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Determination of ofloxacin, norfloxacin, and ciprofloxacin in sewage by selective solid-phase extraction, liquid chromatography with fluorescence detection, and liquid chromatography–tandem mass spectrometry

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Abstract

A solid-phase extraction (SPE) and liquid chromatographic (LC) method was developed for the determination of selected fluoroquinolone (FQ) drugs including ofloxacin, norfloxacin, and ciprofloxacin in municipal wastewater samples. Extraction of the FQs was carried out with a weak cation exchanger SPE cartridge, the Oasis WCX. The cartridge was washed with water and methanol as a cleanup before the FQs were eluted by a mixture of methanol, acetonitrile, and formic acid. Separation of the FQs was achieved by using a Zorbax SB-C$_8$ column under isocratic condition at a flow rate of 0.2 mL/min. Recoveries of the FQs in spiked final effluent samples were between 87 and 94% with a relative standard deviation of less than 6%. Several techniques have been evaluated for the detection of FQs in sewage extracts; they included fluorescence detection and electrospray ionization (ESI) mass spectrometry using either mass-selective detection or tandem mass spectrometry (MS/MS). When they were applied to sewage influent and effluent samples, the LC–MS/MS technique operating in the multiple reaction monitoring (MRM) mode proved to be best suited for the determination of FQs in sewage samples as it provided the highest sensitivity (limit of quantification 5 ng/L) and selectivity. The observation of signal suppression (matrix effect) for some FQs in ESI LC–MS and LC–MS/MS is discussed and a solution is proposed. The three FQs were detected in all the sewage samples tested in this work, with median concentrations between 34 and 251 ng/L.
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1. Introduction

Fluoroquinolones (FQs) are a class of relatively new and entirely man-made, non-steroidal antibiotics/antibacterials. They are used to treat infection in many parts of the body by killing the harmful bacteria or preventing their growth. Norfloxacin (Fig. 1), the first FQ, was synthesized by converting nalidixic acid into a quinolone structure, adding a fluorine atom and a piperazine ring [1]. Other first generation FQs introduced in the 1980s included ciprofloxacin and ofloxacin (Fig. 1). Ofloxacin is a racemic mixture and its active form is the (−) S isomer, levofloxacin. FQs are pharmacologically superior to

nalidixic acid because they exhibit broader activity against Gram (−) and Gram (+) bacteria, less protein binding, higher drug tolerance, lower toxicity, and longer half-life [1]. In Europe and other parts of the world, these three FQs are still heavily used in many human and animal applications. Ciprofloxacin, in its hydrochloride form, is perhaps the most popular FQ and it is one of the top 20 prescription drugs in Canada [2]. This drug has also received considerable attention lately in the USA where it has been approved as one type of antibiotic for the treatment of the inhaled form of anthrax [3].

Due to the extensive use of the FQs in urban centers and the fact that they are largely excreted unchanged [4], significant quantities of these antibacterials can be found in municipal wastewaters as well as in surface waters of the surrounding areas. For instance, ciprofloxacin was detected in hospital
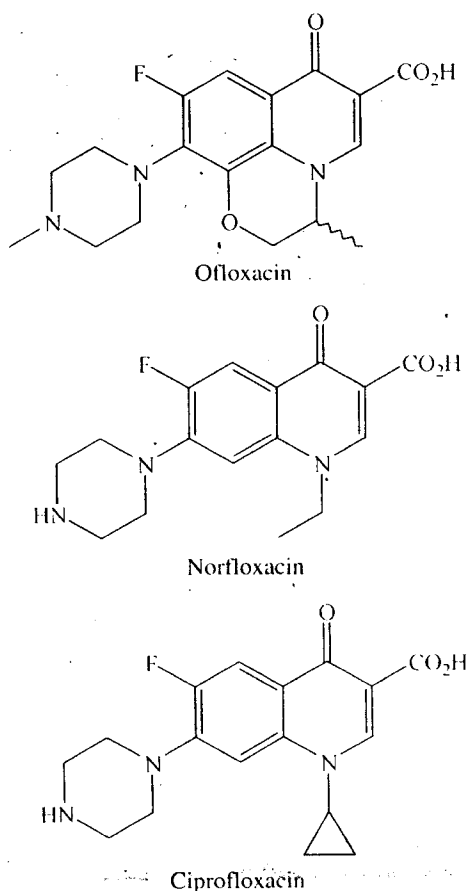
Fig. 1. Molecular structures of ofloxacin, norfloxacin, and ciprofloxacin.

wastewater samples with concentrations varying from 3 to 87 μg/L [4]. Norfloxacin and ciprofloxacin occurred in primary and tertiary treated sewage effluents at levels from 27 to 489 ng/L [5–7]. Ofloxacin was present in sewage effluent at concentrations from 150 to 1081 ng/L [7,8]. Ciprofloxacin, among other therapeutic drugs, has been detected at low ng/L levels in river waters [9]. While the log K_{ow} for norfloxacin and ciprofloxacin were <1, these two FQs have been found in raw and digested sludge samples at low mg/kg levels [6,10]. When stored at ambient temperature, ciprofloxacin was stable for at least 2 weeks in spiked river water [11]. However, little is known about the fate of FQs in the environment except that less than 1% of sarafloxacin, a FQ approved for the prevention of poultry diseases, was eliminated from soils within 80 days [12].

