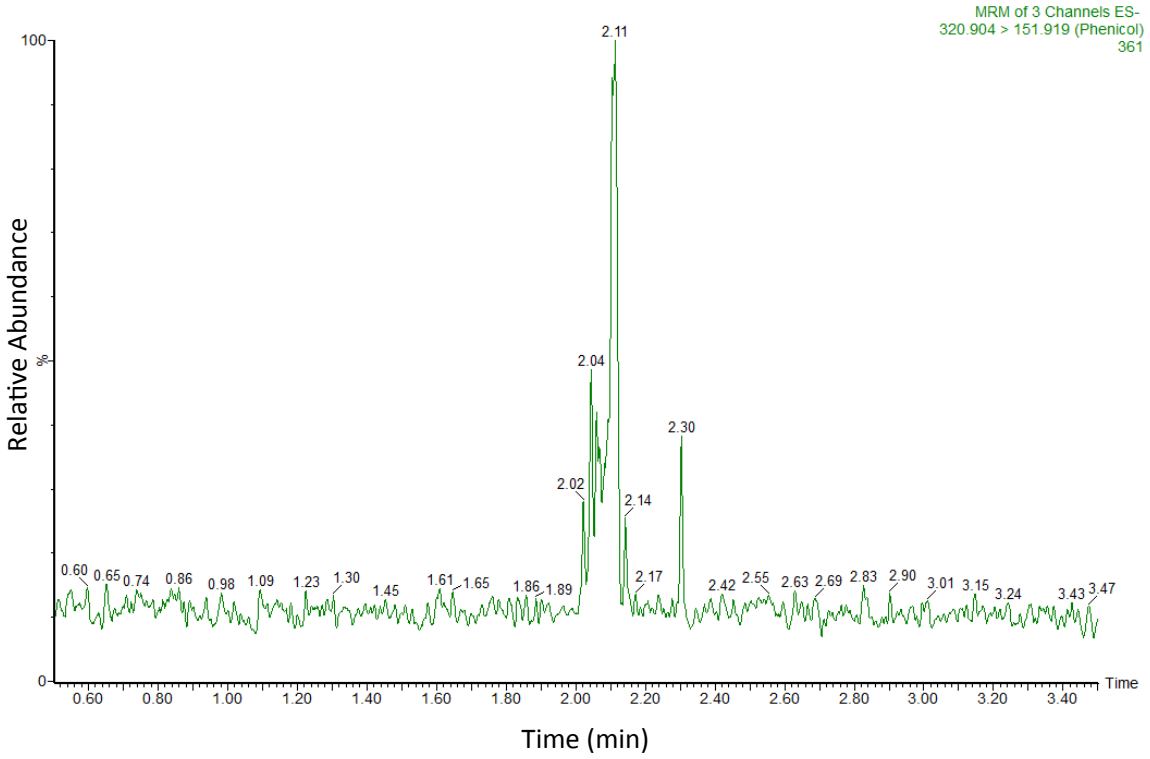


Figure 3.2: Extracted ion chromatogram for chloramphenicol in sample 2

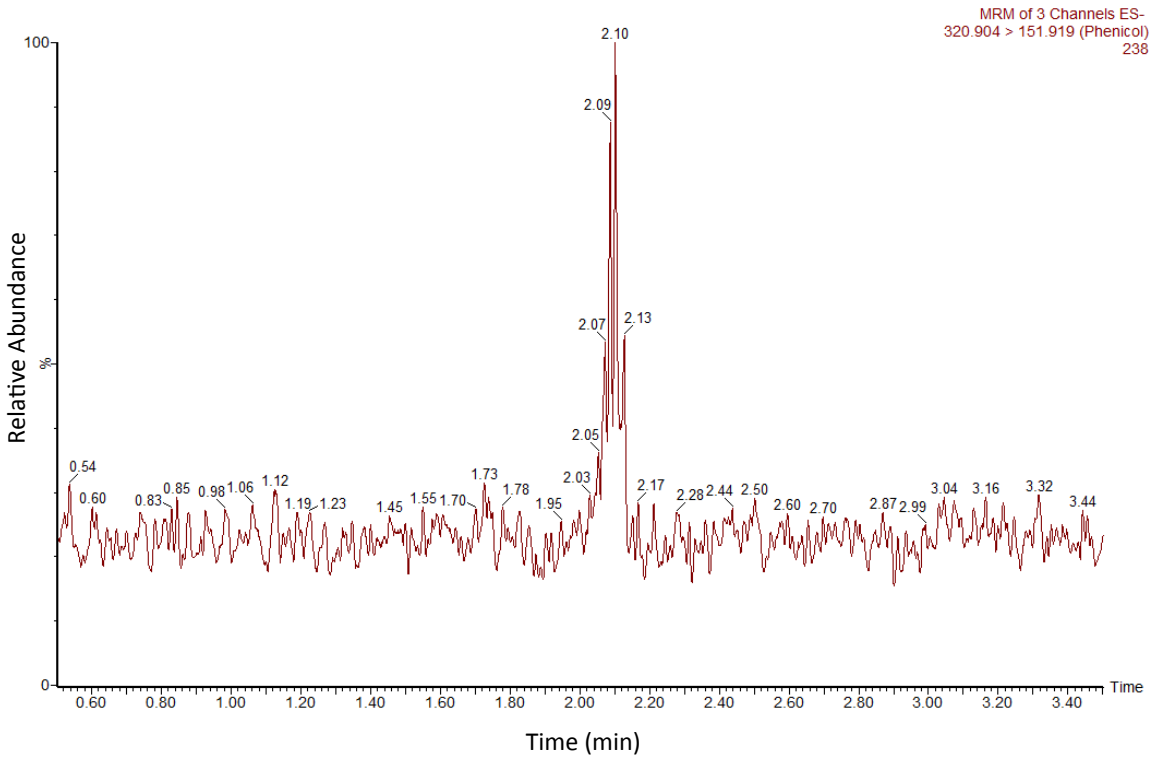


Figure 3.3: Extracted ion chromatogram for chloramphenicol in sample 4

3.2.2 Fluoroquinolones

The fluoroquinolone ELISA had a detection limit of 0.2 ppb in fish and shrimp and is 100% cross-reactive with both enrofloxacin and its primary metabolite ciprofloxacin. The antibody also displays substantial cross-reactivity with several other antimicrobial drugs (Table 3.4). Twenty samples tested positive for fluoroquinolone residues, with concentrations ranging from 1.31 to 4.17 ppb. The average variability in absorbance between duplicate samples was 5.03%.

Table 3.4: Cross-reactivity profile for fluoroquinolone ELISA

Analytes	Cross-reactivity (%)
Enrofloxacin	100
Ciprofloxain	100
Danofloxacin	90
Norfloxacin	45
Enofloxacin	36
Pipemidic acid	31
Ofloxacin	21
Benofloxacin	10
Flumequin	8
Oxolin acid	7

Using LC-MS/MS, enrofloxacin residues were confirmed in two of the five samples that were positive using ELISA. Mass spectrometric analysis of samples 2 and 3 detected 5.95 and 1.22 ppb enrofloxacin, respectively. The confirmed concentration for sample 2 was higher than the concentration detected in that sample using ELISA, and the confirmed concentration for sample 3 was lower than the ELISA concentration for that sample. The multiple reaction monitoring (MRM) extracted ion chromatogram (XIC) for enrofloxacin in samples 2 and 3 are presented in figures 3.4 and 3.5, respectively. In a study published in 2007, the fluoroquinolone enrofloxacin was detected in three out of 30 samples of farm-raised seafood in the United Kingdom in 1994 to 1995 [46].

