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High-performance liquid chromatography method for the simultaneous determination of sulfamethoxazole and trimethoprim in bovine milk using an on-line clean-up column

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Abstract

A bidimensional HPLC method for the simultaneous determination of sulfamethoxazole (SMX) and trimethoprim (TMP) in bovine milk has been developed and validated. After centrifugation, aliquots (150 μl) of milk samples were directly injected to a column-switching HPLC system. At the first step a RAM octyl-BSA column was employed to automatically remove proteins that otherwise would interfere with milk analysis. The mobile phase 0.01 M phosphate buffer pH 6.0:acetonitrile (95:5, v/v) was used in the first 5 min for the elution of milk proteins and then 0.01 M phosphate buffer pH 6.0:acetonitrile (83:17, v/v) for transfer SMX and TMP to the analytical column. The separation of SMX and TMP from one another and from other remaining milk components was performed on an octyl column using the mobile phase 0.01 M phosphate buffer pH 5.0:acetonitrile (82:18, v/v), which were detected by UV at 265 nm. The calibration graphs were linear in the concentration ranges of 25–800 ng/ml and 50–400 ng/ml for SMX and TMP, respectively. The intra- and inter-assay coefficients of variation were less than 15% for both drugs. The validated method was applied to the analysis of milk samples of twelve (two groups of six) cows after administration (intramuscular or subcutaneous) of a single recommended therapeutic dose of the SMX–TMP combination.

Keywords: Restricted access media (RAM); Column-switching; Direct injection; Sulfonamide; Sulfamethoxazole; Trimethoprim; Bovine milk

1. Introduction

Sulfamethoxazole (SMX) (Fig. 1) is a sulfonamide antibiotic of broad spectrum that competitively inhibits the bacterial enzyme dihydropteroate synthetase. Trimethoprim (TMP) (Fig. 1) is a dihydrofolate-reductase inhibitor. In veterinary medicines, trimethoprim is commonly administered in combination with a sulfonamide. Both drugs block the folic acid metabolism and produce a synergistic antibacterial activity [1].

SMX–TMP is commonly used in dairy cattle for treatment or to prevent respiratory infections and mastitis [2]. However, the use of these products may lead to the presence of unwanted residues in milk. Withdrawal times for food animals and milk discard times should be respected to avoid food residues and consequent health problems, such as allergic reactions in hypersensitive individuals, and induction of resistance in strains of pathogenic bacteria.

In Brazil, government authorities have established a monitoring program to determine sulfonamide levels in meat, honey and milk. The maximum residue limit (MRL) in milk has been set at 100 μg of total sulfonamides/kg [3]. The European Agency for the Evaluation of Medicinal Products has set 50 μg/kg as MRL of trimethoprim in meat and milk [4].

A number of analytical HPLC methods have been published which describe the analysis of sulfonamides [5–9] and trimethoprim [10] in milk. Because some of these methods are labor intensive, requiring extensive sample pre-treatments, they cannot be used for routine analysis of numerous samples.

More recently, HPLC methods incorporating restricted access media (RAM) columns linked on-line with analytical columns have been reported for the analysis of analytes in biological fluids by direct injection [11–13]. Recently, we have used RAM–bovine serum albumin (BSA) columns coupled with a C_{18}

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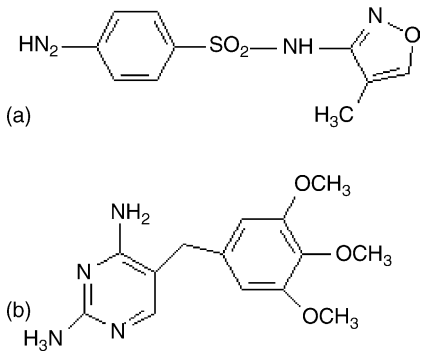

Fig. 1. Chemical structures of (a) sulfamethoxazole and (b) trimethoprim.

analytical column for the analysis of cephalosporin antibiotics in bovine milk by direct injection [14].

The purpose of the work described in this article was to develop a simple, fast and sensitive HPLC method with on-line clean-up RAM column for SMX and TMP, which could be applied for the simultaneous determination of these compounds in cows' milk (centrifuged at 15,000 × *g* at 20 °C for 15 min) after receiving SMX–TMP treatment.

2. Experimental

2.1. General

Acetonitrile was HPLC grade purchased from J.T. Baker (Philipsburg, PA, USA). Water used for the mobile phase was purified through a Milli-Q system (Millipore, São Paulo, Brazil). Bovine serum albumin was purchased from Sigma (fraction V powder minimun 98%; St. Louis, MO, USA).

