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# Determination of sulfite and antimicrobial residue in imported shrimp to the USA

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ABSTRACT

Incorrect usage of grow out and post-harvest products in shrimp aquaculture can lead to problems such as residue from veterinary drugs and melanosis prevention. These residues can be a serious concern for human health during handling and consumption of the shrimp. In an effort to determine if banned antimicrobial drugs were present in commercial shrimp, imported shrimp from India, Thailand, Indonesia, Vietnam, China, Bangladesh and Ecuador were purchased from retail stores in Baton Rouge, LA, USA and screened for the presence of veterinary drug residues (oxytetracycline, nitrofurantoin, chloramphenicol, fluoroquinolone and malachite green) using ELISA test kits. Additional screening with the Alert sulfite detection kit was used to determine if sulfite residue was over the legal limit of 100 ppm. Screening analysis revealed that samples were positive for nitrofurantoin (70 %), malachite green (5 %), oxytetracycline (7 %), and fluoroquinolone (17 %). No samples contained chloramphenicol residue. Using LC-MSMS validation, one sample tested positive for 60 ppm of oxytetracycline and 4 ppb of ciprofloxacin. Most samples tested positive for sulfite residue (43 %), but were within the US Food and Drug Administration (USFDA) limit (10−100 ppm). However, sulfites were not listed on any of labels of the 51 packages of imported shrimp. These drug residues and sulfites can have negative effects on human health. Results of this study confirm that veterinary drug residue is present in imported shrimp sold in the USA and all labeling rules are not followed.

## 1. Introduction

Shrimp is one of the world's most popular shellfish. High demand for shrimp leads to intensive farming, which can lead to bacterial disease problems. To prevent bacterial disease and promote growth, antimicrobial drugs are frequently used. Commonly used antimicrobials include cyclines (e.g. oxytetracycline, chlortetracycline,); quinolones (e.g. enrofloxacin, ciprofloxacin); chloramphenicol; malachite green; and nitrofurans (Roque et al., 2001; Soto-Rodríguez et al., 2006). The abuse of antimicrobial drugs creates several detrimental effects including the spread of the drugs to the environment, bacterial antibiotic resistance, and residue present in seafood (Binh et al., 2018). Some antimicrobials are considered harmful (Vass et al., 2008).

The first broad spectrum antibiotic was chloramphenicol (CAP) which was introduced in 1949 and isolated from *Streptomyces venezuelae* (Hanekamp and Bast, 2015). Chloramphenicol was widely used as veterinary drug as well as human antibiotic. CAP can be considered as carcinogenic when exposed to higher doses. The use of CAP is banned in the US, EU, Japan, China, Canada and Australia due to links to a fatal disease, aplastic anemia, and limited evidence of genetic carcinogenicity (Hanekamp and Bast, 2015).

Nitrofurans are a broad spectrum synthetic antimicrobial which include nitrofurantoin (NIT), furaltadon (FTD), furazolidone (FZD) and nitrofurazone (NFZ); all contain a 5 nitrofuran ring. Nitrofuran is used in aquaculture as a growth promoter and for prevention and treatment of bacterial and protozoan disease (Vass et al., 2008). Although nitrofuran has been banned for livestock and aquaculture use since 1995 by EU (European Commission, 1995), it is still used for human therapy. It was also used as a growth promoter in food-producing animals. However, both WHO and the European Union (EU) are unable to assign a maximum residue limit for NIT because of the potential carcinogenic effects of its residues on human health. Because of the rapid excretion of the NIT and their instability in vitro and in vivo, it is impossible to monitor residues of the parent drug nitrofurantoin directly. Instead, 1-aminohydantoin (AHD), the major metabolite of nitrofurantoin, which is stable in tissue, even after months of long-term storage, is

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selected to monitor. The detection limit is 1 ppb, so this is set at the compliance limit in food (Øye et al., 2019).

Malachite green (MG) has a diverse use as a dye but also feed additive, fungicide, parasiticide, bactericides, and antiprotozics (Srivastava et al., 2004; Bilandžić et al., 2012). Since 1933, it was used in aquaculture due to its effectiveness, low cost, and availability (Bilandžić et al., 2012). It is highly cytotoxic in bacterial and mammalian cell, acting as a liver tumor enhancer and responsible for reproductive abnormalities (Bilandžić et al., 2012). Therefore, the use of MG is not authorized.

Quinolones with fluorine atom are known as fluoroquinolones (FQ) and are banned because of the potential harms including cardiac arrhythmia, renal failure, hemolysis, and thrombocytopenia (Stahlmann, 2002). It was used for the treatment of bacterial disease in aquaculture. In the US no FQs are approved for use in shrimp aquaculture (U.S. Food and Drug Administration (USFDA, 2020a).

Oxytetracycline (OTC) is most widely used in aquaculture for treatment of bacterial diseases such as vibriosis and furnuculosis (Reed et al., 2004). However, it can also cause harmful effects, and histological studies indicate that liver damage might be caused by oxytetracycline. No OTC drugs are approved in the US for shrimp aquaculture (U.S. Food and Drug Administration (USFDA, 2020a).

