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Analytical Methods

A simple and rapid assay based on hot water extraction and liquid chromatography–tandem mass spectrometry for monitoring quinolone residues in bovine milk

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Abstract

A rapid, specific and sensitive procedure for determining residues of eight widespread used quinolone antimicrobials in bovine milk is presented. The method is based on the matrix solid-phase dispersion technique with hot water as extractant followed by LC/MS/MS. The entire sample treatment did not take more than 40 min. Hot water appeared to be an efficient extracting medium, since absolute recoveries of the analytes in milk were 77–90%. The method proved to be robust as matrix effects did not affect significantly the accuracy of the method, as evidenced by analyzing six different batches of milk. Using norfloxacin as surrogate analyte, the accuracy of the method at three different spike levels of the analytes in milk was 93–110% with RSDs not larger than 10%. On the basis of a S/N of 10, estimated LOQs of this method range from 0.3 to 1.5 ng/ml, well below the tolerance levels of quinolones in milk set by the European Union.

Keywords: Fluoroquinolones; Milk; Hot water extraction; Liquid chromatography–tandem mass spectrometry

1. Introduction

Antimicrobials are widely used in dairy cattle management for the treatment and prevention of diseases. The use of antimicrobials may result in drug residues being present in milk, especially if they are not used according to label directions. There are concerns that the widespread usage of antimicrobials may be responsible for the promotion of resistant strains of bacteria (Brady & Katz, 1988; Wegener, Aarestrup, Gerner-Smidt, & Bager, 1999). For this reason, both the EU commission (Commission Regulation (EC) No 508/1999 of 4th March 1999) and the USA Food and Drug Administration (Code of Federal Regulation, Title 21, 2006) have established maximum residue limits (MRLs) of antimicrobials in food.

Immunological or microbial inhibition screening tests are commonly used to determine if antibiotic residues are present in milk. Some drawbacks of screening tests are: they cannot identify which antimicrobials are present in milk, the presence of high somatic cell counts may result in false positives (Tyler et al., 1992; Van Eenennaam et al., 1993), and they may detect antibiotic residues at levels far below the officially mandated safe levels, resulting in the unnecessary destruction of the milk. Therefore, sensitive and specific chemical methods for the identification and quantitation of antibiotic residues in milk need to support screening tests. Public health agencies in many countries rely on detection by MS for unambiguous confirmation of contaminants in foodstuff.

In the past 15 years, the on-line combination of LC and MS has developed into a widely applied and routinely applicable detection and on-line identification approach for LC. The ease of operation and robustness of current LC/MS interfaces based on atmospheric-pressure ionisation enable the application of LC/MS in a large variety of analytical fields. In particular, the MS analysis of residues of antimicrobials in food has greatly benefited from

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these developments and has been the object of several reviews (Andreu, Blasco, & Picò, 2007; Di Corcia & Nazzari, 2002; Gentili, Perret, & Marchese, 2005; Hernández-Arteseros, Barbosa, Compañó, & Prat, 2002; Kotretsou, 2004; Niessen, 1998; Stolker & Brinkman, 2005).

Quinolones are a group of relatively new antimicrobials synthesized from 3-quinolone carboxylic acid. Quinolones show excellent activity against both Gram-positive and Gram-negative organisms, as well anaerobes. They act to inhibit DNA gyrase a key enzyme in DNA replication. Several quinolones were specifically developed for veterinary medicine, i.e., danofloxacin (DAN), enrofloxacin (ENR) and sarafloxacin (SAR). These drugs are used to treat respiratory and enteric bacterial infections in cattle and other food producing animals.

