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Multiclass analysis of antibacterial residues in milk using RP-liquid chromatography with photodiode array and fluorescence detection and tandem mass spectrometer confirmation

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ABSTRACT

A simplified procedure for simultaneous quantification of ceftiofur (CEF), fluoroquinolone (FQ) and sulfonamide (SA) antibacterials in bovine milk was developed. The reverse-phase liquid chromatography (RP-LC) multiclass method for analysis of eleven distinct compounds, from three antibacterial classes, was validated in line with Commission Decision 2002/657/EC. Confirmation of the analytes identities was performed by electrospray mass spectrometry detection. The analytes were extracted from milk matrix by liquid–liquid extraction with acidified ultrapure water and directly analyzed in the chromatograph. The SA compounds were pre-column derivatized with fluorescamine for fluorescence detection. The method provided good results regarding the analytical parameters of linearity, selectivity, sensitivity, precision, recovery, decision limit (CCα), detection capability (CCβ), limit of detection (LOD), limit of quantification (LOQ), stability and robustness. Analytes were extracted by liquid–liquid extraction in the fortified matrix and the compounds identity was confirmed by their precursor ion and fragments through tandem mass spectrometry analysis. Additionally, milk samples from two state capitals in the South Region of Brazil were analyzed by both the quantitative and confirmatory methods. The validation process showed correlation coefficients (r^2) greater than 0.98 for all the analytes, with recovery rates up to 98% for all the studied drugs. LOD and LOQ limits ranged from 8.0 to 20.0 ng mL^{-1} and 10.0 to 32.0 ng mL^{-1}, demonstrating good specificity of the method. The intra-day and inter-day precisions for all the analytes were below or equal to 7.40 and 10.13, respectively. The studied antibacterials were not detected in milk samples. The developed method represents an efficient alternative for multi-residue analysis in milk, being suitable and especially viable for monitoring in developing countries.

1. Introduction

The large-scale application of antibacterials is a matter of concern worldwide. Nowadays, antibacterial medicines are used for the prevention and treatment of infections and for weight gain in cattle. In dairy practice the misuse of these drugs, such as improper observance of withdrawal periods, raises the risk of their residues being present in milk. Antibacterial residues can represent a human health hazard and also cause technological problems in the dairy industry [1].

In Brazil, veterinary drugs such as the fluoroquinolones, sulfonamides and cephalosporins, mainly ceftiofur, are especially applied in dairy cattle for mastitis treatment due to the broad-spectrum activity and low costs of these medicines. Sulfonamides (SAs) are synthetic agents approved for respiratory or gastro-intestinal infections and mastitis in cattle. Ceftiofur (CEF) is an antibiotic of the cephalosporin group, primarily applied for the treatment of clinical mastitis in lactating cows caused by *Escherichia coli* and *Staphylococcus* species. Moreover, the allergenic potential of the β-lactam ring structure is well known. Fluoroquinolones (FQs) are a large synthetic group of antibacterials derived from nalidixic acid, used in cattle for the treatment of respiratory and urinary infections [2].

Residues of these drugs remain in milk and are potentially harmful. In order to minimize the risk to human health, the *Codex Alimentarius* and European Union Legislation have established the safe maximum residue limits (MRLs) for veterinary drugs in

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animal products such as milk [3]. Brazil has an extensive dairy industry, with an estimated yearly production of 30 million liters, mainly for national consumption [4].

The control of veterinary residues in the food chain is one of the main tasks in monitoring programs, and several confirmatory techniques have been used for the determination of CEF, FQs and SAs in milk, notably high performance liquid chromatography (HPLC) coupled to diode array detection (DAD) [5–8], fluorescence detection (FD) [9–11], mass spectrometry (MS) [12], and tandem mass spectrometry (MS/MS) [13–16]. Liquid chromatography coupled to all of these detection techniques has been applied to FQ determination in milk [17]. However, few methods report the analysis of antibacterial residues in Brazilian milk, whereas microbiological and chromatographic analysis of teracyclines and β-lactams are mainly described [18,19].

Major research efforts have been focused on multi-residue chromatographic analysis for the determination of veterinary drugs. In this context, multiclass methodologies are particularly useful. Such methodologies are usually described in the literature for the determination of compounds of a single class of antibacterials in different matrices including foodstuffs [20,21], environmental water [22], soils [23] and biological fluids [24], whereas only a few studies have applied multiclass analysis in milk [25–28]. In these studies, the extraction procedure is generally laborious or time consuming, and expensive equipment is often necessary. Notwithstanding, cost-efficiency is a key point in monitoring programs. Although several chromatographic methods for the quantitative analysis of antibacterial residues in milk are available, most of them lack practicality and economical feasibility.

In this study, a simplified and low-cost procedure for the determination of eleven compounds from three different antibacterial classes (cephalosporin, sulfonamide and fluoroquinolone) was developed and applied. Considering the multi-residue extraction, the results indicate that this achievement represents a novel, effective, less toxic and economically feasible alternative to monitor milk quality in developing countries.

The FQs norfloxacin, ciprofloxacin, enrofloxacin, and danofloxacin, the SAs sulfadoxine, sulfadimethoxine, sulfamerazine, sulfamethoxazole, sulfachlorpyridazine, and sulfathiazole, and CEF were analyzed by LC coupled with FD and DAD. The Brazilian National Monitoring Plan for residue determination in food matrices assumes the MRLs for veterinary drugs in milk set by the European Community. The MRL for SA residues in milk is established at 100 $\mu g\,kg^{-1}$ for each SA compound or the sum of the SA residues. The MRL for CEF, a cephalosporin compound, is set at 100 $\mu g\,kg^{-1}$. The MRL for most of the FQs, such as enrofloxacin, ciprofloxacin and norfloxacin, is also set at 100 $\mu g\,kg^{-1}$, with the exception of danofloxacin which is 30 $\mu g\,kg^{-1}$ [29]. These agents comprising three antibacterial classes were especially chosen for method development to meet demand of the Brazilian National Monitoring Plan for residue determination in milk, considering the large applicability of the selected drugs in dairy practice in the country. As for the cephalosporin agents, ceftiofur (CEF) was selectively chosen considering that no withdrawal period is established after treatment with CEF.

