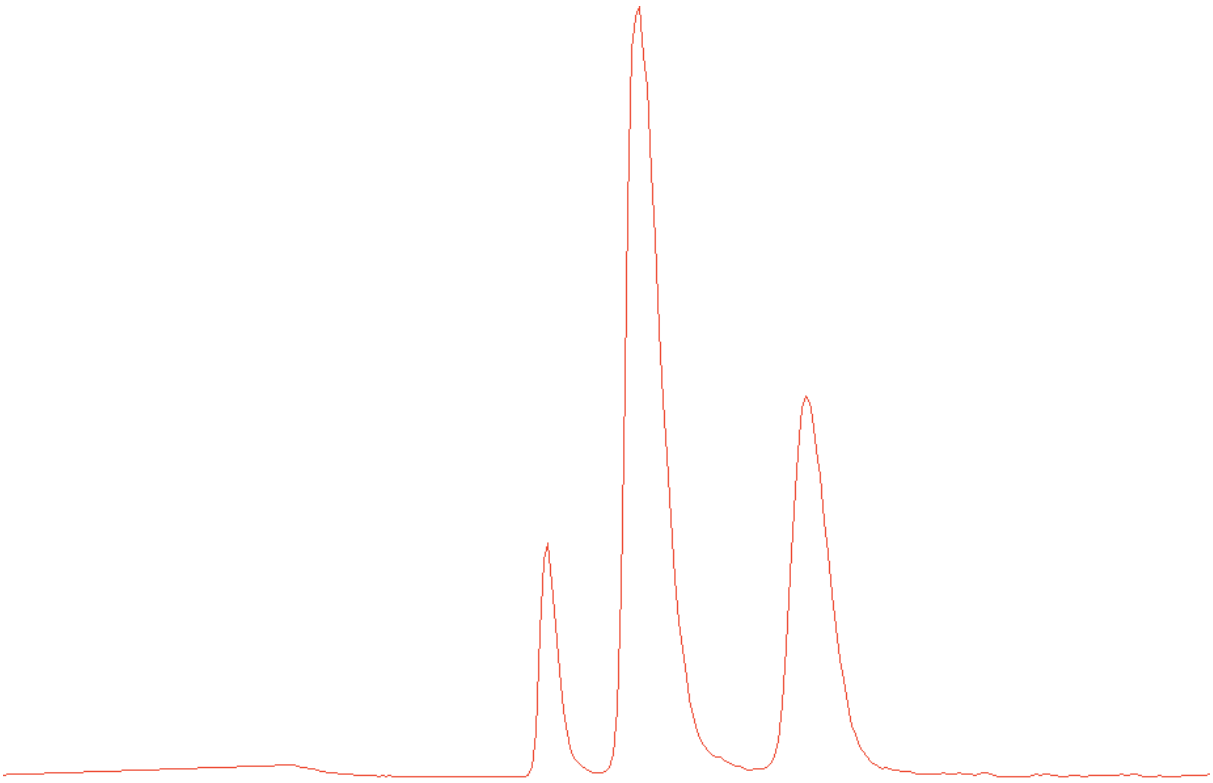
Direct hollow fibre liquid phase membrane extraction and LC-MS determination of selective serotonin reuptake inhibitors in fish tissue



Henrik Engström

Master Thesis in Analytical Chemistry 2013

Centre for Analysis and Synthesis

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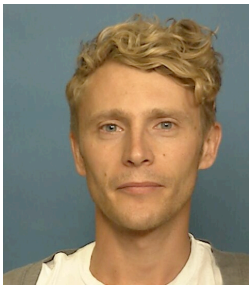

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Master Thesis in Analytical Chemistry

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Abbreviations

ACN	Acetonitrile
ASE	Accelerated solvent extraction
DHE	Di-*n*-hexyl ether
DMSO	Dimethyl sulphoxide
E	Extraction efficiency
E_e	Enrichment factor
FLU	Fluoxetine
GC	Gas chromatography
HAc	Acetic acid
HF-LPME	Hollow fibre liquid phase micro extraction
LC-DAD	Liquid chromatography with diode array detection
LC-FLD	Liquid chromatography with fluorescent detection
LC-MS	Liquid chromatography with mass spectrometric detection
LC-MS/MS	Liquid chromatography with tandem mass spectrometric detection
LC-UVD	Liquid chromatography with ultra violet detection
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantisation
MDL	Method detection limit
MIP	Molecularly imprinted polymers
NH_4AC	Ammonium acetate
$NH_4H_2PO_4$	Ammonium phosphate
norFLU	Norfluoxetine
NSAID	Non steroidal anti-inflammatory drugs
PLE	Pressurised liquid extraction
PPCP	Pharmaceuticals and personal care products
PQL	Practical quantisation limit
Q-TOF	Quadrupole time of flight mass spectrometer
RSD	Relative standard deviation
SBSE	Stir bar sorptive extraction
SD	Standard deviation
SER	Sertraline
SLME	Supported Liquid Membrane Extraction
SPE	Solid phase extraction
SPME	Solid phase micro extraction
SSRI	Selective serotonin reuptake inhibitor
TEA	Triethylamine
WWTP	Waste water treatment plant

