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Trace analysis of fluoxetine and its metabolite norfluoxetine. Part I: Development of a chiral liquid chromatography–tandem mass spectrometry method for wastewater samples

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ABSTRACT

An enantioselective method for the determination of fluoxetine (a selective serotonin reuptake inhibitor) and its pharmacologically active metabolite norfluoxetine has been developed for raw and treated wastewater samples. The stable isotope-labeled fluoxetine and norfluoxetine were used in an extended way for extraction recovery calculations at trace level concentrations in wastewater. Wastewater samples were enriched by solid phase extraction (SPE) with Evolute CX-50 extraction cartridges. The obtained extraction recoveries ranged between 65 and 82% in raw and treated wastewater at a trace level concentration of 50 pM (15–16 ng L^{-1}). The target compounds were identified by the use of chiral liquid chromatography tandem mass spectrometry (LC–MS/MS) in selected reaction monitoring (SRM) mode. The enantiomers were successfully resolved on a chiral α_1-acid glycoprotein column (chiral AGP) with acetonitrile and 10 mM ammonium acetate buffer at pH 4.4 (3/97, v/v) as the mobile phase. The effects of pH, amount of organic modifier and buffer concentration in the mobile phase were investigated on the enantiomeric resolution (R_s) of the target compounds. Enantiomeric R_s-values above 2.0 (1.03 RSD%, $n=3$) were achieved for the enantiomers of fluoxetine and norfluoxetine in all mobile phases investigated. The method was validated by assessing parameters such as cross-contamination and carryover during SPE and during LC analysis. Cross-talk effects were examined during the detection of the analytes in SRM mode. In addition, the isotopic purity of fluoxetine-d_5 and norfluoxetine-d_5 were assessed to exclude the possibility of self-contamination. The interassay precision of the chromatographic separation was excellent, with relative standard deviations (RSD) equal to or lower than 0.56 and 0.81% in raw and treated wastewaters, respectively. The method detection and quantification limits (respectively, MDL and MQL) were determined by the use of fluoxetine-d_5 and norfluoxetine-d_5. The MQL for the single enantiomers ranged from 12 to 14 pM (3.6–4.3 ng L^{-1}) in raw wastewater and from 3 to 4 pM (0.9–1 ng L^{-1}) in treated wastewater. The developed method has been employed for the quantification of (*R*)-fluoxetine, (*S*)-fluoxetine and the enantiomers of norfluoxetine in raw and treated wastewater samples to be presented in Part II of this study.

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1. Introduction

During the last few decades, different active pharmaceutical ingredients (API) from various therapeutic classes of pharmaceutical compounds have been detected in the aquatic environment. Many pharmaceuticals and their metabolites are not completely eliminated during passage through sewage treatment plants and enter the environment mainly through the sewage systems [1,2]. Consequently, pharmaceuticals and their metabolites have been detected in sewage influent and effluent, seawater, rivers, lakes, groundwater and even in tap water [1,3–6]. Many clinically important APIs are chiral compounds, and some drugs are administered as racemic mixtures whereas others are administered as only one of the enantiomers [7,8]. The enantiomers of chiral compounds can interact differently with receptors, enzymes and other chiral molecules [7]. Pharmacological enantiomers should thus be considered to be different chemical compounds from one another as they often mediate different pharmacological activity, potency and pharmacokinetic profiles [9]. As the pharmacological effect might be mediated, to a great extent, by only one of the enantiomers there is a strong need for chiral separation systems in the field of pharmaceutical sciences [10]. During the last recent decades, several achiral

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analytical methods have been developed for trace level analysis of APIs in the aquatic environment [11–13]. However, only a few chiral ones have been developed. These types of analyses are challenges as concentrations of APIs in the aquatic environment are generally low and often found to be as low as the ng L^{-1}-level [14] and in extremely complex environmental samples with a high degree of interference [7].

Fluoxetine, trademarked as Prozac®, is a selective serotonin reuptake inhibitor (SSRI) used as an antidepressant [8]. Fluoxetine is a chiral compound and the commercially available drug is marketed as a racemic mixture of (*R*)- and (*S*)-fluoxetine, where the enantiomers exert equipotent antidepressant effects [15]. However, fluoxetine is metabolized in humans to the chiral and active metabolite, norfluoxetine, where the antidepressant effect is exerted mainly by the (*S*)-enantiomer, which is as potent as the parent pharmaceutical [16]. It has recently been found that significant enantioselectivity in toxicity occurs in the aquatic environment and that the different enantiomers of fluoxetine exert different levels of toxicity on aquatic organisms at different trophic levels [9]. It is therefore highly desirable to consider the enantiomeric differences when evaluating the ecotoxicological effects of chiral pharmaceuticals. Furthermore, the environmental fate may differ for the enantiomers of chiral contaminants as for example the enantiomeric fraction of chiral APIs may be altered during biological sewage treatment [17–19]. This was demonstrated in a Canadian treatment plant where it was found that the relative concentration of (*R*)-fluoxetine was higher in influent (raw) wastewater than in effluent (treated) wastewaters [19]. However, most studies on chiral APIs in the aquatic environment do not determinate the concentrations of the different enantiomers. One of the reasons for this might be the challenge of analyzing the enantiomers at trace level concentrations in extremely complex matrices, such as wastewater [7].

The enantiomers of APIs have been separated by different techniques, such as liquid and gas chromatography and capillary electrophoresis [20]. In liquid chromatography (LC), the enantiomers can be resolved directly by the use of a chiral stationary phase (CSP) [20] or by a chiral mobile phase additive [21]. One of the most used CSPs is silica bound α_1-acid glycoprotein (AGP). Chiral AGP binds drugs with hydrophobic and electrostatic interactions [22] and has been used to separate basic [23], neutral [24], and acidic [25] drug enantiomers. The enantiomers of fluoxetine have previously been resolved by chiral AGP [26]. In addition, a vancomycin-based CSP has been used to determinate the enantiomeric fractions and concentrations of fluoxetine in raw and treated wastewater samples in a Canadian wastewater treatment plant [19].

The aim of this study was to develop a chiral method for trace-level analysis of fluoxetine and norfluoxetine in raw and treated wastewater samples by the use of solid phase extraction (SPE) and chiral HPLC–MS/MS. The scope of the present study was furthermore to investigate if the isotope-labeled standards, fluoxetine-d_5 and norfluoxetine-d_5, could be used to validate key parameters, such as extraction recoveries, at trace-level concentrations in the actual matrix in which the target analytes were present.

2. Materials and methods

2.1. Chemicals and stock solutions

(*R*,*S*)-Fluoxetine (Fig. 1) hydrochloride (analytical standard, Riedel-de Haën), (*R*,*S*)-norfluoxetine (Fig. 1) hydrochloride (≥97%, Sigma), (*S*)-(+)-fluoxetine hydrochloride (≥98%, Sigma) and (*R*,*S*)-[$^2H_5$]-fluoxetine (fluoxetine-d_5) in methanol (drug standard grade, isotopic purity; 98%, Isotec stable isotopes) were all obtained

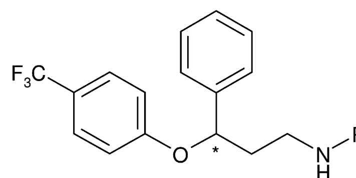

Fig. 1. Fluoxetine and norfluoxetine. The molecular structure of fluoxetine (R=CH_3) and its chiral metabolite norfluoxetine (R=H).

from Sigma–Aldrich (St Louis, MO, USA). (*R*,*S*)-[$^2H_5$]-Norfluoxetine-d_5 hydrochloride (norfluoxetine-d_5) (98%, isotopic purity; 99%) was bought from Toronto Research Chemicals Inc. (North York, Canada). Ammonia solution (25%, *pro analysi*) and glacial acetic acid (*pro analysi*) were obtained from Merck (Darmstadt, Germany). Ammonium acetate (analytical reagent grade) and isopropanol (HPLC grade) were purchased from Fisher Scientific UK Limited (Loughborough, UK). Methanol (isocratic grade) came from BDH, Prolabo (VWR International LLC, West Chester, PA, USA), acetonitrile (for HPLC) was obtained from Sigma–Aldrich (Chemie GmbH, Steinheim, Germany) and formic acid (*pro analysi*) was supplied by Acros Organics (Springfield, NJ, USA). Stock solutions of (*R*,*S*)-fluoxetine hydrochloride, (*R*,*S*)-norfluoxetine hydrochloride, (*R*,*S*)-fluoxetine-d_5 and (*R*,*S*)-norfluoxetine-d_5 were prepared in methanol. A stock solution of (*S*)-fluoxetine hydrochloride was prepared in ethanol (Etax Aa, 99.7%, v/v, Altia Corporation, Rajamäki, Finland). All stock solutions were stored at −18 °C. Working standards were stored in the dark at 8 °C and prepared by the dilution of stock solutions.

