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ANALYSIS OF SULFONAMIDE RESIDUES IN REAL HONEY SAMPLES USING LIQUID CHROMATOGRAPHY WITH FLUORESCENCE AND TANDEM MASS SPECTROMETRY DETECTION

Ádám Tölgyesi [a] [b] , Róbert Berky [b] , Katalin Békési [a] , Szabolcs Fekete [b] , Jenõ Fekete [b] & Virender K. Sharma [c]

[a] Central Agricultural Office Food and Feed Safety Directorate, Food Toxicological National Reference Laboratory, Budapest, Hungary

[b] Department of Inorganic and Analytical Chemistry, Budapest University of Technology and Economics, Budapest, Hungary

[c] Chemistry Department, Florida Institute of Technology, Melbourne, Florida, USA

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Ádám Tölgyesi,[1,2] **Róbert Berky,**[2] **Katalin Békési,**[1] **Szabolcs Fekete,**[2] **Jenõ Fekete,**[2] **and Virender K. Sharma**[3]

[1] *Central Agricultural Office Food and Feed Safety Directorate, Food Toxicological National Reference Laboratory, Budapest, Hungary*
[2] *Department of Inorganic and Analytical Chemistry, Budapest University of Technology and Economics, Budapest, Hungary*
[3] *Chemistry Department, Florida Institute of Technology, Melbourne, Florida, USA*

□   *This paper presents new reversed phase liquid chromatographic methods (HPLC-FLD and LC-MS/MS) for the quantification of sulfonamides in spiked and incurred honey samples. The sample preparation was optimized using Oasis HLB (hydrophilic–lipophilic balance) solid-phase extraction (SPE) cartridge. Elutions of sulfonamides were carried out under acidic, neutral, and basic conditions using methanol. Recoveries under acid condition were in the range from 66.8–90%, which were approximately 10% higher than those obtained under other conditions. The sample clean-up was also tested using Strata-XL cartridges. The HPLC-FLD separation was performed using a Varian C18 column and a ternary (methanol-acetonitrile-phosphate buffer, pH 5) mobile phase resulting good selectivity for the determination. The robustness of the ternary gradient method was evaluated by computer simulation (DryLab). LC-MS/MS separation was carried out on a Kinetex XB core-shell type HPLC column that enabled a low limit of detection (0.01–0.5 µg/kg) and faster separation (6 min). The developed methods were validated in accordance with the European Union Commission Decision 2002/657/EC and were applied successfully for more than four hundred honey samples (under a national monitoring program). The concentrations of sulfadimethoxine, sulfachloropyridazine, and trimethoprim residues in samples were found in a concentration range from 0.03 up to 686 µg/kg.*

Keywords DryLab software, high performance liquid chromatography, honey, kinetex XB HPLC column, optimization, sulfonamides
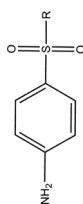
Address correspondence to Ádám Tölgyesi, Central Agricultural Office Food and Feed Safety Directorate, Food Toxicological National Reference Laboratory, Mester utca 81, Budapest 1095, Hungary. E-mail: adam.tolgyesi@t-online.hu
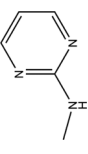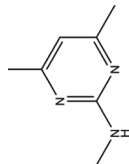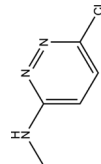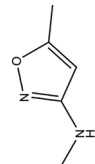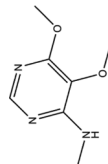
INTRODUCTION

Sulfonamides are synthetic antimicrobial agents, which contain the sulfonamide group (Table 1). They are commonly used for therapy in both agriculture and human practices. In Hungary, eighteen different veterinary medicines contain sulfonamide active agents such as sulfadiazine, sulfadimethoxine, sulfadoxine, sulfachloropyridazine, and sulfamethoxazole. The veterinary residues may cause allergic or toxic reaction to consumers and promote occurrence of antibiotic resistant bacteria. Therefore there is a need to develop analytical methods to monitor pharmaceutical residues in foods of animal origin. In the European Union (EU), sulfonamides belong to group B substances. A maximum residue limit (MRL) has been set as $100 \, \mu g/kg$ for sulfonamides in tissues, milk and fat by EU.[1] However, no permitted limit has been established for sulfonamides in honey, therefore a minimum required performance level (mrpl) as $20 \, \mu g/kg$ was established by the Hungarian Food Toxicological National Reference Laboratory. The detection capability (CCβ) of screening method equals to mrpl concentration of a confirmation method.[2]

High performance liquid chromatographic (HPLC) separations coupled to different detectors have been reported to determine sulfonamides in biological and water matrices.[3,4] Nowadays, liquid chromatography–tandem mass spectrometry (LC-MS/MS) is generally used as the most selective and precise technique for analyzing residues.[5–11] LC-MS/MS methods require the suitable sample preparation in order to avoid the ion suppression effect of matrix compounds, which generally limits the quality of the confirmation of analytes in matrices.[12–15] LC-MS/MS confirmation method on positive real honey samples is available in literature,[6,9,11] but expensive LC-MS/MS instrumentation restricts its use widely. Therefore, an alternate method such as HPLC with fluorescence detection (HPLC-FLD) is needed to determine sulfonamides in biological matrices.[16,17] After derivatization, fluorescence detection can be used for the accurate determination of sulfonamides with enhanced sensitivity.[18–22] However, unless these methods are well optimized, they could not be applied to different kind of honey samples due to matrix interferences, since the honey matrix could be diverse from sample to sample. Although EU directive allows confirmation of group B substances using HPLC-FLD technique,[23] only a few papers demonstrates HPLC-FLD method for sulfonamide determination in incurred honey sample.[21,22] In this present paper, a recently developed HPLC-FLD method was applied for more than three hundred real samples, and added no false positive or negative results. As sulfonamides did not have MRL in honey, the aim is now to reach as low a decision limit (CCα) as possible for them. We have, therefore, also developed an LC-MS/MS method, which could improve the
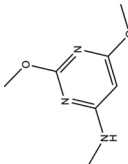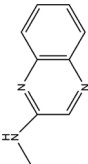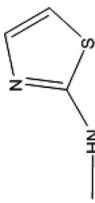
TABLE 1 Structures of Analyzed Sulfonamides and pKa Values