The introduction of chemicals extends beyond the grow out. Melanosis, or black spot, is a quality defect in shrimp and other crustaceans characterized by the discoloration or darkening of the shrimp shell both in wild caught and cultured shrimp that affects marketability (Andrade et al., 2015; Gonçalves and de Oliveira, 2016). The cause of melanosis is polyphenol oxidase enzymes, an endogenous enzyme complex (Andrade et al., 2015) where tyrosinase is the main active enzyme. Melanosis gives an unappetizing appearance in shrimp, but is not harmful to human health (Gonçalves and de Oliveira, 2016). In addition to affecting aesthetics, melanosis also negatively affects the commercial value (Gómez-Guillén et al., 2005) and can result in a significant financial loss due to consumer rejection (Nirmal and Benjakul, 2009). Sodium sulfites or sodium meta-bisulfites are the most widely used inorganic chemicals effective for melanosis control in crustaceans (Nirmal and Benjakul, 2009). Sulfites have been used for decades, and while they are very effective in preventing melanosis, metabisulfite can trigger asthma attacks and allergic reactions (Collins-Williams, 1983). For hypersensitive asthmatics patients, small amount of sulfite can create life threatening conditions. Even contact with sulfites (i.e. during treatment of the shrimp) can be problematic by initiating severe problems such as breathing problem, cyanosis and sometimes death. Due to the potential hazard, the FDA requires that food products exposed to sulfites must include a statement about the presence of sulfites on their labels (U.S. Food and Drug Administration (USFDA, 2001). The label is mandatory if sulfite residue is more than the detectable limit (10 ppm) in shrimp (Rotllant et al., 2002). The FDA has established a regulatory maximum limit of 100 ppm for sulfite residue in shrimp (U.S. Food and Drug Administration (USFDA, 2001). The legal limit varies among countries: in Spain, the sulfite residue limit is 150 ppm, the same as the European regulation (Rotllant et al., 2002); in Australia the limit is 30 ppm (Diei, 1998). Sulfite residue exceeding acceptable limits can occur in shrimp flesh for multiple reasons including excessive sulfite concentrations, longer immersion times, or multiple treatments of sulfite to remove black spots as well as prevent it (Cintra et al., 1999).

As NIT, CAP, MG, FQ, and OTC have been banned in aquaculture in the US, no maximum residual limit (MRL) has set for these in the US (U.S. Food and Drug Administration (USFDA, 2020a); there is a zero tolerance for any residue. Unfortunately, these chemicals and sulfites are still used or overused. The use of antimicrobial drugs is not properly documented and regulated in many exporting countries. The US is a large importer of farmed shrimp, and 90 % of shrimp consumed in the US is imported (Food and Agricultural Organization (FAO, 2019). The top exporters to the US (in order of quantity) are India, Indonesia, Ecuador, Vietnam, China, and Thailand (Food and Agricultural Organization (FAO, 2019). The FDA inspects and rejects shrimp shipments with any trace of antimicrobials or sulfite levels over 100 ppm, but only about 2 percent of imported shrimp are tested by FDA because of budget constraints (Anders and Westra, 2011). Therefore, the objective of this work was to determine if imported shrimp available for sale in local markets in Baton Rouge, LA, US, contained any of these substances. Specific attention was focused on testing for 1) antimicrobials including nitrofuran (NIT), chloramphenicol (CAP), fluoroquinolones (FQ), oxytetracycline (OTC), and malachite green (MG) and 2) sulfite residue.

## 2. Materials and methods

### 2.1. Sample source

Farmed, imported shrimp samples were purchased from multiple locations of grocery stores and box stores with grocery departments in Baton Rouge, LA in winter 2016 and spring 2017 (n = 56 samples). As many different types of shrimp (brand, product type, size count, etc.) were purchased as were available. In instances when only one product type was available, different expiration dates were purchased to represent different lots of shrimp. Samples were not evenly distributed by country, as this was an artifact of what was available for purchase. Some shrimp products were processed in the US, but all originated from other countries (Table 1). Packages were checked for sulfite labels, either in the ingredients or listed in the allergen statement. None of the purchased packages indicated sulfite use and none were labeled as sulfite treated, though none proclaimed to be free of sulfite. After purchasing, shrimp samples were stored at −20 °C (Bermúdez-Almada et al., 1999). The experiment was performed in the Louisiana State University's School of Renewable Natural Resources. Due to availability, timing, and quantity restrictions, a total of 51 samples were screened for sulfite residue and 42 samples were screened for antimicrobial residue (Table 1). If screened positive, samples with adequate quantities were sent for verification testing (Table 2).

### 2.2. Screening for antimicrobial residue

#### 2.2.1. Sample preparation and extraction

Frozen samples were thawed at room temperature for 1 h. To prepare the samples the head and shell of the shrimp were removed and the meat was homogenized to obtain uniformity (Bermúdez-Almada et al., 1999). Individual shrimp from a sample were combined as necessary to create a homogenized sample of adequate weight. ELISA test kits (Bioo Scientific Max Signal® ELISA test kits: Oxytetracycline 1081−01D (OTC); Chloramphenicol 1 step 0.05 ppb 1013−02 (CAP); Nitrofurantoin (AHD) 1070−02 (NIT); FLU 1024−01 (FQ); and MG/LMG 1 step 1019−04A (MG)) were used for antimicrobial drug residue. For Malachite Green screening, this is the approved method and test kit by the US Food Safety and Inspection Service (U.S. Department of Agriculture (USDA, 2016). All test kits used competitive assays. All reagents and solvents were of analytical quality and were mixed to kit specifications provided by BIOO Scientific. Test kits were kept at 5 °C, per manufacturer directions.

