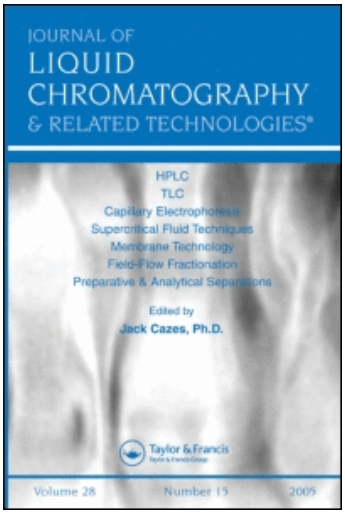

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Samanidou, Victoria F. , Tolika, Evanthia P. and Papadoyannis, Ioannis N.(2008) 'Development and Validation of an HPLC Confirmatory Method for the Residue Analysis of Four Sulphonamides in Cow's Milk According to the European Union Decision 2002/657/EC', Journal of Liquid Chromatography & Related Technologies, 31: 9, 1358 — 1372
To link to this Article: DOI: 10.1080/10826070802019947
URL: http://dx.doi.org/10.1080/10826070802019947

Journal of Liquid Chromatography & Related Technologies®, 31: 1358–1372, 2008

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802019947

Development and Validation of an HPLC Confirmatory Method for the Residue Analysis of Four Sulphonamides in Cow's Milk According to the European Union Decision 2002/657/EC

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Abstract: In the present study, an HPLC method was developed and validated for the determination of four sulphonamides: sulphadiazine (SDZ), sulphaquinoxaline (SQX), suplhamethazine (SMTH), and sulphadimethoxine (SDM). Two of them, SQX and SDM, were determined in cow's milk.

The analytical column, a Kromasil, C_{18} 5 μm, 250 × 4 mm analytical column, was operated at ambient temperature. The mobile phase, a mixture of 0.5% acetic acid as solvent A, CH_3CN as solvent B, and CH_3OH was delivered to the analytical column according to a gradient program. PDA detection was performed for the detection and confirmation of separated analytes with monitoring at 260 nm.

Method validation was performed by means of intra-day (n = 5) and inter-day accuracy and precision (n = 5), sensitivity, and linearity. Limits of detection (LOD) and limits of quantification (LOQ) were 13 and 40 μg/kg, respectively.

Solid phase extraction was applied to remove all matrix interference from milk samples after deproteinization with 8 M HCl. High extraction recoveries (>84%) were achieved using DSC-18 cartridges with CH_3OH-0.5% CH_3COOH as eluent. CCα values were 111.8 and 117.1 μg/kg for SDM and SQX, respectively, and CCβ values were 116.6 and 134.0 μg/kg, respectively. The method was applied to the

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analysis of twenty two milk samples from the local market. SQX was identified in seven of these samples.

Keywords: Sulphonamides, Validation, Milk, HPLC, SPE, 2002/657/EC

INTRODUCTION

Sulphonamides (SAs) are a group of synthetic antibacterial agents widely used in veterinary practice for the treatment of infections and growth promotion of food producing animals. There is a risk of SA residues remaining in animal products if these drugs have been improperly administered or if the withdrawal period has not been observed. Improper use of these sulphonamides in lactary cows has caused, for example, the presence of sulphonamide residues in milk, so that monitoring of such residues in products designated for human consumption and in slaughtered animals has become one of the most important duties for public health agencies. Drug residues may cause allergic or toxic reactions to consumers and promote occurrence of antibiotic resistant bacteria.[1–7]

It has been reported that sulphamethazine produces tumours in rodent bioassay and some evidence on the toxicity of sulphonamides on the thyroid gland has been presented.[8–10] To protect the consumers' health, the European Union has adopted for SAs a maximum residue level (MRL) of 100 μg/kg in the foodstuffs of animal origin.[11] The substances with MRLs (permitted) are contained in group B of Annex I of Council Directive 96/23/EC.[12] Recently, the European Union (EU) has issued a specific regulation decision (2002/657/EC) concerning the performance of methods and the interpretation of results in the official control of residues in products of animal origin.[13] Some new parameters must be calculated as to the limit of decision (CCα) and detection capability (CCβ).[14]

Daily milk consumption is expected to be greater than that of meat and meat products, particularly in infants.[15] Therefore, there is a need for a rapid, sensitive, and selective method for monitoring their residual concentration in milk.[16]

The present work describes a simple, selective, and reliable method for the simultaneous determination and confirmation of four sulphonamides: sulphadiazine (SDZ), sulphaquinoxaline (SQX), suplhamethazine (SMTH), and sulphadimethoxine (SDM). Their chemical structures are shown in Figure 1. Two of them (SDM and SQX) were determined in milk. The extraction of analytes from the matrix is performed with HCl 8 M as a protein precipitation agent and is followed by a simple SPE procedure, using a C_{18} sorbent and a mixture of methanol and acetic acid as eluent. Determination of analytes is then performed by HPLC–diode-array detection. Validation of the method was based on the Commission Decision 2002/657/EC.

