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Determination of quinolones in water samples by solid-phase extraction and liquid chromatography with fluorimetric detection

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Abstract

A method is reported for the determination, in water samples, of 10 quinolones which are used as veterinary drugs. Analytes are isolated from samples by solid-phase extraction (SPE) and analysed by reversed-phase high-performance liquid chromatography using fluorimetric detection. A solid-phase extraction procedure based on retention on HBL OASIS cartridges and elution with a mixture of acetonitrile–water in basic medium is suitable for pre-concentration of the analytes. Pre-concentration factors up to 250 can be obtained. The quinolones are separated with an octyl silica-based column and mobile phases consisting of aqueous oxalic acid solutions and acetonitrile mixtures. The attained detection limits of the whole process are in the ng l^{-1} level when 250 ml of water sample is processed. Recovery rates, from natural water samples spiked at 20–60 ng l^{-1} level, range from 70 to 100% and common standard deviation are about 6–12%.

Keywords: Water analysis; Solid-phase extraction; Quinolones

1. Introduction

During the last decade, it has become evident that numerous drugs widely used in human and veterinary medicine are being introduced into the aquatic environment [1–4]. Urinary or fecal excretion introduce human pharmaceuticals into wastewater that reaches treatment plants. These pharmaceuticals are thus partially removed before treated water reaches aquifers. In contrast, veterinary drugs result in a direct input to soils and subsequently, via manure, to groundwater. Moreover, the use of veterinary drugs in fish farming activities, typically in the form of feed additives, leads to their direct entrance into the aquatic environment.

Antibacterial agents are among the most widely discussed emerging pollutants. Some of them, such as penicillins, tetracyclines, sulfonamides, quinolones, and macrolides have been detected at the ng to low μg l^{-1} levels in the aquatic environment in Europe and North America [5–9]. Although such low concentrations are probably not active to humans, they are potentially hazardous to bacteria and other micro-organisms. In addition, their occurrence in the environment may contribute to the development of drug resistant bacterial strains. To evaluate the fate of these drug residues and to control the quality of the aquatic medium, sensitive analytical methods are needed.

The aim of this study is to develop a simple method for trace determination, in natural water samples, of 10 quinolones which are used as veterinary drugs in food-producing animals. The studied quinolones are: oxolinic acid (OXO), flumequine (FLU), nalidixic acid (NAL), sarafloxacin (SAR), norfloxacin (NOR), danofloxacin (DAN), marbofloxacin (MAR), difloxacin (DIF) and enrofloxacin (ENR) and its metabolite ciprofloxacin (CIP). OXO, FLU and NAL belong to the older generation of quinolone drugs but are still used in veterinary medicine. ENR, CIP, SAR, DAN, MAR, and DIF are second-generation fluoroquinolones and are registered for use in several animals.

The structures of these compounds are shown in Fig. 1. The presence of a carboxylic group makes all these compounds acidic. In addition, the second-generation quinolones have an amino group in the heterocyclic ring (namely

