Comparative study of the LC–MS/MS and UPLC–MS/MS for the multi-residue analysis of quinolones, penicillins and cephalosporins in cow milk, and validation according to the regulation 2002/657/EC

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ABSTRACT

The aim of this study was to develop and validate an analytical method to simultaneously determine European Union-regulated β-lactams (penicillins and cephalosporins) and quinolones in cow milk. The procedure involves a new solid phase extraction (SPE) to clean-up and pre-concentrate the three series of antibiotics before analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS) and ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). LC–MS/MS and UPLC–MS/MS techniques were also compared. The method was validated according to the Directive 2002/657/EC and subsequently applied to 56 samples of raw cow milk supplied by the Laboratori Interprofessional Lleter de Catalunya (ALLIC) (Laboratori Interprofessional Lleter de Catalunya, Control Laboratory Interprofessional of Milk of Catalunya).

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1. Introduction

Antibiotics are widely use in human and veterinary medicine. The β-lactams, which include penicillins and cephalosporins, and quinolones, are the most frequently employed. These drugs are administered at therapeutic doses to treat bacterial infections in food-producing animals, such as cattle, swine, turkey and chicken. They are also used at sub-therapeutic doses as prophylactics or growth promoters, although European Union (EU) legislation has forbidden this practice since 2006 [1]. Milk is widely consumed globally and can contain antibiotics or their residues if the animals that produced it had been treated with the drugs. These residues might have direct toxic effects on consumers through allergic reactions or they may cause indirect problems by promoting bacterial resistance [2,3]. Thus, the use of antibiotics has become an increasingly important public health concern.

The EU has established Maximum Residue Limits (MRLs) for several classes of antibiotics in animal products, such as milk and edible tissues, with the aim of minimising risk to human health [4–6]. In milk, the MRL ranges are between 4 and 30 μg/kg for penicillins, 20 and 100 μg/kg for cephalosporins, and 30 and 100 μg/kg for quinolones.

To enforce these regulations and ensure that consumers are protected from accidental consumption of contaminated milk, it is necessary to have strategies to regularly test large numbers of milk samples. Milk samples are usually examined by rapid screening methods that only indicate whether some antibiotics are present or not and also by immunoassays to determine the type of antibiotics [7]. This strategy is adequate to reduce the number of samples to be quantified and can be applied in routine laboratory analysis. The samples that fail the screening tests are then examined using developed analytical methods that are sensitive enough to monitor and determine drugs in cow milk, thus allowing the identification and quantification of the antibiotic in accordance with EU regulations.

There are several papers on antibiotic analysis in milk in the literature, but most focus on only few compounds or one class of antibiotics [10–15]. Although it is also possible to find multi-class published methods [8,16–23], some are not quantitative or do not determine the quality parameters necessary for validating the method according to European legislation [24]. Quinolones and penicillins regulated except AMOX and PENG, have been previously analysed in milk by ultra-high-performance liquid chromatography–time of flight-mass spectrometry (UPLC–TOF-MS) [19], while other authors [8] simultaneously measured the complete series of quinolones, penicillins and cephalosporins regulated by the EU in raw milk samples using UPLC with TOF to screen 150 veterinary drugs.

Liquid chromatography (LC) coupled to several detectors have been used to analyse samples, although mass spectrometry (MS)

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detection has recently gained popularity since its selectivity and sensitivity enables the determination of drug residues in complex matrices [16,17,20–22]. An optimal method for routine analysis should allow rapid detection of a large number of compounds. In this sense, UPLC which uses small particle size columns and operates at a much higher pressure than LC, has been shown to be a promising solution for batch analysis. Compared to LC, UPLC improves resolution and sensitivity, as well as significantly reducing sample analysis time and mobile phase solvent consumption [8,9,19,25,26].

The aim of this study was to develop a method for the multi-class and multi-residue determination of penicillins, cephalosporins and quinolones regulated by European legislation 37/2010 [5] in cow milk using liquid chromatography–tandem mass spectrometry (LC–MS/MS) and ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). The proposed method consisted of a new solid phase extraction (SPE) step to clean-up and pre-concentrate the three series of antibiotics simultaneously prior to analysis by liquid chromatography. The method was validated according to the Commission Decision 2002/657/EC [24] and subsequently applied to several milk samples from cows treated with antibiotics.

2. Experimental procedures

2.1. Reagents

The standards were purchased from several pharmaceutical firms:

Cephalosporins: cephalexin (LEX) and cefoperazone (PER) (Sigma, St. Louis, MO, USA), cephazolin (ZOL), cephapirin (PIR) and ceftiofur (TIO) (Fluka, Buchs, Switzerland), cefquinome (QUI) (AK Scientific, Inc., USA) and cephalonium (LON) was graciously provided by Schering-Plough Animal Health Corporation (Ireland).
Penicillins: ampicillin (AMPI), dicloxacillin (DICL) and penicillin G (PENG) (European Pharmacopeia, Strasbourg Cedex, France), amoxicillin (AMOX), nafcillin (NAFC) and oxacillin (OXAC) (Sigma, St. Louis, MO, USA), and cloxacillin (CLOX) and piperacillin (PIPE; internal standard (IS)) (Fluka, Buchs, Switzerland).
Quinolones: ciprofloxacin (CIP) (Ipsen Pharma, Barcelona, Spain), enrofloxacin (ENR) (Cenavisa, Reus, Spain), danofloxacin (DAN) (Pfizer, Karlsruhe, Germany), marbofloxacin (MAR) (Vetoquinol, Barcelona, Spain), flumequine (FLU) (Sigma, St. Louis, MO, USA) and pipemidic acid (PIP; internal standard (IS)) (Prodesfarma, Barcelona, Spain).

All reagents were of analytical grade unless indicated. Formic acid (HFo), acetic acid (HAc), trifluoroacetic acid (TFA), acetonitrile (MeCN), methanol (MeOH), sodium dihydrogenphosphate and sodium hydroxide were supplied by Merck. Sodium chloride was supplied by Sigma. Ultrapure water was generated by a Milli-Q system (Millipore).

The solid phase extraction (SPE) cartridges were Oasis HLB (3 cm^3/60 mg) obtained from Waters (Milford, MA, USA) and Strata X (1 cm^3/30 mg; Phenomonex, USA).

2.2. Standards and stock solutions

Individual stock solutions of penicillins and cephalosporins were prepared at a concentration of 100 μg/ml by dissolving in water. The individual stock solutions of quinolones were prepared at a concentration of 500 μg/ml by dissolving in 50 mM acetic acid aqueous solution (MAR, CIP, DAN and ENR). FLU at a concentra-

tion of 100 μg/ml was prepared at a concentration of 100 μg/ml in MeCN.

The individual standard solutions of PIPE and PIP (IS) were prepared by dissolving the internal standards in water and 50 mM acetic acid: MeCN (4:1, v/v) at a concentration of 100 μg/ml and 40 μg/ml, respectively. The working individual standard solutions of IS were prepared at a concentration of 5 μg/ml in Milli-Q water.

