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Enantioselective studies of biodegradation and ecotoxicity of tramadol and its metabolites

Cristiana da Costa Santos

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ENANTIOSELECTIVE STUDIES OF BIODEGRADATION AND ECOTOXICITY OF TRAMADOL AND ITS METABOLITES

Dissertation for Master degree in Environmental Contamination and Toxicology submitted to Instituto de Ciências Biomédicas de Abel Salazar of University of Porto

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POSTER COMMUNICATIONS

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The abstract and poster are in Appendix **B**.

ABSTRACT

Chiral pharmaceuticals are part of a major group of emerging environmental pollutants due to their increasing consumption levels and continuous release to the environment. The continuous disposal of these substances to the environment through wastewater treatment plants (WWTP) and the inappropriate discharge may present potential short and/or long-term risks for humans and other exposed organisms. It is well established that biodegradation and toxicity of chiral pharmaceuticals can be enantioselective. Thus, evaluation of biodegradation and toxicity levels of both enantiomers is crucial for a better understanding of the fate of chiral pharmaceuticals in the environment and the risk associated to this class of pollutants.

Tramadol (T), a chiral pharmaceutical, is a centrally acting synthetic analgesic, structurally related to codeine and morphine, indicated for moderate to severe pain. It is commercialized as a racemate and it is extensively metabolized in the liver through *O*- and *N*- demethylations and by conjugation reactions. Both T enantiomers contribute to the analgesic effect, even though via different mechanisms. Its main metabolite is *O*-desmethyltramadol (*O*-DT), which has higher pharmacologically activity than T itself.

In order to obtain the pure enantiomers of T for biodegradation and ecotoxicity studies, a semi-preparative liquid chromatographic method was developed and optimized for the enantioseparation of T using a homemade amylose 3,5-dimethylphenylcarbamate column coated on to APS-Nucleosil (500 A, 7µm, 20%, w/w) and packed into a stainless-steel 20 x 0.7 cm i.d. chiral column. The optimized chromatographic conditions were the following: mobile phase of hexane/isopropanol/diethylamine (98/2/0.1, v/v/v), injection volume of 10 µL, flow rate of 1.5 mL/min and column oven temperature of 23ºC. After 72 replicates injected and collected, the obtained amounts were 29.3 and 18.1 mg of the first and second T enantiomers eluted, respectively.

This work also presents a validated enantioselective liquid chromatographic method developed to follow the biodegradation of T and its metabolites by activated sludge inoculum. The optimized conditions were achieved using a Lux Cellulose-4 column (150 x 4.6 mm i.d.; 3 µm) and guard column (4.4 x 3.0 mm i.d.) with a mobile phase of ACN:EtOH:DEA (10mM ammonium formate) (50/50/0.1)/ H_2O (38/62, v/v) under isocratic elution. The method was validated for the enantiomers of T and its main metabolite *O*-DT with high selectivity, linearity ($r^2 > 0.99$), intra-day and inter-day precisions (RSD < 5.1%)

and accuracy (93 to 101%). Detection and quantification limits were 40 and 125 ng/mL for T enantiomers and 15 and 40 ng/mL for *O*-DT enantiomers, respectively. The method was applied to a 21-days biodegradation study concerning racemate T and both enantiomers. Racemate T suffered a biodegradation up to 29%, increasing this value in about 10% when added an additional carbon source (sodium acetate). Both T enantiomers biodegradation levels were similar, with and without acetate, ranging between 29 and 33% at the end of the study. This may be an indicator that this process is not enantioselective for T.

Ecotoxicological assessment was also performed in two test ecological relevant organisms (*Daphnia magna* and *Tetrahymena termophila*) with racemate T, *O*-DT and *N*-DT and enantiomers of T and *O*-DT. Racemate *O*-DT and its enantiomers did not showed toxicity for both organisms, while *N*-DT was more toxic to the protozoan. (*R*,*R*)-T presented higher toxicity levels for both *D. magna* and *T. thermophila* indicating enantioselectivity.

Keywords: chiral pharmaceuticals | tramadol | enantioseparation | *O*-desmethyltramadol | *N*-desmethyltramadol | biodegradation | activated sludge | ecotoxicological assays | *Daphnia magna* | *Tetrahymena thermophila*

RESUMO

Os fármacos quirais fazem parte do grupo dos poluentes ambientais emergentes, devido às suas elevadas taxas de consumo e à sua constante libertação para o meio ambiente. O constante descarte destas substâncias para o ambiente pelas estações de tratamento de águas residuais (ETARs), bem como a descarga inapropriada podem levar a potenciais riscos a curto ou longo prazo para humanos e outros organismos expostos. Está bem estabelecido que a biodegradação e a toxicidade dos fármacos quirais podem ser enantioseletivas. Deste modo, a biodegradação e a avaliação da toxicidade de ambos os enantiómeros são cruciais para entender o destino destes fármacos no ambiente e o risco associado a esta classe de poluentes.

O tramadol (T) é um fármaco quiral estruturalmente relacionado com a codeína e a morfina, que atua ao nível do sistema nervoso central no tratamento de dor moderada a severa. É comercializado como racemato e é extensivamente metabolizado no fígado através de *O*- e *N*-desmetilações e de reações de conjugação. Ambos os enantiómeros contribuem para o seu efeito analgésico, apesar de por mecanismos diferentes. O seu principal metabolito é o *O*-desmetil-tramadol (*O*-DT), que apresenta uma maior atividade farmacológica que o próprio T.

De forma a obter os enantiómeros puros do T para estudos de biodegradação e de ecotoxicidade, foi desenvolvido e otimizado um método semi-preparativo de cromatografia líquida para a enantiosepararação dos enantiómeros do T, utilizando uma coluna quiral de 3,5-dimetilfenol-carbamato de amilose caseira revestida em APS-Nucleosil (500 A, 7µm, 20%) e empacotada em aço inoxidável (20 x 0.7 cm i.d.). As condições cromatográficas otimizadas consistem numa fase móvel hexano/isopropanol/dietilamina (98/2/0.1, v/v/v), um volume de injeção de 10 µL, um fluxo de 1.5 mL/min e uma temperatura do forno de coluna de 23ºC. Após 72 injecções, obtiveram-se 29.3 e 18.1 mg do primeiro e do segundo enantiómeros eluídos, respetivamente.

Adicionalmente, foi também validado um método analítico de cromatografia líquida desenvolvido para monitorizar a biodegradação do T e dos seus metabolitos em lamas ativadas. A otimização do método foi conseguida numa coluna quiral Lux Cellulose-4 (150 x 4.6 mm i.d; 3 µm) e respetiva pré-coluna (4.4 x 3.0 mm i.d.), numa fase móvel de acetonitrilo:etanol:dietilamina (formato de amónio 10mM) (50/50/0.1) / H_2O (38/62, v/v)

em eluição isocrática. O método foi validado para os enantiómeros do T e do *O*-DT com boa seletividade, linearidade ($R^2 > 0.99$), precisão (RSD < 5.1%) e exatidão (93 a 101%). Os limites de deteção e quantificação obtidos foram 40 e 125 ng/mL para T, 15 e 40 ng/mL para *O*-DT, respetivamente. O método foi posteriormente aplicado a estudos de biodegradação executados durante 21 dias, para o T e para os seus enantiómeros. O T racemato sofreu uma biodegradação até 29%, sendo que, na presença de uma fonte adicional de energia (acetato de sódio), este valor aumentou cerca de 10%. No caso dos enantiómeros, os seus níveis de biodegradação foram semelhantes com e sem acetato, variando entre 29 e 33%. Isto pode ser indicador de que este processo não é enantioseletivo para o T.

Os efeitos ecotoxicológicos do T, *O*-DT e *N*-DT em mistura racémica, bem como dos enantiómeros do T e do *O*-DT foram avaliados recorrendo a dois organismos-teste ecologicamente relevantes (*Daphnia magna* e *Tetrahymena termophila*). O *O*-DT e os seus enantiómeros não apresentaram toxicidade para ambos os organismos, contrariamente ao *N*-DT, que apresentou uma maior toxicidade para o protozoário. (*R*,*R*)-T apresentou níveis tóxicos mais elevados para ambos os organismos *D. magna* e *T. termophila*, o que demonstra existência de enantioseletividade.

Palavras-chave: fármacos quirais | tramadol | separação enantioseletiva | *O*-desmetil-tramadol | *N*-desmetiltramadol | biodegradação | lamas ativadas | ensaios ecotoxicológicos | *Daphnia magna* | *Tetrahymena thermophila*

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ABBREVIATIONS AND SYMBOLS

AS	Activated sludge
CSP	Chiral stationary phase
CYP450	Cytochrome P450
DEA	Diethylamine
EC_{50}	Half maximal effective concentration
LC-FD	Liquid chromatography with fluorescence detection
ICH	International Conference on Harmonization
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MM	Mineral salts medium
***N*-DT**	*N*-desmethyltramadol
NSAID	Non-steroidal anti-inflammatory drug
***O*-DT**	*O*-desmethyltramadol
OECD	Organization for Economic Cooperation and Development
QC	Quality control
R_T	Retention time
T	Tramadol
TEA	Triethylamine
UPLC-MS/MS	Ultra Performance Liquid Chromatography with Tandem Mass Spectrometry
US EPA	United States Environmental Protection Agency
WWTP	Wastewater treatment plant
λ	Wavelength

Chapter 1

INTRODUCTION

1.1 Pharmaceuticals in the environment

Pharmaceuticals are part of a major group of emerging environmental pollutants, alongside with illicit drugs [1] and pesticides [2,3]. Over the past decades, these compounds became of great concern to the scientific community due to their increasing consumption levels and continuous release to the environment [2,4,5]. Therefore, as a result of their biological activity, they can lead to physiologic alterations and toxic effects in non-target organisms even when present at small amounts [6,7].

Pharmaceuticals reach the environment through several sources, being patient excretion following therapy considered the primary one (**Figure 1**) [5,8,9]. Improper disposal of unused or expired medication and wastewater discharging of contaminated waste from manufacturing facilities are also two of the main pathways of environmental contamination [1,5,8-10]. Other important sources such as aquaculture [5,11], agriculture [1,5,8,12], health institutions and industries [1,9,12-14], landfills and buried waste [9-11] should not be neglected (**Figure 1**). These compounds may appear in the environment unaltered, i.e. as parent compounds, and/or as metabolites, conjugates and wastewater treatment transformation products [1,8,10], which constitutes an additional issue.

Wastewater treatment plants (WWTP) cannot completely eliminate pharmaceuticals from influent wastewaters arriving from the sewage network, since WWTP are not designed to handle highly polar compounds like most pharmaceuticals [2,5,11,13]. Therefore, these residues are discharged into surface waters and may end up in drinking water treatment facilities [5]. Even though pharmaceutical levels usually detected in surface and wastewaters worldwide are in the concentration range of ng/L to μg/L [1,2,5,8,12], long-term exposures at these concentrations for non-target organisms

including humans may lead to adverse effects [8]. Additionally, synergistic effects between different compounds as well as with other common pollutants may occur and lead to toxicity [8,12,13]. Pharmaceuticals belong to the larger group of “pseudo-persistent” drugs [2,8], its environmental levels are constantly renewed due to ongoing worldwide high consumption, even though its degradation rate is quite reasonable [8,10]. This may lead to relevant biological effects resulting from bioconcentration and bioaccumulation of these compounds [15].

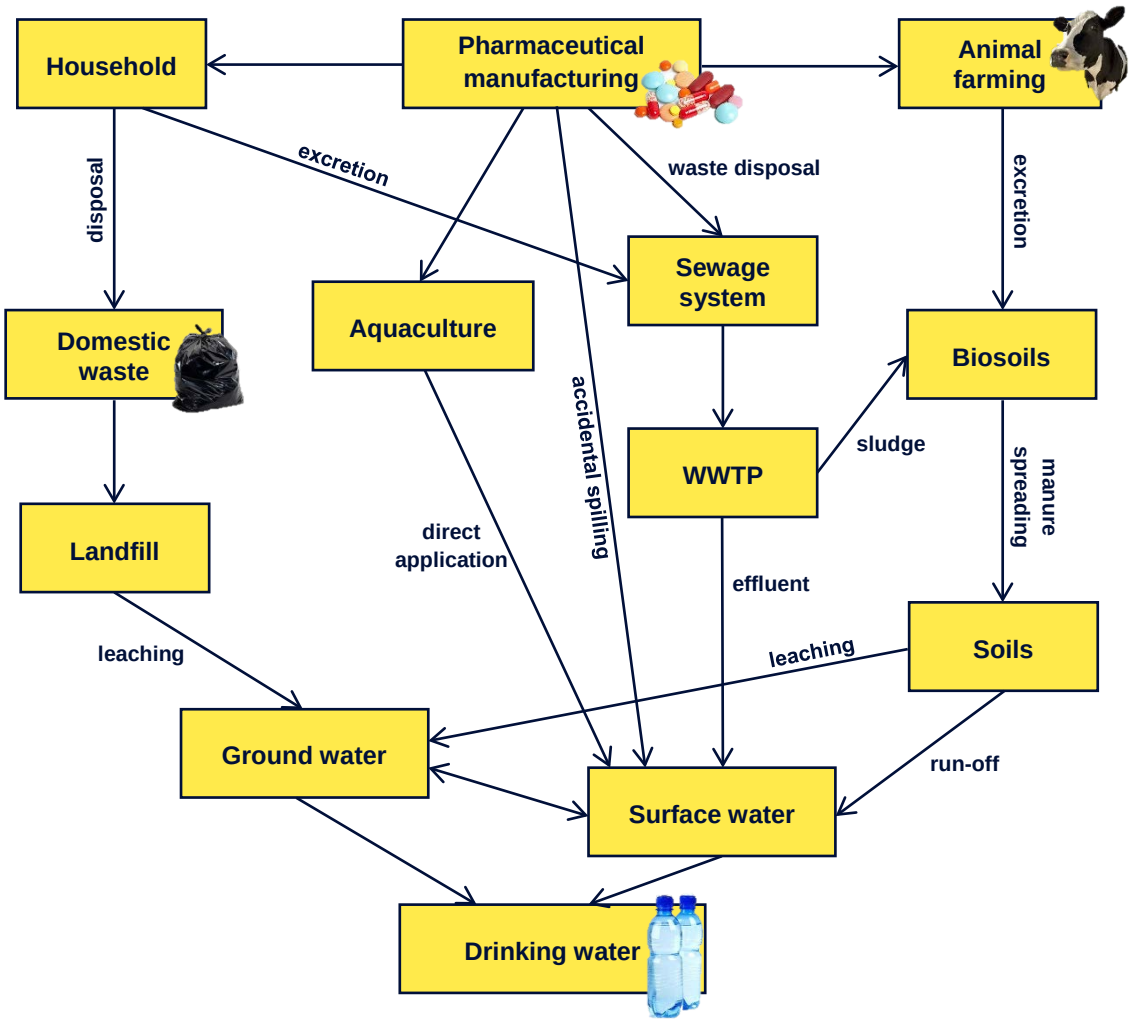

Figure 1. Sources of pharmaceuticals in the environment (adapted from [5]).

Pharmaceuticals are intended to have a specific mode of action in the human body, and thus toxic effects may occur when these substances become in contact with non-target living organisms [16,17]. Aquatic organisms may be the most vulnerable to this type of toxicity due to the fact that they are chronically exposed to contaminated wastewater residues [16]. This topic also includes effects on human life due to food chain

and drinking water contamination [17,18]. The first report of occurrence of pharmaceuticals in treated wastewater happened in the United States of America in 1977, where clofibric acid was found in the range of 0.8-2 μg/L [16,19]. Years later, there were also reports of river contamination in the United Kingdom [20] and ibuprofen and naproxen were detected in Canadian wastewaters [16,21]. Pharmaceuticals of diverse therapeutic classes were also found in Douro River estuary [22] and most recent in the Ave and the Sousa Rivers [23].

Most pharmaceuticals found in the environment are chiral compounds commercialized as enantiomeric mixtures or as single enantiomers [7,24]. Enantiomers may present different pharmacodynamics and pharmacokinetics when interacting with chiral structures as biological systems [1,7,25], which brings out an additional environmental concern. Knowledge about the environmental occurrence of chiral pharmaceuticals in the environment has been increasing with the establishment of enantioselective analytical methods [4,26].

1.1.1 Chiral pharmaceuticals in the environment

Chirality is a key property of about 50% of the pharmaceuticals used nowadays, and it essentially means that a certain object is non-superimposable with its mirror image, being both called enantiomers [7,27,28]. Most common chiral molecules have at least one stereogenic centre, often a carbon atom with four different substituents. Enantiomers have similar physical and chemical properties (melting and boiling points, pH, solubility, etc.), except when they interact with chiral systems [25,29]. Most of its target molecules, such as enzymes, nucleic acids and binding proteins, are chiral. These different interactions with biological systems confer different pharmacodynamics, pharmacokinetic and toxicological properties of the two enantiomers, which may affect living organisms differently [29].

The stereospecific mode of action of some protein receptors leads to a variety of biological responses from chiral pharmaceuticals [29,30]. This occurs due to the preferential bounding of these receptors to one of the enantiomers. According to Lima (1997) [30], biological activity of chiral compounds can be divided into different groups:

a) Both enantiomers have similar activity (e.g., promethazine). In this case, nothing advantageous comes from administrating the pharmaceutical as a single enantiomer, since its activity is the same as the racemate itself;

b) One enantiomer is more active than the other (e.g., warfarin). Even though both enantiomers bind to the receptor at the same site, there is one that binds more strongly;
c) Only one of the enantiomers is active (e.g., α-metildopa). Pharmaceuticals with this property are commercialized in its enantiomeric pure form, since one of the enantiomers has no activity;
d) One enantiomer antagonizes the side effects of the other (e.g., indacrinone);
e) One enantiomer is responsible for the therapeutic action and the other one for the side effects (e.g., L-Dopa). These type of drugs are only commercialized in its enantiomeric pure form;
f) Both enantiomers have activity, but the side effects are associated with only one enantiomer (e.g., ketamine);
g) Enantiomers have different activity (e.g., analgesic dextropropoxyphene and antitussic levopropoxyphene).

Advances in synthesis, analysis and separation of chiral molecules over the years, alongside with better understanding of the different properties of enantiomers of racemates, led to an increased importance of chirality and stereochemistry to pharmaceutical industries [31]. In 1999, the concept “chiral switch” was introduced by Agranat and Caner as the development of a single enantiomer from a chiral drug that has been previously established as a racemate [32,33]. Usually, the new formulation has similar therapeutic indications but this may not always be the case and novel indications may be applied, like lower doses [31]. The main advantages of chiral switching are patient exposure to lower doses, decrease of interindividual variability, drug interactions and side effects, increase in the safety margin and faster onset action [29,33]. Ibuprofen was the first non-steroidal anti-inflammatory drug (NSAID) from its class to be replaced for the enantiopure form [34]. This was due to the fact that the *S*-enantiomer is almost entirely responsible for the anti-inflammatory activity of ibuprofen, being much more potent than the *R*-enantiomer [35,36]. In fact, when administrated, *R*-ibuprofen suffers partial inversion to *S*-ibuprofen [33,36]. Other known examples of chiral switches such as omeprazole and esomeprazole (*S*-omeprazole), citalopram and escitalopram (*S*-citalopram), are presented in **Table 1** [29,31,37].

Table 1. Examples of pharmaceuticals that suffered chiral switch.

RACEMATE	THERAPEUTIC CLASS	ENANTIOPURE FORM	REFERENCE
Ibuprofen	Anti-inflammatory	Dexibuprofen (*S*-ibuprofen)	[38]
Citalopram	Anti-depressant	Escitalopram (*S*-citalopram)	[25]
Ketoprofen	Anti-inflammatory	Dexketoprofen (*S*-ketoprofen)	[39]
Methadone	Analgesic	Levomethadone (*R*-methadone)	[40]
Omeprazole	Proton pump inhibitor	Esomeprazole (*S*-omeprazole)	[41]
Ofloxacin	Antimicrobial	Levofloxacin (*S*-ofloxacin)	[42]
Salbutamol	B2-adrenergic receptor agonist	Levalbuterol (*S*-salbutamol)	[43]
Cetirizine	Antihistaminic	Levocetirizine (*R*-cetirizine)	[29]

In the last years, understanding the environmental risk and toxicity of chiral compounds and its enantiomers gained a relevant role in scientific research [4]. Enantiomers can only be discriminated when present in a chiral environment. Analytical methods used for separation and quantification of enantiomers rely on this principle. Liquid chromatography (LC), gas chromatography, capillary electrophoresis and supercritical fluids chromatography are the main techniques used for enantioselective analysis of these compounds [28,29]. LC is perhaps the tool of excellence for chiral analysis in environmental matrices, due to the wide number of available chiral stationary phases (CSPs) that can be combined with several mobile phases. Additionally, this method can be coupled with several detection methods, such as mass spectrometry, fluorescence, ultraviolet/visible and diode array [29,44,45].

1.2 Biodegradation of pharmaceuticals

Pharmaceuticals reach the environment mainly through effluents discharged of WWTPs. The majority of these compounds are highly polar due to its metabolism in the organism, which makes microbial biodegradation its main removal pathway from influent wastewaters [29]. Microorganisms play an important role in the removal of pharmaceutical residues, since they have the ability to degrade or convert such molecules [46]. This type of biotransformation method has become of great interest in recent years as the data about contamination with pharmaceuticals in aquatic environments has increased.

