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A new and simple method to determine trace levels of sulfonamides in honey by high performance liquid chromatography with fluorescence detection

José Bernal[a,*], María Jesús Nozal[b], Juan José Jiménez[b], María Teresa Martín[b], Esther Sanz[b]

[a] Institute of Industrial Fermentations (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain
[b] I.U.CINQUIMA, Analytical Chemistry Group, Faculty of Sciences, University of Valladolid, Dr. Mergelina s/n, 47011 Valladolid, Spain

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ABSTRACT

A novel method for the simultaneous analysis at trace level of sulfonamides (sulfaguanidine, sulfanilamide, sulfacetamide, sulfathiazole, sulfapyridine, sulfachloropyridazine, sulfamerazine, sulfameter, sulfamethazine, sulfadoxine, sulfadiazine, sulfamonomethoxine, sulfadimethoxine) in honey is described. Methanol has been used in the sample treatment step to avoid the emulsion formation and to break the N-glycosidic bond between sugars and sulfonamides. The determination is carried out by liquid chromatography in gradient elution mode, with fluorescence detection after the on-line pre-column derivatization with fluorescamine. The influence of parameters such as the mobile phase composition, column temperature, pH or injection volume, on the separation has been taken into account and the derivatization step has also been optimized. Recoveries of the compounds on spiked honey samples ranged from 56% for sulfadoxine to 96% for sulfacetamide, with relative standard deviations below 10%. The quantitation limits are between 4 and 15 ng g^{-1}.

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1. Introduction

One of the most widespread and lethal diseases that are affecting honey bees is the American Foulbrood (AFB) [1,2] caused by the spore-forming bacteria *Paenibacillus larvae* spp. *larvae*. Sulfonamides, the structures of which are shown in Fig. 1, are antimicrobial agents widely used in food producing animals as growth promoters as well as for therapeutic and prophylactic purposes [3,4]. Although sulfathiazole was initially recommended to control AFB, at this moment, its use is banned because there were found residues in honey many months after being applied; this fact caused a major concern due to the possible appearance of resistance phenomena in consumers' health. Nevertheless it is known that those compounds and products with similar moiety have still been detected in several honey samples from some countries [5]. For this reason, the analysis of these sulfa drugs in honey nowadays is very important to assure that this natural product does not contain residues in quantities that could imply a risk to the consumer.

Currently, as their use in apicultural work is forbidden, no maximum residue levels (MRLs) for sulfonamides in honey were set in the European Union, which means that those antibiotics, if present, must be below the limit of quantitation (LOQ) of the best analytical method used. But taking the real situation into account, some countries within the European Union have established tolerated levels for these antibiotics, for example Belgium and the United Kingdom have action limits of 20 and 50 ng g^{-1} for total sulfonamides in honey, and Switzerland has set an MRL of 50 ng g^{-1}, referring to the sum of sulfonamides and their metabolites. All these limits can be lowered upon improvement of analytical methods.

A variety of methods have been used or proposed to measure sulfonamide residues in honey and other matrices. Those methods included mainly: colorimetric procedures [6,7], enzyme immunoassays [8,9], thin layer chromatography [10] gas chromatography [11,12], capillary electrophoresis [13] and high performance liquid chromatography [14–34]. HPLC methods are preferred because they provide good qualitative and quantitative information by using different detection systems, although recently MS detection seems to be the most preferred choice [22,31–34]. Nevertheless in applying liquid chromatography methods, the reference to a big matrix effect caused by the presence of sugars is common; sometimes it is not taken into account whereas in other cases avoiding that negative effect implies the inclusion of consuming steps trying to break the glycosidic bond. When a mass spectrometric detector is not available, the fluorescence detector (FLD) is a good alternative, mainly due to its inherent sensitivity, but the target compounds need to be previously derivatized with an appropriate reagent. In this way, post-column derivatization with fluorescamine has been widely applied for the HPLC determination of sulfonamides [23–25], although pre-column derivatization has

* Corresponding author. Tel.: +34 91 2587515; fax: +34 91 5644853.
E-mail address: jbernal@ifi.csic.es (J. Bernal).