As a first step in the studies of occurrence, fate, effects, and risk assessment of FQs, analytical methods were developed for the determination of these compounds in sewage and water samples. A variety of adsorbents including the C_{18} (ODS) phase, the hydrophilic–lipophilic balance (HLB) phase, and the mixed-phase cation exchange phases (MPC and MCX) have been successfully applied to the solid-phase extraction (SPE) of FQs in water samples [5,9,13–15]. Since the cation-exchange phases exhibit more selectivity for compounds with a basic NH group than the reversed-phase adsorbents, they have been exploited to render cleaner extracts of FQs for the more contaminated wastewater samples. Owing to their better sensitivity and

specificity, procedures of liquid chromatography coupled with mass spectrometry (LC–MS) [13–15] or tandem mass spectrometry (LC–MS/MS) [5,7,9,16] procedures with the electrospray ionization technique have been developed for the confirmation and quantitation of FQs. However, workers who have opted for the simple and more available fluorescence detector have also obtained good results [5,15]. In this work, we report a selective SPE procedure using a weak cation-exchanger, the Oasis WCX cartridge. We have also compared the quantitation of FQs in sewage extracts by fluorescence detection, by LC–MS, and LC–MS/MS techniques.

2. Experimental

2.1. Chemicals and reagents

Ciprofloxacin was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Norfloxacin, ofloxacin, formic acid, trifluoroacetic acid (TFA), and hydrochloric acid were products of Sigma–Aldrich (Oakville, Canada). Stock solution of ofloxacin, at 500 μg/mL, was prepared in 20:80 (v/v) methanol/acetonitrile in the presence of 0.2% concentrated HCl. Individual stock solutions of norfloxacin and ciprofloxacin, at 400 μg/mL, were prepared in 1:1 (v/v) methanol/water in the presence of 0.2% concentrated HCl. Mixtures of the three FQs with concentrations from 1 to 20 μg/mL were made up in acetonitrile for spiking purposes. Instrument calibration standards were prepared, by dilution of the above mixtures, for each batch of samples. A labeled ciprofloxacin HCl (2,3-[$^{13}C_3$]carboxyl, 99%, [^{15}N]quinoline, 98%, catalog no. CNLM-7539-S), available from Cambridge Isotope Laboratories at 100 μg/mL in methanol, was used as a surrogate standard for recovery studies.

All solvents (methanol and acetonitrile) were HPLC grade supplied by Caledon Laboratories or Burdick and Jackson. Water was purified by a Milli-Q system (Millipore) and was used to prepare the HPLC mobile phase.

2.2. Sample collection

Composite primary and final sewage effluent samples, over a 24-h period, were collected by the staffs of local sewage treatment plants. The samples, in amber glass bottles, were shipped immediately to our laboratory and were kept at 4 °C in the dark until extraction.

2.3. Solid-phase extraction

Immediately prior to extraction, a sewage sample was filtered through a 90 mm GF/C filter (1.2 μm retention) with a fritted, all-glass filtration device under a slight vacuum. Celite was added as a filter aid to prevent clogging. To each 250 mL of the filtered sample, 100 μL of the ciprofloxacin-$^{13}C_3{}^{15}N$ solution at 1 μg/mL in methanol was spiked as a recovery standard. The sample was acidified to ca. pH 3 with sufficient 1 N HCl. Meanwhile, for each sample, a 6 mL, 150 mg, 30 μm Oasis WCX SPE cartridge (Waters, Milford, MA, USA; part 186002498) was conditioned by washing with 4 mL of methanol followed by 10 mL of water at pH 3. With a solid-phase extraction man-

ifold (Supelco Visiprep 57044 or equivalent), the sample was siphoned through the cartridge at a flow rate between 10 and 15 mL/min by adjusting the vacuum. At the end of the extraction, the cartridge was washed with 100 mL of pH 3 water, then washed with 5 mL of methanol, and the washings were discarded. This was followed by an elution with 10 mL of 20/75/5 (v/v) methanol/acetonitrile/formic acid. This fraction was collected and evaporated to just dryness with a gentle stream of nitrogen, in a water bath maintained at 40 °C. The sample extract was made up to 1 mL with the LC mobile phase (see below) in the presence of an additional 10 μL of formic acid. It was then filtered through a 4 mm nylon syringe filter (0.45 μm) for LC and LC–MS/MS analyses.

2.4. Liquid chromatography with fluorescence detection (LC–FLD)

LC analysis was performed by using an Agilent 1100 series LC system consisting of a solvent degasser (G1322A), a quaternary pump (G1311A), an automatic liquid sampler (G1313A), a thermostatted column compartment (G1316A), and a fluorescence detection (FLD) system (G1321A). Separation of the three FQs was achieved by a Zorbax SB-C_8 column (2.1 mm × 150 mm, 3.5 μm, part no.830990-906) at 35 °C under isocratic condition at a flow rate of 0.2 mL/min. A Zorbax SB-C_8 guard column (2.1 × 12.5 mm, 5 μm) was also in use to protect the analytical column. The mobile phase was a mixture of acetonitrile/methanol/formic acid/water 6/12/0.5/81.5 (v/v). The detector was operated at 278 nm excitation and 445 nm emission.

