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Residues of β-lactams and quinolones in tissues and milk samples. Confirmatory analysis by liquid chromatography–mass spectrometry

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Abstract The aim of this work is to optimize and validate methods for the multiresidue determination of series of families of antibiotics as quinolones, penicillins and cephalosporins included in European regulation in food samples using LC-MS/MS. Different extraction techniques and clean-up applied to antibiotics in meat were compared. The quality parameters were established according with EU guideline. The developed method was applied to 49 positive raw milk samples from animals medicated with different antibiotics; 63% of the analyzed samples were found to be compliant and adequate for human consumption.

Keywords: quinolones, penicillins, cephalosporins, animal tissues, milk, LC-MS/MS.

1. Introduction

Over the last decades the food production systems has changed from small to large-scale intensive farming. One of the consequences of this evolution has been the increasing administration of antimicrobial agents to food producing animals, in order to control the spread of infections in the farm [1]. Veterinary drugs require a rational use in the efficient production of food of animal origin. Some veterinary drugs are use at low levels of concentration as growth promoters; at intermediate levels they can prevent diseases, while high levels are used to treat infected animals [2-3]. As a consequence, the presence if residual amounts of veterinary drugs that remain in the different tissues of medicated animals can increase the risk of adverse effects or antibiotic resistance on people consuming them [4-6].

The possibility to develop antibiotic resistance has prompted European and USA health authorities to strictly limit the levels of these substances in foodstuffs and raw materials, used in food manufactured. To safeguard human health, the EU has established safe maximum residues limits (MRLs) for residues of veterinary drugs in animal tissues entering the human food chain. The establishment of the MRLs in the EU is governed by Commission Regulation (EU) No 37/2010. This Regulation repealing Council Regulation (EEC) 2377/90 and its amendments and regulates the authorized drugs that can be applied for therapeutic veterinary use in animals intended for food production [7-9].

Antibacterial agents, also known as antimicrobials, include synthetic and natural compounds; these last, well-known as antibiotic, are substances of low molecular weight produced by fungi and bacteria such as β-lactams, macrolides or tetracyclines. At the present the term “antibiotic” is used as a synonymous with “antibacterial”, so it includes synthetic drugs such as quinolones. [10-13]. In this work attention is paid to the analysis of quinolones and β-lactams.

Quinolones are synthetic antimicrobial agents used in veterinary and human medicine in special for the treatment of respiratory diseases, urinary tract infections and enteric bacterial infections. Quinolones act principally by inhibiting DNA-gyrase in bacterial cells. These antimicrobial agents have demonstrated broad-spectrum activity against many pathogenic gram-negative and gram-positive bacteria [14-15].

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β-lactams are probably the most widely used class of antibiotics in veterinary medicine for the treatment of bacterial infections of animals used in livestock farming and bovine milk production. β-lactams consist basically of two classes of thermally labile compounds: penicillins and cephalosporins. Both classes contain bulky side chain attached respectively to 6-aminopenicillanic acid or 7-amino cephalosporanic acid nuclei [12, 13]. The modifications made on the side chain have enlarged the antibiotic spectrum to include gram-negative and gram-positive bacteria. The MRLs established in the Commission Regulation (EU) No 37/2010 depend of the substances and on the matrix.

Quinolones range between 100 and 400 μg/kg in beef muscle, and 30-100 μg/kg in milk. Penicillins range between 50-300 μg/kg in beef muscle and 4-30 μg/kg in milk while cephalosporins ranged from 50 μg/kg to 1000 μg/kg in beef muscle and from 20 to 100 μg/kg in milk [7].

The control of abuse is at present based on screening procedures, as immunoassays, which are often too specific and do not distinguish between members of a class of antibiotics. These methods provide only semi-quantitative results and sometimes give rise to false positives, but they are widely used because of their simplicity, sensitivity, speed and cheapness [13]. However, it may be necessary to perform additional confirmatory methods by using alternative analytical techniques, sufficiently selective and sensitive, like LC-MS or LC-MS/MS. According with the Decision 2002/657/EC [16], a number of identification points (IP) must be collected to confirm the identity of a compound and MS detection is almost mandatory to earn the required IPs per compound.

Numerous methods exist to detect and quantify antibiotic residues in milk and edible tissues; however, efforts to improve existing methods remain an active area of research. These methods allow the detection or quantification of antibiotics from single-analyte to multiresidue or multiclass methods, being the objectives different depending on the approach [2,13, 17-27]. In this study, multiresidue methods were developed and optimized to allow the determination of the series of quinolones, penicillins and cephalosporines, regulated by European Union legislation, in muscle and milk of beef. The different steps (extraction, separation and detection) were optimized. A new method, the dispersive-SPE greatly simplifies and accelerates sample clean-up [18, 28-29] and the results were compared with those obtained with conventional methods as SPE. The methods were validated according with Commission Decision 2002/657/EC in terms of linearity, recovery, precision, decision limit, detection capability and limits of detection and quantification.

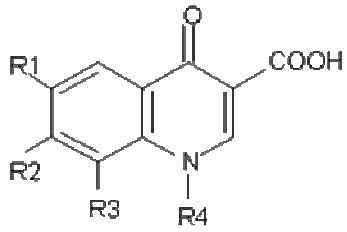
2. Experimental

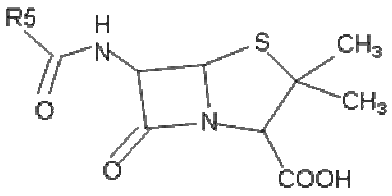
2.1 Reagents

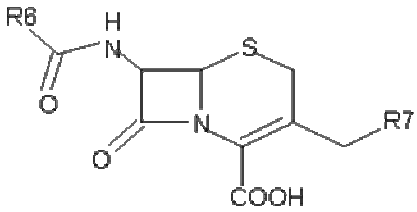
All reagents were of analytical grade unless indicated. Acetic acid (HAc), Formic acid (HFo), trifluoroacetic acid (TFA), acetonitrile (MeCN), methanol (MeOH), sodium dihydrogenphosphate and sodium hydroxide were supplied by Merck. Sodium chloride was supplied by Sigma. Ultrapure water was generated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

The standards were purchased from several pharmaceutical firms: 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). 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). Cloxacillin (CLOX) and piperacillin (PIPE; internal standard (IS)) (Fluka, Buchs, Switzerland). 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 (Irlanda).