Abstract

Selective serotonin reuptake inhibitors (SSRIs) have been found in waste water treatment plant effluents and surface water at detectable concentrations. Although the medical effects and side effects of pharmaceuticals and personal care products are investigated through safety and toxicology studies the potential environmental impacts are less studied, and information concerning ecotoxicological risks and the distribution in sludge, surface water and water living organisms is rather scarce. In this thesis, sertraline (SER), fluoxetine (FLU) and its N-desmethyl metabolite norfluoxetine (norFLU) were chosen as model substances to develop a method for detection. Fish was chosen as the model matrix because fish is a top predator with possible high analyte concentrations because of biomagnification. Hollow fibre liquid-phase membrane extraction (HF-LPME) was used as the extraction, clean-up and enrichment technique and LC-MS was used to detect the SSRIs in fish muscle tissue. This analytical method showed enrichment factors ranging 1500-1800 for fish samples and 3000-6300 for water samples. The R^2-values of the linearity were 0.936, 0.990, 0.966 for norFLU, FLU and SER, respectively. The detection limits of the method for norFLU, FLU and SER were in the range of 130-280 ng L^{-1}. The method was successfully applied to detect the analytes in exposed crucian carp; 1.7 μg g^{-1} FLU and 2.8 μg g^{-1} SER were found after exposure to a 51 μg L^{-1}-mixture for 3 days. The FLU metabolite norFLU was not added in the exposure solution and it was not formed in detectable concentrations during the exposure. In unexposed crucian carp, none of the analytes were detected. The developed analytical method might be extended to estimate the distribution or the fate of norFLU, FLU and SER in other biota or human beings.

Key words: HF-LPME, LC-MS, SSRI, selective, fish

Contents

1. Introduction - SSRI in the environment

Pharmaceuticals and personal care products (PPCPs) are important as they help in the treatment and prevention of disease in both humans and animals. The occurrence and fate of PPCPs in the environment have been recognised as major issues because of their growing use and unknown environmental impacts [1-6]. When excreted, PPCPs and their metabolites reach waste water treatment plants (WWTP), and analysis of effluent waters show that PPCPs are not completely removed [7,8], leading to levels of PPCPs in aquatic environments reaching at least ng L^{-1} concentrations at several locations around the world [9,10].

Depression is among the oldest diseases known to mankind and is among the most serious medical diseases, both to society [11] and to the patient and his/her kins [12]. Since the introduction of selective serotonin reuptake inhibitors (SSRIs), even milder forms of depression are being treated medically [13]. The most commonly prescribed pharmaceuticals for psychiatric illnesses belong to the antidepressants group selective serotonin reuptake inhibitors (SSRIs) [4,5,14-17], including fluoxetine (FLU), the active compound in pharmaceuticals like Fontex and Prozac, and sertraline (SER), active compound in e.g. Sertranatl and Zoloft. SSRIs are designed to be highly active and interact with receptors in humans, but they also have unintended effects on animals in the environment [18]. Although the effects and side effects of the pharmaceuticals are investigated through safety and toxicology studies [19,20], the potential environmental impacts are less studied.

It has been shown that psychiatric drugs are not completely metabolised by the human body and therefor excreted, either as the parent compounds, or as metabolites to wastewater [2,20-23]. Even metabolites, such as norfluoxetine (norFLU), an N-desmethyl product of FLU, although less potent than the parent compound, can be biologically active [17,20,23-26]. Also, SSRIs find their way to aquatic ecosystems through WWTP discharges [27] and sludge [28]. Therefore, it is necessary to determine the presence and levels of SSRIs in biota. Most of the studies on the fate of pharmaceuticals in WWTPs are focused on aqueous samples because biological samples always present a complex matrix with plenty of interferences to be co-extracted. To exclude these interferences to a greater extent, clean-up techniques for the determination of SSRIs in biological samples are necessary. Also, the enrichment of the analytes is an important step since they are present at trace levels of ng g^{-1} [16,20].

1.2 Sample preparation for SSRIs - a review

Very often in analytical chemistry, the analytes are present in a more or less complex matrix, the SSRIs of this study being no exception. Over the years a number of different techniques have been employed to clean up and pre-concentrate the analytes; to remove coexisting unwanted substances and to enrich the analytes. The methodology of choice is somewhat dependent both on the physical properties of the sample, the chemical properties of the analyte, and availability of equipment. Therefore, different analytes in different matrices may be extracted and analysed by different methods. Below is a review of different techniques used, in regard to the matrix.

1.2.1 Sample preparation for SSRIs in water samples

Aqueous samples are the simplest type of matrix to extract from, with not too many interferents available. The most commonly applied extraction technique for aqueous samples is solid phase extraction (SPE) combined with either LC-MS/MS [3,16,21,23] or LC-DAD [22]. Demeestere et al [4] also used SPE with molecularly imprinted polymers (MIP) to analyse SSRI in aqueous samples.

