NEW APPROACH FOR DETERMINATION OF FLUMEQUINE AND OXOLINIC ACID IN AQUEOUS SAMPLES BY IONIC LIQUID-BASED DISPERSIVE LIQUID-LIQUID MICROEXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–FLUORESCENCE DETECTION

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ABSTRACT

A rapid and sensitive analytical method for the determination of oxolinic acid and flumequine in aqueous samples based on dispersive liquid-liquid microextraction using ionic liquids (ILs) was developed. Based on the structural properties of the antibiotic agents studied, two ILs with different functionalities were required: ethyl-dimethyl-(2-methoxyethyl) ammonium tris(pentafluoroethyl)trifluorophosphate ([MOEDEA][FAP]) for extraction and 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]) to adjust the polarity of the medium. The significant experimental factors involved in DLLME were identified and optimized using the experimental design methodology. A Plackett-Burman design was initially used for screening, and a central composite design was used for optimization. The optimized method exhibited good precision, with relative standard deviation values of less than 5 % and limits of detections on the order of 0.1 and 0.3 ng mL^{-1} for the two drugs. The enrichment factors for both antibiotics were 13–33 fold. The proposed method was applied to the analysis of the two antibiotic in spiked surface, river, and wastewater samples.

1 INTRODUCTION

Emerging contaminants or contaminants of emerging concern in water are attracting increasing attention from both the general public and government agencies [1]. This class of contaminants includes a variety of compounds, such as pharmaceuticals, personal care products, endocrine-disrupting chemicals, persistent organic pollutants, veterinary medicines and nanomaterials [2]. Of particular concern are veterinary medicines such as antibiotic agents; the increasing release of antibiotics into the environment has been implicated in the escalation of antibiotic resistance [1]. Quinolones comprise two of the most important families of antibiotic agents and are used widely throughout the world to treat both human and animal diseases. Relevant quantities are also used in veterinary medicine at sub-therapeutic levels as growth promoters and to improve feed efficiency [3, 4]. The release of these antibiotics into aquatic environments primarily occurs via waste treatment plants. These polar drugs are highly soluble in water and are easily transferred to other types of waters [5]. In aquaculture, particularly in salmon farming, antibiotic agents are mainly administered orally in feed [6]. Lai and Lin 2009, showed that the main factor for the degradation of oxolinic acid (OXO) and flumequine (FLU) in pond water and sediment was the natural light, but this degradation is blocked in a darkness condition [7].

Chilean salmon farmers have been battling with intracellular bacterial pathogens, so they have been forced to use a significant amount of antibiotics [6]. Therefore, an environmental point of view, it is necessary to know the situation regarding antibiotic residues in surface water of salmon farming.

The potential impact of releasing high quantities of these substances has been suggested but not proven by collateral effects, such as quantitative and qualitative modifications of bacterial flora or impacts on the food chain [6]. Thus, developing reliable analytical methods for determining antibiotic agent residues at low concentrations in aquatic systems is of critical importance.

Many methods for the determination of quinolones in environmental waters or solid samples have been published [5, 8-16]. Due to the low concentrations of emergent pollutants, preconcentration approaches have also been proposed. Microextraction techniques have evolved from classic sample pretreatment techniques such as solid-phase extraction and liquid-liquid extraction (LLE) [17]. This last technique is widely employed for sample preparation and is based on the partitioning of the target compound between two immiscible phases [18]. However, limitations such as large required sample volumes and the use of toxic solvents make LLE expensive, time-consuming and environmentally unfriendly [19]. Moreover, an extra step of concentrating the extract to a small volume is needed. The demand to reduce solvent volumes and avoid the use of toxic organic solvents in LLE has led to substantial efforts to adapt existing samples [20].

In recent years, ionic liquids (ILs) have gained popularity as environmentally friendly alternatives to traditional organic solvents. In contrast to common molten salts, ILs are salts with a melting point below 100 °C. Room-temperature ILs are a subset of ILs that are liquid at room temperature (25 **°C)** [21]. The immiscibility of some ILs in water and their capacity for dissolving organic species are suitable for LLE [8, 22]. ILs have proven to be excellent tools in methods involving minimum consumption of sample and solvents and as replacements for conventional chlorinated solvents usually used as extractants in conventional dispersive liquid-liquid microextraction (DLLME) [8, 23-25].

