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Quantitative analysis of twelve sulfonamides in honey after acidic hydrolysis by high-performance liquid chromatography with post-column derivatization and fluorescence detection

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Abstract

A quantitative HPLC–fluorescence method for the simultaneous determination of 12 sulfonamides (sulfaguanidine, sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamether, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine and sulfadoxine) in honey was developed and validated. Sample pretreatment included acidic hydrolysis, followed by liquid–liquid extraction and solid-phase extraction on a strong cation exchanger. LC separation was performed in 45 min, with a total analysis time of 60 min. Identification and quantitation were based on retention time and fluorescence intensity, respectively. Peak area ratios of the target analytes and the internal standard were fit to a linear least-squares regression curve with a weighting factor of $1/x$. Limits of detection and quantitation (LOQ) had values of 1 or 2 and 2 or 5 ng/g, respectively. Linearity was obtained with an average coefficient of determination (R^2) higher than 0.997, over a dynamic range from the LOQ value up to 100 ng/g. The method demonstrated good intra- and interbatch precision and accuracy. No interferences with the peaks of interest were observed throughout the chromatographic run. Sample pretreatment provided efficient cleanup, while post-column derivatization with fluorescamine proved to be a reproducible derivatization technique enabling a sensitive and rugged quantitative determination of sulfonamides.
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1. Introduction

Prevention and treatment of bacterial bee diseases such as American foulbroud (*Bacillus larvae*) and European foulbroud (*Streptococcus pluton*) with sulfonamides can lead to residues of these compounds in honey [1]. Residues of these antibacterial drugs in honey are of major concern because of their contribution in development of antibiotic-resistant pathogenic bacteria [2].

No maximum residue levels (MRLs) for sulfonamides in honey were set in the European Union, which means that sulfonamides, if present, must be below the limit of quantitation (LOQ) of the analytical method used [3]. Since LOQs differ between laboratories, some countries within the European Union have established action limits or tolerated levels. Belgium and the United Kingdom for instance have action limits of 20 and 50 ng/g, respectively, referring to the sum of all substances within the sulfonamide-group. France has a limit of 10 ng/g for sulfathiazole. All these limits can and will be lowered upon improvement of analytical methods. Switzerland has established a fixed limit of 50 ng/g, referring to the sum of initial substances (sulfonamides and their metabolites).

Until now, no metabolites have been defined in honey. However, already in 1981 Belliardo noted that concentrations decreased over a period of time [4]. In 1989 Low et al. [5] stated that sulfathiazole disappeared by reaction with reducing-sugar solutions at elevated temperatures. Sheth et al. [6] demonstrated that the free aromatic amino group of

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sulfonamides could react with reducing sugars to form a variety of different sugar-bound compounds, which had a different chromatographic behaviour. Sulfonamides were not destroyed in these reactions, and hydrolysis of most sugar-bound compounds into the free forms could be achieved by acidification. Finally, Schwaiger and Schuch [7] proved the need of an acidic hydrolysis step prior to the analytical determination of sulfonamide residues in honey. They stated this step avoids an underestimation of an actual contamination since considerable amounts of bound sulfonamides are released under the acidic conditions in the stomach.

Over the years a number of colorimetric [8–10], enzyme immuno assay [6,11,12], radio receptor assay (commercially available Charm II test), gas chromatographic [7], thin-layer chromatographic [10,13] and reversed phase high performance liquid chromatographic methods [4,7,8,14–24] for the determination of sulfonamides in honey have been developed. In view of the relevance for this work a brief overview of the HPLC methods is given. Some determine only sulfathiazole [7,8,14,15,23], while others determine simultaneously from 3 up to 16 sulfonamides [4,16–21,24]. Surprisingly, only a limited number of methods include the essential initial acidic hydrolysis step [7,21–23]. Extraction techniques for sulfonamides in honey include dissolution [15,17–19,23], liquid–liquid extraction [7,14,22], solid-phase extraction (SPE) [18,20,21], extraction with an organic solvent [4], extraction with an organic solvent followed by liquid–liquid extraction [8], liquid–liquid extraction followed by solid-phase extraction [16] and a combination of solid-phase extractions [24]. A variety of detection techniques such as UV [4,8,14,17–19], diode array detection (DAD) [15,16], fluorescence after pre-column derivatization with fluorescamine [7,20,23,24] and electrospray ionization tandem mass spectrometry [21,22] have been applied.

A more detailed description of the four methods with an initial acidic hydrolysis step is given below. Schwaiger and Schuch developed a method for the determination of sulfathiazole, but stated that their sample preparation was also applicable to 16 other sulfonamides and their chromatographic conditions could separate 11 sulfonamides [7]. After hydrolysis the sample was adjusted to pH 4.5 with a sodium hydroxide solution. A mixture of acetonitrile, dichloromethane and sodium chloride was added. An aliquot of the organic layer was partially evaporated and diluted with water prior to a derivatization step with fluorescamine. Separation was performed on a Spherisorb ODS2 column with an isocratic run of 10 min. Fluorescence detection was applied at an excitation and emission wavelength of 405 and 495 nm, respectively. The limit of quantitation for sulfathiazole was 15 ng/g.

