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Multiresidue Method for the Determination of Quinolone Antibiotics in Bovine Raw Milk by Capillary Electrophoresis−Tandem Mass Spectrometry

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A new analytical method based on capillary zone electrophoresis−tandem mass spectrometry (CZE−MS/MS) is proposed and validated for the identification and simultaneous quantification of eight quinolones for veterinary use in bovine raw milk. The studied quinolones include danofloxacin, sarafloxacin, ciprofloxacin, marbofloxacin, enrofloxacin, difloxacin, oxolinic acid, and flumequine, whose contents are regulated by the EU Council Regulation no. 2377/90 in animal edible tissues. Different parameters (i.e., separation buffer composition and electrospray conditions) were optimized in order to obtain both an adequate CE separation and a high sensitivity, using experimental design methodology to consider the interactions among the studied variables. MS/MS experiments using an ion trap as analyzer operating in the multiple reaction monitoring mode were carried out to achieve the minimum number of identification points according to the 2002/657/EC European Decision. For the quantification in bovine raw milk samples, a two-step solid-phase extraction procedure was developed using Oasis MAX and HLB cartridges without protein precipitation. Satisfactory results were obtained in terms of linearity (r^2 between 0.989 and 0.992) and precision (RSD below 18%). The limits of detection and quantification (below 6 and 24 ppb, respectively) were in all cases lower than the maximum residues limits tolerated for these compounds in milk, the recoveries ranging from 81 to 110%, indicating the potential of the CZE−MS/MS for the analysis of regulated quinolone antibiotics in the food quality and safety control areas.

In the past few years, the public concern about the utilization of antibiotics in food-producing animals has increased due to the transfer of antibiotic-resistant bacteria to man.[1] This is an increasingly prominent problem because antibiotics are used in animals both to treat infections and as growth promoters and so antibiotic-resistant strains can emerge in both healthy and sick animals. This fact would make useless the antibiotic treatment in common human infections, and some authors[2] have even pointed out that "we are at the gate of an uncertain future in which the conditions that existed before 1940, in the pre-antibiotic era, may recur". Other problems related to the misuse of antibiotics are as follow: (a) they can produce allergic hypersensitivity reaction in some persons; (b) fermentation processes, such as the yogurt elaboration, could fail; and (c) the presence of antibiotics could hide the existence of pathogens in foodstuffs when bacteriological analyses are carried out. For these reasons, it is important to control the antibiotic residue content in edible animal tissues.

An important group of antibiotics are quinolones, which were introduced in the early 1960s with nalidixic acid, and now are still among the most used ones because they are highly active against a wide spectrum of Gram-negative bacteria but also moderately active against Gram-positive bacteria.[3] Their activity is based on the inhibition of bacterial DNA synthesis, and they are widely used in human as well as in veterinary medicine for the treatment of pulmonary, urinary, and digestive infections. The wide application range and the extensive use and misuse of quinolones in veterinary medicine represent a potential hazard because residues of these antibiotics may persist in edible tissues or foodstuffs, such as milk or eggs. Some quinolones have been specifically developed for veterinary medicine, but when metabolized, it is possible to find quinolones intended for human use in food-producing animals, which is the case of enrofloxacin and its major metabolite ciprofloxacin.

The European Union has set maximum residue limits (MRLs) of antibiotics in foodstuffs of animal origin by means of the directive 2377/90/EEC.[4] On the other hand, in order to achieve unambiguous identification of the antibiotic residues, it is necessary to reach the minimum number of identification points established in the 2002/657/EC European decision.[5] Therefore, sufficiently sensitive multiresidue methods that provide reliable determinations of quinolones in foodstuff of animal origin are

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needed in order to implement these directives. Traditionally, quinolones have been determined by liquid chromatography[6,7] using ultraviolet[8,9] or fluorescence detection,[10−13] although for regulatory purposes mass spectrometry[14−17] is the preferred technique due to its intrinsic characterization power. In spite of its possibilities in the analysis of antibiotics,[18] capillary electrophoresis (CE) has been less used so far for the determination of quinolones in foods, mainly with ultraviolet detection,[19−21] although laser-induced fluorescence detection[22] has also been employed. CE−mass spectrometry (CE−MS) is still not much used for quantification purposes[23,24] although some papers indicated the great potential of such a coupling in the determination of pesticides[25−28] and antibiotics.[29] To our knowledge, only in one previous paper was the applicability of CE−MS/MS for the separation of quinolones[30] investigated, but sarafloxacin (SAR), difloxacin (DIF), and oxolinic acid (OXO)—which are EU-regulated quinolones—were not included, no quantification was carried out, and the method was not applied to real samples.

The goal of this work is, therefore, to demonstrate for the first time the possibilities of CE−MS/MS to quantify and identify simultaneously all quinolones for veterinary use in a complex matrix such as bovine raw milk. To achieve this purpose, after selecting the separation conditions, an optimization of the electrospray ionization was carried out using experimental design. Although this methodology is still not actually common in the optimization of CE−MS methods,[25,26] it represents an interesting strategy in contrast to the univariate traditional approach when different parameters are to be optimized in order to consider possible interactions or quadratic effects that can be found among them. In the case of CE−MS coupling, it is not so obvious to know the order in which the optimization of parameters involved in the electrospray phenomena must be carried out or if all of them are really significant for the selected response so as to require a rigorous optimization. The application of experimental design methodology can solve these problems by means of the use of screening and response surface designs. A preliminary screening design can be used to determine the significant and nonsignificant factors on the selected response, in order to reduce their number in the optimization study. Next, response surface designs can be applied to obtain the optimum values for the significant factors in the selected experimental region. By means of the study of the interactions between factors, it is possible to obtain a better knowledge of the electrospray ionization process. In this way, a more efficient spray can be obtained and lower limits of detection are achieved.

