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Analytical Methods

Determination of 21 antibiotics in sea cucumber using accelerated solvent extraction with in-cell clean-up coupled to ultra-performance liquid chromatography-tandem mass spectrometry

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Ciprofloxacin (PubChem CID: 2764)
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ABSTRACT

An accelerated solvent extraction (ASE) with in-cell clean-up method coupled to ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was developed to determine 21 antibiotics in sea cucumber. The analytes include 10 sulfonamides, 4 fluoroquinolones, 3 amphenicols, 2 beta-lactams, 1 lincosamide and trimethoprim. Optimal parameters of ASE method were obtained at 80 °C, 1 static cycle of 5 min with methanol/acetonitrile (1/1, v/v) using 2 g of C18 as adsorbent. Recoveries at 50.1–129.2% were achieved with RSD under 20%. Method detection limits ranged from 0.03 to 2.9 μg kg^{-1}. Compared to the reported ultrasound-assisted extraction method, the proposed method offered comparable extraction efficiency for sulfonamides from sea cucumber, but higher for other categories of antibiotics. This validated method was then successfully applied to sea cucumber samples and 9 antibiotics were detected with the highest concentration up to 57.7 μg kg^{-1} for norfloxacin.

1. Introduction

Veterinary antibiotics are a group of pharmaceuticals and personal care products (PPCPs), which are largely used in livestock and aquaculture industry to treat and prevent diseases or as growth promoter for animals (Cabello, 2006; Van Boeckel et al., 2014). China, the largest producer and user of antibiotics in the world, consumed about 162, 000 tons of antibiotics in 2013 alone, approximately 52% of which was shared by animals (Zhang, Ying, Pan, Liu, & Zhao, 2015; Zhu et al., 2013). It has been estimated that about 75% of the veterinary antibiotics are excreted via urine or feces as parent compounds or bioactive metabolites (Alvarez-Muñoz, Huerta, Fernandez-Tejedor, Rodríguez-Mozaz, & Barceló, 2015; Chee-sanford et al., 2009). As a result, they have been detected at nanogram to microgram per liter levels in the aquatic environment (Gao, Shi, Li, Liu, & Cai, 2012; Pham et al., 2015). Ecotoxicological studies have shown that long term exposure to lower concentration of antibiotics could cause adverse effects on aquatic organisms. For example, the chronic assay with *D. magna* has proven that antibiotic ciprofloxacin can induce impairments on its life-history parameters (Martins et al., 2012). Furthermore, antibiotics can contribute to the emergence of antibiotic resistance bacterial, which will turn into a much bigger problem in the treatment of infectious diseases (Luo et al., 2010). World health organization (WHO) identified antibiotic resistance as one of the three greatest threats to global health in 2014 (WHO, 2014). To protect public health and ensure food safety, European Union (EU), the US Food and Drug Administration (FDA), China and others have regulated maximum residue limits (MRLs) of veterinary drugs in animal origin food.

Recent studies have focused on the determination of antibiotics in aquaculture products such as fish, shrimp and bivalve (Dasenaki & Thomaidis, 2010; Dufresne, Fouquet, Forsyth, & Tittlemier, 2007; Jang, Lee, & Kim, 2015), while little information is available for sea cucumber (*Holothuroidea, Echinoderms*), which is one of the most precious marine foods and commercially important aquatic products with production of more than 100,000 tons in China (Zhao, Guo, Quan, Jiang, & Qu, 2016; Li, Li, Guo, Li, & Zhu, 2015). Sea cucumber is rich in biological components, mainly including proteins, lipids and mucopolysaccharides (Farjami, Nematollahi, Moradi, & Nazemi, 2014), which

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may interfere with sample extraction and instrumental analysis as well as produce matrix effect. So it is a challenge to develop a simple, fast and effective method to analyze antibiotics in sea cucumber samples.

Determination of antibiotics in biological matrices generally comprises sample extraction, clean-up method and instrument analysis. In recent years, high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and ultrahigh-pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) detection have been used as preferential tools in the quantitative analysis of antibiotic residues (Moreno-Bondi, Marazuela, Herranz, & Rodriguez, 2009; Santos & Ramos, 2016). Extraction methods such as liquid-liquid extraction (LLE) (Mottier, Parisod, Gremaud, Guy, & Stadler, 2003), microwave–assisted solvent extraction (MASE) (Dorival-García, Labajo-Recio, Zafra-Gómez, Juárez-Jiménez, & Vílchez, 2015), and ultrasound-assisted extraction (USE) (Dorival-García, Junza, Zafra-Gómez, Barrón, & Navalón. 2016; Zavala, & Reynoso-Cuevas, 2015) present a poor selectivity. As a consequence, appropriate clean-up methods such as solid-phase extraction (SPE), dispersive solid-phase extraction (d-SPE) (Guo et al., 2016) and liquid-liquid partitioning are commonly required to further remove matrix-interfering compounds. Unfortunately, these sample pretreatment methods are often complex to handle, difficult to automate, solvent-consuming and time-consuming. In order to decrease sample handling time and simplify procedures, previous studies reported the accelerated solvent extraction (ASE) with in-cell clean-up method using adsorbents such as modified silica, florisil or alumina to simultaneously extract and purify persistent organic pollutants from environmental samples (Duodu, Goonetilleke, & Ayoko, 2016; Malavia, Santos, & Galceran, 2011). However, this method has been hardly explored to analyze multi-class antibiotics in biological samples.

In this study, a simple, fast and efficient ASE with in-cell clean up method coupled to UPLC-MS/MS for analyzing multiple classes of antibiotics in sea cucumber was developed. Several parameters of the ASE method, such as extraction solvent, temperature, static time, number of cycle, retainer type and retainer mass, were optimized in order to maximize recovery with the minimum organic solvent consumption and analysis time. Furthermore, the proposed method was validated and compared to the reported USE method. At last, the developed method was applied to analyze antibiotics in sea cucumber samples and nine target compounds were detected.