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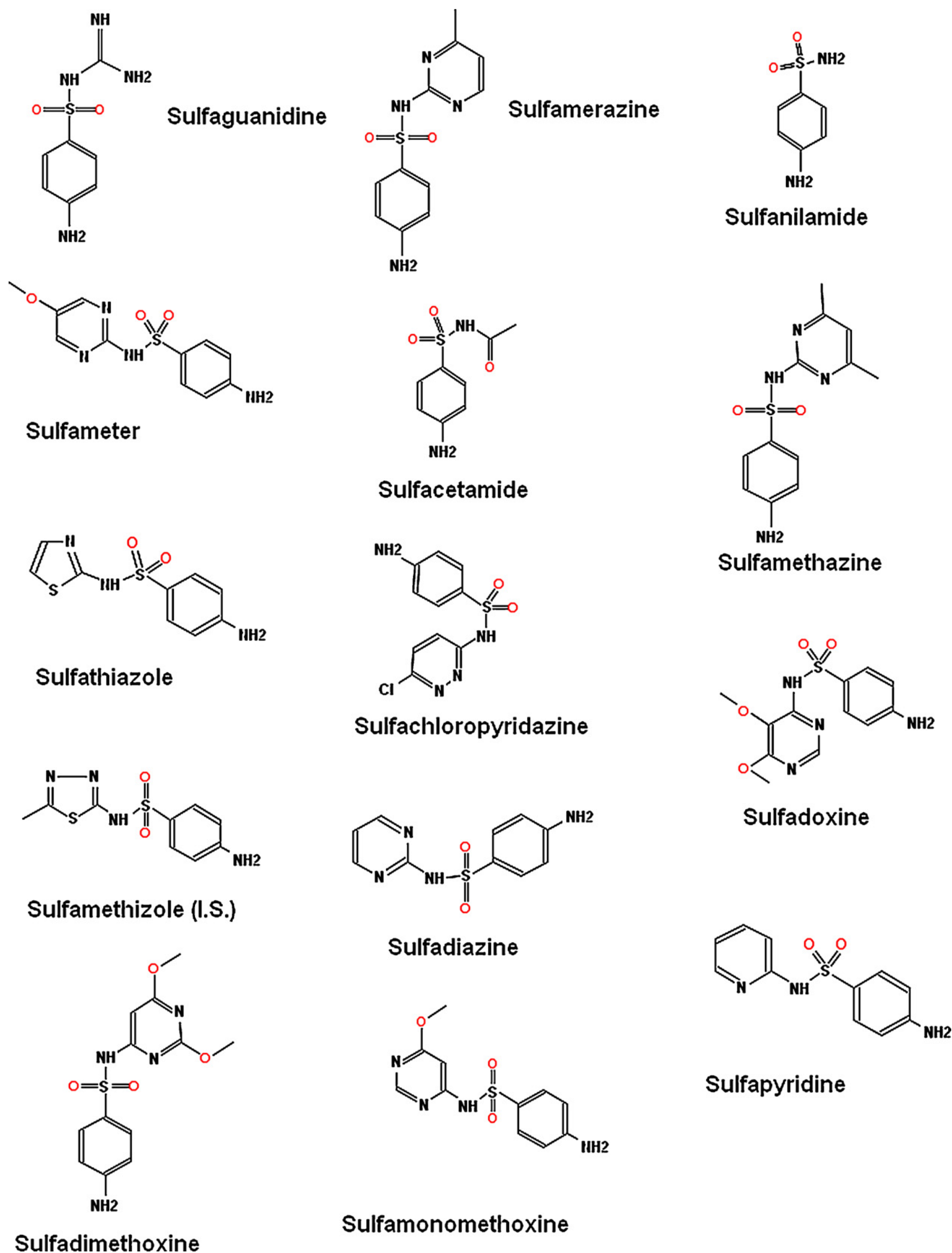

Fig. 1. Chemical structures of the investigated compounds.

been also proposed [26–30]. We have tried to apply some of these methods to honey samples but we met difficulties at the sample preparation step which affected to the recoveries of the compounds. It could be due to the above mentioned matrix effect caused by the N-glycosidic bond. So we have tested other alternatives to sort out this problem.