2.5. Liquid chromatography/mass spectrometry (LC/MS)

LC/MS data of sewage extracts were acquired by a mass-selective detector (Agilent 1100 LC/MSD SL) equipped with an electrospray ionization (ESI) interface. Instrument control, data acquisition, and data editing were managed by the MSD ChemStation Software Rev. B.010.3 under the Windows 2000 environment. The liquid chromatographic system and conditions were identical to those described above for fluorescence detection. After autotuning, the detector was operating in the ESI+ mode using nitrogen as a drying gas at a flow rate of 12 L/min and a temperature of 350 °C. The nebulizer pressure was 30 psi. Operating parameters such as capillary voltage and fragmentor

voltage were further optimized by injecting each individual FQ at 1 μg/mL in the flow injection analysis (FIA) mode. In this work, a single capillary voltage of 4000 V and two fragmentor voltages (120 V for MH$^+$ and 200 V for the confirmation ions) were selected. For each compound in the samples, the MH$^+$ ion and a fragment ion were used in the selected ion monitoring (SIM) mode for the quantitation and confirmation, respectively, as shown in Table 1.

2.6. Liquid chromatography/tandem mass spectrometry (LC–MS/MS)

Samples were also analyzed by a Micromass Quattro Ultima triple quadrupole mass spectrometer (Manchester, UK) equipped with an ESI interface operated in the positive mode. The system consisted of a Waters quaternary pump and a liquid sampler as well. Again, the same column and mobile phase as described above were used. Instrument control and data acquisition were carried out by the MassLynx 4.1 software operated in the Windows XP environment. Nitrogen (>99% purity), generated by an on-line system, was used as the cone and desolvation gas with typical flow rates of 50 and 500 L/h, respectively. Full scan spectra of the FQs were obtained in ESI+ mode by infusing each individual standard at 1 μg/mL using a Harvard syringe pump at a flow rate of 10 μL/min. The capillary voltage was held at 3.3 kV and the cone voltage (Table 2) was adjusted to maximize the response of the precursor ion (MH$^+$) for each compound. In MS/MS mode, the collision gas (argon at 2×10^{-3} mbar) was then turned on and the collision energy was optimized for the maximum production of product ions in the middle quadrupole. Source and desolvation temperatures of 120 and 350 °C, respectively, were used.

Pharmaceuticals in the sample extracts were detected, confirmed, and quantified by MS/MS in multiple-reaction monitoring (MRM) mode. The following transitions (precursor > product) were used for quantitation and confirmation, respectively. Ofloxacin: 362 > 318 and 362 > 261. Norfloxacin: 320 > 276 and 320 > 233. Ciprofloxacin: 332 > 245 and 332 > 288. Labeled ciprofloxacin: 336 > 235 and 336 > 253 (Table 2).

2.7. Quantification

Quantification of FQs using LC–FLD, LC–MS, and LC–MS/MS was carried out by an external standard method. A

Table 1

Major ions (m/z) and relative intensities (%) of selected FQs as observed by the MSD operating in the ESI+ mode

FQ	m/z			
	[MH]$^+$	[MH-H$_2$O]$^+$	[MH-CO$_2$]$^+$	Others
Ofloxacin	362 (100)	344 (25)	318[a] (65)	261 (25) [MH-CO$_2$-C$_3$H$_7$N]$^+$
Norfloxacin	320 (100)	302 (100)	276 (30)	233 (15) [MH-CO$_2$-C$_2$H$_5$N]$^+$
Ciprofloxacin	332 (100)	314 (85)	288 (45)	245 (20) [MH-CO$_2$-C$_2$H$_5$N]$^+$
Ciprofloxacin-^{13}C$_3$^{15}N·HCl	336 (100)	318 (100)	291[b] (30)	235 (25) [MH-C$_3$H$_4$-C$_2$H$_5$N-H$_2$O]$^+$

Fragmentor voltages were 120 V for MH$^+$ and 200 V for the other ions.

[a] The underlined ions were used for confirmation of the FQs.

[b] Loss of ^{13}CO$_2$ (45 u).

Table 2
MRM transitions, cone voltages, and collision energies used in the LC–MS/MS analyses of fluoroquinolones, with approximate retention times

Compound	Retention time (min)	MRM transitions	Cone voltage (V)	Collision energy (eV)
Ofloxacin	12.7	362 > 318 quantitation 362 > 261 confirmation	45.0	18.0
Norfloxacin	13.6	320 > 276 quantitation 320 > 233 confirmation	60.0	15.0
Ciprofloxacin	16.5	332 > 245 quantitation 332 > 288 confirmation	35.0	21.0
Ciprofloxacin-$^{13}C_3^{15}N\cdot HCl$	16.4	336 > 235 quantitation 336 > 253 confirmation	42.0	42.0

three-point calibration curve was generated for each compound at the concentrations of 10, 50, and 100 pg/μL. Sample extracts with FQ concentrations higher than 100 pg/μL were diluted and analyzed again.

