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Research Article

Dispersive liquid–liquid microextraction of quinolones in porcine blood: Optimization of extraction procedure and CE separation using experimental design

A dispersive liquid–liquid microextraction procedure was developed to extract nine fluoroquinolones in porcine blood, six of which were quantified using a univariate calibration method. Extraction parameters including type and volume of extraction and dispersive solvent and pH, were optimized using a full factorial and a central composite designs. The optimum extraction parameters were a mixture of 250 μL dichloromethane (extract solvent) and 1250 μL ACN (dispersive solvent) in 500 μL of porcine blood reached to pH 6.80. After shaking and centrifugation, the upper phase was transferred in a glass tube and evaporated under N_2 steam. The residue was resuspended into 50 μL of water–ACN (70:30, v/v) and determined by CE method with DAD, under optimum separation conditions. Consequently, a tenfold enrichment factor can potentially be reached with the pretreatment, taking into account the relationship between initial sample volume and final extract volume. Optimum separation conditions were as follows: BGE solution containing equal amounts of sodium borate ($Na_2B_4O_7$) and di-sodium hydrogen phosphate (Na_2HPO_4) with a final concentration of 23 mmol/L containing 0.2% of poly (diallyldimethylammonium chloride) and adjusted to pH 7.80. Separation was performed applying a negative potential of 25 kV, the cartridge was maintained at 25.0°C and the electropherograms were recorded at 275 nm during 4 min. The hydrodynamic injection was performed in the cathode by applying a pressure of 50 mbar for 10 s.

Keywords:
Dispersive liquid–liquid microextraction / Experimental design / Fluoroquinolones / Porcine blood
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1 Introduction

Blood has been used as a source of iron and protein of high nutritional and functional quality in human food and animal feed [1]. Porcine blood composition is similar to that of bovine meat. It is approximately composed of 79% water, 18.5% protein, 0.15% fat, 0.07% carbohydrates, and 0.86% minerals, especially heme iron [2]. In Argentina, porcine blood traditionally has been used as an ingredient in many types of sausages due to its gelling properties as well as its ability to improve water holding capacity, and emulsion stability [3].

Fluoroquinolones (FQs) are highly useful antibacterial agents, particularly because their broad-spectrum activity against Gram-positive and Gram-negative bacteria and mycoplasma. FQs are being used as growth promoters and also to improve feed efficiency in food-producing animals at subtherapeutic levels [4].

Because FQs are used in food-producing animals, there are potentially serious health and safety issues related to blood consumption, particularly the risk of the presence of residues of veterinary drugs. Consequently, monitoring of these

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Abbreviations: AS, salicylic acid; **CCD**, central composite design; **CPF**, ciprofloxacin; **DCM**, dichloromethane; **DIF**, difloxacin; **DLLME**, dispersive liquid–liquid microextraction; **DNF**, danofloxacin; **ENF**, enrofloxacin; **ENO**, enoxacin; **FFD**, full factorial design; **FLU**, flumenique; **FQs**, fluoroquinolones; **GTF**, gatifloxacin; **IS**, internal standard; **MRF**, marbofloxacin; **OFL**, ofloxacin ; **PDADMAC**, poly (diallyldimethylammonium chloride); **TCE**, tetrachloroethylene

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compounds in animal blood is imperative for human health protection.

The use of blood for food preparation was approved in Argentina by SENASA (National Service of Agroalimentary Health and Quality). The regulations of SENASA specify all aseptic collection of blood requirements to slaughterhouses but do not express maximum residue limits of veterinary drugs [5].

There are numerous studies and reviews about the determination of FQs in animal tissues [6–11], eggs [12, 13], and bovine milk [14–17], but the number of studies about FQs in porcine plasma or serum is quite small. In general, FQs determination has been made by LC [18–22] and CE [4, 23–25].

In this work, CE was used for the analyte separation because it is a highly efficient separation technique used to determinate charged components. The target FQs have one or two relevant ionizable functional groups, for example, the carboxylic group (pK_{a1} in the range of 5.0–6.5) and the N4 of the piperazine ring placed at position 7 (pK_{a2} in the range of 7.0–8.5). At pH values between pK_{a1} and pK_{a2}, the quinolones are in the zwitterionic form [26, 27].