Structures of the studied substances are shown in **Fig. 1.** The solid phase extraction (SPE) cartridges used in this study were Oasis HLB ($3cm^3$/60mg) obtained from Waters (Milford, MA, USA), Strata X ($1cm^3$/30mg; Phenomonex, USA) and ENV+ Isolute (Symta, Madrid, Spain).







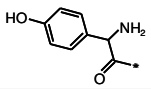
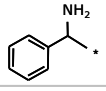
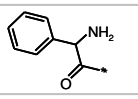
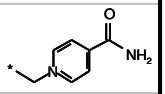
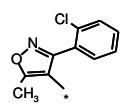
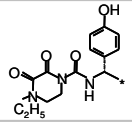
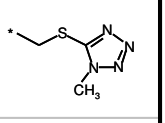
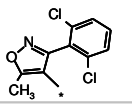
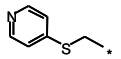
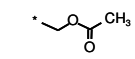
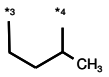
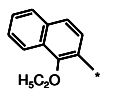
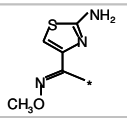
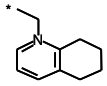
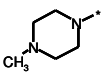
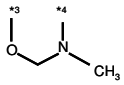
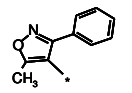
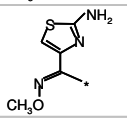
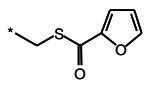
QUINOLONES					PENICILLINS		CEPHALOSPORINS		
Name	R1	R2	R3	R4	Name	R5	Name	R6	R7
CIP			H		AMOX		LEX		
DAN			H		AMPI		LON		
DIF			H		CLOX		PER		
ENR			H		DICL		PIR		
FLU		H			NAFC		QUI		
MAR					OXAC		TIO		
OXO			H		PENG		ZOL		

Fig.1 Structures of the studied antibiotics, classified by families (quinolones, penicillins and cephalosporins).

Dispersive SPE (15 mL) (Agilent Technologies, Palo Alto, CA, USA)) that contain 150 mg PSA, 150 mg C_{18} EC and 900mg $MgSO_4$ following the European Method-EN 15662 was used. 0,45 µm membrane filters of nylon (Scharlau, Sentmenat, Spain) were used for filtering samples.

2.2 Standards and stock solutions

The individual stock solutions of quinolones were prepared at the concentration of 500µg/mL by dissolving the quantity of each compound in MeCN. Individual stock solutions of penicillins and cephalosporins were prepared at a concentration of

100µg/mL by dissolving the quantity of each compound in water. For meat studies, individual stock solutions of each cephalosporin were prepared with concentration of 1000 µg/mL, except for LON prepared at 500 µg/mL and TIO prepared at 250 µg/mL in water. The individual standard solutions of internal standards (IS) (PIPE and PIP) were prepared by the dissolving the quantity of the IS in water and MeCN respectively. PIPE was prepared at the concentration of 500 µg/mL and 100 µg/mL, while PIP was prepared at 100 µg/mL. The working individual standard solutions of IS were prepared at a concentration of 5 µg/mL to use in milk samples and at a concentration of 80 µg/mL to be used in meat. Working solutions (containing a standard mixture separated by families of antibiotics) were prepared at a concentration of 100 MRL and 20 MRL to validate the milk method. Working solution (containing a standard mixture of cephalosporins) was prepared at a concentration of 80 µg/mL to the studies made in meat. Working solutions were used to spike the milk and meat samples. All standard solutions were stored at -20ºC.

Phosphate solutions at 0.05M at pH 8.5 and 9 and also 0.1M at pH 10 were prepared to be added to the milk samples. Phosphate solutions at 0.05M at pH 5 were prepared to be added to the meat samples. Saturated solution of NaCl was prepared with ultrapure water.

Fourteen different extraction solutions were prepared to optimize the extraction of cephalosporins from meat samples. Extraction solvents were prepared with MeCN, water and HAc. The extraction solutions contain from 0% to 40% water. HAc is added to the half of the extraction solutions.

2.3 Instruments

Chromatographic separation was achieved on a Zorbax Eclipse XDB-C8 (5µm, 4.6 × 150 mm) from Agilent Technologies (Waldbronn, Germany), using a pre-column Kromasil C8 (5µm, 4.6 × 15 mm) supplied by Akady (Barcelona, Spain). An HP Agilent Technologies 1100 LC system equipped with an autosampler and a coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex) with a turbo ion spray source was used. The system was controlled by software Analyst v.1.4.2 supplied by Applied Biosystems (Foster City, CA, USA). A Crison 2002 potentiometer (± 0.1 mV) (Crison, Barcelona, Spain) using a Crison 5203 combinated 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 is used and soaked for 15-20 min in MeCN-water mixture (15%) before pH measurements of the mobile phase. Three centrifuges Rotanta 460RS (Hettich Zentrifuguen), Macrotronic SELECTA and Centronic SELECTA (J.P. SELECTA S.A., Abrera, Spain) were used to perform the extraction. The SPE was carried out on a Supelco vacuum manifold for 12 cartridges and Supelco vacuum manifold with disponsable liners for 24 cartridges (Bellefonte, PA, USA) connected to a Supelco vacuum tank. Finally, evaporation to dryness system under a stream of nitrogen was used at the end of sample treatment.

2.4 Procedure

Chromatographic conditions

The mobile phase used in the LC-MS/MS is composed of water and MeCN with 0.1% formic acid in both solvents. The initial mobile phase is composed of H_2O:MeCN (85:15, v/v) with a pH of 3.2. The flow-rate was 1mL/min. **Table 1** shows the gradient used for the separation of analytes in LC. Twenty microlitres aliquots of the extracts were injected in the LC-MS.

Table 1. Gradient used for separation of the three series of antibiotics studied in this work.

