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Enantioselective quantification of fluoxetine and norfluoxetine by HPLC in wastewater effluents

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HIGHLIGHTS

- Enantioseparation of fluoxetine and norfluoxetine on a Chirobiotic V.
- Enantioselective HPLC-FD method for quantification of fluoxetine and norfluoxetine.
- FLX was degraded in wastewater effluent samples.

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ABSTRACT

Microbial degradation is the most important process to remove organic pollutants in Waste Water Treatment Plants. Regarding chiral compounds this process is normally enantioselective and needs the suitable analytical methodology to follow the removal of both enantiomers in an accurate way. Thus, this paper describes the development and validation of an enantioselective High Performance Liquid Chromatography with Fluorescence Detection (HPLC-FD) method for simultaneous analysis of fluoxetine (FLX) and norfluoxetine (NFLX) in wastewater effluents. Briefly, this method preconcentrated a small volume of wastewater samples (50 mL) on 500 mg Oasis MCX cartridges and used HPLC-FD with a vancomycin-based chiral stationary phase under reversed mode for analyses. The optimized mobile phase was EtOH/aqueous ammonium acetate buffer (92.5/7.5, *v/v*) at pH 6.8. The effect of EtOH percentage, buffer concentration, pH, column oven temperature and flow rate on chromatographic parameters was systematically investigated. The developed method was validated within the wastewater effluent used in microcosms laboratory assays. Linearity ($R^2 > 0.99$), selectivity and sensitivity were achieved in the range of 4.0–60 ng mL^{-1} for enantiomers of FLX and 2.0–30 ng mL^{-1} for enantiomers of NFLX. The limits of detection were between 0.8 and 2.0 ng mL^{-1} and the limits of quantification were between 2.0 and 4.0 ng mL^{-1} for both enantiomers of FLX and the enantiomers of its demethylated metabolite NFLX. The validated method was successfully applied and proved to be robust to follow the degradation of both enantiomers of FLX in wastewater samples, during 46 days.

1. Introduction

Fluoxetine (FLX) (Table 1) is a fluorinated pharmaceutical worldwide prescribed as racemic mixture to the treatment of depression and other neurological or psychiatric disorders. It acts by selective inhibition of the serotonin reuptake pump, increasing the extracellular catecholamines, such as serotonine, dopamine and norepinephrine. In the human body FLX is metabolized to norfluoxetine (NFLX), its demethylated active metabolite (Table 1). NFLX is reported to be slightly more active than the parent compound (Stokes and Holtz, 1997). Enantiomers of FLX have a similar potency as inhibitors of the serotonin reuptake pump whereas enantiomers of NFLX act differently, with (*S*)-NFLX being the more potent enantiomer regarding its inhibition capacity (Fuller et al., 1992).

FLX and NFLX have been detected in Waste Water Treatment Plants (WWTP) effluents (Trenholm et al., 2006; Schultz and Furlong, 2008; Vasskog et al., 2008), in surface waters (Gros et al., 2008; Fernández et al., 2010) and during drinking water

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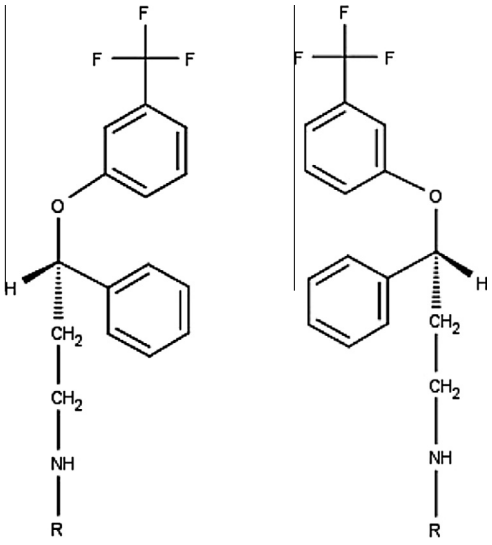
Table 1
Abbreviation and structures of target enantiomers, its molecular formula, molecular weight and pKa value.