The method was validated according to the EU regulation 2002/657/EC [30], and the analyte identities were confirmed by electrospray tandem mass spectrometry analysis. Additionally, milk samples purchased on the Brazilian market were analyzed to verify the method performance.

2. Experimental

2.1. Chemicals and reagents

Standards of the FQs norfloxacin (NOR, 99.0%), ciprofloxacin (CIP, 99.0%), enrofloxacin (ENR, 99.0%), danofloxacin (DAN, 99.0%) were obtained from Fluka (Steinheim, Germany). Ceftiofur (CEF) and the SA standards sulfadoxine (SDX), sulfadimethoxine (SDMX), sulfamerazine (SMR), sulfamethoxazole (SMA), sulfathiazole (STZ), and sulfachlorpyridazine (SCP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The purity of all compounds was greater than 99.0%. The internal standards lomefloxacin (LOM, 99.0%) and sulfapyridine (SPY, 99.0%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fluorescamine (98.0%) was obtained from Sigma–Aldrich (St. Louis, MO, USA). All reagents were of analytical grade unless otherwise indicated. Acetic, formic, and trichloroacetic (TCA) acids were supplied by Merck (Darmstadt, Germany). Acetonitrile (MeCN) and methanol (MeOH) were of HPLC grade and were purchased from Merck (Darmstadt, Germany). Ultrapure water was generated by a Milli-Q Millipore system (Massachusetts, USA).

2.2. Standard and stock solutions

The stock standard solutions of SAs were prepared by precise weighing of the substances and then dissolving them in acetonitrile. The FQ and CEF stock standards were prepared in methanol. All stock solutions of the individual substances at a concentration of 1 $mg\,mL^{-1}$ were stored at $-12\,^{\circ}C$ for no longer than sixty days. Working solutions of the analytes CEF, ENR, CIP, DAN, NOR, SMR, STZ, SMA, SCP, SDMX and SDX, at a concentration of 1 $\mu g\,mL^{-1}$, were prepared daily by diluting aliquots of the stock solutions in ultrapure water. The internal standard solutions of SPY and LOM at a concentration of 0.1 $\mu g\,mL^{-1}$ were prepared separately by diluting aliquots of the stock solutions in ultrapure water. For the CEF analysis, matrix-based standards were prepared by extracting 1 g samples of milk (blank) according to the procedure described in the following section for the method validation procedure.

2.3. Milk samples

Pasteurized bovine milk was obtained from local markets. For validation purposes, milk samples were previously analyzed to verify the absence of the studied analytes. The validation process was carried out with processed milk sample in order to suit a control measure in food quality preventing human health hazards arising from commercial milk.

2.4. Equipment and chromatographic conditions

2.4.1. LC-FD/DAD quantitative analysis

The chromatographic system consisted of a Shimadzu HPLC chromatograph (Kyoto, Japan) equipped with a quaternary pump, on-line degasser, column heater, and diode array and fluorescence detectors connected on-line. Analytical separation was carried out on a Shim-pack CLC-ODS end-capped RP-column (250 × 4.6 mm, 5 μm), at room temperature, fitted with a G-ODS end-capped guard column (10 × 4 mm) of the same packing material, both manufactured by Shimadzu (Kyoto, Japan). A Biomixer QL-901 vortex mixer (Sao Paulo, Brazil) was used to mix and homogenize the milk samples during the sample treatment and fortification steps. A Janetzki K24 centrifuge (Engelsdorf, Germany) was employed. The pH of the mobile phase was verified on a Mettler Toledo MP 220 pH meter (Greifensee, Switzerland).

The UV detection was performed on a diode array detector (DAD) at the maximum wavelength of ceftiofur (280 nm). Programmed fluorescence detection was performed at the maximum excitation/emission wavelength for the FQs (280/440 nm) and SAs (405/495 nm). A gradient elution program was used with the mobile phase combining solvent A (ultrapure water 2% acetic acid (pH 3.0), and solvent B (acetonitrile) as follows: 10% B (1 min), 10–21.4% B (11 min), 40% B (14.5 min), 45.1% B (17 min), 51.1% B

(23 min), 51.9% B (28 min), 70% B (30 min), 100% B (31 min), 100% B (36 min), and 10% B (40 min). The initial condition was maintained for 5 min for reconditioning of the column. The mobile phase flow was set at 1.0 mL min^{-1} and the volume of injection was 20 μL. All data were processed using Shimadzu LC solutions software version 1.21 (Kyoto, Japan).

2.4.2. LC-MS/MS confirmatory analysis

Confirmatory analysis was carried out on an Alliance HT 2795 Separations Module HPLC system manufactured by Waters (Milford, USA), equipped with a Symmetry C-18 analytical column (75 × 4.6 mm, 3.5 μm) guarded by a Symmetry pre-column (10 × 2.1 mm, 3.5 μm) of the same packing material, interfaced to a Micromass Quattro micro API tandem quadrupole mass spectrometer system (Manchester, UK) via an electrospray probe.

A Biomixer QL-901 vortex mixer (Brazil) and an ultracentrifuge with Sigma 1–15 PK cooling system (Osterode, Germany) were also employed. The pH of the mobile phase was verified with a Mettler Toledo MP 220 pH meter (Greifensee, Switzerland).

The initial mobile phase consisted of 98% (A) ultrapure water with 0.1% formic acid and 2% (B) acetonitrile with 0.1% formic acid. Separation was performed through a 20 min gradient elution program, with 3 min for reconditioning of the column, as follows: 2% B (1 min), 2–98% B (15 min), 98% B (17 min), 98–2% B (20 min). The flow rate was set at 0.4 mL min^{-1} and the injection volume was 20 μL.