| Compound | R | pKa | Elution Conditions | | | Recovery at 20, 30 40 µg/kg levels | Within-Laboratory Reproducibility [RSD (%)] | Validation Parameters | | | |
			Acidic	Neutral	Basic			CCα (µg/kg)	CCβ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
SU-diazine		6.4	90.0% ± 6%	76.9% ± 7%	77.5% ± 4%	71.8–77.1%	17.9–29.5%	5.0	7.5	5.0	16.7
SU-methazine		7.4	76.6% ± 5%	65.8% ± 6%	68.5% ± 5%	69.4–79.7%	13.3–26.5%	5.0	7.1	5.0	16.7
SU-chloropyridazine		5.1	68.4% ± 6%	55.3% ± 7%	59.4% ± 6%	72.9–76.5%	19.7–30.2%	10.0	14.7	10.0	33.3
SU-methoxazole		5.7	73.0% ± 5%	60.2% ± 6%	63.7% ± 5%	69.5–71.9%	26.1–33.9%	5.0	7.6	5.0	16.7
SU-doxine		5.8	81.0% ± 7%	66.4% ± 6%	68.1% ± 7%	69.8–71.2%	20.3–32.9%	5.0	7.5	5.0	16.7

(*Continued*)

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TABLE 1 Continued

Compound	R	pKa	Elution Conditions			Validation Parameters					
			Acidic	Neutral	Basic	Recovery at 20, 30 40 μg/kg levels	Within-Laboratory Reproducibility [RSD (%)]	CCα (μg/kg)	CCβ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
SU-dimethoxine		5.9	66.8%±5%	58.8%±6%	60.2%±5%	78.6–84.8%	21.0–34.8%	1.0	1.7	1.0	3.3
SU-quinoxaline		5.5	73.7%±5%	62.8%±7%	72.7%±5%	79.9–82.6%	28.3–34.4%	15	20.0	15	50
SU-thiazole		7.2	Not validated for HPLC-FLD								

Recovery results ($n=3$) on HLB SPE column using different elution conditions and HPLC-FLD technique. Validation parameters ($n=72$).

analytical limits. These two techniques can confirm each other and this comparison is very important when no-MRL substances are analyzed.

This paper reports analysis of sulfonamides in real honey samples using both HPLC-FLD and LC-MS/MS methods for confirmation. Both binary and quaternary pump separations were developed for HPLC-FLD separation using ternary mobile phase. The binary pump separation was optimized by DryLab chromatographic method development software. The sample preparation, which was developed for HPLC-FLD determination, was also tested with LC-MS/MS separation using Strata-XL SPE cartridges. Analyzed sulfonamides were sulfadiazine (SU-diazine), sulfamethazine (SU-methazine), sulfachloro-pyridazine (SU-chloropyridazine), sulfamethoxazole (SU-methoxazole), sulfa-doxine (SU-doxine), sulfadimethoxine (SU-dimethoxine), sulfaquinoxaline (SU-quinoxaline), and sulfathiazole (SU-thiazole) (Table 1). The values of pK_a of analyzed sulfonamides suggest that different forms of sulfonamides exist depending on the pH. The developed methods were validated and the analytical parameters met the requirements of the EU 2002/657/EC decision and validation strategy of LC-GC International.[23,24]

EXPERIMENTAL

Reagents and Samples

HPLC grade acetonitrile, methanol, acetic acid, and Suprapur formic acid (98–100%) were obtained from Merck (Budapest, Hungary). HPLC grade potassium dihydrogen phosphate was purchased from Reanal (Budapest, Hungary). Sulfonamides were obtained from Sigma-Aldrich (Budapest, Hungary). Ammonia solution (25%) was purchased from Schar-lau (Barcelona, Spain). The derivatization reagent, fluorescamine (Figure 1) was obtained from Serva (Heidelberg, Germany). Trimethoprim (TRIM) was obtained from Riedel-de-Haen (Seelze, Germany). Trimethoprim-d$_9$ (TRIM-d$_9$) was purchased from Witega (Berlin, Germany).

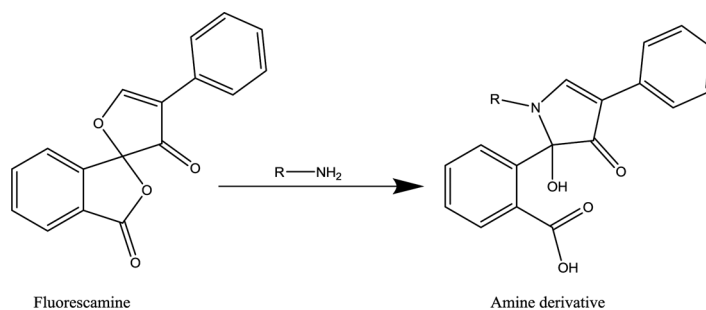Fluorescamine   Amine derivative

FIGURE 1 Derivatization of sulfonamides with fluorescamine.

Stock solutions were prepared by dissolving 25 mg standards (of accurate weight) in 25 mL of methanol to obtain concentrations of 1 mg/mL and were stored at $-20°C$. The stock solutions were stored for one month. For the working standard solutions, 25 μL of the stock solutions were diluted with distilled water to 25 mL in volumetric flasks to obtain a final concentration of 1 μg/mL. Working standard solutions were prepared daily.

