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Ketamine and Norketamine: Enantioseparation, Enantioselective Ecotoxicity and Biodegradation Studies

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Ariana Isabel Pérez Pereira

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Dissertation thesis presented to the University Institute of Health Sciences (IUCS, CESPU) to fulfill the requirements necessary to obtain a Master's Degree in Forensic Laboratory Science and Techniques under the guidance of Professor Doctor Maria Elizabeth Tiritan and Professor Doctor Cláudia Ribeiro.

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DECLARAÇÃO DE INTEGRIDADE

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To my dreams, believes and to the loves of my life,

“The future depends on what we do in the present.”

“It is not non-violence if we merely love those that love us. It is non-violence only when we love those that hate us. I know how difficult it is to follow this grand law of love. But are not all great and good things difficult to do?”

“Carefully watch your thoughts, for they become your words. Manage and watch your words, for they will become your actions. Consider and judge your actions, for they have become your habits. Acknowledge and watch your habits, for they shall become your values. Understand and embrace your values, for they become your destiny.”

“Live as if you were to die tomorrow. Learn as if you were to live forever.”

Mahatma Gandhi

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- A. Pereira, V. Gonçalves, C. Ribeiro, M. E. Tiritan, Multimilligram enantioresolution of norketamine on polysaccharide phase using microporous silica as support, Porto, Portugal, XXIV Encontro Luso Galego de Química, 21-23 November 2018. (Abstract and poster communication in annex 2).

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Oral communication

- A. Pereira, V. Gonçalves, A. S. Maia, C. Ribeiro, M. E. Tiritan, Ketamine and norketamine: enantioseparation, enantioselective ecotoxicity and biodegradation studies, IJUP 2019, 12th Meeting of Young Researches of University of Porto, Porto, Portugal, 13-15 February 2019. (Abstract in annex 4).

ABSTRACT

Ketamine (K), commercialized as a racemate, is used in pediatric and veterinary medicine as an anesthetic. However, it has been abusively used by adolescents and young adults in recreational environments due to its hallucinogenic and sedative effects. K and its main metabolite, norketamine (NK), have been detected either in effluents of wastewater treatment plants (WWTP) and in aquatic environments. Nonetheless, enantioselective biodegradation studies of K as well as enantioselective ecotoxicity of K and NK in diverse aquatic organisms is still unknown.

This work describes the development and validation of an enantioselective liquid chromatography method to quantify the enantiomers of K and NK. The enantiomers were separated using an analytical Lux® 3 µm cellulose-4 column (150 × 4.6 mm internal diameter (I.D.)) under isocratic elution mode. Optimized conditions consisted of ammonium acetate in ultra-pure water (with 0.1 % of diethylamine (DEA)) and acetonitrile (70:30, *v*/*v*) as mobile phase at a flow-rate of 1 mL/min. The method was validated and demonstrated to be precise and accurate with a linearity range of 5 to 50 µg/mL for K and 2.5 to 25 µg/mL for NK. The limit of quantification was 1.25 µg/mL for NK enantiomers and 2.5 µg/mL for K enantiomers. The validated method was employed to follow a 21 days enantioselective biodegradation assay of K by activated sludge (AS). Results showed that K is poorly biodegraded which corroborates to its persistence in the aquatic environment. Also, enantioselectivity biodegradation was not observed and transformation products were not detected.

In order to obtain the pure enantiomers of K and NK for the enantioselective ecotoxicological assays, a semi-preparative enantioseparation method was developed and optimized. The enantioseparation were achieved with an amylose *tris*-3,5-dimethylphenylcarbamate column coated on to APS-Nucleosil (500 A°, 7 µm, 20 %, *w*/*w*; 200 x 70 mm (I.D.)). Optimized conditions allowed recovery of enantiomers higher than 70 %. The enantiomeric purity of the enantiomers was assessed by the analytical method and was close to 100 %, except for K2 (≈ 97 %).

Acute and chronic toxicity assays were performed in two ecological relevant aquatic organisms at different concentrations, including concentrations at environmental level, for racemates and the pure enantiomers of K and NK. The ecotoxicity assays were performed using the crustacean *Daphnia magna* and the protozoan *Tetrahymena thermophila* in accordance with national and international standards (ISO and OECD). NK racemate presented higher mortality for crustacean *D. magna* compared to K. For both compounds mortality increased across gradient of exposure. Considering *T. thermophila,* K demonstrated greater growth inhibition compared to NK. These results demonstrate a species-dependent toxicity. Also, different enantioselective response between enantiomers of K and NK were found at the selected concentrations.

Keywords:
Enantioseparation; Enantioselectivity; Biodegradation Assays; Semi-Preparative Chromatography; *Daphnia magna; Tetrahymena thermophila.*

RESUMO

A cetamina (K), comercializada como racemato, é utilizada na medicina pediátrica e veterinária, como anestésico. No entanto, tem sido usada de forma abusiva por adolescentes e jovens adultos em ambientes recreativos, devido aos seus efeitos alucinogénios e sedativos. K e o seu principal metabolito, a norcetamina (NK), são detetadas nos efluentes de estações de tratamento de águas residuais, e consequentemente, em ambientes aquáticos. No entanto, estudos de biodegradação enantiosseletiva de K, bem como de ecotoxicidade enantiosseletiva de K e NK em diversos organismos aquáticos ainda são desconhecidos.

O presente trabalho descreve o desenvolvimento e validação de um método enantiosseletivo para quantificar os enantiómeros de K e NK por cromatografia líquida. Os enantiómeros foram separados utilizando a coluna analítica, Lux® 3 µm de celulose-4 (150 × 4,6 mm de diâmetro interno (I.D.)) em modo de eluição isocrático. As condições otimizadas foram em acetato de amónio em água ultrapura (com 0,1 % de dietilamina (DEA)) e acetonitrilo (70:30, *v*/*v*) como fase móvel e fluxo de 1 mL/min. O método foi validado e demonstrou ser exato e preciso numa faixa de linearidade de 5 a 50 µg/mL para K e 2,5 a 25 µg/mL para NK. O limite de quantificação foi de 1,25 µg/mL para os enantiómeros de NK e 2,5 µg/mL para os enantiómeros de K. O método validado foi aplicado num ensaio de biodegradação enantiosseletiva de K durante 21 dias em lamas ativadas (AS). Os resultados mostraram que a K foi pouco biodegradada, o que corrobora a sua persistência no meio aquático. Além disso, não se verificou biodegradação enantiosseletiva nem formação de metabolitos ou produtos de transformação.

Com a finalidade de obter os enantiómeros puros de K e NK para os ensaios ecotoxicológicos enantiosseletivos, foi desenvolvido e otimizado um método de enantiosseparação semi-preparativa. A enantiosseparação foi realizada numa coluna de *tris*-3,5-dimetilfenilcarbamato de amilose revestida em APS-Nucleosil (500 A°, 7 µm, 20 %, *w*/*w*; 200 x 70 mm (I.D.)). As condições otimizadas permitiram a recuperação dos enantiómeros acima de 70 %. A pureza

enantiomérica foi avaliada pelo método analítico. Os enantiómeros foram obtidos numa pureza próxima de 100 %, exceto a K2 (≈ 97 %).

Ensaios de toxicidade aguda e crónica foram realizados em dois organismos aquáticos, ecologicamente relevantes, em diferentes concentrações, incluindo concentrações ao nível ambiental, para racematos e enantiómeros puros de K e NK. Os ensaios de ecotoxicidade foram realizados utilizando o crustáceo *Daphnia magna* e o protozoário *Tetrahymena thermophila*, em conformidade com as normas nacionais e internacionais (ISO e OCDE). O racemato NK apresentou maior mortalidade para o crustáceo *D. magna* comparado a K. Para ambos os compostos, a mortalidade aumentou com o gradiente de exposição. Considerando *T. thermophila*, K demonstrou maior inibição do crescimento em relação ao NK. Estes resultados demonstram uma toxicidade dependente da espécie. Além disso, diferentes respostas enantiosseletivas entre os enantiómeros de K e NK foram encontradas nas concentrações selecionadas.

Palavras-Chave:
Enantiosseparação; Enantiosseletividade; Ensaios de Biodegradação; Cromatografia Semi-Preparativa; *Daphnia magna; Tetrahymena thermophila.*

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LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

ACN Acetonitrile

ALP Alprenolol

AM Amphetamine

AS Activated Sludge

ATE Atenolol

CD Chiral Drugs

CE Capillary Electrophoresis

COX Cyclooxygenase

CP Chiral Pharmaceuticals

CSPs Chiral Stationary Phases

DAD Diode Array Detector

DEA Diethylamine

DHNK Dehydronorketamine

EC_{50} Half Maximal Effective Concentration

EF Enantiomeric Fraction

ER Enantiomeric Ratio

EtOH Ethanol

FD Fluorescence Detector

FLX Fluoxetine

GC Gas Chromatography

Hex Hexane

IBU Ibuprofen

ICH International Conference on Harmonization

IPA Isopropanol

ISE Ion Selective Electrodes

ISO International Organization for Standardization

IUPAC International Union of Pure and Applied Chemistry

K Ketamine

K1 First enantiomer eluted from K

K2 Second enantiomer eluted from K

LC Liquid Chromatography

LC_{50} Half Lethal Concentration

LC-DAD Liquid Chromatography-Diode Array Detector

LOD Limits of Detection

LOQ Limits of Quantification

MA Methamphetamine

MEKC Micellar Electrokinetic Chromatography

MeOH Methanol

MET Metoprolol

MM Minimal Salts Medium

MS Mass Spectrometry

NFLX Norfluoxetine

NK Norketamine

NK1 First enantiomer eluted from NK

NK2 Second enantiomer eluted from NK

NMDA Non-Competitive *N*-methyl-D-aspartate Receptors

NSAIDs Nonsteroidal Anti-Inflammatory Drugs

OD Optical Density

OECD Organization for Economic Co-operation and Development

PCP Phenylcyclidine

PHO Propranolol

QC Quality Control

(*R*)-K Arketamine

%RSD Relative Standard Deviation Percentage

RT Room Temperature

SBT Salbutamol

SFC Supercritical Fluids Chromatography

(*S*)-K Esketamine

TEA Triethylamine

UPW Ultra-Pure Water

UV/Vis Ultraviolet-Visible

WFN Warfarin

WWTP Wastewater Treatment Plant

λ_{Em} Emission Wavelength

λ_{Ex} Excitation Wavelength

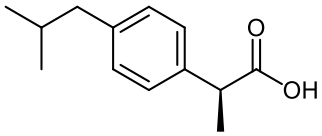
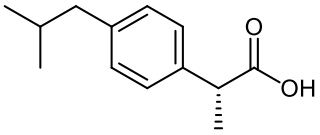
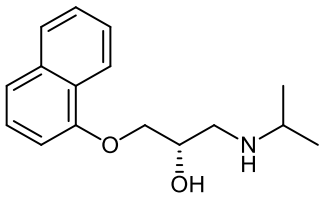
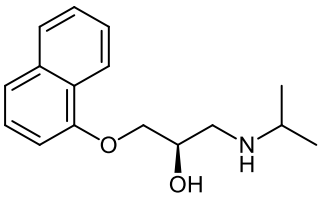
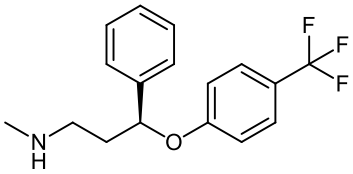
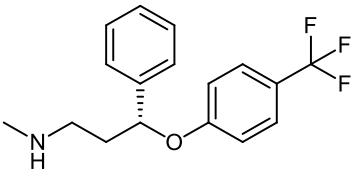
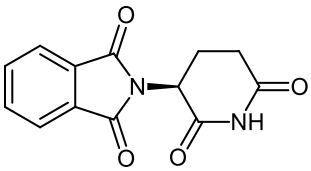
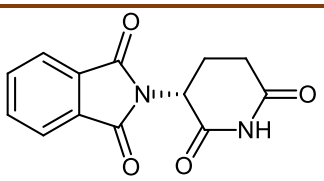
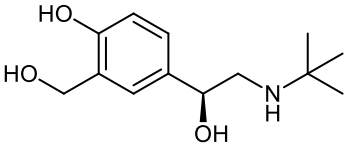
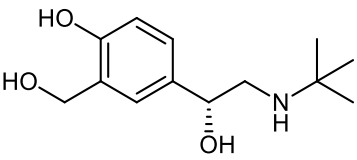
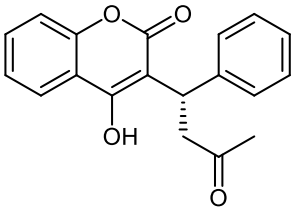
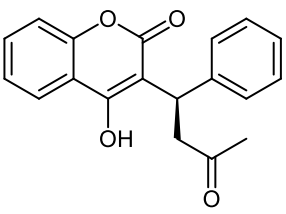
1. INTRODUCTION

1.1. Chiral Pharmaceuticals

Chirality is present whenever a given structure is not superimposed with its image mirror and rotates the plane of polarized light (Caldwell and Wainer 2001; Francotte and Lindner 2006). This is possible, when compounds have one or more stereogenic centers or asymmetry originated by planes or axis. If the rotation is to the right (positive or clockwise) the isomer is designated dextrorotatory (+), but if the rotation is to the left (negative or counterclockwise) it is called levorotatory (-) (Lima 1997; Caldwell and Wainer 2001; Tiritan et al. 2016). According to the International Union of Pure and Applied Chemistry (IUPAC), the absolute structural configuration of the stereoisomers is denominated as *R* or *S* based on the sequence of groups around the sterogenic center, in increasing order of the atomic number (Caldwell and Wainer 2001).

Chirality is an intrinsic property of biological systems, for example in amino acids (*L*-amino acids) which originate the proteins (Müller and Kohler 2004; Hühnerfuss and Shah 2009). Thus, at the biological level, chirality is of high importance since enzymes, receptors and binding molecules are chiral and the interaction with xenobiotic chiral small molecules may lead to different biological responses (Lima 1997; Ribeiro et al. 2012b; Tiritan et al. 2016). Chiral drugs (CD) have at least two enantiomers that present similar chemical, physical and spectroscopic characteristics and may present different pharmacokinetics and pharmacodynamics properties, which translates into enantioselectivity (Lima 1997; Caldwell and Wainer 2001). Thus, enantioselective effects may occur through the enantiomers of CD in bioactivity and/or toxicity events (Lima 1997; Mannschreck and Kiesswetter 2007; Gal 2008; Núñez et al. 2009; Ribeiro et al. 2012b). Some examples of CD and their different pharmacological/toxicological activities are shown in **Table 1.**

Table 1. Examples of CD and their respective pharmacological/toxicological activities.

CD	Pharmacological/Toxicological Activities		References
	(S)-Enantiomer	*(R)*-Enantiomer	
IBU	 *(S)*-Ibuprofen **NSAIDs/COX inhibitor**	 *(R)*-Ibuprofen **Is not a COX inhibitor** **Pro-drug – Chiral Inversion**	(Buser et al. 1999; Winkler et al. 2001)
PHO	 *(S)*-Propranolol **Active Beta-Blocker**	 *(R)*-Propranolol **100 Times Less Active**	(Barrett and Cullum 1968; Stoschitzky et al. 1998)
FLX	 *(S)*-Fluoxetine **Headache Treatment**	 *(R)*-Fluoxetine **Antidepressant**	(Moreira et al. 2014; Ribeiro et al. 2014a)
Thalidomide	 *(S)*-Thalidomide **Teratogenic**	 *(R)*-Thalidomide **Sedative and Hypnotic** **Enantiomerization *in vivo***	(Eriksson et al. 1998; Eriksson et al. 2001)
SBT	 *(S)*-Salbutamol **Inactive or Adverse Effects**	 *(R)*-Salbutamol **Active Bronchodilator**	(Evans et al. 2017)
WFN	 *(S)*-Warfarin **Active Anticoagulant**	 *(R)*-Warfarin **2–5 Times Less Potent**	(Lao and Gan 2012)

COX: cyclooxygenase; FLX: Fluoxetine; IBU: Ibuprofen; NSAIDs: nonsteroidal anti-inflammatory drugs; PHO: Propranolol; SBT: Salbutamol; WFN: Warfarin.

Ideally, CD should be commercialized enantiomerically pure. The advantages of enantiomerically pure formulations are numerous, such as: lower therapeutic dose, greater safety margin, lower interindividual variability, less drug interactions and side effects (Lima 1997; Nguyen et al. 2006; Mannschreck and Kiesswetter 2007; Núñez et al. 2009).

1.1.1. Enantioseparation of Chiral Drugs in Biological Matrices

The enantioseparation of chiral compounds and evaluation of enantiomeric composition (through enantiomeric ratio (ER), and enantiomeric fraction (EF)) (Hashim et al., 2010) have been carried out through different techniques including chiral chromatography, such as liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE) and supercritical fluids chromatography (SFC) (Tiritan et al. 2018).

Chromatographic methods include direct (eg. chiral stationary phases (CSPs) or chiral mobile phase additives) and indirect (derivatization with enantiomerically pure reagents) methods. Regarding indirect method, it is based on the chemical conversion of enantiomers to diastereomers through the reaction with an enantiomerically pure reagent and the employment of an achiral stationary phase (Hashim et al. 2010). Indirect methods can be used for preparative and analytical separation. The chiral separation by direct method consists on temporarily formation of diastereomers in the mobile or stationary phase (Hashim et al. 2010). CSPs for LC are the most common and reliable tool, for enantioseparation and analysis of enantiomeric compositions. In chiral LC, the polysaccharide-based CSPs are one of the most employed CSP for analytical and preparative separation (Matlin et al. 1994; Tiritan et al. 1998; Ikai and Okamoto 2009; Teixeira et al. 2019).

Analysis of chiral pharmaceutical and illicit CD have been described for different biological matrices, such as plasma (eg. amphetamine (AM) (Leis et al. 2003)), hair (eg. 3,4-methylenedioxymethamphetamine (Martins et al. 2006)), blood (eg. methylphenidate (Thomsen et al. 2012)), blood tissues (eg. methadone (Jantos and Skopp 2013)) and whole blood (eg. *N*-methyl-diethanolamine (Rasmussen et al. 2006)), urine (eg. tramadol (Chytil et al.

2009)), liver (eg. 2-ethylidene-l,5-dimethyl-3,3-diphenylpyrrolidine (Moody et al. 2008)) and serum (eg. reboxetine (Öhman et al. 2003)).

1.1.2. Chiral Pharmaceuticals in the Environment

In recent years, drugs have taken on a role of extreme importance in the modern lifestyle, for a high and healthy quality of life (Kümmerer 2010; Basheer 2018). These substances may reach the environment through different sources as veterinary medicine, industrial (productions in large scale and inappropriate discard), hospitals, and residential/domestic effluents. The effluents reach the wastewater treatment plants (WWTP), that are not designed to completely eliminate these small molecules and thus they are still detected in effluents and, consequently, in aquatic environments (**Figure 1**) (Ferrari et al. 2003; Baker and Kasprzyk-Hordern 2013; Ribeiro et al. 2014 ; Ribeiro et al. 2017).

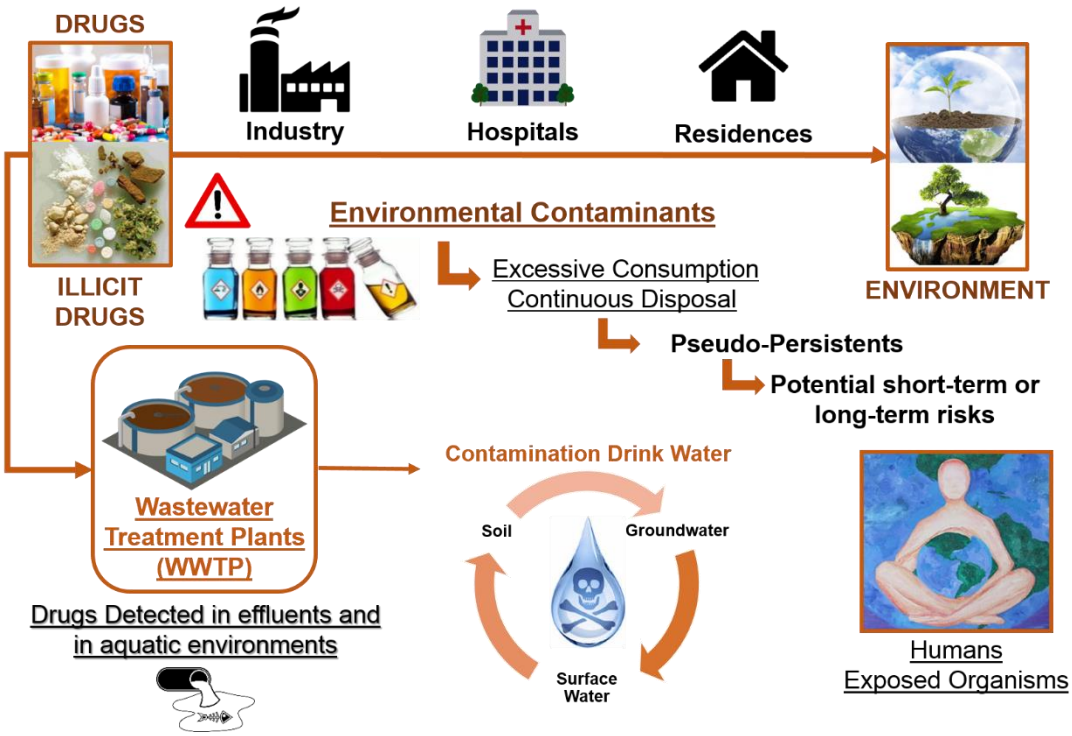


Figure 1. Principal routes of entry of pharmaceuticals into the environment.

Pharmaceuticals and illicit drugs have been detected in the environment in the order of ng/L to µg/L levels (Heberer 2002; Bila and Dezotti 2003; Kümmerer 2010; Ribeiro et al. 2016; Li et al. 2017). The constant interaction between surface water, groundwater and soil, can also lead to the contamination

of drinking water (Kümmerer 2010; Baker and Kasprzyk-Hordern 2013). Besides, the excessive consumption and continuous disposal lead to their persistence or pseudo-persistence, behaving as environmental contaminants (Zuccato et al. 2005; Boleda et al. 2007; Huerta-Fontela et al. 2008; Ribeiro et al. 2016; Li et al. 2017) and posing potential short- and long-term risks to human and other exposed organisms (Kasprzyk-Hordern et al. 2009b). The CD also reach the environment, through the same pathway of achiral drugs (Ribeiro et al. 2016; Rastogi et al. 2017).

