Using chiral liquid chromatography quadrupole time-of-flight mass spectrometry for the analysis of pharmaceuticals and illicit drugs in surface and wastewater at the enantiomeric level

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Abstract

This paper presents and compares for the first time two chiral LC-QTOF-MS methodologies (utilising CBH and Chirobiotic V columns with cellobiohydrolase and vancomycin as chiral selectors) for the quantification of amphetamine, methamphetamine, MDA (methylenedioxyamphetamine), MDMA (methylenedioxymethamphetamine), propranolol, atenolol, metoprolol, fluoxetine and venlafaxine in river water and sewage effluent. The lowest MDLs (0.3-5.0 ng L^{-1} and 1.3-15.1 ng L^{-1} for river water and sewage effluent respectively) were observed using the chiral column Chirobiotic V. This is with the exception of methamphetamine and MDMA which had lower MDLs using the CBH column. However, the CBH column resulted in better resolution of enantiomers (R_s = 2.5 for amphetamine compared with R_s = 1.2 with Chirobiotic V). Method recovery rates were typically >80% for both methodologies. Pharmaceuticals and illicit drugs detected and quantified in environmental samples were successfully identified using MS/MS confirmation. In sewage effluent, the total beta-blocker concentrations of propranolol, atenolol and metoprolol were on average 77.0, 1091.0 and 3.6 ng L^{-1} thus having EFs (Enantiomeric Fractions) of 0.43, 0.55 and 0.54 respectively. In river water, total propranolol and atenolol was quantified on average at <10.0 ng L^{-1}. Differences in EF between sewage and river water matrices were evident: venlafaxine was observed with respective EF of 0.43±0.02 and 0.58±0.02.

Keywords

Chiral; pharmaceutical; illicit drug; river water; wastewater; enantiomer

1.0 Introduction

Complete removal of pharmacologically active a compounds is rarely achieved by sewage treatment processes, as these are biological treatment systems designed to reduce the level of organic substances found in domestic sewage. The incomplete removal of pharmacologically active compounds during sewage treatment results in their sustained emission to the aquatic environment (Ternes 1998; Hirsch, Ternes et al. 1999; Jones, Voulvoulis et al. 2001; Jones, Voulvoulis et al. 2002; Calamari, Zuccato et al. 2003; Glassmeyer, Furlong et al. 2005; Jones, Voulvoulis et al. 2007; Kasprzyk-Hordern, Kondakal et al. 2010). Once in the environment, trace levels of some compounds have been demonstrated to have adverse effects upon aquatic organisms. Previous research has focused upon compounds such as estrogens (Servos, Bennie et al. 2005; McAdam, Bagnall et al. 2010; Racz and Goel 2010) which have been demonstrated to cause feminisation of fish, or fluoxetine which has been shown to accumulate in fish tissue (Chu and Metcalfe 2007). Resultantly, a burgeoning field of research and

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numerous methodologies for the analysis of pharmacologically active compounds within the aquatic environment has developed (Andreozzi, Raffaele et al. 2003; Hilton and Thomas 2003; Hernando, Petrovic et al. 2004; Quintana, Rodil et al. 2004; Castiglioni, Bagnati et al. 2005; Balakrishnan, Terry et al. 2006; Kasprzyk-Hordern, Dinsdale et al. 2007; Batt, Kostich et al. 2008; Berset, Brenneisen et al. 2010; Nödler, Licha et al. 2010; Baker and Kasprzyk-Hordern 2011; López-Serna, Petrović et al. 2011). However, none of the above methods have the capability to resolve chiral drugs. This is surprising considering that approximately 56% of the pharmaceuticals currently in use are chiral and 88% of these are administered in racemic proportions (Lien Ai, Hua et al. 2006).

Growing evidence of stereoselectivity in the aquatic environment demonstrates a need for the monitoring of chiral compounds. Fono and Sedlak (Fono and Sedlak 2005) reported racemic proportions of propranolol (EF, 0.49 - 0.54) in sewage influent but not in effluent (EF, 0.31-0.44). In agreement with this, Nikolai et al. (Nikolai, McClure et al. 2006) reported enantioselective biodegradation of atenolol, metoprolol and propranolol during sewage treatment. Kasprzyk-Hordern et al. (Kasprzyk-Hordern, Kondakal et al. 2010) studied several pharmaceuticals and drugs of abuse including amphetamine, methamphetamine, MDMA and venlafaxine during wastewater treatment and observed their non-racemic composition following treatment. Recently in a profiling study of chiral drugs in wastewater and receiving water, it was observed that stereoselectivity was dependent upon the type of chiral drug, treatment technology used and season (Kasprzyk-Hordern and Baker 2011).

The stereospecific distribution of chiral pharmaceuticals in the environment is an important consideration, particularly in terms of ecotoxicity. In a recent review, it was suggested that single enantiomers of chiral drugs should be considered as separate contaminants due to their differing ecotoxicity within the aquatic environment (Kasprzyk-Hordern 2010). In a study of the sub-lethal effects of the antidepressant fluoxetine on aquatic vertebrates and invertebrates, it has been observed that S-fluoxetine was more toxic to Pimephales promelas than R-fluoxetine (Stanley, Ramirez et al. 2007). However, these authors did not observe the same response for Daphnia magna. The authors suggest that different stereospecific responses may have resulted from different physiology between these two species and the closer homology between mammals and fish could indicate a potential hazard to humans. There is limited data on stereospecific toxicity of chiral drugs as currently toxicity of chiral drugs is only determined in racemic form. The work of Stanley et al. (Stanley, Ramirez et al. 2007) would indicate that this is an inaccurate means of assessment. If this is the case, then it would no longer be sufficient to monitor racemic concentrations of common chiral drugs.

Consideration of the implications of chiral drugs within the aquatic environment is still in its infancy. Few methods exist for the analysis of chiral drug in environmental matrices (Matamoros and Bayona 2006; Nikolai, McClure et al. 2006; MacLeod, Sudhir et al. 2007; Barreiro, Vanzolini et al. 2010; Kasprzyk-Hordern, Kondakal et al. 2010; Hashim and Khan 2011). Therefore the reliability of current chiral methods for the analysis of environmental matrices needs further critique. For example, the Chirobiotic V column with vancomycin as a chiral selector (supplied by Sigma-Aldrich) is widely used for the chiral separation in blood plasma (Siluk, Mager et al. 2007; Kingback, Josefsson et al. 2010; Zuo, Wo et al. 2010). Yet in application with environmental matrices, only MacLeod et al. (MacLeod, Sudhir et al. 2007) utilised the Chirobiotic V column for the quantification of single enantiomers of nine compounds, including: the beta-blockers, atenolol, metoprolol and propranolol and the antidepressant fluoxetine.

The aim of this research was to develop, validate and evaluate highly sensitive and selective multi-residue methodology for the analysis of chiral compounds at enantiomeric levels in

river water and sewage effluent. Samples were analysed using a QTOF mass spectrometer in full scan mode and confirmed with MS/MS. This method of analysis allows for retrospective screening and verification of analytes in the form of new and emerging contaminants and their transformation pathways in the environment. To the authors' knowledge, this is the first report discussing the application of HPLC-QTOF instrumentation for separation of chiral drugs at enantiomeric level with the usage of two chiral columns (Chirobiotic V and CBH). This paper compares and contrasts the method parameters (such as linearity, resolution, detection/quantification limits and recovery rates) for these two columns. This paper hopes to contribute to a new but rapidly expanding area of analytical chemistry.

2.0 Materials and Methods

2.1 Chemicals and Reagents

The reference standards: R/S (±)-amphetamine, S (+)-amphetamine, R/S (±)-methamphetamine, S (+) methamphetamine, R/S (±)-MDA and R/S (±)-MDMA were purchased from LGC Standards (UK): R/S (±)-venlafaxine; R/S (±)-fluoxetine; S (+)-fluoxetine; R/S (±)-atenolol; R/S (±)-metoprolol and R/S (±)-propranolol were purchased from Sigma-Aldrich (UK). All solvents were of HPLC grade and purchased from Sigma-Aldrich. All glassware was silanised with dimethylchlorosilane (5% DMDCS in toluene, Sigma–Aldrich) to minimise sample loss through adsorption of basic analytes onto OH-sites present on glass surface. The internal standards (IS): R/S (±)-amphetamine-d11, R/S (±)-methamphetamine-d14, R/S (±)-MDMA-d5, R/S(±)-MDA-d5 were purchased from LGC standards, whilst R/S(±)-fluoxetine-d5 and R/S(±)-atenolol-d7 were purchased from Sigma-Aldrich. All internal standards were added to the samples before solid-phase extraction (SPE) and upon preparation of calibration standards. Stock solutions of each compound (1 mg mL^{-1}) were prepared in methanol and stored in the dark at −16 °C. Working solutions were prepared by diluting stock solution in mobile phase and stored at 4 °C. Ultrapure water (UP) obtained with PURELAB UHQ-PS Unit (Elga, UK), river water (collected from the River Avon, Salford, Somerset) and wastewater (collected from a local WWTP) were used for method validation.