1.2.2 Sample preparation for SSRIs in semi-solid and solid samples

Semi-solid and solid samples include sewage sludge, soil and sediment. More interfering compounds than in aqueous samples are present, why the extraction needs to be more efficient. Methods for extraction of SSRIs include SPE followed by LC-MS/MS [16], SPE and liquid-liquid extraction (LLE) followed by LC-MS [15], pressurised liquid extraction (PLE), followed by LC-MS [1], and accelerated solvent extraction (ASE) followed by LC-MS/MS [3]. Because of the more complex matrix, membrane extraction is also popular for semi-solid samples, such as liquid phase membrane extraction (LPME) [2,24,28,29] or pressurised hot water extraction (PHWE) followed by LPME [28] in combination with either LC-MS, LC-MS/MS or LC-DAD.

1.2.3 Sample preparation for SSRIs in biological samples

Another complex type of matrix is present in biological samples. Common extraction techniques are SPE combined with LC-MS/MS [8,30], LC-FLD/UVD [31], or GC-MS [32,33], LLE with CG-MS [34] or stir bar sorptive extraction (SBSE) with LC-FLD [35].

Because of the utterly complex matrix, such methodologies as solid phase micro extraction (SPME) [36] or membrane extraction are other good ways of excluding interferents.

1.3 Membrane extraction

In the search for more environmentally friendly yet efficient methods, membrane extraction is a method that lately has received growing attention; target analytes are selectively extracted, extraction and work up is done in one step, and extracted analytes are easily quantitatively transferred to the instrument for final analysis. The use of organic solvents is negligible. The benefits of membrane extraction include high selectivity for target analytes, high enrichment power, as well as being economical and environmentally friendly [37-42].

In membrane extraction a membrane is used to separate the sample (called the donor phase) from the acceptor phase. Target analytes pass from the donor phase, through the membrane and into the acceptor, where they are enriched. Membrane extraction can be made with porous and nonporous membranes and in one, two, or three phase configurations. Examples of porous one phase techniques are filtration and dialysis, while nonporous techniques include two or three phase membrane configurations.

If either the donor or acceptor phase has the same composition as the membrane, only one phase boundary (and one partitioning interface) is created. This is what characterises a two phase system. In a three phase system, on the other hand, the donor and acceptor phases are separated by the membrane phase, to form two phase boundaries and two partitioning interfaces. This membrane phase can be a liquid, a polymer, or a gas. Donor and acceptor phases can be either gas or liquid, aqueous or organic. To create a liquid membrane phase, a support is needed. This support is often a porous hydrophobic membrane, in whose pores the liquid is immobilised. This is called a supported liquid membrane (SLM). Depending on the type of analyte, the system will need optimisation by the use of different phases, to maximise selectivity and enrichment.

1.3.1 Principles of Supported Liquid Membrane Extraction (SLME)

If the target analyte is an acid or a base, a three-phase SLM system is a good choice of extraction method because of its ability to enrich the analyte in the acceptor phase. The ability of acids and bases to exist both in ionic and nonionic form is crucial for SLME, not only for

the process of enrichment, but also for the selectivity of the method. Figure 1 illustrates the general set up of an SLME. Initially the analyte is present, along with a lot of interfering compounds, in the donor phase. The organic membrane phase, immobilised in the pores of the membrane, is usually a solvent of long chain hydrocarbons (*n*-undecane, kerosene or more polar solvents like DHE) for which the analyte has a higher selectivity than for the donor phase. The analyte diffuses into the pores and further on, into the acceptor phase in the lumen of the membrane. By chemical means, the analytes is altered in the acceptor and thus being trapped and enriched inside the fibre.