Taking into account that CD can be market as racemate or in enantiomerically pure forms, they can be found in different enantiomeric compositions in the environment (**Figure 2**) (López-Serna et al. 2013). CD may present a different EF after absorption, metabolization and excretion, and also, during wastewater treatment processes such as photolysis, hydrolysis, volatilization, adsorption and biodegradation (Bila and Dezotti 2003; Kümmerer 2010; Baker and Kasprzyk-Hordern 2013; Ribeiro et al. 2013a; Ribeiro et al. 2014a; Ribeiro et al. 2016). The microbial degradation can promote racemization or enantioisomerization (Richardson 2006; Pérez and Barceló 2008; Ribeiro et al. 2013a; Moreira et al. 2014; Maia et al. 2016; Maia et al. 2018). Additionally, CD as well as their metabolites and transformation products may also be found at different EF in the environment (Kasprzyk-Hordern et al. 2009b; Evans and Kasprzyk-Hordern 2014; Ribeiro et al. 2014b).

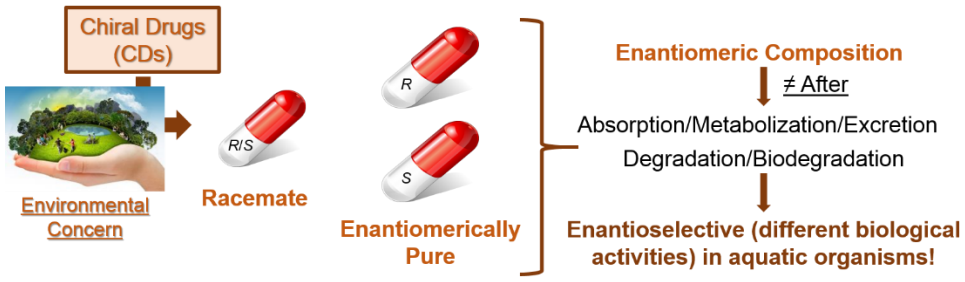


Figure 2. Chiral pharmaceuticals in the environment.

Therefore, CD and their metabolites are current an issue of environmental concern since enantiomers may differ in their biological activity toward to non-target organisms, causing adverse effects (Basheer 2018). This situation makes imperative to carry out enantioselective biodegradation by activated sludge (AS) and ecotoxicological studies considering different species

of aquatic microorganisms for accurate environmental and biological risk assessment (Kasprzyk-Hordern et al. 2009b; a; Ribeiro et al. 2013a; Ribeiro et al. 2014a; Basheer 2018). The occurrence of CD and their EF have been determined in various environmental matrices such as influent and effluent of WWTP, and surface water (Heberer 2002; Kasprzyk-Hordern et al. 2009b; a; Kümmerer 2010; Bagnall et al. 2012; Kasprzyk-Hordern and Baker 2012a; b; Ribeiro et al. 2013a; Ribeiro et al. 2014a; Evans et al. 2015; Ma et al. 2016; Ribeiro et al. 2016) . Briefly, the EF consists of the ratio of the concentration of an enantiomer to the total concentration, corresponding to the relative concentration of one enantiomer (EF of 0.5 and 0 or 1, for racemate and pure enantiomers, respectively) (Tiritan et al. 2018). EF have been used to verify WWTPs efficiency, distinguish of treated and untreated water, consumption of pharmaceuticals or illicit drugs (Hühnerfuss and Shah 2009; Kasprzyk-Hordern et al. 2009b; a; MacLeod and Wong 2010; Ribeiro et al. 2012b; a; Ribeiro et al. 2013b; Ribeiro et al. 2014a; Ribeiro et al. 2014b). EF of CD of different classes have been published (Fono and Sedlak 2005; Hashim et al. 2010), as for example: anticoagulants (eg. WFN (Lao and Gan 2012)), antidepressants (eg. venlafaxine (Gasser et al. 2012; Li et al. 2013), FLX and the main metabolite norfluoxetine (NFLX) (Moreira et al. 2014; Ribeiro et al. 2014a)), beta-blockers (eg. alprenolol (ALP), PHO, atenolol (ATE) and metoprolol (MET) (Ribeiro et al. 2013a; Ribeiro et al. 2013b)), NSAIDs (eg. naproxen and IBU (Ma et al. 2016)), synthetic psychoactive CD (eg. metamphetamine (MA) (Kasprzyk-Hordern and Baker 2012b)), antibiotics (eg. ofloxacin (Camacho-Muñoz et al. 2016)), antihistaminic (eg. fexofenadine (Camacho-Muñoz and Kasprzyk-Hordern 2015)) and bronchodilators (eg. SBT (Evans et al. 2017)).

Nonetheless, most studies regarding the adverse effects and toxicity caused by exposure of non-target organisms to CD are scarce and their stereochemistry is ignored (Ribeiro et al. 2014). Thus, determinations of the EF through the enantioselective analytical methods, as well as enantioselective biodegradation and ecotoxicological studies are urgently needed to assess the real environmental and biological risk of CD (Ribeiro et al. 2012a; b; Ribeiro et al. 2013a; Ribeiro et al. 2013b; Ribeiro et al. 2014 ; Tiritan et al. 2018).

1.1.2.1. Biodegradations Assays

The biodegradation is a biological process and can be enantioselective. The biodegradation assays using AS is an important tool to predict changing in the EF during wastewater treatment (Buser et al. 1999; Hühnerfuss and Shah 2009; MacLeod and Wong 2010; Maia et al. 2017). There are few biodegradation studies of CD already reported, such as beta blockers, antidepressants and their metabolites, anticoagulants, NSAIDs, antibiotics and illicit drugs (Buser et al. 1999; Kasprzyk-Hordern et al. 2009a; Kasprzyk-Hordern and Baker 2012a; Ribeiro et al. 2013b; Ribeiro et al. 2013a; Moreira et al. 2014; Maia et al. 2016).

The pioneering study of enantioselective biodegradation studies by Buser *et al.* (1999) with IBU used WWTP affluent with AS, the EF IBU varied, and almost complete degradation was reported. The degradation profile revealed to be faster for (*S*)-IBU corroborating what is observed throughout the biological process in the WWTP (Buser et al. 1999; Maia et al. 2017). However, Winkler *et al.* (2001) studied the same drug in biofilm reactors, and (*R*)-IBU degraded faster than (*S*)-IBU (active form). This suggests that the main environmental contaminant resulting from the use of IBU may be pharmacologically active (Winkler et al. 2001). Another distinct study by Matamoros *et al.* (2009) revealed non-enantioselective degradation of IBU and naproxen under anaerobic conditions, with removal efficiency between 52 % and 80 % (Matamoros et al. 2009). These three works with the same drug present different results concerning enantioselectivity, possible due to the variability of the biomass that can be different from WWTP to WWTP (Ribeiro et al. 2016).

Recent studies of enantiobiodegradation by AS for ALP, ATE, MET, PHO and FLX demonstrate enantioselectivity of the (*S*)-enantiomer of ALP, PHO and MET, still, for ATE and FLX no enantioselectivity was observed (Ribeiro et al. 2013a; Ribeiro et al. 2013b). Ribeiro *et al.* (2013) observed that ALP degraded must faster than PHO (Ribeiro et al. 2013a). Ribeiro *et al.* (2014) also studied the enantioselective biodegradation of FLX enantiomers in wastewater effluents demonstrating to be almost complete and enantioselective (Ribeiro et al. 2014a). The biodegradation of FLX (racemate and pure enantiomers) with the bacterial strain *Labrys portucalensis* F11 was studied by Moreira *et al.* (2014). The authors observed complete degradation of the enantiomers, without formation of the

NFLX metabolite or occurrence of enantiomerization; in racemate only (*S*)-FLX persisted until the end of the assay (Moreira et al. 2014). Maia *et al.* (2016) performed an enantioselective biodegradation study of ofloxacin enantiomers by an AS consortium. The biodegradation of ofloxacin (racemate) and (*S*)-enantiomer (levofloxacin) was higher than 50 % for both. In addition, the external carbon source increased the biodegradation by 20 %. The formation of the (*R*)-enantiomer during (*S*)-enantiomer biodegradation was verified (Maia et al. 2016). Enantioselective degradation of ofloxacin and levofloxacin by the bacterial strains *Labrys portucalensis* F11 and *Rhodococcus* sp. FP1 was also studied by Maia *et al.* (2018). Strain F11 demonstrated almost complete degradation for both antibiotics when acetate was supplied regularly to the cultures. Racemization was verified in the biodegradation of levofloxacin by strain F11. Enrichment of the (*R*)-enantiomer was observed in FP1 and F11 cultures supplied with ofloxacin (Maia et al. 2018). All these works emphasize the importance of the microbial community in the processes of enantioselective biodegradation.

1.1.2.2. Ecotoxicity

To assess the impact and adverse effects of CD , racemates and their pure enantiomers, acute and chronic toxicity assays have been performed in different organisms, such as algae, luminescence bacteria, plant tissues, and animal cells (Andrés et al. 2009). These assays are important because complement the occurrence studies, providing important results on the effects/impact of these compounds on the environment and on ecologically relevant organisms. In fact, some works have been reporting enantioselective and species dependent toxicity of CD. *Daphnia magna* is a small crustacean typical of freshwater environments, widely distributed throughout the northern hemisphere and South Africa. This organism is commonly used to investigate toxicity of compounds in aquatic ecosystems (Lilius et al. 1995; Stuer-Lauridsen et al. 2000; Wollenberger et al. 2000; Cleuvers 2003; Ferrari et al. 2003; Carlsson et al. 2006; Kim et al. 2007; Cleuvers 2008; Sancho et al. 2016; Li et al. 2017).

Ferrari and collaborators' work demonstrated that the acute toxicity values of diclofenac, clofibric acid and carbamazepine to *D*. *magna* were 224.3 > 13.8 and > 200 mg/L, respectively (Ferrari et al. 2003). Also, the influence on the

behavior, after exposure to drugs such as amphetamine sulfate, caffeine, diazepam, clofibrinic acid, phenobarbital, diphenylhydantoin and carbamazepine were reported to exhibit an acute toxicity level above the mg/L level to these organisms (Lilius et al. 1995; Cleuvers 2003; Kim et al. 2007). Chronic toxicity of antibiotics, antibacterial drugs, anti-inflammatory drugs and NSAIDs, were also reported in *D. magna* at the mg/L level (Stuer-Lauridsen et al. 2000; Wollenberger et al. 2000; Carlsson et al. 2006; Cleuvers 2008). Sancho *et al.* (2016) showed the chronic effects of tebuconazole (fungicide) in *D. magna* assessing the survival, reproduction and growth of organism, after different conditions of exposure (Sancho et al. 2016). Though various works have been reporting effects of pharmaceuticals to exposed organisms, however the enantiomers and evaluation of enantioselective ecotoxicity were ignored. This fact emphasizes the urgency of assays with CD in order to study the enantioselectivity.

Regarding enantioselective ecotoxicity studies, few studies are reported. Andrés *et al.* (2009) study the ATE observing that not harmful at concentrations found in the environment in normally, although *D. magna* and *Pseudokirchneriella subcapitata* (microalgae), nonetheless these CD revealed to be more sensitive to (*S*)-ATE than to (*R*)-ATE (Andrés et al. 2009). Enantioselective toxicity of FLX enantiomers were investigated for *D. magna* and no enantioselectivity was observed (Andrés et al. 2009). However, in a study conducted by Stanley *et al.* (2007), (*S)*-FLX was found to be more toxic to sublethal standardized and behavioral endpoints in *Pimephales promelas* than (*R*)-FLX (Stanley et al. 2007). This was not observed for *D. magna* responses. This study demonstrated that enantiotoxicity can be species dependent. Diao *et al.* (2010) demonstrated that the toxicity effects of lactofen and desethyl lactofen in acute toxicity in *D. magna* is enantioselective (Diao et al. 2010).

T. thermophila is a ciliated protozoan of extreme importance in the community of aquatic microfauna, being responsible for the recycling of organic matter. This organism is often found in freshwater environments. Toxicity assays were performed by Andrés *et al.* (2009) with isomers of dopa, FLX and ATE in *T. thermophila*, *D. magna* and *Pseudokirchneriella subcapitata*. Different levels of toxicity were observed for each enantiomer, suggesting that significant enantioselectivity occurs in aquatic toxicity and that such enantiomeric differences must be considered when evaluating the ecological effects of these

compounds (Andrés et al. 2009). Enantioselective toxicity of FLX was demonstrated for *Pimephales promelas* and *T. thermophila*, where (*S*)-FLX was found to be more toxic than (*R*)-FLX (Stanley et al. 2007; Andrés et al. 2009). However, Andrés *et al.* (2009) proved that (*R*)-FLX was considered more hazardous to *Pseudokirchneriella subcapitata* (Andrés et al. 2009). More recently, Gao *et al.* (2015) investigated the effects of triclosan and triclocarban (antimicrobial agents) on the growth inhibition, cell viability, genotoxicity and multixenobiotic by *T. thermophila* (Gao et al. 2015), however enantioselectivity was not considered.

1.1.3. Ketamine and Norketamine

Ketamine (K) was synthesized in 1962 by Calvin Stevens to replace phenylcyclidine (PCP) behaving as a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, by binding to PCP site (Harrison and Simmonds 1985; Hirota and Lambert 1996; Annetta et al. 2005). This pharmaceutical is used frequently in veterinary and pediatric medicine (Liu et al. 2016). K is chiral and its main human metabolite is norketamine (NK) which has 20 to 30 % of the equivalent activity of the K (Clements et al. 1982) (**Figure 3**). The commercial trade names including Ketalar®, Ketaject®, Ketaset®, Vetalar®, Ketofen®, Ketanest®, Spravato®, Ketamine 500® and Imalgen®, including the racemate and pure enantiomers (Walker 1972; Harrison and Simmonds 1985; Wolff and Winstock 2006; Domino 2010). K is known for its anesthetic, hallucinogenic, sedative and analgesic effects (Clements et al. 1982) (**Figure 3**), nonetheless it is widely misused in recreational environments by adolescents and young adults (Guerreiro et al. 2011; Li et al. 2017). At the recreational level, K is usually called “K”, “vitamin K”, “super K” or “special K” (Walker 1972; Harrison and Simmonds 1985; Wolff and Winstock 2006; Domino 2010; Li et al. 2011).

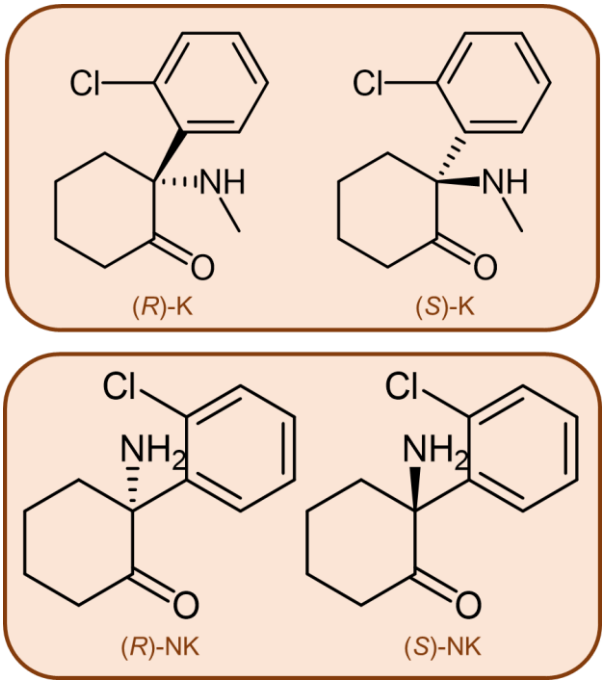


Figure 3. Enantiomers of K ((*R*/*S*)-2-(o-chlorophenyl)-2-(methylamino)cyclohexanone) and NK ((*R*/*S*)-2-(o-chlorophenyl)-2-aminocyclohexanone).

K has two enantiomers (*R*)-K (arketamine) and (*S*)-K (esketamine) (**Figure 3**). Commercially, K is widely distributed as a racemate but more and more in pure enantiomeric forms or in different enantiomeric compositions. The (*S*)-K enantiomer is more active than (*R*)-K (Hong and Davisson 1982; Pai and Heining 2007; Sinner and Graf 2008; Singh et al. 2016), with greater affinity for NMDA receptors. In the illicit form, K is often detected together with other drugs such as AM, MA, cocaine, morphine, methadone, cannabinoids.

Esketamine is often used at the medicinal level, for example in the treatment of psychological disorders (eg. Schizophrenia and Alzheimer's (Kavalali and Monteggia 2012; Zhou and Sheng 2013; Johnson et al. 2015)), treatment of chronic persistent depression (Kavalali and Monteggia 2012; Zhou and Sheng 2013; Johnson et al. 2015; Singh et al. 2016) and chronic pain (Kavalali and Monteggia 2012; Zhou and Sheng 2013; Johnson et al. 2015). Esketamine has a higher affinity for NMDA receptors compared to arketamine (Singh et al. 2016). The present mechanism of action results from non-competitive binding to NMDA receptor, a subfamily of glutamate receptors, acting as a glutamate receptor antagonist, blocking the ion channel of the respective receptor (Kavalali and Monteggia 2012; Zhou and Sheng 2013; Johnson et al. 2015; Singh et al. 2016). The arketamine is the enantiomer with potential effect

hallucinogenic, the effect pretended in illicit consumption. NK enantiomers binds to the PCP site with less affinity than K, evidencing its anesthetic potential in particular at the (*S)*-NK (Holtman Jr et al. 2008; Moaddel et al. 2010).

Regarding administration route pharmacodynamics and pharmacokinetics, little is known about K, its enantiomers and metabolites. After 72 hours of a single dose of K, it is eliminated 90 % in the urine as 2.3 % unchanged, 1.6 % as NK, 16.2 % as dehydronorketamine (DHNK) and 80 % as the conjugated hydroxylated derivatives of K (Domino 2010; Moffat et al. 2011). These compounds have been detected either in human biological (Bolze and Boulieu 1998; Hijazi et al. 2001; Apollonio et al. 2006; Kim et al. 2008; Tabernero et al. 2009; Chen et al. 2010; Harun et al. 2010; Goldberg et al. 2011; Favretto et al. 2013; Leung et al. 2015; Anilanmert et al. 2016; Fassauer et al. 2017; Hasan et al. 2017; Yang et al. 2017; Leung et al. 2018) and in environmental samples (Boleda et al. 2007; Huerta-Fontela et al. 2008; Lin et al. 2010; Baker and Kasprzyk-Hordern 2013; Lai et al. 2013; van Nuijs et al. 2013; Lin et al. 2014; Jiang et al. 2015; Li et al. 2016).

To detect and quantify K and their metabolites, different chromatographic techniques which include GC and LC methods coupled with mass spectrometry (MS), ultraviolet-visible (UV/Vis), diode array (DAD) and fluorescence (FD) detectors have been published (Bolze and Boulieu 1998; Harun et al. 2010; Zhu et al. 2011; Tsui et al. 2012; Favretto et al. 2013; Fassauer et al. 2017). Although less frequent, other methods of CE, micellar elektrokinetic chromatography (MEKC), ion selective electrodes (ISE) and enantioselective methods are also reported (Rosas et al. 2003; Theurillat et al. 2007; Portmann et al. 2010; Goldberg et al. 2011; Porpiglia et al. 2016). These methods were applied to different biological matrices as urine, hair, blood, finger nails, plasma, brain, serum and oral fluids (Bolze and Boulieu 1998; Yanagihara et al. 2000; Hijazi et al. 2001; Kim et al. 2008; Chen et al. 2010; Moaddel et al. 2010; Favretto et al. 2013; Leung et al. 2015; Hasan et al. 2017; Toki et al. 2018).

Over the last few years some reports have been carried out to understand the occurrence of K and NK and their effects in aquatic living organisms and consequently, in the environment. Previous reports indicated that K cannot be removed completely by natural purification processes or by conventional WWTP being present in several environmental matrices (Lin et al.

2010; Baker and Kasprzyk-Hordern 2013; Lin et al. 2014). High concentrations of K in order to 0.1 to 10 µg/L have been detected in effluent samples (Lin et al. 2010; Baker and Kasprzyk-Hordern 2013; van Nuijs et al. 2013; Lin et al. 2014), and K is frequently and persistently detected in the receiving surface water (Lin et al. 2014; Li et al. 2016). Lai *et al.* (2013) studied the estimation of variations (daily and diurnal) of illicit drugs use in wastewater analysis in Hong Kong, including K and NK (Lai et al. 2013). Lin *et al.* (2014) studied the persistence and phototransformation toxicity of K and NK in hospital wastewater and surface water (Lin et al. 2014). In addition, a study has demonstrated that a mass event, such as a music festival, can result in a significant release of K into the environment, detected in surface water (up to 9.53 µg/L) and wastewaters (up to 138 µg/L) (Jiang et al. 2015).

Regarding ecotoxicity of K and NK, acute and chronic assays in aquatic organisms are scarce. Fick *et al.* (2010) used a model to calculate and predict that K can cause a pharmacological effect in fish (Fick et al. 2010). A toxicity assessment report indicated that K and MA delayed blood circulation and hatching time in embryos of medaka fish and altered larval alteration in early life stages (Liao et al. 2015). To assess the ecotoxicological effect of K and NK, Li *et al.* (2017) conducted studies of acute, chronic, photolysis and synergistic toxicity in *D. magna*. K and NK caused acute toxicity to *D. magna* after 48 hours of exposure, with half lethal concentration (LC_{50}) values of 30.93 and 25.35 mg/L, respectively (Li et al. 2017). In a recent work, Félix *et al.* (2018) worked with zebrafish embryos to study the K induction of p53-dependent apoptosis and oxidative stress (Félix et al. 2018).

The enantiomers continued to be ignored and consequently enantioselectivity is not considered for a correct environmental risk assessment. Consequently, the development of enantioselective analytical methods is necessary to evaluate occurrence and biodegradation. Enantioselective toxicological studies of both enantiomers are also scarce.

2. AIMS

The aim of this dissertation is to study the enantioselective biodegradation and ecotoxicity of K ant its main metabolite NK. To achieve these goals, the present dissertation is subdivided into:

- Development and validation of an enantioselective LC-DAD method to quantify the enantiomers of K and NK.
- Enantioselective biodegradation assay of K by AS.
- Development of a semi-preparative LC-DAD method to obtain the enantiomers of K and NK.
- Evaluation of the enantioselective ecotoxicity of K and NK, racemates and the pure enantiomers, in two aquatic environmental relevant organisms (*Daphnia magna* and *Tetrahymena thermophila*), according to the national and international standards (ISO and OECD).