A 47 mm diameter, 0.45 μm nylon membranes used to filter all the mobile phases were also from Millipore.

Glutaraldehyde, potassium dihydrogen phosphate and sodium borohydride were from Merck (Darmstadt, Germany).

Sulfamethoxazole and trimethoprim were generously supplied by Laboratório Teuto-Brasileiro Ltda (Anápolis, GO, Brazil). The purities of the two analytes were up to 99% as stated by the supplier.

The commercial formulation (Trissulfin® injection) containing SMX (200 mg/ml) and TMP (40 mg/ml) was kindly provided by Produtos Veterinários Ouro Fino Ltda, Ribeirão Preto, SP, Brazil. The administration of the SMX–TMP combination and collection of milk samples from the cows were made at Centro de Pesquisas em Sanidade Animal, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, SP, Brazil. Twelve healthy cows were used in the experiment.

2.2. Equipment

The HPLC system consisted of two Shimadzu LC-10ATVP pumps (Kyoto, Japan), with one of the pumps having a FCV-10AL valve for selecting solvent, an SIL 10AVP auto-injector model, a DGU-14A degasser model, a SPD-6AV UV–vis detector (at 265 nm) and a SCL 10AVP interface. A HPLC 7000 Nitronic EA (Sulpelco, St. Louis, MO, USA) six-port valve was used for the automated column switching. Data acquisition was done on a Shimadzu Class-VP Software.

2.3. Columns

The analytical column used (150 mm × 4.6 mm I.D.; octyl-Luna, 10 μm particle size and 120 Å pore size) was packed by the ascending slurry method using methanol for the preparation of the slurry (50 ml) and also for the packing. The packing was carried out at a pressure of 7500 psi [15].

The BSA restricted access media columns (100 mm × 4.6 mm I.D.) were prepared as follows: octyl-silica (Luna, 10 μm particle size and 120 Å pore size) column was packed as the analytical column. After the columns were conditioned for about 12 h with methanol at a flow-rate of 1.0 ml/min, the immobilization of BSA was done in situ, based on the protocol of Menezes and Felix [16]: the columns were first eluted at flow-rate of 1.0 ml/min with 0.05 M phosphate buffer (pH 6.0) (50 ml) before passing a 1.0 mg/ml solution of bovine serum albumin prepared in 0.05 M phosphate buffer (pH 6.0) (25 ml), followed by a 25% (v/v) solution of glutaraldehyde (25 ml). After 5 h, the columns were washed with a 1.0 mg/ml solution of sodium borohydride (10 ml) and then with water (60 ml).

2.4. Solutions

2.4.1. Buffer solutions

The 0.01 M phosphate buffer solutions (pH 5.0 and 6.0) were prepared by dissolving 1.36 g of KH_2PO_4 in 1000 ml of distilled water and the pH was adjusted with a solution of 1 M of sodium hydroxide as required.

2.4.2. Standard solutions

SMX and TMP stock solutions (1 mg/ml) were individually prepared by dissolving approximately 10 mg in 10 ml of methanol. From these stock solutions seven standard solutions were made with methanol to give working calibration solutions of 2.5; 5; 10; 20; 40; 60; 80 μg/ml and 5.0; 7.5; 10; 15; 20; 30; 40 μg/ml for SMX and TMP, respectively. Three quality controls (QC) standard solutions of each drug were individually prepared in the concentrations of 3; 48; 72 μg/ml and 6; 24; 36 μg/ml for SMX and TMP, respectively. Stock solutions were stable for 2 months when stored at + 4 °C and no evidence of degradation of the analytes was observed on the chromatograms in this period.

2.5. Preparation of spiked samples

To prepare the fortified milk samples, aliquots (10 μL) of each of the appropriated standard solutions of SMX and TMP were placed into a culture tubes and the solvent was evaporated under a stream of nitrogen. All milk samples were centrifuged (15,000 × *g* at 20 °C for 15 min) resulting in a thin upper fat layer, a middle aqueous layer and a small cell pellet at the bottom of the centrifuge tube. The middles layers (1 ml) were used to prepare the samples by reconstituting the dried analytes. The solutions were mixed by vortex agitation for 30 s. Aliquots of

