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Determination of a liquid chromatography–tandem mass spectrometry method for the determination of sulfonamides, trimethoprim and dapsone in honey and validation according to Commission Decision 2002/657/EC for banned compounds

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ABSTRACT

This work reports a sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for identification and quantification of seven sulfonamides, trimethoprim and dapsone in honey. The method is based on a solid-phase extraction (SPE) step of the target analytes with Oasis HLB cartridges after acidic hydrolysis of the honey sample to liberate the sugar-bound sulfonamides. Analysis was performed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) in the positive electro-spray ionization (ESI) mode with two different isotopically labeled internal standards with the view to improve the quantitative performance of the method. The method validation has been performed according to the Commission Decision 2002/657/EC; the average recoveries, measured at three concentration levels (1.5, 2.5 and 5.0 μg kg^{-1}), have been estimated in the range 70 to 106% while the respective % relative standard deviations of the within-laboratory reproducibility ranged from 6 to 18%. Mean values of the expanded uncertainties calculated were in the range 22–41% at the 99% confidence level. Decision limit (CCα) and detection capability (CCβ) values were in the ranges 0.4–0.9 and 0.7–1.4 μg kg^{-1}, respectively. Matrix effects have been investigated demonstrating a moderate signal suppression/enhancement for most of the target compounds. The method described has been successfully applied to the analysis of honey samples; sulfamethoxazole, sulfathiazole and trimethoprim were detected in some cases.

1. Introduction

Sulfonamides are a class of antibacterial compounds widely used in veterinary practice; trimethoprim and dapsone, having a similar activity to sulphonamides, are commonly administered in conjunction with some sulfonamides in pharmaceutical preparations. High levels of sulfonamides, dapsone and trimethoprim in food products have been known to cause various adverse effects to human health and contribute to the development and spread of antibiotic resistance [1,2]. In apiculture, sulfonamides have been used to control three serious pests of bees, the European foulbrood, the American foulbrood and nosemosis [3–6].

Sulfonamides, trimethoprim and dapsone are included in the EU legislation establishing maximum residue limits (MRLs) of veterinary drugs as described in the Regulation 2377/90/EC [7] and the associated amendments (EC) 470/2009 [8] and (EU) 37/2010 [9]. In animal tissues and milk, sulfomamides (as a total) cannot exceed 100 μg kg^{-1}, trimethoprim is not allowed to exceed 50 μg kg^{-1} whereas dapsone is prohibited for use in veterinary practice for food producing animals [7–9]. Although honey is specifically classified as a product of animal origin, in the Regulations 2377/90/EC [7] and (EU) 37/2010 [9], no MRLs have been set for sulfonamides and trimethoprim; these compounds, along with dapsone, may be consequently considered as "zero-tolerance" substances in this commodity. Additionally, Annex II of the Directive 2001/110/EC [10] mentions that: "If possible, honey must, as far as possible, be free from any organic or inorganic matters foreign to its composition".

Until recently, some EU countries, Canada and US authorities had set their own national "action limits", "reporting limits" or "tolerance levels" for total sulfonamides and their metabolites in honey [5,6,11–13]. Currently, a EU "zero-tolerance" policy is being applied for residues of sulfonamides in honey since community-coordinated and national monitoring programs conducted in EU member states over the last years [5,6,14,15] revealed the presence of residues of these compounds in honey samples as

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reported in several notifications in the Rapid Alert System for Food and Feed (RASFF) from the Directorate-General for Health and Consumers [16].

The requirements for higher selectivity and sensitivity, as well as the necessity for confirmation, imposed by the legislation on analytical methods for the determination of residues of veterinary drugs (detailed in the Commission Decision CD 2002/657/EC [17] implementing the Council Directive 96/23/EC [18]) have been successfully met by coupling liquid chromatography with mass spectrometry [1,2,19–23]. The determination of sulfonamides in honey by LC–MS and LC–MS/MS has been reported previously [24–32]. The lowest concentrations for validation studies have been in the 10–50 μg kg^{-1} range [5,13,32] but even these levels may be considered too high in view of the "zero-tolerance" policy followed in practice regarding sulfonamides residues in honey and especially in the case of organically produced honey. In addition, there is no report of the simultaneous determination of sulfonamides, trimethoprim and dapsone in honey by LC–MS or LC–MS/MS, although some of these compounds have been simultaneously determined in other animal products [22,33,34].

Therefore, the objective of this work was the development and validation of a simple, selective, reliable and sensitive method for the simultaneous determination – identification and quantification – of residues of seven sulfonamides, trimethoprim and dapsone in honey using LC–MS/MS. Indeed, this is the first work in which the validation for this combination of target compounds/matrix was performed according to the requirements of Decision 2002/657/EC [17] and its amending guideline SANCO 2726/2004 [35] for the determination of residues of "banned and unauthorized" substances.

2. Experimental

2.1. Chemicals and reagents

Certified standards of sulfapyridine (SPD), sulfamethoxazole (SMTX), sulfathiazole (STZ), sulfamerazine (SMR), sulfamethazine (SMZ), sulfadoxine (SDX), sulfadimethoxine (SDT), dapsone (DAP) and trimethoprim (TMP) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Purity of all standards was higher than 99.0%. The deuterated sulfonamide standards sulfathiazole-d4 (d4-STZ) and sulfamethoxazole-d4 (d4-SMTX) (both 98% pure and used as internal standards) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). The chemical structures of the target compounds are given in Table 1.