The MS/MS system was operated in positive electrospray ionization (ESI) mode using nitrogen gas as the nebulization and desolvation gas, and high purity argon gas as the collision gas. The nebulizer gas flow rate was set at the maximum of 90 L h^{-1} while the desolvation gas flow rate was set at 800 L h^{-1}. The desolvation gas and ion source block temperatures were maintained at 350 and 120 °C, respectively. The capillary voltage was set at 3 kV. Confirmatory analysis was performed using multiple reaction monitoring (MRM). A precursor-product ion was chosen for each compound, with two transition ions monitored for qualitative and confirmatory purposes, respectively. The cone voltage and collision energy were optimized for all MRM transitions, each of which had a dwell time of 0.1 s. The ESI-MS/MS conditions were optimized by replicate infusions of each compound individually at a concentration of 1 μg mL^{-1} in acetonitrile. The optimized ESI-MS/MS parameters are shown in Table 1. For data processing, Masslynx software version 4.1 produced by Micromass (Manchester, UK) was used.

2.5. Sample preparation

Aliquots of 1.0 g of pasteurized milk were weighed into a polypropylene tube and fortified with the analytes at the MRL concentrations for the method validation. The spiked samples were stirred for 30 s and allowed to stand for 20 min for analyte-matrix equilibration. After the addition of 2 mL of ultrapure water with 10% TCA, the mixtures were shaken on a vortex mixer for 30 s and transferred to centrifuge glass tubes. The sample tubes were washed with 3 × 0.5 mL of ultrapure water and added to the extracts. The extracts were centrifuged for 40 min at room temperature (25 °C), at 1000 × *g*. The upper phase was transferred to new centrifuge glass tubes for further centrifugation, under the same conditions, in order to suppress interference originating from the protein content. No evaporation step was applied. The supernatants were collected in polypropylene syringes for filtration through a 0.45 μm PTFE membrane, and transferred to glass tubes prior to the derivatization step.

Table 1
Multiple reaction monitoring parameters for the LC-MS/MS analysis. Dwell time of 0.1 s for all MRM transitions.

Compound	M.W. (g mol^{-1})	Precursor ion $[M+H]^+$	Qualitative *m/z* transition	Confirmatory *m/z* transition	Cone voltage (V)
NOR	319.3	320.2	302.2 (17)[a]	276.2 (25)	40
CIP	331.3	332.2	288.2 (17)	245.2 (25)	40
ENR	359.3	360.3	316.2 (25)	245.2 (25)	45
STZ	255.3	256.0	155.9 (15)	107.8 (28)	30
SMR	264.3	265.2	107.7 (15)	156.0 (30)	35
SCP	284.7	285.0	155.9 (25)	107.7 (15)	30
SDX	310.3	311.3	107.7 (18)	140.0 (20)	30
SDMX	310.3	311.0	155.9 (20)	107.8 (25)	35
SMA	253.3	254.0	155.9 (15)	91.6 (25)	30

[a] Collision energy (eV).

2.5.1. Derivatization procedure

To aliquots (1.0 mL) of the extracts containing all analytes, 100 μL of fluorescamine solution with 0.1% in acetone was added for derivatization of the SAs. The mixture was stirred on a vortex mixer for 30 s, and allowed to stand at room temperature (25 °C) for 44 min for the derivatization reaction. Subsequently, 20 μL were injected into the LC-FD system.

2.5.2. Liquid–liquid extraction for LC-MS/MS

To a 1.5 mL Eppendorf flask, 500 μL of pasteurized milk sample were added and fortified with the analytes at their MRL concentrations. After stirring on a vortex mixer, the samples were allowed to stand for 20 min for analyte-matrix equilibration. A volume of 500 μL of methanol–ultrapure water 70:30 (v/v) with 0.1% formic acid was added for the analyte extraction. The samples were mixed for 30 s on a vortex mixer, and centrifuged at 0 °C for 20 min, at 2200 × *g*. Aliquots (500 μL) of the supernatant were then transferred to injection vials and diluted with 500 μL of the initial mobile phase. A volume of 20 μL was injected for the chromatographic analysis.

2.6. Calibration curves

The calibration curves were prepared by fortifying blank milk samples with the working solutions of the standards in the concentration range of 0–200 ng mL^{-1} for STZ, SMR, SCP, SDX, SMA, SDMX, NOR, CIP, ENR, and CEF; and 0–60 ng mL^{-1} for DAN, corresponding to 0.0, 0.5, 1.0, 1.5 and 2.0 times the MRL for each compound in milk. Quantification of fluoroquinolone and sulfonamide residues was performed by internal standardization whereas for the ceftiofur analysis external standardization and matrix-matched calibration were applied. For the SA and FQ analysis, the internal standards (IS) SPY and LOM were fortified at concentrations of 300 ng mL^{-1}. The curves were prepared on three different days and each concentration was analyzed from six replicates. All data were processed by Shimadzu LC solutions software version 1.21.

2.7. Validation study

The LC-FD/DAD method validation was carried out according to Commission Decision 2002/657/EC, determining the selectivity, dynamic range and linearity, sensitivity, precision, trueness (recovery), decision limit (CCα) and detection capability (CCβ), detection limit (LOD), quantification limit (LOQ), stability and robustness [30].

In the selectivity study, 20 blank samples consisting of pasteurized and ultra-heat-treated (UHT) milk (both whole and skimmed) were analyzed to verify possible matrix interferences at the retention time of the analytes. For the calibration, analytical

curves were constructed based on the dynamic range of each analyte in matrix as five-point calibration curves. Linear regression analysis was applied to obtain the regression coefficients and evaluate the sensitivity and linearity of the method. The statistical acceptability of the calibration data was assessed by analysis of variance.

The precision was verified by spiking blank samples with the analytes at three concentration levels, corresponding to 0.5, 1.0, and 1.5 times the MRLs, and evaluating the coefficient of variance (CV) of the calculated concentrations. The intra-day precision was determined by analyzing the analyte at each concentration level six times. The procedure was performed on three different days for inter-day precision. Recovery assays were conduced by analysis of spiked samples at the concentration levels of the calibration curve for each analyte. Each concentration was analyzed six times. The analyte recovery and the coefficient of variance were calculated for all concentration levels.