2.2 Sample Collection, Preparation and Solid-Phase Extraction

River water was collected from the River Avon (Salford, Somerset) during July and October. Wastewater was collected from a wastewater treatment work during July. For both river water and wastewater, each sample was collected into 1 L polypropylene bottles and stored on dry-ice. Prior to solid-phase extraction (SPE), samples were filtered through Whatman GF/F 0.7 µm glass fibre filters (Whatman, UK). The SPE procedure was based on methodology described elsewhere (Kasprzyk-Hordern, Dinsdale et al. 2007; Kasprzyk-Hordern, Kondakal et al. 2010). In brief, HLB cartridges were preconditioned with 2 mL of methanol followed by 2 mL of water at a flow rate of <3 mL min^{-1}. 250 mL of the previously filtered river water samples, or 100 mL of sewage effluent, was spiked with mixed racemic standard containing the following IS (50 ng of each enantiomer): R/S (±)-amphetamine-d11; R/S (±)-methamphetamine-d14; R/S (±)-MDMA-d5; R/S (±)-MDA-d5; R/S (±)-fluoxetine-d5 and R/S (±)-atenolol-d7. The sample was then passed through the cartridge at a flow rate of <6 mL min^{-1}. Analytes were eluted with 4 mL of methanol at a rate of <1 mL min^{-1}. Extracts were then evaporated to dryness with a TurboVap evaporator (Caliper, UK, 40 ºC, N_2, <5 psi) and reconstituted in 0.5 mL of mobile phase. All samples were filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13mm) and transferred to polypropylene 0.3 mL capacity vials (Waters, UK). In addition to IS (spiked at 200 and 500 ng L^{-1} for river water and sewage

effluent respectively), matrix was spiked, with a methanolic stock solution containing a racemic mix of chiral compounds at 50, 100 or 200 ng L^{-1} for river water and 250, 2500 or 5000 ng L^{-1} for sewage effluent and were extracted according to the procedure described above. Three extractions were carried out for each concentration and each extract was injected into the HPLC-QTOF in triplicate.

2.3 Chromatographic and Analytical Conditions

Two multi-residue methods are described here, both utilising an ACQUITY UPLC system (Waters, UK) and a micrOTOFQ (Quadrupole, Time-of-Flight) mass spectrometer (Bruker Daltoniks GmbH, Germany). Two chiral columns were utilised: (i) a Chiral-CBH column, 100 x 2 mm, I.D. 5µm (Chromtech, UK) and Chiral-CBH 10 x 2.0 mm, I.D. 5µm guard column (Chromtech, UK), and (ii) a Chirobiotic V column, 250 x 2.1 mm, I.D. 5µm (Sigma-Aldrich, UK) and 20 x 1.0 mm, I.D. 5 µm guard column (Sigma-Aldrich, UK).

The Chiral-CBH method was based on methodology of Kasprzyk-Hordern et al. (Kasprzyk-Hordern, Kondakal et al. 2010). Separation was undertaken using isocratic conditions, with a mobile phase of 90 % H_2O, 10 % 2-propanol and 1 mM ammonium acetate at a flow rate of 0.075 mL min^{-1}. The apparent pH of the mobile phase was 7. The column was maintained at 25 ºC, the autosampler temperature was 4 ºC, with an optimal chromatographic run time of 65 minutes and the injection volume was 20 µL.

The Chirobiotic V method also used isocratic conditions. Several mobile phases were studied in order to obtain chiral separation and to maintain satisfactory electrospray ionisation (ESI) performance in positive mode. These included: methanol, acetonitrile, 2-propanol and water used either as the key constituent of the mobile phase or as a blend. Mobile phase additives included ammonium acetate and formic acid, which were added at concentrations ranging from 1 to 10 mM and 0.1 to 0.005 % respectively. Flow rates between 0.075–0.2 mL min^{-1} were studied. The optimised chromatographic conditions for this column were: methanol containing 4 mM ammonium acetate and 0.005 % formic acid at a flow rate of 0.1 mL min^{-1}. The column was maintained at 25 ºC, the autosampler temperature was 4 ºC, with an optimal chromatographic run time of 40 minutes, the injection volume was 20 µL.

A micrOTOFQ mass spectrometer equipped with an electrospray ionization source was used for chiral drug identification and quantification. Analyses were performed in positive ion mode with a capillary voltage of 4.5 kV, end plate offset of -500 V; the nebuliser gas pressure was 2.0 bar, and dry gas flow of 8 L min^{-1}, with a dry gas temperature of 200°C. Nitrogen was used as the nebulising gas, provided by a high purity nitrogen generator (Parker Hannifin Ltd, UK). Argon (99.999%) was used as the collision gas during MS/MS experimentation. Hystar software (Bruker Daltonik GmbH) was used to control the Waters ACQUITY system and the micrOTOFQ. Data was processed using DataAnalysis v4.0 and QuantAnalysis v4.0 (Bruker Daltonik GmbH).

2.4 Elution Order of Enantiomers

The elution order of atenolol, metoprolol and venlafaxine enantiomers has previously been established for a Chirobiotic V column under similar chromatographic conditions was used during this study (Liu, Wang et al. 2007; MacLeod, Sudhir et al. 2007). The elution order of atenolol, amphetamine, methamphetamine, MDA and MDMA has also been previously determined for the CBH column (Kasprzyk-Hordern and Baker 2011). The elution order for amphetamine, methamphetamine and fluoxetine using the Chirobiotic V column was determined experimentally using single enantiomeric standards and subsequent comparison with racemic standards.

2.5 Method Validation

Identification of the target compounds was carried out using accurate mass measurements. Subsequent quantification of chiral drugs was carried out by a 13-point multi-component internal standard calibration curve (0-500 µg L^{-1}) produced by serial dilution of a stock solution of compounds (1 mg L^{-1}). The calibration curve was prepared by calculating the ratios between the peak area of each substance and the peak area of the internal standard and was used to determine linearity, range and instrumental detection and quantification. Compass QuantAnalysis software was used to analyse and process all data. The instrument quantitation limit (IQLS/N) was estimated for the concentration of compound that gave a signal-to-noise ratio of 10:1. The instrument detection limit (IDLS/N) corresponded to the concentration that gave a signal-to-noise ratio of 3.3:1. Method detection limits (MDL) and method quantification limits (MQL) for river water and sewage effluent were calculated using Eq. 1 and 2.

$$MDL = \frac{IDL \times 100}{RR \times CF} \quad (1)$$

$$MQL = \frac{IQL \times 100}{RR \times CF} \quad (2)$$

MDL = method detection limit
MQL = method quantification limit
IDL = instrumental detection limit
IQL = instrumental quantification limit
RR = recovery rate
CF = concentration factor (500 for river water, 200 for sewage effluent).

Method validation parameters such as accuracy and precision were determined using calibration standards (50 and 500 µg L^{-1}). These were injected in triplicate each day over a three-day period. Accuracy of the method was assessed as the percentage deviation from the known amount of analyte added to the sample. Precision was evaluated as the relative standard deviation (RSD) of replicate measurements. Both intra- and inter-day reproducibilities of the analytical method were determined.

Resolution (R_s) was determined using Eq. 3, over three concentrations in standards (50, 100 and 200 µg L^{-1}), river water (50, 100 and 200 ng L^{-1}) and sewage effluent (250, 2500 and 5000 ng L^{-1}) for both Chirobiotic V and CBH methods.

$$R_s = \frac{1.177\left(Rt_2 - Rt_1\right)}{{}^1b_{0.5} + {}^2b_{0.5}} \quad (3)$$

R_s = resolution
Rt_1 and Rt_2 = retention times of the first and second eluting enantiomers respectively
${}^1b_{0.5}$ and ${}^2b_{0.5}$ = the peak widths of the first and second eluting enantiomers at half height

Enantiomeric fraction (EF) was calculated using Eq. 4 over the calibration range for each compounds for both Chirobiotic V and CBH methods with both absolute and relative (normalised with internal standard) peak areas.

$$EF = \frac{E(+)}{(E(+) + E(-))} \quad (4)$$

EF = enantiomeric fraction

E(+) = the peak area of the (+) enantiomer
E(-) = peak area of the (-) enantiomer.

In the case where elution order was not known, the following Eq.5 was used.

$$EF = \frac{E1}{(E1 + E2)} \quad (5)$$

E1 = the peak area of the first eluting enantiomer
E2 = peak area of the second eluting enantiomer.

3 Results and Discussion

3.1 The Chiral Separation of Drugs with Chirobiotic V and CBH columns

The aim of this investigation was to develop and compare two new methods for multi-residue separation of chiral drugs in environmental matrices using two chiral columns (Chirobiotic V and CBH) and Acquity UPLC-QTOF instrumentation. The Chirobiotic V column with vancomycin as a chiral selector utilises wide-ranging interactions including hydrogen and hydrophobic bonding, ionic, π-π, dipole and steric interactions and is therefore applicable for compounds with a broad range of physicochemical properties. Enantiomeric resolution of ≥1.0 indicating maximum 2% overlap, which is required for quantitative analysis, was achieved with the Chirobiotic V column for amphetamine, methamphetamine, MDMA, atenolol, metoprolol, propranolol, venlafaxine and fluoxetine (Table 1).

The CBH column with cellobiohydrolase as a chiral selector possesses multiple chiral centres of one configuration as well as mechanisms for ionic, hydrophobic and hydrogen bonding that contribute to the retention process. It is designed primarily for the chiral separation of compounds containing one or more nitrogen atoms in addition to one or more hydrogen donating or accepting groups, thus allowing for enantiomeric resolution of ≥1.0 for a smaller group of compounds. These included amphetamine, methamphetamine, MDA, MDMA, atenolol and venlafaxine (Table 2).

In general, the CBH column provided much better resolution for all amphetamine-like compounds, than the Chirobiotic V column. For example, R_s for amphetamine enantiomers was 2.1 in the case of the CBH column and only 1.1 in the case of the Chirobiotic V column (Tables 1 and 2). In the case of MDMA, R_s was on average >1.9 for the CBH column and only 1.0 in the case of the Chirobiotic V column. Baseline resolution of MDA enantiomers was obtained in the case of the CBH column (R_s, >3.1), while this proved to be impossible with the Chirobiotic V column (R_s, <0.4). The CBH column was also more selective towards certain beta-blockers than the Chirobiotic V column, the R_s for atenolol being on average 7.3 in the case of the CBH column, and only 1.9 for the Chirobiotic V column. Very strong and selective interactions between propranolol and the CBH column, resulted in very long retention times (>90 min). Therefore the Chirobiotic V column was found to be a better choice for the analysis of this compound at enantiomeric level.