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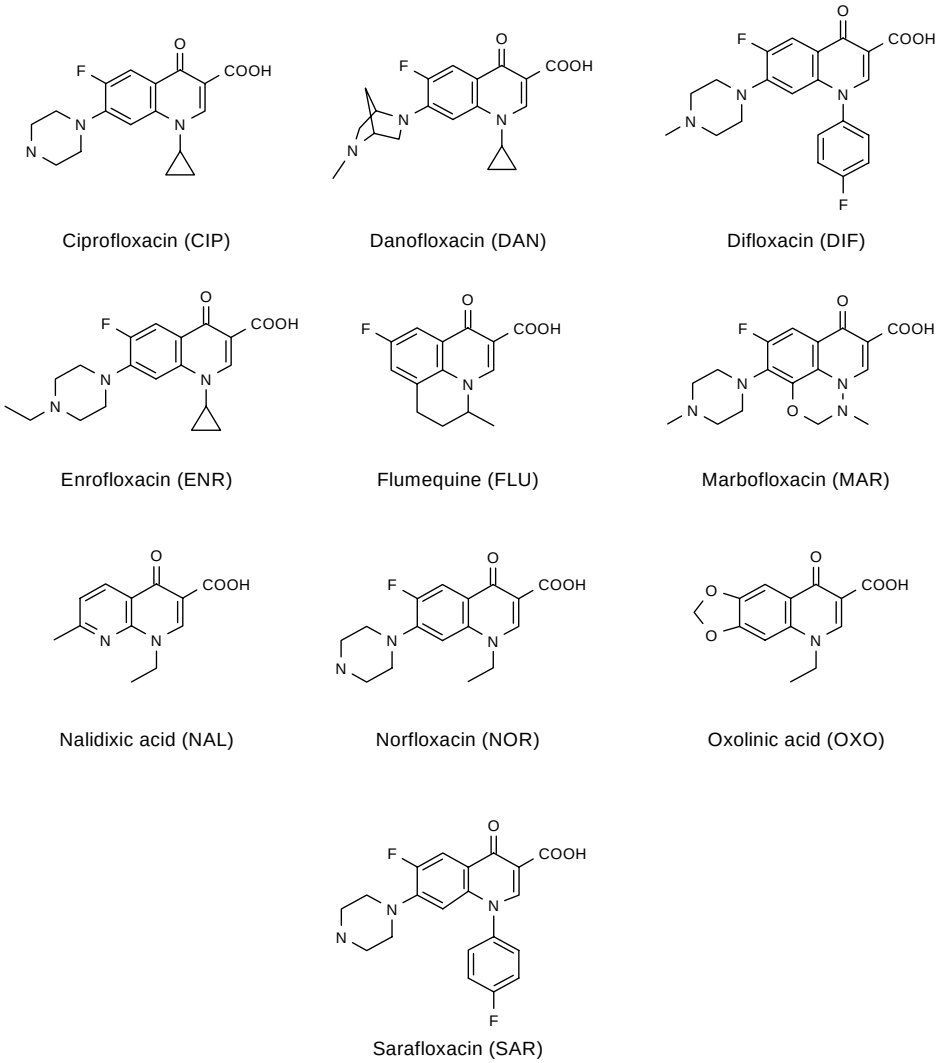

Fig. 1. Structural formulae of the quinolones under study.

piperazinyl). In what follows, the OXO, NAL and FLU are referred to as acidic quinolones (AQs) and those with the heterocyclic group are called piperazinyl quinolones (PQs). The reported values of pK_a for the carboxylic group ranges from 6.0 to 6.8 for AQ and from 5.7 to 6.3 for PQ, whereas those for the protonated amino group are higher (7.6–8.3), and thus, the intermediate form for PQ is a zwitterion [10].

Since quinolones are polar compounds and most of them are highly fluorescent, reversed-phase liquid chromatography with fluorimetric detection is the determination technique mainly used for trace analysis. However, several methods dealing with LC–MS have been reported for confirmatory analysis. Most of the published methods are designed for biological fluids [11,12] and animal tissues [13], whereas, to our knowledge, few methods report the analysis of quinolones in environmental samples. Those that do exist refer mainly to oxolinic acid in seawater and sediments [14,15] and, more recently, to the analysis of PQs (mainly of human use) in urban wastewater and sewage sludge samples [16,17].

The studied method is based on determination by LC-fluorimetry. Although the high sensitivity of the method, the detection limits required for environmental monitoring, can only be achieved by pre-concentration sample preparation techniques, providing high enrichment factors of the target analytes. A study of the conditions for the extraction and pre-concentration of quinolones using commercially available solid-phase extraction (SPE) cartridges is reported. The optimum conditions were used for analysis of quinolones in sea and continental waters.

2. Experimental

2.1. Chemicals and solutions

Ciprofloxacin hydrochloride and enrofloxacin were kindly supplied by Cenavisa (Reus, Spain), norfloxacin by Boral Química (Barcelona, Spain), danofloxacin mesilate by Pfizer (Sandwich, NJ, USA), difloxacin hydrochloride

and sarafloxacin hydrochloride by Abbott (North Chicago, IL, USA), marbofloxacin by Vétoquinol (Lure, France) and nalidixic acid by Impex Química (Barcelona, Spain). Oxolinic acid and flumequine were purchased from Sigma (St. Louis, MO, USA).

Acetonitrile, HPLC gradient grade (Merck, Darmstadt, Germany), and doubly de-ionised water (Milli-Q, Millipore, Molsheim, France) with a resistivity of 18.2 MΩ cm^{-1} were used throughout. All other reagents were of analytical reagent grade.