For those reasons, we have developed and validated a new method to determine sulfonamides in honey samples by using methanol in the sample treatment step which improves significantly the recoveries of the sulfonamides, and we have employed the fluorescence detector as a more economic alternative to the MS detectors.

2. Experimental

2.1. Material and chemicals

The sulfonamides (sulfaguanidine, sufanilamide, sulfacetamide, sulfathiazole, sulfapyridine, sulfachloropyridazine, sulfamerazine, sulfameter, sulfamethazine, sulfadoxine, sulfadiazine, sulfamonomethoxine, sulfadimethoxine, sulfamethizole (I.S.)), fluorescamine and 2- mercaptoethanol were supplied by Sigma–Aldrich Chemie Gbmh (Steinheim, Germany).

Hydrochloric acid (HCl) 37% was purchased from Merck (Darmstadt, Germany).

HPLC-grade methanol and acetonitrile were obtained from Labscan Ltd. (Dublin, Ireland). Sodium acetate (trihydrated), glacial acetic acid and monobasic sodium phosphate (dihydrated) were purchased form Panreac Química S.A. (Barcelona, Spain). Ultrapure water was obtained in a Milli-RO plus system together with a Milli-Q system from Millipore (Bedford, MA, USA). All the solvents and solutions were passed through a 0.45 μm nylon filter from Phenomenex (Torrance, CA, USA) before use. Ultrasonic bath Bransonic 5 was obtained from Scharlau Chemie S.A. (Barcelona, Spain). A 6-port sample concentrator from Alltech Associates (Deerfield, IL, USA) was employed. pH values were measured on pH meter Crison (Barcelona, Spain). Syringe filter (17 mm nylon 0.45 μm) from Nalgene (Rochester, NY, USA) were used.

2.2. Preparation of standards solutions

Stock solutions of the sulfonamides were prepared in methanol at a concentration of 100 mg L^{-1}. Working solutions of pertinent concentrations were made daily by an appropriate combination and a serial dilution of standard solutions with methanol.

All standards and stock solutions were kept in darkness at +4 °C and they were stable at least for one month.

2.3. Instruments and conditions

An Agilent Technologies (Palo Alto, CA, USA) 1100 series HPLC system consisting of a vacuum degasser, a quaternary solvent pump, an autosampler with a column oven and a fluorescence detector with scanning capabilities, all of them controlled by a Chemstation software, was used.

An Ascentis RP- Amide 5 μ 80 A (250 mm × 4.6 mm i.d.) from Supelco (Bellefonte, PA, USA) was used as an analytical column for LC separation.

The mobile phase consisted of (A) sodium acetate 0.020 M (pH 4.5) and acetonitrile in a gradient-elution analysis programmed as follows: 0–15 min, 26% B; 15–20 min, 26–29% B; 20–35 min, 29-41% B; 35–40 min, 41–26% B; 40–45 min, 26%B; at a flow rate of 1 mL min^{-1}. The column temperature was set at 55 °C and the injection volume was 50 μL.

The excitation and emission wavelengths for the determination of the 13 sulfonamides, previously derivatized with fluorescamine, were set at 403 and 492 nm, respectively.

2.4. Honey samples

Multifloral honeys from non-treated beehives were used as blank samples. They were kindly donated by the Centro Apícola Regional (CAR) of Marchamalo (Guadalajara, Spain). Multifloral commercial honey samples were obtained from local markets.

2.5. Sample preparation

After the optimization study described below, the next conditions were selected: 5 g of honey, spiked with the internal standard (sufamethizole), were diluted in 10 mL of methanol, this solution was thoroughly mixed for 5 min using a mechanical shaker, at room temperature, and then passed through a syringe filter. After that, the sulfonamides were on-line pre-column derivatized with fluorescamine using the injection program of the autosampler and an aliquot of 50 μL was injected onto the HPLC-FLD system.

