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A strategy for untargeted screening of macrolides and metabolites in bass by liquid chromatography coupled to quadrupole orbitrap mass spectrometry

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ABSTRACT

An analytical method for the non-target screening of macrolides and metabolites in bass (*Lateolabrax*) was developed using an automated on-line extraction procedure followed by ultrahigh-performance liquid chromatography coupled to electrospray ionization quadrupole Orbitrap high-resolution mass spectrometry (UHPLC Q-Orbitrap). The estimated performance characteristics were satisfied, complying with the requirements of the guidelines specified in European Commission Decision 2002/657/EC. The decision limit ranged from 0.12 μg kg^{-1} to 3.61 μg kg^{-1}, and detection capability ranged between 0.20 μg kg^{-1} and 6.02 μg kg^{-1}. Precision in terms of relative standard deviation (RSD) was under 14% for all compounds, and the extraction recoveries ranged from 81% to 107%. Finally, the method was applied to ten different commercially important bass species and confirmed the presence of ten macrolides and metabolites. Five non-target compounds of robenidine, lincomycin hydrochloride, thiacloprid, fenbendazole, and thiabendazole were elucidated in the untargeted screening.

1. Introduction

Aquaculture contributes to nearly half of the fish consumed by the human population worldwide (Guseo, 2016). In recent years, the demand for bass (*Lateolabrax*) has increased and is still growing rapidly than other aquaculture species (Cordero et al., 2016), especially in Europe and Eastern Asia, because of its characteristic good flavour and high nutritional value (Ryan, Wögerbauer, & Roche, 2016). Because of their high oral bioavailability, rapid absorption and long half-life (Dickson, 2014), macrolides are widely used as fish-feed additives to prevent diseases and for the clinical treatments of bacterial infections (Hammad, Watanabe, Fujii, & Shimamoto, 2012), such as *Chlamydia* spp. and bacterial kidney disease (Yamada, Kikuchi, Tanaka, & Numata, 2012). The accumulation of macrolides and their metabolites in edible fish tissues might have potential threat to human health (Arfè, Blasi, Merlino, & Corrao, 2016), causing allergic reaction, carcinogenic and teratogenic effects as well as increased antibiotic resistance (Entorf, et al., 2016). Macrolide antibiotics have been prohibited from using as feed additives for growth promotion (sub-therapeutic) in European Union (EU) and the United States. Nevertheless, as a result of cross-contamination or illegal use, the unintentional presence of some compounds such as roxithromycin, can be found in edible fish tissues. Furthermore, some of these antibiotics have been detected in Surface Water from the North Canal River (Li, Zhang, Wu, & Zhao, 2014), and Huangpu river and Yangtze estuary (Wang et al., 2016), which are the primary water sources for Beijing and Shanghai (China). Concentrations of roxithromycin and tilmicosin in Surface Water have been detected between 1 and 16 ng L^{-1} and 0.3–48 ng L^{-1}, respectively (Xue, Qi, & Liu, 2015). Since the EU maximum residue levels (MRLs) for spiramycin are based on the sum of spiramycin I and its metabolite neospiramycin I (Council Regulation 37/210), the metabolites must be included in any analytical methods (Granelli & Branzell, 2007). Thus, supervising and controlling macrolide drug residues and their metabolites in edible tissues has received considerable attention in many countries (Mitchell, Ullman, Teel, & Watts, 2015; Sismotto, Paschoal, Teles, de Rezende, & Reyes, 2014; Zhu et al., 2014).