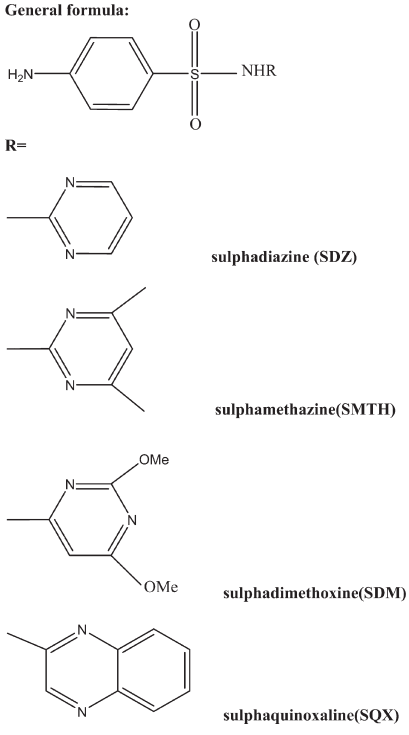

Figure 1. Chemical structures of examined sulphonamides.

EXPERIMENTAL

Chemicals and Reagents

SDZ, SMTH, SDM, and SQX were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were obtained from Carlo Erba (Milano, Italy). Hydrochloric acid, acetic acid, and oxalic acid of analytical grade were supplied by Riedel-de-Haen (Seezle, Germany). Trichloroacetic acid and trifluoroacetic acid 99% were obtained from Aldrich (Steinheim, Germany). High purity water, obtained by a Milli-Q purification system (Millipore, Bedford, MA, USA), was used throughout the study. Milk samples were supplied from local markets.

Instrumentation

A Shimadzu (Kyoto, Japan) quaternary low pressure gradient system was used for chromatographic determination of sulphonamides. The solvent lines were

mixed in an FCV-9AL mixer and an LC-9A pump was used to deliver the mobile phase to the analytical column. Sample injection was performed by an SIL-9A autosampler and detection was achieved by an SPD- M6A Photodiode Array Detector coupled with Data acquisition software Class-M10A. Degassing of solvents was achieved by continuous helium sparking in the solvent flasks through a DGU-2A degassing unit.

The analytical column, a Kromasil C_{18}, 5 μm, 250 × 4 mm, was purchased from MZ-Analysentechnik (Mainz, Germany).

A glass vacuum filtration apparatus obtained from Alltech Associates was employed for the filtration of the buffer solution, using Whatman cellulose-nitrate 0.2 μm membrane filters (Whatman Laboratory Division, Maidstone, England).

A Glasscol small vortexer (Terre Haute, IN, USA) and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the pretreatment of milk samples.

The SPE study was performed on a Vac-Elut vacuum manifold column processor, purchased from Analytichem International, Varian (Harbor City, CA, USA). Nexus cartridges (30 mg/1 cm^3) were supplied from Varian, Discovery DSC-18 (500 mg/3 mL) from Supelco (Bellefonte, PA, USA), and Lichrolut (200 mg/3 mL) from Merck. All evaporations were performed with a Supelco 6-port Mini-Vap concentrator/evaporator (Bellefonte, PA, USA).

Chromatography

The separation of the examined SAs was achieved on a Kromasil, C_{18} 5 μm, 250 × 4 mm analytical column, operated at ambient temperature, with observed backpressure ranging from 240 to 270 kg/cm^2. The mobile phase, a mixture of 0.5% acetic acid as solvent A, CH_3CN as solvent B, and CH_3OH was delivered to the analytical column according to a gradient program, shown in Table 1. The flow rate was 0.8 mL/min. An equilibration time of 8 min was required between runs. The monitoring of the examined SAs was performed at 260 nm.

Preparation of Standards

Stock standard solutions of each SA were prepared in methanol at a concentration of 100 ng/μL. These solutions were found to be stable for three months, when stored refrigerated. Working methanolic standards were prepared from stocks by the appropriate dilution at 0.5, 1, 2, 3, 5, 10, and 20 ng/μL and found to be stable for at least one month.

