HPLC enantioseparation of Tramadol and its metabolites: method validation and application to environmental samples

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2016

Separação enantiomérica por HPLC do Tramadol e seus metabolitos: validação do método e aplicação em amostras ambientais

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Dissertação do 2º Ciclo de Estudos

Conducente ao Grau de Mestre em Controlo de Qualidade – especialização em Ambiente

Trabalho realizado sob a orientação do Professor Doutor Carlos Manuel Magalhães Afonso e Professora Doutora Maria Elizabeth Tiritan

2016

ACKNOWLEDGEMENTS

I would like to thank to Prof. Dr. Carlos Afonso, my supervisor, and Prof.ª Dr.ª Maria Elizabeth Tiritan, my co-supervision, for all the support, patience and dedication during the realization of this seminar and the experimental work. Your unconditional support was very important to me and it was certainly a privilege to work with you and acquire all knowledge you have given to me.

To Prof.ª Dr.ª Cláudia Ribeiro, to Master Alexandra Maia, PhD Student, and to Master Virgínia Gonçalves, Superior technical of IINFACTS, I would like to thank for all the support that given me in experimental work, over this year, and for all the attention and availability in the laboratory's work.

To my parents, my family and my friends which have a lot of patience for me and make me laugh in the certain time. Thanks for the good disposition, sharing moments, by listen to my lamentations and specially the friendship. Without you, everything would be much more difficult, I'm sure!

To my paternal grandmother, who watches differently to this my achievement from the few months, my thanks for everything you gave and helped along this period of my life.

To Parada WWTP, localized in Maia, thank you for cede all effluent and influential samples used, were undoubtedly crucial to the success of the work.

This work was developed at Laboratory of Environmental Research area/Environmental and Applied Chemistry research line of the IINFACTS-CESPU. The author acknowledges the financial support from PARMADRUGS-CESPU-2014. This research was partially supported through national funds provided by FCT – Foundation for Science and Technology, European Regional Development Fund (ERDF) and COMPETE under the projects PEst-C/MAR/LA0015/2013, PTDC/MAR-BIO/4694/2014, and INNOVMAR - Innovation and Sustainability in the Management and Exploitation of Marine Resources, reference NORTE-01-0145-FEDER-000035, Research Line NOVELMAR.

ABSTRACT

The presence of pollutants in the environment is of great interest since it affects the sustainability of the planet and is a major concern of the scientific community in recent decades. Many of these pollutants are chiral pharmaceuticals (CP) which are present as single enantiomeric forms or as a mixture of enantiomers.

CP enantiomers having different configurations, can interact differently with the chiral molecules of the biological systems, and may exhibit different biological activities or be metabolized differently. For the same reasons enantiomers may also present different toxicity and eco-toxicity.

Most of the CP in the environment occur from improper disposal of unused drugs, human excreta and inefficient removal by wastewater treatment plants (WWTP). In addition it is also important to consider the presence and effects of its metabolites in the environment.

Tramadol is a chiral drug, used as racemate in the treatment of pain. It is rapidly absorbed and metabolized in the liver, resulting in the formation of its primary metabolites, *N*-desmethyltramadol (*N*-DT) and *O*-desmethyltramadol (*O*-DT).

In this work a High Performance Liquid Chromatography with Fluorescence Detector (HPLC-FD) method that allows the separation of the enantiomers of Tramadol and its metabolites in environmental matrices is presented.

The optimized conditions to enantioseparation of Tramadol, *N*-DT and *O*-DT were obtained using Lux Cellulose-4 column 150 x 4.6 mm, particle size 3 µm, in isocratic mode with a mixture of 0.1% diethylamine in hexane and ethanol (96:4, *V:V*) and a flow rate of 0,7 mL/min. The method was validated, showing accuracy, good selectivity, linearity (r^2>0.99) and precision in the racemic range of 56 ng/L to 384 ng/L. The detection limit (DL) and the quantification limit (QL) of each enantiomer were: 8 ng/L and 28 ng/L for Tramadol and *N*-DT and 20 ng/L and 56 ng/L for *O*-DT, respectively. The method was applied to the study of effluents and influents of a WWTP and compounds were detected in a concentration range between QL and 325.1 ng/L, in effluent samples and between QL and 357.9 ng/L, in influent samples.

Keywords: Chiral pharmaceuticals | Tramadol | *N*-desmethyltramadol | *O*-desmethyltramadol | Effluent of WWTP | Influent of WWTP

RESUMO

A presença de poluentes no meio ambiente tem um grande interesse desde que afeta a sustentabilidade do planeta e tem constituído uma grande preocupação na comunidade cientifica nas ultimas décadas. Muitos destes poluentes são fármacos quirais (FQ) que estão presentes numa única forma enantiomérica ou como uma mistura de enantiómeros.

Os enantiómeros de um FQ têm configurações diferentes, podem interagir de modo diferente com moléculas quirais dos sistemas biológicos, e podem exibir atividades biológicas diferentes ou serem metabolizados de maneiras distintas. Pelas mesmas razões os enantiómeros podem apresentar diferente toxicidade e ecotoxicidade.

A maior parte dos FQ no ambiente é proveniente do depósito impróprio de fármacos não usados, excreções humanas e remoção ineficiente pelas estações de tratamento de águas residuais (ETAR). Para além da presença dos FQ, é importante considerar também os seus metabolitos e os efeitos ambientais.

Tramadol é um fármaco quiral, comercializado como mistura racémica e utilizado no tratamento da dor. É rapidamente absorvido e metabolizado no fígado, resultando na formação de dois metabolitos primários, *N*-desmetiltramadol (*N*-DT) e *O*-desmetiltramadol (*O*-DT).

Neste trabalho, foi estabelecido um método para a separação e quantificação dos enantiómeros do Tramadol e seus metabolitos por cromatografia líquida de alta eficiência associada à deteção por fluorescência (*HPLC-FD*).

As condições otimizadas da separação enantiomérica do Tramadol, *N*-DT e *O*-DT foram conseguidas usando a coluna Lux Celulose-4 150 x 4.6 mm, tamanho de partícula 3 µm, em modo isocrático com a mistura de 0.1% de dietilamina em hexano e etanol (96:4, *V:V*), e fluxo de 0.7 mL/min. O método validado demonstrou exactidão, boa seletividade, linearidade (r^2>0.99) e precisão num intervalo de 56 ng/L a 384ng/L da mistura racémica. O limite de deteção (LD) e o limite de quantificação (LQ) foram 8 ng/L e 28 ng/L para cada enantiómero do Tramadol e do *N*-DT, 20 ng/L e 56 ng/L para cada enantiómero do *O*-DT, respetivamente. O método foi aplicado no estudo de Efluentes e Influentes de ETAR e os compostos foram detetados num intervalo de concentração entre o LQ e 325.1 ng/L, para amostras de efluentes e entre o LQ e 357.9 ng/L, para amostras de influente.

Palavras-chave: Fármacos Quirais | Tramadol | *N*-desmetiltramadol | *O*-desmetiltramadol | Efluente de ETAR | Influente de ETAR

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List of abbreviations and Symbols

ACN	Acetonitrile
ATE	Atenolol
ATFA	Ammonium trifluoroacetate
CP	Chiral Pharmaceuticals
CSP	Chiral Stationary Phase
DEA	Diethylamine
DL	Detection Limit
DRE	Degree of removal efficiency
E	Enantiomer
EC_{50}	Effect concentration at 50% level
EF	Enantiomeric Fraction
EtOH	Ethanol
FD	Fluorescence
FLX	Fluoxetine
Hex	n-Hexane
HPLC-FD	High Performance Liquid Chromatography with Fluorescence Detector
ICH	International Conference on Harmonization
IPA	Isopropyl Alcohol (Propan-2-ol)
K	Retention Factor
LC	Liquid Chromatography
LC/MS/MS	Liquid Chromatography tandem Mass Spectrometry
LC_{50}	Lethal concentration at 50% level
LOEC	Lowest observed effect concentration
MeOH	Methanol
MS	Mass Spectrometry
***N*-DT**	*N*-desmethyltramadol
NOEC	No observed effect concentration
***O*-DT**	*O*-desmethyltramadol
PHO	Propranolol
QL	Quantification limit
RMN	Nuclear Magnetic Ressonance

Rs	Resolution
RSD	Relative standard deviation
R_T	Retention Time
SPE	Solid Phase Extraction
SSRIs	Selective serotonin re-uptake inhibitors antidepressant
TEA	Triethylamine
TLC	Thin-layer Chromatography
UV/VIS	Ultraviolet-Visible
WWTP	Wastewater Treatment Plant
α	Selectivity or Separation Factor

1. Introduction

1.1. Pharmaceuticals in the environment

Pharmaceuticals are an important class of pollutants due to their increasing consumption and their persistence of in the environment (1). These pollutants can enter in the environment through various routes such as emissions from health institutions, industries, agriculture/aquaculture and households (Figure 1) (1-4).

Often, wastewater treatment plants are unable to completely remove pharmaceuticals and residues are discharged to surface waters and can end up in the drinking water, potentially causing toxic effects to aquatic organisms and humans (1).

Many pharmaceuticals pollutants that exist in the environment are chiral and can be present as a single enantiomer or as enantiomeric mixtures (5). This aspect brings an additional environmental problem because beyond the different behavior of the enantiomers concerning pharmacokinetic and pharmacodynamic, the toxicological and ecotoxicological properties can also be different (6).

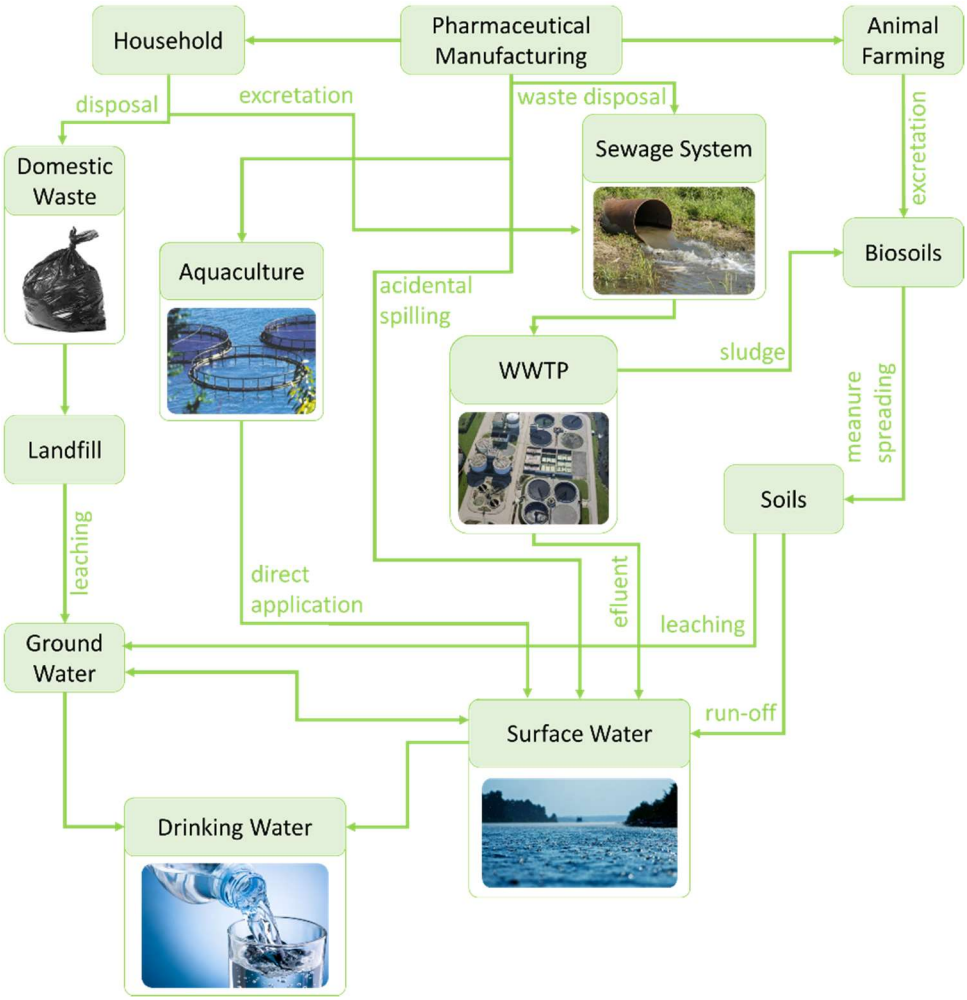

Figure 1: Sources of pharmaceutical products in the environment (1)

1.2. Chiral Pharmaceuticals

Pharmaceuticals are compounds used in humans or animals for medical diagnosis, treatment, or prevention of disease (7). To achieve the desired effects, pharmaceuticals interact with target-molecules which are normally chiral macromolecules, such as enzymes, nucleic acids or membrane-bounded proteins, mediating the biochemical and physiological changes in the body (6). Chiral pharmaceuticals (CP) are used in several areas of medicine, as enantiopure forms or as racemates (4). Enantiomers can interact differently with chiral receptors presenting significant differences in pharmacodynamics, pharmacokinetics and toxicology.

Biological activity of CP can be accomplished in two main different manners: i. both enantiomers have the same activity or ii. enantiomers exhibit different activities. In the case of the enantiomers that can display the same activities, exist cases that one enantiomer are more potent than the other, one enantiomer and one enantiomer have less toxic effects than the other. When both enantiomers of the drug have distinct biological activities, the drug needs to be administrated in an enantiomerically pure form.

Flecainide (antiarrhythmic) and fluoxetine (antidepressant), are examples of CP administrated as racemic mixtures, that both enantiomers having the same biological active. In this group are included other cardiovascular drugs (beta-blockers), agents widely used to the treatment of hypertension, heart failure, arrhythmias, and other diseases. However, there are many CP that enantiomers have different biological activity, one enantiomer with beneficial effects and other with hazardous adverse activity. Example of these drugs are ketamine, *S*-(+)-ketamine is an anesthetic and analgesic but *R*-(-)-ketamine is associated to hallucinations and agitation; naproxen, where *S*-naproxen is used as anti-inflammatory and analgesic and *R*- present hepatotoxicity (4, 8). There are also many example of CP in which the enantiomers differ in the potency of the activity and are commercialized as racemic mixture and as enantiomerically pure form. Racemic citalopram is used in the treatment of depression, but *S*-(+)-citalopram (escitalopram) is 100 times more potent as a serotonin reuptake inhibitor as compared to *R*-(-)-citalopram. Citalopram is commercialized as racemate, and escitalopram marketed in the enantiomerically pure form. Omeprazole that is used in the treatment of dyspepsia, peptic ulcer disease, gastroesophageal reflux disease and Zollinger-Ellison syndrome is market as racemic mixture and as enantiomerically pure form (esomeprazole (*S*-(-)-omeprazole)) (4, 8). Armodafinil (*R*-(-)-modafinil) that is a eugeroic used to treat sleep disorders (4, 8) is another example.

Beta-blockers, also known as β-adrenergic blocking agents, a class of CP that are used for the management of cardiac arrhythmias, usually one enantiomer present higher

potency than the other. For instances, *S*-(-)-propranolol is 100 times more than *R*-(+)-propranolol. However, most of beta-blockers (except timolol*: S*- isomer) are marketed as racemates, such as acebutolol, atenolol, alprenolol, betaxolol, carvediol, metoprolol, labetalol, pindolol and sotalol (4).

Other class of CP is propionic acid derivatives non-steroidal anti-inflammatory drugs (NSAID), which are used as analgesic, anti-inflammatory and anti-pyretic. Ibuprofen, ketoprofen, fenoprofen, are the most known NSAID of this group. Only the *S*-enantiomer of these molecules is active in order to obtain an analgesic and anti-inflammatory effect, and is marketed in racemic and enantiopure form (4). Table 1 shows several CP that are used as racemic mixture or as enantiopure forms.

Table 1: CP that are used as racemic mixture or as enantiopure forms

Class	Commercialized form		Ref.
	Racemate INN	Enantiopure pharmaceutical (INN)	
Calcium channel blocker	Amlodipine	(*S*)-amlodipine (levamlodipine)	(9)
	Verapamil	-	(10)
	-	(+)-diltiazem (diltiazem)	(11)
CNS stimulant	Amphetamine	(*S*)-amphetamine (dextroamphetamine)	(12, 13)
	Methylphenidate	(*R,R*)-methylphenidate (dexmethylphenidate)	(13)
Anaesthetic	Bupivacaine	(*S*)-bupivacaine (levobupivacaine)	(14)
General anaesthetic	Ketamine	(*S*)-ketamine (esketamine)	(14)
Antihistaminic	Cetirizine	(*R*)-cetirizine (levocetirizine)	(12, 13, 15)
Antidepressant	Citalopram	(*S*)-citalopram (escitalopram)	(13, 15, 16)
	Milnacipran	(*S,R*)-milnacipran (levomilnacipran)	(17)
	Venlafaxine	-	(11)
	Fluoxetine	-	(10)
Serotonergic anorectic	Fenfluramine	(*S*)-fenfluramine (dexfenfluramine)	(18)
Beta-adrenoceptor agonist	Formoterol	(*R,R*)-formoterol (arformoterol)	(19)
	Salbutamol	(*S*)-salbutamol (levalbuterol)	(12)
	-	(*S*)- albuterol (albuterol)	(10)
NSAID	Ibuprofen	(*S*)-ibuprofen (dexibuprofen)	(15)
	Ketoprofen	(*S*)-ketoprofen (dexketoprofen)	(15)
	-	(*S*)-naproxen (naproxen)	(11, 20)

(INN: International Nonproprietary Name)

Table 1: CP that are used as racemic mixture or as enantiopure forms (continuation)

Class	Commercialized form		Ref.
	Racemate INN	Enantiopure pharmaceutical (INN)	
Wakefulness-promoting	Modafinil	(*R*)-modafinil (armodafinil)	(13)
Antimicrobial	Ofloxacin	(*S*)-ofloxacin (levofloxacin)	(15)
Hypnotic	Zopiclone	(*S*)-zopiclone (eszopiclone)	(13, 21)
Analgesic	Methadone	(*R*)-methadone (levomethadone)	(22)
	Tramadol	-	(11)
	-	(-)-morphine (morphine)	(8) (14)
Antidiabetic	Rosiglitazone	-	(10)
β-blockers	Atenolol	-	(8)
	Propranolol	-	(11)
	Metoprolol	-	(11)
Anticoagulants	Warfarin	-	(8)
Anti-arrhythmic	Flecanide	-	(11)
Sedative/Hypnotics	Diazepam	-	(11)
Proton pump inhibitors	Omeprazole	(*S*)-omeprazole (esomeprazole)	(12, 15)
	Pantoprazole	-	(11)
	Lansoprazole	-	(11)
Angiotensin-II receptor antagonist	-	(*S*)- valsartan (valsartan)	(11)
	-	(*R*)- losartan (losartam)	(11)
Angiotensin-converting enzyme inhibitors	-	(*S*)- ramipril (ramipril)	(11)
Anaphylaxis	-	(*R*)- epinephrine (epinephrine)	(8)
Anticholonergics	-	(-)-(*S*)- hyoscine (hyoscine)	(8)
Dopaminergic antiparkinsinism agents	-	(*S*)- levodopa (levodopa)	(8)
Antithyroid and Thyroid hormone	-	(*S*)- levothyroxine (levothyroxine)	(8)
Lipid regulating	-	(*R,R*)- atorvastatin (atorvastatin)	(11)

(INN: International Nonproprietary Name)

1.2.1. Toxicity of Chiral Pharmaceuticals

In last two decades the impact of pharmaceuticals on aquatic organism and in the environment has been a growing concern due to acute and long exposure toxicity, but also due its genotoxicity and mutagenicity. This concern is extended to human health due food chain and drinking water contamination (23). When introduced into the environment pharmaceuticals can affect animals that have identical target organs, tissues, cells or biomolecules (24).

Antibiotics are of major concern since they may lead to the development of antimicrobial resistance, and a threat to health. However, even anti-inflammatories have eco-toxicity associated with the development of visceral drop, which occurs when uric acid salts are deposited in organs, leading to the decline of some species populations. This class has also been linked to the fact cause renal dysfunction trout. Many other pharmaceuticals, such as chlorprozamine, fluoxetine, sertaline and propranolol, have shown the ability to impact behavior and reproduction of aquatic organisms (25). Regarding the toxicity of CP, like the pharmacological activity the effect caused by the enantiomers can also differ.

