ENANTIOMERIC DETERMINATION OF CHIRAL DRUGS IN ENVIRONMENTAL WATERS

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UNIVERSITAT ROVIRA i VIRGILI

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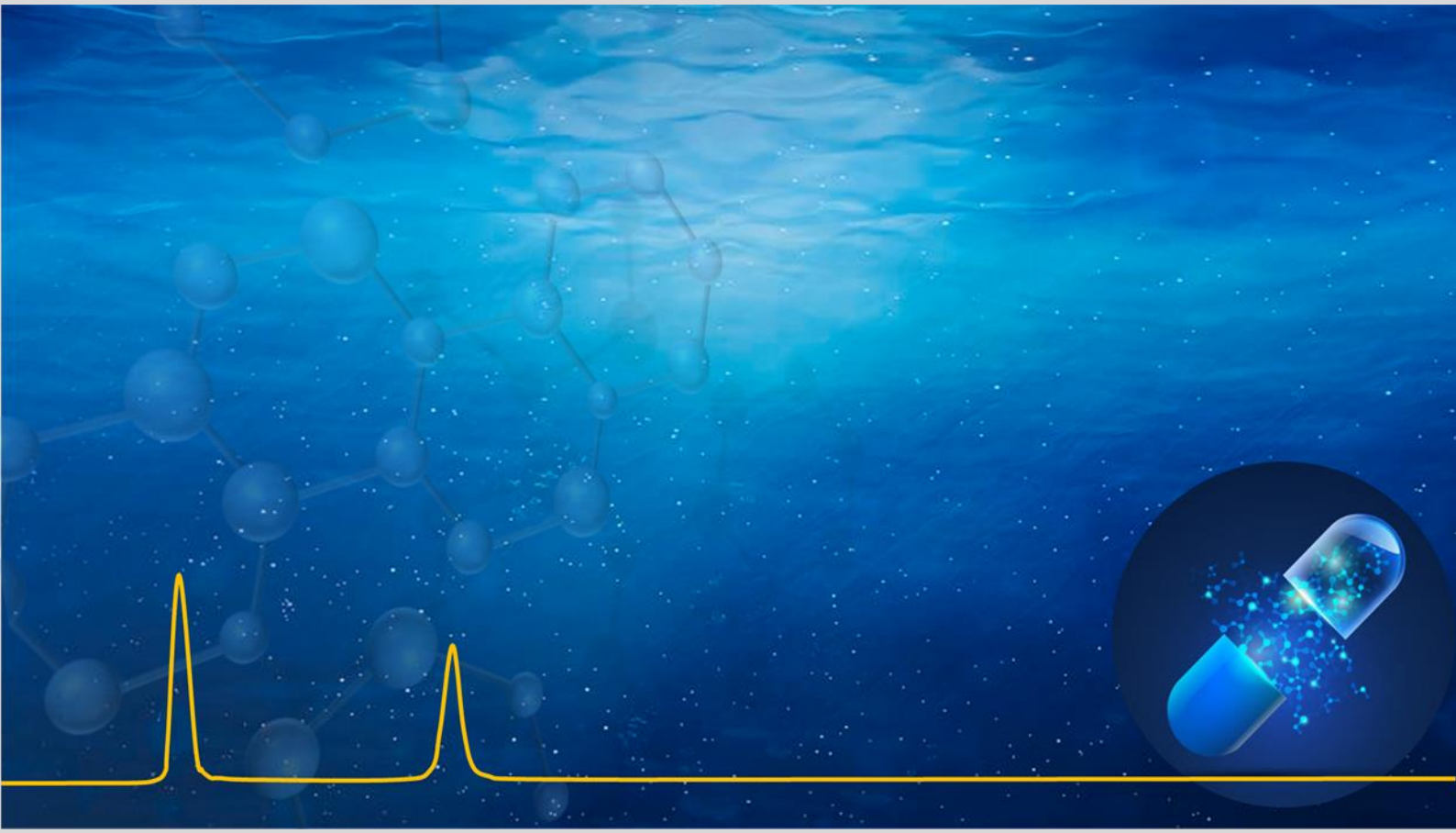

DOCTORAL THESIS
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Enantiomeric determination of chiral drugs in environmental waters

Yandi Fu

DOCTORAL THESIS

Supervised by

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Department of Analytical Chemistry and Organic Chemistry

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WE STATE that the present study, entitled "ENANTIOMERIC DETERMINATION OF CHIRAL DRUGS IN ENVIRONMENTAL WATERS", presented by YANDI FU for the award of the degree of Doctor, has been carried out under our supervision at the Department of Analytical Chemistry and Organic Chemistry of this university.

Tarragona, March 3rd 2022

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ABSTRACT

Chirality plays an important role not only in the pharmaceutical industry, but also in the environmental area. Growing evidence has demonstrated that the enantiomers of chiral drugs in the environment usually present stereoselectivity in their occurrence, fate and toxicity. Therefore, the enantiomeric determination of chiral drugs is of great importance for accurate environmental risk assessment. However, due to the complex matrices and very low concentrations of chiral drugs, their enantiomeric determination at environmental level is still a challenge. To date, solid-phase extraction (SPE) in combination with chiral liquid chromatography (LC) coupled to mass spectrometry (MS) are the techniques of choice for chiral environmental analysis owing to its sensitivity and wide scope of applicability.

Synthetic cathinones, the second largest group of new psychoactive substances, have gained popularity all over the world in recent years. All these drugs are chiral and are commercially available as racemates. Although their occurrence in environmental water has already been reported, there is still a lack of knowledge on their enantiomeric distribution in the environment mainly due to unavailability of sensitive and selective chiral analytical methods. In view of these facts, the present Doctoral Thesis focuses on the development and application of sensitive and selective analytical methods for the enantiomeric determination of cathinones in environmental water samples on the use of SPE followed by chiral LC coupled with high-resolution mass spectrometry (HRMS).

For this purpose, the first section focuses on the development and optimization of enantioseparation methods for these chiral drugs. In addition to cathinones, some β-blockers and omeprazole were also included as analytes to better understand the chiral recognition mechanisms involved and the effect of different chromatographic conditions on chiral separation and retention. Different chiral stationary phases (CSPs), including commercial polysaccharide-based and protein-based CSPs and in-house prepared polymeric CSPs, were evaluated for their chiral recognition abilities in different elution modes (normal phase, reversed phase and polar organic phase). The factors that affected the enantioseparation were tested and optimized to achieve successful enantioseparations.

In the second section, the optimized enantioseparation method was coupled with HRMS for the enantiomeric determination of five cathinones in environmental water samples. To improve the selectivity of the methods, different SPE sorbents – two commercial mixed-mode cation-exchange sorbents (Oasis WCX and Oasis MCX) and a series of in-house prepared sorbents based on molecularly imprinted polymers (MIPs) – were assessed for the extraction of target cathinones. Different SPE parameters were carefully optimized to achieve high recoveries and selectivity for the analytes. Both Oasis MCX and MIP sorbents

offered high selectivity for the extraction of cathinones thanks to their ability to establish the specific interaction with the analytes. The developed methods based on SPE followed by chiral LC-HRMS were successfully applied for the enantiomeric determination of the target cathinones in river water and effluent wastewater samples.

CHAPTER 1. INTRODUCTION

1.1. Chiral Drugs in the Environment

Drugs including legally used pharmaceuticals and illicit drugs are a group of emerging environmental contaminants as they are potentially hazardous to environmental organism and even human health [1]. Over the decades, their consumption has been constantly increasing worldwide, due to changes in clinical practice and rise in age-related and chronic diseases and mental illness [2]. For example, the consumption of anti-depressant drugs and cholesterol-lowering agents increased two and three times in Economic Cooperation and Development (OECD) countries between 2000 and 2017, respectively [2]. Obviously, as the drug consumption increases, the discharge of drugs and their metabolites into the environment also increases, and consequently the occurrence of these compounds in the environment has been of global concern. As a result, research on the occurrence, fate, and effects of drugs in the environment has increased dramatically over the past two decades.

Various drugs and their metabolites have been widely detected in aquatic environment all over the world. A global review of pharmaceuticals in the environment, commissioned by Germany's environment ministry, found that 631 out of 713 pharmaceuticals and transformation products measured were positively detected in the environment of 71 countries covering all continents [3]. A nationwide investigation of the occurrence of 45 pharmaceutically active compounds (PhACs) in 217 surface water samples across China was conducted, and the targeted PhACs were prevalent in all sampled streams [4]. Another monitoring study [5] investigated the occurrence of 111 PhACs in the Danube River and the abstraction wells operating along its banks. 52 of 111 PhACs were detected in the river samples and 32 of the targeted compounds were detected in the well water samples. The concentration levels of drugs detected in the environment vary depending on the country, region, area consumption pattern and manufacturing industry locations [1,3]. For example, ibuprofen and diclofenac were detected in river water in Spain at 0.3 and 0.1μg/L, respectively [6], while maximum concentrations detected in river water in South Africa for ibuprofen and diclofenac were 19.2 and 9.7 μg/L, respectively [7]. Generally, drug residues are present in the environment at concentrations below those that can cause acute toxicity to environmental organisms. However, these environmental contaminants can cause long-term effects on the organisms in environment, as they exhibit the ability to persist in nature and bioaccumulate in living organisms [1]. Various adverse impacts that drugs at environmentally relevant concentrations can have on organisms has been reported, such as causing the development of antibiotic resistance of microorganisms [8], changes in sex ratios of invertebrates [9], inhibition or stimulation of growth in aquatic plant [10], and the effects on the reproduction and predator avoidance behavior of fish, reptiles and invertebrates [11].

Although the studies concerning this emergent class of environmental contaminants have increased exponentially over the last decades, the exact environmental impacts of the drugs are still not completely understood at present. A fact often overlooked is that over 50% of

drugs are chiral and stereoselectivity plays an important role in the environmental risk assessment of chiral drugs. It is well known that the enantiomers of a chiral drug can exhibit significantly different activities in biological systems, and thus each enantiomer is assessed individually during drug design and development. However, such differences are often ignored in the environmental risk assessments of chiral drugs, which might lead to an over- or under-estimation of their ecotoxicity [12]. Thus, investigations on the enantiospecific occurrence, fate and toxicity are crucial to achieve accurate ecological risk assessments for chiral drugs.

The following sections give an overview of chirality and chiral drugs and discuss the enantiomeric occurrence, toxicity, source and fate of chiral drugs in the environment addressing the significance of enantiomeric determination of chiral drugs in the environment.

1.1.1. Overview of chirality and chiral drugs

The term chirality is used to describe the geometric property of a molecule which is non-superimposable with its mirror image. The non-superimposable mirror image isomers are called enantiomers. The enantiomers of a compound have different configurations: they have the same bond connectivities but differ in three-dimensional structure due to different arrangements of the bonds in space. The chirality of a molecule can be attributed to the existence of chiral axis, chiral plane or a helix. Among them, the presence of a chiral center usually generated by a carbon atom with four different substituents is the most common cause of chirality in molecules, e.g. lactic acid (Figure 1). Except carbon atom, nitrogen, phosphorus and sulfur can sometimes form chiral molecules such as methaqualone, cyclophosphamide and omeprazole, respectively.

Two enantiomers have identical physical properties except optical activity in achiral environment, i.e. they have equal but opposite optical rotations. For this reason, the enantiomers are also called optical isomers. The enantiomer rotating plane-polarized light in a right (clockwise) direction is the dextrorotatory (D-isomer) and is given a plus sign (+); whereas that rotating plane-polarized light in a left (counterclockwise) direction is the levorotatory (L-isomer) and is given a minus sign (−). An equal mixture of two enantiomers is called a racemic mixture or racemate with sign (±) or (D, L), which is optically inactive.

In order to specify the spatial arrangement of the substituents at a chiral center, the Cahn-Ingold-Prelog sequence rules are followed to determine if an enantiomer has R (rectus) or S (sinister) configuration by assigning priorities of substituent groups. A priority sequence 1 > 2 > 3 > 4 is assigned to the four substituents according to the atomic number of each atom attached to the asymmetric carbon, with higher atomic number acquiring higher priority. When two identical atoms are attached, their substituents are then inspected in turn, until a substituent of higher atomic number is found. When deciding the ranking order, the group

of lowest priority is placed pointing away from the viewer as shown in Figure 1. Then start counting the remaining three substituents from the highest priority to the lowest one. If the counting follows a clockwise direction, the configuration is designated as R; otherwise in a counter clockwise direction, it is S. It should be noted that the R/S system has no fixed relation to the D/L system. Optical activity of R and S enantiomers has to be measured experimentally with a polarimeter.

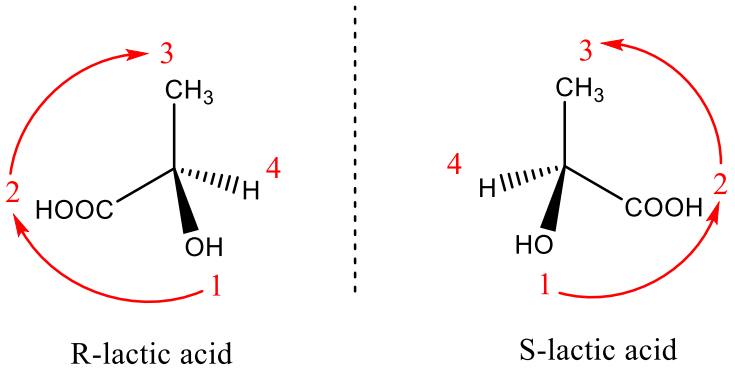

Figure 1. The enantiomers of lactic acid.

Chirality plays a key role in the drug development. As mentioned, most of drugs currently on the market are chiral. The enantiomers of chiral drugs often exhibit differences in biological activities such as pharmacology, toxicology and pharmacokinetics. This results from the fact that enantiomers may stereoselectively interact with chiral molecules such as proteins, enzymes and amino acids in a living organism. This can be easily explained with the example of a drug-receptor model depicted in Figure 2. For chiral drug molecules, in most cases, only one enantiomer (eutomer) is pharmacologically active, and the other enantiomer (distomer) is often not effective or can be harmful. By way of example, the analgesic naproxen is prescribed as S-enantiomer, because the R-enantiomer is suspected to be toxic to liver [13]; S-(−)-propranolol is 100 times more active than its R-(+)-enantiomer in blocking beta adrenergic receptors [14]. From 1992 onwards, the United States Food and Drug Administration (FDA), European Committee for Proprietary Medicinal Products and other drug regulatory authorities have issued guidelines for the development of new chiral drugs [15–18]. These guidelines recommended to evaluate the therapeutic activity of each enantiomer in the various stages of chiral drug development and if possible, market the eutomer instead of the racemate. Consequently, market approval of enantiopure drugs has increased steadily whereas it has significantly declined for racemic drugs [19]. In spite of this, a large number of chiral drugs are still marketed as racemic form.

When administered as a racemate, two enantiomers often exhibit differences in their pharmacokinetic processes involving absorption, distribution, metabolism and excretion, as outlined for pharmacology and toxicology. Especially in metabolism, high degree of stereoselectivity may be observed due to the involvement of the enzymatic system [20]. For example, albuterol, the most widely used β2-adrenoceptor agonist in the treatment of bronchial asthma, is administered in racemic form. It has been reported that R-(−)-albuterol is preferentially metabolized (10 times) compared to S-(+)-albuterol leading to enrichment of excreted albuterol with S-(+)-enantiomer [21]. In addition, some chiral drugs can undergo chiral inversion in humans, i.e. one enantiomer converts into its antipode. The typical examples are nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, ketoprofen and flurbiprofen, in which cases unidirectional chiral inversion of inactive R-enantiomer to active S-enantiomer takes place [22]. As a result of the stereoselective metabolism and chiral inversion occurring in humans, the enantiomeric composition of chiral drugs can change significantly after their administration.

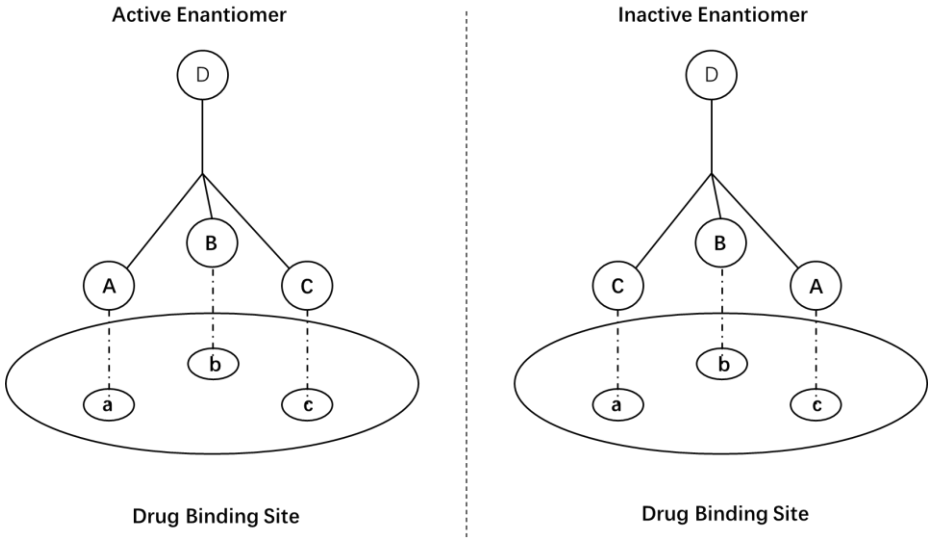

Figure 2. Stereoselectivity of enantiomer-receptor binding.

In chiral analysis, the enantiomeric fraction (EF), as defined in Equation 1, is commonly used to depict the enantiomeric composition of chiral drugs.

$$\mathrm{EF} = \frac{\mathrm{E1}}{\mathrm{E1} + \mathrm{E2}} \tag{1}$$

where E1 and E2 are the concentrations of first and second enantiomer of chiral drugs, respectively. EF equals 1 or 0 in the case of single enantiomer form and 0.5 in the case of racemate.

1.1.2. Source, fate and toxicity of chiral drugs in the environment

Various chiral drugs and their residues have been found in environment such as in rivers, lakes, groundwater, sludge and soil. β-Blockers, NSAIDs, antidepressants and chiral illicit drugs are the most commonly studied chiral drugs. In a monitoring study of 18 chiral pharmaceuticals and their active metabolites (belonging to several therapeutic classes including NSAIDs, antidepressants, antibiotics and β-blockers) in the Guadalquivir River (South Spain), 14 out of 18 chiral pharmaceuticals were detected in analyzed water samples [23]. Among them, NSAIDs were found to be the most abundant compounds with the maximum concentration up to 768 ng L^{-1} (in the case of ibuprofen). High concentration of NSAIDs were also detected in China [24], in United Kingdom [25,26], in Australia [27] and in Japan [28]. NSAIDs were generally found to be enriched with S-(+)-enantiomer in the environment. For example, an obvious excess of S-(+)-ibuprofen was observed in river waters (EF = 0.69−0.71) in China [24], which could be mainly due to the chiral inversion of R-(−)-ibuprofen to S-(+)-ibuprofen *in vivo* leading to enrichment of excreted ibuprofen with S-(+)-enantiomer. In the case of naproxen, the EF values were found to be close to 1.0 [24], since it is administrated in a pure S-(+)-enantiomer form.

β-Blockers such as atenolol, propranolol and metoprolol have also been widely detected in the environment at concentrations ranging from ng/L to μg/L with considerably varying EF values. For example, a significant enrichment of atenolol with S(−)-enantiomer was observed in Guadalquivir River [23]. In contrast, Evans et al. [29] reported that atenolol was present in river water in UK as racemic mixtures. A similar situation was observed for antidepressants such as venlafaxine and fluoxetine. For example, studies on enantiomeric occurrence of fluoxetine in the environment conducted in UK, Spain and China showed that the EF value of fluoxetine varied from 0.51 to 0.88 [23,29–31].

As in human bodies, these chiral drugs can also be enantiomer-specific towards environmental organisms. For example, S-(−)-atenolol shows higher toxicity to *D. magna* than R(+)-atenolol, while R(+)-atenolol is more toxic to protozoan *T. thermophila* than the S(−)-enantiomer [32]; R-(−)-fluoxetine is 30 times more toxic to the *T. thermophila* than S-(+) fluoxetine [32] whereas S-(+)-fluoxetine is 9.4 times more toxic to the fish *P. promelas* than R-(−)-fluoxetine [33]. However, the role of enantiomeric occurrence and toxicity of chiral drugs is often overlooked in their environmental risk assessment, which might lead to under- or over-estimation of toxicity of these chiral contaminants. Therefore, investigating the enantiomeric composition of chiral drugs in the environment is helpful to improve the accuracy in their risk assessment.

Chiral drugs have multiple pathway to enter the environment. Urban domestic wastewater is the major emission source of chiral drugs in the environment [1]. Most

ingested drugs are excreted from the human body primarily via the faeces and urine together with their metabolites, discharged into domestic waste waters and then released into the environment after passage of a wastewater treatment plant (WWTP) where most of these compounds are not completely removed. Hospital effluents and drug manufacturing wastewater also contribute to the distribution of chiral drugs in the environment [34].

During wastewater treatment process in WWTPs, these chiral drugs and their metabolites are subjected biotic and abiotic processes. Whereas abiotic processes such as sorption, photo-degradation and volatilization are generally non-enantioselective, biotic processes such as microbial degradation and metabolism may lead to stereoselective enrichment or depletion of enantiomers of chiral drugs [19]. For example, Bagnall et al. [35] studied the stereoselective degradation of amphetamine and methamphetamine in river microcosm bioreactors in both biotic and abiotic conditions and demonstrated that stereoselectivity and changes in enantiomeric composition were only observed under biotic conditions. Many studies have reported the stereoselective degradation of chiral dugs in the wastewater. For example, López-Serna et al. [23] reported that atenolol was present in racemic form in influent wastewater and the enrichment of S(−)-enantiomer took place during wastewater treatment. In contrast, an enrichment of atenolol with R(+)-enantiomer was reported by Kasprzyk-Hordern et al. [36] during wastewater treatment. The varied stereoselective removal of chiral drugs may be attributed to differences in the type of wastewater treatment technology used in the WWTPs.

It should be noted that the changes in enantiomeric composition of chiral drugs during wastewater treatment may be caused not just by differences in the rate of degradation of enantiomers but also by the chiral inversion of enantiomers. Several studies have demonstrated that certain drugs may undergo chiral inversion in the wastewater [25,27,37]. For example, Camacho-Muñoz et al. [25] reported that only S-(+)-naproxen was detected in the influent of one WWTP of the study (EF=1). However, EF values of naproxen decreased from 1 to 0.89 after wastewater treatment suggesting that S-(+)-naproxen underwent chiral inversion to R-(−)-naproxen during the treatment process. In another study [38], bidirectional but uneven inversion of the profens was confirmed by investigating transformation of single enantiomers of profens in enzymatic membrane bioreactors used for advanced wastewater treatment. For example, 14% of R-(−)-naproxen in the influent was inverted to the S-(+)-naproxen whereas 4% of S-(+)-naproxen was inverted to R-(−)-naproxen. The role of chiral inversion is of critical importance in understanding the fate of chiral drugs in the environment and needs to be studied further.

Once these chiral drugs and their metabolites have been released into the environment from WWTPs, their enantiomeric composition can be further altered as a result of biological degradation processes in the environment. In a study monitoring EF variation of venlafaxine

along a 30 km river stretch, venlafaxine was found to be racemic at the first sampling site, while EF increased to 0.74 at the second sampling site [39]. The changes in the EF of venlafaxine indicating that biodegradation occurred in the river. On the other hand, seasonal variations in enantiomeric composition of chiral drugs in the environment were reported in previous studies [36,40,41]. In a monitoring program undertaken in river water over the period of 9 months, the lowest EF value (EF of 0.40) was recorded for venlafaxine in the December sampling campaign, while the highest EF (0.65) was recorded in the August sampling campaign [36]. The significant temporal variations of EF values observed in the river water might be due to changes in diversity and activity of microorganisms over time.

As a result of stereoselective degradation during human metabolism and also during biological degradation in the wastewater treatment process and in the surface water, chiral drugs are rarely present in the environment with the same enantiomeric composition as their original form. Therefore, enantiomeric profiling of chiral drugs in environment can distinguish between the residues of consumed drugs and unused drugs that have been directly disposed of [42,43].

Comprehensive understanding of the fate of chiral drugs in the environment is still far from complete. Due to the diversity of wastewater treatment, complex environment conditions and variations in nature of chiral drugs, it would not be possible to predict enantiospecific transformations. Enantiomeric determination of chiral drugs in the environment can provide valuable information about their environmental transport and fate.

1.2. Determination of chiral compounds

As discussed above, enantiomers of chiral drugs have to be treated as independent entities. Thus, the development of methods for chiral separation (also called "enantioseparation") is fundamental for drug development as well as environmental and biological analysis. Owing to enantiomers exhibiting identical physical and chemical properties in achiral environments, chiral separations remain challenging and require specialized separation techniques. With regard to analytical chiral separations, the most used technologies include liquid chromatography (LC), gas chromatography (GC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE). Other technologies such as capillary electrochromatography (CEC) and thin layer chromatography (TLC) have also been used to a lesser extent. The advancement of various techniques has led to the constant development in the field of chiral separation.

Chiral separations can generally be categorized into indirect and direct methods. In the indirect methods, enantiomers are converted into diastereomers by derivatization with an enantiomerically pure chiral derivatization reagent, and then separated on achiral phases. This method can be applied only if the enantiomers possess easily derivatizable functional groups such as hydroxyl, amine, carboxylic and carbonyl groups. The frequently used chiral derivatization reagents include cyanuric chlorides, Marfey's reagent, isothiocyanates and naproxen based reagents [44]. The indirect methods are less used now since chiral derivatization is usually time-consuming and laborious and may cause racemization. The direct methods utilize chiral stationary phases (CSPs) or chiral mobile phase additives (CMPAs) to form diastereomeric complexes with analytes. CSPs consist of a chiral selector either covalently bonded or physically adsorbed onto a solid support. In the CMPA approach, the chiral selector is added to the mobile phase and an achiral stationary phase is used. The CMPA approach is an expensive and complex method because it requires a large amount of chiral selectors, which are not recovered and can interfere with the detection mode. Compared with CMPA approach, direct chiral separations using CSPs are more convenient and popular due to its inherent advantages such as simplicity, high reproducibility and flexibility during the analysis of chiral drugs in complex matrices. The discussion of this approach is therefore the focus of the following sections.

The chiral recognition mechanism during the direct enantioseparations is usually explained by the "three-point interaction model" proposed by Easson and Stedman [45]. The key points in the model are that a minimum of three simultaneous interactions between the chiral selector and the enantiomer are required and at least one of them must be stereoselective to form diastereomeric complexes, and thereby enable chiral separation. Any intermolecular interaction, such as hydrogen bonding, hydrophobic interaction, π-π interaction, dipole−dipole interaction, inclusion complexing, steric interaction and ionic interaction, may participate in the chiral recognition process.

The following sections provide an overview and comparison of the main chiral drug enantioseparation techniques including LC, GC, SFC and CE. The advances and limitations of these methods are also discussed.

1.2.1. Liquid chromatography

LC is by far the dominant technique used for the enantioseparation of chiral drugs, which is associated with the numerous combinations of available CSPs and mobile phases modes including normal-phase (NP), reversed-phase (RP) and polar-organic (PO) mode. In addition, the availability of coupling with mass spectrometry (MS) detector broads the applicability of LC to various chiral analytes in the different matrices.

1.2.1.1. Major classes of modern chiral stationary phases

Numerous CSPs have been developed for chiral LC separation. Based on the chiral selectors employed, these CSPs can be mainly divided in: Pirkle-type CSPs, polysaccharide-based CSPs, cyclodextrin-based CSPs, macrocyclic antibiotic-based CSPs, ligand exchange CSPs, protein CSPs and chiral crown ethers. The commonly commercialized CSPs are listed in Table 1. There is no single CSP universal for all the enantioseparations. The proper selection of CSP is key to achieve effective enantioseparations for the given chiral drugs. Although many different types of CSPs are described, the development of new, high efficiency CSPs continues to be of great importance in this field.

Table 1. Commonly commercialized CSPs used in LC.

	Polysaccharide-based CSPs	Trade name	Manufacturer
Pirkle-type	1-(3,5-Dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene	Whelk-O1	Regis, USA
	1-(3,5-Dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene	Whelk-O2	Regis, USA
	N-(3,5-Dintrobenzoyl)-1,2-diphenyl-1,2-diaminoethane	ULMO	Regis, USA
	N-(3,5-Dinitrobenzoyl)-leucine amide	DNB-Leu	Regis, USA
	N-(3,5-Dinitrobenzoyl)-phenylgylcine amide	DNB-PG	Regis, USA
	Dimethyl N-3,5-dinitro-benzoyl-amino-2,2-dimethyl-4-pentenyl phosphonate	α-Burke 2	Regis, USA

Table 1. (Continued)

	Polysaccharide-based CSPs	Trade name	Manufacturer
	3-(3,5-Dinitrobenzoyl)-amino-(R)-3-phenyl-(R)-2-tBu-propanoic acid undecyl ester	β-Gem 1	Regis, USA
	N-Dinitrobenzoyl-(R)-1-naphthylglycine	Sumichiral OA 2500	Sumika, Japan
	3,5-Dinitrophenylaminocarbonyl-(S)-valine	Sumichiral OA-3100	Sumika, Japan
Cyclodextrin-based	β-Cyclodextrin	Cyclobond I 2000	Astec, USA
	Acetyl-β-cyclodextrin	Cyclobond I 2000 AC	Astec, USA
	2,3-Dimethyl-β-cyclodextrin	Cyclobond I 2000 DM	Astec, USA
	Hydroxypropyl-β-cyclodextrin	Cyclobond I 2000 RSP	Astec, USA
	γ-Cyclodextrin	Cyclobond II	Astec, USA
	Acetyl-γ-cyclodextrin	Cyclobond II AC	Astec, USA
Macrocyclic antibiotic-based	Vancomycin	Chirobiotic V	Astec, USA
	Vancomycin	Chirobiotic V2	Astec, USA
	Ristocetin A	Chirobiotic R	Astec, USA
	Teicoplanin	Chirobiotic T	Astec, USA
	Teicoplanin aglycone	Chirobiotic TAG	Astec, USA
Ligand-exchange	(L)-Proline	Chiralpak WH	Daicel, Japan
	N,N-Dioctyl-(D)-alanine	Chiralpak MA(+)	Daicel, Japan
	(L)-Hydroxy-proline	Chiral-Si 100 L-ProCu Chiral-Si 100 L-ValCu	Serva, Germany Serva, Germany
	(L)-Chiral bidentate ligand	CLC-L	Astec, USA
	(D)-Chiral bidentate ligand	CLC-D	Astec, USA
	(L)-Hydroxyproline	Nucleosil Chiral-1	Macherey Nagel, Germany

Table 1. (Continued)

	Polysaccharide-based CSPs	Trade name	Manufacturer
Polysaccharide-based	Cellulose tris(3,5-dimethylphenylcarbamate)	Chiralcel OD	Daicel, Japan
	Cellulose tris(3,5-dimethylphenylcarbamate)	Chiralpak IB	Daicel, Japan
	Cellulose tris(3,5-dimethylphenylcarbamate)	Lux Cellulose-1	Phenomenex, USA
	Cellulose tris(3-chloro-4-methylphenylcarbamate)	Chiralcel OZ	Daicel, Japan
	Cellulose tris(3-chloro-4-methylphenylcarbamate)	Lux Cellulose-2	Phenomenex, USA
	Cellulose tris(4-methylbenzoate)	Chiralcel OJ	Daicel, Japan
	Cellulose tris(3,5-dichlorophenylcarbamate)	Chiralpak IC	Daicel, Japan
	Amylose tris(3,5-dimethylphenylcarbamate)	Chiralpak AD	Daicel, Japan
	Amylose tris(3,5-dimethylphenylcarbamate)	Chiralpak IA	Daicel, Japan
	Amylose tris(3,5-dimethylphenylcarbamate)	Lux Amylose-1	Phenomenex, USA
	Amylose tris(3-chlorophenylcarbamate)	Chiralpak ID	Daicel, Japan
Protein-based	Human serum albumin	Chiralpak HSA	Daicel, Japan
	α1-Acid glycoprotein	Chiralpak AGP	Daicel, Japan
	Cellobiohydrolase	Chiralpak CBH	Daicel, Japan
Chiral crown ether	(+) (18-Crown-6)-2,3,11,12-tetracarboxylic acid	ChiroSil RCA (+)	RStech Corp., Daejeon, Korea
	(+) (18-Crown-6)-2,3,11,12-tetracarboxylic acid	Opticrown RCA (+)	US Mac Corporation, USA
	(S)-18-Crown-6-ether	Crownpak CR (+)	Daicel, Japan
	(R)-18-Crown-6-ether	Crownpak CR (−)	Daicel, Japan
	(S,S)-Pseudo-18-crown-6-ether	Sumichiral OA-8000	Sumika, Japan

Pirkle-type CSPs (also known as Brush CSPs) are characterized by low molecular mass selectors covalently bonded on the silica surface forming an ordered layer. These chiral selectors usually possess π-donor and/or π-acceptor aromatic groups that are capable of forming π-π interactions, hydrogen bonding, dipole-dipole interactions and steric interactions with the enantiomers [46]. This class of CSP has a broad applicability for the enantioseparation of analytes with electron-deficient or electron-rich aromatic moieties [47]. It should be mentioned that the elution order of enantiomers on Pirkle-type CSPs can be reversed by using the same type of CSP but with the opposite absolute configuration. Chemical and thermal inertness makes Pirkle-type CSPs compatible with all commonly used mobile phase [48]. However, donor–acceptor interactions are more favored in nonpolar mobile phase, and thus Pirkle-type CSPs are most often operated in NP mode. Several Pirkle-type CSPs are commercially available such as Whelk-O1, Whelk-O2, ULMO, DNB-Leu, DNB-PG, α-Burke 2, and β-Gem 1 (Table 1). Among them, Whelk-O1 containing 1-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene as chiral selector is the most applied CSP within this group. For example, Perera et al. [49] reported enantioseparation of mephedrone and related cathinones on a Whelk-O1 column in NP mode. In another study, a Whelk-O1 column was used in NP mode for the enantiomeric determination of haloxyfop in soils [50]. In the last decade, various sub-2 μm Pirkle-type CSPs have been developed [51,52]. Compared with conventional columns packed with 5 μm particles, sub-2 μm Pirkle-type CSPs offer the advantages of high efficiency, reduced analysis time, and lower solvent consumption, enabling their application in rapid high-throughput chiral analyses [51,53]. For example, Kotoni et al. [54] prepared a series of 1.7 μm Whelk-O1 columns with different dimensions (lengths of 50, 75, 100, and 150 mm and internal diameters I.D. of 3.0 or 4.6 mm). Their efficiency and enantioselectivity toward 21 racemates were studied and compared with a conventional 5 μm Whelk-O1 column, and the results showed that significantly higher efficiency with shorter analysis time was achieved on 1.7 μm Whelk-O1 columns.

Cyclodextrins are cyclic oligosaccharides composed of six (α-cyclodextrin), seven (β-cyclodextrin) or eight (γ-cyclodextrin) glucopyranose units with the shape of a hollow truncated cone, as shown in Figure 3. The secondary 2- and 3- hydroxyl groups located on the wider rim of the torus and the primary 6-hydroxyl groups located on the narrow rim make the outer surface of the cyclodextrins hydrophilic, while the glucose oxygens and methylene hydrogens make the inner cavity hydrophobic [47]. The chiral recognition mechanism of cyclodextrin is based on inclusion complexation of lipophilic parts of the analytes into the cavity driven by hydrophobic interactions. The hydrogen bonding and dipole–dipole interactions occurring between the polar groups of the analytes and the hydroxyl groups at the outer rims of the CSP also contribute to the chiral recognition [55]. In addition, the hydroxyl groups can be derivatized to construct π-π interactions, ionic interactions and steric

interactions resulting in the broad applicability of the cyclodextrin CSPs [46]. To date, a large variety of cyclodextrin-based CSPs have been commercialized, as shown in Table 1. These columns are capable of working in all elution modes (NP, RP and PO mode), which further increases their applicability. For example, Han et al. [56] assessed the enantioselectivity of a series of cyclodextrin-based CSPs including Cyclobond I 2000, Cyclobond RSP, Cyclobond DM, Cyclobond AC, Cyclobond DMP and Cyclobond SN for 30 chiral furan derivatives in three elution modes, and successful enantioseparations were achieved for 28 of 30 analytes in at least one elution mode. In another study, a Cyclobond I 2000 column was used in RP mode for the enantioseparations of nine amphetamine derivatives, methorphan and propoxyphene [57].

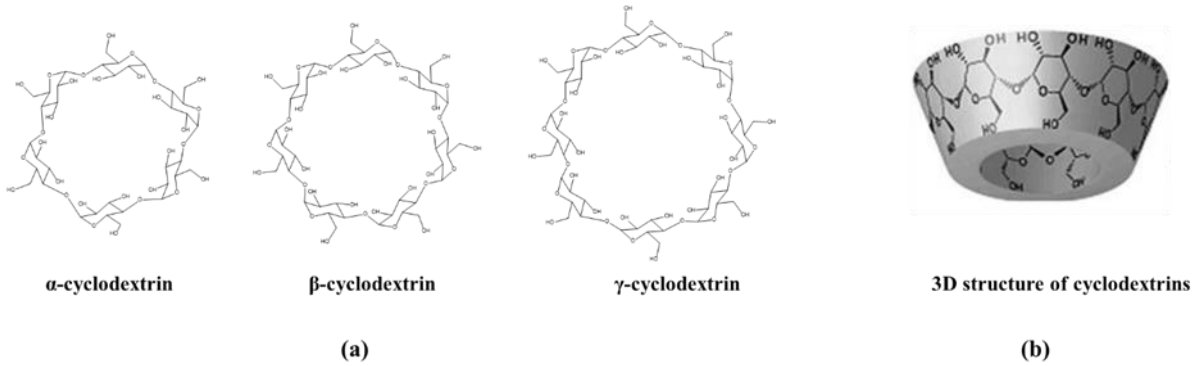

Figure 3. Structures of: (a) α, β and γ-cyclodextrin, (b) cyclodextrins in 3D. Reproduced from [58].

Macrocyclic antibiotic-based CSPs were first introduced by Armstrong and co-workers in 1994 [59]. They possess a wide variety of functional groups such as amine, aromatic, hydroxyl and carboxyl groups, which allow multiple interactions with enantiomers, such as hydrogen and hydrophobic bonding, dipole-dipole, π-π, ionic and steric interactions [60]. This type of CSPs has good stability and capacity and is capable of working in all three elution modes. Due to these properties, macrocyclic antibiotic-based CSPs are widely applied in enantioseparation for various chiral analytes [55,60]. Currently, the most common macrocyclic antibiotic-based CSPs include vancomycin, ristocetin A, teicoplanin and teicoplanin aglycone, which are marketed under the trade name Chirobiotic V, Chirobiotic R, Chirobiotic T and Chirobiotic TAG by Astec, respectively. For example, to study the stereoselective pharmacokinetics of trantinterol in human plasma, a Chirobiotic V column was used in PO mode for the enantioseparation of trantinterol [61]. A Chirobiotic T column was applied for enantioseparation of six quinolones, and the mechanism of chiral recognition was evaluated [62]. In a study on assessing the enantiomeric biodegradation of ofloxacin and levofloxacin in laboratory-scale microcosms, a comparison of Chirobiotic V, Chirobiotic T, Chirobiotic TAG and Chirobiotic R columns were performed in RP mode for the separation

of ofloxacin enantiomers [63]. The results indicated that the best enantioseparation was achieved on Chirobiotic R column.

In the ligand-exchange CSPs, a cation exchanger that carries ions of a transition metal such as copper ion is immobilized on silica supports. The analytes which possess electron-donating functional groups enter the coordination sphere of the metal ion, forming a reversible diastereomeric ternary metal-ion/chiral selector/analytes enantiomers complex [64]. The difference in stability between these diastereomeric complexes results in the enantioseparation. Ligand-exchange CSPs are therefore limited to the enantioseparation of analytes containing two or three electron-donating functional groups, such as hydroxyl, amino and carboxylic functionalities [47]. For this reason, the number of applications with these selectors still remains limited [65]. Examples of commercial these columns, such as Chiralpak WH, Chiralpak MA(+), Chiral-Si 100 L-ProCu, Chiral-Si 100 L-ValCu, CLC-L and CLC-D (shown in Table 1), are most often used in RP mode. For example, in a study on the enantiomeric determination of lactic acid in Ringer-lactate solution, a Astec CLC-D chiral bidentate ligand column was used for the enantioseparation with a mobile phase of 7 mM copper(II) sulfate in 1.0 mM acetic acid (HAc) aqueous solution containing 4% methanol (MeOH) [66]. The enantioseparation of N-arylated histidines was performed on a Chiralpak WH column in RP mode with the mobile phase composed of 0.5 mM copper(II) sulfate aqueous solution and 95–5% 2-propanol in gradient mode [67].

