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Ultrasound-assisted dispersive liquid-liquid microextraction for determination of enrofloxacin in surface waters

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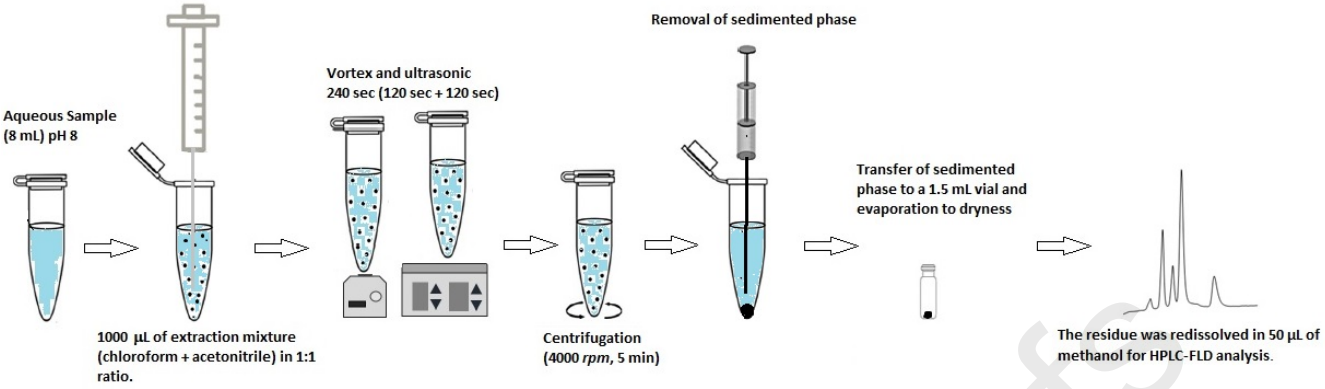
GRAPHICAL ABSTRACT



ABSTRACT

This work describes the development of an HPLC-FLD methodology for the separation of five fluoroquinolones (ciprofloxacin, enrofloxacin, sarafloxacin, norfloxacin and levofloxacin) followed by optimization of the DLLME process for the clean-up and preconcentration of enrofloxacin in samples of seawater and river water. The mobile phase used for the chromatographic separation consisted of methanol: phosphate buffer ($NaHPO_4$ H_2O 0.04 M pH 3 with H_3PO_4 85 %), gradient eluted at a ratio of 20:80 *(v:v)*. The mobile phase flow was maintained at 1.2 mL min^{-1}. For the ultrasonic-assisted dispersive liquid-liquid microextraction (UA-DLLME), the following conditions were used: 8 mL of sample with pH adjusted to 8, extraction solvent: 500 µL of chloroform, dispersive solvent: 500 µL of acetonitrile; samples were vortexed and sonicated for 2 minutes, each. The enrichment factor (EF) was 54.7 and the recovery was 70 %, achieving a limit of detection (LOD) of 0.11 µg L^{-1}. Repeatability and intermediate reproducibility presented values of relative standard deviation (RSD) lower than 2 %. Finally, the optimized method was applied to the analysis of water and enrofloxacin was detected in both water samples with a concentration of 0.20 µg L^{-1} in the river and 0.12 µg L^{-1} in the seawater. However, recovery tests performed to evaluate the water matrices' effects on the extraction performance, presented recoveries of 72±6.1 for river water and 27±8.3 for seawater. These results demonstrate that hereby developed method is only suitable for water samples with a low salinity content.

Keywords: pharmaceuticals; fluoroquinolones; High-performance liquid chromatography; water samples; dispersive liquid-liquid microextraction.

1. INTRODUCTION

Fluoroquinolones (FQ's) are one of the most consumed classes of antibiotics in the world and have been extensively used to prevent or treat bacterial infections and promote yields of animal husbandry [1]. It has been reported that FQ's are not completely metabolized in the human body and are often detected in the environment and wastewater treatment plants (WWTPs) [2,3], being the main source of these antibiotics in the environment [3]. For this reason, FQ's have been detected in river water [4,5], seawater [6], tap water [7], soil [8] and sediment [9].

Among the several FQ's, enrofloxacin (ENRO), ciprofloxacin (CIPRO), sarafloxacin (SARA), norfloxacin (NORF) and levofloxacin (LEVO) are the most common [5,10]. Their physicochemical properties are listed in Table A1. Log K_{ow} is the octanol/water partition coefficient (K_{ow}), which is defined as the ratio of a chemical concentration in the octanol phase to its concentration in the aqueous phase in a two-phase octanol/water system and its values are generally inversely related to water solubility. The carboxylic and fluorine groups contribute to the polar characteristics of FQ's, making them difficult to extract from aqueous matrices. Another important aspect of FQ's structure is the presence of carboxyl and nitrogen functional groups, responsible for the amphoteric properties of these antibiotics. The ionizable functional groups: carboxylic (pK_{a1}) and piperazine nitrogen (pK_{a2}) are responsible for cationic, anionic and zwitterionic species depending on the aqueous pH, affecting greatly their environmental behavior [11].

Table A1. Physical-chemical properties of levofloxacin (LEVO), norfloxacin (NORF), ciprofloxacin (CIPRO), enrofloxacin (ENRO) and sarafloxacin (SARA).

Antibiotics	**pK_{a1}**	**pK_{a2}**	**Log K_{ow}**	**Isoelectric point**	**Reference**
LEVO	5.59	7.94	-2.00	6.77	[12]
NORF	6.20	8.55	-3.78	7.34	[12,13]
CIPRO	5.90	8.89	-2.82	7.50	[12,14]

ENRO	6.27	8.30	-0.83	7.28	[12,13]
SARA	6.00	8.60	-1.26	7.3	[12,13]

K_{ow}: octanol-water partition coefficient.