For OTC, NIT, and FQ, 1 *g* samples were used and for CAP and MG, 3 *g* and 2 *g* samples were used, respectively. All extractions were done using the ELISA test kit method for shrimp for each of the five tests, individually. While all five extraction methods varied, all were vortexed (Miniroto S56 model Fisher Scientific) with sample buffer or appropriate reagent and centrifuged at 4000 rpm for 5−10 min at RT (Sorvall legend x1 r centrifuge Thermo Scientific). Supernatants were transferred to clean tubes and dried in a water bath with nitrogen gas per kit recommendations. When appropriate, other reagents were added. Samples were then vortexed and centrifuged again. Then, samples were dissolved in the sample extraction or balance buffer (provided with test kit). For each sample, 50−100 μl of supernatant, per test, was saved for the ELISA. Extraction were held at −20 °C until the plates could be run. Each

**Table 1**
Imported shrimp samples tested for antimicrobial and sulfite residue.

| Country of Origin | Sample | Product Type* | Processed in US | Sulfite Testing ** | Antimicrobial Testing** |
|---|---|---|---|---|---|
| Bangladesh | 1 | R | | | X |
| | 1 | C, P, D, T | | X | X |
| | 2 | C, P, D, T | Yes | X | X |
| China | 3 | R, T, S | | X | X |
| | 4 | R,T, S | | X | |
| | 5 | R,P, D, T | Yes | X | |
| Ecuador | 1 | R | | X | X |
| | 1 | R, EZ, T | | X | X |
| | 2 | R, P, D, T | | X | X |
| | 3 | R, S, EZ, T | | X | X |
| | 4 | R, P, D, T | | X | X |
| | 5 | R | | X | X |
| | 6 | R, P, D | | X | X |
| India | 7 | R, T, S | | X | X |
| | 8 | R, S, EZ, T | | X | X |
| | 9 | R, P, D, TO | | X | X |
| | 10 | R, P, D, TO | | X | X |
| | 11 | R, P, D, TO | | X | X |
| | 12 | R, P, D, TO | | X | X |
| | 13 | R, EZ, S, T | | X | |
| | 14 | R, P, D, T | Yes | X | |
| | 1 | R, S, EZ, T | | X | X |
| | 2 | R | | X | X |
| Indonesia | 3 | R | | X | X |
| | 4 | C, P, D, T | | X | X |
| | 5 | R, T, S | | X | X |
| | 6 | R,S, T | | X | |
| | 1 | R, EZ, T | | X | X |
| | 2 | R, EZ, T | | X | X |
| | 3 | R, S, EZ, T | Yes | X | X |
| | 4 | R, S, EZ, T | Yes | X | X |
| | 5 | R, S, EZ, T | | X | X |
| | 6 | R, P, D, TO | Yes | X | X |
| | 7 | R | | X | X |
| Thailand | 8 | R, T, S | | X | X |
| | 9 | R, P, D, TO | | X | X |
| | 10 | R, P, TO | | X | X |
| | 11 | R, T, S | | X | X |
| | 12 | R | | X | X |
| | 13 | R, P, TO | | X | X |
| | 14 | R, P, TO | | X | |
| | 15 | R, P, TO | | X | |
| | 16 | R, P, D, TO | | X | |
| | 1 | R, S, EZ, T | | X | X |
| | 2 | R, S, EZ, T | | X | X |
| | 3 | R, P, D, TO | | X | X |
| | 4 | C, P, D, T | | X | X |
| Vietnam | 5 | R, P, D, TO | | X | X |
| | 6 | R, P, D, TO | | X | X |
| | 7 | R, S, EZ, T | | | X |
| | 8 | R, EZ, S | | X | |
| | 9 | R, P, D, TO | | X | |
| | 10 | R, P, D, T | | X | |

* Product type codes: P = peeled; TO = tail off, T = tail on; S = shell on, D = Deveined, EZ = EZ peel, R = raw, C = cooked.
** X = sample was tested.

test kit and shrimp extraction method had a unique detection limit (DL) and dilution factor (DF): OTC DL = 1.5 ppb and DF = 10; CAP DL = 0.025 ppb and DF = 0.5; NIT DL = 0.05 ppb and DF = 2; FQ DL = 0.4 ppb and DF = 10; and MG DL = 0.08 ppb and DF = 1.5.

#### 2.2.2. ELISA

All samples and standards were run in duplicate. The 96 well plates provided were used for the ELISAs. All reagents, wash solutions, antibody solutions, and standards were mixed to kit specifications for each kit immediately prior to loading the plates. The plate was read per kit directions by 450 nm primary filter and/or 630 nm differential filter wavelengths (Bio-Tek Synergy HT Multi-Detection Microplate Reader, VT, USA). Standard curves were constructed using the Bio-Tek program by plotting mean relative absorbance (%) of the standard against the known concentration. Concentrations were measured using the formula provided for each test kit using the standard curve by the Bio-Tek program. Raw absorbance values were analyzed for outliers in the duplicate values. All 42 samples were run in 2017 with proper blanks. For any sample that tested positive for antimicrobial drugs residue, three or four shrimp replicates from the same sample were extracted and run in 2018. Additionally, a solvent control was run.