Time (min)	% A	% B
0	15	85
2	15	85
4	45	55
7	56	44
8.5	56	44
10	15	85
11	15	85

Sample treatment and clean-up (SPE)

Figure 2 shows the scheme of the sample treatment and clean-up of quinolones, penicillins and cephalosporins in milk sample. This method was previously used to determine penicillins in milk [25].

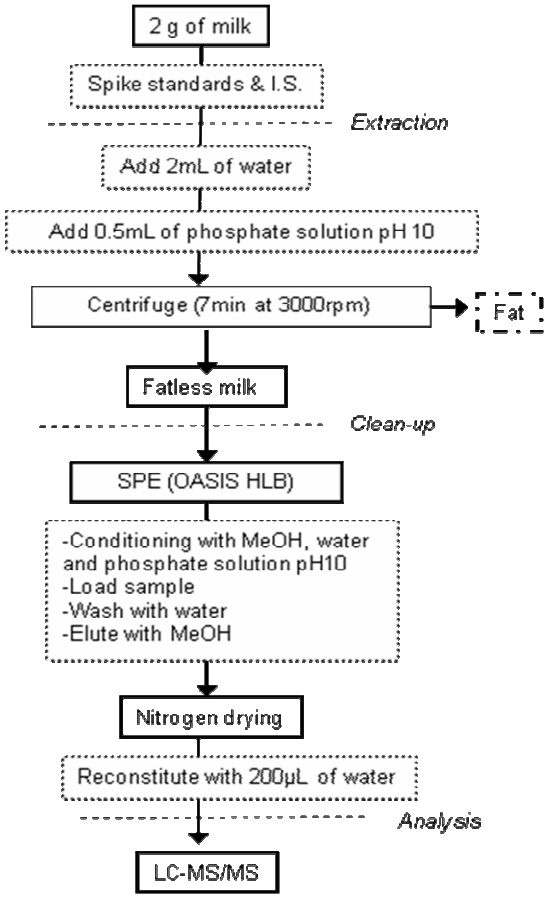


Fig.2. Flow chart for the extraction of antibiotics from raw milk

In summary, the extraction method involves an addition of phosphate solution 0.1 M at pH 10, centrifugation of samples and subsequently an SPE process using Oasis HLB cartridges. The HLB cartridges were activated with 1 mL of methanol, 1 mL of water and 1 mL 0.1 M phosphate solution at pH 10. After samples were passed through the system, the cartridge was cleaned with 3 mL of water in order to decrease the matrix interference. The analytes were eluted with 2 mL of methanol. **Figure 3** shows the procedure for the beef muscle treatment.

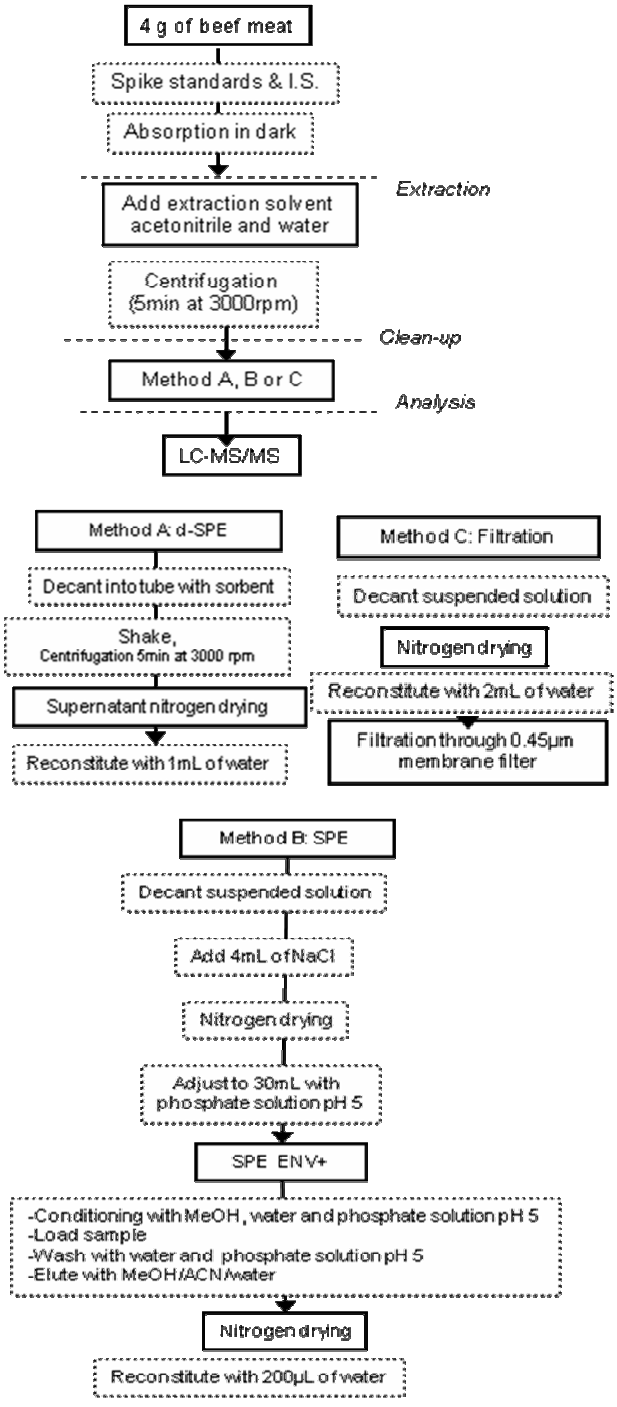


Fig. 3. Flow chart of the comparison of the extraction and clean-up methods for the cephalosporins analysis in beef muscle.

The extraction of the cepahlosporins was made using a MeCN:H_2O mixture with different percentages of MeCN (between 80 and 100%).Three different methods were used in the clean-up of cephalosporins from beef muscle. The method A consists on a dispersive-solid phase extraction, the method B is the classical SPE using a polymeric cartridge (ENV+ Isolute) and the method C is a short and easy method described in the literature [19-20].

