Simultaneous determination of antibiotics residues in edible fish muscle using eco-friendly SPE-UPLC-MS/MS: Occurrence, human dietary exposure and health risk assessment for consumer safety

Heba Shaaban*, Ahmed Mostafa

Department of Pharmaceutical Chemistry, College of Clinical Pharmacy, Imam Abdulrahman Bin Faisal University, King Faisal Road, P.O. Box 182, Dammam 31441, Saudi Arabia

ARTICLE INFO

Handling Editor: Prof. L.H. Lash

Keywords:
UPLC-MS/MS
Aquaculture
Antibiotics
Greenness assessment
Human health risk
Estimated daily intake

ABSTRACT

Occurrence of multi-class antibiotics in edible fish species from the Saudi market was investigated. A fast and sensitive UPLC-MS/MS method for simultaneous determination of selected fluoroquinolones, sulfonamides and macrolides in fish muscle was developed and validated. Sample clean up was performed using solid-phase extraction on Oasis HLB cartridges. The greenness profile of the developed method was evaluated using three assessment tools: analytical Eco-scale assessment method, green analytical procedure index and analytical greenness metric. Detection limits ranged from 0.008 to 0.35 μg/kg. The recovery ranged from 80.1% to 98.6% with RSDs ≤ 12.1%. The mean and maximum concentrations of the detected antibiotics in fish samples ranged between 0.28 and 19.15 and 3.50–112.00 μg/kg, respectively. Human antibiotics (clarithromycin and roxithromycin) were detected in 50% and 27.5% of the samples, respectively. The estimated daily intake for the detected antibiotics ranged from 0.10 to 6.61 ng/kg/BW/day for Saudi adults. The health risk associated with fish consumption was evaluated. The results suggested that fish consumption may not pose a serious risk to consumers in Saudi Arabia. The developed method was fast, sensitive, cost-effective and environmentally friendly.

1. Introduction

Antibiotics are extensively used worldwide for preventing and controlling diseases, not only for humans but also for animals. Antibiotics are utilized in aquaculture mainly for growth promotion [1]. Because of incomplete metabolism of large number of antibiotics in the body, they are excreted into the environmental compartments [2–4] causing potential adverse effects on ecosystem and human health as well. In aquaculture, antibiotics are commonly used and added to the feed or to water. However, the excessive use or abuse of antibiotics could lead to continual release into the aquatic systems [5], thus posing harmful effects to the exposed creatures [6].

Antibiotics can also discharged into the environment through wastewater [7]. These compounds could be excreted as metabolites or parent compounds via urine or feces and discharged through wastewater. Because of the low efficiencies of wastewater treatment plants, especially in the developing countries where the treatment plants are not well designed to remove such compounds, antibiotics residues can reach the environment [8]. The occurrence of antibiotics as metabolites or degradation products in water systems even at low concentrations can develop antimicrobial resistance genes and resistant bacteria [9] which recently have become a global concern. The resistant bacteria can cause diseases that require more expensive, more toxic and in some times less available antibiotics [10]. Because of the harmful effects of antibiotics, they have been globally considered as emerging environmental pollutants. Macrolides, sulfonamides, tetracyclines and quinolones are among the most highly detected antibiotics classes in the environmental samples [4,11].

Several analytical methods have been developed to investigate the occurrence of antibiotics and to measure their levels in fish tissues using liquid chromatography-tandem mass spectrometry (LC-MS/MS) e.g. [12–16] and liquid chromatography-fluorescence detection e.g. [17,18]. Even trace levels of antibiotics (from ng/L to μg/L) can produce antibiotic-resistant bacterial strains that can cause harmful effects on...
human health and the environmental ecosystems as well [19]. Therefore, the investigation on the occurrence and distribution of antibiotics in aquatic edible organisms, in addition to assessment of dietary exposure risk, are of a paramount importance in order to ensure the quality and safety for consumers. The profiles of antibiotics in fish and the assessment of human risk associated with fish consumption have been widely studied in different regions such as South China [20], North China [21,22], Malaysia [23] and Argentina [12].

The behaviour of emerging contaminants (e.g. antibiotics) in the environment is dynamic, hence should be studied and monitored regularly. However, most of such studies are focused in Europe and North America with few reports in Eastern Asia, setting of a gap of knowledge in Africa and Western Asia. This led us to develop a sensitive and reliable in-house UPLC-MS/MS method for simultaneous determination of antibiotics residues in commercial fish of different species (Siganus rivulatus, Scomberomorus commerson, Lethrinus lentjan and Cephalopholis argus) sold in the markets of Saudi Arabia. Additionally, this work aimed to assess the human health risk associated with fish consumption in adult male and female Saudi population for the first time. The present work could provide a baseline information about the occurrence and distribution of sulfonamides, fluoroquinolones and macrolides in different fish species in Saudi Arabia, where no such studies have been conducted so far.

2. Materials and methods

2.1. Analytical standards and reagents

High purity grade (≥ 98 %) antibiotic standards of ciprofloxacin (CPF), enrofloxacin (ENF), marbofloxacin (MRF), norfloxacin (NOF), ofloxacin (OFX), clarithromycin (CLM), lincomycin (LCM), Roxithromycin (ROX), sulfadiazine (SDZ), sulfamethazine (SMZ), sulfamethoxazole (SMX), sulfaphenazole (SPZ), sulfathiazole (STZ) and trimethoprim (TMP) were purchased from Sigma-Aldrich (Steinheim, Germany). Trimethoprim was added in sulfonamides group because it is commonly used in combination with sulfonamides. Isotopically labelled compounds used as internal standards were ofloxacin-d9 (OFX-d9), 13C6-Sulfamethoxazole ([13C6-SMX]) and 13C6-Trimethoprim ([13C6-TMP]). All were also obtained from Sigma-Aldrich (Steinheim, Germany). HPLC grade methanol, ethanol and ethyl acetate were purchased from Merck (Darmstadt, Germany). Formic acid (LC-MS grade) and ethylendiamine tetra-acetic acid disodium salt (EDTA) was obtained from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was produced by a Pure Lab Ultra water system (ELGA, High Wycombe, UK).

Stock standard and isotopically labelled internal standard solutions were prepared individually in methanol at 1 mg/mL and stored at −20 °C until use (approximately one month after preparation). For fluoroquinolones, formic acid was added to enhance their solubility. Multicomponent mixtures were prepared by appropriate dilution from the stock solutions and stored at −20 °C. Working standard solutions for the calibration curve (at 0.5, 5, 10, 25, 50, 100, 200 µg/L) were prepared before each analytical batch.

