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Trace enrichment of (fluoro)quinolone antibiotics in surface waters by solid-phase extraction and their determination by liquid chromatography–ultraviolet detection

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Abstract

A new and simple analytical methodology for the simultaneous analysis of acidic and zwitterionic (fluoro)quinolones in surface waters at trace concentration level is presented. The method is based on the preconcentration of these analytes by a solid-phase extraction procedure and their subsequent quantification by liquid chromatography using ultraviolet detection. The breakthrough volumes of the selected (fluoro)quinolones in four different sorbents—C_{18}, styrenedivinylbenzene (SDB), C_{18}-cation-exchange and SDB-cation-exchange—have been evaluated and varied between 25 and 150 ml depending on the antibiotic and the sorbent used. An exhaustive study of the influence of sample pH on the preconcentration step has been carried out in order to find a suitable procedure for extraction of acidic and zwitterionic FQs in one single step. Under optimum conditions, it was possible to percolate up to 250 ml of water solution onto both C_{18} and SDB-cation-exchange cartridges with quantitative recoveries for all the analytes tested. However, matrix components of the surface water samples analysed negatively affected the recoveries of the analytes in the SDB-cation-exchange cartridge and thus, C_{18} cartridges were finally selected for the analysis of the (fluoro)quinolones in lake and river water. The limits of detection achieved with this procedure varied between 8 and 20 ng l^{-1} proving its suitability for the determination of the (fluoro)quinolones in water samples at a realistic environmental concentration level.

Keywords: Water analysis; Trace analysis; Quinolones; Fluroquinolones; Antibiotics

1. Introduction

In the last decades, antibiotics have been widely used against different diseases in human and veterinarian medicine as well as in industrial farming. Quinolones (Qs), one of the most powerful classes of antibiotics, were initially employed in the treatment of Gram-negative urinary tract infections in humans and animals [1]. Their activity is based on the inhibition of the enzyme DNA gyrase or topoisomerase II, which are responsible for the preservation of the DNA biological activity of bacteria [2]. Fluoroquinolones (FQs) are piperazinyl derivates of the quinolone nadixilic acid and represent the second generation of this family of antibiotics. They are nowadays broadly used in the treatment of a wide

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variety of diseases [3], since they are not only more effective against Gram-negative bacteria but also moderately active against Gram-positive bacteria.

As a consequence of non-adequate treatment of human and animal excretions, antibiotic residues have been present in the environment for long periods of time, leading to the appearance of antimicrobial resistant bacteria. The way in which antibiotics reach the environment depends on the specific use of each compound. In the case of antibiotics used against human diseases, they are mainly spread to surface water through urban waste water. In fact, it has been reported that the actual procedures for waste water treatment are not able to completely remove these compounds [4]. Concerning the antibiotics used in veterinarian medicine, the use of animal excretions for manure has been the main focus of contamination. In this case, antibiotics are directly spread to the soil, and after rainfall, ground and surface water may therefore also become polluted [5].

For this reason, there is an emerging interest among the scientific community on increasing the knowledge of the consequences that the presence of antibiotics in the environment may have on human health [6], paying special attention to antibiotics used against human diseases. Up to now, maximum residue levels (MRLs) for antibiotics in environmental waters have not been established. However, the European Agency for the Evaluation of Medicinal Products (EMEA) and the Food and Drug Administration of the United States (FDA) require environmental risk assessment before placement of these products in the market [7,8]. In general, environmental risk assessment studies require data concerning the concentration of the contaminant in the environment. In many cases the predicted environmental concentrations data (obtained in the laboratory) have to be used for this purpose, as the measured environmental concentrations are not always available [1]. Therefore, there is a clear necessity to develop analytical methods that would allow the measurement of antibiotics at naturally occurring levels. Several articles have been published dealing with the determination of antibiotics in different types of environmental waters [4,9–11], however, the literature available on quinolones is very short and the reported works have only focused on the analysis of the piperazynil derivates in aquatic systems. The presence of ciprofloxacin and norfloxacin in the output of a treatment water plant [12], in hospital waste water [13] and, together with sarafloxacin, in surface waters at concentrations of 0.03, 0.12 and 0.02 $\mu g\ l^{-1}$, respectively [14], has been reported. These methods employed selective detectors such as mass spectrometry or fluorescence detection, as well as a preconcentration step using two solid-phase extraction (SPE) disks or two SPE-cartridges in tandem.

In this paper, a new and simple method for the multiresidue analysis of several quinolones and fluoroquinolones in surface water samples (lake and river) by HPLC using UV detection is presented. Due to the different acid–base properties of the selected analytes, special attention has been given to the development of a solid-phase extraction enrichment procedure suitable for the exhaustive and simultaneous preconcentration of all of them in one single step.

