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Supercritical fluid chromatography time-of-flight mass spectrometry enantiomeric determination of basic drugs in sewage samples

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ABSTRACT

Many pharmacologically active compounds are chiral species, and their therapeutic or toxicological effects might differ between isomers. Herein, we develop a fast and sensitive chiral analysis methodology for the determination of eight pharmaceuticals, considered as emerging environmental pollutants and belonging to two different chemical classes, in wastewater and sludge samples. Compounds were separated using supercritical fluid chromatography (SFC) combined with time-of-flight mass spectrometry (TOF-MS) detection. The stationary phase, the modifier and the additive combined with supercritical carbon dioxide (CO_2), in the SFC mobile phase, played a major effect in the enantiomeric resolution of selected compounds. Moreover, the composition of the mobile phase affected their ionization efficiency in the electrospray ionization source. Methanol (MeOH), containing a 0.1% of ammonia, was used as CO_2 modifier for the separation of compounds in a polysaccharide-type column. Total analysis time was 15.5 min, achieving resolution factors between 1.03 and 2.49 for the eight pairs of enantiomers. In combination with mixed-mode solid-phase extraction and matrix solid-phase dispersion protocols, compounds were determined in wastewater and sludge samples, with limits of quantification in the range of 0.010–0.020 μg L^{-1} and 3.7–11.1 ng g^{-1}, for aqueous and solid samples, respectively. The amine-type drugs (tramadol, propranolol and venlafaxine) were mostly found in wastewater samples, whilst azolic antimycotics were mainly quantified in sludge. The first group of compounds showed enantiomeric fractions significantly different to those existing in the commercial counterpart pharmaceuticals.

1. Introduction

Many bioactive compounds designed by pharmaceutical and agrochemical industries are chiral molecules. Usually, the therapeutic and/or the toxicological effects of these species, on target and non-target organisms, differ significantly between isomers [1]. Even in case of compounds marketed as racemates, sometimes, their metabolization rates and their environmental biodegradability are enantioselective processes [2]. As a result, the enantiomeric fraction (EF) of these species in sewage samples might be different to that existing in the commercial formulations [3,4].

The development of chiral analysis methodologies is an emerging topic to characterize the enantiomeric purity of bioactive compounds, to understand the interaction of chiral compounds with biotic systems (vegetables, bacteria and animals) [2], and to discriminate the sources of some pollutants found in the aquatic media, as it has been reported for amphetamine-like drugs [5]. In the field of environmental analysis, chiral separations methods need to be combined with sensitive determination techniques allowing the quantification of these compounds in the ppb or sub-ppb range. Thus, the hyphenation of chiral separation techniques with mass spectrometry (MS) detection is the preferred approach to cope with complex extracts obtained from this kind of samples [1,2,6,7].

Chiral separations developed under supercritical fluid chromatography (SFC) conditions, are presented as a green alternative to liquid chromatographic (LC) methods [2], particularly to those using normal phase conditions. As separation technique, SFC employs higher flowrates than LC, it requires shorter column equilibration times, and the drop of pressure between both edges of the chromatographic column is lower than for the latter technique [8,9]. On the other hand, the yield of ionization processes, at the electrospray source, is less favorable for CO_2 rich mobile phases than for aqueous solutions of methanol (MeOH), or acetonitrile (ACN), employed in LC [6]. Consequently, hyphenation of SFC and MS requires the use of a make-up solution, whose composition

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and flowrate affect the formation of ions in the ESI source and thus, the limits of quantification (LOQs) of SFC-ESI-MS methods. The make-up solution also prevents compound precipitation after CO_2 decompression [6,7]. In case of SFC-MS interfaces which divide the flow of mobile phase between the ESI source and a back-pressure regulator (BPR), i.e. the pre-BPR splitter design [6], sensitivity might be a challenge in environmental applications. Another challenge in chiral analysis is the development of multianalyte procedures, with separation times in the range of values employed when dealing with non-chiral separations. Despite the fact that the number of applications of chiral LC or SFC separations, combined with MS detection, is increasing steadily, only a reduced number of works consider more than one compound at a time, and just in a few cases, chiral separation methods involve the simultaneous determination of compounds from different families or chemical groups [9–12]. In most cases, a drawback of multianalyte chiral LC methods is the use of long isocratic separations, with run times in the range of 40–60 min [2,3,10,13].

In terms of occurrence, persistence and toxicity, azolic drugs are a relevant family of emerging environmental pollutants [14,15]. Many azolic drugs are chiral species, marketed as racemates and employed mainly as topic antimycotic prescription pharmaceuticals. Moreover, in some cases, they are included in the formulation of certain personal care products (i.e. climbazole, CBZ). In both uses, they are directly discharged into the network of urban sewers during showering. Upon arrival to sewage treatment plants (STPs), they distribute between wastewater and sludge depending on their polarities. The amino group is present in several groups of highly prescribed, chiral pharmaceuticals, such as beta-blockers, psychiatric drugs and pain relief medicaments, administrated as oral pills. Many drugs within the above prescription groups are recognized as ubiquitous in the aquatic environment, as it is the case of venlafaxine (VFX), tramadol (TRA) and some beta-blockers, i.e. propranolol (PRO). Although chiral separation methodologies have been proposed for some of the above compounds [13,16], either their performance was not investigated in combination with MS detection [17], or the chiral analysis of imidazolic and amine drugs, with very different water solubilities and pKa values, in the same chromatographic injection has not been reported, yet.

In this research, we assess the performance of SFC-ESI-MS for the chiral determination of five azolic antimycotics and three basic pharmaceuticals in wastewater and sludge samples obtained from urban STPs. The selection of compounds was made attending to their occurrence in water and sludge, combined with their reported environmental toxicity and risk assessment results [14,18–20]. Moreover, it was attempted to cover the analysis of chiral compounds with different polarities, water solubilities, and presence of basic functionalities with different strength (the weak basicity imidazole ring and the strongly basic amine moiety) in their structures. The aims of the study were to investigate the capability of the SFC-ESI-MS system to separate the enantiomers of selected compounds in the same chromatographic analysis, to achieve enough sensitivity to measure their enantiomeric fractions (EFs) in environmental matrices, and to identify possible variations in their EFs compared to those existing in commercial formulations.

2. Material and methods

2.1. Solvents, standards and sorbents

Methanol (MeOH), HPLC-grade purity, and formic acid (FA, 98%) were acquired from Merck (Darmstadt, Germany). Ammonia, 7 M solution in methanol, and diethyl amine (DEA) were purchased from Acros Organics (Geel, Belgium). Ultrapure deionized water (18.2 MΩ cm^{-1}) was obtained from a Geni U system (Rephile, Shanghai, China).

