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# A Liquid Chromatography–Tandem Mass Spectrometry Method for the Detection of Antimicrobial Agents from Seven Classes in Calf Milk Replacers: Validation and Application

Luca Maria Chiesa[†], Maria Nobile[†], Sara Panseri[*][†], Bartolomeo Biolatti[§], Francesca Tiziana Cannizzo[§], Radmila Pavlovic[†], and Francesco Arioli[‡]

[†] Department of Veterinary Science and Public Health, University of Milan, Via Celoria 10, 20133 Milan, Italy

[§] Department of Veterinary Science, University of Turin, Largo Paolo Braccini 2, 10095 Grugliasco, Italy

[‡] Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy

**Abstract**

Calf milk replacers are low-cost feeds that contain available, digestible protein. During their reconstitution, however, the addition of drugs, such as antibiotics, could make them a very simple route for illicit treatment for therapeutic, preventive, or growth-promoting purposes. We developed an HPLC-MS/MS method, preceded by a unique extraction step, able to identify 17 antibiotics from seven classes (penicillins, tetracyclines, fluoroquinolones, sulfonamides, cephalosporins, amphenicols, and lincosamides) in this matrix. Prior to solid phase extraction (SPE), the sample underwent deproteinization and defatting. The method was fully validated according to Commission Decision 2002/657/EC. Decision limits (CCα) and detection capability (CCβ) were in the ranges of 0.13–1.26 and 0.15–1.47 ng/mL, respectively. Thirty-eight samples were finally analyzed, showing the occasional presence of marbofloxacin (six samples) and amoxicillin (one sample).

**Keywords:**

antibiotics; calf; HPLC-MS/MS; milk replacers

## Introduction

Milk replacers can be an economical and valid source of calf nutrition. Their formulation is carried out to increase the protein level to ensure maximum muscle growth of the calves; to enhance the palatability and, consequently, the ingestion to facilitate the weaning phase; to make energy from diverse sources (starch, soluble fiber and digestible fat) for maximum growth without digestive problems (acidosis, intestinal problems, etc.); to increase the range of supply, both from the point of view of the nutritional characteristics and from the point of view of prices; and to simplify the management of calves' food, due to the availability of products used at each stage (starter, weaning, breeding until 6 months).(1)

Powdered milk replacers are usually made up of skimmed-milk powder, whey protein, vegetable or animal fat, soy lecithin, and vitamin–mineral premix.(2) Fat levels range from 10 to 30%, with 18–22% being the

most common fat levels, which are mainly added as tallow, lard, or coconut oil. Proteins range from 18 to 30% with an average value of approximately 20–22%, preferably of diary origin, but can also include soy protein, soy flour, potato, wheat proteins, and animal plasma protein.(3, 4)

Antibiotics are usually added to preweaned calf milk replacer to treat bacterial infections; to decrease the incidence of scours, morbidity, and mortality; to improve calf growth; improve feed consumption; and to increase average daily gains.(5-7) Among feedstuffs used in animal husbandry, powdered milk replacers are perhaps the most suitable for illegal treatments and, for example, drug administration via this route is very simple, that is, during the reconstitution of milk replacers or immediately before feeding. It should be considered that the antibiotics found in milk replacers might result from prior treatment of cows producing the milk or what the milk replacer was made with, or they may have intentionally been added directly to the feed.(8) Whatever the reason, the overuse of antibiotics in animal husbandry may affect the antibiotic resistance of pathogens(9-11) and the consequent possibility of human infection with resistant bacteria.(12, 13)

The inclusion of veterinary drugs in calf milk replacers constitutes a major health issue for the consumer, particularly as their regulation varies between countries. For example, inclusion in milk replacers of decoquinate, lasalocid, oxytetracycline, chlortetracycline, and neomycin-based medications is approved in the United States. The Food and Drug Administration (FDA) in 2013, however, recommended a 3-year "judicious period" during which utilization of antibiotics should be reduced. Therefore, the presence of these antibiotics in feedstuff can be authorized for therapeutic and prophylactic purposes but is not authorized as a growth promoter.(14)

European legislation does not treat milk replacers individually, but states the conditions under which feedstuffs must be produced, marketed, and employed within the European Union (EU).(15, 16) From 2006, the antibiotics as feed additives are no longer allowed, except coccidiostats and histomonostats.(17) Currently, the use of antibiotics for auxinic purposes has been abolished and is illegal. To minimize the risk to human health, the EU stated maximum residue limits (MRLs) for several classes of antibiotics in milk and edible tissues.(18) However, until now, the EU did not enact regulations about maximum levels of antibiotics either in feedstuff in general or in milk replacers.

