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Original Research Article

A simple liquid chromatography coupled to quadrupole time of flight mass spectrometry method for macrolide determination in tilapia fillets

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ABSTRACT

A method for the identification and quantification of macrolides (erythromycin, josamycin, tilmicosin, tylosin, spiramycin and neospiramycin) in tilapia fillets by liquid chromatography coupled to quadrupole time of flight mass (LC–QToF) spectrometry is presented. Sample preparation was quite simple and low cost: proteins were precipitated and the analytes were extracted with ethanol, extract was defatted with hexane and concentrated by solvent evaporation. The matrix effect was statistically demonstrated during method validation, in which matrix-matched calibration was applied. Matrix effect mechanism was clarified thanks to the capability of QToF mass spectrometer of generating full scan spectra with accurate mass measurement. The limits of quantification were at least 45% lower than the maximum residue limits. The method was able to identify the studied macrolides with relative *m/z* errors lower than 2.5 ppm and to monitor two fragment ions per analyte, which is in accordance with the European Community recommendations for the analysis of contaminants in foods. Samples from the retail market of São Paulo State, Brazil were analyzed by the developed method and none of them presented positive results for the macrolides studied.

1. Introduction

World fish capture reached its apex in the 1990s. In contrast, aquaculture has presented growth rates close to those of the global population (FAO, 2009). Tilapia is the most widely raised species of any farmed fish. In 2004, tilapia was the eighth most popular seafood in the USA, and its production was projected to increase from 1.5 million tons in 2003 to 2.5 million tons in 2010, with a sales value of more than USD 5 billion (FAO, 2010). In this context, tilapia farming presents a very competitive scenario where producers keep the maximum fish density within a single tank. The use of antimicrobials in the production system for therapeutic (disease control) and prophylactic (disease prevention) purposes is almost inevitable because the spread of an eventual bacterial disease in the production tanks would certainly jeopardize production.

In the production of foods of animal origin, the incorrect use of antimicrobials or the disrespect of withdrawal time after treatment can lead to the presence of antibiotic residues in foods. These residues can promote the growth of pathogenic, drug-resistant bacterial strains and also cause allergic reactions in some hypersensitive individuals (Wang, 2009).

Macrolides are highly potent antimicrobials used in veterinary practices against a wide variety of Gram-positive and Gram-negative bacteria. Chemically, they consist of macrocyclic lactone rings with 14 (erythromycin, roxithromycin and clarithromycin), 15 (azithromycin) or 16 (spiramycin, tylosin, tilmicosin and josamycin) carbons linked to the carbohydrate molecules, presenting lipophilic and basic characteristics (Kanfer et al., 1998). In general, macrolide antibiotics present pK_a values between 7.1 and 9.9 (Gobel et al., 2004). These are important characteristics when someone considers the development of methods for the extraction of these substances from food matrices

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and their chromatographic separation. Some macrolide antibiotics are sensitive to low pH and suffer degradation under acidic conditions (Horie, 1995). Fig. 1 illustrates the molecular structures of the macrolides studied in this work.

The World Organization for Animal Health (WHO, 2007) considers three macrolides (josamycin, erythromycin and spiramycin) as "critically important" antimicrobials for fish harvesting because they are essential for fighting against specific infections that affect some species and because there is a lack of therapeutic alternatives.

Codex Alimentarius (2009) including the regional (European Community (EMA, 2011) and MERCOSUR, 2000) and national regulatory agencies (United States Department of Agriculture, 2010); the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA, 2010); and the Japan Minister of Health, Labor and Welfare (JFCRF, 2010) have all established maximum residue limits (MRL) for macrolides in food matrices, and some of them include MRL for fish matrices. Nonetheless, it is important to notice that neither the MERCOSUR (2000), nor the USFDA (2011) or Brazilian (MAPA, 2010) regulatory agencies have approved any macrolide drug for use in aquaculture.

The presence of macrolide residues in food due to their abusive use in veterinary practices has a significant impact on public health and on food international trade, and this has raised concerns in the scientific community and the regulatory agencies (Wang, 2009). Analytical methods for the identification and quantification of these antimicrobials are fundamental for the establishment of effective dose recommendations and withdrawal periods through pharmacokinetic studies and to evaluate the impact of the employment of these substances in aquaculture from an economic and food safety perspective.

Numerous analytical methods for the determination of residues of macrolides in fish and other edible animal tissues have been published recently (Jo et al., 2011; Horie et al., 2003; Wang and Leung, 2007; Lucchetti et al., 2005). However, few scientific papers about the analytical methods for quantifying macrolides in tilapia fillets by the use of liquid chromatography coupled to quadrupole time of flight (LC–QToF) spectrometry have been reported. Mass spectrometry (MS) is a universal detection technique, which is ideal for multi-residue analysis. The most commonly mass analyzers applied for contaminant determination in food matrices are triple-quadrupoles (QqQ). Although QqQ analysers present better sensitivity, QToF mass spectrometers can obtain full scan spectra with medium to high resolution and accurate mass measurement, besides having the capability of performing MS/MS analysis, so they generate spectra of better qualitative information, with enough sensitivity to meet the target of quantifying contaminants at concentrations below their maximum residue levels.

