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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:

Table 1
Chemical formulas, retention times (t_R) and compound-specific LC-ESI–MS/MS parameters for the target species and deuterated internal standards.

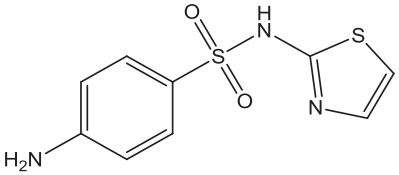
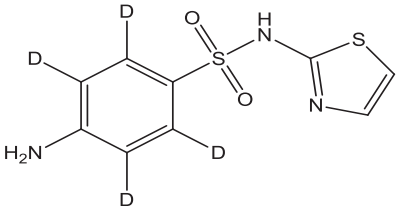
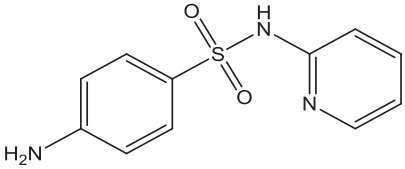
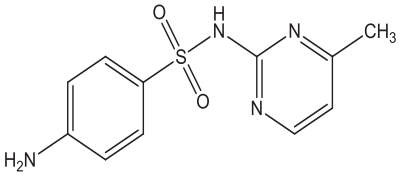
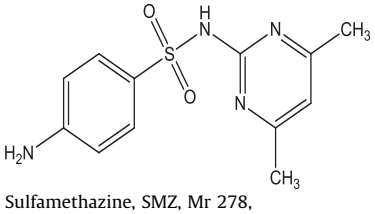
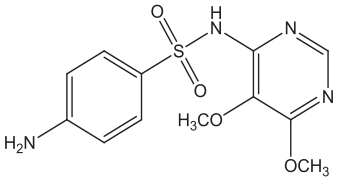
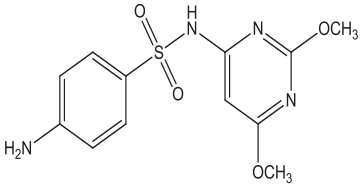
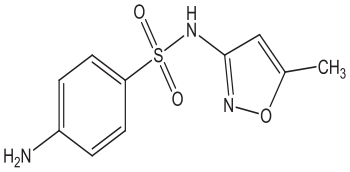
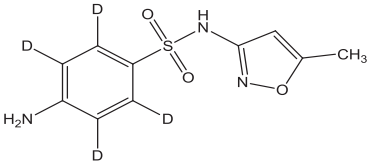
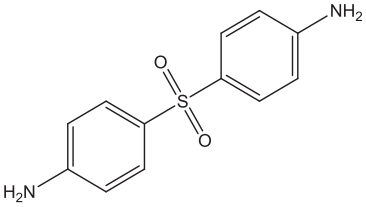
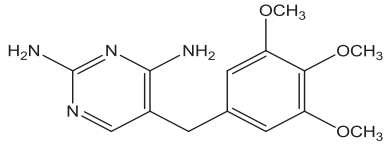
Compounds	Retention times, t_R (min)	Molecular ion $[M+H]^+$	Product ions		Collision energy (V)	[Qualifier]/[Quantifier]
			Quantifier	Qualifier		
 Sulfathiazole, STZ, Mr 255, CAS Nr [72-14-0]	6.1	256	156	92	22	0.59
 Sulfathiazole-d4 (d4-STZ), Mr 259	6.0	260	96	160	22	–
 Sulfapyridine, SPD, Mr 249, CAS Nr [144-83-2]	6.2	250	156	92	25	0.99
 Sulfamerazine, SMR, Mr 264, CAS Nr [127-79-7]	7.2	265	156	92	23	0.60
 Sulfamethazine, SMZ, Mr 278, CAS Nr [57-68-1]	9.9	279	186	124	25	0.67
 Sulfadoxine, SDX, Mr 310, CAS Nr [2447-57-6]	17.8	311	156	92	24	0.45
 Sulfadimethoxine, SDT, Mr 310, CAS Nr [122-11-2]	14.8	311	156	92	24	0.29

Table 1 (*continued*)

Compounds	Retention times, t_R (min)	Molecular ion $[M+H]^+$	Product ions		Collision energy (V)	$\frac{[Qualifier]}{[Quantifier]}$
			Quantifier	Qualifier		
 Sulfamethoxazole, SMTX, Mr 253, CAS Nr [723-46-6]	15.2	254	156	92	20	0.65
 Sulfamethoxazole-d4 (d4-SMTX), Mr 257	15.1	258	112	160	24	–
 Dapsone, DAP, Mr 248, CAS Nr [80-08-0]	13.9	249	92	156	26	0.32
 Trimethoprim, TMP Mr 290, CAS Nr [738-70-5]	6.0	291	230	261	30	1.0

0–3 min: A/B=90:10 (v/v), 3–25 min: A/B=90:10 (v/v)–10:90 (v/v), 25–26 min: A/B=10:90–90:10 (v/v), 26–30 min: A/B=90:10 (v/v).

