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Enantiomeric Separation of Tramadol and Its Metabolites: Method Validation and Application to Environmental Samples

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Abstract: The accurate assessment of racemic pharmaceuticals requires enantioselective analytical methods. This study presents the development and validation of an enantioselective liquid chromatography with a fluorescence detection method for the concomitant quantification of the enantiomers of tramadol and their metabolites, *N*-desmethyltramadol and *O*-desmethyltramadol, in wastewater samples. Optimized conditions were achieved using a Lux Cellulose-4 column 150 × 4.6 mm, 3 µm isocratic elution, and 0.1% diethylamine in hexane and ethanol (96:4, v/v) at 0.7 mL min^{-1}. The samples were extracted using 150 mg Oasis® mixed-mode cation exchange (MCX) cartridges. The method was validated using a synthetic effluent of a laboratory-scale aerobic granular sludge sequencing batch reactor. The method demonstrated to be selective, accurate, and linear ($r^2 > 0.99$) over the range of 56 ng L^{-1} to 392 ng L^{-1}. The detection and the quantification limits of each enantiomer were 8 ng L^{-1} and 28 ng L^{-1} for tramadol and *N*-desmethyltramadol, and 20 ng L^{-1} and 56 ng L^{-1} for *O*-desmethyltramadol. The feasibility of the method was demonstrated in a screening study in influent and effluent samples from a wastewater treatment plant. The results demonstrated the occurrence of tramadol enantiomers up to 325.1 ng L^{-1} and 357.9 ng L^{-1}, in the effluent and influent samples, respectively. Both metabolites were detected in influents and effluents.

Keywords: chiral pharmaceuticals; tramadol; *N*-desmethyltramadol; *O*-desmethyltramadol; wastewater; Lux Cellulose-4 column

1. Introduction

Pharmaceutical compounds are an important group of emergent environmental pollutants due to their high consumption and continuous discharge. These pollutants can enter the environment through various routes, including the direct discharge of sewage from health institutions, industries, agriculture, aquaculture, and households [1,2]. On the other hand, wastewater treatment plants (WWTPs) are unable to completely remove pharmaceutical compounds, and thus their effluents are also sources of these compounds in aquatic systems [2–4]. Therefore, residues (i.e., unchanged compounds and their metabolites) are continuously discharged into surface waters, where they can cause toxic effects

to aquatic organisms and humans [1]. Further, many pharmaceuticals are chiral and are found in environmental matrices as single enantiomers or as enantiomeric mixtures [5]. These aspects produce additional environmental problems due to the potential diverse behavior of the enantiomers concerning their toxicological and ecotoxicological properties [4].

Enantiomers may present similar or different pharmacodynamic properties. In the first case, the drug can be commercialized as a racemic mixture, while in the second situation the drug may be commercialized as a racemic mixture or in an enantiomerically pure form, depending on the biological activities of each enantiomer. Despite the increased interest in chiral pharmaceuticals in the environment, most occurrence studies neglect the enantiomers and consider these compounds as a unique molecular entity [3]. Also, the ecotoxicological effects of chiral pharmaceuticals have been studied only for some therapeutic classes and enantiotoxicity has been poorly investigated. In the work developed by Stanley et al. [6], enantioselective effects were evaluated using the antidepressant drugs Fluoxetine (FLX) concerning the survival of the fish *Pimephales promela* [6]. This study demonstrated that the racemic mixture presents higher toxicity than its enantiomers, and the enantiomer *R* is more toxic that the enantiomer *S* (LC_{50} (rac) = 198 μg L^{-1}; LC_{50} (*S*-FLX) = 216 μg L^{-1}; LC_{50} (*R*-FLX) = 212 μg L^{-1}) [6]. Enantiotoxicity was also evaluated for β-blockers as propranolol: (*S*)-propranolol affected the growth of *P. promela* and demonstrated to be more toxic than the *R* enantiomer [7].

The enantioselective fate of chiral pharmaceuticals in the environment is important, since enantiomers of the same chiral compound can differ in their environmental behavior (e.g., occurrence, distribution, biodegradation and toxicological effects. This requires the development of enantioselective methods for the quantitative assessment of each enantiomer and for the determination of enantiomeric fraction. Different analytical methods have been described for the separation of enantiomers using optical force [8], molecular imprinting [9]. However, gas chromatography mass spectrometry [10], and mainly liquid chromatography coupled with fluorescence, ultraviolet, or mass spectrometry are described [11–13]. Also, chiral membranes have been described for the separation of enantiomers [14].

Tramadol [2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexan-1-ol] (Figure 1) is a chiral pharmaceutical analgesic, structurally related to codeine and morphine, that is used for the treatment of moderate to severe pain [15,16]. It is a synthetic opioid that acts as an agonist by selective activity at the μ-opioid receptors and is commercialized as a racemic mixture. Tramadol is mainly metabolized in the liver by the cytochrome P450 CYP2D6 to *N*-desmethyltramadol (*N*-DT) and *O*-desmethyltramadol (*O*-DT) (Figure 1). *O*-DT, its main active metabolite, has higher pharmacological activity than the parent compound, and shows a higher affinity for the μ-opioid receptors [17,18]. According to Grond and Sablotzki [16], after oral administration, tramadol and its metabolites, *O*-DT and *N*-DT, are excreted via the kidney (≈90%) and via the biliary (1%) in the urine within 24 h at 12% for tramadol, and at 15% and 4% for *O*-DT and *N*-DT, respectively. Beyond the analgesic effects, tramadol has demonstrated other effects, such as antitussive, antidepressant, anti-inflammatory, and immunostimulatory effects [19]. The use of tramadol is associated with some risks, such as possible dependence, addiction, and adverse effects [10]. Some studies have demonstrated that tramadol can induce hepatotoxicity and nephrotoxicity upon acute and chronic exposure to rats, leading to liver and kidney damage [20,21].

Different analytical techniques have been reported for the simultaneous quantification of tramadol and its metabolites in biological samples, such as the brain tissue of mice and rats [22], saliva [23], urine [23,24], amniotic fluid [25], plasma [23,26–39], and environmental samples [11,40], but reports regarding enantiomeric quantification are scarce.

Quantification in WWTPs samples of enantiomers of tramadol and the racemic *O*-DT has been reported [11,40]. Enantiomers of tramadol have been quantified in a concentration of 506 ng L^{-1} to 1320.7 ng L^{-1} in effluent and influent WWTPs samples, respectively [40], and in a WWTP sample in a range of 595 to 798 ng L^{-1} [11]. The metabolite *O*-DT has also been quantified in a WWTP sample, in a concentration range of 801 to 950 ng L^{-1}, but to the best of our knowledge, the quantification of the enantiomers of *N*-DT in the environment has never been reported.