3. MATERIALS AND METHODS

3.1. Chemicals and Equipments

Chromatographic grade solvents, isopropanol (IPA) and methanol (MeOH) were purchased from Fisher Scientific UK (Leicestershire, United Kingdom); ethanol (EtOH) was acquired from Chem-Lab NV (Zedelgem, Belgium); hexane (Hex) was acquired from VWR Chemicals Prolabo (Fontenay-sous-Bois, France) and acetonitrile (ACN) from CARLO ERBA Reagents (Val-de-Reuil, France). Ammonium acetate was purchased from Fluka (Netherlands); diethylamine (DEA) and triethylamine (TEA), both with ≥ 99 % purity, were purchased from Sigma Aldrich (St Louis, MO, USA).

Ultra-pure water (H_2O) (UPW) was obtained from a SG Ultra Clear UV plus equipment. Glass microfibers filter with 0.7 µm porous size were purchased from VWR (Leuven, Belgium).

Standards of the K and NK were acquired from Sigma Aldrich (St Louis, MO, USA). Individual standards stock solutions were prepared at 1 mg/mL in MeOH and stored in amber bottles at – 20º C. For the preparative chromatography, K was obtained from the pharmaceutical NIMATEK, kindly given from Dechra Veterinary Products (Bladel, Netherlands), through the regeneration with 30 % NaOH. NK was acquired from LGC Standards (Luckenwalde, Germany).

Working standard solutions were freshly prepared and obtained by dilution of stock solutions in appropriate solvent depending of the study: minimal salts medium (MM) or MeOH for development and validation of analytical chromatography of K and NK; MeOH and IPA with 0.1 % of DEA or of TEA for semi-preparative chromatography of K and MeOH or EtOH for semi-preparative chromatography of NK; MM for biodegradation studies and standard freshwater for toxicity tests.

A rotavapor (R-210) equipped with vacuum controller (V-850), heating bath (B-491) and vacuum pump (V-700) from BÜCHI SWITZERLAND and Speed Vac purchased from LABCONCO with Centrivap Concentrator, Cold Trap and vacuum pump were used in semi-preparative chromatography.

Regarding to the column used in analytical LC was used a Lux® 3 µm Cellulose-4 with dimensions of 150 x 4.6 mm couple to SecurityGuard™, Guard Cartridge Kit with dimensions of 4 x 3.0 mm I.D., both obtained from Phenomenex® (USA). For semi-preparative LC was used an amylose *tris*-3,5-dimethylphenylcarbamate column coated on to APS-Nucleosil (500 A°, 7µm, 20 %, *w*/*w*) and packed into a stainless-steel 200 x 70 mm I.D. size column ("homemade" column).

LC was LaChrom Merck Hitachi Diode Array Detector (LC-DAD) equipped with a pump (L-7100), an automatic injector (L-7200), DAD (L-7455) and an interface system (D-7000), acquired from Merck Hitachi for the development and validation of the enantioseparation of K and NK analytical method and for optimization and semi-preparative enantioseparation of K and NK. Data acquisition software was HPLC System Manager HSMP-7000, Version 3.0.

LC coupled to the UV/Vis and fluorescence detector (LC-UV/Vis and LC-FD) was also used for the development of the enantioseparation analytical and semi-preparative methods. This equipment was acquired from the Shimadzu UFLC Prominence System, equipped with two pumps (LC-20AD), an automatic injector (SIL-20AC), the column oven (CTO-20AC), a system controller (CBM-20A). The UV/Vis detector SPD-20A and the fluorescence detector RF-10AXL. Data acquisition software was LC Solution, Version 1.24 SP1.

The MM used was prepared with the following reagents: di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), ammonium sulfate ($(NH_4)_2SO_4$), sodium hydroxide (NaOH), manganese(II) sulfate tetrahydrate ($MnSO_4.4H_2O$) and ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) were purchased from Merck (Darmstadt, Germany); potassium dihydrogen phosphate (KH_2PO_4) was acquired from José M. Vaz Pereira, LDA (Budenheim, Germany); ethylenediaminetetraacetic acid disodium salt hydrate ($Na_2EDTA_2.2H_2O$) and calcium chloride ($CaCl_2$) were acquired from PanReac AppliChem ITW Reagents (Germany); sodium sulfate (Na_2SO_4) and cupric sulfate ($CuSO_4$) were acquired from José Manuel Gomes dos Santos, LDA (Odivelas, Portugal); zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) was purchased from Riedel-de Haën (Seelze, Germany); and sodium molybdate dihydrate (Na_2MoO_4) and sulfuric acid (H_2SO_4) 95-97% were acquired from

Sigma Aldrich (St Louis, MO, USA). The sodium acetate used as an external source of feed was acquired from Merck (Darmstadt, Germany).

Biodegradation assays were performed in an Infors HT Ecotron incubator (Fisher Scientific, Portugal) at the temperature 25º C under dark and light conditions. Absorbance was measured using an UV/Vis spectrometer (ATI Unicam, Leeds, England). Centrifuge Heraeus Biofuge Pico, centrifuge Refrigerated Heraeus Biofuge 1.0R, magnetic stirrer multipoint VARIOMAG® and autoclave were also used to performance these works.

Kits for the toxicity tests DAPHTOXKIT F™ MAGNA and PROTOXKIT F™ were acquired from MicroBioTests Inc., (Gent, Belgium). Organisms were incubated in an Infors HT Ecotron incubator (Fisher Scientific, Portugal) under the temperature and illumination indicated in the standard protocols provided with the kits. Absorbance was measured using an UV/Vis spectrometer (ATI Unicam, Leeds, England). Potassium dichromate ($K_2Cr_2O_7$) was used as a reference test in the ecotoxicity assays purchased from José Manuel Gomes dos Santos, LDA (Odivelas, Portugal).

3.2. Enantioseparation optimization and method validation for quantification of Ketamine and its metabolite Norketamine

The chromatographic column selected for enantioseparation was Lux® 3 µm Cellulose-4 couple to the respective SecurityGuard™. Optimization experiments for K and NK were performed in isocratic mode at room temperature (RT). Flow-rate was tested between 0.5 and 1.1 mL/min. Sample injection volume was 10 µL. Mobile phase compositions were 20 mM ammonium acetate in UPW with or without 0.1 % of DEA combined with EtOH or ACN in reverse elution mode (**Table 2**). The emission (λ_{Em}) and excitation (λ_{Ex}) wavelengths for FD detector were studied as following values 290 and 220, 510 and 350 and 510 and 380 nm, respectively for both compounds. DAD or UV/Vis detector was set at 220 nm. All solvents of the mobile phases were previously filtered using a glass microfibers filter with 0.7 µm porous size. The stock solution of K and NK used in the development of enantioseparation was at 1 mg/mL in MeOH for both compounds.

The different working standard solutions of K and NK were freshly prepared by dilution in MeOH at different concentrations.

The analytical chromatography conditions optimized consisted in 20 mM ammonium acetate in UPW with 0.1 % of DEA combined with ACN, 70:30, *v*/*v* as mobile phase, DAD detector at 220 nm, flow-rate of 1 mL/min and injection volume of 10 µL.

Table 2. Experimental conditions of analytical optimization of K and NK.

Equipments	Mobile Phase	Composition (*v*/*v*)	Flow-rate (mL/min)	FD (λ=nm)
LC-DAD	20 mM Ammonium Acetate with 0.1 % DEA: ACN	73/27	1.1	-
		65/35	1.0	
		67/33		
		70/30		
		73/27		
		74/26		
		75/25		
		70/30	0.5	
		67/33		
		65/35		
	20 mM Ammonium Acetate with 0.1 % DEA: EtOH	50/50		
		60/40		
		70/30		
	20 mM Ammonium Acetate: ACN	70/30		
LC-UV/Vis LC-FD	20 mM Ammonium Acetate with 0.1 % DEA: ACN	70/30	1.0	λ_{Em} - 510 λ_{Ex} - 340
				λ_{Em} - 510 λ_{Ex} - 380
				λ_{Em} -290 λ_{Ex} - 220

λ_{Em}: Emission Wavelengths; λ_{Ex}: Excitation Wavelengths.

The optimized method was validated according to the International Conference on Harmonization guidelines (ICH) (1996) and according to previous works (Ribeiro et al. 2013a; Ribeiro et al. 2013b; Moreira et al. 2014; Ribeiro et al. 2014a; Maia et al. 2016). Selectivity, linearity and range, accuracy, recovery, intra- and inter- day precision, and detection and quantification limits were determined. Selectivity was verified by comparing the chromatograms of standards of K and NK dissolved in MeOH with those spiked with AS in MM, to assess the matrix interferences. The linearity was evaluated using calibration

curves performed in MM at six calibration standard mixtures each one in triplicate. The nominal racemate concentrations were 5, 10, 20, 30, 40 and 50 µg/mL for K; and 2.5, 5, 10, 15, 20 and 25 µg/mL for NK. Calibration curves were obtained by linear regression. Limits of detection (LOD) and quantification (LOQ) were calculated from spiked samples through the signal/noise ratio of 3 LOD and 10 for LOQ. Accuracy, intra- and inter-batch precision were determined by analysis in three replicates of three quality control standard solutions (QC) in MM, at three different concentrations within the linearity range. The nominal racemate concentrations were 8, 25 and 35 µg/mL of K and 4, 12 and 17 µg/mL for NK. Precision was expressed as the relative standard deviation (%RSD) percentage of the triplicate measurements and the accuracy of the method was evaluated as the percentage of agreement between the method results and the nominal amount of compound added. Blank samples with MM inoculated with AS were fortified at the three QC concentrations and used for recovery assays. The three different concentrations of K were spiked with AS in MM, previously centrifuged at 13000 rpm for 10 minutes (centrifuge Heraeus Biofuge Pico), after 2 hours of shaking at 110 rpm. The recovery was calculated by comparing the peak areas of the standards in MM with those of similar concentrations from the supernatant of centrifuged aliquots collected from the spiked AS.

3.3. Biodegradation Assays

The AS inoculum used in the biodegradation assays was obtained from the secondary/biological treatment aerated tanks of a municipal WWTP (Ponte de Moreira, Maia, Portugal), which receives domestic and industrial wastewater. AS was collected in amber glass flasks placed in a plastic bag and transported in a styrofoam box with ice and cold accumulators until properly conditioned at 4° C in the laboratory until usage.

The MM used in the biodegradation assays was prepared with the following composition (per 1L of UPW): $Na_2HPO_4.2H_2O$, 2.67 g; KH_2PO_4, 1.4 g; $MgSO_4.7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 0.5 g with 10 mL of a trace elements solution. The trace elements solution was prepared following the composition (per 1L of UPW): $ZnSO_4.7H_2O$ 0.4 g; $Na_2EDTA_2.2H_2O$, 12 g; $CuSO_4$, 0.1 g; NaOH, 2 g; $FeSO_4.7H_2O$, 2 g; Na_2MoO_4, 0.1 g; $MnSO_4.4H_2O$, 0.4 g; Na_2SO_4, 10 g; $CaCl_2$, 1

g; H_2SO_4 95-97 %, 0.5 mL. The sodium acetate stock solution was prepared at final concentration of 1 M (CH_3COONa, 8.203 g in 50 mL of MM). The MM and the sodium acetate solution were previously autoclaved to avoid bacterial growth, manipulated by flame (sterile environment). The solution stock of K racemate was prepared at final concentration of 1 mg/mL in MM.

The AS inoculum was washed with MM before being used in the biodegradation assays, through 3 wash cycles: centrifuge at 4000 rpm for 10 minutes at 4º C (Refrigerated Centrifuge Heraeus Biofuge 1.0R), discard the supernatant and resuspend the pellet in MM. After washing, AS inoculum was autoclaved to perform the assays with the dead cells.

The biodegradation assays were performed in batch mode using 100 mL covered flasks containing 25 mL of MM inoculated with AS inoculum, at an optical density (OD) of ca. 0.3 at 600 nm measured using a spectrophotometer UV/Vis. The K racemate stock solution was added to obtain initial concentration of 40 µg/mL in the flasks of assays. Biodegradation assays were assessed under light and dark (coated flasks with aluminum foil) conditions; in the presence or absence of sodium acetate as an extra carbon source at an initial concentration of 200 µg/mL; and with AS or dead cells (**Table 3**).

Table 3. Experimental conditions of biodegradation assays of K racemate in AS.

Flask	Conditions	Illumination
1, 2, 3	(*R,S)*-K 40 µg/mL + AS + Sodium Acetate	Dark
4, 5, 6		Light
7, 8,9	(*R,S)*-K 40 µg/mL + AS	Dark
10, 11, 12		Light
13, 14	AS + Sodium Acetate (Growth Control)	Dark
15, 16		Light
17, 18, 19	Abiotic (*R,S)*-K 40 µg/mL (Without AS)	Dark
20, 21, 22		Light
23, 24	Dead cells + (*R,S)*-K 40 µg/mL	Dark
25, 26		Light

All experiments were done in triplicate (except experiments with dead cells and control that were done in duplicate), using glass flasks of a volume fourfold the medium volume used to guarantee the aeration of the cultures. The cultures were incubated at 25º C with shaking at 110 rpm. Control assays without inoculation, under light and dark condition, were also included. The assays were

monitored during 21 days by the LC-DAD validated method. Briefly, 1 mL of each sample assays were collected with sterile syringe, centrifuged at 13000 rpm (Centrifuge Heraeus Biofuge) for 20 minutes, approximately 900 µL aliquots of the supernatant was stored in amber vials at – 20º C until LC analysis. Growth was monitored by measuring the OD of the cultures at 600 nm previously to centrifugation. The assays were monitored with the validated analytical method.

3.4. Semi-Preparative Enantioseparation of Ketamine and Norketamine

Semi-preparative enantioseparation optimization of K and NK was performed using the Merck Hitachi LaChrom LC with DAD detector and Shimadzu LC Prominence System with UV/Vis and FD detector. The semi-preparative chromatographic column selected for separation of the enantiomers was an amylose *tris*-3,5-dimethylphenylcarbamate column coated on to APS-Nucleosil.

Three fractions were collected: fraction 1 - corresponding to the first enantiomer eluted from K or NK (K1 or NK1), intermediate fraction – both K1 or NK1 and the second enantiomer eluted (K2 or NK2) and fraction 2 - corresponding to K2 or NK2. These fractions were collected into round-bottom flasks, subjected to evaporation on the rotary evaporator to concentrate the fractions and transferred to glass tubes and evaporated to dryness on a Speedvac. After evaporation the residue was reconstituted in 2 mL of MeOH and stored in amber of vial/flasks at 4º C. The enantiomeric purity of each collected fraction was evaluated using analytical Lux® 3 µm cellulose-4 column through the LC method previously validated, for each enantiomer.

3.4.1. Semi-Preparative Enantioseparation of Ketamine

Enantioseparation optimization for K was performed in isocratic mode. Flow-rate tested were 1.2 and 1.5 mL/min. Mobile phase compositions were Hex with 0.1 % of DEA combined with EtOH or IPA (both with 0.1 % of DEA) and Hex with 0.1 % of TEA combined with IPA with 0.1 % of TEA (**Table 4**). The temperature analysis was also evaluated at different temperatures through the oven column at 20, 23, 27.7, 35 and 40º C. The UV detector was set at

wavelength range fixed in 220 nm or 230 nm. Different λ_{Em} for FD detector were investigated for K as 230, 240, 260, 270, 280 and 300 nm, while λ_{Ex} was set at 220 nm, respectively for both compounds. Different standard stock concentrations of K diluted in different solvents, of 50 µg/mL (MeOH), 1 mg/mL (MeOH), 2 mg/mL (MeOH), 2 mg/mL (IPA with 0.1 % of DEA), 2 mg/mL (IPA with 0.1 % of TEA), 2 mg/mL (EtOH), 10 mg/mL (IPA with 0.1 % of DEA) and 20 mg/mL (IPA with 0.1 % of DEA) were investigated. Sample injection volumes of 5, 10, 15, 20, 25, 50 and 100 µL were evaluated.

Table 4. Experimental conditions of semi-preparative chromatography of K.

Equipments	Mobile Phase	Composition (*v/v*)	Flow (mL/min)	Temperature (° C)	Injection Volume (µL)	UV (λ=nm)	FD (λ=nm)
LC-DAD	Hex with 0.1% of DEA: IPA with 0.1% of DEA	97/3	1.5	RT	10	220	-
		96/4					
		95/5				230	
		90/10				220	
		80/20					
		70/30					
	Hex with 0.1% of DEA: EtOH with 0.1% of DEA	97/3					
		90/10					
		80/20					
		70/30					
LC-UV/Vis LC-FD	Hex with 0.1% of DEA: IPA with 0.1% of DEA	92/8	1.2				
		95/5					
		92/8	1.5				
		97/3			25		
					50		
					100		
					10		λ_{Em} - 280 λ_{Ex} - 220
				35			λ_{Em} - 300 λ_{Ex} - 220
							λ_{Em} - 240 λ_{Ex} - 220
					5		λ_{Em} - 230 λ_{Ex} - 220
				40	10		λ_{Em} - 260 λ_{Ex} - 220
				27.7	25		-
		96/4		23			
				20	5		
					10		
					15		
		95/5			5		
					10		
					15		
	Hex with 0.1% of TEA: IPA with 0.1% of TEA	97/3		RT	10		λ_{Em} - 270 λ_{Ex} - 220
					20		

λ_{Em}: Emission Wavelengths; λ_{Ex}: Excitation Wavelengths; RT: Room Temperature.

The optimized semi-preparative chromatography conditions consisted in Hex with 0.1 % of DEA and IPA with 0.1 % of DEA, 95:5, *v*/*v* as mobile phase, DAD detector at 230 nm, flow-rate of 1.5 mL/min, injection volume of 10 µL and stock solution of K at 20 mg/mL (IPA with 0.1 % of DEA).

3.4.2. Semi-Preparative Enantioseparation of Norketamine

Enantioseparation optimization experiments for NK were performed in isocratic mode. Flow-rate was 1.5 mL/min at RT. Mobile phase compositions consisted of Hex with 0.1 % of DEA combined with EtOH or IPA (both with 0.1 % of DEA) (**Table 5**). The UV detector was set at 220 nm. Different standard stock concentrations of NK diluted in different solvents of 50 µg/mL (MeOH), 1 mg/mL (MeOH), 2 mg/mL (EtOH), 4 mg/mL (EtOH), 6 mg/mL (EtOH), 10 mg/mL (EtOH) and 20 mg/mL (EtOH) were investigated. Sample injection volume of 10, 25, 30, 50 and 100 µL were evaluated.

Table 5. Experimental conditions of semi-preparative chromatography of NK.

Equipment	Mobile Phase	Composition (*v*/*v*)	Injection Volume (µL)
LC-DAD	Hex with 0.1 % of DEA: IPA with 0.1% of DEA	90/10	10
		80/20	50
	Hex with 0.1 % of DEA: EtOH with 0.1% of DEA	90/10	10
		80/20	50
		70/30	10
			25
			30
		65/35	50
			100
		60/40	10
			50

The semi-preparative chromatography conditions optimized consisted in Hex with 0.1 % of DEA and EtOH with 0.1 % of DEA, 65:35, *v*/*v* as mobile phase, DAD detector at 220 nm, flow-rate of 1.5 mL/min, injection volume of 50 µL and stock solution of NK at 10 mg/mL (EtOH).

3.5. Enantioselective Ecotoxicity Assays

The acute and chronic enantioselective ecotoxicity assays with K and NK as racemate and pure enantiomers forms were performed according to procedure provided by MicroBioTests kits in accordance with national and international standards (ISO and OECD). The standard stock solutions of K and NK in racemate forms were 1000 mg/L in MeOH. The standard stock of pure enantiomers of K and NK obtained previously by semi-preparative chromatography, as 1000 mg/L and 500 mg/L, respectively.

3.5.1. Acute Assays in *Daphnia magna*

The acute toxicity tests of *D. magna* were performed followed the procedures described in the kit. The kit consists of a tube with dormant eggs of *D*. *magna* (ephippia) coated with a chitinous capsule (ephippium). Storage for several months without losing viability, in the refrigerator at 5º C (+/- 2º C). These assays occur in accordance with OECD Guideline 202 (OECD Adopted April 2004) and ISO Standard 6341 (ISO Fourth Edition 2012).

Briefly, the preparation of standard freshwater medium consisted on solutions of concentrated salts (sodium bicarbonate ($NaHCO_3$), 129.5 mg; calcium chloride dihydrate ($CaCl_2.2H_2O$), 588 mg; magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 246.5 mg and potassium chloride (KCl), 11,5 mg) diluted in distilled water. Before use the standard freshwater medium was aerated for about 20 minutes with the aid of an aeration pump and a magnetic stirrer. The parameters of medium were measured (temperature, dissolved oxygen, percentage of dissolved oxygen, conductivity, pH) after aerating using a multiparameter waterproof meter. Hatching of ephippia was initiated 3 days prior to the beginning of the toxicity tests, by incubating the eggs in Petri dishes with “standard freshwater” at 20º C under continuous illumination of 6000 lux. To perform a complete test, 120 neonates younger than 24 hours were feed with a suspension of spirulina microalgae 2 hours prior to perform the toxicity test. For a statistically acceptable evaluation of the effects, each test concentration as well as the control was assayed in four replicates each with 5 neonates corresponding to a total of 20 neonates for each test. Concerning to the preparation of K and NK standards (racemate and enantiomerically pure forms) and reference test

($K_2Cr_2O_7$), concentrations were prepared in standard freshwater medium, adding the respective amount of compound. A MeOH control was made in standard freshwater medium. Concentrations used were 0.1, 1, 10, 100 and 1000 µg/L for K and NK racemate and 0.05, 0.5, 5, 50, 500 µg/L for each enantiomer. A reference test (positive control) was performance with $K_2Cr_2O_7$ to assess the viability, the correct execution of assays, and sensitivity of the test organisms at five concentrations (0.32, 0.56, 1, 1.8 and 3.2 mg/L) and 24 or 48 hours EC_{50} calculated.

The ecotoxicity assays are performance in disposable multiwell test plates, each plate is provided with four wells for the controls and four wells for each toxicant concentration.

Plates were then placed in in an Infors HT Ecotron incubator, in darkness at 20° C for 48 hours. The number of dead or immobilized neonates was recorded, versus that of the actively swimming test organisms in each well at 24 hours and at 48 hours. The test was considered valid when the number of dead plus immobile organisms did not exceed 10 % in the controls.

3.5.2. Chronic Assays in *Tetrahymena thermophila*

The chronic toxicity tests of *T*. *thermophila* followed the procedures described in procedure brochure present in the kit. The kit consists of a tube with ciliated protozoan of *T. thermophila* in specific culture medium. Storage for several months without losing viability at RT. These assays were performed in accordance with OECD Guideline 244 (OECD Adopted October 2017).