LC-MS/MS parameters

The LC-MS/MS conditions were optimized by direct injection of each compound individually at a concentration of 10µg/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 4500V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), drying gas (N_2) was heated to 400ºC and introduced at a flow-rate of 6500 ml/min.

Multiple reaction monitoring (MRM) experiments in the positive ionization mode were performed using a dwell time of 60 ms. The ions in MRM mode were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole and analyzed with the second analyzer 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** shows these transitions with their optimum collision energy for the three families of antibiotics studied.

Table 2 [M+H] + ions, quantification and identification transitions for the substances studied in this work and their optimum collision energy.

	m/z	Transition Quantification (CE)*	Transition Identification (CE)
AMOX	366	366 → 114 (28)	366 → 208 (19)
AMPI	350	350 → 106 (26)	350 → 192 (21)
CLOX	436	436 → 160 (20)	436 → 277 (20)
DICL	470	470 → 160 (21)	470 → 311 (22)
NAFC	415	415 → 199 (19)	415 → 256 (21)
OXAC	402	402 → 160 (18)	402 → 243 (18)
PENG	335	335 → 160 (16)	335 → 176 (16)
PIPE(IS)	518	518 → 143 (27)	518 → 160 (16)
PIR	424	424 → 292 (20)	424 → 181 (35)
QUI	529	529 → 134 (20)	529 → 396 (20)
LEX	348	348 → 140 (35)	348 → 158 (15)
LON	459	459 → 152 (30)	459 → 337 (20)
ZOL	455	455 → 323 (15)	455 → 295 (25)
PER	646	646 → 290 (35)	646 → 530 (20)
TIO	524	524 → 285 (30)	524 → 241 (25)
MAR	363	363 → 320 (22)	363 → 345 (30)
CIP	332	332 → 314 (32)	332 → 288 (27)
DAN	358	358 → 340 (31)	358 → 283 (31)
ENR	360	360 → 316 (29)	360 → 342 (29)
FLU	262	262 → 244 (26)	262 → 202 (45)
PIP(IS)	304	304 → 286 (30)	304 → 261 (25)

* Collision energy (CE) in V.

2.5 Quality parameters

In the validation of the method different quality parameters should to be established, as linearity range, recovery, precision, selectivity, decision limit (CCα) and detection capability (CCβ), according to the European Union regulation 2002/657/EC decision [16].

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

The limit of quantification (LOQ) was determined in order to know the lowest point in the calibration curve, because is the lowest concentration of analyte that can be quantified. These LOQ for each antibiotic were determined using spiked milk samples at different concentration levels from 0.001 MRL to 0.1MRL and were prepared in duplicate. LOQ were calculated from a signal-to-noise ratio (S/N) of 10.

Recovery experiments were performed by comparing the analytical results for extracted standard samples of milk and internal standard added before the extraction procedure, with unextracted standards prepared at the same concentrations in blank extract representing 100 % recovery. Recoveries obtained in beef muscle using the three clean-up methods described in the section 2.4. were compared.

The intra-day precision was assessed comparing the results of five replicates prepared the same day at three different concentration levels (0.5 MRL, MRL and 2 MRL). The procedure was repeated to determine the inter-day precision by the comparison between results of samples prepared and analyzed on three different days. The relative standard deviations (%RSD) were calculated.

The decision limit (CCα) 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 (CCβ) means the smallest content of a substance that may be detected, identified and/or quantified in a sample with an error probability of β [16,30]. CCα values were determined by analysing 20 blank samples fortified with quinolones, penicillins and cephalosporines at MRL concentration. CCβ was calculated as the decision limit CCα plus 1.64 times the corresponding standard deviation (β = 5%), supposing that standard deviation at the MRL is similar to that obtained at the CCα level.

3. Results and discussions

3.1 Optimisation of the LC conditions

In order to obtain a separation of the different classes of antibiotics in milk samples, we have taken into account the previously separation of each class of antibiotics in milk. When only quinolones were analysed in milk samples, the initial mobile phase contain 14% of MeCN [24]. With the gradient elution optimised for them, a good separation is obtained in 15 min. When penicillins were the subject of the separation an initial 20% of MeCN was used [25]. The best compromise found allows us to separate penicillins in less than 8 min. The series of cephalosporins were also analysed in milk, and a 15% of MeCN was used as an initial mobile phase, achieving the separation of the substances in less than 8 min. When the series of quinolones, penicillins and cephalosporins should be analysed, to keep chromatographic run times as short as possible a complete separation of the substances is not possible. In this case, the initial mobile phase consists of 15 % MeCN with a 0.1% formic acid and the gradient elution shown in Table 2. In this condition the separation of the 21 drugs analysed is achieved in 10 min. **Figure 4** shows the separation of the three series of antibiotics at the 3 .MRL level each antibiotic.

When samples of beef muscle were analysed to determine cephalosporins the same gradient elution was used, obtaining the complete separation of the drugs in less than 8 min. The separation for 8 cephalosporins at the MRL level is presented in **Fig. 5**.

3.2. Sample treatment and clean-up

In the literature, a lot of sorbents and different conditions, washing and elution steps in SPE were used simultaneously to improve the clean-up and pre-concentration of antibiotics from food, biological tissues and water [31].