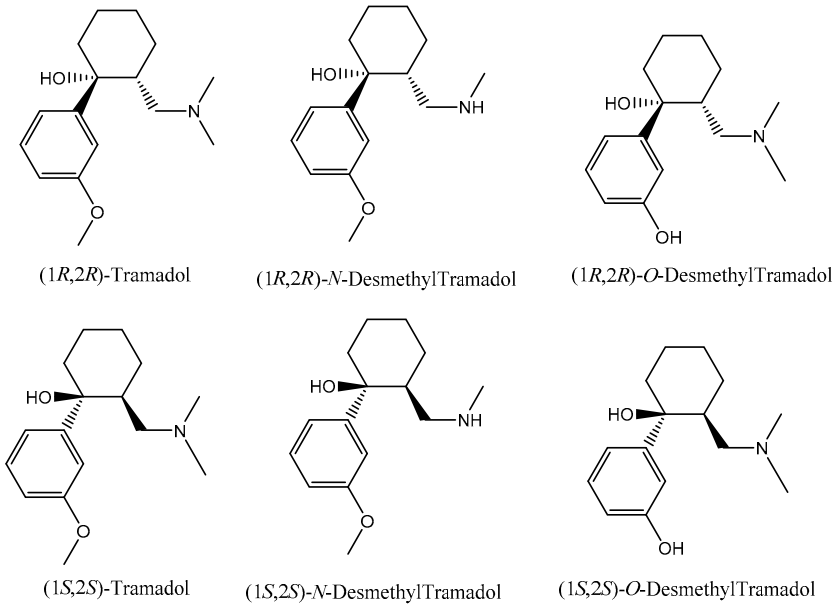

Figure 1. Chemical structure of tramadol enantiomers and its metabolites.

The quantification of tramadol and its metabolites is challenging due to the low levels usually found in environmental matrices and the presence of interferences. Therefore, the aim of this study was to develop a method to enantioresolve the tramadol and its metabolites for further quantification in environmental samples. The method was validated for the simultaneous quantification of the enantiomers of tramadol and its metabolites in the influent and effluent of WWTPs. This is the first report that allows for the simultaneous chemo and enantioseparation of enantiomers of tramadol and its main metabolites, their quantification in environmental samples, and enantiomeric quantification.

2. Materials and Methods

2.1. Standards and Reagents

The tramadol and *O*-DT standards were purchased from Sigma Aldrich (Steinhein, Germany), and the *N*-DT standard was purchased from Lipomed (Arlesheim, Switzerland). The standards present a purity degree above 99%. The tramadol and *O*-DT enantiomers were kindly supplied by Grünenthal (Aachen, Germany). The ethanol (EtOH), acetonitrile (ACN), methanol (MeOH), and propan-2-ol (IPA) HPLC grade were purchased from Fisher Chemical (Leicestershire, UK). Hexane (Hex) HPLC grade was acquired from VWR Chemicals. Diethylamine (DEA), ammonium formate, ammonium acetate, and ammonium trifluoroacetate (ATFA) with ≥99% purity were obtained from Sigma Aldrich. Acetic acid was purchased by Panreac (Barcelona, Spain). Formic acid with 98–100% purity was acquired from MERCK (Darmstadt, Germany). Ultrapure water was supplied by a Milli-Q water system (Ultra Clear UV model). The tramadol, *N*-DT, and *O*-DT stock standard solutions were prepared at 1 mg mL^{-1} of racemic mixture in ethanol. These stock standard solutions were stored at −20 °C in amber bottles. Working solutions were prepared by freshly diluting the stock solutions into an appropriate mobile phase. The standards mixtures and dilution in Hex/EtOH in a proportion of 8:2 (v/v) and in ethanol, in normal elution mode, and reverse elution mode, respectively, were used in a final racemic concentration of 1 μg mL^{-1} for the optimization of the separation of enantiomers. To prepare the synthetic wastewater influent, the following substances were used: Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA·$2H_2O$) (Panreac, Portugal), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (Riedel, Kuffstein, Austria), calcium chloride ($CaCl_2$) (Panreac, Portugal), manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), Iron (II) sulfate hetpahydrate (FeSO4·7H2O), ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$), copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), cobalto (II)

chloride ($CoCl_2$), potassium hydroxide pellets, sodium acetate (CH_3COONa), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), and ammonium chloride (NH_4Cl).

2.2. Instrumental Conditions

Chromatographic analyses were performed using a Shimadzu UFLC Prominence System equipped with two pumps LC-20AD, an autosampler SIL-20AC, a column oven CTO-20AC, a Degasser DGU-20A5, a System Controller CBM-20A, and an LC Solution, Version 1.24 SP1 (Shimadzu Corporation, Tokyo, Japan). The Fluorescence Detector (FD) coupled to the LC System was a Shimadzu RF-10AXL, with the excitation and emission wavelengths set at 275 and 300 nm, respectively. Solid Phase Extraction (SPE) procedures were realized using a Varian vacuum extraction device and Oasis® mixed-mode strong cation exchange (MCX) (150 mg 6cc) and Oasis® hydrophilic-lipophilic balance (HLB) (150 mg 6cc) from Waters Corporation (Milford, MA, USA). A vacuum concentrator, model Centrivap Centrifugal concentrator with a cold trap (−50 °C model) (Labconco, Kansas City, MO, USA) was used to evaporate to dryness the extracts from the SPE procedures.

2.3. Chromatographic Conditions

Several chiral stationary phases (CSP) and chromatographic conditions were attempted. After achieving the first baseline conditions on normal elution mode, the effects of several factors on chromatographic separation were studied, including EtOH percentage, buffer concentration, pH, column oven temperature, and flow rate. The chiral chromatographic columns used were: Astec Chirobiotic TAGTM and Astec Chirobiotic VTM, both with a particle size of 5 µm (150 × 2.1 mm), and supplied by SUPELCO Analytical (Sigma Aldrich, Steinhein, Germany); and Lux Cellulose-2 and Lux Cellulose-4, both with particle size 3 µm (150 × 4.6 mm), and supplied by Phenomenex (Torrance, CA, USA).

Diverse mobile phases in normal, reversed, and polar organic modes of elution were investigated, and different flow rates were attempted. The optimized condition was achieved with a Lux Cellulose-4 CSP with the mobile phase at an isocratic mode of a mixture of 0.1% diethylamine in hexane and ethanol (96:4, v/v) at 0.7 mL min^{-1}. The injection volume was 10 µL. The column oven temperature was set at 23 °C and the autosampler tray temperature was set at 15 °C. The elution order of the compounds was determined by the injection of a solution of racemic compounds separately, namely tramadol, *N*-DT, and *O*-DT. The elution order of each of the enantiomers was determined by the injection of a solution of (−)-enantiomer of the compounds.

2.4. Sample Preparation Procedure

The solid phase extraction was optimized with 250 mL of ultrapure water spiked with 200 µL of a mixture of the three standards in a concentration of 1 µg mL^{-1} of the racemic mixture dissolved in EtOH. Two cartridges, 150 mg Oasis® Mixed-mode strong cation exchange (MCX) and Hydrophilic-Lipophilic Balance (HLB) were tested, and different SPE procedures were realized in order to select and define the most appropriate conditions for the determination of the compounds (higher recoveries and less matrix interferences). The procedures are described in Table 1. Each procedure was realized in triplicate and the pH of the sample adjusted according to the cartridge used. In each procedure the cartridges were sequentially conditioned with solvents described in Table 1. After the loading of the sample, the cartridges were washed with mixtures of the solvents described in Table 1. The cartridges were dried, between the washing and elution steps, under vacuum for 30 min and elution was done in two steps. The resulting extracts of the SPE procedures were evaporated to dryness and reconstituted in Hex/EtOH in the proportions of 8:2 (v/v), and then 20 µL was injected into the HPLC-FD for quantification.