Polysaccharide derivatives, including cellulose derivatives and amylose derivatives, are the most popular CSPs employed in both analytical and preparative separations due to their wide applicability, durability and high loading capacity. Cellulose and amylose are linear polymers with D-glucose units linked via β-1,4'-glycosidic and α-1,4'-glycosidic linkages respectively, as shown in Figure 4. Underivatized cellulose and amylose have low-resolution capacities. The introduction of various substituents on the three hydroxyl groups of each glucose unit can significantly enhance their enantioselectivity. Table 1 shows the commonly used polysaccharide-based CSPs along with their tradenames. As these polymers contain a large number of chiral active sites, the chiral recognition mechanisms of polysaccharide-based CSPs are quite complex and are not yet fully understood [68]. The generally accepted recognition mechanism of the polysaccharide-based CSPs is mainly based on the inclusion of analytes into the chiral cavities of the CSPs driven by additional attractive interactions such as hydrogen bonding, dipole–dipole and π–π interactions [48]. Such interactions are favored in NP mode since nonpolar alkanes, the main component of mobile phase, should not compete with analytes for these interaction sites on CSPs. Thus, enantioseparations on polysaccharide-based CSPs are commonly explored in the NP mode. However, these CSPs can also achieve effective enantioseparations in RP and PO mode using the appropriate mobile phases, which greatly expands the applicability of the polysaccharide-based CSPs. For example, an amylose-based column, Chiralpak IA, was used for enantioseparations of

four proton pump inhibitors (pantoprazole, omeprazole, lansoprazole and rabeprazole) in three elution modes: NP, RP and PO mode [69]. The results showed that the Chiralpak IA column exhibited good enantioselectivity in the three modes, with all analytes successfully resolved. As another example, successful enantioseparations of eight imidazole antifungal drugs (miconazole, econazole, isoconazole, sulconazole, butoconazole, fenticonazole, sertaconazole, and ketoconazole) were achieved on a cellulose-based column, Chiralpak IC, in the three mobile phase modes [70].

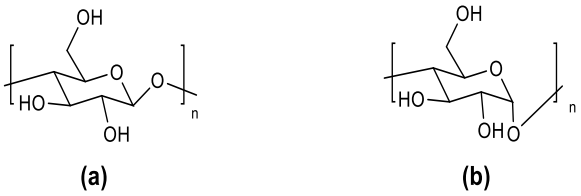

Figure 4. Structures of cellulose (a), amylose (b).

It is well known that proteins are capable of stereoselective interaction with chiral compounds in nature. Therefore, proteins have attracted attention as chiral selectors in separation sciences and a variety of protein CSPs have been developed. The most widely used protein-based CSPs are based on human serum albumin (HSA), α1-acid glycoprotein (AGP) and cellobiohydrolase (CBH) [48]. Such columns are commercially available under the trade name Chiralpak HSA, Chiralpak AGP and Chiralpak CBH (Daicel, Japan), respectively. Similar to polysaccharides, a single protein may contain a variety of chiral centers and different binding sites. The chiral recognition on the protein CSPs is capable of complex mechanisms involving ion-exchange, hydrogen bonding, and hydrophobic interactions [71]. The major disadvantage of this kind CSPs is the limited chemical stabilities making that they are used exclusively in RP mode with restrictions in organic modifier content and pH value of the mobile phase. For example, Jiang et al. [72] reported a LC–MS method for the enantiomeric determination of alprenolol and its active metabolite diacetolol in human plasma using a chiral CBH column in RP mode with a mobile phase of 10 mM ammonium acetate (NH_4Ac) aqueous buffer (pH 5.0)/acetonitrile (ACN) (85/15, v/v). Recently, in a study [73] conducted for the determination of the antihistamine enantiomers in pharmaceutical formulations, successful enantioseparations of eight antihistamines were performed on a Chiralpak AGP column in RP mode. A Chiralpak HSA was used in RP mode for enantioseparation of cetirizine, and the developed method was applied for the assays of cetirizine enantiomers in drops and oral solution [74].

Chiral crown ether selectors are prepared by incorporating various chiral units (e.g., binaphthyl, biphenanthryl or tartaric acid) as chiral barriers into macrocyclic polyethers [55].

These crown ethers have a cavity that exactly matches the size of the protonated primary amine group. Thus, the protonated primary amine can enter into the cavities forming inclusion complexes and the relative stabilities of the resulting diastereomeric complexes are based on additional interactions occurring between the crown ether substituents and the enantiomers of analytes (e.g., hydrogen bonds or steric interactions) [71]. Therefore, chiral crown ether CSPs are mainly restricted to discriminate chiral compounds with a primary amine group, such as amino alcohols [75], amino acids [76] and amino ketones [77], although enantioseparations of secondary amines have also been reported [78]. Of various commercially available chiral crown ether CSPs (Table 1), ChiroSil RCA (+), Opticrown RCA (+), and Crownpak CR (+) column are widely used. Their structures are shown in Figure 5. Lee et al. [79] reported a simultaneous chiral separation method for three iodo-thyronines and two iodo-thyrosines using a Crownpak CR (+) column. In another study, the use of a ChiroSil RCA column for the enantioseparation of thyroxine to measure its enantiomeric composition in pharmaceutical products was also described [80].

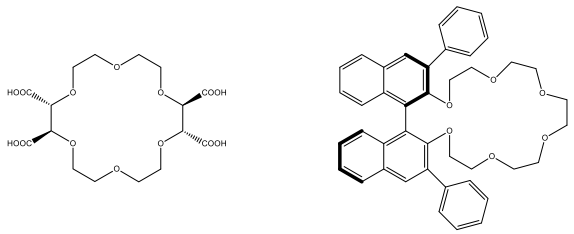

Figure 5. Chemical structures of the chiral crown ethers. (a) (+) (18-Crown-6)-2,3,11,12-tetracarboxylic acid; (b) (+) (3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6-ether.

1.2.1.2. Elution modes in chiral separation

Besides the type of CSPs, the mobile phase is another important factor in those successful chiral separations as it may determine which interaction type is more important for the chiral recognition. As mentioned, mobile phase in chiral separation can be divided into NP, RP and PO mode. In the development of chiral methods of separation, the mobile phase must be selected according to the type of column and analytes.

In NP mode, the mobile phase generally consists of a nonpolar solvent such as n-hexane or n-heptane mixed with an appropriate proportion (up to 40%) of polar mobile phase modifier such as ethanol (EtOH) or isopropanol (IPA). The type and ratio of organic modifier have a considerable effect on the retention and resolution of analytes. It is generally accepted that retention decreases when the content of polar organic modifier increases [81,82]. In addition, basic and/or acidic additives are often required to improve enantioseparations and

peak shapes by minimizing nonspecific retention [82]. In general, the acidic additive such as formic acid (FA), acetic acid (HAc) and trifluoroacetic acid (TFA) is helpful for improving the enantioselectivity for acidic compounds while the basic additive such as diethylamine (DEA) and triethylamine (TEA) is helpful for improving the enantioselectivity for basic ones [81].

As mentioned above, many chiral CSPs are preferentially used with the NP elution mode, as NP mode favors such interaction as hydrogen bonding, dipole–dipole and π–π interactions. However, the mobile phases commonly used in NP are incompatible with MS detection due to their high flammability in ion sources operated at high temperature, which limits its applicability in biological and environmental analysis.

Mobile phases commonly used in RP mode are buffer aqueous solutions mixed with organic modifiers. The organic modifier plays an important role in regulating retention and enantioselectivity. ACN, MeOH, EtOH and IPA are the most frequently used organic modifier, and their relative eluting strength can be ranked as follows: IPA > EtOH > ACN > MeOH [83]. Buffers are commonly used in RP to control the pH values which influence the ionization of both analytes and CSPs and therefore significantly affect the retention and enantioselectivity. Typical buffer systems are phosphate, acetate and formate (ammonium or sodium salts) [84,85]. For example, Perrin et al. [81] reported the effect of pH values of mobile phase on the enantioselectivity of different polysaccharide-based columns towards several β-blockers (propranolol, metoprolol, alprenolol, oxprenolol and pindolol) using 50 mM phosphate aqueous buffer/ACN (60/40, v/v) as the mobile phase. Greatly enhanced enantioseparations was observed with increasing pH from 2 to 9. Besides the control of pH, the buffers may enhance chiral separations via special effect on various types of interactions involved in chiral recognition. For example, the use of TEA/acetate buffer is generally preferred in enantioseparations on cyclodextrin-based CSPs, because they are considered capable of forming inclusion complexes with the cyclodextrins and promote separation [86].

The hydrogen bond interactions are disfavored in RP mode, and the ionic, hydrophobic, steric interactions and in some cases inclusion are primary interactions involved in the chiral recognition process [87]. Compared with NP mode, RP is much more widely used in analytical filed today due to its compatibility with MS detection and possibility of direct injection of biological samples. For example, Rao et al. [88] described an on-line two-dimensional RP LC–MS method using a new chiral Cyclobond column for enantiomeric determination of sertraline enantiomers in rat plasma by direct injection of plasma.

In recent years, the PO mode became increasingly popular because it offers several advantages including improved solubility of analytes, simplicity of solvent evaporation, fast separations and LC-MS compatibility [89,90]. In PO mode, only polar organic solvents such as ACN, alcohols (MeOH, EtOH and IPA), or their mixtures are used as mobile phase. For

the enantioseparations of acidic or basic analytes, an acidic (i.e. FA, HAc or TFA) and/or a basic additive (i.e. DEA or TEA) are generally needed in the mobile phase. For example, Taschwer et al. [91] reported that 40 out of 43 basic psychoactive chiral drugs were successfully separated using Lux Cellulose-2 in PO mode with a mobile phase consisting of ACN/IPA/DEA/FA (95/5/0.1/0.1, v/v/v/v). To date, polysaccharide-based CSPs and cyclodextrin-based CSPs have been extensively explored in PO mode [89,91–95]. The high success rate of enantioseparation has proved the potential of PO mode in chiral separation.

1.2.2. Gas chromatography

The first direct GC enantioseparation on a CSP was reported in 1966, by Gil-Av and his coworkers [96]. They separated several amino acid esters using a CSP based on derivatives of amino acids and dipeptides (N-trifluroacetyl (N-TFA)-l-isoleucine lauryl ester). To date, direct GC enantioseparation methods have been widely reported in various areas including agrochemicals, forensic toxicology, pharmaceutical, food and environmental analysis [97]. Chiral GC method based on CSPs offers the advantages of high separation efficiency, sensitivity, reproducibility, fast analysis and absence of liquid mobile phases [98]. Moreover, chiral GC method development is much more straightforward without the delicate choice of solvents and gradient elution systems, compared to that of LC. However, the application of chiral GC is often limited to the analysis of volatile and thermally stable compounds, such as essential oils, flavors, fragrances, alcoholic beverages, terpenoids, pheromones, pesticide, chiral auxiliaries, chiral catalysts, volatiles pharmaceuticals and amino acids [99]. For non-volatile, highly polar or thermally instable analytes, derivatization with a chiral reagent is needed to facilitate enantioseparation by GC, which is usually time-consuming. For example, in the chiral separation of several amino acids (including 2-amino-1-butanol, phenylglycine, isoleucine, threonine, aspartic acid and valine) by GC using a chiral inorganic mesoporous material as CSP, the amino acids were derivatized into their volatile amino acid derivatives prior to the analysis [100].

Compared with LC, the limited variety of CSPs can be utilized in chiral GC separations. Early on, CSPs used for chiral GC separations were mainly classified into three categories: amino acid derivatives, metal coordination complexes and cyclodextrin derivatives [98,101]. Besides, some other GC CSPs, such as chiral ionic liquids, polysaccharides, and cyclopeptides, have also been developed and applied for the separation of enantiomers [101]. So far, the cyclodextrin-based CSPs are the most widely used for GC enantioseparation [97]. For example, Gao et al. [102] have employed a Hydrodex-β-6TBDM chiral column based on heptakis(2,3-di-O-methyl-6-O-tert-butyldimethylsilyl)-β-cyclodextrin for the enantiomeric determination of polycyclic musks in sewage sludge. Successful enantioseparations of whisky and cognac lactones were achieved on a series of cyclodextrin-based CSPs [103].

An Astec Chiraldex B-DM GC column based on 2,3-di-O-methyl-6-t-butyl silyl derivative of β-cyclodextrin was used for the enantiomeric determination of linalool in teas [104].

There are a variety of detectors available for GC, such as the flame ionization detector (FID), electron capture detector (ECD), thermal conductivity detector (TCD) and MS, allowing GC to meet varied needs in different research areas. For example, Kuang et al. [105] reported the use of GC-ECD for the enantiomeric determination of pyrethroids (cypermethrin and cis-bifenthrin) in Chinese teas. Mifsud et al. [106] developed a sensitive GC-MS method for the enantiomeric determination of fluoxetine and norfluoxetine in urine and plasma samples. In addition, when combined with solid-phase microextraction (SPME) or liquid-phase microextraction (LPME), GC has emerged as an ideal choice for the analysis of chiral volatile compounds in complex matrices [98]. For example, Marsol-Vall et al. [107] reported the enantioselective analysis of chiral terpenoids in nine fruit and vegetable fibers using headspace SPME for extraction of analytes and followed by GC-MS for the enantiomeric analysis. In another study, an analytical method employing dispersive liquid–liquid microextraction (DLLME) followed by chiral GC-MS analysis was successfully applied for the enantiomeric determination of fifteen triazole fungicides in canal water [108].

On the other hand, since the majority of the drugs are less volatile, derivatization is required to convert these compounds into their volatile derivatives prior to GC analyses. This restricts the application of GC in chiral analysis of drugs. Future research in the field of GC chiral separations should aim to develop new versatile CSPs with broader enantioselectivity and high thermal stability, which would greatly expand the applicability of chiral GC separation.

1.2.3. Supercritical fluid chromatography

SFC is considered a powerful tool for chiral separation, as it combines the advantages of GC and LC. SFC generally uses supercritical carbon dioxide (CO_2) as a major component of the mobile phase. The higher diffusivity and lower viscosity of its mobile phase enable fast separation with high efficiency than LC [109]. In addition, SFC is as an environment-friendly technique compared to LC, as much less solvent is consumed during analysis. On the other chiral SFC is able to determine thermally instable and non-volatile compounds that cannot be separated by GC.

The CSPs used in LC can also be used in SFC, which makes SFC to be a strong competitor to LC for chiral separation. Similarly in LC chiral separation, polysaccharides are the most commonly utilized CSPs in SFC separation [109,110]. Other CSPs including cyclodextrins, Pirkle-type, macrocyclic antibiotic and synthetic polymers also play an important role. A lot of studies have reported the successful enantioseparations on SFC

employing various CSPs. For example, Hamman et al. [111] evaluated the chiral recognition ability of a series of commercially available polysaccharide-based CSPs in SFC towards 46 chiral compounds. All the analytes were enantiomerically separated in 2.5 min on at least one of the CSPs. The Chiralpak AD column was proved to be the most useful providing baseline enantioseparation for 21 analytes, followed by Cellulose-1 providing baseline enantioseparation for 18 analytes. As another example, rapid chiral separations of atenolol, metoprolol, propranolol and the zwitterionic metoprolol acid were achieved within 8 min by using SFC with a polysaccharide-based column, namely Chiralpak IB-3 [112]. A number of studies have been conducted to compare enantioselectivites of various CSPs in SFC and LC. For example, Kalíková et al. [113] compared the enantioselectivity of a Chiralcel OD column towards 21 chiral drugs in SFC and LC conditions. The results showed that SFC provided better enantioresolution with shorter analysis times than LC for the majority of analytes. However, examples of enantiomers better resolved under the optimized conditions in LC were also found. Similarly, Khater et al. [114] compared enantioseparations of 24 chiral sulfoxides on seven different polysaccharide-based CSPs in SFC and LC, and results were better in SFC in terms of enantioresolution and analysis time.

As mentioned, a new generation of chiral columns packed with sub-2 μm particles have been developed and employed in ultra-high performance liquid chromatography (UHPLC). Due to the low viscosity of CO_2-based mobile phases, a full compatibility of SFC with these sub-2 μm columns is guaranteed, have greatly broadened the application spectrum of this technique, making modern SFC competitive and complementary to UHPLC. For example, a new sub-2μm Whelk-O1 CSP has been used in SFC for the high-throughput screening of 129 pharmaceutical compounds [115]. The whole screening under fast gradient elution (total analysis time 9 min, including column re-equilibration) was accomplished in less than 24 h, yielding 63% of positive results.

In SFC, CO_2 is generally used as main component of mobile phase due to its low values of critical temperature and pressure (31°C, 7.3 MPa), non-flammable, non-explosive, relative inertness, low-cost, no generation of waste and low toxicity [110]. However, CO_2 has a low polarity, and an appropriate proportion of polar organic modifiers such as ACN, MeOH and IPA are essential for the elution of most chiral compounds [110]. Acidic or basic additives such as TFA and TEA are also frequently added in small concentrations to improve the enantioseparations. This combination of a principal non-polar solvent and a polar co-solvent makes the mobile phase similar to those employed in NP-LC. Some studies reported the transfer of NP-LC chiral separation methods to SFC. For example, Breitbach et al. [116] reported that the enantioseparations of 19 of 21 α-aryl ketones were achieved on cyclofructan-based CSPs in NP-LC, and most enantioseparations obtained in NP-LC could be transferred to SFC. SFC provided much shorter analysis time, but the enantioresolutions obtained on LC were generally better than those on SFC.

Thanks to its high separation efficiency, high-throughput screening capability and environment-friendly mobile phase, SFC has become an attractive alternative to LC for the separation of chiral drugs, especially in pharmaceutical industries at both preparative and analytical scales [117]. Nevertheless, compared to LC, SFC has several limitations that cannot be overlooked. First, despite the addition of polar organic modifiers in mobile phase, the elution strength of mobile phase in SFC is still limited and it is not able to analyze extremely polar compounds. In addition, the lack of reliable SFC instrumentation has slowed down the spread of SFC in routine use. The old generation of SFC instruments suffers from drawbacks such as lack of robustness and repeatability, poor UV sensitivity and inadequate dynamic range, which largely limits the applicability of SFC in many areas of interest [118]. However, as the new generation of instruments are already available in the market, the potential of SFC in chiral separation may increase.

1.2.4. Capillary electrophoresis

Chiral separation in CE is achieved by adding a chiral selector to the background electrolyte (BGE). Reversible and transient diastereoisomeric complexes of differing effective mobility are formed between the enantiomers and chiral selector thereby enabling chiral separation in CE. Chiral CE has gained increasing interest over the past decade as it offers some inherent advantages such as high resolving power, high efficiency, fast analysis speed, low consumption of sample, solvent and chiral selectors, and high flexibility in choosing and changing selector types [119,120].

Various chiral selectors have been applied in chiral CE separation, such as macrocyclic antibiotics, cyclodextrins, peptides and proteins, chiral crown ethers, chiral ligand exchange, chiral ionic liquids and polysaccharides [120]. Among them, cyclodextrins are the most frequently used chiral selectors in CE which is associated to their commercial availability, wide enantioselectivity, UV transparency and stability over a wide pH range [121]. A great variety of cyclodextrin-based chiral CE selectors are available in native, neutral, negatively and positively charged forms, which allows the enantioseparation of a broad spectrum of chiral drugs. For example, carboxymethyl-β-cyclodextrin was used as chiral selector in CE for the enantioseparation of 10 cardiovascular agents and six bronchiectasis drugs [122]. The successful enantioseparation of terbutaline, a β2 adrenergic receptor agonist drug, was performed using various β-cyclodextrins as chiral selectors including native β-cyclodextrin, heptakis(2,6-di-O-methyl)-β-cyclodextrin, heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin, heptakis(2-O-methyl-3,6-di-O-sulfo)-β-cyclodextrin, heptakis(2,3-di-O-methyl-6-O-sulfo)-β-cyclodextrin and heptakis(2,3-di-O-acetyl-6-O-sulfo)-β-cyclodextrin [123]. In addition, the mixtures of chiral selectors can be used in chiral CE separation, which often enhances enantioselectivity significantly and can be applied for the chiral separation of multi-class

analytes [119]. For example, the enantioseparations of ten drugs belonging to several therapeutic classes were carried out by a chiral CE method using a mixture of a hydroxypropyl-β-cyclodextrin and an ionic liquid (1-ethyl-3-methylimidazolium-l-lactate) as chiral selectors [124]. The separation significantly improved for all the analytes using the dual chiral selector system compared to when using hydroxypropyl-β-cyclodextrin alone. Pérez-Alcaraz et al. [125] developed a chiral CE method for the enantiomeric determination of a group of cathinones in urine samples using a mixture of 2-hydroxypropyl β-cyclodextrin and β-cyclodextrin as chiral selectors.

The main drawback of chiral CE is the limitation in its combination with MS detection. Most of chiral selectors employed in chiral CE are nonvolatile, which may cause ion suppression and contamination of the ionization source [126]. To avoid the entrance of nonvolatile chiral selectors commonly used in CE-MS approaches into the ion source, two strategies have been mainly employed: the counter migration technique (CMT) and the partial filling technique (PFT) [125,127]. In the CMT, a charged chiral selector migrates away from the detection system in the opposite direction to the analytes, whereas in the PTF, only a part of the capillary (e.g. 70–90%) is filled with the BGE containing the chiral selector [127]. For example, Pascual-Caro et al. [128] developed a chiral CE-MS method applying CMT with a BGE of 1 M FA/1 M ammonium formate (5/0.1, v/v) containing 0.2% of sulfated γ-CD for the enantiomeric determination of a group of amphetamines and their metabolites in urine samples. In another study, a CE-MS method was developed for the simultaneous enantioselective determination of tetrahydropalmatine and tetrahydroberberine in Corydalis Rhizoma (a botanical drug) using (2-hydroxypropyl)-β-cyclodextrin as the chiral selector and PFT to avoid signal suppression and contamination of the MS detector [129].

Nevertheless, the development of chiral CE-MS methods remains a challenge, which largely limits the application of CE in the chiral analysis of drugs in complex matrices such as environmental and biological samples. The development of MS-compatible chiral selectors will foreseeably be of great significance to facilitate the wide use of CE in chiral separation.

1.3. Enantiomeric determination of chiral drugs in the environment

Growing evidence has demonstrated the stereoselective behaviors of chiral drugs in the environment [19,24,26,28,31]. Therefore, the significance of enantiomeric determination of chiral drugs in the environment has gained increasing attention. Consequently, the number of environmental chiral analysis studies has grown substantially in recent years, as can be seen in Figure 6 that provides an overview of the publications over the years 2002-2021. Over the last five years (2017-2021), the number of publications increased to more than 100 papers against 22 between the years 2002 and 2006. Among the various analytical technology used, chiral LC-MS has been the dominant technique for environmental analysis mainly due to its simplicity, wide applicability and high sensitivity.

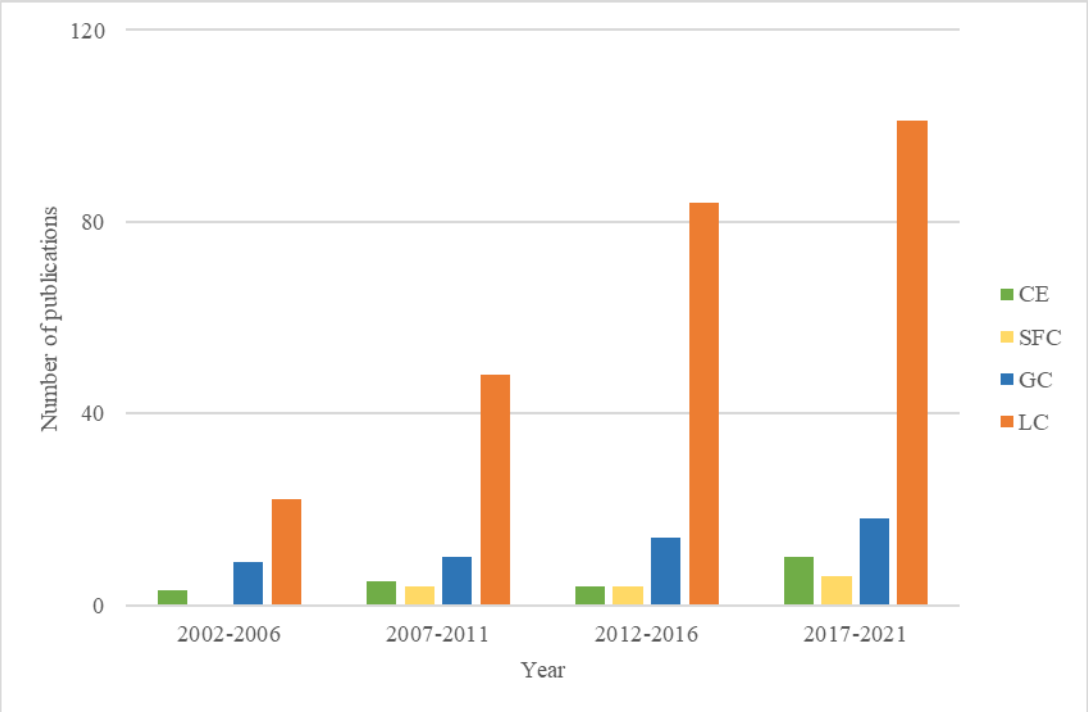

Figure 6. Number of publications related to enantiomeric determination of chiral drugs in environmental samples. Data obtained from Scopus in Jan 2022 using the keywords: enantiomeric, drugs, environment, and name of analytical technology (LC, GC, SFC, or CE).

The latest chiral LC-MS methods developed for environmental applications have been compiled in a review paper published in the journal Trends in Environmental Analytical Chemistry. This review focuses on the development of chiral LC-MS methods applying various CSPs in combination with different elution modes. In addition, diverse applications of chiral LC-MS methods for the environmental analysis using chiral LC-MS are discussed according to the most common groups of chiral drugs. This manuscript can be found in the following section.

1.3.1. Enantiomeric fraction determination of chiral drugs in environmental samples using chiral liquid chromatography and mass spectrometry

ENANTIOMERIC FRACTION DETERMINATION OF CHIRAL DRUGS IN ENVIRONMENTALSAMPLES USING CHIRAL LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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Abstract

When it was recognized that chiral drug residues have stereospecific toxicity towards environmental organisms the attention given to enantiomeric fraction determination of chiral drugs in the environment increased. Among various analytical techniques, chiral liquid chromatography (LC) coupled with mass spectrometry (MS) has been widely used due to its simplicity, wide applicability and high sensitivity. In this review, we aim to provide a comprehensive overview and comparison of the types of chiral stationary phases, elution modes and MS detection techniques employed and address the advances and limitations. The impact of the mobile phase composition on enantioseparation and MS detection are discussed based on the different methods developed. In addition, diverse applications for the enantiomeric fraction determination of chiral drugs in environmental matrices using chiral LC and MS are discussed in depth.

Keywords: chiral drugs, enantiomeric fraction determination, environment, chiral liquid chromatography, mass spectrometry

1. Introduction

In the last decades, there has been increasing concern over the potentially adverse effects of drugs, both pharmaceuticals and illicit drugs, on the environment due to the rapid increase in drug consumption [1,2]. Various drugs and their residues have been found mainly in surface waters, such as rivers and lakes, but also in groundwater, sludge and even drinking water [3–5]. They are released into the environment via a number of pathways at different stages of their life cycle, from their production, to their use and later disposal, but primarily via discharges of wastewater treatment plants (WWTPs) from which drugs are not completely eliminated [2]. What's more, it should be emphasized that more persistent and/or more hazardous metabolites may be formed during these processes [1]. The continuous influx of drugs into the environment from several sources even at trace concentrations has raised concern that long-term exposure to environmental contaminants may have a considerable impact on environmental organisms and human health [1]. Furthermore, complex mixtures of drug contaminants might lead to synergistic interactions resulting in substantially higher overall toxicity [2]. A lot of studies have been conducted to assess the environmental risk caused by such residues and metabolites. For example, exposure to antidepressant fluoxetine at environmentally relevant concentrations has been reported to affect the reproduction and predator avoidance behavior of fish [6,7] and impair the cryptic performances of newly hatched cuttlefish [8]. However, an important factor of environmental drug residues that is not currently considered in the large majority of cases, is chirality, as a high proportion of drugs are chiral.

It is well known that the enantiomers of chiral drugs often exhibit different pharmacodynamics and pharmacokinetics; that is, only one enantiomer may act on targets, while the other may be inactive or may even be harmful [9,10]. Although the use of single enantiomers has expanded during recent decades, a high percentage of them are still being marketed as racemic mixtures [11,12]. Chiral drugs can be enantioselectively degraded during human metabolism and also during biological transformation in the wastewater treatment process and in the receiving environment. This results in a wide variety of enantiomeric compositions [9]. It has also been established that the enantiomers of chiral drugs often exhibit enantioselective toxicity towards environmental organisms. For example, R-(–)-fluoxetine is 30 times more toxic for the protozoan *T. thermophila* than S-(+) fluoxetine [13], whereas S-(+)-fluoxetine is 9.4 times more toxic to *P. promelas* than R-(–)-fluoxetine [14]. For these reasons, chirality must be taken into account for a complete understanding of the

environmental risks of drugs.

Enantiomeric analysis of environmental samples can provide valuable information about the occurrence and the fate of chiral drugs and their metabolites in the environment. Therefore, it contributes to estimating the most accurate potential impact of chiral contaminants on living organisms. In addition, studying the enantiomeric composition of chiral drugs in the environment would help to (i) evaluate the removal efficiency of treatment systems; (ii) track the origin of the drug residues (from consumption, the direct disposal of unused drugs or production waste); (iii) identify illicit sewage discharges; (iv) estimate the community consumption of pharmaceuticals or illicit drugs; and (v) identify the synthesis pathway of chiral drugs.

Due to the complex matrices and very low concentrations of chiral drugs, highly sensitive and selective methods are needed for the environmental analyses. To date, various methods have been developed for enantiomeric fraction (EF) determination of chiral drugs in environmental samples. The first studies involving chiral analyses of environmental samples mostly employed gas chromatography (GC)-mass spectrometry (MS) either with directed enantioselective methods using a cyclodextrin-based chiral stationary phase (CSP) [15,16] or indirect methods using derivatizing reagents [17,18]. However, the use of chiral GC–MS with cyclodextrin-based CSP is often limited to the analysis of volatile and thermally stable compounds. For non-volatile, highly polar or thermally instable analytes, derivatization with a chiral reagent is needed to facilitate enantioseparation by GC–MS, which is usually time-consuming and laborious [11]. Since 2006, liquid chromategraphy (LC) coupled to MS using CSP has been the dominant technique for environmental analysis owing to its simplicity and wide scope of applicability. Therefore, the aim of this review is to outline the current state-of-the-art of enantioselective LC–MS based methods for the determination of chiral drugs in environmental samples.

There have been several other reviews published on chiral drugs in the environment. For example, in the review by Sanganyado et al. [11], the environmental occurrence and fate of chiral drugs are fully discussed; Dogan et al. [19] highlighted the problems and challenges in determination of chiral pharmaceutical residues in wastewater; Ribeiro et al. [20] emphasized the method trends on analysis of chiral drugs in environmental, biodegradation and forensic applications. In this review, we aimed to provide a comprehensive overview of various CSPs that have applied in LC–MS based methods for EF determination of chiral drugs in the environment, including the mechanism of chiral recognition involved under different elution modes, method

development considering compatibility of the mobile phase with MS and their capabilities for the enantioseparation of different kind of drugs. We also discuss advances and limitations of the chiral LC–MS based methods. In addition, we look at various applications for studying the enantiomeric occurrence of common chiral drug groups in environmental matrices.

2. Chiral LC–MS analytical methods

To date, various chiral LC–MS based methods have been developed and applied to make a chiral analysis of different chiral contaminants from different types of environmental matrices, including influent and effluent wastewater, surface water and sludge. In this section, we describe different chiral LC separation and MS detection techniques used in these reported studies. The choice of elution modes and mobile phase optimization are highlighted and discussed based on examples.

2.1. Chromatographic separation

LC has become very popular for the EF determination of chiral drugs mainly due to the availability of numerous commercial CSPs (i.e. polysaccharides, ion-exchangers, macrocyclic antibiotics, chiral crown ethers, proteins, cyclodextrins and Pirkle-type) and the high versatility provided by the different elution modes (normal-phase (NP), reversed-phase (RP), polar-organic phase (PO) or polar-ionic phase (PI) modes) [9]. However, not all the columns are compatible with all the elution modes. Moreover, the compatibility of the mobile phase with MS should also to be taken into account when the chiral LC–MS based method is developed for environmental analysis.

Chiral recognition of enantiomers on CSPs is often explained with a three-point model. It was proposed that three interactions have to take effect and at least one of them must be stereoselective to form diastereomeric complexes, and thereby enable chiral separation. The key step in the process is the formation of transient diastereoisomeric complexes between the enantiomers and the chiral selector. Therefore, the choice of the chiral columns is vital for successful enantioseparations. Furthermore, the elution mode and mobile phase compositions have a large effect on the enantioresolutions as they participate in the interactions.

In environmental analyses the most used CSPs so far are macrocyclic glycopeptides (an antibiotic-based class) and protein-based, followed by polysaccharide-based, and to a lesser extent Pirkle type CSPs. The structures of commonly used commercial chiral columns for each type in chiral LC–MS based method are shown in Table 1. We discuss below their applications in EF determination of different chiral drugs in environmental matrices. Chiral mecha-nisms and method development are also

discussed briefly. Some selected examples of the different columns applied to determine different chiral drugs in environmental matrices are listed in Table 2.

2.1.1. Macrocyclic glycopeptide antibiotics-based CSPs

Macrocyclic glycopeptides, particularly vancomycin and teicoplanin, which are marketed under the trade name Chirobiotic V and Chirobiotic T by Astec1, respectively, are the most commonly used CSPs in the EF determination of chiral drugs in environmental samples. As shown in Table 1, these selectors contain a variety of functional moieties including a peptidic part, carbohydrate units and ionizable carboxylic and amine groups, multiple stereogenic centres and inclusion cavities, which could interact with enantiomers through most of the molecular interactions, such as hydrogen and hydrophobic bonding, dipole-dipole, π–π, ionic and steric interactions. They therefore make it possible to separate a broad range of chiral analytes [21,22]. In addition, the commercial macrocyclic glycopeptide-based columns are capable of working in different elution modes (NP, RP, PO and PI mode), which further increases their applicability.

In environmental analyses, the RP and PI modes are the most frequently used for macrocyclic glycopeptide CSPs, as NP is generally incompatible with MS detection. The PO mode has been demonstrated to be effective only for neutral molecules [22]. In RP mode, the typical mobile phase is composed of organic solvents combined with aqueous buffers with a pH between 3.5 and 7.0. The hydrogen bond interactions are disfavored in aqueous media, and the hydrophobic, steric and ionic interactions are dominant in the enantioseparation process [23]. The type and concentration of organic modifier and buffer, pH and flow rate are the main parameters for regulating retention and enantioselectivity. Methanol (MeOH), ethanol (EtOH) and acetonitrile (ACN) are the most commonly used organic solvents that provide good enantioselectivities for various types of analytes. Generally, retention and enantioresolution versus organic modifier content curves have a U-shape [24], which means that the RP mode exhibits enantioselectivity at both low and high organic solvent content. It is most common to use a high amount of organic modifier, such as 90 %, in mobile phases in environmental analysis [25–27].

The buffer pH value can influence the ionization of both analytes and CSPs, which significantly affects the enantiorecognition capabilities of the stationary phases as well as the peak shape. In one study [28], the effect of the pH values on enantioselectivity towards several drugs, including anticancer drugs, anthelmintic drugs, non-steroidal anti-inflammatory drugs (NSAIDs) and their metabolites (Table 2), was evaluated on Chirobiotic T

Table 1. Structures of commonly utilized commercial chiral columns in chiral LC-MS based methods.

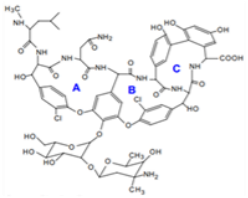
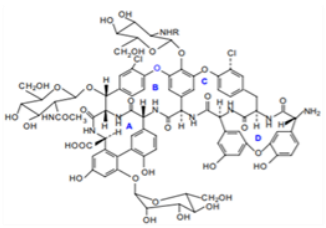
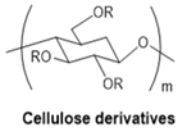
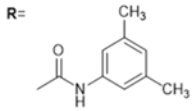
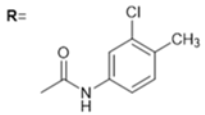
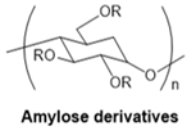
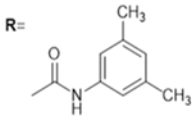
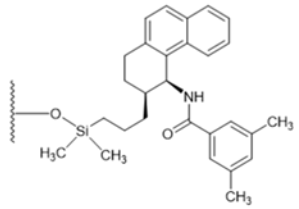
Types of CSPs	Structure	Trade name	Elution mode feasible	Analytes involved	Ref.
Macrocyclic glycopeptides antibiotic-based	 **Vancomycin**[a]	Chirobiotic V	NP, RP, PO, PI	atenolol, metoprolol, propranolol, sotalol, citalopram, fluoxetine, nadolol, pindolol, salbutamol, venlafaxine, mirtazepine, ibuprofen, alprenolol, bisoprolol, albuterol, clenbuterol, carazolol, terbutaline, MDMA, betaxolol, amphetamine, timolol, metham phetamine, norfluoxetine, metoprolol acid, 4 hydroxypropranolol, α-hydroxymetoprolol, O -desmethylvenlafaxine, O -desmethyltramadol, N,O-didesmethylvenlafaxine	[26, 27, 29-32, 34, 38, 41, 66, 69]
	 **Teicoplanin**[a]	Chirobiotic T	NP, RP, PO, PI	chloramphenicol, carboxyibuprofen, naproxen, ketoprofen, ibuprofen, 2-hydroxy ibuprofen, praziquantel, ifosfamide, indoprofen, propranolol, atenolol, nadolol, metoprolol, sotalol	[28, 35]
Protein-based		Chiralpak CBH	RP	amphetamine, methamphetamine, MDA, MDMA, MDEA, HMMA, ephedrine/pseudoephedrine, atenolol, venlafaxine, fluoxetine, norfluoxetine, para -methoxyamphetamin, tramadol, zopiclone, desmethylvenlafaxine, mephedrone, flephedrone, methedrone, methylone, butylone	[34, 38-42, 44, 45, 67, 68, 70]
	-	Chiralpak AGP	RP	ibuprofen, naproxen, ketoprofen, ifosfamide, aminorex, econazole, fexofenadine, tetramisole, tebuconazole, propiconazole, dihydroketoprofen, praziquantel, miconazole, chloramphenicol, 3 -N-dechloroethylifosfamide, 10,11 -dihydro-10-hydroxycarbamazepine.	[46-48]
		Chiralpak HSA	RP	ketoconazole	[48]

Table 1 (Continued)

Types of CSPs	Structure	Trade name	Elution mode feasible	Analytes involved	Ref.
Polysaccharide-based	 Cellulose derivatives	 3,5-dimethylphenylcarbamate	Chiralcel OD-RH	NP, RP, PO	-
			Lux Cellulose-1	NP, RP, PO	
		 3-chloro-4 methylphenylcarbamate	Chiralcel OZ-RH	NP, RP, PO	nadifloxacin, ofloxacin, ofloxacin-N-oxide, prulifloxacin, flumequine, besifiloxacin, desmethylofloxacin, moxifloxacin, ulifloxacin; [50]
	 Amylose derivatives	 3,5-dimethylphenylcarbamate	Chiralpak AD - RH	NP, RP, PO	ibuprofen, naproxen, flurbiprofen [51, 52]
Pirkle-type			Whelk-O 1	NP, RP	flurbiprofen, ketoprofen, ibuprofen, naproxen [30]

[a] Reproduced from Ref. [72].

HMMA, 4-Hydroxy-3-methoxymethamphetamine; MDA, methylenedioxyamphetamine; MDEA, 3,4-methylenedioxy-N-ethyl-amphetamine; MDMA, methylenedioxymethamphetamine; NP, normal-phase; PI, polar-ionic phase; PO, polar-organic phase; RP, reversed-phase.

column, using 10 mM ammonium acetate (NH_4Ac) aqueous buffer/MeOH (10/90, v/v) as the mobile phase. For most of the acidic analytes, enantioresolutions increased with the decreasing pH values from 6.5 to 4.2. The use of a mobile phase at pH 4.2 led to a better enantioseparation and an improved peak shape, and also favored ionization in the positive electrospray ionization (ESI) mode. The better enantioseparations with the lower pH was probably as a result of the enhanced ionic interactions between the carboxylic group of the analytes and the protonated amine group of the chiral selector. Salt, such as NH_4Ac or triethylammonium acetate, is often added to control the pH of the mobile phase, enhance the enantioresolution and improve the ionization in MS [26]. Since higher salt concentrations would suppress the ionization, a buffer not exceeding 20 mM is typically used [26,28,29]. Although in some cases a higher buffer concentration has been used, the lower aqueous content in the mobile phase reduces the potential of the expected ion suppression [30].

The PI mode is a variation of the PO mode in which the mobile phase is composed by 100 % organic polar solvents mainly MeOH and/or EtOH, an acid, most often formic acid (FA) or acetic acid (HAc), and a base, often triethylamine (TEA), as well as volatile salts, often NH_4Ac [31–35]. The mechanism for chiral recognition is predominantly ionic interactions, and therefore, this mode is especially effective for enantioseparation of ionizable analytes [22,23]. Using acidic and/or basic additive in the mobile phase can adjust the ionization of the analyte and the macrocyclic selector, and consequently control the chiral recognition. In general, the acidic additive is beneficial for enhancing the enantioselectivity for basic molecules while the basic additive is generally beneficial for enhancing the enantio-selectivity for acidic ones. For example, the developed LC coupled with tandem MS (MS/MS) using the Chirobiotic V column with mobile phase composed of MeOH modified with 4 mM NH_4Ac and 0.005 % FA provided enantioseparation for 12 basic pharma-ceuticals (Table 2) [31]. In contrast, enantiomers of acidic analytes (ketoprofen, naproxen, fluoro-quinolone and flumequine) were not separated and eluted within a very short time indicating weak interactions with the CSP [31].

The mobile phase composition obviously determines which interactions dominate the chiral recognition process, so that the enantioselectivity varies in different elution modes. For selecting the most suitable elution mode, some studies have compared the performance of macrocyclic glycopeptide columns in different elution modes. For instance, in the above example [31], the enantio-selectivity of the Chirobiotic V column was also evaluated in PO, PI and RP mode towards 15 chiral pharmaceuticals and

their active metabolites. The results showed that the PI mode provided the best enantioseparations, satisfactory retention times and sensitivity. In addition, owing to the ease of coupling to MS and the outstanding sensitivity achieved, PO mobile phases are more favorable for environmental analyses.

The broad applicability of macrocyclic glycopeptide CSPs makes multi-residue analysis possible. In one study [28], a Chirobiotic T column was successfully used in a multi-residue chiral LC–MS/MS method for the simultaneous enantiomeric profiling of 22 chiral pharmaceuticals belonging to several therapeutic classes, including NSAIDs, anthelmintic drugs, anticancer drugs, central nervous system drugs and antifungal drugs in river water and wastewater.

2.1.2. Protein-based CSPs

Protein-bonded CSPs are another widely used CSP in environmental analyses. Among various commercial protein-based CSPs, the most commonly used are cellobiohydrolase (CBH), α1-acid glycoprotein (AGP) and human serum albumin (HSA) [36]. As a single protein may contain a large number of chiral centres and different binding sites, it is capable of multiple interaction involving hydrogen-bonding, hydrophobic interactions and ion-exchange (for charged analytes), which contributes to the chiral recognition process [37]. These CSPs are used exclusively in RP mode with aqueous/organic mobile phases. In addition, these columns are relatively restrictive in terms of the mobile phase composition, e.g. no more than 20% organic modifier is required [38]. This has significant limitations for the sensitivity of the MS detector, particularly with the ESI interface.

CBH is a degrading enzyme with an isoelectric point of 3.9 [39]. It is effective for the enantiomeric separation of basic drugs, in which the chiral recognition process is mainly driven by the ion-exchange between the basic analytes and carboxylic acid residues on the protein [37]. This column is particularly sensitive to pH, ionic strength and organic content in the mobile phase. CBH columns are negatively charged over the column working pH range (4.0–7.0). An increase in mobile phase pH would increase the negative charges of the CSP resulting in an increased affinity for cationic compounds, and consequently, retentions and enantioresolutions for basic compounds. In addition, when the concentration of the organic modifier and buffer solution is decreased, the retention factors generally increase [40]. As shown in Table 2, the typical mobile phase used is composed of isopropanol (IPA)/H_2O (10/90, v/v) containing 1 mM NH_4Ac [34,39,41–45].

AGP is a peptide chain that includes 14 sialic acid residues, resulting in a low isoelectric point of 2.7 [36]. Unlike CBH,

Table 2. Chiral LC-MS based methods employed in the EF determination of chiral drugs in environmental matrices.

Compounds	Environmental matrix	Sample preparation	Analytical methodology	Chromatographic conditions	MDL/MQL	Ref.
Fully enantioseparated (R_s≥1.0): atenolol, metoprolol, propranolol, sotalol, citalopram, fluoxetine **Partially enantioseparated (R_s<1.0):** nadolol, pindolol, salbutamol	Influent Effluent	SPE: Oasis HLB L: 100 mL influent, 500 mL effluent E: 5 mL MeOH Extracts dried with Na_2SO_4 Filt.: 0.22 µm filter Evapor. to dryness Reconst.	LC-(ESI)QTRAP operating in QqQ mode	Chirobiotic V column (250×4.6 mm, 5 µm) MeOH/H_2O (90/10, v/v) with 20 mM NH_4Ac and 0.1% FA (pH 4) 0.5 mL/min	Influent: MDL:0.4-7.5ng/L MQL: 1-25 ng/L Effluent MDL:0.2-7.2ng/L MQL: 1-24 ng/L	[26]
Partially enantioseparated: chloramphenicol, carboxyibuprofen, naproxen, ketoprofen, ibuprofen, 2-hydroxy ibuprofen, praziquantel, ifosfamide, indoprofen **No enantioseparated:** tetramisole, 10,11-dihydro, cephalexin, dihydroketoprofen, 10-hydroxycarbamazepine, 3-N-dechloroethylifosfamide, aminorex, 2-phenylpropionic acid	Influent Effluent River	SPE: Oasis HLB-MAX L: 100 mL wastewater, 200 mL river water, pH=7.5 W: HLB with 2 mL H_2O, MAX with 2 mL of 5% NH_4OH E: 4×1 mL MeOH + 2×1 mL MeOH with 2% FA Evapor. to dryness Reconst.: 0.5 mL MP Filt.: 0.2 µm PTFE	LC-(ESI)QqQ	Chirobiotic T column (250×2.1 mm, 5 µm) 10 mM NH_4Ac aqueous buffer (pH 4.2)/MeOH (70/30, v/v) 0.08 mL/min	MDL: Influent: 0.24-94.1 ng/L Effluent: 0.22-79.9 ng/L River water: 0.12-81.7 ng/L	[28]
Fully enantioseparated: 5 parent compounds: atenolol, propranolol, metoprolol, fluoxetine, venlafaxine 6 transformation products: α-hydroxymetoprolol, metoprolol acid, 4-hydroxypropranolol, O-desmethylvenlafaxine, norfluoxetine, N,O-didesmethylvenlafaxine	River	SPE: Oasis HLB L: 500 mL, pH=4 W: 10 mL H_2O E: 4 mL MeOH + 4 mL MeOH/ACN (50:50, v/v). Evapor. to dryness Reconst.: 0.5 mL MeOH with 0.025% FA Filt.: 0.22 µm PTFE	LC-(ESI)QqQ	Chirobiotic V column (250×4.6 mm, 5 µm) MeOH/H_2O (90/10, v/v) with 10 mM NH_4Ac (pH 4) 0.6 mL/min, 20°C	MDL:0.1-2.2 ng/L MQL: 0.2-7.2 ng/L	[29]
Fully enantioseparated: fluoxetine, alprenolol, metoprolol, propranolol, salbutamol, bisoprolol, O-desmethyltramadol, flurbiprofen, ketoprofen, naproxen, warfarin **Partially enantioseparated:** mirtazepine, venlafaxine, O-desmethylvenlafaxine, ibuprofen **No enantioseparated:** norcocaine, cocaine, benzoylecgonine, amphetamine, methamphetamine, tramadol, N-desmethyltramadol, nebivolol	River	SPE: Oasis MCX L: 500 mL, pH=2 W: 4 mL H_2O with 2% FA E: 4 mL EtOH for acidic analytes; 4 mL EtOH with 5% NH_4OH for basic analytes Evapor. to dryness Reconst.: 250 µL EtOH Filt.: 0.22 µm PTFE	LC-(ESI)QqQ	For basic analytes: Chirobiotic V column (150×2.1 mm, 5 µm) EtOH/10 mM NH_4Ac aqueous buffer (92.5/7.5, v/v) (pH 6.8) 0.32 mL/min, 25°C For acidic analytes (NSAIDs): Whelk-O1 column (250x4.6 mm, 5 µm) MeOH/H_2O (60/40, v/v) with 0.1% FA, 1.0 mL/min room temperature	MDL: 0.01-2.66 ng/L MQL: 0.02-5.71 ng/L	[30]

Table 2. (Continued)

Compounds	Environmental matrix	Sample preparation	Analytical methodology	Chromatographic conditions	MDL/MQL	Ref.
Fully enantioseparated: ibuprofen, fluoxetine, sotalol, propranolol, 4OH propranolol, timolol, albuterol, clenbuterol **Partially enantioseparated:** carazolol, pindolol, betaxolol, metoprolol, atenolol, norfluoxetine **No enantioseparated:** ketoprofen, naproxen, ofloxacin, flumequine	River Influent Effluent	SPE: Oasis HLB L: 500 mL river water, 100 mL wastewater, pH=7.5 E: 4 mL MeOH Evapor. to dryness Reconst.: 0.5 mL MP Filt.: 0.2 µm PTFE	LC-(ESI)QqQ	Chirobiotic V column (250×2.1 mm, 5 µm) MeOH containing 4 mM NH_4Ac and 0.005% FA 0.1 mL/min, 25°C	River water: MDL: 0.09-0.62 ng/L MQL: 0.23-1.65 ng/L Effluent: MDL: 0.09-2.91 ng/L MQL: 0.23-7.75 ng/L Influent: MDL: 0.08-10.03 ng/L MQL: 0.30-26.74 ng/L	[31]
Fully enantioseparated: amphetamine, methamphetamine	River	SPE: Oasis HLB L: 100 mL E: 4 mL MeOH Evapor. to dryness Reconst.: 0.5 mL MP Filt.: 0.2 µm PTFE	LC-(ESI)QTOF	Chirobiotic V column (250×2.1 mm, 5 µm) MeOH containing 4 mM NH_4Ac and 0.005% FA 0.1 mL/min, 25°C	MQL: 4.8-18.5 ng/L	[32]
Fully enantioseparated: amphetamine, methamphetamine, MDA, MDMA, atenolol, propranolol, metoprolol, fluoxetine, venlafaxine	River Effluent	SPE: Oasis HLB L: 250 mL river water, 100 mL effluent E: 4 mL MeOH Evapor. to dryness Reconst.: 0.5 mL MP Filt.: 0.2 µm PTFE	LC–(ESI)QTOF	1) Chiral CBH column (100×2 mm, 5 µm) H_2O/IPA (90/10, v/v) with 1 mM NH_4Ac (pH=7) 0.075 mL/min, 25°C 2) Chirobiotic V column (250×4.6 mm, 5 µm) MeOH containing 4 mM NH_4Ac and 0.005% FA 0.1 mL/min. 25°C	CBH column: River water: MDL: 2.1-10.7 ng/L MQL: 9.1-51.7 ng/L Chirobiotic V column: River water: MDL: 0.2-10.4 ng/L MQL: 0.3-39.0 ng/L Effluent: MDL: 0.6-22.8 ng/L MQL:1.3-85.7 ng/L	[34]
1) **Fully enantioseparated:** methamphetamine, amphetamine, ephedrine/pseudoephedrine, MDMA, MDA **Partially enantioseparated:** 3,4-methylenedioxy-N-ethyl-amphetamine (MDEA), norephedrine 2) **Fully enantioseparated:** alprenolol, propranolol, metoprolol, tramadol, salbutamol, sotalol, terbutaline, venlafaxine, fluoxetine, desmethylvenlafaxine, citalopram, desmethylcitalopram	Influent Effluent Sludge	1) For sludge: MAE: Sample: 1 g/ 3 g (d.w.) Solvent: 20 mL MeOH/H_2O (50:50, v/v), 120 °C Exposure time: 30 min. SPE with Oasis MAX L: 20 mL MAE centrifuged supernatant + 65 mL H_2O, pH=7 E: 4 mL MeOH 2) For wastewater: SPE: Oasis HLB	LC-(ESI)QqQ	1) Chiral CBH (100×2 mm, 5 µm) H_2O/IPA (90/10, v/v) with 1 mM NH_4Ac 0.075 mL/min, 25°C 2) Chirobiotic V column (250×2.1 mm, 5 µm) MeOH containing 4 mM NH_4Ac and 0.005% FA	Influent: MDL: 0.03-28.74 ng/L MQL: 0.03-95.81 ng/L Effluent: MDL: 0.01-32.73 ng/L; MQL: 0.07-109.08 ng/L	[38]

Table 2. (Continued)

Compounds	Environmental matrix	Sample preparation	Analytical methodology	Chromatographic conditions	MDL/MQL	Ref.
Partially enantioseparated: norfluoxetine		L: 50 mL wastewater, pH=7 E: 4 mL MeOH In both cases: Evapor. to dryness Reconst.: 0.5 mL MP Filt.: 0.2 µm PTFE				
Fully enantioseparated: amphetamine, methamphetamine, mephedrone, MDA, MDMA, HMA, norfluoxetine, venlafaxine, pseudoephedrine, zopiclone, para-methoxyamphetamin **Partially enantioseparated:** MDEA, HMMA, tramadol, fluoxetine, ephedrine, norephedrine, desmethylvenlafaxine	Influent	SPE: Oasis HLB L: 100 mL W: 3 mL H_2O E: 4 mL MeOH Evapor. to dryness Reconst.: 0.5 mL MP Filt.: 0.2 µm PTFE	LC-(ESI)QqQ	Chiral CBH column (100×2.0 mm, 5 µm) 1 mM NH_4Ac aqueous buffer (pH=6.4) /MeOH (85:15, v/v) 0.1 mL/min, 25°C	MDL: 0.1-61.2 ng/L MQL: 0.1-320.8 ng/L	[39]
Fully enantioseparated: mephedrone, flephedrone, methedrone, methylone, butylone	River Effluent	SPE: Oasis MCX L: 500 mL river water, 250 mL effluent W: 10 mL MeOH E: 5 mL 5% NH_4OH in MeOH Evapor. to dryness Reconst.: 1 mL MP Filt.: 0.45 µm PTFE	LC-(HESI)Orbitrap	Chiral CBH (150x2 mm, 5 µm) 1 mM NH_4Ac aqueous buffer /MeOH (98/2, v/v) 0.4 mL/min, 30°C	River: MDL: 0.3-1.2 ng/L MQL: 1.0 -2.9 ng/L Effluent: MDL: 0.8-2.5 ng/L MQL: 2.3 - 6.0 ng/L	[40]
Fully enantioseparated: propranolol, sotalol, salbutamol, metoprolol, atenolol, mirtazapine, citalopram, desmethylcitalopram, MDMA, MDA, MDEA, amphetamine, fluoxetine, venlafaxine, O-desmethylvenlafaxine **Partially enantioseparated:** methamphetamine, alprenolol	Sludge Receiving water	SPE: Oasis HLB L: 50 mL E: 4 mL MeOH Evapor. to dryness Reconst.: MP	LC-(ESI)QqQ	Chiral CBH column (100x2 mm, 5 µm) H_2O/ IPA (90/10, v/v) with 1 mM NH_4Ac 0.075 mL/min, 25°C Chirobiotic V column (250x2.1 mm, 5 µm) MeOH containing 4 mM NH_4Ac and 0.005% FA 0.1 mL/min	Influent: MDL: 0.03-28.74 ng/L MQL: 0.11-95.81 ng/L Effluent: MDL: 0.02-30.80 ng/L MQL: 0.07-109.08 ng/L	[41]
Fully enantioseparated: 3-N-dechloroethylifosfamide, dihydroketoprofen, ibuprofen, naproxen, chloramphenicol, ifosfamide, aminorex, fexofenadine, tetramisole, 10,11-dihydro-10-hydroxy carbamazepine	Surface Effluent	SPE: Oasis HLB-MAX L: 500 mL river water, 250 mL effluent W: HLB with 2 mL H_2O, MAX with 2 mL of 5 % NH_4OH E: 4×1 mL MeOH for HLB-MAX,	LC-(ESI)QqQ	Chiral AGP (100×2 mm, 5 µm) 10 mM NH_4AC aqueous buffer (pH 6.7) /ACN (99/1, v/v)	Surface: MDL: 0.04-34.7 ng/L MQL: 0.14-118 ng/L	[46]

Table 2. (Continued)

Compounds	Environmental matrix	Sample preparation	Analytical methodology	Chromatographic conditions	MDL/MQL	Ref.
Partially enantioseparated: ketoprofen **No enantioseparated:** carboxyibuprofen, mandelic acid, 2-hydroxyibuprofen, 2-phenylpropionic acid, cephalexin, indoprofen		2×1 mL MeOH with 2 % FA for MAX Evapor. to dryness Reconst.: 0.5 mL MP Filt: 0.2 µm PTFE			Effluent: MDL: 0.08-23.2 ng/L MQL: 0.29-94.8 ng/L	
1) **Fully enantioseparated:** ketoconazole 2) **Fully enantioseparated:** econazole, tebuconazole **Partially enantioseparated:** miconazole, propiconazole	Influent Effluent Sludge	Sludge: ultrasound-assisted extraction & SPE: HLB Wastewater: SPE: HLB L: 100 mL wastewater, 200 mL river water, pH=3.2 E: MeOH Evapor. to dryness Recons.: H_2O/MeOH (90:10, v/v)	LC-(ESI)QqQ	1) Chiral HSA column (2 × 100 mm,5 µm) ACN/H_2O (10:90, v/v) with 10 mM NH_4Ac (pH 7.0) 0.25 mL/min, 25°C 2) Chiral AGP column (4 × 100 mm, 5 µm) 10 mM NH_4Ac aqueous buffer (pH 7.0)/ ACN Gradient elution, 0.3 mL/min, 25°C	MQL: Influent: 0.5-10 Effluent: 0.3-5 Sludge: 3-29 ng/g	[48]
Fully enantioseparated: nadifloxacin, ofloxacin, ofloxacin-N-oxide, prulifloxacin, flumequine, besifiloxacin **Partially enantioseparated:** desmethyl-ofloxacin, moxifloxacin ulifloxacin;	River Influent Effluent	SPE: Oasis HLB L: 50 mL wastewater, 100 mL river water W: 1 mL H_2O E: 4 mL MeOH Evapor. to dryness Reconst.: 0.5 mL MP Filt.: 0.2 µm PTFE	LC-(ESI)QqQ	Chiralcel OZ-RH column (150x2.1 mm, 5 µm) 10 mM NH_4Ac aqueous buffer /MeOH (1/99 v/v) with 0.05% FA 0.1 mL/min, 30°C	Influent: MDL: 0.2-33.9 ng/L MQL: 2.2-33.9 ng/L Effluent: MDL: 0.3-36.2 ng/L MQL: 1.6-36.2 ng/L River: MDL: 0.3-33.9 ng/L MQL: 2.6-33.9 ng/L	[50]
Fully enantioseparated: ibuprofen, naproxen, flurbiprofen	River water	SPE: Oasis HLB L: 500 mL, pH=4 W: 10 mL H_2O E: 5.5 mL MeOH + 2.5 mL MeOH/Acetone (1:1, v/v) Evapor. to dryness Reconst.: 0.5 mL MeOH with 0.025% FA Filt.: 0.22 µm PTFE	LC-(ESI)QqQ	Chiralpak AD-RH column (150×4.6 mm, 5 µm) 10 mM NH_4Ac aqueous buffer (pH 5)/ACN (65/35, v/v) 0.4 mL/min, 25°C	MDL:0.35-11.1 ng/L MQL: 1.1–37.1 ng/L	[51]

Table 2. (Continued)

Compounds	Environmental matrix	Sample preparation	Analytical methodology	Chromatographic conditions	MDL/MQL	Ref.
Fully enantioseparated: ibuprofen, naproxen, ketoprofen	Influent Effluent	SUPRAS-based microextraction 3.8 mL THF+ 76mg decanoic acid + 72.2 mL sewage Magnetically stir: 900 rpm, 10 min Centrifugation: 3500 rpm, 5 min withdraw 50 µL extract	LC-(ESI)QTRAP	Sumichiral OA-2500 THF/50 mM NH_4Ac in MeOH (90/10, v/v) a variable flow: 0- 18 min, 0.5 mL/min, then 1.2 mL/min, 25°C	MDL: 0.5–1.2 ng/L	[58]

ACN, acetonitrile; AGP, α1-acid glycoprotein; CBH, cellobiohydrolase; d.w., dried weight; E, elution; ESI, electrospray ionization; EtOH, ethanol; FA, formic acid; HMMA, 4-Hydroxy-3-methoxymethamphetamine; HSA, human serum albumin; IPA, isopropanol; L, loading; LC, liquid chromatography; MAE, microwave assisted extraction; MDA, methylenedioxyamphetamine; MDEA, 3,4-methylenedioxy-N-ethyl-amphetamine; MDL, method detection limit; MDMA, methylenedioxymethamphetamine; MeOH, methanol; MP, mobile phase; MQL, method quantification limit; NH_4Ac, ammonium acetate; NH_4OH, ammonium hydroxide; NSAIDs, non-steroidal anti-inflammatory drugs; QqQ, triple quadrupole; QTOF, quadrupole-time of flight; SPE, solid-phase extraction; THF, tetrahydrofuran; W, washing.

AGP-based CSPs exhibit an extremely broad enantioseparation capability that covers a wide variety of acidic, basic and neutral drugs. The chiral recognition mechanism of AGP is mainly based on hydrogen-bonding, hydrophobic and ionic interactions [37]. The mobile phase composition is almost the same as that of CBH columns. Enantioselectivity and retention are also easily regulated by changing the mobile phase pH and composition. Due to its broad enantioselectivity, the AGP column has been widely used in the field of environmental chiral analysis and can be used for multi-residue analysis [37,46,47]. For example, a broad group of chiral pharmaceutically active compounds (Table 2) including NSAIDs, anthelmintic drugs, anticancer drugs, gastrointestinal drugs, antibacterial drugs, antifungal drugs and antihistamine drugs has been determined in wastewater and river water using the Chiralpak AGP column with a mobile phase of 10 mM NH_4Ac aqueous solution modified with 1% ACN (pH 6.7) [46].

HSA contains two major binding sites, warfarin-azapropazone and indol-benzodiazepine, as well as several minor sites, and preferentially resolves acidic compounds [37]. To our knowledge, there is only one application of the HSA column in environmental analysis: the EF determination of ketoconazole in wastewater and sludge using LC–MS/MS with the Chiralpak HSA column and a mobile phase of ACN/H_2O (90/10, v/v) containing 10 mM NH_4Ac at pH 7.0 [48] (Table 2).

2.1.3. Polysaccharide-based CSPs

Cellulose and amylose derivatives are the most useful polysaccharide-based CSPs, and have been widely used for both preparative and analytical separations due to their wide chiral recognition and high loading capacity. Table 1 shows the structures of the selected cellulose and amylose derivatives that are commercially available.

The mechanism of chiral recognition of the polysaccharide CSPs involves hydrogen-bonding, hydrophobic, π-π and dipole-dipole interactions [49]. These interactions are relatively weak and are more effective in NP mode. Therefore, enantioseparations on polysaccharide-based columns are primarily explored in the NP mode that is not MS compatible, and thus the applications of polysaccharide CSPs for chiral pharmaceuticals in environmental analysis are quite limited [50–52]. These applications are employed in RP mode. Indeed, by careful optimization of mobile phase conditions, successful enantioseparations can also be achieved under RP or PO modes with polysaccharide type CSPs [53].

The organic modifier has a significant impact on the regulation of retention and enantioselectivity with polysaccharide CSPs under RP mode. It is common to use ACN or MeOH as an organic modifier. MeOH as a protic solvent competes with the analytes for the hydrogen-bonding sites of the CSPs, while ACN as aprotic solvent should not interfere with hydrogen-bonding interactions between analytes and CSPs. Therefore, MeOH and ACN would result in significantly different enantioseparations and they should be carefully evaluated during method development. For instance, MeOH-based mobile phase was demonstrated to be more effective than ACN-based mobile phase when the Chiralcel OZ-RH column was used for enantioseparation of (fluoro)quinolones and their metabolites in one study [50]; meanwhile ACN with 10 mM NH_4Ac aqueous solution (pH 5.0) (35/65, v/v) provided satisfactory enantioseparations for NSAIDs in other studies [51,52]. In general, the retention times of analytes decrease when the concentration of the organic modifier is increased.

When the polysaccharide CSPs are used, another important consideration is the control of the ionic behavior of the analytes since there are no ionizable groups in the polysaccharide to interact with charged analytes. Otherwise, a charged analyte is mainly distributed into a polar RP solvent, which weakens the interaction with the CSP, and eventually results in a poor enantioseparation [53]. For a neutral analyte, a mobile phase of water-organic modifier mixture is sufficient to obtain satisfactory enantioseparations [54]. For example, Zhou et al. [55] described enantioseparation methods for 19 neutral pharmaceutical compounds on six different polysaccharide-based columns under the RP mode. The results revealed that a mixture of water and

organic solvent (mainly ACN, or MeOH, EtOH and IPA in several cases) with gradient elution mode achieved enantioseparations for all the target compounds. For an acidic analyte, using an acidic mobile phase is essential for suppressing the dissociation of the analyte and thus minimizing ionic interactions [54]. For example, Ma et al. [51] evaluated the effect of mobile phase pH values (3–6) on the enantioseparations of ibuprofen, naproxen and flurbiprofen when the Chiralpak AD-RH column is used. The results showed that both the enantioresolution and retention time increased with the decreasing pH values due to their progressive suppression of the ionization of the analytes (Fig. 1). Consequently, the mobile phase at pH 3, 4 and 5 provided full baseline enantioseparations for all the analytes. However, considering that the higher signal-to-noise ratios and shorter analysis times were achieved at higher pH values, the pH of buffers was determined and adjusted to 5 as a compromise (see Table 2). For basic analytes, a basic mobile phase is preferable to suppress the dissociation of analytes. The enantioseparations of weakly basic analytes could be achieved with a mobile phase composed of an ammonium bicarbonate buffer and organic modifier [56]. For enantioseparations of strongly basic analytes, adjusting the pH of mobile phase to a higher value, such as 9, by adding diethylamine (DEA) are often required. However, the addition of DEA can significantly suppress analyte response in positive ESI mode even at a very low level in the mobile phase [56]. In addition, most silica supports are unstable at pH > 7. Therefore, the use of polysaccharide CSPs is limited in applications for basic analytes.

2.1.4. Pirkle-type

Pirkle-type CSPs are made of low-molecular-mass selectors that are bonded on the silica support forming an ordered layer (brush-type). They are therefore also referred to as brush-type CSPs [21]. The Whelk-O1 is the most widely used of the various Pirkle-type CSPs. As shown in Table 1, the Whelk-O1 selectors bear a strong π-acceptor aromatic group (3,5-dinitrobenzamide) and a π-donor aromatic moiety (naphthyl fragments), which are close to the stereogenic centre, providing sites capable of forming strong hydrogen-bonding, π-π and dipole-dipole interactions with the enantiomers [57]. In addition, the bulky groups or rigid structural elements can further enhance the enantiorecognition process via steric interacttions [36]. Therefore, this kind of CSP has an extremely broad applicability for the enantioseparation of analytes possessing an aromatic moiety with a hydrogen-bond acceptor group located close to the stereogenic centre. A significant advantage of the Pirkle CSPs is the ability to invert elution order by using the same type of CSP but with the opposite absolute configuration [57]. These CSPs are mainly used in the NP mode to enhance attractive interactions, but they are able to work in RP mode as well.

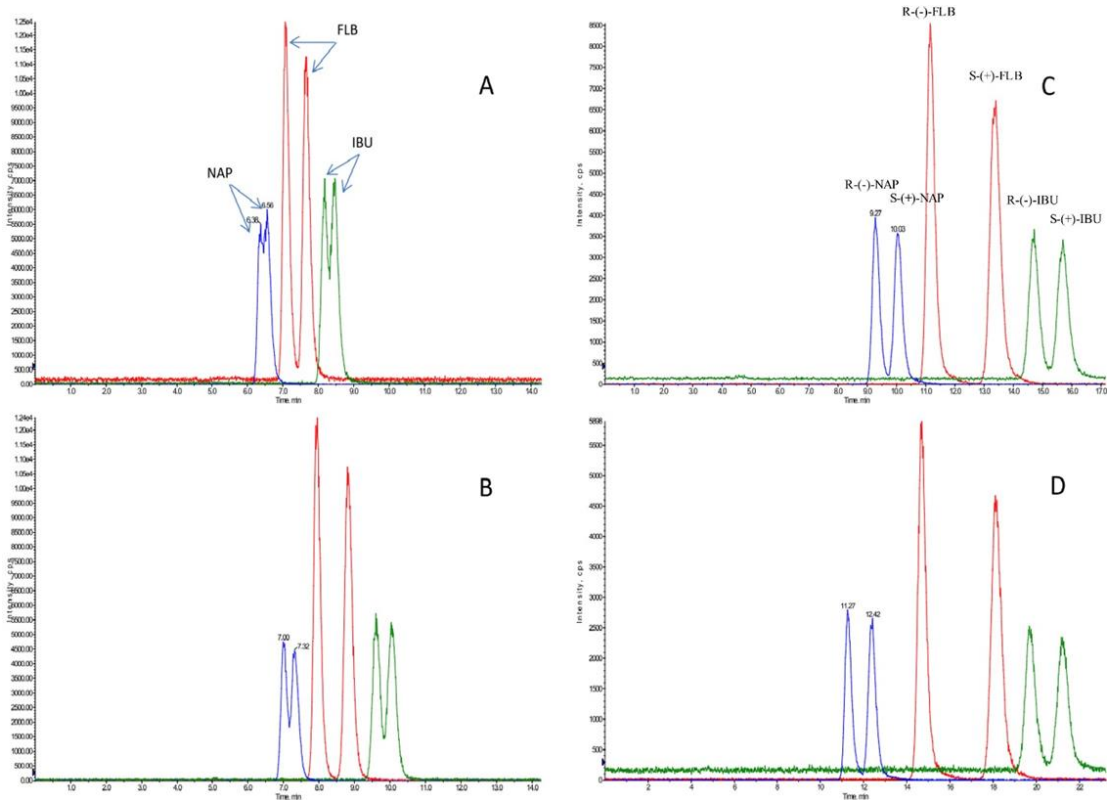


Fig. 1. Effect of different pH values of buffers in the mobile phase on the simultaneous enantioseparation of ibuprofen, naproxen and flurbiprofen using a Chiralpak AD-RH column. (A) pH = 6.0; (B) pH = 5.5; (C) pH = 5.0; (D) pH = 4.0; Mobile phase: ACN/10 mM NH_4Ac aqueous solution (35/65, v/v), flow rate: 0.4 mL/min. Reproduced from [51] with permission of Elsevier.

The applications of Pirkle-type columns in environmental chiral analysis are currently very limited. This kind of columns are effective for enantioseparation of acidic analytes such as NSAIDs, which could be complementary to macrocyclic glycol-peptide-based columns. For example, in one study [30], a Whelk-O1 column was applied for EF determination of NSAIDs (flurbi-profen, ketoprofen, ibuprofen and naproxen) and warfarin in surface water using LC–MS/MS with a mobile phase of MeOH/H_2O/FA (60/40/0.1, v/v/v), while a Chirobiotic V column was used in deter-mination of antidepressants, β-blockers and the others basic chiral drugs. In another study [58], ibuprofen, naproxen and ketoprofen were determined in influent and effluent wastewater using another Pirkle-type column, namely Sumichiral OA-2500, with a mobile phase composed of tetra-hydrofuran (THF)/50 mM NH_4Ac in MeOH (90/10, v/v).

2.2. Mass spectrometry detection

To date, EF determination of chiral drugs and their metabolites in environment-tal samples is still a significant challenge due to the very low concentrations of the

target drugs present and the complex environmental matrices with a large variety of non-target compounds. Although some studies have reported for the EF determination of drugs using LC and spectrophotometric techniques, such as ultraviolet (UV) [59,60] and fluorescence detection (FD) [61,62], LC–MS, and especially LC–MS/MS has generally been the technique of choice due to its sensitivity and specificity.

Although the LC–MS techniques employed are sensitive, uncertainties in the EF determination and quantification of chiral drugs might be introduced as a result of inadequate enantioselective separation, matrix effects and poor quantification. Firstly, it should be emphasized that sufficient chromatographic enantioseparation is obligatory since a pair of enantiomers have the same *m/z*. However, the method development including the selection of elution mode and mobile phase composition is limited considering the compatibility with MS analyzers. As discussed above, NP solvent systems are generally not suitable for MS detection due to the possible explosion hazard, despite the fact that many chiral columns are preferentially used with the NP elution mode. In addition, the salts and additives used in the mobile phase have to be volatile, which has implications for the enantioseparation and ionization of the compounds. Therefore, the requirement of compatibility with MS involves an extra challenge for achieving successful enantioseparations.

Moreover, enantioselective signal suppression or enhancement caused by matrices can occur, resulting in faulty analytical results in enantiomeric compositions. For instance, Camacho-Muñoz et al. [46] reported an enantioselective matrix effect of 10,11-dihydro-10-hydroxycarbamazepine, prazi-quantel and chloramphenicol in effluent wastewater: e.g. in the case of chloramphenicol, signal suppression accounting for 41.3 % and 18.5 % was observed for 1R,2R-(–)- and 1S,2S-(+)-enantiomers respectively. To overcome this issue, isotopically labelled internal standards should be used during the enantiomeric quantification. Furthermore, an appropriate clean-up step in the sample extraction is beneficial to minimize matrix effects.

Among various MS analyzers, triple quadrupole (QqQ) is the most used analytical technology in environmental chiral analyses, as shown in Table 2. Numerous examples of LC–MS/MS (QqQ) applications have been reported [28–31,39,41–44,50,51]. An example of this method determined five chiral pharmaceuticals (atenolol, propranolol, metoprolol, fluoxetine and venlafaxine) as well as six of their metabolites in surface water using a Chirobiotic V column in RP mode. The method detection limit (MDL) and method quantification limit (MQL) ranged between 0.1–2.2 ng/L and 0.2–7.2 ng/L, respectively [29].

Further improvements in selectivity were achieved by using hybrid high-resolution mass spectrometry (HRMS), such as quadrupole-time of flight (QTOF). Improved mass accuracy provides more

reliable identification of analytes in complex matrices. Moreover, QTOF analyzers provide the capability of operating in full-scan mode to capture all of the ions in one run, thus making retrospective analysis possible for target, post-target and non-target analytes. This is especially useful for monitor break-down products of chiral drugs in environmental analyses [34]. Several studies involving enantioselective analyses of multi-class drugs using the chiral LC-QTOF approach have been published [32,34]. Although the method based on QTOF provides increased selectivity compared to the QqQ method, the sensitivity of the QTOF method was found to be lower. To demonstrate this, Kasprzyk-Hordern et al. [44] reported that instrument detection limits (IDLs) ranged from 0.025 to 0.1 mg/L when LC–MS/MS (QqQ) methods were employed with the Chiralpak CBH column in RP mode for EF determination of methamphetamine, amphetamine, methylenedioxy-amphetamine (MDA) and methylenedioxy-methamphetamine (MDMA) in wastewater. Meanwhile, higher IDL values ranging from 1.25 to 5.0 mg/L were observed when the QTOF analyzer was used with the same column and mobile phase composition [34].

Currently, the high-resolution single-stage Orbitrap analyzer is increasingly used in research laboratories and has been extended to chiral environmental analyses. These analyzers provide higher resolution (from 70 000 up to 280 000 at 200 *m/z*) and mass accuracy (< 1 ppm with internal calibration), which significantly affects the selectivity and thus provides the required sensitivity gain, compared to TOF analyzers (10 000–40 000 at full width at half maximum) [63]. Furthermore, high-energy collision-induced dissociation (HCD) fragmentation is available for Orbitrap to generate all-ion-fragmentation data for additional confirmation. Souchier et al. [27] developed a chiral LC-Orbitrap-HRMS method in full-scan mode for the EF determination of metoprolol and three of its major metabolites (metoprolol acid, α-hydroxymetoprolol and O-desmethylmeto-prolol) in influent and effluent wastewater, obtaining instrument quantification limit (IQL) values ranging from 0.5 to 1.1 mg/L. More recently, a LC-HRMS (Orbitrap) method developed with a Chiralpak CBH column was applied for the EF determination of a group of cathinones (mephedrone, flephedrone, methedrone, methylone, and butylone) in river water and effluent wastewater. In this case, IDLs and IQLs ranged from 0.1 to 0.5 mg/L and from 0.3 to 1.2 mg/L, respectively [40].

3. Applications of chiral LC–MS based methods for EF determination of chiral drugs in environmental samples

After being administered, chiral drugs are subjected to complex metabolic processes, such as absorption, distribution and metabolism, and are consequently excreted into the sewage system either as parent unaltered compounds or in the form of metabolites or conjugates [11]. For example, 40–50 % atenolol is excreted in urine unchanged [2]. However, significant stereoselectivity can occur during the

metabolism process, which would change the enantiomeric composition of these drugs. These chiral drugs are introduced into sewage systems either by human excretion or due to disposal from the manufacturing process, and then incompletely eliminated after undergoing biotic and abiotic remove processes in WWTPs [1]. In general, biotic processes such as microbial degradation and metabolism can lead to stereoselective enrichment or depletion of enantiomers of chiral drugs, while abiotic processes such as sorption, photo-degradation and volatile-zation are generally non-stereoselective [47]. Once these chiral compounds have been released into the environment (such as surface water) from WWTPs, further enantioselective degradation can take place due to microbially mediated transformation. As a result, these chiral drugs are not present in the environment with the same enantiomeric composition as their original form.

Tracing the occurrence of each enantiomer for chiral drugs in the environment is helpful for providing information about their environmental transport and fate and for assessing the accurate impact of chiral drug residues towards environmental organisms. To date, a large number of studies have been published whose research area is focusing on the chiral drugs that are frequently used and are thus of environmental concern, including β-blockers, NSAIDs, anti-depressants and illicit drugs. Fig. 2 compiles the results obtained from these monitoring studies in the form of a range of EF values reported for selected chiral drugs in the environment among different countries. It should be remembered that EF = 1 or 0 indicates a single enantiomer, while EF = 0.5 indicates the racemate form [64]. We discuss these applications below according to the most common groups of chiral drugs.

3.1. β-blockers

The β-blockers, a class of drugs used to treat cardiovascular disorders, are among the most frequently used chiral drugs. Most β-blockers are marked as racemic mixtures, and the enantiomers show stereoselectivity in the pharmacodynamic and pharmaco-kinetic behaviors: S-(−)-enantiomers usually exhibit much higher potency in cardiac β-blocking than R-(+)-enantiomers [45].

The EF determination of β-blockers has mainly employed macrocyclic glycopeptide columns. For example, Xu et al. [35] applied the Chirobiotic T column in PO mode with the mobile phase of MeOH containing 5 mM NH_4Ac and 0.005 % FA for EF determination of 5 common β-blockers (propranolol, atenolol, nadolol, metoprolol and sotalol) in wastewater, canal water, soil, sediment and sludge samples. In another study, the Chirobiotic V column was used in RP mode for EF determination of six β-blockers in effluent [65]. In several studies, protein-based columns (CBH and AGP columns) showed enantioselectivity for certain β-blockers. For example, enantioseparation of atenolol was carried

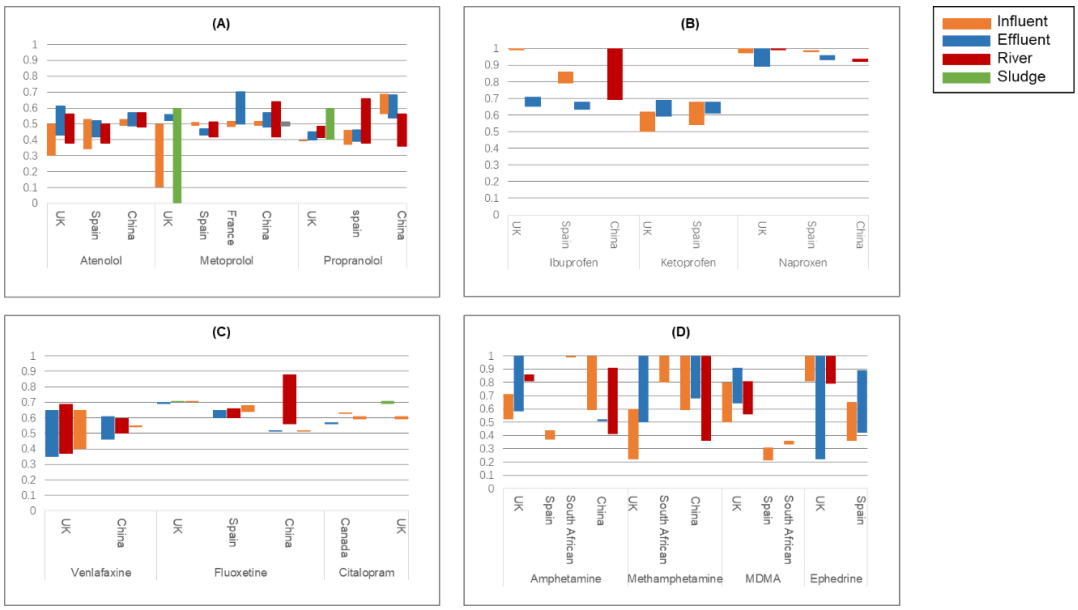


Fig. 2. The range of EF values reported for selected chiral drugs in the environment among different countries. (A) β-blockers; (B) NSAIDs; (C) antidepressants; (D) illicit drugs.

out with a CBH column and a mobile phase of H_2O/IPA (90/10, v/v) containing 1 mM NH_4Ac (pH = 5) [42,45]. Some studies have compared the performance of the Chirobiotic V and chiral CBH column for β-blockers. Evans et al. [38] reported that alprenolol, atenolol, metoprolol, propranolol and sotalol were enantioseparated on Chirobiotic V column under RP mode, while no analytes were separated on the CBH column. In another study [34], the Chirobiotic V column was also proven to be more selective than the CBH column for metoprolol and propranolol, while the CBH column provided much better enantioseparation for atenolol.

Based on the monitoring studies [29-31,38,41,45,65] conducted in different regions, β-blockers were found to be abundant in wastewater and even in river water, especially atenolol, metoprolol and propranolol, with various EF values reported in the environment (see Fig. 2A). In a study [42] conducted in Spain, the enantiomers of atenolol were determined in influent at concentrations from 538 to 1322 ng/L with EF values ranging from 0.34 to 0.53. It is expected that S-(−)-atenolol is enriched due to the preferential metabolism of R-(+)-enantiomer in humans. A similar situation for metoprolol and propranolol in influent wastewater was observed in another study [38] in which an enrichment of S-(−)-enantiomer took place. In contrast, López-Serna et al. [31] reported that atenolol, metoprolol and satalol were present in influent wastewater as racemic mixtures (the EF values were close to 0.5). This observation could be attributed to the diversity of metabolism stereoselectivity

among individuals or disposal from the manufacturing process. In addition, some studies have researched the enantiomeric fate of certain β-blockers during the wastewater treatment process, applying chiral LC–MS based methods, and the results indicated that the stereoselective removal depends strongly on the type of wastewater treatment technology used. In a monitoring study [45] undertaken in seven WWTPs in the UK, it was observed that atenolol has higher stereoselectivity during treatment in the WWTPs implementing activated sludge processes than those implementing trickling filters, which could be attributed to the different consortia of microorganisms used in the two treatment processes. However, it should be noted that different stereoselective chiral-drug removal processes can even occur among WWTPs that use the same treatment technology. In the eight major WWTPs studied in Beijing, China, while an obvious enrichment of S-(+)-metoprolol was detected as a result of preferential degradation of R-(−)-enantiomer in one of the WWTPs, which employed anaerobic/anoxic/oxic, a membrane bio-reactor, and joint disinfection with sodium hypochlorite and ozone, no or weak stereoselectivity was observed in the other two WWTPs that employed the same treatment technology [66].