High-resolution mass spectrometry couple to high-performance liquid chromatography (HPLC-HRMS) has shown merits in meeting the targeted multi-residue analysis of veterinary drugs and metabolites in animal tissues (Berendsen, Stolker, Nielen, & Nielen, 2013; Gómez-Pérez, Plaza-Bolaños, Romero-González, Martínez-Vidal, & Garrido-Frenich, 2012; Kaklamanos, Vincent, & von Holst, 2013; Kaufmann, Butcher, Maden, Walker, & Widmer, 2014; Singh et al., 2012). New analytical opportunities arose with the development of Orbitrap mass

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spectrometers (Kaklamanos, Vincent, & von Holst, 2013; Kaufmann & Walker, 2013), or time-of-flight (TOF) analyzers providing high specificity and selectivity over the full spectrum based on accurate mass lists or collision induced dissociation (CID) spectra (Boix et al., 2014; Guidi, Tette, Fernandes, Silva, & Gloria, 2017). Using selective screening by data-dependent fragmentation acquisition (DDA) and powerful confirmation by highly specific product ion scan (Wang, Leung, Chow, Chang, & Wong, 2015; Yang et al., 2015). Wang, Chow, Chang, and Wong (2014) developed a Q-TOF screening with a HR spectra library of 500 compounds. Data-dependent fragmentation approaches are superior for target screening (Cai, Ting, & Jin-lan, 2016), but the data are limited as a result of the pre-determined nature of list transitions (Jia, Chu, Ling, Huang, & Chang, 2014). These techniques are "blind" to any compound present in samples but not included in the list of monitored analytes (Cherta et al., 2015). Consequently, by retrospective analysis, it is unfeasible for searching metabolites (Jia, Chu, Chang, & Zhang, 2015; Zomer & Mol, 2015), transformation products, and new compounds suspected to be present in the samples (Gómez-Nieto, Gismera, Sevilla, & Procopio, 2017; Jia et al., 2014; Jia, Ling, Lin, Chang, & Chu, 2014). Another disadvantage is that signals are only obtained a single scan, and no information of the chromatographic peak profiles are obtained for the fragments (Chin, Eyres, & Marriott, 2015).

Lacking of methodologies for non-targeted data acquisition for identification of macrolides and metabolites in bass demand for new developments to fulfill the requirements of the control program (Fu, Zhao, Lu, & Xu, 2017; Knolhoff & Croley, 2016; Knolhoff, Zweigenbaum, & Croley, 2016). In the present study, we have developed an analytical strategy for the quantitative untargeted screening of macrolides, their metabolites and other undesirable substances in fish using an automated on-line QuEChERS extraction procedure followed by UHPLC/ESI Q-Orbitrap analysis. The macrolides and their metabolites were selected based on their worldwide use and frequency of occurrence in the fish tissue. After the method was validated, it was applied to analyze 126 bass samples. To our knowledge, a retrospective Q-Orbitrap screen of macrolides and their metabolites in bass firstly represented in this paper.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Sodium sulfate anhyfrous (Na_2SO_4), potassium carbonate (K_2CO_3), sodium hydrogen carbonate ($KHCO_3$), formic acid, and ammonium formate were of analytical grade and purchased from J.T. Baker (Phillipsburg, NJ, USA). Primary-secondary amine (PSA) and QuE Z-Sep$^+$ sorbent (zirconium oxide based) were provided by Supelco (Bellefonte, PA, USA). Ethylene diamine tetraacetic acid disodium salt (Na_2EDTA) was purchased from Merk (Darmstadt, Germany). Deionized ultra pure water (> 18.2 MΩ cm resistivity) was purified on a Milli-Q Plus Water System (Millipore, Brussels, Belgium). All commercial standards were purchased from RIKILT (Community Reference Laboratory, Wageningen, Netherlands), Dr. Ehrenstorfer GmbH (Augsburg, Germany), European Pharmacopoeia (EDQM, Strasbourg, France), Sigma-Aldrich (Steinheim, Germany), USP Reference Standards (Maryland, United States), LGC Standards (Teddington, UK) or Witega (Berlin, Germany). Macrolides are divided into three groups according to the number of atoms in the lactione nucleus: 14-membered-, 15-membered-, and 16-membered macrolides compounds. The internal standards used were erythromycin A-$^{13}C_2$ for 14-membered macrolides, azithromycin-d3 for 15-membered macrolides, and tilmicosin-d3 for 16-membered macrolides.