The analytical methods for monitoring the presence of undesirable pharmacological principles must have high sensitivity and selectivity. As feedstuffs are heterogeneous matrices with different protein, fatty acid, neutral lipid (phospholipids, glycerides, and sterols), glucide, vitamin, and mineral contents, the occurrence of interferences is a major issue in the analysis. The methods used for the detection of drug residues in these matrices often need intensive steps for the preparation and extraction of samples to improve the analysis performances. Studies in the literature on antibiotics in powdered calf milk or feed in general include a rapid multiresidue and multiclass screening method for 50 antimicrobials in feed,(19) some about detection of chloramphenicol and florfenicol in powdered milk,(20-22) and one about a specific class of antibiotics, as well as fluoroquinolone residues, but in powdered infant formulas,(23) and many more on milk for human consumption.(24)

The purpose of this study was the development of a unique extraction and HPLC-MS/MS analysis method that is able to identify antibiotics from different classes in calf powdered milk with the aim of improving residue control and preventing possible consequences for animal and consumer welfare. The considered antibiotics included amoxicillin and ampicillin (penicillins); chlortetracycline, doxycycline, oxytetracycline, and tetracycline (tetracyclines); ciprofloxacin, enrofloxacin, lomefloxacin, and marbofloxacin (fluoroquinolones); sulfadimidine and sulfathiazole (sulfonamides); cephalexin and cefquinome

(cephalosporins); florfenicol and its metabolite florfenicol amine (amphenicols); and lincomycin (lincosamides). The chemical structures of the seven antimicrobial classes are shown in Figure 1. The method validation was made according to Commission Decision 2002/657/EC,(25) and the application of the analysis to 38 real samples of powdered bovine milk was performed.
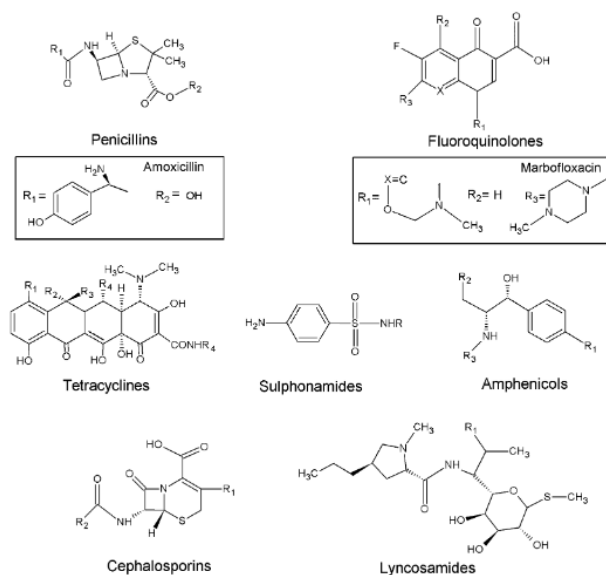


Figure 1.
Structures of the seven classes of antimicrobial agents. The substituents of the two antibiotics found in the samples are shown in the boxe

## Materials and Methods

Chemicals and Reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid (98–100%) and hydrochloric acid (37%) were obtained from Riedel-de Haën (Sigma-Aldrich). Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). The extraction cartridges (Oasis HLB 3 mL, 60 mg) were provided by Waters (Milford, MA, USA). Amoxicillin, ampicillin, cefalexin, cefquinome sulfate, florfenicol, florfenicol amine, lomefloxacin hydrochloride, ciprofloxacin, enrofloxacin, marbofloxacin, tetracycline hydrochloride, doxycycline hyclate, chlortetracycline hydrochloride, oxytetracycline, lincomycin, sulfathiazole sulfadimidine, and enrofloxacin-$d_5$ as the internal standard (IS) were purchased from Fluka.