The use of IL-DLLME, introduced by Zhou Q., Bai H., Xie G., Xiao J., in 2008 [26], has thus reduced both the amount of organic solvent required [1,27-29]. Previous studies demonstrated the applicability of this approach for evaluation of fluoroquinolones [8,9,23]. However, the extraction time is relatively elevated and [8], probably due to chemical differences of fluoroquinolones. A recent approach is to use a mixture of two ILs in DLLME procedure [21]. In this case, one IL serves as the extraction medium, and a second is used to modify the solubility of the analytes in the aqueous medium, resulting in more efficient extraction and replacing the NaCl typically added to the aqueous sample. This approach was not previously tested for quinolones but it was showed excellent results for the detection of other emergent organic contaminants, such as non-steroidal anti-inflammatory drugs in aqueous samples [21].

In this study, we propose an environmentally friendly, rapid and sensitive IL-DLLME approach for the determination of the two most used antibiotics in the Chilean salmon industry, FLU and OXO, in surface, river and waste water samples using HPLC with fluorescence detection (FD). Due to the chemical properties of quinolones, two ILs were employed to obtain reliable results: ([MOEDEA][FAP]) for extraction and [BMIM][BF_4] to adjust the polarity of the medium. Several experimental factors in this extraction approach were optimized using experimental design methodology. Finally, under the established optimal conditions, the method was successfully applied to determine trace levels of the two antibiotics in river and wastewater samples.

2 MATERIALS AND METHODS

2.1 Reagents

All reagents used were of analytical grade or higher. FLU and OXO were obtained from Sigma-Aldrich (St. Louis, MO, USA). Standard solutions of the drugs were prepared at 25 µg mL^{-1} in acetonitrile and ultrapure water (ρ = 18 MΩ cm^{-1}) from a Millipore Milli-Q system (MQ water) for a working solution. For the mobile phase, oxalic acid dihydrate, methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). 1-Butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]), 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]), 1-hexyl-3-methylimidazolium hexafluorophosphate ([HMIM][PF_6]), ethyl-dimethyl-propylammonium bis(trifluoromethylsulfonyl)imide ([NEMMP][NTF]), ethyl-dimethyl-(2-methoxyethyl)ammonium tris(pentafluoroethyl) trifluorophosphate

([MOEDEA][FAP]), and 1-methyl-3-octylimidazolium hexafluorophosphate [MOIM][PF_6] were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Apparatus and equipment

A Hettich EBA 20 centrifuge (Hettich Lab. Technology, Tuttlingen, Germany) was used to accelerate phase separation in 15-mL conical centrifuge tubes. A Radwag AS 60 analytical balance (RADWAG Wagi Elektroniczne, Radom, Poland) was used to weigh the standard drugs. A Thermolyne Maxi-Mix II Vortex Mixer (Thermo Scientific, Waltham, MA, USA) was used for the extraction of the analytes in DLLME. The HPLC analyses were performed on a Jasco LC Net II system equipped with a quaternary gradient pump (PU-2089 U plus), a DAD (MD-2018), a fluorescence detector (FP-2020), and a column thermostat (CO-2060) (Easton, MD, USA). The analytes were separated on a Kinetex-Phenomenex reversed-phase (Torrance, CA, USA) C-18 column. The mobile phase consisted of oxalic acid (0.1 N), methanol and acetonitrile (50/25/25) at a flow rate of 1 mL min^{-1} at 30 °C. The injection volume was 20 µL. Fluorescence detection with excitation and emission wavelengths of 324 and 366 nm, respectively, were used for the two drugs.

2.3 IL-DLLME procedure

The working solution (100 ng mL^{-1} each analyte) was first adjusted to pH 2.5 using H_3PO_4. Then, 10 mL of the working solution was placed in a 15-mL screw-capped conical-bottom graduated glass centrifuge tube. A 96-µL aliquot of [BMIM][BF_4] was injected into the sample solution to alter the polarity of the sample, which was then stirred manually to promote mixing. DLLME was performed by rapidly injecting 300 **µL** of a mixture of ([MOEDEA][FAP] (82 **µL**) and acetonitrile (218 **µL**) into the water sample using a syringe. The rapid injection of the extraction mixture produced a cloudy sample solution, which was subsequently vortexed for 20 s and centrifuged for 8 min at 4000 rpm. The upper aqueous phase was removed with a syringe, and the sedimented phase (≈ 82 **µL**) was withdrawn using a 50-µL microsyringe. Finally, 20 **µL** of sediment IL was injected into the HPLC system. All measurements were performed in triplicate, and the syringe was rinsed with acetonitrile to remove residual analytes and ILs.

2.4 Real sample preparation

For method validation, samples from wastewater treatment plants (WWTPs) and river and sea water were used. Both samples were collected within the city of Santiago (Chile). WWTP samples were collected from the influent and effluent of the plant. The river water sample was collected from the Mapocho River. Seawater samples were collected from Reloncaví Gulf in the city of Puerto Montt from two different zones near to salmon farms. All three matrices were stored in polypropylene bottles and frozen until analysis. For analysis, the WWTP samples were filtered through 2.7-µm followed by 1-µm glass fiber filters and then further filtered through 0.45-µm nylon Whatman membrane filters. River and seawater samples were only filtered through 0.45-µm nylon membrane filters. Gros M., Rodríguez-Mozaz S., Barceló D., described a similar sample pretreatment in 2013 [13].

3 RESULTS AND DISCUSSION

Several variables can affect the efficiency of the DLLME procedure, and experimental design methodology was employed to decrease the number of experiments required to optimize the procedure. First, the statistically significant factors in the extraction step were identified using a Placket-Burman design and optimized using a second-order central composite design.

3.1 Preliminary study

In the first step, five different ILs were evaluated as potential extractants: [BMIM][PF_6], [HMIM][PF_6], [NEMMP][NTF], [MOIM][PF_6] and [MOEDEA][FAP]. Before the addition of each IL, [BMIM][BF_4] was first added to the water sample to change the polarity of the aqueous system and improve analyte extraction [20]. DLLME was then performed according to the procedure described in Section 2.3, and the results are presented in Figure 1. [MOEDEA][FAP] resulted in the highest recoveries for both quinolones. By contrast, [NEMMP][NTF] was unsuitable because of the low recoveries of the two drugs obtained. [HMIM][PF_6] was also not appropriate due to co-elution of this IL with OXO (see Figure 1A). [MOEDEA][FAP] was selected for the next set of experiments.

The abilities of four organic solvents, namely, methanol, acetonitrile, ethanol and acetone, to disperse [MOEDEA][FAP] in the aqueous phase were evaluated. The effect of these dispersants on the extraction of the analytes was quantitatively evaluated, and the results are presented in Figure 1B. All dispersants exhibited similar effects on the extraction of the analytes, with the higher recovery obtained for FLU. Acetonitrile resulted in the best extraction efficiencies for the two drugs and was therefore employed in subsequent experiments.

