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Multi-residue and multi-class method for the determination of antibiotics in bovine muscle by ultra-high-performance liquid chromatography tandem mass spectrometry

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ABSTRACT

A multi-residue quantitative screening method covering 41 antibiotics from 7 different families, by ultra-high-performance–liquid-chromatography tandem mass spectrometry (UHPLC–MS/MS), is described. Sulfonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol are simultaneously detected after a simple sample preparation of bovine muscle optimized to achieve the best recovery for all compounds. A simple sample treatment was developed consisting in an extraction with a mixture of acetonitrile and ethylenediaminetetraacetic acid (EDTA), followed by a defatting step with n-hexane. The methodology was validated, in accordance with Decision 2002/657/EC by evaluating the required parameters: decision limit (CCα), detection capability (CCβ), specificity, repeatability and reproducibility. Precision in terms of relative standard deviation was under 20% for all compounds and the recoveries between 91% and 119%. CCα and CCβ were determined according the maximum residue limit (MRL) or the minimum required performance limit (MRPL), when required.

1. Introduction

In food producing animals, antibiotics are widely used and administrated as feed additives and in drinking water not only to treat and prevent diseases but also to illegally stimulate animal growth (Laxminarayan et al., 2013; Wassenaar, 2005).

The continuous use of these drugs carries the risk of their presence in edible tissues which, for consumers, can be responsible for toxic effects and allergic reactions in hypersensitive individuals (Le Bizec, Pinel, & Antignac, 2009, Marazuela and Bogialli, 2009). It can also result in the development of resistant strains of bacteria that might compromise the efficiency of antibiotics used for treatment of animals (Laxminarayan et al., 2013). When that occurs it became difficult to treat serious diseases, increasing the negative effects in animal welfare and consequently severe consequences for productivity and economy. Furthermore, the potential spread of resistant strains of bacteria from animals to humans can have the same effect when using antibiotics as human medicines (Doyle & Erickson, 2006). These concerns make the analysis of antibiotic residues in food producing animals an important field in food safety. To control abusive situations, and because food safety is a key police priority for the European Commission (Commission of the European Communities, 2000); several official documents were settled down to regulate the control of veterinary drugs in products of animal origin. The Council Directive 96/23/EC (European Commission, 1996) determines the measures to monitor certain substances and residues of veterinary medicines in living animals and in animal products. This directive foresees laboratorial control. For permitted veterinary drugs, tolerance levels were established as maximum residue limits (MRLs) in foodstuff of animal origin and listed in the EU Commission Regulation 37/2010 (European Commission, 2009; European Commission, 2010). For non-authorized substances there are no tolerance levels but, for some compounds, to harmonize the analytical performance of the methods, a minimum required performance limit (MRPL) had been set (European Commission, 2002; SANCO, 2007). The MRPL level is not a concentration obtained from toxicological data, but is only related with analytical performance. The European Decision 2002/657/EC (European Commission, 2002) describes the requirements for the performance and validation of the analytical methods employed in the official residues control. To fulfill such requirements it is important to have sensitive and specific analytical methodologies capable of monitoring the use or potential abuse of these drugs in the field of animal husbandry, ensuring that MRL levels are respected. The concern about having efficient screening methods is increasing and also about the improvement of cost-effectiveness of analytical procedures (Kaufmann, 2009; Martos et al., 2010; Reig & Toldrá, 2008). Typically the methods used in laboratory are multi-detection of related compounds, usually

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from the same family of antibiotics. That means that a single sample, to be analyzed for different groups of antibiotics, became part of a time consuming process that can last weeks. The delayed final result is associated with high cost and turns to be questionable in terms of usefulness of the result. This efficiency can be gathered in multi-class and multi-detection methods based on liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) being the tool of choice, providing the required degree of confidence for veterinary residue analysis in biological samples (Kaufmann, 2009; Le Bizec et al., 2009). Nowadays, the use of ultra-high performance liquid chromatography (UHPLC) provides numerous advantages in terms of resolution, sensitivity and also in minimizing time of analysis which is an important feature when running numerous samples in routine laboratories (DeBrabander et al., 2009; Geis-Asteggiante et al., 2012; Lehotay et al., 2012; Malik, Blasco, & Picó, 2010). Despite that, the simultaneous determination of antibiotics from different pharmacologic families in complex biological matrices, such as bovine muscle, has several constrains mainly related with the differences in physicochemical properties of the compounds (DeBrabander et al., 2009; Kinsella, O'Mahony, Cantwell, Furey, & Danaher, 2009).

In the literature, only few methods, combining multi-detection and multi-class in a quantitative screening method for bovine muscle, are available. Martos et al. (2010) describe an LC–MS/MS method for the screening of 39 compounds from 7 families of antibiotics, although not validated. Granelli, Elgerud, Lundström, Ohlsson, and Sjöberg (2009) presented an LC–MS/MS method for the determination of 19 compounds, from 5 classes. A group of the US Department of Agriculture (Geis-Asteggiante et al., 2012; Lehotay et al., 2012) described a qualitative screening method for the determination of more than 100 compounds in bovine muscle and/or in the kidney, by UHPLC–MS/MS, including not only antibiotics, but also several other drugs, such as anthelmintics, thyreostatics, beta-agonists, hormones, NSAIDS and tranquilizers. Although proved to be efficient for screening purposes, the validation presented is not based on European Commission requirements (European Commission, 2002). Recently, multi-detection methods for the analysis of veterinary drugs using liquid chromatography coupled with time-of-flight mass spectrometry (LC–ToF-MS) have been published (Peters, Bolck, Rutgers, Stolker, & Nielen, 2009) and UHPLC–ToF-MS (Kaufmann, Butcher, Maden, & Widmer, 2008). One of the main advantages is the possibility of analyzing an unlimited number of analytes in a single run, since the detection by ToF-MS is not limited by dwell time (Stolker, Zuidema, & Nielen, 2007). Nevertheless, although it can be applied for screening and quantification purposes it cannot be used as confirmatory methods due to the requirements of legislation (European Commission, 2002) and always obliges the confirmation of positive findings using a MS/MS detector.