Ecotoxicological effects of CP has been studied, but only in some therapeutic classes and enantioselective toxicity has been poorly explored, some data are shown in Table 2. The enantioselective studies must be carried out more frequently and involving chiral drugs from different therapeutic classes for a better understanding of the toxic effects, as the racemic mixture may behave differently the two enantiomers.

Table 2: Enantioselective ecotoxicological effects of CP in some species

Class/ Pharmaceutial	Specie (Taxonomic Group)	Concetration	Ecotoxicity effect	Ref.
Selective serotonin re-uptake inhibitors antidepressant (SSRIs)				
Fluoxetine	*Ceriodaphnia dubia* (Crustacean)		(*R*)-FLX more toxic	(26, 27)
	Daphnia magna (Crustacean)		(*S*)-FLX more toxic	(26, 27)
		429 µg/L (*R*-FLX)	Reproduction [NOEC (21 d)]	(28)
		444 µg/L (*S*-FLX)		
		430 µg/L (racemic)		
		170 µg/L (*R*-FLX)	Reproduction [LOEC (21 d)]	(28)
		195 µg/L (*S*-FLX)		
		174 µg/L (racemic)		
	Pimephales promela (Fish)	212 µg/L (*R*-FLX)	[LC_{50} (48 h)]	(28)
		216 µg/L (*S*-FLX)		
		198 µg/L (racemic)		
		170 µg/L (*R*-FLX)	Growth [LOEC (7 d)]	(28)
		51 µg/L (*S*-FLX)		
		53 µg/L (racemic)		

(EC_{50}: Effect concentration at 50% level; LC_{50}: Lethal concentration at 50% level; LOEC: Lowest observed effect concentration; NOEC: No observed effect concentration)

Table 2: Enantioselective ecotoxicological effects of CP in some species (continuation)

Class/ Pharmaceutial	Specie (Taxonomic Group)	Concetration	Ecotoxicity effect	Ref.
Fluoxetine	*Pimephales promela* (Fish)	118 µg/L (*R*-FLX)	Growth [NOEC (7 d)]	(28)
		9 µg/L (*S*-FLX)		
		9 µg/L (racemic)		
Paroxitine	*Daphnia magna* (Crustacean)	35.0 mg/L	Immobilization* [EC_{50} (48 h)] (* main metabolite)	(26)
Cardiovascular Drugs **β-Adrenoceptor blocking drugs**				
Propranolol	(Algae)	-	(*S*)-PHO is the most toxic	(26)
	Ceriodaphnia dubia (Crustacean)	-	(*S*)-PHO is the most toxic	(26, 27)
	Pimephales promela (Fish)	-	(*S*)-PHO affects the growth	(29)
		-	(*S*)-PHO is the most toxic	(26, 27)
Atenolol	*Ceriodaphnia dubia* (Crustacean)	-	More sensitive to (*S*)-ATE	(30)
	Daphnia magna (Crustacean)	-	More sensitive to (*S*)-ATE	(30)

(EC_{50}: Effect concentration at 50% level; LC_{50}: Lethal concentration at 50% level; LOEC: Lowest observed effect concentration; NOEC: No observed effect concentration)

1.2.2. Methods for quantification of chiral pharmaceuticals

Quantification of CP can be performed by several analytical techniques such as gas and liquid chromatography, electrochemical sensors and biosensors, thin-layer chromatography (TLC) and even by nuclear magnetic resonance (RMN) (6). Enantiomers are distinguishable if and only if they are placed in a chiral environment, and all methods to separate or discriminate enantiomers are based on this principle. However for enantioselective analysis of CP in environmental matrices, chromatography is the most reliable methodology.

1.2.2.1. Liquid Chromatography

Liquid chromatography (LC) is a technique which can operate with many types of detectors, such as liquid chromatography / mass spectrometry (LC/MS) and liquid chromatography tandem mass spectrometry (LC/MS/MS), ultraviolet-visible (UV/Vis), diode array, fluorescence (FD), and electrochemical. LC/MS and LC/MS/MS play an important role in environmental analysis, due to its versatility, sensitivity, and selectivity (6) being the technique most used to quantify CP in the environment.

LC is an excellent tool for chiral analysis since there are a high number of commercial columns for LC able to work in different elution mode. Several different chiral columns are marketed for LC as Pirkle type, crown ethers, ligand exchangers, cyclodextrins, polysaccharides, proteins, macrocyclic antibiotics glycopeptides, among others (6). However mass spectrometry detection present same limitations in type of elution mode and the additives that can be used.

1.3. Tramadol

Tramadol [2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexan-1-ol] (Figure 2), is a CP that acts as centrally acting analgesic, via an opioid mechanism and a nonopioid mechanisms, structurally related to codeine and morphine, used for the treatment of moderate to several pains (31, 32). It is a synthetic opioid that acts as agonist by selective activity at the μ-opioid receptors commercialized as racemic mixture and its major active metabolite, O-DT, shows higher affinity for the μ -opioid receptor and has twice the analgesic potency of the parent drug. The analgesic effect of tramadol can be contribution of inhibiting the reuptake of norepinephrine and serotonin, that occurs due the nonopioid mechanism, where predominate in (*S,S*)-Tramadol. The opioid mechanism involves (*R,R*)-Tramadol, that is a component opioid with weak affinity of the parent drug (31, 33, 34). Apart from analgesic effects, tramadol has been found to produce several positive effects such as antitussive, antidepressant, anti-inflammatory and immunostimulatory effects (35).

It is a drug that had low incidence of adverse effects. These side effects include seizures, respiratory depression, nausea, vomiting, dry mouth, dizziness, fatigue, sweating, drowsiness, orthostatic hypotension, tiredness and constipation (31, 32, 36). The abuse potential is lower, but are being reported (33). The use of this CP carries some risks, as possible dependence, addiction and withdrawal symptoms (37).

Tramadol in plasma, is almost (about 60 to 70%) and quickly absorbed and metabolized in the liver, resulting in the formation of its primary chiral metabolites, *N*-

desmethyltramadol (*N*-DT) and *O*-desmethyltramadol (*O*-DT), represented in Figure 2, catalized by the cytochrome P450, mostly isoenzyme CYP2D, and further conjugation with glucuronic acid and sulfate, the mechanism of human metabolic pathway are represented in Figure 3 (37-39), in this way about 30 to 40% of the tramadol drug is excreted by the urine in its original form. *O*-DT is the main metabolite that is pharmacologically active and responsible for the analgesic effect of tramadol (40, 41).

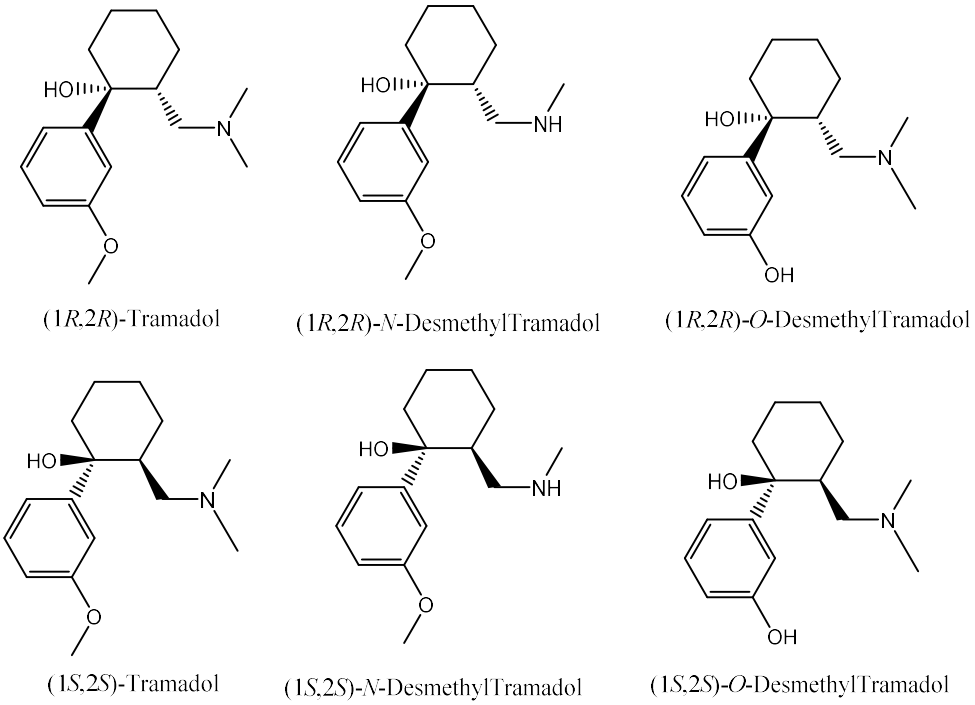

Figure 2: Chemical structure of enantiomers of Tramadol and of its metabolites

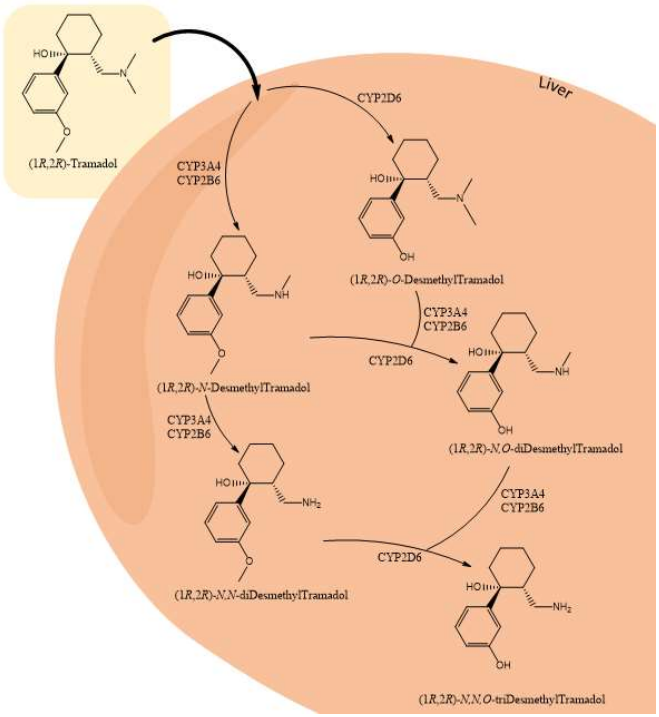

Figure 3: Human Metabolic Pathway of Tramadol (37)

Simultaneous quantification of Tramadol and its metabolites in brain tissue of mice and rats (42), saliva (43), urine (43, 44), amniotic fluid (45) and plasma (39, 43, 46-58) as matrix with resource of different analytical techniques have been reported, using diverse methods (35). Quantification of the enantiomers of tramadol and its metabolites has also been reported. Table 3 presented a summary of the methods, that include chiral stationary phase (CSP) and the mobile phase used, used to quantify tramadol enantiomers and its metabolites by LC.

Table 3: Methods used to separate enantiomerically and quantify enantiomers of tramadol and its metabolites

Analyte	CSP	Detector	Mobile Phase [Proportion (v/v)]	Matrix	Ref.
Tramadol, *N*-DT and *O*-DT	Chriralpak AD	LC-MS/MS	Hex/EtOH/DEA [97/3/0.1]	Human plasma	(48)
Tramadol and *O*-DT	Lux Cellulose-2 and Lux Cellulose-2 security guard column	LC-MS/MS	Hex/IPA/DEA [90/10/0.1]	Human plasma	(59)
	Chriralpak AD	LC-MS/MS	Hex/EtOH [97/3] 5 mM TEA	Human plasma	(60)
Tramadol	Chiralpack CBH	UPLC-MS/MS	1mM ammonium acetate aqueous/MeOH [85/15]	WWTP influent	(61)
	Chirobiotic V	LC-MS/MS	MeOH/ 0.005% Formic Acid/ 4mM ammonium Acetate	WWTP samples	(62)

According to the literature the enantioseparation of three compounds was achieved by Ceccato *et all*. (48) using a Chriralpak AD, a polysaccharide derivates CSP, with Hexane (Hex)/ Ethanol (EtOH)/Diethylamide (DEA) 97:3:0.1 (*v:v:v*) as mobile phase, a flow rate of 1 mL/min and a temperature of 25 ± 0.1 °C. This separation was achieved using a LC-MS/MS, this technique combine the outstanding separation of LC with the qualitative capabilities of Mass Spectrometry (63), which is a detector with a high sensitivity. The enantioseparation of each compound is presented in Figure 4, an Selected Reaction Monitoring (SRM) ion chromatogram, enantioseparation is achieved without chemoseparation.

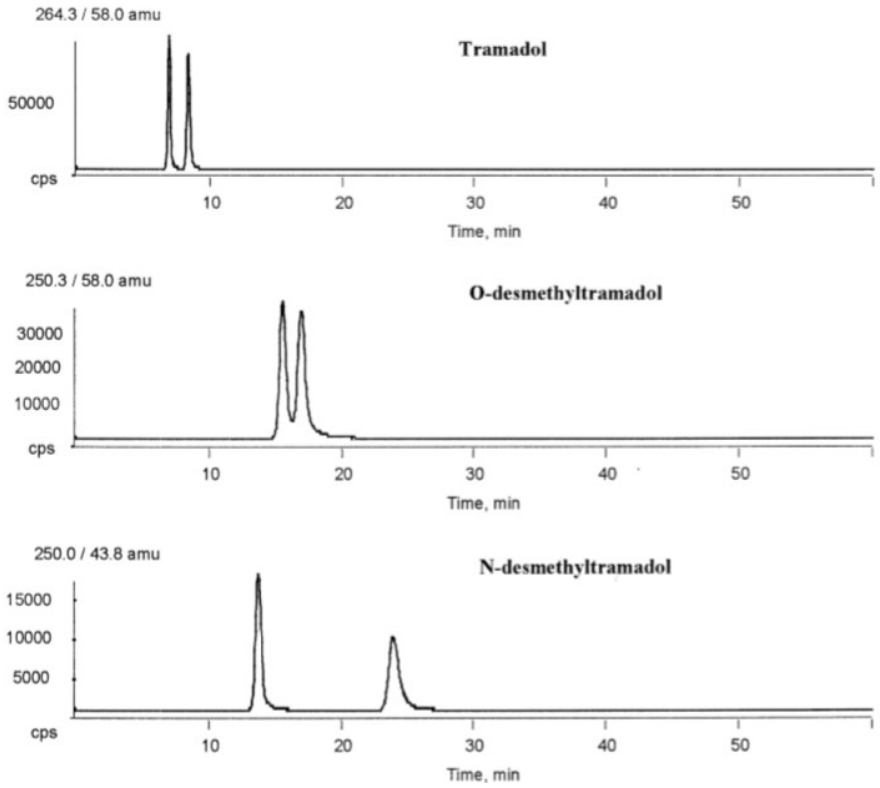

Figure 4: Enantioseparation of Tramadol, *N*-DT and *O*-DT by Ceccato *et all.* (48)

The methods that Chytil *et all*. (59) and Musshoff et all. (60) reported only achieved the enantioseparation of Tramadol and *O*-DT. Both methods use and MS detector, and conditions demonstrated in Table 3, both used polysaccharide derivates CSP. The remaining methods shown in Table 3, just separated the enantiomers of Tramadol. Evans *et all*. (62) using Chiralpack CBH CSP, an protein based CSP and Castrignano *et all*. (61) using Chirobiotic V CSP, an macrocyclic glycopeptides antibiotics CSP.

1.3.1. Tramadol in the environment

Tramadol has been quantified in environmental sample in the range of ng/L to μg/L. Catrignano *et al.* (61) and Evans *et al.* (62) quantified Tramadol in samples of WWTP (Table 4). The metabolite O-DT has also been quantified.

Table 4: Tramadol and its metabolite O-DT in the environmental

Analyte	Concentration (ng/L)	Sample	Ref.
Tramadol	595 ± 22 – 798 ± 39	WWTP	(61)
	1320.7 ± 59.3	Influent	(62)
	506.0 ± 46.6	Effluent	(62)
***O*-DT**	801 ± 6 – 950 ± 21	WWTP	(61)

Ecotoxicological effects of Tramadol and of its primary metabolites have already. Been reported (Table 5). However the eocotoxicological effects of the enantiomers has never been reported.

Table 5: Ecotoxicological effects of Tramadol and of its metabolites

Analyte	Specie (Taxonomic Group)	Concetration	Ecotoxicity effect	Ref.
Tramadol	(Crustacean)	73 mg/L (racemic)	[LC_{50} (48 h)]	(63)
		130 mg/L (racemic)	[LC_{50} (< 96 h)]	(63)
***N*-DT**	*Vibrio Fischeri* (Bacteria)	10 mg/L	Bioluminescence inhibition (41 %)	(64)

(LC_{50}: Lethal concentration at 50% level)

1.4. Objectives

The objective of this study was to develop and validate a high performance liquid chromatography with fluorescence detection (HPLC-FD) methodology to separate, identify and quantify the enantiomers of Tramadol and its metabolites, *N*-DT and *O*-DT.

1.4.1. Project commitments and outline of the thesis

The understanding of the fate of Tramadol, its metabolites and transformation products in environmental matrices is extremely necessary to the development of enhanced environmental risk assessment studies. Although this matter is quite discussed currently in scientific research circles and is being amply studied, there are still important information gaps concerning CP.

The planning of this work had the following purposes:

- ✓ Development and validation of an analytical HPLC-FD method for enantioseparation, identification and quantification of Tramadol, *N*-DT and *O*-DT.
- ✓ Application of the developed chromatographic method to monitoring samples from WWTP.

This work is structured comprehending four main topics: introduction; material and methods; results and discussion and conclusions.

2. Material and Methods

2.1. Instrumental conditions

High Performance Liquid Chromatography with Fluorescence Detection (HPLC-FD) was used to quantification Tramadol and their primary metabolites in wastewater samples.

The chromatographic equipment used in analytical analysis was a Shimadzu UFLC Prominence System equipped with two Pumps LC-20AD, an Autosampler SIL-20AC, column oven CTO-20AC, a Degasser DGU-20A5, a System Controller CBM-20A and a LC Solution, Version 1.24 SP1 (Shimadzu). The Fluorescence Detector (FD) coupled to the LC System was a Shimadzu RF-10AXL.

2.2. Standards and reagents

Tramadol and *O*-DT standards were purchased from Sigma Aldrich, *N*-DT standard was purchased from Lipomed. The standards present a purity degree above 99%. Tramadol, *N*-DT and *O*-DT stock standard solutions were prepared at 1 mg/mL of racemic mixture in ethanol. These stock standard solutions were stored at -20 ºC in amber bottles. All the work standard solutions were obtained by dilutions of the stock solutions performed in appropriated solvents to analyses, accordingly to the mobile phase.

The ethanol (EtOH), acetonitrile (ACN), methanol (MeOH) and propan-2-ol (IPA) HPLC grade were purchased from Fisher Chemical. Hexane 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. Formic acid with 98-100% purity bought from MERCK. Ultrapure water was supplied by a Milli-Q water system.

2.3. Enantiomeric Separation

Standards mixture and dilution in Hex/EtOH in proportion 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 for the optimization of the enantioseparation.

The chiral chromatographic columns used were: Astec Chirobiotic TAG, Astec Chirobiotic V, Lux Cellulose-2 and Lux Cellulose-4. Astec Chirobiotic TAG and Astec Chirobiotic V have particle size 5 µm, with dimension of 150 x 2.1 mm, from Sulpelco Analytical (sigma-aldrich). Lux Cellulose-2 and Lux Cellulose-4 have particle size 3 µm, with dimension of 150 x 4.6 mm, from Phenomenex. Diverse mobile phases in normal, reversed, polar organic mode of elution, and in different flow rate were attempted (Tables

6, 7 and 8). The analysis was executed with an injection volume of 10 µL, a column oven temperature of 23 ºC and a autosampler tray temperature of 15ºC, in some cases oven temperature were 27 ºC. The fluorescence detector was set to an excitation wavelength of 275 nm and an emission wavelength of 300 nm.

