Journal of Chromatography A, 1227 (2012) 105–114

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

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

Trace analysis of fluoxetine and its metabolite norfluoxetine. Part II: Enantioselective quantification and studies of matrix effects in raw and treated wastewater by solid phase extraction and liquid chromatography–tandem mass spectrometry☆

Victoria K.H. Barclay[a], Niklas L. Tyrefors[a], I. Monika Johansson[a,b], Curt E. Pettersson[a,*]

[a] Division of Analytical Pharmaceutical Chemistry, Uppsala University, BMC Box 574, SE-751 23 Uppsala, Sweden
[b] Medical Products Agency, Box 26, SE-751 03 Uppsala, Sweden

ARTICLE INFO

Article history:
Received 14 October 2011
Received in revised form
20 December 2011
Accepted 23 December 2011
Available online 5 January 2012

Keywords:
Matrix effects
LC–MS/MS
Chiral analysis
Wastewater
Isotope-labeled compound
Fluoxetine and norfluoxetine

ABSTRACT

The isotope-labeled compounds fluoxetine-d_5 and norfluoxetine-d_5 were used to study matrix effects caused by co-eluting compounds originating from raw and treated wastewater samples, collected in Uppsala, Sweden. The matrix effects were investigated by the determination of matrix factors (MF) and by a post-column infusion method. The matrix factors were determined to be 38–47% and 71–86% for the enantiomers of norfluoxetine-d_5 and fluoxetine-d_5, respectively. The influence of matrix effects when quantifying the enantiomers of the active pharmaceutical ingredient and the metabolite in wastewater samples with LC–MS/MS is discussed and methods to overcome the problem are presented. The enantiomeric concentrations of fluoxetine and its human metabolite norfluoxetine, quantified by a one-point calibration method, were 12–52 pM (3.5–16 ng L^{-1}) in raw wastewater and 4–48 pM (1.2–15 ng L^{-1}) in treated wastewater. Furthermore, the calculated enantiomeric fractions (EF) of the substances were found to be between 0.68 and 0.71 in both matrices. Neither the EF values for fluoxetine nor those for norfluoxetine were significantly different in the raw wastewater compared to the treated wastewater. Interestingly, the concentration of (*S*)-fluoxetine was found to be higher than the concentration of (*R*)-fluoxetine in both raw and treated wastewater. These results are different from other results presented in the literature, which shows that the relative concentrations of the enantiomers of a chiral active pharmaceutical ingredient might be significantly different in wastewater samples from different treatment systems. We report, for the first time, the concentrations of the enantiomers of norfluoxetine in wastewater samples. The concentrations of (*S*)-norfluoxetine were found to be higher than the concentration of (*R*)-norfluoxetine in the raw as well as in the treated wastewater samples.

1. Introduction

Fluoxetine (launched as Prozac® by Eli Lilly) is a common selective serotonin re-uptake inhibitor (SSRI) and one of the most frequently prescribed antidepressant drugs [1]. Fluoxetine is one of several active pharmaceutical ingredients (API) that have been detected and quantified at trace level concentrations in the aquatic environment [2–5]. Nowadays, it is widely known that pharmaceutical residues and their metabolites are not completely eliminated during their passage through sewage treatment plants. Consequently, many APIs and human metabolites are released by the sewage treatment plants to the receiving surface water and to the aquatic environment [6,7]. Fluoxetine and its metabolite norfluoxetine have been quantified in Europe and North America in both raw and treated wastewater [2,4,5]. These substances have also been quantified in river water [4,8]. Moreover, fluoxetine has been found in several U.S. streams [9] and even in drinking water [10].

The distribution, seasonal variations and fate of pharmaceutical residues in the aquatic environment are currently under scrutiny. Environments like these often contain high amounts of interferences and matrix compounds, and generally only trace amounts of the target analytes [11]. Mass spectrometry is considered to be the technique of choice for the detection of APIs in the aquatic environment owing to its inherent selectivity and sensitivity. However, matrix effects, caused by co-eluting matrix compounds, might result in signal suppression of the analyte response in the MS interface. These suppression effects are probably mainly caused by the presence of non-volatile compounds in the spray with the analyte

☆ Parts of this manuscript were presented as a poster at the 59th ASMS Conference on Mass Spectrometry and Allied Topics in Denver, CO, USA, June 5–9, 2011.

* Corresponding author. Tel.: +46 18 4714340; fax: +46 18 4714393.
E-mail address: Curt.Pettersson@farmkemi.uu.se (C.E. Pettersson).

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.12.084

[12]. Any changes in the signal response of the target analytes might decrease the overall accuracy of the method, i.e. faulty analytical results would be obtained. However, matrix effects can be reduced by efficient removal of the matrix compounds from the samples [12] or compensated for, but not eliminated, by the use of stable isotope-labeled internal standards during quantification [13,14].

Nowadays it is widely known that the enantiomers of an API or a metabolite can have different pharmacological properties, for example different potency [15] and moreover different toxicological profiles. However, the enantiomers might also possess different eco-toxicological effects on aquatic living organisms [16,17]. It is, therefore, of great importance to consider the concentrations of the separate enantiomers when conducting eco-toxicological risk assessments for aquatic living organisms. Interestingly, the enantiomeric fractions of some APIs have been shown to be altered during their passage through sewage treatment plants. For example, the β-blocker propranolol was reported to be racemic in influent (raw) wastewater but non-racemic in effluent (treated) wastewater [18]. A further reason for conducting enantioselective quantifications of pharmaceutical residues in the aquatic environment is that the enantiomeric fraction (EF) of APIs might be used as a marker for biologically mediated degradation [19]. Although there are several reasons for the interest in chiral analysis of APIs in environmental samples, the knowledge of the environmental occurrence, fate and effects of the separate enantiomers of APIs and metabolites is still scarce. The reason for this might partly be that there are several analytical challenges for the enantioselective quantifications of APIs in complex environmental matrices (e.g. wastewater) and today only few chiral analytical methods for environmental trace-level determinations have been developed [11,20]. Furthermore, even fewer enantioselective analytical methods have been developed for studying chiral metabolites in the aquatic environment [21,22]. Even so, as metabolites might be biologically active, persistent and contribute to the overall ecotoxicity, they should also be considered in environmental risk assessments and in drug monitoring programs.

The (*R*)- and (*S*)-enantiomers of fluoxetine have approximately equipotent pharmacological effects [23]. In the aquatic environment, however, the (*S*)-enantiomer has been shown to be more toxic to a teleost fish than the (*R*)-enantiomer [16], whereas (*R*)-fluoxetine is more toxic to a crustacean than to a specific protozoan species [17]. The commercially available pharmaceutical product of fluoxetine contains a racemic mixture. In humans, fluoxetine is metabolized by *N*-demethylation to the pharmacologically active metabolite norfluoxetine. (*S*)-Norfluoxetine is just as potent as the parent compound and about 20 times more potent as an SSRI than (*R*)-norfluoxetine [15,24]. The separate enantiomers of fluoxetine have been quantified in raw and treated wastewater from a Canadian wastewater treatment plant [3]. The enantiomers were likely to be subjected to biologically mediated treatment, as the relative concentration of (*R*)-fluoxetine to (*S*)-fluoxetine was higher in raw wastewater than in treated wastewater [3].

The aim of this study was to evaluate the matrix effects caused by the matrix compounds in the wastewater samples on the responses of the enantiomers of fluoxetine and norfluoxetine in the mass spectrometry ionization source. An advantage by using the isotope-labeled compounds, fluoxetine-d_5 and norfluoxetine-d_5, which are not expected to be found in environmental matrices, is that the matrix effects can be determined in the actual matrix in which the target compounds are quantified. The validated method was applied for quantification and determination of the enantiomeric composition of fluoxetine and the active metabolite norfluoxetine, in raw and treated wastewater. To the best of the authors' knowledge, the enantiomers of the active metabolite norfluoxetine have not previously been quantified in wastewater samples.