Racemic mixtures of miconazole (MCZ), CBZ, cis- TRA, their isotopically labeled analogues (miconazole-d_5, MCZ-d_5; climbazole-d_4, CBZ-d_4; and Tramadol-^{13}C-d_3, TRA-^{13}C-d_3), sertaconazole (STZ), tioconazole (TCZ), fenticonazole (FTZ), PRO, and VFX were acquired from Sigma-Aldrich (Milwaukee, WI, USA) and Toronto Research Chemicals (Ontario, Canada). The S-form of PRO (PRO-S) was purchased from Sigma-Aldrich. Chemical structures, log D values at neutral pH, and acidity constants of selected compounds are provided as supplementary information, Fig. S1. Individual standards of each compound were prepared in MeOH. Further mixtures were made in the same solvent. Enantiomers of target compounds were identified with codes 1 and 2, attending to their elution order under final working SFC conditions. Their identities were not confirmed, with the exception of those of PRO.

Polypropylene syringes (12 mL volume), and polyethylene frits (20 µm pore size, 15 mm diameter) for matrix solid-phase extraction (MSPD) were supplied by International Sorbent Technology (Mid Glamorgan, UK). The dispersant sorbent (C_{18}-bonded silica) was obtained from Agilent Technologies (Santa Clara, CA, USA). Primary and secondary amine (PSA), used as clean-up co-sorbent in the MSPD of sludge samples, was purchased from Sigma-Aldrich. Oasis mixed-mode (reversed-phase and cationic exchanger) cartridges (OASIS MCX-150 mg) were provided by Waters (Milford, MA, USA). This sorbent was employed for solid-phase extraction (SPE) of target compounds from water samples. Bond Elut Jr strong cation exchanger cartridges (SCX-150 mg) were acquired from Agilent Technologies.

2.2. Samples and sample preparation

Grab wastewater samples were obtained from several urban STPs located in Galicia (Northwest Spain) during year 2021. After reception, samples were spiked with the selection of labeled surrogate standards (SSs), adjusted at pH 3 using HCl (0.1 M solution), mixed with a 20% of MeOH, filtered and concentrated using MCX SPE cartridges, under conditions reported elsewhere for triazolic compounds [21]. In brief, water aliquots (200–400 mL) were passed through the cartridge, previously conditioned with MeOH and water at pH 3. Thereafter, the sorbent was rinsed with ultrapure water adjusted at pH 3 (5 mL), and dried using a stream of nitrogen. Neutral and acidic compounds were rinsed with 2 mL of MeOH containing a 0.1% of FA. Target compounds were recovered using 2 mL of MeOH: NH_3 (98:2). This extract was filtered (0.22 µm pore size) and stored at 4 °C until analysis.

Sludge samples (solid wastes after primary and secondary wastewater treatment) were obtained from six different STPs. After reception, they were frozen and lyophilized. Freeze-dried fractions (0.5 g) were spiked with SSs and allowed to stand overnight. Thereafter, they were thoroughly mixed with 2 g of C_{18} in a mortar, using a pestle, and packed in a polypropylene syringe containing 1 g of PSA as co-sorbent of acidic compounds [22]. The MSPD syringe was placed on top of a SCX cartridge. The modular combination of sorbents was first rinsed with MeOH (10 mL), which were discarded. Thereafter, the MSPD syringe, containing the packed samples, was de-attached from the SCX cartridge. Moderate and strong basic compounds were recovered from the latter sorbent using MeOH: NH_3 (98:2). This extract (5 mL) was evaporated to 2 mL, filtered, and maintained at 4 °C until analysis.

2.3. SFC-MS determination conditions

Compounds were determined using a QTOF-MS spectrometer (Agilent 6550 model), furnished with an electrospray ionization source (ESI), and coupled to 1260 infinity II SFC system from

Table 1
Retention times, quantification ions ([M+H]$^+$), determination coefficients (R^2), limits of quantification (LOQs), resolution factors (Rs) and peak widths for target compounds and their isotopically labeled analogues.

Compound	Retention time (min)	[M+H]$^+$(m/z)	R^2 (2–250 µg L^{-1})	LOQ (µg L-1)	Rs	Peak width (min)
PRO-R[a]	8.31	260.165	0.999	2	2.33	0.271
PRO-S[b]	8.86		0.999	2		0.203
TRA-1[a]	3.88	264.1963	0.999	1	1.37	0.44
TRA-2[b]	4.47		0.999	1		0.417
VFX-1[a]	4.36	278.212	0.999	1	1.09	0.417
VFX-2[b]	4.82		0.999	1		0.429
CBZ-1[c]	4.73	293.1057	0.999	1	1.25	0.406
CBZ-2[d]	5.23		0.998	1		0.395
TCZ-1[e]	7.68	386.9892	0.999	1	1.96	0.293
TCZ-2[f]	8.23		0.999	1		0.259
MCZ-1[e]	8.51	416.9905	0.999	1	2.17	0.327
MCZ-2[f]	9.24		0.999	1		0.35
STZ-1[e]	11.61	437.0049	0.999	1	1.03	0.372
STZ-2[f]	11.98		0.999	1		0.35
FTZ-1[e]	12.41	455.0752	0.999	1	2.49	0.485
FTZ-2[f]	13.45		0.999	1		0.35
TRA-^{13}C-d_3-1[a]	3.91	268.2182	-		1.36	0.417
TRA-^{13}C-d_3-2[b]	4.48		-			0.429
CBZ-d_4-1[c]	4.67	297.1302	-		1.34	0.383
CBZ-d_4-2[d]	5.23		-			0.44
MCZ-d_5-1[e]	8.45	422.0227	-		2.03	0.338
MCZ-d_5-2[f]	9.16					0.361

[a to f]Identify the surrogate standard associated to each compound.

the same supplier. The SFC was connected to the ESI source using a pre-BPR splitter interface, with an auxiliary sheath pump. Three different polysaccharide-based columns were tested for the separation of target compounds. They were the Lux Amylose-1 (3,5-dimethylphenyl carbamate), Lux i-Amylose-3 (3-chloro-5-methylphenyl carbamate) and Lux i-Cellulose-5 (3,5-dichlorophenyl carbamate) (150 mm x 3.0 mm, 3 µm) columns acquired from Phenomenex (Torrance, CA, USA). In the first case, the stationary phase was coated to the surface of silica particles, whilst the other two columns contained immobilized phases. Columns were used directly, without pre-column, connected to a metallic prefilter (0.2 µm). Under final working conditions, the Amylose-1 column was used. The mobile phase flowrate was adjusted at 1.5 mL min^{-1}, the column was maintained at 40 °C, and the injection volume was set at 2 µL. The mobile phase consisted of CO_2 (phase A) and MeOH with a 0.1% of NH_3 (phase B). Its composition was programmed as follows: 2% B (0–0.2 min), 8% B (0.3–6 min), 18% B (8–12 min), 25% B (12.1–13 min), 2% B (13.1–15.5 min). MeOH (0.5% in FA), at 0.1 mL min^{-1}, was employed as make-up solution, combined with the column flow before the splitter. The BPR pressure was set at 140 bar (60 °C), and the splitter was connected to the ESI source using a 0.5 m length, 0.050 mm i.d., silica capillary. The ESI source was operated in the positive mode (ESI+), using nitrogen as nebulizing and drying gas (35 psi and 15 L min^{-1}, 200 °C, respectively). The voltages of the ESI needle and the fragmentor were set at 3.5 KV and 380 V, respectively.

The QTOF-MS instrument was operated in the 2 GHz, extended Dynamic range resolution MS mode, with continuous recalibration of the *m/z* axis using the ions 121.0508 and 922.0098. The *m/z* ratio corresponding to the [M+H]$^+$ ion of each compound was extracted using a window of 10 ppm and employed for quantitative purposes, Table 1. Product ion scan spectra were used for confirmation purposes when required. Table S1 summarizes the most intense product ions of target compounds.

2.4. Characterization of SFC-ESI-MS methods

Performance of analytical procedures applied to wastewater and sludge analysis was characterized using chromatographic parameters, extraction efficiencies (EEs), matrix effects (MEs), accuracy and limits of quantification (LOQs). The efficiency of chiral separations was evaluated with resolution factors (Rs), calculated as defined in Eq. (1), being tr_2 and tr_1 the retention times of the later and earlier eluting enantiomer of each analyte. Baseline peak widths are represented by w_1 and w_2. Rs values above 1.0 indicate overlapping percentages below 2%, for equal intensity peaks. Values above 1.5 correspond to baseline separation.

$$Rs = 2(tr_2 - tr_1)/(w_1 + w_2), \quad (1)$$

The enantiomeric fractions (EFs) of chiral compounds are calculated as defined in Eq. (2) [4,5], with E_1 and E_2 corresponding to concentrations measured for the earlier and the later eluting enantiomer of each compound.

$$EF = E_1/(E_1 + E2), \quad (2)$$

The EEs of employed sample preparation procedures (SPE and MSPD for water and sludge samples, respectively) were defined as the ratio between the response (peak area without correction with responses obtained for SSs) of each compound in spiked samples and in extracts from same matrix, fortified at the end of the sample preparation process. MEs (%) represent the ratio between the slopes of calibration curves obtained for spiked extracts, from freeze-dried sludge and wastewater samples, and those corresponding to solvent-based standards. The closer the normalized ratio to 100%, the lower the variation between the efficiency of compound ionization in sample extracts versus solvent-based standards [23]. The accuracy of the overall method was evaluated dividing the difference of concentrations measured for spiked and non-spiked fractions of each sample by the added level. Concentrations were calculated against calibration curves obtained for solvent-based standards.

3. Results and discussion

3.1. Optimization of SFC-ESI-TOF-MS conditions

The success of environmental chiral SFC methods depends on two factors: the performance of the separation process itself, and the achieved LOQs, which are mainly controlled by the efficiency of compound ionization at the ESI source. In the earlier steps of

this study, chiral columns were operated using linear gradients between 2 and 30% of three different modifiers: MeOH, EtOH or ACN, either combined with ammonium acetate ($AcONH_4$, 5 mM) or DEA (0.1%), as neutral and basic additives, respectively. The total analysis time, including column re-equilibration, was limited to 10 min.

The retention and the enantiomeric resolution of antimycotic drugs were mainly affected by the modifier combined with CO_2. Their retention times varied in the following order: MeOH < EtOH < ACN, whilst enantiomeric resolution increased as follows: ACN < EtOH < MeOH. Fig. S2A illustrates this behavior using CBZ as model compound using the Amylose-1 column. Out of three tested columns, the Amylose-1 and the Cellulose-5 succeeded in separating (Rs > 1) the enantiomers of the five imidazole drugs, whilst the Amylose-3 failed to resolve those of STZ and TCZ. Retention and resolution between enantiomers of PRO, TRA and VFX were affected by the stationary phase of the chiral column, and the additive mixed with MeOH in the mobile phase. Better resolution and shorter retention times were observed using DEA instead of $AcONH_4$, as MeOH additive. Fig. S2B illustrated this behavior for the enantiomers of TRA using the Amylose-1 column. The effect of the additive was coherent with the strong basicity of amine-type compounds, and the acidic character of supercritical CO_2 [7]. Likely, DEA shields the non-stereospecific interactions of these strong bases with residual silanol groups in the stationary phase [8]. On the other hand, maintaining the same composition of the make-up solution (MeOH 0.5% in FA, 0.5 mL min^{-1}), DEA caused a significant attenuation in the ionization efficiency of all compounds (amine-type drugs and the imidazolic antimycotics) compared to that observed using $AcONH_4$ as additive in the mobile phase. Lower responses are linked to the high proton affinity of DEA [7], which limits the ionization of the compounds in the ESI (+) mode. This drawback was overcome replacing DEA by NH_3 (both 0.1%). The basicity of the latter additive was enough to improve the resolution between enantiomers of tertiary amine drugs in comparison to $AcONH_4$, without compromising their ionization efficiency as noticed for DEA, Fig. S2B. Moreover, the primary adduct between neutral molecules and the ammonium cation has been reported to evolve generating the $[M+H]^+$ species in the ESI source [24]. Whatever the exact mechanism enhancing compounds ionization, differences observed between the use of NH_3 and DEA in this study, are in agreement with previous results reported by Hamman et al. during determination of basic drugs by SFC-ESI-MS [24]. Using the same combination of modifier and additive (MeOH, 0.1% in NH_3), it was verified that the Cellulose-5 chiral column failed to resolve the isomers of PRO, TRA and VFX. The Amylose-3 separated the enantiomers of PRO and those of VFX; however, broad tailing peaks were observed for the latter species. On the other hand, it failed to resolve the stereoisomers of TRA. Thus, the Amylose-1 column was selected to continue with the study.