Table 6: Experimental conditions using Macrocyclic glycopeptides antibiotics CSP

Column	Elution mode	Mobile Phase	Proportion (v/v)	Flow (mL/min)
Chirobiotic TAG	Normal	Hex/EtOH	80/20	0.75
		Hex/EtOH	90/10	0.75
	Reversed	MeOH (0.1%M ATFA) / H_20	50/50	1
		MeOH (0.1%M ATFA) / H_20	25/75	1
		MeOH (0.1%M ATFA) / H_20	75/25	1
		ACN (0.1%M ATFA) / H_20	50/50	0.5
		ACN (0.1%M ATFA) / H_20	25/75	0.75
		EtOH (0.1%M ATFA) / H_20	50/50	0.5
		EtOH (0.1%M ATFA) / H_20	25/75	0.5
		MeOH/H_20	50/50	075
		MeOH/H_20	25/75	0.75
		MeOH/H_20	75/25	0.75
		MeOH/H_20	10/90	0.75
Chirobiotic V	Reversed	EtOH/10 mM aqueous ammonium acetate buffer [pH=5.3]	92.5/7.5	0.32
		EtOH/10 mM aqueous ammonium acetate buffer [pH=6.8]	80/20	0.32
		EtOH/10 mM aqueous ammonium acetate buffer [pH=6.8]	92.5/7.5	0.32
		MeOH/4 mM aqueous ammonium acetate buffer [pH=5.3]	95/5	0.1

Table 7: Experimental conditions using Polysaccharide derivate Cellulose-2 CSP

Column	Elution mode	Mobile Phase	Proportion (v/v)	Flow (mL/min)
Cellulose-2	Normal	Hex/IPA/DEA	90/10/0.1	0.5
		Hex/IPA/DEA	95/5/0.1	0.5
		Hex/EtOH/DEA	96/4/0.1	0.7
		Hex/EtOH/DEA	96/4/0.1	0.5
		Hex/IPA/DEA	90/10/0.1	0.5
		Hex/IPA/DEA	90/10/0.05	0.5
		Hex/IPA/DEA	96/4/0.05	0.5
		Hex/IPA/DEA	96/4/0.05	0.7*
		Hex/IPA/DEA	94/6/0.05	0.3*
		Hex/IPA/DEA	94/6/0.1	0.3*
	Reversed	ACN (5 mM ammonium formate):DEA/H_2O	35:0.1/65	0.5
		ACN (5 mM ammonium formate):DEA/ H_2O	30:0.1/70	0.5
		ACN (5 mM ammonium formate):DEA/ H_2O	20:0.1/80	0.7
		ACN (5 mM ammonium formate):DEA/ H_2O	25:0.1/75	0.7
		EtOH (5 mM ammonium formate):DEA/ H_2O	40:0.1/60	0.5
		EtOH (5 mM ammonium formate):DEA/ H_2O	40:0.1/60	0.7*
		ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	16.36:28.63:0.1/55	0.5*
		ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	16.36:28.63:0.05/ 55	0.5*
		ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	14.55:25.45:0.05/ 60	0.5*
		ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	12.73:22.27:0.1/65	0.7*
		ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	14.54:25.46:0.1/60	0.5*
		ACN:EtOH (5mM ammonium formate):DEA/ H_2O	8:32:0.1/60	0.5*

(* T=27ºC)

Table 8: Experimental conditions using Polysaccharide derivate Cellulose-4 CSP

Column	Elution mode	Mobile Phase	Proportion (v/v)	Flow (mL/min)
Cellulose-4	Normal	Hex/IPA	90/10	0.5
		Hex/IPA/DEA	90/10/0.1	0.5
		Hex/IPA/DEA	90/10/0.1	0.5*
		Hex/IPA/DEA	92/8/0.1	0.5
		Hex/IPA/DEA	94/6/0.1	0.5
		Hex/IPA/DEA	96/4/0.1	0.5
		Hex/EtOH/DEA	90/10/0.1	0.5
		Hex/EtOH/DEA	94/6/0.1	0.5
		Hex/EtOH/DEA	96/4/0.1	0.5
		Hex/EtOH/DEA	98/2/0.1	0.5
		Hex/EtOH/DEA	96/4/0.1	0.5
		Hex/EtOH/DEA	95/5/0.1	0.5
		Hex/EtOH/DEA	95/5/0.1	0.7
		Hex/EtOH/DEA	96/4/0.1	0.7
	Reversed	ACN (5 mM ammonium formate)/ H_2O:DEA (pH=8)	45/55	0.5
		ACN (5 mM ammonium formate)/ H_2O	45/55	0.5
		H_2O (20 mM ammonium formate)/ACN pH=8 [adjusted with DEA]	50/50	0.5
		ACN (5 mM ammonium formate):DEA/EtOH	50:0.1/50	0.5
		ACN (5 mM ammonium formate):DEA/EtOH	45:0.1/55	0.5
		H_2O (20 mM ammonium formate) : DEA/ACN	50:0.1/50	0.5
		H_2O (20 mM ammonium formate) : DEA/ACN	60:0.1/40	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	45:0.1/55	0.5
		EtOH (10 mM ammonium formate) : DEA/ H_2O	45:0.1/55	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	35:0.1/65	0.5

(* T=27ºC)

Table 8: Experimental conditions using Polysaccharide derivate Cellulose-4 CSP (continuation)

Column	Elution mode	Mobile Phase	Proportion (v/v)	Flow (mL/min)
Cellulose-4	Reversed	ACN (5 mM ammonium formate) : DEA/ H_2O	30:0.1/70	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	40:0.1/60	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	50:0.1/50	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	45:0.1/55	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	45:0.1/55	0.5*
		ACN (5 mM ammonium formate) : DEA/ H_2O	40:0.1/60	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	45:0.08/55	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	35:0.08/65	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	25:0.08/75	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	30:0.08/70	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	45:0.05/55	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	35:0.05/65	0.5
		ACN (5 mM ammonium formate) : DEA/ H_2O	30:0.05/70	0.5
		EtOH (15 mM ammonium formate)/ H_2O pH=8.19 [adjusted with DEA]	50/50	0.5
		EtOH (15 mM ammonium formate)/ H_2O pH=8.03 [adjusted with DEA]	40/60	0.5
		EtOH (15 mM ammonium formate)/ H_2O pH=7.96 [adjusted with DEA]	50/50	0.5
		EtOH (10 mM ammonium formate)/ H_2O pH=7.96 [adjusted with DEA]	50/50	0.5

(* T=27ºC)

Table 8: Experimental conditions using Polysaccharide derivate Cellulose-4 CSP (continuation)

Column	Elution mode	Mobile Phase	Proportion (v/v)	Flow (mL/min)
Cellulose-4	Reversed	EtOH (10 mM ammonium formate)/ H_2O pH=8.13 [adjusted with DEA]	45/50	0.5
		EtOH (10 mM ammonium formate) : DEA/ H_2O	45:0.1/55	0.5
		EtOH (10 mM ammonium formate) : DEA/ H_2O	50:0.1/50	0.5
		EtOH (10 mM ammonium formate) : DEA/ H_2O	47:0.1/53	0.5
		EtOH (10 mM ammonium formate) : DEA/ H_2O	40:0.1/60	0.5
		EtOH (10 mM ammonium formate: DEA/ H_2O	50:0.1/50	0.5
		EtOH (10 mM ammonium formate) : DEA/ H_2O	45:0.1/55	0.5
		MeOH (5 mM ammonium formate)/ H_2O	45/55	0.5
		MeOH (5 mM ammonium formate) : DEA/ H_2O	50:0.1/50	0.5
		MeOH (5 mM ammonium formate) : DEA/ H_2O	45:0.1/55	0.5
		MeOH (5 mM ammonium formate) : DEA/ H_2O	45:0.05/55	0.5
		MeOH (5 mM ammonium formate) : DEA/ H_2O	60:0.05/40	0.5
		MeOH (5 mM ammonium formate) : DEA/ H_2O	55:0.05/45	0.5
		MeOH (5 mM ammonium formate) : DEA/ H_2O	45:0.05/55	0.5
		MeOH (5 mM ammonium formate) : DEA/ H_2O	45:0.05/55	0.7
		MeOH (5 mM ammonium formate) : DEA/ H_2O	45:0.02/55	0.5
		ACN:EtOH (10 mM ammonium formate):DEA/ H_2O	20:20:0.1/65	0.5
		ACN:EtOH (10 mM ammonium formate):DEA/ H_2O	17.5:17.5:0.1/65	0.5
		ACN:EtOH (10 mM ammonium formate):DEA/ H_2O	21:9:0.1/60	0.5

(* T=27ºC)

Table 8: Experimental conditions using Polysaccharide derivate Cellulose-4 CSP

(continuation)

Column	Elution mode	Mobile Phase	Proportion (v/v)	Flow (mL/min)
Cellulose-4	Reversed	ACN:EtOH (10 mM ammonium formate):DEA/ H_2O	31.5:31.5:0.1/55	0.5
		ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	35:20:0.1/45	0.5
		ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	28.6:14.5:0.1/55	0.5
		ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	25.5:14.5:0.1/60	0.5

(* T=27ºC)

2.3.1. Calculations

The tables of results relative to the enantiomeric separation includes values of retention factor (K), separation factor (α) and resolution (Rs). These parameters were calculated by the following formulas.

The retention factor is calculated used the retention time of the analyte (R_T) by the formula (1).

(1) $K = \frac{R_T - R_{T\,0}}{R_{T\,0}}$~

(where $R_{T\,0}$ is the dead time of the run)

Separation factor was calculated according to formula (2).

(2) $\alpha = \frac{K_1}{K_2}$

Resolution is represented in formula (3) and in formula (4). Formula (4) were used to calculate resolution.

(3) $Rs = \frac{\sqrt{N}}{4}\,(\alpha - 1)\left(\frac{K}{1+K}\right)$

N: Theorical plates = $16\left(\frac{R_T}{W}\right)^2$

(where W = 4 σ, is the width of the peak; σ is the standard deviation)

(4) $Rs = 2 \times \frac{R_{T\,1} - R_{T\,2}}{(W_1 + W_2)}$, w_1 and w_2 are the width at the base of the peaks of the first and second enantiomer eluted, respectively.

2.4. Sample preparation

2.4.1. Solid-phase extraction (SPE)

Samples used to this procedure were obtained from WWTP of Parada (North of Portugal). The selection of the best cartridge for the SPE procedure was done by a spike of 200 µL of a mixture of three standards in a concentration of 1 µg/mL of the racemic mixture, dissolved in EtOH, in 250 mL of ultrapure water acidified with H_2SO_4 to pH=2, or basified with NaOH to pH=7.5, in the sample used to the procedure with Oasis® cartridge from Ireland. The cartridge used were Oasis® MCX 150 mg 6cc and Oasis® HLB 150 mg 6cc.

In order to obtain a higher recovery ratio, various procedures were attempted, presented in Table 9, with the steps shown on Figure 5. Loading samples were done simultaneously using a vacuum manifold system connected to a vacuum pump and percolated through the cartridges at a constant flow rate of 10 mL min−1. The drying step, was, under vacuum for 30 min to dry out residual water and equal for all procedures. Elution was done in two steps, the eluate from the first step was rejected and only the eluated from the second step was used. In all procedures the assays were done in triplicate.

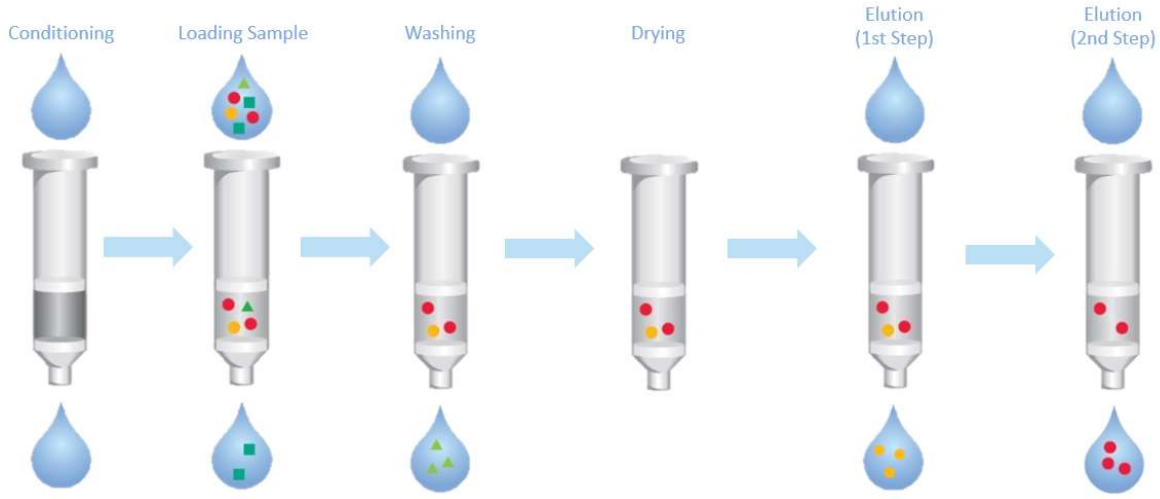

Figure 5: Steps of SPE procedure (65)

After the elution procedures, the eluted were evaporated to dryness and reconstituted in Hex/EtOH in the proportions of 8:2 (*v:v*) and 20 µL was used for the HPLC-FD quantification using the optimized enantioseparation method. The recovery of each compound is calculated by Recovery (%) = $\frac{A_{peak\ of\ spiked\ sample}}{A_{peak\ of\ compounds\ in\ solvent}} \times 100.$

Table 9: Experimental conditions of SPE

Procedure	Cartridge	Conditioning	Washing	Elution (1st Step)	Elution (2nd Step)	Ref.
1	MCX	8 mL MeOH 8 mL H_2O	8 mL H_2O 8 mL MeOH	8 mL 5% NH_4OH solved in ACN/MeOH 60/40	-	(66)
2	MCX	8 mL MeOH 8 mL H_2O	8 mL 2% Formic Acid	8 mL MeOH	8 mL methanoic solution of 5% NH_4OH	(67)
3	MCX	8 mL MeOH 8 mL H_2O	8 mL 2% Formic Acid	12 mL methanoic solution of 10% NH_4OH	-	(67)
4	MCX	8 mL EtOH 8 mL H_2O	8 mL 2% Formic Acid	8 mL EtOH	8 mL ethanoic solution of 5% NH_4OH	(67)
5	MCX	8 mL EtOH 8 mL Formic acid 2%	8 mL 2% Formic Acid	8 mL ethanoic solution of 0,6 % of Formic Acid	8 mL ethanoic solution of 5% NH_4OH	(68, 69)
6	MCX	8 mL EtOH 8 mL H_20 (pH=2 adjusted with HCl)	8 mL 2% Formic Acid	8 mL EtOH	8 mL ethanoic solution of 5% NH_4OH	(70)
7	HLB	10 mL MetOH 10 mL H_20	10 mL H_20	10 mL MeOH	-	(70)
8	MCX	8 mL MeOH 8 mL H_20	8 mL 2% Formic Acid	12 mL methanoic solution of 10% NH_4OH	-	(67)
9	MCX	8 mL EtOH 8 mL H_20	8 mL 2% Formic Acid	8 mL EtOH	8 mL ethanoic solution of 5% NH_4OH	(67)
10	MCX	8 mL EtOH 8 mL 2% Formic Acid	8 mL 2% Formic Acid	8 mL ethanoic solution of 0,6 % of Formic Acid	12 mL ethanoic solution of 5% NH_4OH	(68, 69)
11	MCX	8 mL EtOH 8 mL 2% Formic Acid	8 mL 2% Formic Acid	12 mL ethanoic solution of 5% NH_4OH	-	(68, 69)
12	MCX	8 mL EtOH 8 mL 2% Formic Acid	8 mL 2% Formic Acid	8 mL ethanoic solution of 0,6 % of Formic Acid	12 mL ethanoic solution of 5% NH_4OH	(68, 69)

2.4.2. Synthetic wastewater Influent

After optimization of the SPE procedure in ultra-pure water, the best condition was used to validate the method with sample of synthetic wastewater. The synthetic wastewater influent was prepared by the mixture of various reagents. Initially was preparated the Vischniac Trace element Solution, the reagents an amount used to prepare this solution was presented in Table 10.

Table 10: Reagents used in preparation of Vischniac Trace Element Solution

Reagent	g/100 mL	Reagent	g/100 mL
$EDTA.2H_2O$	6.377	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.11
$ZnSO_4.7H_2O$	2.2	$CuSO_4.5H_2O$	0.157
$CaCl_2$	0.554	$CoCl_2$	0.0879
$MnCl_2.4H_2O$	0.506	KOH (pellets)	Adjust pH to 6
$FeSO_4.7H_2O$	0.499		

($EDTA.2H_2O$: Ethylenediaminetetraacetic Acid Disodium Salt Dihydrate; $ZnSO_4.7H_2O$: Zinc Sulfate Heptahydrate; $CaCl_2$: Calcium Chloride; $MnCl_2.4H_2O$: Manganese (II) Chloride Tetrahydrate; $FeSO_4.7H_2O$: Iron (II) Sulfate Heptahydrate; $(NH_4)_6Mo_7O_{24}.4H_2O$: Ammonium Molybdate Tetrahydrate; $CuSO_4.5H_2O$: Copper (II) Sulfate Pentahydrate; $CoCl_2$: Cobalt (II) Chloride; KOH: Potassium Hydroxide)

All reagents except the KOH were weighed into a beaker and dissolved in 80 ml of ultrapure water. The only way of all compounds dissolve well are adjust pH to 6.0 with KOH pellets. The solution was poured into a 100 ml flask and made up to volume with water. After use the solution was stored in a refrigerator at temperature of 4 ° C for future use. The synthetic wasterwater was prepared with Vischniac Trace element Solution and another reagent mixture, which involves the substances presents in Table 11.

Table 11: Reagents used in preparation of Synthetic wastewater

Reagent	m (g) /250 mL
NaAc	0.121
$MgSO_4.7H_2O$	0.021
KCl	0.008
Na_2HPO_4	0.014
KH_2PO_4	0.007
NH_4Cl	0.044
Vischniac trace elemnt solution (mL/L)	0.234

(NaAc: Sodium Acetate; $MgSO_4.7H_2O$: Magnesium Sulfate Heptahydrate; KCl: Potassium Chloride; Na_2HPO_4: Sodium Hydrogenphosphate; KH_2PO_4: Potassium Dihydrogenphosphate; NH_4Cl: Ammonium Chloride)

2.5. Method validation

2.5.1. Selectivity

Method selectivity was verified with the analysis of the synthetic wastewater matrix. A solution including diverse reagents, prepared as described in “2.4.2”. The matrix passed through a SPE procedure, dried and reconstituted in Hex/EtOH 8:2 (*v:v*). The resulting chromatogram was then compared with two others chromatograms, one referring to the same matrix spiked with the Tramadol, *N*-DT and *O*-DT standards in the final concentrations of 410 ng/mL of the racemic mixture, with the same treatment prior to injection, and other referring to 200 μL aliquots the mix of compounds in concentration of 410 ng/mL of the racemic mixture standard dried and reconstituted in Hex/EtOH 8:2 (*v:v*).

2.5.2. Linearity

Calibration curves were performed with 200μl of a racemic mixture of seven different racemic concentrations (56 ng/mL, 112 ng/mL, 168 ng/mL, 224 ng/mL, 280 ng/mL, 336 ng/mL and 384 ng/mL) spiked in 250mL acidified synthetic wasterwater. For each concentration, SPE procedure was processed in triplicate. The mentioned curves were obtained by linear regression corresponding to the correlation between the peak area and the nominal concentration.

2.5.3. Accuracy

Method accuracy was determined to each analyte using three different racemic concentrations (150 ng/mL, 300 ng/mL and 410 ng/mL) in a spiked matrix in triplicate and expressed as relative standard deviation (RSD). The protocol followed was similar to the one mentioned in 2.5.1.

2.5.4. Precision

Method precision was deliberated by the analysis of three different racemic concentrations (the same used in accuracy), in triplicate, of the mix of the compounds in spiked matrix and expressed as relative standard deviation (RSD) of intra-day (three determinations executed in the same day) and inter-day (three determinations executed in three different days) assays.

2.5.5. Recovery

The recovery rate of the analytical method was determined to each analyte using three different racemic concentrations (the same used in accuracy and precision), in triplicate, both in the spiked matrix and in solvent, in order to compare the

extraction/recovery capability of the method. The results were expressed in recovery percentage.

2.5.6. Detection Limit (DL) and Quantification Limit (QL)

Detection limit and quantification limit were determined based on signal/noise. Determination of signal-to-noise ratio is 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 as a signal-to-noise ratio of 10:1 is typically considered to estimate the QL.

