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# Dual enantioselective LC–MS/MS method to analyse chiral drugs in surface water: Monitoring in Douro River estuary

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ABSTRACT

This work presents the development of an enantioselective method to quantify chiral drugs (CDs) in surface water and its application in the Douro River estuary monitoring. Different classes of CDs were targeted, including 23 compounds, namely beta-blockers, antidepressants, one $beta_2$-adrenergic agonist, non-steroidal anti-inflammatory drugs, stimulants, and some illicit drugs as cocaine (COC) and its metabolites, and amphetamines. The analytical method was based on an innovative application of solid phase extraction (SPE), followed by liquid chromatography-tandem mass spectrometry (LC–MS/MS) using a triple quadrupole analyzer. The ground-breaking approach of SPE consists in the use of Oasis® MCX cartridges to pre-concentrate 500 mL of water samples, allowing the simultaneous extraction of acidic, basic and neutral analytes, rather than the conventional recovery of basic compounds only. Two chiral columns were used for enantiomeric separation in reverse elution mode, a Chirobiotic™V and a Pirkle type Whelk-O® 1, for basic and acidic compounds, respectively. The method validation demonstrated good linearity ($r^2 > 0.99$), selectivity and sensitivity, with method detection limits between 0.01 and 2.66 ng $L^{-1}$ and method quantification limits between 0.02 and 5.71 ng $L^{-1}$. The developed method was successfully applied to monitor daily variations along one week in surface waters collected in 5 locations of the Douro River estuary. Tramadol (TRM) and its metabolite *N*-desmethyltramadol (NDT), presented high concentrations near the affluent of a tributary river, while the second eluted enantiomer of *O*-desmethyltramadol (ODT) was found at high concentrations at the mouth of the Douro River. The metabolite NDT was quantified at higher concentrations than TRM. Venlafaxine (VNF) was found at high concentrations near the affluent of the same tributary river, but its metabolite, *O*-desmethylvenlafaxine (ODV), was found at concentrations 3 times higher. COC was found every day at all sampling points along the estuary, with slight variations.



## 1. Introduction

Over the last decades, a wide range of organic compounds has been constantly released into the aquatic compartments, becoming an environmental concern due to their (pseudo)persistence and potential toxicity [1,2]. These pollutants are generally found in different environmental aqueous matrices at concentrations in the range of ng $L^{-1}$ to μg $L^{-1}$ [3]. They reach the environment through different pathways, including the elimination of drugs by industry, disposal of unused medicines, illegal discharges, leaching from agriculture, and through excretion after veterinary and human

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use, the last one considered the main source of pharmaceuticals and illicit drugs in the environment [2]. Besides excretion of parent drugs, phase I metabolites are also excreted, as well as phase II metabolites, which can be hydrolysed into the parent drug by microbial enzymes, or persist after wastewater treatment in both the aqueous and suspended solid phases [4]. In fact, the wastewater treatments plants (WWTPs) are not designed to complete eliminate this type of residues, being their effluents the most important source of environmental contamination by pharmaceuticals, illicit drugs and their metabolites [5]. Thus, these residues may reach the environmental compartments, such as surface water, groundwater and soil, which are interconnected. These contaminants represent a major environmental and public health concern due to their potential effects on both aquatic organisms and human health, in this case through the possible entry into drinking water resources and into the food chain [2,3]. Although the concentrations of this type of drugs and their metabolites usually found in surface water are considered low, their potential effects on fauna, flora and human health, especially with prolonged exposure, must be considered and deeply studied, especially in vulnerable populations [3].

Many of these contaminants are chiral and their enantiomers may exhibit different biological properties. Chiral drugs (CDs) are marketed as racemate or enantiomerically pure and the enantiomeric composition may differ after absorption, metabolism and excretion. Additionally, the degradation/biodegradation in the WWTPs depends on their stereochemistry and may lead to stereoselective enrichment or racemization [6]. Therefore, CDs can be detected in the environment as enantiomeric mixtures and/or pure enantiomers [7,8]. Although the occurrence of chiral pharmaceuticals and illicit drugs in environmental waters has been extensively investigated in the last decades, most studies neglect the role of their stereochemistry [9]. However, the enantiomeric fraction (EF) is crucial to understand the fate of CDs and their complete lifecycle in the environment, as well as for an accurate environmental risk assessment [8]. Furthermore, the knowledge about the enantiomeric profile of CDs is a powerful tool to give insights about the biodegradation occurring in WWTPs, the identification of illicit discharges and the consumption pattern of many substances (e.g., illicit drugs and pharmaceuticals used as recreational drugs) [10–12].

The multi-residue enantioselective environmental analysis is still challenging due to the constraints related to the demanding sample preparation and enantioselective separation of different classes of CDs using the same extraction and chromatographic conditions [2]. More than one decade ago, MacLeod et al. [13] developed the first multi-class enantioselective LC–MS/MS method to analyse 3 classes of pharmaceuticals in wastewater, using a Chirobiotic™V. Other multi-residue enantioselective analytical methods have been developed to determine pharmaceutical in environmental matrices [8,14], as well as drugs of abuse [5,15]. In 2012, Kasprzyk-Hordern et al. [15] developed the first enantioselective LC–MS/MS method to determine multi-class pharmaceuticals and drugs of abuse in wastewater. In the last years, some reports have been published in this topic [2,9]. This work presents a validated analytical method based on solid-phase extraction (SPE) with Oasis® MCX cartridges to extract acidic, neutral and basic CDs, followed by a dual enantioselective LC–MS/MS analytical method for the quantification of 23 compounds (pharmaceuticals and illicit drugs, and some of their phase I metabolites), using two chiral stationary phases (CSPs). Chirobiotic™V CSP was selected due to its ability to enantioseparate neutral molecules, amides, acids, esters and amines, and Whelk-O® 1 CSP due to its high enantioselectivity for acidic compounds such as profens [9]. This validated dual analytical method was applied to monitor diverse therapeutic classes of pharmaceuticals and various illicit substances (beta-blockers, selective serotonin and noradrenaline reuptake inhibitors, a beta$_2$-adrenergic agonist, non-steroidal anti-inflammatory drugs (NSAIDs), and illicit drugs) in surface water of Douro River estuary. This work describes for the first time the pre-concentration of basic, acidic and neutral compounds using Oasis® MCX cartridges and the dual chiral analyses with Chirobiotic™ V and Whelk-O® 1 CSPs. The use of the Whelk-O® 1 CSP was never reported before in analysis enrolling environment matrices.

## 2. Material and methods

### 2.1. Chemicals

Acetonitrile (ACN) and methanol (MeOH) MS grade, and ethanol (EtOH) HPLC grade were purchased from Fisher Scientific (UK). Formic acid (98–100%) (FA), chloroform ($CHCl_3$) and ammonia were acquired to EMSURE (Germany); ammonium trifluoroacetate and ammonium acetate were obtained from Fluka (Netherland); acetic acid (100%), ethyl acetate (EtOAc) and sulfuric acid 95–97% were respectively purchased from HiPerSolv Chromanorm (France), LiChrosolv and Sigma-Aldrich (Germany). Fluoxetine (FLX), alprenolol hydrochloride (ALP), metoprolol tartrate (MET), tramadol hydrochloride (TRM), propranolol hydrochloride (PHO), salbutamol hemisulfate (SBT), benzoylecgonine (BE), mirtazepine (MZP), ibuprofen (IBU), ketoprofen (KET), naproxen (NPX), warfarin (WARF), bisoprolol hemifumarate (BSP), nebivolol (NEV), *O*-desmethylvenlafaxine (ODV), and the internal standards PHO-$D_7$, FLX-$D_5$ and KET-$D_5$ were purchased from Sigma-Aldrich (Steinhein, Germany). Venlafaxine hydrochloride (VNF) and flurbiprofen (FLB) were supplied by Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). *O*-desmethyltramadol (ODT) was purchased from Fluka Analytical (Switzerland). *N*-desmethyltramadol (NDT), amphetamine (AM), methamphetamine (MA), norcocaine (NCOC), cocaine (COC), and COC-$D_3$ were acquired from Lipomed (Arlesheim, Switzerland). All reference standards were >98% purity (Table S1). Individual standard solutions were prepared for all compounds, which were then diluted to prepare an ethanolic stock solution containing a mixture of all target compounds with a concentration of 100 μg mL$^{-1}$, which was stored at −4 °C.

### 2.2. Solid-phase extraction (SPE)

For optimization of SPE, method development and validation, water samples from the headwaters of Leça River (41°19′N 8°24′20″O) were collected to be used as a blank matrix.