The diversity of environmental matrices, trace concentrations and cost involved in the analysis of pharmaceutical compounds are obstacles that makes it imperative the development of rapid, simple, sensitive, selective and more accessible analytical methods [15]. More sophisticated techniques, such as ultraperformance liquid chromatography-tandem mass spectrometer (UHPLC/MS-MS), have been used to analyze FQ's, obtaining a low detection limit in the range of 25 ng L^{-1} and 33 ng L^{-1} [16]. The disadvantages of these techniques are the high cost of instrumentation and maintenance, generally associated to these equipment's. In the last years, fluorescence techniques have been applied in several studies due to their efficiency, low-cost and capacity to detect low concentrations [17–19]. Therefore, the use of HPLC-FLD [14,20] has been chosen as an alternative due to the fact that is faster, easier to use and more accessible in common laboratories. Despite the use of a robust technique such as UHPLC-MS and HPLC-MS, preconcentration and cleaning techniques are still necessary to improve the analytical performance [21].

In the field of environmental analysis, there are many protocols for enrichment and clean-up of water samples: liquid-liquid extraction [22], solid-phase extraction [23], QuEChERS [9], pressurized liquid extraction (PLE) [24], molecular imprinted polymer extraction (MIPs) [25], stir bar sorptive extraction [26]. As an alternative, microextraction techniques have experienced great development lately, such as solid-phase microextraction (SPME) [27], liquid-phase microextraction (LPME) [22], dispersive liquid-liquid microextraction (DLLME) [28,29], hollow fiber dispersive liquid-phase microextraction (HF-DLLME) (Lopes et al., 2017), ultrasound-assisted ionic liquid dispersion liquid-liquid microextraction (IL-US-DLLME) [30]. In general, the use of alternative microextraction techniques for the sample preparation reduces the number of errors that usually result from

the multi-stage procedures and limits the negative impact on the environment and the health of the analytical chemists performing laboratory work [31].

DLLME is a simple and fast technique, with many advantages over conventional extraction methods, among them, the low volume of organic solvents (μL), high surface area between the extraction solvent and the aqueous sample and the high enrichment factor [32]. Succinctly, a few microliters of a specific organic solvent (extracting solvent), with high miscibility in the extracting and aqueous phases, are quickly injected into the sample, producing high turbulence cloud. This turbulence causes the formation of micro drops, which are dispersed throughout the aqueous sample. After the formation of a cloudy solution, the equilibrium state is reached, the mixture is centrifuged and the sedimented phase collected. Then, the sedimented phase is evaporated, redissolved in a solvent suitable for HPLC (acetonitrile or methanol) and the analyte is analyzed. Modified DLLME has been applied for the effective removal of fluoroquinolones from water. Among the modifications is ultrasound [33], ice-water bath [30] and hollow fiber-supported [29]. However, the development of fast, simple, and low-cost methodologies for pharmaceutical analysis is fundamental for the monitoring of emerging pollutants, such as FQs in surface waters. The aim of the present work is the development of an HPLC-FLD method for the separation of five FQ's, as well as the optimization of a UA-DLLME procedure for separation, clean-up, and concentration of ENRO in environmental aqueous samples. Although the DLLME method was not developed for the extraction of all five FQ's, it was important the development of an HPLC methodology able to separate them to avoid future interference that could occur from the analysis of environmental water samples. Various parameters affecting the extraction efficiency, including type and volume of extracting and dispersive solvent, sample pH, and extraction time, were investigated.

2. MATERIAL AND METHODS

2.1 Reagents and Standards

Fluoroquinolone antibiotics LEVO (≥ 98.0 %), NORF (≥ 98.0 %), CIPRO (≥ 98.0 %), ENRO (≥ 98.0 %) and SARA (≥ 98.0 %) were provided by Sigma-Aldrich (Brazil). All of the HPLC grade organic solvents (≥ 99.9 %) used (methanol, acetonitrile and dichloromethane, chloroform, tetrachloroethylene, acetone, isopropanol) were from Merck (Darmstadt, Germany) and all other standard analytical grade reagents employed (ammonium hydroxide, orthophosphoric acid, sodium phosphate monobasic, disodium EDTA) were from Isofar (Rio de Janeiro, Brazil). The ultrapure water used was obtained through Milli-Q systems from Merck Millipore (Darmstadt, Germany). The individual stock solutions of LEVO, CIPRO, ENRO and SARA standards were prepared in methanol and the NORF in acetonitrile at the concentration of 100 mg L^{-1}. Successive dilutions were prepared in methanol.

2.2 Instrumentation

The analysis was performed on a Shimadzu High-Performance Liquid Chromatograph coupled to a Fluorescence Detector (HPLC-FLD). This device consists of a DGU-20A5R degasser, two LC-20AT high pressure pumps coupled to a fluorescence detector, model RF-20A also from Shimadzu®, with an excitation length of 280 nm and emission length 450 nm, column oven CTO-10AS and an injector with a capacity of 20 µL. To control the equipment and obtain the data, a microcomputer and LCsolution® software version 1.25 SP4 of Shimadzu were used. The column used for separation was Luna C18, Phenomenex® (5 µm, 250 x 4.6 mm). The column temperature was maintained at 35 ° C. The mobile phase consisted of methanol: phosphate buffer ($NaHPO_4 \cdot H_2O$ a 0.04 mol L^{-1},

adjusted to pH 3 with H_3PO_4 85 %). Table A2 shows the chromatographic conditions adopted for the optimization and validation of the methodology of this work.