After the optimization of the SPE procedure in ultra-pure water, the best condition was used to validate the method using a synthetic wastewater.

Table 1. Experimental conditions of SPE.

Procedure	Cartridge	Conditioning	Washing	Elution (1st Step)	Elution (2nd Step)
1	MCX	8 mL MeOH 8 mL H_2O	8 mL H_2O 8 mL MeOH	8 mL of 5% NH_4OH solved in ACN/MeOH 60:40	-
2	MCX	8 mL MeOH 8 mL H_2O	8 mL 2% Formic Acid	8 mL MeOH	8 mL of 5% NH_4OH solved in MeOH
3	MCX	8 mL MeOH 8 mL H_2O	8 mL 2% Formic Acid	12 mL of 10% NH_4OH solved in MeOH	-
4	MCX	8 mL EtOH 8 mL H_2O	8 mL 2% Formic Acid	8 mL EtOH	8 mL of 5% NH_4OH solved in EtOH
5	MCX	8 mL EtOH 8 mL Formic acid 2%	8 mL 2% Formic Acid	8 mL of 0.6% Formic Acid solved in EtOH	8 mL of 5% NH_4OH solved in EtOH
6	MCX	8 mL EtOH 8 mL H_2O (pH = 2 adjusted with HCl)	8 mL 2% Formic Acid	8 mL EtOH	8 mL of 5% NH_4OH solved in EtOH
7	HLB	10 mL MetOH 10 mL H_2O	10 mL H_2O	10 mL MeOH	-
8	MCX	8 mL MeOH 8 mL H_2O	8 mL 2% Formic Acid	12 mL of 10% NH_4OH solved in MeOH	-
9	MCX	8 mL EtOH 8 mL H_2O	8 mL 2% Formic Acid	8 mL EtOH	8 mL of 5% NH_4OH solved in EtOH
10	MCX	8 mL EtOH 8 mL 2% Formic Acid	8 mL 2% Formic Acid	8 mL of 0.6% Formic Acid solved in EtOH	12 mL of 5% NH_4OH solved in EtOH
11	MCX	8 mL EtOH 8 mL 2% Formic Acid	8 mL 2% Formic Acid	12 mL of 5% NH_4OH solved in EtOH	-
12	MCX	8 mL EtOH 8 mL 2% Formic Acid	8 mL 2% Formic Acid	8 mL of 0.6% Formic Acid solved in EtOH	12 mL of 5% NH_4OH solved in EtOH

MCX: Mixed-mode strong cation exchange; HLB: Hydrophilic-Lipophilic Balance Cartridge.

2.5. Method Validation

The analytical method was validated following the analytical performance parameters established by the International Conference on Harmonisation (ICH) validation guidelines in terms of selectivity, linearity, accuracy, intra-day precision, inter-day precision, and limits of detection and quantification [41]. For the validation of the method, a synthetic wastewater influent was used. The synthetic wastewater was prepared as follows: initially take Vischniac Trace Element Solution consisting in a mixture of the following substances: $EDTA{\cdot}2H_2O$ (6.377 g), $ZnSO_4{\cdot}7H_2O$ (2.2 g), $CaCl_2$ (0.554 g), $MnCl_2{\cdot}4H_2O$ (0.506 g), $FeSO_4{\cdot}7H_2O$ (0.499 g), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.11 g), $CuSO_4{\cdot}5H_2O$ (0.157 g), and $CoCl_2$ (0.0879 g) in 100 mL of water and adjust the pH to 6 with KOH. All substances except the KOH were weighed into a beaker and dissolved in 80 mL of ultrapure water. To dissolve all compounds, the pH was adjusted to 6.0 with KOH pellets. The solution was poured into a 100 mL flask and made up to volume with water. The solution was stored in a refrigerator at a temperature of 4 °C for future use. The synthetic wastewater, 250 mL, was prepared with Vischniac Trace Element Solution (0.234 mL) and another mixture, which involves the substances CH_3COONa (0.121 g), $MgSO_4{\cdot}7H_2O$ (0.021 g), KCl (0.008 g), Na_2HPO_4 (0.014 g), KH_2PO_4 (0.007 g), and NH_4Cl (0.044 g).

Selectivity was verified by comparing the chromatograms of a synthetic wastewater matrix, a matrix spiked with the standards, and standard in solvent.

Linearity was performed using 200 µL of a racemic mixture at seven different racemic concentrations (56 ng mL^{-1}, 112 ng mL^{-1}, 168 ng mL^{-1}, 224 ng mL^{-1}, 280 ng mL^{-1}, 336 ng mL^{-1}, and 392 ng mL^{-1}) spiked in 250 mL of acidified synthetic wastewater realized in triplicate, and each one was injected and analyzed in duplicate. For each concentration, the SPE procedure was processed in triplicate. The calibration curves were obtained by linear regression corresponding to the correlation between the peak area and the nominal concentration.

The detection limit (DL) and quantification limit (QL) were determined based on signal/noise. The determination of the signal-to-noise ratio was performed by comparing measured signals from

samples with low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected or quantified. A signal-to-noise ratio of 3:1 is generally acceptable to estimate the DL, and a signal-to-noise ratio of 10:1 is typically considered to estimate the QL.

Accuracy, precision, and recovery assays were estimated by three quality controls (QCs) standard solutions using the racemic concentrations (150 ng mL^{-1}, 300 ng mL^{-1}, and 410 ng mL^{-1}) in spiked synthetic waste water, in triplicate. Accuracy was determined as the percentage of agreement between the method's results and the nominal amount of added compound. Precision was expressed by the relative standard deviation (% RSD) of the replicate measurements.

The recovery rate results from the ratio of standards in spiked matrix by the optimized SPE and in solvent, and the results were expressed in recovery percentage.

2.6. Application of Developed HPLC-FD Method in WWTP Samples

Water samples of the influent, previously treated through solid filtration and some chemical treatments, and of the final effluent of the secondary clarifier of the WWTP of Parada, localized in Maia, North of Portugal, were collected on the 26th and 27th of June 2016 in pre-rinsed amber glass bottles (2 L) and transported at 4 °C to the laboratory.

After the collection of the samples, these were filtered under vacuum and acidified with H_2SO_4 to pH = 2 and preconcentrated by SPE with optimized conditions, according to the procedures established in Section 2.4.

In order to confirm the presence of tramadol, *N*-DT, and *O*-DT, a crossmatch test was realized, adding 20 µL of the standards mixture at a racemic concentration of 1 µg mL^{-1} to the samples.

The enantiomeric fraction (EF) and the degree of removal efficiency (DRE) were calculated with the following Formulas (1) and (2), respectively.

$$EF_1 = \frac{Concentration\ E_1}{Concentration\ E_1 + Concentration\ E_2} \text{ or } EF_2 = \frac{Concentration\ E_2}{Concentration\ E_1 + Concentration\ E_2}. \quad (1)$$

E_1 and E_2: the first and second enantiomer eluted, respectively.

$$DRE = \frac{C_{analyte\ in\ effluent}}{C_{analyte\ in\ influent}} \times 100. \quad (2)$$

3. Results and Discussion

3.1. Enantiomeric Separation

Different types of chiral columns are commercially available [42–46]. The most important types of chiral selectors pointed to: Pirkle type, polysaccharide derivatives, cyclodextrin, protein, and macrocyclic glycopeptides antibiotics-based CSP [42,47,48]. Due the versatility and suitability for all elution modes, polysaccharides or macrocyclic antibiotics CSP are usually the first choice to start the trial–error challenge to achieve the desired separation of enantiomers [47,49–52]. In a tentative attempt to obtain the best result of chemo and enantioseparation of the enantiomers of tramadol and its metabolites (*O*-DT and *N*-DT), CSP based on macrocyclic glycopeptides antibiotics and polysaccharide derivates were evaluated in normal, polar, and reversed elution modes.