The SPE was performed using a Visiprep™ SPE Vacuum Manifold (Sigma-Aldrich) and OASIS® MCX (Mixed mode Cation eXchange) cartridges (150 mg, 6cc) obtained from Waters (Portugal). In the optimization of the SPE conditions, the procedure was performed using 500 mL of water sample, acidified with $H_2SO_4$ to pH 2 and spiked with 250 μL of an ethanolic solution containing all target compounds with a concentration of 75 ng L$^{-1}$. Apart from these set conditions, different protocols were tested (Table S2) in order to optimize the SPE procedure, aiming to recover all the target CDs with a wide range of p*K*a values. The conditions tested included the conditioning step (with EtOH or MeOH or using the cartridge without previous conditioning), the washing step (different solvents used) and the elution steps (one or two steps, depending on the washing step used). Briefly, the optimized procedure started with the sample loading using the cartridge without conditioning, which was then washed with 4 mL of ultrapure water acidified with 2% FA and dried for approximately 30 min. After drying, two elutions were performed, the first elution with 4 mL of EtOH and the second elution with 4 mL of an ethanolic solution of 5% $NH_4OH$. The first and second elutions were analysed

respectively on the Whelk-O® 1 and Chirobiotic™ V CSPs, the first for analysis of acidic/neutrals and the second for basic compounds. The eluates were evaporated in a CentriVap Benchtop Vacuum Concentrators (Labconco®), then reconstituted with 250 μL of EtOH and filtered using 0.22 μm polytetrafluoroethylene (PTFE) syringe filters (Membrane Solutions, Texas, USA).

### 2.3. Ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS)

Chromatographic analysis was performed using a Shimadzu UHPLC Nexera System equipped with two Pumps LC-30AD, an Autosampler SIL-30AC, an Oven CTO-20AC, a Degasser DGU-20A5, a System Controller CBM-20A, a LC Solution Version 5.41SP1 (Shimadzu Corporation, Tokyo, Japan) and a triple quadrupole mass spectrometer detector Shimadzu LCMS-8030 coupled to the LC System.

A Chirobiotic™ V CSP (150 mm × 2.1 mm i.d., 5 μm particle size) and a Whelk-O® 1 CSP (250 mm x 4.6 mm i.d., 5 μm particle size) were used. The optimized mobile phase for Chirobiotic™ V CSP was a solution of EtOH/10 mM aqueous ammonium acetate buffer (92.5/7.5, *v/v*) at pH: 6.8 and using a flow rate of 0.32 mL $min^{-1}$, as published elsewhere [8]. The optimized mobile phase for Whelk-O® 1 CSP consisted in a mixture of MeOH/$H_2O$ (60/40, *v/v*) acidified with 0.1% FA, with a flow rate of 1.0 mL $min^{-1}$. The elution mode was isocratic for both columns. Column oven temperatures were 25 °C for Chirobiotic™ V and room temperature for Whelk-O® 1. The autosampler temperature was set at 15 °C. The volume of injection was 10 μL.

For LC–MS/MS, an electrospray ionization (ESI) source operating in positive and negative ionization mode was used. The direct injection of individual standard solutions of each compound at a concentration of 10 mg $L^{-1}$ was performed in both ionization modes to select the precursor ion through full scan mode (Table S3). Afterwards, the selection of the most abundant fragments and the optimization of the mass spectrometer parameters was performed by direct injection of individual standard solutions at 1 mg $L^{-1}$ for each compound. These parameters were optimized as 1.5 V for capillary voltage, 2.0 $dm^3$ $min^{-1}$ for nebulizing gas flow, 12.5 $dm^3$ $min^{-1}$ for drying gas flow, 300 °C for source temperature, and 475 °C for desolvation temperature. Argon was used as collision induced dissociation (CID) gas at 230 kPa.

Most of the target compounds were analysed in the positive ionization mode ($[M+H]^+$) with the exception of IBU, KET, FLB and NPX which were analysed in the negative ionization mode ($[M-H]^-$). For most target CDs, two selected reaction monitoring (SRM) transitions between the ion precursor and two of the most abundant ions fragments allowed the quantification (SRM1) and the unequivocal identification (SRM2 and ratio SRM1/SRM2) according to the EU Commission Decision 2002/657/EC as performed elsewhere [8], together with the retention time. Some of the target analytes in this study only had one transition (SRM1), which was used for quantification.

EF was used to express the enantioselectivity where [E1] and [E2] designate the concentrations of the first and second enantiomers eluted from the chiral column, respectively. When the configurations of the eluted enantiomers were known, it was assigned as (*S*) and (*R*) for each enantiomer. The following equations were used to calculate the EF:

$$EF = \frac{[E1]}{([E1]+[E2])} \quad (1)$$

or

$$EF = \frac{[S]}{([S]+[R])} \quad (2)$$

### 2.4. Method validation

The method was validated according to previous works [8], considering the following parameters: selectivity, linearity and range, limits of detection and quantification, accuracy, precision and recovery. Selectivity was verified by comparing the chromatograms of standards dissolved in EtOH, extracts of surface water of the Douro River, and extracts of spiked and non-spiked (blanks) surface water of the Leça River. To evaluate the accuracy and the intra and inter-batch precision [16], surface water samples of Douro River were spiked at three different concentrations (32.5, 65 and 130 ng $L^{-1}$), with three standard solutions (quality control, QC), in triplicate (n = 9). The precision of the method was expressed through the relative standard deviation (RSD) of the replicate measurements. The recovery was calculated by comparing the peak areas of the SPE extracts of each fortified matrix after subtracting the blank signal, with the respective standard solution with the same theoretical concentration. Linearity and range were evaluated using calibration curves prepared in triplicate with a set of seven different standard concentrations of the enantiomers in the enriched samples: 7.5; 15; 30; 60; 75; 90; 120 ng $L^{-1}$. The possible carryover was assessed by injecting the mobile phase solvent between each set of standards. Method detection limits (MDLs) and the method quantification limits (MQLs) were calculated through the signal-to-noise (S/N) ratio of 3.3 for MDL and 10 for MQL of extracts of spiked water samples.

### 2.5. Water sampling

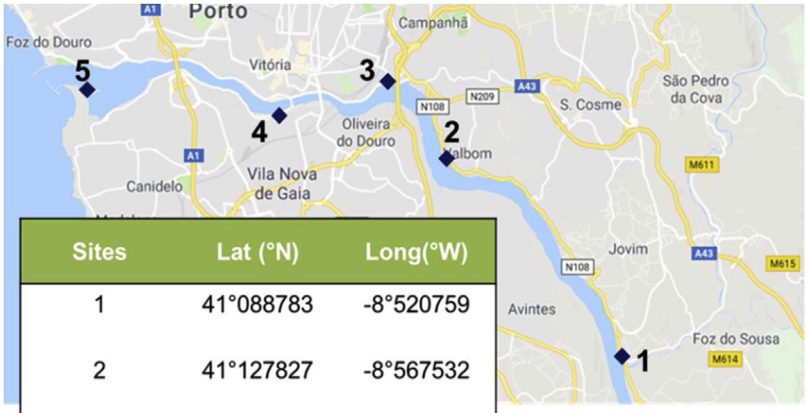
Water samples from the Douro River estuary (Fig. 1) were collected in pre-washed amber glass bottles (1000 mL) approximately one meter deep and as far from the shore as possible, transported at 4 °C and processed within 24 h in the laboratory. After reception, the samples were acidified to pH 2, vacuum filtered through 0.45 μm membrane filters (Whatman™, United Kingdom), pre-concentrated as described above and 10 μL of the reconstituted extract were analysed by the validated dual enantioselective LC–MS/MS analytical method. The sampling campaigns were performed between July 25th (Wednesday) and July 31th, 2018 (Tuesday).

## 3. Results and discussion

### 3.1. Optimization of enantiomeric separation

The enantioseparation of the target compounds was performed with two different CSPs, namely Chirobiotic™ V and Whelk-O® 1, both in reversed elution mode. The Whelk-O® 1 CSP was selected for the separation of the NSAIDs, and as expected, all these pharmaceuticals were successfully enantioseparated, with great resolutions between 4.00 and 28.1, except for IBU (0.68), in a total run time of 90 min (Fig. 2, Table 1). The anticoagulant WARF presented enantioseparation in both CSPs, but it was analysed using the Whelk-O® 1, since this pharmaceutical eluted in the same fraction of the NSAIDs in the sample preparation procedure. The Whelk-O® 1 CSP showed an excellent enantioselectivity and resolution for WARF (α = 1.63 and Rs = 32.3) (Fig. 2 and Table 1).

