Analytica Chimica Acta xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Determination of chiral pharmaceuticals and illicit drugs in wastewater and sludge using microwave assisted extraction, solid-phase extraction and chiral liquid chromatography coupled with tandem mass spectrometry

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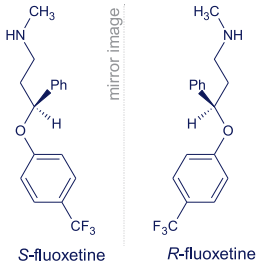
HIGHLIGHTS

- This is the first method for enantiomeric profiling of chiral drugs (cPACs) in solid & liquid matrices.
- Analysis of both liquid & solid matrix is critical to do mass balance of cPACs in wastewater.
- Chiral PACs are often non-racemic in wastewater matrices.
- Enantiomeric composition of PACs differs in liquid and solid wastewater matrices.
- Not analysing the solid fraction of wastewater may lead to over-estimation of cPAC's removal rates.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 1 November 2014
Received in revised form 14 March 2015
Accepted 24 March 2015
Available online xxx

Keywords:
Chiral drugs
Pharmaceuticals
Illicit
Wastewater
Sludge
Chiral chromatography coupled with mass spectrometry

ABSTRACT

This is the first study presenting a multi-residue method allowing for comprehensive analysis of several chiral pharmacologically active compounds (cPACs) including beta-blockers, antidepressants and amphetamines in wastewater and digested sludge at the enantiomeric level. Analysis of both the liquid and solid matrices within wastewater treatment is crucial to being able to carry out mass balance within these systems. The method developed comprises filtration, microwave assisted extraction and solid phase extraction followed by chiral liquid chromatography coupled with tandem mass spectrometry to analyse the enantiomers of 18 compounds within all three matrices. The method was successfully validated for 10 compounds within all three matrices (amphetamine, methamphetamine, MDMA, MDA, venlafaxine, desmethylvenlafaxine, citalopram, metoprolol, propranolol and sotalol), 7 compounds validated for the liquid matrices only (mirtazapine, salbutamol, fluoxetine, desmethylcitalopram, atenolol, ephedrine and pseudoephedrine) and 1 compound (alprenolol) passing the criteria for solid samples only. The method was then applied to wastewater samples; cPACs were found at concentration ranges in liquid matrices of: 1.7 ng L^{-1} (metoprolol) – 1321 ng L^{-1} (tramadol) in influent, <LOD (desmethylcitalopram and metoprolol) – 506 ng L^{-1} in effluent, and in solid matrix digested sludge: 0.4 ng g^{-1} (metoprolol) – 275 ng g^{-1} (citalopram). Enantiomeric profiling revealed that studied compounds were present in analysed samples in non-racemic composition. Furthermore, enantiomeric composition of studied analytes differed in liquid and solid matrices. This demonstrates that not

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http://dx.doi.org/10.1016/j.aca.2015.03.039

analysing the solid fraction of wastewater may lead to over-estimation of the removal rates of cPACs as well as possible misrepresentation of the enantiomeric fraction of the compounds as they leave the wastewater treatment plant. Consequently risks from cPACs entering the environment might be higher than anticipated.

1. Introduction

Developments in pharmacology and an ageing population have led to a steady increase in the prescription of pharmaceuticals worldwide. Once metabolised and excreted pharmacologically active compounds (PACs) and their metabolites enter the sewage system and if not removed or degraded, they are then pumped into effluent receiving water bodies and/or are incinerated or spread on agricultural land if adsorbed on to solids.

Pharmaceuticals and illicit drugs are routinely identified in wastewater. The data produced is often used to inform risk assessments [1,2] and develop and test prediction models [3]. However, these concentrate on pharmacologically active compounds (PACs) present in the aqueous matrix and they do not consider the fraction of PACs adsorbed on to the sediments and suspended solids which may still be bioavailable to organisms and/or may desorb in the future, possibly due to a change in matrix or environmental conditions. Methods have been developed recently in order to tackle this knowledge gap and to verify concentration levels of PACs in sediments [4–8], sludge [7], colloids [9], settable particulate matter [10] and suspended matter [6,11] as well as other solid matrices such as soil [12]. Corresponding models have also emerged to take into account this additional data [13].

Many PACs, prescribed, over the counter and illicit, are chiral. Enantiomers often have widely disparate pharmaco-dynamics and -kinetics in humans [14]. This may result in the excreted parent drug and/or metabolites having a different enantiomeric composition to the ingested drugs [14]. The fate of chiral PACs (cPACs) within the environment can be affected by micro-organisms and this process has been shown to be, at least in part, stereoselective. The enantiomeric composition of cPACs has also been demonstrated to be significant in the toxicity of several environmentally relevant species. S(+)-fluoxetine and S(−)-atenolol were found to significantly inhibit the growth of *Tetrahymena thermophila*, a freshwater protozoan, more than R(−)-fluoxetine and R(+)-atenolol. Whilst R(+)-atenolol increased mortality rates in *Pseudokirchneriella subcapitata*, an algae, significantly more than S(−)-atenolol [16]. Stanley et al. [17,18] have also demonstrated enantiomer selective toxicity in fluoxetine and propranolol towards *Pimephales promelas* and fathead minnow, with S(+)-fluoxetine and S(−)-propranolol being the more toxic enantiomers [17,18]. All three of these studies also exposed *Daphnia magna* to the enantiomers of atenolol, fluoxetine and propranolol but found no enantioselective toxicity towards this species.

Consequently, in more recent years, attention has been placed on the verification of enantiomeric composition of cPACs within wastewater and the effluent receiving waters [19–34]. However, these studies all concern themselves with the liquid fraction of the environment. Only one study has extracted and quantified antifungals from wastewater solid material [35]. This gap in the methodology may result in an under estimation of the effects of cPACs within an aquatic environment, if they adsorb to solid material. It may also be the case that bacterial populations associated with the solid material, and the process under which it is digested at wastewater treatment plants (WWTPs), may alter the enantiomeric fraction compared to both what it was on solid material in the influent as well as what is currently being recorded in the liquid fraction. Therefore, it is of the greatest importance to develop analytical capability to undertake stereoselective analysis of chiral compounds in solid matrices.

This study aims to develop a novel comprehensive analytical method for multi-residue identification and quantification of 23 cPACs (including several beta-blockers, antidepressants and amphetamines, Table S1) at enantiomeric level in both the liquid and solid fractions of wastewater. To the authors knowledge, such a method has never been previously reported. It is anticipated that this technique could then be validated and adapted for other solid matrices such as biological matrices and soils where these PACs may also be found due to, for example, the disposal of digested sludge on agricultural land. The targeted analytes have been identified based on high prescription/consumption rates within the UK, widespread environmental occurrence and biological effects, their chirality and the ability to resolve enantiomers via chiral HPLC.

2. Materials and methods

2.1. Chemicals and materials

The reference standards: R/S (±)-amphetamine, R/S (±)-methamphetamine, R/S (±)-MDA (3,4-methylenedioxyamphetamine) and R/S (±)-MDMA (3,4-methylenedioxy-methamphetamine), were purchased from LGC Standards (Teddington, UK); 1R,2S (+)-ephedrine, 2R,2S (−)-ephedrine, 1S,2S (+)-pseudoephedrine, 2S,2S (−)-pseudoephedrine, R/S (±)-norephedrine and R/S (±)-venlafaxine, R/S (±)-fluoxetine, R/S (±)-norfluoxetine, R/S (±)-*O*-desmethylvenlafaxine, 1R,2R/1S,2S (±)-tramadol, R/S (±)-atenolol, R/S (±)-metoprolol and R/S (±)-propranolol, R/S (±)-alprenolol, R/S (±)-sotalol, R/S (±)-salbutamol, R/S (±)-mirtazapine, R/S (±)-citalopram, R/S (±)-mexiletine, R/S (±)-terbutaline, were purchased from Sigma–Aldrich (Gillingham UK). R/S (±)-MDEA (3,4-methylenedioxy-*N*-ethyl-amphetamine) was purchased from LGC (Middlesex, UK) and R/S (±)-desmethylcitalopram was purchased from TRC (Toronto, Canada). All solvents were of HPLC grade and were 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 the glass surface. The surrogate/internal standards (SS/IS): R/S (±)-amphetamine-d11, R/S (±)-methamphetamine-d14, R/S (±)-MDMA-d5, R/S (±)-MDA-d5 were purchased from LGC standards (Middlesex, UK), whilst R/S(±)-atenolol-d7 were purchased from Sigma–Aldrich (Gillingham, UK). R/S(±)-propranolol-d7, R/S(±)-metoprolol-d7, R/S(±)-sotalol-d6, R/S(±)-salbutamol-d6 were purchased from TRC (Toronto, Canada). 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 the stock solution in mobile phase, and storing them at −16 °C. Solid phase extraction was carried out with the use of a vacuum manifold (Sigma–Aldrich, UK). Oasis 60 mg MCX, 60 mg MAX and HLB 60 mg cartridges were purchased from Waters (Waters, UK). Samples were eluted into silanised borosilicate glass tubes (12 × 75 mm) (Fisher, UK) and evaporated with a turbovap LV concentration workstation (Caliper, UK). Solid matrix samples were extracted using a MARS 6 microwave (CEM, UK).

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2.2. Sample collection and preparation

Digested cake and grab wastewater samples were collected in a single visit to a UK wastewater treatment plant, whose characteristics are shown in Table S2. They were transported back to the laboratory in cool boxes packed with ice blocks and frozen immediately upon arrival. All influent, effluent and digested sludge samples were collected simultaneously and therefore should not be interpreted as representing the same body of water. The cake was frozen, then dry frozen (Scanvac dryer, Scientific Laboratory Supplies, UK) and stored at −20 °C. Wastewater was also stored at −20 °C.

2.2.1. Microwave assisted extraction (MAE) for solid matrix

Method development for MAE firstly identified conditions which had been reported as successful within the literature for related compounds, methods and matrices. Mass of sample, solvent volume and type, temperature and exposure time as well as pH were identified as crucial parameters. Table 1 summarises the iterations trialled.

The following conditions resulted in the most effective MAE recovery: 20 mL methanol:water (1:1), heated to 120 °C for 30 min. Both 1 g and 3 g of freeze-dried sample were used for method validation purposes.

2.2.2. Solid-phase extraction (SPE)

2.2.2.1. SPE for liquid matrix. The wastewater samples (50 mL) were filtered and then spiked with SS/IS (1 μg L^{-1}) before being passed through Oasis HLB cartridges, with reversed phase sorbent, conditioned with 2 mL methanol, followed by 2 mL deionised water and eluted in 4 mL methanol.

2.2.2.2. SPE for solid matrix. Method development for the solid matrix SPE included trialling 3 types of SPE adsorbents (Table 2). Initially Oasis HLB 60 mg cartridges were used in accordance with the liquid matrix methodology described above, with the sample eluent diluted to no more than 10% methanol.

MCX cartridges were also trialled using a method previously successful in non-chiral analysis of pharmaceuticals on suspended particulate matter [11], these are packed with a mixed-mode polymeric sorbent for extracting basic compounds with cation-exchange groups. In brief, cartridges were primed with 2% formic acid in water, and after extraction of cPACs from wastewater they were washed with 2% formic acid in water and then again with 0.6% formic acid in water prior to elution with 7% ammonium hydroxide in methanol.

Following this, Oasis MAX cartridges were trialled, using the same methodology as the HLB cartridges described above. These are packed with a mixed-mode polymeric sorbent for extracting acidic compounds with anion-exchange groups.

In all cases, the sample (wastewater or MAE extract) was deposited on the SPE cartridges at a rate of <6 mL min^{-1}, eluents were evaporated to dryness with a TurboVap evaporator (Caliper, UK, 40 °C, N_2, <5 psi) and reconstituted in 0.5 mL of mobile phase.

Table 1
MAE method development.

Parameter	Iterations trialled
Mass	1 g, 3 g
Solvent type	Methanol, water, methanol:water (1:1)
Solvent volume	10, 20, 50 mL
Temperature	90, 120, 150 °C
Exposure time	15, 30 min

All samples were filtered through 0.2 μm PTFE filters (Whatman, Puradisc, 13 mm) and transferred to 0.5 mL capacity polypropylene vials (Waters, UK).

2.3. LC–MS/MS

All samples were analysed using chiral HPLC performed on Waters ACQUITY UPLC® system (Waters, UK). The chiral separation was carried out with two columns: CBH, an enzyme based column packed with Cellobiohydrolase (100 × 2 mm, I.D. 5 μm, Sigma–Aldrich, UK) with a 0.2 μm, 2.1 mm in-line column filter, and Chirobiotic V (250 × 2.1 mm, I.D. 5 μm, Sigma–Aldrich, UK) packed with an antibiotic Vancomycin with a 20 × 1.0 mm, I.D. 5 μm guard column.