In the first attempt, macrocyclic glycopeptides antibiotics CSP, namely the Chirobiotic TAG (Telcoplanin Aglycone CSP) and Chirobiotic V (Vancomycin CSP), were tested. These CSP can be used in normal, reversed, polar organic, and polar ionic elution modes [3]. The separation of the enantiomers of tramadol was also reported in Chirobiotic V CSP [40]. Several mobile phases were attempted to enantioseparate tramadol, *N*-DT, and *O*-DT. Regarding Chirobiotic TAG CSP, only partial separation of the enantiomers of tramadol was achieved with MeOH (0.1% ATFA)/H_2O (75:25 (v/v)) as the mobile phase and a flow rate of 1 mL min^{-1}, with elution time around 7 min (data not shown).

Chirobiotic V CSP presented only a partial enantioseparation of *N*-DT with EtOH with 10 mM aqueous ammonium acetate buffer (pH = 5.3) (92.5:7.5; v/v) as the mobile phase, and a flow rate of 0.1 mL min^{-1} (data not shown). The poor results led to the abandonment of the trial and error in this type of CSP.

Polysaccharide derivates CSP have a broad application in the separation of enantiomers of chiral pharmaceuticals, and can also be used within various elution modes. The columns Cellulose-2 (Cellulose *tris* (3-chloro-4-methylphenylcarbamate)) and Cellulose-4 (Cellulose *tris* (4-chloro-3-methylphenylcarbamate)) CSP were selected for this study. The difference between Cellulose-2 and Cellulose-4 is only the position of the substituents of the aromatic ring of the carbamate. Table 2 shows the best results relative to the enantiomeric separation achieved with these columns, and includes values of retention factor (K), separation factor (α), and resolution (Rs).

Table 2. Results of the enantiomeric separation of tramadol and of its metabolites on Cellulose chiral stationary phases (CSP).

Column	Elution Mode	Mobile Phase: Proportion (v/v)	Tramadol				*N*-DT				*O*-DT			
			K_1	K_2	α	Rs	K_1	K_2	α	Rs	K_1	K_2	α	Rs
Lux Cellulose-2	Normal	Hex/IPA/DEA: 90:10:0.1 **	0.73	0.89	1.22	1.00	1.13	1.62	1.44	3.02	1.55	3.04	1.96	5.53
		Hex/IPA/DEA: 90:10:0.05 **	4.31	4.66	1.08	0.92	4.53	5.57	1.23	2.75	9.86	14.7	1.49	4.10
		Hex/EtOH/DEA: 96:4: 0.1 **	0.98	1.07	1.11	1.12	1.91	-	1.00	-	3.56	4.16	1.17	2.42
	Reversed	ACN (5 mM ammonium formate/0.1% DEA)/H_2O: 35:65 *	5.75	6.13	1.07	1.49	4.29	-	1.00	-	1.29	1.42	1.11	0.949
		ACN (5 mM ammonium formate/0.1% DEA)/H_2O: 30:70 *	9.98	10.6	1.07	1.89	7.19	-	1.00	-	1.91	2.11	1.11	1.31
Lux Cellulose-4	Reversed	ACN (5 mM ammonium formate/0.1% DEA)/H_2O: 35:65 **	6.32	7.21	1.14	2.79	10.7	11.4	1.07	1.26	1.25	1.66	1.33	2.31
		MeOH (5 mM ammonium formate/0.05% DEA)/H_2O: 55:45 **	10.7	11.9	1.12	2.18	4.62	4.83	1.05	0.236	2.43	2.85	1.17	1.43
		ACN:EtOH (10 mM ammonium formate/0.1% DEA) /H_2O: 17.5:17.5:65 **	8.91	10.1	1.13	2.69	4.65	4.92	1.06	0.36	2.07	2.65	1.28	2.62
	Normal	Hex/EtOH/DEA—96:4:0.1 ***	0.74	0.920	1.24	2.16	1.43	1.70	1.19	1.85	2.48	3.33	1.34	4.16

K_1: Retention Factor of enantiomer 1; K_2: Retention Factor of enantiomer 2; α: Separation Factor; Rs: Resolution; * flow rate of 0.3 mL/min; ** flow rate of 0.5 mL/min; *** flow rate of 0.7 mL/min.

The separation of the enantiomers of *N*-DT and *O*-DT in Celullose-2 using Hex/IPA/DEA (90:10:0.1 (v/v/v)) as the mobile phase presented good results of α (1.44 and 1.964) and Rs (3.02 and 5.53), respectively. However, the separation of the enantiomers of tramadol did not present baseline separation. A reversed elution mode was attempted in different mobile phases and the best separation of enantiomers was obtained using ACN with 5 mM ammonium formate and 0.1% of DEA and H_2O (30:70 (v/v)). The chromatograms obtained with these mobile phases are presented in Figure 2. In this elution mode, the separation of the enantiomers *N*-DT was not achieved and the chromatographic run was longer than 45 min.

Regarding the Cellulose-4 CSP, the majority of mobile phases attempted presented the separation of the enantiomers of the target compounds. The best result using the reversed elution mode was achieved with ACN (5 mM ammonium formate/0.1% DEA)/H_2O (35:65 (v/v)) as the mobile phase (Figure 3); all target compounds presented enantioseparation, but none were chemoselective with tramadol and *N*-DT, which can be good results and conditions for analyses by mass spectrometry analysers but are not satisfactory for UV and FD detectors.

A mobile phase with MeOH and EtOH was used instead of ACN. Figure 4 presents the results with MeOH (5 mM Ammonium formate/0.05% DEA)/H_2O (55:45 (v/v)) with a flow rate of 0.5 mL min^{-1}. In this mobile phase, it is possible to observe a good separation of the enantiomers for *O*-DT, partial enantioseparation of *N*-DT, and baseline enantioseparation of tramadol, but long retention times (RT) (RT >50 min).