The Chirobiotic V column proved to be more selective than CBH column for antidepressants (fluoxetine and venlafaxine). For example, baseline separation of venlafaxine and fluoxetine was recorded for the Chirobiotic V (R_s = >4.3 and >2.2 respectively). The CBH column allowed for good separation of venlafaxine (R_s = 0.9 - 1.0) and no satisfactory separation of fluoxetine.

The study showed that the impact from environmental matrix appeared to have little effect upon enantiomer resolution. The reproducibility of resolution of enantiomers over three

concentrations in standards (50, 100 and 200 µg L^{-1}), river water (50, 100 and 200 ng L^{-1}) and sewage effluent (250, 2500 and 5000 ng L^{-1}) was consistent for both the Chirobiotic V and CBH column methods (Table 1 and 2). The inter-concentration RSD of R_s for both methods was ≤11% in both standards and environmental matrices. This was with the exception of metoprolol and propranolol in sewage effluent matrix (Chirobiotic V column method) which had average inter-concentration R_s of 1.6±0.4 (RSD = 22.9 %) and 2.2±0.4 (RSD = 18.7 %) respectively. Examples of enantiomeric resolution in spiked river water are detailed for the Chirobiotic V and CBH columns respectively in Figures 1 and 2.

3.2 Method Validation

Linearity and limits of detection data are presented in Tables 1 and 2. Average linearity for all compounds showed R^2 of 0.997 using both the Chirobiotic V and CBH columns. The IDL and IQL for the Chirobiotic V column ranged from <0.3 – 4.0 µg L^{-1} and 0.5 – 15.0 µg L^{-1}. Furthermore excluding MDMA, the MDL and MQL for river water matrices ranged from <0.2 – 5.5 ng L^{-1} and <0.3 – 18.5 ng L^{-1} respectively (Table 1). The MDL and MQL for sewage effluent matrices, excluding MDMA, ranged from 0.6 – 14.2 ng L^{-1} and 1.3 – 47 ng L^{-1} respectively (Table 1). In comparison, the IDL and IQL for CBH were typically higher, ranging respectively from 1.25– 5 µg L^{-1} and 5 – 25 µg L^{-1} (Table 2), probably as the CBH mobile phase was 90% aqueous and would have caused lower MS signal in comparison to that of the Chirobiotic V method utilising organic mobile phase. MDMA was an exception as it was found to have slightly lower IQL with the CBH column method (12.5 µg L^{-1} for both enantiomers) than with the Chirobiotic V column (15.0 µg L^{-1} for both enantiomers). Furthermore, the MQL in river water for MDMA separated with the CBH column was lower (R (-) = 26.8 and S (+) = 25.6 ng L^{-1}) when compared to the Chirobiotic V column (E1 = 85.7 and E2 = 81.9 ng L^{-1}). In comparison with triple-quadrupole analysers, which have better sensitivity due to analysis of targeted fragmentation ions, the IDL/IQLs presented here were unsurprisingly higher. Kasprzyk-Hordern et al. (Kasprzyk-Hordern, Kondakal et al. 2010) reported IDLs for amphetamine, methamphetamine, MDA and MDMA of 0.025 – 0.1 µg L^{-1}. However, the advantages of utilising QTOF over triple-quadrupole analysers include the screening for non-target metabolites and break-down products that can be carried out. High throughput analytical techniques employing QTOF are developing into important tools for the analysis of environmental matrices (Ibanez, Sancho et al. 2008; Helbling, Hollender et al. 2010; Hernandez, Bijlsma et al. 2011). Studies such as these demonstrate that in order to progress research in this field, it is necessary to analyse a broader range of compounds in addition to their metabolites and microbial transformation products.

The detection limits of this study, although higher than in the cases utilising triple quadrupoles, are comparable or lower than other analytical methodologies utilising QTOF. For example, in a multi-residue (non-chiral) method for 29 pharmaceuticals using HLB SPE methodology with Acquity UPLC coupled to a QTOF-Micro (Waters Corp., USA) IDLs ranged from 0.5 – 10 µg L^{-1} (Petrovic, Gros et al. 2006). These authors observed IDLs of 1, 0.2 and 2 µg L^{-1} for atenolol, metoprolol and propranolol respectively. During this study (Table 1), the IDLs for atenolol, metoprolol and propranolol were 1.2, 0.3 and 0.3 µg L^{-1} respectively (for individual enantiomers) using the Chirobiotic V method. Furthermore, Petrovic et al. (Petrovic, Gros et al. 2006) observed MDLs of 50, 15 and 100 ng L^{-1} for atenolol, metoprolol and propranolol respectively in sewage influent. In this study, the MDLs were lower for sewage effluent using the Chirobiotic V column and were: 5.3 and 5.0 ng L^{-1} for S (-) and R (+) atenolol respectively; 0.6 and 0.7 ng L^{-1} enantiomers of metoprolol respectively and 1.0 and 1.4 ng L^{-1} in the case of S (-) and R (+) propranolol respectively.

The enantiomeric fractions of all studied compounds for both methodologies are detailed in Table 3 and were calculated using Equations 4 and 5. Upon calculation of absolute EF (based upon peak areas of the target compound alone), deviation from 0.5 indicating racemic solution, could be witnessed with both methodologies. This was particularly evident with amphetamine (absolute EF 0.58±0.08 and 0.57±0.05 for Chirobiotic V and CBH respectively). The absolute EF for atenolol was 0.54±0.03 and 0.43±0.06 for Chirobiotic V and CBH respectively. Normalising peak areas with internal standard resulted in relative EFs that were closer to 0.5.

Precision and accuracy data for the Chirobiotic V and CBH column methodologies are detailed in Tables 4 and 5. Over three days and across the range of concentrations tested (50 – 500 µg L^{-1}) intra-day and inter-day precision was on average 4.6 and 7.7 % respectively for the Chirobiotic V column, which was similar to the CBH column intra-day and inter-day precision over three-days (on average 5.7 and 7.6 % respectively). Over three days and across the range of concentrations assessed, intra-day and inter-day accuracy was on average 90 % and 91 % respectively for the Chirobiotic V column. For the CBH column, over three days intra-day and inter-day precision were both higher, on average 101 %. Recovery rates for spiked river water matrices were typically >80 % for both the Chirobiotic V and CBH column methodologies (Table 6). Recovery rates for spiked sewage effluent matrices were comparable to those of river water and were typically ~80 %, over the range of spiked concentrations.

In summary, although the accuracy of LC-chiral-QTOF has been demonstrated to be less than equivalent LC-chiral-quadrupole methodology (MacLeod, Sudhir et al. 2007; Kasprzyk-Hordern, Kondakal et al. 2010), overall precision and accuracy was suitable for environmental analysis, given the complexity of the matrices. Application of the correct chiral column and applying recovery correction, meaningful quantification of environmental matrices could be performed. The findings of this study suggest that the Chirobiotic V methodology can be used successfully for the quantification of amphetamine, methamphetamine, metoprolol, propranolol, fluoxetine, atenolol, metoprolol and venlafaxine in environmental matrices. Furthermore the CBH methodology can be used for the quantification of amphetamine, MDA, MDMA, atenolol, and venlafaxine in these same matrices. With the use of QTOF technology, retrospective and non-targeted analysis is achievable and is a distinct advantage over the use of triple-quadrupole mass spectrometers. QTOF-MS methodology could also be used to monitor for break-down products in conjunction with routine targeted analysis.

3.3 The Identification and Confirmation of Chiral Drugs in the Environment using QTOF

Using the CBH column method no chiral drugs were detected or quantified in river water matrix. The MQLs for this method were not as low as those when using the Chirobiotic V column. However, the Chirobiotic V method was capable of quantifying venlafaxine, propranolol and atenolol in both river and sewage effluent matrices (Table 7). In river water, the total concentration of propranolol was just below the MQL 1.7 ng L^{-1} and the EF was 0.45, this was within the range of propranolol concentrations and EFs reported by Fono and Sedlak et al. (Fono and Sedlak 2005). The concentration of total venlafaxine was just above the MQL (19.3 ng L^{-1}) and the EF was 0.58. These results similar to Kasprzyk-Hordern and Baker (Kasprzyk-Hordern and Baker 2011) who reported near racemic proportions of venlafaxine in river water at a total concentration of 10 ng L^{-1}. The average total concentration of atenolol was 30.0 ng L^{-1} and the EF was 0.47; and again falls within the range observed by Kasprzyk-Hordern and Baker (Kasprzyk-Hordern and Baker 2011). Direct comparison for venlafaxine has not been possible as the order in which the enantiomers elute is not known for the CBH column method.