The blank and incurred honey samples used in the study originated from the Hungarian residue control monitoring program in the period from September 2009 to May 2011 and were stored at $-20°C$ until subjected to analysis. Samples were analyzed within two weeks. Blank samples were analyzed previously and did not contain sulfonamide residues above the limit of detection. Spiked samples were prepared by fortifying blank samples with different volumes of working standard solution. Incurred samples originated from animals treated by medicine containing sulfonamide active agent. A certified reference real honey sample, which contained 110 μg/kg sulfaquinoxaline and 103 μg/kg sulfathiazole, was obtained from FAPAS (York, UK).

Equipments and Instruments

SPE vacuum manifolds were obtained from Merck (Budapest, Hungary). The nitrogen evaporator was a Caliper TurboVap LV (Hopkinton, MA, USA) and the shaker was an IKA KS125 (Janke & Kunkel, Staufen, Germany). Oasis HLB (6 mL, 200 mg, 30 μm) SPE cartridges were purchased from Waters Corp. (Budapest, Hungary). Quaternary pump HPLC-FLD instrument was a HP 1100 LC system that includes a G1322A degasser, a G1311A quaternary pump, a G1313A auto sampler, a G1316A column thermostat and an Agilent FLD 1321A fluorescence detector. Data acquisition and analysis were performed by ChemStation A.10.02 (1757) software. The LC-MS/MS system was an Agilent 6410A Triple Quad equipped with Agilent 1200 HPLC and Agilent 6410A mass selective detector with Agilent multimode ion source (G1978B) (Palo Alto, CA, USA). Data analysis was performed using Agilent Mass Hunter B 01.04 software. In case of verification test of HPLC-FLD method, a HP 1100 binary pump HPLC was used. The dwell volume of binary instrument was determined as 1.3 mL. Validation results were evaluated by InterVal 3 (version 3.1.2) software (EU-RL Berlin, Germany). DryLab software for computer simulation was obtained from Molnar Institute (Berlin, Germany).

Sample Preparation for HPLC-FLD Determination

The honey sample (5.0 g) was weighed into a 50 mL centrifuge tube and dissolved in 15 mL distilled water by vortex-mixing for 1 min. Acetic

acid (300 µL) was then added to the sample, followed by vortex-mixing for 20 s. Sample was hydrolyzed in acetic acid solution by shaking it at 700/min speed for 40 min at pH 2.3 and ambient temperature. Hydrolyzed sample was cleaned-up and concentrated on an Oasis HLB (6 mL, 200 mg) cartridge, which was previously conditioned with 6 mL methanol, 6 mL water, and 6 mL 2% (*v/v*) acetic acid in water (pH 2.3). The sample was passed through the cartridges drop wise. This steps was followed by washing two times with 6 mL 2% (*v/v*) acetic acid in water (pH 2.3), then dried under vacuum for 60 s. The sample was eluted with 5 mL methanol that contained 2% (*v/v*) acetic acid. The eluted sample was evaporated to dryness at 45°C under a gentle nitrogen stream and re-dissolved in 1.0 mL 25 mM potassium dihydrogen phosphate buffer (pH 5). Finally, the sample was filtered through a 0.45-µm Phenex nylon filter (Gen-lab Ltd., Budapest, Hungary) and transferred into the HPLC vial.

The same procedure was performed for the method development process under a basic elution (methanol-25% ammonia solution, 95:5, *v/v*) and a neutral elution (methanol without pH adjustment) conditions.

HPLC-FLD Method

Sulfonamides were separated on a Varian OmniSpher C-18 (250 mm × 4.6 mm, 5 µm) (BST Corp., Budapest, Hungary) column using a ternary linear gradient elution mode and quaternary pump HPLC. Solvent A contained 25 mM potassium dihydrogen phosphate in water (pH 5), solvent B was 100% methanol, and solvent C was 100% acetonitrile. The gradient program started from 55% (*v/v*) of A, 35% (*v/v*) of B, and 10% (*v/v*) of C. Solvent A decreased from 55% to 40%, and solvent B increased from 35% to 50% over 30 min, while 10% of C was held from 0 to 30 min. After 30 min solvent C was increased to 50% within 2 min, while solvent A was decreased to 0%. This setting was held from 32 min to 37 min and applied to elute non-polar matrices compounds from the column. The flow rate was set as 0.8 mL/min and the complete analysis time was 37 min. Column temperature was not optimized; therefore, the column thermostat was set at 26°C. An online pre-column derivatization was carried out with applying injector program and 4 mg/mL (0.4%) fluorescamine solution in acetonitrile (Figure 1). In the program, the injector needle drew 20 µL sample, followed by taking up 20 µL fluorescamine solution. The needle mixed the two solutions in the loop for 1 min and derivatized for 15 min before injecting into mobile phase. Before the injector program began, the instrument was in post run mode for 10 min. The excitation and emission wavelengths of the FLD detector were set as 420 nm and 480 nm, respectively.

LC-MS/MS Method

The LC-MS/MS separation was carried out using Kinetex XB C18 (100 mm × 3 mm, 2.6 μm) core-shell type HPLC column (Gen-lab Ltd, Budapest, Hungary) with 0.1% formic acid in water/acetonitrile (80/20, v/v, pH 2.6) mobile phase. Isocratic elution mode was applied with a flow rate of 0.8 mL/min. The injection volume was 10 μL, the analysis time was 6 min. The column thermostat was set 35°C. The MS/MS parameters are summarized in Table 2. The multimode ion source was set into the positive ESI mode. The ESI parameters were: drying gas temperature: 350°C, drying gas flow: 5 L/min, vaporizer: 250°C, nebulizer pressure: 413.7 kPa (60 psi), capillary voltage: 2500 V, charging voltage: 2000 V. Nitrogen was collision and drying gas. The pressure of the collision gas was 1.07 Pa.

