Simultaneous determination of 8 fluoroquinolone antibiotics in sewage treatment plants by solid-phase extraction and liquid chromatography with fluorescence detection

L. Shi, X. F. Zhou, Y. L. Zhang and G. W. Gu

ABSTRACT

Fluoroquinolones (FQs) are among the most important antibiotics used in human and veterinary medicines. A simple and effective analytical method based on reversed-phased liquid chromatography with fluorescence was developed and validated for the simultaneous determination of eight FQs in wastewater at trace level. Aqueous samples were extracted using Anpel^{TM} MEP cartridges where they were subsequently eluted by formic acid in methanol. The aqueous extracts were analyzed by gradient elution Liquid Chromatography with Fluorescence Detection (LC–FLD), whose mobile phase was composed of acetonitrile and 10 mM tetrabutyl ammonium bromide (TBAB). The limits of detection (LOD) and the relative standard deviation (RSD) were as low as 0.11–1.06 μg/L and 2–5%, respectively. The presented method was successfully applied to quantify FQs in the influent and effluent of several typical Sewage Treatment Plants (STPs) in Shanghai. For the extraction of 100 mL influent and 500 mL effluent sewage water samples, recoveries obtained were between 79–109% and 80%–105%, respectively. 7 FQs were occurred and identified in the STPs with the concentrations varying from 7 ng/L to 1 μg/L. Norfloxacin, ciprofloxacin and lomefloxacin were the most frequently detected antibiotics occurring in the wastewaters. The analytical procedure developed may be used for more in-depth studies on the occurrences and the fate of these commonly used pharmaceuticals in the sewage treatment plants and in the aquatic environment.

Key words | antibiotics, fluoroquinolones, liquid chromatography with fluorescence detection, PPCPs, water analysis

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INTRODUCTION

In the last decades, public and scientific concern about the relevance of trace amounts of pharmaceuticals and personal care products (PPCPs) that occur in the environment has been continuously increasing (Kolpin *et al.* 2002; Boyd *et al.* 2003; Ellis 2006). Fluoroquinolone antibacterial agents are powerful and effective groups of synthetic antibiotics in human and veterinary medicines worldwide. Among the leading FQs for human and animal treatment, ciprofloxacin (CIP) and norfloxacin (NOR) are the major human-use FQs, each contributing about 0.03 and 0.12 μg/L in the output of a treatment water plant in hospital wastewater (Golet *et al.* 2001). It is now generally recognized that the treatment processes applied in STPs do not fully eliminate these compounds, as a consequence, FQs are expected to enter the environment for long periods of time, mainly either via human excretion into wastewaters or via dispersion of manure onto agricultural soils (Golet *et al.* 2002). Once the substances enter the rivers and lakes, they may pose a risk to the aquatic environment.

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Many chromatographic methods have been published for the analysis of FQs in biological matrixes (Schneider *et al.* 2007*a*,*b*); however to our knowledge, there is less method available to analyze trace amounts of several FQs in environmental samples (Revert *et al.* 2003; Turiel *et al.* 2003; Prat *et al.* 2004; Karthikeyan & Meyer 2006; Vieno *et al.* 2006; Lee *et al.* 2007). Because of the low concentrations occurring in the environment, enrichment and sample cleanup are difficult. Therefore, a comprehensive method to enrich various FQs with reliable recoveries is still awaited.

In this paper, a new and simple method for the multi-residue analysis of 8 FQs in wastewater samples by LC–FLD is explored. Due to the different acid-base properties of the selected compounds, special attention has been given to the development of solid-phase extraction enrichment procedures, other specific aims of our study were to develop and validate a specific and sensitive LC–FLD method and to check the power of the method to analyze urban wastewaters.

MATERIALS AND METHODS

Chemicals and reagents

Reference compounds of FOs were purchased from Sigma-Aldrich, including norofloxacin (NOR), pefloxacin mesylate dehydrate (PEF), ciprofloxacin (CIP), lomoxacin (LOM), danofloxacin (DANO), enrofloxacin (ENRO), difloxacin (DIF) and sarafloxacin (SAR), whose features studied were shown in Table 1. Standard solutions of 200 mg/L were prepared in a water: acetonitrile mixture (1:1) containing 0.2% v/v hydrochloric acid and stored at −20°C. Standard solutions were renewed monthly. Working standard mixtures of 100 μg/L to 1 μg/L were prepared in mobile phase, stored at +4°C, and renewed weekly.