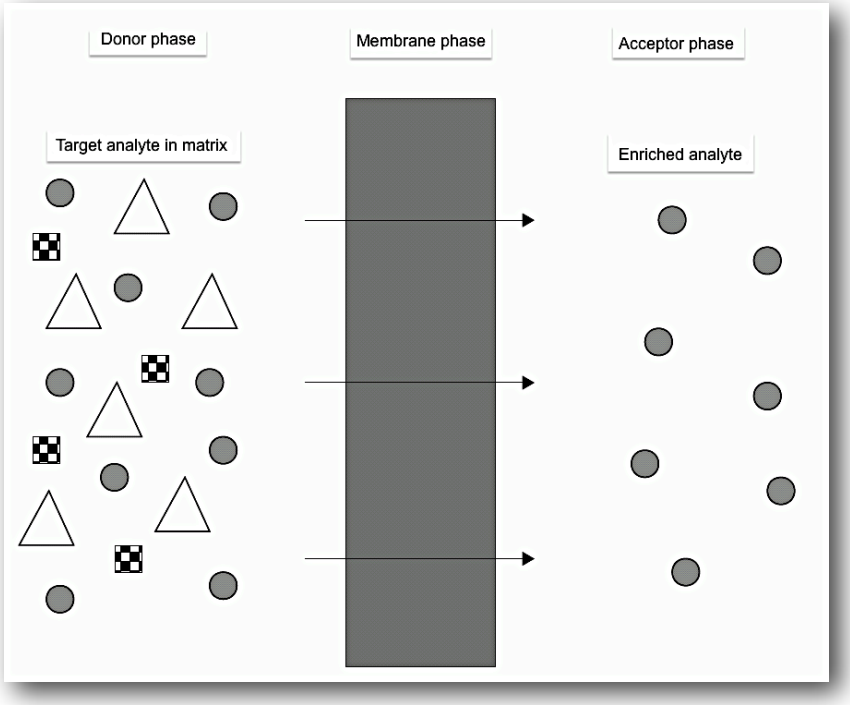


Figure 1. General set up for an SLME: the target analyte diffuses from the donor phase, through the membrane phase, and into the acceptor phase where it is enriched.

1.3.2 SSRI Hollow Fibre Liquid Phase Membrane Extraction (HF-LPME)

Hollow fibre liquid phase membrane extraction is a three phase supported liquid extraction technique, where the support membrane is a hollow polymer fibre. This technique is a good choice for extracting acidic or basic pharmaceuticals. By utilising a pH-gradient, nonionised target analytes will diffuse from the donor phase into the organic phase in the pores of the membrane. To ionise the analyte is a matter of pK_a. In the case of basic compounds, pH in the donor phase needs to be well above pK_a, and vice versa in the acceptor phase. From the organic phase the analyte will continue into the acceptor phase inside the membrane, where it

will be ionised by acid-base reactions and immobilised because of the charged forms inability to dissolve in the organic phase. After extraction the analyte is readily transferred in a quantitative way to an analytical instrument. Figure 2 describes the principle of HF-LPME of fluoxetine from a pH-adjusted donor. Uncharged FLU readily diffuses into the organic solvent in the membrane phase and into the acceptor phase inside the fibre. The low pH of the acceptor phase protonates FLU, making it ionic. Once charged, FLU no longer diffuses back into the organic solvent, but gets trapped and enriched in the acceptor phase.

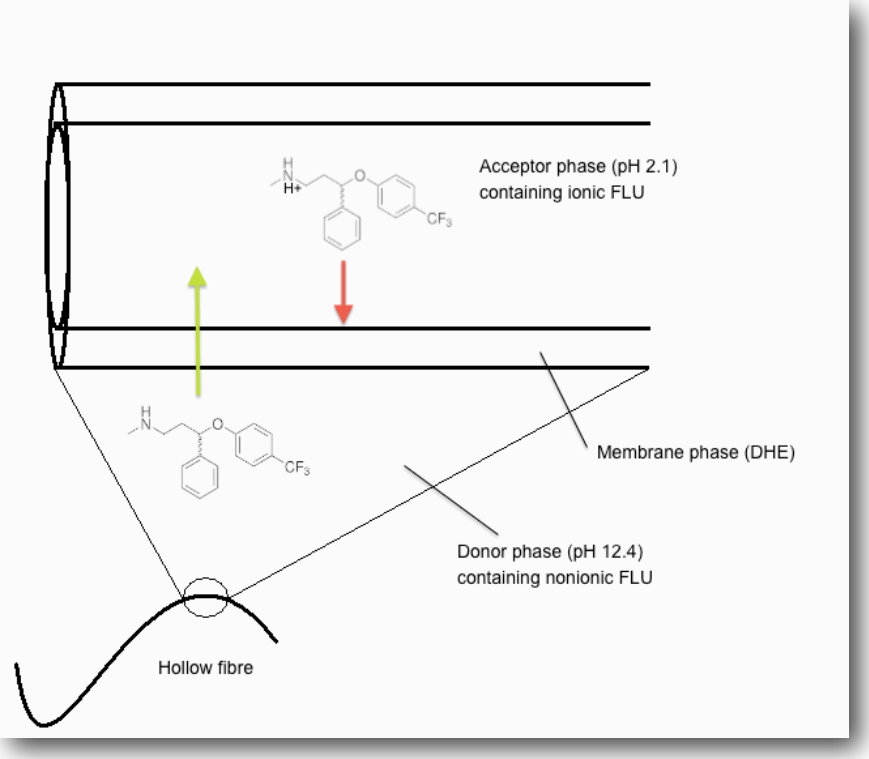


Figure 2. Principle of HF-LPME of the basic target analyte FLU. pH in the donor phase is well above pKa to ensure nonionic analytes. Uncharged FLU readily diffuses into the organic solvent in the membrane phase and into the acceptor phase inside the fibre. Here, the low pH turns FLU ionic. Once charged, FLU will no longer diffuse into the organic solvent, but gets trapped and enriched in the acceptor phase.

