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The development and validation of a rapid method for the determination of antimicrobial agent residues in milk and meat using ultra performance liquid chromatography coupled to quadrupole – Orbitrap mass spectrometry

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ABSTRACT

A new multi-class method has been developed for the identification and quantification of the residues of 26 antibiotics from different classes (sulfonamides, macrolides, tetracyclines, penicillins, and quinolones) in milk and meat by ultra performance liquid chromatography coupled to hybrid quadrupole – high resolution Orbitrap mass spectrometry (UPLC–qOrbitrap). The sample preparation included extraction of two analytical portions with acetonitrile and 5% trichloroacetic acid, respectively, followed by centrifugation and filtration. The method was validated over three days at 50% of MRL (maximum residue limit) set in the European Union. Experiments on spiked meat and milk samples showed that the average recovery of the antibiotics ranged from 83% to 112%, and the coefficients of variation were between 8.9% and 39%.

1. Introduction

Antibiotics are widely used in veterinary medicine to treat and prevent bacterial diseases. Extensive and improper application of these drugs can lead to residues in animal products, which present a hazard for human health, potentially causing allergic reactions in sensitive humans and reducing the effectiveness of antibiotics to treat diseases, due to the occurrence of new strains of antibiotic-resistant bacteria. European Union (EU) legislation has established the maximum residue limits (MRLs) of the veterinary drugs in edible animal tissues in order to protect the consumers [1]. The EU Council Directive 96/23/EC [2] requires implementing the control system for certain veterinary drugs in live animals and in animal products. In order to ensure the fulfillment of these regulatory requirements regarding the control of antibiotics residues, it is necessary to employ sensitive, selective and accurate analytical methods.

Several techniques have been proposed for the analysis of antibiotics in foodstuffs, including microbial or immunoassays [3], capillary electrophoresis (CE) [4] and high performance liquid chromatography (HPLC) [5–8] with fluorescence (FLD), ultraviolet (UV), diode array detectors (DAD), and mass spectrometric (MS) [9–12] detection. Despite the availability of several analytical techniques, currently there is an increasing demand for fast, sensitive, and reliable multi-class multi-residue methods, which could reduce costs and achieve high sample throughput. High resolution mass spectrometry (HRMS) is a promising approach for routine screening and confirmation of antibiotics residues in animal origin products. Several methods have been published for the determination of veterinary drug residues applying time-of-flight mass spectrometry [13–16] and Orbitrap mass spectrometry [17–20]. These methods provide a high level of selectivity, however, in most cases the sensitivity is lower in comparison to HPLC coupled with tandem quadrupole mass spectrometry [21]. The previously described Orbitrap mass spectrometric procedures for the analysis of antibiotics residues were performed in full scan mode and confirmed the great potential of this type of mass spectrometry for the analysis of drug residues in objects of animal origin [22]. The recently introduced Q-Exactive mass spectrometer provides the possibility of combining the high resolution detection of

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analytes of interest specific for the Orbitrap mass analyzer with an enhanced selectivity and sensitivity ensured by tandem mass spectrometry, thus providing a more capable alternative to the existing MS approaches [23,24].

The objective of this work was to develop a rapid, sensitive, and reliable method for the determination of 26 antibiotic residues from different classes (sulfonamides, macrolides, tetracyclines, penicillins, and quinolones) in milk and meat by ultra high performance liquid chromatography coupled to hybrid quadrupole – high resolution Orbitrap mass spectrometry (UPLC–qOrbitrap). The sample preparation procedure involved a simple extraction step without any further treatment of the sample extract. The method was comprehensively validated over three days at 50% of MRL (maximum residue limit) set in the European Union in accordance to the Guidelines for the validation of screening methods for residues of veterinary medicines, issued by the European Community reference laboratories and representing the comprehensive interpretation of the European Commission Decision 2002/657/EC [25,26]. To the best of our knowledge this is the first time that a highly selective and sensitive hybrid quadrupole – Orbitrap mass spectrometric method is described for analysis of antibiotics in meat and milk.

2. Experimental

2.1. Materials and reagents

HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). ACS grade formic acid was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water (18.2 MΩ) was generated by a Millipore Milli-Q System (Billerica, MA, USA). Analytical standards such as erythromycin, josamycide, lincomycin, spiramycine, tylosin (macrolides), ampicillin, cloxacycline, dicloxacillin, oxacillin (penicillins), sulfadimethoxine, sulfadimidine, sulfachloropyridazine, sulfamethiazole, sulfathiazole (sulfanilamides), doxycycline, oxytetracycline, tetracycline (tetracyclines), ciprofloxacin, difloxacin, enrofloxacin, flumequine and nalidixic acid (quinolones) were of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA). Danofloxacin, chlortetracycline and marbofloxacin were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Individual 1 mg mL^{-1} stock solutions of each analyte in methanol were prepared and stored at −18 °C. Working standard solutions were prepared in methanol at concentration ratios according to the MRLs in milk and meat. The working solutions were used for spiking blank samples and stored at +4 °C for 1 month. The bovine milk and meat samples originating from the Latvian official residue control program (January 2012–December 2012) were thoroughly homogenized and stored at −20 °C until analysis.