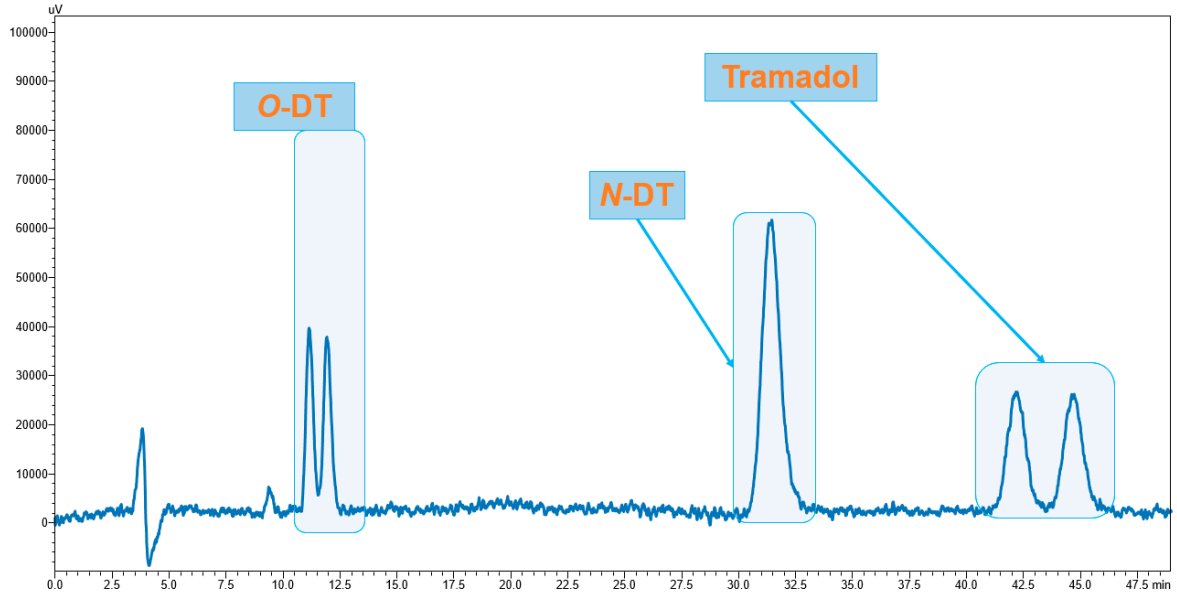

Figure 2. Chromatogram of a mixture of the three analytes using ACN (5mM ammonium formate/ 0.1% DEA)/H_2O 30:70 (v/v) as the mobile phase at a flow rate of 0.5 mL min^{-1} a using Lux Cellulose-2 column.

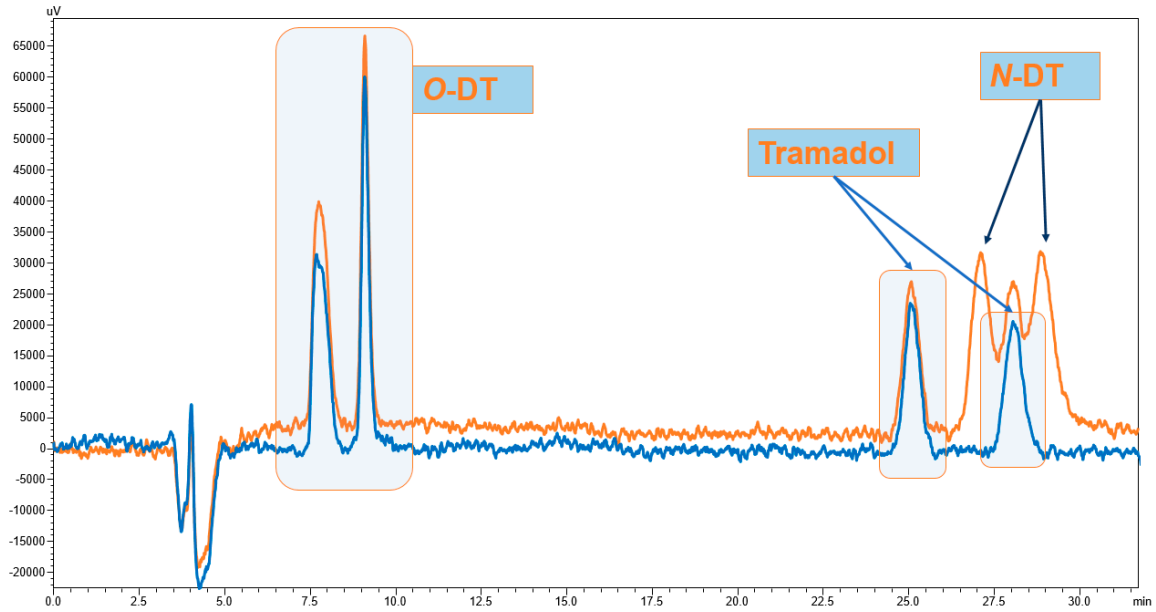

Figure 3. Chromatogram of a mixture of the three analytes (orange line) and of a mixture of O-DT and tramadol (blue line) using ACN (5 mM ammonium formate/0.1% DEA)/ H_2O (35:65 (v/v)) as the mobile phase at a flow rate of 0.5 mL min^{-1} using a Lux Cellulose-4 column.

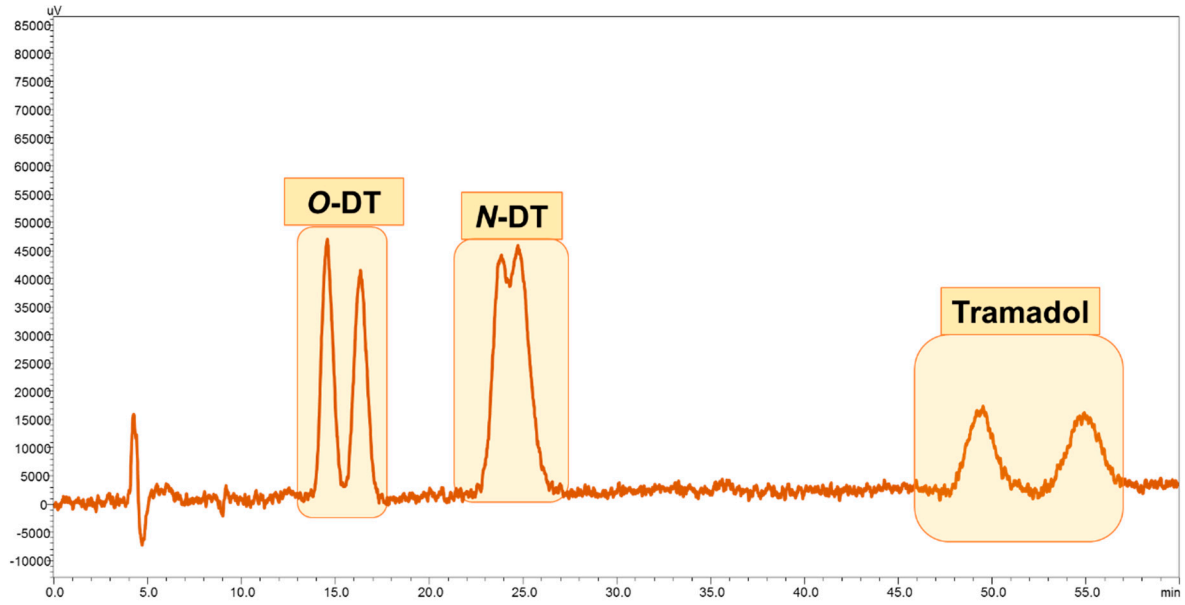

Figure 4. Chromatogram of a mixture of the three analytes using MeOH (5 mM Ammonium formate/0.05% DEA)/H_2O (55:45 (v/v)) as the mobile phase at a flow rate of 0.5 mL min^{-1} using a Lux Cellulose-4 column.