So far, three methods based on MS/MS detection aimed at monitoring residues of quinolones in bovine milk have been proposed. Volmer, Mansoori, and Locke (1997) conducted a quite interesting and exhaustive study concerning the potential of the LC/MS/MS technique for trace analysis of quinolones in several biological matrices, including milk. However, they made no effort in elaborating a simple and rapid sample preparation procedure by taking advantage of the high specificity offered by MS/MS detection and followed a previously reported laborious procedure developed for monitoring quinolones in milk by LC with UV detection (Hormazabal & Yndestadt, 1994). Later, Van Hoof et al. (2005) elaborated and validated a LC/MS/MS method for identifying and quantifying eight quinolones in muscle tissue, aquaculture products and milk. They made use of a conventional sample treatment protocol, that is milk protein precipitation, centrifugation, analyte extraction, another centrifugation step before clean-up of the extract with a C_{18} solid-phase extraction cartridge. Recently, a Spanish researcher group designed an original and innovative method based on capillary electrophoresis–tandem MS for determining eight quinolones in bovine raw milk (Lara, García-Campaña, Alés-Barrero, Bosque-Sendra, & García-Ayuso, 2006). The sample treatment elaborated by the authors is rather laborious as it consists of (i) milk defattening; (ii) analyte solid-phase extraction (SPE) by an Oasis MAX cartridge; (iii) eluate drying; (iv) a second SPE with an Oasis HLB cartridge, after residue reconstitution; (v) eluate drying; and (vi) filtration of the reconstituted residue before injection in the CE system.

Recently, we have proposed two sensitive LC/MS confirmatory methods for determining residues of sulfonamide antibacterials (Bogialli, Curini, Di Corcia, Nazzari, & Polci, 2003) and aminoglycoside antimicrobials (Bogialli et al., 2005) in milk. These methods involve a simple and rapid sample treatment procedure that couples positive features of the matrix solid-phase dispersion (MSPD) technique, i.e. simplicity and intimate contact between the extractant and the matrix, to those offered by heated water as extractant. Besides to be a cheap and environmentally friendly solvent, water is able to selectively extract analytes by suitably controlling the extraction temperature (Hawthorne, Yang, & Miller, 1994). In essence, this method consists of: (i) dis-