3. Results and discussion

3.1. Liquid chromatographic separation and fluorescence detection of FQs

Based on several reported cases of chromatographic separation of FQs using the C_{18} stationary phase [5,13,17], our initial work with a fluorescence detector was done on a Zorbax SB C_{18} column. In an effort to reduce tailing in the LC of FQs, the pH of the mobile phase was lowered to values below the pK_as of the analytes by the addition of an acid. Phosphoric acid, TFA, acetic 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. As we shall use the same chromatographic procedure for further LC–MS/MS studies, mobile phases containing phosphoric acid and phosphate buffers were avoided. Under the conditions that we have tested with the C_{18} column, good resolution for the three FQs could only be achieved with a mixture of water and acetonitrile in the presence of TFA; substituting TFA with formic acid yielded unresolved and tailing peaks, especially for sewage extracts. However, since TFA has been shown to exhibit ion suppression in LC–MS work [18], especially with electrospray ionization, its use in this work was also avoided. Replacing the C_{18} column with a C_8 column produced much better selectivity for the FQs, particularly in the presence of methanol [19]. Indeed, all three compounds were baseline resolved under isocratic conditions at 35 °C using a mixture of acetonitrile, methanol, formic acid, and water (see Section 2.4). This solvent mixture, however, had to be replaced every 2 weeks as formic acid was slowly depleted by the reaction with methanol to form methyl formate, leading to longer retention times and broader peaks for the FQs. After the assay of each batch of sewage samples, the analytical column and the guard column were detached and flushed separately with, respectively, 10 and 5 mL of acetonitrile. This will remove the less polar coextractives that may still be adsorbed on the column and prolong the optimal column performance. The

effect of column temperature on the separation of FQs was not studied.

FLD was very sensitive for the detection of the norfloxacin and ciprofloxacin at 278 nm excitation and 445 nm emission as reported by Golet et al. [5]. Instrument detection limits for these two compounds were estimated to be 5 pg on column for an S/N ratio of 10. Ofloxacin, on the other hand, has a sensitivity ca. 10-fold lower. Detector response was linear for ofloxacin with concentrations from 10 to 200 pg/μL, and for norfloxacin and ciprofloxacin with concentrations from 1 to 50 pg/μL ($r^2 > 0.999$). The co-elution of ciprofloxacin and the labeled ciprofloxacin precluded the use of the latter as a surrogate standard in the FLD work.

3.2. Selective solid-phase extraction

SPE procedures using the ODS reversed phase and the Oasis HLB cartridges have been successfully applied to the quantitative extraction of FQs in water samples. In these cases, FQs and most of the other extracted organics are eluted from the solid phase by methanol. For sewage samples, the coextractives may cause severe interference when a less selective detection method such as FLD is used. It may also produce erratic quantitative results in ESI LC–MS/MS analysis due to the "matrix effect" (Section 3.5).

The use of mixed-mode adsorbents (reversed-phase and strong cation-exchange) such as the Oasis MCX cartridge and the Varian MPC disks is supposed to produce better method specificity than the ODS and HLB phases, due to the different mechanisms in the solute–sorbent interactions. For example, after the extraction of a sewage sample with the MCX cartridge, the neutral and acidic compounds in the sample can be removed by an elution with methanol as a cleanup step. Those components with a basic NH group such as the FQs are still bound to the cationic stationary phase; they can be subsequently removed by an elution with methanol in the presence of a base. To our knowledge, none of the procedures in the literature have fully exploited this capability of the mixed-mode adsorbents, as they all opted for simplicity in a single elution step.

In our preliminary work with the MCX cartridges, we were unable to obtain >60% recovery for the FQs from spiked distilled water or final effluent samples with the Oasis MCX cartridge, even if 20% ammonium hydroxide in methanol was used in

the elution step. Low recoveries of FQs in our case were not attributable to losses in the methanol cleanup step, as no FQs were found in that fraction. While the addition of ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) to fortified distilled water sample prior to extraction improved the recoveries of FQs, it did not help in the cases of sewage samples. As other workers have also encountered even lower recoveries (32%) for ciprofloxacin and ofloxacin with the MCX cartridge [9], further work on this adsorbent was abandoned. Using the Oasis WCX cartridge (a weak cation exchanger with a mixed-mode sorbent), on the other hand, we were able to obtain recoveries of >80% (Section 3.4) for all three FQs from spiked effluent samples in the absence of the chelating agent. Our extraction method is unique not only because of the successful application of a new adsorbent but also due to the inclusion of the methanol cleanup step which selectively removed the acidic and neutral interferences in sewage samples, thereby producing a cleaner extract that displayed much less signal suppression in the LC–MS/MS qunatitation step (Section 3.5).

3.3. ESI+ LC–MS and LC–MS/MS detection and confirmation

At acidic pHs, the parent ions observed for the FQs in ESI+ mode are invariably the protonated molecular ions $[MH]^+$. With the MSD, the compound dependent parameters were optimized by varying the capillary voltage (from 2000 to 4000 V in steps of 250 V) and the fragmentor voltage (from 80 to 200 V in steps of 20 V and from 200 to 300 V in steps of 25 V) using the flow injection analysis (FIA). While maximum responses of the MH^+ ions were observed with a fragmentor voltage of 120 V, few characteristic fragment ions of the FQs in the m/z range from 200 to 300 u were formed at this voltage. Fragmentation started to occur when the fragmentor voltage was increased to 160 V and a voltage of 200 V was considered optimal for the production of the fragment ions for confirmation of the FQs [20]. Fragmentor voltages in excess of 250 V produced a large number of product ions at lower m/z which were not characteristic of the parent compounds. In contrast, at a fragmentor voltage of 120 V, the responses of the MH^+ ions for the three FQs increased with increasing capillary voltage until the maximum value of 4000 V was reached and this value was used for all compounds in subsequent work.