2. Experimental

2.1. Reagents

Norfloxacin (NOR), cinoxacin (CIN), oxolinic acid (OXO), nalidixic acid (NAL) and flumequine (FLU) were obtained from Sigma–Aldrich (St. Louis, MO, USA); enrofloxacin (ENR), enoxacin (ENO) and ciprofloxacin (CIP) were purchased from Bayer (Leverkusen, Germany) and danofloxacin (DAN) from Pfizer (Groton, CT, USA). Stock standard solutions ($1\ g\ l^{-1}$) were prepared in acetonitrile containing 2% of ammonia and stored at $-18\,^{\circ}C$. The ultrapure water system used was purchased from Millipore (Paris, France) and all other chemicals used were of analytical reagent grade obtained from Merck (Darmstad, Germany).

2.2. Instruments and materials

Solid phase extraction (SPE) was carried out on a Chromabond Vacuum mainfold for 24 columns from Macherey–Nagel (Duren, Germany) connected to a vacuum pump from Barnant (Barrington, USA). The

3M Empore Extraction disk cartridges of octadecylsilica (C_{18}), poly-styrenedivinylbenzene (SDB), and their corresponding mixed cartridges with cation-exchange sorbent (MPC and SDB-RPS) were purchased from Varian Inc. (CA, USA). The (fluoro)quinolones separation was carried out using a Polarity™ dC_{18} HPLC column (150×3.0 mm, 3 μm) from Waters (MA, USA) and a HPLC–DAD instrument from Dionex (CA, USA) consisting in a GP40 HPLC gradient pump and an UVD305 DAD detector.

2.3. Procedures

2.3.1. Liquid chromatography

LC separations were performed using injection volumes of 200 μl and linear gradient elution as follows: from 96% A (diluted formic acid pH 2.5) and 4% B (acetonitrile) to 50% A and 50% B in 25 min, returning to initial conditions in 5 min. ENO, NOR, CIP, DAN and ENR were monitored at 275 nm whereas CIN, OXO, FLU and NAL were monitored at 255 nm. Quantification was performed using external calibration and peak area measurements. All UV spectra were obtained by subtracting the background spectra (just before each peak) to the spectra at the maximum of the peak.

2.3.2. Solid phase extraction

The C_{18} disk cartridges were conditioned with 2×1 ml of methanol, 1×1 ml of pure water and 1×1 ml of a 2 m*M* sodium acetate buffer solution (pH 4). Water sample pH was adjusted to pH 4 and 250 ml were percolated through the cartridge using the Visiprep large volume sampler tubes supplied by Sigma Aldrich NV (Bornem, Belgium). Then the cartridges were washed with 3×500 μl of acetate buffer (pH 4) solution containing 15% of acetonitrile and vacuum dried for 5 min. Subsequently, analytes were eluted with 3×1 ml of a solution consisting of 6% of ammonia in methanol. Finally, sample extracts were evaporated to dryness under nitrogen and redissolved in 250 μl of diluted formic acid (pH 2.5) for final HPLC analysis.

2.3.3. Sample preparation

Lake water (Miranda Lake, Antwerpen) and river water (Oude Nete, Antwerpen) were spiked at the 25 ng l^{-1} concentration level of each (fluoro)quinolone and were adjusted to pH 4. Before the SPE procedure described above, samples were prefiltered through a 0.45-μm filter to remove suspended matter.

3. Results and discussion

3.1. Chromatographic separation and trace enrichment of (fluoro)quinolones

As already mentioned, the concentration at which (fluoro)quinolones are present in environmental waters is extremely low making necessary the development of very sensitive analytical methods suitable for monitoring these analytes in the low nanogram per litre concentration level. Keeping this in mind, chromatographic conditions and injection volume were optimised in order to obtain the maximum signal-to-noise ratio for all the (fluoro)quinolones. The best separation performance was achieved using a specially designed C_{18} column for separation of polar analytes (Polarity dC_{18}, 150×3.0 mm, 3 μm). As can be observed in Fig. 1, an excellent separation with good resolution and peak shapes for all analytes was achieved, allowing the use of an injection volume up to 200 μl without disturbing it. Subsequently, calibration curves were performed and the analytical parameters were established (Table 1). The limits of detection, calculated as three-times the signal-to-noise ratio, varied within the 4–10 μg l^{-1} concentration range, making necessary the development of an SPE procedure, which enabled us to reach a preconcentration factor of around 1000 times, with the aim of achieving limits of detection in the low ng l^{-1} range.