Degradation of pharmaceuticals is usually substrate specific and stereoselective, which varies among compounds [2,17]. Thus, the biodegradation studies of chiral substances should always consider the enantiomers. The rates of adsorption to activated sewage sludge during WWTP treatment are dependent on the hydrophobic and electrostatic interactions between pharmaceuticals and microorganisms and can also be enantioselective [16,47]. The remaining residues and relevant metabolites are released into surface waters, where they became diluted. For that reason, pharmaceuticals are present in these environments at low concentrations (range of ng/L to μg/L) [17,48] and with different enantiomeric fraction (EF) [4]. These concentrations could be negligible if these discharges were punctual, which is not the case. There is a continuous release of contaminated effluents into aquatic environments, posing a potential risk to aquatic organisms [17]. It is important to understand if these compounds are toxic for aquatic species in the found concentration ranges and if the toxicity is enantioslective.

1.3 Tramadol

Tramadol hydrochloride [2-[(dimethylamino)-methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride] [49-51] is a chiral pharmaceutical structurally related to morphine and codeine [51-53]. It was originally developed in 1962 as an easy-to-synthesize alternative to morphine [51,54] and has been used as a centrally acting analgesic to treat moderate to severe pain [51,53-55]. Tramadol (T) acts as both an opioid agonist and a serotonin and norepinephrine reuptake inhibitor [56], which provides a dual-action analgesic effect (opioid and non-opioid) [52,56]. In fact, due to its low affinity to μ- and κ- opioid receptors, the agonist effect exerted on these receptors is weak, leading to the obstruction of serotonin and norepinephrine reuptake, responsible for the inhibition of pain transmission in the spinal cord [52].

T is commercialized as a racemate and has two chiral centres, consisting of a mixture of the two enantiomers (−)-T ((*R*,*R*)-T) e (+)-T ((*S*,*S*)-T) [53,56]. Both enantiomers contribute to the dual mechanism described above through different but complementary processes [49,50,55]. The (+) enantiomer has a higher affinity to opioid receptors and is a more potent inhibitor of serotonin reuptake, whereas the (−) enantiomer preferentially inhibits norepinephrine reuptake [50,52,55]. It has been shown that T can also produce other positive responses besides analgesia, such as antitussive, antidepressant, anti-inflammatory and immunostimulatory effects [52,57].

After oral administration, T is rapidly absorbed and distributed in the body [51,57]. Its bioavailability is approximately 70% due to first-pass hepatic metabolism and about 20% is bound to plasma proteins [51,52,55,57]. It is extensively metabolized in the liver through *O*- and *N*-demethylations (phase I reactions) and conjugation reactions [56,58], all catalysed by CYP450 enzymes (**Figure 2**). Its main metabolite (M1) is *O*-desmethyltramadol (*O*-DT) and has higher pharmacological activity than the parent compound, as well as higher affinity to the μ-opioid receptor [49,54], being the major responsible for the analgesic efficacy of T. This compound and its metabolites are mainly excreted via the kidneys, with a mean elimination half-life of about 6 hours [51]. Approximately 30% of the parent drug is excreted unchanged in the urine [52,57].

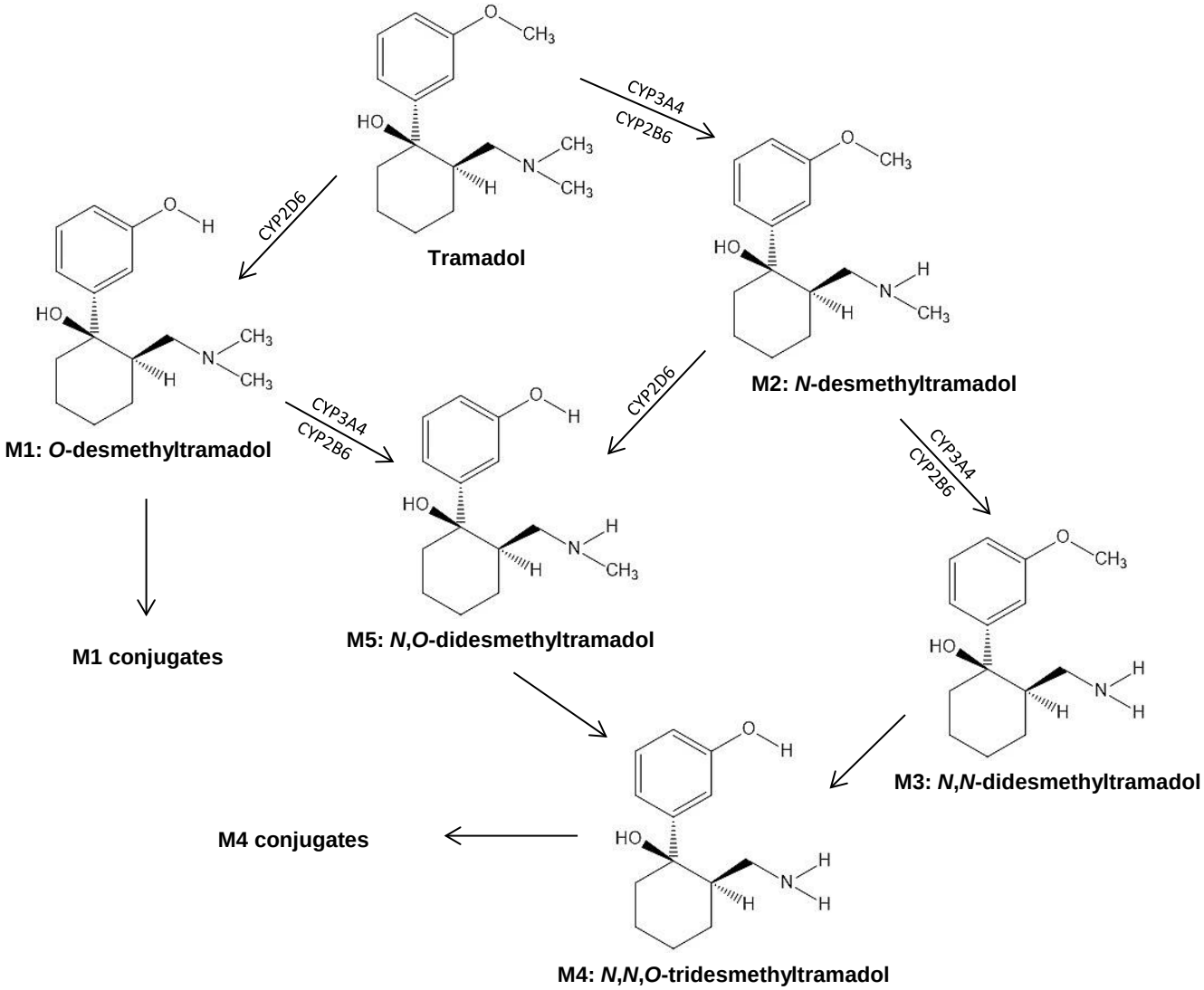

Figure 2. Main metabolic pathways of T.

The most common adverse effects that may occur upon therapeutic use of T include seizures, nausea and vomiting, dry mouth, dizziness, respiratory depression, drowsiness, orthostatic hypotension and constipation [51,52,59]. As others opioid-like drugs, T may cause dependence when used abusively, leading to addiction and withdrawal symptoms [51,52,56].

T and its main metabolites have been simultaneous quantified by LC in different biological matrices, such as saliva [60], urine [60-62], amniotic fluid [63] and plasma [60,62,64-67]. **Table 2** shows LC chiral chromatographic methods for enantioseparation of T and its metabolites.

Table 2. LC analytical methods for enantioseparation and quantification of T enantiomers and its metabolites in biological and environmental matrices.

Analyte	Method	CSP	Mobile phase	Matrix	Reference
T, M1	LC-MS/MS	Lux Cellulose-2	Hexane:Isopropanol:DEA (90:10:0.1)	Plasma	[65]
T, M1	HPLC-FD	Chiralpak AD	Isohexane:Ethanol:DEA (97:2.8:0.1)	Plasma, urine	[50]
T, M1, M2, M5	LC-MS/MS	Chiralpak AD	Isohexane:Ethanol:DEA (97:2.8:0.1)	Plasma	[68]
T, M1, M2	HPLC-FD	CHIRAL-AGP	0.03M diammonium hydrogen phosphate buffer: acetonitrile: TEA (98.9:1:0.1)	Plasma	[58]
T, M1, M2	HPLC-FD	Chiralcel OD-R	0.05M phosphate buffer (1M sodium perchlorate):acetonitrile:N,N-dimethyloctylamine (74.8:25:0.2)	Plasma	[69]
T, M1	HPLC-FD	Chiralcel OD-R	0.05M sodium dihydrogen phosphate buffer (0.2M sodium perchlorate, 0.09M TEA):acetonitrile (80:20)	Plasma	[49]
T, M1	LC-MS/MS	Chiralpak AD	Hexane:Ethanol (97:3) 5mM TEA	Plasma	[70]
T	UPLC-MS/MS	Chiralpak CBH	1mM ammonium acetate:methanol (85:15)	WWTP influent	[71]
T	LC-MS/MS	Chirobiotic V	Methanol: 0.005% formic acid: 4mM ammonium acetate	WWTP wastewater	[72]
T, M1, M2	HPLC-FD	Lux Cellulose-4	Hexane:ethanol:DEA (96:4:0.1)	WWTP wastewater	[53]

(DEA: diethylamine; FD: fluorescence detetor; HPLC: high performance liquid chromatography; LC: liquid chromatography; MS/MS: tandem mass spectrometry; M1:*O*-desmethyltramadol ; M2: *N*-desmethyltramadol; T: tramadol; TEA: triethylamine UHPLC: ultra-high performance liquid chromatography; WWTP: wastewater treatment plant)

Data concerning the presence of T in the environment is scarce, since most published studies concerns biological matrices. Nonetheless, Evans *et al*. (2015) detected T in WWTP wastewater samples in concentrations up to 1320.7 ± 59.3 ng/L (influent) and 506 ± 46.6 ng/L (effluent) [72]. In Portugal, Silva *et al*. (2017) demonstrated the occurrence of T enantiomers up to 325.1 and 357.9 ng/L in influent and effluent wastewater samples, respectively [53]. Regarding biodegradation studies, there are no reports regarding T and its metabolites to the best of our knowledge.

Ecotoxicological assays are used as biological monitoring tools due to their capacity of providing complementary data on the occurrence of pollutants on contaminated environments. Nevertheless, the ecotoxicological impact of pharmaceuticals is still poorly understood but essential to improve prediction of environmental risks. Besides, most occurrence studies neglect the enantiomers and consider these compounds as a unique molecular entity. Ecotoxicological assays can be performed using acute and/or chronic exposures, according to the work objectives. Therefore, acute ecotoxicological assays are advocated with short time tests, evaluating parameters as mortality, immobilization, luminescence [73,74], being these considered for most authors as screening methods in toxicological evaluations [75,76]. For chronic studies, the several stages of life of the test organisms are integrated, using endpoints as reproduction rate, growth, changing frequency and embryonic malformations [74].

The ecotoxicological effects of the enantiomers of T and *O*-DT and racemic *N*-desmethyltramadol (*N*-DT) on two aquatic microorganisms from two different trophic levels, the freshwater crustacean *Daphnia magna* (consumer) and the ciliate protozoan *Tetrahymena thermophila* (reducer) were never evaluated. Both species were adopted as test organisms by the Organization for Economic Cooperation and Development (OECD) and by the United States Environmental Protection Agency (US EPA) due to their specific characteristics: *D. magna* has a short lifecycle, high sensitivity to a variety of chemicals and it is easy to manipulate; *T. thermophila* is the second most important group of biota in activated sludge of WWTPs.

1.4 Objectives

The main objectives of this thesis were to:

- To develop and validate a LC with fluorescence detection (LC-FD) method for the separation of the enantiomers of T and its main metabolites, and its application to a

monitor study concerning the biodegradation of racemate T and its enantiomers in an activated sludge inoculum during 21 days;

- To develop a semi-preparative LC method to obtain T enantiomers;

- To verify the susceptibility of ecological relevant organisms to T and *O*-DT racemates and enantiomers at environmental concentrations, as well as racemic *N*-DT using two different test organisms approved by OECD, *D. magna* and *T. thermophila*.

Chapter 2

MATERIAL AND METHODS

2.1 Chemicals and equipments

Chromatographic analysis were performed using a Shimadzu UFLC Prominence System equipped with two pumps LC-20AD, an autosampler SIL-20AC, a column oven CTO-20AC, a degasser DGU-20A5, a system controller CBM-20A and an LC Solution, Version 1.24 SP1 (Shimadzu Corporation, Tokyo, Japan). The fluorescence detector coupled to the HPLC system was a Shimadzu RF-10AXL and the UV/visible was a Shimadzu SPD-20A.

Tramadol hydrochloride (*rac*-tramadol), *O*-DT and *N*-DT were purchased from Sigma-Aldrich (Steinheim, Germany), with a purity degree above 98%. Isolated enantiomers of T and *O*-DT were kindly donated by Grünenthal, GmbH.

All solvents were of HPLC grade. EtOH, isopropanol (IsopOH) and methanol (MetOH) were purchased from Fisher Scientific UK (Leicestershire, United Kingdom); hexane (Hex) was acquired from VWR Chemicals Prolabo and acetonitrile (ACN) from CARLO ERBA Reagents (Val-de-Reuil, France). Diethylamide (DEA) and triethylamide (TEA) were purchased from Merck (Darmstadt, Germany); and trifluoroacetic acid (TFA) from ACROS Organics (Geel, Belgium).

Sodium phosphate dibasic dihydate ($Na_2HPO_4.2H_2O$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), ammonium sulfate ($(NH_4)_2SO_4$), sodium hydroxide (NaOH), iron sulfate heptahydrate ($FeSO_4.7H_2O$), manganese sulfate tetrahydrate ($MnSO_4.4H_2O$) were purchased from Merck (Darmstadt, Germany). Potassium phosphate monobasic (KH_2PO_4) and copper sulphate ($CuSO_4$) were acquired from José M. Vaz Pereira Lda. (Sintra, Portugal). Ethylenediaminetetraacetic acid disodium salt hydrate ($Na_2EDTA_2.2H_2O$) and calcium chloride ($CaCl_2$) were bought from Panreac ITW

Companies (Barcelona, Spain). Sodium sulphate (Na_2SO_4) was purchased from José Manuel Gomes dos Santos, Lda. (Lisboa, Portugal) and magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) from Riedel-deHaen (Bucharest, Romania). The additional solvents, sodium molibdate ($MoNa_2O_4$) and sulfuric acid (H_2SO_4) were acquired from Sigma-Aldrich, now Merck (Darmstadt, Germany).

2.2 Preparative enantioseparation of T

During method optimization, standard solutions of T were prepared in EtOH and in the initial mobile phase Hex/EtOH (0.1% DEA) in a proportion of 80/20 (v/v), in a racemate concentration of 1 mg/mL. Separation of the main metabolites of T (*O*-DT and *N*-DT) was also attempted, even though only T enantiomers were successively separated and collected. The dilutions of these compounds were also performed in the same conditions as the parent compound.

The chiral chromatographic column used for semi-preparative separation of T was a homemade amylose 3,5-dimethylphenylcarbamate column coated on to APS-Nucleosil (500 A, 7µm, 20%, w/w) and packed into a stainless-steel 20 x 0.7 cm i.d. size column. Diverse mobile phases in normal mode of elution, different flow rates and injection volumes were attempted (**Table 3**). The optimized conditions were achieved with a column oven temperature of 23ºC, a flow rate of 1.5 mL/min and an injection volume of 10 µL. The FD was set to excitation and emission wavelengths of 275 nm and 300 nm, respectively, coupled with an UV/visible detector with a wavelength of 275 nm (maximum absorption of T). Fractions were collected into round-bottom flasks and the remaining solvent was evaporated using a vacuum rotary evaporator.

Table 3. Experimental conditions tested for the optimization of the enantioseparation semi-preparative HPLC method of T.

MOBILE PHASE	PROPORTION (v/v)	FLOW (mL/min)	INJECTION VOLUME (µL)	OVEN TEMPERATURE (ºC)
Hex (0.1% DEA)/ EtOH (0.1% DEA)	96/4	1.0	20	-
Hex (0.1% DEA)/ EtOH (0.1% DEA)	98/2	1.0	20	-
Hex (0.1% DEA)/ IsopOH (0.1% DEA)	96/4	1.0	20	-

Table 3. (continuation)

MOBILE PHASE	PROPORTION (v/v)	FLOW (mL/min)	INJECTION VOLUME (µL)	OVEN TEMPERATURE (ºC)
Hex (0.1% DEA)/ IsopOH (0.1% DEA)	96/4	1.5	20	-
Hex (0.1% DEA)/ IsopOH (0.1% DEA)	96/4	2.0	20	-
Hex (0.1% DEA)/ IsopOH (0.1% DEA)	98/2	1.5	20	-
Hex (0.1% DEA)/ IsopOH (0.1% DEA)	98/2	2.0	20	-
Hex (0.1% DEA; 0.1% TFA)/ IsopOH (0.1% DEA; 0.1% TFA)	98/2	1.5	20	-
Hex (0.1% DEA; 0.1% TFA)/ IsopOH (0.1% DEA; 0.1% TFA)	50/50	1.5	20	-
Hex (0.1% DEA; 0.1% TFA)/ IsopOH (0.1% DEA; 0.1% TFA)	80/20	1.5	20	-
Hex (0.1% DEA)/ IsopOH (0.1% DEA)	98/2	1.5	30	-
Hex (0.1% DEA)/ IsopOH (0.1% DEA)	98/2	1.5	40	-
Hex (0.1% DEA)/ IsopOH (0.1% DEA)	98/2	1.5	10	23

2.3 Development and validation of the enantioseparation method of T and its metabolites *O*-DT and *N*-DT by LC-FD to follow the biodegradation study

Standards mixtures were prepared in Hex/EtOH/DEA (80/20/0.1, v/v/v) and in EtOH for normal phase elution mode at a racemate concentration of 5 µg/mL, and in ultra-pure water and mineral salts medium (MM) (see 2.4 Biodegradation assays) for reverse phase elution mode, at a racemate concentration of 1 µg/mL and 0.5 µg/mL.

The chiral chromatographic column used was a Lux Cellulose-4 with a dimension of 150 x 4.6 mm i.d. and 3 µm particle size, with the respective guard column of 4.4 x 3.0 mm i.d., both purchased from Phenomenex (Torrance, United States). Diverse mobile phases, in both normal and reversed mode of elution, different flow rates and injection volumes were attempted (**Table 4**). The optimized conditions were achieved with a column oven temperature of 25ºC, an autosampler tray temperature of 15ºC, a flow rate of

0.5 mL/min and an injection volume of 10 µL, under isocratic elution with ACN:EtOH:DEA (10mM ammonium formate) (50:50:0.1) / H_2O (38/62, v/v). The FD was set on high sensitivity (4 times) with excitation and emission wavelengths of 275 nm and 305 nm, respectively.

Table 4. Experimental conditions tested for the development and optimization of the enantioseparation method of T and its metabolites.

Elution mode	Mobile phase	Proportion (v/v)	Flow (mL/min)	Injection volume (µL)	Oven temperature (°C)
Normal	Hex (0.1% DEA)/ EtOH (0.1% DEA)	96/4	0.7	10	23
	Hex (0.1% DEA)/ IsopOH (0.1% DEA)	96/4	0.7	10	23
Reverse	ACN (0.05% DEA)/ H_2O	35/65	0.5	10	23
	ACN (0.1% DEA)/ H_2O	35/65	0.5	10	23
	ACN (0.01% DEA)/ H_2O	35/65	0.5	10	23
	ACN (0.05% TEA)/ H_2O	35/65	0.5	10	23
	ACN (0.1% TEA)/ H_2O	35/65	0.5	10	23
	ACN (0.01% TEA)/ H_2O	35/65	0.5	10	23
	MetOH (0.05% DEA)/ H_2O	35/65	0.5	10	23
	MetOH (0.05% DEA)/ H_2O	50/50	0.5	10	23
	MetOH (0.05% DEA)/ H_2O	25/75	0.5	10	23
	MetOH (0.05% DEA)/ H_2O	40/60	0.5	10	23
	ACN (5mM ammonium formate, 0.1% DEA)/ EtOH	45/55	0.5	10	23
	ACN:EtOH (50:50) (5mM ammonium formate, 0.1% DEA)/ EtOH	45/55	0.5	10	23
	ACN:EtOH (50:50) (5mM ammonium formate, 0.1% DEA)/ H_2O	35/65	0.5	10	23
	ACN:EtOH (50:50) (15mM ammonium formate, 0.1% DEA)/ H_2O	35/65	0.5	10	23
	ACN:EtOH (50:50) (10mM ammonium formate, 0.1% DEA)/ H_2O	35/65	0.5	10	25
	ACN:EtOH (50:50) (10mM ammonium formate, 0.1% DEA)/ H_2O	37/63	0.5	10	25
	ACN:EtOH (50:50) (10mM ammonium formate, 0.1% DEA)/ H_2O	38/62	0.5	10	25

Table 4. (continuation)

Elution mode	Mobile phase	Proportion (v/v)	Flow (mL/min)	Injection volume (µL)	Oven temperature (°C)
Reverse	ACN:EtOH (50:50) (10mM ammonium formate, 0.1% DEA)/ H_2O	38/62	0.5	20	25
	ACN:EtOH (50:50) (10mM ammonium formate, 0.1% DEA)/ H_2O	38/62	0.5	30	25

2.3.1 Method validation

Method validation was performed for T and its main metabolite *O*-DT, according to International Conference on Harmonization (ICH) guidelines [77] and considering the following parameters: limits of detection (LOD) and quantification (LOQ), linearity, selectivity, precision, accuracy and recovery. The mixture of standards of T and *O*-DT was prepared in MM.

2.3.1.1 Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ were calculated based on the signal-to-noise ratio. The ratios were determined by comparing measured signals from samples with known low concentrations of each enantiomer (T and *O*-DT) with those of blank samples and establishing the minimum concentration at which the analyte could be reliably detected or quantified. Signal-to-noise ratios of 3:1 and 10:1 were considered for estimating the detection and quantification limits of each enantiomer, respectively, and were calculated according to the following equation:

$$S/N = \frac{2H}{h}$$

where H represents the height of the peak corresponding to the analyte (signal) and h is the background noise calculated one minute before and one minute after the peak [77,78].

