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A simple hollow fiber renewal liquid membrane extraction method for analysis of sulfonamides in honey samples with determination by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A sensitive and precise analysis using hollow fiber renewal liquid membrane (HFRLM) extraction followed by high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) is described for determination of five sulfonamides in honey samples. In this procedure, the organic solvent introduced directly into the sample matrix extracts the sulfonamides and carries them over the polypropylene porous membrane. An organic solvent is immobilized inside the polypropylene porous membrane, leading to a homogeneous phase. The stripping phase at higher pH in the lumen of the membrane promotes the ionization of the target compounds releasing them to this phase. The most important parameters affecting the extraction efficiency were optimized by multivariable designs (pH and sample mass, pH and buffer for stripping phase, extraction temperature and time, type and volume of extractor solvent and use of salt to saturate the sample). Detection limits in the range of 5.1–27.4 $\mu g\,kg^{-1}$ and linearity coefficient of correlation higher than 0.987 were obtained for the target analytes. The results obtained for the proposed method show that HFRLM–LC–MS/MS can be used for determination of the five sulfonamides studied in honey samples with excellent precision, accuracy, practicality and short analysis time.

1. Introduction

According to European Council Directive 2001/110/EC, honey is a natural sweet substance produced by bees (*Apis mellifera*) from nectar of plants, secretions of living parts of plants, or excretions of plant-sucking insects on the living parts of plants. The bees collect these materials and transform them by combining them with specific substances of their own. After, deposit, dehydrate, store and leave them in honeycombs to ripen and mature [1]. Chemically speaking, honey is a highly hygroscopic and very concentrated aqueous solution of sugars. As an analytical sample, honey is a very complex matrix. Its composition depends strongly on the plant species from which the nectar or honeydew was collected, and other factors such as environmental conditions and climate [2].

Because of its rich composition, honey presents appreciable value for human health. However, these benefits can be reduced due to the presence of toxins, pollutants and contaminants introduced during its manipulation or coming from the environment due to agriculture or apiculture. Honey can be contaminated in a direct way (contaminants like pesticides, pharmaceutical residues, acaricides, bee repellents, wood protectants and wax foundations coming from beekeeping) or in an indirect way (contaminants like pesticides, polychlorinated biphenyls, bacteria, heavy metals, polyaromatic hydrocarbons and radionuclides coming from agriculture practices or the environment in general) [2]. Among the direct contaminants are those that belong to several pharmaceutical classes, where antibiotics such as the sulfonamides are including.

The sulfonamide residue determination in food is of concern because of their potential carcinogenic character and the possibility of development of antibiotic resistance in humans [3].

Different analytical methods based on a Purospher Star RP-18ec column with fluorescence (RP-HPLC-FL) or by tandem mass spectrometry (RP-LC–ESI-MS/MS) detection has been reported for determining residues of sulfonamides in food samples. Most of these methods involve a pretreatment with hydrolysis followed by solid phase extraction (SPE). However, these techniques are considered expensive and laborious, and frequently result in high blank levels, i.e., high noise signal to blank samples, causing ionic suppres-

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sion due to the matrix and reducing the limit of detection obtained by signal-to-noise ratio because of an inefficient sample clean-up [4–7].

Recently, extraction techniques based on supported liquid membranes (SLM) have attracted attention due to such advantages as low acquisition and operational costs, operational simplicity, zero effluent discharge, high selectivity, use of small amounts of sample and a combination of extraction and stripping in a single step [8–10]. The mass transfer process of target analyte from a donor to an acceptor solution through a liquid membrane involves several stages, which include: solubilization of the analyte in the extractor solvent and interaction of the extractor solvent with the donor solution/membrane interface, diffusion of the analyte through the liquid membrane and ionization of the analyte at the membrane/acceptor solution interface with the release of the analyte to the acceptor solution. This mechanism enables the transfer process to be carried out even at low analyte concentrations, and even against an analyte concentration gradient, known as facilitated transport [11].