The extraction of FQs in porcine blood is a difficult task because the analyte is immersed in a complex biological matrix, which consists of highly concentrated erythrocytes, immunological cells, proteins, lipoproteins, lipids, hormones, and unknown compounds. Moreover, FQs exist in two distinct forms as they pass through the blood stream: (i) a fraction that is noncovalently bound to proteins (as albumin or α_1-acid glycoprotein) or other blood components, and (ii) a free fraction that is believed to represent the active form of many drugs, which usually crosses cell membranes or binds to receptors [28, 29].

The analysis of free drug fractions is engaging in clinical chemistry and pharmaceutical science as a means for controlling and studying the effects of drugs on the body. To determine the free fraction of drugs, methods such us equilibrium dialysis, ultrafiltration, and affinity extraction [30–32] should be used. In the determination of FQs residues in porcine blood it is important to measure the total amount of drug, free and bound protein fraction.

The commonly utilized techniques for sample clean–up and FQs extraction from porcine plasma or serum involve SPE using commercial cartridges (Sep–Pak C–18 or Oasis HLB) [18, 19, 21, 23–25, 33] conventional liquid–liquid extraction using ACN and phosphoric acid [20] and precipitation cleanup including extraction with acidic ACN, coagulation with ammonium acetate, and centrifugation [22]. Conventional liquid–liquid extraction can be used to prepare samples for analysis by CE but is slow and a laboriously intensive technique because it requires extensive amounts of hazardous organic solvents and greats samples volumes. On the other hand, SPE is an efficient extraction technique, but the sample requires a previous cleanup to be adequately conditioned.

The dispersive liquid–liquid microextraction (DLLME) technique was reported for the first time for Rezaee et al. in 2006 [34] and it has been widely used. The advantages of DLLME technique are simplicity of operation, rapidity, low cost, high recovery, and great enrichment factor. There are some methods based on DLLME to extract FQs from water samples [35] and biological samples such as swine muscle, chicken liver, and raw cow milk [7, 11, 15]. Although these matrices are different from porcine blood, DLLME, it is a good strategy to extract FQs in porcine blood as an alternative to conventional techniques. It is noteworthy that, to the best of our knowledge, there are not methods to determine FQs in porcine blood using DLLME as extraction technique.

This paper presents a novel pretreatment of porcine blood, based on DLLME, to sample cleanup and extract simultaneously nine FQs. The FQs analyzed were flumenique (FLU), difloxacin (DIF), enrofloxacin (ENF), marbofloxacin (MRF), ofloxacin (OFL), ciprofloxacin (CPF), danofloxacin (DNF), enoxacin (ENO), and gatifloxacin (GTF). The first six FQs were quantified by a univariate calibration method. The quantification of the last three needs to be carried out with chemometrics tools because the overlapping with porcine blood components. As will be demonstrated, the advantages of this method are the simplicity of operation, rapidity, low cost, high–recovery, high enrichment factor, and environmental benignity fitting the requirements of the green analytical chemistry.

The CE analysis was realized using dynamic coating capillary with high molecular mass poly (diallyldimethylammonium chloride) (PDADMAC) to obtain a constant EOF and guarantee excellent migration time precision.

Additionally, the use of the internal standard (IS) is a common practice in CE techniques to solve problems related to lack of precision of the migration time (t_m) of the analytes and their peak areas. Salicylic acid (AS, pK_a 2.97) was selected as IS because it migrates faster than EOF at pH higher than of 3.97 using capillary mentioned above and reverses polarity, and it presents good absorbance in a wide range of wavelength. Moreover, there is little probability that appears in porcine blood and interferes with the extraction and detection of FQs.

To obtain complete separation between the nine FQs and the IS in CE analysis and to increase the extraction of FQs by DLLME, experimental design including both full factorial (FFD) and central composite design (CCD) were performed. The combination of microextraction and experimental design significantly simplifies sample processing and allowed a reduction in the number of optimization experiments.

2 Materials and methods

2.1 Chemicals and reagents

Ultrapure water was obtained from Millipore (Bedford, MA, USA). Sodium tetraborate, disodium hydrogen phosphate, hydrochloric acid (HCl), sodium hydroxide (NaOH), DMF, dichloromethane (DCM), tetrachloroethylene (TCE) and TCA were purchased from Cicarelli (San Lorenzo, Argentina). The polymer PDADMAC was purchased from Sigma–Aldrich

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Inc. (St. Louis, USA). LC grade methanol (MeOH) and ACN were obtained from J.T. Baker (Deventer, Netherlands).