Martel and Zeggane [23] described a method for the determination of sulfathiazole only. An aliquot of the hydrolyzed sample was taken and a mixture of sodium acetate, sodium citrate buffer and internal standard solution was added prior to a derivatization step with fluorescamine. Separation was performed on a Hypersil BDS C_{18} column with an isocratic run of 10 min. Fluorescence detection was applied at an excitation and emission wavelength of 405 and 495 nm, respectively. The limit of quantitation was 10 ng/g.

Kaufmann et al. [21] developed a method for the simultaneous determination of 16 sulfonamides and some other antibiotics. After hydrolysis and dilution with a citric acid solution and filtration, a portion of the honey filtrate was adjusted to pH 3.5–4.5 with ammonia and subsequently loaded onto a conditioned Oasis HLB SPE column. Elution was performed with acetonitrile, followed by partial evaporation and dilution with the mobile phase. Separation was performed on a Nucleosil C_{18} HD column with a gradient run of 19 min. Electrospray ionization (ESI) tandem mass spectrometry with a multi-reaction monitoring (MRM) program was applied as detection technique. The limit of detection varies from 0.4 to 11 ng/g, depending on the compound. Although this LC–MS–MS method with MRM traces is highly selective, quantitative analysis is somewhat impractical because, depending on the origin of the honey, each time a different calibration is necessary due to matrix-related ionization suppression. As a consequence multiflower honeys are very hard to quantify using this method.

Verzegnassi et al. developed a qualitative method for the simultaneous detection of 10 sulfonamides [22]. After hydrolysis and adjustment to pH 6.5 with a saturated disodium hydrogenphosphate solution, a mixture of acetonitrile and dichloromethane was added twice. The combined organic layers were diluted with dichloromethane. An aliquot was evaporated to dryness. After addition of an internal standard, the residue was redissolved in a mixture of water-acetonitrile and filtered. Separation was performed on a Nucleosil C_{18} HD column with an overall gradient run time of 23 min. Electrospray ionization tandem mass spectrometry with selected reaction monitoring (SRM) transitions was applied as detection technique. Since this is a qualitative method, an exact detection limit could not be given, but the authors stated that low nanograms per gram levels could be detected. The authors extensively addressed the issue of matrix-induced suppression of ionization in LC–ESI-MS–MS and concluded that even matrix-matched calibration curves could not be used for the quantitation of sulfonamide residues in honey using their sample preparation method.

We present a fully validated HPLC–fluorescence method, combined with prior acidic hydrolysis and post-column derivatization, for the simultaneous determination of 12 sulfonamides (Fig. 1) in honey down to levels far below currently fixed limits. The method was applied to honey samples from a Belgian honey processing company and to Belgian honey samples collected by the Centre of Agricultural Research from the Scientific Institute of the Flemish Community.

2. Experimental

2.1. Instrumentation

All experiments were carried out on a LaChrom HPLC system from Merck–Hitachi (Tokyo, Japan) consisting of a