The concentrations of chiral drugs observed in sewage effluent are detailed in Table 7. In this matrix, the average total propranolol concentration was 77.0 ng L^{-1} and the EF was 0.43. This is in agreement with Fono and Sedlak et al. (Fono and Sedlak 2005) who reported EF ranging from 0.31 to 0.44 and MacLeod et al. (MacLeod, Sudhir et al. 2007) who reported EF for propranolol of ~0.4. Average total venlafaxine concentration was 106.5 ng L^{-1} and EF constituted 0.43. Atenolol was recorded at the highest concentration of all compounds (931 ng L^{-1}). The EF of this compound was 0.55. The E2 MDMA enantiomer was detected in sewage effluent at levels >MDL (19.9 ng L^{-1}), however this concentration can only be viewed semi-quantitatively. It is likely that E2 MDMA corresponds with R (-)-MDMA as Kasprzyk-Hordern and Baker (Kasprzyk-Hordern and Baker 2011) found this enantiomer to be enriched during sewage treatment. Metoprolol was also detected at just above the MQL (3.6 ng L^{-1}), the EF for this compound was 0.54. It is likely that E1 and E2 for metoprolol correspond respectively with the S (-) and R (+) stereoisomers; as metoprolol is structurally related to atenolol and propranolol which both elute in this order (Figure 1).

It is demonstrated here that chiral pharmaceuticals and illicit drugs in the aquatic environment are non-racemic in proportion. Furthermore, there was a pronounced difference in EF between the same compound in sewage effluent and river water. For example, the enrichment of the respective S (-) and R (-) enantiomers of propranolol (EF=0.43±0.02) and venlafaxine (0.43±0.02) that was seen in sewage effluent was not witnessed in river water to the same degree. In fact, these compounds in river water were closer to racemic proportions, the greatest evidence for which being with venlafaxine that demonstrated slight enrichment of the S (+) enantiomer (0.58±0.02). Differences in EF between sewage and river water matrices were also evident with atenolol: respective EF of 0.55±0.00 and 0.47±0.02 was observed. These observations could indicate that stereo-selective mechanisms during sewage treatment are different from those occurring in the aquatic environment.

Quantification of chiral pharmaceuticals and drugs was done on an accurate mass basis; therefore further confirmation using MS/MS was desirable. The parameters utilised to achieve MS/MS spectra are detailed in Table 8. The MS/MS spectra obtained for venlafaxine, propranolol and atenolol in pure standard, river water and sewage effluent are detailed in Figure 3. Confirmation was done based on purity, fit and reverse fit (Equation 6, 7 and 8) using Bruker Data Analysis software. Comparison of ion ratios as per confirmation criteria of Council Directive 96/23/EC was also conducted (of 12 August 2002).

$$P = 100\frac{A^2}{UL} \quad (6)$$

$$F = 100\frac{A^2}{TL} \quad (7)$$

$$R = 100\frac{A^2}{UR} \quad (8)$$

Where:
A=∑ of the product of the intensities of the unknown and the library spectrum
U=∑ of the square of the intensities of the unknown spectrum
L=∑ of the square of the intensities of the library spectrum
T=∑ of the square of the intensities of the unknown spectrum where the library spectrum has intensity above 0
R=∑ of the square of the intensities of the library spectrum where the unknown spectrum has intensity above 0

In both river water and sewage effluent P, F and R scores for propranolol and atenolol were >70% (Table 9), which has been suggested to be an acceptable threshold for trace analysis using library matching (Hopley, Bristow et al. 2008). The presence of E2 MDMA in sewage effluent could also be confirmed, as the P, F and R scores were 98 (Figure 4). Metoprolol was only detected in sewage effluent, matching scores for this compound were low (P = 52 and R = 53) for E1 (Table 9; Figure 4) and E2 metoprolol failed to match at all. Poor matching is likely to be have been contributed to by the low concentration of the compound in the environmental samples thus influencing the calculation matching scores as a result of signal noise (Pihlainen, Sippola et al. 2003). For venlafaxine, whilst P, F and R scores were >78% in river water, P scores were 46 and 59% for the S (+) and R (-) respectively. This could have been due to the isolation width of the collision cell which was ±3Da for the generation of MS/MS spectra. Thus compounds of similar mass derived from river water and sewage effluent could have contributed to these spectra. Furthermore, there was a statistical difference between the P, F and R scores of each enantiomer (ttest $p<0.05$). The P, F and R scores were higher for the R (-) venlafaxine in both river water and sewage effluent. This could indicate more co-eluting compounds where entering the collision cell with the S (+) enantiomer than the R (-).

The presence of venlafaxine and metoprolol in sewage effluent as well as further confirmation of the other compounds was done by comparison of ion ratios between environmental samples and a 100 µg L^{-1} standard (Table 10) as per Council Directive 96/23/EC criteria (of 12 August 2002). For example, the ion ratio between the first and second fragmentation ions for venlafaxine was 1.31. In river water this ion ratio was 1.27 for S (+) and 1.39 for R (-) and represents a deviation from that of the standard of -3.05 and 6.11 % respectively. Similarly in sewage effluent, the deviation from that of the standard was 7.63 % for both enantiomers. In fact all compounds had ion ratios between the first and second fragmentation ions that deviated <20 % from the 100 µg L^{-1} standard. These ions ratios could therefore be used as unique identifiers for confirmation of the compounds analysed here.

4.0 Conclusion

The effective application of a chiral HPLC-QTOF-MS methodology has been demonstrated for the analysis of environmental matrices, achieving resolution typically >1.0 for both methods. Using the Chirobiotic V or CBH column methodologies amphetamine, methamphetamine, MDA, MDMA, propranolol, atenolol, metoprolol, fluoxetine and venlafaxine could be quantified in environmental matrices. The Chirobiotic V column method gave lower MDLs for more compounds, however, precision and accuracy were comparable and were <8 % and >90 % respectively for both methodologies. Recoveries in river water and sewage effluent were typically >80 %. The Chirobiotic V methodology was used successfully for the quantification of amphetamine, methamphetamine, metoprolol, propranolol, fluoxetine, atenolol, metoprolol and venlafaxine in environmental matrices. Furthermore the CBH methodology can be used for the quantification of amphetamine, MDA, MDMA, atenolol, and venlafaxine in the same matrices. Subsequently, atenolol and venlafaxine were quantified in river water, showing average total concentrations of 30.1 and 19.3 ng L^{-1} respectively. Furthermore EF for atenolol and venlafaxine were 0.47 and 0.58 indicating slight enrichment of the S (-) and S (+) enantiomers respectively. In river water propranolol was detected just below the MDL. In sewage effluent, propranolol, atenolol, metoprolol and venlafaxine were quantified with average total concentrations of 77.0 and 1090.7, 12.6 and 106.5 ng L^{-1} respectively. The EF for propranolol, atenolol, metoprolol and venlafaxine were 0.43, 0.55, 0.54 and 0.43 respectively, thus indicating that the river environment effects change in ratio

for chiral drugs. The presence of pharmaceuticals and illicit drugs detected and quantified in environmental samples were successfully confirmed using MS/MS confirmation. In sewage effluent E2-MDMA (likely to be R (-)-MDMA) was detected just below the MDL. The use of a QTOF mass spectrometer has distinct advantages over quadrupole analysers as this methodology could also be used to monitor break-down products and be used for non-target screening in conjunction with routine targeted quantification.

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Table 1. The method validation parameters for chiral drugs using Chirobiotic V including linearity, resolution, detection and quantification limits.

Compound		Instrumental Parameters								River Water						Sewage Effluent				
		[a]Rt (mins)	Linearity range µg L^{-1}	[b]R^2	[c]$IDL_{s/n}$ µg L^{-1}	[d]$IQL_{s/n}$ µg L^{-1}	[e]R_s 50 µg L^{-1}	[e]R_s 100 µg L^{-1}	[e]R_s 200 µg L^{-1}	[f]MDL_{calc} ng L^{-1}	[g]MQL_{calc} ng L^{-1}	R_s 25 ng L^{-1}	R_s 50 ng L^{-1}	R_s 100 ng L^{-1}	MDL_{calc} ng L^{-1}	MQL_{calc} ng L^{-1}	R_s 25 ng L^{-1}	R_s 250 ng L^{-1}	R_s 500 ng L^{-1}
Amphetamine	**S (+)**	22.2	0.5-500	0.999	1.0	2.6	1.2 ±0.1	1.1 ±0.1	1.2 ±0.1	1.8	4.8	1.3 ±0.1	1.2 ±0.1	1.3 ±0.1	4.4	11.5	1.2 ±0.1	1.2 ±0.0	1.2 ±0.1
	R (-)	23.4	0.5-500	0.996	1.0	2.7				1.8	5.0				4.6	12.4			
Methamphetamine	**S (+)**	31.2	0.25-500	0.999	3.0	10.0	1.1 ±0.0	1.1 ±0.0	1.2 ±0.3	5.5	18.3	1.3 ±0.0	1.3 ±0.1	1.1 ±0.0	14.2	47.3	1.2 ±0.0	1.0 ±0.1	0.9 ±0.0
	R (-)	32.6	0.25-500	0.999	2.5	10.0				4.6	18.5				11.9	47.6			
MDMA	**E1**	35.4	5-500	0.997	4.0	15.0	1.0 ±0.1	1.1 ±0.0	1.0 ±0.0	9.6	35.8	0.9 ±0.0	0.9 ±0.0	0.9 ±0.0	22.8	85.7	1.0 ±0.0	1.0 ±0.1	0.9 ±0.0
	E2	37.0	5-500	0.994	4.0	15.0				10.4	39.0				21.8	81.9			
Propranolol	**S (-)**	24.4	[h]0.25-100/5-500	[h]0.999/0.995	0.2	0.5	2.4 ±0.0	2.3 ±0.1	2.0 ±0.1	0.4	1.2	2.3 ±0.1	2.4 ±0.1	2.1 ±0.1	1.0	2.6	2.7 ±0.1	2.2 ±0.2	1.8 ±0.0
	R (+)	27.1	[h]0.25-100/5-500	[h]0.999/0.992	0.3	0.6				0.6	1.4				1.4	3.4			
Atenolol	**S (-)**	35.0	[h]0.5-100/5-500	[h]0.998/0.998	1.2	2.5	2.0 ±0.1	1.9 ±0.0	1.8 ±0.1	2.2	4.7	1.8 ±0.1	1.9 ±0.1	1.8 ±0.1	5.3	11.0	1.8 ±0.0	1.8 ±0.2	1.6 ±0.0
	R (+)	38.2	[h]0.25-100/5-500	[h]0.998/0.999	1.1	2.5				2.1	4.8				5.0	11.4			
Metoprolol	**E1**	22.1	[h]0.25-100/5-500	[h]0.999/0.993	0.3	0.5	1.8 ±0.1	1.6 ±0.0	1.4 ±0.1	0.2	0.4	2.0 ±0.1	1.8 ±0.1	1.6 ±0.1	0.6	1.3	2.1 ±0.1	1.5 ±0.1	1.3 ±0.1
	E2	24.0	[h]0.25-100/5-500	[h]0.998/0.992	0.3	0.5				0.2	0.3				0.7	1.3			
Venlafaxine	**S (+)**	28.9	[h]0.25-100/5-500	[h]0.993/0.999	1.0	3.8	4.4 ±0.2	4.6 ±0.2	4.3 ±0.1	2.2	8.1	3.8 ±0.1	4.3 ±0.0	3.9 ±0.0	3.9	14.4	4.7 ±0.0	4.2 ±0.0	3.8 ±0.0
	R (-)	34.6	[h]0.25-100/5-500	[h]0.995/0.999	1.2	3.8				2.5	7.9				4.8	15.1			
Fluoxetine	**S (+)**	32.9	[h]0.25-100/5-500	[h]0.999/0.996	0.4	1.0	2.4 ±0.1	2.3 ±0.1	2.2 ±0.1	0.8	2.0	2.6 ±0.3	2.5 ±0.1	2.5 ±0.3	2.6	6.5	2.5 ±0.1	2.3 ±0.1	2.2 ±0.0
	R (-)	36.1	[h]0.25-100/5-500	[h]0.999/0.996	0.4	1.3				0.8	2.5				2.4	7.6			