A 20 μL aliquot was injected onto the column and quantitative analysis was based on peak area measurements as ratios toward the peak area of internal standard.

Table 1. Gradient program for the elution of the examined SAs

t (min)	A: CH_3COOH 0.5%	B: CH_3CN	C: CH_3OH
0	65	5	30
5	50	5	45
12	50	5	45
15	30	5	60

Method Validation According to European Commission Decision 2002/657/EC

The proposed method was validated according to the European Commission Decision 2002/657/EC using spiked milk samples, since certified reference materials (CRM) for SAs in milk were not available. From the performance characteristics enacted by EU, linearity, accuracy, precision, sensitivity, and stability were examined using spiked milk samples at various concentrations.

The linearity response of SAs was first studied using standard solutions, using ten working standards injected three times, covering the entire working range of 0.5–20 ng/μL. In milk samples, linearity response was examined by fortifying matrix samples with a series of mixed standards of the examined SAs, covering a broad range from 40 to 200 μg/kg. Calibration curves were constructed with these samples, injected three times, using analyte/internal standard peak area ratio. The calculations for the limits of detection (LODs) were based on the standard deviation of y-intercepts of regression analysis (σ) and the slope (S), using the following equation $LOD = 3.3\ \sigma/S$. Limits of quantitation (LOQs) were calculated by the equation $LOQ = 10\ \sigma/S$. Precision and accuracy, expressed in terms of SAs recovery from milk samples, was studied by analyzing spiked samples at three concentration levels (40, 60, and 100 μg/kg). Intra-assay precision was studied by five replicate measurements at these concentration levels, while inter-assay precision was conducted during routine operation of the system over the period of five consecutive days. Recovery was calculated as the percentage of the found mass of the analyte on the spiked sample toward the mass that was initially spiked, and was tested after replicate analysis of spiked samples in various concentrations.

The decision limit, CC*a*, was calculated as the mean measured concentration at the MRL (100 μg/kg tissue) plus 1.64 times the SD of within-day precision at this concentration. The detection capability, CC*b*, was calculated as CC*a* plus 1.64 times the SD of within-day repeatability at CC*a*. Statistical analysis was performed at the 95% confidential level and the number of replicate analyses was 20.

Peak identification was performed by spectral information provided by the diode array detector.

The selectivity of the procedure as interference from endogenous compounds was investigated by the analysis of ten different blank milk samples. For monitoring the stability of the examined SAs in standard solutions, stock solutions of the examined SAs and a mixture of a working solution were stored at $+4^{\circ}C$ in the dark, after analyzing one fresh aliquot.

Sample Preparation

Isolation from Matrix

Aliquots of 1 mL from homogenized and pasteurized milk were placed into 10 mL centrifuge tubes. Fortified samples were prepared by adding 200 μL of SAs standard working solutions at different concentration levels (0.5, 1, 2, 3, 5, 10 ng/μL), to the milk samples that were subsequently homogenized in a vortexer for 2 min, and 3 mL of hydrochloric acid 8 M was added. The mixtures were then centrifuged at 9000 rpm for 10 min and the supernatants were removed.

To optimize extraction of SAs from milk samples, a variety of protein precipitation solvents, such as oxalic acid, trichloroacetic acid, trifluoroacetic acid, H_2SO_4, CH_3CN were tested. For the purification step, the supernatants were applied to preconditioned SPE cartridges after filtration through 0.2 μm Whatman filter papers. The optimized SPE protocol was subsequently applied to isolate SAs from the samples.

Clean up Procedure by SPE

Solid phase extraction was used as a purification step after extraction of SAs from milk. Three different sorbents were tested, Abselut Nexus by Varian, Discovery by Supelco, and Lichrolut by Merck. Cartridges were preconditioned by flushing 2 mL of methanol and 2 mL of water. Then, the supernatants were applied by allowing them to pass through the bed without suction. A mixture of CH_3OH/CH_3COOH 0.5% (50:50 v/v) was chosen as the eluting solvent. The samples were subsequently evaporated to dryness under a nitrogen stream in a water bath at $35^{\circ}C$ and the residues were dissolved in 100 μL of methanol. Aliquots of 20 μL of the resulting sample were injected into the HPLC system.