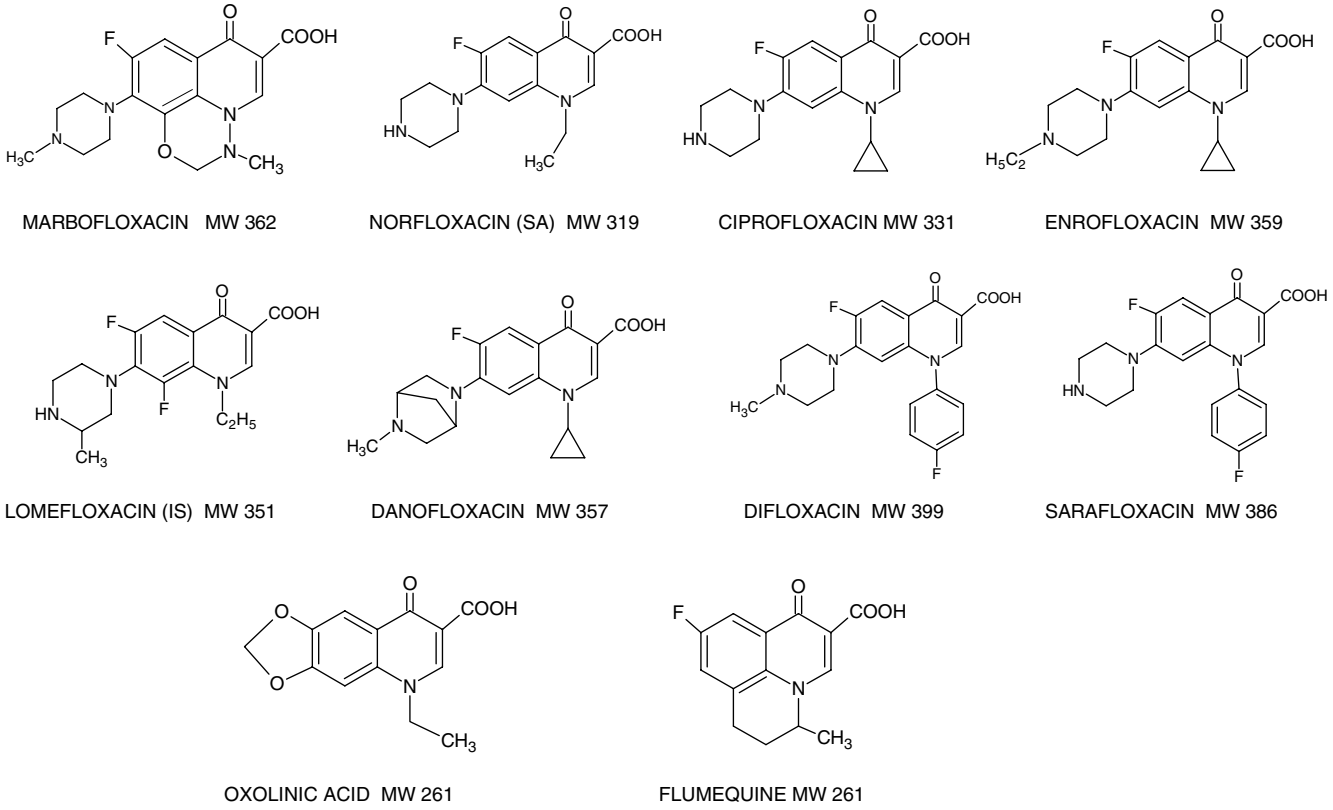

Fig. 1. Chemical structures of selected quinolone antimicrobials.

persion of the biological matrix onto a solid support by blending the sample and the support with a mortar and pestle; (ii) filling a column with this material; (iii) flowing through the MSPD column a suitable volume of water heated at a selected temperature; and (iv) pH adjustment and filtration of the aqueous extract before injecting a relatively large volume of it into a reversed-phase LC column. It has to be pointed out that the entire sample treatment procedure described above does not require more than 40 min to be completed.

The aim of this work has been that of designing a LC-tandem MS method for monitoring residues of eight quinolones (Fig. 1) in bovine whole milk at tolerance levels set by the EU. The sample treatment protocol was the same as described above. A Directive regulating MRLs in milk of three of the quinolones selected, i.e., SAR, difloxacin (DIF) and oxolinic acid (OXO) has been not yet enacted by the EU. Nevertheless, we included them in this study because quinolones have such an outstanding effectiveness that their illegal use in certain veterinary applications is very likely.

2. Experimental

2.1. Reagents and chemicals

Lomefloxacin (LOM), norfloxacin (NOR), SAR, OXO, flumequine (FLU) and marbofloxacin (MAR) were purchased from Sigma, St. Louis, MO. DAN and DIF were from Riedel-de Haën, Seelze Germany. Ciprofloxacin (CIP) and ENR were provided by Fluka, Buchs, Switzerland. LOM and NOR were used respectively, as internal standard (IS) and surrogate analyte (SA). Individual stock solutions of the analytes, the SA and the IS were made by dissolving each compound in acetonitrile/water (1:1, v/v) to obtain 0.1 μg/μl concentration. These solutions were stored at 4 °C in amber glass bottles. Composite working standard solutions of the target compounds were obtained by mixing the above solutions and diluting with suitable volumes of water. A 6 ng/μl working standard solution of the SA was prepared in the same way, while that of the IS was prepared at concentration of 12 ng/μl. When unused, all working solutions were stored at 4 °C in the dark and renewed after 1 month of use.

Sand (Crystobalite, 40–200 mesh size), a material obtained by heating silica at about 1500 °C, was from Fluka. Methanol "Plus" of gradient grade was obtained from Carlo Erba (Milan, Italy). For LC, distilled water was further purified by passing it through a Milli-Q Plus apparatus (Millipore, Bedford, MA). Formic acid (95%, purity) was from Sigma.

2.2. Milk samples

Pasteurized, homogenized whole bovine milk were from retail markets. Before use, any sample was analyzed by this method to ascertain the absence of the drugs considered.