Sagristá et al. [43] used this technique in 2010 to determine NSAIDs in WWTP sewage sludge and in 2012 [28] to determine SSRIs in the same matrix. Huang used this technique in 2012 [44] to determine NSAIDs in fish. Vasskog et al. [2] used it to detect SSRI in sewage sludge. Even though being an extraction technique on the rise, to the best our knowledge, only very few pieces of work [2,14,24,28,29] lend themselves to extracting SSRI with HF-LPME, and non to extracting SSRI with HF-LPME from fish.

1.3.2.1 The enrichment factor

How well the analyte is concentrated in the acceptor phase, the enrichment, is crucial to the efficiency of HF-LPME, and is defined as [38,42]:

$$E_e = \frac{C_{Ae}}{C_{Di}} \qquad (1)$$

where C_{Ae} is the analyte concentration in the acceptor phase at equilibrium, and C_{Di} is the initial analyte concentration in the donor phase.

The extraction efficiency (E) of HF-LPME is defined in a similar way [45]:

$$\left.\begin{aligned} E &= \frac{m_{Ae}}{m_{Di}} \cdot 100 \\ m &= C \cdot V \end{aligned}\right\} \Rightarrow E = \frac{C_{Ae} \cdot V_A}{C_{Di} \cdot V_D} \cdot 100$$

and in combination with equation (1):

$$E = E_e \cdot \frac{V_A}{V_D} \cdot 100 \qquad (2)$$

where, C_{Ae} and m_{Ae} are the analyte concentration and mass in the acceptor phase at equilibrium, respectively; C_{Di} and m_{Di} are the initial analyte concentration and mass in the donor phase, respectively, and V_A and V_D are the acceptor and donor volumes, respectively.

1.3.2.2. The partition coefficient

In three phase HF-LPME, the analytes are extracted from the sample solution to the acceptor solution, passing through the organic solvent in the membrane phase, see Figure 2. As the analyte reaches equilibrium between the three phases, the partition coefficient of acceptor-donor, K_{AD}, depends on the conditions of the donor and the acceptor [45]. K_{AD} is defined as:

$$K_{AD} = \frac{C_{Ae}}{C_{De}} = \frac{m_{Ae} \cdot V_D}{m_{De} \cdot V_A} \qquad (3)$$

where C_{Ae} and C_{De} are the analyte concentrations at equilibrium in the acceptor and donor phases, respectively, and m_{Ae} and m_{De} are the analyte masses at equilibrium in the acceptor and donor phases, respectively.

In the same way, the partition coefficient of fish tissue/donor phase, K_{FD}, and membrane phase/donor phase, K_{MD}, can be defined as:

$$K_{FD} = \frac{C_{Fe}}{C_{De}} = \frac{m_{Fe} \cdot V_D}{m_{De} \cdot m_F} \quad (4)$$

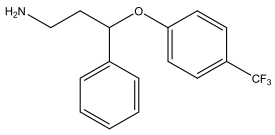
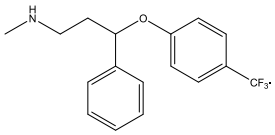
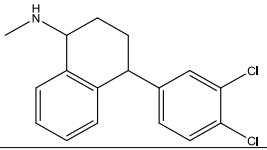
$$K_{MD} = \frac{C_{Me}}{C_{De}} = \frac{m_{Me} \cdot V_D}{m_{De} \cdot V_M} \quad (5)$$

where C_{Fe}, , C_{De} and C_{Me} are the analyte concentrations at equilibrium in the fish, donor phase, and membrane phase, respectively; m_{Fe}, m_{De} and m_{Me} are the analyte masses at equilibrium in the fish, donor phase, and membrane phase, respectively, and m_F, V_D and V_M are the mass of the fish, the volume of the donor phase and the volume the membrane phase, respectively.

1.4 The analytes

The target analytes of this study are the SSRIs sertraline (SER) and fluoxetine (FLU), plus a desmethylated metabolite of FLU; norfluoxetine (norFLU). The most important chemical properties of the analytes are compiled in Table 1.

Table 1. Chemical properties of the target analytes, norFLU, FLU and SER.

Name	Structure	Mw	pK_a	Log K_{ow}	Solubility mg L^{-1}	Ref.
Norfluoxetine		295.12	9.05	4.07	60.3	[24]
Fluoxetine		309.13	10.05	4.05	3.52	[14]
Sertraline		305.07	9.47	5.29	4.07	[14]

1.5 Aim of this study

SSRIs have widely been found in WWTP effluents and surface water at different concentrations. For example, citalopram, paroxetine, fluoxetine and sertraline were extracted from sewage sludge by HF-LPME and analysed by LC/MS by Sagristà et al. in 2012 [28]. Although the medical effects of PPCPs are investigated through safety and toxicology studies [19,20], the potential environmental impacts are less studied, and information concerning ecotoxicological risks and the distribution in sludge, surface water and water living organisms is rather scarce. Being top predators, fish are especially subjected to high concentrations because of biomagnification. To investigate this, a new method which can consecutively extract and detect different SSRIs and metabolites in fish is needed. To our knowledge, no previous studies for the analysis of SSRIs using HF-LPME in fish have been published.