2.2. Instrumentation and conditions for the UPLC-qOrbitrap analyses

The UPLC-qOrbitrap system was a Thermo Q-Exactive hybrid mass spectrometer (Bremen, Germany) coupled to a Thermo Scientific Accela UPLC system (Zwingen, Switzerland). Instrument control as well as the raw data interpretation and targeting/quantification of the analytes of interest were performed by ThermoXcalibur™ and TraceFinder™ software (version 3.0). Separation was achieved using a Phenomenex Kinetex C18(2) column (50 mm × 2.1 mm, 2.6 μm) using a gradient elution. The mobile phase A was a 0.1% aqueous formic acid solution and the mobile phase B was a 0.1% solution of formic acid in methanol. The initial mobile phase of the effective gradient consisted of 80% A and 20% B and was kept constant for 0.5 min. From 0.5 min to 5 min the percentage of B increased from 20% to 100%, holding it until 6.5 min, returning to 20% B by 6.6 min and holding at 20% B until 10 min. The flow rate of the mobile phase was 400 μL min^{-1} and the injection volume was 10 μL. The column and sample temperatures were 40 °C and 10 °C, respectively.

High resolution mass spectrometry using the hybrid quadrupole – Orbitrap mass spectrometer was employed to detect the analytes. Heated electrospray ionization (HESI-II) was selected for the ionization of the compounds, operating in positive mode. The heater temperature was set at 450 °C, the temperature of capillary of the ESI interface (HESI-II) at 270 °C, and the electrospray voltage at 3.20 kV. Nitrogen was employed as sheath gas (50 arbitrary units) and auxiliary gas (10 arbitrary units). The tube lens voltage was adjusted to 90 V. The mass spectrometer was operated in Full MS – data dependent MS^2 (dd-MS^2) mode. This type of scanning comprises a full MS scan (the selected range was from 200 to 1000 *m/z* and the resolution was 70,000 full width half maximum (FWHM)) with an isolation window applied, followed by a data dependent scan (resolution of 17,500 FWHM) with the fragmentation energy applied. The target capacity of the C-trap was always defined at 1×10^6 charges and the maximum injection time was limited to 120 ms. External calibration of the Q-Exactive mass spectrometer was performed daily over the mass range of *m/z* 50–2000 according to the guidelines provided by the instrument supplier. The accurate masses for the precursor and transition ions, collision energies, and the retention times for the analyzed antibiotics are shown in Table 1.

2.3. Sample preparation

Samples (2.0 g) were weighed in two separate 50 mL polypropylene centrifuge tubes. The control samples were fortified with the appropriate volumes of the standard solution in order to obtain levels corresponding to 50% of EU MRL. The first part of sample was treated with 8 mL of 5% trichloroacetic acid (TCA) aqueous solution. Samples were vortexed for 1 min, shaken for 10 min, and centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was decanted and filtered through filter paper (Sartorius Filter Discs, grade 388). The tube containing the second part of sample was treated with 8 mL of acetonitrile (ACN). Samples were vortexed for 1 min, shaken for 10 min, and centrifuged at 4000 rpm for 10 min at 4 °C. The ACN extract (6 mL) was evaporated to dryness in a Zymark TurboVap (Massachusetts, USA) evaporator at 50 °C under nitrogen. The samples were redissolved in 300 μL of the TCA extract obtained from the first portion of the samples using a vortex, transferred to chromatography vials, and an aliquot of 10 μL was injected into the UPLC-qOrbitrap system.

2.4. Method validation

For the estimation of accuracy, blank milk and meat samples were fortified with a mixture of antibiotics corresponding to 50% of the MRLs set in the European Union. Twenty replicate test portions were analyzed. Analysis of the 20 portions was carried out on three separate occasions for each matrix, obtaining 60 results for each of the analytes and the matrix. For the estimation of the method precision, repeatability and reproducibility within the laboratory were calculated.

3. Results and discussion

3.1. Optimization of the LC–high resolution MS parameters

Optimization of the liquid chromatography parameters was performed in order to obtain the best separation and peak intensities of the analytes. Addition of formic and acetic acid in concentration