Compound	Stereoconfiguration	Formula	M_w (g mol^{-1})	pKa
Fluoxetine (FLX) R = CH_3 Norfluoxetine (NFLX) R = H	 (S) (R)	$C_{17}H_{18}F_3NO$ $C_{16}H_{16}F_3NO$	309.33 295.30	10.05 9.05

treatment processes (Focazio et al., 2008; Schultz and Furlong, 2008; Benotti et al., 2009; Bruce et al., 2010). Both FLX and NFLX have been detected in fish tissues from municipal effluent-dominated streams (Brooks et al., 2005; Chu and Metcalfe, 2007; Ramirez et al., 2007; Ramirez et al., 2009). One of those studies reported NFLX in higher frequency and concentrations than FLX (Ramirez et al., 2009) and another reported only the presence of the metabolite in high frequency (Ramirez et al., 2007). There are some works that analyse the concentration of each enantiomer of FLX in both raw and treated wastewaters, although the NFLX concentration is not addressed. In a recent study, Barclay et al. found that (*S*)-FLX and (*S*)-NFLX had a slightly higher concentration than (*R*)-FLX and (*R*)-NFLX in both raw and treated wastewaters, with no significant differences in enantiomeric fractions (EF) of both FLX and NFLX (Barclay et al., 2012). On the other hand, MacLeod et al. reported that (*R*)-FLX had a higher concentration in both raw and treated wastewaters with a higher degradation of (*R*)-FLX during the WWTP process, which led to an enrichment of the (*S*)-FLX in the effluent and to a significant variation in EF (MacLeod et al., 2007).

Recently, FLX and NFLX were proposed in a list of 10 pharmaceuticals potentially dangerous for the environment (Santos et al., 2013). FLX was reported as toxic at low concentrations to several aquatic species and as endocrine disruptor (Brooks et al., 2003; Foran et al., 2004; De Andrés et al., 2009; Gonzalez-Rey and Bebianno, 2013). A recent study reported FLX as toxic to a freshwater fish, the fathead minnows (*Pimephales promelas*), after exposure to this compound, with NFLX being detected in their brain tissues (Schultz et al., 2011). Paterson and Metcalfe found that NFLX can achieve higher concentration than FLX in fish after their exposure to FLX, and underlined the bioaccumulation of FLX and NFLX in such organisms (Paterson and Metcalfe, 2008). NFLX was also reported to be responsible for spawning and parturition effects in bivalves (Fong and Molnar, 2008). Enantioselective toxicity of FLX was also demonstrated using this species, being (*S*)-FLX more toxic than the (*R*)-FLX (Stanley et al., 2007). FLX was reported as highly toxic to protozoan and daphnids species, being the protozoan more affected by the enantioselectivity than daphnids (De Andrés et al., 2009). In that study, (*S*)-FLX was more toxic to both species, although (*R*)-FLX exhibited greater toxicity to the crustacean than to the protozoan species (De Andrés et al., 2009). Therefore, the presence of such compounds in the environment and their enantioselectivity associated with the biodegradation and ecotoxicity are important concerns (De Andrés et al., 2009; Stanley and Brooks, 2009). In this context, analytical methods able to quantify FLX and NFLX enantiomers are essential to achieve accurate data regarding their fate and the enantiomeric risk assessment. Enantioselective HPLC is a fast, selective and efficient technique that is usually performed with a stationary phase able to discriminate enantiomers, but validated of enantioselective methods for environmental analysis is scarce and need more investigation. Additionally the systematic investigation to optimize the best chemo and enantioresolution, normally, is not performed with systematic variation of the chromatographic conditions. Despite the several commercial chiral stationary phases (CSP) available, polysaccharides and macrocyclic antibiotics are normally preferred (Hashem et al., 2011) because of their broad application and the possibility to operate in normal, reversed, polar organic or polar ionic mode (Cass et al., 2003; Sousa et al., 2004; Fernandes et al., 2012a; Fernandes et al., 2012b; Ribeiro et al., 2013a).

This work presents the development and validation of a Solid Phase Extraction (SPE) procedure followed by an enantioselective HPLC method using Chirobiotic V and fluorescence detection to monitor the degradation of FLX enantiomers by wastewater and the possible formation of its metabolite NFLX. To the best of our knowledge, this is the first report demonstrating the chemoselectivity and enantioselectivity on separation of FLX and its demethylated metabolite NFLX by HPLC with fluorescence detection. Thus, the chromatographic conditions achieved allowed the validation of a method for quantification of the target compounds in wastewater effluents. It is important to emphasise that the validated method can be adjusted for diverse environmental matrices and even for biological matrices, such as plasma and tissues.