In brief, the preparation of standard freshwater medium based on solutions of concentrated salts ($NaHCO_3$, 96 mg; $CaSO_4.2H_2O$, 60 mg (two flasks); $MgSO_4.7H_2O$, 123 mg and KCl, 4 mg) diluted in distilled water. Before the use of the standard freshwater medium, it was aerated for about 20 minutes with the aid of an aeration pump and a magnetic stirrer. The parameters of medium were measured (temperature, dissolved oxygen, percentage of dissolved oxygen, conductivity, pH) after aerating using a multiparameter waterproof meter. Regarding to the preparation of K and NK standards (racemate and enantiomerically pure forms) and reference test ($K_2Cr_2O_7$), concentrations of the different test compounds were prepared in standard freshwater medium by

adding the respective amount of compound. Concentrations used were 0.1, 1, 10, 100 and 1000 µg/L for K and NK racemate and 0.05, 0.5, 5, 50, 500 µg/L for each enantiomer. A reference test (positive control) was performance with $K_2Cr_2O_7$ to assess the viability and sensitivity of assays. The stock solution of $K_2Cr_2O_7$ was prepared at 0.1 mg/mL (100 mg/L). The concentrations of $K_2Cr_2O_7$ were 5.6, 10, 18, 32 and 56 mg/L. For preparation of ciliates inoculum, a 500 µL stock culture was taken with a sterile syringe and transferred into a 1.5 mL plastic cuvette. OD at 440 nm was adjusted to 0.040 with distilled water. Food suspension was prepared by adding reconstitution medium. To perform the assay disposable 1 cm polystyrol spectrophotometric cells containing 40 µL food suspension and 40 µL ciliate inoculum *T. thermophila* and control *vs* tested compounds were incubated in an Infors HT Ecotron incubator at 30º C in darkness, for 24 or 28 hours. OD measurements at 0, 24 and 28 hours were performance using a spectrophotometer at 440 nm. For the test to be acceptable, the OD in the controls after 24 or 28 hours of incubation must show a decrease of the T0 value by at least 60 %.

3.6. Statistical Analysis

For statistical purposes, the immobilization or growth inhibition for racemate and individual enantiomers were first checked for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). Since these parameters followed a non-parametric distribution the Kruskal-Wallis analysis of variance was used. The differences were considered statistically significant at $p < 0.05$. *D. magna* and *T. thermophila* EC_{50} reference test with $K_2Cr_2O_7$ were estimated using probit analysis.

4. RESULTS AND DISCUSSION

4.1. Enantioseparation Optimization of Ketamine and its metabolite Norketamine

The enantioseparation of K and NK was performed using the commercial column Lux® 3 µm Cellulose-4, a polysaccharide-based CPS cellulose *tris*(4-chloro-3-methylphenylcarbamate) in reverse elution mode.

For the enantioseparation optimization different mobile phases composition and flow-rates were evaluated. First conditions were 20 mM ammonium acetate in UPW with 0.1 % of DEA and EtOH (50:50, *v*/*v*) as mobile phase at a flow-rate of 0.5 mL/min, injection volume of 10 µL, at 220 nm and RT. However, under these conditions enantioseparation was not observed (**Figure 4**).

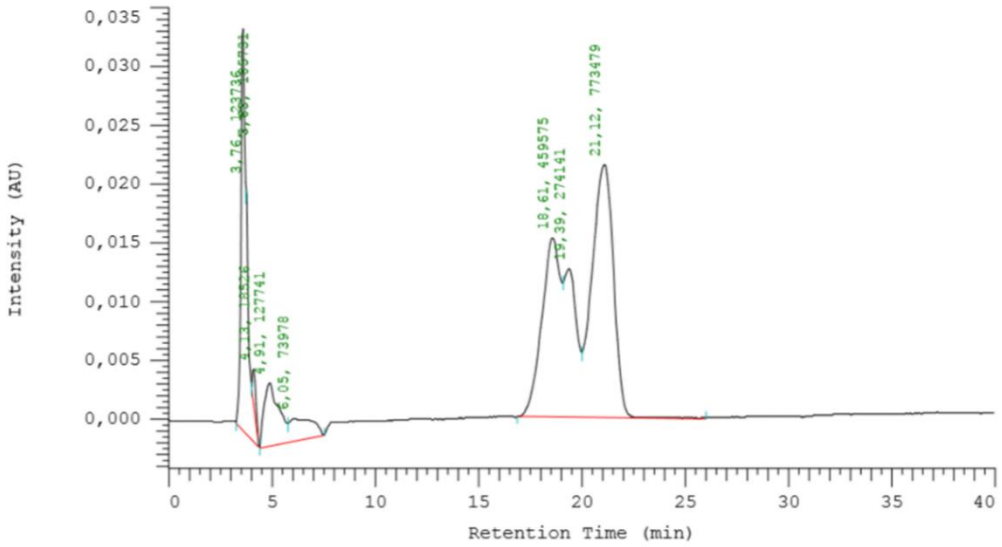


Figure 4. Chromatogram of the enantioseparation optimization of K and NK in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1 % DEA) and EtOH (50:50, *v*/*v*); flow-rate: 0.5 mL/min; injection volume: 10 µL; detector: 220 nm. The concentration of standard solution was 50 µg/mL in MeOH.

Thus, EtOH was replaced by ACN in the mobile phase and different proportions were tested maintaining the other conditions. Mobile phase consisted in 20 mM ammonium acetate in UPW (without 0.1 % of DEA) and ACN (70:30, *v*/*v*), however enantioseparation for all enantiomers still did not occur (**Figures 5**).

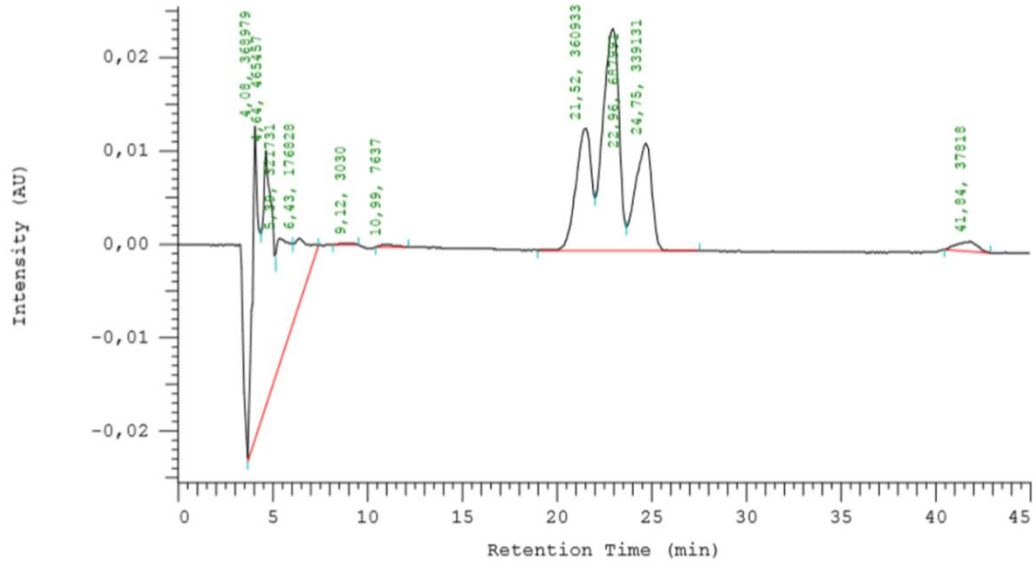


Figure 5. Chromatogram of enantioseparation optimization of K and NK in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW and ACN (70:30, *v*/*v*); flow-rate: 0.5 mL/min; injection volume: 10 µL; detector: 220 nm. The concentration of standard solution was 50 µg/mL in MeOH.

In order to improve enantioseparation, variation of the flow-rate to 0.5 mL/min with 20 mM ammonium acetate in UPW (with 0.1 % of DEA) and ACN (70:30, *v*/*v*) as mobile phase was tested under these conditions, good enantioseparation and enantioresolution was observed, however though at longer retention times (**Figure 6**).

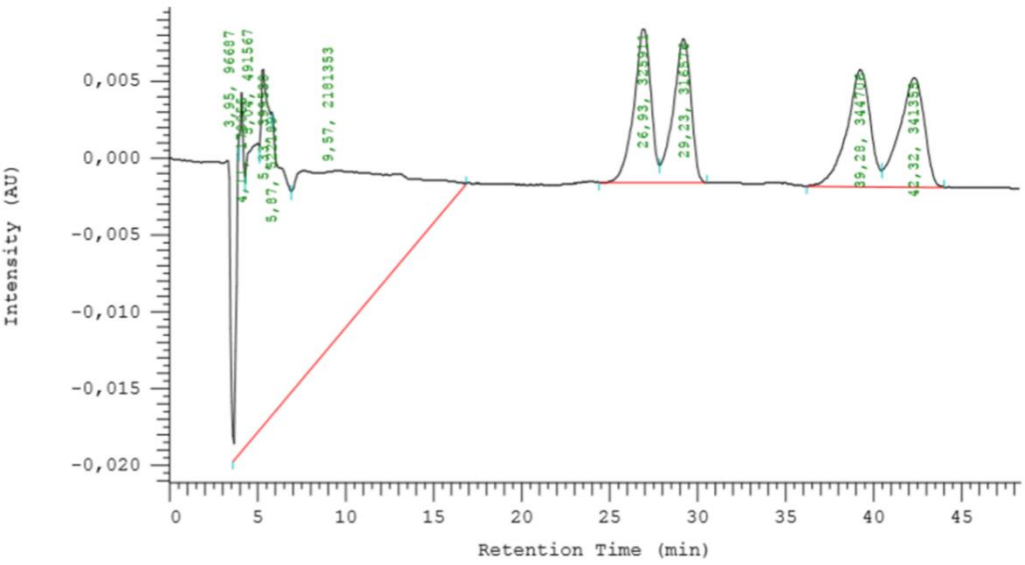


Figure 6. Chromatogram of enantioseparation optimization of K and NK in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (with 0.1 % of DEA) and ACN (70:30, *v*/*v*); flow-rate: 0.5 mL/min; injection volume: 10 µL; detector: 220 nm. The concentration of standard solution was 50 µg/mL in MeOH.

Thus, to reduce retention time of the analytes while maintaining enantioseparation different proportion (*v*/*v*) of this mobile phase were tested at different proportions of 75:25, 74:26 and 73:27. **Figure 7** show the chromatogram with 20 mM ammonium acetate in UPW (with 0.1 % of DEA) and ACN (75:25, *v*/*v*) as mobile phase. However, despite the good enantioselective and resolutions, the time analysis still high.

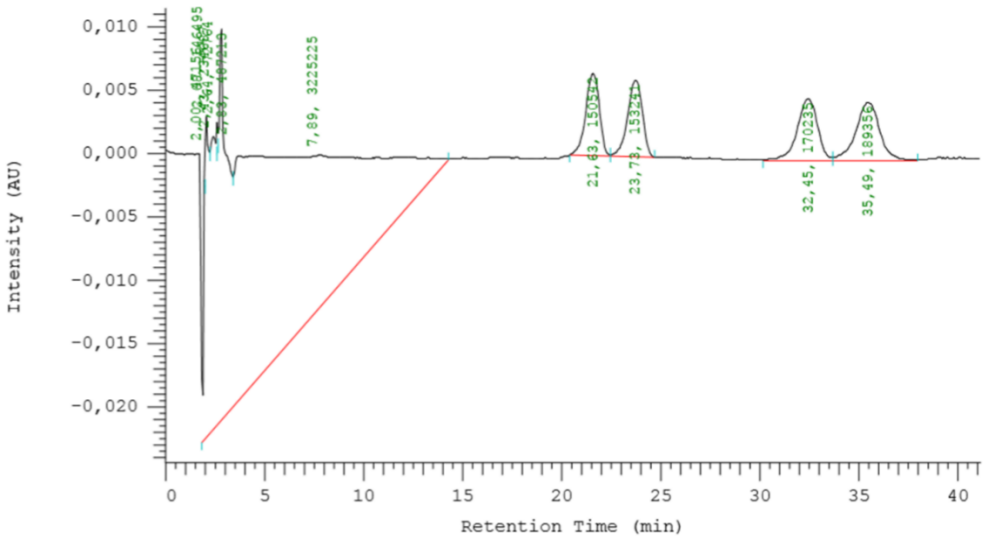


Figure 7. Chromatogram of enantioseparation of K and NK in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1 % DEA) and ACN (75:25, *v*/*v*); flow-rate: 1 mL/min; injection volume: 10 µL; detector: 220 nm. The concentration of standard solution was 50 µg/mL in MeOH.

Thus, to reduce retention time of the analytes the amount of ACN was increased. The optimized conditions were established as mobile phase 20 mM ammonium acetate in UPW (with 0.1 % of DEA) and ACN (70:30, *v*/*v*) as mobile phase, flow-rate of 1 mL/min and injection volume of 10 µL. Under these conditions good enantioseparation was achieved and at lower retention times. The retention times obtained for NK and K enantiomers were 13, 14, 19 and 20 minutes, respectively (**Figure 8**). These conditions were selected for further validation of the method.

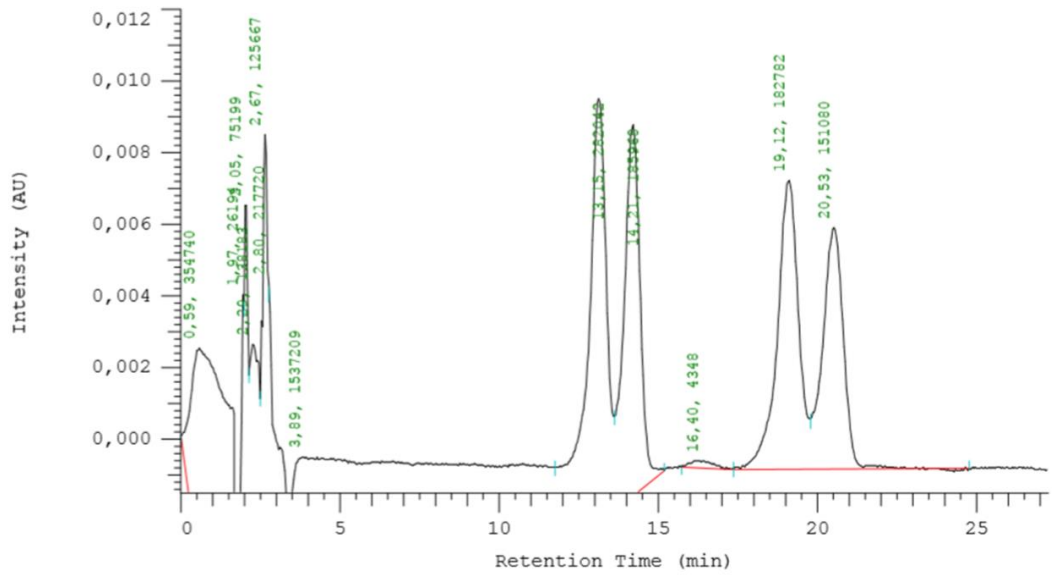


Figure 8. Chromatogram of the enantioseparation of K and NK in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1 % DEA) and ACN (70:30, *v*/*v*); flow-rate: 1 mL/min; injection volume: 10 µL; detector: 220 nm. The concentration of standard solution was 50 µg/mL in MeOH.

4.2. Validation of the method for quantification of Ketamine and Norketamine

Selectivity of the method was verified by comparison of the chromatograms with standards of K and NK in MeOH, blank matrix and spiked blank matrix with AS in the presence and in the absence of AS inoculum after 2 hours incubation. The nominal racemate concentrations used for K and NK were 50 and 25 µg/mL, respectively. Although the matrix is composed by several ionizable salts present in the MM, these salts did not interfere with the chromatographic performance of polysaccharide-based CPS.

The linearity was assessed for NK and K enantiomers ranging from their LOQ to 12.5 and 25 µg/mL respectively for each enantiomer (**Table 6**), and analyzed by linear regression using the peak areas and the theoretical concentrations. Concentration range, calibration equation and correlation level (R^2 > 0.99) obtained for each enantiomer (**Figures 9 to 12** and **Table 6**) presented values in accordance with the requirements specified by the international guidelines (ICH).

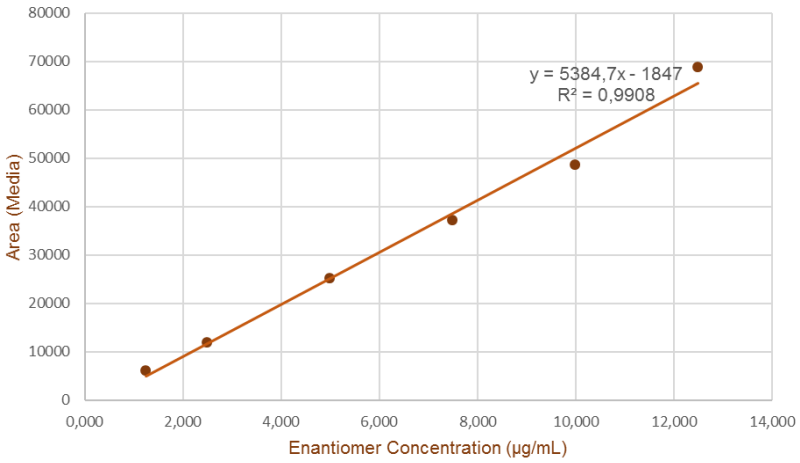


Figure 9. Calibration curve of NK_E1 (the first enantiomer eluted of NK).

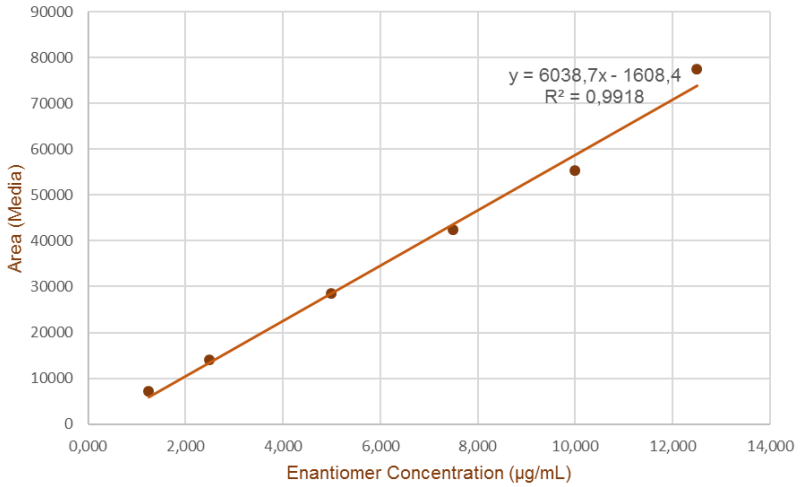


Figure 10. Calibration curve of NK_E2 (the second enantiomer eluted of NK).

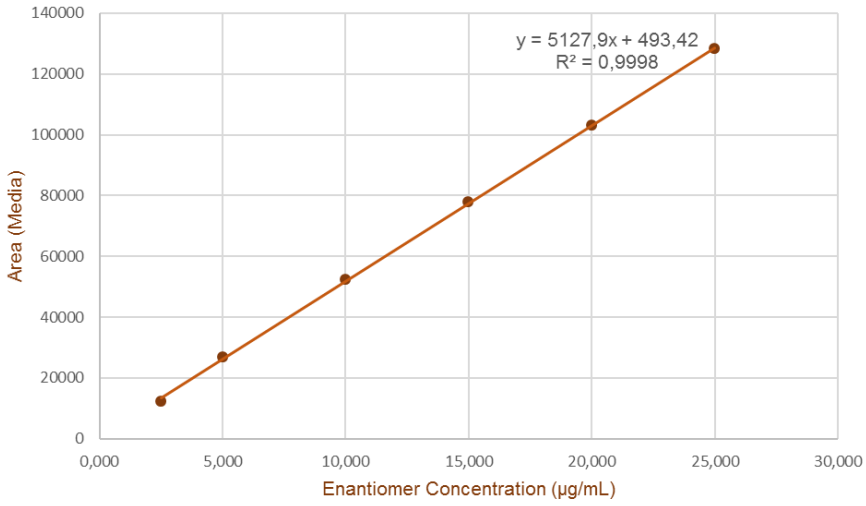


Figure 11. Calibration curve of K_E1 (the first enantiomer eluted of K).

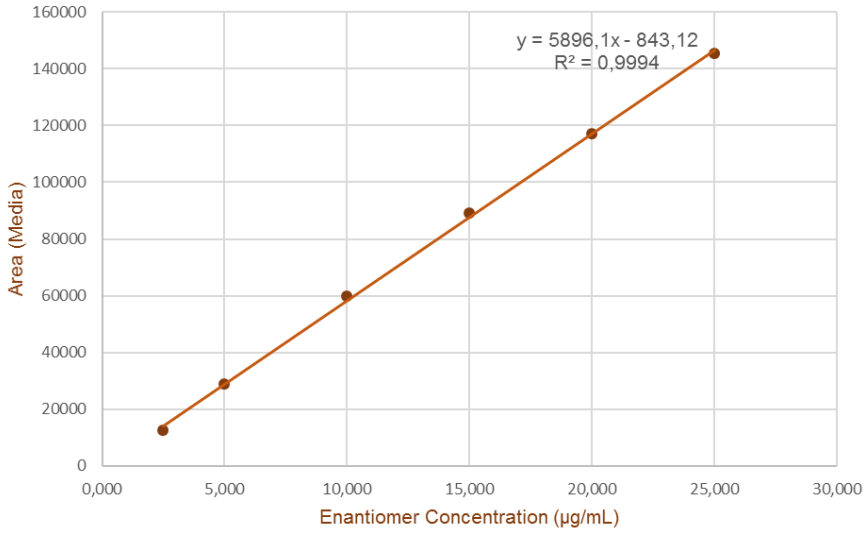


Figure 12. Calibration curve of K_E2 (the second enantiomer eluted of K).

Table 6. Linearity data, LOD and LOQ for K and NK enantiomers.

Enantiomer	Linearity			Limits	
	Range (µg/mL)	Linear Regression	Correlation Level (R^2)	LOD (µg/mL)	LOQ (µg/mL)
NK_E1	1.25-12.5	$y = 5384.7x - 1847$	0.9908	0.5	1.25
NK_E2	1.25-12.5	$y = 6038.7x - 1608.4$	0.9918	0.5	1.25
K_E1	2.5-25	$y = 5127.9x + 493.42$	0.9998	0.5	2.5
K_E2	2.5-25	$y = 5896.1x - 843.12$	0.9994	0.5	2.5

Table 7. Accuracy, intra and inter-day precision and recovery obtained for K and NK enantiomers.