2.6. Application of developed HPLC-FD method in WWTP samples

The Samples were collected in 26th and 27th of June of 2016 in Parada wastewater treatment plant (WWTP), localized in Maia, Portugal. The samples were collected in influent and effluent of the WWTP, two times of day during days.

After collection of the samples, these were filtered under vacuum and acidified with H_2SO_4 to pH=2 and preconcentrated by SPE, using a volume of sample of 250 mL, according to the procedures established in section 2.4.1 with injection of 20 µL.

In order to understand if the compounds present in the samples are Tramadol, *N*-DT and *O*-DT a crossmatch test was realized. This test consist in adding a certain volume, in this case 20 µL, of the mixture of the standards, with a known racemic concentration, 1 µg/mL, in a vial with the samples, in order to identify better what is compound and what is impurity.

2.6.1. Calculations

The data obtained in quantification allowed to determine the enantiomeric fraction (EF) and degree of removal efficiency (DRE). It is possible calculate these compounds trough the formula (1) and (2), respectively.

(1) $$EF=\frac{Concentration\ E_{1/2}}{Concentration\ E_1 + Concentration\ E_2}$$

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

3. Results and Discussion

3.1. Enantiomeric Separation

Different types of chiral columns are commercial avaiable (72-76). The most important types of chiral selectors pointed to: Pirkle type, polysaccharide derivatives, cyclodextrin, protein, macrocyclic glycopeptides antibiotics-based and others based on synthetic polymers (72, 77, 78). The most useful and broadly applied are the CSP based on polysaccharide, macrocyclic antibiotics and Pirkle type (72, 75, 76, 79, 80). Due the versatile and suitable for all elution modes of polysaccharides or macrocyclic antibiotics as chiral selector many authors start the trial-error challenge with these CSP (77, 81-91). The range of application of polysaccharide-based CSP is broader than macrocyclic antibiotics-based CSP concerning the number of compounds enantioseparated by both CSP (75, 80, 82). In a tentative to obtain the best result of chemo and enantioseparation 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.

3.1.1. Macrocyclic glycopeptides antibiotics CSP

These CSP have a great diversity of structures which contain a variety of functional group, multiple stereogenic centers and inclusion cavities. These aspects show the success of these columns. The applicability of these CSP has increased due the possibility to use multimodal elution conditions (71).

In the first attempted to achieve enantioseparation of Tramadol and its metabolites (*O*-DT and *N*-DT) macrocyclic glycopeptides antibiotics CSP, namely the Chirobiotic TAG (Telcoplanin Aglycone CSP) (Figure 6) was selected. This column is formed by covalently bonding between the aglycon part of teicoplanin and silica via linkage chains. This column was used in normal and reversed elution mode, the results obtain are represented in Table 12.

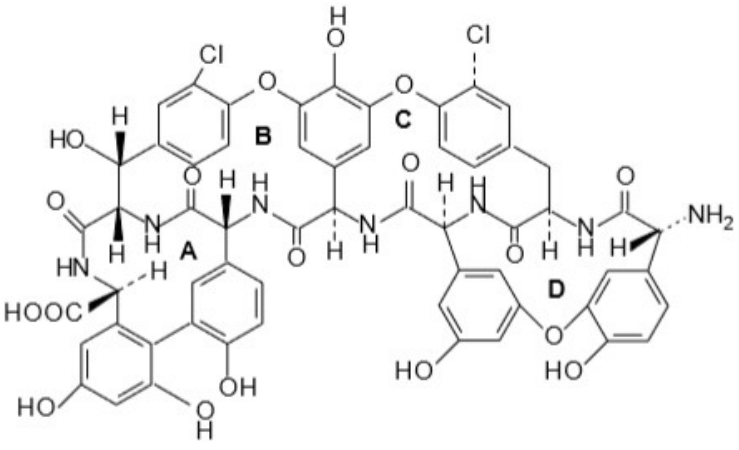

Figure 6: Chemical Structure of Chirobiotic TAG (92)

Table 12: Results of enantiomeric separation of Tramadol on Chirobiotic TAG CSP

Analyte	Elution Mode	Mobile Phase	Proportion (v/v)	$R_{T\,1}$ (24)	$R_{T\,2}$ (24)	K_1	K_2	α	Rs
Tramadol	Normal	Hex / EtOH	80:20**	1.405	-	-	-	1.0	-
		Hex / EtOH	90:10**	2.058	-	-	-	1.0	-
	Reversed	MeOH (0.1%M ATFA) / H_2O	50:50***	1.483	-	1.806	-	1.0	-
		MeOH (0.1%M ATFA) / H_2O	25:75***	1.889	-	2.638	-	1.0	-
		MeOH (0.1%M ATFA) / H_2O	75:25***	6.786	7.009	6.749	7.003	1.038	0.024
		EtOH (0.1%M ATFA) / H_2O	50:50*	27.991	-	19.210	-	1.0	-
		EtOH (0.1%M ATFA) / H_2O	25:75*	30.437	-	19.360	-	1.0	-
		ACN (0.1%M ATFA) / H_2O	50:50*	13.273	-	8.068	-	1.0	-
		ACN (0.1%M ATFA) / H_2O	25:75**	21.246	-	14.850	-	1.0	-

($R_{T\,1}$: Retention time of enantiomer 1; $R_{T\,2}$: Retention time of enantiomer 2; K_1: Retention Factor of enantiomer 1; K_2: Retention Factor of enantiomer 2; α: Separation Factor; Rs: Resolution; * flow rate of 0.5 mL/min; ** flow rate of 0.75 mL/min; *** flow rate of 1 mL/min)

Considering normal elution mode the separation of Tramadol wasn't achieved with any composition of mobile phase. Due the results with normal elution mode, it decided change to reversed elution mode. In this elution mode, only partial enantioseparation of Tramadol was achieved with MeOH (0.1%M ATFA)/H_2O 75:25 (*v:v*) as mobile phase, in a flow rate of 1 mL/min, a column oven in a temperature of 23 °C and with elution time around 7 minutes. Figure 7 presented the chromatogram of this result. The separation (α= 1.038) and resolution (Rs= 0.024) were not satisfactory. When the percentage of water was increased the elution time also increased but did not improve enantioseparation. . For this reason this CSP were abandoned.

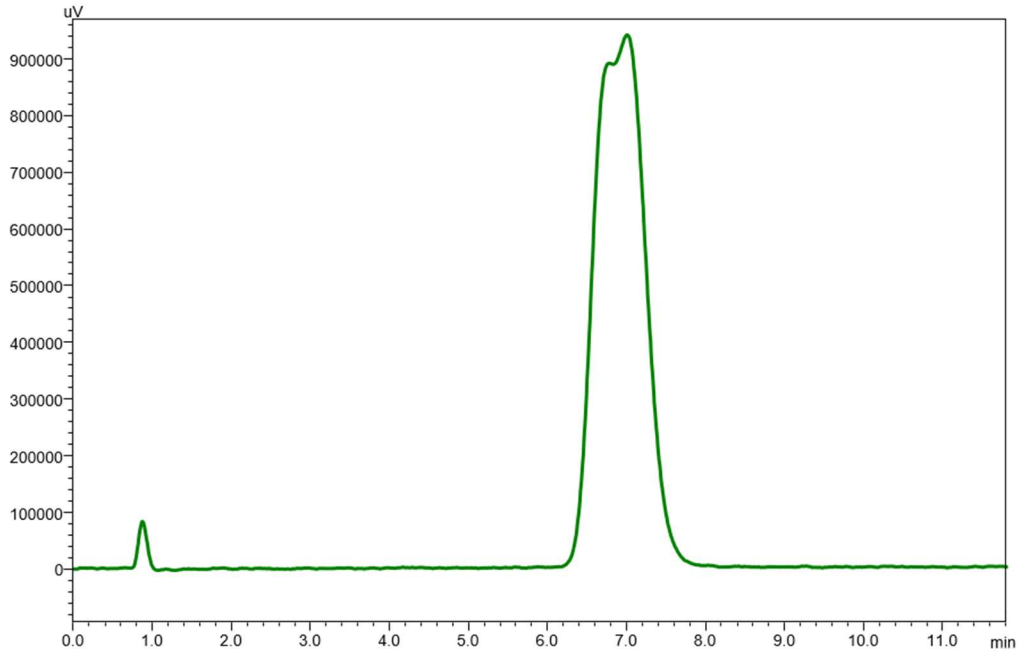

Figure 7: Chromatogram obtain using MeOH (0.1%M ATFA)/H_2O 75:25 (*v:v*) as mobile phase with Chirobiotic TAG CSP.

Regarding Chirobiotic V (Vancomycin CSP), with aromatic rings and peptide linkages that provide some rigidly to the molecule and provide good interactions with small molecules (Figure 8) (93). The enantioseparation of Tramadol has been reported (62). Several mobile phases were attempted in this CSP in order to improve the results form literature. The results are presented in Table 13. Several mobile phase in reversed elution mode were attempted and only partial enantioseparation of the enantiomers of *N*-DT was achieved (Figure 9).

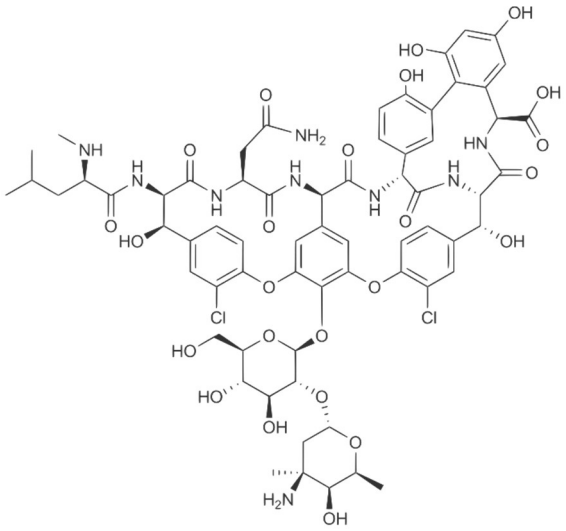

Figure 8: Chemical Structure of Chirobiotic V (92)

Table 13: Results of enantiomeric separation of Tramadol and its metabolites on Chirobiotic V CSP

Analyte	Elution mode	Mobile Phase	Proportion (v/v)	$R_{T\,1}$ (24)	$R_{T\,2}$ (24)	K_1	K_2	α	Rs
Tramadol	Reversed	MeOH/4 mM aqueous ammonium acetate buffer [pH=5.3]	95/5*	>40		-	-	-	-
		EtOH/10 mM aqueous ammonium acetate buffer [pH=5.3]	92.5/7.5**	13.43	-	6.25	-	1.0	-
		EtOH/10 mM aqueous ammonium acetate buffer [pH=6.8]	80/20**	9.21	-	3.88	-	1.0	-
		EtOH/10 mM aqueous ammonium acetate buffer [pH=6.8]	92.5/7.5**	13.69	-	6.34	-	1.0	-
***N*-DT**	Reversed	EtOH/10 mM aqueous ammonium acetate buffer [pH=5.3]	92.5/7.5**	23.38	24.38	11.30	11.83	1.04	0.111
		EtOH/10 mM aqueous ammonium acetate buffer [pH=6.8]	92.5/7.5**	7.73	-	3.05	-	1.0	
***O*-DT**	Reversed	EtOH/10 mM aqueous ammonium acetate buffer [pH=5.3]	92.5/7.5**	14.02	-	7.67		1.0	-
		EtOH/10 mM aqueous ammonium acetate buffer [pH=6.8]	92.5/7.5*	8.70	-	3.56	-	1.0	-

($R_{T\,1}$: Retention time of enantiomer 1; $R_{T\,2}$: Retention time of enantiomer 2; K_1: Retention Factor of enantiomer 1; K_2: Retention Factor of enantiomer 2; α: Separation Factor; Rs: Resolution; * flow rate of 0.1 mL/min; ** flow rate of 0.32 mL/min)

In comparison with the literature the mobile phase MeOH/4 mM aqueous ammonium acetate buffer [pH=5.3, adjusted with 0.005% of formic acid] (62) did not improved the separate the Tramadol, and the retention time of Tramadol was superior that 40 min, what is an unthinkable to diary analyses. The enantioseparation of the metabolites was not attempted in mobile phase. In attempted to improve the mobile conditions in order to achieve enantioseparation of Tramadol and its metabolites and also to use a more eco-friendly solvent, ethanol with 10mM aqueous ammonium acetate buffer [pH=5.3] in a proportion of 92.5:7.5 (*v:v*) and with a flow rate of 0.1 mL/min, was attempted. Partial enantioseparation of *N*-DT was achieved, but Tramadol and *O*-DT did not present any degree of enantioseparation (Figure 9). The enantioseparation of *N*-DT had a poor resolution (Rs=0.111) and a weak separation factor (α = 1.04). The results presented in Table 12 and 13, led to an abandonment of this type of chiral columns in the trial and error.

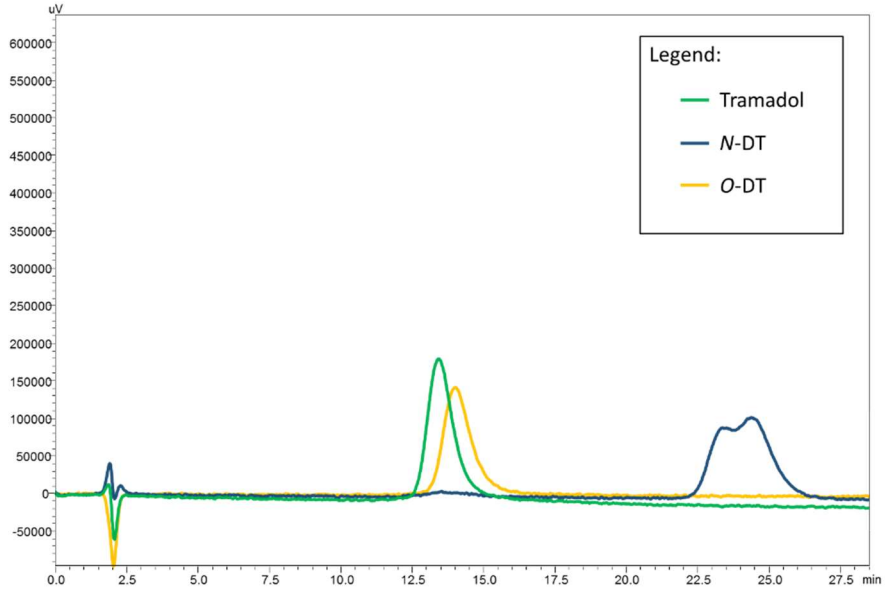

Figure 9: Chromatogram using EtOH/10 mM aqueous ammonium acetate buffer [pH=5.3] 92.5:7.5 (*v:v*) and with a flow rate of 0.1 mL/min as mobile phase

3.1.2. Polysaccharide derivates CSP

Polysaccharide derivates CSP have a broad application in enantioseparation of CP and can be use in normal, reversed and polar elution mode, what increase the rate of application. The particle size (3 to 2.5 μm) allows greater speed and power of resolution of the column (92, 94). Chytil *et all*. (59) reported the enantioseparation of Tramadol and *O*-DT, using Lux Cellulose-2 CSP (Figure 10) using Hex/IPA/DEA 90:10:0.1 (*v:v:v*) as mobile phase with a flow rate of 1.3 mL/min. The first trial in this type of CSP was based in this report (59). The results are presented in Table 14, referring to Tramadol and the metabolites.

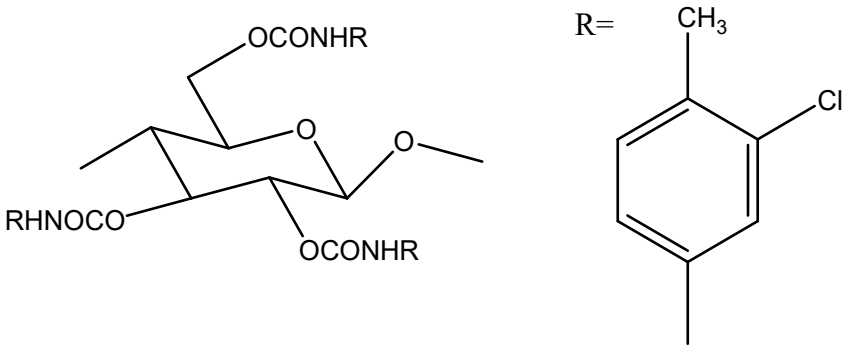

Figure 10: Chemical Structure of Lux Cellulose-2

The enantioseparation of Tramadol in Celullose-2 was not achieved with baseline resolution (Rs=1.196 and α=1.120). Tramadol with different proportion of hexane and

the organic modifier (ETOH or IPA) as mobile phase have a partial enantioseparation, with a poor resolution (Rs < 1.5).

The enantioseparation of *N*-DT and *O*-DT, in normal phase elution mode presented, good results of α and Rs. The chromatograms of the best results in normal elution mode tested in this column are presented in Figure 11. However the enantioseparation of Tramadol did not presented the desired result. Considering that, the enantioseparation of the three compounds, was not achieved in CSP in normal elution mode, the next trial was carried out with reversed elution mode.

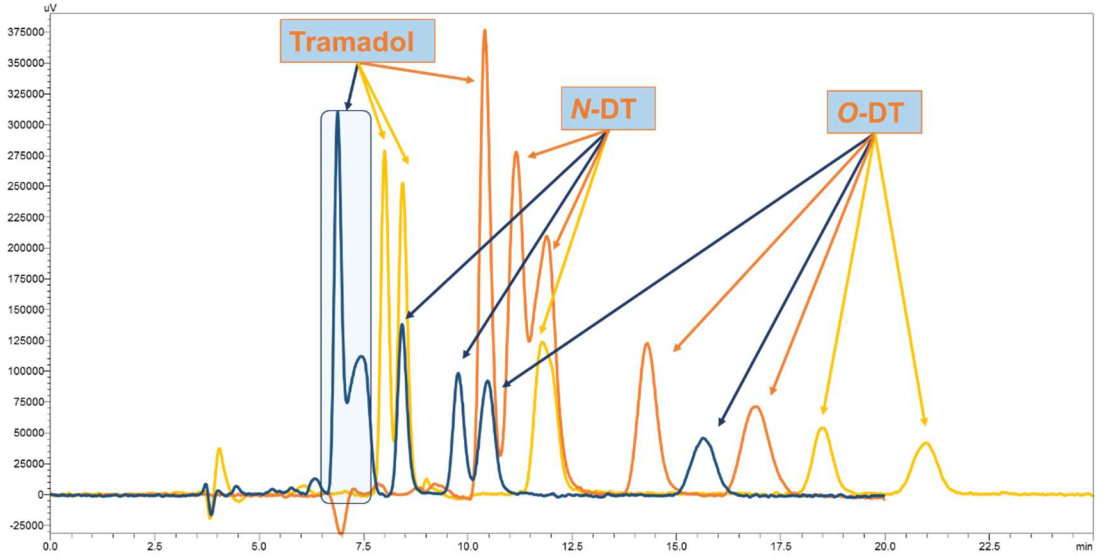

Figure 11: Chromatogram obtain using Hex/IPA/DEA 90/10/0.1 (v/v/v) (**blue line**), Hex/IPA/DEA 90/10/0.05 (v/v/v) (**oranje line**) Hex/EtOH/DEA 96/4/0.1 (v/v/v) (**yellow line**)

Table 14: Results of enantiomeric separation of Tramadol in normal elution mode on Cellulose-2 CSP

Mobile Phase	Proportion (v:v)	Tramadol						*N*-DT						*O*-DT					
		$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs
Hex/IPA/DEA	90:10:0.1**	6.761	7.39	0.725	0.886	1.222	1.003	8.337	10.263	1.131	1.623	1.435	3.019	9.543	15.131	1.55	3.044	1.964	5.529
Hex/IPA/DEA	95:5:0.1**	8.253	9.059	1.118	1.325	1.185	0.368	11.329	14.818	1.907	2.802	1.469	3.257	17.069	31.065	3.416	7.038	2.060	7.117
Hex/IPA/DEA	90:10:0.05**	10.459	11.141	4.311	4.657	1.080	0.924	11.974	14.242	4.527	5.573	1.231	2.754	11.617	16.793	9.859	14.698	1.491	4.104
Hex/IPA/DEA	96:4:0.05**	7.7	8.049	1.048	1.141	1.089	0.848	10.22	11.293	1.59	1.862	1.171	1.792	13.013	13.937	2.461	2.706	1.100	1.219
Hex/IPA/DEA	96:4:0.05***#	6.021	6.6	1.132	1.337	1.181	0.864	8.446	-	1.99	-	1.0	-	11.745	13.936	3.159	3.975	1.181	2.336
Hex/IPA/DEA	94:6:0.05**	7.439	7.857	0.885	0.991	1.120	1.196	1.309	-	1.472	-	1.0	-	13.846	18.749	2.509	3.751	1.495	4.971
Hex/IPA/DEA	94:6:0.05*#	12.501	12.792	1.014	1.061	1.046	0.155	16.05	-	1.585	-	1.0	-	21.827	-	2.516	-	1.0	.
Hex/IPA/DEA	94:6: 0.1*#	12.251	12.748	0.97	1.05	1.082	0.538	16.222	-	1.608	-	1.0	-	21.843	-	2.512	-	1.0	-
Hex/EtOH/DEA	96:4: 0.1***	6.139	7.632	1.19	1.723	1.448	0.323	9.014	11.743	2.215	3.188	1.439	3.134	14.514	20.055	4.15	6.116	1.474	5.89
Hex/EtOH/DEA	96:4: 0.1**	8.001	8.44	0.978	1.086	1.110	1.119	11.783	-	1.912	-	1.0	-	18.448	20.891	3.56	4.164	1.170	2.418

($R_{T\,1}$: Retention time of enantiomer 1; $R_{T\,2}$: Retention time of enantiomer 2; 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; # oven temperature at 27°C)

Within this elution mode many combination of mobile phase were attempted. The results are shown in Table 15.