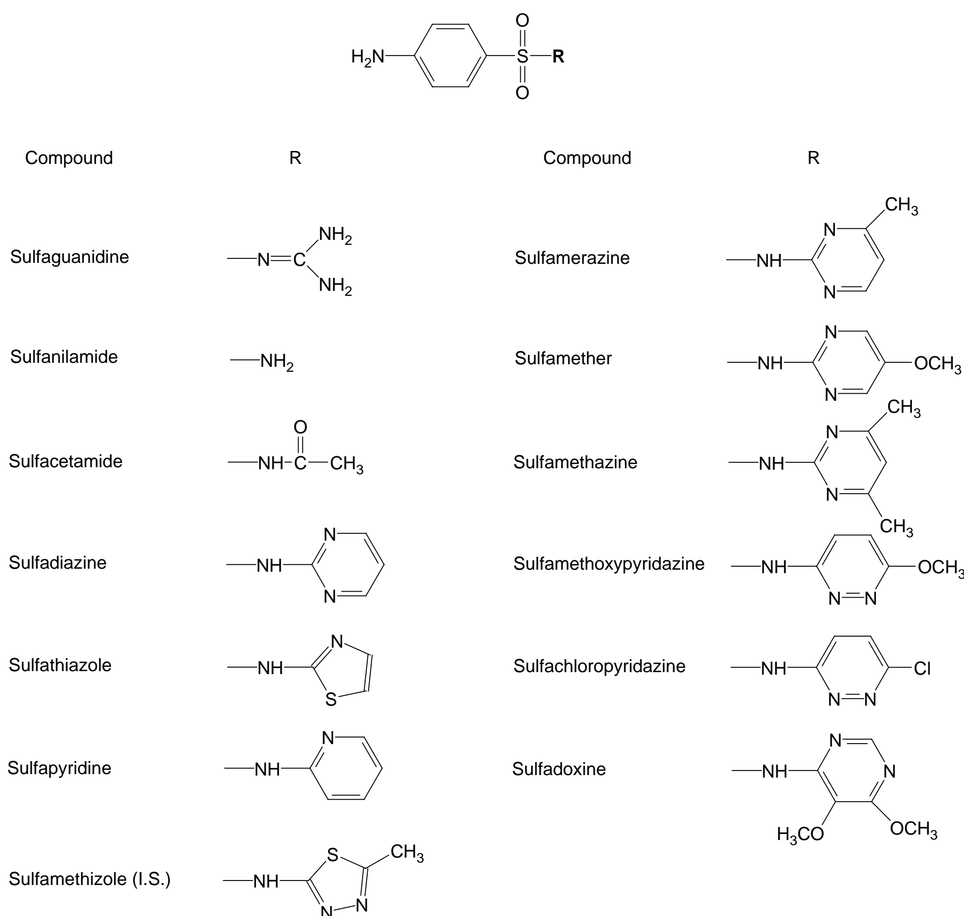

Fig. 1. Chemical structures of the studied sulfonamides.

L-7612 solvent degasser, a L-7100 pump with low pressure gradient accessory, a L-7200 autosampler, a L-7360 column oven, a L-7485 fluorescence detector and a D-7000 interface. The post-column reagent was delivered by a L-6200 pump from Merck–Hitachi. All data were acquired and analyzed using the Multi-HSM software. Centrifugation of the samples was performed in a MSE Mistral 2000 centrifuge (Breda, The Netherlands). Evaporation under nitrogen was conducted in a TurboVap LV evaporator from Zymark (Hopkinton, MA, USA). Mixing of the samples was performed by a rotary mixer from Labinco (Breda, The Netherlands) and a Mistral mixer from Lab-Line (Melrose Park, IL, USA).

2.2. Chemicals and reagents

Sulfaguanidine, sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfapyridine, sulfamethizole (internal standard), sulfamether, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, sulfisoxazole, sulfamethoxazole, and *p*-aminobenzoic acid were purchased from Sigma (St. Louis, MO, USA). Sulfamerazine and sulfadoxine were obtained from Riedel-de Haen (Seelze, Germany) and sulfadimethoxine from Fluka (Buchs, Switzerland). Reagent-grade anhydrous sodium acetate, sodium dihydrogenphosphate monohydrate, anhydrous sodium sulfate, 2 M hydrochloric acid, 5 M sodium hydroxide, orthophosphoric acid 85% and 2-mercaptoethanol were purchased from Merck (Darmstadt, Germany). Fluorescamine and ammonium hydroxide solution 10% were obtained from Fluka (Buchs, Switzerland). Acetic acid was purchased from Vel (Leuven, Belgium). HPLC-grade water, methanol and acetonitrile were obtained from Biosolve (Valkenswaard, The Netherlands).

2.3. Honey samples

Honey samples were of different geographical origin (single flower as well as multiflower). The samples were stored at 4 °C in the dark.

2.4. Preparation of standard solutions

Individual primary stock solutions of all standards were prepared in methanol at a concentration of 1 mg/mL and stored in the dark at −20 °C until use. Secondary stock solutions, ranging from 1 to 50 μg/mL, were prepared by mixing the individual primary stock solutions of the 12 determined sulfonamides and *p*-aminobenzoic acid and further dilution

with methanol. These stock solutions were stored protected from light at 4 °C. Working solutions, ranging from 20 to 2000 ng/mL, were prepared by dilution of the stock solutions with methanol and stored in the dark at 4 °C. Blank honey samples were fortified with the working solutions to make honey standards at concentrations of 1, 2, 5, 10, 20, 50 or 100 ng/g.

A primary stock solution of the internal standard was prepared in methanol at a concentration of 1 mg/mL and stored in the dark at −20 °C until use. A secondary stock solution and a working solution of the internal standard at a concentration of 20 μg/mL and 400 ng/mL were prepared by dilution with methanol and were stored protected from light at 4 °C. A 75-μl volume of this working solution was added to each sample, giving a final internal standard concentration of 20 ng/g.