The extraction materials used were MEP (Anpel^{TM}, 60 mg, 3cc), LC-18 (supleco, 60 mg, 3cc), ENVI-18 (supleco, 60 mg, 3cc), ENVI Chromp (supleco, 200 mg, 3cc), Oasis HLB (Waters, 30 mg, 3cc), Oasis MAX (Waters, 30 mg, 3cc), and Oasis MCX (Waters, 30 mg, 3cc).

All of the solvents were reagent grade or higher in quality. HPLC-grade water, acetonitrile, and methanol (MeOH) were purchased from Sigma-Aldrich. *Ortho*-Phosphoric acid (*o*-H3PO4) 85%, hydrochloric acid (HCl) 32%, Triethylamine and tetrabutyl ammonium bromide (TBAB) were obtained from Fluka AG (Buchs, Switzerland).

Sample collection

Primary and tertiary wastewater effluents were collected from different urban wastewater treatment plants in Shanghai, china. The descriptions of different wastewater treatment plant were shown in Table 2. Primary effluents were collected after primary clarification; Tertiary effluents were collected after advanced treatment with contact filtration. Samples were collected in amber glass bottles and immediately adjusted to pH 3 using 1% *ortho*-Phosphoric acid solution to reduce biological activity, and then stored in the dark at + 4°C until analysis.

Table 1 | Features of selected fluoroquinolones

Compounds	Ab.	CAS NO.	Molecular Formula	M.W. (g mol^{-1})
Norfloxacin	NOR	110871-86-8	$C_{19}H_{22}F_2N_4O_3$	392.40
Ciprofloxacin	CIP	85721-33-1	$C_{17}H_{18}FN_3O_3$	331.34
Pefloxacin mesylate dihydrate	PEF	70458-95-6	$C_{17}H_{20}FN_3O_3{\cdot}CH_4O_3S{\cdot}2H_2O$	465.49
Lomefloxacin hydrochloride	LOM	98079-52-8	$C_{17}H_{19}F_2N_3O_3{\cdot}HCl$	387.81
Enrofloxacin	ENRO	93106-60-6	$C_{19}H_{22}FN_3O_3$	359.39
Danofloxacin	DANO	112398-08-0	$C_{19}H_{20}FN_3O_3$	357.38
Sarafloxacin hydrochloride	SAR	91296-87-6	$C_{20}H_{17}F_2N_3O_3{\cdot}HCl$	421.83
Difloxacin hydrochloride	DIF	91296-86-5	$C_{21}H_{19}F_2N_3O_3{\cdot}HCl$,	435.85

Table 2 | Information about the studied sewage treatment plants (STPs) in shanghai

Location	Average daily flow ($*10^4 m^3.D^{-1}$)	Received sewage	Treatment process	Effluent discharge
A	5.67	Urban wastewater	A/A/O* or MBR†	Surface water
B	3.11	Urban wastewater	CAS‡	Surface water
C	34.43	Urban and industrial wastewater	UNITANK	Surface water
D	170	Urban and industrial wastewater	CBF§	Surface water
E	50	Urban and industrial wastewater	A/O	Surface water

*Anaerobic/anoxic/aerobic.
†Membrane biological reactor.
‡Conventional activated sludge.
§Chemical and biological flocculation.

Extraction procedure

FQs were extracted from wastewaters using MEP SPE disk cartridges preconditioned with 5 mL of methanol and 10 mL of water at pH 3. Samples (100 mL of primary and 500 mL of tertiary) of effluent at pH 3 were percolated through the disk cartridge at a flow of 1 mL/min using a vacuum manifold (Supelco; USA). After extraction, the disk cartridges were vacuum-dried for 30 min to remove the residual water in the cartridges. Compounds were then eluted using 6 mL of 2% formic acid in MeOH, and analyzed in a week.

Accuracy was determined by recovery studies on MEP disk cartridges by spiking wastewater samples. Six replicate analyses were performed for RSD test, which 20ng and 100 ng of FQs standard mixtures were spiked into primary and tertiary effluent samples (100 mL and 500 mL, respectively) for recovery test.