Qualification

In the case of the HPLC-FLD separation, qualification was based on the retention time and both excitation and emission spectrum of a compound. The sulfonamide residues in incurred samples were also identified with standard additions. Identifications in the LC-MS/MS separation were based on the ion ratios, which are the intensity ratio of qualifier and quantifier ion transitions.

Verification of the Method Robustness

The ternary eluent separation was modeled on an HP 1100 binary HPLC using DryLab software.[25] In the experimental study we set three factors (gradient time, column temperature, different organic modifiers). The levels of gradient time and column temperature were 15 and 45 min and 15 and 45°C, respectively. The three different organic modifiers were: 100% methanol, methanol/acetonitrile 50/50 (v/v), and 100% acetonitrile. In the gradient program, two solvents were mixed. Solvent A was 25 mM phosphate buffer in water (pH = 5)/organic modifier (90/10, v/v) and solvent B was 25 mM phosphate buffer in water (pH = 5)/organic modifier (10/90, v/v). The gradient program started from 5% solvent B then increased to 100% over the different gradient times. This experimental design required 12 measurements, which included all settings.

RESULTS AND DISCUSSION

General Conditions of HPLC-FLD Separation

Honey is a very complex matrix containing a number of interfering compounds. Fluorescence detection was therefore applied to achieve more

TABLE 2 MS/MS Parameters and CCα, LOD, and LOQ Values of LC-MS/MS Determination

Segment	Time (min)	ΔEMV	Compound	Precursor Ion	Product Ions	Dwell Time	Fragmentor	CE	CCα (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
1	0–2.0	150V	TRIM-d9	[M+H]$^+$ 300.3	234.2	75 ms	70V	25V		ISTD	
			TRIM	[M+H]$^+$ 291.2	230.4 123.0	75 ms 75 ms	90V	25V 25V	0.5	0.5	1.7
			SU-methazine	[M+H]$^+$ 279.2	156.2 124.2	75 ms 75 ms	80V	15 V 30V	0.3	0.3	1.0
			SU-thiazole	[M+H]$^+$ 256.1	156.1 92.1	75 ms 75 ms	80V	10V 30V	0.5	0.5	1.7
			SU-diazine	[M+H]$^+$ 251.1	156.1 92.1	75 ms 75 ms	80V	15V 30V	0.3	0.3	1.0
			SU-pyridine	[M+H]$^+$ 250.1	156.1	75 ms	80V	15V		ISTD	
2	2.0–4.5	150V	SU-doxine	[M+H]$^+$ 311.1	156.1 92.1	75 ms 75 ms	80V	15V 30V	0.01	0.01	0.03
			SU- chloropyridazine	[M+H]$^+$ 256.1	156.1 92.1	75 ms 75 ms	80V	10V 30V	0.1	0.1	0.3
			SU-methoxazole	[M+H]$^+$ 254.1	156.1 92.1	75 ms 75 ms	80V	15V 30V	0.5	0.5	1.7
3	4.5–6.0	300V	SU-dimethoxine	[M+H]$^+$ 311.1	156.1 92.1	200 ms 200 ms	80V	20V 30V	0.03	0.03	0.1

Scan type: MRM, Ion mode: ESI +.
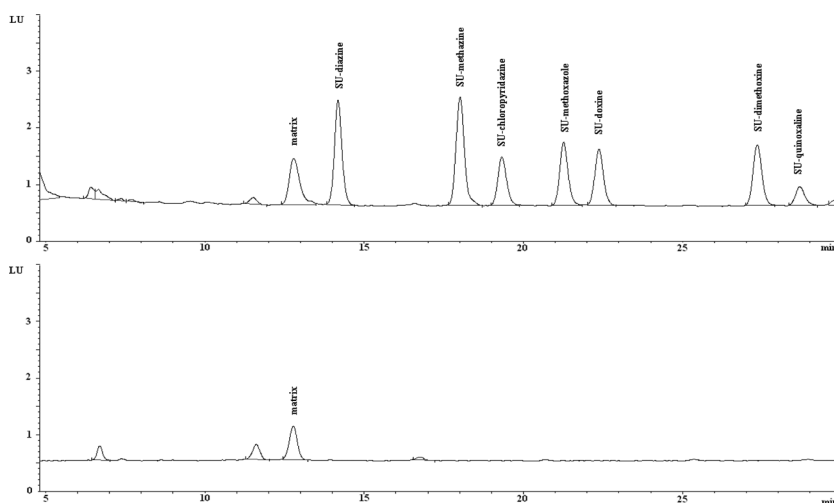
FIGURE 2 HPLC-FLD chromatograms of a spiked (20 μg/kg for all studied sulfonamides) and a blank honey sample (below) using ternary mobile phase.

selective separation compared to the diode array detection. The selection of the excitation and emission wavelengths was performed to minimize interferences from the matrix solutes in blank chromatogram (Figure 2) in order to fulfill the performance criteria of EU standards.[23] A long analytical column (250 mm × 4.6 mm) was used to obtain the highest resolution between sulfonamides and matrices and to avoid the column overload. A slow gradient elution was applied to maximize the resolution between compounds.

The HPLC separation was optimized by injecting a prepared spiked sample (at 20 μg/kg concentration for all sulfonamides) and by using different mobile phases. A use of only one organic modifier (methanol or acetonitrile) in the mobile phase did not result in selective separation. An eluent as a mixture of methanol and 25 mM potassium dihydrogen phosphate in water (pH 5) gave a matrix peak interference during the analysis of sulfadiazine. However, a mobile phase containing acetonitrile and phosphate buffer showed no matrix interference in analyzing sulfadiazine, but the resolution between sulfadimethoxine and sulfaquinoxaline was not acceptable. Therefore, both methanol and acetonitrile were important as organic modifiers for increasing the selectivity of the chromatographic separation. Acetonitrile enhanced the resolution between more polar compounds while methanol increased the selectivity between non-polar ones. Using a ternary mobile phase consisted of methanol-acetonitrile-25 mM phosphate buffer (pH 5), resulted in appropriate resolution (Figure 2).