2. Materials and methods

2.1. Chemical reagents and materials

All standards of antibiotics with high purity grade (≥98.0%) were acquired from Dr. Ehrenstorfer (Augsburg, Germany) except for sulfamonomethoxine from Sigma-Aldrich (St. Louis, MO, USA). Sulfadoxine-D4, sulfamethoxazole-^{13}C6, enrofloxacin-D5, trimethoprim-D3, and chloramphenicol-D5 were used as surrogate standards. Atrazine-D5 was used as an internal standard.

HPLC grade methanol (MeOH), acetonitrile (ACN) and *n*-hexane were obtained from Sigma-Aldrich. Analytical reagent formic acid (99.0%) and ammonium formate (99.0%) were purchased from Tianjin Kermel Chemical Reagent Co., Ltd (Tianjin, China). Diatomaceous earth was bought from Dionex (Thermo Fisher Scientific, MA, USA) and was dealt with muffle furnace for 4 h at 350 °C to discharge impurities. SKR series silica-based reversed phase chromatography media C18 (mean particle diameter 50 μm, carbon loading 17.5%) was purchased from Jinan Bona Biological Technology CO., Ltd. (Shandong, China), soaked in MeOH for 6 h. Primary secondary amine (PSA) bulk powder was obtained from Bonna-Agela Technologies Inc. (Tianjin, China). Alumina-neutral (Al-N) was purchased from Waters Crop. (Milford, MA, USA). Syringe filters of 0.22 μm were purchased from Tianjin Jinteng Experiment Co., Ltd (Tianjin, China). Water was purified using a Heal Force system from Heal Force Bio-meditech Holdings Limited Co., Ltd (Shanghai, China). KQ5200B-type ultrasonic cleaner was obtained from Kunshan ultrasonic instruments Co., Ltd (Jiangsu, China).

2.2. Standard solutions

Standard stock solutions were prepared by dissolving each standard in MeOH at a concentration of 1000 μg mL^{-1}. Standard work mixture containing thiamphenicol, florfenicol, chloramphenicol, sulfamethoxazole, sulfapyridine, sulfamethyldiazine, sulfadimethoxine, sulfaguanidine, sulfamethazine, sulfamonomethoxine, sulfachlorpyridazine, sulfathiazole, sulfadiazine, penicillin G sodium salt, amoxicillin trihydrate, lincomycin hydrochloride monohydrate, trimethoprim, norfloxacin, enrofloxacin, ciprofloxacin and enoxacin was prepared by diluting the stock solutions in MeOH at a concentration of 1 μg mL^{-1}. Work solutions of surrogate standards and internal standard were prepared by diluting stock solution in MeOH or ACN depending on their solubility. They were stored in darkness in a refrigerator at −20 °C.

2.3. Sample pretreatment

The ASE method was performed on a Dionex™ ASE™ 350 system (Thermo Fisher Scientific). Before extraction, 0.5 g of the freeze-dried sea cucumber samples spiked with 100 μL of 0.5 μg mL^{-1} surrogate standards were transferred into a stainless-steel extraction cell, and were fortified at 200 μg kg^{-1} by pipetting 100 μL of 1 μg mL^{-1} standard work mixture. To prevent the metal from clogging, an extraction cell filter was placed into the cell outlet. Extraction conditions such as extraction solvent, temperature, static time, number of cycle, retainer type and retainer mass were optimized. Samples were finally extracted at 80 °C (heating time of 5 min), 1500 psi with MeOH/ACN (1/1, v/v), applying 1 static cycle of 5 min with a flushed volume of 60% and purged time of 60 s. The extraction time was about 15 min per sample. The final extract was then solvent-reduced to 1 mL under gentle flow of nitrogen gas, adding 100 μL of 0.5 μg mL^{-1} internal standard. Finally the solution was filtered and transferred to a HPLC vial for further instrument analysis.

2.4. UPLC-MS/MS conditions

The chromatographic analyses were performed on an Acquity UPLC I-Class system (Waters, MA, USA). Separations were achieved on an Xterra C_{18} column (3.5 μm particle size, 2.1 × 100 mm, 125 Å average pore diameter, Waters) at 40 °C. The analytes were separated with a mobile phase consisting of water containing 0.1% formic acid and 0.1% ammonium formate (eluent A) and MeOH/ACN (1/1, v/v) (eluent B) at a flow rate of 0.3 mL per min. The elution gradient started with 5% of eluent B, and then kept at 5% (eluent B) for 1 min, the percentage linearly increased to 90% in 24 min. After that, it readjusted to the initial conditions in 1 min and equilibrated for 4 min.

The mass spectrometric analyses were carried out using a Xevo TQ-S triple quadrupole mass spectrometer (Waters). The analytes were determined using multiple reaction monitoring (MRM) mode of at least two transitions. The source of the mass spectrometer was operated in positive or negative modes with electrospray ionization (ESI) source. Capillary voltage was set at 3.0 kV for all the analytes. Source temperature was set at 150 °C, desolvation temperature was at 350 °C, collision gas flow was at 0.25 mL min^{-1}, cone and desolvation gas flow (nitrogen) were at 150 and 650 L h^{-1}, respectively. Collision-induced dissociation energy with argon was optimized for each MS/MS chosen transition (Table 1).

2.5. Method performance

The method was evaluated by matrix effect, specificity, method detection limit (MDL), linearity, recovery, precision, and comparison against the reported USE method. Matrix effect was calculated by comparing the slopes of matrix-matched calibration curves and