Precision results were used to calculate the decision limit ($CC\alpha$) and detection capability ($CC\beta$), approaching the permitted limit of the substance and the standard deviation obtained for inter-day precision. The LOD values were determined using a signal-to-noise ratio of 3 (ratio between the peak intensity and the baseline noise), through analysis of extracted milk samples spiked with the analytes at eight concentration levels, ranging from 1 to 50 ng mL^{-1}. The LOQ values were established as the lowest concentration levels for which the method was validated with an accuracy and precision that felt within the ranges recommended by the Commission of the European Communities [29,30].

The analyte stability was determined in solution and in the matrix at the MRL concentrations. The stability of the individual stock standard solutions and fortified samples was verified at 8 and -12 °C over 12 weeks. Robustness studies were conducted through variations of analyst and matrix. The assays were performed by two analysts with samples of pasteurized milk and UHT skimmed milk.

Each analyst variation was applied to the analysis of twelve samples, comprising six pasteurized milk samples and six UHT skimmed milk samples. Analytes were added to the milk at their respective permitted limits. The results were evaluated in terms of analyte recoveries.

Matrix effects were evaluated through comparison of the chromatograms of spiked matrix extracts with the chromatograms of standard solutions of the analytes in ultrapure water. The analytical signals were verified at the MRL concentrations and the recoveries were calculated for each compound. The assay was conducted using three spiked samples for each group (matrix and solution), with three replicates per sample. The significance of the results was assessed using the student's *t*-test, by comparison of the means of independent groups. A p value of <0.05 was considered significant.

The LC-MS/MS method was performed to achieve the unambiguous identification of the analytes, through multiple reaction monitoring full-scan mass spectrometry analysis. For the LC-MS/MS confirmatory method, the qualitative $CC\beta$ was assessed by analyzing twenty milk samples fortified with the analytes at their LOQ concentrations using the quantitative method. For analyte confirmation an established point system for identification of the compounds was applied. According to Decision 2002/657/EC, the ion ratio criteria for veterinary drugs must achieve three points [30].

2.8. Analysis of milk samples from the South Region of Brazil

In order to verify the performance of both quantitative and confirmatory methods, bovine milk samples produced and commercialized in the South Region of Brazil were analyzed following the proposed procedures. Liquid and powdered milk, raw, pasteurized and UHT milk, with different fat contents, were analyzed. For the LC-FD/DAD analysis, fifty samples were randomly obtained from local markets in Florianópolis, Santa Catarina State. For the LC-MS/MS analysis, fifty samples were purchased from retail markets in Porto Alegre, Rio Grande do Sul State.

3. Results and discussion

3.1. Aqueous extraction and chromatographic analysis

Residues of basic veterinary drugs in food matrices are usually extracted by pollutant organic solvents such as methanol and acetonitrile. In our work, we first applied methanol and acetonitrile as extraction solvents, as reported in several papers. We then compared the analytical signals (peak intensities) obtained for both aqueous and organic extractions, and no significant increase was observed for the signal intensity of the analytes. Thus, we selected ultrapure water as the extractor solvent and TCA was used for the acidification, this compound generally being used for protein precipitation in milk [9,15,17,25].

The insoluble proteins and the associated lipid content of the pasteurized milk were removed by centrifugation. In order to enhance the selectivity of the fluorescence and diode array detections, an additional centrifugation step was applied to the supernatants. Since no purification step was performed, the supernatants were filtered twice through a 0.45 μm PTFE membrane prior to the chromatographic analysis. This procedure allowed the removal of potential matrix interference in the chromatograms.

For the fluorimetric detection of the SA compounds, a derivatization reaction was required. This step was performed using fluorescamine, a non-fluorescent compound widely applied for the derivatization of primary amines [10,11]. It is relevant to note that when the derivatization reaction was performed with an organic extract, some deformations were observed in the chromatographic peaks of the sulfonamide compounds. Broadened and tailing peaks were observed for all these analytes, and some of the misshaped peaks also showed coelution. On the other hand, when using the aqueous extract for the derivatization step, an increase in the peak resolutions of these compounds was achieved. Therefore, acidified ultrapure water was selected as the extraction solvent. The derivatization step had no effect on the detection of other analytes, as the analytical signal of CEF and FQs showed no variation after reaction with fluorescamine.

The separation of the FQ and SA compounds on the fluorescence chromatogram was carried out preventing overlapping of the analytes peaks. In the array diode chromatogram, the elution gradient was optimized for separation of CEF, SAs and FQs at distinct retention times. The analytes were added to the samples at their MRL concentrations, and after the extraction procedure, the chromatographic signals were evaluated as analyte recovery. For all the extraction solvents, the analyte signals could be detected on both detectors, although no concentration step was applied.

The antibacterial compounds were separated and identified on the chromatograms, and no significant increases in the analyte peaks were observed, regardless of the extraction solvent applied. Thus, good recovery rates were obtained for all of the antibacterials for the ultrapure water extraction, since the cephalosporin compound, the FQs and the SAs were identified and separated by liquid chromatography. The chromatograms for the SAs, FQs and CEF in the milk samples, are presented in Fig. 1.