One of the main difficulties in the development of an analytical method for a complex matrix is the sample treatment, which becomes more complicated when several analytes with different chemical properties are implied. This is the case for this specific study, in which we intended to carry out a multiresidue method for all the veterinary quinolones, regulated by the EU, among which appear two well-differentiated types: acidic and zwitterionic quinolones. Particularly, in the determination of these antibiotics in milk samples, the proposed methods usually involve solid-phase extraction (SPE) after protein precipitation using organic solvents in conjunction with strong organic or inorganic acids.[31,32] These conditions can affect in a different way the retention of quinolones in the selected solid-phase cartridge; hence, procedures to make the retention conditions compatible for chemically different quinolones should be developed to obtain satisfactory recoveries in a multiresidue method.

In this paper, we propose a specific and sensitive method based on the use of CE coupled to tandem mass spectrometry for the identification and simultaneous quantification of all quinolones for veterinary use. Previously, a direct SPE procedure in two steps was developed for the regulated quinolones in milk, without protein precipitation, achieving good recoveries for all the analytes. With the present study we have provided the following: (a) a sensitive and selective CE−MS/MS method for the identification and quantification of eight quinolones below the MRLs established by the EU legislation; (b) an exhaustive knowledge about the electrospray ionization process by means of a rigorous study of the instrumental and chemical variables involved by using experimental design methodology; (c) a new strategy to solve the problem of sample treatment when acidic and zwitterionic quinolones are simultaneously determined in bovine raw milk samples. These results demonstrate the possibilities of CE−MS/MS within the European framework of confirmatory methods for the analysis of antibiotics in foodstuffs.

EXPERIMENTAL SECTION

Chemicals. All chemicals and solvents were of analytical grade. Acetonitrile, methanol, ammonium hydroxide, and sodium

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Table 1. Main Parameters of the MS/MS Method and Obtained Identification Points.

	segment time (min)								
	0−13.0	13.0−13.9			13.9−15.1			15.1−18.0	
	LOM	DAN	SAR	CIP	MAR	ENR	DIF	OXO	FLU
width (*m/z*)	1	1	1	1	1	1	1	1	1
cutoff (*m/z*)	96	98	106	91	99	98	110	71	71
amplitude (V)	0.7	0.7	0.7	0.6	0.6	0.7	0.7	0.5	0.5
precursor ion	352.1	358.1	386.0	332.1	363.1	360.1	400.0	262.0	262.0
fragment ions	334.1, 308.1, 288.2, 265.1	340.1, 314.2	368.1, 342.2, 299.1	314.1, 288.2, 245.1	345.1, 320.0, 276.1	342.1, 316.1, 245.1	382.1, 356.2, 299.3	244.0	244.1
identification points	7	4	5.5	5.5	5.5	5.5	5.5	2.5	2.5

hydroxide were supplied by Panreac (Madrid, Spain). 2-Propanol, formic acid, and ammonium acetate were purchased from Merck (Darmstad, Germany). Danofloxacin (DAN), SAR, and DIF were obtained from Riedel-de Haën (Seelze, Germany). Flumequine (FLU) and lomefloxacin (LOM) were purchased from Sigma (St. Louis, MO). Ciprofloxacin (CIP), enrofloxacin (ENR), and OXO were acquired from Fluka (Buchs, Switzerland). Marbofloxacin (MAR) was kindly supplied by Vetoquinol S.A. (Madrid, Spain). Distilled water (18.2 MΩ cm^{-1}) was deionized by using a Milli-Q system (Millipore, Bedford, MA). A mixture of 100 μg/mL of each quinolone was prepared in MeCN/H_2O (1:1) (v/v) and stored at 4 °C in an amber-colored bottle. The working solutions were made by dilution with water. Two different extraction cartridges containing Oasis MAX and HLB (60 mg, 3 cm^3) (Waters, Milford, MA) were used.

CE Conditions. CE experiments were carried out with an HP^{3D} CE instrument (Agilent Technologies, Waldbron, Germany) equipped with a UV−visible detector working at 275 nm with a bandwidth of 20 nm. Separation was carried out in a bare fused-silica capillary 96 cm total length × 50 μm inner diameter (360-μm outer diameter) from Polymicro Technologies (Phoenix, AZ). Injections were made at the anodic end using a pressure of 50 mbar for 75 s (~3% of the total capillary volume, 60 nL). The electrophoretic separation was achieved with a voltage of 25 kV (normal mode) with an initial ramp of 0.3 min. The running buffer was an aqueous solution of 70 mM ammonium acetate adjusted to pH 9.1 with 5 N ammonium hydroxide. The temperature of the capillary was kept constant at 25 °C. Under these conditions, the capillary current was ~35−40 μA avoiding corona discharge and disruptions of the CE separations. Before the first use, the capillary was conditioned by flushing with 1 M NaOH for 10 min at 60 °C, then with water for 5 min, and finally with the background electrolyte solution for 20 min. A pressure of 1 bar was applied. At the beginning of each day, the capillary was prewashed with a N_2 pressure of 7 bar for 4 min with 0.1 M NaOH, 3 min with water, and 5 min with running buffer. After each run, the capillary was prewashed at 7 bar for 1 min with buffer to maintain an adequate repeatability of run-to-run injections.

ESI Interface. The Agilent coaxial sheath-liquid sprayer was used for CZE−MS coupling (Agilent Technologies). The fused capillary was mounted in a way that the tip just protruded from the surrounding steel needle ~1/4 of the capillary outer diameter. The sheath liquid consisted of 2-propanol/water/formic acid (50:49:1 v/v/v) and was delivered at a flow rate of 3 μL/min by a PU-2085 Jasco high-pressure pump (Jasco Analítica, Madrid, Spain), equipped with a splitter 1:100. The ESI voltage was set to −4 kV. Other electrospray parameters at optimum conditions were as follows: nebulizer pressure at 10 psi, dry gas flow at 6 L/min, and dry gas temperature at 150 °C. In order to avoid the entrance of air bubbles during the injection step due to the Venturi effect caused by the nebulizer gas, it was necessary to carry out the preconditioning and the sample injection with the nebulizer pressure and the ESI voltage set at zero, and a delay of 2 min was introduced after the sample injection before setting the optimum spray gas and ESI voltage.

