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Comprehensive Study of Antidepressants and
Benzodiazepines in Environmental Matrices: Chiral
Biodegradation and Analytical Method Development

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Submitted in fulfilment of the requirements of the Degree of
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Abstract

A number of drugs, including antidepressants and benzodiazepines, are currently of environmental concern due to their widespread use and presence in aquatic ecosystems. This thesis initially focuses on the environmental literature associated with their pharmacology, physicochemical properties, chiral impact, and methods of extraction and analysis. The persistence of these compounds in the environment raises significant concerns about their potential ecological consequences. Understanding their enantiomeric compositions and environmental fates is crucial, as enantiomers can exhibit differing toxicity, bioactivity, and persistence. Environmental studies have consistently revealed the presence of these compounds in various ecosystems, where they can adversely affect aquatic organisms and disrupt the food chain. To accurately quantify these pharmaceuticals in environmental samples, advanced extraction and analysis methods, such as solid-phase extraction (SPE) and liquid chromatography-mass spectrometry (LC-MS), are essential.

Chiral chromatographic separations were performed for antidepressants and benzodiazepines, with adjustments to mobile phase conditions, such as buffer pH and ethanol content, proving beneficial for enantiomeric resolution. However, further research is needed to achieve optimal separation for all compounds. The biodegradation of these pharmaceuticals in wastewater systems was also investigated, revealing the critical role of microbial activity in their degradation. The study emphasized the importance of characterizing microbial communities, as they are central to understanding the environmental fate and stereoselective transformations of chiral pharmaceuticals.

The investigation into benzodiazepines, including diazepam, oxazepam, temazepam, and etizolam, highlighted their biodegradability and transformation pathways. Microbial-mediated biodegradation was found to be a vital process in reducing the concentrations of these compounds, with abiotic degradation pathways also contributing to their overall breakdown. Key factors such as pH, temperature, microbial activity, and co-occurring contaminants were shown to influence their degradation dynamics. These findings underscore the importance of optimizing wastewater treatment systems to mitigate the environmental and human health risks associated with benzodiazepine contamination.

Similarly, the study of antidepressants, such as fluoxetine and venlafaxine, revealed efficient biodegradation pathways driven by microbial activity, with stereoselective processes influencing the degradation of individual enantiomers. The impact of environmental parameters, such as pH and dissolved oxygen levels, on microbial efficiency was highlighted, along with the identification of bacterial taxa capable of metabolising these compounds. These findings demonstrate the critical role of microbial consortia in the removal of antidepressants from wastewater, with implications for reducing their ecological footprint and ensuring the safety of aquatic ecosystems.

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Author's Declaration

"I declare that, except where explicit reference is made to the contribution of other, that this thesis is the results of my own work and has not been submitted for any other degree at University of Glasgow or any other institution"

Signature

Printed name: Ali Ibrahim Alawi

Definitions/Abbreviations

Abbreviation	Full Term	Definition / Context
α	Separation factor (selectivity factor)	Ratio of retention factors in chromatography, measure of enantioselectivity.
α-HETIZ	α -hydroxyetizolam	
AAFS	American Academy of Forensic Sciences	
ACN	acetonitrile	
Bioreactor	Biotic reactor	A controlled vessel or system designed to support a biologically active environment, in which microorganisms, cells, or enzymes carry out biochemical reactions. Conditions such as temperature, pH, aeration, and nutrient supply are carefully regulated to optimize growth and activity.
BZs	Benzodiazepines	Psychoactive drugs used in medicine; commonly detected in forensic toxicology.
CR-RF	Cold Room 4°C	
CYP450	Cytochrome P 450	A large family of enzymes responsible for the metabolism of many xenobiotics and drugs.
DAD	Diode Array Detector	A type of UV-Vis detector used in HPLC/LC systems. It records absorbance across a range of wavelengths simultaneously, allowing both quantification and spectral confirmation of analytes.
DIAZ	diazepam	
DO	Dissolved Oxygen	The amount of oxygen present in water, measured in mg/L. Critical for microbial activity in bioreactors and biodegradation studies.
e1	enantiomer 1	
e2	enantiomer 2	
ee%	enantiomeric excess	A quantitative measure of enantiomeric purity, expressed as the difference in percentage between the two enantiomers. Indicates the degree of chiral imbalance.
EF	enantiomeric fraction	A measure of the relative abundance of one enantiomer compared to the total amount of both enantiomers.
ETIZ	etizolam	
EtOH	ethanol	
FA	formic acid	
FLX	fluoxetine	
FZ	Freezer -20°C	
GC	Gas Chromatography	
IPA	isopropanol	
K	Retention Factor (Capacity Factor)	Chromatography measure of analyte retention relative to void volume.
LC	Liquid Chromatography	

Abbreviation	Full Term	Definition / Context
LOD	Limit of Detection	lowest concentration of an analyte that can be detected.
LOQ	Limit of Quantification	lowest concentration of an analyte that can be reliably quantified with acceptable accuracy and precision.
MAOIS	Monoamine Oxidase Inhibitors	Class of antidepressants inhibiting MAO enzyme.
MeOH	methanol	
MS	Mass spectrometer	
N	Number of Theoretical Plates (Column Efficiency)	A measure of column performance in chromatography, representing how efficiently the column can separate compounds. Higher N values indicate sharper peaks and better resolution, which is critical in separating enantiomers.
N/A	Not available	
NDIZ	nordiazepam	
NFLX	norfluoxetine	
OD	Optical Density	A spectrophotometric measure of light absorption by a sample, commonly at 600 nm (OD600) to estimate microbial growth in biodegradation experiments.
ODV	O-desmethylvenlafaxine	
OXZ	oxazepam	
Praz	prazepam	
QC	Quality Control	Procedures ensuring reliability and accuracy in lab testing.
R-	R-enantiomer	
R-FLX	R-Fluoxetine	
R-NFLX	R-Norfluoxetine	
R-ODV	R-O-desmethylvenlafaxine	
R-VEN	R-Venlafaxine	
Rs	Resolution	Measure of chromatographic separation between peaks.
RT	Room temperature 22°C	
S-	S-enantiomer	
S-FLX	S-Fluoxetine	
S-NFLX	S-Norfluoxetine	
S-ODV	S-O-desmethylvenlafaxine	
S-VEN	S-Venlafaxine	
SNRIs	Serotonin-Noradrenaline Reuptake Inhibitors	Another class of antidepressants that block the reuptake of both serotonin and norepinephrine. Often prescribed when SSRIs are ineffective.
SPE	Solid Phase Extraction	Sample preparation method used for drug and toxin analysis.
SSRIs	Selective serotonin reuptake inhibitors	A major class of antidepressants that block serotonin reuptake, increasing serotonin levels in the brain.
SWGTOX	Scientific Working Group for Forensic Toxicology	

Abbreviation	Full Term	Definition / Context
SWW	Synthetic wastewater, abiotic reactor	Laboratory-prepared wastewater that mimics the chemical composition of real wastewater, used in controlled biodegradation studies.
TCA	Tricyclic Antidepressants	Class of antidepressant drugs; or an organic acid used in sample preparation.
TEA	triethylamine	
TEM	temazepam	
TSS	Total suspended solids	Water quality parameter in environmental toxicology studies.
VEN	venlafaxine	
WWTP	Wastewater treatment Plant	Facility for wastewater processing.

Chapter 1. Introduction

1.1. Statement of the problem

In recent years, pharmaceutical consumption, particularly antidepressants and benzodiazepines, has increased substantially, improving clinical outcomes but also raising environmental concern due to the continuous release of drug residues into wastewater systems. [1] Following administration, many compounds are excreted unchanged or as metabolites and enter wastewater treatment plants (WWTPs), where conventional treatment may not fully remove them.

Consequently, parent drugs and metabolites can persist and be discharged into receiving waters, creating potential ecological impacts and secondary public-health concerns (e.g., disturbance of aquatic organisms and selection pressures on microbial communities). [2,3] Despite increasing attention from regulators and researchers, major uncertainties remain regarding the fate, transformation, and biological relevance of pharmaceutical residues under environmentally realistic conditions. [4–6]

A central challenge is that many pharmaceuticals are chiral, and their enantiomers can differ in pharmacological activity, toxicity, and environmental behaviour. [7,8]

In environmental systems, stereoselective processes may occur because microbial communities can transform or degrade enantiomers at different rates, potentially altering persistence and shifting toxicity profiles over time. [9,10] However, for many therapeutically important chiral drugs and their metabolites, there is still limited understanding of (i) the extent to which removal is driven by biotic versus abiotic processes, (ii) whether transformation is enantioselective, and (iii) which conditions favour persistence versus attenuation in aqueous matrices relevant to wastewater. [11–14]

Progress is also constrained by analytical limitations. Robust assessment of stereoselective fate requires validated enantioselective LC-MS methods with sufficient selectivity, sensitivity, and precision in complex matrices, alongside appropriate experimental designs that distinguish biological transformation from non-biological effects such as partitioning or physicochemical loss. [14–17] These gaps are especially relevant for widely used chiral compounds and metabolites, including benzodiazepines (e.g., oxazepam and temazepam) and key

antidepressant residues, where comparatively fewer studies have addressed enantiomer-specific behaviour under controlled reactor conditions that model wastewater environments. [18–20]

Accordingly, this thesis addresses the above limitations by investigating the biodegradation and transformation of selected antidepressant and benzodiazepine residues (including chiral compounds and metabolites) under controlled synthetic-wastewater conditions, supported by enantioselective LC-MS method development/validation and comparative abiotic versus biotic reactor experiments. This integrated approach is intended to improve interpretation of pharmaceutical attenuation processes, including the potential for stereoselective changes during microbial processing, and to strengthen the evidence base needed for environmental monitoring and treatment-relevant assessment of chiral pharmaceutical contaminants.

1.2.Aims and objectives

1.2.1. Aim

The aim of this project is to investigate the behaviour and degradation of selected antidepressants and benzodiazepines in environmental matrices, with emphasis on enantiomer-specific reduction and transformation, and to develop validated analytical and extraction methods that improve assessment of persistence, treatment removal potential, and environmental exposure implications.

1.2.2. Objectives

- Develop a reliable analytical method for the precise differentiation, separation, and quantification of enantiomers of antidepressants and benzodiazepines in complex environmental matrices.
- Investigate the degradation of antidepressants and benzodiazepines by microorganisms in simulated environmental matrices, to assess their fate and potential for transformation.
- Identify and characterize the bacteria responsible for the biodegradation process using 16S rRNA sequencing, aiming to understand the microbial communities involved in the degradation.
- Investigate the influence of environmental parameters, such as pH, total suspended solids, and optical density, on the degradation efficiency of the target compounds in abiotic and biotic reactors, to understand the environmental fate and potential risk in different scenarios.
- Understand the behavior, stability, and transformation pathways of antidepressants and benzodiazepines in various environmental matrices, including their potential metabolites or transformation products, and provide crucial data on their persistence and environmental impact under varying conditions.

Chapter 2. Background and Literature Review

2.1. Pharmaceuticals in the Environment

Pharmaceuticals in the environment have emerged as a significant concern due to their extensive use and improper disposal. Antidepressants (ADs) are among the most frequently prescribed medications for mental health conditions, yet residues of these drugs often enter the environment through wastewater discharge or discarded pharmaceuticals. Selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) have been shown to disrupt aquatic ecosystems by altering the behaviour, feeding, and reproduction of aquatic organisms, ultimately leading to ecological imbalances. [21–24]

Beyond ecological effects, the occurrence of antidepressants in environmental matrices raises potential risks to human health. Chronic exposure to low concentrations in drinking water or contaminated food may pose unknown health consequences. Some antidepressants have also been linked to the promotion of antibiotic-resistant bacteria, intensifying public health concerns. [11,25–27]

Benzodiazepines (BZs), including commonly detected compounds such as alprazolam and diazepam, also present environmental and societal risks. They can reach water bodies through excretion or improper disposal, with documented adverse effects on aquatic organisms. [28] From a public health perspective, benzodiazepine misuse can lead to addiction, overdose, and cognitive impairment, while careless disposal increases the risk of diversion into illicit markets, reinforcing cycles of drug abuse. [29]

Addressing these challenges requires integrated strategies. Advanced wastewater treatment technologies are needed to improve the removal of pharmaceutical residues. Public awareness campaigns can encourage responsible medication disposal, reducing environmental contamination. [26]

As detailed in Sections 0 and 2.3.3, ADs undergo metabolic transformation within the human body, leading to the production of various metabolites. For instance, fluoxetine (FLX) is metabolized into norfluoxetine (NFLX), and venlafaxine (VEN) is converted into desvenlafaxine (O-desmethylvenlafaxine). These metabolites have been detected in environmental samples, indicating their persistence beyond human excretion. [30]

Benzodiazepines are metabolized into multiple compounds, with diazepam converting into nordiazepam (desmethyldiazepam), oxazepam, and temazepam. These metabolites are not only present in the human body but have also been identified in various environmental matrices, reflecting their environmental persistence. [31]

Pharmaceuticals undergo various environmental transformation processes such as photodegradation, oxidation, and biodegradation. These reactions can generate transformation products that differ significantly in chemical structure and properties from their parent drugs and metabolites, sometimes resulting in compounds with greater stability or altered biological activity. [14,32]

The primary pathways introducing ADs and BZs into the environment include effluent discharges from wastewater treatment plants, excretion by humans and animals, and various other sources. Consequently, these compounds and their transformation products have been detected in surface waters, groundwater, drinking water, soils, sediments, and even within aquatic and terrestrial organisms. [33,34]

2.1.1. Drugs metabolites and transformation products

Antidepressants can undergo biotic and abiotic transformations in the environment. These transformations can result in the formation of metabolites and transformation products with potentially distinct chemical properties and biological activities from the parent drugs. [14,32] While benzodiazepines can undergo various transformation processes in the environment, resulting in the formation of metabolites and transformation products. [14,32]

2.1.2. Sources, occurrence and fate

The principal environmental sources of antidepressants are wastewater discharges from sewage treatment plants and excretion by humans and animals. Antidepressants and their metabolites have been detected in surface waters, groundwater, and even drinking water sources. [14,35,36] Their persistence in these matrices can result in prolonged exposure of aquatic organisms. The environmental fate of antidepressants is influenced by their chemical characteristics, surrounding conditions, and the presence of microbial communities capable of degradation.

Benzodiazepines enter the environment mainly through wastewater discharges and excretion associated with therapeutic or illicit use. Parent compounds and their metabolites have been reported in surface and groundwater, where they may bioaccumulate in aquatic organisms. Their persistence is likewise dependent on environmental conditions and the availability of microorganisms capable of facilitating degradation. Table 2-1 lists microorganisms documented to degrade pharmaceutical compounds. [15,37]

Table 2-1: List of micro-organisms reported to degrade pharmaceutical compounds.

Group	Degrading microbes	Pharmaceutical compound	Author and Year	Ref
Agaricomycetes	<i>Trametes versicolor</i> <i>Phanerochaete chrysosporium</i>	Naproxen	G. Feijoo et al. 2012	[38]
Agaricomycetes	<i>Trametes versicolor</i> <i>Ganoderma lucidum</i> <i>Trametes versicolor</i> <i>Trametes versicolor</i>	Carbamazepine Carbamazepine Clofibric acid Ibuprofen	G. Feijoo et al. 2012 and Marco-urrea et al. 2009	[38] [39] [39] [39]
Gammaproteobacteria	<i>Pseudomonas sp Strain CE22</i>	Cefalexin	B Lin & et al., 2015	[40]
Actinobacteria	<i>Microbacterium sp</i>	Sulfamethazine	E Topp et al. 2013	[41]
Gammaproteobacteria	<i>Pseudomonas psychrophila HA-4</i>	Sulfamethoxazole	B Jiang & et al., 2014	[42]
Actinobacteridae	<i>Actinoplanes sp</i>	Diclofenac	B Jiang et al. 2014	[42]
Gammaproteobacteria	<i>Raoultella ornithinolytica</i> <i>B6 Pseudomonas aeruginosa</i> <i>Pseudomonas sp. P16</i> <i>Stenotrophomonas sp. 5LF</i> <i>19TDL</i>	Ketoprofen	MM Ismail et al. 2016	[43]
Agaricomycetes	<i>Phanerochaete sordida</i>	Mefenamic acid	T Hata et al. 2010	[44]

The occurrence of antidepressants, benzodiazepines, and their transformation products in environmental matrices has raised serious ecological and public health concerns. [45,46] These residues are frequently detected in rivers, lakes, and other aquatic systems, often at concentrations that may negatively affect aquatic organisms. [47,48]

Mitigating these challenges requires improvements in wastewater treatment technologies. Conventional treatment processes are often insufficient for complete removal, resulting in the persistence of these substances in treated effluents. Advanced methods, including oxidation processes and membrane filtration, have

shown greater effectiveness in reducing pharmaceutical contaminants in wastewater. [49]

Further research is needed to elucidate the fate and behavior of these pharmaceuticals in natural systems. Understanding their degradation pathways, persistence, and bioaccumulation potential is crucial for developing effective mitigation strategies. [19,50]

2.1.3. Effects of pharmaceuticals on aquatic organisms

Tackling the impact of pharmaceuticals on aquatic life demands an integrated strategy that combines advancements in wastewater treatment, the design of environmentally friendly medications, and increased public awareness regarding the safe disposal of unused drugs. [51,52] Continuous monitoring and scientific investigation of the ecotoxicological effects of pharmaceuticals in aquatic systems are essential to deepen our understanding of the threats they pose and to establish effective measures for protecting the environment and preserving aquatic biodiversity. [47,48]

2.1.4. Antidepressants and environmental issues

Pharmaceuticals have emerged as a significant group of environmental contaminants, with their presence widely detected in aquatic systems through multiple pathways such as wastewater effluents, hospital waste, industrial discharges, and agricultural runoff. Urban wastewater represents a major source, carrying pharmaceutical residues from human excretion and the improper disposal of unused drugs, while the extensive use of antibiotics in livestock farming contributes additional loads through surface runoff. These contaminants persist in the environment, where they pose risks to aquatic organisms and may accelerate the spread of antibiotic-resistant bacteria. [51,53]

The continuous rise in global pharmaceutical consumption - driven by population growth and lifestyle changes - has further intensified this challenge, leading to the identification of hundreds of active pharmaceutical compounds in rivers, coasts, and other aquatic regions. Among these, antidepressants are particularly prevalent due to their high prescription rates and excretion of both parent drugs and active metabolites, resulting in increased risks of bioaccumulation and ecological disruption. [19,54]

Once released into the environment, the degradation and fate of pharmaceuticals are influenced by diverse factors, including their physical and chemical properties, stereoselectivity, concentration, microbial community diversity, and surrounding conditions such as temperature, light exposure, and pH. [51] For instance, hydrophobic drugs are more likely to partition into sludge during water treatment, whereas water-soluble compounds may remain in effluents and persist in receiving waters.

2.1.5. Benzodiazepines and Environmental Issues

Benzodiazepines and other pharmaceuticals can pose environmental issues when their residues find their way into the environment through various pathways.

[21,28,55] One of the primary contributors to this problem is the improper disposal of medications by patients, such as flushing them down the toilet or throwing them in the trash. Additionally, pharmaceutical manufacturing processes and inadequate wastewater treatment can also lead to the release of these compounds into the environment. [31]

Once in the environment, benzodiazepines can persist and accumulate in water bodies and soil. [56] Their presence in aquatic ecosystems can potentially impact aquatic organisms, including fish and other aquatic life. Studies have shown that exposure to trace amounts of these drugs can affect behaviour, reproduction, and development in some species. There are also concerns about the potential for these compounds to enter the food chain, leading to exposure in animals and, ultimately, humans. [57]

Benzodiazepines are released into the environment primarily via wastewater effluents, hospital discharges, and the improper disposal of unused medications. Their persistence in aquatic systems, together with other pharmaceuticals such as antibiotics and antidepressants, may promote selective pressure on microbial communities, thereby contributing to the development of antibiotic-resistant bacteria. This represents a growing concern for both human and animal health. [58,59]

Addressing the environmental issues related to benzodiazepines requires a comprehensive approach involving various stakeholders, including healthcare providers, patients, pharmaceutical manufacturers, and policymakers. Promoting

proper medication disposal methods, improving wastewater treatment facilities to remove pharmaceutical residues, and raising awareness about the environmental impact of pharmaceuticals are some of the steps that can be taken to mitigate these issues. [18,60,61]

Diazepam, its metabolites, and etizolam, along with its metabolite, contribute to the environmental issues associated with benzodiazepines. Faulty disposal of unused pharmaceutical products, including diazepam and etizolam, can lead to the release of these compounds into the environment. The main active metabolites of diazepam, such as desmethyldiazepam (nordiazepam) and temazepam, as well as the active metabolite of etizolam, α -hydroxyetizolam, can also enter the environment through similar pathways. [62]

When released into aquatic systems via wastewater effluents or unsuitable disposal practices, these substances may remain in the environment and threaten aquatic life. [63] Studies have shown that even at trace levels, benzodiazepines and their metabolites can impact aquatic life, affecting behaviour and reproduction in some species. [64,65] Moreover, the potential for these drugs to accumulate in the food chain raises concerns about human exposure through the consumption of contaminated aquatic organisms. [52,66–68]

The presence of benzodiazepines and their metabolites in the environment also raises the possibility of contributing to antibiotic resistance. As these drugs can interact with bacterial communities in environmental settings, there is a potential for the development and spread of antibiotic-resistant bacteria, which can have serious implications for public health. [62,69]

Addressing the environmental issues related to benzodiazepines, including diazepam and its metabolites, and etizolam and its metabolite, requires collective efforts. Proper disposal practices, improved wastewater treatment, and raising awareness among healthcare providers, patients, and the general public about the environmental impact of pharmaceuticals are critical steps in minimizing the environmental footprint of these drugs. Additionally, continued research and monitoring are vital to understanding the extent of contamination and potential risks, guiding effective strategies to safeguard both the environment and human health. [5,28,68]

Table 2-2 summarises the most relevant reviews that frame the context of this thesis, highlighting advances in understanding the occurrence, fate, biodegradation, and enantioselective analysis of pharmaceuticals, while also identifying critical gaps that this work seeks to address.

Table 2-2: Key Review Articles Relevant to Chiral Pharmaceuticals and Biodegradation in the Environment.

Title	Year	Review Type	Relevance to Thesis	Ref
Occurrence of chiral bioactive compounds in the aquatic environment	2017	Narrative	Highlights the presence of chiral pharmaceuticals in aquatic systems and stresses the need for enantioselective monitoring.	[52]
Chiral analysis of pesticides and drugs of environmental concern: biodegradation & enantiomeric fraction	2017	Critical	Discusses enantioselective biodegradation and EF analysis, aligning with the thesis focus on stereoselective behaviour of BZs and ADs.	[70]
Pharmaceuticals in the environment: Biodegradation and effects on natural microbial communities.	2015	Narrative	Explores microbial roles in pharmaceutical breakdown, supporting the thesis's use of 16S rRNA sequencing.	[13]
Review on fate and mechanism of removal of pharmaceutical pollutants from wastewater using biological approach	2017	Critical	Provides insights into biological removal mechanisms, contextualising synthetic wastewater biodegradation experiments.	[51]
Pharmaceutical compounds in aquatic environments—occurrence, fate and bioremediation prospective	2021	Critical	Situates this thesis within broader pharmaceutical fate and remediation research, showing how it fills gaps in enantioselective analysis.	[71]
Chiral pharmaceuticals: Environment sources, potential human health impacts, remediation technologies and future perspective	2018	Experimental	Connects environmental chemistry with risk assessment and remediation, framing the forensic toxicology relevance of this thesis.	[72]

2.2. Antidepressants (ADs)

2.2.1. ADs classification

ADs, (Table 2-3), have been classified according to their effects on the central nervous system (CNS) into four main categories which include tricyclic antidepressants (TCAs), (e.g. amitriptyline), monoamine oxidase inhibitors (MAOIs) (e.g. mirtazapine), selective serotonin reuptake inhibitors (SSRIs) (e.g. fluoxetine) and serotonin-norepinephrine reuptake inhibitors (SNRIs) (e.g. duloxetine). [73] Moreover, MAOIs and TCAs are referred as classical antidepressants, second-generation antidepressants are divided into SSRIs and SNRIs. [73,74] Figure 2-1 displays the chemical structures of antidepressants in this study.

2.2.2. Physico-chemical properties of ADs

Understanding the physicochemical properties of the pharmaceuticals such as SSRIs and SNRIs, is essential for optimizing their therapeutic use and developing effective analytical methods. [75,76] SSRIs and SNRIs are generally lipophilic, facilitating their ability to cross the blood-brain barrier and exert central nervous system effects. [77] However, their solubility can vary; for instance, some SSRIs are water-soluble, enhancing their absorption and distribution. These drugs are typically weak bases. In acidic environments, they are more likely to exist in their neutral form, while in basic conditions, they tend to ionize. This ionization behaviour influences their absorption, distribution, and elimination. These drugs are typically weak bases. In acidic environments, they are more likely to exist in their ionized form, while in basic conditions, they remain predominantly neutral. This ionization behavior directly influences their absorption, distribution, and elimination, since neutral molecules are absorbed more readily across lipid membranes, whereas ionized forms are eliminated more efficiently. [76]

A thorough understanding of these properties is crucial in analytical toxicology for several reasons. Firstly, it can be important for method development; knowledge of a drug's solubility and lipophilicity informs the selection of appropriate solvents and extraction techniques, ensuring efficient sample preparation.

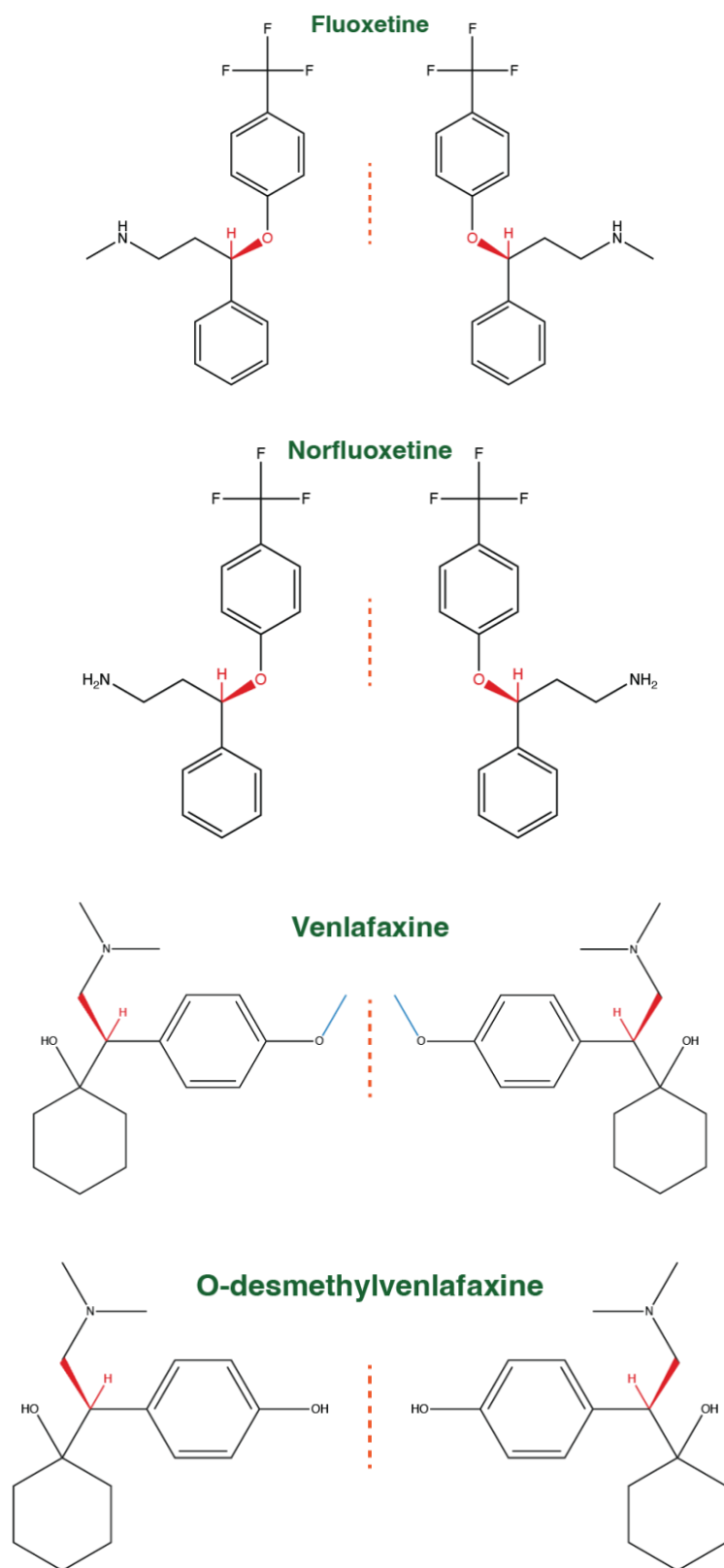


Figure 2-1: Chemical structures of ADs enantiomers.

Secondly, drugs detection and quantification; awareness of a drug's ionization state at different pH levels aids in optimizing chromatographic conditions and detector settings, leading to accurate detection and quantification.

Thirdly, it can help for interpretation of results; Understanding how these drugs behave under various physiological conditions allows for more accurate interpretation of toxicological findings, which is essential for clinical and forensic applications. By tailoring analytical methods to the specific physicochemical properties of SSRIs and SNRIs, toxicologists can enhance the reliability and accuracy of their analyses, ultimately improving patient care and safety. [78]

A comprehensive understanding of the physicochemical properties of SSRIs and SNRIs is essential in analytical toxicology for several reasons:

- Method development: knowledge of a drug's solubility and lipophilicity informs the selection of appropriate solvents and extraction techniques, ensuring efficient sample preparation. For instance, the solubility and distribution coefficient (octanol/water at pH 7.4) of a drug influence its absorption, distribution, metabolism, and excretion (ADME) properties, which are critical for developing effective analytical methods. [79]
- Detection and quantification; awareness of a drug's ionization state at different pH levels aids in optimizing chromatographic conditions and detector settings, leading to accurate detection and quantification. The ionization state affects the drug's behaviour during chromatographic separation, impacting retention time and detector response. [79]
- Interpretation of results; understanding how these drugs behave under various physiological conditions allows for more accurate interpretation of toxicological findings, which is essential for clinical and forensic applications. Physicochemical properties such as solubility and distribution coefficients are crucial for characterizing chemicals and developing drug formulations, ultimately optimizing bioavailability and therapeutic efficacy. [80]

By tailoring analytical methods to the specific physicochemical properties of SSRIs and SNRIs, toxicologists can enhance the reliability and accuracy of their analyses, ultimately improving patient care and safety. [79] Table 2-3 shows general physiochemical properties of most common antidepressants.

Table 2-3: Physio-chemical properties of common antidepressants, SSRIs, SSNRIs and other.

Drug	Class	CAS number	MW (g/mol)	pKa	log Kow	Photolysis t _{1/2}	Solubility in water (mg/mL)	Melting point (°C)
Fluoxetine	SSRI	54910-89-3	309.33	10.05	1.22	122	14	179
Norfluoxetine	SSRI metabolite	56161-73-0	295.33	~9.5–10	~1–2	-	-	-
Paroxetine	SSRI	61869-08-7	329.14	10.32	1.37	0.67	5.4	147
Citalopram	SSRI	59729-33-8	324.16	9.59	1.39	39	2	182
Sertraline	SSRI	79617-96-2	305.07	9.47	1.37	23	3.8–4.2	-
Fluvoxamine	SSRI	54739-18-3	318.16	9.39	1.21	0.57	-	120
Venlafaxine	SNRI	93413-69-5	277.41	9.4	3.10	4–6	572	215–217
ODV	SNRI metabolite	93413-62-8	263.38	~9.4	~2–3	-	-	-
Duloxetine	SNRI	116539-59-4	297.42	9.70	4.68	-	3	-
Mirtazapine	Other AD	85650-52-8	265.36	7.1–7.3	2.90	-	1.1	114
Amitriptyline	TCA	50-48-6	277.41	9.76	4.92	34–85	14	187
Clomipramine	TCA	303-49-1	314.86	8.98	5.19	-	70–125	189.5
Nortriptyline	TCA	72-69-5	263.38	10.1	4.51	0.74	slight	213

(-) Unavailable data, [81–83]

2.2.3. Chirality of ADs

In recent years, there has been a concerted effort to reduce the production of racemic drugs, reflecting scientific concerns about their effects on biological systems. [84,85] Chirality plays a significant role in pharmacology, as many antidepressants are chiral compounds. [86] For example, fluoxetine and venlafaxine are commonly administered as racemic mixtures, containing equal parts of two enantiomers. In contrast, other second-generation antidepressants are formulated as single enantiomers to enhance therapeutic specificity. [87] Unlike achiral drugs, which do not occur as enantiomeric pairs, chiral drugs can exist as stereoisomers with distinct three-dimensional arrangements. These differences can lead to stereoselective interactions with biological targets (receptors, enzymes, transporters) and may also influence physicochemical behaviour and environmental fate. It should be noted that achiral drugs can still vary in behaviour due to factors such as ionisation state, formulation/solid form, and metabolism, but they do not present enantiomer-specific effects. [87] The clinical implications of using racemic mixtures versus enantiomerically pure drugs are profound. Each enantiomer in a racemic mixture can exhibit different pharmacological activities. [88] One enantiomer, known as the eutomer, may provide the desired therapeutic effect, while the other, the distomer, could be less active or even cause adverse reactions. For instance, in the case of albuterol, the (R)-enantiomer is responsible for its bronchodilatory effect, whereas the (S)-

enantiomer may contribute to side effects. Additionally, enantiomers can differ in their pharmacokinetic profiles, including absorption, distribution, metabolism, and excretion, which can influence dosing regimens. [89] For example, the (S)-enantiomer of citalopram, known as escitalopram, has been shown to be more potent and may require lower doses compared to the racemic mixture. [90] Beyond clinical considerations, the environmental impact of chiral pharmaceuticals is complex. [91,92] Enantiomers can degrade at different rates in the environment, leading to the accumulation of one enantiomer over the other. [93] This can result in non-racemic mixtures in water bodies, potentially affecting aquatic organisms. Moreover, the toxicity of enantiomers to wildlife can vary, with one enantiomer possibly being more harmful to certain species. Understanding these differences is crucial for accurate environmental impact assessments and for developing strategies to mitigate potential ecological risks associated with pharmaceutical contaminants. [94]

Enantioselective analysis is crucial in pharmacology to understand the distinct behaviors of drug enantiomers. [87] According to Maia et al. (2017), the percentage of antidepressants studies up to 2017 in respect to enantioselective analysis was 27%, covering 8 different drug classes. [70] Moreover, between 2017 and 2023, several additional studies have been conducted in this area; however, the exact number is difficult to determine because relevant research is scattered across different disciplines and databases, and many reports do not explicitly indicate enantioselective approaches. These studies have explored various aspects, including the efficacy, safety, and metabolic pathways of individual enantiomers. For instance, research has compared the effectiveness of escitalopram (S-enantiomer) with other antidepressants, highlighting its superior efficacy and tolerability in treating major depressive disorder. [95–99]

The increasing number of enantioselective analysis studies reflects a growing global concern regarding the distinct pharmacological effects of individual enantiomers in chiral antidepressants. Developing and optimizing enantiomer separation techniques for environmental samples is crucial for accurately evaluating the environmental fate and potential risks associated with chiral antidepressants. Such advancements will enhance our understanding of how these substances behave in natural settings and inform strategies to mitigate their environmental impact. [93]

Fluoxetine (SSRI) and venlafaxine (SNRI) have been prescribed as racemic drugs. [100,101] In the case of fluoxetine both enantiomers (S and R) react identically to block serotonin reuptake while both venlafaxine enantiomers work differently, R-venlafaxine blocks serotonin and norepinephrine reuptake to pre-synaptic whereas S-venlafaxine strongly blocks serotonin reuptake. The chemical structures of S,R-fluoxetine and S,R-venlafaxine are shown in Figure 2-1. [87,102]

Citalopram (SSRI) was previously administered as a racemic drug; however, it has recently been marketed in its pure enantiomeric form (S-citalopram), due to differences in potency for blocking serotonin reuptake - a process known as a chiral switch. [87,103] Another pure enantiomer is duloxetine (SNRI) and marketed as S-duloxetine, even though, another form of the drug reacts similarly with blocking norepinephrine and serotonin reuptake, but S-duloxetine is higher effective than R-duloxetine by double. [87]

For paroxetine (SSRI) and sertraline (SSRI) both manufactured as pure enantiomers, each containing two stereogenic carbons. Paroxetine is produced in the trans configuration (3R,4S), as shown in its chemical structure. [104–106] In contrast, sertraline is produced in the cis configuration, with the S-enantiomer at carbon 1 and carbon 4, as demonstrated in the Figure 2-2. Owing to its selectivity, the cis configuration of sertraline is preferred over the transform. It has been reported that the R-configuration at carbon 1 combined with the S-configuration at carbon 4 of sertraline effectively blocks serotonin, dopamine, and norepinephrine, whereas the inverse configuration is more selective for inhibiting norepinephrine. [87]

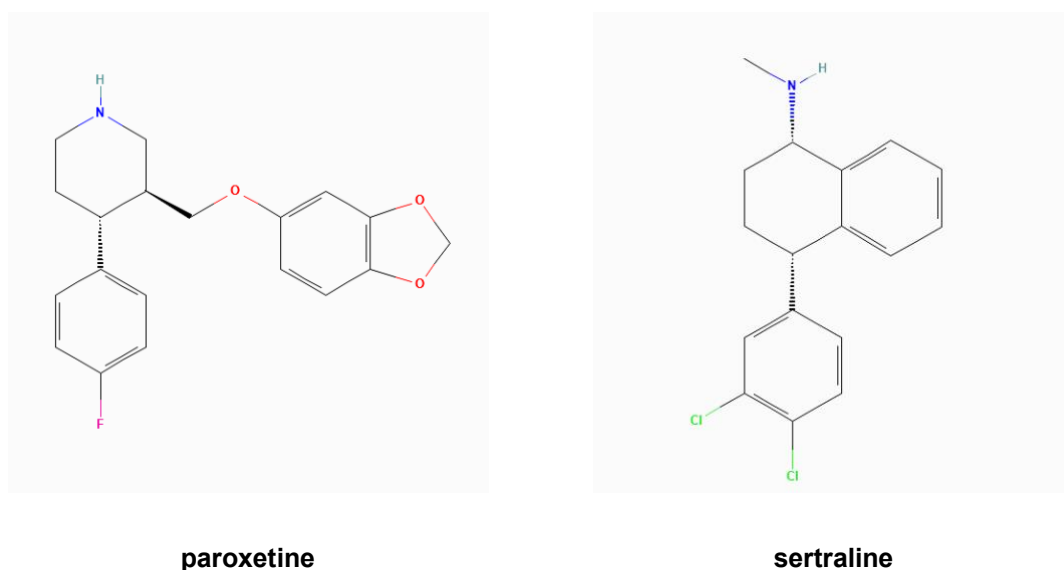


Figure 2-2: Chemical structures of Paroxetine and sertraline.

2.2.4. ADs stability

According to Kwon et al. (2006), FLX exhibited remarkable stability across various aquatic samples, including water/sediment systems and activated medium. [15] Its stability remained unaffected even under varying lighting conditions. [15] However, it was reported that FLX was not stable in wastewater. [107] Photolysis of fluoxetine was also examined, and five degradants were detected when exposed to UV. [108] Moreover, most antidepressants listed in the Table 2-4 seem to be stable in respect to photodegradation, except fluvoxamine. [109] It is important to note that one wastewater treatment approach for degrading pharmaceutical compounds is UV irradiation. [110] This process may generate additional potentially harmful compounds in the environment.

Generally, degradation rate relies on the medium that analyte interfere in, for example, buffered water, lake waters, synthetic humic water and basic aqueous solution. In the case of paroxetine, the drug seems to be less stable in basic aqueous solution compared to other types of solutions. [111] Another important point that should be considered is temperature stability. It was reported that FLX and VEN were degraded when stored at 4 °C in a refrigerator for 72 h under highly acidic conditions, with a loss of 15.1% in wastewater samples. However, when samples were kept at natural pH, both drugs showed greater instability, decreasing by about 50%. [112]

2.2.5. ADs pharmacology

2.2.5.1. Pharmacokinetics

SSRIs and SNRIs are primarily metabolised in the liver by cytochrome P450 (CYP) enzymes; however, defining a complete CYP profile for antidepressant biotransformation remains challenging because multiple isoenzymes contribute and activity varies with genetic polymorphisms. [113] Key pharmacokinetic parameters (e.g., protein binding, excretion pathways, and elimination half-life) for selected antidepressants are summarised in Table 2-4.

FLX is completely metabolized in the liver (CYP2D6) and other enzymes by transforming fluoxetine to the main active metabolite norfluoxetine (NFLX) by N-demethylation and illustrated in Figure 2-3. [113,114] From a stereochemistry perspective, as mentioned above, fluoxetine is marketed as a racemate which has

S- and R- forms with the same proportion. Hence, S-FLX and R-FLX metabolized to its main active compounds S-NFLX and R-NFLX. It was reported that NFLX has exact effect to the parent drug. [114] S-enantiomer and R-enantiomer of fluoxetine have identical potency on the inhibiting of serotonin reuptake while S-enantiomer of norfluoxetine has higher potency than R-enantiomer in the same effect, which may reach to 20 times. Both enantiomers of fluoxetine have different pharmacokinetic properties such as half-life and metabolism rate. [114] It was stated that the concentration of S-FLX and S-NFLX were metabolized faster than R-form over period of time of treatment. [74,87] The reason for this may be attributed to the enzyme's selective binding to the 3D structure of enantiomers, leading to distinct pharmacokinetic properties owing to their diverse natures. [113]

Table 2-4: Antidepressants stability in different conditions.

Drug	UV	VIS	Wastewater	anaerobic sewage	Ref
Fluoxetine	Unstable (photolysis)	Unstable	Variable/Unstable during storage & sewer transport (loss within 24–72 h reported; matrix-dependent)	Data limited; in-sewer anaerobic transformation reported for many APIs but drug-specific evidence sparse	[115–117]
Paroxetine	Unstable (photolabile in sunlight/UV)	Unstable (UV–Vis/photoinduced)	Limited / condition-dependent (degrades under AOPs; storage stability not well resolved)	No robust drug-specific data found	[118,119]
Citalopram	Unstable (≈100% loss in 4 h under UV in lake water)	Unstable (photoactive under UV–Vis; theoretical & experimental support)	Often persistent in water; strong adsorption to sediments (appearing “stable” there)	No robust drug-specific data found	[120–122]
Sertraline	Unstable (photodegrades; pseudo-first-order)	Unstable under photo-processes (fast with UV/ZnO; VIS-only less documented)	Relatively stable/persistent in wastewater; poor removal; stable in storage in some tests	Likely stable/persistent (no clear anaerobic decay signal reported)	[122–124]
Fluvoxamine	Unstable (photodegradation kinetics reported)	Unstable (photolabile under simulated sunlight)	No robust data found	No robust data found	[125,126]
Duloxetine	Unstable (UV photolysis; TPs identified)	Unstable (visible-light photolysis shown)	No robust data found	No robust data found	[127,128]
Venlafaxine	Unstable (direct & self-sensitized photolysis)	Unstable (UV–Vis/photo-AOPs degrade VBX)	Unstable in sewers/wastewater over time; temperature & matrix dependent	Transforms under in-sewer (often anaerobic) conditions; rate lower than in oxic/UV	[115,129,130]
Mirtazapine	Unstable (photolysis/forced-degradation)	Unstable (photolysis under VIS/photostrass)	No robust data found	No robust data found	[127,128]

Citalopram is metabolized through demethylation to form desmethylcitalopram (DCIT), similar to fluoxetine, which also produces an active metabolite. [87] The S-enantiomer of citalopram (S-CIT) is approximately six times more potent than S-DCIT, whereas R-DCIT shows greater potency than its parent compound. [87] Because of the higher potency of the S-enantiomer and the dose-dependence and side effects associated with the R-enantiomer, citalopram has recently been marketed as a pure chiral drug in the S-form rather than as a racemic mixture. This process is referred to as a “chiral switch”. [87] Reports indicate that the S-enantiomer is about 30 times more effective than the R-enantiomer in blocking serotonin reuptake [87], and in some studies, it has been shown to be up to 100 times more potent. [103] These differences in drug potency may be influenced by pharmacogenomic variations affecting the enzymes involved. [95]

Table 2-5: CYP450 metabolizer level of antidepressants.

Drug	Chiral forms	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Fluoxetine	S-FLX	+++	++	+	+++	+
	R-FLX	++	++	++	+++	+
Paroxetine	-	+	+	+	+++	+
Citalopram	S-CIT	-	-	++	+	++
	R-CIT	-	-	++	+	++
Sertraline	-	-	-	-	-	-
Fluvoxamine	-	+++	++	+++	+	++
Duloxetine	-	++	-	-	++	-
Venlafaxine	S-VEN	+	-	+	+++	+
	R-VEN	+	-	+	++	
Mirtazapine	-	+	-	-	+++	++

(-) = Unavailable data

The level of metabolizer Lower (+), Medium (++) and Higher (+++) [74,87,113,131]

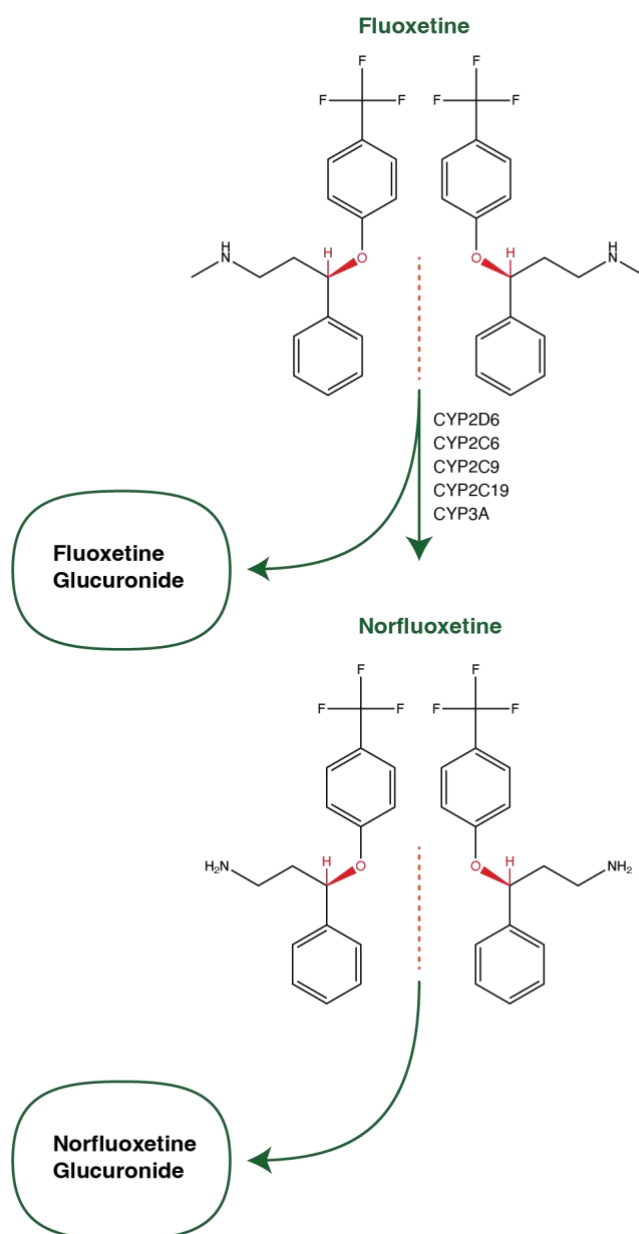


Figure 2-3: Fluoxetine metabolism pathway.

VEN is primarily metabolized in the liver by CYP2D6 and CYP3A4 into three major metabolites: O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDVEN), and N,O-didesmethylvenlafaxine (DDVEN). Similar to fluoxetine (FLX), VEN is administered as a racemic mixture containing both S- and R-enantiomers for the treatment of depression. However, unlike FLX, the parent drug VEN is more potent than its metabolites in inhibiting serotonin reuptake, Figure 2-4. [87]

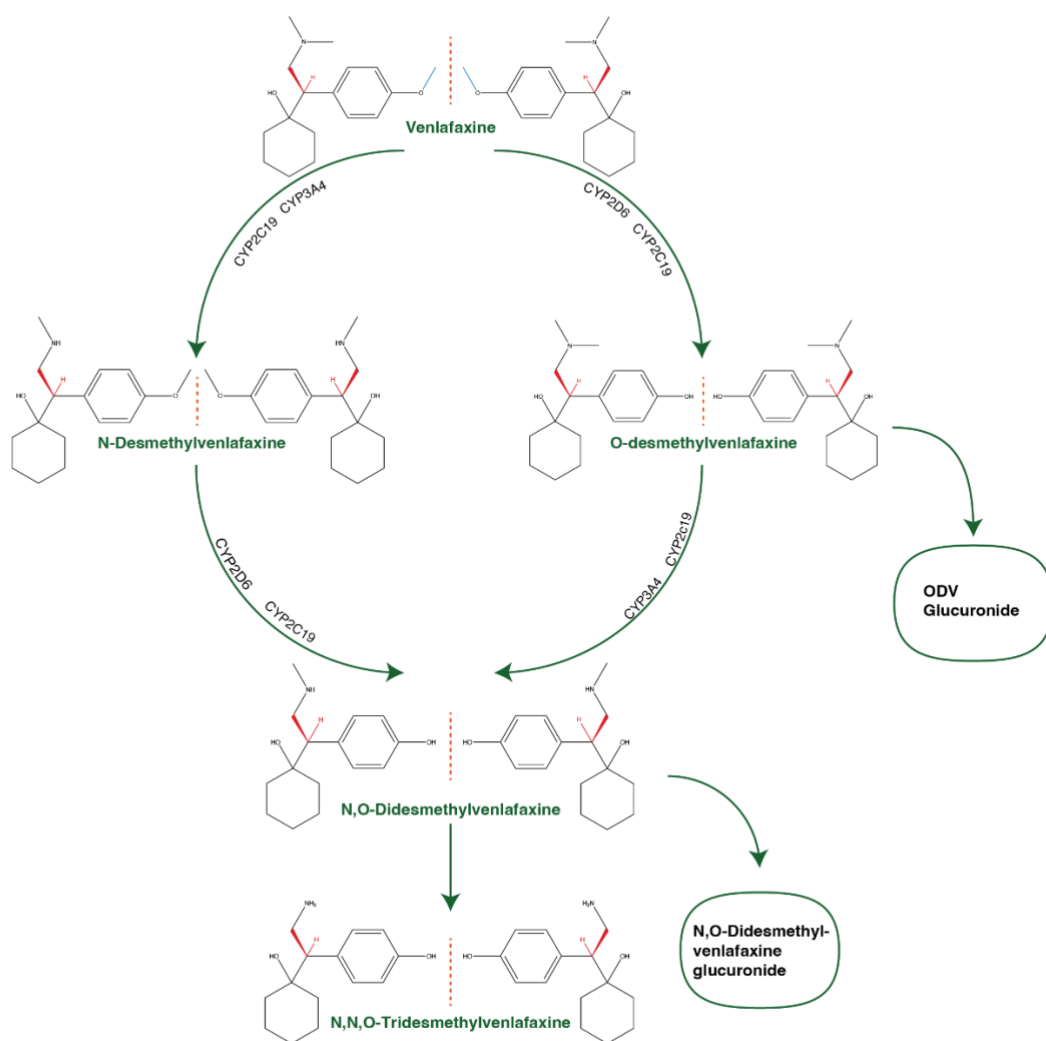


Figure 2-4: Venlafaxine metabolism pathway.

2.2.5.2. Pharmacodynamics

SSRIs are commonly prescribed for the treatment of obsessive-compulsive disorder (OCD), premenstrual dysphoric disorder (PMDD), and bulimia nervosa. SNRIs, on the other hand, are used to manage major depressive disorder, panic disorder, and generalized anxiety disorder. [87]

In terms of mechanism of action, second-generation antidepressants (SSRIs and SNRIs) were approved as safer alternatives to tricyclic antidepressants (TCAs), largely because TCAs are associated with more adverse side effects. [132] SSRIs specifically act by selectively blocking the reuptake of serotonin. They bind to serotonin transporter proteins, leading to an increased concentration of serotonin in the synaptic cleft. This higher serotonin level enhances the likelihood of

activating postsynaptic receptors within the central nervous system (CNS). SNRIs function in a similar way but with broader activity: they block the reuptake of both serotonin and norepinephrine, thereby increasing the levels of both neurotransmitters in the synaptic cleft. [132]

The most common adverse effects associated with SSRIs and SNRIs include dry mouth, nausea, vomiting, diarrhea, dyspepsia, anxiety, insomnia, and headache. Compared to TCAs, SSRIs are generally safer overall, although no significant differences have been observed among individual SSRIs. [133]

In contrast, mirtazapine (TeCA) exhibits a distinct side-effect profile. When co-administered with alcohol or benzodiazepines, it enhances sedation. Its common adverse effects include increased appetite and weight gain, dizziness, headache, hypotension, skin rashes, nightmares, agitation, mania, hallucinations, paraesthesia, convulsions, tremors, and hyponatraemia. [74]

As shown in Table 2-6, most SSRIs, including fluoxetine, paroxetine, and citalopram, demonstrate high protein binding (80-95%), which increases the potential for drug-drug interactions. Fluoxetine is distinctive in having a prolonged elimination half-life (1-6 days, extended further by norfluoxetine), allowing once-daily dosing but also contributing to accumulation. Paroxetine, by contrast, has a shorter half-life (~21 h), making plasma concentrations more sensitive to missed doses. Citalopram occupies an intermediate position with a half-life of about 36 h and somewhat lower protein binding (~80%). [134]

The second part of Table 2-6 highlights differences among other antidepressants. Sertraline and fluvoxamine show moderate half-lives (15-26 h), whereas duloxetine and venlafaxine exhibit shorter elimination (8-17 h and 5-11 h, respectively), often requiring careful dose titration. Mirtazapine, a tetracyclic antidepressant, has a variable half-life (20-40 h) and moderately high protein binding (85%), with predominant urinary excretion. These differences are clinically relevant when considering dosing schedules, onset of action, withdrawal symptoms, and safety in patients with hepatic or renal impairment. [135–138]

Table 2-6: ADs pharmacokinetics data.

Drug	Peak plasma concentrations	Protein binding	Excretion	Elimination $t_{1/2}$
Fluoxetine	6 to 8 h	95%	80% Urine 20% faeces	1-4 days for FLX 4-6 days for NFLX
Paroxetine	5 h	95%	64% urine 36% faeces	21 h
Citalopram	2 to 4 h	80%	-	36 h
Sertraline	4.5 to 8.4 h	98%	50% urine 50% faeces	26 h
Fluvoxamine	3 to 8 h	80%	Urine	15 h
Duloxetine	6 h	96%	80% 20%	8 to 17 h
Venlafaxine	2 to 4 hours	27%	98% urine	5 to 11 h
Mirtazapine	2 h	85%	75% urine 15% faeces	20 to 40 h

(-) = Unavailable data [83]

2.2.5.3. Drug interactions

SSRIs have been reported to inhibit some benzodiazepines elimination in human body. For example, fluvoxamine is found to inhibit diazepam and midazolam elimination. However, it was reported that venlafaxine induces elimination of alprazolam and diazepam. [133] With respect to antibiotics, FLX was involved in causing hallucination when administrated with nitrazepam during treating with clarithromycin owing to inhibition of cytochrome P450. [133] Generally, the administration of the same neurotransmitter type antidepressants may produce synergistic interaction, such as VEN. [74]

2.2.6. Biodegradation of Antidepressants

In the past few years, the scientific community focused on the effect of steroid drugs on the ecosystem, whereas, the impact of non-steroidal drugs on environment have not been fully elucidated. [13] In the literature, several studies were conducted with the aim of detecting SSRIs and SNRIs in environment. [11,32,113,139] Several studies showed the occurring of SSRIs and SNRIs degradation in environmental samples, however, it is still ambiguous to confirm whether it occurred by chemical or biological processes. [11] Furthermore, applying microorganisms in ecosystem was established last decade when used for removing contaminants during wastewater treatment. [51] Although, the total view of knowledge about biodegradation of antidepressants by microorganism is still unclear. [13]

SSRIs are mostly stable in water when compared to other antidepressant classes. [140] The most common SSRIs detected in nature is fluoxetine which is one of the

microcontaminants. [13] Several studies have been conducted to trace fluoxetine and its main active metabolite in environmental samples. However, monitoring showed fluoxetine appeared with high stability that reached to around 9 months. [140] In contrast, it was reported that fluoxetine's half-life in water ranged between 6-10 days, (Table 2-6). [13] Furthermore, to obtain accurate mechanism of degradation action rate should be slow. [141]

More recent attention has focused on environmental contamination by pharmaceuticals. Only a few studies indicated the impact of pharmaceutical stereochemistry in the natural environment.[142,143] Consequently, understanding of the contamination with respect to the chiral perspective may play an important role regarding risk assessment. [86] Monitoring of chiral drugs degradation and metabolic products in the human body generally has become more comprehensive due to developing of analytical procedures and development of different fields. Various studies have been conducted to provide filling the gap in these pharmaceuticals, in respect to stereochemistry, that will be discussed in next section.

2.2.7. Chiral biodegradation

There is a degree of uncertainty regarding the diversity of chiral pharmaceutical compounds and their ecological impacts. Chiral pharmaceuticals have been detected in aquatic organisms regardless of whether they were introduced as racemic mixtures or pure enantiomers. [11] Considering enantiomer-specific biodegradation is essential for accurate risk assessment due to the complexity of environmental factors involved. [70]

FLX, particularly the S-enantiomer, has been identified as hazardous to aquatic species. [114] The S-form of FLX is significantly more toxic to *Tetrahymena thermophila* than the R-form, while its primary metabolite, S-NFLX, shows lower potency compared to the R-enantiomer. [114] However, studies on FLX biodegradation in environmental matrices remain limited, and it is unclear whether observed degradation occurs via chemical or biological processes. [13] The continued detection of FLX in environmental and drinking water samples indicates limitations in current wastewater treatment systems, In the past few years, the scientific community focused on the effect of steroid drugs on the ecosystem, whereas, the impact of non-steroidal drugs on environment have not been fully

elucidated. [13] In the literature, several studies were conducted with the aim of detecting SSRIs and SNRIs in environment. [11,32,87,139] Several studies showed the occurring of SSRIs and SNRIs degradation in environmental samples, however, it is still ambiguous to confirm whether it occurred by chemical or biological processes. [11] Furthermore, applying microorganisms in ecosystem was established last decade when used for removing contaminants during wastewater treatment. [144] Although, the total view of knowledge about biodegradation of antidepressants by microorganism is still unclear. [13]

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In one of the first studies on chiral biodegradation, Moreira et al. (2014) demonstrated that *Labrys portucalensis strain F11*, a Gram-negative bacterium, preferentially degraded R-FLX, resulting in enrichment of the S-enantiomer. [11] In contrast, other studies have reported higher concentrations of R-FLX in both treated and untreated wastewater, suggesting its slower degradation rate compared to the S-enantiomer. [145,146] These findings highlight the variability of

enantioselective degradation across environmental conditions. Table 2-8 summarizes key review articles on pharmaceuticals in the environment.

Table 2-7: Microbes involved in enantiomers ADs.

Drugs	Microbes	Ref.
FLX NFLX	<i>Daphnia magna</i> and <i>Pimephales promelas</i>	[147]
FLX	<i>Pseudomonas aeruginosa</i>	[148]
	<i>Staphylococcus aureus</i>	
	<i>Escherichia coli</i>	
FLX NFLX	<i>Labrys portucalensis strain F11</i>	[11]
FLX	<i>Escherichia coli</i>	[59]
FLX	<i>D. magna</i> <i>T. thermophila</i>	[32]
FLX Sertaline	<i>Escherichia coli</i>	[149]
Sertaline Tetracycline	<i>Escherichia coli</i> APEC_O2	[26]

Additionally, in activated sludge systems, S-FLX was shown to degrade faster than R-FLX. This observation aligns with enantioselective patterns seen in human metabolism and excretion processes, Table 2-5.

The ecological relevance is further underscored by the activity of S-NFLX, the active metabolite of FLX, which exhibits enantioselectivity and may pose risks to aquatic ecosystems due to its potency and persistence. [114]

Table 2-8: Important review papers published on pharmaceuticals in the environment studies within the period 2002-2022.

Review	Year	Ref
Chiral switches: A Review	2011	[150]
Chiral separations: A review of current topics and trends	2012	[65]
Chiral HPLC for efficient resolution of enantiomers	2008	[151]
Chirality of modern antidepressants: An overview	2017	[152]
Advances and applications of chiral resolution in pharmaceutical field	2022	[51]
Stereochemistry and drug efficacy and development: Relevance of chirality to antidepressant and antipsychotic drugs	2002	[13]

Table 2-9: Summarised extraction details of antidepressants.

Analyte	Sample	Hollow Fiber-Liquid Phase Microextraction				Instrument	Validation			Author & year	Ref
		Type of HP-LFME	Organic Solvent	Acceptor phase	Length (cm) id (mm) × wt (mm) × ps (μm)		LOD	Linear range ng/mL	Recovery %		
PROX FLUV Mianserin CIT	BM Water B M Water B M Water B M Water	Three phases	polyphenyl-methylsiloxane	10mM HCl	1.8 1.2 × 2 × 0.2	CE	N/A	N/A	20 62 38 52 18 58 22 37	Jin et al. 2018	[149]
MIRT	Plasma	Two phase Accurel Q3/2 polypropylene	-	toluene	7 0.6 × 2 × 0.2	HPLC	6.25	6.25–625	32	Schlüsener et al. 2015	[54]
amitriptyline, imipramine SER	Biological	N/A Q3/2 Accurel KM polypropylene	n-dodecane	0.1M of H ₃ PO ₄	8.8 0.6 × 2 × 0.2	HPLC	0.5 and 0.7 μg L ⁻¹	5–500	68 68 65	Evans et al. 2017	[53]
S-MIRT R- MIRT S-Metabolite R-Metabolite	Urine	- polypropylene hollow	n-hexyl ether	0.01 mol/L acetic acid solution	8 -	CE	N/A	62.5-2500 62.5- 1250	75.9 80 23.9 23.6	Calisto et al. 2009	[140]
FLX FLUV PROX SER CIT	sewage sludge	N/A plasmaphan polypropylene	dihexyl ether	purified water/for mic acid at	28	LC-MS	N/A	0.5–250	57.2 26.2 61.4 58.4 71.4	Barra Caracciolo et al. 2015	[13]
FLX NFLX	Plasma	Three phase Accurel Q3/2 polypropylene	n-Hexyl ether	HCL	7 0.6 × 2 × 0.2	HPLC	5	5-500	70.9 59.7	Benotti et al. 2009	[141]
FLX FLUV PROX SER	sewage sludge	N/A plasmaphan polypropylene	dihexyl ether	purified water/for mic acid	28 N/A	HPLC/MS-MS	N/A	1-250	N/A	Barra Caracciolo et al. 2015	[13]

Analyte	Sample	Hollow Fiber-Liquid Phase Microextraction				Instrument	Validation			Author & year	Ref
		Type of HP-LFME	Organic Solvent	Acceptor phase	Length (cm) id (mm) × wt (mm) × ps (μm)		LOD	Linear range ng/mL	Recovery %		
CIT escitalopram											
CIT PROX FLX SER NFLX	sewage sludge	Three phases 50/280 Accurel polypropylene	DHE	0.1 M ammonium phosphate	20 0.28 × 0.5 × 0.1	LC-MS	6 ng g ⁻¹	-	72.2 80.4 85.8 85.5 84.4	Moreira et al. 2014	[11]
FLX NFLX	Human plasma	Two phase Accurel Q3/2 polypropylene	n-octanol and n-hexyl ether from	Nortriptyline hydrochloride	0.37 0.6 × 2 × 0.2	GCMS	3 5	10-500 15-500	3.78 5.16	Andrés-Costa et al. 2017 & Silva et al. 2015	[114] [153]
FLX NFLX	Whole blood	Three phase Q3/2 Accurel KM polypropylene	dodecane	0.1 M formic acid	8 0.6 × 2 × 0.2	GCMS	10 10	20-1200	57 34	Ford et al. 2016	[154]
FLX OLZ	Plasma Urine Plasma Urine	Three phase Accurel Q3/2 polypropylene	1-octanol	10 mM HCl	2.8 1.2 × 2 × 0.2	(HPLC/UV)	0.3 0.5 0.2 0.5	2-500	88.7 82.6 87.5 93.2	Andrés-Costa et al. 2017	[114]
S- and R-CIT	Water urine	Three phase Q3/2 Accurel polypropylene	1-octanol	HCl	- 0.6 × 2 × 0.2	CE	10 20	30-300 60-300	71 for S&R R, 59 / S, 58	Baker et al. 2011	[155]
FLX SER PROX FLUV CIT	Plasma Urine Plasma Urine Plasma Urine Plasma Urine	Three phase sweeping-MEKC Accurel Q3/2 polypropylene	phenetole	acetic acid	3 0.6 × 2 × 0.2	CE	1.55 μg L ⁻¹	1.5-200 1.5-200 2.0-200 5.0-200 0.6-200	92.3 94.8 89.3 94.0 95.7 91.6 100 91.5 110 109	Petrie et al. 2018	[156]

N/A= Not available, id= id=internal diameter, wt= wall thickness, ps= pore size, CE= Capillary Electrophoresis, BM= Breast Milk, LOD= Limit of Detection.

2.2.8. Extraction of Antidepressants

Sample preparation and extraction methods have advanced in parallel with improvements in analytical instrumentation, together enabling greater selectivity and sensitivity. Recent progress in extraction techniques has also highlighted the importance of green chemistry, defined by Paul Anastas as “the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances”. [157]

When analysing environmental samples such as soil, it is essential to consider the presence of co-contaminants and naturally occurring substances, including humic acids, which can interfere with the detection and quantification of target analytes. To minimize these matrix effects, appropriate clean-up steps are required. [158] The following section introduces the most commonly applied and recent methodologies for the extraction of antidepressants. [158]

2.2.8.1. Liquid-Liquid Extraction (LLE)

One of the most common traditional sample preparation techniques is Liquid-Liquid Extraction (LLE). Despite its extensive use, several drawbacks limit its efficiency and routine application. The method requires relatively large volumes of often toxic organic solvents, making it costly, environmentally unfriendly, and inconsistent with current “green chemistry” principles. Moreover, LLE is generally time-consuming, as it involves multiple manual handling steps and prolonged shaking or mixing stages to achieve phase separation (Figure 2-5). These limitations frequently result in variable and lower recovery rates compared to more modern extraction techniques, largely due to losses during solvent transfer, emulsification, and evaporation [159–161]

In a study by A. Ribeiro et al. (2013), the method yielded recovery rates exceeding 88%; however, this was achieved only after a shaking time of 2 hours, which underscores the labor-intensive and time-consuming nature of the procedure. [139] Similarly, other investigations confirmed that LLE could achieve satisfactory recovery when applied to drugs of abuse in wastewater

matrices. [162] However, its reproducibility and efficiency are strongly influenced by sample complexity and operator expertise.

To overcome some of these challenges, LLE has frequently been used in combination with Solid-Phase Extraction (SPE), particularly for complex matrices such as wastewater, surface water, and biological fluids. This hybrid approach improves selectivity, reduces matrix interferences, and enhances method robustness. For instance, the simultaneous determination of selective serotonin reuptake inhibitors (SSRIs) from wastewater using LLE followed by SPE has been successfully reported, demonstrating both good recovery and reduced matrix effects. [163–165]

2.2.8.2. Solid phase extraction (SPE)

The solid phase extraction (SPE) method is one of the more practical ways of extracting pharmaceuticals from biological and environmental matrices. Optimization of the SPE methods assistance to gain optimum selectivity and recovery to overcome the limitation of using LLE. [112] Thus, other SPE types may help to resolve this analytical issue with enantiomer antidepressants, e.g. Oasis MAX or HLB stationary phases. [158]

The SPE, (Figure 2-5) is a widely employed sample preparation technique in analytical toxicology, designed to extract, purify, and concentrate analytes from complex biological and environmental matrices such as blood, urine, tissues, and wastewater. By using a solid sorbent material, SPE provides selective retention of target compounds while enabling efficient removal of co-extracted interferences. This process not only enhances analyte recovery but also improves the sensitivity and specificity of downstream instrumental analyses, such as liquid chromatography-mass spectrometry (LC-MS) and gas chromatography–mass spectrometry (GC-MS). [166,167]

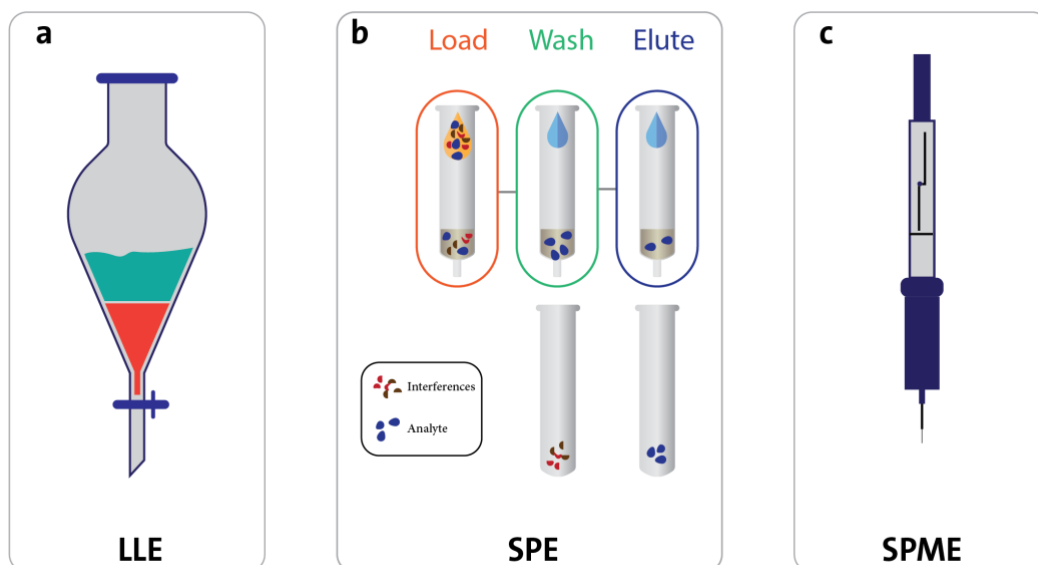


Figure 2-5: Illustrative images of LLE, SPE and SPME.

In analytical toxicology, SPE is extensively utilized for the detection and quantification of drugs, metabolites, and toxic substances. Its applications include: SPE is widely used to isolate a range of drugs from biological samples, enabling comprehensive toxicological screenings. For example, a unified SPE protocol has been developed to extract multiple drug classes simultaneously from serum and urine, which is then analysed using high-performance liquid chromatography with diode-array detection (HPLC-DAD). [168] SPE supports the accurate quantification of specific drugs and their metabolites. A prominent application is an SPE method combined with liquid chromatography–tandem mass spectrometry (LC-MS/MS), which has been optimized to detect 40 benzodiazepines and three Z-drugs in blood and urine samples. [169] SPE is highly effective at removing interfering substances from complex matrices, which enhances the accuracy and reliability of toxicological analyses. This cleanup capability is essential for biological samples that may contain multiple potential interferences. [168]

The choice of sorbent in SPE is critical and depends on the chemical properties of the analytes and the sample matrix. Common types of SPE sorbents include: Non-polar or reversed-phase sorbents, such as C18 and C8 bonded silica, are used to retain non-polar compounds from polar matrices. These sorbents operate on the basis of hydrophobic interactions,

which makes them particularly effective for extracting hydrophobic analytes from aqueous samples. [170]

Polar or normal-phase sorbents, including materials such as silica, alumina, and Florisil, are employed to retain polar compounds from non-polar matrices. The retention mechanism is driven by polar interactions between the analyte and the sorbent surface, most notably hydrogen bonding and dipole–dipole forces. In practice, this means that analytes with functional groups such as hydroxyl (-OH), amino (-NH₂), or carbonyl (C=O) can strongly interact with the polar sites on the sorbent, while non-polar components of the matrix are washed away. As a result, polar sorbents provide effective clean-up of complex, non-polar matrices by selectively retaining the analytes of interest. Once bound, the analytes can be released (eluted) using an appropriate polar solvent, enabling both isolation and concentration prior to instrumental analysis. [169]

Ion-exchange sorbents are specifically designed to capture charged analytes via electrostatic interactions. They are divided into cation-exchange and anion-exchange types based on the target analyte's charge. Ion-exchange SPE is especially useful for isolating ionic compounds from complex matrices. [171]

Mixed-mode sorbents combine the characteristics of both reversed-phase and ion-exchange functionalities. This duality offers enhanced selectivity, making mixed-mode sorbents suitable for complex sample matrices that contain analytes with diverse chemical properties. [170,172]

Several key factors impact the efficiency and success of solid-phase extraction (SPE) in analytical toxicology:

- Choosing the appropriate sorbent is essential, as it must align with the chemical properties of both the analyte and the sample matrix. Proper sorbent selection enhances the retention and subsequent elution of target compounds. [173]

- The pH of the sample can influence the ionization state of the analyte, affecting how it interacts with the sorbent. Adjusting the sample pH to favour either a neutral or charged state, as required, can improve analyte retention on the sorbent. [169]
- Selecting the right elution solvent is crucial for effectively desorbing the analyte from the sorbent. Solvents with suitable polarity and pH are chosen to efficiently elute target compounds without co-eluting unwanted matrix components. [174]
- The flow rate of the sample through the SPE cartridge impacts the interaction time between the analyte and sorbent. An optimal flow rate ensures adequate retention and efficient elution of analytes. [174]
- The volume of the sample being processed influences the capacity of the sorbent and the analyte's concentration. When processing larger volumes, a proportional increase in sorbent mass may be necessary to maintain extraction efficiency. [174]

In addition, Evans & et al. (2014) stated that, several studies were conducted on SPE-Oasis HLB to enantiomers separation, with LC-MS. However, the Oasis HLB is not a selective cartridge which reflect increasing of matrix effects, hence, enantiomers recovery may significantly reduce. increasing of matrix effect might cause chiral separation. [158] SPE is frequently the most common extraction method for environmental samples due to the level of clean-up required prior to analysis. [86]

2.2.8.3. Solid phase micro extraction (SPME)

Solid Phase Microextraction (SPME) is a solvent-free sample preparation technique that employs a fiber coated with a specific extracting phase to isolate analytes from various media, including liquids, gases, and solids. Developed in the early 1990s by Professor Janusz Pawliszyn, SPME has become a widely adopted method in analytical chemistry due to its efficiency and environmental benefits. [175]

The SPME process involves exposing a coated fiber - typically with materials such as polydimethylsiloxane (PDMS), divinylbenzene (DVB), or Carboxen - to the sample matrix. Analytes partition between the sample and the coating based on their affinity for the extracting phase. After a designated extraction period, the fiber is retracted and transferred to an analytical instrument, such as a gas chromatograph or mass spectrometer, where the analytes are thermally desorbed for analysis. [164,165,176]

SPME is applicable to a wide range of samples, regardless of their volatility and concentrations, and is compatible with chromatographic instruments. The technique operates on the principle of establishing equilibrium between the sample and the fiber coating. The properties of the coated fiber play a crucial role in achieving efficient extraction, depending on the type of analytes being analyzed. Selecting the appropriate fiber coating enhances sensitivity and selectivity for target compounds, making SPME a versatile and effective tool in analytical procedures. [175]

However, there are certain drawbacks associated with the use of SPME, one of these, its availability in the market with limited options, selectivity and thermal stability. [175] An important part is selecting the appropriate stationary phase to target compounds of interest. For example, Polydimethylsiloxane (PDMS) is one of the common fiber types used to extract non-polar analytes with thermostability and immobilization. Another common stationary phase used to extract mixtures of compounds is carboxen/polydimethylsiloxane (CAR/PDMS). [177] To avoid these disadvantages, SPME has been developed to be more applicable extraction method to induce its thermostability and extraction efficiency. New phases based on nanomaterials have been developed to improve SPME, e.g. carbon nanotubes (CNTs), graphene, nanoporous carbon. [175,177]

Furthermore, SPME can be classified into three formats - in-tube, in-needle, and in-tip - which are applied across diverse matrices, including biological fluids (blood, plasma, urine) for drug and metabolite extraction, environmental samples (air, water) for volatile and semi-volatile compounds, and clinical or forensic specimens for high-throughput bioanalysis. [176,178]

There are several parameters that help to succeed optimum extraction which absolutely effect the sample extraction coating fiber, period time of the extraction and applied temperature and ionic strength [179]. On-tip SPME is one of the more recent extraction methods, which is developed from SPE that loads on packed sorbents in pipette tips. [180]

However, this extraction method was developed to extract drugs from biological matrices yet its applications to extract pharmaceuticals from environmental matrices are still limited. [180] It is important to consider that the capacity of the pipette is 200 μL that will lead to repeat separation of the sample and dispensing cycles manually in few steps. The method requires repeated manual cycles of sample separation and dispensing in a few steps. In addition, sample pretreatment, such as filtration and dilution, must be performed before introducing the matrices into the tips. These requirements represent major drawbacks of the extraction technique and may limit its suitability for use with complex environmental matrices. [180]

2.2.8.4. Liquid phase microextraction (LPME)

Liquid-phase microextraction (LPME), (Figure 2-6), an innovative technique derived from liquid-liquid extraction (LLE) over three decades ago by Audunsson, has become a green, effective tool for sample preparation. [181] [173] LPME generally includes two approaches: direct immersion extraction (DI) and headspace extraction (HS). [182] A popular variant, hollow fiber liquid-phase microextraction (HF-LPME), is especially advantageous due to its minimal solvent consumption - often only a few microliters - and its compatibility with various analytical techniques. This versatility has made HF-LPME a preferred choice in many laboratories. [183]

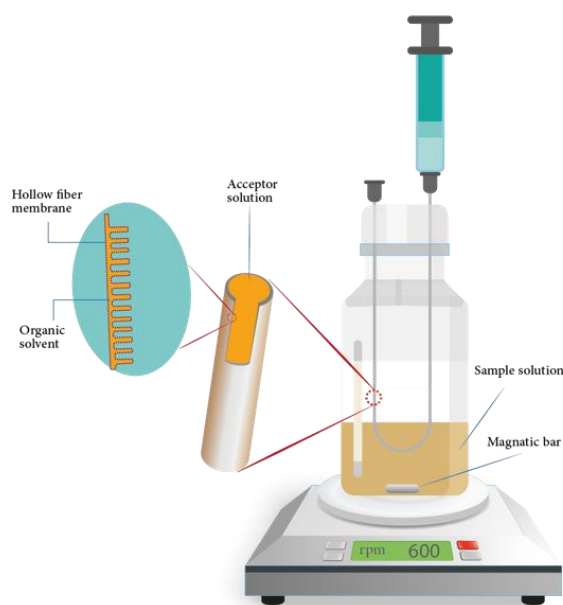


Figure 2-6: Illustrative picture of HF-LFME.