2.3.1.2 Linearity

Calibration curves were performed with a racemate of each compound (T and *O*-DT) in MM. The nominal racemate concentrations used for T were 250, 300, 400, 450, 500, 600 ng/mL; and for *O*-DT 80, 110, 150, 200, 250, 300 and 350 ng/mL. Each concentration was prepared in triplicate. Linearity was determined by linear regression between peak area and the known prepared concentration.

2.3.1.3 Selectivity

Selectivity was evaluated by comparison of chromatograms obtained by injection of T and *O*-DT standard solutions prepared in ultrapure water with those prepared in MM in the absence and in the presence of activated sludge inoculum. Samples composed by MM with activated sludge inoculum were spiked with T and *O*-DT at nominal racemate concentrations of 450 and 310 ng/mL, respectively, and centrifuged at 14 000 rpm for 10 min, after two hours of shaking at 110 rpm. The supernatant aliquots were collected and injected, in order to obtain the corresponding chromatograms to compare with the same concentrations of standard samples prepared in MM.

2.3.1.4 Precision

Intra- and inter-day precisions, expressed as relative standard deviation percentages (%RSD), were estimated using three quality control standard solutions (QCs) prepared at three different concentrations within the linearity range, all in triplicate. The nominal racemate concentrations used for T were 320, 420 and 550 ng/mL; and for *O*-DT 130, 230 and 330 ng/mL.

2.3.1.5 Accuracy

Method accuracy was calculated as the agreement percentage between method quantitative results of the three QCs and the real amount of compound added. The linear regression equation obtained for linearity was used for calculation of the actual concentration of compound present in the standards mixture.

2.3.1.6 Recovery

The recovery rate was calculated for each enantiomer using the different racemate concentrations in triplicate. Samples composed by MM with activated sludge inoculum were spiked with T and *O*-DT at the three QCs concentrations (the same used in precision and accuracy) and centrifuged at 14 000 rpm for 10 min, after two hours of shaking at 110 rpm. The supernatant aliquots were collected and used to calculate the recovery

percentage of those spiked samples and compared with the same concentrations of standard samples prepared in MM.

2.4 Biodegradation assays

The biodegradation assays were performed accordingly to work published elsewhere [79,80]. The activated sludge (AS) inoculum used in the biodegradation assays was collected from the secondary treatment aerated tanks of a municipal WWTP (Parada, Maia, Portugal) and preserved in amber glass flasks at 4 ºC until usage. The inoculum was washed with MM for three centrifuge cycles (4000 rpm, 10 min) prior to its use. The final pellet was used to prepare the pre-inoculum for the assays. The composition per litre of the MM used is presented in **Tables 5** and **6**.

MM was previous prepared without adding the trace elements solution, and sterilized alongside with the solution itself. Both solutions are ideal to grow bacteria, so it is important to guarantee that the only microorganisms present in the assay are the ones provided by the AS inoculum. Sterile trace elements solution was then added to the medium also in a sterilized environment (flame) and preserved at 4ºC. Sodium acetate solution (CH_3COONa) at concentration 1M was also prepared and sterilized.

Table 5. Reagents needed to prepare the mineral salts medium.

REAGENT	m (g) PER LITRE
$Na_2HPO_4.2H_2O$	2.67
KH_2PO_4	1.40
$MgSO_4.7H_2O$	0.20
$(NH_4)_2SO_4$	0.50
Trace elements solution	10 mL

($Na_2HPO_4.2H_2O$: sodium phosphate dibasic dihydate; KH_2PO_4: potassium phosphate monobasic; $MgSO_4.7H_2O$: magnesium sulfate heptahydrate; $(NH_4)_2SO_4$: ammonium sulfate)

Table 6. Reagents needed to prepare the trace elements solution.

REAGENT	m (g) PER LITRE
NaOH	2.0
$Na_2EDTA_2.2H_2O$	12.0
$FeSO_4.7H_2O$	2.0
$CaCl_2$	1.0
Na_2SO_4	10.0
$ZnSO_4$	0.4
$MnSO_4.4H_2O$	0.4
$CuSO_4.5H_2O$	0.1
$Na_2MoO_4.2H_2O$	0.1
H_2SO_4 98%	0.5 mL

(NaOH: sodium hydroxide; $Na_2EDTA_2.2H_2O$: ethylenediaminetetraacetic acid disodium salt hydrate; $FeSO_4.7H_2O$: iron sulfate heptahydrate; $CaCl_2$: calcium chloride; Na_2SO_4: sodium sulfate; $ZnSO_4$: zinc sulfate; $MnSO_4.4H_2O$: manganese sulfate tetrahydrate; $CuSO_4.5H_2O$: copper sulfate pentahydrate; $Na_2MoO_4.2H_2O$: sodium molybdate dihydrate; H_2SO_4: sulfuric acid)

The biodegradation assays were performed in batch mode using 100 mL covered sterile flasks containing 25 mL of MM (with or without sodium acetate, CH_3COONa) inoculated with 1 mL of AS previously washed, giving an optical density of ca. 0.3 at 600 nm. Individual stock solutions of standards were prepared in H_2O at concentration of 25 µg/mL for T racemate and 12.5 µg/mL for the enantiomers and stored at -20 ºC in amber vials. Work solutions were prepared freshly by dilution of stock solutions in MM. The compounds were added in order to obtain final concentrations of 500 ng/mL and 250 ng/mL for racemate T and its enantiomers, respectively. Biodegradation in the presence of an additional carbon source (CH_3COONa) was also investigated at a concentration of 200 mg/L. Each condition was prepared in duplicate, using glass flasks of a four-time higher volume to assure the necessary aeration of the cultures. All experiments were incubated at 25 ºC with constant shaking (110 rpm). Two control assays were also performed: one without inoculum (abiotic) and other with dead AS cells. Aliquots of 1 mL were collected every 2/3 days, by perforation of the lid with a syringe and needle. The assays were monitored during 21 days using the validated LC-FD method, by injecting 10 µL aliquots obtained after centrifuging 800 µL samples at 14000 rpm for 10 min. The other 200 µL were used to measure the optical density (O.D) at a wavelength of 600 nm using a 96-wells plate.

2.5 Ecotoxicity assays

2.5.1 *Daphnia magna* acute immobilization test

Toxicity tests with *Daphnia magna* were performed using the DAPHTOXKIT F^{TM} *MAGNA* (MicroBioTests Inc., Gent, Belgium), which contains all the necessary materials to perform six complete acute bioassays, including the dormant eggs of *D. magna* (ephippia) protected by a chitinous capsule (ephippium). These eggs can be stored for long periods of time at 4 ºC without losing their viability.

The experiments were carried out in on multiwell plates provided, with *D. magna* hatched from ephippia. The compounds and corresponding concentrations used were 0.2, 2, 20, 200 and 2000 μg/L for racemate T, *O*-DT and *N*-DT; 0.1, 1, 10, 100 and 1000 μg/L for the enantiomers of T and *O*-DT. These solutions were diluted in a medium called "standard freshwater", which was prepared by adding the concentrated salt solutions provided in the kit to distilled water up to 2 L. This medium is composed by sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), magnesium sulphate ($MgSO_4$) and potassium chloride (KCl), according to the formula recommended by the International Standardization Organization (ISO) for the acute toxicity test with Daphnia magna (ISO 6341). Hatching of eppiphia was initiated 3 days prior to the beginning of the toxicity tests, by incubating the eggs in Petri dishes with "standard freshwater" at 20ºC under continuous illumination of 6000 lux. To perform a complete test, 120 neonates younger than 24h were needed. For a statistically acceptable evaluation of the effects, each test concentration as well as the control was assayed in four replicates. The multiwell plates are provided with a column of "rinsing wells" to prevent dilution of the compound in the multiwell cups during the transfer of the test organisms from the hatching petri dish to the test plate. Each row of the multiwell plate corresponded to one toxicant dilution. 10 mL of "standard freshwater" were transferred into each well of the control row and 10 mL of the respective compound concentration into each well of the corresponding row, in the sequence of increasing toxicant concentrations.

The neonates transfer was executed with the aid of a micropipette. First, about 25 Daphnids were transferred into each "rinsing well". Then exactly 5 neonates were brought from the "rinsing wells" into each corresponding test well corresponding to a total of 20 neonates for each test. After this, each multiwell plate was sealed with a parafilm strip and covered with on tightly. Plates were then placed in the incubator at 20ºC in the dark for 48h. To score the results, the number of dead or immobilized neonates was recorded, versus that of the actively swimming test organisms in each well at 24h and at 48h. The

test was considered valid when the number of dead plus immobile organisms did not exceed 10% in the controls.

In order to check the correct execution of the test procedure and the sensitivity of the test organisms, a reference test with potassium dichromate was also performed. The five concentrations used were 3.2, 1.8, 1, 0.56 and 0.32 mg/L. From the data obtained in this quality control test, the 24h EC_{50} was calculated.

2.5.2 *Tetrahymena thermophila* growth inhibition test

Freshwater toxicity tests with *Tetrahymena thermophila* were conducted for racemate T and *O*-DT as well as for each of its enantiomers, using the PROTOXKIT F™ (MicroBioTests Inc., Gent, Belgium). An additional test was performed for racemic (*N*-DT. Each PROTOXKIT F contains all the necessary materials to perform six complete 24h assays in duplicate. This test consists in a multi-generation growth assay based on the conversion of substrate into ciliate biomass. The concentrations tested were the same as for the *D. magna* acute immobilization test: 0.2, 2, 20, 200 and 2000 μg/L for racemate T, *O*-DT and *N*-DT; 0.1, 1, 10, 100 and 1000 μg/L for the enantiomers of T and *O*-DT. A control without compound was also performed, in order to guarantee the validity of the test.

The medium called “standard freshwater” used to prepare the compound concentrations was prepared according to the protocol provided, adding the concentrated salt solutions included in the kit to deionized water up to 1L. This medium is composed by sodium bicarbonate ($NaHCO_3$), calcium sulphate ($CaSO_4$), magnesium sulphate ($MgSO_4$) and potassium chloride (KCl). Disposable 1 cm polystyrol spectrophotometric cells containing *Tetrahymena thermophila*, food substrate and toxicant were incubated in the dark for 24h at 30 ºC. While normal proliferating cell cultures clear the substrate suspension in 24h, inhibited culture growth is reflected by remaining turbidity. The degree of inhibition was quantified by optical density measurements of the turbidity at a wavelength of 440 nm.

In order to check the correct performance of the test and the sensitivity of the test organisms, a reference test with potassium dichromate ($K_2Cr_2O_7$) was also executed. The five concentrations used were 56, 32, 18, 10 and 5.6 mg/L. From the data obtained in this quality control test, the 24h EC_{50} was calculated.

Chapter 3

RESULTS AND DISCUSSION

3.1 Semi-preparative enantioseparation of T by LC

In this work, a semi-preparative LC was used to separate the enantiomers of T. Its main metabolites *O*-DT and *N*-DT were also included in the optimization process, even though enantioseparation was not achieved for these two compounds. Silva *et al*. (2017) reported the analytical separation of T enantiomers and its main metabolites *O*-DT and *N*-DT using a CSP in a mobile phase of 0.1% DEA in Hex/EtOH (96/4, v/v) under isocratic elution [53]. The mobile phase conditions were used in this work as a starting point for the optimization of the preparative method, increasing the flow rate to 1.0 mL/min and the injection volume to 20 μL. FD and UV/visible detectors were set in parallel for a better understanding of the compounds behaviour in the corresponding chromatographic conditions. **Figure 3** presents the chromatograms obtained for the three compounds in the initial conditions.

Considering the analytical conditions mentioned before, only the separation of T was partially achieved with retention times (R_T) of 15 and 17 min for the first and second T enantiomers, respectively (**Figure 3**). Dilution in EtOH or in mobile phase did not modify the chromatographic profile for this compound; however, in the second case, the peaks presented higher UV/visible intensity. In order to improve the peak shape and the baseline separation (chromatographic resolution), the percentage of EtOH in the mobile phase was reduced to 2%. This modification in the mobile phase composition led to a decrease in peak intensities and to higher retention times, which did not improve chromatographic resolution (data not shown).

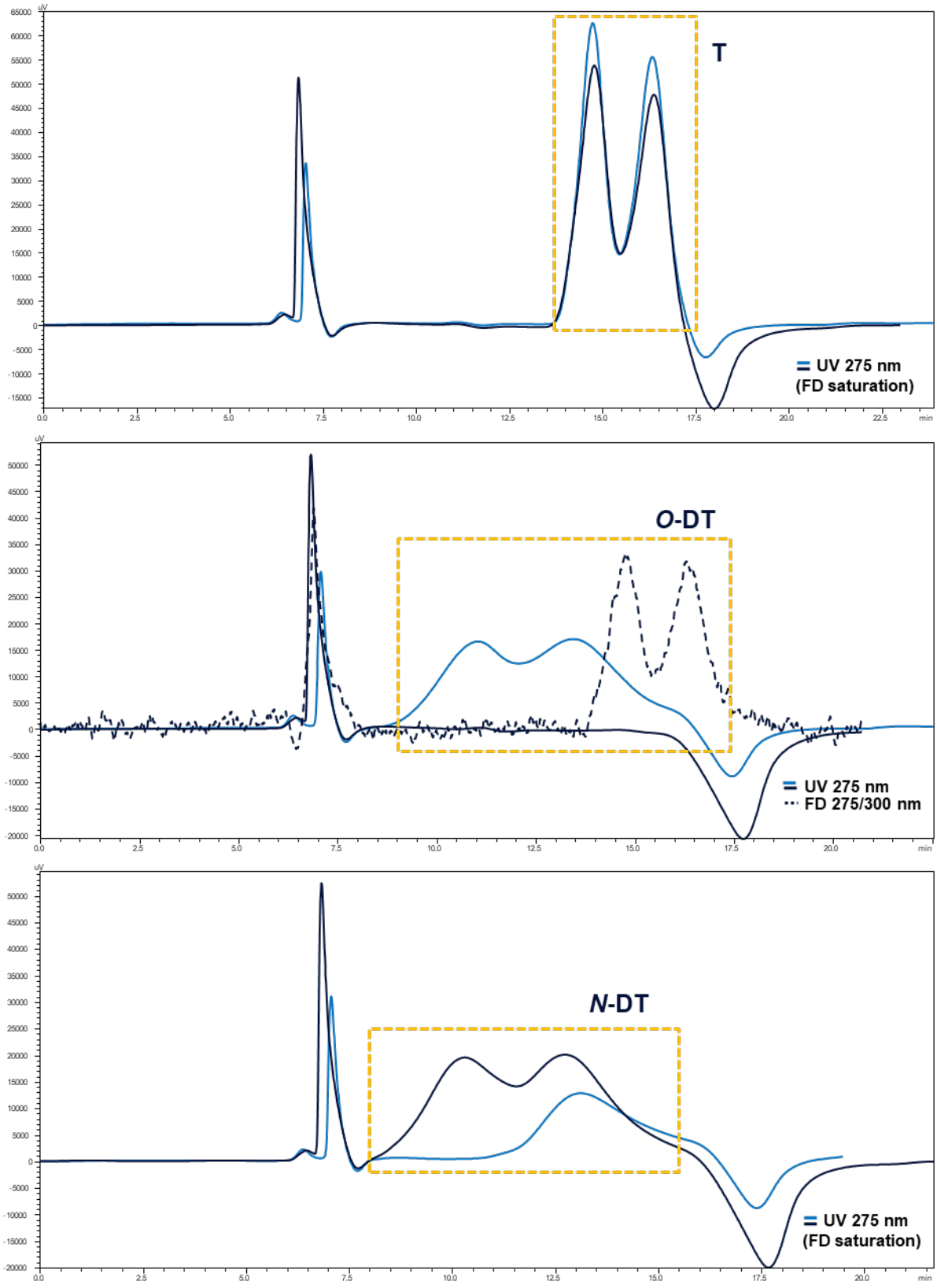

Figure 3. Chromatograms of T, *O*-DT and *N*-DT prepared in EtOH (**dark blue**) and in Hex/EtOH (80/20) with 0.1% DEA (**light blue**). Conditions: mobile phase Hex/EtOH (0.1% DEA) (96/4, v/v); flow rate 1.0 mL/min; $\lambda_{exc/em}$ = 275/300 nm (fluorescence); λ = 275 nm (UV/visible).

EtOH was replaced by IsopOH in the mobile phase, at the initial proportions (96/4, v/v), maintaining all the remaining conditions. The chromatograms obtained for T and *N*-DT are presented in **Figure 4** (separation of *O*-DT enantiomers did not occur with this mobile phase – data not presented).

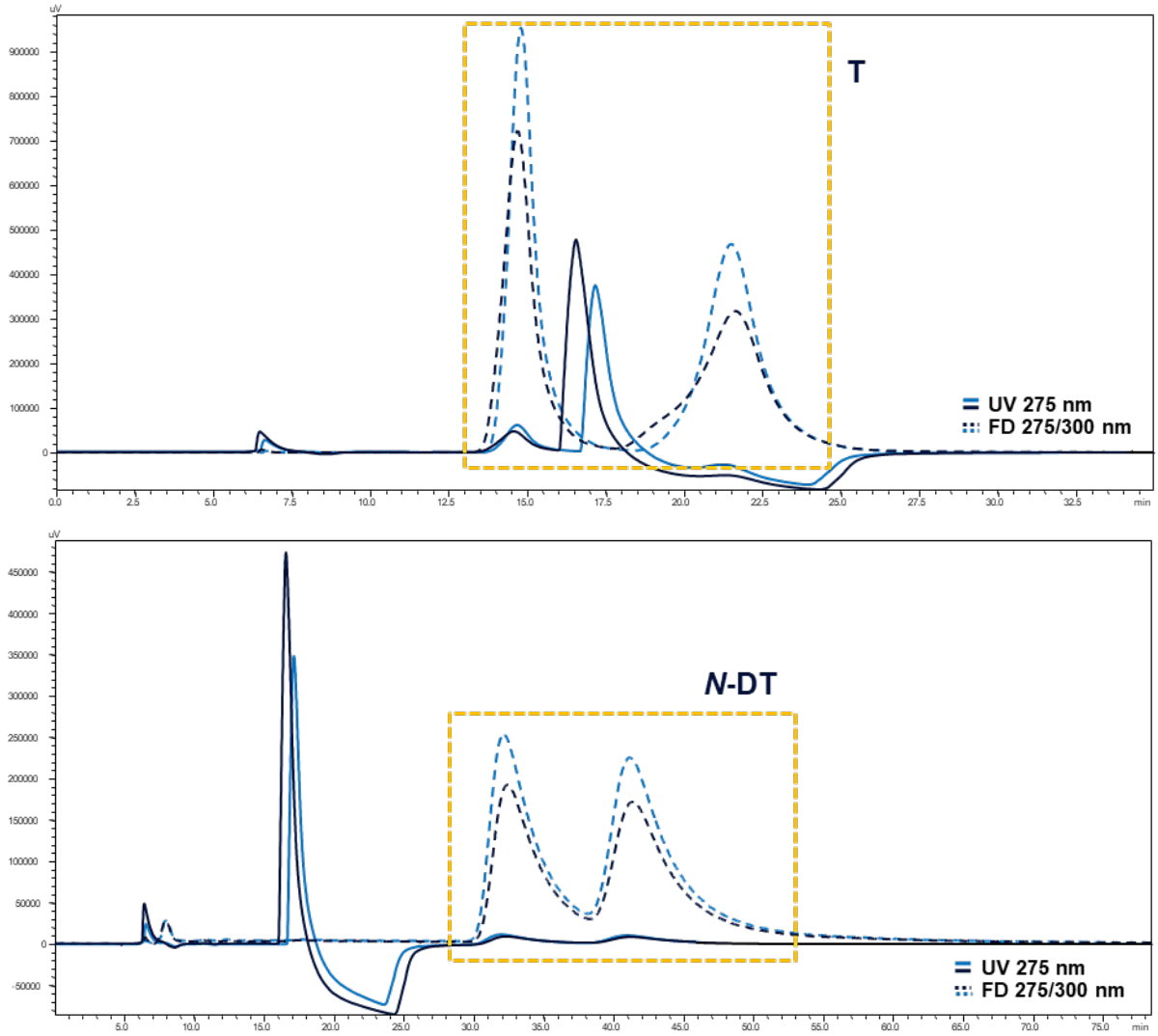

Figure 4. Chromatograms of T and *N*-DT prepared in EtOH (**dark blue**) and in Hex/EtOH (80/20) with 0.1% DEA (**light blue**). Conditions: mobile phase Hex/IsopOH (0.1% DEA) (96/4, v/v); flow rate 1.0 mL/min; $\lambda_{exc/em}$ = 275/300 nm (fluorescence); λ = 275 nm (UV/visible).

Since semi-preparative LC requires higher flow rates, variations of flow rate were attempted using only the standards of T and *N*-DT in Hex/EtOH/DEA. The metabolite *O*-DT was not analysed since it was not possible to separate its enantiomers in this mobile phase. The best results were obtained with a flow rate of 1.5 mL/min, since the separation for both compounds were similar to that with lower flow rate, but with better retention times: 10 and 14 min for T enantiomers, 21 and 26 min for *N*-DT (**Figure 5**). This mobile

phase showed good baseline resolution for both compounds. Using the flow rate of 1.5 mL/min, new injections of the three compounds in Hex/EtOH/DEA were made with 2% less of isopropanol in the mobile phase, in an attempt of optimizing the retention times without undermining resolution. Once more, separation of *O*-DT enantiomers was not verified and so, only the chromatograms obtained for T and *N*-DT are presented in **Figure 5**.