2.2. Sample collection and preparation

In this study, a total of forty fish samples including four species of the most widely consumed fish in Saudi Arabia were collected from local markets and aquaculture facilities in the Eastern province, Saudi Arabia between March and April 2021. The collected fish species included Cephalopholis argus (Hamour) (n = 10), Scomberomorus commerson (Kanad) (n = 10), Lethrinus lentjan (Shaov) (n = 10) and Siganus rivulatus (Saf) (n = 10). The extraction and clean-up process were conducted using sonication followed by a solid phase extraction (SPE).

Fish muscle was separated from each fish sample and ground in a mortar. Then, 0.5 g of fish muscle was weighed and placed in 15-mL polypropylene centrifuge tube. To fish samples, an isotope labelled antibiotics mixture, 5 mL ethyl acetate and 0.01 g EDTA were added. Then, the mixture was vortexed for 1 min, sonicated for 10 min and centrifugated for 5 min at 3500 rpm. EDTA was used as a chelating agent to prevent the chelation of fluoroquinolones with metal ions present in glassware [24,25]. For clean-up, SPE was performed using HyperSep™ Glass Block Vacuum Manifold purchased from Thermo Scientific™ (Dreieich, Germany). After centrifugation, the aliquots of the supernatants were loaded into Oasis HLB cartridges (60 mg, 3 mL) obtained from Waters Corporation (Milford, USA). The cartridges were pre-conditioned with 3 mL methanol followed by 3 mL ultrapure water. After sample loading and washing, the analytes were eluted using 5 mL methanol and evaporated to dryness under a stream of nitrogen. Finally, the residues were dissolved in 500 µL of the mobile phase and subjected to UHPLC-MS/MS for analysis after being filtered through 0.2 µm nylon syringe filter (Thermo Scientific, USA).

2.3. UPLC-MS/MS analysis

The analysis was performed using a liquid chromatography system (Shimadzu Nexera X2 UPLC, Japan) equipped with an automatic degasser, binary pump and an autosampler. The chromatographic analysis was carried out using Hypersil™ Gold C18 column (30 mm, 2.1 mm, 1.9 µm) at 30 °C. A guard column from Thermo Scientific (San Jose, USA) was used. The UPLC flow was maintained at 500 µL/min. The mobile phase was composed of ethanol (A) and 0.1 % formic acid in water (B) (v/v). A gradient elution was used starting with 5 % ethanol, increased to 100 % at 10 min, reduced to 5 % at 14 min and equilibrated for 6 min before the next injection. The injection volume was 2 µL. Blank samples (5:95 (v/v) ethanol: 0.1 % formic acid in water) were run every ten samples to verify a clean background chromatogram.

The UPLC system was connected to triple quadrupole mass spectrometer 8050 (Shimadzu, Japan). The electrospray ionization source was operated in the positive ionization mode. LabSolutions® (Shimadzu, Japan) was used for system control and data processing. Multiple reaction monitoring (MRM) was used for data acquisition. Two MRM transitions were used for analytes identifications based on the retention time and the ratio of the two MRM transitions (i.e. within ± 20 % of the ratio in the reference standards). The retention time was accepted when the value was within ± 2.5 % for that of the respective standard. The most intense MRM transition (in case of positive identification of a target analyte) was used for analytes quantification and the second most intense transition was used for confirmation purposes. The following parameters were used for tuning: interface temperature-350 °C; heat block temperature-200 °C; desolvation line temperature-250 °C; nebulizer gas-nitrogen at 3 L/min; drying gas-nitrogen at 10 L/min; interface current-0.1 µA. Argon gas (Airgas, USA) was used for the collision cell dissociation. ATN-1050 nitrogen gas generator (Shimadzu, Kyoto, Japan Scientific) was used to supply nitrogen and air. Because collision energy (CE) is an important factor that strongly affects the collision behaviour, optimal CE was automatically selected for the studied antibiotics and the internal standards in the range of 10–60 eV based on compound optimization function. The MS/MS parameters used for quantification and structure confirmation of the studied analytes are illustrated in Table S1. The MRM transitions of the studied analytes are presented in Fig. S1.

2.4. Method validation

To ensure the quality, consistency and reliability of the analytical results, validation procedures were performed for the developed UPLC-MS/MS method.

Matrix-matched calibration was used to evaluate the linearity. The calibration curves were prepared by spiking blank fish extracts (previously determined to be free from the studied antibiotics) at seven concentration levels in the range of 0.5–200 µg/L. The quantitation was...
performed based on the internal standard method in which the ratios of peak areas of the analytes and the internal standard were plotted against the concentration of each respective analyte. The correlation coefficients \( r^2 \) obtained by least squares linear regression model was used to evaluate the linearity of the method. The accuracy of the developed method was expressed in terms of recovery. Blank samples were spiked with a mixture of the studied antibiotics at three concentrations levels \((1, 10, \text{ and } 100 \, \mu g/kg)\) with six replicates to verify recovery percentages. The intra-day precision \((n = 6)\) and the inter-day precision \((n = 6)\) were performed to evaluate the repeatability of the developed method. The precision was expressed as the relative standard deviations (RSDs). The limits of detection (LODs) and limits of quantification (LOQs) were measured according to the ICH guidelines [26], which were based on signal-to-noise ratio of 3:1 and 10:1 respectively.

2.5. Data analysis

For the statistical analysis and calculations, Statistical Package for Social Science (SPSS version 22) (IBM Corp., Armonk, NY, USA) and Excel program were used. The results were expressed as mean, maximum, range and frequency. Results below LODs were considered zero for calculation of mean value. For dietary risk assessment, if values were below the LOD, the concentrations were set to LOD/2 as per the criteria stated in [27]. SPSS was used to evaluate Pearson’s correlation between concentrations of the detected antibiotics. The level of significance was set at \( p = 0.05 \) and 0.01 as indicated.

2.6. Human health risk assessment

The estimated daily intake (EDI; \( \text{ng/kg of bw/day} \)) of the detected antibiotics were calculated using the following equation: EDI = \((C \times IR)/BW (1)\) where \( C \) (\( \mu g/g \)) represents the measured concentration of each antibiotic in the analyzed fish samples. For representing the average and the worst-case scenarios, mean and maximum concentrations were used, respectively. IR is the daily consumption rate of fish (g/day), considering 23.25 g/day for Saudi adults as per a questionnaire-based dietary survey conducted in Saudi Arabia [28]. BW (kg) refers to the body weight, considering 67.4 and 61.9 kg for adult Saudi male and female, respectively [29].