2.3. Extraction apparatus

The design of the laboratory made extraction apparatus used in this work was very similar to that shown in a previous paper (Crescenzi et al., 1999), with the exception that nitrogen was bubbled in water to eliminate any trace of dissolved oxygen and the analyte containing water leaving the extraction cell was collected in a calibrated glass tube instead of a sorbent cartridge. Briefly, the extraction apparatus consisted of a LC single pump to force water to pass through the extraction cell, a gas-chromatography oven containing a 5 m pre-heating stainless steel coil and an extraction cell (8.1 cm × 8.3 mm i.d. stainless-steel column). Twenty micrometer pore size polyethylene frits (Alltech, Sedriano, Milan, Italy) were located above and below the matrix/sand material.

2.4. Sample preparation

Fifteen milliliter of milk was spiked with known variable amounts of the analytes and 250 μl of the SA solution. Under continuous agitation, 15 min was allowed for equilibration at room temperature. Thereafter, 1.5 ml of milk was poured in a porcelain mortar containing 6 g of sand, and the mixture was blended with the pestle for *ca.* 10 min, until an apparently dry and homogeneous material was obtained. This blend was then packed into the extraction cell. To ensure homogeneous packing of the cell, close attention was paid to pour the material into the tube in 3–4 aliquots, tapping firmly the tube for 10–15 s after addition of each aliquot. Any void space remaining after packing the solid material was filled with sand. The tube was then put into the oven and conditioned at 90 °C for 10 min. Five milliliter of water was then passed through the cell at 1 ml/min flow rate to extract the analytes and the SA. After the addition of 50 μl of the IS, the extract was acidified to *ca.* pH 4.5 with ca 70 μl of 1 mol/l formic acid. After filtration through a glass fiber filter (1.2 μm pore size, 2.5 cm diameter, Alltech), 200 μl of the final extract was injected into the LC column. By following the procedure described above, the guard column was replaced with a new one after more than 200 injections of extracts.

2.5. LC/MS/MS instrumentation and conditions

The liquid chromatograph consisted of a Waters pump (Model 600 E, Millford, IL), a 200 μl injection loop, an Alltima 5 μm C-18 guard cartridge (7.5 × 4.6 mm i.d., Alltech), a 5 μm C-18 analytical column (250 mm × 4.6 mm i.d., Alltech); the chromatograph was interfaced by an electrospray ion (ESI) source to a benchtop triple-quadrupole mass spectrometer (Model Micromass Quattro Micro API, Waters). Mobile phase component A was a methanol/acetonitrile (70:30, v/v) mixture, while component B was water. Both components were acidified with 20 mmol/l formic acid. At 1.0 ml/min, the mobile phase gradient profile was as follows (t in min): t_0, $A = 20\%$; t_{10}, $A = 30\%$; t_{12}, $A = 100\%$; t_{15},

$A = 100\%$; t_{16}, $A = 20\%$; t_{25}, $A = 20\%$. A diverter valve led the effluent into the ion source with a flow of 400 μl/min only after 5 min from the beginning of the chromatographic run. High-purity nitrogen was used as drying and curtain gases; high-purity argon was used as collision gas. Nebulizer gas was set at 650 l/h while the cone gas at 50 l/h; the probe and desolvation temperatures were maintained at 120 °C and 350 °C, respectively. The gas pressure in the collision cell was 3 mbar. The ESI source was operated in the positive ion mode and MS data acquisition was performed in the multi reaction monitoring mode, selecting two precursor ion to product ion transitions for each target compound (Table 1). Capillary voltage was 3000 V, extractor voltage was 2 V. Declustering potential, collision energy and others transmission parameters were optimized for each analyte and are reported in Table 1. Mass axis calibration of each mass-resolving quadrupole Q_1 and Q_3 was performed by infusion of a sodium and ceasium iodide solution at 10 μl/min. Unit mass resolution was set and maintained in each mass-resolving quadrupole by keeping a full-width at half-maximum of approximately 0.7 μm. All the source and instrument parameters for monitoring quinolones were optimized by standard solutions at 5 μg/ml of each analyte infused at 10 μl/min by a syringe pump.