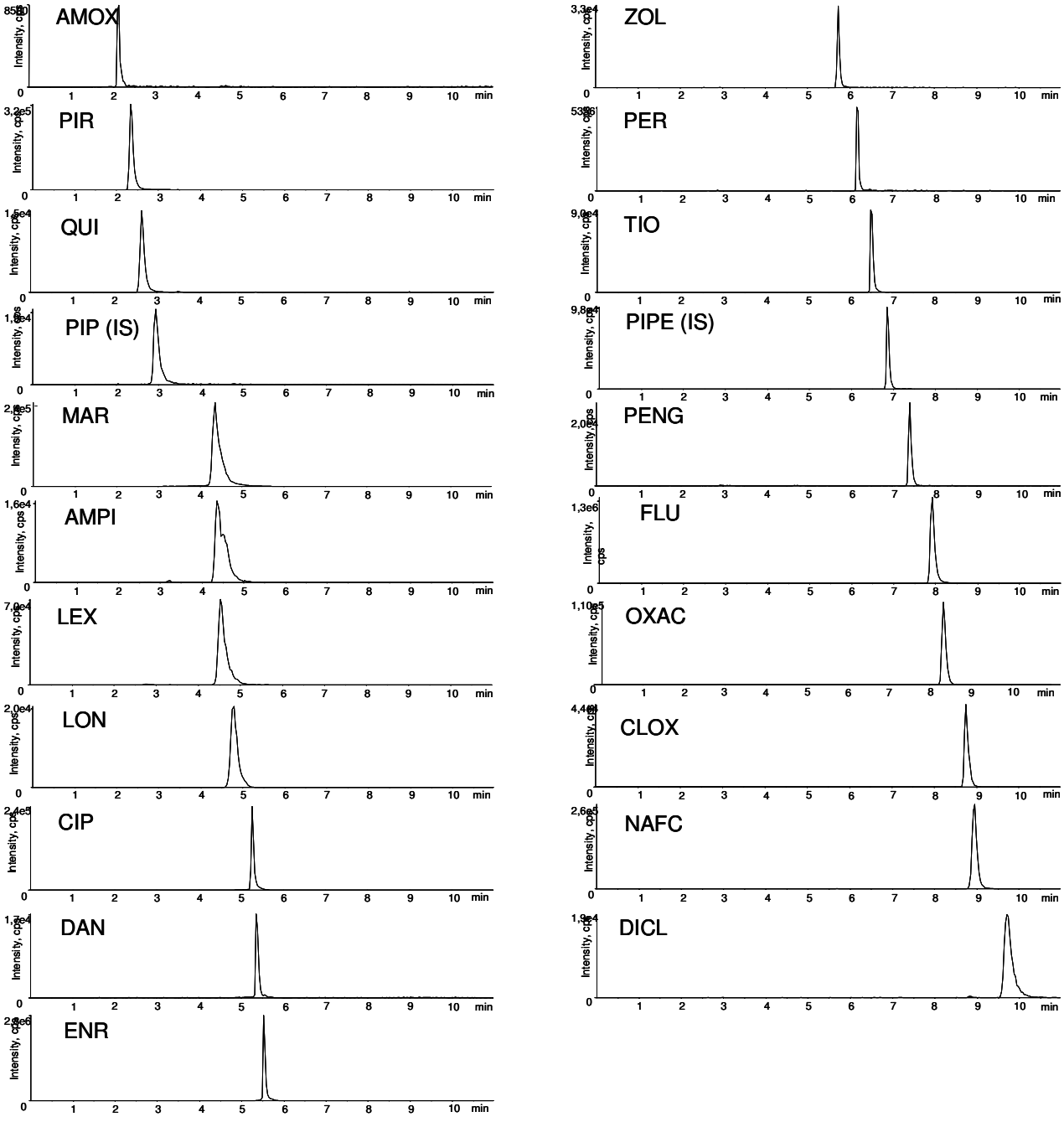


Fig.4. LC-MS/MS chromatogram, in MRM mode, of spiked samples of milk at 3 MRL each antibiotic. Peaks: 1) AMOX, 2) PIR, 3) QUI, 4) PIP, 5) MAR, 6) AMPI, 7) LEX, 8) LON, 9) CIP, 10) DAN, 11) ENR, 12) ZOL, 13) PER, 14) TIO, 15) PIPE, 16) PENG, 17) FLU, 18) OXAC, 19) CLOX, 20) NAFC and 21) DICL.

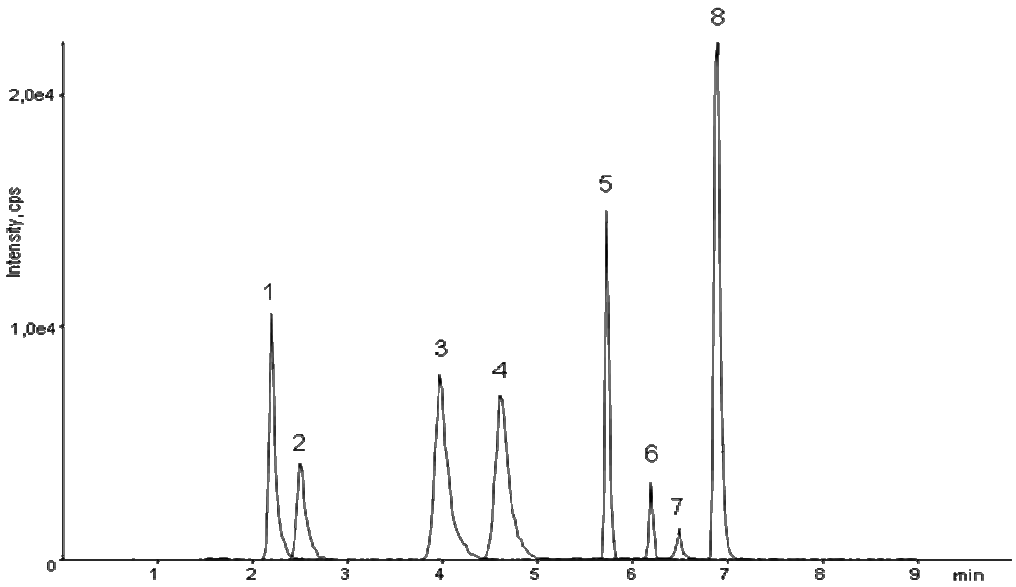


Fig.5. LC-MS/MS chromatogram, in MRM mode, of spiked samples of beef meat at MRL each antibiotic. Peaks: 1) PIR, 2) QUI, 3) LEX, 4) LON, 5) ZOL, 6) PER, 7) TIO and 8) PIPE.

From previous studies we concluded that the best results in the extraction of quinolones from several tissues are obtained when polymeric sorbents are used, as is the case of Strata X and ENV+ Isolute. To analyse quinolones in milk, maximum recovery with minimum interference has been obtained using Strata X [24]. Recoveries higher than 80% were obtained for all studied quinolones.