Table A2. Chromatographic conditions used in gradient elution of Fluoroquinolones

Parameters	**Conditions**
Column	Luna C_{18}, Phenomenex® (250 x 4.6 mm, particles of 5 µm)
Oven temperature	35 °C
Mobile phase	MeOH/ phosphate buffer ($NaHPO_4.H_2O$ at 0.04 M pH 3 with H_3PO_4 85 %), elution gradient: 0.01 – 7.50 minutes 20/80 (v/v) 7.51 – 9.00 minutes 25/75 (v/v) 9.01 – 25 minutes 35/65 (v/v) 25.01 – 28 minutes 20/80 (v/v)
Injection volume	20 µL
Flow	1.2 mL min^{-1}
λ_{exc} and λ_{ems}	280 nm and 450 nm

The mobile phase was filtered through 0.45µm nitrocellulose membrane filters (Millipore). An MX-S mini vortex and a USC-1400A ultrasound were used for shaking during the extraction process. Each four sample replicates were analyzed two times.

2.3 DLLME procedure

Sample aliquots with 8 mL and pH adjusted to 8 using NH_4OH 1 % (*v/v*). Then, a 1000 µL mixture containing the extracting solvent (chloroform) and dispersive solvent (acetonitrile) in 1:1 ratio, was added to each sample/standard, aliquot the mixture was vortexed and sonicated for 240 s (120 s + 120 s). After the formation of a cloudy solution, due to the dispersion of chloroform droplets in the aqueous sample, tubes were centrifuged at 4000 *rpm* for 5 min. The sedimented phase was collected, transferred to a 1.5 mL vial, and evaporated to dryness. The residue was redissolved in 50 µL of methanol for further HPLC-FLD analysis. DLLME was performed in quadruplicate and each sample was analyzed using HPLC two times.

2.4 Determination of fluoroquinolones in environmental water samples

Samples were collected in July 2018 in the city of São Luís, Brazil, one consisted of a freshwater and another had saline characteristic (sea water). For the collection, the rules of sampling, depth, flow and cleaning of materials used were taken into consideration. The vessel used in the collection was disinfected and the samples transferred into an amber flask with a volume of 1 L and stored and refrigerated at approximately 4 °C. Samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore), previously to the DLLME procedure described in section 2.3. The UA-DLLME-HPLC-FLD method was applied directly to the environmental samples. The evaluation of the water matrix influence on the extraction was performed by spiking known amounts of ENRO on both water samples and subjecting them to the previously optimized extraction procedure. Spiked concentrations used were 1.24 μg L^{-1} and 3.16 μg L^{-1}. At least four replicates were performed for each of the two levels of fortification studied. The recovery percentage was calculated by the ratio between the experimentally determined average concentration and the corresponding expected concentration.

3 RESULTS AND DISCUSSIONS

3.1 Optimization of chromatographic conditions

Initially, an isocratic process was performed starting from the chromatographic conditions [14]. The separation of the five FQ's were inadequate, with overlapping chromatographic peaks (Fig. A1-a). The reverse peak shown in Fig. A1-a is due to a drop in HPLC pressure, associated to the sample injection. Due to overlapping chromatographic peaks, several attempts were made to define the best chromatographic conditions. The following variables were considered: elution mode, mobile phase, mobile phase

concentration, flow, and temperature, as presented in Table A2. The best chromatographic condition (Fig. A1-b) was obtained using gradient elution, in which the concentration of the mobile phase varied throughout the chromatographic run, showing the good separation of the 5 FQ's.

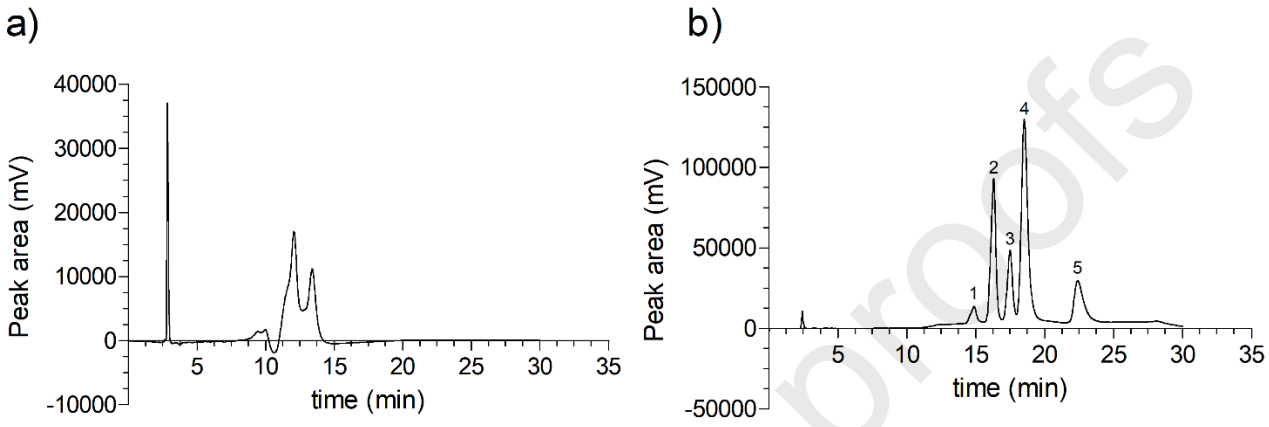


Fig. A1 a) chromatogram of the 5 fluoroquinolones: LEVO, NORF, CIPRO, ENRO and SARA at concentration of 50 µg L^{-1} obtained using an isocratic elution MeOH (15 %)/phosphate buffer (85 %); flow: 1.0 mL min^{-1}; temperature: 25 °C; HPLC-FLD and b) chromatogram of the 5 fluoroquinolones LEVO (1), NORF (2), CIPRO (3), ENRO (4), SARA (5) at concentration of 50 µg L^{-1} obtained using a gradient elution MeOH/phosphate buffer; flow: 1.2 mL min^{-1}; temperature: 35 °C; HPLC-FLD detection; λ_{exc} 280nm and λ_{ems} 450nm. Note that the y-axis is different in all graphs for better visualization.

An analytical technique able to separate these 5 FQ's is fundamental for the quantification of only ENRO in environmental matrices, since all 5 FQ's have been detected in water samples, and the optimization of the extraction procedure might extract the other FQ's, interfering in the quantification of ENRO. Also, this work might be an important contribution to the study of antibiotics in the environment.

3.2 Analytical performance of the HPLC-FLD method

An external analytical curve, without DLLME, was constructed by the relationship between the analytical signal and the concentration of the substance of interest. For this, four replicates of six concentrations of ENRO ranging from 10 to 300 μg L^{-1} were analyzed. The values were used to obtain the linear regression equation by least-squares method. The coefficient of determination (r^2) was used to evaluate the adequacy of the representation of the mathematical model expressed by the linear equation. The performance of the HPLC-FLD method was calculated based on the values of r^2, LOD, and linearity (Lin (%) = 100-RSD_b, where RSD_b is the relative standard deviation of slope of the curve). The LOD was calculated from each calibration curve as $a + 3s_{y/x}$, where a is the intercept of the regression line and $s_{y/x}$ is the statistical parameter that estimates the random errors in the y-axis.

A value of r^2 greater than 0.99 was obtained for ENRO calibration curve (Table A3), which was considered satisfactory. The data from the analytical curve is in Table A3, demonstrating adequate linearity, with a limit of detection of 7.68 μg L^{-1}.

Table A3. Quantitative parameters for typical ENRO analytical curve obtained by HPLC–FLD.

FQ	Slope[a]	Intercept[a]	r^2	LOD (μg L^{-1})	LOQ (μg L^{-1})
ENRO	21756 ± 771.1	102573 ± 40269	0.999	7.68	25.59

[a] (mean value ± standard deviation). n=4.

3.3 Optimization of extraction conditions

3.3.1 Selection of the extracting and dispersive solvents

In DLLME several factors affect the extraction efficiency. One of these factors is the choice of the appropriate extracting solvent. Organic solvents with a higher density than water, good chromatographic behavior and high extraction capacity of the analyte of interest are generally used in these extractions [20,34]. Among them, halogenated hydrocarbons are generally selected as extracting solvents because of their high density. In this work, the following extracting solvents (tetrachloroethylene, chloroform, dichloromethane) were

tested [35,36]. Thus, 110 µL of each extracting solvent together with 500 µL of acetonitrile (as dispersive solvent) were added to an aliquot of 8 mL of water fortified with 10 µg L^{-1} of ENRO adjusted to pH 8 with NH_4OH 1.0 % (*v*/*v*) solution. The DLLME procedure was performed as described in section 2.3

The dichloromethane/acetonitrile mixture added to the sample did not allow to form a clear and well-defined phase separation. The chloroform as solvent extractor showed a clear separation of the sedimented phase, allowing to obtain a higher peak area for ENRO (Fig. A2).

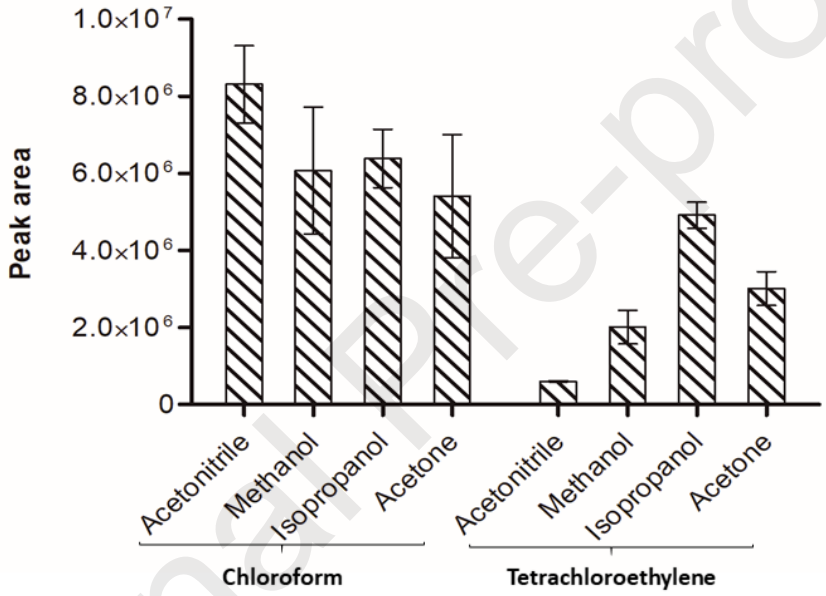


Fig. A2 Efficiency of dispersive and extracting solvents in ENRO extraction using DLLME. Experimental conditions: 8 mL of sample fortified with 10 µg L^{-1}; pH = 8; stirring time 120 s + 120 s; extracting solvent: 110 µL; dispersive solvent: 500 µL; dispersive: extracting ratio 1:4.5. (n=4).