All standards were of analytical grade. AS, FLU, DIF, ENO, OFL, and GTF were provided by Sigma-Aldrich (Munich, Germany). ENF, CPF, and DNF were purchased from Fluka (St. Gallen, Switzerland), and MRF was obtained from Molekula (Gillingham, UK).

2.2 Standard solutions preparation

Stock standard solutions of FQs were prepared in MeOH with a concentration level of 2000 μg/mL and were maintained under refrigeration at 4°C in the dark. Working standard solutions were prepared daily by appropriate dilution of the stock standard solutions in a mixture of water–ACN (70:30, v/v). NaOH solution was prepared at a concentration of 0.1 mmol/L and HCl was prepared at a concentration of 0.1 and 2.0 mmol/L. These solutions were used to adjust the pH of BGE and porcine blood.

2.3 Instrumentation and optimum experimental conditions

All the CE experiments were carried out on an Agilent CE system (Agilent Technologies, Waldbronn, Germany) equipped with an on-column DAD. The separations were performed in the anionic mode (i.e. cathode in the inlet and anode at the outlet), employing an uncoated fused-silica capillary (MicroSolv Technology Corporation, Eatontown, USA) with an inner diameter of 75 μm and a total length of 40 cm (31.5 cm effective length), which was dynamically coated with PDADMAC. Separation was performed applying a negative potential of 25 kV, using a BGE solution containing equal amounts of sodium borate ($Na_2B_4O_7$) and disodium hydrogen phosphate (Na_2HPO_4) with a final concentration of 23 mmol/L containing 0.2% of PDADMAC and adjusted to pH 7.80. The cartridge was maintained at 25.0°C, and the electropherograms were recorded at 275 nm during 4 min. The hydrodynamic injection was performed in the cathode by applying a pressure of 50 mbar for 10 s.

To reduce the analysis time and to improve the EOF velocity precision we propose the use of CE with a dynamic coating of a fused-silica capillary with PDADMAC, a linear saturated positive polyelectrolyte polymer that is transparent throughout most of the UV-visible spectrum. The total time required for capillary pretreatment, coating, and equilibration with buffer is only ca. 10 min. Therefore, the capillary was coated using 1% v/v PDADMAC solution, which was prepared in the BGE solution. The coating was performed at the beginning of every working day by following the methodology presented by Fritz and Steiner [36].

Between runs, the capillary was successively flushed with 0.1 mol/L of NaOH, ultrapure water and BGE for 3 min. At the end of the day the capillary was washed with 0.1 mol/L of NaOH and ultrapure water for 5 min, and finally, it was air dried for 3 min.

The pH of the solutions was determined employing an Orion 410 A potentiometer equipped with Ag/AgCl pH-meter (Hanna Instruments, Inc., Woonsocket, USA). All solutions were filtered through 0.45 μm Nylon membranes (Sartorius AG, Göttingen, Germany) before use.

2.4 Software

The CE ChemStation software (Agilent Technologies) was employed for the CE instrument control and data acquisition. Experimental design and desirability function calculations were performed with Design-Expert 8.0.5 [37, 38].

2.5 Optimization of the electrophoretic and extraction conditions

The selected FQs have one or two relevant ionizable functional groups. Depending on the medium pH and its pK_a values, the FQs may be in neutral, positive, or negative form. Figure 1 depicts the pK_a values and structure of each FQs analyzed, where the similarity of functional groups and closeness pK_a are appreciated. The efficiency in the electrophoretic separation and DLLME extraction depend on the ionic form of the FQs. Therefore, separation and extraction conditions must be cautiously optimized, specially the pH value. Optimum electrophoretic and extraction conditions were set by experimental design and optimization using desirability function (for more information, see Supporting Information).

A CCD was used to optimize the separation of target compounds (nine FQs and the IS) and consisted in combining the following experimental variables: electrolyte concentration, pH, and the content of PDADMAC in the BGE. After modeling responses, the resolution between all peaks and analysis time, the optimization was conducted using the desirability function.

Two experimental designs were performed to optimize the efficiency of DLLME procedure. First, a FFD consisted in combining categorical factors as a type of extraction and dispersive solvent, and precipitation of protein. Afterward, a CCD consisted in combining numerical factors as sample pH and extraction, and dispersive volume was performed. Finally, the multiple response criterions using the desirability function was successfully used to optimize the extraction efficiency of the nine FQs.

