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Food contaminant analysis at high resolution mass spectrometry: Application for the determination of veterinary drugs in milk

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ABSTRACT

Veterinary drugs (VDs) can remain in milk as a consequence of their use in livestock. In order to control the levels of VD residues in milk, screening methodologies can be applied for a rapid discrimination among negative and non-negative samples. In a second stage, non-negative samples are classified as negative or positive samples by using a confirmation method. Pre-target screening methods in low resolution MS (LRMS) are normally applied, but the number of analytes is limited, whereas the information obtained by full scan acquisition in high resolution mass spectrometry (HRMS) is improved. Here, three screening methods (running time < 4 min) based on Orbitrap, quadrupole-time of flight (QqTOF) and triple quadrupole (QqQ) have been compared, using in all cases ultra-high performance liquid chromatography (UHPLC). For HRMS, the identification of the VDs was based on retention time (RT) and accurate mass measurements. Confirmation was based on the monitoring of fragments generated without precursor selection. The performance characteristics of the screening method provided reliable information regarding the presence or absence of the compounds below an established value, including uncertainty region and cut-off values. Better results in terms of cut-off values ($\leq$5.0 $\mu g\,kg^{-1}$, except for spiramycin with a cut-off of 13.4 $\mu g\,kg^{-1}$ for milk samples and 43.1 $\mu g\,kg^{-1}$ for powdered milk based, emamectin with a cut-off of 42.2 $\mu g\,kg^{-1}$ for milk samples and doxycycline, with a cut-off value of 15.8 $\mu g\,kg^{-1}$ in powdered milk-based infant formulae) and uncertainty region were obtained using the Orbitrap-based screening method, which was submitted to further validation and used to analyze different real milk samples. The proposed method can be used in routine analysis, providing reliable results.

1. Introduction

The inadequate use of veterinary drugs (VDs) in livestock may leave residues in edible tissues or in food of animal origin, such as milk, egg and meat [1,2]. The presence of VDs in foodstuff may cause toxic effects on consumers [3,4], specially in the most vulnerable risk group: infants [5,6]. For this reason, the European Union (EU) and the US Government agencies have set maximum residue limits (MRLs) for VDs in food, including milk, and some VDs have been banned in milk-producing animals [7,8]. However, there is not any legislation applicable for VDs in baby food so far.

Different sensitive and specific methods have been reported [9–12] to monitor VDs in foodstuffs. Bearing in mind the high number of samples to be analyzed in short periods of time in routine laboratories, and the fact that most of the samples fulfil the established MRLs, screening methods can be applied for rapid identification [13]. Screening methods provide a qualitative binary response and samples can be classified among negative and non-negative samples, considering as non-negative samples those containing one or more analytes above a pre-established concentration level. Then, non-negative samples must be re-analyzed by a confirmation/quantification method to determine the final concentration in the positive samples.

The majority of screening methods employed in routine analysis detect the presence of analytes previously known and included within a defined MS method (pre-target screening); and using low-resolution mass spectrometry (LRMS) instruments, such as quadrupole (Q) [14], triple quadrupole (QqQ) [10,12,15–20] and ion trap (IT) analyzers [21], applying selected reaction monitoring (SRM) [12,15,17,22], precursor-ion scan [20] or/and neutral loss scan [19]. However, pre-target screening methods are often insufficient because only a limited number of analytes are monitored, and, thus, other non-target analytes, which could be present in the samples, would not be detected. Full-scan acquisition offers a

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complete information dataset, but its sensitivity in LRMS is deficient. By contrast, time of flight (TOF), hybrid quadrupole-TOF (QqTOF) and Orbitrap, medium/high mass resolution (MR/HRMS) analyzers, provide the selectivity and sensitivity required for efficient and wide-range screening, allowing the accurate mass measurement of any ionizable component in the sample [23]. The full scan data provided by MR/HRMS allows testing the presence of compounds in the sample by extracting any desired exact mass [24]. In this way, data can be acquired without any previous knowledge (no analyte-specific information) about the compounds present in the sample [25].

Despite QqTOF has been used for multi-screening analysis of VDs in food [25,26], TOF mass resolution can be insufficient in complex food matrices due to not resolved isobaric interferences [23]. This problem may be overcome by the use of Orbitrap since its high resolving power (up to 100,000 full width at half maximum, FWHM, depending on the acquisition time) can help to discriminate matrix interferences, improving mass accuracy measurements and the confidence on the screening results. On the other hand, it has been reported that the wider dynamic range over which accurate masses can be determined, implies that Orbitrap analyzer may be more suited for identification/quantification purposes than TOF analyzer [23,27]. Despite of these advantages, only two papers have reported VDs screening in honey and animal tissues employing Orbitrap [23,28], and only one with quantification purposes [27].

In this study, three screening methods using LRMS, MRMS and HRMS have been compared in terms of uncertainty region and cut-off values for the determination of 29 VDs from different families in milk and powdered milk-based infant formulae. Ultra-high performance liquid chromatography (UHPLC) was coupled to the different analyzers using a running time < 4 min. Sample extraction was based on a reported method based on the QuEChERS methodology (quick, easy, cheap, effective, rugged and safe), without sample clean-up. The most promising method was subsequently submitted to further validation for quantification purposes.