In turn, tetrachloroethylene as a solvent extractor presented results lower than the obtained using chloroform. This can be justified by the K_{ow} value reported for ENRO in Table A1. Based on these results, chloroform seemed to be a better choice as extracting solvent.

Although chloroform seemed to be a better choice as extracting solvent in the presence of acetonitrile as dispersive solvent, the extraction of ENRO using different dispersive solvents was evaluated, for both chloroform and tetrachloroethylene, as extracting solvents. Dispersive solvents tested were methanol, acetonitrile, acetone and isopropanol [37,38].

For the selection of dispersive solvent, from the eight combinations, the best extraction results (Fig. A2), evaluated by the peak area, was obtained using acetonitrile as dispersive solvent and chloroform as extracting solvent.

3.3.2 Selection of extracting solvent: dispersive solvent ratio

After defining acetonitrile as the dispersive solvent and chloroform as the extracting solvent, the next step in the optimization of the DLLME conditions involves choosing the ratio between the extracting and dispersive solvent. The ideal ratio between extracting and dispersive solvent volumes should ensure high extraction efficiency. This ratio directly affects the formation of the dispersive mixture, the degree of dispersion of the extracting solvent in the aqueous phase and the extraction efficiency. After the extraction and dispersive solvents were defined in the previous sections, the best extracting: dispersive solvent ratio was investigated. The proportions tested were 1:10, 1:7, 1:4.5 and 1:1 (extracting solvent: dispersive solvent) [33,36], the volume of the dispersive solvent, in this case, acetonitrile (500 µL), was kept fixed, varying only the volume of the extractive solvent, in this case, chloroform. Fig. A3 (a) shows the concentrations of the pharmaceutical extracted using different ratios, with chloroform as extracting solvent and acetonitrile as dispersive solvent.

The ratio 1:4.5 and 1:10 demonstrated good extraction efficiency resulting in ENRO concentration values between 372 µg L^{-1} and 376 µg L^{-1}. Using the 1:7 ratio the

concentration value obtained were very low, being between the ratios tested the one with worst extraction efficiency. The 1:1 ratio tested allowed to obtain the best results for the ENRO extraction, reaching a concentration of 663 μg L^{-1}. Based on these results, the 1:1 ratio was chosen for following tests.

3.3.3 Selection of extracting mixture volume

The extracting mixture volume can be increased to improve the extraction efficiency. This relationship directly affects the formation of the turbid solution, the degree of dispersion of the extracting solvent in the aqueous phase and also the extraction efficiency [39]. The volume of extracting mixture may influence the enrichment factor of DLLME. Therefore, the volumes of the tested mixture were: 610 μL, 800 μL, 1000 μL; 1500 μL, 2000 μL, 2500 μL. The results presented in Fig. A3 (b) showed that the increase in the volume of the extracting mixture improved the extraction of ENRO up to the volume of 1000 μL. When the mixture volume increased to 1500 μL, there was a decrease in the concentration of ENRO, decreasing even more for a volume of 2500 μL. Moreover, the increase in the mixture volume also increases the volume of the sedimented phase for subsequent drying, making it harder to evaporate. Considering the results obtained, the volume of extracting mixture chosen for the following experiments was 1000 μL.

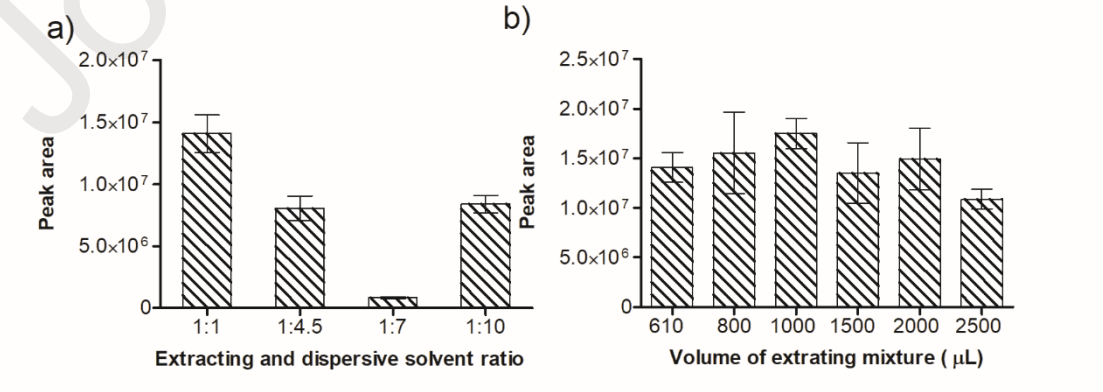


Fig. A3 a) efficiency of extracting and dispersive solvent ratio in ENRO extraction using DLLME. Experimental conditions: 8 mL of sample fortified with 10 μg L^{-1} of ENRO; pH = 8; stirring time 4 min; dispersive solvent: 500 μl of acetonitrile; extracting solvent: chloroform (variable volume) and b) efficiency of volume of extracting and dispersive solvent mixture in ENRO extraction using DLLME. Experimental conditions: 8 mL of sample fortified with 10 μg L^{-1} of ENRO; pH = 8; stirring time 120 s + 120 s; dispersive solvent: acetonitrile; extracting solvent: chloroform; extracting and dispersive solvent ratio 1:1. (n=4).