Once the SFC column, the mobile phase modifier and the additive were selected, different mobile phase gradients and temperatures (30–45 °C) were tested. Under final conditions described in Material and Methods section, compounds were separated in 15.5 min, with Rs values between 1.03 (STZ enantiomers) and 2.59 (FTZ enantiomers), Table 1. Total analysis time employed in this research was between twice [25] and four times [13] lower than those required in previous multianalyte methods using chiral LC as separation technique, under isocratic conditions; and in the range of values reported in other multianalyte SFC-MS applications [12]. Same SFC conditions were also tested to resolve the racemates of over the counter metabolites of TRA and VFX: N-desmethyl TRA and O-desmethyl VFX. Fully overlapped peaks were observed for N-desmethyl TRA enantiomers. The two isomers of O-desmethyl VFX were only partially resolved (Rs 0.7), but well separated from the stereoisomers of TRA (they appeared at longer retention times, c.a. 2 min). The latter is a relevant achievement since both compounds are ubiquitous in wastewater and they share identical MS and MS/MS spectra, with a single intense product ion ($[C_3H_8N]^+$) at *m/z* 58 [11].

As make-up solution, we considered the use of MeOH with a 0.5% of FA. The effect of its flowrate (from 0.1 to 0.75 mL min^{-1}) in the responses of target compounds was first investigated with solvent-based standards. Obtained data are provided as supplementary information, Fig. S3A. Increasing the make-up flowrate reduced the responses for all compounds without affecting other chromatography parameters, such as retention time repeatability, peak width or Rs factors. Depending on the compound, normalized responses were reduced between 20 and 50% when increasing the make-up flowrate from 0.1 to 0.75 mL min^{-1}, Fig. S3A. Thus, the dilution factor introduced by the increase in the make-up flowrate was partially compensated by a more effective ionization process. The trend observed for solvent-based standards, was similar to that noticed for a spiked sludge extract, Fig. S3B. Thus, the working value for this parameter was set at 0.1 mL min^{-1}. It is worth noting that, the make-up flowrate and the additive employed in the mobile phase are not independent parameters. As example, using MeOH (0.1% in DEA) in the mobile phase, higher responses were obtained for a make-up flow rate of 0.75 mL of MeOH:FA (99.5:0.5) than considering 0.1 mL min^{-1}, figure not shown. In this case, it is assumed that the increase in the amount of FA introduced in the ESI source, through the make-up solution, compensates in part the negative effect of DEA in the formation of $[M+H]^+$ ions.

Under optimized conditions, the repeatability of the injection, calculated as relative standard deviation of peak areas ($n = 5$, 10 µg L^{-1} standard), stayed between 1 and 4%. Relative standard deviations associated to the retention times of compounds varied between 0.1 and 1.02% for consecutive injection of calibration standards (2–250 µg L^{-1}), and between 0.2 and 1.3% for a quality control standard (100 µg L^{-1}) injected several times ($n = 16$ injections) during a 48 h sequence. Instrumental LOQs varied between 1 and 2 µg L^{-1}. The plots of normalized responses (compound peak area/SS peak area) versus concentration fitted a linear model, with determination coefficients above 0.998, within the range of concentrations from 2 to 250 µg L^{-1} per enantiomer, Table 1. Fig. 1 shows a chromatogram for a standard mixture of all compounds at a concentration level of 10 µg L^{-1} per isomer. Chromatograms obtained with the other two chiral columns tested in this research, under same SFC-MS conditions, are provided as supplementary information, Fig. S4.

3.2. Performance of analytical methodologies

The original mixed-mode SPE procedure, previously reported for extraction of antimycotic drugs from wastewater [21], was re-evaluated for the selection of compounds involved in this research. Polar, strong basic amine drugs were effectively retained by the mixed-mode sorbent during cartridge loading and clean-up steps; however, losses of moderately basic compounds (i.e. CBZ enantiomers) were noticed during the removal of neutrals with MeOH. This drawback was solved replacing MeOH by MeOH 0.1% in FA, as washing solvent, which reinforced the interaction between weak basic compounds and the negatively charged sites in the sorbent. The EEs of the SPE procedure for raw and treated wastewater were in the range from 82 to 113%, with standard deviations (SDs) below 11%, Table S2. The only exception was FTZ. The average EEs for the enantiomers of this compound in raw wastewater were 68%, Table S2. For this particular matrix, FTZ was partially lost due to breakthrough problems during concentration of 200 mL sample aliquots, Table S2. For freeze-dried sludge samples, the EEs of the modular MSPD procedure were between 83 and 117%, with standard deviations lower than 7%, Table S2.