*(adapted from [181])

Compared to traditional methods like LLE and SPE, HF-LPME offers several distinct advantages. [184] By reducing solvent volume and simplifying sample preparation, HF-LPME not only cuts costs but also lessens the environmental impact of extraction processes. Additionally, HF-LPME delivers high sensitivity for a wide range of analytes, making it suitable for numerous applications. Nonetheless, HF-LPME encounters challenges with complex matrices, such as biological and environmental samples, where larger molecules can clog the fiber's porous structure, sometimes lowering recovery rates. [185]

While HF-LPME shows high recovery rates for certain analytes under optimized conditions, some compounds, such as FLX and its enantiomers, display variable recoveries influenced by factors like pH and solvent selection. Although research has increasingly explored LPME for environmental applications, studies specifically examining antidepressants in environmental matrices, such as sewage sludge, are still limited. Current findings suggest further refinement is necessary to achieve consistently high recoveries in these complex samples, highlighting the potential for advancing HF-LPME in environmental analysis. [109,185–187] Further details of its

applications, particularly the determination of antidepressants using HF-LPME as the extraction method, are summarized in the Table 2-9.

Despite the increasing application of chiral pharmaceuticals in biological matrices, their impact on the ecosystem has often been overlooked. However, the fate of pharmaceutical enantiomers is now becoming a matter of concern. Existing research indicates that the number of enantioselective validated methods is insufficient to provide a comprehensive understanding of the impact of chiral pharmaceuticals in the ecosystem. [86]

The development of robust, validated analytical methods for the determination of pharmaceutical enantiomers in environmental samples is therefore essential. Such methods are pivotal for accurate risk assessment, as they provide insights into the potential ecological risks of chiral pharmaceuticals and support evidence-based decision-making for their sustainable management. [86]

Environmental matrices have many challenges, for example, pharmaceuticals and their metabolites in the environment are usually in concentration as low as ng/L levels. [188] Therefore, the trace analysis of a low number of drugs requires further optimization of the steps involved. Another factor that important to be considered is samples complexities, that may introduce interferences which needs more steps to applied prior to be injected into instruments. [158]

2.2.9. Analysis of Antidepressants

2.2.9.1. GC-MS

Gas Chromatography (GC) is one of the most commonly used analytical techniques for the determination of drugs in various matrices. It has been widely applied due to several advantages, including short separation times, high efficiency, good sensitivity, and the absence of a solvent-based mobile phase. In contrast to HPLC, GC does not require pH optimization, which simplifies method development. [22]

Optimizing the chiral separation of pharmaceuticals has become an important aspect of GC analysis. Indirect chiral separation is often employed because the commercial availability of chiral stationary phases remains limited.

Cyclodextrin-based phases are commonly used for the enantiomeric separation of pesticides, while metal- β -diketonate polymers are applied for metal complexes. [113] However, GC presents several challenges for chiral separation: achieving high-resolution chromatograms can be difficult, derivatization of the analytes is often necessary, and the procedure can be time-consuming. In addition, compounds may undergo thermal decomposition due to the high operating temperatures of GC. [22]

Antidepressants, including FLX and its primary metabolite NFLX, are organic compounds with relatively low melting points (<187 °C; Table 2-3). These physicochemical properties render them unsuitable for direct chiral separation by GC, as they lack sufficient volatility. [22,113] To overcome this limitation, derivatization is required. For example, (S)-(-)-N-trifluoroacetylpropyl chloride has been used as a derivatizing agent to achieve enantiomeric separation of FLX and NFLX in biological matrices. In another study, β -cyclodextrin was employed as the derivatization agent with the same analytes. [113]

To the best of our knowledge, no studies published between 2020 and 2025 report GC-MS enantioselective separation of antidepressants in environmental matrices. Recent enantioselective analyses in wastewater rely on LC-based approaches (e.g., online SPE-chiral-LC-MS/MS and 2D-LC), reflecting the limited availability of chiral GC columns and the methodological challenges of applying GC to these compounds. [189–191] However, the limited availability of chiral GC columns, along with the challenge of separating multiple compound classes on a single column, remains a major obstacle. These limitations underscore the need for improved chiral separation techniques in environmental analysis to enable more reliable assessment of chiral pharmaceuticals. [192,193]

Table 2-10: Chiral separation of pharmaceuticals and drugs of abuse.

No	Drugs	Column	Mobile phase	Author & year	Ref
1	R/S(±)-salbutamol, R/S(±)-propranolol hydrochloride, R/S(±)-atenolol, R/S(±)-amphetamine, R/S(±)- MDMA, R/S(±)- chlorpheniramine maleate and R/S(±)-FLX	Screening Chirobiotic V and Chirobiotic V2 (250 × 2.1 mm; 5 µm)	Final mobile phase conditions were MeOH containing 1 mM AA and 0.01% acetic acid operated under isocratic conditions at a flow rate of 0.2 mL min ⁻¹	Petrie et al. 2018	[156]
2	atenolol, metoprolol and FLX	Astec Chirobiotic V (150, 4.6 mm, I.D., 5-µm particle size)	EtOH/MeOH (50:50, v/v) with 0.075 % of triethylamine and 0.225 % of acetic acid to adjust the pH to 6.7, at isocratic mode	Ribeiro et al. 2013	[194]
3	FLX	Astec CBV (25 cm × 2.1 mm, 5 µm particle size)	(pH 6.5, 0.06 mL min ⁻¹ , 25 °C) composed of 70% of EtOH, 30% of ultra-pure water (HQ water), 4 mM of AAC and 0.005% of FA under isocratic conditions	Andrés-Costa et al. 2017	[114]
4	FLX, NFLX	Nonchiral Spherisorb ODS2 (15 cm × 0.4 cm, 5µm)	A was ® and solution B was tetramethylammonium chloride (0.3%, pH 2.8). The gradient program was: 1– 9 min, 1:99 (A:B); 9–10 min, from 1:99 (A:B) to 5:95 (A:B); 10–22 min, 5:95 (A:B); 22–23 min, return to initial conditions. The flow rate was kept at 1ml min ⁻¹ .	Unceta et al. 2007	[195]
5	MDMA and MDA Amphetamine and methamphetamine Ephedrine and pseudoephedrine VEN, Atenolol	ACQUITY UPLC BEH C18, 150x1mm, 1.7µm column	A (pH 2.9): 79.7% H ₂ O, 20% MeOH, 0.3% CH ₃ COOH and mobile phase B (pH 3.30): 99.7% MeOH, 0.3% CH ₃ COOH at a flow rate of 0.04 mL min ⁻¹ and	S. Evans, J. Bagnall 2017	[196]
6	VEN, FLX And other	Chirobiotic V column, 250 × 4.6 mm, I.D. 5 µm (Advanced Separation Technologies, USA) with a guard column (20 × 4.0 mm, I.D. 5 µm)	Not available	Ma et al. 2016	[197]
7	VEN, FLX And other	Chirobiotic V column, 250 4.6 mm, I.D. 5 µm	Separation was undertaken using isocratic conditions, with a mobile phase of 90% H ₂ O, 10% 2-propanol and 1 mM AAC at a flow rate of 0.075 mL min ⁻¹ .	Bagnall et al. 2012	[198]
8	VEN, FLX And other	Chirobiotic V 250×2.1 mm, I.D. 5 µm column and Chiro- biotic V 20×1.0 mm, I.D. 5	4 mM NH ₄ Ac, 0.005 % FA and 99.995 % MeOH working in isocratic mode at a flow rate of 0.1 mL min ⁻¹ .	López-Serna et al. 2013	[199]
9	(±)-VEN, (±)-FLX, (±)-ODV, and other	chirobiotic V column (vancomycin, 250 × 2.1 mm; 5 µm)	MeOH containing 0.005% HCOOH and 4 mM NH ₄ Oac at 0.1 mL min ⁻¹ .	S. Evans, J. Bagnall 2017	[196]
10	FLX, NFLX, VEN, ODV	Chirobiotic V (250 × 2.1 mm; 5 µm, Sigma–Aldrich, UK) packed with an antibiotic Vancomycin	Not available	Evans et al. 2015	[200]

2.2.9.2. LC-MS

Chiral pharmaceuticals in environmental samples are often polar and non-volatile compounds. Thus, difficulties arise when attempting GC analysis [202]. Consequently, HPLC combined with different detectors has been applied in last decade, due to its suitability with a wide range of compounds. [157] Since 2006, LC-MS/MS has been employed to achieve chiral separations of pharmaceuticals in environmental matrices. [86] LC-UV is generally not used for trace analysis of environmental matrices due to sample complexity and low concentration level of targeted drugs. Alternatively, LC combined with mass spectrometry has become the preferable instrument to determine pharmaceuticals traces in the environmental matrices due to its sensitivity and selectivity, versatility, and its applicability for the sensitive quantification of pharmaceuticals at trace levels. [158]

Enantiomer separation of antidepressants can be achieved using direct and indirect methods. Derivatisation may be required when analysed by LC combined with fluorescence detection (FD). [113] For example, fluorescent agents, such as, NBD-COCl and DBD-COCl were selected to gain enantiomer separation by direct chiral HPLC-FD. [203] In addition, β -cyclodextrin, Ovomucoid, Chiralcel ODR, Cyclobond and Chiralcel OD are most common equipped stationary phase with direct HPLC method to obtain enantiomers separation of FLX and its main metabolite. [113]

On the other side, several derivatization agents were selected to develop enantiomer separation of antidepressants, e.g. S- naproxen, (S)-N-(4-nitrophenoxy carbonyl) phenylalanine methoxyethyl ester [(S)-NIFE], Marfey's reagent and 1-fluoro-2,4- dinitrophenyl-L- methionine amide (FDNP-L-Met-NH₂) when using indirect chiral HPLC method. [113]

All three stationary phases were applied with chiral pharmaceuticals in environmental samples, e.g. protein-based, polysaccharide-based and macrocyclic antibiotic-based CSPs. Previously, in respect to chiral pharmaceuticals biodegradation studies, the common chiral mode used is reversed phase elution mode. However, recently, polar ionic mode has

established and been applied in several applications. [86,107] It was reported that FLX and VEN were separated with a mixture of drugs in wastewater and urban water using a vancomycin-based CSP under polar ionic mode, Table 2-10. Moreover, a protein-based CSP in reversed phase mode also with river water and wastewater. [199] and [86] Furthermore, a mixture of therapeutic drug monitoring compounds, encompassing over 80 drugs, including antibiotics, steroids, and more, was successfully extracted from environmental matrices using solid-phase extraction (SPE) and analysed using LC-MS/MS. The method's validation was performed with a detection limit at the ng/L level. [202] This application utilises the highly selectivity of the instrument. However, the chiral separation of multi-drugs is limited. The Table 2-10 Summarised chiral separation of antidepressants by HPLC.

2.3. Benzodiazepines (BZs)

2.3.1. Introduction

Over the past 60 years, benzodiazepines, benzodiazepines - a group of psychoactive drugs - have become among the most frequently prescribed medications worldwide. Their popularity stems from their rapid onset of action, effectiveness in treating a broad spectrum of neurological and psychiatric conditions, and their generally favorable safety profile when administered appropriately. [204,205]

Benzodiazepines are widely prescribed for the management of anxiety-related disorders, including panic disorder, social anxiety disorder, generalized anxiety disorder, and specific phobias. They exert a calming effect by enhancing inhibitory neurotransmission mediated by γ -aminobutyric acid (GABA), thereby reducing excessive worry, anxiety, and panic symptoms. [29,206–208] Beyond anxiety, they are also valuable in the short-term treatment of insomnia, where they facilitate sleep onset and improve sleep quality.

In addition, benzodiazepines have well-established roles as anticonvulsants, particularly in the acute management of epilepsy and seizure disorders. For example, midazolam is used as a short-acting emergency intervention to stop prolonged or repeated seizures and reduce neurological complications. [207,208] Their muscle relaxant properties have also been exploited in clinical practice to alleviate muscle spasms and stiffness in conditions such as cerebral palsy, multiple sclerosis, and spinal cord injuries.

Despite their therapeutic benefits, there are ongoing concerns regarding the risks associated with benzodiazepine use, including tolerance, dependence, withdrawal, and potential misuse. These issues necessitate continued research into their pharmacology, clinical applications, and long-term safety. [207,209]

Benzodiazepines are generally classified according to their elimination half-life and duration of effect, which provides a framework for optimal prescribing and dosing. [207,209]

This classification highlights how pharmacokinetic differences guide the clinical application of benzodiazepines. Short-acting agents are valuable in acute or emergency situations, such as panic attacks, sleep-onset difficulties, or seizure control, due to their rapid onset and short duration of action. Intermediate-acting benzodiazepines provide moderate coverage, making them suitable for conditions like insomnia, generalized anxiety, or pre-surgical sedation. In contrast, long-acting benzodiazepines offer sustained therapeutic effects and are therefore preferred for chronic disorders, including anxiety, epilepsy, muscle spasms, and alcohol withdrawal. By linking half-life and duration of action to therapeutic use, this classification underscores the importance of pharmacokinetics in tailoring treatment to individual patient needs, Table 2-11. [210] Even though the classification offers a framework for comprehending their therapeutic use, it is essential for medical experts to individually adapt treatment strategies for each patient, taking into account aspects including age, medical history, and potential drug interactions. [204]

Table 2-11: Classification of benzodiazepines based on half-life, duration of action, representative examples, and common therapeutic applications.

Class	Half-life	Typical Duration	Examples	Therapeutic Uses	Ref.
Short-acting	< 12 hours	Rapid onset, short effect	Alprazolam, midazolam	Acute anxiety, panic attacks, sleep onset issues, emergency seizure control	[206]
Intermediate-acting	12–24 hours	Moderate duration	Lorazepam, temazepam	Insomnia, generalized anxiety, pre-surgical sedation	[204]
Long-acting	> 24 hours	Prolonged effect	Diazepam, clonazepam	Chronic anxiety, epilepsy, muscle spasm relief, alcohol withdrawal	[204]

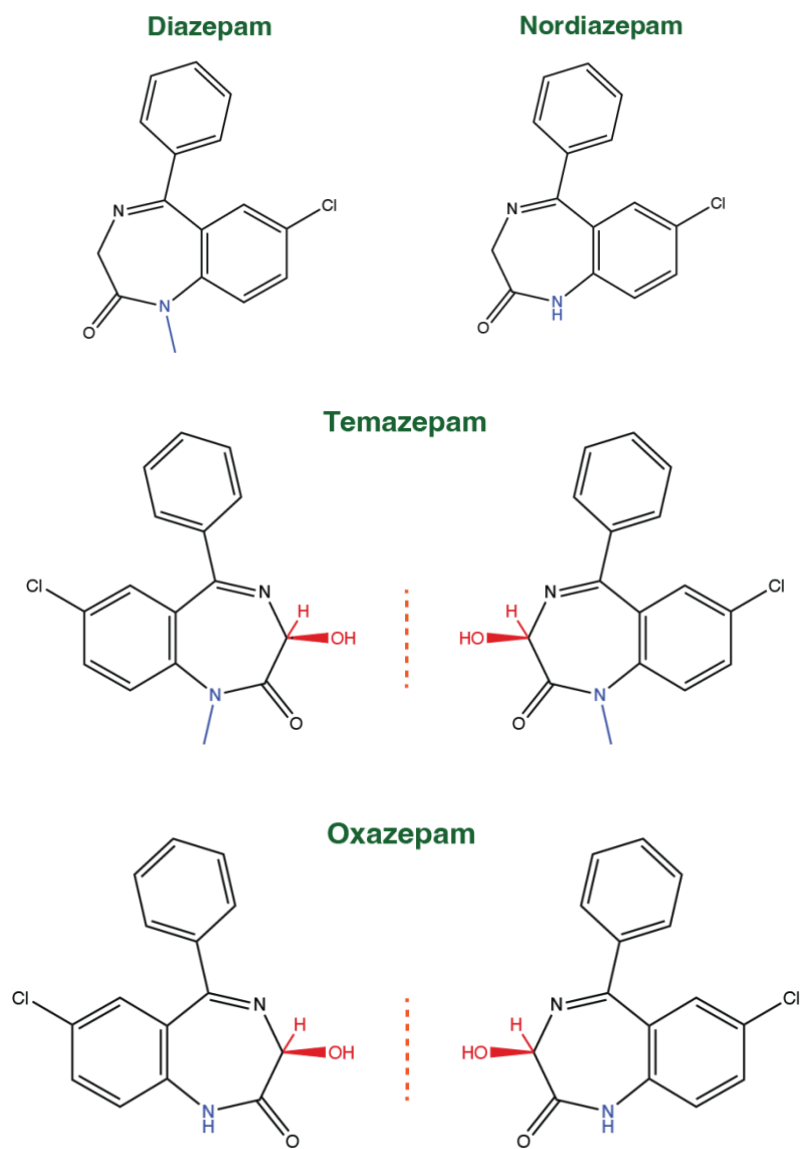


Figure 2-7: Diazepam and its main metabolites chemical structures.

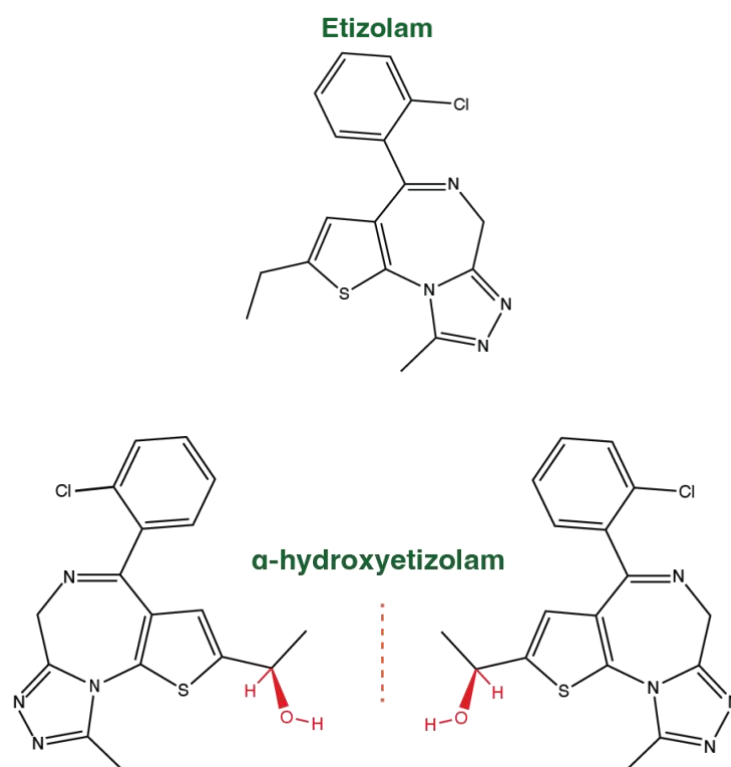


Figure 2-8: Etizolam and its main metabolite chemical structures.

2.3.2. Physico-chemical properties of benzodiazepines

Diazepam, 7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one, a classic benzodiazepine, exists as a white to yellow crystalline powder, practically insoluble in water, but highly soluble in organic solvents. These characteristics enable the formulation of various pharmaceutical preparations, including tablets, injections, and oral solutions, catering to different routes of administration and clinical requirements. [211]

Table 2-12: Physio-chemical proprieties information for benzodiazepines.

Drug	MW g/mol	MF	pKa	LogP	Melting Point °C	Boiling Point °C	Half-Life h
Diazepam	284.74	C ₁₆ H ₁₃ ClN ₂ O	3.4	3.0	131-134	295-299	20-50
Nordiazepam	270.71	C ₁₅ H ₁₁ ClN ₂ O	5.5	2.9	216-217	462.60	40-100
Oxazepam	286.71	C ₁₅ H ₁₁ ClN ₂ O ₂	7.9	2.2	205-206	420	5.7-10.9
Temazepam	300.74	C ₁₆ H ₁₃ ClN ₂ O ₂	7.7	2.8	119-121	300	3.5-18
Etizolam	342.8	C ₁₇ H ₁₅ ClN ₄ S	7-9	2.6	147-148	147-148	3.5-6
α-hydroxyetizolam	358.8	C ₁₇ H ₁₅ ClN ₄ OS	7-9	1.3	595	-	-

(-) = Unavailable data [83]

Etizolam is a thienodiazepine derivative with the chemical name 4-(2-chlorophenyl)-2-ethyl-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine. Its unique structure combines a thiophene ring fused to a diazepine ring. [212] In its natural state, etizolam is a crystalline, off-white powder. It is sparingly soluble in water (~0.03 mg/mL) but readily soluble in organic solvents such as ethanol and acetone, properties that influence its formulation into tablets and oral solutions. [213] These physicochemical characteristics are important for understanding its solubility, stability, and pharmacokinetic behaviour.

Several review papers published between 2018 and 2022 have examined different aspects of etizolam, reflecting the growing scientific and public health interest in this compound. These reviews include a short summary of etizolam and its metabolites [213], an overview of its pharmacology and patterns of non-medical use [214], a discussion of its emergence as a novel psychoactive benzodiazepine (NPS) [215], and an assessment of its toxicological and public health risks within the broader context of designer benzodiazepines. [204] Collectively, these studies emphasize both the therapeutic potential and the significant concerns surrounding the misuse and regulation of etizolam.

2.3.3. Benzodiazepines pharmacology

2.3.3.1. Benzodiazepines pharmacokinetics

Pharmacokinetics describes how drugs are absorbed, distributed, metabolized, and eliminated. For benzodiazepines such as diazepam and etizolam, understanding these processes is essential for optimizing dosing regimens and predicting clinical effects. [216,217]

When administered orally, diazepam is well absorbed, and peak plasma concentrations are generally reached within 1-2 hours. This relatively rapid absorption contributes to its usefulness in treating acute anxiety or seizure episodes, though interindividual differences may occur due to factors such as gastric emptying time and gastrointestinal motility. [218]

Diazepam exhibits a substantial volume of distribution (V_d), approximately 0.8 to 1.0 liters per kilogram (L/kg), indicating extensive distribution throughout body tissues. [216,219] Its high lipid solubility facilitates rapid penetration across the blood-brain barrier, leading to swift central nervous system effects. Additionally, diazepam tends to accumulate in adipose tissue, contributing to its prolonged half-life. [31]

Diazepam is extensively metabolised in the liver by the cytochrome P450 enzymes, particularly CYP2C19 and CYP3A4. These enzymes catalyse several biotransformation processes, resulting in the creation of active metabolites such as desmethyldiazepam, temazepam, and oxazepam. [216] Desmethyldiazepam, the principal metabolite, possesses high pharmacological action and contributes to the overall therapeutic effects of diazepam. [216]

Diazepam is extensively metabolized in the liver by CYP2C19 and CYP3A4 to active metabolites including nordiazepam, temazepam, and oxazepam. These metabolites are then conjugated and excreted renally, with approximately 70–80% of the administered dose eliminated in the urine, mostly as glucuronide conjugates, and less than 2% excreted unchanged in the urine. [218] The terminal elimination half-life of diazepam ranges from 20 to 50 hours, influenced by age, genetics, and concurrent medications that affect hepatic metabolism. [220]

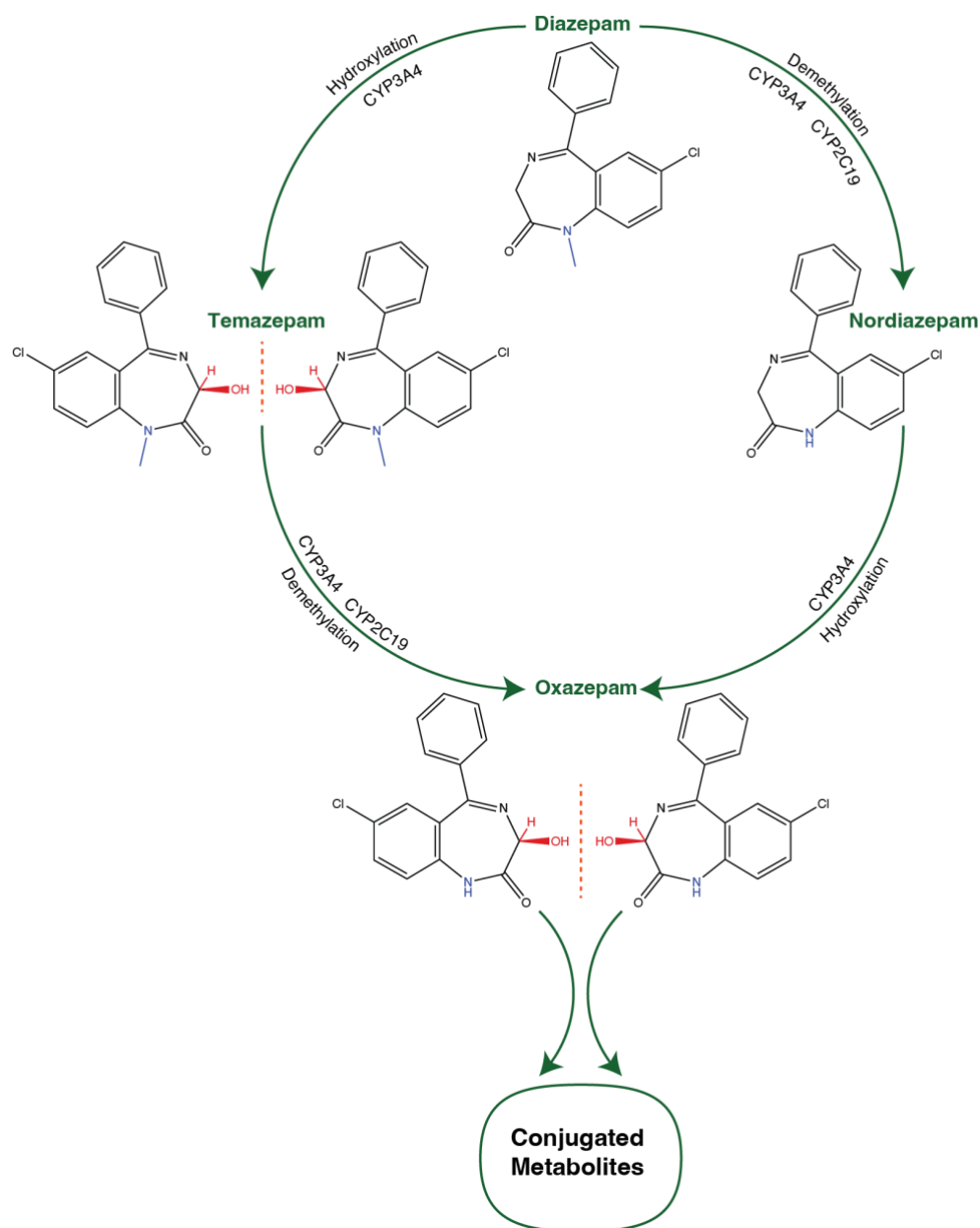


Figure 2-9: Diazepam metabolism pathway.

Etizolam is rapidly absorbed after oral administration, achieving peak plasma concentrations within 1 to 2 hours. Its high oral bioavailability of approximately 93% enhances its effectiveness as a short-acting anxiolytic and hypnotic agent. [221]

Similar to diazepam, etizolam has a wide volume of distribution, indicating extensive tissue distribution and a propensity to pass the blood-brain barrier, resulting in its effects on the central nervous system. [221]

Etizolam is metabolised by cytochrome P450 enzymes, primarily CYP3A4, in the liver. The main metabolite of etizolam is α -hydroxyetizolam, which is produced through hydroxylation. This metabolite is expected to significantly contribute to the pharmacological effects of etizolam, despite ongoing study, Figure 2-10. [222]

The elimination half-life of etizolam and its metabolites ranges between about 3.5 and 6 hours. Etizolam's relatively short half-life makes it suitable for short-term treatment, especially in acute anxiety and panic situations. [221]

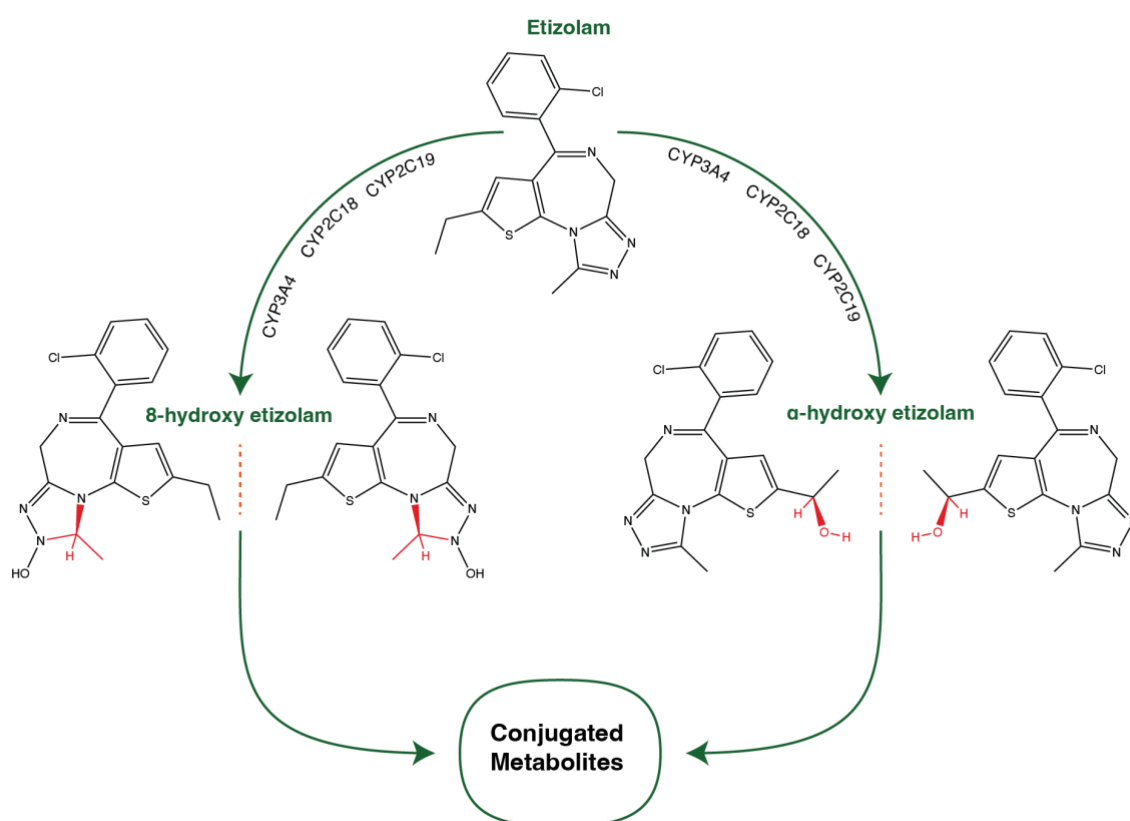


Figure 2-10: Etizolam metabolism pathway.

2.3.3.2. Benzodiazepines pharmacodynamics

Benzodiazepines produce their clinical effects primarily through modulation of GABA-A receptor function in the central nervous system, resulting in enhanced inhibitory neurotransmission and reduced neuronal excitability. These actions explain their major therapeutic uses, including anxiolysis, sedation, muscle relaxation, and anticonvulsant activity. [62]

Within this class, clinically important differences are driven largely by pharmacokinetics rather than pharmacodynamic mechanism. Diazepam is a long-acting agent with prolonged clinical activity due to the formation of active metabolites, supporting its use where sustained effects are required (e.g., chronic anxiety, seizure control, and muscle spasm management). [223] In contrast, etizolam (a thienodiazepine) acts via the same GABA-A receptor pathway but has a shorter half-life and typically faster onset, making it more suitable for short-term management of anxiety or insomnia; however, it may also be associated with a greater tendency toward tolerance with repeated use. [224]

Accordingly, despite shared receptor-level effects, diazepam and etizolam differ in potency, onset, duration, and adverse-effect risk, and selection should consider patient factors and the risks of tolerance, dependence, and withdrawal. [62]

2.3.3.3. Benzodiazepines interactions

Both diazepam and etizolam are metabolized primarily by cytochrome P450 enzymes, particularly CYP3A4 and CYP2C19. Consequently, co-administration of drugs that inhibit or induce these enzymes can significantly alter benzodiazepine plasma levels, affecting both therapeutic efficacy and risk of adverse effects. [216] Clinicians should therefore exercise caution when prescribing benzodiazepines to patients already receiving other CYP450 substrates, inhibitors, or inducers.

In addition to drug-drug interactions, both diazepam and etizolam carry a well-documented risk of tolerance and dependence when used beyond the recommended duration. Typically, tolerance can develop within a few weeks,

and dependence is more likely with continuous use extending into several months. [216,222,225] Abrupt discontinuation or rapid dose reduction may provoke withdrawal symptoms such as anxiety, insomnia, tremors, and, in severe cases, seizures. To minimize these risks, benzodiazepines are generally prescribed for short-term use (2-4 weeks), and gradual dose tapering is advised during discontinuation. [225,226]

Given these concerns, safe prescribing requires not only an understanding of their pharmacokinetics but also careful consideration of each patient's clinical profile, concurrent medications, and history of substance use. This individualized approach helps balance therapeutic benefits against the risks of dependence, interactions, and withdrawal. [193]

2.3.4. Determination of Benzodiazepines

The study conducted by Stamper et al. (2017) investigated the prevalence of benzodiazepines during football events in a city, analysing a total of 30 WWTP samples. The validated method enabled the determination of ten benzodiazepines in WWTP influent using LC-MS/MS, with chromatographic separation achieved on a Synergi Hydro-RP column on a Synergi Hydro-RP column (150 × 3.0 mm; 4 µm; 80 Å). Sample preparation employed Phenomenex Strata Screen-C SPE cartridges (55 µm, 70 Å) with a capacity of 150 mg/3 mL. This study is particularly relevant as it demonstrated the ability to detect multiple benzodiazepines in complex wastewater matrices, highlighting both the applicability and the challenges of multi-residue analysis in environmental monitoring. [227]

Similarly, Asimakopoulos et al. (2017) analysed 10 wastewater and freshwater samples for 89 pharmaceuticals and micropollutants. Chromatographic separation was performed using a Zorbax SB-Aq column (150 × 2.1 mm; 3.5 µm) and a Luna C18 column (150 × 4.6 mm; 3 µm), both serially connected to a Javelin guard column. Additionally, a Kinetex F5 column (50 × 2.1 mm; 1.7 µm) was coupled with a Security Guard ULTRA C18 guard column (2.1 mm, sub-2 µm core-shell). SPE was performed using

Oasis HLB cartridges, demonstrating a comprehensive strategy suitable for broad-spectrum drug detection in complex environmental samples. [228]

In another study, Gómez-Canela et al. (2021) performed an extensive characterisation of 76 pharmaceuticals and metabolites in wastewater, including FLX, DIAZ, and VEN. Separation was achieved using a CORTEX T3 column (100 × 2.1 mm; 1.6 µm particle size), with a binary mobile phase of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient started at 95% A and 5% B, increasing to 60% B in 7 min, then to 50% B in 12 min, and finally to 100% B in 3 min, before returning to initial conditions. Flow rates of 300, 350, and 400 µL/min were tested, with 300 µL/min providing optimal resolution and selectivity. Sample preparation again relied on Oasis HLB cartridges. This work is important because it provided an optimised LC-MS/MS protocol for antidepressants and benzodiazepines in environmental matrices, with improved selectivity and reproducibility. [229]

For the extraction of etizolam, optimisation of the CEREX® PSAX plate protocol was required, as α-hydroxyetizolam could not be detected using the original method. The issue was attributed to the strong second wash solvent (70:30 v/v methanol:water), which caused analyte loss. To resolve this, the solvent composition was modified to 50:50 (v/v) methanol:water, considerably improving the recovery of α-hydroxyetizolam without compromising selectivity for other analytes. [230]

Additionally, the reconstitution solvent was adjusted to 60:40 (v/v) methanol:water to align with the LC conditions of the original synthetic cannabinoid method. While this modification improved the recovery of etizolam and its metabolites, it led to retention issues for certain synthetic cathinones. Nevertheless, this optimisation was a critical step toward developing a reliable LC-MS/MS method for designer drugs, including etizolam and its major metabolite, ensuring accurate and reproducible results. [213,231]

2.4. Conclusion

This literature review has provided a comprehensive overview of antidepressants and benzodiazepines, with emphasis on their pharmacology, physicochemical and chiral properties, environmental occurrence, and the analytical techniques used for their detection. These drugs are critical in the treatment of mental health disorders, but their widespread and long-term use has led to frequent detection in wastewater and surface waters, raising concerns about ecological risks and forensic implications.

The review of physicochemical and chiral characteristics has shown that enantiomers may differ significantly in toxicity, persistence, and degradability, underscoring the importance of enantioselective approaches in environmental analysis. Environmental monitoring studies further confirm the occurrence of these pharmaceuticals in aquatic systems, sometimes at concentrations capable of affecting aquatic organisms and contributing to bioaccumulation in the food chain. These findings highlight the urgent need for analytical methods that are both sensitive and selective, capable of distinguishing enantiomers and providing accurate assessments of environmental fate.

Despite this progress, important knowledge gaps remain. Research has given limited attention to the enantiomeric behaviour of these compounds in environmental contexts. Their degradation pathways under realistic biotic and abiotic conditions remain poorly understood, and the role of microbial communities in facilitating or inhibiting these transformations is only superficially characterised. Furthermore, the combined influence of environmental parameters such as pH, temperature, oxygen availability, and nutrient levels on degradation kinetics and stability has not been systematically evaluated.

This thesis seeks to address these gaps by developing and validating an enantioselective LC-MS method for the detection and quantification of

selected antidepressants and benzodiazepines, evaluating their biodegradation in synthetic wastewater under controlled conditions, identifying microbial taxa involved in their transformation through 16S rRNA sequencing, and assessing how environmental parameters influence degradation kinetics and compound stability. By integrating enantioselective analytical chemistry with microbial ecology, the work aims to generate essential data on the persistence, transformation, and microbial interactions of chiral pharmaceuticals in wastewater systems. In doing so, it contributes to environmental risk assessment and advances forensic toxicology by improving understanding of the environmental behaviour of psychoactive drugs and their implications for both public health and casework interpretation.

Chapter 3. Method development and validation for antidepressants and benzodiazepines

3.1. Introduction

The detection and analysis of pharmaceutical residues, particularly antidepressants and benzodiazepines, in environmental matrices such as wastewater are critical for assessing ecological and human health risks. Human waste, discharge and industrial waste releases lead to bioaccumulation of toxic chemicals and harm aquatic life and humans. These chemicals can enter into the aquatic systems through excretion by humans and animals, illegal dumping, and industrial activities. To cope with these challenges, strong analytical methods for detecting and quantifying these compounds in complex environmental samples needs to be developed and validated. [232,233]

Method development in this context involves selecting appropriate analytical techniques, optimising chromatographic conditions, and refining sample preparation procedures to accommodate the unique chemical properties of target analytes. Due to their sensitivity and specificity, techniques such as GC-MS and LC-MS are commonly employed to screen pharmaceutical pollutants. GC-MS is typically used for volatile and semi-volatile compounds while LC-MS is used for non-volatile labile or polar compounds. The techniques allow detection of contaminants and probable environmental assessment. [227,230,234–236]

Validation of these analytical methods is a structured process that establishes their reliability and efficacy. Guidelines provided by organizations such as the Scientific Working Group for Forensic Toxicology (SWGTOX) and the American Academy of Forensic Sciences (AAFS) outline critical performance characteristics, including accuracy, precision, limits of detection (LOD) and quantification (LOQ), linearity, range, and specificity. [237–239] Adhering to these standards ensures that the methods produce consistent and reproducible results across diverse environmental matrices, thereby meeting regulatory requirements and supporting informed decision-making. [240–242]

Quality assurance and control are integral components of method development and validation. Regular calibration of instruments using certified reference materials, inclusion of control samples, and adherence to standardized operating procedures are essential practices. [243] These measures maintain the accuracy and reliability of analytical results, thereby supporting effective environmental monitoring and forensic investigations. By implementing these protocols, laboratories can effectively monitor and assess the presence of pharmaceutical residues in environmental samples, contributing to informed decision-making and the development of strategies to mitigate potential health risks. [237,243,244]

Stability studies are conducted to shed light on the degradation patterns of drugs under different environmental conditions, unveiling potential degradation products and their environmental significance. Solid-phase extraction methods are developed and validated for efficient pharmaceutical extraction, while the most suitable SPE cartridges are selected to ensure maximum recovery and sensitivity during analysis. [245]

Furthermore, this chapter delves into the crucial role of bioreactors in pharmaceutical wastewater treatment. Through meticulous preparation and setup, bioreactors harness microbial activity to effectively degrade pharmaceutical residues, presenting a sustainable and eco-friendly approach to wastewater treatment. Environmental testing, encompassing parameters such as pH and optical density of bacteria, is conducted to assess the performance of bioreactor systems. [109] To understand the microbial communities involved in pharmaceutical degradation, DNA extraction and taxonomy analysis are performed, shedding light on the key players responsible for pollutant removal within the bioreactors. [246]

This chapter provides a comprehensive overview of methodologies for pharmaceutical analysis in synthetic wastewater and bioreactors, integrating analytical chemistry, environmental toxicology. It frames the development and validation of an enantioselective LC-MS workflow for antidepressants and benzodiazepines, details green, matrix-tolerant extraction/clean-up strategies suited to complex environmental samples, and outlines controlled

biotic and abiotic degradation assays in synthetic wastewater. In parallel, it describes the microbial community analyses (16S rRNA sequencing) used to associate stereoselective transformation with specific taxa and the parameter studies (pH, temperature, redox/oxygen, nutrients) used to quantify effects on degradation kinetics and compound stability. Together, these methods operationalize the chapter's objective: to generate high-quality, enantiomer-resolved fate data that support environmental risk assessment and strengthen forensic interpretation of psychoactive pharmaceuticals in aquatic systems.

3.2. Aim and objectives

3.2.1. Aim

This chapter aims to present an integrated methodology for the comprehensive analysis of pharmaceutical compounds in environmental matrices, covering various aspects such as method development, chiral separation, stability studies, and bioreactor setup. The focus includes the validation of LC-DAD and LC-MS methods for accurate and sensitive analysis.

3.2.2. Objectives

- Develop and optimise methods for preparing and analysing antidepressants and benzodiazepines in synthetic wastewater, including chiral separation for enantiomeric analysis.
- Investigate drug stability and degradation under defined conditions, including storage stability (freezer, refrigerated, room temperature, and so on).
- Validate SPE methods for pharmaceutical extraction and recovery from environmental matrices, following SWGTOX and ICH guidelines.
- Establish controlled biotic and abiotic reactors to study the biodegradation of target antidepressants and benzodiazepines. The reactors will be operated under aerobic and anaerobic conditions, with systematic

monitoring of pH, optical density, and biomass concentration (TSS used as a proxy for microbial load).

- Develop and validate LC-DAD and LC-MS methods for accurate quantification of chiral and achiral pharmaceuticals. Validation will be conducted according to international guidelines, with focus on selectivity, sensitivity, reproducibility, and applicability to environmental samples.

3.3. Materials and methods

3.3.1. Materials

3.3.1.1. General laboratory equipment

A range of standard laboratory instruments was employed throughout the experimental work to ensure accuracy, reproducibility, and proper sample handling. Table 3-1 provides an overview of the general laboratory equipment used.

Table 3-1: General laboratory equipment used in the project.

	Equipment	Specification
1	pH Meter	pH/conductivity meter MPC 227 Mettler Toledo/ ORION STAR A215 (Thermo Scientific)
2	Spectrophotometer	spectrophotometer Thermo (Helios ZETA UV-VIS) and (Hach Lange DR 6000)
3	Oxygen dissolved	Thermo Scientific Orion Orion Star A113 Benchtop Dissolved Oxygen Meter
4	Balance	Mettler Toledo ME
5	Autoclave	Thermo
6	Shaker	Stuart (orbital shaker SSL1)
7	Oven	Thermo
8	SPE kit	Fisher
9	Incubator	Fisher
10	Freezer	Thermo Scientific
11	Fridge	Thermo Scientific
12	Stirring incubator	SI500, from Kisker Biotech
13	Ultrasonic bath	VWB2
14	Homogenizer	FastPrep Homogenizer FastPrep-24™ Classic
15	Centrifuge	Megafuge 16 Centrifuge
16	Vortex mixer	Biologix Vortex Mixer
17	Nitrogen evaporator and sample concentrator	Techne (Dri-Block DB-3)
18	Microbiological safety cabinet	Class II Microbiological safety Cabinet for Cytotoxics Manipulation.
19	Thermometer	ThermoPRO
20	Filtration system	2000mL Vacuum Filtration Apparatus (Borosilicate Glass), Suction Filter Device.
21	Water system	Triplered (Nanopure)
22	Cotton wool Roll	Fisher Scientific, non abs
23	Disposable culture tubes	Fisher brand 16X 125mm
24	Oasis MAX	6cc 150mg Extraction cartages
25	Oasis HLB	3cc 60mg Extraction cartages
26	Oasis MCX	Extraction cartages
27	FastDNA™ SPIN Kit for Soil	FastPrep-24™ Classic from MP Biomedicals
28	Glass microfiber filters	GF/C Diameter 47 mm
29	Filters	0.45µm PTFE Syringe Filters
30	Mobile phase filtration system	Nylon membrane 0.45µm, 45mm

3.3.1.2. Analytical columns

Various analytical columns were employed throughout this project to meet the distinct objectives of each study. A reversed-phase C18 column (Ascentis Express, 90 Å) was used in the stability studies of antidepressants and benzodiazepines. This column was chosen for its reliable retention of moderately polar compounds under conventional reversed-phase conditions, making it highly suitable for assessing stability, recovery, and precision.

For enantioselective biodegradation studies, two chiral columns (Astec Chirobiotic V and V2) were employed. These columns were specifically selected for their ability to resolve enantiomers of fluoxetine, norfluoxetine, venlafaxine, O-desmethylvenlafaxine, and benzodiazepines (including etizolam metabolites). Their use was essential in exploring stereoselective transformation and degradation behaviour, which are discussed in detail in Chapter 4 and Chapter 5.

In addition, a capillary column for GC was utilised during drug screen monitor. Detailed specifications of all columns employed are summarised in Table 3-2.

Table 3-2: Analytical columns list.

Name	Type	Column's features	Cat	Company
Astec Chirobiotic V2	Chiral column	250mm, 2.1mm, I.D., 5µm particle size	15020AST	Supelco, UK
Astec Chirobiotic V	Chiral column	150mm, 2.1mm, I.D., 5µm particle size	1109AST	Supelco, UK
Ascentis Express 90 Å	C18	150mm, 2.1mm, I.D., 5µm particle size	50518-U	Supelco, UK
ZB-Semi Volatiles	GC-MS capillary column	(30 m x 0.25 mm I.D., 0.25 µm film thickness)	7HG-G027-11	Phenomenex

3.3.2. Chemicals and reagents

Rac-fluoxetine hydrochloride (FLX), rac-norfluoxetine hydrochloride (NFLX), rac-venlafaxine hydrochloride (VEN), rec-O-desmethylvenlafaxine hydrochloride (ODV), R-fluoxetine and S-fluoxetine and α-hydroxyetizolam were obtained from Sigma-Aldrich, while S-ODV and R-ODV were obtained from Syncom in the Netherlands and venlafaxine enantiomers and norfluoxetine enantiomers were purchased from Santa Cruz Biotechnology,

USA. Methanol, acetonitrile (HPLC grade), ammonium hydroxide 25% (in water), ethyl acetate and acetone were obtained from Fisher, Germany. Acetic acid and formic acid, as well as the org 24598 lithium salt, which was used as an internal standard, were purchased from Sigma-Aldrich. Ammonium acetate, toluene, and 0.45 µm nitrocellulose were obtained from VWR.

Diazepam, nordiazepam, temazepam, oxazepam, prazepam, ethanol, 1-isopropanol (HPLC grade), ammonium hydroxide 25% (in water), acetone was obtained from Fisher, Germany. Acetic acid, formic acid, Sodium bicarbonate, etizolam and deuterated internal standards (fluoxetine-d6, norfluoxetine-d6 oxalate, venlafaxine-d6, (±)-o-desmethylvenlafaxine-d6, diazepam-d5, nordiazepam-d5, oxazepam-d5, temazepam-d5, etizolam-d3) were also purchased from Sigma-Aldrich. Ammonium acetate and 0.45µm Nitrocellulose were obtained from VWR. In addition, etizolam and norfluoxetine were purchased from Chiron, Norway, standards and internal standards (ISD) details listed in Appendix 2.

3.3.3. Standards and solution preparation

3.3.3.1. Preparation of stock standards

Individual stock standard solutions were accurately prepared for each standard by diluting or dissolving the listed ADs and BZs, shown in Table 3-3 in methanol, aiming to achieve a concentration of 1 mg/mL. The preparation process involved adding 5 mg of each standard to separate 5 mL volumetric flasks and then using methanol to fill the flasks up to the mark. To ensure thorough mixing, the flasks for each drug were shaken several times. Subsequently, each stock solution was carefully transferred into dedicated amber glass bottles to protect the samples from light-induced degradation. To maintain the stability of the stock solutions and preserve the integrity of the standards, all bottles were labelled then stored at a temperature of -80°C until further analysis.

Table 3-3: List of the ADs and their enantiomers and BZs in the project.

ADs	Enantiomers	BZs
Fluoxetine	S-FLX & R-FLX	Etizolam
Norfluoxetine	S-NFLX & R-NFLX	α -hydroxyetizolam
Venlafaxine	S-VEN & R-VEN	Diazepam
ODV	S- ODV & R-ODV	Nordiazepam
		Oxazepam
		Temazepam

3.3.3.2. Preparation of standard solutions

Individual solutions of each drug were prepared at final concentration of 10 $\mu\text{g/mL}$ in methanol. This was achieved by transferring 1 mL from each stock solution of ADs, BZs at a concentration of 1 mg/mL to a 100 mL volumetric flask. The solutions were then diluted to the mark with methanol, following the dilution equation outlined in Equation 3-1.

All prepared solutions were properly labelled and stored at -20°C until further analysis to ensure stability and prevent degradation.

$$C_1 V_1 = C_2 V_2$$

Where: V_1 = volume of working solution

C_1 = concentration of working solution

V_2 = final volume of new point of standard

C_2 = final concentration of the new point of standard

Equation 3-1: Dilution calculation equation

3.3.3.3. Preparation of internal standard solutions

A working solution with a concentration of 10 $\mu\text{g/mL}$ was prepared for both the deuterium-labelled and non-deuterium internal standards, as detailed in section 3.3.3.2. The concentration of the internal standard was selected to be equivalent of the medium QC sample, (Table 3-4).

Table 3-4: Target analytes and corresponding ISDs.

ADs ISD		BZs ISD	
Fluoxetine	Fluoxetine-D6	Etizolam	Etizolam-D3
Norfluoxetine	Norfluoxetine-D6	Diazepam	Diazepam -D5
Venlafaxine	Venlafaxine-D6	Nordiazepam	Nordiazepam -D5
ODV	ODV-D6	Oxazepam	Oxazepam-D5
FLX, NFLX, VEN and ODV (For LC-DAD)	Org 24598 lithium salt	Temazepam	Temazepam-D5
		5 above Benzos (For LC-DAD)	Prazepam

3.3.3.4. Preparation of calibrations standards

Calibration standards were prepared by diluting a stock solution of the analyte, initially at 10 µg/mL, to generate a series of seven calibration points at concentrations of 1.2 µg/mL, 1 µg/mL, 0.8 µg/mL, 0.6 µg/mL, 0.4 µg/mL, 0.2 µg/mL, and 0.1 µg/mL. Additionally, an internal standard working solution, with a concentration of 0.3 µg/mL, was added to each calibration point using methanol. These calibration standards are essential for accurate quantification and calibration of the analytical instrument.

3.3.3.5. Preparation of quality control standards

QCs played a crucial role in the analysis, serving as reference points for bias and accuracy calculations. Alongside calibration standards and samples, QCs were prepared and analysed to ensure the reliability of the results. These QCs were further subjected to testing during biodegradation batches to verify the accuracy of the obtained data. While the working solutions used for QCs differed from the calibration standards, their preparation followed the same procedure. For the QCs, two levels were established from the standard solution of 10 µg/mL, specifically at concentrations of 0.3 µg/mL and 0.9 µg/mL.

3.3.4. Environmental Study

3.3.4.1. Preparation of Synthetic Wastewater

Synthetic wastewater was prepared in the laboratory to simulate the composition and complexity of authentic wastewater for experimental purposes. A mixture of chemical compounds, as illustrated in Figure 3-1 was dissolved in 1 litre of deionized water to prepare the solution. The selection of these compounds was based on previous studies and their relevance to the project research objectives. [247] 1 mL of the previously prepared trace metal solution was also added into the bulk media to ensure the presence of essential trace elements. After thorough mixing, the synthetic wastewater was subjected to autoclaving, which not only sterilized the media but also promoted stability by eliminating any potential contaminants. Autoclaving is a crucial step to maintain consistency and reproducibility in subsequent experiments. [248]

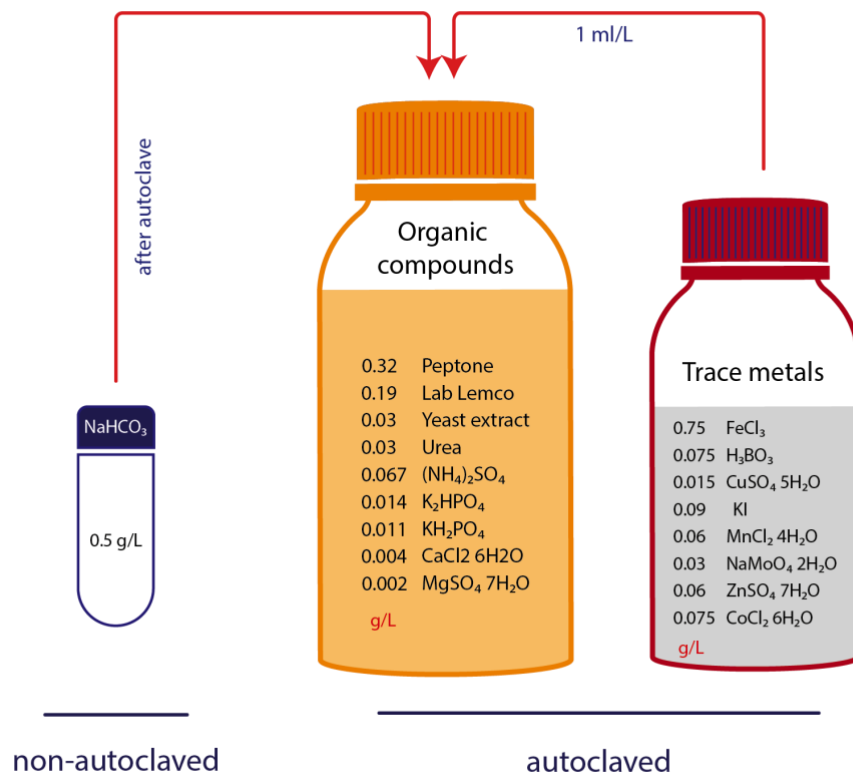


Figure 3-1: Chemical compounds to be used to create synthetic wastewater.

Following the autoclaving process and cooling down, 0.5 g of NaHCO₃ was added to the synthetic wastewater. It is a significant buffering agent found in synthetic wastewater, and its inclusion helps mimic the buffering capacity observed in real-world scenarios. The pH of the bulk media was measured within the range of 7-8. This pH range is commonly found in various types of natural wastewater and maintaining it in the synthetic wastewater ensures a more accurate representation of real environmental conditions.

3.3.4.2. Stock bioreactors

Bioreactors play a pivotal role in treating pharmaceutical-laden wastewater by leveraging biological processes to degrade complex pharmaceutical compounds into simpler, less harmful substances. [249,250]

In this study, specialised bioreactors were meticulously designed and assembled to cultivate bacterial cultures under controlled environmental

conditions, ensuring reproducibility and optimal microbial activity throughout the experimental period. [251–254]

The bioreactors for cultivating stock bacteria were equipped with essential components, including filters, 2 L Duran amber bottles, silicone tubing, Hidom 4.0 W aquarium adjustable air pumps with twin valves (model HD-603), and air stones (Figure 3-2). The air pumps ensured consistent circulation and maintained a steady flow rate within the system. Inside each covered bottle, two silicone tubes were installed: one connected to an air stone to introduce air bubbles, thereby enhancing oxygen availability for the bacterial culture, and the other serving as a sampling port for collecting samples to monitor environmental parameters. Regular monitoring of parameters such as pH, dissolved oxygen, TSS, and optical density was conducted to assess the system's performance. [255,256]

To maintain sterile conditions and minimise contamination, all tubing, glassware, and bottles were washed with laboratory detergent, rinsed thoroughly with deionised water, and sterilised prior to assembly. Heat-stable items were autoclaved, while heat-sensitive components were disinfected using 70% ethanol and assembled aseptically.

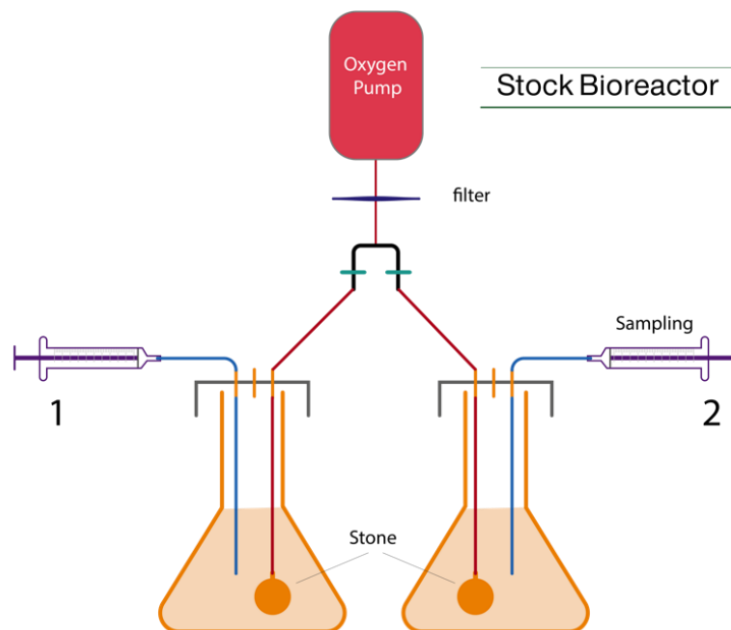


Figure 3-2: The bioreactors setting for stock bacteria.

Following sterilization, synthetic wastewater was introduced into the Duran bottles. This synthetic medium was formulated to closely replicate authentic wastewater conditions, providing an appropriate environment for bacterial cultivation.

To sustain bacterial growth and optimise the degradation study, the synthetic wastewater in each bioreactor was replaced once per week (weekly medium renewal), supplying fresh nutrients and maintaining stable operating conditions.

3.3.4.3. Samples preparation and collection

Influent wastewater samples were collected from the Sewage Works in Govan, a well-established wastewater treatment plant in Glasgow operated by Scottish Water, Scotland, UK. Samples were drawn from the activated sludge system reservoir, representing the initial stage of wastewater treatment.

Upon collection, samples were transported to the laboratory under controlled conditions and stored at 4 °C to prevent degradation or alteration of pharmaceutical compounds. To remove larger particulate matter, samples were filtered using Whatman™ glass microfiber filters, which are effective for high-solids-content samples. Subsequently, to eliminate microbial interference, the filtered samples were passed through 0.45 µm PTFE syringe filters, removing remaining microbes. The prepared samples were then stored at 4 °C until extraction stage.

To ensure accurate pharmaceutical analysis, a drug screen monitor was applied to identify and quantify pharmaceutical compounds present in influent wastewater samples. This preliminary screening step was essential for distinguishing target pharmaceuticals from naturally occurring compounds and other potential interferences, thereby improving the reliability and selectivity of the analysis. Wastewater samples collected from a treatment plant (WWTP) were processed using SPE with Oasis HLB cartridges. A 200 mL aliquot of wastewater was passed through the cartridge at a flow rate of 10 mL/min, enabling adsorption of analytes onto the sorbent material. The

cartridge was then dried under a continuous stream of nitrogen for at least 30 minutes to remove residual water and solvents. Elution was performed in two steps: first with 2 mL of ethyl acetate, followed by 8 mL of acetone. The combined eluents were evaporated under a gentle nitrogen stream at 25 °C, and the residue was reconstituted to 0.1 mL. For derivatization, 0.1 mL of MSTFA (N-methyl-N-(trimethylsilyl)trifluoroacetamide) was added, and the mixture was heated at 80 °C for 60 minutes to produce volatile derivatives suitable for GC-MS analysis. [257–260]

GC-MS analysis was conducted under optimized conditions. Samples were introduced in splitless mode with a split ratio of 50, and the injector was maintained at 280 °C. Separation was achieved using a ZB-Semi Volatiles capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) with helium as the carrier gas at a constant flow of 1.3 mL/min. The oven program began at 90 °C (1 min hold), increased to 130 °C at 25 °C/min, then ramped to 280 °C at 4 °C/min, with a final hold of 5 minutes, giving a total run time of 45.1 minutes and a solvent delay of 4.5 minutes. Detection was performed with electron impact ionization (70 eV), using an ion trap temperature of 220 °C, interface temperature of 280 °C, and a mass scan range of 40-500 m/z to identify and quantify drug residues in wastewater samples. [257]

3.3.4.4. Cultivating bacteria in synthetic wastewater

The reactors were inoculated with varying proportions of mixed liquor sourced from the activated sludge system of a municipal wastewater treatment plant operated by Scottish Water. The mixed liquor, a rich source of microbial communities necessary for biodegradation, was combined with sterilized synthetic wastewater (SWW) media in controlled proportions to evaluate the effect of inoculum concentrations on reactor performance. Specifically, the combinations included 1% mixed liquor with 99% SWW, 2.5% mixed liquor with 97.5% SWW, 5% mixed liquor with 95% SWW, and 10% mixed liquor with 90% SWW. These ratios were designed to study microbial activity and system efficiency under varying conditions.

The inoculated samples were incubated at room temperature for one week, ensuring the stability of microbial activity under ambient conditions.

Continuous agitation at 120 rpm was maintained to promote proper mixing and aeration, both of which are critical for maximising microbial contact with the substrate and ensuring uniform distribution of nutrients and oxygen throughout the reactors. [261] Figure 3-3 illustrated microbial community at phylum level for cultivated samples.

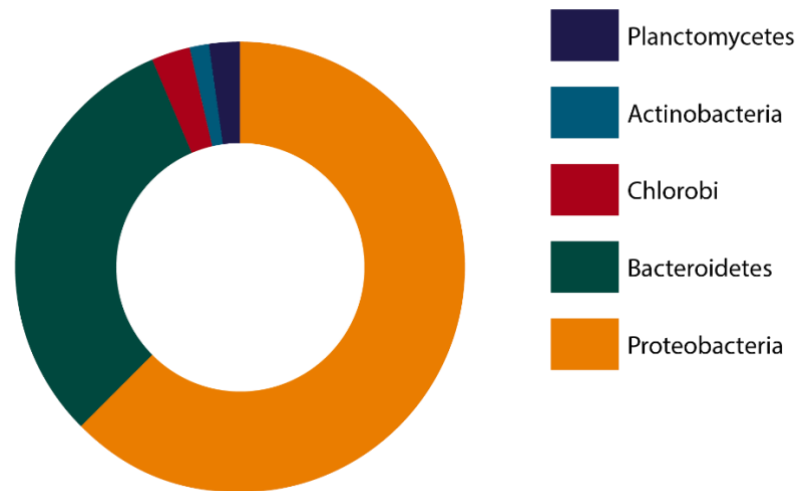


Figure 3-3: Relative abundance of the total microbial community at phylum level for cultivated samples.

3.3.4.5. Initial environmental parameters studies

Initiating a pilot study for bioreactors necessitates the careful selection and control of environmental parameters to ensure optimal microbial activity and process efficiency. Key parameters include temperature, pH, dissolved oxygen levels, and mixing intensity. Key parameters to monitor include:

A. Total suspended solids

Determining Total Suspended Solids (TSS) is a fundamental procedure for assessing biomass in activated sludge samples. [262] The process begins by heating glass weighing dishes at 104 ± 1 °C for at least three hours to ensure complete removal of residual moisture. This extended heating time is necessary to guarantee constant weight and avoid underestimation of mass caused by incomplete drying. After cooling, the filters were pre-weighed using an analytical balance to establish their initial mass.

Well-mixed wastewater samples (2 mL) were then filtered in triplicate through these pre-weighed filters. The filters, retaining the suspended solids, were

dried overnight at 104 ± 1 °C to eliminate residual moisture. Once dried and cooled, they were re-weighed to determine the final mass. TSS was calculated as the difference between the initial and final filter weights, normalized to the sample volume, and expressed as mg/L. [263] This method quantifies the concentration of suspended particles, including microbial cells and particulate matter. In pharmaceutical-contaminated wastewater, the presence of drugs may indirectly influence TSS measurements by altering microbial growth and biomass production or through adsorption onto particulates. Pharmaceuticals can alter microbial activity and growth, potentially increasing or decreasing biomass, and may also adsorb onto particulate matter, changing the apparent solid fraction. Therefore, drug presence should be considered when interpreting TSS results in biodegradation studies. [264–266] TSS concentration was calculated according to the following equation:

$$TSS (mg/L) = \left[\frac{W2 - W1}{V} \right] \times 1000$$

Where:

W1 = Initial weight of the empty filter (mg)

W2 = Final weight of the filter with dried suspended solids (mg)

V = Volume of the water sample filtered (in liters)

Equation 3-2: The TSS equation.

B. pH

Throughout the study, 3 mL samples were collected daily from all reactors to measure pH using the Mettler Toledo MPC227 pH/conductivity meter. To maintain aseptic conditions and prevent contamination, all sample collections were conducted within a microbiological safety cabinet, ensuring sample integrity and accurate pH measurements. Regular pH monitoring allows for timely adjustments to sustain favourable conditions for microbial growth and efficient biodegradation. [267,268]

Throughout the study, 3 mL samples were collected once daily at a fixed time from all reactors to measure pH using the Mettler Toledo MPC227

pH/conductivity meter. To maintain aseptic conditions and prevent contamination, all sample collections were performed within a microbiological safety cabinet, ensuring sample integrity and accurate pH measurements.

Regular pH monitoring was critical to maintain favourable conditions for microbial growth and efficient biodegradation. In this study, the target pH range was 7-8, as this range is generally optimal for the activity of diverse microbial consortia involved in pharmaceutical degradation. [267,268] Adjustments were made as necessary to sustain this range throughout the experimental period.

C. Optical density

To monitor bacterial growth, 3 mL samples were collected daily and transferred to a 10 mm cuvette for spectrophotometric analysis. The Thermo Helios ZETA UV-VIS spectrophotometer was set to measure absorbance at 600 nm (OD_{600}), a standard wavelength for assessing bacterial growth due to its minimal interference from media components and its ability to accurately reflect cell density. [269,270] This methodology aligns with established protocols, ensuring consistency and comparability in experimental results. [263,264] [270] OD_{600} measurements provide valuable insights into microbial biomass concentration by assessing the turbidity of the culture. Regular monitoring of OD_{600} helps evaluate microbial growth dynamics and overall health, which are critical indicators of the system's capacity to degrade pharmaceuticals. [269,271]

D. Temperature

Temperature plays an essential role in microbial metabolism and reaction kinetics. Maintaining an optimal temperature range is essential, as deviations can impact microbial growth rates and enzymatic activities. [272] The temperature was carefully controlled and maintained at room temperature and monitored daily using a thermometer (ThermoPro) to ensure consistent experimental conditions. [248,273]

E. Dissolved Oxygen

Dissolved oxygen (DO) levels were measured regularly for the samples collected from the stock reactors to ensure optimal conditions for bacterial growth and activity. DO measurements were conducted using calibrated probes to provide accurate and reliable data throughout the study.

3.3.5. Biodegradation study

In this study, biodegradation experiments were conducted using three distinct reactor setups: (1) Control Reactors (2) Abiotic Reactors and (3) Biotic Reactors. Figure 3-4 illustrates the biodegradation experiment setup and Table 3-5 shows the experiment study stages.

Key operational parameters, including pH, TSS, dissolved oxygen levels, and optical density, were meticulously monitored and optimised throughout the experiments. These parameters ensured stable reactor conditions, supporting accurate assessments of both abiotic and biotic degradation mechanisms. This comprehensive experimental design enabled a detailed understanding of the interplay between abiotic and biotic factors in pharmaceutical degradation. The findings contribute valuable insights for enhancing the efficiency of wastewater treatment systems.

The Figure 3-5 illustrates the sampling schedule for a biodegradation study conducted over a 14-day period, with sampling time points on Day 0, Day 1, Day 2, Day 3, Day 7, Day 10, and Day 14. At each time point, samples were collected in triplicate to ensure data reliability. This systematic approach ensured consistent and accurate data collection across both biotic and abiotic conditions. The repeated sampling process at regular intervals facilitated the analysis of the biodegradation process over time, enabling a detailed understanding of changes in the target substances.



Figure 3-4: Biodegradation experiment set.

Table 3-5: Experiment stages.

Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
FLX	NFLX	VEN	ODV	DIAZ	ETIZ
S-FLX	S-NFLX	S-VEN	S-ODV	TEM	α -HETIZ
R-FLX	R-NFLX	R-VEN	R-ODV	OXAZ	

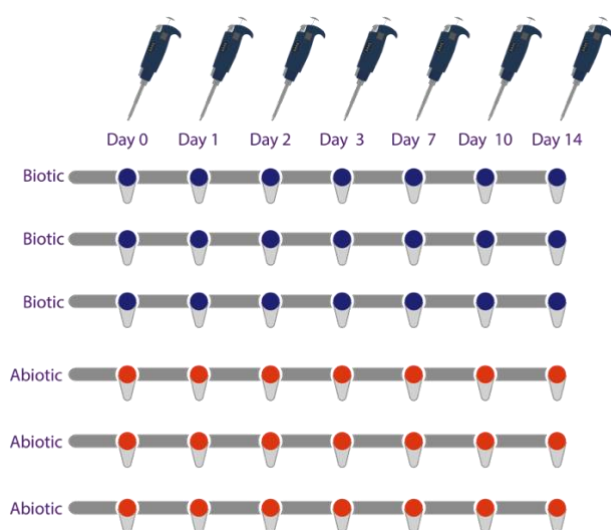


Figure 3-5: Experiment overview in the time scale.

3.3.5.1. Free drug reactors

Control reactors containing free drugs solutions were established to investigate the natural degradation behaviour of selected antidepressants and benzodiazepines. Two types of control systems were designed: biotic reactors, which were inoculated with microbial cultures obtained from a stock bioreactor, and abiotic reactors, which consisted of sterile synthetic wastewater and were maintained under the same environmental conditions but without microbial inoculation. These control setups allowed for the differentiation between biologically driven and chemically induced degradation processes. Samples from both reactor types were collected at

predetermined intervals for environmental analysis and assessment of degradation, as illustrated in Figure 3-6 and Figure 3-7.

To ensure consistency across experimental setups, methanol was added to the control reactors in the same concentration used in the spiked drug reactors. Methanol played a dual role: it served as a carbon source for certain bacteria and was also used as a solvent in the preparation of analytical standards. While it can stimulate microbial activity, excessive levels may be toxic and inhibit bacterial growth. [273] Therefore, careful control and monitoring of methanol concentrations were essential to maintain optimal bioreactor performance and ensure reliable experimental outcomes. [274,275]

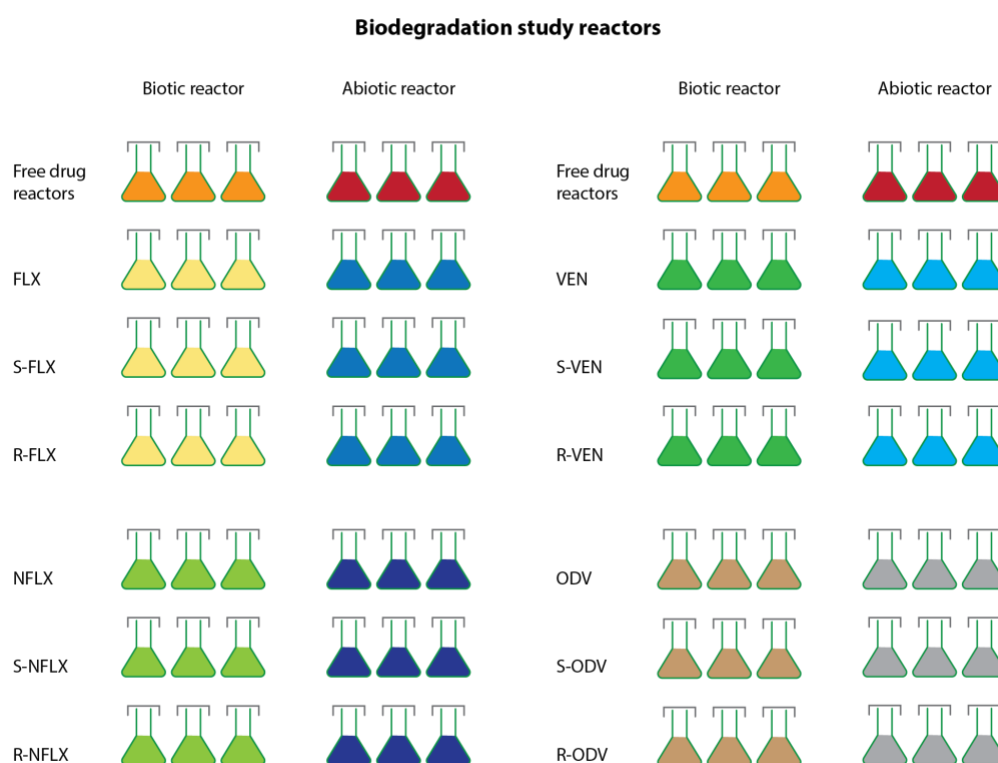


Figure 3-6: ADs biodegradation experimental setting.

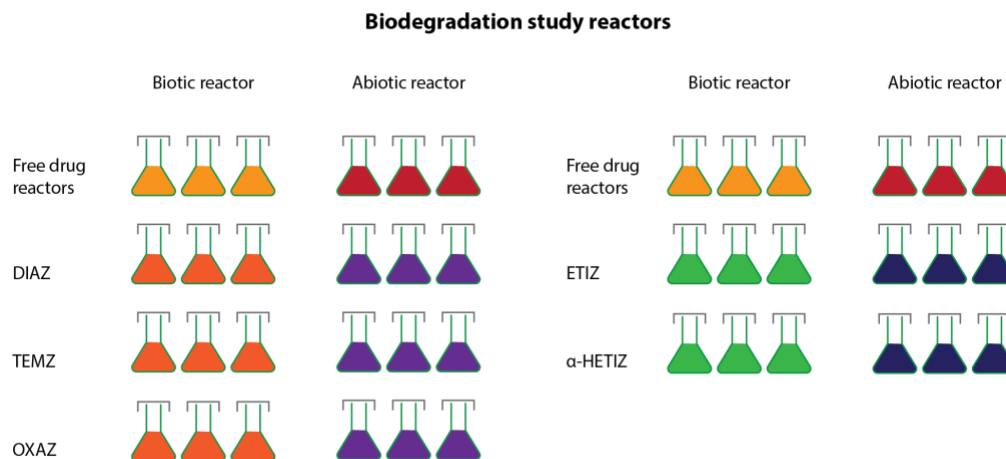


Figure 3-7: BZs biodegradation experimental setting.

3.3.5.2. Abiotic reactors

In this group, the reactors contained no biological components, serving exclusively as abiotic controls. Instead, they were filled only with SWW. To evaluate the abiotic degradation of pharmaceutical compounds, the reactors were spiked with antidepressants and benzodiazepines at a concentration of 1 $\mu\text{g/mL}$ for each drug. The setup and details are illustrated in Figure 3-6 and Figure 3-7. All spiked drugs were prepared using methanol as a solvent, following the procedure outlined in section 3.3.3.2. This design enabled the assessment of non-biological degradation mechanisms, such as hydrolysis, photolysis, or other abiotic interactions, independent of microbial activity. This design not only enabled the assessment of non-biological degradation mechanisms, such as hydrolysis, photolysis, or other abiotic interactions, but also provided a basis for comparing results with other reactor setups and evaluating the stability of pharmaceuticals in solutions.

