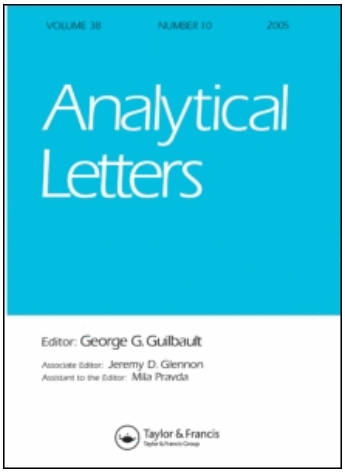

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J. L. Conkle [a]; C. V. Lattao [b]; J. R. White [a]; R. L. Cook [b]
[a] Department of Oceanography and Coastal Sciences, Louisiana State University, Baton Rouge, Louisiana, USA [b] Department of Chemistry, Louisiana State University and Southern University of Baton Rouge, Baton Rouge, Louisiana, USA

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CHROMATOGRAPHY

Pharmaceutical Analysis for Environmental Samples: Individual and Simultaneous Determination of Ciprofloxacin, Ofloxacin and Norfloxacin Using an HPLC with Fluorescence and UV Detection with a Wetland Soil Matrix

J. L. Conkle,[1] C. V. Lattao,[2] J. R. White,[1] and R. L. Cook[2]
[1]Department of Oceanography and Coastal Sciences, Louisiana State University, Baton Rouge, Louisiana, USA
[2]Department of Chemistry, Louisiana State University and Southern University of Baton Rouge, Baton Rouge, Louisiana, USA

Abstract: Two HPLC methods were developed for individual and simultaneous determination of ciprofloxacin, norfloxacin and ofloxacin for use in laboratory experiments producing large numbers of samples (100 s to 1000 s). Individual compound detection produced retention times between 1.5 and 2 min and simultaneous detection between 6.5 to 8 min. The methods are compatible with complex geomatrices, e.g. a wetland soil. These methods provide 1) detection limits in the low parts per-billion range; 2) decrease in retention times of 5–10 times for single compounds, and up to 2 times for simultaneous detection over published methods; and 3) require no solid phase extraction.

Keywords: Direct injection, fluoroquinolone, soil

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Address correspondence to J. R. White, Wetland and Aquatic Biogeochemistry Laboratory, Department of Oceanography and Coastal Sciences, Louisiana State University, Baton Rouge, LA 70803, USA. E-mail: jrwhite@lsu.edu or R. L. Cook, Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA. E-mail: rlcook@lsu.edu

1. INTRODUCTION

Pharmaceuticals, including fluoroquinolone antibiotics, have been detected in surface waters around the world (Batt, Kim, and Aga 2007; Batt, Snow, and Aga 2006; Conkle, White, and Metcalfe 2008; Golet et al. 2001; Nakata et al. 2005). The fate of these compounds in the environment needs further investigation, specifically pertaining to sorption, desorption, transport, and biotic and abiotic degradation (White, Belmont, and Metcalfe 2006).

Methods have been developed for the determination of ofloxacin (OFL), norfloxacin (NOR), and ciprofloxacin (CIP) in sewage using an HPLC (Carlucci 1998; Golet et al. 2001; Lee, Peart, and Svoboda 2007; Samanidou, Demetriou, and Papadoyannis 2003). However, these methods require solid phase extraction (SPE) and have retention times in excess of 10 min. Laboratory scale experiments aimed at elucidating removal mechanisms yield large numbers of samples (ranging from 100 s to 1000 s), which, when combined with long preparation and analysis times, makes such approaches impractical for most laboratories. The motivation behind the presented study was to reduce the time required to analyze fluoroquinolone antibiotic as well as developing a method which is effective for complex environmental matrices while, at the same time, have detection limits appropriate for environmental analysis. All three of these compounds have similar structures and properties, which can make separation of each compound more challenging in the presences of the others.

Therefore, the goal of this research, using standard HPLC equipment (with UV and fluorescence detection), was to develop a method for individual and simultaneous analysis of CIP, NOR, and OFL (Figure 1), with the following criteria: 1) shortened retention times, 2) elimination of the need for SPE, and 3) capable of providing detection at parts per billion ($\mu g\,L^{-1}$) up to high parts per million ($mg\,L^{-1}$).