Enantiomer	Concentration (µg/mL)	Validation Parameters			
		Accuracy (%)	Recovery (%)	Intra-Day Precision (%RSD)	Inter-Day Precision (%RSD)
NK_E1	2	113.6	75.4	6.1	6.7
	6	94.8	81.9	8.1	8.4
	8.5	106.9	67.9	4.5	4.8
NK_E2	2	105.4	69.5	6.3	8.8
	6	101.2	76.2	8.7	8.8
	8.5	105.9	64.0	5.9	6.1
K_E1	4	86.6	61.0	9.4	10.5
	12.5	104.1	86.9	2.3	3.4
	17.5	102.3	71.1	1.8	2.7
K_E2	4	85.9	50.1	10.4	11.3
	12.5	104.1	79.3	2.8	2.9
	17.5	103.6	69.4	3.6	3.8

Accuracy, intra- and inter-batch precisions and recovery were determined at three QC standard solutions with enantiomeric concentrations of 2, 6 and 8.5 µg/mL for NK and 4, 12.5 and 17.5 µg/mL for K (**Tables 6 and 7**).

Accuracy percentages for NK were 113.6, 94.8 and 106.9 for the first enantiomer eluted and 105.4, 101.2 and 105.9 for the second enantiomer eluted (**Table 7**). For K, the accuracy percentages were 86.6, 104.1 and 102.3 for the first enantiomer eluted and 85.9, 104.1 and 103.6 for the second enantiomer eluted (**Table 7**). Intra- and inter-day precisions were expressed as % RSD and were lower than 10.4 % and 11.3 %, respectively (**Table 7**). Precision values obtained were in agreement with those demanded by the ICH (up to 20 %). Recovery percentages varied between 50.1 and 86.9 %. LOD achieved was 0.5 for both enantiomers of K and NK. LOQ was 1.25 for NK enantiomers and 2.5 for K enantiomers (**Table 6**). These values proved to be suitable for monitoring of the target enantiomers during the biodegradation assays.

4.3. Enantioselective Biodegradation Assays

The enantioselective biodegradation of K racemate was monitored using the above mentioned validated LC-DAD method. The biodegradation assays were performed with K racemate added at initial concentration of 40 µg/mL to the MM inoculated with the AS. The capacity of the AS to degrade the compound tested was investigated in the present and in the absence of sodium acetate as an external carbon and energy source, under dark and light conditions. The abiotic and dead cells experiments were performed under the same conditions. AS was obtained from the aerated tanks of a municipal WWTP selected due to its high microbial diversity. The biodegradation assay was monitored for 21 days (**Figure 13**). To construct the biodegradation patterns of K at the different experimental conditions the concentration of K enantiomers were monitored by the enantioselective validated LC method and converted into degradation % according to the following formula:

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

where $\%\,D_t$ is the degradation percentage at day t; C_t is the concentration at day t and C_0 is the initial concentration (day 0).

The biodegradation profiles were similar for both enantiomers in all experimental conditions and enantioselective degradation was not observed. Under tested conditions, K racemate was poorly degraded. Adsorption of K enantiomers to the dead cells was not observed neither the formation of metabolites (flasks 23 to 26 see **Figure 13**). These data are in accordance to monitoring reports that have been shown the occurrence and the persistence of K in aquatic environment (Lin et al. 2010; Baker and Kasprzyk-Hordern 2013; Lai et al. 2013; van Nuijs et al. 2013; Lin et al. 2014; Jiang et al. 2015; Li et al. 2016). Nonetheless, various studies have been shown that microbiological degradation is an important degradation pathway for the reduction of the content in organic compounds in effluent wastewaters. Besides, it has been shown that biodegradation of chiral compounds can be enantioselective leading to changes in the EF. In fact, the EF profile has been used to distinguish between treated and illegal untreated discharges of wastewaters. Microbiological degradation can also lead to the enrichment of one of the enantiomers. Nonetheless, microbiological community of AS is different among WWTPs and even over time in the same WWTP. This can influence biodegradation pathways.

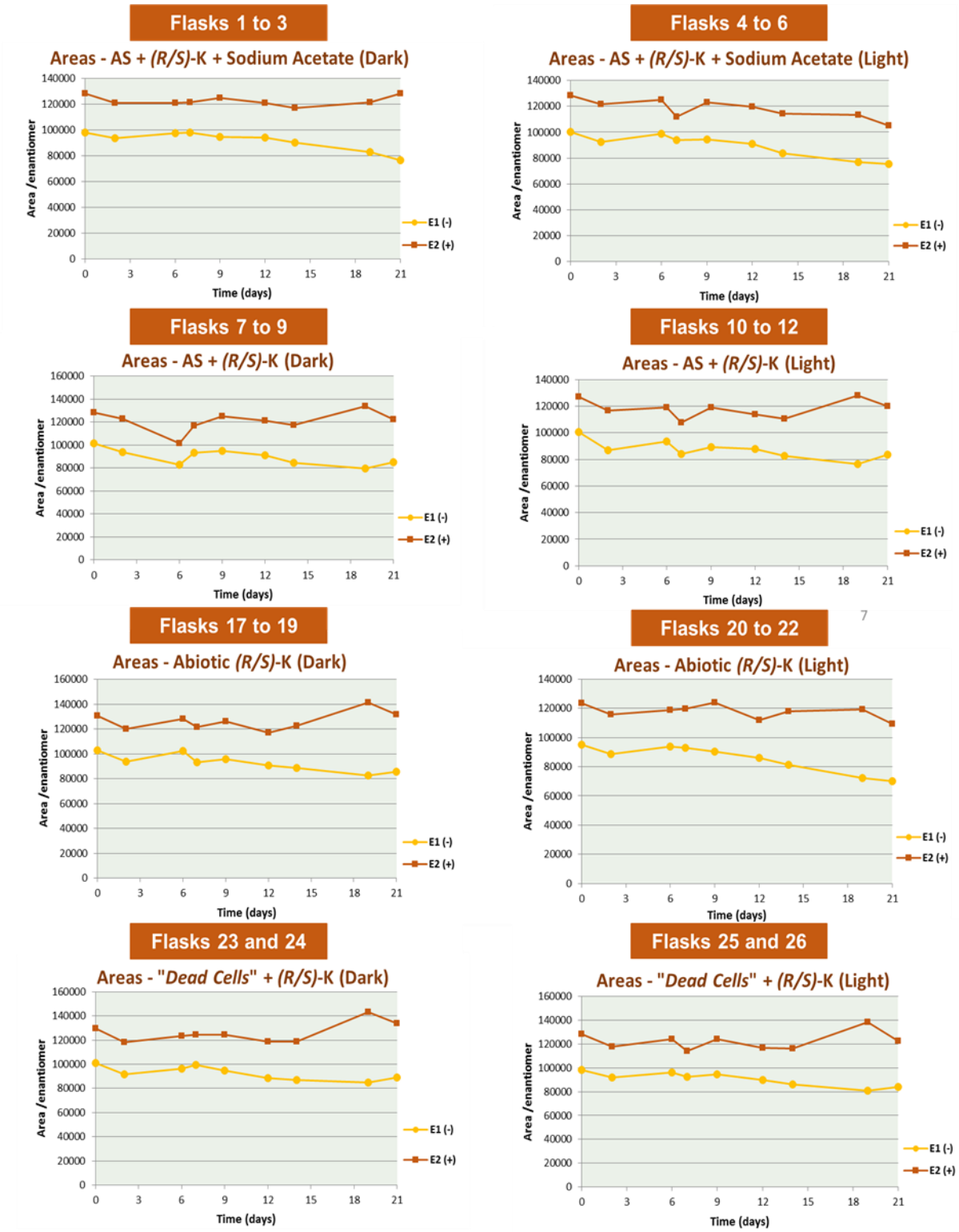


Figure 13. Enantioselective biodegradation assays with K racemate at 40 µg/mL by AS monitoring during 21 days at different experimental conditions: (Flasks 1 to 3) AS, sodium acetate, dark; (Flasks 4 to 6) AS, sodium acetate, light; (Flasks 7 to 9) AS, dark; (Flasks 10 to 12) AS, light; (Flasks 17 to 19) abiotic, dark; (Flasks 20 to 22) abiotic, light; (Flasks 23 and 24) dead cells, dark; (Flasks 25 and 26) dead cells, light.

4.4. Semi-Preparative Enantioseparation

The development of the semi-preparative enantioseparation of K and NK was performed with a “house made” semi-preparative chromatographic column (amylose *tris*-3,5-dimethylphenylcarbamate) under normal elution mode approach by LC.

4.4.1. Semi-Preparative Enantioseparation of Ketamine

Considering the optimization at RT in LC-DAD with Hex with 0.1 % of DEA and EtOH with 0.1 % of DEA, 90:10 *v*/*v* as a mobile phase at a flow-rate of 1.5 mL/min and injection volume of 10 µL, enantioseparation was not observed. The Hex with 0.1 % of DEA and IPA with 0.1 % of DEA as a mobile phase with different proportion (*v*/*v*) (such as 97:3, 95:5, 92:8) and flow-rate (1.2 and 1.5 mL/min), UV/Vis detector at 220 nm, injection volume of 10 µL were evaluated. Enantioseparation was achieved in those conditions (**Figure 14**).

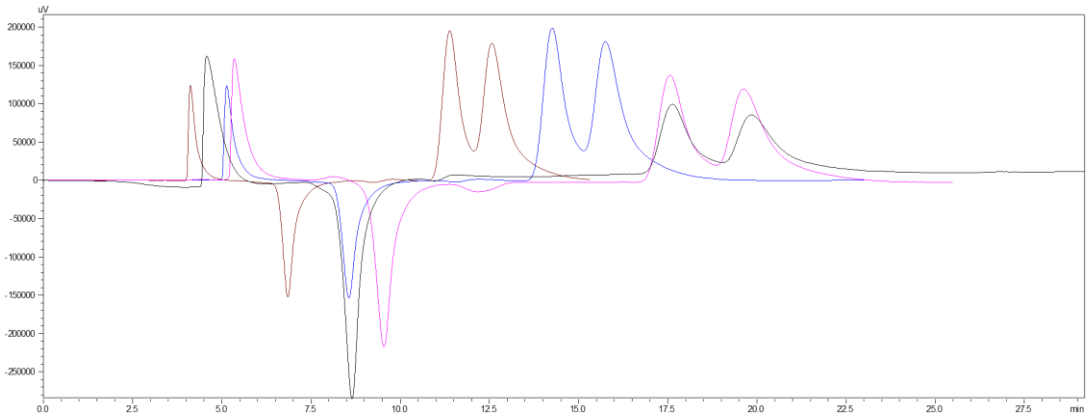


Figure 14. Chromatogram showing the separation of K enantiomers in the semi-preparative column (amylose *tris*-3,5-dimethylphenylcarbamate) by LC-UV/Vis at RT. Mobile phase: Hex (0.1 % DEA) and IPA (0.1 % DEA); injection volume: 10 µL; detector: 220 nm. Standard solution at 2 mg/mL (IPA with 0.1 % DEA). Legend: Blue Line - 92:8, *v*/*v* and flow-rate: 1.2 mL/min; Pink Line - 95:5, *v*/*v* and flow-rate: 1.2 mL/min; Black Line - 97:3, *v*/*v* and flow-rate: 1.5 mL/min; Brown Line - 92:8, *v*/*v* and flow-rate: 1.5 mL/min.

The variation of proportion of mobile phases shows that the percentage of IPA with 0.1 % of DEA influenced the intensity of peaks and the resolution (**Figure 14**). The variation of flow-rate of 1.2 and 1.5 mL/min with a proportion of 92:8 (*v*/*v*) show that retention times was higher with lower flow-rate. The temperature was also studied using the oven column (such 27.7 and 40º C) with the same

conditions, but with injection volume at 25 µL, however, the results are similar, only influence the retention times of the enantiomers (**Figure 15**).

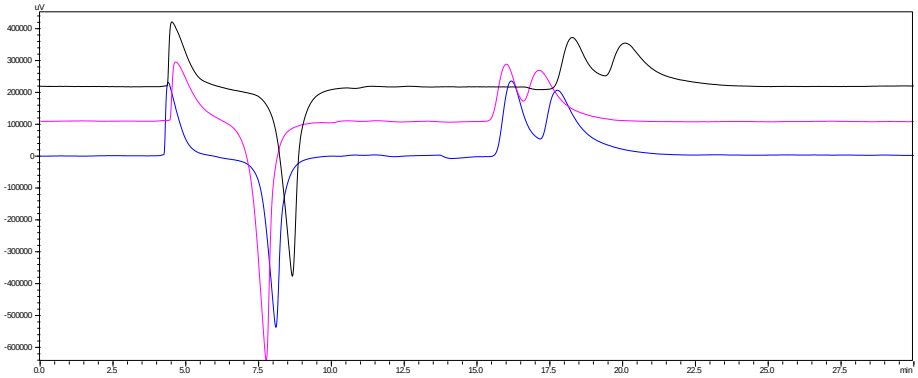


Figure 15. Chromatogram showing the separation of K enantiomers in the semi-preparative column (amylose *tris*-3,5-dimethylphenylcarbamate) by LC-UV/Vis. Mobile phase: Hex (0.1 % DEA) and IPA (0.1 % DEA); injection volume: 25 µL; flow-rate: 1.5 mL/min; detector: 220 nm. Standard solution at 2 mg/mL (IPA with 0.1% DEA). Legend: Blue Line – (96:4, *v*/*v*); oven column: 27.7º C; Pink Line - (97:3, *v*/*v*); oven column: 40º C; Black Line - (97:3, *v*/*v*); at RT.

After several development using different combinations and composition of mobile phase, flow-rates, and injection volume (see **Table 4**), the optimization of chromatographic conditions were: Hex (with 0.1 % of DEA) and IPA (with 0.1 % of DEA) (95:5, *v*/*v*) as a mobile phase, flow-rate of 1.5 mL/min. Thus, within these conditions different concentration of K at different injection volume (such as 5, 10 and 15 µL) were tested (**Figure 16 and 17**). The standard solution of K was diluted in IPA (with 0.1 % of DEA) at 10 and 20 mg/mL. The strategy of separation consists of a set between injection volumes and different concentrations of K, aiming to reach the concentration limit of the sample, taking into account the load capacity of the column.

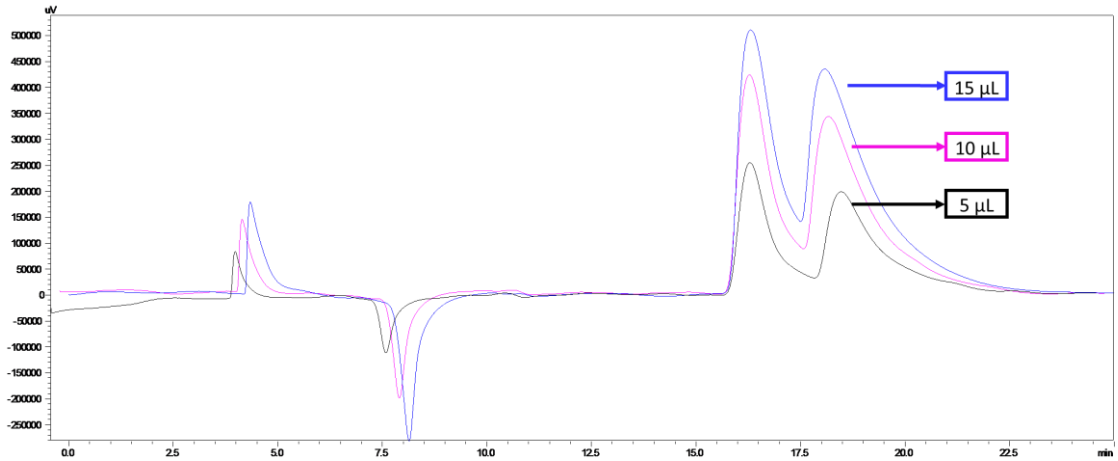


Figure 16. Chromatogram showing the separation of K enantiomers in the semi-preparative column (amylose *tris*-3,5-dimethylphenylcarbamate) by LC-UV/Vis at RT. Mobile phase: Hex (0.1 % DEA) and IPA (0.1 % DEA) (95:5, *v*/*v*); flow-rate: 1.5 mL/min; detector: 220 nm; oven

column: 20º C. Standard solution at 10 mg/mL (IPA with 0.1 % DEA). Legend: Blue Line – injection volume: 15 µL; Pink Line - injection volume: 10 µL; Black Line - injection volume: 5 µL.

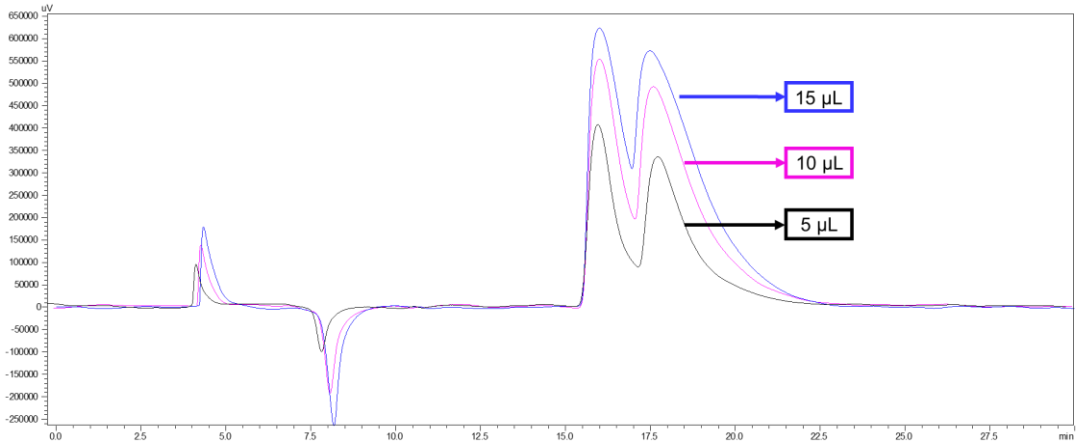


Figure 17. Chromatogram showing the separation of K enantiomers in the semi-preparative column (amylose *tris*-3,5-dimethylphenylcarbamate) by LC-UV/Vis at RT. Mobile phase: Hex (0.1 % DEA) and IPA (0.1 % DEA) (95:5, *v*/*v*); flow-rate: 1.5 mL/min; detector: 220 nm; oven column: 20º C. Standard solution at 20 mg/mL (IPA with 0.1 % DEA). Legend: Blue Line – injection volume: 15 µL; Pink Line - injection volume: 10 µL; Black Line - injection volume: 5 µL.

The optimized conditions for enantioseparation was established with injection volume of 10 µL at 20 mg/mL (**Figure 18**).

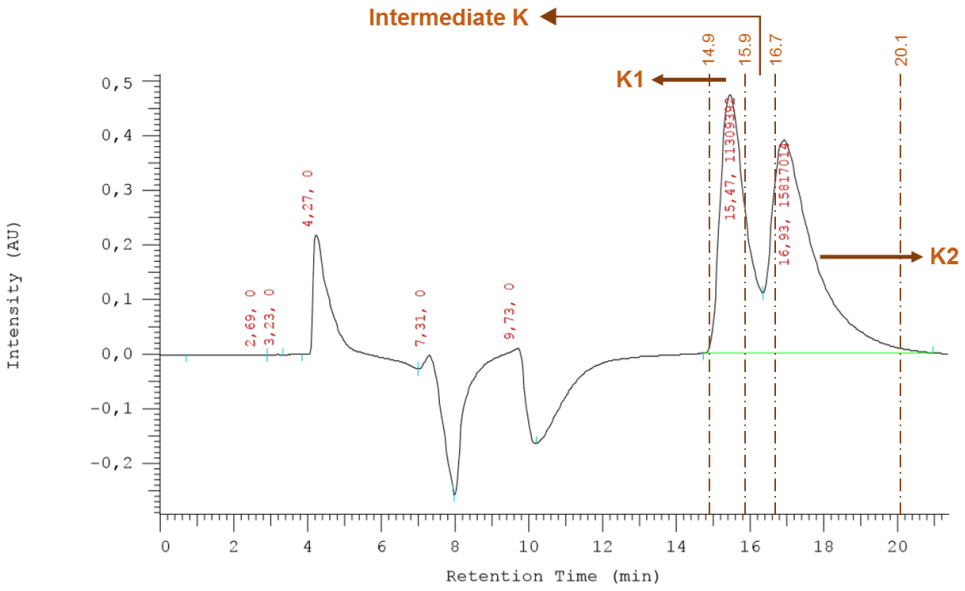


Figure 18. Chromatogram showing the separation of K enantiomers in the semi-preparative column (amylose *tris*-3,5-dimethylphenylcarbamate) by LC-DAD at RT. Mobile phase: Hex (0.1 % DEA) and IPA (0.1 % DEA) (95:5, *v*/*v*); injection volume: 10 µL; flow-rate: 1.5 mL/min; detector: 230 nm. Stock solution concentration at 20 mg/mL (IPA with 0.1 % DEA).

Figure 18 showed an example of chromatogram of separation of K enantiomers, with the collections times of fractions separated by semi-preparative LC-DAD. Fraction K1 was collected from 14.9 until 15.9 minutes and fraction K2 was collected from 16.7 to 20.1 minutes. The collection times of

fractions were selected taking account the enantioseparation of enantiomers of K which does not obtained with a higher resolution and either complete separation of compounds. So, the collection of intermediated fraction was performed to avoid the contamination of K1 and K2. After fraction collection and treatment of these fractions, the enantiomeric purity was evaluated with the analytical method previously validated (**Figure 19 to 22**).

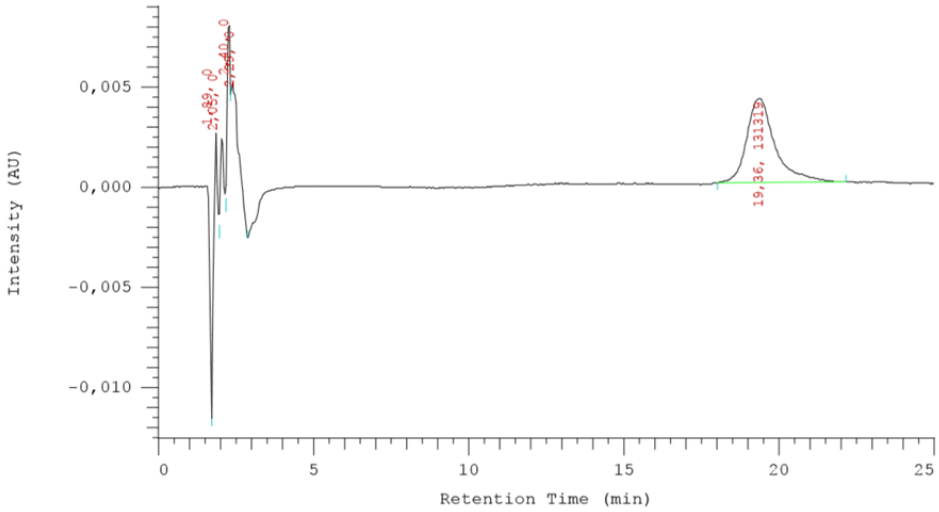


Figure 19. Chromatogram of the analysis of fraction K1 in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1 % DEA) and ACN (70:30, *v*/*v*); flow-rate: 1 mL/min; injection volume: 10 µL; detector: 220 nm.