The major fragment ions of each FQ resulted from the loss of H_2O (18 u), CO_2 (44 u), C_2H_5N or the partial piperazine ring (43 u), C_3H_4 or the cyclopropyl ring (40 u, for the labeled ciprofloxacin only), or a combination of the above, from its parent ion (Table 1). For ofloxacin, the loss of C_3H_7N or the partial piperazine ring with a methyl group (57 u) was also observed. These assignments are consistent with the chemical structures of the FQs (Fig. 1) and they agreed with the spectra of FQs reported by Ballesteros et al. [21] where characteristic fragment ions such as m/z 314, 288, 245 as well as m/z 302, 276, 233 were observed for ciprofloxacin and norfloxacin, respectively. Based on the response of MH^+, the MSD is linear for the FQs in the concentration range from 5 to 100 pg/μL with $r^2 > 0.9993$. Instrument detection limits (for a S/N of 10) were estimated to be 15 pg for norfloxacin and ciprofloxacin and 10 pg for ofloxacin.

With tandem MS/MS under collision-induced dissociation conditions, the operating conditions were optimized for the maximum yield of the principal product ions, which resulted from the loss of CO_2, C_2H_5N, C_3H_7N (for ofloxacin only), C_3H_4 (for the labeled ciprofloxacin only), or a combination of the above, from its precursor ion MH^+. Such fragmentation patterns were again consistent with those reported by Golet et al. [5] for ciprofloxacin and other FQs. Another common product ion observed for the FQs was derived from the loss of H_2O from the precursor. Since the loss of water is a common process under LC–MS conditions, the transition $MH^+ > [MH–H_2O]^+$ was not used for either quantitative or confirmative purposes. The MRM transitions used for the quantitation and confirmation of each FQ are given in Table 2. Linear detector response was obtained from standard solutions from 5 to 100 pg/μL. The instrument detection limits for the FQs were ca. 1 pg for ofloxacin and 5 pg for norfloxacin and ciprofloxacin.

3.4. Method performance

Recoveries of the FQs in a bulk sewage effluent sample at two spiking levels are shown in Table 3. The mean recoveries are in the range of 87–94% with coefficients of variation between 2 and 6. The recoveries were blank subtracted for norfloxacin and ciprofloxacin at the lower spiking level. For reasons unknown to us, the addition of Na_2-EDTA to spiked sewage samples prior to extraction resulted in a drop of recoveries to <40%. In contrast, for spiked distilled water samples, the recoveries of FQs were >85 and <50% with and without added Na_2-EDTA, respectively. All sewage samples in this work were then extracted in the absence of the chelating agent. Using the LC–MS/MS procedure, the limit of quantification (LOQ) for the FQs is estimated to be 2 ng/L for ofloxacin and 10 ng/L for norfloxacin and ciprofloxacin, with a sample volume of 250 mL.

3.5. Application to sewage samples and comparison of detection methods

A number of 24-h composite sewage samples (primary and final effluents) have been collected from nearby sewage treatment plants in southern Ontario and processed by the described method. The fluorescence detector has been used to analyze those extracts where the labeled ciprofloxacin was not added

Table 3
Mean percent recoveries and coefficient of variation (C.V.), in parentheses, of fluoroquinolones in spiked final effluent samples

Fluoroquinolone	Spiking level (ng/L)	%Recovery (C.V.)
Ofloxacin	1000	94 (4)
	100	87 (6)
Norfloxacin	500	92 (2)
	50	93 (5)[a]
Ciprofloxacin	500	89 (2)
	50	87 (3)[a]

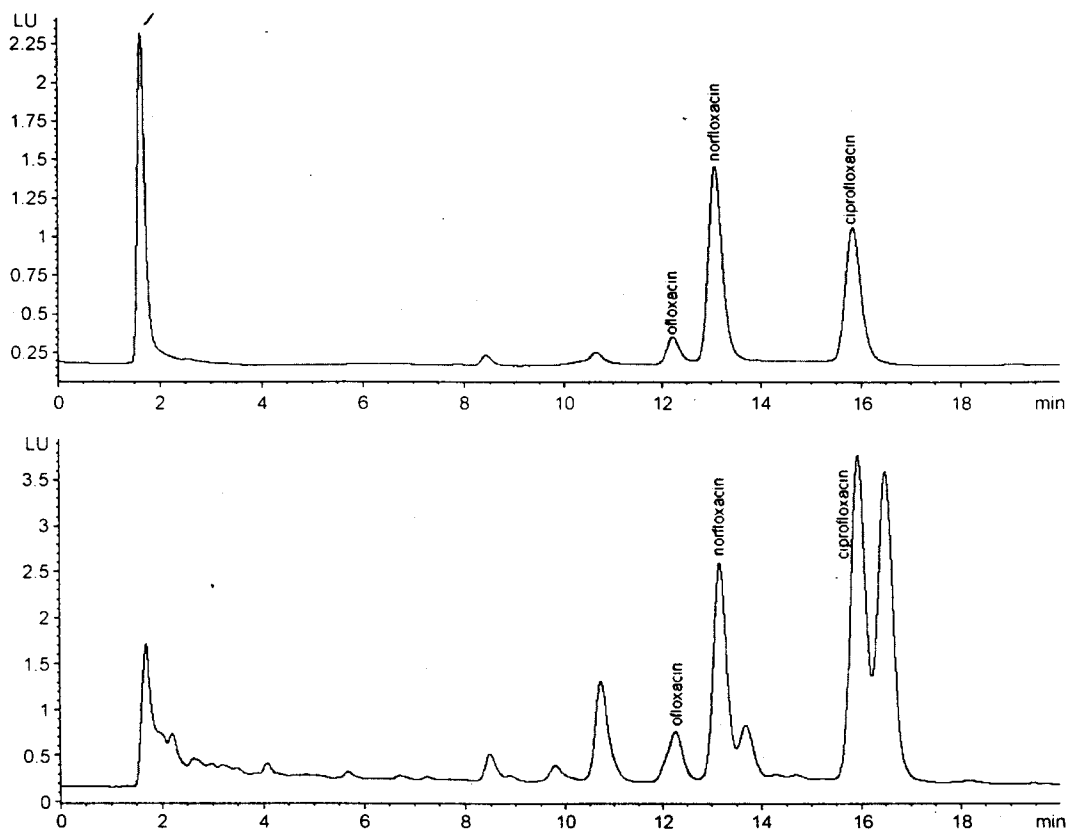Replicate of six determinations.