3.3.5.3. Biotic reactors

This group of reactors contained SWW supplemented with 250 μL of biomass, which was collected from the stock bioreactor described in section 0. The biotic reactors were spiked with antidepressants and benzodiazepines at the same concentrations as the abiotic reactors, specifically 1 $\mu\text{g/mL}$ for each drug. The setup and preparation details are illustrated in Figure 3-6 and Figure 3-7. This design allowed for the assessment of biodegradation processes facilitated by the introduced microbial communities, enabling direct

comparison with the abiotic reactors to understand the role of biological activity in pharmaceutical degradation.

To ensure consistency and minimize external influences, all reactors were covered with foil and maintained at a constant temperature of 22 ± 1 °C throughout the study. The contents of each reactor were agitated at a speed of 120 rpm using an orbital shaker over 14 days, as illustrated in Figure 3-9. This setup facilitated uniform mixing and aeration, providing consistent environmental conditions for reliable experimental results.

To monitor biodegradation progress and evaluate environmental changes, 8 mL samples were collected daily from each reactor. These samples underwent immediate testing for key parameters, including pH and OD, to track microbial activity, Figure 3-5.

Before pharmaceutical analysis, all collected samples were filtered to remove particulate matter and impurities. The filtered samples were then processed using SPE cartridges, which efficiently isolated and concentrated the target compounds for subsequent analysis. [276]

To further investigate the microbial response to spiked drugs, samples collected on days 0, 7, and 14 were pooled to ensure sufficient DNA yield for sequencing and to capture overall shifts in microbial community composition during the biodegradation process. This approach focused on broad trends in bacterial community responses rather than day-to-day fluctuations. For the biotic reactors, bacterial DNA was extracted using the FastDNA™ SPIN Kit for Soil, which was selected because it reliably yields high-quality DNA suitable for 16S rRNA-based taxonomic profiling. [277] A detailed extraction protocol is provided in Appendix 1; however, briefly, the procedure involved mechanical cell disruption, binding of nucleic acids to a silica-based matrix, and subsequent washing and elution to obtain purified DNA.

The bacterial community was then profiled to assess compositional changes and responses to pharmaceutical spiking. Coupled with LC-MS results, this

analysis provided valuable insights into the interactions between bacterial taxa and pharmaceutical compounds.

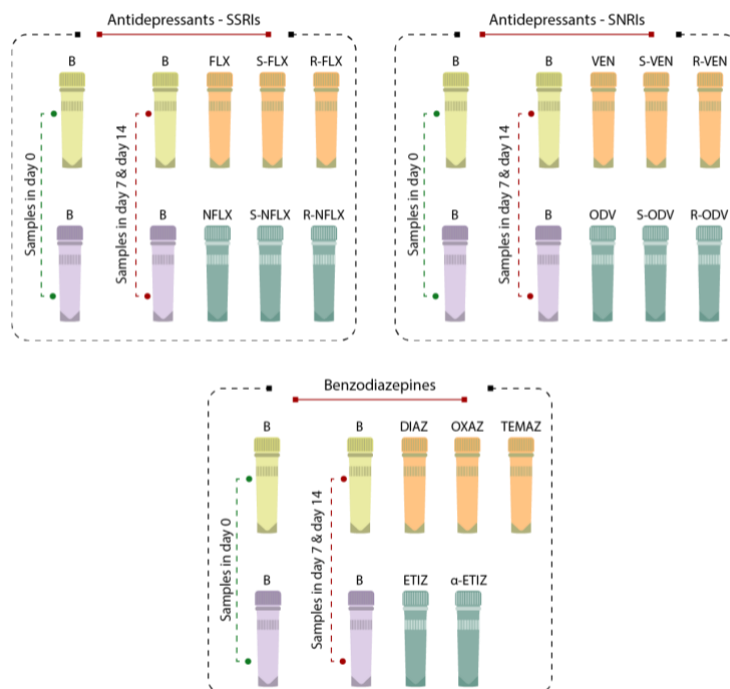


Figure 3-8: Samples Collected for the 16S taxonomy analysis for bacteria.

To maintain sample integrity and stability, all collected samples were stored at -80°C until they were ready for LC-MS analysis (sections 4.3.10 and 5.3.10). This stringent storage condition ensured the preservation of chemical and biological properties, enabling accurate and reliable results during the subsequent stages of the study. Figure 3-10 demonstrates Overview of the biodegradation study, illustrating the processes and methodologies applied to analyse the degradation of ADs and BZs over the experimental period.

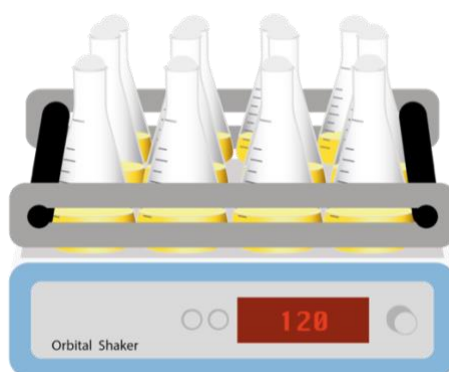


Figure 3-9: The reactors set on the shaker.

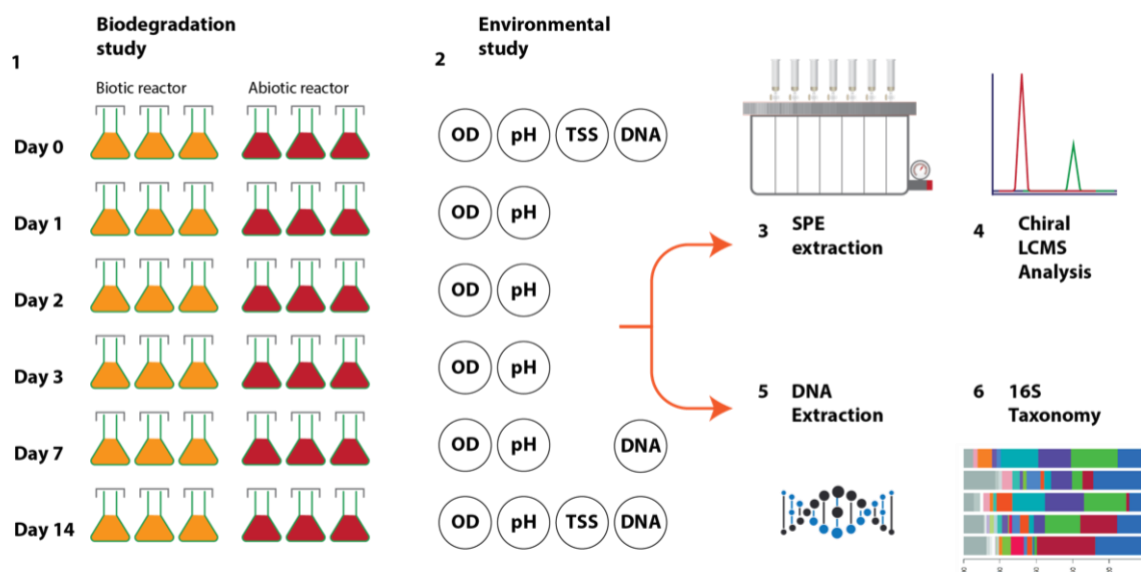


Figure 3-10: Biodegradation study diagram.

3.3.5.4. Polyomics test

Biodegradation and biotransformation of pharmaceuticals in wastewater are primarily driven by bacterial activity. [278] To identify and characterize the bacterial strains involved, 16S rRNA gene sequencing was conducted, targeting the V3/V4 regions, a standard approach for microbial community analysis. The analysis was performed at the Glasgow Polyomics Centre, where library preparation, quality control, and sequencing were carried out using the Illumina MiSeq system with 2x300 bp paired-end reads, ensuring a sequencing depth of at least 100,000 reads per sample. This approach provided detailed taxonomic profiling of the microbial communities in the bioreactors. [279]

During the experiment, samples were collected from each biotic reactor on Day 0, Day 7, and Day 14. To preserve DNA integrity, the samples were stored in sterilized polypropylene-copolymer screw-cap tubes at -80 °C until analysis, as shown in Figure 3-8. The preservation process ensured that the bacterial composition remained intact, enabling accurate downstream analysis.

The microbial community within the biomass of the bioreactors was analysed using molecular biology techniques. The V3/V4 region of the 16S rRNA gene

was amplified using polymerase chain reaction (PCR) with primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). This amplification allowed for the identification of bacterial strains involved in biodegradation and biotransformation processes. The data gathered through 16S sequencing provided valuable insights into the microbial diversity and composition in the bioreactors, highlighting the specific bacteria responsible for degrading pharmaceutical compounds. [280]

3.3.5.5. Bioinformatic analysis process

A. software:

Microbiome bioinformatics were performed with QIIME 2 that was installed on Linux system via a virtual machine. Raw sequence data were demultiplexed and quality filtered using the q2-demux plugin followed by denoising with DADA2 then classify reads (via q2-dada2). [281] An up-to-date web browser, such as the latest version of Firefox or Chrome, is needed for visualizations using QIIME 2 View.

B. Hardware:

For the Qiime2 analysis, a robust hardware setup was employed to effectively handle the computational demands. The system specifications included 16 GB of memory, a Graphics NV117 card, and a disk capacity of 1.5 TB for storage. The processing power was provided by an Intel® Xeon® CPU E5-1607 v3 @ 3.10GHz x4, ensuring efficient data processing. The operating system used was Linux, specifically Ubuntu 22.04, which provided a stable and reliable environment for running the Qiime2 analysis.

This powerful hardware configuration allowed complex data analyses, including 16S rRNA gene sequencing, taxonomy classification, and diversity analysis, to be performed with enhanced speed and accuracy. The ample memory capacity and efficient processor capabilities facilitated the handling of large datasets, ensuring smooth and efficient Qiime2 workflows. Moreover, data visualization and interpretation were made a seamless experience with the contribution of the Graphics NV117 card, which accelerated graphical tasks. [246,282]

3.3.6. Instrumentation

3.3.6.1. LC-DAD System and Operating Conditions

All prepared samples were initially analysed using a Dionex Ultimate 3000 HPLC system equipped with a diode array detector (DAD) and a variable wavelength UV detector (VWD-3400) (Dionex Corporation, Sunnyvale, USA). Data acquisition and instrument control were carried out using Chromeleon v. 6.80 SP2 Build 2212 (Dionex Corporation). This system was primarily applied in method development and preliminary screening experiments.

3.3.6.2. LC-MS System and Operating Conditions

All samples were prepared using SPE cartridges, with eluates dried under nitrogen and reconstituted in the mobile phase prior to injection. The chromatographic system consisted of an Advion Avant Series HPLC (models A-2155, A-2112, A-2046 and A-2031-D) equipped with a quaternary pump. A UV detector (200-400 nm) was applied only during preliminary method screening, while final analyses relied on mass spectrometry detection.

Detection was achieved using an Advion Expression L Compact Mass Spectrometer (CMS) with electrospray ionization (ESI) operated in positive polarity, as the target drugs are basic compounds that readily protonate and produce strong, stable signals under ESI⁺. The molecular characteristics, precursor ions, and ionization modes of the target antidepressants, benzodiazepines, and their metabolites are summarized in Table 3-6. Data were acquired in both Total Ion Chromatogram (TIC) mode for broad scans (m/z 40–500) and Selected Ion Monitoring (SIM) mode for sensitive quantification of the target analytes. Acquisition was initiated after the void volume to minimize solvent front interference.

The ion source conditions were optimized to ensure consistent sensitivity and reproducibility across all analytes. Parameters included a gas temperature of 350 °C with a gas flow of 8 L/min, nebulizer pressure of 40 psi, capillary temperature of 250 °C, and capillary voltage of +4,000 V in positive mode (–3,500 V in negative mode when required). Additional settings were a source gas temperature of 200 °C, source voltage offset of 20 V, source voltage span of 30 V, fragmentor voltage of 75 V, cell accelerator voltage of 3 V, and

an ESI voltage of 3,500 V. A dwell time of 200 ms was applied during SIM acquisitions. These optimized parameters ensured efficient ionization, stable signal performance, and accurate quantification of the target compounds.

Mass spectrometric data acquisition, peak integration, and quantitative analysis were performed using MassExpress v. 6.7.15.1 (Advion Interchim Scientific). Calibration curves were generated from matrix-matched standards, and recovery and stability were confirmed with quality control samples. Additional statistical analysis and peak integration were conducted using Microsoft Excel and Python libraries (Pandas and NumPy), ensuring reliability and reproducibility of the method.

Table 3-6: Molecular information, precursor ions, and ionization mode for antidepressants and benzodiazepines analysed in this study.

Drug	Molecular formula	MW	Precursor m/z	Ionization mode
Fluoxetine	C ₁₇ H ₁₈ F ₃ NO	309.33	310.1413	POSITIVE
S-FLX (Enantiomer 1)				
R-FLX (Enantiomer 2)				
Norfluoxetine	C ₁₆ H ₁₆ F ₃ NO	295.3	296.1257	POSITIVE
S-NFLX (Enantiomer 1)				
R-NFLX (Enantiomer 2)				
Venlafaxine	C ₁₇ H ₂₇ NO ₂	277.4	278.2115	POSITIVE
S-VEN (Enantiomer 1)				
R-VEN (Enantiomer 2)				
O-D-venlafaxine	C ₁₆ H ₂₅ NO ₂	263.37	264.1958	POSITIVE
S-ODV (Enantiomer 1)				
R-ODV (Enantiomer 2)				
Diazepam	C ₁₆ H ₁₃ ClN ₂ O	284.74	285.0789	POSITIVE
Oxazepam	C ₁₅ H ₁₁ ClN ₂ O ₂	286.71	287.0582	POSITIVE
Tenazepam	C ₁₆ H ₁₃ ClN ₂ O ₂	300.74	301.0738	POSITIVE
Nordiazepam	C ₁₅ H ₁₁ ClN ₂ O	270.71	271.0633	POSITIVE
Etizolam	C ₁₇ H ₁₅ ClN ₄ S	342.8	342.0705	POSITIVE
α-hydroxyetizolam	C ₁₇ H ₁₂ Cl ₂ N ₄ O	359.2	358.0388	POSITIVE

3.3.7. Analytical Method Development

3.3.7.1. Selection and Application of Chromatographic Columns

This study employed three chromatographic columns -two chiral and one non-chiral- to achieve the distinct analytical objectives of enantioselective separation and stability assessment. The choice of columns was influenced by both the methodological requirements of the research and practical

considerations such as instrument availability during the COVID-19 pandemic, which temporarily limited access to analytical facilities at the University.

A. Chiral columns

Achieving effective chiral separation of racemic drug compounds requires the careful selection of an appropriate chiral stationary phase (CSP) in HPLC, as the choice directly influences the resolution and selectivity of enantiomers. Among the available CSPs, vancomycin-based columns, such as the Astec CHIROBIOTIC™ V and V2, have been widely reported to provide efficient enantioselective separations, particularly for antidepressants like FLX, VEN, and their metabolites. [283,284]

In the initial stages of the study, achieving chiral separation of racemic compounds, particularly for the parent drugs and their metabolites, proved challenging. To overcome this, a longer and upgraded Astec CHIROBIOTIC™ V2 column was selected, as it offered improved resolution and enhanced performance compared with the standard version. This upgrade enabled a more comprehensive and reliable analysis of drug biodegradation, with the detailed results presented in Chapter 4 and Chapter 5.

During the initial phase of the study, chiral separation of racemic compounds, including both parent drugs and their metabolites, was performed using the Astec CHIROBIOTIC™ V column. However, consistent and well-resolved enantiomeric separation was not achieved for all compounds under investigation, as the resolution and reproducibility were limited under the tested conditions. To enhance chromatographic performance, a longer and upgraded Astec CHIROBIOTIC™ V2 column was subsequently employed. Improved resolution, peak symmetry, and reproducibility were obtained with this column, allowing for a more accurate and comprehensive evaluation of the enantioselective biodegradation of the target analytes. Detailed results of these optimized separations are presented in Chapter 4 and Chapter 5.

The CHIROBIOTIC™ V column is based on the antibiotic vancomycin, a macrocyclic glycopeptide with multiple chiral centers. Its unique structure facilitates chiral recognition through various non-covalent interactions, including hydrogen bonding, π - π stacking, dipolar interactions, and steric complementarity. With a pore size of 100 Å, the CHIROBIOTIC™ V column is highly effective for separating secondary and tertiary amines in the polar ionic mode. It is particularly noted for its stability and higher sample capacity, making it suitable for a wide range of applications. [85,285]

The CHIROBIOTIC™ V2 column represents an improved version of the same stationary phase, incorporating modified bonding chemistry and an enlarged 200 Å pore size. These enhancements increase selectivity, loading capacity, and performance in both polar ionic and polar organic modes. The V2 column provides superior separation of basic and larger molecules and is particularly compatible with LC-MS applications. [286]

When comparing their performance in chiral separation of antidepressants, the V2 column's enhanced selectivity and increased capacity make it more suitable for challenging separations. Its larger pore size allows for better accommodation of larger molecules, potentially improving the separation of complex antidepressants. Both columns can operate in various modes, providing flexibility in method development; however, the V2 column's design offers distinct advantages in the polar ionic and polar organic modes, which are increasingly popular due to their compatibility with mass spectrometry detection and preparative liquid chromatography. [284]

B. Non-Chiral column

A reversed-phase C18 (Ascentis Express, 90 Å) column was used for stability study which was applied in LC-DAD. This column was selected for its robustness, reproducible retention, and suitability for moderately polar compounds. It provided stable chromatographic conditions for evaluating parameters such as precision, accuracy, and recovery in accordance with SWGTOX and ICH guidelines. The use of the C18 column also ensured experimental continuity during periods of limited instrument access and laboratory restrictions imposed by the pandemic.

3.3.7.2. Mobile phase Optimization

This section describes the systematic optimization of chromatographic conditions used for both enantiomeric and non-chiral separation of the target antidepressants and benzodiazepines. Separate optimization approaches were employed depending on the analytical purpose—either to achieve stereochemical resolution or to evaluate compound stability and method performance.

A. Enantiomeric Separation

To achieve optimal chiral resolution of antidepressants and their metabolites, several chromatographic parameters were systematically investigated. The optimization focused on the influence of ethanol content, aqueous composition, buffer concentration, and pH within the mobile phase. Variations in methanol, ethanol, and water ratios, together with controlled levels of ammonium acetate and formic acid, were tested to establish conditions that promoted effective enantiomeric separation under polar ionic and polar organic modes.

A total of 19 mobile phase methods were developed and tested using the Astec CHIROBIOTIC™ V2 column, as summarized in Table 3-7. Each method was assigned a numerical identifier (Method 1-19) and differed in solvent composition, ionic strength, and organic content, resulting in pH values typically ranging between 6.4 and 6.7. These variations were designed to systematically examine solvent polarity, buffer capacity, and ion-pairing effects on enantiomeric resolution.

For each condition, racemic mixtures of fluoxetine and venlafaxine, together with their principal metabolites norfluoxetine and O-desmethylenlafaxine, were analyzed at a target concentration of 1 µg/mL. Enantiopure standards (S-FLX, R-FLX, S-NFLX, R-NFLX) were also examined to confirm peak identity and aid in resolution assessment.

All mobile phases were freshly prepared, filtered through 0.45 µm nylon membranes, and sonicated for 15 minutes to ensure homogeneity and minimize baseline noise. The LC column oven was maintained at 20 °C, and

an injection volume of 2 μL was applied. Chromatographic runs were performed in positive ionization mode under the same instrumental conditions to maintain consistency.

Three key chromatographic parameters, retention factor (k), selectivity (α), and resolution (R_s), were determined to evaluate enantioselective performance. These parameters were calculated according to Equation 3-3 to Equation 3-5 and used to interpret separation efficiency across the tested mobile-phase systems. In chromatographic method development, k values in the approximate range of 2–10 are generally considered optimal, providing sufficient retention without excessively long analysis times; $k < 1$ indicates weak retention, whereas $k > 10$ may result in unnecessarily prolonged runs. Selectivity (α) describes the relative separation between enantiomers, where $\alpha = 1$ indicates no selectivity and $\alpha > 1$ indicates separation potential; in practice, $\alpha \geq 1.10$ – 1.20 is typically associated with useful enantioselectivity. However, baseline separation ultimately depends on resolution (R_s), which integrates selectivity, retention, and column efficiency, with $R_s \geq 1.5$ commonly accepted as indicative of baseline separation. [287–289]

$$\text{A- Selectivity } (\alpha) = \frac{k_2}{k_1}$$

Where:

k_1 is the retention factor of the first enantiomer.

k_2 is the retention factor of the second enantiomer ($k_2 > k_1$).

Equation 3-3: Selectivity equation.

$$\text{B- Retention factor } (k) = \frac{t_R - t_0}{t_0}$$

Where:

t_R is the retention time of the analyte.

t_0 is the retention time of an unretained peak (dead time).

Equation 3-4: Retention factor equation.

$$C\text{- Resolution (Rs)} = \frac{2 \times (\Delta tR)}{W_1 + W_2}$$

$$\text{Or alternatively: } Rs = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_2}{1 + k_1}$$

Where:

$\Delta tR = tR_2 - tR_1$ is the difference in retention times of the two enantiomers.

W_1 and W_2 are the baseline widths of the two enantiomer peaks.

N is the theoretical plate number.

α is the selectivity factor.

Equation 3-5: Resolution equation.

Table 3-7: Mobile phases composition for optimisation chiral separation study.

Method	EtOH (%)	H ₂ O (%)	MeOH (%)	Other solvents	Ammonium Acetate	FA (%)	Note
1	35	15	50	-	4 mM	0.005	
2	30	40	30	-	4 mM	-	
3	35	30	35	-	4 mM	0.005	
4	28	30	42	-	4 mM	0.005	
5	30	15	55	-	-	-	
6	30	20	50	-	-	-	
7	-	10	30	30% ACN + 30% IPA	4 mM	0.05	
8	20	70	-	10% ACN	4 mM	0.05	
9	-	-	50	50% ACN	4 mM	0.05	
10	-	80	-	10% ACN + 10% IPA	4 mM	0.05	
11	10	80	10	-	4 mM	0.05	
12	15	75	10	-	4 mM	0.05	
13	25	50	25	-	4 mM	0.05	
14	-	-	100		4 mM	0.005	
15	-	65	-	35% CAN	-	0.01	(A) Water (B) ACN (65:35)
20	10	65	25		4 mM		
17	45	10	45	-	4 mM	0.05	
18	-	-	-	100% ACN	10 mM	-	
19	15	15	55	15% Isopropanol			

B. Non-Chiral Separation

For non-chiral analyses such as stability assessment and quantitative method validation a reversed-phase C18 column (Ascentis Express, 90 Å) was used to achieve reliable retention and consistent quantification of the

target drugs. The mobile phase was optimized to provide stability and sensitivity rather than stereoselectivity.

A binary gradient elution was employed using Solvent A (0.1 % formic acid in water) and Solvent B (acetonitrile with 0.1 % formic acid) at a flow rate of 0.7 mL/min. The column oven was maintained at 40 °C, and the injection volume was 20 µL. These conditions produced sharp, symmetrical peaks and stable baselines suitable for quantitative LC-DAD analysis.

3.3.7.3. LC method development

To optimise the chiral separation of FLX, its primary metabolite NFLX, VEN, and its main metabolite ODV, together with selected benzodiazepines, including DIAZ, NDIAZ, OXAZ, TEM, ETIZ, and its metabolite α -HETIZ using LC-DAD, a series of isocratic mobile phase conditions were systematically evaluated. Chromatographic analyses were performed on an Astec CHIROBIOTIC™ V2 column (250 mm × 2.1 mm, 5 µm particle size), selected for its high chiral recognition capacity and reproducible performance across a wide range of analyte structures.

Flow rates of 0.2 mL/min and 0.5 mL/min were tested, maintaining a stable system pressure of approximately 60 bar to ensure consistent elution and column integrity. The autosampler was maintained at 8 °C to preserve sample stability, while the column oven was set between 21–22 °C to provide reproducible retention times and symmetrical peak profiles.

Detection was conducted using a diode array detector (DAD) set at a wavelength of 230 nm, selected to maximize the sensitivity of both antidepressants and benzodiazepines while minimizing baseline noise and spectral interference. This wavelength offered a suitable balance between analyte absorbance and signal stability, ensuring reliable quantification and qualitative evaluation.

These chromatographic conditions were carefully refined to achieve precise, reproducible, and enantioselective separation of the target antidepressants, benzodiazepines, and their metabolites. The optimized parameters established under these LC-DAD conditions were subsequently verified and

further refined using LC-MS, which provided enhanced selectivity, sensitivity, and confirmation of enantiomeric identity. [290]

3.3.7.4. The optimum condition methods in LC-MS

The LC-MS system was operated using an autosampler, coupled with an Astec CHIROBIOTIC™ V2 column. It was selected over alternative vancomycin-based stationary phases because its larger pore size and improved selectivity provide superior resolution for structurally complex compounds such as antidepressants (e.g., FLX, VEN) and benzodiazepines (e.g., DIAZ, etizolam), along with their metabolites. This makes it especially advantageous for enantioselective separations where baseline resolution is critical.

Sample injection was performed at 2 µL to prevent column overloading, which can lead to peak broadening, loss of resolution, and reduced reproducibility. The system employed isocratic elution with optimized mobile phases tailored to the chemical properties of the analytes. For antidepressants, the mobile phase consisted of 30% methanol, 30% ethanol, and 40% water containing 4 mM ammonium acetate, chosen to provide suitable ionic strength and polarity for enantiomeric resolution. For benzodiazepines, the mobile phase consisted of 30% methanol, 30% ethanol, and 40% H₂O with 4 mM ammonium acetate, selected during the initial method optimisation to provide suitable ionic strength and polarity for effective enantiomeric separation. Following optimisation with antidepressants, the same chromatographic framework was adapted and further optimised for benzodiazepines, resulting in a mobile phase composed of 15% H₂O, 15 mM ammonium acetate, 15% acetonitrile (ACN), 15% isopropanol (IPA), and 55% methanol to achieve improved chiral resolution for this compound class. The flow rate was set to 1.0 mL/min, with column temperature controlled between 21-22 °C to ensure consistent retention times and peak symmetry.

The mass spectrometer operated in positive polarity mode with electrospray ionization (ESI+). This was chosen because most target drugs in this study (antidepressants and benzodiazepines) are basic in nature, readily

protonated under ESI+ conditions, and thus produce stronger, more stable signals compared to negative ionization. While ESI- can be useful for acidic pharmaceuticals, it is less effective for the analytes investigated here. The capillary temperature was maintained at 250 °C, with a capillary voltage of 180 V. Additional source parameters included a source voltage offset of 20 V, a source voltage span of 30 V, and a gas temperature of 180 °C. Additional source parameters included a source voltage offset of 20 V, a source voltage span of 30 V, and a gas temperature of 200°C. The ESI voltage was set to 3500 V, optimizing ionization for detection of the analytes. These optimized conditions ensured sensitive, precise, and reproducible enantioselective separation of the studied pharmaceuticals.

Data acquisition was performed in both selected ion monitoring (SIM) and full scan modes, enabling sensitive quantification of target analytes while maintaining broad-spectrum detection of potential metabolites and transformation products. Acquisition was initiated after the void volume to minimize solvent front interference and ensure accurate peak identification.

Mass spectral data were collected using Advion Interchim Scientific's Mass Express software. Subsequent data processing and interpretation were conducted with Microsoft Excel and Python libraries (Pandas and NumPy), allowing for robust peak integration, quantification, and statistical evaluation. This workflow ensured reliable interpretation of the enantioselective separation and biodegradation behaviour of the studied antidepressants and benzodiazepines.

3.3.8. Method validation

This chapter presents the validation of the developed analytical method, which was carried out in accordance with the guidelines established by the Scientific Working Group for Forensic Toxicology (SWGTOX) and other internationally recognized standards. [291] The validation process was designed to ensure the method's reliability, accuracy, precision, and selectivity for the intended forensic toxicology applications. Parameters such as linearity, sensitivity, recovery and stability were systematically evaluated to

confirm the method's suitability for routine analysis in complex biological and environmental matrices.

3.3.8.1. Linearity

To validate the linearity of HPLC-DAD and LC-MS methods for antidepressants and benzodiazepines, calibration standards and quality control (QC) samples were prepared in accordance with SWGTOX (2013) and ICH Q2(R1) guidelines. [291,292] Fresh working solutions of each analyte (10 µg/mL in methanol, section 0) were used to prepare calibration standards, while an internal standard (ISD) solution at a fixed concentration was prepared in methanol (Section 3.3.3.3).

Seven calibration standards (ST1-ST7) were prepared at concentrations ranging from 0.1-1.2 µg/mL, Table 3-8. Each was obtained by diluting the working solution with methanol, spiking with ISD (0.3 µg/mL), and adjusting to a final volume of 1 mL. A blank containing 50 µL of ISD in 950 µL of methanol was also prepared. QC samples at 0.3 µg/mL (QC_0.3, low) and 0.9 µg/mL (QC_0.9, high) were prepared in the same manner.

Each calibration level, QC, and blank was injected in triplicate to assess repeatability. The mean analyte-to-ISD peak area ratios were calculated, and the relative standard deviation (RSD) was determined to evaluate precision Equation 3-6. Calibration curves were constructed by plotting the mean peak area ratios against nominal concentrations. Linearity was confirmed by calculating the correlation coefficient (R^2), with acceptance defined as $R^2 \geq 0.995$, consistent with ICH Q2(R1). [291,292]

This design ensures compliance with internationally recognized validation standards, demonstrating that the method achieves the required selectivity, linearity, and precision for reliable quantification of ADs and BZs in environmental matrices.

Table 3-8: Linearity solution preparation.

	Conc. µg/ml	Standards µl	ISD µl	methanol	Total µl
ST1	1.2	120	50	830	1000
ST2	1	100	50	850	1000
ST3	0.8	80	50	870	1000
ST4	0.6	60	50	890	1000
ST5	0.4	40	50	910	1000
ST6	0.2	20	50	930	1000
ST7	0.1	10	50	940	1000
B	0	0	50	950	1000
QC_0.9	0.9	90	50	860	1000
QC_0.3	0.3	30	50	920	1000

Relative standard deviation (RSD)

$$= \left(\frac{\text{The average of standard deviation}}{\text{The average of target analytes ratio}} \right) \times 100$$

Equation 3-6: Relative standard deviation equation.

3.3.8.2. Limit of Detection and Lower Limit of Quantification

The Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ) for each antidepressant and benzodiazepine were determined following standard analytical validation guidelines. The LOD was defined as the lowest analyte concentration producing a signal-to-noise (S/N) ratio greater than 3, while the LLOQ was defined as the lowest concentration meeting the acceptance criteria for accuracy and precision, corresponding to a S/N ratio greater than 10, Equation 3-7. [291]

Calculations were performed using Advion Mass Express software, which compared analyte peak intensities with baseline noise. Specifically, the peak height (signal) was divided by the standard deviation of the baseline noise, in line with ICH Q2(R1) and SWGTOX (2013) recommendations. This systematic approach ensured reproducible and robust estimation of LOD and LLOQ values for all analytes, supporting their applicability in forensic and environmental toxicology studies. [291,292]

$$\text{Signal to noise S/N ratio} = \frac{\text{The height of mass fragment ion in the analyte}}{\text{The noise in the basement of background}}$$

Equation 3-7: Signal to noise S/N ratio equation.

3.3.8.3. Precision and accuracy

To evaluate the precision and accuracy of the analytical method for antidepressants and benzodiazepines, quality control (QC) samples were prepared at two concentration levels: 0.3 µg/mL (low) and 0.9 µg/mL (high). The QC samples were analyzed in triplicate over three consecutive days to assess both intra-day and inter-day precision.

Method performance was considered acceptable if the measured concentrations fell within ±15% of the nominal values, in accordance with established analytical validation guidelines (e.g., ICH Q2(R1), SWGTOX 2013). The optimized method described here was applied consistently throughout the study. Detailed precision and accuracy results are reported in Chapter 4 and Chapter 5.

3.3.8.4. Selectivity

Selectivity refers to the ability of the analytical method to accurately identify and quantify the target antidepressants (ADs: FLX, NFLX, VEN, O-desmethylenlafaxine) and benzodiazepines (BZs: DIAZ, NDIAZ, TEM, OXAZ, etizolam, α-hydroxyetizolam) in the presence of other matrix components.

To assess potential interferences, blank samples (synthetic wastewater without spiked analytes) were first analyzed. This confirmed that no significant peaks appeared at the retention times of the target compounds, demonstrating the absence of endogenous interferences.

Next, standard solutions containing known concentrations of each analyte were analyzed to establish their characteristic retention times and detector responses, serving as reference points. To further mimic real conditions, blank wastewater samples were spiked with known amounts of the target ADs and BZs. These spiked samples were examined to verify that the

method could reliably resolve and quantify the analytes without interference from co-eluting substances.

Finally, chromatograms from blank, standard, and spiked samples were carefully compared. The analytes were well resolved, with distinct retention times, confirming method selectivity. Where necessary, mass spectrometry confirmation was applied to verify analyte identity and exclude potential interferences. Detailed chromatographic data and spectral comparisons are presented in Chapter 4 and Chapter 5.

3.3.8.5. Carryover

Carryover was evaluated by injecting triplicates of 5 µL of a 10 µg/mL benzodiazepine mixture into the LC-MS system and analysing them using a validated chromatographic method. Immediately after the high-concentration injections, blank samples consisting of the mobile phase were injected to detect any potential carryover from the previous analyses, using the Equation 3-8.

$$\text{Carryover (\%)} = \frac{\text{Signal in Blank}}{\text{Signal in High-Concentration Sample}} \times 100$$

Equation 3-8: Carryover equation.

3.3.9. Extraction method

An SPE procedure was developed and optimized for the recovery of antidepressants and benzodiazepines from environmental matrices. The method design considered key experimental factors, including cartridge type, analyte concentration, sample matrix, and temperature, to ensure effective extraction and applicability in both environmental and forensic toxicology contexts. [291,292]

To evaluate extraction efficiency, two different SPE cartridges were tested: Oasis HLB and MCX. The HLB cartridge, composed of a hydrophilic–lipophilic balanced sorbent, is widely applied for broad analyte recovery across a range of polarities. MCX (mixed-mode cation exchange) was assessed to enhance retention of basic compounds through cation-exchange interactions (Figure 3-11 and Figure 3-12). Prior to use, all cartridges were

conditioned with 2 mL of methanol followed by 2 mL of 2% formic acid in water to ensure proper equilibration. By comparing recovery, reproducibility, and extract cleanliness across these cartridges, the study aimed to identify the most suitable option for extracting ADs and BZs. [155]

Water and synthetic wastewater were chosen as test matrices to represent both clean and complex environmental conditions. Quality control (QC) samples at two levels 0.3 µg/mL (low) and 0.9 µg/mL (high) were prepared according to section 3.3.3.5. Each QC level was spiked with the internal standard (ISD) to support accurate quantification. Triplicate preparations were carried out for each matrix and QC condition to enable robust evaluation of extraction efficiency, reproducibility, and matrix effects during the SPE optimisation process.

Prepared samples were carefully loaded onto the conditioned cartridges. To eliminate interfering substances, washing was performed using 2 mL of 2% formic acid/H₂O (pH 2) followed by 2 mL of 0.6% formic acid/MeOH (pH 2), each at a flow rate of 3 mL/min.

Target analytes were eluted using 3 mL of 7% ammonium hydroxide/MeOH at a flow rate of 3 mL/min. The eluates were evaporated under a gentle stream of nitrogen gas (2-10 psi) and reconstituted in 0.3% acetic acid/5% MeOH/H₂O. All samples were filtered through 0.2 µm PTFE filters before HPLC-DAD or LC-MS injection. [155]

To assess extraction efficiency, two environmental factors were systematically evaluated: temperature and pH. Temperature experiments were conducted at 30 °C, 40 °C, and 50 °C to investigate how thermal conditions influence analyte, cartridge interactions and recovery. In parallel, pH adjustments were made to 3, 6, and 9 to evaluate the impact of ionization state on retention and elution, particularly given the amphoteric nature of many antidepressants and benzodiazepines. The combination of these variables provided critical insight into optimizing SPE conditions for maximum recovery and reproducibility. [293–295] For biodegradation studies and

stability assurance, all prepared extracts were stored at -80 °C until analysis by LC-DAD and LC-MS.

This optimized SPE procedure, adapted in part from Baker and Kasprzyk-Hordern (2011), provided a structured approach to drug extraction, allowing systematic evaluation of cartridge type, matrix composition, analyte level, temperature, and pH. Recovery was calculated according to the Equation 3-9, ensuring accurate assessment of extraction efficiency. Detailed results for recovery, linearity, and sensitivity are presented in Chapter 4 and Chapter 5. [112,155,156,230,296,297]

$$\text{Recovery (\%)} = \left(\frac{\text{Measured Concentration (after SPE)}}{\text{Spiked Concentration (before SPE)}} \right) \times 100$$

Equation 3-9: Recovery equation.

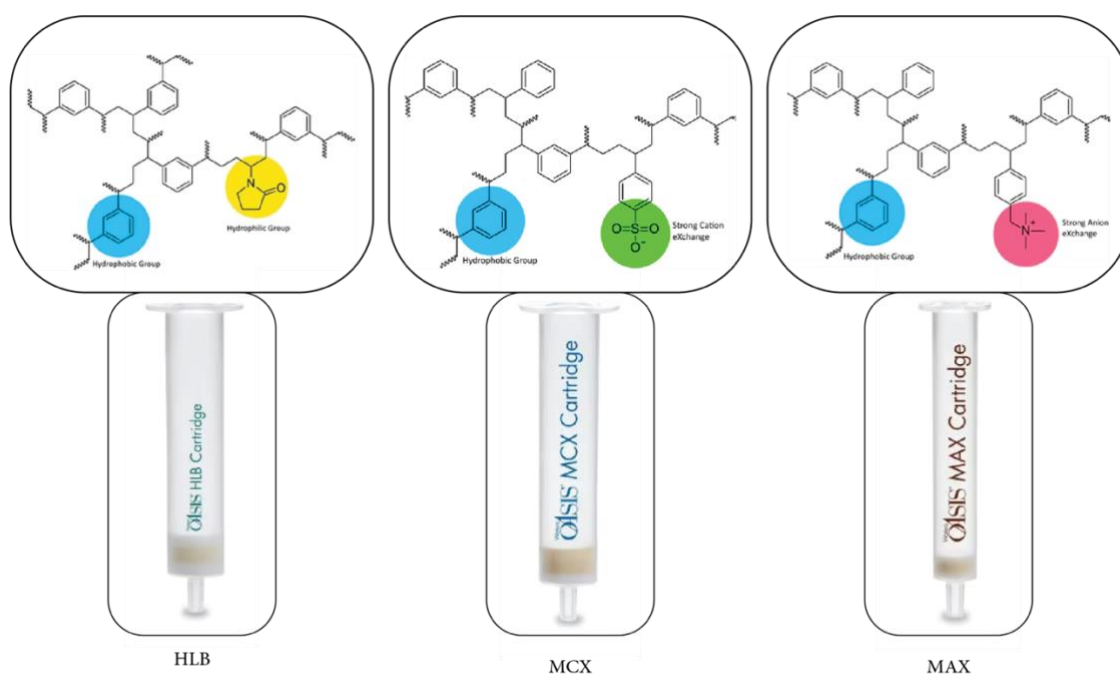


Figure 3-11: SPE selected and their chemical structures.

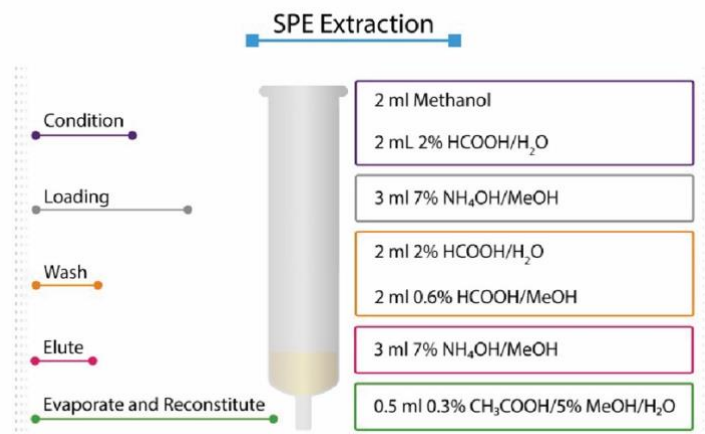


Figure 3-12: Final optimised solid-phase extraction (SPE) workflow used for ADs and BZs.

3.3.10. Stability study

The stability study was designed to evaluate the chemical integrity and recovery of antidepressants under different storage conditions and time intervals. Stock solutions were prepared in methanol (or a suitable solvent) and diluted to working concentrations. Quality control (QC) levels at 0.3 µg/mL (low) and 0.9 µg/mL (high) (prepared as described in section 3.3.3.5) were aliquoted into amber vials to prevent photodegradation and stored at three controlled temperatures: -20 °C (frozen, FZ), 4 °C (cold room, CR), and 22 °C (room temperature, RT).

Stability testing was scheduled at multiple time points: initial (day 0), short-term (day 1, day 2, day 3), medium-term (week 1, week 2, week 4), and long-term intervals (week 24, week 48), as illustrated in Figure 3-13.

Analyses were conducted using validated chromatographic methods (HPLC-DAD), optimized for each drug with respect to mobile phase, flow rate, and column type. Calibration standards ensured accurate quantification, and validation parameters included specificity, linearity, accuracy, and precision. For chiral antidepressants, a chiral column was employed to resolve enantiomers.

Recovery was calculated at each time point by comparing measured concentrations with initial QC levels, while degradation rates were determined to establish drug half-lives and stability profiles.

This structured approach provided stability data under both controlled laboratory and real-world simulated conditions, enabling the identification of degradation mechanisms such as hydrolysis or oxidation, and supporting recommendations for appropriate storage and handling of pharmaceuticals.

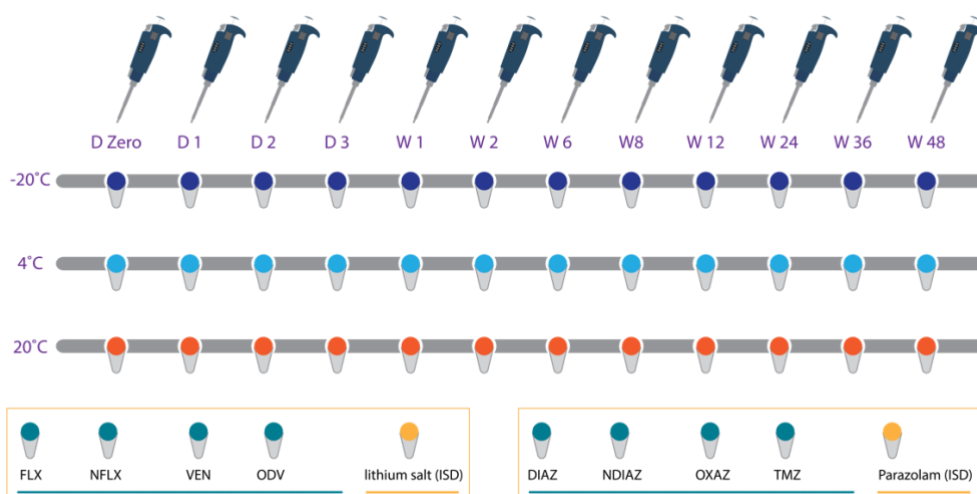


Figure 3-13: Schematic overview of the stability study design.

3.4. Results and discussion

3.4.1. Environmental study

3.4.1.1. pH

Maintaining an optimal pH range is essential for effective bacterial activity in wastewater treatment processes. Most bacteria involved in these processes thrive within a pH range of 6.5 to 8.5, with the ideal range often cited as 7.0 to 7.4. Deviations from this range can inhibit microbial activity, leading to reduced treatment efficiency or process failure. Regular pH monitoring ensures timely adjustments to sustain favourable conditions for microbial growth and efficient biodegradation. [267,268]

During the optimisation process, pH levels in the synthetic wastewater bioreactors were closely monitored and adjusted incrementally with acid or

base additions. Baseline values typically ranged between 6.5 and 7.0. Bacterial growth, as reflected by optical density measurements, was most active within the narrower range of 7.5-8.0. Below pH 6.5 or above 8.5, microbial activity and viability declined significantly, confirming the importance of maintaining a stable neutral to slightly alkaline environment. [298]

3.4.1.2. Temperature

Temperature is another key parameter influencing microbial community activity. In wastewater treatment systems, temperatures between 20 °C and 35 °C are generally regarded as optimal for sustaining microbial growth. [272,299]

In this study, the bioreactors were maintained at 22 °C ± 1 °C using precise monitoring instruments. This controlled range is consistent with previous reports that identified 20-35 °C as favourable for biodegradation processes. Maintaining a stable temperature ensured reproducibility of microbial activity and contributed to consistent system performance across the experimental period.

In this study, reactor pH was monitored continuously and adjusted as needed. Growth, assessed by OD and other biomass indicators, showed highest activity between pH 7.5 and 8.0. Below pH 6.5 or above pH 8.5, microbial growth declined significantly, consistent with literature trends.

3.4.1.3. Dissolved Oxygen

Dissolved Oxygen Levels are fundamental, especially in aerobic bioreactors. Adequate oxygen supply ensures that aerobic microorganisms can effectively metabolise substrates. DO levels in the reactors were consistently monitored throughout the study to ensure optimal conditions for microbial activity. The measured DO levels ranged between 1.4 mg/L and 2.4 mg/L across all samples. This range indicates sufficient oxygen availability to support aerobic bacterial growth and biodegradation processes, aligning with the requirements for maintaining effective microbial activity in the reactors. DO levels are typically maintained above 2 mg/L to prevent oxygen limitation,

which could lead to reduced microbial activity and process efficiency. [256,299]

3.4.1.4. TSS

The average TSS in the system was measured at 2.6 mg/L, with a relative standard deviation (RSD%) of 15%, indicating moderate variability. This variability may be attributed to minor inaccuracies in pipetting during sample preparation. According to the Standard Methods published by the American Public Health Association (APHA), and the Water Environment Federation (WEF), TSS levels in similar samples typically range from 2 to 200 mg/L. The measured average in this study falls within this expected range, indicating consistency with established benchmarks. [300]

Monitoring TSS is crucial for assessing biomass levels in the system, ensuring they remain within optimal ranges to support microbial activity and prevent operational issues such as clogging or reduced treatment efficiency. Regular monitoring and precise handling techniques can further enhance data reliability and system performance. [262]

3.4.2. Method development

3.4.2.1. Optimisation of chiral liquid chromatography

A. Chiral separation of ADs

- Ethanol content effect

In the early phase of the study, chiral separation of FLX, NFLX, VEN, and ODV was conducted using the Astec CHIROBIOTIC™ V column to evaluate the suitability of the vancomycin-based stationary phase for antidepressant compounds under polar organic and polar ionic conditions.

Chiral separation of antidepressants such as FLX, NFLX, VEN, and ODV is inherently challenging and requires careful optimization of chromatographic conditions, particularly the mobile phase composition. Various analytical approaches have been reported for enantiomer resolution of these compounds, including chiral high-performance liquid chromatography HPLC CE, and GC with chiral stationary phases [87,288]. Among these, vancomycin-based chiral HPLC columns were selected for this study due to their proven efficiency and versatility in pharmaceutical and environmental applications.

Initially, the Astec CHIROBIOTIC™ V column was employed with a methanol-based mobile phase containing 4 mM ammonium acetate and 0.005% formic acid. This composition yielded satisfactory separation for FLX, VEN, and ODV; however, the resolution of NFLX enantiomers remained inadequate, Figure 3-14. [301] Peak overlapping and unstable retention were observed, indicating limited chiral recognition and insufficient interaction with the stationary phase. The use of pure organic solvents such as MeOH and CAN without ionic modifiers further reduced enantioselectivity, confirming that ammonium acetate and acidic additives are essential to facilitate chiral recognition and improve resolution.

These results suggested that while the CHIROBIOTIC™ V column provided partial enantiomeric discrimination, its 100 Å pore size and lower phase capacity limited its performance for structurally complex antidepressants and their metabolites. Consequently, a longer and enhanced version of the

column- the CHIROBIOTIC™ V2 - was later adopted to improve chromatographic resolution and reproducibility.

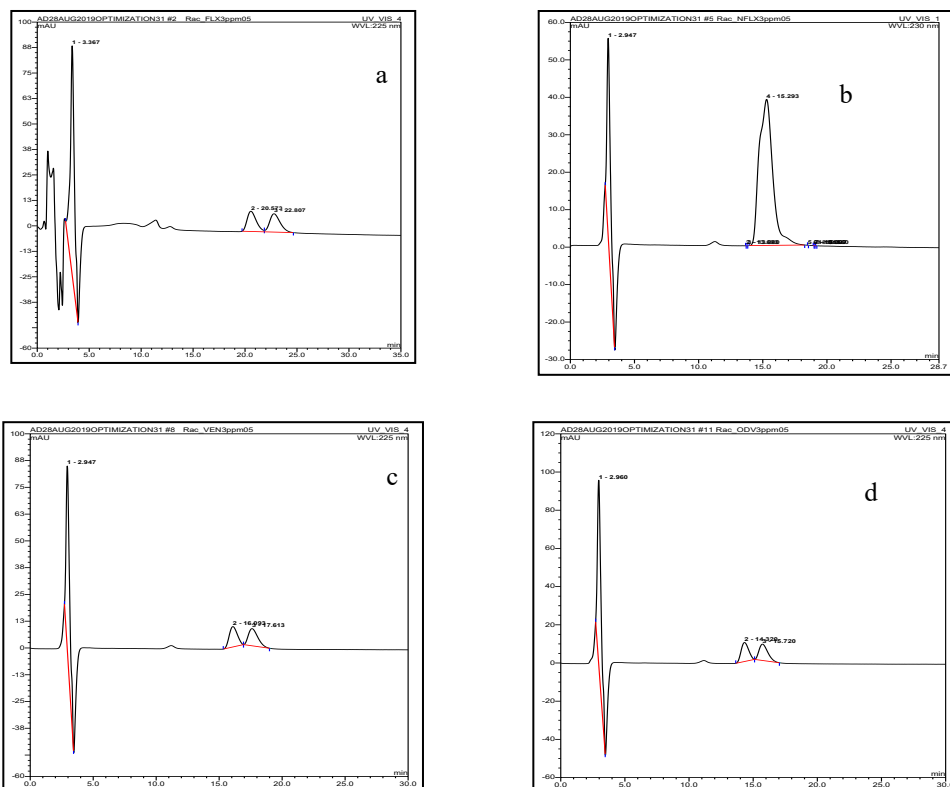


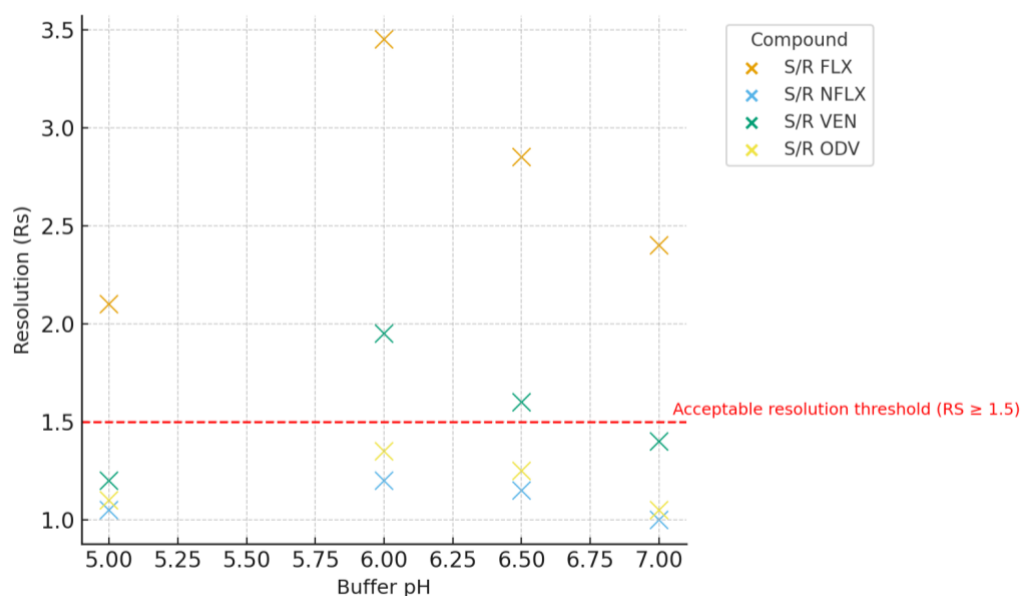
Figure 3-14: (a)FLX, (b) NFLX, (c) VEN and (d) ODV chiral separations.

Since buffer composition strongly influences chiral recognition, pH adjustments were explored within the range of 5.0-6.5. Notably, increasing pH to 6.0 improved resolution across all racemic antidepressants, with the best outcome observed using 17.5% EtOH at pH 6.0. Conversely, lowering the buffer to pH 5.0 resulted in reduced enantioselective resolution (Figure 3-15)

To support these observations, differences in resolution values across pH conditions were statistically evaluated by calculating RSD% of replicate injections ($n = 3$). An $RSD \leq 10\%$ was considered acceptable, in line with chromatographic method validation standards. The results confirmed that pH 6.0 provided the most consistent separation, with R_s values showing minimal variability compared to pH 5.0 and 6.5. These findings emphasize the critical role of buffer pH in achieving reproducible and effective chiral separations, Table 3-9.

Table 3-9: Effect of buffer pH on enantiomeric resolution and precision of FLX, NFLX, VEN, and ODV, n=3.

Compound	pH 5.0 (RS, RSD%)	pH 6.0 (RS, RSD%)	pH 6.5 (RS, RSD%)	pH 7.0 (RS, RSD%)
S/R FLX	2.10 (12%)	3.45 (6%)	2.85 (10%)	2.40 (15%)
S/R NFLX	1.05 (14%)	1.20 (12%)	1.15 (15%)	1.00 (18%)
S/R VEN	1.20 (17%)	1.95 (8%)	1.60 (14%)	1.40 (18%)
S/R ODV	1.10 (16%)	1.35 (9%)	1.25 (13%)	1.05 (20%)

**Figure 3-15: Effect of buffer pH on enantiomeric resolution.**

A series of experiments were conducted to optimize the mobile phase composition for the enantioselective separation of antidepressants and their metabolites, namely FLX, NFLX, VEN, and ODV, using vancomycin-based chiral stationary phases. The optimization involved systematic evaluation of solvent composition, buffer strength, and pH to identify the most effective chromatographic conditions for simultaneous separation of these racemic compounds, Table 3-7.

Several solvent systems were screened, including MeOH, EtOH, ACN, and IPA, individually and in mixed ratios, with the addition of 4 mM ammonium acetate and trace formic or acetic acid. The results showed that purely organic mobile phases (e.g., 100% MeOH or ACN) failed to achieve chiral resolution, emphasizing the necessity of polar modifiers and buffering

components to enable effective ion-pairing and stereoselective interactions between analytes and the vancomycin-based stationary phase. [11,301,302]

Among the tested systems, an EtOH:MeOH mixture produced the most consistent and satisfactory results for multiple compounds. For FLX, the EtOH:MeOH mobile phase achieved excellent enantiomeric separation ($\alpha = 5.31$) and resolution ($RS = 3.08$). Similarly, NFLX displayed accepted separation under the same conditions ($\alpha = 3.08$; $RS = 3.08$), confirming that both compounds respond favorably to this solvent polarity range. The same mobile phase also provided reasonable separation for ODV ($\alpha = 1.3$; $RS = 1.3$). In contrast, VEN performed better in a different mixture containing 10% IPA, 10% ACN, and 80% H₂O (IPA:ACN:H₂O), which yielded improved enantiomeric resolution ($\alpha = 1.9$; $RS = 1.9$). These results illustrate that although EtOH–MeOH mixtures provide general applicability, compound-specific polarity differences can significantly affect chiral interactions and separation efficiency, Table 3-10 and Figure 3-16. [303,304]

Initial separations were performed using the Astec CHIROBIOTIC™ V column (150 mm × 2.1 mm, 5 μ m). While acceptable separation was achieved for FLX and VEN, consistent baseline resolution across all analytes was not obtained, likely due to limited surface interaction area. To enhance performance, a longer and upgraded Astec CHIROBIOTIC™ V2 column (250 mm × 2.1 mm, 5 μ m) was introduced. The V2 column, with improved silica bonding and a larger 200 Å pore size, provided increased selectivity and capacity compared with the original V column, particularly benefiting larger or more basic molecules such as antidepressants. The extended column length enhanced peak capacity, improved enantioselectivity, and yielded sharper, more symmetrical peaks, Figure 3-17. [11,302,305]

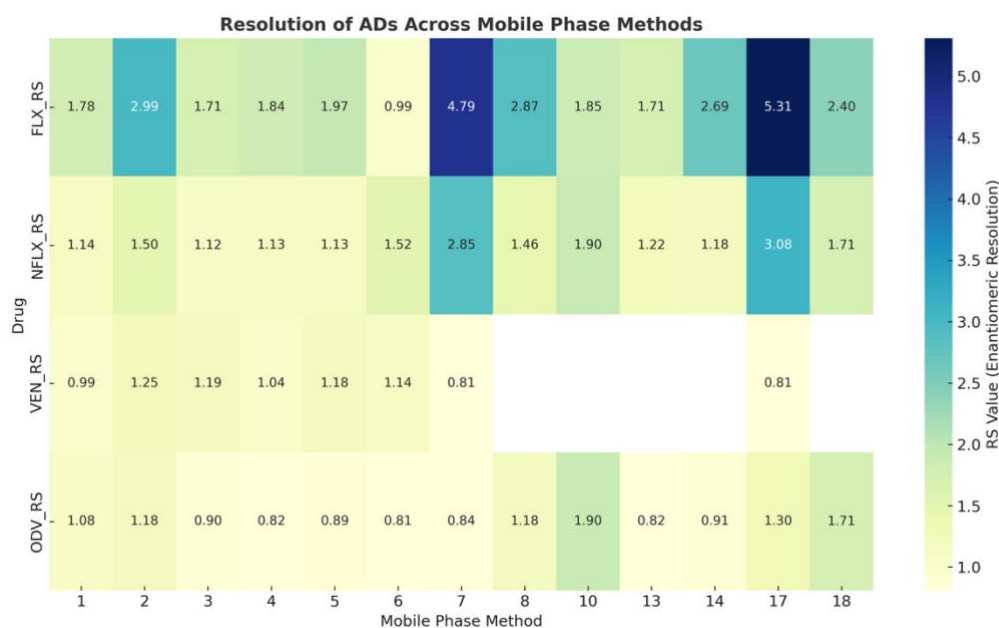


Figure 3-16: Resolution (RS) of antidepressants (FLX, NFLX, VEN, ODV) across different mobile phase methods.

- Final Optimized Mobile Phase of ADs

Through comparative analysis of nineteen mobile phase formulations, Method 2 - consisting of 30% MeOH : 30% EtOH : 40% H₂O with 4 mM ammonium acetate - was identified as the optimal composition for simultaneous chiral separation of all four antidepressants. This combination provided a balanced polarity and moderate ionic strength, facilitating stable ion-pairing and efficient stereochemical interactions with the vancomycin-based stationary phase. The ammonium acetate buffer improved peak shape and resolution by minimizing secondary interactions, while the mixed organic solvents ensured appropriate elution strength. Under these conditions, FLX achieved excellent resolution (RS = 2.99), and NFLX, VEN, and ODV reached acceptable separations (RS ≥ 1.2-1.5) with reproducible retention and low RSD (< 10%), confirming method reliability and analytical robustness.

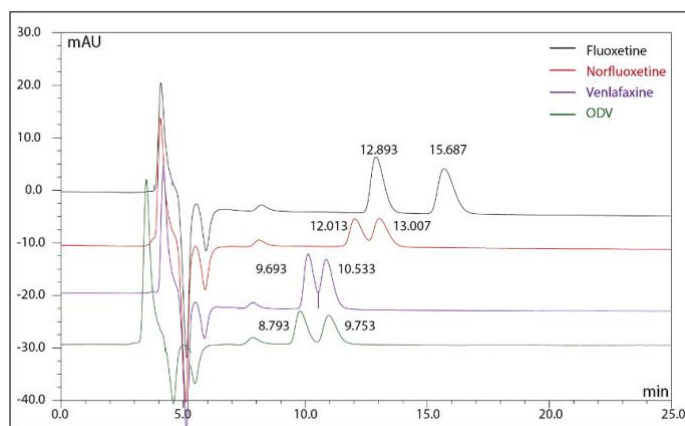


Figure 3-17: Racemic separation of the antidepressants Using Astec CHIROBIOTIC™ V2 column.

Table 3-10: Enantiomeric resolution and precision (RSD%) of FLX, NFLX, VEN, and ODV under different mobile phase methods, (n=3).

Method	FLX	NFLX	VEN	ODV	Acceptance
1	1.78	1.14	0.99	1.08	VEN & NFLX not accepted
RSD%	9.5	12.2	14.8	8.1	
2	2.99	1.5	1.25	1.18	Accepted (best overall)
RSD%	7.4	6.7	7.7	2.9	
3	1.71	1.12	1.19	0.9	VEN & NFLX not accepted
RSD%	8.6	11.5	12.8	10.2	
4	1.84	1.13	1.04	0.82	ODV not accepted
RSD%	6.2	13.7	9.9	14.6	
5	1.97	1.13	1.18	0.89	VEN & ODV not accepted
RSD%	9.1	8.8	15.4	11.9	
6	0.99	1.52	1.14	0.81	Not accepted (poor RS & high RSD)
RSD%	12.3	14.7	16.2	10.5	
7	4.79	2.85	0.81	0.84	Excellent FLX/NFLX, VEN/ODV poor
RSD%	5.8	7.3	10.8	13.4	
8	2.87	1.46	invalid	1.18	NFLX not accepted
RSD%	8.9	12.6		11.3	
10	1.85	1.9	invalid	1.9	Marginal (RSD borderline)
RSD%	11.5	13.8		12.4	
13	1.71	1.22	invalid	0.82	Not accepted (ODV RS<1.5, high RSD)
RSD%	10.8	14.2		15.6	
14	2.69	1.18	invalid	0.91	NFLX & ODV not accepted
RSD%	8.3	16.7		12.9	
17	5.31	3.08	0.81	1.3	Excellent FLX/NFLX, VEN/ODV poor
RSD%	6.1	5.9	14.2	13.7	
18	2.4	1.71	invalid	1.71	Borderline (ODV RSD ~10%)
RSD%	9.7	8.5		10.2	

Table 3-11: antidepressants chiral separation optimization methods.

Drugs	mobile phase	TEA	AA	Acetic acid	k1	k2	α	RS
FLX	MeOH	0.001	-	0.005	8.34	10.99	1.32	2.69
FLX	45% EtOH:45%MeOH:10%H ₂ O	-	4mM		7.31	11.25	1.54	5.31
FLX	30% IPO:30%ACN:30MEOH	-	-		3.35	5.38	1.60	4.79
FLX	10%ACN:20%EtOH:70% H ₂ O	-	4mM		9.75	12.68	1.30	2.87
FLX	10% IPA:10%ACN:80 H ₂ O	-	10mM		18.77	24.41	1.30	1.85
FLX	10% EtOH:10%MeOH:80% H ₂ O		4mM		23.84	-1.00	0.04	Invalid
FLX	30% EtOH:30%MeOH:40% H ₂ O		4mM		8.72	11.36	1.30	2.99
FLX	25% EtOH:25%MeOH:50% H ₂ O		4mM		2.18	2.67	1.22	1.71
FLX	10%ACN:25MeOH: H ₂ O	-	4mM		13.86	16.77	1.21	2.4
NFLX	MeOH	0.001	-	0.005	8.20	9.19	1.12	1.18
NFLX	45% EtOH:45%MeTH:10% H ₂ O	-	4mM		9.09	11.72	1.29	3.08
NFLX	30% IPA:30%ACN:30MEOH	-	-		3.27	4.42	1.35	2.85
NFLX	10%ACN:20% EtOH:70% H ₂ O	-	4mM		10.83	12.94	1.19	1.46
NFLX	10% IPA:10%ACN:80 H ₂ O	-	10mM AA		23.31	28.01	1.20	1.9
NFLX	10% EtOH:10%MeOH:80% H ₂ O		10mM		25.56	-1.00	0.04	Invalid
NFLX	30% EtOH:30%MeOH:40% H ₂ O		4mM		9.60	11.39	1.19	1.5
NFLX	25% EtOH:25%MeOH:50% H ₂ O		4mM		2.48	2.85	1.15	1.22
NFLX	10%ACN:25MeOH: H ₂ O	-	4mM		15.14	17.36	1.15	1.71
ODV	MeOH	0.001	-	0.005	5.49	6.05	1.10	0.91
ODV	45% EtOH:45%MeTH:10% H ₂ O	-	4mM		4.81	5.56	1.16	1.3
ODV	30% IPA:30%ACN:30MEOH	-	-		2.86	3.36	1.17	0.84
ODV	30% EtOH:30%MeOH:40% H ₂ O	-	4mM		2.32	2.49	1.08	1.18
VEN	30%EthO:30%MeOH:40% H ₂ O	0.001	4mM	0.005	6.15	6.75	1.10	1.25
VEN	45%EtOH:45%MeOH:10% H ₂ O	-	4mM		5.40	5.94	1.10	0.81

B. Chiral separation of BZs

As is known, DIAZ and etizolam are non-chiral compounds, whereas several of their metabolites - including OXAZ, TEM, and α -HETIZ - possess one or more chiral centers. The presence of chirality in these metabolites is analytically significant, as their enantiomers can differ in pharmacological activity, toxicity, and metabolic stability. Therefore, achieving effective chiral separation of benzodiazepine metabolites is essential for accurate toxicological and forensic interpretation.

To establish a reliable method for enantiomeric separation, several mobile phase compositions were systematically evaluated using vancomycin-based chiral stationary phases under polar organic conditions. The tested systems

varied in organic solvent composition, polarity, and ammonium acetate concentration to determine optimal elution behavior and enantioselectivity for TEM, OXAZ, and α -HETIZ. The results are summarized in Table 3-12.

Among the tested conditions, the mobile phase consisting of 55% methanol (MeOH), 15% acetonitrile (ACN), 15% water (H₂O), and 15% isopropanol (IPA) with 4 mM ammonium acetate (AA) provided the best overall performance. This composition achieved excellent enantioselectivity (α = 1.25) and baseline resolution (R_s = 2.10, RSD = 4.9%) for α -hydroxyetizolam, indicating a robust and reproducible separation. The balanced polarity and ionic strength of this system enhanced analyte–stationary phase interactions through hydrogen bonding and ionic pairing with the vancomycin moieties, facilitating stable chiral recognition.

In contrast, temazepam and oxazepam demonstrated poor chiral resolution under all tested conditions. TEM achieved only partial separation (R_s < 1), while OXAZ enantiomers co-eluted, suggesting weak stereochemical interaction with the chiral sites of the stationary phase. The limited separation efficiency for these compounds may be attributed to their relatively simple molecular structures and lower polarity, which reduce their interaction potential with the glycopeptide chiral selector. These findings are consistent with reports indicating that certain benzodiazepine analogues exhibit poor enantioselectivity under similar chromatographic conditions. [76,83–85]

Importantly, to the best of our knowledge, no previous study has reported the chiral separation of α -hydroxyetizolam. This research successfully established the first validated LC method capable of resolving its enantiomers, marking a novel contribution to the field of chiral forensic and pharmaceutical analysis.

Although the developed method achieved satisfactory separation for α -hydroxyetizolam, further optimization is still needed for TEM and OXAZ. Future work could focus on modifying the organic solvent ratios, adjusting buffer concentrations, or exploring alternative vancomycin-based or teicoplanin-based chiral stationary phases to enhance resolution.

Temperature and flow rate adjustments may also improve peak sharpness and reproducibility.

Across all experiments, ammonium acetate was identified as a critical component of the mobile phase, stabilizing ion-pairing interactions and improving chiral selectivity. The use of moderate flow rates (1.0 mL/min) and controlled column temperature (21–22 °C) provided symmetrical peaks and stable retention times without significantly increasing runtime.

In conclusion, the final optimized chiral separation method for benzodiazepine metabolites employed a mobile phase of 55% MeOH, 15% ACN, 15% H₂O, and 15% IPA containing 4 mM ammonium acetate on a vancomycin-based stationary phase. This composition achieved reliable and reproducible baseline separation for α -hydroxyetizolam, while partial separation was observed for TEM and unresolved peaks for OXAZ. The success of this method in separating α -hydroxyetizolam represents a novel analytical achievement, providing a foundation for future research on the chiral behavior and biodegradation of benzodiazepines and their metabolites, Table 3-12 and Figure 3-19.

Table 3-12: Chromatographic parameters (k_1 , k_2 , α , R_s , and RSD%) for the chiral separation of benzodiazepine metabolites.

Drug	Mobile phase (v/v) + buffer	k_1	k_2	α	R_s	RSD% (R_s)
TEM	10% ACN : 20% EtOH : 70% H ₂ O + 4 mM AA	2.33	2.59	1.11	0.35	6.01
TEM	10% IPA : 10% ACN : 80% H ₂ O + 4 mM AA	3.92	4.35	1.11	0.77	5.42
TEM	55% MeOH : 15% ACN : 15% H ₂ O : 15% IPA + 4 mM AA	3.2	3.47	1.08	0.65	7.22
α -HETIZ	10% ACN : 20% EtOH : 70% H ₂ O + 4 mM AA	4.6	5.05	1.1	1.1	8.43
α -HETIZ	10% IPA : 10% ACN : 80% H ₂ O + 4 mM AA	5.7	6.3	1.11	1.25	6.84
α -HETIZ	55% MeOH : 15% ACN : 15% H ₂ O : 15% IPA + 4 mM AA	5.5	6.5	1.25	2.1	4.95
OXAZ	10% ACN : 20% EtOH : 70% H ₂ O + 4 mM AA	2.55	2.63	1.03	0.12	11.66
OXAZ	10% IPA : 10% ACN : 80% H ₂ O + 4 mM AA	3.45	3.52	1.02	0.1	12.13
OXAZ	55% MeOH : 15% ACN : 15% H ₂ O : 15% IPA + 4 mM AA	Not achieved				

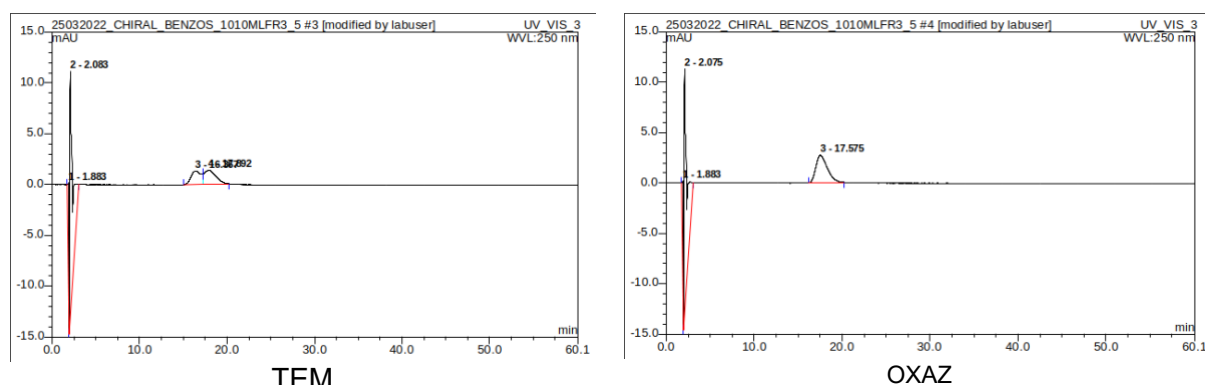


Figure 3-18: TEM and OXAZ enantiomers separation.

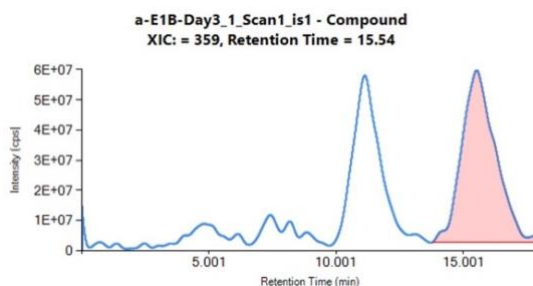


Figure 3-19: α -HETIZ enantiomers separation.

3.4.3. Method validation

3.4.3.1. Linearity

Calibration curves for ADs and BZs were prepared and validated over the same working range. Seven calibration levels spanning 0.1–1.2 $\mu\text{g/mL}$ (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 $\mu\text{g/mL}$) were freshly prepared in methanol and analysed in triplicate ($n = 3$). Calibration curves were constructed by plotting the mean peak-area ratio (analyte/ISD) against nominal concentration and fitting the data using linear regression. Linearity was evaluated using R^2 supported by inspection of residuals and back-calculated performance across the calibration range. The calibration levels and regression outputs for ADs and BZs are summarised in section in 3.3.8.1.

Calibration was performed with matrix-matched standards spanning 0.10–1.20 $\mu\text{g/mL}$ (ST7–ST1), with QCs at 0.30 and 0.90 $\mu\text{g/mL}$ ($n=3$). All analytes met SWGTOX linearity criteria ($R^2 \geq 0.99$ recommended): benzodiazepines showed $R^2 = 0.995$ – 0.999 and antidepressants $R^2 = 0.995$ – 0.998 , indicating excellent proportionality across the validated range. Replicate precision

across the curve was strong (RSD 4.6–7.1%), supporting homoscedastic behavior over this interval. Where appropriate, weighting (e.g., 1/x) was applied during regression to minimize high-end leverage; back-calculated calibrator accuracies were within typical SWGTOX limits ($\pm 15\%$, $\pm 20\%$ at the LOQ), confirming a robust quantitative model. QC recoveries at 0.30 and 0.90 $\mu\text{g/mL}$ fell within acceptance, further verifying linear performance in-matrix, Table 3-13, Figure 3-20. [291]

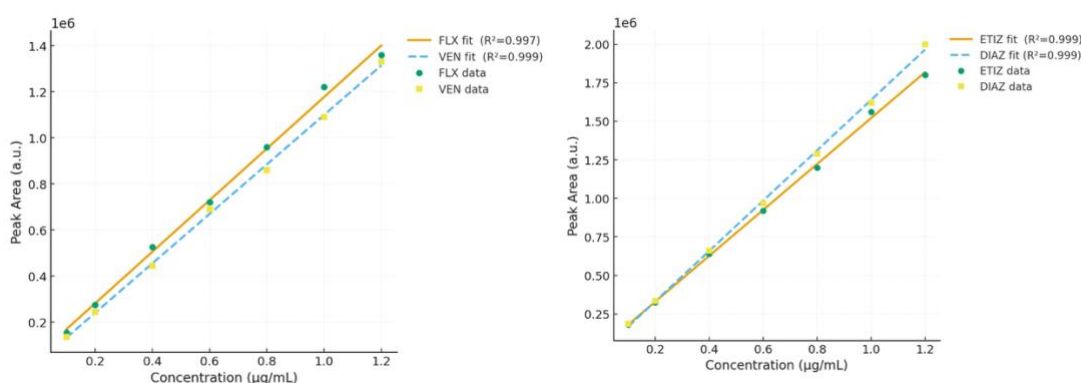


Figure 3-20: calibration caurves of ADs and BZs.

Table 3-13: The correlation coefficient, LOD and LOQ of benzodiazepines and antidepressants.

Drug	LOD (μg/mL)	LOQ (μg/mL)	R ²	RSD% (n = 3)
Benzodiazepines				
Diazepam	2.6	7.87	0.999	4.8
Nordiazepam	3.85	11.68	0.995	6.3
Oxazepam	2.87	8.7	0.997	5.9
Temazepam	2.17	6.57	0.999	4.6
Etizolam	1.95	5.8	0.998	5.2
α-Hydroxyetizolam	2.25	6.75	0.997	5.8
Antidepressants				
Fluoxetine	0.041	0.126	0.995	7.1
Norfluoxetine	0.052	0.157	0.996	6.9
Venlafaxine	0.083	0.251	0.998	5.7
O-Desmethylvenlafaxine	0.068	0.204	0.997	6.1

3.4.3.2. Limit of Detection and Limit of Quantification

The limits of detection (LOD) and limits of quantification (LOQ) were established in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation (2013;

updated 2020). [291] Both statistical and empirical criteria were applied to ensure the reported limits reflect the true analytical capability of the LC-MS method.

Initially, preliminary LOD and LOQ values were estimated from the calibration curve using the signal-to-noise ratio (S/N) approach, with approximate thresholds of $S/N \approx 3$ for LOD and $S/N \approx 10$ for LOQ. These instrumental estimates were subsequently verified by replicate injections ($n = 3$) of fortified matrix samples at the calculated LOQ concentrations. The experimental validation required that precision not exceed 20 % RSD and accuracy remain within ± 20 % of the nominal concentration. All analytes satisfied these criteria, confirming the reliability of the determined detection limits.

For benzodiazepines and their metabolites, the LODs ranged from 1.95 – 3.85 $\mu\text{g/mL}$, with corresponding LOQs between 5.80 – 11.68 $\mu\text{g/mL}$. These values are fully consistent with the sensitivity typically achievable using a single-quadrupole LC-MS operating in selected-ion monitoring (SIM) mode for structurally related benzodiazepines. The precision of replicate measurements remained between 4.6 - 6.3 % RSD, meeting SWGTOX's recommended maximum variability of 20 % at the LOQ level.