2. Materials and methods

2.1. Chemicals and stock solutions

(*R*,*S*)-Fluoxetine hydrochloride (analytical standard, Riedel-de Haën), (*R*,*S*)-norfluoxetine hydrochloride (≥97%), *S*-(+)-fluoxetine hydrochloride (≥98%) and (*R*,*S*)-[$^2H_5$]-fluoxetine in methanol (fluoxetine-d_5, drug standard grade, isotopic purity; 98%, Isotec stable isotopes) were all purchased from Sigma–Aldrich (St Louis, MO, USA). (*R*,*S*)-[$^2H_5$]-Norfluoxetine hydrochloride (norfluoxetine-d_5, 98%, isotopic purity; 99%) and (*S*)-norfluoxetine (98%) were acquired from Toronto Research Chemicals Inc. (North York, Canada). Formic acid (*p.a.*) was from Acros Organics (Morris Plains, NJ, USA), glacial acetic acid (*p.a.*) and ammonia solution (25%, *p.a.*) were purchased from Merck (Darmstadt, Germany). Ammonium acetate (analytical reagent grade) was obtained from Fisher Scientific UK Limited (Loughborough, UK). The organic solvent acetonitrile (E Chromasolv® for HPLC) was obtained from Sigma–Aldrich (St Louis, MO, USA) and methanol (for HPLC, isocratic grade) was bought from BDH, Prolabo (VWR International LLC, West Chester, PA, USA).

Stock solutions of the racemic standards (*R*,*S*)-fluoxetine hydrochloride, (*R*,*S*)-norfluoxetine hydrochloride, (*R*,*S*)-fluoxetine-d_5, (*R*,*S*)-norfluoxetine-d_5 as well as (*S*)-norfluoxetine were all prepared in methanol. Ethanol (Etax Aa, 99.7% v/v, Altia Corporation, Rajamäki, Finland) was used for the preparation of a stock solution of (*S*)-fluoxetine hydrochloride. These stock solutions were stored in the freezer (−18 °C). The working standards were prepared by diluting the stock solutions with a mixture of methanol and Millipore water (20:80, v/v). The working standards were stored at 4 °C in the dark.

2.2. Experimental

2.2.1. Sample preparation and solid phase extraction

Grab samples of influent (raw) and effluent (treated) wastewater were collected in amber glass bottles at Kungsängsverket, a municipal wastewater treatment system located in Uppsala, Sweden. The plant receives water from approximately 160 000 inhabitants and the sewage is treated by mechanical, biological and chemical purification. The collected raw and treated wastewater samples were filtered through a glass fiber filter with a pore size of 0.7 μm (purchased from Millipore, Billerica, MA, USA). During the quantification studies, filtration of the water samples was carried out within 3 h of sampling. After the fortification of standards and internal standards, the pH of the water samples was adjusted to 4 with formic acid in Millipore water (50:50, v/v). The samples were stored at 2 °C until subjected to solid phase extraction, which was conducted within 30 h of sampling. Samples of 200.0 mL of raw or 500.0 mL of treated wastewater were extracted using Evolute CX-50 cartridges (200 mg, 6 mL) obtained from Biotage (Uppsala, Sweden). Each cartridge was conditioned with methanol (6.0 mL), Millipore water (6.0 mL) and then equilibrated with formic acid in Millipore water (6.0 mL, 2:98, v/v), after that, the wastewater samples were applied to the Evolute CX-50 cartridges with a flow rate of approximately 5 mL min^{-1}. The cartridges were washed twice, once with formic acid in Millipore water (6.0 mL, 2:98, v/v) and then, again, with methanol (4.0 mL). The analytes were eluted with a mixture of methanol and 25% ammonia solution (8.0 mL, 95:5, v/v,) and the extracts were then evaporated to dryness at 40 °C under a gentle steam of nitrogen. Mobile phase (250 μL) was used to reconstitute the dried residues, and each sample was filtered through disposable PVDF (polyvinylidene fluoride) syringe filters with a pore size of 0.45 μm (Whatman Inc, Piscataway, NJ, USA). The procedure for the sample preparation and the method

development of the solid phase extraction are described in detail in Part I of this study [21].

The glassware utilized for the environmental samples was washed in a laboratory glassware washer (Miele G7783, Miele, Inc. Gutersloh, Germany) before usage. The washing process included two prewashing cycles, one washing step (with a maximum temperature of 85 °C), four rinse cycles and one drying step. The detergent used was Neodisher FLA (Chemische Fabrik Dr. Weigert GmbH & Co. KG, Hamburg, Germany).

2.2.2. Chiral LC–MS/MS analysis

The chromatographic system used was an Agilent 1100 HPLC system equipped with a degasser, binary pump and an auto sampler from Agilent Technologies Inc. (Palo Alto, CA, USA). The chiral analyses were performed using a chiral AGP column (100 mm × 2.0 mm, with 5 μm particle size) from ChromTech Ltd (Congleton, UK). Two AGP columns (batch numbers 96-09 and 1030) were used in this study. In order to protect the analytical AGP column from high affinity impurities and small particles, an 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 column. The mobile phase composition was a mixture of 10 mM ammonium acetate buffer (pH 4.4) and acetonitrile (97:3, v/v). The flow rate and the injected sample volume were set to 0.22 ml min^{-1} and 10 μL, respectively. The chiral separations were conducted at ambient temperature.

The analytes were detected by a Quattro Micro mass spectrometer (Waters Corporation, Milford, MA, USA) with an electrospray ionization (ESI) interface working in positive ion mode. The peaks were recorded in selected reaction monitoring (SRM) mode. The precursor and product ions for the SRM transitions (m/z) were optimized as follows; fluoxetine 310 → 44, fluoxetine-d_5 315 → 44, norfluoxetine 296 → 134 and norfluoxetine-d_5 301 → 139, and the dwell time was set to 0.25 s. The capillary voltage was set to 3.0 kV, the cone voltage to 15 or 18 V and the collision energy was varied between 6 and 11 V. The desolvation temperature and desolvation flow rate were set to 450 °C and to 13 × 10^3 mL min^{-1}, respectively. The cone gas was used at a flow rate of 1.7 × 10^3 mL min^{-1} and the source temperature was set to 100 °C. Nitrogen was utilized as the nebulizer, desolvation and cone gas and argon was used for collision induced dissociation in the collision cell. The MassLynx software program 4.1 (Waters Corporation, Milford, MA, USA) was employed for the data acquisition and peak integration. For detailed information about the development of the analytical method, see Part I of this study [21].

2.2.3. Determination of matrix effects

Possible suppression of ionization in the electrospray ion source caused by the co-elution of the analytes in the chromatographic system was investigated. The peak areas of fluoxetine or norfluoxetine, at a racemic concentration of 0.10 μM, were plotted as a function of increasing concentration (up to 2.0 μM) of the isotope-labeled standards, i.e. fluoxetine-d_5 or norfluoxetine-d_5. In addition to this, the ratio of fluoxetine to fluoxetine-d_5 was plotted as a function of increasing concentration of norfluoxetine-d_5 and the ratio of norfluoxetine to norfluoxetine-d_5 was plotted as a function of increasing concentration of fluoxetine-d_5. The experiments were conducted by injecting the standards dissolved in the mobile phase (n = 3). To evaluate whether the analytes were subjected to signal suppression, the 95% confidence interval of the slopes (b) obtained from the plots was calculated. An analyte was considered to be subjected to signal suppression if the slope was significantly different from zero.

Ion suppression effects, in the mass spectrometer ion source, caused by matrix ions from the environmental samples were evaluated using two different protocols. In both protocols, unspiked raw and treated wastewater samples were extracted by the Evolute CX-50 SPE cartridges according to the method described in Section 2.2.1. These extracted samples of raw and treated wastewater were used as blank matrices as the ion suppression effects were evaluated for fluoxetine-d_5 and norfluoxetine-d_5, which were not found in the wastewater samples.

The first protocol involved post column continuous infusion [25] of 0.50 μM fluoxetine-d_5 or 0.50 μM norfluoxetine-d_5 in the mobile phase conducted with the aid of the inbuilt syringe pump on the Quattro Micro mass spectrometer. The infusion flow rate was set to 10 μL min^{-1} and the infused compound was mixed with the HPLC column effluent (0.22 ml min^{-1}) in a tee before entering the ESI interface and the mass spectrometer. One sample of the mobile phase, or extracted raw or treated wastewater was injected into the LC system, the injected sample volume was set to 10 μL. The SRM response for the infused compound was monitored for 30 min from the point of sample injection of the blank matrix.

