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Multiresidue antimicrobial determination in Nile tilapia (*Oreochromis Niloticus*) cage farming by liquid chromatography tandem mass spectrometry

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ABSTRACT

Aquaculture production has been sharply increasing in Brazil in the last years. Many classes of antimicrobials are commonly used in aquaculture worldwide to treat infections caused by a variety of bacterial pathogens and they are also used as a growth promoter. However, these intense uses may cause environmental contamination and bacterial resistance. A method was developed and validated for simultaneous assessment of 12 drugs of different antimicrobial classes (chloramphenicol, florfenicol, oxytetracycline, tetracycline, chlortetracycline, sulfadimethoxine, sulfathiazole, sulfamethazine, enrofloxacin, ciprofloxacin, norfloxacin, and sarafloxacin) on the Nile tilapia's muscle (*Oreochromis niloticus*). This study presents the development of a rapid method using ultrafiltration by Captiva cartridges and liquid chromatography tandem mass spectrometry triple quadrupole (Agilent 6430 - Agilent Technologies) in a negative mode for florfenicol and a positive mode for the others. The sample pretreatment involves extraction with 5 g of muscle fish, 1 mL of 0.1 M Na_2EDTA and 24 mL of acetonitrile: water (0.1% formic acid; 70:30), and purification by Captiva cartridges, followed by the determination of all compounds in a single run. Sulfadimethoxine-d6 was used as an internal standard to obtain more reliable results. The developed method was validated based on Eurachem Guide: The fitness for purpose of analytical methods, with the calibration curves carried out at blank samples spiked, matrix-matched calibration (MMC). The limits of quantification were lower than 4.3 μg kg^{-1} for all compounds; calibration curve showed linearity at the work range, recovery ranged from 83.8% to 110.1%, and accuracy was lower than 5.5%. The developed analytical method was successfully applied in 36 fish samples collected in 4 fish farms in the most important producing region of São Paulo State, Brazil.

1. Introduction

The use of antimicrobials in food-producing animals has generated considerable interest because the widespread administration of these drugs may lead to the development of resistant human pathogens. A large increase in the demand for fish products has been occurring since the last century. This has led to a concomitant increase in high-intensity aquaculture, characterized by high stock density and volume and the heavy use of formulated feeds containing antimicrobials, among other substances. Therefore, accurate and sensitive determination of antimicrobial residue is now a necessity.

Antimicrobials are frequently used in veterinary practice for the treatment of bacterial infections and the promotion of animal growth to improve the livestock productivity. Although an adequate withdrawal time is prescribed, in the last decade, concerns have been raised regarding public health issues over the occurrence of antimicrobials through the food chain (Kan and Meijer, 2007). These residues may include the non-altered parent compound and metabolites and/or conjugates (Cháfer-Pericás et al., 2010). Because of the misuse, the antimicrobial residues in products of animal origin brought a concern to consumers. The residue of this kind of drugs can be directly toxic or even cause allergic reactions in some hypersensitive individuals. In addition, low-level doses of antimicrobial in foodstuff consumed for long periods can lead to the spread of drug-resistant microorganisms (Lopes, 2012).

Aquaculture has an annual growth of about 9% worldwide; in Brazil, the growth production of fish farming reached 60.2% only between 2007 and 2009 (FAO, 2009). Alone, the Nile tilapia (*Oreochromis niloticus*) production increased by 105% in just seven years (2003–2009) (Brasil, 2013a). Brazil has great potential for the development of aquaculture because of its 8400 km of seacoast and 5.5 million hectares of fresh water bodies, which contains approximately 12% of the fresh water available

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in the planet (FAO, 2012). In Brazil, 82.3% of the total fish production comes from continental aquaculture, and Nile tilapia is the most farmed aquatic species. Its production was 155,451 t in 2010, which corresponded to more than 39% of the total fish production (Brasil, 2012).

Nile tilapia cage farming in Brazil is characterized by high stocking densities (80 to 120 kg m^{-3}), with mortality rates ranging from 10 to 20% (Ayroza et al., 2014; Sabbag et al., 2007) whereas in other countries, densities range from 2 to 50 kg m^{-3} (Chakraborty et al., 2010; Gibtan et al., 2008; Ouattara et al., 2003; Watanabe et al., 1992; Yi et al., 1996). These conditions, processes of grading, capturing and transporting fish, poor water quality and all stressful factors increase the risks of disease outbreaks (Conte, 2004) and become the system increasingly dependent on chemical inputs, especially antimicrobials (Garcia et al., 2013). Moreover, the Brazilian farmers have only two available antimicrobials licensed for aquaculture; florfenicol and oxytetracycline (CPVS, 2013); because of lack of options, these commercial drugs are used repeatedly in the same production cycle.

The maximum residue limits (MRLs) are set based on scientific studies that ensure no long-term risks for consumers; they are relatively low at the level range from μg kg^{-1} to ng kg^{-1}, and because of this, analytical methods should be sensitive enough and able to detect the compounds unequivocally.