Optimization of Derivatization Step for HPLC-FLD

Pre-column derivatization step was optimized using different concentrations of fluorescamine solution, prepared in acetonitrile. Standard solutions ($n = 2$), which contained studied sulfonamides in $20 \, \mu g/kg$, were derivatized for 10 min by applying 0.5, 2, 4, and 8 mg/mL fluorescamine. Derivatized sulfonamides were injected into the HPLC column. The areas of the peaks obtained were integrated. Higher concentration level of fluorescamine increased the detector responses, but no significant difference was observed between 4 and 8 mg/mL. The areas of sulfonamides varied from 1.0–8.6 unit using 0.5 mg/mL fluorescamine. The peak areas were ranged in 4.8–34.3 and 5.2–37.8 for 4 and 8 mg/mL, respectively. Hence, 4 mg/mL concentration of fluorescamine was tested to see if the time of derivatization makes any differences. The derivatization was optimized by varying the reaction time as 1, 5, 15, and 30 min. An enhancement in the peak areas was observed in applying longer derivatization time, however the improvement was negligible between 15 and 30 min. Areas were in the range of 5.3 to 31.0 unit in the use of 15 min derivatization time. Comparatively, 30 min derivatization led to areas in the range from 6.4–33.2 unit. In further development of the method, 4 mg/mL fluorescamine level for 15 min derivatization time was applied to avoid overloading the column and to obtain shorter analysis time.

Optimization of Sample Preparation for HPLC-FLD

In honey matrix the N-glycoside bond formed between hydroxy groups of sugars and amino group of sulfonamides, which needs to be hydrolyzed prior to clean-up.[7] Initially, 5.0 g honey was dissolved in 15 mL water and $300 \, \mu L$ acetic acid was added. After shaking the sample in the acidic phase for 40 min, the sulfonamides could be deconjugated from N-glycoside form. This was confirmed using incurred samples that contained sulfadimethoxine residue.

Washing of the SPE column was done by using 2% acetic acid in water (pH 2.3). Each mL of the eluted solvent was analyzed for sulfonamides. No detectable level of sulfonamide was found. The pirrolidone monomer groups of HLB sorbent interact strongly with ionic sulfonamides at acidic pH and therefore sulfonamides were not eluted using acidic water.

An elution of sulfonamides from HLB cartridge was optimized using methanol under different pH conditions. Methanol without pH control and methanol with acidic (methanol–acetic acid, 98:2, v/v) or basic pH control (methanol -25% ammonia solution, 90:10, v/v) were applied. Elution conditions were tested with spiked samples ($n = 3$) and with an incurred one. In case of the spiked samples, neutral and basic elutions

resulted in similar recovery (55.3%–76.9% and 59.4%–77.5%, respectively). However, recoveries were improved (Table 1) under acidic methanol for elution (66.8%–90.0%). A well-homogenous incurred sample containing 97 μg/kg sulfadimethoxine, confirmed by an independent accredited laboratory using LC-MS/MS method, was cleaned on HLB SPE cartridge and eluted under both neutral and acidic conditions. The levels of sulfadimethoxine were 87.5 μg/kg and 79.5 μg/kg under acid and neutral methanol elution conditions, respectively. Acidic elution was thus better than that of elution under neutral condition. Interestingly, the same difference was observed between spiked and incurred samples' recovery on sulfadimethoxine. An 8% improvement in recovery was found by using acidic elution compared to neutral elution for both spiked and incurred samples (Table 1).

After the validation, the optimized solid-phase extraction clean-up was tested using polymeric Phenomenex Strata-XL (6 mL, 200 mg, 100 μm) cartridges (Gen-lab Ltd., Budapest, Hungary) and resulted in reasonably appropriate results. The absorbent of Strata-XL is also a copolymer of divinylbenze and N-vinylpyrrolidone monomer groups as Oasis HLB; however, it has large pore size that protects the cartridge from the clogging.

Validation of HPLC-FLD Method

Method was validated in accordance with EU Commission Decision 2002/657/EC[23] using InterVal 3 software. InterVal has planned and evaluated the validation. The software allowed the simultaneous validation of matrices and required reduced number of samples.[26–28] In the present study, only one matrix was validated, but the validation protocol, used in this experiment, can be extended for other matrices in the future. The software enables the application of several factors at different levels. The following factors were evaluated during the validation process: operators and a lot of equipment. All factors were investigated at two levels. Selectivity was proven by analyzing and comparing blank and spiked samples. More than one hundred different blank honey samples were analyzed, and there were not any interfering compounds observed in blank chromatograms where sulfonamides were expected (Figure 2). The resolutions (Rs) between sulfonamides were Rs > 1.5 in all cases. More than three hundred real samples were analyzed using this method. All of the positive samples were confirmed by LC-MS/MS by an independent accredited laboratory using an unknown method. By using this new HPLC-FLD method, no false positive and false negative results were found.