Composition of Powdered Milk Replacer

For the method validation, we used a commercially available complete milk replacer for calves being weaned. The chosen milk replacer, which was proven free of antibiotics, contained whey powder, vegetable oils (palm and coconut oil), hydrolyzed wheat protein, soy protein concentrate, calcium carbonate, and magnesium sulfate. The analytical constituents were crude protein, 21%; oils and fats, 16%; crude fiber, 0.3%; crude ash, 9%; calcium, 0.8%; sodium, 0.8%; phosphorus, 0.7%; lysine, 1.5%; methionine, 0.4%; and cysteine, 0.4%. Additives included vitamin A (40000 IU/kg), vitamin D3 (5000 IU/kg), vitamin E

(200 mg/kg), vitamin B1 (6 mg/kg), vitamin B6 (4 mg/kg), vitamin C (158 mg/kg), vitamin K (4 mg/kg), and niacin (40 mg/kg). The formulation also contained the following quantities per kilogram: iron monohydrate sulfate, 273.6 mg; copper pentahydrate sulfate, 39.3 mg; manganese monohydrate sulfate, 141.7 mg; zinc monohydrate sulfate, 230.2 mg; potassium iodide, 2.62 mg; and sodium selenite, 0.66 mg. All of the information about the feedstuff compositions came from the manufacturer's certificates.

Standard Solutions

Stock solutions (1 mg/mL) for each standard were prepared in methanol and kept at −20 °C. Working solutions in methanol, containing each of the studied analytes at concentrations of 10 and 100 ng/mL, were prepared daily. Each working solution was maintained at −20 °C during the method validation procedures.

Sample Extraction

The preliminary deproteinization and defatting steps were performed using the protocol of Wang et al.,[(26)] which was slightly modified with regard to the relative amounts of matrix and reagents, and acetonitrile was substituted by methanol. The whole procedure was as follows: One gram of powdered milk was reconstituted in 10 mL of water, and 1 mL of this solution was spiked with the internal standard to a final concentration of 2 ng/mL. The sample was vortexed and then sonicated for 10 min, followed sequentially by deproteinization through the addition of methanol (10 mL), 2 min of shaking, and 10 min of sonication. After the addition of sodium chloride (2 g), samples underwent 2 min of shaking and 10 min of centrifugation at 4500*g*. The supernatant was transferred into a 50 mL polytetrafluoroethylene centrifuge tube and defatted with 2 × 7 mL of *n*-hexane extraction. Each time, after centrifugation at 2500*g*, the *n*-hexane layer was removed. The methanol/water layer was evaporated by a vacuum rotary evaporator at 40 °C and was then reconstituted in 5 mL of water for further purification and extraction using Oasis HLB cartridges under vacuum. The SPE cartridges were preconditioned with 3 mL of methanol, 3 mL of 0.5 M HCl, and 3 mL of Milli-Q water. The samples were loaded, and then the cartridges were washed with 3 mL of water and 3 mL of methanol/water (20:80, v/v). Finally, samples were eluted using 5 mL of methanol and were collected in a 15 mL polypropylene tube. The solvent was evaporated with a rotary vacuum evaporator. The dried extract was reconstituted in 200 μL of methanol/0.1% aqueous formic acid (10:90 v/v) and then transferred to an autosampler vial. The injection volume was 10 μL.[(27)]

HPLC-MS/MS Analyses

HPLC analysis was carried out with an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) that was made up of a Surveyor MS quaternary pump with a degasser, a Surveyor AS autosampler with a column oven/ and a Rheodyne valve with a 20-μL loop. Analytical separation was carried out using a reverse-phase HPLC column/ 150 mm × 2 mm i.d., 4 μm, Synergi Hydro RP, with a 4 mm × 3 mm i.d. C18 guard column (Phenomenex, Torrance, CA, USA). The mobile phase used in the chromatographic separation consisted of a binary mixture of solvents A (0.1% aqueous formic acid) and B (MeOH). The elution started with 98% A, which was maintained for 5 min, followed by a linear gradient to 50% A at 22 min. Subsequently, the mobile phase was gradually increased to 95% B at 24 min and then held constant until 29 min. The mobile phase was returned to initial conditions at 31 min, with equilibration time that included the interval from 31 to 40 min. The run was performed at 0.2 mL/min. The mass spectrometer was a triple-quadrupole TSQ Quantum MS (Thermo Fisher) equipped with an electrospray interface (ESI) that was set in the positive (ESI+) electrospray ionization mode for all analytes except florfenicol, for which it was set in the negative (ESI−) mode. Acquisition parameters were optimized in the electrospray mode by direct continuous pump–syringe infusion of the standard solutions of analytes at a concentration of 1 μg/mL, a flow rate of 20