Pestiscan-grade methanol was purchased from Lab-Scan (Dublin, Ireland). LC–MS grade acetonitrile and NH3 (25% w/w) were obtained from Merck (Darmstadt, Germany). Formic acid, (HCOOH), (85% w/w) and HCl (37% w/w), were from Sigma-Aldrich (Steinheim, Germany) and citric acid monohydrate from Riedel-de-Haën (Seelze, Germany). The water used was purified with a Milli-Q water purification system from Millipore (Bedford, MA, USA). The cartridges used for solid-phase extraction were Oasis HLB (200 mg, 6 mL; Waters, Milford, MA, USA).

2.2. Standard solutions

Primary individual standard stock solutions of the analytes (400 mg L^{-1}) were prepared in methanol and stored at −20 °C in glass ambered bottles. Individual intermediate standard solutions of the analytes (10 mg L^{-1}) were prepared by appropriate dilution of the stock solutions with methanol and stored at −20 °C. Mixed standard working solutions (1 and 0.1 mg L^{-1}) were prepared from the intermediate standard solutions by dilution with aqueous mobile phase (0.1% v/v HCOOH in water).

Individual standard stock solutions of the internal standards – SMTX-d4 and STZ-d4 (1000 mg L^{-1}) – were prepared in methanol and stored at −20 °C in glass ambered bottles. Individual intermediate standard solutions of the internal standards (10 mg L^{-1}) were prepared by appropriate dilution of the stock solutions with methanol and stored at −20 °C. Standard working mixtures containing both internal standards (0.100 mg L^{-1}) were prepared daily in ultra-pure water from the intermediate standard solutions.

Eight mixed calibration solutions containing all the analytes in the concentration range 0.5–100 μg L^{-1} and the internal standards at a fixed concentration of 10 μg L^{-1} were prepared daily by serial dilution of the mixed standard working solutions in aqueous mobile phase (0.1% v/v HCOOH in water).

2.3. Matrix calibration

Eight matrix-matched calibration solutions containing all the analytes in the range 0.5–100 μg L^{-1} and the internal standards at a fixed concentration of 10 μg L^{-1} were prepared by subjecting "blank" honey samples to SPE (according to the procedure described in Section 2.4) and spiking of the extract with the appropriate volumes of the mixed standard working solutions to achieve the required final concentrations of the matrix-matched calibration solutions.

2.4. Sample extraction

5.0 g ± 0.1 g of honey was accurately weighted in a 50 mL PTFE centrifuge tube and spiked with 100 μL of the 0.100 mg L^{-1} internal standard working mixture. 15 mL of a 2 mol L^{-1} HCl solution was added and the sample was sonicated for 45 min at 35 °C. Then, 30 mL of a 0.3 mol L^{-1} citric acid solution were added and the sample was centrifuged at 3000 rpm for 5 min. The supernatant solution was collected, adjusted to pH ~ 4 with NH_3 and diluted to 250 mL with purified water. Solid-phase extraction was performed on Oasis HLB cartridges using a Visiprep vacuum manifold (Supelco) which enabled parallel extraction of up to 12 samples. The cartridges were conditioned with 3 mL MeOH and 3 mL of a 0.5 mol L^{-1} HCl solution. The diluted honey solutions were percolated through the cartridges at a flow rate of 3 mL min^{-1}. The Oasis HLB cartridges were rinsed with 2 mL of H_2O, vacuum dried for 15 min and the retained analytes were eluted with 2 × 3 mL MeOH into a test tube containing 0.5 mL of aqueous mobile phase (0.1% v/v HCOOH in water). The eluate was concentrated in a rotary evaporator system at 40 °C to near dryness and the solution was reconstituted up to a final volume of 1.0 mL with aqueous mobile phase. Before LC analysis the sample was filtered through a 0.22 μm filter (Millipore).

2.5. Instrumentation

The LC–tandem MS system consisted of a Surveyor LC quaternary pump, a solvent degasser, a Surveyor autosampler and a TSQ Quantum Ultra triple-quadrupole mass spectrometer with an ESI (Ion Max API) interface (ThermoElectron, San Jose, CA, USA); data acquisition was performed using XCalibur 1.4 software (ThermoElectron Corporation).

The chromatographic separation was performed on a Xterra MS C_{18} column (2.1 mm × 150 mm, 3.5 μm particle size) in combination with a Xterra MS C_{18} guard column (2.1 mm × 10 mm, 3.5 μm particle size) (Waters, Milford, MA). The column temperature was maintained at 28 °C. A multi-step binary elution gradient was applied using two mobile phases: phase A: Milli-Q-water containing 0.1% (v/v) HCOOH; phase B: acetonitrile containing 0.1% (v/v) HCOOH. The flow rate was 0.2 mL min^{-1} and a volume of 20 μL was injected for both standard and sample solutions. Separation of the target compounds was achieved with the linear gradient: