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Restricted access material as sorbent for in-tube-LC-UV to determine sulfonamides in milk samples

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Abbreviation: in-tube SPME, in-tube solid phase microextraction; **HPLC**, high performance liquid chromatography; **RAM**, restricted access material; **UV**, ultraviolet detection; **DAD**, diode array detection; **LLE**, liquid–liquid extraction; **SPE**, solid-phase extraction; **ACN**, acetonitrile; **SAs**, sulfonamides; **STZ**, sulfathiazole; **SMT**, sulfamethazine; **SDT,** sulfadimethoxine; **MRL**, maximum residue limit; **CV**, coefficient of variation.

Abstract

The capillary packed with restricted-access materials (RAM) was developed for in-tube solid-phase microextraction and liquid chromatography analysis (in-tube SPME/LC-UV) for the determination of sulfonamides (sulfathiazole, sulfamethazine and sulfadimethoxine) in milk samples. The in-tube SPME variables (sample pH, diluted diluted sample volume, and desorption procedure) were optimized to improve the sensitivity of the method and short the analysis time. The proposed method presented a linear range from 30 ng mL^{-1} (limit of quantification - LOQ) to 150 ng mL^{-1}, with the correlation coefficients (r) above 0.998. The accuracy values were adequate for all the sulfonamides (> 85%), and the interassay precision data presented coefficient of variation lower than 13%. Based on analytical validation results, the in-tube SPME/LC-UV method was a promising alternative for the determination of sulfonamides in milk samples.

Keywords: HPLC, in-tube SPME, milk sample, restricted access material (RAM).

1. Introduction

Antibiotics are widely used in veterinary medicine practice for the treatment of diseases involving bacterial infections [1]. Sulfonamides (SAs) are a group of synthetic antibiotics associated with chemotherapeutics in bacterial and protozoan infections in veterinary medicine practice. Moreover, they are often used in animal food as growth promoters and to prevent and treat a series of diseases related to animal feeding, such as infectious diseases of the digestive and respiratory tracts [1,2].

SAs remain in edible animal products like meat, milk, egg, and fish should be a result of their illegal use, excessive administration, and inappropriate withdrawal period. Residues of these compounds in food are pointed out as potential carcinogenic agents, not to mention the possible development of antibiotic resistance in humans as well as severe allergic reactions [3-5].

The Brazilian government authorities have established a monitoring program for the analysis of SAs levels in meat, honey, and milk samples. The Brazilian regulatory agency has determined that the maximum residue limit (MRL) allowed in milk is be 100 μg of total sulfonamides/kg [6,7].

High performance liquid chromatography (HPLC) with UV and DAD detection has been commonly applied to determine several SAs residues in milk samples [1,2,8,9]. However, due the milk matrix complexity, it is not possible inject these samples directly into HPLC system.

A sample preparation step is usually necessary to eliminate the majority of endogenous compounds and to concentrate the drugs that often exist at low concentrations in these complex matrices. This should help increase the selectivity and sensitivity of analytical methods. Conventional sample preparation techniques, such as liquid-liquid extraction (LLE) and solid phase extraction (SPE) usually use organic solvent, and require some step that increase the analysis time [3,6,10].

Recent trends in biological sample preparation have focused on miniaturized analytical systems, to simplify automation and allow for high-throughput performance and online coupling with analytical instruments. Online analytical systems require small biological samples and consume extremely low amounts of organic solvent or even no solvent at all. Minimizing biological sample preparation steps not only diminishes the sources of error, but also reduces analysis time and cost. In this context, online solid-phase microextraction (in-tube SPME) coupled with HPLC or liquid chromatography coupled to mass spectrometry (LC-MS) is worthy of mention [11].

In-tube SPME is an effective solvent-free sample preparation technique that uses a capillary column as the extraction device. In this technique, organic compounds in aqueous samples are directly extracted and concentrated into the stationary phase of capillary columns by repeated draw/eject cycles of the sample solution before being transferred to the liquid chromatographic column. In-tube SPME is fast to operate, easy to automate, solvent-free, and inexpensive [12]. This miniaturized sample preparation technique has been used for the determination of various compounds, such as drugs and environmental contaminants, and has been coupled with LC [12-15], LC/MS [16], and LC-MS/MS [17].

Restricted access materials (RAM) are stationary phases that are able to exclude the macromolecules (endogenous compounds) from biological matrix. These compounds interact only with the outer surface of the particle support coated with hydrophilic groups. Consequently, the adsorption of matrix proteins is minimized. The hydrophilic barrier enables the permeation of small molecules, such as drugs, through the hydrophobic part of the stationary phase, while macromolecules are excluded by physical or chemical means, or a combination of both [13,18-21].

In this work, in-tube SPME/LC system with RAM capillary as the extraction device was evaluated for the simultaneous determination of sulfathiazole (STZ), sulfamethazine (SMT) and sulfadimethoxine (SDT) in milk samples (Figure 1).

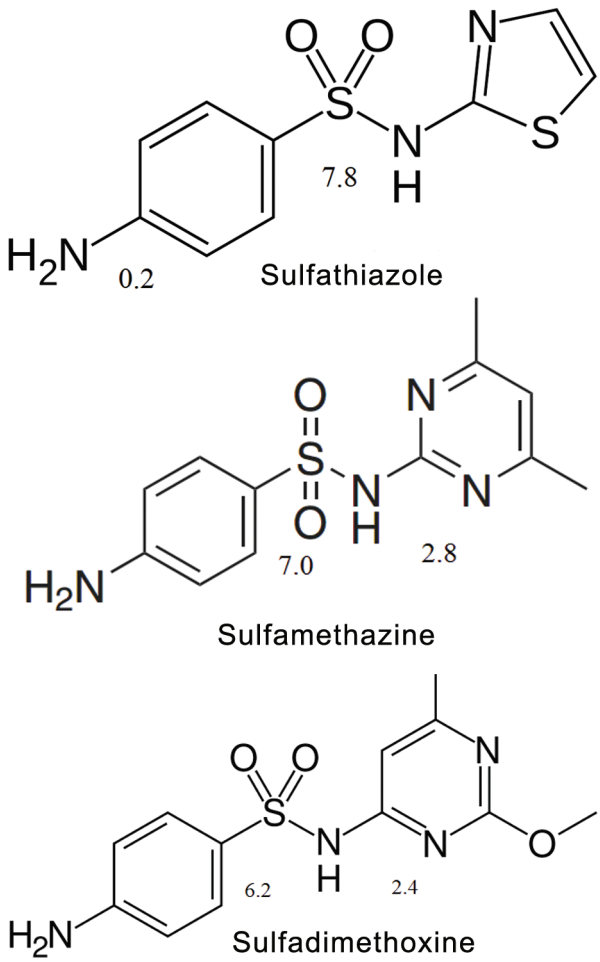

Figure 1. Sulfonamides and their corresponding pKa values.

2. Experimental

2.1. Reagents and analytical standards

The SAs (sulfathiazole, sulfamethazine and sulfadimethoxine) and primidone analytical standards were supplied by Sigma Aldrich (St. Louis - USA). Primidone was used as internal standard for SAs analysis in milk samples.

The standard solutions were prepared by diluting the SAs stock solutions (1 mg mL^{-1}) in methanol. These solutions were stable at a temperature of −20 ºC. The water used to prepare the mobile phase had been previously purified in a Milli-Q system (Millipore, São Paulo, Brazil). Methanol and acetonitrile HPLC grade were purchased from J.T. Backer (Phillipsburg, USA). Glutaraldehyde, potassium dihydrogen phosphate, and sodium borohydride were furnished from Merck, (Darmstadt, Germany). Trifluoroacetic acid HPLC grade was furnished from Fisher Scientific (Leics, UK).

2.2. Chromatographic conditions

The SAs were analyzed on the high-performance liquid chromatographic system Varian 230 ProStar (Varian, California, EUA) equipped with a DAD detector operating at λ = 269 nm. The separations were achieved on an analytical reversed-phase column C18 ChromSep HPLC (Varian, 250 mm x 3.0 mm, i.d.) by using a linear gradient from 10% to 30% of phase A (ACN/ methanol 60:40, v/v) with phase B (water with pH adjusted to 4 by means of TFA addition), at a flow rate of 0.8 mL min^{-1}. The mobile phase had been filtered and degassed prior to use.

2.3. Preparation of the milk sample

Blank milk samples (skimmed milk, 500 µL) spiked with an internal standard (IS) (primidone, 15 L, 30 µg mL^{-1}), as well as standard SAs solutions at different concentrations were used during the development and validation of this method. Before, the in-tube SPME-LC procedure, the proteins of milk samples (500 µL) were precipitated with acetonitrile (1:2 v/v). The supernatant was collect, and the sample solution was vaporized. The dried extract was reconstituted with buffer solution.

2.4. RAM (bovine serum albumin – SCX) capillary

The RAM phase capillary was prepared according to a previous work [19-21]. To this end, SAX particles (45µm) were slurred in methanol and packed into 50 mm (length) of polyether ether ketone (PEEK) tubing (1/16 in. O.D. and 0.02 in. I.D.). The capillary column was capped at both ends by a 1/16 in. (1 in. = 2.54 cm) zero-volume union fitted with a 10µm frit. After this procedure, the capillary was conditioned with phosphate buffer (0.05 mol L^{-1}, pH 6.0) at a flow-rate of 1.0 mL min^{-1} for 20 minutes. Bovine serum albumin (BSA) immobilization was accomplished *in situ*, on the basis of the protocol proposed by Menezes and Felix[20] and

by other authors [21]. Initially, phosphate buffer solution (0.05 mol L^{-1}, pH 6.0) was percolated through the capillary at a flow rate of 1.0 mL min^{-1} for 20 min, followed by BSA solution 1.0 mg mL^{-1} (prepared in phosphate buffer solution) for 30 min, deionized water for approximately 20 minutes, and two portions of 10 mL glutaraldehyde (25% v/v). After a repose period of 5 h, the column was percolated with a sodium borohydride solution ($NaBH_4$ 1.0 mg mL^{-1}) until a pH value of 10 was reached. After a further repose period 2 h, the stationary phase was washed with deionized water for 1 h. The RAM–BSA column was stored in phosphate buffer solution (0.05 mol L^{-1}, pH 7.4) at 4ºC.

2.5. In-tube solid phase microextraction

The RAM capillary was fixed in the place of the injection loop of the LC system. The capillary connections were facilitated by placing MicroTight sleeves at each end of the capillary (Figure 2).

The sample diluted in buffer solution was vortexed for 10 s before the extraction procedure, which was

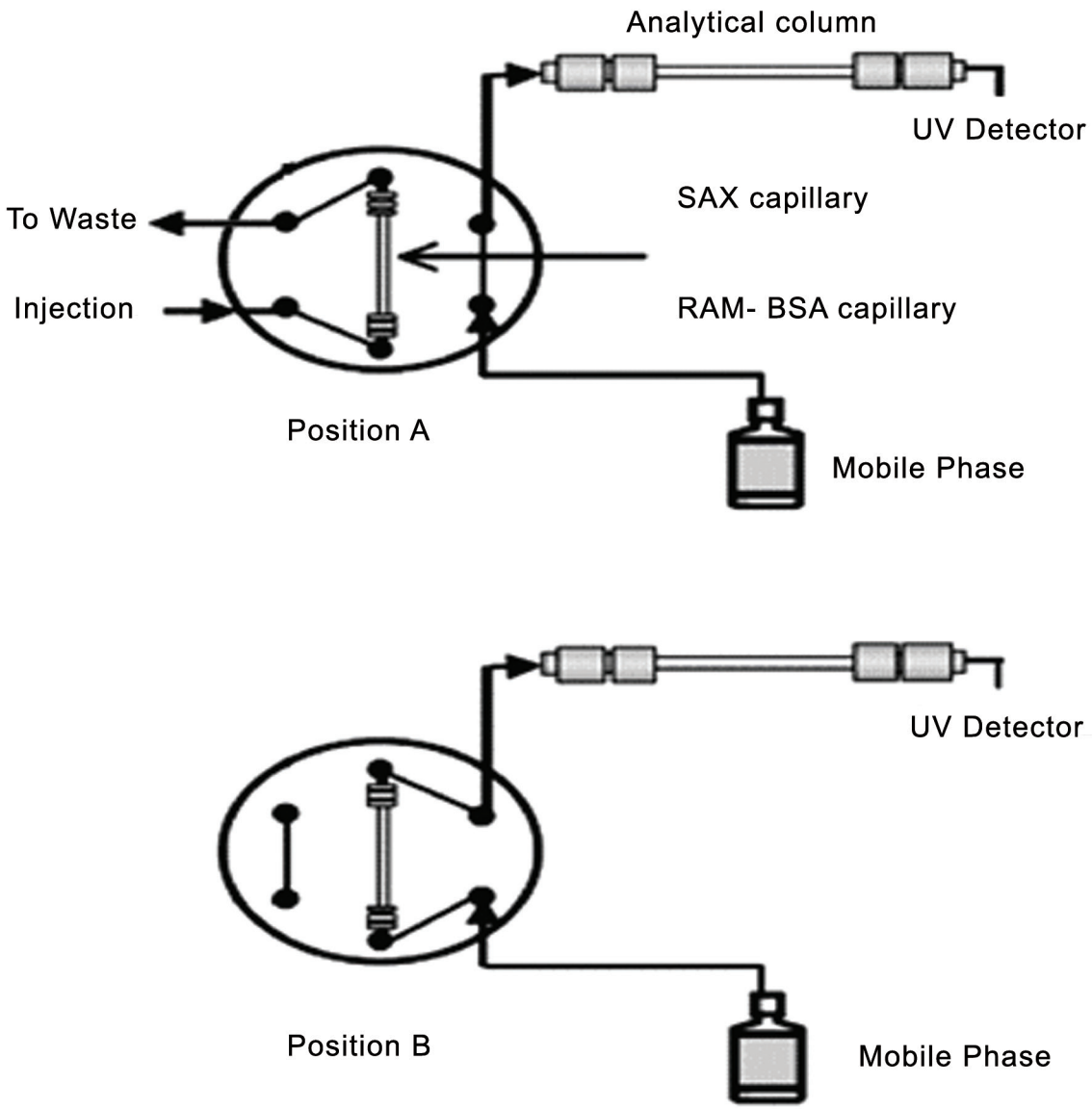

Figure 2. Schematic design of in-tube SPME system.

carried out with the six-port valve in the load position. The analytical column was conditioned with the mobile phase. After the preparation step, the milk sample was directly injected into the RAM capillary (2.4 item). Extraction of the sample was accomplished by manual injection through the capillary (position A). After the extraction, the valve was switched to the inject position (position B), the mobile phase eluted the analytes (desorption process) from the RAM capillary to the analytical column. The in-tube SPME variables such as sample pH, diluted sample volume, and desorption procedure were optimized to improve the sensitivity of the method.

2.6. Analytical validation

The in- tube SPME method was validated by using SAs free milk sample, spiked with standard solutions of the analytes at different concentrations, considering the safe maximum residue limits (MRL). The linearity was evaluated by calibration curves constructed by using linear regression of the ratio between the SAs and the internal standard (Y) peak areas versus the nominal milk SAs concentrations (X, ng mL^{-1}). Accuracy and interassay precision values were determined by calibration curves, by means of quintuplicate in- tube SPME assays of the blank samples spiked with the analytes. Accuracy values were calculated by comparison between the SAs concentrations added to the samples and the SAs concentrations measured by means of the calibration curves.

3. Results and Discussion

3.1. Developed capillaries

The developed RAM capillary column consists of SCX particles with the outer surface covered with BSA. This makes the external surface of the particles compatible with a protein of the samples that cannot penetrate into small pores [13].

The RAM materials based on BSA immobilization present a protein network on the outer hydrophilic surface instead of the polymer. SCX phase at the internal surface are responsible for the interaction with the small analytes. RAMs have been developed as suitable tools for direct, repetitive injection of simple pre-treated biological samples into the analytical system, so they are ideal for automation, purification, and pre-concentration.

In the present work, the straightflush mode was used for SAs preconcentrarion from the milk samples. The straightflush mode is the simplest mode applied to processing of the biological samples using the column-switching configuration. First, the sample is injected into the pre-column, where undesirable components are directly discharged to waste. By rotating the six-port selection valve, the fraction containing the target analytes is transmitted to the analytical column, and the analytes are separated [18].

For SAs determination in milk samples, the samples were directly injected into the RAM column after their preparation. To increase the RAM column life time, skimmed milk was used during the development step.

This procedure allowed for repetitive injections, of milk sample; i.e., about 40 extractions, using the same RAM column, without significant loss of the RAM column extraction efficiency.

3.2. In-tube SPME conditions

The in-tube SPME variables, such as sample pH, diluted sample volume, and desorption conditions (solvent and solvent volume) were optimized, in order to establish the partition equilibrium of the SAs in shorter analysis times.

The pH value has a significant effect on the extraction procedure (partition equilibrium), especially for drugs containing a pH-dependent dissociable group. Thus the milk samples, prepared as previously described, were diluted with 500 µL 0.05 mol L^{-1} phosphate buffer solutions using different pH values (3.0, 4.0, 5.0, and 6.0). The milk samples diluted with 500 µL of phosphate

buffer solution at pH 3.0 (0.05 mol L^{-1}) presented the highest extraction efficiency (Figure 3 a). At pH 3.0, the interactions between sulfonamides and SCX particles is favored. Silica sorbent could be damaged, at pH values lower than 3 as well as above 7.