As mentioned before, the analytes selected in this study can be grouped in acidic quinolones (FLU, OXO, CIN and NAD) and piperazinyl quinolones (ENO, NOR, CIP, DAN and ENR). The basic structures as well as the ionisation equilibria according to their acid–base properties are shown in Fig. 2. As can be observed, the (fluoro)quinolones have different acid–base properties and all of them can be present as different species in aqueous solution [2]. In acidic conditions, FLU, OXO, CIN and NAD will be neutral whereas, ENO, NOR, CIP, DAN and ENR

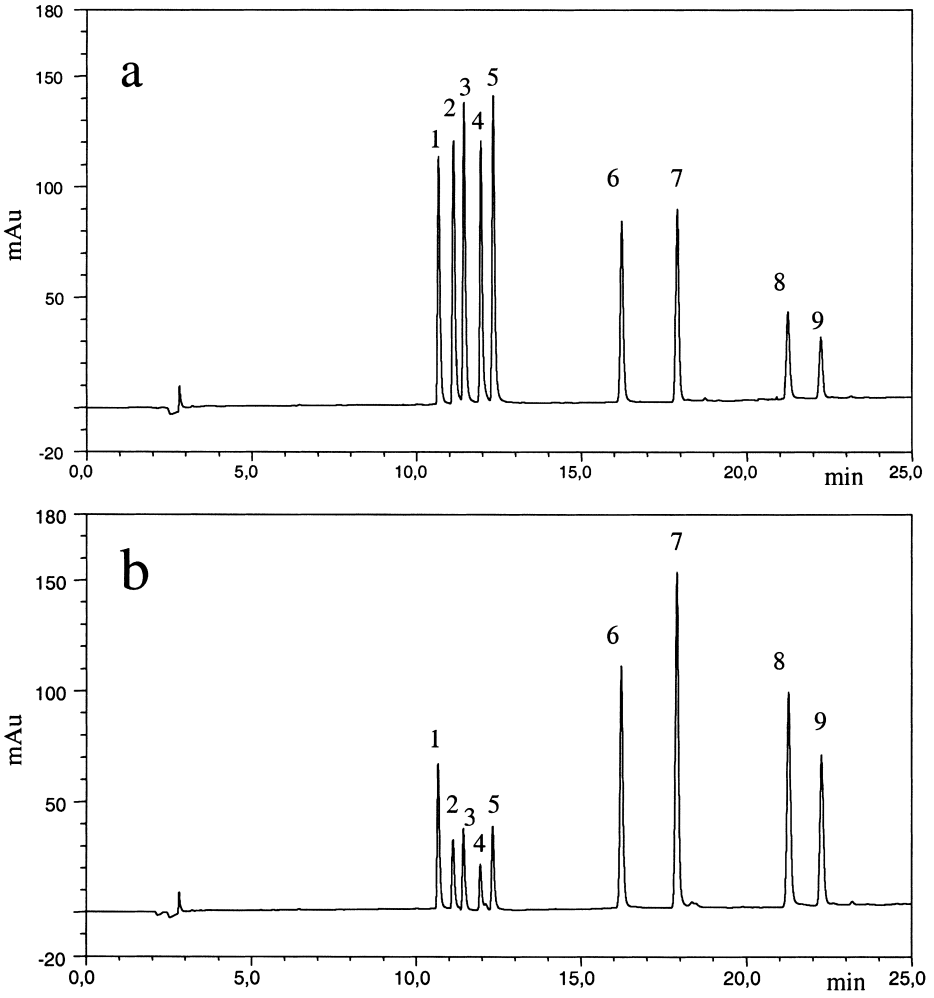


Fig. 1. Chromatograms obtained at 275 nm (a) and 255 nm (b) using the Polarity dC_{18} column in the optimised conditions. 1-ENO, 2-NOR, 3-CIP, 4-DAN, 5-ENR, 6-CIN, 7-OXO, 8-FLU, 9-NAL (concentration level 250 μg l^{-1}, injection volume 100 μl). Chromatographic conditions: see Experimental section.

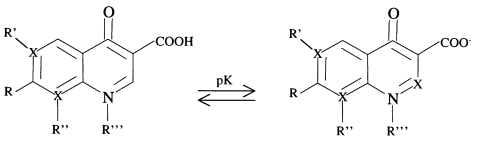
which are zwitterions would be in the cationic form. In order to find the most suitable sorbent for the preconcentration of both groups in a single SPE procedure, four different sorbents were evaluated: C_{18}, SDB and their corresponding mixed phases with a cation exchanger MPC and SDB-RPS.