Stock standard solutions (100 mg l^{-1}) of the quinolones were prepared by dissolving the compounds in 0.01 M aqueous nitric acid (or 0.01 M aqueous NaOH for oxolinic acid, nalidixic acid and flumequine). Solutions were stored in dark glass bottles at 4 °C and were stable over 3 months. Working standard solutions were freshly prepared by dilution.

Mobile phases of several compositions were prepared by mixing acetonitrile with the appropriate volume of aqueous oxalic acid buffer solutions previously filtered through a 0.22 μm nylon filter.

The SPE cartridges used were: OASIS HLB 30 mg (Waters, Milford, MA, USA).

All glassware used for experiments was soaked in 10% nitric acid for 24 h and rinsed with doubly de-ionised water.

2.2. Samples

Samples were collected in the NW Mediterranean basin. Seawater sample (S1) were from a fish factory at Ebre river delta (Tortosa, Spain). Fresh waters from wells (S2 and S3) were provided by the Department of the Environment of the Generalitat de Catalunya: S2 was taken from a rural livestock-producing area in the Ter river basin (Empordà, Spain), whereas S3 belongs to an urban and agricultural area in the Llobregat basin, close to Barcelona (Spain). Samples were acidified to pH 2, filtered through a 0.45 μm pore size filter (Whatman, Maidstone, UK) and stored in dark glass bottles at 4 °C. Prior to the analysis, samples were allowed to reach room temperature. For recovery studies, water samples were spiked with an appropriate amount of standard solution of each compound, shaken and set aside for 2 h before analysis.

2.3. Apparatus

The chromatographic system was an Agilent 1100 Series (Palo Alto, CA, USA), consisting of a quaternary pump HP 1100 Series and a Rheodyne model 7725 injector equipped with a 20 μl injection loop. Detection was performed on a fluorescence Agilent Series 1100 detector, which may be programmed for full-spectra acquisition. For the separation, a 250 mm × 4.6 mm; 5 μm Inertsil C_8 column (Alltech, Deerfield, IL, USA) equipped with a similar guard-column was used.

pH was measured on a CRISON GLP 21 pH meter (Alella, Barcelona, Spain), equipped with an Ag/AgCl combined glass electrode, CRISON 52-02.

For pre-concentration, a Minipuls 3 peristaltic pump (Gilson, Villiers-le-Bel, France) was used to pump the solvents and the samples through the cartridges. PTFE tubing (0.5 mm i.d.) and Tygon tubes were used for this purpose.

2.4. Procedure

SPE cartridges were conditioned with 5 ml methanol followed by 10 ml water at 2 ml min^{-1}. Next, 5 ml aqueous buffer at the same pH value as samples were pumped at 2 ml min^{-1}. For the retention of analytes on the cartridge, the sample pH was adjusted to selected value (5.5) immediately before percolation through the SPE-cartridge. Two hundred and fifty to 500 ml of sample was pumped through the cartridge at a flow rate of 10 ml min^{-1}. After loading, the cartridge was rinsed with 10 ml water, dried and eluted with 2 ml 0.01 M NaOH–acetonitrile (75:25) at a flow rate of 0.5 ml min^{-1}. The eluate was injected (20 μl) into the LC system.

The separation was performed using binary gradient elution. Mobile phase A was 10 mM oxalic acid buffer at pH 4–acetonitrile (89:11 (v/v)) and mobile phase B acetonitrile. The elution profile was as follows: mobile phase A for 12 min, gradient elution from 12 to 25% acetonitrile in 9 min and from 25 to 45% acetonitrile in 8 min and a post-time of 6 min to back to initial conditions. The mobile phase flow rate was set at 1.5 ml min^{-1} and the separation was carried out at room temperature. The excitation/emission wavelengths selected were: 297/507 nm up to minute 12 (for MAR); 280/450 nm from minute 12 to minute 23 (for NOR, CIP, DAN, ENR, SAR and DIF); 263/380 nm from minute 23 to minute 28 (for OXO); 248/361 nm from minute 28 up to the end (for NAL and FLU).