3. Results and discussion

3.1. Sample preparation

Honey is a complex matrix, mainly composed of sugars, which are the major responsible of the matrix effect in its analysis. They can form a very stable bond with the sulfonamides, the N-glycosidic bond. This bond makes more difficult the extraction of the sulfa compounds [5], and it has to be broken by chemical methods, frequently time-consuming steps, to obtain good recoveries. So we have tried to find a simple sample treatment which allows us to solve this problem.

In the bibliographic revision, they were found some methods [18,19,25,26] based in the hydrolysis of the N-glycosidic bond (it was usually employed 1 M HCl) to convert sugar-bonded into unbounded forms, for determining several sulfonamides in honey by HPLC with post-column derivatization and fluorescence detection. We applied this sample preparation step to our samples but the obtained recoveries were in our opinion not adequate for almost all the sulfonamides.

As we did not get good results, we tested other proposed procedures with the aim of obtaining higher recoveries and at the same time, decreasing the analysis time and reducing the higher loss of analytes usually observed in the evaporation step which were usually employed to remove the initial solvent and then redissolve the obtained extract in a more suitable one.

The first attempt consisted of suppressing this evaporation step, so 5 g of blank honey spiked with the sulfonamides and the internal standard (sulfamethizole) were directly dissolved in 10 mL of 1 M HCl, then the compounds were derivatized and afterwards, the obtained extract was injected into the HPLC system. With this treatment, they were obtained slightly higher recoveries for the sulfonamides, between 25% (sulfachloropyridazine) and 55% (sulfanilamide) but the results were not good enough.

So a new modification was tested, in this one methanol was used for some reasons, to break the N-glycosidic bond between sugars and the sulfonamide group, to decrease the surface tension avoiding the emulsion formation and also because it was used to dissolve the sulfonamides. So, a sample of 5 g of blank honey spiked with the sulfonamides and the internal standard was diluted in 10 mL of methanol, the solution was mixed for 5 min in a mechanical shaker at room temperature and passed through a syringe filter, then the sulfonamides were derivatized with fluorescamine and injected into the chromatographic system. As it can be seen in Table 1, the recoveries of the 13 sulfonamides were notably improved, ranging from 56% for sulfadoxine to 96% for sulfacetamide with relative standard deviations (RSD) below 10%. So, it has been demonstrated that the use of methanol has allowed obtaining higher recoveries for all the compounds, this fact corroborates the statement regarding to the effectiveness of methanol to break the N-glycosidic bond, and subsequently that the extraction of the sulfonamides from the honey was improved.

3.2. Chromatographic conditions

To optimize the chromatographic separation, mixtures of the 13 sulfonamides and the internal standard were analyzed. The inter-

Table 1
Recovery determined on spiked honey samples at three concentration levels.

Compounds	Absolute recovery (mean (%) ± RSD (%), *n* = 5)		
	20 ng/g	50 ng/g	100 ng/g
Sulfadoxine	56.5 ± 5.8	59.1 ± 5.1	58.7 ± 6.0
Sulfanilamide	83.2 ± 8.8	84.3 ± 8.2	83.5 ± 7.5
Sulfacetamide	94.5 ± 3.2	96.5 ± 3.8	95.8 ± 4.1
Sulfadiazine	73.6 ± 4.7	75.1 ± 5.6	76.4 ± 5.9
Sulfapyridine	67.8 ± 3.5	68.9 ± 3.5	68.1 ± 3.3
Sulfathiazole	85.0 ± 3.5	87.2 ± 2.4	81.8 ± 2.9
Sulfamerazine	61.5 ± 3.8	62.1 ± 4.5	63.9 ± 4.0
Sulfamethazine	67.2 ± 3.2	69.8 ± 3.4	70.6 ± 3.9
Sulfamonomethoxine	91.0 ± 2.9	92.3 ± 3.6	93.2 ± 4.2
Sulfameter	71.9 ± 4.6	74.1 ± 4.1	72.9 ± 4.6
Sulfachloropyridazine	94.9 ± 4.0	95.5 ± 4.6	94.7 ± 4.1
Sulfaguanidine	89.2 ± 3.7	90.4 ± 3.1	91.2 ± 3.8
Sulfadimethoxine	85.5 ± 7.8	87.2 ± 8.3	89.1 ± 7.5