For antidepressants and their metabolites, including FLX, NFLX, VEN, and ODV, considerably lower instrumental limits were achieved due to their higher ionization efficiency under electrospray positive mode. LODs were within 0.041 - 0.083 $\mu\text{g/mL}$, and LOQs ranged from 0.126 - 0.251 $\mu\text{g/mL}$, with repeatability between 5.7 - 7.1 % RSD ($n = 3$). These results reflect the system's high sensitivity and reproducibility despite the moderate mass-spectrometric performance typical of single-quadrupole instruments.

Across both drug classes, ammonium acetate (4 mM) in the mobile phase played an important role in stabilizing ion-pair interactions and improving analyte protonation efficiency, which directly enhanced signal-to-noise ratios and detection sensitivity.[307] All analytes produced well-defined peaks without baseline interference at their respective LOQs, confirming adequate

method selectivity. The calculated LOD and LOQ results of benzodiazepines and antidepressants are presented 3.4.3.2.

The developed LC-MS method demonstrated excellent linearity ($R^2 \geq 0.995$), precision ($RSD < 10\%$), and sensitivity, with limits of detection ($LOD = 0.041\text{--}3.85\text{ }\mu\text{g/mL}$) and quantification ($LOQ = 0.126\text{--}11.68\text{ }\mu\text{g/mL}$) that comply with SWGTOX (2013) validation criteria ($R^2 \geq 0.99$; $RSD < 15\%$). These performance parameters are comparable to, and in some cases superior to, previously published LC-MS methods for benzodiazepines and antidepressants. Reported LODs for diazepam, oxazepam, and temazepam typically range between $2\text{--}5\text{ }\mu\text{g/mL}$, while antidepressants such as fluoxetine and venlafaxine exhibit LODs of $0.03\text{--}0.08\text{ }\mu\text{g/mL}$ using single- or triple-quadrupole instruments under ESI(+) mode. [308]

The method's detection limits and reproducibility indicate sufficient robustness for forensic and environmental applications, including post-mortem toxicology and wastewater monitoring. In environmental studies, antidepressants and benzodiazepines have been reported at concentrations between $0.1\text{--}50\text{ }\mu\text{g/mL}$, confirming that the present method provides adequate sensitivity for trace-level quantification and enantioselective degradation analysis in real-world matrices. The inclusion of 4 mM ammonium acetate as a volatile buffer enhanced ion-pair stabilization and protonation efficiency, improving signal-to-noise ratio and ensuring stable, interference-free baselines across all analytes. [8,309]

3.4.4. Extraction method

SPE procedure was systematically optimized to identify the most effective sorbents and experimental conditions for the extraction of the selected benzodiazepines (DIAZ and ETIZ) and antidepressants (FLX and VEN) from aqueous (H_2O) and synthetic wastewater (SWW) matrices. Three commercial SPE cartridges were evaluated - Oasis HLB (Hydrophilic-Lipophilic Balanced), Oasis MCX (Mixed-Mode Cation Exchange), and Oasis MAX (Mixed-Mode Anion Exchange) - across three pH levels (3, 6, and 9). The optimization aimed to maximize recovery and precision according to SWGTOX (2013) validation standards, which consider recoveries between

70 - 120% and RSD below 15% as acceptable. In addition, the effect of evaporation temperature (30°C, 40°C, and 50°C) on analyte stability and recovery was examined, with 40°C found to yield the most consistent results across all analytes and matrices, as shown in Figure 3-21. [291]

For benzodiazepines, the performance of the sorbents varied significantly depending on pH and matrix type. The MCX cartridge consistently provided the highest extraction efficiencies for both diazepam (DIAZ) and etizolam (ETIZ). For DIAZ, recoveries exceeded 95% in H₂O under all tested conditions, with the highest value of 97.0% (RSD 3.7%) at pH 9. In SWW, recoveries remained above 90%, peaking at 97.8% (RSD 3.5%) at pH 3. Similarly, ETIZ exhibited excellent recovery performance using MCX, reaching 95.4% (RSD 3.3%) in H₂O and 95.8% (RSD 2.3%) in SWW at pH 3. These findings demonstrate the robustness of MCX in retaining basic drugs under acidic conditions through cation-exchange interactions between the protonated analytes and the sulfonic acid functional groups of the sorbent. [291]

In contrast, HLB showed moderate performance for benzodiazepines, with recoveries increasing with pH due to partial ionization of the compounds. For DIAZ, recoveries ranged from 72.23% (RSD 9.9%) at pH 3 to 81.4% (RSD 6.34%) at pH 9 in H₂O, and from 80.0% (RSD 10%) to 83.1% (RSD 6.02%) in SWW. For ETIZ, recoveries varied between 78.2% (RSD 6.12%) and 84.28% (RSD 5.27%) in H₂O, with the highest recovery achieved at pH 6, while in SWW the maximum recovery was 81.7% (RSD 9.20%) at pH 9. Although HLB performance was within SWGTOX acceptance limits, the precision was lower compared to MCX. MAX, on the other hand, yielded invalid results for both DIAZ and ETIZ under all tested conditions, confirming that the anion-exchange mechanism of this cartridge is unsuitable for weakly basic benzodiazepines. These observations are consistent with the findings of Samanidou et al. (2015) and Jang et al. (2018), who reported superior performance of MCX for benzodiazepines extracted from aqueous and biological matrices.

The optimization of antidepressant extraction revealed different trends based on the physicochemical characteristics of FLX and VEN. HLB cartridges provided the best recoveries for FLX in both matrices, confirming their suitability for moderately polar and amphiphilic molecules. In H₂O, HLB achieved a maximum recovery of 94.8% (RSD 3.62%) at pH 6, while in SWW recoveries of 86.8% (RSD 3.8%) and 87.3% (RSD 3.1%) were observed at pH 6 and 9, respectively. These results confirm the strong hydrophilic–lipophilic balance of the sorbent, enabling effective interaction with both the aromatic and polar moieties of FLX. Comparable recoveries (90–95%) for FLX using HLB have been reported in previous LC-MS studies. [8,310]

For VEN, recoveries were more dependent on matrix and pH. In H₂O, HLB performed well, giving the highest recovery of 84.4% (RSD 7.71%) at pH 6, but recoveries decreased at higher pH values, reaching 73.71% (RSD 7.82%) at pH 9. In SWW, HLB provided consistent recoveries (71–82%) with slightly higher RSD values. Conversely, MCX performed comparably or better for VEN in more complex matrices: the best recovery of 84.6% (RSD 9.22%) was achieved at pH 6 in SWW, indicating partial cationic interaction of the protonated amine group. MAX displayed variable and mostly poor performance for VEN, with recoveries only reaching 78.9% (RSD 8.71%) at pH 9 in H₂O, and lower or invalid results under other conditions.

Across all analytes, matrix effects were more pronounced in SWW than in H₂O, as expected due to the presence of organic and ionic components that influence analyte - sorbent interactions. Nevertheless, the optimized methods produced recoveries and precision within the SWGTOX acceptance limits, confirming their reliability and reproducibility. [291]

Based on the optimization outcomes, the optimal extraction conditions were established as follows: for benzodiazepines, the MCX cartridge at pH 3 in SWW yielded the highest recoveries - 97.80% for DIAZ and 95.81% for ETIZ with RSD values below 4%. For antidepressants, HLB at pH 6 in H₂O produced the best recovery for FLX (94.81%, RSD 3.62%), while MCX at pH 6 in SWW was optimal for VEN (84.61%, RSD 9.22%). These optimized methods achieved recoveries comparable to, or exceeding, those reported in

previous studies and fulfilled the precision and accuracy requirements outlined by SWGTOX. [291]

Matrix effects were evident for both analytes, with H₂O generally providing higher recoveries and better precision compared to SWW. Despite the challenges posed by the complex SWW matrix, HLB consistently delivered excellent results for FLX, while MCX showed superior performance for VEN in SWW. For FLX, the optimal SPE method involves using HLB cartridges at pH 6 for both H₂O and SWW, achieving recoveries of 94.76% and 86.83% with RSD values of 3.61% and 3.78%, respectively. For VEN, the best method involves using MCX cartridges at pH 6 in SWW, achieving the highest recovery (84.56%) with an RSD of 9.21%, while HLB remains a competitive alternative in cleaner matrices like H₂O.

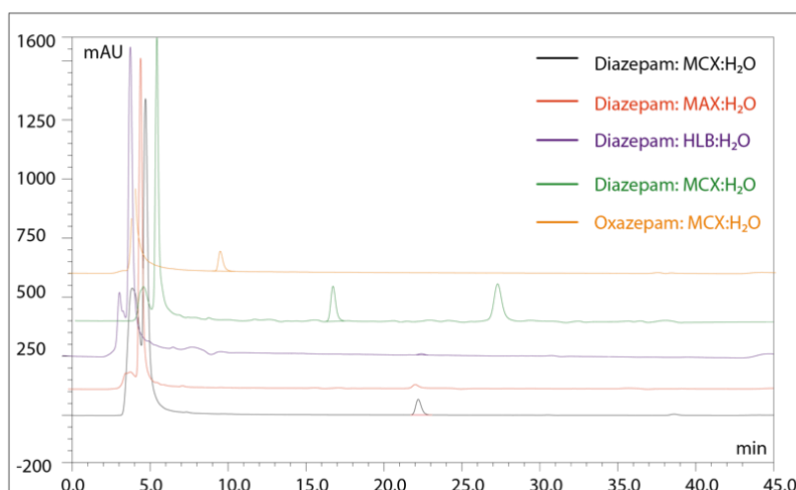


Figure 3-21: Extracted drugs using SPE cartridges.

3.4.4.1. Final Extraction method

SPE method employed Oasis HLB cartridges for antidepressants and Oasis MCX cartridges for benzodiazepines, providing effective and precise extraction of the target analytes. The procedure began with conditioning the cartridge using 2 mL of methanol to activate and wet the sorbent, followed by 2 mL of 2% formic acid in water (HCOOH/H₂O) to equilibrate the stationary phase and promote optimal analyte retention. This step ensured that the sorbent was fully prepared for efficient interaction with the analytes during the sample loading stage.

The sample loading was performed using 3 mL of 7% ammonium hydroxide in methanol ($\text{NH}_4\text{OH}/\text{MeOH}$), allowing the analytes to interact effectively with the functional sites of the sorbent. To remove matrix impurities without affecting analyte recovery, a two-step washing procedure was applied: the first wash with 2 mL of 2% $\text{HCOOH}/\text{H}_2\text{O}$ eliminated polar interferences, while the second wash with 2 mL of 0.6% HCOOH/MeOH further purified the sample by removing non-polar residues.

Elution was carried out with 3 mL of 7% $\text{NH}_4\text{OH}/\text{MeOH}$, which disrupted the ionic and hydrophobic interactions between the analytes and the sorbent, releasing the target compounds into the eluent. The eluates were then evaporated under a gentle stream of nitrogen gas (N_2) at 40°C to concentrate the analytes while minimizing potential thermal degradation. The dried residues were reconstituted in 0.5 mL of the mobile phase to ensure compatibility with subsequent LC-MS analysis.

This optimized SPE protocol provided high recovery and precision, producing clean extracts and stable analyte responses that met SWGTOX validation criteria. The workflow improved reproducibility, reduced matrix effects, and maximized analyte detectability. Figure 3-12 illustrates the final optimized SPE procedure.

Table 3-14: The recovery% and RSD% of ADs and BZs extraction in different parameters, (n=3).

Diazepam					Fluoxetine				
Sorbent	matrices	pH	Recovery %	RSD%	Sorbent	matrices	pH	Recovery %	RSD%
HLB	H ₂ O	3	72.21	9.87	HLB	H ₂ O	3	90.38	3.64
HLB	H ₂ O	6	76.01	7.81	HLB	H ₂ O	6	94.76	3.61
HLB	H ₂ O	9	81.38	6.4	HLB	H ₂ O	9	87.26	3.02
MCX	H ₂ O	3	95.7	3.99	MCX	H ₂ O	3	82.02	7.62
MCX	H ₂ O	6	95.81	2.73	MCX	H ₂ O	6	83.43	5.84
MCX	H ₂ O	9	97.04	3.72	MCX	H ₂ O	9	81.99	6.22
MAX	H ₂ O	3	invalid	invalid	MAX	H ₂ O	3	70.77	9.04
MAX	H ₂ O	6	invalid	invalid	MAX	H ₂ O	6	84.77	9.15
MAX	H ₂ O	9	invalid	invalid	MAX	H ₂ O	9	72.05	8.74
HLB	SWW	3	79.98	9.99	HLB	SWW	3	76.73	9.96
HLB	SWW	6	78.34	8.84	HLB	SWW	6	86.83	3.78
HLB	SWW	9	83.07	6.01	HLB	SWW	9	87.31	3.09
MCX	SWW	3	97.76	3.45	MCX	SWW	3	84.7	5.19
MCX	SWW	6	91.39	2.49	MCX	SWW	6	76.63	9.69
MCX	SWW	9	90.96	2.4	MCX	SWW	9	83.78	6.25
MAX	SWW	3	invalid	invalid	MAX	SWW	3	invalid	invalid
MAX	SWW	6	invalid	invalid	MAX	SWW	6	57.78	6.52
MAX	SWW	9	invalid	invalid	MAX	SWW	9	invalid	invalid
Etizolam					Venlafaxine				
Sorbent	matrices	pH	Recovery %	RSD%	Sorbent	matrices	pH	Recovery %	RSD%
HLB	H ₂ O	3	78.16	6.14	HLB	H ₂ O	3	82.07	6.06
HLB	H ₂ O	6	84.75	5.72	HLB	H ₂ O	6	84.4	7.73
HLB	H ₂ O	9	83.74	8.78	HLB	H ₂ O	9	73.67	7.75
MCX	H ₂ O	3	93.9	2.71	MCX	H ₂ O	3	83.75	6.99
MCX	H ₂ O	6	93.62	3.14	MCX	H ₂ O	6	70.45	5.33
MCX	H ₂ O	9	95.43	3.32	MCX	H ₂ O	9	75.33	9.76
MAX	H ₂ O	3	invalid	invalid	MAX	H ₂ O	3	invalid	invalid
MAX	H ₂ O	6	invalid	invalid	MAX	H ₂ O	6	invalid	invalid
MAX	H ₂ O	9	invalid	invalid	MAX	H ₂ O	9	78.96	8.71
HLB	SWW	3	77.87	8.96	HLB	SWW	3	71.93	8.02
HLB	SWW	6	71.35	6.1	HLB	SWW	6	82.38	8.88
HLB	SWW	9	81.65	8.97	HLB	SWW	9	80.74	8.9
MCX	SWW	3	95.78	2.27	MCX	SWW	3	83.66	7.98
MCX	SWW	6	93.87	2.71	MCX	SWW	6	84.56	9.21
MCX	SWW	9	91.21	3.54	MCX	SWW	9	84.52	6.44
MAX	SWW	3	invalid	invalid	MAX	SWW	3	64.52	8.95
MAX	SWW	6	invalid	invalid	MAX	SWW	6	66.29	9.63
MAX	SWW	9	invalid	invalid	MAX	SWW	9	77.5	7.88

3.4.5. Stability Study

Stability testing was conducted to evaluate the integrity of analytes during storage and handling, following the recommendations of SWGTOX and the ICH guideline Q1A(R2) on the stability testing of new substances and products. These international protocols are essential to ensure that analyte concentrations remain reliable throughout the analytical workflow and accurately reflect real-world environmental and forensic sample handling conditions. [291,292] However, due to the restrictions and time limitations imposed by the COVID-19 pandemic, it was not possible to conduct the freeze–thaw stability study. Consequently, stability assessment was limited to short-term and post-preparative stability evaluations under controlled laboratory conditions. [291,292]

3.4.5.1. Antidepressants stability

The stability of FLX was evaluated under freezer (–20 °C, FZ), refrigerator (4 °C, CR), and room temperature (22 °C, RT) storage conditions over a 48-week period. At Day 0, recovery values were set at 100% for all conditions. During the initial period (Days 1-3), FLX remained highly stable, with recoveries between 92.2% and 99.3% across all conditions and RSD values ranging from 2.4% to 7.4%, indicating minimal degradation. By the end of Week 1, only a slight reduction was observed, particularly at room temperature, where recovery decreased to 85.2%, compared with 95.8% in the freezer and 88.2% in the refrigerator.

From Week 2 onwards, a more noticeable decline in stability was observed, particularly at elevated temperatures. At Week 2, recoveries were 86.8% (FZ), 80.9% (CR), and 75.5% (RT), while by Week 12 recoveries had decreased further to 73.0%, 61.1%, and 53.3%, respectively. This progressive degradation continued into the long-term phase, with recoveries by Week 24 reduced to 66.9% under freezer storage, 55.8% under refrigeration, and 40.8% at room temperature. At the final time point of Week 48, substantial degradation was evident, particularly at room temperature. FLX recoveries were 57.8% (FZ), 53.1% (CR), and only 25.6% (RT). When expressed as overall percentage decrease from Day 0 to Week 48, FLX

losses amounted to 42.2% under freezer storage, 46.9% under refrigeration, and 74.4% at room temperature. RSD values throughout the study remained within 2.0-7.6%, generally meeting SWGTOX acceptance criteria (<15%), although higher variability was consistently noted at room temperature.

These results confirm that FLX is relatively stable when stored under frozen or refrigerated conditions, but undergoes pronounced degradation at room temperature over extended periods. The marked decline observed at RT after 12 weeks and the steep drop to 25.6% by 48 weeks indicate that ambient storage is unsuitable for preserving sample integrity. This outcome is consistent with previous reports in pharmaceutical and forensic literature, which highlight the role of hydrolysis and oxidative pathways in the accelerated degradation of psychoactive drugs at elevated temperatures. [113,311,312]

In line with SWGTOX (2013) validation recommendations, these findings emphasise the need to establish sample storage protocols that preserve analyte integrity throughout the analytical workflow. Similarly, the ICH Q1A(R2) stability testing guidelines stress the importance of defining long-term storage conditions, recommending lower temperatures to minimise chemical instability. The present data reinforce these recommendations by showing that freezer storage offers the most reliable preservation of FLX, with losses limited to ~42% over 48 weeks, compared with ~74% degradation at room temperature, Table 3-15 and Figure 3-22.

Moreover, Future studies should extend stability assessments to FLX metabolites such as NFLX, which may follow different degradation kinetics and thus impact forensic interpretation in long-term storage scenarios.

Table 3-15: Rac-FLX and Rac-VEN recovery%, (n=3).

Drugs	FLX			VEN		
	FZ	CR	RT	FZ	CR	RT
Day 0	100	100	100	100	100	100
RSD%	5.20	5.20	5.20	4.12	4.12	4.12
day 1	99.34	98.80	94.19	101.88	96.85	93.44
RSD%	2.40	3.78	7.37	2.56	3.20	7.16
day 2	99.27	99.26	97.01	99.83	91.33	95.84
RSD%	2.61	3.73	5.96	2.20	3.11	8.08
day 3	96.67	94.44	92.22	102.14	95.34	92.07
RSD%	2.41	2.64	6.84	2.68	2.78	8.60
week 1	95.79	88.18	85.19	95.69	91.33	85.49
RSD%	1.85	3.42	7.45	2.75	2.53	2.60
week 2	86.84	80.87	75.53	96.20	87.93	74.48
RSD%	2.59	3.33	7.62	1.93	3.66	6.40
week 4	86.67	76.67	71.11	92.27	84.00	68.27
RSD%	2.07	3.61	5.65	2.38	2.52	5.80
week 8	81.11	69.22	66.67	90.00	79.65	54.10
RSD%	2.94	3.53	6.48	2.93	2.75	6.72
week 12	73.00	61.11	53.33	73.45	63.00	50.58
RSD%	2.27	2.86	6.21	3.28	3.07	7.33
week 24	66.89	55.78	40.78	66.20	51.93	41.69
RSD%	3.43	3.34	7.47	3.15	3.59	7.43
week 48	57.78	53.11	25.56	65.48	47.58	31.14
RSD%	3.56	3.56	6.59	2.93	2.79	8.16
Overall Decrease (%)	42.22	46.89	74.44	34.52	52.42	68.86

Regarding to FLX enantiomers (S-FLX and R-FLX) was investigated under similar conditions for up to 60 days. At Day 0, recovery values were set at 100% for all conditions. Both enantiomers remained close to 100% during the first three days across all storage conditions, with recoveries ranging from 87.6% to 108.7%. RSD values during this early phase were generally within acceptable limits (<15%), although higher variability was noted at certain points, particularly for S-FLX under refrigerated conditions (RSD 25.8% at Day 7) and at room temperature (RSD 64.8% at Day 19), Figure 3-23 and Figure 3-24 and Table 3-16.

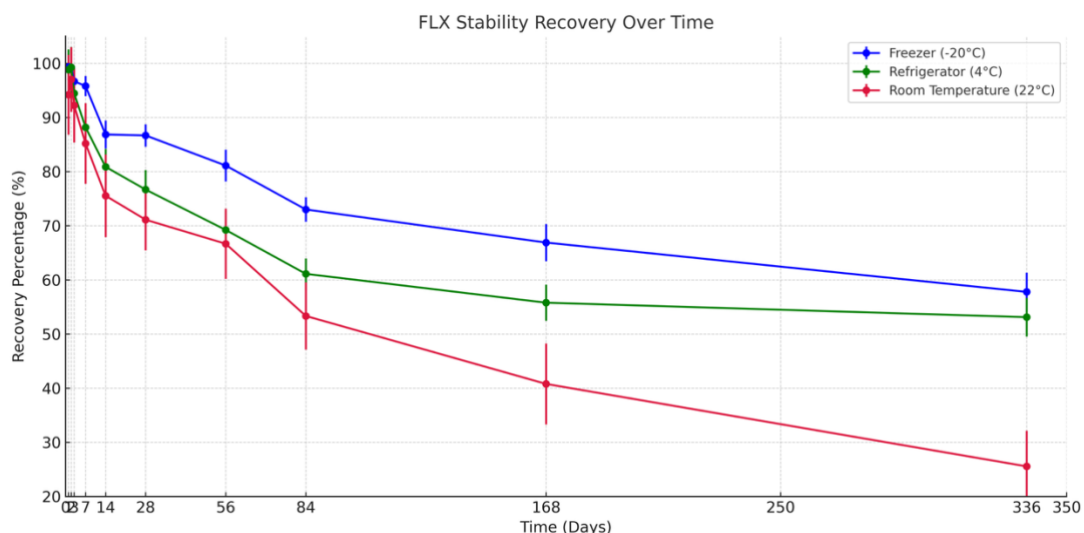


Figure 3-22: FLX stability recovery%.

By Day 30, recoveries declined further, with S-FLX ranging from 82–86% across conditions and R-FLX between 85–90%. At the final time point (Day 60), S-FLX exhibited recoveries of 80.4% (FR), 76.0% (RF), and 63.3% (RT), while R-FLX retained 86.1% (FR), 89.4% (RF), and 81.8% (RT). When expressed as overall decrease from Day 0 to Day 60, S-FLX losses amounted to 19.6% under freezer storage, 24.0% under refrigeration, and 36.3% at room temperature, whereas R-FLX showed smaller decreases of 13.9%, 10.6%, and 18.2%, respectively. These findings indicate that R-FLX is more stable than S-FLX, particularly under room temperature storage.

The higher susceptibility of S-FLX to degradation may be explained by stereoselective chemical reactivity, with the S-enantiomer more prone to hydrolysis or oxidative transformation than the R-form. Such stereoselective instability has been documented for other chiral pharmaceuticals, where enantiomers differ in their physicochemical properties, metabolic pathways, or interactions with environmental matrices. The greater variability (higher RSD) observed for S-FLX at room temperature further suggests that degradation pathways may be less predictable under ambient conditions.

[313]

These findings demonstrate that while both FLX enantiomers are relatively stable under frozen and refrigerated conditions for up to 60 days, degradation is accelerated at room temperature, with S-FLX particularly affected. The data align with SWGTOX guidelines, which recommend stability assessment for enantiomers independently, and with ICH Q1A(R2), which emphasises evaluating stability under multiple storage conditions. For forensic and environmental toxicology applications, freezer storage (-20°C) remains the preferred condition for preserving both enantiomers, although R-FLX shows greater resilience across all conditions. Future studies should extend stability testing to longer timeframes and include degradation product profiling to better understand stereoselective pathways. These results align with general pharmaceutical storage guidelines, which recommend lower temperatures to maintain drug stability. The European Medicines Agency highlights the importance of carefully defined storage conditions to preserve product quality over its shelf life. While specific studies on FLX enantiomer stability under these conditions are limited, the observed trend of increased degradation at higher temperatures is consistent with the known behaviour of chiral pharmaceuticals. [313]

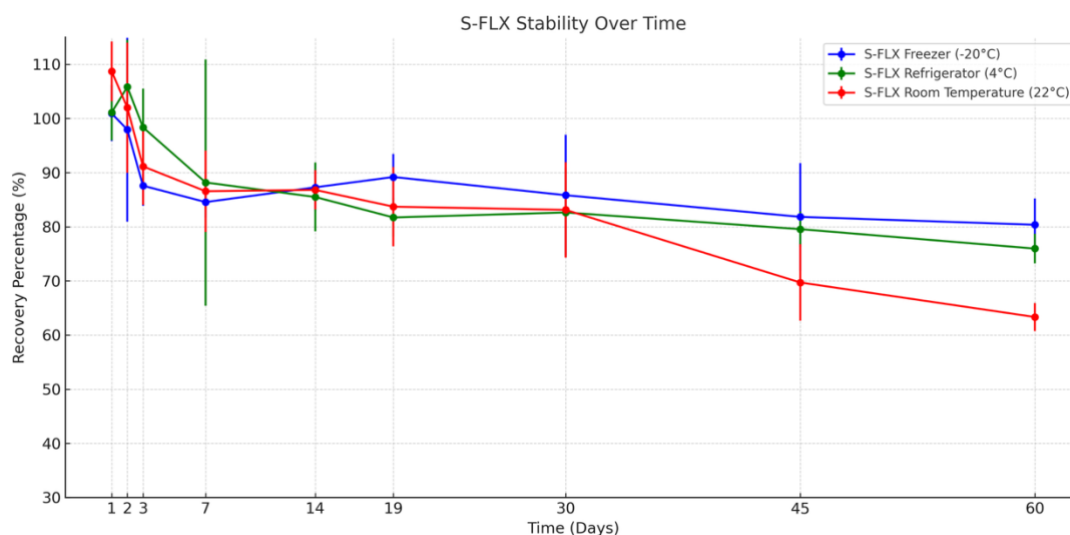
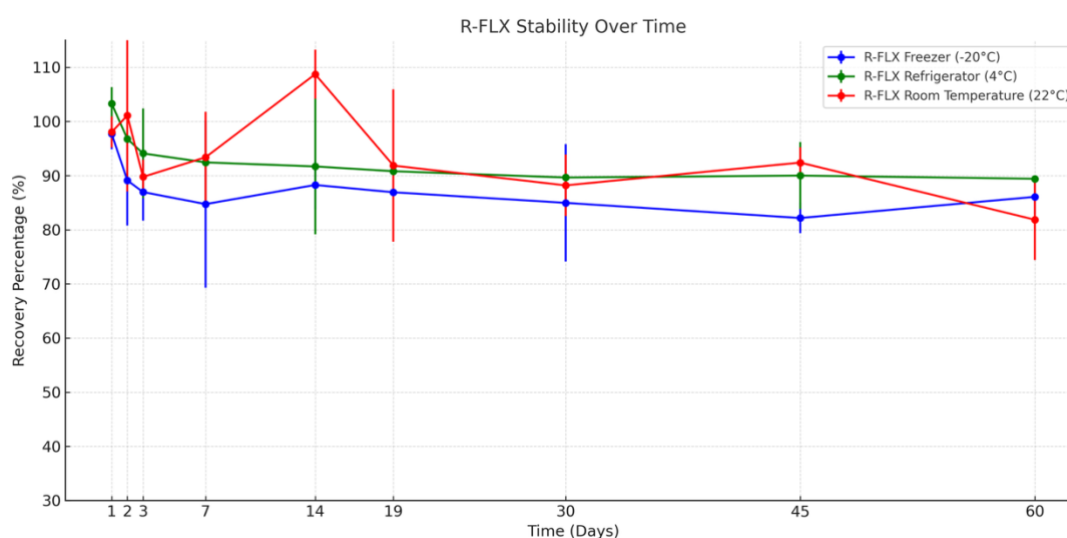


Figure 3-23: S-FLX recovery%.

Table 3-16: FLX enantiomers stability Recovery %, n=3.

Drugs	S-FLX			R-FLX		
Conditions	FR	RF	RT	FR	RF	RT
day 0	100	100	100	100	100	100
RSD%	7.23	5.82	8.42	3.12	3.12	3.12
day 1	100.91	101.11	108.69	97.70	103.29	98.03
RSD%	2.07	5.02	5.87	2.93%	5.82	8.42
day 2	97.92	105.82	101.98	89.10	96.77	101.11
RSD%	17.33%	8.17%	11.79%	9.36%	27.42%	13.88%
day 3	87.57	98.35	91.14	86.95	94.09	89.75
RSD%	4.22%	7.26%	7.80%	6.10%	8.80%	3.56%
day 7	84.55	88.17	86.55	84.73	92.43	93.38
RSD%	3.33%	25.80%	8.70%	18.23%	8.21%	8.99%
day 14	87.27	85.49	86.80	88.26	91.68	108.72
RSD%	4.99%	7.44%	4.19%	5.06%	13.70%	4.15%
day 19	89.18	81.71	83.70	86.91	90.80	91.84
RSD%	4.75%	4.34%	64.76%	23.22%	32.77%	15.32%
day 30	85.82	82.63	83.11	84.96	89.64	88.19
RSD%	12.99%	9.86%	10.59%	12.73%	28.74%	6.45%
day 45	81.82	79.55	69.70	82.16	90.00	92.39
RSD%	12.13%	4.10%	10.15%	3.42%	6.90%	3.06%
day 60	80.36	75.96	63.32	86.07	89.41	81.84
RSD%	6.02%	3.59%	4.07%	3.76%	63.50%	9.06%
Overall Decrease (%)	19.64	24.04	36.32	13.93	10.59	18.16

**Figure 3-24: R-FLX recovery%.**

The stability of NFLX enantiomers was evaluated over 60 days under same conditions. Recovery percentages were measured at multiple intervals, providing insights into the stability profiles of the enantiomers, Figure 3-25 and Figure 3-26.

For S-NFLX, the freezer condition ($-20\text{ }^{\circ}\text{C}$) demonstrated the greatest stability, with recoveries remaining above 76% throughout the study, starting at 101.5% on Day 1 and decreasing to 79.4% by Day 60. Under refrigerated storage ($4\text{ }^{\circ}\text{C}$), recoveries declined from 114.3% on Day 1 to 69.1% at Day 60, indicating moderate degradation. The elevated Day 1 value ($>100\%$) is likely attributable to analytical variability or matrix effects and was not excluded from the analysis, as the overall recovery trend was consistent with other replicates. At room temperature ($22\text{ }^{\circ}\text{C}$), recoveries dropped from 93.8% on Day 1 to 68.3% by Day 14, stabilising around this level thereafter, suggesting pronounced degradation under ambient conditions.

For R-NFLX, similar trends were observed. The freezer condition ($-20\text{ }^{\circ}\text{C}$) again provided the highest stability, with recoveries starting at 97.2% on Day 1 and declining to 81.3% by Day 60. At $4\text{ }^{\circ}\text{C}$, recoveries decreased from 102.7% on Day 1 to 74.7% by Day 60, showing a gradual loss of stability. At room temperature, recoveries fell from 102.5% on Day 1 to 66.7% at Day 60, again reflecting substantial degradation under ambient conditions.

Taken together, these results highlight that both enantiomers exhibit greater stability at lower temperatures, with freezer storage ($-20\text{ }^{\circ}\text{C}$) consistently providing the most reliable preservation. Notably, S-NFLX demonstrated greater instability at room temperature compared with R-NFLX, indicating potential stereoselective differences in degradation pathways, Table 3-17.

[314]

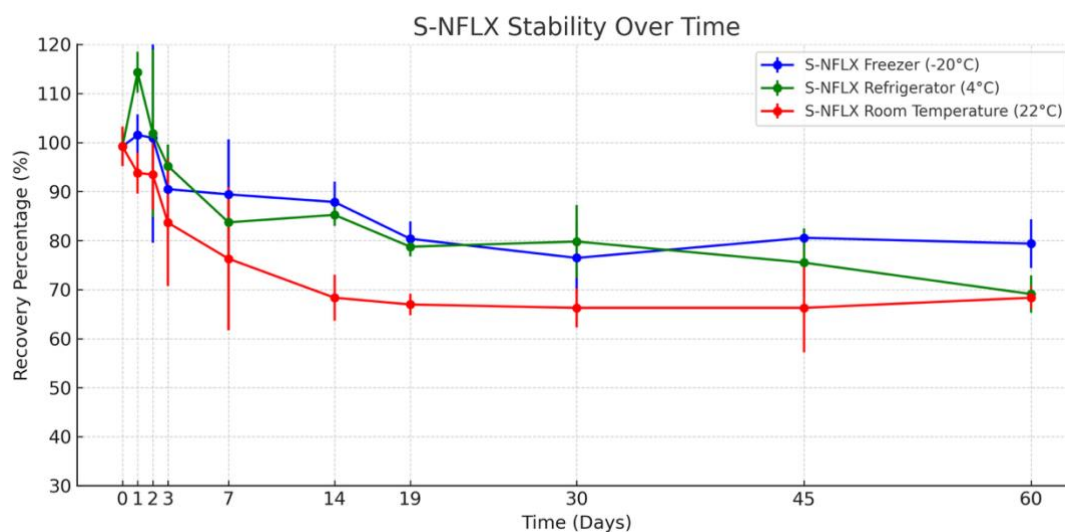


Figure 3-25: S-NFLX recovery%.

Table 3-17: NFLX enantiomers stability recovery % n=3.

Drugs	S-NFLX			R-NFLX		
Conditions	FR	RF	RT	FR	RF	RT
day 0	100	100	100	100	100	100
RSD%	12.01	12.01	12.01	8.02	8.02	8.02
day 1	101.52	114.32	93.77	97.23	102.65	102.49
RSD%	4.19%	4.19%	4.19%	5.93%	5.93%	5.93%
day 2	100.95	101.86	93.46	100.65	101.32	97.20
RSD%	21.39%	17.08%	7.29%	50.42%	10.04%	9.74%
day 3	90.49	95.20	83.65	90.68	95.22	94.08
RSD%	7.82%	4.36%	12.93%	18.43%	4.34%	9.29%
day 7	89.41	83.71	76.29	84.32	95.99	95.81
RSD%	11.25%	7.10%	50.59%	5.65%	58.98%	66.30%
day 14	87.86	85.23	68.34	82.84	86.12	83.12
RSD%	4.17%	2.24%	4.69%	7.04%	invalid	8.73%
day 19	80.36	78.74	66.96	86.86	90.85	90.14
RSD%	3.54%	1.97%	2.19%	6.49%	39.78%	3.29%
day 30	76.45	79.80	66.27	93.19	79.80	70.07
RSD%	7.11%	7.42%	3.99%	11.59%	8.10%	12.81%
day 45	80.56	75.49	66.27	91.59	74.32	70.36
RSD%	1.82%	6.96%	9.06%	8.45%	8.70%	6.40%
day 60	79.37	69.09	68.34	81.31	74.71	66.69
RSD%	5.00%	3.83%	2.21%	2.92%	8.39%	2.91%
Overall Decrease (%)	20.63	30.91	31.66	18.69	25.29	33.31

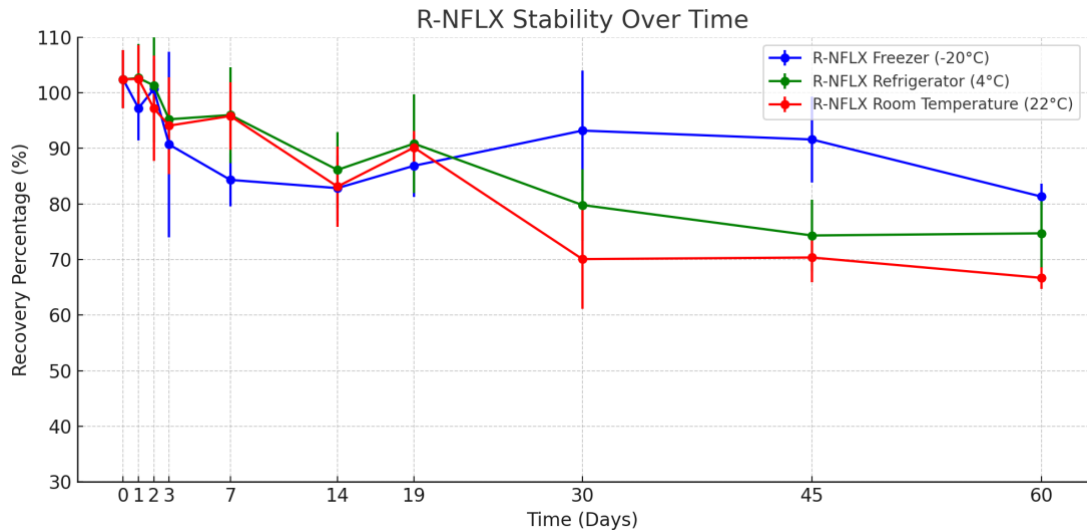


Figure 3-26: R-NFLX recovery%.

The stability of VEN was assessed under similar conditions over a 48-week period. At Day 0, recovery values were set at 100% across all conditions. During the early phase (Days 1-3), VEN remained highly stable, with recoveries ranging from 92.1% to 102.1%. RSD values were generally low (2.2–8.6%), meeting acceptance criteria, although slightly higher variability was noted under room temperature storage, Figure 3-27.

By Week 1, recoveries remained above 90% under freezer (95.7%) and refrigerated conditions (91.3%), while recovery at room temperature had declined to 85.5%. By Week 2, degradation became more evident, with recoveries of 96.2% (FZ), 87.9% (CR), and 74.5% (RT). The trend continued through Week 4, with recoveries of 92.3%, 84.0%, and 68.3%, respectively.

From Week 8 onwards, substantial degradation was observed, particularly under room temperature storage. At this stage, VEN recovery was 90.0% (FZ), 79.7% (CR), and 54.1% (RT). By Week 12, recoveries had decreased to 73.5%, 63.0%, and 50.6%. At longer intervals, the decline became more pronounced. At Week 24, recoveries were reduced to 66.2% (FZ), 51.9% (CR), and 41.7% (RT). At the final time point of Week 48, VEN retained 65.5% under freezer storage, 47.6% under refrigeration, and only 31.1% at room temperature, corresponding to overall decreases of 34.5%, 52.4%, and 68.9%, respectively.

RSD values across all intervals ranged from 1.9% to 8.2%, remaining within the SWGTOX acceptance threshold of <15% and confirming the consistency of the analytical method. The higher RSD values at room temperature reflect the increased variability associated with accelerated degradation under ambient storage.

These findings align with existing literature, which also reports temperature-dependent degradation of VEN. For example, immediate-release VEN suspensions stored at 23 °C retained ~99.9% of their original concentration after 28 days, while samples stored at 5 °C retained 100.5%, highlighting the protective effect of lower temperatures. Similarly, the development of stability-indicating methods for VEN has demonstrated the importance of robust analytical validation, with RSD values typically below 2% confirming high precision. [315,316]

This study demonstrates that VEN undergoes progressive degradation over extended storage, with the rate of decline strongly influenced by temperature. Freezer conditions (–20 °C) preserved analyte stability most effectively, while room temperature storage resulted in pronounced degradation and higher variability. These results underscore the importance of appropriate storage protocols for maintaining VEN's integrity in forensic and pharmaceutical contexts, in agreement with SWGTOX and ICH recommendations.

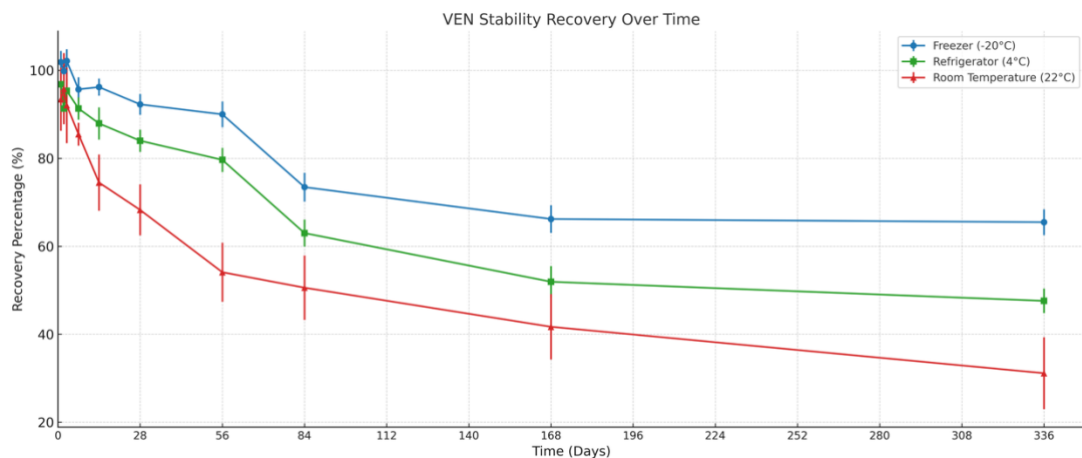


Figure 3-27: VEN stability recovery%.

3.4.5.2. Benzodiazepines stability

The stability of DIAZ, NDIAZ, OXAZ, and TEM was assessed under similar storage conditions over a period of 90 days. At Day 0, recovery values were set at 100% for all analytes across conditions.

All four benzodiazepines demonstrated relatively high stability during the initial days, with recoveries generally close to or above 90%. On Day 1, DIAZ exhibited recoveries of 92.2% (FR), 104.5% (RF), and 103.3% (RT), while NDIAZ showed slightly lower values (80.2 - 87.6%). OXAZ displayed values above 100% across conditions (109 -113%), likely reflecting matrix or ionisation variability rather than true concentration increases. TEM showed good stability with recoveries between 95.6% and 98.4%. By Day 7 and Day 14, moderate fluctuations were observed across analytes. Notably, OXAZ maintained >100% recoveries under freezer and refrigerated storage but began to decline at room temperature (101.2% to 102.2% at Day 14). In contrast, DIAZ and NDIAZ displayed moderate declines by Day 14, with recoveries ranging from approximately 86–94%. This decline is consistent with the reported thermal instability of benzodiazepines, particularly those containing N-4 oxides, 7-nitro groups, α -hydroxy ketones, and N-methyl- α -hydroxy ketones, which are more prone to thermal degradation, Table 3-18 and Figure 3-28. [317,318]

By Day 42, a more marked decline in stability was apparent. DIAZ recoveries decreased to 81.9% (FR), 75.2% (RF), and 92.0% (RT). NDIAZ showed recoveries of 84.9% (FR), 87.3% (RF), and 88.1% (RT), indicating moderate stability. OXAZ declined more steeply, particularly under room temperature storage, with recoveries of 66.2% (FR), 54.1% (RF), and 54.6% (RT). TEM also exhibited reduced stability at this point, with recoveries of 62.0–76.3%. By Day 60, DIAZ had degraded substantially, with recoveries of 46.2% (FR), 48.1% (RF), and 44.6% (RT). NDIAZ showed a similar trend, declining to 50.8% (FR), 42.0% (RF), and 47.5% (RT). In contrast, OXAZ displayed greater apparent stability at this time point, with recoveries of 81.9% (FR), 96.3% (RF), and 71.1% (RT). TEM also retained higher recoveries compared to DIAZ and NDIAZ (60.1–75.4%).

At 90 days, all four analytes had undergone extensive degradation, with recoveries converging around ~40-46% across conditions. DIAZ recoveries were 46.3% (FR), 44.0% (RF), and 42.7% (RT), while NDIAZ displayed values of 44.9% (FR), 43.1% (RF), and 45.0% (RT). OXAZ and TEM similarly decreased to ~40 - 49%, with only small differences between conditions at this late stage.

The results demonstrate that all four benzodiazepines are subject to progressive degradation over time, with temperature having a marked effect on stability in the short and mid-term phases. In the early phase, freezer and refrigerated storage offered better preservation, particularly for DIAZ and NDIAZ. OXAZ and TEM, however, displayed variable behaviour, with OXAZ appearing anomalously stable (recoveries >100%) in the early phase but declining more sharply by Day 42. By Day 90, all analytes had degraded to ~40-46% of their initial concentrations regardless of storage condition, indicating that extended storage even under frozen conditions leads to significant analyte loss.

These findings are consistent with reports in the literature describing benzodiazepines as moderately unstable over extended storage, especially in aqueous solutions, due to susceptibility to hydrolysis and photodegradation. The large variability observed for OXAZ in the early phase (>120% recoveries) likely reflects ion enhancement effects or analytical variability, emphasising the importance of replicate analysis and careful data interpretation. The relatively higher mid-term stability of OXAZ and TEM at Day 60 compared with DIAZ and NDIAZ suggests structural differences may confer varying resistance to degradation pathways. [319–321]

It is important to note that data became invalid for all drugs at week 4. This might be due to factors like analytical limitations or changes in the sample matrix that affected the accuracy of measurements. Figure 3-29 peaks separation of benzodiazepines.

From an environmental and toxicological perspective, these results highlight the critical importance of low-temperature storage to minimise analyte

degradation in the short term, although long-term storage beyond 60 days leads to substantial losses across all benzodiazepines. This aligns with SWGTOX and ICH) recommendations.

Table 3-18: DIAZ and main metabolites stability recovery %, n=3.

Drugs	DIAZ			NDIAZ			OXAZ			TEM		
Conditions	FR	RF	RT	FR	RF	RT	FR	RF	RT	FR	RF	RT
day 0	100	100	100	100	100	100	100	100	100	100	100	100
RSD%	3.15	11.58	4.92	4.05	4.05	4.05	7.04	7.04	7.04	2.12	2.12	2.12
day 1	92.22	104.52	103.31	80.19	87.58	82.14	109.32	109.7	103.27	95.55	95.66	98.36
RSD%	6.78	0.74	10.08	4.6	7.44	6.77	3.36	5.38	7.93	1.65	1.33	1.51
day 2	99.03	82.39	93.99	78.66	92.94	91.22	113.15	127.47	123.85	94.44	80.9	101.69
RSD%	9.29	1.87	7.86	8.9	9.61	6.59	6.13	7.04	11.15	11.43	5.81	9.48
day 3	96.21	92.15	103.75	99.52	89.88	86.88	110.42	105.04	100.34	88.8	94	90.3
RSD%	6.05	5.75	7.04	7.17	9.45	10.51	4.79	11.75	9.69	2.94	3.55	9.41
day 7	104.07	91.26	93.95	90.81	84.76	80.9	113.82	106.88	101.2	98.66	98.16	90.77
RSD%	7.12	4.64	5.53	5.84	2.16	11.36	5.89	6.51	5.27	4.61	11.35	8.34
day 14	102.38	86.54	94.53	90.12	87.91	67.07	99.54	105.29	102.22	93.27	93.72	92.4
RSD%	8.38	4.13	4.69	15	0.73	7.61	2.81	7.54	7.6	0.72	2.93	1.99
day 30	invalid	invalid	invalid	invalid	invalid	invalid	invalid	invalid	invalid	invalid	invalid	invalid
RSD%	invalid	invalid	invalid	invalid	invalid	invalid	invalid	invalid	invalid	invalid	invalid	invalid
day 42	81.97	75.24	92.04	84.91	87.33	88.06	66.24	54.06	54.56	62.04	76.29	69.14
RSD%	10.18	8.01	3.42	1.9	7.06	5.55	11.82	1.87	1.68	10.3	8.05	2.1
day 60	46.23	48.06	44.55	50.83	41.97	47.54	81.94	96.31	71.09	75.4	72.9	60.11
RSD%	3.79	2.77	4.75	9.57	5.87	3.32	1.88	2.34	1.78	11.81	11.73	5.89
day 90	46.26	44.04	42.71	44.9	43.07	45.03	48.91	46.63	40.22	44.29	43.58	45.84
RSD%	4.05	11.73	7.46	2.47	9.94	1.63	9.96	3.76	1.89	2.59	3.91	1.87

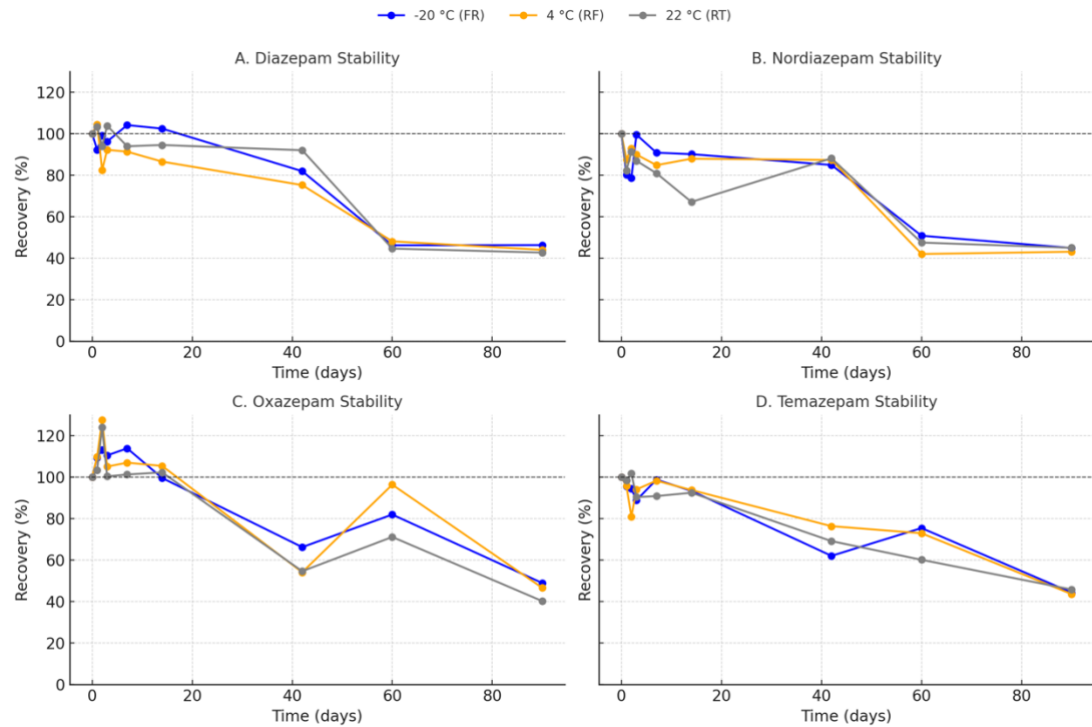
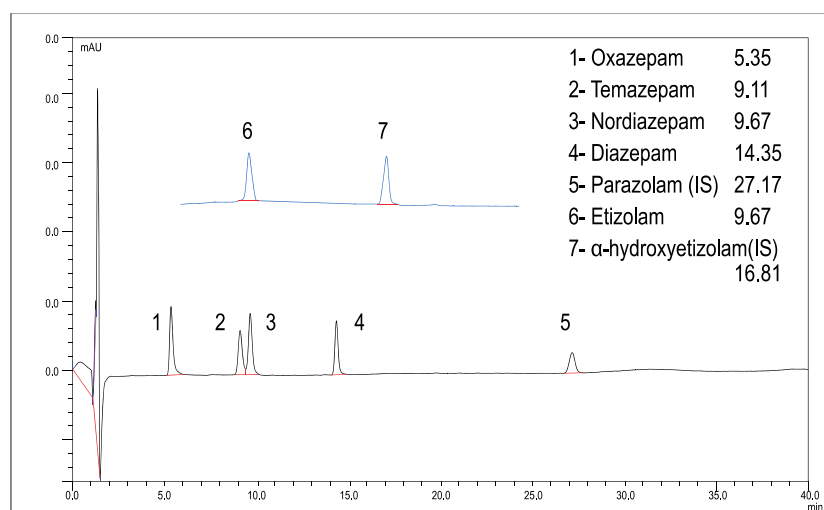
**Figure 3-28: DIAZ and the main metabolites stability results.**

Table 3-19: Etizolam standards stability recovery %, n=3.

Conditions	RT	RSD%	4C°	RSD%	-20C°	RSD%
day 0	100.00	0.80	100.00	0.59	100.00	0.26
day 1	97.39	0.13	96.53	1.12	103.41	0.19
day 2	98.10	23.75	96.53	0.59	104.04	0.16
day 3	99.50	6.73	98.13	11.15	104.21	0.62
day 8	103.01	3.22	111.69	1.02	114.34	1.64
day 14	97.62	0.70	95.07	2.23	103.79	2.36
day 21	98.10	1.37	103.34	1.37	103.44	0.54
day 28	95.60	2.96	99.05	3.54	102.12	1.98
day 35	94.18	6.42	91.85	1.49	98.79	0.61
day 42	96.23	3.69	92.03	7.12	95.41	5.57
day 60	88.38	4.03	94.32	2.86	97.37	7.08

**Figure 3-29: Etizolam and DIAZ, main metabolites and ISD peaks.**

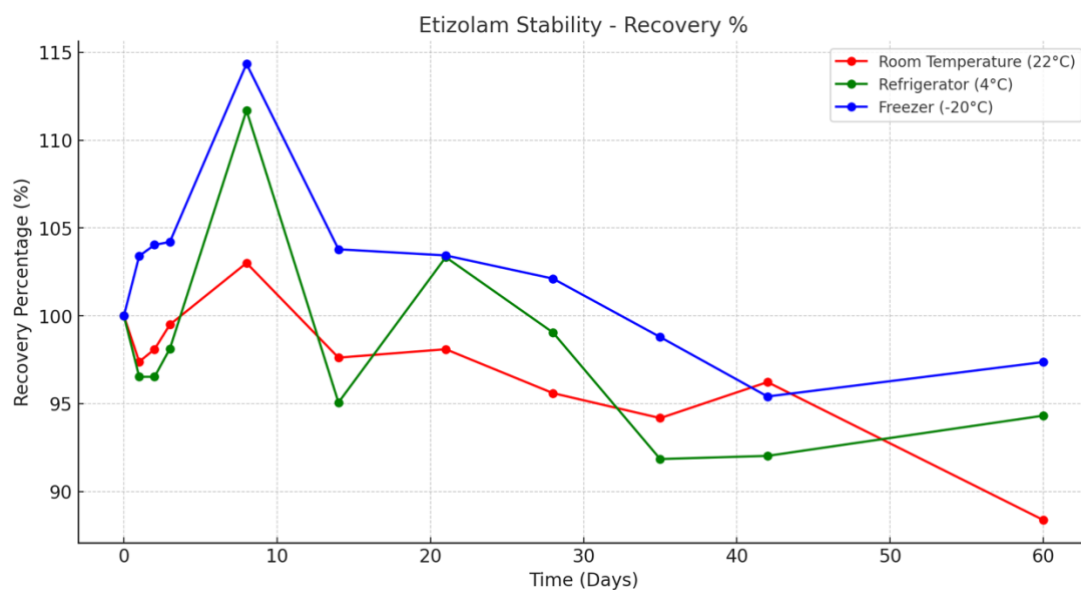


Figure 3-30: Etizolam recovery%.

The stability of etizolam in methanol varies with storage temperature, as evidenced by recovery percentages and RSD over a 60-day period. At room temperature (22°C), the recovery percentage declines to approximately 88.38% by day 60, with notable variability (RSD peaking at 23.75% on day 2), indicating significant degradation and imprecision. In refrigerated conditions (4°C), recovery decreases to 94.32% by day 60, with moderate RSD values generally below 7.12%, suggesting better stability than at room temperature but still some degradation. Storage in a freezer (-20°C) maintains recovery percentages above 97.37% throughout the 60 days, with the lowest RSD values, reflecting optimal stability and precision. It can be shown at the Table 3-19 and Figure 3-30.

These findings align with existing literature on benzodiazepine stability. For instance, a study on the long-term stability of benzodiazepines in blood reported that compounds like DIAZ and OXAZ exhibited minimal degradation (0–10%) over six months when stored at freezing temperatures, whereas significant losses occurred at room temperature. [322] Additionally, certified reference materials of etizolam in methanol are recommended to be stored frozen to ensure stability, Figure 3-30.

3.5. Conclusion

The method development and validation processes in this study demonstrated a systematic approach to optimising conditions for environmental studies, chiral separation, and the stability evaluation of targeted compounds. By addressing critical parameters such as pH, temperature, dissolved oxygen (DO), and total suspended solids (TSS), the environmental study successfully established optimal conditions for microbial activity and biodegradation efficiency in synthetic wastewater systems. The findings underscore the importance of maintaining a favourable pH range (7.5-8.0), room temperature ($22^{\circ}\text{C} \pm 1^{\circ}\text{C}$), and adequate DO levels (1.4-2.4 mg/L) for aerobic reactors. Monitoring these parameters ensures robust microbial performance and reliable biodegradation processes.

The chiral separation study effectively optimized chromatographic conditions to enhance enantiomeric resolution for antidepressants and benzodiazepines. Adjusting mobile phase compositions, such as ethanol-methanol ratios and buffer pH, significantly improved separation outcomes. For instance, increasing ethanol content and maintaining a buffer pH of 6 facilitated superior resolution for FLX and NFLX enantiomers. The implementation of extended chiral columns (e.g., 25 cm) further improved enantioselectivity. However, the study revealed challenges in resolving certain compounds, such as OXAZ and VEN, highlighting the need for additional column and mobile phase optimizations. These findings demonstrate the intricacies of chiral separation and the necessity of tailored approaches for different analytes.

The stability study provided valuable insights into the long-term preservation of targeted compounds under various storage conditions. Freezing at -20°C consistently offered the best stability for antidepressants (e.g., FLX and NFLX) and benzodiazepines (e.g., DIAZ and TEM), with recovery percentages exceeding 90% over extended periods. Refrigerated conditions (4°C) provided moderate stability, while room temperature (22°C) resulted in significant degradation, particularly for compounds like NDIAZ and OXAZ. These observations align with existing literature, confirming the importance of

cold storage for preserving the integrity of such compounds in analytical studies.

The study's method validation steps ensured the reliability of the developed techniques. Calibration curves with high correlation coefficients ($R^2 > 0.99$) demonstrated excellent linearity across tested concentration ranges. The LOD and LOQ further validated the sensitivity of the methods. SPE optimization identified the MCX cartridge as the most effective for benzodiazepines, while HLB cartridges were preferred for antidepressants. Recovery rates and precision across different matrices reinforced the robustness of the extraction.

Chapter 4. Biodegradation study of antidepressants in the environmental matrix using SPE-LC-MS

4.1. Introduction

In recent years, the presence of pharmaceuticals and drugs of abuse in the environment has raised significant concerns for both ecological integrity and human health. [323,324] The improper disposal and excretion of these chemicals have led to their detection in water sources, emphasizing the need for better management as our society becomes increasingly reliant on prescription drugs and illicit substances. [323–325] Pharmaceuticals, including hormones, antidepressants, and antibiotics, are frequently found in water bodies, prompting concerns about potential environmental effects and the emergence of antibiotic resistance. Additionally, illicit substances including cocaine, amphetamines, and opioids enter the environment through wastewater discharge and improper disposal, posing risks to aquatic life and ecosystem stability. [323,325] Understanding the fate, transport, and impacts of these pollutants is crucial for effective environmental protection and conservation.

4.2. Aim and objectives

To achieve a novel analytical toxicology sample investigation strategy, additional methods must be developed to provide quantification and green analytical chemistry while adhering to the validation requirements for controlled and illegal synthetic antidepressants. Consequently, the following are the objectives of the study:

- To investigate the biodegradation of selected antidepressants in synthetic wastewater by quantifying parent compound removal, degradation rate constants, and the formation of transformation products in bioreactor systems under environmentally relevant conditions.
- To identify and evaluate key environmental and operational factors influencing antidepressant biodegradation in synthetic wastewater, including biotic and abiotic processes.

- To assess the role of microbial communities in antidepressant biodegradation by characterizing dominant bacterial taxa using 16S rRNA gene sequencing and correlating microbial abundance with degradation efficiency.
- To develop and validate a chiral SPE-LC-MS method for the separation and quantification of fluoxetine, norfluoxetine, venlafaxine and ODV in wastewater matrices.
- To investigate enantioselective degradation and transformation pathways of antidepressant enantiomers under controlled biotic and abiotic conditions in synthetic wastewater

4.3. Materials and methods

4.3.1. Chemicals and reagents

All the ADs standards and deuterium standards at the concentration of 1 mg/mL, and 0.1 mg/mL, respectively, were purchased from the supplier mentioned in section 3.3.2.

4.3.2. General laboratory equipment

All the general laboratory equipment were mentioned in 3.3.1.1.

4.3.3. Preparation of internal standards

Internal standards Table 3-4 at the concentration of 100 µg/mL and the preparation method mentioned in section 3.3.3.3.

4.3.4. Preparation of working solutions

Working solutions of 10 µg/mL were monthly prepared in MeOH and the preparation method mentioned in section 3.3.3.2.

4.3.5. Preparation of synthetic wastewater (SWW)

SWW preparation method mentioned in section 3.3.4.1.

4.3.6. Preparation of the bioreactors

The bioreactor setup used in this study was previously described in Section 0. and was applied consistently in the experiments full technical details are available in Chapter 3.

4.3.7. pH monitor

In each day, during the study all the study stages, 3mL of the reactor were collected to measure pH by using pH meter.

4.3.8. Optical density

A 3 mL of cultivated samples were collected daily and transferred to a 10 mm cuvette. The spectrophotometer was set up at 600 nm to obtain optimum results of growth rate of bacteria. [270]

4.3.9. Total Suspended Solids

The concentration of TSS in the bioreactor and environmental water samples was determined following a modified procedure based on APHA 2540 D.

[263] The modification involved adjusting the sample volume to accommodate high-solids microreactor systems.

Glass-fiber filters (GF/F, pore size 0.45 μm) were cleaned, rinsed with deionized water, and placed in pre-weighed glass or aluminum dishes. Filters were dried in an oven at 104 ± 1 °C for 1 h (or overnight for enhanced drying), cooled in a desiccator for 30 min, and weighed using an analytical balance with a precision of ± 0.1 mg to record the initial weight (W_1).

Each sample was thoroughly mixed to ensure uniform suspension. An aliquot of 2 mL (or an adjusted volume depending on the expected TSS concentration) was filtered through the pre-weighed filters using a vacuum or gravity filtration system. Highly turbid samples were diluted appropriately, and the dilution factor was recorded. Filtration was performed in triplicate to improve analytical precision and reproducibility.

After filtration, the filters containing retained solids were re-dried at 104 ± 1 °C for 24 h (or overnight for consistency), cooled again in a desiccator for 30 min, and re-weighed to determine the final weight (W_2).

The TSS concentration was calculated according to Equation 3-2. The average of triplicate measurements was reported as the final TSS value. This method ensured accurate quantification of suspended matter by maintaining controlled drying temperatures, preventing moisture interference during weighing, and applying replicate measurements for improved accuracy and reproducibility. [263,264]

4.3.10. LC-MS method

In this study, chiral LC-MS analysis was employed to quantify and characterize the biodegradation and biotransformation of selected antidepressants and their enantiomers. The analytical separation was achieved using an Astec Chirobiotic V2 column (250 mm $\times$ 2.1 mm I.D., 5 μm

particle size) under isocratic conditions, optimized to enhance enantioselective resolution. The mobile phase consisted of 30% methanol, 30% ethanol, and 40% water containing 4 mM ammonium acetate, at a flow rate of 0.5 mL/min and column oven temperature of 20-25 °C. An injection volume of 20 µL was used.

Detection was performed using positive electrospray ionization (ESI⁺) mode, with a UV detector range of 200-400 nm and mass spectrometric detection optimized for antidepressant compounds. Ion source parameters are summarized in Table 4-1 and Table 4-2.

All instrument parameters were optimized to ensure high sensitivity and reproducibility across analyses. Method performance (linearity, precision, and recovery) was validated using calibration standards and quality-control samples.

Table 4-1: LC parameters

LC	Parameters
Flow rate	0.5 mL/min
Oven temp.	20-25°C
Injection volume	20 µL
Solvent A%	30% MeOH, 30% Ethanol, 40% H ₂ O and 4mM Ammonium Acetate
Solvent B%	0%
Mode	Isocratic
UV detector	Start Wavelength 200 nm End Wavelength 400 nm

Table 4-2: Mass spectrometry parameters (ESI-positive mode).

Ion Source Parameters	
Settings for tune mix using source type ESI Positive.	
Parameter	Value
Polarity	Positive
Capillary Temperature	250 °C
Capillary Voltage	180 V
Source Voltage Offset	20 V
Source Voltage Span	30 V

4.4. Results and discussion

4.4.1. Environmental Monitoring Parameters

4.4.1.1. TSS study

The analysis of TSS in the biotic and abiotic bioreactors over a 14-day experimental period revealed distinct variations among the tested compounds. TSS measurements were used to monitor microbial proliferation and particulate formation during the biodegradation process. The Day 0 TSS values represented baseline background solids present in the medium prior to microbial inoculation, rather than generated biomass.

In the biotic control (free-drug bioreactor), TSS increased substantially from 90 mg/L on Day 0 to 198 mg/L on Day 14, reflecting active microbial growth and particulate accumulation in the absence of pharmaceutical exposure. This established a reference for natural microbial activity within the system.

In comparison, fluoxetine-amended bioreactors exhibited only a modest increase in TSS, from 205 mg/L to 220 mg/L, suggesting that the high initial TSS reflected pre-existing particulates from drug solubilisation, and that subsequent microbial stimulation or particulate generation was limited. The smaller relative increase implied that microbial activity reached an early equilibrium due to the compound's initial concentration and medium conditions. A marked enantioselective effect was observed when comparing individual enantiomers.

The S-fluoxetine bioreactor showed the most significant TSS rise, from 26.7 mg/L to 259.1 mg/L, indicating strong microbial proliferation or particulate formation associated with this enantiomer. The R-fluoxetine bioreactor also exhibited a substantial increase, from 86.7 mg/L to 248.7 mg/L, though slightly less pronounced than the S-form. These results suggest that S-fluoxetine stimulated greater microbial activity, likely due to preferential biodegradation or stereospecific microbial affinity, consistent with reports of enantioselective biotransformation of chiral antidepressants in environmental systems. [32,326]

Regarding the metabolites, NFLX exhibited a moderate TSS increase from 70 mg/L to 114 mg/L, indicating limited microbial or particulate response. However, both enantiomers, S-norfluoxetine and R-norfluoxetine-produced substantial TSS increases, from 46.7 mg/L to 207.4 mg/L and 45 mg/L to 220 mg/L, respectively. This pattern suggests that the biotransformation products also supported microbial proliferation, with both enantiomers exerting comparable influences on biomass accumulation.

The observation that both S- and R-enantiomers of fluoxetine and norfluoxetine produced higher increases in TSS than their racemic mixtures indicates enantioselective interactions within the bioreactor environment. This behaviour likely arises from differences in biodegradation kinetics and microbial affinity toward each enantiomer. Studies have shown that microbial enzymes often display stereoselectivity, favouring one enantiomer over the other as a substrate, which can lead to differential rates of transformation, biomass proliferation, and particulate generation. [326,327]

Several factors could contribute to the observed differences in TSS, including the compound's biodegradability, hydrophobicity, and sorption characteristics, as well as variations in microbial community composition, pH, and nutrient availability. The more hydrophobic enantiomers (e.g., S-fluoxetine) may preferentially adsorb to biomass surfaces, enhancing microbial colonisation and apparent TSS increases, whereas more polar forms may remain in solution. These combined processes result in enantioselective particulate accumulation within the bioreactor.

While the enantiomer-specific effects on TSS are evident, it is important not to attribute these changes solely to fluoxetine degradation without considering other influencing factors such as biofilm development, extracellular polymeric substance (EPS) production, and microbial aggregation. Notably, norfluoxetine and its enantiomers caused greater TSS increases than fluoxetine, suggesting that the metabolite may exert a stronger stimulatory effect on microbial growth or particulate formation, possibly due to its enhanced bioavailability and lower toxicity.

Furthermore, compounds with lower initial TSS levels (e.g., S-fluoxetine) exhibited more pronounced relative increases during the 14-day period, while those with higher initial TSS levels (e.g., racemic fluoxetine) showed smaller proportional changes. These findings highlight that both enantioselectivity and initial reactor conditions must be considered when interpreting TSS variations, reinforcing the complexity of chiral pharmaceutical interactions in biotic degradation systems, (Table 4-3).

Table 4-3: Presents the TSS results of antidepressants and their metabolites and enantiomers, (n = 3).

Compound	Day 0			Day 14		
	mean mg/L	SD	RSD%	mean mg/L	SD	RSD%
Free drug bioreactor	90.00	10.00	11%	198.33	59.65	30%
Rac-Fluoxetine	205.00	55.00	27%	220.37	55.18	25%
S-fluoxetine	26.67	5.77	22%	259.05	6.87	3%
R-fluoxetine	86.67	25.17	29%	248.72	16.81	7%
Rac-Norfluoxetine	70.00	10.00	14%	114.29	37.72	33%
S-norfluoxetine	46.67	5.77	12%	207.44	56.74	27%
R-norfluoxetine	45.00	5.00	11%	220.00	37.12	17%
Rac-Venlafaxine	40.00	0.00	2%	446.03	62.10	14%
S-VEN	140.00	10.00	7%	203.81	36.16	18%
R-VEN	252.50	47.50	19%	200.00	26.96	13%
Rac-ODV	80.00	25.00	25%	60.51	6.73	11%
S-ODV	175.00	25.00	14%	221.57	20.66	9%
R-ODV	230.00	10.00	4%	212.24	26.30	12%

TSS was determined by gravimetric filtration and drying in accordance with Standard Methods 2540 D (APHA/AWWA/WEF). [263]

VEN exhibited a dramatic increase in TSS, rising from 40.00 mg/L to 446.03 mg/L, with the RSD% increasing from 2% to 14%. This pronounced elevation indicates intense microbial proliferation and particulate generation within the bioreactor. The increase in RSD over time reflects the growing heterogeneity of biomass and particle distribution as microbial consortia adapted to the presence of VEN, leading to variable accumulation patterns across replicates. [328,329]

The enantiomers of VEN displayed distinct and contrasting trends, confirming enantioselective interactions between the compounds and the microbial environment. The S-VEN bioreactor showed a moderate TSS increase, from

140.00 mg/L to 203.81 mg/L, accompanied by a rise in RSD from 7% to 18%. This pattern suggests that the S-enantiomer moderately stimulated microbial growth and sorption onto suspended particles but introduced variability in biomass distribution, possibly due to uneven microbial utilisation or differences in bioavailability.

Conversely, R-VEN demonstrated a decrease in TSS, from 252.50 mg/L to 200.00 mg/L, with RSD declining from 19% to 13%, indicating a stabilisation of the system over time. The reduction in TSS may result from enhanced biodegradation or weaker adsorption of R-VEN onto biomass compared to its S-form. Such divergence between enantiomers aligns with previously documented enantioselective biodegradation behaviour of chiral antidepressants, where microbial enzymes preferentially transform one optical isomer due to differences in stereochemistry, hydrophobicity, and interaction with active sites. [330–332]

These findings suggest that the racemic VEN mixture induced substantial overall biomass proliferation, while its individual enantiomers exhibited opposing particulate dynamics - S-VEN enhancing biomass accumulation and R-VEN contributing to reduced suspended matter. This behaviour supports the concept that stereochemistry influences biodegradation kinetics and sorption equilibria within bioreactor systems, with S-VEN showing greater persistence and microbial interaction potential.

ODV exhibited an overall decrease in TSS from 80.00 mg/L to 60.51 mg/L, with the RSD decreasing from 25% to 11%, reflecting improved measurement precision and a reduction in microbial or particulate levels within the bioreactor. This observation suggests that, unlike other antidepressants, racemic ODV may have contributed less to microbial proliferation or biomass generation, possibly due to its limited bioavailability or slower microbial transformation rate.

The enantiomers of ODV, however, displayed distinct and more variable behaviours. The S-ODV bioreactor showed an increase in TSS from 175.00 mg/L to 221.57 mg/L, accompanied by a decrease in RSD from 14% to 9%,

indicating that microbial growth and particulate formation became more uniform over time. This improvement in precision may be attributed to the progressive adaptation of microbial consortia to the S-ODV substrate, leading to more consistent biodegradation and biomass production across replicates.

In contrast, the R-ODV bioreactor demonstrated a slight decrease in TSS, from 230.00 mg/L to 212.24 mg/L, while RSD increased from 4% to 12%, suggesting greater heterogeneity and reduced consistency in system performance over time. This variability could stem from uneven sorption behaviour or lower microbial affinity for the R-enantiomer, which is generally less biodegradable than the S-form. Additionally, some fluctuations in replicate measurements may have resulted from minor pipetting or sample-handling errors during TSS preparation or measurement, which are known sources of analytical uncertainty in bioreactor studies involving low-concentration environmental samples. [262,264]

Such enantioselective differences in TSS trends are consistent with earlier findings on chiral antidepressants, where one enantiomer stimulates microbial proliferation while the other undergoes slower or inconsistent transformation, resulting in differential biomass formation and suspended solids accumulation. Overall, these findings emphasise that both stereochemical configuration and experimental precision critically influence biodegradation kinetics and particulate dynamics in bioreactor systems.

4.4.1.2. pH measurement and OD

A. pH measurements of FLX and its metabolite

The provided data show the pH values of the biotic and abiotic reactors and the optical density OD readings taken daily during the 14-day biodegradation study of FLX and its enantiomers (S-FLX and R-FLX), as well as NFLX and its enantiomers (S-NFLX and R-NFLX), Table 4-4.

Throughout the 14-day experimental period, both the biotic and abiotic systems exhibited variations in pH, but the biotic bioreactor consistently maintained a more stable pH than the abiotic (SWW). This stability can be attributed to microbial buffering capacity, where bacteria regulate pH through

metabolic processes such as organic acid production, nutrient assimilation, and proton exchange mechanisms. In contrast, the abiotic SWW system, lacking microbial regulation, exhibited larger pH fluctuations caused by chemical interactions such as hydrolysis and CO₂ absorption from the atmosphere.

For instance, in the case of FLX, a slight decrease in pH was observed in both systems over the 14 days, but the bioreactor showed low variability (RSD%), confirming steady-state microbial conditions. Conversely, the abiotic SWW displayed higher RSD values, indicating unstable chemical equilibrium. A similar pattern was observed for S-FLX and R-FLX, where the biotic system maintained a more consistent pH profile, while the abiotic reactors showed irregular shifts, likely due to the absence of microbial buffering. The same trend was evident for NFLX and its enantiomers (S-NFLX and R-NFLX). [333–335]

These results confirm that pH stability in the biotic system was driven by microbial activity, which moderated local acid-base changes, while abiotic systems were more chemically reactive. Since pH strongly influences drug ionisation, solubility, and microbial enzyme activity, maintaining stable pH conditions was critical for reliable biodegradation and enantioselectivity throughout the experiment. [334]

The OD measurements provided complementary information on bacterial growth dynamics during the biodegradation process. Overall, OD values increased progressively in both the SWW and biotic reactors, confirming microbial proliferation and adaptation to the pharmaceutical substrates. However, the rate and pattern of increase differed between systems and compounds, reflecting variations in biodegradability and microbial utilisation efficiency.

In the biotic bioreactors, OD increased steadily after an initial lag phase (Days 0–3), representing microbial acclimatisation to the drug-enriched environment. The sharp OD rise observed between Days 4 and 10 corresponded to the exponential growth phase, where active biodegradation

likely occurred. Reactors containing S-FLX and S-NFLX displayed the most pronounced OD increases, consistent with their greater biological affinity and higher biodegradation rates. By contrast, R-enantiomer reactors showed slower OD progression, suggesting reduced microbial uptake or mild inhibitory effects.

In the abiotic systems, only minor OD changes were detected, confirming that observed optical increases in biotic reactors were due to biological growth rather than abiotic particle formation. By Day 14, OD values plateaued in most reactors, indicating the stationary phase, where substrate depletion and waste accumulation limited further microbial proliferation.

The correlation between stable pH and increasing OD reinforces that microbial metabolism played a central role in both maintaining reactor conditions and facilitating enantioselective degradation. These combined results demonstrate that biotic systems not only supported sustained microbial activity but also exhibited stereochemical selectivity, with S-enantiomers generally promoting greater microbial adaptation and biomass growth. [269,270]

B. pH measurements of VEN and its metabolite

The pH values of biotic and abiotic reactors, along with the optical density OD measurements for bacteria, taken daily during the biodegradation study of VE) and its enantiomers S-VEN and R-VEN and ODV and its enantiomers S-ODV and R-ODV, Table 4-5.