Alternatively, two sample injections can be made, one for PQs using mobile phase A for 12 min and gradient elution from 12 to 25% acetonitrile in 9 min, and a second run for AQs with isocratic elution (32% acetonitrile). Wavelengths selected in the second run were: 263/380 nm up to minute 7 (for OXO) and 248/361 from this time (for NAL and FLU). For identification purposes, full-emission spectra were recorded in the range 375–550 nm (or full-excitation spectra from 250 to 425 nm).

3. Results and discussion

3.1. Chromatographic separation

In previous studies, we reported the use of a C_8 silica-based reversed-phase column and mobile phases consisting of acetonitrile–water mixtures containing oxalic acid as a buffer, for the separation of a series of 7-piperazinylquinolones [18] and also for the determination of flumequine

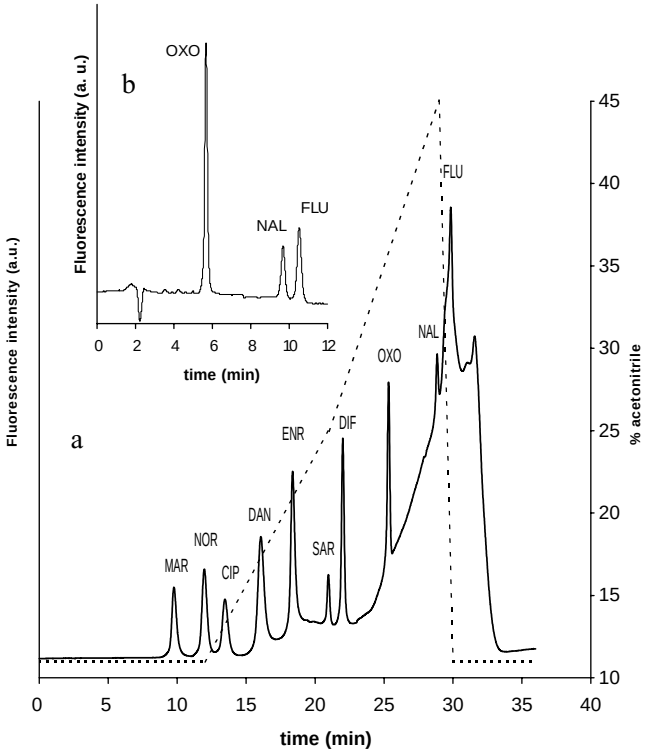

Fig. 2. Chromatogram of a standard mixture of quinolones. (a) Gradient elution, at a flow rate of 1.5 ml min^{-1}. The dashed line indicates gradient profile. (b) Isocratic elution (32% ACN). Twenty μg l^{-1} of each quinolone, except MAR (100 μg l^{-1}) and DAN (4 μg l^{-1}).

and oxolinic acid [19]. Since OXO and FLU are more hydrophobic than the piperazinyl derivatives, the chromatographic conditions for the separation of each group were different. Acetonitrile content ranged from 10 to 25% for piperazinyl derivatives and was about 40% for FLU and OXO.

The use of an Inertsil C_8 column and a suitable elution gradient program led to a good separation of the 10 quinolones studied in this work in just one chromatographic run. However, when injecting very diluted solutions, and thus working in highly sensitive conditions, some distortions in the baseline were observed, which made difficult the determination of the three AQs (see Fig. 2a). This may be due to the gradient profile, since there is a sharp change in the mobile phase composition at about 21 min. Therefore, to allow quantification of low concentrations of AQs, an alternative method involving two injections is proposed. The first separation, using the previously described gradient profile to minute 29, allows the determination of the seven PQs. The second run, which is performed isocratically at 32% acetonitrile, is used for the AQs. In the latter, no interference due to the PQs is observed, since the mobile phase makes them elute quickly at the dead time. Moreover, PQs are not detected at the wavelengths used in the second run. The chromatogram obtained under these conditions is shown in Fig. 2b.

The limits of detection for the chromatographic method based on the two runs, calculated as the concentrations corresponding to a signal of three times the standard deviation of baseline, are in the range 0.1–0.5 μg l^{-1} for NOR, CIP, ENR, SAR, NAL and FLU, about 0.05 μg l^{-1} for DAN, DIF and OXO, and about 1 μg l^{-1} for MAR, which is the less fluorescent quinolone. In order to achieve the maximum sensitivity, detection was performed by wavelength programming so that each analyte was detected at its optimal wavelengths (see procedure).