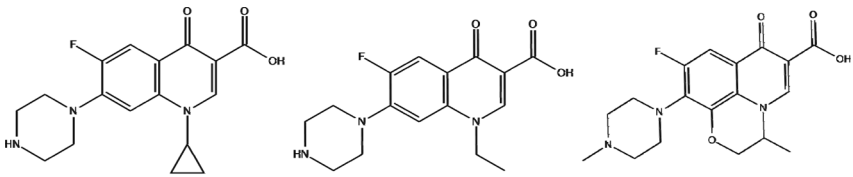

Figure 1. Structure of the three compounds for which analytical methods were developed.

2. EXPERIMENTAL

2.1. Materials

CIP, NOR, and OFL (HPLC grade) were obtained from Sigma-Aldrich (St. Louis, MO) in powder form. Acetonitrile and water were purchased from Mallinckrodt chemicals. Methanol (HPLC grade), glacial acetic acid (biochemical grade, 99.8%), and sodium azide (99%) were obtained from Acros Organics. Sodium acetate (anhydrous, 99.7%) and calcium chloride (anhydrous, 96.0%) were purchased from Fisher Scientific and Sigma-Aldrich respectively. All solvents used were HPLC grade. Triethylammonium (TEAP) phosphate buffer solution (pH = 3), as well as, citric acid and sodium citrate monobasic (both anhydrous, ultra grade $\geq$99.5%) were supplied by Fluka Bio Chemika. An 18 mΩ resistivity water filter with a 0.1 μm filtering device (Modular Water Systems, United States Filter Corporation) was used to treat all water used in the stock, standard electrolyte solution and sample solution preparation.

2.2. UV and Fluorescence

UV-vis (on a Cary 50Bio, Palo Alto, CA) and fluorescence (on a Fluorolog, Horiba Jobin Yvon, Edison, NJ) characterization was carried on all three antibiotics. The UV-vis and fluorescence data yielded absorbance and emission maxima of 272, 273 and 289 nm and 421, 414 and 458 nm for CIP, NOR, and OFL, respectively. CIP and NOR have similar absorbance and emission spectra, while OFL has a higher range.

2.3. Chromatography

The liquid chromatographic system used in this study consisted of an Agilent 1100 (Santa Clara, CA). This LC instrument was equipped with the following parts: solvent degasser (G1379A), quaternary pump (G1311A), automatic liquid sampler (G1329A), temperature controlled column compartment (G1316A), DAD detector (G1315B), and fluorescence detector (G1321A). The instrument was fitted with a Zorbax (Santa Clara, CA) eclipse XDB C18 (4.6 mm × 150 mm × 5 μm) column, and a Phenomenex (Torrance, CA) C18 guard column (4.0 mm × 3.0 mm × 5 μm).

For the analysis of CIP, NOR, and OFL as separate components, a mobile phase consisting of sodium acetate (pH 3), acetonitrile (ACN) and TEAP (10 mM). The TEA solution was added to solutions to minimize peak tailing (Zendelovska and Stafilov 2005; Snider, Kirkland,

and Glajch 1997). A 60/40 v/v ratio sodium acetate to ACN was used for CIP and NOR, while a 70/30 ratio was used for OFL. For simultaneous analysis of all three fluoroquinolones, an aqueous citric acid buffer (pH 2.5), ACN, methanol (MeOH) in a 82/8/10 v/v ratio mobile phase was utilized (a modified adaptation from Canada-Canada, Espinosa-Mansilla, and de la Pena 2007). A column temperature of 35°C, a flow rate of 1 mL min^{-1}, and an injection volume of 20 μL were used. For UV-vis detection a wavelength of 280 nm and $\lambda_{ex}/\lambda_{em}$, of 280/450 nm was found to be optimal for fluorescence detection. All methods were run in isocratic mode and used a direct injection of aqueous samples without prior sample pretreatment. All samples were injected into the HPLC in triplicate.