Linearity was tested between 0 and 80 μg/kg using a five points calibration curve (0, 10, 20, 40, and 80 μg/kg). The determination coefficients

(r^2) were higher than 0.9974. The proper linear detector responses suggest that the high level of fluorescamine (0.4%) did not make a destructive effect on the detection and efficiency. Recovery was investigated at 20, 30, and 40 µg/kg levels. The 24 parallel samples were prepared at each level under 3 days (8 samples at each level per one day). All of 72 fortified samples were analyzed at three levels during the validation. Average relative recoveries between levels were found in the range of 69.4–84.8% for sulfonamides (Table 1). The acceptable range at these levels is 60–115%.[24] The relative standard deviation (RSD%) of peak areas obtained from consecutive samples defines the precision of the method.[29] High precision results in low RSD%. Within-laboratory reproducibility was determined as the precision of method at the fortification levels and was found between 13.3% and 34.8% at the spiking levels (Table 1). According to EU standards, the precision of a method under 100 µg/kg level should be as low as possible; therefore, the within-laboratory reproducibility fulfills the EU directive and the LC-GC strategy.[23,24]

Decision limit (CCα) of a confirmatory method (α = 1) means the limit at and above which it can be concluded that a sample is non-compliant with an error of 1%. Decision limits (CCα) and limit of detections (LODs) were determined by analyzing twenty different blank samples, and were calculated as three times the signal-to-noise ratio and were found from 1 µg/kg to 15 µg/kg for sulfonamides. Limit of quantifications (LOQs) were determined as 3.33 × LOD (Table 1). The detection capability (CCβ) was calculated as the value of the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility at decision limit and was found between 1.7 and 20 µg/kg for studied sulfonamides (Table 1).

According to the EU 2002/657/EC, the decision limit of a banned substance should be lower than the mrpl and the detection capability must be lower or equal to the mrpl. As shown in Table 1, both decision limits and the detection capabilities met the conditions of EU Directive. Decision limit and limit of detection were confirmed by analyzing 20 samples, which had been fortified to the individually calculated values. CCα and LOD were accepted for a compound when the signal-to-noise ratios for spiked samples were higher than three.

LC-MS/MS Analysis

In March 2011, the optimized sample preparation was tested on an Agilent 6410A LC-MS/MS. Modifications on SPE clean-up were made as follows: (1) only Strata-XL (6 mL, 200 mg) cartridges were used for SPE; (2) two internal standards at 4 µg/kg concentration (TRIM-d_9 for TRIM and SU-pyridine for sulfonamides expect SU-dimethoxine) were added to the samples at the end of sample preparation to calibrate the

ionization source response; and (3) after the SPE procedure samples were evaporated to dryness and were re-dissolved in 0.5 mL 0.1% (v/v) aqueous formic acid solution.

The optimization of ion transitions in 6410A MS/MS detector was carried out using flow injection analysis (FIA) and different MS settings.[13–15,26,27] The injector outlet of HPLC was directly connected to the multimode ion source (MMI) of MS detector. MMI was tested in both ESI and APCI modes. ESI resulted in higher responses and, therefore, MMI was set into ESI mode during the optimization. A mobile phase of methanol −0.1% formic acid in water (70/30, v/v) was used at a flow rate of 0.3 mL/min. Individually standard solutions diluted in methanol (1 μg/mL) were injected into the eluent and the precursor ions were scanned using MS2 scan mode in the MS detector. The intensity of selected precursor ions was maximized by searching the optimal fragmentor voltage between 70 and 150 V. The ion traces were then optimized using product ion scan mode. Precursor ions were fragmented in the collision cell using different collision energies (CE) between 0 and 30 V. MS/MS detector was set into MRM (multiple reaction monitoring) mode. The two most intense ion transitions were applied for a compound (Table 2). The strongest ion trace was used for quantification and the less intense for qualification.

The LC-MS/MS separation was carried out using the recently introduced Kinetex XB core-shell type HPLC column. In the present study, Kinetex XB was very much usable for LC-MS/MS analysis, which reduced the analysis time for the first time to 6 min with an enhanced sensitivity (Figure 3). Kinetex phases were developed by using sol–gel processing techniques in which homogeneous porous shell was grown on the solid silica core. They are engineered to provide enhanced resolution, sensitivity, and lifetime.[30] Moreover, core-shell type columns enable a reduction in solvent consumption. This XB column has protective butyl side chains that make it resistant to pH. This column has been developed specially for basic molecules,[26,27] and was not used for sulfonamides previously.

The quantification of compounds using the LC-MS/MS technique was performed by using both internal and external standard methods. Analysis of real samples showed that SU-pyridine is not usable as an ISTD for SU-dimethoxine. SU-dimethoxine was quantified with an external standard method. All studied sulfonamides could be quantified, except SU-quinoxaline. The accuracy of SU-quinoxaline was not acceptable due to a high ion enhancement, which was observed during the validation. However, SU-thiazole and TRIM were detectable along with other sulfonamides in the LC-MS/MS method. Sulfonamide active agent medicines always contain TRIM that gain the effectiveness of sulfonamides. Therefore, the detection of sulfonamide residue in honey could be confirmed by the appearance of TRIM in real samples.
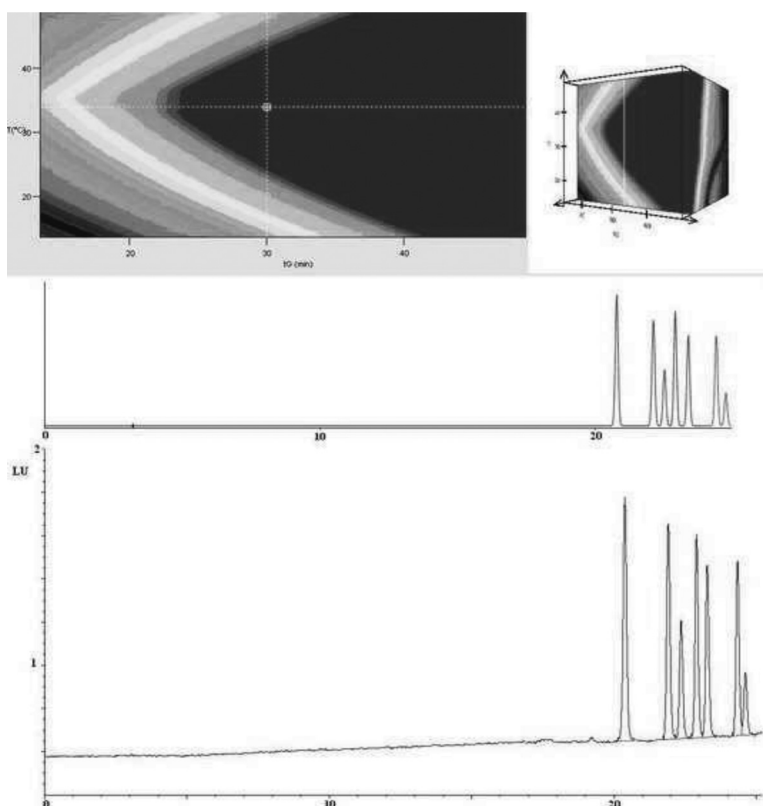
FIGURE 3 3D resolution map created by DryLab software (print screen), the predicted (upper) and the real chromatograms. The darkest color shows the setting where the resolution is higher than 1.5. Compounds from left to right: SU-diazine, SU-methazine, SUchloropyridazine, SU-methoxazole, SU-doxine, SU-dimethoxine, and SUquinoxaline.