Working solutions (containing a standard mixture for each family of antibiotics) were prepared at a concentration of 100 MRL and 20 MRL to validate the method. For the preliminary studies, individual stock solutions were diluted to a concentration of 10 μg/ml in Milli-Q water. Working solutions were used to spike the milk samples. All standard solutions were stored at −20 °C.

Phosphate solutions 0.05 M at pH 8.5 and 9, and 0.1 M at pH 10 were prepared for addition to the milk samples.

2.3. Instruments

Liquid chromatography separation was performed on a Zorbax Eclipse XDB-C8 column (5 μm, 4.6 mm × 150 mm) from Agilent Technologies using a pre-column Kromasil C8 (5 μm, 4.6 mm × 15 mm) supplied by Akady (Barcelona, Spain). The column used for separation by UPLC–MS/MS was an Acquity UPLC BEH Shield RP 18 (1.7 μm, 2.1 mm × 50 mm) from Waters (Ireland).

An HP Agilent Technologies 1100 LC system equipped with an autosampler and coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex) with a turbo ionspray source was used. The system was controlled by the Analyst v.1.4.2 software from Applied Biosystems (Foster City, CA, USA).

Chromatographic analysis by UPLC was performed using Acquity-Ultra Performance LC-Waters system equipped with an autosampler. The mass spectrometer was the same as that used by LC. The system was controlled by the Analyst v.1.4.2 software from Applied Biosystems (Foster City, CA, USA) and the Acquity Console to control the UPLC.

A Crison 2002 potentiometer (±0.1 mV) (Crison, Barcelona, Spain) using a Crison 5203 combined pH electrode from Orion Research (Boston, MA, USA) was used to measure the pH of the phosphate solution and of the mobile phase. The electrode was stored in water when not used and soaked for 15–20 min in a MeCN–water mixture (15%) before pH measurements of the mobile phase.

A Rotanta 460RS (Hettich Zentrifuguen) centrifuge was used to perform the extraction. The SPE step was carried out on a Supelco vacuum manifold with 12 cartridges and a Supelco vacuum manifold with disposable liners for 24 cartridges (Bellefonte, PA, USA) connected to a Supelco vacuum tank. Evaporation to dryness was performed under a stream of nitrogen at the end of sample treatment.

2.4. Procedure

2.4.1. Sample treatment and clean-up (SPE)

To achieve a single method valid for analysing the three families of antibiotics, different methods from the literature were tested.

2.4.1.1. Method 1. The following method was based on an article of Becker [17] with some modifications. Raw milk samples were used to optimise and validate the method. Specific volumes of antibiotic working solutions were added to 2.0 g of whole milk to give appropriate concentration of each antibiotic. The IS, PIPE and PIP were added at a concentration of 100 μg/kg.

After vortexing for 1 min, the milk samples were kept at room temperature for 10 min and centrifuged (1500 × g for 10 min). MeCN was added (to defat milk) to obtain a final volume of 15 ml and the samples were vortexed for 15 s. After the solutions were

centrifuged (1500 × g for 15 min), the supernatants were transferred into tubes.

4 mL of a saturated sodium chloride solution was added to avoid foaming during the MeCN evaporation. MeCN was evaporated completely under a stream of nitrogen and then mixed with 15 ml of 0.05 M phosphate solution at pH 9 to obtain a pH from 8.5 to 8.7, depending on the milk sample used.

Oasis HLB cartridges were used and pre-conditioned with 2 mL of methanol, 2 mL of water and 2 mL of 0.05 M phosphate solution at pH 8.5. The samples were passed through the cartridge. The clean-up solution was 3 mL of phosphate solution at pH 8.5, 1 mL water and 1 mL 3% MeCN:H_2O in order to decrease matrix interference. The analytes were eluted with 3 mL of MeCN:MeOH:H_2O (30:40:30, v/v/v).

The elution fraction obtained from SPE was evaporated to dryness under a stream of nitrogen. 200 μl of water were added to dissolve the residue to be injected into the LC system.

2.4.1.2. Method 2. This method was used previously to determine penicillin levels in milk [10]. In summary, the extraction method involved addition of 0.5 mL of phosphate solution 0.1 M at pH 10, centrifugation of samples and an SPE process using HLB cartridges. The HLB cartridges were activated with 1 mL of methanol, 1 mL of water and 1 mL of 0.1 M phosphate solution at pH 10. After samples were passed through the system, the cartridge was cleaned with 3 mL of water to decrease matrix interference. The analytes were eluted with 2 mL of methanol.

2.4.1.3. Method 3. This method was previously applied to test quinolones [11]. The extraction procedure consisted of adding 2 mL of Milli-Q water to fortified milk samples and subsequent application of solid phase extraction on a Strata X cartridge. The cartridges were conditioned by passing 2 mL of MeOH and 2 mL of Milli-Q water. In the washing step, the clean-up solutions and order of addition were as follows: 1 mL of Milli-Q water, 1 mL of 0.5% TFA:MeOH (9:1, v/v), 1 mL of Milli-Q water, 1 mL of 1% TFA:MeCN (9:1, v/v) and finally, 1 mL of Milli-Q water. The analytes were eluted using 2 ml of 1% TFA:MeCN (25:75, v/v).

2.4.1.4. Method 4. This method was a mix of the three methods described above and consisted of weighing 2 g of sample and adding appropriate volumes of antibiotic working solutions. After vortexing for 1 min, the milk samples were kept at room temperature for 10 min and centrifuged (1500 × g for 10 min). The defatted milk was mixed with 15 ml of 0.05 M phosphate solution at pH 9.

The Oasis HLB cartridges were preconditioned with 2 mL of methanol, 2 mL of water and 2 mL of 0.05 M phosphate solution at pH 8.5. The samples were loaded onto the cartridge. The clean-up solution was 3 mL of phosphate solution at pH 8.5, 1 mL of water and 1 mL of 3% MeCN in H_2O. The analytes were eluted with 3 mL of MeCN:MeOH:H_2O (30:40:30, v/v/v).

The elution fraction obtained from SPE was evaporated to dryness under a stream of nitrogen. 200 μl of water were added to dissolve the residue to be injected into the LC system.

2.4.2. Chromatographic conditions

The mobile phase of LC–MS/MS was composed of water and MeCN with 0.1% formic acid in both solvents. The initial mobile phase was composed of H_2O:MeCN (85:15, v/v) with a pH of 3.2. The flow-rate was 1 mL/min. In the case of UPLC–MS/MS, the mobile phase and flow used were the same as those for LC but the initial mobile phase consisted of H_2O:MeCN (88:12, v/v).

Table 1 shows the gradient used for the separation of analytes in LC and UPLC. 20 μl aliquots of the extracts were injected into the LC–MS while 6 μl aliquots of the filtered extracts were injected into the UPLC.

Table 1
Gradients used for the separation of the substances studied by LC and UPLC.