2.4. Preparation of Standard Solutions

Stock solutions with concentrations of 80 mg L^{-1} were prepared in triplicate. In addition, each stock solution contained 100 mg L^{-1} sodium azide (to remove possible biological components) and 0.01 M $CaCl_2$ (to mimic the ionic strength of environmental samples). Stock solutions were used to create a 9-point standard curve for each fluoroquinolone. The standard solution was subsequently diluted with water to yield concentrations spanning three orders of magnitude from 0.05 to 80 mg L^{-1}. The 80 mg L^{-1} stock solutions were also used to create the 10-point standard curve for the simultaneous method, with standard solution concentrations ranging from 0.04 to 20 mg L^{-1}. All standards were prepared in triplicate from the stock solutions prior to their use and each standard was injected into the instrument in triplicate. When standards and stock solution were not in use they were stored at 4°C in darkness. Peak area was used for determination of compound concentrations, not height.

2.5. Method Validation with a Wetland Soil

These methods were tested using an environmental matrix; soil from a local wetland classified as an Arat Silty Clay Loam, which is a fine silty, siliceous, non-acid, thermic Typic Hydraquent (Trahan, Bradley, and Nolde 1990). This particular soil was chosen because it belongs to the same class of soil as in a nearby treatment wetland that receives treated wastewater containing pharmaceuticals; its parameters are shown in Table 1. Woody and root materials were removed and the soil was homogenized and refrigerated at 4°C prior to experimental analysis. A 20 mL aliquot of solution containing 5 mg L^{-1} of CIP, NOR, and/or OFL (the remainder of the solution composition was identical to that of the

Table 1. Parameters from the Bayou Castine wetland soil used for environmental method application. All standards used in method development were prepared in triplicate and injected in triplicate. (±values represent standard deviation)

Parameter	Value
Cation Ex Capacity ($cmol_c\,kg^{-1}$)[‡]	19.8 ± 0.8
Moisture content (%)	65 ± 0.0
pH	6.9 ± 0.2
Organic matter (%)[†€]	18.6 ± 1.0
Total Carbon ($g\,kg^{-1}$)[¥€]	88.4 ± 3.3
Total Phosphorus ($mg\,kg^{-1}$)[€]	474.2 ± 15.8
Total Nitrogen ($g\,kg^{-1}$)[¥€]	6.2 ± 0.2
Clay (%)	31.3

[‡](Sumner and Miller 1996).
[†]Loss on ignition.
[€]Dry soil basis.
[¥](White and Reddy 2000).

standard solutions) was added to glass vials containing 50 mg of field moist soil. Blank vials containing only the antibiotic solution were prepared to account for sorption to the glass scintillation vials, which is essential for mass balance calculations. Four replicates of each treatment were prepared and shaken for 5 days. At the end of the incubation, samples were centrifuged and a 2 mL aliquot was extracted for analysis using the methods presented in this study. Blanks containing only the spiked solution demonstrated that there was little if any sorption to the glass vials.

3. RESULTS AND DISCUSSION

3.1. Overall Chromatographic Performance

In recent years the presence of pharmaceuticals in the environment has been a topic of growing concern. While there is a need for data obtained in the field with regards to compound identification, transport, and fate, controlled lab studies can provide a baseline for understanding compound interactions in the environment. We developed two methods for the analysis of three fluoroquinolone compounds using an HPLC with UV and fluorescence detection that improve upon previous methods by decreasing analysis times.

For the methods developed in this work, the retention times for analysis of individual compounds (1.5 – 1.7 min) are significantly shorter than

those obtained with the multiple compound detection method (6.5–8.0 min) (Table 2). A short retention time for individual compound detection is important when performing laboratory studies that require hundreds of samples, such as sorption and desorption experiments. However, when analyzing compounds simultaneously there is a significant increase in retention time. The method recently developed by Canada-Canada, Espinosa-Mansilla, and de la Pena (2007) demonstrated retention times of 7.8–9.7 min for the same compounds when analyzed simultaneously along with twelve other fluoroquinolone compounds. Therefore, when only analyzing these three compounds, the two methods presented herein provide at a minimum: 1) 4.5–6.4 times shorter retention times for individual compounds, and 2) 14–17% shorter retention times for simultaneous compound detection over previously published methods (Canada-Canada, Espinosa-Mansilla, and de la Pena 2007; Golet et al. 2001; Lee, Peart, and Svoboda 2007).