The present paper describes the development and validation of a simple and effective quantitative screening method by UHPLC–MS/MS for the simultaneous detection of 41 antibiotic compounds from sulfonamides, tetracyclines, penicillins, macrolides, quinolones, trimethoprim and chloramphenicol in bovine muscle. Validation procedure followed the requirements from the European Commission Decision 2002/657/EC (European Commission, 2002) in order to apply the method in routine analysis.

2. Material and methods

2.1. Reagents, solvents and standard solutions

All reagents and solvents used were of analytical grade with the exception of chemicals used for the mobile phase, which were of high-performance liquid chromatography grade. Methanol, acetonitrile and formic acid were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma-Aldrich (Madrid, Spain). All standards of sulfonamides, tetracyclines, penicillins, macrolides, quinolones, trimethoprim and chloramphenicol were supplied by Sigma-Aldrich (Madrid, Spain). The individual standards are listed in Table 1. Six internal standards were used: demethyltetracycline for tetracyclines, penicillin V for penicillins, lomefloxacin for quinolones, roxithromycin for macrolides, sulfameter for sulfonamides and for trimethoprim and chloramphenicol-d5 for chloramphenicol. All the internal standards were provided by Sigma-Aldrich. For all substances, stock solutions of 1 mg mL^{-1} were prepared by weighing the appropriate amount of standard, diluted in methanol, and storing at −20 °C. Suitable dilutions were also prepared to have convenient spiking solutions for both the validation process and the routine analysis.

2.2. Instrumentation

For the sample preparation, the following equipment was used: Mettler Toledo PC200 and AE100 balances (Greifensee, Switzerland), Heidolph Reax 2 overhead mixer (Schwabach, Germany), Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), Turbovap Zymark Evaporator (Hopkinton, MA, USA) and Whatman Mini-Uniprep PVDF 0.45 μm filters (Clifton, NJ, USA). Chromatographic separation and mass spectrometry detection were performed with a Xevo TQ MS — Acquity UHPLC system coupled to a triple quadrupole tandem mass spectrometer from Waters (Milford, MA, USA). The electrospray ion source in positive (ESI+) and negative (ESI−) modes was used with data acquisition in multiple reaction monitoring (MRM) mode and analyzed using Masslynx 4.1 software (Waters). The MRM optimized conditions are presented in Table 1. The UHPLC system consisted of a vacuum degasser, an autosampler and a binary pump equipped with an analytical reverse-phase column Acquity HSS T3 2.1 × 100 mm with 1.8 μm particle size (Waters). The mobile phases used were: [A] formic acid 0.1% (v/v) in water and [B] acetonitrile. The gradient program used, at a flow rate of 0.45 mL min^{-1}, was: 0–5 min from 97% [A] to 40% [A]; 5–9 min from 40% to 0% [A]; 9–10 min from 0% back to 97% [A]; 11–12 min 97% [A]. The column was maintained at 40 °C, the autosampler at 10 °C and the injection volume was 20 μL.

2.3. Sample preparation

A portion of 2.0 ± 0.05 g of minced and mixed bovine muscle sample was weighed into a 20 mL glass centrifuge tube. The internal standard solution was added, then vortexed for 30 s and allowed to stand in the dark for at least 10 min.

Afterwards, twelve different extraction procedures were tested; the list of them and the main steps are presented in Table 2.

The liquid extraction was performed by shaking the sample with the solvent using a Reax shaker for 20 min followed by centrifugation for 15 min at 3100 *g*. The supernatant was transferred into a new tube and, for extractions *ADry*, *MDry* and *EaDry* evaporated to dryness under a gentle stream of nitrogen, at 40 °C. For the extract samples *A*, *M* and *Ea* the evaporation were just until 0.5 mL. Procedures *AHxDry*, *MHxDry*, *EaHxDry*, *AHx*, *MHx* and *EaHx* followed a defat step by adding 3 mL of n-hexane to the supernatant obtained after centrifugation. The extracts were vortexed for 30 s and centrifuged for 15 min at 3100 *g*. The n-hexane layer was discarded and, for extractions *AHxDry*, *MHxDry* and *EaHxDry* they were evaporated to dryness under a gentle stream of nitrogen, at 40 °C. For extract samples *AHx*, *MHx* and *EaHx* the evaporation was just until 0.5 mL. In all procedures, the residue was redissolved with mobile phase A (400 μL) or added to the 0.5 mL of final extract, filtered through a 0.45 μm PVDF Mini-uniprep™, transferred to vials and injected into the UHPLC–MS/MS under MRM optimized conditions for each compound (Table 1).

2.4. Validation procedure

The validation procedure followed that of described by the EU Commission Decision 2002/657/EEC (European Commission, 2002). According to those requirements, specificity, recovery, repeatability,