Significantly, varying EF values were often observed in different surface water samples. For example, it was reported that EF values of atenolol, propranolol and sotalol ranged between 0.38−0.50, 0.38−0.66 and 0.41−0.65, respectively in the Guadalquivir River [31]. This might have resulted from the different microbial populations involved in the stereoselective degradation occurring in the environment. Kasprzyk-Hordern et al. [45] reported seasonal variations of EF values of atenolol in receiving water: the lowest EF value was recorded for atenolol in August (0.39 ± 0.00) and the highest in March (0.56 ± 0.09). The higher stereoselectivity observed in the receiving water over the spring/summer time might be due to the potentially higher activity of microorganisms.

3.2. Non-steroidal anti-inflammatory drugs

NSAIDs are among the most frequently prescribed drugs worldwide. Most NSAIDs, such as ibuprofen and ketoprofen, are marketed as racemic mixtures despite the stereoselectivity of their pharmacokinetics between enantiomers. Naproxen is marketed as the S-(+)-enantiomer due to the known hepatotoxicity of the R-(−)-enantiomer [9,58]. The enantiomeric occurrence of NSAIDs in the environment has been conducted by applying LC–MS based methods with various chiral columns including macrocyclic glycopeptides [31,39], protein-based [46,47], polysaccharide-based [51,52] and Pirkle type CSPs [30,58]. As an example, in a monitoring study undertaken in southwest England, Camacho-Muñoz et al. [47] used a Chiral AGP column with a mobile phase composed of 10 mM NH_4Ac with 1 % of ACN (pH 6.7) for EF determination of

several NSAIDs, including ibuprofen, ketoprofen, naproxen, chloramphenicol and their metabolites.

NSAIDs were generally found to be enriched with S-(+)-enantiomer in wastewater matrices (see Fig. 2B). For example, an obvious enrichment of S-(+)-ibuprofen was observed in influent (EF = 0.79−0.86), which could be due to the unidirectional chiral inversion of inactive R-ibuprofen to active S-ibuprofen in vivo [58]. In the case of naproxen, the EF values in influent wastewater were close to 1.0 [47,58], since it is dosed in a pure enantiomeric form of the S-(+)-enantiomer.

During wastewater treatment, stereoselective removal of NSAIDs has often been reported. For example, Camacho-Muñoz et al. [47] reported that the EF values of ketoprofen in influent wastewater were close to the racemic mixture (0.56 ± 0.06), whereas a higher EF value of 0.64 ± 0.05 was observed in effluent wastewater. This was attributed to the preferential degradation of R-(−)-enantiomer. In addition, the authors also reported that the EF values of naproxen decreased from 1 to 0.89 ± 0.02 in one WWTP of the study and from 0.97 ± 0.02 to 0.89 ± 0.03 in another WWTP after wastewater treatment. This could suggest that chiral inversion occurs during the treatment process.

3.3. Antidepressants

A number of studies have been performed to determine the enantiomeric composition of chiral antidepressants, mainly focused on venlafaxine and fluoxetine, in various environmental matrices by applying a Chiral CBH or a Chirobiotic V column. For instance, Duan et al. [66] studied the enantiomeric composition of venlafaxine and fluoxetine as well as three β-blockers in eight major WWTPs in Beijing using a Chirobiotic V column with a mobile phase composed of MeOH/H_2O (85/15, v/v) containing 10 mM NH_4Ac (pH = 4). In another study [45], venlafaxine was enantioseparated using a Chiral CBH column and mobile phase composed of H_2O/IPA (90/10, v/v) containing 1 mM NH_4Ac (pH = 5). According to the comparison studies [34,38], it can be concluded that the Chirobiotic V column was more selective than the CBH column for fluoxetine and venlafaxine.

Venlafaxine and fluoxetine were found to be omnipresent in both the surface water and wastewater. The enrichment of S-(+)-enantiomer for fluoxetine has been observed in the UK [38,41], Spain [31] and China [66], with EF values ranging from 0.51 to 0.88. However, the EF of venlafaxine varied significantly in different environmental water samples (see Fig. 2C). For example, in a monitoring program performed in seven WWTPs in the UK over the period of nine months, the results revealed that the EFs of venlafaxine were 0.35−0.65 in influent and 0.46−0.69 in effluent as a result of weak stereoselective removal during wastewater treatment [45]. A recent study [29] conducted in China reported that EF values of venlafaxine were

close to 0.5 in river water both in the wet season and the dry season in or between rivers (EF = 0.54 ± 0.06 in the wet season and 0.55 ± 0.03 in the dry season).

3.4. Illicit drugs

The enantiomeric profiling of chiral illicit drugs, such as amphetamine-like drugs, ephedrines and cathinones, has attracted extensive attention due to the increasing consumption of the drugs and different potencies between enantiomers. Most of these drugs are taken as racemates, with a few exceptions, such as methamphetamine. This drug is commonly marketed as the pure S-(+)-enantiomer. However, racemic methamphetamine can also be abused depending on the synthetic route. To date, for the EF determination of illicit drugs studies [38,41-45,67,68] have mainly used the CBH column with the typical RP mobile phase (such as, H_2O/IPA (90/10, v/v) containing 1 mM NH_4Ac (pH = 5)). In a few cases, macrocyclic glycopeptide columns (Chirobiotic V or Chirobiotic V2) have been applied for EF determination of certain amphetamines [30,32,33,69]. Nonetheless, some studies have shown that the CBH column was more effective for chiral illicit drugs than macrocyclic glycopeptide columns [34,38,39]. For example, Bagnall et al. [34] reported that the CBH column provided much better resolution for methamphetamine, amphetamine, MDMA and MDA than the Chirobiotic V column.

Diverse enantiomeric occurrences of illicit drugs in the environment have been found among different countries (see Fig. 2D). Xu et al. [69] traced the enantiomeric occurrence of amphetamine and methamphetamine in wastewaters and river waters across China using the chiral LC–MS/MS (QqQ) method. The results indicated that there was considerable amphetamine and methamphetamine abuse in many major cities in China, and both amphetamine and methamphetamine generally showed a predominance of S-(+)-enantiomer in river water and wastewater. Archer et al. [70] also found the enrichment of the S-(+)-enantiomer of amphetamine and methamphetamine in influent wastewater in South Africa. In contrast, the enrichment of the R-(−)-enantiomer of amphetamine was reported in the studies conducted in the UK [45] and Spain [42]. The spatial variation of EF values may result from the different trends in illicit drug abuse among countries. As a good example, in a monitoring study [71] performed in eight European cities, racemic meth-amphetamine was detected in Oslo wastewater samples (EF = 0.49 ± 0.02), whereas S-(+)-methamphetamine was the predominant enantiomer in other European cities. The authors concluded that this was due to different synthetic or trafficking routes used for methamphetamine in Oslo where this drug is mainly available as racemate.

EF determination of illicit drugs in wastewater provides a valuable method for estimating the community-wide consumption of these drugs based on wastewater-based epidemiology (WBE). Compared with the traditional epidemiological tools,

such as population surveys, WBE can be used to carry out more comprehensive and real-time monitoring at a lower cost and in less time [70]. However, it should be emphasized that the overestimation of consumption could result from the direct disposal of unused drugs and production waste. Therefore, it is important to identify the source of chiral drug residues in wastewater to minimize the uncertainty in WBE-based estimation. As mentioned above, many of the chiral illicit drugs (e.g. amphetamines and MDMA) are often abused as racemates. After consumption, the enantiomeric composition of chiral drugs is deviated from 0.5 (racemic form) as a result of the stereoselective metabolism of different enantiomers in the human body. Therefore, if the chiral drug is in racemic form in the wastewater, it might have resulted from direct disposal of the drug rather than its consumption. For example, S-(+)-MDMA is preferentially metabolized, resulting in the enrichment of MDMA with R-(−)-enantiomer [31]. In a monitoring campaign in Dutch cities conducted by Emke et al. [43], MDMA was found to be enriched in wastewater with R-(−)-MDMA with $EF = 0.68 \pm 0.04$ during the 2010 sampling campaign, suggesting that the MDMA found in 2010 resulted from abuse of the drug rather than direct disposal of the unused drug. In contrast, in the 2011 sampling campaign, extremely high levels of MDMA were found to be racemic ($EF = 0.51$) indicating direct disposal of unused MDMA possibly as a result of a police raid at a nearby illegal production facility. Similarly, in another study [71] undertaken in the UK, mephedrone was reported to be enriched with the R-(+)-enantiomer in wastewater, suggesting that the drug had been consumed rather than disposed of directly.

In addition, enantiomeric profiling of chiral illicit drugs can help to identify whether drug residues in wastewater are derived from direct use or metabolic formation from other drugs, which is also important in the estimation of their consumption. MDA, as an example, is an abused drug and is also a metabolite of MDMA [71]. Similarly to MDMA, MDA is abused in racemic form and S-(+)-MDA undergoes preferential metabolism leading to enrichment of excreted MDA with R-(−)-enantiomer. However, if the presence of MDA resulted from MDMA abuse, an enrichment of S-(+)-MDA should be expected in urine as a result of the preferential metabolism of S-(+)-MDMA. Kasprzyk-Hordern et al. [67] reported that the MDA in influent wastewater was enriched with S-(+)-enantiomer, indicating that the presence of MDA might be linked to the metabolism of MDMA rather than direct MDA consumption. The ratio of MDMA to MDA was also tested to confirm the hypothesis. Similarly, in a 7-year study conducted in Australia [33], the enantiomeric composition of amphetamine and methamphetamine as well as the amphetamine/methamphetamine ratio were investigated. The results suggested that the consumption of methamphetamine was the major source of amphetamine residues in wastewater samples.

For those illicit drugs that also have medical usage, such as amphetamine, methamphetamine and ephedrine, another important consideration in their consumption estimation is the differentiation between medical uses and illicit uses. For example, amphetamine can be excreted as a result of the metabolism of certain pharmaceuticals, such as selegiline (R-(−)-amphetamine), clobenzorex (S-(+)-amphetamine) and fenproporex (S(+)/R(−)-amphetamine) [67]. Due to different uses of these chiral drugs (both medical and illicit), estimation of their abuse via enantiomeric analysis of wastewater is difficult. In one study [71], enantioselective profiling was combined with consumption data of legal prescription use from official health statistics to research the consumption of illicit drugs in Europe. The authors proposed that the presence of amphetamine and methamphetamine in Bristol mainly resulted from illegal use since the contribution of estimates from the legal sources was negligible, while the presence of ephedrine was reasonably attributed to its medical use.

Thus, determining the presence of enantiomers is a potential tool for many applications. The development of efficient and accurate chiral LC–MS based methods for environmental analysis would certainly be attractive.

4. Conclusions

The chiral LC–MS based method is the technique of choice for EF determination of chiral drugs in complex environmental matrices. This could largely be attributed to the various chiral columns that are commercially available, the flexible mobile phase combination and sensitive MS detection. The selection of the chiral column and mobile phase composition is fundamental to provide the sufficient enantioresolutions for chiral drugs. These processes are still complicated and time-consuming. An important consideration during the optimization of enantio-separation, is the compatibility of the mobile phase with the MS analyzer, which implies an extra challenge for satisfactory enantioseparations. In addition, further improved sensitivity would be needed for the LC–MS based method, due to the very low concentration of the target drugs present in environmental matrices.

In recent years, a rapidly increasing number of studies have applied diverse chiral LC–MS based methods for the EF determination of chiral drugs in environmental matrices to assess the enantiomeric distribution and fate of chiral drug residues in the wastewater treatment process and the environment, and to provide real-time information on the community-wide consumption of illegal drugs. Nevertheless, the data available are still limited and inconsistent in some cases. As various chiral LC–MS methods are being continually developed, the EF determination could be expanded to a wide range of chiral drugs. It shows a great potential to provide a fast, cheap and accurate way to investigate the consumption of various

chiral drugs. Furthermore, the information offered by EF determination of chiral drugs would be very valuable to completely understand the enantioselective behaviors and impacts of chiral drugs in the environment. In general, the development of EF determination of chiral drugs in the environment by chiral LC–MS based method will increasingly attract interest in many areas.

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CHAPTER 2. OBJECTIVE

The objective of the present Doctoral Thesis is the development of analytical methods based on the use of SPE in combination with LC-HRMS to determine the enantiomeric composition of chiral drugs in environmental samples including river water and effluent wastewater.

To achieve this objective, enantioseparation methods based on LC using different CSPs coupled to HRMS will be optimized and investigated. Moreover, different SPE strategies will be evaluated to promote the enrichment of the analytes and selective determinations.

CHAPTER 3. EXPERIMENTAL, RESULTS AND DISCUSSION

As previously discussed in the Introduction of this Thesis, the enantiomeric determination of chiral drugs in the environment is crucial to better understand their environmental occurrence, fate and toxicity. Chiral drugs sold as racemic mixtures generally undergo enantioselective metabolism and transformation in the human body, WWTPs and the environment, which would alter the enantiomeric composition of these drugs [1,2]. Enantiomeric profiling of chiral drugs in the environment offers a distinction between drug consumption and direct disposal, which is important for estimating the community-wide consumption of these drugs based on waste water epidemiology (WBE) [1]. Changes in EF values of these chiral contaminants in the treatment process can be also an indicator of the removal efficiency of treatment systems [3]. Therefore, the development of analytical methods for the enantiomeric determination of chiral drugs is of great interest in various fields.

Due to the very low concentrations of the target drugs present and the complex environmental matrices, enantiomeric determination of chiral drugs and their metabolites in environmental samples is still a significant challenge. To date, SPE combined with chiral LC-MS has been the dominant technique for environmental analysis owing to its simplicity, sensitivity and wide scope of applicability [4,5]. The research in this Thesis mainly focuses on the development of new selective and sensitive analytical methods applying these techniques for the enantiomeric determination of chiral drugs in environmental samples. The Doctoral Thesis has been developed in the research group of Chromatography and Environmental Applications at the Universitat Rovira i Virgili in Tarragona, which has extensive experience in the determination of organic contaminants in different kinds of environmental samples although these are the first studies involving chiral LC.

This chapter includes the experimental part and results from the different studies that have been carried out through the course of this Thesis. Most of these results have already been published in international scientific journals, and therefore they are presented in article format. The results are divided in two sections that include the papers published and some additional results that did not result in a paper. In each section, a brief introduction is included to establish the context of the research, and the most notable results are also discussed after the papers. The list of the published papers resulting from this Thesis is included in Appendix II.

In the first section, different enantioselective methods for the enantiosepration of β-blockers, omeprazole and cathinones has been developed. Different chromatographic columns were evaluated for their chiral recognition abilities for the targeted chiral drugs in different elution modes. The factors that affected the enantioseparation were tested and optimized to achieve successful enantioseparations.

In the second section, SPE followed by LC-HRMS methods have been developed and applied for the enantiomeric determination of cathinones in different environmental water samples. SPE sorbents including Oasis MCX, Oasis WCX and in-house synthesized molecularly imprinted polymers (MIPs) were evaluated for the selective extraction of a group of cathinones in environmental samples.

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3.1. Enantioseparation of chiral drugs

It is well known that enantiomers of chiral drugs often exhibit differences in pharmacology, toxicology and pharmacokinetics and thus have to be treated as independent entities [1]. The promotion of chiral separation is of great significance in various fields such as the pharmaceutical industry, clinical analysis and environmental analysis. The chiral separation technique has progressed since the early 1980s [2]. To date, different analytical technologies have been applied for the enantioseparation of chiral drugs including LC, GC, SFC and CE. Among them, as discussed in the Introduction, LC using CSPs is the most widely used technique mainly due to the availability of numerous commercial CSPs (i.e. polysaccharide-based, Pirkle-type, cyclodextrin-based, macrocyclic antibiotics, ligand exchange, proteins and chiral crown ethers) and the high versatility provided by the different elution modes (NP, RP, PO or PI modes) [3,4]. This section focuses on the enantioseparation of a group of chiral drugs including β-blockers, omeprazole and cathinones by LC using different CSPs.

These studies were the first investigations of our research group using chiral LC with CSPs for chiral separation. In order to understand and verify the influence of different chromatographic conditions on chiral separation and retention, we first selected several β-blockers and omeprazole whose enantioseparations and chiral recognition mechanisms involved in have been already well documented in previous studies. Then we added the cathinones of interest to the list of target analytes, as they have gained popularity as new psychoactive substances (NPSs) and their enantioseparation was less studied.

For this purpose, in the first study, two kinds of chiral columns were selected: a polysaccharide-based column (Lux Cellulose-1) which is universal, and a protein-based column (Chiralpak CBH) which is effective for the enantioseparation of basic drugs and used exclusively in RP mode enabling couple to MS. Their chiral recognition abilities towards the target analytes were evaluated and compared in PO mode and RP mode, respectively. The effect of mobile phase composition and flow rate on the enantioseparations were tested and optimized. Moreover, particular attention was paid to the compatibility of the mobile phase with MS detection in the development of the method to enable coupling with a MS detector for enantiomeric determination of chiral drugs in environmental matrices in future research. The result obtained from this study has been published in the journal Chirality 32 (2020) 876-884, and is presented in the following section.

The second study presents the evaluation of four in-house synthesized polymeric materials as CSPs for the enantioseparation of β-blockers, omeprazole, cathinones and ibuprofen in NP mode. These polymeric materials were prepared based on poly(divinylbenzene-co-vinylbenzyl chloride) microspheres modified with (1S, 2S)-(-)-1,2-diphenylethylenediamine (DSA 35), (1S, 2S)-(-)-1,2-di-1-naphtyl-ethylenediamine (DSA 37), D-phenylaniline methylester (SD 099) and L-phenylaniline methylester (SD 100),

respectively. This work was carried out in collaboration with Dr. Daniela Salas, former PhD student of our research group, Dr. Stuart Dargo and Prof. Peter A.G. Cormack from University of Strathclyde in Glasgow, Scotland (United Kingdom), where these polymeric materials were synthesized with the aim of obtaining chiral material for chromatographic separation. This study is not presented in article form considering its preliminary state, and more experimental work is still needed.

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3.1.1. Comparison of polysaccharide -based and protein -based chiral liquid chromatography columns for enantioseparation of drugs

COMPARISON OF POLYSACCHARIDE-BASED AND PROTEIN-BASED CHIRAL LIQUID CHROMATOGRAPHY COLUMNS FOR ENANTIOSEPARATION OF DRUGS

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Abstract

Two different columns—Lux Cellulose-1 and Chiralpak CBH—were evaluated for their chiral recognition abilities for eight drugs comprising three β-blockers, one antiacid, and four cathinones in polar-organic elution mode and reversed-phase elution mode, respectively. The factors that affected the enantioseparation were tested and optimized to develop a suitable chiral separation method whose LC conditions are compatible with MS detection. In polar-organic elution mode with the Lux Cellulose-1 column, methanol and acetonitrile were tested as the main components of the mobile phase. In addition, the effects of adding isopropanol as organic modifier, acidic additives (formic acid), and basic additives (diethylamine) were evaluated. In reversed-phase elution mode with the Chiralpak CBH column, the effect of type and concentration of organic modifier (isopropanol, acetonitrile, and methanol), the mobile phase pH (6.4 and 5.0), and buffer concentration (1 mM-20 mM ammonium acetate) were evaluated. The best enantioseparation was achieved with the Chiralpak CBH column with a mobile phase composed of 5 mM ammonium acetate aqueous (pH = 6.4)/methanol (95/5, v/v) at a flow rate of 0.1 mL/min and a temperature of 30°C. Under these conditions, six of eight chiral drugs were baseline separated.

Keywords: chiral drugs, chiralpak CBH column, enantioseparation, Lux Cellulose-1 column, polar-organic mobile phase, reversed-phase

1. Introduction

Chiral separation has recently attracted much attention, not only in the pharmaceutical field but also in the environmental and biological areas as well. This is due to the increasing evidence of pharmacological, pharmacokinetic, and toxicological differences between enantiomers. Over half of the drugs currently on the market are chiral [1], and for these, one enantiomer is usually pharmacologically active, while the other may be less active or even can be harmful [1-3]. For example, S-propranolol is 100 times more active than R-propranolol [3]. However, many chiral drugs are still sold as racemic mixtures because of the difficulty of chiral separation, the high cost of production, and the similar pharmacological effects between enantiomers [4]. As a result, there is increasing demand for the enantioselective determination of chiral drugs [5].

It is well known that enantiomeric analysis in biological systems is decisive to understand the stereoselective implications, therapeutic use, and toxicology of pharmaceuticals [6]. For example, stereoselective disposition was observed for carvedilol – an antihypertensive drug – with higher excretion rates for the S-enantiomer by monitoring its enantiomer concentration in human plasma [7]. On the contrary, the role of stereoselectivity is normally neglected in environmental studies. However, enantiomers of chiral drugs often exhibit stereoselectivity in environmental occurrence, fate, and toxicity [1]. For example, Stanley et al. [8] reported that S-fluoxetine was 9.4 times more toxic for *P. promelas* than R-fluoxetine. Therefore, it is also important to investigate the enantio-meric composition of chiral drugs in the environment to provide a more realistic risk assessment of chiral contaminants [1].

LC using chiral stationary phases (CSPs) is the most commonly used technique for the enantioselective determination of chiral drugs [2]. Several chiral stationary phases have been designed using different chiral selectors (ie, polysaccharides, proteins, macrocyclic antibiotics, cyclodextrins, Pirkle type, ion exchangers, and chiral crown ethers). Of these, polysaccharide-based CSPs are the most frequently used due to their wide chiral recognition and high loading capacity [9,10]. Various polysaccharide-based columns have been developed and are commercially available, such as the Lux series (eg, Lux Cellulose-1) from Phenomenex (Torrance, California) and the Daicel Chiralpak and Chiralcel series (eg, Chiralpak IA and Chiralcel OD) from Chiral Technologies (Exton, Pennsylvania). Polysaccharide-based columns can be used in normal-phase (NP), reversed-phase (RP), or polar-organic (PO) mode. As the NP mode is favorable for its principal mechanisms of chiral recognition (ie, hydrogen bonding interaction), most chiral separations with polysaccharide phases are performed in NP mode using hexane and alcohol modifiers as mobile phase components [11-13]. However, these mobile phases are not compatible with MS

detection. There are several studies showing that polysaccharide-based chiral columns in PO mode can achieve successful enantioseparations for a wide range of chiral compounds [9,10,14]. The PO mobile phase made up of polar-organic solvents such as acetonitrile (ACN), alcohols, or a mixture of them offers several advantages including improved solubility of analytes, favorable peak shapes, and LC-MS compatibility.

Protein-bonded CSPs, which are used exclusively in RP mode, have become popular due to the character of the chiral selector that can be changed by a simple modification of the mobile-phase composition, allowing a wide range of enantiomers to be separated [15,16] Nowadays, a broad variety of protein-based columns have been commercialized, of which the Chiralpak CBH and the Chiralpak AGP columns appear to be the most used so far.

The major aim of this study is to evaluate the effect of different parameters on the enantioseparation of eight drugs comprising β-blockers, antacid, and a group of new psychoactive drugs named cathinones. A polysaccharide-based column (Lux Cellulose-1) and a protein-based column (Chiralpak CBH) were examined for their chiral recognition abilities in PO mode and RP mode, respectively. The compatibility of the mobile phase with LC-MS detection was taken into account in the development of the method with a view to allowing coupling with an MS detector for determining individual drug enantiomers in environmental and biological matrices in future research.

2. Materials and methods

2.1. Chemicals and solutions

The standard analytes—(±)-atenolol, R-(+)-atenolol, (±)-propranolol hydro-chloride, R-(+)-propranolol hydro-chloride, (±)-metoprolol (+)-tartrate salt, and (±)-omeprazole—were purchased from Sigma-Aldrich (St. Louis, Missouri), and (±)-2-methylmethcathinone hydro-chloride (2-MMC HCl), (±)-3-methyl-methcathinone hydrochloride (3-MMC HCl), (±)-4-methylmethcathinone hydro-chloride (4-MMC HCl,) and (±)-4-methyl-N-ethylcathinone hydrochloride (4-MEC HCl) were purchased from LGC Standards (Luckenwalde, Germany). The structures of these compounds are shown in Figure S1.

ACN, methanol (MeOH), and isopropanol (IPA) of HPLC grade were purchased from J. T. Baker (Deventer, The Netherlands). Reagent-grade diethylamine (DEA) from Scharlau (Barcelona, Spain) and formic acid (FA) from Honeywell (Augsburg, Germany) were used as mobile phase additives. Analytical grade ammonium acetate (NH_4Ac) was supplied by Sigma-Aldrich, and the acetic acid used to adjust the pH of the mobile phases was from J. T. Baker. Ultrapure water for the preparation of the mobile phase was obtained using a water purification system (Veolia, Sant Cugat del Vallès, Spain).

Individual stock standard solutions (1 mg/mL) were prepared in MeOH. Working

solutions of each compound (20 μg/mL) were prepared by diluting the stock solution with mobile phase. All stock and working solutions were protected from light and stored in a refrigerator at 4°C.

2.2. LC instrument and chromatographic conditions

All the analyses were performed with an Agilent 1100 series LC system (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, a binary pump, a temperature controller, and a diode array detector. Chemstation software (Agilent Technologies) was used for data acquisition and data handling. All the analyses were performed at 30°C under isocratic conditions at a flow rate of 0.1 mL/min. UV data were collected at 220 nm or 254 nm. The injection volume was 5 μL.

The Lux Cellulose-1 column (150 × 4.6 mm, 3 μm) from Phenomenex (Torrance, California) was used in PO mode. MeOH and ACN were tested as the main components of the mobile phase. In addition, the effect of the concentration of IPA as organic modifier and the addition of FA and/or DEA as acidic/basic additives in the mobile phase were investigated. The optimum mobile phase was ACN/IPA/FA/DEA (90/10/0.1/0.1, v/v/v/v).

The Chiralpak CBH column (150 × 2 mm, 5 μm) from Daicel (Illkirch, France) was used in RP mode with a mobile phase consisting of a mixture of aqueous buffer and an organic modifier. The effects of the organic modifier, mobile phase pH, and buffer concentration were evaluated. The satisfactory enantioseparations with appropriate retention times were achieved with the mobile phase consisting of 5 mM NH_4Ac aqueous solution (pH = 6.4)/MeOH (95/5, v/v).

3. Results and discussion

Two different columns (Lux Cellulose-1 and Chiralpak CBH) were evaluated using two different elution modes (PO mode and RP mode) to separate enantiomers of eight drugs. Various LC methods for the enantioseparation of β-blockers (atenolol, propranolol, and metoprolol) and omeprazole have been reported [3,17-19], as the different pharmacological effects between their enantiomers are well known. However, the LC enantioseparations of cathinones have been less studied because of their novelty. Silva et al. [20] have reviewed the methods developed for cathinones enantioseparation, but in most cases, the mobile phase systems are incompatible with MS detection. In the previous studies on chiral separation of cathinones, the Lux Cellulose-1 column was only used in NP mode [21], and the Chiralpak CBH column was only used for chiral separation of 4-MMC [22].

3.1. Optimization of chiral separation with the Lux Cellulose-1 column

The Lux Cellulose-1 column contains cellulose tris (3,5-dimethylphenyl carbamate) as the chiral selector, which is coated on silica gel. In the Lux Cellulose-1 column, the derivatized glucose units are regularly arranged along the helical axis

with the substituents creating a helical groove. The polar carbamate groups, which are known to be the most important adsorbing site for chiral recognition, are located inside the chiral grooves, while the hydrophobic aromatic moieties are located outside the polymer chain [23]. Enantiomers are discriminated by enantioselective inclusion into chiral cavities, which maybe mediated via formation of hydrogen bonding and dipole-dipole interactions with C═O or NH of the carbamate groups as well as via steric and π-π interactions with the phenyl rings [21,24]. In our case, functional groups of OH and NH for β-blockers, S═O and NH for omeprazole and NH and C═O for cathinones, could be contributed to the interaction with the carbamate groups on CSP, resulting in chiral recognition. In addition, all the target analytes have phenyl ring groups providing an additional stabilizing effect to the analyte-CSPs complex.

In the present study, the Lux Cellulose-1 column was used in PO mode, which is compatible with MS detection. The optimization of the mobile phase was conducted according to previous studies [24,25]. Following the literature, MeOH and ACN were tested as the main components of the mobile phase. The effect of the organic modifier (IPA), acidic additives (FA), and basic additives (DEA) was evaluated to find the optimal composition of the mobile phase. The initial flow rate was set at 0.6 mL/min. However, retention times were quite short, and no analytes showed any chiral separation. Since some studies have reported successful enantioseparations of a wide variety of analytes at very low flow rates (eg, 0.075, 0.08, and 0.1 mL/min) [22,26-28], this was reduced to 0.1 mL/min for all the analyses.

Nonetheless, with the initial mobile phase for method development—100% ACN—no analytes showed any chiral separation, and β-blockers and omeprazole did not elute as a well-defined peak during the analysis time (60 min).

3.1.1. Effect of organic modifier concentration

The alcoholic modifier is traditionally considered to be the elective modulator of the eluotropic power because it competes with the analyte for the hydrogen bonding sites of the CSPs and thus modifies retention and enantioselectivity [29]. For this reason, the effects of the addition of 10% IPA and 20% IPA to the mobile phase were evaluated. As expected, when the IPA content increased, the retention times of the target analytes decreased, but no improvement in resolution was observed.

3.1.2. Effect of acidic and/or basic additives

Acidic and basic additives are often used to minimize peak broadening and improve enantioresolution by minimizing non-specific retention [30]. The influence of acidic additives on enantioseparation was investigated in the mobile phase by adding 0.1% FA to ACN with different ratios of IPA (0%, 10%, and 20%). The presence of FA in

the mobile phase led to a significant decrease in retention time for all the analytes. However, no enantioseparation was observed.

In order to evaluate the influence of basic additives on enantioseparation, instead of 0.1% FA, the same concentration of DEA was added to the mobile phase. 4-MMC was partially separated (R_s = 1.1) with the mobile phase of ACN/DEA (100/0.1, v/v). In the case of propranolol, baseline separation (R_s = 1.3 or 1.5) was achieved after the addition of IPA (either 10% or 20%) to the mobile phase, while a very broad single peak was obtained without IPA in the mobile phase. No enantioseparation was observed for the other six compounds.

The results showed that no satisfactory separations were achieved with the presence of either FA or DEA in the mobile phase. In some cases, the simultaneous presence of both a basic and an acidic additive in the mobile phase improved the enantioseparation [25,30] Therefore, the influence of a combination of 0.1% FA and 0.1% DEA on enantioseparation was also studied in the mobile phase of ACN with different ratios of IPA. The results of enantioseparation are shown in Table 1. It was observed that β-blockers and omeprazole were separated with the simultaneous presence of both 0.1% FA and 0.1% DEA in 100% ACN, while enantiomers of cathinones remained unresolved. In most cases, by increasing the IPA concentration in the mobile phase, the retention time and resolution decreased, and the peaks of the enantiomers became narrower. According to the results (Table 1), ACN/IPA/FA/DEA (90/10/0.1/0.1, v/v/v/v) was found to provide the best combination between a satisfactory resolution, short retention time, and good peak shapes, although the enantiomers of the cathinones were not separated.

Table 1. Enantioseparation results obtained on Lux Cellulose-1 column with presence of both 0.1% FA and 0.1% DEA in ACN based mobile phase.

	ACN/FA/DEA (100/0.1/0.1,v/v/v)				ACN/IPA/FA/DEA (90/10/0.1/0.1,v/v/v/v)				ACN/IPA/FA/DEA (80/20/0.1/0.1,v/v/v/v)			
Compound	t_1 (min)	t_2 (min)	α	R_s	t_1 (min)	t_2 (min)	α	R_s	t_1 (min)	t_2 (min)	α	R_s
Atenolol	41.05	52.01	1.33	3.6	21.67	26.02	1.32	2.9	14.31	16.50	1.35	2.2
Propranolol	26.65	33.77	1.38	6.0	16.83	22.50	1.66	7.5	12.72	16.92	1.91	8.3
Metoprolol	16.92	28.13	2.30	12.8	12.80	18.70	2.30	10.2	10.46	14.06	2.90	8.9
Omeprazole	15.01	15.55	1.08	1.3	12.69	13.01	1.08	1.0	11.44	-	-	-
2-MMC	13.42	-	-	-	11.92	-	-	-	10.56	-	-	-
3-MMC	13.86	-	-	-	11.98	-	-	-	10.98	-	-	-
4-MMC	14.35	-	-	-	12.03	-	-	-	10.13	-	-	-
4-MEC	12.54	-	-	-	10.85	-	-	-	9.66	-	-	-

3.1.3. Selection of the main solvent

Instead of ACN, MeOH as the main solvent was also tested with the addition of different ratios of IPA (0%, 10%, and 20%) and 0.1% of both FA and DEA. No better results were obtained. A maximum of two analytes were separated during the optimization process for the MeOH-based mobile phase. Therefore, the optimum composition of the mobile phase for the enantioseparation of the target drugs using the Lux Cellulose-1 column was ACN/IPA/FA/DEA (90/10/0.1/0.1, v/v/v/v) (Table 1). With this mobile phase, the enantiomers of four of eight analytes were separated; however, none of the studied cathinones was enantioseparated.

3.2. Optimization of chiral separation with the Chiralpak CBH column

The Chiralpak CBH column contains a protein cellobiohydrolase (CBH) as the chiral selector, which is immobilized on 5 μm particles of silica gel. As a single protein may contain a variety of chiral centers and different binding sites, the chiral recognition on the column is capable of complex mechanisms involving ion exchange, hydrogen bonding, and hydrophobic interactions [22]. Particularly, CBH is effective for the enantiomer separation of basic drugs, in which the key chiral recognition mechanism has been attributed to the ion-exchange between the carboxylic acid residues on the protein and basic analytes [31]. This column is used in RP mode with a mobile phase consisting of a mixture of aqueous buffer and an organic modifier, so it can easily be coupled with MS detection. In order to achieve the best enantioseparation for the target analytes, the following parameters were optimized in this study: type and concentration of organic modifier (IPA, ACN, and MeOH), mobile phase pH (6.4 and 5.0), and buffer concentration (1 mM - 20 mM NH_4Ac).

3.2.1. Effect of organic modifier concentration

Based on previous studies [26,27], initially 1 mM NH_4Ac in water was chosen as the aqueous phase. In the first instance, the organic modifier concentration of the mobile phase was optimized. The IPA concentrations of 2%, 5%, and 10% were evaluated, and the results are shown in Table 2. With the mobile phase composed of 1 mM NH_4Ac aqueous/IPA (98/2, v/v), the enantiomers of six drugs were separated within 40 minutes with R_s ranging from 1.4 for 4-MEC to 17.7 for atenolol. In fact, metoprolol was separated, but the second enantiomer was eluted after 75 minutes. In the case of propranolol, no peak was eluted within the analysis time. On increasing the organic content from 2% to 5%, retention time decreased, as did enantioresolution, for all the analytes. Meanwhile, retention time increased and resolution decreased for certain analytes on increasing the organic content from 5% to 10%. A mobile phase consisting of 5% organic modifier was chosen because it provided the shortest retention times with acceptable resolutions for most of the analytes.

Table 2. Enantioseparation results obtained on Chiralpak CBH column with mobile phase consisting of 1 mM NH_4Ac buffer and different ratio of IPA (2%, 5%, 10%).

	1 mM NH_4Ac aqueous/IPA (98/2, v/v)				1 mM NH_4Ac aqueous/IPA (95/5, v/v)				1 mM NH_4Ac aqueous/IPA (90/10, v/v)			
Compound	t_1 (min)	t_2 (min)	α	R_s	t_1 (min)	t_2 (min)	α	R_s	t_1 (min)	t_2 (min)	α	R_s
Atenolol	21.97	39.79	2.00	17.7	19.58	35.27	2.01	17.0	21.82	40.44	2.05	18.7
Propranolol	-	-	-	-	-.	-	-	-	-	-	-	-
Metoprolol	39.86	> 75	-	-	31.34	> 75	-	-	31.31	> 75	-	-
Omeprazole	14.77	18.47	1.33	4.3	10.84	13.15	1.31	3.3	7.78	8.74	1.26	2.1
2-MMC	19.38	25.88	1.43	4.8	17.45	22.68	1.39	4.5	20.40	25.67	1.32	4.0
3-MMC	21.08	24.73	1.22	3.8	18.40	21.11	1.19	3.3	22.34	25.10	1.15	2.9
4-MMC	20.81	23.52	1.16	2.9	18.18	20.34	1.15	2.6	22.35	25.07	1.15	2.9
4-MEC	20.44	21.51	1.06	1.4	17.01	17.80	1.06	1.1	21.60	22.64	1.06	1.3

3.2.2. Effect of buffer concentration with 5% IPA

The effect of the buffer concentration on separation was studied by varying the concentration of NH_4Ac with 5% IPA in the mobile phase. Figure 1 presents the chromatograms of the trend of the enantioseparation of 2-MMC and 3-MMC, which show that a higher buffer concentration resulted in shorter retention times, lower enantioresolutions, and narrower peaks. As a compromise, a concentration of 5 mM was selected as the optimal buffer concentration.

3.2.3. Effect of mobile phase pH

The buffer pH can affect not only the dissociation/protonation of analytes but also the ionization of functional groups of the chiral selector or even of the residual silanol groups of the silica gel support, thus providing a variety of possible stereoselective interactions [32]. The effect of pH was evaluated using a mobile phase consisting of 5 mM NH_4Ac buffer and 5% IPA. A solution of 5 mM NH_4Ac in water had a pH of 6.4, and this same solution was adjusted to pH 5 with acetic acid. When the pH was reduced from 6.4 to 5.0, the retention times and enantiomeric resolution for all analytes significantly decreased except for propranolol and omeprazole. For example, R_s for atenolol decreased from 14.7 to 1.8, and t_2 reduced from 22.65 to 5.27 minutes when the mobile phase pH decreased from 6.4 to 5.0. In the case of propranolol, the use of a mobile phase at pH 5 led to baseline separation (R_s = 26.9) within 60 minutes, while at pH 6.4, no peak was eluted in that time. In spite of this, since the R_s decreased for the rest of the analytes

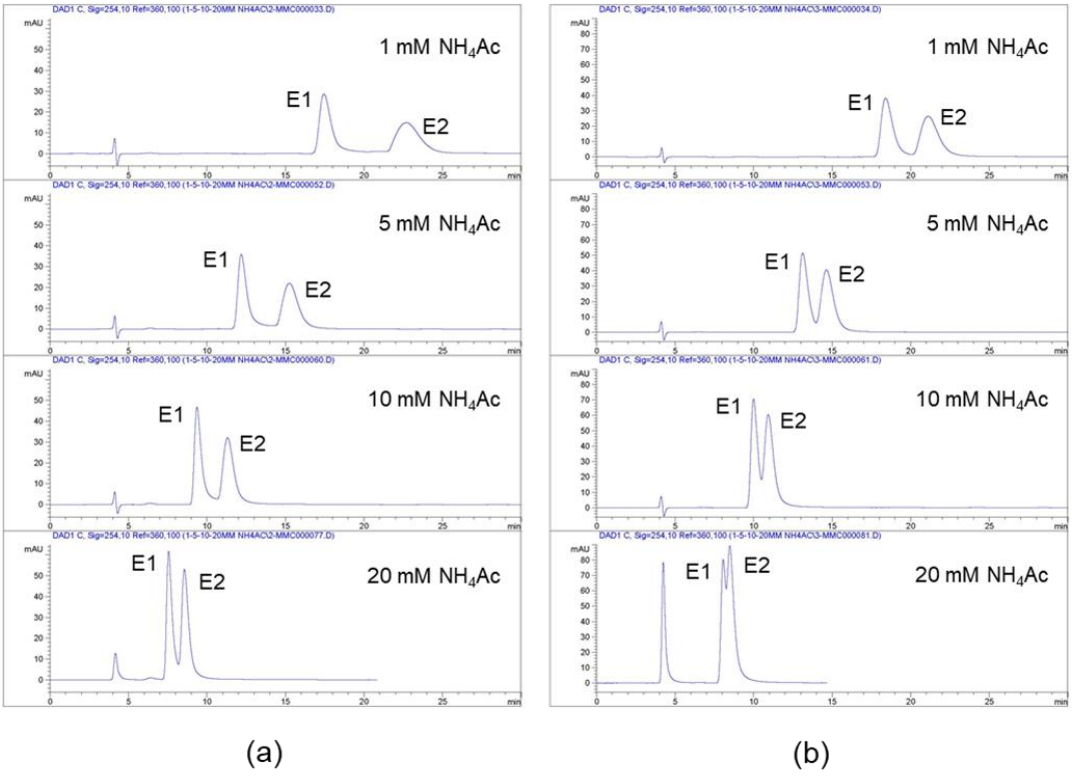

Figure 1. Effect of buffer concentration on enantioseparation using Chiralpak CBH column. (a): 2-MMC; (b): 3-MMC

on decreasing the pH of the mobile phase from 6.4 to 5.0, a pH of 6.4 was chosen.

3.2.4. Selection of organic modifier type

The effect of organic modifier type was also evaluated. MeOH and ACN were used instead of IPA, and the results are shown in Table S1. With the replacement of IPA by ACN or MeOH, the R_s for all the compounds decreased (but they were still larger than 1.9), except for 2-MMC and 3-MMC. In the case of 2-MMC and 3-MMC, the replacement of IPA by ACN or MeOH gave rise to a significant increase in resolution: R_s increased from 3.6 to 6.3 and 5.6 for 2-MMC, respectively, and from 1.9 to 4.5 and 4.0 for 3-MMC, respectively (Figure S2). No significant differences in R_s were found between MeOH and ACN, but MeOH provided shorter retention times for most of the analytes. Even though the enantiomers of 4-MEC were partially separated with IPA, the resolution was quite poor (R_s = 0.8). Taking these results into account, 5% MeOH was selected as a compromise. Castrignanò et al. [22] also reported that better chiral recognition was achieved for 56 drugs using MeOH rather than ACN or IPA with the Chiralpak CBH column.