The goal of this study is therefore to evaluate HF-LPME as extraction, clean-up and enrichment technique followed by LC/MS-analysis for the simultaneous determination of norfluoxetine, fluoxetine and sertraline in fish tissue. As far as possible, the method follows that of Huang [44]. To adapt the method to fit SSRIs as analytes, the work of Sagristà et al [28] was taken into consideration. Ultimately the work in this thesis could help in providing a way to monitor SSRIs in biota.

2. Experimental

2.1 Chemicals and reagents

Fluoxetine hydrochloride, sertraline hydrochloride, norfluoxetine hydrochloride, dihexylether (DHE), ammonium acetate (NH_4AC) reagent grade were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Acetonitrile (ACN) gradient grade was obtained from Honeywell B&J brand (Sleeze, Germany). Reagent sodium hydroxide was obtained from Scharlau Chemie S.A. (Barcelona, Spain). Acetic acid glacial (HAc), phosphoric acid (85 % pure) and ammonium phosphate reagent grade were purchased from Merck (Darmstadt, Germany). Dimethyl sulphoxide (DMSO) was purchased from Thermo Scientific (Rockford, IL, USA). Ultra-pure water was produced by a MilliQ water purification system (Millipore, Billerica, MA, USA), and was used throughout the experiments.

Individual stock standard solutions containing 100 mg L^{-1} of fluoxetine and sertraline and 80 mg L^{-1} of norfluoxetine were prepared in DMSO. For the optimisation and application of the methods, firstly a stock solution containing 10 mg L^{-1} each of all three SSRIs studied was

prepared in water, secondly working solutions of this mixture were prepared by appropriate dilution in water. Calibration curves for LC/MS were prepared by diluting the mixture in the acceptor phase used for HF-LPME. The HF-LPME acceptor buffer was 0.1 M ammonium phosphate solution, pH 2.1. The organic phase was DHE. The LC mobile phase buffer was 0.01 M ammonium acetate, pH adjusted to 4 with acetic acid, 6.5 M. Solutions with a high DMSO content were stored in darkness at ambient temperature, all other solutions in darkness at 4 ºC to prevent photo degradation of the analytes.

2.2 Fish tissue samples

Cod (*Gadus morhua*) was chosen as the fish used for developing the method. A fresh cod was purchased locally and stored at -18 ºC.

Crucian carp (*Carassius carassius*) was chosen for validating the developed method. Live young crucian carps were collected from an unexposed pond on the Revinge field, Lund, Sweden.

2.3 Sampling

2.3.1 Spiked water samples

While optimising the LC-MS method, and to investigate the matrix effect, spiked water was used. The water samples were spiked with 10 mg L^{-1} stock solution, resulting in C_{Di}s of 0.2, 0.5, 0.8, 2, 4, 10, 20, 100 and 200 µg L^{-1}.

2.3.2 Spiked cod samples

During early development of the method frozen fresh cod was used. An appropriate mass of cod muscle tissue from the side of the fish was mixed with an equal mass water. The mixture was homogenised for 10 minutes using a Polytron mixer (Kinematica GmbH, Lucerne, Switzerland), see Figure 3. 1.0 g samples of the fish mixture were placed in brown wide mouth bottles, spiked, and homogenised another 2 minutes with 19.5 mL of water. This slurry was left to equilibrate overnight, before 30 mL of water was added and stirred with magnetic stir bars for extraction.

Figure 3. The fish/water mixture was homogenised for 10 minutes using a Polytron mixer.

Later on lyophilised cod was used. Muscle tissue from the fish was cut into pieces of approximately 0.5 g each. These samples were lyophilised for 24 h before put in brown wide mouth bottles, spiked and homogenised for 10 minutes with 20 mL of water. This slurry was left to equilibrate overnight, before 30 mL of water was added and stirred with magnetic stir bars for extraction.

2.3.3 Exposed crucian carp samples

Crucian carp was acclimatised in the lab environment for four weeks in a flow-through tap water system before transferred into a 40 L tank where they were exposed to 51 µg L^{-1} of FLU and SER. After three days the fish was removed from the water, decapitated, and wiped with a Kleenex tissue.

The young crucian carp is a rather small fish, of approximately 2.5 g each, why the heads were removed and put in a batch of their own. The rest of the fish was cut into small pieces and randomised, packed in portions of approximately 0.5 g each. These samples were lyophilised for 24 h before put in brown wide mouth bottles and homogenised for 10 minutes with 20 mL of water. This slurry was left to equilibrate overnight, before 30 mL of water was added and stirred with magnetic stir bars for extraction.