[a] Rt is the retention time, [b] R^2 is the correlation coefficient, [c] $IDL_{s/n}$ is the instrumental detection limit based on signal to noise, [d] $IQL_{s/n}$ is the instrumental quantification limit based on signal to noise, [e] R_s is the chromatographic resolution, [f] MDL_{calc} is the calculated method detection limit, [g] MQL_{calc} is the calculated method quantification limit, [h] low and high range calibrations used due to lack on linearity of over the full range

Table 2. The method validation parameters for chiral drugs using CBH including linearity, resolution, detection and quantification limits.

Compound			Instrumental Parameters				River Water							
		[a]R_t (mins)	Linearity range	[b]R^2	[c]$IDL_{s/n}$	[d]$IQL_{s/n}$	[e]R_s			[f]MDL_{calc}	[g]MQL_{calc}	R_s		
			µg L^{-1}		µg L^{-1}	µg mL^{-1}	25 µg L^{-1}	50 µg L^{-1}	100 µg L^{-1}	ng L^{-1}	ng L^{-1}	25 ng L^{-1}	50 ng L^{-1}	100 ng L^{-1}
Amphetamine	**R (-)**	28.9	0.5-500	0.996	2.5	5.0	2.1±0.1	2.2±0.0	1.9±0.1	4.8	9.7	2.5±0.2	2.4±0.3	2.5±0.4
	S (+)	33.9	0.5-500	0.995	2.5	5.0				5.0	10.0			
Methamphetamine	**R (-)**	29.9	2.5-500	0.996	2.5	12.5	1.2±0.2	1.2±0.2	1.2±0.1	4.1	20.6	1.3±0.1	1.2±0.2	1.2±0.1
	S (+)	32.9	2.5-500	0.993	2.5	12.5				3.6	18.1			
MDA	**R (-)**	43.5	1.75-500	0.998	1.25	5.0	3.1±0.3	3.4±0.2	3.2±0.2	2.4	9.6	3.3±0.4	3.4±0.2	3.2±0.2
	S (+)	52.8	1.75-500	0.998	1.25	5.0				2.3	9.1			
MDMA	**R (-)**	40.2	12.5-500	0.995	5.0	12.5	2.7±0.5	1.9±0.1	1.9±0.1	10.7	26.8	3.1±0.2	2.3±0.2	2.2±0.1
	S (+)	46.7	12.5-500	0.996	5.0	12.5				10.2	25.6			
Atenolol	**R (+)**	28.6	0.5-500	0.998	1.3	12.5	7.2±0.2	7.4±0.6	7.5±0.3	2.3	22.9	7.5±0.5	7.5±0.2	7.4±0.3
	S (-)	46.5	0.5-500	0.999	1.3	12.5				2.1	20.7			
Venlafaxine	**E1**	28.4	5-500	0.995	5.0	25.0	0.9±0.1	0.9±0.1	1.0±0.1	10.3	51.7	0.9±0.1	0.9±0.1	1.0±0.1
	E2	30.8	5-500	0.997	5.0	25.0				9.6	47.9			

[a] Rt is the retention time, [b] R^2 is the correlation coefficient, [c] $IDL_{s/n}$ is the instrumental detection limit based on signal to noise, [d] $IQL_{s/n}$ is the instrumental quantification limit based on signal to noise, [e] R_s is the chromatographic resolution, [f] MDL_{calc} is the calculated method detection limit, [g] MQL_{calc} is the calculated method quantification limit,

Table 3. The absolute and relative EF of calibration standard using Chirobiotic V and CBH methodologies

Compound	Chirobiotic V		CBH	
	[c]Abs	[d]Rel	Abs	Rel
Amphetamine	[a]0.58±0.08	[a]0.51±0.03	[a]0.57±0.07	[a]0.51±0.05
Methamphetamine	[a]0.52±0.06	[a]0.54±0.06	[a]0.54±0.04	[a]0.49±0.03
MDMA	[b]0.47±0.03	[b]0.49±0.03	[a]0.53±0.03	[a]0.51±0.04
Propranolol	[a]0.54±0.04	[a]0.50±0.02		
Atenolol	[a]0.54±0.03	[a]0.51±0.01	[a]0.43±0.06	[a]0.51±0.04
Metoprolol	[b]0.52±0.01	[b]0.54±0.02		
Venlafaxine	[a]0.49±0.03	[a]0.50±0.03	[b]0.46±0.04	[a]0.52±0.03
Fluoxetine	[a]0.46±0.04	[a]0.49±0.04		

[a] EF calculated using Equation 4, [b] EF calculated using Equation 5, [c] Abs is the absolute EF, [d] Rel is the relative EF (peak areas are normalised using internal standards)

Table 4. The intra and inter-day precision (%) observed over three days using the Chirobiotic V and CBH columns

Compound		Intra-day precision (%)												Inter-day precision (%)			
		50 µg L^{-1}						500 µg L^{-1}						50 µg L^{-1}	500 µg L^{-1}	50 µg L^{-1}	500 µg L^{-1}
		Chirobiotic V			CBH			Chirobiotic V			CBH			Chirobiotic V		CBH	
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3				
Amphetamine	**S (+)**	1	3	1	8	11	13	8	10	3	4	9	13	2	8	9	9
	R (-)	3	4	4	8	5	3	3	10	7	6	10	12	3	10	6	9
Methamphetamine	**S (+)**	5	4	2	7	11	11	6	6	3	1	1	3	7	6	10	4
	R (-)	4	5	4	9	9	3	6	3	2	4	7	7	7	9	9	9
[a]Venlafaxine	**Chiro (S (+)) / CBH (E1)**	2	2	4	3	2	4	7	3	2	2	3	4	9	14	6	5
	Chiro (R (-))/CBH (E2)	2	3	9	3	3	15	9	4	4	1	6	1	11	14	10	5
Fluoxetine	**S (+)**	6	5	1	-	-	-	5	5	3	-	-	-	6	7	-	-
	R (-)	4	3	3	-	-	-	9	3	5	-	-	-	9	17	-	-
MDA	**S (+)**	-	-	-	9	1	6	-	-	-	4	3	3	-	-	5	4
	R (-)	-	-	-	6	3	3	-	-	-	5	6	6	-	-	1	3
[b]MDMA	**Chiro (E1)/CBH (S (+))**	4	5	3	10	8	9	7	4	7	7	6	4	9	10	15	5
	Chiro (E2)/CBH (R (-))	2	6	7	10	3	9	5	20	17	3	8	4	10	16	11	5
[c]Metoprolol	**E1**	11	6	0	-	-	-	4	4	5	-	-	-	11	9	-	-
	E2	9	9	4	-	-	-	3	3	1	-	-	-	11	2	-	-
Atenolol	**S (-)**	2	0	5	7	5	4	2	1	6	2	2	2	3	4	7	11
	R (+)	4	4	5	5	7	4	3	4	2	2	2	1	5	5	6	4
Propranolol	**S (+)**	9	7	1	-	-	-	2	7	2	-	-	-	10	7	-	-
	R (-)	7	5	3	-	-	-	2	1	2	-	-	-	8	2	-	-

[a] Elution order known only for Chirobiotic V. [b] Elution order only known for CBH. [c] Neither elution order is known for Chirobiotic V or CBH

Table 5. The intra and inter-day accuracy (%) observed over three days using the Chirobiotic V and CBH columns