The CBH column is relatively restrictive regarding mobile phase composition e.g. no more than 20% organic modifier is recommended and pHs between 3 and 7 are allowed [36]. However, it shows the best chiral recognition towards amphetamine-related compounds. A successful method for the resolution of amphetamines has already been developed by Kasprzyk-Hordern and Baker [20] and it was utilised in this work without any further development.

Method development was carried out for the Chirobiotic V column. This column can be used in reverse or normal phase, however previous results [28] had elicited resolution of enantiomers in some of the target compounds with methanol, 0.005% formic acid and 4 mM ammonium acetate so this mobile phase composition was used as the basic method to improve upon, iterations trialled are included: proportion of water (0, 2.5, 5, 7.5, 10, 20%), ammonium acetate (1, 2, 4, 6, 8, 10, 20, 30 mM) and formic acid (0, 0.001, 0.0025, 0.005, 1 and 2%).

The best enantiomeric separation and sensitivity were observed in the following conditions: methanol, 4 mM ammonium acetate and 0.005% formic acid.

All analytes were identified and quantified using a Xevo TQD Triple Quadrupole Mass Spectrometer (Waters, UK), equipped with an electrospray ionisation source in positive ion mode. Nitrogen was used as the nebulising gas, supplied by a high purity nitrogen generator (Peak Scientific, UK). Argon (99.998%) was the collision gas supplied by a BOC cylinder. MassLynx 4.1 (Waters, UK) was used to control the Waters ACQUITY system and the Xevo TQD. Data processing was carried out using the TargetLynx software (Waters, UK). A valley drop method was used to integrate partially resolved peaks. The sensitivity was monitored whilst adjusting the nebulising gas flow rate to 100, 250 and 500 L h^{-1} as well as the source temperature between 90 and 250 °C to achieve the greatest sensitivity with the chosen mobile phase, each injection was 20 μL. Optimised MS parameters were as follows: the capillary voltage set at 3.49 kV, source temperature at 150 °C, desolvation gas flow at 300 L h^{-1}. Nitrogen was used as nebulising and desolvation gas, while argon was used as a collision gas.

2.4. Quantification and confirmation

Each compound was quantified in multiple reaction monitoring (MRM mode), using the protonated molecular ion as the precursor ion (Table 3). The most abundant product ion was used for quantification (in most cases), whilst confirmation was carried out using the lesser (second) abundant product ion. In addition the ratio of quantifier to confirmatory ion was used according to limits set by EC guidelines [37] i.e. ratios ≥50% ± 20%, >20–50% ± 25%, >10–20% ± 30% and ≤10% ± 50%. Deuterated surrogate/internal standards (SS/IS) were used to compensate for ion suppression/enhancement, loss during

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Table 2
SPE conditions trialled.

SPE sorbent	Eluting agent	pH	Comments
HLB	Methanol	7	High back pressure in LC column
MCX	Ammonium hydroxide	12	Poor chromatography
MAX	Methanol	7	No high back pressure, good chromatography

sample preparation and/or stereoselective mechanisms during sample preparation; where exact deuterated matches were not available, the closest match (both in retention time and structure) was substituted.

2.5. Validation of analytical method

2.5.1. Validation of instrumental parameters – LC–MS/MS method

Linearity of analytical methods was determined using a 15 point calibration curve for each enantiomer ranging from 0.025 $\mu g\,L^{-1}$ to 250 $\mu g\,L^{-1}$, injected three times. Linearity was accepted if the curve demonstrated $R^2 \geq 0.997$. Interday and intraday precision and accuracy was determined at 0.5 $\mu g\,L^{-1}$, 5 $\mu g\,L^{-1}$, 50 $\mu g\,L^{-1}$ and 250 $\mu g\,L^{-1}$, by repeated injection within 24 h and across three days, respectively. Acceptable tolerance limits were placed at the EC guideline levels [37] i.e. for concentrations 10–100 $\mu g\,L^{-1}$ the RSD $\leq$20%, for standards >100 $\mu g\,L^{-1}$ RSD $\leq$15%, and accuracy should not exceed −20% or +10% of the standard concentration. Instrumental detection limits ($IDL_{S/N}$) and instrumental quantification limits ($IQL_{S/N}$) were calculated using the signal to noise approach i.e. IDL was the lowest concentration at which the quantification ion S/N $\geq$ 3 assuming a linear response, IQL was the lowest concentration at which the quantification ion S/N $\geq$ 10 and the confirmatory ion S/N $\geq$ 3 assuming a linear response.

Resolution was calculated using Eq. (1), $R_s \geq 1$ was deemed adequate for quantification. Enantiomeric fraction (EF) was calculated using Eq. (2), EF accuracy was considered acceptable if ±0.05 of the true value. Retention times were recorded and relative retention times were also calculated (see Eq. (3)).

$$R_s = \frac{t_{r2} - t_{r1}}{0.5(w1 + w2)} \quad (1)$$

where R_s is the resolution of two enantiomers, t_{r2} is the retention time of the second eluting enantiomer, t_{r1} is the retention time of the first eluting enantiomer, $w1$ is the base width of the first eluting enantiomer, $w2$ is the base width of the second eluting enantiomer.

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

where EF is the enantiomeric fraction. When calculating relative enantiomeric fractions, $E1$ is the concentration of (+)-enantiomer or first eluted enantiomer, $E2$ is the concentration of (−)-enantiomer or second eluting enantiomer. Peak areas were also used instead of concentrations to calculate absolute enantiomer fractions (not adjusted with surrogate/standard).

$$t_{\mathrm{rrel}} = \frac{t_r}{\mathrm{IS}t_r} \quad (3)$$

where $t_{r\ \mathrm{rel}}$ is the relative retention time, t_r is the retention time recorded for the analyte, IS t_r is the retention time recorded for the internal standard.

2.5.2. Validation of analytical method – MAE-SPE-LC-MS/MS method

The method was validated at 5 $ng\,g^{-1}$, 50 $ng\,g^{-1}$ and 100 $ng\,g^{-1}$ of target analytes (each enantiomer) for digested sludge, and 5 $ng\,L^{-1}$, 50 $ng\,L^{-1}$ and 250 $ng\,L^{-1}$ in wastewater. At each concentration triplicate samples were either spiked before MAE, before SPE or after SPE to assess the impact of these steps on analyte recovery. This was carried out on 1 g and 3 g of matrix or 50 mL of wastewater. Sludge and wastewater samples were also run with only deuterated compounds to assess concentrations of target analytes in studied matrices.

In addition, the relative ion intensities were assessed according to the EC Directive guidelines [37] i.e. for relative intensities $\geq$50% ± 20%, >20% to 50% ± 25%, >10% to 20% ± 30%, $\leq$10% ± 50%. Resolution, enantiomeric fractions and relative retention times were calculated, using Eqs. (1)–(3), for three whole drug concentrations (10 $ng\,L^{-1}$, 100 $ng\,L^{-1}$, 500 $ng\,L^{-1}$ in the case of wastewater or 10 $ng\,g^{-1}$, 100 $ng\,g^{-1}$ or 200 $ng\,g^{-1}$ for sludge) spiked into wastewater influent, effluent and digested sludge. Method detection limits (MDLs) and method quantification limits (MQLs) were determined using Eqs. (4)–(7). The $IDL_{S/N}$, $IQL_{S/N}$ were used to generate MDL_{calc} and MQL_{calc}.

Table 3
MRM conditions.

Drug	CV	MRM (Q)	CE (Q)	MRM (I)	CE (I)	$I_{Q:I}$
Alprenolol	44	250.0 > 115.9	16	250.0 > 97.9	18	0.4
Amphetamine	18	136.2 > 91.1	16	136.2 > 119.1	8	1.0
Atenolol	38	266.9 > 145.0	30	266.9 > 190.1	16	1.2
Citalopram	46	325.1 > 262.1	18	325.1 > 109.9	26	0.9
Desmethylcitalopram	46	311.4 > 109.0	27	311.4 > 262.0	18	3.4
Desmethylvenlafaxine	32	264.2 > 106.9	34	264.2 > 133.0	26	0.5
Ephedrine/ pseudoephedrine	23	166.1 > 148.1	12	166.1 > 133.1	21	1.0
Fluoxetine	34	310.2 > 44.0	10	310.2 > 148.1	10	16.7
MDA	21	180.0 > 163.1	11	180.0 > 105.1	22	3.8
MDEA	18	209.0 > 164.0	12	209.0 > 164.0	20	4.5
MDMA	24	194.1 > 163.1	13	194.1 > 105.1	24	2.6
Methamphetamine	24	150.2 > 91.1	19	150.2 > 119.1	10	1.5
Metoprolol	42	268.3 > 116.1	20	268.3 > 121.1	22	2.6
Mexiletine	26	180.0 > 104.9	20	180.0 > 120.9	16	2.2
Mirtazapine	44	266.1 > 195.0	26	266.1 > 72.0	18	1.0
Norephedrine	23	152.2 > 134.1	10	152.2 > 117.1	16	1.0
Norfluoxetine	56	296.3 > 134.1	6			
Propranolol	40	260.0 > 183.3	18	260.0 > 116.8	16	136.3
Salbutamol	30	240.1 > 148.0	18	240.1 > 166.0	14	0.3
Sotalol	30	272.9 > 133.0	28	272.9 > 212.9	18	0.8
Terbutaline	66	226.1 > 76.9	32	226.1 > 152.0	24	2.4
Tramadol	28	264.0 > 58.0	45	264.0 > 120.7	46	10.6
Venlafaxine	27	278.2 > 260.1	12	278.2 > 121.0	32	1.6
Amphetamine-d5	18	141.0 > 92.9	16			
Atenolol-d7	44	274.3 > 145.1	30			
Citalopram-d6	46	331.0 > 109.0	28			
Fluoxetine-d5	26	315.2 > 136.2	20			
MDA-d5	21	185.1 > 168.1	11			
MDEA-d5	18	214.0 > 164.0	12			
MDMA-d5	24	199.1 > 165.1	13			
Methamphetamine-d5	24	155.0 > 121.0	11			
Metoprolol-d7	42	275.4 > 123.1	20			
Mirtazapine-d7	30	273.0 > 209.1	19			
Propranolol-d7	42	267.3 > 123.2	18			
Salbutamol-d9	51	249.5 > 231.0	12			
Sotalol-d6	32	279.0 > 134.2	36			

CV – cone voltage; CE – collision energy; $I_{Q:I}$ – ion ratio.

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$$\mathrm{MDL_{MAE\text{-}SPE\text{-}LC\text{-}MS/MS}} = \frac{\mathrm{S.IDL} \times 100}{\mathrm{Av.Rec} \times (\mathrm{CF} \times \text{discarded fraction})} \quad (4)$$

where S.IDL is the $IDL_{S/N}$ multiplied by the solvent volume used, in this case 30 mL, divided by the mass of matrix used, in this case 1 or 3 g. Av.Rec is the average absolute recovery for that compound. The concentration factor is the ratio between the initial volume (30 mL) and the final volume in mobile phase, in this case 0.5 (mL), therefore CF = 60. The discarded fraction is to compensate for taking a known volume post centrifugation; in this case 50% was discarded so the concentration factor is multiplied by 2.

$$\mathrm{MQL_{MAE\text{-}SPE\text{-}LC\text{-}MS/MS}} = \frac{\mathrm{S.IQL} \times 100}{\mathrm{Av.Rec} \times (\mathrm{CF} \times \text{discarded fraction})} \quad (5)$$

This equation is set out the same as Eq. (4), however the S.IQL is the $IQL_{S/N}$ multiplied by the solvent volume used, in this case 30 mL, divided by the mass of matrix used, in this case 1 or 3 g.

$$\mathrm{MDL_{SPE\text{-}LC\text{-}MS/MS}} = \frac{\mathrm{IDL} \times 100}{\mathrm{Av.Rec} \times \mathrm{CF}} \quad (6)$$

where Av. Rec is the average absolute recovery recorded and CF is the concentration factor between the sample taken and the final volume in mobile phase, in this case 50–0.5 mL so CF = 100.

$$\mathrm{MQL_{SPE\text{-}LC\text{-}MS/MS}} = \frac{\mathrm{IQL} \times 100}{\mathrm{Av.Rec} \times \mathrm{CF}} \quad (7)$$

This is set out the same as Eq. (6).

Analyte recovery was calculated using Eq. (8) by comparing the initial spike concentration with the measured concentration after extraction and analysis. Known concentrations of racemic standards were applied to the liquid matrices before SPE and also after SPE, whereas solid matrix samples were spiked before MAE and after MAE (before SPE) to identify the analyte recovery associated with each step of the process.

$$\mathrm{Rec_{MAE-SPE-LC-MS/MS}[\%]} = \left(\frac{\mathrm{PA_{SPE}} - \mathrm{PA_b}}{\mathrm{PA_c}}\right) \times 100 \quad (8)$$

where PA_{SPE} is the peak area of an analyte from the sample spiked before SPE, PA_b is the peak area of the (unspiked) blank sample, PA_c is the peak area from the quality control sample (at the same concentration of that which PA_{SPE} was spiked at). Alternatively, concentrations were also used, instead of peak areas, to calculate relative recoveries.