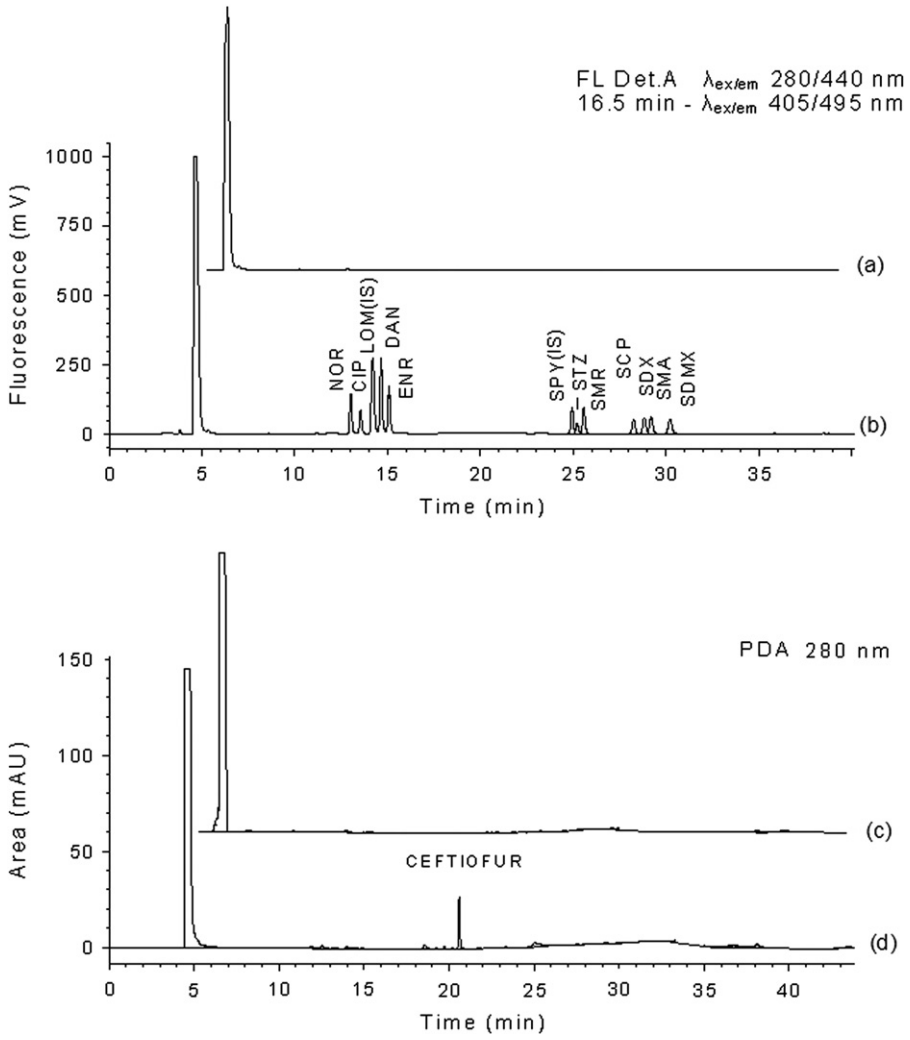

Fig. 1. Chromatograms: (a)–(b) FQs and SAs in milk by LC-FD; (c)–(d) CEF in milk by LC-DAD; (a)–(c) blank milk sample; (b)–(d) fortified milk sample. Fortifications levels: NOR, CIP, ENR, CEF, STZ, SMR, SCP, SDX, SMA, SDMX (100 ng mL^{-1}); DAN (30 ng mL^{-1}); (IS) LOM and SPY (300 ng mL^{-1}).

The use of a high polarity solvent allowed the extraction of eleven residues from three classes of antibacterial agents in the fortified milk samples, at their MRL concentrations. The liquid–liquid extraction by acidified ultrapure water comprises an effective and less toxic procedure. Moreover, the extraction of pharmaceutical drugs applying less toxic solvents in lower quantities and the use of automated extraction techniques are prospective trends for the multi-residue analysis of food matrices.

The use of an organic mobile phase composed of acetonitrile permitted a better analytical separation in comparison with the organic phase composed of methanol, a more polar solvent. The aqueous mobile phase (A) acidified with acetic acid at pH 2.5 showed an increased resolution for the chromatographic peaks. Through minor changes in the solvent strength it was possible to create an elution gradient program for the separation of eleven analytes in a single chromatographic run.

3.2. Validation of the multiclass method

In this study we validated the LC-FD/DAD method in line with the requirements of the European Community. The validation parameters were approached according to Decision 2002/657/EC regarding the performance criteria for analytical methods. The linearity and sensitivity of the method were assessed through the calibration curves. Table 2 summarizes the linear regression analysis of the calibration data.

As shown in Table 2, good correlation coefficients were obtained for all analytical curves, with r^2 values exceeding 0.98 for all compounds. The regression coefficient obtained for the slope was representative of the sensitivity of the method, with low values being obtained for the standard deviation. In the selectivity study, the analysis of twenty milk samples from different origins showed no matrix interferences around the retention times of the analytes, as can be seen by the blank chromatograms in Fig. 1.

The European Decision 2002/657/EC determines that for substances analyzed at a mass fraction of 100 $\mu g\,kg^{-1}$, the reproducibility CV should not exceed 23%. Regarding the trueness of the quantitative methods, the analyte recoveries at mass fraction $\geq 10\,\mu g\,kg^{-1}$ should fall within the range of -20 and $+10$%. Thus, the accuracy of a confirmation method should range from 80 to 110%.

The MRL concentrations for the SAs, FQs and CEF in milk, along with the precision and recovery results are shown in Table 3. According to the 2002/657/EC regulation, the CVs obtained in the precision study and the analyte recovery rates for the LC-FD/DAD method are acceptable for all of the analytes in the milk matrix. The mean recovery was above 98% for all of the antibacterials, and only the enrofloxacin compound showed an overrated recovery of

Table 2
Linear regression analysis for the calibration curves. y=slope x C+intercept (y=analyte peak area/IS peak area; C=analyte concentration).

Compound	Mean ± S.D.; n=3			Working range[a] (ng mL^{-1})
	Slope	Intercept	r^2	
NOR	0.0061 ± 0.0001	0.0115 ± 0.0120	0.994	0–200
CIP	0.0026 ± 0.0002	0.1239 ± 0.0322	0.984	0–200
DAN	0.0281 ± 0.0011	0.0424 ± 0.0232	0.991	0–60
ENR	0.0056 ± 0.0002	0.0559 ± 0.0191	0.995	0–200
STZ	0.0019 ± 0.0000	0.0202 ± 0.0058	0.992	0–200
SMR	0.0069 ± 0.0001	0.0401 ± 0.0164	0.983	0–200
SCP	0.0033 ± 0.0003	0.0903 ± 0.0340	0.988	0–200
SDX	0.0053 ± 0.0001	0.0079 ± 0.0012	0.981	0–200
SMA	0.0057 ± 0.0003	0.0323 ± 0.0300	0.993	0–200
SDMX	0.0044 ± 0.0004	0.0209 ± 0.0068	0.988	0–200
CEF[b]	0.0122 ± 0.0003	0.1798 ± 0.0040	0.992	0–200

[a] Dynamic range representing 0–2 times the MRL value.
[b] External standardization (y=analyte peak area).