2. Experimental

2.1. Chemicals and materials

Ethanol (EtOH), methanol (MeOH) and acetonitrile (ACN) (HPLC grade) were purchased from Fisher Scientific UK Limited

(Leicestershire, UK). Acetic acid 100% (HAc) Chromanorm (HPLC grade) and triethylamine ⩾99% (TEA) were obtained from VWR International (Fontenay-sous-Bois, France). Ammonium acetate and sulphuric acid were purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (Steinhein, Germany), respectively. Ultrapure water was supplied by a Milli-Q water system. HPLC grade solvents were filtered with 0.45 μm glass microfiber filters (Whatman™).

Fluoxetine hydrochloride (FLX), (*S*)-(+)-fluoxetine hydrochloride ((*S*)-FLX), (*R*)-(−)-fluoxetine hydrochloride ((*R*)-FLX) and norfluoxetine (NFLX) were purchased from Sigma–Aldrich (Steinhein, Germany). (*S*)-norfluoxetine ((*S*)-NFLX) was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). All reference standards were of >98% purity.

Stock solutions of the individual compounds were prepared by dissolution of known amounts of each compound in EtOH, to obtain a concentration of ca. 1000 μg mL^{-1} of the enantiomeric mixtures of FLX, NFLX and (*S*)-NFLX, and of ca. 500 μg mL^{-1} of the individual enantiomers of FLX.

The cartridges used for SPE were Oasis® MCX (Mixed-mode Cation exchange) (500 mg, 6 mL) acquired from Waters (Milford, Massachusetts, USA).

2.2. Equipment

Chromatographic analysis were performed using a Shimadzu UFLC prominence system equipped with two Pumps LC-20AD, an Autosampler SIL-20AC, an Oven CTO-20AC, a Degasser DGU-$20A_5$, a System Controller CBM-20A and a LC Solution, Version 1.24 SP1 (Shimadzu). A Shimadzu RF-10AXL Fluorescence Detector (FD) was coupled to the LC System, with the excitation and emission wavelengths set at 230 and 290 nm, respectively. A vancomycin-based CSP, Chiral HPLC Astec Chirobiotic™ V, 5 μm (15 cm × 0.46 cm ID) supplied by SUPELCO analytical (Sigma–Aldrich, Steinhein, Germany) was used in the chromatographic analysis.

2.3. Chromatographic conditions

Several chromatographic conditions were attempted. After achieving the first baseline conditions on reversed mode, the effects of several factors on the chromatographic separation were studied, including EtOH content, buffer concentration, pH, column oven temperature and flow rate. The optimized mobile phase was EtOH/10 mM aqueous ammonium acetate buffer (92.5/7.5, *v/v*), pH 6.8, performed at isocratic mode with a flow rate of 1.0 mL min^{-1} and the column oven set at 20 °C. The pH adjustments were done with pH meter HI 2210 pH/°C bench meter from Hanna Instruments. Volume of injection was 20 μL.

The elution order of enantiomers of FLX was determined by the injection of a solution of each enantiomer of FLX separately, namely (*S*)-FLX and (*R*)-FLX. The elution order of enantiomers of NFLX was determined by the injection of a solution of (*S*)-NFLX.

2.4. Sample collection and microcosms tests at laboratory scale

Water samples of the final effluent of the secondary clarifier of WWTP of Parada, localized on the North of Portugal, were collected at July 2012 in pre-rinsed amber glass bottles (2 L) and transported at 4 °C to the laboratory. The degradation assays were performed into 2 L pre-rinsed glass bottles covered with aluminium foil with 400 mL of wastewater sample spiked with the enantiomeric mixture of FLX to obtain a concentration of each enantiomer of 25 ng mL^{-1}. These assays were done in quadruplicate on a shaker (130 rpm) at 25 °C. Non spiked controls were assayed at the same conditions. The concentration of each enantiomer was monitored during 46 days using the described SPE-HPLC-FD validated method.

2.5. Solid phase extraction

Off-line SPE was performed on Oasis® MCX 500 mg sorbent cartridges using a Varian vacuum extraction device. The optimized SPE procedure was carried out accordingly to work published elsewhere (Madureira et al., 2010). Briefly, the cartridges were conditioned sequentially with 8 mL of methanol and 8 mL of ultrapure water, at a flow rate of 1 mL min^{-1}. The effluent wastewater samples were acidified at pH 2, adjusted with sulphuric acid, and then were percolated through the cartridges at a constant flow rate of 10 mL min^{-1} using a vacuum manifold system connected to a vacuum pump. Afterwards, the cartridges were washed with 8 mL of aqueous solution of 2% formic acid and then dried under vacuum for 30 min to dry out residual water. Elution was performed in two steps, the first with 8 mL of methanol to eluate acids and neutrals, the second with 8 mL of methanolic solution of 5% ammonium hydroxide to eluate the basic compounds, at 1 mL min^{-1}. The extracts of the second elution were evaporated to dryness in a vacuum concentrator, model Centrivap Centrifugal concentrator with cold trap (−50 °C model) (Labconco, Kansas City, USA). The residues were reconstituted in 500 μL of EtOH, and 20 μL were injected into the HPLC system.