2.6. Analysis of wastewater samples

Grab samples were collected from the influent, effluent and digested sludge at the WWTP. The liquid grab samples were collected in a clean container and transferred to plastic bottles for transportation, on ice, and storage, at −20 °C. Digested sludge was collected in a plastic sampling bag, sealed and transported and stored under the same conditions as the liquid matrices. All the samples were collected at the same time and should not be interpreted as the same body of waste. The validated analytical procedures described in Section 3 and shown in Fig. 1, were applied to samples prepared in triplicate, which were then injected and analysed in triplicate.

3. Results and discussion

3.1. Sample preparation

3.1.1. Microwave assisted extraction for solid matrix

MAE was demonstrated to successfully extract the chosen analytes from complex matrices such as digested sludge. In brief, increased temperature did appear to improve recovery with complex matrices from an average recovery across all the compounds analysed of 22.5% at 90 °C for 30 min to 37.4% recovery at 120 °C. Although interestingly initial trials using silica as a

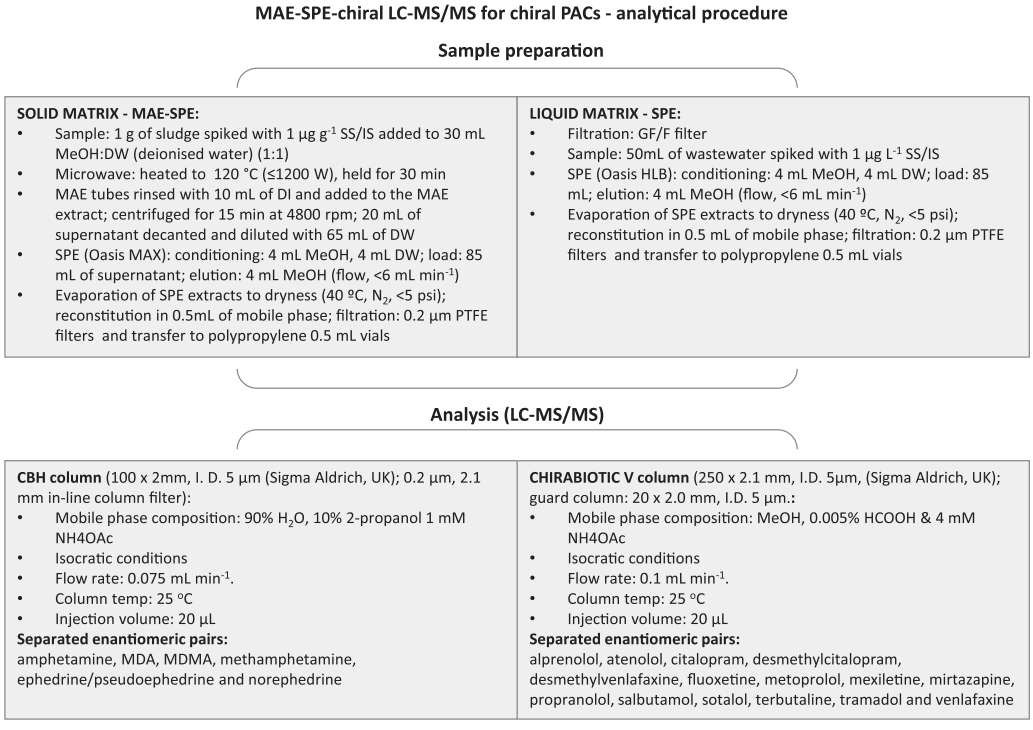

Fig. 1. MAE–SPE-chiral LC–MS/MS for chiral PACs – analytical procedure.

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matrix found lower temperatures performed better, 120 °C for 30 min resulted in 50.6% recovery with 90 °C resulting in 102%. With both matrices 150 °C resulted in lower recoveries for some compounds, with an average recovery of only 51% in silica, suggesting degradation was occurring at this temperature. For complex matrices 120 °C was the most successful temperature. At 120 °C, as well as lower temperatures, 30 min extraction time had improved recovery as compared to 15 min, 37% and 29%, respectively.

The mass of the sample did not appear to have a great effect on the recovery. In the case of analysis of samples where lower concentrations are expected, 3 g of matrix would be suggested to improve the MDL; however, 3 g of matrix is more difficult to process. After centrifugation a semi-solid hydrophobic layer was formed between the solid matrix and the solvent, whilst the dry matrix also absorbed the solvent; a larger mass of sample increased these challenges making it difficult to decant a known volume for SPE. Therefore, 1 g was preferred for this method. Compounds with lower log *P* values tended to be best extracted with 1:3 methanol:water mixture, amphetamine based compounds average recovery was 101% at 1:3 methanol:water and 91% at a ratio of 1:1; however, this had a significant effect on the recovery of more hydrophobic compounds, with recoveries of the anti-depressants falling from 80% to 34%. Therefore, a 1:1 mixture was settled upon as this had limited impact on the recovery of the more hydrophilic compounds. In general it was found the less solvent used the better the recovery, however, the difference was minimal (1 g with 10 mL had an average recovery of 30% and with 30 mL 27%) and less solvent made it considerably harder to cleanly remove the known volume for SPE without disturbing the matrix and/or removing the hydrophobic layer on top of it. It was decided that 30 mL of solvent was the lowest volume which could be used consistently with both 3 g and 1 g of matrix.

The final method is as follows (Fig. 1): replicates of digested sludge (1 g each), after being spiked with SS/IS (at concentration 1 $\mu g\,g^{-1}$), were placed into the MAE tubes with 15 mL of methanol and 15 mL deionised water and microwaved (CEM 1400 microwave, CEM, UK) between 0 W and 1200 W to allow for a controlled ramp to 120 °C, this was held for 30 min before allowing to cool to room temperature. The samples were transferred to a centrifuge, the MAE tubes were rinsed out with 10 mL of deionised water, which was added to the centrifuged containers. The samples were then centrifuged (VWR, Radnor, USA) for 15 min at 4800 rpm. A fixed volume (20 mL) of solvent was decanted and diluted with 65 mL of deionised water ready for SPE.

If analysis of individual chiral drugs was to be carried out, solvents, exposure time etc. could be tailored more to specific analytes, however, a compromise was made here to ensure extraction of the widest range of analytes possible.

3.1.2. Solid phase extraction

3.1.2.1. SPE for MAE extracts. The chromatography was successful when Oasis HLB cartridges were used after MAE (for solid matrix), i.e. resolution of enantiomers was sufficient for quantification (see Fig. S1a), however, the backpressure recorded in the column increased with each subsequent injection, (see Fig. S2). This was not observed in the case of liquid matrices where extraction with HLB was carried out. This back pressure increase suggested matrix clean-up by SPE using Oasis HLB cartridges had not been sufficient for MAE extracts and interfering compounds were retained on the chiral LC column causing the increased backpressure.

MCX sorbents were also found to be successful in removing the interfering compounds (from MAE extracts) which had been retained in the column, and therefore no increase in backpressure was observed, however, cPACs have to be eluted from MCX cartridges in methanol modified with ammonium hydroxide. Despite evaporation and re-elution in mobile phase the high pH resulted in very poor resolution for all the compounds as well as loss of chiral recognition (see Fig. S1b). This has been demonstrated not to be an issue for C_{18} stationary phase and non-chiral reversed phase chromatography [11], however, chiral columns are particularly sensitive to even small pH changes and the presence of charged molecules in the mobile phase impacts on both retention time and resolution of enantiomers.

MAX cartridges proved to be the most effective when coupled with chiral LC–MS. Despite low recoveries, good chromatographic separation of enantiomers was achieved and no build-up of back pressure was observed (see Fig. S1c).

The optimised method for the extraction of analytes from MAE extracts is therefore as follows (Fig. 1): 20 mL of MAE centrifuged supernatant, diluted with 65 mL of deionised water, was passed through Oasis MAX cartridge pre-conditioned with 4 mL of methanol followed by 4 mL of deionised water and eluted with 4 mL methanol.

3.1.2.2. SPE for liquid matrix. Oasis HLB cartridges provided successful extraction of analytes from wastewater. The method involved filtering 50 mL wastewater samples (GF/F, Whatman, UK) then spiking with SS/IS (1 $\mu g\,L^{-1}$). These were then passed through Oasis HLB cartridges, conditioned with 2 mL methanol, followed by 2 mL deionised water and eluted in 4 mL methanol (Fig. 1). However, it has to be remembered that this method, due to the use of GF/F filters, allows for the measurement of the analytes present in the liquid phase of wastewater and not any compounds adsorbed onto suspended particulate material (SPM). Previous analysis of SPM suggests that the amphetamine based compounds, venlafaxine and tramadol, are not significantly bound to SPM ($\leq$3.4% of the total concentration was found on SPM), however, fluoxetine was largely found on the SPM (59.1% of the total concentration) so further analysis of SPM is required to determine an accurate whole matrix concentration [11].

3.2. LC–MS/MS

3.2.1. Chiral LC–MS/MS with CBH column

The method for separation of enantiomers on the CBH column was used as reported elsewhere (Kasprzyk-Hordern and Baker [21]). 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 column was maintained at 25 °C and the injection volume was 20 μL (Fig. 1).

The following compounds were separated with this column: amphetamine, MDA, MDMA, methamphetamine, ephedrine/pseudoephedrine and norephedrine (Figs. S6–8).

3.2.2. Chiral LC–MS/MS with Chirobiotic V column

Increased water content in the mobile phase increased elution of the compounds, however, also decreased the signal to noise ratio and the enantiomeric resolution in most compounds, with the notable exception of norfluoxetine. Increased ammonium acetate concentration increased the retention times of most compounds and with up to 10 mM the resolution was also improved, however, no compounds which were previously below the quantification threshold (resolution $\geq$ 1) improved enough to become quantifiable. Retention times and resolution were best at 0.001% formic acid, but again none of the compounds whose resolution was too poor to quantify became quantifiable between 0.001 and 0.005%. There was no trend related to signal to noise for ammonium acetate and formic acid at these concentrations. Therefore, the final mobile phase composition chosen for the Chirobiotic V column

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Table 4
Performance data for LC–MS/MS method – sample diluent.