The beta-blockers, antidepressants and salbutamol and the others basic CDs were analysed according to conditions established before [9]. A chromatographic run of less than 30 min in the Chirobiotic™ V allowed the detection of all basic CDs and the enantioseparation of 10 compounds, namely SBT, ALP, BSP, MET, PHO, FLX, MZP, VNF, ODV, and NDT (Fig. 3 and Table 1). The $beta_2$-adrenergic agonist (SBT) and the antidepressants (FLX, VNF, MZP, ODV) were enantioseparated with α and Rs ranging between 1.09–1.20 and 0.59–1.38, respectively. The standards of COC, BE




| Sites | Lat (°N) | Long(°W) |
|---|---|---|
| 1 | 41°088783 | -8°520759 |
| 2 | 41°127827 | -8°567532 |
| 3 | 41°140468 | -8°596753 |
| 4 | 41°138911 | -8°620612 |
| 5 | 41°136485 | -8°660477 |

**Fig. 1.** Location of the sampling sites and respective GPS coordinates in the Douro River estuary, Portugal.

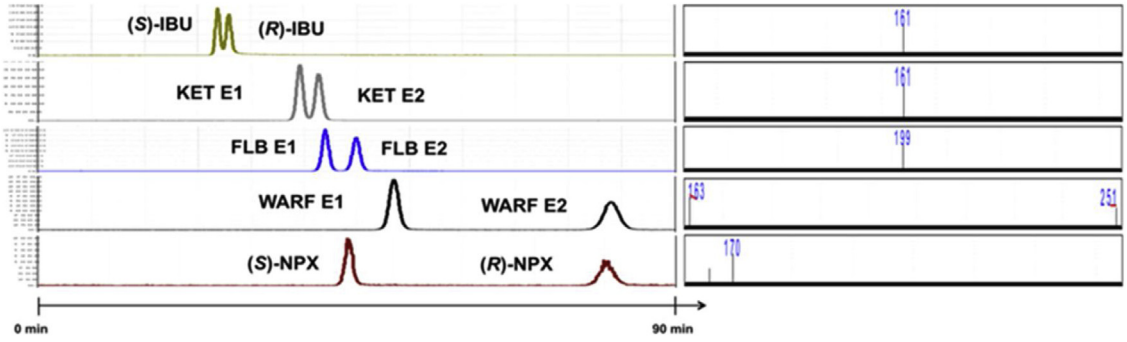



**Fig. 2.** Chromatograms of IBU, KET, FLB, WARF and NPX. Conditions: Whelk-O® 1CSP, 250 mm x 4.6 mm i.d., 5 μm particle size; Mobile phase: MeOH/ultrapure water (60:40) and 0.1% acetic acid; Flow rate of 1.0 mL $min^{-1}$; Column at room temperature; Volume injection 10 μL.

**Table 1**
Retention time and resolution (Rs) of the target CDs.

| CSP | Compound | Retention time (1 st enantiomer) | Retention time (2nd enantiomer) | $k_1$ | $k_2$ | α | Rs |
|---|---|---|---|---|---|---|---|
| Chirobiotic™V | COC | 6.4 | – | 0.60 | – | – | – |
| | NCOC | 5.5 | – | 0.22 | – | – | – |
| | BE | 5.5 | – | 2.67 | – | – | – |
| | AM | 8.5 | – | 9.75 | – | 1.00 | – |
| | MA | 12.4 | – | 23.96 | – | 1.00 | – |
| | TRM | 10.0 | – | 9.10 | – | 1.00 | – |
| | ODT | 19.5 | 22.7 | 11.3 | 13.4 | 1.18 | 1.74 |
| | NDT | 8.1 | – | 9.11 | – | 1.00 | – |
| | SBT ((*R*)-SBT*) | 7.5 | 8.8 | 6.50 | 7.80 | 1.20 | 1.53 |
| | ALP((*S*)-ALP*) | 19.4 | 22.6 | 4.78 | 5.72 | 1.20 | 1.47 |
| | BSP (BSP E1*) | 9.2 | 10.4 | 8.16 | 9.44 | 1.16 | 1.51 |
| | MET ((*S*)-MET*) | 9.9 | 11.4 | 49.0 | 56.5 | 1.15 | 1.31 |
| | NEV | 14.9 | – | 5.98 | – | 1.00 | – |
| | PHO ((*S*)-PHO*) | 10.5 | 12.3 | 9.50 | 11.3 | 1.19 | 1.41 |
| | FLX ((*S*)-FLX*) | 11.5 | 13.0 | 7.00 | 8.05 | 1.15 | 1.38 |
| | MZP (MZP E1*) | 12.7 | 14.9 | 2.00 | 2.54 | 1.27 | 0.77 |
| | VNF ((*S*)-VNF*) | 13.6 | 14.8 | 27.6 | 30.0 | 1.09 | 0.59 |
| | ODV (ODVE1*) | 14.0 | 14.1 | 10.3 | 11.2 | 1.09 | 0.74 |
| Whelk-O® 1 | FLB (FLB E1*) | 27.0 | 29.9 | 5.75 | 6.50 | 1.13 | 7.06 |
| | KET (KET E1*) | 24.6 | 26.4 | 1.86 | 2.06 | 1.11 | 4.00 |
| | NPX ((*S*)-NPX*) | 44.1 | 80.0 | 39.4 | 81.1 | 2.06 | 28.1 |
| | WARF (WARF E1*) | 33.5 | 53.9 | 36.1 | 59.0 | 1.63 | 32.3 |
| | IBU ((*S*)-IBU*) | 16.8 | 17.9 | 4.09 | 4.42 | 1.08 | 0.68 |

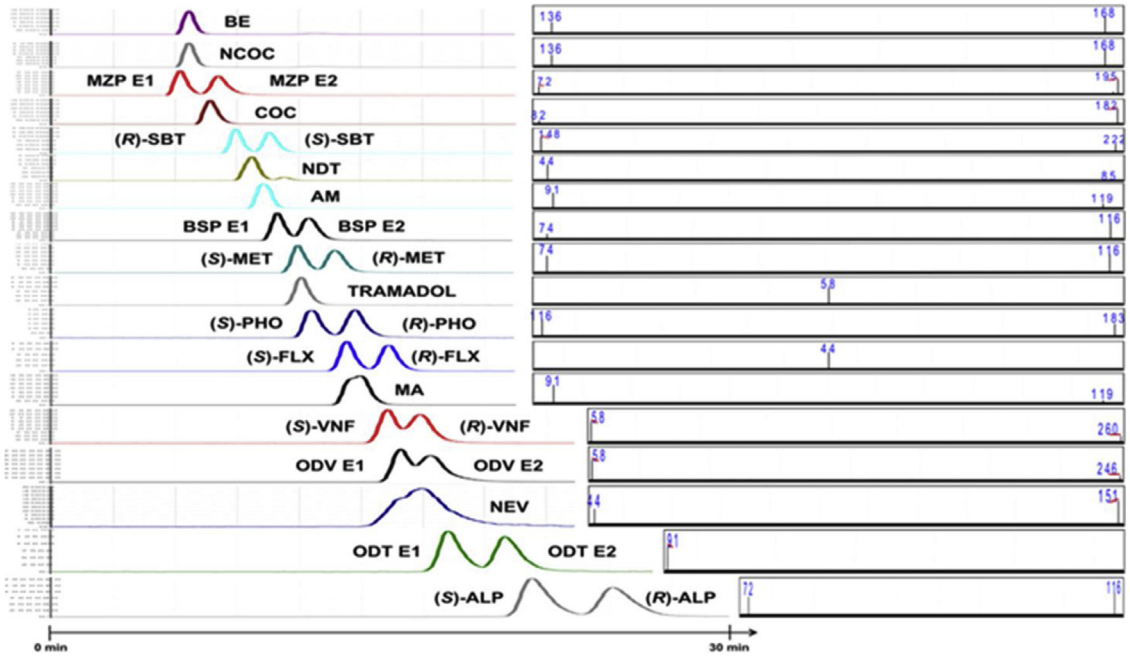



**Fig. 3.** Chromatograms of BE, NCOC, MZP, COC, SBT, NDT, AM, BSP, MET, TRM, PHO, FLX, MA, VNF, ODV, NEV, ODT and ALP. Conditions: Chirobiotic™V CSP, 150 mm x 2.1 mm i.d., 5 μm particle size; Mobile phase: EtOH/10 mM aqueous ammonium acetate buffer with pH 6.8; Flow rate of 0.32 mL $min^{-1}$; Column oven 25 °C; Volume injection 10 μL.

and NCOC were enantiomerically pure, therefore the method was validated for only one enantiomer. Despite the analgesic TRM and its metabolite NDT were determined as unique molecular entities since they were not enantioseparated, the metabolite ODT was enantioseparated with a great resolution (Rs = 1.74). The enantiomers of CDs belonging to the class of stimulants of central nervous system and illicit drugs as AM and MA, were also not enantioseparated under the optimized conditions, but they were also included in the validated method to be analysed as unique molecular entities.

### 3.2. Solid-phase extraction (SPE)

The sample pH and the volume for reconstitution were adopted from a previous work of our team [8]. Oasis® MCX cartridges were selected due to their high selectivity for basic compounds. The innovative approach selected for the sample preparation in this work was based on the fact that in the traditional SPE procedure with Oasis® MCX cartridges, the first eluate containing the acidic compounds is usually rejected and only the basic compounds are recovered on the second elution. In order to attempt to recovery the basic, acidic and neutral compounds using one single cartridge and a simple protocol, different conditions for conditioning, washing and elution steps were tested, to establish the overall best procedure for all target compounds. The requirement of the conditioning step was also investigated, by testing the procedures with and without cartridges conditioning (Scheme 1).