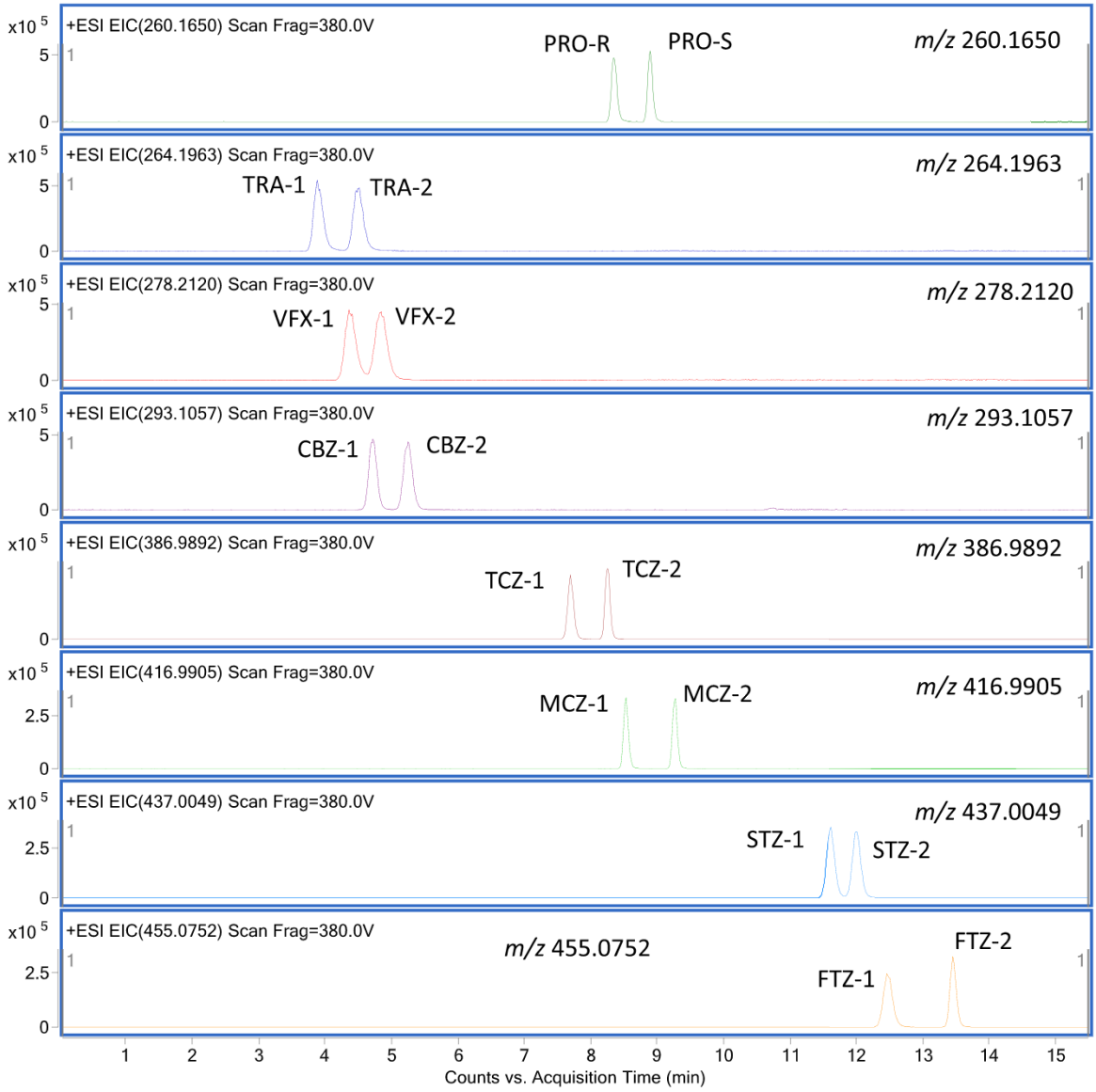

Fig. 1. Extracted ion chromatograms (mass window 10 ppm) for the $[M+H]^+$ ion of target compounds (10 μg L^{-1} per enantiomer) under optimized SFC-ESI-TOF-MS conditions using the Lux amylose-1 column with methanol (0.1% in NH_3) as modifier and additive of supercritical CO_2, respectively. Column temperature and mobile phase flow rate were 40 °C and 1.5 mL min^{-1}. The mobile phase gradient is given in Section 2.1.

The assessment of MEs became a major issue in this research. Changes in the efficiency of ionization between solvent-based standards and sample extracts affect the accuracy of the method; moreover, enantioselective MEs are a serious limitation when aiming to identify possible variations in the EFs of chiral compounds. Table 2 summarizes the MEs obtained for the most complex matrices involved in this research: raw wastewater and sludge. In the first case, MEs varied between 99 and 115%. Considering the definition of MEs reported in the Material and Methods section, these values point out to minor changes in the efficiency of compounds ionization between SPE extracts and solvent-based standards. For extracts obtained from sludge, moderate signal suppression (MEs in the range from 62 to 80%) was noticed for some species. The maximum differences between MEs for the enantiomers of a given pharmaceutical remained below 10%, Table 2.

Table 3 summarizes results obtained during evaluation of the accuracy of the SFC-ESI-TOF-MS method for water and sludge samples. For the first matrix, individual recoveries of each species, in the three considered water types, stayed between 80 and 115%, except in case of FTZ enantiomers with a value of 77% for the wastewater sample. It is worth noting that for the river water, the concentrated sample volume was increased to 400 mL, with satisfactory recoveries. The SD values associated to recoveries obtained for spiked water samples remained below 8%. In case of sludge, the enantiomers of VFX and MCZ showed overall recoveries between 120 and 133%, with associated SDs below 10%. For the rest of species, recoveries were in the range from 95 to 120%. The LOQs of the two analytical procedures are also shown in Table 3. Reported values were estimated from instrumental LOQs, considering the ratio between sample amount (200 mL and 0.5 g for wastewater and sludge, respectively) and the final volume (2 mL) of extracts obtained using SPE and MSPD extraction protocols. These ratios were corrected with EEs and MEs, when they stayed below 80%. The LOQs in raw wastewater were between 0.010 and 0.020 μg L^{-1}. Similar LOQs have been reported using chiral LC combined with a TOF-MS instrument [13], whilst lower values were achieved replacing the above system by a QqQ instrument [11]. Rice et al. [9] developed a SFC-QqQ-MS method for the determination of large se-

Table 2
Assessment of MEs (%) for raw wastewater and sludge samples. Normalized responses between slopes of calibration curves obtained for spiked extracts from raw wastewater, or sludge, and solvent-based standards (2–100 μg L^{-1}). Values within parenthesis are the standard deviations for slopes of calibration curves for matrix-matched standards.

Compound	Raw wastewater	Sludge
PRO-R	106% (1)	72% (4)
PRO-S	107% (1)	82% (5)
TRA-1	108% (2)	91% (7)
TRA-2	106% (1)	93% (6)
VFX-1	100% (3)	92% (6)
VFX-2	99% (3)	94% (6)
CBZ-1	115% (2)	107% (6)
CBZ-2	112% (2)	106% (5)
TCZ-1	104% (5)	70% (5)
TCZ-2	103% (2)	62% (5)
MCZ-1	104% (3)	81% (6)
MCZ-2	114% (2)	73% (4)
STZ-1	113% (3)	78% (6)
STZ-2	112% (1)	84% (6)
FTZ-1	108% (3)	80% (6)
FTZ-2	115% (3)	80% (5)

lection of non-chiral and nine chiral drugs in wastewater samples. The latter group included two compounds common to those considered in this study: PRO and TRA. For the 2nd species, authors failed to separate both enantiomers; moreover, their SPE method provided non-satisfactory recoveries for TRA in spiked wastewater samples [9]. In case of PRO, enantiomeric resolution was similar to that reported in this study, with equivalent LOQs (0.01-0-05 μg L^{-1}) despite using a more sensitive mass analyzer. LOQs compiled in Table 3 for sludge varied between 3.7 and 11.1 ng g^{-1}, referred to the dry sludge matrix. The linear response range of the method extended up to 2.5 μg L^{-1} and 1000 ng g^{-1} for wastewater and sludge, respectively.