MS and MS/MS Conditions. MS was performed using an Agilent 1100 Series LC/MSD SL mass spectrometer (Agilent Technologies) equipped with an ion trap analyzer. The mass spectrometer was operated in the positive ion mode and scanned at 190−410 *m/z* (target mass 360 *m/z*, compound stability 100%) at 13 000 *m/z*/s. In the MS experiments, the trap parameters were selected in ion charge control mode using a target of 50 000, maximum accumulation time of 300 ms, and five averages per experiment. In MS/MS experiments, the maximum accumulation time was set at 50 ms with two averages per experiment in order to obtain 10 spectra per peak. Fragmentation was carried out by means of collision-induced dissociation with the helium present in the trap for 40 ms in multiple reaction monitoring (MRM) mode. MS/MS parameters are summarized in Table 1 and MS/MS spectra and their corresponding structures are shown in Figure 1.

Sample Preparation. Bovine raw milk was taken from a local farm. Before the SPE procedures, the milk samples were treated as follows: A 5-g aliquot of bovine raw milk was accurately weighed in a test tube and spiked with quinolones at different concentration levels. A 400-μL aliquot of concentrated NH_3 was added and the mixture vortexed for ~10 s. A 2.5-mL aliquot of this mixture was centrifuged at 18 000 rpm for 10 min in two different Eppendorf vials, and then the solid fat was separated from the aqueous portion with a spatula. A 2-mL aliquot of the resulting sample was percolated through an Oasis MAX cartridge (60 mg, 3 mL), which was previously activated with 2 mL of methanol and 2 mL of 2% NH_3 solution in water. After sample percolation, the cartridge was washed with 2 mL of 2% NH_3 solution in water and 1 mL of 2% NH_3 solution in acetonitrile to eliminate salts and proteins. Finally, the cartridge was vacuum-dried and the elution was carried out with 3 mL of 2% formic acid in MeOH. The eluent was collected in a 4-mL vial and concentrated under a gentle nitrogen current at 40 °C until dryness. The extract was reconstituted in 3 × 1 mL of 50 mM phosphate buffer at pH

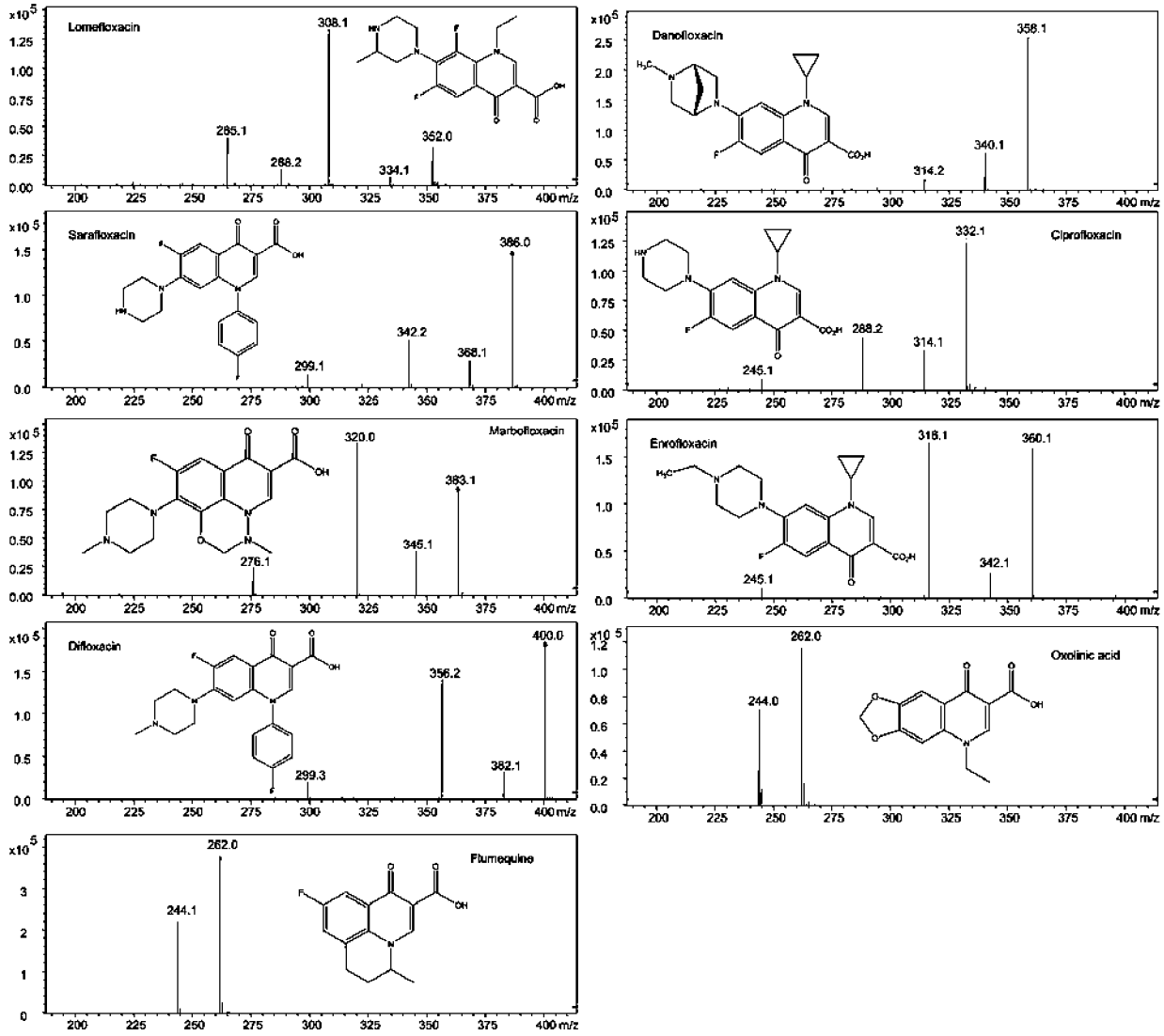

Figure 1. MS/MS spectra of the studied quinolones and their corresponding structures.