3.3.4 Effect of sample pH and chelating agent

The influence of pH on extractions is more significant when the analytes of interest have one or more ionizable groups [32]. FQ's are amphoteric compounds which have two functional groups charged oppositely with $pK_1 \approx 6$ (carboxyl group) and $pK_2 \approx 9$ (amino group) (Table A1) [40]. With the change of pH, FQ's, such as ENRO, can vary as the cationic, anionic, and intermediate loaded forms, influencing the extraction efficiency. The distribution of an analyte from an aqueous phase in a hydrophobic organic solvent is greater for a molecule that is neutral [33].

If we assume that at the isoelectric point the positive and negative charges of the antibiotic are equivalent, this point would be the most appropriate for its extraction. For the evaluation of pH in the extraction of ENRO from aqueous solution, pH between 6 and 9.7 were tested and compared with results obtained for pH=8 (used in the previous experiments). To an aliquot of 8 mL water fortified with 10 μg L^{-1} of ENRO with pH adjusted to values between 6 and 9.7, 1000 μL of 1:1 extracting mixture was added. Furthermore, EDTA was used as a chelating agent and an evaluation of the analyte recovery with increasing concentrations of EDTA was performed [35]. To an aliquot of 8 mL of

EDTA solution at each concentration tested (0.05 M, 0.1 M and 0.2 M, with pH adjusted to 8). As shown in Fig. A4 (a), pH has an effect in the extraction of ENRO from water. Higher extraction efficiency was confirmed at pH 8. Moreover, as the pH is increased above 8, there was a decrease in extraction efficiency because ENRO was negatively charged. At pH 7 and lower, ENRO was positively charged, decreasing the extraction efficiency. Thus, the pH of aqueous solution should continue to be adjusted to pH 8 to increase the extraction efficiency of ENRO using DLLME. Franziska et al. (2014) verified an increase of ciprofloxacin recovery with an increase of EDTA concentration, although for other FQ's the opposite was observed. Our results showed, that comparing the results in the absence and presence of EDTA, that the addition of the chelating agent decreased the recovery in the extraction efficiency (Fig. A4 (b)). Therefore, the addition of EDTA to the extraction was not performed.

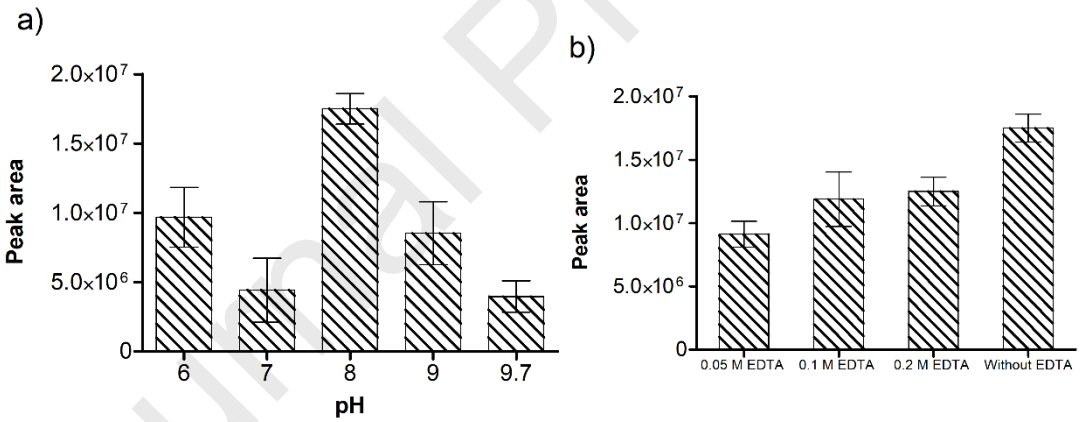


Fig. A4 a) Evaluation of aqueous solution pH in the extraction efficiency of ENRO using DLLME. Experimental conditions: 8 mL of sample fortified with 10 µg L^{-1} of ENRO; stirring time: 4 min; dispersive solvent: acetonitrile; extracting solvent: chloroform; extracting and dispersive solvent ratio 1:1; volume of extracting mixture: 1000 µL and b) Effect of EDTA in ENRO extraction from aqueous sample using DLLME. Experimental

conditions: 8 mL of EDTA solution at different concentrations at pH 8 and fortified with 10 μg L^{-1} of ENRO; stirring time 120 s + 120 s; dispersive solvent: acetonitrile; extracting solvent: chloroform; extracting and dispersive solvent ratio 1:1; volume of extracting mixture: 1000 μL. (n=4).

3.3.5 Effect agitation time

The stirring time effect was evaluated vortexing between 0 seconds and 5 minutes. The stirring time is defined as the time interval between the injection of the extracting: dispersive mixture and the beginning of the centrifugation. The times 0 seconds, 30 seconds, 1 minute and two minutes have been tested, maintaining the sonication time of 2 minutes.

Finally, to evaluate the influence of the agitation using vortex and ultrasound in the extraction efficiency several experiments were performed. Initially, experiments using only ultrasound (120 s) or vortex (120 s) were carried out and compared with extraction efficiency using sequential agitation with vortex (120 s) plus sonification (120 s). The procedure consisted in adding to an aliquot of 8 mL of water fortified with 10 μg L^{-1} 1000 μL of 1:1 extracting: dispersive mixture and agitation which consisted in vortex plus ultrasound in sequence using 0 s – 0 s; 30 s – 30 s; 60 s – 60 s and 120 s – 120 s.