**Table 2**
Shrimp samples positive for at least one antimicrobial residue (2018 testing) (X). **X** were sent for further LC-MSMS analysis if sufficient sample amount was available.

| Sample ID | FQ | MG | NIT | CAP | OTC |
|---|---|---|---|---|---|
| Bangladesh 1 | | | X | | |
| China 3 | | | X | | **X** |
| Ecuador 1 | | | **X** | | |
| India 1 | **X** | | **X** | | |
| India 3 | | | X | | |
| India 5 | | | **X** | | |
| India 6 | | | X | | |
| India 7 | | | X | | |
| India 10 | | | X | | |
| India 11 | | | X | | |
| India 12 | | | X | | |
| Indonesia 2 | | | X | | |
| Indonesia 3 | | | X | | |
| Indonesia 4 | | | X | | |
| Indonesia 5 | | **X** | | | |
| Thailand 2 | X | | X | | |
| Thailand 3 | X | | X | | |
| Thailand 4 | **X** | | | | |
| Thailand 5 | | | X | | |
| Thailand 6 | | | X | | |
| Thailand 7 | | | X | | |
| Thailand 8 | | | **X** | | |
| Thailand 9 | | | | | **X** |
| Thailand 11 | | | X | | |
| Thailand 12 | X | | X | | **X** |
| Thailand 13 | | | X | | |
| Vietnam 1 | | | X | | |
| Vietnam 2 | | | X | | |
| Vietnam 3 | **X** | | X | | |
| Vietnam 4 | | | X | | |
| Vietnam 5 | **X** | | **X** | | |
| Vietnam 7 | | X | X | | |

### 2.3. Residue analysis

Of the samples that tested positive for one of the drug residues in 2018, 11 were delivered frozen to Eurofins Central Analytical Laboratories, New Orleans, LA (Eurofins). Not all samples that tested positive had enough shrimp left to meet the minimum amount required for testing. A total of 11 samples were sent with a minimum of 100 g per test per sample (Table 2). Eurofins is accredited A2LA ISO/IEC 17025:2005 2993−01. Established and approved FDA methods were used for all validation: FQ by FDA Laboratory Information Bulletin (LIB) 4298 (Turnipseed et al., 2006); NIT metabolites (liquid chromatography/-tandem mass spectrometry (LC-MSMS)) by FDA/ CFSAN (U.S. Food and Drug Administration (USFDA, 2004); OTC (LC-MSMS) by AOAC 995.09 (MacNeil et al., 1996); and MG (total, LC-MSMS) by FDA LIB 4395 (U.S. Department of Agriculture (USDA, 2016). For FQ, NIT, and MG, the testing limit was 1.0 ppb, and for OTC the testing limit was 10 ppb.

As seen in the screening, different shrimp within a package did not always have similar results, but the exact shrimp that was positive in 2018 was used up in that analysis. Other shrimp from the sample package had to be selected.

### 2.4. Screening for sulfite residue

Frozen shrimp was thawed at 4 °C for 1 h. before starting experiment. From each unique sample (n = 51), 10 replicate shrimp were randomly selected for testing. The head and shell of the shrimp were removed. To determine residue sulfite levels, the Alert Sulfite detection kit (Neogen Corporation #9500) was used. This method is correlated with Monier-Williams AOAC method (Horwitz, 2000). One drop of the activator solution was applied to the whiter thorax area next to the removed head of the shrimp. Next, one drop dye reagent was added to the moistened meat. After one minute, the color change was observed. If the blue dye did not change color, shrimp had sulfite levels below 10 ppm (detection limit). If the blue dye turned violet, shrimp were treated with sulfite, but the sulfite level did not exceed 100 ppm (0−100 ppm). If no color remained from the dye, sulfite level exceeded 100 ppm (>100 ppm). Positive control and negative control shrimp treated with known amounts of bisulfite were also tested to verify the color change with known levels of sulfite exposure. The three-color observations were assigned a score of 1−3, with 1: <10 ppm, 2: 10−100 ppm, and 3: >100 ppm. The scores were averaged for all 10 shrimp and results are reported in mean score ± SD. A sample was considered positive for sulfite if any of the 10 shrimp scored a 2 or 3.

## 3. Results

### 3.1. Antimicrobial residue

#### 3.1.1. Oxytetracycline (OTC)

The detection limit for the OTC ELISA was 1.5 ppb in shrimp, and cross reactivity of the antibody with OTC is 100 %. All negative controls tested negative, and all standards were positive (1.5 ppb–4.5 ppb). In 2017, five separate samples screened positive. Therefore, three to four separate shrimp replicates from the original five samples were rerun in 2018. Only 3 samples (7.1 %) screened positive for OTC residue in 2018 (Fig. 1); two of those came from Thailand (15.4 % of Thai samples) and one from China (33.3 % of Chinese samples). Sample Thailand 9 had three replicates tested again. One was below detection, and two were positive. Thailand 12 had four replicates tested in 2018, and all four were positive. China 3 had four replicates tested in 2018, and two were below the detection limit, but the other two were positive (Table 2). Only China 3 samples tested positive by LC-MSMS, and it was 60 ppb. However, the detection limit of the LC-MSMS was 10 ppb so the other two samples, Thailand 9 and 12, might have been below this level or the specific shrimp sent were negative.

#### 3.1.2. Chloramphenicol (CAP)

The detection limit for the CAP ELISA was 0.025 ppb in fish and shrimp. All negative controls tested negative, and all standards were positive (0.05 ppb–4.5 ppb). No shrimp samples were above the test range limit for CAP (0%) (Fig. 1 and Table 2).

#### 3.1.3. Nitrofurantoin (NIT)

The detection limit for the NIT ELISA was 0.05 ppb in fish and shrimp. Specificity or cross reactivity of the antibody with NIT is 100 %. All negative controls tested negative, and all standards were positive (0.5 ppb–6.4 ppb). Almost all samples tested positive in 2017, so all were reanalyzed in 2018 with a solvent control. In 2018 over 70 % of the shrimp samples were still positive for NIT residue over the detection limit with a residue range of 0.4–4.4 ppb (Table 2). In both years, the blank controls were always negative. Shrimp which had NIT residue were imported from Bangladesh (100 % of samples), China (33 %), Ecuador (100 %), India (67 %), Indonesia (60 %), Thailand (77 %), and Vietnam (86 %) (Fig. 1). However, none of the samples sent for further anaylsis tested positive in 2019. Unfortunately, not all samples could be sent for confirmation.