μL/min, and an MS pump rate of 100 μL/min. The following conditions were used: capillary voltage, 3.5 kV; ion-transfer capillary temperature, 340 °C; nitrogen as sheath and auxiliary gases at 30 and 10 arbitrary units, respectively; argon as the collision gas at 1.5 mTorr; and peak resolution, 0.70 Da at full-width half-maximum (fwhm). Three diagnostic product ions were chosen for each analyte and internal standard. The acquisition was made in multiple reaction monitoring (MRM) mode. The selected diagnostic ions, one of which was chosen for the quantitation, and the collision energies are reported in Table 1. Acquisition data were recorded and elaborated using Xcalibur software from Thermo Fisher.

**Table 1. MS/MS Conditions for the MRM Acquisitions of Investigated Antibiotics**

| analyte | precursor ion[a] ($m/z$) | product ions$_{CE}$ ($m/z$) | ESI |
|---|---|---|---|
| amoxicillin | 366 | **114 (80)$_{20}$**, 134 (21)$_{31}$, 349 (100)$_7$ | (+) |
| ampicillin | 350 | **106 (100)$_{18}$**, 114 (14)$_{29}$, 160 (14)$_{14}$ | (+) |
| cefalexin | 348 | 158 (63)$_5$, **174 (100)$_{15}$**, 191 (23)$_6$ | (+) |
| cefquinome | 529 | **134 (100)$_{15}$**, 324 (43)$_{15}$, 396 (44)$_{10}$ | (+) |
| ciprofloxacin | 332 | 268 (16)$_{22}$, **288 (100)$_{17}$**, 314 (94)$_{21}$ | (+) |
| enrofloxacin | 360 | 245 (49)$_{26}$, **316 (100)$_{18}$**, 342 (29)$_{21}$ | (+) |
| lomefloxacin | 352 | **265 (100)$_{23}$**, 288 (16)$_{19}$, 308 (63)$_{16}$ | (+) |
| marbofloxacin | 363 | 72 (83)$_{23}$, **320 (100)$_{15}$**, 345 (18)$_{21}$ | (+) |
| florfenicol | 356 | 169 (1)$_{39}$, 185 (35)$_{21}$, **336 (100)$_{12}$** | (−) |
| florfenicol amine | 248 | 130 (24)$_{23}$, 134 (8)$_{28}$, **230 (100)$_{11}$** | (+) |
| chlortetracycline | 479 | 154 (39)$_{27}$, **444 (100)$_{21}$**, 462 (69)$_{16}$ | (+) |
| doxycycline | 445 | 321 (10)$_{31}$, 410 (8)$_{24}$, **428 (100)$_{19}$** | (+) |
| oxytetracycline | 461 | 337 (26)$_{29}$, **426 (100)$_{19}$**, 443 (52)$_{12}$ | (+) |
| tetracycline | 445 | 154 (38)$_{30}$, **410 (100)$_{19}$**, 427 (43)$_{14}$ | (+) |
| lyncomicin | 407 | **126 (100)$_{16}$**, 359 (10)$_{18}$, 389 (5)$_{28}$ | (+) |
| sulfathiazole | 256 | 92 (50)$_{27}$, 108 (45)$_{25}$, **156 (100)$_{15}$** | (+) |
| sulfadimidine | 279 | 108 (32)$_{26}$, 124 (39)$_{265}$, **186 (100)$_{18}$** | (+) |
| enrofloxacin-$d_5$ (IS) | 365 | 245 (49)$_{32}$, **321 (100)$_{27}$**, 347 (46)$_{19}$ | (+) |

a[M + H]$^+$ for all compounds except [M − H]$^−$ for florfenicol. Ions for quantitation are in bold. The values in parentheses represent the relative intensities (%). CE, collision energy, subscripted and expressed in volts.