Time (min)	%A	%B
LC–MS/MS		
0	15	85
2.0	15	85
4.0	45	55
7.0	56	44
8.5	56	44
10	15	85
11	15	85
UPLC–MS/MS		
0	12	88
0.28	12	88
2.36	45	55
3.06	45	55
3.40	12	88
3.64	12	88

2.4.3. LC–ESI-MS/MS parameters

The LC–ESI-MS/MS conditions were optimised by individual direct injection of each compound at a concentration of 10 μg/ml and a flow-rate of 0.05 mL/min. The turbo ion spray source was in positive mode with the following settings: capillary voltage 4500 V, nebuliser gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units) and drying gas (N_2) was heated to 400 °C and introduced at a flow-rate of 6500 mL/min. Table 2 shows the declustering potential (DP), focusing potential (FP) and entrance potential (EP) optimised to detect compounds with higher signals.

Multiple reaction monitoring (MRM) experiments in the positive ionisation mode were performed using a dwell time of 60 ms. The ions in the MRM mode were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole and analysed with the second analyser of the instrument. N_2 4 (arbitrary units) was used in CAD. Two transitions were followed for each analyte, one was used for quantification and the other for identification. Table 2 also shows these transitions with their optimum collision energy. For UPLC–MS/MS, a dwell time of 40 ms was used to detect all the analytes.

2.5. Quality parameters

The validation of the method was carried out using spiked raw milk samples. The quality parameters established were linearity range, recovery, precision, selectivity, decision limit (CC_α), detection capability (CC_β), limit of detection (LOD) and limit of quantification (LOQ) according to the EU regulation 2002/657/EC [24] and the Food and Drug Administration (FDA) guideline for bioanalytical assay procedure [6].

The linearity was tested from the calibration curves prepared from spiked milk samples at a concentration ranging from the LOQ of each analyte and 3 MRL. Each level was prepared in duplicate. PIPE and PIP were the internal standards used at a concentration of 100 μg/kg. The calibration curves were constructed using analyte/internal standard peak area ratio versus concentration of analyte/internal standard ratio.

The limit of detection (LOD) is the lowest concentration of analyte that the analytical process can reliably differentiate from background levels, while the limit of quantification (LOQ) is the lowest concentration of analyte that can be quantified. These were determined using spiked milk samples at different concentrations from 0.001 MRL to 0.1 MRL and were prepared in duplicate. LOD and LOQ values were calculated from a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Recovery experiments were performed by comparing the results for extracted standard samples of milk and internal standards added before the extraction procedure with the

Table 2
[M+H]+ ions, optimised parameters of the mass spectrometer and quantification and identification transitions for the substances studied and their optimum collision energy.

	m/z	SIM (Q₁ multiple ions/Q₁)				Transition quantification (CE)[a]	Transition identification (CE)
		IS	DP	FP	EP		
AMOX	366	4500	40	150	6	366 → 114 (28)	366 → 208 (19)
AMPI	350	4500	65	150	6	350 → 106 (26)	350 → 192 (21)
CLOX	436	4500	40	140	7	436 → 160 (20)	436 → 277 (20)
DICL	470	4500	50	150	8	470 → 160 (21)	470 → 311 (22)
NAFC	415	4500	50	120	9	415 → 199 (19)	415 → 256 (21)
OXAC	402	4500	40	160	9	402 → 160 (18)	402 → 243 (18)
PENG	335	4500	40	150	7	335 → 160 (16)	335 → 176 (16)
PIPE(IS)	518	4500	40	175	5	518 → 143 (27)	518 → 160 (16)
PIR	424	4500	40	150	5	424 → 292 (20)	424 → 181 (35)
QUI	529	4500	40	175	5	529 → 134 (20)	529 → 396 (20)
LEX	348	4500	30	125	5	348 → 140 (35)	348 → 158 (15)
LON	459	4500	30	125	5	459 → 152 (30)	459 → 337 (20)
ZOL	455	4500	40	175	5	455 → 323 (15)	455 → 295 (25)
PER	646	4500	50	200	11	646 → 290 (35)	646 → 530 (20)
TIO	524	4500	50	200	5	524 → 285 (30)	524 → 241 (25)
MAR	363	4500	45	200	10	363 → 320 (22)	363 → 345 (30)
CIP	332	4500	45	200	10	332 → 314 (32)	332 → 288 (27)
DAN	358	4500	45	200	10	358 → 340 (31)	358 → 283 (31)
ENR	360	4500	45	200	10	360 → 316 (29)	360 → 342 (29)
FLU	262	4500	38	200	10	262 → 244 (26)	262 → 202 (45)
PIP(IS)	304	4500	50	200	10	304 → 286 (30)	304 → 261 (25)

[a] Collision energy (V).

non-extracted standards prepared at the same concentrations in blank extracts representing 100% recovery. The concentration range was from LOQ and 3 MRL for each compound.

Intra-day precision was assessed by comparing the results of five replicates prepared the same day at three different concentrations (0.5 MRL, 1 MRL and 2 MRL). The procedure was repeated to determine inter-day precision by comparing results from samples prepared and analysed on three different days. The relative standard deviations (%RSD) were calculated.

The decision limit (CC$_\alpha$) is the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. Detection capability (CC$_\beta$) means the smallest content of a compound that may be detected, identified and/or quantified in a sample with an error probability of β [24,27]. CC$_\alpha$ values were determined by analysing 20 blank samples fortified with quinolones, penicillins and cephalosporins at MRL concentrations. CC$_\beta$ was calculated as the decision limit CC$_\alpha$ plus 1.64 times the corresponding standard deviation (β = 5%), supposing that the standard deviation at the MRL is similar to that obtained at the CC$_\alpha$ level.

2.6. Matrix effect

Matrix effect was evaluated by preparing standard solutions of analytes studied and solutions of analytes in milk. Milk from four different batches was used to evaluate the matrix effect. Aliquots of the corresponding milk were subjected to SPE and the extracts obtained were spiked separately with PENG, PIPE and PIP. Ten replicates were prepared of each batch of milk. Ten standard solutions of PENG, PIPE and PIP were prepared in water at the same concentration as the matrix-matched solutions. All samples were analysed by LC and UPLC.

2.7. Positive samples

56 samples that tested positive in the screening test were obtained from the "Control Laboratori Interprofessional Lleter de Catalunya (ALLIC)" in different batches. The effectiveness of the developed method was checked by analysing the samples with the M2 method. The samples were labelled as M1–M56. The samples were assessed in duplicate when there were sufficient amounts.

3. Results and discussion

3.1. Optimisation of the LC conditions

Co-eluting compounds originating from the matrix can enhance or suppress signals. When analytes and matrix compounds enter the ion source simultaneously, the ionisation efficiency of the analyte might be affected. Therefore, improvements in chromatographic separation, complete separation of analytes is not required in MS/MS detection, could result in decreased amounts of matrix compounds co-eluting with the analyte and thus, reduce the matrix effect. Consequently, chromatographic gradient conditions were adjusted to keep chromatographic run times as short as possible and also to achieve nearly complete separation of all analytes. The optimised LC conditions are described in Section 2.4.3. Fig. 1 shows the separation of the antibiotics at their MRL levels by LC and UPLC. As shown in Fig. 1A, LC–MS/MS separated penicillins, cephalosporins and quinolones in less than 10 min. To use UPLC, the LC gradient was modified by a gradient converter software included in the UPLC system. The change of the gradient was necessary as the column and working pressure of the equipments were different. With the new gradient, analysis of the 21 drugs was achieved in less than 4 min (Fig. 1B).