2.2. Experimental

2.2.1. Sample preparation

Grab samples of influent (raw) wastewater and effluent (treated) wastewater were collected from the municipal wastewater treatment system Kungsängsverket in Uppsala, Sweden, in 2.5 L clean amber glass bottles. All glassware used in this study was washed with detergent (Neodisher FLA, Chemische Fabrik Dr. Weigert GmbH & Co. KG, Hamburg, Germany) in a Miele G7783 laboratory glassware washer (Miele Inc., Gutersloh, Germany) before usage. The washing procedure consisted of two prewashing cycles, one washing cycle (with a maximum temperature of 85 °C), four rinse cycles (including three steps with deionized water) and one drying step. The sample bottles were transported for about 20 min and upon arrival at the laboratory, the water was immediately filtered through glass fiber filters (pore size 0.7 μm from Millipore, Billerica, MA, USA) and stored at 2 °C until sample extraction.

Aliquots of 200 mL of raw wastewater and 500 mL of treated wastewater were spiked with (*R*,*S*)-fluoxetine-d_5 and (*R*,*S*)-norfluoxetine-d_5 and the pH was adjusted to 4 with 100 or 150 μL of 50.0% formic acid in Millipore water. The water samples were extracted with the use of a 12-port vacuum manifold by solid phase extraction (SPE) with Evolute CX-50 cartridges (200 mg, 6.0 mL, and a mean particle size of 50 μm) obtained from Biotage (Uppsala, Sweden). Initially, Oasis MCX extraction cartridges (150 mg, 6.0 mL, and mean a particle size of 30 μm) from Waters Corporation (Milford, MA, USA), were also tested. Each Evolute CX-50 cartridge was conditioned with methanol (6.0 mL) and Millipore water (6.0 mL) and then equilibrated with 2.0% formic acid in Millipore water (6.0 mL). The water samples were transferred using pieces of polytetrafluoroethylene (PTFE) tubing (Biotage, Uppsala, Sweden) which were placed in the sample containers and connected to the cartridges with column adaptors (Biotage, Uppsala, Sweden). The application of the samples to the SPE cartridges was performed under vacuum, with a flow rate of approximately

5 mL min^{-1}. The cartridges were washed with 2.0% formic acid in Millipore water (6.0 mL) followed by methanol (4.0 mL), and were allowed to dry for 10 min. Methanol/25% ammonium hydroxide (95/5, v/v, 8.0 mL) was used to elute the analytes and the extracts were evaporated at 40 °C until dry under a gentle steam of nitrogen. The dry residues were reconstituted in 250.0 μL mobile phase and filtered through 4 mm disposable polyvinylidene fluoride (PVDF) syringe filters with a pore size of 0.45 μm (Whatman Inc., Piscataway, NJ, USA).

The extraction recoveries with Oasis MCX and Evolute CX-50 cartridges were determined for fluoxetine-d_5 and norfluoxetine-d_5. The cartridges were conditioned with methanol (6.0 mL) and Millipore water (6.0 mL), and then equilibrated with 2.0% formic acid in Millipore water (6.0 mL) whereupon the water samples were applied. The cartridges were washed with 2.0% formic acid in Millipore water (6.0 mL) and the analytes were eluted with methanol/25% ammonium hydroxide (95/5, v/v, 8.0 mL). This extraction procedure is referred to as Method A. In Method B, the cartridges were conditioned and equilibrated as in Method A, where after the samples were applied to the extraction cartridges. The cartridges were then washed with 2.0% formic acid in Millipore water (6.0 mL), followed by an additional wash with methanol (6.0 mL). The analytes were eluted with methanol/25% ammonium hydroxide (95/5, v/v, 8.0 mL) as in Method A.

A breakthrough study of fluoxetine-d_5 and norfluoxetine-d_5 was performed to ensure that the analytes did not pre-elute during the washing step with methanol in Method B. Duplicates of raw (200 mL) and treated (500 mL) wastewater samples were spiked with the isotope-labeled compounds to a concentration of 1.0 nM. The wastewater samples were extracted with Evolute CX-50 and fractions of 1 or 2 mL of methanol from the washing step were collected. The fractions were evaporated, reconstituted and analyzed by the LC–ESI-MS/MS system as described in Sections 2.2.2 and 2.2.3.

2.2.2. *Enantiomeric separation of fluoxetine and norfluoxetine*

The chromatographic system used was an Agilent 1100 Series HPLC system equipped with degasser, binary pump and autosampler from Agilent Technologies Inc. (Palo Alto, CA, USA). The method development of the chromatographic separation was performed on a chiral α_1-acid glycoprotein column. The AGP column dimensions used were 100 mm × 2.0 mm (AGP100.2) with a particle size of 5 μm (ChromTech Ltd., Congleton, UK). AGP columns prepared from different batches of chiral AGP gave slightly different retention for the enantiomers even though preparation conditions of mobile phases were uniform. However, results given in a certain figure or table have been obtained from the same stationary phase and batch number. The flow rate was set to 0.22 mL min^{-1}. The analytes were monitored either by UV at 226 nm or by MS/MS. The MS/MS conditions are described in Section 2.2.3. Data acquisition and the peak integration were performed with Agilent ChemStation Rev.A.10.02 (Agilent Technologies, Santa Clara, CA, USA) and with MassLynx software, v. 4.1 (Waters Corporation, Milford, MA, USA).

The AGP 100.2 column was coupled to a UV detector during the development of the enantiomeric separation method. The retention factors (k), peak resolution (R_s), plate numbers (N) and separation factors (α), were evaluated by the injection of (R,S)-fluoxetine, (R,S)-norfluoxetine and/or (S)-fluoxetine. All samples were injected as triplicates. The chromatographic conditions were studied using a mobile phase consisting of ammonium acetate buffer and acetonitrile. The ammonium acetate buffers were prepared using stock solutions of 1.0 M ammonium acetate and 1.0 M acetic acid. A 'pH Meter 744' from Metrohm (Herisau, Switzerland) was used to measure pH. To study the enantiomeric separation, the pH and the total concentration of added ammonium acetate (C_{NH_4Ac}) in the buffer were varied. The effect of pH on separation was studied from pH 4.0 to 5.4 in the buffers (C_{NH_4Ac} = 10 mM). Moreover, the effect of the buffer concentration on enantiomeric separation was studied in the range from 10 to 100 mM ammonium acetate. Buffers containing 10, 20 and 50 mM ammonium acetate were prepared by the dilution of a 100 mM ammonium acetate buffer with Millipore water. The pH was 4.38 ± 0.02 in these different buffers and the composition acetonitrile/ammonium acetate buffer was 3/97 (v/v). The amount of organic modifier (acetonitrile) in the mobile phase was also studied in the range from 2.0% (v/v) to 4.0% (v/v) in ammonium acetate buffer (pH 4.4, C_{NH_4Ac} = 10 mM).

Table 1
MS/MS conditions. SRM transitions for fluoxetine, norfluoxetine and the isotope-labeled compounds.