In the second protocol, the responses of fluxetine-d_5 or norfluoxetine-d_5 in the presence of matrix ions were compared with the responses of fluoxetine-d_5 and norfluoxetine-d_5 dissolved in the mobile phase. The dry residues from extracted raw or treated wastewater were reconstituted in 250 μL of 0.50 μM fluoxetine-d_5 or 0.50 μM norfluoxetin-d_5 dissolved in the mobile phase. The samples were filtered through the PVDF syringe filters and injected into the LC–MS/MS system. Each sample was injected three times. As references, filtered standard solutions of 0.50 μM fluoxetine-d_5 or 0.50 μM norfluoxetine-d_5 in mobile phase, were injected (n = 3) into the LC–MS/MS system. The matrix factors (MF) were calculated by dividing the peak area of the compound in the presence of matrix ions by the peak area achieved from the compound dissolved in the mobile phase [26,27].

2.2.4. Quantification of the enantiomers of fluoxetine and norfluoxetine in raw and treated wastewater

A one-point calibration method was used to measure the enantiomers of fluoxetine and norfluoxetine in raw and treated wastewater. The analytes were quantified by comparing the peak area of the "naturally" occurring enantiomer with the peak area of the respective isotope-labeled compound, for which the concentration was known. The first eluted peaks of fluoxetine-d_5 and norfluoxetine-d_5 were used to quantify the first eluted enantiomers of fluoxetine and norfluoxetine, respectively. Furthermore, the second eluted peaks of fluoxetine-d_5 and norfluoxetine-d_5 were used to quantify the second eluted enantiomers of fluoxetine and norfluoxetine. Volumes of 200 mL filtered raw wastewater samples (n = 6) and 500 mL of filtered treated wastewater samples were spiked with fluoxetine-d_5 and norfluoxetine-d_5 to a concentration of the separate enantiomers of 250 pM. The wastewater samples were extracted and analyzed by SPE and LC–MS/MS, as described in Sections 2.2.1 and 2.2.2. Thus, the enantiomers of fluoxetine and norfluoxetine were quantified by a direct comparison of the peak areas of the analyte and the peak area of the isotope-labeled compound for which the concentration were known to be 250 pM. During the quantification studies, procedural blanks of 200 mL or 500 mL Millipore water were extracted in parallel with the spiked wastewater samples to detect possible cross-contamination during the sample handling.

The linearity expressed as the correlation coefficient (R^2) of the calibration curves was determined to confirm that the response of the enantiomers of fluoxetine and norfluoxetine in extracted wastewater samples was linear, within the expected concentration interval. Filtered raw and treated wastewater was divided into aliquots of 200 or 500 mL, respectively. The water samples were spiked with standards of (*R*,*S*)-fluoxetine and (*R*,*S*)-norfluoxetine to five concentrations, zero (no addition), 125, 250, 375 and

500 pM, for the single enantiomers of each substance. Duplicates of samples were prepared at each concentration. Fluoxetine-d_5 and norfluoxetine-d_5 were added as internal standards to a concentration of 250 pM of the separate enantiomers. The first eluted enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 were used as internal standards (IS) for the first eluted enantiomers of fluoxetine and norfluoxetine, respectively. In the same way, the second eluted enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 were used as IS for the second eluted enantiomers of fluoxetine and norfluoxetine, respectively. The samples were extracted and analyzed as described in Sections 2.2.1 and 2.2.2. The linearity was determined by plotting the ratio of the peak area of the analyte to the peak area of the IS as a function of the added concentration of the analyte in the wastewater samples.

2.2.5. Calculation of enantiomeric compositions

The enantiomeric fraction was used as a measurement of the enantiomeric composition of fluoxetine, norfluoxetine and the isotope-labeled compounds in raw and treated wastewater samples as well as in standards of mobile phase. In addition, the enantiomeric excess was determined for the "naturally" occurring fluoxetine and norfluoxetine in the water samples. The enantiomeric fractions (EF) were calculated by the use of Eq. (1), in which the peak area or concentration of the (*S*) and (*R*)-enantiomers of fluoxetine, norfluoxetine and the isotope-labeled standards are denoted by (*S*) and (*R*), respectively.

$$\mathrm{EF} = \frac{(S)}{(S)+(R)} \quad (1)$$

The EF values calculated from the peak areas are denoted EF_a and the EF values calculated by the use of the concentrations are denoted EF_c. Statistical comparisons between mean EF values in different matrices were evaluated by two-tailed *t*-tests at the 0.05 level.

The enantiomeric excess (e.e.) of fluoxetine and norfluoxetine was determined as $|F(S) - F(R)|$, where $F(S)$ was the mole fraction of (*S*)-fluoxetine or (*S*)-norfluoxetine and $F(R)$ was the mole fraction of (*R*)-fluoxetine or (*R*)-norfluoxetine.

3. Results and discussion

In Part I of this study [21], the SPE method (using Evolute CX-50 cartridges) and the chiral chromatographic separation (using the CSP AGP) and mass spectrometric detection method was developed. The method was validated with respect to; extraction recoveries for the enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 in raw and treated wastewater samples, method accuracy, interassay precision of the chiral separation system in wastewater matrices, method detection limit (MDL), method quantification limit (MQL), cross-contamination, carryover, cross-talk and isotopic purity of fluoxetine-d_5 and norfluoxetine-d_5 [21].

In this part of the study, Part II, we extend the validation and evaluate the matrix effects in the mass spectrometric electrospray ionization source caused by the (I) co-elution of the analytes and the isotope-labeled standards and (II) the matrix compounds in the extracted raw and treated wastewater samples. The latter is explored by post-column infusion as well as by determination of the matrix factors. The effect of the matrix compounds on the separate enantiomers is furthermore evaluated by comparing the enantiomeric fractions of fluoxetine-d_5 and norfluoxetine-d_5 in raw and treated wastewater with the enantiomeric fractions in matrix free samples. Finally, the matrix effects are compensated for by the use of the isotope-labeled internal standards and the concentrations and the enantiomeric composition of the "naturally" occurring enantiomers of fluoxetine and norfluoxetine in raw and treated wastewater is reported.

Table 1
Retention times for the analytes in raw and treated wastewater. Relative standard deviations (RSD %, $n = 6$) for "naturally" occurring enantiomers of fluoxetine and norfluoxetine in raw and treated wastewater samples. The enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 were used for identification of the enantiomers of fluoxetine and norfluoxetine. The experimental conditions are described in Sections 2.2.1 and 2.2.2.

Compound	#	Raw wastewater[a]		Treated wastewater[b]	
		t_R (min)	RSD %	t_R (min)	RSD %
First eluting enantiomers					
(*S*)-Fluoxetine-d_5	*1*	5.6	1.0	4.6	0.5
(*S*)-Fluoxetine	*2*	5.7	1.2	4.7	0.6
(*S*)-Norfluoxetine-d_5	*3*	5.5	0.9	4.7	0.5
(*S*)-Norfluoxetine	*4*	5.6	1.0	4.8	0.2
Second eluting enantiomers					
(*R*)-Fluoxetine-d_5	*5*	8.7	1.1	6.8	0.8
(*R*)-Fluoxetine	*6*	9.0	1.6	7.0	0.8
(*R*)-Norfluoxetine-d_5	*7*	8.8	0.9	7.1	0.8
(*R*)-Norfluoxetine	*8*	9.0	1.8	7.3	1.8

[a] Chiral AGP, batch number 96-09.
[b] Chiral AGP, batch number 1030.

3.1. Criteria for positive identification of the enantiomers of fluoxetine and norfluoxetine in wastewater samples

In the present study, the α_1-acid glycoprotein column (chiral AGP) was used to separate the enantiomers of the target analytes. The chromatographic method was developed in Part I of this study and the mobile phase was acetonitrile and 10 mM ammonium acetate buffer at pH 4.4 (3/97, v/v). (*S*)-Fluoxetine was found to elute before (*R*)-fluoxetine [21] and the elution order of norfluoxetine was in the present study determined to be the (*S*)-enantiomer before the (*R*)-enantiomer.