3.3. Distribution in environmental samples

Chiral SFC-TOF-MS, combined with SPE and MSPD sample preparation methodologies, was applied to investigate the concentrations, and the EFs, of target compounds in sewage samples. Positive identifications were based on retention time and $[M+H]^+$ match (maximum differences 0.1 min and 10 ppm, respectively) with calibration standards. For compounds close to the LOQs of the method, their identities were confirmed in a second injection, using target MS/MS conditions reported in Table S1. Table 4 summarizes the concentrations measured for seven pairs of wastewater samples (raw and treated wastewater) obtained from four different STPs. Pharmaceuticals were ubiquitous in the environment of STPs. As a general observation, those showing high logD values (see Fig. S1) were mostly associated to sludge, whilst the amine-type pharmaceuticals were mainly found in water samples, Tables 4 and 5. Despite this global trend, one of the processed sludge samples contained a concentration of TRA enantiomers around 1000 ng g^{-1}, Table 5.

The concentrations of the enantiomers of TRA and VFX in wastewater ranged between 0.11 and 1.0 μg L^{-1}. Although data compiled in Table 4 correspond to grab samples, they confirm the poor biodegradability of both compounds at STPs. CBZ and PRO enantiomers were often above the LOQs of the method in wastewater; however, their concentrations remained one order of magnitude below those of TRA and VFX. Finally, MCZ enantiomers were just noticed in two raw wastewater samples, whilst TCZ, STZ and FTZ remained undetected.

As regards the sludge matrix, VFX and the imidazole antimycotic drugs were noticed in all samples above method LOQs. Given aside the high concentration of TRA found in sludge sample code S2, pollutants displaying the highest levels were MCZ and FTZ. The total concentration (sum of enantiomers) of the first compound varied between 0.44 and 1.57 μg g^{-1}. These concentrations are in the range of values found in sludge samples from other geographic areas, such as South America [26] and Asia [27]. In case of MCZ, they stay 3-order of magnitude above the lowest predicted non-effect concentration (PNEC) level compiled in the NORMAN ecotoxicity database for MCZ in freshwater sediments [28].

The EFs of above compounds in the set of processed samples are provided as supplementary information, Table S3. Fig. 2 shows the Box-Whisker plot for EFs measured in the set of processed sludge and wastewater samples. PRO, TRA and VFX showed EFs significantly different from 0.5. In the first case, environmental samples were enriched significantly in the latter eluting isomer (S-form), with average and median EF values of 0.40 and 0.41, respectively. This enantiomeric pattern agrees with that reported by Bagnall et al. [13] for environmental water samples. Rice et al. [9] have reported a value of 0.40 for the EF or PRO in raw and treated wastewater samples, but without identifying which enantiomeric form presented the higher concentration. The EFs of TRA showed a very narrow distribution of values, with an average of 0.53, which matched also the median value. In case of VFX, average and me-

Table 3
Accuracy of the analytical procedures for water and sludge samples. Recoveries obtained using solvent-based calibration standards, $n = 3$ replicates. Procedural LOQs estimated for raw wastewater and sludge.

	Water type (addition level)			Sludge (addition level)		LOQs	
Compound	Raw wastewater (0.5 μg L^{-1})	Treated wastewater (0.25 μg L^{-1})	River water (0.05 μg L^{-1})	(250 ng g^{-1})	(125 ng g^{-1})	Raw wastewater (μg L^{-1})	Sludge (ng g^{-1})
PRO-R	104% (2)	92% (5)	108% (4)	112% (6)	103% (7)	0.020	11.1
PRO-S	104% (2)	92% (5)	112% (5)	100% (4)	95% (8)	0.020	9.8
TRA-1	104% (2)	103% (4)	104% (2)	106% (1)	106% (4)	0.010	4.4
TRA-2	107% (2)	101% (3)	101% (4)	102% (2)	111% (5)	0.010	4.3
VFX-1	98% (5)	100% (5)	108% (2)	127% (8)	133% (7)	0.010	4.3
VFX-2	95% (3)	100% (5)	107% (3)	106% (3)	125% (4)	0.010	4.3
CBZ-1	115% (2)	95% (5)	100% (3)	105% (4)	112% (6)	0.010	3.7
CBZ-2	115% (2)	96% (4)	101% (3)	103% (1)	111% (7)	0.010	3.8
TCZ-1	110% (2)	89% (5)	98% (2)	98% (2)	117% (6)	0.010	5.7
TCZ-2	108% (2)	90% (7)	103% (2)	106% (4)	119% (6)	0.010	6.5
MCZ-1	108% (2)	88% (5)	102% (5)	125% (2)	128% (5)	0.010	4.9
MCZ-2	109% (1)	87% (6)	102% (3)	123% (5)	125% (4)	0.010	5.5
STZ-1	110% (2)	86% (8)	98% (2)	107% (4)	112% (7)	0.010	4.7
STZ-2	111% (1)	87% (8)	100% (2)	117% (7)	112% (5)	0.010	4.8
FTZ-1	98% (3)	77% (7)	88% (3)	102% (4)	108% (6)	0.015	5.0
FTZ-2	100% (2)	77% (8)	93% (4)	117% (4)	110% (6)	0.015	5.0

Table 4
Concentrations (μg L^{-1}) measured in wastewater samples. Average values for triplicate extractions, with standard deviations within parenthesis. Empty cells correspond to concentrations below procedural LOQs in wastewater.