Compound	SRM transition (m/z)	Cone voltage (V)	Collision energy (V)
Fluoxetine	310 → 44	15	11
Fluoxetine-d_5	315 → 44	18	11
Norfluoxetine	296 → 134	15	7
Norfluoxetine-d_5	301 → 139	15	6

MS/MS detection was used during the analysis of wastewater samples (during extraction recovery determinations and method validation). For the MS/MS system, a direct in-line high-pressure filter with a replaceable cap frit (4 mm, 0.5 μm, Restek, Bellefonte, PA, USA) and a 10 mm × 2.0 mm Chiral-AGP guard column (ChromTech Ltd., Congleton, UK), were connected to the analytical column in order to protect it from unsolved and high affinity impurities. The separation was performed under isocratic conditions with acetonitrile/10 mM ammonium acetate buffer (3/97, v/v) as the mobile phase at a flow rate of 0.22 mL min^{-1}. The separation was performed under ambient temperature and the injected sample volume was set to 10 μL.

2.2.3. *Mass spectrometry*

The LC system was coupled to a Quattro Micro Mass Spectrometer (Waters Corporation, Milford, MA, USA) equipped with Z spray and an electrospray ionization (ESI) interface operating in positive ion mode. The analytes were detected in selected reaction monitoring (SRM) mode and the system was tuned for each compound. The MS parameters were optimized by direct infusion of working standards of each compound separately. The most abundant product ion produced for each precursor ion was recorded. The SRM conditions for fluoxetine, norfluoxetine and the isotope-labeled compounds are listed in Table 1. The dwell time was 0.25 s and the capillary voltage was set to 3.0 kV. To ensure efficient desolvation of the formed droplets with the high water content (the amount of organic modifier were ranging from 2.0 to 4.0% in the mobile phase), the desolvation temperature was set to 450 °C and the desolvation flow rate to 13 × 10^3 mL min^{-1}. The cone gas was supplied at a flow rate of 1.7 × 10^3 mL min^{-1} and the source temperature was 100 °C. Nitrogen was used as the nebulizer, desolvation and cone gas and argon was used as the collision gas.

2.2.4. *Method validation*

The following validation parameters were determined: the extraction recoveries, accuracy, interassay precision (i.e. repeatability) of the chromatographic system (with respect to the retention), method detection limit (MDL), method quantification limit (MQL), cross-contamination, carryover, cross-talk and isotopic purity of fluoxetine-d_5 and norfluoxetine-d_5. Since the isotope-labeled compounds were not found in the wastewater samples, the extraction recoveries, MDL, MQL and the interassay precision were determined for the isotope-labeled compounds in raw and treated wastewater.

The accuracy and precision of the developed method were determined by performing recovery experiments [27,28] at two different spiking levels (50.0 and 500.0 pM) in raw and treated wastewater. For the extraction recovery determinations, wastewater samples were filtered and one set of samples (*Set 1*) was spiked with (*R,S*)-fluoxetine-d_5 and (*R,S*)-norfluoxetine-d_5 to a concentration of 1.0 nM (310 and 300 ng L^{-1}, respectively) or to a concentration of 0.10 nM (31 and 30 ng L^{-1}, respectively) in raw (200 mL) and treated (500 mL) wastewaters. The samples were extracted according to the analytical procedure given in Method A or Method B. The signals were monitored by LC–ESI-MS/MS. The second set (*Set 2*) was spiked after extraction. *Set 2* was prepared as follows: the wastewater samples were filtered whereupon 200 mL of raw wastewater, or 500 mL of treated wastewater were extracted by SPE. The eluates from the SPE procedure were spiked with (*R,S*)-fluoxetine-d_5 and (*R,S*)-norfluoxetine-d_5 and the signals were monitored the same way as for *Set 1*. The recoveries were calculated by comparing the peak areas from *Sets 1* and *2* and are presented as percentages [29].

Six replicates of raw and treated wastewater samples, with respective volumes of 200 and 500 mL, were spiked with fluoxetine-d_5 and norfluoxetine-d_5 and the interassay precision of the retention times was determined for the isotope-labeled compounds in the different matrices.

All sample handling was performed in such way that the risk of sample contamination was minimized. Stock solutions and working standards were not prepared in the same fume hood as the one in which the wastewater samples were extracted. Cross-contamination during solid phase extraction, as well as carryover and cross-talk effects during LC–MS/MS analysis were studied to make sure that the analytes truly originated from the wastewater treatment plant and not from the standards in the laboratory. Cross-contamination during sample handling was assessed by the integration of procedural blanks of 200 mL or 500 mL of Millipore water in parallel with extraction of all wastewater samples and standards. The extracts were analyzed in the same way as the wastewater and standard samples. Carryover in the analytical system was assessed by the injection of Millipore water into the LC–MS/MS system at regular intervals in-between the samples. Possible cross-talk effects in the MS/MS system were analyzed by the separate injection of the racemic standards at the highest enantiomeric concentration used in the study (1.0 μM). Cross-talk were studied by monitoring the three other SRM channels than the SRM channel for the injected compound for peaks with signal-to-noise ratios above the MDL for respective compound. The isotopic purity of the isotope-labeled compounds was examined by spiking 200 or 500 mL of Millipore water with fluoxetine-d_5 and norfluoxetine-d_5 to a concentration of 0.50 nM (160 and 150 ng L^{-1}, respectively). The extracted water samples were analyzed and the SRM channels for fluoxetine and norfluoxetine were monitored to identify peaks above the MDL.

The MDL and MQL were evaluated according to the guidelines produced by the International Conference on Harmonization (ICH) for the validation of analytical procedures [30] and the American Food and Drug Administration's guide to bioanalytical method validation [31]. The MDL was defined as the concentration of the analyte in the sample resulting in a peak height that was three times as high as the average peak to peak amplitude of the background noise. The MQL can be defined as the concentration of the analyte in the sample resulting in a peak height five or ten times as high as the average peak to peak amplitude of the background noise. In addition, the analyte peak of MQL was defined as a reproducible and defined peak with a precision of 20%. The MDL and MQL for fluoxetine and norfluoxetine were determined by spiking raw and treated wastewater with the enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 to an enantiomeric concentration of 250.0 pM (78.6 and 75.1 ng L^{-1}, respectively). The reconstituted eluates from the extracted wastewater samples were diluted with the mobile phase and analyzed. The response–concentration relationships were plotted to estimate the MDL and MQL for the four analytes. Another extracted sample was diluted to the concentrations corresponding to those of the MDL and MQL and analyzed. The precision of the MQL was also determined, $n = 3$. The peak heights from the isotope-labeled compounds were thus used for the direct determination of the MDL and MQL for fluoxetine and norfluoxetine.

3. Results and discussion

3.1. Solid phase extraction

Some of the analytical challenges with emerging contaminants in the aquatic environment arise because of the trace level concentrations and the complex matrix in which the APIs tend to be detected and quantified in. Solid phase extraction and solid phase microextraction are the most commonly used extraction techniques for the enrichment and cleaning of environmental samples [12]. However, different approaches have been employed to determine the extraction recoveries for the analytes of interest in environmental matrices. Recovery experiments have been performed during method development through the use of blank matrices (i.e. in matrices in which the analytes have not been detected), e.g. in well water [32] and surface water [33]. One concern with this approach is that the extraction recoveries are not always the same in different types of water matrices [34,35], hence, the extraction efficiency might be under- or overestimated in comparison with the true matrix. Another approach taken to obtain extraction recoveries has been to spike the water samples with higher amounts of the analytes than the naturally occurring concentrations become negligible [36]. However, extraction recoveries are not necessarily the same in different concentrations ranges. A further approach has been to compensate for the "naturally" occurring quantities of substances by subtracting the un-spiked measured concentrations from the spiked ones before performing recovery calculations [34,36]. In addition, the more time consuming standard addition method has been used for extraction recovery determinations in the true matrix [27]. However, if stable isotope-labeled compounds are commercially available, determination of validation parameters, such as the extraction recovery, can be simplified by using these compounds. As isotope-labeled compounds have almost the same physical and chemical properties as the non-labeled compounds, and as they are not found in wastewater samples, the deuterated compounds can be used for recovery calculations. In this study fluoxetine-d_5 and norfluoxetine-d_5 were used to determinate the extraction recoveries for fluoxetine and norfluoxetine at trace level concentrations in raw and treated wastewater.