For each sorbent, the conditioning and elution conditions were optimised and a first study of the breakthrough volume (BTVs, maximum volume that can be preconcentrated with quantitative recovery of the analyte) for the (fluoro)quinolones in the different cartridges was performed. Different solutions of 1 μg of each analyte contained in 25, 50, 100, 150, 200 and 250 ml of acidified pure water (pH 2.5) were loaded in each cartridge and, after allowing them to dry for 5 min, the analytes were eluted using a methanol solution containing 6% ammonia. After evaporation, the extracts were redissolved in 250 μl of diluted formic acid for final quantification. The BTVs obtained in each case are shown in Table 2. As can be expected due to the polarity of the analytes under study, the BTVs were in general not very high, making necessary to improve them in order to get the preconcentration factor required. With this purpose,

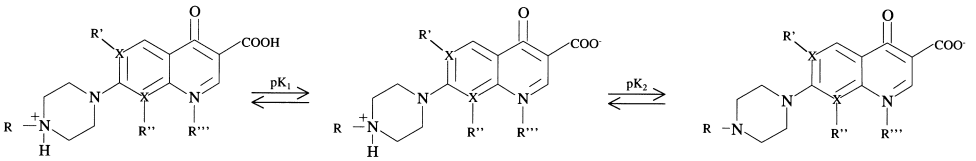
Table 1
Characteristics and limits of detection of the HPLC–UV determination of the nine (fluoro)quinolones

FQ	Calibration equation C: FQ concentration ($\mu g\ l^{-1}$)	R^2	Linearity[a] ($\mu g\ l^{-1}$)	LOD ($\mu g\ l^{-1}$)	Acquisition wavelength (nm)
ENO	$0.0113C+0.0437$	0.9976	10–3000	6.0	275
NOR	$0.0123C+0.0049$	0.9983	10–3000	5.7	275
CIP	$0.0105C+0.00471$	0.9989	10–3000	4.3	275
DAN	$0.0105C+0.0412$	0.9957	10–3000	6.2	275
ENR	$0.0117C+0.0882$	0.9970	10–3000	4.0	275
CIN	$0.0081C-0.0599$	0.9979	10–3000	7.5	255
OXO	$0.0090C-0.0064$	0.9984	10–3000	5.1	255
FLU	$0.0114C-0.0482$	0.9964	10–3000	8.2	255
NAL	$0.0078C-0.0735$	0.9981	10–3000	9.9	255

[a] Concentration range studied.



Acidic quinolones: CIN, OXO, FLU, NAL



7-Piperazinyl quinolones: ENO, NOR, CIP, DAN, ENR

Fig. 2. Structures and ionization equilibria of acidic and piperazinyl (fluoro)quinolones.

the effect of sample pH and the influence of the charge of the analyte molecule on the interactions with the different sorbents, were investigated. The reported values of pK_1 and pK_2 for the piperazinyl quinolones are in the 5.5–6.0 and 7.7–8.5 range, respectively, and the pK_a of the acidic quinolones

Table 2
Breakthrough volumes[a] (ml) obtained for the nine (fluoro)quinolones in the different sorbents at acidic pH

	ENO	NOR	CIP	DAN	ENR	CIN	OXO	FLU	NAL
C_{18}	100	100	150	150	200	150	250	>250	>250
SDB	25	25	25	50	100	>250	>250	>250	>250
MPC	150	150	150	150	200	50	150	150	200
SDB-RPS	100	100	100	100	100	100	100	100	100

[a] The values correspond to the minimum volume for which the recovery decreased to a value lower than 85.

range between 6.0 and 6.9 [15]. Therefore, the 2.5–10 pH range was covered in this study. All experiments were performed using a 250-ml sample volume and the experiments at basic values (pH 9 and 10) were carried out only using the C_{18} and SDB cartridges as the analytes will not interact at this pH with the cation exchanger present in the mixed cartridges.

Fig. 3 shows the recoveries obtained for piperazinyl (a,b) and acidic (a′,b′) (fluoro)quinolones at the different pH values using the reversed-phase sorbents, C_{18} (a,a′) and SDB (b,b′). From this study and from the BTVs study reported above, several conclusions could be derived. First, at basic pH it can be observed that the anionic species of both the acidic and piperazinylic quinolones are least retained in comparison to the cationic, zwiterionic and neutral species. However, different behaviour between both groups is observed at acidic pH. In this case, the acidic quinolones, present as uncharged species in the loading solution, are better retained on SDB than on C_{18} (Fig. 3b′,a′, respectively). This fact is not surprising since it is known that polymeric sorbents offer higher affinity for polar analytes because aromatic rings in the polymeric matrix produce intense $\pi-\pi^*$ interactions. However, this fact does not occur for the piperazinyl quinolones, which are mainly present as cationic molecules at acidic pH. As can be seen (Fig. 3a,b), lower recoveries were obtained when the SDB sorbent was used, suggesting