For assessing the human health risk, the hazard quotient (HQ) for each antibiotic was calculated using the following equation: HQ = EDI/ADI (2). ADI is the acceptable daily intakes (\( \mu g/kg \text{ bw/day} \)). HQ < 1 % indicates a negligible risk and HQ > 1 % indicates a potential risk for human health [30]. Since fish samples are often detected with multi-residues of antibiotics, it is necessary to perform a cumulative risk assessment by calculating the hazard index (HI) which is an indication of chronic dietary exposure due to the detected antibiotics. The HI value was determined by summing up the HQ value for each detected analyte in every antibiotic class under study.

3. Results and discussion

3.1. Method optimization and validation

On the basis of the literature and the authors’ experience, sub-2 \( \mu m \) fully porous C18 narrow diameter short column was used for analysis. This column could provide a better peak shape, high resolution, short retention times, reduced solvent consumption and waste generated [31, 32]. Because the toxicity of acetonitrile according to the US Environmental Protection Agency (EPA), ethanol was selected in this study as a green organic modifier because of its minor toxicity and derivation from renewable sources [33]. To select the proper composition of the mobile phase, different compositions of the mobile phase were tried to achieve the best separation of the selected antibiotics in the shortest possible time (7 min) with good peak shape. It was found that adding 0.1 % formic acid in the mobile phase could significantly improve the peak shape and achieve good separation for the analytes.

The developed multi-class antibiotics residues method was validated through the linearity, accuracy, precision, LODs and LOQs. The validation results of the developed UPLC-MS/MS method are summarized in Table 1. The correlation coefficients \( r^2 \) were 0.9980–0.9995 for all studied antibiotics, indicating high linearity of the method. The LODs and LOQs were 0.008–0.35 and 0.03–1.50 \( \mu g/kg \), respectively. The recoveries for the studied antibiotics ranged between 80.1 % and 98.6 % with RSDs ≤ 12.1 % for all studied antibiotics, indicating satisfactory precision of the developed method (Table 1). The validation results of the developed UPLC-MS/MS method indicated that the analytical method was efficient and reliable for simultaneous trace determination of antibiotics in fish muscle samples.

3.2. Evaluating the greenness level of the developed UPLC-MS/MS method

For evaluating the greenness level of the developed method, three greenness assessment methods were used; namely analytical Eco-Scale, green analytical procedure Index (GAPI) and analytical greenness metric (AGREE).

Analytical Eco-Scale tool was employed to assess the greenness level of analytical procedures in terms of the quantity of hazards [34]. This approach is based on giving penalty points to the amount and type of reagents used, hazards, energy consumed and waste generated. Then subtracting the penalty points from a value of 100. The excellent green method is scored with a value of 75. A score higher than 50 indicates that the method is acceptable and the method is considered inadequate, if the Eco-scale value is lower than 50. A high Eco-Scale score was mainly attributed to the amount and type of the consumed solvents. In this study, the calculated penalty points for the developed method was 75 out of 100, indicating that the developed method was excellent green (Table 2).

GAPI [35] allows any research to compare the greenness of analytical methods, thus enabling quick selection of the greenest procedure for a particular study. It represents the greenness level of the method through five fields. Each field represents a certain aspect of the developed method. Fields can be shaded green, yellow or red depending on the ecological impact for each step. The field is shaded green if certain requirements are fulfilled. In this study, GAPI pentagons has four green fields, seven yellow fields and four red fields (Table 2). One of the red fields is associated with the use of UPLC-MS/MS (>1.5 kW per sample) for analysis. LC-MS/MS is the technique of choice for trace determination of analytes in complex matrices such as fish, thus its use in the presented application is inevitable. Another pentagon is shaded red because of the need for sample preparation. The step of extraction and clean-up is also necessary in case of complex matrices to reduce matrix effect. Overall, the developed method has low environmental impact because the use of hazardous solvents was eliminated and the generated waste was minimized. It is worthy to mention that the developed method can be used for qualification and quantification as well.

AGREE is another approach of evaluating the environmental impact of the developed method [36]. This approach is based on using a simple automated software freely available and can be downloaded through a free website link provided in an AGREE article [36]. The AGREE pictogram contains twelve sections equivalent to 12 principles of green analytical chemistry (GAC). Each section and the middle zone of the AGREE pictogram can be colored from red to green based on the level of the method greenness. The total score of the method is automatically calculated ranging from zero to one according to the method greenness and the score appeared in the middle zone of the AGREE pictogram. In this study, the AGREE score is 0.64 (Table 2) which indicates that the method is eco-friendly and do not pose harmful impact on the environment.
The mean and maximum concentrations observed were 26.87 and 46.90 µg/kg, respectively. Marbofloxacin was the least detected fluoroquinolones (10 % detection rate). The highest concentration of the individual fluoroquinolones was observed for enrofloxacin (112 µg/kg) which was higher than the maximum concentrations of other fluoroquinolones detected in the analyzed fish samples (3.5–60.1 µg/kg). The overall detection frequency of sulfonamides in all analyzed samples was 80 %, including sulfamethoxazole (52.5 %), sulfamethazine (40 %), sulfathiazole (22.5 %) and sulfadiazone (20 %). Their mean concentrations in commercial fish were 6.63, 6.47, 1.81 and 0.28 µg/kg, respectively. Trimethoprim was less frequently detected in the analyzed samples (17.5 %) at a concentration ranged from lower than LOD to 6.7 µg/kg. Macrolides were detected at a lesser extent compared to other studied groups. Clarithromycin was the highest detected macrolide (found in half of the analyzed fish samples), followed by roxithromycin (27.5 %) at a concentration ranged from lower than LOD to 6.7 µg/kg. Macrolides were detected at a lesser extent compared to other studied groups. Clarithromycin was the highest detected macrolide (found in half of the analyzed fish samples), followed by roxithromycin (27.5 %) at a concentration ranged from lower than LOD to 6.7 µg/kg.

3.3. Occurrence of antibiotics in commercial fish

In this study, five fluoroquinolones, three macrolides, five sulfonamides and trimethoprim were investigated in commercial fish from the Saudi market. The mean concentrations, ranges, 95th percentile concentrations and detection frequencies for the detected antibiotics in all fish samples are shown in Table 3. A total of thirteen antibiotics (out of 14) were detected in the analyzed samples. Sulfaphenazole was not found in every fish sample (100 % antibiotics positives in sampled fish). At least, one antibiotic was detected in any of the analyzed fish samples. In this study, five fluoroquinolones, three macrolides, five sulfonamides and trimethoprim were investigated in commercial fish from the Saudi market. The mean concentrations, ranges, 95th percentile concentrations and detection frequencies for the detected antibiotics in all fish samples are shown in Table 3. A total of thirteen antibiotics (out of 14) were detected in the analyzed samples. Sulfaphenazole was not found in every fish sample (100 % antibiotics positives in sampled fish). At least, one antibiotic was detected in any of the analyzed fish samples.