Compound		Intra-day Accuracy (%)												Inter-day Accuracy (%)			
		50 µg L^{-1}						500 µg L^{-1}						50 µg L^{-1}	500 µg L^{-1}	50 µg L^{-1}	500 µg L^{-1}
		Chirobiotic V			CBH			Chirobiotic V			CBH			Chirobiotic V		CBH	
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3				
Amphetamine	**S (+)**	104	100	100	109	107	103	103	114	109	114	103	115	101	109	106	112
	R (-)	100	98	97	112	103	108	87	93	80	123	122	119	98	87	108	122
Methamphetamine	**S (+)**	97	109	99	132	117	121	102	94	98	109	118	115	102	89	124	114
	R (-)	91	103	102	115	115	105	111	95	98	96	94	89	99	102	112	93
[a]Venlafaxine	**Chiro (S (+)) / CBH (E1)**	90	81	75	76	78	68	128	109	96	94	97	90	82	113	74	94
	Chiro (R (-))/CBH (E2)	93	80	74	86	93	87	137	113	102	97	106	102	82	118	89	101
Fluoxetine	**S (+)**	101	104	111	-	-	-	76	73	82	-	-	-	105	77	-	-
	R (-)	98	99	117	-	-	-	76	68	97	-	-	-	104	81	-	-
MDA	**S (+)**	-	-	-	115	115	114	-	-	-	106	107	110	-	-	115	108
	R (-)	-	-	-	108	108	108	-	-	-	101	102	102	-	-	108	102
[b]MDMA	**Chiro (E1)/CBH (S (+))**	65	56	54	71	81	61	75	66	82	102	103	99	59	75	71	102
	Chiro (E2)/CBH (R (-))	57	48	48	70	79	65	64	73	78	94	94	89	52	71	71	92
[c]Metoprolol	**E1**	100	95	81	-	-	-	85	104	95	-	-	-	93	94	-	-
	E2	104	93	86	-	-	-	99	102	99	-	-	-	95	100	-	-
Atenolol	**S (-)**	94	90	94	120	112	127	85	80	81	101	88	113	92	82	119	101
	R (+)	87	85	91	97	101	107	83	76	83	94	92	101	88	81	102	96
Propranolol	**S (-)**	90	91	76	-	-	-	80	88	75	-	-	-	87	80	-	-
	R (+)	84	90	78	-	-	-	88	87	85	-	-	-	85	87	-	-

[a] Elution order known only for Chirobiotic V. [b] Elution order only known for CBH. [c] Neither elution order is known for Chirobiotic V or CBH

Table 6. The average recovery rates (±%RSD) observed for the overall analytical protocol using Chirobiotic V or CBH (n=9).

Compound	Chirobiotic V										CBH					
	Enantiomer		River Water			Mean	Sewage Effluent			Mean	Enantiomer		River Water			Mean
			25 ng L^{-1}	50 ng L^{-1}	100 ng L^{-1}		125 ng L^{-1}	1250 ng L^{-1}	2500 ng L^{-1}				25 ng L^{-1}	50 ng L^{-1}	100 ng L^{-1}	
Amphetamine	**E1**	**S (+)**	105±8	110±2	113±6	109±7	109±3	115±3	114±4	113±4	**E1**	**R (-)**	101±10	101±10	108±7	104±9
	E2	**R (-)**	104±5	109±4	113±6	108±6	104±6	113±2	108±8	109±6	**E2**	**S (+)**	105±7	94±9	102±9	100±9
Methamphetamine	**E1**	**S (+)**	107±6	110±6	110±6	109±6	93±12	106±3	118±5	106±12	**E1**	**R (-)**	132±6	118±8	115±4	112±9
	E2	**R (-)**	104±8	111±7	110±6	108±8	97±13	103±6	115±7	115±14	**E2**	**S (+)**	138±12	138±6	138±7	138±8
MDA	**E1**		-	-	-	-	-	-	-	-	**E1**	**R (-)**	114±2	107±7	95±5	105±9
	E2		-	-	-	-	-	-	-	-	**E2**	**S (+)**	113±4	113±6	104±8	110±7
MDMA	**E1**		66±12	85±7	82±7	84±17	61±15	102±10	105±1	88±26	**E1**	**R (-)**	77±21	106±8	96±6	93±17
	E2		64±15	81±8	87±7	77±16	73±17	94±10	108±8	92±19	**E2**	**S (+)**	78±20	110±12	105±7	98±18
Propranolol	**E1**	**S (-)**	81±10	74±9	85±5	81±9	91±4	96±2	88±3	92±4			-	-	-	-
	E2	**R (+)**	71±9	67±7	87±7	76±14	75±12	84±2	76±6	78±9			-	-	-	-
Atenolol	**E1**	**S (-)**	95±4	104±4	120±5	107±11	108±1	116±4	115±7	113±5	**E1**	**R (+)**	116±6	112±9	102±3	109±9
	E2	**R (+)**	92±4	110±5	112±4	104±10	95±5	115±5	118±6	109±11	**E2**	**S (-)**	126±7	122±5	116±10	121±8
Metoprolol	**E1**		101±9	100±5	108±5	104±7	73±0	79±3	73±4	76±5			-	-	-	-
	E2		116±8	113±3	107±4	112±7	87±10	77±3	65±5	75±13			-	-	-	-
Venlafaxine	**E1**	**S (+)**	86±9	78±7	80±12	82±10	93±14	119±1	126±5	115±14	**E1**		90±10	79±10	87±11	85±11
	E2	**R (-)**	88±8	78±6	84±6	84±9	104±0	115±6	109±12	110±8	**E2**		99±13	88±13	87±11	85±11
Fluoxetine	**E1**	**S (+)**	83±10	90±5	106±5	90±12	67±10	64±5	76±6	69±10			-	-	-	-
	E2	**R (-)**	92±21	93±5	82±12	89±15	86±20	67±9	73±6	74±15			-	-	-	-

Table 7. Average concentration and EF (enantiomeric fraction) of chiral drugs observed in river water and sewage effluent (river water n=6, sewage effluent n=4)

Compound	July 2011 (Chirobiotic V)								Oct 2011 (CBH)			
	Enantiomer		River water (ng L^{-1})			Sewage effluent (ng L^{-1})			Enantiomer		River water (ng L^{-1})	
			MQL	Mean	EF	MQL	Mean	EF			MQL	Mean
Amphetamine	**E1**	**S (+)**	4.8	<MQL		11.5	<MQL		**E1**	**R (-)**	9.7	<MQL
	E2	**R (-)**	5.0	<MQL		12.4	<MQL		**E2**	**S (+)**	10.0	<MQL
Methamphetamine	**E1**	**S (+)**	18.3	<MQL		47.3	<MQL		**E1**	**R (-)**	20.6	<MQL
	E2	**R (-)**	18.5	<MQL		47.6	<MQL		**E2**	**S (+)**	18.1	<MQL
MDA	**E1**								**E1**		9.6	<MQL
	E2								**E2**		9.1	<MQL
MDMA	**E1**		35.8	<MQL		85.7	<MQL		**E1**		26.8	<MQL
	E2		39	<MQL		81.9	19.9±6.2		**E2**		25.6	<MQL
Propranolol	**E1**	**S (-)**	1.2	0.9±0.1	[a]0.45±0.04	2.6	46.5±2.5	[a]0.43±0.02				
	E2	**R (+)**	1.4	0.8±0.1		3.4	30.5±5.9					
Atenolol	**E1**	**S (-)**	4.7	15.8±1.3	[a]0.47±0.02	11	497.6±11.5	[a]0.55±0.00	**E1**	**R (-)**	22.9	<MQL
	E2	**R (+)**	4.8	14.2±1.4		11.4	593.1±22.9		**E2**	**S (+)**	20.7	<MQL
Metoprolol	**E1**		0.4	<MQL		1.3	1.7±0.1	[b]0.54±0.02				
	E2		0.3	<MQL		1.3	1.9±0.1					
Venlafaxine	**E1**	**S (+)**	8.1	10.8±0.8	[a]0.58±0.02	14.4	43.9±5.0	[a]0.43±0.02	**E1**		51.7	<MQL
	E2	**R (-)**	7.9	8.5±1.0		15.1	62.6±8.3		**E2**		47.9	<MQL
Fluoxetine	**E1**	**S (+)**	2.0	<MQL		6.5	<MQL					
	E2	**R (-)**	2.5	<MQL		7.6	<MQL					

[a] EF calculated using Equation 4, [b] EF calculated using Equation 5

Table 8. The mass, mass accuracy and MS/MSMS parameters associated with the compounds of study