2.6. Method validation parameters

The method was validated according to the International criteria (Validation of Analytical Procedures: Text and Methodology Q2(R1), 1996) and previous work (Madureira et al., 2009; 2010; Ribeiro et al., 2012; Ribeiro et al., 2013a; 2013b) considering the following parameters: selectivity, linearity and range, limits of detection (LOD) and quantification (LOQ), accuracy, recovery and precision. After collection, 50 mL of water samples were vacuum filtered through a 0.45 μm Glass microfiber filters (Whatman™) for matrix calibration standards. Filtered samples were immediately spiked and submitted to SPE procedure.

Selectivity was verified by comparing the chromatograms of standards dissolved in EtOH, standards extracted from the spiked wastewater and extracts of wastewater. Linearity and range were evaluated using calibration curves prepared in triplicate with a set of six different standard concentrations of the spiked wastewater: 2; 4; 7; 10; 20; 30 ng mL^{-1} for enantiomers of the NFLX and 4; 8; 14; 20; 40; 60 ng mL^{-1} for enantiomers of the FLX. Calibration curves were drawn by the injection of 20 μL of the reconstituted 500 μL extract dissolved in ethanol. LOD and LOQ were calculated from spiked samples through the signal-to-noise (S/N) ratio of 3 for LOD and 10 for LOQ.

Three quality control (QC) standard solutions, with three different concentrations (3.5, 15 and 28 ng mL^{-1} for enantiomers of the NFLX and 6.0, 30 and 56 ng mL^{-1} for enantiomers of the FLX), each one in triplicate, were prepared to assess the accuracy and intra- and inter-batch precision. The accuracy of the method was evaluated as the percentage of agreement between the concentrations of the standards analysed in the SPE extracts and the nominal concentration (Validation of Analytical Procedures: Text and Methodology Q2(R1), 1996; Bioanalytical method validation: Guidance for Industry, 2001; Madureira et al., 2009; 2010). Precision was expressed by the relative standard deviation (RSD) of the replicate measurements. Fortified matrices at the same QC concentrations were used for the recovery assays and the evaluation of efficiency of the extraction. It was calculated by comparing the peak areas of the standards from the spiked matrix with those of similar concentrations in ethanolic standard solutions.

3. Results and discussion

3.1. Chiral chromatographic separation

Several attempts using different CSP and elution modes were done to achieve the best separation of enantiomers of FLX and NFLX in a short chromatographic run. Chirobiotic V, a CSP based on vancomycin, a macrocyclic antibiotic, has demonstrated great versatility due to its several types of molecular interactions namely ionic, H-bond, π–π, dipole, hydrophobic and steric, and its several inclusion sites that are important to the enantioselectivity. The molecular recognition mechanism is not yet completely understood and there are no publications about detailed NMR studies or X-ray crystallographic studies in combination with analytical chiral separations (Scriba, 2012). Non conclusive studies were taken to understand mechanisms involved in the relation structure – separation using various classes of analytes (Scriba, 2012). This CSP is compatible with all modes of mobile phase conditions. However, polar ionic and reversed modes were selected due to the basic nature of the enantiomers.