The electrospray settings were optimized by infusing a 1 mg L^{-1} standard solution of each analyte – prepared in a 50:50 (v/v) methanol–water mixture fortified with 0.1% (v/v) HCOOH – into the ESI source at a flow rate of 0.02 mL min^{-1}, merging – via a T-piece connector – to the LC eluent flowing at the constant rate of 0.2 mL min^{-1}. The intensity of the selected parent ion was monitored and optimized by sequentially varying the following parameters: spray voltage, sheath gas pressure, auxiliary gas pressure, ion transfer capillary temperature. All target analytes were detected in positive ionization mode.

Selection of the MS/MS conditions (i.e., optimization of the collision energies and selection of the appropriate SRM transitions) was performed individually for each analyte with direct infusion of a 1 mg L^{-1} standard solution – prepared in a 50:50 (v/v) methanol–water mixture fortified with 0.1% (v/v) HCOOH – at a flow rate of 0.05 mL min^{-1}. From the MS/MS optimization, the two most intense and characteristic precursor/product ion transitions were selected for operation in the SRM mode for target compounds and internal standards. The selected precursor/product ion transitions together with the retention times of the compounds are presented in Table 1. The effects of the Q1 peak width (FWHM; full width half maximum), the dwell time and the scan width on the detection sensitivity were further investigated by analyzing the matrix-matched solution of the lowest calibration level containing 0.5 μg L^{-1} of all the target species.

The optimized operational LC-ESI(+)–MS/MS and detection conditions were: spray voltage, 4 kV; sheath gas pressure (N_2), 40 (arbitrary units); auxiliary gas pressure (N_2), 15 (arbitrary units); ion transfer capillary temperature, 350 °C; collision gas pressure (Ar), 1.0 mTorr; Q1 peak width, 0.2 Da FWHM; Q3 peak width, 0.7 Da FWHM; dwell time, 400 ms; scan width, 0.1 Da.

2.6. Method validation

The method was validated as a quantitative confirmatory method according to the Commission Decision 2002/657/EC [17] and its amending guideline SANCO/2004/2726 [35]. Identification criteria were established for each analyte by monitoring retention times and relative ion intensities of matrix-matched standard calibration solutions (data in Table 1).

The performance parameters evaluated in the validation study were: specificity, sensitivity, linearity, precision (repeatability and within-laboratory reproducibility), trueness (in terms of recovery), decision limit (CCα) and detection capability (CCβ) [17,35–37]. The specificity was tested by analyzing twenty organically-grown honey samples of different origin. The linearity of the method was evaluated by regression analysis both of solvent calibration and matrix-matched calibration solutions using the ratio of the standard area (derived from the most intense transition) to internal standard area against the analytes concentrations, except in the case of TMP where external calibration (i.e., peak area of the most intense transition against

concentration) was used. d4-STZ was chosen as internal standard for STZ, SPD, SMR and SMZ and d4-SMTX was selected as the internal standard for SMTX, DAP, SDX and SDT. This selection was based on the closeness of the retention times of the target compounds and of the internal standards. Matrix-matched calibration and solvent calibration solutions responses were used to evaluate matrix effects.

For the recovery experiments, "blank" honey samples (5 g) were spiked with appropriate amounts of the standard working mixture of analytes and of the working mixture of deuterated standards to achieve the concentration levels of 1.5, 2.5 and 5.0 μg kg^{-1} for the target analytes and 2 μg kg^{-1} for the internal standards. The spiked samples were allowed to remain at room temperature for at least 1 h in order for the sulfonamides to sufficiently bind to the sugars in the honey [13,31]. Six replicates per spiking level were analyzed during the same day along with a matrix-matched calibration curve prepared as described previously. Each series, consisting of a matrix calibration curve and 18 spiked samples, were prepared on three different days (a total of 54 spiked samples) varying time, operator and calibration/operation status of the LC–MS/MS. Trueness (in terms of percent recoveries) and precision (repeatability and within-laboratory reproducibility in terms of the respective % relative standard deviations) were determined from these series of experiments.

The decision limit (CCα) and the detection capability (CCβ) were determined from the calibration curves obtained from "blank" honey samples spiked at five concentration levels of 0.5, 1.0, 1.5, 2.5, 5.0 μg kg^{-1} – six replicates per level – and subjected to SPE, using the ratio of the signal of the less intense transition of the target analyte to that of the internal standard applicable in each case.