#### 3.1.4. Fluoroquinolone (FQ)

The detection limit for the FQ ELISA rapid method was 0.4 ppb in fish and shrimp. Cross reactivity of the antibody for enrofloxacin, ciprofloxacin, difloxacin, and sarafloxacin is 100 %. All negative controls tested negative, and all standards were positive (0.4 ppb–4.5 ppb). Fluoroquinolone residue was detected in 16.7 % of samples during screening (Table 2). Shrimp detected to have FQ residue were imported from India (8.3 % of samples), Thailand (30.8 % of samples), and

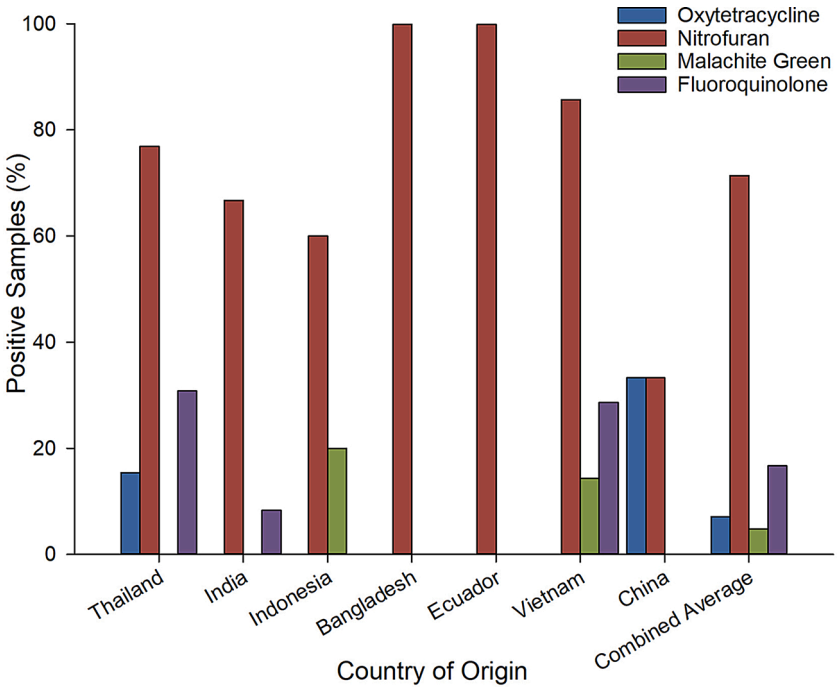


**Fig. 1.** Imported shrimp positive for antibiotic residue by country. All values are percent of positive samples (individual countries) or averaged percent positive (combined average). No chloramphenicol residue was detected.

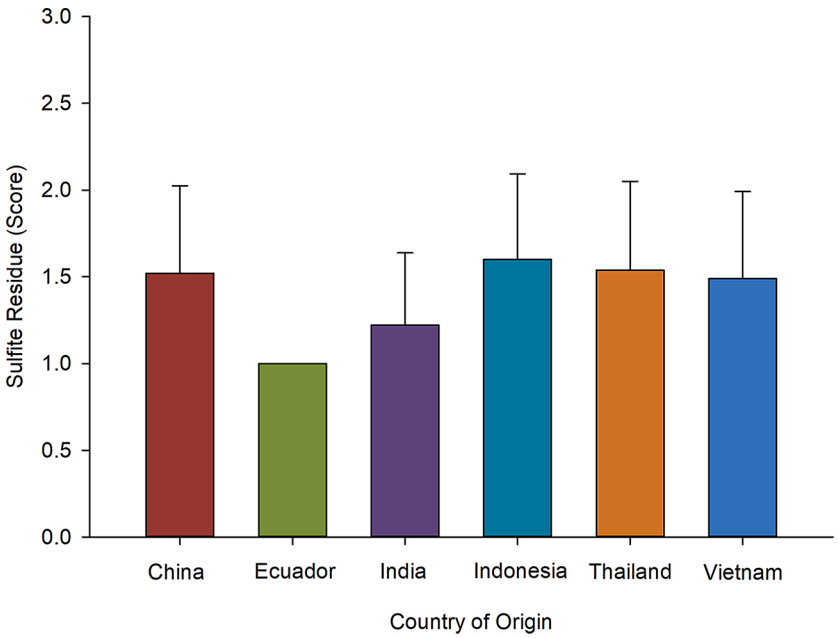


**Fig. 2.** Average sulfite residue in imported shrimp (Error bars = S.D.; sample n: China = 5, Ecuador = 1, India = 14, Indonesia = 6, Thailand = 16, and Vietnam = 9; 10 shrimp per sample).

Vietnam (28.6 % of samples) (Fig. 1). From the further analysis with LC-MSMS, Thailand 12 had 4 ppb of Ciprofloxacin.

#### 3.1.5. Malachite green (MG)

The detection limit for the malachite green ELISA was 0.08 ppb in fish and shrimp. Cross reactivity of the antibody with MG is 100 %. All negative controls tested negative, and all standards were positive (0.05 ppb–4.5 ppb). In 2017, 15 samples were above or near detection limits, so they were rerun in 2018, including a solvent control and using the high background extraction method. In 2018, MG residue was detected in 2 out of 42 sample (4.8 %) with residue ranges of 1.6 to > 4.5 ppb (Table 2). Only one of the two samples had enough for LC-MSMS analysis, and came back negative. However, that sample was near the threshold for LC-MSMS. The ELISA test is approved for screening by USDA (U.S. Department of Agriculture (USDA, 2016). Shrimp which had malachite green residue were imported from Vietnam and Indonesia.