Polar ionic mode consists in a 100% of polar organic solvent and the addition of salt, that makes it buffered, which is important to allow the separation of basic compounds (Hashem et al., 2011). Several proportions of EtOH/MeOH from 50/50 to 100/0 (*v*/*v*) with 0.075% TEA and 0.225% HAc were attempted. These first trials were done on the basis that a polar ionic mode combines the principles of both reversed and polar organic mode. FLX enantiomers were enantioresolved using EtOH/MeOH (50/50, *v*/*v*), 0.075% TEA, 0.225% HAc, pH 6.8, that are similar conditions of our previous work (Ribeiro et al., 2013b), however NFLX enantiomers were not enantioresolved with this mobile phase (Fig. 1). So, reversed mode condition was attempted with many proportions of EtOH/15 mM aqueous ammonium acetate buffer (pH 5.2)/ACN, and only led to enantioseparation of FLX and partial enantioseparation of NFLX (data not shown). The alternative use of MeOH/15 mM aqueous ammonium acetate buffer (90/10, *v*/*v*) did neither result in an ideal enantioresolution of NFLX nor any degree of separation between (*R*)-NFLX and (*S*)-FLX. The introduction of EtOH in the latter mobile phase (MeOH/EtOH/15 mM aqueous ammonium acetate buffer, 45/45/10, *v*/*v*) improved the enantioresolution of both compounds and led to a partial resolution between NFLX and FLX. As such, MeOH was almost totally substituted by EtOH (MeOH/EtOH/15 mM aqueous ammonium acetate buffer, 5/85/10, *v*/*v*), achieving a better resolution of both pairs of enantiomers. The content of MeOH was completely substituted by EtOH to achieve a baseline separation (Fig. 2). Besides, according to the green chromatography concept, combination of EtOH and ammonium acetate is considered the best mobile phase choice concerning environment impact (Sandra et al., 2010). At this point, different chromatographic conditions were evaluated, namely EtOH content, buffer concentration, pH, column oven temperature and flow rate, in order to improve the chromatographic parameters to allow the monitorization of WWTP effluents.

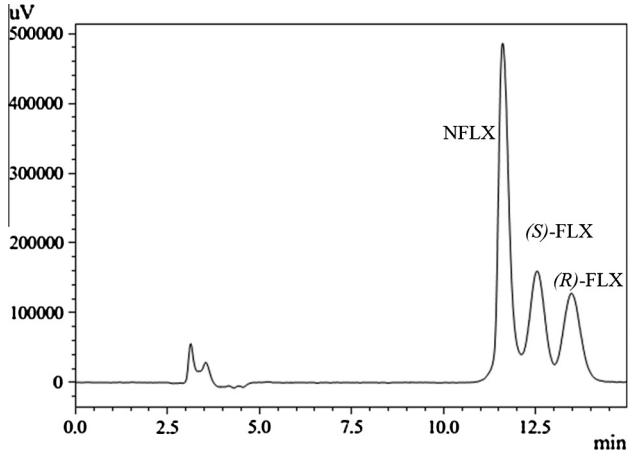


Fig. 1. Chromatogram demonstrating the optimization process of the method, with the following chromatographic conditions: EtOH/MeOH (50/50, *v*/*v*), 0.075% TEA, 0.225% HAc, pH 6.8; flow 0.8 mL min^{-1}; column oven temperature 20 °C.

3.1.1. Effect of ethanol content

EtOH content was the most important factor to achieve chemo and enantioselectivity of FLX and NFLX. Fig. 2 represents the chromatograms obtained with the variation of EtOH content, showing that as the percent of EtOH increases, the chemoselectivity and enantioresolution of enantiomers improved. Fig. 2a shows that a content of 95% of EtOH leads to a very good resolution, however with high elution time and peak broadening. Fig. 2b represents the chromatogram corresponding to the best compromise between the enantioseparation and the elution time, with 92.5% of EtOH. On the contrary, Fig. 2c shows that the decrease of ethanol percentage to 90% leads to a shorter elution time but with loss of chemoselectivity with poor resolution of (*R*)-N-FLX and (*S*)-FLX.

3.1.2. Effect of buffer concentration

The variation of buffer concentration had a slight effect on the resolution (Table 2). The shortening of the elution time required an increase in the buffer concentration to 10 mM (Table 2). This concentration of aqueous ammonium acetate buffer combined a good resolution with a lower chromatographic run time and consequently less solvent consumption.

3.1.3. Effect of pH

The decrease of pH lowered the enantioresolution at a great extent. The pH adjustment of the mobile phase plays an important role in the optimization of the chromatographic separation to achieve the best resolution, due to the basic nature of target compounds and the functional groups of the CSP that strongly interact by ionic interactions (Beesley and Lee, 2009). In order to achieve the highest resolution combined with a short run time, the pH