3.2. Preliminary study

In this study, commercial whole milk samples were used to test the four methods described above. This milk was used because it was easier to obtain and also contained less fat than raw milk and, therefore, easier to treat. The results of the study are given in Fig. 2. Method 1 (M1) had very high recoveries for most of the analytes, but too low for AMOX, AMPI, CIP and LEX. Moreover, its results were scattered. With method 3 (M3) using an acidic medium, some analytes were not observed, possibly due to degradation. It was a fast method, but the recoveries of cephalosporins were lower than those obtained with method 2 (M2). Method 4 (M4) had intermediate recoveries, but the recovery was only around 20% for AMOX. Furthermore, the elution solution had a large percentage of water (30%), making the evaporation slower and the method very long. The best method was M2 and although it did not elicit the best recoveries for penicillins, these recoveries were still quite good. In
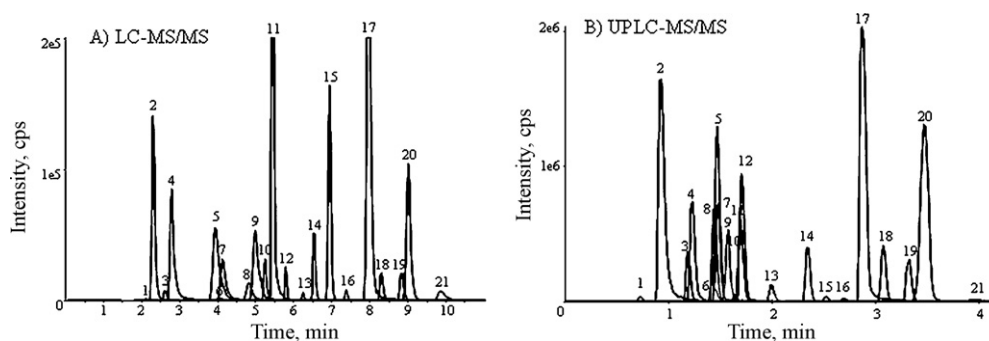
Fig. 1. Chromatogram obtained from a milk sample fortified at the MRL level using: (A) LC–MS/MS; (B) UPLC–MS/MS. Peaks: (1) AMOX, (2) PIR, (3) QUI, (4) PIP, (5) MAR, (6) AMPI, (7) LEX, (8) LON, (9) CIP, (10) DAN, (11) ENR, (12) ZOL, (13) PER, (14) TIO, (15) PIPE, (16) PENG, (17) FLU, (18) OXAC, (19) CLOX, (20) NAFC and (21) DICL.
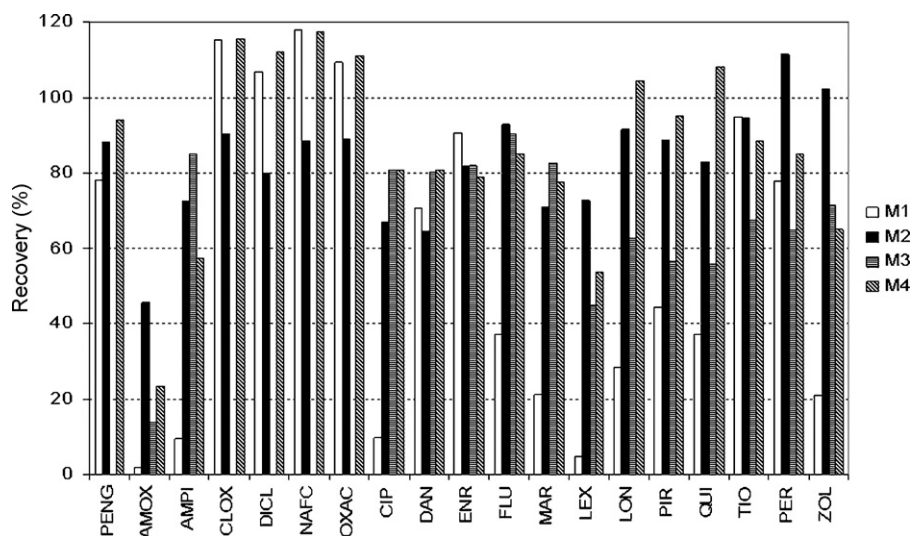
Fig. 2. Recoveries obtained by application of the different methods tested. Study made with commercial milk.
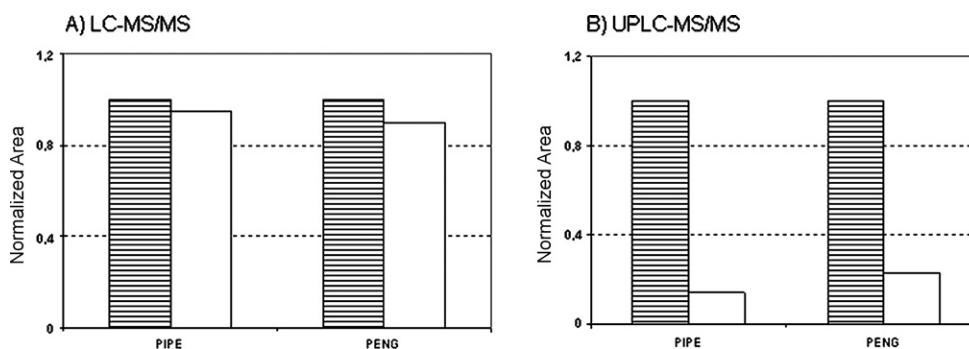
Fig. 3. Matrix effect on the response of PIPE and PENG in milk using: (A) LC–MS/MS and (B) UPLC–MS/MS.

addition, the best results for AMOX and LEX were obtained by this method.

3.3. Optimisation of the extraction method

Some experiments using M2 were performed to confirm whether commercial and raw milk samples gave similar results. The recoveries were similar for most of the analytes, but there were noticeable differences (∼40%) in compounds like DICL, PIR, CIP and ENR. As a consequence, the following studies were carried out using raw milk.

The evaporation step was also optimised. Air and nitrogen were tested and the recoveries obtained for quinolones and penicillins were similar for both. However, using nitrogen as the drying gas gave better results for cephalosporins, where differences higher than 20% were obtained for LON and ZOL.

3.4. Matrix effect

Some researchers have focused on optimising sample preparation to reduce matrix effect, while others have assessed the level of matrix effect and compensated for the alteration in signal using an internal standard (IS), often a stable isotope-labelled analogue of the drug [28–31] or, if it is lacking, a structural analogue. However, in a multi-component analysis, finding an appropriate IS for every analyte might be difficult or impossible. In this study, two

Table 3
Quality parameters obtained for cephalosporins by LC–MS/MS and UPLC–MS/MS.