The addition of an extra extractor solvent amount directly into the sample simultaneously with the hollow fiber liquid membrane procedure was proposed by Ren et al. [12,13]. This modification of the SLM technique is called hollow fiber renewal liquid membrane (HFRLM) and results in a solution with a high aqueous/organic solvent ratio. Due to the wetting affinity of the organic phase and hydrophobic membrane, a thin organic film of solvent develops at the interface between the donor phase and the membrane. The shear force due to the sample agitation causes the formation of organic microdroplets on the surface of the liquid membrane layer, which separate from the surface of the liquid membrane. At the same time, the organic microdroplets present in the sample greatly increase the contact area between the extractor solvent and the sample. Simultaneously, these microdroplets are reintroduced into the solvent film renewing the liquid membrane, which can accelerate the mass transfer rate, significantly reducing mass transfer resistance in the hydrodynamic boundary layer at the donor phase/membrane interface. An additional organic phase in the donor phase is necessary only for the renewal, and continuously replenishes the loss of membrane liquid due to its solubility and emulsification to avoid liquid membrane degradation. On the other hand, the resistance of back-extraction on the lumen side of the membrane can be greatly reduced by chemical reactions [14].

Recently, HFRLM has been applied with success in our research group for determination of cadmium in aqueous samples using polypropylene and poly(dimethylsiloxane) membranes. With both membranes low limits of detection were obtained [11,14]. On a continuation of these studies, a HFRLM procedure was developed and used with high performance liquid chromatography–tandem mass spectrometry. This method was validated for determination of antibiotics belong to sulfonamide group in honey samples.

2. Experimental procedure

2.1. Instrumentation

The chromatographic analyses were performed with a Quattro Micro API quadrupole mass spectrometer coupled to an Alliance 2690 liquid chromatograph (Waters, Manchester, UK). The quadrupole mass spectrometer was equipped with a Z-spray source for positive electrospray ionization (ESI). Capillary and cone voltages were set at 3.5 kV and 35 V, respectively, and the temperature source was kept at 120 °C while the desolvatation temperature was held at 450 °C. Nitrogen was used as cone and desolvating gas at flow rates of 50 and 680 L h^{-1}, respectively. The mass spectrometer was operated in the MS/MS mode using multiple reaction monitoring (MRM). Pure argon (99.8%) from Air Liquide (White Martins, Rio de Janeiro, Brazil) was used as collision gas at a constant pressure of 2×10^{-3} mbar. Table 1 summarizes the acquisition window definition, the masses of parent and daughter ions that were monitored, the optimized collision induced dissociation (CID) voltages and physical–chemical characteristics of the compounds.

Table 1
Selected ion transitions, instrumental parameters and pK_a for the sulfonamides.

Compound	pK_a	SMR transitions (m/z)	Cone voltage (V)	Collision energy (eV)
SD	6.50	251.30 → 156.1[b]	28	15
		251.30 → 91.7[a]	28	30
SMX	5.81	254.25 → 160.1[b]	40	16
		254.25 → 107.8[a]	40	20
STZ	7.24	256.22 → 156.0[b]	27	16
		256.22 → 108.0[a]	27	20
SM	6.98	265.29 → 172.2[b]	28	16
		265.29 → 156.0[a]	28	18
SMP	6.70	281.16 → 155.7[b]	28	13
		281.16 → 91.3[a]	28	25

[a] Quantification ion.
[b] Confirmation ion.

The chromatographic separation was carried out with a Nova-Pak C18-A HPLC column (150 mm × 3.9 mm i.d., 4 μm) from Waters (Manchester, UK), maintained at 20–30 °C. The mobile phase consisted of methanol (solvent A) and aqueous solution with 0.1% of formic acid and 10 mmol L^{-1} of ammonium acetate (solvent B). Gradient elution was applied at a flow rate of 0.4 mL min^{-1} as follows: initial conditions of 30% A held for 3 min, increased linearly to 50% A in 5 min, increased linearly to 70% A in 2 min, held at 70% A during 3 min, returned to initial conditions in 0.1 min and maintained for 6 min.

2.2. Reagents and standards

All chemicals were of analytical grade and were used without prior purification. Milli-Q water (Millipore, Bedford, MA, USA) with 18.2 MΩ cm^{-1} conductivity was used to prepare all solutions and mobile phases. Pentanol (Aldrich, Steinheim, Germany), hexanol (Aldrich), heptanol (Aldrich), 1-octanol (Merck, Darmstadt, Germany), cyclohexanol (Merck), butanol (Merck), ethyl acetate (Merck), and toluene (Tedia, Rio de Janeiro, Brazil) were evaluate as extracting solvents. The sulfonamide standards, including sulfadiazine (SD), sulfamethoxazole (SMX), sulfathiazole (STZ), sulfamerazine (SM) and sulfamethoxypyridazine (SMP) were purchased from Sigma–Aldrich. Stock solutions were prepared in methanol and working solutions were prepared in methanol:water (50:50 v/v) and stored at −18 °C.

HPLC-grade methanol (JT Baker, Deventer, Holland) and formic acid (Merck) were used as mobile phase. Ammonium sulfate (Synth, Diadema, Brazil) was used to modify the sample ionic strength.

2.3. Optimization strategies

The optimization of the parameters affecting the antibiotics extraction using the HFRLM was performed using multivariate designs. A triangular surface mixture design was used to define the best extracting organic solvent (pentanol, 1-octanol or cyclohexanol) for the liquid extraction. A central composite design was applied to study the influence of sample mass, solvent volume, and extraction time and temperature on the extraction efficiency. A univariate study to determine the influence of sample pH and type of buffer in the acceptor phase on the efficiency of the antibi-

otic extraction from honey samples was also made. In order to maximize the simultaneous antibiotic extraction, for the three optimization designs, the geometric average of the peak areas for the antibiotics were used as the response for optimization in the computer programs, since good levels of detection for all antibiotics were obtained. Furthermore, similar results were obtained when the peak areas for each antibiotic were used as the response for the optimization. The experimental data were processed using the Statsoft Statistica 6.0 computer program (StatSft Inc., Tulsa, OK, USA).

2.4. Optimized sample preparation

Extraction of antibiotics from honey was performed using a HFRLM three-phase system. Into a 20 mL vial were added 0.625 g of honey, 10 g of ammonium sulfate, 10 mL of 0.05 mol L^{-1} acetate buffer at pH 5.0 and 300 μL of 1-octanol:pentanol (55:45 v/v). A Q 3/2 Accurel polypropylene hollow fiber (600 μm id, 200 μm wall thickness and 0.2 μm pore size) purchased from Membrana GmbH (Wuppertal, Germany) was carefully cut into 8 cm pieces and the ends of each piece were connected to the needles of 250 μL microsyringes. Each segment of membrane was cleaned in acetone and dried before use. One of these microsyringes contained 100 μL of the acceptor solution (carbonate buffer, 0.05 mL^{-1}, pH 10.0) which was used to fill the lumen of the hollow fiber. The vial containing the sample was introduced into the temperature-controlled bath unit at an appropriate temperature. The extraction was carried out by placing the hollow fiber in the sample vial. After stirring (1200 rpm) the system for a predetermined time, the acceptor solution was collected by the second microsyringe and 20 μL of this solution was injected into the LC–MS/MS.

2.5. Validation

The performance of the method was evaluated using the following figures of merit: sensitivity, linearity, linear range, repeatability and intermediate precision, relative recovery, detection and quantification limits, stability and selectivity. Analytical curves were constructed to estimate the linear ranges, correlation coefficients and detection and quantification limits for the proposed HFRLM–LC–MS/MS method. The linearity of the method was evaluated by calculation of the regression line and expressed by the coefficient of correlation (R). The detectability of the method was evaluated by determining the limit of detection (LOD) and quantification (LOQ), where the limits of detection and quantification were calculated as three and 10 times the signal-to-noise ratio, respectively. Precision was calculated as relative standard deviation (RSD) considering acceptability criteria precisions less than 20%, evaluate at 100 μg kg^{-1}. Repeatability was assessed by six determinations in one day. Intermediate precision was assessed by six determinations on three separate days.

The relative recovery was evaluated with separately prepared individual and mixed working solutions of all standards over the linear dynamic range at three different concentration levels, i.e. low (50 μg kg^{-1}), medium (200 μg kg^{-1}) and high (400 μg kg^{-1}). Relative recovery was assessed by five repetitions per concentration.

Stability of the stock solutions was tested monthly and compared with freshly prepared working solutions.

3. Results and discussion

3.1. Effect of salt, pH sample and acceptor phase

First, the effect of salt addition on the extraction efficiency was studied. Sodium chloride, potassium sulfate and ammonium sulfate (10 g for each salt) were added to saturate the sample. Because of the effect of ammonium sulfate on the sample pH, all experiments to study the effect of salt on the extraction efficiency were carried out at pH closed to 5.0, which correspond to the ammonium sulfate solution pH. All of them showed a positive effect on the extraction efficiency. However, the ammonium sulfate presented a more significant effect. A possible explanation is the high solubility and the presence of double charge in the ammonium sulfate which considerably increase the ionic strength of the solution. Despite the difference in ionic strength of the sample solution and the stripping solution (acceptor phase), there was no osmotic process observed in the proposed system.

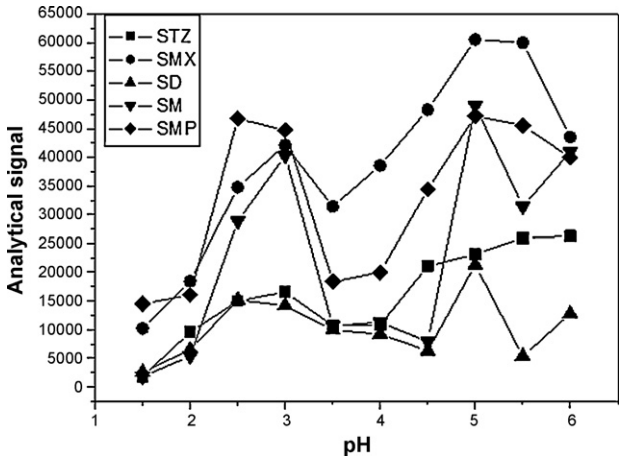

Fig. 1. Effect of sample pH on extraction by HFRLM with polypropylene membrane. Experimental conditions: 300 μL of 1-octanol, 10 g of ammonium sulfate, 10 mL of buffer at the respective pH, 250 μg kg^{-1} of each sulfonamide, 0.5 g of honey sample and acceptor phase of 100 μL of carbonate buffer at pH 10.

The sample pH has significant influence on the extraction efficiency due to the possible ionization of the analytes. For the proposed extraction, to occur, the analytes should be, necessarily, in their molecular forms to promote their interaction with the organic solvent and, therefore, an adequate extraction. Fig. 1 shows that, with lower sample pH, the extraction efficiency is reduced, probably because of the protonation of the amine group of each analyte studied. On the other hand, with higher sample pH enhancements in the analytical signal for all antibiotics studied were obtained. A pH of 5 was thus fixed for the rest of the study.

The efficiency of the HFRLM three-phase system depends on the equilibrium between the analytes from the donor phase and the organic solvent, as well as between the analytes from the organic solvent and the acceptor phase. In this case, it is important that this equilibrium is shifted to the acceptor phase, to trap the analytes in this phase. In this study the extraction procedure was carried out using acceptor phase pH two units above the target analytes pK_a (Table 1). This experimental condition promotes the ionization of the analytes in the organic solvent and acceptor phase interface and, consequently, traps the analytes in the acceptor phase due to the absence of interaction between the ionized analytes and the organic solvent. The influence of different buffers on the extraction efficiency was evaluated. The analytes showed different behaviors for each buffer, however, the carbonate buffer presented the best compromise, as can be seen in Fig. 2.