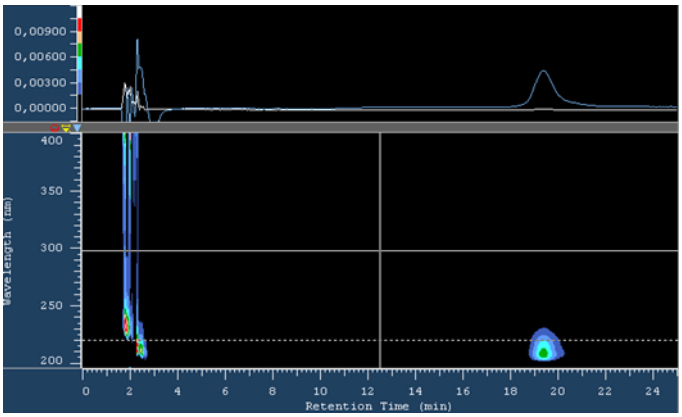


Figure 20. Chromatogram with absorption spectra of the analysis of fraction K1 in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1 % DEA) and ACN (70:30, *v*/*v*); flow-rate: 1 mL/min; injection volume: 10 µL; detector: 220 nm.

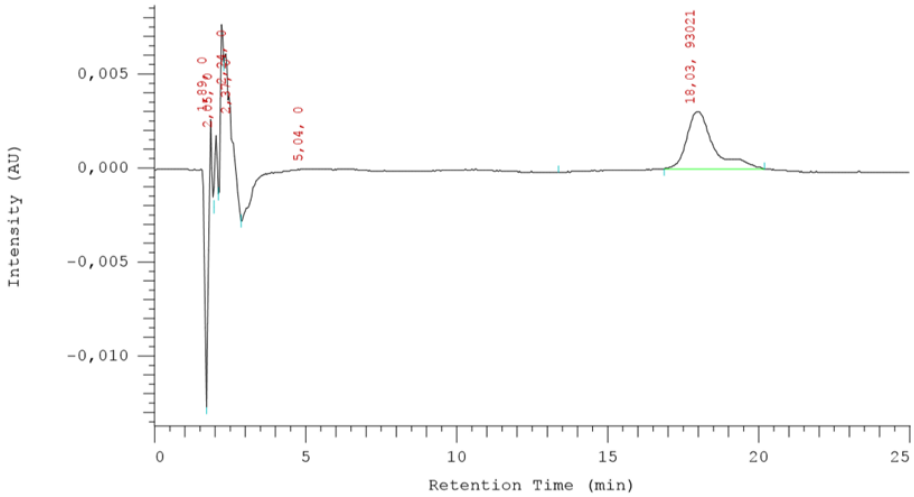


Figure 21. Chromatogram of the analysis of fraction K2 in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1 % DEA) and ACN (70:30, *v*/*v*); flow-rate: 1 mL/min; injection volume: 10 µL; detector: 220 nm.

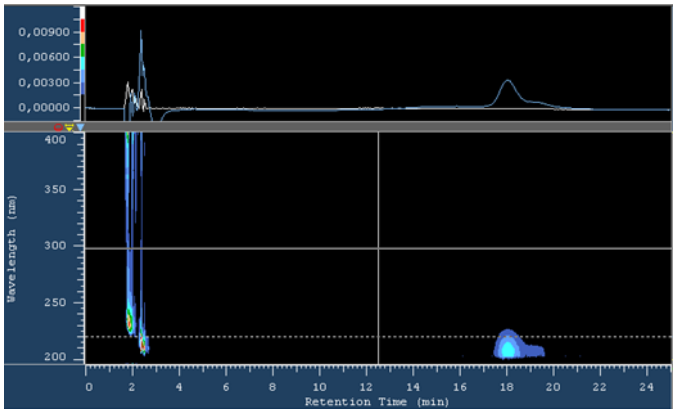


Figure 22. Chromatogram with absorption spectra of the analysis of fraction K2 in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1 % DEA) and ACN (70:30, *v*/*v*); flow-rate: 1 mL/min; injection volume: 10 µL; detector: 220 nm.

Regarding the efficiency of the semi-preparative chromatography method, 6 mg of racemic K was separated corresponding to 3 mg injected of each enantiomer, 2.51 mg of the first enantiomer eluted (K1) and 2.26 mg of the second enantiomer eluted (K2) were obtained. Recovery percentage was 84 % and 75 % for K1 and K2, respectively (**Table 8**). Thirty injections of racemic K were performed, and only the first enantiomer eluted was obtained in a purity close to 100 % (**Figures 19 and 20**), while the second enantiomer presented an enantiomeric purity above 97 % (**Figures 21 and 22**).

Table 8. Results of semi-preparative chromatography of K.

Enantiomer	Standard Solution 20 mg/mL K (IPA with 0.1 % DEA)			
	Theorical Concentration (mg)	Linear Equation	Recovery Concentration (mg)	Recovery (%)
K_E1	3	$y = 5127.9x + 493.42$	2.51	84
K_E2		$y = 5896.1x - 843.12$	2.26	75

4.4.2. Semi-Preparative Enantioseparation of Norketamine

Considering the optimization at room temperature in LC-DAD with Hex with 0.1 % of DEA and IPA with 0.1 % of DEA, 80:20, *v*/*v* as a mobile phase, flow-rate of 1.5 mL/min, volume injection of 50 µL and the detector fixed at 220 nm, partial enantioseparation was observed (**Figure 23**). Considering Hex with 0.1 % of DEA and EtOH with 0.1 % of DEA, 90:10, *v*/*v* as a mobile phase, flow-rate of 1.5 mL/min and volume injection of 10 µL, a baseline enantioseparation was observed, but with long time of analysis. The time between the enantiomers elution shows a difference of 10 minutes (**Figure 24**). Hence, the mobile phase composed by Hex with 0.1 % of DEA and EtOH with 0.1 % of DEA was study in different proportion, injection volume and injection of different concentrations of standard solutions.

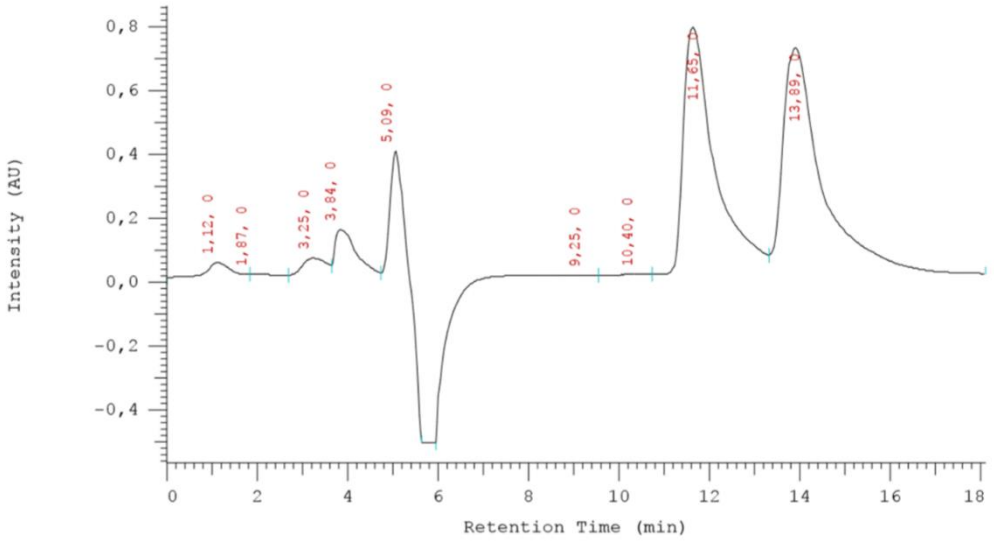


Figure 23. Chromatogram showing the separation of NK enantiomers in the semi-preparative column (amylose *tris*-3,5-dimethylphenylcarbamate) by LC-DAD at RT. Mobile phase: Hex (0.1 % DEA) and IPA (0.1 % DEA) (80:20, *v*/*v*); flow-rate: 1.5 mL/min; injection volume: 50 µL; detector: 220 nm. Standard solution at 2 mg/mL (EtOH).

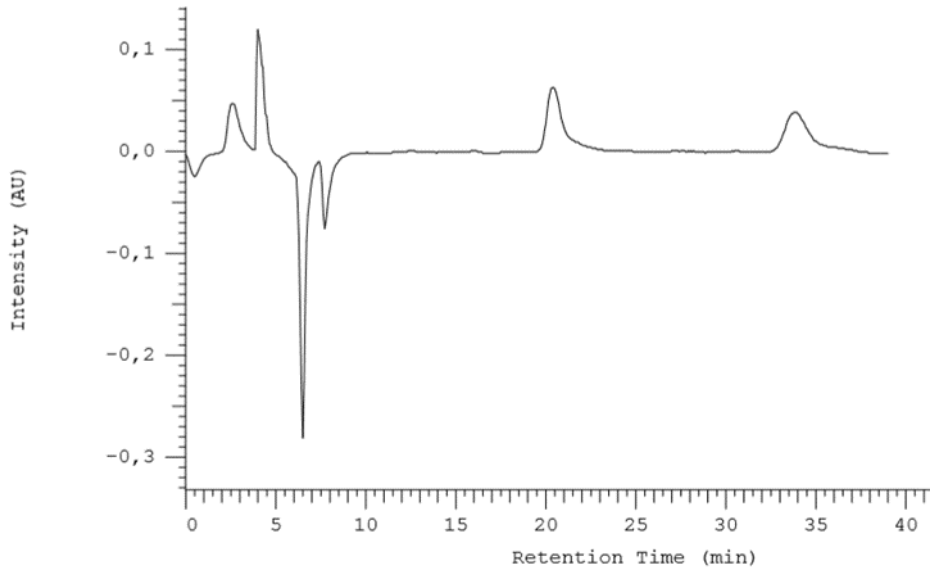


Figure 24. Chromatogram showing the separation of NK enantiomers in the semi-preparative column (amylose *tris*-3,5-dimethylphenylcarbamate) by LC-DAD at RT. Mobile phase: Hex (0.1 % DEA) and EtOH (0.1 % DEA) (90:10, *v*/*v*); flow-rate: 1.5 mL/min; injection volume: 10 µL; detector: 220 nm. Standard solution at 1 mg/mL (MeOH).

After several attempts using different combinations and composition of mobile phase and injection volume (**Table 5**), Hex with 0.1 % of DEA and EtOH with 0.1 % of DEA, 65:35, *v*/*v* as a mobile phase, at a flow-rate of 1.5 mL/min, was established as a optimized mobile phase. Subsequently, different concentrations of NK at different volume injection, such as 25, 50 and 100 µL were evaluated. **Figure 25** represent the chromatogram with 50 µL of volume injection. The standard solution of NK was diluted in (EtOH) at 2, 4, 6, 10 and 20 mg/mL. The strategy of separation consists of a set between injection volumes and different concentrations of NK, aiming to reach the load capacity of the column.

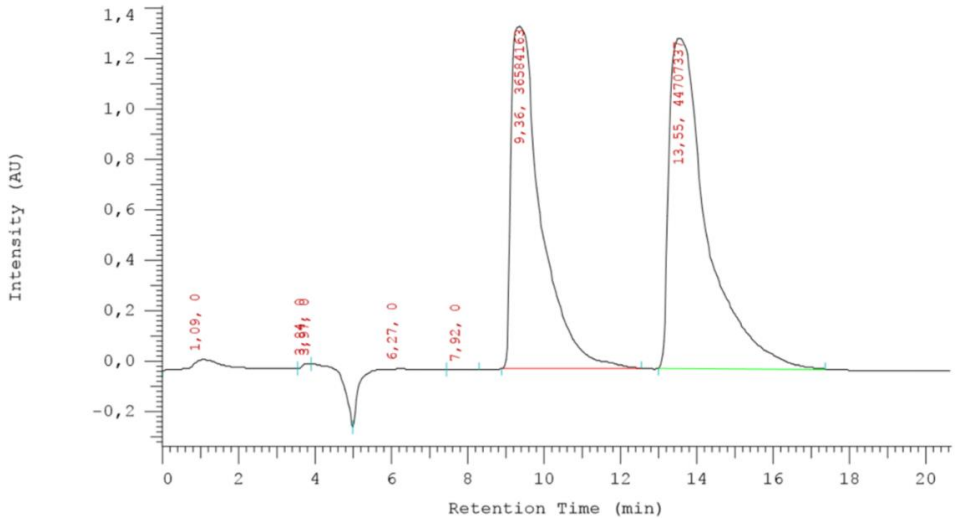


Figure 25. Chromatogram showing the separation of NK enantiomers in the semi-preparative column (amylose *tris*-3,5-dimethylphenylcarbamate) by LC-DAD at RT. Mobile phase: Hex (0.1 % DEA) and EtOH (0.1 % DEA) (65:35, *v*/*v*); flow-rate: 1.5 mL/min; injection volume: 50 µL; detector: 220 nm. Standard solution at 6 mg/mL (EtOH).

The volume injection of 50 µL and concentration of 10 mg/mL (EtOH), both enantiomers were eluted in 19 minutes. **Figure 26** showed an example of chromatogram of separation of NK enantiomers, with the collections times of fractions. Fraction NK1 was collected from 9 until 10.5 minutes and fraction NK2 was collected from 13.3 to 18.6 minutes. The collection times of fractions were selected taking account the time of elution and the chromatographic parameters (alpha and resolution) that allow high load of the column and high enantiomeric purity of the fractions. Therefore, to avoid the contamination of fractions an intermediate fraction was collected from 10.5 to 13.3 minutes.

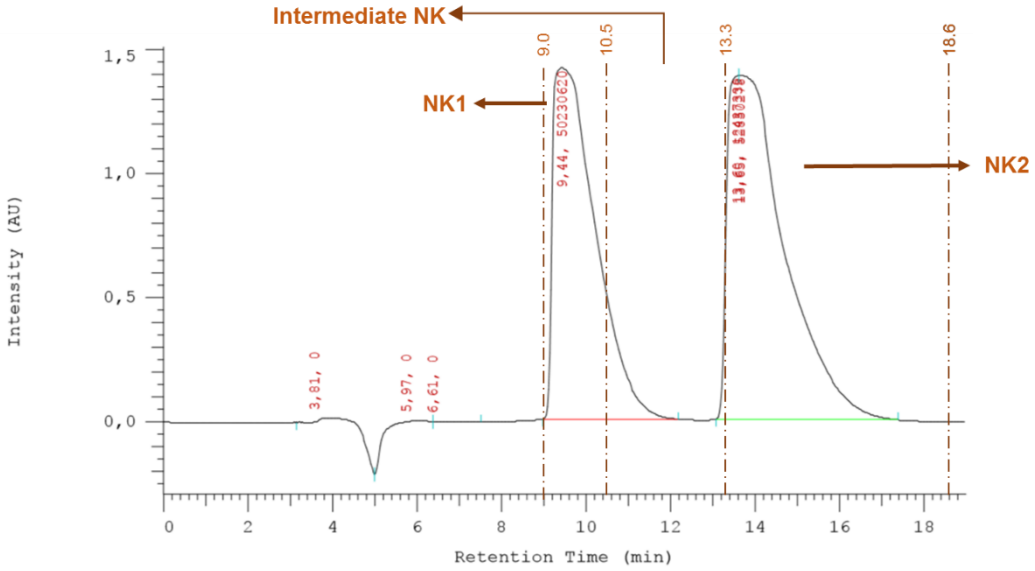


Figure 26. Chromatogram showing the separation of NK enantiomers in the semi-preparative column (amylose *tris*-3,5-dimethylphenylcarbamate) by LC-DAD at RT. Mobile phase: Hex (0.1

% DEA) and EtOH (0.1 % DEA) (65:35, *v*/*v*); flow-rate: 1.5 mL/min; injection volume: 50 µL; detector: 220 nm. Stock solution concentration at 10 mg/mL (EtOH).

The enantiomeric purity and the yield of the factions were evaluated by the analytical method previously validated (**Figures 27 to 30**).

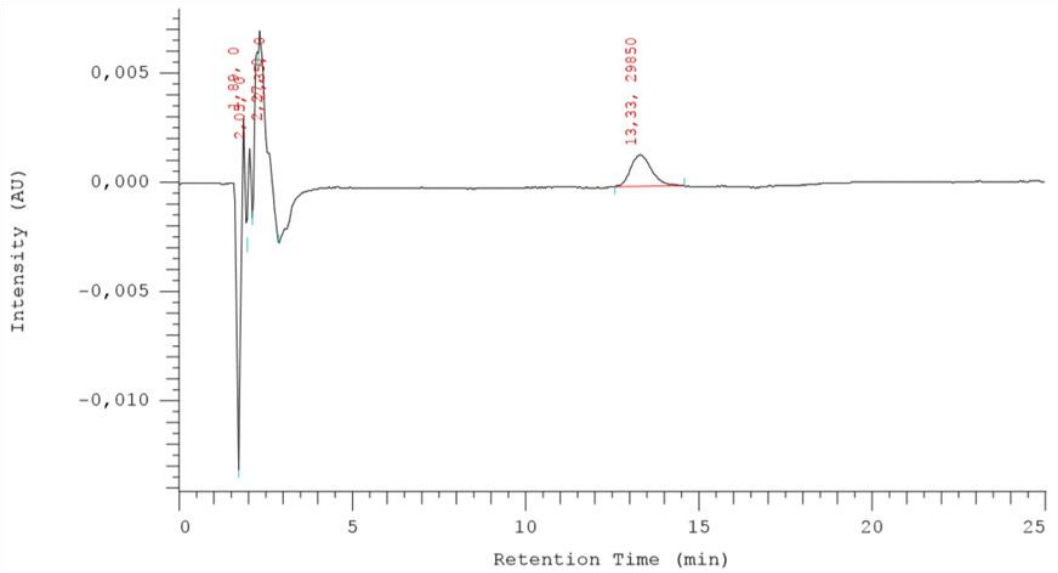


Figure 27. Chromatogram of the analysis of fraction NK1 in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1 % DEA) and ACN (70:30, *v*/*v*); flow-rate: 1 mL/min; injection volume: 10 µL; detector: 220 nm.

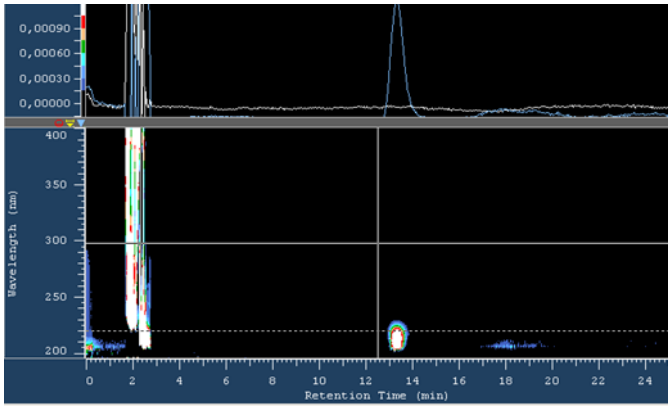


Figure 28. Chromatogram with absorption spectra of the analysis of fraction NK1 in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1 % DEA) and ACN (70:30, *v*/*v*); flow-rate: 1 mL/min; injection volume: 10 µL; detector: 220 nm.

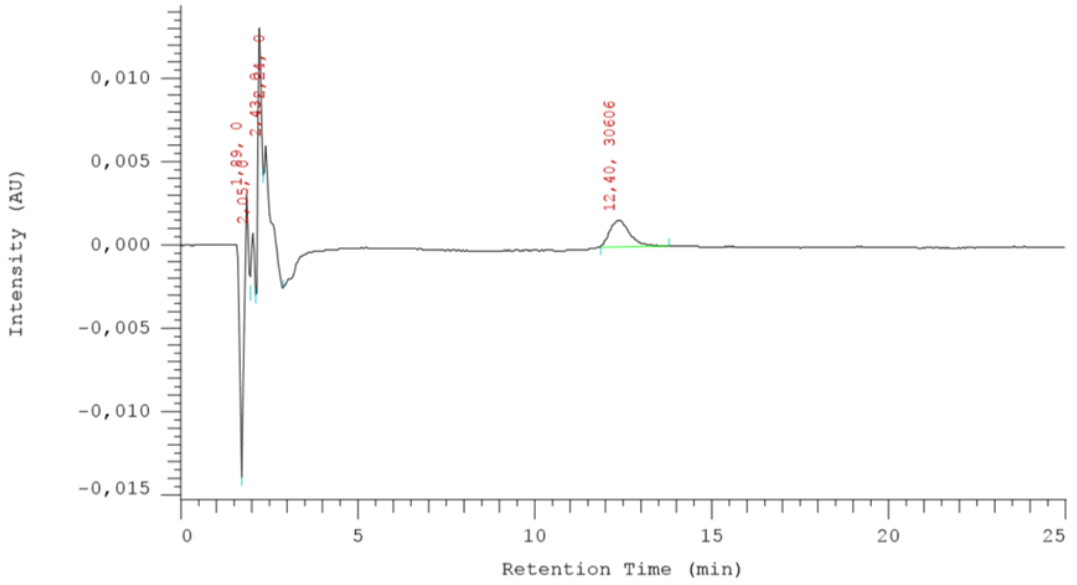


Figure 29. Chromatogram of the analysis of fraction NK2 in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1 % DEA) and ACN (70:30, *v*/*v*); flow-rate: 1 mL/min; injection volume: 10 µL; detector: 220 nm.