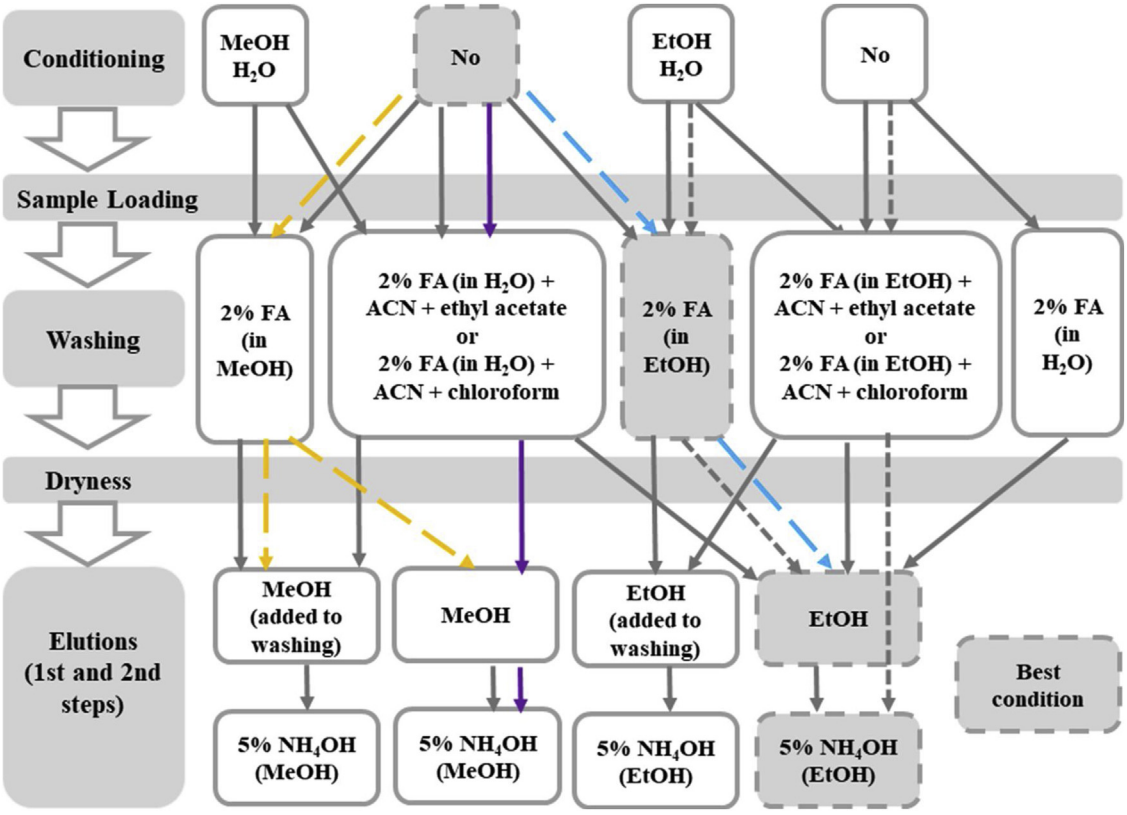
Fig. 4 shows the recoveries obtained for the first and second elution steps, after conditioning the cartridge with MeOH or EtOH, washing with 2% FA in ultrapure water and eluting firstly with MeOH or EtOH and secondly with 5% $NH_4OH$ in MeOH or EtOH. It was found that the acidic compounds (NSAIDs) and the neutral WARF was recovered in the first elution, with a good performance for the NSAIDs regardless of the organic solvent used, MeOH (25–90%) or EtOH (22–110%). In the second elution the recoveries using 5% $NH_4OH$ in MeOH and 5% $NH_4OH$ in EtOH were respectively in the range 15–85 and 28–98%.

Both organic solvents (MeOH and EtOH) gave similar recovery rates as eluting solvents, and therefore EtOH was selected due to its safer use to the operator and its ecofriendlyness. Moreover, attempting to decrease the solvent consumption, the time needed for sample preparation and to provide a more ecofriendly methodology, skipping of the conditioning step was tested (Fig. 5). This assay allowed to conclude that the conditioning step with either MeOH or EtOH did not have an improving effect on the recoveries in comparison to the procedure without conditioning. Thus, the method could be optimized without conditioning the cartridge, reducing the time spared and turning the sample preparation method more ecofriendly.

Afterwards, the possible loss of compounds in the washing step was verified, with the aim of optimize the procedure, either by using dispersive liquid-liquid microextraction (DLLME) to extract the aqueous washing solvent (2% FA in ultrapure water) or by using acidified organic solvents (2% FA in MeOH and EtOH) in the washing step. The DLLME of the acidified ultrapure water used as washing solvent, was performed with 1 mL of ACN as dispersive solvent and 50 μL of chloroform or EtOAc as extracting solvent [17], which were then collected from the bottom (chloroform) or from the top (EtOAc, after freezing the aqueous phase), respectively. These experiments suggested that the washing process using 2% FA in ultrapure water did not lead to any loss of the compounds. Otherwise, when MeOH or EtOH were used in the washing step, a significant loss of the compounds was observed (data not shown).

In the further experiments, the solvent used in the washing step was 2% FA in ultrapure water, and then the elution step was also tested with both 5% $NH_4OH$ in MeOH and EtOH (Fig. 6), with EtOH presenting the best overall results.

The method validation was performed using the optimized SPE procedure, skipping the conditioning step and using EtOH and 5% $NH_4OH$ in EtOH as solvents for the first and second elutions, respectively, representing one less step and a reduction of solvent consumption, making the method faster and more environmental friendly. The overall recoveries using the optimized SPE conditions, are presented in Table 2.




**Scheme 1.** SPE procedures attempted, with the best conditions highlighted.

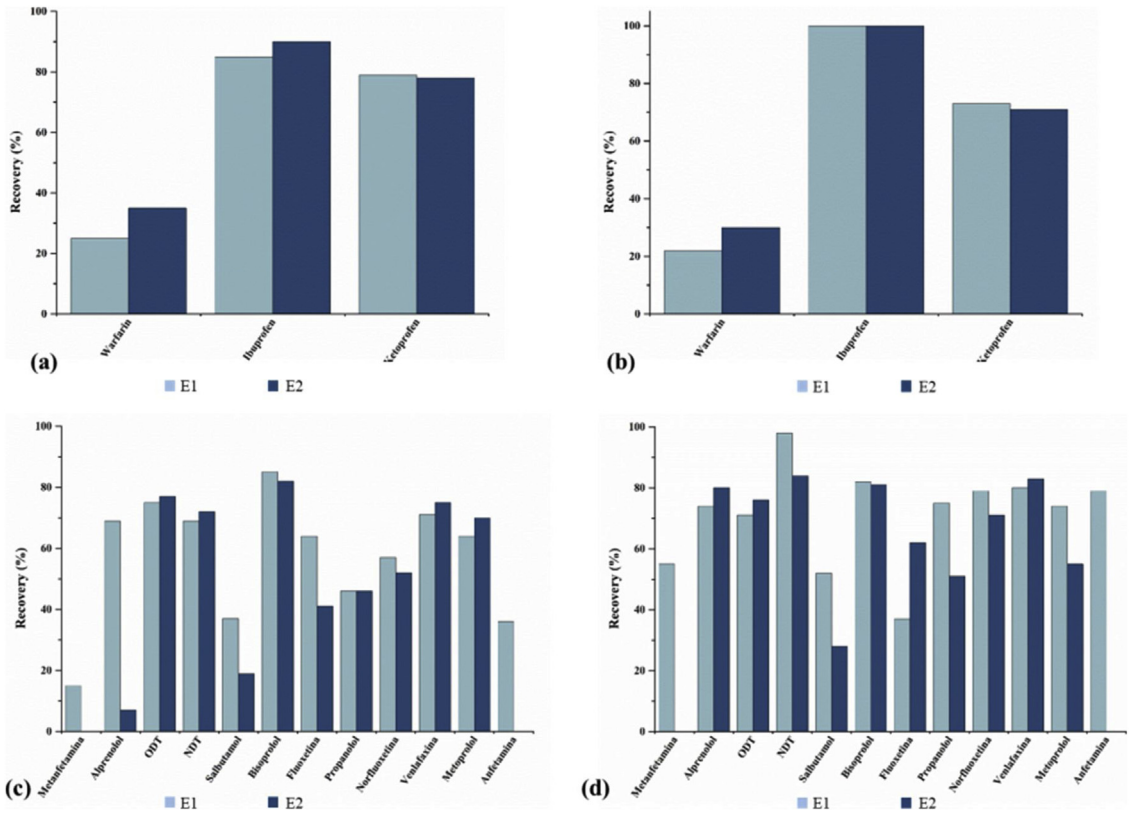



**Fig. 4.** Recoveries of each enantiomer (first eluted E1 and second eluted E2), obtained after: (a) conditioning with MeOH, washing with 2% FA in ultrapure water and eluting with MeOH; (b) conditioning with EtOH, washing with 2% FA in ultrapure water and eluting with EtOH; (c) eluting with 5% $NH_4OH$ in MeOH, after step (a); and (d) eluting with 5% $NH_4OH$ in EtOH, after step (b). (a) and (b) were analysed using the Whelk-O® 1 column, whereas (c) and (d) were analysed using the Chirobiotic™ V column.