3.2. Pre-concentration of analytes by SPE

Because of the acid–base characteristics of quinolones [10], SPE of the analytes is expected to be strongly pH dependent and thus, the effect of pH on retention of quinolones was evaluated. Preliminary studies were performed by pumping through commercial Oasis HLB cartridges (30 mg) 100 ml of aqueous standard solutions buffered at several pH values, i.e. from 2 to 12, and containing all the studied quinolones at concentration levels ranging from 25 to 250 μg l^{-1}, depending on the analyte. Twenty μl of the percolated solution was injected into the LC system.

Fig. 3 shows the variation of retention as a function of the sample pH for three selected quinolones: OXO (a model compound for AQ) and DIF and CIP, which are model compounds for strongly retained PQs (DIF) and moderately retained PQs (CIP). AQs, which are neutral molecules in the acid pH range, showed quantitative retention up to pH 8–9, whereas at higher pH values retention decreased and no retention was found at pH 12, a pH at which AQs are in anionic form. The retention behaviour of PQs can also be explained according to their acid–base properties, thus, they exhibited maximum retention at pH values between 5.5 and 8.5, when

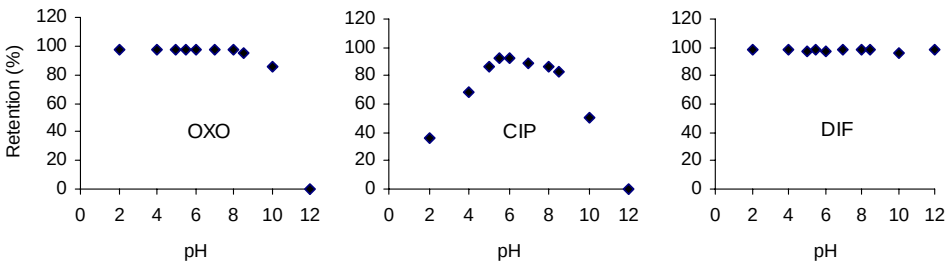

Fig. 3. Effect of pH on retention of quinolones on Oasis HLB cartridges (30 mg). Processed volume: 100 ml.

zwitterionic species prevailed. However, the extraction profile depends on the individual quinolone and seems mainly related to analyte polarity. The most polar compounds, NOR and CIP, were the least retained analytes with about 10% losses in the optimum pH range (5.5–7), whereas retention for most quinolones are higher than 98% in the pH range 5.5–9. SAR and DIF showed strong affinity towards the sorbent phase, as they exhibited high retention in the whole pH range assayed, from 2 to 12, regardless of their acid–base speciation. This behaviour may be related to the fact that they have an additional phenyl group (see Fig. 1) which increases their interaction with the *N*-vinylpyrrolidone-divinylbenzene phase of the cartridge.

These studies proved that adjusting the pH of the sample to between 5.5 and 7 allows us to achieve an efficient retention of the whole set of analytes on an Oasis HLB phase. In order to minimise the complexing ability of quinolones towards metal ions [20] that may be present in natural waters, the selected value to perform the loading of the sample on the cartridges was pH 5.5, which can be easily adjusted with acetic acid/acetate buffer.

To choose a proper eluent for the retained quinolones, 100 ml of aqueous standard solutions containing the whole set of analytes and buffered at pH 5.5 was pre-concentrated, rinsed, and then stripped with acetonitrile–water mixtures. Two separate eluate fractions of 2 ml were collected and 20 μl of each fraction injected into the chromatographic system.

Elution with the initial mobile phase (acetonitrile–aqueous buffer (89:11), pH 4) led to low or no recoveries for most quinolones, except NOR, CIP and MAR. Although recoveries increased as acetonitrile content did, more than 25% acetonitrile in the eluting solution was not favourable for the chromatographic separation, since it resulted in peak broadening and losses in response. Addition of sodium hydroxide to the eluting solvent strongly improved recovery rates and quinolones were satisfactorily eluted using 0.01 M NaOH aqueous solution (pH 12)/acetonitrile mixtures containing 20–25% AN. In these conditions, the extract can be injected into the chromatograph with no additional treatments. Elution was almost complete, i.e. more than 90%, with just 2 ml of eluent, except for SAR, which is the most retained quinolone. Pre-concentration of large volumes, up to 500 ml, provided good recoveries for most of the analytes whereas some losses of PQ were observed when 1000 ml water were processed. As a whole, CIP, NOR and SAR show lower recoveries than other quinolones, but some differences were found in the retention behaviour of NOR and CIP in comparison with SAR. Whereas CIP and NOR recovery rates may be explained by an incomplete sorption onto the solid phase in the retention step rather to low efficiency in the elution, SAR behaviour seems to be due