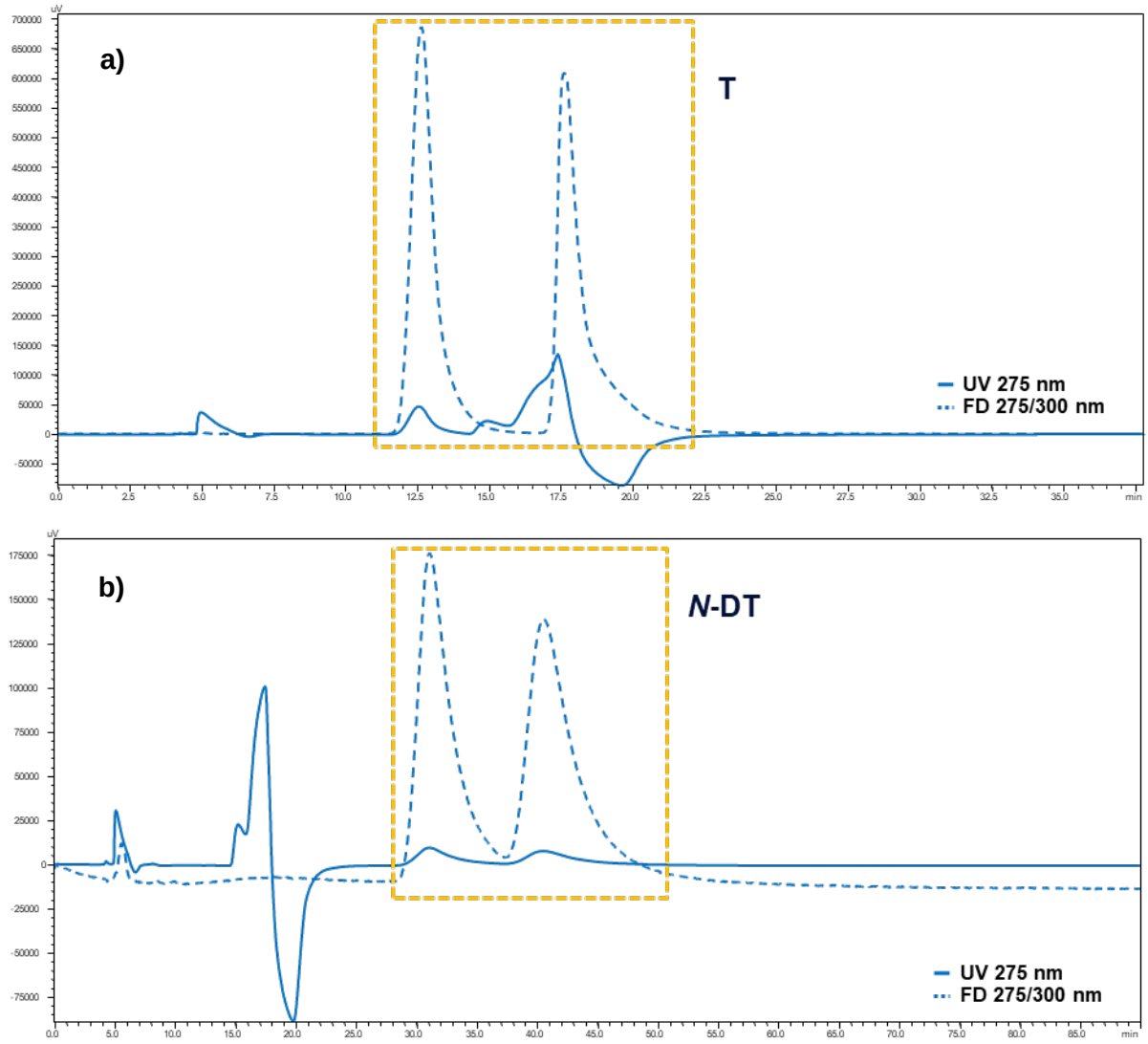

Figure 5. Chromatograms of a) T and b) *N*-DT prepared in Hex/EtOH (80/20) with 0.1% DEA. Conditions: mobile phase Hex/IsopOH (0.1% DEA) (98/2, v/v); flow rate 1.5 mL/min; $\lambda_{exc/em}$ = 275/300 nm (fluorescence); λ = 275 nm (UV/visible).

Contrarily to what happened with EtOH, the decrease of IsopOH percentage in the mobile phase improved significantly the enantioseparation of T and its metabolite *N*-DT with good baseline separation. As demonstrated in **Figure 5**, the mobile phase with only 2% of IsopropOH led to higher resolution for both compounds, even though retention times suffered small modifications (13 and 18 min for tramadol; 31 and 40 min for *N*-DT).

According to Mosiashvili *et al*. (2013), adding a small amount of acid to a mobile phase that already have a basic addictive (e.g. DEA) can improve the enantioseparation of some compounds [81]. In order to test this effect for T and its metabolites, 0.1% of trifluoroacetic acid (TFA) was added to the mobile phase Hex/IsopOH/DEA (98/2/0.1, v/v/v). Nevertheless, the chromatograms obtained with this condition did not improve enantioseparation (data not presented) and TFA was eliminated from the mobile phase.

Even though good enantioseparation of T was already achieved, some additional adjustments were made, mainly in the dilution solvent of the compound standards. New racemate solutions of T and *N*-DT were diluted in the optimized mobile phase Hex/IsopOH/DEA at the same concentration (1 mg/mL) in different volume proportions (v/v/v): 98/2/0.1, the exact proportions used for the mobile phase; 80/20/0.1, for direct comparison with the prior standards; 80/20, for verifying the influence of DEA in the separation.

The chromatographic profiles obtained with different solvent proportions were all identical (data not shown), demonstrating that standard solvent dilution was not influencing enantioseparation. T is a polar organic compound with low solubility in nonpolar compounds such as hexane. On the other hand, it has high solubility in water, ethanol and methanol [82]. Consequently, and since the objective of this section was to collect pure T enantiomers, the dilutions were prepared in EtOH and injected in order to compare with those already analysed. Once again, the resulting chromatograms were similar in terms of peak intensity and profile (data not presented).

In a semi-preparative chromatography, the ideal conditions are to work with the maximum concentration at the higher injection volume possible, in order to obtain better separation efficiency. For this reason, different concentrated standards of racemate T were prepared in EtOH (2, 3, 4, 5, 6, 8 and 10 mg/L) and injected in different volumes (10, 20, 30, 40 μL). The optimized conditions for semi-preparative enantioseparation of T are presented in **Table 7**. Column oven temperature was set on 23ºC and injection volume on 10 μL.

Table 7. Optimized conditions of the semi-preparative method developed for the T enantioseparation.

PARAMETER	VALUES
Mobile phase (v/v/v)	Hex/IsopOH/DEA (98/2/0.1)
Injection volume	10 µL
Column oven temperature	23ºC
Flow rate	1.5 mL/min
UV/visible detection wavelength	275 nm
Fluorescence detection wavelengths	275 nm (excitation), 300 (emission)

After complete optimization of the method, the first fractions were collected with a racemate T concentration of 10 mg/mL (*O*-DT and *N*-DT were not used for this part of the procedure). **Figure 6** shows the chromatograms obtained for this concentration in EtOH under the optimized LC conditions.

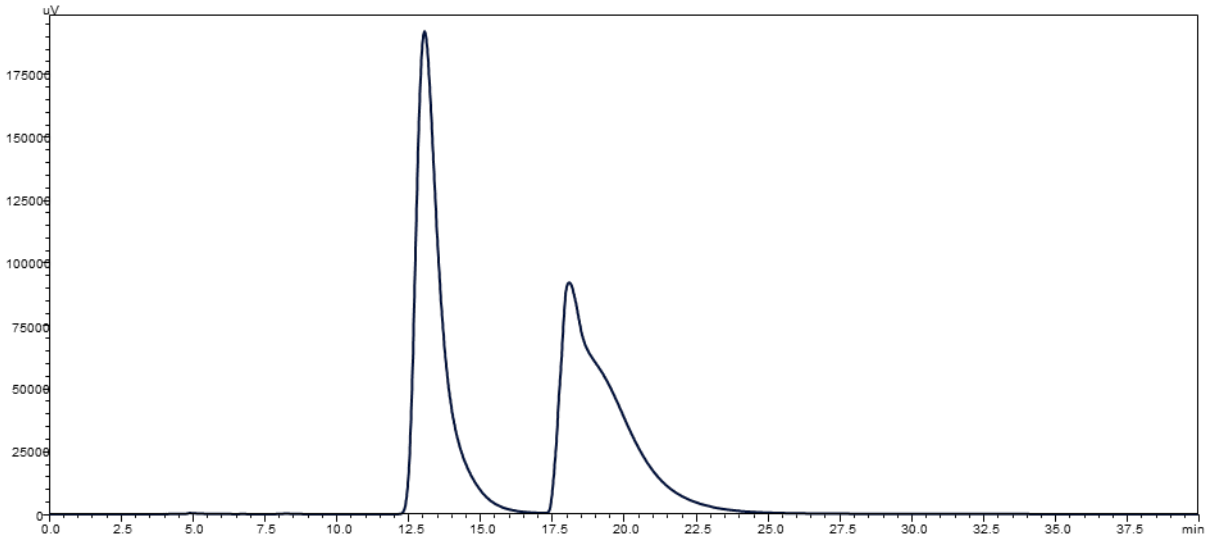

Figure 6. Chromatograms obtained for T at a concentration of 10 mg/mL in EtOH under the optimized conditions. Conditions: mobile phase Hex/IsopOH (0.1% DEA) (98/2, v/v); flow rate 1.5 mL/min; λ = 275 nm (UV/visible).

Even though the increase of the concentration led to a slight deployment in the second peak of T (**Figure 6**), the baseline separation was not prejudice. In this manner, the first fractions were collected according to retention times of the enantiomers: from 12 to 16 min for the first one (F1), 16 to 17.5 min for the second one (F2) and 17.5 to 27.5 min for the third (F3), according to UV/visible detection. Five injections of T standard (10

mg/mL) were performed and collected to three different round-bottom glass flasks. After collecting all the fractions, the remaining solvent was evaporated in the rotary evaporator (close to dryness) and reinjected. The fraction chromatograms obtained are displayed in **Figure 7**.

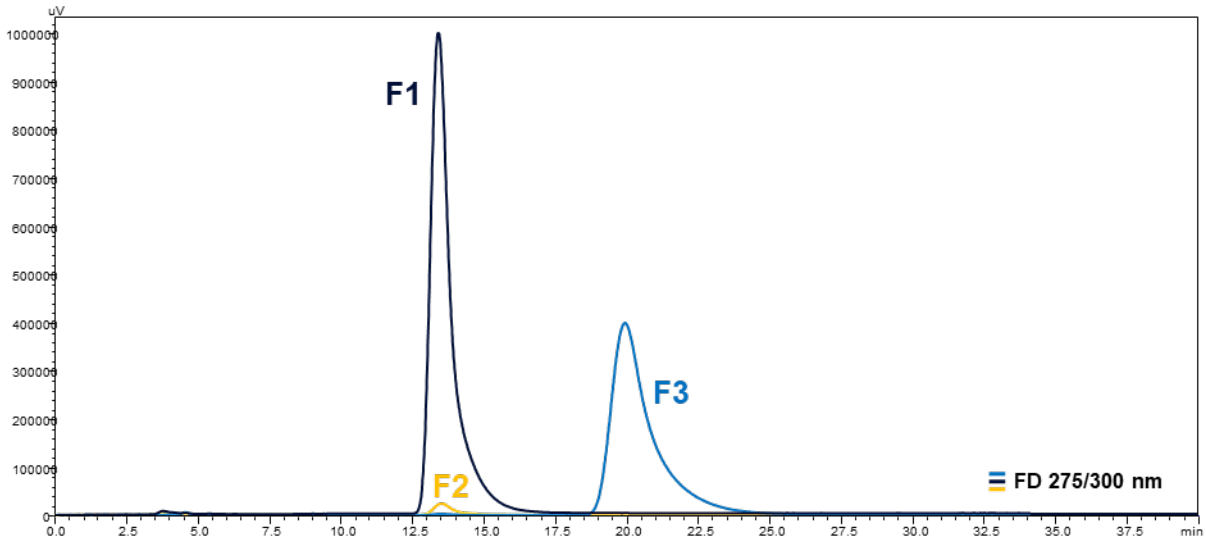

Figure 7. Comparison of chromatograms obtained for the collected fractions (**F1**, F2 and **F3**) of T at a concentration of 10 mg/mL in EtOH (5 injections). Conditions: mobile phase Hex/IsopOH (0.1% DEA) (98/2, v/v); flow rate 1.5 mL/min; $\lambda_{exc/em}$ = 275/300 nm (fluorescence); λ = 275 nm (UV/visible).

The fractions that correspond to the separated enantiomers (F1 and F3) were enantiomerically pure (**Figure 7**). In face of these results, new and higher concentrated T standards were prepared in EtOH (15, 20, 25, 30, 50, 60, 65, 70, 80 mg/mL) (**Figure 8**). The final concentration used for collecting fractions was set on 70 mg/mL and the same procedure used for the prior concentration was applied.

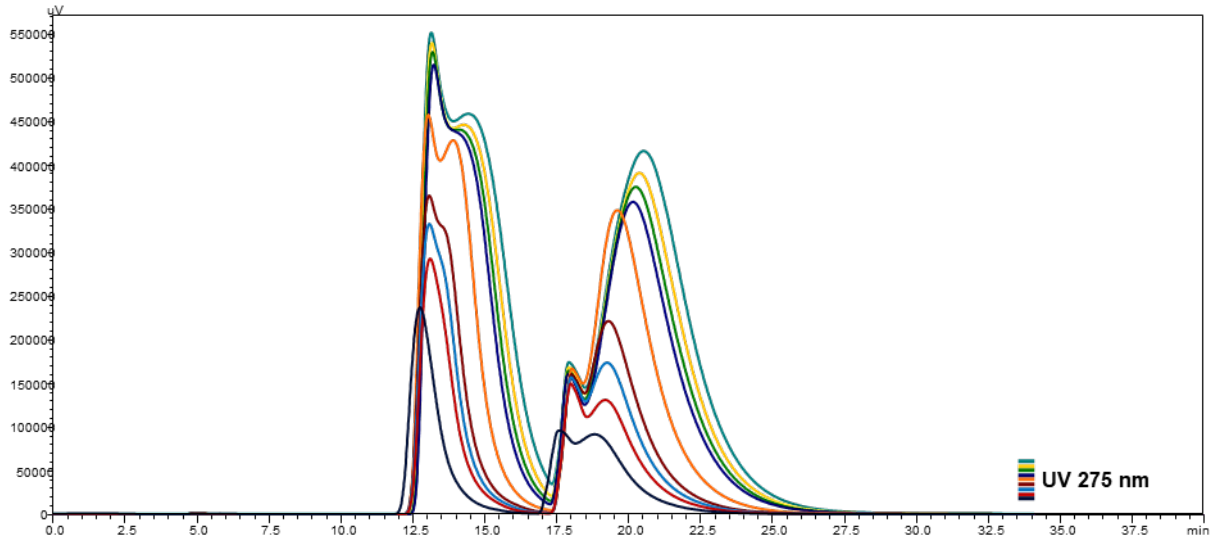

Figure 8. Comparison of chromatograms obtained for T at different concentrations in EtOH (**15**, **20**, **25**, **30**, **50**, **60**, **65**, **70** and **80** mg/mL). Conditions: mobile phase Hex/IsopOH (0.1% DEA) (98/2, v/v); flow rate 1.5 mL/min; λ = 275 nm (UV/visible).

Figure 9 shows the chromatograms obtained for the collected enantiomers of T and consequent evaporation purification procedures at a final concentration of 70 mg/mL, after 72 injections. In theory, since a racemate consists in identical amounts of two enantiomers, each injection contributes with 0.7 mg of racemate T gives approximately 0.35 mg of each enantiomer. After 72 injections, 25.2 mg of each T enantiomer were expected. After evaporating the solvent until dryness and weighing the glass flasks with the solid obtained, the following mass was obtained: 30.2 mg for the first enantiomer and 20.6 mg for the second. Recovery percentages were calculated and are presented in **Table 8**.

Table 8. Recovery percentages obtained for T enantiomers after 72 injections.

PARAMETER	ENANTIOMER 1	ENANTIOMER 2
Nominal mass (mg)	25.2	25.2
Experimental mass (mg)	29.3	18.1
Recovery (%)	116	72

According to **Table 8**, the first enantiomer was not pure, since it presented a recovery of 116%. Compound losses are usual in this type of technique and further procedures should be made in order to evaluate purity more precisely.

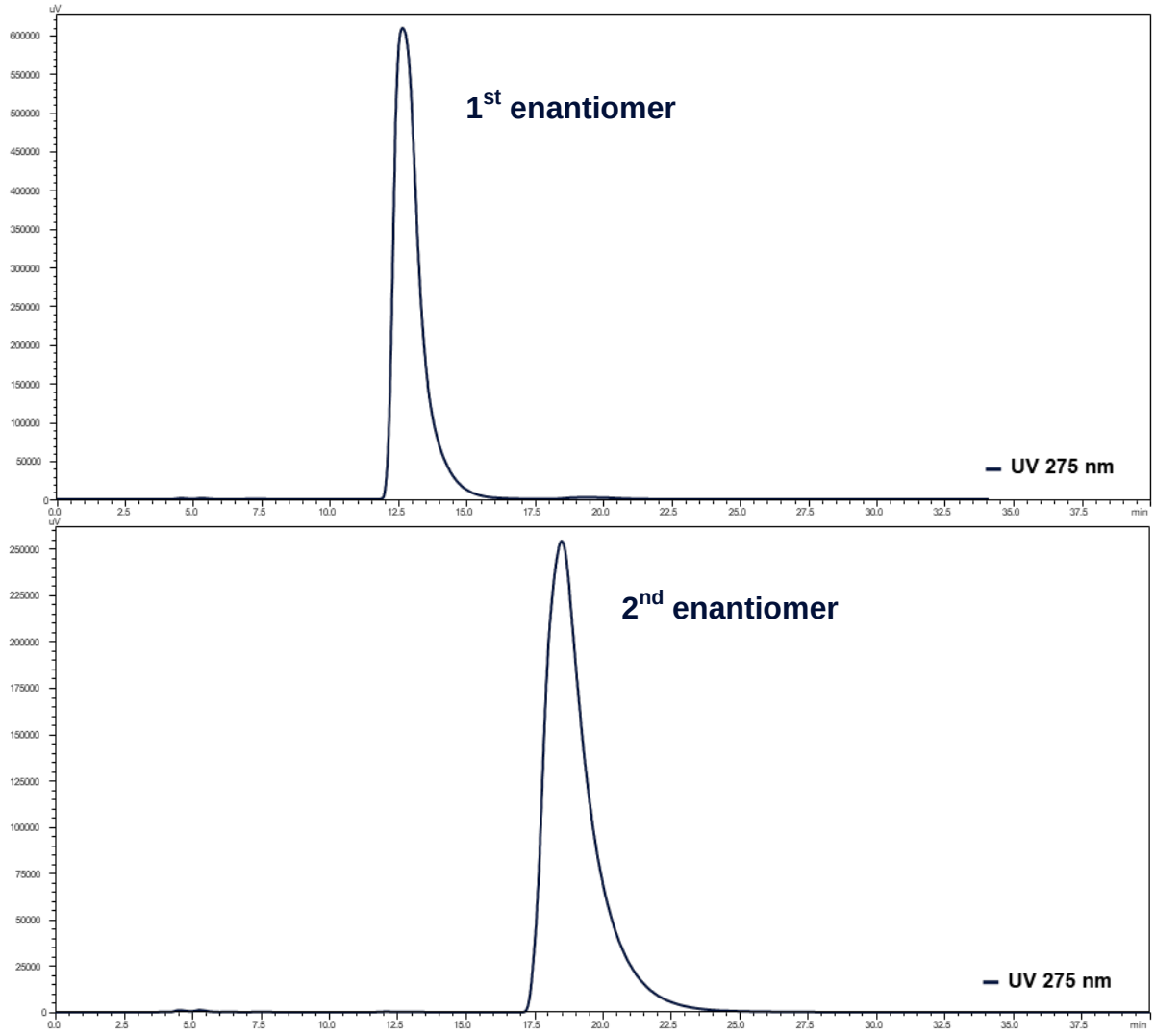

Figure 9. Chromatograms obtained for the collected enantiomers of T in EtOH (72 injections). Conditions: mobile phase Hex/IsopOH (0.1% DEA) (98/2, v/v); flow rate 1.5 mL/min; λ = 275 nm (UV/visible).

3.2 Development and validation of the enantioseparation method of T and its metabolites *O*-DT and *N*-DT

Different types of CSPs are commercially available [83,84]. The chiral column used in the development of the LC-FD method for the enantioseparation of T and its metabolites was Lux Cellulose-4. This column is a polysaccharide derivative CSP with a

broad application in methods developed to analyse chiral pharmaceuticals and can be used in normal, reversed and polar elution modes [45]. Its linear chlorinated cellulose phenylcarbamate structure provides specific interactions that influence the CSPs ability to separate and discriminate between enantiomers [85]. Silva *et al*. (2017) reported the enantioseparation of T and its main metabolites *O*-DT and *N*-DT using a Lux Cellulose-4 CSP (**Figure 10**) under isocratic elution, with a mobile phase of 0.1% DEA in hexane/ethanol (96/4, v/v) and a flow rate of 0.7 mL/min [53]. These conditions were also used in this work as a starting point for the optimization of the method, as for the semi-preparative LC (**Figure 11**).

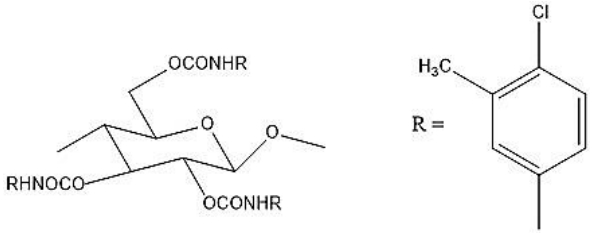

Figure 10. Chemical structure of Lux Cellulose-4 CSP.