[a] Blank subtracted.

Fig. 2. LC-fluorescence chromatograms of a standard (upper trace) and an extract of a primary effluent (lower trace) showing the presence of the FQs. The retention times of ofloxacin, norfloxacin, and ciprofloxacin are ca. 12.2, 13.1, and 15.8 min, respectively.

as a surrogate. An example is shown in Fig. 2. Due to the lack of specificity of this detector, useful quantitative results could only be obtained in about one-third of the samples due to the presence of interference in the extract. The fluorescence detector was, as mentioned earlier, much less sensitive for the detection of ofloxacin, thus making it difficult to obtain accurate results of this FQ in some extracts. This detection technique also precluded the use of the labeled ciprofloxacin as a recovery surrogate as it coeluted with the unlabeled compound. For the above reasons, it was concluded that this detector was not best suited for sewage samples. However, this detection technique is simple and quick to set up and may be useful for the analysis of FQs in less contaminated matrices such as surface waters and in spiked samples for biodegradation and toxicity studies.

Although not a very popular detector in environmental analysis, the MSD operating in the ESI+ mode provided useful data for the determination of FQs in nearly all sewage extracts. It was necessary though to apply various fragmentor voltages in order to maximize the response of the MH⁺ ions and to generate fragment ions for confirmation. Due to its limitation of a single quadrupole design, the SIM technique is more susceptible to interference by coextractives, especially when the concentrations of the target compounds are low. Also, the confirmation of norfloxacin may sometimes be doubtful since the relative intensity of its qualifying ion is low.

The ESI+ LC–MS/MS operating in MRM mode offered a highly sensitive and specific technique for the detection of FQs.

Even at low pg/µL levels, this technique is capable of providing the most reliable qualitative and quantitative results of FQs in sewage extracts due to the unique transitions between the precursor and product ions of a compound that filter out most of the chemical noises. Fig. 3 depicts the LC–MS/MS chromatograms of the FQs and the surrogate in an extract of a primary effluent, showing virtually no interference. Since the quantitation and confirmation MRM transitions for ofloxacin, norfloxacin, and ciprofloxacin (Table 2) were observed at the expected retention times, the presence of these FQs in the sample was confirmed. As reported earlier by Renew and Huang for the determination of antibiotics in wastewaters [14], quantitative results obtained by this detection method still have to be treated with caution, as suppressed detector response (matrix effect) of FQs was observed in one of our samples. Lower response of a FQ was likely caused by the less efficient desolvation and ionization of an analyte in the sample matrix, due to the presence of coextractives as a result of their incomplete removal in the cleanup steps.