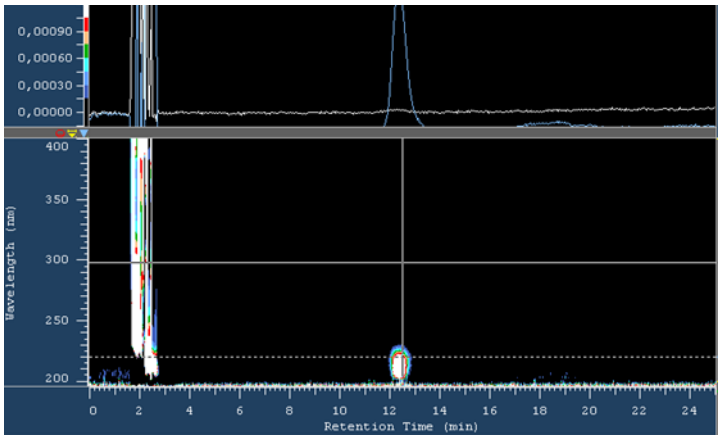


Figure 30. Chromatogram with absorption spectra of the analysis of fraction NK2 in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1 % DEA) and ACN (70:30, *v*/*v*); flow-rate: 1 mL/min; injection volume: 10 µL; detector: 220 nm.

Regarding the efficiency of the semi-preparative chromatography method, 3 mg of racemic NK was separated corresponding to 1.5 mg injected of each enantiomer, 1.19 mg of the first enantiomer eluted (NK1) and 1.12 mg of the second enantiomer eluted (NK2) were obtained. Recovery percentage was 80 % and 74 % for NK1 and NK2, respectively. Six injections of racemic NK were performed and both enantiomers eluted from NK were obtained in a purity close to 100 % (**Figures 27 to 30**).

Table 9. Results of semi-preparative chromatography of NK.

Enantiomer	Injection Volume (µL)	Standards Solutions 2[a]/4[b]/6[c]/10[d]/20[e] mg/mL NK (EtOH)			
		Theorical Concentration (mg)	Linear Equation	Recovery Concentration (mg)	Recovery (%)
NK_E1	25[d]	0.375	$y = 5384.7x - 1847$	0.12	32
NK_E2			$y = 6038.7x - 1608.4$	0.13	35
NK_E1	50[a-d]	1.9	$y = 5384.7x - 1847$	0.69	36
NK_E2			$y = 6038.7x - 1608.4$	0.89	47
NK_E1	50[d]	1.5	$y = 5384.7x - 1847$	1.19	80
NK_E2			$y = 6038.7x - 1608.4$	1.12	74
NK_E1	100[d]		$y = 5384.7x - 1847$	0.45	30
NK_E2			$y = 6038.7x - 1608.4$	0.68	45
NK_E1	100[e]	4.7	$y = 5384.7x - 1847$	2.34	50
NK_E2			$y = 6038.7x - 1608.4$	1.88	40

a: 2 mg/mL NK (EtOH); b: 4 mg/mL NK (EtOH); c: 6 mg/mL NK (EtOH); d: 10 mg/mL NK (EtOH); e: 20 mg/mL NK (EtOH).

4.5. Enantioselective Ecotoxicity Assays

Toxicity of K and NK racemate and possible enantiomer-dependent toxicity were evaluated for two aquatic organisms: *D. magna* and *T. thermophila*. The ecotoxicity assays were performed using standard kits commercialized, in accordance with national and international standards (ISO and OECD) (OECD Adopted April 2004; Adopted October 2017; ISO Fourth Edition 2012). These kits contain all the material necessary for the realization of the acute and chronic bioassays of short duration, containing the test biota in their inactive or immobilized forms, ready to use, without the need to maintain live stocks of the species under test. These organisms are widely used in ecotoxicological studies due to their short lifecycle, high sensitivity to a variety of chemicals and ease of manipulation in the laboratory (Wollenberger et al. 2000; Carlsson et al. 2006; Kim et al. 2007; Stanley et al. 2007; Cleuvers 2008; Andrés et al. 2009; Diao et al. 2010; Li et al. 2017).

4.5.1. *Daphnia magna* acute immobilization test

The acute immobilization toxicity assays were performed on *D. magna* for 48 hours. The concentration ranged between 0.1 to 1000 µg/L for K and NK racemate and from 0.05 to 500 µg/L for enantiomers. These concentrations were selected based on reported monitoring data in order to evaluate toxicity of K and NK racemate and their enantiomers at environmental relevant concentrations. For validation of the assay, the number of dead plus immobile organisms did not exceed 10 % in the controls. A reference test with $K_2Cr_2O_7$ was performance to assess the viability of assays. 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. According to ISO 6341, the EC_{50} calculated for $K_2Cr_2O_7$ is acceptable if its value is situated in the range 0.6 – 2.1 mg/L. The EC_{50} at 48 hours of exposure was of 0.67 mg/L. Thus, the reference test is valid and demonstrated the correct execution of the test procedure, as well as the good sensitivity of the test animals.

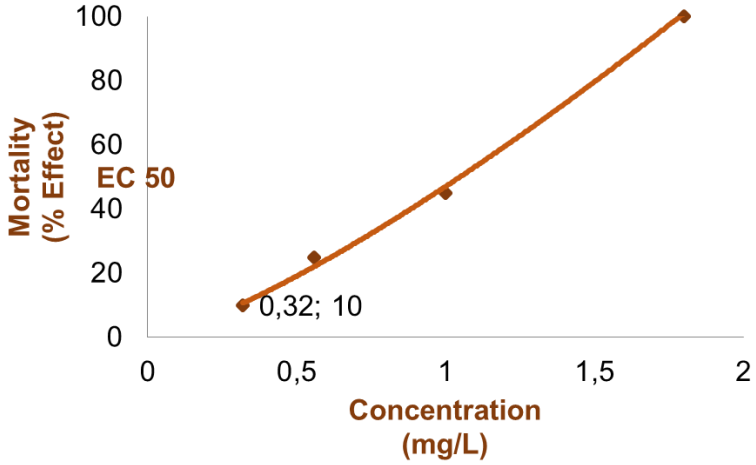


Figure 31. Potassium dichromate acute toxicity curve at the end of 48 hours exposure.

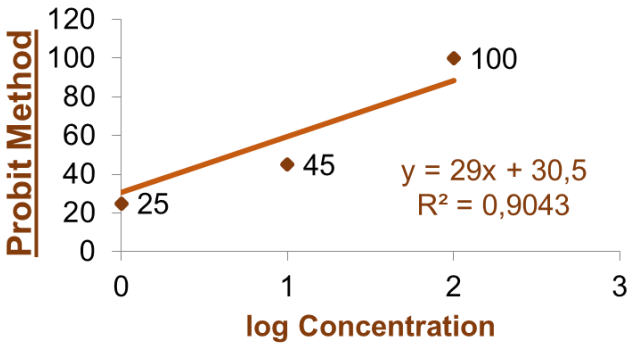


Figure 32. Probit method of acute toxicity of potassium dichromate at the end of 48 hours of exposure. EC_{50} of 0.672 mg/L.

The percentage of immobilization/ mortality results for the racemate K and NK are reported in **Tables 10 and 11**, respectively. Percentage of mortality of K and NK racemate were compared between the control and exposure concentrations. Regarding toxicity of K all concentrations presented percentage of mortality similar to the control except at the highest concentration (1000 µg/L) (**Table 10, Figure 33**). Nonetheless, no statistically significant differences were found between control and this concentration. On the other hand, higher percentage of mortality was verified for NK in all range of concentrations compared to the control and gradient dependent (**Table 11, Figure 34**). No statistically significant differences were found between control and the selected exposure concentrations. Nevertheless, NK presented higher percentage of mortality compared with K for all range of concentrations (**Figure 35**). These results show higher susceptibility of *D. magna* to the metabolite of K, NK, rather than to K.

Regarding assays with enantiomers of K, toxicity for both enantiomers were not statistically different from the control, nevertheless, K1 showed higher percentage of mortality compared to K2 at the highest concentrations, i.e., 5, 50 and 500 µg/L (**Table 10, Figure 33**). Regarding enantiomers of NK2, also both enantiomers did not showed percentage of mortality statistically different of the control, nonetheless, NK2 showed higher mortality compared to NK1 for all concentrations tested (**Table 11, Figure 34**).

To verify possible enantioselective toxicity of K1 and NK2 to *D. magna*, a preliminary assay was performed with K1 and NK2 at 1000 and 10000 µg/L (1 and 10 mg/L). The immobilization percentage was of 20 % and 10 % for K1 and NK2 at 10000 µg/L, respectively. However, further tests must be performed at higher concentrations and with both enantiomers to corroborate the previous results and verify enantioselective toxicity.

Table 10. Percentage of immobilization/mortality of *D. magna* after 48 hours of exposure for the racemate K and enantiomers K1 and K2.

Racemate	K	Enantiomers	K1	K2
Concentration (µg/L)	Mortality (% Effect)	Concentration (µg/L)	Mortality (% Effect)	Mortality (% Effect)
0.1	0	0.05	0	0
1	5	0.5	0	0
10	0	5	10	0
100	5	50	15	5
1000	15	500	10	0

Table 11. Percentage of immobilization/mortality of *D. magna* after 48 hours of exposure for the racemate NK and enantiomers NK1 and NK2.

Racemate	NK	Enantiomers	NK1	NK2
Concentration (µg/L)	Mortality (% Effect)	Concentration (µg/L)	Mortality (% Effect)	Mortality (% Effect)
0.1	15	0.05	5	5
1	15	0.5	0	10
10	15	5	5	5
100	25	50	0	0
1000	20	500	0	10

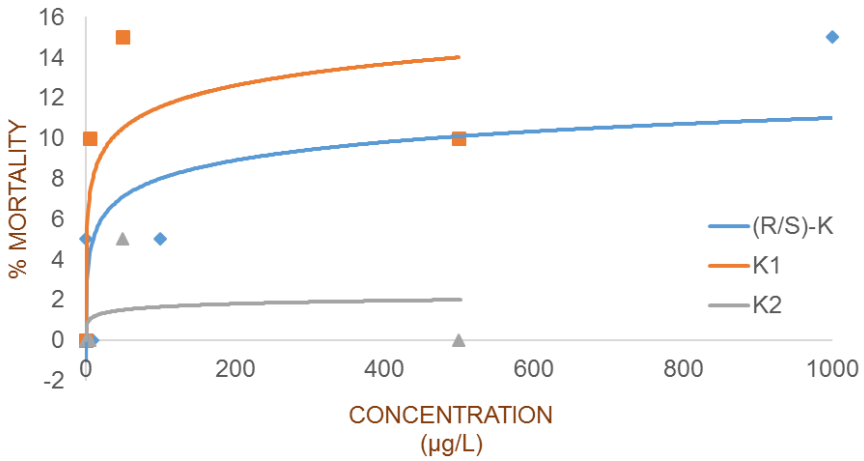


Figure 33. Percentage of mortality of K racemate, K1 and K2 for *D. magna* acute immobilization toxicity test at the end of 48 hours exposure.

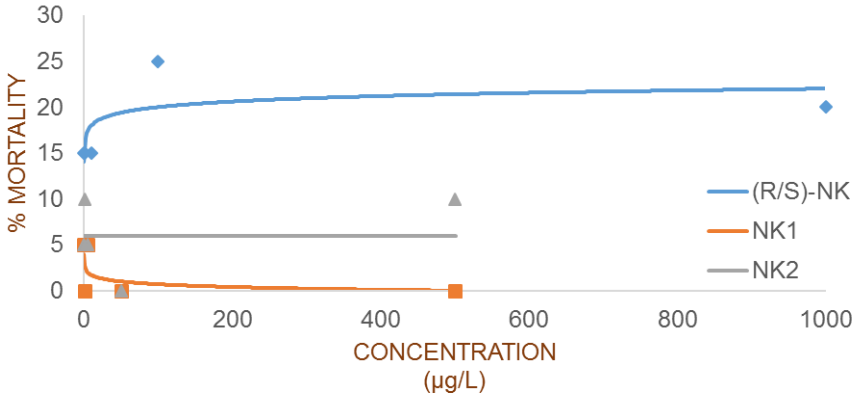


Figure 34. Percentage of mortality of NK racemate, NK1 and NK2 for *D. magna* acute immobilization toxicity test at the end of 48 hours exposure.

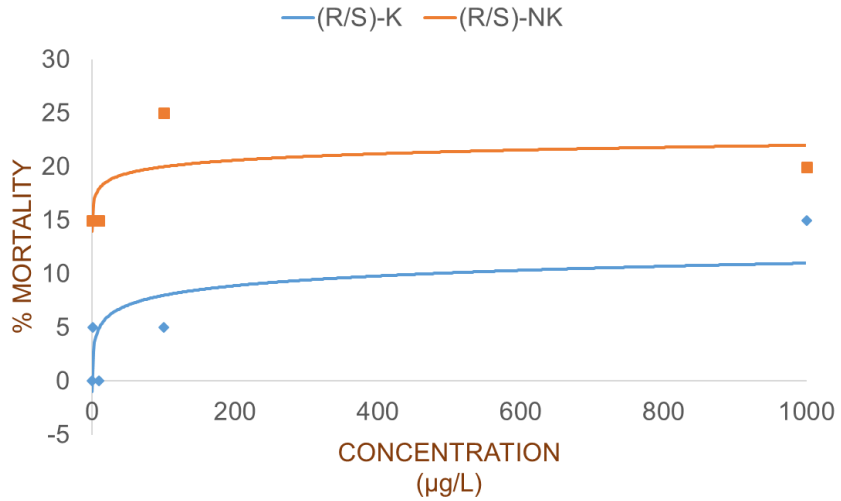


Figure 35. Comparison of percentage of mortality of K and NK racemates for *D. magna* acute immobilization toxicity test at the end of 48 hours exposure.

4.5.2. Chronic Enantioselective Ecotoxicity Assays in *Tetrahymena thermophila*

The chronic toxicity assays were performed on *T. thermophila* for 28 hours. The concentration ranged between 0.1 to 1000 µg/L for K and NK racemate and from 0.05 to 500 µg/L for enantiomers. The control in all assays were acceptable (≈ 60 % of growth inhibition). The reference test with $K_2Cr_2O_7$ was performance to assess the viability of assays. The assays occur well, the percentage of inhibition to 28 hours is adequate (**Table 12 and 13**). The 24 hours EC_{50} value calculated for this compound was 14.3 mg/L. The reference test is

acceptable and demonstrated the correct execution of the test procedure, as well as the good sensitivity of the test animals.

Regarding toxicity of K, except for the 0.1 and 10 µg/L, all other concentrations presented growth inhibition though not statistically different from the control (**Table 12**). On the contrary, NK racemate did not demonstrated at all range of concentration growth inhibition of the *T. thermophila* (**Table 12**). Thus, K appears to be more toxic to *T. thermophila* rather than its metabolite. Regarding assays with enantiomers, enantioselectivity was not observed at these range of concentrations for both K and NK enantiomers (**Table 13**). Nevertheless, both K2 and NK1 demonstrated growth inhibition at 0.05 µg/L that was not verified with the other pair of enantiomers. These results demonstrate possible enantioselective toxicity. To verify possible toxicity of K1 and NK2 to *T. thermophila*, a preliminary assay was performed with K1 and NK2 at 1000 and 10000 µg/L (1 and 10 mg/L). The percentage of growth inhibition exceed 60 % for both K1 and NK2 at 10000 µg/L. However, further tests must be performed at higher concentrations and both enantiomers to corroborate these preliminary results and verify enantioselective toxicity.

Table 12. Percentage of growth inhibition of *T. thermophila* for the chronic toxicity test of K and NK racemates and potassium dichromate (reference test) at the end of 24 hours and 28 hours of exposure.

Compound	% Growth Inhibition	Concentrations				
		C1	C2	C3	C4	C5
K	(0-24)	6.164	28.176	16.101	26.918	30.440
	(0-28)	-7.100	4.091	-2.166	6.739	6.498
NK	(0-24)	6.283	30.105	10.209	8.377	9.686
	(0-28)	-13.684	-7.632	-24.211	-11.053	-7.105
Potassium Dichromate	(0-24)	-41.245	-8.171	79.767	96.887	61.089
	(0-28)	-1.452	4.979	71.784	84.440	70.954

Concentrations of K and NK - C1: 0.1 µg/L; C2: 1 µg/L; C3: 10 µg/L; C4: 100 µg/L; C5: 1000 µg/L. Concentrations of potassium dichromate - C1: 5.6 mg/L; C2: 10 mg/L; C3: 18 mg/L; C4: 32 mg/L); C5: 56 mg/L).

Table 13. Percentage of growth inhibition of *T. thermophila* for the chronic toxicity test of K and NK enantiomers and potassium dichromate (reference test) at the end of 24 hours and 28 hours of exposure.

Compound	% Growth Inhibition	Concentrations				
		C1	C2	C3	C4	C5
K1	(0-24)	-109.728	-123.346	-98.833	-121.012	-87.938
	(0-28)	-23.237	-33.195	-31.120	-31.535	-26.556
K2	(0-24)	-57.836	-75.000	-74.627	-99.627	-97.761
	(0-28)	14.971	-3.839	-12.092	-13.436	-11.708
NK1	(0-24)	9.155	-51.056	-46.831	-61.620	-21.831
	(0-28)	2.292	-3.333	-16.667	-27.292	-16.250
NK2	(0-24)	-137.611	-88.053	-96.460	-135.841	-107.080
	(0-28)	-35.062	-32.099	-58.519	-57.037	-62.469
Potassium Dichromate	(0-24)	-41.245	-8.171	79.767	96.887	61.089
	(0-28)	-1.452	4.979	71.784	84.440	70.954

Concentrations of K1, K2, NK1 and NK2 - C1: 0.05 µg/L; C2: 0.5 µg/L; C3: 5 µg/L; C4: 50 µg/L; C5: 500 µg/L. Concentrations of potassium dichromate - C1: 5.6 mg/L; C2: 10 mg/L; C3: 18 mg/L; C4: 32 mg/L); C5: 56 mg/L).

Both ecotoxicity tests showed that K and its main metabolite, NK, at these range of concentrations show low levels of acute (*D. magna)* and chronic (*T. thermophila*) toxicity, in racemate and enantiomeric pure form. Nonetheless, the results with these two ecological relevant organisms showed different sensibility towards K and NK racemates. NK presented a higher percentage of mortality for the crustacean *D. magna* compared to K while K demonstrated greater percentage of growth inhibition for *T. thermophila* compared to NK.

Ecotoxicity of pharmaceuticals and illicit drugs are scarce and currently assessed for the racemates. Also, metabolites are usually not considered as well as different organisms to evaluate species dependent toxicity. In this sense, environmental risk assessment data need to be re-evaluated as they assume that chiral pollutants are found in the ecosystems as racemates. These ecotoxicity

assays demonstrated different susceptibility of the two selected organisms to K and NK racemates as well as possible enantiomer dependent toxicity, nonetheless, higher concentrations for both racemates and enantiomers must be investigated to corroborate these preliminary studies.

5. CONCLUSIONS AND FURTHER PERSPECTIVES

The current and growing evolution of the chemical industry has contributed to an urgent environmental problem. In fact, due to chemical pollution, numerous substances, including chiral pharmaceuticals and illicit drugs, have been detected in the environment, behaving as environmental contaminants. The excessive consumption and the continuous disposal of these substances into all ecosystems may present potential short-term and/or long-term risks for humans and life cycle of exposed organisms. The knowledge about CD, their metabolites and biodegradation behavior end ecotoxicity is scarce but crucial for an accurate risk assessment. Chiral compounds may undergo enantioselective metabolism in humans and in biodegradation during wastewater treatment leading to changes in their enantiomeric composition.

K, used in pediatric and veterinary medicine as an anesthetic, and its major metabolite NK have been detected either in effluents of WWTP and in aquatic environments and required more investigation.

An enantioselective LC to quantify the enantiomers of K and NK was developed and validated under isocratic elution mode. The method was successfully applied to follow a 21 days enantioselective biodegradation assay of K by AS. The analytical method developed demonstrated to be precise and accurate and can be applied to follow enantioselective process in other matrices, such bioreactor, wastewater and even biological matrices. The optimized mobile phase conditions are also compatible to mass spectra analyzer.

The biodegradation studies regarding the racemate of the K in different conditions, with and without addition of sodium acetate; dark and light showed similar results which supports to its persistence reported in the aquatic environment. The metabolite NK was not detected in these assays and both enantiomers of K shows similar behavior.

The pure enantiomers of K and NK for the enantioselective ecotoxicological assays obtained by the developed and optimized semi-preparative method. The optimized conditions allowed recovery of enantiomers higher than 70 % and the enantiomeric purity that allow to use the enantiomers for enantioselective ecotoxicological assays.

Acute and chronic toxicity assays were performed in the crustacean *D. magna* and the protozoan *T. thermophila* in accordance with national and international standards (ISO and OECD). NK racemate presented higher mortality for crustacean *D. magna* compared to K. For both compounds mortality increased across gradient of exposure. Considering *T. thermophila,* K demonstrated greater growth inhibition compared to NK. A species-dependent toxicity was observed. Enantioselective response between enantiomers of K and NK were also observed at the selected concentrations. Nevertheless, further studies at higher concentrations should be done to confirm these results.

As further work, the configuration of the enantiomers achieved by semi-preparative chromatography method need to be confirm.

All ecotoxicity studies will be update and amplify to others methods of analysis and other organisms, including zebrafish.

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ANNEX

Annex 1

Annex 1. A. Pereira, V. Gonçalves, C. Ribeiro, M. E. Tiritan, Cetamina e norcetamina: ensaios de toxicidade aguda em *Daphnia magna* e de toxicidade crónica em *Tetrahymena thermophila*, XII Jornadas Científicas IUCS and III Congresso APCF, Porto, Portugal, 24-25 May 2018. (Abstract and poster communication).

JORNADAS CIENTÍFICAS DE CIÊNCIAS FORENSES, CIÊNCIAS BIOMÉDICAS, CIÊNCIAS DA NUTRIÇÃO E BIOQUÍMICA DO INSTITUTO UNIVERSITÁRIO DE CIÊNCIAS DA SAÚDE

III CONGRESSO DA ASSOCIAÇÃO PORTUGUESA DE CIÊNCIAS FORENSES

CETAMINA E NORCETAMINA: ENSAIOS DE TOXICIDADE AGUDA EM *DAPHNIA MAGNA* E DE TOXICIDADE CRÓNICA EM *TETRAHYMENA THERMOPHILA*

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Introdução: Diversas substâncias entre as quais fármacos e drogas ilícitas têm sido detetadas no ambiente e consideradas contaminantes ambientais [1- 5]. De facto, devido ao seu elevado consumo e continuo descarte estas substâncias são classificadas como pseudo-persistentes e podem apresentar potenciais riscos a curto e a longo prazo para o Homem e outros organismos expostos [1, 6]. A cetamina (K) é utilizada na medicina pediátrica e veterinária como anestésico, apresenta potentes efeitos alucinogénios, sedativos e analgésicos, e tem sido utilizada de forma abusiva por adolescentes e jovens adultos em ambientes recreativos [2]. Após consumo, a K e o seu principal metabolito, norcetamina (NK), são excretadas e entram nas redes de esgotos hospitalares e domésticas, estando presentes nos efluentes das estações de tratamento de águas residuais (ETAR) e consequentemente em meios aquáticos [1, 2, 6]. Assim, torna-se urgente avaliar a ecotoxicidade da K e da NK para uma correta avaliação do risco.