All UV standard curves achieved $>0.995\,R^2$ value for concentrations ranging from 0.055 (CIP, NOR) or 0.11 (OFL) to 80 mg L^{-1} for individual compound detection. UV detection was effective over the entire range tested for individual compounds. However, fluorescence detection of individual compounds was only linear at the lower end of the range tested ($\sim$0.06 to 1.5 mg L^{-1}). This indicates that UV analysis is best when either unsure of compound concentration or the known range varies from $\mu g\,L^{-1}$ to mg L^{-1}.

For the simultaneous method, the standard curve was linear up to the concentration of 20 mg L^{-1} for both UV and fluorescence. The R^2 values was near 1.0 for UV analysis during simultaneous detection and >0.995 for CIP and OFL fluorescence. However, NOR was only 0.989 (Table 2) indicating that fluorescence detection of NOR may be inferior to UV detection.

There did not appear to be any degradation of the compounds, as indicated by the absence of significant peaks other than the target compounds during both sample and standard analysis (Figs. 2 and 3). It should be noted that both methods are isocratic in nature, and hence, can be run on an HPLC instrument with a single channel pump. In comparison, the method developed by Canada-Canada, Espinosa-Mansilla, and de la Pena (2007) requires, at a minimum, a three channel pump. In addition, it was found that the mobile phase is required to be at a pH below 3 in order to resolve NOR and OFL satisfactorily. All standard samples were prepared in triplicate and injected into the HPLC in triplicate to account for variation in standard preparation and the detector.

There is a small signal between 1.1–1.6 minutes that is attributed to the HPLC water that was detected during UV analysis (Fig. 2). This signal appears during fluorescence analysis as well, but has a much smaller

Table 2. Method development calibration curves of CIP, NOR & OFL using UV and fluorescence. (±values represent standard deviation)

Method		Retention time (min)	Slope	Intercept	R^2	Range tested (mg L^{-1})	LOQ (mg L^{-1})[†]
UV							
Single	CIP	1.520 ± 0.000	125.63 ± 1.93	28.55 ± 20.59	0.999 ± 0.000	0.0570 ± 0.008–80.833 ± 0.684	0.024
	NOR	1.511 ± 0.000	124.61 ± 0.67	19.40 ± 9.68	0.999 ± 0.000	0.0597 ± 0.004–79.200 ± 1.266	0.033
	OFL	1.704 ± 0.000	52.86 ± 0.42	9.53 ± 2.65	0.999 ± 0.000	0.1093 ± 0.025–79.933 ± 0.133	0.017
Triple	CIP	8.041 ± 0.077	91.55 ± 0.49	−7.29 ± 0.45	1.000 ± 0.000	0.1637 ± 0.123–20.342 ± 0.158	0.037
	NOR	7.000 ± 0.063	88.41 ± 1.34	−7.32 ± 1.63	1.000 ± 0.000	0.1567 ± 0.112–19.742 ± 0.283	0.035
	OFL	6.509 ± 0.057	35.59 ± 0.16	−2.9 ± 0.19	1.000 ± 0.000	0.0533 ± 0.003–19.950 ± 0.000	0.078
Fluorescence							
Single	CIP	1.552 ± 0.001	590.99 ± 22.27	−6.92 ± 3.91	0.999 ± 0.000	0.0653 ± 0.001–1.362 ± 0.280	0.065
	NOR	1.542 ± 0.000	613.80 ± 16.41	5.81 ± 1.76	0.997 ± 0.001	0.0597 ± 0.004–0.931 ± 0.357	0.03
	OFL	1.737 ± 0.000	432.09 ± 15.24	9.83 ± 2.28	0.997 ± 0.001	0.0827 ± 0.003–1.599 ± 0.003	0.024
Triple	CIP	8.088 ± 0.075	350.97 ± 3.35	19.8 ± 4.87	0.999 ± 0.000	0.1637 ± 0.123–20.342 ± 0.158	0.011
	NOR	7.026 ± 0.063	356.53 ± 4.60	81.68 ± 4.62	0.989 ± 0.000	0.1533 ± 0.113–19.742 ± 0.283	0.019
	OFL	6.533 ± 0.057	61.47 ± 0.30	−3.65 ± 0.40	1.000 ± 0.000	0.0533 ± 0.003–19.950 ± 0.000	0.061