7 and percolated through an Oasis HLB cartridge (60 mg, 3 cm^3), which was previously conditioned with 2 mL of methanol and 2 mL of water. After application of the extract, the cartridge was rinsed with 2 mL of water and vacuum-dried. The quinolones were eluted from the column with 3 mL of MeOH. The eluate was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was reconstituted in 250 μL of 1 N NH_3 solution in water containing 1 μg/mL lomefloxacin as internal standard (IS). Finally the extract was filtered through a 0.22-μm nylon membrane and injected in the CE system.

Software. UV−visible electropherograms were acquired using the software provided with the HP ChemStation version A.09.01. The MS spectrometer was controlled by a PC running the Esquire software 4.1 from Bruker Daltonics (Bremen, Germany). The StatGraphics Plus Software 5.1 (Statistical Graphics, Rockville, MD) was used to generate the experimental designs and data processing.

RESULTS AND DISCUSSION

Electrophoretic Separation. Previous to the coupling with the mass spectrometer, an optimization of the electrophoretic separation was carried out using a UV−visible detector. Some important considerations have to be taken into account when a CE method is developed before its coupling with mass spectrometry detection. For example, a volatile buffer of low conductivity (i.e., electric current below 50 μA) is required to avoid plugging of the dielectric capillary between the spray chamber and the mass spectrometer, as well as to obtain a stable electrospray. As was previously reported and according with the pK values,[30] adequate separation between quinolones can be achieved with basic buffers; hence, for this reason, only ammonium acetate and ammonium carbonate buffers were studied, selecting the first one because better peak shapes were obtained. With this buffer, a systematic study of the pH effect was carried out between pH 8 and 10; finally, pH 9.1 was chosen because at this value the best resolution among the eight quinolones occurred. Although comigrating quinolones can be separated by the mass spectrometer, a minimum resolution is preferred to avoid ionization suppression and because it is easier to insert time segments with different MS/MS conditions in the MRM mode. The optimized buffer concentration was 70 mM as it was found to provide a good compromise among peak shape, electrical current intensity, and analysis time. The separation voltage was also optimized, and a value of 25 kV was selected

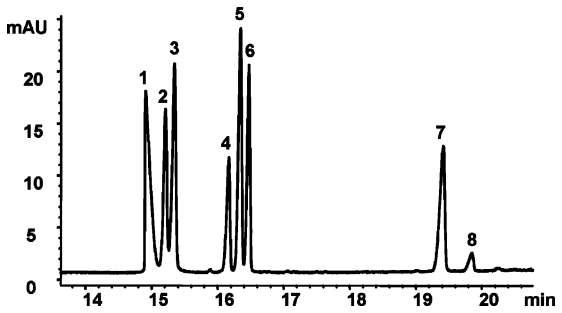

Figure 2. Separation of the quinolones. Optimized conditions using CE−UV−visible: 70 mM ammonium acetate at pH 9.1; separation voltage, 25 kV; capillary temperature, 25 °C; wavelength, 275 nm; injection, 50 mbar × 10 s; capillary length, 96.5 cm. Peak identifications: (1) DAN, (2) SAR, (3) CIP, (4) MAR, (5) ENR, (6) DIF, (7) OXO, and (8) FLU (100 μg/mL of each quinolone).

because higher voltages increased the electric current intensity up to values higher than 50 μA and no significant improvement in analysis time and resolution were observed. Finally, the temperature of the capillary was set to 25 °C because lower temperatures worsened the resolution among MAR, ENR, and DIF, and higher temperatures worsened the resolution among DAN, SAR, and CIP. The addition of organic modifiers—such as acetonitrile or methanol—did not improve the resolution between the quinolones; therefore, they were not employed. Figure 2 shows an electropherogram using UV−visible detection at optimum separation conditions.

CE−ESI-MS(/MS) optimization. First, it was necessary to select the organic solvent present in the sheath liquid. Mixtures of acetonitrile, methanol, ethanol, and 2-propanol with water were tested, all of them at 1:1 v/v and containing 1% formic acid. The best results in terms of intensity of MS signals for the studied quinolones were obtained using a mixture 2-propanol/water. Under these conditions, and with the aim to detect the significant factors related to the electrospray conditions that influence the analytical response, a half-fraction factorial screening design 2^{6-1} in two blocks plus three central points per block (38 runs) was carried out. The selected response was the signal-to-noise ratio of DAN as this is the quinolone with the lowest MRL. The ranges for the different factors studied in this design were as follows: nebulizer pressure (2−10 psi), dry gas flow (2−10 L/min), dry gas temperature (50−300 °C), sheath liquid flow rate (2−8 μL/min), percentage of 2-propanol in the sheath liquid (20−80%) and percentage of formic acid in the sheath liquid (0.2−1.5%). The studied intervals for these parameters were imposed by different constraints such as stability of the spray or instrumental limitations. Although direct infusion has been used in the study of the variables influencing the electrospray using a screening design,[25] in the present work, we have used for the first time a more realistic approach that implies the study of their effects under CE separation conditions. The study and the conclusions from the design are in fact obtained at the same instrumental conditions that will be applied in further experiments using CE−MS to establish the analytical method. Moreover, in order to obtain useful results, it is necessary to use the same response in the screening design to select the significant factors and in the response surface design to optimize these variables. From the screening design, a Pareto Chart (Figure 3) is obtained, in which neither the percentage of formic acid nor the dry gas temperature was found significant in the studied range for the signal-to-noise ratio of DAN. A possible explanation to understand the nonsignificant effect of the formic acid could be that even when 0.2% formic acid is used, DAN appears totally in its cationic form, so an increase in this value does not improve the signal-to-noise ratio. A similar explanation could be suggested to understand the nonsignificant effect of the dry gas temperature; a complete desolvation was obtained even at 50 °C. For further experiments, these values were fixed close to the central point of the experimental design that is 1% formic acid and 150 °C as dry gas temperature, considering that extreme values of the selected experimental region must be avoided because slight deviations of the operating conditions could affect the robustness of the method. Other interesting conclusions were the signs (positive or negative) of the main effects on the response. As we can see in Figure 3, surprisingly, an increase of the 2-propanol percentage produced a negative effect in the response; however, the opposite effect was expected since a higher content of 2-propanol should cause a better desolvation process. The rest of the main factors had an expected sign, for example, the increase in the sheath liquid flow rate produced a lower response maybe due to an increase of the dilution of the DAN into the spray. Nebulizer pressure and dry gas flow had a positive effect on the response. These facts can be explained by considering that higher nebulizer pressures produce smaller droplets in the spray, and, therefore, the desolvation process takes place more easily; a similar result can be observed if the dry gas flow is increased. It is most important to note that the interactions between percentage of 2-propanol−dry gas flow and nebulizer pressure−dry gas flow were found significant, showing the convenience of utilizing the experimental design methodology in the multivariate optimization process. Figure 4 shows these interactions among the significant effects. In Figure 4a it can be seen that when the percentage of 2-propanol in the sheath liquid is low, a higher dry gas flow is necessary to obtain better signal-to-noise ratios. This behavior could be explained considering that it is more difficult to evaporate water than 2-propanol; therefore, a higher dry gas flow is necessary to achieve a complete desolvation of DAN molecules. Figure 4b shows that the best results in terms of signal-to-noise ratios are obtained when high nebulizer pressure and dry gas flow are applied. In this situation, smaller droplets in the spray are obtained, as a consequence of the high nebulizer pressure, and a complete desolvation is achieved, due to the high dry gas flow. The limitation is imposed by the stability of the spray, because if the nebulizer pressure and the dry gas flow are too high, the capillary tip can dry off, especially when the percentage of 2-propanol is high as well, and it would not be possible to close the electric circuit.