Several SPE disposable systems and protocols were tested to analyse penicillins in milk [25]: Oasis HLB, Bond Elut C18, Isolute ENV+, SDB-RPS and Oasis MAX. For these sorbents, the SPE conditions were optimized. High extractions ranging from 50 to 90% for the different penicillins were obtained with the different sorbent studied, excepted for AMOX and AMPI. These two penicillins only present recoveries higher than 50% with Bond Elut C18 and Oasis HLB cartridges. The recoveries values are lightly lower with Bond Elut C18, and for this reason, Oasis HLB cartridges were selected to the determination of penicillins in milk.

In previous studies, we concluded that Oasis HLB also present the best recoveries when cephalosporins were analysed in milk. From the literature review, we can see that Oasis HLB provide efficient extraction with optimal recoveries, equal retention and also history of batch to batch reproducibility [17, 23, 32, 33]. Taking into account the results obtained the different cartridges and series of antibiotics, the Oasis HLB cartridges were chosen to analyse the three classes of antibiotics, subject of this study.

Using the clean-up method explained in the section 2.4, the recoveries of the three series of antibiotics have been determined by comparing analytical results of extracted samples with antibiotics spiked after the extraction procedure, representing 100% recovery. Samples were analysed by LC-MS/MS. As can be observed in **Fig. 6**, the cephalosporins present in general the best results, while poor results were obtained for quinolones. The most part of the substances present recoveries around or higher than 80%, except for AMOX, CIP, DAN and ENR.

In previous works the determination of quinolones and penicillins in meat samples was optimized for different tissues and animals [26, 27, 34, 35]. In order to obtain a multiresidue, multiclass method for quinolones, penicillins and cephalosporins in meat samples, it is necessary to study the behaviour of the cephalosporins in meat samples.

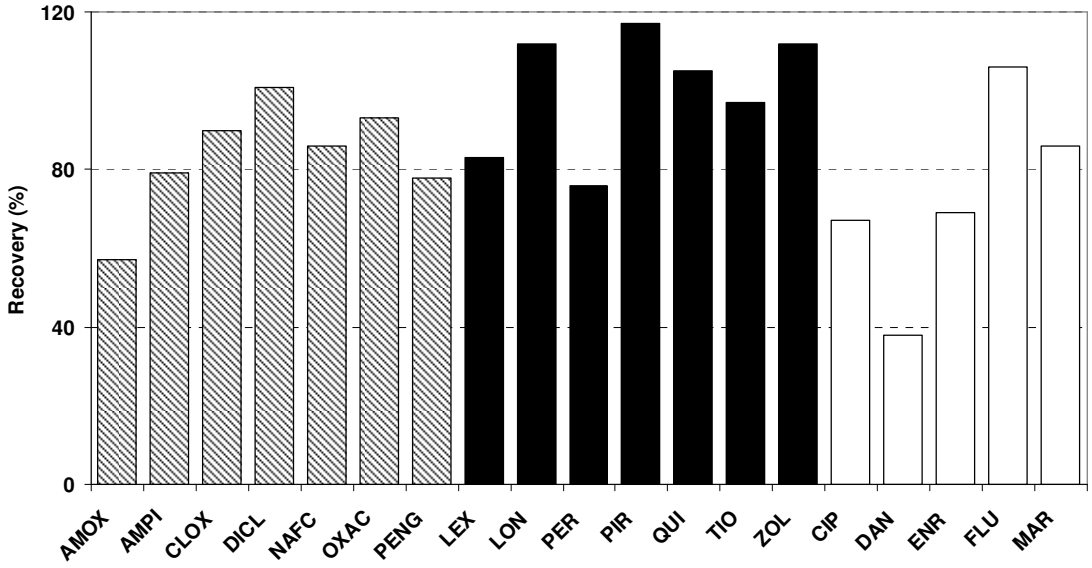


Fig.6. Recoveries (%) of antibiotics classified by families in milk samples.

To extract cephalosporins from the beef muscle, different mixtures of MeCN and water were used. Three different methods for clean-up were applied. A new method, the dispersive-SPE (d-SPE, method A) used in the literature for the clean-up of samples with pesticides has been applied for the cephalosporins. The method is based on MeCN extraction/partition of the analytes followed by the removal of water and proteins by salting out with sodium chloride and magnesium sulphate. Then the d-SPE, which involves the addition of small amounts of a bulk sorbent to the extracts, is applied. This method greatly simplifies and accelerates sample clean-up and the results were compared with those obtained with conventional methods as SPE (method B), and with a method applied to different drugs that is considered easy and rapid (method C) [19-20]. **Figure 7a** shows the effect of the percentage of MeCN on the recovery of the cephalosporin ZOL. As can be observed, comparable results are obtained using all three clean-up methods when the extraction is made with 80% of MeCN. In this case, recoveries of 75% are obtained. Higher % of MeCN gives worst recoveries for d-SPE and lower % of MeCN decrease the recovery of methods B and C. **Figure 7b** shows the recoveries obtained for the cephalosporins using 80% of MeCN. It can be observed that the method A is an alternative and useful method for the clean-up of cepaholosporins, because the best recoveries were obtained. The d-SPE clean-up procedure allows the use of smaller amounts of organic solvent and provides high recovery rates for drugs covering a wide polarity range.

3.3 Ion mass detection

The coupling of LC with MS is a powerful tool for identification and quantification of drugs in biological samples. In the case of the antibiotics substances studied in this work ESI+ mode was used. When SIM mode was applied, the most prominent ion for every compound is the protonated molecular ion $[M+H]^+$. MRM mode exhibited the highest selectivity and sensitivity using LC-MS/MS. For quinolones the most abundant product ions corresponded to $[M+H]^+$, $[M+H-H_2O]^+$ and $[M+H-CO_2]^+$. The transition $[M+H]^+ \rightarrow [M+H-H_2O]^+$ was used as quantification transition for CIP, DAN and FLU, while $[M+H]^+ \rightarrow [M+H-CO_2]^+$ was used for MAR and ENR.

