Microchemical Journal 185 (2023) 108217

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Automatised on-line SPE-chiral LC-MS/MS method for the enantiomeric determination of main fluoroquinolones and their metabolites in environmental water samples

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ARTICLE INFO

Keywords:
Antibiotics
Metabolites
Enantiomers
On-line solid-phase extraction
Chiral LC-MS/MS
Wastewater

ABSTRACT

Fluoroquinolones are antibiotics of significant environmental concern their extended use not only in human medicine but also in veterinary medicine and not only as therapeutic agents but also to promote livestock growth and in aquaculture. Some fluoroquinolones and their metabolites are chiral compounds. Therefore, for a proper environmental risk assessment, enantioselective analytical methods are required. In this work, an analytical method has been developed and validated for the first time automatised enantioselective determination of environmental significant fluoroquinolones and their metabolites in wastewater and surface water samples. Target fluoroquinolones were selected ~~by~~ considering their extended use in human (ciprofloxacin and ofloxacin) and veterinary (flumequine) medicine. The analytical method was based on on-line solid-phase extraction-chiral liquid chromatography-tandem mass spectrometry. Analysis, including sample extraction and chiral LC-MS/MS determination, was carried out in just 14 min. The method was validated for its application to surface water and effluent and influent wastewater. Accuracy values were in the range from 61.4 to 122 % in wastewater and from 73.4 to 119 % in surface water. Precision, expressed as relative standard deviation, was lower than 13.6 % for all the compounds and sample matrices. Method quantification limits were in the range from 0.2 to 50 ng/L for all the compounds in wastewater and surface water. Method application to wastewater and surface water samples revealed the enantioselective transformation of LEV into (R)-OFL in surface water and the prevalence of OH-FLU D2 with respect to OH-FLU D1 in influent wastewater.

1. Introduction

There is an increasing concern about the overuse and misuse of antibiotics. Such practices are resulting in an increasing antibiotic resistance what is threatening the treatment of common infection diseases [1]. Fluoroquinolones are an antibiotic class of special environmental relevance because of their persistence and extended use in human and veterinary medicine [2,3]. They have been included in the list of critically important antimicrobials by the World Health Organization [4] and ciprofloxacin (CIP) and ofloxacin have also been included in the 3rd [5] and 4th [6] watch lists, respectively, of substances for Union-wide monitoring in the field of water policy [5]. CIP can also be released to the environment as a metabolite of the veterinary drug enrofloxacin [2,7]. Fluoroquinolones are used not only as therapeutic agents but also to promote livestock growth and in aquaculture [8]. They have been reported to be one of the most frequently detected class of antibiotics, together with tetracyclines, in Portuguese surface–groundwater [7] and the antimicrobials at the highest concentrations in hospital wastewater (up to 13.78 μg L^{-1} for CIP and up to 14.38 μg L^{-1} for ofloxacin (OFL)) [9]. Concentrations in urban wastewater have been reported to be at least one order of magnitude lower than in hospital wastewater due to CIP and OFL are mainly used in hospitals [9]. They are considered recalcitrant compounds that can stimulate antibacterial resistance even at low concentrations [10].

Analytical methods reported for the determination of pharmaceuticals, including fluoroquinolone antibiotics, in environmental water samples are commonly based on off-line solid-phase extraction (SPE) [11–16]; dispersive solid-phase extraction [17,18]; ionic liquid-based dispersive liquid–liquid microextraction [19]; QuEChERS (quick, easy, cheap, effective, rugged, and safe) approach method [20]; and solid–liquid extraction after sample lyophilisation [21]. Analytical determination is commonly carried out by LC-MS/MS [12–16] due to its high

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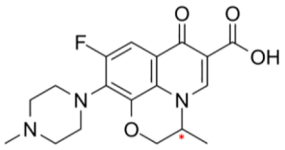
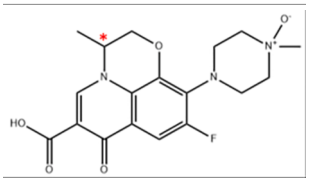
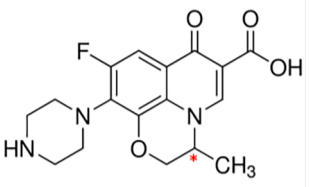
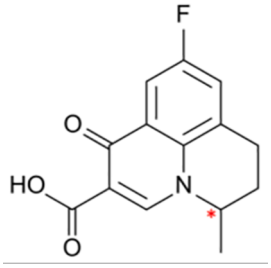
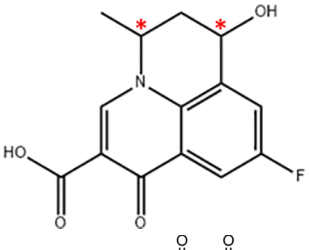
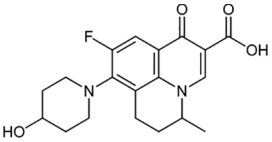
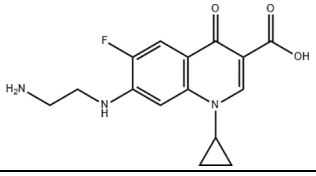
https://doi.org/10.1016/j.microc.2022.108217
Received 23 August 2022; Received in revised form 7 November 2022; Accepted 21 November 2022
Available online 24 November 2022

selectivity and sensitivity [11]. The European Commission proposes the application of SPE followed by LC-MS/MS for the determination of the antibiotics included in the watch list of substances to monitor in surface water [5].