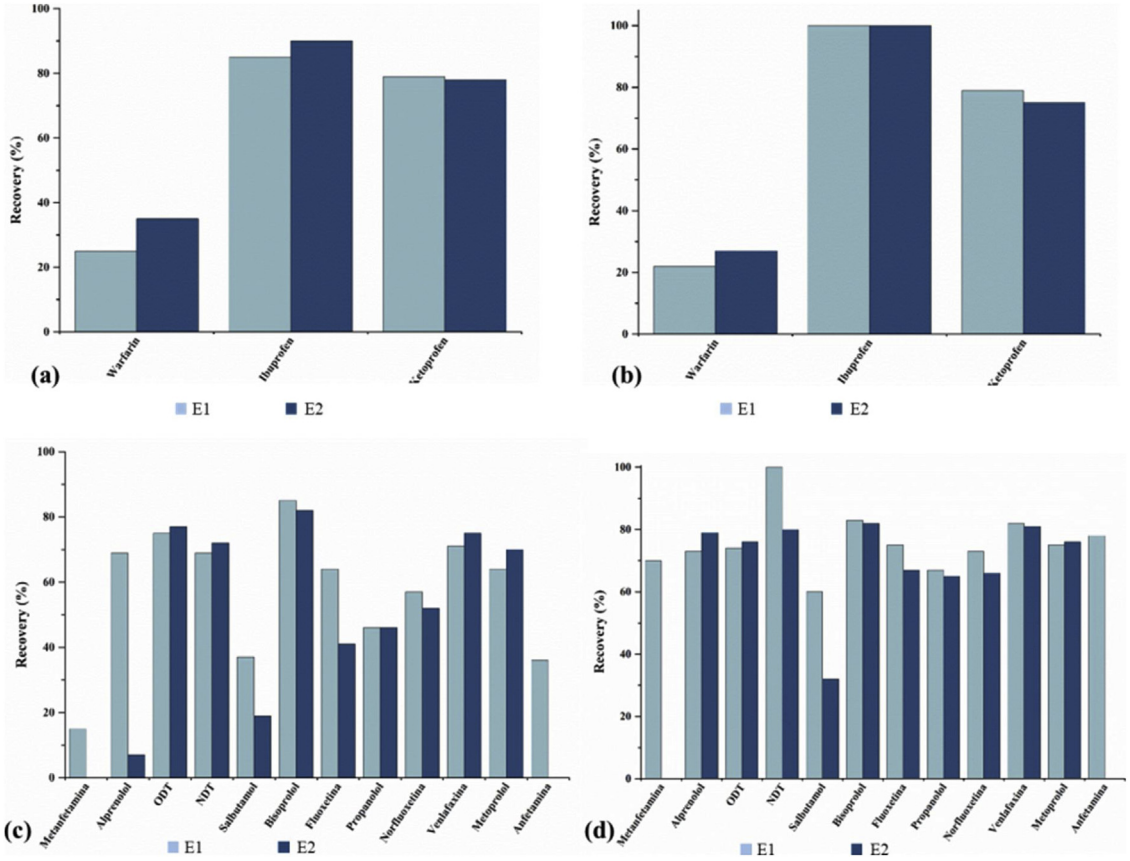



**Fig. 5.** Recoveries of each enantiomer (first eluted E1 and second eluted E2), obtained after: (a) conditioning with MeOH, washing with 2% FA in ultrapure water and eluting with MeOH; (b) no prior cartridge conditioning, washing with 2% FA in ultrapure water and eluting with MeOH; (c) eluting with 5% $NH_4OH$ in MeOH, after step (a); and (d) eluting with 5% $NH_4OH$ in MeOH, after step (c). (a) and (b) were analysed using the Whelk-O® 1 column, whereas (c) and (d) were analysed using the Chirobiotic™V column.

### 3.3. Method validation

The method was validated according to previous works [8,18] considering the following parameters: selectivity, linearity and range, MDLs, MQLs, accuracy, recovery and precision. The calibration curves (Table S4) were performed using internal calibration method, by spiking the samples with isotopically labelled internal standards, before SPE procedure. Different groups of target analytes were set and one internal standard was selected for each group of compounds, as described elsewhere [8]. The injection of the reconstituted extracts gave correlation coefficients between 0.995 and 0.998 in the range of linearity (Table S4). No carryover was observed. The MDLs were between 0.01 and 2.66 ng $L^{-1}$ and the MQLs were between 0.02 and 5.71 ng $L^{-1}$. The method accuracy for the quantification of the target CDs is presented in Table S4 and ranged from 75.4 to 122%.

### 3.4. Quantification of the target chiral compounds in five sampling point of Douro River estuary

In general, anthropogenic stress in the Douro River estuary results from population growth, industrial development, WWTPs effluents discharged into the estuary or its tributaries, and illicit discharges [18].

The five sampling points were selected based on previous works [18,19] and samples were collected for one week and analysed using the optimized and validated method here described, to study the daily variations on their concentrations and EFs. Some of the compounds were not detected under the optimized conditions, namely MZP, PHO, NEV, NPX and WARF. Some compounds, including BSP, MET, FLX, VNF, ODV and the first enantiomer of FLB, were detected every day at all sampling points. SBT and ALP were the single compounds that were detected and quantified only once in the sampling location 3. The EF values were calculated (Table S5) for all the compounds that were enantioseparated in either Chirobiotic™V or Whelk-O® 1 CSPs, with MET, VNF and ODV showing variations during the week. The temporal changes on the enantiomeric profiles of CDs in surface water can result from variations on EF of CDs in the WWTP effluents, contamination by sewage and direct disposal of drugs, which would undergo stereoselective metabolism when consumed, but studies on this subject are limited.

#### 3.4.1. Central nervous system stimulants - illicit drugs and metabolites

COC is one of the most potent stimulants in the central nervous system. In the liver, COC is rapidly metabolized by an enzyme to its major metabolite BE, which is excreted in the urine as 45% of the administered dose and also NCOC, a common metabolite in the urine and blood. COC is also partially excreted in the urine as a non-metabolised drug (1–9% of the administered dose) [20].

The COC and its metabolites were quantified with similar concentrations, but COC was detected every day in all sites at similar concentrations (Fig. 7). Higher concentrations of the metabolites in comparison to COC were expected due to the metabolism pattern of COC and the reported levels of BE usually higher than COC in wastewater [21]. However, similar results in surface waters were reported before, suggesting a different pattern of degradation of these compounds in the receiving the environment. Other reason that may also contribute to the higher levels or persistence of COC in comparison to its metabolites can be its direct disposal [20].