Sample code	Compound									
	PRO-R	PRO-S	TRA-1	TRA-2	VFX-1	VFX-2	CBZ-1	CBZ-2	MCZ-1	MCZ-2
RW1	0.035 (0.005)	0.039 (0.003)	0.48 (0.03)	0.43 (0.02)	0.24 (0.01)	0.26 (0.01)	0.122 (0.009)	0.123 (0.008)		
TW1	0.051 (0.005)	0.086 (0.006)	0.48 (0.03)	0.42 (0.03)	0.24 (0.01)	0.29 (0.02)	0.035 (0.002)	0.036 (0.001)		
RW2	0.048 (0.003)	0.071 (0.004)	0.71 (0.05)	0.63 (0.05)	0.27 (0.02)	0.30 (0.01)	0.075 (0.004)	0.075 (0.003)		
TW2	0.048 (0.006)	0.068 (0.005)	0.40 (0.03)	0.35 (0.02)	0.18 (0.02)	0.20 (0.01)	0.029 (0.002)	0.030 (0.001)		
RW3	0.023 (0.002)	0.031 (0.002)	0.27 (0.03)	0.23 (0.01)	0.13 (0.01)	0.14 (0.01)	0.023 (0.001)	0.024 (0.002)	0.013 (0.001)	0.013 (0.002)
TW3			0.24 (0.01)	0.21 (0.01)	0.110 (0.008)	0.122 (0.004)	0.019 (0.001)	0.020 (0.002)		
RW4			0.50 (0.03)	0.41 (0.02)	0.21 (0.01)	0.23 (0.01)	0.068 (0.003)	0.070 (0.005)	0.032 (0.003)	0.026 (0.002)
TW4	0.021 (0.002)	0.031 (0.001)	0.34 (0.02)	0.30 (0.02)	0.15 (0.01)	0.17 (0.01)	0.022 (0.002)	0.022 (0.004)		
RW5	0.031 (0.001)	0.038 (0.003)	1.04 (0.02)	0.92 (0.03)	0.21 (0.01)	0.24 (0.01)	0.036 (0.002)	0.042 (0.002)		
TW5	0.029 (0.002)	0.029 (0.002)	0.75 (0.06)	0.63 (0.02)	0.25 (0.01)	0.26 (0.02)	0.025 (0.002)	0.031 (0.003)		
RW6			0.52 (0.06)	0.49 (0.02)	0.38 (0.02)	0.54 (0.03)	0.028 (0.001)	0.032 (0.002)		
TW6			0.56 (0.04)	0.48 (0.02)	0.41 (0.03)	0.52 (0.03)				
RW7	0.028 (0.002)	0.037 (0.002)	0.82 (0.09)	0.66 (0.03)	0.32 (0.01)	0.39 (0.02)	0.025 (0.001)	0.029 (0.002)		
TW7	0.021 (0.002)	0.027 (0.002)	0.75 (0.05)	0.66 (0.03)	0.25 (0.01)	0.31 (0.01)				

RW, raw wastewater.
TW, treated wastewater.

Table 5
Concentrations (ng g^{-1}, referred to dry weight) obtained in sludge samples. Average values for triplicate extractions, with standard deviation data. Empty cells correspond to concentrations below procedural LOQs in freeze-dried sludge.

Compound	Sample code					
	S1	S2	S3	S4	S5	S6
PRO-R	91.1 (0.9)				26.3 (2.1)	
PRO-S	113 (2)				37.5 (2.6)	
TRA-1	29.8 (1.7)	1067 (23)	136 (6)	92.2 (8.0)		
TRA-2	23.2 (1.9)	997 (30)	123 (4)	80.3 (8.2)		
VFX-1	20.4 (1.5)	133 (5)	112 (7)	43.8 (3.5)	11.8 (1.0)	8.7 (0.6)
VFX-2	23.0 (1.8)	133 (7)	119 (6)	50.6 (3.5)	12.8 (0.9)	9.2 (0.5)
CBZ-1	11.5 (0.9)	48.2 (2.4)	48.7 (3.5)	32.9 (2.7)	14.7 (1.0)	10.6 (0.9)
CBZ-2	12.8 (0.8)	48.8 (3.2)	47.7 (4.1)	35.1 (1.9)	15.1 (1.2)	11.1 (0.9)
TCZ-1	13.7 (0.9)	33.8 (2.7)	34.6 (2.7)	10.8 (0.9)	112 (7)	55.4 (4.8)
TCZ-2	13.8 (0.9)	41.1 (3.8)	45.0 (4.0)	10.5 (1.1)	110 (8)	68.8 (5.5)
MCZ-1	213 (11)	264 (15)	283 (19)	493 (20)	797 (35)	291 (16)
MCZ-2	229 (10)	303 (27)	310 (25)	418 (27)	782 (42)	305 (22)
STZ-1	20.2 (0.5)	34.5 (0.9)	39.6 (3.0)	4.8 (0.5)	74.7 (8.1)	10.9 (0.9)
STZ-2	25.4 (1.2)	48.5 (2.1)	56.6 (3.9)	5.0 (0.6)	73.6 (5.4)	11.0 (0.8)
FTZ-1	132 (7)	106 (6)	119 (9)	68.2 (5.4)	173 (12)	158 (9)
FTZ-2	153 (6)	137 (9)	154 (12)	72.9 (6.0)	165 (13)	150 (11)

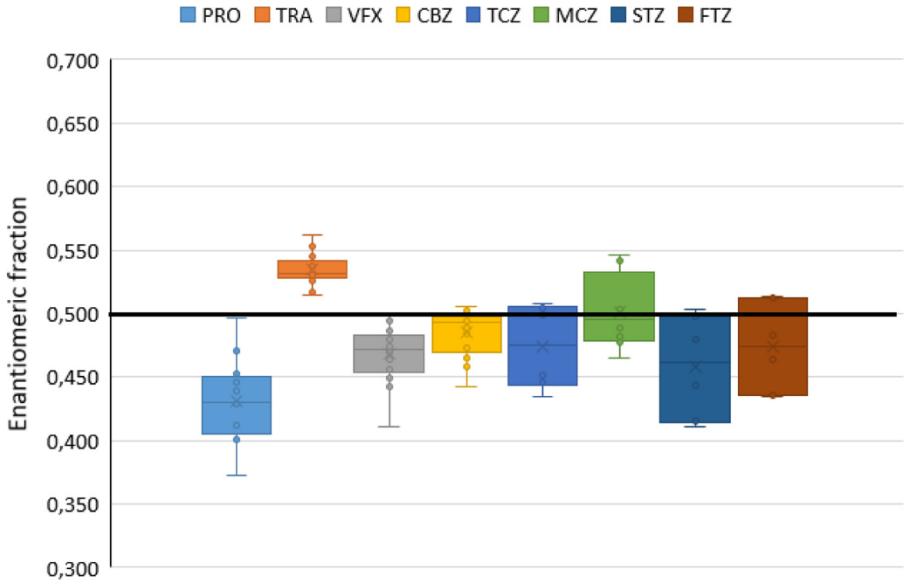

Fig. 2. Box-Whisker plot of enantiomeric fractions (EFs) for target compounds in samples (wastewater and sludge) above method LOQs.