### 3.2. Sulfite residue

In order to determine if sulfite abuse (>100 ppm residue level) could be occurring in shrimp imported into the United States and available for retail purchase, 51 samples from 6 different countries were tested (10 shrimp per sample for a total of 510 shrimp). All positive control shrimp treated with bisulfite to manufacturer recommendations scored a 2 (10−100 ppm; n = 30). All negative control shrimp known to be sulfite free scored a 1 (<10 ppm; n = 30). With the exception of Ecuador, each country had shrimp with a sulfite residue between 10−100 ppm. Ecuador had the lowest (1.0 ± 0.0) score and Indonesia had the highest sulfite residue score (1.6 ± 0.4). Of both the raw and cooked shrimp from China (5 samples, total = 50 shrimp), 52 % (26 out of 50) were positive for sulfite residue (score: 1.52 ± 0.5). Of these, 50 % of the shrimp were cooked. Of the shrimp from India, 22.14 % were positive for sulfite residue (score: 1.22 ± 0.4). All the samples from India were raw. One sample from each India, Thailand, and China were processed in the USA, and two of those (India and China) were below the detectable limit for sulfite. The Thai shrimp sample processed in the US tested positive for sulfite residue, but sulfite was not included on the label. Indonesian shrimp samples were both raw (n = 5) and cooked (n = 1), and 60 % tested positive for > 10 ppm sulfite. These shrimp had the highest average score (1.6 ± 0.4) (Fig. 2). Twenty-five percent of the shrimp from Indonesia that tested positive for sulfite were cooked. For shrimp from Thailand (all raw), 53 % were positive for sulfite residue >10 ppm (score: 1.53 ± 0.5). One shrimp in the Thailand samples tested positive for >100 ppm. However, additional shrimp replicates from that sample were run, and no other single shrimp tested positive for sulfite residue >100 ppm. Forty-nine percent of Vietnam shrimp samples (score: 1.48 ± 0.5) were positive for sulfite residue >10 ppm, which included raw and cooked samples. Overall, one shrimp contained more than 100 ppm sulfite residue, 43.5 % of shrimp contained 10−100 ppm, and 56.3 % of shrimp contained less than 10 ppm of sulfite residue.

## 4. Discussion

### 4.1. Antimicrobial residue

In this experiment, shrimp from 7 countries: Bangladesh, China, Ecuador, India, Indonesia, Thailand, and Vietnam were tested for five different antimicrobial residues and sulfite residue. Using every unique sample available for retail purchase in 2017, oxytetracycline, nitrofurantoin, fluoroquinolone and malachite green were all detected. These results are not surprising considering in 2017, 43 shrimp lines were rejected for banned antibiotics from Vietnam (12), India (12), China (11), Thailand (7) and Hong Kong (1) (USFDA, 2017). Shrimp have been rejected by FDA every year during the period between 2002–2020 due to the presence of antimicrobial drugs, with the highest rejections occurring in 2015, mostly from Vietnam, India, Thailand, China, Hong Kong, and Bangladesh (U.S. Food and Drug Administration (USFDA, 2020b). With only a small amount of seafood inspected, antimicrobial residue is still a major problem in imported shrimp.

OTC is a widely used antibiotic, and in this study only 3 samples tested positive for OTC, and those shrimp samples were from Thailand and China. In addition to testing positive for OTC, samples from Thailand also tested positive for NIT and FQ. In 2002 and 2003, the European Union detected nitrofuran metabolites in shrimp originating from Thailand (Tittlemier et al., 2007). Some shrimp were imported from India, Thailand, or China but processed in the United States (Table 1). Of these, several samples from Thailand still tested positive for FQ and/or NIT. In our study, shrimp from China were only positive for

NIT drugs, and these findings are similar with previous work. In the first quarter of 2018, 5 shipments were refused out of the 135 of total seafood entry lines where shrimp contained banned antibiotics; those five shipments were all from China (FDA, 2020b). The use of NIT, CAP, and OTC in Chinese aquaculture has also been reported (Liu et al., 2017).

Only one source of shrimp from Bangladesh was found in stores around Baton Rouge, LA. This shrimp sample was positive for NIT. In January and May 2018, Bangladesh shrimp were rejected due to presence of NIT (FDA, 2020b). For future work, it would be useful to try to find more lines of shrimp from Bangladesh.

In our research, NIT and FQ were also present in shrimp from Vietnam and India, though OTC and CAP residue was not found. Thuy and Loan (2011) reported the most commonly used antibiotics in shrimp farming in Vietnam are FQ, OTC, sulfonamides, and diaminopyramidines, and shrimp are regularly checked for antibiotic residue by the authorities in order to try to control antibiotic usage. In Vietnam, antibiotic residues were found in the surrounding environments of shrimp ponds including norfloxacin, oxolinic acid, sulfamethoxazole, and trimethoprim (Thuy and Loan, 2011). CAP was used in shrimp farming in northern Vietnam (Chi et al., 2017), although in the present study chloramphenicol was absent in shrimp originating from Vietnam. Previously fish and shrimp collected from different fish markets of Vietnam were positive for different types of antibiotic residue such as quinolones, sulfonamide, β-lactam, and triemethoprim (Uchida et al., 2016).