3.1.1. Extraction recovery determinations for the enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 in raw and treated wastewater

During extraction recovery determinations, the isotope-labeled compounds were either added to raw water or to treated water before or after extraction (*Sets 1* and *2*, respectively) and the recoveries were determined as described in Section 2.2.4. This approach was developed by Matuszewski et al. [29] for the determination of extraction recoveries in biological fluids. The procedure was subsequently applied in recovery calculations in blank surface waters during development of a method for the determination of basic pharmaceuticals in wastewater and surface water samples [37]. The methodology was, in this study used for the determination of extraction recoveries in raw or treated wastewater.

Table 2A
Extraction recoveries in raw and treated wastewater. Extraction recoveries and relative standard deviations, RSD%, for fluoxetine-d_5 and norfluoxetine-d_5 in raw or treated wastewater extracted with Evolute CX-50 according to Method B. Extraction recoveries determined at an enantiomeric concentration of 500 pM. Experimental details are given in Section 2.2.1.

Compound	Treated wastewater			Raw wastewater
	Oasis MCX Method A[a] Recovery ± RSD (%)	Evolute CX-50 Method A[a] Recovery ± RSD (%)	Evolute CX-50 Method B[b] Recovery ± RSD (%)	Evolute CX-50 Method B[a] Recovery ± RSD (%)
(*S*)-Fluoxetine-d_5	70 ± 13	85 ± 9	96 ± 11	87 ± 8
(*R*)-Fluoxetine-d_5	73 ± 11	80 ± 7	99 ± 12	89 ± 13
Norfluoxetine-d_5 E1	74 ± 7	77 ± 10	66 ± 20	87 ± 10
Norfluoxetine-d_5 E2	79 ± 9	70 ± 14	73 ± 17	83 ± 17

[a] $n = 4$.
[b] $n = 3$.

Table 2B
Extraction recoveries in raw and treated wastewater. Extraction recoveries and relative standard deviations, RSD%, for fluoxetine-d_5 and norfluoxetine-d_5 in raw or treated wastewater extracted with Oasis MCX and Evolute CX-50 according to Method A or Method B. Extraction recoveries determined at an enantiomeric concentration of 50 pM. Experimental details are given in Section 2.2.1.

Compound	Raw wastewater Evolute CX-50 Method B[a] Recovery ± RSD (%)	Treated wastewater Evolute CX-50 Method B[a] Recovery ± RSD (%)
(*S*)-Fluoxetine-d_5	65 ± 25	76 ± 11
(*R*)-Fluoxetine-d_5	66 ± 24	77 ± 14
Norfluoxetine-d_5 E1	77 ± 24	72 ± 10
Norfluoxetine-d_5 E2	76 ± 30	82 ± 11

[a] $n = 5$.

Two types of polymer based solid phase extraction cartridges were evaluated for their usefulness in the extraction of fluoxetine and norfluoxetine from wastewater, Oasis MCX and Evolute CX-50. Oasis MCX is a mixed mode cartridge with reversed phase and strong cation exchange properties. This sorbent was previously used by Batt et al. for the extraction of fluoxetine and norfluoxetine from wastewater samples [38]. Evolute CX-50 is a water-wettable, resin-based sorbent with mixed-mode functionalities, with retention mechanisms arising from a combination of hydrophobic, hydrophilic and cation change interactions. Initially, extraction recovery experiments were conducted with Oasis MCX (150 mg, 6.0 mL) following the analytical procedure given in Method A (described in Section 2.2.1). The mean extraction recoveries for the sum of the enantiomers were determined to be 71% for fluoxetine-d_5 and 77% for norfluoxetine-d_5 in 500 mL treated wastewater. The extraction recoveries and relative standard deviations (RSD%) for the single enantiomers are given in Table 2A. The calculated recoveries for fluoxetine-d_5 were lower than for fluoxetine (101 ± 4%), as previously reported by Batt et al. [38]. Furthermore, the RSD values in this study were higher for treated wastewaters than the RSD values obtained earlier with Oasis MCX [38]. This was probably due to slightly different extraction procedures and because the extraction recoveries were studied at lower concentrations in the study presented here. However, in this study the extraction recoveries for norfluoxetine-d_5 were slightly higher and the RSD values lower than those previously reported for norfluoxetine (66 ± 11%) by Batt et al. [38]. Recovery studies conducted with Evolute CX-50 cartridges (200 mg, 6.0 mL) and Method A resulted in an increase in the extraction recovery to 83% for fluoxetine-d_5 (this value being the mean value for the sum of the enantiomers) and a slight decrease for norfluoxetine-d_5, to 74% (mean value for the sum of the enantiomers) in 500 mL treated wastewater (Table 2A). The extraction method using Oasis MCX was therefore not selected. Thus, the Evolute CX-50 extraction cartridge was chosen for further optimization of the extraction method.

One of the benefits with ion exchange interactions in the solid phase extraction sorbent is that the cartridge can often be washed efficiently with organic solvents such as methanol to remove interfering compounds from the matrix. To ensure that cleaner extracts were obtained, the Evolute CX-50 cartridges were washed with 6.0 mL of 2.0% formic acid followed by 6.0 mL of methanol before elution of the analytes. The extraction recoveries for fluoxetine-d_5 and norfluoxetine-d_5 obtained with the Evolute CX-50 cartridges following the analytical procedure given in Method B (Section 2.2.1) are shown in Table 2A. The recoveries were generally higher for fluoxetine-d_5 than for the more polar compound norfluoxetine-d_5. In addition, higher extraction recoveries were obtained in treated wastewater for fluoxetine-d_5 than in the raw wastewaters. Surprisingly, the opposite was observed for norfluoxetine-d_5.

The addition of the washing step with methanol resulted in visibly cleaner extracts and, more importantly, enhanced the sensitivity for the substances (experiments performed in treated wastewater). The methanol wash was shown to be crucial for the detection of the “naturally” occurring enantiomers of norfluoxetine (Fig. 2). Samples of treated wastewater, collected at the same date and time, were extracted with Evolute CX-50 according to the analytical procedures given in either Method A or Method B. No peaks of the enantiomers of norfluoxetine in treated wastewaters were detected in the chromatograms when the washing step was excluded (Fig. 2A). The enantiomers of norfluoxetine were, however, detectable when the methanol step was included in the procedure (Fig. 2B). The peaks were detected with signal-to-noise (S/N) ratios of 10 for the first eluted enantiomer of norfluoxetine and 4 for the second eluted enantiomer of norfluoxetine. Another noteworthy result was that the unidentified impurities in the SRM channel of norfluoxetine, which were detected at a retention time of about 3.8 min, were eliminated with the additional methanol step. The improved detectability of norfluoxetine was probably a result of that cleaner extracts being achieved when the methanol wash was included, which resulted in less ion suppression and hence improved the signal to noise ratios for the enantiomers of norfluoxetine. Washing with methanol did not result in any breakthroughs for the isotope-labeled compounds when up to 6.0 mL of methanol was used. In addition, Method B yielded visibly clearer eluates than Method A; an additional washing step using 4.0 mL methanol was therefore incorporated in the method. A schematic overview of Method B and the used analytical method for the analysis is given in Fig. 3.