The SRM transitions in the tandem mass spectrometer and the retention times in the SRM chromatograms were used to identify the peaks of the "naturally" occurring (*S*)-fluoxetine, (*R*)-fluoxetine, (*S*)-norfluoxetine and (*R*)-norfluoxetine. The retention times of the enantiomers present in the wastewater samples were directly compared with the retention times of the spiked isotope-labeled enantiomers from the same chromatographic run (Table 1). The average difference between the "naturally" occurring enantiomers and the labeled ones ranged between 1.8 and 3.3% in raw wastewater and between 0.9 and 3.1% in treated wastewater. Moreover, the first eluting enantiomers of fluoxetine, fluxoetine-d_5, norfluoxetine and norfluoxetine-d_5 (compounds *1–4*, Table 1 and Fig. 1) all co-eluted. Co-elution of the second enantiomers of the four target analytes was also observed (compounds *5–8*, Table 1 and Fig. 1). Hence, the first four and the four last eluted enantiomers, (*1–4*) and (*5–8*), respectively, were separated according to their *m*/*z* ratio in the mass spectrometer. Consequently, the MS/MS detector was set to scan over the chosen variable parameters and mass ranges (as explained in Section 2.2.2.) for the four analytes in the same time window. Two SRM transitions of each target analyte are often used for positive identification. However, for the benefit of additional data points in the chromatograms only one SRM transition was used for each compound. The exclusion of an identifier ion was also considered to be acceptable since the retention and SRM transitions of the isotope-labeled standards could be used for the identification of the enantiomers of fluoxetine and norfluoxetine present in the wastewater samples.

3.2. Determination of matrix effects in the electrospray ion source

3.2.1. Determination of ion suppression effects caused by the co-elution of analytes and isotope-labeled standards

It is of great importance to investigate possible matrix effects on the target analytes in the LC–MS interface. Thus, the co-elution of

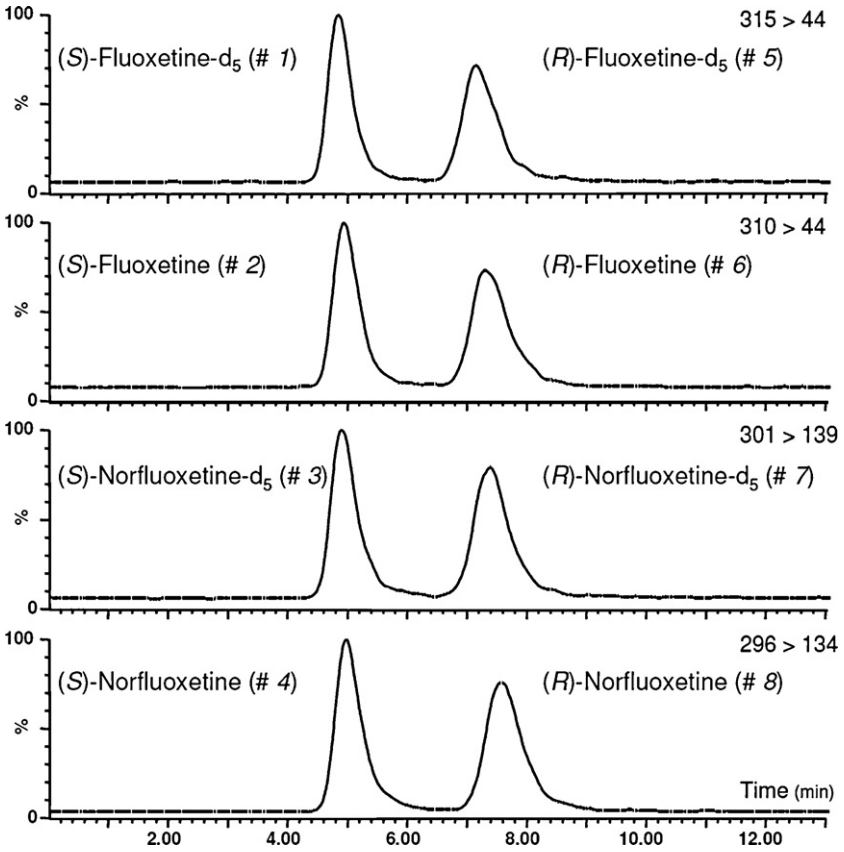

Fig. 1. LC–MS/MS chromatograms of (*R*,*S*)-fluoxetine-d_5, (*R*,*S*)-fluoxetine, (*R*,*S*)-norfluoxetine-d_5 and (*R*,*S*)-norfluoxetine. The compound numbers (*1*–*8*) are given in brackets. The experimental details are presented in Section 2.2.2.

the respective first or second eluted enantiomers of fluoxetine and norfluoxetine, together with the co-elution of the isotope-labeled compounds (compounds *1*–*4* and *5*–*8*, respectively, in Table 1 and Fig. 1), was studied with respect to the suppression of ionization in the electrospray source.

No visible trend, in terms of a decrease in the peak areas, was found for the enantiomers of fluoxetine (*2*, *6*) as the concentration of fluoxetine-d_5 (*1*, *5*) or norfluoxetine-d_5 (*3*, *7*) was increased. A representative plot is given in Fig. 2A in which the peak area of (*R*)-fluoxetine (*6*) was plotted as a function of the concentration of (*R*)-norfluoxetine-d_5 in the injected sample (*7*). It was also found that the peak areas of the enantiomers of norfluoxetine (*4*, *8*) were not suppressed as increased concentrations of norfluoxetine-d_5 (*3*, *7*) co-eluted with norfluoxetine (data not shown). However, increased concentrations of the fluoxetine-d_5 enantiomers (*1*, *5*) resulted in slightly decreased peak areas of the enantiomers of norfluoxetine (*4*, *8*), as can be seen for (*S*)-norfluoxetine (*4*) in Fig. 2B. The peak area of (*S*)-norfluoxetine (*4*) was suppressed by 13% as the concentration of (*S*)-fluoxetine-d_5 (*1*) increased from zero to 1.0 μM.

The peak area ratio of (*S*)-norfluoxetine to (*S*)-norfluoxetine-d_5 (*4*/*3*) remained constant within the studied concentration interval for (*S*)-fluoxetine-d_5 (*1*) as the slope (*b*) in the plot was not significantly different from zero ($b = 0.039 \pm 0.058$), Fig. 2C. Thus, the reduction in the response of norfluoxetine (*4*, *8*), caused by increasing the concentrations of fluoxetine-d_5 (*1*, *5*), could be compensated for by the use of norfluoxetine-d_5 (*3*, *7*) as the internal standard. These observations are in agreement with other studies where it has been shown that mutual suppression between active pharmaceutical ingredients and their corresponding isotope-labeled standards occurs in ESI-MS [14]. The ratio of the peak areas of (*R*)-norfluoxetine to (*R*)-norfluoxetine-d_5 (*8*/*7*) was also found to remain constant ($b = -0.013 \pm 0.039$) with increasing concentrations of (*R*)-fluoxetine-d_5 (*5*). Thus, the observed suppression of ionization for norfluoxetine by fluoxetine-d_5 should not affect quantification. Liang et al. [28] have shown that the ESI suppression of some drugs by their corresponding co-eluting isotope-labeled standards, could be compensated for by the use of an appropriate concentration of the labeled standard during quantification. Thus, by the use of the labeled standards, the response factors could be kept constant and calibration curves linear. In addition, in the present study, the peak area ratios of (*S*)-fluoxetine/(*S*)-fluoxetine-d_5 (*2*/*1*) and (*R*)-fluoxetine/(*R*)-fluoxetine-d_5 (*6*/*5*) remained constant as the slopes in the plots were not significantly different from zero ($b = 0.025 \pm 0.10$ and $b = 0.049 \pm 0.063$, respectively) as the concentration of co-eluting (*S*)-norfluoxetine-d_5 (*3*) or (*R*)-norfluoxetine-d_5 (*7*) was raised (plots not shown).