The sample volume is intrinsically related to the amount of extracted analytes, which depends of the stationary phase capability. Herein, the diluted sample volume was evaluated from 50 to 250 µL. The extraction efficiency increased from 50 up to 200 µL. After this value, the extraction efficiency decreased, probably due the partial desorption of the drugs (Figure 3b).

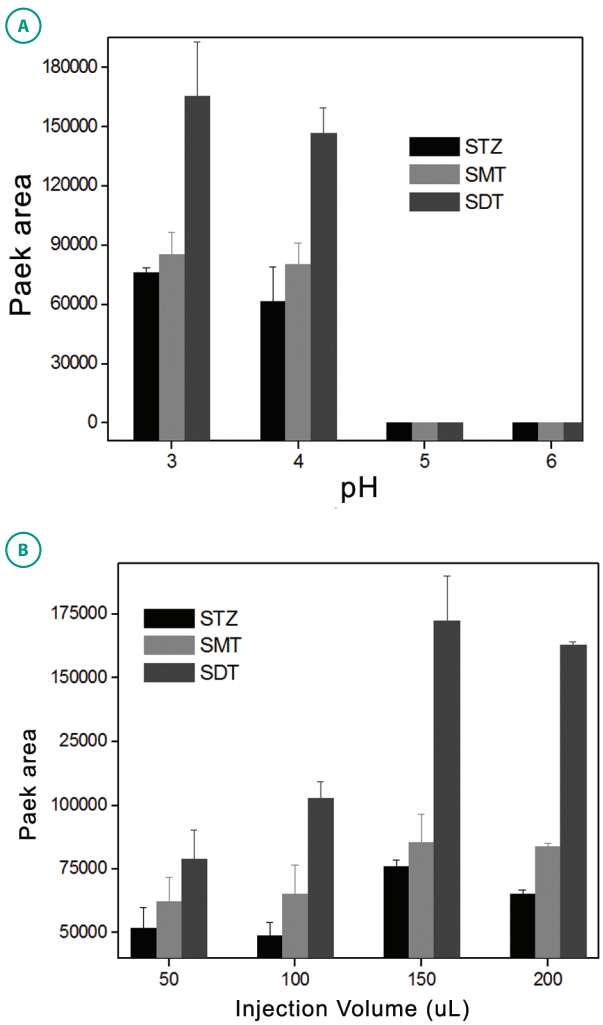

Figure 3. Effect of the variables on in-tube SPME performance. (a) pH sample, (b) diluted sample volume. STZ: sulfathiazole, SMT: sulfamethazine, and SDT: sulfadimethoxine.

Although the salting-out effect (NaCl addition to the sample) increase the in-tube SPME efficiency, this procedure could be block the capillary. The salt solubility in water–organic mixtures is lower than in pure water. Thus, the influence of salt addition to the sample matrix on the performance of in-tube SPME was not evaluated.

The online elution was carried out by redirecting the mobile phase through the capillary (dynamic desorption), where the six-port valve was switched to the injection position, and the mobile phase transported the desorbed drugs to the LC column. The desorption process (drugs elution) was evaluated by using different solvents; i.e., methanol, buffer solution, water, and mobile phase. The highest extraction efficiency were achieved with dynamic desorption using the mobile phase.

After the chromatographic analysis, the LC six-port valve was switched to the inject position, and the RAM capillary column was conditioned with water (500 µL) prior to the subsequent sample injection, to avoid carryover effect.

3.3. Analytical validation of the RAM in-tube SPME/LC – UV method

The selectivity of the developed method was demonstrated by representative chromatograms of reference milk blank samples, and of reference milk blank samples spiked with the target drugs, (Figure 4). The chromatogram evidence the ability of the method to measure the drugs in the presence of endogenous milk components.

The in tube SPME/LC - UV presented a linear range from 30 (LOQ) to 150 ng mL-1 with coefficient of determination, with the correlation coefficients (r) above 0.998. LOQ values were determined as the lowest concentration in the calibration curve in which the coefficient of variation (CV) was lower than 20%, based on a signal-to-noise ratio of about 10. Each point of the calibration curve was performed in replicate (n = 5).