nal standard method was used to obtain more reproducible results. First of all, a standard which contained the 14 compounds which were previously derivatized was injected, under the chromatographic conditions that we had previously used to analyze some sulfonamides by direct UV detection. The mobile phase employed was a mixture of sodium acetate 0.02 M (pH 4.5) in water and acetonitrile (50:50, v/v), used in isocratic mode. The flow rate was 1 mL min^{-1}, the injection volume was 20 μL and the temperature was set at 45 °C. Under these conditions all the compounds were not separated, most of them coeluted. We studied the influence of each chromatographic parameter in the separation for an isocratic mode but we could not separate the 14 compounds in a reasonable analysis time, so we decided to use a gradient elution analysis.

As the main goal was to separate all the compounds in the shortest time as possible, many gradients were tested, they are summarized in Table 2. It should be remarked that the best separation was achieved with gradient 9.

Table 2
Gradient elution programs tested for the separation of the sulfonamides with a mobile phase which consisted of (A) sodium acetate 0.020 M (pH 4.5) and (B) acetonitrile at 55 °C.

1			2			3		
t (min)	%A	%B	*t* (min)	%A	%B	*t* (min)	%A	%B
0	70	30	0	70	30	0	75	25
25	70	30	26	70	30	15	75	25
30	50	50	40	60	40	25	65	35
35	70	30	43	70	30	32	75	25
40	70	30	48	70	30	37	75	25
4			5			6		
4			**5**			**6**		
t (min)	%A	%B	*t* (min)	%A	%B	*t* (min)	% A	%B
0	73	27	0	75	25	0	75	25
15	73	27	15	75	25	15	75	25
25	65	35	25	62	38	25	68	32
35	40	60	35	50	50	35	40	60
42	73	27	40	75	25	42	75	25
47	73	27	45	75	25	47	75	25
7			**8**			**9**		
t (min)	%A	%B	*t* (min)	%A	%B	*t* (min)	%A	%B
0	75	25	0	75	25	0	74	26
15	75	25	15	75	25	15	74	26
20	70	30	20	73	28	20	71	29
35	45	55	35	60	40	35	59	41
42	75	25	40	75	25	40	74	26
47	75	25	45	75	25	45	74	26

3.2.1. Selection of the emission and excitation wavelengths

For the fluorescence detection of the 13 sulfonamides, it was necessary a derivatization step. The reagent which was chosen after the bibliographic revision [22–29] was fluorescamine. This reactive was dissolved in a mixture of diacid phosphate (0.021 M) and acetonitrile (3:1, v/v) at pH 3. It was also found in the literature that 2-mercaptoethanol had been usually added to the derivatization solution. To check if this reagent, in honey analysis, improved the results, we have made some tests. The obtained results showed no advantages on this matrix. So, as there were not seen real benefits provided by its use, the employ of 2-mercaptoethanol was discarded.

Once each sulfonamide standard was derivatized, the solutions were scanned in a spectrofluorimeter to obtain the emission and excitation wavelengths. The wavelengths which provided the highest signal for all the sulfonamides were λ_{ex} 403 nm and λ_{em} 492 nm and no interference was observed from the derivatization reagent in the detection of the compounds.

3.2.2. Influence of the pH

Considering the poor stability of sulfonamides outside the pH range 3.5–6.0, some experiments were carried out between these pH values. It was observed that at pH values close to 3.5, the retention times increased, whereas at pH close to 6.0 the retention times decreased causing in several cases the coelution of several sulfonamides. So, pH 4.5 was selected because it provided the best separation in a shortest time.