	LEX	LON	PER	PIR	QUI	TIO	ZOL
LC–ESI-QqQ							
MRL (μg/kg)	100	20	50	60	20	100	50
LOD (μg/kg)	0.3	0.3	0.5	0.1	0.2	0.3	0.15
LOQ (μg/kg)	1	1	1.25	0.3	0.5	0.5	0.3
Calibration curve	$y = 0.506x - 0.0123$	$y = 0.953x + 0.0058$	$y = 0.026x + 0.0020$	$y = 3.090x + 0.0069$	$y = 0.431x - 0.0033$	$y = 0.407x + 0.0089$	$y = 0.248x + 0.0192$
Regression (r)	0.995	0.994	0.993	0.993	0.991	0.993	0.990
Recovery (%)	87 ± 2	110 ± 3	81 ± 3	107 ± 3	98 ± 3	102 ± 3	99 ± 5
Precision (%RSD)							
Intra day (n = 15)[a]	6–9	8–12	6–10	7–12	6–8	4–10	8–13
Inter day (n = 45)[a]	5–8	6–13	11–14	8–12	8–13	7–10	11–14
CC_α	114	25	59	69	24	110	61
CC_β	127	29	67	78	29	120	72
UPLC–ESI-QqQ							
MRL (μg/kg)	100	20	50	60	20	100	50
LOD (μg/kg)	0.3	0.06	0.3	0.2	0.02	0.75	0.25
LOQ (μg/kg)	1	0.2	1.25	0.5	0.06	2.5	0.5
Calibration curve	$y = 0.890x + 0.2341$	$y = 3.131x + 0.1309$	$y = 0.308x + 0.0103$	$y = 5.134x + 0.5362$	$y = 1.817x + 0.0236$	$y = 0.500x + 0.0681$	$y = 1.190x + 0.1341$
Regression (r)	0.992	0.992	0.992	0.993	0.997	0.991	0.991
Recovery (%)	95 ± 4	100 ± 3	89 ± 3	100 ± 6	112 ± 2	91 ± 3	101 ± 5
Precision (%RSD)							
Intra day (n = 15)[a]	6–9	6–13	7–10	6–10	6–12	7–12	8–11
Inter day (n = 45)[a]	6–13	8–13	9–11	9–15	9–12	7–14	10–13
CC_α	110	24	59	66	23	113	59
CC_β	120	28	68	72	26	126	67

[a] The intra-day and the inter-day data showed are the minimum and maximum values obtained in analysis of the samples prepared at 0.5 MRL, 1 MRL and 2 MRL.

Table 4
Quality parameters obtained for penicillins by LC–MS/MS and UPLC–MS/MS.

	AMOX	AMPI	CLOX	DICL	OXAC	NAFC	PENG
LC–ESI-QqQ							
MRL (μg/kg)	4	4	30	30	30	30	4
LOD (μg/kg)	0.1	0.1	<0.1	0.15	<0.1	0.03	0.04
LOQ (μg/kg)	0.3	0.3	0.15	0.5	0.1	0.1	0.1
Calibration curve	$y = 0.383x - 0.00018$	$y = 3.22x - 0.0012$	$y = 0.74x - 0.0014$	$y = 0.727 - 0.0176$	$y = 5.490x + 0.0088$	$y = 1.380x + 0.0081$	$y = 1.960x + 0.0037$
Regression (r)	0.990	0.993	0.995	0.995	0.992	0.996	0.995
Recovery (%)	55 ± 3	79 ± 2	90 ± 2	99 ± 2	83 ± 3	99 ± 3	81 ± 1
Precision (%RSD)							
Intra day (n = 15)[a]	6–10	7–11	7–9	6–9	4–8	7–12	4–7
Inter day (n = 45)[a]	10–14	8–13	6–8	8–9	6–8	8–9	7–9
CC_α	5	4.6	34	34	35	33	4
CC_β	6	5.3	38	38	40	36	5
UPLC–ESI-QqQ							
MRL (μg/kg)	4	4	30	30	30	30	4
LOD (μg/kg)	0.3	0.1	0.15	2.4	0.1	0.03	0.2
LOQ (μg/kg)	<1	0.4	0.4	9	0.3	0.1	0.4
Calibration curve	$y = 0.699x - 0.0043$	$y = 4.449x - 0.0161$	$y = 1.251x + 0.0036$	$y = 0.729 - 0.0069$	$y = 1.642x + 0.0056$	$y = 9.134x + 0.6741$	$y = 1.992x - 0.0019$
Regression (r)	0.994	0.995	0.990	0.993	0.990	0.992	0.995
Recovery (%)	52 ± 3	85 ± 2	96 ± 3	99 ± 3	96 ± 4	108 ± 6	88 ± 2
Precision (%RSD)							
Intra day (n = 15)[a]	4–13	6–9	5–11	3–9	5–10	4–10	3–9
Inter day (n = 45)[a]	7–13	7–13	10–13	9–11	10–13	7–12	6–12
CC_α	4.7	4.7	35	35	36	35	5
CC_β	5.4	5.4	39	40	41	40	5

[a] The intra-day and the inter-day data showed are the minimum and maximum values obtained in analysis of the samples prepared at 0.5 MRL, 1 MRL and 2 MRL.

IS were chosen to correct for matrix effect: PIPE and PIP. PIPE was chosen as an IS for the quantification of β-lactams (cephalosporins and penicillins), while PIP was chosen as the IS for quantifying quinolones.

During the quantification of β-lactams (penicillins and cephalosporins) by UPLC–MS/MS, some inconsistencies were observed in the calibration curves, where linear correlation between the analyte/IS (PIPE) areas and the concentration ratio was only good for PENG. There were no such problems with LC–MS/MS analysis since chromatographic separation was different from that of UPLC and there were no matrix components co-eluting with the analytes that could suppress the ions. Regarding the analyte sig-

nal for PENG and its IS (PIPE), there was poor reproducibility of the signal and either ion suppression.

All data from β-lactams were treated without using the IS (PIPE) and a significant increase in the linearity of the calibration curves was observed, except for PENG, where linearity did not improve. Unfortunately, this did not give satisfactory precision. These results are not surprising given the lack of an IS, which plays a very important role in compensating for variation resulting from samples, instrument, manipulation or matrix effect. The β-lactams were analysed with PIP as the IS, which significantly increased the linearity of the calibration curves of all β-lactams except PENG.

Table 5
Quality parameters obtained for quinolones by LC–MS/MS and UPLC–MS/MS.