Table 3
MRL values for the compounds in milk. Intra-day and inter-day precisions and recovery data.

Compound	MRL (μg kg^{-1})	Intra-day precision[a] CV (%) (n=18)	Inter-day precision[a] CV (%) (n=54)	Recovery[b] (%) (n=24)
NOR	100	1.41–4.45	2.61–4.83	99.2
CIP	100	1.14–7.40	3.13–7.60	99.6
DAN	30	2.24–7.30	2.34–8.90	99.6
ENR	100	1.33–4.02	3.47–4.90	100.3
STZ	100	1.29–4.92	4.76–8.27	99.5
SMR	100	1.01–3.69	2.15–8.36	98.8
SCP	100	2.82–5.52	3.34–10.13	98.6
SDX	100	1,21–4.15	1.75–6.13	98.6
SMA	100	1.33–3.23	4.15–5.23	98.7
SDMX	100	1.14–3.05	2.46–7.93	98.7
CEF	100	2.02–4.85	4.32–5.42	99.7

[a] Maximum and minimum CV values for the analytes at 0.5, 1.0, 1.5 times the MRL concentrations.
[b] Overall mean recovery for the analyte fortification at 0.5, 1.0, 1.5 and 2.0 times the MRL value.

100.3%. Nonetheless, the obtained recovery for this analyte meets the European criteria.

The LOD values were assessed on the basis of a minimal accepted value of the signal-to-noise ratio of 3:1. The LODs for the spiked extracts ranged between 4 and 20 ng mL^{-1} for the FQs and CEF, respectively. The LOD for all the SAs was 14 ng mL^{-1}. The LOQs were defined as the lowest concentration of the analyte that could be determined with acceptable precision and accuracy under the stated conditions of the method. The decision limit (CCα) and the detection capability (CCβ) were assessed through the reproducibility data, calculated at the MRL values for the analytes. The CCα and CCβ parameters and the analytical limits for the LC-FD/DAD method are summarized in Table 4.

According to EU legislation, the decision limit (CCα) corresponds to the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. For substances with a permitted limit, such as the antibacterials, a non-compliant decision is assessed through an analytical detection above the MRL concentration. For all of the residues studied, the CCα value determined is very close to the MRL value.

The antibacterials analyzed were stable for 12 weeks at temperatures of 8 and −12 °C, in both the matrix and solution. The intensity of the analytical signals (chromatographic peaks)

Table 4
Analytical parameters. Limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα), and detection capability (CCβ).

Compound	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	CCα (ng mL^{-1})	CCβ (ng mL^{-1})
LC-FD				
NOR	8	50	102.4	105.4
CIP	8	50	105.1	110.2
DAN	4	15	38.0	46.0
ENR	8	50	105.6	111.2
STZ	14	50	113.5	127.0
SMR	14	50	103.5	107.0
SCP	14	50	105.4	110.8
SDX	14	50	106.6	113.2
SMA	14	50	108.5	117.0
SDMX	14	50	104.6	109.2
LC-DAD				
CEF	20	50	107.0	114.0

Table 5
Analyte recoveries for fortified pasteurized and UHT skimmed milk obtained by two analysts in the robustness assays.

Compound	Average recoveries[a] (%)				p[b] value
	Pasteurized milk		UHT skimmed milk		
	Analyst 1	Analyst 2	Analyst 1	Analyst 2	
NOR	96.26	97.00	99.26	96.42	0.496
CIP	103.78	104.67	106.90	105.73	0.104
DAN	109.92	107.12	105.12	108.10	0.448
ENR	100.27	98.47	99.48	98.96	0.887
STZ	102.71	103.37	101.35	102.67	0.297
SMR	107.07	108.24	107.70	107.02	0.777
SCP	105.14	105.02	107.00	102.31	0.846
SDX	114.41	114.84	112.43	114.04	0.237
SMA	98.24	102.31	101.68	100.69	0.706
SDMX	102.39	99.82	98.94	100.51	0.456
CEF	95.86	98.36	96.47	95.22	0.460

[a] n=24.
[b] Student's t-test p value.

showed no significant decreased (p value ≥ 0.05) for all the analytes.

The method robustness was assessed through a double factor variation in the analytical conditions. The results were evaluated in terms of analyte recovery by varying the analyst and the matrix conditions. Pasteurized and UHT skimmed milk samples were analyzed at the MRL fortification level of the analytes. The recovery rates for both variations are shown in Table 5. The student's t-test was applied to evaluate the differences for the matrix and the analyst variations.

In the statistical analysis, the p value for each antibacterial showed no significant difference between the mean recoveries obtained on varying the analyst and matrix, since the p values were greater than 0.05. Thus, the method was sufficiently robust for the analysis of pasteurized and UHT semi-skimmed milk, carried out by two different analysts, in accordance with the EC requirements.

In the matrix effects analysis, the analyte recovery was calculated at the MRL concentration, based on the analytical signal obtained. The student's t-test was applied to verify the statistical significance of the different treatments. The analytical signals for the standard solutions were assumed to represent 100% of the analyte recovery. The mean recoveries for spiked extracts were then calculated. The results are summarized in Table 6.

Signal suppressions or enhanced intensities in analytical detections are expected due to matrix interference [16]. The matrix effects

can be compensated for by using internal standardization, in which the internal standard compound is added at the same concentration for all points of the analytical curve, therefore being submitted to the same analytical procedures as the analytes. In the case of CEF, the matrix effects were particularly relevant since the analyte quantification was performed through external standardization and matrix-matched calibration. Thus, the calibration applied could not quantify this analyte with acceptable trueness. However, application of the student's *t*-test for the comparison of means showed no significant difference between the analyte recoveries for the matrix extracts and aqueous solution. Therefore, the calibration method is considered to be adequate for CEF quantification.