One approach to evaluate signal suppression due to matrix effects is to use a standard addition method, as described by Miao et al. [16]. In this work, we have attempted to counteract this problem by reanalyzing the extract with a series of dilutions. The matrix effect was presumably eliminated at the point where the response of the analyte decreased in proportion to the dilution. An example of this exercise is shown in Fig. 4, where the extract of a primary effluent was first analyzed undiluted, and then after a two-fold dilution (analyzed
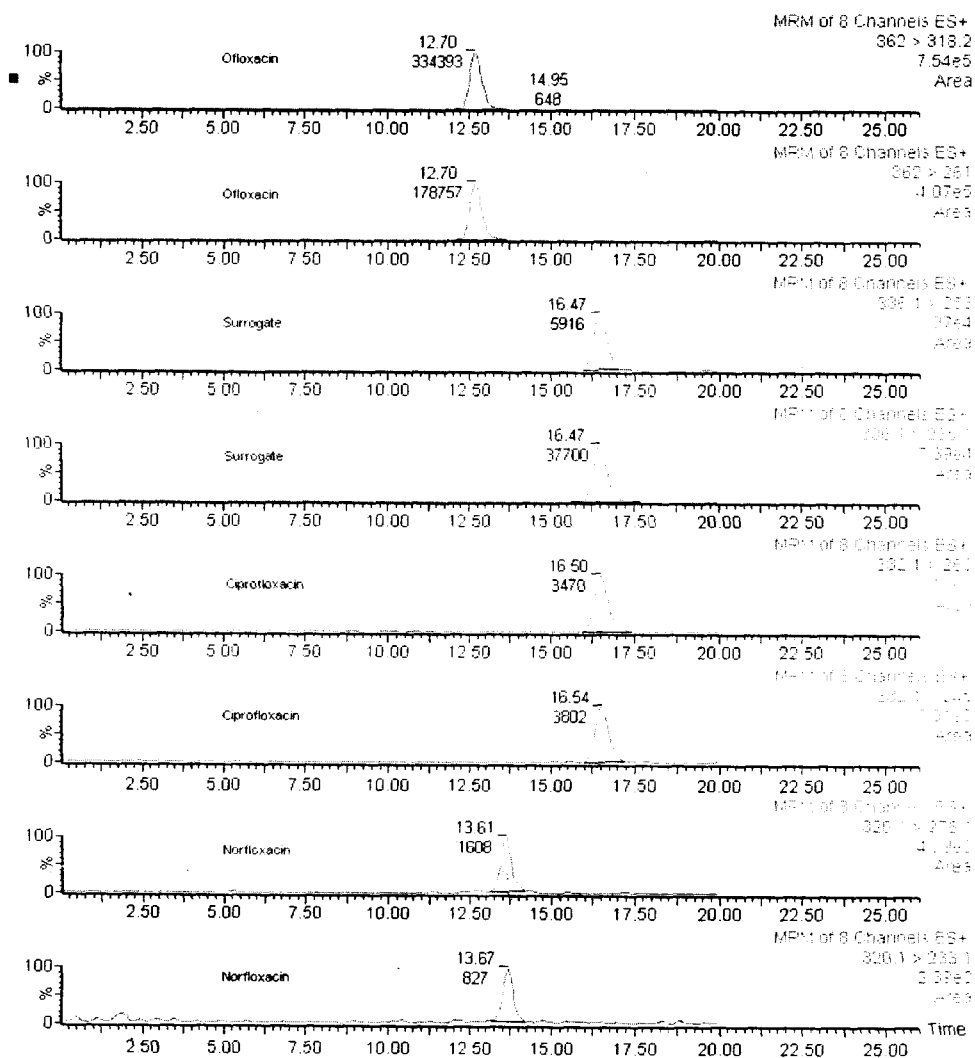
Fig. 3. MRM transitions (quantitation and confirmation) for the monitoring of FQs in a primary effluent sample. Traces 1 and 2 (from top), ofloxacin, 3 and 4, labeled ciprofloxacin, 5 and 6, ciprofloxacin, 7 and 8, norfloxacin.

twice to confirm reproducibility of results) and a four-fold dilution. It can be seen that the detector responses of norfloxacin and ciprofloxacin in the diluted samples decreased in proportion to the dilution, indicating minimal or no matrix effects for these two compounds. However, the responses of ofloxacin and the labeled ciprofloxacin were severely suppressed in the original sample extract, as indicated by a six-fold and three-fold increase, respectively, in peak area after a two-fold dilution. The detector responses of these two chemicals decreased by 50% as expected when the extract was further diluted by a factor of two. A similar suppression in response was also observed for ofloxacin and the labeled ciprofloxacin in the same undiluted extract when the MSD was used. Previous studies have shown that the matrix effects observed in LC-ESI–MS/MS declined with chromatographic retention time [22]. Therefore, it would be expected that ofloxacin, which elutes first from the LC column, would exhibit a greater degree of signal suppression than the later eluting analytes. The fact that signal suppression was only observed for ofloxacin in that case suggested that the wastewater extracts obtained by the present SPE procedure removed most

of the coextractives (such as humic substances [14]) that caused the matrix effect. Unfortunately, the presence (or absence) and extent of the matrix effect was unpredictable; sewage extracts in this work were therefore reanalyzed after dilution in order to ensure that the reported results were not affected by this matrix effect.

Using the present SPE procedure and the LC–MS/MS technique in the final analysis, the levels of ofloxacin, norfloxacin, and ciprofloxacin found in the primary and final effluent samples collected from eight Canadian sewage treatment plants are shown in Table 4. In all cases, the labeled ciprofloxacin was used as a surrogate standard and its recovery in sewage samples ranged from 65 to 95%. The three FQs detected had concentrations ranging from the low to the high ng/L levels in all samples, suggesting the widespread use of these antibacterials in those municipalities. With median concentrations of 34 ng/L in the final effluent and 60 ng/L in the primary effluent, norfloxacin was usually present at a lower concentration than the other two compounds. In comparison, the median concentrations of ciprofloxacin and ofloxacin in these samples were 146 and
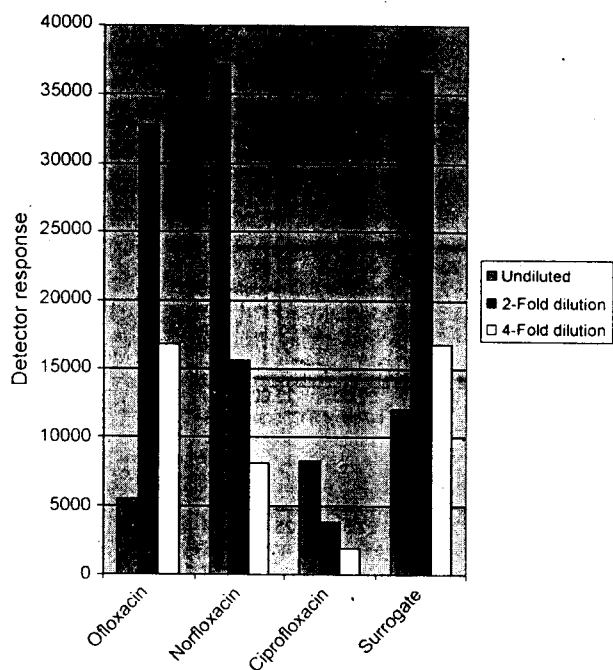
Fig. 4. An example of the matrix effect on the response of fluoroquinolones in a primary sewage effluent extract as measured under ESI+ LC–MS/MS MRM conditions. Area counts in arbitrary units.