2.6 Sample preparation (DLLME procedure)

Porcine blood was hygienically collected in six different slaughterhouses located in Santa Fe, Argentina. Blood was divided into aliquots in plastic bottles and stored at –20°C until analysis. To extraction of nine FQs, an aliquot of 500 μL of raw blood was adjusted to pH 6.80 by the addition of

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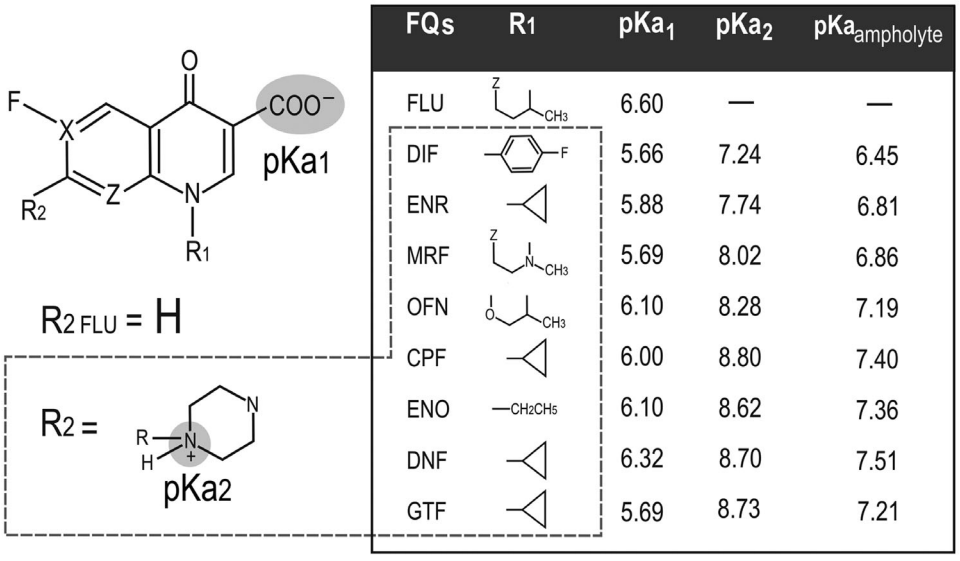

FQs	R1	pKa_1	pKa_2	$pKa_{ampholyte}$
FLU		6.60	—	—
DIF		5.66	7.24	6.45
ENR		5.88	7.74	6.81
MRF		5.69	8.02	6.86
OFN		6.10	8.28	7.19
CPF		6.00	8.80	7.40
ENO	—CH_2CH_5	6.10	8.62	7.36
DNF		6.32	8.70	7.51
GTF		5.69	8.73	7.21

Figure 1. General structure of the quinolones with its corresponding pK_a values.

40 μL of HCl 0.2 mol/L. Then, 1250 μL of ACN and 250 μL of DCM were rapidly inserted into the sample by a micropipette. With the aim of assuring a complete dispersion and to favor the extraction, the mixture was vortexed for 1 min. The extracting phase was separated by centrifugation at 10 000 rpm for 5 min and collected in a glass tube. Finally, the solvent was evaporated to dryness under a nitrogen stream, and the residue was redissolved in 50 μL of a mixture of water–ACN (70:30 v/v) and injected into the CE system. Consequently, a tenfold enrichment factor can potentially be reached with the pretreatment, taking into account the relationship between initial sample volume and final extract volume.

3 Results and discussion

3.1 Optimization of the CE separation

Due to a significant amount of analytes with similar physicochemical properties, and considering that the objective of this work is to achieve the complete analyte separation in the shortest analysis time, it was necessary to assure a constant and reproducible EOF. To solve this challenge we used a capillary coating with PDADMAC, where a stable and almost constant anionic EOF is achieved.

The criterion used to define the complete separation of the analytes was the analysis of the resolution. The resolution can be defined according to Eq. (1):

$$R = 2\frac{(t_{m2} - t_{m1})}{(w_1 + w_2)} \quad (1)$$

where tm_1 and tm_2 are the migration times of the consecutive peaks, and w_1 and w_2 are the electrophoretic peak widths at half height (in time). When the resolution is higher than 1.50, the two species are considered to be completely resolved at the baseline.

The pH value is an important parameter for the separation. At pH less than 7.00, most of the FQs are in neutral or positive form, and overlapping peaks or reduced separations can be obtained. On the other hand, at pH upper than 8.00, FQs are in negative form, and in our experience the separation was not complete (there were overlapping of peaks). At the same time, under conditions of higher pH, BGE concentration and a significant amount of PDADMAC, they appeared problems associated with high current, causing the need to limit the pH values used in the CCD for CE separation.