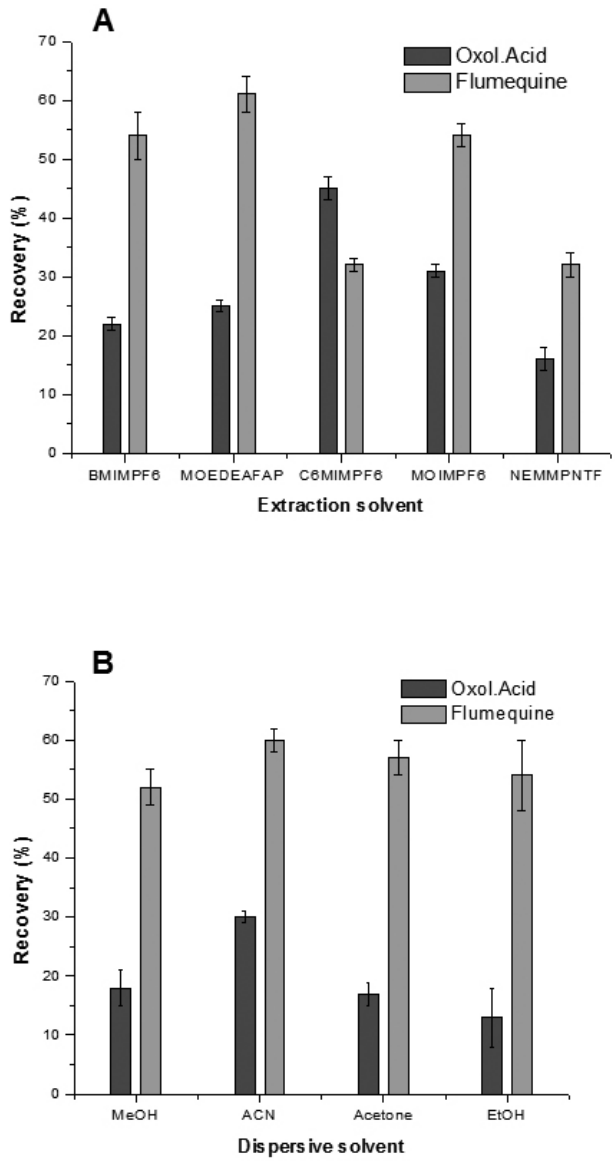

Figure 1 (A) Effect of different ILs as extraction solvents on drug recovery. (B) Effect of the dispersive solvent on drug recovery (5 mL total sample volume, 100 µL of IL, and 200 µL [BMIM][BF_4], mixed by vortexing for 20 s, followed by 5-min centrifugation at 4000 rpm).

3.2 Optimization of the DLLME procedure

3.2.1 Screening design

A Plackett-Burman design was employed due to the large number of variables to be tested. For each experimental factor, two levels chosen based on preliminary experiments were considered [21], and factors with effects higher than the experimental error were considered significant. In this study, the variables considered were the extractant volume, sample volume, NaCl concentration, stirring rate, and extraction time. Previous studies have indicated that these factors can influence the performance of the DLLME procedure. The results presented in Table 1 demonstrate that the volume of [NEMMP][FAP] (extractant volume) was significant for both compounds. This variable influences the efficiency of the extraction process because a major volume of the IL phase ensures higher transference between the two solvents. Similarly, centrifugation time was also significant because centrifugation improves the phase separation and recovery of the IL phase containing the quinolones. By contrast, [BMIM][BF_4] volume and sample volume were significant only for OXO and FLU, respectively. A higher volume of [BMIM][BF_4] increases the solubility of OXO in the IL phase and improves its extraction but has no significant effect on FLU, which is insoluble in water.

Table 1. Variables selected for optimization of the IL-DLLME method

Factors	Coded Factors	Levels		Effect	
		-1	1	Flumequine	Oxolinic acid
[NEMMP][FAP] volume (mL)	IL	30	100	S	S
[BMIM][BF_4] volume (µL)	BF4	0	200	NS	S
Sample volume (mL)	Vol	4	10	S	NS
NaCl (%)	NaCl	0	5	NS	NS
Centrifugation time (min)	Time	2	8	S	S

S: significant ($p < 0.05$); NS: not significant.

3.2.2 Optimization design

Based on the results obtained in the previous section, [NEMMP][FAP] volume, [BMIM][BF_4] volume and sample volume were considered significant factors for the optimization of the extraction of FLU and OXO. The centrifugation time was adjusted to the "+1" level to maximize the recovery of the IL phase and decrease the factors for the optimization step. The non-significant factors were adjusted to the "-1" level (see Table 1). A significant model was obtained for the recoveries of both compounds ($p < 0.05$; $R^2 > 94.0$), and the response surfaces obtained with a central composite design are presented in Figure 2. Figure 2A and 2B indicate that the maximal extraction was reached in the experimental domain for both quinolones; however, their optimal conditions differed. To achieve a compromise for the simultaneous extraction of both quinolones, the desirability function was used, and the surface obtained is presented in Figure 2C. The optimal conditions for this process were 9.6 mL of [BMIM][BF_4], 82 µL of [NEMMP][FAP] and 9.7 mL of sample.

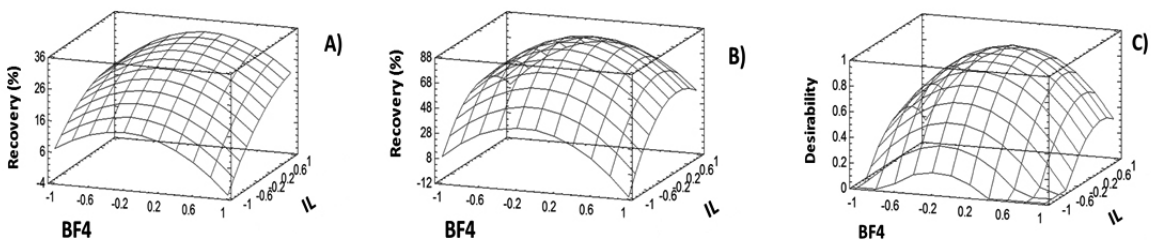

Figure 2 Response surfaces for A) oxolinic acid, B) flumequine and C) the desirability function obtained during the optimization procedure.