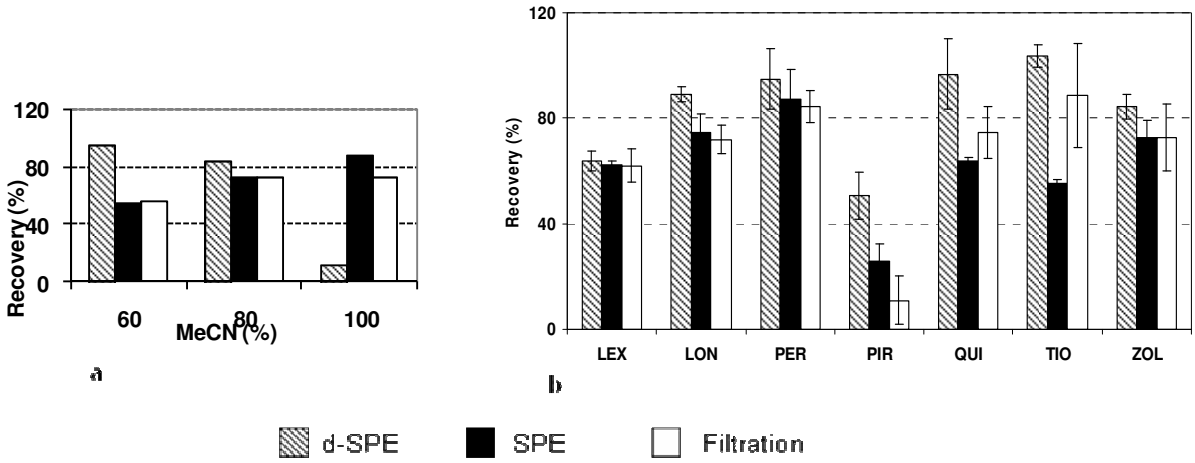


Fig.7. a) Recoveries (%) of the ZOL in meat sample using the different clean-up procedures, as a function of the percentage of MeCN used in the extraction step. b) Recoveries (%) of the cephalosporins in meat sample using the different clean-up procedures, at the optimised % MeCN conditions.

As an illustrative example, the product ions from CIP are shown in **Fig. 8a**.

The basic structure of penicillin consists of a thiazolidinic ring condensed on a β-lactame ring, to which a lateral chain is linked. The *m/z* 160 ion is the common fragment obtained for all penicillins. The product ion of these compounds at *m/z* 160 corresponds to the thiazilidinic ring those fragment is $[C_6H_{10}O_2NS]$. Also, the fragment $[M+H^+-159]$ is characteristic. The ion 160 gives a fragment of m/z 114 due to the loss of the carboxylic group from the 160 fragment. **Figure 8b** shows, as an illustrative example, the mass spectra of AMOX.

In the case of cepahalosporins, the $[M+H]^+$ is a common ion obtained and also the ions obtained from the fragmentation of the substances in the β-lactam ring. **Figure 8c** shows the mass spectra of LEX.

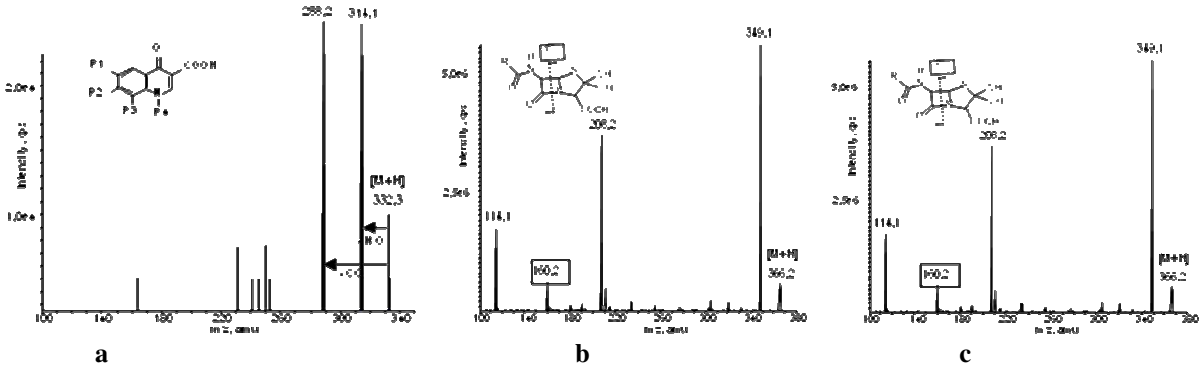


Fig. 8. a) Mass spectra of CIP in product ion scan mode of m/z of 332. b) Mass spectra of AMOX in product ion scan mode of m/z of 366. c) Mass spectra of LEX in product ion scan mode of m/z of 348.

3.4 Analysis of raw milk samples

In order to analyse different samples of milk from animals previously medicated with antibiotics, and to assure that the results are good enough, the optimised method should be validated.

In order to validate the method there have been assessed the next parameters according to the European Union regulation 2002/657/EC and some of them from the FDA guidelines for bio analytical procedure [8,16]: limit of detection (LOD), limit of quantification (LOQ), linearity, recovery, precision intra- and inter-day, decision limit (CCα) and detection capability (CCβ).

The linearity was evaluated using calibration curves whose correlation coefficients were higher than 0.990 in milk samples. The obtained LOD and LOQ were lower than the MRL for the 19 studied substances, from two to three magnitude order.

The accuracy of the method was assessed by recovery test. 17 analysed drugs presented recoveries higher than 65% while AMOX and DAN present lower recoveries. By families, better recoveries have been obtained for cephalosporins while the quinolones in milk presented the worst values. The intra- and inter-day precisions also were evaluated for all substances. Both parameters were lower than 15% as is regulated by FDA guidelines for bioanalytical procedure. The CCα and CCβ were also established. The results sorted by families can be observed in the **Fig. 9**.

Samples provided by the "Laboratory Interprofessional lleter de Catalunya (ALLIC)" were positive in the screening of control made in this laboratory. All samples contained drugs residues; most samples of the 49 analyzed contained penicillins (69%), 28% have been founded positive in cephalosporins and in the rest of samples quinolones were identified.

By substances, the most frequent three drugs found in the milk samples were PENG (31%), AMOX (24%) and PIR (16%). Among samples analysed, only two samples were positive in ENR.