Among the detected fluoroquinolones, the detection frequency of enrofloxacin in the commercial fish was the highest (65 %), followed by ciprofloxacin (47.5 %) and norfloxacin (45 %). Their mean concentrations in the analyzed samples were 11.25, 7.13 and 6.65 µg/kg respectively. Marbofloxacin was the least detected fluoroquinolones (10 % detection rate). The highest concentration of the individual fluoroquinolones was observed for enrofloxacin (112 µg/kg) which was higher than the maximum concentrations of other fluoroquinolones detected in the analyzed fish samples (3.5–60.1 µg/kg). The overall detection frequency of sulfonamides in all analyzed samples was 80 %, including sulfamethoxazole (52.5 %), sulfamethazine (40 %), sulfathiazole (22.5 %) and sulfadiazone (20 %). Their mean concentrations in commercial fish were 6.63, 6.47, 1.81 and 0.28 µg/kg, respectively. Trimethoprim was less frequently detected in the analyzed samples (17.5 %) at a concentration ranged from lower than LOD to 6.7 µg/kg. Macrolides were detected at a lesser extent compared to other studied groups. Clarithromycin was the highest detected macrolide (found in half of the analyzed fish samples), followed by roxithromycin (27.5 % detection rate) and lincomycin was the least detected macrolide (5 %) (Table 3). The mean and maximum concentrations of macrolides in all analyzed samples ranged from 0.28 to 19.15 µg/kg for fluoroquinolones, 112.00 µg/kg for macrolides.

### Table 1

| Antibiotics class | Analyte | R² | LOD | LOQ | Recoveries (%, n = 6) | Inter-day Precision (%RSD) | Intra-day Precision (%RSD) |
|------------------|---------|----|-----|-----|-----------------------|-----------------------------|----------------------------|
|                   |         |    |     |     | Low Medium High       | Low Medium High             | Low Medium High             |
| **Fluoroquinolones** |         |    |     |     |                       |                             |                           |
| CPF   | 0.9980  | 0.05 | 0.17 | 89.6 | 93.8 | 95.8 | 10.8 | 6.7 | 7.9 | 11.1 | 10.6 | 8.5 |
| ENF   | 0.9991  | 0.16 | 0.55 | 97.3 | 98.9 | 98.6 | 9.1  | 6.8 | 5.2 | 6.4  | 5.7  | 9.3 |
| MRB   | 0.9990  | 0.22 | 0.75 | 88.3 | 90.4 | 97.5 | 5.3  | 6.5 | 9.5 | 12.1 | 11.3 | 11.2 |
| NOF   | 0.9987  | 0.25 | 0.8  | 92.4 | 96.8 | 98.1 | 6.2  | 8.2 | 8.9 | 7.9  | 8.1  | 9.6 |
| OFX   | 0.9980  | 0.15 | 0.5  | 97.5 | 98.3 | 99.1 | 4.9  | 3.7 | 6.5 | 3.3  | 2.6  | 4.9 |
| **Macrolides**    |         |    |     |     |                       |                             |                           |
| LCM   | 0.9985  | 0.35 | 1.5  | 80.1 | 85.6 | 84.9 | 11.1 | 10.0 | 9.9 | 8.4  | 9.1  | 9.5 |
| ROX   | 0.9990  | 0.01 | 0.05 | 80.1 | 82.5 | 90.5 | 7.6  | 7.8 | 6.5 | 7.6  | 8.6  | 7.9 |
| **Sulfonamides**  |         |    |     |     |                       |                             |                           |
| SDZ   | 0.9984  | 0.01 | 0.05 | 88.8 | 89.3 | 91.5 | 7.9  | 6.4 | 6.9 | 11.9 | 10.7 | 10.1 |
| SMZ   | 0.9991  | 0.02 | 0.07 | 89.9 | 95.2 | 98.1 | 8.6  | 9.1 | 7.9 | 10.7 | 10.6 | 9.8 |
| **Sulfonamides**  |         |    |     |     |                       |                             |                           |
| SMX   | 0.9987  | 0.09 | 0.05 | 80.8 | 82.3 | 81.3 | 9.5  | 7.0 | 7.5 | 9.7  | 8.2  | 8.5 |
| SPZ   | 0.9992  | 0.008| 0.03 | 80.3 | 85.6 | 80.9 | 7.2  | 7.8 | 6.1 | 5.4  | 5.1  | 5.5 |
| STZ   | 0.9989  | 0.02 | 0.05 | 82.5 | 85.9 | 84.7 | 3.9  | 4.5 | 4.2 | 5.0  | 4.6  | 4.8 |
| TMP   | 0.9995  | 0.02 | 0.05 | 91.6 | 96.4 | 97.0 | 2.9  | 3.1 | 3.8 | 4.9  | 3.9  | 4.4 |

1. LOD and LOQ are in µg/kg.
2. The precision was expressed as the relative standard deviations (RSDs). Samples were spiked at low, intermediate and high concentrations (1, 10 and 100 µg/kg, respectively).

### Table 2

| Analytical Eco-Scale score | GAPI | AGREE |
|---------------------------|------|-------|
| Hazards (reagents/ instruments) | Penalty | points |
| Ethanol | 4 |
| Methanol | 6 |
| Formic acid | 6 |
| Ethyl acetate | 4 |
| UPLC-MS/MS | 2 |
| Occupational hazards | 0 |
| Waste | 3 |
| Total penalty points | 25 |
| Total score | 75 |

3.4. Comparison of antibiotics concentrations in different fish species

The highest number of detected antibiotics was found in Siganus rivulatus and Scomberomorus commerson (13), followed by Lethrinus lentjan (12) and Cephalopholis argus (11). The detection frequency of...
each antibiotic grouped by fish species is presented in Fig. 1. Marbofloxacin was not detected in Cephalopholis argus fish species and lincomycin was not detected in any of Cephalopholis argus and Lethrinus lentjan samples. The concentration of individual detected antibiotics were widely varied among the four fish species (Fig. 2a). Mean and maximum concentrations of individual antibiotics ranged between 0.10 and 19.00 and 0.50–105.50 µg/kg (Cephalopholis argus), 0.09–29.5 and 0.80–112.00 µg/kg (Lethrinus lentjan), 0.75–113.09 and 0.50–110.20 µg/kg (Siganus rivulatus) and 0.24–19.53 and 0.50–103.00 µg/kg (Scomberomorus commerson) (Table 3).