Method Validation

After a preliminary screening of a few samples of reconstituted milk to search the "blank" milk, the validation was performed according to the criteria of European Commission Decision 2002/657/EC.(25) For each analyte, the method performance was assessed as follows: through its qualitative parameters, as well as molecular identification in terms of retention time (RT) and transition ion ratios, specificity, and selectivity; through its quantitative parameters, such as linearity, recovery, accuracy in terms of trueness, and precision expressed as the intra- and interday repeatability; and through the analytical limits, that is, decision limit (CCα) and detection capability (CCβ), as clarified in the document SANCO/2004/2726 revision 4.(28) Finally, we evaluated robustness, matrix effect, and stability of antibiotics in the standard solutions and in the spiked samples.

# Results and Discussion

Method Performance

The specificity was good as 20 blank samples were analyzed and no interferences were found in the region of expected elution of the target analyte.

Selectivity showed a good compliance with the relative retention times, which in our case were within 2.5% tolerance for each analyte related to its standard. Moreover, the three chosen transitions showed an ion ratio within the recommended tolerances(25) when compared with the standards.

Validation was performed on three analytical series obtained by fortification of milk samples with each of the analytes (matrix validation curves). Each series had six replicates for three concentration levels that were previously chosen according to the minimum concentration detectable with our instrumentation: 0.1, 0.2, and 0.3 ng/mL for lomefloxacin, lyncomicin, sulfathiazole, and sulfadimidine; 0.3, 0.6, and 0.9 ng/mL for ampicillin, cefalexin, florfenicol amine, ciprofloxacin, marbofloxacin, tetracycline, doxycycline, and oxytetracycline; 0.5, 1.0, and 1.5 ng/mL for amoxicillin, cefquinome, and florfenicol; and 1.0, 2.0, and 3.0 ng/mL for chlortetracycline. All correlation coefficients of the matrix validation curves were >0.99, demonstrating a good fit for all antibiotics.

The HPLC-MS/MS chromatograms and the ion spectra of the antibiotics, at the lowest concentration level of the validation, and of the internal standard (2 ng/mL) are shown in Figure 2.
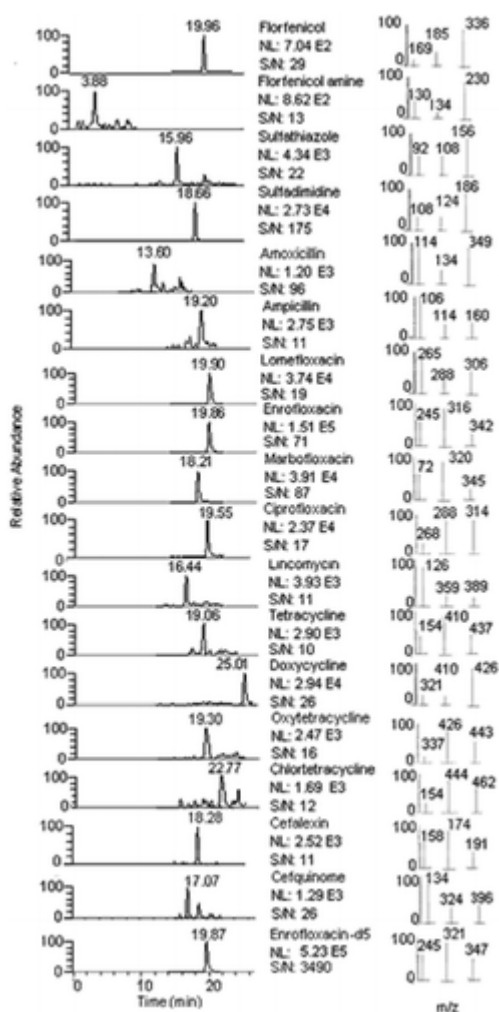
Figure 2. Chromatograms performed in matrix and related ion spectra of antibiotics at the lowest concentration level of validation (0.1 ng/mL for lomefloxacin, lyncomicin, sulfathiazole, and sulfadimidine; 0.3 ng/mL for ampicillin, cefalexin, florfenicol amine, ciprofloxacin, marbofloxacin, tetracycline, doxycycline, and oxytetracycline; 0.5 ng/mL for amoxicillin, cefquinome, and florfenicol, 1.0 ng/mL for chlortetracycline) and of the internal standard (2 ng/mL).