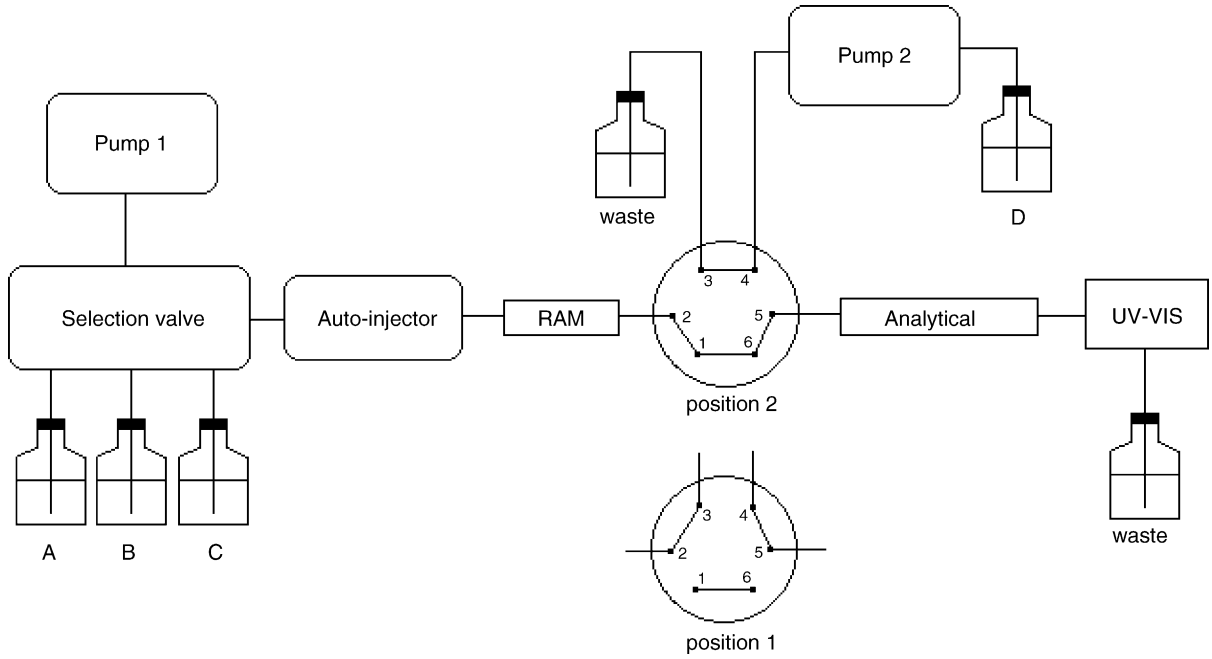

Fig. 2. Schematic diagram of the column-switching HPLC system.

250 μL were transferred to auto-sampler vials and 150 μL were injected into the column-switching HPLC system. Skimmed milk was used in the development and validation steps while whole bovine milk was used in the blind test.

2.6. Column-switching procedure

The column-switching system used is illustrated in Fig. 2. The position of the column-switching device alternated between positions 1 and 2 and was controlled through the timed events using the Class-VP Software. Initially, the column-switching was set to position 1 and the milk sample was injected into the RAM column. The time sequence used is listed in Table 1. The six-port valve remained in this position for 11.50 min while the macromolecules were discharged into the waste. At the same time, the analytical column was conditioned with the mobile phase delivered by pump 2. Then, the valve changed to position 2 redirecting the flow from the waste to the analytical column; the transfer of the effluent fraction containing the analytes took between 11.50 and 14.50 min. At the end of this time, the valve was switched back to position 1 for cleaning and conditioning the RAM column while SMX and TMP were analyzed on the octyl analytical column. The flow-rate used was of 1.0 ml/min and the absorbance was measured at 265 nm. Analysis time was 32 min.

2.7. Method validation

Method validation was carried out according to internationally accepted criteria [17]: linearity, selectivity, accuracy and precision, recovery, limit of quantification (LOQ), limit of detection (LOD) and stability.

2.7.1. Linearity

The linearity curves of SMX and TMP were assessed by preparing calibration standards (25, 50, 100, 200, 400, 600, 800 ng/ml and 50, 75, 100, 150, 200, 300, 400 ng/ml for SMX and TMP, respectively) in triplicate using blank skimmed milk previously centrifuged at 15,000 × *g* at 20 °C for 15 min. SMX and TMP calibration curves were constructed