Some fluoroquinolones and their metabolites are chiral compounds what can influence their environmental behaviour. Although physical and chemical processes affect in the same way to both enantiomers, they can act differently with other chiral molecules (such as proteins: receptors or enzymes), which can result in different biological and toxicological behaviours [22,23] and pharmacokinetic and pharmacodynamic responses. To the date, only two methods have been reported for the enantioselective determination of fluoroquinolones in environmental water samples [24,25]. One of them is limited to flumequine (FLU), mainly used in veterinary medicine [26], and its metabolite 7-hydroxyflumequine (OH-FLU) [25]. They had to be separately analysed as their enantioselective determination required two different chiral LC columns. The other method allows the LC chiral separation of five fluoroquinolones and three of their metabolites after off-line SPE but do not include the determination of OH-FLU [24].

Automatised on-line SPE has emerged as a promising sample treatment technique for trace analysis of pharmaceuticals in liquid environmental samples. It allows to process samples in short times; to

Table 1
Physical-chemical properties of the target compounds.

Compound	Abbreviation	Molecular structure	MW (g mol^{-1})	pKa
Ofloxacin	**OFL**		361.4	5.19[a]
Ofloxacin-*N*-oxide	OFL *N*-OX		377.4	5.19[b]
N-Desmethylofloxacin	DM-OFL		347.3	5.19[b]
Flumequine	**FLU**		261.3	5.70[a]
7-Hydroxyflumequine	OH-FLU		277.3	5.63[b]
Ciprofloxacin	**CIP**		360.4	6.43[a]
Desethyleneciprofloxacin	DES-CIP		305.3	6.4[b]

Parent compounds are marked in bold; MW: molecular weight. *Chiral center.
[a] Rocha et al., 2015 [3]; [b]: https://www.chemicalbook.com/.

minimise the loss of analytes that can occur in multi-step sample treatments; to reduce labour intensity in comparison to off-line sample treatment techniques that usually require evaporation and reconstitution steps [11]; and to improve health and safety of the analyst. In addition, it allows reducing solvent consumption, required sample volume, plastic waste generation and analysis cost, as cartridges are reusable, in comparison to off-line SPE. Nevertheless, only a few on-line SPE-LC-MS/MS methods have been reported for the determination of antibiotics in environmental water samples [27–34]. None of them include the enantioselective determination of chiral antibiotics.

The aim of this paper was to develop an automatised on-line SPE-chiral-LC-MS/MS analytical method suitable to be applied for the enantioselective determination of environmentally relevant human and veterinary fluoroquinolones and their metabolites. Target compounds included two chiral fluoroquinolones ((±)-OFL and (±)-FLU), three of their chiral metabolites ((±)-7-hydroxyflumequine (OH-FLU), (±)-desmethyl-ofloxacin (DM-OFL) and (±)-ofloxacin-*N*-oxide (OFL *N*-OX)), an achiral fluroquinolone (CIP) and its achiral metabolite desethylenociprofloxacin (DES-CIP) (Table 1). Selection of parent fluoroquinolones was carried out considering their high use as human (CIP and OFL) and veterinary (FLU) fluoroquinolone antibiotic drugs. This fact makes them suitable to be used as indicators of fluoroquinolone pollution. The analytical method was validated for its application to influent and effluent wastewater and surface water.

2. Experimental

2.1. Chemicals and reagents

Standards of CIP (≥98.0 %), (±)-OFL (≥98.0 %), (±)-FLU (≥98.0 %) and S-(-)-OFL (also named levofloxacin (LEV)) (≥98.0 %) were purchased from Sigma-Aldrich (Steinheim, Germany). DES-CIP (≥98.0 %) and (±)-OFL *N*-OX (≥98.0 %) were supplied by Toronto Research Chemicals (Toronto, Canada). (±)-DM-OFL (≥95.0 %) was provided by LGC Standards (Montevideo, Uruguay). (±)-OH-FLU (≥95.0 %) was purchased from Key Organics (Camelford, United Kingdom). (±)-Ofloxacin-d_3 ((±)-OFL-d_3, ≥99.0 %), used as internal standard (I.S.), was purchased from Sigma-Aldrich (Steinheim, Germany). Ammonium formate ($HCOONH_4$) was supplied by Sigma-Aldrich (Madrid, Spain). Ammonium acetate was provided by Scharlab (Barcelona, Spain). Formic acid (HCOOH) was provided by Panreac (Barcelona, Spain). The reagents were of high analytical grade and purity. LC-MS-grade acetonitrile (ACN), water and methanol (MeOH) were supplied by Biosolve BV (Valkenswaard, the Netherlands). Individual 1000 mg L^{-1} (equivalent to 500 mg L^{-1} for each enantiomer) stock standard solutions were prepared in MeOH. A mix stock standard solution, containing all analytes at 0.5 mg L^{-1} (each enantiomer) except *S*-(-)-OFL and I.S., was prepared by diluting individual stock solutions in water. A individual 1 mg L^{-1} stock solution of *S*-(-)-OFL was prepared in MeOH. It was used to elucidate the elution order of (±)-OFL enantiomers. Mixture working solutions used for method optimization and validation were prepared by dilution of mix stock solutions in water. Stock and working solutions were stored at −20 °C.

2.2. Sample collection and pretreatment

Influent and effluent composite wastewater samples were collected from seven WWTPs located in Andalusia (South of Spain) to test method applicability. Composite samples were obtained by mixing hourly sample volumes collected by an automatic device (Sigma 900 MAX Portable Sampler) during a 24-hour period. Surface water samples were collected from five different Andalusian streams. Samples were collected in glass flasks and transported to the laboratory into boxes containing cool accumulators. When required, they were stored frozen until analysis. Samples were filtered through a 0.22 μm cellulose syringe filter. Filtered sample (950 μL) was transferred to an automatic injector vial and 50 μL of I.S. solution at 25 ng mL^{-1} (each enantiomer) was added to the vial.