3.3 Analytical features and applications to actual samples

The analytical features were obtained under the extraction conditions using the optimized IL-DLLME method. The proposed method was evaluated by characterizing its analytical performance in terms of linearity, precision, recovery, limit of detection (LOD), and limit of quantification (LOQ). Calibration plots of each analyte, prepared at six concentration levels, were linear in the range of 0.5–30 ng mL^{-1} for FLU and in the range of 1.5–30 ng mL^{-1} for OXO, with correlation coefficients (r) of 0.9986 and 0.9984, respectively. For LOD and LOQ determination, signal-to-noise (S/N) ratios of 3 and 10, respectively, were employed. The repeatability, described as the percent relative standard deviations (RSDs) of the results from six replicate experiments using two different concentrations of drugs, were in the range of 3–5 % for real samples. The results are presented in Table 2. The recovery values (R) were also investigated for six replicate experiments performed under the determined optimal conditions. We observed that the optimized extraction process was highly efficient, with good recoveries ranging from 87 % to 95 % for the two levels (Table 2). Real samples were examined to validate the applicability of the developed IL-DLLME method and to evaluate the matrix effects for the extraction of quinolones.

Table 2. Analytical features of quinolone extraction by IL-DLLME.

Analyte	Retention time (min)	Linear range (ng mL^{-1})	r	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	R ±RSD (%) 2 ng mL^{-1}	R ±RSD (%) 16 ng mL^{-1}
Oxolinic acid	2.7	1.5–30	0.99837	0.3	1	87 ± 5	88 ± 3
Flumequine	4.2	0.5–30	0.99855	0.1	0.3	92 ± 3	95 ± 3

RSD = Repeatability with n = 6
R = Recovery

Table 3 presents the concentration and recovery of the two studied analytes spiked into samples from the Mapocho River, wastewater treatment plants (WWTPs) in Santiago, and surface waters of the Gulf of Reloncaví near salmon farms. No analytes were detected in the blank extraction of the matrix samples (Figure 3). In a case of surface waters, these results are similar to those reported, where both antibiotics were not detected in marine sediments [6]. Regarding wastewater, other types of fluoroquinolones were found [30]. The recovery ranged from 91 to 104 % for the three different matrixes. However, comparing the results for the WWTP and Reloncaví samples with those for the river water samples reveals a slight increase in the detected levels of FLU and OXO. This increase might be due to the complexity of the sample water treatment and, in the case of the Gulf of Reloncaví samples (sample B), the anthropogenic actions of the artisanal fishing sector and tourism. These results indicate that the effect of the matrix on the recovery of the analytes is negligible compared with spiked Nanopure water (Tables 2 and 3).

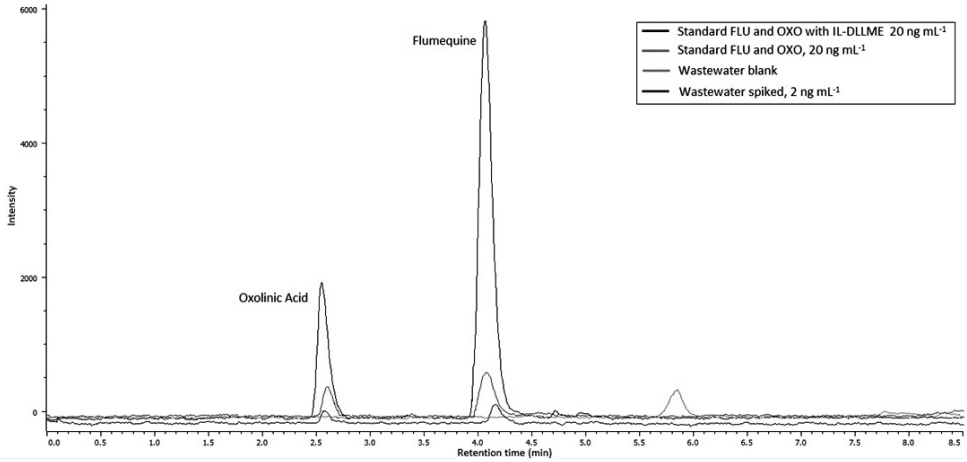

Figure 3 Chromatograms of fluorescence detection of standards of FLU and OXO (red line: without IL-DLLME; black line: with IL-DLLME) and wastewater samples (green line: blank; blue line: spiked).