Table 6
Matrix effects analysis.

Compound	Average recoveries[a] (%)		p^b value
	Aqueous solution	Matrix extract	
NOR	96.83	95.60	0.098
CIP	103.03	103.26	0.998
DAN	109.69	107.97	0.367
ENR	105.43	102.97	0.181
STZ	94.14	91.70	0.112
SMR	104.90	100.84	0.073
SCP	101.98	98.80	0.074
SDX	112.59	105.53	0.061
SMA	104.43	100.98	0.098
SDMX	99.62	97.25	0.128
CEF	96.59	92.79	0.071

[a] Samples per group at triplicate ($n=18$).
[b] Student's *t*-test *p* value.

3.3. Qualitative parameters

Although the European Community recognizes the LC technique coupled to fluorescence or diode array detections as confirmatory methods for the analysis of residues and contaminants in food matrices, additional information on the molecular structure of the compounds was obtained in order to provide an unambiguous identification of the analytes studied.

In the ESI-MS/MS system operating in positive mode, the analytes were identified on the basis of their precursor ions and *m/z* transitions. For the analysis of Group B substances using the MRM detection mode, Decision 2002/657/EC establishes a three-point identification system for analyte confirmation, in which for each precursor ion one point is added, whereas for each *m/z* transition (ion product) monitored 1.5 points are added [28]. For the confirmatory analysis, after extraction and chromatographic separation, one precursor ion and two *m/z* transitions were selected and monitored for each one of the compounds. Thus, the identities of the analytes were confirmed by obtaining a total of four identification points for each compound. The chromatograms obtained in the multiple reaction monitoring of a milk sample fortified with the analytes at their MRL concentrations are presented in Fig. 2.

During the direct infusion of individual solutions of the analytes for optimization of the electrospray mass spectrometry parameters, the analytes CEF and DAN showed low intensity

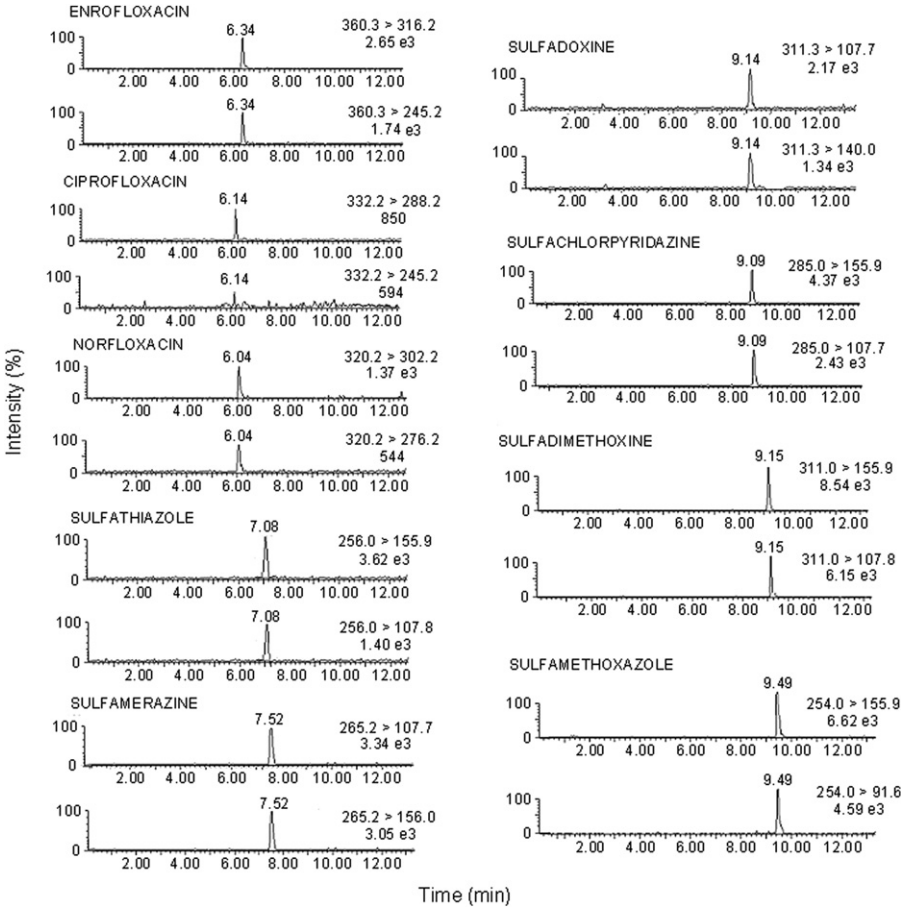

Fig. 2. MRM chromatograms for pasteurized milk sample fortified with SAs and FQs at 100 ng mL^{-1}.

transitions to confirm the qualifier ion, even though their precursor ions had been determined. This finding verifies that in some cases the fluorimetric and the diode array detections can demonstrate improved selectivity compared with mass spectrometry. Notwithstanding, the FD and DAD automation is much more accessible. Indeed, the validated quantitative method employing LC-FD/DAD is suitable as a confirmatory method for the analysis of CEF and DAN in milk, in accordance with the European Commission requirements. Therefore, these compounds were not analyzed in the LC-MS/MS system.

According to Decision 2002/657/EC, the CCβ value indicates the lowest concentration at which a substance can be detected and identified in a sample with an error probability β. For qualitative confirmatory methods, the EC considers acceptable a β error of up to 5% for the analysis of substances with permitted limits. Therefore, the qualitative CCβ value was determined by analyzing twenty milk samples fortified at the respective LOQ of the quantitative method for each antibacterial residue. The detection was considered at a signal to noise ratio of 3:1. The chromatograms of a spiked milk sample for CCβ analysis is shown in Fig. 3.

In the CCβ analysis, a signal to noise ratio of 3:1 was obtained for all of the samples analyzed, with detection of both qualitative and confirmatory transitions, as shown in Fig. 3.