2.6. Quantitation

Absolute recovery of each analyte and the SA added to any milk sample was assessed by summing the ion current profiles relative to the transitions considered, normalizing them to the peak area of the IS, and comparing these ratios to those obtained by injecting a related blank sample extract to which the analytes and the SA were added post-extraction. We followed this procedure to obviate matrix effects that provoked significant variations of the analyte ion signal intensities, as compared to those of an authentic standard solution. The accuracy of the method at different analyte concentrations were estimated in an analogous way, with the difference that normalized signals of targeted compounds were related to that of the SA.

Table 1
Time-scheduled multi reaction monitoring conditions for detecting selected quinolone antimicrobials in bovine milk

Compound	MRM transition (*m/z*)	Cone voltage (V)	Collision energy (eV)	Retention window (min)
Marbofloxacin	363 > 320	25	12	5–16
	363 > 72		20	
Norfloxacin (SA)[a]	320 > 276	28	20	–
Ciprofloxacin	332 > 288	25	18	–
	332 > 314		–	
Enrofloxacin	360 > 316	30	20	–
	360 > 342		–	
Danofloxacin	358 > 314	30	15	–
	358 > 340		20	
Lomefloxacin (IS)[b]	352 > 308	28	15	–
Difloxacin	400 > 356	30	20	–
	400 > 382		–	
Sarafloxacin	386 > 342	30	15	–
	386 > 368		20	
Oxolinic acid	262 > 216	22	28	16–21
	262 > 244	20	15	
Flumequine	262 > 202	20	30	–
	262 > 244		15	

[a] SA = surrogate analyte.
[b] IS = internal standard.

3. Results and discussion

3.1. Optimization of the extraction conditions

As water is heated at high temperatures, its surface tension, viscosity and polarity progressively decrease. Heated water, thus, becomes an efficient medium for extracting from a given matrix even those organics that are scarcely soluble in water at ambient temperature. On the other hand, a risk inherent to the use of hot water as extractant is that it could decompose those compounds that are thermolabile and/or prone to hydrolytic attack. Therefore, we evaluated the temperature effect on recoveries of the selected quinolones (including NOR candidate for use as SA) by performing extractions at various temperatures. The aim of this study was also that of finding the minimum extraction temperature able to give good recovery of the analytes and the lowest amount of matrix components that could contaminate the ion source and/or interfere with the rest of the analysis. For this study, a milk sample was spiked with the analytes and the SA at 100 ng/ml level and 5 ml of water was passed through the extraction cell at 1 ml/min flow-rate. At each temperature, three extractions were carried out and results are reported in Table 2. With the exception of the two most hydrophilic quinolones, i.e. NOR and CIP, raising the temperature of the extractant from 60 to 90 °C had the effect of improving remarkably the extraction yield of the targeted compounds. A further increase of the extraction temperature resulted in some decrease of the analyte recovery, may be due to some decomposition occurring at 120 °C.

Table 2
Extraction yield of quinolone antimicrobials in bovine milk by varying the extraction temperature and the extractant volume

Compound	Recovery[a], % (RSD, %)				
	60 °C, 5 ml	90 °C, 5 ml	120 °C, 5 ml	90 °C, 4 ml	90 °C, 6 ml
Marbofloxacin	83 (5)	82 (4)	75 (7)	76 (5)	82 (6)
Norfloxacin (SA)[b]	72 (7)	79 (4)	70 (6)	82 (4)	85 (6)
Ciprofloxacin	85 (3)	87 (4)	71 (7)	83 (5)	89 (5)
Enrofloxacin	74 (7)	90 (9)	76 (12)	77 (11)	90 (9)
Danofloxacin	56 (8)	86 (7)	73 (9)	75 (10)	83 (6)
Difloxacin	58 (8)	90 (5)	70 (6)	79 (5)	92 (3)
Sarafloxacin	64 (7)	87 (8)	74 (10)	78 (9)	87 (7)
Oxolinic acid	63 (8)	77 (6)	57 (11)	66 (8)	79 (6)
Flumequine	67 (10)	82 (8)	53 (12)	70 (11)	80 (10)

Spike level: 100 ng/ml.
[a] Mean values from triplicate measurements.
[b] SA = surrogate analyte.