LC and FLD conditions

Separation was performed on a HITACHI liquid chromatograph (HPLC) equipped with a HITACHI L-2485 fluorescence detector (FLD) and Ezchrom Elite workstation. The FLD excitation wavelength was 278 nm and emission wavelength 445 nm. The LC column was Kromasil ODS C18 (250 mm × 4.6 mm, 5 μm). Eluent A was acetonitrile, and eluent B was a 10 mM tetrabutyl ammonium bromide (TBAB) solution (pH 3.0). Elution started with 4%A, followed by a 8 min isocratic elution, and a 8 min linear gradient to 15% B, followed by a 10 min isocratic elution, and a 5 min linear gradient to 25% B, then decreased to the initial conditions in 5 min, followed by an equilibration time of 6 min. Analyses were performed at a flow rate of 1 mL/min and sample inject volume was 20 μL.

RESULTS AND DISCUSSION

Chromatographic separations

Because the inherent fluorescence of the FQs enables very sensitive and specific detection, fluorescence detection (FLD) was selected as the main identification and quantification technique at an excitation wavelength and an emission wavelength of 278 nm and 445 nm, as reported by Golet *et al.* (2001).

Based on several reported cases of chromatographic separation of FQs using the C18 stationary phase, our work initial with a kormosil C18 column. In an effort to reduce tailing in the LC of FQs, the pH of the mobile phase was set to values below the pKas of the analytes by the addition of acid. Phosphoric acid, phosphate buffer, acetic acid, critic acid and formic acid have been used to protonate the amino groups of the FQs and the residual silanol groups of the stationary phase, so that their interaction and thus peak asymmetry could be reduced. Though mobile phases containing phosphoric acid and phosphate buffers could acquire good result, they were avoided as they would cause jam when mixed with organic solvent such as acetonitrile or methanol. Ion-pair were added to the mobile phase to restrain the disassembly of the FQs molecule. tetrabutyl ammonium bromide (TBAB) used as an ion-pair can change the reservation factor of the FQs to the C18 stationary phase, which result in the good separations of FQs.

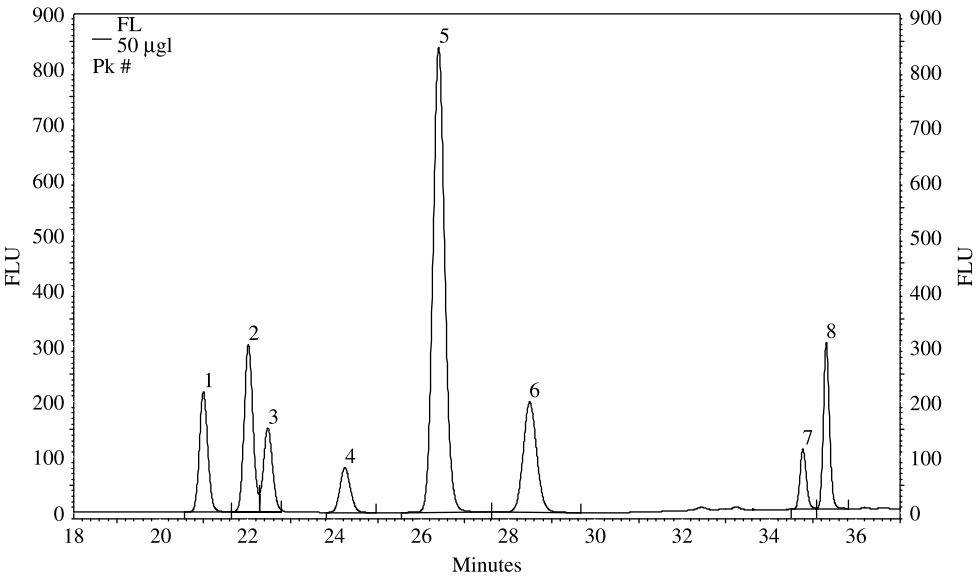

Figure 1 | Liquid chromatogram of standards mixture of 8 FQs (mobile phase:A acetonitrile; B 10 mmol/L tetrabutyl ammonium bromide 1. NOR; 2. PEF; 3. CIP; 4. LOM; 5. DANO; 6. ENRO; 7. SAR; 8. DIF).

Different mass concentrations of the TBAB solvent were studied. Good result were achieved when the mass concentrations of the TBAB were above 10 mmol/L, and the pH value of the mobile phase should be adjusted to 3.0.Under the conditions that we have tested with the C18 column, good resolution for the eight FQs could be achieved with a mixture of water and acetonitrile in the presence of TBAB (Figure 1).