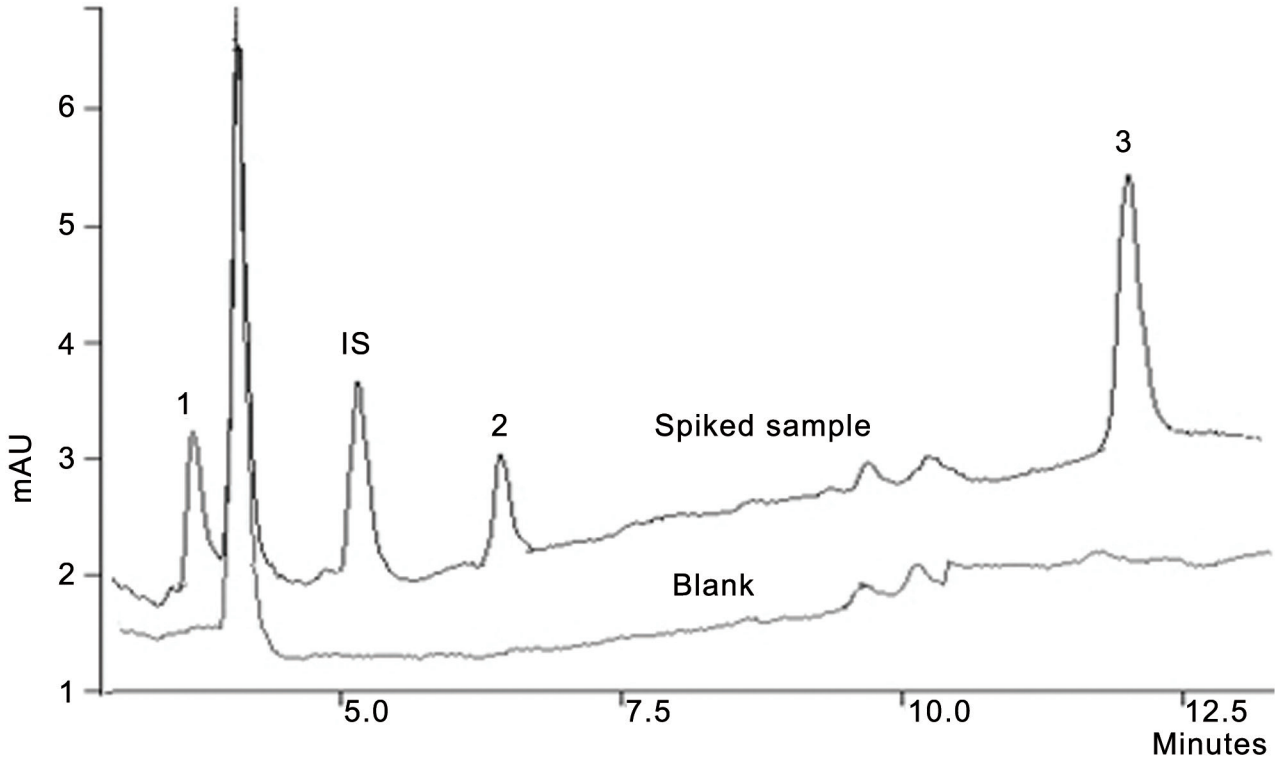

Figure 4. Representative chromatograms of the reference milk blank sample and of the reference milk blank sample spiked with the target analytes (100 ng g $^{-1}$): 1: sulfathiazole, 2: sulfamethazine, 3: sulfadimethoxine, and IS: internal standard (Primidone).

The interassays precision evaluated three different levels presented CV values ranged from 3.8% to 12.8% (Table 1).

Table 1. Interassay precision (CV) and accuracy of the RAM/LC - UV method for sulfonamides determination in milk samples.

Sulfonamides	Added concentration (ng g $^{-1}$)	Accuracy (%) (n = 5)	CVa (%) (n = 5)
sulfathiazole	150	104	7.3
	100	109	7.5
	30	118	9.0
sulfametazine	150	107	3.8
	100	108	7.3
	30	85	12.8
sulfadimethoxine	150	98	2.5
	100	98	8.9
	30	90	8.0

[a]coefficient of variation.

4. Conclusions

RAM (BSA-SCX) capillary not only exclude the endogenous compounds of milk samples, but also concentrate the sulfonamides. Consequently, the automate in-tube SPME-LC system increases the selectivity and the sensibility of the method, and reduces the sample preparation step and the analysis time.

This method presents LOQ values lower than those established as MRLs (100 µg kg^{-1}) in food of animal origin. Considering the evaluated analytical validation parameters, the developed method should be useful to determine sulfonamides in milk samples.

Acknowledgments

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