Besides affecting the extraction yield of the target compounds, the water volume passing through the extraction cell can influence the sensitivity of the method, as this method does not include any concentration step of the extract. For the purpose of finding the minimum volume of water able to extract efficiently the analytes, experiments were performed by spiking a milk sample with the analytes and the SA at 100 ng/ml level and extracting at 90 °C with increasing water volumes. Experiments were made in triplicate and results are visualized in Table 2. As can be seen, extracting with more than 5 ml of water did not increase significantly analyte recovery. Thus, the best compromise between method sensitivity and extraction yield was that of passing through the extraction cell 5 ml of water heated at 90 °C.

3.2. Matrix effect

When analyzing contaminants in foodstuff matrices by LC/MS with an ESI source, a "negative" matrix effect, or less commonly, a "positive" matrix effect is the rule more than the exception. To obviate this drawback, the common practice is today that of using analyte-fortified control matrix extracts as reference standards. However, it is possible that the extent of the matrix effect can vary by varying the source of a given biological matrix. In this case, using a generic analyte-fortified control matrix extract as reference standard will affect analyte quantitation in incurred samples. Moreover, recovery of the analytes could be significantly affected from sample to sample due to variable matrix effects. Therefore, for all the analytes considered as well as the SA and the IS, we conducted a study aimed at assessing variations of the matrix effect (if present) by varying the sources of milk samples. For this purpose, we selected six batches of milk from six different producers. After dividing each sample in two aliquots, one was spiked with the analytes and the SA, while the other aliquot was left intact. Thereafter, they were extracted in duplicate by two analysts. After extraction and before extract filtration, uncontaminated milk extracts were spiked with the same amounts of analytes and the SA used to contaminate the other six milk samples before extraction. Estimation of the recovery was performed as described in Section 2.6 and results are reported in Table 3.

Mean absolute recovery of targeted compounds in milk ranged between 81% and 87% with RSDs not larger than 14%. These data indicated that the method was satisfactorily reliable and robust. Depending on the particular analyte, data of the ion signal intensities showed that matrix effects influenced significantly the response of the MS detector. Anyway, these matrix effects were not dependent on the particular sample of milk, as RSDs were in any case not higher than 13%. Therefore, analyte-fortified control extracts could be used as reference standards to circumvent matrix effects, so improving the accuracy of the analysis of quinolones in incurred milk samples.

Table 3
Matrix effects on recoveries and relative ion signal intensities of quinolone antimicrobials on analyzing in duplicate six different bovine milk samples

Compound	Recovery[a], % (RSD, %)	Signal intensity[b], % (RSD, %)
Marbofloxacin	84 (11)	66 (10)
Norfloxacin (SA)[c]	83 (10)	71 (12)
Ciprofloxacin	82 (11)	68 (11)
Enrofloxacin	85 (9)	70 (13)
Danofloxacin	86 (12)	76 (12)
Lomefloxacin (IS)[d]	–	66 (11)
Difloxacin	86 (12)	84 (9)
Sarafloxacin	84 (11)	89 (9)
Oxolinic acid	81 (13)	110 (12)
Flumequine	87 (14)	50 (13)

Spike level: 100 ng/ml.
[a] Mean values from duplicate analyses of six milk samples from different producers. For each milk sample, analyte recoveries were estimated by comparing their signals to those obtained by injecting an extract of the same milk sample contaminated post-extraction by quinolones.
[b] Mean values obtained by injecting in duplicate six different milk extracts spiked post-extraction with the analytes and relating their signals to those obtained by injecting a pure standard solution.
[c] SA = surrogate analyte.
[d] SI = internal standard.