[‡]limit of quantitation (S/N = 10), LOQs were calculated from the standard solutions, not sample analysis.

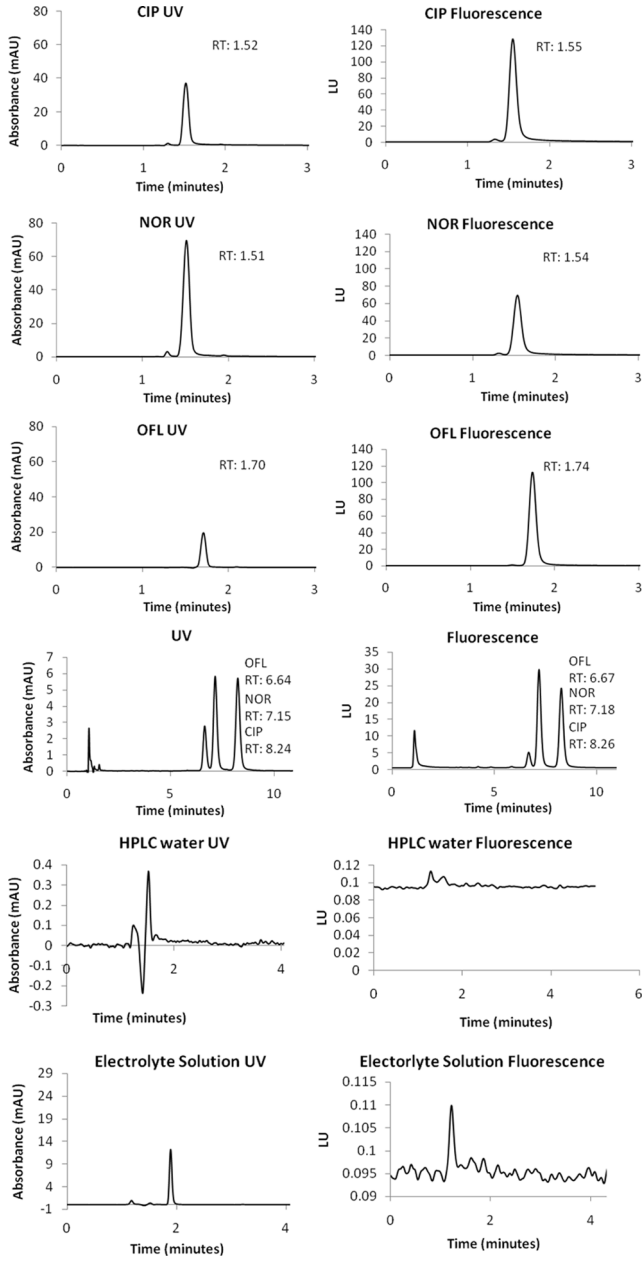

Figure 2. UV and Fluorescence signals of individual and simultaneous compound detection at $1\,\mathrm{mg\,L^{-1}}$ (RT = Retention Time). Chromatograms were randomly picked and RT are only for that individual run.