Across the 14-day incubation, pH varied between the abiotic (SWW) and biotic (bioreactor) systems, with the biotic reactors showing a more stable pH profile (lower %RSD) for most compounds. This stability is consistent with biological buffering and alkalinity-mediated control of acidity generation/consumption in mixed microbial systems, where biochemical processes (e.g., nitrification and carbon transformations) can moderate net pH drift when buffering capacity is sufficient. In contrast, abiotic SWW lacks biological buffering, so small shifts in dissolved constituents (e.g., CO₂

exchange, ionic strength, matrix composition) can translate into greater short-term pH variability. [334,336,337]

Compound-specific trends were modest but informative. For example, VEN showed a slight pH decrease in both systems, but the bioreactor maintained lower variability, consistent with a comparatively stable biotic matrix. Because pH governs (i) speciation of ionisable pharmaceuticals and (ii) microbial enzyme activity and community performance, differences in pH stability between biotic and abiotic reactors should be considered when interpreting degradation rates and any apparent enantioselective behaviour. [338]

The same trend was observed for the enantiomers S-VEN and R-VEN, where the biotic system displayed a more consistent pH compared to the abiotic system. The presence of bacteria in the bioreactor likely played a role in maintaining a stable pH environment. A similar pattern can be seen for ODV and its enantiomers, S-ODV and R-ODV. The biotic system showed a more constant pH, while the abiotic system exhibited fluctuations. Again, this indicates the impact of microbial activity on pH regulation in the bioreactor. Regarding the OD measurements for bacteria, they provide information on bacterial growth and activity. Throughout the 14-day study, the OD values generally increased in both the SWW and the bioreactor, suggesting bacterial proliferation and metabolic activity. [338]

Table 4-4: Environmental monitor results of ADs (SSRIs), n=3.

Drugs		Blank			FLX			S-FLX			R-FLX			N-FLX			S-NFLX			R-NFLX		
	Test	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD
Day 0	Mean	8.03	8.02	0.03	8.16	8.07	0.03	8.08	7.97	0.05	8.08	7.99	0.05	7.60	7.53	0.04	8.06	8.02	0.02	8.08	8.03	0.03
	SD	0.23	0.29	0.00	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.04	0.01	0.01	0.01	0.01	0.02	0.01	0.00	0.02	0.02	0.00
	RSD%	2.89	3.60	14.10	0.12	0.00	8.47	0.07	0.07	12.89	0.12	0.51	17.68	0.15	0.15	19.09	0.21	0.14	5.59	0.29	0.25	0.00
Day 1	Mean	8.44	8.40	0.13	8.40	8.21	0.15	8.48	8.25	0.16	8.47	8.26	0.14	8.21	8.12	0.13	8.53	8.36	0.08	8.53	8.37	0.09
	SD	0.16	0.21	0.03	0.01	0.01	0.04	0.01	0.02	0.01	0.01	0.04	0.02	0.01	0.02	0.00	0.02	0.02	0.00	0.02	0.02	0.02
	RSD%	1.92	2.53	24.94	0.07	0.12	26.65	0.07	0.19	8.74	0.12	0.46	16.67	0.12	0.19	3.01	0.24	0.24	5.25	0.18	0.18	22.66
Day 2	Mean	8.37	8.55	0.10	8.82	8.78	0.15	8.48	8.47	0.15	8.45	8.47	0.16	8.26	8.45	0.12	8.50	8.61	0.13	8.51	8.57	0.15
	SD	0.29	0.14	0.01	0.11	0.03	0.01	0.01	0.01	0.02	0.06	0.01	0.01	0.04	0.05	0.02	0.01	0.05	0.01	0.01	0.01	0.05
	RSD%	3.50	1.63	12.26	1.25	0.30	4.83	0.12	0.12	16.44	0.72	0.07	5.76	0.50	0.53	18.24	0.14	0.58	4.93	0.12	0.07	32.03
Day 3	Mean	8.46	8.57	0.08	8.38	8.66	0.14	8.48	8.53	0.15	8.48	8.56	0.15	8.21	8.31	0.09	8.51	8.62	0.11	8.50	8.62	0.11
	SD	0.16	0.18	0.01	0.03	0.02	0.02	0.07	0.08	0.02	0.03	0.02	0.01	0.03	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02
	RSD%	1.93	2.07	9.94	0.32	0.24	13.53	0.77	0.91	11.60	0.35	0.27	8.72	0.32	0.14	6.47	0.07	0.18	10.63	0.24	0.07	15.71
Day 7	Mean	8.61	8.54	0.11	8.68	8.40	0.13	8.72	8.25	0.19	8.71	8.15	0.17	8.75	8.68	0.09	8.49	8.47	0.10	8.45	8.43	0.10
	SD	0.14	0.14	0.02	0.01	0.07	0.02	0.05	0.09	0.04	0.04	0.04	0.01	0.01	0.03	0.02	0.01	0.00	0.02	0.02	0.01	0.02
	RSD%	1.59	1.61	16.83	0.12	0.84	13.65	0.52	1.05	20.99	0.40	0.49	3.13	0.13	0.35	17.68	0.07	0.00	18.07	0.18	0.14	19.87
Day 10	Mean	8.55	8.44	0.07	8.88	8.73	0.12	8.68	8.38	0.18	8.66	8.44	0.27	8.41	8.41	0.09	8.53	8.40	0.06	8.34	8.40	0.07
	SD	0.18	0.15	0.03	0.01	0.05	0.03	0.04	0.11	0.03	0.03	0.03	0.04	0.01	0.06	0.01	0.03	0.01	0.02	0.06	0.09	0.01
	RSD%	2.12	1.72	39.08	0.07	0.54	25.21	0.48	1.28	15.45	0.37	0.31	13.74	0.14	0.66	8.54	0.31	0.12	30.20	0.67	1.07	10.30
Day 14	Mean	8.72	8.46	0.14	8.89	8.64	0.16	8.66	8.27	0.19	8.65	8.37	0.21	8.49	8.36	0.09	8.54	8.40	0.14	8.60	8.35	0.15
	SD	0.20	0.13	0.03	0.01	0.03	0.01	0.03	0.18	0.03	0.03	0.04	0.02	0.06	0.03	0.02	0.07	0.06	0.03	0.02	0.03	0.00
	RSD%	2.31	1.56	23.53	0.06	0.29	4.38	0.31	2.18	15.71	0.31	0.50	8.30	0.69	0.36	20.81	0.81	0.68	18.86	0.18	0.35	3.07

Table 4-5: Environmental monitor results of ADs (SNRIs), n=3.

Drugs		Blank			VEN			S-VEN			R-VEN			ODV			S-ODV			R-ODV		
	Test	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD
Day 0	Mean	8.03	8.02	0.03	8.32	8.33	0.0243	7.99	7.93	0.016	7.98	7.92	0.01	7.66	7.55	0.04	8.15	8.06	0.05	8.14	8.06	0.04
	SD	0.23	0.29	0.00	0.032146	0.01	0.0045	0.01	0.005	0.0031	0.01	0.01	0.00	0.02	0.01	0.01	0.01	0.01	0.00	0.02	0.01	0.01
	RSD%	2.89	3.60	14.10	0.39%	0.12%	18.53%	0.13%	0.07%	18.70%	0.13%	0.15%	18.72%	0%	0%	17%	0.12%	0.07%	9.75%	0.19%	0.07%	17.36%
Day 1	Mean	8.44	8.40	0.13	8.63	8.57	0.134	8.49333	8.3	0.143	8.50	8.28	0.13	8.21	8.12	0.12	8.42	8.22	0.13	8.40	8.27	0.12
	SD	0.16	0.21	0.03	0.01	0.040	0.0087	0.00577	0	0.0036	0.01	0.01	0.01	0.01	0.01	0.00	0.04	0.01	0.02	0.08	0.01	0.01
	RSD%	1.92	2.53	24.94	0.12%	0.47%	6.51%	0.07%	0.00%	2.52%	0.14%	0.12%	4.40%	0%	0%	3%	0.52%	0.14%	18.31%	0.97%	0.07%	11.55%
Day 2	Mean	8.37	8.55	0.10	8.49	8.487	0.0867	8.13	8.26	0.0723	8.19	8.30	0.07	8.22	8.29	0.10	8.74	8.75	0.08	8.67	8.71	0.11
	SD	0.29	0.14	0.01	0.07	0.006	0.005	0.06245	0.053	0.0021	0.03	0.02	0.00	0.02	0.00	0.01	0.06	0.02	0.01	0.10	0.01	0.00
	RSD%	3.50	1.63	12.26	0.82%	0.07%	5.81%	0.77%	0.64%	2.88%	0.31%	0.18%	2.82%	0%	0%	6%	0.67%	0.20%	7.52%	1.20%	0.07%	3.70%
Day 3	Mean	8.46	8.57	0.08	8.557	8.533	0.0873	8.43333	8.52	0.108	8.46	8.49	0.10	8.36	8.34	0.09	8.57	8.62	0.09	8.59	8.61	0.10
	SD	0.16	0.18	0.01	0.035119	0.0058	0.0145	0.01528	0.032	0.003	0.01	0.01	0.00	0.04	0.02	0.01	0.03	0.01	0.00	0.03	0.01	0.01
	RSD%	1.93	2.07	9.94	0.41%	0.07%	16.57%	0.18%	0.38%	2.78%	0.14%	0.12%	3.72%	0%	0%	8%	0.29%	0.12%	0.75%	0.29%	0.13%	13.22%
Day 7	Mean	8.61	8.54	0.11	8.5267	8.35	0.105	8.43	8.32	0.1007	8.45	8.34	0.09	8.61	8.64	0.09	8.71	8.49	0.08	8.71	8.45	0.10
	SD	0.14	0.14	0.02	0.100167	0.01	0.0176	0.21656	0	0.0142	0.05	0.03	0.03	0.03	0.00	0.01	0.01	0.02	0.01	0.01	0.01	0.01
	RSD%	1.59	1.61	16.83	1.17%	0.12%	16.74%	2.57%	0.00%	14.06%	0.63%	0.35%	33.83%	0%	0%	8%	0.11%	0.20%	11.18%	0.11%	0.14%	10.58%
Day 10	Mean	8.55	8.44	0.07	8.71	8.4433	0.1423	8.43	8.296	0.0857	8.47	8.31	0.38	8.33	8.33	0.09	8.85	8.57	0.12	8.85	8.51	0.13
	SD	0.18	0.15	0.03	0.096437	0.0115	0.0086	0.24249	0.025	0.0176	0.08	0.03	0.51	0.06	0.01	0.00	0.01	0.03	0.02	0.02	0.01	0.02
	RSD%	2.12	1.72	39.08	1.11%	0.14%	6.06%	2.88%	0.30%	20.56%	0.89%	0.39%	###	1%	0%	2%	0.11%	0.34%	15.84%	0.17%	0.12%	14.95%
Day 14	Mean	8.72	8.46	0.14	8.53	8.44	0.171	8.55333	8.4967	0.1183	8.67	8.51	0.13	8.47	8.36	0.09	8.88	8.62	0.16	8.90	8.58	0.18
	SD	0.20	0.13	0.03	0.0989	0.02645	0.0241	0.28868	0.012	0.0232	0.02	0.03	0.02	0.07	0.04	0.01	0.02	0.03	0.04	0.01	0.01	0.00
	RSD%	2.31	1.56	23.53	1.15%	0.31%	14.07%	3.38%	0.14%	19.59%	0.23%	0.31%	14.04%	1%	0%	15%	0.17%	0.33%	23.87%	0.11%	0.07%	2.07%

4.4.2. Validation of Analytical Method

4.4.2.1. Linearity

Linearity was evaluated as part of the analytical method validation using the calibration procedure described in the Methods section (matrix-matched standards prepared across the working range and quantified using analyte-to-internal standard peak area ratios). For FLX, the calibration curves for the racemate and both enantiomers showed a strong proportional relationship between response and concentration. The coefficients of determination (R^2) were 0.989 for Rac-FLX, 0.989 for S-FLX, and 0.995 for R-FLX (Figure 4-1), supporting acceptable linear behaviour across the evaluated range. Small differences in slope between the racemate and individual enantiomers reflect minor differences in detector response/sensitivity that can occur in chiral LC-MS assays and do not affect the suitability of the calibration within the working range. In line with validation best practice, linearity was judged based on overall calibration performance (including acceptable quantitative behaviour across the range) rather than relying on R^2 alone.

NFLX calibration curves for the racemate and both enantiomers also demonstrated strong linear behaviour across the evaluated concentration range, with a clear proportional relationship between LC-MS response (peak area ratio) and concentration. The R^2 were 0.999 for Rac-NFLX, 0.992 for S-NFLX, and 0.994 for R-NFLX (Figure 4-2), supporting acceptable linearity for quantitative analysis. Minor differences in slope between the racemate and individual enantiomers indicate small variations in detector response/sensitivity, which is expected in chiral LC-MS quantification and did not compromise calibration suitability within the working range.

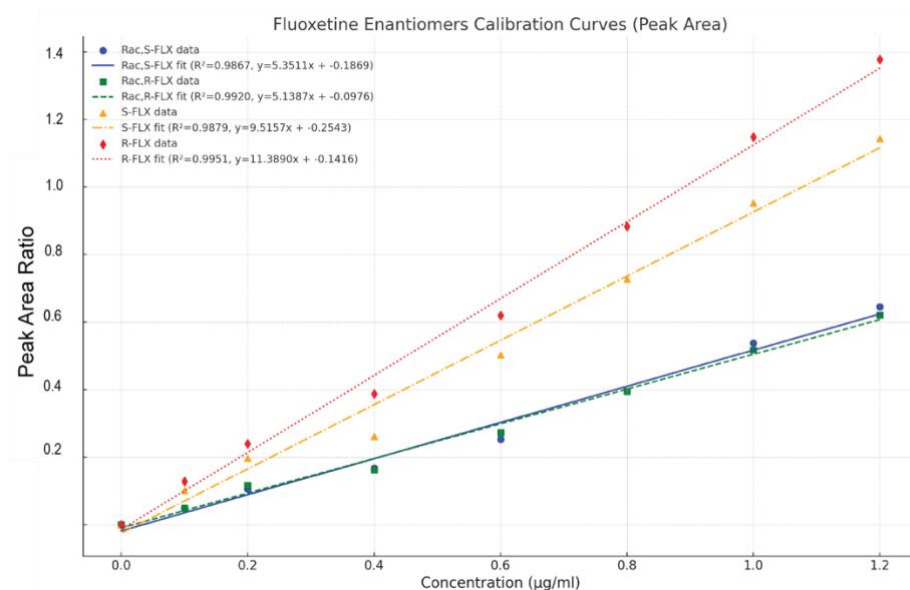


Figure 4-1: Calibration curves of Rac-FLX and its enantiomers.

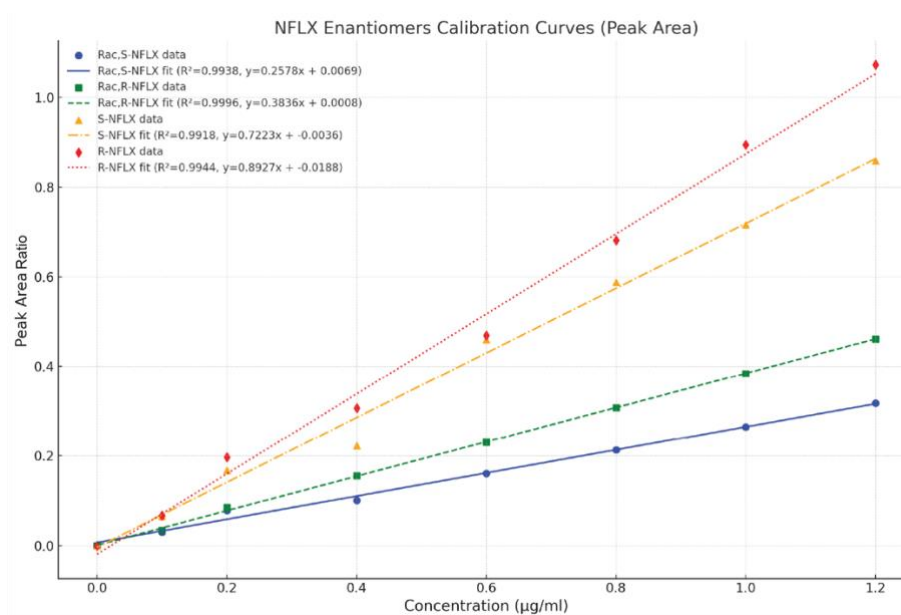


Figure 4-2: Calibration curves of Rac-NFLX and its enantiomers.

The calibration curves for VEN, including the racemate (Rac-VEN) and both enantiomers, showed strong linearity across the evaluated concentration range, indicating a clear proportional relationship between LC-MS response (peak area ratio) and concentration. The coefficients of determination (R^2) were 0.993 for Rac-VEN, 0.992 for S-VEN, and 0.991 for R-VEN (Figure 4-3), supporting acceptable linear performance for quantitative analysis. Minor differences in slope among the racemate and enantiomers likely reflect small variations in detector

response/sensitivity, which is expected in chiral LC-MS measurements and did not compromise calibration suitability within the working range.

The calibration curves for ODV, including the racemate (Rac-ODV) and both enantiomers, demonstrated good linearity across the evaluated concentration range, supporting a proportional relationship between LC-MS response (peak area ratio) and concentration. The coefficients of determination (R^2) were 0.969 for Rac-ODV, 0.991 for S-ODV, and 0.990 for R-ODV (Figure 4-4).

Although Rac-ODV showed a slightly lower R^2 than the individual enantiomers, the model still indicates acceptable linear behaviour within the working range, while S-ODV and R-ODV exhibited excellent linearity. Small differences between the racemate and enantiomer fits may reflect increased chromatographic/ionisation complexity when the combined racemic signal is quantified (e.g., broader peak shape or greater sensitivity to integration/baseline), but overall, the results support the method's suitability for quantitative determination of ODV and its enantiomers.

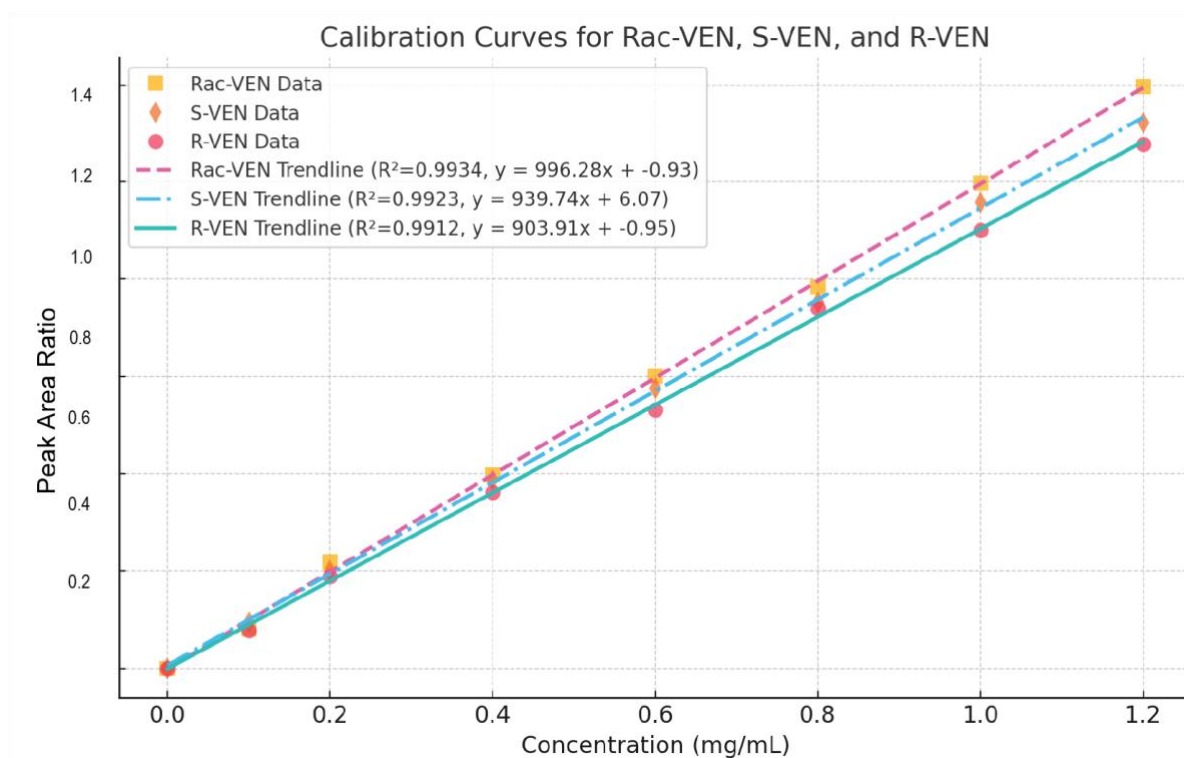


Figure 4-3: Calibration curves of Rac-VEN and its enantiomers.

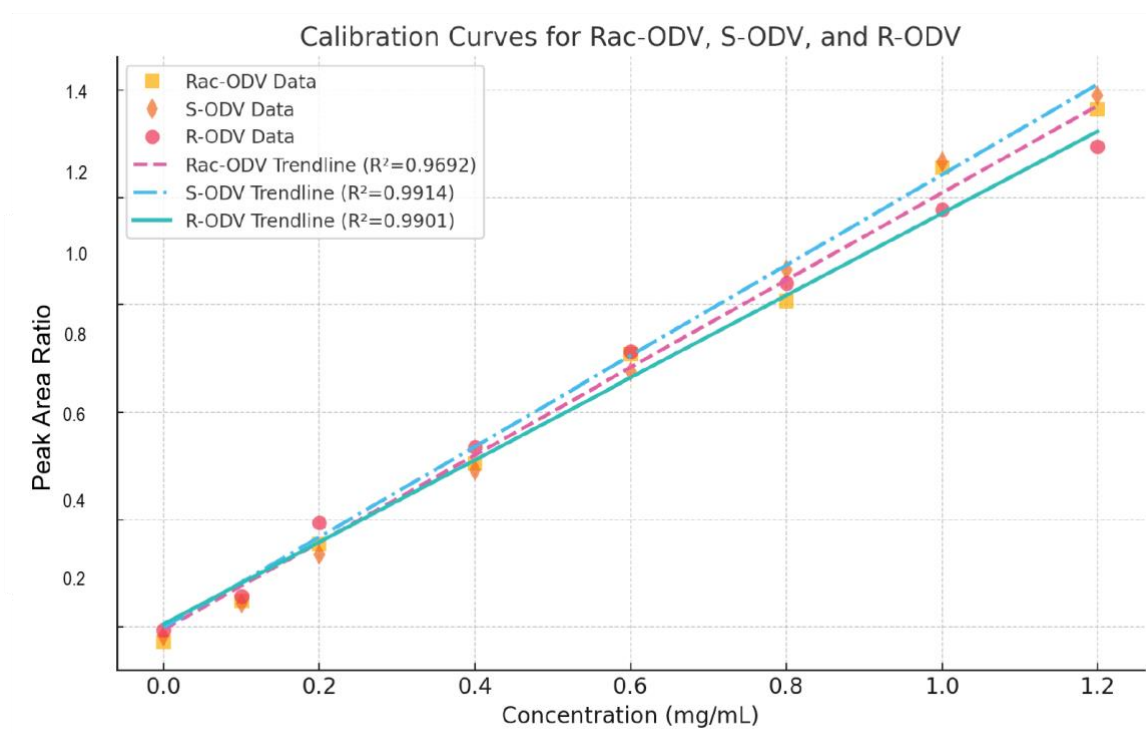


Figure 4-4: Calibration curves of Rac-ODV and its enantiomers.

4.4.2.2. Precision and Accuracy

Accuracy and precision were evaluated for FLX, NFLX, VEN, ODV and their enantiomers at two QC levels (0.300 and 0.900 $\mu\text{g/mL}$) (Table 4-6). Accuracy was expressed as percent of nominal concentration (i.e., bias), and precision was assessed as intra-day and inter-day %RSD. Acceptance criteria were defined in line with SWGTOX and internationally recognised bioanalytical/analytical validation guidance, where quantitative performance is typically considered acceptable when accuracy (bias) is within $\pm 15\%$ of nominal ($\pm 20\%$ at the LLOQ) and precision is $\leq 15\%$ RSD ($\leq 20\%$ at the LLOQ). [291,292]

For FLX and NFLX, the method demonstrated strong quantitative performance across both QC levels. Accuracy values were close to nominal (e.g., $\sim 97.5\text{--}100.2\%$), and precision was excellent, with low intra-day and inter-day variability ($\leq \sim 2.4\%$ and $\leq \sim 2.6\%$ RSD, respectively). These findings indicate good repeatability and intermediate precision for the quantification of FLX/NFLX enantiomeric species within the tested range.

In contrast, VEN and ODV showed reduced accuracy at the evaluated QC levels (approximately 83.5–89.0%), although precision remained moderate (intra-day ~6.5–7.3% RSD; inter-day ~7.2–7.9% RSD). While the %RSD values remain within common validation limits, the lower accuracy indicates a systematic bias for VEN/ODV under the applied conditions (e.g., matrix effects and/or ionisation-related differences relative to the internal standard), and therefore VEN/ODV quantitative results should be interpreted more cautiously, or the method further optimised to improve trueness at these concentrations.

Table 4-6: Accuracy and precision results for Antidepressants, n=3.

Compound	Concentration (µg/mL)	Accuracy (%)	Intra-day Precision (RSD, %)	Inter-day Precision (RSD, %)
S-Rac-Fluoxetine	0.300	99.5	2.1	2.4
	0.900	98.8	1.8	2.2
R-Rac-Fluoxetine	0.300	100.2	2.0	2.3
	0.900	99.0	2.2	2.5
S-Fluoxetine	0.300	99.8	2.0	2.2
	0.900	98.5	1.9	2.1
R-Fluoxetine	0.300	99.7	2.1	2.4
	0.900	99.1	2.3	2.3
S-Rac-Norfluoxetine	0.300	98.9	2.3	2.5
	0.900	97.5	2.0	2.3
R-Rac-Norfluoxetine	0.300	99.3	2.4	2.4
	0.900	98.8	2.2	2.6
S-Norfluoxetine	0.300	99.0	2.1	2.3
	0.900	98.2	2.2	2.4
R-Norfluoxetine	0.300	99.1	2.3	2.5
	0.900	98.7	2.4	2.6
Rac-Venlafaxine	0.300	89.0	6.5	7.2
	0.900	85.5	6.8	7.5
S-Venlafaxine	0.300	88.5	6.7	7.3
	0.900	87.0	6.9	7.4
R-Venlafaxine	0.300	88.0	7.0	7.6
	0.900	84.8	7.2	7.8
Rac-ODV	0.300	87.5	7.1	7.7
	0.900	86.9	7.3	7.9
S-ODV	0.300	86.0	6.8	7.4
	0.900	83.5	6.9	7.6
R-ODV	0.300	85.8	7.0	7.5
	0.900	85.0	7.1	7.7

Overall, the accuracy/precision results in Table 4-6 support robust quantification for FLX and NFLX, while indicating that VEN and ODV require additional attention to improve accuracy despite acceptable precision under the current LC-MS conditions.

4.4.2.3. Selectivity

Blank samples, showed no detectable peaks at the retention times of the target compounds, indicating no matrix interference. When analysing matrix samples spiked with known concentrations of the analytes, the peaks were clearly distinguishable from background noise, further demonstrating the method's ability to selectively quantify the target compounds, Figure 4-5.

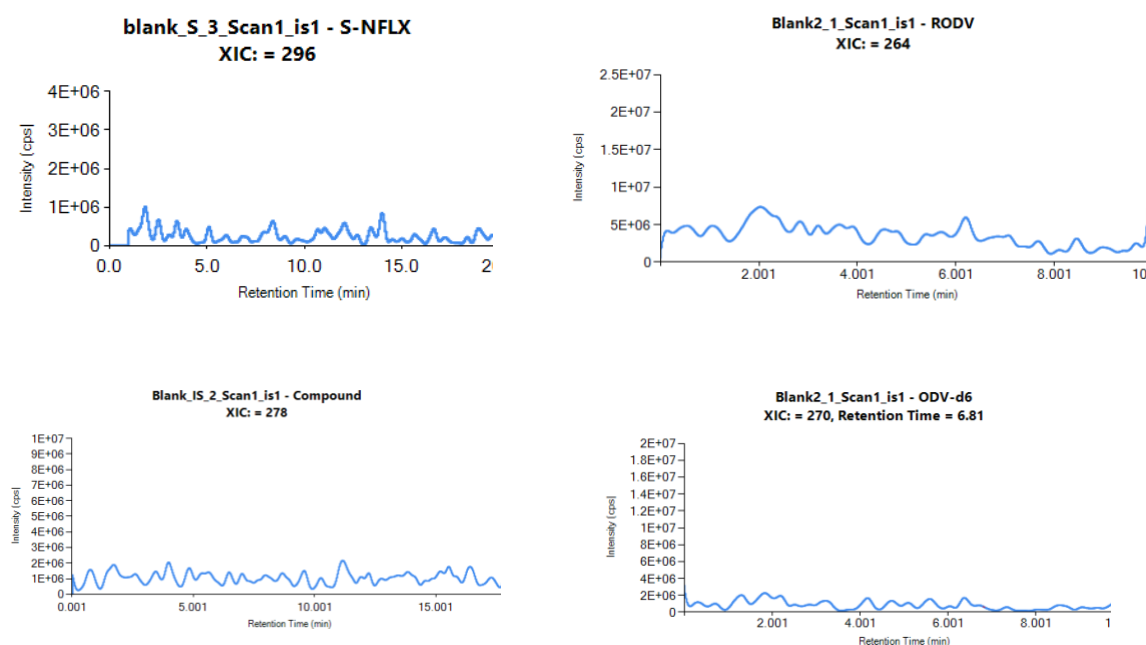


Figure 4-5: Representative examples of ADs and IS used for method selectivity.

4.4.2.4. Limits of Detection (LOD) and Quantification (LOQ)

For FLX, LOD and LOQ determined using the S/N approach indicated the highest sensitivity among the target analytes (LOD 10 ng/mL; LOQ 25–30 ng/mL), with good repeatability demonstrated by low variability (%RSD 4.8-6.7%). NFLX showed comparable performance, with LOQ values within the same range (25–30 ng/mL) and similarly low %RSD, supporting consistent quantification under the applied conditions.

For VEN and ODV, sensitivity was slightly lower (LOD 15 ng/mL; LOQ 26–29.5 ng/mL) and variability was modestly higher (up to 8.9% RSD), indicating slightly reduced precision at low levels compared with FLX/NFLX. This difference is consistent with compound-dependent response behaviour in LC–ESI–MS, where analyte structure, ionisation efficiency, and matrix effects can influence signal stability and therefore LOD/LOQ performance.

Overall, the LC-MS method provided reliable detection and quantification for all analytes across the validated range. Best performance (lowest LOD/LOQ and lowest %RSD) was obtained for FLX/NFLX, while VEN/ODV remained quantitatively acceptable but showed slightly higher variability and marginally higher LOQs, which should be considered when interpreting results near the lower end of the calibration range, (Table 4-7).

Table 4-7: LOD and LLOQ of antidepressants.

Compound	LOD (ng/mL)	SD (LOD)	RSD% (LOD)	LLOQ (ng/mL)	SD (LLOQ)	RSD% (LLOQ)
S-Rac-Fluoxetine	10	0.7	6.7	25	1.3	5.1
R-Rac-Fluoxetine	10	0.8	6.7	25	1.4	5.4
S-Fluoxetine	10	0.5	5	25	1.2	4.8
R-Fluoxetine	10	0.8	6.4	30	1.5	5.5
S-Rac-Norfluoxetine	12.5	0.8	7.3	26.5	1.5	5.7
R-Rac-Norfluoxetine	12.5	0.9	7.5	27.5	1.6	5.8
S-Norfluoxetine	12.5	0.7	6.7	25	1.3	5
R-Norfluoxetine	12.5	0.6	5.5	26	1.4	5.3
Rac-Venlafaxine	15	1.2	8.9	29.5	1.9	6.4
S-Venlafaxine	15	1	8.3	28	1.7	6.1
R-Venlafaxine	15	0.9	8.3	26	1.6	6.1
Rac-ODV	15	0.7	6.1	27	1.4	5.2
S-ODV	15	0.6	5.2	25	1.2	4.7
R-ODV	15	0.5	4.9	25	1.1	4.4

4.4.2.5. Carryover

Carryover was assessed by injecting a blank sample immediately after the ULOQ (1.2 µg/mL) and examining the extracted-ion chromatograms at the expected retention times of FLX, NFLX, VEN, and ODV. For all analytes, no peaks were detected within the integration windows in the post-ULOQ blanks and signal intensities remained below the established LOD, indicating no detectable carryover under the applied LC-MS conditions. Calibration levels were analysed in triplicate (n

= 3). These results support reliable successive injections and confirm robust system performance for quantitative analysis (Figure 4-6).

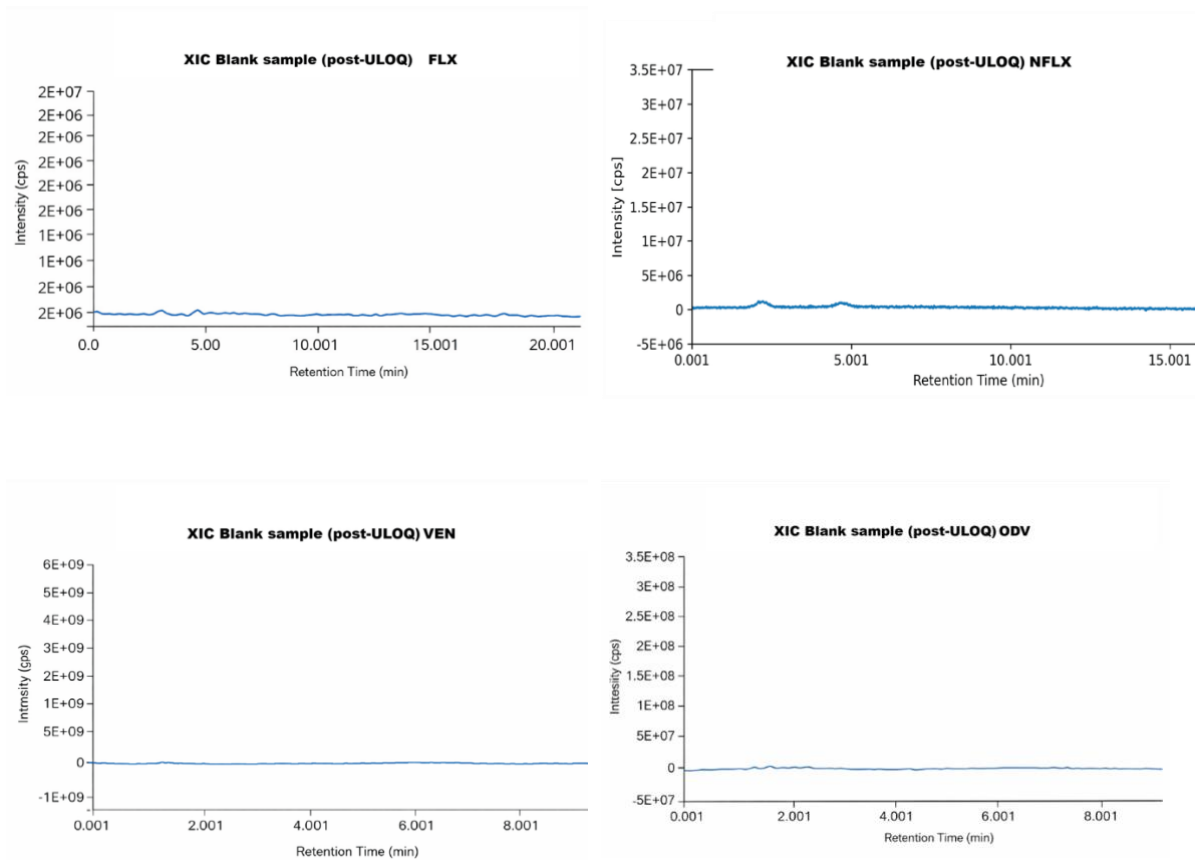


Figure 4-6: Carryover assessment (post-ULOQ blanks) for target antidepressants.

4.4.3. FLX and its enantiomerS Taxonomy

4.4.3.1. Free drugs reactors

The blank (control) reactors showed clear shifts in community structure between Day 0 and Day 14 across stages 1-6 (Figure 4-7). At Day 0, the profiles were dominated by *Proteobacteria* classes (notably *Alpha*-, *Beta*-, and *Gamma*-*proteobacteria*) together with substantial contributions from *Bacteroidetes* classes. By Day 14, multiple stages displayed increased relative contributions from *Planctomycetota*/*Planctomycetes*-related classes and *Actinobacteria*, alongside reduced fractions of some *Proteobacteria* classes. Importantly, the magnitude of these changes varied between stages, indicating that even without drug amendment,

the reactors undergo time-dependent succession and stage-to-stage variability in community composition. [339]

These baseline shifts are consistent with well-described microbial dynamics in wastewater/bioreactor systems, where community composition can change over time due to a combination of deterministic controls (e.g., operational conditions and resource availability) and stochastic processes (e.g., ecological drift/colonisation effects), even under broadly controlled settings. Establishing this control trajectory is important because it provides the reference against which any drug-amended community changes should be interpreted. [340]

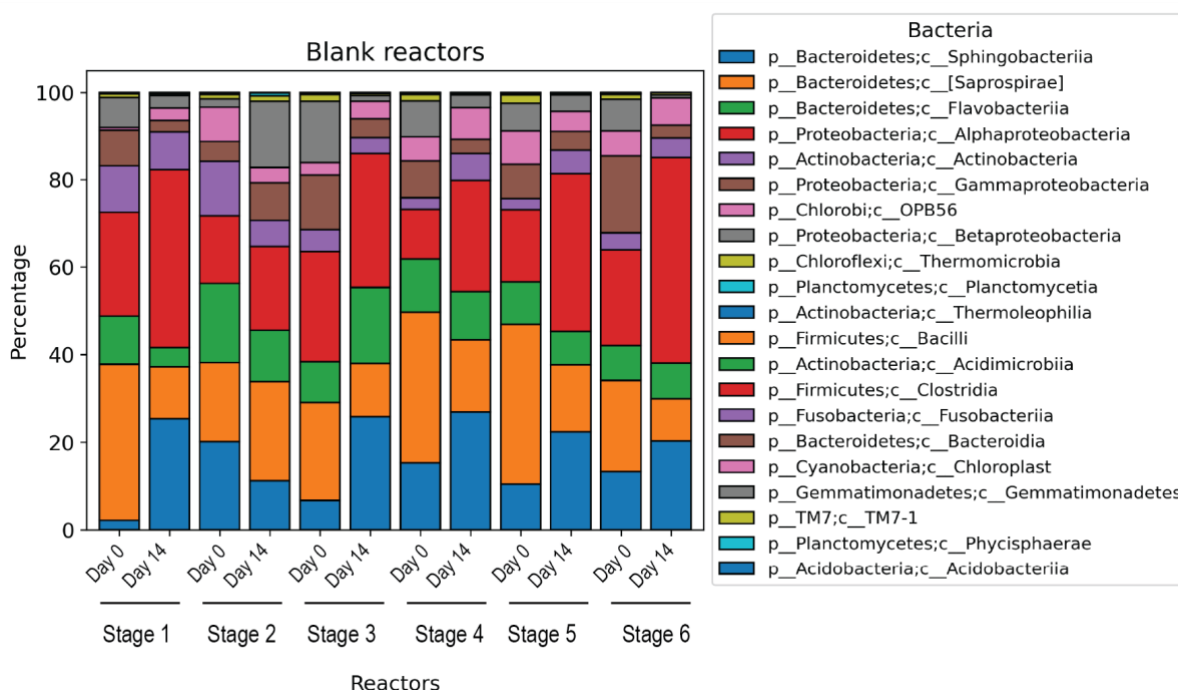


Figure 4-7: Taxonomy results for control reactors across all study stages.

4.4.3.2. FLX and NFLX bioreactors 16S Taxonomy

This section evaluates whether exposure to Rac-FLX and its individual enantiomers (S-FLX and R-FLX) altered the bacterial community structure relative to the blank (drug-free) reactors (Table 4-8 and Figure 4-8). To focus on overall community response rather than fine day-to-day variation, the 16S rRNA analysis was performed on composite samples: for each treatment, Day 7 and Day 14 aliquots were pooled, and the triplicate reactors were also combined ($n = 3$ per condition) prior to sequencing. This pooling strategy was adopted to manage 16S sequencing

costs while still capturing the *late-stage* community profile; consequently, the reported percentages represent relative abundance in the pooled sample and are interpreted comparatively (i.e., shifts in dominance/structure), rather than as time-resolved trajectories with replicate variance.

At Day 14 in the blank reactor, the community was dominated by *Proteobacteria* (40.71%) and *Bacteroidetes* (25.41%), with smaller contributions from *Actinobacteria* (8.79%) and *Chlorobi* (2.83%) (Table 4-8 and Figure 4-8). Following exposure to racemic FLX, *Bacteroidetes* increased to 32.05% (notably within *Sphingobacteriia*) while *Proteobacteria* decreased to 35.75%. *Actinobacteria* also decreased to 5.97%, and *Planctomycetota* increased to 6.73%. Together, these shifts indicate that FLX imposed a selective pressure that favoured specific *Bacteroidetes*-associated groups while reducing the relative contribution of *Proteobacteria* and *Actinobacteria* under the tested conditions.

Comparable but not identical patterns were observed for the individual enantiomers. In the S-FLX reactors, *Bacteroidetes* increased to 28.10% and *Proteobacteria* to 35.86%, whereas in the R-FLX reactors *Bacteroidetes* reached 27.52% and *Proteobacteria* 36.87% (Table 4-8 and Figure 4-8). When considered alongside the blank profile, all FLX treatments were associated with a higher *Bacteroidetes* contribution than the control, with the largest enrichment occurring under racemic FLX (32.05%), followed by S-FLX (28.10%) and R-FLX (27.52%). This supports the interpretation that the racemate produced the strongest overall community shift, potentially reflecting combined or additive effects of both enantiomers on microbial selection (Figure 4-8).

These observations are consistent with reports that racemic pharmaceuticals can exhibit distinct biodegradation behaviour and ecological effects compared with their individual enantiomers, reinforcing the value of considering stereochemistry when interpreting environmental biological responses. [32] In addition, enantioselective biodegradation of fluoxetine has been reported in microbial systems, indicating that stereochemistry can influence microbial interactions and transformation behaviour. [11,341,342] In the present dataset, the community profiles in Figure 4-8 suggest enantiomer-dependent differences in the distribution of secondary taxa (beyond the

dominant phyla), which may reflect differential selective pressures and/or differences in microbial utilisation or tolerance mechanisms between S-FLX and R-FLX.

[342,343] Finally, evidence that fluoxetine may become enriched in one enantiomer under environmental/analytical contexts, and that enantiomers can differ in biological effects, supports the broader implication that the dominant enantiomer present may modulate ecological impact. [199]

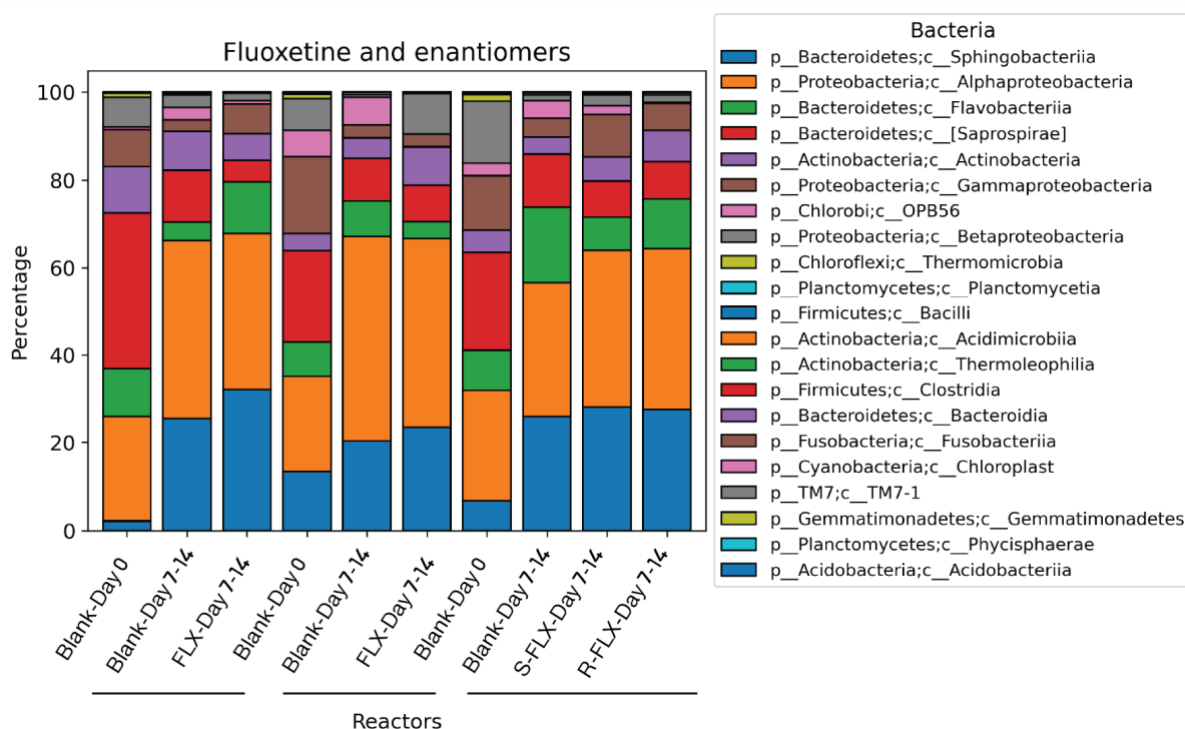


Figure 4-8: FLX bioreactors 16S taxonomy results.

In NFLX reactors, the influence of NFLX and its enantiomers (S-NFLX and R-NFLX) on microbial community composition was evaluated over a 14-day incubation period and compared with blank reactor controls. Because of the high cost associated with 16S rRNA sequencing, biomass samples collected at Day 7 and Day 14 (each in triplicate) were pooled prior to DNA extraction and sequencing. Consequently, the reported Day 14 taxonomy profiles represent the combined late-stage microbial community structure rather than independent time-point measurements.

In the blank reactor, *Bacteroidetes* dominated the microbial community at Day 0 (20.09%), followed by *Proteobacteria* (15.43%), with moderate contributions from *Actinobacteria* (12.53%) and *Chlorobi* (7.82%), while *Chloroflexi* remained minor (0.95%). By the pooled late-stage sample (Day 7 + Day 14), *Bacteroidetes*

decreased to 11.26%, whereas *Proteobacteria* increased to 19.15%, indicating natural community succession and potential enrichment of taxa associated with intrinsic degradation or adaptation processes in the absence of pharmaceutical stress.

Exposure to NFLX produced clear compositional shifts. Bacteroidetes and Proteobacteria remained dominant (17.46% and 18.99%, respectively), while *Chlorobi* increased to 9.72% relative to the blank reactor. The persistence of *Chloroflexi* at low abundance (~0.92%) and the modest reduction in *Actinobacteria* compared with Day 0 suggest selective ecological pressure imposed by NFLX, potentially favouring taxa capable of tolerating or transforming the metabolite. Distinct yet partially overlapping responses were observed for the individual enantiomers.

In the S-NFLX reactor, *Bacteroidetes* increased markedly to 45.71%, while *Proteobacteria* (1.49%) and *Actinobacteria* (3.92%) declined substantially, indicating strong selective enrichment of Bacteroidetes-associated populations.

In the R-NFLX reactor, *Bacteroidetes* also dominated (48.19%), but *Proteobacteria* (5.22%) and *Actinobacteria* (5.09%) remained slightly more abundant than in S-NFLX, suggesting a more balanced microbial structure and potentially weaker selective pressure.

The consistently low abundance of *Chlorobi* and *Chloroflexi* across enantiomer-treated systems implies secondary or specialised ecological roles in transformation pathways rather than primary degradation activity. These enantioselective community responses align with previous studies reporting stereochemistry-dependent microbial adaptation and degradation behaviour of chiral pharmaceuticals. [343,344]

Overall, NFLX and its enantiomers induced stronger community restructuring than FLX, particularly through enrichment of *Bacteroidetes*, which may reflect enhanced microbial adaptation to the metabolite. These findings emphasise the importance of metabolic transformation products and stereochemistry when evaluating the

environmental fate and ecological impact of antidepressants in wastewater-related systems (Figure 4-9).

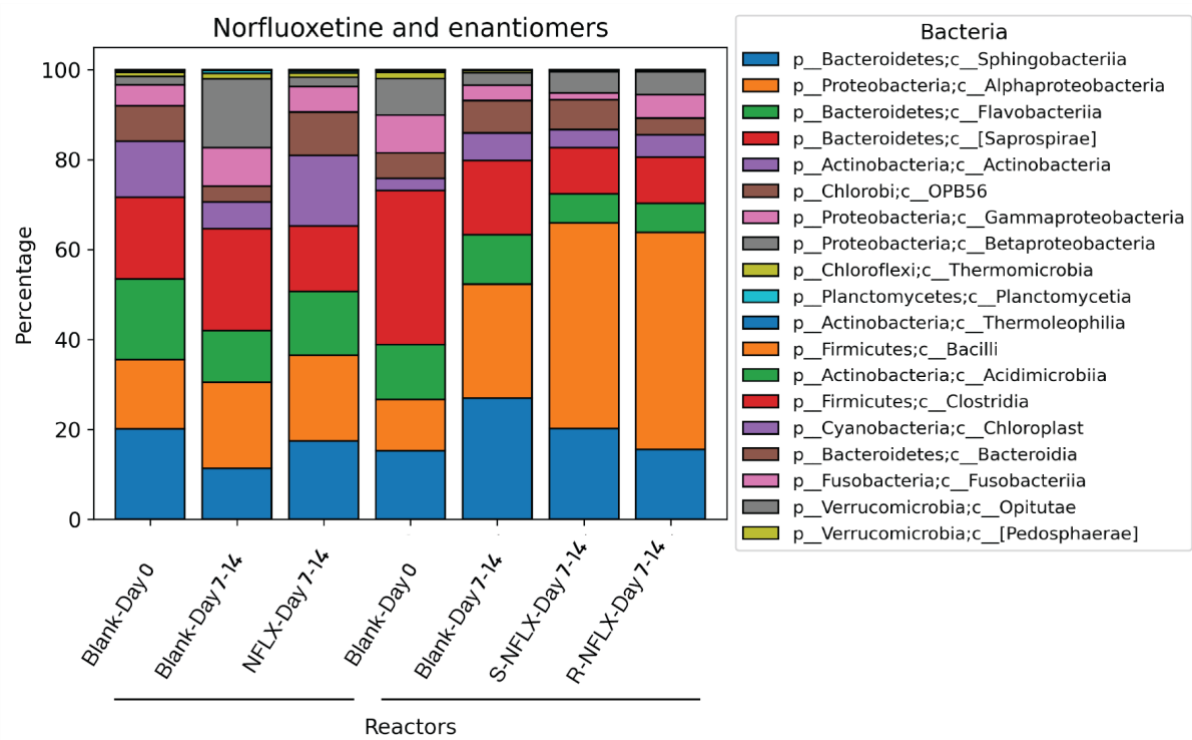


Figure 4-9: NFLX and enantiomers bioreactors 16S Taxonomy results.

Table 4-8: Percentages of the abundance of bacteria in the reactors.

Reactor	Sphingobacteriia	Alphaproteobacteria	Flavobacteriia	Saprospirae	Actinobacteria	Gammaproteobacteria	OPB56	Betaproteobacteria	Thermomicrobia	Other
B-d0	2.16	23.79	10.93	35.63	10.59	8.34	0.55	6.80	0.89	<1
B-d14	25.41	40.71	4.33	11.82	8.79	2.53	2.83	2.83	0.46	<1
FLX*	32.05	35.75	11.84	4.93	5.97	6.73	0.71	1.72	0.18	<1
B-d0	13.29	21.84	7.87	20.89	3.91	17.58	5.85	7.22	1.07	<1
B-d14	20.31	46.82	8.15	9.70	4.62	2.93	6.17	0.69	0.47	<1
FLX **	23.49	43.14	3.89	8.35	8.69	2.82	0.04	9.18	0.32	<1
B-d0	6.72	25.18	9.19	22.45	5.00	12.44	2.88	14.06	1.52	<1
B-d14	25.88	30.68	17.24	12.15	3.82	4.26	3.98	1.28	0.52	<1
S-FLX	28.10	35.86	7.55	8.25	5.50	9.63	1.94	2.52	0.36	<1
R-FLX	27.52	36.87	11.33	8.50	7.06	6.14	0.14	1.78	0.46	<1
B-d0	20.09	15.43	17.97	18.12	12.53	4.69	7.82	1.87	0.95	<1
B-d14	11.26	19.15	11.62	22.62	5.95	8.64	3.48	15.20	1.24	<1
NFLX	17.46	18.99	14.28	14.57	15.58	5.62	9.72	2.13	0.92	<1
B-d14	15.25	11.37	12.21	34.32	2.67	8.43	5.66	8.14	1.41	<1
B-d	26.90	25.41	11.03	16.42	6.14	3.47	7.19	2.79	0.46	<1
S-NFLX	20.20	45.71	6.45	10.38	3.92	1.49	6.69	4.77	0.25	<1
R-NFLX	15.60	48.19	6.50	10.22	5.09	5.22	3.64	5.13	0.25	<1

B=Free drugs reactors.

d0= day zero

d14=day 14

FLX*= stage 1

FLX**-stage 6

Day 0 profiles represent baseline (pre-adaptation) community composition. For 16S rRNA taxonomy, triplicate reactors were pooled per treatment, and Day 7 and Day 14 samples were combined (equal contributions) to produce an integrated late-stage community profile, reflecting cost-efficient sequencing design and emphasising overall treatment effects rather than day-to-day variation

4.4.4. Biodegradation of Fluoxetine

4.4.4.1. Biodegradation of racemic fluoxetine

The objective of this study was to investigate the biodegradation of racemic fluoxetine (Rac-FLX) in both abiotic (SWW) and biotic (bioreactor) systems over a 14-day period. Samples were analysed by chiral LC-MS using an Astec Chirobiotic™ V2 column to achieve enantiomeric separation, and S-FLX and R-FLX were quantified using peak area ratios. Figure 4-10 demonstrates the successful chiral separation of Rac-FLX under the applied conditions and presents the mass spectra of each enantiomer within the racemic mixture.

In the abiotic reactor, the concentrations of S-FLX and R-FLX on Day 0 were 0.866 µg/mL and 0.722 µg/mL, respectively. Over the course of 14 days, the concentration of S-FLX remained relatively stable, with a slight decrease observed on Day 7 and Day 14. On the other hand, R-FLX showed a more significant decrease in concentration over time, with a 75% reduction observed on Day 14. The pH in the abiotic reactor ranged from 8.16 to 8.89 and remained relatively constant throughout the study

In the biotic reactor, the Day 0 concentrations of S-FLX and R-FLX were lower than in the abiotic reactor, with mean concentrations of 0.541 and 0.496 µg/mL, respectively (n = 3). Over the 14-day period, both enantiomers showed substantial decreases in concentration: S-FLX declined by approximately 71% by Day 14, while R-FLX decreased by approximately 62%. The biotic reactor pH ranged from 8.07 to 8.78 and remained relatively stable throughout the study; no clear association between the minor pH fluctuations and the time-course of enantiomer attenuation was evident. [35]

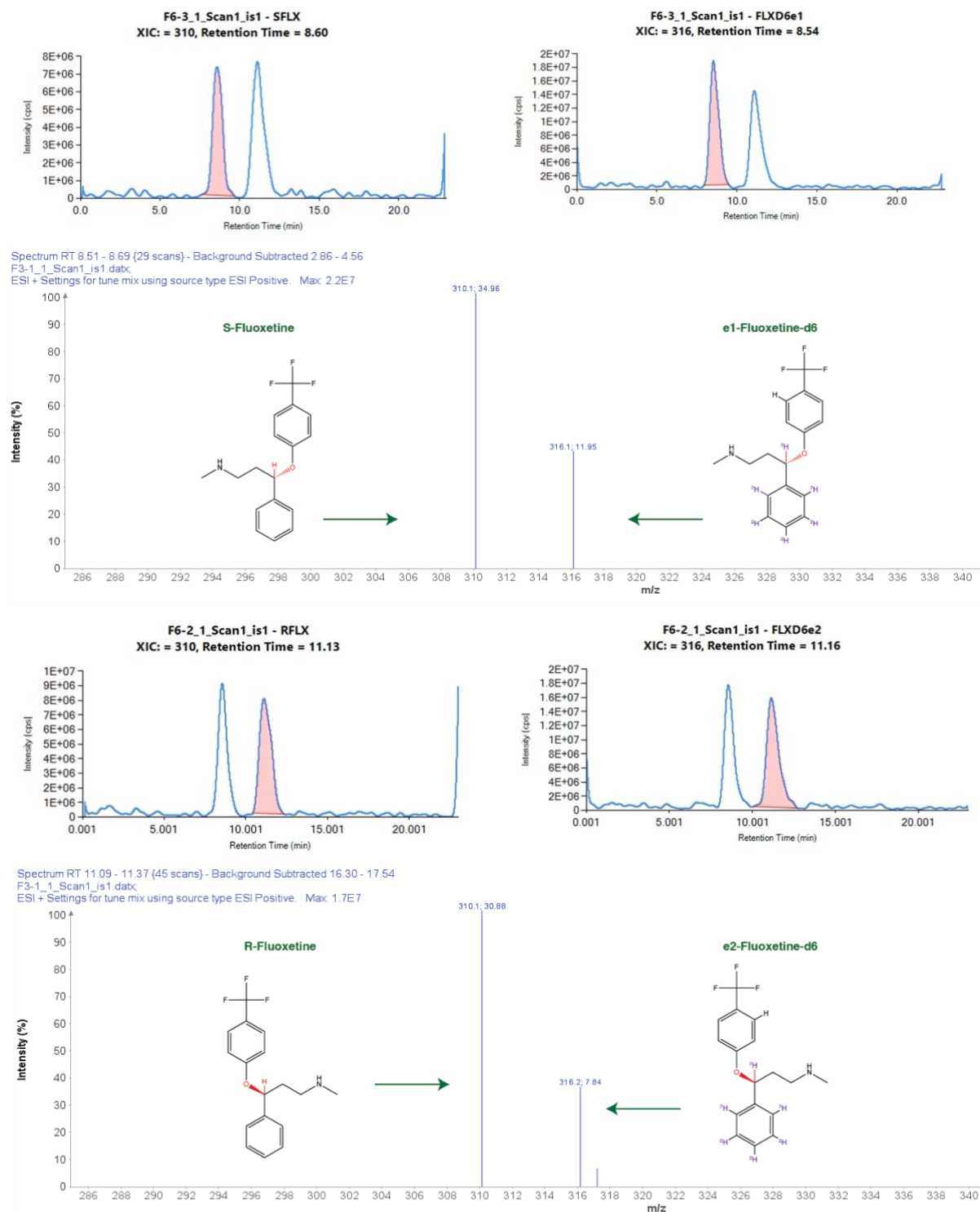


Figure 4-10: Representative extracted-ion chromatograms (XICs) showing chiral separation of fluoxetine (FLX) and its ISD (FLX-d6. Peaks corresponding to the S- and R-enantiomers are indicated.

The comparison between abiotic and biotic reactors indicates different stereochemical removal behaviour. In the abiotic reactor, R-FLX declined more rapidly than S-FLX, whereas in the biotic reactor the two enantiomers decreased at more similar rates, leading to convergence of their concentrations over time. Rather than indicating racemization, this convergence is more consistently interpreted as reduced net enantioselectivity in the biotic system. In mixed microbial consortia, simultaneous biodegradation pathways with different (or opposing) stereopreferences can yield an overall removal profile that appears nearly non-enantioselective. In addition, biomass/EPS partitioning and time-dependent matrix composition can alter the apparent aqueous-phase enantiomer balance without requiring chemical interconversion between enantiomers. These observations therefore suggest that microbial activity and reactor conditions (Section X; operating pH, temperature, aeration/mixing, and matrix composition) moderated stereoselective behaviour, resulting in similar apparent depletion rates for S-FLX and R-FLX. [345]

Although the concentrations of S-FLX and R-FLX converged in the biotic reactor, this pattern should not be interpreted as racemization based on concentration data alone. True racemization requires chemical interconversion ($S \leftrightarrow R$), which would need dedicated confirmation (e.g., incubation of a single enantiomer and tracking formation of the opposite enantiomer, supported by mass balance and appropriate controls). Instead, the convergence observed here is more plausibly explained by reduced net enantioselectivity in the mixed microbial community, where multiple biodegradation pathways with different stereopreferences can result in similar apparent depletion rates, and/or by time-dependent matrix and partitioning effects (e.g., sorption to biomass/EPS) that influence aqueous-phase concentrations without requiring enantiomer interconversion. Overall, the results support the conclusion that microbial activity and reactor conditions modulated stereoselective behaviour during Rac-FLX biodegradation, but they do not provide direct evidence of racemization, Figure 4-11 and Table 4-9. [346–349]

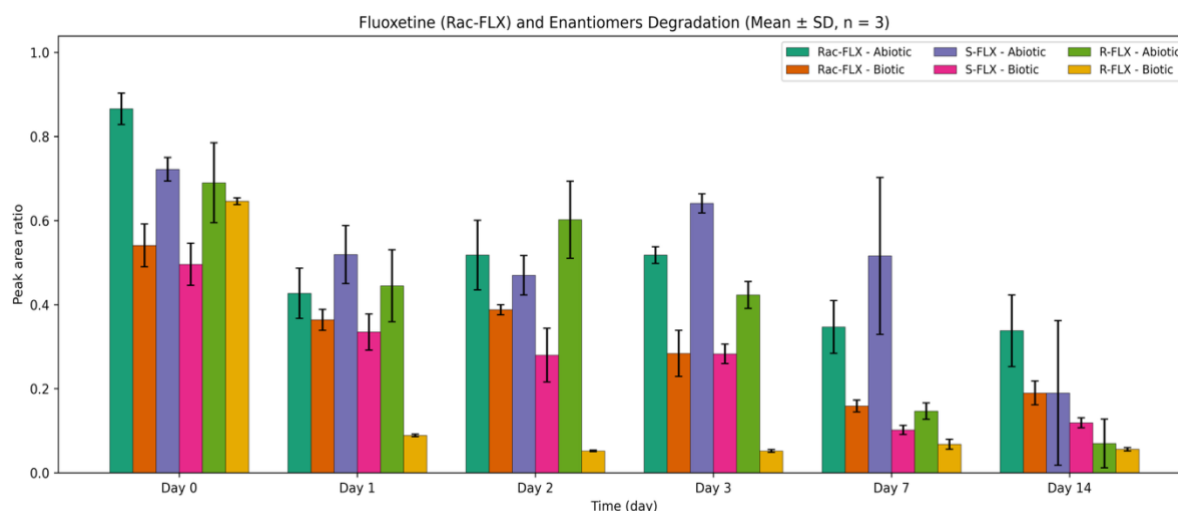


Figure 4-11: FLX and its enantiomers degradation study, n=3)

The %RSD values were generally low across most time points ($n = 3$), indicating favourable analytical reproducibility for Rac-FLX and the individual enantiomers in both abiotic and biotic systems. Nevertheless, several data points showed elevated %RSD, most notably in the biotic reactor at Day 1 for S-FLX (%RSD 36.97) and at low-response stages where mean peak area ratios were small, as well as in the abiotic reactor at Day 7 and Day 14 for S-FLX and R-FLX (e.g., S-FLX Day 14 %RSD 90.50; R-FLX Day 14 %RSD 82.73). These higher %RSD values are most plausibly attributed to a combination of (i) reduced signal intensity as concentrations approached the lower end of the working range, which amplifies small integration differences into large %RSD values, and (ii) time-dependent matrix effects in SWW (ion suppression/enhancement) that can vary between replicates and affect peak integration and response stability. Therefore, while the overall trends are robust, individual late-stage abiotic points with very high %RSD should be interpreted cautiously and given lower weight in mechanistic interpretation, Table 4-9.

The difference in degradation behaviour between enantiomers observed in the abiotic reactor suggests stereoselective attenuation under abiotic conditions, with R-FLX showing a different time-course compared with S-FLX. Such behaviour is consistent with abiotic processes (e.g., sorption–desorption dynamics, hydrolysis/oxidation pathways, and changing chemical equilibrium in SWW) that can affect one enantiomer more than the other through subtle differences in physicochemical interactions and time-dependent matrix composition. In contrast,

the biotic reactor showed a more comparable decline in S-FLX and R-FLX over time, indicating that biological processes reduced the extent of stereoselective divergence observed under abiotic conditions.

The more balanced behaviour in the biotic reactor can be explained without invoking racemization. A more parsimonious interpretation is that microbial transformation pathways in the bioreactor produced broadly similar net removal kinetics for both enantiomers, either because multiple taxa/enzymes act on both forms or because community-level metabolism reduces enantiomer-specific differences that are more apparent under purely abiotic conditions. In addition, increased biological mixing, biomass-associated partitioning, and more stable pH buffering in the biotic system can reduce time-dependent analytical variability and minimise apparent enantiomer-specific fluctuations that may occur in the abiotic matrix.

The observed differences between abiotic and biotic patterns can be discussed alongside the microbial community profile from the taxonomy results. In the biotic system, the convergence in S-FLX and R-FLX behaviour is consistent with the presence of metabolically versatile microbial groups capable of transforming xenobiotics and supporting co-metabolic degradation processes. In particular, *Bacteroidetes* and *Proteobacteria* were dominant phyla in reactors exposed to FLX and its enantiomers, and these groups are widely associated with broad catabolic capacity and biodegradation of complex organic compounds, including pharmaceuticals. The enrichment of these groups under FLX/enantiomer exposure supports their likely contribution to the observed biodegradation trends and the reduced enantiomer-to-enantiomer divergence in the biotic system. This interpretation aligns with reports that bacteria from these phylogenetic groups can participate in FLX transformation under environmental/engineered conditions. [342]

Although enantioselective biodegradation of FLX has been reported for specific isolates (e.g., documented enantiomer preferences in bacterial degradation studies), the present dataset does not provide direct evidence of enantiomer interconversion. Instead, the key finding is that biotic conditions dampened the stereoselective differences seen in SWW, consistent with community-driven biodegradation acting on both enantiomers and producing a more comparable net loss profile. Reported strain-level enantioselectivity in the literature remains relevant as mechanistic

support for why enantiomer-specific responses can occur, but the most defensible conclusion from these reactors is that the microbial community produced an overall biodegradation regime in which both enantiomers were removed with broadly similar efficiency over the incubation period. [11]

In contrast, the abiotic reactor exhibited larger enantiomer-to-enantiomer divergence and substantially higher variability at later time points, indicating that, in the absence of microbial regulation, SWW chemistry and matrix behaviour play a stronger role in shaping apparent enantiomer profiles. Taken together, these results support the conclusion that microbial activity is a key driver of reproducible attenuation trends for Rac-FLX enantiomers, while abiotic conditions are more susceptible to stereoselective effects and time-dependent analytical/matrix variability, particularly when concentrations approach low levels where %RSD inflation is expected.

Table 4-9: FLX and its enantiomers degradation, n=3.

Days		Rac-FLX				S-FLX		R-FLX	
		S-FLX		R-FLX		S-FLX		R-FLX	
		Abiotic	Biotic	Abiotic	Biotic	Abiotic	Biotic	Abiotic	Biotic
	R ²	0.990	0.990	0.992	0.992	0.990	0.990	0.995	0.995
Day 0	Mean	0.866 *	0.541	0.722	0.496	0.690 *	0.821	0.604	0.646
	RSD%	4.314	9.392	3.862	10.081	13.783	19.340	5.190	1.242
Day 1	Mean	0.427	0.364	0.519	0.335	0.445	0.060	0.677	0.089
	RSD%	13.97	6.854	13.281	12.802	19.211	36.971	13.052	3.410
Day 2	Mean	0.518	0.388	0.470	0.280	0.602	0.045	0.794	0.052
	RSD%	15.96	3.101	9.980	22.742	15.202	26.262	3.750	3.242
Day 3	Mean	0.518	0.284	0.641	0.283	0.423	0.044	0.449	0.052
	RSD%	3.802	19.361	3.540	8.142	7.588	7.722	30.231	6.291
Day 7	Mean	0.347	0.159	0.516	0.102	0.147	0.065	0.223	0.068
	RSD%	18.061	8.962	36.160	10.70	13.211	6.231	35.352	17.492
Day 14	Mean	0.338	0.190	0.190	0.119	0.070	0.051	0.047	0.056
	RSD%	25.171	14.951	90.501	9.891	82.730	19.423	9.841	6.940

* The rac-FLX represents total concentration (S + R). S- and R-FLX represent individual enantiomers and therefore are not expected to match rac-FLX. Racemate and enantiomers were quantified in separate analytical runs, which may introduce minor between-run differences.

4.4.4.2. Enantiomeric excess of FLX

The enantiomeric excess (%ee) provides insights into the chiral behavior of FLX in abiotic versus biotic environments, revealing differences in stability, predictability, and enantiomeric preferences. In abiotic systems, the fluctuating %ee highlights the impact of external factors like pH and temperature, whereas in biotic systems,

metabolic processes stabilize the %ee with consistent preferences. This distinction is crucial as it underscores the role of biological systems in modulating chiral behavior, informing applications in environmental analysis, drug design, and biotransformation studies.

Enantiomeric excess (%ee) is a crucial parameter used to evaluate the difference in the amount of two enantiomers of a chiral compound. It can be calculated using the Equation 4-1.

In this study, the enantiomeric excess of chiral FLX was monitored in both abiotic (SWW) and biotic (bioreactor) systems over the study period. In the abiotic reactors, %ee fluctuated considerably over time, shifting from negative values (−10.61% and −19.58%) to positive values (up to 28.03%). The magnitude and range of %ee were also greater in the abiotic reactors than in the biotic reactors, indicating more pronounced temporal variability under abiotic conditions.

$$\text{Enantiomeric excess (\%ee)} = \frac{eS - eR}{eS + eR} * 100$$

Where:

e(S) = Concentration of the S-enantiomer

e(R) = Concentration of the R-enantiomer

Equation 4-1: Enantiomeric excess (%ee) calculation equation.

The stability of enantiomeric excess (%ee) in the abiotic reactors was relatively low, as the preference between S-FLX and R-FLX shifted over time without a consistent directional trend. Such variability is unlikely to arise from biological processes, since the abiotic systems were maintained under controlled and constant experimental conditions. Instead, the observed fluctuations are more plausibly attributed to physicochemical influences, including minor variations in pH, temperature, matrix composition, and time-dependent changes in ionisation efficiency or sorption behaviour, all of which can affect apparent enantiomer ratios in LC-MS based measurements. Similar non-systematic %ee variability under abiotic conditions has been reported for other chiral pharmaceuticals in aqueous matrices, where chemical

transformation and partitioning processes dominate over stereoselective mechanisms. [32]

In contrast, the biotic reactors exhibited comparatively stable %ee values over time (0.18-22.98%), indicating a more consistent enantiomeric preference. Across sampling points, the %ee values generally indicated a modest but reproducible preference toward S-FLX, suggesting the presence of stereoselective microbial metabolism rather than random physicochemical fluctuation. Microorganisms are known to possess enantioselective enzymes capable of preferential transformation of one enantiomer, resulting in stabilised %ee profiles during biodegradation. This behaviour has been widely documented for chiral pharmaceuticals, including fluoxetine and other antidepressants, where microbial communities mediate stereoselective degradation and reduce temporal variability in enantiomeric distribution compared with abiotic systems. Collectively, these findings indicate that biotic processes promote controlled stereoselective transformation, whereas abiotic conditions primarily reflect non-selective physicochemical variability in enantiomer ratios.

The stability of the %ee in the biotic reactors is higher, as the preference for one enantiomer over the other remains relatively consistent at each time point. The %ee values consistently show a preference for one enantiomer, indicating a more predictable behaviour. The presence of living organisms and their metabolic activities in the biotic reactors likely plays a significant role in influencing the enantiomeric preference and leading to the more stable results observed.

The comparison between the abiotic and biotic reactors reveals distinct differences in the enantiomeric excess behaviour of fluoxetine. The abiotic reactors exhibit more variable and unpredictable preferences, with fluctuations between positive and negative values and no clear trend over time. On the other hand, the biotic reactors show a more consistent and stable preference for one enantiomer over the other, with relatively stable %ee values.

The negative enantiomeric excess (%ee) values observed in the abiotic reactor indicate a higher concentration of the R-enantiomer compared to the S-enantiomer.

This suggests that the abiotic conditions may favor the degradation or transformation of the S-enantiomer, leading to an excess of the R-enantiomer. Such stereoselective processes can result from various factors, including chemical reactions or interactions with surfaces that preferentially affect one enantiomer over the other. [350] In contrast, the biotic reactor shows positive %ee values, indicating a higher concentration of the S-enantiomer. This implies that microbial activity in the biotic reactor may preferentially degrade or transform the R-enantiomer, leading to an excess of the S-enantiomer. Microorganisms can exhibit stereoselective behaviour due to the chiral nature of their enzymes, which can preferentially interact with one enantiomer over the other.

The presence of an active microbial community in the biotic reactors is likely to be a key driver of the more consistent and predictable %ee profiles. In biological systems, transformation is mediated by enzyme-catalysed pathways that can be stereoselective, resulting in a reproducible preference for one enantiomer as the community adapts to the substrate and dominant metabolic routes stabilise. By contrast, in the abiotic reactors, where enzymatic selectivity is absent, apparent fluctuations in %ee are more plausibly governed by physicochemical processes (e.g., sorption–desorption dynamics and time-dependent matrix composition) and analytical factors such as variable electrospray ionisation efficiency at low concentrations, rather than by a consistent stereoselective mechanism. Understanding these contrasts is important when interpreting the environmental fate of chiral pharmaceuticals such as fluoxetine, because enantiomer-specific behaviour is often more reliably expressed under biotic conditions, whereas abiotic systems may display greater variability due to non-biological controls on measured enantiomer ratios. [8,351]

Table 4-10: %ee of FLX in abiotic / biotic reactors.

Days	Abiotic reactor			Biotic reactor		
	S-FLX	R-FLX	%ee	S-FLX	R-FLX	%ee
Day 0	0.866	0.722	9.071	0.541	0.496	4.342
Day 1	0.427	0.519	-9.730	0.364	0.335	4.151
Day 2	0.518	0.471	4.862	0.388	0.282	16.172
Day 3	0.518	0.641	-10.610	0.284	0.283	0.180
Day 7	0.347	0.516	-19.581	0.159	0.102	21.841
Day 14	0.338	0.191	28.033	0.190	0.119	22.980

4.4.4.3. Fluoxetine enantiomers

A. Biodegradation of S-FLX

To assess the biodegradation behaviour of a single fluoxetine enantiomer, S-fluoxetine (S-FLX) was incubated in parallel abiotic (SWW) and biotic (bioreactor) systems for 14 days. Quantification was performed by chiral LC–MS using the Astec Chirobiotic™ V2 column, with response calculated as the peak-area ratio). The chromatographic separation and confirmatory MS information for S-FLX are shown in Figure 4-12.

In the abiotic reactor, the initial S-FLX concentration on Day 0 was 0.690 µg/mL, decreasing to 0.070 µg/mL by Day 14 (≈90% reduction). In the biotic reactor, the Day 0 concentration was 0.821 µg/mL, decreasing to 0.051 µg/mL by Day 14 (≈94% reduction). Across the study, pH remained within a narrow alkaline range in both systems (8.08–8.72 in SWW; 7.97–8.53 in the biotic reactor), indicating that the observed decline was not driven by major pH drift (Figure 4-11 and Table 4-9).

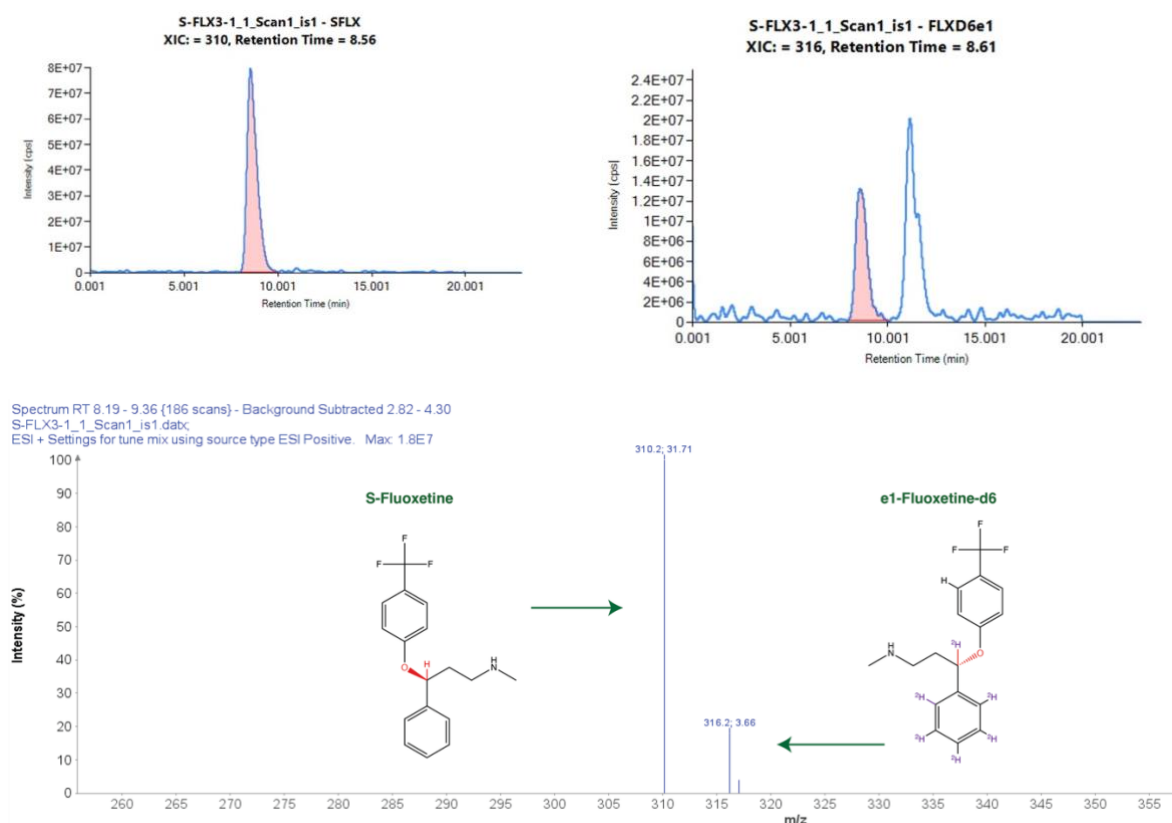


Figure 4-12: Representative extracted-ion chromatograms (XIC) and mass spectra for S-FLX and the FLX-d6.