To determine the accuracy and precision of the developed method (Method B), recovery experiments were also conducted at a lower spiking level of 50 pM for the enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 in both raw and treated wastewater, Table 2B. The observed recoveries were in the range of 65–82% at 50 pM. The recoveries obtained at the concentration of 500 pM (Table 2A) were

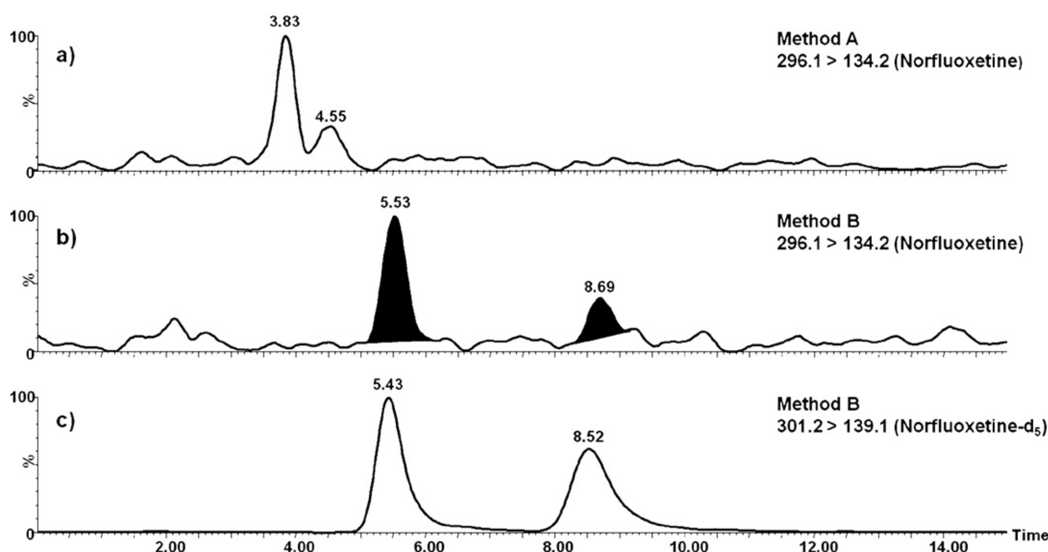

Fig. 2. The impact of methanol in SPE on the detection of norfluoxetine. Analysis of the enantiomers of norfluoxetine and norfluoxetine-d_5 in 500 mL of treated wastewater with LC–ESI-MS/MS using two different solid phase extraction methods. The conditions used in the LC–MS/MS analysis are described in Sections 2.2.2 and 2.2.3. (A) SRM channel for norfluoxetine. Extraction performed with Evolute CX-50 according to Method A (Section 2.2.1). (B) SRM channel for norfluoxetine. Extraction performed with Evolute CX-50 and with an additional step incorporated in the washing procedures involving 6 mL methanol (Method B, described in Section 2.2.1). (C) SRM channel for norfluoxetine-d_5 and the same experimental conditions as in (B).

higher, except for the enantiomers of norfluoxetine-d_5 in treated wastewater. The RSD% values were, as expected, also higher at the low spiking level, except for norfluoxetine-d_5 in treated wastewater.

3.2. Chiral LC–MS/MS analysis

During the method development, fluoxetine and norfluoxetine co-eluted under all investigated conditions and the compounds were separated in accordance with their mass-to-charge ratios

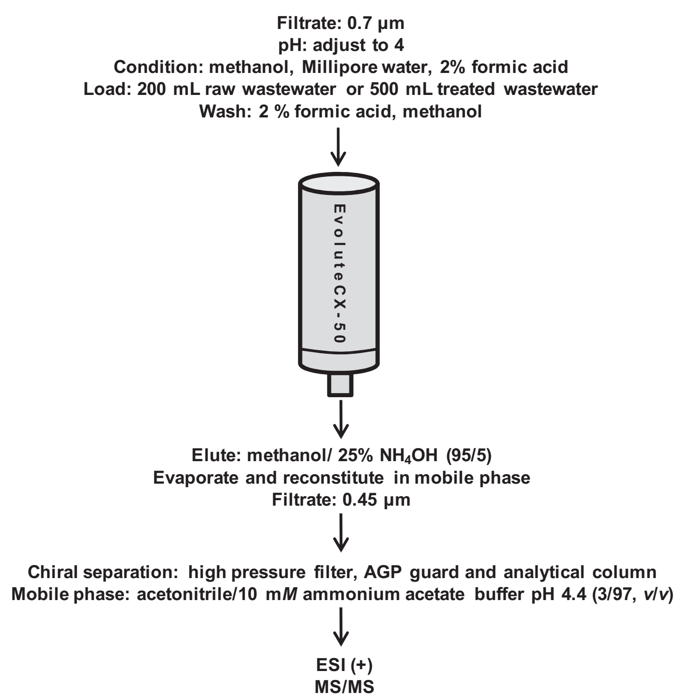

Fig. 3. Schematic overview of the analytical method. The analytical protocol of the developed SPE-LC–MS/MS method. Experimental details are given in Sections 2.2.1–2.2.3.

(m/z) by the mass spectrometer. To compensate for ion suppression owing to co-elution of fluoxetine and norfluoxetine, and owing to interfering matrices, the isotope-labeled compounds, fluoxetine-d_5 and norfluoxetine-d_5, were selected as internal standards in Part II of this study. With the intention of selecting the appropriate chromatographic conditions for the enantiomeric separation, studies were made of the pH in the sample solution and of the pH, amount of organic modifier and total concentration of ammonium acetate in the mobile phase.

3.2.1. The enantiomeric elution order of fluoxetine and norfluoxetine

The enantiomers of fluoxetine and norfluoxetine were baseline-resolved under all investigated chromatographic conditions and (*S*)-fluoxetine was found to elute before (*R*)-fluoxetine (Fig. 4). The elution order of the enantiomers of norfluoxetine could not be determined as the enantiomerically pure forms of norfluoxetine were not available. The first and second eluted enantiomers of norfluoxetine are therefore referred to as norfluoxetine E1 and norfluoxetine E2, respectively.

3.2.2. The effect of the mobile phase pH on the enantiomeric separation

The effect on the enantiomeric separation of fluoxetine and norfluoxetine when increasing the pH in the ammonium acetate buffer from 4.0 to 5.4 is demonstrated in Table 3. A significant effect on retention was observed for fluoxetine and norfluoxetine when increasing the pH; the retention factors increased from 1.21 (0.41 RSD%) to 14.4 (0.18 RSD%) for (*S*)-fluoxetine and from 1.13 (0.30 RSD%) to 14.3 (0.16 RSD%) for norfluoxetine E1. This change in retention is probably caused by an increased coulombic attraction between the positively charged analytes and the negatively charged protein. In the literature, the isoelectric point of unbound, native α_1-acid glycoprotein is 2.7 [39,40] and the recommended pH range for the column is 4–7. Raising pH within this interval might affect the overall conformation of the protein and, moreover, increase the negative net charge of the protein and, therefore, augment the cationic exchange capacity. The charges of fluoxetine (pK_a 10.05) and norfluoxetine (pK_a 9.05) are also affected by the pH, however the charge of the analytes is positive within the pH range

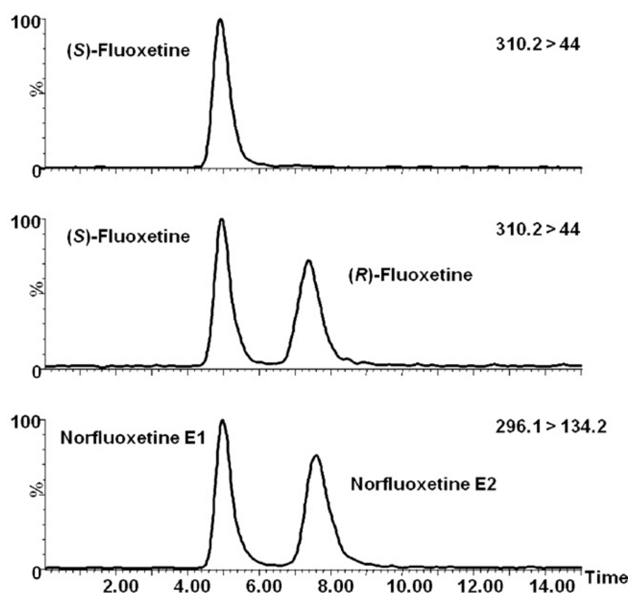

Fig. 4. Elution order of the enantiomers of fluoxetine and norfluoxetine. SRM chromatograms of (*S*)-fluoxetine and racemic standards of fluoxetine and norfluoxetine. E1 and E2 are the first and second eluted enantiomers of norfluoxetine, respectively (the chromatographic conditions are described in Section 2.2.2).