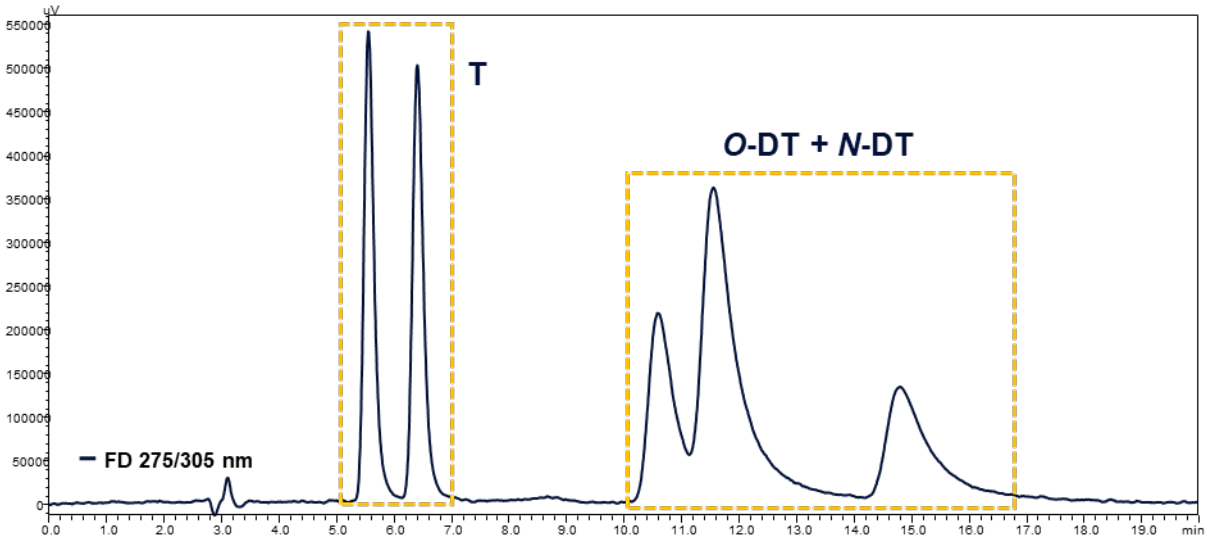

Figure 11. Chromatogram obtained for T, *O*-DT and *N*-DT at a concentration of 5 μg/mL in EtOH. Conditions: mobile phase Hex/EtOH (0.1% DEA) (96/4, v/v); flow rate 0.7 mL/min; $\lambda_{exc/em}$ = 275/305 nm.

Contrarily to what was expected, *O*-DT and *N*-DT did not present separation under the published conditions [53], since the peaks of *O*-DT were overlapped with the ones of *N*-DT (**Figure 11**). In an effort of improving chromatographic resolution, the EtOH in the mobile phase was replaced by IsopOH. Considering that the enantioseparation for the two metabolites still did not occur (data not shown), the next trial was carried out under reverse phase mode of elution.

According to the same author, a mobile phase with good resolution for T and *O*-DT is ACN (0.05% DEA)/ H_2O (35/65, v/v), with an injection volume of 10 µL and a flow rate of 0.5 mL/min [86]. Standards mixture with a concentration of 1 µg/mL was prepared in MM and injected. Separation of T and *N*-DT was not achieved (**Figure 12**) and so, different percentages of DEA were tested, in order to understand its influence. This modification did not improve enantiomeric separation of the three compounds (data not presented). After varying the volume proportions in the mobile phase and still not obtain better results, the organic solvent in the mobile phase was replaced by MeOH. Separation of T and its metabolites did not occur under these conditions (data not shown).

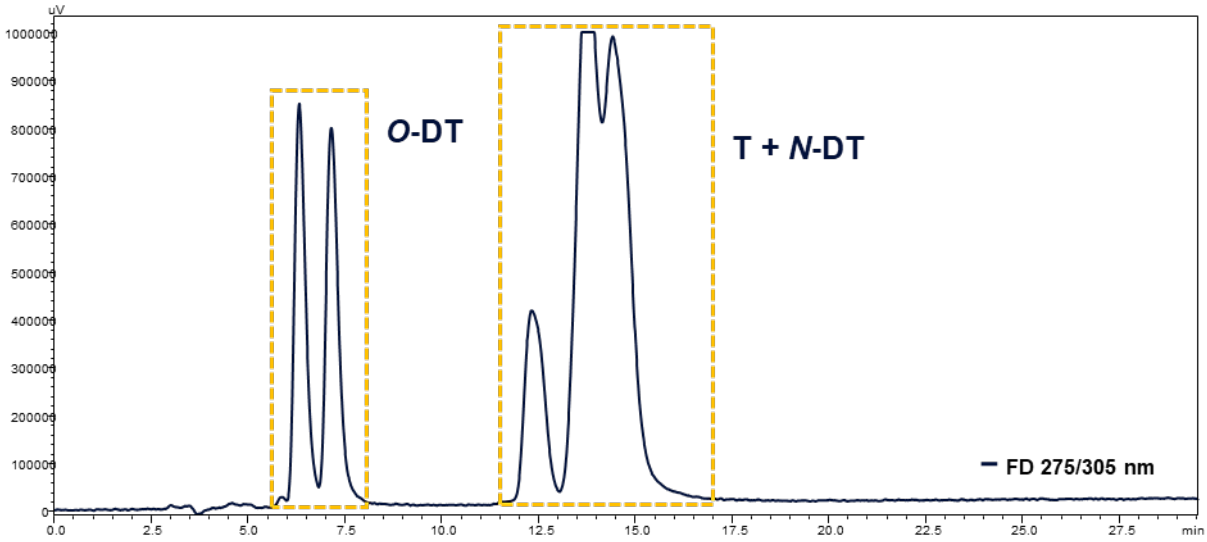

Figure 12. Chromatogram obtained for T, *O*-DT and *N*-DT at a concentration of 1 µg/mL in MM. Conditions: mobile phase ACN (0.05% DEA)/H_2O (35/65, v/v); flow rate 0.5 mL/min; $\lambda_{exc/em}$ = 275/305 nm.

Other attempts for improving enantioseparation were tested according to conditions investigated by Silva, C. (2016) (see Table 4 in Section 2.3) [86]. The mixture of two organic solvents, as ACN and EtOH, in equal proportions and the addition of ammonium formate to the mobile phase led to good enantioseparation of T and *O*-DT. Variations on the mobile phase volume proportions were attempted in order to choose the most advantageous (data not presented). The same chromatographic run, *N*-DT did not present baseline resolution in optimized condition and it was not included in the validation of the method (**Figure 13**). The optimized conditions for T and *O*-DT are presented in **Table 9**. The retention times obtained for *O*-DT and T enantiomers were 11, 13, 29 and 32 minutes, respectively.

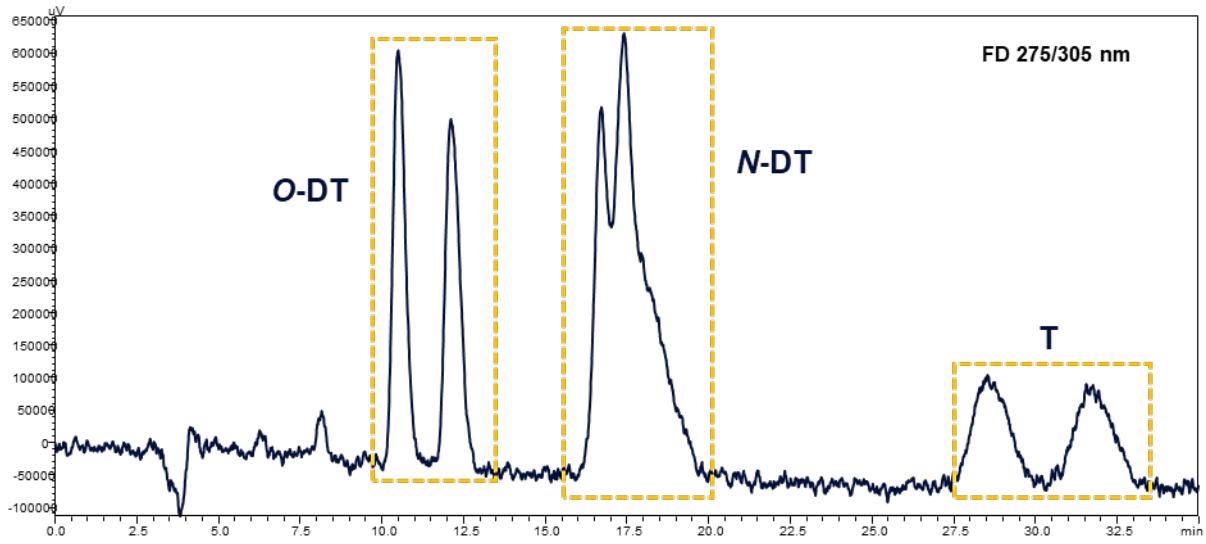

Figure 13. Chromatogram obtained for T, *O*-DT and *N*-DT at a concentration of 1 µg/mL in MM under optimized conditions. Conditions: mobile phase ACN:EtOH:DEA (10mM ammonium formate) (50:50:0.1)/ H_2O (38/62, v/v); flow rate 0.5 mL/min; $\lambda_{exc/em}$ = 275/305 nm.

Table 9. Optimized conditions of the analytical method developed for T and *O*-DT enantioseparation.

PARAMETER	VALUES
Mobile phase (v/v)	ACN:EtOH:DEA (10mM ammonium formate) (50:50:0.1)/ H_2O (38/62)
Injection volume	10 µL
Column oven temperature	25ºC
Autosampler tray temperature	15ºC
Flow rate	0.5 mL/min
Fluorescence detection wavelengths	275 nm (excitation), 305 (emission) Sensitivity High 4x

Order of elution of the compounds was determined by injecting known standards of racemate and of each enantiomer under the same mobile phase conditions. The R_{Ts} obtained were 11, 13, 29 and 32 min for (–)-*O*-DT, (+)-*O*-DT, (–)-T and (+)-T, respectively.

3.2.1 Method validation

The main goal of validating an analytical method is to demonstrate that the process used is suitable to the purpose required, giving an overall knowledge on the capabilities of the procedure [77]. In this work, to validate de LC-FD method developed and optimized to enantioseparate the enantiomers of T and of its main metabolite *O*-DT, the following parameters were determined: LOD, LOQ, linearity, selectivity, precision, accuracy and recovery.

3.2.1.1 Limits of detection (LOD) and quantification (LOQ)

According to the International Conference on Harmonization Q2A, the LOD 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 LOQ is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy [87]. In this work, LOD and LOQ were calculated based on the signal-to-noise ratio. Determination of this 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 [77]. Signal-to-noise ratios of 3:1 and 10:1 are typically considered acceptable for estimating the LOD and LOQ, respectively. **Table 10** shows the instrumental LOD and LOQ values of each enantiomer of T and *O*-DT in MM. These values proved to be suitable for monitoring of the target enantiomers during the biodegradation progression.

Table 10. Limits of detection and quantification of each enantiomer of T and *O*-DT.

ANALYTE	ENANTIOMER	LOD (ng/mL)	LOQ (ng/mL)
T	(−)-T	40	125
	(+)-T	40	125
O-DT	(−)-*O*-DT	15	40
	(+)-*O*-DT	15	40

3.2.1.2 Linearity

The linearity of an analytical procedure consists in its ability (within a given range) to obtain results that are directly proportional to the concentration (amount) of analyte in the sample [87]. The range referred is the interval between the upper and the lower

concentrations (amounts) of analyte in the sample, including these concentrations, for which it has been demonstrated that the method has a suitable level of precision, accuracy and linearity [77,87]. According to ICH Q2B, for the establishment of linearity of an analytical method a minimum of five different concentrations is recommended [77]. If there is a linear relationship, the results should be statistically analysed by appropriate mathematical methods, such as a linear regression.

In order to obtain the calibration curves for each enantiomer, seven different nominal racemate concentrations for each compound were used: 250, 300, 350, 400, 450, 500, 600 ng/mL for T; and 80, 110, 150, 200, 250, 300 and 350 ng/mL for *O*-DT. Each concentration were prepared and injected in triplicate. The calibration curves for T and *O*-DT enantiomers, as well as the linear regression equations, are represented in **Figures 14 to 17**. **Table 11** summarizes the linearity data obtained for all analytes. Linearity was achieved with a good correlation coefficient for all compounds ($R^2 \geq 0.996$).

Table 11. Linearity data obtained for T and *O*-DT enantiomers in the HPLC-FD method.

ANALYTE	RANGE (ng/mL)	CALIBRATION CURVE	CORRELATION COEFFICIENT (R^2)
(−)-T	125 - 300	$y = 51264x + 543748$	0.9974
(+)-T	125 - 300	$y = 49028x + 557871$	0.9960
(−)-*O*-DT	40 – 175	$y = 70523x + 372692$	0.9995
(+)-*O*-DT	40 – 175	$y = 7 \times 10^7 x + 327723$	0.9989

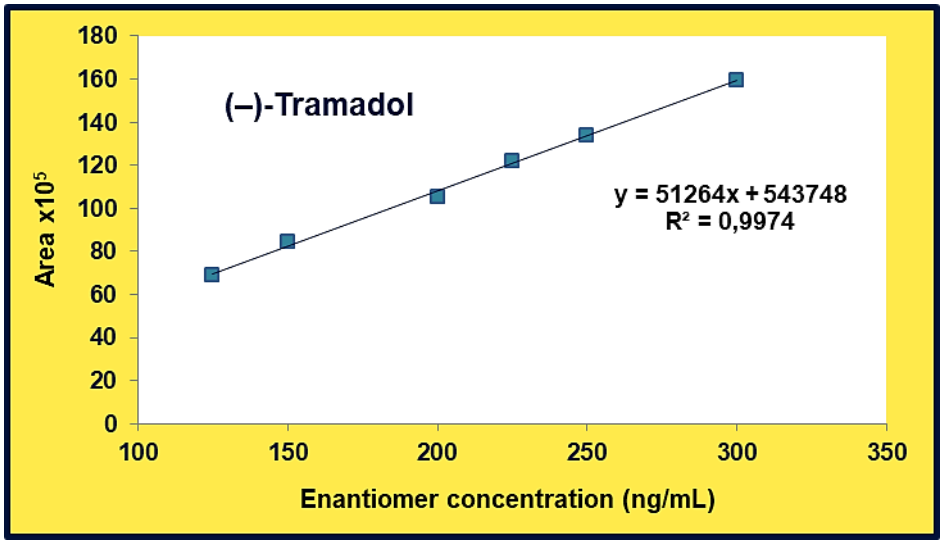

Figure 14. Calibration curve and linear regression for (−)-T.

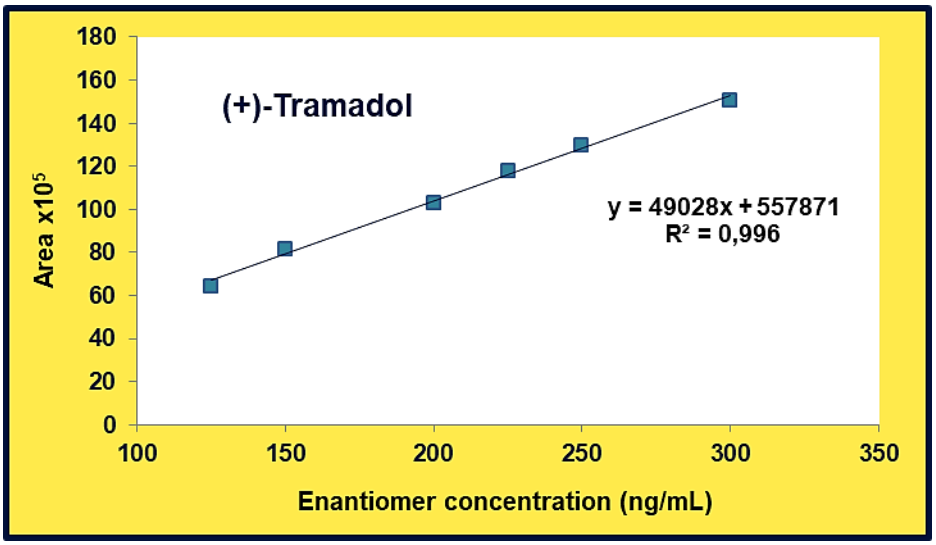

Figure 15. Calibration curve and linear regression for (+)-T.

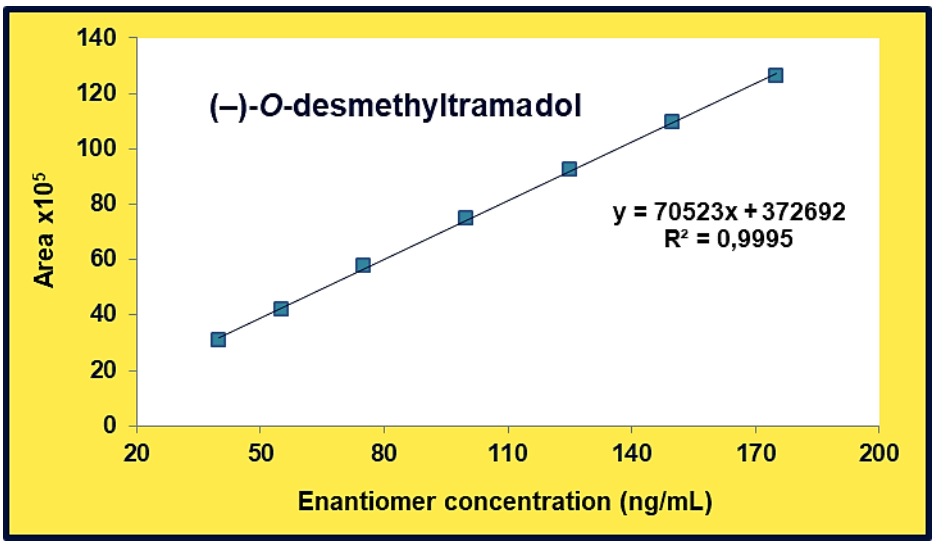

Figure 16. Calibration curve and linear regression for (–)-*O*-DT.

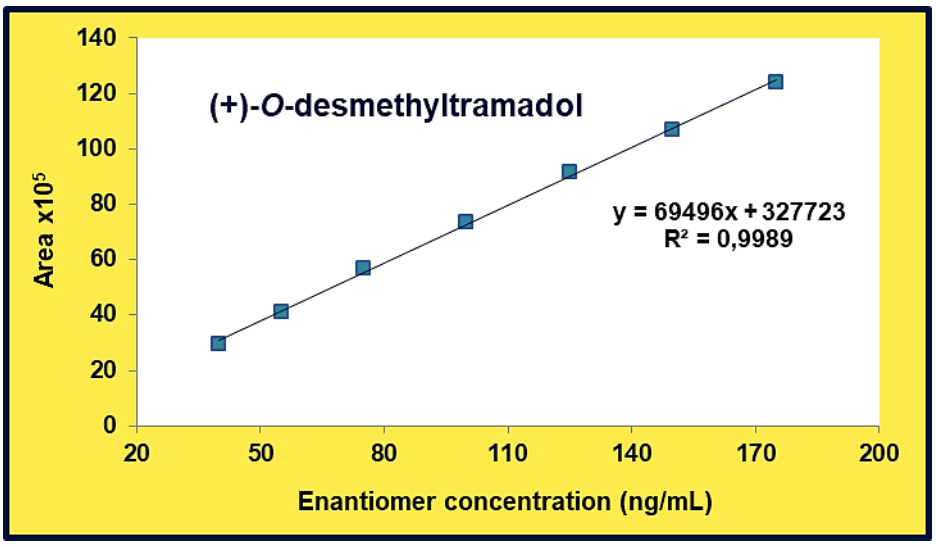

Figure 17. Calibration curve and linear regression for (+)-*O*-DT.

3.2.1.3 Selectivity

Selectivity (or specificity) is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, matrix interferences, etc. [87]. It is important to evaluate this parameter in environmental studies due to the potential presence of other substances in the matrix. Selectivity of the method was verified through comparison of chromatograms of the standard compounds with those of spiked matrix (AS inoculum) in MM. The nominal racemate concentrations used for T and *O*-DT were 450 and 310 ng/mL, respectively. Chromatograms of the standards in ultrapure water and in MM, in order to understand if the MM had any influence in the separation of the compounds, as well as those of the spiked matrix in the presence and in the absence of AS inoculum after 2h-incubation, are represented in **Figures 18 and 19** to verify matrix interferentes.

Preparation of standards mixture in ultrapure water or in MM did not influence the stability, the separation of T and O-DT and did not showed interferences in the same retention times of the analytes (**Figures 18 and 19**).The method was validated preparing the standards in MM. Although the matrix content included several ionisable salts present in the medium, these salts did not interfere with the chromatographic performance of the method.