179 ng/L (for final effluent samples), and 251 and 148 ng/L (for primary effluent samples), respectively. In general our results for the three FQs are consistent with those reported earlier for Canadian [16] and other sewage samples [5,6,8]. As indicated by the median values, there was a >40% reduction in concen-

trations for norfloxacin and ciprofloxacin between the final and the primary effluents. However, for ofloxacin we have observed a higher median value in the final effluent than in the primary effluent and the anomaly was not caused by the matrix effect. A de-conjugation of metabolites (through microbial activity in the sewage treatment processes), similar to the release of estrogens in unbounded form in the treated effluent as observed by Ternes et al. [23], is a plausible explanation for the higher ofloxacin levels in the final effluents. This newly developed method is being used in the monitoring of the FQs in municipal wastewater samples.

Acknowledgements

Table 4
Concentrations of fluoroquinolones (ng/L) in sewage primary and final effluents

Sample	Ofloxacin (ng/L)	Norfloxacin (ng/L)	Ciprofloxacin (ng/L)
Plant A final	216	19	170
Plant A primary	182	76	721
Plant B final	282	32	208
Plant B primary	92	56	291
Plant C final	289	34	392
Plant C primary	288	90	481
Plant D final	32	66	62
Plant D primary	42	126	84
Plant E final	141	72	235
Plant E primary	75	64	231
Plant F final	87	34	121
Plant F primary	113	56	270
Plant G final	105	10	42
Plant G primary	212	40	171
Plant H final	548	36	106
Plant H primary	2148	53	165
Range final	32–548	10–72	42–392
Median final	179	34	146
Range primary	42–2148	40–126	84–721
Median primary	148	60	251

Results were obtained by LC–MS/MS.

References

[1] F. Van Bambeke, J.-M. Michot, J. Van Eldere, P.M. Tulkens, Clin. Microbiol. Infect. 11 (2005) 256.

[2] Top Prescription Drugs, Rx Canada Plus, 2004. http://www.pharmacycan.com.

[3] M.N. Swartz, N. Engl. J. Med. 345 (2001) 1621.

[4] A. Hartmann, A.C. Alder, T. Koller. R.M. Widmer, Environ. Toxicol. Chem. 17 (1998) 377.

[5] E.M. Golet, A.C. Alder, A. Hartmann, T.A. Ternes, W. Giger, Anal. Chem. 73 (2001) 3632.

[6] E.M. Golet, I. Xifra, H. Siegrist, A.C. Alder, W. Giger, Environ. Sci. Technol. 37 (2003) 3243.

[7] S. Castiglioni, R. Bagnati, D. Calamari, R. Fanelli, E. Zuccato, J. Chromatogr. A 1092 (2005) 206–215.

[8] R. Andreozzi, M. Raffaele, P. Nicklas, Chemosphere 50 (2003) 1319–1330.

[9] D. Calamari, E. Zuccato, S. Castiglioni, R. Bagnati, R. Fanelli, Environ. Sci. Technol. 37 (2003) 1241.

[10] E.M. Golet, A. Strehler, A.C. Alder, W. Giger, Anal. Chem. 74 (2002) 5455.

[11] E. Turiel, A. Martin-Esteban, G. Bordin, A.R. Rodriguez, Anal. Bioanal. Chem. 380 (2004) 123.

[12] J.R. Marengo, R.A. Kok, K. O'Brien, R.R. Velagaleti, J M. Stam, Environ. Toxicol. Chem. 16 (1997) 462.

[13] S. Reverté, F. Borrull, E. Pocurull. R.M. Marcé, J. Chromatogr. A 1010 (2003) 225.

[14] J.E. Renew, C.-H. Huang, J. Chromatogr. A 1042 (2004) 113.

[15] H. Nakata, K. Kannan, P.D. Jones, J.P. Geisy, Chemosphere 58 (2005) 759.

[16] X.-S. Miao, F. Bishay, M. Chen, C.D. Metcalfe, Environ. Sci. Technol. 38 (2004) 3533–3541.

[17] B.-H. Kim, N.H. Choi, J.H. Ok, J. Chromatogr. Sci. 40 (2002) 369.

[18] T.M. Annesley, Clin. Chem. 49 (2003) 1041.

[19] V.F. Samanidou, C.E. Demetriou, I.N. Papadoyannis, Anal. Bioannal. Chem. 375 (2003) 623.

[20] R. Hindle, C.-K. Meng, Application Notes AN00046008. Agilent Technologies. Wilmington, DE, USA, 2004.

[21] O. Ballesteros, V. Sanz-Nebot, A. Navalón, J.L. Vilchez, J. Barbosa, Chromatographia 59 (2004) 543.

[22] B.K. Choi, D.M. Hercules, A.I. Gusev, Fresenius J. Anal. Chem. 369 (2001) 370.

[23] T.A. Ternes, M. Stumpf, J. Mueller, K. Haberer, R.-D. Wilken, M. Servos, Sci. Total Environ. 225 (1999) 81.