3. Results and discussion

3.1. LC-ESI(+)–MS/MS method development

Different chromatographic conditions were tested for their efficiency to separate the target compounds aiming at optimum peak shape, good resolution and high sensitivity. Mobile phases consisting of acetonitrile and water, both fortified at 0.1% (v/v) formic acid, provided the best sensitivity for the majority of the tested compounds. Several gradient elution programs (with varying starting compositions and gradient slopes) were evaluated in order to achieve a good resolution. The selected elution protocol allowed the chromatographic run to be divided into two time segments with fewer SRM transitions to be monitored in each segment; this, in turn, allowed the application of longer dwell times for each transition with a concomitant increase in sensitivity. Additionally, base separation between the peaks of SDX and SDT was obtained — necessary since these two isomers share the same scan filter (i.e., identical molecular and product ions, Table 1). A gradient washing step (about 7 min) along with a 5 min re-equilibration step at the initial mobile phase composition were included to avoid carry-over effects, to prolong column life-time and to maintain a stable and reproducible separation.

Electrospray ionization in the positive mode [30–32] and negative mode [13], atmospheric pressure photoionization [25] and laser diode thermal desorption atmospheric pressure chemical ionization in the negative mode [38] have been applied for the analysis of the sulfonamide group of compounds. In this case, electrospray ionization in the positive mode has been selected for the ionization of the target compounds since negative ionization provided lower overall sensitivity. MS/MS fragmentation of the target compounds has been investigated by recording the full-scan product ion spectrum of each analyte as a function of the collision energy. Common fragment ions with *m/z* values of 92, 108 and 156 derived from the sulfonamidic part of the sulfonamide molecules, appear at the highest abundances at their respective ESI(+) full scan product spectra (data not shown). The ESI(+)–MS/MS product ion spectrum of sulfamethazine (SMZ) was differentiated giving as more abundant product ions the ions at *m/z* 186 $([M+H]-93)^+$ and 124 $([M+H]-155)^+$, originating from the heterocyclic aromatic group of the compound [1,2]. Based on the ESI(+)–MS/MS product ion scan spectrum of TMP (Fig. 1), fragmentation of this compound produced the main product ions at *m/z* 261, 230, 275, and 123.

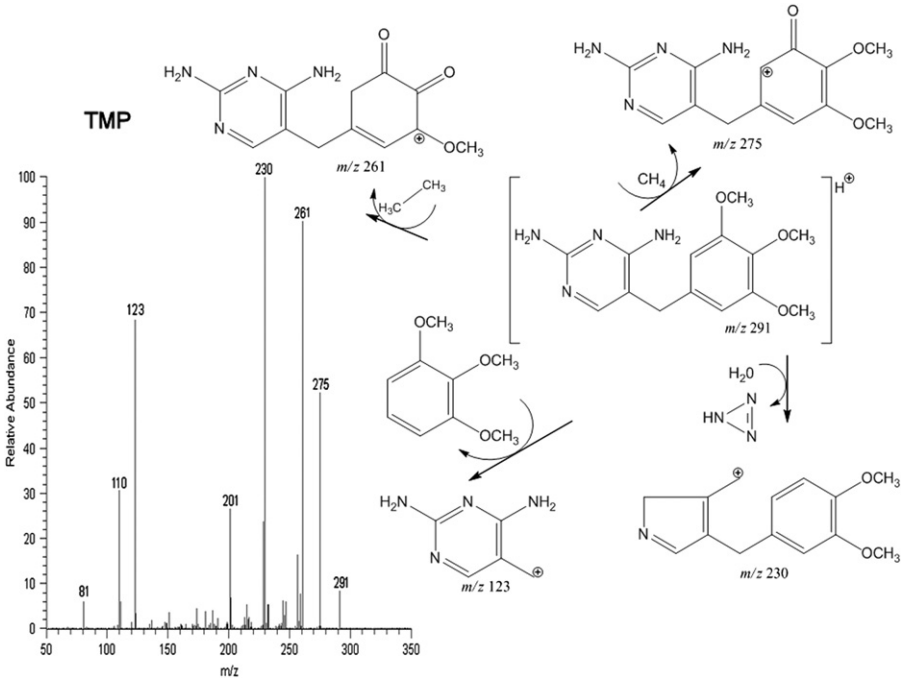


Fig.1. ESI(+)–MS/MS spectrum and tentative fragmentation pattern of TMP.

The proposed fragmentation pattern along with the tentative structures of its main product ions (shown in Fig. 1), are in agreement with the elemental compositions derived from the QqTOF-MS/MS analysis of TMP described elsewhere [39,40]. Based on the MS/MS product ion spectra of the target compounds, the molecular ions and the two most intense product ions have been selected for data acquisition fulfilling the EU requirements for 4 identification points (IPs) [17]; selected precursor and product ions, collision energies and qualifier-to-quantifier ion ratios are included in Table 1.