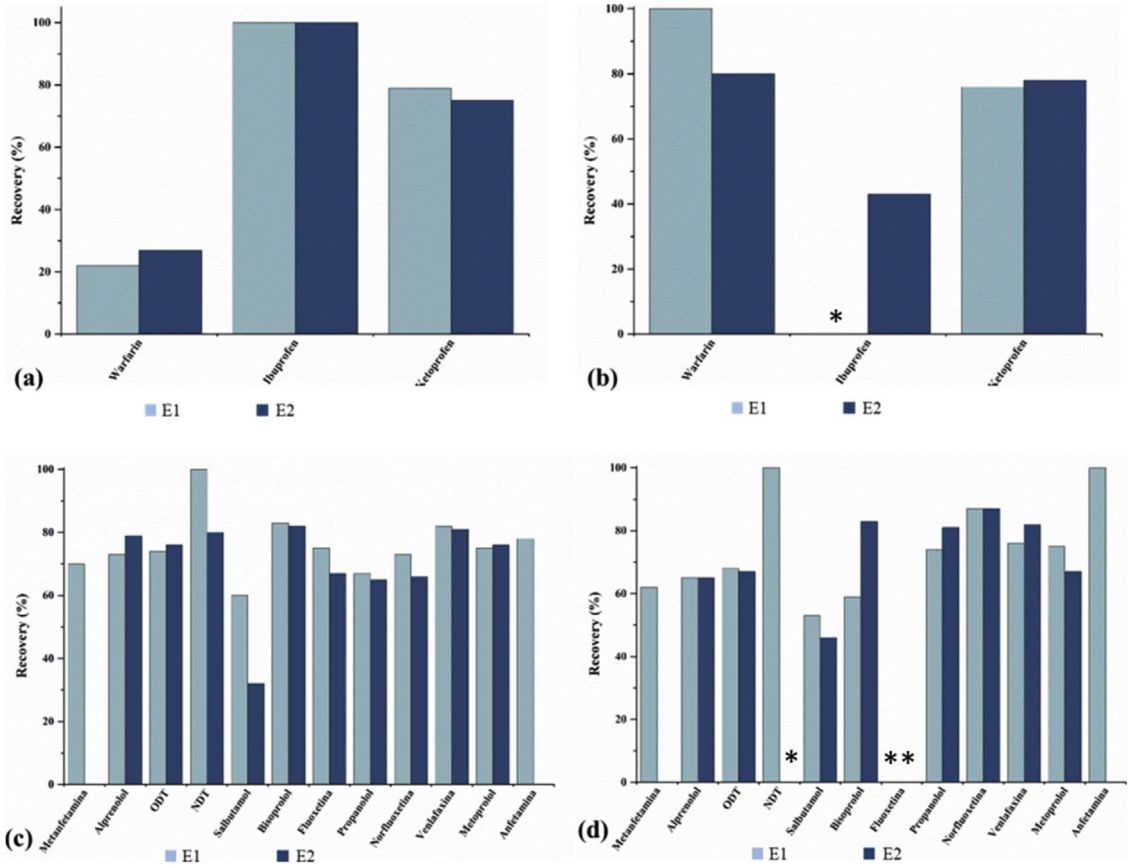



**Fig. 6.** Recoveries of each enantiomer (first eluted E1 and second eluted E2), obtained after: (a) no prior cartridge conditioning, washing with 2% FA in ultrapure water and eluting with 4 mL of MeOH; (b) no prior cartridge conditioning, washing with 2% FA in ultrapure water and eluting with EtOH; (c) eluting with 5% $NH_4OH$ in MeOH, after step (a); (d) eluting with 5% $NH_4OH$ in EtOH, after step (b). (a) and (b) were analysed using the Whelk-O® 1 column, whereas (c) and (d) were analysed using the Chirobiotic™ V column (* not determined.

Estimation of drug use at the community level from the concentrations of the parent drugs or their major metabolites measured in wastewater has become an increasingly accepted and extended tool, complementary to the methods traditionally used for this purpose [11]. Other study performed over a year in a major river in the UK has suggested that the high frequency of detection of COC and BE could be related to a high and steady consumption of COC in the studied region [5]. In that study, the range of concentrations of COC was similar to the present report, but BE was quantified up to 72.4 ng $L^{-1}$, meaning 6 times higher than the maximum values registered in our study. On the contrary, NCOC which was quantified in 4 of the 5 sampling points in Douro River, was not detected in any river water sample collected in that study in UK [5].

AM is a sympathomimetic derivative of phenethylamine that has been used in the treatment of obesity, narcolepsy, and hypotension but is now primarily used due to its stimulant activity. MA is a derivative of AM and is also excreted primarily unchanged and partially as AM [22].

AM and MA were analysed as unique molecular entities (Fig. 8). The AM was determined at higher concentrations between Saturday and Tuesday, which can be ascribed to the consumption at the weekend. Regarding MA, it was mostly determined from Wednesday, but it was not detected at all sites. Similar values were found for AM in Spain, but the concentrations of MA were higher than those reported in that study [20].

### 3.4.2. Analgesic

TMR is an opioid analgesic used to relieve moderate to severe pain. Its metabolism begins in the liver, resulting in the formation of its major chiral metabolites, ODT and NDT. Regarding TMR and its metabolites, only the metabolite ODT was enantioseparated under the conditions used in the present work (Fig. 9). Enantiomers of TMR and NDT were treated as unique molecular entities. TMR and NDT presented higher concentrations at the mouth of the Sousa River (sampling point 1), whereas the second eluted enantiomer of ODT was found at high concentrations at the mouth of the Douro River. The NDT metabolite was quantified at higher concentrations than TMR, which may indicate a high excretion and persistence of this metabolite in environment. TMR and its metabolite were detected at high concentration in our previous report concerning WWTP effluents [23].

### 3.4.3. $Beta_2$-adrenergic agonist

The presence of SBT was verified only at sampling point 3 on Thursday (Fig. 10). A previous study in WWTPs of Milano, reported similar results, the SBT showing some unexpected peak in one day, very high in relation to the days before and after [24]. This result may indicate an illegal discharge.

SBT is used to relief bronchoconstriction in asthma and other pulmonary diseases and the metabolism of SBT is stereoselective. Since the (*R*) enantiomer is the one with the highest concentration, in the quantification in the surface waters, it indicates that the biodegradation in WWTP is lightly enantioselective.

### 3.4.4. Beta-blockers

Regarding the beta-blockers, BSP was the compound found at highest concentration, mainly the second enantiomer eluted, giving

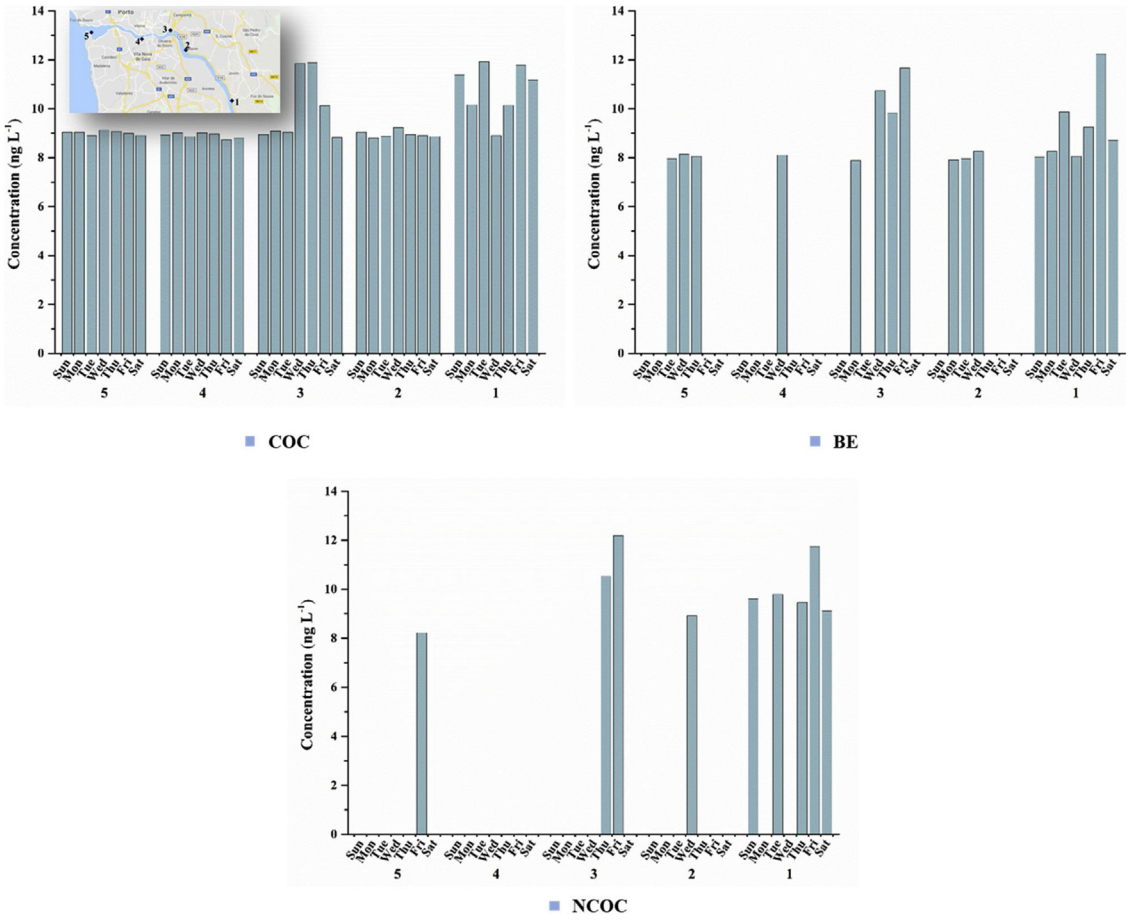



**Fig. 7.** Variation of concentration (ng $L^{-1}$) of COC and its metabolites BE and NCOC in five sampling sites of Douro River estuary.