The enantiomeric fractions based on concentrations (EF_c, Eq. (1)) for fluoxetine (*2*, *6*) and norfluoxetine (*4*, *8*) were, as expected, not affected by increasing the concentrations of norfluoxetine-d_5 (*3*, *7*) and fluoxetine-d_5 (*1*, *5*), respectively. The average EF_c values were 0.50 ($n = 18$) for fluoxetine (3.3 RSD %, plot not shown) as well as for norfluoxetine (2.9 RSD %, Fig. 2C), as the concentration of the respective norfluoxetine-d_5 (*3*, *7*) or fluoxetine-d_5 (*1*, *5*) enantiomers was increased.

In conclusion, the peak area ratios of the analyte to the corresponding isotope-labeled standards remained constant as the concentration of the co-eluting compound, i.e. fluoxetine-d_5 or norfluoxetine-d_5, was increased, as did the EF_c values.

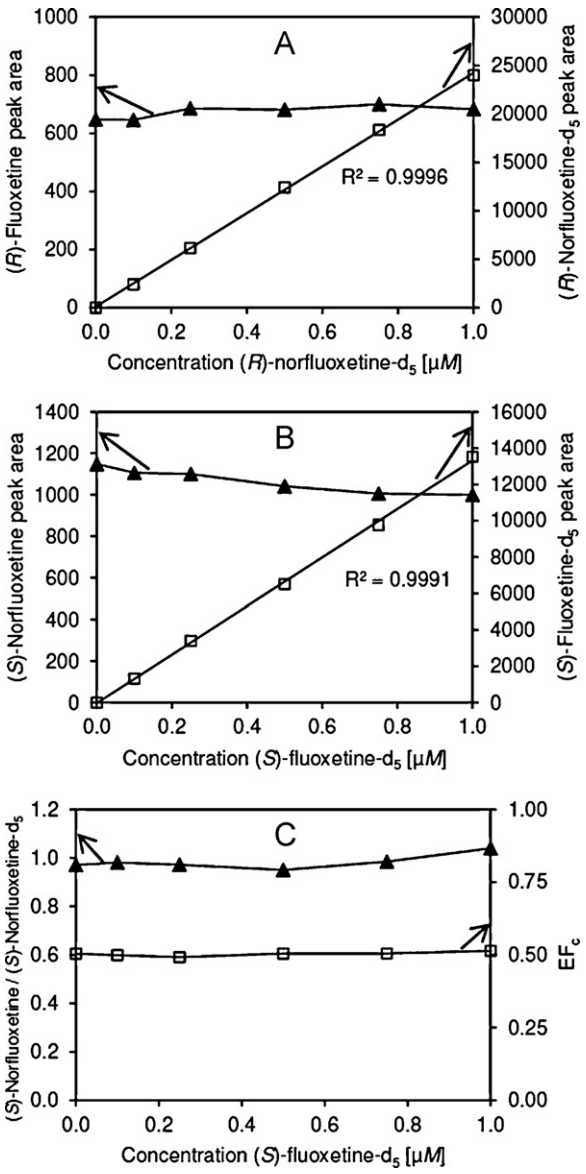

Fig. 2. Determination of ion suppression caused by the co-elution of analytes and the isotope-labeled standards. (A) Mean peak areas of (*R*)-fluoxetine (filled triangles, $n = 3$) at a concentration of 50 nM plotted as a function of increased concentration of co-eluting (*R*)-norfluoxetine-d_5 (open squares). (B) The mean peak areas of (*S*)-norfluoxetine (filled triangles, concentration 50 nM, $n = 3$) were found to decrease as the concentration of the co-eluting (*S*)-fluoxetine-d_5 was increased (open squares). (C) The peak area ratio of (*S*)-norfluoxetine to (*S*)-norfluoxetine-d_5 (triangles) at a constant concentration (50 nM) as well as enantiomeric fraction (EF_c) of norfluoxetine (open squares) plotted as a function of increased concentration of co-eluting (*S*)-fluoxetine-d_5. The experimental conditions are described in Section 2.2.3.

Consequently, the ion suppression caused by the co-elution of the analytes and the internal standards would not affect quantification or the determined EF_c values in e.g. wastewater samples.

3.2.2. Determination of ion suppression effects caused by matrix compounds in the extracted raw and treated wastewater

Two different experimental protocols were employed to study whether the matrix compounds in the wastewater suppressed the ionization of fluoxetine-d_5 and norfluoxetine-d_5. The post-column infusion system [25] provided information about the ion suppression effects as a function of time, i.e. as the interferences eluted from the AGP column. The second protocol was used to calculate the matrix factors (MFs, Section 2.2.3.) [26,27]. The matrix effects were studied on fluoxetine-d_5 and norfluoxetine-d_5 instead of the target analytes, as isotope-labeled compounds have almost the same retention times and ionization efficiency and are subjected to the same ion suppression as their analogs [29]. An important aspect of using the isotope-labeled standards and not the target analytes is that the wastewater can be used as a true blank matrix since the isotope-labeled compounds were neither expected nor found in the wastewater samples.

Table 2
Matrix factors, correlation coefficients and quantified concentrations in raw and treated wastewater. The matrix factors (MF %) for fluoxetine-d_5 and norfluoxetine-d_5 in raw and treated wastewater samples, $n = 3$. Linearity expressed as correlation coefficients (R^2) from calibration curves and mean concentrations [pM] ($n = 6$) of the "naturally" occurring enantiomers of fluoxetine and norfluoxetine in raw and treated wastewater obtained by one-point calibration. Relative standard deviations (RSD %) are given in brackets. Experimental details are described in Sections 2.2.3 and 2.2.4.

Compound	Raw wastewater MF % (RSD %)	Treated wastewater MF % (RSD %)
(*S*)-Fluoxetine-d_5	71 (10)	83 (3)
(*R*)-Fluoxetine-d_5	79 (8)	86 (5)
(*S*)-Norfluoxetine-d_5	47 (2)	38 (4)
(*R*)-Norfluoxetine-d_5	46 (6)	38 (9)

Compound	Raw wastewater R^2	Treated wastewater R^2
(*S*)-Fluoxetine	0.9825	0.9961
(*R*)-Fluoxetine	0.9968	0.9990
(*S*)-Norfluoxetine	0.9936	0.9964
(*R*)-Norfluoxetine	0.9939	0.9926

Compound	Raw wastewater [pM] (RSD %)	Treated wastewater [pM] (RSD %)
(*S*)-Fluoxetine	52 (13)	48 (5)
(*R*)-Fluoxetine	21 (18)	19 (10)
(*S*)-Norfluoxetine	27 (13)	9 (14)
(*R*)-Norfluoxetine	12 (11)	4 (12)

As presumed in the post-column infusion experiment, the injected mobile phase did not cause any suppression of ionization of fluoxetine-d_5 or norfluoxetine-d_5 (Fig. 3A and B). However, the matrix compounds in extracted raw and treated wastewater samples gave rise to ion suppression of fluoxetine-d_5 and norfluoxetine-d_5, and the most pronounced effects on the signals were noted after about 2 min. Thereafter, the SRM responses increased gradually and the recovery time for the ESI signal, i.e. the time taken to return to the non-suppressed level, was about 12 min for fluoxetine-d_5 and about 9 min for norfluoxetine-d_5 (Fig. 3). After the point of recovery, fluctuations in the responses were, however, clearly apparent until about minutes 20 and 23 for norfluoxetine-d_5 and fluoxetine-d_5, respectively. Interestingly, in the present study, there seemed to be no difference in the trends associated with ion-suppression caused by the raw and treated wastewater extracts (Fig. 3A and B). This observation might be attributable to a smaller volume (200 mL) of the raw wastewater having been extracted, with a higher excess of matrix compounds, than the treated wastewater (500 mL) with, most likely, less of the matrix compounds.