Column	Compound	Internal standard	R^2	t_r	$t_{r\ rel}$	R_s	EF_{abs}	EF_{rel}	Accuracy	Precision RSD (%)		Ion ratio	$IDL_{S/N}$ ($\mu g\,L^{-1}$)	$IQL_{S/N}$ ($\mu g\,L^{-1}$)
										Intra-day	Inter-day			
CBH	R(−)-amphetamine	R(−)-amphetamine-d5	0.998	24.4 ± 0.5	1.0 ± 0.0	2.1 ± 0.4	0.4 ± 0.0	0.5 ± 0.0	95.7 ± 6.4	2.6	3.3	94.2 ± 13.0	0.30	1.01
	S(+)-amphetamine	S(+)-amphetamine-d5	0.998	30.2 ± 0.5	1.0 ± 0.0				95.7 ± 5.9	3.2	3.3	90.4 ± 11.5	0.34	1.14
	1S,2R(+)-ephedrine	R(−)-methamphetamine-d5	0.997	21.1 ± 0.2	1.2 ± 0.0	1.8 ± 0.4	0.6 ± 0.2	0.5 ± 0.1	102.4 ± 3.9	4.0	3.8	9.2 ± 1.1	0.97	3.24
	1R,2S(−)-ephedrine	S(+)-methamphetamine-d5	0.997	24.0 ± 0.2	1.1 ± 0.0				102.9 ± 3.0	2.3	2.8	8.8 ± 2.6	1.83	6.11
	R(−)-MDA	R(−)-MDA-d5	0.999	39.8 ± 0.7	1.0 ± 0.0	4.5 ± 1.2	0.5 ± 0.2	0.5 ± 0.0	97.2 ± 4.8	1.3	1.8	26.7 ± 2.1	0.27	0.91
	S(+)-MDA	S(+)-MDA-d5	0.999	51.2 ± 0.7	1.0 ± 0.0				97.6 ± 4.6	2.0	2.5	26.4 ± 2.1	0.31	1.03
	R(−)-MDEA	R(−)-MDEA-d5	0.997	35.6 ± 0.8	1.0 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	94.6 ± 6.8	4.4	6.2	25.1 ± 5.0	0.25	0.83
	S(+)-MDEA	S(+)-MDEA-d5	0.997	37.5 ± 0.8	1.0 ± 0.0				94.9 ± 5.2	2.9	3.8	27.3 ± 11.7	0.25	0.82
	R(−)-MDMA	R(−)-MDMA-d5	0.998	35.1 ± 0.7	1.0 ± 0.0	1.7 ± 0.3	0.5 ± 0.1	0.5 ± 0.0	95.1 ± 4.5	2.0	2.2	34.9 ± 6.3	0.05	0.17
	S(+)-MDMA	S(+)-MDMA-d5	0.998	42.4 ± 0.8	1.0 ± 0.0				95.8 ± 4.3	2.2	2.5	34.8 ± 5.8	0.05	0.18
	R(−)-methamphetamine	R(−)-methamphetamine-d5	0.998	25.1 ± 0.6	1.0 ± 0.0	1.0 ± 0.1	0.5 ± 0.2	0.5 ± 0.0	97.5 ± 5.5	3.7	3.9	68.4 ± 7.2	0.10	0.32
	S(+)-methamphetamine	S(+)-methamphetamine-d5	0.998	28.1 ± 0.7	1.0 ± 0.0				97.5 ± 5.0	3.8	3.9	66.9 ± 5.9	0.11	0.35
	E1- norephedrine	R(−)-methamphetamine-d5	0.997	21.7 ± 0.2	1.2 ± 0.0	0.9 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	102.3 ± 3.4	3.9	3.3	4.6 ± 0.9	1.64	5.47
	E2- norephedrine	S(+)-methamphetamine-d5	0.997	23.4 ± 0.2	1.2 ± 0.0				102.7 ± 5.1	4.9	5.4	4.7 ± 1.2	1.63	5.42
	1R,2R(−)-pseudoephedrine	R(−)-methamphetamine-d5	0.997	22.4 ± 0.1	1.1 ± 0.0	2.8 ± 0.8	0.5 ± 0.1	0.5 ± 0.0	103.4 ± 5.2	4.1	5.3	9.4 ± 2.2	1.38	4.60
	1S,2S(+)-pseudoephedrine	R(−)-methamphetamine-d5	0.997	27.5 ± 0.3	1.0 ± 0.0				103.1 ± 4.3	4.4	4.3	8.5 ± 2.0	1.46	4.85
Chirobiotic V	S(−)-alprenolol	S(−)-metoprolol-d7	0.998	25.2 ± 0.3	0.9 ± 0.0	1.3 ± 0.4	0.5 ± 0.0	0.5 ± 0.0	93.6 ± 8.1	1.9	4.6	38.9 ± 13.2	0.02	0.07
	R(+)-alprenolol	R(+)-metoprolol-d7	0.997	27.3 ± 0.3	0.9 ± 0.0				91.1 ± 10.3	2.0	3.6	33.2 ± 7.7	0.03	0.09
	S(−)-atenolol	S(−)-atenolol-d7	0.999	44.3 ± 0.6	1.0 ± 0.0	1.4 ± 0.6	0.5 ± 0.0	0.5 ± 0.1	99.0 ± 3.3	2.4	3.3	49.8 ± 4.0	1.85	6.16
	R(+)-atenolol	R(+)-atenolol-d7	0.999	48.5 ± 0.7	1.0 ± 0.0				97.8 ± 3.9	3.4	4.1	48.9 ± 4.7	1.70	5.65
	S(+)-citalopram	S(+)-citalopram-d7	0.999	57.8 ± 1.1	0.9 ± 0.2	1.2 ± 0.3	0.5 ± 0.1	0.5 ± 0.1	98.0 ± 7.6	2.6	6.0	1.0 ± 1.0	0.09	4.57
	R(−)-citalopram	R(−)-citalopram-d7	0.998	62.9 ± 0.9	0.8 ± 0.2				98.4 ± 5.2	3.0	5.0	0.7 ± 0.6	0.11	4.83
	S(+)-desmethylcitalopram	S(+)-citalopram-d7	0.997	55.7 ± 0.5	0.8 ± 0.2	4.0 ± 1.5	0.5 ± 0.0	0.5 ± 0.0	101.1 ± 7.0	2.0	7.1	25.9 ± 4.6	0.12	0.41
	R(−)-desmethylcitalopram	R(−)-citalopram-d7	0.997	71.7 ± 0.8	1.0 ± 0.1				102.0 ± 7.3	1.8	5.8	23.9 ± 7.8	0.18	0.61
	R(−)-desmethylvenlafaxine	S(−)-propranolol-d7	0.998	28.7 ± 0.5	0.9 ± 0.0	1.4 ± 0.5	0.5 ± 0.1	0.7 ± 0.2	92.5 ± 12.2	1.9	10.4	44.7 ± 5.1	0.19	0.62
	S(+)-desmethylvenlafaxine	R(+)-propranolol-d7	0.999	32.1 ± 0.3	0.9 ± 0.0				101.1 ± 5.3	3.2	4.4	44.3 ± 7.2	0.23	0.78
	S(+)-fluoxetine	S(−)-propranolol-d7	0.999	39.8 ± 0.3	1.3 ± 0.0	1.2 ± 0.2	0.5 ± 0.0	0.5 ± 0.1	96.4 ± 7.7	2.4	7.5	5.6 ± 1.0	0.01	0.04
	R(−)-fluoxetine	R(+)-propranolol-d7	0.997	43.2 ± 0.3	1.2 ± 0.0				98.4 ± 7.4	2.2	7.7	5.6 ± 1.0	0.01	0.05
	S(−)-metoprolol	S(−)-metoprolol-d7	0.997	28.4 ± 0.3	1.0 ± 0.0	1.2 ± 0.3	0.5 ± 0.0	0.5 ± 0.0	99.6 ± 3.0	2.5	2.8	38.3 ± 3.8	0.01	0.03
	R(+)-metoprolol	R(+)-metoprolol-d7	0.999	31.3 ± 0.3	1.0 ± 0.0				99.0 ± 3.5	2.5	3.5	41.0 ± 9.1	0.01	0.04
	R(−)-mirtazapine	R(−)-mirtazapine-d7	0.998	18.5 ± 0.3	0.4 ± 0.0	1.9 ± 0.4	0.5 ± 0.1	0.6 ± 0.2	93.4 ± 7.5	1.9	3.0	85.9 ± 8.4	0.12	0.40
	S(+)-mirtazapine	S(+)-mirtazapine-d7	0.998	23.1 ± 0.4	0.5 ± 0.0				97.1 ± 4.7	5.5	4.9	88.9 ± 7.1	0.28	0.93
	S(+)-norfluoxetine	S(−)-propranolol-d7	0.958	32.4 ± 0.3	1.0 ± 0.0	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	124.5 ± 38.1	5.3	20.5		0.37	1.25
	R(−)-norfluoxetine	R(+)-propranolol-d7	0.992	33.4 ± 0.2	1.0 ± 0.0				107.9 ± 15.0	4.0	10.7		0.28	0.92
	S(−)-propranolol	S(−)-propranolol-d7	0.999	31.2 ± 0.2	1.0 ± 0.0	1.5 ± 0.7	0.5 ± 0.0	0.5 ± 0.1	98.8 ± 2.6	2.1	2.8	49.5 ± 12.2	0.01	0.05
	R(+)-propranolol	R(+)-propranolol-d7	0.999	34.8 ± 0.2	1.0 ± 0.0				98.5 ± 4.4	3.5	7.1	53.9 ± 12.4	0.02	0.06
	S(+)-salbutamol	S(−)-propranolol-d7	0.999	23.1 ± 0.3	0.7 ± 0.2	1.2 ± 0.4	0.5 ± 0.0	0.6 ± 0.2	100.4 ± 6.1	2.0	6.3	34.8 ± 2.3	0.13	0.45
	R(−)-salbutamol	R(+)-propranolol-d7	0.999	26.0 ± 0.3	0.7 ± 0.2				99.6 ± 5.5	3.7	5.6	35.4 ± 2.7	0.16	0.53
	E1-sotalol	E1-sotalol-d6	0.998	35.3 ± 0.3	1.0 ± 0.0	1.4 ± 0.3	0.5 ± 0.0	0.5 ± 0.0	97.3 ± 5.2	2.3	5.4	62.4 ± 6.2	0.16	0.53
	E2-sotalol	E2-sotalol-d6	0.998	39.6 ± 0.4	1.0 ± 0.0				96.0 ± 5.9	2.3	5.7	62.5 ± 6.3	0.16	0.53
	R(−)-terbutaline	R(+)-propranolol-d7	0.997	28.4 ± 0.2	0.9 ± 0.2	2.3 ± 2.1	0.5 ± 0.1	0.5 ± 0.2	113.4 ± 27.1	6.0	22.8	47.1 ± 15.4	1.35	4.51
	S(+)-terbutaline	S(−)-propranolol-d7	0.971	28.3 ± 0.4	0.9 ± 0.2				100.9 ± 20.7	6.9	21.5	45.5 ± 19.2	1.81	6.03
	E1-tramadol	R(+)-propranolol-d7	0.997	28.8 ± 0.4	0.9 ± 0.2	1.1 ± 0.2	0.8 ± 0.1	0.7 ± 0.2	97.3 ± 8.2	2.1	8.3	0.3 ± 0.1	0.02	0.06
	E2-tramadol	S(−)-propranolol-d7	0.997	31.8 ± 0.7	0.9 ± 0.2				103.8 ± 11.2	3.1	9.4	0.5 ± 0.4	0.09	0.28
	R(−)-venlafaxine	R(+)-propranolol-d7	0.997	31.6 ± 0.5	1.0 ± 0.1	1.1 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	98.1 ± 9.3	2.2	9.1	23.8 ± 3.2	0.01	0.03
	S(+)-venlafaxine	S(−)-propranolol-d7	0.997	35.2 ± 0.4	1.0 ± 0.0				99.6 ± 10.1	2.6	10.0	23.9 ± 3.7	0.01	0.04

Note: (1) performance parameters (linearity retention time, resolution, enantiomeric fractions and ion ratios) were calculated for average values for the calibration protocol as a whole, above the ID. Whole calibration is 63 injections in total (2) accuracy and precision were calculated for two concentrations 50 and 250 $\mu g\,L^{-1}$; elution orders shown have either been demonstrated by comparing single enantiomer t_r or assumed to be the same as in other published work, using a comparable mobile phase and same column type. These are: alprenolol [38], citalopram, desmethylcitalopram [39], venlafaxine, desmethylvenlafaxine [40], metoprolol [41], mirtazapine [42], salbutamol [43] and terbutaline [44]. Linearity range for individual compounds: IDL-250 $\mu g\,L^{-1}$.

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Table 5
Performance data for SPE-LC-MS/MS method - wastewater influent.