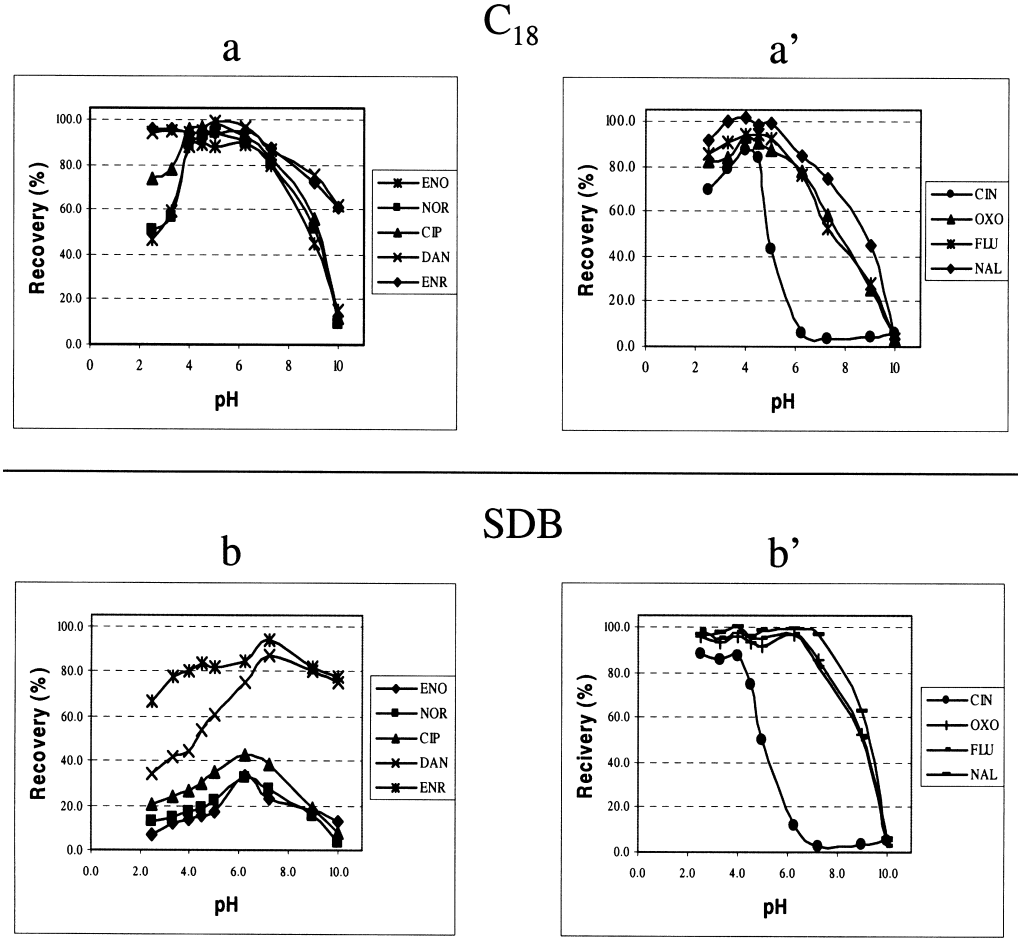


Fig. 3. Influence of pH in the recoveries of piperazinyl (a,b) and acidic (a′,b′) (fluoro)quinolones in C_{18} (a,a′) and SDB (b,b′) after percolation of 250 ml of water solution.

that a cationic charge in the quinolone molecule negatively affects the above mentioned π−π* interactions. Finally, it is important to point out that, although the C_{18} and SDB sorbents are stable between the 2 and 10 pH range, their affinity for the analytes under study considerably increased in all cases when pH values equal or higher than than 4 were used. Additionally, using pH values between pK1 and pK2 of the piperazinylic quinolones has shown to be preferable for the preconcentration of these analytes by a reversed-phase mechanism. It is important to take this fact into account as typically sample pH is adjusted to very acidic values, far from the p$K_a$of the molecules, in order to ensure that they will be in the desired chemical form. However, as demonstrated in this study, this very acidic pH may not be optimum for preconcentration purposes.

Fig. 4 shows the results obtained using the MPC (a,a′) and the SDB-RPS (b,b′) mixed cartridges. It is clear that the cation-exchange mechanism can be used to retain the piperazinyl quinolones (Fig. 4a,b) over a wide range of pH values, as high recoveries are obtained when the analytes are loaded both at acidic pH (cationic species) and neutral pH (zwiterionic form). It seems that the presence of the negative charge in the zwitterionic molecule does not affect the cation-exchange mechanism. Concerning the acidic quinolones (Fig. 4a′,b′), the recoveries