2.3. On-line SPE-chiral LC-MS/MS

Automatised sample extraction, enrichment and analytical determination was carried out in an Agilent 1290 Infinity II online SPE-liquid chromatographic system coupled to an Agilent 6495 triple quadrupole (QqQ) mass spectrometer. The LC system included an autosampler with a 100-μL loop and a binary pump for chromatographic elution. The on-line SPE system included a 900-μL loop, a quaternary pump and a two-position 10-port switching valve. The valve allowed simultaneously working with two SPE cartridges, one in loading or conditioning position and the other in elution position. On-line SPE was carried out in reverse-phase Bond Elut Online PLRP-S (12.5 mm × 4.6 mm i.d., 15–20 μm) cartridges (Agilent, Santa Clara, CA, USA).

Chromatographic separation was carried out in a Chiralcel OZ-RH (150 mm × 4.6 mm i.d., 5 μm) column (Daicel, Japan) thermostated at 30 °C and protected by a Chiralcel OZ-RH (10 mm × 4 mm i.d., 5 μm) guard cartridge (Daicel, Japan). The chemical structure of the selected chiral stationary phase can be seen in Fig. S1. Loading, elution, and conditioning programs are summarized in Table S1 in Supplementary Materials. First, on-line SPE sample loop was charged with 700 μL of sample. Then, the sample was transferred to the SPE cartridge by passing water through the loop for 2 min at a flow rate of 2 mL min^{-1}. After sample loading, the switching valve was changed to elution mode allowing mobile phase transferring retained compounds to the analytical column. Mobile phase was composed by 10 mM $HCOONH_4$ containing HCOOH (0.05 %, v/v) (solvent A) and MeOH (solvent B). Elution was carried out in isocratic mode (solvent A: 8 %, v/v; solvent B: 92 %, v/v) at a flow rate of 1.5 mL min^{-1} for 12 min. While loaded SPE cartridge is being eluted, the previously used SPE cartridge is washed and conditioned to be used for the next sample. Cartridges were washed and conditioned by passing pure MeOH at a flow rate of 2 mL min^{-1} for 4.4 min. Then MeOH was linearly replaced by water in 0.1 min, held for 3.4 min. MS/MS analysis was carried out in multiple reaction-monitoring mode (MRM) with ESI source operating in positive mode. The following settings were used: fragmentor, 166 V; capillary voltage, 4000 V; nebuliser pressure, 40 psi; sheath gas flow rate, 12 L min^{-1}, sheath gas temperature, 350 °C; drying gas flow rate, 11 L min^{-1} and drying gas temperature, 250 °C. Two MRM transitions were monitored for each compound. The most abundant transition was used for quantification and the other transition for confirmation. Retention times and MS/MS parameters for each compound can be seen in Table 2. MassHunter Quantitative Analysis Software (Agilent, Santa Clara, USA) was used for data processing.

3. Results and discussion

3.1. Optimisation of the enantiomeric separation

Enantiomeric separation was optimised by testing different types of chiral stationary phases, mobile phase composition (type and proportion of organic modifier, buffer concentration and aqueous phase pH) and flow rates as they are the main factors affecting enantioselective LC separations [35,36]. Separation efficiency was evaluated in terms of enantioresolution (R_s) that was calculated by applying the equation: $R_s = 2(t_{R2}\text{-}t_{R1})/(w_1 + w_2)$ where t_R and w correspond to retention time and peak width, respectively. Optimisation was carried out with an aqueous 0.5 mg L^{-1} mixture standard solution. Chiral columns tested were a protein-based column (Chiralpak AGP (100 mm × 2.1 mm i.d., 5 μm particle size) (Daicel, Tokyo, Japan)); a macrocyclic antibiotic-based column (Astec Chirobiotic V (250 mm × 2.1 mm i.d., 5 μm particle size) (Sigma Aldrich, Steinheim, Germany)) and a cellulose-based column (Chiralcel OZ-RH (150 mm × 4.6 mm i.d., 5 μm particle size) (Daicel, Tokyo, Japan)). They were tested by applying isocratic elution

Table 2
Retention times and MS/MS parameters.

Compound	Retention time (min)	Precursor ion *(m/z)*	Product ions (quantifier/ qualifier) *(m/z)*	CE (eV)	Ion ratio
OFL	*(S)*-Ofloxacin (LEV): 8.75 *(R)*-Ofloxacin: 10.72	362.2	318.2/261.2	20/ 28	74.2
OFL *N*-OX	E1: 8.74 E2: 10.87	378.4	317.2/361.2	20/ 20	84.0
DM-OFL	E1: 5.16 E2: 6.13	348.3	303.9/329.9	20/ 20	74.9
FLU	E1: 7.49 E2: 9.24	262.3	244.0/202.0	20/ 36	60.5
OH-FLU	D1: 5.08 D2: 5.43	278.3	260.0/217.9	12/ 28	57.9
CIP	7.10	332.1	231.0/314.1	40/ 16	86.0
DES-CIP	3.94	306.3	268.0/216.9	28/ 44	84.5
OFL-d_3 (IS)	E1: 6.29 E2: 8.11	365.4	321.2/261.1	20/ 28	91.4

CE: collision energy; D1: first eluting diastereomer; D2: second eluting diastereomer; E1: first eluting enantiomer; E2: second eluting enantiomer; IS: internal standard.

with water and MeOH mixed at different proportions (10, 30, 50, 70 and 90 %, v/v). The best enantiomeric resolutions were achieved by isocratic elution with water:MeOH (10:90, v/v) in the Chiralcel OZ-RH column. Then, water was replaced by $HCOONH_4$ aqueous solutions at concentrations in the range from 1 to 10 mM (1, 2.5, 5, 7.5 and 10 mM). No significant difference was observed neither in retention times nor in enantioresolution. Because of that, the highest tested concentration was selected (10 mM) because of its stronger buffer capacity.