Compound	t_r	$t_{r\ rel}$	R_s	EF_{abs}	EF_{rel}	Precision RSD (%)		Ion ratio	Absolute SPE-LC-MS/MS recovery (%)	Relative SPE-LC-MS/MS recovery (%)	MDL_{calc} ($ng\,L^{-1}$)	MQL_{calc} ($ng\,L^{-1}$)
						Intra-day	Inter-day					
S(−)-alprenolol	24.4 ± 0.2	0.9 ± 0.0	0.9 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	8.08	6.1	37.6 ± 0.8	98.1 ± 20.6	159.3 ± 39.7	0.07	0.24
R(+)-alprenolol	26.4 ± 0.2	0.9 ± 0.0				10.9	14.6	39.6 ± 0.6	97.6 ± 19.4	198.9 ± 49.2	0.14	0.47
R(−)-amphetamine	22.2 ± 0.2	1.0 ± 0.0	1.1 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	2.4	5.5	90.5 ± 2.1	78.7 ± 11.9	107.8 ± 1.2	0.38	1.28
S(+)-amphetamine	25.4 ± 0.3	1.0 ± 0.0				2.3	3.7	90.4 ± 1.0	86.6 ± 13.7	111.1 ± 1.5	0.39	1.32
S(−)-atenolol	40.9 ± 0.4	1.0 ± 0.0	1.0 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	23.0	10.0	49.1 ± 2.4	48.7 ± 18.2	21.4 ± 18.6	17.40	58.00
R(+)-atenolol	44.5 ± 0.4	1.0 ± 0.0				50.8	74.6	48.8 ± 3.0	32.14 ± 12.1	19.5 ± 8.8	28.74	95.81
S(+)-citalopram	52.2 ± 0.9	0.9 ± 0.0	1.0 ± 0.1	0.8 ± 0.2	0.8 ± 0.2	9.0	9.7	1.0 ± 0.2	174.9 ± 49.2	91.9 ± 14.1	0.24	13.07
R(−)-citalopram	56.7 ± 1.1	0.9 ± 0.0				6.2	10.0	0.9 ± 0.2	176.4 ± 37.3	95.1 ± 11.4	0.31	13.69
S(+)-desmethylcitalopram	52.2 ± 0.9	0.9 ± 0.0	2.3 ± 0.2	0.5 ± 0.0	0.5 ± 0.0	5.5	5.4	26.9 ± 0.6	169.2 ± 28.1	99.7 ± 6.0	0.36	1.21
R(−)-desmethylcitalopram	56.7 ± 1.1	0.9 ± 0.0				9.1	10.7	27.4 ± 0.9	181.6 ± 29.5	102.4 ± 10.6	0.50	1.68
R(−)-desmethylvenlafaxine	27.6 ± 0.3	0.9 ± 0.0	1.0 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	10.6	9.1	42.7 ± 1.9	292.9 ± 179.2	142.6 ± 21.3	0.32	1.05
S(+)- desmethylvenlafaxine	30.0 ± 0.3	0.9 ± 0.0				5.9	8.5	43.1 ± 1.6	100.8 ± 19.4	99.2 ± 12.8	1.16	3.85
1S,2R(+)-ephedrine	18.5 ± 0.2	0.8 ± 0.0	20.0 ± 1.6	0.5 ± 0.1	0.5 ± 0.1	2.3	4.5	7.9 ± 2.7	61.4 ± 6.6	120.2 ± 21.0	0.16	0.02
1R,2S(−)-ephedrine	21.9 ± 0.7	0.9 ± 0.0				2.6	4.6	9.6 ± 1.5	80.3 ± 20.4	94.8 ± 6.9	0.23	0.01
S(+)-fluoxetine	37.9 ± 0.3	1.4 ± 0.0	1.0 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	9.3	9.8	5.8 ± 0.1	88.6 ± 13.7	120.4 ± 22.1	0.07	0.22
R(−)-fluoxetine	41.1 ± 0.1	1.4 ± 0.0				6.8	7.6	5.7 ± 0.1	89.9 ± 14.0	91.2 ± 13.3	0.08	0.26
R(−)-MDA	33.3 ± 0.4	1.0 ± 0.0	1.3 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	3.2	3.2	24.2 ± 0.6	80.8 ± 10.8	100.6 ± 2.1	0.33	1.13
S(+)-MDA	39.9 ± 0.4	1.0 ± 0.0				2.8	3.1	24.0 ± 0.5	86.3 ± 13.9	103.1 ± 3.1	0.36	1.19
R(−)-MDMA	31.6 ± 0.3	1.0 ± 0.0	1.0 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	1.5	1.8	29.0 ± 0.3	98.1 ± 14.7	97.4 ± 1.2	0.05	0.17
S(+)-MDMA	36.1 ± 0.3	1.0 ± 0.0				2.2	2.0	29.4 ± 0.5	101.9 ± 16.6	97.4 ± 2.1	0.05	0.18
R(−)-methamphetamine	23.1 ± 0.2	1.0 ± 0.0	0.7 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	1.9	2.6	69.3 ± 0.8	84.4 ± 9.6	106.2 ± 1.3	0.12	0.38
S(+)-methamphetamine	25.2 ± 0.3	1.0 ± 0.0				3.0	3.9	70.1 ± 1.0	85.3 ± 10.1	107.5 ± 1.7	0.13	0.41
S(−)-metoprolol	27.2 ± 0.2	1.0 ± 0.0	1.0 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	2.2	2.2	38.4 ± 0.6	85.6 ± 13.8	87.2 ± 4.7	0.06	0.18
R(+)-metoprolol	29.6 ± 0.2	1.0 ± 0.0				3.2	2.9	38.5 ± 0.9	77.6 ± 14.7	87.1 ± 5.4	0.08	0.27
R(−)-mirtazapine	16.8 ± 0.5	0.4 ± 0.0	1.6 ± 0.4	0.6 ± 0.0	0.5 ± 0.0	14.7	16.1	84.9 ± 3.2	57.3 ± 12.8	72.5 ± 14.6	0.40	1.32
S(+)-mirtazapine	21.3 ± 1.5	0.5 ± 0.0				11.2	15.1	83.8 ± 3.8	81.7 ± 14.1	81.5 ± 16.0	1.17	3.89
E1-norephedrine	18.7 ± 0.2	0.8 ± 0.0	7.5 ± 0.3	0.3 ± 0.1	0.5 ± 0.0	10.0	15.1	4.0 ± 0.3	50.3 ± 5.8	86.3 ± 8.7	0.33	0.01
E2-norephedrine	19.9 ± 0.2	0.8 ± 0.0				3.7	8.6	4.4 ± 0.5	108.6 ± 30.3	74.4 ± 33.2	0.15	0.02
S(−)-propranolol	29.8 ± 0.2	1.0 ± 0.0	1.1 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	4.6	4.7	52.3 ± 1.5	77.8 ± 16.3	109.4 ± 6.2	0.09	0.30
R(+)-propranolol	33.0 ± 0.3	1.0 ± 0.0				3.1	3.0	52.0 ± 1.4	110.9 ± 24.8	108.9 ± 5.4	0.08	0.26
1R,2R(−)-pseudoephedrine	19.9 ± 0.2	0.9 ± 0.0	22.7 ± 1.8	0.7 ± 0.1	0.6 ± 0.1	7.1	8.5	9.6 ± 1.5	52.3 ± 7.0	76.85 ± 10.2	0.26	0.01
1S,2S(+)-pseudoephedrine	22.9 ± 0.2	0.9 ± 0.0				5.1	9.2	12.3 ± 1.2	143.6 ± 63.6	56.6 ± 3.83	0.10	0.03
S(+)-salbutamol	22.1 ± 0.1	0.7 ± 0.0	1.2 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	8.7	10.1	36.9 ± 0.1	30.4 ± 9.0	44.5 ± 5.8	2.20	7.32
R(−)-salbutamol	25.0 ± 0.2	0.8 ± 0.0				12.4	14.7	38.1 ± 0.9	35.7 ± 11.1	51.4 ± 12.5	2.22	7.41
E1-sotalol	33.5 ± 0.3	1.0 ± 0.0	1.1 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	2.2	6.5	59.5 ± 2.5	120.4 ± 26.5	94.8 ± 7.9	0.66	2.20
E2-sotalol	37.4 ± 0.3	1.0 ± 0.0				7.6	4.4	56.9 ± 2.4	130.4 ± 30.8	94.3 ± 9.9	0.61	2.05
E1-tramadol	28.1 ± 0.3	0.9 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	8.9	12.7	0.3 ± 0.0	99.1 ± 28.0	138.8 ± 34.1	0.09	0.29
E2-tramadol	30.0 ± 0.3	0.9 ± 0.0				6.8	11.5	0.4 ± 0.0	99.7 ± 16.7	102.4 ± 15.5	0.43	1.43
R(−)-venlafaxine	30.1 ± 0.6	1.0 ± 0.0	1.1 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	4.6	8.2	23.1 ± 1.1	99.7 ± 64.4	148.3 ± 36.3	0.03	0.11
S(+)-venlafaxine	33.1 ± 0.6	1.0 ± 0.0				2.7	8.5	22.5 ± 1.6	108.2 ± 69.8	112.4 ± 37.3	0.04	0.12

Note: all performance parameters (retention time, resolution, accuracy, precision, ion ratios and SPE recoveries) were calculated for two concentrations 50 and 250 $ng\,L^{-1}$ which were prepared in triplicate, $n = 6$.

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Table 6
Performance data for SPE-LC–MS/MS method – wastewater effluent.

Compound	t_r	$t_{r\ rel}$	R_s	EF_{abs}	EF_{rel}	Precision RSD (%)		Ion ratio	Absolute SPE-LC-MS/MS recovery (%)	Relative SPE-LC-MS/MS recovery (%)	MDL_{calc} ($ng\,L^{-1}$)	MQL_{calc} ($ng\,L^{-1}$)
						Intra-day	Inter-day					
S(−)-alprenolol	24.9 ± 0.1	0.9 ± 0.0	1.0 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	6.8	6.0	37.5 ± 0.5	252.4 ± 32.5	187.3 ± 31.1	0.03	0.09
R(+)-alprenolol	27.2 ± 0.1	0.9 ± 0.0				9.1	8.9	39.6 ± 0.6	222.0 ± 29.9	170.3 ± 30.8	0.06	0.21
R(−)-amphetamine	25.3 ± 2.6	1.0 ± 0.0	1.1 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	4.7	6.8	89.3 ± 2.2	107.4 ± 35.3	100.7 ± 29.2	0.28	0.94
S(+)-amphetamine	28.9 ± 3.2	1.0 ± 0.0				3.1	12.8	89.7 ± 2.3	83.7 ± 17.9	112.6 ± 5.7	0.41	1.36
S(−)-atenolol	42.1 ± 0.1	1.0 ± 0.0	1.0 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	27.6	27.3	45.8 ± 2.3	25.9 ± 17.9	55.1 ± 21.4	32.73	109.08
R(+)-atenolol	45.9 ± 0.1	1.0 ± 0.0				28.2	26.7	45.6 ± 2.6	30.0 ± 16.1	54.8 ± 24.6	30.80	102.68
S(+)-citalopram	53.3 ± 0.3	0.9 ± 0.0	1.0 ± 0.1	0.8 ± 0.0	0.7 ± 0.1	8.2	7.9	1.0 ± 0.1	205.0 ± 42.7	82.3 ± 20.4	0.21	11.15
R(−)-citalopram	58.0 ± 0.5	0.9 ± 0.0				8.7	8.0	0.9 ± 0.1	205.1 ± 38.5	76.9 ± 17.7	0.27	11.78
S(+)-desmethylcitalopram	53.3 ± 0.3	0.9 ± 0.0	2.4 ± 0.3	0.5 ± 0.0	0.5 ± 0.0	8.7	8.4	27.3 ± 0.4	212.1 ± 33.3	73.2 ± 18.2	0.29	0.96
R(−)-desmethylcitalopram	58.0 ± 0.5	0.9 ± 0.0				7.7	7.7	26.9 ± 0.7	228.4 ± 30.3	77.3 ± 19.3	0.40	1.34
R(−)-desmethylvenlafaxine	28.2 ± 0.1	0.9 ± 0.0	1.0 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	25.5	23.2	41.3 ± 1.3	240.6 ± 35.2	124.1 ± 21.5	0.38	1.28
S(+)-desmethylvenlafaxine	30.7 ± 0.1	0.9 ± 0.0				7.2	6.6	41.3 ± 1.1	169.2 ± 19.7	127.0 ± 9.3	0.08	2.30
1S,2R(+)-ephedrine	22.2 ± 0.3	0.8 ± 0.0	26.3 ± 1.2	0.5 ± 0.0	0.6 ± 0.0	5.5	16.7	10.5 ± 0.8	132.2 ± 14.6	120.1 ± 21.0	0.07	0.25
1R,2S(−)-ephedrine	25.8 ± 0.3	0.9 ± 0.0				1.6	6.9	11.3 ± 0.5	127.4 ± 13.4	94.8 ± 6.9	0.14	0.48
S(+)-fluoxetine	39.1 ± 0.1	1.4 ± 0.0	1.0 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	25.0	22.7	5.8 ± 0.1	139.2 ± 19.8	108.7 ± 22.6	0.04	0.14
R(−)-fluoxetine	42.3 ± 0.1	1.4 ± 0.0				7.2	6.9	5.8 ± 0.1	141.4 ± 222.7	108.3 ± 16.2	0.05	0.17
R(−)-MDA	38.5 ± 4.0	1.0 ± 0.0	1.2 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	3.7	9.1	21.4 ± 5.8	126.3 ± 39.4	89.8 ± 18.1	0.21	0.72
S(+)-MDA	44.7 ± 5.5	1.0 ± 0.0				3.9	8.7	23.1 ± 2.4	123.5 ± 39.2	96.9 ± 27.9	0.25	0.83
R(−)-MDMA	36.4 ± 4.0	1.0 ± 0.0	0.9 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	2.2	7.2	27.7 ± 1.5	134.7 ± 42.1	89.2 ± 18.6	0.04	0.13
S(+)-MDMA	41.0 ± 4.7	1.0 ± 0.0				2.8	6.2	27.2 ± 1.8	124.3 ± 39.2	99.2 ± 39.1	0.04	0.14
R(−)-methamphetamine	26.6 ± 2.8	1.0 ± 0.0	1.0 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	5.7	10.4	67.9 ± 2.0	114.4 ± 37.3	102.9 ± 15.1	0.09	0.28
S(+)-methamphetamine	29.0 ± 3.0	1.0 ± 0.0				3.7	10.9	68.4 ± 1.8	130.8 ± 44.8	109.3 ± 6.1	0.08	0.27
S(−)-metoprolol	27.9 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	7.7	6.4	37.9 ± 0.9	131.2 ± 16.3	87.2 ± 4.7	0.04	0.12
R(+)-metoprolol	30.4 ± 0.1	1.0 ± 0.0				7.7	6.5	38.0 ± 0.9	138.1 ± 15.1	86.2 ± 7.0	0.05	0.15
R(−)-mirtazapine	16.7 ± 0.1	0.5 ± 0.0	1.4 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	8.9	9.5	80.4 ± 3.1	150.4 ± 25.7	105.8 ± 24.1	0.40	1.32
S(+)-mirtazapine	20.6 ± 0.2	0.5 ± 0.0				8.2	8.3	81.5 ± 3.7	163.4 ± 30.3	120.1 ± 17.2	0.86	2.86
E1-norephedrine	22.4 ± 0.3	0.8 ± 0.0	10.1 ± 1.3	0.6 ± 0.1	0.6 ± 0.1	3.3	26.1	21.2 ± 0.8	87.2 ± 17.1	86.3 ± 8.7	0.19	0.63
E2-norephedrine	23.7 ± 0.3	0.8 ± 0.0				2.3	59.4	17.5 ± 11.1	76.2 ± 12.0	74.4 ± 33.2	0.21	0.71
S(−)-propranolol	30.7 ± 0.1	1.0 ± 0.0	1.1 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	9.1	7.7	53.2 ± 1.0	136.2 ± 15.1	108.8 ± 9.9	0.05	0.17
R(+)-propranolol	33.0 ± 0.3	1.0 ± 0.0				6.9	5.7	53.5 ± 1.0	147.1 ± 17.2	110.6 ± 8.1	0.06	0.20
1R,2R(−)-pseudoephedrine	23.8 ± 0.2	0.9 ± 0.0	25.2 ± 2.3	0.4 ± 0.0	0.4 ± 0.0	3.5	14.0	11.0 ± 0.3	89.2 ± 7.0	76.9 ± 10.2	0.15	0.52
1S,2S(+)-pseudoephedrine	27.2 ± 0.3	0.9 ± 0.0				2.8	14.0	9.5 ± 1.8	133.0 ± 46.2	56.6 ± 3.8	0.11	0.36
S(+)-salbutamol	22.8 ± 0.1	0.7 ± 0.0	1.2 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	5.7	6.0	37.7 ± 0.9	68.4 ± 12.2	46.9 ± 20.9	0.98	3.26
R(−)-salbutamol	25.6 ± 0.1	0.8 ± 0.0				5.6	5.0	38.1 ± 0.9	60.7 ± 13.9	64.2 ± 13.6	1.31	4.36
E1-sotalol	34.5 ± 0.1	1.0 ± 0.0	1.2 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	8.2	8.6	56.2 ± 2.0	151.1 ± 21.9	94.8 ± 8.1	0.53	1.76
E2-sotalol	38.5 ± 0.1	1.0 ± 0.0				8.4	8.4	56.9 ± 2.4	174.1 ± 24.6	94.3 ± 10.9	0.46	1.53
E1-tramadol	28.7 ± 0.1	0.9 ± 0.0	0.8 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	8.3	8.3	0.3 ± 0.0	172.9 ± 26.0	136.0 ± 29.0	0.05	0.16
E2-tramadol	30.7 ± 0.1	0.9 ± 0.0				7.4	9.0	0.4 ± 0.0	179.6 ± 21.3	142.0 ± 14.3	0.24	0.79
R(−)-venlafaxine	30.4 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	8.3	9.2	21.6 ± 1.0	220.4 ± 20.5	132.1 ± 22.8	0.02	0.07
S(+)-venlafaxine	33.3 ± 0.2	1.0 ± 0.0				8.8	11.3	21.6 ± 1.0	177.7 ± 29.8	117.6 ± 43.1	0.03	0.11