The effect of the Q1 peak width (range 0.1–0.7 Da (FWHM)), the dwell time (range 20–400 ms) and the scan width (range 0.01–0.1 Da) on the detection sensitivity were also investigated. The selected final LC–MS/MS conditions offered a good compromise between selectivity and sensitivity, both required in the analysis of low concentration levels of target compounds in the difficult honey matrix.

3.2. Sample extraction procedure

The sample extraction procedure involved acidic hydrolysis of the sugar bound sulfonamides [11,13,31] followed by a SPE clean-up step. The efficiency of the different sample extraction steps was investigated by evaluating the recovery of "blank" honey samples spiked with 5 μg kg^{-1} of the target compounds. The effect of the pH of the honey sample after acidic hydrolysis was studied in the range 2–7; the highest recoveries of sulfonamides occurred when the sample was adjusted to pH 4 at which target species were in their isoelectric form (accordingly to their respective pK_a values [41]). Addition of citric acid solution to the acidified honey solution was necessary to prevent overshooting of pH in the subsequent adjustment with NH_3 (which was preferred to NaOH to avoid any formation of sodium adducts of the target compounds during their ionization). During our initial experiments, it was observed that that the cartridges were often clogged by undissolved material – probably wax in the honey sample [13] – resulting in low recovery values; therefore, after pH adjustment and prior to the SPE procedure, the sample solution was diluted to 250 mL to minimize the likelihood of the sulfonamides re-binding to sugars and to prevent blocking of the SPE cartridges. Additionally, the SPE cartridges were conditioned with methanol followed by 0.5 mol L^{-1} HCl and extraction was performed as quickly as possible to prevent any re-binding of sulfonamides to sugars during extraction. Elution of the target compounds was performed with methanol since better recoveries were obtained compared to acetonitrile. The methanolic eluate was collected along with 0.5 mL of aqueous mobile phase (0.1% v/v HCOOH in water) to avoid sulphonamides binding to any co-eluted sugars in the following rotary evaporation step.

3.3. Method validation

3.3.1. Specificity, linearity, sensitivity

The specificity of the method was tested by analyzing a number of twenty "blank" honey samples. In these "blank" sample assays, no interfering peaks overlapping with the analyte peaks were observed in the SRM chromatograms, demonstrating adequate specificity for the trace analysis of the target compounds. The signal-to-noise ratios were greatly improved by the use of a more "narrow" resolution window setting the peak width at 0.2 Da (FWHM) on the first quadrupole (Q1). SRM chromatograms of a "blank" honey sample and of a "blank" sample spiked with 2.5 μg $kg^{-1}$of all the compounds are illustrated in Fig. 2(a) and (b), respectively.

Calibration curves were constructed for both solvent and matrix matched calibration in the range 0.5–100 μg L^{-1}. In the case of matrix-matched calibration solutions, the concentration range 0.5–100 μg L^{-1} corresponded to 0.1–20 μg kg^{-1} of the target compounds in the honey sample. The parameters of the linear regression equations: slope (a), intercept (b), standard deviation of the slope (s_a), standard deviation of the intercept (s_b) and the correlation coefficient (r^2) were calculated. In all cases $r^2 > 0.99$, indicating satisfactory linearity in the concentration range studied. The linear regression parameters along with the instrumental limits of detection (LODs), (where $LOD = 3s_b/a$), calculated from the matrix-matched calibration solutions, are given in Table 2. The LODs were