3.2. Effect of extractor solvent

In the proposed method, the polarity between the organic solvent and the sulfonamides should be similar, so a high partition coefficient could be reached. The effect of some extractor solvents on the extraction efficiency was studied. Alcohols were selected in this study because of their relative polarity in relation to the ana-

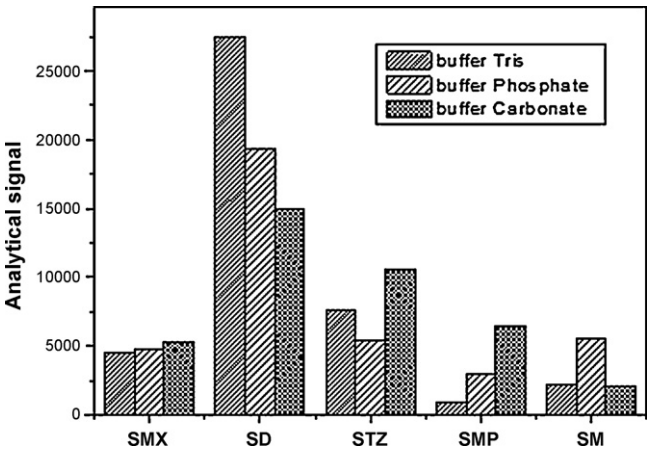

Fig. 2. Influence of the different buffers of the acceptor phase on the extraction efficiency. Experimental conditions: 300 μL of 1-octanol, 10 g of ammonium sulfate, 10 mL of acetate buffer at pH 5.0, 250 μg kg^{-1} of each sulfonamide, 0.5 g of honey sample and acceptor phase of 100 μL of the respective buffer at pH 10.

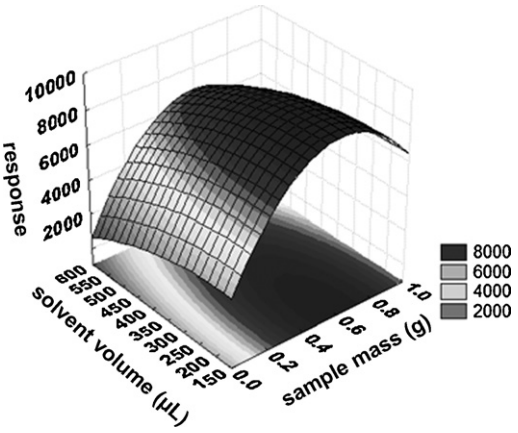

Fig. 4. Study of sample mass and organic solvent effect on the extraction efficiency of the sulfonamides by HFRLM. Experimental conditions: 10 g of ammonium sulfate, 10 mL of acetate buffer (pH 5.0), 250 μg kg^{-1} of each sulfonamide and acceptor phase of 100 μL of carbonate buffer (pH 10).

lytes. 1-Butanol, 1-pentanol, 1-hexanol, cyclohexanol, 1-heptanol and 1-octanol were studied. The effect of toluene or ethyl acetate as extractor solvent on the extraction efficiency was also studied. Preliminary studies showed that 1-pentanol, cyclohexanol and 1-octanol presented the most significant effects on the extraction efficiency. The mixture of these alcohols was optimized through triangular surface mixture design, where 1-octanol, pentanol and cyclohexanol were evaluated individually, through binary mixtures with 33% and 67% (v/v) of one solvent and a ternary mixture containing 33% of each solvent. The results obtained from this design can be seen in Fig. 3. Here, there is a region in which the response reaches high values, corresponding to the use of 1-octanol:1-pentanol (55:45 v/v). This condition was selected to continue the optimization of the proposed method.