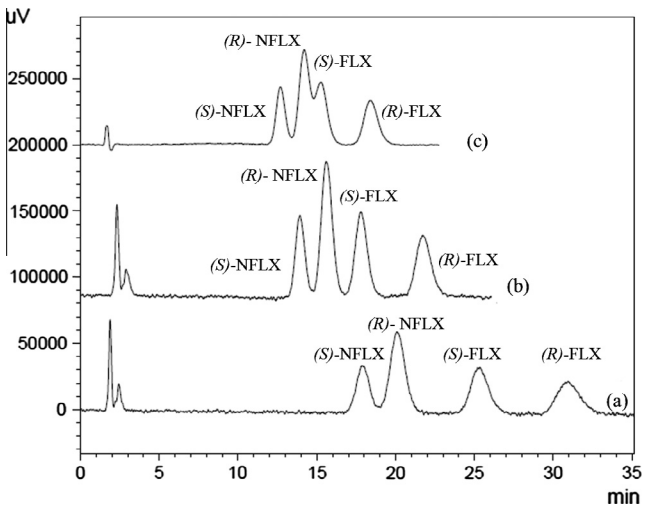


Fig. 2. Chromatograms showing the separation in the following order of (*S*)-NFLX, (*R*)-NFLX, (*S*)-FLX and (*R*)-FLX, with the constant chromatographic conditions: pH 6.8; flow 1.2 mL min^{-1}; column oven temperature 20 °C. Mobile phase: (a) EtOH/15 mM aqueous ammonium acetate buffer (95/5, *v*/*v*); (b) EtOH/15 mM aqueous ammonium acetate buffer (92.5/7.5, *v*/*v*); (c) EtOH/15 mM aqueous ammonium acetate buffer (90/10, *v*/*v*).

Table 2
Chromatographic parameters (Rs) obtained for the variation of a single chromatographic condition, when the other conditions were: EtOH/10 mM aqueous ammonium acetate buffer (92.5/7.5, *v/v*), pH 6.8; flow 1.0 mL min^{-1}; column temperature 20 °C.

Resolution/enantioresolution	Rs (*S*)-NFLX/(*R*)-NFLX	Rs (*R*)-NFLX/(*S*)-FLX	Rs (*S*)-FLX/(*R*)-FLX
Buffer concentration			
2.5 mM	1.61	1.94	3.44
5 mM	1.52	1.78	2.90
10 mM	1.50	1.58	2.79
pH			
6	0.86	0.76	1.90
6.4	0.98	1.20	2.09
6.8	1.50	1.58	2.79
Temperature			
15 °C	1.64	1.59	2.85
20 °C	1.50	1.58	2.79
25 °C	1.34	1.50	2.66
Flow			
0.8 mL min^{-1}	1.61	1.61	3.00
1.0 mL min^{-1}	1.50	1.58	2.79
1.2 mL min^{-1}	1.35	1.39	2.46

Chromatographic conditions: Chiral HPLC Astec Chirobiotic™ V, 5 μm (15 cm × 0.46 cm ID) λ exc/em = 230/290 nm.

6.8 was considered the optimal value (Table 2), since this CSP has to work in the range of pH of 3.5–7.0.

3.1.4. Effect of column oven temperature

Temperature slightly affected the resolution, with the higher temperature lowering the resolution (Table 2). As the column temperature decreased, higher enantioselectivity was achieved. A good resolution joined with a short elution time and a minimal consumption of the mobile phase was achieved with the column oven set at 20 °C.

3.1.5. Effect of flow rate

The flow rate had a slight effect on the resolution (Table 2), being set at 1.0 mL min^{-1} to achieve a good resolution and a compromise between the run chromatographic time and solvent consumption.

3.1.6. Overall chromatographic factors

After several attempts using different combinations of mobile phase and pH values, optimized chromatographic conditions within a reasonable elution time were achieved. EtOH/10 mM aqueous ammonium acetate buffer (92.5/7.5, *v/v*), pH 6.8 at isocratic mode, with a flow rate of 1.0 mL min^{-1} and the column oven temperature kept at 20 °C were considered the optimized conditions. EtOH content and pH were the most important chromatographic variable affecting the resolution between NFLX and FLX and their enantiomers, as can be observed in Table 2. The results indicate that FLX can be easier separated on Chirobiotic V than NFLX under all the chromatographic conditions tested. The *N*-methylation in FLX seems to be the responsible for this better enantioresolution, being the interactions with the primary amine of NFLX and CSP the disturbing factor for the enantioselectivity. Interesting was the achiral separation of the second eluted peak of NFLX ((*R*)-NFLX) and the first one of FLX ((*S*)-FLX), which depended in a great extent on the variation of EtOH content and pH, such as the enantioseparations.