Figure 10 shows LC-MS/MS chromatograms corresponding to the analysis of positive milk samples. **Figure 10a** presents the results obtained for one of the samples positive in ENR. In this sample is also observed the peak corresponding to the CIP, main metabolite of ENR, as can be proved by the corresponding confirmatory chromatogram. **Figure 10b** shows the results of one sample positive to PENG, together with the identification chromatogram.

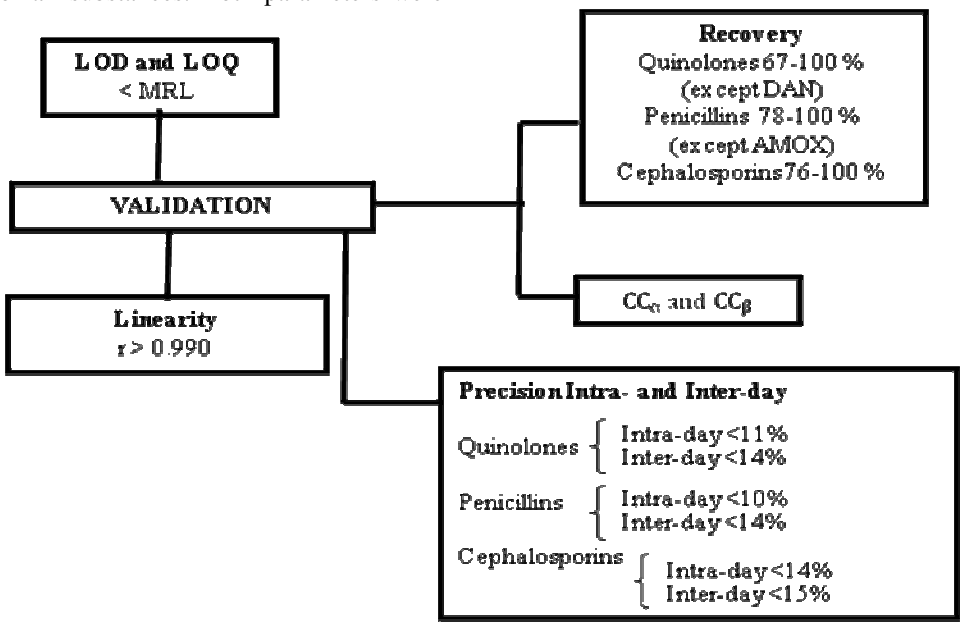


Fig. 9. Quality parameters of the developed method in raw milk for the antibiotic determination.

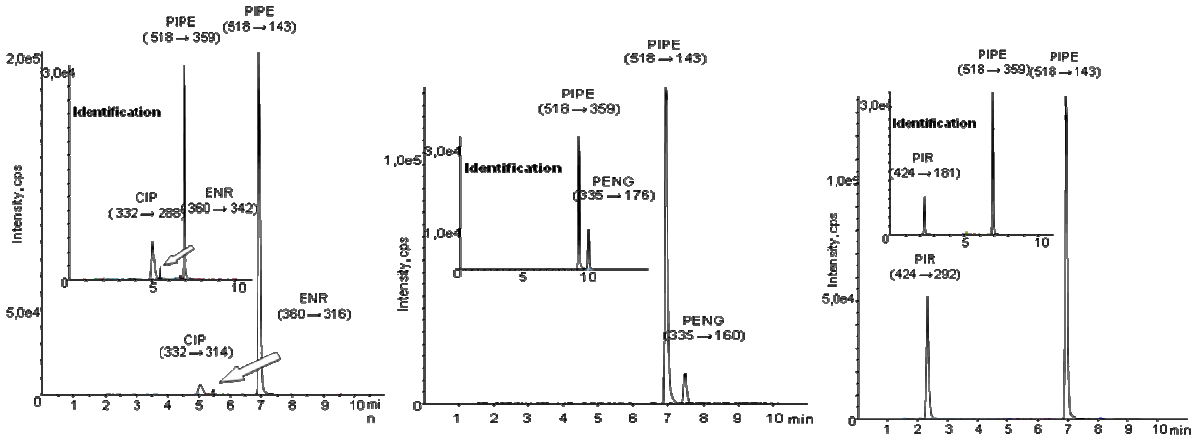


Fig. 10. Ion reconstituted chromatogram obtained in the analysis of non compliant raw milk samples using LC/MS/MS in MRM mode showing quantification and identification transitions. a) ENR and metabolite, CIP. b) PENG. c) PIR.

Figure 10c shows one sample containing PIR together with the corresponding identification chromatogram.

Among the analysed samples, 63% of the samples were found to be compliant with an error probability of 5%. This means that although some antibiotics are found in the analysed samples, the most part of samples are considered adequate to human consumption, because the concentration is lower that the corresponding MRL (**Table 3**).

4. Conclusions

In this work multiresidue methods were developed and optimised to allow the analysis of quinolones, penicillins and cephalosporins included in the European Union regulations in samples of milk and tissues of beef.

Different methodologies of extraction and clean-up were explored for beef muscle. Different cartridges were studied for the clean-up of antibiotics in milk samples.

Table 3. MRL of the studied substances (Normative 37/2010) in milk and muscle of beef.

QUINOLONES			PENICILLINS			CEPHALOSPORINS		
Name	MRL (µg/kg)		Name	MRL (µg/kg)		Name	MRL (µg/kg)	
	Beef milk	Beef muscle		Beef milk	Beef muscle		Beef milk	Beef muscle
CIP	100	100	AMOX	4	50	LEX	100	200
ENR			AMPI	4	50	LON	20	-
DAN	30	200	CLOX	30	300	PER	50	-
DIF	-	400	DICL	30	300	PIR	60	50
FLU	50	200	NAFC	30	300	QUI	20	50
MAR	75	150	OXAC	30	300	TIO	100	1000
OXO	-	100	PENG	4	50	ZOL	50	-

Appropriate quality parameters were obtained in milk samples using LC-MS/MS. The optimised method was applied to determined regulated antibiotics in milk samples from animals medicated with several drugs. 63% of the samples were found to be compliant and adequate for human consumption.

5. Acknowledgements

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