In this elution mode, the best enantioseparation and resolution were achieved to Tramadol (Tables 15), with Rs values higher than 1.5.

Figure 12 presented the chromatograms of the best results using the reversed elution mode on Cellulose-2 CSP. The increase of H_2O percentage improved the enantioseparation of Tramadol and *O*-DT, but does not improved the separation of *N*-DT enantiomers. It was tested different percentages of DEA, in order to understand how influenced the separation. The decrease of DEA percentage in mobile phase decreased the enantioseparation of the compounds. Despite the good results in the enantioseparation of the enantiomers the simultaneous separation of all the enantiomers was not achieved in this CSP, in normal and reversed mode of elution. Thus, the next step was to attempt a similar CSP was in normal and reversed elution modes.

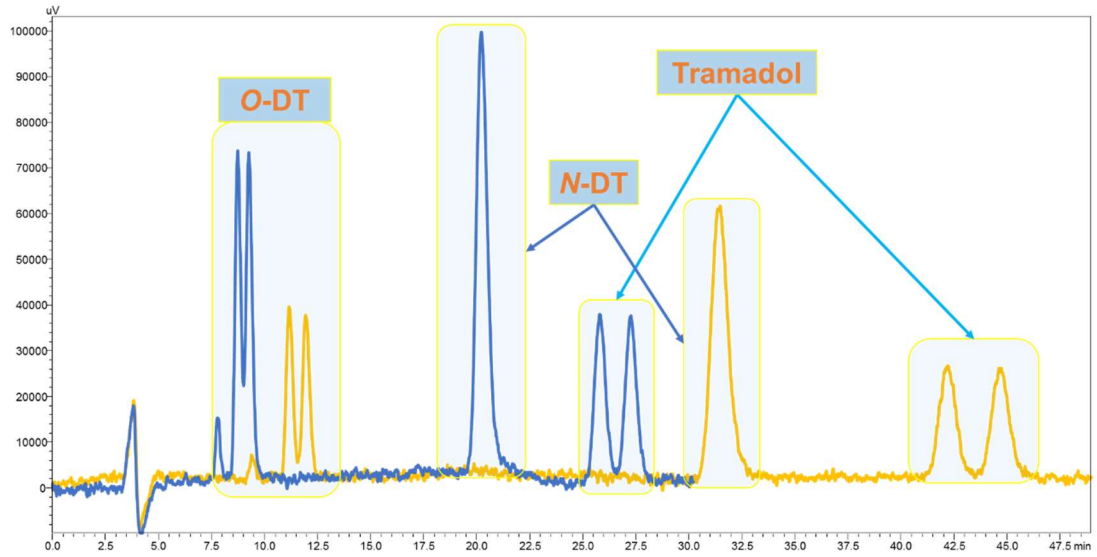

Figure 12: Chromatogram obtain using ACN(5mM ammonium formate):DEA/H_2O 35:0.1/65 (v/v/v) (**blue line**) and ACN(5mM ammonium formate):DEA/H_2O 30:0.1/70 (v/v/v) (**oranje line**)

Table 15: Results of enantiomeric separation of Tramadol in reversed elution mode on Cellulose-2 CSP

Mobile Phase	Proportion (v/v)	Tramadol						*N*-DT						*O*-DT					
		$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs
ACN (5 mM ammonium formate): DEA/ H_2O	35:0.1/65*	25.8	27.275	5.745	6.131	1.067	1.486	20.222	-	4.287	-	1.0	-	8.741	9.266	1.285	1.423	1.107	0.949
ACN (5 mM ammonium formate): DEA/ H_2O	30:0.1/70*	42.175	44.723	9.976	10.639	1.066	1.887	31.464	-	7.188	-	1.0	-	11.155	11.934	1.903	2.106	1.107	1.305
ACN (5 mM ammonium formate): DEA/ H_2O	20:0.1/80**	>60		-	-	1.0	-	>60		-	-	1.0	-	17.398	20.062	5.153	6.094	1.183	2.318
ACN (5 mM ammonium formate): DEA/ H_2O	25:0.1/75*	55.078	56.627	18.767	20.041	1.068	1.341	38.51	-	12.821	-	1.0	-	10.952	12.13	2.931	3.353	1.144	1.728
EtOH (5 mM ammonium formate): DEA/ H_2O	40:0.1/60**#	39.945	-	13.199	-	1.0	-	27.702	29.979	8.847	9.657	1.092	1.221	11.56	-	3.109	-	1.0	-
ACN:EtOH (5 mM ammonium formate):DEA/H_2O	16.36:28.63: 0.1/55*#	21.168	22.087	4.509	4.748	1.053	0.645	18.58	-	3.835	-	1.0	-	8.411	8.961	1.189	1.332	1.120	0.724
ACN:EtOH (5 mM ammonium formate):DEA/H_2O	16.36:28.63: 0.05/55*#	17.302	17.891	3.617	3.465	0.958	0.289	8.96	-	1.312	-	1.0	-	7.513	7.886	0.939	1.035	1.102	0.31
ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	14.55:25.45: 0.05/60*#	26.491	27.487	5.819	6.076	1.044	0.474	12.059	-	2.104	-	1.0	-	9.456	10.113	1.434	1.603	1.118	0.73

($R_{T\,1}$: Retention time of enantiomer 1; $R_{T\,2}$: Retention time of enantiomer 2; 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; # oven temperature at 27°C)

Table 15: Results of enantiomeric separation of Tramadol in reversed elution mode on Cellulose-2 CSP (continuation)

Mobile Phase	Proportion (v/v)	Tramadol						*N*-DT						*O*-DT					
		$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs
ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	12.73:22.27: 0.1/65**#	41.747	43.631	13.888	14.56	1.048	1.055	34.339	-	11.246	-	1.0	-	10.432	11.426	2.72	3.075	1.131	1.267
ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	14.54:25.46: 0.1/60*#	34.004	35.495	7.82	8.207	1.049	0.916	28.996	-	6.521	-	1.0	-	10.827	11.744	1.809	2.046	1.131	1.068
ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	8:32:0.1/ 60*#	46.273	47.852	10.857	11.261	1.037	0.508	39.448	-	9.108	-	1.0	-	12.952	14.03	2.319	2.595	1.119	0.864

($R_{T\,1}$: Retention time of enantiomer 1; $R_{T\,2}$: Retention time of enantiomer 2; 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; # oven temperature at 27°C)

Cellulose-4 CSP (Figure 13) was selected to search the enantioseparation of the target compounds. The only difference between Cellulose-2 and Cellulose-4 is the position of substituents of aromatic ring of the carbamate. The initially tests in this CSP was using reversed elution mode. Table 16 presented the results of Tramadol, *N*-DT and *O*-DT, using ACN with ammonium formate and DEA in different proportions.

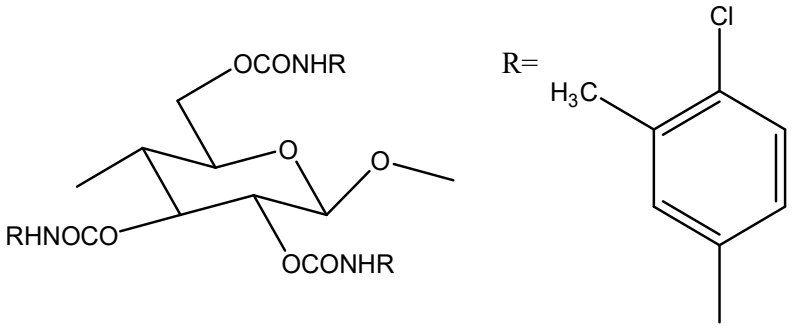

Figure 13: Chemical Structure of Lux Cellulose-4

The majority of mobile phases attempted presented enantioseparation of the target compounds individually. The best result was achieved with ACN (5mM ammonium formate) : DEA/H_2O 35:0.1/65 as mobile phase (Figure 14), all target compounds presented enantioseparation but no chemoselective with Tramadol and *N*-DT, which is a good results and conditions for analyses by mass spectrometry analysers but not satisfactory for UV and FD detectors. For this reason, MeOH and EtOH were used instead of ACN.

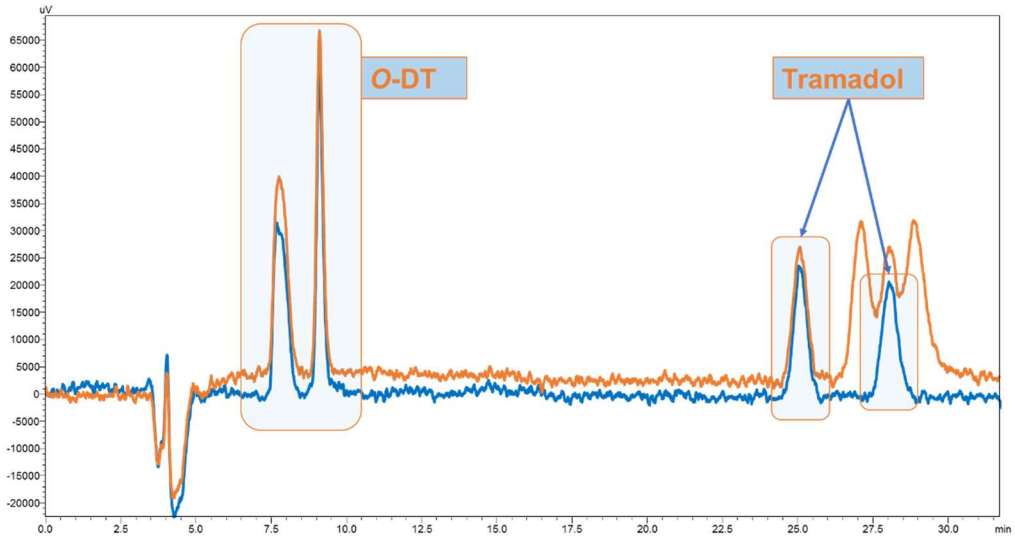

Figure 14: Chromatogram of mixture of three analytes (**orange line**) and of mixture of *O*-DT and Tramadol (**blue line**) using ACN (5 mM ammonium formate) : DEA/ H_2O 35:0.1/65 (v/v/v) as mobile phase with a flow rate of 0.5 mL/min and an oven temperature of 23 °C

Table 16: Results of enantiomeric separation of Tramadol in reversed elution mode, using ACN, on Cellulose-4 CSP

Mobile Phase	Proportion (v/v)	Tramadol						*N*-DT						*O*-DT					
		$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs
ACN (5 mM ammonium formate) : DEA/ H_2O	50:0.1/50*	10.405	11.282	1.690	1.916	1.134	1.928	9.727	10.204	1.542	1.666	1.080	0.678	5.832	6.48	0.513	0.681	1.327	1.6
ACN (5 mM ammonium formate) : DEA/ H_2O	40:0.1/60*	17.015	18.808	3.215	3.659	1.138	2.662	18.496	19.600	3.639	3.916	1.076	1.326	6.794	7.895	0.683	0.956	1.400	2.397
ACN (5 mM ammonium formate) : DEA/ H_2O	35:0.1/65*	25.03	28.078	6.315	7.206	1.141	2.794	27.094	28.838	10.688	11.440	1.070	1.256	7.862	9.091	1.245	1.657	1.331	2.312
ACN (5 mM ammonium formate) : DEA/ H_2O	45:0.1/55*	12.665	13.847	2.252	2.556	1.135	2.149	13.557	14.032	2.48	2.671	1.077	1.101	6.2	7.046	0.592	0.810	1.368	1.733
ACN (5 mM ammonium formate) : DEA/ H_2O	45:0.1/55*#	12.708	13.82	2.262	2.547	1.126	2.068	13.291	13.928	2.411	2.574	1.068	1.003	6.272	7.072	0.61	0.815	1.336	1.666
ACN (5 mM ammonium formate) : DEA/ H_2O	45:0.08/55*	11.19	12.244	1.897	2.17	1.144	1.251	10.517	-	1.746	-	1.0	-	6.186	6.804	0.601	0.762	1.268	0.915
ACN (5 mM ammonium formate) : DEA/ H_2O	30:0.08/70*	36.931	41.52	8.213	9.358	1.139	2.842	19.545	-	3.853	-	1.0	-	7.761	8.892	0.936	1.218	1.313	0.943
ACN (5 mM ammonium formate) : DEA/ H_2O	35:0.05/65*	3.875	4.5	2.69	3.285	1.221	1.025	3.861	4.384	1.03	1.305	1.267	0.908	3.693	-	1.107	-	1.0	-
ACN (5 mM ammonium formate) : DEA/ H_2O	30:0.05/70*	5.164	-	1.638	-	1.0	-	3.718	4.235	0.611	0.835	1.367	0.762	4.002	-	1.044	-	1.0	-

($R_{T\,1}$: Retention time of enantiomer 1; $R_{T\,2}$: Retention time of enantiomer 2; K_1: Retention Factor of enantiomer 1; K_2: Retention Factor of enantiomer 2; α: Separation Factor; Rs: Resolution; * flow rate of 0.5 mL/min; # oven temperature at 27°C)

Table 17 presented the results of the separations of Tramadol, *N*-DT and *O*-DT using an alcohol in the mobile phase, respectively.

According to the results presented in Table 17 and Figure 15, the best result was obtained using MeOH (5mM Ammonium formate) : DEA/ H_2O 55:0.05/ 45 (*v:v/v*) with a flow rate of 0.5 mL/min and an oven temperature of 23 °C. In this mobile phase it is possible to observe partial separation of *N*-DT, baseline separation of Tramadol, but with retention time (R_T > 50 min) and *O*-DT with good enantioseparation. The influence of amount of DEA and of ammonium formate, was also verified. The variation of concentration of ammonium formate did not influence the separation, but the decrease of DEA percentage did not help the enantioseparation of compounds, with exception of decrease of DEA percentage in a mobile phase with MeOH.

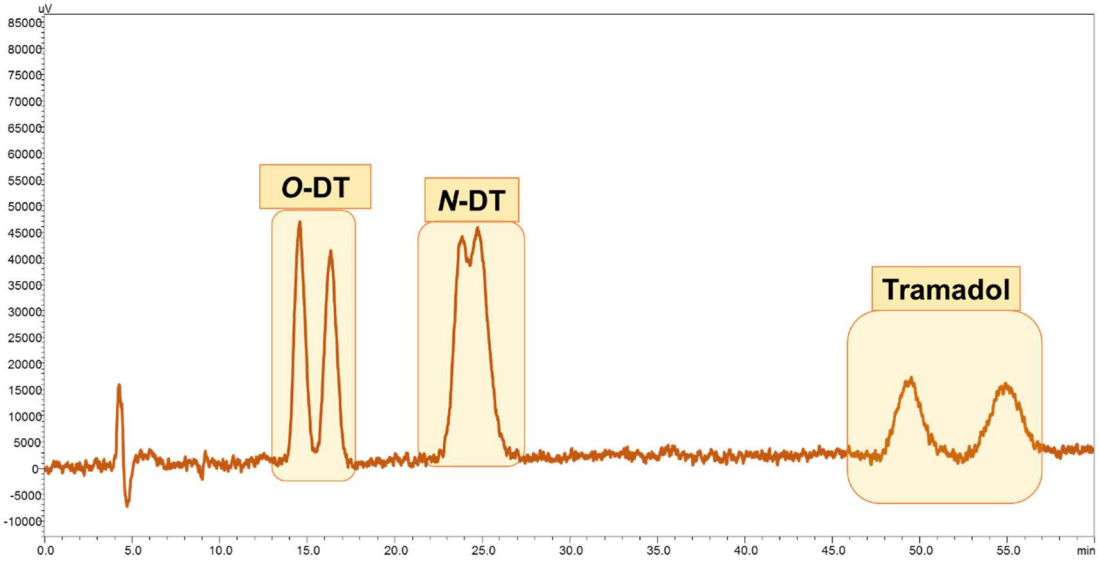

Figure 15: Chromatogram of mixture of three analytes using MeOH (5 mM ammonium formate) : DEA/ H_2O 55:0.05/ 45 (*v:v/v*) as mobile phase with a flow rate of 0.5 mL/min and an oven temperature of 23 °C

Table 17: Results of enantiomeric separation of Tramadol in reversed elution mode, using an alcohol, on Cellulose-4 CSP at a flow-rate of 0.5mL/min

Mobile Phase	Proportion (v/v)	Tramadol						*N*-DT						*O*-DT					
		$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs
EtOH (15 mM ammonium formate)/ H_2O pH=8.19 [adjusted with DEA]	50/50	8.817	9.133	1.263	1.344	1.064	0.393	4.89	-	0.250	-	1.0	-	5.323	-	0.356	-	1.0	-
EtOH (15 mM ammonium formate)/ H_2O pH=8.03 [adjusted with DEA]	40/60	13.87	14.806	2.521	2.758	1.094	0.808	5.809	-	0.472	-	1.0	-	6.031	6.361	0.529	0.613	1.159	0.139
EtOH (10 mM ammonium formate)/ H_2O pH=7.96 [adjusted with DEA]	50/50	13.967	14.918	2.559	2.801	1.095	0.943	6.204	-	0.576	-	1.0	-	6.711	7.145	0.701	0.811	1.157	0.467
EtOH (10 mM ammonium formate) : DEA/ H_2O	45:0.1/55	35.156	38.223	7.742	8.504	1.098	1.939	27.82	28.859	5.917	6.176	1.044	0.54	11.639	13.009	18.940	2.235	0.118	1.422
EtOH (10 mM ammonium formate) : DEA/ H_2O	50:0.1/50	21.264	22.813	4.214	4.594	1.090	1.328	17.979	-	3.409	-	1.0	-	8.82	9.607	1.163	1.356	1.166	0.98
EtOH (10 mM ammonium formate) : DEA/ H_2O	47:0.1/53	28.322	30.684	5.939	6.517	1.097	1.763	22.752	23.402	4.574	4.733	1.035	0.188	10.287	11.383	1.520	1.789	1.177	1.169

($R_{T\,1}$: Retention time of enantiomer 1; $R_{T\,2}$: Retention time of enantiomer 2; K_1: Retention Factor of enantiomer 1; K_2: Retention Factor of enantiomer 2; α: Separation Factor; Rs: Resolution)

Table 17: Results of enantiomeric separation of Tramadol in reversed elution mode, using an alcohol, on Cellulose-4 CSP (contituation)

Mobile Phase	Proportion (v/v)	Tramadol						*N*-DT						*O*-DT					
		$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs
EtOH (5 mM ammonium formate)/ H_2O pH=7,96 [adjusted with DEA]	50/50	13.953	14.881	2.554	2.790	1.092	0.869	3.942	5.981	2.249	3.928	1.747	3.238	6.644	7.073	0.685	0.794	1.159	0.349
MeOH (5 mM ammonium formate)/ H_2O	45/55	>60		-	-	-	-	>60		-	-	-	-	28.199	32.814	5.75	6.877	1.196	1.777
MeOH (5 mM ammonium formate) : DEA/ H_2O	50:0.1/50	>60		-	-	-	-	>60		-	-	-	-	18.503	21.335	3.437	4.117	1.198	1.729
MeOH (5 mM ammonium formate) : DEA/ H_2O	60:0.05/ 40	30.063	32.999	6.159	6.859	1.114	1.929	16.291	-	2.88	-	1.0	-	10.698	11.816	1.548	1.814	1.172	1.192
MeOH (5 mM ammonium formate) : DEA/ H_2O	55:0.05/ 45	49.505	54.886	10.671	11.94	1.119	2.181	23.836	24.713	4.619	4.826	1.045	0.236	14.562	16.332	2.433	2.850	1.171	1.433
MeOH (5 mM ammonium formate) : DEA/ H_2O	45:0.05/ 55	49.505	54.886	10.671	11.990	1.119	2.181	23.836	24.713	4.619	4.826	1.045	0.236	14.562	16.332	2.433	2.850	1.172	1.433
MeOH (5 mM ammonium formate) : DEA/ H_2O	45:0.02/ 55	77.683	86.688	17.7	19.87	1.122	2.179	22.736	-	4.433	-	1.0	-	17.98	20.473	3.306	3.928	1.188	0.968

($R_{T\,1}$: Retention time of enantiomer 1; $R_{T\,2}$: Retention time of enantiomer 2; K_1: Retention Factor of enantiomer 1; K_2: Retention Factor of enantiomer 2; α: Separation Factor; Rs: Resolution)

The last tentative of enantioseparation of the target compounds in this mode of elution was to join two organic solvents, like ACN with EtOH. The results of this tentative are presented in Table 18.