2.2. Standards solutions

Individual macrolides, metabolites and the corresponding isotopically labeled analogues stock solutions at a concentration of 1 mg mL^{-1} were prepared by weighing the appropriate amount of standard, diluting it in methanol, and storing in tightly closed amber vessels at −20 °C. Intermediate working solutions and the mixtures of working internal standards were prepared at 5 μg mL^{-1} in methanol from stock solutions. Mixtures of working solutions were prepared daily as a mixture of all analytes through the dilution of stock solutions and were used immediately after preparation.

2.3. Sample collection and preparation

Ten different commercially important bass species were considered in this study: three of them were from the Lateolabracidae family, 16 roughskin sculpin (*Trachidermus fasciatus*), 19 Japanese seabass (*Lateolabrax japonicus*), and 8 blackfin seabass (*Lateolabrax latus*),3 from the Moronidae family, 17 European seabass (*Dicentrarchus labrax*), 6 white bass (*Morone chrysops*) and 13 striped bass (*Morone saxatilis*), and 4 from the Centrarchidae family, 14 Guadalupe bass (*Micropterus treculii*), 13 spotted bass (*Micropterus punctulatus*), 14 largemouth bass (*Micropterus salmoides*), and 6 smallmouth bass (*M. dolomieu*). A total of 126 frozen skinless boned bass filets were collected from different retail commercial outlets and stored at −80 °C. Approximately 25 g bass muscle tissue was removed and homogenized with a Polytron PT-3100 (Kinematica, Luzern, Switzerland) for 60 s till no chunk larger than 1/5 in. was left and stored at −20 °C prior to analysis.

All automated sample preparation steps for the macrolides and metabolites determination were performed using a dual-head MultiPurpose Sampler (Gerstel MPS XL) equipped with a ^{m}VORX vortex, disposable pipettes extraction (DPX) Option, and CF-100 dual position centrifuge (Gerstel, Columbia, MD, USA). Accurately one gram of test portions was weighted into 10 mL centrifuge tubes (VWR International, Mississauga, Ontario, Canada). Then, 10 μL mixture of internal standard solution was added, and the samples were vortex mixed for 60 s. Afterwards, samples were held for 20 min at 4 °C in the dark, then 5 mL of acetonitrile/water solution (84/16, *v/v*) with 0.1 M of EDTA was added to each tubes. Next, the mixtures were vigorously homogenized using the ^{m}VORX vortex mixer for 1 min atmaximum speed (3200 rpm). Sodium acetate anhydrous (1.00 g), potassium carbonate (0.25 g), and sodium hydrogen carbonate (0.25 g) were added to the DPX tip (5 mL), to induce phase separation, and then 2 mL of samples were aspirated into a DPX tip three times from the bottom followed by an equilibration time of 30 s. Empty 5 mL volume DPX tip was purchased from Gerstel (Columbia, MD, USA). The sorbent powdered sorbent is dispersed between two frits (0.45 μm filter), one located at the upper portion of the pipettes tip and the other located at the bottom. The DPX tip acts as a filter removing the salt particulate matter from the solution. The extracts were then centrifuged at 2264 × *g* for 5 min to precipitate the debris, and 1 mL aliquots of the supernatant were transferred into a 5 mL DPX tip (Fig. S1, Electronic Supplementary Material). DPX Cleanup was performed by adding a mixture of 32 mg of Z-Sep$^+$, 75 mg of PSA, and 0.25 g of Na_2SO_4, which were subsequently vortexed for 1 min and centrifuged (2264 × *g*, 5 min). An aliquot of the final upper phases (200 μL) was transferred into a Mini-UniPrep vial, to which 300 μL of methanol, 500 μL of 8 mM ammonium formate were added, and 5 μL of the diluted extract was injected into the UHPLC Q-Orbitrap system for analysis.

2.4. Instrumentation

Chromatographic separation was performed on a Dionex Ultimate 3000 UHPLC system (Dionex Corporation, Sunnyvale, CA, USA). Analytes were separated on an Hypersil Gold aQ C18 column (100 mm × 2.1 mm, 1.9 μm) connected with the guard column Accucore aQ C18 (10 mm × 2.1 mm, 1.9 μm) both from Thermo Fisher Scientific, (San Jose, USA). Mobile phase A and B were water and methanol, respectively, both containing formic acid and 4 mM

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