The next step was the optimization of the significant factors once identified. This task was carried out by means of a Doehlert design[33] plus three central points (23 runs). This kind of response surface design is scarcely used in analytical chemistry[34−36] in spite of advantages such as its high efficiency compared with other

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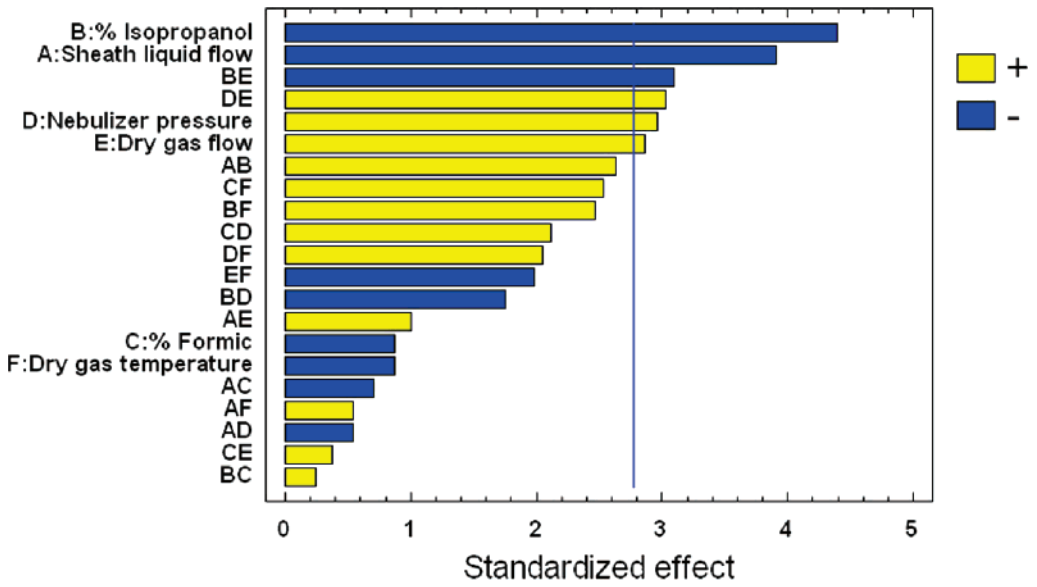

Figure 3. Pareto chart showing the significant effects on the DAN signal-to-noise ratio from the screening design in the study of important variables for ESI conditions. (+) Positive effects on the response; (−) negative effects on the response. Black line shows the limit of decision to consider the significance of the factors (based on the standardized effect = estimated effect/standard error, *P*-value = 0.05 at 95% of confidence level).

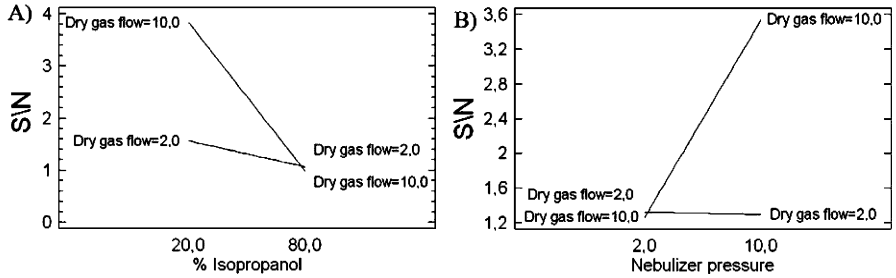

Figure 4. Interaction plots for (A) % 2-propanol−dry gas flow and (B) nebulizer pressure−dry gas flow.