#### 4.1.1. Oxytetracycline (OTC)

There was wide variability even within a single sealed package of shrimp testing positive or negative for OTC. The variability within a shrimp sample is likely an indication of shrimp mixing from various aquaculture facilities along the supply chain. While no safe MLR is set in the US, the WHO has set it at 0.2 ppm (World Health Organization (WHO, 2002). So while the shrimp in our study (Screened: >1.5 ppb and LC-MSMS: 60 ppb) should be rejected at US customs, these shrimp would be acceptable under the WHO MRL. However, these shrimp could be a serious concern for consumers who are allergic or sensitive to OTC. These consumers would not expect to encounter OTC in shrimp.

OTC is the most commonly used antibiotic for the treatment of vibriosis and necrotizing hepatopancreatitis in shrimp farms (Wang et al., 2004; Nogueira-Lima et al., 2006). A previous study suggested that OTC residue is not detectable in muscle tissue of *Penaeus chinensis* after 96 h from the administration of OTC mixed feed when concentrations of OTC were 2000 mg kg feed (Wang et al., 2004). The presence of OTC residue in our study indicates that either improper doses of OTC might have been used or withdrawal time was not maintained before harvesting. In addition to overuse of OTC resulting in tissue residue, bacterial resistance to OTC has been reported in shrimp farms in the Philippines where isolated *Vibrio* were highly resistant to OTC (Tendencia and de la Peña, 2001). About 95 % of OTC is passed through the host organism to the surrounding environment (Serrano, 2005), and this could be another route for exposure if shrimp are not directly given OTC. Previous work in Vietnam found OTC residue in shrimp and fish collected from a domestic fish market where all shrimp samples (13 out of 13) and some fish samples (5 out of 15) were positive for tetracycline (Pham et al., 2015). Similarly, in Iran, both raw (100 %) and fried (44 %) rainbow trout were positive for OTC residue, indicating that withdrawal time was not adequately maintained and that frying cannot destroy OTC residue (Sharafati-Chaleshtori et al., 2013).

#### 4.1.2. CAP

While no CAP residue was found in our study, the use of chloramphenicol in shrimp farming in Asia has been reported (Gräslund and Bengtsson, 2001). CAP use is prohibited in many countries including the US, Canada, China, Japan, and Australia; no maximum residual limit is set for CAP (European Commission, 2009; Wongtavatchai et al., 2004). While samples in this study were negative for CAP, it has been found in shrimp samples imported into the USA, and companies from Brazil, China, Indonesia, Malaysia, Venezuela, and Vietnam are under an import alert and subject to detention without physical examination due to the presence of chloramphenicol in previous shipments (U.S. Food and Drug Administration (USFDA, 2017). The lack of detection could be due to adequate withdrawal time before harvesting or increased adherence to the regulations banning CAP.

#### 4.1.3. NIT

Our screening results results indicate that exporting countries are not adhering to the NIT ban. Other studies also found that while use of NIT is banned by the US and EU, shellfish farms in Asia and Latin America still use it (Conti et al., 2015). Vass et al. (2008) found the nitrofuran metabolites furazolidon (AOZ) and nitrofurazone (SEM) in *Penaeus monodon, Macrobrachium rosenbergii,* and *Penaeus vannamei,* with residue ranges of >1 ppb to 150 ppb. The 150 ppb was found in *Penaeus monodon* imported from India (Vass et al., 2008). Many consignments of shrimp and prawns from Bangladesh were rejected by the USFDA and European Commission because of the presence of nitrofuran drugs (Shamsuzzaman and Biswas, 2012). Shrimp shipments from China, India, United Arab Emirates, Malaysia, Canada, and Bangladesh were rejected several times even in 2018, due to presence of nitrofuran (U.S. Food and Drug Administration (USFDA, 2018).

None of our samples sent for further LC-MSMS anaylsis tested positive in 2019. Unfortunately, not all samples could be sent for confirmation as insufficient or no quantities remained. All samples were in storage over 3 years which could affect the metabolites, and previous work has only looked at stability over 100–300 days (Hurtaud-Pessel et al., 2006). Additionally, work has found certified labs to have a range with LC-MSMS of the same sample from 0.1 to 1 ppb (Hurtaud-Pessel et al., 2006). Many of our samples were 1−3 ppb in 2017. Recent work by Øye et al. (2019) had a false-positive for AHD, and they have new recommendations with washing of samples to prevent this. Additional work is needed to understand the level of NIT entering the US food chain through shrimp and to improve the accuracy of testing to ensure false positives do not lead to unnecessary rejections but any NIT contamination is detected and stopped. Nothing remained of the samples with contrary ELISA and LC-MSMS results for further analysis, but additional research focused on detecting NIT is important to protect business interests and human safety.

#### 4.1.4. FQ

The FQ Ciprofloxacin residue was detected in one sample from Thailand at 4 ppb and seven in the ELISA screening. Previous work in Italy found FQ was also detected in the tissue of seabass, gilthead seabream, and fish feed using ELISA kits (Conti et al., 2015) where concentrations of FQ in fish muscle tissue was 3.87 % and in feed 0.68 %. In Vietnam, fish and shrimp samples collected from domestic fish market were also positive for FQ residue with detections by both LC/MS and ELISA methods (Pham et al., 2015).