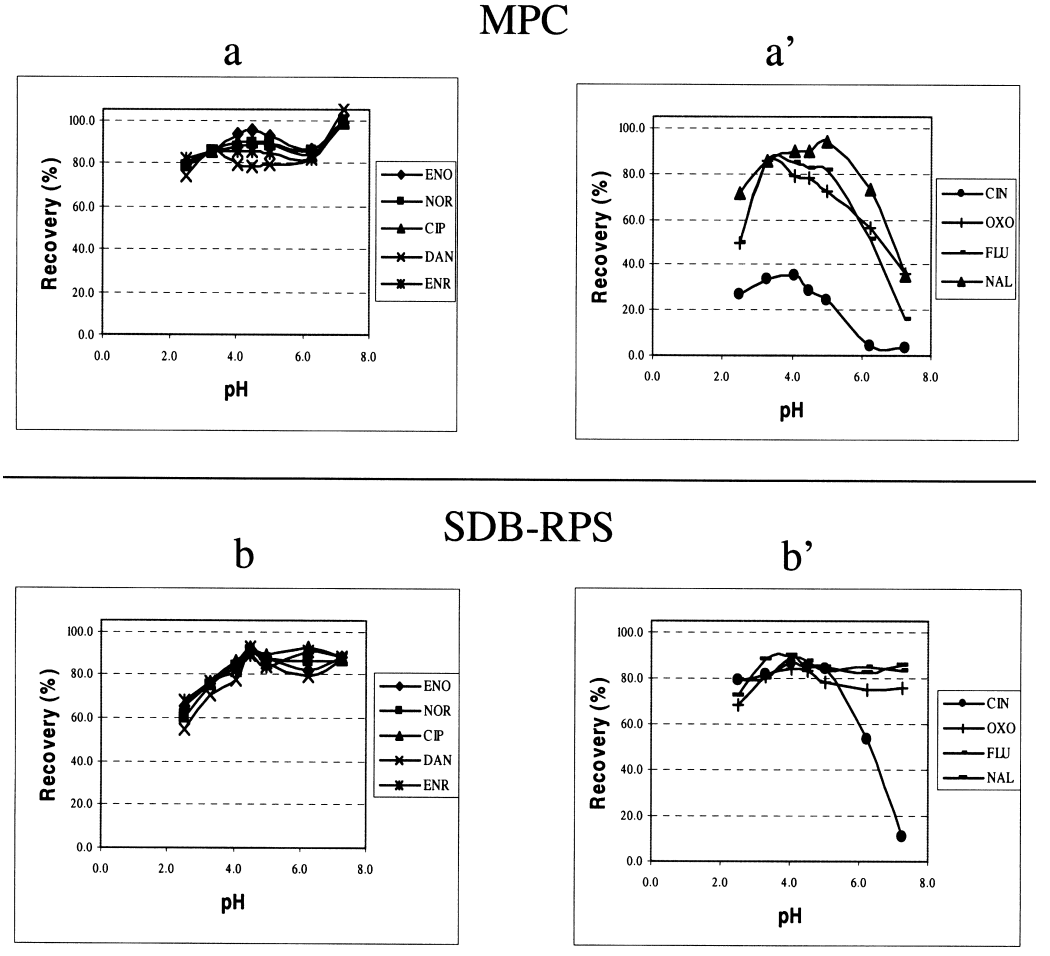


Fig. 4. Influence of pH in the recoveries of piperazinyl (a,b) and acidic (a′,b′) (fluoro)quinolones in MPC (a,a′) and SDB-RPS (b,b′) after percolation of 250 ml of water solution.

slightly decreased with respect to those previously obtained, which can be attributed to the lower amount of reversed-phase sorbent present in these cartridges. Finally, it can be seen that, as well as in the case of SDB and C_{18}, pH values equal or higher that 4 are preferable for preconcentration purposes.

Apart from these theoretical comments, from this study it can be concluded that both C_{18} (at pH 4) and SDB-RPS (at pH 4.5), cartridges can be used for the preconcentration of acidic and piperazinylic quinolones in a single SPE step. Both cartridges allow the preconcentration of 250 ml of water solution with quantitative recoveries for all the (fluoro)quinolones.

3.2. Analysis of water samples

It is known that matrix compounds present in real samples may affect the interaction of the analytes with the sorbent used in solid-phase extraction processes. In some cases, matrix constituents may form complexes with the target compounds, preventing their interaction with the sorbent or, more frequently, matrix components (usually present at high concentration levels) interact with the sorbent reducing the number of free sites available for the retention of the analytes. These facts may affect the breakthrough volumes obtained using standards in pure water and thus, an evaluation of breakthrough volumes of the selected (fluoro)quinolones in river and lake waters using the selected sorbents was carried out (constant mass of 1 μg). In this study, the results obtained using the mixed cartridge (SDB-RPS) were not satisfactory as the recoveries of ionic compounds dropped considerably (some of them were not recovered at all) using only 50 ml of sample volume. These results suggest that metal cations present in these samples interfere strongly in the interaction of the piperazinyl quinolones with the cation-exchange sorbent. However, quantitative recoveries were obtained when C_{18} cartridge was used and no effect of sample matrix on the BTVs of analytes was observed in this case, in the range of volumes evaluated (up to 250 ml of sample). Thus, it was decided to perform the SPE on C_{18} cartridges as a final enrichment procedure, as these cartridges allow the achievement of the preconcentration factor required for trace analysis (1000 times).