Generally, the extraction time in DLLME is fast, and the steady state can be reached very quickly because the finely dispersed drops of extracting solvent. The extracting solvent droplets provide a large contact surface area between the extracting solvent and the aqueous sample. Comparing the results obtained (Fig. A5 (a)) it was possible to conclude that vortex is a much more efficient way of agitation when compared to ultrasound. If we use sequential agitation, we increase even more the extraction efficiency, although time also duplicates. Since sequential agitation seemed to increase the extraction efficiency, variation of total combined time was also performed. Stirring time (vortex plus ultrasound) was

evaluated from 0 to 120 seconds, for each type of agitation. Excessive agitation time can disturb the steady state and affect the extraction efficiency. Considering the results obtained (Fig. A5-b)) a significant increase in extraction can be obtained using conjugated vortex for 120 s followed by ultrasound for another 120 s.

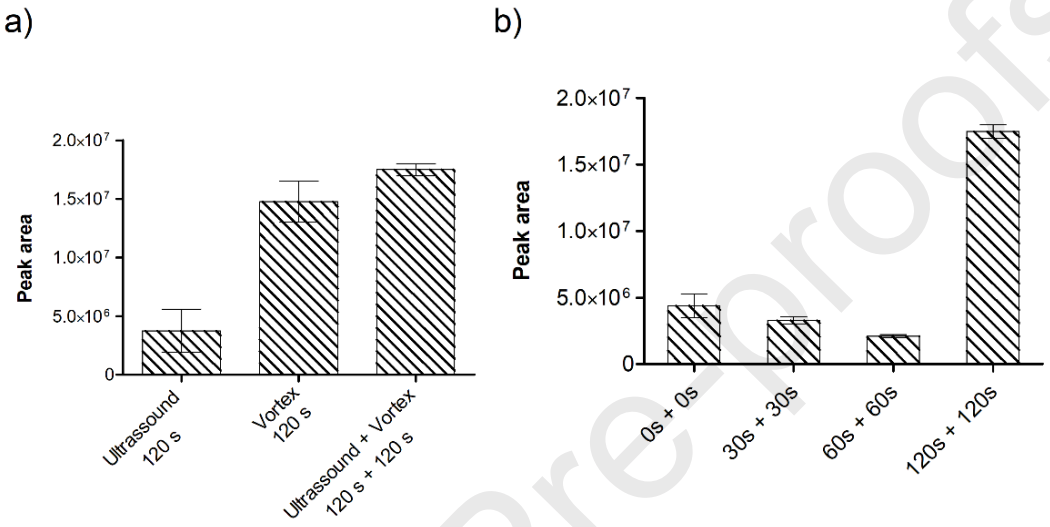


Fig. A5 a) Agitation type selection at a fixed time of 120 min and b) effect of extraction time in the extraction efficiency of ENRO using DLLME. Experimental conditions: 8 mL of sample fortified with 10 µg L^{-1} of ENRO and pH adjusted to 8; dispersive solvent: acetonitrile; extracting solvent: chloroform; extracting and dispersive solvent ratio 1:1; volume of extracting mixture: 1000 µL (n=4).

3.4 Analytical Performance UA-DLLME-HPLC-FLD

In order to obtain a calibration curve that considers of the DLLME-HPLC-FLD, four standards were prepared in triplicates with concentrations ranging from 0.13 µg L^{-1} to 3.16 µg L^{-1} and subjected to the previously optimized DLLME procedure in ultrapure water. The obtained UA-DLLME-HPLC-FLD calibration curve parameters are presented in Table

A4. The UA-DLLME-HPLC-FLD obtained optimum linearity for ENRO. The results of r^2 were 0.998, showing a very strong correlation.

Table A4. Quantitative parameters for the ENRO analytical curve obtained by UA-DLLME–HPLC–FLD.

Calibration Equation	r^2	Lin (%)	LOD (µg L^{-1})	LOQ (µg L^{-1})	Repeatability	EF	Recovery (%)
389022x + 70.435	0.998	99.82	0.11	0.36	1.6[a]; 1.4[b]	54.7±1.4 [b,c]	70±2.9 [b,c]

[a] Value obtained for a 1.13 µg L^{-1}concentration (n=4)
[b] Value obtained for a 3.16 concentration (n=4).
[c] Mean value ± standard deviation (n=4).
Extraction condition: sample volume: 8 mL, extracting solvent: chloroform, dispersive solvent: acetonitrile, extracting and dispersive solvent ratio 1:1, 1000 µL, stirring time: 120 s + 120 s.

Through this curve the limit of detection, enrichment factor and recovery were calculated. The ER is given by ER (%) = EF × V_{ACN}/V_{sample} × 100%, where V_{ACN} is the volume of acetonitrile used to redissolved the dry sediment phase and V_{sample} is the volume of sample used in the extraction [20]. The detection limit was calculated from the calibration curve as a + 3 $s_{y/x}$, where *a* is the regression line interception and $s_{y/x}$ is the statistical parameter that estimates the random errors on the *y*-axis (signal). Linearity, Lin (%) = 100-RSD_b, where RSD_b is the relative standard deviation (in percentage) of the slope. It is important to refer that there was a decrease in the detection limit from 7.68 µg L^{-1}, without DLLME (Table A3) to 0.11 µg L^{-1} after the application of DLLME, showing to be a highly effective technique for the quantification of ENRO at low concentrations.

3.5 Matrix effect

3.5.1 Analysis of environmental water samples

From the analyzed samples, ENRO was detected in the river water at concentration of 0.20 µg L^{-1} and the seawater at concentration of 0.12 µg L^{-1} (Table A5). These values

confirm results reported in literature where enrofloxacin and other fluoroquinolones were often detected in Brazilian waters [41–43].

Table A5. Recovery rates obtained for the ENRO DLLME from environmental water samples.