The join of these two solvents does not improve the enantioseparation. The best mobile phase was constituted with ACN:EtOH(10mM ammonium formate):DEA/ H_2O in a proportion of 17.5:17.5:0.1/65 (*v:v:v/v*) with a flow rate of 0.5 mL/min and an oven temperature of 23 °C (Figure 16). Tramadol and *O*-DT presented good resolution (Rs > 1.5) but *N*-DT presented only partial enantioseparation. Due these reasons, this elution mode was abandoned and the further tests were made in normal elution mode.

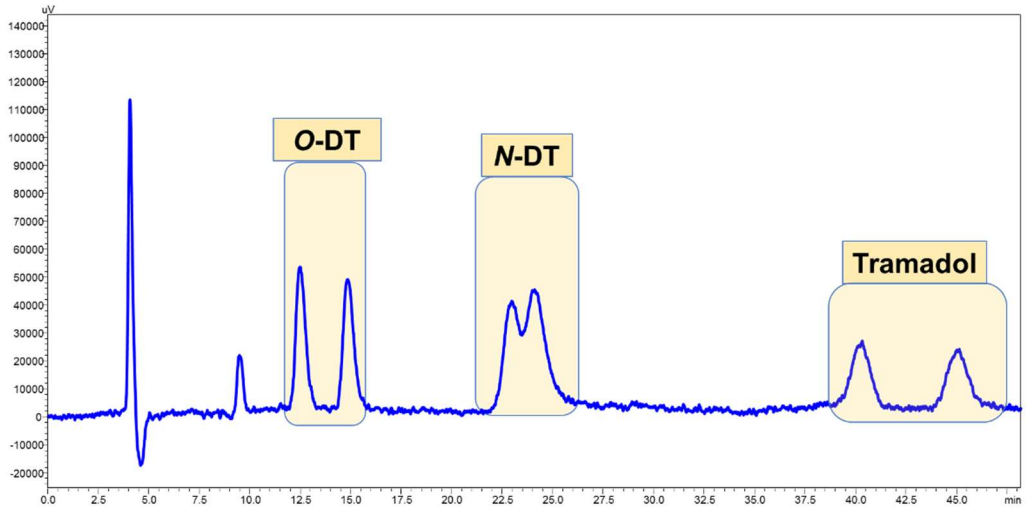

Figure 16: Chromatogram of mixture of three analytes using ACN:EtOH (10 mM ammonium Format):DEA/ H_2O 17.5:17.5:0.1/65 (*v:v:v/v*) as mobile phase with a flow rate of 0.5 mL/min and an oven temperature of 23 °C

Many different mobile phases were evaluated on Cellulose-4 CSP in normal elution mode. Table 19 shows the results of this elution mode for Tramadol, *N*-DT and *O*-DT.

Normal elution mode allowed enantioseparation with high resolutions (1.848 < Rs < 4.161) and good selectivity (1.187 < α < 1.344). EtOH and IPA were used as organic modifier and DEA as ionic suppressor. The enantio and chemoselective were achieved using Hex/EtOH/DEA as mobile phase, in a proportion of 96:4:0.1 (*v:v:v*), and in a flow of 0.7 mL/min. The chromatogram presented in Figure 17 shows the excellent chromatographic parameters. These conditions were used to validate the chromatographic method for further quantification of Tramadol and its metabolites *N*-DT and *O*-DT in influent and effluents of WWTP.

Table 18: Results of enantiomeric separation of Tramadol and its metabolites in reversed elution mode, using a mixture of organic solvents, on Cellulose-4 CSP, at a flow-rate of 0.5 mL/min

Mobile Phase	Proportion (v/v)	Tramadol						*N*-DT						*O*-DT					
		$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs
ACN:EtOH (10 mM ammonium formate):DEA/ H_2O	20:20:0.1/65	24.888	27.49	5.13	5.771	1.125	2.166	15.657	16.156	2.856	2.979	1.043	0.114	9.445	10.933	1.326	1.693	1.277	1.876
ACN:EtOH (10 mM ammonium formate):DEA/ H_2O	17.5:17.5:0.1 /65	40.319	45.116	8.912	10.091	1.132	2.69	22.962	24.096	4.645	4.923	1.060	0.36	12.481	14.837	2.068	2.647	1.280	2.619
ACN:EtOH (10 mM ammonium formate):DEA/ H_2O	21:9:0.1/60	20.287	22.441	4.033	4.567	1.132	1.966	13.977	-	2.467	-	1.0	-	8.374	9.61	1.077	1.384	1.285	1.809
ACN:EtOH (10 mM ammonium Format):DEA/ H_2O	31.5:31.5: 0.1/55	>40		-	-	-	-	30.238	31.893	6.429	6.836	1.063	0.272	14.57	17.949	2.58	3.410	1.322	3.188
ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	35:20:0.1/ 45	11.801	12.716	1.939	2.166	1.117	1.529	12.039	12.579	1.959	2.092	1.068	0.719	6.242	6.844	0.554	0.704	1.271	1.144
ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	28.6:14.5: 0.1/55	21.105	23.368	4.168	4.722	1.133	2.417	21.252	22.548	4.244	4.563	1.075	1.083	7.901	9.091	0.949	1.243	1.310	2.064
ACN:EtOH (5 mM ammonium formate):DEA/ H_2O	25.5:14.5: 0.1/60	31.479	35.432	6.697	7.664	1.144	2.583	30.928	33.079	6.539	7.063	1.080	1.443	9.428	10.946	1.305	1.676	1.284	2.691

($R_{T\,1}$: Retention time of enantiomer 1; $R_{T\,2}$: Retention time of enantiomer 2; K_1: Retention Factor of enantiomer 1; K_2: Retention Factor of enantiomer 2; α: Separation Factor; Rs: Resolution)

Table 19: Results of enantiomeric separation of metabolites of Tramadol in normal elution mode on Cellulose-4 CSP

Mobile Phase	Proportion (v/v)	Tramadol						*N*-DT						*O*-DT					
		$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs	$R_{T\,1}$ (min)	$R_{T\,2}$ (min)	K_1	K_2	α	Rs
Hex/IPA	90/10*	9.206	9.839	0.185	0.266	1.438	0.856	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Hex/IPA/DEA	90/10/0.1*	6.639	7.19	0.278	0.384	1.381	1.238	NT	NT	NT	NT	NT	NT	10.385	10.907	0.999	1.111	1.112	1.051
Hex/IPA/DEA	90/10/0.1*#	6.482	7.167	0.241	0.367	1.523	1.111	NT	NT	NT	NT	NT	NT	10.38	10.97	0.973	1.093	1.123	1.278
Hex/IPA/DEA	92/8/0.1*	6.923	7.984	0.675	0.931	1.379	1.122	NT	NT	NT	NT	NT	NT	12.616	18.667	2.052	2.306	1.124	1.19
Hex/IPA /DEA	94/6/0.1*	7.41	8.699	0.727	1.104	1.519	2.723	NT	NT	NT	NT	NT	NT	16.137	17.523	2.903	3.238	1.115	1.281
Hex/IPA /DEA	96/4/0.1*	7.217	8.766	0.747	1.122	1.502	2.737	NT	NT	NT	NT	NT	NT	19.384	21.539	3.694	4.215	1.141	1.277
Hex/EtOH/DEA	90/10/0.1*	6.118	6.653	0.491	0.622	1.267	1.687	7.486	8.00	0.822	0.948	1.153	1.397	8.033	9.004	0.958	1.194	1.246	2.424
Hex/EtOH/DEA	94/6/0.1*	6.944	7.672	0.658	0.833	1.266	2.068	9.137	9.948	1.181	1.373	1.163	1.828	11.354	13.042	1.712	2.115	1.235	3.226
Hex/EtOH/DEA	98/2/0.1*	9.415	11.165	1.308	1.417	1.083	4.886	14.757	16.864	2.538	3.043	1.199	3.112	ND	ND	ND	ND	ND	ND
Hex/EtOH/DEA	95/5/0.1*	7.112	7.881	0.706	0.891	1.262	1.365	9.672	10.635	1.320	1.551	1.175	1.571	12.747	15.537	2.058	2.727	1.325	3.075
Hex/EtOH/DEA	95/5/0.1**	5.083	5.605	0.696	0.870	1.250	1.258	6.878	7.58	1.295	1.529	1.181	1.495	9.02	11.017	2.010	2.676	1.331	2.923
Hex/EtOH/DEA	96/4/0.1*	7.592	8.131	0.769	0.895	1.164	1.953	10.729	11.868	1.500	1.766	1.177	1.805	15.511	18.619	2.615	3.339	1.277	3.416
Hex/EtOH/DEA	96/4/0.1**	5.398	5.95	0.742	0.920	1.240	2.159	7.536	8.369	1.432	1.700	1.187	1.848	10.771	13.413	2.476	3.328	1.344	4.161

($R_{T\,1}$: Retention time of enantiomer 1; $R_{T\,2}$: Retention time of enantiomer 2; K_1: Retention Factor of enantiomer 1; K_2: Retention Factor of enantiomer 2; α: Separation Factor; Rs: Resolution; * flow rate of 0.5 mL/min; ** flow rate of 0.7 mL/min; # oven temperature of 27 °C, NT: Not tested; ND: Not detected)

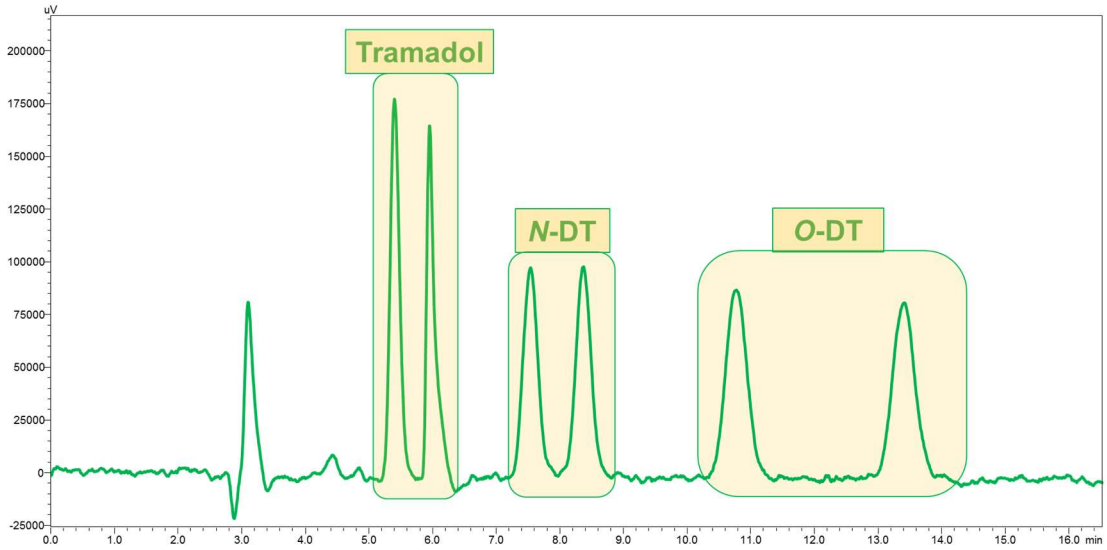

Figure 17: Chromatogram using Hex/EtOH7DEA 96/4/0.1 (v/v/v) with a flow of 0.7 mL/min as mobile phase

3.2. Solid-phase extraction optimization

The best conditions for retention of target compounds in the SPE procedure was established with ultrapure water as matrix.

The results of the different SPE procedures are after injection and comparing with standards of 1 μg/mL of the mixture of three analytes, are presented in Table 20, the numeration of the procedure agrees with procedure described in Table 9 (section 2.4.1., page 22)

Table 20: Recovery of each SPE procedure

Procedure	Cartridge	Recovery (%)						Procedure	Cartridge	Recovery (%)					
		Tramadol		*N*-DT		*O*-DT				Tramadol		*N*-DT		*O*-DT	
		E1	E2	E1	E2	E1	E2			E1	E2	E1	E2	E1	E2
1	MCX	34	36	34	35	30	51	7	HLB	34	42	27	24	20	27
2	MCX	44	48	23	26	23	52	8	MCX	66	65	54	50	52	46
3	MCX	20	20	19	19	25	12	9	MCX	79	80	82	79	84	69
4	MCX	23	22	22	22	30	20	10	MCX	76	78	79	79	74	77
5	MCX	60	60	90	98	78	60	11	MCX	70	70	76	69	69	71
6	MCX	38	38	52	53	43	29	12	MCX	105	102	77	82	106	59

(Enantiomer 1 and 2: the first and second enantiomer eluted, respectively)

Procedure 12 (Table 20) presented the best recovery of the compounds, this procedure were performed using Oasis® MCX cartridge 150 mg 6cc. This procedure was selected procedure for applying to WWTP sample (Figure 18).

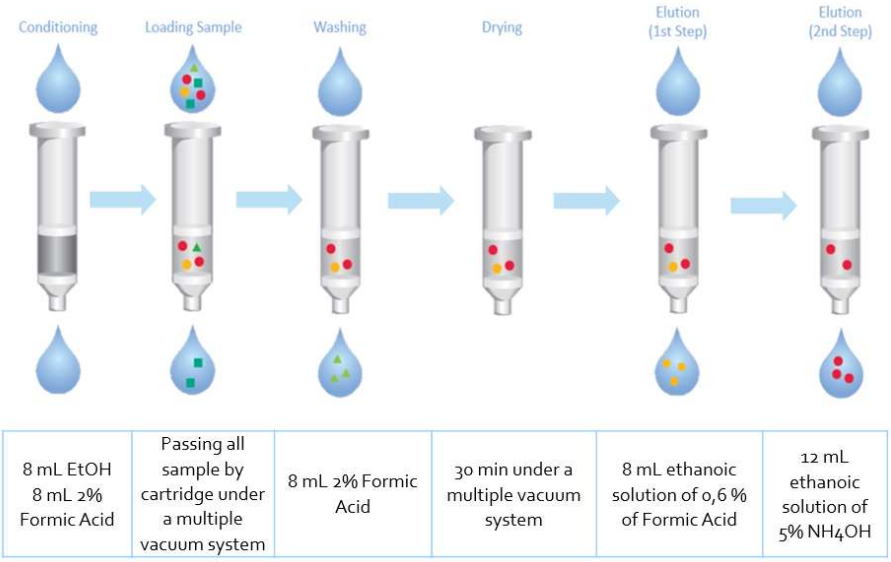

Figure 18: Optimized SPE procedure

The SPE procedures were performed in a sample without spiked and with a spiked sample, in triplicate, with a mixture of Tramadol, *N*-DT and *O*-DT with a racemic concentration of 1 μg/mL.

The recovery of each compound is calculated by the formula previously mentioned, and in order to quantify only the compounds and not the interfering the area of peak in spiked matrix is calculated by the following formula: $A_{real\ (peak\ in\ sipked\ matrix)} = A_{pea\ \ of\ spiked\ sample} - A_{peak\ of\ nonspiked\ sample}$. The results is presented in Table 21.

Table 21: Recovery in WWTP sample

Analyte	Tramadol		*N*-DT		*O*-DT	
Enantiomer	1	2	1	2	1	2
Recovery (%)	81	88	69	64	81	74

(Enantiomer 1 and 2: the first and second enantiomer eluted, respectively)

3.3. Method validation

According to the International Conference in Harmonization Q2B (95) the goal of the validation of an analytical method is to demonstrate that the method is suitable to the objective required. In order to proceed with the validation of the LC-FD method

developed for the quantification of the enantiomers of Tramadol and its metabolites the following parameters were considered: selectivity; linearity; accuracy; precision (intra-day and inter-day), detection limit, and quantification limit. Every parameter of the validation has specific definition and objective.

3.3.1. Selectivity

Selectivity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present, as impurities, degradation products, matrix interferences, etc. To assess the selectivity of the developed method and given its importance in environmental studies due to the potential presence of other substances in the matrix. The protocol followed included the analysis of a synthetic WWTP sample, previously treated by SPE procedure. The chromatogram obtained is represented in Figure 19, under the optimized chromatographic conditions referred in section 3.1.2.

To guarantee the selectivity the matrix spiked with the mixture of Tramadol, *N*-DT and *O*-DT were also analyzed, each one in a final concentration of 410 ng/mL of the racemic mixture, Figure 20 represents the chromatogram obtained for the matrix spiked. Figure 21 represents the chromatogram obtained for mix of three compounds in solvent (Hex/EtOH 8:2 (*v:v*)), in order to understand if the interferences of the synthetic influences in the separation of the compounds. The comparison between the three chromatograms presented certificates that the synthetic influent matrix does not represent any interference.