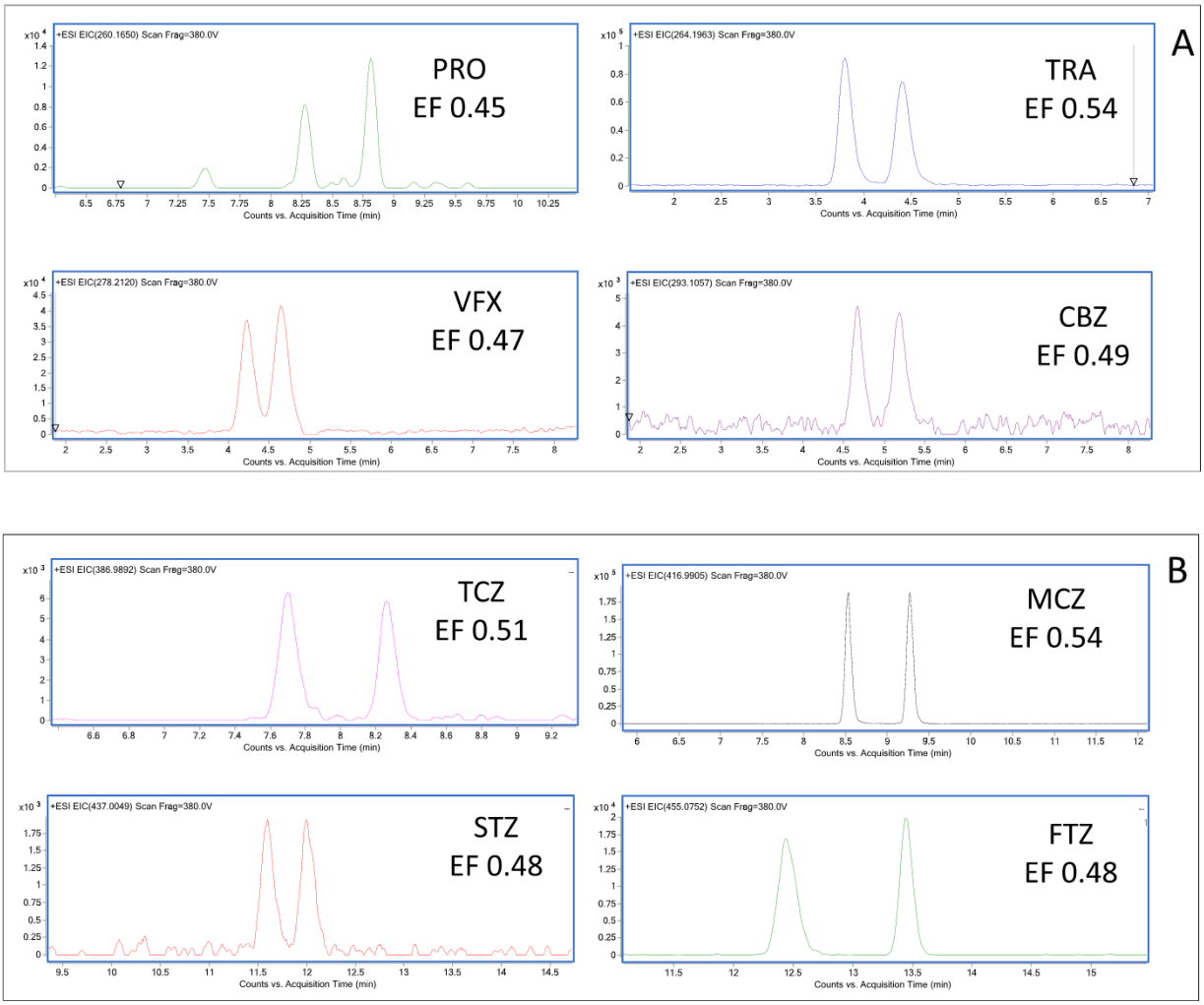

Fig. 3. Chromatograms for target compounds in non-spiked samples of wastewater (A, sample code TW2; PRO, TRA, VFX and CBZ) and sludge (B, sample code S4; TCZ, MCZ, STZ, FTZ), with EFs, calculated as ratio of concentration for the first eluting enantiomer and the sum of concentrations of both forms. Same SFC conditions as in Fig. 1.

dian EFs were below 0.5. In summary, the three pharmaceuticals showed a non-racemic distribution in the set of analyzed environmental samples. Their EFs were also calculated in three commercial medicaments, in this case, average values ranged from 0.501 to 0.507. We assume that changes in the EFs of these compounds did not occur at STPs, but they are the result of a differential metabolization between enantiomers. As regards the group of antimycotic pharmaceuticals (mainly found in sludge samples) their EFs showed a higher dispersion than those of amine-type pharmaceuticals. Considering the number of positive samples, no significant variations were noticed in their EFs compared to that expected for racemates (EF 0.5). As these compounds are mainly used in topic applications; thereafter, if they are partially biodegraded at STPs, EFs data obtained in this research suggest that the process was not enantioselective for investigated compounds. Fig. 3 shows the extracted ion chromatograms corresponding to the $[M+H]^+$ ion of each compound in two non-spiked samples of wastewater (Fig. 3A) and sludge (Fig. 3B), with the experimental EF values measured for each compound.

4. Conclusion

The SFC-QTOF-MS methodology optimized in this research permitted the rapid resolution of a selection of eight chiral compounds, with shorter analysis time and lower consumption of organic solvents than those employed in previous chiral applications using LC as separation technique. To the best of our knowledge, this research describes for the first time, the simultaneous enantiomeric resolution of azolic and amine-type drugs. Retention and enantiomeric resolution of the latter group of compounds were sensitive to the additive combined with MeOH in the mobile phase. Ammonia (0.1% in MeOH) provided the best balance between enantiomeric resolution and ionization efficiency of target compounds in the ESI source of the MS spectrometer. SFC-QTOF-MS combined with extraction-concentration methods isolating basic species from environmental samples, permitted to determine the concentrations and the EFs of target compounds in wastewater and sludge samples, with minor or moderate signal suppression effects. Residues of the three considered amine drugs (PRO, TRA and VFX) showed a non-racemic distribution in the investigated environmental samples, whilst EF values of azolic drugs remained around 0.5%. Very likely, enantioselective metabolization, rather than chiral selective biodegradation at STPs, is the responsible of the non-racemic distribution of PRO, TRA and VFX in the processed samples.

Declaration of Competing Interest

Authors declare no competing interest.

CRediT authorship contribution statement

M. Cobo-Golpe: Investigation, Methodology, Writing – original draft. **M. Ramil:** Supervision, Writing – review & editing. **R. Cela:** Project administration, Funding acquisition, Writing – review & editing. **I. Rodríguez:** Conceptualization, Supervision, Funding acquisition, Writing – original draft.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463088.

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