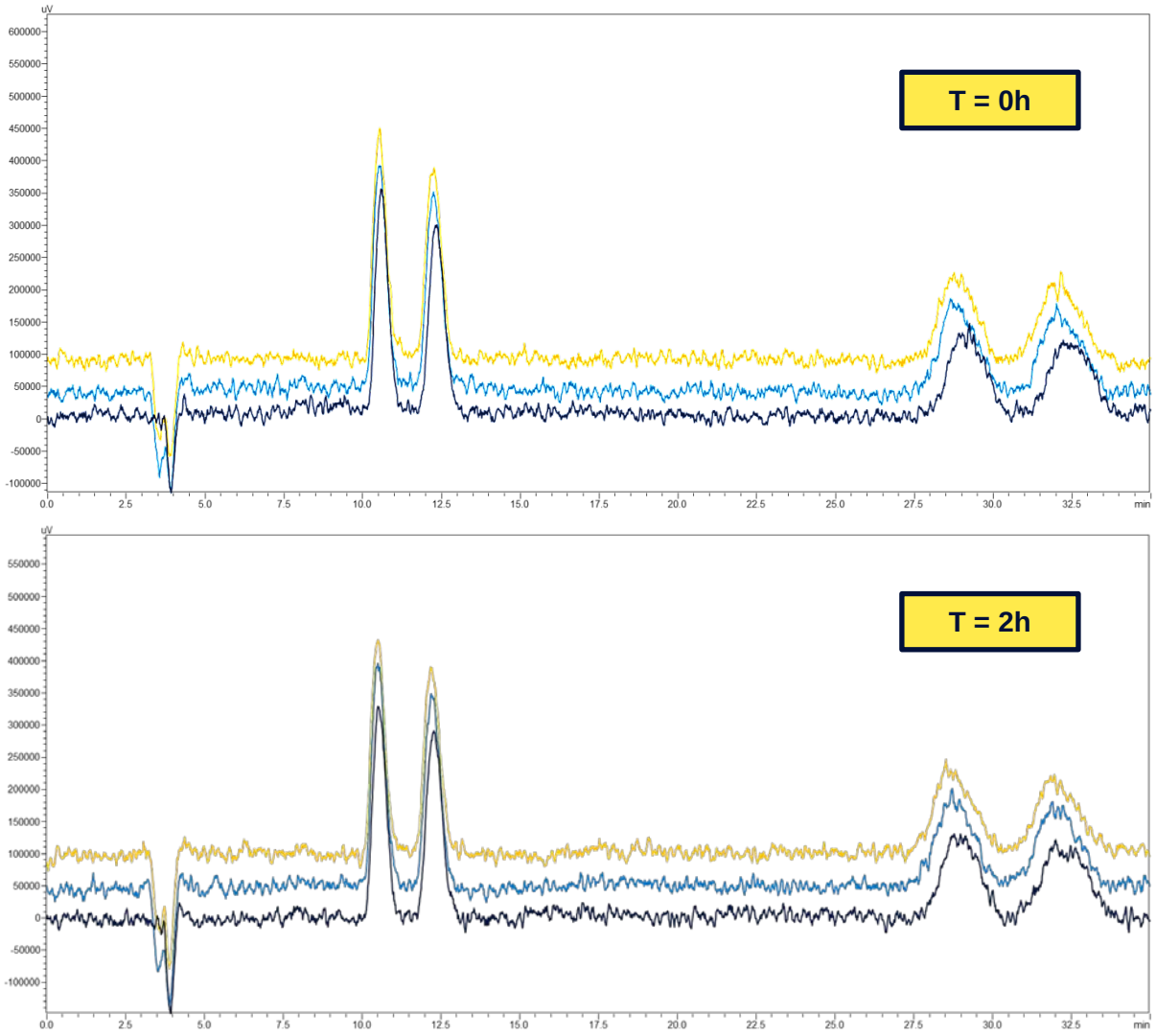

Figure 18. Comparison of the chromatograms of samples of T and *O*-DT prepared in ultrapure water (**dark blue**) and in MM with (**yellow**) and without AS inoculum (**light blue**), before and after 2h-incubation at 110 rpm. Conditions: mobile phase ACN:EtOH:DEA (10mM ammonium formate) (50:50:0.1) / H_2O (38/62, v/v); flow rate 0.5 mL/min; column oven temperature 20ºC; $\lambda_{exc/em}$ = 275/305 nm.

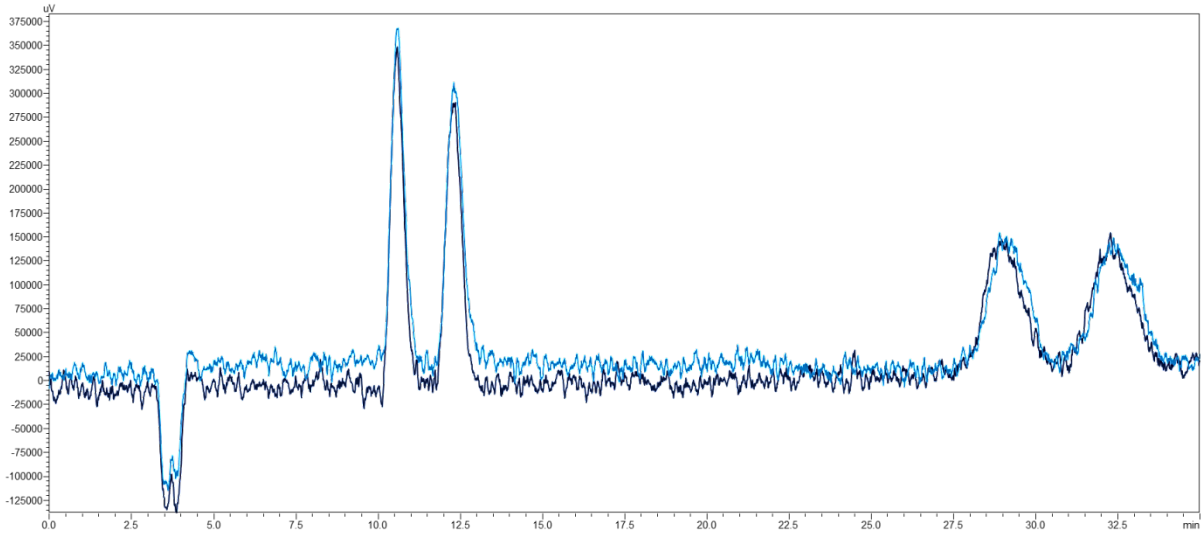

Figure 19. Comparison of the chromatograms of the standards mixture of T and *O*-DT prepared in ultrapure water (**dark blue**) and in MM (**light blue**). Conditions: mobile phase ACN:EtOH:DEA (10mM ammonium formate) (50:50:0.1) / H_2O (38/62, v/v); flow rate 0.5 mL/min; column oven temperature 20ºC; $\lambda_{exc/em}$ = 275/305 nm.

3.2.1.4 Precision

According to the International Conference in Harmonization Q2A, 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 [87]. Precision may be evaluated at three levels: repeatability (or intra-day precision), intermediate (or inter-day) precision and reproducibility, and it is usually expressed as the variance, standard deviation or coefficient of variation of a series of data [77,87]. In this work, precision was determined considering repeatability, which expresses the precision under the same operating conditions over a short period of time, and inter-day precision, that expresses within-laboratories variations (in this case, different days).

Both intra- and inter-day precisions were evaluated using nine determinations covering the linear range of the procedure and expressed as relative standard deviation percentage (% RSD). Three different QCs were prepared and injected in triplicate for both nominal racemate compounds: 320, 420 and 550 ng/mL for T; 130, 230 and 330 ng/mL for *O*-DT. Intra-day precision was studied by analysing nine determinations for each quality control solution, all performed in the same day, while inter-day precision was evaluated in three different days. **Table 12** summarizes the obtained values of % RSD for T and *O*-DT enantiomers for both levels of precision studied.

The results show that repeatability (intra-day precision) ranged from 1.42 and 2.32% for T and from 1.60 and 2.67% for *O*-DT enantiomers. Values for inter-day precision were lower than 5.10 and 4.88% for T and *O*-DT enantiomers, respectively. For both compounds, the % RSD values obtained are according to those demanded by international guidelines (under 20%) [77], which means that the developed method is precise.

3.2.1.5 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value determined by the method and the nominal concentration of the analyte, expressed in percentage [87]. Like precision, accuracy should be evaluated across the specified range of the method, using a minimum of nine determinations over a minimum of three concentration levels (e.g. 3 concentrations/ 3 replicates each) [77]. The concentrations used to evaluate accuracy were the same as for precision, also in triplicate. Results obtained are summarized in **Table 12**.

According to International Conference on Harmonization Q2B, accuracy percentage values for pharmaceutical compounds should be between 70 and 130% [77]. The values obtained ranged from 91 to 105% and from 91.8 to 102% for T and *O*-DT enantiomers, respectively, showing that the method presents accuracy within acceptable values.

3.2.1.6 Recovery

Recovery assessment was used to estimate the potential losses of the analytes by sorption to biomass, which might affect their quantification in the biodegradation assays employed. The recovery percentage was obtained comparing the peak area of each analyte after the extraction process with those of standard solutions prepared at the same concentrations in solvent. The three quality control standard concentrations used for precision and accuracy were also applied to recovery, each prepared in triplicate. Table 13 shows the recovery percentages for each enantiomer of T and *O*-DT.

For T enantiomers, recovery ranged from 98.4 and 100%; for *O*-DT enantiomers the values varied between 99.4 and 101% (**Table 12**). Therefore, the developed method showed excellent recovery rates.

Table 12. Intra- and inter-day precisions, accuracy and recovery obtained for T and *O*-DT enantiomers in the HPLC-FD method.

Analyte	Nominal enantiomer concentration (ng/mL)	Enantiomer	1ST Day		2ND Day		3RD Day		Inter-day RSD (%)	Recovery (%)	
			Intra-day RSD (%)	Accuracy (%)	Intra-day RSD (%)	Accuracy (%)	Intra-day RSD (%)	Accuracy (%)		Dead cells	Live cells
T	160	(–)-T	2.96	104	1.89	97.8	2.00	101	3.35	98.7	98.6
		(+)-T	3.57	103	1.33	95.3	2.07	97.4	3.97	98.5	98.4
	210	(–)-T	0.86	105	2.38	93.0	1.03	101	5.10	100	99.6
		(+)-T	1.59	101	2.75	91.0	1.47	99.5	4.81	100	99.2
	275	(–)-T	1.78	103	1.61	98.9	1.98	98.2	2.71	100	100
		(+)-T	2.04	102	2.56	97.1	1.77	97.1	2.96	100	100
O-DT	65	(–)-*O*-DT	2.91	102	3.44	92.2	1.68	94.9	4.70	99.6	101
		(+)-*O*-DT	1.83	98.8	2.10	88.5	3.21	92.2	4.88	99.4	100
	115	(–)-*O*-DT	1.51	101	2.76	92.5	1.60	97.1	3.84	100	101
		(+)-*O*-DT	1.79	100	2.89	91.8	1.62	96.5	3.98	101	100
	165	(–)-*O*-DT	1.80	101	1.67	95.9	2.12	98.4	2.76	101	101
		(+)-*O*-DT	1.29	99.2	1.19	95.6	2.30	96.3	2.28	100	100

3.3 Biodegradation assays

The validated enantioselective chromatographic method was applied to monitor the biodegradation assays of T and its enantiomers using an AS inoculum. Compounds were added individual and separately at an initial nominal concentration of 500 ng/mL and 250 ng/mL for racemate and enantiomers, respectively, into MM inoculated with AS inoculum, in the absence and in the presence of an additional carbon source, sodium acetate. The selected inoculum (AS from aerated tanks of a municipal WWTP) presents an elevated microbial diversity and potential of previous exposure to such compounds, being the ideal model to this type of assays [80].

In order to construct the biodegradation patterns for each compound, the concentration of T and its enantiomers were monitored by the enantioselective validated LC method and converted into biodegradation % according to the following formula:

$$\%\,\mathrm{BD_t} = 100 - \left[\frac{\mathrm{C_t}}{\mathrm{C_0}} \times 100\right]$$

where $\%\,\mathrm{BD_t}$ is the biodegradation percentage at day t; $\mathrm{C_t}$ is the concentration at day t and $\mathrm{C_0}$ is the initial concentration (day 0). **Table 13** presents the O.D. measured at 600 nm (OD_{600nm}) and the biodegradation percentage obtained from the equation above for racemate T and for its enantiomers, as well as for the abiotic control assays. The biodegradation patterns monitored during 21 days are shown in **Figures 20 and 21**.

Table 13. Data obtained from the biodegradation assays.

		DAYS	0	1	2	6	7	9	12	14	16	19	21
Abiotic controls	Without AS inoculum	OD_{600nm}	0.003	0.001	0.000	0.000	0.002	0.000	0.000	0.000	0.003	0.116	0.105
		%BD (-)-T	0.0	0.6	0.6	0.4	0.8	0.0	0.1	0.2	0.2	0.8	0.5
		%BD (+)-T	0.0	1.0	1.5	0.1	0.2	0.6	0.1	0.7	0.4	0.7	0.5
	With dead AS cells	OD_{600nm}	0.224	0.211	0.208	0.233	0.257	0.256	0.243	0.241	0.240	0.246	0.235
		% BD (-)-T	0.0	0.1	0.4	37.5	2.1	10.5	5.4	2.5	9.0	6.0	9.1
		% BD (+)-T	0.0	0.3	0.4	39.5	1.2	15.2	4.2	2.0	11.0	4.3	12.9
rac-T	Without acetate	OD_{600nm}	0.237	0.256	0.176	0.071	0.100	0.078	0.079	0.074	0.060	0.081	0.053
		%BD (-)-T	0.0	6.9	10.0	18.0	18.7	21.1	28.9	25.6	24.9	27.3	29.9
		%BD (+)-T	0.0	8.6	12.4	17.4	19.4	24.3	29.2	20.4	24.3	25.0	28.6
	With acetate	OD_{600nm}	0.242	0.250	0.132	0.086	0.105	0.068	0.120	0.064	0.088	0.064	0.062
		% BD (-)-T	0.0	9.3	19.0	26.3	30.1	31.9	34.7	24.6	35.1	38.7	32.0
		% BD (+)-T	0.0	10.2	18.7	25.3	29.6	33.2	34.8	23.5	35.6	38.8	32.1
(-)-T	Without acetate	OD_{600nm}	0.236	0.252	0.140	0.081	0.099	0.088	0.069	0.073	0.083	0.083	0.052
		% BD	0.0	0.3	12.6	12.8	19.6	22.8	24.2	21.9	24.9	28.5	25.3
	With acetate	OD_{600nm}	0.233	0.334	0.179	0.082	0.081	0.080	0.072	0.115	0.069	0.075	0.079
		% BD	0.0	16.8	9.3	24.0	15.7	30.5	29.0	33.4	28.4	32.5	30.5
(+)-T	Without acetate	OD_{600nm}	0.213	0.265	0.161	0.082	0.078	0.080	0.079	0.071	0.055	0.052	0.070
		%BD	0.0	3.0	10.9	22.5	29.0	25.0	25.4	29.7	30.8	31.0	31.7
	With acetate	OD_{600nm}	0.234	0.266	0.157	0.084	0.084	0.081	0.062	0.128	0.070	0.090	0.072
		%BD	0.0	10.3	15.3	16.3	16.4	25.7	26.3	25.1	26.9	29.0	29.0

(OD_{600nm}: optical density at a wavelength of 600 nm; % BD: biodegradation percentage)

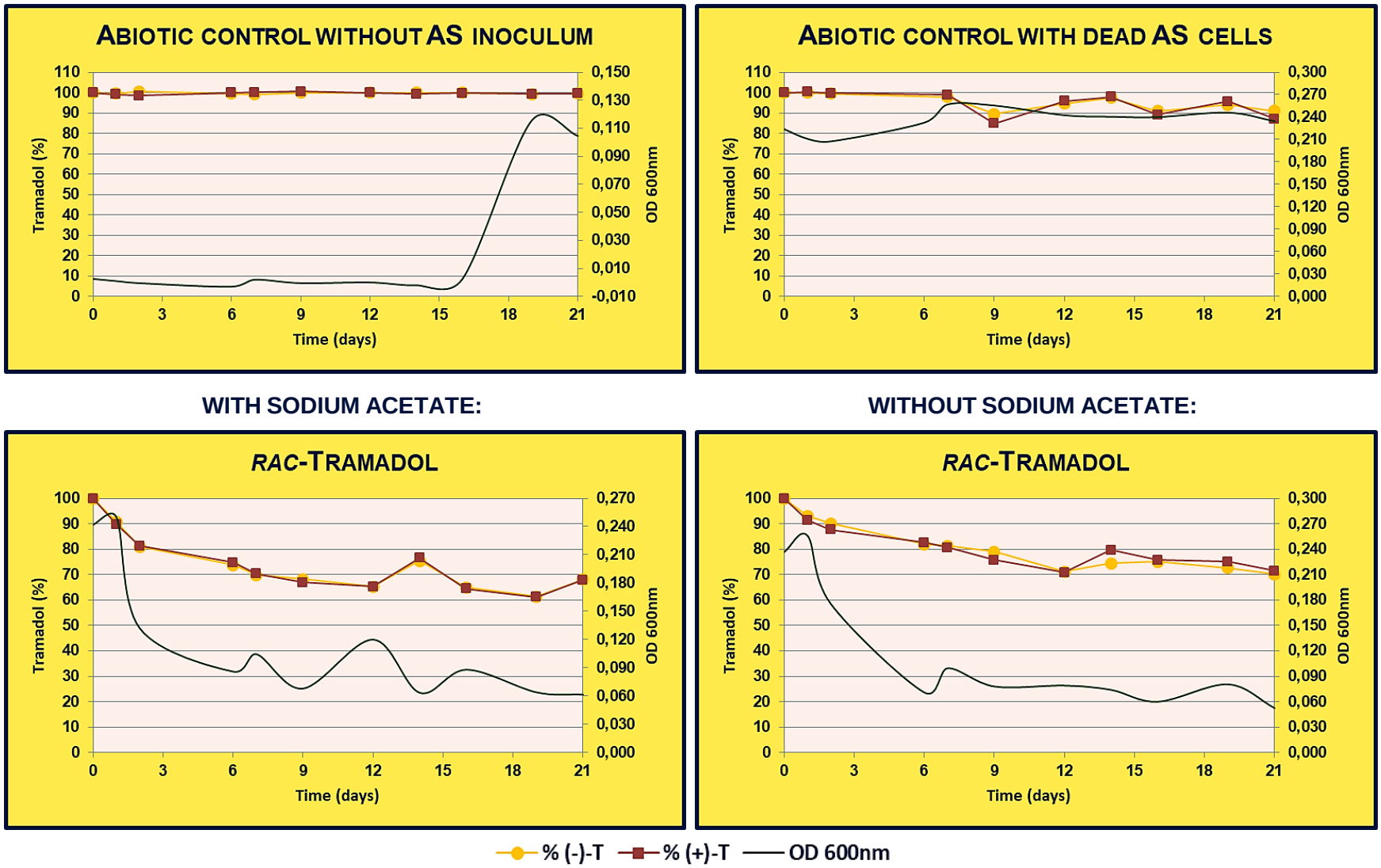

Figure 20. Biodegradation patterns for the abiotic control assays (with and without AS inoculum) and for racemate T.

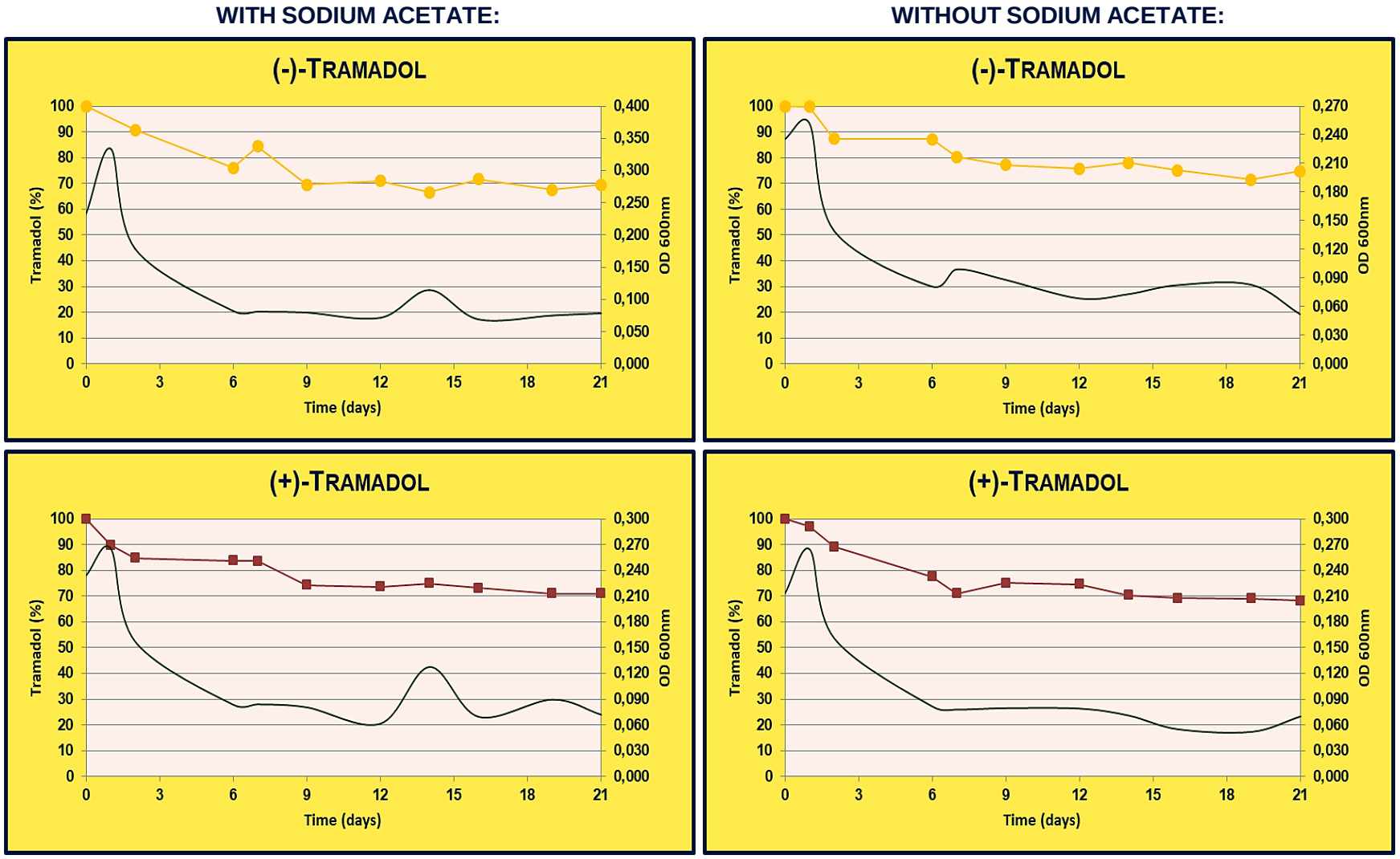

Figure 21. Biodegradation patterns for T enantiomers (-)-T and (+)-T.