The RSD varied across time points and between systems. In the biotic reactor, variability was higher at early time points (e.g., Day 1: 36.97%; Day 2: 26.26%) but decreased markedly by Day 7 (6.23%) and remained moderate at Day 14 (19.42%). This trend is consistent with biologically active systems, where initial heterogeneity in biomass distribution and short-term microbial adaptation can increase replicate variability before the community stabilises. In parallel, pH was monitored throughout the experiment (Table X); differences in pH between abiotic and biotic reactors may have influenced FLX ionisation and microbial activity, thereby contributing to the observed variation in degradation behaviour across conditions.

In contrast, the abiotic reactor showed moderate %RSD at most time points (e.g., Day 0: 13.78%; Day 3: 7.59%; Day 7: 13.21%) but exhibited a marked increase at Day 14 (82.73%) despite a low mean response (0.070). This elevated Day-14 variability is most plausibly explained by end-point effects at low residual levels, where small absolute differences between replicates can generate large relative variability. It may also reflect reduced signal near the quantification limit, minor baseline/integration differences, and/or small sampling differences in suspended material in the abiotic matrix. Accordingly, the Day-14 %RSD increase is interpreted as an artefact of very low remaining S-FLX rather than evidence of inconsistent degradation behaviour.

Comparing biodegradation behaviour between Rac-FLX and the single-enantiomer experiment indicates a similar overall decrease in S-FLX over time in both designs. Any small differences in apparent removal between Rac-FLX and the single-enantiomer system may reflect interactions between enantiomers in the racemate (e.g., competition for sorption sites or microbial uptake/enzymatic capacity) and the fact that the racemate experiment simultaneously tracks both S-FLX and R-FLX under identical conditions. Overall, the results confirm that S-FLX is removed under both abiotic and biotic conditions, with enhanced removal in the biotic reactor supporting a contribution from microbial activity in wastewater-like systems.

B. Biodegradation of R-FLX

A similar approach was used to investigate the biodegradation of R-Fluoxetine (R-FLX). Over a period of 14 days, the experiment was conducted in both biotic and

abiotic reactors with the goal of learning more about how these react to the presence of S-FLX. The chiral separation of R-FLX and mass spectrum is shown in Figure 4-13.

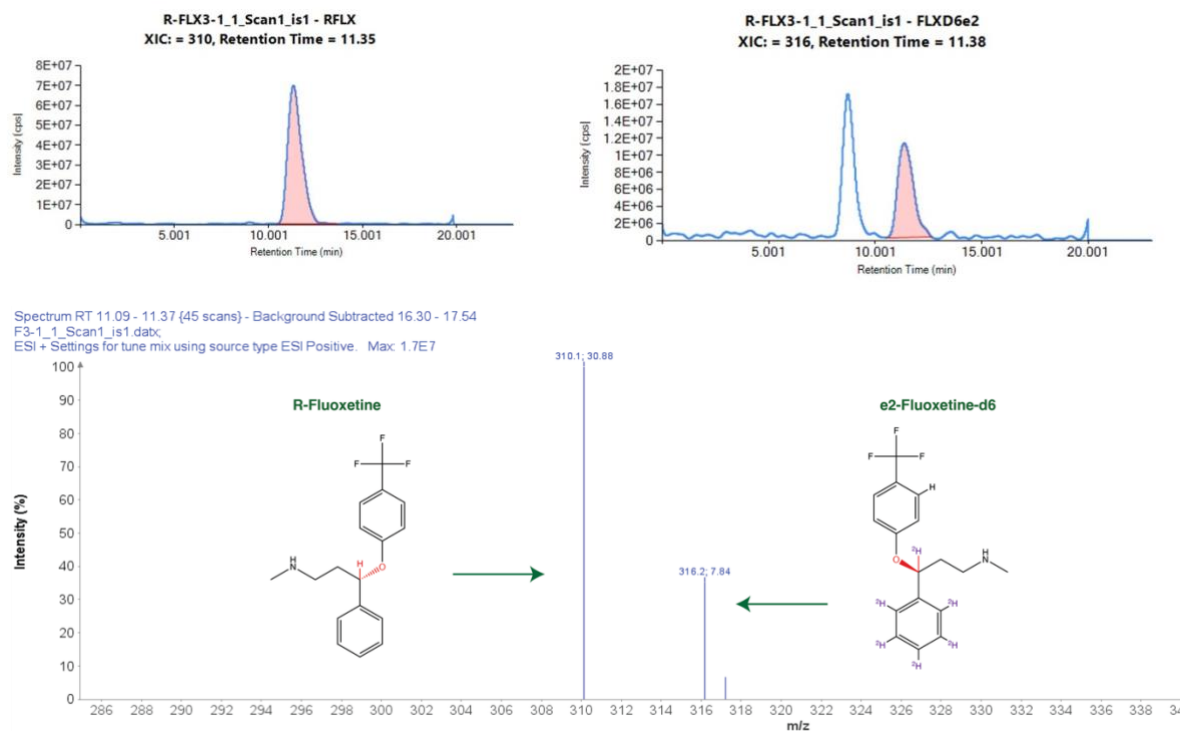


Figure 4-13: Representative extracted-ion chromatograms (XIC) and mass spectra for R-FLX and the FLX-d6.

In both systems, R-FLX concentrations decreased over time, consistent with progressive removal during the 14-day experiment (Figure 4-11 and Table 4-9). However, replicate variability (%RSD) differed between the abiotic and biotic reactors and varied across time points.

In the abiotic reactor, R-FLX started at 0.604 $\mu\text{g/mL}$ (Day 0; %RSD 5.19%) and showed an overall decrease to 0.047 $\mu\text{g/mL}$ by Day 14 (%RSD 9.84%), corresponding to an approximate 92% reduction. Intermediate measurements showed some short-term fluctuations (e.g., Day 1: 0.677 $\mu\text{g/mL}$; Day 2: 0.794 $\mu\text{g/mL}$), which is reflected in the elevated variability at later midpoints (Day 3: %RSD 30.23%; Day 7: %RSD 35.35%). This higher %RSD likely reflects matrix/sampling heterogeneity and analytical variability (e.g., minor differences in suspended material, extraction/injection variability, and integration effects), rather than a true

increase in concentration. The pH in the abiotic reactor remained relatively stable (8.08–8.71) throughout the study.

In the biotic reactor, R-FLX began at 0.646 µg/mL (Day 0; %RSD 1.24%) and decreased rapidly to 0.089 µg/mL on Day 1 (%RSD 3.41%), reaching 0.056 µg/mL by Day 14 (%RSD 6.94%), again representing an overall ~92% reduction. Replicate variability remained comparatively low across most time points (Day 2: %RSD 3.24%; Day 3: %RSD 6.29%), with the highest variability observed at Day 7 (%RSD 17.49%), which is consistent with biological systems where microbial activity, biomass distribution, and ongoing community adaptation can introduce transient heterogeneity. The pH in the biotic reactor also remained stable (7.99–8.56).

The results of this study provide further evidence of the biodegradability of R-FLX and its potential transformation in both abiotic and biotic environments. The enhanced degradation of R-FLX in the biotic reactor once again highlights the essential role of microorganisms in the removal of pharmaceuticals from wastewater systems.

Regarding the biodegradation trend, R-FLX in (Rac-FLX) might exhibit variations in R-FLX degradation rate due to the presence of S-FLX and other components in the mixture, whereas single enantiomer (R-FLX) offers a clear view of the essential biodegradation trend of the pure R-FLX enantiomer, enabling a more accurate assessment of its degradation kinetics and behaviour as an individual enantiomer.

In terms of enantiomer ratios, R-FLX in Rac-FLX has a fixed ratio of S-FLX to R-FLX according to the initial composition of the mixture, which could potentially impact the observed degradation rates of R-FLX during biodegradation. In contrast, single enantiomer (R-FLX) eliminates the influence of other enantiomers, maintaining a constant ratio of R-FLX at 1:1, as it focuses solely on studying the pure enantiomer independently. [11,72]

Across the 14-day period, the single-enantiomer incubations showed greater overall attenuation than the racemic run. In the abiotic system, Rac-FLX decreased from 0.866 to 0.338 (≈61% reduction) for S-FLX and from 0.722 to 0.190 (≈74% reduction) for R-FLX, whereas single S-FLX decreased from 0.690 to 0.070 (≈90%

reduction) and single R-FLX from 0.604 to 0.047 ($\approx 92\%$ reduction). In the biotic system, Rac-FLX decreased from 0.541 to 0.190 ($\approx 65\%$ reduction) for S-FLX and from 0.496 to 0.119 ($\approx 76\%$ reduction) for R-FLX, while single S-FLX decreased from 0.821 to 0.051 ($\approx 94\%$ reduction) and single R-FLX from 0.646 to 0.056 ($\approx 91\%$ reduction).

This pattern suggests that the racemic matrix can alter apparent removal relative to single-enantiomer exposure. Plausible explanations include competitive effects (both enantiomers accessing similar enzymes/uptake routes), time-dependent partitioning to biomass/EPS that differs between runs, and/or matrix-driven analytical variability when residual levels become low. These interpretations are consistent with the broader literature that enantioselective outcomes can depend on treatment conditions, microbial consortia composition, and matrix interactions. [102]

Replicate variability (%RSD) differed across time points and between systems, reflecting both biological heterogeneity and end-point effects at low residual levels. In the single S-FLX experiment, the biotic reactor showed higher %RSD early (Day 1: 36.97%; Day 2: 26.26%) but improved markedly by Day 7 (6.23%) and remained moderate at Day 14 (19.42%), consistent with microbial systems where adaptation and non-uniform biomass distribution can elevate early variability before stabilisation. In contrast, the abiotic reactor showed moderate %RSD at most time points (e.g., Day 0: 13.78%; Day 3: 7.59%; Day 7: 13.21%) but exhibited a pronounced increase at Day 14 (%RSD 82.73%) when the mean response was very low (0.070). This high Day-14 %RSD is most plausibly explained by relative-error inflation near low residual levels, where small absolute differences between replicates produce large %RSD, potentially exacerbated by minor integration/baseline differences and small sampling heterogeneity in suspended material. Accordingly, the Day-14 %RSD increase is interpreted as an artefact of low remaining S-FLX rather than evidence of inconsistent degradation behaviour.

These results provide a practical framework for interpreting the biodegradation data mechanistically, beyond reporting concentration decreases alone. First, they help distinguish abiotic from biotic contributions to stereochemical removal. The stronger enantioselectivity observed in the abiotic Rac-FLX experiment (greater attenuation of R-FLX relative to S-FLX) suggests that non-biological processes in synthetic

wastewater, together with selective partitioning phenomena, can favour the loss of one enantiomer over the other. In contrast, the biotic reactor produced a reduced net enantioselectivity, consistent with microbially mediated pathways acting on both enantiomers and moderating any stereopreference at the system level. [20]

Second, the findings emphasise why an enantiomer-resolved fate assessment is needed, rather than relying only on total FLX decline. Changes in stereochemical metrics such as %ee can provide additional evidence about the dominant attenuation mechanisms during treatment and in environmental matrices. In particular, %ee trends can reflect enantioselective biotransformation and/or time-dependent partitioning behaviour, which may not be apparent when only total concentrations are reported.

Third, the results guide how to evaluate the “racemization” question appropriately. The convergence of S-FLX and R-FLX concentrations over time is not sufficient evidence of racemization. True racemization requires chemical interconversion ($S \leftrightarrow R$), which should be confirmed using targeted experiments, such as incubating a single enantiomer and monitoring the appearance of the opposite enantiomer under suitable controls and mass-balance considerations.

In terms of real-life relevance, fluoxetine in wastewater treatment plants and receiving waters is typically present as a mixture (often alongside other pharmaceuticals), so stereochemical behaviour can influence exposure and risk assessment. If treatment processes remove enantiomers differently, the enantiomeric profile discharged to rivers may differ from the profile entering the plant, and the remaining enantiomer (s) may show different persistence or biological effects. This is one reason enantiomer-specific monitoring is increasingly used in environmental fate studies of chiral pharmaceuticals. EF/%ee trends can also help interpret whether observed attenuation is dominated by biotransformation or by partitioning/sorption processes, particularly when concentrations approach low residual levels where relative variability increases. More broadly, reporting enantiomer-resolved behaviour strengthens the environmental and scientific relevance of the study because stereochemistry is a recognised driver of distinct transformation pathways and biological effects for many chiral pharmaceuticals. [102]

4.4.5. Biodegradation of Norfluoxetine

4.4.5.1. Biodegradation of racemic norfluoxetine

The present study aimed to investigate the biodegradation of fluoxetine's main metabolite, racemic norfluoxetine (Rac-NFLX), in both abiotic and biotic reactors. The biodegradation study of Racemic norfluoxetine (Rac-NFLX), which consists of two enantiomers (S-NFLX and R-NFLX), was conducted using both abiotic and biotic reactors. The aim was to investigate the degradation of the FLX metabolite's enantiomer over a period of 14 days. The chiral separation of Rac-NFLX and mass spectrum is shown in Figure 4-14 and Figure 4-15 demonstrates NFLX and its enantiomers biodegradation.

The initial concentrations of S-NFLX and R-NFLX on Day 0 were determined in both abiotic and biotic reactors. In the abiotic system, S-NFLX was measured at 0.574 µg/mL (%RSD = 16.23), while R-NFLX was 0.302 µg/mL (%RSD = 21.74). Lower initial concentrations were observed in the biotic reactor, where S-NFLX and R-NFLX were 0.300 µg/mL (%RSD = 4.76) and 0.086 µg/mL (%RSD = 21.69), respectively. The starting pH values were comparable between systems (abiotic = 7.60; biotic = 7.53), indicating similar initial chemical environments (Table 4-12).

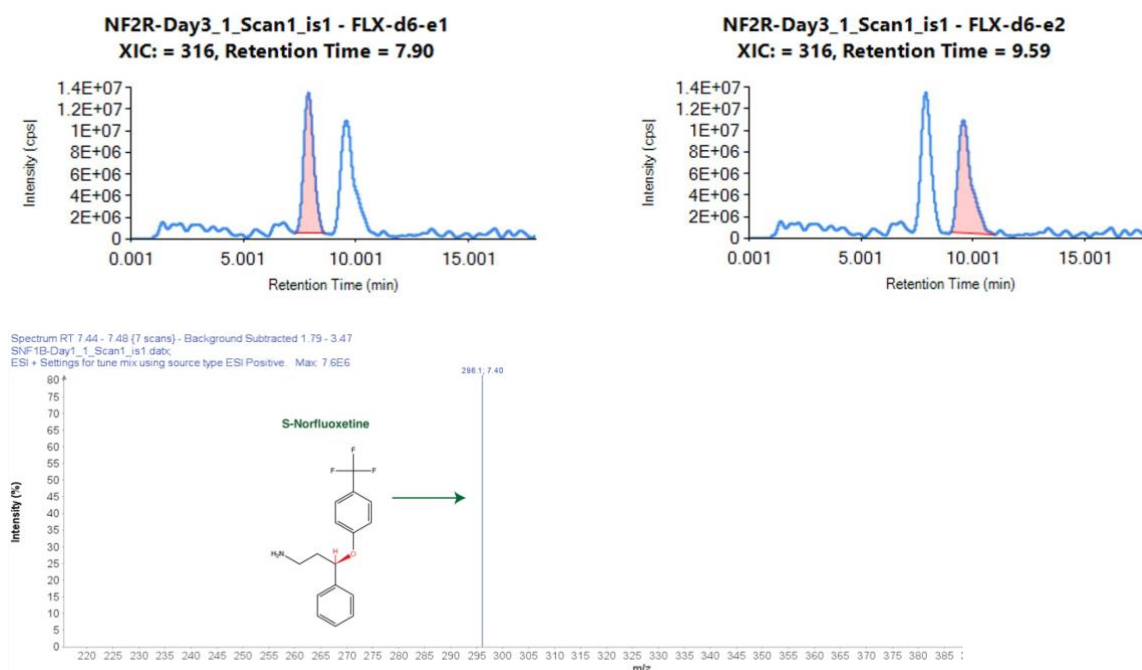
By Day 1, both enantiomers showed decreased concentrations in each reactor. In the abiotic reactor, S-NFLX declined to 0.515 µg/mL (%RSD ≈ 20.0) and R-NFLX to 0.205 µg/mL (%RSD ≈ 21.0), accompanied by an increase in pH to 8.21. In the biotic reactor, S-NFLX decreased to 0.171 µg/mL (%RSD = 15.41) and R-NFLX to 0.014 µg/mL (%RSD = 2.86), with pH rising slightly to 8.12.

From Day 2 onward, both S-NFLX and R-NFLX were below the analytical limit of detection (LOD = 12.5 ng/mL; Section 4.4.2.4) in both reactors. Therefore, the absence of detectable signal should be interpreted as concentrations falling below the method detection capability rather than complete disappearance of the compounds. The persistence of non-detectable levels through Days 3, 7, and 14 indicates rapid attenuation of NFLX enantiomers under both abiotic and biotic conditions.

The gradual increase in pH observed in both systems (abiotic: 7.60–8.49; biotic: 7.53–8.45) indicates modest alkalisation during incubation. Because similar pH trends were observed in the abiotic reactor (i.e., in the absence of biomass), the pH elevation cannot be attributed solely to microbial activity and may instead reflect buffering capacity, degradation by-products, or physicochemical equilibration within the synthetic wastewater matrix.

Although the biotic reactor showed slightly faster early-stage attenuation, particularly for R-NFLX, the subsequent decline to below the LOD in both systems suggests that NFLX removal was governed by a combination of abiotic processes (e.g., sorption, hydrolysis, or other transformation pathways) alongside biotic mechanisms. This mixed removal behaviour aligns with reports that antidepressant metabolites can attenuate rapidly in aqueous systems through coupled physicochemical and microbial pathways.

Overall, these findings demonstrate that NFLX enantiomers undergo rapid depletion to sub-detectable levels within 48 h under the tested conditions, with only limited differentiation between abiotic and biotic removal after Day 2. This highlights the importance of analytical sensitivity and early-time sampling when assessing the environmental fate of rapidly transforming pharmaceutical metabolites.



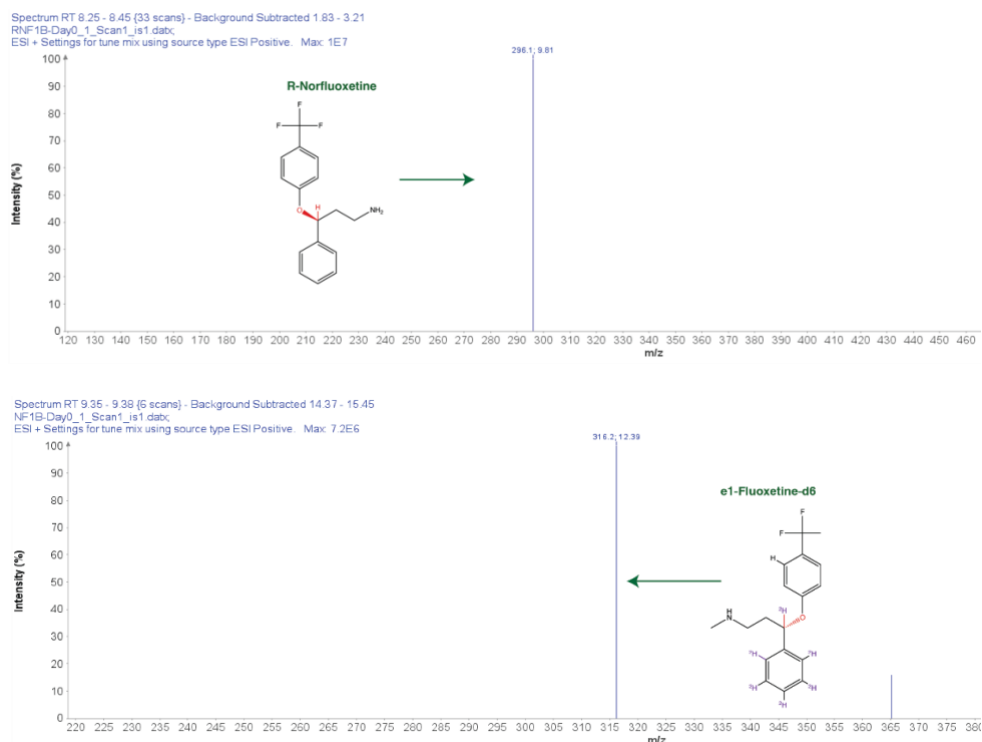


Figure 4-14: Representative extracted-ion chromatograms (XIC) and mass spectra for NFLX and the NFLX-d6.

Table 4-11: NFLX and its enantiomers degradation, n=3.

Days	Rac-NFLX					S-NFLX	R-NFLX		
	S-NFLX		R-NFLX						
		Abiotic	Biotic	Abiotic	Biotic	Abiotic	Biotic	Abiotic	Biotic
Day 0	R ²	0.994	0.994	0.999	0.999	0.992	0.992	0.994	0.994
	Mean	0.574	0.300	0.302	0.086	0.714	0.452	0.379	0.313
	RSD%	16.23	4.76	21.74	21.69	9.87	5.61	12.43	12.08
Day 1	Mean	0.515	0.171	0.205	0.014	0.618	0.076	0.305	ND
	RSD%	20.01	15.41	21.08	2.86	12.88	6.27	8.15	
Day 2	Mean	ND	ND	ND	ND	0.439	ND	0.309	ND
	RSD%					1.80		11.2	
Day 3	Mean	ND	ND	ND	ND	0.460	ND	0.239	ND
	RSD%					13.34			
Day 7	Mean	ND	ND	ND	ND		ND		ND
	RSD%								
Day 14	Mean	ND	ND	ND	ND	ND	ND	ND	ND
	RSD%								

* ND= Not Detected

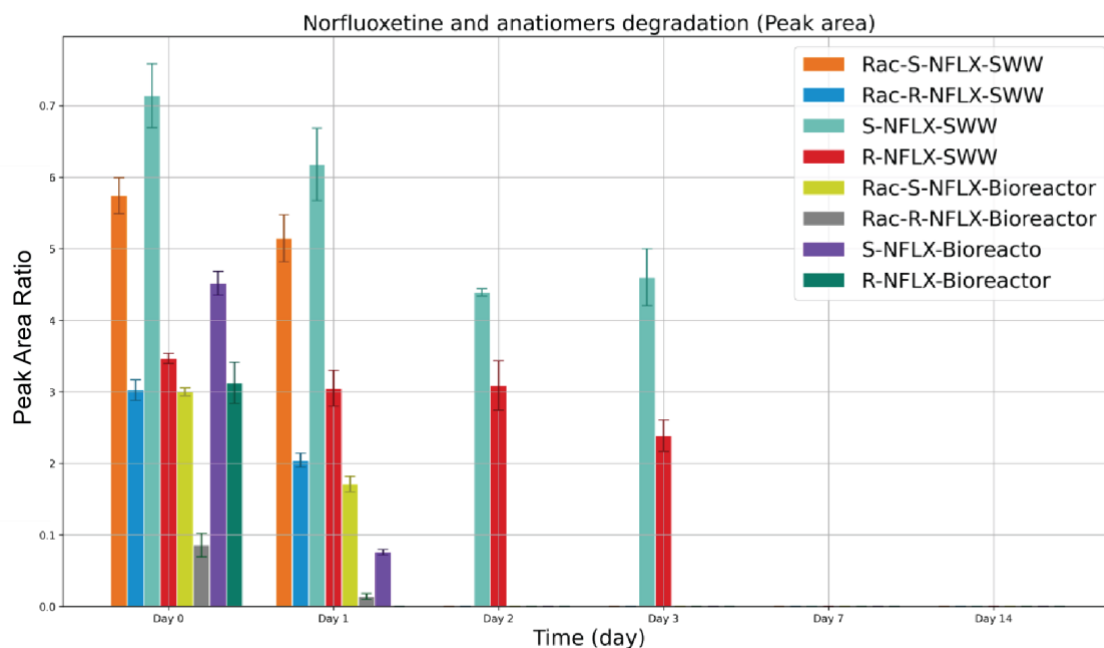


Figure 4-15: NFLX and its enantiomers degradation study.

4.4.5.2. Enantiomeric excess of NFLX

The %ee results indicate stereoselective behaviour of NFLX in both reactors. In the biotic reactor, %ee was already positive at Day 0 (55.44%) and increased to 84.87% by Day 1, indicating enrichment of S-NFLX over time. The abiotic reactor also showed a smaller increase in %ee (31.05% at Day 0 to 43.06% at Day 1). The marked difference in Day 0 %ee between reactors is most plausibly attributable to initial condition effects rather than biological “formation” of S-NFLX, including slight deviations from an ideal 50:50 racemate in the spiking solution and/or rapid, early partitioning immediately after dosing (e.g., differential sorption to biomass and suspended solids in the biotic reactor) before full mixing/equilibration and sampling. After Day 0, the stronger increase in %ee in the biotic system is consistent with preferential attenuation of one enantiomer under biologically active conditions, although contributions from abiotic processes (e.g., sorption or transformation) are also indicated by the non-zero %ee observed in the abiotic reactor.

The comparison between the results of the biotic and abiotic reactors for spiked norfluoxetine reveals interesting insights into the enantioselective behaviour of

the two systems. In the biotic reactors, S-NFLX shows a remarkable increase in enantiomeric excess over time, with %ee values rising from 55.44% at Day 0 to an impressive 84.87% at Day 1. This significant increase indicates a strong preference for the formation of S-NFLX over R-NFLX in the presence of specific biocatalysts, enzymes, or microorganisms. On the other hand, the abiotic reactors also exhibit an increase in enantiomeric excess, but the rise is more moderate, with %ee values going from 31.05% at Day 0 to 43.06% at Day 1. The comparison highlights the much higher stereoselectivity of the biotic reactors compared to the abiotic ones. The biotic system's ability to produce S-NFLX with a substantially higher enantiomeric excess suggests potential applications in chiral transformations and pharmaceutical synthesis, Table 4-12.

The enantiomeric excess (%ee) results for spiked norfluoxetine (NFLX) provide a sensitive indicator of enantioselective behaviour in the two systems. In this context, an increase in %ee in favour of S-NFLX is best interpreted as enrichment of the S-enantiomer in the measured (aqueous/extractable) phase, which can arise from preferential loss of R-NFLX (e.g., stereoselective biotransformation) and/or enantioselective partitioning (e.g., sorption to solids/biomass), rather than “formation” of S-NFLX.

In the biotic reactor, %ee increased strongly from 55.44% (Day 0) to 84.87% (Day 1), indicating rapid S-enrichment during the first 24 h. This pattern is consistent with wastewater biotic matrices where microbial consortia and their enzymes can display stereoselective transformation, producing non-racemic residues and time-dependent enantiomeric profiles. Such enantioselective behaviour has been reported for fluoxetine/norfluoxetine in environmental and wastewater-related contexts, where biological processing can shift enantiomeric signatures over short timescales. [352,353]

In the abiotic reactor, %ee also increased but more moderately (31.05% to 43.06%, Day 0 to Day 1). A smaller shift under abiotic conditions suggests that non-biological drivers (e.g., pH-dependent speciation, selective sorption to suspended matter or reactor surfaces, and other matrix-driven partitioning effects) can influence the

apparent enantiomer balance, but typically with weaker net enantioselectivity than in biologically active systems. [352,353]

The Day-0 difference (biotic %ee vs abiotic %ee) can occur immediately after spiking because the biotic reactor contains biomass and extracellular polymeric substances that introduce additional sorption and microenvironments. Rapid, preferential partitioning of one enantiomer to solids/biomass can shift the measured aqueous/extractable enantiomer ratio even before substantial biodegradation has occurred. Minor practical factors (mixing intensity, sampling timing, and matrix heterogeneity) can further accentuate small early differences. Therefore, the Day-0 offset should be interpreted primarily as a matrix/partitioning effect, while the Day-1 increase (especially in biotic) supports stronger enantioselective removal processes. [352,353]

Enantiomeric profiling (EF/%ee) is increasingly used because total concentration alone can mask stereoselective fate. If treatment or receiving waters enrich one enantiomer, the discharged mixture may differ in persistence and biological effects, since enantiomers of chiral pharmaceuticals often differ in potency and ecotoxicity. Field and wastewater studies have reported non-racemic signatures for fluoxetine/norfluoxetine in wastewater matrices, supporting the relevance of enantiomer-resolved interpretation. [354,355]

Table 4-12: %ee of NFLX in biotic / abiotic reactors.

	Abiotic reactor			Biotic reactor		
	S-NFLX	R-NFLX	%ee	S-NFLX	R-NFLX	%ee
Day 0	0.574	0.302	31.050	0.3	0.086	55.440
Day 1	0.515	0.205	43.056	0.171	0.014	84.865
Day 2- Day 14	ND					

ND= Not Detected

4.4.5.3. Biodegradation of norfluoxetine enantiomers

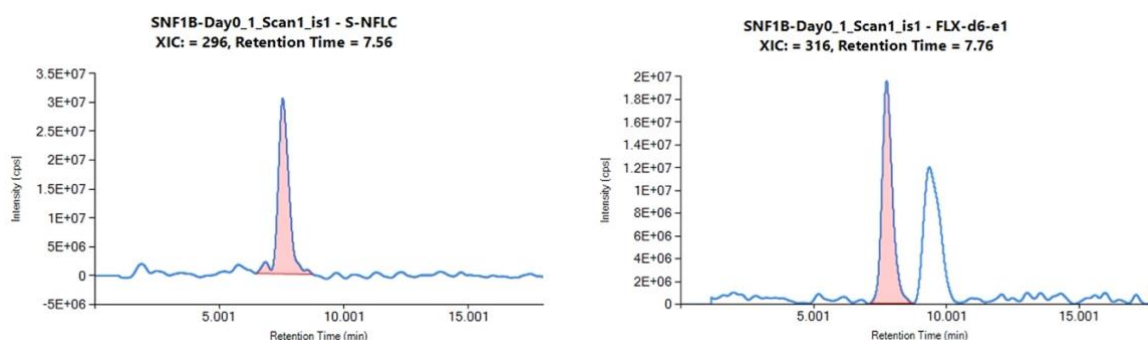
A. Biodegradation of S-NFLX

The biodegradation of S-norfluoxetine (S-NFLX), the principal metabolite of S-fluoxetine, was investigated in parallel abiotic (SWW) and biotic (bioreactor) systems over 14 days. Enantiomeric separation and identification were performed using the

same chiral LC-MS method described previously (Section 4.4.2.4), employing the Astec Chirobiotic™ V2 column, with quantification based on peak-area ratios. The chromatographic separation and confirmatory mass spectra for S-NFLX are shown in Figure 4-16.

On Day 0, S-NFLX was quantified in both systems, with mean concentrations of 0.714 µg/mL in the abiotic reactor (%RSD = 9.87) and 0.452 µg/mL in the biotic reactor (%RSD = 5.61). Reactor pH values were similar (8.06 abiotic; 8.02 biotic). The slightly lower Day-0 concentration in the biotic reactor likely reflects matrix/partitioning effects (e.g., sorption to biomass/EPS or solids) and/or small differences in effective aqueous phase recovery, rather than a true difference in the spiked dose. Overall, replicate precision at Day 0 was acceptable in both systems (Table 4-11).

By Day 1, S-NFLX decreased in both reactors. In the abiotic reactor, the mean concentration was 0.618 µg/mL (%RSD = 12.88), whereas in the biotic reactor it declined more sharply to 0.076 µg/mL (%RSD = 6.27). Over the same interval, pH increased to 8.53 (abiotic) and 8.36 (biotic). The markedly faster decrease in the biotic reactor is consistent with additional biotic removal processes (biotransformation and/or enhanced partitioning to biomass) operating alongside any abiotic losses.



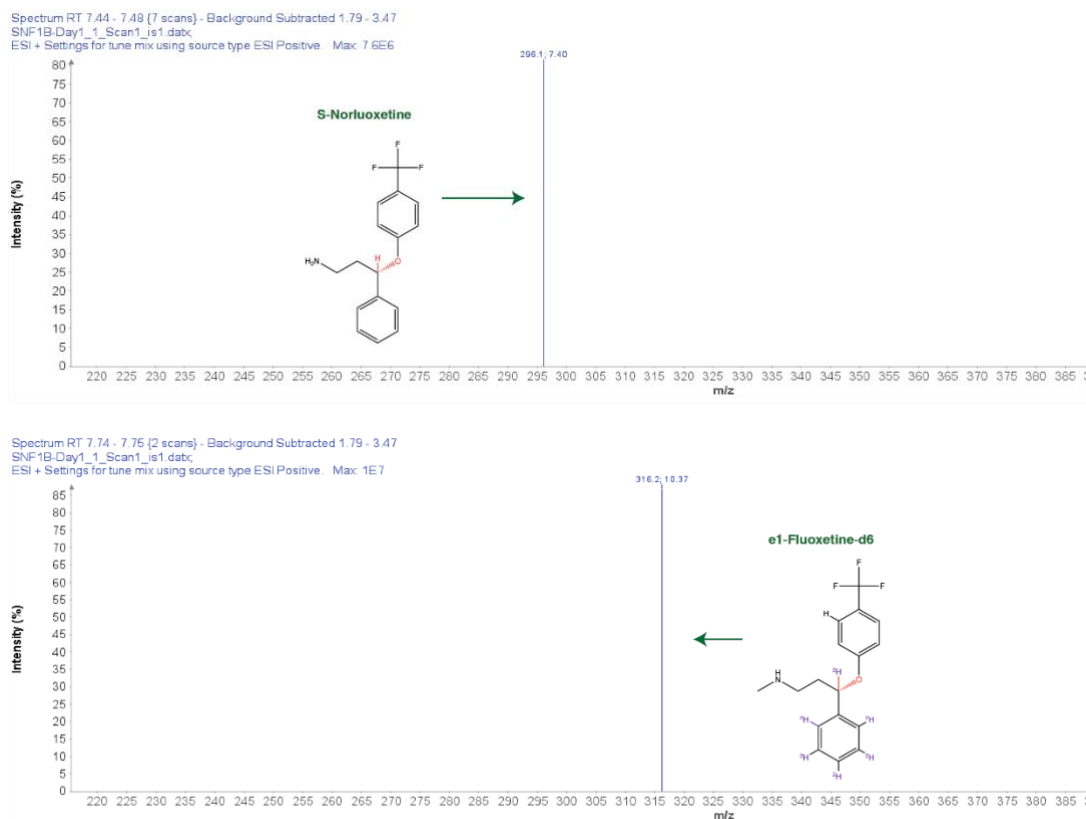


Figure 4-16: Representative extracted-ion chromatograms (XIC) and mass spectra for S-NFLX and the FLX-d6.

On Day 2, S-NFLX in the abiotic reactor further decreased to 0.439 $\mu\text{g/mL}$ (%RSD = 1.8). In the biotic reactor, S-NFLX was not detected (ND). Here, ND indicates that the signal was below the method LOD (12.5 ng/mL; Section 4.4.2.4) and therefore could not be reliably distinguished from baseline noise. From Day 3 onward (Days 3, 7, and 14), S-NFLX was ND in both reactors. Importantly, “ND” does not necessarily mean complete absence; it indicates that residual concentrations (if present) were < LOD under the applied analytical conditions.

Overall, S-NFLX removal occurred in both systems, but the biotic reactor showed earlier attenuation to below the LOD, consistent with the contribution of biological processes. The abiotic reactor exhibited a more gradual decline before also falling below the LOD at later time points. pH remained within a mildly alkaline range during the experiment; however, given that both systems became ND despite similar pH conditions, pH change alone is unlikely to explain the disappearance, and the

findings are more consistent with time-dependent transformation and/or partitioning processes in each matrix.

The comparison of S-NFLX biodegradation in Rac-NFLX and single-enantiomer S-NFLX systems reveals both shared trends and mechanistic differences under abiotic and biotic conditions. In both experimental sets, the initial S-NFLX concentrations were higher in the abiotic reactors than in the biotic reactors. This difference is most plausibly explained by early microbial uptake, sorption to biomass/extracellular polymeric substances (EPS), or rapid biotransformation occurring immediately after incubation in the biotic system, rather than true formation differences at time zero. Similar early-phase decreases driven by biomass interaction and microbial metabolism have been widely reported for chiral pharmaceuticals in activated sludge and bioreactor environments. [32,302]

Therefore, the present results are consistent with the published literature and mechanistically expected, rather than anomalous. From an analytical-precision perspective, %RSD values were generally higher in Rac-NFLX than in single S-NFLX experiments. This is reasonably attributed to the greater chemical and chromatographic complexity of racemic mixtures, which can introduce variability in extraction efficiency, ionisation, and peak integration compared with pure enantiomer systems. Despite this, %RSD values remained within acceptable analytical ranges, supporting the reliability of the biodegradation trends observed in the biotic reactors.

Overall, the combined Rac-NFLX and single-enantiomer experiments demonstrate that microbial activity governs the environmental fate of S-NFLX, producing rapid and extensive degradation that aligns with previously reported behaviour for chiral antidepressants in wastewater treatment. While the presence of the second enantiomer in Rac-NFLX may slightly influence degradation kinetics through competition, co-metabolism, or stereoselective enzyme interactions, the dominant outcome in both systems is efficient microbial removal.

B. Biodegradation of R-NFLX

The biodegradation of R-NFLX, the R-enantiomer of fluoxetine's primary metabolite, was investigated in parallel abiotic and biotic reactor systems over 14 days.

Enantiomeric separation and confirmatory mass spectral identification were achieved using the previously validated chiral LC–MS method (Figure 4-17).

At Day 0, R-NFLX concentrations were comparable between reactors, measuring 0.379 µg/mL (%RSD = 12.43) in the abiotic system and 0.313 µg/mL (%RSD = 12.08) in the biotic system, with similar initial pH values (8.08 and 8.03, respectively). These %RSD values indicate acceptable analytical precision at the starting point.

By Day 1, the abiotic concentration declined modestly to 0.305 µg/mL (%RSD = 8.15), whereas R-NFLX became non-detectable in the biotic reactor. Considering the validated LOD of 12.5 ng/mL (Section 4.4.2.4), this non-detection indicates removal below the analytical detection threshold rather than absolute disappearance. The simultaneous increase in pH to 8.53 (abiotic) and 8.37 (biotic) suggests that microbial transformation, rather than pH variation alone, governed the rapid loss observed in the biotic system.

During Days 2–3, R-NFLX continued to decline gradually in the abiotic reactor (0.309 µg/mL; %RSD = 11.2 and 0.239 µg/mL; %RSD ≈ 0.004, respectively), while remaining below detection in the biotic reactor. By Days 7 and 14, R-NFLX was non-detectable in both systems, indicating near-complete attenuation over time. Because concentrations fell below the method LOD, these later-stage observations should be interpreted as residual levels below quantifiable limits rather than definitive absence.

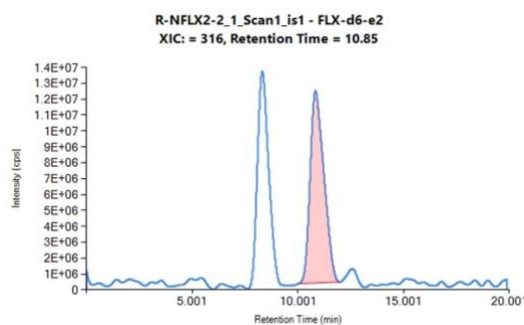
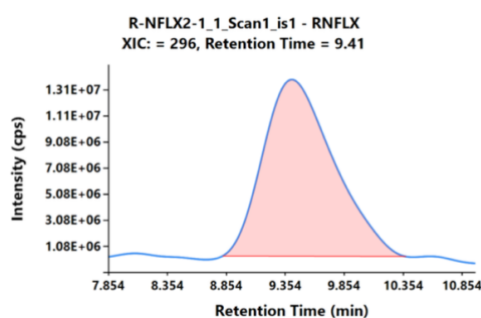
The biodegradation of R-norfluoxetine (R-NFLX), the R-enantiomer of fluoxetine's primary metabolite, was investigated in parallel abiotic and biotic reactors over 14 days. Enantiomeric separation and confirmatory mass spectral identification were achieved using the validated chiral LC–MS method (Figure 4-17).

At Day 0, R-NFLX concentrations were comparable between reactors, measuring 0.379 µg/mL (%RSD = 12.43) in the abiotic system and 0.313 µg/mL (%RSD = 12.08) in the biotic system, with similar initial pH values (8.08 and 8.03, respectively). By Day 1, the abiotic concentration decreased modestly to 0.305 µg/mL (%RSD = 8.15), whereas R-NFLX was reported as non-detectable (ND) in the biotic reactor. Here, ND indicates that concentrations fell below the method LOD (Section 4.4.2.4), and should be interpreted as removal below analytical detection capability rather

than absolute loss of mass. Over Days 2–3, R-NFLX continued to decline gradually in the abiotic reactor ($0.309\ \mu\text{g/mL}$, %RSD = 11.2; and $0.239\ \mu\text{g/mL}$, %RSD $\approx$ 0.004), while remaining ND in the biotic reactor. By Days 7 and 14, R-NFLX was ND in both systems, indicating that residual concentrations—if present—were below the method detection/quantification capability.

Across the experiment, pH showed only minor increases and remained within a similar range in both reactors (e.g., Day 1: 8.53 abiotic vs 8.37 biotic), suggesting that pH drift was not the primary driver of the contrasting removal profiles. Overall, the much faster decline in the biotic reactor indicates that microbial activity was the dominant removal mechanism, whereas abiotic processes alone produced a slower attenuation pattern.

Comparing R-NFLX behaviour within the racemic metabolite mixture (Rac-NFLX) and as a single enantiomer provides further context on stereochemical effects. In the racemate experiment, R-NFLX decreased gradually under abiotic conditions but became ND in the biotic reactor by Day 1, consistent with rapid microbial attenuation. In the single-enantiomer experiment, R-NFLX again showed slower abiotic decline but fell below the LOD in the biotic reactor by Day 3, confirming efficient microbial removal of the R-enantiomer regardless of whether it was present alone or within a racemic mixture. Collectively, these findings support that biotic transformation primarily governs environmental attenuation of R-NFLX, while abiotic mechanisms contribute secondary, slower removal.



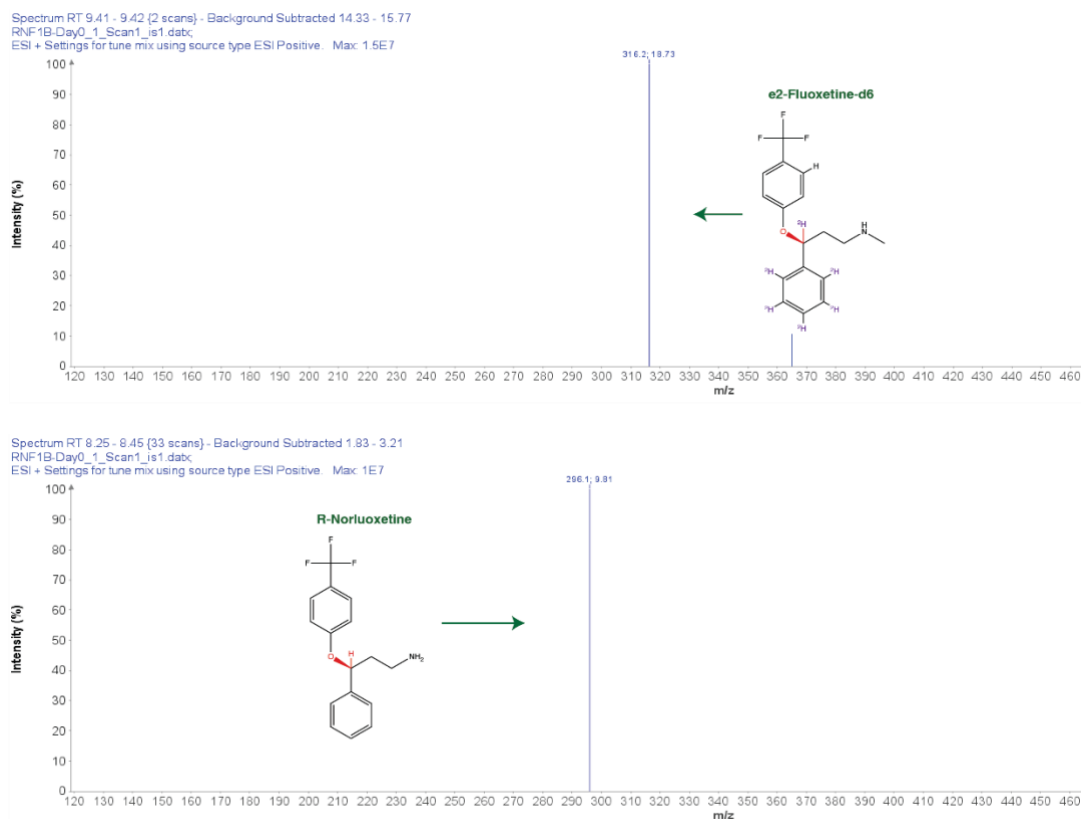


Figure 4-17: Representative extracted-ion chromatograms (XIC) and mass spectra for R-NFLX and the FLX-d6.

The comparison of R-norfluoxetine (R-NFLX) behaviour across the racemate (Rac-NFLX) and single-enantiomer experiments indicates that microbial activity is the dominant driver of rapid attenuation of this metabolite in aqueous systems. In the single R-NFLX run, initial concentrations were comparable between reactors at Day 0 (0.379 µg/mL in the abiotic system vs 0.313 µg/mL in the biotic system). By Day 1, however, R-NFLX was non-detectable in the biotic reactor, while it remained measurable in the abiotic reactor (0.305 µg/mL). This provides a clear quantitative contrast between systems: biotic concentrations fell below the detection limit by Day 1, whereas abiotic removal was slower and gradual. Importantly, “non-detectable” (ND) should be interpreted as below the validated method LOD (12.5 ng/mL; Section 4.4.2.4) rather than as evidence of complete mass loss; ND is therefore an analytical statement about method sensitivity rather than an absolute statement of disappearance.

These observations are consistent with the wider literature on chiral pharmaceuticals, where biotic wastewater treatment processes commonly reduce concentrations and may also alter enantiomeric composition (EF/%ee) via biodegradation/biotransformation, while abiotic processes alone are typically slower and often governed by partitioning/sorption with comparatively limited chemical transformation. [356] For fluoxetine/norfluoxetine specifically, enantioselective behaviour during treatment and in environmental matrices has been reported, supporting the interpretation that microbial transformation is a key driver and that stereochemistry can influence apparent attenuation patterns. [302,356]

From an environmental perspective, these findings strengthen fate and risk interpretation in real systems. If R-NFLX is removed rapidly under biotic conditions but persists longer under abiotic conditions, then attenuation in wastewater treatment plants and receiving waters will be expected to depend strongly on biological activity (e.g., biomass abundance, acclimation, redox conditions, temperature, and matrix composition). Practically, this supports the use of enantioselective monitoring (EF/%ee alongside concentration) as an interpretive tool to help distinguish biotransformation from non-biological effects when tracking fluoxetine residues through treatment and into the environment. More broadly, because fluoxetine and norfluoxetine are environmentally relevant and have documented biological effects in aquatic organisms, characterising both the rate of attenuation and any stereochemical shifts during treatment adds ecological meaning to the dataset. [356–358]

Finally, the rapid decline to <LOD at the tested $\mu\text{g/mL}$ spike level does not exclude concentration- or system-dependence in kinetics (e.g., enzyme saturation/inhibition, co-metabolism limitations, and non-linear sorption). Under higher loadings or less active microbial conditions, persistence could plausibly increase. This can be evaluated by repeating the experiment across multiple spike levels and, where feasible, tracking both aqueous and sorbed phases to support a fuller mass balance. [100,189]

4.4.6. VEN and ODV bioreactors 16S Taxonomy

4.4.6.1. Free drugs reactors

The “Blank-day 0” reactors represent the free-drug biotic reactors at Day 0 (baseline) for each stage. The “Blank-day 14” reactors represent the pooled late time-point samples (Day 7 + Day 14 combined) from the same free-drug reactors, used to summarise the community composition at the later phase of incubation. Across all stages, the bacterial community profiles show clear shifts between baseline (Blank-day 0) and the pooled late samples (B-d14), indicating time-dependent changes in relative abundance of multiple taxa. Overall, *Sphingobacteriia* and *Alphaproteobacteria* remain among the dominant classes across stages, although their relative abundances vary with time and stage. For example, in Stage 1, the relative abundance of *Sphingobacteriia* increased from 2.16% (B-d0) to 25.41% (B-d14; pooled Day 7 + Day 14), while *Alphaproteobacteria* increased from 23.79% to 40.71% (Table 4-13).

4.4.6.2. VEN and ODV bioreactors 16S Taxonomy

The drugs and their enantiomers being compared are VEN, S-VEN, and R-VEN at Day 14. The relative abundances of bacterial classes in the drug and enantiomer-spiked reactors are as follows: for VEN at Day 14, *Sphingobacteriia* comprises 34.07%, and *Alphaproteobacteria* accounts for 29.44%, making them dominant classes. For S-VEN at Day 14, *Sphingobacteriia* is at 22.26%, and *Alphaproteobacteria* is at 41.41%, indicating their dominance. For R-VEN at Day 14, *Sphingobacteriia* is at 19.56%, and *Alphaproteobacteria* is at 46.16%, making them dominant classes. Comparing VEN with S-VEN and R-VEN, there are differences in the bacterial class distribution, particularly in the relative abundances of *Sphingobacteriia* and *Alphaproteobacteria*. S-VEN and R-VEN show distinct effects on the bacterial community, with the enantiomers leading to different changes compared to the racemic mixture (VEN).

The analysis of microbial community composition in reactors exposed to VEN and its enantiomers, S-VEN and R-VEN, reveals substantial shifts over a 14-day period. In the blank reactor at Day 0 (B-d0), the dominant classes were Saprospirae (35.63%) and *Alphaproteobacteria* (23.79%), with notable contributions from *flavobacteriia*

(10.93%) and *Actinobacteria* (10.59%). By Day 14 (B-d14), there was a marked increase in *Alphaproteobacteria* to 40.71% and *Sphingobacteriia* to 25.41%, while *Saprospirae* decreased to 11.82%. These changes suggest a natural succession in microbial communities, possibly due to environmental factors or resource availability, Figure 4-18.

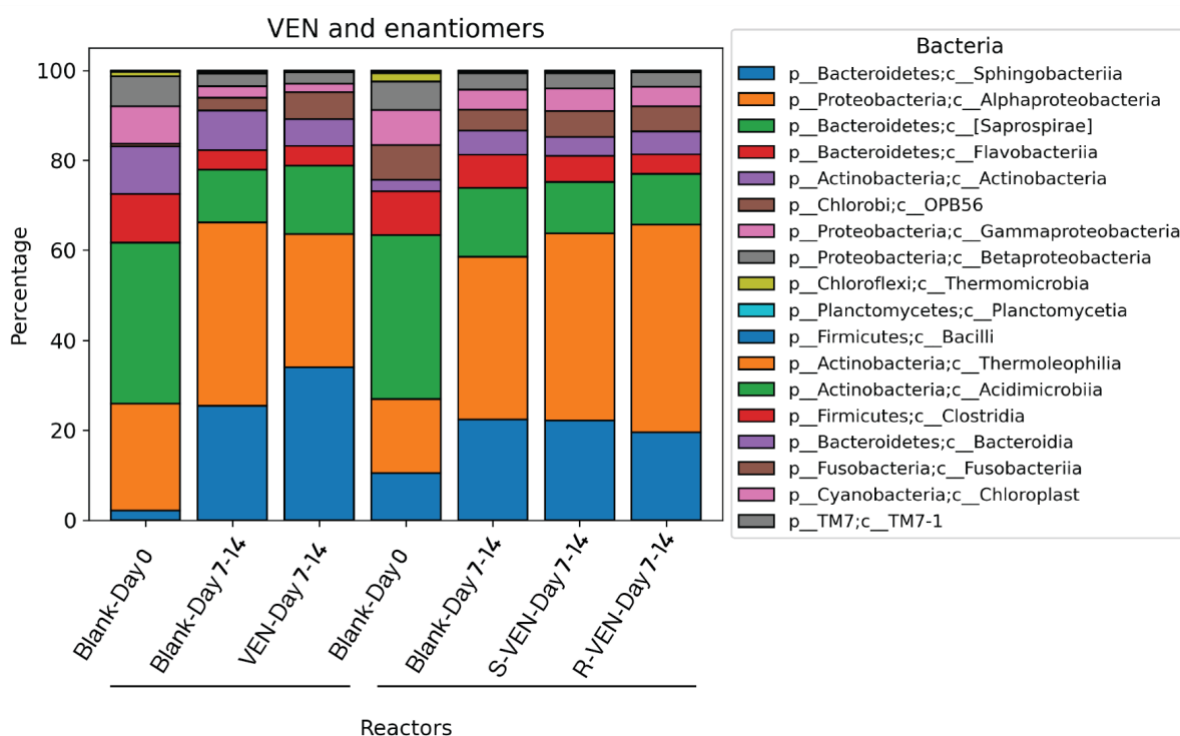


Figure 4-18: VEN and its enantiomers taxonomy results.

Upon introduction of VEN, the microbial profile exhibited notable alterations. *Sphingobacteriia* became the dominant class at 34.07%, followed by *Alphaproteobacteria* at 29.44%. The increase in *Sphingobacteriia* suggests their potential role in venlafaxine degradation. Conversely, *saprospirae* decreased to 15.24%, indicating a reduced role under VEN exposure. Other classes such as *actinobacteria* (5.89%) and *flavobacteriia* (4.51%) also showed declines, highlighting the selective impact of VEN on microbial community structure.

The enantiomers S-VEN and R-VEN elicited distinct microbial responses. In the S-VEN treatment, *alphaproteobacteria* dominated at 41.41%, with *Sphingobacteriia* at 22.26%. This suggests that *Alphaproteobacteria* are more responsive to the S-

enantiomer. In the R-VEN treatment, *Alphaproteobacteria* increased further to 46.16%, while *sphingobacteriia* were at 19.56%. The higher abundance of *Alphaproteobacteria* in R-VEN indicates a stronger microbial adaptation to the R-enantiomer. Other classes, including *saprospirae*, *Flavobacteriia*, and *Actinobacteria*, remained relatively consistent between the enantiomer treatments, suggesting that their roles are less influenced by the stereochemistry of VEN.

On the other hand, the analysis of microbial community composition in reactors exposed to ODV and its enantiomers, S-ODV and R-ODV, reveals significant shifts over a 14-day period. In the blank reactor at Day 0 (B-d0), the dominant classes were saprospirae (18.13%) and sphingobacteriia (20.09%), with notable contributions from alphaproteobacteria (15.43%) and flavobacteriia (17.97%). By Day 14 (B-d14), there was a marked increase in Betaproteobacteria to 15.2% and alphaproteobacteria to 19.15%, while sphingobacteriia decreased to 11.26%. These changes suggest a natural succession in microbial communities, possibly due to environmental factors or resource availability, Figure 4-19.

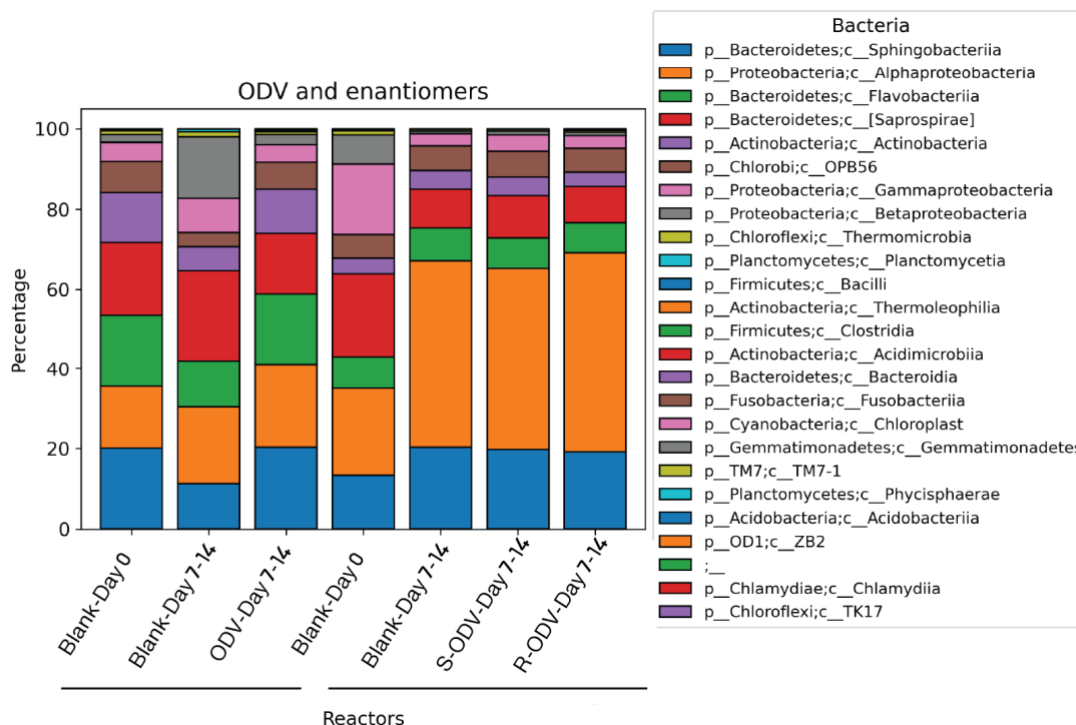


Figure 4-19: ODV and its enantiomers taxonomy results.

Upon introduction of ODV, the microbial profile exhibited notable alterations. alphaproteobacteria became the dominant class at 20.77%, followed by sphingobacteriia at 20.35%. The increase in alphaproteobacteria suggests their potential role in ODV degradation. Conversely, saprospirae decreased to 15.11%, indicating a reduced role under ODV exposure. Other classes such as Actinobacteria (11.1%) and flavobacteriia (17.67%) also showed declines, highlighting the selective impact of ODV on microbial community structure.

The enantiomers S-ODV and R-ODV elicited distinct microbial responses. In the S-ODV treatment, alphaproteobacteria dominated at 45.44%, with sphingobacteriia at 19.79%. This suggests that alphaproteobacteria are more responsive to the S-enantiomer. In the R-ODV treatment, alphaproteobacteria increased further to 49.93%, while sphingobacteriia were at 19.18%. The higher abundance of alphaproteobacteria in R-ODV indicates a stronger microbial adaptation to the R-enantiomer. Other classes, including saprospirae, flavobacteriia, and Actinobacteria, remained relatively consistent between the enantiomer treatments, suggesting that their roles are less influenced by the stereochemistry of ODV.

Initially dominant sphingobacteriia exhibited a decrease in relative abundance over time in control reactors. However, their presence remained significant in reactors treated with VEN and ODV, indicating a potential role in the degradation of these compounds.

Conversely, saprospirae and actinobacteria showed variable responses, generally declining in abundance upon exposure to VEN and ODV. This trend suggests they are less involved in the degradation processes of these compounds.

These findings align with previous research highlighting the persistence of venlafaxine in aquatic environments and the challenges associated with its degradation. For instance, studies have reported that VEN exhibits resistance to degradation, with half-lives ranging from several days to weeks, depending on environmental conditions. [359] Additionally, advanced oxidation processes have been explored to enhance the breakdown of VEN, underscoring the need for effective treatment strategies to mitigate its environmental impact. [360]

In Figure 4-18 and Figure 4-19, microbial community composition is presented at the class level for blank controls and reactors spiked with VEN/ODV and their enantiomers. B-d0 refers to the blank reactor at Day 0 (baseline), whereas B-d7–14 refers to the *blank control sample pooled/composited from Days 7 and 14*. Likewise, the drug-spiked bars labelled Day 7–14 represent the corresponding pooled/composited community over the later sampling window, rather than a single Day-14 measurement.

In the blank control at baseline (B-d0), the community was dominated by *Saprospirae* (35.63%) and *Alphaproteobacteria* (23.79%), with additional contributions from *Flavobacteriia* (10.93%) and *Actinobacteria* (10.59%). In the pooled late-time blank (B-d7-14), the profile shifted towards *Alphaproteobacteria* (40.71%) and *Sphingobacteriia* (25.41%), while *Saprospirae* decreased to 11.82%. This indicates that community structure changed over time even in the absence of drug exposure, consistent with normal community succession under the reactor conditions. [361]

Against this time-shifted background, the VEN-spiked reactor (VEN-d7–14) showed a profile in which *Sphingobacteriia* (34.07%) and *Alphaproteobacteria* (29.44%) were the most abundant classes. When the single enantiomers were tested, both S-VEN and R-VEN were associated with a stronger relative dominance of *Alphaproteobacteria* (41.41% and 46.16%, respectively) and a lower proportion of *Sphingobacteriia* (22.26% and 19.56%, respectively) compared with VEN. Overall, these patterns suggest that VEN exposure, and potentially VEN stereochemistry, coincided with measurable shifts in community composition, particularly in the balance between *Alphaproteobacteria* and *Sphingobacteriia*. However, these data indicate association rather than proving which taxa directly drive transformation, because relative abundance shifts can also reflect indirect ecological responses (e.g., competitive release, altered resource availability, or tolerance). [362,363]

For the ODV series, the blank control at Day 0 (B-d0) contained substantial proportions of *Sphingobacteriia* (20.09%), *Flavobacteriia* (17.97%), *Alphaproteobacteria* (15.43%), and *Saprospirae* (18.13%). In the pooled late blank (B-d7-14), the distribution changed, with increases in *Alphaproteobacteria* (19.15%) and *Betaproteobacteria* (15.2%), alongside a reduction in *Sphingobacteriia*

(11.26%), again supporting time-dependent community succession under control conditions.

In the ODV-spiked reactor (ODV-d7-14), *Alphaproteobacteria* (20.77%) and *Sphingobacteriia* (20.35%) were co-dominant, with other classes (e.g., *Saprospirae* 15.11%, Actinobacteria 11.1%, *Flavobacteriia* 17.67%) remaining appreciable. In contrast, the single-enantiomer reactors showed a pronounced enrichment of *Alphaproteobacteria* in both cases (45.44% for S-ODV and 49.93% for R-ODV), while *Sphingobacteriia* remained at a similar level (19.79% and 19.18%, respectively). Taken together, the ODV results mirror the VEN pattern in that single-enantiomer exposures were associated with a stronger dominance of *Alphaproteobacteria* than the parent compound, suggesting that stereochemistry may influence how the reactor community is structured during the later phase. [361]

Across both drug series, the blank controls demonstrate that community composition shifts over time even without drug, so comparisons should be made against the appropriate pooled blank (B-d7-14) rather than baseline alone. Relative to the pooled blank, VEN/ODV exposure coincided with distinct community profiles, and the single-enantiomer reactors (S- and R-forms) were consistently associated with greater *Alphaproteobacteria* dominance than the parent compound under the same pooled time window. Because the Day 7-14 bars represent pooled samples, these plots provide a late-phase summary and may mask day-specific dynamics; therefore, the findings are best interpreted as evidence of time-dependent community restructuring with drug- and stereochemistry-associated differences, rather than definitive identification of degraders.

Table 4-13: VEN, ODV and their enantiomers bioreactors taxonomy.

index	Sphingobacteriia	Alphaproteobacteria	Saprospirae	Flavobacteriia	Actinobacteria	OPB56	Gammaproteobacteria	Betaproteobacteria	Thermomicrobia	Other
B-d0	2.16	23.79	35.63	10.93	10.59	0.55	8.34	6.80	0.89	<1
B-d14	25.41	40.71	11.82	4.33	8.79	2.83	2.53	2.83	0.46	<1
VEN	34.07	29.44	15.24	4.51	5.89	5.99	1.95	2.55	0.24	<1
B-d0	20.09	15.43	18.13	17.97	12.53	7.82	4.70	1.87	0.95	<1
B-d14	11.26	19.15	22.62	11.62	5.95	3.48	8.64	15.20	1.24	<1
ODV	20.35	20.77	15.11	17.67	11.10	6.70	4.33	2.53	0.64	<1
B-d0	10.51	16.48	36.31	9.74	2.58	7.73	7.85	6.29	1.91	<1
B-d14	22.44	36.01	15.34	7.48	5.40	4.56	4.51	3.66	0.42	<1
S-VEN	22.26	41.41	11.49	5.80	4.22	5.75	5.07	3.35	0.31	<1
R-VEN	19.56	46.16	11.24	4.38	5.06	5.65	4.29	3.23	0.28	<1
B-d0	13.29	21.84	20.89	7.87	3.91	5.85	17.58	7.22	1.07	<1
B-d14	20.31	46.82	9.70	8.15	4.62	6.17	2.93	0.69	0.47	<1
S-ODV	19.79	45.44	10.63	7.54	4.60	6.46	4.02	0.95	0.44	<1
R-ODV	19.18	49.93	9.00	7.50	3.68	5.91	3.02	0.84	0.48	<1

B=Free drugs reactors.

d0= day 0

d14=day 14

4.4.7. Biodegradation of VEN

4.4.7.1. Biodegradation of racemic venlafaxine

Racemic venlafaxine (Rac-VEN) was spiked into parallel abiotic (SWW) and biotic (bioreactor) systems and monitored over 14 days. Analysis was performed by LC–MS using the Astec Chirobiotic™ V2 column, with quantification based on the peak-area ratio of VEN to the internal standard (VEN-d6). Representative chromatograms and confirmatory mass spectra are shown in Figure 4-20, and the time-course concentrations (mean and %RSD) are summarised in Table 4-14.

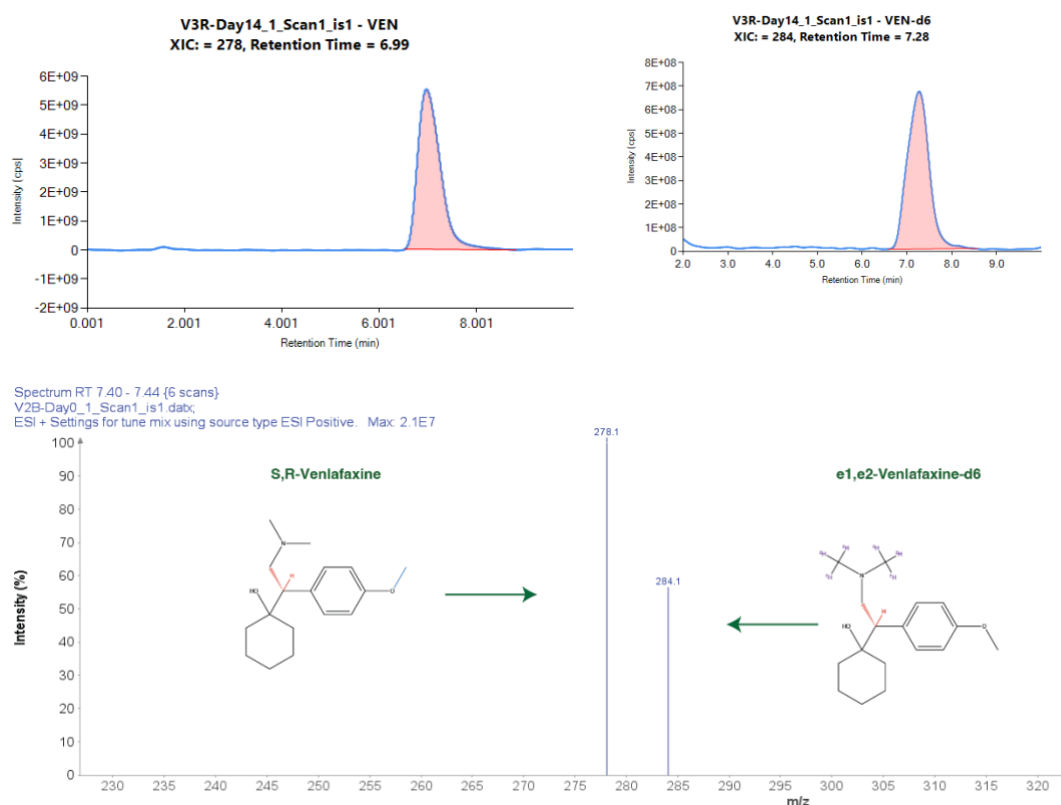


Figure 4-20: VEN/VEN-D6 chromatograms and mass spectrum.

Day 0, the mean Rac-VEN concentration was 0.600 µg/mL in the abiotic reactor and 0.478 µg/mL in the biotic reactor (Table 4-13). Because Day-0 sampling reflects the post-spike starting condition, this between-reactor offset is more plausibly explained by experimental and matrix factors (e.g., minor variability in spike delivery, mixing/aliquot heterogeneity, and matrix-

dependent recovery associated with biomass and dissolved organic matter) rather than biodegradation, which would be expected to manifest as a progressive decline over time. Similar considerations are widely recognised in environmental and wastewater analyses of polar/ionisable pharmaceuticals, where apparent early differences can reflect partitioning and extraction/recovery behaviour in addition to transformation. [364]

Analytical sensitivity for Rac-VEN was LOD = 15 ng/mL (0.015 µg/mL) and LOQ = 29.5 ng/mL (0.0295 µg/mL) (4.4.2.4). Therefore, any “non-detectable” result should be interpreted as < LOD (below the method’s detection capability) rather than complete loss of mass. Over the study period, Rac-VEN showed an overall decrease in both reactors (Table 4-13), consistent with combined attenuation processes. The early time-course exhibited some non-monotonic behaviour (e.g., the biotic Day-1 mean of 1.090 µg/mL), which can occur when distribution between aqueous and solids phases, equilibration, and matrix effects influence measured aqueous concentrations and extraction efficiency, particularly in biotic systems containing suspended biomass. Such behaviour is consistent with the broader understanding that abiotic removal of ionisable organic micropollutants is often governed by partitioning/sorption and limited chemical transformation, whereas biotic systems can additionally support biotransformation, with observed concentrations reflecting the net effect of these concurrent processes. [364]

From Day 7 onwards, Rac-VEN concentrations were substantially lower and similar in both reactors (0.177 vs 0.176 µg/mL at Day 7; 0.215 vs 0.216 µg/mL at Day 14 for abiotic and biotic, respectively), indicating pronounced attenuation by mid-experiment under both conditions (Table 4-13 and Figure 4-21). The %RSD values were generally lower at later time points (e.g., 4.68% and 2.29% at Day 14 for abiotic and biotic, respectively), supporting improved analytical consistency as the system approached a steadier concentration range (Table 4-13). Overall, the data indicate that Rac-VEN attenuation occurs under both abiotic and biotic conditions, with interpretation focused on the time-course pattern (rather than absolute Day-0 differences) providing the most robust basis for comparing reactor behaviour.

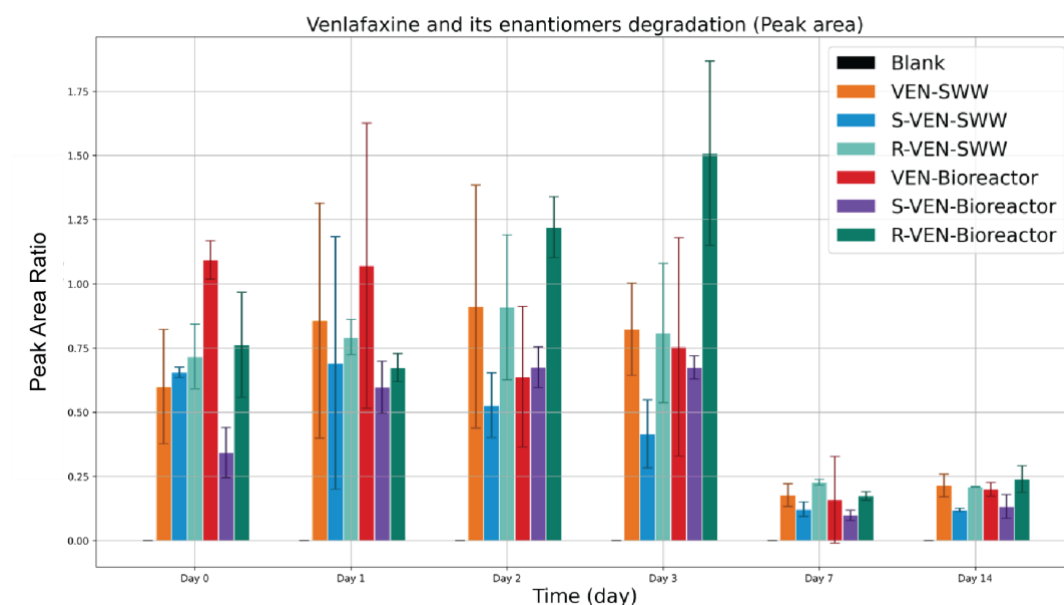


Figure 4-21: VEN and its enantiomers degradation study in biotic and abiotic, n=3.

Table 4-14: VEN and enantiomers degradation study, n=3.

Days		VEN		S-VEN		R-VEN	
		Abiotic	Biotic	Abiotic	Biotic	Abiotic	Biotic
	R ²	0.993	0.993	0.992	0.992	0.991	0.991
Day 0	Mean	0.600	0.478	0.655	0.343	0.717	0.763
	RSD%	11.53	27.00	1.280	11.360	13.96	21
Day 1	Mean	0.857	1.090	0.692	0.598	0.792	0.674
	RSD%	17.65	17.00	29.000	6.940	6.91	6.42
Day 2	Mean	0.911	0.652	0.527	0.675	0.908	1.221
	RSD%	17.32	13.00	9.840	4.870	24.86	7.87
Day 3	Mean	0.823	0.749	0.415	0.674	0.808	1.508
	RSD%	7.20	18.30	12.940	2.750	24.16	19.36
Day 7	Mean	0.177	0.176	0.123	0.099	0.227	0.174
	RSD%	5.20	20.00	8.250	6.940	3.6	6.6
Day 14	Mean	0.215	0.216	0.120	0.133	0.210	0.239
	RSD%	4.68	2.29	1.900	12.650	0.4	15.85

The pH remained relatively stable throughout (approximately 8.32–8.63), suggesting that large pH shifts were unlikely to be the primary driver of the observed profiles. This pH range is compatible with stable operation of biological wastewater processes and is commonly within the functional window reported for nitrifying/activated sludge systems. [365]

Finally, interpretation of Rac-VEN behaviour should be placed in the context of chiral pharmaceuticals in wastewater and surface waters. Enantiomeric profiling (e.g., EF/%ee combined with concentrations) has been recommended as a diagnostic tool to help distinguish biotransformation from non-biological processes during treatment and environmental transport. [366] Enantioselective patterns for venlafaxine and related residues have also been documented in wastewater and environmental matrices, supporting the premise that biological processing can influence both concentrations and stereochemical signatures. [367]

4.4.7.2. Venlafaxine enantiomers

A. S-VEN

S-VEN attenuation was evaluated in parallel abiotic and biotic reactors over 14 days. Concentrations were derived from the peak-area ratio (S-VEN/VEN-d6) using the validated LC-MS method (Figure 4-22) and are reported as mean $\pm$ RSD% (Table 4-14). At Day 0, S-VEN was higher in the abiotic reactor (0.655 $\mu\text{g/mL}$; RSD 1.28%) than in the biotic reactor (0.343 $\mu\text{g/mL}$; RSD 11.36%). Because Day-0 values represent the immediate post-spike/mixing condition, this offset is more plausibly explained by matrix-dependent recovery and early partitioning (e.g., interactions with biomass/suspended solids and dissolved organic matter affecting apparent aqueous concentration and extraction efficiency) rather than biodegradation, consistent with the known influence of sludge–water partitioning and matrix effects on pharmaceutical measurements in reactor and wastewater systems. [345,368,369]

From Day 0 to Day 3, concentrations did not show a simple monotonic decline. In the abiotic reactor, S-VEN decreased overall from 0.655 to 0.415 $\mu\text{g/mL}$, whereas in the biotic reactor S-VEN increased relative to Day 0 (0.343 to 0.675–0.674 $\mu\text{g/mL}$ at Days 2–3). This early divergence is most consistent with system equilibration (redistribution between aqueous and solid/biomass phases followed by partial release) combined with normal analytical variability (notably the higher Day-1 RSD in the abiotic reactor, 29%), and does not support interpreting the biotic reactor as showing “faster

degradation” during the first 72 h because concentrations were not consistently decreasing.