3.2.3. Temperature

Some tests were made varying the temperature between 20 and 60 °C at 5 °C steps to study the influence of this parameter. It was observed a variation of the retention times and peak symmetries according to the column temperature changes. As it was expected, with the increase in the temperature the retention times slightly decreased. It can be also pointed out that a loss of symmetry was observed at low temperatures. The best results were achieved at 55 °C, because the peaks were narrow and the best separation between the compounds was observed.

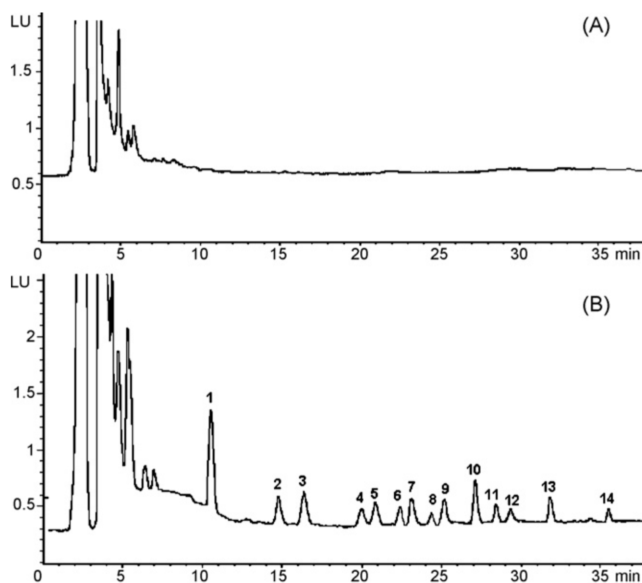

Fig. 2. HPLC-FLD chromatograms of (A) 5 g of a multifloral honey sample, diluted in 10 mL of methanol and (B) 5 g of a multifloral honey sample, diluted in 10 mL of methanol spiked with 50 ng g^{-1} ((1) sulfadoxine, (2) sulfanilamide, (3) sulfacetamide, (4) sulfadiazine, (5) sulfapyridine, (6) sulfathiazole, (7) sulfamerazine, (8) sulfamethizole (I.S.), (9) sulfamethazine, (10) sulfamonomethoxine, (11) sulfameter, (12) sulfachloropyridazine, (13) sulfaguanidine and (14) sulfadimethoxine).

Table 3
Linearity studies ($n = 5$) and LOQ values for the 13 sulfonamides.

Compounds	Analytical range (ng/g)	$R^2 \pm s_{y/x}$	$a \pm s_a$	$b \pm s_b$	LOQ (ng/g)
Sulfadoxine	4–100	0.999 ± 0.002	0.01 ± 0.02	0.0367 ± 0.0003	4
Sulfanilamide	8–100	0.999 ± 0.002	0.04 ± 0.02	0.0303 ± 0.0003	8
Sulfacetamide	12–100	0.991 ± 0.002	0.03 ± 0.03	0.0149 ± 0.0010	12
Sulfadiazine	8–100	0.992 ± 0.001	0.04 ± 0.02	0.0102 ± 0.0006	8
Sulfapyridine	6–100	0.999 ± 0.001	0.02 ± 0.01	0.0231 ± 0.0004	6
Sulfathiazole	8–100	0.999 ± 0.001	0.02 ± 0.01	0.0186 ± 0.0004	8
Sulfamerazine	8–100	0.997 ± 0.001	0.02 ± 0.02	0.0178 ± 0.0006	8
Sulfamethazine	15–100	0.999 ± 0.001	−0.02 ± 0.01	0.0174 ± 0.0005	15
Sulfamonometoxine	4–100	0.999 ± 0.001	0.04 ± 0.01	0.0163 ± 0.0002	4
Sulfameter	15–100	0.998 ± 0.001	0.03 ± 0.01	0.0077 ± 0.0002	15
Sulfachloropyridazine	12–100	0.998 ± 0.001	0.02 ± 0.01	0.0044 ± 0.0001	12
Sulfaguanidine	6–100	0.994 ± 0.002	0.04 ± 0.01	0.0057 ± 0.0002	6
Sulfadimetoxine	12–100	0.998 ± 0.001	0.01 ± 0.01	0.0042 ± 0.0001	12

$s_{y/x}$: Standard deviation of the vertical distances of the points from the line.
a: intercept with the y axis.
s_a: standard deviation of the intercept.
b: slope of the line.
s_b: standard deviation of the slope.