3.3. Accuracy and intra-day precision

The accuracy and intra-day precision of the method was assessed at three different concentrations corresponding to one-half of the MRL, the MRL and 1.5 times the MRL set by EU. MRLs of three of the quinolones considered, i.e. SAR, DIF and OXO, in milk have been not yet regulated by the EU. For them, we assigned arbitrarily MRLs of 50 ng/ml. At each analyte concentration, six measurements were performed with the criterion of adding 100 ng/ml of the surrogate analyte (NOR) before analyte extraction. Analyte quantitation was performed as reported in Section 2.6, that is by using milk extracts spiked with the analytes and the SA at the same nominal concentration levels as reference standards. Results are reported in Table 4, while a typical MRM LC/MS/MS chromatogram of milk spiked

Table 4
Accuracy (%)[a] and precision (RSD = %) data on analyzing quinolone antimicrobials in bovine milk at concentration equal or close to MRLs set by EU

Compound	MRL/2	MRL	1.5 MRL
Marbofloxacin (75)[b]	99 (7)	101 (7)	102 (5)
Ciprofloxacin (100)[c]	102 (5)	106 (4)	104 (5)
Enrofloxacin (100)[c]	106 (9)	109 (7)	106 (8)
Danofloxacin (30)	96 (10)	101 (6)	101 (7)
Difloxacin (50)[d]	106 (8)	110 (6)	105 (7)
Sarafloxacin (50)[d]	101 (10)	103 (7)	99 (6)
Oxolinic acid (50)[d]	93 (9)	96 (7)	101 (8)
Flumequine (50)	103 (8)	101 (6)	103 (7)

[a] Mean values from six measurements.
[b] MRLs expressed as ng/ml.
[c] The MRL for enrofloxacin set by the EU comprises also its metabolite (ciprofloxacin).
[d] For those quinolones whose tolerance limits have not yet established by the EU, arbitrary MRLs of 50 ng/ml were assigned by us.