Table 1
Time events for the column-switching procedure and mobile phases

Time (min)	Pump	Event	Valve position
0.00–5.00	Pump 1 (eluent A)	Milk proteins are excluded by RAM column	1
	Pump 2 (eluent D)	Conditioning of the analytical column	
5.01–14.60	Pump 1 (eluent B)	Elution of retained analytes from the RAM	1
11.50–14.50	Pump 1 (eluent B)	Analytes are transferred to the analytical column	2
14.51–32.00	Pump 2 (eluent D)	Analysis of the SMX and TMP	1
14.60–22.00	Pump 1 (eluent C)	Washing of RAM column	1
22.01–32.00	Pump 1 (eluent A)	Conditioning of RAM column	1

Pump 1: eluents (A) KH_2PO_4 0.01 M pH 6.0:CH_3CN (95:5, v/v); (B) KH_2PO_4 0.01 M pH 6.0:CH_3CN (83:17, v/v); (C) CH_3CN:H_2O:isopropanol (75:15:10, v/v), flow rate: 1.0 ml/min, pump 2: eluent (D) KH_2PO_4 0.01 M pH 5.0:CH_3CN (82:18, v/v), flow rate: 1.0 ml/min; λ: 265 nm.

by plotting the peak area against the concentration of each drug.

2.7.2. Selectivity

The interference of endogenous compounds was assessed by analyzing drug-free milk samples, milk fortified with SMX and TMP and milk samples obtained from cows treated with the SMX–TMP combination.

2.7.3. Precision and accuracy

Inter- and intra-day variability of the method was determined by analyzing replicates of three QC samples (30, 480, 720 ng/ml and 60, 240, 360 ng/ml for SMX and TMP, respectively). Five samples of each concentration were prepared in milk on three non-consecutive days.

2.7.4. Recovery

The extraction recoveries of each drug from milk and transfer efficiencies from the RAM column to the analytical column were estimated using spiked skimmed milk prepared at the same concentrations of the three QC samples used for the determination of intermediate precision. The spiked milk samples were prepared as in the sample preparation item and the efficiencies of extractions were calculated by comparing the peak areas of SMX and TMP with those of similar concentration of methanolic standard solutions.

2.7.5. Limits of quantification and detection

The acceptance criteria for the LOQ were that the precision and the accuracy for three extracted samples be under 20% variability. LOD was calculated taking a signal-to-noise ratio of 3 as criteria. LOQ and LOD were measured by preparing fortified milk samples with serial diluted solutions.

2.7.6. Stability

The stability of SMX and TMP in milk samples was evaluated by comparing assay results in fortified samples at three different concentrations (QC samples) and by analyzing aliquots of the same samples after storage in two different conditions. All stability determinations were assessed by preparing a set of samples from a freshly made stock solution of skimmed milk. The chemical stability of the QC milk samples containing SMX and TMP and free-drug milk samples (matrix) were tested in the following conditions: (a) sitting at room temperature for 4 h (bench-top stability) and 24 h (auto-sampler stability), respectively; (b) stored at −20 °C and exposed to three freeze-thaw cycles; (c) stored at −20 °C for 2 months (long-term stability). Stability was also evaluated for stock solutions stored and refrigerated at +4 °C for at least 2 months. The analytes were considered stable if the variation of the concentrations between the assays were less than 15% of initial time response.

2.8. Application of the method

2.8.1. Spiked whole milk samples

The efficiency of the method for analyzing and quantifying samples of whole milk spiked with SMX and TMP was evaluated. Pasteurized milk samples of different brands were acquired at local markets in São Carlos, SP, Brazil. The described method was applied to six-spiked milk samples prepared with unknown concentrations by a different analyst (blind test).

2.8.2. Elimination time study

Complementary, the application of the method was demonstrated for the SMX and TMP quantification by analyzing raw milk samples of diary cows after intramuscular (i.m.) or subcutaneous (s.c.) administration. Two groups (A and B) of six cows were used for this experiment. In order to study the time needed to complete elimination, a single i.m. (A) or s.c. injection (B) of Trissulfin® was applied at the therapeutic dose (200 mg/15 kg for SMX and 40 mg/15 kg for TMP). In order to quantify SMX and TMP, milk samples (20 ml) were collected before injections (blanks) and for the following intervals after administration: 12, 24, 36, 48, 72, 84 and 96 h. The sampling times were based on routine farm milking. Milk samples were stored at −20 °C after collection and assayed within 4 weeks. The raw milk samples (1 ml) were placed into culture tubes and centrifuged at 15,000 × *g* at 20 °C for 15 min and then, from the middles layers aliquots of 250 μl were transferred to auto-sampler vials and 150 μl were injected into the HPLC system. In the assay, the QC samples (skimmed milk) used among the raw milk samples were prepared as in the topic spiked sample preparation.