Standard curve, lineral range and quantification and quality control

Working standards of 0.5 to 250 µg/L were sequence injected; calibration curves were prepared by plotting the peak area versus the analyte concentration. Good linearity was observed over 1–2 orders of magnitude with correlation factors of $R^2 > 0.99$ (Table 3). Because of the large range in linearity, the response factor method was preferred over the least-squares method for determining linearity. Using the response factor approach, the ratios of detector response (sensitivity) of each FQs versus analyte concentration were calculated. Limits of detection (LOD) and limits of quantification (LOQ) for FQs were calculated first on the basis of the standard deviation ($n = 6$) of the analysis of a FQs standard mixture of 50 ng. LOD and LOQ were defined as 3 and 10 times the standard deviation of the FQs measurements, respectively. As a result of this, we defined

Table 3 | Method validation parameters

Compounds	Linearity range (µgl^{-1})	Calibration equation	R^2	LOD (µgl^{-1})	LOQ (µgl^{-1})	RSD (%), $n = 6$
NOR	0.5–250	$C = 4.33 \times A \times 10^{-7} - 0.568$	0.9999	0.35	1.15	3.20
PEF	0.5–250	$C = 2.90 \times A \times 10^{-7} + 0.8488$	0.9980	1.06	3.54	4.28
CIP	1.0–250	$C = 5.58 \times A \times 10^{-7} - 0.3584$	0.9999	0.11	0.35	2.76
LOM	2.5–250	$C = 9.09 \times A \times 10^{-7} + 0.9832$	0.9981	0.15	0.49	2.05
DANO	0.25–100	$C = 7.70 \times A \times 10^{-8} + 0.7058$	0.9977	0.16	0.54	4.12
ENRO	1.0–250	$C = 2.81 \times A \times 10^{-7} + 0.8389$	0.9979	0.19	0.62	4.43
SAR	1.0–250	$C = 1.12 \times A \times 10^{-6} + 1.0294$	0.9990	0.88	2.92	4.80
DIF	2.5–250	$C = 4.22 \times A \times 10^{-7} + 1.1116$	0.9981	0.29	0.96	3.55

A: Peak area; C: Concentration of compound.

the LOD and LOQ to be 0.11 to 1.06 μg/L and 0.35 to 3.54 μg/L. The overall precision of the method is indicated by a relative standard deviation (RSD, $n = 6$) generally below 5%.

Method performance of SPE

Solid-phase extraction using C18 SPE cartridge has often been used for the enrichment and cleanup of FQs from biological samples. However, for extracting FQs from wastewater samples, C18 SPE cartridges were not shown to present good recovery efficiencies. A variety of other extraction materials were tested: (1) apolar sorbent including LC-18 (supleco, 60 mg, 3cc) and ENVI-18 (supleco, 60 mg, 3cc), (2) polymeric sorbents such as MEP (Anpel^{TM}, 60 mg, 3cc), ENVI Chromp (supleco, 200 mg, 3cc) and Oasis HLB (Waters, 30 mg, 3cc), (3) ion changer sobents including Oasis max (Waters, 30 mg, 3cc) and Oasis mcx (Waters, 30 mg, 3cc). The recoveries test were carried out under two extraction systems of pH 3 and 10 (Figure 2). Under extraction system of pH3, polymeric sorbents such as MEP, Chromp, HLB and MEP, present good recovery efficiencies, while under extraction system of pH10, only MCX material presents good recovery efficiencies exceeding