During the validation the recoveries at level 2, 3, and 4 µg/kg levels varied between 84.6 and 117.9%, which meet the criteria of 2002/657/EC decision. The reproducibility was found in overall range of 8.4–31.1% for both sulfonamides and TRIM. The identification was confirmed using the calculated ion ratios (qualify/quantify ratio) in samples and standard solutions. The decision limit could be reduced along with LOD to 0.01–0.5 µg/kg for the sulfonamides and TRIM (Table 2), which are lower than those reported earlier.[6,7,9,11] These results met the EU standards.

The accuracy of our developed LC-MS/MS method was proven by analyzing ten real samples (sample 1–10), which were measured in parallel to an independent accredited laboratory using an unknown LC-MS/MS method. Both LC-MS/MS methods identified the same compounds. TRIM, SU-thiazole, SU-chloropyridazine, SU-methazine, and SU-dimethoxine were detected in applications both LC-MS/MS methods (Table 3). In sample

TABLE 3 Comparison of the Developed LC-MS/MS Method to an Independent Laboratory's LC-MS/MS Results Analyzing Ten Real Samples

Sample	Our Results	Independent Laboratory Results
1	SU-thiazole 1.7 μg/kg	SU-thiazole 2 μg/kg
2	SU-dimethoxine 1.8 μg/kg	SU-dimethoxine 3 μg/kg
3	SU-dimethoxine 4.1 μg/kg	SU-dimethoxine 4 μg/kg
	TRIM 2.1 μg/kg	TRIM 1 μg/kg
4	SU-dimethoxine 0.2 μg/kg	SU-dimethoxine 1 μg/kg
5	SU-dimethoxine 5.9 μg/kg	SU-dimethoxine 6 μg/kg
	TRIM 3.4 μg/kg	TRIM 2 μg/kg
6	SU-dimethoxine 0.5 μg/kg	SU-dimethoxine 1 μg/kg
7	SU-dimethoxine 0.4 μg/kg	SU-dimethoxine 1 μg/kg
8	SU-dimethoxine 3.3 μg/kg	SU-dimethoxine 4 μg/kg
	SU-methazine 1.3 μg/kg	SU-methazine 1 μg/kg
	TRIM 0.9 μg/kg	TRIM 1 μg/kg
9	SU-dimethoxine 0.3 μg/kg	SU-dimethoxine 1 μg/kg
10	SU-dimethoxine 0.7 μg/kg	SU-dimethoxine 1 μg/kg
	SU-chloropyridazine 0.8 μg/kg	SU-chloropyridazine 2 μg/kg
	TRIM 1.1 μg/kg	TRIM 1 μg/kg

1, SU-thiazole had 1.7 μg/kg concentration, determined in the present study, which is similar to 2 μg/kg residue, determined by independent laboratory. All of other samples (sample 2–10) contained SU-dimethoxine. In the case of samples 2, 3, 5, 8, and 10, concentration of SU-dimethoxine was between 0.7 and 5.9 μg/kg, which is again similar to 1–6 μg/kg SU-dimethoxine, quantified by the independent laboratory. For sample 4, 6, 7, and 9, lower amount of SU-dimethoxine was detected. Using our LC-MS/MS method, 0.2–0.5 μg/kg SU-dimethoxine was found. The independent laboratory quantified these samples as containing 1 μg/kg SU-dimethoxine. Sample 3, 5, 8, and 10 contained TRIM. We detected 0.9–3.4 μg/kg of TRIM while the other laboratory quantified the TRIM between 1 and 2 μg/kg. Some differences in concentrations could be caused by the inhomogenity of samples and the deviation in methods. In sample 8 and 10 SU-methazine and SU-chloropyridazine were also detected, respectively. The concentrations of these residues were between 0.8 and 1.3 μg/kg detected by our method. Comparatively, independent laboratory determined 1 and 2 μg/kg SU-methazine and SU-chloropyridazine, respectively (Table 3).