During the MF determinations, the chromatographic runtime was set to 30 min as no internal standard could be used to compensate for the fluctuations in the ESI responses that were observed in the post-column infusion chromatograms (Fig. 3). It was noted that the compound that was more polar, norfluoxetine-d_5, was more suppressed than fluoxetine-d_5 in both raw and treated wastewater, Table 2. This observation is in agreement with previous studies where it has been shown that polar analytes are more subjected to ion suppression than less polar ones [25]. Furthermore, the MFs of the enantiomers of norfluoxetine-d_5 ranged between 38 and 47% and between 71 and 86% for the enantiomers of fluoxetine-d_5. In

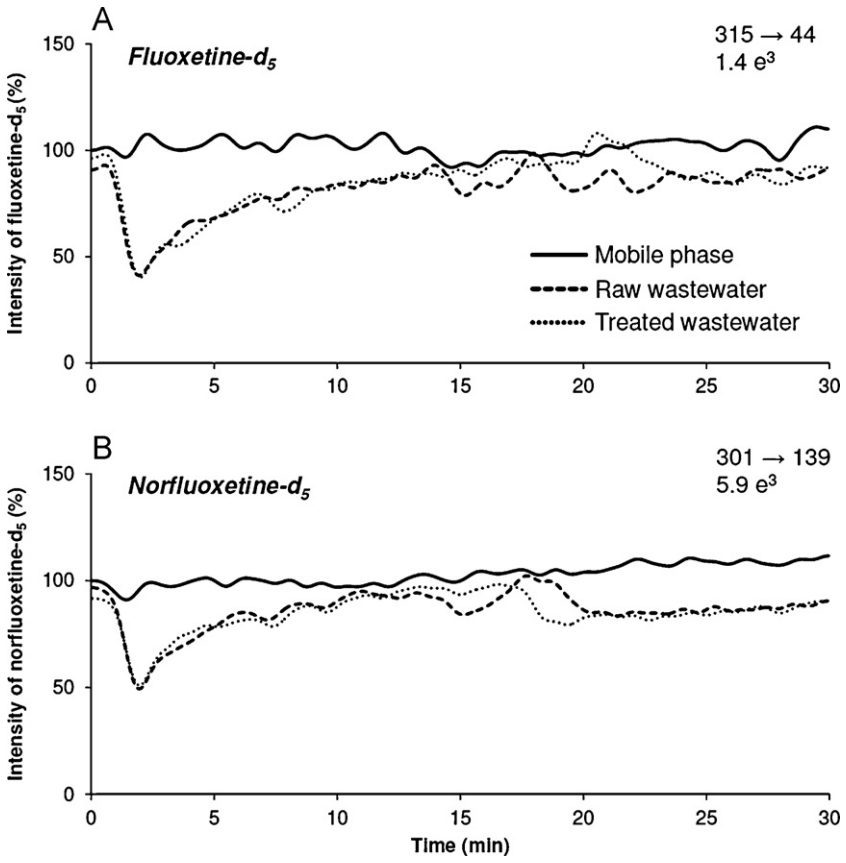

Fig. 3. LC–MS/MS infusion chromatograms of (A) fluoxetine-d_5 and (B) norfluoxetine-d_5. ESI-signals for the infused isotope labeled compounds (0.50 μM) when 10 μL of mobile phase or extracted raw or treated wastewater was injected into the HPLC system. The experimental details are presented in Section 2.2.3.

other studies, ion suppression has been determined for inter alia APIs and herbicides in environmental matrices, and in these studies similar degrees of ion suppression have been observed [29,30]. For some of the analytes considered, the ion suppression (derived by linear regression) has been shown to be over 70% in SPE extracts of wastewater samples [29,30]. In the present study, the highest observed ion suppression was 62% (MF 38%) for norfluoxetine-d_5 in treated wastewater SPE extracts. A disadvantage with high suppression of ionization is that the detection and quantification limits of the methods are increased.

It should be emphasized that in our previous study [21], the method detection limits of the enantiomers of norfluoxetine were significantly decreased, i.e. the peaks of "naturally" occurring norfluoxetine became detectable, when a washing step of 6 mL of methanol was incorporated in the SPE extraction procedure. The washing step most probably lowered the amount of the matrix compounds in the extracts, and therefore, the ion suppression decreased. Thus, the development of selective extraction methods is of great importance when analyzing trace level compounds in complex matrices. However, the effect of ion suppression on quantification can be compensated for by the use of stable isotope-labeled standards [14,28,29].

To summarize, there were no significant differences between the MFs for (*S*)-fluoxetine-d_5 and (*R*)-fluoxetine-d_5 or (*S*)-norfluoxetine-d_5 and (*R*)-norfluoxetine-d_5 in either raw or treated wastewater. However, the enantiomers of norfluoxetine-d_5 were more subjected to ion suppression than the enantiomers of fluoxetine-d_5. Moreover, by post-column infusion of fluoxetine-d_5 and norfluoxetine-d_5 it was concluded that the extracted raw and treated wastewater samples affected the signals of the isotope-labeled compounds in the ESI source for up to 23 min.

3.2.3. Determination of matrix effects on EF_a values for fluoxetine-d_5 and norfluoxetine-d_5

Harner et al. [31] introduced EF as a useful descriptor for the relative concentrations of enantiomers in environmental samples. An enantiomeric mixture with an EF value that is not equal to 0.50 contains an excess of one of the enantiomers over the other (Eq. (1)). However, it must be stressed that the value of the enantiomeric fraction might be highly affected by matrix effects, i.e. ion suppression or ion enhancement, when MS is used for detection. Then, the EF value reflects the changes in matrix effects during the analysis rather than changes in the enantiomeric fraction of the target compounds.

In the presented study, we investigated the effects of the two different matrices, i.e. raw and treated wastewater, on the EF_a values (i.e. EF values based on peak areas) for fluoxetine-d_5 and norfluoxetine-d_5, in comparison with EF_a values for the isotope-labeled compounds in a sample free from matrix compounds, i.e. the mobile phase. Thus, by using fluoxetine-d_5 and norfluoxetine-d_5, which were not found in the wastewater samples, the determined EF_a values could be compared.

To measure the enantiomeric fractions without matrix compounds originating from the raw or treated wastewater samples, the EF_a values were determined for the analytes dissolved in the mobile phase (0.50 μM). The EF_a values were 0.50 for fluoxetine (1.6 RSD %) and norfluoxetine (0.88 RSD %), and 0.51 for fluoxetine-d_5 (1.8 RSD %) and norfluoxetine-d_5 (2.1 RSD %), $n = 10$.

The mean EF_a values for fluoxetine-d_5 in raw and treated wastewater were determined to be 0.45 (8.2 RSD %) and 0.49 (5.7 RSD %), respectively (Fig. 4A). These EF_a values were significantly lower than for the racemic standard dissolved in mobile phase (EF_a 0.51, 1.8 RSD %) ($0.0002 \leq p \leq 0.0006$). The shift in EF_a

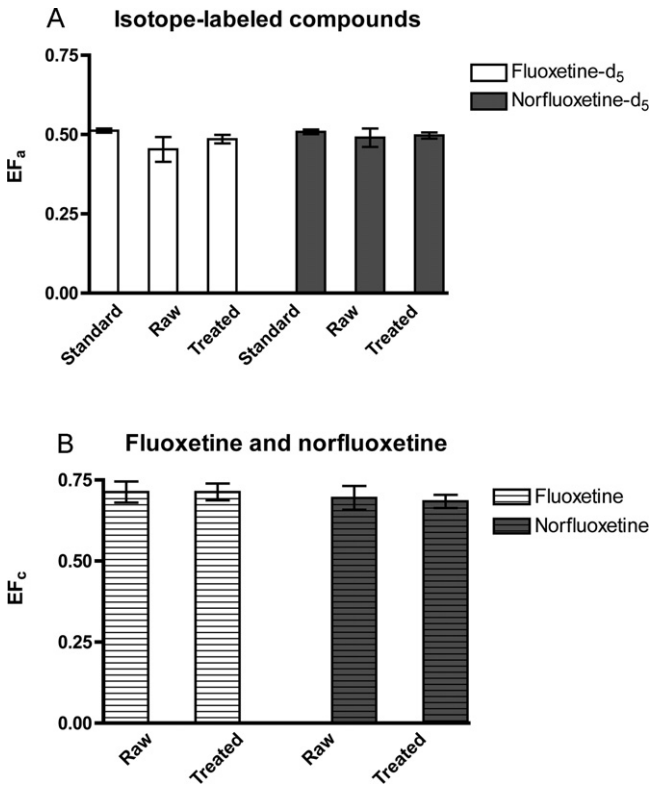

Fig. 4. EF values for the isotope-labeled compounds and "naturally" occurring fluoxetine and norfluoxetine in raw and treated wastewater. The error bars display the 95% confidence intervals. The experimental details are given in Sections 2.2.4 and 2.2.5. (A) Mean EF_a values of fluoxetine-d_5 and norfluoxetine-d_5 dissolved in mobile phase (standard), and in raw and treated wastewaters ($n = 10$ for the standards, $n = 6$ for the raw and the treated wastewater). (B) EF_c values of "naturally" occurring fluoxetine and norfluoxetine in raw and treated wastewaters. The EF_c values were calculated from concentrations obtained by one-point calibration, $n = 6$.