One of the main problems involved in the analysis of complex matrices such as muscle fish is the residue extraction procedure. Some simple, clean and rapid extraction methods are based on solid–liquid extraction (Romero et al., 2007) or solid-phase extraction (Gehring et al., 2006; Zhu et al., 2001). A widely used method for screening analysis is immunoaffinity chromatography (Senyuva and Gilbert, 2010), but it shows little selectivity. Metal chelate affinity chromatography (Cooper et al., 1998) has high throughput as it can be carried out on-line HPLC, on the other hand it can show co-eluting matrix interferences. Although the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method (Lopes, 2012) is being used successfully, there are problems with matrix interferences too. Subcritical water extraction (Wang et al., 2008) presents high selectivity, but thermolabile compounds could be decomposed. Other extraction procedures reported in the literature are a technique based on magnetic molecularly imprinted polymer (Chen et al., 2009), a pressurized liquid extraction (Liu et al., 2013), and a solid-phase microextraction (Tsai et al., 2009). In these procedures, extraction and clean up were processed in one-step, but they usually have poor reproducibility, sensibility and throughput. There is not any study about Captiva cartridges in analysis of antimicrobial residue in fish muscle. The chosen extraction procedures should be appropriate for the analysis intended and the reality of the laboratory, so factors such as less reagents consumption, labor availability and equipment acquisition are crucial. Simple and rapid methods stand out in this context because they are not dependent on high investments and also generate less hazardous waste and they are friendly to the environment.

The liquid chromatography tandem mass spectrometry triple quadrupole (LC–MS/MS) is an excellent analytical tool because of its high specificity and sensitivity; however, matrix components may influence the analyte response, so sample preparation is required, which is mainly a cleanup to minimize the negative effect.

The aim of this study was to develop and validate a rapid method using ultrafiltration by Captiva cartridges and liquid chromatography tandem mass spectrometry triple quadrupole (LC–MS/MS) and to determine the presence and quantify 12 antimicrobial residues in Nile tilapia of cage farming in the most important producing region of São Paulo State, Brazil.

2. Material and method

2.1. Chemicals

The solvents used were: methanol and acetonitrile HPLC grade purchased from Tedia Company Inc. (Fairfield, OH, USA), 99.5% formic acid from JT Baker (Phillipsburg, USA), Na2EDTA from Sigma-Aldrich (Dorset, UK). The water used was purified using a Milli-Q system from Millipore (Bedford, USA).

The antimicrobials were selected based on the Brazilian National plan of residues and contaminants (Brazil, 2009). The analytical standards of 97% oxytetracycline (OTC), 97.5% tetracycline (TC), 93% chlortetracycline (CTC), 99.5% ciprofloxacin (CFX), 99.0% enrofloxacin (EFX), 97.2% sarafloxacin (SAR), 99% norfloxacin (NFX), 98.0% sulfathiazole (STZ), 99.4% sulfadimethoxine-d6 (SDM-d6), 98.0% florfenicol (FF) were purchased from Sigma-Aldrich (St. Louis, USA), 99.5% sulfadimethoxine (SDM) and 99.5% sulfamethazine (SMZ) were purchased from ChemService (West Chester, USA) and 98.5% chloramphenicol (CAP) was purchased from Dr. Ehrestorfer GmbH (Augsburg, Germany).

Stock standard solutions of individual compounds (100 μg mL^{-1}) were prepared in methanol and stored at −20 °C in amber bottles up to six months. A multicompound working standard solution (1000 μg L^{-1}) was prepared using appropriate dilution of the stock solutions with water, stored under refrigeration (T below 5 °C) and renewed weekly.

2.2. Sampling and sample preparation

Samples were collected at four Nile tilapia cage farms located in Ilha Solteira hydroelectric reservoir, São Paulo, formed by Paraná and Grande rivers in Brazil. All units were georeferenced using GPS-Aquaread AP 5000 AgSolv (Kent, England) (Fig. 1).

In each cage farm, three cages were selected by giving preference to those who were under antimicrobial medication. Triplicates of Nile tilapias per cage were selected, totaling 36 samples. These fish were packed individually in plastic bags, stored in a Styrofoam box with ice and transported to the laboratory. At the laboratory, the muscle with the skin was chopped and crushed with dry ice using a blender with a glass cup. After proper labeling and conditioning in vials, they were stored at −18 °C for later extraction procedure, which was performed until 15 days after the sample collection.

Five g of sample was placed in a teflon tube of 50 mL with a screw cap. As an internal standard, 50 μL of sulfadimethoxine-d6 1.0 μg mL^{-1} was added to the sample, 1 mL of 0.1 M Na_2EDTA solution and 24 mL of acetonitrile:water (70:30, v/v) with 0.1% formic acid solution were included. The mixture was homogenized for 5 min by ultraturrax Marconi model MA102 (Piracicaba, Brazil) and then centrifuged in a Hitachi CF16RXII centrifuge (Hitachinaka, Japan) for 5 min at 1370 g. Afterwards, 500 μL of supernatant was eluted in Captiva ND (non-drip) filter cartridges of 3 mL 0.2 μm, polyvinyldifluoride and polypropylene from Agilent Technologies (Wilmington, USA), using a Manifold Supelco Visiprep System (Pennsylvania, USA) into the 2 mL vial, which was analyzed using LC–MS/MS.

2.3. LC–MS/MS analyses

The analyses were carried out in the LC–MS/MS system: Liquid Chromatograph 1200 from Agilent Technologies (Wilmington, USA) equipped with a binary pump and an automatic sampler G1367C. The chromatographic separations were carried out using Agilent Zorbax Eclipse Plus C18 (3 × 100 mm; 3.5 μm) column. The mobile phases were Milli-Q® water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient program started at 5% B with linear gradient until 95% B in 13 min and then constant for 3 min. After running for 15 min, the re-equilibrium time (post-time) was 10 min using 5% B. The flow remained constant at 0.4 mL min^{-1}, the column temperature was fixed at 30 °C, and the injection volume was 10 μL.

To preserve the sensitivity, florfenicol was injected separately in a negative ionization mode from other antimicrobials. The conditions were the same; only the gradient program was changed to the following: it started at 30% B until 2 min, followed by a start of the linear