3. Results and discussion

3.1. Chromatographic conditions

Methods incorporating RAM columns coupled on-line with HPLC analytical columns have been developed and take advantage of the minimal sample preparation. This type of packing has shown excellent efficiency for protein exclusion of human plasma [15,18], but analysis of drugs in milk without tedious and time consuming pre-treatment procedures remains a challenge. Column-switching techniques have been widely used for on line clean-up of biological samples but the number of methods employing RAM columns for analysis of exogenous compounds in milk samples is, however, limited [10,16,19–22].

In a recent work [14], we described a simple on-line clean-up procedure for low volumes (50–200 μl) of milk (skimmed and whole) containing polar and highly hydrophilic antibiotics (cephalosporins) by the use of different RAM columns. During method development, when successive centrifuged whole milk samples were injected lost of precision on the retention factor was observed probably due to lipid adsorption by the RAM column. Then, to increase the life time of the RAM column, skimmed milk was used during the development step.

A simultaneous determination of sulfonamides and trimethoprim in egg, milk and meat was described by Aerts at al. [8]. In this work, for the raw milk analysis, the samples were centrifuged and they report that more than 95% of both drugs were recovered from the aqueous phase indicating that these drugs are not lost in the fat layer in the centrifugation step.

In our previous experience with RAM columns [14,15], excellent elution profile of human plasma and bovine milk pro-

teins was obtained using pH >6.0, with or without an organic modifier (methanol or acetonitrile). Complete exclusion of proteins was achieved within 5 min. And, also, the addition of 2–5% of organic modifier improved the clean-up of samples by avoiding undesired adsorption of lipids and proteins on the surface of hydrophobic phases. Otherwise, the RAM column may be clogged by proteins, leading to an increase in back-pressure [15].

In this work, we used an octyl-RAM BSA column with column-switching system in order to develop a simple, rapid and sensitive bidimensional HPLC method for the simultaneous determination of SMX and TMP in raw milk samples (defatted by centrifugation at 15,000 × *g* at 20 °C for 15 min). Due to the low selectivity of the RAM column, it was coupled to an octyl analytical column. Using the column-switching technique, it was possible to achieve a highly selective separation of analytes from endogenous compounds of milk by cutting the fraction containing SMX and TMP and transferring it to the analytical column.

In the preliminary experiments, the octyl-RAM BSA column was directly connected to an UV detector in order to evaluate the protein exclusion profile of the milk samples. The exclusion step was complete when the protein chromatographic band returned to the baseline. The proteins of the milk samples were excluded before 5 min at a flow rate of 1.0 ml/min using 0.01 M phosphate buffer pH 6.0:acetonitrile (95:5, v/v); under these conditions, the analytes were retained in the RAM column (Fig. 3a).

The percentage of acetonitrile (16–20%) in the mobile phase (B) used for the transfer of SMX and TMP to the analytical column was also evaluated. The weak acid SMP (p*K*a 6.1) and the weak base TMP (p*K*a 7.3) co-eluted as a narrow band in the RAM column with a mobile phase made up of 0.01 M phosphate buffer pH 6.0:acetonitrile (83:17, v/v). A suitable retention time "window", defined by the exact time needed for switching the six-port valve, was selected for transferring SMX and TMP from the RAM to the analytical column (Fig. 3b). Except for the analyte fraction, all the eluate from RAM column was directed to waste.

The injection volume of the milk samples was of 150 μL to get the desired sensitivity. Retention time of SMX and TMP on the octyl column was adjusted as a function of pH and percentage of organic modifier. The best chromatographic condition was obtained with 0.01 M phosphate buffer pH 5.0:acetonitrile (82:18, v/v).

3.2. Validation of the assay

The column-switching procedure described provided adequate clean-up of milk samples. The typical chromatograms of blank and spiked milk sample are shown in Fig. 4. No endogenous compounds interfered with the detection of SMX and TMP.

The calibration curves were linear for the calibration ranges of 25–800 ng/ml and 50–400 ng/ml for SMX and TMP, respectively. The following regression equations and correlation coefficients were obtained: (a) SMX ($y = 1.95 \pm 0.003 \times 10^{-3}x + 1.51 \pm 1.32$; $r^2 = 0.9997$) and (b) TMP ($y = 8.90 \pm 0.021 \times 10^{-3}x - 3.60 \pm 1.75$; $r^2 = 0.9997$).