Also, the total concentrations of each antibiotic class as per fish species are presented in Fig. 2b. The concentrations of fluoroquinolones were higher than those of sulfonamides and macrolides in Cephalopholis argus, Lethrinus lentjan and Scomberomorus commerson, in which the mean concentrations were 27.77, 32.75 and 30.83 µg/kg, respectively. The highest concentration of fluoroquinolones was found in Scomberomorus commerson (121.1 µg/kg), followed by Lethrinus lentjan (112.0 µg/kg), Cephalopholis argus (105.0 µg/kg) and Siganus rivulatus (56.30 µg/kg). This finding can be attributed to the widespread use of fluoroquinolones as feed additive in aquaculture [20]. Enrofloxacin was the highest detected antibiotic in all analyzed fish species with overall detection frequency 65 %, including Siganus rivulatus and Lethrinus lentjan (70 %) and Cephalopholis argus and Lethrinus lentjan (50 %). Enrofloxacin is a broad spectrum antimicrobial agent widely available in the market for veterinary use in many countries (Guidi et al., 2018). Although the FDA approval of using enrofloxacin in poultry and

Table 3

Concentrations (µg/kg) of the detected antibiotics in the analyzed fish samples.

| CPF | ENF | MRF | NOF | OFX | CLM | LCM | ROX | SDZ | SMZ | SMX | SPZ | STZ | TMP |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **Mean** | 7.47 | 13.55 | nd | 3.28 | 19.00 | 14.98 | nd | 13.10 | 0.15 | 2.08 | 0.10 | nd | 1.50 |
| **95th percentile** | 22.89 | 63.82 | nd | 16.10 | 20.35 | 37.75 | nd | 24.95 | 0.82 | 9.50 | 0.37 | nd | 2.40 |
| **Range** | nd-25.5 | nd-105.5 | nd | nd-24.5 | nd-20.5 | nd-43.5 | nd | nd-27.5 | nd-1.5 | nd-14.5 | nd-0.5 | nd-2.5 | nd-2.5 |
| **Frequency (%)** | 50 % | 50 % | nd | 30 % | 20 % | 60 % | nd | 40 % | 10 % | 40 % | 40 % | nd | 30 % |

**Cephalopholis argus (n = 10)**

| Mean | 5.74 | 14.23 | 3.50 | 9.69 | 29.50 | 17.42 | nd | 12.35 | 0.25 | 11.29 | 10.78 | nd | 2.70 |
| **95th percentile** | 20.27 | 63.82 | nd | 16.10 | 20.35 | 37.75 | nd | 24.95 | 0.82 | 9.50 | 0.37 | nd | 2.40 |
| **Range** | nd-60.1 | nd-112.0 | nd-3.5 | nd-35.4 | nd-29.5 | nd-31.5 | nd | nd-23.5 | nd-2.5 | nd-106.0 | nd-102.4 | nd | 4.32 |
| **Frequency (%)** | 40 % | 70 % | 10 % | 50 % | 10 % | 50 % | nd | 20 % | 10 % | 50 % | 50 % | nd | 20 % |

**Lethrinus lentjan (n = 10)**

| Mean | 4.12 | 2.24 | 2.40 | 85.10 | 10.00 | 66.00 | 20.8 | 92.80 | 4.80 | 104.30 | 113.9 | nd | 4.10 |
| **95th percentile** | 16.37 | 7.10 | 1.47 | 40.30 | 9.05 | 22.13 | 20.8 | 45.09 | 2.33 | 55.95 | 67.08 | nd | 4.10 |
| **Range** | nd-28.9 | nd-9.8 | nd-1.5 | nd-52.3 | nd-9.5 | nd-24.3 | nd-20.8 | nd-46.9 | nd-3.5 | nd-100.5 | nd-110.2 | nd | 0.43 |
| **Frequency (%)** | 40 % | 70 % | 20 % | 50 % | 20 % | 6 % | 10 % | 30 % | 30 % | 50 % | 50 % | nd | 20 % |

**Scomberomorus commerson (n = 10)**

| Mean | 9.45 | 14.97 | 0.50 | 4.26 | 9.83 | 19.53 | 17.50 | 11.10 | 0.24 | 2.06 | 2.81 | nd | 0.77 |
| **95th percentile** | 19.37 | 7.10 | 1.47 | 40.30 | 9.05 | 22.13 | 20.8 | 45.09 | 2.33 | 55.95 | 67.08 | nd | 1.23 |
| **Range** | nd-40.2 | nd-103.0 | nd-0.5 | nd-17.5 | nd-15.9 | nd-25.5 | nd-17.5 | nd-21.3 | nd-1.1 | nd-15.6 | nd-9.5 | nd | 3.95 |
| **Frequency (%)** | 40 % | 70 % | 10 % | 50 % | 30 % | 30 % | 10 % | 20 % | 30 % | 20 % | 70 % | nd | 20 % |

**All (n = 40)**

| Mean | 7.13 | 11.25 | 1.60 | 6.65 | 13.38 | 15.08 | 19.15 | 17.46 | 0.28 | 6.47 | 6.63 | nd | 1.81 |
| **95th percentile** | 19.37 | 7.10 | 1.47 | 40.30 | 9.05 | 22.13 | 20.8 | 45.09 | 2.33 | 55.95 | 67.08 | nd | 1.23 |
| **Range** | nd-36.0 | nd-103.0 | nd-0.5 | nd-17.5 | nd-15.9 | nd-25.5 | nd-17.5 | nd-21.3 | nd-1.1 | nd-15.6 | nd-9.5 | nd | 3.95 |
| **Frequency (%)** | 47.5 % | 65 % | 10 % | 45 % | 20 % | 50 % | 5 % | 27.5 % | 20 % | 40 % | 52.5 % | nd | 17.5 % |

Results below the LOD were reported as “not detected (nd)”.

**Fig. 1.**: Detection frequency of the investigated antibiotics grouped by fish species.

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aquaculture was withdrawn in 2005 because of the possibility of causing resistance, it is still approved for use in some food producing animals [37]. The enrofloxacin residues may reach fish by different routes of contamination, one of them is the illegal use of enrofloxacin in aquaculture. Additionally, the use of enrofloxacin in aviculture can result in its release in the environment through waste streams leading to fish contamination. The use of enrofloxacin in aquaculture throughout the world is well documented in the literature [38]. The detection of enrofloxacin in edible fish has been reported in other studies; however, the present study shows lower levels than those reported by [22] and [39] in China, and higher than those reported by [40] in Spain and by [38] in Brazil.