RESULTS AND DISCUSSION

Chromatography

The multi step gradient elution yielded optimum separation of the four studied SAs within 20 min. Retention times of the examined analytes were 5.400 for

SDZ, 7.426 for SMTH, 15.092 for SDM, and 16.079 for SQX. A typical chromatogram is shown in Figure 2. Under the conditions described above, two of the studied SAs (SDM and SQX) were well resolved while the other two (SDZ and SMTH) eluted at the same time as the endogenous compounds. Resolution factors (R_s) were calculated according to the formula: $R_s = 2(t_2 - t_1)/(t_{w1} + t_{w2})$, where t_1 and t_2 are the retention times and t_{w1} and t_{w2} the baseline peak widths of successive peaks. Resolution factors were 1.4 for SDZ-SMTH, 6.5 for SMTH-SDM, and 1.0 for SDM-SQX.

Due to a deterioration of the column, the retention times in milk samples were slightly higher:18.216 min for SDM and 19.630 min for SQX (Figure 3).

Sample Preparation

For the precipitation of milk proteins oxalic acid, trichloroacetic acid, trifluoroacetic acid, H_2SO_4, CH_3CN, HCl, acetone, and CH_3CN were examined. As already mentioned after extraction of SAs, an extra purification step with SPE was necessary. Optimum extraction includes CH_3OH/CH_3COOH 0.5% (50:50 v/v) as the eluting solvent. To avoid the presence of unknown peaks at chromatograms of blank milk samples, three different cartridges were studied: Nexus, Discovery, and Lichrolut. Results are presented in Table 2.

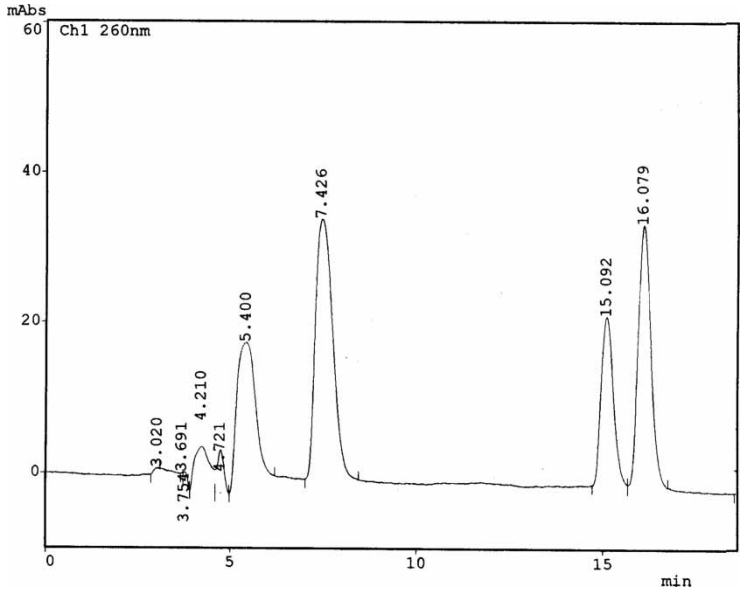

Figure 2. High performance liquid chromatogram of standard solutions of sulphonamides. Peaks: 5.400 min (SDZ), 7.426 min (SMTH), 15.092 min (SDM), 16.079 min (SQX).

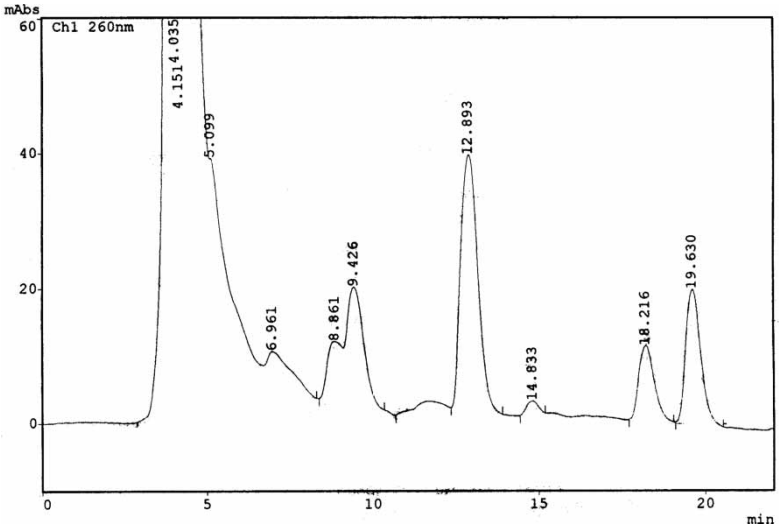

Figure 3. High performance liquid chromatogram of sulphonamides in spiked milk sample. Peaks: 18.216 min (SDM), 19.630 min (SQX).