The effect of the buffer concentration was also confirmed when MeOH was used as the organic modifier in a similar trend as when IPA was used: by increasing the buffer concentration in the mobile phase, retention time and resolution decreased in

most cases (Table S2). Finally, an aqueous solution of 5 mM NH_4Ac (pH = 6.4) with 5% MeOH was chosen as a compromise. With the optimized mobile phase, six of eight target drugs were baseline separated within 45 minutes, and the chromatograms are shown in Figure 2. The enantioseparation of propranolol was not resolved because very strong interactions between propranolol and the CBH column resulted in very long retention times (>120 min).

3.3. Comparison of the Lux Cellulose-1 and Chiralpak CBH column performances

According to the results obtained, atenolol, metoprolol, and omeprazole were enantioseparated in both columns. However, the Chiralpak CBH column provided better chiral separation for these target analytes than the Lux Cellulose-1 column. For example, R_s for atenolol enantiomers was 9.3 in the case of the Chiralpak CBH column and only 2.9 in the case of the Lux Cellulose-1. In addition, 2-MMC, 3-MMC, and 4-MMC showed no enantioseparations in the Lux Cellulose-1 column, whereas they were enantioseparated with the Chiralpak CBH. Experiments carried out with the Lux Cellulose-1 column only showed higher enantioselectivity for propranolol. In summary, the Chiralpak CBH column in RP mode provided better enantioseparation for the target analytes than the Lux Cellulose-1 column in PO mode. The more successful chiral separation on the Lux Cellulose-1 column might be achieved in NP mode, but this possibility was not examined in the present study because NP mode is not compatible with MS detection. Moreover, in the previously reported method [22] on the chiral separation of 4-MMC using CBH column, a similar resolution (R_s = 1.4) was achieved; however, the suitability for other cathinones was not tested.

The Lux Cellulose-1 and Chiralpak CBH columns were used in PO and RP mode, respectively. Using PO mode offers several advantages, including enhanced solubility of polar analytes, favorable peak shape, and more compatibility with the solvent from previous extractions. However, the mobile phases have large eluent strengths leading to shorter retention times (ie, limited interaction with the CSPs), and therefore, the success rate of enantioseparation is lower in this mode [9]. RP conditions are especially useful for determining the enantiomeric ratios of drugs in biological matrices [33] because they are aqueous and can be directly injected. Moreover, this involves the use of less costly solvents.

The elution order of atenolol and propranolol enantiomers was studied on two columns by spiking racemic standard solution with R-(+)-enantiomer. In the case of atenolol, the same elution order was obtained on both columns with the R-(+)-enantiomer eluted before the S-(−)-enantiomer, which indicated that the R-(+)-enantiomer binds to the two CSPs with lower affinity than its S-(−)-enantiomer. In

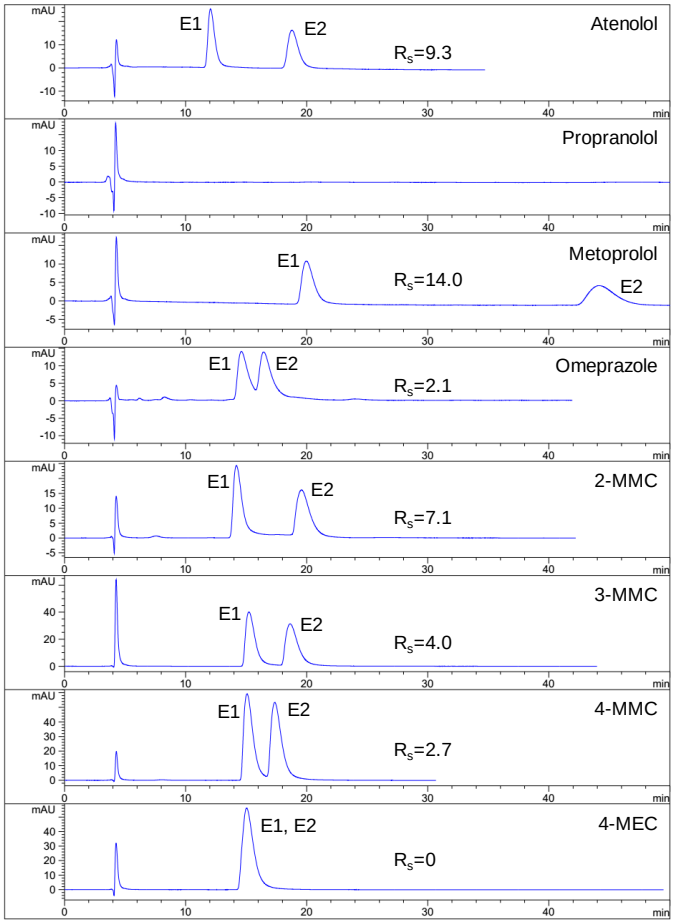

Figure 2. Chromatograms of the eight chiral drugs on Chiralpak CBH column with the mobile phase of 5mM NH_4Ac buffer (pH 6.4)/MeOH (95/5, v/v).

the case of propranolol, the R-(+)-enantiomer also eluted before the S-(−)-enantiomer on the Lux Cellulose-1 column. As propranolol was not separated on the Chiralpak CBH column with the optimal mobile phase, its elution order was not determined on this column.

4. Conclusions

In this paper, the recognition ability of two chiral columns (Lux Cellulose-1 and Chiralpak CBH, which are polysaccharide and protein-based, respectively) towards the enantiomers of eight chiral drugs was studied in PO elution mode and RP elution mode, respectively.

With the Lux Cellulose-1 column, by increasing the concentration of organic modifier, the retention time and enantioresolution decreased. The addition of 0.1% FA or 0.1% DEA alone did not bring about as much improvement as a combination of both. As a result, the optimized mobile phase was ACN/IPA/FA/DEA (90/10/0.1/0.1, v/v/v/v). With this mobile phase, β-blockers were baseline separated, and omeprazole was partially separated, while cathinones were not separated.

With the Chiralpak CBH column, by increasing the concentration of organic modifier and buffer solution, the retention time and enantioresolution decreased. The decrease of mobile phase pH led to a significant reduction in retention time and enantioresolution. The optimized mobile phase was 5 mM NH_4Ac aqueous solution (pH = 6.4)/MeOH (95/5, v/v). With this mobile phase, the enantiomers of six of eight studied drugs were baseline separated.

In summary, the Chiralpak CBH column in RP mode was more effective for the target compounds than the Lux Cellulose-1 in PO mode. In addition, since the solvents and additives used are MS compatible, both chiral LC methods developed in this study can be directly coupled with an MS detector for the enantiomeric determination of chiral drugs at low concentration.

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Supplementary data

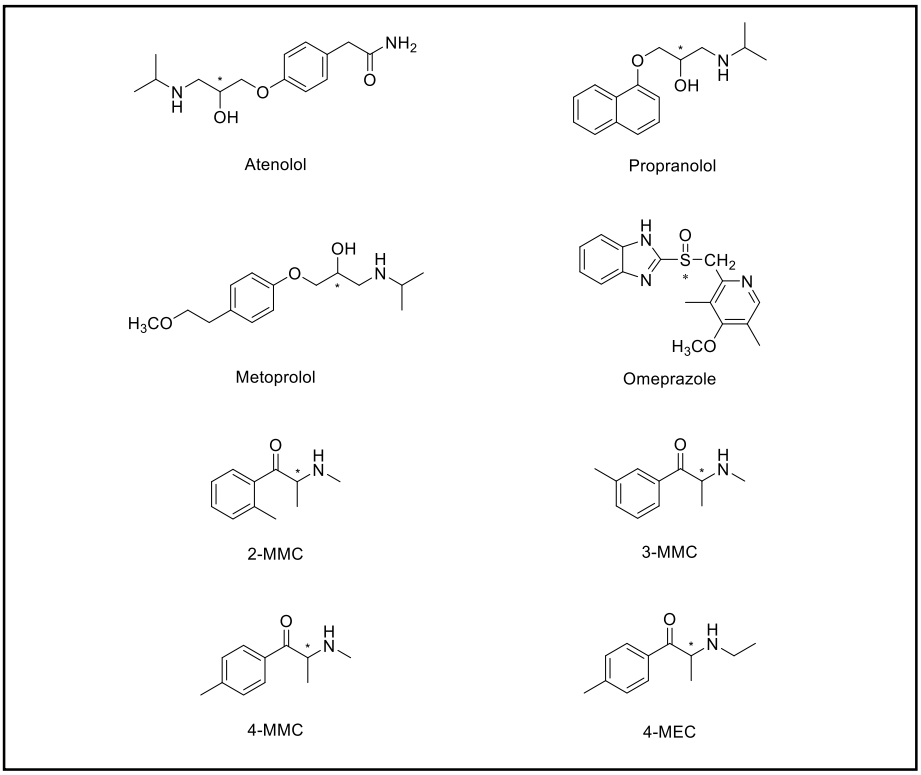

Figure S1. Structures of target analytes. Chiral center was indicated by asterisks.

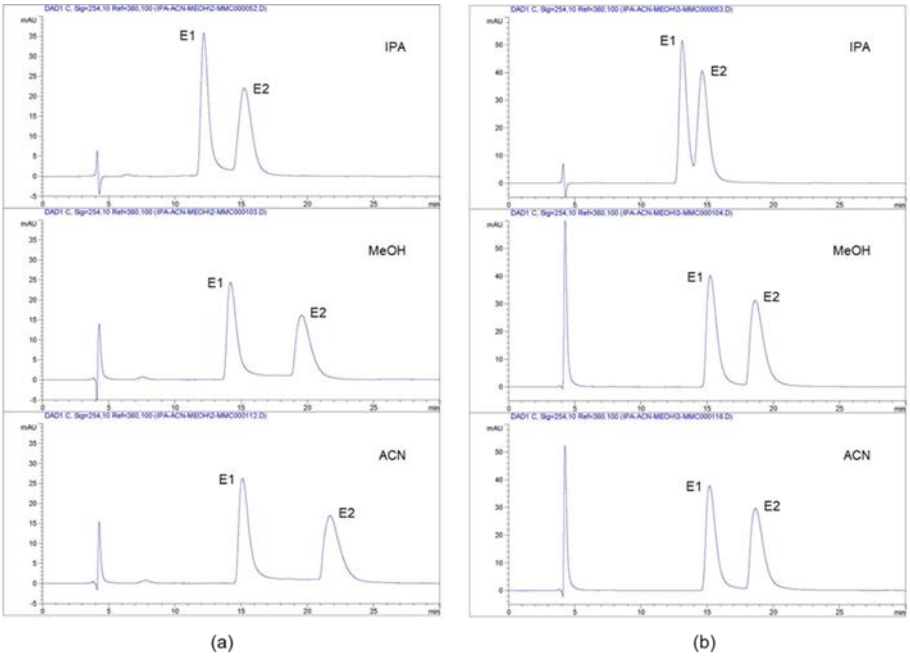

Figure S2. Effect of organic modifier type on enantioseparation of 2-MMC (a) and 3-MMC (b) using Chiralpak CBH column.

Table S1. Enantioseparation results obtained on Chiralpak CBH column with mobile phase consisting of 5 mM NH_4Ac buffer and 5% of different type of organic modifier.

	5 mM NH_4Ac aqueous/IPA (95/5, v/v)				5 mM NH_4Ac aqueous/ACN (95/5, v/v)				5 mM NH_4Ac aqueous/MeOH (95/5, v/v)			
Compound	t_1 (min)	t_2 (min)	α	R_s	t_1 (min)	t_2 (min)	α	R_s	t_1 (min)	t_2 (min)	α	R_s
Atenolol	12.27	22.65	2.28	14.7	13.60	20.45	1.69	8.8	12.06	18.78	1.85	9.3
Propranolol	-	-	-	-	-	-	-	-	-	-	-	-
Metoprolol	21.51	61.18	3.28	17.7	21.07	46.18	2.45	15.0	19.96	44.21	2.53	14.0
Omeprazole	10.47	12.26	1.28	3.0	13.49	15.02	1.16	1.9	14.61	16.45	1.17	2.1
2-MMC	12.18	15.24	1.38	3.6	15.09	21.70	1.58	6.3	14.19	19.57	1.52	5.6
3-MMC	13.12	14.63	1.17	1.9	15.18	18.66	1.30	4.5	15.23	18.63	1.30	4.0
4-MMC	12.97	14.56	1.18	2.5	15.15	17.07	1.17	2.4	15.07	17.36	1.20	2.7
4-MEC	12.45	13.02	1.07	0.8	15.02	-	-	-	15.04	-	-	-

Table S2. Enantioseparation results obtained on Chiralpak CBH column with mobile phase consisting of 5% MeOH and different buffer concentration at pH 6.4.

	1 mM NH_4Ac aqueous/MeOH (95/5, v/v)				5 mM NH_4Ac aqueous/MeOH (95/5,v/v)				10 mM NH_4Ac aqueous/MeOH (95/5,v/v)			
Compound	t_1 (min)	t_2 (min)	α	R_s	t_1 (min)	t_2 (min)	α	R_s	t_1 (min)	t_2 (min)	α	R_s
Atenolol	19.94	29.35	1.60	9.4	12.06	18.78	1.85	9.3	10.07	14.83	1.77	8.1
Propranolol	-	-	-	-	-	-	-	-	73.97	-	-	-
Metoprolol	32.15	74.70	2.47	15.4	19.96	44.21	2.53	14.0	15.31	32.13	2.48	16.7
Omeprazole	10.95	12.37	1.19	2.0	14.61	16.45	1.17	2.1	14.06	15.53	1.14	1.5
2-MMC	20.60	27.21	1.38	7.1	14.19	19.57	1.52	7.1	10.82	14.77	1.57	6.5
3-MMC	20.45	24.38	1.24	4.2	15.23	18.63	1.30	4.0	11.47	13.86	1.32	3.7
4-MMC	21.03	23.58	1.15	2.5	15.07	17.36	1.20	2.7	11.39	12.88	1.20	2.3
4-MEC	21.97	-	-	-	15.04	-	-	-	11.35	-	-	-

3.1.2. Evaluation of chiral recognition capabilities of four in-house synthesized polymeric chiral stationary phases

1. Introduction

The advances in enantioseparation are mainly associated with the development of chiral stationary phases (CSPs). The types of CSPs available today can be divided into two main categories based on the selector mass. The first type of CSP consists of low-molecular-mass selectors, which are usually immobilized on a silica gel forming a surface layer of chiral molecules [1]. Pirkle-type, cyclodextrin-based CSPs, macrocyclic antibiotics, ligand-exchange CSPs and chiral crown ethers belong to this class. The second type of CSP consists of optically active polymers, which are further divided into synthetic and natural polymers like proteins and polysaccharides [2].

Although a large number of CSPs have been available commercially, the development of new CSPs continues to be a field of research with great importance to satisfy the increasing needs for the efficient separation of enantiomers for analytical and preparative applications. In recent years, synthetic polymeric CSPs have attracted much attention due to several of their attractive properties such as high loading capacities, high stability and the possibility to obtain the opposite absolute configuration of the chiral selectors [3]. Moreover, the availability of a great variety of monomers, the diversity of polymerization methods, and the simplicity of chemical modifications of the CSPs have contributed to the development of polymeric CSPs [3,4].

To date, various CSPs derived from optically active synthetic polymers have been developed for the efficient separation of enantiomers. For example, Gasparrini et al. [4] developed the synthetic polymeric CSPs based on N,N'-(1S,2S)-1,2-cyclohexanediyl-bis-2-propenamide (P-CAP), and Armstrong et al. [5] reported the synthetic polymeric CSPs based on N,N'-[(1R,2R)-1,2-diphenyl-1,2-ethanediyl] bis-2-propenamide. These polymeric CSPs showed excellent chiral recognition abilities towards a diverse number of chiral compounds when used in the normal-phase (NP) and polar-organic (PO) elution modes, which led them to be commercialized as the P-CAP column and P-CAP-DP column, respectively.

In the present study, we investigate the chiral recognition capabilities of four new polymeric CSPs based on (1S, 2S)-(-)-1,2-diphenylethylenediamine, (1S, 2S)-(-)-1,2-di-1-naphtyl-ethylenediamine, D-phenylaniline methylester and L-phenylaniline methylester, respectively with a variety of chiral drugs by LC under NP mode. These polymeric materials were prepared by functionalizing poly(divinylbenzene-co-vinylbenzyl chloride) (poly(DVB-co-VBC)) microspheres with corresponding monomers. The effects of the acidic additives, organic modifier, and the flow rate on enantioseparation were investigated.

2. Experimental

2.1. Chemicals and solutions

The standard analytes—(±)-atenolol, (±)-propranolol, (±)-metoprolol, (±)-omeprazole and (±)-ibuprofen—were purchased from Sigma-Aldrich (St. Louis, MO, USA), and (±)-mephedrone, (±)-methylone, (±)-flephedrone and (±)-pentedrone were purchased from LGC Standards (Luckenwalde, Germany). The structures and pK_a values of these compounds are shown in Figure 1.

Individual stock standard solutions were prepared in methanol (MeOH) at 1 mg/mL, and kept at -20°C. Working solutions of each compound (20 µg/mL) were prepared weekly by diluting the stock solution with mobile phase, and kept at 4°C.

MeOH, ethanol (EtOH) and hexane of HPLC grade were purchased from J. T. Baker (Deventer, The Netherlands). Trifluoroacetic acid (TFA) from Honeywell (Augsburg, Germany) were used as mobile phase additives. Ultrapure water for the preparation of the mobile phase was obtained using a water purification system (Millipore, Billerica, MA, USA).

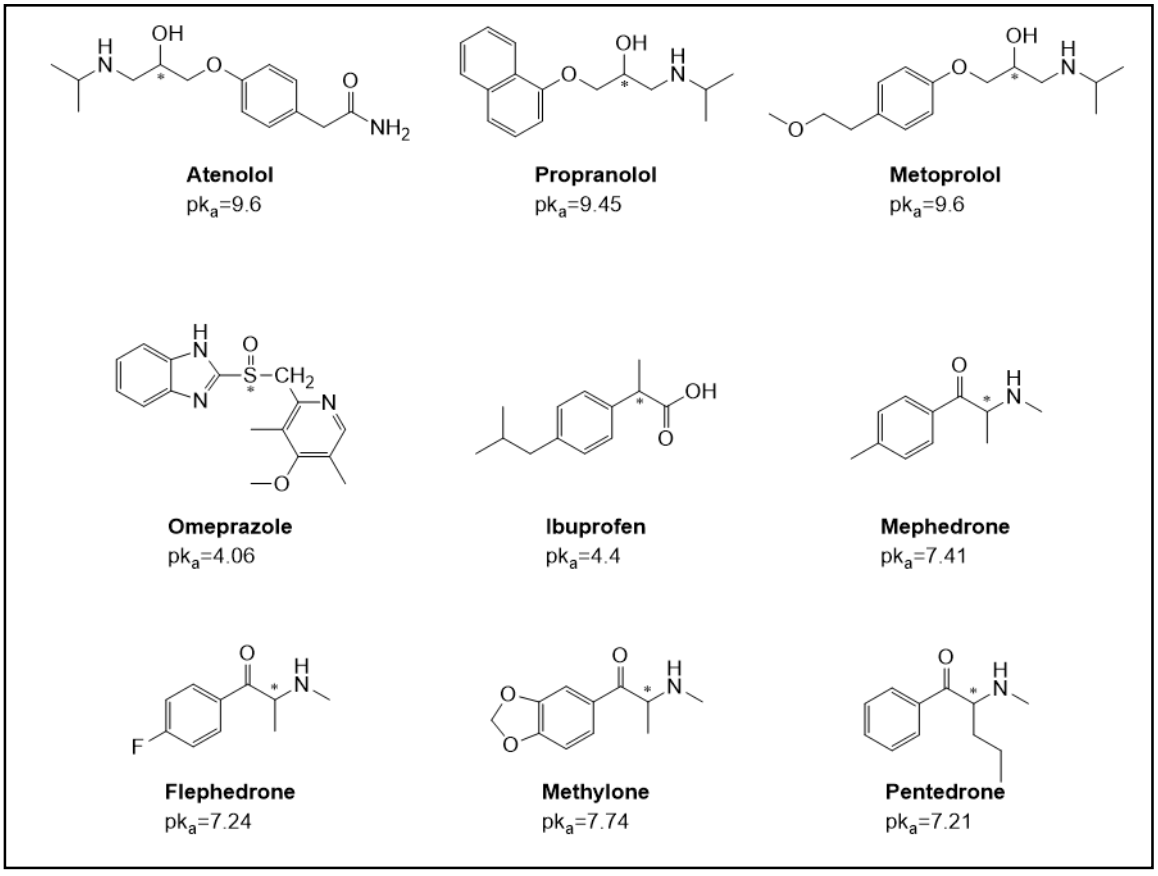

Figure 1. Structures and pK_a values of analytes. Chiral centers are indicated by asterisks.

2.2. Synthesis of the polymeric CSPs

These polymeric CSPs were synthesized by the following procedures (shown in Figure 2): i) poly(DVB-co-VBC) microspheres were synthesized by the precipitation polymerisation of DVB and VBC monomers; ii) in the case of DSA 35 and DSA 37, the poly(DVB-co-VBC) microspheres were chemically modified with 1,2-diphenylethylenediamine and 1,2-di-1-naphtyl-ethylenediamine, respectively, while in the case of SD 099 and SD 100, the poly(DVB-co-VBC) microspheres were hypercrosslinked and then chemically modified with D-phenylaniline methylester and L-phenylaniline methylester, respectively. The resulting particles were packed into 50 × 4.6 mm LC stainless-steel columns.

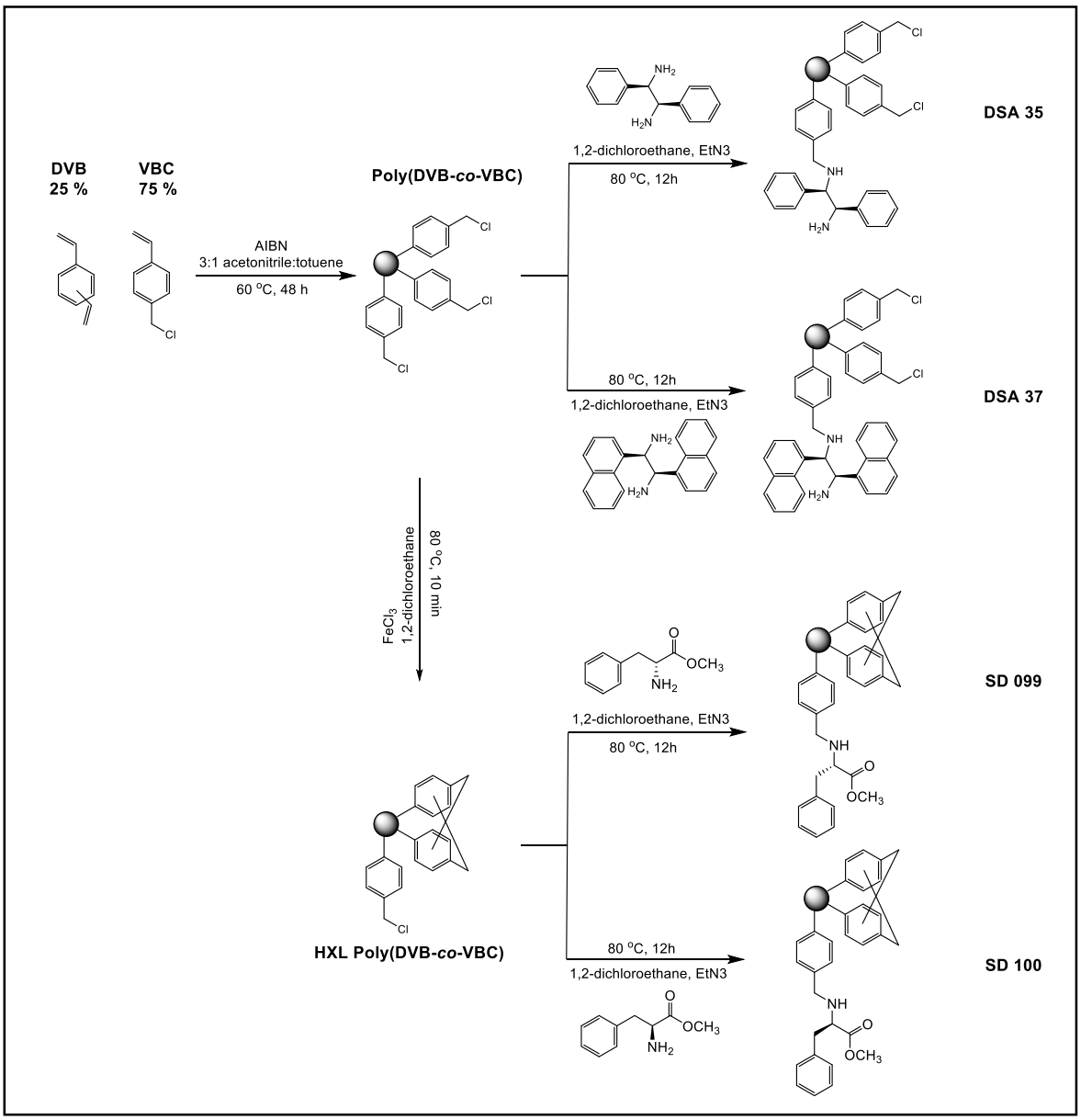

Figure 2. Scheme of the syntheses of four polymeric CSPs (DSA 35, DSA 37, SD 099 and SD 100).

2.3. LC instrument and chromatographic conditions

The evaluation of the chiral recognition capabilities of the four synthesized CSPs was carried out using an Agilent 1100 series LC system (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, a binary pump, a temperature controller, and a diode array detector. Chemstation software (Agilent Technologies) was used for data acquisition and data handling. UV data were collected at 220 nm or 254 nm. The columns were maintained at 30 °C, the injection volume was 5 μL and the analysis time was 60 min. The initial flow rate was set at 0.2 mL/min.

The performance of SD 099, SD 100, DSA 35 and DSA 37 columns were investigated in NP mode using hexane as the main components of the mobile phase. The effect of the concentration of EtOH as organic modifier and the addition of TFA as acidic additives in the mobile phase were assessed on the better performing columns DSA 35 and DSA 37.

3. Results and discussion

Initially, the column SD 099, SD 100 DSA 35 and DSA 37 were tested in the NP mode with the mobile phase of hexane/EtOH (90/10, v/v) at the flow rate of 0.2 mL/min. The NP mode was selected since the similar polymeric CSPs produced successful enantioseparations in NP mode in previous studies [4,5]. As a result, no analyte was enantioseparated on all the columns, and slight better results were obtained on DSA 35 and DSA 37 columns in terms of retention. In the case of SD 099 and SD 100, atenolol, flephedrone, metoprolol and propranolol were not retained on the columns, while other analytes (ibuprofen, omeprazole, mephedrone, pentedrone and methylone) were not eluted in 60 min. In the case of DSA 35 and DSA 37, methylone and propranolol were eluted as single peaks at 19 and 32 min, respectively, although the other analytes were not eluted in 60 min (Table 1). In view of this, DSA 35 and DSA 37 were selected to optimize chromatographic conditions including the addition of an acidic additive (TFA), the concentration of organic modifier (EtOH), and the flow rate.

3.1. Effect of acidic additives

In NP mode, an acidic (such as formic acid (FA) or TFA) or basic additive (such as diethylamine (DEA)) is often added to minimize peak broadening and improve enantioseparations by minimizing non-specific retention. In the present study, the effect of acidic additives in the mobile phase on the enantioseparations was investigated by adding 0.1% TFA to hexane/EtOH (90/10, v/v). The presence of TFA in the mobile phase led to a significant decrease in retention time for all the analytes except for metoprolol both on DSA 35 and DSA 37 columns (Table 1), but no successful enantioseparation was achieved. It should be noted that, in the case of DSA 37 column, 3 cathionones – flephedrone, pentedrone

and mephedrone – showed a sign of enantioseparation with a shoulder peak, as shown in Figure 3(a).

Table 1. The effect of acidic additives (TFA) in the mobile phase on the retention times of analytes on the DSA 35 and DSA 37 columns at the flow rate of 0.2 mL/min.

Analytes	Retention time (min)			
	DSA 35		DSA 37	
	Hexane/EtOH (90/10, v/v)	Hexane/EtOH/TFA (90/10/0.1, v/v/v)	Hexane/EtOH (90/10, v/v)	Hexane/EtOH/TFA (90/10/0.1, v/v/v)
Atenolol	>60	>60	>60	>60
Flephedrone	>60	29.8	>60	19.2
Ibuprofen	>60	2.2	>60	4.7
Mephedrone	>60	26.0	>60	16.8
Methylone	>60	>60	>60	>60
Metoprolol	19.3	2.2	17.2	1.9
Omeprazole	>60	>60	>60	>60
Pentedrone	>60	18.7	>60	12.1
Propranolol	32.1	>60	26.3	>60

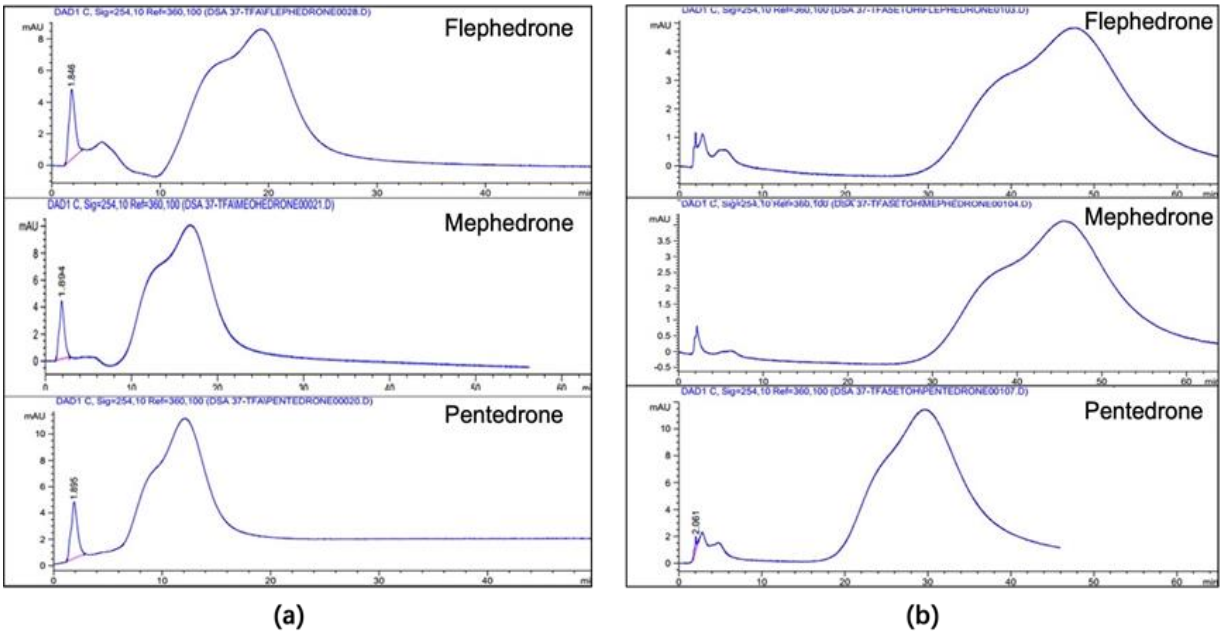

Figure 3. The chromatograms of flephedrone, mephedrone and pentedrone on the DSA 37 column with the mobile phase of (a) hexane/EtOH/TFA (90/10/0.1, v/v/v) and (b) hexane/EtOH/TFA (95/5/0.1, v/v/v) at the flow rate of 0.2 mL/min.

3.2. Effect of organic modifier concentration

An alcohol, such as EtOH or isopropanol (IPA), is generally the polar mobile phase modifier of choice in NP mode to increase the elution strength of the mobile phase and modify the enantioselectivity. Considering that the retention times of some analytes were quite short when using the mobile phase of hexane/EtOH/TFA (90/10/0.1, v/v/v), the concentration of EtOH was decreased to 5%. The results are shown in Table 2. On decreasing the organic content from 10% to 5%, the retention times increased significantly on both columns in most cases. However, no improvement in enantioseparation was observed. The shoulder peaks were also observed for flephedrone, mephedrone and pentedrone with the mobile phase of hexane/EtOH/TFA (95/5/0.1, v/v/v) in the case of DSA 37 column, but the peaks were quite wide, as shown in Figure 3(b).

Table 2. The effect of organic modifier (EtOH) concentration in the mobile phase on the retention times of analytes on the DSA 35 and DSA 37 columns at the flow rate of 0.2 mL/min.

Analytes	Retention time (min)			
	DSA 35		DSA 37	
	Hexane/EtOH/TFA (90/10/0.1, v/v/v)	Hexane/EtOH/TFA (95/5/0.1, v/v/v)	Hexane/EtOH/TFA (90/10/0.1, v/v/v)	Hexane/EtOH/TFA (95/5/0.1, v/v/v)
Atenolol	>60	>60	>60	>60
Flephedrone	29.8	48.6	19.2	47.7
Ibuprofen	2.2	2.9	4.7	7.0
Mephedrone	26.0	45.5	16.8	45.5
Methylone	>60	>60	>60	>60
Metoprolol	2.2	2.5	1.9	2.0
Omeprazole	>60	>60	>60	>60
Pentedrone	18.7	29.0	12.1	29.3
Propranolol	>60	>60	>60	>60

3.3. Effect of flow rate

The effect of flow rate on the enantioseparations were evaluated by increasing the flow rate to 0.6 mL/min when using hexane/EtOH/TFA (95/5/0.1) as mobile phase. As shown in Table 3, with the higher flow rate, the retention times decreased for all the analytes, except for atenolol, omeprazole and propranolol, which did not elute in 60 min. For the other analytes, a single peak was observed during analysis time on both columns.

It should be pointed out that atenolol, omeprazole and propranolol were strongly retained on the columns and were not eluted in 60 min in any case. To investigate the enantioselective

capabilities of the two columns for these strong retained analytes, further increasing EtOH content mobile phase (hexane/EtOH/TFA (85/15/0.1, v/v/v)) was tested at the flow rate of 0.6 mL/min. As a result, all the analytes were eluted in 60 min, but no enantioseparation was achieved.

Table 3. The effect of flow rate on the retention times of analytes on the DSA 35 and DSA 37 columns with the mobile phase of Hexane/EtOH/TFA (95/5/0.1, v/v/v).

Analytes	Retention time (min)			
	DSA 35		DSA 37	
	0.2 mL/min	0.6 mL/min	0.2 mL/min	0.6 mL/min
Atenolol	>60	>60	>60	>60
Flephedrone	48.6	20.2	47.7	18.1
Ibuprofen	2.9	3.4	7.0	2.4
Mephedrone	45.5	17.0	45.5	15.5
Methylone	>60	47.9	>60	41.3
Metoprolol	2.5	1.6	2.0	1.6
Omeprazole	>60	>60	>60	>60
Pentedrone	29.0	10.3	29.3	9.7
Propranolol	>60	>60	>60	>60

4. Conclusions

The chiral recognition abilities of four in-house made columns (DSA 35, DSA 37, SD 099 and SD 100) were evaluated towards nine analytes belonging to different groups in NP mode with hexane as the main components of the mobile phase. DSA 35 and DSA 37 performed better than SD 099 and SD 100 ones in terms of retention in the preliminary tests, and thus were selected to optimize chromatohraphic conditions such as the effect of the organic modifier concentration, acidic additives and flow rate. No baseline enantioseparation was achieved in any case. With the mobile phase of hexane/EtOH/TFA (95/5/0.1, v/v/v) and hexane/EtOH/TFA (90/10/0.1, v/v/v) at the flow rate of 0.2 mL/min, 3 cathinones (flephedrone, pentedrone and mephedrone) showed a sign of enantioseparation with a shoulder peak on the column DSA 37.

Further studies should be conducted to optimize the enantioseparation conditions, such as using heptane as main component of mobile phase, using other alcohols (IPA) as organic modifiers, and adding basic additives (DEA). In addition, strategies to improve the CSP synthesis process or the use of longer columns should also be explored in future studies.

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3.1.3. Discussion of results

Although the results of the experimental part of the studies included in this section have been already discussed in their respective papers, the current section presents and discusses the most important aspects of these results. The previous studies present the development of chiral LC methods applying different chiral columns in combination with different elution modes, for the enantioseparations of eight drugs comprising three β-blockers (atenolol, propranolol and metoprolol), omeprazole, and four cathinones (2-MMC, 3-MMC, 4-MMC and 4-MEC).

In these studies, the main attention is focused on the effect of mobile phase compositions on the enantioseparations. The Lux Cellulose-1 column based on cellulose tris(3,5-dimethylphenylcarbamate) was explored in PO mode to be able to couple to MS. MeOH and ACN were tested as the main components of the mobile phase. In addition, the effects of adding IPA as organic modifier, acidic additives (FA), and basic additives (DEA) were evaluated. The results showed that acidic and basic additives have the most significant impact on the enantioseparations. It is generally believed that the acidic additive is beneficial for enhancing the enantioselectivity for basic molecules while the basic additive is beneficial for enhancing the enantioselectivity for acidic ones [1,2]. Since all the analytes in the first study exhibited a basic character, the improve of enantioseparation of analytes due to the addition of a basic additive may be expected. The basic additives in the mobile phase can probably mask non-enantioselective strong interactions with residual silanol groups on the CSPs, thereby decreasing non-selective retention and improving enantioresolution [1]. As expected, the addition of 0.1% FA alone in the mobile phase of ACN did not bring about any improvement in enantioseparations, while the addition of 0.1% DEA alone led to the successful enantioseparation for 4-MMC (R_s= 1.1). However, after the addition of 0.1% FA to the mobile phase already containing DEA, retention of 4-MMC increased and the enantioseparation was lost. In contrast to the case of 4-MMC, the enantiomers of three β-blockers and omeprazole were not resolved with the mobile phase of ACN containing only 0.1% DEA, whereas baseline separations were achieved for all of them after the addition of 0.1% FA to the mobile phase. The observed results demonstrate that the effect of acidic and basic additives on the chiral recognition is much more complex and further studies are required for a complete understanding of underlying mechanisms of these observations.

The organic modifier concentration and the type of main solvent also have an important effect on the retention and enantioseparation. In most cases, by increasing the organic modifier (IPA) content in the mobile phase, the retention time and resolution decreased, and the peaks of the enantiomers became narrower. This is because IPA can weaken interactions between analytes and CSPs by competing with enantiomers for hydrogen bonding with CSPs, and thus retention decreases, as occasionally does enantioselectivity. ACN as the main solvent provided better enantioseparations compared with MeOH. A maximum of two

analytes were separated with the MeOH-based mobile phase, while four analytes were resolved with the ACN-based mobile phase. MeOH as a protic solvent competes with the analytes for the hydrogen-bonding sites of the CSPs, while ACN as aprotic solvent should not interfere with hydrogen-bonding interactions between analytes and CSPs. The obtained result agrees with previous reports [3,4] that the ACN-based mobile phases performed better than the equivalent MeOH-based in chiral recognition of various chiral drugs using different polysaccharide-based columns in PO mode. In contrast, Matthijs et al. [5] demonstrated that MeOH-based mobile phase was more effective than ACN-based mobile phase when a polysaccharide-based column (Chiralcel OZ-RH) was used for enantioseparation of (fluoro)quinolones and their metabolites. MeOH and ACN would result in significantly different enantioseparations and they should be carefully evaluated during method development.

The enantioselectivity of Chiralpak CBH column (protein-based) was evaluated in RP mode. In order to achieve the best enantioseparation for the target analytes, the following parameters were optimized: type and concentration of organic modifier (IPA, ACN, and MeOH), mobile phase pH (6.4 and 5.0), and buffer concentration (1 mM-20 mM NH_4Ac). Among the above-mentioned parameters, the pH value of the mobile phase was found to be the main parameter affecting enantioseparation as it affects the ionization of analytes and the chiral selectors. CBH has an isoelectric point of 3.9. Therefore, CBH columns are negatively charged over the column working pH range (4.0–7.0). An increase in mobile phase pH would increase the degree of negative charge of the chiral selector resulting in an increased affinity for positively charged analytes, and consequently, retentions and enantioresolutions for basic analytes. As expected, when the pH value was reduced from 6.4 to 5.0, the retention times and enantiomeric resolution for all the analytes significantly decreased except for propranolol and omeprazole.

The addition of buffer salt plays a key role in controlling the pH of mobile phase and ionization of analytes, thus affecting both the retention and enantioselectivity [6]. The results demonstrated that lower buffer concentration resulted in longer retention times, higher enantioresolutions and broader peaks. The effect of organic modifier type may change from case to case. For example, the enantiomers of 4-MEC were partially separated using IPA as organic modifier (R_s= 0.8), while its enantiomers were not resolved using either MeOH or ACN. In contrast, much better enantioseparations were achieved for 2-MMC and 3-MMC when using ACN or MeOH instead of IPA. On increasing the organic content from 2% to 10%, retention time decreased, as did enantioresolution in most cases.

A comparison between the results obtained with the Lux Cellulose-1 and Chiralpak CBH column indicated that more successful enantioseparations were obtained with CBH column. Specifically, enantiomers of four cathinones were not separated on the Lux Cellulose-1

column and eluted within a very short time indicating weak interactions with the CSPs, whereas they were enantioseparated on the Chiralpak CBH except 4-MEC. The low success rate of enantioseparation achieved with Cellulose-1 column in PO mode was due to the large eluent strengths of the mobile phases which resulted in quite short retention times and poor enantioselectivities. Interestingly, a study reported the successful enantioseparations of 19 cathinones (including 2-MMC, 3-MMC, 4-MMC and 4-MEC) on a Lux Cellulose-2 column based on cellulose tris(3-chloro-4-methylphenylcarbamate) in PO mode with a mobile phase consisting of ACN/IPA/FA/DEA (95/5/0.1/0.1, v/v/v/v) [7]. Lux Cellulose-2 and Lux Cellulose-1 have the same cellulose backbone but differ in the substituents on the phenyl moiety. It demonstrated that the nature of the substituents on the phenyl moieties have a significant effect on chiral recognition, highlighting the complexity and unpredictability in chiral separations.