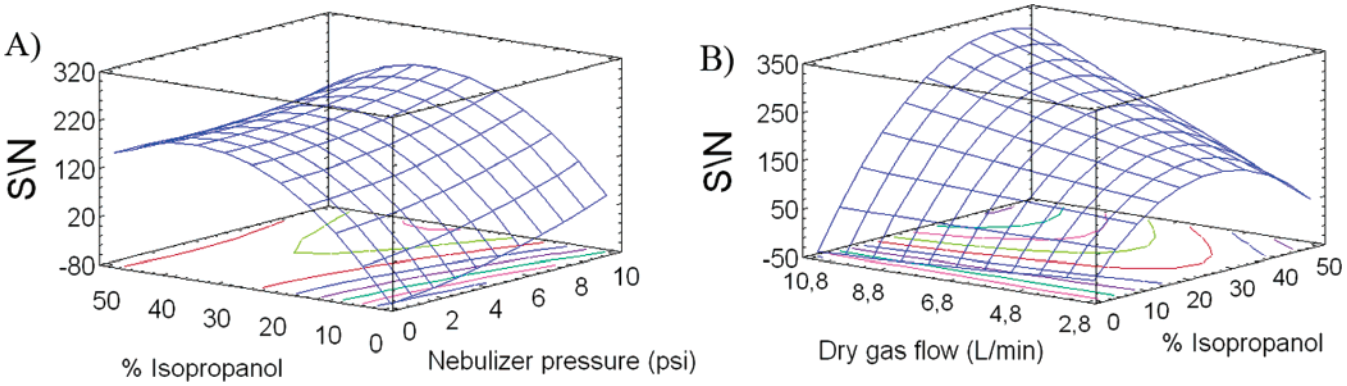

Figure 5. Estimated response surfaces obtained in the multioptimization procedure using Doehlert design. (A) % 2-propanol−nebulizer pressure and (B) dry gas flow−% 2-propanol.

designs like Box-Behnken or central composite designs, and because of the possibility to study a higher number of levels for the most significant variables, taking into account the observed effects from the screening design. In our case, the percentage of 2-propanol in the sheath liquid (0−50%) and the sheath liquid flow (2−8 μL/min) were studied at seven levels, the nebulizer pressure (2−10 psi) at five levels, and the dry gas flow (3−9 L/min) at three. The analytical response to be optimized was the same as in the previous screening design. The response surfaces corresponding to the significant interactions are shown in Figure 5, as optimum values: a percentage of 2-propanol in the sheath liquid of 50%, nebulizer pressure of 10 psi, sheath liquid flow of 3 μL/min, and dry gas flow of 6 L/min. The lack of fit *P*-value for the model was 50% and the determination coefficient (R^2) was 88%, indicating that the predicted model fitted well the experimental data.

Once the spray conditions were established using MS mode, the optimization of the MS/MS mode was carried out. Taking

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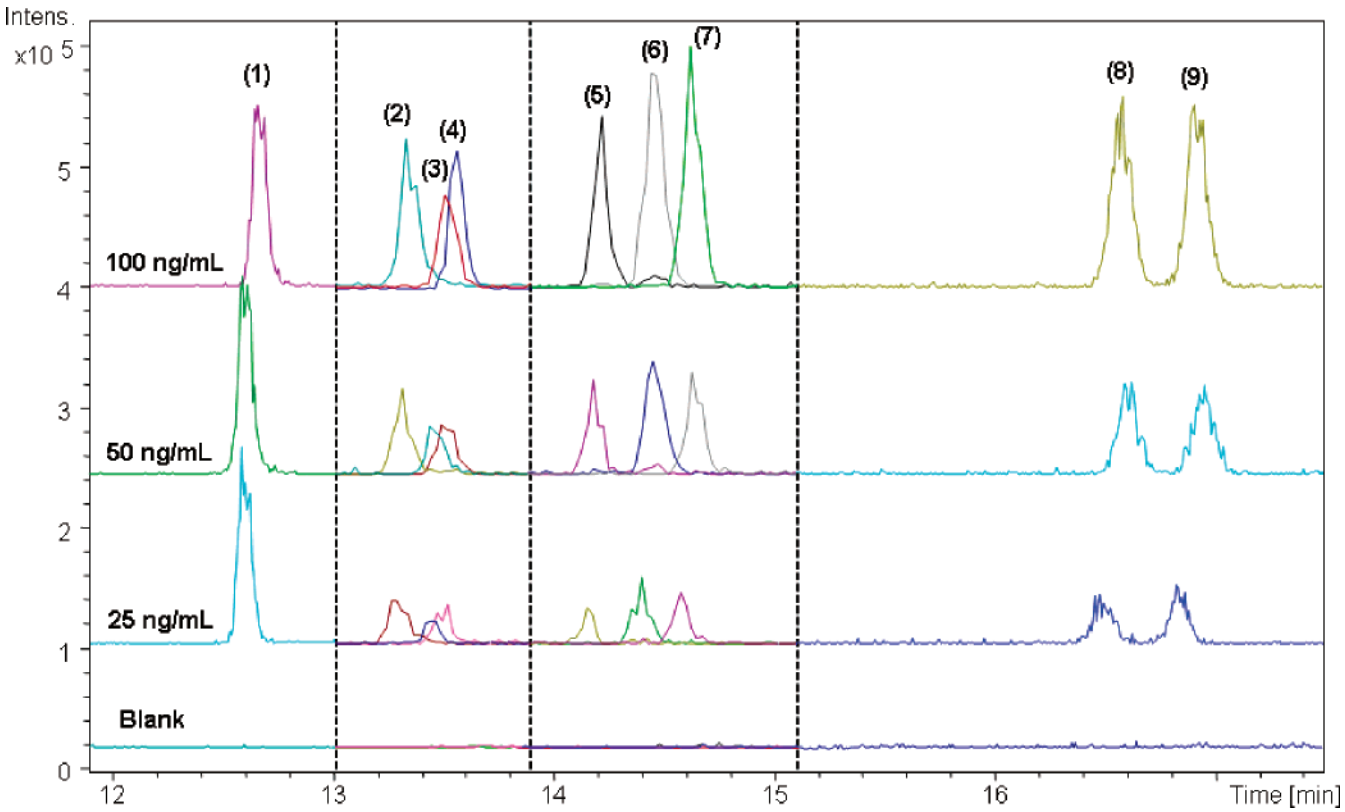