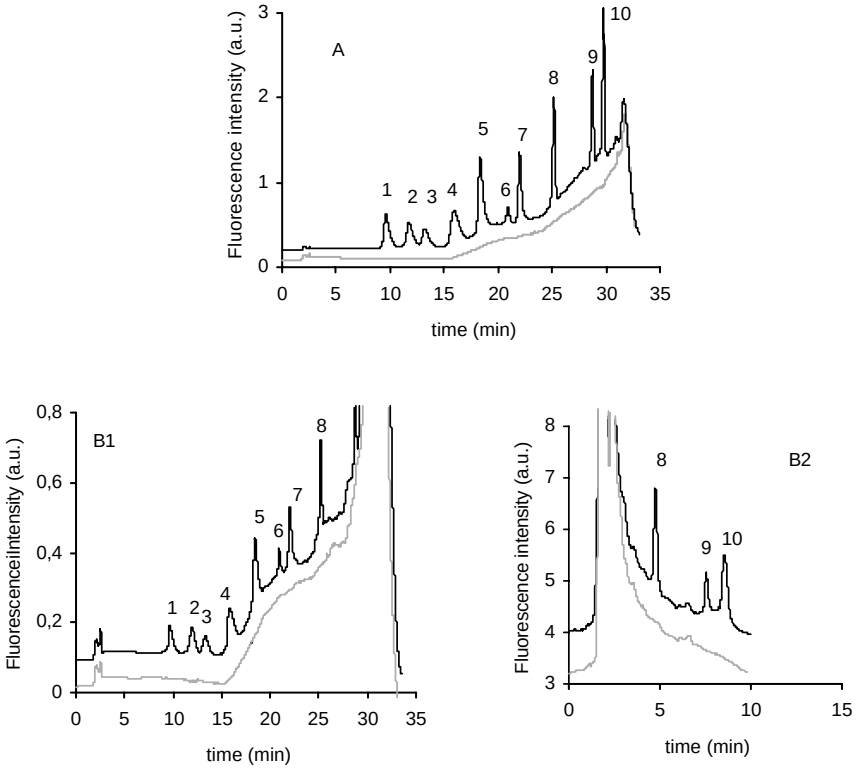

Fig. 4. Chromatograms of extracts from 250 ml of sample S2. (A) unspiked sample and sample spiked with MAR (1.5 μg l^{-1}), NOR, CIP, ENR (0.3 μg l^{-1}), SAR, DIF, OXO (0.2 μg l^{-1}), DAN (0.1 μg l^{-1}) NAL, FLU (0.6 μg l^{-1}). (B1 and B2) Sample spiked with MAR (0.25 μg l^{-1}), NOR, CIP, ENR, NAL, FLU (0.06 μg l^{-1}), SAR, DIF, OXO (0.04 μg l^{-1}), DAN (0.02 μg l^{-1}). Peaks: 1, MAR; 2, NOR; 3, CIP; 4, DAN; 5, ENR; 6, SAR; 7, DIF; 8, OXO; 9, NAL; 10, FLU.

to an incomplete elution caused by the strong interaction between this analyte and the Oasis HBL polymeric phase.