2.4 LC-MS method

Analysis was performed on a Micromass ZMD single quadrupole mass spectrometer (Micromass Ltd, Manchester, UK) with an electrospray interface connected to an Agilent Hewlett Packard Series 1100 HPLC system, consisting of degasser, pump and auto sampler (Hewlett Packard, Karlsruhe, Germany). Injection volume was 4 µL for all samples, using needle wash between each pick up, and flow rate was 0.3 mL min^{-1}. The ZMD was controlled by MassLynx 4.0 software (Micromass Ltd, Manchester, UK), whether as the HPLC system was controlled by a Hewlett Packard remote control. The ZMD was run in positive-ion mode, with capillary voltage 3.6 kV, cone voltage 10 V for norFLU and 15 V for FLU and SER. ESI source block temperature was 150 °C, desolvation temperature 350 °C, desolvation gas N_2 at a flow of 540 Lh^{-1}. Further settings were: extractor voltage 5 V, ion energy 0.9 eV, Rf lens voltage 0.2 V, low mass resolution 17.5, high mass resolution 9.1, and multiplier 672. Selective ion monitoring was used to detect ions with m/z ratios of 296 (norFLU), 310 (FLU) and 306 (SER).

The chromatographic separation was performed on a Thermo Scientific ODS-2 Hypersil column with particle size 5 µm, 2.1×250 mm (Thermo Scientific, Waltham, MA, USA). Mobile phases used were A (ACN:NH_4AC buffer 5:95) and B (ACN:NH_4AC buffer 95:5). To increase separation and shorten runtime a gradient was used, see Table 2. A calibration curve was acquired using standard solution of the three analytes of concentrations 0, 1000, 3000, 5000, 7500 and 10000 µg L^{-1}. To test repeatability of the method, a standard solution of the three analytes (4000 µg L^{-1}) was analysed three times before sample analysis.

Table 2. Mobile phases and gradient for the chromatographic separation of norFLU, FLU and SER on a Thermo Scientific ODS-2 Hypersil column with particle size 5 μm, 2.1×250 mm. Gradient curve indicates the slope of the curve, where 1 is no slope and 6 is 50% slope.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow (mL min^{-1})	Gradient curve*
0.0	50	50	0.3	1
0.5	60	40	0.3	6
2.0	60	40	0.3	1
2.1	50	50	0.3	6
9.0	50	50	0.3	1

2.5 HF-LPME method

The hollow fibre membranes used were PP50/280 Accurel polypropylene with a wall thickness of 50 μm, 0.1 μm pore size and inner diameter of 280 μm (Membrana GmbH, Wuppertal, Germany). The fibre was cut into 20 cm long pieces to carry approximately 10 μL of acceptor phase. Each fibre was filled with fresh acceptor buffer using a 0.5 mL (29G x 1/2" - 0.33 x 12 mm) Myjector syringe (Terumo Medical Corporation, Elkton, MD, USA), see Figure 4a. The fibre was sealed by melting its ends on an electric soldering iron. The fibre was immersed in DHE for 1 min to let the pores saturate with organic solvent, and then washed in water for 30 sec (Figure 4b) before being tied into a loop, attached to a copper wire (Figure 5a) and put into the brown wide mouth bottles containing the donor phase (Figure 5b). Extraction parameters were as follows: 50 mL samples at pH 12.4 with NaOH were stirred at 660 rpm using magnetic stirrer (RO 10 Power, IKA Werke, Staufen, Germany) for 5 h.

After extraction, the fibre was picked out from the samples, gently wiped with a Kleenex® tissue and both ends were cut open using a scalpel. The acceptor phase was evacuated by an air filled syringe into a 2 mL vial with μL inset. The volume of acceptor was determined by the mass difference of the vial prior to and after the filling. The samples were immediately taken to final analysis.

Figure 4a. The hollow fibre membrane was cut into 20 cm long pieces and filled fresh acceptor buffer. The ends were sealed by melting...

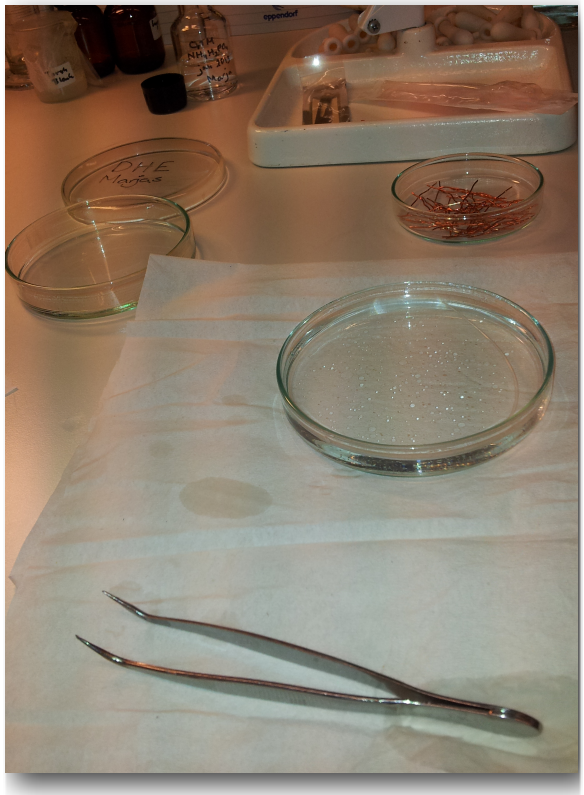


Figure 4b. ...and then the fibre was immersed in DHE for 1 min to let the pores saturate with organic solvent, and then washed in water for 30 sec.