Note: all performance parameters (retention time, resolution, accuracy, precision, ion ratios and SPE recoveries) were calculated for two concentrations 50 and 250 $ng\,L^{-1}$ which were prepared in triplicate, $n = 6$.

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Table 7
Performance data for MAE-SPE-LC-MS/MS method – digested sludge (1 g).

Compound	t_r	$t_{r\ rel}$	R_s	EF_{abs}	EF_{rel}	Precision RSD (%)		Ion ratio	Absolute MAE-SPE-LC-MS/MS recovery (%)	Relative MAE-SPE-LC-MS/MS recovery (%)	MDL_{calc} ($ng\,g^{-1}$)	MQL_{calc} ($ng\,g^{-1}$)
						Intra-day	Inter-day					
S(−)-alprenolol	28.4 ± 0.1	0.9 ± 0.0	1.3 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	15.2	15.9	37.9 ± 1.9	15.0 ± 0.9	82.7 ± 10.2	0.14	0.46
R(+)-alprenolol	31.1 ± 0.2	0.9 ± 0.0				10.2	22.5	67.9 ± 3.2	19.3 ± 1.8	85.0 ± 2.2	0.14	0.48
R(−)-amphetamine	26.5 ± 0.4	1.0 ± 0.0	2.9 ± 0.3	0.5 ± 0.0	0.5 ± 0.0	13.2	11.6	88.1 ± 5.1	6.1 ± 3.1	77.1 ± 10.2	4.92	16.56
S(+)-amphetamine	30.4 ± 0.5	1.0 ± 0.0				15.2	13.1	89.4 ± 6.9	6.6 ± 1.9	75.5 ± 10.0	5.15	17.28
S(−)-atenolol	44.0 ± 7.8	1.0 ± 0.2	1.4 ± 0.3	0.5 ± 0.1	0.5 ± 0.1	16.3	35.0	0.4 ± 0.2	26.0 ± 9.2	25.8 ± 19.1	7.12	23.70
R(+)-atenolol	48.2 ± 8.6	1.0 ± 0.2				32.7	45.9	2.6 ± 1.0	24.5 ± 10.1	18.6 ± 11.5	7.55	25.15
S(+)-citalopram	71.1 ± 0.9	1.0 ± 0.0	1.1 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	10.2	24.8	1.3 ± 0.2	97.5 ± 21.9	137.6 ± 79.7	0.09	4.69
R(−)-citalopram	75.0 ± 5.3	1.0 ± 0.0				8.4	22.1	1.4 ± 0.1	53.2 ± 14.1	98.0 ± 54.8	0.21	9.09
S(+)-desmethylcitalopram	61.5 ± 0.5	0.8 ± 0.0	2.6 ± 0.1	0.5 ± 0.2	0.6 ± 0.1	8.3	25.2	3.9 ± 0.1	33.5 ± 7.8	55.6 ± 36.0	0.36	1.22
R(−)-desmethylcitalopram	76.4 ± 0.7	0.9 ± 0.0				6.2	7.2	3.8 ± 0.2	30.6 ± 14.5	26.2 ± 2.0	0.42	1.99
R(−)-desmethylvenlafaxine	34.4 ± 0.3	1.0 ± 0.0	0.9 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	10.3	35.7	41.4 ± 1.6	24.6 ± 3.8	114.5 ± 34.0	0.75	2.51
S(+)-desmethylvenlafaxine	36.8 ± 0.3	0.9 ± 0.0				11.2	14.5	40.8 ± 2.6	22.8 ± 3.0	108.5 ± 24.9	1.02	3.41
1S,2R(+)-ephedrine	27.7 ± 0.0	0.8 ± 0.0	2.6 ± 0.3	0.8 ± 0.1	0.7 ± 0.1	32.9	30.7	9.5 ± 2.1	–	–	–	–
1R,2S(−)-ephedrine	30.5 ± 0.0	0.9 ± 0.0				14.3	72.3	15.3 ± 3.3	–	–	–	–
S(+)-fluoxetine	45.4 ± 0.3	1.3 ± 0.0	1.2 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	10.3	19.3	5.8 ± 0.1	17.1 ± 3.6	9.0 ± 2.6	0.07	0.23
R(−)-fluoxetine	49.4 ± 0.4	1.2 ± 0.0				9.6	32.8	5.7 ± 0.4	15.7 ± 3.1	47.9 ± 8.5	0.09	0.30
R(−)-MDA	40.0 ± 0.7	1.0 ± 0.0	4.2 ± 0.3	0.5 ± 0.0	0.5 ± 0.0	10.8	10.5	26.3 ± 1.7	12.2 ± 3.6	67.2 ± 7.0	2.21	7.46
S(+)-MDA	47.7 ± 0.9	1.0 ± 0.0				8.1	7.7	25.4 ± 2.6	7.5 ± 3.1	68.3 ± 5.6	4.14	13.74
R(−)-MDMA	38.3 ± 0.7	1.0 ± 0.0	1.2 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	5.7	7.2	30.6 ± 2.5	21.7 ± 5.9	71.6 ± 3.9	1.43	4.75
S(+)-MDMA	43.7 ± 0.8	1.0 ± 0.0				9.2	8.2	30.9 ± 2.5	17.3 ± 6.7	71.2 ± 6.4	1.79	5.96
R(−)-methamphetamine	28.0 ± 0.5	1.0 ± 0.0	1.9 ± 0.2	0.5 ± 0.0	0.5 ± 0.0	6.0	5.1	70.0 ± 1.4	13.7 ± 4.3	84.4 ± 4.8	0.73	2.34
S(+)-methamphetamine	30.9 ± 0.5	1.0 ± 0.0				7.8	6.7	68.9 ± 1.7	14.3 ± 4.4	83.7 ± 6.2	0.77	2.45
S(−)-metoprolol	31.9 ± 0.2	1.0 ± 0.0	1.2 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	10.9	17.6	37.7 ± 1.1	14.3 ± 1.1	103.0 ± 14.1	0.07	0.22
R(+)-metoprolol	35.1 ± 0.2	1.0 ± 0.0				10.7	17.8	38.3 ± 0.7	27.1 ± 2.5	105.7 ± 15.6	0.05	0.15
R(−)-mirtazapine	25.2 ± 0.4	0.7 ± 0.0	2.7 ± 0.2	0.5 ± 0.0	0.3 ± 0.0	11.4	17.8	83.5 ± 1.3	39.1 ± 6.5	243.3 ± 115.4	0.31	1.02
S(+)-mirtazapine	34.1 ± 0.7	0.9 ± 0.0				4.3	35.2	84.5 ± 5.0	38.4 ± 4.5	172.9 ± 73.0	0.73	2.44
E1-norephedrine	27.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	33.4	28.2	25.6 ± 2.3	17.2 ± 16.4	119.9 ± 103.9	9.54	31.52
E2-norephedrine	30.5 ± 0.0	0.8 ± 0.0				32.2	27.4	23.5 ± 3.2	12.5 ± 12.7	86.6 ± 79.3	13.05	43.38
S(−)-propranolol	35.1 ± 0.3	1.0 ± 0.0	1.4 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	9.1	23.1	53.5 ± 0.6	23.6 ± 3.1	93.5 ± 8.7	0.06	0.20
R(+)-propranolol	39.5 ± 0.2	1.0 ± 0.0				5.8	20.5	56.2 ± 0.8	25.8 ± 4.7	105.0 ± 9.2	0.07	0.23
1R,2R(−)-pseudoephedrine	27.7 ± 0.0	0.9 ± 0.0	1.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	23.6	22.4	13.3 ± 1.6	9.4 ± 4.2	94.0 ± 18.4	15.54	51.62
1S,2S(+)-pseudoephedrine	30.5 ± 0.0	0.9 ± 0.0				12.1	24.7	13.8 ± 2.9	3.1 ± 0.6	44.1 ± 5.4	44.53	148.5
S(+)-salbutamol	25.6 ± 0.2	0.7 ± 0.0	1.9 ± 0.2	0.5 ± 0.0	0.6 ± 0.1	20.5	51.7	37.1 ± 5.3	0.2 ± 0.0	0.8 ± 0.2	65.03	225.1
R(−)-salbutamol	29.0 ± 0.2	0.7 ± 0.0				45.1	47.7	41.7 ± 6.2	0.2 ± 0.0	0.5 ± 0.2	80.03	265.1
E1-sotalol	40.0 ± 0.3	1.0 ± 0.0	1.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.2	8.1	28.6	58.6 ± 2.9	9.7 ± 2.7	122.0 ± 11.4	1.64	5.47
E2-sotalol	44.7 ± 0.3	1.0 ± 0.0				9.4	34.2	55.9 ± 2.1	9.1 ± 2.7	142.9 ± 20.2	0.76	5.87
E1-tramadol	34.7 ± 0.3	1.0 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	12.9	41.7	0.3 ± 0.0	31.6 ± 10.4	209.3 ± 41.1	0.05	0.18
E2-tramadol	36.8 ± 0.4	0.9 ± 0.0				13.3	21.9	0.4 ± 0.1	25.3 ± 2.9	323.7 ± 47.0	0.34	1.13
R(−)-venlafaxine	37.6 ± 0.4	1.1 ± 0.0	1.0 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	14.4	24.5	20.5 ± 1.0	37.5 ± 4.7	137.0 ± 36.0	0.03	0.08
S(+)-venlafaxine	40.5 ± 0.4	1.0 ± 0.0				14.3	22.1	20.1 ± 0.9	48.5 ± 7.1	183.0 ± 37.3	0.03	0.08

Note: all performance parameters (retention time, resolution, accuracy, precision, ion ratios and MAE recoveries) were calculated for two concentrations 50 and 100 $ng\,g^{-1}$ which were prepared in triplicate, $n = 6$.