2.5. Sample preparation

Sample pretreatment of fortified honey and unknown honey specimens was performed by hydrolysis of the sample, followed by a liquid–liquid extraction and a solid-phase extraction. A 1.5-g honey sample was dissolved in 1.5 mL of 2 M hydrochloric acid. The sample was shaken on a rotary mixer for 45 min. A volume of 75 μL of internal standard solution and/or working standard solution was added to the sample. The pH was adjusted to 5 by adding 550 μL 5 M sodium hydroxide and 750 μL of a 1.2 M sodium acetate solution. The sample was vortexed for 30 s and extracted with 8 mL of acetonitrile (30 min on a rotary mixer). The mixture was then centrifuged for 10 min at 875 × g and 7.85 mL of the upper organic layer was transferred in another centrifuge tube and dried with 5 g anhydrous sodium sulfate. This tube was shaken on a rotary mixer for 5 min and centrifuged for 10 min at 875 × g. Six milliliters of the upper organic layer was transferred to a tube and evaporated to dryness at 45 °C under a gentle stream of nitrogen. The residue was reconstituted in 5 mL 1% (v/v, in water) acetic acid and loaded onto a Bond Elut SCX (500 mg, 3 mL, 40 μm) SPE column (Varian, Harbor City, CA, USA) conditioned with 3 mL of methanol and 5 mL of 1% (v/v, in water) acetic acid. The column was washed with 3 mL of 1% (v/v, in water) acetic acid and 3 mL of methanol. The cartridge was allowed to run dry for 5 min. Elution was performed with three times 1 mL of a mixture of 2% (v/v, in water) ammonium hydroxide solution–methanol (1:2, v/v). The eluate was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 200 μL 1% (v/v, in water) acetic acid, vortexed for 1 min and centrifuged for 2 min at 875 × g before injection of 50 μL into the chromatographic system.

2.6. Liquid chromatography–fluorescence detection

Chromatographic separation was performed on a Purospher Star RP-18 endcapped column (5 μm, 150 mm × 4.6 mm) (Merck), fitted with a Purospher Star RP-18 endcapped guard column (5 μm, 4 mm × 4 mm) (Merck, Darmstadt, Germany). Gradient elution with a mixture of 0.020 M acetate buffer (0.0819% (w/v), sodium acetate in water, adjusted to pH 4.75 with acetic acid)–acetonitrile (98:2, v/v) (solvent A) and a mixture of the same acetate buffer–acetonitrile (68:32; v/v) (solvent B), at a flow rate of 0.7 mL/min, was applied. The initial gradient conditions were 2% solvent B, increasing to 35% solvent B in 31 min, with a final composition of 75% solvent B in 10 min. The column was flushed for 7 min at 95% solvent B. Initial gradient conditions were re-established immediately and the column was equilibrated for 12 min. The post-column reagent, a mixture of fluorescamine–2-mercaptoethanol–acetonitrile–0.021 M phosphate buffer (0.276%, w/v, sodium dihydrogenphosphate monohydrate in water, adjusted to pH 3.0 with orthophosphoric acid 85%) (0.025:0.2:25:75, w/v/v/v) was stored in the dark at 4 °C, and applied at a flow rate of 0.2 mL/min. The reaction coil consisted of a PTFE tubing for HPLC (10 m × 1/16 in. o.d. × 0.25 mm i.d.; in. = 2.54 cm). The temperature of the column and the reaction coil was 45 °C. Detection was performed at an excitation wavelength of 420 nm and an emission wavelength of 485 nm.

2.7. Data analysis

Calibration, using internal standardization, was done over a concentration range from 2 or 5 to 100 ng/g. For each standard curve, five or six different concentrations were used. Peak area ratios of the target analytes and the internal standard were calculated for each concentration. These data were fit to a linear least-squares regression curve with a weighting factor of $1/x$.

2.8. Validation

The following criteria were used to evaluate the method: sensitivity, linearity, intra- and interbatch precision, accuracy, recovery, stability and selectivity.

The sensitivity of the method was evaluated by determining the limit of detection (LOD) and quantitation (LOQ). LOD was defined as the concentration with a signal-to-noise ratio of at least three, while LOQ was the lowest standard with a signal-to-noise of at least 10 and acceptable precision [relative standard deviation (R.S.D.) less than 15%]. Both parameters were determined empirically by analysis of a series of decreasing concentrations of the sulfonamide-fortified honey in multiple replicates.

The linearity of the method was evaluated by calculation of the regression line by the method of least squares and expressed by the coefficient of determination (R^2). Based on residual plots a $1/x$ weighting factor was applied. Linearity of each of the compounds was determined with at least five concentration levels not including the blank matrix.

Precision was evaluated over the linear dynamic range at three different concentration levels, i.e. low (LOQ), medium (20 ng/g) and high (100 ng/g). Intrabatch precision was assessed by five determinations per concentration in 1 day.

Interbatch precision was assessed by five determinations per concentration on five separate days. Precision was expressed as R.S.D.

Accuracy was evaluated with separately prepared individual and mixed stock and working solutions of all standards and the internal standard over the linear dynamic range at three different concentration levels, i.e. low (5 ng/g), medium (20 ng/g) and high (100 ng/g). Accuracy was assessed by five determinations per concentration on five separate days and was expressed by its average and R.S.D.

The absolute recovery for each analyte was also determined at low (5 ng/g), medium (20 ng/g) and high (100 ng/g) concentration. The standard working solutions were added to the honey samples before and after sample pretreatment. Since quantitation was performed by the peak area ratios of the target analytes and the internal standard, the internal standard working solution was always added after sample pretreatment. The resultant peak area ratios were compared. The recovery of the internal standard was calculated at a concentration of 20 ng/g by addition of the internal standard working solution before and after sample pretreatment, and the resultant peak area ratio was compared. Recovery was expressed by its average and R.S.D.