Figure 6. CE−MS/MS electropherograms at optimum conditions obtained after SPE of milk samples spiked at different concentration levels. Peak identifications: (1) LOM (1μg/mL, IS), (2) DAN, (3) SAR, (4) CIP, (5) MAR, (6) ENR, (7) DIF, (8) OXO and (9) FLU.

into account that some peaks could not be well-resolved, the MRM mode was the preferred option. For fragmentation experiments, a cutoff of 27% of the precursor mass, i.e., the minimum m/z of the fragment ion able to be trapped by the analyzer, was set. Quinolones were fragmented using the SmartFrag option that automatically ramps the fragmentation energy from 30 to 200% of the excitation amplitude. The fragmentation amplitude was manually varied and optimized by monitoring the intensities of the fragment ions in order to reach a maximum and also allowing us to see the precursor ion in the trap. With these conditions, the losses of CO_2 and H_2O can be observed for DAN, SAR, CIP, ENR, and DIF. MAR exhibited losses of CH_3CO and H_2O, and OXO and FLU only exhibited the loss of H_2O. Unfortunately, it was not possible to achieve the minimum number of identification points required by the EU[5] in the case of OXO and FLU; even if MS^3 experiments were intended, no additional fragment ions were observed.

Before optimizing the sample treatment procedure, the selection of the IS was carried out. The use of a compound with properties similar to those of the studied analytes can be most useful for quantification purposes in CE−MS.[24] LOM was selected as IS because it is a quinolone for human use; therefore, it is not supposed to be present in bovine raw milk samples; its migration time is adequate because it does not increase the analysis time and does not interfere with quinolone or matrix peaks.

Study of Sample Treatment. In order to apply the optimized method in bovine raw milk, it was necessary to develop a sample treatment procedure. Different strategies were adopted to extract quinolones from biological fluids including direct solvent extraction after pH adjustment of the sample and extraction after protein precipitation. Direct solvent extraction was not applied because a strong emulsification of the milk samples was observed, as reported by Idowu and Peggins.[37] These authors proposed a method based on protein precipitation by the addition of acetonitrile and phosphoric acid, offering good recoveries for ENR and CIP; unfortunately this method is not suitable for acidic quinolones, due to the use of dichloromethane in acidic conditions, an extra preconcentration not being achieved. The utilization of solid-phase cartridges permits a sensitivity increase, preconcentrating the sample and eliminating matrix interferences. This alternative has proved to be suitable for the determination of quinolones in meat,[38] milk,[39] eggs,[40] wastewaters,[41,42] and biological fluids.[43] In this work, a rigorous study was carried out testing three different cartridges: a cationic exchange cartridge (MCX), a polymeric phase cartridge (HLB), and an anionic exchange cartridge (MAX), all from Oasis. The SPE procedures for MAX and HLB cartridges are those described in the sample preparation section. When MCX cartridges were used, the procedure was as follows: preconditioning of the cartridges with 2 mL of MeOH plus 2 mL of water, loading of the sample, washing with 2 mL of 2% formic acid in

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Table 2. Statistics and Performance Characteristics of the Proposed Method[a]

analyte	linearity range (ng/mL)	intercept	slope (mL/ng)	r^2	LOD (ng/mL)	LOQ (ng/mL)	MRL (ng/mL)
DAN	18−400	0.078	0.0086	0.992	4	18	30
SAR	24−400	0.043	0.0072	0.991	6	24	ne[b]
CIP	17−400	0.158	0.0079	0.989	5	17	100[c]
MAR	17−400	0.052	0.0088	0.991	4	17	75
ENR	18−400	0.035	0.0160	0.990	5	18	100[c]
DIF	17−400	0.018	0.0135	0.990	5	17	ne
OXO	19−400	0.068	0.0137	0.992	5	19	ne
FLU	18−400	0.070	0.0134	0.992	5	18	50

[a] In all cases, the lack-of-fit *P*-values were higher than 5%. Concentration (ng/mL) versus relative peak areas. [b] ne, not established in milk samples. [c] MRL (ENR + CIP) = 100 ng/mL.

water, and a final elution with 3 mL of 2% NH_3 solution in MeOH. The other steps were similar to those described for the other cartridges.

It was necessary to control the pH of the sample in order to achieve an optimum retention of the quinolones. With MCX cartridges, it was possible to retain quinolones from an acidic pH to pH 7, and with HLB cartridges, the optimum retention was obtained at pH 7 observing elution of quinolones at acidic and basic pH values. In the case of MAX, the retention of all quinolones was complete just above pH 9; at pH 7, it would be possible to retain all quinolones except CIP. These results could explain the low recovery obtained for this analyte with this type of cartridge.[38] Considering that the pH of milk is between 6 and 7, it would be possible to apply milk samples directly into the cartridge without the need of controlling the pH in the case of MCX and HLB cartridges. If MAX cartridges are used, however, it is necessary to add 2% NH_3 solution to the milk sample. After sample centrifugation at 13 000 rpm for 10 min and fat elimination, the aqueous portion was loaded in the different cartridges using the procedures described in the Experimental Section. No peaks were observed when MCX cartridges were used, perhaps due to the complexation of quinolones with proteins. Attempts to eliminate proteins by precipitation with strong acids in organic solvents did not provide good results; therefore, these cartridges were not used further. In the case of HLB cartridges, good peak shapes were obtained but the recoveries were ~50% for all quinolones, maybe because a previous cleanup is necessary in order to eliminate the remaining fat and proteins. When MAX cartridges were used, the recoveries were higher than 90% for all quinolones, but quite bad peak shapes were obtained because the extracts were highly conductive and stacking was not produced. From these facts, we suggested that a step where only the neutral and zwitterionic substances were retained might solve this problem. For these reasons, a two-step SPE procedure was established: in the first one, MAX cartridges are used to eliminate proteins and the remaining fat by means a washing step using acetonitrile with 2% NH_3 solution, and the cleaner extracts obtained are used later for a second step using HLB cartridges to eliminate salts. This procedure showed good results in terms of recoveries and peak shapes, and a preconcentration factor of 8 was achieved; therefore, it was adopted for the validation of the optimized method in bovine raw milk samples. Total ion electropherograms of bovine raw milk samples spiked at different concentration levels are shown in Figure 6.