This article presents a simple method for the identification and quantification of six macrolides (erythromycin, josamycin, tilmicosin, tylosin, spiramycin and neospiramycin) in tilapia fillets by liquid chromatography coupled to a hybrid mass spectrometry system composed of quadrupole time of flight mass analyzers (LC–QToF). After validation, the method was applied to analyze samples from the retail market of São Paulo State, Brazil.

2. Materials and methods

2.1. Samples

Blank samples of refrigerated tilapia fillets were supplied by the School of Agronomical and Veterinary Sciences, Julio de Mesquita State University (UNESP – Jaboticabal, SP, Brazil), which guaranteed the absence of macrolide residues. These samples were separated into portions of approximately 500 g and stored at -25 ± 2 °C before being analyzed. The analytical signal of the tested macrolides proved to be stable for a period of ten days when ground, blank tilapia fillets were fortified with them at MRL concentrations and stored at -25 ± 2 °C.

Twenty samples were acquired from the retail market of São Paulo State (Brazil) and stored at -25 ± 2 °C before analysis. Twelve of the samples were entire fish obtained from "fish and pay pond" establishments (from the cities of Espirito Santo do Pinhal, Batatais and Ribeirão Preto) and were filleted before freezing, four were frozen fillets from large chain supermarkets (from the city of Campinas), and four were refrigerated, whole fish obtained from street fairs (from cities of Campinas, Jaguariuna and São Paulo), which were also filleted before freezing. All the samples were analyzed within a maximum period of 10 days of storage.

2.2. Chemicals and reagents

Reagent grade ethanol (Synth, Brazil) and LC grade n-hexane (Omnisolv, USA) were used in the extraction and clean up processes. LC grade methanol (MeOH) (Burdick & Jackson, USA), glacial acetic acid (HAc) (Merck, Brazil), and deionized water (purified by a Gehaka OS20 LX System, Brazil) were used to compose the mobile phase for liquid chromatography.

Primary–secondary amine (PSA), florisil, neutral aluminum oxide, and octadecilsilane (C_{18}) (Sigma–Aldrich, Germany) were tested as dispersive solid phase extraction (DSPE) and matrix solid-phase dispersion (MSPD) adsorbents.

Polyvinylidene difluoride (PVDF) hydrophilic and polytetrafluorethylene (PTFE) membranes (Millipore – USA) with 0.22 μm pore size were used to filter the aqueous and organic mobile phase solutions, respectively. PVDF hydrophilic syringe filters (Millipore – USA), with a 33 mm diameter and 0.22 μm pore size, were applied to filter the sample extracts before chromatographic injection.

Macrolide analytical standards used in this study were: erythromycin A (96.7%, U.S. Pharmacopeia, USA); roxithromycin (97.6%, Sigma–Aldrich, Switzerland); neospiramycin I (97.7%, Waco, Japan); spiramycin (88.9%, Fluka, Germany); tilmicosin (86.5%, Fluka, Germany); josamycin (100%, Fluka-Biochemika, Japan) and tylosin tartrate (98.0%, Dr. Ehrenstofer, Germany).

2.3. Standard solutions

All standard solutions were prepared in LC grade methanol. Stock solutions were prepared at the concentration of 1000 μg mL^{-1} and stored in tightly closed amber vessels at -25 °C for a maximum period of 3 months. Working solutions were prepared daily as a mixture of all macrolides through the dissolution of stock solutions and were used immediately after preparation. The final concentrations of macrolides in the working solutions were: 1.2 μg mL^{-1} for erythromycin; 4.0 μg mL^{-1} for spiramycin and neospiramycin; 2.0 μg mL^{-1} for tylosin and 1.0 μg mL^{-1} for tilmicosin and josamycin. The roxithromycin working solution was prepared separately at the concentration of 4.0 μg mL^{-1} and used as an internal standard. It was observed that the analytical signal of the stock solutions remained stable for a period of approximately 4 months when stored in tight closed amber vessels at -25 °C.

2.4. Equipment

A BL 2105 analytical balance (Sartorius, Germany) was used to weigh the reagents and standards. Samples were ground with a Walita RI 2044 mini food processor (Philips, Brazil). An Ultrasonic Cleaner (Cole Parmer, USA), a Himac CR21 centrifuge (Hitachi, Japan), an AP56 Vortex Agitator (Phoenix, Brazil) and a Centrivap

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