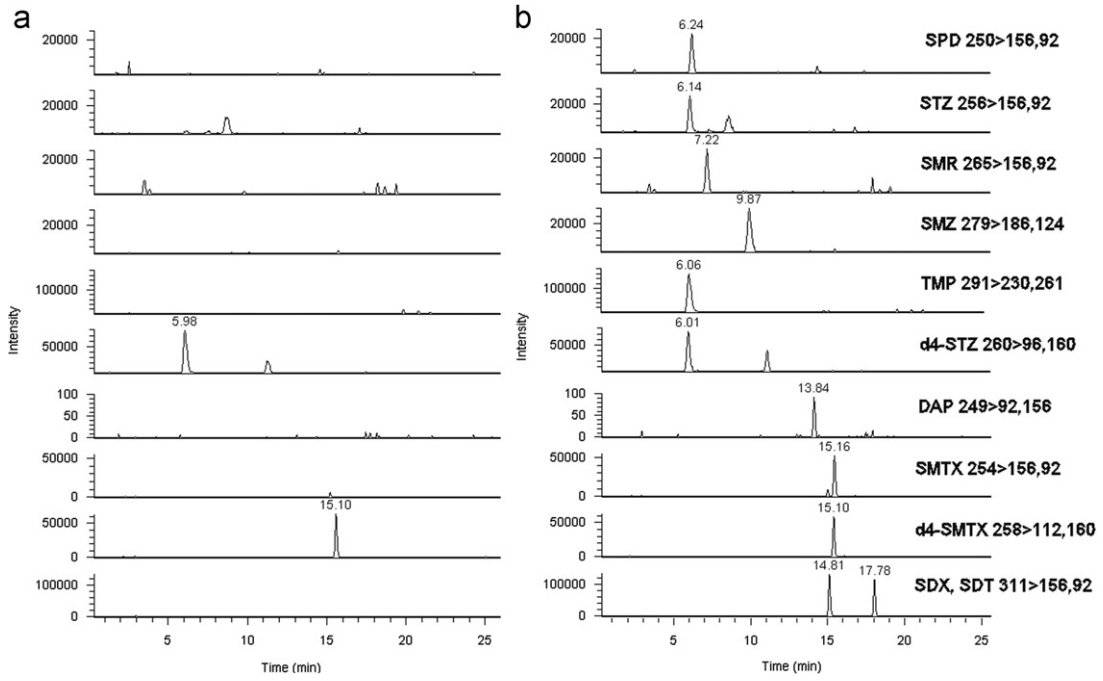


Fig.2. SRM chromatograms of: (a) a "blank" honey sample spiked with 2 μg kg^{-1} of the internal standards and subjected to SPE, and; (b) a "blank" honey sample spiked with 2 μg kg^{-1} of the internal standards and with 2.5 μg kg^{-1} of all the compounds and subjected to SPE.

Table 2
Slope (a), intercept (b), standard deviation of the slope (s_a), standard deviation of the intercept (s_b), correlation coefficient (r^2) and instrumental limit of detection (LOD) obtained in matrix matched calibration solutions in the range 0.5–100 μg L^{-1}.

Compound	$(a \pm s_a)$	$(b \pm s_b)$	r^2	LOD (μg L^{-1})
SPD	0.0117 ± 0.0003	0.0105 ± 0.0027	0.994	0.7
SMR	0.0179 ± 0.0004	0.01311 ± 0.00465	0.996	0.8
STZ	0.0193 ± 0.0003	0.00948 ± 0.00182	0.998	0.3
SMZ	0.0340 ± 0.0004	0.0122 ± 0.0103	0.999	0.9
SMTX	0.0308 ± 0.0002	0.00883 ± 0.00254	0.9994	0.3
TMP	0.0484 ± 0.0012	0.0344 ± 0.00404	0.995	0.3
DAP	0.0435 ± 0.0002	0.0163 ± 0.0058	0.9998	0.4
SDX	0.140 ± 0.002	0.0813 ± 0.0413	0.998	0.9
SDT	0.200 ± 0.001	0.0578 ± 0.0332	0.9998	0.5

Table 3
Method validation data: % recovery ($\overline{R}_m$%), repeatability (RSD_r%), within-laboratory reproducibility (RSD_R%), "Horrat"(H).

Compound	1.5 μg kg^{-1}				2.5 μg kg^{-1}				5.0 μg kg^{-1}			
	$\overline{R}_m$%[a]	RSD_r%[b]	RSD_R%[c]	H	$\overline{R}_m$%[a]	RSD_r%[b]	RSD_R%[c]	H	$\overline{R}_m$%[a]	RSD_r%[b]	RSD_R%[c]	H
SPD	98	12	13	0.58	103	8	9	0.41	103	12	14	0.66
STZ	99	10	10	0.44	106	10	11	0.51	93	6	6	0.27
SMR	98	10	11	0.51	101	8	9	0.41	105	10	11	0.51
SMZ	99	11	11	0.50	104	12	13	0.59	106	11	12	0.55
TMP	86	11	12	0.54	82	12	11	0.50	86	10	11	0.48
SMTX	103	11	11	0.48	100	11	10	0.46	102	9	10	0.48
DAP	70	17	16	0.73	74	18	18	0.80	73	17	17	0.78
SDX	81	9	10	0.45	78	11	11	0.51	78	12	13	0.58
SDT	74	11	12	0.54	73	12	13	0.57	71	12	13	0.58

[a] Average % recovery obtained during 3 days ($n=3\times6=18$ assays per spiking level).
[b] Percent relative standard deviation under repeatability conditions ($n=3\times6=18$ assays per spiking level).
[c] Percent relative standard deviation under within-laboratory reproducibility conditions ($n=3\times6=18$ assays).

between 0.3–0.9 μg L^{-1} (equivalent to 0.06–0.18 μg kg^{-1} in the honey sample) demonstrating the high sensitivity of detection.