The multiple chiral analyte separation using a single analytical method is a challenge in case of simultaneous separation of metabolite(s) and the parent compound. A recent work using a chiral α_1-acid glycoprotein column was able to enantioseparate FLX and NFLX enantiomers although those enantiomers of the different compounds were overlapped (Barclay et al., 2011). There are several reports on the enantioseparation of FLX using many CSP as Chirobiotic V (MacLeod et al., 2007), polysaccharide and protein-based (Olsen et al., 1998; Zhou et al., 2005). However, the chemoselectivity and enantioselectivity of FLX and NFLX were only reported with the use of pre-column chiral derivatization (Unceta et al., 2007; Higashi et al., 2010), achiral-chiral method with Chirobitic V as CSP (Borges et al., 2009) or two polysaccharide CSP on-line coupled (Pichini et al., 1996), which are laborious and time-consuming methods. The use of a single CSP to achieve the referred separation was done with cyclodextrin CSP (Yee et al., 2000) and polysaccharide CSP (Silva et al., 2009; Bueno et al., 2011). However, those methods presented poor resolution or used ACN in the mobile phase. To the best of our knowledge, this is the first report involving the chemo and enantioseparation of FLX and its demethylated metabolite NFLX in a single run, using a Chirobiotic V CSP and a fluorescence detector, with the advantages of short analysis, the use of an eco-friendly solvent and no need of derivatization or columns coupling.

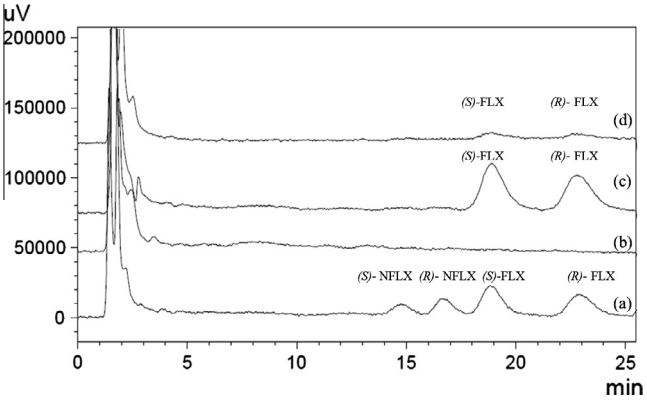


Fig. 3. Chromatograms of enantiomers of NFLX and FLX recovered from the spiked wastewater matrix (a), extract of blank wastewater (b), enantiomers of FLX recovered from the spiked wastewater matrix corresponding to day 0 (c), and enantiomers of FLX recovered from the spiked matrix corresponding to day 46 (d).

3.2. Method validation

3.2.1. Selectivity and matrix interferences

Chromatograms of standards dissolved in EtOH, recovered from the spiked matrix and extracts of wastewater were compared concerning the chromatographic parameters. Fig. 3 shows chromatograms of enantiomers of NFLX and FLX. The standards recovered

Table 3
Calibration curve equations, accuracy, intra- and inter-batch precision, recovery.

Compound	Calibration curve equation	Nominal concentration (ng mL^{-1})	1st day		2nd day		3rd day		Inter-day RSD (%) $n = 9$	Recovery (%)
			Accuracy (%)	RSD (%) $n = 3$	Accuracy (%)	RSD (%) $n = 3$	Accuracy (%)	RSD (%) $n = 3$		
(*S*)-NFLX		3.5	106	17.9	107	7.20	98.5	16.7	13.2	62.5
	$y = 48510.1x + 72723.4$	15	81.8	11.5	86.2	9.85	87.8	11.3	9.97	84.1
		28	85.4	5.53	83.2	3.63	84.8	14.2	7.92	91.1
(*R*)-NFLX		3.5	104	15.3	104	5.44	104	7.75	9.04	99.1
	$y = 100903.4x - 156686.0$	15	94.9	7.81	100	8.12	97.1	4.91	6.61	102
		28	92.4	3.63	92.5	2.98	83.5	12.6	8.06	103
(*S*)-FLX		6.0	76.0	7.90	82.3	13.9	82.4	11.5	10.7	53.1
	$y = 100795.1x + 25261.7$	30	87.1	6.82	87.6	2.01	84.6	5.95	4.88	65.9
		56	91.2	3.16	81.3	8.71	80.4	5.00	8.03	67.6
(*R*)-FLX		6.0	111	8.20	108.2	6.21	116	3.32	6.11	69.4
	$y = 88222.9x - 54079.1$	30	89.3	3.94	88.8	8.53	83.6	6.84	6.59	67.2
		56	89.1	3.44	78.7	9.91	77.0	5.16	8.99	66.8

from the spiked wastewater matrix (Fig. 3a) demonstrated that the matrix effect is minimal when compared with the extract of blank wastewater (Fig. 3b) and enantiomers of FLX recovered from the spiked matrix at days 0 and 46 of the microcosms laboratory experiments (Fig. 3c and d).