Regarding sulfonamides, their concentrations were found to be higher in Siganus rivulatus species (maximum concentration 110.2 µg/kg) compared to other species. The concentrations of macrolides detected in Cephalopholis argus (mean= 14.23 µg/kg) and Scomberomorus commerson species (mean= 9.83 µg/kg) were found to be higher than the concentrations of sulfonamides but lower than those of fluoroquinolones (Fig. 2b). Clarithromycin and roxithromycin, widely used in human medication, were also detected in all fish species at mean concentrations ranged between 14.98 and 66.00 and 11.10–92.80 µg/kg in the analyzed fish species (Table 3). A previous study reported the occurrence of clarithromycin and roxithromycin in edible fish from Argentina [12] in concentrations ranged from below LOD to 34.6 µg/kg. Also, the presence of roxithromycin in fish muscle from China has been reported [22] at a maximum concentration of 1076 µg/kg and 3.9 µg/kg for fish samples collected from Baiyangdian Lake and urban rivers in Nanjing, respectively. Occurrence of these antibiotics in edible fish indicating the low efficiency of wastewater treatment.

This is the first study to investigate the occurrence of fluoroquinolones, sulfonamides and macrolides in fish from the Saudi market and there are no previous studies to be compared in Saudi Arabia at the regional level. Generally, the residue levels of fluoroquinolones found in this study were quite similar to those from North China [22], lower than those in fish collected from Tai Lake, China [41] and slightly higher than those from Brazil [42]. Additionally, the levels of sulfonamides and macrolides in this study were found to be higher than those reported in South China [20]. This variation may be due to the difference in the pollution levels of fluoroquinolones in different regions and the difference in the analyzed fish species as well.

3.5. Correlations among the concentrations of detected antibiotics in commercial fish

Co-administration of antibiotics can lead to cumulative and potential synergistic health effects, therefore the correlation among the concentrations of antibiotics detected in the analyzed fish samples was investigated. Pearson’s correlation was used to describe the linear relationship among the two bivariate data i.e. how much the two variables correlate in terms of similarity or significance in a dataset. A value > 0.50 was considered to correlate the two bivariate data points, irrespective of the correlation whether it was positive or a negative value. Any value approaching “0” was declared a poor correlation. In this study, the Pearson’s correlation was constructed for the detected antibiotics in the analyzed fish samples (n = 40). A high correlation at P = 0.01 and P = 0.05 was observed for antibiotics from the same class and different classes as well. The highest positive correlation was found for the following antibiotics: NOR-LCM, OFX-CLM, CPF-LCM, CLM-STZ, LCM-SMZ and LCM-SDZ, in which the Pearson correlation was 1 (P = 0.01). The next significantly high positive correlation is observed for SMZ-MRF pair with a value of 0.955 (P = 0.05) and for NOF-ROX pair with a value of 0.885 (P = 0.01). Likewise, high significant positive correlation values of 0.495 and 0.433 (P = 0.01) were observed for the pairs of NOR-SMZ and SDZ-SMZ, respectively. None of the remaining pairs showed positive correlation. The correlation data of the investigated antibiotics are presented in Table 4.
3.6. Occurrence of antibiotics and their maximum residue limits (MRLs)

Generally, occurrence of antibiotics residues in edible fish may be responsible for allergic reactions, bacterial resistance and toxic effects on human health. Taking the MRLs established by the European Commission into consideration (Table S2), we observed that three antibiotics (out of 14) exceeded the MRLs in at least one fish species. In particular, enrofloxacin exceeded the established MRL (100 µg/kg) in 7.5% of the samples. Also, the MRL for sulfamethazine and sulfamethoxazole (100 µg/kg) were exceeded in 5% of the analyzed fish samples. Considering the fish species, enrofloxacin, sulfamethazine and sulfamethoxazole exceeded the MRLs in 30% of Lethrinus lentjan, 20% in Siganus rivulatus and 10% in Cephalopholis argus and Scomberomorus commerson.

It is worth mentioning that we found norfloxacin, clarithromycin, and roxithromycin in the analyzed fish samples, which do not have an established MRL. Detecting antibiotics which do not have a MRL is alarming because the risk to the public health is unknown and their presence at sufficient concentrations may cause hazardous effects for consumers who use the products treated with such antibiotics [43]. Moreover, these antibiotics are not approved by the U.S. Food and Drug Administration [44].

### Table 4

Pearson’s correlation for the detected antibiotics in the analyzed fish samples (n = 40).

|   | CPF | ENF | MRF | NOF | OFX | CLM | LCM | ROX | SDZ | SMZ | SMX | STZ | TMP |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| CPF | 1   |     |     |     |     |     |     |     |     |     |     |     |     |
| ENF | .100 | 1   |     |     |     |     |     |     |     |     |     |     |     |
| MRF | .221 | .545 | 1   |     |     |     |     |     |     |     |     |     |     |
| NOF | .006 | .155 | .996 | 1   |     |     |     |     |     |     |     |     |     |
| OFX | .972 | .382 | .058 |     | 1   |     |     |     |     |     |     |     |     |
| CLM | .245 | .284 | .101 | 1.000 |    | 1   |     |     |     |     |     |     |     |
| LCM | .297 | .225 | .690 |     |     |     |     | 1.000 |    |     |     |     |     |
| ROX | .149 | .426 | .885* | .949 | .374 |     |     |     |     |     |     |     |     |
| SDZ | .662 | .191 | .000 | .205 | .465 |     |     |     |     |     |     |     |     |
| SMZ | .829 | .802 | .449 | .201 | .740 | .166 | .423 |     |     |     |     |     |     |
| SMX | .025 | .106 | .955* | .495** | .399 | .225 | 1.000** | .309 | .433** |     |     |     |     |
| CLM | .879 | .516 | .045 | .003 | .328 | .318 | .356 | .005 |     |     |     |     |     |
| SDZ | .136 | .196 | .677 | .146 | .674 | .018 | .103 | .074 | 1   |     |     |     |     |
| STZ | .423 | .572 | .527 | .426 | .097 | .943 | .637 | .543 | .664 |     |     |     |     |
| TMP | .326 | .374 | b   | .541 | b   | 1.000* | b   | .294 | .229 | .597 | 1   |     |     |
| SDZ | .391 | .321 | .268 |     |     |     |     | .443 | .554 | .118 |     |     |     |
| STZ | .511 | .726 | .449 | .551 | .610 | .944 | .393 | .529 | .646 | .353 |     |     |     |

**Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at the 0.05 level (2-tailed).**

b. Cannot be computed because at least one of the variables is constant.