The influence of mobile phase pH (pH 3, 5, 6 and 7) in enantioresolution was tested. The mobile phase was composed of $HCOONH_4$ (10 mM) and MeOH (10:90, v/v). Previously the pH of 10 mM $HCOONH_4$ solution was adjusted to pH 3, 5, 6 and 7 by the addition of HCOOH or ammonium hydroxide solutions. The best enantiomeric separations and sensitivity were obtained at pH 5 and 6. At lower pH values peak intensities decreased At pH values higher than 6, R_s decreased. Then pH range from 5 to 6 was thoroughly studied by adjusting pH of 10 mM $HCOONH_4$ solution to pH 5.2, 5.4, 5.6, and 5.8. The best results for most of the compounds were obtained at pH 5.4 (Fig. S2). At higher pH values (5.6 and 5.8), enantiomers could be separated by their Rs decreased. This fact was especially significant in the case of DM-OFL which enantiomers could not be separated at pH values higher than 5.4. Enantioresolution of DM-OFL increased with the decrease of pH values but chromatographic signals of the other compounds, especially FLU peaks decreased. Because of that, the acidification of 10 mM $HCOONH_4$ solution to pH 5.4 with HCOOH was selected as mobile phase aqueous solvent. That pH value was achieved by the addition of HCOOH to 10 mM $HCOONH_4$ solution at a proportion of 0.05 % v/v.

Once the composition of the aqueous solution was optimised, the influence of the type of organic modifier was studied as it can affect enantioresolution [36,37]. Both on-line SPE cartridges and chiral column manufacturers recommend the use of ACN or MeOH as organic modifiers. Because of that, once tested the use of MeOH, it was replaced by ACN to evaluate its influence on enantioresolution. No significant difference on enantioresolution was observed between MeOH and ACN. Therefore, MeOH was selected as organic modifier because its lower toxicity and price. Once chiral column and mobile phase solvents were optimised, the influence of isocratic elution at different proportions of 10 mM $HCOONH_4$ solution (0.05 % HCOOH, v/v):MeOH were tested (90 %, 92 % and 95 % MeOH) on enantioresolution was evaluated. The Rs of OFL *N*-OX and FLU increased with the increase of MeOH from 90 % to 95 % v/v (see data in Table S1 in Supplementary Materials). Nevertheless, Rs of OFL and DM-OFL improved when MeOH was increased from 90 % to 92 % but decreased when MeOH was increased to 95 %. Because of that the proportion of MeOH in mobile phase was fixed at 92 %. Gradient elution was not tested in spite it is commonly applied in multiresidue methods to short run times. Nevertheless, chiral LC multiresidue methods usually have to be carried out by isocratic elution to achieve a proper enantioseparation [35]. Finally, the influence of mobile phase flow rate on Rs was tested in the range from 0.6 to 1.5 mL min^{-1}. Flow rates were selected taking into account typical flow rates (from 0.5 to 2.5 mL min^{-1}) and pressure limitation (<300 bar) recommended by chiral column manufacturer. The increase of flow rates resulted in higher peak intensities and better peak shapes. Nevertheless, due to column pressure limitation, the highest flow rate tested was 1.5 mL min^{-1} (column pressure: 216 bar). That value was selected as the optimum flow rate.

Therefore, isocratic elution with a 10 mM HCOOH solution containing HCOOH (0.05 %, v/v) and MeOH (8:92 v/v) at a flow rate of 1.5 mL min^{-1} was selected as the best elution conditions. Under such conditions, Rs values in the range from 0.55 to 1.41 were obtained for all the compounds (Table 3). Diastereomers of OH-FLU could be separated but not their enantiomers. Xue et al. [25] achieved the separation of the four enantiomers of OH-FLU by normal-phase LC using a Lux 5 μm Cellulose-4 (250 mm × 4.6 mm i.d. × 5 μm, Phenomenex, USA) column. Nevertheless, enantiomers of FLU had to be separately analysed in a different chromatographic run as reverse-phase mode using a different column (Lux 5 μm Cellulose-2 (250 mm × 4.6 mm i.d., 5 μm, Phenomenex, USA)) was required [25].

3.2. MS/MS optimisation

MS/MS parameters were optimised by direct infusion into the mass spectrometer of individual aqueous standard solutions at 500 μg L^{-1}. Standard solutions were transferred from the LC without column to the mass spectrometer by means of the optimised mobile phase. Optimisation was carried out in both positive and negative ionisation modes. The best results for all the compounds were obtained in positive mode. The optimised LC-MS/MS parameters can be seen in Table 2. The two most abundant product ions were monitored for each compound. The most abundant transition was used for quantification and the other one for confirmation.