The intra- and inter-day precision and accuracy of the method were determined by analyzing five replicates of three QC milk samples representing the entire range of the calibration curves (low, medium and high concentrations) on three non-consecutive days. The results of precision are expressed as coefficients of variation and the accuracies were evaluated by back-calculation and expressed as the percent of deviation between found and added concentrations of SMX and TMP. The results of precision and accuracy parameters are described in Table 2.

The extraction recovery for both drugs in skimmed milk was determined by comparing the peak areas of the SMX and TMP in methanol solution at three different levels with those QC milk samples at corresponding concentrations. The extraction and transfer efficiencies were excellent for both compounds analyzed at the three quality controls levels (Table 2).

The LOQ was 25 and 50 ng/ml for SMX and TMP, respectively. The LOD, based on a signal-to-noise ratio of 3:1, were 15 and 25 ng/ml for SMX and TMP, respectively.

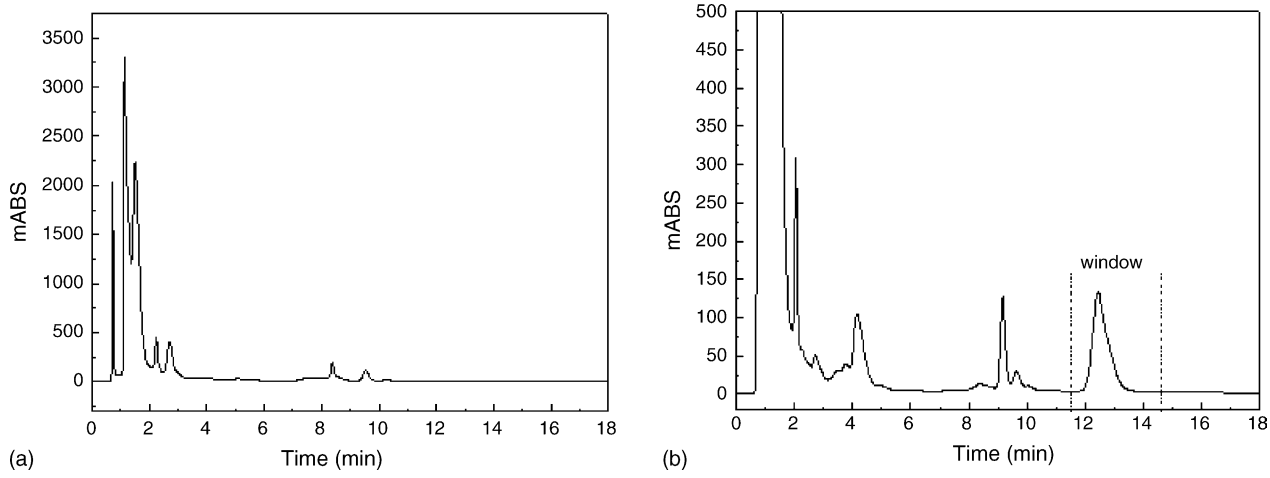

Fig. 3. Typical chromatograms of (a) exclusion profile of milk free drug and (b) milk spiked with SMX and TMP (10 μg/ml) showing the window time used. Chromatographic conditions: octyl-RAM BSA (100 mm × 4.6 mm I.D., 10 μm); mobile phase, 0.01 M phosphate buffer pH 6.0:acetonitrile (95:5, v/v); flow rate, 1.0 ml/min.