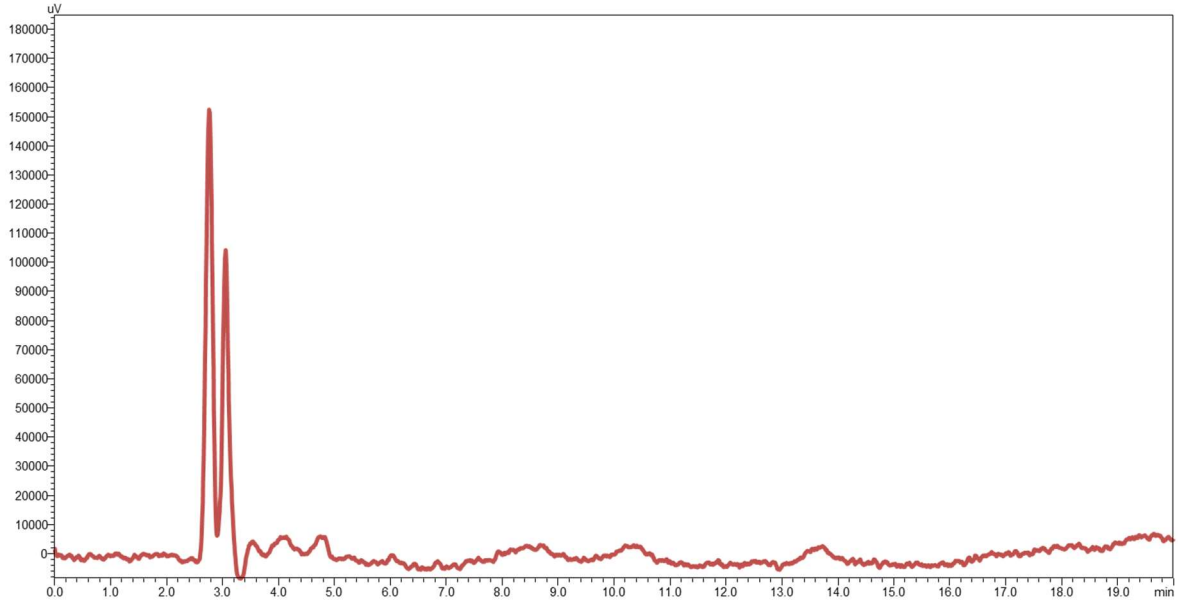

Figure 19: Chromatogram of Synthetic Influent; mobile phase: Hex/EtOH/DEA 96:4:0,1 (*v:v:v*) ; flow rate = 0.7 mL/min; column oven temperature = 23 ºC

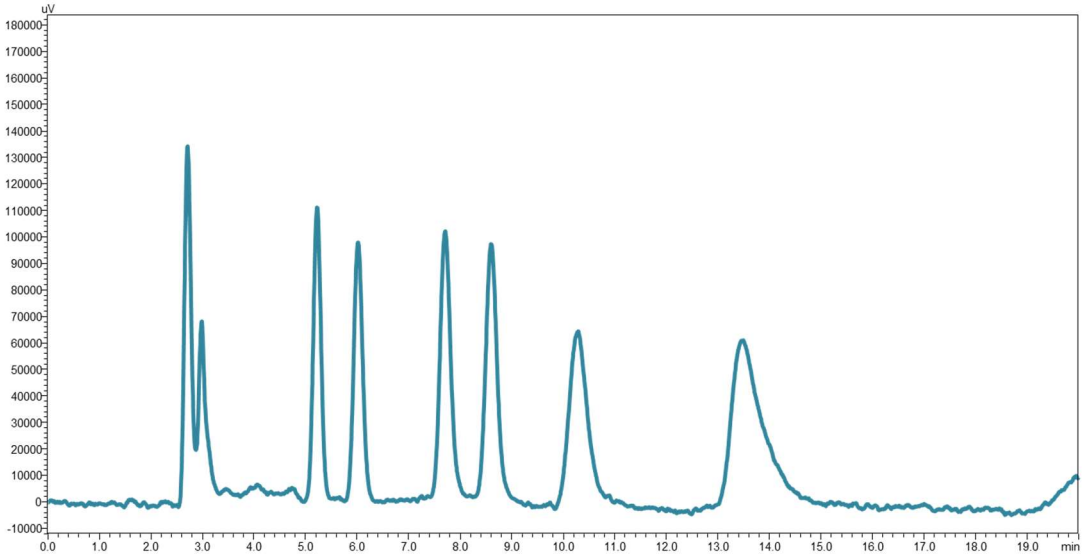

Figure 20: Chromatogram of mixture of compounds, at 410 ng/mL of racemic mixture, spiked in Synthetic Influent; mobile phase: Hex/EtOH/DEA 96:4:0.1 (*v:v:v*) ; flow rate = 0.7 mL/min; column oven temperature = 23 ºC

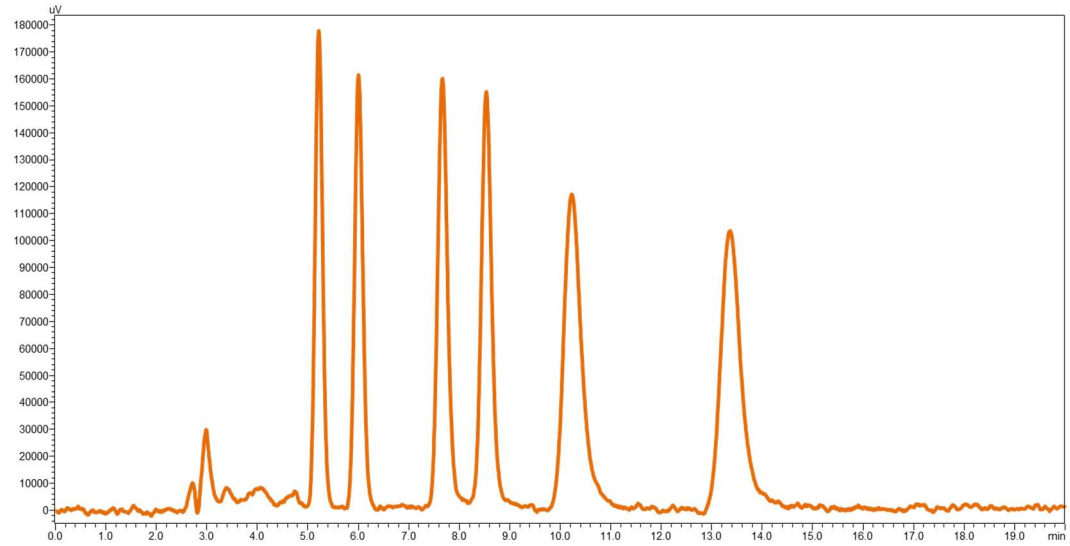

Figure 21: Chromatogram of mix of compounds in solvent in racemic concentration of 410 ng/mL; mobile phase: Hex/EtOH/DEA 96:4:0.1 (*v:v:v*) ; flow rate = 0.7 mL/min; column oven temperature = 23 ºC

3.3.2. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. To an analytical LC method to be considered able to

quantify analytes there must be a linear relation between the concentration and the absorbance of the chromatographic peaks within the linear range of the procedure. In the linearity assay of this work was used a concentration range considering five different concentration levels. According ICH (95) a minimum of five different concentration levels is required to demonstrate linearity within the linear range. The results should be statistically manipulated with appropriate methods such as the linear regression. In order to construct the calibration curves to each compound were prepared the racemic concentrations of 70 ng/mL, 140 ng/mL, 210 ng/mL, 280 ng/mL, 350 ng/mL, 420 ng/mL and 480 ng/mL spiked in synthetic wasterwater in triplicate and each one was injected and analyzed in duplicate. To the calibration curve was used 5 concentration, different for each compound, and in some cases, for each enantiomer of the same compound. To the calibration curve of enantiomers of Tramadol and *N*-DT were used a range of nominal concentration between 35 and 210 ng/mL and to the calibration curve of each enantiomer of *O*-DT were used a range of 70 to 245 ng/mL.

The calibration curves as well as the curve equations are represented in figures 22 - 27, for enantiomer 1 of T, enantiomer 2 of T, enantiomer 1 of *N*-DT, enantiomer 2 of *N*-DT, enantiomer 1 of *O*-DT and enantiomer 2 of *O*-DT respectively. The correlation coefficients were always higher than 0.99.

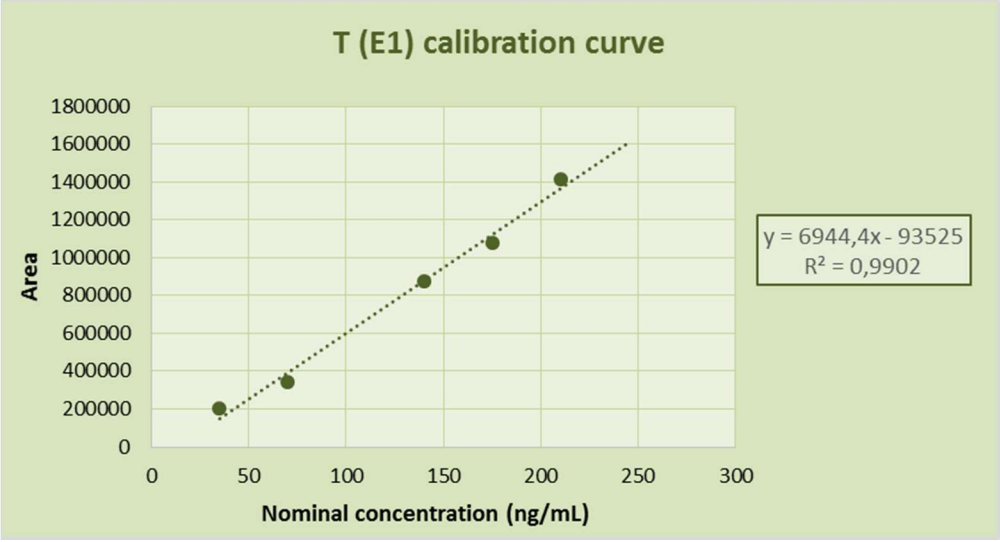

Figure 22: Calibration curve of enantiomer 1 of Tramadol

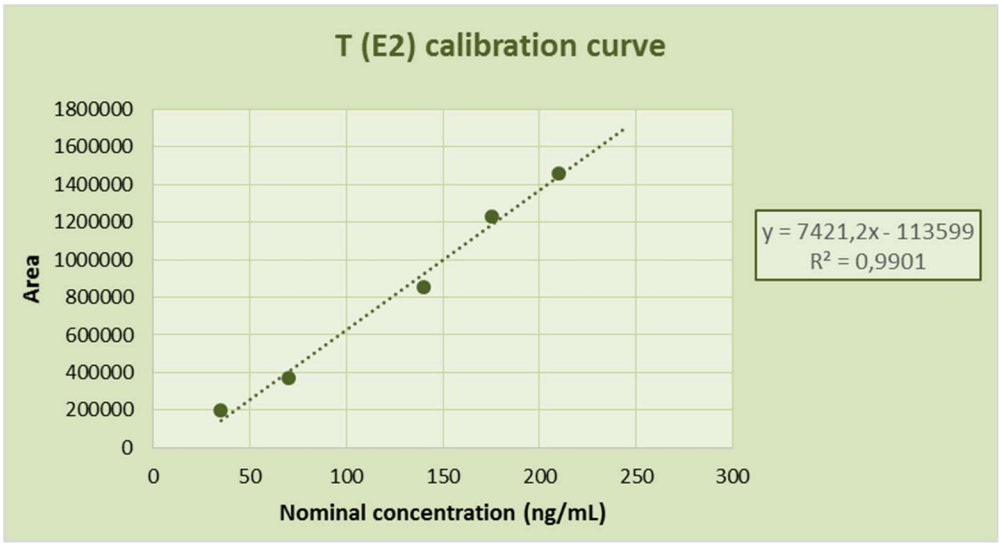

Figure 23: Calibration curve of enantiomer 2 of Tramadol

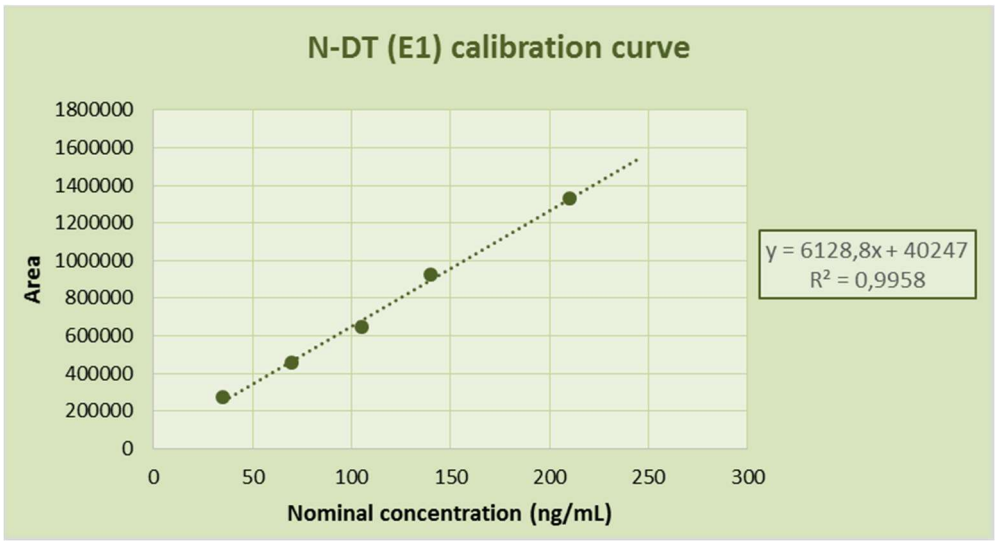

Figure 24: Calibration curve of enantiomer 1 of *N*-DT

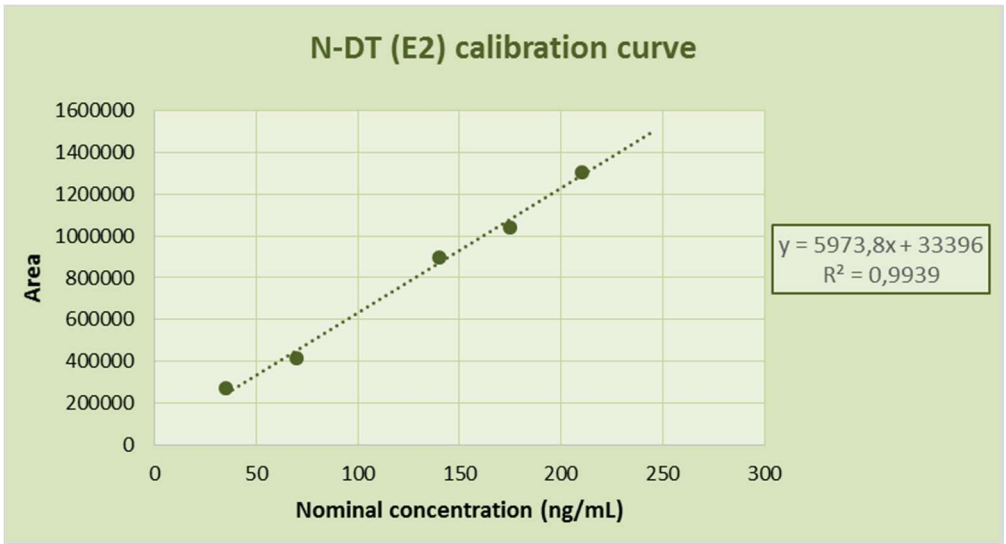

Figure 25: Calibration curve of enantiomer 2 of *N*-DT

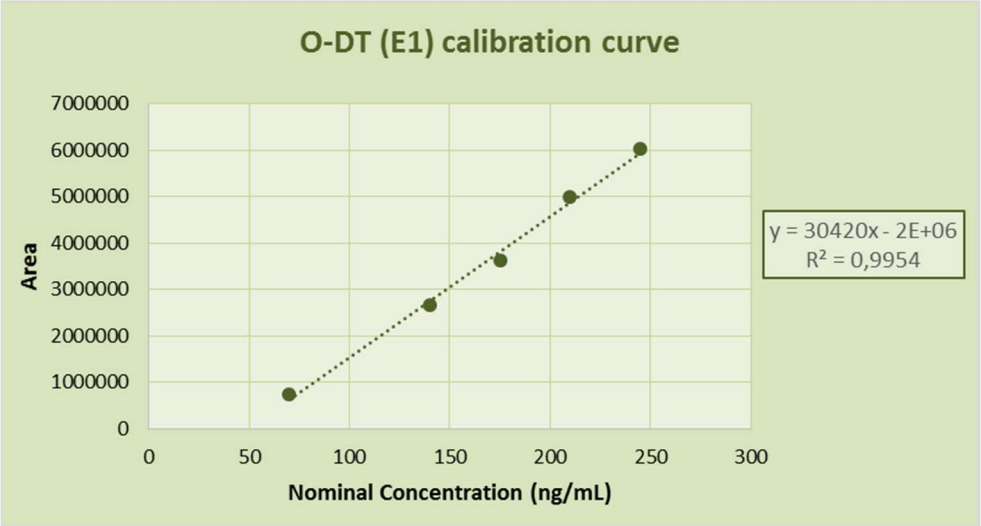

Figure 26: Calibration curve of enantiomer 1 of *O*-DT

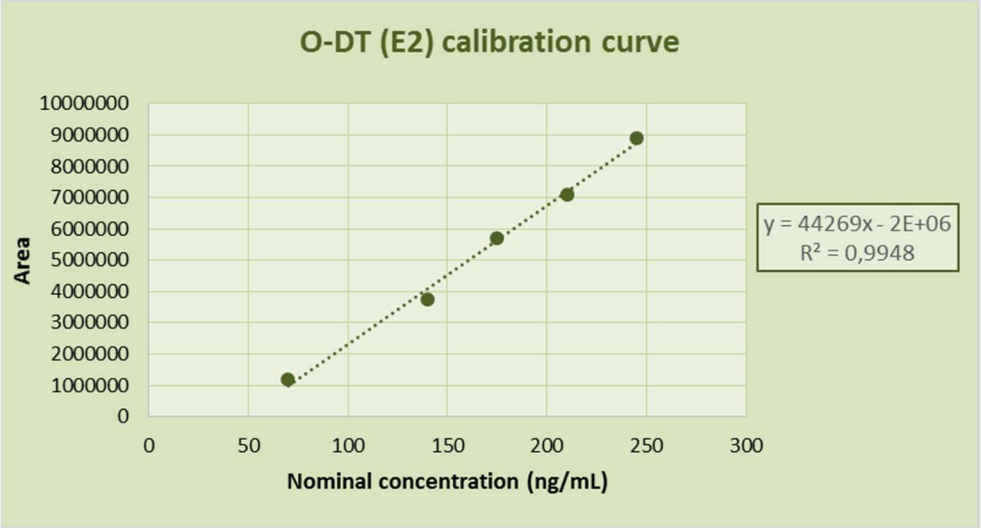

Figure 27: Calibration curve of enantiomer 2 of *O*-DT

3.3.3. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The accuracy of an analytical method expresses the concordance between a value determined by the analytical method and the real value of analyte in the sample. Accuracy must be evaluated with a minimum of three different concentration levels in triplicate (nine determinations) according Q2B guideline (95) and calculated by the relation between the concentration obtained by the peak area of the standards and the nominal concentrations. The following racemic concentrations were used: 150 ng/mL, 300 ng/mL and 410 ng/mL. Results for the accuracy assay are exhibit in Table 22. ICH (95) recommends accuracy values for pharmaceutical compounds

between 70% and 130%. Through the Table 22 it is possible see that the method is accurate, once the values are within the aforementioned range.

3.3.4. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. In the validation of this work the method precision was evaluated considering the parameters repeatability (intra-assay precision) and intermediate precision (inter-assay precision) and the values expressed in % of relative standard deviation (RSD). Following ICH (95) recommendations both precision parameters were calculated using nine determinations. Consequently, three different concentrations were prepared, each one in triplicate. Intra-day precision was studied by the analyses of the nine determinations, all performed in the same day with similar work conditions (operator, equipment, temperature and humidity). Inter-day precision was studied by the analyses of the nine determinations performed along three different days, maintaining the conditions operator and equipment and modifying the conditions temperature and humidity. Table 22 presents the values of RSD obtained for repeatability and intermediate precision. It is also a precise method.

3.3.5. Recovery

The recovery rate refers to the extraction ability of the method and relates to the fraction of analyte added to a test sample. The percentage of recovery was obtained comparing the peak area ratio of the compound after a SPE procedure to the ones prepared at the same concentrations in solvent. Three different concentrations were used and prepared each one in triplicate, as done before for accuracy and precision determination. Table 22 presents the values of percentage of recovery obtained for each enantiomer of Tramadol and its metabolites.

Table 22: Recovery, accuracy, intra- and inter-day precision of each enantiomer

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

(E:Enantiomer)

3.3.6. Detection Limit (DL) and Quantification Limit (QL)

The detection limit (DL) of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value and the quantification limit (QL) is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. In this work the

detection and quantification limits were calculated by the signal/noise ratio. According ICH Q2B (95) the determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the DL. A signal-to-noise ratio of 10:1 is suitable for estimating the QL. Table 23 summarizes the instrumental DL and QL values of each enantiomer of the different compounds in solvent. Figure 28 represents two stacked chromatograms corresponding to the concentration of instrumental DL (orange line), and instrumental QL (green line).