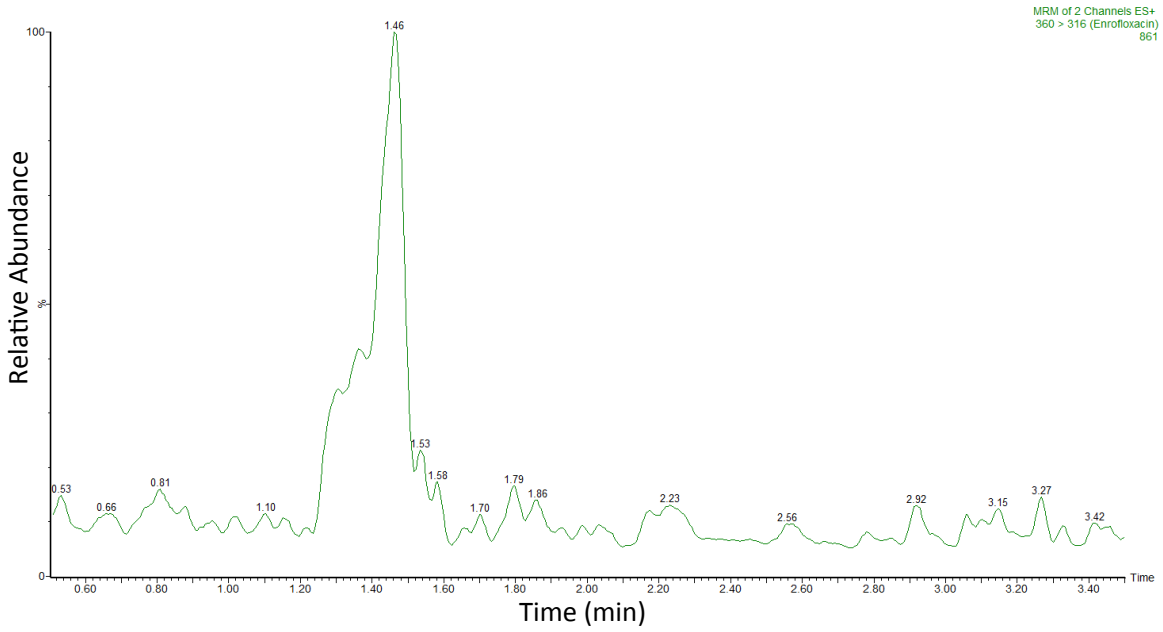


Figure 3.4: Extracted ion chromatogram for enrofloxacin in sample 2

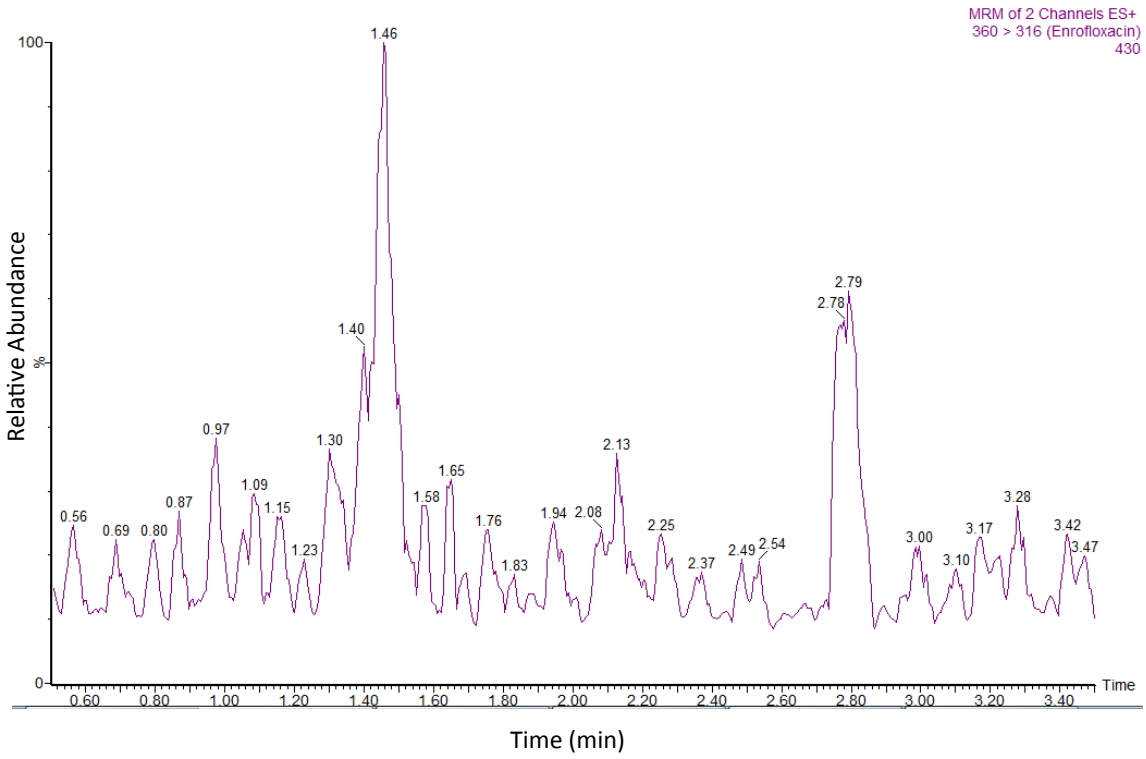


Figure 3.5: Extracted ion chromatogram for enrofloxacin in sample 3

3.2.3 Malachite Green

The detection limit for the malachite green ELISA was 0.1 ppb in shrimp. The antibody exhibits cross-reactivity of 100% with both malachite green and its primary metabolite, leucomalachite green. The antibody is also considerably cross-reactive with crystal violet, another triphenylmethane dye that is commonly used in aquaculture, at 42% (Table 3.5). Twenty-five samples tested positive for malachite green residues, with concentrations ranging from 0.35 to 3.76 ppb. The average variability in absorbance between duplicate samples was 14.6%. Five positive ELISA samples were analyzed for leucomalachite green using LC-MS/MS, and residues were not confirmed in any of the five samples.

Table 3.5: Cross-reactivity profile for malachite green ELISA

Analytes	**Cross-reactivity (%)**
Malachite Green	100
Leucomalachite Green	100
Crystal Violet	42
Leucocrystal Violet	<0.01
Diethylstilbestrol	<0.01
Sulfumethazine	<0.01
Chloramphenicol	<0.01
Furazolidone	<0.01

A study published in 2003 reported the malachite green metabolite leucomalachite green was detected in 13 out of 18 trout samples purchased from markets in the Netherlands at concentrations ranging from 1.3 to 14.9 ng g^{-1} [48]. Leucomalachite green was also detected in eight out of 12 trout samples purchased