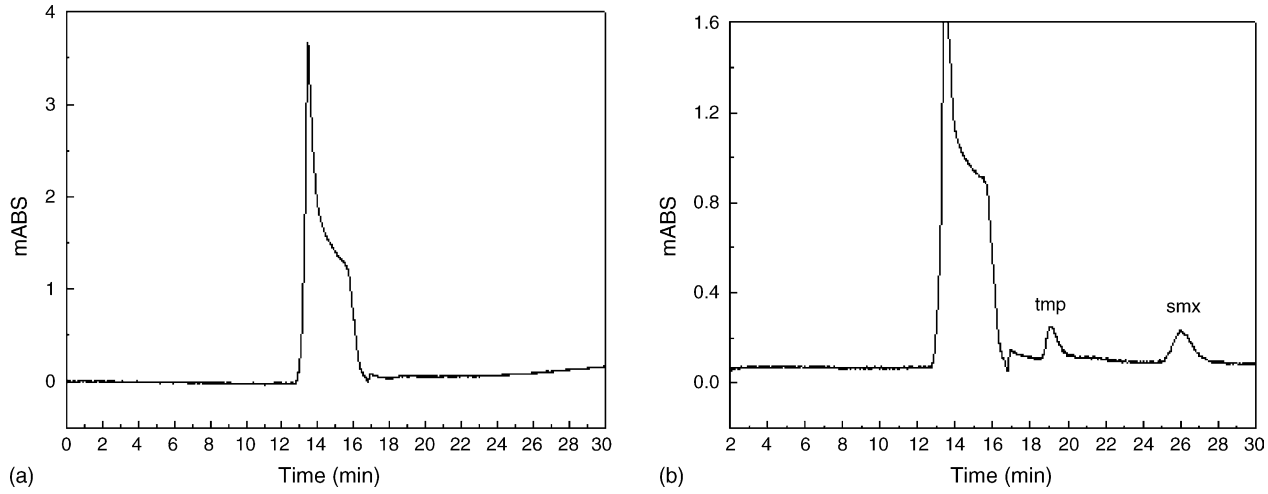

Fig. 4. Typical chromatograms of (a) milk free drug and (b) milk spiked with SMX (30 ng/ml) and TMP (60 ng/ml). Chromatographic conditions: octyl-RAM BSA (100 mm × 4.6 mm I.D., 10 μm); analytical column (octyl-Luna, 150 mm × 4.6 mm I.D., 10 μm). For details, see Table 1.

Table 2
Accuracy, intra- and inter-day variability and recovery for the assay of sulfamethoxazole and trimethoprim in bovine milk

Drug (ng/ml)	1st day[a]		2nd day[a]		3rd day[a]		Recovery[a] (%)
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	(mean ± S.D.)
Sulfamethoxazole							
30	96.4	5.4	90.1	4.3	100.8	4.5	94.4 ± 4.1
480	99.9	1.0	100.1	1.4	97.7	2.6	103.5 ± 3.2
720	99.1	1.8	101.2	2.3	97.2	2.8	97.5 ± 2.6
Trimethoprim							
60	104.5	5.5	91.6	3.4	91.5	6.2	94.7 ± 2.1
240	102.2	2.1	102.6	3.6	103.7	2.9	95.3 ± 3.5
360	100.8	2.0	94.1	5.2	101.1	1.7	101.0 ± 2.8

[a] $n = 5$.

3.3. Stabilities

The stability procedures aimed to evaluate all possible conditions the milk samples might suffer during collection, handling and analysis. Under all conditions studied, both drugs proved to be stable. The permanence in the auto-sampler tray for 24 h at room temperature (approximately 22 °C) had no significant effect on the quantitative determination of SMX and TMP in milk samples. The same can be said for samples analyzed from the three freeze-thaw cycles and 2 months storage at −20 °C (Table 3). The stability of milk samples (matrix) was evaluated under different conditions: stored at +4 °C for 120 h and at −20 °C for 2 months. No sign of degradation was observed in the blank milk samples under these conditions. Also, SMX and TMP stock solutions prepared in methanol proved to be chemically stable after storage at +4 °C for at least 2 months.

Table 3
Stability assays at −20 °C (freeze-thaw cycles and long-term) of spiked samples

Drug (ng/ml)	1st cycle (24 h)		2nd cycle (72 h)		3rd cycle (120 h)		2 months	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Sulfamethoxazole								
30	96.5	6.5	93.4	4.2	92.1	3.9	94.8	4.9
480	99.2	4.2	97.5	3.9	95.8	1.7	102.5	5.3
720	96.7	3.4	97.9	3.7	103.7	2.8	93.6	1.8
Trimethoprim								
60	93.9	7.3	91.6	5.4	95.3	3.5	89.9	3.5
240	100.3	3.5	98.9	2.1	96.4	4.4	95.5	5.0
360	98.4	6.4	94.9	3.0	93.7	5.6	94.4	2.1

Table 4
Precision and accuracy for the determination of SMX and TMP in blinded milk samples

Milk samples	SMX			TMP		
	Concentration (ng/ml)	CV (%)	Accuracy (%)	Concentration (ng/ml)	CV (%)	Accuracy (%)
1	30	6.8	94.0	50	8.5	97.2
2	60	6.9	98.9	100	5.4	98.6
3	100	1.6	98.5	160	6.8	102.7
4	160	2.3	103.0	200	6.2	100.8
5	300	2.8	90.1	260	3.4	98.6
6	680	0.8	93.7	340	2.4	100.5

Table 5
SMX and TMP residue levels in milk from six cows after intramuscular and subcutaneous injections as a single dose