Since the ionic interactions of vancomycin-based CSP like Chirobiotic V are very important concerning the interaction sites of polar and ionisable analytes, the components of wastewater effluent after typical secondary treatment such as suspended and dissolved material can interfere with chiral recognition and slightly affect the resolution. Thus, the values for resolution and enantioresolution (Rs) slightly decreased in the spiked matrix. Despite the decrease on the values of the chromatography parameters, Chirobiotic V was able to resolve efficiently all the enantiomers recovered from the spiked matrix and allowed the selectivity for the monitorization purpose (Fig. 3).

3.2.2. Linearity and range

The linearity assay was performed ranging from 2.0 (LOQ) to 30 ng mL^{-1} in the case of NFLX and 4.0 (LOQ) to 60 ng mL^{-1} in the case of FLX. The values of LOD (between 0.8 and 2.0 ng mL^{-1}) and LOQ referred above are suitable to monitoring the target compounds in microcosm assays at laboratory scale.

3.2.3. Accuracy and precision

Accuracy and recovery rates ranged from 76.0% to 116% and from 53.1% to 103%, respectively, as described in Table 3. These values are suitable for quantitative determinations in complex matrix samples (Validation of Analytical Procedures: Text and Methodology Q2(R1), 1996, Madureira et al., 2009; 2010).

The precision of the method was evaluated by determining the intra- and inter-batch assays. The results demonstrated that this method is precise, with RSD values lower than 17.9% for intra-batch precision and lower than 13.2% for inter-batch precision (Table 3). This is in agreement with International criteria, which recommends RSD values lower than 20% for complex matrices (Validation of Analytical Procedures: Text and Methodology Q2(R1), 1996).

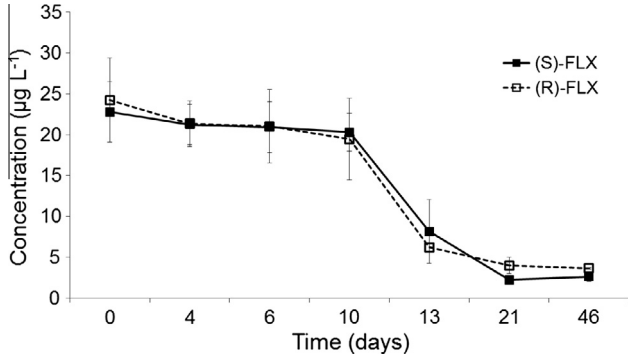


Fig. 4. Degradation pattern (46 days) of (*S*)- (—■—) and (*R*)- (- -□- -) enantiomers of FLX by wastewater spiked samples at an initial supplementation of 25 ng mL^{-1} of each enantiomer. These samples were able to remove both enantiomers of fluoxetine in a non-enantioselective manner, after a lag phase of 10 days.

3.3. Microcosms tests at laboratory scale

The enantioselective validated method was applied to monitor the degradation of enantiomers of the antidepressant FLX and the possible formation of enantiomers of its active metabolite NFLX. The removal of enantiomers of FLX was successfully quantified. Fig. 4 shows the degradation behaviour of enantiomers of FLX spiked in the wastewater effluent, during 46 days. The degradation was not enantioselective and it is observed an initial lag phase of ca. 10 days for both enantiomers, from which the degradation accelerated until the day 21, being 65–75% of removal achieved until the day 13 (Fig. 4). Regarding the metabolite formation, the FLX biotransformation in its metabolite NFLX, was not observed. These results are in accordance with a previous study performed in our laboratory concerning biodegradation of FLX and other pharmaceuticals by activated sludge collected from a WWTP (Ribeiro et al., 2013b).

4. Conclusions

The enantioselective SPE-HPLC-FD method using Chirobiotic V column demonstrated to be accurate and precise to quantify in a single run the enantiomers of FLX and the enantiomers of its demethylated metabolite NFLX in wastewaters. The optimized mobile phase used a green choice with ethanol as organic solvent. The selectivity and LOQ achieved by the fluorescence detector were adequate for monitoring the degradation assays at environmental level. This validated method was successful applied in the biodegradation assays using real wastewater samples spiked with FLX. Trace of FLX remained until 46 days of the assays, however non-enantioselective degradation and no formation of enantiomers of the metabolite NFLX were observed. This is in accordance with previous publications which enforce that the degradation pattern and the enantioselectivity depend very much on the microorganism phylogenies.

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