River and lake water samples were then spiked at 25 ng l^{-1} in order to evaluate the suitability of the developed procedure for the determination of the FQs at real environmental concentration levels. Samples were prepared as described in the Procedure section and blank water samples were also analysed in each case. The obtained chromatograms are shown in Fig. 5 (grey traces). As can be seen, due to the lack of selectivity of the C_{18} sorbent and the DAD detector employed here, the dissolved organic matter provoked a large increase in the baseline in the middle of the chromatograms hindering the identification of any of the FQs. Therefore, and in order to provide more selectivity to the SPE step, a sequential elution procedure for interferents and FQs was optimised by using different mixtures of acetonitrile and aqueous solutions at the optimum retention pH of the analytes. It was found that a washing step consisting of 3×500 μl of an acetate buffer solution (pH 4) containing 15% of acetonitrile allows the removal of a high degree of the interfents present in the water samples. Higher contents of acetonitrile and higher volumes of washing solution were also tested but they produced losses in the recoveries of the FQs.

None of the FQs studied was found in the analysis of the blank lake water samples whereas a high peak at the same retention time than cinoxacin was clearly detected in the blank river water samples. Then, the corresponding UV spectrum (obtained as described in the Experimental section) was compared to that recorded for cinoxacin, and a very low match percentage (lower than 40%) was found between them, discarding the assignment of this peak to cinoxacin. The chromatograms obtained in the analysis of the spiked lake and river water samples are overlaid in Fig. 5 (black traces). As can be observed, the included washing step in the SPE procedure was very effective and, although complete removal of matrix interferents was not achieved, all the selected FQs could be determined in lake water (Fig. 5a). In the case of river water (Fig. 5b), only cinoxacin (peak 6) could not be quantified due to the presence of the unknown peak at the same retention time, completely preventing the determination of this analyte. The spectra of the target peaks in the chromatograms were recorded and compared with the corresponding antibiotic in each case. Match