Water samples	Concentration determined μg L^{-1}	Recovery spiking level 1.24 μg L^{-1}	Recovery spiking level 3.1
River water	0.20	72±6.15	120 ±4.59
Sea water	0.12	27±8.36	32±2.65

Extraction conditions: sample volume: 8 mL with pH adjusted to 8, extracting solvent: chloroform, dispersive solvent: acetonitrile, extracting and dispersive solvent ratio 1:1, 1000 μL, stirring time – 120 s + 120 s.

The extraction recovery values were obtained from fortification of environmental water samples using two ENRO concentration levels: 1.24 μg L^{-1} and 3.16 μg L^{-1}. Recovery results ranged from 72-120 % for the river water and 27-32 % for the seawater, as shown in Table A5. These recovery results showed that the analyzes carried out in river water are within the established parameters. However, the sea water recovery results were below the established values, probably due to the salty characteristic of the matrix, since during the optimization process of the DLLME, the salting-out effect did not show significant improvement in ENRO extraction, but even decreased the extraction efficiency. The coefficient of variation (*RSD*, %) range up to 8.46 %, therefore, the extraction procedure can be considered precise.

3.6 Comparison with other methods

The comparison of DLLME with other methods used for quantification of ENRO in water samples, considering the following parameters: % recovery, detection limit (LOD), sample volume (mL) and enrichment factor are presented in Table A6.

Table A6. Comparison of DLLME–HPLC–FLD with other methods used for the quantification of enrofloxacin in water samples.

Method	Recovery (%)	LOD (μg L^{-1})	Sample volume (mL)	EF	Reference
SPE-HPLC-DAD	87.8	100	500	*n.a*	[44]
UA-IL-DLLME-LC-FLD	89.0	0.01	10	122	[45]
SPE-HPLC-UV	80.0	0.1	500	210	[46]
N-CDs-FLD assay	96.5	160	5	*n.a*	[47]
SPE-LC-MS/MS	91.1	0.003	500	*n.a*	[48]
EME-HPLC-FLD	82.0	0.07	*n.a*	65	[49]
UA-DLLME-HPLC-FLD	70.0	0.11	8	54	This study

SPE: Solid-phase extraction; UA-IL: Ultrasound-assisted ionic liquid; N-CDs: Carbon dots; EME: Electromembrane extraction.
n.a: not applicable

When comparing the UA-DLLME-HPLC-FLD method, it was possible to observe that it has many advantages, for example, the recovery is similar concerning the other works reported in Table A6, evidencing that the method is effective for the quantification of ENRO in water samples. The limit of detection obtained was comparable to the obtained by other authors, being better than the obtained by Ašperger et al., 2009 and Xingjia Guo et al., 2019 and better % Recovery when compared with Ramos-Payán et al. 2013.

Comparing to results obtained by Wagil et al. [48] our LOD was higher, however, the lower limit was achieved by using an MS/MS detector, which due to the high cost of acquisition and maintenance it may become unviable. Another advantage was the low volume of sample needed for the extraction procedure when compared to the work published and showed in Table A6.

Our work was applied in a simple and fast extraction process, using a vortex followed by ultrasound, in opposition to the work described by Vázquez et al., 2012, despite having a good enrichment factor and low detection limit, it presented a method with multi-steps (vortex, ultrasound and ice-water bath), The use of many extraction steps has numerous disadvantages, for example, the difficulty of applying the method, the long extraction time and many errors associated.

Ultrasonic-assisted dispersive liquid-liquid microextraction is an innovative sample preparation method that attracted more attention and provides high enrichment factors from low volumes of water samples. In general, the DLLME method has many advantages compared to other extraction techniques, among them we can mention the speed, simplicity, low cost, efficiency and environmentally friendly, since it uses a reduced amount of organic solvents, generating less amount of residue, showing which is a viable sample preparation method for ENRO analysis in water samples and can be applied for the determination of other fluoroquinolones.

4 CONCLUSIONS

A methodology based on ultrasonic-assisted dispersive liquid-liquid microextraction followed by high-performance liquid chromatography with fluorescence detection (UA-DLLME–HPLC–FLD) was developed and optimized for the analysis of fluoroquinolone (ENRO) in water samples. The optimized UA-DLLME–HPLC–FLD methodology provides low detection limits (0.11 μg L^{-1}), enrichment factor (54.7) and high extraction recoveries (72 % to 120 %) for river water. Also, recovery tests proved that the river water sample matrix does not interfere in the extraction efficiency, although for the seawater, the presence of salt decreases considerably the extraction efficiency. Thus, this method is only suitable for freshwater samples.

The DLLME method has many advantages when compared to other traditional methods of sample preparation for waste determination, such as good percentages of recovery and simplicity of operation. The activities developed in this work demonstrated that the method is fast, low cost and efficient, proving to be a promising technique for the detection of such pollutants in waters. In addition, it involved a small number of steps, consuming low volumes of solvents, being environmentally friendly.

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Appendix A. Supplementary data

Complementary data and additional information on the application of the method in real samples can be found in the Support Information.

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Ultrasound-assisted dispersive liquid–liquid microextraction for determination of enrofloxacin in surface waters

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HIGHLIGHTS

- UA-DLLME was applied for clean-up and concentration of ENRO in aqueous samples.
- UA-DLLME–HPLC–FLD provides low detection limits (0.11 µg L^{-1}) for river water.
- Recovery results in river water sample ranged from 72-120 %.
- ENRO was detected in the river water analyzed at concentration of 0.20 µg L^{-1}.