A CCD was used to obtain the optimum separation, and consisting of 17 experiments, three of which were central points. The experimental points were performed for duplicated with a total of 34 experiments (design matrix is shown in Supporting Information Table 1). The combinations of the selected independent variables were in the following ranges: BGE concentration 15–25 mmol/L, pH 7.00–8.00, content of PDADMAC 0.10–0.30% v/v. These ranges were selected based on prior knowledge about the system under study and were limited by the physical constraints of the instrument and buffer systems [39]. All experiments were performed in random order (the software used allowed the constructions arrays in this way) to minimize the effects of uncontrolled factors that may introduce a bias in the measurements. The evaluation consisted of analyzing a standard stock solution containing the nine FQs (10.00 μg/mL). In each case, the peak resolutions between FQs were evaluated fitting polynomial models. The model coefficients were computed by backward multiple regression and validated by the ANOVA [38].

The desirability function was used to optimize the multiple response systems [40] with the criteria of maximize the resolution between adjacent peaks. Finally, values of the design variables that maximize the global desirability were chosen as the optimal experimental conditions, resulting in 23.00 mmol/L of a mixture that contains equal amounts of sodium borate and disodium hydrogen phosphate, pH 7.80 with 0.2% of PDADMAC. The suggested optimal conditions

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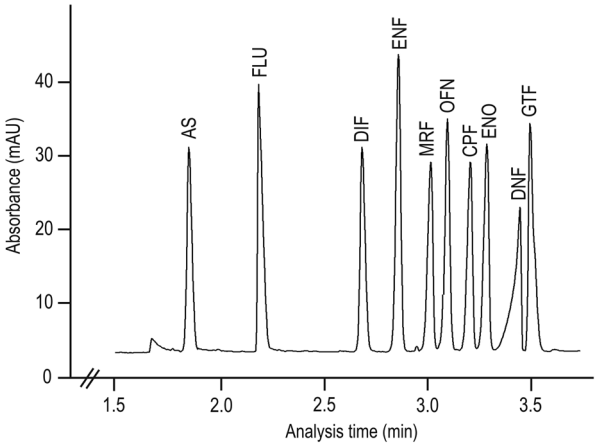

Figure 2. Electropherogram of a standard solution of nine FQs at 10.00 μg mL/L, obtained under optimum separation conditions: BGE solution containing equal amounts of $Na_2B_4O_7$ and Na_2HPO_4 with a final concentration of 23 mmol/L containing 0.2% of PDADMAC and adjusted to pH 7.80. Separation was performed applying a negative potential of 25 kV, temperature 25°C, hydrodynamic injection (applying 50 mbar 10 s) and detection at 275 nm.

were then experimentally corroborated, obtaining electropherogram as presented in Fig. 2. As can be seen in Fig. 2, there is a complete separation between all analytes. Although the peaks were very close, the criteria of a resolution greater than 1.50 were fulfilled.

3.2 Optimization of DLLME

Some factors affect the extraction process, including type and volume of extraction and dispersive solvents, centrifugation time, sample volume, and sample conditioning. The aim of sample condition is changed analyte or sample properties (ionic form, oxidation state, ionic strength, or relativity permittivity of the sample) to increase the extraction efficiency by the addition of several types of substances as a salt solution, solvents, acid, or bases solutions.

An FFD was built to identify the factors that affect the extraction efficiency of nine FQs. The design consisted of eight experiments that corresponded to combinations of categorical factors: type of extraction solvent (DCM or TCE), type of dispersive solvent (ACN or MeOH), and protein precipitation (yes or no) (design matrix is shown in Supporting Information Table 2). To evaluate these parameters, 235 μL of extraction solvent, 1500 μL of dispersion solvent, and 50 μL of TCA 20%v/v (when it was appropriate according to the design), were added to 500 μL of porcine blood containing the nine FQs (10.00 μg/mL). In this sense, we analyzed three responses: phase separation, evaporation rate, and efficiency extraction of nine FQs. Before the injection in CE system, all extracts were inspected visually to evaluate the separation phase. Then, the evaporation rate was measured and finally the extraction efficiency was calculated comparing the areas of extracted FQs with those obtained by injecting a standard solution of FQs. After the analysis, it was decided to work with ACN as a dispersive solvent because it allows better phase separation and fast evaporation rate.