During the optimization of enantioseparations for both Lux Cellulose-1 and Chiralpak CBH column, the compatibility of the mobile phase with the MS analyzer was taken into account, such as using volatile salts and acidic/basic additives. This could pose a limitation to the mobile phase choices and an extra challenge for satisfactory enantioseparations. However, when switching from LC-DAD to LC-MS analysis, the current LC conditions can be directly used without modifications, which is significant in environmental and biological analysis. To date, several chiral LC method using MS compatible mobile phases have been reported for the enantioseparation of β-blockers and omeprazole [8-11], but rarely for cathinones. For example, Meetani el al. [12] developed a chiral LC-UV method for the enantiomeric determination of 18 synthetic cathinones using an Astec Cellulose DMP column. Satisfactory enantioseparations were obtained for all the analytes, but they were performed in NP mode using mobile phase of hexane/IPA/TEA (99/1/0.1, v/v/v), which is incompatible with MS detection. Similarly, Kadkhodaei et al. [13]. reported the successful use of a Lux Amylose-1 column in NP mode with the mobile phase of hexane/IPA/DEA (90/10/0.1, v/v/v) for the enantioseparation of 60 cathinones along with other new psychoactive substances (NPSs). Recently, successful enantioseparations of 33 out of 49 cathinones were performed using a Trefoil CEL1 column based on cellulose tris(3,5-dimethylphenylcarbamate), but also in NP mode with a mobile phase of hexane/butanol/DEA (100/0.3/0.2, v/v/v) [14]. Chiral CSPs, especially polysaccharide-based, may achieve a high success rate in enantioseparation in NP mode, but the mobile phase systems are incompatible with MS detection, which would largely limit their applications in trace analysis in complex matrices.

In order to contribute to the development of new CSPs, four polymeric CSPs (DSA 35, DSA 37, SD 099 and SD 100) were synthesized in the second study. Their chiral recognition capabilities were investigated in NP mode. NP mode was selected as similar polymeric CSPs have been reported to produce efficient enantioseparations of a variety of chiral drugs when

used in the NP mode [15,16]. Hexane was used as the main component of the mobile phase. The effects of the organic modifier, acidic additives and flow rate on enantioseparation were investigated. The results showed that no analyte was enantioseparated on all the columns. However, it should be mentioned that 3 cathinones – flephedrone, pentedrone and mephedrone – showed a sign of enantioseparation with a shoulder peak on the DSA 37 column after the addition of 0.1% TFA as acidic additives in the mobile phase.

The poor results obtained with the synthesized polymeric CSPs may be caused by the column being too short. In general, a longer column would lead to better enantioseparation. Therefore, one strategy is to use longer columns in future research. In addition, the synthesis process of polymeric CSPs is crucial for their chiral recognition abilities. Thus, improved synthesis method with proper control of various parameters such as the degree of polymerization, grafting density and polymer architecture is highly desirable for the preparation of polymeric CSPs in the future study. Moreover, it can be observed that the retention times are quite long and the peaks are broad in most cases when applying these polymeric CSPs in NP mode. Thus, using these polymeric CSPs in PO or RP mode may be feasible to get better results.

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3.2. Enantiomeric determination of cathinones in environmental water samples

Due to the growing awareness of stereoselective environmental fate and toxicity of chiral drugs, enantiomeric determination of chiral drugs in the environment has gained increasing attention over the last decade. So far, certain chiral drugs have been extensively studied such as β-blockers, venlafaxine, ibuprofen and amphetamines, and their enantiomeric occurrence in environmental matrices is well documented [1-4]. In spite of this, there is a lack of knowledge of the enantiomeric distribution of most frequently used chiral drugs in the environment mainly due to unavailability of sensitive and selective analytical methods [2]. Cathinones, more commonly known as "bath salts", belong to new psychoactive substances (NPSs). In the last 10 years, more than 130 new cathinone derivatives have been synthesized, making them the second largest group of NPS after cannabinoids [5]. However, despite their great popularity, their enantiomeric determination in the environment was less reported. For this reason, the research in this section has focused on the development of two analytical methods based on SPE followed by LC-HRMS for the enantiomeric determination of a group of cathinones in environmental water samples.

As shown in the study presented in the previous section (Section 3.1.1), the Chiralpak CBH column employing in RP mode provided good enantioselectivity towards some cathinone analytes, and therefore this column was selected for the enantioseparation of cathinone analytes in both studies included in this section. Since the LC enantioseparation conditions developed in the previous study are MS compatible, they can be directly applied in LC-MS analysis. Thus those LC conditions were used as the basis to be improved on in both studies included in this section. Once the chromatographic conditions were set up, an Orbitrap-HRMS analyzer was used for identification and quantification of target cathinones in method validation and analysis of environmental samples.

As discussed in the Introduction, chiral drugs are present in the environment at trace levels and environmental matrices are quite complex. For this reason, efficient sample preparation to remove interference components and to concentrate target analytes is an essential part in the analytical process. For environmental water samples, SPE is the most commonly used sample preparation technique [6]. A wide range of sorbents are currently available. In addition, it is well known that the selection of the suitable sorbent is crucial to achieve successful extraction.

In the first study, two mixed-mode cation-exchange sorbents (Oasis WCX and Oasis MCX cartridge) were examined to extract target cathinones from environmental water samples. Oasis MCX, a strong cation-exchange sorbent, contains surface functional groups of sulfonic acid, while Oasis WCX, a weak cation-exchange sorbent, contains surface functional groups of carboxylic acid. These sorbents were chosen in view of the fact that cathinones are basic and would be retained favorably on the negatively charged sorbents via ion-exchange interactions, thereby contributing to greater selectivity. Different SPE

parameters were carefully optimized to achieve high recoveries and selectivity for the analytes. The performance of the two sorbents were compared, and the one provided better results was chosen for further analysis. The developed method based on SPE followed by LC-HRMS was applied for the analysis of river water and effluent wastewater samples.

Motivated by the promising results obtained in our first work, our efforts then focused on further improving the selectivity and sensitivity of the analytical methods for the enantiomeric determination of cathinones in environmental waters. During the past decades, the use of molecularly imprinted polymers (MIPs) as SPE sorbents have attracted considerable attention because of their highly selective extraction of the analytes of interest [7,8].

With this in mind, in the second study included in this section, novel MIPs using (1R,2S)-(−)-ephedrine or (1S,2S)-(+)-pseudoephedrine as dummy template were synthesized and compared for the extraction of targeted cathinones from river water samples. This work was done in collaboration with Dr. Federica Pessagno and Dr. Panagiotis Manesiotis, from Queen's University of Belfast (United Kingdom), who prepared the new MIP sorbents. To achieve higher extraction recoveries for the selected analytes, different parameters were optimized. The developed method was applied to determine the enantiomeric composition of targeted analytes in river water samples.

The results obtained from these two studies have been published in the Journal of Chromatography A 1626 (2020) 461359 and Microchemical Journal 175 (2022) 107100, respectively, and are presented in the following sections.

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3.2.1. Enantiomeric determination of cathinones in environmental water samples by liquid chromatography-high resolution mass spectrometry

ENANTIOMERIC DETERMINATION OF CATHINONES IN ENVIRONMENTAL WATER SAMPLES BY LIQUID CHROMATOGRAPHY-HIGH RESOLUTION MASS SPECTROMETRY

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Abstract

The enantiomeric determination of chiral drugs in the environment is of emerging concern since their enantiomers often exhibit stereoselectivity in environmental occurrence, fate and toxicity. In this study, a method based on solid-phase extraction followed by chiral liquid chromatography and high-resolution mass spectrometry has been developed for the enantiomeric determination of a group of cathinones in river water and effluent wastewater. The enantioseparation was carried out using a Chiralpak CBH column in reversed-phase mode, and optimised by evaluating the effects of flow rate, buffer concentration and organic modifier. Under optimal conditions, good enantioseparations ($R_s \geq 1.2$) were achieved for all the analytes. Two mixed-mode cation-exchange sorbents (Oasis WCX and Oasis MCX) in solid-phase extraction were evaluated in river water. Oasis MCX sorbent showed better performance with apparent recoveries ranging from 57 to 91% and matrix effect ranging from -10 to 15%. It is worth noting that a shifting of retention times and loss of enantioresolutions in environmental water samples was observed for all the analytes when the Oasis WCX sorbent was used. The method was validated with river water and effluent wastewater samples and its overall performance was satisfactory. The method quantification limits for all the analyte enantiomers ranged from 1.0 to 2.9 ng/L in river water, and from 2.3 to 6.0 ng/L in effluent wastewater. The repeatability and reproducibility values, expressed as % relative standard deviation (n = 5) were less than 15%. The method was then applied to the analysis of river water and effluent wastewater. The racemic methylone and methedrone (EF=0.49 and 0.46, respectively) were detected at low ng/L in some of the river water samples.

Keywords: cathinones, enantiomeric determination, environmental waters, chiral liquid chromatography, high-resolution mass spectrometry, mixed-mode ion-exchange SPE

1. Introduction

Over recent years various chiral drugs have been found in environmental waters as a result of human excretion and incomplete removal by wastewater treatment plants (WWTPs) [1]. They can become stereoselectively degraded during human metabolism and also during biological degradation in the wastewater treatment process and in the surface water [2]. The enantiomeric determination of drugs in the environment is crucial in order to obtain specific information regarding the degradation processes within different ecosystems. As a result of stereoselective degradation, the enantiomeric enrichment or depletion of chiral drugs may also occur. There is a clear evidence that the enantiomers of the same compound can exhibit stereoselectivity in toxicity towards aquatic organisms due to different pharmacological activity. For example, De Andrés et al. [3] have reported that S(−)-atenolol shows higher toxicity to *D. magna* than R(+)-atenolol, while R(+)-atenolol is more toxic to *T. thermophila* than the S(−)-enantiomer. Investigating the enantiomeric composition of chiral drugs in the environment is therefore fundamental for improving accuracy in risk assessment of chiral contaminants. In addition, the enantiomeric analysis of drugs in wastewater would help to identify whether a drug residue originated from consumption, the direct disposal of unused drugs or manufacturing waste [1]. Emke et al. [4], for instance, investigated the enantiomeric composition of 3,4-methylenedioxymethamphetamine (MDMA) in influent wastewater in Utrecht and proved that extremely high mass loads of MDMA (present in racemic mixtures) during the sampling campaign were due to the direct disposal of unused MDMA, probably resulted from a police raid at a nearby illegal production facility.

Synthetic cathinones have become very popular in recreational drug markets all over the world [5]. These designer drugs are sold mainly via on-line stores as “bath salts” and are labelled “not for human consumption” to circumvent legal regulations on marketing [6]. All these compounds are chiral and are commercially available as racemates. As with many chiral drugs, the pharmacological potency of the enantiomers of these psychoactive substances can vary. For instance, S(−)-methcathinone exhibits more potent stimulating effects on the central nervous system than R(+)-methcathinone [7]. Some studies have been conducted to monitor the presence of cathinones in different environmental waters [8-11]. However, there are few studies on its enantiomeric determination at environmental level, and in those only one cathinone – mephedrone – was included [12-14].

Over the years, the enantioseparations of cathinones have been carried out using different chiral columns including polysaccharide-based [15-18], ion-exchange [19], Pirkle-type [20] and protein-based [12-14]. In this study a protein-based column – the Chiralpak CBH – was selected because our previous study [21], which

compared the performance of polysaccharide-based and protein-based columns for the enantioseparation of various drugs on LC-UV, showed that it provided satisfactory enantioseparation for the cathinone included in the study. In the Chiralpak CBH column, a protein cellobiohydrolase (CBH) was immobilised on 5 μm silica particles as the chiral selector. This column is used exclusively in reversed-phase mode, allowing it to be coupled with mass spectrometry (MS) detection. The CBH column can provide effective enantioseparation for basic compounds including primary and secondary amines, in which the key chiral recognition mechanism can involve ion-exchange, hydrophobic interactions and hydrogen bonding [12].

Due to the extremely complex matrices and low concentrations of chiral drugs, liquid chromatography (LC) coupled with MS is often used in enantiomeric determination at environmental level. To date, most of the related work has used analysers such as triple quadrupole (QqQ) [12,22-24], while some has used quadrupole time-of-flight (QTOF) [25,26]. Orbitrap high-resolution mass spectrometry (HRMS) is rarely utilised for enantioselective environmental analysis [27]. Orbitrap instruments provide enhanced selectivity linked to improved mass accuracy, enabling the sensitive detection of analytes in complex matrices. Moreover, Orbitrap-HRMS analysers operate in full-scan mode, thus making retrospective analysis possible [28]. Orbitrap-HRMS is therefore also considered to be a powerful tool for identifying and quantifying chiral drugs in environmental matrices.

Considering that cathinones are polar basic compounds that possess an amino group, mixed-mode cation-exchange sorbents may be suitable for use in solid-phase extraction (SPE) since they are able to exploit both ion-exchange and reversed-phase interactions [29]. Both Oasis WCX and Oasis MCX, which are mixed-mode weak and strong cation-exchange sorbents respectively, have been used to extract cathinones from environmental waters in previous studies. For instance, in a previous study from our group [8], when the performance of both sorbents in the extraction of 12 cathinones from environmental waters in a non-chiral analysis study was compared, the best results were achieved with the Oasis WCX cartridge. Meanwhile Senta et al. [10] achieved excellent recoveries using Oasis MCX to extract 11 cathinones along with other amphetamine-like drugs in environmental waters. On the basis of these studies and bearing in mind the unpredictability of chiral analysis, both cartridges (Oasis WCX and Oasis MCX) were assessed in this study.

The current study aims to develop an analytical method for the enantiomeric determination of a group of cathinones in environmental samples (river water and effluent wastewater). The developed method is based on SPE followed by LC–

Orbitrap-HRMS using a Chiralpak CBH column. To our knowledge, this is the first time the enantiomeric determination of a group of cathinones at environmental level has been reported.

2. Experimental

2.1. Standards and materials

The target cathinone standards – (±)−4-methylmethcathinone HCl (mephedrone), (±)-flephedrone, (±)−4-methoxymethcathinone HCl (methedrone), (±)−3,4-methylenedioxymethcathinone HCl (methylone) and (±)-butylone HCl (butylone) – were purchased from LGC Standards (Luckenwalde, Germany). The structures and pK_a values of these analytes are shown in Fig. 1S.

The solid standards were diluted in methanol (MeOH) to prepare stock solutions at 1000 or 2000 mg/L depending on the compound and stored in the freezer at −20 °C. Working solutions were prepared by diluting the stock solution with mobile phase. The solvents MeOH, acetonitrile (ACN) and isopropanol (IPA) of HPLC grade were purchased from J.T. Baker (Deventer, Netherlands). Ultrapure water was obtained from a Synergy® UV water purification system (Millipore, Billerica, MA, USA). Formic acid (HCOOH) was from Honeywell (Augsburg, Germany). Analytical grade ammonium acetate (NH_4Ac) and ammonium hydroxide (NH_4OH) were from Sigma-Aldrich (St. Louis, MO, USA). SPE cartridges Oasis WCX and Oasis MCX (500 mg) were purchased from Waters Corp. (Milford, MA, USA).

2.2. Sampling

River water samples were collected from the River Ebre in the region of Catalonia, and effluent wastewater samples were collected from WWTPs in the Tarragona area (north-east Spain). The samples were collected in plastic containers and stored at −20 °C until analysis. Prior to analysis, the samples were filtered through a 1.2 μm glass-fibre membrane filter and subsequently through a 0.45 μm nylon membrane filter (Whatman, Maidstone, UK).

2.3. Solid-phase extraction

Two types of cartridges were assessed during the SPE optimisation: Oasis WCX and Oasis MCX (500 mg). The optimal loading volumes were 500 mL for river water and 250 mL for effluent wastewater samples.

The WCX cartridges were conditioned with 10 mL of MeOH, and with 10 mL of ultrapure water (pH=7) for equilibration. The filtered samples adjusted to pH 7 were percolated through the cartridges. The cartridges were then rinsed with 5 mL of MeOH. The analytes were subsequently eluted with 5 mL of 5% HCOOH in MeOH.

The MCX cartridges were conditioned as WCX cartridges, but adjusting the water for equilibration to pH=3 with HCOOH. The filtered samples were also adjusted to pH 3 in order to protonate the basic analytes.

After loading the samples, the cartridges were washed with 10 mL of MeOH. The analytes were then eluted with 5 mL of 5% NH_4OH in MeOH.

In both cases the eluates were evaporated to dryness employing a vacuum concentrator (MiVac, Genevac, Ipswich, UK). Finally, the extracts were reconstituted with 10 mL of mobile phase for Oasis WCX and 1 mL of mobile phase for Oasis MCX, vortex-mixed for 1 min and filtered through a 0.45 μm PTFE syringe filter prior to injection.

2.4. Instrumentation and chromatographic conditions

Optimisation of the enantioseparation was carried out using an Agilent 1100 series LC system (Waldbronn, Germany) equipped with a degasser, a binary pump, a column oven and a diode array detector. The column used was a Chiralpak CBH (150 × 2 mm, 5 μm) with a Chiralpak CBH guard column (10 × 2 mm, 5 μm), both supplied by Daicel (Illkirch, France). The injection volume was 5 μL, the column temperature 30 °C and UV detection was performed at 230 or 254 nm.

The Chiralpak CBH column was employed under reversed-phase mode with mobile phase composed of a buffer solution and an organic modifier. After optimisation, the optimal conditions involved the use of the mobile phase consisting of 1 mM NH_4Ac aqueous solution/MeOH (98/2, v/v) in isocratic mode at a flow rate of 0.4 mL/min.

Once the chromatographic conditions were optimised, an Accela 1250 UHPLC System coupled to an Exactive Orbitrap™ mass spectrometer (Thermo Scientific, Bremen, Germany) was used for the SPE optimisation, method validation and analysis of environmental samples. The UHPLC instrument is equipped with a quaternary pump, an automatic injector and a column oven. The LC system was coupled to HRMS instrument with a heated electrospray ionisation (HESI) source and a higher-energy collisional dissociation (HCD) cell. The chromatographic conditions used in the LC–HRMS were the same as in the LC–UV and the analysis time was 18 min. The column oven and sample tray inside the automatic injector were set at 30 °C and 10 °C respectively. In the ion source, cathinones were ionised in positive mode. The data were acquired in a single time window by alternating two scan events: (1) a full scan (100–500 m/z) at 50,000 FWHM with 250 ms of injection time; (2) a fragmentation scan at 10,000 FWHM with 50 ms of injection time. The optimum collision voltage was 15 eV. For quantification, the protonated molecules were measured with a mass error $\leqslant$ 5 ppm, while for confirmation, the selected fragments and ion ratios were taken into account.

The optimal HRMS parameters were: capillary voltage, 30 V; spray voltage, 3 kV; skimmer voltage, 20 V; tube lens voltage, 70 V; sheath gas, 50 AU (adimensional units); auxiliary gas, 5 AU; and heater and capillary temperatures, 350 °C. The probe

position adjustment was side to side 0, vertical B and micrometre 1.

2.5. Validation

Instrumental linearity was investigated by injecting standard solutions in triplicate at seven concentrations in a range from the instrumental quantification limit (IQL) to 40 μg/L for each enantiomer. The instrumental detection limit (IDL) and IQL were experimentally measured with standard solutions and corresponded to the concentrations that gave a signal-to-noise ratio (S/N) of 3 and 10 respectively. The signal intensity at the IDLs should be higher than 1×10^3.

The apparent recovery (R_{app}) was expressed as the ratio between the measured concentration in the extract obtained when a sample was spiked before the SPE and the theoretical concentration.

The matrix effect (ME) was assessed using the following equation:

$$ME=(C_{POST-SPIKED}/C_{STD} - 1) \times 100 \quad (1)$$

Where $C_{POST-SPIKED}$ is the concentration obtained from the sample extract spiked after the SPE, and C_{STD} is the theoretical concentration. A positive value indicates signal enhancement, while a negative value indicates signal suppression.

The method detection limit (MDL) and method quantification limit (MQL) were first estimated from the IDL and IQL by applying the SPE concentration factor and R_{app}. The values were then confirmed by testing three replicated river and effluent wastewater samples spiked at the estimated concentration level and checking that they met the S/N criteria and that the signal intensity was higher than 1×10^3.

Accuracy, intra-day precision (repeatability) and inter-day precision (reproducibility) were assessed by determining five spiked samples at 40 ng/L for river water and 80 ng/L for effluent wastewater. Accuracy, expressed as relative recovery, was determined as the percentage of the mean experimental concentration and the actually spiked concentration. Repeatability and reproducibility were expressed as percentage of the relative standard deviation (RSD) the same day and different days respectively.

The enantiomeric fraction (EF) was calculated using the following equation:

$$EF=\frac{E1}{E1 + E2} \quad (2)$$

where E1 and E2 are the peak areas of the first and second eluting enantiomers respectively. EF equals 1 or 0 in the case of enantiopure compounds and 0.5 in the case of racemates.

3. Results and discussion

3.1. Optimisation of enantioseparation

As mentioned earlier, a previous study by our group [21] showed that the Chiralpak CBH column provided satisfactory enantioseparations for some cathinone compounds with a mobile phase of 1 mM NH_4Ac/MeOH (95/5, v/v) at a flow rate of 0.1 mL/min. These separation conditions

were therefore used as the basis to be improved on. The following parameters were investigated in the present study: flow rate (0.1, 0.4 and 0.5 mL/min), type of organic modifier (IPA, ACN and MeOH), concentration of organic modifier (2%, 5% and 10%) and buffer concentration (1–10 mM NH_4Ac).

With the initial conditions, all the analytes were enantioseparated. However, the retention times were quite long (up to 45 min). In the first instance, higher flow rates (0.4 and 0.5 mL/min) were tested. As expected, by increasing flow rate the retention times decreased and the peaks became narrower. Consequently all the analytes achieved the highest resolution at a flow rate of 0.4 mL/min. Buffer concentration is another main parameter that affects both retention and enantio-resolution. A lower buffer concentration (1 mM) resulted in higher enantioresolutions with longer retention times. Therefore 1 mM of NH_4Ac was selected. By increasing the organic content (MeOH) from 2% to 10% in the mobile phase, its elution strength increased, and retention and enantio-resolution decreased in most cases. Of the three organic modifiers studied (IPA, ACN and MeOH), MeOH was selected as it provided slightly better resolutions for most of the analytes.

In conclusion, good enantioseparation of the target cathinones was carried out with a mobile phase composed of an aqueous solution of 1 mM NH_4Ac with 2% MeOH at a flow rate of 0.4 mL/min. With the optimal chromatographic conditions, all the analytes were baseline separated within 18 min (Fig. 1). Although some of them eluted at the close retention times, they were able to be separated by their different protonated molecules in the Orbitrap analyser.

3.2. Optimisation of mass spectrometry detection

In order to achieve the highest sensitivity for all the analytes, HRMS parameters were optimised by injecting a mixture standard solution using the optimal chromatographic conditions. Since all the target analytes presented basic functional groups in their structures, the analyses were performed in positive ionisation mode. The optimal HRMS instrument parameters are described in Section 2.4.

For quantification, $[M + H]^+$ was selected for all the analytes. Table 1 presents the exact and accurate mass of the diagnostic ions, as well as the accurate mass of fragment ions monitored for confirmation purposes. In order to achieve two abundant fragment ions for each analyte, collision energy (10–60 eV) was optimised by injecting individual standard solutions. As a compromise, 15 eV was adopted for all the studied analytes. Two fragment ions were achieved for all the target analytes except flephedrone, for which only one characteristic fragment ion was observed. Fig. 2S shows the proposed fragmentation pathway of the studied analytes.

3.3. Solid-phase extraction

The optimisation of the SPE procedure was carried out in accordance with a study

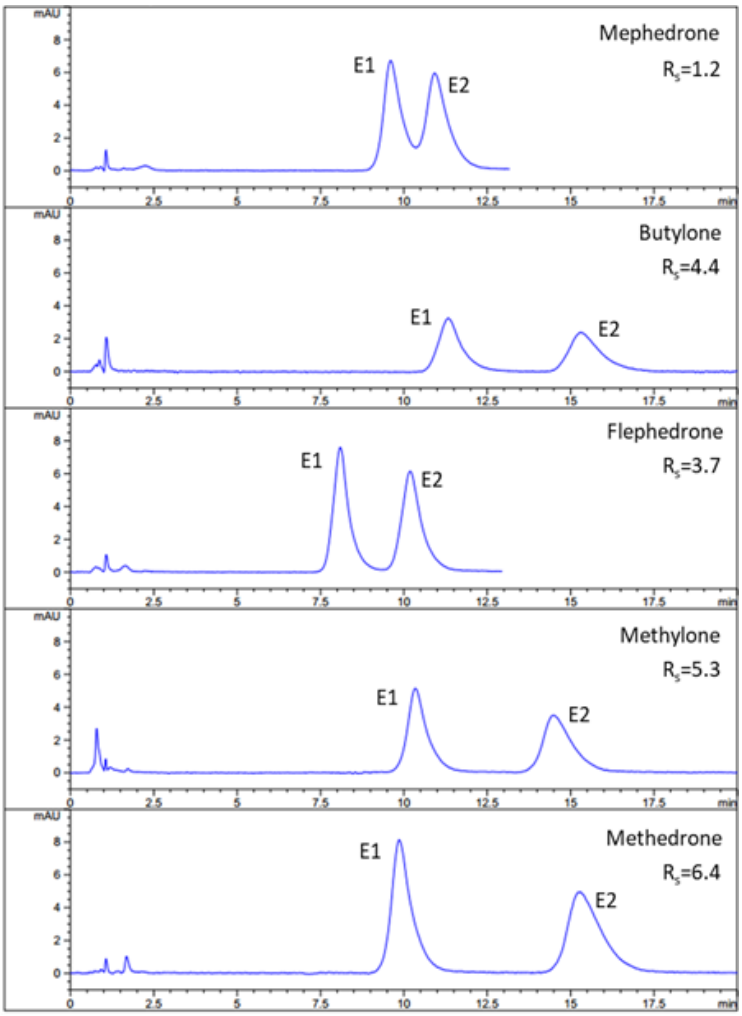

Figure 1. LC-UV chromatograms of the enantioseparation of the target analytes at 5 mg/L on the Chiralpak CBH column with the mobile phase of 1mM NH_4Ac buffer/MeOH (98/2, v/v) at a flow rate of 0.4 mL/min.

Table 1. Diagnostic ions and fragments monitored for the two enantiomers of each compound.

Compound	Diagnostic ion $[M+H]^+$			Fragment 1		Fragment 2	
	Formula	Exact mass m/z	Accurate mass m/z	Formula	Accurate mass m/z	Formula	Accurate mass m/z
Mephedrone	$C_{11}H_{16}NO$	178.12264	178.12291	$C_{11}H_{14}N$	160.11243	$C_{10}H_{11}N$	145.08894
Butylone	$C_{12}H_{16}NO_3$	222.11247	222.11288	$C_{12}H_{14}NO_2$	204.10255	$C_{11}H_{12}NO$	174.09187
Flephedrone	$C_{10}H_{13}FNO$	182.09757	182.09816	$C_{10}H_{11}FN$	164.08759	-	-
Methylone	$C_{11}H_{14}NO_3$	208.09682	208.09743	$C_{11}H_{12}NO_2$	190.08691	$C_{10}H_{10}NO$	160.07617
Methedrone	$C_{11}H_{16}NO_2$	194.11755	194.11794	$C_{11}H_{14}NO$	176.10735	$C_{10}H_{11}NO$	161.08371

[8] with the protocol detailed in Section 2.3 that evaluated both Oasis WCX and Oasis MCX cartridges in the non-chiral analysis of cathinones in different types of environmental water samples, with only the effect of different volumes of washing and elution solutions being tested.

Recoveries were initially evaluated using 500 mL of ultrapure water. After conditioning, 500 mL of ultrapure water (adjusted at pH=3 for Oasis MCX and at pH=7 for Oasis WCX) spiked with a standard solution at 40 ng/L was loaded. To optimise the MeOH volume in the washing step, two volumes were tested: 5 and 10 mL. Finally, the analytes were eluted with three fractions of 5 mL of MeOH containing 5% HCOOH in the case of Oasis WCX or 5% NH_4OH in the case of Oasis MCX. All the elution fractions and washing solutions were collected, evaporated to dryness and reconstituted with 1 mL of mobile phase. In the case of Oasis WCX, washing with 10 mL of MeOH, the loss of analytes in this step was no higher than 10% and recoveries ranged from 75% to 96%. With 5 mL of MeOH, no analytes were lost in the washing step and the recoveries were comparable (≈ 100%), as can be seen in Table 2. In the case of Oasis MCX, there was no great difference in R_{app} when using 5 or 10 mL of MeOH (R_{app} ranged from 76 to 97% and from 73 to 103% respectively). In all cases, no analytes were found in the second and third elution fractions, and therefore the elution volume was fixed at 5 mL.

Before selecting the volume of washing solution (MeOH), we moved on to river water to investigate its impact on the ME and R_{app} in the matrix. 500 mL of river water spiked with analytes at 40 ng/L was loaded. The protocol followed for this sample was the same as that shown above. Unexpectedly, in the case of Oasis WCX it was observed that the peak retention times measured in the river water samples were significantly decreased compared to those measured in standard solution, as were the enantioresolutions. This issue was further explored by diluting an appropriate volume of extract with mobile phase to get a different dilution factor (defined as the ratio between the final volume after dilution and the initial volume of the SPE extract), and then injecting it into LC–HRMS. The effect of the dilution factor on retention times is shown in Fig. 2. As the dilution factor increased, the retention times obtained were closer to those in standard solutions. This indicated that the shifting of retention times could be attributed to the influence of the sample matrices in this type of column. Interestingly, such effect was not observed in the previous study [8] when Oasis WCX was used to extract the cathinone analytes in different environmental waters and reversed-phase chromatography to determine them. Although some studies have also reported the shifting of retention times in matrix [30,31], this is the first report to present the phenomenon in chiral analysis. A reasonable explanation is that the interferences present in river water might compete with active sites of column for binding to analytes, thus shifting forward the retention time. The interference of matrices in LC-behaviours should depend

Table 2. R_{app} and ME obtained when 500 mL of ultrapure water or river water spiked with the analyte mixture at 40 ng/L were extracted with the Oasis WCX and MCX cartridges using 5 mL or 10 mL of MeOH in the washing step.

Compound	Ultrapure water				River water			
	WCX		MCX		WCX		MCX	
	5 mL of MeOH	10 mL of MeOH	5 mL of MeOH	10 mL of MeOH	5 mL of MeOH		10 mL of MeOH	
	R_{app} (%)	R_{app} (%)	R_{app} (%)	R_{app} (%)	ME (%)	R_{app} (%)	ME (%)	R_{app} (%)
Mephedrone E1	105	79	82	84	62	144	12	83
Mephedrone E2	103	81	91	88	58	137	8	79
Butylone E1	102	96	97	103	49	142	14	85
Butylone E2	105	92	95	100	39	120	12	89
Flephedrone E1	101	77	76	73	52	145	-7	61
Flephedrone E2	102	76	80	78	43	121	-10	57
Methylone E1	103	75	95	101	52	134	11	89
Methylone E2	103	76	96	99	44	126	4	86
Methedrone E1	123	89	96	101	48	163	15	91
Methedrone E2	127	92	95	102	54	154	7	82

n=3,
RSD ≤ 15%

on the nature of the matrices and the analytes, the sample treatment methods and the instrumental parameters, including the mobile phase and column used in the analysis [30]. In addition, the eluates of analytes with a dilution factor of 10 were considered to be comparable to those in standard solution (the retention time varied by less than 1 min). We therefore decided to reconstitute the extracts from SPE with 10 mL mobile phase for Oasis WCX. Obviously, the high reconstitution volume would result in higher MDLs and MQLs. To improve MDLs and MQLs, higher injection volumes (10, 15, 20 and 25 µL) were tested. However, when the injection volume increased, the retention times and enantioresolutions decreased again, as expected. Therefore, the injection volume was fixed at 5 µL for the Oasis WCX cartridge. Even washing with 5 mL of MeOH and reconstituting the extract to 10 mL, significant signal enhancement (ranging from 39 to 62%) was observed for all the analytes, with recoveries ranging from 98 to 163%, as shown in Table 2. When the washing solution volume was increased from 5 to 10 mL, MEs were slightly decreased while recoveries were significantly decreased for all the analytes (the results with 10 mL of MeOH are not shown in the case of Oasis WCX). Therefore 5 mL of MeOH was selected in the washing step for Oasis WCX.

In contrast as regards Oasis MCX, the matrices did not interfere with the retention times of analytes. Consequently the dried extracts of river water samples were reconstituted with 1 mL of mobile phase for

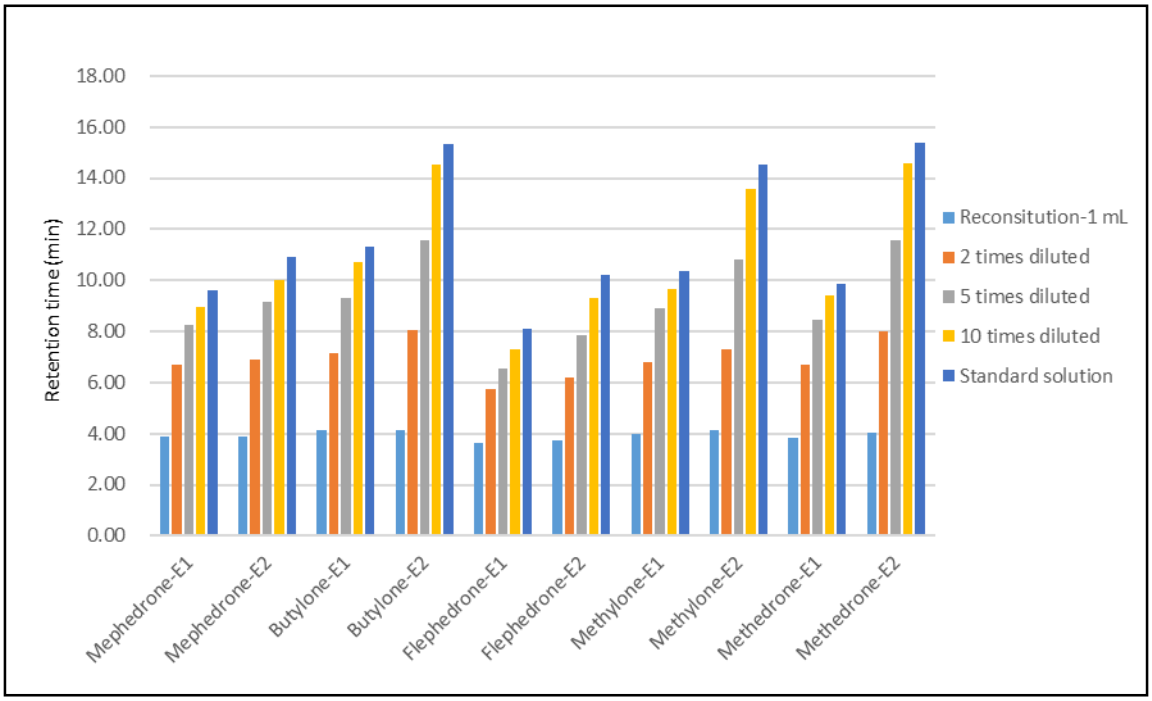

Figure 2. The influence of matrices on the retention times of analytes in river watersamples with the Oasis WCX cartridge.

Oasis MCX. Higher injection volumes (10, 15, 20 and 25 μL) were also tested with Oasis MCX. As a higher injection volume would result in higher ME, 10 μL of injection volume was selected as a compromise. With 10 mL of MeOH in the washing step, ME ranged from −10 to 15% and good recoveries (>75%) were obtained for all the analytes except for the enantiomers of flephedrone (R_{app}=61%, 57%, respectively) (Table 2). When the washing solution volume was decreased from 10 to 5 mL, a slight increase of MEs was observed and there was no significant difference in R_{app} (the results with 5 mL of MeOH are not shown in the case of Oasis MCX). Therefore 10 mL of MeOH was used to clean up for Oasis MCX.

Comparing the performance of both sorbents, the above results indicate that Oasis MCX behaved better than Oasis WCX in terms of recovery, ME, MDL and MQL, and therefore this was selected for further analysis. The recovery and ME values are comparable with the previous study [8] in which Oasis WCX sorbent was finally selected to extract cathinones in the environmental water samples. In another study [12] in which Oasis HLB sorbent was used as SPE sorbent to extract only one cathinone (mephedrone) along with other chiral drugs from influent wastewater sample; in this case, higher recoveries of mephedrone enantiomers were obtained, although higher ME values were also observed. The lower ME in our study could be attributed the effective washing step.

3.3. Method validation

For the instrumental validation, IDLs and IQLs were determined using S/N under the optimal conditions (with 10 μL of injection volume) and ranged from 0.1 to 0.5 μg/L and 0.3 to 1.2 μg/L respectively. Higher IDLs and IQLs could be due to a high aqueous content (98%) in the mobile phase and is therefore not optimal for effective ionisation in the ion source. The linearity was good from IQL to 40 μg/L for each enantiomer, with all determination coefficients exceeding 0.996.

The Oasis MCX cartridge was selected on the basis of the analysis of river water. We were also interested in the applicability of the developed method for effluent wastewater, so the R_{app} and ME were evaluated with this too. Other method validation parameters including MQLs, MDLs, accuracy, repeatability (intra-day) and reproducibility (inter-day) were also determined with both river water and effluent wastewater samples. Method validation data for the analytes are presented in Table 3.

ME and R_{app} in effluent wastewater were evaluated using 250 mL effluent wastewater spiked with the analytes at 80 ng/L. The decrease in loading volume for effluent wastewater was due to the higher complexity of the matrix which would decrease the recoveries as well as would result in much higher ME [32]. As shown in Table 3, ion suppression was observed for all the analytes except for the enantiomers

Table 3. Retention time (t_R) and validation parameters of the method based on SPE with Oasis MCX followed by LC-HRMS for the enantiomeric determination of the target cathinones in environmental water samples.

Compound	t_R (min)	EF	River water[a]						Effluent wastewater							
			MDLs[b] (ng/L)	MQLs[b] (ng/L)	Accur[c] (%)	Rep[c] RSD (%)	Repro[c] RSD (%)	EF[c]	MDLs[b] (ng/L)	MQLs[b] (ng/L)	ME[d] (%)	R_{app}[d] (%)	Accur[d] (%)	Rep[d] RSD (%)	Repro[d] RSD (%)	EF[d]
Mephedrone E1	9.60	0.49	0.5	1.3	87	9.3	13.1	0.48	1.2	3.0	-8	66	102	7.4	6.6	0.48
Mephedrone E2	10.92		0.5	1.3	94	8.4	12.6		1.2	3.0	-9	67	96	6.2	8.9	
Butylone E1	11.33	0.47	0.7	1.2	102	6.9	7.9	0.46	1.5	2.5	-5	79	88	11.1	14.6	0.47
Butylone E2	15.31		0.7	1.2	96	7.7	12.6		1.6	2.6	-7	76	91	9.4	13.5	
Flephedrone E1	8.09	0.47	0.3	1.0	110	10.2	10.7	0.47	0.8	2.3	-32	50	97	10.4	7.2	0.46
Flephedrone E2	10.19		0.4	1.1	108	6.4	9.8		0.8	2.3	-36	52	105	8.9	9.1	
Methylone E1	10.34	0.48	0.7	1.6	93	9.7	8.3	0.48	1.8	4.1	-14	68	112	11.5	12.3	0.48
Methylone E2	14.51		0.6	1.5	97	9.3	7.6		1.8	4.1	-8	68	107	12.7	9.6	
Methedrone E1	9.86	0.49	1.1	2.6	106	11.6	8.5	0.50	2.5	6.0	4	80	93	9.6	13.7	0.48
Methedrone E2	15.30		1.2	2.9	98	10.8	5.5		2.4	5.6	6	85	85	8.0	14.3	

[a] R_{app} and ME for river water spiked at 40 ng/L are presented in Table 2;
[b] RSD $\leq$ 12%;
[c] spiked at 40 ng/L;
[d] spiked at 80 ng/L.

of methedrone, and in all cases the ME values were lower than 15% in agreement with the SANTE/12,682/2019 criteria [33] of ME ≤ 20%, except for the enantiomers of flephedrone. In the case of flephedrone, significant signal suppression accounting for 32% and 36% was observed for first and second enantiomers respectively, which resulted in lower R_{app} values (50% and 52% respectively). Nonetheless, the R_{app} values of the other analytes were satisfactory (ranging from 66% to 85%). In general, both the ME and R_{app} were acceptable and comparable to those obtained with river water during SPE optimisation.

The MDLs in river water ranged from 0.3 to 1.2 ng/L and the MQLs from 1.0 to 2.9 ng/L. Because of the lower loading volume (250 mL), these values increased in effluent wastewater, with MDLs and MQLs ranging from 0.8 to 2.5 ng/L and 2.3 to 6.0 ng/L respectively. The values were comparable with those reported in the previous non-chiral analysis study [8] utilising the Orbitrap-HRMS analyser as well. Kasprzyk-Hordern et al. [12] reported the MQLs for mephedrone enantiomers of 2.6 ng/L when 100 mL of influent wastewater was analysed, whereas in this study the MQLs for mephedrone enantiomers were 1.3 and 3.0 ng/L when 500 mL river water and 250 mL effluent water were analysed respectively (Table 3). Considering the more complex matrix and lower loading volume of influent water sample, they achieved lower MQLs values than our case which could be attributed to the higher recoveries (higher ME as well) as mentioned and higher sensitivity of QqQ analyser. However, the Orbitrap-HRMS analyser could provide enhanced selectivity owing to the significantly improved mass accuracy [28].