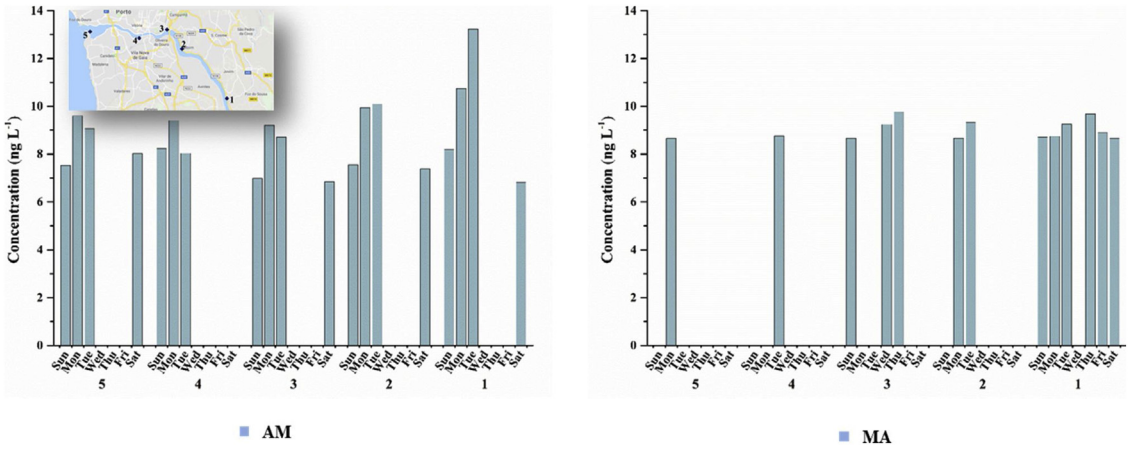



**Fig. 8.** Variation of concentration (ng$^{TM}$ $L^{-1}$) of AM and MA in five sampling sites of Douro River estuary.

an EF lower than 0.5 during the whole week at all sampling points, except on Sunday at sampling point 5 (Fig. 11).

The highest concentrations of BSP were found at sampling point 1. BSP is an antihypertensive and it is indicated to relieve high blood pressure, stable chronic heart failure and angina. It is considered a lipossoluble compound and the (*S*) enantiomer has a degradation time of 1.4 times greater than the (*R*) enantiomer. The enantiomer (*R*) is also the one with the highest renal clearance [25].

MET was also found in all sampling points, albeit at lower concentrations than BSP at sampling point 1. The enantiomer (*S*) was found at higher concentration than (*R*) at all sampling points and every day of the week, except for Wednesday at sampling point 1. This enrichment of (*S*)-MET was also observed in another study reporting the monitoring of surface waters [26].

ALP was detected at low concentration at sampling point 3 on Friday in a nearly racemic form and only the (*R*)-ALP was detected and quantified on Monday and Thursday. ALP is used for the treatment of angina pectoris but it is not marketed in Portugal, thus its determination can be ascribed to tourism in this area, namely tourists coming from Asia where this pharmaceutical is sold.

Beta-blockers such as PHO and NEV are two highly prescribed pharmaceuticals in Portugal, according to information obtained

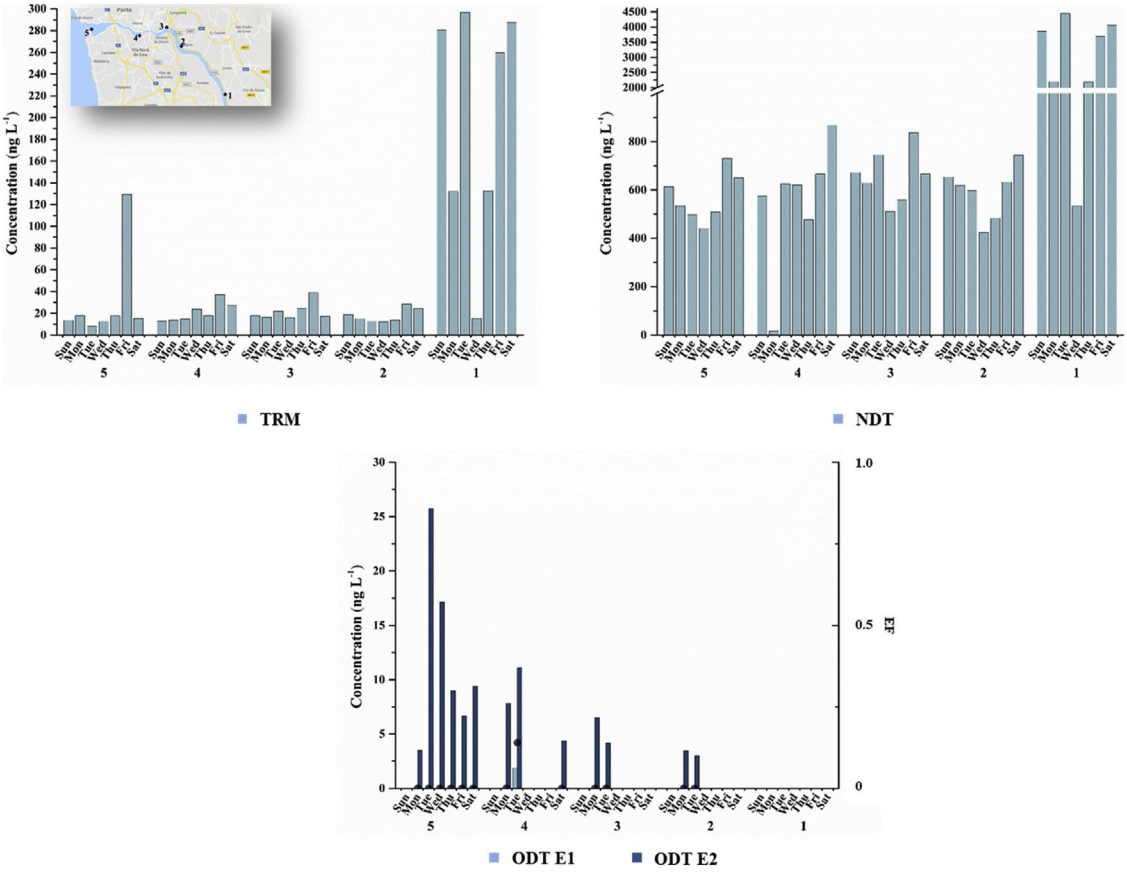



**Fig. 9.** Variation of concentration (ng $L^{-1}$) of TRM and metabolites (NDT and ODT) in five sampling sites of Douro River estuary.

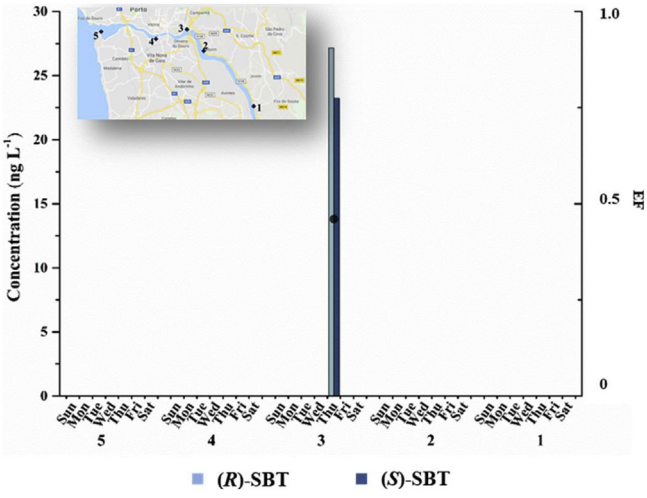



**Fig. 10.** Variation of concentration (ng $L^{-1}$) of the enantiomers of SBT in five sampling sites of Douro River estuary.

through INFARMED. However, it was not possible to detect them since they were below the MDLs.

### 3.4.5. Antidepressant

Fig. 12 shows the range of concentration (ng $L^{-1}$) of each enantiomer of the antidepressant FLX, VNF and its metabolite ODV.

FLX was found at low concentrations (up to 14 ng $L^{-1}$), similar to other studies [26,27], it is an ubiquitous compound as it was quantified at all sampling sites, confirming the persistence of this compound as already reported [26]. FLX was found with EF close to racemate. FLX is used for treatment of depression, obsessive compulsive disorder, bulimia nervosa and panic disorder, and improperly used for weight control. The (*S*) enantiomer is reported to have greater toxicity than the (*R*) enantiomer [28].

Regarding VNF, higher concentrations were found at the mouth of the Sousa River (sampling point 1), and the first enantiomer at higher concentrations on Sunday, Tuesday and Friday. Regarding sampling points 2–5, VNF was found at very low concentrations and the second eluted enantiomer was predominant. The ODV metabolite was found at higher concentrations than VNF. The first eluted enantiomer was the most prevalent. Another study on the enantiomeric profile of CDs has already shown the higher concentration of this metabolite in comparison to the parent compound, however in that report both VNF and ODV were found with EF near to 0.5 [26].

The (*R*)-enantiomer of VNF demonstrates greater serotonin reuptake inhibition properties, while the (*S*) inhibits the reuptake of both monoamines and is highly metabolised in humans. Demethylation to ODV is the main route of first pass metabolism of VNF and ODV is excreted unchanged [29]. Some studies have described a possible enantioselective metabolism of VNF to ODV with selection for (*S*) enantiomer or (*R*) enantiomer, but most pharmacokinetic studies of VNF do not distinguish between enantiomers. ODV has antidepressant activity and an ODV salt is a drug approved by the Food and Drug Administration (FDA).