3.3. Analysis of spiked natural water

To evaluate the performance of the method, three spiked natural water samples (see Section 2.2) were analysed following the proposed method. Analysis of the original samples revealed no measurable quinolones. Spiked amounts were 20–250 ng l^{-1} for all quinolones, except DAN (10–60 ng l^{-1}) and MAR (100–250 μg l^{-1}). Fig. 4 shows chromatograms corresponding to SPE extracts from 250 ml of sample S2 (groundwater) and the same water spiked with quinolones at two concentration levels. A comparison of chromatogram A with a chromatogram from standard solutions showed no significant matrix effects on the separation and determination of the quinolones. In contrast, for the sample spiked at the lower concentration level (chromatogram B1), a broad matrix peak become relevant after elution of OXO (about 27 min). The same trend was observed for samples S1 and S3. This peak, due to humic compounds and other naturally occurring fluorescent substances in water, has no effect on the analysis of the PQs, but affects the determination of AQs—which are the last eluted quinolones—at the low μg l^{-1} level when working with just one run. However, the method based on two runs allows detection and quantification of nine of the 10 quinolones studied at concentrations below 100 ng l^{-1}, as can be seen in chromatograms B1 and B2. The relatively poor fluorescence of MAR prevents its determination at these low levels. The earlier broad eluting peak in chromatogram B2, which is a matrix peak, is separated from those of the analytes, and it does not interfere with the determination of the AQs. Chromatograms from sample S1 (seawater) spiked with quinolones at about 10–100 ng l^{-1} (Fig. 5) are similar to those obtained from sample S2 (groundwater).

The fluorescence detector used in this study allows the acquisition of spectra throughout the chromatogram, and although fluorimetry is not so powerful as MS as confirmatory technique, this option permits to check the identity of peaks eluting at the retention times of the analytes. Taking into account that quinolones are detected as the native compounds, not as derivatives, the information provided by the fluorescence spectra is quite valuable in terms of selectivity. Although this detection mode usually results in some increase in the background noise, the differences are not dramatic. Chromatograms obtained using both detection modes proved that the spectral acquisition mode led to detection limits about two times higher than those attained using detection at a single wavelength pair.

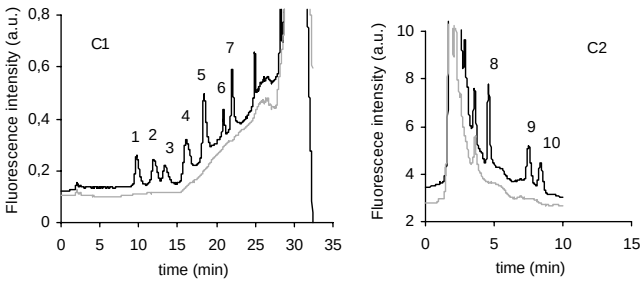

Fig. 5. Chromatograms of extracts from 250 ml of sample S1 and the same sample spiked with MAR (0.25 μg l^{-1}), NOR, CIP, ENR, NAL (0.06 μg l^{-1}), SAR, DIF, OXO, FLU (0.04 μg l^{-1}), DAN (0.02 μg l^{-1}).

Table 1
Recovery data (%) from spiked natural water samples

	Spiking level (ng l^{-1})	S1	S2	S3
MAR	250	90 (6)[a]	91 (8)[a]	93 (9)[a]
NOR	200	79 (5)	82 (6)	77 (8)
	60	72 (9)	79 (8)	75 (15)
CIP	200	82 (4)	85 (5)	79 (4)
	60	88 (6)	80 (8)	81 (9)
DAN	20	95 (6)	89 (7)	88 (10)
ENR	200	88 (5)	93 (5)	85 (3)
	60	90 (7)	87 (6)	80 (7)
SAR	150	72 (6)	74 (4)	79 (6)
	40	76 (8)	72 (6)	70 (7)
DIF	150	92 (3)	96 (4)	88 (4)
	40	82 (7)	90 (6)	84 (5)
OXO	150	97 (3)	94 (4)	95 (4)
	40	96 (5)	92 (6)	90 (8)
NAL	200	90 (5)	94 (4)	92 (4)
	60	84 (8)	89 (9)	90 (20)
FLU	150	94 (3)	92 (4)	91 (4)
	40	99 (7)	95 (6)	88 (9)

Processed volume: 250 ml.
[a] Mean value and standard deviation ($n = 4$).

Recoveries (Table 1) were satisfactory when up to 250 ml of natural samples were processed. However, when 500 ml samples were processed, recoveries from S2 and S3 significantly decreased, probably due to the presence of a higher amount of organic matter in these samples. Therefore, to avoid loss of analytes, a sample volume of 250 ml is recommended.

In conclusion, SPE with the LC-fluorimetry is a useful procedure for quinolone analysis in natural waters, which allows the determination of these pharmaceuticals at the lower ng l^{-1} level when processing 250 ml of sample. Fluorimetric detection has the additional advantage that the spectra of the eluting peaks can be used to confirm the identity of the analytes.

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