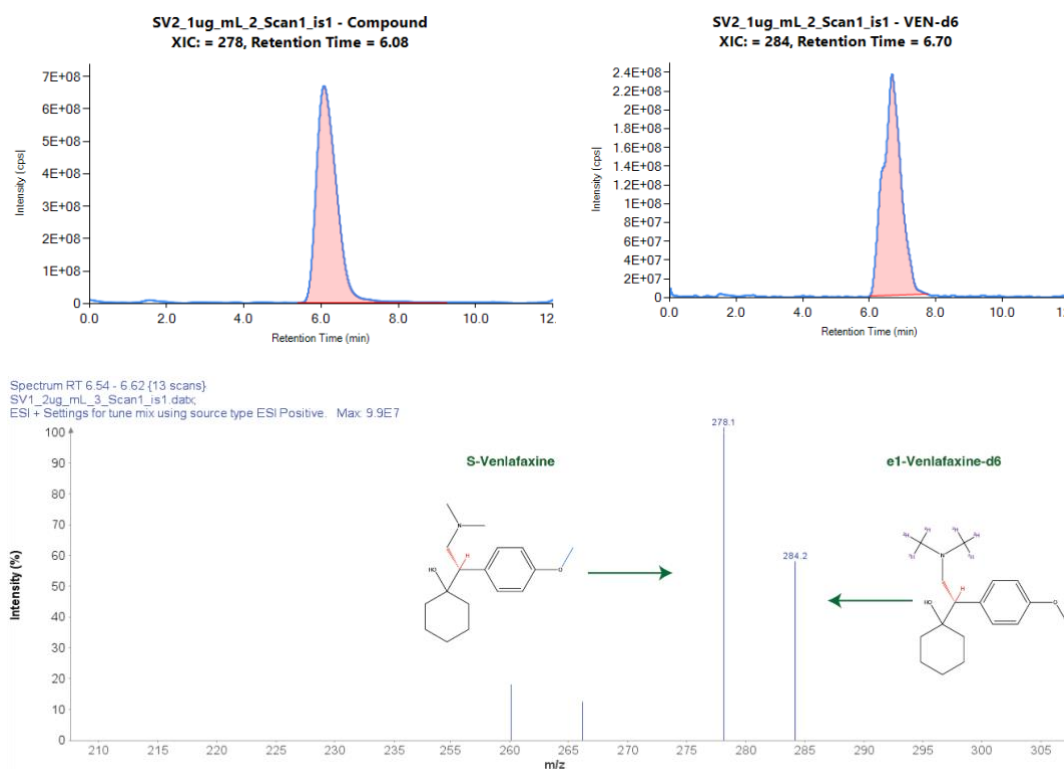


Figure 4-22: S-VEN/VEN-d6 chromatogram and mass spectrum.

The dominant attenuation occurred between Day 3 and Day 7, when S-VEN dropped sharply in both reactors to similarly low levels (0.123 $\mu\text{g/mL}$ abiotic; 0.099 $\mu\text{g/mL}$ biotic), corresponding to an approximate reduction of ~70% (abiotic) and ~85% (biotic) relative to Day-3 values. Concentrations then remained low and comparable at Day 14 (0.120 $\mu\text{g/mL}$ abiotic; 0.133 $\mu\text{g/mL}$ biotic), suggesting an apparent plateau consistent with slow residual removal and/or ongoing phase redistribution. Overall, the convergence of concentrations by Days 7–14 indicates that both non-biological processes (especially partitioning and limited transformation) and biotic processes (biotransformation) contributed to attenuation under these conditions, with any incremental biotic effect most evident during the Day 3–7 interval rather than during the initial equilibration phase. This mixed behaviour is consistent with reports that wastewater treatment systems can reduce concentrations

through biodegradation/biotransformation while sorption/partitioning can substantially shape apparent aqueous trends. [364,368,369]

Method precision was evaluated using %RSD of replicate measurements at each time point (Table 4-14). With the exception of Day 1 in the abiotic reactor (29% RSD), most values were $\leq \sim 13\%$ in both reactors, supporting the robustness of the time-course interpretation. The elevated Day-1 abiotic %RSD likely reflects short-term heterogeneity immediately after spiking/equilibration (mixing/partitioning and matrix-dependent recovery) rather than a true shift in degradation behaviour. This interpretation is consistent with common bioanalytical expectations for precision (typically $\leq 15\%$ CV for QC levels and $\leq 20\%$ at the LLOQ), which places the later time points within typical performance while indicating that the Day-1 abiotic value should be treated cautiously. [292]

Across the experiment, pH remained within 7.93-8.72, a range generally compatible with activated-sludge microbial activity; therefore, the observed concentration profile is more consistent with combined process kinetics and matrix/partitioning effects than with a strong pH-driven change. [279,364,368,369]

B. R-VEN

R-VEN attenuation was evaluated in parallel abiotic and biotic reactors over 14 days. Concentrations were derived from the peak-area ratio (S-VEN/VEN-d6) using the validated LC-MS method (Figure 4-22) and are reported as mean $\pm$ RSD% (Table 4-14). Concentrations were derived from the peak-area ratio of R-VEN to the isotopically labelled internal standard (VEN-d6) using the validated LC-MS method (Figure 4-23), and results are reported as mean with %RSD for replicate measurements (Table X). The chromatograms show a well-defined R-VEN peak ($RT \approx 7.06$ min) with the corresponding VEN-d6 peak ($RT \approx 7.30$ min); minor background features in the internal-standard trace are consistent with matrix contributions, but the use of an isotopically labelled internal standard helps correct for signal drift and matrix-dependent ion suppression/enhancement during quantification.

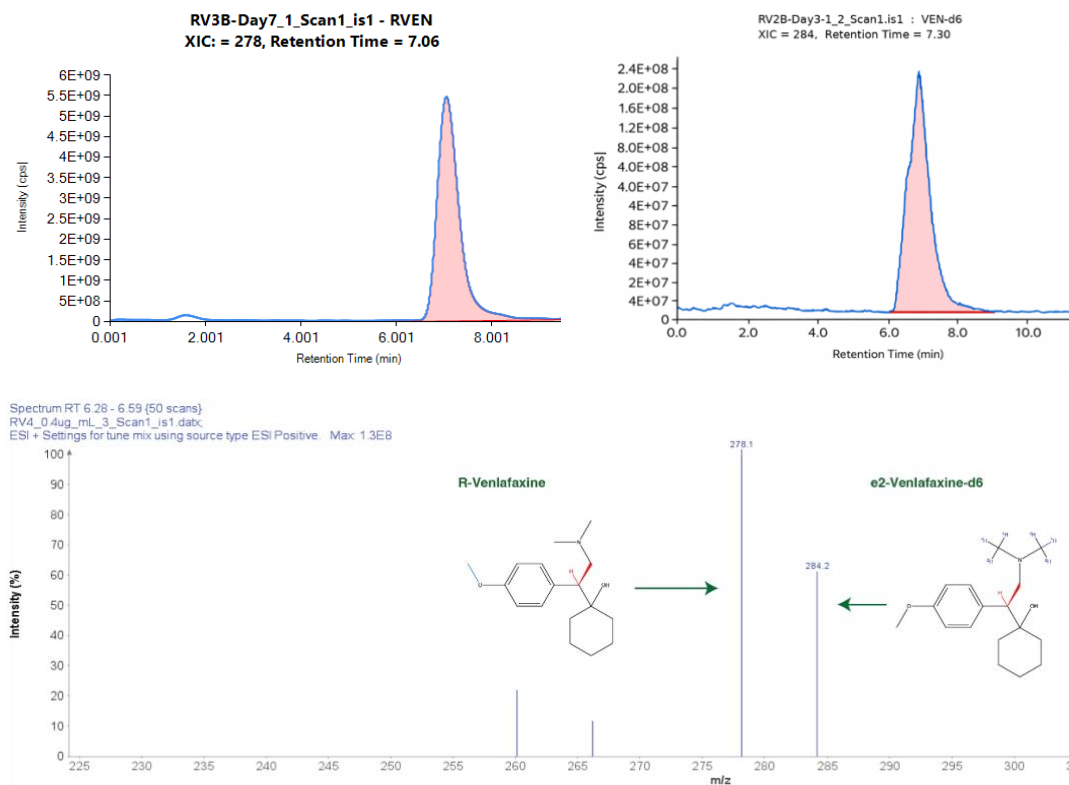


Figure 4-23: R-VEN/VEN-D6 chromatogram and mass spectrum.

At Day 0, R-VEN concentrations were similar between reactors (0.717 $\mu\text{g/mL}$ abiotic; 0.763 $\mu\text{g/mL}$ biotic). During the early phase (Days 0-3), concentrations did not decrease monotonically: R-VEN increased to 0.908 $\mu\text{g/mL}$ in the abiotic reactor by Day 2 and to 1.221-1.508 $\mu\text{g/mL}$ in the biotic reactor by Days 2–3. Because these changes occur immediately after spiking and during the initial equilibration period, they are most plausibly explained by phase redistribution and matrix/recovery effects (e.g., sorption to and subsequent desorption from biomass/suspended solids, changes in dissolved organic matter interactions, and time-dependent extraction efficiency) rather than true net formation or “negative degradation.” Such behaviour is well recognised for pharmaceuticals in sludge/wastewater systems, where apparent aqueous concentrations can shift as partitioning equilibria establish and matrix effects stabilise.

The dominant attenuation occurred after Day 3. Between Day 3 and Day 7, R-VEN declined sharply in both reactors—from 0.808 to 0.227 $\mu\text{g/mL}$ in the abiotic reactor (~72% reduction) and from 1.508 to 0.174 $\mu\text{g/mL}$ in the biotic

reactor (~88% reduction). By Day 14, concentrations remained low (0.210 µg/mL abiotic; 0.239 µg/mL biotic), indicating an apparent plateau consistent with slower residual removal and/or continued redistribution between phases. Overall, both abiotic mechanisms (partitioning and limited chemical transformation) and biotic mechanisms (microbially mediated biotransformation) likely contributed to attenuation, with the clearest incremental biotic effect expressed during the Day 3–7 interval when the decline was steeper in the biologically active system. This mixed contribution is consistent with the broader understanding that WWTP-relevant biotic processes can drive transformation of pharmaceuticals while non-biological processes, especially sorption/partitioning, can strongly influence apparent aqueous trends.

Throughout the experiment, pH remained within a relatively narrow range (7.98–8.67), suggesting that the observed time-course is more consistent with process kinetics and matrix effects than with a major pH-driven shift. This pH range is generally compatible with activated-sludge microbial function reported in biodegradation contexts. [335,369]

Most time points in the study showed acceptable repeatability, with notably improved precision once the reactors had equilibrated (e.g., Day 7: 3.6% abiotic and 6.6% biotic; Day 14: 0.4% abiotic). Higher variability was observed during the early phase, particularly in the abiotic reactor at Days 2–3 (24.86% and 24.16% RSD) and in the biotic reactor at Day 0 and Day 3 (21% and 19.36% RSD), which is consistent with short-term heterogeneity immediately after spiking/equilibration (mixing and partitioning effects) and matrix-dependent analytical uncertainty. [292]

In conclusion, the study successfully demonstrated the biodegradation of Rac-VEN and its enantiomers in both biotic and abiotic reactors, with the biotic reactor exhibiting a faster degradation rate. Despite attempts to separate the enantiomers of Rac-VEN using chiral liquid chromatography (chiral-LC) columns, the separation could not be achieved in the samples analysed. [370] This limitation underscores the need for more effective analytical methods to distinguish between enantiomers during degradation

studies. Overall, this study provides valuable insights into the fate of Rac-
VEN, S-*VEN*, and R-*VEN* under different environmental conditions and
highlights the importance of considering both biotic and abiotic processes in
the assessment of drug degradation in wastewater treatment systems.

4.4.8. Biodegradation of ODV

4.4.8.1. Rac-ODV

Rac-ODV was monitored in parallel abiotic and biotic reactors over 14 days using the validated LC-MS workflow with a chiral stationary phase. Concentrations were calculated from the ODV/ODV-d6 peak-area ratio (triplicate injections at each time point; Table 4-15). Under the chromatographic conditions used for this run, the S-ODV and R-ODV peaks were not baseline-resolved and showed partial co-elution. Because accurate enantiomer-specific integration depends on sufficient chromatographic resolution (i.e., reliable valley placement and minimal cross-contribution between adjacent peaks), the combined area of the overlapping S-ODV and R-ODV signal was integrated and reported as total ODV (Rac-ODV). The same approach was applied to the internal standard, where the two closely eluting ODV-d6 features were integrated as a single internal-standard response. This strategy allows robust total-ODV quantification, but it cannot be used to infer enantioselective behaviour (e.g., changes in enantiomeric fraction) until full separation is achieved and revalidated, Figure 4-24. [292]

At Day 0, Rac-ODV measured 0.735 µg/mL (abiotic) and 0.595 µg/mL (biotic) (Table 4-15). Because Day-0 samples reflect the immediate post-spike state, this between-reactor offset is more plausibly attributed to matrix and recovery effects and early distribution/partitioning (e.g., interaction with biomass/suspended solids and dissolved organic matter in the biotic matrix affecting apparent aqueous recovery), rather than biodegradation. This interpretation is reinforced by the subsequent short-term pattern: from Day 0 to Day 3, concentrations do not decrease monotonically in the biotic reactor (0.595 → 0.637 → 0.605 → 0.574 µg/mL), which is consistent with system equilibration and redistribution rather than immediate net removal.

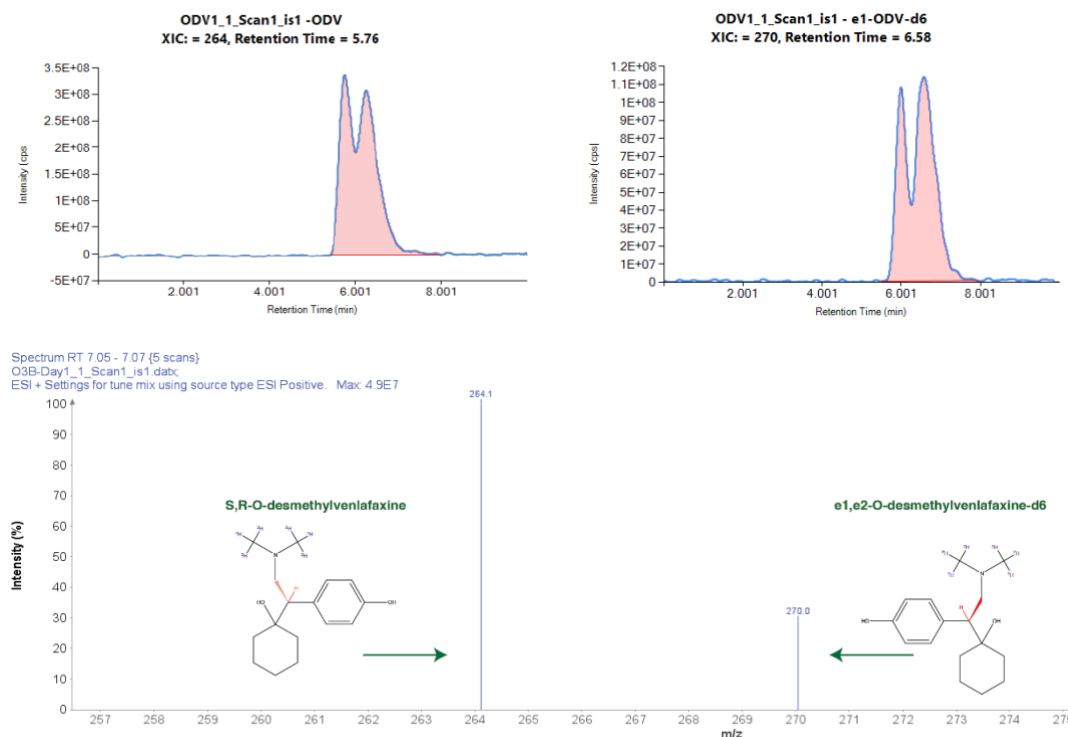


Figure 4-24: ODV/ODV-d6 chromatogram and mass spectrum.

Across the full study period, however, Rac-ODV attenuated substantially in both reactors. In the abiotic system, concentrations decreased from 0.735 $\mu\text{g/mL}$ (Day 0) to 0.206 $\mu\text{g/mL}$ (Day 14), while in the biotic system they decreased from 0.595 $\mu\text{g/mL}$ (Day 0) to 0.259 $\mu\text{g/mL}$ (Day 14) (Table 4-15). The largest step-change occurred between Day 3 and Day 7, when Rac-ODV dropped to 0.227 $\mu\text{g/mL}$ (abiotic) and 0.271 $\mu\text{g/mL}$ (biotic), after which concentrations remained comparatively low through Day 14. Given that the biotic reactor does not show consistently lower concentrations than the abiotic reactor at matched time points (Days 1–14), these data support the conclusion that both abiotic mechanisms (e.g., partitioning and/or slow chemical transformation) and biotic processes (microbially mediated transformation) may contribute to observed attenuation, but this particular racemate dataset does not provide strong evidence for faster net removal in the biotic reactor on concentration alone.

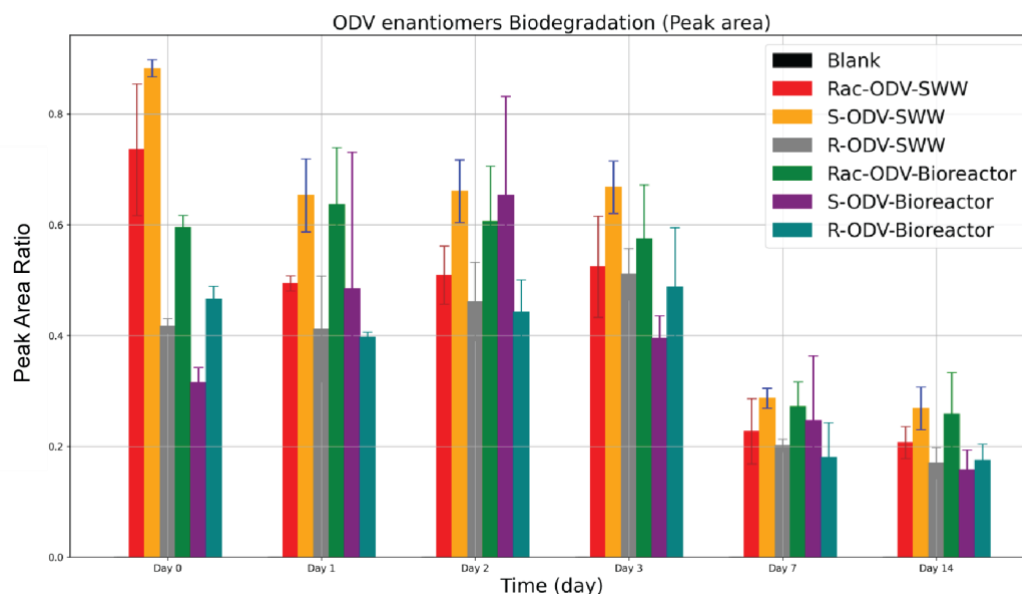


Figure 4-25: Rac-ODV, S-ODV and R-ODV degradation in bioreactor and synthetic reactor (peak area).

Table 4-15: ODV and enantiomers degradation study.

Days		ODV		S-ODV		R-ODV	
		Abiotic	Biotic	Abiotic	Biotic	Abiotic	Biotic
	R ²	0.969	0.969	0.991	0.991	0.950	0.950
Day 0	Mean	0.735	0.595	0.882	0.315	0.417	0.465
	RSD%	12.11	2.78	1.57	4.37	2.01	3.17
Day 1	Mean	0.494	0.637	0.653	0.484	0.412	0.397
	RSD%	2.06	12.04	8.81	24.99	14.47	1.41
Day 2	Mean	0.509	0.605	0.660	0.653	0.461	0.442
	RSD%	7.67	12.34	4.77	13.40	5.81	18.95
Day 3	Mean	0.524	0.574	0.668	0.395	0.510	0.488
	RSD%	12.98	12.68	6.18	5.54	5.66	13.79
Day 7	Mean	0.227	0.271	0.287	0.246	0.201	0.180
	RSD%	18.89	12.09	4.90	27.32	3.45	20.44
Day 14	Mean	0.206	0.259	0.268	0.157	0.169	0.175
	RSD%	10.08	20.95	10.93	10.80	9.76	9.82

Most values in the study fall within an acceptable range for time-course interpretation, although variability increased at some later points (e.g., Day 7 abiotic and Day 14 biotic). In line with common bioanalytical validation expectations (typically $\leq 15\%$ for routine QC precision and $\leq 20\%$ near the lower limit of quantification), these %RSD values support the overall trend

while indicating that selected points should be interpreted cautiously when differences between reactors are small. [292]

The reactor pH remained mildly alkaline and relatively stable (abiotic 8.21–8.61, biotic 8.12–8.64). This range is not, by itself, evidence of rapid chemical breakdown; rather, it indicates that pH drift is unlikely to be the dominant driver of the observed time-course. Nevertheless, pH can influence abiotic transformation pathways (e.g., hydrolysis rates) for susceptible compounds, and therefore it is appropriate to treat pH as a contextual factor rather than a primary causal explanation unless supported by dedicated hydrolysis/kinetic experiments. [333,336,369]

Complete chiral resolution for racemic ODV was not achieved within the available time window for this experimental phase. In addition, pandemic-related laboratory access constraints and implementation of mitigation arrangements required work across different laboratory settings, limiting the opportunity to undertake iterative optimisation (e.g., mobile-phase additives/strength, temperature, flow rate, and injection solvent) followed by full revalidation. Accordingly, enantioselective quantification of ODV (separate S-ODV and R-ODV peak integration and reporting of EF/%ee) is designated as future work, building directly on the present dataset, which reliably reports total Rac-ODV attenuation using combined-peak integration.

4.4.8.2. ODV enantiomers

A. S-ODV

S-ODV degradation was evaluated in parallel abiotic and biotic reactors over 14 days ($n = 3$), using the validated LC-MS workflow and a chiral stationary phase. However, the two isotope-labelled internal-standard enantiomer peaks (e1-ODV-d6 and e2-ODV-d6) were not fully resolved. To ensure consistent and reproducible quantification despite this limitation, Rac-ODV was therefore reported using combined-peak integration (Figure 4-26 and Table 4-15).

On day 0, the concentration of S-ODV was 0.882 $\mu\text{g/mL}$ (RSD%= 1.57) in the abiotic reactor and 0.315 $\mu\text{g/mL}$ (RSD%= 4.37) in the biotic reactor. The

lower concentration in the biotic reactor indicates that biodegradation may have already started, leading to a decrease in the S-ODV concentration compared to the abiotic reactor.

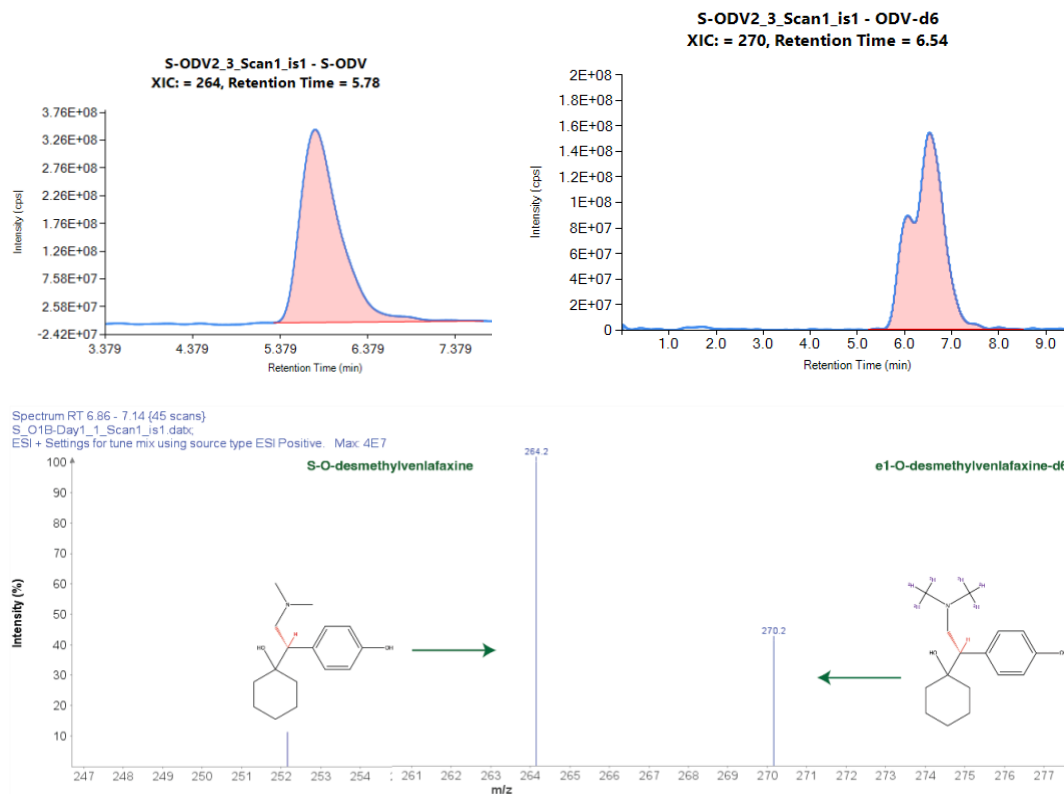


Figure 4-26: S-ODV/ODV-d6 chromatogram and mass spectrum.

During the study, the concentrations of S-ODV exhibited variations in both the abiotic and biotic reactors. On day 1, the concentration of S-ODV in the abiotic reactor was 0.653 $\mu\text{g/mL}$ (RSD%= 8.81), while in the biotic reactor, it was 0.484 $\mu\text{g/mL}$ (RSD%= 24.99). This indicates that the biotic reactor displayed a higher degradation rate compared to the abiotic reactor.

On day 2 and day 3, similar trends were observed, with the concentration of S-ODV in the abiotic reactor slightly higher than in the biotic reactor. This suggests that some degradation of S-ODV may have occurred in the abiotic reactor as well, possibly due to abiotic processes or chemical transformation.

As the study progressed, the concentrations of S-ODV decreased in both reactors. On day 7, the concentration of S-ODV was 0.287 $\mu\text{g/mL}$ (RSD%=

4.9) in the abiotic reactor and 0.246 µg/mL (RSD%= 27.32) in the biotic reactor. By day 14, the concentration further decreased to 0.268 µg/mL (RSD%= 10.93) in the abiotic reactor and 0.157 µg/mL (RSD%= 10.80) in the biotic reactor.

The pH values of the reactors ranged from 8.06 to 8.62 in the biotic reactor and from 8.16 to 8.86 in the abiotic reactor. These pH ranges indicate a slightly alkaline environment. [334,337]

The results indicate that S-ODV undergoes degradation and transformation in both abiotic and biotic reactors. The biotic reactor showed a higher degradation rate compared to the abiotic reactor, suggesting the presence of microorganisms capable of metabolizing S-ODV. However, the significant variability in the concentration and RSD values in the biotic reactor suggests that the degradation process might be influenced by various factors, such as microbial activity, environmental conditions, and interferences from other compounds.

B. R-ODV

R-ODV was evaluated in parallel abiotic and biotic reactors over 14 days following a 1 µg/mL spike (triplicate reactors). Concentrations were quantified using the peak-area ratio of R-ODV/ODV-d6 (Table 4-15). Because the chiral method did not fully resolve the two ODV-d6 isotopologue/enantiomer peaks under the available optimisation window, the internal standard response was integrated as a single combined ODV-d6 peak for all calculations (i.e., e1-ODV-d6 + e2-ODV-d6), ensuring consistent quantification across time points, Figure 4-27.

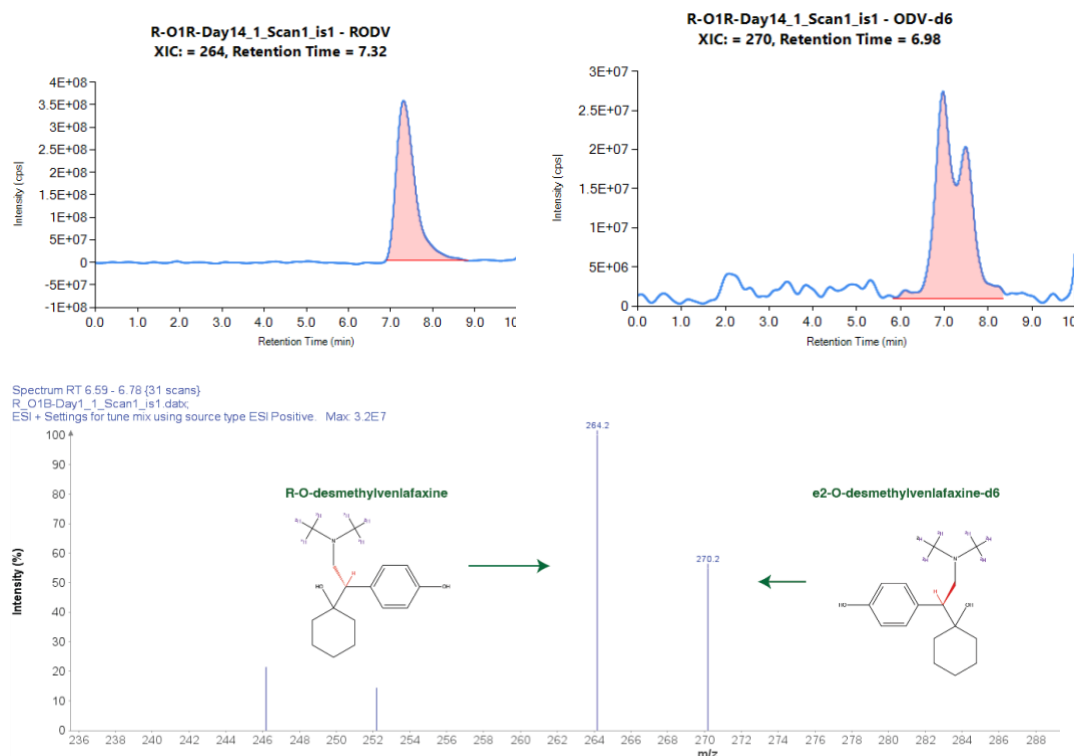


Figure 4-27: R-ODV/ODV-d6 chromatogram and mass spectrum.

At Day 0, R-ODV concentrations were similar between reactors (0.417 $\mu\text{g/mL}$ in the abiotic reactor vs 0.465 $\mu\text{g/mL}$ in the biotic reactor; RSD 2.01-3.17%), indicating comparable starting conditions. During Days 0-3, concentrations showed modest increases in both reactors (abiotic: 0.417 to 0.510 $\mu\text{g/mL}$; biotic: 0.465 to 0.488 $\mu\text{g/mL}$). This early pattern is most consistent with system equilibration and phase redistribution (e.g., partitioning between aqueous phase and reactor solids/biomass and subsequent release), rather than a clear net removal signal, particularly because the direction of change is not a monotonic decrease.

The principal attenuation occurred after Day 3. By Day 7, R-ODV decreased sharply to 0.201 $\mu\text{g/mL}$ (abiotic) and 0.180 $\mu\text{g/mL}$ (biotic), representing an approximate 60% reduction relative to Day-3 levels in both systems. Concentrations remained low at Day 14 (0.169 $\mu\text{g/mL}$ abiotic; 0.175 $\mu\text{g/mL}$ biotic), indicating an apparent plateau and broadly similar residual levels in the two reactors. Overall, these profiles suggest that both abiotic mechanisms (e.g., partitioning and limited transformation) and biotic processes (biotransformation) contributed to attenuation under the conditions

tested; however, because concentrations converged at Days 7-14 and differences between reactors were small, the dataset does not support a strong, consistent biotic enhancement for R-ODV removal across the full time course.

Precision was evaluated using %RSD at each time point (Table 4-15). Most measurements showed acceptable repeatability (typically $\leq \sim 15\%$), supporting interpretation of the overall time-course trend. A small number of points showed higher variability, most notably the biotic reactor at Day 7 (20.44% RSD) and Day 2 (18.95% RSD), so comparisons between reactors at those specific time points should be interpreted cautiously when absolute differences are small. Reactor pH remained mildly alkaline and relatively stable (biotic 8.06–8.58; abiotic 8.14–8.90), indicating that pH drift is unlikely to be the primary driver of the observed concentration changes; pH is therefore treated as contextual rather than a causal explanation in the absence of dedicated abiotic kinetic testing.[334,335,337]

Collectively, the results indicate that R-ODV undergoes measurable attenuation in both abiotic and biotic systems, with the dominant decline occurring between Days 3 and 7 and comparable residual concentrations by Day 14. Further work that includes phase-resolved measurements (aqueous + sorbed) and expanded optimisation of the chiral/ISD separation would strengthen mechanistic interpretation and allow more definitive assessment of enantioselective behaviour.

Overall, the results from the studies indicate that Rac-ODV, S-ODV and R-ODV, the main metabolites of venlafaxine, undergo degradation and transformation in both abiotic and biotic reactors. The biotic reactors consistently exhibited a higher degradation rate compared to the abiotic reactors, suggesting the presence of microorganisms capable of metabolising the compounds. The pH variations between the reactors may have influenced the microbial activity and degradation rate.

A limitation common to both studies is the inability to separate the enantiomers of the metabolites due to the limitations of the enantiomer separation method used. This limitation introduces uncertainties in the data

analysis, as the combined peak areas of the enantiomers were used for quantification.

In conclusion, these studies provide valuable insights into the biodegradation and transformation of Rac-ODV, S-ODV, and R-ODV in wastewater treatment systems. The findings emphasise the significance of microbial activity in the degradation process and suggest the potential for using biotic reactors for the removal of these pharmaceutical compounds.

4.4.9. Peak Height ratio study of ODV

Both peak area and peak height ratios for Rac-ODV demonstrate a decreasing trend in the concentration of Racemic ODV over time in both the abiotic and biotic reactors, Figure 4-28. On the other hand, there may be differences in the absolute values of the concentrations obtained using peak area and peak height ratios. This can occur due to various factors such as the sensitivity of the analytical technique, potential variations in detector responses, or differences in the integration methods used for peak area and peak height calculations.

4.4.9.1. S-ODV

In S-enantiomer both peak area and peak height ratios also demonstrate a decreasing trend in the concentration of S-ODV over time in both the abiotic and biotic reactors, this indicates that biodegradation processes are occurring in both systems. There may be differences in the absolute values of the concentrations obtained using peak area and peak height ratios. This could be due to variations in the sensitivity of the analytical technique or differences in detector responses. Moreover, both peak area and peak height measurements show varying degrees of precision and reproducibility. For example, the RSD% values for peak area measurements range from 1.57% to 10.93%, while the RSD% values for peak height measurements range from 3.18% to 28.8%. Relatively high RSD% values observed for certain measurements, particularly for peak height ratios, Figure 4-28.

4.4.9.2. R-ODV

Both peak area and peak height ratios show a consistent concentration of R-ODV over time in both the abiotic and biotic reactors. The concentrations remain relatively stable without significant variations. In contrast, there may be differences in the absolute values of the concentrations obtained using peak area and peak height ratios. These differences could be due to variations in the sensitivity of the analytical technique or differences in detector responses.

Based on RSD% results, both peak area and peak height measurements show varying degrees of precision and reproducibility. For example, the RSD% values for peak area measurements range from 2.01% to 5.81%, while the RSD% values for peak height measurements range from 1.41% to 20.44%. Notably, the RSD% values for peak height measurements are generally higher compared to peak area measurements. This suggests that peak height ratios may be more variable and less reproducible compared to peak area ratios. In addition, there appears to be a discrepancy between the R-ODV concentrations on Day 1 in the abiotic reactor when comparing peak height and peak area ratios. The RSD% value for peak area measurements is significantly higher (14.47%) compared to the RSD% value for peak height measurements (8.31%). This discrepancy indicates potential issues with reproducibility or variability in the measurements on Day 1, Figure 4-28.

In conclusion, both peak area and peak height ratios offer insights into the general trend of R-ODV in the reactors. However, it is crucial to comprehend the sources of differences and discrepancies to ensure accurate assessment. To achieve this, further investigations should focus on enhancing measurement techniques and implementing rigorous data analysis. By employing improved separation techniques and carefully evaluating integration methods, more robust and reliable data can be obtained for a comprehensive understanding of the degradation and transformation processes of R-ODV in the studied systems.

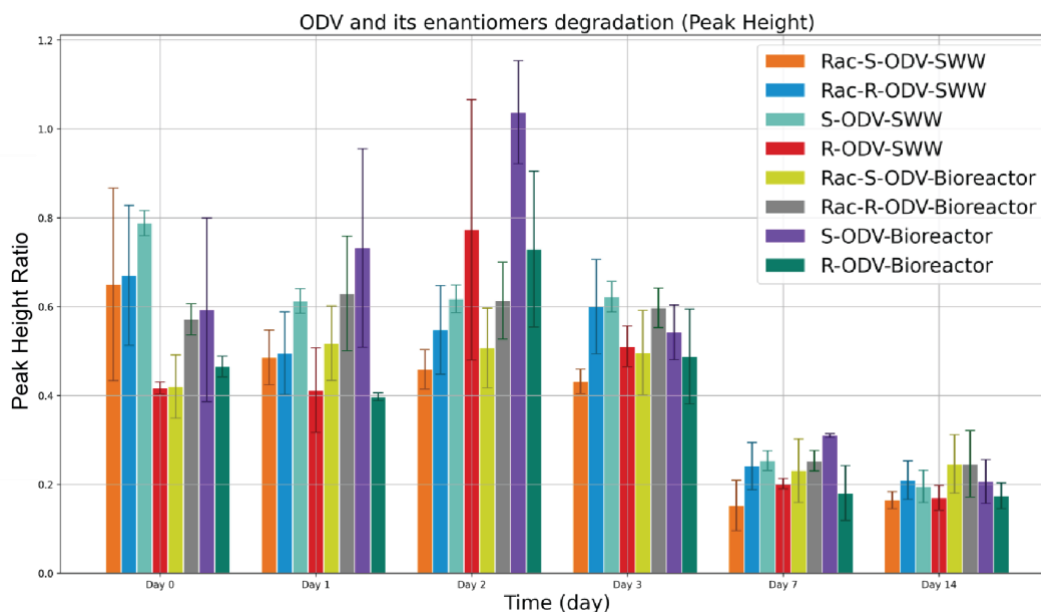


Figure 4-28: ODV, S-ODV, and R-ODV degradation in biotic and biotic systems (peak height).

4.5. Conclusion

This chapter investigated the fate of four antidepressant-related analytes in wastewater-relevant systems, FLX and NFLX, and VEN with its major metabolite ODV. Biodegradation and transformation were evaluated under controlled abiotic (synthetic wastewater, SWW) and biotic (bioreactor) conditions using time-course sampling across the incubation period and quantitative LC-MS analysis. Where chromatographic resolution allowed, enantiomeric behaviour was examined to assess whether biotic conditions produced evidence of stereoselective transformation.

Across the study, the biotic reactors consistently showed greater attenuation than the abiotic SWW controls, indicating that microbial activity was a key driver of removal and/or transformation under the applied reactor conditions. In abiotic controls, concentration changes were generally smaller and more gradual, consistent with non-biological losses such as slow chemical transformation and/or partitioning to reactor surfaces and particulate matter. For VEN and ODV, both systems demonstrated measurable attenuation, but the stronger and more consistent decline in the biotic reactors supports a biologically mediated contribution to their fate in wastewater-like

environments. For FLX/NFLX, the overall profiles similarly supported microbial involvement, although interpretation of enantiomer-specific behaviour was constrained in places by chromatographic resolution and/or standards-related limitations.

A key analytical outcome of this chapter is that chiral separation was not achievable for all targets at all stages, which limits the certainty of enantiomer-specific conclusions for some compounds and highlights a practical constraint relevant to environmental interpretation. Where enantiomeric resolution was achieved, the data suggested that biotic conditions can perturb enantiomeric balance, consistent with stereoselective biotransformation, whereas abiotic controls tended to show more stable enantiomeric patterns. Where enantiomeric resolution was not achievable, conclusions were appropriately based on overall (non-enantioselective) concentration trends, and the need for stronger enantiomer assignment in future work is acknowledged.

Beyond the mechanistic findings, this work has practical implications for wastewater monitoring and treatment design. First, the validated LC-MS approach, where chiral resolution was achievable, enables distinction between racemic residues and enantiomer-enriched profiles, improving wastewater surveillance and reducing the risk of over-interpreting total concentration trends that may mask stereoselective removal. Second, the direct comparison of abiotic and biotic systems supports treatment optimisation by indicating when removal is likely dominated by microbial transformation rather than physicochemical loss pathways, informing decisions related to operational stability, biomass performance, and retention time. Third, the observation that racemates and single enantiomers can exhibit different apparent behaviour emphasises that risk assessment based only on racemic standards may underestimate persistence of specific enantiomers; incorporating stereochemistry can therefore strengthen environmental fate modelling and exposure estimates. Finally, the identification of compounds or metabolites showing slower attenuation under abiotic conditions highlights targets that may require enhanced treatment or polishing steps.

Although a number of studies have examined antidepressants and benzodiazepines in wastewater and some have reported chiral signatures for selected compounds, fewer have combined (i) quantitative assessment of parent drugs and key metabolites, (ii) controlled comparison of abiotic versus biotic reactors, and (iii) evaluation of racemic mixtures alongside single-enantiomer experiments to interpret stereoselective biodegradation. By integrating these elements, this chapter provides mechanistic evidence for how stereochemistry and microbial activity influence removal in wastewater-relevant systems, and offers a framework that can be applied in future monitoring, treatment evaluation, and environmental risk assessment studies.

Chapter 5. Biodegradation study of benzodiazepines in the environmental matrices using SPE-LC-MS

5.1. Introduction

Benzodiazepines are widely prescribed psychoactive pharmaceuticals and are frequently detected in wastewater treatment effluents and surface waters, raising concerns about their persistence and potential environmental impacts. [207,372] Compounds such as diazepam, lorazepam, and etizolam, together with their metabolites, have been reported in aquatic environments, where they may pose risks to non-target organisms. [23,71,373,374]

The stereochemical nature of benzodiazepines adds further complexity to their environmental behaviour, as individual enantiomers can exhibit different pharmacological activities, toxicities, and transformation pathways. Despite this, environmental studies have largely relied on achiral analytical approaches, limiting understanding of enantioselective fate and biodegradation processes. [17]

This chapter investigates the biodegradation behaviour of selected benzodiazepines in environmental matrices using SPE-LC-MS, with particular emphasis on chiral LC-MS analysis. The application of chiral analytical techniques enables the separation and quantification of individual enantiomers of compounds such as α -hydroxyetizolam, temazepam, and oxazepam, providing insight into their enantioselective degradation and biotransformation under controlled experimental conditions. [98,372,375]

5.2. Aim and objectives

The aim of this chapter is to investigate the biodegradation and transformation of selected benzodiazepines (diazepam, etizolam, and their major metabolites) in wastewater systems, with particular emphasis on enantioselective behaviour under controlled biotic and abiotic conditions. Advanced chiral analytical techniques are employed to elucidate degradation

kinetics, stereoselective transformation pathways, and the role of microbial communities in influencing the environmental fate of these compounds.

- To evaluate the biodegradation of benzodiazepines in synthetic wastewater by quantifying parent compound removal, degradation rate constants, and transformation product formation in laboratory-scale bioreactors under environmentally relevant conditions.
- To investigate the influence of microbial activity on benzodiazepines biodegradation and correlating degradation behaviour with changes in bacterial community composition.
- To characterise microbial communities involved in the biodegradation process using 16S rRNA gene sequencing, enabling identification of dominant taxa potentially associated with benzodiazepines biodegradation.
- To characterise the bioreactor microbial communities using 16S rRNA gene sequencing, in order to identify dominant taxa and assess their potential association with benzodiazepine biodegradation.
- To develop, optimise, and validate chiral analytical methods using chiral LC-MS and chiral HPLC-DAD for the separation and quantification of benzodiazepines enantiomers.
- To investigate enantioselective degradation and transformation of α -hydroxyetizolam in biotic and abiotic systems using SPE-LC-MS.

5.3. Materials and methods

5.3.1. Chemicals and reagents

All benzodiazepines reference standards and corresponding deuterated internal standards standards and deuterium standards at the concentration of 1 mg/mL, and 0.1 mg/mL, respectively, were obtained from the supplier specified in section 3.3.2.

5.3.2. General laboratory equipment

General laboratory equipment used in this study is listed in Table 3-1.

5.3.3. Preparation of internal standards

Internal standard solutions (100 µg/mL) were prepared as described in section 3.3.3.3, (Table 3-4).

5.3.4. Preparation of working solutions

Working solutions (10 µg/mL) were prepared monthly in methanol following the procedure outlined in section 3.3.3.2.

5.3.5. Preparation of synthetic wastewater

Synthetic wastewater was prepared according to the protocol described in section 3.3.4.1.

5.3.6. Preparation of the bioreactors

The bioreactor configuration, operating conditions, and inoculation procedure were identical to those described in section 3.3.4.1. and were applied consistently throughout the experiments in this chapter.

5.3.7. Spiking BZs into the reactors

Benzodiazepines were introduced into the bioreactors by spiking a known concentration of each target compound into the synthetic wastewater following reactor stabilisation. This controlled spiking approach enabled reproducible assessment of biodegradation kinetics and transformation behaviour under defined experimental conditions. The spiking procedure and reactor configuration are illustrated in Figure 5-1.

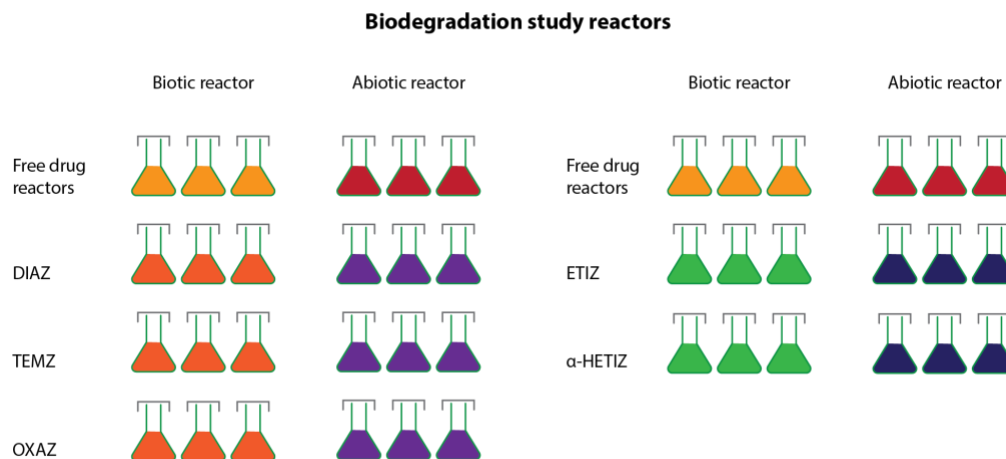


Figure 5-1: BZs biodegradation experimental setting.

5.3.8. Sample Collection for Microbial Taxonomy Analysis

The sampling strategy was designed to monitor bacterial community composition and development throughout the experiment. Samples were collected at three key time points: Day 0, Day 7, and Day 14. On Day 0, baseline samples were collected prior to the introduction of benzodiazepines. These samples represent the initial microbial community structure (from the stock bioreactor). After the benzodiazepines were introduced, additional samples were collected on Day 7 and Day 14 to observe changes in microbial taxonomy over time, Figure 5-2 shows the sampling method.

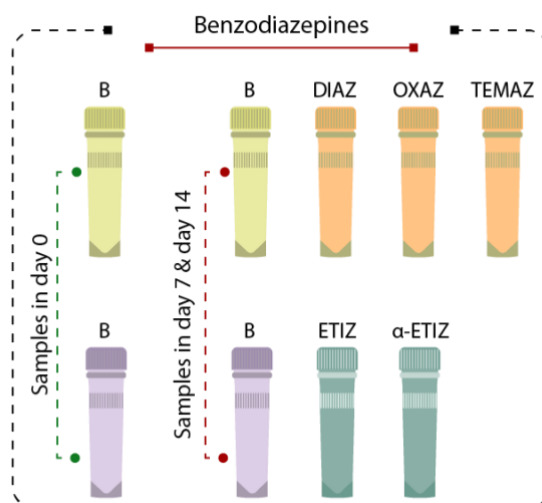


Figure 5-2: BZs samples collecting method for the 16S taxonomy analysis for bacteria.

5.3.9. Operational monitoring (pH, OD₆₀₀ and TSS)

During the biodegradation study, bioreactor performance was monitored daily using pH, (OD₆₀₀), and TSS. For each reactor, 3 mL of sample was collected per day to measure pH using a calibrated pH meter. Bacterial growth was assessed by measuring OD at 600 nm using a 10 mm cuvette as described previously. [270] TSS measurements were performed following the procedure reported in section 3.4.1.

5.3.10. LC-MS method

In this study, chiral LC-MS was employed to monitor the biodegradation and biotransformation of benzodiazepine enantiomers. Enantiomeric separation was achieved using an Astec Chirobiotic V2 column (250 mm × 2.1 mm I.D., 5 µm), enabling accurate resolution and quantification of chiral analytes during reactor incubation. The optimised chromatographic and mass spectrometric conditions are summarised in Table 5-1 and Table 5-2.

The method was optimised and validated in accordance with SWGTOX recommendations and established analytical validation guidance. Performance evaluation included selectivity, linearity, accuracy, precision, LOD, LOQ, and carryover, confirming robust enantiomeric separation and reliable quantification of benzodiazepines in aqueous matrices.

Table 5-1: LC parameters

LC	Parameters
Flow rate	0.5 mL/min
Oven temp.	40°C
Injection volume	20 µL
Solvent A%	55% MeOH, 15% ACN, 15% H ₂ O , 15%IPA, 4mM Ammonium Acetate in
Solvent B% & 0%	-
Mode	Isocratic
UV detector	Start Wavelength 200 nm End Wavelength 400 nm

Table 5-2: Mass spectrum parameters

Ion Source Parameters	
Settings for tune mix using source type ESI Positive	
Parameter	Value
Polarity	Positive
Capillary Temperature	250 °C
Capillary Voltage	180 V
Source Voltage Offset	20 V
Source Voltage Span	30 V
ESI Source	
Source Gas Temperature	200 °C
ESI Voltage	3500 V

5.4. Results and discussion

The degradation behaviour of the investigated benzodiazepines was evaluated under controlled abiotic and biotic conditions to determine the relative contributions of physicochemical and microbial processes. Time-resolved concentration profiles obtained by LC-MS analysis enabled assessment of attenuation rates, metabolite formation, and system-dependent variability across the incubation period. The results presented in this section focus on experimentally observed degradation trends and their mechanistic interpretation within wastewater-relevant environments. [376]

To ensure that the biodegradation trends and enantiomeric profiles reported in this chapter are analytically robust, benzodiazepines and metabolites were monitored using a validated SPE-chiral LC-MS workflow. Method optimisation and validation were undertaken in line with SWGTOX recommendations for forensic toxicology method validation and internationally recognised analytical validation guidance. Validation characteristics were selected to match the intended use of the assay and included assessment of selectivity/specificity, calibration performance (linearity and range), accuracy, precision, and sensitivity (LOD/LOQ), consistent with the principles described in ICH Q2(R2). [291,292]

The interpretation of attenuation behaviour in abiotic and biotic reactors must also consider operational and environmental factors that can influence apparent removal, including pH, temperature, light exposure, microbial activity, and matrix composition. These factors can affect reaction kinetics,

transformation routes, and analytical response through changes in sample composition. Accordingly, the Results and Discussion section compares trends between abiotic and biotic conditions and between parent compounds and metabolites, while accounting for time-dependent matrix effects and reactor dynamics. [376]

This chapter therefore presents an integrated evaluation of benzodiazepine behaviour under controlled reactor conditions, combining quantitative chemical measurements with interpretation of system factors that may explain differences between experimental conditions.

5.4.1. Environmental studies

5.4.1.1. TSS study

The total suspended solids (TSS) test was conducted on two types of bioreactors: free drug bioreactors and spiked drug bioreactors. The TSS measurements were taken at the beginning of the experiment (day 0) and at the end of the experiment (day 14). The TSS values obtained for each bioreactor and drug compound are summarised in the Table 5-3.

At day 0, the free drug bioreactor exhibited the lowest TSS of 90 mg/L with a relatively low RSD of 11%, indicating consistent initial measurements. In comparison, the benzodiazepines had higher initial TSS values, reflecting greater suspended solids accumulation. Diazepam showed the highest Day 0 TSS at 310 mg/L, with a moderate RSD of 23%, indicating more variability than the free drug bioreactor. Oxazepam had the lowest initial TSS among the benzodiazepines at 70 mg/L, similar to the free drug bioreactor, but with a much higher RSD of 29%, demonstrating significant variability in measurements. Temazepam and etizolam displayed intermediate TSS values of 145 mg/L and 155 mg/L, respectively, with temazepam showing a lower RSD (10%) compared to etizolam (29%). α -hydroxyetizolam, a metabolite of etizolam, started with the lowest TSS among the benzodiazepines at 30 mg/L, but with the highest variability, reflected by an RSD of 33%.

By day 14, all reactors exhibited increased TSS values, with significant differences in RSD trends. The control bioreactor saw its TSS rise to 198.33 mg/L, with an RSD increasing to 30%, suggesting greater variability over time. In contrast, the benzodiazepine reactors showed diverse trends. Diazepam's TSS increased slightly to 340 mg/L, but its RSD dropped to 12%, indicating improved consistency. Oxazepam experienced a substantial increase in TSS to 217.95 mg/L, with its RSD reducing to 22%, reflecting better reliability compared to Day 0. Temazepam and etizolam showed TSS increases to 233.22 mg/L and 250 mg/L, respectively. Temazepam's RSD rose moderately to 25%, while etizolam's RSD dropped to 15% showing improved precision for the later compound. α -hydroxyetizolam showed a dramatic increase in TSS to 206.67 mg/L, with its RSD decreasing to 16%, reflecting a significant improvement in consistency compared to its initial variability, **Error! Reference source not found..**

Table 5-3: TSS results of benzodiazepines.

Compound	Day 0			Day 14		
	TSS mg/L (n=3)	SD	RSD%	TSS mg/L (n=3)	SD	RSD%
Free drug bioreactor	90.00	10.00	11%	198.33	59.65	30%
Diazepam	310.00	70.00	23%	340.00	40.00	12%
Oxazepam	70.00	20.00	29%	217.95	48.85	22%
Temazepam	145.00	15.00	10%	233.22	58.98	25%
Etizolam	155.00	45.00	29%	250.00	37.50	15%
α-hydroxyetizolam	30.00	10.00	33%	206.67	33.33	16%

The TSS results confirm the presence of particulate material in the bioreactors and indicate that suspended solids accumulated over time in several conditions. TSS is a gravimetric measure of particulate matter retained on a filter and is commonly used to track solids loading and biomass-associated particulates in wastewater systems. The associated %RSD values provide an indication of replicate variability and, therefore, the stability of solids distribution and sampling consistency across time points. [377–379]

A notable outcome was the increase in TSS in the α -hydroxyetizolam-amended reactors from 30 mg/L on Day 0 to 206.67 mg/L on Day 14, accompanied by a reduction in variability (%RSD from 33% to 16%). This

pattern is consistent with progressive system stabilisation, where suspended solids become more uniformly distributed across replicates as the reactor matures. Importantly, an increase in TSS should be interpreted as a change in particulate content rather than direct proof of chemical degradation. In biologically active systems, rising TSS can reflect biomass growth, increased production of extracellular polymeric substances (EPS), and the formation or accumulation of particulate-associated material during microbial adaptation to xenobiotic exposure. [380]

Variations in TSS and %RSD across reactors are plausibly influenced by (i) differences in microbial growth and community adaptation, (ii) operational conditions that modulate microbial activity (e.g., pH, temperature, oxygen availability), and (iii) practical sources of analytical variability such as sampling heterogeneity and filtration/gravimetric handling. Standardised sampling and adherence to recognised TSS methods are therefore critical for minimising measurement variability and ensuring comparability between time points and reactor conditions. [262] Overall, the TSS trends provide complementary evidence of evolving reactor solids dynamics under pharmaceutical exposure and support interpretation of bioreactor performance alongside chemical concentration profiles and microbial data.

5.4.1.2. pH measurement

By Day 14, measurable shifts in pH were observed across all conditions (Table 5-4), indicating progressive changes in the reactor environment during incubation. In the free-drug control, pH increased to 8.50 in SWW and 8.45 in the bioreactor, suggesting an overall trend toward mild alkalinisation. Similar patterns were recorded for the spiked conditions: etizolam reached pH 8.40 (SWW) and 8.30 (bioreactor), while α -hydroxyetizolam increased to 8.60 (SWW) and 8.50 (bioreactor). Diazepam maintained the highest pH values at Day 14 (8.60 in SWW and 8.55 in the bioreactor). Oxazepam and temazepam also showed moderate increases, with SWW values reaching 8.50 and 8.55, and bioreactor pH increasing to 8.35 and 8.40, respectively.

Overall, these results indicate that the systems evolved from initial conditions toward mildly alkaline environments by Day 14. Such pH drift is consistent

with combined effects of microbial metabolism, buffering capacity, and ongoing chemical transformations, and may differ slightly between SWW and bioreactor conditions due to biological activity and reactor dynamics. [334,335]

pH is a key factor influencing the behaviour of diazepam and its metabolites because it can affect both chemical stability (e.g., hydrolytic transformation) and biological processes (e.g., microbial activity). Under alkaline conditions, hydrolytic reactions may be promoted for some benzodiazepines, particularly through reactions involving the benzodiazepine ring system and carbonyl-containing functional groups, although the extent of transformation is compound-dependent. [279,319,381,382]

Importantly, pH effects on ionisation should be interpreted carefully for diazepam. Diazepam behaves primarily as a weak base, therefore at mildly alkaline pH (~8-9) it is expected to remain largely neutral rather than more ionised. Consequently, pH-related differences in attenuation are more appropriately discussed in terms of changes in reactivity, solubility behaviour, and microbial activity, rather than assuming increased ionisation automatically enhances degradation. Hydroxylated metabolites such as oxazepam and temazepam may respond differently due to increased polarity and functionalisation. [319,381,383,384]

Finally, apparent concentration changes should be interpreted alongside non-biological processes such as sorption and matrix interactions, which may contribute to losses independent of true biodegradation. [318,385]

Table 5-4: Environmental monitor results, n=3.

	Drugs	Blank			Etiz			α-Etiz			DIAZ			OXAZ			TEM		
	Test	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD	pH SWW	pH bioreactor	OD
Day 0	Mean	8.03	8.02	0.03	7.59	7.51	0.04	8.08	8.01	0.01	8.34	8.32	0.04	8.09	7.98	0.06	8.00	7.94	0.02
	SD	0.23	0.29	0.00	0.02	0.01	0.00	0.03	0.01	0.00	0.03	0.02	0.01	0.01	0.01	0.01	0.01	0.03	0.01
	RSD%	2.89	3.60	14.10	0.20	0.08	4.92	0.40	0.12	22.33	0.30	0.24	17.25	0.07	0.07	13.02	0.07	0.33	31.77
Day 1	Mean	8.44	8.40	0.13	8.20	8.11	0.12	8.53	8.40	0.12	8.59	8.53	0.12	8.47	8.33	0.10	8.50	8.32	0.12
	SD	0.16	0.21	0.03	0.01	0.02	0.00	0.01	0.02	0.00	0.02	0.01	0.01	0.01	0.03	0.00	0.01	0.01	0.01
	RSD%	1.92	2.53	24.94	0.12	0.26	3.65	0.07	0.25	3.44	0.20	0.12	5.85	0.12	0.35	1.47	0.07	0.07	9.23
Day 2	Mean	8.37	8.55	0.10	8.44	8.37	0.10	8.26	8.59	0.08	8.45	8.46	0.08	8.24	8.51	0.13	8.19	8.29	0.07
	SD	0.29	0.14	0.01	0.11	0.01	0.01	0.13	0.02	0.01	0.02	0.02	0.01	0.04	0.02	0.03	0.10	0.03	0.01
	RSD%	3.50	1.63	12.26	1.26	0.07	7.29	1.58	0.20	11.02	0.20	0.18	15.13	0.51	0.24	21.76	1.21	0.37	13.48
Day 3	Mean	8.46	8.57	0.08	8.13	8.29	0.09	8.54	8.64	0.08	8.57	8.51	0.09	8.50	8.56	0.10	8.46	8.51	0.10
	SD	0.16	0.18	0.01	0.15	0.01	0.01	0.02	0.01	0.01	0.04	0.03	0.01	0.01	0.02	0.01	0.01	0.01	0.01
	RSD%	1.93	2.07	9.94	1.85	0.14	14.58	0.27	0.13	10.69	0.47	0.36	9.48	0.14	0.18	8.66	0.14	0.07	12.15
Day 7	Mean	8.61	8.54	0.11	8.81	8.70	0.09	8.51	8.51	0.08	8.53	8.48	0.09	8.62	8.35	0.10	8.39	8.45	0.08
	SD	0.14	0.14	0.02	0.02	0.02	0.02	0.03	0.02	0.01	0.02	0.01	0.01	0.05	0.02	0.00	0.19	0.02	0.02
	RSD%	1.59	1.61	16.83	0.24	0.18	19.00	0.38	0.18	14.47	0.20	0.12	12.67	0.52	0.28	1.41	2.22	0.24	18.83
Day 10	Mean	8.55	8.44	0.07	8.44	8.43	0.09	8.49	8.49	0.09	8.62	8.27	0.14	8.65	8.41	0.13	8.42	8.42	0.07
	SD	0.18	0.15	0.03	0.01	0.03	0.01	0.09	0.12	0.02	0.06	0.02	0.02	0.02	0.03	0.02	0.18	0.04	0.01
	RSD%	2.12	1.72	39.08	0.07	0.41	16.78	1.05	1.40	18.34	0.75	0.18	14.11	0.27	0.34	12.66	2.13	0.48	9.46
Day 14	Mean	8.72	8.46	0.14	8.40	8.30	0.09	8.59	8.39	0.16	8.57	8.38	0.12	8.66	8.33	0.16	8.60	8.51	0.13
	SD	0.20	0.13	0.03	0.01	0.04	0.01	0.02	0.03	0.02	0.13	0.03	0.01	0.04	0.03	0.02	0.26	0.01	0.02
	RSD%	2.31	1.56	23.53	0.07	0.42	8.75	0.20	0.30	14.39	1.52	0.32	10.08	0.47	0.32	12.24	3.00	0.07	15.92

5.4.2. Etizolam and its metabolite 16S taxonomy

5.4.2.1. Free drug bioreactor

In the free drug bioreactor (control), the observed changes in bacterial community composition from Day 0 to Day 14 reflect natural community succession under stable reactor conditions in the absence of pharmaceutical exposure (Table 5-5 and Figure 5-3). At Day 0, the community was mainly composed of wastewater-associated classes from *Bacteroidetes* and *Proteobacteria*, including *Saprospirae*, *Flavobacteriia*, *Sphingobacteriia*, and *Alphaproteobacteria*, with *Actinobacteria* present as a consistent secondary group. By Day 14, the relative abundance of these dominant classes redistributed, with a clearer enrichment of *Alphaproteobacteria* alongside continued representation of *Bacteroidetes* classes, indicating progression toward a more reactor-adapted community structure over time. These temporal shifts are consistent with activated-sludge studies showing that community composition varies during reactor operation due to ecological selection and assemblage processes even when external stressors are absent. [386,387]

This shift suggests that microbial communities can change dynamically over time due to factors such as nutrient availability, competitive interactions, and adaptation to operational conditions, rather than remaining static during incubation. [388,389] Experimental and full-scale wastewater research has shown that temporal changes in substrate and growth conditions can alter community composition and favor taxa with higher competitive fitness under the prevailing environment. [388,389] The observed enrichment of *Alphaproteobacteria* by Day 14 is therefore interpreted as a likely response to reactor selection pressures and functional niche advantages, consistent with reports describing *Proteobacteria* as major functional contributors in wastewater systems and frequently abundant across a range of treatment processes. [390,391]

5.4.2.2. Etizolam and its metabolite taxonomy

The introduction of etizolam and its primary metabolite α -hydroxyetizolam into the bioreactors was associated with measurable shifts in the bacterial community structure after 14 days, compared with the free-drug control reactors. Overall, the drug-exposed reactors showed an increased representation of taxa affiliated with *Proteobacteria* and *Actinobacteria*, while a reduction was observed in *Firmicutes*

under the same conditions. These results indicate that exposure to etizolam-related compounds can influence the relative abundance of dominant bacterial groups during bioreactor incubation. The bacterial composition is presented in Table 5-5 and Figure 5-3 at the class level (16S rRNA taxonomy) within each major phylum.

The proliferation of proteobacteria and *Actinobacteria* suggests that these bacterial groups may possess metabolic pathways enabling them to degrade or tolerate etizolam and its metabolites. [392] The increased presence indicates a potential role in the bioremediation of environments contaminated with such compounds. On the other hand, the reduction in firmicutes implies that etizolam and α -hydroxyetizolam may exert toxic effects on certain bacterial taxa, leading to a decline in their populations. [393] This shift could disrupt the ecological balance within the microbial community, potentially affecting essential functions such as nutrient cycling and organic matter decomposition.

The study examines the impact of etizolam and its metabolite, α -hydroxyetizolam, on bacterial communities within bioreactors over a 14-day period. In the control bioreactors, free drug reactors, the initial microbial population was predominantly composed of sphingobacteria, *Flavobacteria*, *Saprospirae*, and *Actinobacteria*, collectively accounting for over 68% of the community. After 14 days, natural microbial succession led to a decrease in sphingobacteria and *Flavobacteria*, while *Saprospirae* and *Alphainproteobacteria* increased in relative abundance. These shifts suggest that, in the absence of external stressors, microbial communities undergo predictable changes over time, likely driven by factors such as nutrient availability and interspecies interactions.

In contrast, bioreactors spiked with etizolam exhibited significant alterations in microbial composition by Day 14. There was a notable increase in proteobacteria and *Actinobacteria* compared to the control reactors. This enrichment indicates that etizolam may serve as a selective agent, promoting the growth of bacterial taxa capable of metabolising or tolerating the compound. The ability of these bacteria to thrive suggests potential pathways for the biodegradation of etizolam, highlighting their role in mitigating pharmaceutical contamination in wastewater treatment systems.

Similarly, reactors exposed to α -hydroxyetizolam demonstrated an increase in proteobacteria and *Actinobacteria* by Day 14. The presence of this metabolite appears to exert selective pressure, favouring bacterial groups adept at degrading or withstanding its effects. The parallel responses observed in both etizolam and α -hydroxyetizolam spiked reactors underscore the adaptability of certain microbial taxa to pharmaceutical compounds, which may have implications for the efficiency of bioremediation processes.

Comparing these findings to the control reactors suggests that the introduction of etizolam and α -hydroxyetizolam may alter the natural succession of microbial communities over the incubation period. While control reactors showed time-dependent shifts consistent with typical bioreactor maturation, the spiked reactors exhibited changes in the relative abundance of several bacterial groups, indicating a potential drug-associated selection effect. The selective enrichment of specific taxa following pharmaceutical exposure suggests that these compounds may influence microbial community dynamics and could potentially impact the overall functionality of bioreactor ecosystems. [394]

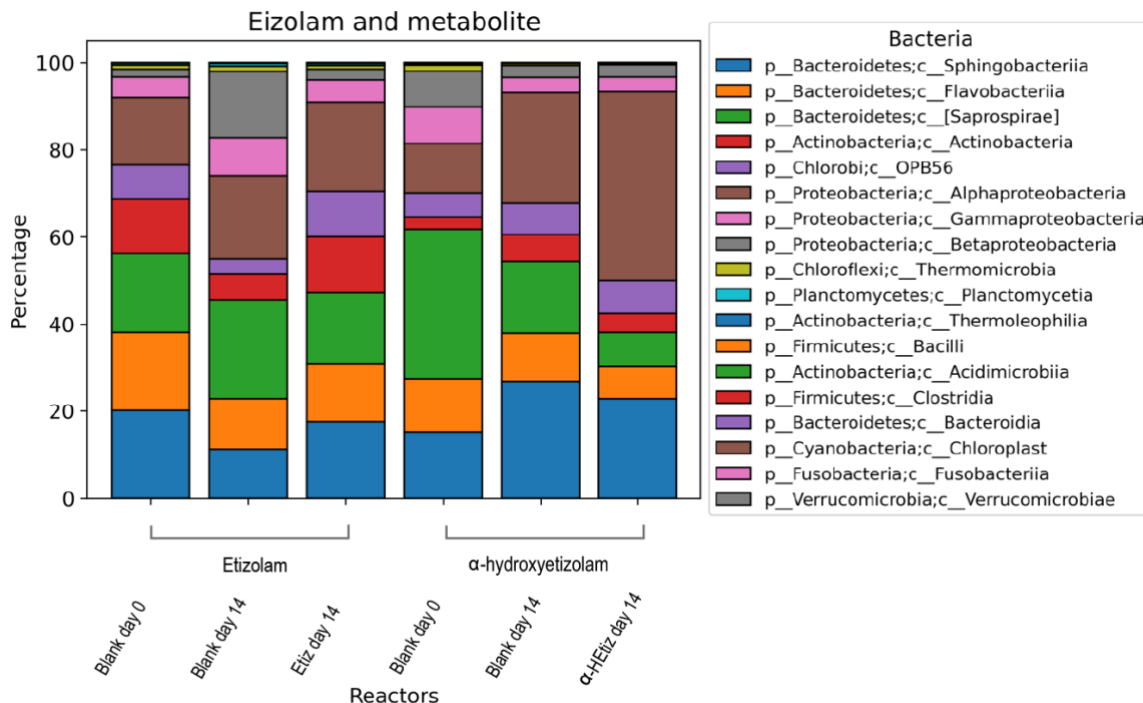


Figure 5-3: Etizolam and α -hydroxyetizolam 16S taxonomy.

Table 5-5: Etizolam and its metabolite bioreactors 16S taxonomy.

reactor	stage	Spingobacteriia	Flavobacteriia	Saprospirae	Actinobacteria	OPB56	Alphaproteobacteria	Gammaproteobacteria	Betaproteobacteria	Thermomicrobia	Other
B-d0	2	20.09	17.97	18.12	12.53	7.82	15.43	4.69	1.87	0.95	< 1 %
B-d14	2	11.26	11.63	22.63	5.95	3.48	19.15	8.64	15.2	1.22	< 1 %
ETIZ	2	17.55	13.25	16.39	12.84	10.4	20.45	5.18	2.43	0.88	< 1 %
B-d0	4	15.25	12.21	34.32	2.67	5.66	11.37	8.43	8.14	1.41	< 1 %
B-d14	4	26.9	11.03	16.42	6.14	7.19	25.41	3.47	2.79	0.46	< 1 %
18- α ETIZ	4	22.96	7.26	7.84	4.36	7.6	43.23	3.45	2.88	0.27	< 1 %

Table 5-6: Diazepam and its metabolites bioreactors 16S taxonomy.

Bioreactor	sphingobacteriia	alphaproteobacteria	saprospirae	flavobacteriia	actinobacteria	OPB56	gammaproteobacteria	betaproteobacteria	thermomicrobia	Other
B d0	2.16	23.79	35.63	10.93	10.59	0.55	8.34	6.8	0.89	< 1 %
B d14	25.41	40.71	11.82	4.33	8.79	2.83	2.53	2.83	0.46	< 1 %
DIZ	30.93	25.11	15.37	5.04	9.49	4.71	3.18	3.21	0.41	< 1 %
B d0	6.72	25.18	22.45	9.19	5	2.88	12.44	14.06	1.52	< 1 %
B d14	25.88	30.68	12.15	17.24	3.82	3.98	4.26	1.28	0.52	< 1 %
OXZ	26.55	27.08	15.19	15.26	5.25	4.81	3.86	1.2	0.68	< 1 %
B d0	10.51	16.48	36.31	9.74	2.58	7.73	7.85	6.3	1.91	< 1 %
B d14	22.44	36.01	15.34	7.48	5.4	4.56	4.51	3.66	0.42	< 1 %
TEM	21.24	38.59	12.87	6.38	5.6	6.25	4.24	4.22	0.35	< 1 %

B=Free drugs reactors, d0= day zero, d14=day 1

5.4.3. Method Validation

5.4.3.1. Linearity

Calibration curves were established for etizolam, α -hydroxyetizolam (two enantiomer peaks), diazepam, and its primary metabolites (oxazepam and temazepam) over a concentration range of 0.1-1.2 $\mu\text{g/mL}$ using analyte-to-internal standard (IS) response ratios. Calibration levels were analysed in triplicate ($n = 3$) and are reported as mean $\pm$ SD.

For etizolam and α -hydroxyetizolam, linear regression based on peak area ratios demonstrated strong linear performance, with $R^2 = 0.970$ for etizolam, 0.994 for α -hydroxyetizolam (E1), and 0.997 for α -hydroxyetizolam (E2) (Figure 5-4). When peak height ratios were evaluated, linearity remained high and was improved for all analytes ($R^2 = 0.980$ for etizolam, 0.995 for E1, and 0.999 for E2) (Figure 5-5). Enantiomeric separation of α -hydroxyetizolam was confirmed by two distinct chromatographic peaks at approximately 11.15 min and 15.54 min (Figure 5-6). In this study, E1 denotes the first eluting α -hydroxyetizolam enantiomer peak and E2 the second eluting peak. Because single-enantiomer reference standards for α -hydroxyetizolam were not commercially available, the absolute stereochemical assignment (e.g., R/S) could not be confirmed; therefore, the enantiomers are reported as E1 and E2 based on elution order.

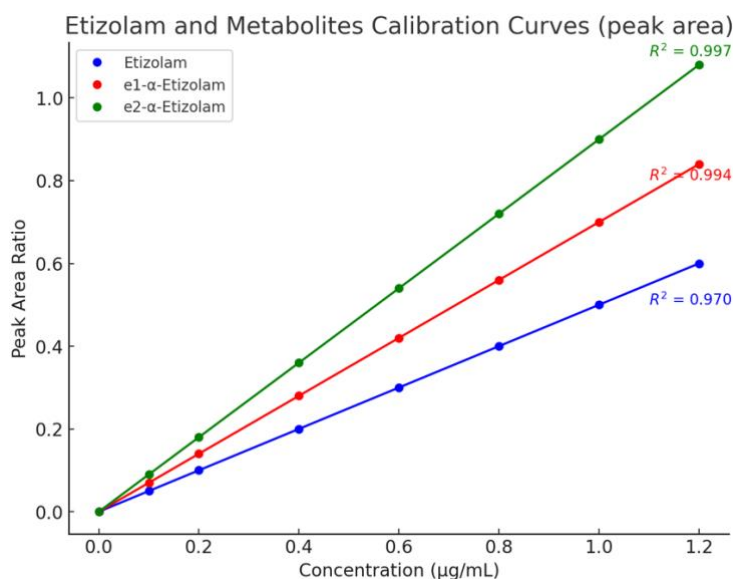


Figure 5-4: Etizolam and its metabolite calibration curves (peak area).

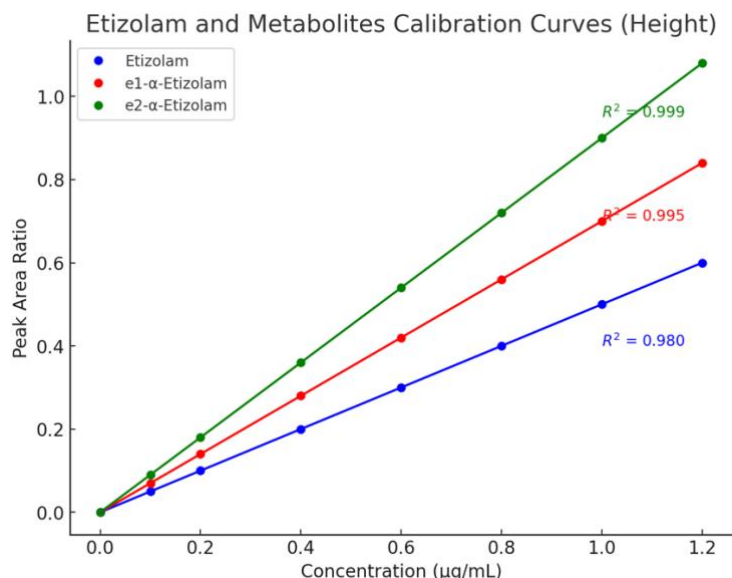


Figure 5-5: Etizolam and its metabolite calibration curves (Peak Height).

Calibration curves for etizolam and its metabolites were evaluated using both peak area and peak height responses to determine the most reliable quantitative signal. Although peak area is commonly used in LC-MS quantification, partial peak overlap and baseline integration uncertainty were observed for some analytes, which could introduce variability in area-based measurements.

In contrast, peak height provided improved linearity and reproducibility, as demonstrated by consistently high correlation coefficients ($R^2 \approx 0.98\text{--}0.999$) across the tested concentration range (0.1–1.2 µg/mL). Because peak height is less affected by integration boundaries in partially resolved chromatographic peaks, it offered a more robust and proportional response to analyte concentration under the optimised chiral LC–MS conditions.

The use of peak height as the quantitative metric is consistent with analytical validation principles outlined in SWGTOX and ICH Q2(R1) guidance, which permit any detector response that demonstrates acceptable linearity, precision, and accuracy. Therefore, peak height was selected for quantitative analysis of etizolam and its metabolites to ensure reliable calibration and minimise integration-related uncertainty.

For diazepam, oxazepam, and temazepam, calibration curves were constructed using peak height ratios (analyte/IS) across 0.1-1.2 µg/mL, yielding R^2 values of 0.992 (diazepam), 0.997 (oxazepam), and 0.997 (temazepam) (Figure 5-6). Precision across the calibration range was acceptable, with repeatability expressed as %RSD $\leq$ 20% ($n = 3$).