3.3. Online SPE optimisation

The influence of sample load volume and type of washing solvents were evaluated. Optimisation was carried out with influent wastewater spiked with the target compounds at 20 μg L^{-1} each. Sample load volumes were tested in the range from 100 to 1000 μL. As can be seen in Fig. 1, signals of all the compounds increased significantly with the increase of sample volume from 400 μL to 700 μL. At higher volumes, response remained constant for most of the compounds, except for FLU and DM OFL enantiomers whose signals decreased. Therefore, 700 μL was selected as sample volume load. MeOH, ACN and MeOH:ACN (50:50, v/v) were tested as washing solvents for used cartridges. Their washing efficiency was evaluated by means of injection of pure water after injection of an influent wastewater sample spiked at 50 μg L^{-1} for each compound. No carry over was observed after cartridge washing with the tested solvents. Therefore, MeOH was selected because its lower price and toxicity and because it is also used as mobile phase solvent. After washing, cartridge was conditioned by passing pure water (Table S1).

3.4. Method validation

The method was validated in influent and effluent wastewater and in surface water in terms of linearity, method detection limits (MDLs),

Table 3
Enantioresolution (Rs), linearity (R^2), method detection limits (MDL) and method quantification limits (MQL) for each sample matrix.

Compound	Rs	Influent wastewater			Effluent wastewater			Surface water		
		R^2	MDL (ng L^{-1})	MQL (ng L^{-1})	R^2	MDL (ng L^{-1})	MQL (ng L^{-1})	R^2	MDL (ng L^{-1})	MQL (ng L^{-1})
(*S*)-OFL (LEV)	1.22	0.992	0.15	0.50	0.997	0.15	0.50	0.998	0.06	0.20
(*R*)-OFL		0.997	15.0	50.0	0.992	3.00	10.0	0.996	3.00	10.0
OFL *N*-OX E1	1.08	0.996	0.30	1.00	0.997	0.30	1.00	0.995	0.30	1.00
OFL *N*-OX E2		0.995	0.30	1.00	0.994	0.30	1.00	0.993	0.30	1.00
DM-OFL E1	0.57	0.998	1.00	50.0	0.992	1.00	10.0	0.996	1.00	10.0
DM-OFL E2		0.995	1.00	50.0	0.995	1.00	10.0	0.990	1.00	10.0
FLU E1	1.41	0.996	0.10	0.33	0.995	0.06	0.20	0.994	0.06	0.20
FLU E2		0.999	0.26	0.86	0.993	0.10	0.33	0.991	0.10	0.33
OH-FLU D1	0.55	0.996	3.00	10.0	0.995	3.00	10.0	0.990	15.0	50.0
OH-FLU D2		0.997	3.00	10.0	0.992	3.00	10.0	0.990	15.0	50.0
CIP	–	0.995	0.15	0.20	0.997	0.15	0.20	0.993	0.15	0.20
DES-CIP	–	0.991	3.00	10.0	0.991	3.00	10.0	0.990	15.0	50.0

Rs: enantioresolution; R^2: correlation coefficients; D1: first eluting diastereomer; D2: second eluting diastereomer; E1: first eluting enantiomer; E2: second eluting enantiomer.

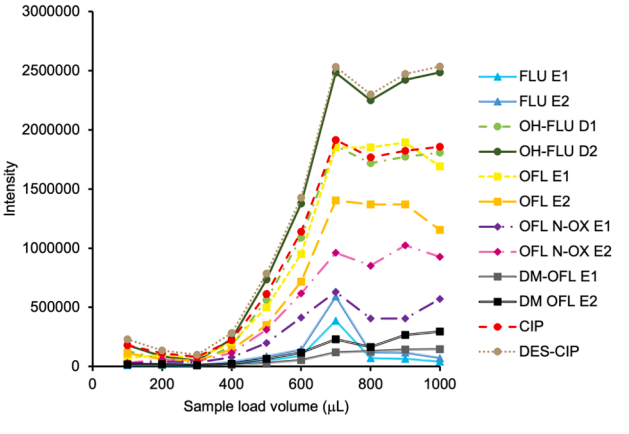


Fig. 1. Influence of sample load volume on sensitivity.

method quantification limits (MQL), accuracy, precision, and selectivity. Previously, the presence of matrix effect (ME) in each type of matrix was evaluated. It was quantified at three concentration levels (0.1 µg L^{-1}, 10 µg L^{-1} and 25 µg L^{-1}) by comparison of the peak area of spiked samples ($A_{spiked\ sample}$), after subtracting the peak area obtained from non-spiked sample ($A_{non\text{-}spiked\ sample}$), and in water standard solutions ($A_{standard}$) following equation: ME (%) = $(A_{spiked\ sample} - A_{non\text{-}spiked\ sample} - A_{standard})/A_{standard}) \times 100$). Signal suppression was observed for all compounds in all matrices (Table 4). Similar ME were reported by the only one reported method for the enantioselective determination of fluoroquinolones in aqueous environmental samples [24]. Therefore matrix-matched calibration curves were used for quantification. In addition, IS solution was added to samples before analytical determination as an additional ME correction.