suggests that the matrix affects the enantiomers of fluoxetine-d_5 in different ways. (*S*)-Fluoxetine-d_5 was, consequently, relatively more suppressed than (*R*)-fluoxetine-d_5 by the matrix. Hence, by using the peak areas or concentrations achieved from quantifications without an ideal internal standard (e.g. an isotope-labeled standard), the EF values in complex matrices might be under or overestimated. For norfluoxetine-d_5, the EF_a values in raw wastewater were not significantly different from the EF_a in the mobile phase ($p \geq 0.07$), Fig. 4A. Thus, the enantiomers of norfluoxetine-d_5 in raw wastewater were not affected significantly different by co-eluting interferences from the wastewater matrix. However, the enantiomers of norfluoxetine-d_5 were subjected to different degree of ion suppression by the matrix compounds in the treated wastewater. The obtained EF_a value in treated wastewater was significantly lower than the EF_a obtained for norfluoxetine-d_5 dissolved in mobile phase ($p \leq 0.02$), Fig. 4A.

The shift in the EF_a values between the different matrices indicates that the matrix compounds in the MS interface suppressed the enantiomers differently. However, as described above in Section 3.2.2, there were no significant differences in the MF % values between the enantiomers of fluoxetine-d_5 and norfluoxetine-d_5, respectively. It should be noted that the wastewater used for the experiments in the present section, were not collected on the same date as the matrices used for the MF % determinations. The different results obtained by the matrices obtained from different days suggest that the raw and treated wastewater give rise to different matrix effects on the enantiomers of fluoxetine-d_5 and norfluoxetine-d_5. By the visual inspections of the color of the water, it could be concluded that the composition of the wastewater samples was different from day to day.

In conclusion, the determined EF_a values did not reflect the actual enantiomeric composition of fluoxetine-d_5 in the environmental samples. Thus, matrix effects should be determined and furthermore eliminated or compensated for when enantiomeric compositions are to be reported for active pharmaceutical ingredients and metabolites in wastewater samples.

3.3. Quantification of the enantiomers of fluoxetine and norfluoxetine by one-point calibration

The isotope-labeled standards were used in an extended way for the direct quantification of the fluoxetine and norfluoxetine enantiomers in wastewater samples. According to Peters and Maurer one-point calibration is often used in routine chemical analysis [32]. The one-point calibration approach applied in the present study (experimental described in Section 2.2.4.) used the isotope-labeled standards to compensate for the matrix effects [29] discussed above (Section 3.2). Moreover according to Namiesnik et al. [33], the losses of the isotope-labeled internal standards are of the same order of magnitude as the analyte in every step of the analysis [33]. Another way of performing quantifications in complex matrices such as wastewater, is by the standard addition method. Disadvantages of the standard addition method are, however, that it is time consuming and expensive as solid phase extraction cartridges and other laboratory utensils are consumed at high rate.

A prerequisite for performing one-point calibration is that the concentration-response function is linear. The linearity of the analytical method developed was determined in both raw and treated wastewater samples. The obtained correlation coefficients (R^2) of the standard curves were between 0.9825 and 0.9990 (Table 2) within the concentration interval 0–500 pM. The R^2 values were slightly higher in treated wastewater than in raw water with the exception of (*R*)-norfluoxetine. As the concentration-response functions obtained were linear (calibration curves not shown), one-point quantifications were conducted for (*S*)-fluoxetine and (*S*)-norfluoxetine as well as for (*R*)-fluoxetine and (*R*)-norfluoxetine in the raw and treated wastewater samples. The concentrations of the enantiomers of fluoxetine and norfluoxetine were between 12 and 52 pM (3.5–16 ng L^{-1}) in raw wastewater and between 4 and 48 pM (1.2–15 ng L^{-1}) in treated wastewater (Table 2). The sum of the concentrations of the enantiomers of norfluoxetine was significantly higher in the raw wastewater (39 pM/12 ng L^{-1}) than in the treated wastewater (13 pM/3.8 ng L^{-1}). The raw and treated wastewater samples were not, however, collected on the same date, and consequently do not represent the same plug of water passing through the sewage system. Hence, the concentrations obtained might reflect the efficiency of the sewage treatment or the natural fluctuations of the metabolite concentrations in the wastewater treatment plant. For fluoxetine, no significant difference was found between the concentrations of the sum of the enantiomers in raw (72 pM/22 ng L^{-1}) and treated (67 pM/21 ng L^{-1}) wastewater.

The total concentrations of the respective enantiomers of fluoxetine and norfluoxetine were in the same concentration ranges as found in other studies performed in Europe [2,5] and in North America [3,4]. Interestingly, the predicted environmental concentrations (PEC) of (*R*,*S*)-fluoxetine in raw wastewater in Spain have been determined to be in the range from 80 to 200 ng L^{-1} [34], however the concentrations found in this study were slightly lower.

The precision of the quantifications, given as the RSD, was between 11 and 18% for the raw wastewater ($n = 6$) and between 5 and 14% for the treated wastewater ($n = 6$), Table 2. The RSD values of the determined concentrations were below 15% for all enantiomers in both matrices, with the exception of (*R*)-fluoxetine in raw wastewater, for which the RSD was 18%. All observed RSD

values were, however, within the given precision (RSD) for the lower limit of quantification according to the guidelines for bioanalytical methods given by the Food and Drug Administration (FDA) [35]. Currently, there are no harmonized guidelines for the validation of analytical methods developed to quantify pharmaceutical residues in wastewater samples. However, according to the FDA guidelines for bioanalytical methods [35], the precision, expressed as the relative standard deviation of the determined concentrations, should not exceed 15% with the exception of the lower limit of quantification or sometimes denoted as method quantification limit (MQL), for which the RSD for the measurements must not exceed 20% [35]. The MQLs for the four enantiomers as well as the accuracy of the method in raw and treated wastewater were determined in our previous study [21]. The quantified concentrations of the analytes were higher than those previously determined MQLs, with the exception for (*R*)-norfluoxetine in raw wastewater for which MQL was 14 pM [21].

3.4. Enantiomeric fractions and enantiomeric excess of fluoxetine, norfluoxetine in raw and treated wastewater with compensation for matrix effects

In order to compensate for ion suppression the isotope-labeled compounds were used as internal standards when the EF_c values were calculated for fluoxetine and norfluoxetine present in the wastewater samples. The EF_c values obtained for fluoxetine were 0.71 (e.e. 0.43) in both raw and treated wastewater respectively (Fig. 4B). For norfluoxetine, the EF_c was 0.69 (e.e. 0.39) in raw wastewater and 0.68 (e.e. 0.37) in treated wastewater. It could be concluded that there were no significant differences ($0.5 \leq p \leq 1.0$) between EF_c in raw or treated wastewater for either fluoxetine or norfluoxetine.

Fluoxetine is one of few APIs for which the separate enantiomers have been determined in raw and treated wastewater samples [3]. As already mentioned above, the measured concentrations of fluoxetine in this study were in the same concentration range as those from the study conducted in Canada [3]. The EF values (EF_c 0.71 in both raw and treated wastewater) were, however, significantly different from those obtained by MacLeod et al. in Edmonton, Canada, where the EF were approximately 0.21 in raw wastewater and approximately 0.31 in treated wastewater [3]. MacLeod et al. also reported a shift in EF, where the relative concentration of (*R*)-fluoxetine was higher in the raw wastewater than in the treated wastewater. In the present study, however, the concentration of (*S*)-fluoxetine was higher than the concentration of (*R*)-fluoxetine, and the EF was not significantly different in raw and treated wastewater. The enantiomeric fractions in raw and treated wastewater have been reported not only to be compound-dependent but also that the enantioselective degradation of pharmaceuticals can be different in different treatment systems depending on the prevailing anaerobic or aerobic conditions [36]. For example, the active pharmaceutical ingredient (*S*)-ibuprofen has been shown to degrade faster than (*R*)-ibuprofen under aerobic conditions whereas the degradation was not enantioselective under anaerobic conditions [36]. The EF values might, therefore, vary for different wastewater samples, depending on the treatment and on the origin of the samples. Considering that enantioselectivity in toxicity has been shown to be of significance in the aquatic environment [17], these differences between aquatic concentrations of (*S*)-fluoxetine and (*R*)-fluoxetine in Edmonton and Uppsala is interesting and might be of importance for water-living microorganisms.