Stability of the stock solutions was tested monthly by injection of freshly prepared working solutions. Analyte stability during pretreatment was determined at the following stages: reconstitution in 1% acetic acid after liquid–liquid extraction, 5 days at 4 °C; final extract redissolved in 1% acetic acid, 7 days at 4 °C, and 24 h at room temperature.

Selectivity was investigated in respect of sample pretreatment, post-column derivatization and chromatographic stability. A number of possible interfering compounds were investigated.

3. Results and discussion

3.1. Sample preparation

The procedure of Schwaiger and Schuch for hydrolysis of the sugar-bound sulfonamides was applied, but the hydrolysis time was slightly extended to 45 min to ensure completeness of the reaction [7]. The high concentration of salt enhances the separation of the aqueous and the acetonitrile layer in the liquid–liquid extraction step. Anhydrous sodium sulfate had to be added to eliminate all traces of water, containing a very high amount of salt compromising the retention of some sulfonamides (sulfacetamide and sulfamethizole) on the SCX column.

3.2. LC–fluorescence method development

The separation of 12 compounds and the internal standard was achieved in 45 min. All compounds differed at least 0.9 min in retention time, resulting in baseline separation, with exception of the couple sulfamethazine-sulfamethoxypyridazine, which have a minimal overlap. A column wash and reequilibration period allowed the injection of samples every 60 min. Post-column derivatization was used to enhance the sensitivity and selectivity, and maximize separation efficiency of the compounds (the latter in contrast with pre-column derivatization). The stability of the LC method was evaluated by calculation of the variation of retention times. R.S.Ds., calculated from retention times obtained over 25 injections, proved to be less than 0.9% for all compounds, indicating very good chromatographic stability (Table 1). Fig. 2 shows chromatograms of a blank sample (A), the same sample fortified at 20 ng/g for all sulfonamides and *p*-aminobenzoic acid (B) and a positive sample containing 8 ng/g sulfathiazole and 16 ng/g sulfamethazine (C).

Table 1
HPLC–fluorescence method parameters

Compound	t_R (min)	Variation t_R (R.S.D., %; $n = 25$)
Sulfaguanidine	6.10	0.28
Sulfanilamide	7.00	0.30
Sulfacetamide	12.29	0.82
Sulfadiazine	19.50	0.32
Sulfathiazole	24.01	0.38
Sulfapyridine	25.91	0.29
Sulfamerazine	28.00	0.25
Sulfamethizole (I.S.)	30.99	0.63
Sulfamether	34.49	0.26
Sulfamethazine	36.21	0.17
Sulfamethoxypyridazine	37.10	0.20
Sulfachloropyridazine	39.57	0.32
Sulfadoxine	44.65	0.12

3.3. Calibration and validation

Sulfamethizole was chosen as internal standard based on its structure, retention behaviour and very low prevalence as residue in honey.

The complete method was evaluated according to the criteria described in the Section 2. Table 2 provides the LODs, LOQs and calibration results for all analytes. LOD and LOQ values of 1 or 2 and 2 or 5 ng/g, respectively, were obtained.

Linearity was obtained with an average coefficient of determination (R^2, weighting factor, $1/x$) higher than 0.997, over a dynamic range from LOQ to 100 ng/g for each of the analytes (Table 2). Variation on the calibration intercept and slope indicate absence of constant error and matrix effect, respectively.

Precision and accuracy of the method were evaluated at three concentrations over the linear dynamic range (low, medium, high). Table 3 includes the concentrations tested and results for both validation parameters. Intrabatch precision for all compounds proved to be less than 10%. Interbatch precision was less than 7% for all compounds, except for sulfacetamide, which had a variation of 12.4% for the medium concentration level. Accuracy was between 98 and 107%, with a variation of less than 9% for all compounds, except

Table 2
LODs, LOQs and calibration results

Compound	LOD (ng/g)	LOQ (ng/g)	Calibration intercept (mean ± S.D., $n = 5$)	Calibration slope (mean ± S.D., $n = 5$)	R^2	Linear dynamic range (ng/g)
Sulfaguanidine	1	2	0.0175 ± 0.0113	0.0972 ± 0.0075	0.9990	2–100
Sulfanilamide	1	2	0.0189 ± 0.0220	0.1535 ± 0.0099	0.9997	2–100
Sulfacetamide	2	5	−0.0254 ± 0.0302	0.0510 ± 0.0057	0.9976	5–100
Sulfadiazine	1	2	0.0127 ± 0.0139	0.0681 ± 0.0047	0.9992	2–100
Sulfathiazole	1	2	0.0065 ± 0.0086	0.0873 ± 0.0051	0.9994	2–100
Sulfapyridine	1	2	−0.0342 ± 0.0422	0.0872 ± 0.0054	0.9986	2–100
Sulfamerazine	1	2	0.0113 ± 0.0076	0.0725 ± 0.0040	0.9994	2–100
Sulfamether	2	5	0.0002 ± 0.0082	0.0338 ± 0.0026	0.9995	5–100
Sulfamethazine	2	5	0.0115 ± 0.0345	0.0766 ± 0.0045	0.9989	5–100
Sulfamethoxypyridazine	2	5	−0.0131 ± 0.0195	0.0198 ± 0.0011	0.9987	5–100
Sulfachloropyridazine	2	5	−0.0068 ± 0.0133	0.0192 ± 0.0013	0.9997	5–100
Sulfadoxine	2	5	−0.0099 ± 0.0172	0.0150 ± 0.0008	0.9991	5–100