3.3. Effect of solvent volume and sample mass

The volume of organic solvent used to extract the analytes from the sample is an important parameter not only to extract the analytes but also to regenerate the liquid membrane. Thus, the extraction efficiency can be influenced due to the interaction between the sample mass and the organic solvent volume. Fig. 4 shows that there is a compromise between the sample mass and solvent volume. After increasing the sample mass a higher volume of organic solvent is necessary to maintain the extraction efficiency.

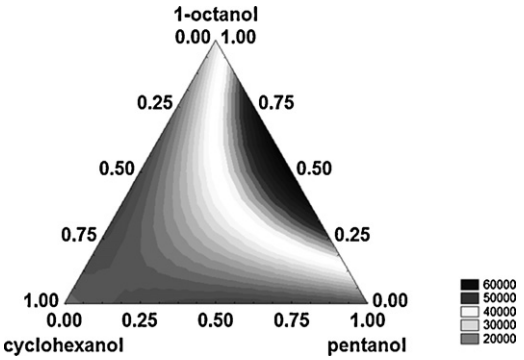

Fig. 3. Study of the effect of solvent choice on sulfonamides extraction by HFRLM with polypropylene membrane and determination by LC–MS/MS. Experimental conditions: 300 μL of solvent, 10 g of ammonium sulfate, 10 mL of acetate buffer at pH 5.0, 250 μg kg^{-1} of each sulfonamide, 0.5 g of honey sample, and acceptor phase: 100 μL of carbonate buffer at pH 10.

Probably, this fact occurs because some part of the organic solvent can be retained by the sample matrix. On the other hand, the use of too much organic solvent can promote a dilution of the analytes in the extractor phase, reducing the analytical signal. In accordance with the statistical treatment (Fig. 4) there is an optimum region that represents maximum extraction and adequate method robustness. Thus, 0.625 g of honey sample and 300 μL of 1-octanol:1-pentanol (55:45 v/v) were selected for the rest of this study.

3.4. Effect of extraction time and temperature

Similar to other microextraction techniques, HFRLM is an equilibrium process controlled by diffusion of the analytes. Thus, normally, the HFRLM needs a long extraction time to reach the equilibrium condition [15] and to have a maximum amount of the analytes extracted. Another important parameter affecting the extraction efficiency in HFRLM is the extraction temperature which influences sample viscosity and solvent solubility. On the other hand, the temperature of the extraction process can cause the degradation (degeneration) of the liquid membrane in the SLM. However, in the case of the HFRLM technique, this degradation should not occur because of the excess of solvent present in the sample, which constantly renews the liquid membrane. These parameters were optimized in this study and the results are shown in Fig. 5.

Fig. 5 indicates an increment in the analytical signal is obtained by increasing both the extraction temperature and the extraction time. Probably, this effect is caused due to an increase in analyte diffusion through the membrane. However, for some extraction temperature and time combinations the analytical signal decreases, probably because of target compound degradation, as they are thermally unstable when exposed to higher temperatures. In accordance with Fig. 5, there is a maximum analytical signal region corresponding to the best values of temperature and time extraction. Besides, Fig. 5 shows that small temperature and time variations during the extraction procedures do not affect the analytical signal and, therefore, the proposed method presents adequate robustness. Thus, the selected temperature and time for extraction were, respectively, 40 min and 58 °C.

3.5. Calibration and validation

The HFRLM–LC–MS/MS method was evaluated according to the criteria described in Section 2.5. Table 2 provides the lim-