Table 3. Study of the Procedure Precision of the Proposed Method.

n		DAN	SAR	CIP	MAR	ENR	DIF	OXO	FLU
		Intraday RSD (%)							
6	50 ng/mL	5	12	11	14	8	11	9	10
	100 ng/mL	8	8	9	9	6	7	9	8
	200 ng/mL	6	14	11	6	10	6	5	7
18	mig time (min)	13.1	13.3	13.3	13.9	14.2	14.3	16.2	16.5
	RSD (%)	0.5	0.7	0.6	0.5	0.6	0.6	0.6	0.7
		Interday RSD (%)							
18	50 ng/mL	12	14	16	15	12	15	15	12
	100 ng/mL	18	16	18	12	10	9	14	12
	200 ng/mL	17	15	10	17	12	16	13	12
54	mig time (min)	13.4	13.6	13.7	14.3	14.6	14.8	16.9	17.2
	RSD (%)	2.2	2.3	2.4	2.5	2.6	2.7	3.4	3.5

Validation of the Method. The whole analytical method was validated in terms of linearity, limits of detection (LOD), limits of quantification (LOQ), repeatability, intermediate precision, and trueness, by means of recovery studies. Calibration curves for the quinolones with CE−MS/MS were obtained using clean bovine raw milk samples spiked before SPE treatment. The linearity of the response was established from five calibration levels corresponding to 25, 50, 100, 200, and 400 ng/mL, intending to establish the MRLs in the middle of the linear calibration range. In all cases, 1 μg/mL LOM was added as IS. Two replicates were prepared at each concentration level, and each one was injected in triplicate. Calibration curves were established by considering the relative peak areas (ratio analyte peak vs IS peak). Although a previous calibration curve using standard solutions showed that the MS response was linear at least 2 orders of magnitude (i.e., from 100 ng/mL to 10 μg/mL), the studied working range was considered appropriate since real milk samples with higher contents only occur rarely. A comparison between the calibration curve with standard solutions in 1 N NH_3 solution and the matrix calibration curve with spiked samples was performed using a Student *t*-test with a confidence level of 95%, showing that no significant differences exist between the slopes and intercept, so the matrix does not produce systematic errors for some analytes, making it possible to use directly the standard calibration curve for the quantification of DAN, SAR, MAR, and DIF in bovine milk samples, without significant errors. For the other quinolones, significant differences were found, so a matrix calibration curve

Table 4. Recovery for Each Quinolone at Different Spiked Levels in Bovine Raw Milk Samples[a]

$n = 6$	DAN	SAR	CIP	MAR	ENR	DIF	OXO	FLU
50 ng/mL	91 (9)	85 (9)	81 (4)	92 (9)	88 (5)	99 (11)	101 (9)	99 (6)
100 ng/mL	87 (11)	87 (9)	81 (10)	93 (13)	88 (11)	94 (8)	99 (10)	99 (8)
200 ng/mL	102 (5)	99 (7)	91 (5)	105 (17)	101 (9)	107 (16)	110 (12)	106 (8)

[a] Mean recovery (RSD).

with spiked and treated samples would be necessary in order to take into account the matrix effect or the losses due to the sample treatment.

The LOD was considered as the minimum analyte concentration yielding an S/N ratio equal to three. The LOQ was adopted as the lowest analyte concentration yielding a signal 10 times greater than the noise. To evaluate the noise, a blank sample was treated with the developed SPE procedure and no matrix peaks were found comigrating with the analytes. It is important to notice that the obtained LOQs are below the MRLs for all quinolones. The statistic parameters calculated by least-squares regression and the performance characteristics are presented in Table 2.

The precision of the whole method was evaluated in terms of repeatability and intermediate precision. Repeatability was assessed on the same day by means of repetitive application of the SPE procedure to two samples (experimental replicates) at each concentration level (50, 100, and 200 ng/mL), and each one was injected in triplicate (instrumental replicates). Intermediate precision was assessed for three consecutive days with a similar procedure for repeatability studies. The results, expressed as relative standard deviation (RSD) of relative peak areas and migration times, are given in Table 3. As can be observed, acceptable precision was obtained in all cases.

In order to test the efficiency of the sample treatment, recovery studies at three concentration levels (50, 100, and 200 ng/mL) were carried out. Two replicates were prepared at each concentration level and each one was injected in triplicate. Absolute recoveries were calculated by comparing relative peak areas of milk samples spiked before the SPE procedure with relative peak areas of milk samples spiked after the SPE procedure. Recoveries higher than 80% were obtained in all cases with acceptable RSD. The results are shown in Table 4.

CONCLUSION

A sensitive and specific CE−MS/MS method is described for the determination of eight quinolones for veterinary use in bovine raw milk samples below the MLRs as legislated by the EU. The proposed method eliminates the need to separate quantification and confirmation procedures as required by most published methods for quinolones; moreover, it presents the advantages of the application of CE as separation technique such as simplicity, low cost, and analysis time (up to three samples per hour can be analyzed) combined with the characteristics inherent to MS detection. The detailed study carried out to select and optimize the significant variables that affect the electrospray ionization by means of experimental designs appears to be a suitable alternative to the traditional univariate optimization. Using this strategy, it is possible to achieve a most efficient ionization in order to obtain limits of detection below the established MLRs. The utilization of SPE in the sample treatment without any doubt proves to be a valuable alternative for liquid−liquid extraction procedures even without the necessity to precipitate proteins. This sample treatment avoids the use of organic solvents or chlorinated acids, harmful for the environment. The developed method could be satisfactorily applied as a routine procedure to identify and quantify quinolones for veterinary use in laboratories of food quality and safety control, due to its robustness and feasibility, demonstrating the possibilities of CE−MS/MS, scarcely used so far for quantification purposes.

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