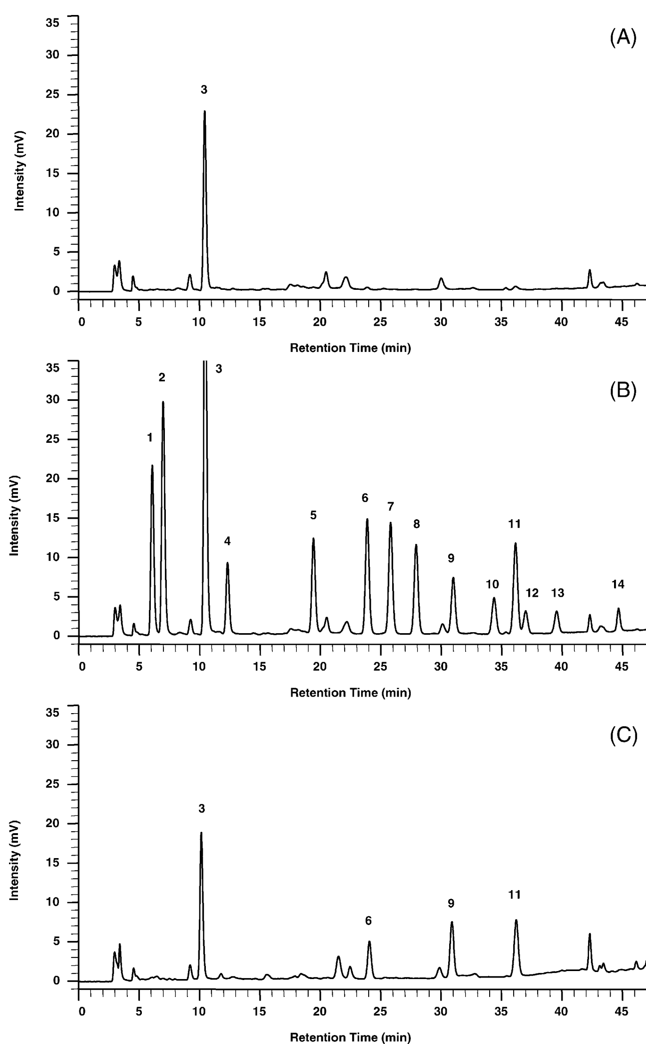

Fig. 2. Chromatograms of a blank sample (A), the same sample fortified at 20 ng/g for all sulfonamides and *p*-aminobenzoic acid (B) and a positive sample containing 8 ng/g sulfathiazole and 16 ng/g sulfamethazine (C). Peaks: 1 = sulfaguanidine, 2 = sulfanilamide, 3 = *p*-aminobenzoic acid, 4 = sulfacetamide, 5 = sulfadiazine, 6 = sulfathiazole, 7 = sulfapyridine, 8 = sulfamerazine, 9 = sulfamethizole (internal standard), 10 = sulfamether, 11 = sulfamethazine, 12 = sulfamethoxypyridazine, 13 = sulfachloropyridazine and 14 = sulfadoxine.

sulfaguanidine, which had a slightly higher result for the high concentration level.

For evaluation of long-term reproducibility of this method, three incurred samples containing sulfathiazole were analysed five times in a period of 5 months. Results were 16.35 ± 0.91, 48.34 ± 1.17 and 70.11 ± 2.34 ng/g (mean ± S.D.). This indicates that the method can achieve full hydrolysis of the sulfonamide-sugar bound and reproducible measurements, even after a long storage period of the honey samples at 4 °C.

The absolute recovery of the analytes with the whole procedure was also determined at three concentrations (low, medium, high). An overview of the results obtained is given in Table 4. Absolute recoveries varied between 37 and 67%. The recovery was mainly determined by the liquid–liquid extraction step, while the SPE step had a recovery of nearly 100% (data not shown). The range in recoveries reflects the polarity of the compounds and range of pK_a values of the sulfonamide-group. The recovery did not depend on the concentration. Variation of the results was less than 15% for all compounds. The variation reflected the multiple cleanup steps of the sample. The low recovery and its variaton stressed the need for a suitable internal standard. In fact, the previous validation parameters clearly demonstrated that all drawbacks of the low absolute recovery and its variation were resolved by application of the internal standard.

Under the stated conditions, stock solutions proved to be stable for at least 6 months. No significant loss or deterioration for any of the compounds of interest was observed at the specified stages of sample treatment.