The instrumental linearity was also evaluated on six-point calibration curves in the solvent containing a constant concentration of the internal standards (2 ng/mL), with the initial analyte concentration equal to the minimum detectable for each group up to 10 ng/mL. The fit of these curves was good, as all the correlation coefficients were >0.99.

Calibration curves were made in matrix to quantitate the analytes eventually detected during the application of the method. The levels were the same used for the assessment of the instrumental linearity. All of these curves had a good fit, too, with a correlation coefficient >0.99.

The trueness, expressed by recovery, was evaluated using the data from the validation points of the three analytical series, in terms of a percentage of the measured concentration with respect to the spiked concentration. The recoveries ranged between 89 and 111%.

The precision was evaluated by calculating the relative standard deviation of the results obtained for six replicates of each analyte at three concentration levels of the three analytical series, performed on three different days. It was expressed as the coefficient of variability (CV) in terms of intra- and interday repeatability, which never exceeded 20 and 22%, respectively. These CVs were ≤22%, as proposed by Thompson,(29) and thus represent good method performance.

In Table 2, the analytical limits, recoveries, and precision are shown. CCα and CCβ, which were calculated as described in SANCO/2004/2726 revision 4(28) using parallel extrapolation to the x-axis at the lowest experimental concentration, were in the orders of magnitude 0.1–1.0 ng/mL.

**Table 2. Validation Parameters for Antibiotics[a]**

| analyte | CCα (ng/mL) | CCβ (ng/mL) | concentration level (ng/mL) | recovery % (n = 18) | repeatability intraday (CV; n = 6) | repeatability interday (CV; n = 18) |
|---|---|---|---|---|---|---|
| amoxicillin | 0.78 | 1.04 | 0.5, 1.0, 1.5 | 104, 96, 101 | 18, 8, 7 | 22, 17, 9 |
| ampicillin | 0.47 | 0.62 | 0.3, 0.6, 0.9 | 101, 99, 100 | 20, 13, 9 | 21, 15, 9 |
| cefalexin | 0.53 | 0.80 | 0.3, 0.6, 0.9 | 102, 97, 101 | 19, 19, 16 | 22, 21, 18 |
| cefquinome | 0.75 | 0.93 | 0.5, 1.0, 1.5 | 103, 91, 109 | 19, 11, 8 | 20, 12, 9 |
| ciprofloxacin | 0.41 | 0.52 | 0.3, 0.6, 0.9 | 95, 105, 98 | 14, 15, 11 | 16, 16, 12 |
| enrofloxacin | 0.13 | 0.17 | 0.1, 0.2, 0.3 | 100, 100, 100 | 8, 8, 7 | 15, 15, 8 |
| lomefloxacin | 0.18 | 0.27 | 0.1, 0.2, 0.3 | 97, 103, 98 | 20, 14, 16 | 22, 21, 18 |
| marbofloxacin | 0.45 | 0.58 | 0.3, 0.6, 0.9 | 103, 97, 101 | 17, 11, 8 | 20, 16, 9 |
| florfenicol | 0.69 | 0.88 | 0.5, 1.0, 1.5 | 98, 101, 100 | 11, 12, 8 | 16, 17, 9 |
| florfenicol amine | 0.38 | 0.47 | 0.3, 0.6, 0.9 | 93, 107, 98 | 6, 11, 10 | 12, 15, 11 |
| chlortetracycline | 1.26 | 1.47 | 0.1, 0.2, 0.3 | 92, 103, 98 | 7, 5, 7 | 11, 11, 10 |
| doxycycline | 0.46 | 0.64 | 0.3, 0.6, 0.9 | 104, 96, 101 | 18, 16, 12 | 22, 21, 13 |
| oxytetracycline | 0.41 | 0.52 | 0.3, 0.6, 0.9 | 102, 98, 101 | 10, 8, 8 | 16, 15, 9 |
| tetracycline | 0.48 | 0.63 | 0.3, 0.6, 0.9 | 89, 111, 96 | 20, 9, 10 | 21, 12, 10 |
| lyncomicin | 0.15 | 0.19 | 0.1, 0.2, 0.3 | 101, 99, 100 | 17, 13, 11 | 20, 17, 12 |
| sulfathiazole | 0.16 | 0.21 | 0.1, 0.2, 0.3 | 104, 96, 101 | 20, 10, 9 | 21, 17, 10 |
| sulfadimidine | 0.13 | 0.15 | 0.1, 0.2, 0.3 | 101, 99, 100 | 8, 4, 6 | 11, 9, 7 |