In order to improve the separation of enantiomers and decrease the retention time, two polar organic elution modes were attempted with ACN and EtOH, but separation of enantiomers was not improved (data not shown). The best mobile phase was constituted with ACN/EtOH (10 mM ammonium formate/0.1% DEA)/H_2O in a proportion of 17.5/17.5:65 (v/v/v) with a flow rate of 0.5 mL min^{-1} (Figure 5). Tramadol and *O*-DT presented good resolution (Rs) (Rs >1.5), but *N*-DT presented only a partial separation of enantiomers. Further tests were made in normal elution mode.

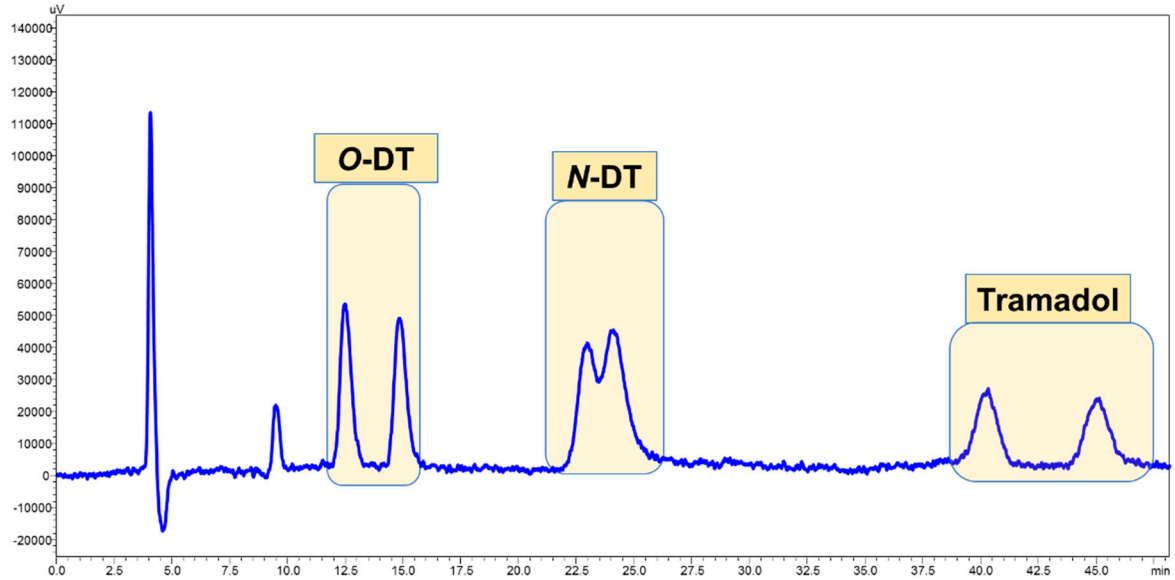

Figure 5. Chromatogram of a mixture of the three analytes using ACN/EtOH (10 mM ammonium Format/0.1% DEA)/ H_2O (17.5:17.5:65 (v/v/v)) as the mobile phase with a flow rate of 0.5 mL min^{-1} using a Lux Cellulose-4 column.

Many different mobile phases were evaluated on a Cellulose-4 CSP in normal elution mode. EtOH and IPA were used as the organic modifier and DEA as the ionic suppressor. Enantio and chemoselective properties were achieved using a mixture of 0.1% diethylamine in Hex and EtOH as the mobile phase, in a proportion of 96:4, and in a flow rate of 0.7 mL min^{-1}. Figure 6 shows the chromatogram obtained. These conditions were used to validate the chromatographic method for the further quantification of the enantiomers of tramadol and its metabolites *N*-DT and *O*-DT in the influent and effluent of a WWTP. It was possible to determine the order of elution of the enantiomers of tramadol and *O*-DT: the first enantiomer eluted of both compounds is the (−)-*S*,*S*-enantiomer.

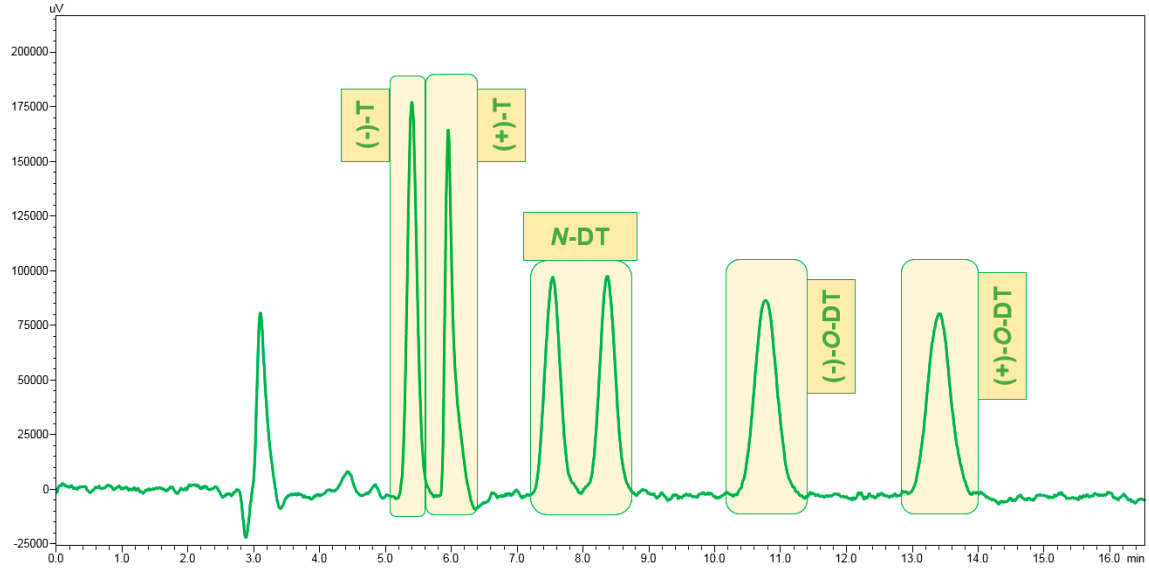

Figure 6. Chromatogram of a mixture of the three compounds using a mixture of 0.1% diethylamine in Hex/EtOH, (96:4 (v/v)) as the mobile phase at a flow of 0.7 mL min^{-1} using a Lux Cellulose-4 column.

Ceccato et al. [28] reported the enantiomeric determination of tramadol and its main metabolite *O*-DT in human plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Chiralpak AD column; nevertheless, the separation of enantiomers was not achieved in the same chromatographic run [28]. Other studies have reported the enantiomeric separation of tramadol and *O*-DT using similar columns in human plasma and environmental matrices, such as the effluent or influent of WWTPs [11,40,53,54]. However, this work reports for the first time the chemo and enantioseparation of enantiomers of tramadol and its two main metabolites in less than 15 min.

3.2. Optimization of the Sample Preparation Procedure

MCX and HLB cartridges with different conditions were tested using ultrapure water in order to establish the best procedure for sample preparation, i.e., the best recoveries for all compounds. The recoveries obtained for each enantiomer are shown in Figure 7.

The procedure 12 demonstrated the best overall recoveries for all compounds with the Oasis® MCX 150 cartridge, and was selected for the method's validation with a synthetic influent and for the quantification of the compounds in the WWTP samples.