The use of TCA to perform protein precipitations decreases blood pH significantly and changed FQs to their positive form, and therefore, the efficiency of the extraction was extremely small. Based on these results, it was decided not to make the protein precipitation with this reagent. However, the pH factor will be considered in the next experimental design.

Moreover, when all the electropherograms were analyzed, nonidentified endogenous peaks at different migration times were observed. Some of these signals interfere with the detection and quantitation of ENO, DNF, and GTF; therefore, for these analytes, efficient extraction values could not be obtained. Thereby, for the other FQs (FLU, DIF, ENF, MRF, OFN, and CPF) both DMC and TCE have the same extraction efficiency, but we decided to use DCM because TCE is more harmful to the environment.

To define the range of pH that could be used in the CCD; an experiment that consisted of adding aliquots of 50 μL HCl (0.1 mmol/L) to 7.00 mL of porcine blood, and measuring the pH value after each addition, was performed. The initial pH value of blood was 7.40 (upper limit in the next CCD). As HCl is added to the sample, the pH decreases and at pH 6.60, the first clots start to appear (Fig. 3). So, pH 6.60 was defined as the lower limit in the pH range in CCD. Simultaneously, the pH corresponding to ampholyte's pK_a is contained in the proposed pH range of CCD, where FQs have a neutral form (fifth column of Table in Fig. 1) and assure an optimum efficiency of extraction.

Therefore, to determinate the best combinations of factors that ensure the optimum efficiency of FQs, a CCD was performed consisting of 17 experiments (design matrix is shown in Supporting Information Table 3). The factors were changed in the following ranges: volume of extraction solvent (DMC) 200–270 μL, volume of dispersive solvent (ACN) 1250–1750 μL, and pH between 6.60 and 7.40.

The aim of optimization procedure was to find the DLLME conditions that provide the maximum extraction

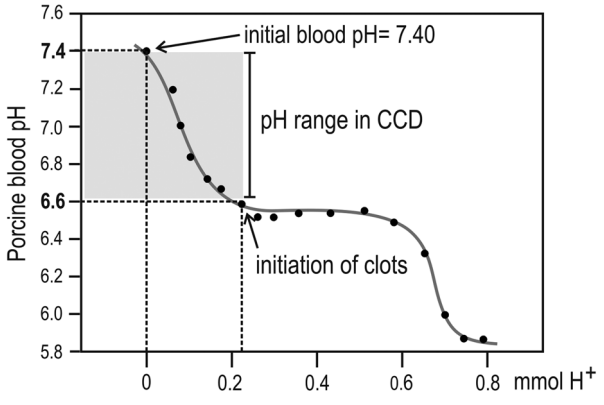

Figure 3. Experiment performed to determine the pH of blood clotting.

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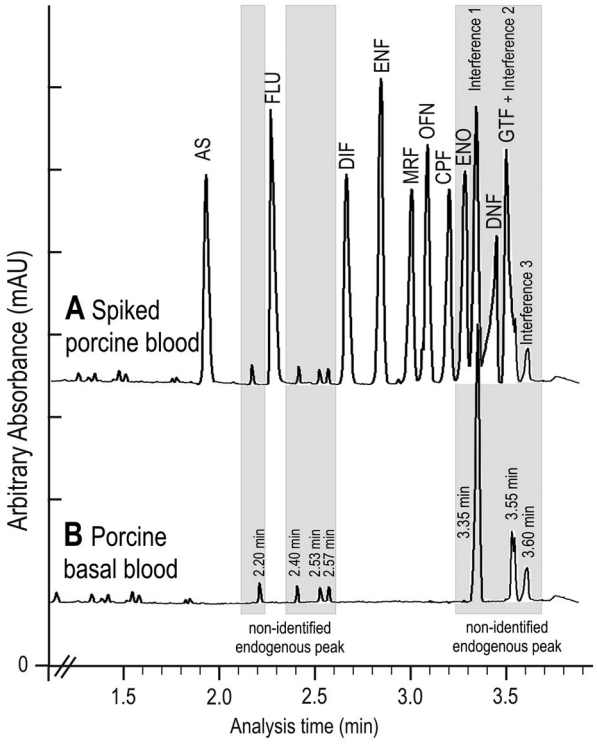