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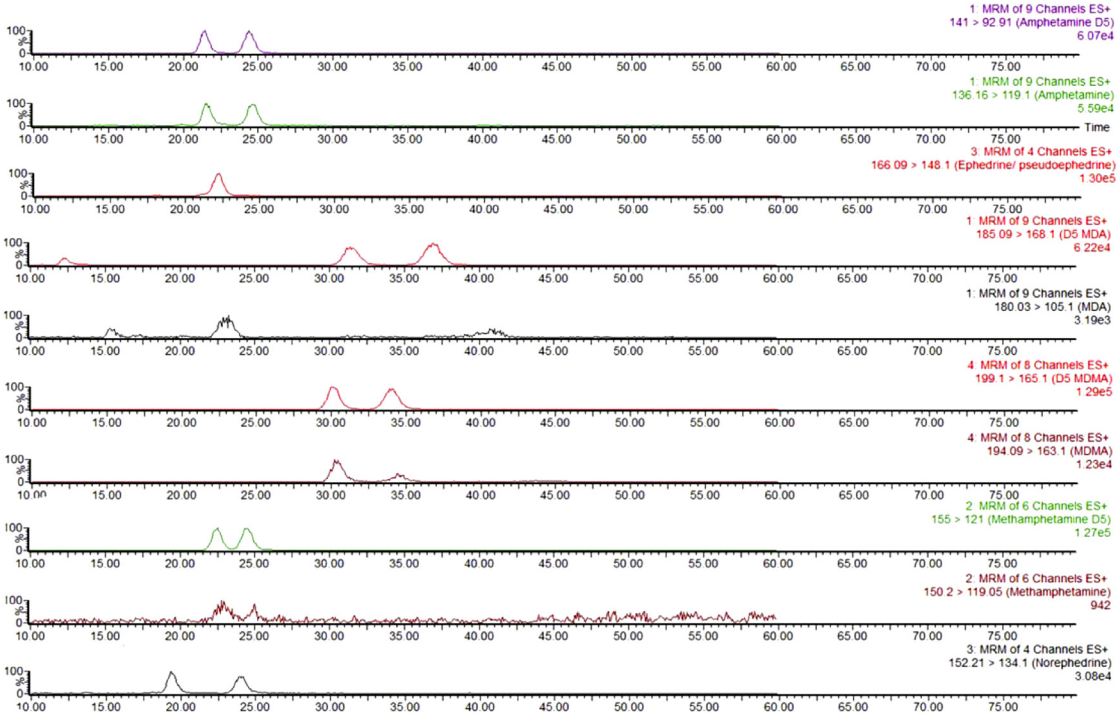

Fig. 2. Mass chromatograms of chiral drugs present in wastewater influent analysed with CBH column.

was 100% methanol, 0.005% formic acid and 4 mM ammonium acetate.

In general the method development results highlight how longer retention times can produce better resolution, however, these two factors must be balanced in order to maintain a practical analytical technique, the current method for the target analytes is 80 min long. Although many iterations of additives and mobile phases were trialled, and trends were noted, none of the compounds previously unsuccessfully resolved with methanol, 0.005% formic acid and 4 mM ammonium acetate could be brought to above the relevant quantification thresholds and the method therefore remained unchanged.

The optimised chromatographic conditions used for separation of enantiomers in Chirobiotic V utilised a mobile phase composed of methanol, 0.005% formic acid and 4 mM ammonium acetate. Separations were undertaken under isocratic conditions and a flow rate of 0.1 mL min^{-1}. The column temperature was 25 °C and the injection volume was 20 μL (Fig. 1).

The following compounds were separated with this column: alprenolol, atenolol, citalopram, desmethylcitalopram, desmethylvenlafaxine, fluoxetine, metoprolol, mexiletine, mirtazapine, propranolol, salbutamol, sotalol, terbutaline, tramadol and venlafaxine (Figs. S3–5).

3.3. Validation of analytical methods

3.3.1. Validation of instrumental parameters

Results of the calibration curves are detailed in Table 4. Good linearity of response ($R^2 \geq 0.997$) within the studied concentration range was achieved for most compounds with the exception of terbutaline and norfluoxetine. These analytes consistently fell outside of tolerance limits set and will therefore not be considered further (results are included in the relevant table for illustrative purposes only).

Resolution ($R_s \geq 1.00$) was also achieved for all the enantiomers except MDEA ($R_s = 0.59–0.65$), norephedrine (0.81–0.94) and norfluoxetine (0.83–0.87). For a few compounds, including methamphetamine, desmethylvenlafaxine, fluoxetine and citalopram, higher concentrations, resulting in larger peak areas, resulted in reduced resolution, however, this effect is considered to be negligible at environmentally relevant concentrations. Absolute EF values were usually within the range of 0.45–0.55 with the exception of amphetamine, ephedrine, norfluoxetine and tramadol, however, all of these were brought to within this range when average relative EF values were calculated, with the exception of tramadol. However, several compounds' relative enantiomeric fractions were not between 0.45 and 0.55, although their absolute enantiomeric fractions were. These include salbutamol, mirtazapine and desmethylvenlafaxine. Accuracy was good throughout and both inter- and intra-day precision was generally <10%. The ion ratio standard deviations were consistently well within tolerance limits set for all compounds. IDLs were usually between 0.01 and 1.83 μg L^{-1} and IQLs between 0.03 and 1.16 μg L^{-1}.

3.3.2. Validation of analytical methods

Almost all of the compounds were successfully calibrated, passing all of the criteria set out in Section 2.5, only exceptions were MDEA, terbutaline and norfluoxetine which were therefore not carried forward to validation. These three compounds all suffer from poor chromatographic resolution. Terbutaline co-eluted with another similarly structured compound resulting in multiple, overlapping peaks which impacted identification and quantification. MDEA and norfluoxetine did not achieve adequate resolution. During method development it was known that norfluoxetine requires 10% water in the mobile phase to achieve good enantiomeric resolution, however, this had significant impacts on the resolution and sensitivity of many other compounds, therefore, this compound could not be analysed alongside the other compounds resolved by the Chirobiotic V.

3.3.2.1. SPE-chiral LC–MS/MS. Results for the validation of the SPE-chiral LC–MS/MS method in wastewater influent and effluent can be found in Tables 5 and 6, respectively. In brief, the retention time

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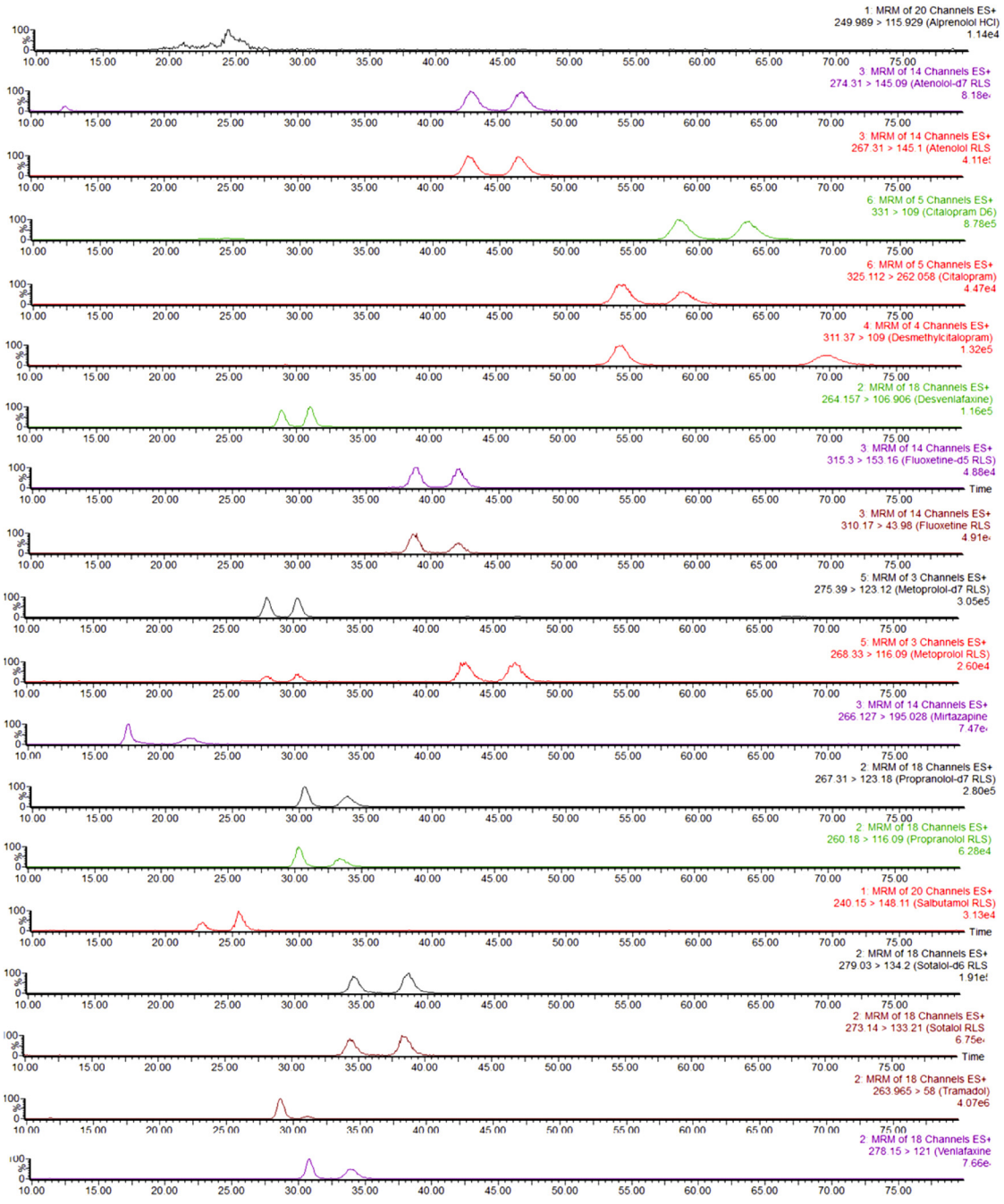

Fig. 3. Mass chromatograms of chiral drugs in wastewater influent analysed with Chirobiotic V column.

for all the compounds from the liquid matrices is not impacted by more than 4 min compared to sample diluent, except in the cases of citalopram, desmethylcitalpram and MDA in influent and effluent as well as S(+)-MDMA and S(+)-amphetamine in influent only. Changes in retention times were compensated for by using internal standards (see relative retention times in Tables 5 and 6).

Enantiomeric resolution was maintained above 1 for all compounds at the three studied concentrations in wastewater influent and effluent except alprenolol ($R_s = 0.9$), MDMA ($R_s = 0.9$), tramadol ($R_s = 0.8$) and methamphetamine ($R_s = 0.7$). In order to undertake quantification of enantiomers, their resolution has to be ≥ 1. Therefore, compounds having $R_s < 1$ were treated on a semi-quantitative basis.

Several absolute EFs deviated from 0.5, however, their relative EF values were close to 0.5, with the exception of citalopram (EF = 0.8), tramadol (0.6), desmethylvenlafaxine (0.4) in influent, as well as citalopram (0.7) and pseudoephedrine (0.4) in effluent. In addition, although their absolute EF values were close to 0.5, desmethylvenlafaxine (0.4) and fluoxetine (0.6) EF values deviated from racemic in influent and ephedrine (0.6) and tramadol (0.4) in effluent. No deuterated analogues were available for these compounds and this result suggests that although there is no stereoselective degradation of the target analytes stereoselective matrix effects do appear to have occurred in the internal standard and therefore impacted the relative enantiomeric fraction. Interday and intraday precision for both matrices was high, ranging from 1.5% (MDMA) to 74.6% (atenolol), although most were less than 10%. Relative ion intensities were consistently within tolerance levels for both wastewater influent and effluent.

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Table 8
Wastewater analysis.