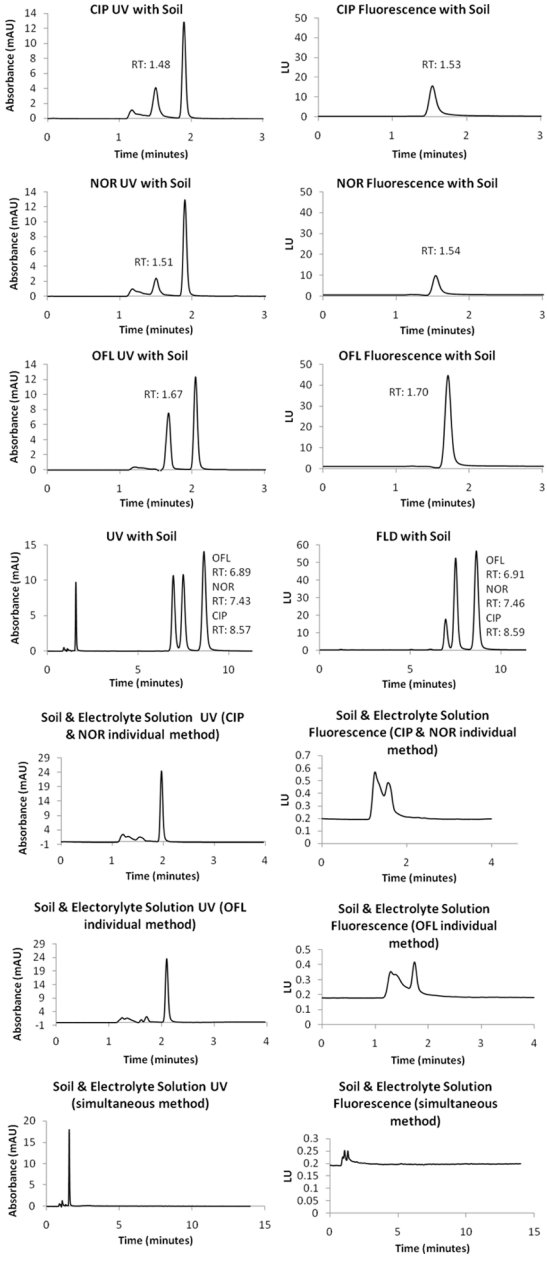

Figure 3. UV and fluorescence signals of individual compound detection with a wetland soil at $5\,\mathrm{mg\,L^{-1}}$ loading. Chromatograms were randomly picked and RT are only for that individual run.

response. This HPLC water signal is mainly noticeable during UV analysis when compound concentrations are low and is observed during environmental applications (Fig. 3).

3.2. Buffer Solutions

The use of sodium acetate buffer (pH = 3) in the individual analysis of antibiotics or citrate buffer (pH = 2.5) in the simultaneous separation has a two-fold purpose. The mobile phase pH is below the pKa's of the fluoroquinolones and prevents ionization of the molecules. For example, CIP has the following reported pKa's: carboxylic group (5.85 – 6.35), amino (8.24 – 8.95) and the other two N groups (5.05, 3.64) (De Witte et al. 2007). In addition the use of low pH ($2.0 < pH < 2.5$) minimizes the presence of free unprotonated silanol groups of silica-based columns. Previous methods employed phosphoric acid (Zendelovska and Stafilov 2005), citric acid (Canada-Canada, Espinosa-Mansilla, and de la Pena 2007), formic acid and trifluoracetic acid (De Witte et al. 2007; Lee, Peart, and Svoboda 2007). Moreover, triethylammonium from TEAP exchanges with less strongly retained ions such as sodium cations, thereby reducing the amount of free ionized silanol groups and suppresses the access of fluoroquinolones to residual silanols (Snider, Kirkland, and Glajch 1997; Zendelovska and Stafilov 2005). All of the previous reasons result in peak shape improvement and decrease in retention times for the fluoroquinolones tested.

3.3. Mobile Phase Ratios

For individual compound analysis a 60/40 mobile phase consisting of sodium acetate and ACN was used for CIP and NOR while a 70/30 ratio was used for OFL. When all three compounds are run with same amount of ACN in the mobile phase, the first fluoroquinolone to elute is OFL. If a 60:40 (A/B) mobile phase for ofloxacin is used, a potential problem arises especially at very low ppb level detection, where ofloxacin may completely overlap with early eluters from the matrix solution. Therefore, the 70/30 ratio is needed for proper OFL separation.