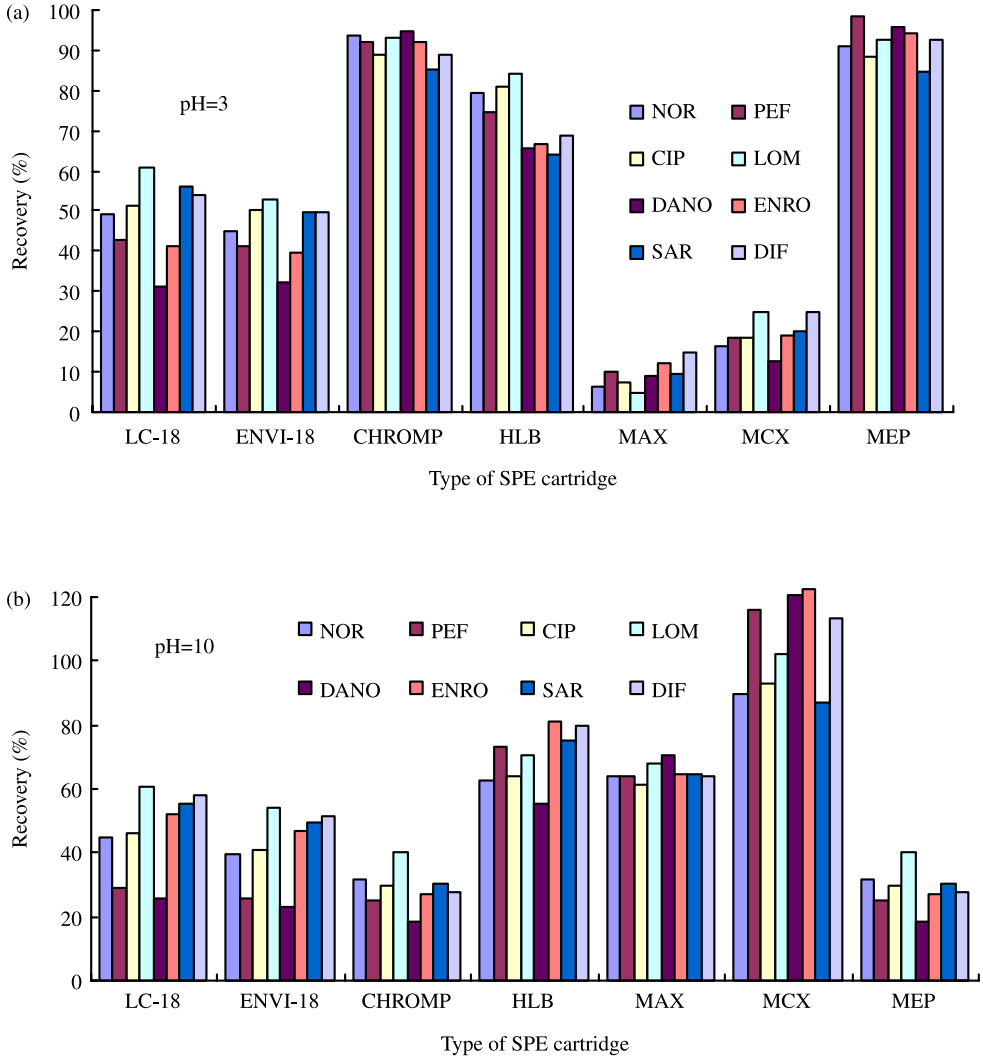

Figure 2 | Recoveries of different type of SPE cartridges. (a) pH of samples were adjusted to 3 by phosphoric acid; (b) pH of samples were adjusted to 10 by 1 M sodium hydroxide.

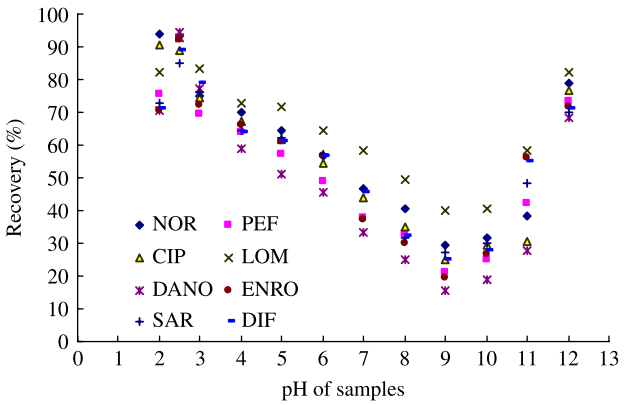

Figure 3 | Influences of pH in the recoveries of FQs in SPE with 100 ng mixture of standard spiked in 100 ml deioned water.

80%. C18 SPE cartridge offered recovery efficiencies lower than 60% at either pH environment. In comparison to C18 materials, the MEP disk cartridge material is silica-based sorbent consisting of divinylbenzene and vinylpyrrolidone copolymer, and the MCX disk cartridge is consisting of vinylpyrrolidone and a strong cation exchanger (benzenesulfonate). The specific structure of MEP and MCX result in a good combination of the group of FQs by either adsorption or ion-changer. Take economic factor into consideration, MEP disk cartridge was chosen to be the SPE material studied.