According to the results presented in **Table 13** and in **Figures 20** and **21**, biodegradation occurred faster in the first 6 days, corresponding to about 50% of the total degradation reached at the end of the experiments. After that, biodegradation occurred slowly until the end of the assay. In the presence of an additional carbon source, the compounds in general were consumed faster than when in the absence of sodium acetate. This may be due to a higher metabolic activity of the cells when in presence of a readily available growth substrate.

Regarding racemate T (**Figure 20**), it suffered a biodegradation up to 29% increasing this value in about 10% when added sodium acetate. Both enantiomers present similar biodegradation patterns, being the total degradation only 2 to 3% higher in the presence of acetate. In the assays with isolated T enantiomers (**Figure 21**), the biodegradation levels were also identical, with and without acetate, ranging between 29 and 33% at the end of the study. For both enantiomeric compounds, the enantioselectivity of the degradation process was the same in the presence of an additional energy source as in its absence. For the (+)-enantiomer, biodegradation without acetate was slightly superior in the end of the study (≈3%). This can be negligible and justified with data quantification errors.

In the abiotic control assays without AS inoculum, only a small extent of degradation of T occurred (under 2%), which suggests that the degradation levels observed in the biotic conditions are mediated only by microbial activity. However, in the control with dead AS cells, the biodegradation pattern is a little irregular but with very low degree of removal.

In this study, the AS inoculum was able to partially degrade T and its enantiomers, but no significant enantioselective pattern was observed. When dealing with low concentration of an organic compound, the addition of an easily degradable carbon source like sodium acetate may reduce the threshold level needed to activate degradation enzymes [79,88].

Regarding O.D. of the abiotic control assays, the results were not as expected. In the non-inoculated assays, OD values increased significantly after day 16. This happens when there is growth of microbial activity, which may indicate an outside contamination of the flasks in question. However, biodegradation levels were not affected by this possible contamination. Contrarily, in the assays with the enantiomers OD varied identically alongside with biodegradation, which makes it even harder to explain the punctual alteration in the control experiments.

3.4 Ecotoxicity assays

3.4.1 *Daphnia magna* acute immobilization test

Daphnia magna is a freshwater crustacean adopted as a test organism by the Organization for Economic Cooperation and Development (OECD) and by the United States Environmental Protection Agency (US EPA). It is widely used in ecotoxicological studies due to its short lifecycle, high sensitivity to a variety of chemicals and ease of manipulation in the laboratory [89-91]. Additionally, its transparency allows for the observation of inner anatomical structures and its asexual reproduction by parthenogenesis permits the generation of clones [91], being these two characteristics important for chronic toxicity studies. In this work, *D. magna* was used in acute toxicity assays performed with the DAPHTOXKIT F^{TM} *MAGNA* kit, based on the OECD guideline 202 – "Daphnia sp. Acute Immobilisation Test".

The DAPHTOXKIT F^{TM} *MAGNA* makes use of the dormant eggs to allow acute toxicity tests to be performed without the need of culturing and maintenance of live stocks of *D. magna* organisms. In order to check the correct execution of the test procedure and the sensitivity of the test animals, a quality control test was performed with the reference chemical potassium dichromate. The mortality percentages were calculated based on the number of dead or immobilized neonates, versus that of the actively swimming test organisms in each well. From the data obtained, a 24h EC_{50} and a 48h EC_{50} was calculated using the specific DAPHTOXKIT computer program provided alongside with the kit. The reference test data is presented in **Table 14** and in **Figure 22**.

Table 14. Number of immobile *Daphnias* for both 24 and 48h exposures obtained for the reference test (each exposure A, B, C, D is one replicate).

		CONTROL		C1		C2		C3		C4		C5	
Compound	**Exposure (h)**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**
$K_2Cr_2O_7$	A	0	1	0	0	1	2	1	4	4	5	4	5
	B	0	1	0	1	1	3	3	4	5	5	5	5
	C	0	0	0	1	2	2	4	5	5	5	5	5
	D	0	0	0	0	0	1	1	3	5	5	5	5
	Total	**0**	**2**	**0**	**2**	**4**	**8**	**9**	**16**	**19**	**20**	**19**	**20**
	% effect	**0**	**10**	**0**	**10**	**20**	**40**	**45**	**80**	**95**	**100**	**95**	**100**

(C1 = 0.32 mg/L; C2 = 0.56 mg/L; C3 = 1 mg/L; C4 = 1.8 mg/L; C5 = 3.2 mg/L)

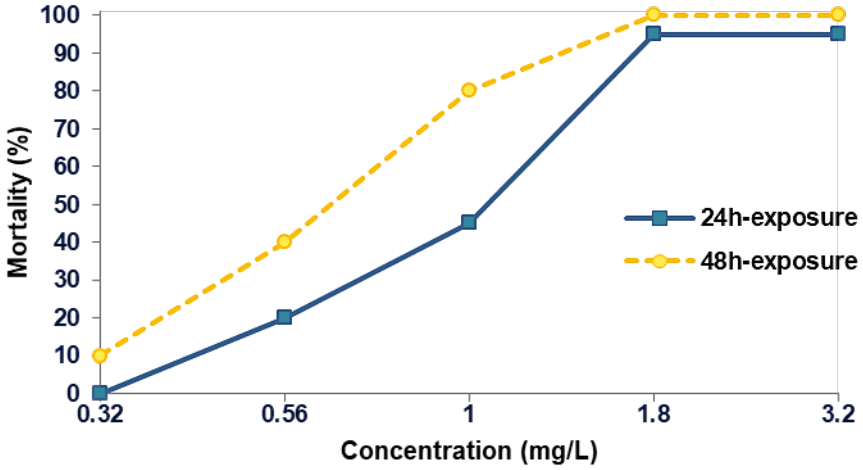

Figure 22. Toxicity of dichromate potassium in *D. magna* (reference test).

According to the results presented in **Table 14**, the reference test was considered valid for both time exposures since it fulfils the criterion needed: the number of dead plus immobile organisms did not exceeded 10% in the controls. As expected, higher concentrations led to higher toxicity for *D. magna* (**Figure 22**). According to ISO 6341, the 24h EC_{50} calculated for potassium dichromate is acceptable if its value is situated in the range 0.6 – 2.1 mg/L. The values obtained for 24h EC_{50} and 48h EC_{50} were 1.00 mg/L and 0.69 mg/L, respectively. The reference test is valid and demonstrated the correct execution of the test procedure, as well as the good sensitivity of the test animals.

Mortality results for the racemate compounds (T, *O*-DT, *N*-DT) and for T and *O*-DT enantiomers are reported in **Tables 15** and **16**, respectively.

Table 15. Number of immobile *Daphnias* for both 24 and 48h exposures obtained for racemate compounds (each exposure A, B, C, D is one replicate).

		CONTROL		C1		C2		C3		C4		C5	
Compound	**Exposure (h)**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**
rac-T	A	0	0	0	1	0	0	0	0	0	1	0	1
	B	0	1	0	1	0	1	0	0	0	0	0	1
	C	0	1	0	0	0	1	0	0	0	0	0	1
	D	0	0	0	0	0	0	0	1	0	0	0	1
	Total	0	2	0	2	0	2	0	1	0	1	0	4
	% effect	0	10	0	10	0	10	0	5	0	5	0	20
rac-ODT	A	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	1	0	0	0	0
	C	0	1	0	0	0	1	0	0	0	0	0	1
	D	0	0	0	0	0	0	0	1	0	0	0	0
	Total	0	1	0	0	0	1	0	2	0	0	0	1
	% effect	0	5	0	0	0	5	0	10	0	0	0	5
rac-NDT	A	0	0	0	1	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0	1	0	1	0	0
	D	0	0	0	0	0	0	0	1	0	0	0	0
	Total	0	0	0	1	0	0	0	2	0	1	0	0
	% effect	0	0	0	5	0	0	0	10	0	5	0	0

(C1 = 0.2 µg/L; C2 = 2 µg/L; C3 = 20 µg/L; C4 = 200 µg/L; C5 = 2000 µg/L)

Table 16. Number of immobile *Daphnias* for both 24 and 48h exposures obtained for T and *O*-DT enantiomers (each exposure A, B, C, D is one replicate).

		CONTROL		C1		C2		C3		C4		C5	
Compound	**Exposure (h)**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**
(-)-T	A	0	0	0	1	0	0	0	0	0	0	1	2
	B	0	0	0	1	0	0	0	0	0	0	0	1
	C	0	0	0	1	0	2	0	0	0	0	1	1
	D	0	0	0	0	0	1	0	0	0	0	1	1
	Total	0	0	0	3	0	3	0	0	0	0	3	5
	% effect	0	0	0	15	0	15	0	0	0	0	15	25
(+)-T	A	0	0	0	0	0	0	0	1	0	0	0	0
	B	0	0	1	1	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	1	1	2	0	0	0	0
	D	0	0	0	1	1	1	0	0	0	0	0	0
	Total	0	0	1	2	1	2	1	3	0	0	0	0
	% effect	0	0	5	10	5	10	5	15	0	0	0	0
(-)-ODT	A	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0	1	0	0	0	0
	D	0	0	0	1	0	0	0	1	1	1	0	0
	Total	0	0	0	1	0	0	0	2	1	1	0	0
	% effect	0	0	0	5	0	0	0	10	5	5	0	0
(+)-ODT	A	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	1	0	0
	C	0	0	0	0	0	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0	0	0	1	0	0
	% effect	0	0	0	0	0	0	0	0	0	5	0	0

(C1 = 0.1 µg/L; C2 = 1 µg/L; C3 = 10 µg/L; C4 = 100 µg/L; C5 = 1000 µg/L)

All the experiments were considered valid, since mortality of all controls did not exceed 10%. In a 24h-exposure, T and its enantiomers did not present toxicity for *D. magna*. Higher immobilization effect was observed for racemate T after 48h exposure to the most concentrated T solution analysed (2000 µg/L) (**Table 15**). The same pattern was observed for the assays with (–)-T at this concentration, where 15% of the organisms were found immobile after 24h and an additional 10% after 48h (**Table 16**). The smaller concentrations of this enantiomer also showed toxicity after a 48h exposure. For (+)-T,

toxicity was observed only for the three least concentrated solutions (0.1, 1 and 10 μg/mL). Additional replicates should be performed in order to confirm these results.

The effect percentages obtained for racemate *O*-DT, (−)-*O*-DT, (+)-*O*-DT and *N*-DT were less than or equal to the ones of the respective controls (**Table 15**), which indicates that in these conditions, these compounds did not present toxicity for *D. magna*. Though additional replicates should be performed in order to confirm the results of *rac*-T and of its enantiomers, *rac*-T seems to be more toxic to *D. magna* than its metabolite *O*-DT.

3.4.2 *Tetrahymena thermophila* growth inhibition test

Tetrahymena thermophila is a freshwater protozoan ciliate that plays a key role in the recycling of organic matter. Ciliates are a main component of benthic microfauna communities and are, next to bacteria, the second most important group of biota in activated sludge of WWTPs.

After incubation of *T. thermophila* in the dark at 30ºC, the OD was measured at a wavelength of 440 nm. For the test to be acceptable the OD of the controls after a 24h-incubation must show a decrease of the initial value by at least 60% (i.e. the final OD shall be 40% or less of the initial OD). If this doesn't occur, a second OD scoring of all the test cells must be attempted 2 to 4h later. If the new OD readings of the controls fulfil the 60% decrease criterion, this indicates a slightly slower growth of the ciliates, which does not affect the test results. Consequently, the bioassay is still considered as being valid on the basis of the second set of OD values. In this study, the criterion was not fulfilled after a 24h-incubation for all the compounds, so new readings were attempted 4h later.

In order to check the correct performance and sensitivity of the test organisms, a quality control assay was performed with the reference chemical potassium dichromate. Despite its low toxicity to *Tetrahymena*, this negatively charged metal complex has a wide acceptance as a reference chemical in ecotoxicological testing. From the data obtained, a 24h EC_{50} was calculated using the specific PROTOXKIT computer program provided alongside with the kit. The reference test data is presented in **Table 17**.

Table 17. OD_{440nm} values obtained for the *T. thermophila* growth inhibition reference test.

	Exposure time (h)	OD_{440nm}						
		Replicates	Control	C1	C2	C3	C4	C5
$K_2Cr_2O_7$	0	1	0.7230	0.8130	0.6690	0.7090	0.6710	0.6530
		2	0.6830	0.7830	0.6810	0.7020	0.6610	0.6620
		Mean	0.7030	0.7980	0.6750	0.7055	0.6660	0.6575
	24	1	0.3740	0.7780	0.6180	0.6690	0.6320	0.5870
		2	0.3670	0.7370	0.6570	0.6620	0.6430	0.5430
		Mean	0.3705	0.7575	0.6375	0.6655	0.6375	0.5650
	28	1	0.2730	0.7980	0.6510	0.6880	0.6350	0.3310
		2	0.2550	0.7840	0.6700	0.6670	0.6300	0.3220
		Mean	0.2640	0.7910	0.6605	0.6775	0.6325	0.3265

(C1 = 56 mg/L; C2 = 32 mg/L; C3 = 18 mg/L; C4 = 10 mg/L; C5 = 5.6 mg/L)

According to the results presented in **Table 17**, the reference test was considered valid since it fulfils the criterion needed: the OD of the controls suffered a decrease of the initial value by 63% after 28h-incubation. Inhibited culture growth is reflected by remaining turbidity, since the cells cannot clear the substrate into ciliate biomass, which is translated in relatively constant OD values. In the reference test performed, culture cells were only capable of converting the substrate in the presence of the lowest concentration studied (5.6 mg/L), due to the high toxicity of potassium dichromate. The 24h EC_{50} value calculated for this compound was 5.48 mg/L, which is close to the one provided in the kit (7.21 mg/L) and it is within the confidence limits presented (1.47 – 19.79 mg/L). The reference test is not only acceptable, but it demonstrated the correct execution of the test procedure, as well as the good sensitivity of the test animals.

OD values obtained for the studied compounds, racemates and enantiomers, are presented in **Tables 18** and **19**. From these values, the inhibition percentages were calculated using the following equation:

$$\%\ \text{growth inhibition} = \left(1 - \frac{\Delta \mathrm{OD}_{(\mathrm{C}_n)}}{\Delta \mathrm{OD}_{\mathrm{C0}}}\right) \times 100$$

where $\Delta \mathrm{OD}_{(\mathrm{C}_n)}$ represents the difference in optical density for each concentration (n = 1, 2, 3, 4, 5) between the end and the beginning of the test and $\Delta \mathrm{OD}_{\mathrm{C0}}$ is the difference in optical density in the control tests. The results are summarized in **Table 20**.

Table 18. OD_{440nm} values obtained for the *T. thermophila* growth inhibition test with racemate compounds.

	Exposure time (h)	OD_{440nm}						
		Replicates	Control	C1	C2	C3	C4	C5
rac-T	0	1	0.7520	0.7880	0.8140	0.7880	0.7820	0.8130
		2	0.7780	0.8050	0.7810	0.8340	0.8340	0.7950
		Mean	0.7650	0.7965	0.7975	0.8110	0.8080	0.8040
	24	1	0.3490	0.6270	0.5280	0.5060	0.5180	0.5590
		2	0.3470	0.6480	0.5240	0.6820	0.6550	0.6080
		Mean	0.3480	0.6375	0.5260	0.5940	0.5865	0.5835
	28	1	0.2550	0.3980	0,3619	0.2450	0.2840	0.3580
		2	0.2380	0.4380	0,3590	0.4940	0.4190	0.3650
		Mean	0.2465	0.4180	0,3605	0.3695	0.3515	0.3615
rac-*O*-DT	0	1	0.6527	0.6403	0.6604	0.6543	0.6574	0.6767
		2	0.6280	0.6590	0.6590	0.6603	0.6755	0.6519
		Mean	0.6404	0.6497	0.6597	0.6573	0.6665	0.6643
	24	1	0.2939	0.1532	0.1486	0.1826	0.1761	0.1811
		2	0.2859	0.1654	0.2930	0.4088	0.1439	0.1635
		Mean	0.2899	0.1593	0.2208	0.2957	0.1600	0.1723
	28	1	0.2169	0.1291	0.1242	0.1326	0.1740	0.1793
		2	0.2394	0.1521	0.2089	0.2664	0.1523	0.1703
		Mean	0.2282	0.1406	0.1666	0.1995	0.1632	0.1748
rac-*N*-DT	0	1	0.7550	0.7679	0.7710	0.7915	0.7874	0.7097
		2	0.7811	0.7678	0.7903	0.7909	0.8096	0.7566
		Mean	0.7681	0.7679	0.7807	0.7912	0.7985	0.7332
	24	1	0.4466	0.5493	0.5481	0.6033	0.5486	0.5395
		2	0.5195	0.5200	0.5645	0.5731	0.5883	0.5609
		Mean	0.4831	0.5347	0.5563	0.5882	0.5685	0.5502
	28	1	0.2589	0.4430	0.4449	0.5069	0.4653	0.4499
		2	0.3197	0.2881	0.4295	0.4763	0.4608	0.4223
		Mean	0.2893	0.3656	0.4372	0.4916	0.4631	0.4361

(C1 = 2000 µg/L; C2 = 200 µg/L; C3 = 20 µg/L; C4 = 2 µg/L; C5 = 0.2 µg/L)

Table 19. OD_{440nm} values obtained for the *T. thermophila* growth inhibition test with T and *O*-DT enantiomers.

	Exposure time (h)	OD_{440nm}						
		Replicates	Control	C1	C2	C3	C4	C5
(–)-T	0	1	0.7950	0.8320	0.8380	0.8750	0.8520	0.8380
		2	0.8060	0.8230	0.8270	0.8620	0.8250	0.8350
		Mean	0.8005	0.8275	0.8325	0.8685	0.8385	0.8365
	24	1	0.4560	0.6720	0.6410	0.6720	0.6310	0.6820
		2	0.5040	0.6560	0.6580	0.6960	0.6280	0.6520
		Mean	0.4800	0.6640	0.6495	0.6840	0.6295	0.6670
	28	1	0.2720	0.4830	0.4680	0.5080	0.4460	0.4500
		2	0.3300	0.5180	0.5560	0.5520	0.4490	0.4400
		Mean	0.3010	0.5005	0.5120	0.5300	0.4475	0.4450
(+)-T	0	1	0.7970	0.8210	0.8380	0.8230	0.7890	0.7930
		2	0.8260	0.8190	0.8350	0.8270	0.8250	0.8100
		Mean	0.8115	0.8200	0.8365	0.8250	0.8070	0.8015
	24	1	0.5470	0.6610	0.6890	0.6020	0.5410	0.5770
		2	0.5400	0.6360	0.5770	0.6180	0.6180	0.5540
		Mean	0.5435	0.6485	0.6330	0.6100	0.5795	0.5655
	28	1	0.3220	0.4670	0.4570	0.4180	0.3990	0.3990
		2	0.3201	0.4330	0.3880	0.4460	0.4640	0.3920
		Mean	0.3211	0.4500	0.4225	0.4320	0.4315	0.3955
(–)-*O*-DT	0	1	0.6218	0.6480	0.6351	0.6256	0.6617	0.6537
		2	0.6605	0.6290	0.6398	0.6542	0.6609	0.6316
		Mean	0.6412	0.6385	0.6375	0.6399	0.6613	0.6427
	24	1	0.3339	0.4349	0.2986	0.2911	0.3254	0.3340
		2	0.3431	0.3536	0.3000	0.2650	0.3393	0.3775
		Mean	0.3385	0.3943	0.2993	0.2781	0.3324	0.3558
	28	1	0.2425	0.3336	0.1990	0.1919	0.2149	0.1668
		2	0.2491	0.3019	0.2175	0.1774	0.2388	0.2350
		Mean	0.2458	0.3178	0.2083	0.1847	0.2269	0.2009

(C1 = 1000 µg/L; C2 = 100 µg/L; C3 = 10 µg/L; C4 = 1 µg/L; C5 = 0.1 µg/L)

Table 19. (continuation)

	Exposure time (h)	OD_{440nm}						
		Replicates	Control	C1	C2	C3	C4	C5
(+)-*O*-DT	0	1	0.6500	0.6770	0.6870	0.7010	0.6920	0.6610
		2	0.6940	0.7050	0.6920	0.6900	0.7130	0.6910
		Mean	0.6720	0.6910	0.6895	0.6955	0.7025	0.6760
	24	1	0.3070	0.3250	0.3130	0.3410	0.4120	0.2330
		2	0.3920	0.4540	0.3470	0.3450	0.1810	0.2260
		Mean	0.3495	0.3895	0.3300	0.3430	0.2965	0.2295
	28	1	0.2440	0.2340	0.2170	0.2470	0.2830	0.1860
		2	0.2890	0.2950	0.2350	0.2380	0.1880	0.2140
		Mean	0.2665	0.2645	0.2260	0.2425	0.2355	0.2000
		2	0.2491	0.3019	0.2175	0.1774	0.2388	0.2350
		Mean	0.2458	0.3178	0.2083	0.1847	0.2269	0.2009

(C1 = 1000 µg/L; C2 = 100 µg/L; C3 = 10 µg/L; C4 = 1 µg/L; C5 = 0.1 µg/L)

Table 20. OD decrease and growth inhibition percentages obtained for the *T. thermophila* growth inhibition test.