For all compounds in both environmental matrices, the accuracy values were between 85 and 112% and repeatability and reproducibility values ranged from 6.2 to 12.7% and 5.5 to 14.6% respectively, fulfilled the method validation criteria [33] in which the acceptable relative recovery is in the range of 70–120% with repeatability and reproducibility RSD ≤ 20%.

The EF values were determined at each calibration point in triplicate. Racemic standards showed a mean EF of 0.49 for mephedrone and methedrone, 0.47 for butylone and flephedrone, and 0.48 for methylone. The EFs of each analyte spiked in both matrices are also reported in Table 3. For all the compounds no change in the EF was observed, implying no stereoselectivity occurring during sample treatment.

In summary, the accuracy, ME, repeatability and reproducibility of the developed method meet the criteria of SANTE/12682/2019 for all the analyte enantiomers in river water and effluent wastewater samples, except for ME of flephedrone enantiomers in effluent wastewater (ME=−32 and −36, respectively). In addition, the R_{app}, MDL and MQL were satisfactory comparing with previous studies [8,12]. Therefore, the method can be applied for the enantiomeric

detection and quantification of the target cathinone analytes in river water and effluent wastewater.

3.4. Analysis of environmental samples

Three different river water samples from the Ebre River and three different effluent wastewater samples from WWTPs of Tarragona area were analysed with the validated method. The enantiomers of methylone and methedrone were detected in the river water samples, and the mass error was below 2.5 ppm in all cases.

Both enantiomers of methylone were detected in one river water sample at similar concentration levels, 5.1 and 5.3 ng/L respectively. Of the family of cathinones, methylone is very commonly found in environmental water. For example, Fontanals et al. [8] reported methylone concentrations ranging from 1.8 to 4.7 ng/L and 3.0 to 17.9 ng/L in river water and effluent wastewater respectively. Similarly, in another study by Kinyua et al. [34], methylone was found in two influent sewage samples from Switzerland, ranging from 0.6 to 2.5 ng/L.

The enantiomers of methedrone were detected in one river water sample at 3.8 and 4.4 ng/L respectively. This observation is in agreement with the study by Fontanals et al. [8], in which methedrone was detected at 1.8 ng/L in one river water sample. In all the effluent wastewater samples, a peak was observed whose accurate mass of molecular ion matched methedrone (error<5 ppm), and the retention time matched the second enantiomer of methedrone (within the tolerance limit of 0.1 min). However, no fragment ions appeared. Therefore, confirmation of this compound cannot be supported. No other analytes were detected in the effluent wastewater samples. This could be attributed to the relatively high MDLs and MQLs in effluent wastewater and the more complicated matrices that would interfere with the detection of the target analytes.

For the methylone and methedrone found in river water samples, the EF values obtained were 0.49 and 0.46 respectively, suggesting that no enantioselective metabolism occurred during the various processes or that the compounds were from the disposal of unused drugs. However, the information on enantioselectivity in terms of metabolic behaviour and distribution in the environment is too limited to enable confirmation of this hypothesis. As mentioned above amongst cathinones, only for mephedrone few studies on enantiomeric profiling in environmental water have been published, and these arrived at no consistent conclusions: Castrignanò et al. [12,13] found mephedrone enriched with E1 during the analysis of influent wastewater in the UK, suggesting that enantioselective metabolism may occur in humans, and Archer et al. [14] observed mephedrone was present in racemic mixtures (EF = 0.52 ± 0.01) during an analysis of South African wastewaters. More comprehensive research needs to be undertaken not only as regards the enantiomeric profiling of cathinones but

also into their stereoselective pharmacokinetics.

Flephedrone, mephedrone and butylone were not detected in any sample in the present study. Mephedrone has previously been detected in the UK [9,11-13], Italy [11,35], Spain [8,36] and other European cities [9,10], in varied concentrations ranging from <MQL to 110 ng/L. However, flephedrone and butylone have rarely been detected in environmental waters in previous studies, implying low consumption within the studied area. For example, flephedrone was only detected in some of the weekend samples (collected from Friday to Monday) in the UK among the seven European cities investigated and at levels ranging between 4.4 and 25.8 ng/L, whereas butylone was not detected in any city [11]. In another study [10] conducted in Croatia, flephedrone was found at rather low levels (<MDL - 4 ng/L) in river water and wastewater, while butylone was not detected in any sample. Thus the presence of cathinones in environmental waters is still random.

4. Conclusions

In this study a method based on SPE followed by LC–Orbitrap-HRMS was developed, validated and applied to the enantiomeric determination of different cathinones in river water and effluent wastewater samples.

The enantioseparation was successfully achieved using a Chiralpak CBH column in reversed-phase mode after optimising buffer concentration, organic modifier and flow rate. All the analytes were enantioseparated ($R_s \geq 1.2$) with the optimal mobile phase consisting of 1 mM NH_4Ac aqueous solution /MeOH (98/2, v/v) at a flow rate of 0.4 mL/min.

For the analysis of environmental water samples, Oasis MCX was selected because it behaved better than Oasis WCX in terms of recovery, ME, MDL and MQL. In the case of the Oasis WCX sorbent, the retention times of analytes measured in the matrix significantly decreased compared to those measured in standard solution, as were those for enantioresolution. This is the first time that the shifting of retention times caused by complex matrices in the chiral analysis has been reported.

The validated method was successfully applied in the enantiomeric determination of cathinones in river water and effluent wastewater. The enantiomers of methylone and methedrone were detected at low ng/L concentration levels in river water. The EF values for methylone and methedrone in river water were determined to be 0.49 and 0.46 respectively, suggesting that they were present in racemic mixtures in river water.

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Supplementary Data

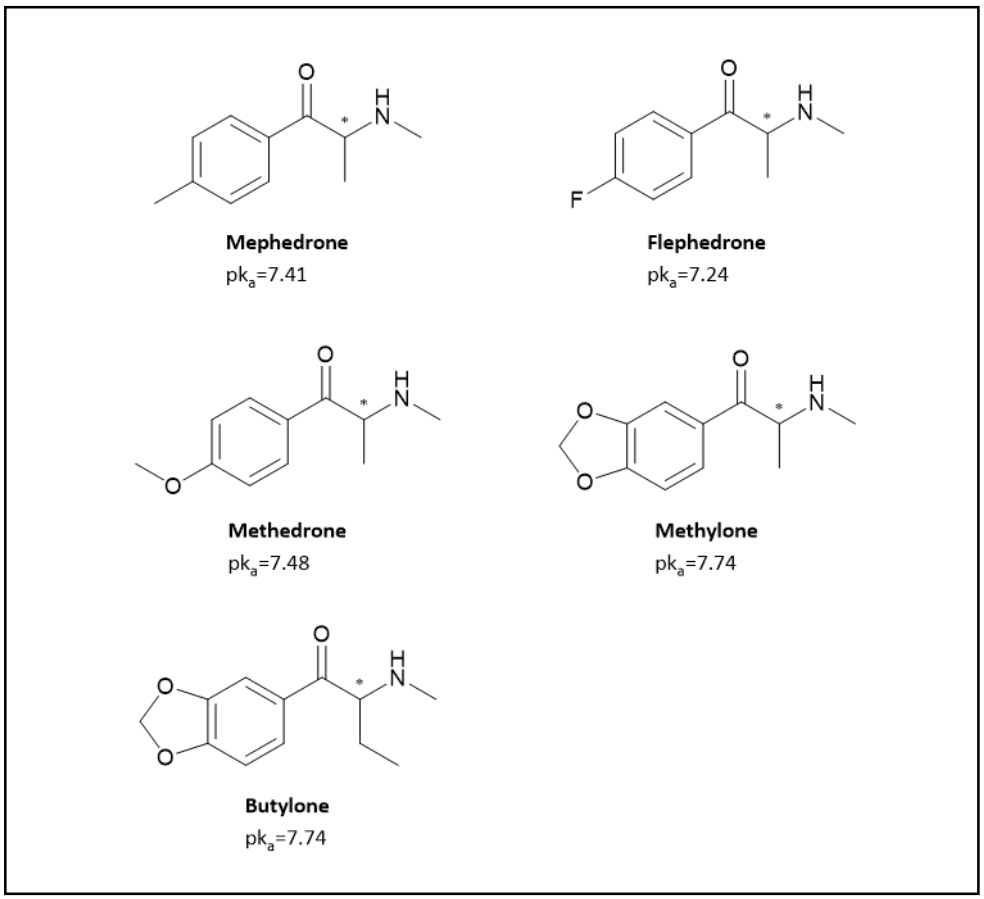

Figure 1S. Structures and pk_a values of target analytes. Chiral centres were indicated by asterisks.

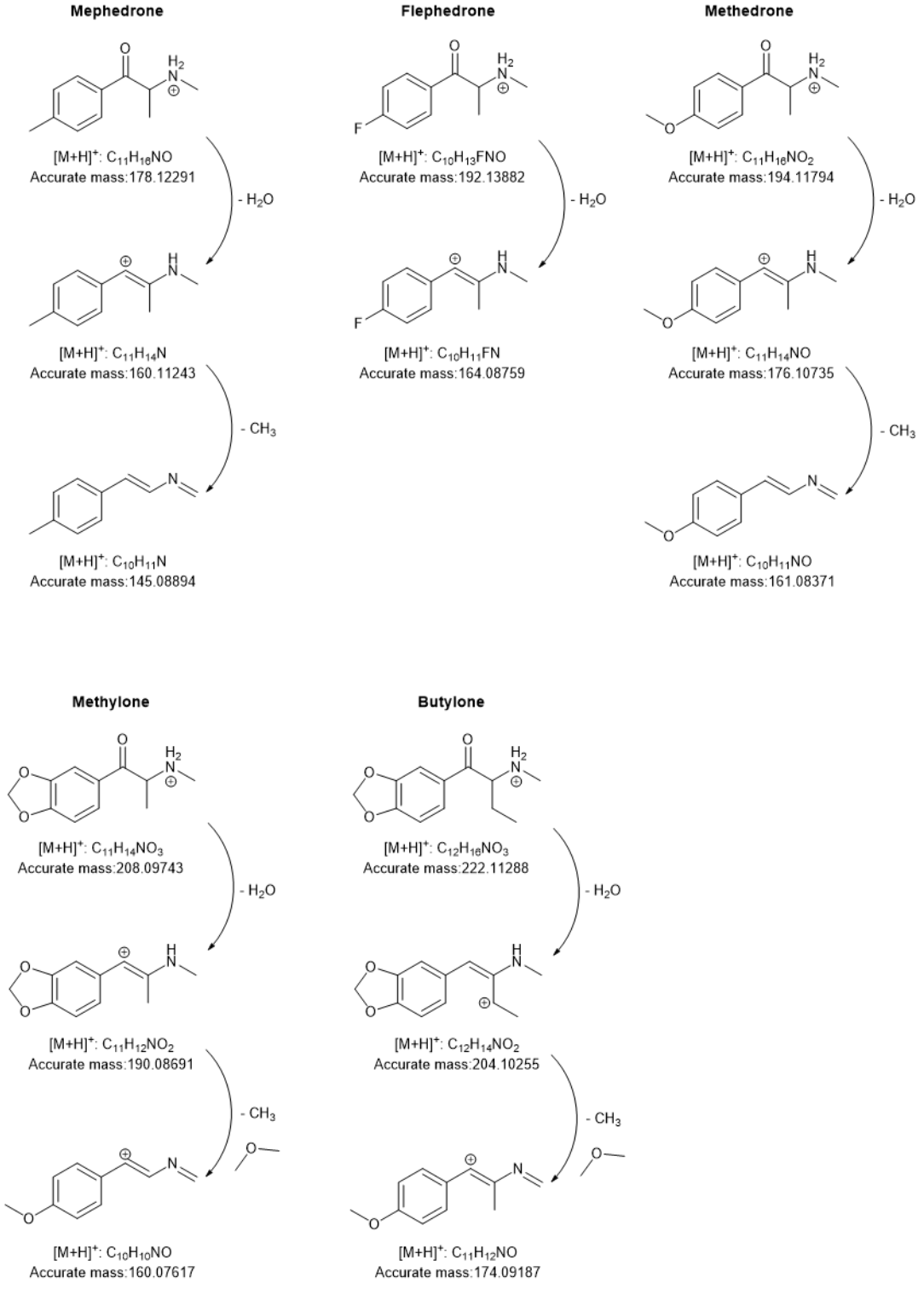

Figure 2S. Proposed fragmentation pathway of the studied analytes.

Table 1S. R_{app} and ME obtained when 500 mL of ultrapure water or river water spiked with the analyte mixture at 40 ng/L were percolated through the Oasis WCX and MCX cartridges using 5mL or 10 mL of MeOH in the washing step.

Compound	Ultrapure water				River water			
	WCX		MCX		WCX		MCX	
	5 mL of MeOH	10 mL of MeOH	5 mL of MeOH	10 mL of MeOH	5 mL of MeOH		10 mL of MeOH	
	R_{app} (%)	R_{app} (%)	R_{app} (%)	R_{app} (%)	ME (%)	R_{app} (%)	ME (%)	R_{app} (%)
Mephedrone E1	105	79	82	84	62	144	12	83
Mephedrone E2	103	81	91	88	58	137	8	79
Butylone E1	102	96	97	103	49	142	14	85
Butylone E2	105	92	95	100	39	120	12	89
Flephedrone E1	101	77	76	73	52	145	-7	61
Flephedrone E2	102	76	80	78	43	121	-10	57
Methylone E1	103	75	95	101	52	134	11	89
Methylone E2	103	76	96	99	44	126	4	86
Methedrone E1	123	89	96	101	48	163	15	91
Methedrone E2	127	92	95	102	54	154	7	82

n=3;
RSD ≤ 15%

3.2.2. Preparation and evaluation of molecularly imprinted polymers as selective SPE sorbents for the determination of cathinones in river water

PREPARATION AND EVALUATION OF MOLECULARLY IMPRINTED POLYMERS AS SELECTIVE SPE SORBENTS FOR THE DETERMINATION OF CATHINONES IN RIVER WATER

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Abstract

In this study, a series of molecularly imprinted polymers (MIPs) were synthesised using (1R,2S)-(–)-ephedrine or (1S,2S)-(+)-pseudoephedrine as template, methacrylic acid as functional monomer and ethylene glycol dimethacrylate as cross-linking agent. A highly selective and sensitive method using the best performing MIP as sorbent in the solid-phase extraction (SPE) followed by liquid chromatography-high resolution mass spectrometry was established for the enantiomeric determination of five cathinones in river water samples. The SPE parameters (sample loading volume and pH, washing and elution solutions) were optimised so that recoveries and selectivities for the analytes were high. The enantioselectivity of MIPs towards the enantiomers of the analytes during the SPE process was also investigated. Under optimal conditions, the method developed provided satisfactory recoveries ranging from 67.6 to 83.2% with a negligible matrix effect ranging from -5.5 to 1.8 %. The detection limits ranged from 0.3 to 0.8 ng/L and quantification limits ranged from 1.0 to 2.0 ng/L. The application of the method to the analysis of the river water samples indicated that methedrone and butylone were present at low concentration levels in river water samples.

Keywords: Cathinones, enantiomeric determination, molecularly imprinted polymer, solid-phase extraction, environmental water

1. Introduction

Nowadays the determination of drug residues in environmental waters is still a challenge that requires highly sensitive and selective analytical methods, due to the very low drug concentrations and extremely complex matrices. Efficient sample preparation can remove interference components and concentrate target analytes prior to instrumental analyses and so plays an important role in improving method sensitivity and selectivity. Solid-phase extraction (SPE) with various sorbents is the most commonly used sample preparation technique [1]. However, classical SPE sorbents, such as silica-based sorbents modified with C_8 or C_{18} chains and hydrophilic-hydrophobic balance (HLB), lack selectivity since analytes are retained via non-specific interactions. Thus, other compounds in the sample are co-extracted with target analytes, leading to significant matrix effects (ME), particularly when liquid chromatography-mass spectrometry (LC-MS) is used [2]. Mixed-mode ion-exchange sorbents may show selectivity towards ionic analytes when a clean-up step is included. However, molecularly imprinted polymers (MIPs) are the sorbents which have attracted considerable attention as promising SPE sorbents that have high selectivity during the preparation of environmental samples [3,4].

MIPs are highly cross-linked and porous synthetic polymers with high selectivity to the target analytes. They are obtained by polymerising functional and cross-linking monomers around a template molecule, which is usually the target analyte. Afterwards, the template molecules are removed to leave cavities complementary to the target molecule in size, shape and functional groups. Thus, MIPs are able to selectively bind target molecules from the complex matrix, with fewer interferences than classical sorbents. In addition, MIPs have the advantage that they have a high loading capacity, are easy to prepare and are remarkably robust under a wide range of operating conditions, which increases their applicability [3]. However, the main obstacle in the application of the traditional MIPs is the template bleeding during the analysis resulting from incomplete template elution from polymeric matrix, which could cause overestimated results [5]. To overcome this, the use of structural analogue as the template, called a “dummy template”, has been proposed to prepare MIPs. Besides, the introduction of dummy templates is a useful solution when the target molecule is expensive or difficult to synthesise [6]. To date, the application of dummy MIPs in the SPE (MISPE) of various drugs has been reported in other studies [7–9].

An emerging class of new psychoactive substances (NPS), cathinones have gained great popularity in illicit drug markets all over the world [10]. The ease of online purchase makes them difficult to regulate. After consumption, cathinones are released into the environment via the sewage system. Determining their residues in environmental samples is a fast and cheap method for estimating their community

consumption [11]. However, it should be noticed that all cathinones are chiral and are usually marketed as racemates. Their enantiomeric composition is often different from the racemic form after excretion as a consequence of the stereoselective metabolism [12]. Monitoring the enantiomeric composition of chiral drugs in the environment can distinguish between the residues of consumed drugs and unused drugs that have been directly disposed, which would make the estimation of consumption more accurate [13,14]. It would also help to identify the synthesis pathway of chiral drugs [15].

Methods based on SPE followed by LC-MS have been applied to determine cathinones at environmental level [11,16–19]. An important consideration during the extraction of chiral drugs is that stereoselectivity can occur since the enantiomers might show different affinities for the sorbents, which would result in a deviation from its initial enantiomeric fraction (EF). In particular, MIPs have already been shown to have a great potential for the enantioseparations of various chiral drugs [20–22]. Therefore, the stereoselectivity of MIPs towards the enantiomers should be assessed during the sample extraction.

The aim of the present study is to establish a highly sensitive and selective method for the enantiomeric determination of a group of cathinones in river water applying MISPE followed by LC–(Orbitrap) high resolution mass spectrometry (HRMS). For this purpose, novel MIPs using (1R,2S)-(−)-ephedrine or (1S,2S)-(+)-pseudoephedrine as dummy template were synthesised and compared for the extraction of cathinones. Ephedrine was chosen as a dummy template molecule because its structure is similar to that of cathinones and it is easy to obtain. Although MIPs employing ephedrine as template have been wildly reported in fundamental molecular imprinting studies [23–25] and as LC stationary phases [22,26–28], their application as SPE sorbent has been rarely reported [29,30], and to the best of our knowledge, this is the first time applying it in the extraction of cathinones.

2. Experimental

2.1. Chemicals and solvents

For the preparation of the MIP and corresponding non-imprinted polymer (NIP) based SPE cartridges, methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), (1R,2S)-(–)-ephedrine, (1S,2S)-(+)-pseudoephedrine, chloroform, 1-hydroxycyclohexyl phenyl ketone (IRG184), acetic acid (HAc), empty polypropylene SPE cartridges (3 mL) and 20 μm porous polyethylene frits were purchased from Sigma Aldrich (Gillingham, UK). The cathinone standards – (±)-flephedrone, (±)-3,4-methylenedioxymethcathinone (methylone), (±)-4-methylmethcathinone (mephedrone), (±)-butylone and (±)-4-methoxymethcathinone (methedrone) – were purchased from LGC Standards (Luckenwalde, Germany), while the standards of (±)-atenolol, (±)-

propranolol, (±)-naproxen and diclofenac, which were used to prove the specific selectivity of the MIPs towards cathinones, were purchased from Sigma–Aldrich (St. Louis, MO, USA). The structures and pK_a values of these analytes and ephedrine are shown in Fig. 1. Individual stock standard solutions were prepared in methanol (MeOH) at 1 or 2 mg mL^{-1}, and kept at -20°C. Working solutions were prepared by diluting the stock standard solutions with mobile phase, and kept at 4°C.

MS grade acetonitrile (ACN), MeOH and water for the LC-HRMS mobile phase were purchased from Scharlab (Barcelona, Spain), and HPLC grade ACN and MeOH were purchased from J. T. Baker (Deventer, The Netherlands). Analytical-grade ammonium hydroxide (NH_4OH) and ammonium acetate (NH_4Ac) were obtained from Sigma–Aldrich. Formic acid (FA) and

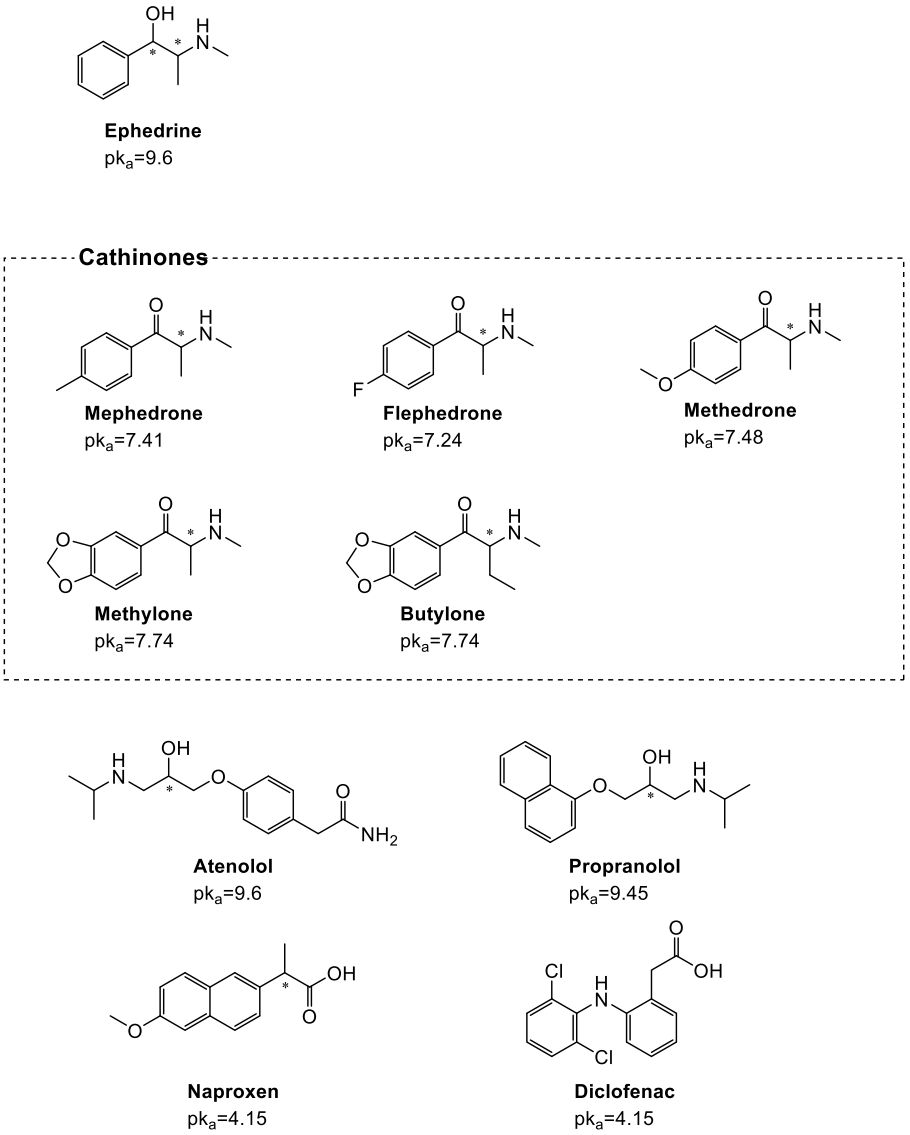

Fig. 1. Structures and pK_a values of ephedrine (template) and the analytes. Chiral centers are indicated by asterisks.

HAc were purchased from Honeywell (Augsburg, Germany). Ultrapure water was produced through a water purification system (Merk-Millipore, Billerica, MA, USA).

2.2. Synthesis of imprinted polymers

Imprinted and non-imprinted polymers were prepared by photochemically initiated free radical polymerisation. The compositions of all the polymers prepared are presented in Table 1. Briefly, the template and the selected functional monomer were transferred into a glass vial and mixed with the porogen (chloroform). Upon complete dissolution, the cross-linker was added followed by the initiator. The resulting pre-polymerisation solutions were ultrasonicated for 5 min, purged with argon and then hermetically sealed. The vials were then placed in the chamber of a UVP CX-2000 UV curing reactor (UVP, Jena, Germany) and irradiated at 365 nm for 3 h at room temperature. The resulting rigid monoliths were coarsely ground and washed with MeOH in a Soxhlet apparatus for 24 h to remove the template and any unreacted monomers. The coarse polymer particles were further ground using a mortar and pestle, wet-sieved with acetone (to further ensure template removal), and the 25-50 μm fraction was collected, dried and stored at room temperature for further experiments. The corresponding NIPs were prepared in a similar fashion, although the template was not added to the pre-polymerisation mixture.

2.3. MISPE procedure

100 mg of each polymer (MIP/NIP (R,S) 1:4, MIP/NIP (R,S) 4:4, MIP/NIP (S,S) 1:4 and MIP/NIP (S,S) 4:4) was packed into a 3 mL empty polypropylene SPE cartridge. Parameters such as the volume and pH of the loading sample, and the type and volume of the washing and elution solution were systematically investigated for the effect they might have on the extraction recovery and selectivity.

The optimised MISPE protocol using the best MIP is as follows: the MIP cartridge is conditioned with 3 mL of ultrapure water (pH=7); 100 mL of sample (pH=7) is loaded onto the cartridges; the cartridges are then washed with 1 mL of 0.02% HAc in MeOH;

Table 1. Amounts of all reagents used for the synthesis of MIPs.

Polymer	(1R,2S)-(–)-ephedrine (mmol)	(1S,2S)-(+)-pseudoephedrine (mmol)	MAA (mmol)	EDGMA (mmol)	Chloroform (mL)	IRG184 (g)
MIP (R,S) 1:4	1	-	4	20	5.60	0.0380
MIP (R,S) 4:4	4	-	4	20	5.60	0.0380
MIP (S,S) 1:4	1	-	4	20	5.60	0.0380
MIP (S,S) 4:4	-	4	4	20	5.60	0.0380

subsequently, 1 mL of 5% NH_4OH in MeOH is used as elution solution; the eluates are evaporated to dryness using a MiVac vacuum concentrator (Genevac, Ipswich, UK), reconstituted with 1 mL of mobile phase and filtered through a 0.45 µm PTFE syringe filter prior to analysis.

Before SPE, the river water samples were consecutively filtered through a 1.2 µm glass-fiber membrane filter and a 0.45 µm nylon membrane filter (both from Whatman, Maidstone, UK).

2.4. Instruments and chromatographic conditions

SPE development experiments were performed by analysing the extracts using an Agilent 1100 series LC system (Waldbronn, Germany) equipped with a diode array detector (DAD). To identify and quantify analytes, a non-chiral analysis method was used with a Brisa LC2 C_{18} column (150 mm × 4.6 mm i.d., 5 µm) (Teknokroma, Sant Cugat del Vallès, Spain). The mobile phase consisted of 0.1% FA in water (solvent A) and 0.1% FA in ACN (solvent B). The gradient was as follows: 10% to 15% B in 10 min, to 30% B in 5 min, to 100 % B in 3 min, hold for 2 min, and then back to initial conditions in 2 min and hold for 5 min. The flow rate was 0.4 mL min^{-1} and the injection volume was 20 µL. The column was maintained at 35 °C, and UV detection was operated at 230 or 254 nm.

A chiral analysis method was used with a Chiralpak CBH column (150 mm × 2 mm i.d., 5 µm) and a Chiralpak CBH guard column (10 mm × 2 mm i.d., 5 µm), both purchased from Daicel (Illkirch, France) to verify the stereoselectivity of MIP cartridges towards the analytes and determine the enantiomeric composition of analytes in river water samples. The enantioseparation conditions were selected using the criteria established in a previous study by our group [17]. Briefly, the enantioseparations were carried out using a mobile phase of 1 mM NH_4Ac aqueous solution/MeOH (98/2, v/v) at 0.4 mL min^{-1} in isocratic mode. The column was maintained at 30 °C, and the analysis time was 10 min.

For method validation and analyses of river water samples, an Accela 1250 LC system coupled to an Exactive Orbitrap™ mass spectrometer (Thermo Scientific, Bremen, Germany) was used. The LC system consisted of an automatic injector, a quaternary pump and a column oven. The separations were carried out under the same chromatographic conditions used in the LC-DAD. The HRMS system was equipped with a heated electrospray ionisation (HESI) source operating in the positive ionisation mode. For the non-chiral analysis method, the optimal ion-source parameters were: sheath gas flow rate, 40 AU (adimensional units); auxiliary gas flow rate, 2 AU; spray voltage, 3.5 kV; skimmer voltage, 20 V; tube lens voltage, 75 V; capillary voltage, 30 V; capillary temperature, 350°C; heater temperature, 350°C. For the chiral analysis method, the HRMS parameter values were the same as above except the sheath gas flow rate and auxiliary gas flow rate, which

were 50 and 5 AU, respectively.

The data were acquired in a single time window by alternating two scan events: (1) a full scan at 50,000 FWHM with 250 ms of injection time; and (2) a fragmentation scan at 10,000 FWHM with 50 ms of injection time using a HCD cell voltage of 15 eV. The analytes were identified based on the retention time (with a tolerance of 0.1 min) and the accurate mass of diagnostic and two fragment ions (shown in Table 1S) (with a mass tolerance of 5 ppm). Ion ratios between the selected fragment ions and diagnostic ions were monitored for purposes of confirmation.

3. Results and discussion

3.1. Preparation and characterisation of the imprinted polymers

Ephedrine was used as dummy template as ephedrine and cathinone have similar structures since ephedrine contains amine and alcohol, whereas cathinone contains amine and carbonyl (see Fig. 1). In addition, in the formation of the template-monomer complex, as reported earlier [28] there is hydrogen bonding and electrostatic interactions between the carboxylic groups in the MAA and amine groups of either ephedrine or cathinones. There can be secondary interactions to the carbonyl or hydroxyl groups but these are much weaker.

The MIPs were prepared using photochemical bulk polymerisation approach in which many different template, monomers, crosslinkers as well as solvents are feasible to be polymerised. Thus, all the tested compositions outcame with final monolithic polymers. Moreover, FT-IR spectra (shown in Figure 1S) proved the incorporation of all the monomers during the polymerisation, and thus, the functional groups in the MIPs available for interaction during the MISPE. After this, the monolith was properly crushed to provide particulate material in form of irregular shape but with narrow size distribution (see scanning electronic microscope (SEM) image in Figure 2S) able to be packed for MISPE.

3.2. Optimisation of chromatographic conditions and MS detection

For the non-chiral analysis of cathinones, a Brisa LC2 C_{18} column (150 × 4.6 mm, 5 μm) was tested with a mobile phase of 0.1% FA in water (solvent A) and 0.1% FA in ACN (solvent B) under various gradient profiles. The best separation in a reasonable analysis time (less than 16 minutes) was achieved with the gradient described in Section 2.4 (Fig. 3S).

After the chromatographic conditions had been optimised, Orbitrap-based HRMS parameters were also investigated to maximise the response of the analytes, and the optimal values are described in Section 2.5. For all the analytes, the protonated ion $[M + H]^+$ was measured for quantification.

With the optimal LC–(Orbitrap) HRMS conditions, the instrumental limits of detection (IDLs), corresponding to the concentrations that gave a signal-to-noise ratio (S/N) of 3 and a signal intensity higher than 1×10^3, ranged from 0.05 to 0.1 μg L^{-1}. The instrumental quantification limits

(IQLs), defined as the concentrations that produce a S/N of 10, ranged from 0.1 to 0.25 μg L^{-1}. Good linearity (r^2 > 0.996) between IQLs and 100 μg L^{-1} was obtained for all the analytes.

3.3. MISPE optimisation

A preliminary test was conducted to compare the performance of four MIP cartridges – MIP (R,S) 1:4, MIP (R,S) 4:4 , MIP (S,S) 1:4 and MIP (S,S) 4:4 – with the initial protocol. That is, conditioning with 3 mL of 10 % HAc in MeOH and 3 mL of ultrapure water (pH=7), loading with 100 mL of ultrapure water spiked with 5 cathinones at 0.1 mg L^{-1}, washing with 1 mL of 0.02% HAc in MeOH and eluting with 1 mL of 10 % HAc in MeOH. The eluates were evaporated to dryness, then reconstituted with 1 mL of mobile phase and injected to LC-DAD. The results indicated that MIP (R,S) 1:4 and MIP (S,S) 4:4 performed better than MIP (R,S) 4:4 and MIP (S,S) 1:4 in terms of both recovery and selectivity. Therefore, MIP (R,S) 1:4 and MIP (S,S) 4:4 cartridges were selected to optimise such SPE conditions as the volume and pH value of the loading sample, and the type and volume of the washing and elution solutions.

3.3.1. MISPE optimisation with ultrapure water

Initially, ultrapure water spiked with 5 cathinones at 0.1 mg L^{-1} was used to evaluate the extraction recoveries. First, the loading volume was set to 100 mL, and the effect of the pH of the loading sample was evaluated with 1 mL of 0.02% HAc in MeOH as washing solution and 1 mL of 10% HAc in MeOH as elution solution. The pH of the loading sample is an important parameter since the retention mechanism of the analytes on the MIPs is based on ionic interactions between the amine moieties of cathinones and the carboxylic acid groups in the MIPs. The effect of the sample pH on the recovery was investigated at pH 3 and pH 7. With both MIP cartridges, the recoveries for all the analytes at pH 3 (3.1-6.4% in the case of MIP (R,S) 1:4 and 2.0 - 4.9% in the case of MIP (S,S) 4:4) were found to be much lower than those at pH 7 (62.2 - 73.4% in the case of MIP (R,S) 1:4 and 68.4 - 86.6% in the case of MIP (S,S) 4:4). The carboxylic acid groups in MIPs have a pK_a of about 5, and the cathinone analytes have a pK_a between 7.2 and 7.8. At pH 3, although the amino group of the cathinones are fully protonated, the carboxylic acid groups of MIPs are primarily in their non-ionised form. In contrast, at pH 7 both the carboxylic acid in the polymer and the amine moieties of cathinones are ionised, which promotes ionic interactions. Thus, the retention of cathinones on the MIPs are more effective at pH 7 than at pH 3. Therefore, the pH of the loading samples was set at 7 in the successive experiments.

Then different washing solutions were tested: 1 or 2 mL of MeOH, and 1 mL of 0.02% HAc in MeOH. The similar trends were observed for both MIP (R,S) 1:4 and MIP (S,S) 4:4 sorbents. Recoveries were good in all the tests. When pure MeOH was

used as the washing solution, there was no obvious difference in recoveries between MIP and NIP cartridges, which indicated that pure MeOH was not strong enough to disrupt the non-specific interactions between the MIPs and cathinones. By comparison, when 1 mL of 0.02% HAc in MeOH was used as the washing solution, MIP cartridges showed much higher recoveries than NIP cartridges. By way of example, the results of MIP (R,S) 1:4 are shown in Fig. 2. On the basis of the results, 1 mL of MeOH with 0.02% HAc was selected as the washing solution. The elution solution was optimised by applying three fractions of 1 mL of 10% HAc in MeOH. The results indicated that 1 mL of 10% HAc in MeOH was sufficient to elute all the analytes both for MIP (R,S) 1:4 and MIP (S,S) 4:4 cartridges.

Subsequently, we increased the sample loading volume to 200 mL which significantly decreased the recoveries (from 50% to 75%) of all the analytes on both cartridges (the results are detailed in Fig. 4S). Thus, the loading volume was set at 100 mL.

Besides cathinones, four other pharmaceuticals (atenolol, propranolol, naproxen and diclofenac) with very different structures (shown in Fig. 1) from cathinones were analysed to confirm the selectivity of the MIP cartridges. With the optimised protocol, the recoveries of these four compounds were lower than 30%. The poor retention of the four pharmaceuticals further demonstrated the selectivity of the MISPE protocol towards cathinones.

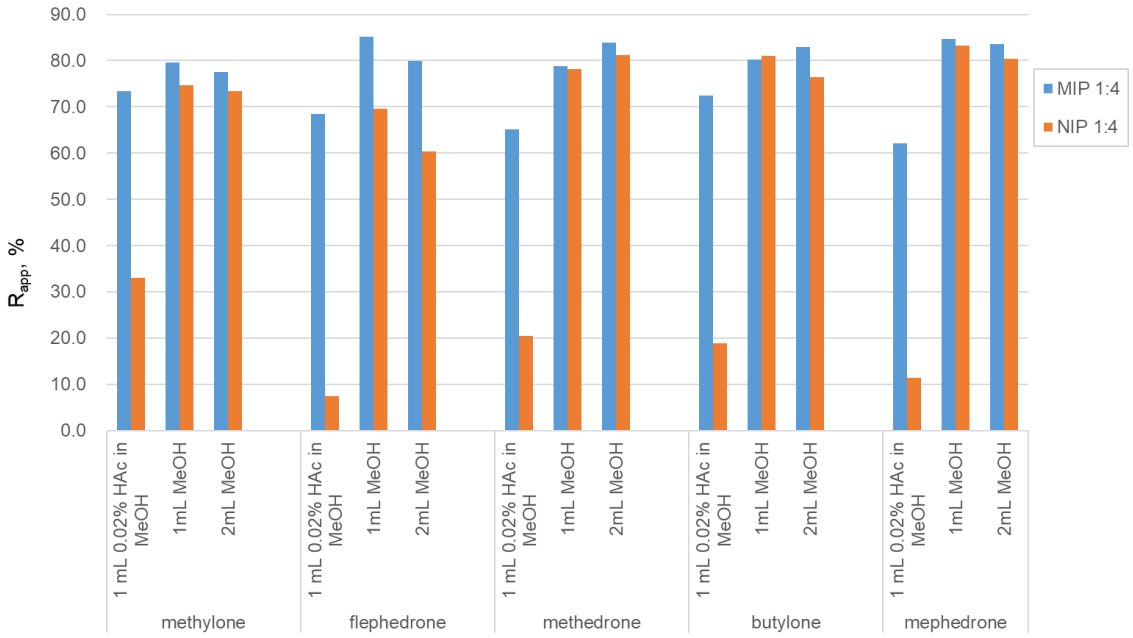

Fig. 2. The effect of different washing solutions on the R_{app} for all the analytes with MIP/NIP 1:4 sorbents.

In the next step, we used an Orbitrap analyser to evaluate the recovery at lower concentration levels and ME. Firstly, 100 mL of ultrapure water spiked with the analytes at 0.4 μg L^{-1} was percolated following the above protocol. Recoveries were good, ranging from 61.7 to 78.5 % for MIP (R,S) 1:4 and 67.6 to 83.7 % for MIP (S,S) 4:4, which were comparable with those obtained at higher concentrations in LC-DAD.

3.3.2. Evaluation of the stereoselectivity of MIP sorbents

To evaluate the stereoselectivity of MIP sorbents during the SPE process, the above extracts were also analysed using the chiral chromatographic method. Under the optimised conditions described in Section 2.4, baseline enantioseparations ($R_s \geq 1.2$) were achieved for mephedrone, flephedrone, methylone and methedrone, while partial enantioseparation was achieved for butylone ($R_s = 0.8$). One example of the enantioselective chromatograms of the analytes obtained with a MIP (R,S) 1:4 cartridge is shown in Fig. 3. The chiral analysis showed that the EF values of all the analytes in the eluates were around 0.5 except for butylone (EF = 0.45), which indicates that there was no stereoselectivity during the MISPE procedure. In the case of butylone EF, the slight deviation from 0.5 is due to the poor enantioseparation of butylone. Although the MIP was synthesised with one stereoisomer, the MIP did not show any stereoselectivity towards the analytes.