2. Experimental

2.1. Reagents and chemicals

Commercial VD standards were supplied by Riedel-de Haën (Seelze, Germany), Dr. Ehrenstorfer (Augsburg, Germany), Fluka (Steinheim, Germany), and Sigma (Madrid, Spain). Additional information related to suppliers for each analyte and reference solutions preparation can be found in the Electronic Supplementary Material (ESM).

HPLC-grade acetonitrile and methanol (MeOH, LC–MS quality) were supplied by Sigma and Scharlab (Barcelona, Spain), respectively. Anhydrous magnesium sulphate, acetic acid (purity > 97%), formic acid (purity > 98%) and sodium acetate were purchased from Panreac (Barcelona, Spain). Na_2EDTA was supplied by Merck (Darmstadt, Germany). Ultrapure water, obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA) was used for the preparation of all aqueous solutions.

For accurate mass calibration, different solutions of caffeine, peptides, ultramark 1600, formate sodium and leukine–enkephalin were used in QqTOF and Orbitrap. Specific details can be found in the ESM.

2.2. UHPLC–Orbitrap-MS analysis

The separation of the analytes was carried out using a Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) equipped with an analytical column Hypersil GOLD aQ C18 column (100 mm × 2.1 mm, 1.7 μm particle size) from Thermo (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase consisted of 0.05% (v/v) formic acid in water (eluent A) and MeOH (eluent B). The analysis started with 50% of eluent A, which was linearly decreased up to 0% in 1.50 min. This composition was held during 2.0 min before returning to the initial composition in 0.1 min, followed by a re-equilibration time of 0.4 min (total running time = 4.0 min). The flow rate was 0.3 mL min^{-1} and the column temperature was set at 30 °C. Aliquots of 10 μL of the sample extract were injected into the chromatographic system.

The UHPLC system was coupled to an Orbitrap mass spectrometer (Exactive™, Thermo Fisher Scientific, Bremen, Germany) operating with a heated electrospray interface (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA), in positive ionization mode (ESI+) using the following operation parameters: spray voltage, 4 kV; sheath gas (N_2, >95%), 35 (adimensional); auxiliary gas (N_2, >95%), 10 (adimensional); skimmer voltage, 18 V; tube lens voltage, 95 V; heater temperature, 305 °C; and capillary temperature, 300 °C. The mass spectra were acquired using an acquisition function as follows: resolution, high (equivalent to a mass resolving power of 50,000 FWHM at m/z 200); automatic gain control (AGC), balance (target value of 1×10^6), and scan speed, 2 Hz. Mass range in the full scan experiments was set at m/z 90–1000. In order to confirm the presence of VDs in the non-negative samples, product ions or fragments were produced in the higher energy collisional dissociation (HCD) collision cell, operating with N_2 (>95%) and setting the collision energy at 35 eV. All the analyses were performed without lock mass. Data were acquired using external calibration mode. Data acquisition and processing were carried out using Xcalibur 2.2.1 (Thermo Fisher Scientific, Les Ulis, France) with Qual and Quanbrowser. Genesis peak detection was applied.

2.3. UHPLC–QqTOF-MS analysis

The analyses were carried out by using an Acquity UPLC system using an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size), both from Waters (Milford, MA, USA). The mobile phases and chromatographic program were identical to those described in Section 2.2, except for the cleaning/equilibration stage, which was reduced to 1.5 min (total running time = 3 min).

The UHPLC system was coupled to a QqTOF equipped with an ESI + interface using the following parameters: cone voltage, 30 V; capillary voltage, 3.5 kV; desolvation temperature, 350 °C; source temperature, 120 °C; cone gas (N_2, >95%), 80 L h^{-1}; and desolvation gas (N_2, >95%), 600 L h^{-1}. The microchannel plate (MCP) detector potential was set at 2000 V. Resolution was at least 8000 FWHM at m/z of the lock mass. Dynamic range enhancement (DRE) was switched on. Mass range in the full scan experiments was set at m/z 90–1000 with a scan time of 0.050 s and interscan time of 0.025 s, acquiring in the centroid mode. In order to carry out the generation of product ions for confirmation purposes, the collision energy employed in the collision-induced dissociation (CID) collision cell was set at 35 eV, using Ar (>99.99%). All the analyses were performed using a lock spray with internal lock mass of a solution of leucine–enkephaline (^{12}C $[M+H]^+$, m/z 556.2771) delivered to the ESI source at 10 μL min^{-1}. Data acquisition and processing was performed using MassLynx 4.1 software with QuanLynx program (Waters).

2.4. UHPLC–QqQ-MS/MS analysis

The UHPLC system was identical to that described in Section 2.3, and it was coupled to a Waters Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK) operating in ESI+. The chromatographic conditions and MS parameters are specified in our previous work [19].