		CONTROL	C1	C2	C3	C4	C5
rac-T	$\Delta OD_{(0\text{-}28h)}$	0.5185	0.3785	0.4371	0.4415	0.4565	0.4425
	OD decrease (%)	67.78	47.52	54.80	54.44	56.50	55.04
	Growth inhibition (%)	-	27.00	15.71	14.85	11.96	14.66
(−)-T	$\Delta OD_{(0\text{-}28h)}$	0.4995	0.3270	0.3205	0.3385	0.3910	0.3915
	OD decrease (%)	62.40	39.52	38.50	38.98	46.63	46.80
	Growth inhibition (%)	-	34.53	35.84	32.23	21.72	21.62
(+)-T	$\Delta OD_{(0\text{-}28h)}$	0.4905	0.3700	0.4140	0.3930	0.3755	0.4060
	OD decrease (%)	60.43	45.12	49.49	47.64	46.53	50.66
	Growth inhibition (%)	-	24.56	15.59	19.87	23.44	17.22
rac-O-DT	$\Delta OD_{(0\text{-}28h)}$	0.4122	0.5091	0.4932	0.4578	0.5033	0.4895
	OD decrease (%)	64.37	78.36	74.75	69.65	75.51	73.69
	Growth inhibition (%)	-	-23.50	-19.64	-11.06	-22.10	-18.75
(−)-*O*-DT	$\Delta OD_{(0\text{-}28h)}$	0.3954	0.3208	0.4292	0.4553	0.4345	0.4418
	OD decrease (%)	61.67	50.23	67.33	71.14	65.69	68.74
	Growth inhibition (%)	-	18.87	-8.56	-15.15	-9.89	-11.74
(+)-*O*-DT	$\Delta OD_{(0\text{-}28h)}$	0.4055	0.4265	0.4635	0.4530	0.4670	0.4760
	OD decrease (%)	60.34	61.72	67.22	65.13	66.48	70.41
	Growth inhibition (%)	-	-5.18	-14.30	-11.71	-15.17	-17.39
rac-N-DT	$\Delta OD_{(0\text{-}28h)}$	0.4788	0.4023	0.3435	0.2996	0.3355	0.2971
	OD decrease (%)	62.34	52.39	44.00	37.87	42.00	40.52
	Growth inhibition (%)	-	15.97	28.26	37.42	29.93	37.95

All the experiments were considered acceptable, since they all fulfil the decrease criterion, i.e. the controls final OD was 40% or less of the initial one for all compounds (**Table 20**). The concentrations applied to these assays were environmental concentrations, i.e. possible to be found in the environment. At these amounts, T and its metabolites, as well as the respective enantiomers, did not show high toxicity for *T.* thermophila. Also, values of EC_{50} were not possible to obtain since at these environmental concentrations the higher growth inhibition (%) was up to 38%.

There was a slight difference in toxicity between the two T enantiomers (**Figure 23**). Values obtained for (−)-T were higher than for (+)-T (except for 1 µg/L), which may indicate a certain enantioselectivity of the protozoan to the toxicity of these enantiomers,

with the sensitivity to the (–) enantiomer being an order of magnitude higher. Also, single enantiomers of T showed higher growth inhibition (%) than the racemate. Additional experiments should be performed in order to confirm this hypothesis.

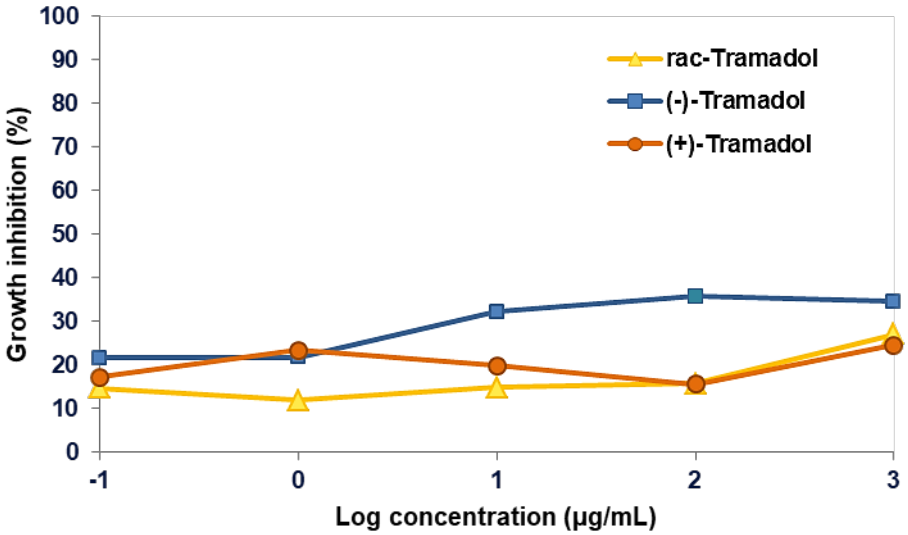

Figure 23. Growth inhibition percentages obtained for T, (–)-T and (+)-T.

Considering *O*-DT, some growth inhibition percentages were negative, which means that not only growth inhibition did not occur, but there was also an increase on the protozoan activity. This showed that not only the compounds in question were not toxic for the tested organisms, but perhaps they had the capability of using these compounds as additional substrate. This happened for racemic *O*-DT and for both enantiomers (–)-*O*-DT and (+)-*O*-DT (**Figure 24**).

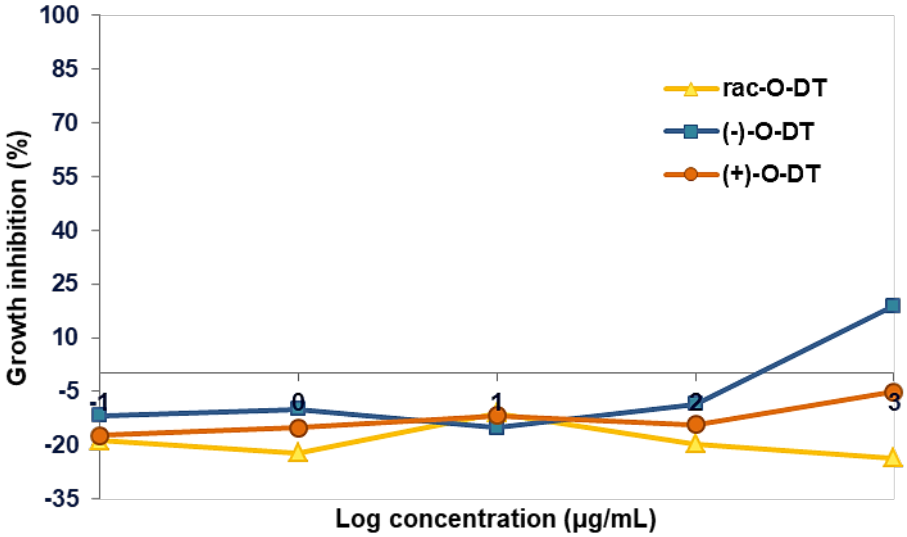

Figure 24. Growth inhibition percentages obtained for *O*-DT, (–)-*O*-DT and (+)-*O*-DT.

For *N*-DT, toxicity was higher than for *O*-DT metabolite (**Figure 25**). According to the data presented, and given the studied conditions, this metabolite showed higher toxicity for *T. thermophila* than O-DT but similar to T enantiomers.

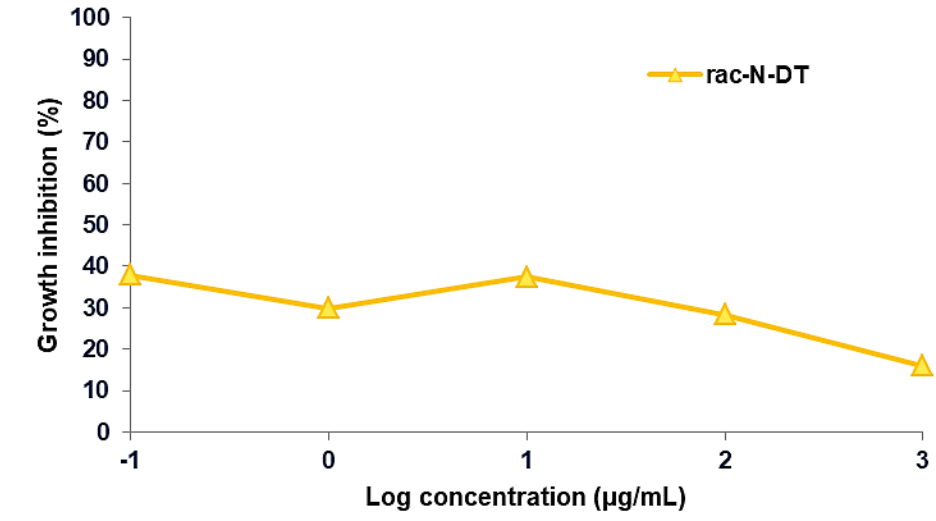

Figure 25. Growth inhibition percentages obtained for *N*-DT.

Chapter 4

CONCLUSIONS AND FUTURE PERSPECTIVES

T enantiomers were successfully separated and collected by a semi-preparative LC method developed in normal phase mode of elution. The optimized chromatographic conditions were the following: mobile phase of Hex/IsopOH/DEA (98/2/0.1, v/v/v), injection volume of 10 μL, flow rate of 1.5 mL/min and column oven temperature of 23ºC.

An LC-FD method was developed and validated for the enantioseparation of T and its primary metabolite *O*-DT. The optimized conditions were achieved with a Lux Cellulose-4 as CSP and ACN:EtOH:DEA (10 mM ammonium formate) (50:50:0.1) / H_2O (38/62, v/v) as mobile phase in isocratic mode. The flow rate was 0.5 mL/min, with an injection volume of 10 μL and a column oven temperature of 20ºC. The retention times obtained for *O*-DT and T enantiomers were 11, 13, 29 and 32 minutes, respectively. The chromatographic was validated in order to follow the biodegradation of the enantiomers of T by AS in different conditions. The validated method showed high selectivity, linearity (R^2 > .99), intra- and inter-day precisions (RSD < 5.1%) and accuracy (93 to 101%). LOD and LOQ were 40 ng/mL and 125 ng/mL for T enantiomers, 15 ng/mL and 40 ng/mL for *O*-DT enantiomers, respectively.

The ability of the AS inoculum to degrade the target compounds, in the absence and in the presence of sodium acetate, was evaluated during 21 days. T and its enantiomers suffered degradation under the studied conditions. Even though the addition of an extra carbon source (sodium acetate) led to a slight increase of overall biodegradation, it did not influence the biodegradation pattern related to enantioselectivity. Both enantiomers presented identical patterns of biodegradation, which might be an indicator that this process is not enantioselective for T.

Ecotoxicity assays performed with *D. magna* and *T. thermophila* showed higher percentages of toxic effect of (-) T than for the (+)-enantiomer, indicating possible enantioselectivity. *O*-DT and its enantiomers did not present significant toxicity for neither the test organisms.

These results are of high importance and demonstrated the importance of considering enantioselectivity when evaluating environmental pollutants toxicity and for risk assessment.

Regarding biodegradation, in fact, this study showed that T is not extensively biodegraded and various reports have demonstrated the occurrence of T in aquatic systems. Also, *N*-DT showed higher toxicity than *O*-DT metabolite, which indicates the importance to investigate the occurrence of *N*-DT in surface waters, its biodegradation and to evaluate *N*-DT toxicity to other ecological aquatic relevant organisms and the possibility of the enantioselectivity in the ecotoxicity.

As future perspectives it would be interesting to investigate the possible ecotoxicological effects of other T metabolites, besides *O*-DT and *N*-DT, such as *N*,*O*-didesmethyltramadol (M5). Biodegradation studies performed with AS samples from other WWTPs (different regions) would also be an important support to the data here obtained. The identification of the metabolites in the biodegradation process is another challenge for further studies.

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APPENDIX A

molecules

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Review

Chiral Drug Analysis in Forensic Chemistry: An Overview

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Abstract: Many substances of forensic interest are chiral and available either as racemates or pure enantiomers. Application of chiral analysis in biological samples can be useful for the determination of legal or illicit drugs consumption or interpretation of unexpected toxicological effects. Chiral substances can also be found in environmental samples and revealed to be useful for determination of community drug usage (sewage epidemiology), identification of illicit drug manufacturing locations, illegal discharge of sewage and in environmental risk assessment. Thus, the purpose of this paper is to provide an overview of the application of chiral analysis in biological and environmental samples and their relevance in the forensic field. Most frequently analytical methods used to quantify the enantiomers are liquid and gas chromatography using both indirect, with enantiomerically pure derivatizing reagents, and direct methods recurring to chiral stationary phases.

Keywords: chiral drugs; forensic chemistry; enantiomers; pharmaceuticals; illicit drugs

1. Introduction

Chiral compounds are asymmetric three dimensional molecules with one or more stereogenic centers or asymmetry originated by planes or axis that gives two non-superimposable mirror images molecules, called enantiomers [1]. In an achiral environment, a pair of enantiomers shares similar physical and chemical properties, however, in a chiral environment such as living organisms, enantiomers may exhibit different biological activities and/or toxicity due to enantioselective interactions [2–4]. Separation of enantiomers has gained relevance in forensic chemistry and has been applied in the analysis of biological fluids, environmental samples and in the control of illicit drug preparations [5–9]. Figure 1 summarizes the applications of chiral analysis in forensic chemistry.

APPENDIX B

Biodegradation studies of tramadol and its metabolites using an activated sludge inoculum followed by enantioselective liquid chromatography

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Chiral pharmaceuticals are part of a major group of emerging environmental pollutants due to their increasing consumption levels and continuous release to the environment [1,2]. Tramadol (T) is a centrally acting synthetic analgesic, structurally related to codeine and morphine, indicated for moderate to severe pain [3,4]. It is commercialized as a racemate and it is extensively metabolized in the liver through *O*- and *N*- demethylations and by conjugation reactions [3,5]. Both T enantiomers contribute to the analgesic effect, even though via different mechanisms [3,6]. Its main metabolite is *O*-desmethyltramadol (*O*-DT), which has higher pharmacologically activity than T itself [4,6].This work presents a validated enantioselective liquid chromatographic method developed to evaluate the biodegradation of T and its metabolites by activated sludge inoculum. The optimized conditions were achieved using a Lux Cellulose-4 column (150 x 4.6 mm i.d.; 3 µm particle size) and guard column (4.4 x 3.0 mm i.d.) under isocratic elution. The method was validated for the enantiomers of T and its main metabolite *O*-DT with high selectivity, linearity ($r^2 > 0.99$), intra-day and inter-day precisions (RSD < 5.1%) and accuracy. Detection and quantification limits were 0.08 µg mL^{-1} and 0.25 µg mL^{-1} for T, 0.03 µg mL^{-1} and 0.08 µg mL^{-1} for *O*-T, respectively. The method was applied to a 21-days biodegradation study concerning racemate T and both enantiomers. Racemate T suffered a biodegradation up to 29%, increasing this value in about 10% when added an additional carbon source (sodium acetate). Both T enantiomers biodegradation levels were similar, with and without acetate, ranging between 29 and 33% at the end of the study.

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Biodegradation studies of tramadol and its metabolites using an activated sludge inoculum followed by enantioselective liquid chromatography

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INTRODUCTION

Chiral pharmaceuticals are part of a major group of emerging environmental pollutants due to their increasing consumption levels and continuous release to the environment [1,2]. The majority of these compounds reach the environment mainly through wastewater treatment plants (WWTPs), which makes microbial biodegradation its main removal pathway from influent wastewaters [3,4]. Microorganisms play an important role in the removal of pharmaceutical residues, since they have the capability to degrade and/or convert such molecules using them as their main carbon source [4]. When in contact with biological systems, enantiomers may present different pharmacodynamics, pharmacokinetic and toxicological properties [5]. For that reason, when studying the biodegradation of a chiral molecule, it is important to understand the behaviour of the racemate and of the enantiomers alone.

Tramadol (**T**) is a centrally acting synthetic chiral analgesic, structurally related to codeine and morphine, indicated for moderate to severe pain (**Figure 1**) [6]. It is commercialized as a racemate and its main metabolite, *O*-desmethyltramadol (***O*-DT**), has higher pharmacologically activity than T itself (**Figure 1**) [6,7].

This work presents a validated enantioselective HPLC-FL (High Performance Liquid Chromatography with Fluorescence detection) method used to monitor the biodegradation of T and its enantiomers in an activated sludge (AS) inoculum during 21 days.

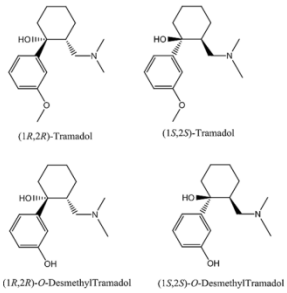


Figure 1: Chemical structure of target compounds.

METHODS AND MATERIALS

CROMATOGRAPHIC CONDITIONS

A HPLC – Shimadzu UFLC Prominence System was used and the optimized conditions were achieved using a Lux Cellulose-4 column (150 x 4.6 mm i.d.; 3 µm particle size) and guard column (4.4 x 3.0 mm i.d.) under isocratic elution.

Column: Lux Cellulose-4

Mobile phase: ACN:EtOH:DEA (10 mM ammonium formate) (50:50:0.1) / H_2O (37/63)

Column oven: 25ºC

Flow rate: 0.5 mL/min

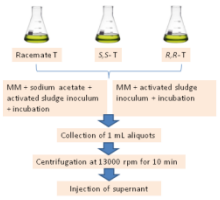
FL Detection: λ_{exc} = 275 nm; λ_{em} = 305 nm (Sensitivity high 4x)

Validation of the method was performed for T and its main metabolite O-DT, according to International Conference on Harmonization (ICH) guidelines. The parameters considered were the following: linearity, selectivity, accuracy, precision, recovery, limits of detection (LOD) and quantification (LOQ).

For method validation, mixture of standards of T and *O*-DT were prepared in minimum salts medium (MM).

BIODEGRADATION STUDY

A 21-days biodegradation study concerning racemate T and both enantiomers was performed in the following conditions:



The biodegradation studies were performed in MM either suplemented with sodium acetate or without and inoculated with an activated sludge (AS) inoculum from WWTP in Parada, Maia, Portugal. AS were previously washed, in order to obtain an optical density of ca. 0.3 at λ = 600 nm. Aliquots were collected every 3 days. Racemate T was used at 500 ng/mL and each enantiomer at 250 ng/mL.

The biodegradation was followed by monitoring the target compounds with the HPLC–FD method .

RESULTS AND DISCUSSION

Tables 1 and 2: Validation data obtained for the HPLC-FL method.

Analyte	Calibration curve	Correlation coefficient (R^2)	LOD [ng/mL]	LOQ [ng/mL]
(1R,2R)-T	$y = 5 \times 10^7 x + 543748$	0,9974	80	250
(1S,2S)-T		0,9960	80	250

Analyte	Concentration [ng/mL]	Recovery (%)	Accuracy (%)	Precision Intra-day %RSD	Precision Inter-day %RSD
(1R,2R)-T	160	98,65	100,88	2,29	3,35
	210	100,21	99,88	1,42	5,10
	275	100,17	100,03	1,79	2,71
(1S,2S)-T	160	98,53	98,52	2,32	3,97
	210	100,05	97,26	1,94	4,81
	275	100,11	98,59	2,12	2,96
(1R,2R)-*O*-DT	65	99,58	96,27	2,67	4,70
	115	100,33	96,72	1,95	3,84
	165	100,57	98,39	1,86	2,76
(1S,2S)-*O*-DT	65	99,35	93,16	2,38	4,88
	115	100,56	96,10	2,10	3,98
	165	100,17	97,03	1,60	2,28

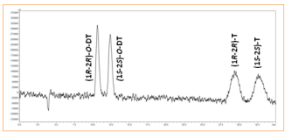


Figure 2: Chromatogram of T and *O*-DT standards (500 ng/mL).

- The retention times for *O*-DT and T enantiomers were 11, 13, 29 and 32 minutes, respectively (**Figure 2**).
- Linearity was achieved with good correlation coefficient for all compounds ($R^2 \geq 0,996$) (**Table 1**).
- Accuracy ranged from 93.2 and 100.9 % and recovery from 98.5 to 100.6 % (**Table 2**).
- *rac*-T suffered a biodegradation up to 29%, increasing this value in about 10% when added sodium acetate.
- Both T enantiomers biodegradation levels were similar, with and without acetate, ranging between 29 and 33% at the end of the study.

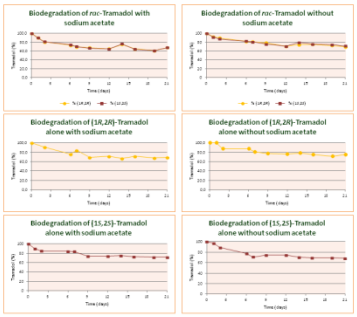


Figure 3: Biodegradation of *rac*-T and its enantiomers (expressed in %) based on quantification by HPLC-FL.

CONCLUSIONS

The HPLC-FD method was validated for the enantiomers of **T** and its main metabolite ***O*-DT** with high selectivity, linearity ($r^2 > 0,99$), intra-day and inter-day precisions (RSD < 5,1%) and accuracy. Detection and quantification limits were 80 ng mL^{-1} and 250 ng mL^{-1} for **T**, 30 ng mL^{-1} and 80 ng mL^{-1} for ***O*-DT**, respectively.

The ability of the AS inoculum to degrade the target compounds, in the absence and in the presence of sodium acetate, was evaluated during 21 days. T and its enantiomers suffered degradation under the studied conditions, even though the addition of an extra carbon source (sodium acetate) did not influence the biodegradation pattern. Both enantiomers presented identical patterns of biodegradation, which might be an indicator that this process is not enantioselective for tramadol.

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