Robustness of HPLC-FLD Method Using Binary Pump HPLC

In the developed HPLC-FLD separation the ratio of acetonitrile (10%) in ternary mobile phase was kept constant while methanol was changed from 35% (v/v) to 50% in 30 min (see paragraph 2.4). Therefore the ratio of organic modifiers (methanol/acetonitrile) was changed during the
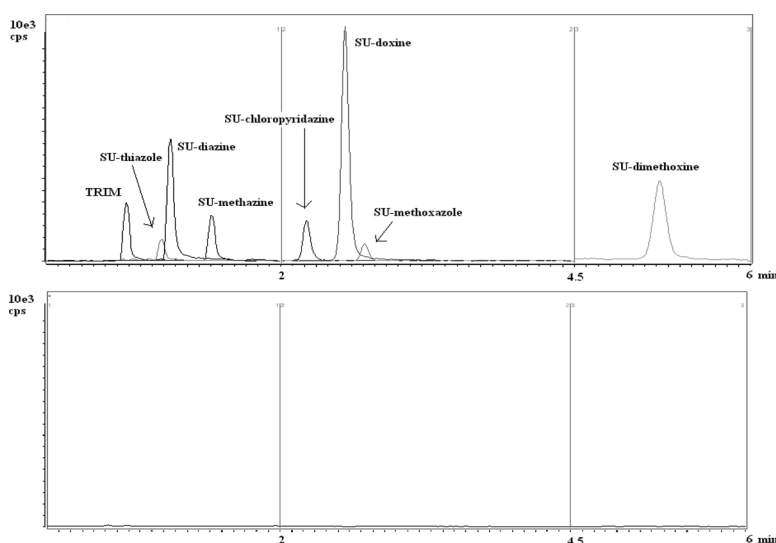
FIGURE 4 LC-MS/MS total ion chromatogram (TIC) of a spiked (2 μg/kg for all studied sulfonamides and trimethoprim) honey sample (upper) and a blank honey sample.

separation, which was carried out using quaternary pump. Generally, a ternary mobile phase is used in a binary pump system; the organic modifier is a mixture of two organic solvents such as methanol and acetonitrile. In this case, the ratio of organic solvents cannot be changed during the analysis, and, therefore, it would be difficult to reproduce this developed ternary gradient method. Consequently, we developed an additional HPLC-FLD method which uses binary pump separation. The gradient program was optimized with the help of DryLab software. The new 3D model (gradient time, temperature, terner composition) of DryLab was applied to find the optimum and robust condition (Figure 4).

On the basis of the computer prediction, the optimal separation requires 30 min gradient time, 34°C column temperature and an organic modifier composition containing methanol/acetonitrile (85/15, v/v). Solvent A is 25 mM phosphate buffer in water (pH = 5)/organic modifier (90/10, v/v) and solvent B is 25 mM phosphate buffer in water (pH = 5)/ organic modifier (10/90, v/v). In the gradient, solvent B starts from 5% and increases up to 100% over 30 min. The optimized parameters were checked by injecting a prepared spiked honey into HPLC. The experimental and predicted chromatograms were in good agreement (Figure 4). Therefore, a selective separation can be carried out using the binary system.

The aforementioned optimized binary pump separation was not applied to real sample analysis; consequently, no validation was performed for honey.

Analysis of Real Samples Using HPLC-FLD

The described quaternary pump HPLC-FLD method was successfully tested for real samples to confirm sulfonamide residues in different honey samples from March 2010 to March 2011. All positive samples contained only sulfadimethoxine residue. The advantage of the developed HPLC-FLD method is that it did not give any false positive results of sulfonamides in honey samples. The positive results of sulfadimethoxine using the HPLC-FLD method were determined between 1 and 686 μg/kg (our results with the developed method). However, the concentration range of confirmation level using LC-MS/MS technique, conducted by an independent accredited laboratory, was from 1 to 509 μg/kg. The small differences in results of two techniques may be due to the analytical procedure including inhomogeneity of the samples applied to analyzed samples for sulfonamides.

A FAPAS certified reference real honey sample was also analyzed using the described method. The acceptable ranges were 61–158 μg/kg and 58–148 μg/kg for sulfaquinoxaline and sulfathiazole, respectively. These wide ranges highlight the difficulty of sulfonamide determination in honey matrix between laboratories. The quality and quantity information were not provided before analysis. The detected concentrations were 123 μg/kg and 107 μg/kg for sulfaquinoxaline and sulfathiazole, respectively. Although HPLC-FLD method has been not validated for sulfathiazole, its determination was also successful.

The wide acceptable range of sulfonamides in FAPAS certified reference material shows that the reproducibility of different methods is not suitable. This could be the reason of the high RSD%, which was observed during the validation.

Analysis of Real Samples Using LC-MS/MS

Our LC-MS/MS method has already been used for 107 real samples in national monitoring program since March 2011. The detected values were: TRIM (0.44–342 μg/kg), SU-chloropyridazine (0.2–244 μg/kg) and SU-dimethoxine (0.03–460 μg/kg).

CONCLUSIONS

An optimized sample preparation and a new subsequent high performance liquid chromatographic method with fluorescence detection was developed with the aim of quantifying sulfonamides in honey samples. In SPE clean-up, elution with acidic methanol resulted in ~10% higher recoveries for analyzed sulfonamides than basic and neutral conditions. The

most selective HPLC separation was obtained using a mobile phase containing three solvents (methanol, acetonitrile, and phosphate buffer) at pH 5 in a slow gradient elution at flow rate of 0.8 mL/min. Method was validated in accordance with EU Commission Decision 2002/657/EC and all parameters met the EU standard.

The HPLC-FLD method has successfully been applied for real samples in Hungarian residue control monitoring program since March 2010. More than three hundred samples were analyzed and no false positive or false negative results were detected. The determination of sulfaquinoxaline and sulfathiazole in the certified reference FAPAS real honey sample was also successful with the developed HPLC-FLD method.

The developed sample preparation was applicable to the determination of sulfonamides using LC-MS/MS method. Significantly, this technique could improve the analytical limits for sulfonamides by a factor of 10. The simultaneous determination of trimethoprim with sulfonamides was also possible applying an LC-MS/MS method.

Both methods have now been accredited by the National Accreditation Board (NAT), and therefore, further confirmation measurement from other accredited laboratory is not necessary at this time.

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