3.4. Simultaneous Method

The ability to simultaneously detect fluoroquinolone compounds allows us to analyze samples that are representative of environmental

Table 3. UV and Fluorescence results obtained from method application on Bayou Castine soils that validate the application of these methods on environmental matrices. Each compound demonstrated high loading to the soil, with low concentrations remaining in the aqueous phase. Values obtained for both UV and Fluorescence were similar. (±values represent standard deviation)

	Initial mass (μg)	Mass on soil (μg)	Mass in solution (μg)	Conc. In soil ($g\,kg^{-1}$)	Conc. In solution ($mg\,L^{-1}$)	% in Solution	% on Soil	UV Retention time
UV								
CIP	97.99 ± 0.08	93.07 ± 2.55	4.92 ± 2.53	4.06 ± 0.08	0.25 ± 0.13	5.02 ± 2.58	94.98 ± 2.58	1.50 ± 0.00
NOR	98.39 ± 0.08	91.97 ± 1.21	6.42 ± 1.21	4.01 ± 0.20	0.33 ± 0.06	6.53 ± 1.23	93.47 ± 1.23	1.50 ± 0.00
OFL	98.34 ± 0.08	87.30 ± 2.61	11.04 ± 2.55	3.81 ± 0.11	0.56 ± 0.13	11.23 ± 2.59	88.77 ± 2.59	1.67 ± 0.00
	Initial mass (μg)	Mass on soil (μg)	Mass in solution (μg)	Conc. In soil ($g\,kg^{-1}$)	Conc. In solution ($mg\,L^{-1}$)	% in Solution	% on Soil	Fluorescence retention time
Fluorescence								
CIP	97.99 ± 0.08	92.34 ± 2.85	5.65 ± 2.83	4.03 ± 0.07	0.29 ± 0.14	5.77 ± 2.89	94.23 ± 2.89	1.54 ± 0.00
NOR	98.39 ± 0.08	89.47 ± 1.42	8.92 ± 1.34	3.90 ± 0.15	0.45 ± 0.07	9.07 ± 1.37	90.93 ± 1.37	1.54 ± 0.00
OFL	98.34 ± 0.08	88.36 ± 2.47	9.98 ± 2.41	3.85 ± 0.11	0.51 ± 0.12	10.15 ± 2.45	89.85 ± 2.45	1.70 ± 0.00

conditions, where multiple compounds may be present in a water sample. The single methods developed for individual compounds would not allow for simultaneous detection of all three compounds because CIP and NOR have similar retention times. The method for simultaneous detection was developed to ensure that there was separation between CIP and NOR.

This simultaneous method provides a closer examination of pharmaceutical and soil interaction in lab microcosm experiments. The method performed well when used to analyze the three compounds in the presence of a wetland soil. There was no interference observed during simultaneous method sample analysis (Fig. 3).

3.5. Analysis of Soil Samples

Neither interference nor degradation was observed during the soil sample analysis, as indicated by the absence of significant peaks other than the target compounds, a sodium azide at ~2 min and the low background signal (Fig. 3). Blanks with soil and electrolyte solution showed no additional peaks associated with soil for either method during both UV and fluorescence detection (Fig. 3). A mass balance was calculated, taking into account any compound sorbed to the surface of the vial, to determine the sorption rates of each compound onto the soil. Peak retention times for the environmental matrix samples were within 2.0% of the coefficient of variation from those observed for the standards (Table 3 and Fig. 3). Both methods produced corresponding results that showed high sorption to the wetland soil. For both UV and fluorescence at 5 mg L^{-1}, the three compound exhibited high sorption to the wetland soil, with both CIP and NOR sorbing >90% and OFL ~88% (Table 3).

4. CONCLUSION

In comparison to previously published methods, the methods developed in this work: 1) allow for faster analytical throughput, with retention times that are 4.5–6.4 times shorter for individual compounds and 14–17% shorter for simultaneous detection of three antimicrobial pharmaceuticals (CIP, NOR, and OFL); 2) remove the necessity of time consuming solid phase extraction if detection down to the low part per billion is desired; and 3) require minimal HPLC hardware, especially in the isocratic mode.

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