3.2.4. Injection volume

On account of the low concentrations expected in honey, the possibility of enhancing the detection limits by injecting high sample volumes was considered. So, in the established conditions, volumes from 20 to 100 μL of a standard solution of 200 μg L^{-1} were injected, the obtained results showed that, for injection volumes higher than 50 μL, the chromatographic peaks began to be somewhat deformed and the S/N ratio did not improve. In consequence, an injection volume of 50 μL was adopted as optimum.

3.2.5. Derivatization

The next stage was the selection of the amount of the derivatization reagent. For that purpose, some tests were made by varying the volume ratio between fluorescamine solution and the sample, from volume ratio 0.5:1 to 3:1.

The highest signal was obtained for the volume ratio 1.5:1. For higher ratios, the signal did not increase, and for lower ratios the peak areas decreased and the signal was somewhat deformed.

It was also examined the option between pre and post-column derivatization The best results were obtained when the derivatization was done by the instrument (pre-column) due to the better reproducibility obtained. For this purpose, we have employed the injector program of the autosampler, included in the system software.

So 50 μL of sample and 75 μL of derivatization reagent were mixed twice in the seat of the injector and, afterwards, 50 μL of the mixture were injected into the HPLC system.

The proposed chromatographic conditions generated narrow and reproducible chromatographic peaks, as it can be seen in Fig. 2.

3.3. Validation of the method

Validation was carried out following the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guidelines [35,36], the International Union of Pure and Applied Chemistry (IUPAC) technical report of 2002 [37] and document SANCO/10476/2003 [38], determining selectivity, limits of quantitation and detection, linearity, precision and trueness.

To check the selectivity of the method, they were injected extracts from blank and spiked multifloral honey samples. It can be deduced from Fig. 2 that there were no matrix interferences.

The LOQ was determined by injecting a number of extracts of multifloral honey samples ($n = 20$) and measuring the magnitude of the background analytical response. We estimated experimentally the LOQ as ten times the signal-to-noise ratio (S/N). The values obtained are in Table 3. As it can be observed, the obtained LOQ values were around 10 ng g^{-1} for all the studied sulfonamides.

Matrix-matched calibration standard curves were used to quantify sulfonamides residues in honey. Multifloral blank honey samples were spiked with variable amounts of sulfonamides, for an analytical range between 10 and 100 ng g^{-1} and they were treated according to the procedure described above. Those spiked honey samples, containing also sulfamethizole (200 ng g^{-1}) as an internal standard, were analysed with the chromatographic method previously described.

The obtained extracts were considered as standards to get the calibration graphs. Concentration versus the ratio of the areas (sulfamide/sulfamethizole) was plotted to prepare the matrix-matched calibration curves of each individual set of standard series. They were obtained graphs that were straight lines, of intercept not significantly ($p < 0.05$) different from zero which confirmed the linearity through the range studied and the lack of bias. The determination of coefficient values (R^2) were >0.99, as it can be observed in Table 3.

Recovery and precision were determined on spiked samples at three concentration levels low (20 ng g^{-1}), medium (50 ng g^{-1}) and high (100 ng g^{-1}). The mean recoveries ranged from 56% to 96% as it could be seen in Table 1

3.4. Application of the method

The developed method was applied onto the analysis of the sulfonamides in several multifloral commercial honey samples and they were not detected residues in any of the analyzed samples.

4. Concluding remarks

A sensitive, simple and precise HPLC-FLD method for the determination of 13 sulfonamides in honey samples has been developed.

The addition of methanol in the sample treatment procedure avoids interferences from matrix, reduces sample treatment time and allows the obtention of good recoveries.

The quantitation limits for all the compounds were around 10 ng g^{-1}, which allows the proposed procedure to be a useful tool for sulfonamide trace analysis in honey samples.

They were not found residues of these sulfonamides in the commercial honey samples analyzed.

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