Linearity was evaluated in the range from 1 ng/L to 75 µg L^{-1}. Thirteen-point matrix-matched calibration curves were prepared in triplicate for each matrix by spiking each type of sample (influent and effluent wastewater and surface water) with the target analytes and subjecting them to the analytical method. MDLs and MQLs were calculated from samples spiked at low concentration levels. MDLs were fixed at concentrations providing signal to noise ratios of 3. MQLs were fixed at concentrations providing signal to noise ratios of 10. For some compounds and matrices poor Rs was obtained at concentrations providing signal to noise ratios of 10. In such cases, MQL values were increased to concentrations providing Rs values of at least 0.55. MQL values for most of the compounds and matrices were in the range from 0.20 to 10 ng/L (Table 3). MDL value for CIP (0.20 ng/L in all matrices) is 445-fold lower than the maximum acceptable MDL (89.0 ng/L) fixed by the European Union for its determination in surface water [5]. MQLs values for most of the compounds and matrices were in the range from 5 to 10-fold lower than those reported by Castrignanò et al. for the determination of OFL, FLU, DM-OFL and OFL *N*-OX enantiomers and achiral CIP and DES-CIP in environmental water samples by off line SPE [24] and 24,000 and 1000-fold lower than those reported by Xue et al. for the determination of FLU and OH-FLU enantiomers, respectively, in water samples by off line SPE (8 and 10 µg L^{-1}, respectively) [25]. Calibration curves were linear in the concentration range from MQL value of each compound to 25 µg L^{-1} in all matrices (Table S3 in Supplementary Materials). Correlation coefficients were higher than 0.99 (Table 3).

Accuracy and precision were evaluated from samples spiked at three concentration levels (0.1 µg L^{-1}, 10 µg L^{-1} and 25 µg L^{-1}) in triplicate.

Table 4
Matrix effect (ME%) for each sample matrix.

Compound	Influent wastewater			Effluent wastewater			Surface water		
	0.1 µg L^{-1}	10 µg L^{-1}	25 µg L^{-1}	0.1 µg L^{-1}	10 µg L^{-1}	25 µg L^{-1}	0.1 µg L^{-1}	10 µg L^{-1}	25 µg L^{-1}
(*S*)-OFL (LEV)	−78.3	−40.7	−36.2	−65.1	−49.7	−36.2	−85.6	−34.3	−16.5
(*R*)-OFL	−70.6	−28.6	−25.0	−60.0	−44.5	−37.1	−81.4	−24.4	−4.95
OFL *N*-OX E1	−84.8	−32.2	−23.8	−82.8	−44.4	−28.3	−89.5	−39.2	−20.6
OFL *N*-OX E2	−82.8	−27.8	−20.7	−81.6	−45.0	−6.96	−90.8	−32.6	−11.8
DM-OFL E1	−73.0	−61.7	−50.5	−81.1	−60.6	−49.6	−85.4	−75.9	−64.9
DM-OFL E2	−61.5	−35.5	−30.3	−63.6	−46.1	−21.4	−72.1	−57.0	−42.3
FLU E1	−109	−43.5	−24.4	−107	−54.7	−39.9	−107	−65.4	−48.5
FLU E2	−109	−37.5	−22.7	−109	−54.8	−23.7	−112	−56.9	−37.0
OH-FLU D1	−87.3	−61.0	−53.0	−83.1	−66.6	−59.2	−92.3	−94.8	−93.1
OH-FLU D2	−79.2	−42.2	−31.0	−75.1	−55.9	−30.0	−92.2	−88.8	−82.9
CIP	−76.7	−41.5	−31.6	−75.6	−50.3	−20.1	−77.5	−46.7	−26.8
DES-CIP	−103	−48.7	−38.2	−150	−67.5	−45.6	−92.4	−90.4	−86.1

D1: first eluting diastereomer; D2: second eluting diastereomer; E1: first eluting enantiomer; E2: second eluting enantiomer.

Accuracy was calculated by comparison of the concentration obtained from spiked samples using matrix-matched calibration curves ($C_{spiked\ sample}$), after blank correction (C_{blank}), with the spike concentration ($C_{spike\ concentration}$) by applying equation: A (%) = ($C_{spiked\ sample}$ - C_{blank}) × 100/$C_{spike\ concentration}$. Precision was calculated as inter-day repeatability. It was expressed as relative standard deviation (RSD, %). Accuracy values were in the range from 61.4 % to 122 % whereas RSD values were below 13.6 % for all the compounds, concentration levels and matrices (Table 5). Method selectivity was evaluated by comparison of the chromatograms of spiked and non-spiked surface water and wastewater samples. No peak was observed at the retention times of the target compounds. In Fig. 2 it can be seen the chromatograms of a standard solution and spiked and non-spiked influent wastewater samples. Only two methods have been reported for the enantiomeric determination of fluoroquinolones in environmental waters [24,25]. Resolution values achieved by the proposed method (1.22, 1.08, 0.57 and 1.41 for OFL, OFl *N*-OX, DM-OFL and FLU, respectively, (Table 3)) are higher than those reported by Castrignanò et al. (0.89, 1.07, 0.56 and 1.10 for OFL, OFl *N*-OX, DM-OFL and FLU, respectively) [24]. Xue et al., 2018 [25] reported R_s for FLU and OH-FLU higher than 2, but they were separately analysed, FLU was analysed by reverse-phase chromatography elution mode whereas OH-FLU was analysed by normal-phase chromatography elution mode. Longer run times were required, 30 min for FLU analysis and 20 min for OH-FLU analysis [25].

3.5. Greenness assessment of the analytical procedure

On-line SPE method proposed is greener than the widely reported off-line SPE methods for the determination of pharmaceuticals in environmental water samples. No plastic waste is generated; less energy consumption is required because no evaporation step is needed; safety of the operators is improved as sample treatment and analytical determination are automatized. Two greenness assessment tools, GAPI [38] and AGREE [39], have been applied to evaluate the greenness degree of the whole analytical procedure, from sampling to analytical determination. GAPI tool takes into account sample preparation (collection, preservation, transport, storage, type of method (direct or indirect), scale of extraction, solvents/reagents used and additional treatments required); reagents and solvents used (amounts, health and safety hazards); and instrumentation (energy consumption, occupational hazard, waste production and waste treatment) [38]. The assessment criteria in AGREE method is taken from 12 principles of green analytical chemistry detailed in Table S5. GAPI and AGREE pictograms corresponding to the greenness assessment of the proposed procedure can be seen in Figs. S3 and S4, respectively. The score for each criteria in AGREE pictogram is detailed in Fig. S5. These assessment tools are more valuable when comparing procedures for the determination of the same target compounds in the same type of samples [38,39]. As mentioned above, only the method proposed by Castrignanò et al. [30] is comparable in terms of similar target compounds and samples. The application of GAPI and AGREE tools to such method would results in similar scores for sample preparation, solvents used and instrumentation, but poorer results would be obtained in terms of occupational hazard, waste production and waste treatment because off-line SPE is applied for sample extraction.