3.3.2. Accuracy

Method accuracy – trueness and precision – was evaluated by recovery studies, using fortified "blank" honey samples spiked with the target compounds. Six replicates were performed per day ($m=6$) at each of the three concentration levels (1.5, 2.5 and 5.0 μg kg^{-1}) ($p=3$) for three different operating days ($q=3$). The percent recovery, R%, was calculated using the formula: $R\% = (C_c/C_s)\times100$, where C_c is the analyte concentration in the spiked samples, C_s is the analyte concentration added at a "blank" honey extract after the extraction and before the LC–MS/MS analysis. C_c and C_s were both calculated from the matrix matched calibration curves using the peak area ratio of each analyte versus the respective internal standard (except for TMP). The total of 18 spiked samples per spiking level have been subjected to one way analysis of variance (ANOVA) to estimate the mean recovery at each level; mean recovery values, $\overline{R}_m$%, ranged from 70 to 106% at the three concentration levels (data on Table 3), complying with the requirements for trueness in the CD 2002/657/EC [17].

The method repeatability and within-laboratory reproducibility were also estimated via ANOVA; experimental F values, F_{exp} (calculated as between-days variance over within-day variance) were in all cases lower than the theoretical F value, $F_{theor\ (2,\ 15,\ 0.05)}$ equal to 3.68. The mean repeatability of the method (expressed as % relative standard deviation, RSD_r%) as well as the within-laboratory reproducibility (expressed as % relative standard deviations, RSD_R%) both ranged from 6 to 18% at the three concentration levels for all the compounds (data shown in Table 3), fulfilling the criteria of CD 2002/657/EC (i.e., $RSD\% \leq 22\%$) [17].

The "modified" or "truncated" Horwitz equation (recommended by Thompson for concentrations lower than 120 μg kg^{-1} [42]) was applied for the estimation of the "target" standard deviation s_H ($s_H=0.22C$, where C is the concentration of the compound (in μg kg^{-1})) and, consequently, of the respective "target" relative standard deviations, RSD_H. The "Horrat" measure, H, is defined as:

$$H = \frac{RSD_R}{RSD_H} \tag{1}$$

where RSD_R is experimental standard deviation of the within-laboratory reproducibility. The "Horrat" values obtained were ≤ 1 for all the compounds (Table 3); a practical requirement for intra-laboratory validation is that the "Horrat" should be in the range 0.2–1 [43].

3.3.3. Uncertainty

The experimental design applied during method validation allowed the estimation of the measurement uncertainty from validation data according to the LGC/VAM protocol [44]. Considering that the type B contributions to uncertainty are of minor significance, the standard uncertainty, $u(Y)$, can be calculated using the following equation:

$$u(Y)^2 = u(P)^2 + u(\overline{R}_m)^2 \tag{2}$$

where $u(P)$ is the uncertainty associated with the method precision and $u(\overline{R}_m)$ is the uncertainty associated with the recovery of the method. Relative uncertainties can be estimated using the formula

$$\frac{u(Y)}{Y} = \sqrt{\left[\frac{u(P)}{P}\right]^2 + \left[\frac{u(\overline{R}_m)}{\overline{R}_m}\right]^2} \tag{3}$$

The term $u(P)/P$ can be expressed using the relative standard deviation of the within laboratory reproducibility, RSD_R, obtained from the ANOVA test at each spiking level [44].

A t-test using the formula $t=(1-(u(\overline{R}_m)/\overline{R}_m))$, where $u(\overline{R}_m)$ is the standard deviation of the recoveries per spiking level, was applied to estimate if $\overline{R}_m$ was significantly different from 1. In the cases of SPD, STZ, SMR, SMZ and SMTX, the t values were lower than the coverage factor $k=2.33$ [35] and subsequently $\overline{R}_m$ values were not statistically different from 1 at the three spiking levels. Therefore, no further correction for bias was necessary and the uncertainties associated with the recoveries, were calculated using the relative standard deviations, $u(\overline{R}_m)/\overline{R}_m$. The % expanded uncertainties, U%, derived from the formula:

$$U\% = k(u(Y)/Y\%) \quad (4)$$

using a coverage factor $k=2.33$ [35] ranged from 23 to 26%, from 24 to 35% and from 15 to 38% at the three respective spiking levels. In the cases of DAP, SDX, SDT and TMP the t values were higher than the coverage factor $k=2.33$ at the three spiking levels tested and subsequently $\overline{R}_m$ values were statistically different from 1. For these compounds, if recovery correction of results is to be applied, the contribution of bias in the total uncertainty can be calculated using the relative standard deviations of the mean recoveries,$(u(\overline{R}_m)/\overline{R}_m)$; the respective % expanded uncertainties, U%, ranged in the case of SDX from 27 to 35%, in the case of SDT from 31 to 33%, in the case of DAP from 38 to 44% and in the case of TMP from 28 to 32% for the three spiking levels tested. If no correction for recovery is applied, the uncertainty associated with recovery, $u(\overline{R}_m)'$, should be calculated using the formula [44]:

$$u(\overline{R}_m)' = \sqrt{\frac{1-\overline{R}_m}{k} + u(\overline{R}_m)^2} \quad (5)$$

In this case, the % expanded uncertainties, U'%, calculated for these compounds, ranged in the case of SDX from 36 to 45%, in the case of SDT from 48 to 54%, in the case of DAP from 56 to 58% and in the case of TMP from 34 to 37%.