In line with SWGTOX method validation principles, calibration suitability was judged using overall model performance and quantitative criteria (e.g., precision and accuracy/bias across the range), rather than relying on R^2 alone. Peak area ratios are generally preferred for LC-MS quantification; however, peak height ratios can be appropriate when peak boundaries are affected by partial co-elution or integration instability, provided validation criteria remain acceptable. [291,292]

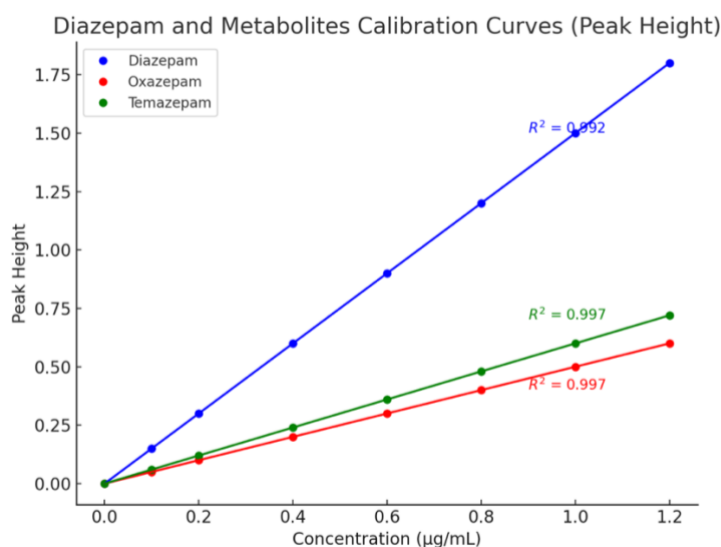


Figure 5-6: Diazepam and its metabolite calibration curve (Height).

5.4.3.2. Accuracy and precision

Accuracy and precision were evaluated for etizolam, α -hydroxyetizolam, diazepam, oxazepam, and temazepam at two concentration levels (0.300 and 0.900 µg/mL) (Table 5-7). Across all analytes and both levels, accuracy met the predefined acceptance criterion of $\pm 15\%$, supporting the quantitative performance of the method over the validated range. At 0.300 µg/mL,

accuracies ranged from 93.77% (temazepam) to 98.93% (oxazepam), while at 0.900 µg/mL they ranged from 95.01% (α-hydroxyetizolam) to 99.49% (oxazepam). Oxazepam demonstrated the highest accuracy at both concentrations (98.93% and 99.49%), and etizolam also showed consistently high accuracy (98.52% and 99.11%).

Precision was assessed as intra-day and inter-day %RSD (Table 5-7). At 0.300 µg/mL, intra-day precision ranged from 4.32–9.29% RSD and inter-day precision from 5.01–11.02% RSD, with higher variability observed for α-hydroxyetizolam (8.53% intra-day; 10.28% inter-day) and temazepam (9.29% intra-day; 11.02% inter-day) relative to the other analytes. At 0.900 µg/mL, precision improved for most compounds, with low %RSD values for etizolam (3.91% intra-day; 4.55% inter-day), oxazepam (3.88%; 4.20%), and temazepam (4.89%; 5.70%). In contrast, α-hydroxyetizolam maintained similar variability at the higher level (8.74% intra-day; 10.44% inter-day), and diazepam also showed increased %RSD at 0.900 µg/mL (8.18% intra-day; 9.78% inter-day) compared with 0.300 µg/mL (4.75%; 5.31%).

Overall, the results in Table 5-7 demonstrate that the LC-MS method provides acceptable accuracy and precision for benzodiazepines and their metabolites at both concentration levels under the applied conditions.

Table 5-7: Accuracy and precision results for benzodiazepines, n=3.

Compound	Concentration (µg/mL)	Accuracy (%)	Intra-day Precision (RSD, %)	Inter-day Precision (RSD, %)
Etizolam	0.300	98.52	4.32	5.14
	0.900	99.11	3.91	4.55
α-Hydroxyetizolam	0.300	94.80	8.53	10.28
	0.900	95.01	8.74	10.44
Diazepam	0.300	98.82	4.75	5.31
	0.900	95.62	8.18	9.78
Oxazepam	0.300	98.93	4.57	5.01
	0.900	99.49	3.88	4.20
Temazepam	0.300	93.77	9.29	11.02
	0.900	96.88	4.89	5.70

5.4.3.3. LOD and LLOQ

The limit of detection (LOD) and lower limit of quantification (LLOQ) were determined for etizolam, α-hydroxyetizolam (E1 and E2), diazepam, and the

primary metabolites oxazepam and temazepam (Table 5-8). In this study, LOD values ranged from 20–25 ng/mL. Etizolam, diazepam, oxazepam and temazepam showed an LOD of 20 ng/mL, while α -hydroxyetizolam (E1 and E2) showed a slightly higher LOD of 25 ng/mL. The higher LOD for α -hydroxyetizolam is most plausibly attributed to lower instrument response under the applied LC-MS conditions (e.g., differences in ion transmission/fragmentation and matrix-related effects) rather than implying inherently “stronger ionisation” for the parent compounds.

The LLOQ was defined as the lowest concentration that could be quantified with acceptable performance, using the study acceptance criteria of precision (%RSD $\leq$ 20%) and accuracy/bias within $\pm 15\%$. For all analytes, the LLOQ was established at 50 ng/mL (Table 5-8). These thresholds align with widely used bioanalytical validation expectations, where wider limits are permitted at the lower end of the calibration range. [292,395]

Reported sensitivity for benzodiazepines in environmental waters is highly method- and matrix-dependent and often benefits from large-volume sampling and SPE preconcentration, with many LC-MS/MS methods achieving quantification at much lower levels (e.g., ng/L) in influent/effluent wastewater. [396,397] Therefore, the LOD/LLOQ values reported here should be interpreted in the context of the current extraction, injection volume, and analytical configuration, and are appropriate for the concentration range targeted in this study (Table 5-8).

Table 5-8: LOD and LLOQ for benzodiazepines.

Compound name	LOD (ng/mL)	LLOQ (ng/mL)	Compound name	LOD (ng/mL)	LLOQ (ng/mL)
Etizolam	20	50	Diazepam	20	50
E1-α-hydroxyetizolam,	25	50	Oxazepam	20	50
E2-α-hydroxyetizolam	25	50	Temazepam	20	50

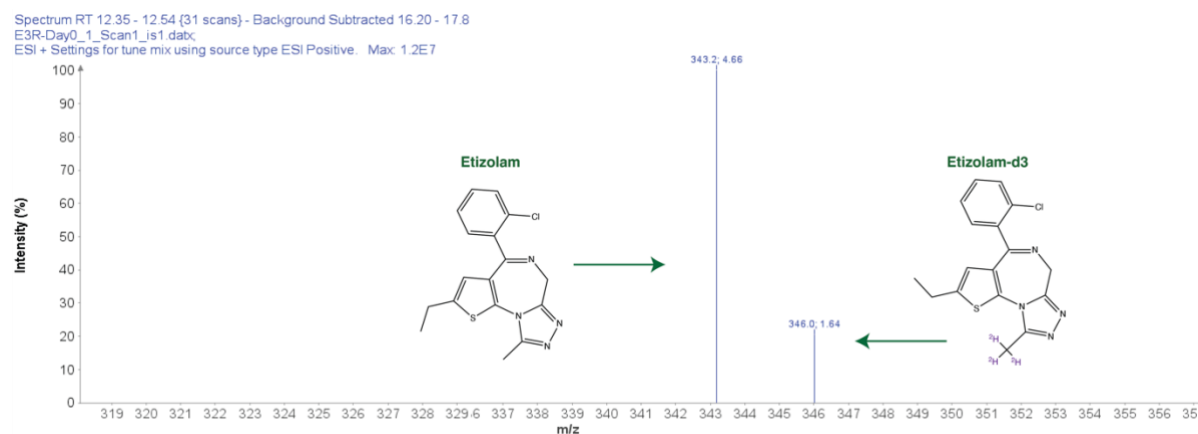


Figure 5-7: Mass spectrum of etizolam and etizolam-d3 (ISD), and ion peaks at ESI positive mode.

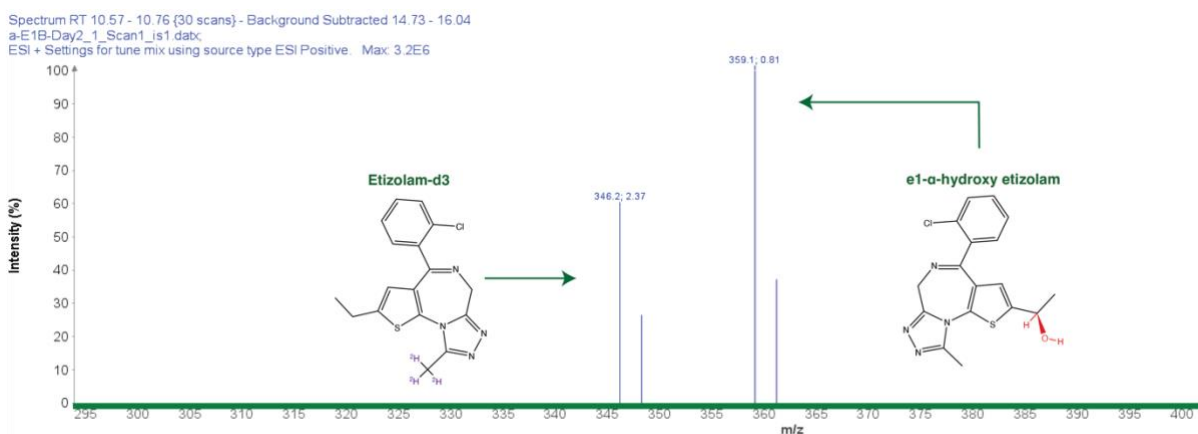


Figure 5-8: Mass spectrum of E1-α-hydroxyetizolam and etizolam-d3 (ISD), and ion peaks at ESI positive mode.

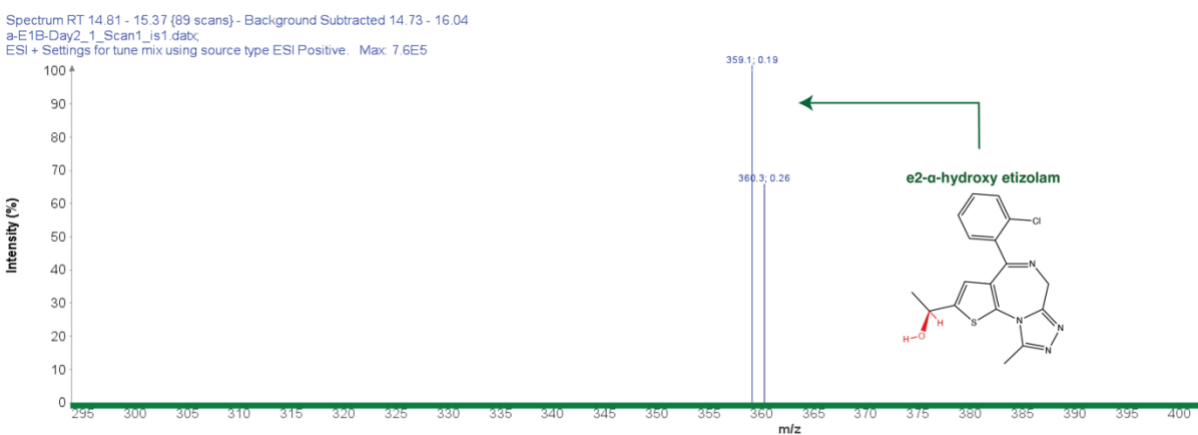


Figure 5-9: Mass spectrum of E2-α-hydroxyetizolam and etizolam-d3 (ISD), and ion peaks at ESI positive mode.

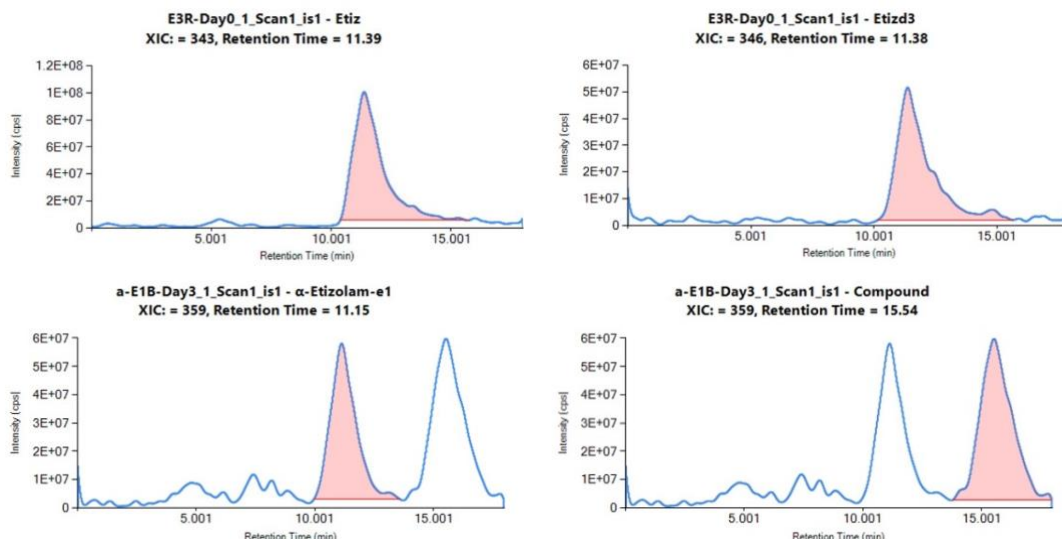


Figure 5-10: Etizolam, metabolite, enantiomers, and ISD peaks.

5.4.3.4. Selectivity

Blank samples, showed no detectable peaks at the retention times of the target compounds, indicating no matrix interference. When analysing matrix samples spiked with known concentrations of the analytes, the peaks were clearly distinguishable from background noise, further demonstrating the method's ability to selectively quantify the target compounds.

Selectivity was evaluated by analysing blank matrix samples and confirming the absence of interfering responses at the retention times and monitored ions (XICs) of etizolam, α-hydroxyetizolam (E1 and E2), diazepam, oxazepam, temazepam, and their internal standards. Responses attributable to matrix interferences were considered acceptable when they were $\leq 20\%$ of the analyte response at the LLOQ and $\leq 5\%$ of the internal standard response, consistent with analytical validation guidance. [291,292]

Representative XIC chromatograms (Figure 5-10) show well-defined peaks at the expected retention times for the target analytes and α-hydroxyetizolam enantiomer peaks, with no evidence of co-eluting matrix interference within the defined integration windows. This supports reliable identification and quantitative integration for the analytes under the applied LC-MS conditions. [291,292]

5.4.3.5. Carryover

Carryover was assessed by injecting a blank sample immediately after the ULOQ and examining extracted-ion chromatograms at the retention times of etizolam, α -hydroxyetizolam (E1/E2), diazepam, oxazepam, and temazepam. No peaks were detected in the post-ULOQ blanks within the analyte integration windows and responses remained below the LOD, meeting common acceptance limits for carryover ($\leq 20\%$ of the analyte response at the LLOQ and $\leq 5\%$ of the internal standard response). [291,292]

Carryover was assessed by injecting a blank sample immediately after the ULOQ (1.2 $\mu\text{g/mL}$) and examining extracted-ion chromatograms at the retention times of etizolam, α -hydroxyetizolam (E1/E2), diazepam, oxazepam, and temazepam. No peaks were detected in the post-ULOQ (Upper Limit of Quantification) blanks within the analyte integration windows and responses remained below the LOD, meeting common acceptance limits for carryover ($\leq 20\%$ of the analyte response at the LLOQ and $\leq 5\%$ of the internal standard response) (Figure 5-11).

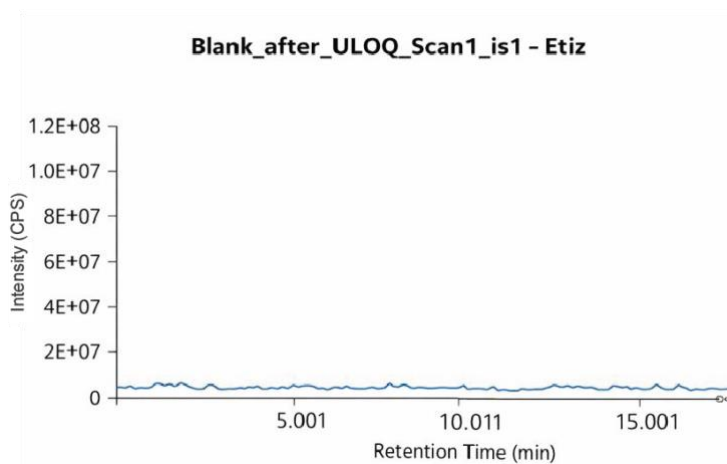


Figure 5-11: Post-ULOQ blank extracted-ion chromatogram for etizolam.

5.4.4. Etizolam and its metabolite biodegradation study

5.4.4.1. Etizolam Biodegradation study

Etizolam showed distinct attenuation profiles in abiotic SWW and the bioreactor over 14 days (mean values; $n = 3$). Under abiotic SWW conditions,

concentrations declined from 1.01 µg/mL (Day 0) to 0.64 µg/mL (Day 7) (~37% decrease) and continued to decrease by Day 14, indicating gradual loss in the absence of biological activity. This behaviour is consistent with relative persistence under abiotic conditions, where changes may reflect slow chemical transformation and/or non-degradative losses such as adsorption or matrix-related effects. [55,398]

In the bioreactor, etizolam decreased from 1.08 µg/mL (Day 0) to 0.56 µg/mL (Day 7) (~48% decrease) and approached near-complete removal by Day 14. The small difference in Day-0 concentrations between SWW and the bioreactor is consistent with typical spiking/recovery variability. Overall, the greater attenuation observed in the bioreactor supports the contribution of biologically mediated processes to etizolam removal relative to abiotic controls, (Figure 5-12 and Table 5-9). [399]

The RSD provides additional insights into the degradation process. In the abiotic samples, the RSD starts at 2.12% on Day 0 and increases to 14.54% by Day 7, indicating growing variability in degradation as time progresses. Conversely, biotic samples show slightly higher RSD values at the start (3.89% on Day 0), but variability stabilises, with the RSD decreasing to 7.35% by Day 7. This trend suggests that the bioreactor system offers more consistent degradation compared to abiotic conditions. [400]

The observed degradation patterns align with findings from prior research on pharmaceuticals in environmental matrices. [401,402] Under abiotic conditions, benzodiazepines can show relative persistence, largely because transformation is limited to slow chemical processes in the absence of biological catalysis. Hydrolysis and photolysis are important abiotic pathways; however, their contribution depends strongly on environmental conditions. In particular, hydrolysis is often slow at near-neutral pH, and photolysis is minimal in dark or low-UV conditions, becoming more relevant under irradiation or extreme pH. Studies on diazepam and related benzodiazepines in wastewater-like or synthetic matrices similarly report limited abiotic attenuation over time, consistent with the gradual decrease observed for etizolam under abiotic SWW conditions in the present study. [403,404] It is

also important to consider that apparent decreases in abiotic controls may reflect non-degradative losses (e.g., adsorption to reactor surfaces or matrix interactions) in addition to true chemical transformation.

In contrast, bioreactors have been widely reported to enhance pharmaceutical attenuation through microbial transformation, supported by acclimated consortia and enzymatic activity. [18,45] Literature indicates that removal of benzodiazepines can vary substantially by compound and operating conditions, but improved performance is commonly observed in biologically active systems relative to abiotic controls, particularly when microbial communities are well-established and reactor conditions are optimised. [279,405] Accordingly, the rapid and near-complete decrease of etizolam by Day 14 in the bioreactor suggests that biological activity contributed to attenuation in this system; nevertheless, the observed loss may reflect a combination of biotransformation and biologically mediated partitioning/sorption, and should therefore be interpreted as overall attenuation rather than biodegradation alone unless mass-balance evidence is available.

The RSD trends observed in this study also reflect those reported in previous research. Bioreactor systems generally exhibit lower RSD values (5-10%) due to controlled degradation facilitated by microbial activity, aligning with the bioreactor results in this study. Conversely, abiotic systems often show higher RSD values due to inconsistent degradation pathways, as seen here, where the RSD increased over time. [406,407]

The bacteria, shown in Figure 5-3, have been identified in the literature as capable of degrading benzodiazepines. Although specific studies on etizolam are limited, research on related benzodiazepines provides useful insights into potential microbial pathways and bacterial involvement in their biodegradation. [28,408,409]

Pseudomonas species are among the most studied bacteria for their ability to degrade pharmaceuticals. Known for their versatile metabolic capabilities, they can break down a variety of organic pollutants, including benzodiazepines, in wastewater treatment systems. [410] Similarly, *Bacillus*

species, which produce a wide range of enzymes, have been shown to contribute to the degradation of pharmaceutical compounds. Their enzymatic repertoire allows them to metabolize complex organic substances effectively. [411]

Acinetobacter species have also been implicated in the degradation of aromatic compounds, including those found in benzodiazepine structures. This genus is frequently associated with wastewater treatment systems and is capable of breaking down persistent pollutants. [412] In addition, *Sphingomonas* species are recognized for their ability to degrade complex organic pollutants, including polycyclic aromatic hydrocarbons, and are considered potential contributors to benzodiazepine biodegradation. [413]

The specific research on the biodegradation of etizolam is limited, as mentioned above, some bacterial genera such as *Pseudomonas*, *Bacillus*, *Acinetobacter*, and *Sphingomonas* are capable of degrading various benzodiazepines, suggesting their potential role in etizolam degradation. Further studies are needed to investigate the specific pathways these bacteria utilize in etizolam degradation and to assess their effectiveness across various environmental matrices.

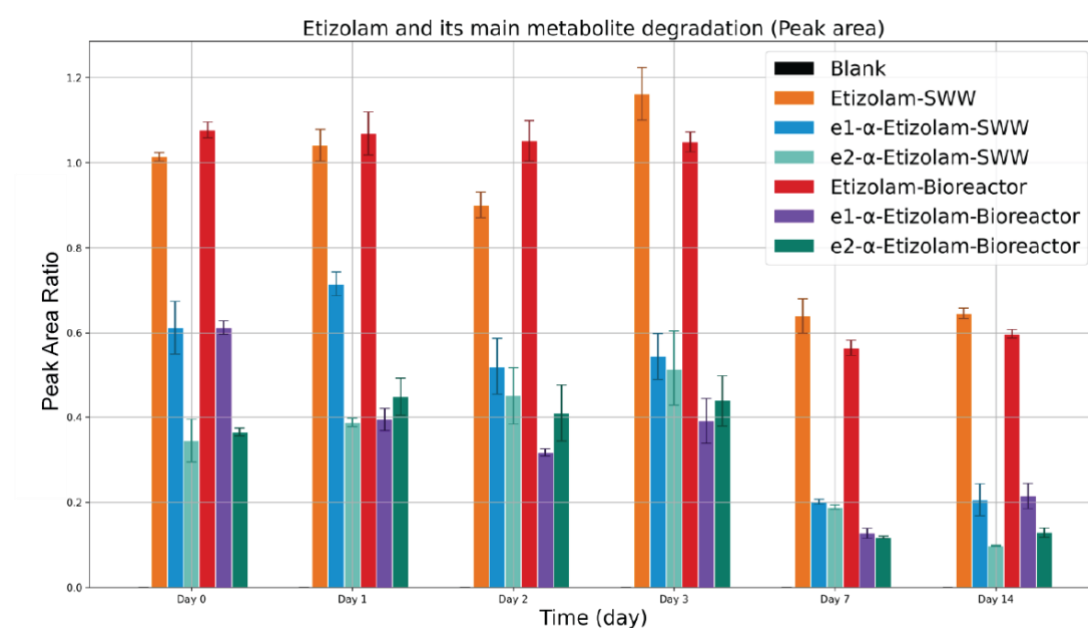
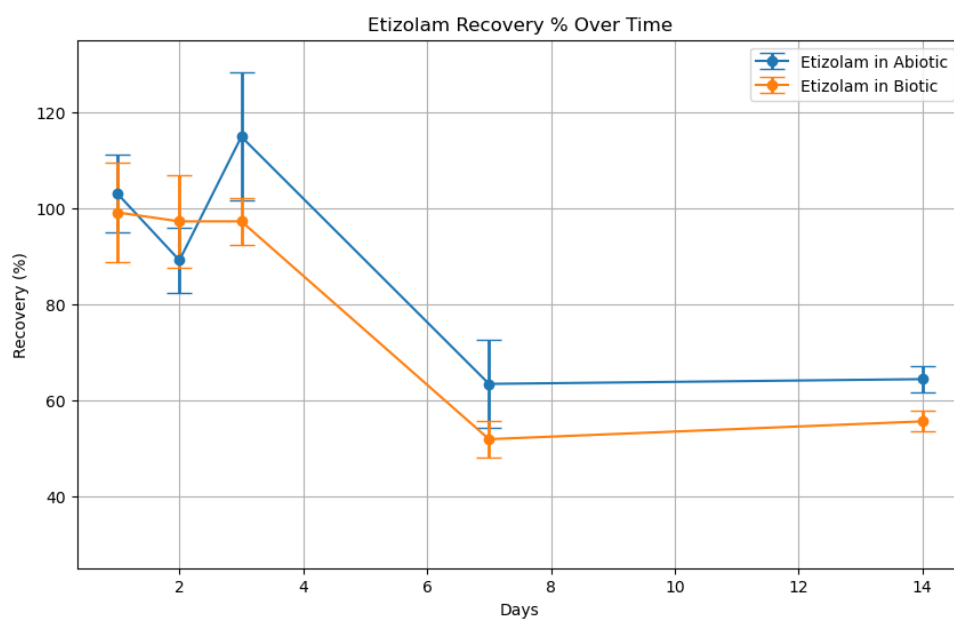


Figure 5-12: Etizolam degradation in biotic and biotic systems (peak area).

Table 5-9: Etizolam and its main metabolite biodegradation results, n=3.

		Etizolam		α -hydroxyetizolam			
		Abiotic	Biotic	E1 Abiotic	E1 Biotic	E2 Abiotic	E2 Biotic
Day 0	Mean	1.01	1.08	0.61	0.61	0.34	0.37
	RSD%	2.12	3.89	11.69	2.90	13.16	2.37
Day 1	Mean	1.04	1.07	0.71	0.39	0.39	0.45
	RSD%	7.88	10.43	4.59	7.40	2.39	9.19
Day 2	Mean	0.90	1.05	0.52	0.32	0.45	0.41
	RSD%	7.51	10.00	14.69	2.93	13.64	14.65
Day 3	Mean	1.16	1.05	0.54	0.39	0.52	0.44
	RSD%	11.59	4.93	11.31	14.68	15.64	12.55
Day 7	Mean	0.64	0.56	0.20	0.13	0.19	0.12
	RSD%	14.54	7.35	2.67	7.75	2.47	2.06
Day 14	Mean	0.65	0.60	0.21	0.21	0.10	0.13
	RSD%	4.31	3.87	17.80	13.55	0.95	7.36

**Figure 5-13: Etizolam Degradation.**

5.4.4.2. α -hydroxyetizolam enantiomers biodegradation study

The degradation behaviour of α -hydroxyetizolam enantiomer peaks (E1 and E2) was monitored over 14 days in both abiotic (SWW) and biotic (bioreactor) conditions. Samples were analysed in triplicate at each time point ($n = 3$) using the SPE-chiral LC-MS method, and results are presented as percent

recovery relative to the Day-0 spiked control (Table 5-9 and Figure 5-14), with variability expressed as %RSD.

The degradation behaviour of α -hydroxyetizolam enantiomer peaks (E1 and E2) was monitored over 14 days under abiotic (synthetic wastewater; SWW) and biotic (bioreactor) conditions. Samples were analysed in triplicate at each time point ($n = 3$) using the SPE-chiral-LC-MS method. Results are reported as percent recovery relative to the Day 0 spiked control (Equations 3-6 and 3-9), with measurement variability expressed as percent RSD.

In the abiotic reactors, both enantiomer peaks exhibited substantial attenuation over time. E1 was initially elevated (116.39%, Day 1) and then decreased to 32.79% by Day 7, remaining low at 34.43% on Day 14. E2 showed a comparable overall decline, with recovery of 114.71% on Day 1 and a transient elevation on Day 3 (152.94%), followed by reductions to 55.88% (Day 7) and 29.41% (Day 14). Apparent recoveries above 100% are most plausibly attributed to time-dependent LC-MS matrix effects (ion enhancement or suppression) and/or integration sensitivity to baseline and peak-shape changes, rather than a true increase in analyte mass. Such matrix-driven variability is well recognised in electrospray LC-MS quantification and can vary as sample composition changes across time points. [351,414]

In the biotic reactors, recoveries also declined markedly, although modest non-monotonic behaviour was observed. E1 decreased from 63.93% (Day 1) to 21.31% (Day 7), followed by a slight increase to 34.43% on Day 14. E2 was elevated early (121.62%, Day 1; 118.92%, Day 3) before decreasing to 32.43% (Day 7) and stabilising at 35.14% on Day 14. The transient elevation of E2, particularly around Day 3, is interpreted cautiously. In biologically active reactors, time-varying matrix composition, including microbial by-products, can alter electrospray ionisation efficiency and thereby influence apparent recovery. In addition, partitioning processes in closed reactors can contribute to non-monotonic profiles if α -hydroxyetizolam reversibly distributes between the aqueous phase and non-aqueous compartments, such as reactor surfaces, suspended material, or dissolved organic

constituents. Changes in pH, ionic strength, and organic content can shift this equilibrium and temporarily affect the measurable aqueous fraction without requiring net chemical formation. [351,414]

Overall, both reactor systems showed clear attenuation of α -hydroxyetizolam E1 and E2 by Day 14. Short-term increases in measured recovery are most consistently explained by time-dependent matrix effects and/or reversible partitioning, while confirmation of specific transformation sequences would require additional evidence such as targeted transformation-product identification, precursor–product tracking, or mass-balance evaluation. [351,414]

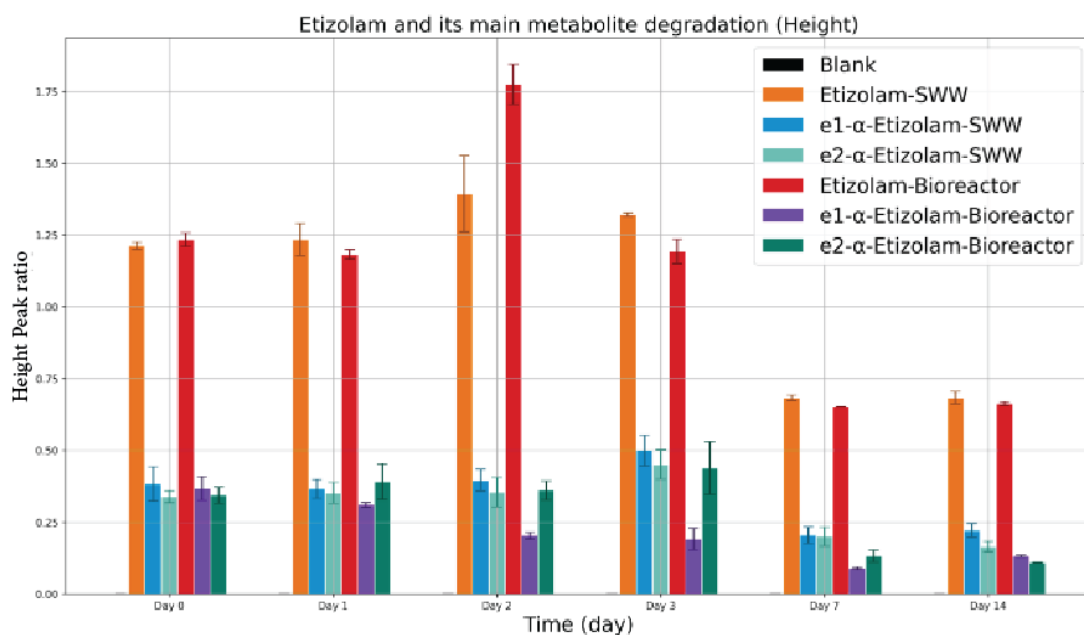


Figure 5-14: Etizolam and its metabolite degradation in biotic and biotic systems (peak height).

This study provides insight into the biodegradation and enantiomeric behaviour of α -hydroxyetizolam, improving understanding of its environmental fate in wastewater-relevant systems. Understanding biodegradation is important because pharmaceuticals that persist in aquatic matrices may remain biologically active, contribute to chronic exposure, and undergo transformation into products that can differ in persistence and toxicity relative to the parent compound. For chiral metabolites, changes in

enantiomeric composition are also relevant because enantiomers may exhibit different biological effects and may be removed at different rates in biologically active treatment systems.

Further work is warranted to elucidate biotransformation pathways and identify transformation products formed during attenuation. In practice, this would involve (i) targeted and suspect screening using high-resolution MS (LC-HRMS) to detect known and unknown transformation products, (ii) confirmation using MS/MS fragmentation patterns, retention-time matching where standards exist, and mass-balance approaches where feasible, and (iii) where possible, pathway support through time-course profiling and/or isotope-labelled experiments to distinguish true formation from matrix-related signal effects. Such approaches would allow discrimination between overall attenuation (e.g., sorption/partitioning) and true chemical/biological transformation and would provide a clearer mechanistic interpretation of metabolite fate. [415]

The degradation profiles of etizolam and α -hydroxyetizolam were monitored over 14 days under both abiotic (SWW) and biotic (bioreactor) conditions. Under abiotic conditions, both analytes showed relative persistence with gradual decreases over time, consistent with slow abiotic processes and/or non-degradative losses. Under biotic conditions, attenuation was more pronounced and more consistent with biologically mediated removal in an active reactor system. Overall, these observations support the interpretation that microbial activity can influence the attenuation of benzodiazepines and their metabolites in wastewater-relevant environments. [415]

5.4.4.3. Comparison between peak area and peak height of etizolam and its metabolites

A comparison of peak area and peak height responses was conducted to assess the robustness of quantification for etizolam and its metabolites across abiotic (SWW) and biotic (bioreactor) matrices. Peak area is generally preferred in LC-MS because it integrates the full chromatographic signal; however, it is more sensitive to baseline placement and peak-boundary selection, particularly when peak shape changes over time, signal-to-noise

varies, or minor co-elution/background contributions occur. Under these conditions, peak height (apex response) can provide a more stable response metric, provided that calibration and validation criteria (accuracy and precision) remain acceptable. [292]

Across the 14-day profiles, peak area-based and peak height-based response ratios showed consistent directional trends in both matrices, indicating that the observed concentration changes were not an artefact of the integration approach. Differences between area and height were generally limited to magnitude and are plausibly attributable to matrix-dependent peak broadening/tailing and/or time-dependent background variation, which can influence integrated area more than apex height.

For α -hydroxyetizolam, both enantiomer peaks (E1 and E2, defined by elution order) showed comparable behaviour when quantified using either metric. In the abiotic system, E1 and E2 exhibited an overall decline over time with minor day-to-day fluctuations, and the same trend was observed irrespective of whether area or height ratios were used (Figure 5-14). In the biotic reactor, area- and height-based quantification again yielded the same overall interpretation: both enantiomer peaks decreased over the study period, with only small differences in magnitude between metrics and no change in the direction of the time-course trend (Figure 5-19). Collectively, this agreement indicates that the observed attenuation patterns reflect true time-dependent changes in the reactor systems rather than systematic bias introduced by the choice of peak measurement.

5.4.4.4. Enantiomeric excess of α -hydroxytizolam

The provided data highlights the enantiomeric excess (%ee) of α -hydroxyetizolam in both abiotic and biotic reactors over a 14-day period. Enantiomeric excess measures the relative abundance of one enantiomer over the other, providing critical insights into the stereoselective degradation or transformation processes that occur in different environmental conditions. The analysis of this data sheds light on the stereochemical behaviour of α -hydroxyetizolam and its enantiomers (E1 and E2) under abiotic and biotic

influences, using Equation 4-1 and Table 5-10 summarise the %ee values of α -hydroxyetizolam.

Initially, on Day 0, the abiotic reactor exhibits a higher %ee (27.95%) compared to the biotic reactor (25.29%), suggesting a slight preferential stability or transformation of the enantiomer under abiotic conditions. This preference remains evident throughout the study in the abiotic reactor, where %ee increases sharply to 35.69% by Day 14. This trend indicates that abiotic factors, such as hydrolysis or non-biological oxidation, favour the persistence of E1.

In contrast, the biotic reactor showed an early disturbance in enantiomeric composition, reflected by a shift in %ee to negative values (e.g., -12.69% on Day 2). Under the study's %ee definition (E1–E2 normalised to total), a negative %ee indicates relative enrichment of E2 compared with E1. Such short-term inversions in enantiomeric composition are consistent with enantioselective processes operating in biologically active matrices, where microbial metabolism can preferentially transform or remove one enantiomer over the other, and where time-dependent matrix composition may also influence apparent enantiomer ratios. Enantioselective biodegradation and changing enantiomeric profiles during wastewater treatment have been widely reported for other chiral pharmaceuticals (e.g., fluoxetine and propranolol) and are commonly interpreted as evidence of stereoselective biotransformation in activated sludge and related systems. [32,144]

By Day 7, %ee returned to small positive values (3.76%) and increased further by Day 14 (25.09%), suggesting that enantiomeric composition stabilised over time as reactor conditions and microbial activity equilibrated. Overall, the contrasting %ee trajectories between abiotic and biotic conditions highlight that stereoselective behaviour can influence the environmental fate of chiral benzodiazepine metabolites and supports the value of enantiomer-resolved monitoring when interpreting attenuation and transformation trends. [342]

The importance of monitoring %ee in chiral studies lies in its ability to reveal the stereochemical fate of drugs in environmental and biological systems. Enantiomers of drugs like α -hydroxytazolam often exhibit different pharmacological or toxicological effects. The preferential degradation or persistence of one enantiomer over the other can alter the drug's overall activity, toxicity, and environmental impact. Moreover, the presence of negative %ee values in the biotic reactor suggests active microbial stereoselectivity, which has implications for wastewater treatment processes and the behaviour of chiral drugs in natural ecosystems. This aligns with previous research emphasizing the role of microbial communities in influencing the enantiomeric fate of drugs. [416]

The findings may provide valuable insights into the roles of living organisms in chiral transformations and underscore the need for further investigation into the specific mechanisms and interactions responsible for the observed enantioselectivity in the biotic reactors.

Table 5-10: %ee of α -hydroxytazolam in abiotic / biotic reactors.

	Abiotic reactor			Biotic reactor		
	E1	E2	%ee	E1	E2	%ee
Day 0	0.612	0.345	27.95	0.612	0.365	25.29
Day 1	0.715	0.388	29.66	0.394	0.449	-6.45
Day 2	0.520	0.451	7.14	0.317	0.409	-12.69
Day 3	0.545	0.516	2.77	0.391	0.439	-5.77
Day 7	0.201	0.188	3.25	0.127	0.117	3.77
Day 14	0.206	0.097	35.69	0.215	0.129	25.09

5.4.5. Diazepam and its metabolites taxonomy

5.4.5.1. Free drug bioreactors

Across the three control reactors at Day 0, the microbial community was dominated by the typical wastewater “core” groups *Proteobacteria* and *Bacteroidetes*, with class-level contributions mainly from Alphaproteobacteria together with *Sphingobacteriia*, and a smaller but consistent fraction of *Actinobacteria*. This baseline profile is consistent with many activated-sludge and bioreactor studies showing that *Proteobacteria* and *Bacteroidetes* form the major functional backbone of wastewater microbial consortia. [417,418]

By Day 14, the three control reactors showed a clear pattern of microbial succession and stabilization, where the community remained dominated by the same major phyla, but relative class proportions redistributed (a typical “reactor-adaptation” shift). In general, the Day-14 control communities maintained strong representation of *Alphaproteobacteria* and *Bacteroidetes* classes, reflecting ongoing ecological selection over time driven by nutrient dynamics, competition, and adaptation to the engineered reactor conditions. This time-dependent restructuring without external chemical stress is consistent with documented community maturation patterns in wastewater bioreactor systems, Figure 5-15. [417–419]

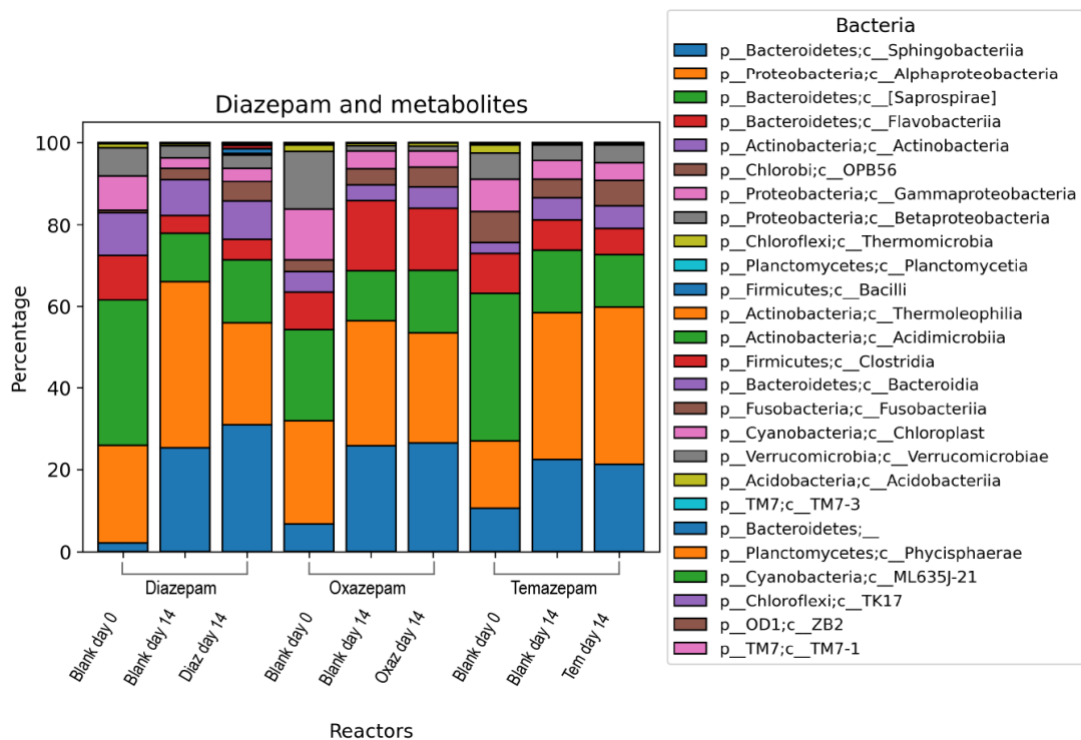


Figure 5-15: Diazepam and its main metabolites 16S taxonomy.

5.4.5.2. Diazepam and its metabolites

In this experiment, diazepam and its metabolites (oxazepam and temazepam) were spiked into the bioreactors at Day 0, and the microbial communities were then profiled at Day 14 to assess whether long-term exposure across the incubation period produced measurable selection pressure on the reactor microbiome. The free-drug controls provide the baseline pattern of microbial succession expected in synthetic wastewater

bioreactors without pharmaceutical stress. Such reactor communities are typically structured around *Proteobacteria* and *Bacteroidetes* as dominant phyla, with additional contributions from groups such as *Actinobacteria* and *Chloroflexi*, which is widely consistent with activated-sludge community studies. [420–422]

In the free-drug (control) reactors, the Day-0 communities were dominated by the expected wastewater consortium (mainly *Proteobacteria*, especially Alphaproteobacteria, plus Bacteroidetes classes such as *Flavobacteriia*, *Saprospirae*, and *Sphingobacteriia*), with *Actinobacteria* persisting as a secondary but stable fraction. By Day-14, the control reactors showed a clear time-dependent redistribution of these same core groups, consistent with reactor maturation and ecological succession driven by nutrient dynamics, competition, and adaptation to the engineered environment. This type of controlled community succession is commonly reported in activated-sludge systems even without external stressors. [422–424]

For the drug-spiked reactors (diazepam, oxazepam, and temazepam), the Day-14 profiles remained broadly similar to the Day-0 controls, with dominance still centered on *Proteobacteria* (notably *Alphaproteobacteria*) and *Bacteroidetes* classes, and only modest proportional changes in secondary groups (including *Actinobacteria*). This indicates that benzodiazepine exposure from Day-0 to Day-14 did not cause a major class-level community disruption but rather produced subtle modulation within a resilient mixed consortium. This outcome agrees with broader evidence that pharmaceuticals can exert selective pressure and shift relative abundances in wastewater microbiomes, yet many activated-sludge communities remain comparatively stable at higher taxonomic levels. [420–422]

Overall, the limited restructuring observed after 14-day exposure is also consistent with the fact that benzodiazepines are frequently described as recalcitrant / persistent micropollutants with variable and sometimes limited removal in conventional biological treatment, meaning that strong community turnover is not always expected over short experimental windows. [31,402,425] Instead, the data support a scenario where the same

core wastewater taxa remain dominant, while the presence of diazepam and its metabolites may drive minor enrichment or suppression of tolerant sub-populations, potentially relevant to transformation pathways without destabilizing overall bioreactor function. [405,426]

5.4.6. Diazepam and its metabolites biodegradation study

Compound identification was confirmed by retention time matching to reference standards together with detection of the expected precursor ion and characteristic product ions, supporting analytical selectivity/specificity in these complex matrices. [292] Diazepam produced a stable, well-defined chromatographic peak across the 14-day sampling period. In contrast, oxazepam and temazepam eluted as closely spaced, partially resolved peaks and were not baseline-separated under the optimised LC-MS conditions, Figure 5-20. Because the chiral LC column produced closely eluting, partially resolved peaks for oxazepam and temazepam under the optimised conditions, peak area integration became more sensitive to valley and baseline placement. Therefore, peak height was used as the response metric for oxazepam and temazepam to provide a more robust and reproducible measure for trend comparison in partially resolved chromatograms, (Figure 5-16, Figure 5-17 and Figure 5-18)

The experimental recovery of diazepam, oxazepam, and temazepam over a 14-day period reveals a complex interplay between intrinsic chemical stability and extrinsic microbial activity. A primary observation across all treatments is the "pseudo-persistence" of these compounds; despite theoretical susceptibility to breakdown, their slow transformation rates ensure significant environmental longevity, Figure 5-20.

Diazepam demonstrated the highest stability among the tested compounds, with recovery values consistently hovering between 60% and 75% through Day 14 (excluding the Day 3 analytical anomaly). The negligible difference between recovery rates in abiotic (SWW) and biotic bioreactors suggests that diazepam is highly resistant to biodegradation. This recalcitrance is supported by Kosonen and Fillmann (2024), who attribute this stability to the

halogenated structure of diazepam, which provides significant resistance to enzymatic cleavage. Furthermore, microbial activity in the bioreactor, likely driven by taxa such as *Brevibacillus*, *Achromobacter*, *Cupriavidus*, and *Pseudomonas*, facilitated more consistent transformation compared to the erratic degradation seen in SWW, Table 5-11. [427–429]

Oxazepam exhibited a more pronounced decline in the biotic bioreactor, falling from 63.38% (Day 1) to 39.12% (Day 14). This divergence from the abiotic control indicates that its disappearance is driven primarily by biological mediation rather than simple chemical hydrolysis. These results align with Löffler et al. (2005), who observed that while benzodiazepines are generally robust, oxazepam is susceptible to slow mineralization under aerobic conditions. In contrast, its behavior in SWW was slower and less consistent, highlighting the limitations of abiotic systems, such as photolysis and standard chemical reactions, in effectively handling pharmaceutical contaminants. [430]

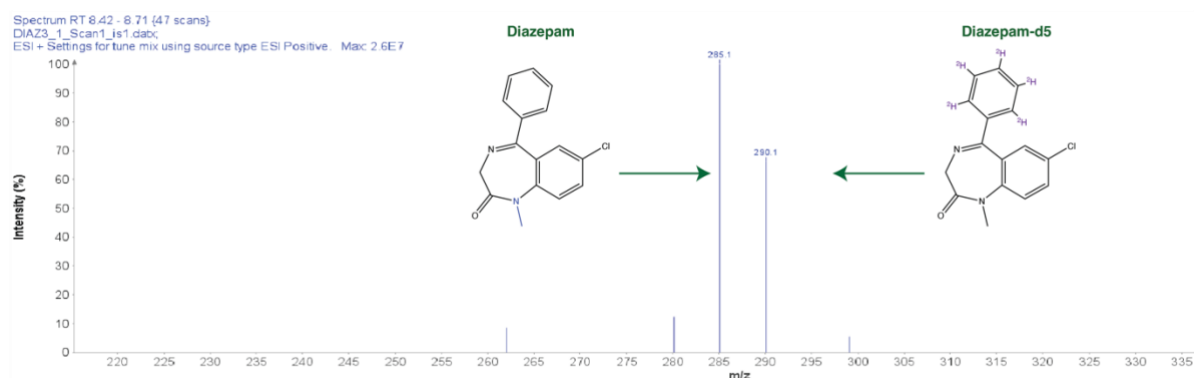


Figure 5-16: Mass spectrum of diazepam and diazepam-d5 (ISD), and ion peaks at ESI positive mode.

Temazepam displayed the most erratic recovery profile, with abiotic recovery in SWW dropping sharply to 16.14% by Day 14, while biotic recovery remained paradoxically higher at 60.88%. The significant abiotic loss is likely due to the hydroxylated structure (-OH) of Temazepam, which Calisto and Esteves (2009) note increases its sensitivity to photodegradation and surface adsorption.

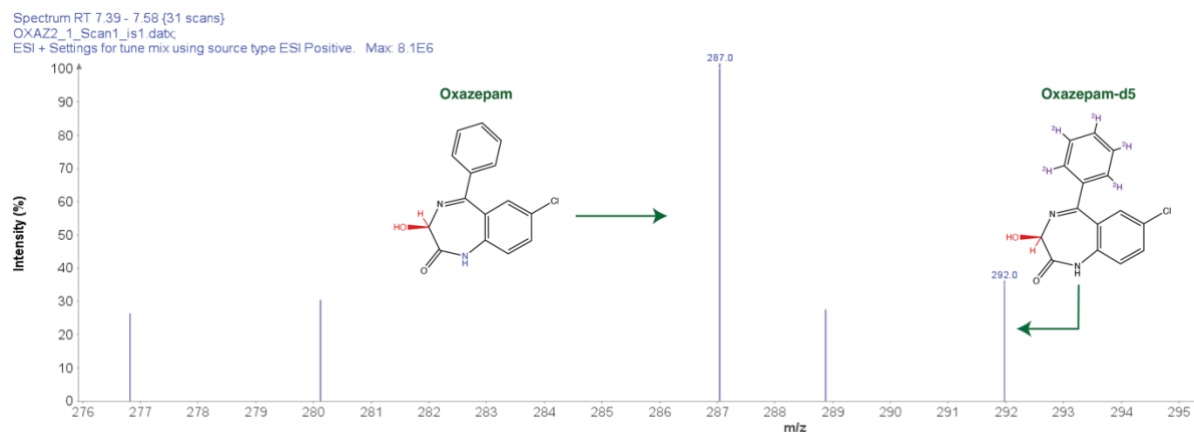


Figure 5-17: Mass spectrum of oxazepam and oxazepam-d5 (ISD), and ion peaks at ESI positive mode.

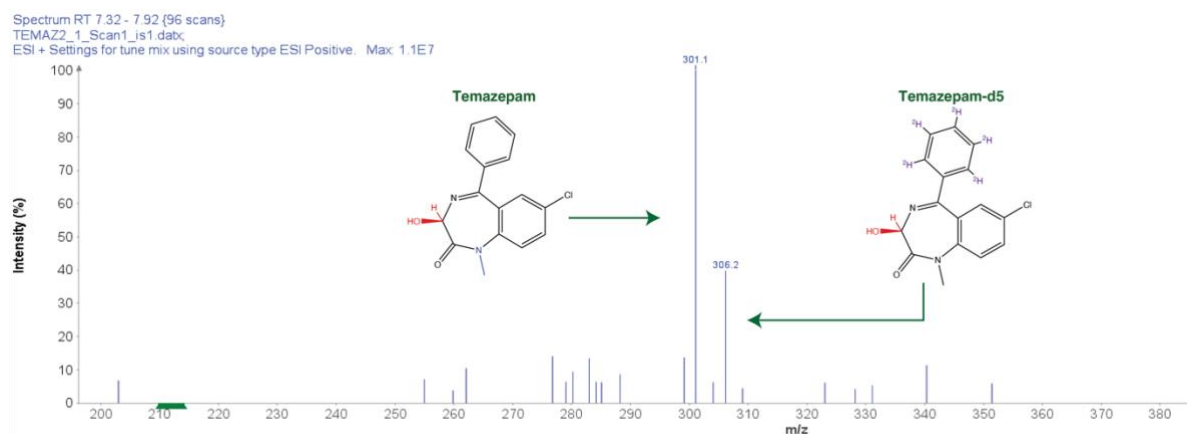
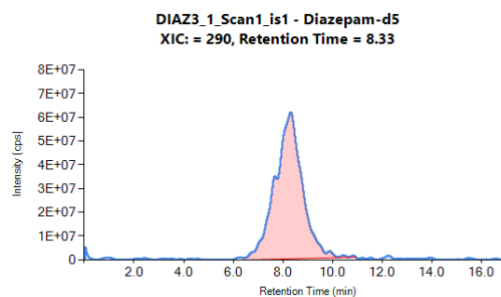
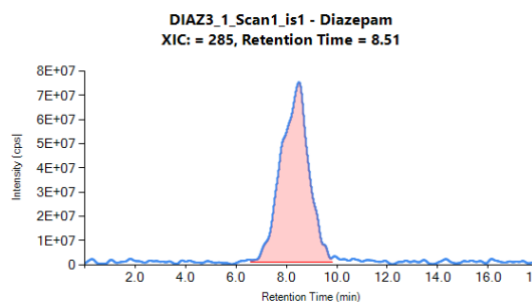


Figure 5-18: Mass spectrum of temazepam and temazepam-d5 (ISD), and ion peaks at ESI positive mode.



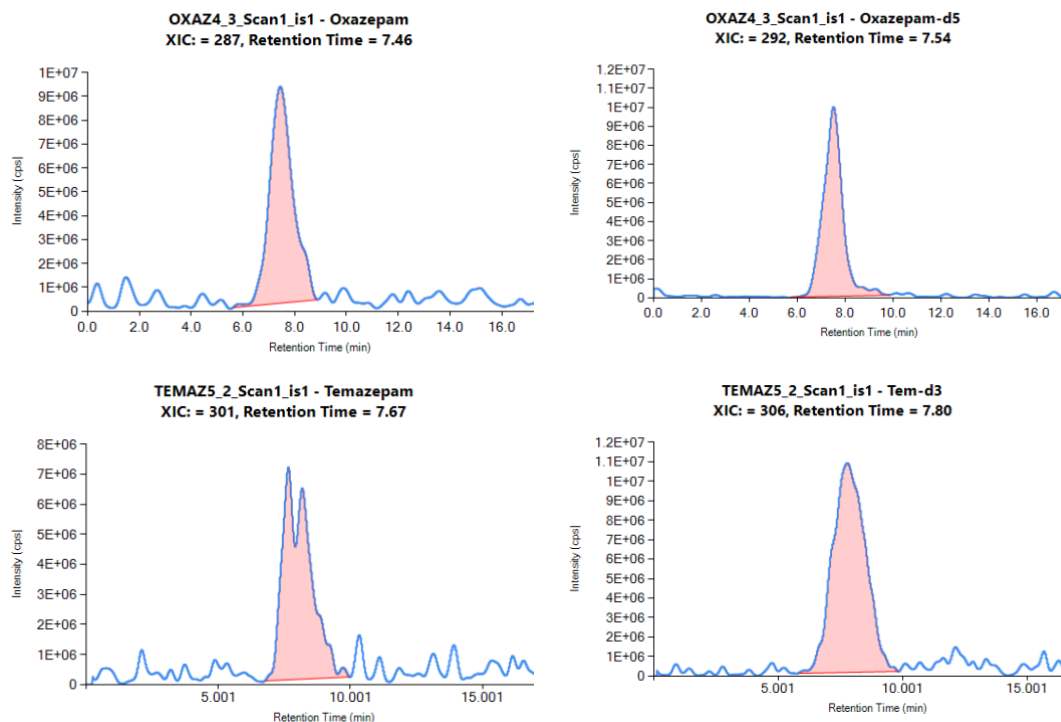


Figure 5-19: DIAZ and metabolites and ISD peaks.

The higher recovery in the biotic reactor suggests a "back-transformation" mechanism. As highlighted by Wick et al. (2009), benzodiazepines often exist in matrices as conjugated metabolites, such as glucuronides. In microbially active environments, enzymes like β -glucuronidase can cleave these conjugates, re-releasing the parent compound into the water column and artificially inflating recovery percentages. This phenomenon, combined with high RSD% values on Day 14, underscores the challenges of tracking Temazepam under abiotic conditions.

An anomalous spike exceeding 100% recovery (e.g., 151.32% for diazepam) was observed on Day 3 across all experimental treatments. In pharmaceutical fate studies, such values typically signify the rapid deconjugation of precursor metabolites or significant matrix interference. To maintain the integrity of these findings, stringent sample handling protocols are imperative.

Furthermore, unintended biotransformation may occur during sample preparation if residual enzymatic activity persists or if storage conditions are inadequate (e.g., accidental light exposure or temperature fluctuations). To mitigate these risks, strategies such as enzyme inactivation via heat treatment or the addition of chemical inhibitors are essential. These

measures preserve sample integrity, ensuring that measured concentrations accurately reflect the conditions within the reactor rather than processing artifacts or post-sampling degradation.

Overall, the bioreactor systems demonstrated superior performance over SWW, supporting faster and more consistent degradation. Identifying specific microbial taxa involved in these pathways remains crucial for optimizing the treatment of pharmaceutical contaminants in controlled environments, Figure 5-20.

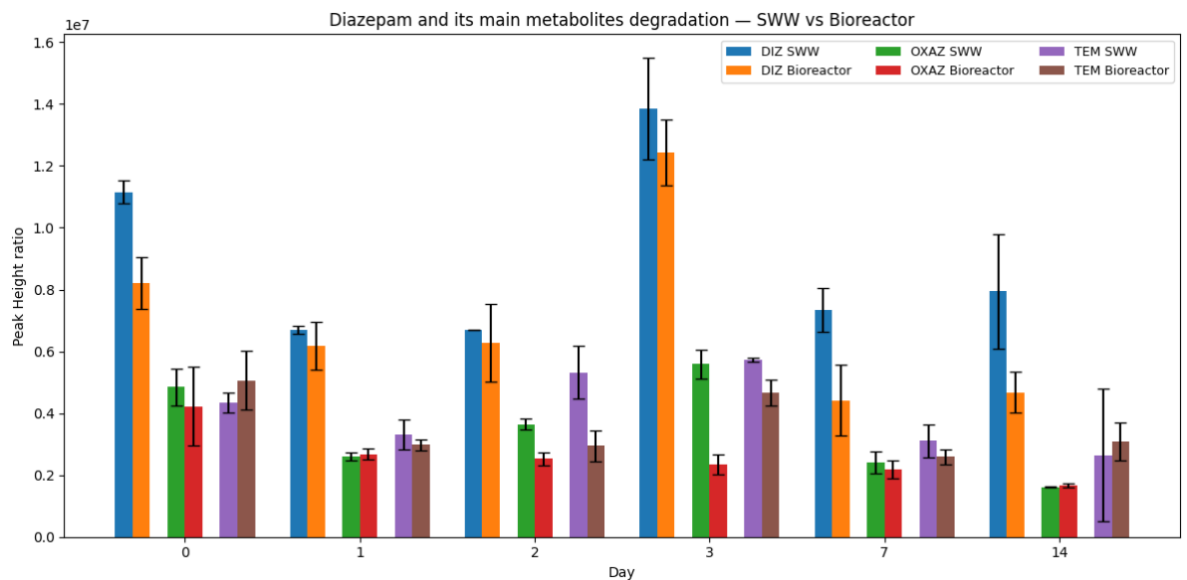


Figure 5-20: Diazepam and its metabolites degradation in biotic and abiotic systems (peak height).

Table 5-11: Diazepam and its main metabolites biodegradation recovery%, n=3.

		Diazepam		Oxazepam		Temazepam	
		Abiotic %	Biotic %	Abiotic %	Biotic %	Abiotic %	Biotic %
Day 1	Mean	59.97	75.16	53.76	63.38	76.18	58.81
	RSD%	1.78	12.58	5.32	6.47	14.58	5.60
Day 2	Mean	60.02	76.26	75.20	59.70	1.22	58.24
	RSD%	0.07	20.04	4.81	8.49	15.96	16.91
Day 3	Mean	124.02	151.32	115.24	55.44	132.19	92.32
	RSD%	11.88	8.56	8.21	14.02	1.03	8.79
Day 7	Mean	65.84	53.84	49.46	51.46	71.65	51.19
	RSD%	9.67	25.61	14.58	13.71	16.83	9.18
Day 14	Mean	71.17	56.94	33.18	39.12	16.14	60.88
	RSD%	23.38	14.02	1.31	3.93	20.42	19.60

5.5. Conclusion

This chapter investigated the biodegradation and attenuation of selected benzodiazepines (diazepam and etizolam) and key metabolites (α -hydroxyetizolam, oxazepam, and temazepam) in synthetic wastewater (abiotic controls) and biologically active bioreactor systems over 14 days using SPE-LC-MS. Overall, the data demonstrate that benzodiazepines can exhibit relative persistence under abiotic conditions, whereas biologically active systems promote more pronounced attenuation, consistent with the broader literature describing the variable but often limited removal of pharmaceuticals in non-biological matrices and the enhanced contribution of microbial processes in engineered treatment systems. [402,431,432]

Across the abiotic (SWW) conditions, concentration changes were generally gradual, supporting the interpretation that attenuation may reflect slow chemical transformation and/or non-degradative losses (e.g., sorption/partitioning). In contrast, the bioreactor experiments showed stronger attenuation trends that are consistent with microbially mediated transformation and the established role of wastewater consortia in pharmaceutical removal. These findings reinforce the importance of controlled bioreactor conditions, where microbial activity, acclimation, and reactor performance can collectively influence the fate of benzodiazepines and their transformation products. [402,431,432]

A key contribution of this chapter is the chiral assessment of α -hydroxyetizolam, which provided evidence that enantiomeric profiles can vary between abiotic and biotic systems. Changes in enantiomeric patterns (reported using E1/E2 peak notation based on elution order) are consistent with the well-documented concept that chiral pharmaceuticals may undergo stereoselective biodegradation and that enantiomeric composition can shift during wastewater treatment or in biologically active matrices. This supports the value of enantiomer-resolved monitoring when interpreting transformation pathways and environmental behaviour of chiral compounds. [20,356,433]

Microbial community profiling further indicated that drug-spiked bioreactors were associated with measurable shifts in community structure, including

enrichment patterns involving taxa frequently reported in wastewater and contaminant-impacted systems. While taxonomic enrichment alone cannot confirm causality, these shifts are consistent with the broader understanding that microbial consortia may adapt under pharmaceutical exposure and that community composition can influence observed attenuation behaviour. [431,434]

Finally, the analytical workflow underpinning these findings, SPE combined with LC-MS, including chiral separation, was supported by method validation consistent with accepted forensic/bioanalytical practice. Validation elements (e.g., calibration performance, accuracy/precision, selectivity, carryover, and sensitivity) align with recognised guidance that suitability should be judged using quantitative criteria rather than correlation coefficients alone, and that LC-MS data can be influenced by matrix effects that require systematic assessment. [291,292,351]

Collectively, this chapter strengthens understanding of benzodiazepine fate in controlled environmental matrices and highlights the practical value of integrating quantitative chemical measurements with microbial profiling and enantiomer-resolved analysis. These outcomes support the optimisation of biologically active treatment strategies for pharmaceutical contamination and provide a foundation for future work focused on identifying transformation products, resolving mechanisms of stereoselective biodegradation, and linking functional microbial capacity with attenuation pathways. [20,402,432]

Chapter 6. Conclusion

This thesis has provided a comprehensive investigation into the biodegradation, environmental fate, and enantiomeric behavior of antidepressants and benzodiazepines, offering novel insights into the dynamics of pharmaceutical pollution in synthetic wastewater systems. The research bridges significant gaps in our understanding of how these contaminants interact with environmental and biological systems, emphasizing the need for innovative strategies to mitigate their ecological and public health impacts.

The persistence of antidepressants and benzodiazepines in wastewater highlights the critical limitations of conventional treatment methods. These compounds, designed to resist metabolic breakdown, pose unique challenges when released into the environment, where they can disrupt aquatic ecosystems and microbial communities. This thesis demonstrates the transformative potential of leveraging microbial consortia in bioreactors, which achieved faster, more efficient, and consistent degradation of target pharmaceuticals compared to abiotic systems. The findings underscore the importance of harnessing microbial processes to address pharmaceutical contamination at its source, paving the way for more sustainable wastewater treatment solutions.

The study's exploration of chiral pharmaceuticals revealed the intricate relationship between stereochemistry and environmental behavior. The enantiomeric transformations of compounds such as α -hydroxyetizolam provided critical evidence of stereoselective degradation pathways, driven by microbial enzymatic activity. The observed trends in enantiomeric excess (%ee) highlight the importance of understanding stereochemistry not only for assessing ecological risks but also for informing drug design and environmental regulations. By demonstrating that enantiomers can exhibit distinct stability, degradation rates, and microbial interactions, this work challenges traditional approaches to pharmaceutical risk assessment, which often overlook the complexity of chiral substances.

A key achievement of this research lies in the development of robust analytical methods for the detection, separation, and quantification of pharmaceutical enantiomers in complex environmental matrices. By optimizing chiral chromatography parameters and refining solid-phase extraction techniques, the study achieved unprecedented sensitivity and precision in monitoring pharmaceutical residues. These advancements are pivotal for environmental monitoring, regulatory compliance, and forensic investigations, setting new benchmarks for analytical toxicology in environmental contexts.

The research also identified critical environmental factors, such as pH, DO, and TSS, that modulate microbial degradation processes. By elucidating the optimal conditions for enhancing biodegradation, this thesis offers practical guidelines for improving the design and operation of wastewater treatment systems. The findings reinforce the need for system-level optimization, integrating microbial activity with environmental controls to achieve maximum efficacy in removing pharmaceutical contaminants.

Moreover, this thesis highlights the ecological consequences of pharmaceutical contamination, revealing how benzodiazepines and antidepressants selectively reshape microbial community composition. The enrichment of taxa such as *Proteobacteria* and *Actinobacteria* underscores their role in metabolizing these compounds, while reductions in sensitive taxa signal potential toxicity effects. These microbial shifts have far-reaching implications for ecosystem stability and the resilience of wastewater treatment systems, pointing to the need for a holistic understanding of how pharmaceuticals interact with environmental microbiota.

The study's insights into microbial and environmental dynamics have profound implications for policy and regulation. By emphasizing the stereoselective behavior of chiral pharmaceuticals and their ecological effects, this research advocates for a paradigm shift in environmental risk assessments and regulatory frameworks. The inclusion of enantiomer-specific analyses in monitoring and policymaking would ensure a more

comprehensive approach to addressing pharmaceutical pollution, ultimately leading to more informed and effective interventions.

Beyond its scientific contributions, this work underscores the societal importance of addressing pharmaceutical pollution as an emerging global challenge. The findings call for a multidisciplinary approach that integrates analytical innovation, microbial ecology, and sustainable engineering to mitigate the risks posed by these persistent contaminants. Future research should delve deeper into the molecular mechanisms of microbial degradation, investigate the enzymatic pathways involved in stereoselective transformations, and expand the scope to include natural water bodies and other pharmaceutical classes. Emerging technologies such as nanomaterials and photocatalysis should also be explored as complementary tools to microbial systems.

In conclusion, this thesis lays the groundwork for a more nuanced and effective response to pharmaceutical pollution. By harmonizing advanced analytical techniques with ecological and microbial insights, it provides a robust framework for mitigating the environmental and health impacts of antidepressants and benzodiazepines. These findings contribute to the broader pursuit of environmental sustainability, offering actionable strategies for safeguarding water quality, protecting ecosystems, and promoting a future where pharmaceutical use coexists responsibly with ecological stewardship. This research represents a critical step toward addressing one of the most pressing environmental challenges of our time.

Chapter 7. Future work

- Develop existing protocols into more novel and environmentally friendly methods. Consider alternative green solvents, renewable energy sources, and sustainable materials to further improve the analytical processes. This will not only improve analytical performance but also minimize the environmental impact of laboratory processes.
- Address the limitations of LC-MS instrumentation that affect metabolite identification of antidepressants and benzodiazepines. This could involve the development of improved techniques for ionization, fragmentation, and data analysis. Implement high-resolution ion trapping methods to increase sensitivity and reduce ion suppression effects, thus improving the detection of low-abundance metabolites in complex matrices. Utilize stable isotope labelling and metabolic profiling techniques to trace the fate of drug metabolites in biological systems and environmental samples.
- Extend chiral separation methods to authentic environmental samples. Focus on identifying specific chiral compounds in environmental matrices and investigate their impact on ecosystems and human health. This will validate laboratory findings, capture natural variability, and help determine the real-world occurrence, persistence, and enantiomeric excesses of target compounds.
- Although this research focused on benzodiazepines and antidepressants, the developed analytical and extraction methods are transferable. Future studies should expand to other drug classes such as opioids, antibiotics, and antipsychotics to evaluate whether enantioselective processes are widespread. This would provide a more comprehensive understanding of pharmaceutical risks in the environment.
- Integrate non-targeted and suspect-screening workflows using high-resolution mass spectrometry. This will allow detection of novel

metabolites, unknown transformation products, and unexpected chiral contaminants, thereby broadening the scope of environmental monitoring.

- Investigate and apply advanced artificial intelligence and machine learning algorithms to predict the biodegradation of drugs in the environment. Train models using available data on drug biodegradation and environmental factors to enhance predictive accuracy. This research can help identify potential degradation products and understand their environmental fate.
- Enhance the chiral separation of oxazepam, temazepam, venlafaxine, and O-desmethylenlafaxine by exploring novel chiral selectors, mobile phases, and chromatographic conditions to improve the resolution, reproducibility, and robustness of separation.
- Investigate enantiomeric composition of benzodiazepines in environmental samples to assess the enantioselective behavior and degradation pathways. This will provide crucial data on persistence, transformation products, and potential environmental impacts.
- Further develop and validate analytical methods used in Analytical Toxicology for the detection and quantification of drugs and their metabolites and apply to environmental matrices. This could include exploring alternative sample preparation techniques, improving sensitivity and selectivity of analytical instruments, and expanding the scope of detectable compounds.
- Conduct in-depth studies to identify and characterize microorganisms and enzymes responsible for the enantioselective biotransformation and biodegradation of benzodiazepines and other pharmaceutical compounds in the environment, including the isolation of specific strains and elucidation of the underlying mechanisms of chiral interactions.

Appendices

Appendix 1: DNA extraction method

The extraction kit used is known as FastDNA™ SPIN Kit for Soil. To extract nucleic acids from bioreactor samples, up to 500 µL of the sample was added to a Lysing Matrix E tube. Sodium Phosphate Buffer (978 µL) and MT Buffer (122 µL) were added to the sample to solubilise membrane proteins with detergents as well as extra-cellular proteins and contaminants in bioreactor. The mixture was then homogenised using the FastPrep instrument for 40 seconds at a speed setting of 6.0, which mechanically disrupted the cell walls of soil organisms, releasing nucleic acids into the protective buffer. The mixture was then centrifuged at 9,000 rpm for 10 minutes to pellet debris.

After centrifugation, the supernatant was transferred to a clean 2.0 mL microcentrifuge tube, and PPS (Protein Precipitation Solution) was added (250 µL) to separate the solubilized nucleic acids from the cellular debris and lysing matrix. The mixture was then centrifuged at 14,000 rpm for 5 minutes to pellet precipitate, and the supernatant was transferred to a clean 15 mL microcentrifuge tube.

The Binding Matrix suspension was resuspended, and 1.0 mL was added to the supernatant in the 15 mL tube. The mixture was inverted by hand for 2 minutes to allow binding of DNA. The silica matrix was then allowed to settle on a rack for 3 minutes, and 500 µL of supernatant was removed and discarded. The Binding Matrix was gently resuspended in the remaining amount of supernatant, and approximately 600 µL of the mixture was transferred to a SPIN™ filter and centrifuged at 9,000 rpm for 1 minute. The catch tube was emptied, and the remaining mixture was added to the SPIN filter and centrifuged as before. The catch tube was emptied again.

Next, 500 µL of prepared SEWS-M was added to the mixture, and the mixture was centrifuged at 14,000 rpm for 1 minute to desalt with ethanol and additional detergents, removing impurities by centrifuging through the SPIN Filter bucket while the purified DNA is still bound to the silica. The SPIN Filter was then centrifuged again at 9,000 rpm for 2 minutes without addition of any

liquid to “dry” the matrix of residual wash solution. The catch tube was discarded with a new, clean catch tube.

The SPIN Filter was air-dried for 5 minutes at room temperature to remove residual ethanol. The Binding Matrix (above the SPIN Filter) was gently resuspended in 100 μ L of DES (Dnase/Pyrogen-Free Water). The purified nucleic acids eluted from the silica with collapse of cation bridges because low salt elution solution rehydrates both the silica and the DNA. The mixture was then centrifuged at 14,000 rpm for 1 minute to bring eluted DNA into the clean catch tube. The SPIN Filter was discarded, and the DNA was ready for S16 metagenomics. The purified DNA was stored at -80°C until use. The method was obtained from <https://www.mpbio.com/uk>.

Appendix 2: List of standards and internal standards details.

Standards/ ISTD	CAS-number
Etizolam-D3	200-659-6
Fluoxetine-D6	200-659-6
Norfluoxetine-D6 oxalate	-
venlafaxine-d6	1062606-12-5
(±)-O-Desmethylvenlafaxine-D6	1062605-69-9
Diazepam	65854-76-4
Nordiazepam	65891-80-7
Oxazepam	65854-78-6
Temazepam	136765-51-0
Prazepam	2955-38-6
Nordiazepam	1088-11-5
α-Hydroxyetizolam	64546-10-7
Fluoxetine-D6	200-659-6
Fluoxetine-D5	1173147-79-9
Diazepam -D5	65854-76-4
Nordiazepam -D5	65891-80-7
Oxazepam-D5	65854-78-6
Temazepam-D5	136765-51-0
Fluoxetine hydrochloride	56296-78-7
Venlafaxine hydrochloride	99300-78-4
(1S)-(-)-Camphanic chloride for chiral derivatization, LiChropur™, ≥98.0%	39637-74-6
(S)-Norfluoxetine	126924-38-7
®-Norfluoxetine	130194-43-3
R-(-)-Fluoxetine hydrochloride	114247-09-5
S-(+)-Fluoxetine hydrochloride	114247-06-2
Norfluoxetine hydrochloride	57226-68-3
O-Desmethylvenlafaxine	93413-62-8
Diazepam solution	439-14-5
Oxazepam solution	604-75-1
Temazepam solution	200-659-6
Desmethyldiazepam	1088-11-5
Etizolam solution	40054-69-1
org 24598 lithium salt	722456-08-8

**Appendix 3: Articles, chapter, and posters published during the study.
(Not directly related to the thesis).**

A. Articles

- 1- Alawi, A. I., Dhabbah, A. M., Morrison, C., Ben-Jaber, S., AlAngari, W. A., Bin Jassas, M., & Badjah-Hadj-Ahmed, Y. (2022). Indirect chiral separation of crystal methamphetamine seized in Saudi Arabia using GC-MS. *Australian Journal of Forensic Sciences*, 1-14.
- 2- Dhabbah, A. M., Ben-Jaber, S. & Alawi, A. I. (2020) Development of Gold Nanocomposite Surfaces for Estimating Trace Concentrations of Amphetamine Compounds," - Research and Studies Center, King Fahd Security College, (Arabic edition).
- 3- Dhabbah, A. M., Badjah-Hadj-Ahmed, A. Y., Alawi, A. I., Al Angari, W. A., & Alrayes, B. F. (2018). Screening of psychoactive components in fresh khat using direct analysis in real time-time of flight-mass spectrometry. *The Saudi Journal of Forensic Medicine and Sciences*, 1(3), 45.

B. Book chapter

- 4- Calum Morrison, Ali I. Alawi (2023). 8.17 Chiral Gas Chromatography. In *Comprehensive Chirality* (pp. 333-353). Elsevier Ltd.
<https://doi.org/10.1016/B978-0-32-390644-9.00072-X>

C. Conference presentation

- 5- Ali I. Alawi, Calum Morrison and Charles Knapp, Unraveling Etizolam and Its Primary Metabolite Biodegradation: A Chiral LC-MS Analysis in Forensic and Environmental Research, the II. International Conference of Forensic Sciences, Ankara, Turkey, Nov 2023.

D. Posters

- 6- Ali I. Alawi, Calum Morrison, and Charles Knapp Method development and stability study of described drugs, School of Medicine, Dentistry and Nursing Postgraduate Research Day, 17 May 2021.
- 7- Ali I. Alawi, Calum Morrison, and Charles Knapp, Chiral separation of Fluoxetine and Venlafaxine and their main metabolites: an

optimization study, the 5th Scottish Students Forensic Research Symposium, Dundee, 17 Nov 2019.

E. Attendance

- 8- TIAFT 2022 VIRTUAL, The 58th Annual Meeting of the International Association of Forensic Toxicologists, Virtual conference, (2022).
- 9- Arab Toxicologists Association Special Interest Group (ATA-SIG) – Society of Toxicology (SOT) Virtual Annual Reception Meeting, (2021).
- 10-The Fifth International Conference of the Arab Society for Forensic Sciences and Forensic Medicine, Virtual conference, (2021).
- 11-The Forensics Middle East & Africa, Virtual event, (2020).
- 12-The 11th Scottish Symposium on Environmental Analytical Chemistry, University of Aberdeen, Royal Society of Chemistry, Analytical Division, Scottish Region, (2019).
- 13-The 10th Scottish Symposium on Environmental Analytical Chemistry, University of Glasgow, Royal Society of Chemistry, Analytical Division, Scottish Region, (2018).

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