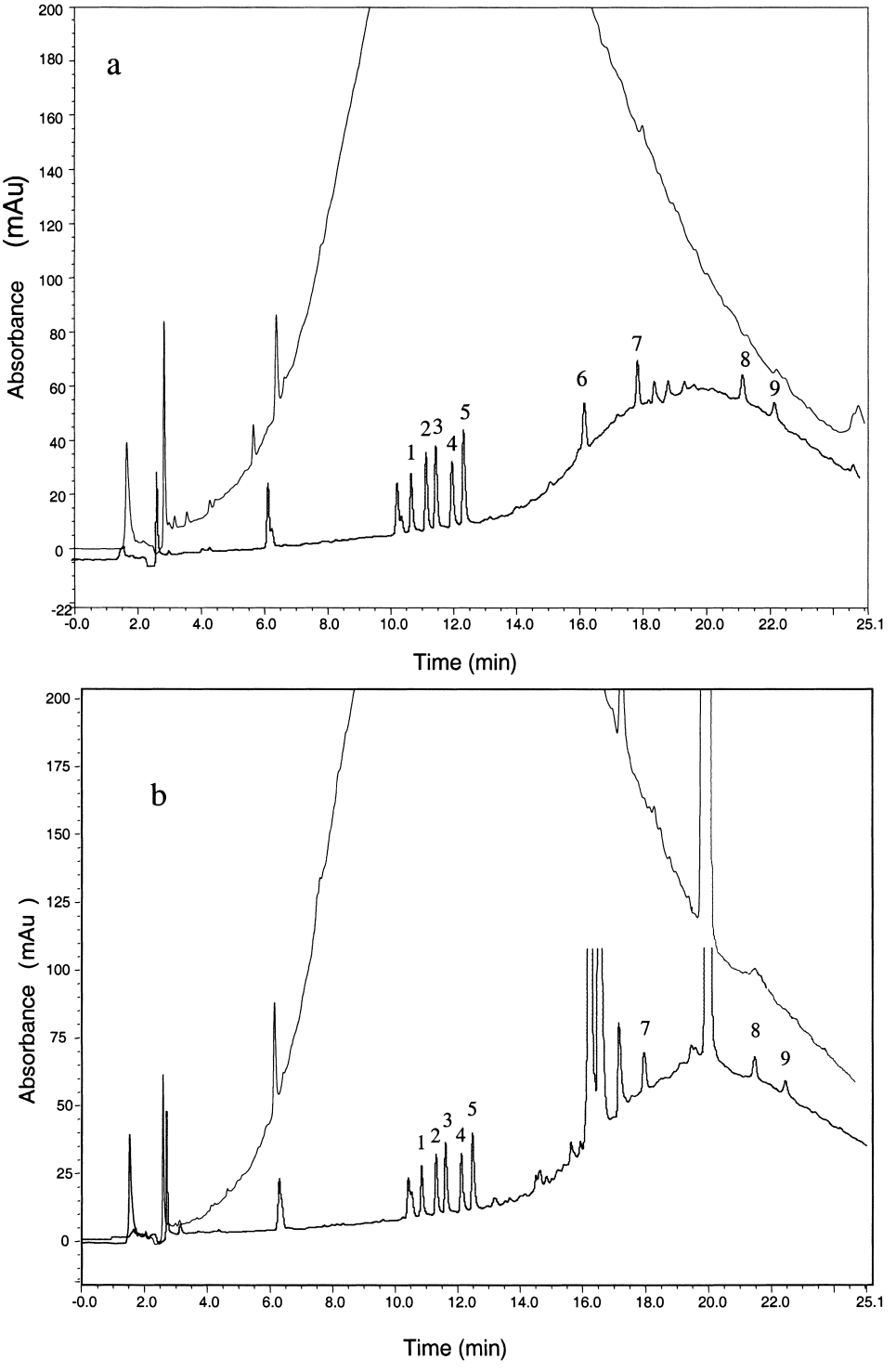


Fig. 5. Chromatograms (275 nm) obtained for lake (a) and river (b) water samples spiked at 25 ng l^{-1} of each (fluoro)quinolone after SPE on C_{18} cartridges, not including (grey traces) and including (black traces) the washing step. For peak identification see Fig. 1. Chromatographic conditions: see Experimental section.

Table 3
Average recoveries[a] (*R*) and relative standard deviations (RSD) obtained after solid-phase extraction of 250 ml of spiked surface water samples (25 ng l^{-1})

(Fluoro)quinolone	Lake water			River water		
	R (%)[a]	RSD (%)	LOD (ng l^{-1})	*R* (%)[a]	RSD (%)	LOD (ng l^{-1})
ENO	89.0	8.2	12	87.6	7.5	13
NOR	92.3	7.5	10	88.3	7.9	11
CIP	95.6	6.8	9	96.2	8.2	10
DAN	98.1	7.0	11	93.8	6.8	12
ENR	94.3	5.8	8	98.5	6.5	9
CIN	87.3	11.2	10	n.d.[b]	–	–
OXO	95.4	9.5	8	92.8	8.7	10
FLU	93.9	8.8	10	96.3	9.1	13
NAL	101.3	7.2	15	97.8	8.1	20

Limits of detection (LOD) achieved by the proposed method.
[a] Average of five independent determinations.
[b] Not detected.

percentages higher than 95% were obtained in all cases. These results prove that, although the organic content of a surface water sample might vary from source to source, the efficiency of the washing step provides high selectivity to the developed method, making it useful for these kind of samples.

Mean recoveries for FQs and relative standard deviations (shown in Table 3) were calculated in each case from five different analyses carried out within the same day. As can be seen, with the exception of cinoxacin in river water samples, quantitative recoveries were obtained in all cases with RSD values lower than 13%, demonstrating the accuracy and repeatability of the method at this concentration level. The limits of detection (also shown in Table 3) were calculated as three times the signal-to-noise ratio and varied within the range 8–15 ng l^{-1} for lake water and 8–20 ng l^{-1} in river water. These values make the proposed methodology suitable for the analysis of the selected FQs in surface waters at their real concentration level in the environment.

It is important to stress that, although the SDB-RPS sorbent was not appropriate for the samples selected in the present study, it may be successfully employed for the analysis of waters with a lower content in cationic species (soft samples).

4. Conclusions

A simple method for the determination of nine acidic and piperazinyl fluoroquinolones in a single analysis has been developed. The procedure involves an efficient trace-enrichment step by SPE (preconcentration factor of 1000), which allows the determination of the analytes by HPLC using UV detection as an alternative to MS or fluorescence detectors.

A deep study on the suitability of different sorbents (C_{18}, SDB, SDP-RPS and MPC) for the preconcentration of these analytes has been carried out, and it was concluded that sample pH values equal or higher than 4 are preferable in all cases for preconcentration purposes. The best results were obtained using the C_{18} and SDB-RPS and a sample pH of 4 and 4.5, respectively. However, the use of the C_{18} cartridge is preferable for the analysis of hard water samples, as the presence of a high amount of metal cations strongly interfere in cation-exchange retention mechanism.

The developed procedure was successfully applied to the determination of the nine FQs in lake and river water samples at the low ng l^{-1} concentration level. The recoveries and limits of detection obtained proved the suitability of this procedure for the control of fluoroquinolones in surface water and to provide real data of the occurrence of these compounds in the environment.

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