Thus, the CCβ value was determined at a concentration of 20 ng mL^{-1} for the sulfonamide and fluoroquinolone antibacterials, with a estimated certainty of 100%. Moreover, the selectivity of the LC-MS/MS analysis was determined by analyzing 20 blank samples of both pasteurized and ultra-heat-treated milk, of whole and skimmed types. As has been well documented, no interference was observed in the chromatograms due to the high selectivity of the MRM mode of the mass spectrometer.

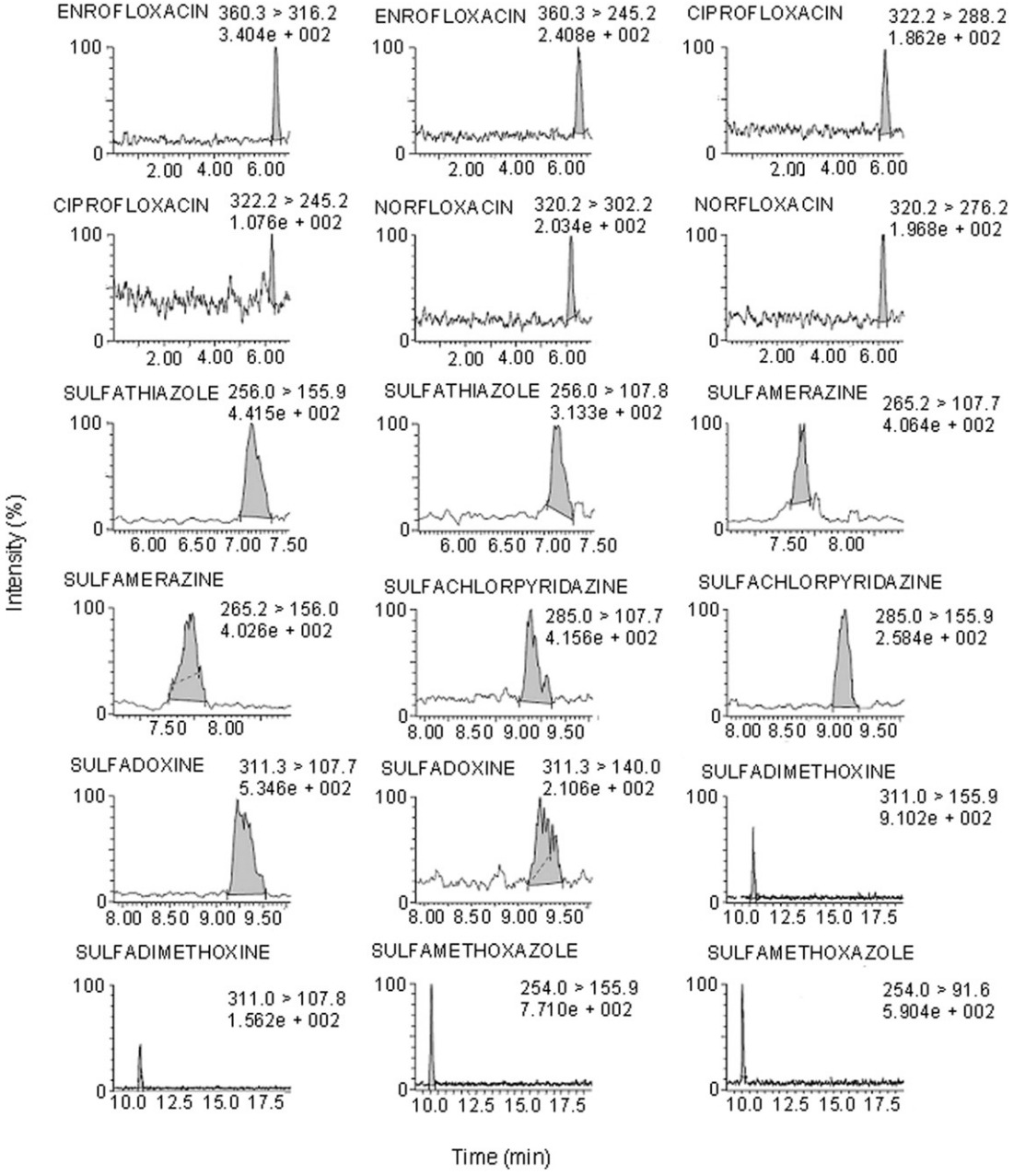

Fig. 3. Chromatograms of LC-MS/MS analysis for CCβ determining. Milk sample fortified with the SA and FQ compounds at 20 ng mL^{-1}.

3.4. Analysis of real samples from South Region of Brazil

To verify the performance of the validated method, 32 samples of UHT milk, 23 samples of pasteurized milk and 2 of raw milk, obtained in Santa Catarina State, were analyzed using the developed procedure. No residues of the studied analytes were detected in the analyzed samples. Nevertheless, the quantitative method showed good applicability for the analysis of different milk matrices.

The applicability of the LC-MS/MS confirmatory method was also verified. The analysis was performed with liquid and powdered milk samples, acquired in Porto Alegre, Rio Grande do Sul State. Among the fluid milk samples, 2 pasteurized milk samples and 41 UHT milk samples were acquired, and 5 samples of powdered milk were obtained. Additionally, two raw milk samples were analyzed. For all of the samples analyzed there was no detection of any of the residues, whereas the *m*/*z* qualitative transitions of the sulfonamide and fluoroquinolone compounds were not detected by the multiple reaction monitoring of the milk samples analyzed. Moreover, the method developed demonstrated good performance for the analysis of milk samples from different origins.

4. Conclusion

In this paper we presented a multiclass methodology to monitor milk quality in developing countries. A simple and less toxic procedure using water extraction and liquid chromatography coupled to fluorescence and diode array detection was successfully applied for simultaneous analysis of CEF, FQs and SAs residues in bovine milk. The method meets international criteria of validation performance, being especially suitable for residue monitoring in milk in developing countries, where the cost-benefit matter is normally mandatory.

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