	CIP	DAN	ENR	FLU	MAR
LC–ESI-QqQ					
MRL (μg/kg)	100	30	100	50	75
LOD (μg/kg)	<0.1	0.03	<0.1	0.06	0.2
LOQ (μg/kg)	0.1	0.1	0.1	0.15	0.5
Calibration curve	$y = 1.360x - 0.0053$	$y = 0.185x - 0.0089$	$y = 15.2x - 1.300$	$y = 40.0 + 3.16$	$y = 5.65x - 0.569$
Regression (r)	0.992	0.991	0.990	0.996	0.995
Recovery (%)	71 ± 2	39 ± 2	68 ± 3	101 ± 5	81 ± 4
Precision (%RSD)					
Intra day ($n = 15$)[a]	5–12	6–14	7–11	5–13	3–5
Inter day ($n = 45$)[a]	8–11	9–12	10–14	10–12	10–14
CC_α	109	37	112	59	82
CC_β	119	44	124	68	89
UPLC–ESI-QqQ					
MRL (μg/kg)	100	30	100	50	75
LOD (μg/kg)	<0.1	0.03	0.3	<0.05	0.375
LOQ (μg/kg)	0.1	0.1	0.5	0.05	0.75
Calibration curve	$y = 1.525x - 0.0262$	$y = 0.175x - 0.0118$	$y = 12.2x - 0.994$	$y = 34.5 + 3.672$	$y = 4.57x - 0.385$
Regression (r)	0.994	0.995	0.995	0.990	0.990
Recovery (%)	95 ± 3	49 ± 5	79 ± 3	99 ± 6	88 ± 3
Precision (%RSD)					
Intra day ($n = 15$)[a]	7–10	8–13	10–13	9–11	6–9
Inter day ($n = 45$)[a]	7–9	10–13	8–13	8–14	7–10
CC_α	108	36	109	57	84
CC_β	116	41	119	64	93

[a] The intra-day and the inter-day data showed are the minimum and maximum values obtained in analysis of the samples prepared at 0.5 MRL, 1 MRL and 2 MRL.
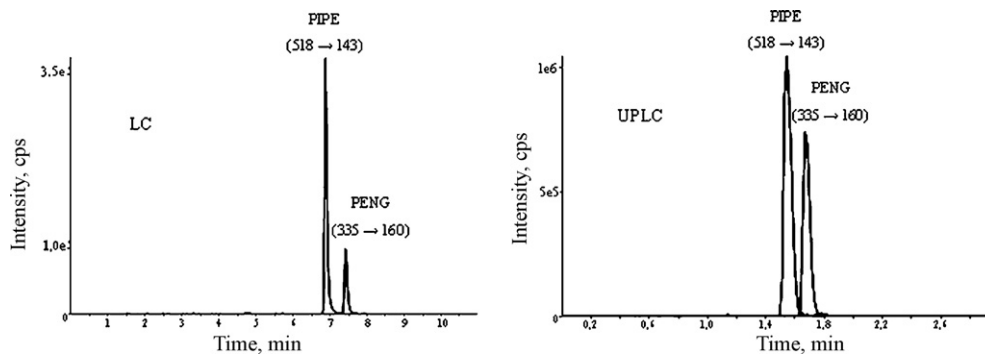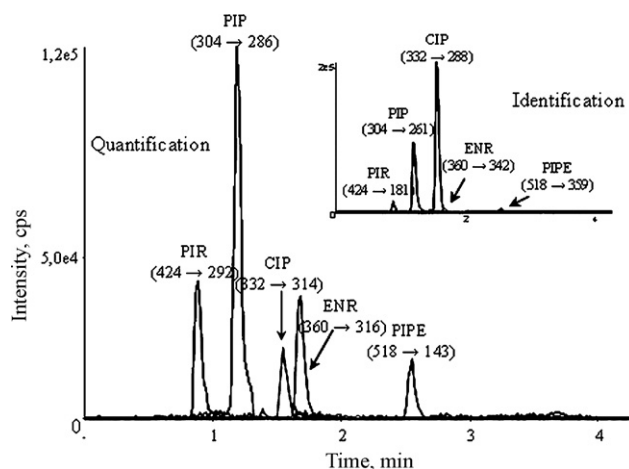

Fig. 4. Chromatograms obtained for a non compliant sample of raw milk in PENG by LC–MS/MS and UPLC–MS/MS.
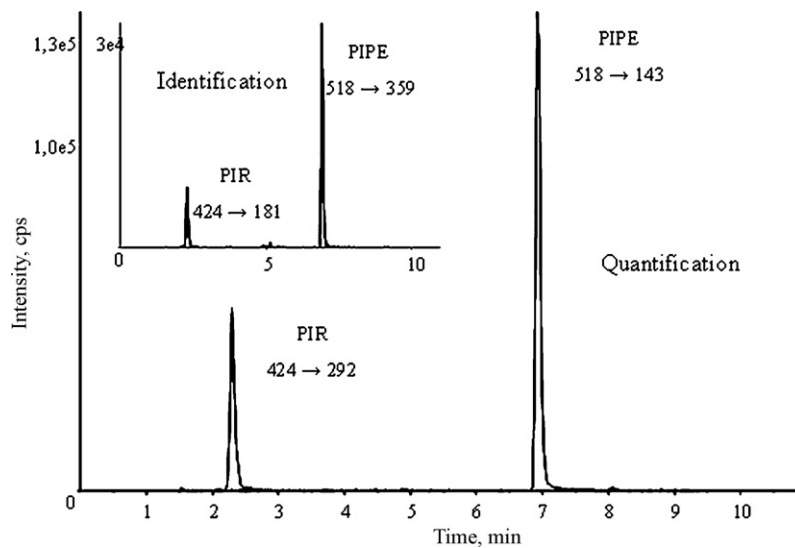
Fig. 5. Chromatogram obtained for a positive sample of raw milk in PIR by LC–MS/MS.

Table 6
Analysis of positive milk samples by LC–MS/MS and UPLC–MS/MS.

	Antibiotic found	Concentration (μg/kg)	
		LC–MS/MS	UPLC–MS/MS
M1	AMOX, PER	1.3 (0.1), 39 (4)	1.1 (0.1), 55 (8)
M2	PENG	5.9 (0.2)	6.9 (0.7)
M3	AMOX	1.2 (0.2)	1.2 (0.4)
M4	AMOX	7.0 (0.2)	7.1 (0.2)
M5	PENG	19.0 (0.3)	20.0 (0.1)
M6	PENG	14.0 (0.4)	14.0 (0.1)
M7	PENG	29.0 (0.3)	28 (2)
M8	AMOX	3.0 (0.5)	3.2 (0.5)
M9	AMOX	4.8 (0.6)	6.2 (1.8)
M10	LEX	36 (12)	37 (18)
M11	LEX	66 (4)	60 (13)
M12	LEX	128 (15)	136 (41)

Fig. 6. Chromatogram obtained for a positive sample of raw milk in PIR and ENR by UPLC–MS/MS.

Possible matrix effect on the signals for PIPE (IS) and PENG was evaluated by studying the difference between the mass spectrometric signal for PIPE and PENG in standard solution and the signal for these compounds in a biological matrix, such as milk. Milk from four different batches was used to evaluate the matrix effect, using the same samples for LC and UPLC.

Fig. 3 shows the normalised area for PIPE and PENG for samples analysed by LC–MS/MS (Fig. 3A) and UPLC–MS/MS (Fig. 3B). The areas for PIPE and PENG in milk and standard solutions obtained by LC–MS/MS did not show significant differences. However, the areas for PIPE and PENG were very much lower in milk samples than in standard solutions for UPLC–MS/MS analysis. These differences could be attributed to the matrix effect, which can be calculated using the modified version of the equation described by Matuszewki [31,32]:

$$\% \ \text{matrix effect} = \left(\frac{A_{\text{milk}}}{A_{\text{standard solution}}} - 1 \right) \times 100$$

where A_{milk} is the area for the analyte in milk and $A_{\text{standard solution}}$ is the area for the compound in standard solution. Therefore, around 80% matrix effect was observed for PENG and PIPE with UPLC. However, the same evaluation performed with PIP did not show any ion suppression. Thus, PIP was used to quantify β-lactams undergoing UPLC.