Compound	Formula	Theoretical Mass	Experimental Mass	Error		MSMS parameters		
	[M]	$[M+H]^+$	$[M+H]^+$	mDa	ppm	Isolation width	Collision energy	Internal Standard
***RS*(±)-Amphetamine**	$C_9H_{13}N$	136.1121	136.1118	-0.3	-2.2	3	12	*RS*(±)-Amphetamine-d11
***RS*(±)-Methamphetamine**	$C_{10}H_{15}N$	150.1277	150.1275	-0.2	-1.3	3	12	*RS*(±)-Methamphetamine-d14
***RS*(±)-MDA**	$C_{10}H_{13}NO_2$	180.1019	180.1019	0.0	0.0	3	9	*RS*(±)-MDA-d5
***RS*(±)-MDMA**	$C_{11}H_{15}NO_2$	194.1176	194.1208	3.2	16.5	3	12	*RS*(±)-MDMA-d5/RS(±)-MDA-d5
***RS*(±)-Propranolol**	$C_{16}H_{21}NO_2$	260.1645	260.1625	-2.0	-7.7	3	14	*RS*(±)-Atenolol-d7
***RS*(±)-Atenolol**	$C_{14}H_{22}N_2O_3$	267.1703	267.1697	-0.6	-2.2	3	12	*RS*(±)-Atenolol-d7
***RS*(±)-Metoprolol**	$C_{15}H_{25}NO_3$	268.1907	268.1885	-2.2	-8.2	3	18	*RS*(±)-Atenolol-d7
***RS*(±)-Venlafaxine**	$C_{17}H_{27}NO_2$	278.2115	278.2102	-1.3	-4.7	3	13	*RS*(±)-Methamphetamine-d14
***RS*(±)-Norfluoxetine**	$C_{16}H_{16}F_3$ NO	296.1257	296.1276	1.9	6.4	3	8	RS(±)-Fluoxetine-d5
***RS*(±)-Fluoxetine**	$C_{17}H_{18}F_3NO$	310.1413	310.1412	-0.1	-0.3	3	8	RS(±)-Fluoxetine-d5
***RS*(±)-Amphetamine-d11**	$C_9H_2D_{11}N$	147.1811	147.1799	-1.2	-8.2			
***RS*(±)-Methamphetamine-d14**	$C_{10}HD_{14}N$	164.2156	164.2143	-1.3	-7.9			
***RS*(±)-MDA-d5**	$C_{10}H_8D_5NO_2$	185.1333	185.1326	-0.7	-3.8			
***RS*(±)-MDMA-d5**	$C_{11}H_{10}D_5NO_2$	199.1489	199.1511	2.2	11.0			
***RS*(±)-Atenolol-d7**	$C_{14}D_7H_{15}N_2O_3$	274.2143	274.2135	-0.8	-2.9			
RS(±)-Fluoxetine-d5	$C_{17}D_5H_{13}F_3NO$	315.1727	315.1720	-0.7	-2.2			

Table 9. Identification of target analytes using library matching by MS-MS spectra the Purity (P), Fit (F) and Reverse Fit (R) for the spectra are illustrated in Figures 3 and 4. Spectra from matrix were compared against pure standard.

Matrix	Compound	Enantiomer	Match score		
			Purity	Fit	Reverse fit
River Water	**Venlafaxine**	**S (+)**	78	81	82
		R (-)	82	86	87
		S+R (±)	80	84	85
	Propranolol	**S (-)**	79	89	83
		R (+)	75	86	78
		S+R (±)	80	87	82
	Atenolol	**S (-)**	95	98	95
		R (+)	94	96	94
		S+R (±)	95	97	95
Sewage effluent	**Venlafaxine**	**S (+)**	46	61	60
		R (-)	59	75	77
		S+R (±)	53	68	69
	Propranolol	**S (-)**	85	94	89
		R (+)	81	92	85
		S+R (±)	89	94	90
	Atenolol	**S (-)**	98	99	98
		R (+)	98	98	98
		S+R (±)	98	99	98
	Metoprolol	**E1**	52	83	53
		E2	[a]n/m	[a]n/m	[a]n/m
	MDMA	**E1**	<MDL	<MDL	<MDL
		E2	98	98	98

[a] n/m = no match

Table 10. The MSMS confirmation parameters for river water and sewage effluent matrices using comparison of ion ratios of the precursor ion (P), the 1st fragmentation ion (F1) and the 2nd fragmentation ion (F2). Ion ratios in environmental samples were compared with that of the pure standard.

Compound	Enantiomer	M/Z		Ion Ratios			Ion ratio deviation from the 100 µg L^{-1} Standard (%)	
		1st Fragmentation Ion	2nd Fragmentation Ion	100 µg L^{-1} Standard	River Water	Sewage Effluent	River Water	Sewage Effluent
				F1:F2	F1:F2	F1:F2	F1:F2	F1:F2
Venlafaxine	**S**				1.27	1.41	-3.05	7.63
	R	215.1418±0.01	121.0635±0.01	1.31	1.39	1.41	6.11	7.63
	S+R				1.35	1.43	3.05	9.16
Propranolol	**S**				1.76	1.66	-6.88	-12.17
	R	183.0802±0.01	157.0636±0.01	1.89	2.07	1.95	9.52	3.17
	S+R				1.76	1.94	-6.88	2.65
Atenolol	**S**				1.09	1.16	-8.4	-2.52
	R	190.0860±0.01	145.0626±0.01	1.19	1.12	1.16	-5.88	-2.52
	S+R				1.1	1.16	-7.56	-2.52
Metoprolol	**E1**					1.06		-4.09
	E2	159.0798±0.01	133.0640±0.01	1.1		0.98		-11.08
	E1+E2					0.99		-10.14
MDMA	**E2**	163.0718±0.01	135.0424±0.01	1.61		1.29		-20.06

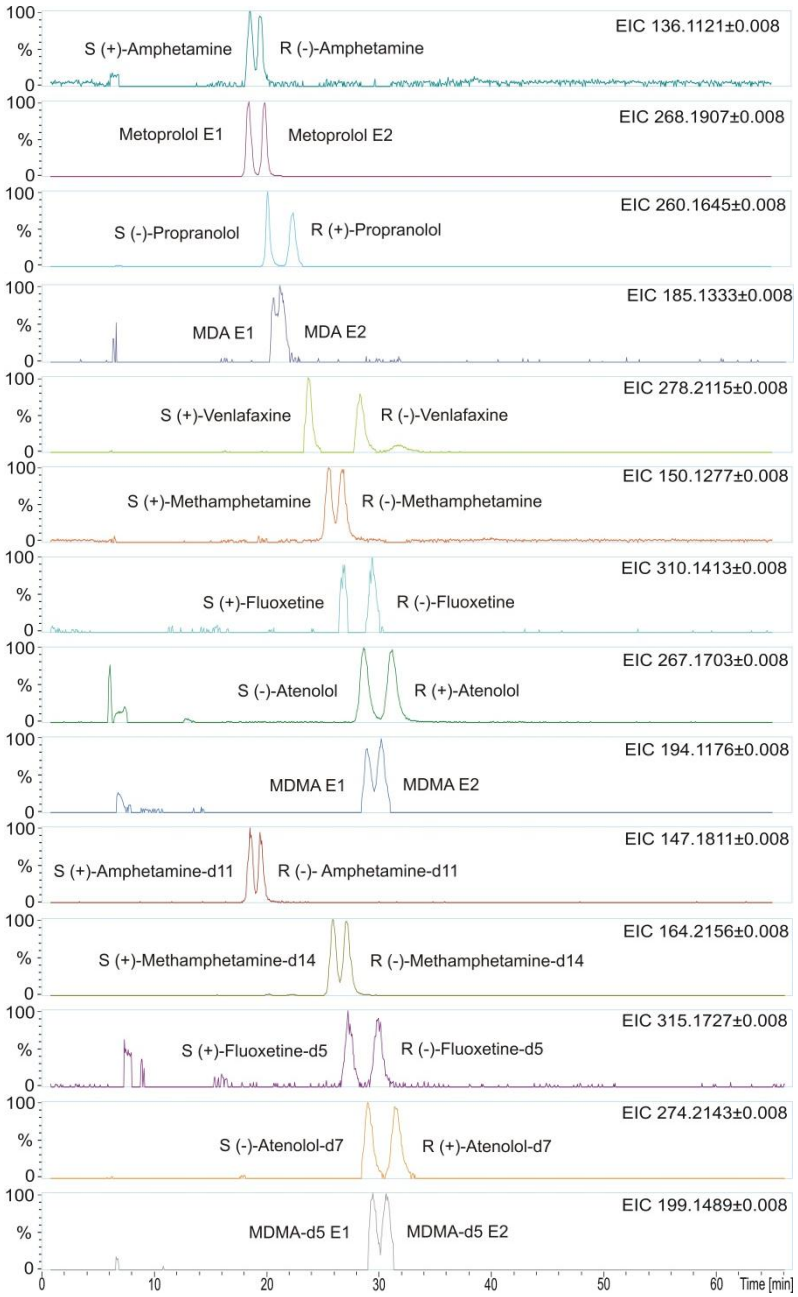

Figure 1. HPLC-MS chromatograms of chiral drugs spiked into river water, extracted by SPE and analysed using the Chirobiotic V column (concentration 100 µg L^{-1}, retention time is presented in minutes)