investigated. Furthermore, the retention factor was increased more rapidly for (*R*)-fluoxetine than for (*S*)-fluoxetine at pH values above 4.0, and therefore, the selectivity factor went up as the pH rose, too. This indicates that the cationic-exchange sites on the CSP have stereoselective binding properties, which has been demonstrated with other amines in earlier studies [39,41]. A similar pattern was observed for norfluoxetine. In addition to this, small improvements in efficiency were observed for the enantiomers of fluoxetine and norfluoxetine by altering the pH from 4.0 to 5.4. The plate numbers increased from 910 (7.8 RSD%) to 1130 (15 RSD%) for (*R*)-fluoxetine and from 1000 (2.4 RSD%) to 1250 (5.9 RSD%) for norfluoxetine E2. The overall effect of increasing the pH in the mobile phase was, therefore, an increase in the enantiomeric resolution. The resolution between the enantiomers was greater than 2.2 (2.4 RSD%) in all mobile phases investigated and spanned the range from that value up to 5.6 (5.9 RSD%) for the enantiomers of fluoxetine, and between 2.4 (1.8 RSD%) and 6.1 (1.3 RSD%) for the enantiomers of norfluoxetine.

3.2.3. The effect of the sample solution pH on zone broadening

In order to improve the efficiency of the chromatographic system it is possible to inject the sample, dissolved in a solvent in which the retention factor of the analyte is higher than the retention factor obtained in the mobile phase [42]. The plate number was studied by varying the pH of the sample solution between 3.9 and 6.6 to improve the column performance. The buffer pH of the sample solution was found to affect the zone broadening of fluoxetine and the most effective peaks were found when the sample was dissolved in the same buffer as the mobile phase, i.e. 10 mM ammonium acetate buffer at pH 4.4. As anticipated, a small decrease in plate numbers for (*S*)-fluoxetine, from 940 (1.4 RSD%) to 870 (0.30 RSD%), was observed when the injected sample was changed from 10 mM ammonium acetate buffer at pH 4.4 into the mobile phase, i.e. acetonitrile in 10 mM ammonium acetate buffer at pH 4.4 (3/97, v/v).

3.2.4. The impact of amount of organic modifier in the mobile phase on the enantiomeric separation

Acetonitrile was used as organic modifier for the regulation of retention and resolution of the enantiomers of fluoxetine and norfluoxetine. An advantage with chiral AGP is that this type of stationary phase can be used with different type and content of organic modifier to optimize chiral separations. The content of organic modifier is a compromise between the analyte retention, chiral selectivity and the stability of the protein. In general, a concentration of up to 15% of organic modifier is recommended [43]. An increase in the proportion of acetonitrile from 2.0% (v/v) to 4.0% (v/v) in the mobile phase was shown to decrease the retention factor for the second eluted enantiomer of fluoxetine and norfluoxetine by 42 and 37%, respectively. It has been demonstrated in previous studies that hydrophobic interactions play an important role in the binding of basic drug enantiomers with the AGP [41]. Moreover, small amounts of organic modifiers, e.g. acetonitrile, in the mobile phase are known to modify the chromatography of the solutes owing to a decrease in the hydrophobic interactions of the analytes with the protein based CSP [40,41]. The observed reduction in retention, brought about by the increasing the amounts of organic modifier, is in accordance with previous studies on enantiomeric separation of amines with AGP [40,41,44]. The hydrophobic interactions were most probably stereoselective as the increased quantity of organic modifier decreased the enantiomeric separation factors and resolution. The separation factors were decreased from 1.65 (0.058 RSD%) to 1.42 (0.10 RSD%) and

Table 3
The impact of the mobile phase pH on the enantiomeric separation of fluoxetine and norfluoxetine. The retention factors (k), separation factors (α), plate numbers (N) and the enantiomeric resolution (R_s) are shown, with the precision in brackets (RSD%), for the fluoxetine and norfluoxetine enantiomers, $n = 3$. The mobile phase was acetonitrile and 10 mM ammonium acetate buffer at pH 4.0, 4.4, 4.9 or 5.4 (97:3, v/v). The enantiomers were separated on an AGP column and detected at 226 nm by UV detection.

Compound	pH 4.0	pH 4.4	pH 4.9	pH 5.4
(*S*)-Fluoxetine				
k	1.21 (0.41)	2.29 (1.8)	6.34 (0.30)	14.4 (0.18)
N	1060 (2.3)	710 (1.8)	1170 (0.94)	1410 (6.2)
(*R*)-Fluoxetine				
k	1.92 (0.24)	4.14 (1.5)	12.1 (0.20)	29.0 (0.22)
N	910 (7.8)	750 (11)	940 (7.3)	1130 (15)
Fluoxetine				
α	1.59 (0.17)	1.81 (0.27)	1.90 (0.12)	2.01 (0.052)
R_s	2.2 (2.4)	3.0 (3.0)	4.5 (2.4)	5.6 (5.9)
Norfluoxetine E1				
k	1.13 (0.30)	2.39 (4.1)	6.25 (0.15)	14.3 (0.16)
N	1160 (3.6)	1100 (17)	1310 (4.9)	1610 (8.2)
Norfluoxetine E2				
k	1.87 (0.39)	4.31 (2.4)	12.3 (0.18)	29.5 (0.071)
N	1000 (2.4)	950 (9.7)	1230 (8.7)	1250 (5.9)
Norfluoxetine				
α	1.65 (0.10)	1.81 (1.8)	1.97 (0.16)	2.07 (0.12)
R_s	2.4 (1.8)	3.5 (4.4)	5.2 (2.7)	6.1 (1.3)

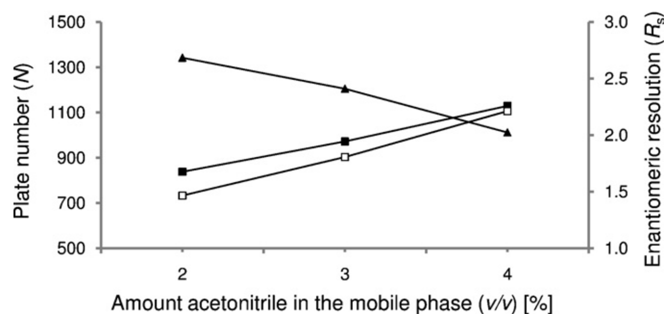

Fig. 5. The impact of the amount of organic modifier in the mobile phase on N and R_s. The enantiomeric resolution (filled triangles) of fluoxetine decreased as the amount of acetonitrile in the mobile phase increased. During the same chromatographic conditions, the plate numbers (N) increased for both (S)-fluoxetine (filled squares) and (R)-fluoxetine (open squares).

from 1.72 (0.10 RSD%) to 1.44 (0.074 RSD%) for the fluoxetine and norfluoxetine enantiomers, respectively, as the proportion of acetonitrile was increased from 2.0% (v/v) to 4.0% (v/v). Under the same chromatographic conditions, the plate numbers increased for the enantiomers of fluoxetine as demonstrated in Fig. 5. The general effect on the plate numbers was similar for the enantiomers of norfluoxetine. The overall effect on the enantiomeric separation was, however, a decrease in the enantiomeric resolution from 2.7 (0.29 RSD%) to 2.0 (1.03 RSD%) for fluoxetine (Fig. 5) and from 3.1 (0.47 RSD%) to 2.2 (0.58 RSD%) for norfluoxetine.