from markets in the United Kingdom in 1994 to 1995 at concentrations ranging from <0.5 to 96 ng g^{-1} [49]

3.2.4 Nitrofurans

The detection limit for the nitrofuran ELISA was 0.05 ppb for fish and shrimp. The antibody exhibits 100% cross-reactivity with the primary metabolite of furaltadone, 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) and negligible cross-reactivity with other compounds. No samples tested positive for nitrofuran residues. The average variability in absorbance between duplicate samples was 2.27%. Although no nitrofuran residues were detected in this study, their presence in shrimp has been documented in literature. Nitrofuran metabolites were detected in Thai-originating shrimp sampled been 2000 and 2003 in the European Union [46,50]. A Swiss study published in 2003 detected nitrofuran residues in shrimp from Asian countries in 54 out of 157 samples at 0.2–150 ng g^{-1} [51].

The results from the ELISA screening and LC-MS/MS confirmation of drug residues in imported shrimp samples are presented in Table 3.6. Four of the five shrimp samples analyzed using mass spectrometry were found to contain detectable levels of veterinary drug residues, with one sample containing multiple residues. Chloramphenicol and fluoroquinolones were detected using confirmatory methods. The most frequently observed residue by LC-MS/MS detection was chloramphenicol, followed by fluoroquinolones. Malachite green was not present in any of the five samples, although it was detected using ELISA.