Selectivity is a key point in developing a HPLC– fluorescence method. Although fluorescence detection lacks the selectivity of some other detection techniques (e.g. tandem mass spectrometry), sample cleanup and chromatographic performance can compensate for this drawback. The combination of liquid–liquid extraction with strong cation-exchange solid-phase extraction and derivatization with fluorescamine effected the selection and detection of medium to non-polar molecules containing a primary amine function. A typical chromatogram obtained after analysis of a blank

Table 3
Precision and accuracy data

Compound	Intrabatch precision (R.S.D.%; $n = 5$)			Interbatch precision (R.S.D.%; $n = 5$)			Accuracy (mean, % ± R.S.D., %; $n = 5$)		
	Low LOQ	Medium (20 ng/g)	High (100 ng/g)	Low LOQ	Medium (20 ng/g)	High (100 ng/g)	Low (5 ng/g)	Medium (20 ng/g)	High (100 ng/g)
Sulfaguanidine	1.90	3.09	2.20	2.73	5.66	4.05	98.8 ± 8.7	98.6 ± 7.9	98.9 ± 10.2
Sulfanilamide	2.58	1.96	0.90	3.86	3.28	5.98	106.0 ± 5.0	103.4 ± 3.4	103.8 ± 4.9
Sulfacetamide	2.29	3.68	3.47	4.64	12.41	6.97	98.6 ± 8.3	102.1 ± 4.9	101.8 ± 6.1
Sulfadiazine	8.30	4.08	1.35	4.39	2.98	4.26	101.5 ± 2.0	99.3 ± 7.2	99.0 ± 7.5
Sulfathiazole	3.75	3.99	1.18	3.74	3.42	3.37	104.0 ± 2.4	104.1 ± 3.5	101.4 ± 5.7
Sulfapyridine	3.81	6.06	4.86	2.17	4.65	5.10	100.8 ± 6.0	104.2 ± 7.4	103.6 ± 8.0
Sulfamerazine	2.53	1.26	1.27	6.94	3.62	3.02	103.6 ± 5.3	105.8 ± 3.5	106.2 ± 2.8
Sulfamether	2.68	3.92	0.32	6.50	2.92	4.01	102.6 ± 4.1	99.5 ± 4.4	100.1 ± 8.6
Sulfamethazine	3.84	5.68	3.41	6.21	3.31	2.76	104.5 ± 7.9	103.7 ± 6.9	104.6 ± 4.4
Sulfamethoxypyridazine	9.79	4.36	1.62	3.78	2.03	3.29	102.8 ± 4.7	102.0 ± 2.6	102.9 ± 3.5
Sulfachloropyridazine	5.09	1.47	1.31	3.77	1.76	4.67	103.0 ± 3.1	99.6 ± 5.9	99.9 ± 7.0
Sulfadoxine	6.17	3.23	2.00	5.11	3.09	1.67	103.3 ± 6.0	100.1 ± 2.6	99.5 ± 4.4

Table 4
Absolute recovery results

Compound	Absolute recovery (mean, % ±R.S.D., %; $n = 4$)		
	Low (5 ng/g)	Medium (20 ng/g)	High (100 ng/g)
Sulfaguanidine	38.2 ± 14.2	40.7 ± 3.2	39.9 ± 9.4
Sulfanilamide	62.8 ± 5.3	63.4 ± 7.0	66.8 ± 9.1
Sulfacetamide	37.4 ± 2.0	44.5 ± 12.3	44.9 ± 10.0
Sulfadiazine	52.7 ± 12.2	59.8 ± 7.9	59.9 ± 9.9
Sulfathiazole	58.5 ± 5.6	62.0 ± 7.6	61.1 ± 7.6
Sulfapyridine	56.0 ± 10.9	57.1 ± 7.7	58.0 ± 9.6
Sulfamerazine	58.6 ± 6.3	63.2 ± 3.9	63.4 ± 7.1
Sulfamethizole (I.S.)	n/a	55.3 ± 8.7[a]	n/a
Sulfamether	56.0 ± 11.0	63.8 ± 10.3	64.0 ± 7.9
Sulfamethazine	64.3 ± 4.1	64.8 ± 6.6	63.4 ± 9.2
Sulfamethoxypyridazine	56.5 ± 13.5	61.3 ± 3.6	62.7 ± 7.6
Sulfachloropyridazine	55.3 ± 11.8	58.5 ± 12.7	60.1 ± 11.1
Sulfadoxine	58.4 ± 5.6	63.5 ± 5.0	65.2 ± 8.3

n/a: not applicable.
[a] $n = 24$.