The validation data obtained from each spiking level (i.e. mean recovery, $\overline{R}_m$, and standard deviation values of the within laboratory reproducibility s_R, derived from the ANOVA test described in Section 3.3.2 (18 experiments per spiking level)), have been subjected to a second ANOVA (54 spiking experiments) to test whether the recoveries were significantly different between the three spiking levels. Experimental F values, F_{exp}, for all the target compounds were lower than the theoretical F value, $F_{theor\ (2,\ 51,\ 0.05)}$ of 3 (i.e, F_{exp} (STZ), 1.82; F_{exp} (SMTX), 0.37; F_{exp} (SDX), 0.54; F_{exp} (SDT), 1.76; F_{exp} (SPD), 1.0; F_{exp} (SMR), 1.91; F_{exp} (SMT), 1.62; F_{exp} (DAP), 0.74; F_{exp} (TMP), 0.96). Since recoveries tested at the three spiking levels were not significantly different, a great mean recovery, $\overline{R}_M$, was calculated as the average recoveries of the three levels (Table 4) along with the respective RSD% values of repeatability and within-laboratory reproducibility. Based on the approach for the estimation of the uncertainty described above and using Eqs. (2)–(4), values of the % relative standard deviation of the within-laboratory reproducibility, $RSD_{M,R}$%, and expanded uncertainties, U_M% ($k=2.33$), of each target compound were obtained ranging from 10 to 17% and from 22 to 41%, respectively (Table 4). Especially for the cases of DAP, SDX, SDT and TMP when no correction for recovery is applied, the uncertainty associated with recovery, $u(\overline{R}_M)'$ should be calculated using the formula (5), yielding % expanded uncertainty values, U'_M%, in the range 32 to 57% (Table 4).

3.3.4. Decision limits (CCα) and detection capability (CCβ)

The decision limit (CCα) is defined as the level over which it can be decided that a sample is not compliant with a probability of error equal to α [17]. The detection capability (CCβ) is the lowest content of the target species that can be detected, identified and quantitatively determined in a sample with an error probability equal to β [17]. The CCα ($\alpha=1$%) and the CCβ ($\beta=5$%) of the present method – based on the concept of "zero-tolerance substances" – was calculated according to the equations proposed by Antignac [45] and Kaufmann [46]. The derived CCα, CCβ values are shown in Table 5.

Table 4
Mean recoveries, R_M%, % relative standard deviations, $RSD_{M,R}$% of the within-laboratory reproducibility and expanded uncertainties, U_M%, of the target species.

Compound	R_M% [a]	$RSD_{M,R}$% [b]	U_M% [c]	U_M'% [d]
SPD	101	12	29	–
STZ	101	10	24	–
SMR	101	11	22	–
SMZ	103	12	29	–
TMP	85	11	27	32
SMTX	101	10	24	–
DAP	72	17	41	57
SDX	79	11	27	41
SDT	74	12	30	47

[a] Average % recoveries of the three concentration levels ($n=3\times3\times6=54$ assays).
[b] % relative standard deviations of the within-laboratory reproducibility of the three concentration levels.
[c] Expanded uncertainties at 99% confidence level, $k=2.33$ for % relative standard uncertainties calculated using Eq. (4).
[d] Expanded uncertainties at 99% confidence level, % confidence level, $k=2.33$, for % relative standard uncertainties calculated using Eqs. (4) and (5).

Table 5
Decision limits (CCα) and detection capabilities (CCβ) of the method towards the target species.

Compound	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
SPD	0.7	1.2
STZ	0.5	0.8
SMR	0.5	0.8
SMZ	0.7	1.2
TMP	0.4	0.9
SMTX	0.4	0.7
DAP	0.9	1.4
SDX	0.4	0.8
SDT	0.5	0.9

3.4. Matrix effects

Matrix effects (generally recognized as a suppression or enhancement of the analytical signal due to co-eluting matrix components) have been widely studied and recognized as a source of error in quantitative LC–MS/MS analysis of veterinary drugs in animal food products [20–23]. Matrix effects are known to be both compound- and matrix-dependent. In this work, matrix effects were investigated by calculating the % signal enhancement or suppression, C%, according to the formula:

$$C\% = 100\times(a_m/a_s-1) \quad (6)$$

where: a_s is the slope of the calibration plot with calibration solutions in solvent and a_m is the slope of the calibration plot with matrix-matched calibration solutions.