Table 3. Application of the IL-DLLME method to spiked samples.

Analytes	Spiked level (ng mL^{-1})	Measured levels (ng mL^{-1})				
		River water	WWTPs		Gulf of Reloncaví (Salmon farm water)	
			Influent	Effluent	A	B
Oxolinic acid	2	1.82 ± 0.09 15.2 ± 0.4	2.1 ± 0.1	1.85 ± 0.09	1.84 ± 0.06	2.02 ± 0.09
	16		16.7 ± 0.8	16.3 ± 0.8	16.0 ± 0.4	16.5 ± 0.7
Flumequine	2	1.85 ± 0.07 16.2 ± 0.4	2.1 ± 0.1	1.95 ± 0.08	1.91 ± 0.05	2.0 ± 0.1
	16		16.2 ± 0.7	16.1 ± 0.7	16.1 ± 0.3	16.4 ± 0.7

Finally, the proposed method was compared to previously reported methods for the extraction of quinolones and fluoroquinolones from aqueous samples (Table 4). The new method exhibited several improvements, particularly in the simplicity of the extraction process, because no additional energy is needed (e.g., ultrasound-assisted) for extraction [8, 9] and the complete replacement of chlorinated organic solvents [9, 23] by ILs, which are green and environmentally friendly solvents.

4. CONCLUSIONS

A new analytical method that uses IL-DLLME in combination with HPLC-FD for the determination of FLU and OXO in natural water and wastewater samples was developed. The new method does not require additional energy for extraction, in contrast to some previous reports of IL-DLLME for antibiotics involving ultrasound-assisted extraction and subsequent cooling of the sample, which increase the time required for the extraction process. Our results also indicated that using [BMIM][BF_4] as an additional semipolar IL can further increase the efficiency of the extraction process, through changing the polarity of the extraction system and decreasing the miscibility of the analytes in the aqueous phase. In addition, the new method does not require chlorinated organic solvents for quantitative extractions and, consequently, does not generate toxic waste. The multivariate optimization enabled the successful determination of the optimal conditions for the main operational parameters considered during IL-DLLME. The newly developed method exhibits a large linear range and good repeatability, precision, and accuracy for FLU and OXO. The new method also provides several other advantages, such as simplified and fast operation and very low consumption of organic solvent. Finally, the method was applied to detection in three different aqueous matrixes. No serious matrix effect was observed, and good recoveries (> 91 %) were obtained at two different quinolone concentrations in spiked real samples. Therefore, we conclude that our IL-DLLME method, in conjunction with HPLC-FD, is a rapid, efficient and green analytical method.

Table 4. Comparison of DLLME methods for quinolones in aqueous samples

Method	Analytes	Chromatographic technique	Pretreatment (min)	Extraction/dispersive solvent	Sample volume (mL)	LOD (µg L^{-1})	Recovery (%)	RSD (%)	Ref.
US-IL-DLLME	[a]FQs	HPLC-FD	19	[b]$[C_8MIM][PF_6]$/MeOH	10	800-13000	85–107	5–9	[8]
US-DLLME	Ofloxacin Norfloxacin Enrofloxacin Lomefloxacin	HPLC-UV	7	CCl_4/MeOH	8	0.14-0.81	83–111	1–5	[9]
Vortex-DLLME	[c]Qs	UPLC-DAD	> 13	$CHCl_3$/ ACN	5	1.91-106	78–117	1–6	[23]
IL-DLLME	Oxolinic acid Flumequine	HPLC-FD	8.5	[NEMMP][FAP]/ACN	9.7	0.2-0.35	87–95	3–5	This work

a) Fluoroquinolones
b) 1-Octyl-3-methylimidazolium hexafluorophosphate
c) Quinolones (nº analytes)

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