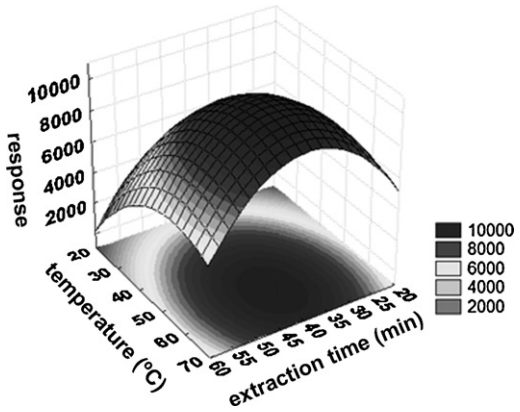

Fig. 5. Effect of temperature and time on sulfonamide extraction by HFRLM and determination by LC–MS/MS. Experimental conditions: 300 μL of 1-octanol:1-pentanol (55:45 v/v), 10 g of ammonium sulfate, 10 mL of acetate buffer (pH 5.0), 250 μg kg^{-1} of each sulfanomide, 0.625 g of honey sample, and acceptor phase of 100 μL of carbonate buffer (pH 10).

its of detection (LOD) and quantification (LOQ), as well as the calibration parameters for all studied analytes. LOD and LOQ of 5.1–27.4 μg kg^{-1} and 16.8–82.2 μg kg^{-1}, respectively, were obtained. These results confirm the applicability of the proposed method to determine sulfonamides in honey samples. Linearity was obtained with an average coefficient of correlation (R) higher than 0.987.

Precision and relative recovery of the method were evaluated at three concentrations within the linear dynamic range.

Table 2
LOD, LOQ, calibration results and MRL.

Compound	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	R	Linear range (μg kg^{-1})	MRL[a,b] (μg kg^{-1})
SD	11.1	36.7	0.987	25–500	100
SMX	27.4	82.2	0.989	50–500	–
STZ	8.7	29.0	0.987	25–500	100
SM	5.1	16.8	0.981	16–500	–
SMP	12.9	39.0	0.994	25–500	–

–, not found.
[a] Values obtained in accordance with the Control of Residues and Contaminants in Honey Program – PNCRC/2009 – Brazil [16].
[b] Maximum residue limit.

Table 3 includes the concentrations tested and results for these validation parameters. Repeatability for all compounds proved to be less than 15%. Intermediate precision was less than 13% for all compounds. Relative recoveries were between 80.9% and 103.1%, with a variation of less than 15% for all compounds.

Under the stated conditions, stock solutions proved to be stable for at least 4 months. No significant loss or deterioration for any of the compounds of interest was observed at the sample treatment stages. The proposed method presented good selectivity, especially considering the complexity of the matrix. Typical chromatograms obtained using the proposed method with a blank and with a spiked honey sample are shown in Fig. 6. These chromatograms clearly demonstrate that none of the peaks occurring in the blank sample interfered with any of the compounds being analyzed.