3.2.5. The effect of the buffer concentration in the mobile phase on the enantiomeric separation

Hydrophobic and ionic interactions result in some of the equilibria that can exist between the analyte and the complex protein in the CSP. A general characteristic of hydrophobic interactions is that the interactions increase with an increase in the ionic strength, whereas a characteristic of ionic interactions is that the interactions decrease as the ionic strength goes up. Consequently, the ionic strength of the buffer in the mobile phase can affect the binding properties of the protein and hence also the retention and resolution of the analytes [40,45,46]. The effect of the ionic strength, i.e. the total concentration of ammonium acetate in the mobile phase, on the enantiomeric separation of fluoxetine was studied by altering the buffer concentration from 10 to 100 mM. The plate numbers went up from 1040 (1.2 RSD%) to 1410 (1.3 RSD%) with the increased buffer concentration for the enantiomers of fluoxetine (Fig. 6). The retention factors rose from 2.29 (0.037 RSD%) to 2.61 (0.19 RSD%) and from 3.57 (0.054 RSD%) to 3.73 (0.15 RSD%) for (S)-fluoxetine and (R)-fluoxetine, respectively, with buffer concentrations up to 50 mM. Above concentrations of 50 mM, the retention factors for the first and second eluted enantiomers of fluoxetine slightly decreased to 2.46 (0.092 RSD%) and 3.35 (0.086 RSD%), respectively. However, the increase in the retention factor for the first eluting enantiomer of fluoxetine was higher than the second one, and consequently, the separation factor decreased from 1.56 (0.030 RSD%) to 1.36 (0.0061 RSD%) and the enantiomeric resolution from 2.67 (0.31 RSD%) to 2.14 (0.48 RSD%). The overall increase in retention implies that the hydrophobic interactions between the enantiomers and the AGP protein are more strongly influenced by the ionic strength than the ionic interactions. Hence, the ionic strength can be used to control the hydrophobic interactions which are in agreement with other studies [45]. A low total concentration of ammonium acetate in the mobile phase was however preferable to make the mobile phase compatible with MS/MS detection, as this enables one to obtain a high response in the MS detector. A concentration of 10 mM ammonium acetate in the mobile phase was chosen as this gave satisfactory plate numbers and a high resolution (Fig. 6).

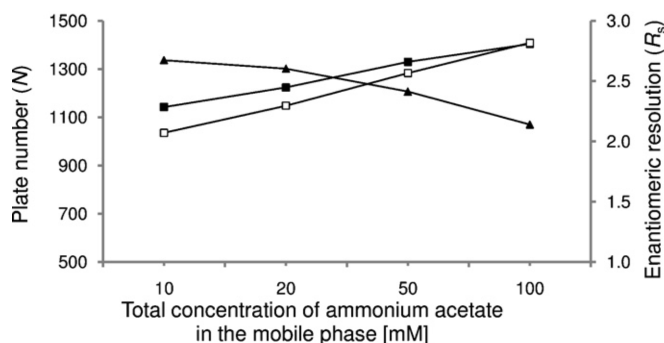

Fig. 6. The influence of the ammonium acetate buffer concentration on N and R_s. The plate numbers (N) increased for (S)-fluoxetine (filled squares) and (R)-fluoxetine (open squares) with the increased concentration of ammonium acetate in the mobile phase. As the buffer concentration was raised from 10 to 100 mM, the enantiomeric resolution (filled triangles) of fluoxetine decreased.

Table 4
The chromatographic precision. Interassay precision, given in terms of the relative standard deviation, for the retention times of the enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 in raw and treated wastewater, $n = 6$.

Compound	Raw wastewater RSD%	Treated wastewater RSD%
(S)-Fluoxetine-d_5	0.38	0.42
(R)-Fluoxetine-d_5	0.41	0.73
Norfluoxetine-d_5 E1	0.31	0.32
Norfluoxetine-d_5 E2	0.56	0.81

3.3. Method validation

As yet, no harmonized validation guidelines have been developed for the trace analysis of APIs in wastewater samples. In this study, the precision, cross-contamination, carryover, cross-talk, isotopic purity of the isotope-labeled standards, the method detection limit and the method quantification limit were determined in addition to the extraction recovery. This ought to add knowledge about the validation procedure of methods developed for the trace level analysis of APIs in complex matrices and thereby facilitating the development of standardized guidelines.

3.3.1. Precision of the enantiomers of fluoxetine-d_5 and norfluoxetine-d_5

The SRM transitions presented in Table 1 were used, together with the retention times in the chromatographic systems, for the identification of the enantiomers of fluoxetine and norfluoxetine in raw and treated wastewater samples. The interassay precision of the method was determined for the retention times of fluoxetine-d_5 and norfluoxetine-d_5 in raw ($n = 6$) and treated wastewater samples ($n = 6$). The repeatability, expressed in RSD% was obtained in the range 0.31–0.56% for raw wastewater and in the range 0.32–0.81% for treated wastewater (Table 4). For positive identification of fluoxetine and norfluoxetine in the wastewater samples, the retention times of the analytes were compared directly with those of the enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 from the same chromatographic run.

3.3.2. Cross-contamination, carryover, cross-talk and isotopic purity of the isotope labeled standards

The high sensitivity obtained with MS/MS drastically increases the risk of carryover during analysis affecting precision and accuracy. Furthermore, cross-contamination during sample handling and solid phase extraction is a major factor that might

Table 5
The sensitivity of the method. MQL and MDL for the enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 in raw and treated wastewater. The concentrations are given in pM. For the MQL, the S/N values are given as the mean and the precision as the RSD%.

Compound	MQL (pM)	S/N (n = 3)	RSD% (n = 3)	MDL (pM)	S/N (n = 1)
Raw wastewater					
(S)-Fluoxetine-d_5	12.4	14.1	8.8	3.0	3.0
(R)-Fluoxetine-d_5	12.4	16.0	18.0	3.0	3.4
Norfluoxetine-d_5 E1	12.1	9.7	4.9	2.4	3.7
Norfluoxetine-d_5 E2	14.3	11.7	15.4	2.4	3.1
Treated wastewater					
(S)-Fluoxetine-d_5	3.0	10.3	8.9	1.0	3.3
(R)-Fluoxetine-d_5	3.0	9.5	19.6	2.0	3.9
Norfluoxetine-d_5 E1	4.0	8.0	18.7	2.0	2.8
Norfluoxetine-d_5 E2	4.0	7.7	22.2	2.0	3.2

decrease the reliability of the method. Thus, investigation of cross-contamination is a necessity as the risk of it occurring is particularly high during sample preparation, especially during the elution and the evaporation steps [29,47]. The above-mentioned contamination risks were, therefore, assessed during the method validation.

Carryover or memory effects that are caused by trapped residues in the autosampler or in the column can be mistaken for false positives or can randomly affect the chromatograms, which might have a major impact on the accuracy of the method and on its precision. Carryover can be observed by the injection of blanks at regular intervals during the LC–MS/MS analysis. Millipore water samples were analyzed after high calibration standards and, subsequently, at frequent intervals to ensure that carryover from the auto sampler and/or the separation column did not occur. No carryover or memory effects were observed in the LC–MS/MS system in this study. Memory effects caused by late eluting interferences are more difficult to detect when analyzing samples in the SRM mode but might give rise to matrix effects such as ion enhancement or suppression. Eventual differences of this kind can, however, be compensated for by using deuterated internal standards. The possibility of cross-talk effects was investigated by injecting one of the analytes at a time and monitoring the other SRM transitions for ions. No cross-talk effects were observed between the SRM channels at the highest concentration (1.0 μM) used in Part II of this study. Furthermore, no evidence of cross-contamination during sample handling or solid phase extraction was found as no peaks were identified for the target analytes or fluoxetine-d_5 and norfluoxetine-d_5 in the extracted Millipore water. Additionally, the isotopic-labeled compounds fluoxetine-d_5 and norfluoxetine-d_5 did not cause any detectable peaks for fluoxetine and norfluoxetine in concentrated Millipore water. The method adopted in this study was therefore considered to minimize the contamination risk during sample handling as well as during the LC–MS/MS analysis.