### 3.4.6. Non-steroidal anti-inflammatory drugs (NSAIDs)

In the case of NSAIDs, it was possible to detect FLB residues (<MQL) at sampling point 5, although it was only possible to detect the first enantiomer. KET was also detected in residual quantities (<MQL) mostly at sampling point 5, but every day and in racemic

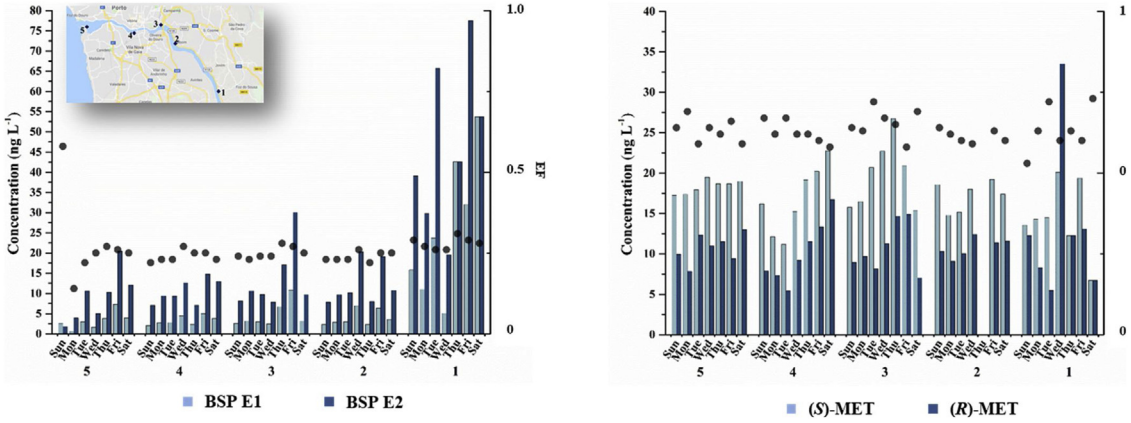



**Fig. 11.** Variation of concentration (ng $L^{-1}$) of the enantiomers of BSP and MET in five sampling sites of Douro River estuary.

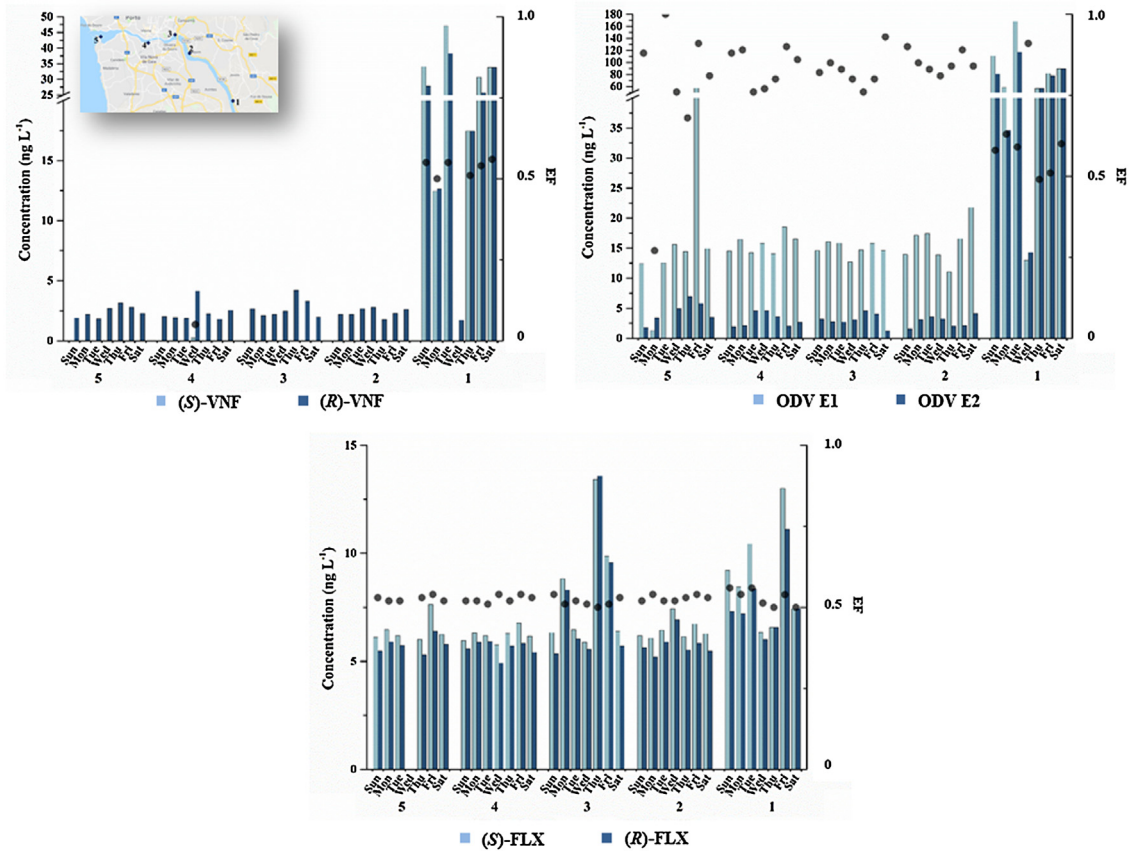



**Fig. 12.** Variation of concentration (ng $L^{-1}$) of the enantiomers of VNF, its metabolite ODV and FLX in five sampling sites of Douro River estuary.

form. The IBU was detected every day at all sampling points and based on the observation of the chromatograms, it was possible to estimate an EF = 0.5. NPX was not detected in the present study.

## 4. Conclusions

A sensitive analytical method based on SPE followed by LC–MS/MS was developed and optimized for simultaneous analysis of several CDs in surface water of the Douro River estuary. The preconcentration and clean-up of the samples in the SPE procedure were achieved using OASIS® MCX cartridges, by using an innovative procedure that was able to extract compounds with a wide range of p*K*a values (basic, neutrals and acidic), allowing to increase the number of analytes in one single extraction process that did not require cartridge conditioning, sparing time for sample preparation, and used the eco-friendly solvent EtOH in the elution steps, minimizing the environmental impact and, consequently, meeting the guidelines of green analytical chemistry procedures. The SPE-LC–MS/MS method validation was performed according to the international criteria and good results were obtained for selectivity, linearity, MDL, MQL, precision and accuracy.

The developed method was applied in the analysis of 35 surface water samples collected in Douro River estuary. From the 23 target compounds, only 5 (MZP, PHO, NEV, NPX and WARF) were not detected. The TMR metabolite NDT was the CD found at the highest concentration (up to 4444 ng $L^{-1}$). COC was found at similar concentrations every day at all sampling points and NCOC and

**Table 2**
Recoveries (%) and relative standard deviation (RSD, %) obtained with the optimized conditions.

| Compound | Recovery (%) | RSD (%) |
|---|---|---|
| COC | 79.3 | 13.0 |
| NCOC | 74.3 | 12.4 |
| BE | 74.3 | 11.9 |
| *R/S* (±)-AM | 63.3 | 14.2 |
| *R/S* (±)-MA | 58.7 | 14.1 |
| *R/S* (±)-TRM | 77.3 | 11.6 |
| ODT E1 | 72.3 | 11.9 |
| ODT E2 | 76.0 | 7.3 |
| *R/S* (±)-NDT | 71.7 | 9.9 |
| (*S*)-SBT | 68.7 | 6.2 |
| (*R*)-SBT | 54.7 | 6.8 |
| (*S*)-ALP | 73.7 | 13.2 |
| (*R*)-ALP | 80.7 | 9.7 |
| BSP E1 | 80.7 | 10.1 |
| BSP E2 | 76.3 | 11.0 |
| (*S*)-MET | 72.7 | 8.5 |
| (*R*)-MET | 75.3 | 9.0 |
| NEV | 65.2 | 18.8 |
| (*S*)-PHO | 71.0 | 7.1 |
| (*R*)-PHO | 66.3 | 0.5 |
| (*S*)-FLX | 65.0 | 7.5 |
| (*R*)-FLX | 58.3 | 9.5 |
| MZP E1 | 77.3 | 23.3 |
| MZP E1 | 80.7 | 22.1 |
| (*S*)-VNF | 80.7 | 11.9 |
| (*R*)-VNF | 75.0 | 14.2 |
| ODV (ODVE1*) | – | – |
| FLB E1 | 98.3 | 15.2 |
| FLB E2 | 99.0 | 15.1 |
| KET E1 | 104.3 | 8.7 |
| KET E1 | 107.0 | 7.8 |
| (*S*)-NPX | 96.0 | 13.4 |
| (*R*)-NPX | 105.7 | 11.6 |
| WARF E1 | 117.7 | 11.1 |
| WARF E2 | 104.0 | 2.4 |

BE were found at some locations, with concentrations in the same order of COC. AM and MA were found at similar concentrations at some locations. The EF values for the majority of the compounds were different than 0.5. The results of the monitoring programme give valuable information for further environment monitoring and shows the importance of multi-residue enantioselective analytical methods for accurate evaluation of risk.

## Acknowledgment

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2019.03.032.

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