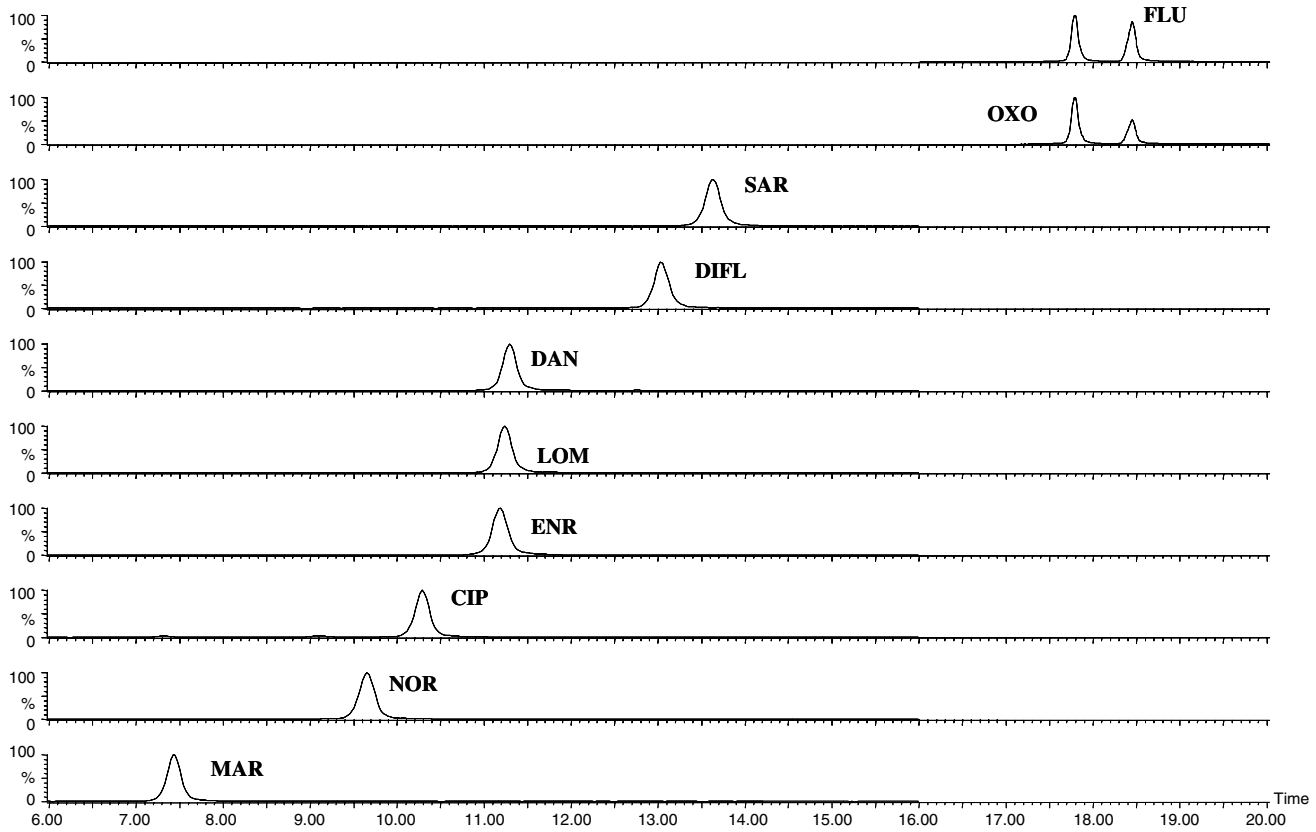

Fig. 2. MRM LC/ESI-MS/MS from analysis of a bovine whole milk sample spiked with quinolone antimicrobials at one half of the tolerance level set by the European Union. For those quinolones (sarafloxacin, difloxacin and oxolinic acid) whose tolerance limits have not yet established by the EU, we assigned arbitrary maximum residue limits of 50 ng/ml. MAR = marbofloxacin (37.5 ng/ml); NOR = norfloxacin (surrogate analyte, 100 ng/ml); CIP = ciprofloxacin (50 ng/ml); ENR = enrofloxacin (50 ng/ml); LOM = lomefloxacin (internal standard); DAN = danofloxacin (15 ng/ml); DIF = difloxacin (25 ng/ml); SAR = sarafloxacin (25 ng/ml); OXO = oxolinic acid (25 ng/ml); FLU = flumequine (25 ng/ml).

with quinolones each one at one-half of the tolerance levels is visualized in Fig. 2. Recoveries varied between 93% and 110% with relative standard deviations not higher than 10%.

3.4. Limits of detection and quantification of the method

LOQs of the method for the eight quinolones considered in milk were estimated from the MRM LC/MS/MS chromatogram shown in Fig. 2. After extracting the sum of the ion currents of the two transitions selected for each analyte, the resulting trace was smoothed twice by applying the mean smoothing method (MassLynx 4.0 Software, Waters). Thereafter, the peak height-to-averaged background noise ratio was measured. The background noise estimate was based on the peak-to-peak baseline near the analyte peak. LOQs were then calculated on the basis of a minimal accepted value of the signal-to-noise ratio (S/N) of 10. As calculated by us, quinolones can be quantified in milk at levels ranging between 0.3 (marbofloxacin) and 1.5 ng/ml (flumequine).

3.5. Linear dynamic range

Under the instrumental conditions reported in Section 2, the linear dynamic range of the ESI/MS/MS detector was estimated for target compounds. Amounts of each analyte equal to 0.3, 0.6, 1.8, 3.6, 7.2 and 15 ng (covering a concentration range between 2 and 250 ng/ml in milk) and a constant amount of 24 ng of the IS were injected from a milk extract sample spiked with the analytes post-extraction. At each analyte amount, three replicate measurements were made. Signal against amount-injected curves were then constructed by averaging the peak area resulting from the sum of the signals for the two precursor ion to product ion transitions of each analyte and relating this area to that of the internal standard. Results showed that ion signals of the eight quinolones were linearly correlated with r^2 ranging between 0.993 and 0.996.

4. Conclusion

This work has shown that an environmentally friendly and inexpensive solvent, such as water, can be successfully used for extracting quinolone antibacterials from milk. Compared to other LC-tandem MS methods quoted in the literature, our method is much simpler and faster. Confirmation of the presence of one particular quinolone in milk could be accomplished in <1 h upon sample receipt, after suitable adjustment of the chromatographic conditions.

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