Other factors, such as the pH system of the SPE, elution solvent, were also studied. The recovery efficiencies first decrease fast with the increasing of pH from 3 to 9, then increased with increasing of pH. Under the extraction system of pH 2–3, high and stability recovery efficiencies of 8 FQs were acquired (Figure 3). At the working pH of 2–3, the cationic form of FQs is predominant because p*Ka* values for the carboxylic group are between 5.9 and 6.3 and for the amino groups on the piperazine moiety, between 7.9 and 10.2. The decomposing of FQs molecules were restrained similar to that in chromatography separations.

Several solvent mixtures were tested for the elution of FQs from MEP disk cartridges. Aqueous solutions present better recovery efficiencies than organic solvents. Due to difficult evaporation of aqueous solution, a variety of organic solutions containing either ammonia or fomic acid or acetic acid at different concentration levels (1% and 5%) were evaluated. Finally, a 6 mL 2% formic acid in MeOH was selected as the best extraction eluent.

Recoveries of FQs of 100 mL spiked influent samples and 500 mL tertiary effluent samples (Table 4) ranged from 79 to 109% and from 80 to 105%, respectively.

Analysis of water samples

The described method was successfully applied to determine the FOs concentration level in different urban wastewaters around Shanghai, China. Figure 4 shows a typical LC–FLD chromatogram obtained from an extracted influent sample. 7 FQs, including NOR, CIP, LOM, PEF, DANO, ENRO and DIF, were detected in STPs, while SAR didn't occur in any of the STPs. Commonly consumed FQs, namely CIP and NOR and LOM, could be identified in primary and tertiary effluents by means of their fluorescence spectra analysis. Determination of FQs in extracts of primary and tertiary effluents showed trace level of human-use FQs of NOR, LOM and CIP, with concentrations

Table 4 | Recoveris of FQs obtained from different water matrices of fortified mixed standards

Compounds	100 ml deioned water spiked 20 ng (%)	100 ml deioned water spiked 100 ng (%)	100 ml influent spiked 100 ng (%)	500 ml effluent spiked 50 ng (%)
NOR	91.2	97.1	100.2	105.5
PEF	98.7	102.0	97.7	87.6
CIP	88.6	95.1	93.2	100.3
LOM	92.5	96.0	109.2	84.0
DANO	96.0	102.6	97.9	90.2
ENRO	94.4	101.5	97.4	96.5
SAR	84.6	89.4	79.2	96.2
DIF	92.7	97.3	85.9	80.0

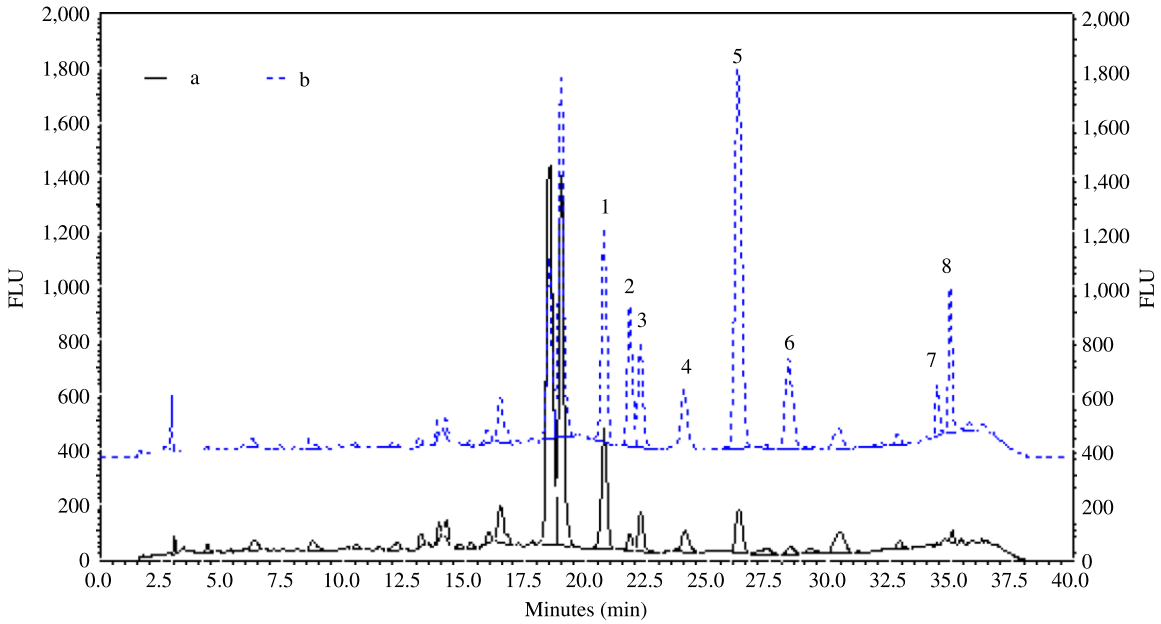

Figure 4 | Chromatograms obtained for water samples. (a) 100 ml influent of STP-B; (b) 100 ml influent of STP-B with 100 ng mixture standard spiked.