Subsequently, 100 mL of river water spiked at 0.4 μg L^{-1} was also analysed following the same protocol as for ultrapure water. When the chromatographic method with the C_{18} column was used for non-chiral analysis of analytes in the extracts, good recoveries (57.2 - 75.9 % in the case of MIP (R,S) 1:4 and 63.4 - 84.9 % in the case of MIP (S,S) 4:4) were attained for all the analytes. In addition, lower ME ranging from −8.1% to 3.4% in the case of MIP (R,S) 1:4 and from -6.6 to 4.1 % in the case of MIP (S,S) 4:4 were obtained. However, when the CBH column was used for the determination of enantiomeric composition of analytes in the extracts, the analyte peaks shifted forward significantly and enantioseparations were lost with both MIP sorbents. This phenomenon was also observed in one of our previous studies [17] where the same cathinone analytes were extracted using an Oasis WCX cartridge with 5 mL of 5% FA in MeOH as elution solution injected with the same CBH column. Considering that just such a problem was solved by changing the elution solution to 5 mL of 5% NH_4OH in MeOH in the previous study [17], we tried 1 mL of the same elution solvent (5% NH_4OH in MeOH) in the MISPE. As expected, the retention time and enantioseparation were not interfered. Moreover, the recovery values attained using this elution solution were similar and the difference in the recoveries between MIP and its respective NIP were notable (Table 2). Moreover, the ME encountered (-9.0 - 1.1% in the case of MIP (R,S) 1:4 and -5.5 - 1.8 % in the case of MIP (S,S) 4:4) were similar to those

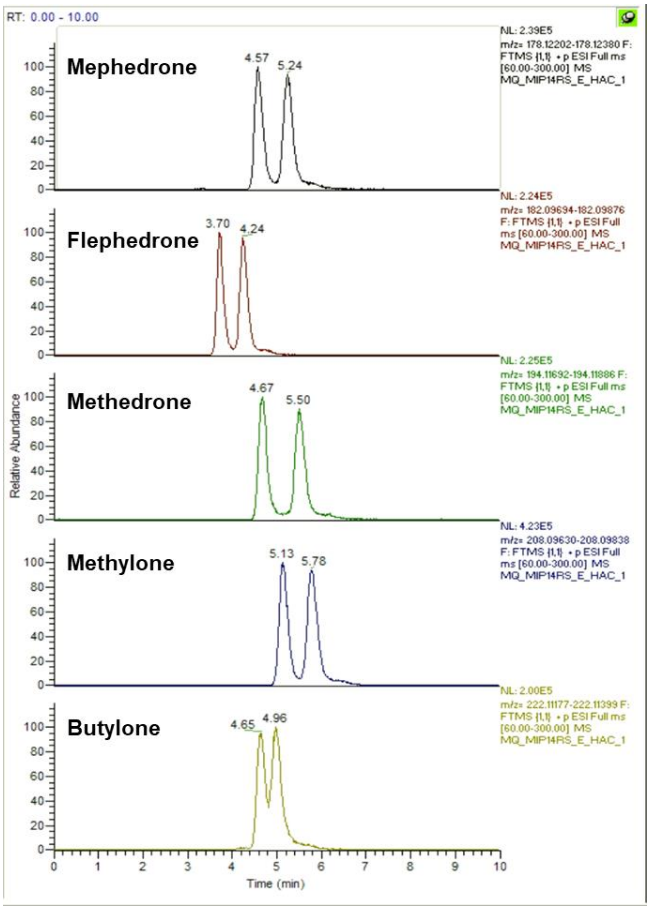

Fig. 3. Enantioselective chromatograms of the analytes spiked in ultrapure water at 0.4 μg L^{-1} extracted by SPE using MIP (R,S) 1:4 followed by LC-HRMS with CBH column. See the text for experimental conditions.

obtained when 1 mL of 10% HAc in MeOH was used as elution solution. This effect on the CBH column could be explained by interferences in the river water which can be eluted with 10% HAc in MeOH, and might compete with analytes to bind to active sites of the CBH column, thus leading to the lack of retention of the cathinones. Consequently, 1 mL of 5% NH_4OH in MeOH was selected as the elution solution.

3.3.3. Selection of the MIP sorbent

Comparing the performance of MIP (R,S) 1:4 and MIP (S,S) 4:4 sorbents, MIP (R,S) 1:4 sorbent showed higher selectivity in comparison to that of corresponding NIP. However, MIP (S,S) 4:4 provided improved recoveries (67.6 - 83.2 %) with good selectivity. For this reason, subsequent experiments were conducted using MIP (S,S) 4:4 as the SPE sorbent. The recovery values obtained with MIP (S,S) 4:4 are comparable to those of other studies [16,17,31] reporting the extraction of cathinones with commercial SPE cartridges. However, much lower ME values between -5.5 and 1.8% were achieved in the present study. For example, in a previous study [16]

Table 2. Comparison of recovery (R_{app}) obtained when 100 mL of river water spiked with the analytes at 0.4 μg L^{-1} were extracted with MIP (R,S) 1:4, MIP (S,S) 4:4 and their NIP cartridges using the optimal protocol that includes elution with 1 mL 5% NH_4OH in MeOH.

Compounds	MIP (R,S) 1:4	NIP (R,S) 1:4	MIP (S,S) 4:4	NIP (S,S) 4:4
	R_{app} (%)	R_{app} (%)	R_{app} (%)	R_{app} (%)
Methylone	75.8	19.6	79.5	47.3
Flephedrone	58.6	6.0	67.6	15.7
Methedrone	72.4	12.8	79.0	42.3
Butylone	81.5	21.7	83.2	55.0
Mephedrone	65.2	12.9	78.3	33.3

in which the Oasis WCX sorbent was used to extract a group of synthetic cathinones from river water samples, signal suppression (from -16% to - 28%) was observed for flephedrone, methylone, methedrone and butylone in spite of washing with 5 mL of MeOH. In another study [31], signal suppression ranging from -13% to -17% was observed for flephedrone, methylone, methedrone, mephedrone and butylone in river water samples, when the Oasis MCX sorbent was applied. The lower ME values in the present study indicated high selectivity of the MIP towards cathinones since the river water samples were cleaned-up efficiently.

The EF values of each analyte in river water sample extracts obtained using the optimal protocol were also determined and compared to those measured in the standard solution. The EFs of each analyte spiked in river water samples were in the range 0.45 - 0.49. For all the analytes no apparent change in the EF was observed, so the MIP (S,S) 4:4 cartridges showed the same retention capacity towards both enantiomers.

3.4. Validation

The method that used MIP (S,S) 4:4 as SPE sorbent followed by LC-HRMS with a C_{18} column was validated with river water samples by evaluating the method detection limits (MDLs), method quantification limits (MQLs), accuracy, repeatability (intra-day precision) and reproducibility (inter-day precision). All validation data are presented in Table 3.

MDLs and MQLs, defined as the minimum concentration with a S/N of 3 and 10 respectively, were determined by analysing a series of spiked water samples. The MDLs in river water were in the range 0.3 - 0.8 ng L^{-1} and the MQLs were in the range 1.0 - 2.0 ng L^{-1}. The values were somewhat higher than those reported in the previous studies due to the lower loading volume. For instance, in previous studies [16] the MDLs and MQLs reported were 0.08 - 0.15 ng L^{-1} and 0.25 - 1.5 ng L^{-1} for

Table 3. Validation parameters of the non-chiral analysis method based on SPE with MIP (S,S) 4:4 followed by LC-(Orbitrap) HRMS for the determination of the target cathinones in river water samples.

Analytes	MDL (ng L^{-1})	MQL (ng L^{-1})	Accuracy[a] (%)	Repeatability[a] RSD (%)	Reproducibility[a] RSD (%)
Mephedrone	0.5	2.0	91	9.3	11.1
Butylone	0.5	1.5	87	11.9	9.1
Flephedrone	0.3	1.0	116	8.8	7.4
Methylone	0.8	1.5	95	13.5	14.7
Methedrone	0.8	2.0	104	10.4	12.4

[a] spiked at 0.4 μg L^{-1}.

river water, when 250 mL of river water was analysed using an SPE (Oasis WCX)-LC-(Orbitrap)HRMS technique. Nevertheless, MDLs and MQLs obtained in our study were still considered to be suitable for environmental analysis.

Accuracy, repeatability and reproducibility were assessed by determining five replicates of river water samples spiked with cathinones at 0.4 μg L^{-1}. As a measure of accuracy, relative recoveries were calculated as the percentage of the mean experimental concentration and the theoretical concentration. They were between 87 and 116% for all the analytes. Repeatability and reproducibility, expressed as the relative standard deviation (%RSD) of these replicate analyses in one day and five successive days, respectively, ranged from 8.8 to 13.5% and 7.4 to 14.7%, respectively.

In summary, the method developed showed satisfactory MDL and MQL values, accuracy, repeatability and reproducibility, and therefore can be used to detect target cathinones in river water.

4. Application of the analytical method in river water analysis

The validated method was used to analyse river water samples collected from the Ebre River. Methedrone and butylone were detected in the river water samples after meeting all the confirmation criteria (mass error, tolerance and ion ratio). Fig. 4 displays one example of the extracted ion chromatograms of the protonated ion (A) and the two fragments (B, C) for one of the river water samples analysed.

Methedrone and butylone were detected in all the river water samples at concentrations between <MQL - 3.5 ng L^{-1} and <MQL - 2.4 ng L^{-1} respectively. These values are in agreement with a previous study [16] with samples from the same river, in which butylone was detected below MQL and methedrone was detected at concentrations of up to 1.8 ng L^{-1} in river water samples.

Mephedrone, flephedrone and methylone were not found in any river water samples. Indeed, after passing through a waste water treatment plant (WWTPs), these drugs are rarely present in river water samples. For example, Senta et al. [31] reported that flephedrone, mephedrone and methylone were detected at concentrations of up to 4.2, 3.2 and 1.6 ng L^{-1} in raw wastewater, while mephedrone and flephedrone were found in neither effluent wastewater nor in river water and only methylone was detected at 1.8 ng L^{-1} in one of the river water samples.

The enantiomeric composition of the analytes detected in river water samples was checked by injecting the eluates into CBH column. The extracted ion chromatograms of the enantiomers of methedrone and butylone are shown in Fig. 5. The EF values for methedrone and butylone found in river water samples were 0.48 ± 0.01 and 0.44 ± 0.01, respectively. They were not significantly different from those recorded in the validation, which means that methedrone and butylone were present in racemic form in the river water samples. The EF value determined for methedrone was consistent with our previous study [17], which suggests that little stereoselective degradation occurred during various processes or that the cathinones present in the river water came from the direct disposal of unused drugs [15].

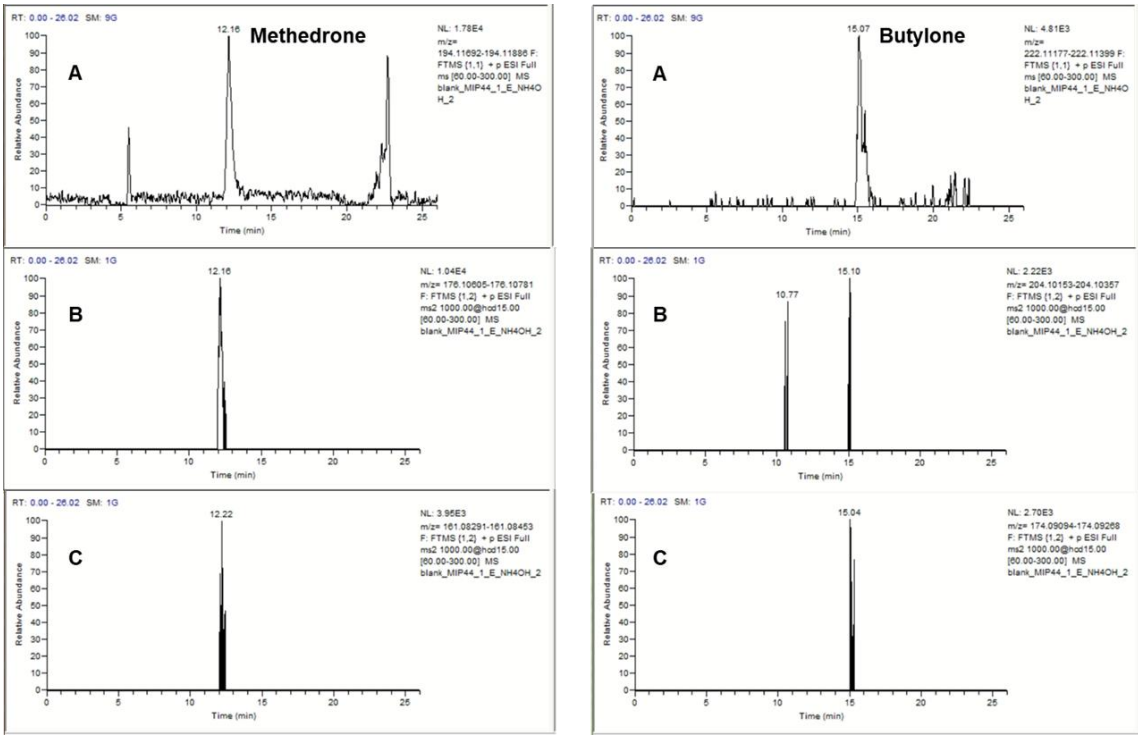

Fig. 4. Extracted ion chromatograms of the protonated ion (A) and the two fragments (B, C) for a river water sample extracted by SPE with MIP (S,S) 4:4 and analyzed with LC-HRMS with a C_{18} column.

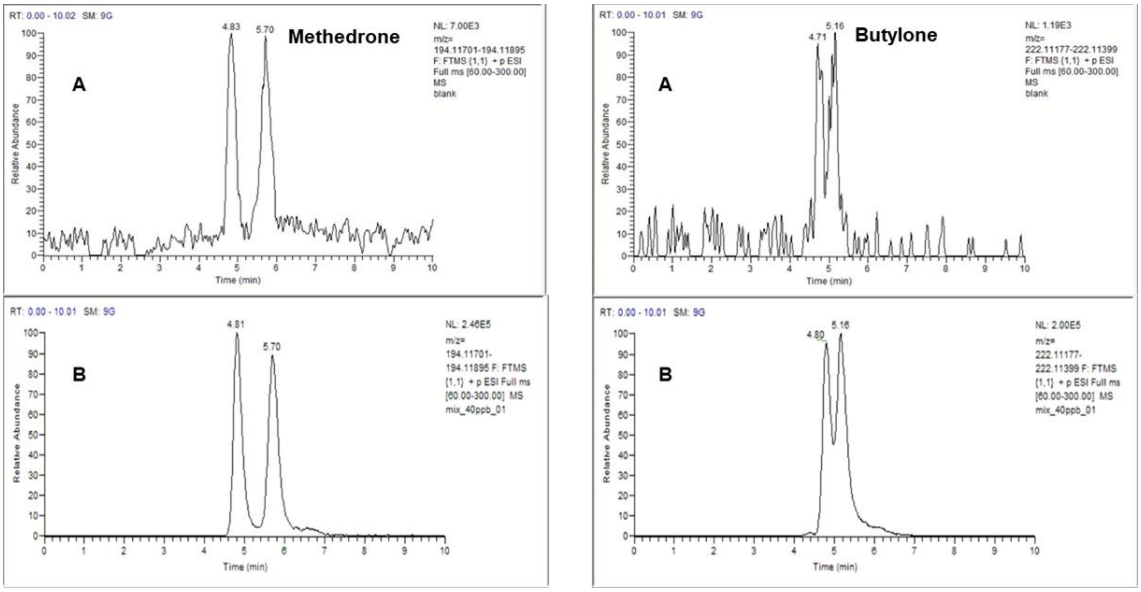

Fig. 5. Extracted ion chromatogram of the enantiomers of methedrone and butylone obtained when 100 mL of (A) Ebro River water sample (B) ultrapure water spiked at 0.4 μg L^{-1} were extracted by SPE with MIP (S,S) 4:4 and analysed by LC-HRMS with a CBH column.

5. Conclusions

A series of MIPs were synthesised and evaluated as sorbent for the extraction of five cathinones. The MIP (S,S) 4:4 with (1S,2S)-(+)-pseudoephedrine as template and a template/monomer molar ratio of 4:4 has been shown to provide best recoveries. Then, a highly selective and sensitive method was developed using MIP (S,S) 4:4 as sorbent in SPE followed by LC-(Orbitrap)HRMS. It was successfully applied to determine five cathinones from river water samples. Racemic methedrone and butylone were detected in Ebre River water samples at concentrations of up to 3.5 and 2.4 ng L^{-1}, respectively.

The good recoveries achieved with high selectivity in the study indicate that using MIPs as the selective sorbent in SPE followed by LC-HRMS analysis can be a promising method for determining these chiral drugs in the environment.

Acknowledgements

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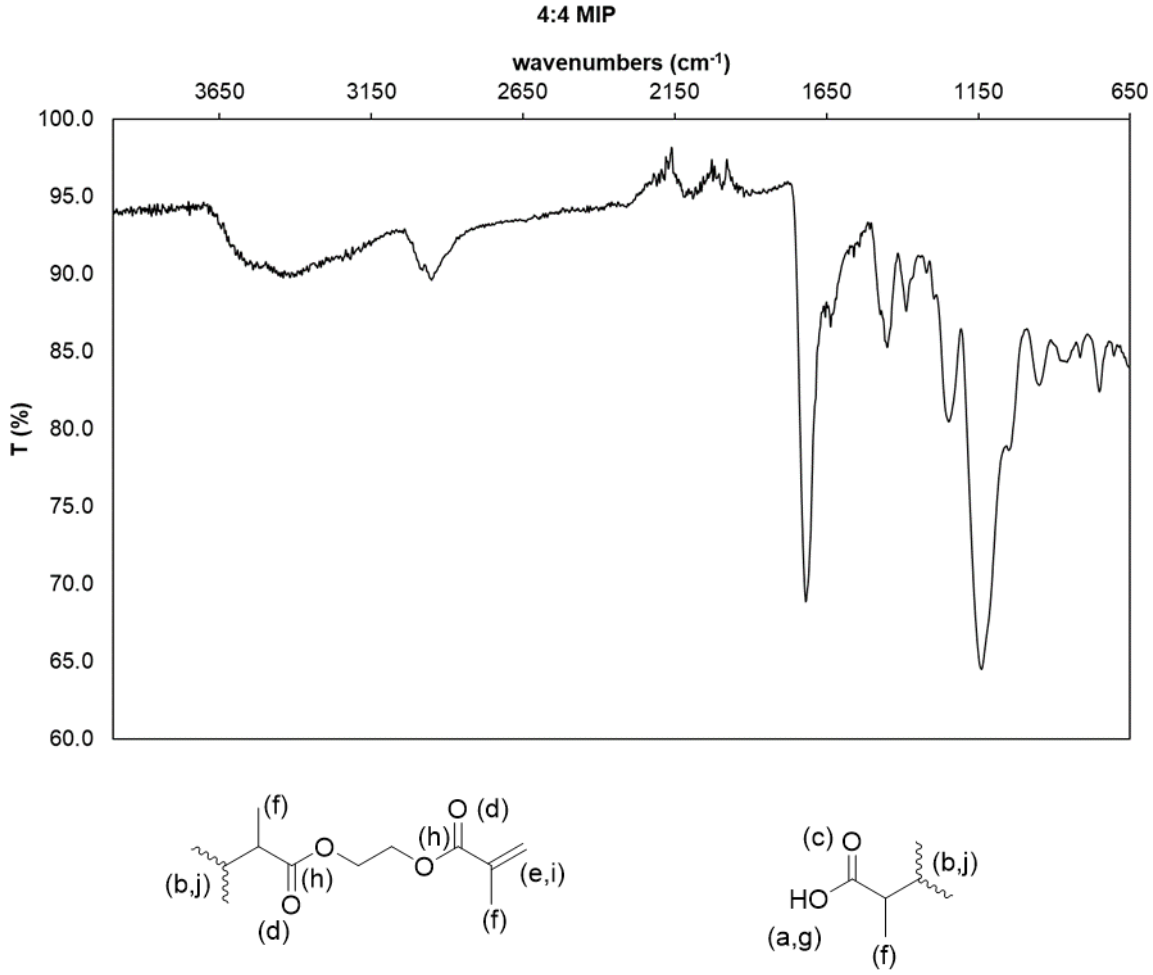
Supplementary data

Table 1S. Retention time (t_R), diagnostic ions and two fragments monitored of analytes.

Compound	t_R[a] (min)	Diagnostic ion $[M+H]^+$			Fragment 1		Fragment 2	
		Formula	Exact mass m/z	Accurate mass m/z	Formula	Accurate mass m/z	Formula	Accurate mass m/z
Mephedrone	15.97	$C_{11}H_{16}NO$	178.12264	178.12291	$C_{11}H_{14}N$	160.112173	$C_{10}H_{11}N$	145.086494
Butylone	15.08	$C_{12}H_{16}NO_3$	222.11247	222.112478	$C_{12}H_{14}NO_2$	204.10255	$C_{11}H_{12}NO$	174.09187
Flephedrone	9.87	$C_{10}H_{13}FNO$	182.09757	182.09785	$C_{10}H_{11}FN$	164.08748	C_9H_8FN	149.06415
Methylone	9.21	$C_{11}H_{14}NO_3$	208.09682	208.09734	$C_{11}H_{12}NO_2$	190.08691	$C_{10}H_{10}NO$	160.07617
Methedrone	12.12	$C_{11}H_{16}NO_2$	194.11755	194.11789	$C_{11}H_{14}NO$	176.10693	$C_{10}H_{11}NO$	161.08371

[a] retention time on LC2 C_{18} column.



	Band ($\bar{\nu}$/cm^{-1}):	Assignation	Functional group
a	3300-2500	O-H stretching	carboxylic acid
b	2950	C-H stretching	alkane
c	1760	C=O stretching	carboxylic acid (not visible as ester C=O dominates)
d	1718	C=O stretching	ester
e	1634	C=C stretching	alkene
f	1453	C-H bending	alkane methyl group
g	1390	O-H bending	carboxylic acid
h	1250; 1144	C-O stretching	ester
i	950	C=C bending	alkene
j	751	C-H bending	monosubstituted

Fig. 1S. Example of FT-IR spectrum of MIP (S,S) 4:4 and its band assignation list.

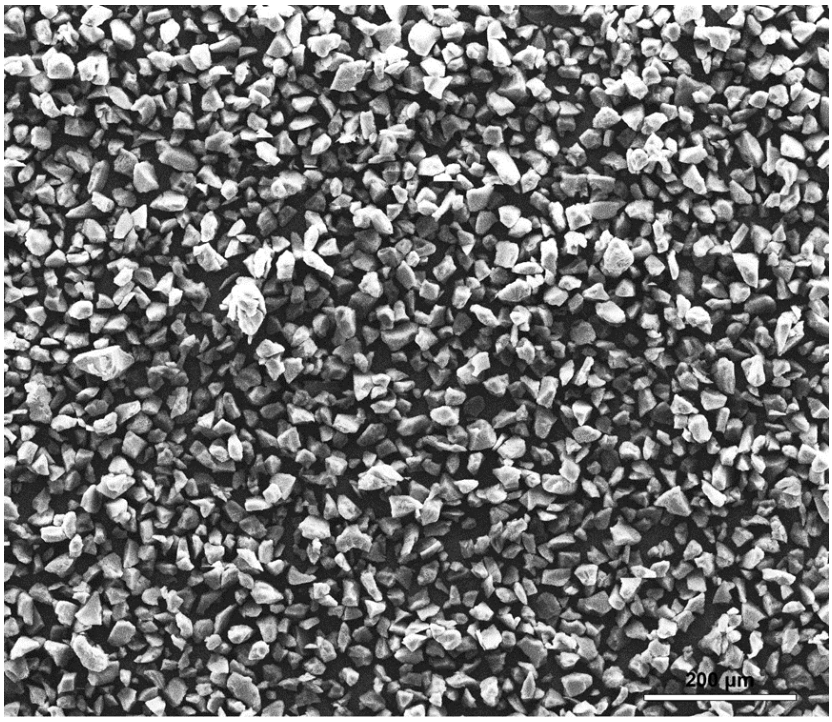


Fig. 2S. Example of SEM image of MIP (S,S) 4:4.

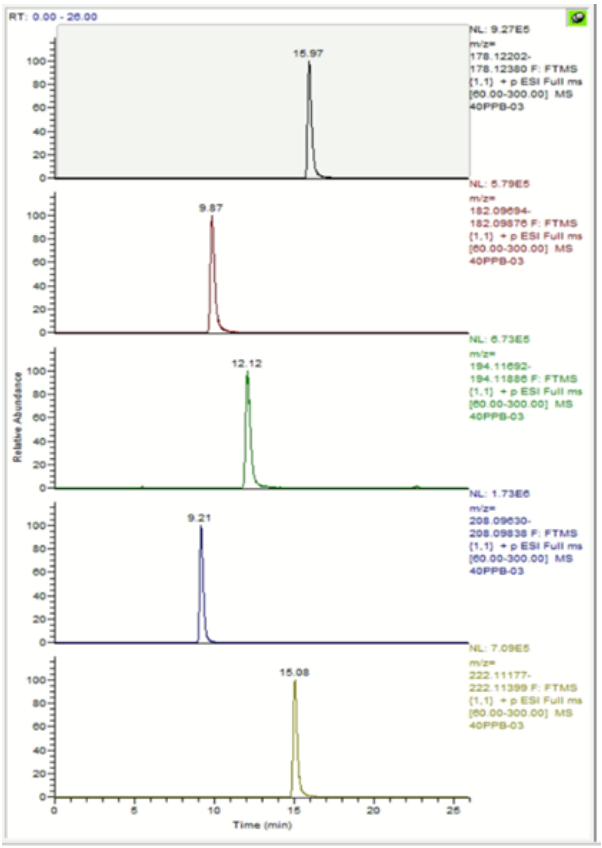


Fig. 3S. LC-HRMS chromatograms of the separation of cathinone analytes on the LC2 C_{18} column.

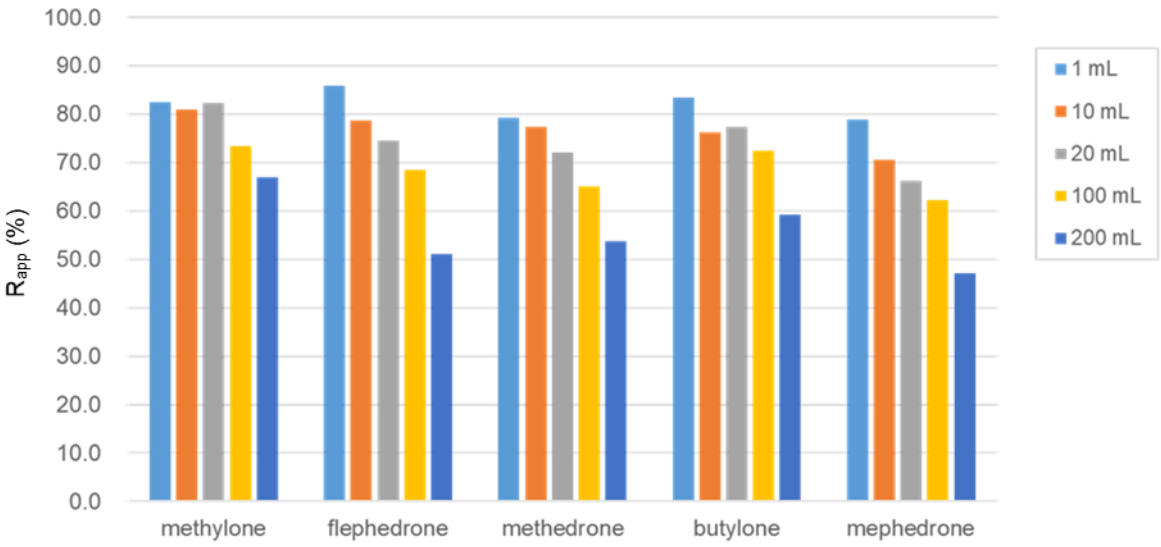


Fig. 4S. The effect of loading sample volume on the $\%R_{app}$ for all the analytes with MIP 1:4 sorbents.

3.2.3. Discussion of results

This section outlines the most important aspects of two methods based on the use of SPE as a clean-up step and enrichment technique in combination with chiral LC-HRMS for the enantiomeric determination of five cathinones (mephedrone, flephedrone, butylone. methylone and methedrone) in environmental water samples. To our knowledge, these are the first studies reporting the enantiomeric determination of a group of cathinones in environmental waters. In previous studies, only one cathinone – mephedrone – has been reported for its enantiomeric determination at the environmental level, along with other chiral drugs [1-3].

In the first study the identification and quantification of cathinones was undertaken with a chiral analysis method, while in the second study a non-chiral analysis method was used for the same purpose and a chiral analysis method was used for the determination of their enantiomeric composition. The enantioseparation conditions used in both studies were established based on the previous study in Section 3.1.1 that achieved successful enantioseparations for a group of cathinones with the use of Chiralpak CBH column in RP mode. The effect of flow rate, type and concentration of organic modifier and concentration of buffer salt were investigated, and good enantioseparations were achieved for all the target cathinones with the optimal mobile phase of 1 mM NH_4Ac aqueous solution/MeOH (98/2, v/v) at a flow rate of 0.4 mL/min. In the non-chiral analysis method developed in the second study, a Brisa LC2 C_{18} column was used under gradient elution (mobile phase A: 0.1% FA in water; mobile phase B: 0.1% FA in ACN).

To improve the selectivity of the methods, different SPE sorbents – two commercial mixed-mode cation-exchange sorbents (Oasis WCX and Oasis MCX) and a series of in-house prepared MIP sorbents – were assessed for the extraction of cathinones from river water and effluent wastewater samples. Both the mixed-mode cation-exchange and MIP sorbents offered high selectivity for the extraction of cathinones thanks to their ability to establish the specific interactions with the analytes. Oasis WCX and Oasis MCX sorbents are capable of retaining basic analytes through cation-exchange and reversed-phase interactions. The MIP sorbents applied in the second study were synthesized using (1R,2S)-(–)-ephedrine or (1S,2S)-(+)-pseudoephedrine as template (Figure 1). The synthesized MIPs possess specific cavities with high affinities towards a group of compounds structurally related to ephedrine. Since cathinones have similar structures as ephedrine, they are able to enter the cavities driven by the ionic interactions and hydrogen bondings between the carboxylic groups in the cavities of the MIP and amine groups of cathinones.

Since ionic interactions play a key role in the retention of analytes on both mixed-mode ion-exchange and MIP sorbents, the selection of the adequate pH conditions is significant important in the SPE protocol. In the case of Oasis MCX, the pH of the sample was adjusted to 3, at which cathinones (pK_a between 7.2 and 7.8) are fully protonated thus promoting ionic

interactions with sulfonic acid moieties ($pK_a < 1$) of the MCX sorbent. In the case of Oasis WCX and MIPs, the pH of the sample was adjusted to 7, at which cathinones were partially protonated giving rise to a relatively weak ionic interaction with carboxylic acids moieties ($pK_a \approx 4.5$) on the WCX or MIP sorbents.

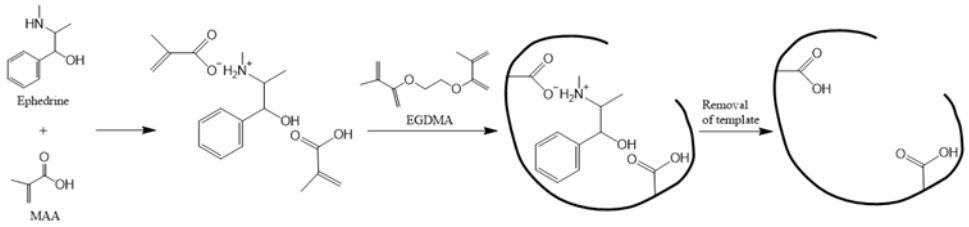


Figure 1. Schematic representation of the synthesis of the MIP using ephedrine as template.

Specific interactions between sorbents and analytes allowed the inclusion of an effective wash step with organic solvents to reduce the non-specific interactions. With regard to the mixed-mode cation-exchange sorbents, 5 mL (in the case of WCX) or 10 mL (in the case of MCX) of pure MeOH was used in the washing step to remove acidic and neutral interferences which were retained on the sorbents only by reversed-phase interactions. In the case of MIPs, pure MeOH was not strong enough to disrupt the non-specific interactions since the ionic interaction would also occur between the basic interferences and carboxylic groups on the MIPs. As a result, 1 mL of 0.02% HAc in MeOH as washing solution ensured a selective extraction of cathinones on MIP sorbents.

In addition, it is worth mentioning that the shifting of retention times and loss of enantioseparations were observed in sample matrix in the case of Oasis WCX and in the case of MIPs when using 10% FA in MeOH as elution solution, while such an effect was not observed in the case of Oasis MCX or in the case of MIPs when using 5% NH_4OH in MeOH as elution solution. As already stated, this phenomenon could be explained because interferences in the river water which were not removed in the washing solution and coeluted with the analytes, might compete with analytes to bind to active sites of the CBH column, thus leading to the lack of retention of the cathinones. It should be noticed that such effect was not observed when the same extract was injected into C_{18} column indicating that such interference of matrices in LC-behaviors should depend not only on sample treatment methods and the nature of the matrices, but also on the column used for the analysis. In this way, Oasis MCX sorbent outperformed WCX sorbent for the extraction of cathinones from environmental water samples, and thus was selected for subsequent analysis in the first study.

During the MISPE process, special attention was paid to the evaluation of the

stereoselectivity towards the enantiomers of cathinones since MIPs were synthesized with one stereoisomer and the enantiomers of cathinones might show different affinities for the sorbents, which could potentially lead to erroneous measurement of their EF values. The chiral analysis showed that the EF values of all the analytes in the eluates were around 0.5, which indicates that there was no stereoselectivity during the MISPE procedure. This could be due to that the SPE cartridges have not enough sorbents or low enantiodiscrimination capacity.

Under the optimal SPE conditions, both Oasis MCX and the best performing MIP cartridge – MIP (S,S) 4:4 cartridges – provided satisfactory recoveries with minimal ME values for the target cathinones. Comparing the performance of the two extraction methods developed for the extraction of cathinones from river water samples, the MCX SPE protocol provided higher recoveries for all the analytes except for flephedrone, while MIP provided lower ME values (-10 - 15% in the case of MCX; -5.5 - 1.8% in the case of MIP). However, it should be noted that lower river water volume (100 mL) was loaded in the case of MISPE resulting in less matrix interferences, while it was 500 mL in the case of MCX SPE protocol. The lower loading capacity of MIP cartridges could be resulted from the lower amount of sorbents packed (100 mg in the case of MIP; 500 mg in the case of MCX). Nevertheless, both developed methods are suitable for application to environmental samples since they gave MDLs at low ng/L and satisfactory recoveries. Recently, Bade et al. [4] reported the use of a Clean Screen® DAU cartridge (mixed-mode hydrophobic and cation exchange) for the extraction of 10 cathinones along with 11 other new psychoactive substances (NPSs) from influent wastewater samples (50 mL). Although good recoveries in the range of 82–119% were obtained, very high ME values ranging from 32% to 335% (different ME expression was used: ME < 100%, signal suppression; ME > 100%, signal enhancement) were observed before the correction by internal standards. In another recent study [5], Oasis MCX was used for the extraction of a group of cathinones along with other NPSs from wastewater. However, the proposed protocol did not include a washing step which resulted in high ME values ranging from 46.9% to 164% (ME < 100%, signal suppression; ME > 100%, signal enhancement) [5].

Both developed methods in this section were applied for the enantiomeric determination of cathinones in river water samples, and the method using MCX SPE was also applied for effluent wastewater samples. Of the compounds studied, methylone, methedrone and butylone were detected in river water samples at few ng/L, and the EF values obtained were around 0.5 in all cases. The occurrence of these compounds in the aquatic environment has been reported in several studies [6-8]. However, these studies almost exclusively utilized non-chiral analytical methods, and there was little information on the enantiomeric composition in the environment. In previous studies, mephedrone was reported to be enriched with first enantiomer during the analysis of influent wastewater in the UK [1,2],

while in another study, racemic mephedrone was observed during an analysis of South African wastewaters [9]. Recently, Langa et al. [10] developed a GC-MS method for the enantiomeric determination of several classes of NPSs (including amphetamines, cathinones, ketamine metabolite and derivatives of piperazine) in Portuguese surface waters of Douro River estuary and effluent wastewaters samples, and in the case of cathinones, only one enantiomer of butylone was detected in the river water sample.

Both studies in this section provide highly sensitive and selective methods for the enantiomeric determination of cathinones in environmental water samples, and the results obtained extend the knowledge on the enantiomeric occurrence of cathinones in the environment. To fully understand their enantioselective behaviors in the environment, more extensive research still needs to be undertaken.

References

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CHAPTER 4. CONCLUSIONS

The major conclusions that can be drawn from the studies presented in this Doctoral Thesis can be summarized as follows:

1. The Chiralpak CBH column (protein-based) in RP mode was more effective than the Lux Cellulose-1 (polysaccharide-based) in PO mode for the chiral separation of target compounds comprising three β-blockers, omeprazole, and four cathinones.

2. In the PO mode with the Lux Cellulose-1 column, the addition of acidic and basic additives has the most significant impact on the enantioseparations. With the optimal mobile phase of ACN/IPA/FA/DEA (90/10/0.1/0.1, v/v/v/v), the enantiomers of four analytes (three β-blockers and omeprazole) were separated.

3. In RP mode with the Chiralpak CBH column, the pH value of the mobile phase plays a key role in successful enantioseparations. With the optimal mobile phase of 5 mM NH_4Ac aqueous solution (pH = 6.4)/MeOH (95/5, v/v), the enantiomers of six analytes (three β-blockers and three cathinones) were separated.

4. Both chiral separation methods developed using Chiralpak CBH column or Lux Cellulose-1 column can be directly coupled with HRMS detection for enantiomeric determination of chiral drugs at low concentration, since the solvents and additives used in the mobile phase are MS compatible.

5. In the analytical methods based on SPE followed by LC–(Orbitrap)HRMS developed for the enantiomeric determination of a group of cathinones in environmental water samples, satisfactory enantioseparations were achieved for all the target cathinones when a Chiralpak CBH column was used in RP mode.

6. High selectivity in the extraction of cathinones has been achieved by using a commercial mixed-mode strong cation-exchange sorbent (Oasis MCX) and an in-house MIP sorbent thanks to the effective washing step.

7. For the extraction of cathinones from environmental water samples, Oasis MCX behaved better than Oasis WCX in terms of recovery and ME.

8. The shift in retention times and loss of enantioseparations caused by sample interferences were observed in the chiral analysis of environmental water samples, when Oasis WCX was employed in the SPE procedure. Such effect was also observed when MIP sorbents was employed with 1 mL of 10% FA in MeOH as the elution solution.

9. Among a series of MIPs synthesized using (1R,2S)-(–)-ephedrine or (1S,2S)-(+)-pseudoephedrine as dummy template, the MIP (S,S) 4:4 with (1S,2S)-(+)-pseudoephedrine as template and a template/monomer molar ratio of 4:4 was found to

provide best recoveries with negligible matrix effect as SPE sorbents for the extraction of cathinones from river water samples.

10. Both methods developed in this Doctoral Thesis to determine the enantiomers of a group of cathinones achieved low limits quantification limits, at few ng/L, enabling their determination in environmental water samples.

11. The highly sensitive and selective methods were successfully applied in the enantiomeric determination of five cathinones in river water and effluent wastewater. Racemic methylone, methedrone and butylone were detected in river water samples at low ng/L concentration levels.

12. The enantiomeric profiling of a group of cathinones was reported for the first time in this Thesis, and the results obtained extend the knowledge on the enantiomeric occurrence of cathinones in the environment.

APPENDIX

Appendix I. List of abbreviations

2-MMC	2-methylmethcathinone
3-MMC	3-methylmethcathinone
4-MMC	4-methylmethcathinone
4-MEC	4-methyl-N-ethylcathinone
ACN	Acetonitrile
AGP	α1-acid glycoprotein
BGE	Background electrolyte
C_{18}	Octadecyl-silica
C_8	Octyl-silica
CBH	Cellobiohydrolase
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CMPA	Chiral mobile phase additive
CMT	Counter migration technique
CSP	Chiral stationary phase
DAD	Diode array detector
DEA	Diethylamine
DLLME	Dispersive liquid–liquid microextraction
DVB	Divinylbenzene
ECD	Electron capture detector
EF	Enantiomeric fraction
EGDMA	Ethylene glycol dimethacrylate
ESI	Electrospray ionization
EtOH	Ethanol
FA	Formic acid
FD	Fluorescence detection
FDA	United States Food and Drug Administration
FID	Flame ionization detector
FT-IR	Fourier-transform infrared spectroscopy
FWHM	Full width at half maximum

GC	Gas chromatography
HAc	Acetic acid
HCD	Higher energy collisional dissociation
HESI	Heated electrospray ionization
HLB	Hydrophilic-hydrophobic balance
HRMS	High resolution mass spectrometry
HSA	Human serum albumin
HXL	Hypercrosslinked
IDL	Instrumental detection limit
IPA	Isopropanol
IQL	Instrumental quantification limit
IRG184	1-hydroxycyclohexyl phenyl ketone
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid-phase microextraction
MAA	Methacrylic acid
MCX	Strong cation-exchange
MDA	3,4-Methylenedioxyamphetamine
MDMA	Methylenedioxymethamphetamine
MDPV	Methylenedioxypyrvalerone
ME	Matrix effect
MeOH	Methanol
MIP	Molecularly imprinted polymer
MISPE	Molecularly imprinted solid-phase extraction
MDL	Method detection limit
MQL	Method quantification limit
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NH_4Ac	Ammonium acetate
NIP	Non-imprinted polymer
NP	Normal-phase

NPSs	New psychoactive substances
NSAID	Non-steroidal anti-inflammatory drugs
OECD	Organisation of Economic Cooperation and Development
PFT	Partial filling technique
PhACs	Pharmaceutically active compounds
PI	Polar-ionic phase
PO	Polar-organic phase
PTFE	Polytetrafluoroethylene
QqQ	Triple quadrupole
QTOF	Quadrupole-time of flight
R_{app}	Apparent recovery
RP	Reversed-phase
R_s	Resolution
RSD	Relative standard deviation
SFC	Supercritical fluid chromatography
S/N	Signal-to-noise ratio
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
TCD	Thermal conductivity detector
TEA	Triethylamine
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
UHPLC	Ultra high performance liquid chromatography
UK	United Kingdom
UV	Ultraviolet
WBE	Wastewater-based epidemiology
WCX	Weak cation-exchange
WWTP	Wastewater treatment plant

Appendix II. List of publications

1. Y. Fu, F. Borrull, N. Fontanals, R.M. Marcé, Comparison of polysaccharide-based and protein-based chiral liquid chromatography columns for enantioseparation of drugs, Chirality 32 (2020) 876-884.
2. Y. Fu, F. Borrull, R.M. Marcé, N. Fontanals, Enantiomeric determination of cathinones in environmental water samples by liquid chromatography-high resolution mass spectrometry, Journal of Chromatography A 1626 (2020) 461359.
3. Y. Fu, F. Borrull, R.M. Marcé, N. Fontanals, Enantiomeric fraction determination of chiral drugs in environmental samples using chiral liquid chromatography and mass spectrometry, Trends in Environmental Analytical Chemistry 29 (2021) e00115.
4. Y. Fu, F. Pessagno, P. Manesiotis, F. Borrull, N. Fontanals, R.M. Marcé, Preparation and evaluation of molecularly imprinted polymers as selective SPE sorbents for the determination of cathinones in river water. Microchemical Journal 175 (2022) 107100.