As shown from the results summarized in Table 3, optimum results are obtained by using HCL 8 M as the precipitation agent.

Method Validation According to 2002/657/EC

The described method was fully validated according to 2002/657/EC guidelines. The results of figures of merit that were investigated are described in the following paragraphs.

Table 2. Recoveries of the examined SAs after solid-phase extraction in extracts using various SPE protocols

		Recovery (%)			
SPE sorbent	Eluent	SDZ	SMTH	SDM	SQX
Nexus	CH_3CN-CH_3COOH 0.5%	33.3	28.6	25.7	23.6
Nexus	CH_3CN	49.4	11.5	22.8	22.3
Lichrolut	CH_3CN	34.9	58.7	53.7	52.2
Lichrolut	CH_3CN-CH_3COOH 0.5%	38.7	37.6	29.4	24
DSC-18	CH_3CN-CH_3COOH 0.5%	44.5	44.5	38.3	33.9
DSC-18	CH_3CN	47.8	40.5	36.7	34.6
DSC-18	CH_3OH	68	67	60.7	55.3
DSC-18	CH_3OH-CH_3COOH 0.5%	100.7	105.8	92.17	84.4

Table 3. Recoveries of the examined SAs using various deproteinization/extraction media

	Recovery (%)	
Deproteinization extraction media	SDM	SQX
25% TCA	54	<10
CH_3CN	24	<10
Oxalic acid 7.5%	37	22
25% TFA	<10	<10
HCl 2M	56	38
HCl 6M	60	43
HCl 8M	88	52

Linearity and Sensitivity

Calibration curves constructed both for standard solutions and for milk samples after appropriate pre-treatment, were obtained by least squares linear regression analysis of the peak area ratio of analyte to internal standard versus analyte injected amount. The method was linear up to 200 μg/kg for all SAs in both standard solutions and milk samples. All calibration data as well as LOD and LOQ are presented in Table 4. Regression equations revealed good correlation coefficients ranging between 0.996 and 0.998 over the examined range.

Selectivity

The application of the whole procedure to ten blank milk samples in order to verify the method selectivity demonstrates that some interference was detected at the retention times of SDZ and SMTH.

Precision and Accuracy

The precision of the method based on within-day repeatability was assessed by replicate (n = 5) measurements from three spiked milk samples at 40, 60, and 100 μg/kg. Relative recovery rates from the spiked samples were determined at three different concentrations by comparing the peak area ratios for extracted SAs and the values derived from the respective calibration curve.

The between-day precision of the method was established using milk samples at the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of five consecutive days. RSD values for all the examined SAs were between 3.4% and 10.5%, while relative recovery rates were for greater than 99%.

Table 4. Calibration and sensitivity data of the examined SAs in standard solutions

Analyte	Slope (ng^{-1})	Intercept	Correlation coefficient	LOD	LOQ	Upper limit
Standards				(ng)	(ng)	(ng/μL)
SDZ	4736.1 ± 227.6	85376.28 ± 24004.65	0.996	16.7	50.2	20
SMTH	8959.0 ± 393.7	−5457.1 ± 37934.18	0.996	14.0	42.0	15
SDM	4659.6 ± 199.3	−7008.96 ± 19199.90	0.996	13.6	40.8	20
SQX	7650.0 ± 325.5	−13771.2 ± 31366.44	0.996	13.5	40.5	20
Milk				(ng)μg/kg	(ng)μg/kg	μg/kg
SDM	1855.4 ± 85.8	−5391.0 ± 7365.9	0.997	13.2	39.7	200
SQX	3385.1 ± 131.9	−33972.5 ± 13608.1	0.998	13.4	40.2	200

Table 5. Within and between-day precision and accuracy of the developed method for the determination of SAs in milk samples after solid-phase extraction

		Within-day (n = 5)			Between-day (n = 5)		
Analyte	Added (μg/kg)	Measured ±SD (μg/kg milk)	RSD	Recovery (%)	Measured ±SD (μg/kg milk)	RSD	Recovery (%)
SDM	40	44.92 ± 2.44	5.4	112.3	44.49 ± 3.37	7.6	111.2
	60	62.94 ± 2.95	4.7	104.9	59.55 ± 5.58	9.4	99.2
	100	99.95 ± 3.4	3.4	99.9	104.15 ± 8.29	8.0	104.2
SQX	40	48.40 ± 2.5	5.2	121.0	49.19 ± 5.17	10.5	123.0
	60	68.42 ± 3.1	4.5	114.0	61.46 ± 5.89	9.6	102.4
	100	101.79 ± 6.9	6.8	101.8	104.47 ± 10.09	9.7	104.5