3.3.3. *Sensitivity of the enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 in raw and treated wastewaters*

The method detection and quantification limits were estimated for the enantiomers of the isotope-labeled compounds in raw and treated wastewater. Samples of raw (200 mL) and treated wastewaters (500 mL) were spiked with fluoxetine-d_5 and norfluoxetine-d_5 to the enantiomeric concentration of 250 pM before the extraction with Evolute CX-50. The values for the MDL and MQL, given in Table 5, were first calculated using the definition of the peak given a signal-to-noise ratio of 3 and 5 or 10, for the MDL and MQL, respectively [30,31]. MDL values in the range 1.0–3.0 pM (0.31–0.94 ng L^{-1}) and MQL values in the range 3.0–14.2 pM (0.94–4.3 ng L^{-1}) were obtained for the analytes. When extracts containing these concentrations were injected, the figures for the signal-to-noise ratios given in Table 5 were obtained. The definition for the MQL in bioanalytical methods may also be used. Then, the precision at the MQL must not exceed 20%. The RSD values ranged from 4.9 to 22.2%, and these values illustrate the uncertainty associated with the analysis of low concentrations of APIs in a complicated matrix like wastewater. In these obtained values, the variance in the solid phase extraction, large volumes (200 mL, 500 mL), low concentrations, evaporation step, and analysis with LC–MS/MS were all included. These analytical steps are normally performed without any isotopically labeled internal standards or sometimes with some general internal standard added after extraction and before the LC–MS/MS analysis.

It can be noted that the MDL/MQL values decreased when an additional washing step involving methanol was included in the sample preparation. A much cleaner extract was then obtained, probably with less ion suppression taking place in the ionization step of the compounds when leaving the LC column and entering the mass spectrometer.

4. Conclusions

It has been demonstrated that the stable isotope-labeled compounds are suitable for the determination of extraction recoveries of environmental contaminants in complex matrices, such as wastewater, in which the analytes are often detected. The recoveries were determined at trace-level concentrations in the actual matrix and by the extended use of stable isotope-labeled compounds. Fluoxetine and norfluoxetine were extracted from wastewater samples collected in a sewage treatment plant in Uppsala Sweden, by the use of Evolute CX-50 SPE cartridges. It was demonstrated that the introduction of an additional washing step involving methanol during the sample extraction was crucial for the detection of the enantiomers of norfluoxetine. Furthermore, a direct chiral separation method has been developed for fluoxetine and its metabolite norfluoxetine by the use of α_1-acid glycoprotein as the chiral stationary phase. This is, to the best of our knowledge, the first time the enantiomers of norfluoxetine have been resolved using chiral AGP. The method that has been developed and validated can be employed to provide important information about the distribution of the enantiomers of fluoxetine and norfluoxetine in the aquatic environment.

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References

[1] T. Heberer, Toxicol. Lett. 131 (2002) 5.
[2] T.A. Ternes, Water Res. 32 (1998) 3245.
[3] D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, H.T. Buxton, Environ. Sci. Technol. 36 (2002) 1202.

[4] T. Heberer, K. Schmidt-Bäumler, H.-J. Stan, Acta Hydrochim. Hydrobiol. 26 (1998) 272.
[5] T. Vasskog, T. Anderssen, S. Pedersen-Bjergaard, R. Kallenborn, E. Jensen, J. Chromatogr. A 1185 (2008) 194.
[6] M.J. Benotti, R.A. Trenholm, B.J. Vanderford, J.C. Holady, B.D. Stanford, S.A. Snyder, Environ. Sci. Technol. 43 (2008) 597.
[7] C. Wong, Anal. Bioanal. Chem. 386 (2006) 544.
[8] H.P. Rang, M.M. Dale, J.M. Ritter, Pharmacology, 4th ed., Churchill Livingstone, Edinburgh, 1999.
[9] F.D. Andrés, G. Castañeda, Á. Ríos, Chirality 21 (2009) 751.
[10] T. Nakanishi, N. Yamakawa, T. Asahi, N. Shibata, B. Ohtani, T. Osaka, Chirality 16 (2004) S36.
[11] S.D. Richardson, Anal. Chem. 81 (2009) 4645.
[12] M. Kostopoulou, A. Nikolaou, TrAC-Trends Anal. Chem. 27 (2008) 1023.
[13] S.D. Richardson, Anal. Chem. 82 (2010) 4742.
[14] S. Öllers, H.P. Singer, P. Fässler, S.R. Müller, J. Chromatogr. A 911 (2001) 225.
[15] M.G. Scordo, E. Spina, M.-L. Dahl, G. Gatti, E. Perucca, Basic Clin. Pharmacol. Toxicol. 97 (2005) 296.
[16] A. Claesson, B. Danielsson, U. Svensson, Läkemedelskemi, 2nd ed., Apotekarsocieteten, Kristianstad, 1996.
[17] H.-P.E. Kohler, W. Angst, W. Giger, C. Kanz, S. Müller, M.J.-F. Suter, Chimia 51 (1997) 947.
[18] L.J. Fono, D.L. Sedlak, Environ. Sci. Technol. 39 (2005) 9244.
[19] S.L. MacLeod, P. Sudhir, C.S. Wong, J. Chromatogr. A 1170 (2007) 23.
[20] T.J. Ward, B.A. Baker, Anal. Chem. 80 (2008) 4363.
[21] A. Ghassempour, H.Y. Aboul-Enein, J. Chromatogr. A 1191 (2008) 182.
[22] R. Kaliszan, A. Nasal, M. Turowski, Biomed. Chromatogr. 9 (1995) 211.
[23] M. Enquist, J. Hermansson, J. Chromatogr. 519 (1990) 285.
[24] C. Vandenbosch, D.L. Massart, W. Lindner, J. Pharm. Biomed. Anal. 10 (1992) 895.
[25] J. Hermansson, I. Hermansson, J. Chromatogr. A 666 (1994) 181.
[26] K. Gyimesiné Forrás, Acta Pharm. Hung. 72 (2002) 205.
[27] A. Lajeunesse, C. Gagnon, S. Sauveĭ, Anal. Chem. 80 (2008) 5325.
[28] E. Gracia-Lor, J.V. Sancho, F. Hernández, J. Chromatogr. A 1217 (2010) 622.
[29] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[30] International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceutical for Human Use, Validation of Analytical Procedures: Text and Methodology Q2(R1), Geneva, 2005.
[31] Guidance for Industry Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), Rockville, 2001.
[32] S. Babić, D. Mutavdžić Pavlović, D. Ašperger, M. Periša, M. Zrnčić, A. Horvat, M. Kaštelan-Macan, Anal. Bioanal. Chem. 398 (2010) 1185.
[33] A.L.N. van Nuijs, I. Tarcomnicu, L. Bervoets, R. Blust, P. Jorens, H. Neels, A. Covaci, Anal. Bioanal. Chem. 395 (2009) 819.
[34] M.M. Schultz, E.T. Furlong, Anal. Chem. 80 (2008) 1756.
[35] M. Gros, M. Petrovicĭ, D. Barceloĭ, Anal. Chem. 81 (2008) 898.
[36] N.M. Vieno, T. Tuhkanen, L. Kronberg, J. Chromatogr. A 1134 (2006) 101.
[37] J.C. Van De Steene, W.E. Lambert, J. Chromatogr. A 1182 (2008) 153.
[38] A.L. Batt, M.S. Kostich, J.M. Lazorchak, Anal. Chem. 80 (2008) 5021.
[39] E. Arvidsson, S.O. Jansson, G. Schill, J. Chromatogr. 591 (1992) 55.
[40] S. Allenmark, Chromatographic Enantioseparation: Methods and Applications, 2nd ed., Ellis Horwood Series in Analytical Chemistry, London, 1991.
[41] H. Xuan, D.S. Hage, J. Sep. Sci. 29 (2006) 1412.
[42] D. Vukmanic, M. Chiba, J. Chromatogr. 483 (1989) 189.
[43] T. Michishita, P. Franco, T. Zhang, J. Sep. Sci. 33 (2010) 3627.
[44] K. Balmér, B.-A. Persson, G. Schill, J. Chromatogr. 477 (1989) 107.
[45] S. Allenmark, B. Bomgren, H. Borén, J. Chromatogr. 316 (1984) 617.
[46] S. Hjerten, Methods Biochem. Anal. 27 (1981) 89.
[47] N.C. Hughes, E.Y.K. Wong, J. Fan, N. Bajaj, AAPS J. 9 (2007) E353.