PIPE and PENG have similar retention times in UPLC. We think that their matrix effect are compensated and for this reason PENG is the only β-lactam that offers a good linearity in its quantification when PIPE is used as IS.

No matrix effect was observed for LC and subsequently, β-lactam quantification was conducted using PIPE as the internal standard instead of PIP.

3.5. Method validation

The optimised extraction method was validated for penicillins, cephalosporins and quinolones according to the European Union regulation 2002/657/EC [24] and the FDA guideline for bioanalytical assay procedure [6].

3.5.1. Linearity range

Linearity was evaluated using calibration curves (prepared in blank milk spiked with antibiotics before SPE) where the relationships between peak area and concentration are represented. Due to the problems observed for PIPE in UPLC, PIP was used as the IS for all analytes except PENG. In LC, no problems were found with PIPE, which was therefore used as the IS for β-lactams, while PIP was used for quinolones. As shown in Tables 3–5 for cephalosporins, penicillins and quinolones, respectively, all compounds presented good linearity in the concentration range studied (from 0.1 to 3 MRL) with a correlation coefficient higher than 0.990 for both LC and UPLC.

3.5.2. LOD and LOQ

LOD and LOQ values were determined in milk samples spiked at different levels, taking signal-to-noise ratios of 3 and 10, respectively. As displayed in Tables 3–5, LOD values ranged from 0.03 to 0.5 μg/kg and LOQ values from 0.1 to 1.25 μg/kg using LC. On the other hand, with UPLC, the range of LOD was 0.02–0.75 μg/kg and 0.1–2.5 μg/kg for LOQ, except for DICL, which had a high LOQ value of 9 μg/kg. This high value could be because DICL was the last compound to be eluted and a broadening peak was obtained. In any case, the results obtained were always lower than the MRLs.

3.5.3. Accuracy

The accuracy of the method was assessed by a recovery test. The recovery of the different compounds was calculated by calibration and external curve comparison. In Tables 3–5, the results for all antibiotics are shown with their associated standard deviation (SD). All drugs analysed by LC–MS/MS had recoveries higher than 70% with the exception of AMOX (55%), ENR (68%) and DAN (39%). When drugs were evaluated by UPLC–MS/MS, the recoveries were higher than 75% except for AMOX (52%) and DAN (49%).

3.5.4. CC_α and CC_β

The revised criteria of 657/2002/EC [24] introduced CC_α and CC_β to replace the LOD and LOQ, respectively. These parameters were established for each compound at their MRL. Tables 3–5 show CC_α values with an error of 5% (probability of false non-compliance ≤5%) and CC_β values with an error of β = 5% (probability of false compliance ≤5%). Comparable results were obtained for drugs screened by LC–MS/MS and UPLC–MS/MS.

3.5.5. Precision studies

The precision of the method was evaluated in terms of repeatability (same day – intra-day precision) and intermediate precision (different days – inter-day precision). The intra-day and inter-day precision of the methods were evaluated at three concentrations. To quantify the concentration of samples, the calibration curves were used. The repeatability values expressed as RSD % were lower than 15%, which is within the acceptance criteria of the FDA [6] for validating analytical methods (Tables 3–5).

Table 7
Samples of milk analysed by LC–MS/MS or UPLC–MS/MS.

	Antibiotic found	Concentration (μg/kg).
LC–MS/MS		
M13	AMOX	7 (2)
M14	AMOX	4.7
M15	AMOX	46
M16	CLOX, LON	51.5 (0.9), 4.4(0.2)
M17	PIR	11.3 (0.7)
M18	PENG	3.5 (0.4)
M19	PIR	7.5 (0.8)
M20	AMPI, DICL	2.9 (0.3), 2.5 (0.2)
M21	ENR, CIP	11.1 (0.1), 22.7 (0.9)
M22	ENR, CIP	11.3 (0.4), 24.1(0.5)
M23	PIR	6.3 (0.6)
M24	PIR	5.6 (0.2)
M25	PIR	8.4 (0.6)
M26	PENG	4.1 (0.3)
M27	PIR	8.0 (0.4)
M28	PENG	1.6 (0.0)
M29	AMOX	19.0 (4.0)
M30	AMOX	10.0 (0.7)
M31	AMPI, DICL	5.95 (0.02), 6.2 (0.4)
M32	AMOX, PER	6.5 (1.3), 7.7 (0.4)
M33	PIR	9.6
M34	PENG	6.5
M35	PIR	22.5 (0.8)
M36	PENG	0.95 (0.05)
M37	PENG	3.7 (0.2)
M38	PIR	15 (2)
M39	PENG	3.4 (0.6)
M40	PENG	2.7 (0.2)
M41	PENG	3.8 (0.2)
M42	PENG	3.8 (0.2)
M43	PENG	3.5 (0.1)
M44	PENG	5.1 (0.1)
M45	AMOX	1.9 (0.4)
M46	PENG	14.5
M47	PENG	13 (2)
M48	AMOX	42 (4)
UPLC–MS/MS		
M49	PIR, ENR, CIP	5.1, 4.8, 7.6
M50	AMOX	2.1 (0.3)
M51	PENG	3.3 (0.5)
M52	PIR	3.6 (0.4)
M53	AMPI, CLOX	2.6 (0.2), 17.7 (0.9)
M54	ENR, CIP	3.5 (0.1), 18 (2)
M55	PIR, ENR, CIP	3.5 (0.6), 7.4 (0.3), 28(2)
M56	PENG	1.2 (0.1)

3.5.6. Applicability of the method

56 samples from animals treated with β-lactams or quinolones were studied. The samples were analysed in duplicate when there were sufficient amounts. To see if the LC–MS/MS and UPLC–MS/MS techniques were comparable, 12 samples were analysed using both techniques. Table 6 shows the results of these samples. The results obtained with the two techniques were similar except for M9, a sample that contained AMOX (6.2 μg/kg) that was non-compliant with UPLC (CC$_\beta$ = 5.4 μg/kg), while the value found (4.8 μg/kg) is compliant with LC (CC$_\beta$ = 6 μg/kg). Since both LC and UPLC detected similar concentrations of antibiotics in the 12 samples evaluated, the remaining samples were only analysed by one of the techniques. Table 7 gives the results of the rest of the samples analysed by LC–MS/MS or UPLC–MS/MS. As seen in Tables 6 and 7, all the milk samples contained at least one antibiotic, while residues of more than one antibiotic from the same or different families were found in 8 samples, indicating multiple uses of β-lactams and quinolones. Five samples, M21, M22, M49, M54 and M55, were found positive for the quinolones ENR and its metabolite CIP.

Among all the samples, 38 were found to be fit for human consumption, according to European Union regulation (Tables 6 and 7). Only 18 samples were considered non-compliant with current EU legislation, having an error probability of β because the concentra-

tion calculated in these samples were higher than the CC$_\beta$ values determined previously. AMOX, PENG and PIR were the most common drugs found in the 56 samples. 39% of the non-complaint samples contained AMOX and 44% had PENG.