3.6. Method application

The method was applied to the determination of the target compounds in influent and effluent wastewater and surface water. Parent compounds (*S*)-OFL (LEV), FLU enantiomers and CIP were detected in all the analysed samples (Table S6). OFL *N*-OX enantiomers were also detected but at concentrations lower than their MQL values. The enantiomer (*S*)-OFL was quantified in all the samples at concentrations in the range from 1.17 ng/L in surface water to 15.1 ng/L in influent wastewater whereas (*R*)-OFL was detected in surface water at concentrations lower than its MQL values but not in wastewater. The higher concentrations of (*S*)-OFL can be explained by the drug administration not only of the racemic drug but also of the pure enantiomer (*S*)-OFL (levofloxacin) whereas the higher concentrations (*R*)-OFL in surface water could be due to a transformation of (*S*)-OFL once released into the environment. The enantiomeric fraction (EF) of FLU was approximately 0.5 in all the analysed samples. The EF was calculated using equation: EF = $E_1/(E_1 + E_2)$, where E_1 and E_2 are the concentrations of the first and second eluted enantiomers, respectively. Nevertheless, whereas OH-FLU D2 was detected in all influent wastewater samples at concentrations in the range from 19.4 to 85.9 ng/L and in one effluent wastewater sample (at 41.3 ng/L), OH-FLU D1 was not detected in any sample. Nevertheless, a higher number of samples must be analysed to obtain reliable conclusions about their behaviour in WWTPs and in the environment.

4. Conclusions

This work represents the first analytical method for automatised sample extraction and chiral determination of relevant fluroquinolones in complex environmental water samples. The method has been optimised and validated for the determination of three fluoroquinolones,

Table 5
Accuracy (A%) and precision, expressed as relative standard deviation (RSD%), at three concentration levels for each sample matrix.

Compound	Influent wastewater						Effluent wastewater						Surface water					
	0.1 μg L^{-1}		10 μg L^{-1}		25 μg L^{-1}		0.1 μg L^{-1}		10 μg L^{-1}		25 μg L^{-1}		0.1 μg L^{-1}		10 μg L^{-1}		25 μg L^{-1}	
	A (%)	RSD (%)	A (%)	RSD (%)	A (%)	RSD (%)	A (%)	RSD (%)	A (%)	RSD (%)	A (%)	RSD (%)	A (%)	RSD (%)	A (%)	RSD (%)	A (%)	RSD (%)
(*S*)-OFL (LEV)	76.9	2.6	116	5.9	95.5	0.6	65.8	1.7	98.6	2.5	77.4	3.0	104	1.9	101	2.6	94.4	1.7
(*R*)-OFL	80.5	10.6	111	12.0	99.1	4.1	102	11.5	114	5.2	99.8	5.6	107	3.3	113	2.4	102	1.9
OFL *N*-OX E1	61.5	3.6	114	4.2	97.3	2.0	89.9	2.6	98.4	3.6	79.5	4.4	92.3	1.1	103	1.5	95.3	1.6
OFL *N*-OX E2	112	2.3	114	3.8	96.6	2.8	97.8	10.5	114	4.1	98.4	3.4	93.4	11.0	118	2.4	97.9	2.9
DM-OFL E1	75.6	4.7	105	3.8	98.9	2.4	108	9.1	111	4.1	94.3	8.7	86.0	6.7	116	1.8	98.6	8.5
DM-OFL E2	116	10.4	102	4.3	96.4	4.4	78.6	5.6	98.3	3.0	104	6.4	115	13.6	102	9.2	101	8.9
FLU E1	78.5	6.8	109	6.1	97.0	3.7	70.0	3.8	98.3	4.3	71.6	3.4	73.4	8.1	111	10.2	96.8	9.8
FLU E2	68.9	8.9	102	11.2	97.9	8.7	62.4	3.5	97.2	2.8	104	1.8	99.5	11.8	116	5.2	97.4	8.7
OH-FLU D1	96.5	4.4	98.6	3.5	78.2	3.9	61.4	3.9	96.6	4.4	63.1	5.1	116	9.2	106	9.6	99.5	5.7
OH-FLU D2	108	5.0	98.1	1.0	95.8	3.1	71.3	5.0	96.8	3.7	106	5.2	81.1	11.6	100	10.3	101	5.0
CIP	102	1.4	114	5.3	97.7	3.8	122	13.6	98.6	2.4	106	3.5	118	1.1	102	7.1	101	6.2
DES-CIP	68.4	12.9	98.4	7.7	96.8	2.7	65.0	1.0	98.3	7.5	106	3.2	102	8.1	119	3.5	96.7	10.1

D1: first eluting diastereomer; D2: second eluting diastereomer; E1: first eluting enantiomer; E2: second eluting enantiomer.