Table 3.6. Concentration of veterinary drugs (ppb) in shrimp as detected by ELISA and confirmed using LC-MS/MS

Sample	Analyte	ELISA	Confirmation
1	Chloramphenicol	ND	0.43
	Enrofloxacin	1.47	ND
	Leucomalachite green	2.08	ND
2	Chloramphenicol	ND	0.49
	Enrofloxacin	1.64	5.95
	Leucomalachite green	0.72	ND
3	Chloramphenicol	ND	ND
	Enrofloxacin	2.22	1.22
	Leucomalachite green	1.04	ND
4	Chloramphenicol	ND	0.30
	Enrofloxacin	1.79	ND
	Leucomalachite green	2.18	ND
5	Chloramphenicol	ND	ND
	Enrofloxacin	1.59	ND
	Leucomalachite green	1.90	ND

ND = none detected

The correlation between residue concentrations from ELISA screening and confirmatory testing is generally poor [52]; however, drug residue kits can be useful for qualitative screening of the compounds in shrimp tissue. The discrepancies between detected concentrations in ELISA and confirmatory methods are thought to be due to cross-reactivity with other compounds, matrix effects, differences in detection limits of the two methods, or contamination of ELISA samples.

Biological matrices are extremely complex, making the analysis of biological samples challenging. High levels of interfering factors can lead to unexpected ELISA results. Analysis is often hampered by the presence of endogenous compounds in the sample; the interferences are often present in higher concentrations than that of the

target analytes and thus may mask their presence. Sample preparation is an important aspect of bioanalytical estimation because biological samples are comprised of many components that can interfere with good separations and or good mass spectrometer signals.

In addition to matrix effects, the presence of interfering compounds from the same group as the analyte can affect the correlation between ELISA and chromatographic results. Enrofloxacin, the main target drug of the fluoroquinolone ELISA, was chosen for confirmatory testing. The fluoroquinolone ELISA was highly cross-reactive with several fluoroquinolones that are commonly used in aquaculture but were not analyzed chromatographically, thereby possibly contributing to the failure to detect enrofloxacin using LC-MS/MS. The malachite green ELISA was cross-reactive with gentian violet, another triphenylmethane dye that is toxic to humans and frequently used in aquaculture, but gentian violet was not analyzed chromatographically.

Contamination of ELISA samples was a possibility owing to the sensitivity of immunoassays. The failure to detect chloramphenicol using ELISA, even in spiked samples and despite having a good standard curve, suggests the extraction procedure or extraction buffers may have been inadequate. The results of this study emphasize why ELISA results have to be confirmed quantitatively.

CHAPTER 4. SUMMARY AND CONCLUSIONS

The objective of this research was to quantify veterinary drug residues in imported, farm-raised shrimp and make this information available to the general public in order evaluate the effectiveness of current imported seafood testing, thus contributing to the collective data on the topic. The use of unapproved veterinary drugs to treat shrimp in aquaculture systems results in the accumulation of drug residues in the edible tissues of the shrimp. These illegal drug residues can negatively impact human health, making routine testing imperative in order to protect consumers.

The results of this study confirm the presence of illegal veterinary drug residues in shrimp sold at the retail level in the United States. Ninety-two percent of imported, farm-raised shrimp samples tested positive for at least one drug that is banned for use in food-producing animals in the United States. Two of the four drugs considered in this study were detected using ELISA: fluoroquinolones and malachite green. The fluoroquinolone enrofloxacin was confirmed in two out of five samples using LC-MS/MS. Malachite green could not be detected using confirmatory methods. Chloramphenicol was not detected using ELISA, but was detected in three out of five samples using LC-MS/MS.

The residue concentrations detected in this study are within the ranges described in the few published reports regarding veterinary drug residues in fish and shrimp purchased at the retail level. Considering the above, ELISA can be a useful tool for the qualitative screening of shrimp muscle tissue for drug residues, but the results of this study suggest that more evaluation of commercial kits is necessary. Further testing is needed to investigate the safety of other imported, farm-raised aquaculture species and

to determine the extent of imported seafood contamination. Wild-caught shrimp from the United States tested negative for all veterinary drugs considered.

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