Fig. 4 illustrates, as an example, the chromatogram obtained for a sample positive for PENG by LC–MS/MS and UPLC–MS/MS. The sample contained a high concentration (19 μg/kg) of this penicillin and was non-compliant (CC$_\beta$ = 5 μg/kg), thus not fit for human consumption.

Figs. 5 and 6 show chromatograms of samples M35 and M55 analysed by only one of the techniques studied and with the corresponding confirmatory chromatogram. Both samples were considered compliant because their residue concentrations were lower than the calculated CC$_\beta$. Fig. 5 shows the results of a sample containing 22.5 μg/kg of the cephalosporin PIR, while Fig. 6 gives the results of a sample containing 3.5 μg/kg of PIR and 7.4 μg/kg of ENR. The metabolite of ENR, CIP, was also found at a higher concentration (28 μg/kg) than that of its parent compound, demonstrating that ENR is metabolized mostly into CIP. However, the concentration detected was lower than the EU-regulated MRL.

4. Conclusions

A multi-class, multi-residue method was developed and validated for the simultaneous determination of 19 antibiotics regulated by the European legislation 37/2010/EC in raw cow milk using LC–MS/MS and UPLC–MS/MS. The method simultaneously detected substances from three families of antibiotics with acceptable quality parameters. The recoveries of the antibiotics were higher than 70%, except for AMOX and DAN. The LOD and LOQ values were lower than the established MRLs and the other parameters were also in accordance with European regulation. UPLC technology demonstrated significant advantages with respect to speed, sensitivity and resolution, making it an attractive option for the analysis of antibiotics in milk. However, the fast gradient used in UPLC promoted matrix effects by reducing chromatographic separation between analytes and endogenous milk compounds. Among all the samples analysed (56 real positive samples provided by the "Laboratori Interprofessional Lleter de Catalunya" (ALLIC) Barcelona, Spain), 38 of them (∼70%) were found to be fit for human consumption. AMOX and PENG were the most common residues found. 39% of the non-complaint samples contained AMOX and 44% contained PENG.

References

[1] EU (2003) Council Regulation No 1831/2003 of the European Parliament and of the Council of 22 September 2003, Off. J. Eur. Commun. L268 (2003) 29.
[2] A.M. Hammerum, O.E. Heuer, C.H. Lester, Y. Agers, A.M. Seyfarth, H.D. Emborg, N. Frimodt-Moller, D.L. Monnet, Int. J. Antimicrob. Agents 30 (2007) 466.
[3] A. Fabrega, J. Sánchez-Céspedes, S. Soto, J. Vila, Int. J. Antimicrob. Agents 31 (2008) 307.
[4] The European Agency for the Evaluation of Medicinal Products. http://www.emea.europa.eu/htms/vet/mrls/a.htm.
[5] Commission of the European Communities, Diario Oficial de las Comunidades Europeas No. 37/2010 L15/1, December 22, 2009.
[6] US Department of Health and Human Services, Centre for Drug Evaluation and Research, Centre for Veterinary Medicine, May 2001. http://www.fda.gov/cder/guidance/index.htm.

[7] F. Davis, S.P.J. Higson, Pediatr. Res. 67 (2010) 476.
[8] D. Ortelli, E. Cognard, P. Jan, P. Edder, J. Chromatogr. B 877 (2009) 2363.
[9] J.L. Martínez-Vidal, A. Garrido-Frenich, M.M. Aguilera-Luiz, R. Romero-González, Anal. Bioanal. Chem. 397 (2010) 2777.
[10] M. Martínez-Huélamo, E. Jiménez-Gámez, M.P. Hermo, D. Barrón, J. Barbosa, J. Sep. Sci. 32 (2009) 2385.
[11] M.P. Hermo, E. Nemutlu, S. Kir, D. Barrón, J. Barbosa, Anal. Chem. Acta 613 (2008) 98.
[12] E.A. Christodoulou, V. Samanidou, J. Sep. Sci. 30 (2007) 2421.
[13] N.V. Hoof, K.D. Wasch, L. Okerman, W. Reybrtoeck, S. Poelmans, H. Noppe, H.D. Brabander, Anal. Chem. Acta 529 (2005) 265.
[14] M. Becker, E. Zittlau, M. Petz, Eur. Food Res. Technol. 217 (2003) 449.
[15] S. Riediker, A. Rytz, R.H. Stadler, J. Chromatogr. A 1054 (2004) 359.
[16] L. Kantiani, M. Farré, M. Sibum, C. Postigo, M. López de Alda, D. Barceló, Anal. Chem. 81 (2009) 4285.
[17] M. Becker, E. Zittlan, M. Petz, Anal. Chim. Acta 520 (2004) 19.
[18] M.M. Aguilera-Luiz, J.L. Martínez Vidal, R. Romero-González, A. Garrido Frenich, J. Chromatogr. A 1205 (2008) 10.
[19] A.A.M. Stolker, P. Rutgers, E. Oosterink, J.J.P. Lasaroms, R.J.B. Peters, J.A. van Rhijn, M.W.F. Nielen, Anal. Bioanal. Chem. 391 (2008) 2309.
[20] S.B. Turnipseed, W.C. Andersen, C.M. Karbiwnyk, M.R. Madson, K.E. Miller, Rapid Commun. Mass Spectrom. 22 (2008) 1467.
[21] S. Ghidini, E. Zanardi, G. Varisco, R. Chizzolini, Food Addit. Contam. 20 (2003) 528.
[22] D.M. Holstage, B. Puschner, G. Whitehead, F.D. Galey, J. Agric. Food Chem. 50 (2002) 406.
[23] M.C. Moreno-Bondi, M.D. Marazuela, S. Herranz, E. Rodríguez, Anal. Bioanal. Chem. 395 (2009) 921.
[24] Official J. European Communities, Diario Oficial de las Comunidades Europeas No. 2002/657/EC, August 17, 2002.
[25] A. de Villiers, F. Lestremau, R. Szucs, S. Gelebart, F. David, P. Sandra, J. Chromatogr. A 1127 (2006) 60.
[26] D. Guillarme, J. Ruta, S. Rudaz, J.L. Veuthey, Anal. Bioanal. Chem. 397 (2010) 1069.
[27] E. Verdon, D. Hurtaud-Pessel, P. Sanders, Accred. Qual. Assur. 11 (2006) 58.
[28] J. Zrostlikova, J. Hajslová, J. Poustka, P. Begany, J. Chromatogr. A 973 (2002) 13.
[29] J. Kang, L.A. Hick, W.E. Price, Rapid Commun. Mass Spectrom. 21 (2007) 4065.
[30] M. Villagrasa, M. Guillamón, E. Eljarrat, D. Barceló, J. Chromatogr. A 1157 (2007) 108.
[31] E. Chambers, D.M. Wagrowski-Diehl, Z. Lu, J.R. Mazzeo, J. Chromatogr. B 852 (2007) 22.
[32] B.K. Matuszewski, M.L. Constanze, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.