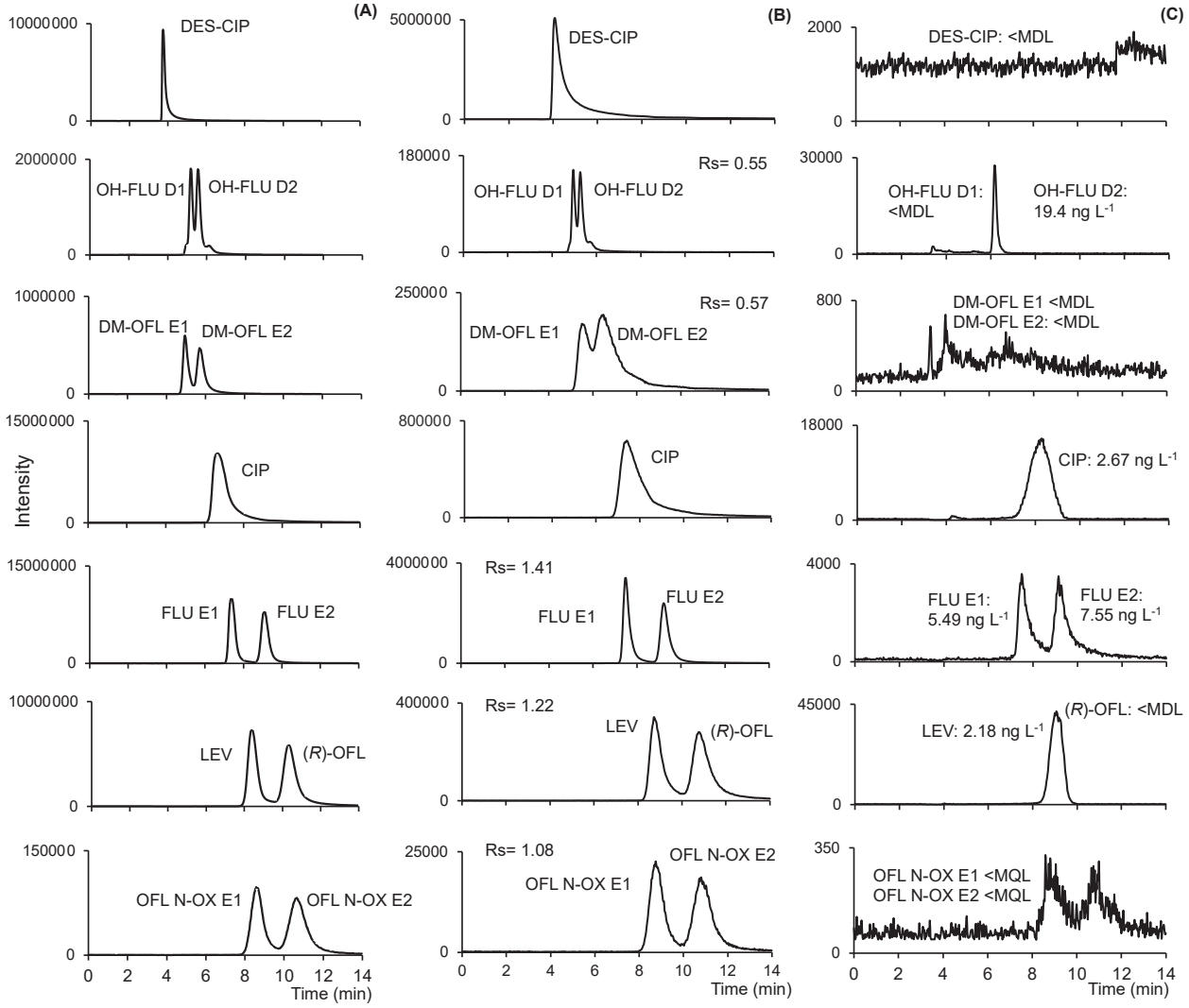


Fig. 2. MRM chromatograms of a 0.5 μg/L standard solution (A), and of an influent wastewater sample spiked at 0.5 μg/L(B) and non-spiked (C).

two of them of wide use in human medicine and the other in veterinary medicine, and four of their metabolites. Five of the seven target compounds were chiral compounds. Total analysis time was 14 min. The method was validated for its application to influent and effluent wastewater and surface water. Enantioresolution was in the range from 0.55 to 1.41 for all the chiral compounds and sample matrices. Good accuracy values (from 61.4 to 122 %), MQLs (lower than 50 ng/L for all the compounds in all the matrices) and precision (RSD% < 13.6) were obtained. The application of the method revealed the transformation of LEV to (R)-OFL in surface water and the prevalence of OH-FLU D2 with respect to OH-FLU D1 in influent wastewater. The proposed method reveals the applicability of on-line SPE in the enantioselective determination of fluoroquinolones by chiral LC-MS/MS. It can constitute a promising tool in the automatisation of analytical methods reducing labour intensity, processing time, solvent consumption, and waste generation even in complex analytical determinations such as enantiomeric separations.

CRediT authorship contribution statement

Carmen Mejías: Investigation, Visualization, Writing – original draft. **Juan Luis Santos:** Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft. **Julia Martín:** Methodology, Writing – original draft. **Irene Aparicio:** Conceptualization, Resources, Validation, Writing – review & editing. **Esteban Alonso:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the Ministerio de Ciencia e Innovación-Agencia Estatal de Investigación (grant number: PID2020-117641RB-I00, funded by MCIN/AEI/10.13039/501100011033) and by the Junta de Andalucía, Consejería de Economía y Conocimiento (grant reference: US-1254283). C Mejías acknowledges the University of Seville for her predoctoral grant (VI PPIT-US 2021 II.2A).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2022.108217.

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