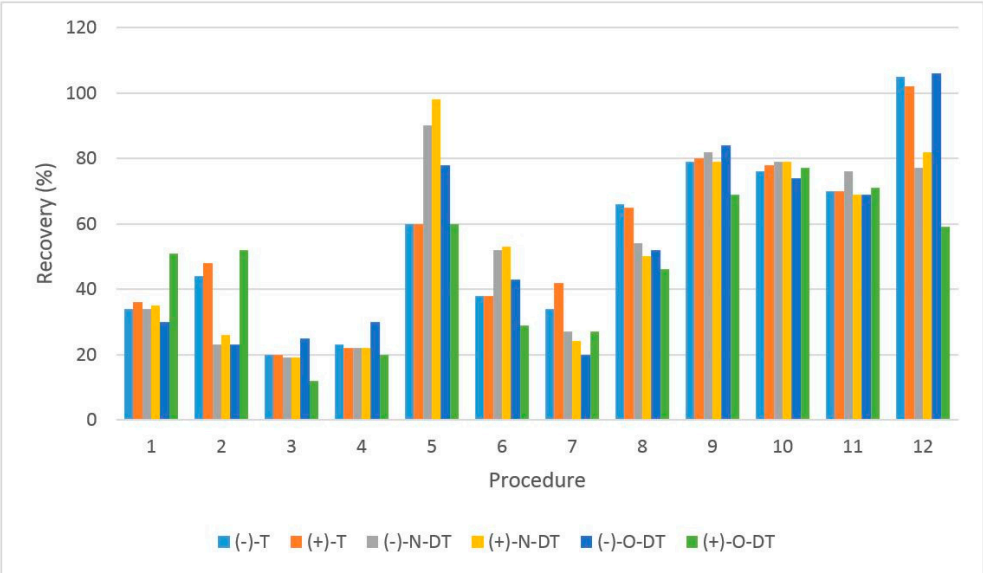

Figure 7. Recoveries obtained for each SPE procedure.

3.3. Method Validation

For the method's validation, the following parameters were considered: selectivity; linearity; accuracy; precision (intra-day and inter-day), DL, and QL.

The selectivity of the developed method included the analysis of a synthetic WWTP sample previously treated by a SPE procedure (Figure 8: red line); the matrix spiked with the mixture of tramadol, *N*-DT, and *O*-DT, each one at a final concentration of 410 ng mL^{-1} of the racemic mixture (Figure 8: green line); and a mixture of the three compounds in solvent, Hex/EtOH 8:2 (v/v) (Figure 8: orange line). The comparison between the three chromatograms demonstrated the selectivity of the method.

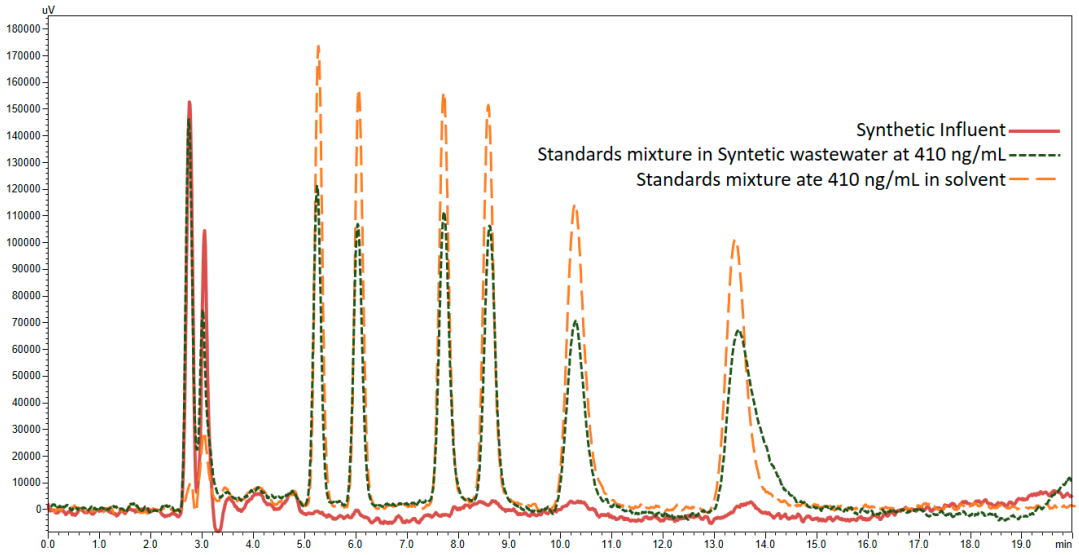

Figure 8. Chromatogram of a synthetic influent (red line), standards mixture in synthetic wastewater at 410 ng mL^{-1} (green line), and a standards mixture at 410 ng mL^{-1} in solvent (orange line); mobile phase: 0.1% diethylamine in hexane and ethanol (96:4, v/v); the flow rate is 0.7 mL min^{-1}.

Concerning the linearity, the calibration curve of the enantiomers of tramadol and *N*-DT in a range of nominal concentrations between 28 and 168 ng mL^{-1} and for each enantiomer of *O*-DT between 56 and 196 ng mL^{-1} demonstrated correlation coefficients higher than 0.99 (Table 3). The instrumental DL and QL and method DL and QL values of each enantiomer of the target compounds in synthetic influent were up to 25 ng L^{-1} and 56 ng L^{-1}, respectively (Table 3).

Table 3. Linearity, range, instrumental detection and quantification limits, and method detection and quantification limits.

Analyte	E	Nominal Conc. Range (ng L^{-1})	Calibration Curve Equation	r^2	IDL (ng L^{-1})	IQL (ng L^{-1})	MDL (ng L^{-1})	MQL (ng L^{-1})
Tramadol	(−)-T	28–168	y = 6944.4x − 93525	0.9902	10	35	8	28
	(+)-T		y = 7421.2x − 113599	0.9901	10	35	8	28
N-DT	(−)-*N*-DT	28–168	y = 6128.8x + 4247	0.9958	10	35	8	28
	(+)-*N*-DT		y = 5973.8x + 33396	0.9939	10	35	8	28
O-DT	(−)-*O*-DT	56–196	$y = 30420x - 2 \times 10^6$	0.9954	25	70	20	56
	(+)-*O*-DT		$y = 44269x - 2 \times 10^6$	0.9948	25	70	20	56

(E: enantiomer; IDL: instrumental detection limit; IQL: instrumental quantification limit; MDL: method detection limit; MQL: method quantification limit).

The accuracy and recovery rates ranged from 70.3% to 108.7% and from 78.3% to 99.4%, respectively, as shown in Table 4. These values are within the range for quantitative determinations in complex matrix samples [41,55]. The precision of the method was evaluated by determining intra- and inter-day assays. The results demonstrated that this method is precise, with RSD values lower than 13.8% for intra-day precision and lower than 16.7% for inter-day precision (Table 4). This in agreement with international criteria, which recommend RSD values lower than 20% for complex matrices [41,55].

Table 4. Recovery, accuracy, and intra- and inter-day precision of each enantiomer.