Cows	Sampling time (h)											
	SMX (ng/ml), subcutaneous			SMX (ng/ml), intramuscular			TMP (ng/ml), subcutaneous			TMP (ng/ml), intramuscular		
	12	24	36	12	24	36	12	24	36	12	24	36
1	243	26.5	ND	121	<LQ	ND	82.2	<LQ	ND	126	<LQ	ND
2	344	41.7	ND	92.0	ND	ND	89.6	<LQ	ND	154	65.0	ND
3	332	38.4	ND	162	<LQ	ND	105	<LQ	ND	189	90.8	ND
4	240	<LQ	ND	277	31.9	ND	51.7	ND	ND	202	91.8	ND
5	215	<LQ	ND	198	<LQ	ND	63.4	ND	ND	193	71.7	ND
6	274	35.3	ND	174	<LQ	ND	78.5	<LQ	ND	167	57.3	ND

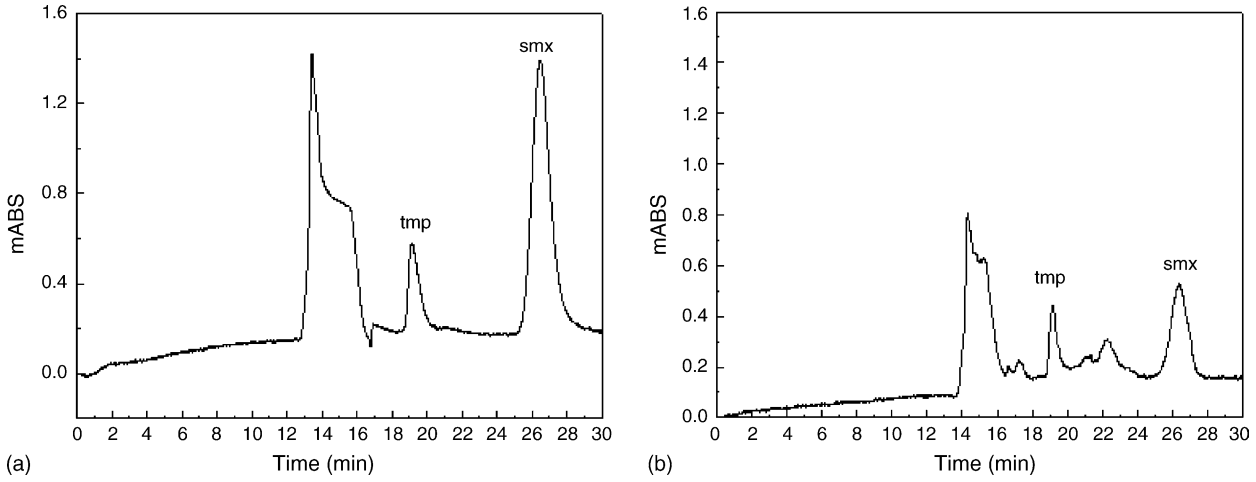

Fig. 5. Typical chromatograms of (a) whole milk (blinded test) and (b) raw milk (elimination study). Chromatographic conditions: octyl-RAM BSA (100 mm × 4.6 mm I.D., 10 μm); analytical column (octyl-Luna, 150 mm × 4.6 mm I.D., 10 μm). For details, see Table 1.

3.4. Application of the method

The method was applied first for the determination of six-blinded milk samples spiked with concentrations unknown to the analyst. The injections were made in triplicate and the accuracy and precision data are given in Table 4.

The validated method has been successfully applied to monitor the SMX and TMP residues in milk samples from cows receiving a combination of SMX–TMP. The results are presented in Table 5. No SMX and TMP residues were found in milk at 36 h after administration. In both cases, no manual sample pre-treatment (except for centrifugation) was required. Typical chromatograms of SMX and TMP in whole and raw milk are shown in Fig. 5.

4. Conclusions

A bidimensional HPLC method has been developed for the rapid and precise determination of SMX and TMP in bovine milk defatted by centrifugation (15,000 × *g* at 20 °C for 15 min). The simplicity of the procedure, the short analysis time (32 min) and, high sensitivities make the use of RAM columns particularly attractive for the direct injection of reduced-fat milk samples. This method was sensitive, accurate and precise. The results described in this paper showed that this procedure was successfully applied to the measurement of residue levels of SMX and TMP in different milk samples, and with that it was possible to establish the withdrawal time after intramuscular and subcutaneous administrations of a SMX–TMP combination. The

octyl-RAM BSA column had excellent lifetime and at least 200 milk samples (about 30 ml) were analyzed without evidence of changes in its performance.

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