In conclusion, higher concentrations of (*S*)-fluoxetine than (*R*)-fluoxetine were observed in the raw wastewater (EF_c 0.71, e.e. 0.43). It might be relevant to compare these enantiomeric compositions with those obtained from bioanalytical studies. The concentrations of (*S*)-fluoxetine have been shown to be higher than those of (*R*)-fluoxetine in urine from patients treated with (*R,S*)-fluoxetine for major depression [37]. In the same study, the excreted concentration of (*S*)-norfluoxetine was higher than the concentration of (*R*)-norfluoxetine [37]. The EFs for norfluoxetine were determined to be 0.69 (e.e. 0.39) in raw wastewater and 0.68 (e.e. 0.37) in treated wastewater. Thus, the concentration of the more pharmacologically active enantiomer, (*S*)-norfluoxetine, was higher than the concentration of the less active one, (*R*)-norfluoxetine, in raw as well as in treated wastewater.

4. Conclusions

It has been demonstrated that matrix effects, arising from co-eluting compounds originating from the wastewater samples, could be evaluated by the extended use of the stable isotope-labeled compounds, fluoxetine-d_5 and norfluoxetine-d_5. The ESI-signals in the MS interface for fluoxetine-d_5 and norfluoxetine-d_5 were suppressed for about 15 min by the extracted raw and treated wastewater samples. It was also found that the signals of the enantiomers of norfluoxetine-d_5 were suppressed to a greater extent than those from the less polar fluoxetine-d_5.

The enantiomers of fluoxetine-d_5 and norfluoxetine-d_5 were suppressed to different extents, thus EF values determined without a proper internal standard might be over or underestimated in environmental matrices. In the present study, the matrix effects were compensated for during quantification by the use of isotope-labeled internal standards. The concentration of (*S*)-fluoxetine was determined to be higher than the concentration of (*R*)-fluoxetine in both raw and treated wastewater. In addition, the EF values were determined to be 0.71 in the two matrices, thus there was no indication of enantioselective microbial degradation in the sewage treatment plant. Interestingly, in the present study the EF values obtained were significantly different from the EF values obtained for fluoxetine in wastewater samples in other studies [3].

To the best to the authors' knowledge, this is the first time the human metabolite norfluoxetine has been enantioselectively quantified in wastewater samples. The EF values for norfluoxetine in influent and effluent wastewater, determined to be 0.69 and 0.68, respectively, were significantly higher than the EF of racemic mixtures ($2 \times 10^{-17} < p < 3 \times 10^{-15}$).

Acknowledgments

Therése Andersson is acknowledged for skillful assistance in conjunction with this work. The employees at the sewage treatment plant, Kungsängsverket in Uppsala, are also acknowledged for their kind assistance during sample collection.

References

[1] K.D. Oakes, A. Coors, B.I. Escher, K. Fenner, J. Garric, M. Gust, T. Knacker, A. Küster, C. Kussatz, C.D. Metcalfe, S. Monteiro, T.W. Moon, J.A. Mennigen, J. Parrott, A.R.R. Péry, M. Ramil, I. Roennefahrt, J.V. Tarazona, P. Sánchez-Argüello, T.A. Ternes, V.L. Trudeau, T. Boucard, G.J. Van Der Kraak, M.R. Servos, Integr. Environ. Assess. Manag. 6 (2010) 524.
[2] A.L.N. van Nuijs, I. Tarcomnicu, W. Simons, L. Bervoets, R. Blust, P.G. Jorens, H. Neels, A. Covaci, Anal. Bioanal. Chem. 398 (2010) 2211.
[3] S.L. MacLeod, P. Sudhir, C.S. Wong, J. Chromatogr. A 1170 (2007) 23.
[4] A. Lajeunesse, C. Gagnon, S. Sauvé, Anal. Chem. 80 (2008) 5325.
[5] T. Vasskog, T. Anderssen, S. Pedersen-Bjergaard, R. Kallenborn, E. Jensen, J. Chromatogr. A 1185 (2008) 194.
[6] T. Heberer, Toxicol. Lett. 131 (2002) 5.
[7] T.A. Ternes, Water Res. 32 (1998) 3245.
[8] M. Gros, M. Petrović, D. Barceló, Anal. Chem. 81 (2009) 898.
[9] 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.
[10] M.J. Benotti, R.A. Trenholm, B.J. Vanderford, J.C. Holady, B.D. Stanford, S.A. Snyder, Environ. Sci. Technol. 43 (2009) 597.
[11] C. Wong, Anal. Bioanal. Chem. 386 (2006) 544.

[12] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.
[13] C.T. Viswanathan, S. Bansal, B. Booth, A. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, Pharm. Res. 24 (2007) 1962.
[14] L.E. Sojo, G. Lum, P. Chee, Analyst 128 (2003) 51.
[15] R.W. Fuller, H.D. Snoddy, J.H. Krushinski, D.W. Robertson, Neuropharmacology 31 (1992) 997.
[16] J.K. Stanley, A.J. Ramirez, C.K. Chambliss, B.W. Brooks, Chemosphere 69 (2007) 9.
[17] F. De Andrés, G. Castañeda, Á. Ríos, Chirality 21 (2009) 751.
[18] L.J. Fono, D.L. Sedlak, Environ. Sci. Technol. 39 (2005) 9244.
[19] N.H. Hashim, S. Shafie, S.J. Khan, Environ. Technol. 31 (2010) 1349.
[20] T. Poiger, H.-R. Buser, M.D. Müller, M.E. Balmer, I.J. Buerge, Chimia 57 (2003) 492.
[21] V.K.H. Barclay, N.L. Tyrefors, I.M. Johansson, C.E. Pettersson, J. Chromatogr. A 1218 (2011) 5587.
[22] H.-R. Buser, T. Poiger, M.D. Müller, Environ. Sci. Technol. 33 (1999) 2529.
[23] D.T. Wong, F.P. Baymaster, L.R. Reid, R.W. Fuller, K.W. Perry, Drug Dev. Res. 6 (1985) 397.
[24] D.T. Wong, F.P. Bymaster, L.R. Reid, D.A. Mayle, J.H. Krushinski, D.W. Robertson, Neuropsychopharmacology 8 (1993) 337.
[25] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.
[26] D.L. Buhrman, P.I. Price, P.J. Rudewicz, J. Am. Soc. Mass Spectrom. (1996) 1099.
[27] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[28] H.R. Liang, R.L. Foltz, M. Meng, P. Bennett, Rapid Commun. Mass Spectrom. 17 (2003) 2815.
[29] L.G. Freitas, C.W. Götz, M. Ruff, H.P. Singer, S.R. Müller, J. Chromatogr. A 1028 (2004) 277.
[30] J.A. Magnér, T.E. Alsberg, D. Broman, Anal. Bioanal. Chem. 395 (2009) 1481.
[31] T. Harner, K. Wiberg, R. Norstrom, Environ. Sci. Technol. 34 (2000) 218.
[32] F.T. Peters, H.H. Maurer, Anal. Chem. 79 (2007) 4967.
[33] A. Mechlinska, L. Wolska, J. Namiesnik, TrAC Trends Anal. Chem. 29 (2010) 820.
[34] M. Carballa, F. Omil, J.M. Lema, Chemosphere 72 (2008) 1118.
[35] 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.
[36] V. Matamoros, M. Hijosa, J.M. Bayona, Chemosphere 75 (2009) 200.
[37] G.A. Torok-Both, G.B. Baker, R.T. Coutts, K.F. McKenna, L.J. Aspeslet, J. Chromatogr. 579 (1992) 99.