Table 23: Detection and Quantification limits of each enantiomer of the analytes

Analyte	Enantiomer	DL (ng/L)	QL (ng/L)
Tramadol	1	8	28
	2	8	28
***N*-DT**	1	8	28
	2	8	28
***O*-DT**	1	20	56
	2	20	56

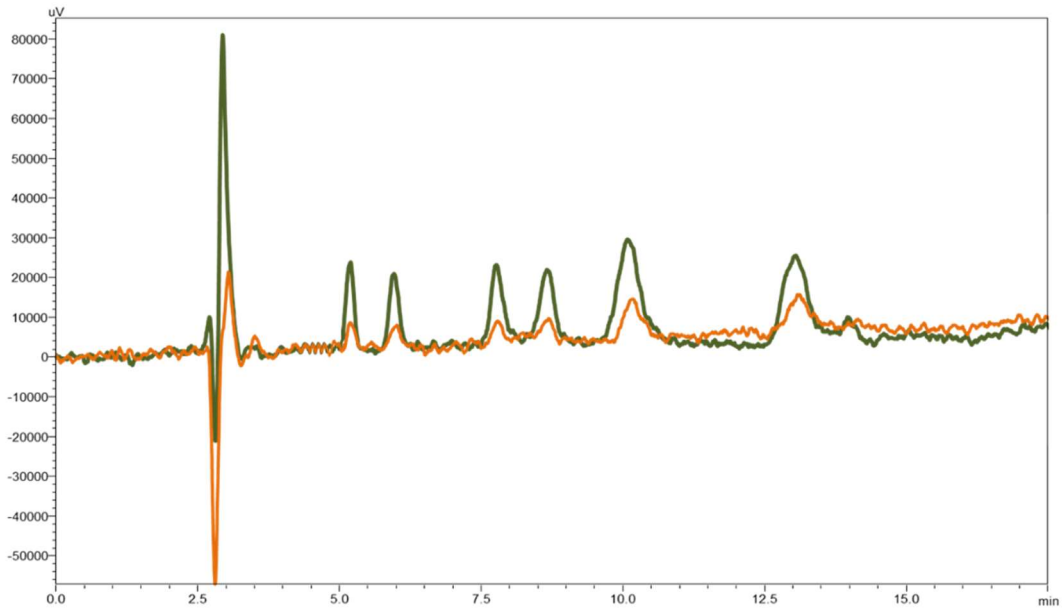

Figure 28: Chromatogram of the detection limite (**orange line**) and quantification limite (**green line**)

3.4. Application of developed LC-FD method in WWTP samples

The method developed and validated was used to quantify sample from effluent and influent of WWTP. After collected the samples, acidification and the subsequent process SPE, the samples were analyzed using the optimized conditions previously established. Table 24 presents the results obtained in influent and effluent of WWTP of Parada, North of Portugal.

Table 24: Concentration of analytes present in samples collected

Sample nº	Sample	Concentration (ng/L)					
		Tramadol		*N*-DT		*O*-DT	
		E1	E2	E1	E2	E1	E2
1	Effluent	ND	<QL	<QL	<QL	ND	ND
	Influent	ND	ND	ND	ND	ND	ND
2	Effluent	ND	ND	ND	ND	ND	ND
	Influent	ND	60.6	<QL	<QL	ND	ND
3	Effluent	235.8	118.7	<QL	43.7	60.8	57.7
	Influent	357.9	233.6	<QL	63.9	69.7	86.7
4	Effluent	325.1	314.9	<QL	62.1	71.6	95.4
	Influent	350.0	233.8	<QL	72.7	69.5	106.7

(<QL: under quantification limit; ND: not detected; E1 and E2 are the first and second enantiomers eluted, respectively)

Crossmatch with standards was performed to certify presence of analytes in the samples.

The results from Table 24 shows that the target compounds were not quantified in the effluent and in influent in first day of sample collection, expect the second enantiomer of Tramadol in the sample collected in the afternoon (60.6 ng/L).

Regarding samples collected on the day 2, all enantiomers of the compounds could be quantified, except the enantiomer 1 of *N*-DT, which were under QL. Although with some interfering providing by the matrix, it is possible see the presence of each enantiomer of each compound. The chromatograms presented in Figure 29 the samples collected in morning of day 2 and in Figure 30, samples collected in afternoon.

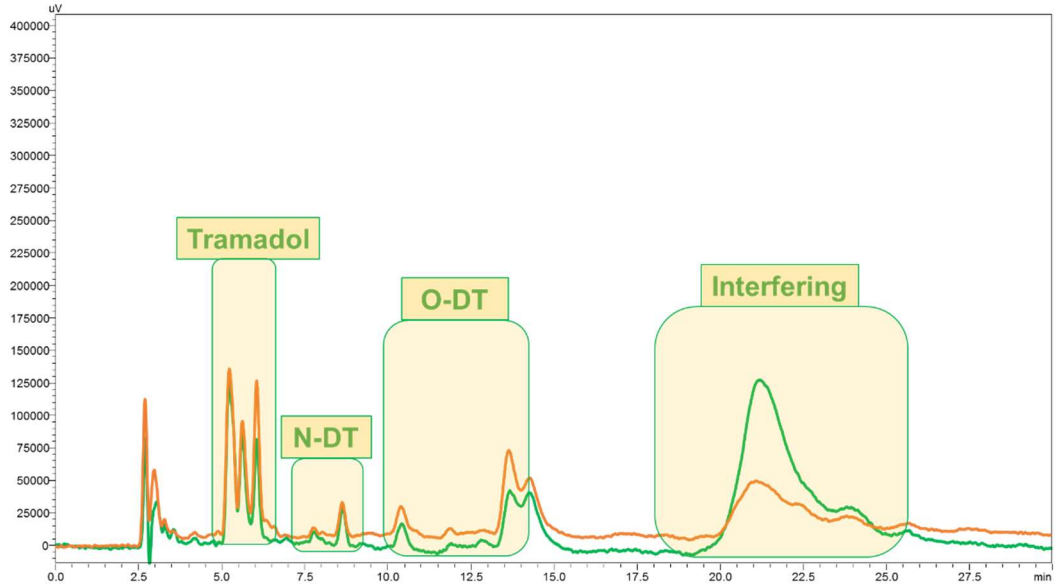

Figure 29: Chromatogram of sample 3, influent sample (**orange line**) effluent sample (**green line**)

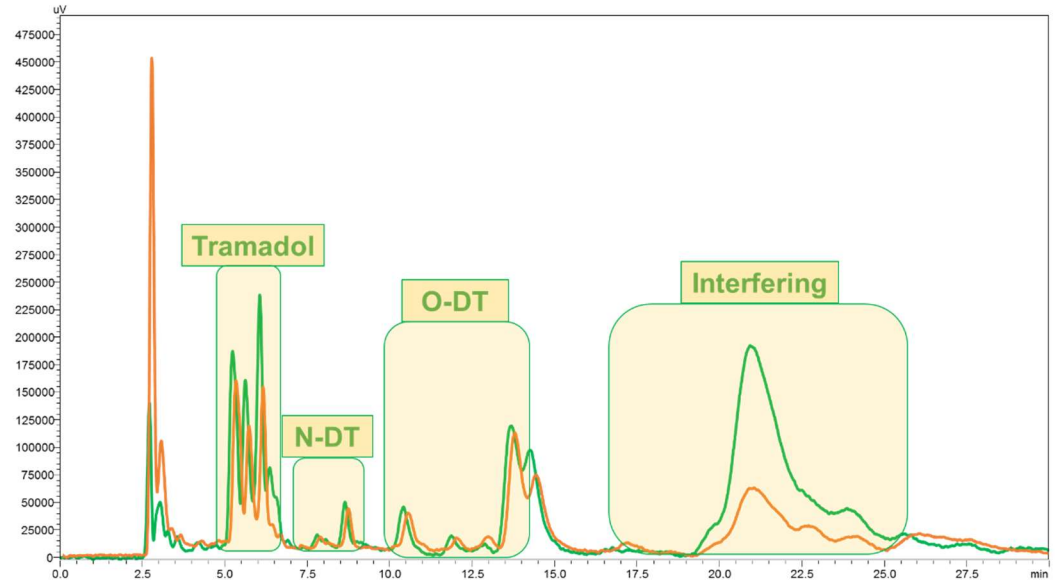

Figure 30: Chromatogram of sample 4, influent sample (**orange line**) effluent sample (**green line**)

Chromatograms of the cross match test with an overlap of sample chromatogram are presented in Figure 31, 32, 33 and 34.

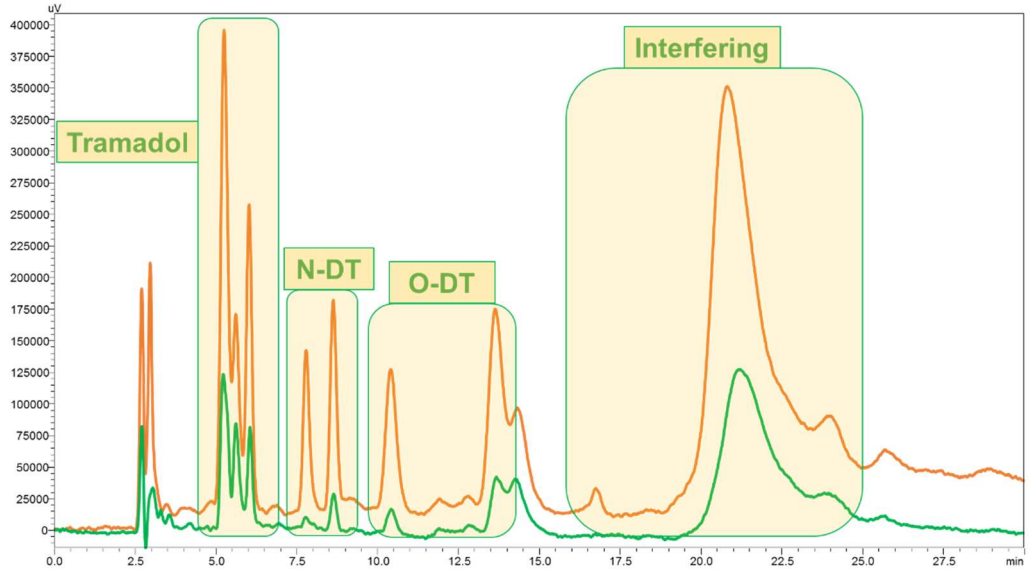

Figure 31: Chromatogram of efluent sample nº 3 (**green line**) and crossmatch test with the sample (**orange line**)

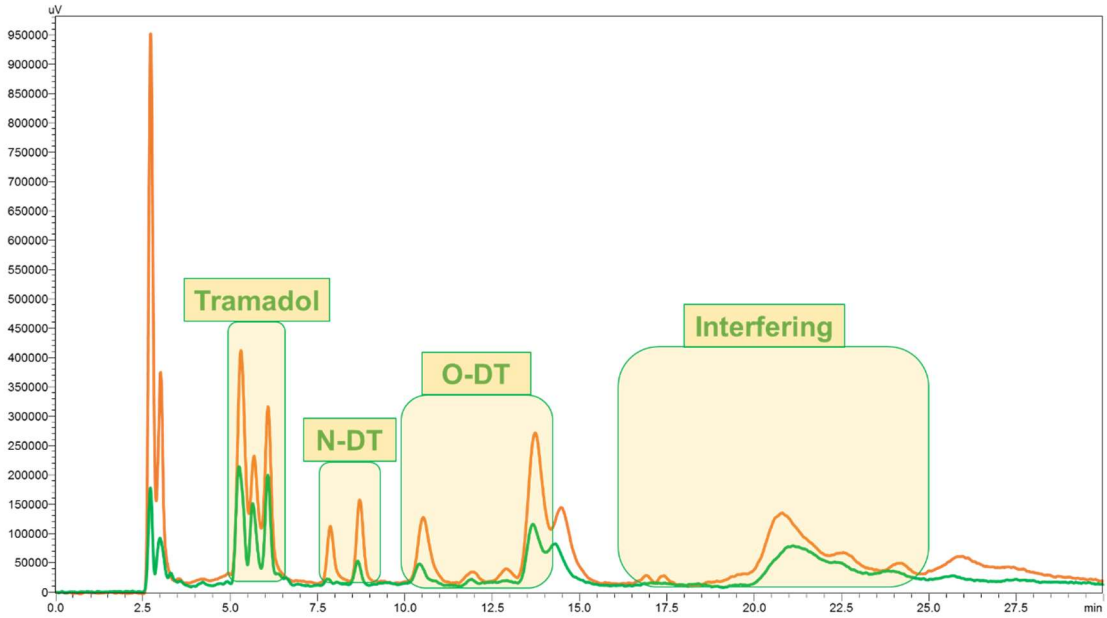

Figure 32: Chromatogram of influent sample nº 3 (**green line**) and crossmatch test with the sample (**orange line**)

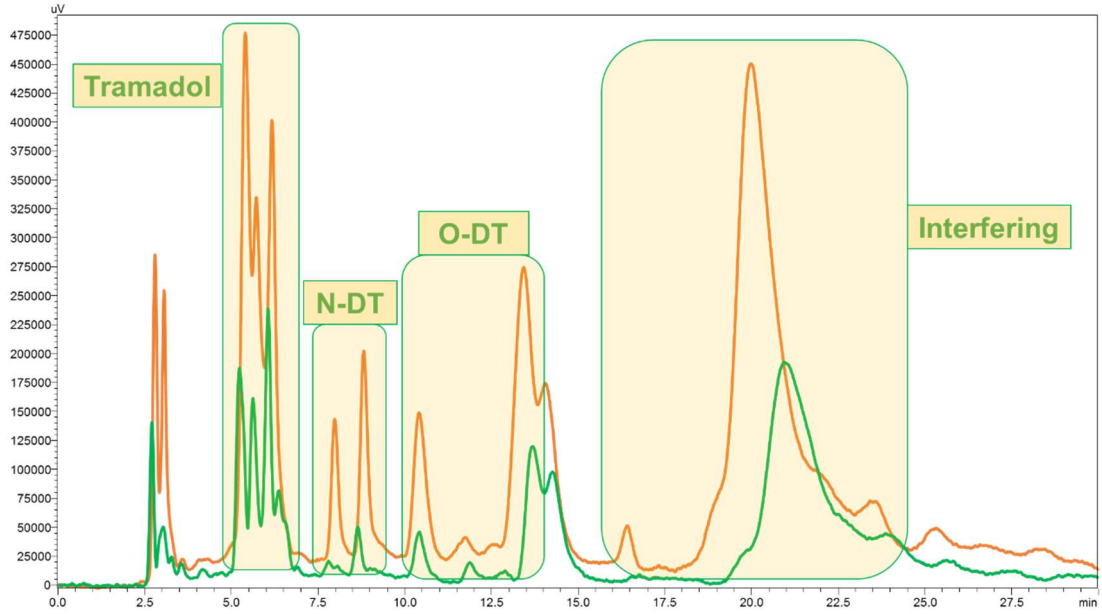

Figure 33: Chromatogram of efluent sample nº 4 (**green line**) and crossmatch test with the sample (**orange line**)

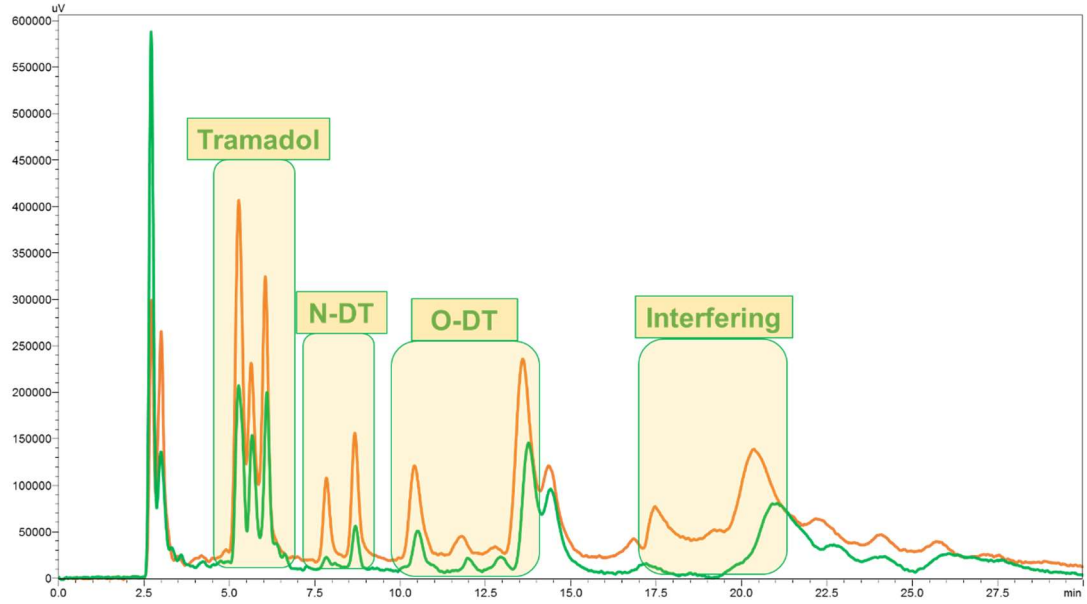

Figure 34: Chromatogram of influent sample nº4 (**green line**) and crossmatch test with the sample (**orange line**)

The presence of interfering in the sample matrix difficult the quantification of the analytes. Although this difficult, in the case of Tramadol the interfering appears between the enantiomers and in the case of *O*-DT the interfering affect the quantification of second the enantiomer 2, it was possible to predict the concentration of the enantiomers of Tramadol, *N*-DT and *O*-DT in WWTP.

Table 25 present results of EF, the fraction can only range from 0 to 1.0, if EF = 0.5 means that is present in racemic mixture.

Table 25: EF of analytes in the samples

Sample nº	Sample	EF					
		Tramadol		*N*-DT		*O*-DT	
		E1	E2	E1	E2	E1	E2
3	Effluent	0.67	0.33	-	≈1.00	0.51	0.49
	Influent	0.61	0.39	-	≈1.00	0.45	0.55
4	Effluent	0.51	0.49	-	≈1.00	0.43	0.57
	Influent	0.60	0.40	-	≈1.00	0.39	0.61

(E1: enantiomer 1; E2: enantiomer 2)

Through the values of EF it is possible see if the analyte are present in the racemic mixture. The second enantiomer eluted of *N*-DT present a value of EF next to 1.0, once the first enantiomer was under QL. Tramadol were presented in the samples in proportion next to racemic mixture, but is evident that enantiomer 1 were present in a major concentration. *O*-DT is the analyte which is mostly very close to the value 0.5, what revel that this analyte are presented in proportion next to racemic mixture. .

Table 26 shows the results of DRE, through this value is possible predict if the removal of pollutants.

Table 26: DRE values of the analytes in the samples

Sample nº	DRE (%)					
	Tramadol		*N*-DT		*O*-DT	
	E1	E2	E1	E2	E1	E2
3	65.9	50.8	-	68.4	87.2	66.6
4	92.9	134.7	-	85.4	103.0	89.4

(E1: enantiomer 1; E2: enantiomer 2)

In the first day the compounds were not found in all the samples and are not possible calculate this value, but it is possible using the samples of second day. According to these results it is possible conclude that in a majority of the times, the elimination treatment is inefficient (DRE<60%).

4. Conclusions

A LC-FD method was developed and validated for the identification and quantification the enantiomers of Tramadol and its primary metabolites, *N*-DT and *O*-DT in influent and effluent of WWTP. Search for ideal CSP and mobile phase conditions for enantioseparation of all enantiomers was allowed the further validation and quantification of all target compounds. The optimized conditions were achieved with Lux Cellulose-4 as CSP and Hex/EtOH/DEA 96/4/0.1 (v/v/v) as mobile phase in an isocratic mode and a flow of 0.7 mL/min. The separation of all enantiomers (six compounds) has been obtained with a run time shorter than 15 minutes, with good resolution, Rs>1.5, and enantioselectivity, α>1.1.

SPE procedure for pre-concentration and cleanup using MCX cartridge allowed quantification in 250 mL of sample volume.

The validated method demonstrated selectivity, with good linearity (r^2>0.99) and demonstrated precision (intra-day: 0.3<RSD<13.8; inter-day: 5.4<RSD<16.7), in the racemic range of 56 ng/L – 384 ng/L. The values of accuracy and precision for the quality control of the highest concentration of *O*-DT it should be adjusted. The QL achieved were 28 ng/L for each enantiomer of Tramadol and of *N*-DT and 56 ng/L for each enantiomer of *O*-DT.

A several sample from effluent and influent WWTP were analysed and the compounds were detected in a concentration range below to QL to 325.1 ng/L and 357.9 ng/L, respectively. Enantiomers of Tramadol and *O*-DT revels an EF next to racemic mixture (EF≈0.5), due the enantiomer 1 of *N*-DT presented in a concentration under of QL, the EF is approximately to 1.0.

Samples 3 present a range of DRE between 50.8% and 87.2%, the sample 4 present a range of DRE between 85.4% and 134.7%.

As future perspectives it would be interesting to investigate the elution order of the enantiomers in order to confirm which enantiomer is at higher concentration. The enantio ecotoxicological effects of Tramadol and its metabolites would be also important for further environmental risk evaluation studies. Increasing of sample analyses with different WWTP form different regions are also important to support the data achieved in this work.

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