honey sample is shown in Fig. 2A. The chromatogram displays one major peak at 10.4 min and a few minor peaks. The major peak was identified as *p*-aminobenzoic acid, a compound naturally occuring in honey. The other peaks were not identified. Fig. 2B shows the chromatogram of the same blank sample fortified at 20 ng/g for all compounds and *p*-aminobenzoic acid. This chromatogram clearly demonstrates that none of the peaks occuring in the blank sample interfered with any of the compounds. Based on their structure, a number of possibly interfering compounds were investigated, i.e. sulfanilic acid, sulfisoxazole, sulfamethoxazole, sulfadimethoxine and sulfaquinoxaline. None of these compounds interfered in the analysis of any of the 12 sulfonamides. Sulfanilic acid eluted in the solvent front. Sulfisoxazole eluted after 41.59 min. Sulfisoxazole was not included in this assay because the compound was not stable in the alkaline solution obtained after elution of the SCX column. Sulfamethoxazole eluted after 43.12 min. It showed similar behaviour as the compounds included in this assay (data not shown), but could not be determined with this chromatographic run because in some cases it partially overlapped with an interfering peak. Sulfadimethoxine and sulfaquinoxaline both eluted later than 47 min and consequently outside the detection window.

Finally, the method was applied to samples from a Belgian honey processing company and to Belgian honey samples collected by the Centre of Agricultural Research from the Scientific Institute of the Flemish Community. The method fulfilled our analytical standard criteria. No interferences in the analyses of the sulfonamides were observed. The sensitivity and linear dynamic range of the method were relevant to monitor the presence of 12 sulfonamides in honey samples. The method achieved precise and accurate measurements of the compounds of interest in honey.

4. Conclusion

The described procedure provided a precise, accurate and sensitive method for the quantitation of sulfonamides

in honey samples. Sample pretreatment by combination of liquid–liquid extraction and solid-phase extraction provided sufficient cleanup of the honey samples prior to HPLC–fluorescence analysis. Post-column derivatization with fluorescamine proved to be a reproducible derivatization technique for sulfonamides. HPLC–fluorescence detection proved to be a sensitive and selective technique, allowing simultaneous quantitative analysis of 12 sulfonamides of interest in honey at levels under, at, and above the various concentration limits within the European Union.

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References

[1] P.S. Milne, Nature 155 (1945) 335.
[2] N.A. Botsoglou, D.J. Fletouris (Eds.), Drug Residues in Foods, Marcel Dekker, New York, 2001, p. 27.
[3] Commission of the European Communities, Council Regulation EEC 2377/90. Off. J. L. 224 (1990) 1.
[4] F. Belliardo, J. Apic. Res. 20 (1981) 44.
[5] N.H. Low, J.L. Standish, P. Sporns, Can. Inst. Food Sci. Technol. J. 22 (1989) 212.
[6] H.B. Sheth, V.A. Yaylayan, N.H. Low, M.E. Stiles, P. Sporns, J. Agric. Food Chem. 38 (1990) 1125.
[7] I. Schwaiger, R. Schuch, Dtsch. Lebensm.-Rundsch 96 (2000) 93.
[8] C.P. Barry, G.M. Maceachern, J. Assoc. Off. Anal. Chem. 66 (1983) 4.
[9] D.P. Schwartz, J. Sherma, J. Assoc. Off. Anal. Chem. 69 (1986) 72.
[10] J. Sherma, W. Bretschneider, M. Dittamo, N. Dibiase, D. Huh, D.P. Schwartz, J. Chromatogr. 463 (1989) 229.
[11] C.A. Thomson, P. Sporns, J. Food Sci. 60 (1995) 409.
[12] W. Heering, E. Usleber, R. Dietrich, E. Märtlbauer, Analyst 123 (1998) 2759.
[13] E. Neidert, Z. Baraniak, A. Sauve, J. Assoc. Off. Anal. Chem. 69 (1986) 641.
[14] U. Jurgens, Z. Lebensm. Unters. Forsch. 174 (1982) 208.
[15] T.G. Diaz, A.G. Cabanillas, F. Salinas, Anal. Lett. 23 (1990) 607.
[16] M. Horie, K. Saito, N. Nose, H. Nakazawa, J. AOAC Int. 75 (1992) 786.
[17] P. Vinas, C.L. Erroz, A.H. Canals, M.H. Cordoba, Chromatographia 40 (1995) 382.
[18] A. Szymanski, Chem. Anal. 45 (2000) 521.
[19] R.D. Caballero, J.R. Torres-Lapasio, J.J. Baeza-Baeza, M.C. Garcia-Alvarez-Coque, J. Liq. Chromatogr. Rel. Technol. 24 (2001) 117.
[20] A. Posyniak, T. Sniegocki, J. Zmudzki, Bull. Vet. Inst. Pulawy 46 (2002) 111.
[21] A. Kaufmann, S. Roth, B. Ryser, M. Widmer, D. Guggisberg, J. AOAC Int. 85 (2002) 853.
[22] L. Verzegnassi, M.C. Savoy-Perroud, R.H. Stadler, J. Chromatogr. A 977 (2002) 77.
[23] A.C. Martel, S. Zeggane, J. Liq. Chromatogr. Rel. Technol. 26 (2003) 953.
[24] G.F. Pang, Y.Z. Cao, C.L. Fan, J.J. Zhang, X.M. Li, Z.Y. Li, G.Q. Jia, Anal. Bioanal. Chem. 376 (2003) 534.