Objetivos: O presente trabalho tem como objetivo principal avaliar a toxicidade K e da NK em organismos aquáticos. Para isso, recorreu-se a dois organismos diferentes utilizados em ensaios de toxicidade: o crustáceo *Daphnia magna* e o protozoário *Tetrahymena thermophila*.

Material e Métodos: No presente estudo foram realizados ensaios de toxicidade aguda e crónica da K e NK em organismos de água doce em conformidade com as normas nacionais e internacionais (ISO e OECD). A *Daphnia magna* foi a espécie utilizada nos ensaios de toxicidade aguda de 48h, avaliando-se a imobilidade/mortalidade. Por sua vez, a *Tetrahymena thermophila* foi a espécie utilizada nos ensaios de toxicidade crónica de 28h, avaliando-se a inibição do crescimento através da medição das densidades óticas (OD). A K e a NK foram utilizados em concentrações ambientais na ordem das g/L.

Resultados: A partir dos resultados obtidos nos ensaios toxicológicos agudos e crónicos verificou-se diferente sensibilidade dos dois organismos em relação à K e NK. Nos ensaios toxicológicos agudos, a NK apresenta maior mortalidade para o crustáceo *Daphnia magna* em comparação com a K. Para os ensaios toxicológicos crónicos, a K demonstrou maior inibição de crescimento para o *Tetrahymena thermophila* comparativamente com a NK. Os resultados nas concentrações utilizadas mostraram uma toxicidade ligeiramente maior do que a verificada no controlo, em ambos os ensaios de toxicidade. As concentrações mais elevadas de K (1000 µg/L) e NK (1000 µg/L e 100 µg/L) causaram maior mortalidade na *Daphnia magna*, enquanto que as concentrações mais elevadas de K (1000 µg/L; 10 µg/L e 1 µg/L) e NK (1000 µg/L; 100 µg/L e 1 µg/L) causaram maior inibição de crescimento no *Tetrahymena thermophila*.

Conclusão: O presente estudo permitiu concluir que, de um modo geral a K e o seu principal metabolito, NK, nas concentrações utilizadas nos ensaios apresentam baixos níveis de toxicidade aguda (*Daphnia magna*) e crónica (*Tetrahymena thermophila*). O metabolito NK apresentou um nível maior de toxicidade que o fármaco K nos ensaios com a *Daphnia magna*.

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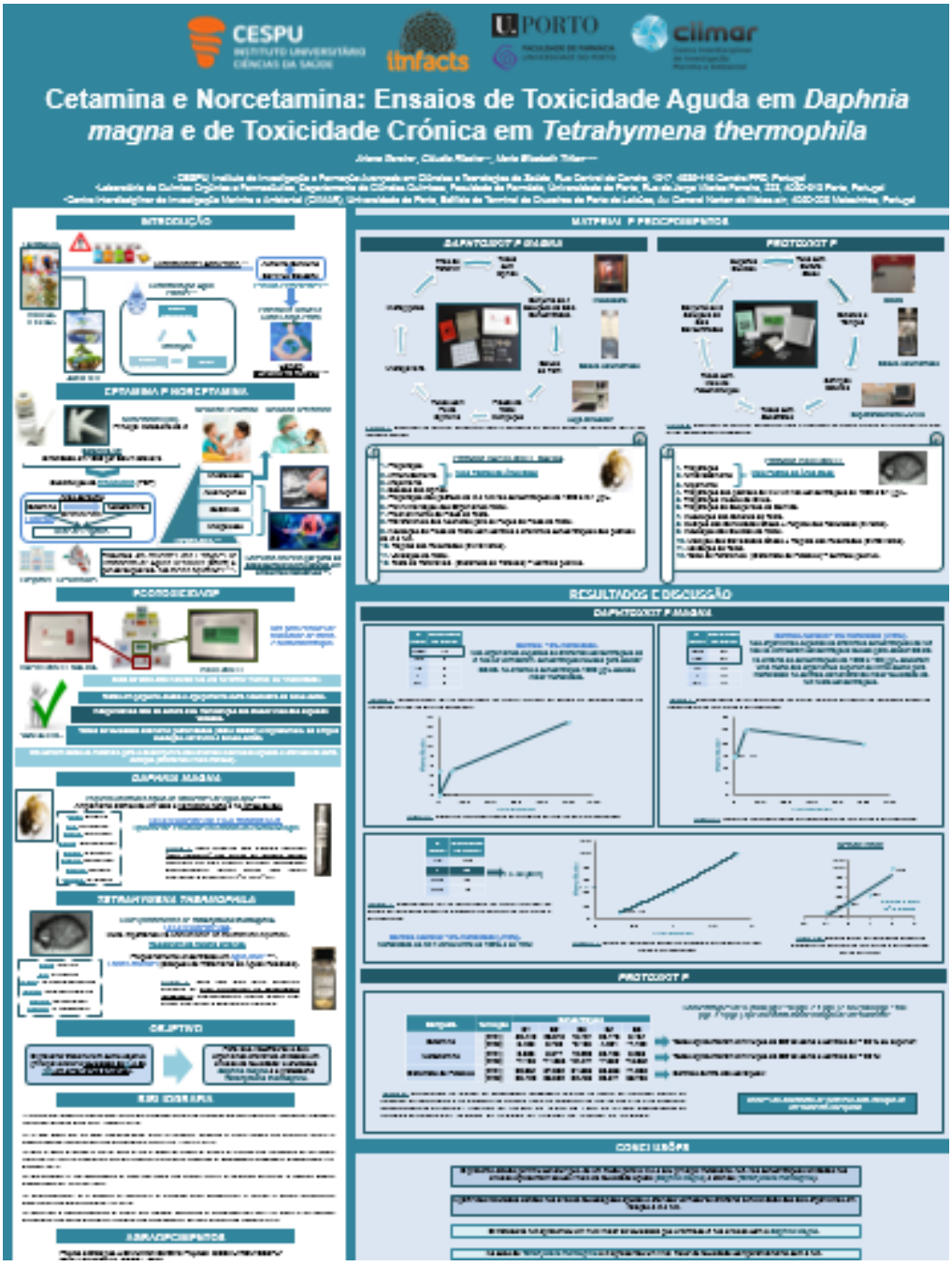
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Cetamina e Norcetamina: Ensaios de Toxicidade Aguda em Daphnia magna e de Toxicidade Crónica em Tetrahymena thermophila
INTRODUÇÃO
CETAMINA E NORCETAMINA
ACCORDSIDAYP
DAPHNIA MAGNA
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MATERIAL E PROCEDIMENTOS
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Annex 2

Annex 2. A. Pereira, V. Gonçalves, C. Ribeiro, M. E. Tiritan, Multimilligram enantioresolution of norketamine on polysaccharide phase using microporous silica as support, Porto, Portugal, XXIV Encontro Luso Galego de Química, 21-23 November 2018. (Abstract and poster communication).

Multimilligram enantioresolution of norketamine on polysaccharide phase using microporous silica as support

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The current and growing evolution of the chemical industry has contributed to an urgent environmental problem, since numerous substances, including drugs and illicit drugs, have been detected in the environment, behaving as environmental contaminants [1,2]. Excessive consumption and continuous disposal of these substances may present potential short-term or long-term risks for humans and other exposed organisms [1,3]. Ketamine (K) is used in pediatric and veterinary medicine as an anesthetic, however, its abusively used by adolescents and young adults in recreational environments due to its hallucinogenic and sedative effects has been increased [2]. After consumption, K and its main metabolite, norketamine (NK), are excreted into the sewage networks and have been detected in effluents from wastewater treatment plants (WWTP) and in aquatic environments [1-3]. Thus, it is urgent to evaluate the ecotoxicity of K and NK, either as racemate or in the enantiomeric pure forms, for a correct risk assessment. In ours previous studies, the acute and chronic toxicity of K and NK in aquatic organisms (both in their racemic form) was evaluated. In acute toxicological assays, NK presents a higher mortality for crustacean *Daphnia magna* compared to K. For chronic toxicological assays, K demonstrated greater growth inhibition for *Tetrahymena thermophila* compared to NK. However the ecotoxicity of the enantiomers of K and NK have never been evaluated (either acute or chronic toxicity). This work presents the preparative enantioseparation of NK in order to obtain the enantiomers for further enantioselective ecotoxicity studies. Preparative chromatography was performed using the Merck Hitachi LaChrom HPLC with DAD detector at 220 nm using the semi-preparative amylose 3,5dimethylphenylcarbamate 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 at an isocratic mobile phase consisting of n-hexane (0.1% DEA (diethylamine)) and ethanol (0.1% DEA) (65:35) and flow-rate of 1.5 mL/min. The chromatographic separation was performed with a concentrated solution of 10 mg/mL NK in ethanol and injection volume of 50 µL. In this chromatographic separation three fractions were collected: fraction 1 - corresponding to the first enantiomer eluted from NK (NK1), intermediate fraction - containing mostly NK1 and some residues of the second enantiomer eluted (NK2) and fraction 2 - corresponding to NK2. The enantiomeric purity was evaluated using analytical lux® 3 µm cellulose 4 column (150 × 4.6 mm column size) for each enantiomer and injection volume of 10 µL, fraction 1 and 2 showed enantiomeric ratio higher than 99% for NK1 and NK2. Acute and chronic enantioselective toxicity tests of NK in *Daphnia magna* organisms and the protozoan *Tetrahymena thermophila* will be carried out in accordance with national and international standards (ISO and OECD).

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Multimilligram enantioresolution of ketamine and norketamine on polysaccharide phase using microporous silica as support

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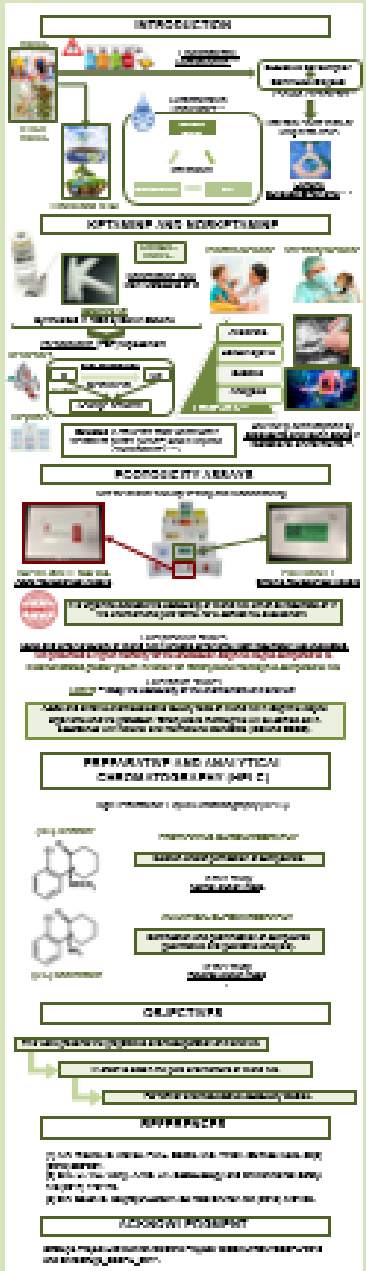

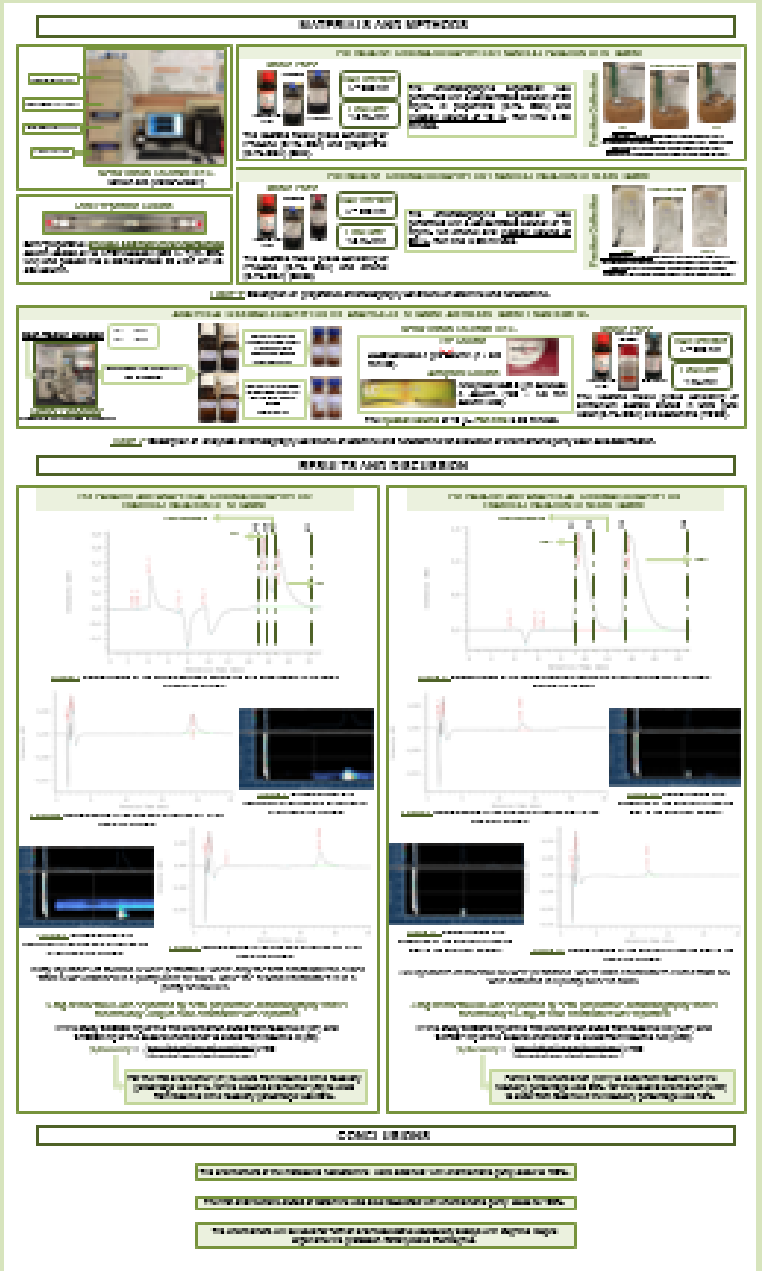

Annex 3

Annex 3. A. Pereira, V. Gonçalves, A. S. Maia, C. Ribeiro, M. E. Tiritan, Enantioselective biodegradation assays of ketamine in activated sludge, XIII Jornadas Científicas IUCS and IV Congresso APCF, Porto, Portugal, 11-12 April 2019. (Abstract and poster communication).

ENANTIOSELECTIVE BIODEGRADATION ASSAYS OF KETAMINE IN ACTIVED SLUDGED

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Introduction: Several substances including chiral drugs have been detected in the environment and are considered environmental contaminants [1,2]. Indeed, due to their high consumption and continued disposal these substances are classified as pseudo-persistent and may present potential short- and long-term risks for man and other organisms exposed [1,3]. Ketamine (K), used in pediatric and veterinary medicine as an anesthetic, has potent hallucinogenic effects, sedatives and analgesics, and has been used abusively by adolescents and young adults in recreational settings [2]. After consumption, K and its main metabolite, norketamine (NK), are excreted into the sewer and domestic sewage networks, being present in effluents from wastewater treatment plants (WWTP) and consequently in aquatic environments [1- 3]. As K is chiral and commercialized as racemate, it is urgent to evaluate the enantioselectivity in biodegradation of K and NK. The pattern of biodegradation in activated sludge (AS) can give important information for further correct evaluation of the risk and persistence of these substances in the environment [4].
Aims: Evaluation of the enantioselective biodegradation of K in activated sludge and/or formation of metabolites, including the enantiomers of NK.
Material and methods: The enantioselective biodegradation assays was performed using AS under different experimental conditions (AS/dead cells, light/dark, with and without sodium acetate) during 21 days, incubation at 25ºC with shaker at 110 rpm. Racemate K was used at final concentration of 40 µg/mL in assays flasks. Biodegradation assays was monitored by a validated enantioselective HPLC method with DAD detection. The method was validated using a minimal salts medium inoculated with AS as matrix.
Results: The enantioselective biodegradation assays showed very low percentages of biodegradation of racemate K in AS, regardless of the experimental conditions. Metabolite formation of K was not verified.
Conclusions: The biodegradation assays with racemate K presents low percentage of biodegradation, which corroborates with the persistence of K in aquatic environment. The non-formation of metabolites reinforces the previous idea. Enantioselectivity was not observed.

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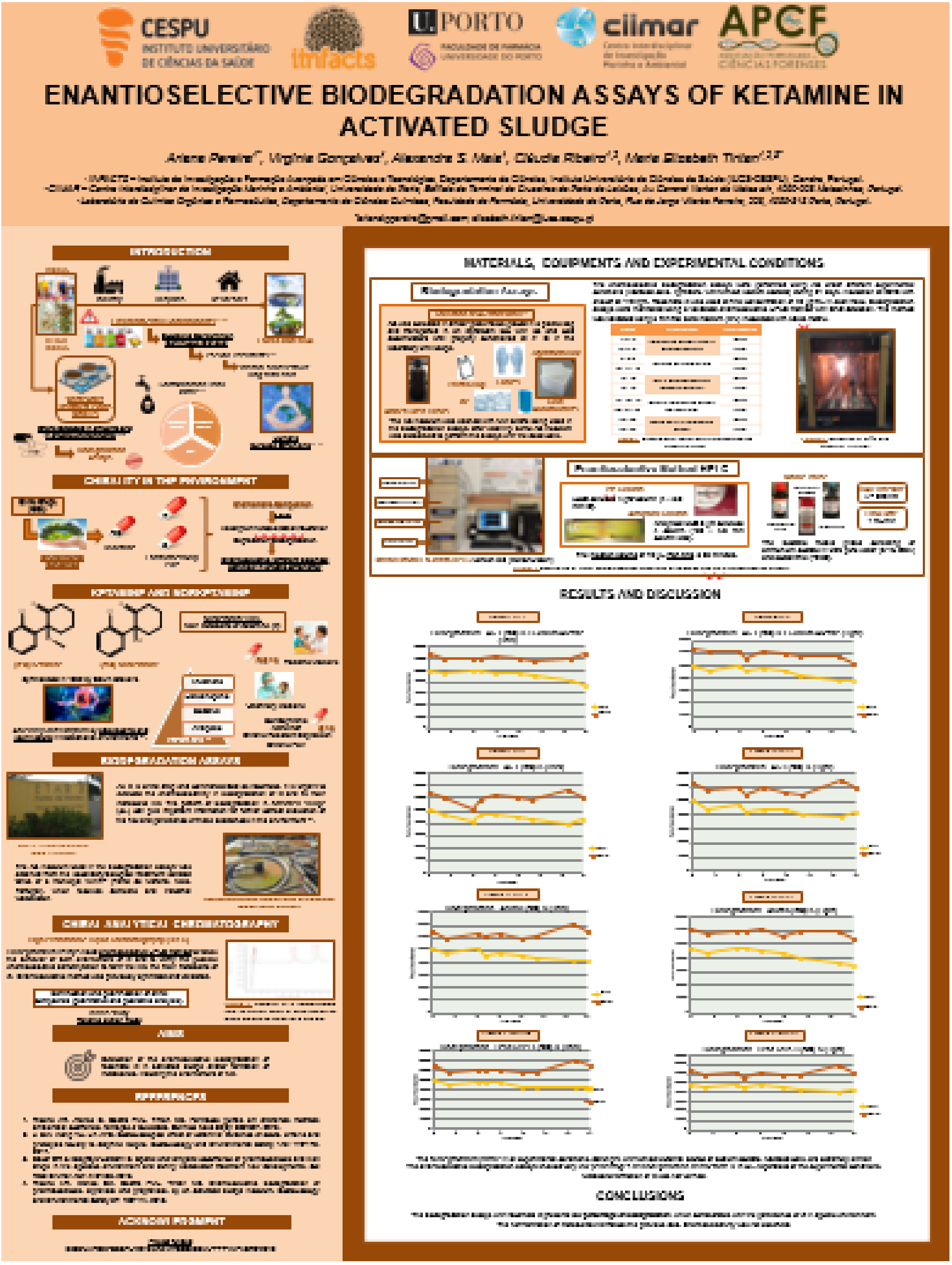
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ENANTIOSELECTIVE BIODEGRADATION ASSAYS OF KETAMINE IN ACTIVATED SLUDGE
MATERIALS, EQUIPMENTS AND EXPERIMENTAL CONDITIONS
RESULTS AND DISCUSSION
CONCLUSIONS

Annex 4

Annex 4. A. Pereira, V. Gonçalves, A. S. Maia, C. Ribeiro, M. E. Tiritan, Ketamine and norketamine: enantioseparation, enantioselective ecotoxicity and biodegradation studies, IJUP 2019, 12th Meeting of Young Researches of University of Porto, Porto, Portugal, 13-15 February 2019. (Abstract).

- ## 15364 | Ketamine and norketamine: enantioseparation, enantioselective ecotoxicity and biodegradation studies

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The current and growing evolution of the chemical industry has contributed to an urgent environmental and forensic problem, since numerous substances, including drugs and illicit drugs, have been detected in the environment, behaving as environmental contaminants [1]. Excessive consumption and continuous disposal of these substances may present potential short-term and/or long-term risks for humans and other exposed organisms [1]. Ketamine (K) is used in pediatric and veterinary medicine as an anesthetic, however, its increasingly being used abusively by adolescents and young adults in recreational environments due to its hallucinogenic and sedative effects. After consumption, K and its main metabolite, norketamine (NK), are excreted into the sewage networks and have been detected in effluents from wastewater treatment plants (WWTP) and in aquatic environments [1]. However, their enantioselective toxicity effects are unknown. This work describe the development and validation of a high performance liquid chromatography (HPLC-DAD) chromatographic method to quantify the enantiomers of K and NK. The method was applied to followed the biodegradation assay of K by activated sludge. The pure enantiomers of K and NK, were also achieved by semi-preparative chromatography using amylose carbamate derivative as chiral stationary phase. The enantiomeric purity of the enantiomers was assessed by the analytical method previously performed and validated. To assess the impact and adverse effects of these substances, racemates and the pure enantiomers of K and NK, acute and chronic toxicity assays were performed in aquatic organisms, in crustacean Daphnia magna and in protozoan Tetrahymena thermophila in accordance with national and international standards (ISO and OECD).

Acknowledgment
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