**Table 5**

Estimated daily intake (EDI, ng/kg/bw/day), acceptable daily intake (ADI, µg/kg/bw/day) and associated hazard quotient (HQ) of each antibiotic in all analyzed fish samples (n = 40) evaluated for male and female adults.

| Analyte | Acceptable daily intake (ADI)* | Estimated daily intake (EDI) | Estimated daily intake (EDI) | Estimated daily intake (EDI) | Estimated daily intake (EDI) | Estimated daily intake (EDI) | Estimated daily intake (EDI) | Estimated daily intake (EDI) | Estimated daily intake (EDI) | Estimated daily intake (EDI) | Estimated daily intake (EDI) | Estimated daily intake (EDI) | Estimated daily intake (EDI) |
|---------|--------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|         | Mean concentrations           | Maximum concentrations      | Mean concentrations         | Maximum concentrations      | Mean concentrations         | Maximum concentrations      |
|         | Male | Female | Male | Female | Male | Female | Male | Female | Male | Female | Male | Female | Male | Female |
| CPF     | 2   |        | 2.46 | 2.68 | 20.73 | 22.57 | 1.23E-03 | 1.34E-03 | 1.04E-02 | 1.13E-02 |     |     |     |     |
| ENF     | 2   |        | 3.88 | 4.22 | 38.64 | 42.07 | 1.94E-03 | 2.11E-03 | 1.93E-02 | 2.10E-02 |     |     |     |     |
| MRF     | —   |        | .55  | .60  | 1.21  | 1.31  | —             | —             | —             | —             |     |     |     |     |
| NOF     | —   |        | 2.29 | 2.50 | 18.04 | 19.64 | 1.15E-03 | 1.25E-03 | 9.02E-03 | 9.82E-03 |     |     |     |     |
| OFX     | 3.2 |        | 4.61 | 5.02 | 10.18 | 11.08 | 1.44E-03 | 1.57E-03 | 3.18E-03 | 3.46E-03 |     |     |     |     |
| T. Quinolones | 13.79 | 15.02 | 88.8 | 96.67 | HI (ΣHQs) | 5.76E-03 | 6.27E-03 | 4.19E-02 | 4.56E-02 |     |     |     |     |
| CLM     | 0.2 |        | 5.20 | 5.66 | 15.01 | 16.34 | 2.60E-02 | 2.83E-02 | 7.50E-02 | 8.17E-02 |     |     |     |     |
| LCM     | 30  |        | 6.61 | 7.19 | 7.18  | 7.81  | 2.20E-04 | 2.40E-04 | 2.39E-04 | 2.60E-04 |     |     |     |     |
| ROX     | —   |        | 6.02 | 6.56 | 16.18 | 17.62 | —             | —             | —             | —             |     |     |     |     |
| T. Macrolides | 17.83 | 19.41 | 38.37 | 41.77 | HI (ΣHQs) | 2.62E-02 | 2.85E-02 | 7.52E-02 | 8.20E-02 |     |     |     |     |
| SDZ     | 20  |        | 0.10 | 0.11 | 1.21  | 1.31  | 4.83E-06 | 5.26E-06 | 6.04E-05 | 6.57E-05 |     |     |     |     |
| SMZ     | 20  |        | 2.23 | 2.43 | 36.57 | 39.81 | 1.12E-04 | 1.21E-04 | 1.83E-03 | 1.99E-03 |     |     |     |     |
| SMX     | 130 |        | 2.29 | 2.49 | 38.01 | 41.39 | 1.76E-05 | 1.91E-05 | 2.92E-04 | 3.18E-04 |     |     |     |     |
| STZ     | —   |        | .62  | .68  | 1.55  | 1.69  | —             | —             | —             | —             |     |     |     |     |
| TMP     | 4.2 |        | 0.13 | 0.14 | 2.31  | 2.52  | 3.02E-05 | 3.29E-05 | 5.50E-04 | 5.99E-04 |     |     |     |     |
| T. Sulfonamides | 5.37 | 5.85 | 79.65 | 86.72 | HI (ΣHQs) | 1.65E-04 | 1.78E-04 | 2.73E-03 | 2.97E-03 |     |     |     |     |

* The data was obtained from the guidelines of the Joint FAO/WHO Expert Committee on Food Additives, the European Medicines Agency and the U.S. Food and Drug Administration.

** Not available
highlights that the lack of control in the use of antibiotics can lead to the contamination of the aquatic systems.

3.7. Human health risk assessment

Detection of multiple antibiotics residues in commercial fish and occurrence of some antibiotics at levels exceeding the MRLs necessitate the assessment of their health risk to consumers. Present is the first report evaluating the dietary exposure to antibiotics in adult male and female Saudi population due to fish consumption. Table 5 shows the EDI, ADI, and HQ values at the average and the worst-case scenarios of exposure to the studied antibiotics. Among the studied antibiotics classes, fluoroquinolones showed the highest EDI of 88.8 and 96.7 ng/kg/bw/day for male and female adults, respectively (Table 5). The relative contributions of each antibiotic to the total daily intakes (calculated from the maximum EDIs) are shown in Fig. 3a. Also, the relative contribution of antibiotics classes to the total daily intakes is as follows: fluoroquinolones (42.9%) > macrolides (38.5%) > sulfonamides (18.6%) (Fig. 3b). Among all the antibiotics, norfloxacin constituted 18.7% of the total antibiotics dietary intake for both male and female adult Saudi population.

The EDI value of each antibiotic in Siganus rivulatus, Scomberomorus commerson, Lethrinus lentjan and Cephalopholis argus was far below their respective ADI (Table 6), suggesting that their consumption could not pose a serious health risk for adult consumers. There are no previous studies assessing the risk for antibiotics intake by fish to be compared in Saudi Arabia. But generally, in the current scenario, none of the studied antibiotics was over the ADI. Additionally, HQ value was calculated for each antibiotic to assess the potential health risk to antibiotics from dietary exposure. The highest HQ value was observed for clarithromycin (HQ = 0.075, that is 0.038% of ADI at the worst case scenario), but it is still much lower than 1, indicating that it can not pose a potential human health risk from fish intake. Hazard index (HI) values were also calculated assuming that each antibiotic in the same group has a similar toxicological mode of action. The HI values of fluoroquinolones, macrolides and sulfonamides were in the range of 1.65 × 10^{-4} to 8.20 × 10^{-2}. These values are much lower than 1 which indicated that there is a negligible human health risk associated with the exposure to antibiotics due to fish consumption in Saudi Arabia.