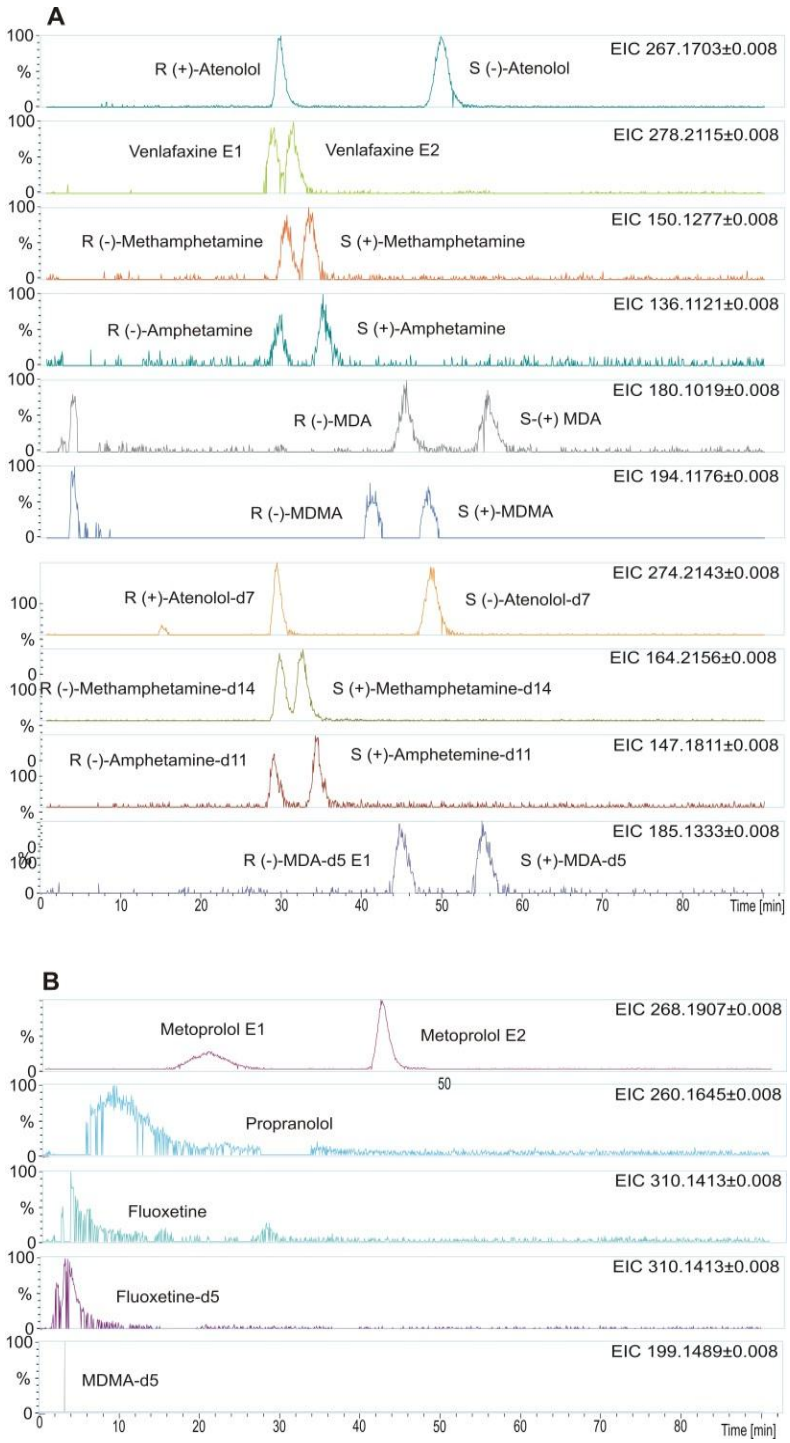

Figure 2. HPLC-MS chromatograms of chiral drugs spiked into river water, extracted by SPE and analysed using the CBH column (concentration 100 µg L^{-1}, retention time is presented in minutes) A = successfully separated compounds, B = unsuccessfully separated compounds

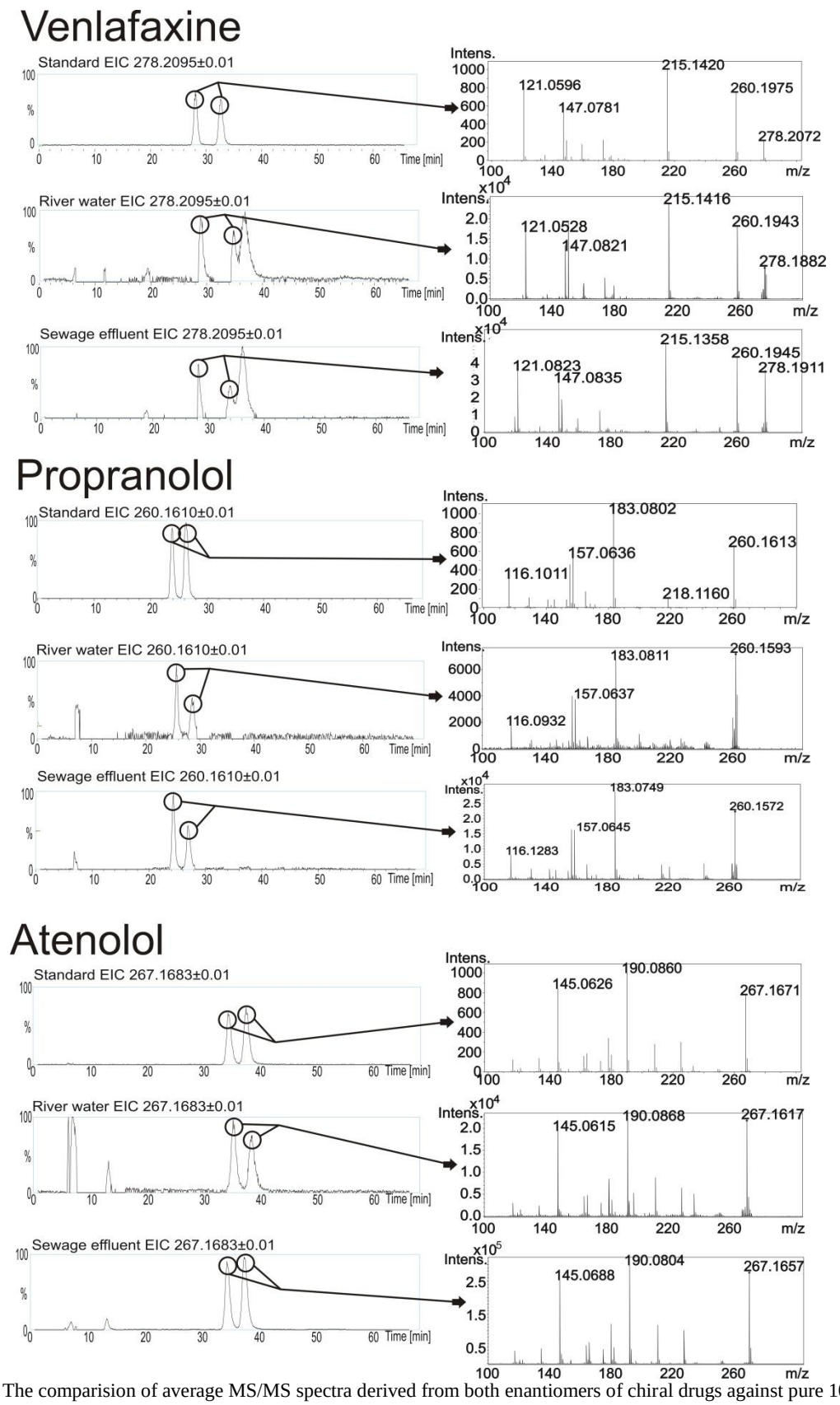

Figure 3. The comparision of average MS/MS spectra derived from both enantiomers of chiral drugs against pure 100 µg L^{-1} standard of chiral drugs quantfied in river water and sewage effluent

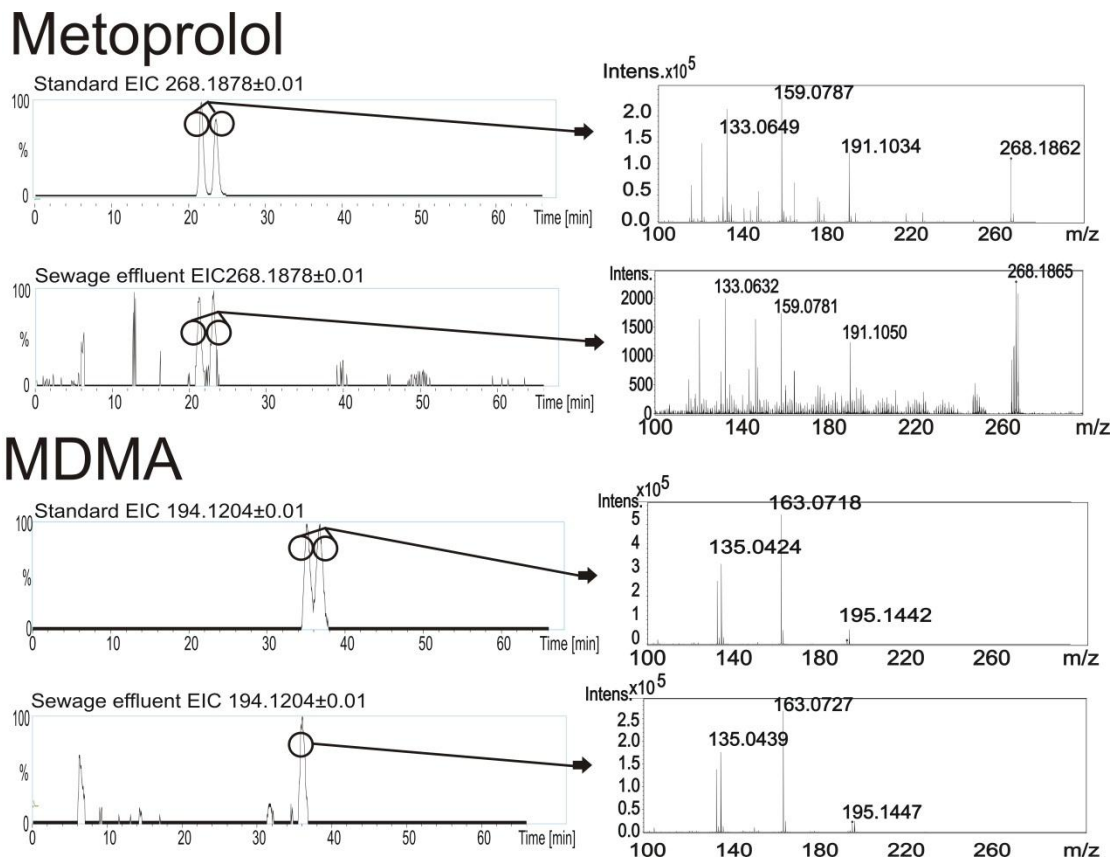

Figure 4. The comparision of average MSMS spectra derived from both enantiomers for metoprolol and only E2 for MDMA against pure100 µg L^{-1} standard of chiral drugs detected in sewage effluent