Table 6. Calculating errors a and b as well as the limit of decision (CC_a) and capability of detection (CC_b) at the MRL enacted by the EU at 100 μg/kg milk

Analytes	Added (μg/kg milk)	Measured ±SD (μg/kg milk)	RSD	Recovery (%)	Error α (1.64*SD)	CC_α (μg/kg)
SDM	100	111.2 ± 7.2	6.5	111.2	11.8	111.8
SQX	100	92.5 ± 10.4	11.2	92.5	17.1	117.1
Analytes	Added (μg/kg milk)	Measured ±SD (μg/kg milk)	RSD	Recovery (%)	Error β (1.64*SD)	CC_β (μg/kg)
SDM	111.8	121.9 ± 2.9	2.4	109.0	4.8	116.6
SQX	117.1	124.1 ± 10.3	8.3	106.0	16.9	134.0

The results of the within-day repeatability and the between-day precision of the method applied to fortified matrix samples are illustrated in Table 5.

Decision Limit and Detection Capability

In compliance with the 2002/657/EC decision, the validation procedure includes the determination of two novel criteria CC*a* (limit of decision) and CC*b* (capability of detection), defined respectively as "the limit at and above which it can be concluded with an error probability of *a*, that a sample is non-compliant" (greater than the MRL for group B substances), and "the smallest content of the substance that may be detected, identified,

Table 7. Results of the analysis of various commercial milk samples

Milk sample	SQX (μg/kg)	Similarity factor
Full-cream pasteurized (3.5%)		
1a	5.6	0.9775
2	N.D.	
3	N.D.	
Low-fat pasteurized (1.5%)		
1b	N.D.	
Skimmed pasteurized (0.0%)		
1c	N.D.	
4a	N.D.	
Full-cream evaporated (7.5%)		
5a	39.7	0.9902
5b	6.2	0.9149
6a	3.8	0.9909
6b	N.D.	
7a	3.0	0.9533
7b	N.D.	
7c	13.8	0.9149
Reduced-fat evaporated (1.5%)		
7d	28.8	0.9886
7e	N.D.	
High pasteurized full-cream (3.5%)		
8	N.D.	
9a	N.D.	
9b	N.D.	
7f	N.D.	
High pasteurized low-fat (1.5%)		
9c	N.D.	
Ultra high temperature treated (1.5%)		
(10	N.D.	
Ultra high temperature treated (0.3%)		
4b	N.D.	

and/or quantified in a sample with an error probability of *b*." For group B substances, errors must be $<5\%$.

The CC*a* values calculated by spiking 20 blank milk samples at MRL (100 μg/kg) were 111.8 μg/kg for SDM and 117.1 μg/kg for SQX. CC*b* values calculated by analyzing 20 blank spiked samples at corresponding CC*a* levels for each analyte were 116.6 μg/kg and 134.0 μg/kg. Results are presented in Table 6.

Eight different types of milk samples, purchased from the local market were analyzed following the proposed method in order to investigate the presence of the examined SAs. Milk samples included full cream pasteurized (3.5%), low fat pasteurized (1.5%), skimmed pasteurized (0.0%), full cream evaporated (7.5%), reduced fat evaporated (1.5%), high pasteurized full cream (3.5%), high pasteurized low fat (1.5%), and ultra high temperature treated (1.5%) and (0.3%). The results are presented in Table 7. Identification of antibiotics was performed by means of a PDA detector. The similarity factors were greater than 0.9149.

CONCLUSIONS

The herein described confirmatory method is a simple validated assay, which can be readily adapted by any laboratory for the quality control and the quantitative determination of residues of the four examined sulphonamides in milk. Validation was performed according to the European Union regulation 2002/657/EC for the validation of an analytical method for residues in animal products.

The results of the validation process demonstrate that the method is suitable for application in European Union statutory veterinary drug residue surveillance programmes.

The four investigated SAs were resolved within 20 min. LOQ values achieved were matching to MRL values.

The CC*a* values calculated by spiking 20 blank milk samples at MRL (100 μg/kg) were 111.8 μg/kg for SDM and 117.1 μg/kg for SQX. CC*b* values calculated by analyzing 20 blank spiked samples at corresponding CC*a* levels for each analyte were 116.6 μg/kg and 134.0 μg/kg.

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Received December 3, 2007
Accepted December 26, 2007
Manuscript 6245