A The recovery and intra- and interday repeatability values are presented in reference to the three validation levels.

We evaluated robustness using the approach of Youden,(25) which has a fractional factorial design. It was observed in eight different trials by fortifying eight blank milk-replacer samples at the lowest validation concentration, changing slightly (±10%) the values of seven factors that may affect the outcome of the analysis. The factors included the methanol volume for the deproteinization milk step; the HCl concentration for the conditioning of the SPE columns; the percentage of methanol in the washing solution of the SPE columns; the volume of the methanolic solution used for washing the SPE columns; the elution volume of the SPE columns; the evaporation temperature of the extract; and the resuspension volume of the dry extract. Applying the Fisher test to compare the standard deviation of the differences between high- and low-value settings for each experimental parameter with the standard deviation of the method carried out under within-laboratory reproducibility condition,(25) none of the parameters showed a significant variation in the concentration measurements, so demonstrating the method robustness.

The matrix effect was evaluated through the approach of Matuszewski et al.(30) that compares the peak areas of samples fortified after extraction with pure solutions prepared in the mobile phase containing equivalent amounts of the analyte of interest. The percentage ratio determines the extent of the matrix effect for each analyte. It ranged from 93 to 114%, indicating a very low ion suppression and sometimes a low ion enhancement.

Stability was evaluated by testing spiked samples and standard solutions over time from 1 week to 1 month under defined storage conditions (−20 °C), and quantitation of components was determined by comparison to freshly prepared standards.

All of the analytes in the working solutions and in the samples showed an acceptable stability (CV ≤ 2%) until 1 month of storage at −20 °C, except amoxicillin and cefquinome. After 1 week, the concentration of these two antibiotics decreased more than the acceptable value. We therefore decided to daily prepare the working solutions.

This paper describes a sensitive, selective, and robust multiclass method for antimicrobials in calf milk replacers. A number of studies are retrievable in the literature on the determination of single classes of antibiotics as well as β-lactam antibiotics in milk for human use(31, 32) and fluoroquinolones both in dairy milk(33) and in powdered milk for infants.(23) The only multiclass method for antibiotics regarding feedstuffs is a qualitative screening method on feed different from milk replacers. The validation levels are usually 5–100 times higher than our analytical limits, except for florfenicol and doxycycline.(19)

Application of the Method

The use of medicated feeds is most common in intensive production.(16) Although the studied antimicrobials are authorized, they must be absent in nonmedicated feed. To demonstrate the efficacy of the proposed method, 38 anonymous samples collected from different farms in Lombardy (Italy) were subjected to analysis. Only one sample was positive for amoxicillin at a concentration of 1.26 ng/mL, whereas six samples contained marbofloxacin residues ranging from 0.52 to 0.91 ng/mL, with an average concentration of 0.74 ± 0.15 ng/mL. In Figure 1 the substituents of these two antimicrobial agents are itemized in the boxes.

Tetracyclines were not found, in contrast to our previous work on urine,(27) in which this class of antibiotics was present in almost all samples. These data could be a demonstration of the good quality of the milk replacers analyzed as milk is the major tetracycline excretion route after urine (50–80%) and bile

(10–20%).(34) Considering that calves eat from 400 to 2500 g of powdered milk per day, the control of residues in this noninvasive matrix is, however, still of concern due to the practicality of illicit administration of drugs via this route.

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