Figure 4. Electropherograms of (A) a porcine blood spiked with nine FQs at 1.00 μg/mL, (B) a porcine basal blood, extracted under the optimal conditions of DLLME: pH 6.80, 1250 μL of ACN and 250 μL of DCM, using 500 μL of sample. The gray areas represent the migration time of the nonidentify endogenous peaks.

recovery of the nine FQs and the minimum number of interfering substances. The efficiency of extraction of each FQs (analyzing 500 μL of porcine blood containing the nine FQs in the level of 1.00 μg/mL) for all experiments was fitted to polynomial models. The model coefficients were calculated by backward multiple regression and validated by the ANOVA

The criteria that were followed by the optimization of the individual responses maximized all responses, giving more importance to the smallest recoveries (the aim of this is to increase the priority to maximize these recoveries) and minimizing the number of unknown peaks. Under the optimization criteria mentioned above, the experimental conditions corresponding to a maximum in the desirability function ($D = 0.48$) are pH 6.80, 1250 μL of ACN and 250 μL of DCM, using 500 μL of porcine blood.

Figure 4A and B shows the electropherogram of a spiked porcine blood and a porcine basal blood, respectively, obtained under the optimal conditions of DLLME. As shown in these electropherograms there were nonidentified endogenous peaks at different migration times. In the first region (between 0 and 2.6 min) these peaks not interfered with target analytes (AS and FLU). However, in the other region (between 2.6 and 4.0 min) there were interferences (called as 1, 2, and 3) that are overlapping with ENO, DNF, and GTF, impeding obtained extraction efficiency values of these analytes. For this reason, recoveries of these FQs were estimated using the theoretical percent of FQs in the pH of the extraction (see before Table 1).

The suggested values during the optimization procedure were experimentally corroborated, and the recoveries obtained using 500 μL of porcine blood spiked with 1.00 μg/mL of the FQs were between 53.4% (FLU) and 95.8% (ENF) (Table 1).

The analysis of Table 1 allowed concluding about the importance of the pH in the extraction. Consequently, under the optimum pH value of 6.80, the extraction efficiencies were greater for the FQs that have its ampholyte's pK_a near to 6.80, because they were in a neutral form, being more soluble in an organic solvent. For example, at pH 6.80, only the 40% of FLU was in its neutral form, which explains its low extraction percentage (53.4%). Notice that the neutral form of ENF was 99.5%, given the greater extraction percentage of 95.8%.

Moreover, this extraction method allows measuring the amount of free FQs and the fraction that can be released from proteins. The HCl added to the sample to reach to desirable pH value changes the charge of molecules and breaks the electrostatic bonds between the protein and FQs, allowing the release of FQs. Moreover, the use of ACN disorganizes

Table 1. Extraction percentage of each FQs obtained under optimal experimental conditions and amount of positive, negative, and neutral species at optimum extraction pH 6.80

Fluoroquinolone	Extraction (%)	FQs(+) at pH 6.80 (%)	FQs (*n*) at pH 6.80 (%)[a)]	FQs(–) at pH 6.80 (%)
FLU	53.4	0.0	40.0	60.0
DIF	72.1	0.0	77.8	22.2
ENF	95.8	0.5	99.5	0.0
MRF	91.6	2.4	97.6	0.0
OFL	86.3	17.9	82.1	0.0
CPF	79.1	21.1	78.9	0.0
ENO[b)]	–	22.2	77.8	0.0
DNF[b)]	–	29.8	70.2	0.0
GTF[b)]	–	13.5	86.5	0.0

[a)] Only the percent of negative form can be extracting with the development method.
[b)] Extraction efficiency cannot be obtained in porcine samples with the development extraction method.

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the protein tertiary structure causing their denaturation and FQs bound to the protein could be release.

4 Concluding remarks

The detailed study carried out to optimize the electrophoretic separation and extraction of target analytes using of experimental design and response surface methodology enhanced by the application of the desirability function, allowed the successful determination of the optimal DLLME and separation conditions.

A novel extraction and preconcentration strategies, based on DLLME can be implemented with the aim of extract FLU, DIF, ENF, MRF, OFN, CPF, ENO, DNF, and GTF in porcine blood samples obtaining an enrichment factor of 10.

The whole method is simple, selective for most analytes, inexpensive and between two and eight times faster than the methods presented in the bibliography [4, 18–25]

Finally, in a future work, ENO, DNF, and GTF will be quantified applying chemometrics tools, and the full validation of the DLLME method presented here will be performed.

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