Compound	Influent		Effluent		Digested sludge	
	Concentration (ng L^{-1})	EF	Concentration (ng L^{-1})	EF	Concentration (ng g^{-1})	EF
Alprenolol	220.7 ± 2.1[b]	0.5 ± 0.0[b]	202.0 ± 5.7[b]	0.5 ± 0.0[b]	19.8 ± 6.1	0.7 ± 0.2
Amphetamine	280.3 ± 4.1	0.5 ± 0.0	22.0 ± 0.0	0.6 ± 0.0	7.9 ± 1.7	0.3 ± 0.1
Atenolol	447.0 ± 18.3	0.5 ± 0.0	76.5 ± 4.9	0.5 ± 0.0	2.4 ± 1.2	0.4 ± 0.0
Citalopram	650.0 ± 50.0	0.6 ± 0.0	53.0 ± 3.7	0.7 ± 0.0	121.0 ± 60.2	0.6 ± 0.0
Desmethylcitalopram	2.3 ± 0.9	1.0 ± 0.0	<MDL	–	167.0 ± 29.0[b]	0.6 ± 0.1[b]
Desmethylvenlafaxine	706.7 ± 18.6	0.5 ± 0.1	288.7 ± 11.4	0.5 ± 0.0	18.4 ± 6.4	0.5 ± 0.0
Ephedrine	20.2 ± 28.6	0.0 ± 0.0	1.3 ± 0.2	0.0 ± 0.0	<MDL[b]	–
Fluoxetine	51.0 ± 4.3	0.7 ± 0.0	26.0 ± 0.0	0.7 ± 0.0	85.6 ± 9.4[b]	0.7 ± 0.1[b]
MDA	17.0 ± 1.4	0.6 ± 0.0	42.7 ± 1.3	0.5 ± 0.0	2.2 ± 0.6	0.3 ± 0.0
MDMA	34.3 ± 1.3	0.7 ± 0.0	45.3 ± 0.5	0.9 ± 0.0	16.3 ± 0.7	0.4 ± 0.0
Methamphetamine	34.0 ± 1.41	0.6 ± 0.0	28.0 ± 0.0	0.5 ± 0.0	3.18 ± 0.5	0.5 ± 0.1
Metoprolol	1.7 ± 0.5	0.3 ± 0.2	<MDL	–	0.4 ± 0.1	0.3 ± 0.3
Mirtazapine	117.3 ± 3.7	0.3 ± 0.0	64.0 ± 2.5	0.2 ± 0.0	217.2 ± 89.4[b]	0.5 ± 0.1[b]
Norephedrine	359.3 ± 56.3	0.0 ± 0.0	52.7 ± 2.1[a]	0.3 ± 0.0[a]	48.9 ± 33.4[b]	0.1 ± 0.1[b]
Propranolol	105.0 ± 0.8	0.4 ± 0.0	35.3 ± 0.9	0.4 ± 0.0	59.9 ± 9.7	0.5 ± 0.1
Pseudoephedrine	479.7 ± 28.5	1.0 ± 0.0	30.0 ± 0.2[a]	0.2 ± 0.1[a]	<MDL[b]	–
Salbutamol	284.7 ± 27.9	0.5 ± 0.0	460.7 ± 11.5	0.5 ± 0.0	<MDL[b]	–
Sotalol	245.7 ± 19.6	0.5 ± 0.0	146.3 ± 9.0	0.5 ± 0.0	6.7 ± 3.4	0.5 ± 0.1
Tramadol	1320.7 ± 59.3	0.7 ± 0.1	506.0 ± 46.6	0.7 ± 0.0	30.5 ± 7.7	0.7 ± 0.1
Venlafaxine	352.7 ± 29.5	0.5 ± 0.0	220.3 ± 17.3	0.5 ± 0.0	83.2 ± 18.5	0.5 ± 0.1

Samples prepared in triplicate and injected in triplicate, $n = 9$.
[a] One of the enantiomers was <MQL and should be treated on a semi-quantitative basis.
[b] The compound did not pass all the validation criteria and should be considered semi-quantitative.

Absolute SPE recovery in influent ranged from 30.4% (salbutamol) to 292.9% (desmethylvenlafaxine). Relative SPE recoveries in influent were all adequate between 56.6% (pseudoephedrine) and 138.8% (tramadol), although there were three exceptions: alprenolol (159.3 and 198.9% for each enantiomer), atenolol (21.4 and 19.5% for each enantiomer) and salbutamol (46.9 and 51.4% for each enantiomer). Effluent absolute SPE recoveries ranged between 25.9% (atenolol) and 252.4% (alprenolol). Several of the absolute recoveries were greater than 150%, although the corresponding relative recoveries were much lower suggesting significant ion enhancement took place. This was not the case in the influent which highlights the importance of validating all types of matrices to be analysed as large discrepancies can occur between comparable matrices and relatively cleaner matrices should not be assumed to pose fewer challenges for analysis. Relative SPE recoveries were generally acceptable, ranging between 56.4% (pseudoephedrine) and 142.0% (tramadol), the only exceptions to this range was atenolol (55.1% and 54.8 for each enantiomer), alprenolol (187.3% and 170.3% for each enantiomer) and salbutamol (46.9 and 64.2 for each enantiomer). There are some cases of absolute recoveries being quite different between enantiomers. This is thought to be a result of temporal separation during the chromatography followed by ion suppression and/or enhancement of one or both of the enantiomers.

Ion ratios for all the compounds in the liquid matrices are within the thresholds suggesting no co-eluting compounds are compromising the analysis. MDLs in wastewater influent were between 0.05 ng L^{-1} and 28.74 ng L^{-1}, MQLs ranged from 0.03 ng L^{-1} to 95.81 ng L^{-1}. MDLs in wastewater effluent were between 0.01 ng L^{-1} and 32.73 ng L^{-1}, MQLs ranged from 0.07 ng L^{-1} to 109.08 ng L^{-1}. The majority of MDLs and MQLs were below 1 ng L^{-1}, isolated cases, notably atenolol, citalopram and salbutamol, were significantly high though. The MQLs in the liquid matrices are all suitable for wastewater analysis, illustrating that despite a complex matrix this sample preparation and analytical method are suitable for wastewater concentrations of this magnitude.

The validation of the analytical method illustrates that the sample preparation methods chosen are appropriate.

3.3.2.2. MAE–SPE-chiral LC–MS/MS. Results for the MAE-chiral LC–MS/MS validation using 1 g of matrix are detailed in Table 7. Ephedrine, pseudoephedrine and norephedrine all suffered from several issues including poor accuracy and precision and they are not considered validated for this method; the results are included for illustrative purposes only, in addition salbutamol's recovery was insufficient to be quantifiable. In brief, the retention times are impacted by this matrix and/or the method more than for the liquid matrices, however, due to the use of surrogate standards, changes in retention times are compensated for. Five compounds' EF values deviated from racemic in solid matrices including citalopram and desmethylcitalopram (0.6), mirtazapine (0.3), fluoxetine (0.2), tramadol (0.6) and venlafaxine (0.4). Intraday precision ranged between 4.3 and 33.4%, however, was generally below 15%. Inter-day precision ranged between 5.1 and 45.9%, although was generally less than 30%. Relative ion intensities were consistently within tolerance levels. Absolute recoveries are generally, relatively low, often between 10% and 50%, however, most relative recoveries were between 65% and 140%, exceptions however include atenolol (25.8 and 18.6% for each enantiomer), desmethylcitalopram (55.6 and 26.2% for each enenatiomer), fluoxetine (9.0 and 47.9% for each enantiomer), mirtazapine (243.3 and 172.9% for each enantiomer), and tramadol (209.3 and 323.7% for each enantiomer). Compounds in standard solutions were also passed through the cartridges without matrix (Table S3) which confirmed that the MAX cartridges are poor at retaining and/or eluting these compounds.

The sample preparation was suitable to remove co-eluting compounds whilst maintaining the resolution at a variety of concentrations. The cartridges, as discussed above, do not retain and/or elute the basic compounds efficiently, resulting in poor absolute recoveries. However, despite this, the majority are well compensated for by their internal standards, highlighting the importance of these internal standards. Compounds which do not meet the standards set for relative recoveries and/or relative enantiomeric fractions did not have direct deuterated analogues as surrogate standards, and therefore these discrepancies may be addressed with the introduction of new surrogate standards (if available), being direct deuterated or C13-analogues of target

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analytes. MDLs ranged between 0.080 and 7.12 ng g^{-1}. The MQLs, ranging from 0.080 ng g^{-1} (S(+)-venlafaxine) to 25.2 ng g^{-1} (R(+)-atenolol), appear to be appropriate for the concentrations found in the sludge samples suggesting the sample size chosen is adequate.

3.4. Wastewater analysis

The methods described above were applied to influent and effluent wastewater and digested sludge, and represent the first example of analysis of a suite of compounds from all three matrices in the wastewater treatment system at the enantiomeric level. Mass chromatograms of chiral drugs present in wastewater influent and analysed with both CBH and Vancomycin V columns are shown in Figs. 2 and 3 as examples. Concentrations in the influent ranged between 1.7 ng L^{-1} (metoprolol) and 1320.7 ng L^{-1} (tramadol), however, effluent levels appeared to be lower for all compounds apart from MDA, MDMA and salbutamol (Table 8). The samples were all taken simultaneously, so it is impossible to comment on removal rates as they do not represent the same body of wastewater as it flows through the WWTP. However, the results do indicate that even closely related compounds do not appear to have similar degradation pathways. For example, atenolol and sotalol appear at relatively high concentrations in the influent and effluent, with little having been adsorbed to the digested sludge, whereas a relatively high concentration of propranolol has adsorbed to the digested sludge.

Enantiomeric fractions of ten compounds in the influent significantly varied from the EF recorded in the validation, suggesting human metabolism and biological action within the sewerage system had altered the enantiomeric fraction from the either racemic or single enantiomer ingested drug. In the effluent, after the biological action of activated sludge, EFs of 8 compounds were significantly different from those measured in a racemic mixture. Nine compounds: amphetamine, desmethylcitalopram, ephedrine, MDA, MDMA, methamphetamine, mirtazapine, norephedrine and pseudoephedrine had significantly different EF values in the influent and effluent, which indicates stereoselective processes occurring during activated sludge treatment.

The digested sludge, which had undergone activated sludge action and anaerobic digestions as well, contained all the validated compounds at quantifiable concentrations, except ephedrine, pseudoephedrine and salbutamol; concentrations ranged from 0.4 ng g^{-1} (metoprolol) to 275.2 ng g^{-1} (citalopram) even though many are polar. This analysis of digested sludge does highlight the potential for an overestimation in the success rate of activated sludge removing pharmaceuticals, such as in the cases of citalopram and fluoxetine where low concentrations are found in the effluent, however, relatively high concentrations are in the digested sludge. For the first time the examination of the enantiomeric fraction of these compounds in the solid matrices indicates that EFs for four compounds (alprenolol, MDA, MDMA, norephedrine and tramadol) significantly deviated from the racemic EF. In addition 9 compounds analysed had significantly different EFs in the effluent and the digested sludge demonstrating that biological processes which occur in the sludge, either during activated sludge treatment or during anaerobic digestion, result in a different EF when compared to the one in the water fraction. This indicates that either differing microorganism populations or the change in conditions instigates a different metabolic pathway which results in a different EF. It is worth noting that different microbial communities are present during aerobic activated sludge treatment and anaerobic digestion and therefore different stereoselective metabolic pathways were expected in this work. This is particularly stark in comparing effluent and digested sludge enantiomeric fractions of MDA (0.5 and 0.3, respectively), MDMA (0.9 and 0.4, respectively), alprenolol (0.5 and 0.7) and mirtazapine (0.2 and 0.5, respectively). Further work is currently being undertaken to understand stereoselective degradation of chiral PACs during wastewater treatment.

4. Conclusions

This work has demonstrated for the first time a method which can identify and quantify a range of chiral pharmaceuticals and illicit drugs at enantiomeric level from wastewater influent, effluent and digested sludge. These methods were evaluated through a series of parameters recommended by the EC Directive [37] and in addition the resolution and enantiomeric fraction, both absolute and relative, were assessed. To the authors' knowledge this is also one of the most rigorous validation tests of chiral wastewater analysis published to date. A combination of excellent instrument sensitivity, concentration steps within the sample preparation and good recoveries have resulted in low quantification levels within the anticipated environmental range.

Based on the results above, the following compounds are considered to have been successfully calibrated and validated for all three matrices: amphetamine, methamphetamine, MDMA, MDA, venlafaxine, desmethylvenlafaxine, citalopram, metoprolol, propranolol and sotalol. Mirtazapine, salbutamol, fluoxetine, desmethylcitalopram, atenolol, ephedrine and pseudoephedrine passed all validation criteria in liquid matrices, although in solid matrices they did not pass all the criteria and should be considered on a semi-quantitative basis only. In addition, alprenolol did not pass all the criteria in the liquid matrices and should be considered semi-quantitative in this matrix, although fully quantitative in the solid matrix.

The results indicate that chiral PACs are present in all wastewater matrices and that the EF values are often not racemic, suggesting current risk assessment procedures assessing whole drugs for toxicity in environmental matrices may not be appropriate, and that biological wastewater treatments are capable of changing the enantiomeric fraction.

Acknowledgements

Sian Evans would like to acknowledge the University of Bath for funding her PhD studies. Support from EPSRC (Project No. EP/I038608/1) and NERC (Project No. NE/I000534/1) is also acknowledged. The authors would like to thank Wessex Water for their assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2015.03.039.

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Please cite this article in press as: S.E. Evans, et al., Determination of chiral pharmaceuticals and illicit drugs in wastewater and sludge using microwave assisted extraction, solid-phase extraction and chiral liquid chromatography coupled with tandem mass spectrometry, Anal. Chim. Acta (2015), http://dx.doi.org/10.1016/j.aca.2015.03.039