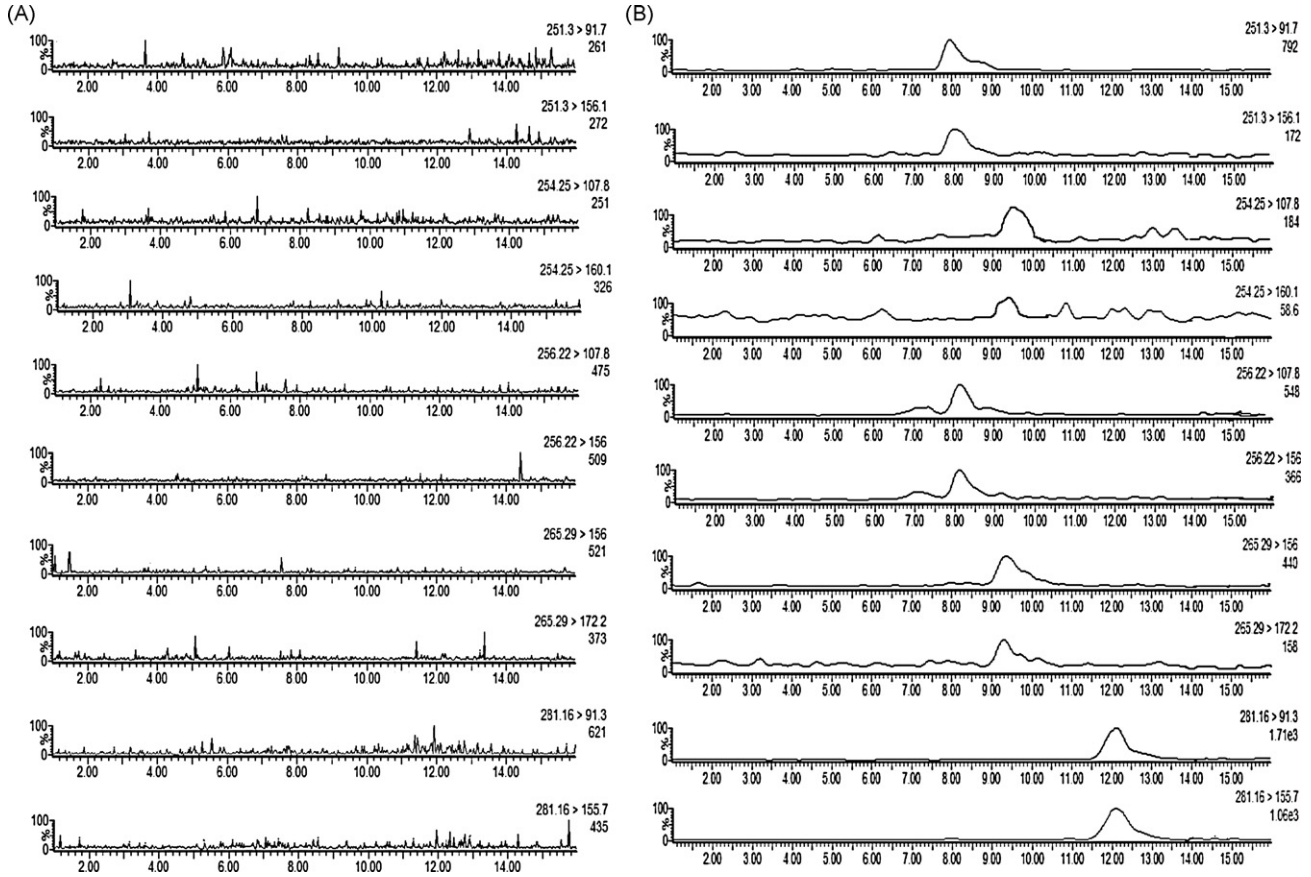

Fig. 6. (A) Blank sample chromatogram obtained after extraction by HFRLM and determination by LC–MS/MS and (B) after spiking the sample with 200 μg kg^{-1} of each standard sulfonamide. Experimental conditions: 300 μL of 1-octanol:1-pentanol (55:45 v/v), 10 g of ammonium sulfate, 10 mL of acetate buffer (pH 5.0), 0.625 g of honey sample and acceptor phase of 100 μL of carbonate buffer at pH 10.

Table 3
Precision and accuracy of the proposed method.

Compound	Relative recovery (%)			Precision (%)	
	50 μg kg^{-1}	100 μg kg^{-1}	400 μg kg^{-1}	Repeatability 100 μg kg^{-1} n = 6	Intermediate precision 100 μg kg^{-1} n = 6
SD	90.9–96.4	92.3–117.8	101.8–114.8	9.2	8.2
SMX	84.8–105.7	92.4–110.5	94.1–99.8	12.7	9.5
STZ	82.9–100.8	102.1–96.9	83.6–100.1	13.4	8.4
SM	80.9–100.9	103.1–111.0	90.0–103.3	13.9	9.9
SMP	83.2–100.8	89.9–106.6	94.8–112.7	14.6	12.5

4. Conclusions

The HFRLM–LC–MS/MS method to determine sulfonamides in honey matrices provided low LOQ (μg kg^{-1}) and good recovery, precision and linearity. The proposed method presents the advantages and drawbacks of HF-LPME: it is simple, effective, low cost, uses μL of organic solvents and almost free of matrix effects. To the best of our knowledge, this constitutes the first study that applies HFRLM for the extraction of organic compounds in a concentration procedure.

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