It is worthy mentioning that antibiotics can develop resistant bacteria at low concentrations, even lower than their current MRLs [45]. Additionally, the occurrence of mixtures of antibiotics can cause chronic effects on human health and the combination of various antibiotics can induce multi-drug bacterial resistance even at low concentration, which is considered a serious issue worldwide. Furthermore, the combined effect of antibiotics and other human or environmental pollutants is still unknown [46]. Therefore, a regular monitoring of the occurrence of antibiotics in the aquatic organisms is of a paramount importance in order to ensure the consumers’ safety. To our knowledge, this is the first report for a wide variety of antibiotics, from both veterinary and human uses, in commercial fish samples from the Saudi Arabian market.

4. Conclusion

The developed UPLC-MS/MS method in the present study allowed the simultaneous trace determination of antibiotics from multiple classes in fish muscle from different species. The method was validated in terms of the linearity of calibration curves, accuracy, precision, LODs and LOQs. The LODs varied from 0.008 to 0.35 µg/kg while LOQs ranged from 0.03 to 1.5 µg/kg. Most individual antibiotics concentrations in the analyzed fish muscle samples were below their respective MRL, except for enrofloxacin, sulfamethazine and sulfamethoxazole, and none of the studied antibiotics exceeded their ADI. The presence of more than one antibiotic in most of analyzed fish samples triggers the need of more studies to investigate the potential human health risk associated with consumption of food contaminated with mixed contaminants. This is important especially for antibiotics which known to develop bacterial resistance. This point is currently considered a hot topic by the World Health Organization. Educating people on the proper use and disposal of antibiotics is very important to minimize the possibility of bacterial resistance.

Funding

This work was funded by the Deanship of Scientific Research, Imam
Table 6
Estimated daily intake and associated hazard quotient for each antibiotic grouped by fish species*

| Analyte            | Cephalopholis argus (n = 10) | Lethrinus lentjan (n = 10) | Siganus rivulatus (n = 10) | Scomberomorus commerson (n = 10) |
|--------------------|------------------------------|-----------------------------|---------------------------|---------------------------------|
|                    | Female | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| CPF                | 4.97E-03 | 4.40E-03 | 1.13E-02 | 1.04E-02 | 5.43E-03 | 4.98E-03 | 1.93E-02 | 1.78E-02 |
| ENF                | 1.97E-02 | 1.81E-02 | 2.10E-02 | 1.92E-02 | 1.84E-02 | 1.69E-03 | 1.93E-02 | 1.78E-02 |
| MRF                | -      | -     | -       | -     | -       | -     | -       | -     |
| NOF                | 4.60E-03 | 4.23E-03 | 6.65E-03 | 6.11E-03 | 9.82E-03 | 9.02E-03 | 3.29E-03 | 3.02E-03 |
| OFX                | 2.41E-03 | 2.21E-03 | 3.46E-03 | 3.18E-03 | 1.12E-03 | 1.02E-03 | 1.87E-03 | 1.71E-03 |
| CLM                | 8.17E-02 | 7.50E-02 | 5.92E-02 | 5.43E-02 | 4.56E-02 | 4.19E-02 | 4.79E-02 | 4.00E-02 |
| LCM                | 0.00E+00 | 0.00E-00 | 0.00E-00 | 0.00E-00 | 2.60E-04 | 2.39E-04 | 2.19E-04 | 2.01E-04 |
| ROX                | -      | -     | -       | -     | -       | -     | -       | -     |
| SDZ                | 2.82E-05 | 2.59E-05 | 4.70E-05 | 4.31E-05 | 6.57E-05 | 6.04E-05 | 2.07E-05 | 1.90E-05 |
| SMZ                | 2.72E-04 | 2.50E-04 | 1.99E-03 | 1.83E-03 | 1.89E-03 | 1.73E-03 | 2.93E-04 | 2.69E-04 |
| SMX                | 1.44E-06 | 1.33E-06 | 2.96E-04 | 2.72E-04 | 3.18E-04 | 2.92E-04 | 2.74E-05 | 2.52E-05 |
| STZ                | -      | -     | -       | -     | -       | -     | -       | -     |
| TMP                | 2.24E-04 | 2.05E-04 | 7.15E-05 | 6.57E-05 | 4.47E-05 | 4.1E-05  | 5.99E-04 | 5.50E-04 |

| Hazard quotient (HQ) | Cephalopholis argus (n = 10) | Lethrinus lentjan (n = 10) | Siganus rivulatus (n = 10) | Scomberomorus commerson (n = 10) |
|----------------------|-------------------------------|-----------------------------|---------------------------|---------------------------------|
| CPF                  | 0.94                          | 0.86                        | 0.30                      | 0.28                            |
| ENF                  | 94.44                         | 86.22                       | 38.64                     | 33.8                             |
| MRF                  | 92.0                          | 84.5                        | 13.30                     | 12.21                           |
| NOF                  | 7.70                          | 7.07                        | 35.77                     | 30.86                           |
| OFX                  | 16.34                         | 15.01                       | 11.83                     | 10.87                           |
| CLM                  | 10.33                         | 9.49                        | 23.87                     | 21.59                           |
| LCM                  | 5.45                          | 5.00                        | 39.81                     | 36.57                           |
| ROX                  | 0.19                          | 0.17                        | 25.67                     | 23.57                           |
| SDZ                  | 0.56                          | 0.52                        | 1.31                      | 1.21                            |
| SMZ                  | 4.54                          | 5.00                        | 31.57                     | 30.97                           |
| OFX                  | 0.94                          | 0.86                        | 5.50                      | 5.28                            |
| STZ                  | -                             | -                           | -                         | -                               |
| TMP                  | -                             | -                           | -                         | -                               |

* EDI and HQ calculated at the worst-case scenario (at maximum concentrations).

Figure captions:

Abdulrahman Bin Faisal University (Grant No. 2016-218-Pharm).

CRediT authorship contribution statement
- **Heba Shaaban**: Conceptualization, Methodology, Formal analysis, Writing – original draft, Funding acquisition, Project administration, Visualization, Supervision. **Ahmed Mostafa**: Methodology, Formal analysis, Validation, editing.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability
Data will be made available on request.

Acknowledgments
The authors would like to thank the Deanship of Scientific Research, Imam Abdulrahman Bin Faisal University for funding this project.

Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.11.010.

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