Analyte	Nominal Concentration (ng mL^{-1})	E	1st Day		2nd Day		3rd Day		Inter-Day RSD (%)	Recovery (%)
			Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)		
Tramadol	75	(−)-T	89.6	7.7	88.5	4.9	94.3	3.1	6.5	87.1
		(+)-T	90.6	8.3	91.4	3.7	93.3	5.9	6.4	88.9
	150	(−)-T	80.1	4.6	80.6	1.6	82.1	6.5	4.9	78.4
		(+)-T	81.0	6.7	80.4	0.3	82.9	5.8	5.4	78.3
	205	(−)-T	105.6	1.0	82.2	8.0	97.9	8.5	12.5	97.8
		(+)-T	70.3	1.1	84.0	3.8	90.0	8.4	11.7	83.2
N-DT	75	(−)-*N*-DT	99.1	8.2	82.0	8.8	105.7	3.5	11.7	99.2
		(+)-*N*-DT	114.1	8.8	91.2	3.9	100.0	8.9	11.7	99.4
	150	(−)-*N*-DT	98.3	1.7	91.9	6.2	97.5	6.1	5.8	97.6
		(+)-*N*-DT	87.8	5.6	81.3	1.5	90.6	6.7	6.8	88.2
	205	(−)-*N*-DT	77.0	6.3	85.6	4.0	105.4	7.9	16.7	85.9
		(+)-*N*-DT	79.4	4.9	89.6	3.7	108.7	7.4	16.3	89.8
O-DT	75	(−)-*O*-DT	94.9	5.0	92.5	8.2	96.9	4.5	14.8	72.2
		(+)-*O*-DT	96.0	6.8	97.1	8.9	97.5	9.2	9.5	83.6
	150	(−)-*O*-DT	97.0	9.9	93.2	5.0	96.3	7.8	10.6	87.4
		(+)-*O*-DT	99.9	4.6	98.5	7.2	99.4	3.6	5.9	98.1
	205	(−)-*O*-DT	81.7	7.0	90.0	4.5	97.9	3.8	14.9	82.0
		(+)-*O*-DT	90.2	5.9	95.6	6.3	106.3	8.9	14.5	92.2

E: Enantiomer; RSD: relative standard deviation.

3.4. Application of Developed LC-FD Method in WWTP Samples

The optimized and validated method was applied for the quantification of the enantiomers of the selected compounds in the effluent and influent of WWTPs. Table 5 presents the results and the EFof the first enantiomer eluted. The EF range is from 0 to 1.0, if EF = 0.5, this means that analyte is present as a racemic mixture.

Table 5. Concentration and enantiomeric fraction of analytes present in samples collected and degree of removal efficiency.

		Sample nº	1		2	
			Effluent	**Influent**	**Effluent**	**Influent**
Concentration (ng L^{-1})	Tramadol	(−)-T	235.8	357.9	325.1	350.0
		(+)-T	118.7	233.6	314.9	233.8
	N-DT	(−)-*N*-DT	<QL	<QL	<QL	<QL
		(+)-*N*-DT	43.7	63.9	62.1	72.7
	O-DT	(−)-*O*-DT	60.8	69.7	71.6	69.5
		(+)-*O*-DT	57.7	86.7	95.4	106.7
Enantiomeric Fraction	Tramadol	EF1	0.67	0.61	0.51	0.60
	N-DT	EF1	≈0	≈0	≈0	≈0
	O-DT	EF1	0.51	0.45	0.43	0.39
DRE (%)	Tramadol	(−)-T	65.9		92.9	
		(+)-T	50.8		134.7 *	
	N-DT	(−)-*N*-DT	NC		NC	
		(+)-*N*-DT	68.4		85.4	
	O-DT	(−)-*O*-DT	87.2		103.0 *	
		(+)-*O*-DT	66.6		89.4	

<QL: under quantification limit; NC: not calculated; E1 and E2 are the first and second enantiomers eluted, respectively; EF1: enantiomeric fraction of the first enantiomer eluted; DRE, degree of removal efficiency. * enrichment in the effluent.

All enantiomers of the target compounds could be quantified in the effluent and influent samples, except for the first enantiomer of *N*-DT, which was under the QL.

According to Stamer et al. [56] and Payne et al. [57], the biotransformation of the racemic tramadol administered by oral drops in a concentration of 1.5 mg/kg [57] and intravenously in a concentration of 3 mg/kg [56] leads to higher quantities of the enantiomers (−)-tramadol and (−)-*O*-DT than the (+)-enantiomers in the human organism. In this study, regarding the influent and effluent samples, the first eluted enantiomer, (−)-tramadol, was also quantified at higher concentrations than the (+)-enantiomer. However, in the case of the metabolite *O*-DT, the results demonstrated an EF close to 0.5 (nearly racemic) or a higher concentration of the enantiomers (+)-*O*-DT (EF = 0.39), which indicates that the transformation of tramadol in the environment is different than the metabolism in the human organism and is enantioselective. Data about the EF of the *N*-DT metabolite in human excretions are not available. The metabolite *N*-DT presented an EF1 near to zero, which indicated that one enantiomer presented a higher amount than its antipode. The EF of the metabolite *N*-DT in the environment was never reported before.

Paar et al. [58] mentioned that the percentage of excretion by humans is 12%, 15%, and 4% for tramadol, *O*-DT, and *N*-DT, respectively. In this work, tramadol was present at higher concentrations than its metabolite *O*-DT in all the wastewater samples analyzed.

The DRE results predicted the removal of pollutants to some extent, and have demonstrated the low efficiency of WWTP to eliminate tramadol and its metabolites. Regarding the samples, (+)-tramadol and (−)-*O*-DT were enriched in the effluent, which indicated again the enantioselectivity in the transformation process of WWTP.

4. Conclusions

The enantioselective LC-FD method using a Lux Cellulose-4 column demonstrated to be accurate and precise to quantify the enantiomers of tramadol and its primary metabolites, *N*-DT and *O*-DT, in wastewater samples. The optimized conditions were achieved using 0.1% of DEA with Hex/EtOH 96:4 (v/v) as the mobile phase in an isocratic elution mode at a flow of 0.7 mL min^{-1}. The separation

of all enantiomers (six compounds) has been obtained in less than 15 min, with good resolution and enantioselectivity. The QL achieved was 28 ng L^{-1} for each enantiomer of tramadol and *N*-DT and 56 ng L^{-1} for each enantiomer of *O*-DT. The validated method was successfully applied in the quantification of the enantiomers of real wastewater samples.

Various samples from the effluent and influent of WWTPs were analyzed, and the compounds were detected in a concentration range below the QL, at 325.1 ng L^{-1} and 357.9 ng L^{-1}, respectively. The enantiomers of tramadol and *O*-DT reveal different values of EF, and *N*-DT presented an EF approximately to zero. The DRE rates of this study reveal that the efficiency of a WWTP to remove these compounds is low. This study contributes to the urgent need of the development of enantioselective methods for racemic pharmaceuticals' quantification in environmental matrices, and to further environmental risk evaluation with the consideration of both the enantiomers of tramadol and its major metabolites.

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Author Contributions: Maria Elizabeth Tiritan and Carlos Afonso conceived and designed the experiments; Cátia Silva, Cláudia Ribeiro, Alexandra Maia, and Virgínia Gonçalves performed the experiments; Maria Elizabeth Tiritan, Carlos Afonso, and Cláudia Ribeiro analyzed the data; Maria Elizabeth Tiritan and Carlos Afonso contributed reagents/materials/analysis tools; and Maria Elizabeth Tiritan, Cátia Silva, Cláudia Ribeiro, and Carlos Afonso wrote the paper. Authorship must be limited to those who have contributed substantially to the work reported.

Conflicts of Interest: The authors declare no conflict of interest.

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