between 56 and 1163 ng/L, 24 and 735 ng/L and 14 and 444 ng/L, respectively (Table 5). These results are consistent with the preference of FQs in USA (Nakata *et al.* 2005) (NOR < 45 ng/L, LOM: < 41 ng/L and CIP: < 19 ng/L), Switzerland R. (Andreozzi *et al.* 2003) (NOR:26–553 ng/L, LOM: < 5.0 ng/L and CIP: 62–568 ng/L), France (NOR: 50–70 ng/L, LOM:180–290 ng/L and CIP:60 ng/L), Sweden (NOR:30 ng/L, LOM:130 ng/L and CIP:30 ng/L), and Italia (NOR:60–70 ng/L, LOM:180–320 ng/L and CIP:40–20 ng/L) (Andreu *et al.* 2007). Some of the veterinarian-use FQs, such as ENRO, DANO, and DIF, were also detected in urban wastewaters. Elimination rates during wastewater treatment based on measured FQs concentrations in influent and effluents varied between 70 and 100%. These findings clearly show that the load of FQs in wastewaters is reduced considerably during wastewater treatment, but complete removal is not achieved. Due to highly adsorption of FQs to the sludge, residual amounts of the FQs are emitted into ambient waters.

Table 5 | Occurence of fluoroquinolones in the STPs in shanghai, china

	A ($\mu g l^{-1}$)			B ($\mu g l^{-1}$)		C ($\mu g l^{-1}$)		D ($\mu g l^{-1}$)		E ($\mu g l^{-1}$)	
STPs	Influent	Effuent1	Effluent2	Influent	Effluent	Influent	Effluent	Influent	Effuent1	Influent	Effluent
NOR	0.663	0.256	0.265	1.163	0.216	0.232	0.121	0.279	0.097	0.133	0.056
PEF	0.055	0.005	0.010	0.093	0.004	0.009	0.008	0.021	< LOD	0.008	0.003
CIP	0.155	0.094	0.084	0.444	0.064	0.042	0.018	0.070	0.014	0.032	0.020
LOM	0.735	0.291	0.207	0.712	0.405	0.262	0.032	0.271	0.036	0.305	0.024
DANO	0.025	0.004	0.012	0.095	0.003	0.011	0.003	0.012	0.002	0.007	0.003
ENRO	ND	ND	ND	0.069	< LOD	0.020	< LOD	ND	ND	0.007	0.007
SAR	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DIF	0.032	< LOD	ND	0.082	< LOD	0.016	0.006	0.052	0.018	0.024	0.005

ND: not detected; < LOD: below limit of detection.

CONCLUSIONS

Solid-phase extraction followed by reversed-phase chromatography with fluorescence detection proved to be a new, specific, and quantitative method for the determination of trace amounts of a wide variety of FQs antibacterial agents in wastewater effluents. Enrichment using the MEP disk cartridges, as compared to a wide range of other materials, was shown to be the most appropriate extraction procedure for FQs in wastewaters, and high and stability recovery efficiencies of 8 FQs were acquired at the working pH of 2–3.

Out of the 8 investigated FQs compounds, the ciprofloxacin (CIP) and norfloxacin (NOR) could be determined quantitatively in urban wastewater treatment plant effluents. Both of these compounds are derived from human-use medication. Some of the investigated veterinarian-use FQs, such as ENRO, DANO, and DIF, were detected in urban wastewater. The overall removal of FQs from the aqueous phase during wastewater treatment was found to be efficient, though incomplete, thus allowing trace amounts to be emitted into the receiving waters.

The present method is easily applicable, because sample enrichment is simple and fast, the separation is reliable, and detection is highly sensitive. The analytical method described can serve as an ideal tool to obtain detailed information on the occurrence, behavior, and fate of FQs antibacterial agents in the aquatic environment.

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