#### 4.1.5. MG

Similar to our results (4.8 % of samples with residue >1.6 ppb), previous work found malachite green in the tissue of rainbow trout and Atlantic salmon, with fish tissue accumulating persistent amounts of residue from MG (Srivastava et al., 2004). There is no established MRL for MG due to its carcinogenic nature, and in the US, Canada and UK, the use of MG in food production, including aquaculture, is not allowed. In 2017, around 1695 pounds of catfish was recalled by U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) for public health concern due to MG adulteration (USDA, 2017). The two samples positive for MG (range 1.6 < 4.5 ppb) should not have been allowed into the country.

### 4.2. Sulfite

Shrimp were purchased from retailers without knowing if they were

treated with any compound to prevent black spot. Many (43 %) tested positive for sulfite residuals, indicating that they had been treated with sulfite, but only one individual shrimp was over the limit of 100 ppm. Some of the imported shrimp did not test above 10 ppm, so this could be due to low treatment dose, short immersion time, storage, rinsing, or time on ice in the process. It is possible that some of these shrimp that tested >10 ppm may have been between 10–100 ppm when they first entered the supply chain, but all would still test safe for consumption (for those without a sulfite-triggered health condition). Of concern for consumption, no package of imported shrimp included sulfite in the label. Sulfite labeling is required if sulfite residue is greater than 10 ppm in shrimp (Rotllant et al., 2002). According to the USFDA (2001), the finished product should contain a declaration about using sulfite agent or the product should not contain detectable levels of sulfite. Importing countries have not adhered to the regulation, and this type of violation can have severe effects on human health. For hypersensitive asthmatic patients, small amount of sulfite can create life threatening conditions.

Hardisson et al. (2002) found the sulfite content in the edible portion of frozen prawn of Spain and shrimp of Venezuela ranged from 12.8−546 ppm and 10.7–380.7 ppm, respectively. The lower ranges are similar to our current results, however only one shrimp from Thailand exceeded the limit of 100 ppm. Sulfite levels in shrimp from Spain had excessive levels between 182–579 ppm (Steinhart at al., 1995). The imported shrimp samples in this project all tested much lower than some previous studies (Rıo Utrabo et al., 1994; Armentia et al., 1994).

Besides initial treatment, the storage of the shrimp could also affect residue sulfite levels. In ice storage, residue level is lower because sulfite is soluble in water and leaches into the ice water bath (Finne et al., 1986; Cintra et al., 1999; Gómez-Guillén et al., 2005). Cintra et al. (1999) reported that sulfite residue was high (around 138 ppm) just a few hours after shrimp were caught and treated. Another study found that concentrations reduced by 50 % after 2 days of ice storage (Finne et al., 1986). All of our imported shrimp samples were frozen. It was reported that during freezing and in frozen storage residual sulfite level decreased by 17 % (Finne et al., 1986). Crustaceans washed before storage have lower sulfite residual levels (Gonçalves and de Oliveira, 2016). The imported shrimp were industrially processed, and this could lower sulfite residues in our shrimp. Goncalves and de Olivera (2016) found storage time may also reduce sulfite residue. Additionally, unpeeled or shell-on shrimp contain higher sulfite content compare to peeled shrimp (Finne et al., 1986), and in the current experiment, only the muscle tissue was tested. Many of the shrimp in 10−100 ppm range were purchased cooked; this is a concern because sulfite residue in cooked shrimp is more threatening than when present in raw shrimp as raw shrimp are further washed and processed.

Most domestic shrimp packaging carriers a “may contain sulfite” label, even when the shrimp is known to be sulfite free for consumer safety. The shrimp tested in this study would be safe for most consumers. However, sulfite residue was present and with no warning label, these shrimp products would be a health concern for consumers sensitive to sulfites. For consumers that know they have a health condition triggered by sulfite, they should only purchase shrimp from a known source guaranteed to be sulfite free. However, sulfite residue could be eliminated by shrimp harvesters and producers by using sulfite-free melanosis prevention products that exist on the market such as 4-hexylresorcinol based products (Frankos et al., 1991; Selçuk and Özden, 2017).

## 5. Conclusion

In Baton Rouge, LA, the majority of imported shrimp available for retail purchase in 2016 and 2017 came from India or Thailand (57 %), compared to the broader US with around 43 % of imported shrimp from India and Thailand (US FDA, 2017). In this research, with NIT, MG, FQ, and OTC detected, residue from more than one antimicrobial was sometimes present in the same shrimp sample (e.g. FQ and NIT were present in Thailand, India, and Vietnam originated shrimp samples (Table 2), and 43 % of imported shrimp contained 10–100 ppm of sulfite. The presence of antimicrobial and sulfite residue in shrimp indicates that exporting and importing country’s testing is insufficient as residue was found in shrimp that were already in the USA market and while safe for consumption according to FDA regulations, there is still concern that none of the imported shrimp products included sulfite on their labels. Proper steps need to be taken by importing countries to change the common practice of using antimicrobial drugs in shrimp farming. Additional efforts should be directed at determining where the contamination is occurring. Exporting governments could strictly prohibit the sale of banned veterinary drugs, provide training for shrimp farmers to improve awareness and try using alternatives. Importing countries need to improve the testing of seafood consignments. Future work should research the same brands and countries of origin for the shrimp to see how common residue violations are over time. Additional shrimp samples from countries with limited samples (e.g. Ecuador and Bangladesh) should be tested. Finally, additional work in needed for NIT to ensure any adulterated product is prevented from entering the US, but also that false positives do not lead to unnecessary rejections.

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## CRediT authorship contribution statement

**Murshida Khan:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Julie A. Lively:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Writing - review & editing.

## Declaration of Competing Interest

The authors report no declarations of interest.

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## Appendix A. Supplementary data

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