The C% values (Fig. 3), are mean figures obtained from the matrix-matched calibration curves of the twenty representative "blank" honey samples used in the specificity and linearity studies. Matrix effects were low ($-25\% < C\% < +25\%$) for the sulfonamides (SMR, SMZ, SMTX, SDX, SDT, STZ and SPD) and higher ($C\% < -40\%$ or $C\% > +40\%$) for DAP and TMP. In this work,

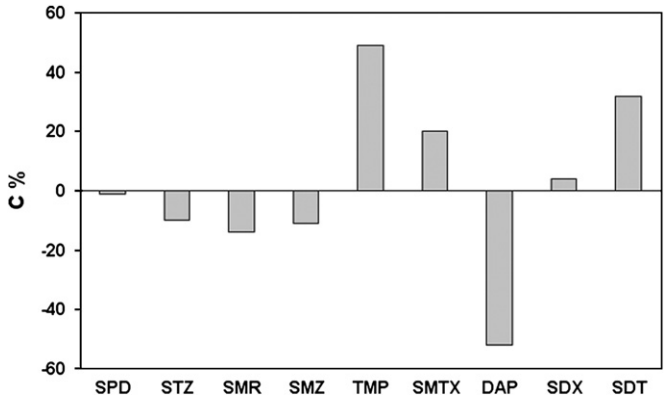


Fig. 3. Matrix effects for the target compounds.

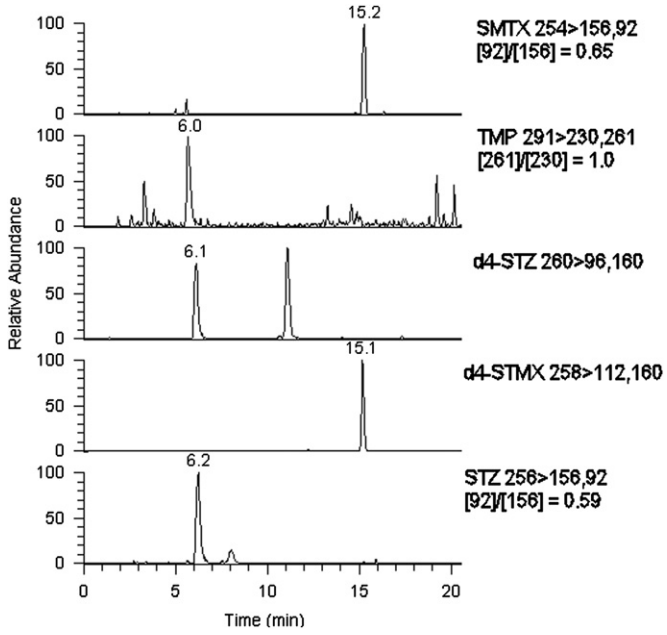


Fig. 4. SRM chomatograms of a honey sample with positive findings of STZ ($5.3 \pm 1.2\ \mu g\ kg^{-1}$) and SMTX ($1.8 \pm 0.4\ \mu g\ kg^{-1}$) and minor traces of TMP (estimated to $0.4 \pm 0.1\ \mu g\ kg^{-1}$). The intensity ratios of the monitored ions for the detected compounds in the sample are included.

quantification was based on matrix matched calibration solutions and, therefore, matrix effects were efficiently minimized.

3.5. Method applicability to honey samples

The applicability of the method described has been evaluated by analyzing thirty honey samples, both of Greek origin and imported, collected from the local markets. Confirmation criteria were that the retention times of the compounds in the sample to be within $\pm 2.5\%$ of the respective retention times in matrix-matched calibration solutions and the intensity ratios of the product ions in the sample to be within the maximum permitted tolerance levels of the respective ratios in matrix-matched calibration solutions (Table 1) according to CD 2002/657/EU [17].

Based on these criteria, only two samples were found positive in STZ, SMTX while TMP was detected in both cases but its concentration was below the CCβ. Typical SRM chromatograms of an incurred honey sample are illustrated in Fig. 4 in which ion ratios calculated for the unambiguous identification of the detected compounds are also shown. For the two positive samples, STZ was determined at the concentrations 5.3 and 5.9 $\mu g\ kg^{-1}$, respectively, SMTX at concentrations 1.8 and 3.4 $\mu g\ kg^{-1}$, respectively, while the estimated concentration of TMP were 0.4 and 0.6 $\mu g\ kg^{-1}$.

4. Conclusions

In this work, a relatively simple, specific and sensitive method has been developed and validated, based on the concept of "zero-tolerance" substances, for the simultaneous determination of selected sulfonamides, dapsone and trimethoprim in honey. The validation data demonstrated that the method complied with EU current legislation requirements in terms of trueness and precision achieving low CCα and CCβ values. The method has been successfully applied in the routine analysis of honey samples

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