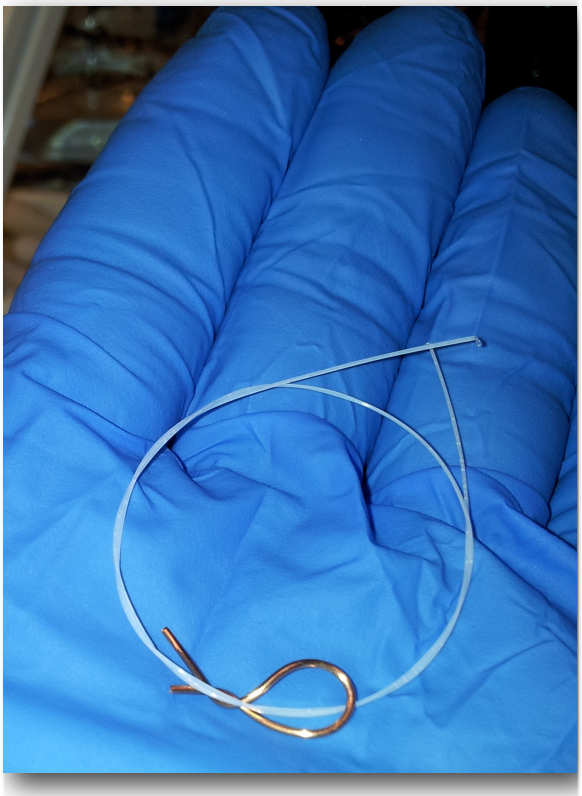


Figure 5a. When filled with acceptor phase, sealed and pores saturated with organic solvent, the fibre was tied into a loop, attached with a copper wire…

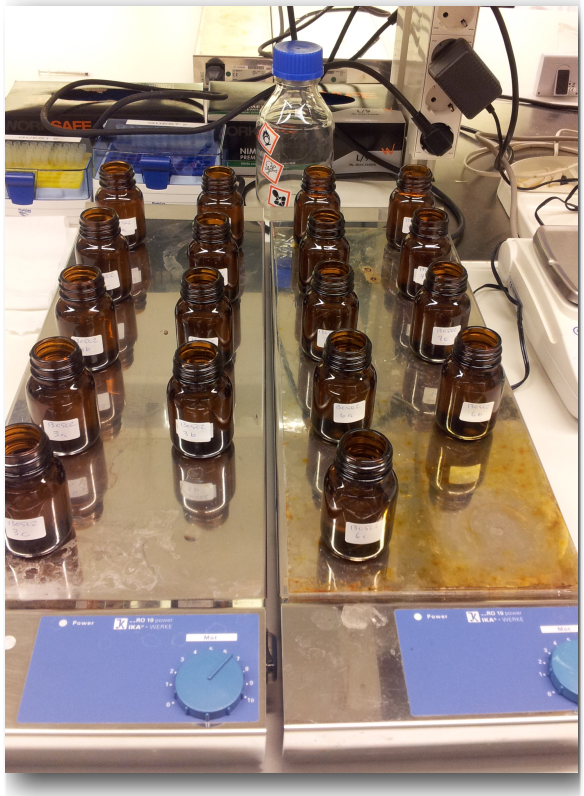


Figure 5b. ...and put into the brown wide mouth bottles containing the samples for 5 h extraction at 660 rpm.

3. Results and discussion

3.1 Optimisation of methods

Optimisations of the method were adapted from Sagristà et al. [28]; HPLC mobile phase ACN and NH_4AC buffer at pH 4 and donor phase pH 12.4. For the trapping and enrichment inside the fibre to take place, protonation of amines need to take place, therefore $NH_4H_2PO_4$ at pH 2.1 was chosen as acceptor phase. Extraction optimisations were adapted from Huang [44]; extraction time 5 h, sampling solvent water and the fish to be lyophilised.

3.1.1 LC separation

Several instrument setups were tested in order to optimise the chromatographic separation of the three analytes norFLU, FLU and SER. An Alliance Waters 2695 Separations Module (Waters, Milford, MA, USA) with the Thermo Scientific ODS-2 Hypersil column, in connection with the Micromass ZMD achieved the best chromatographic separation (Figure 6). This setup was also fully compatible with the MassLynx 4.0 software. However, the needle of Waters 2695 is constructed so that it picks up sample on the side, some 5 mm up from the needlepoint, rather than at the point itself, and thus is unable to reach a sample less than 50 µL of volume (Figure 7).

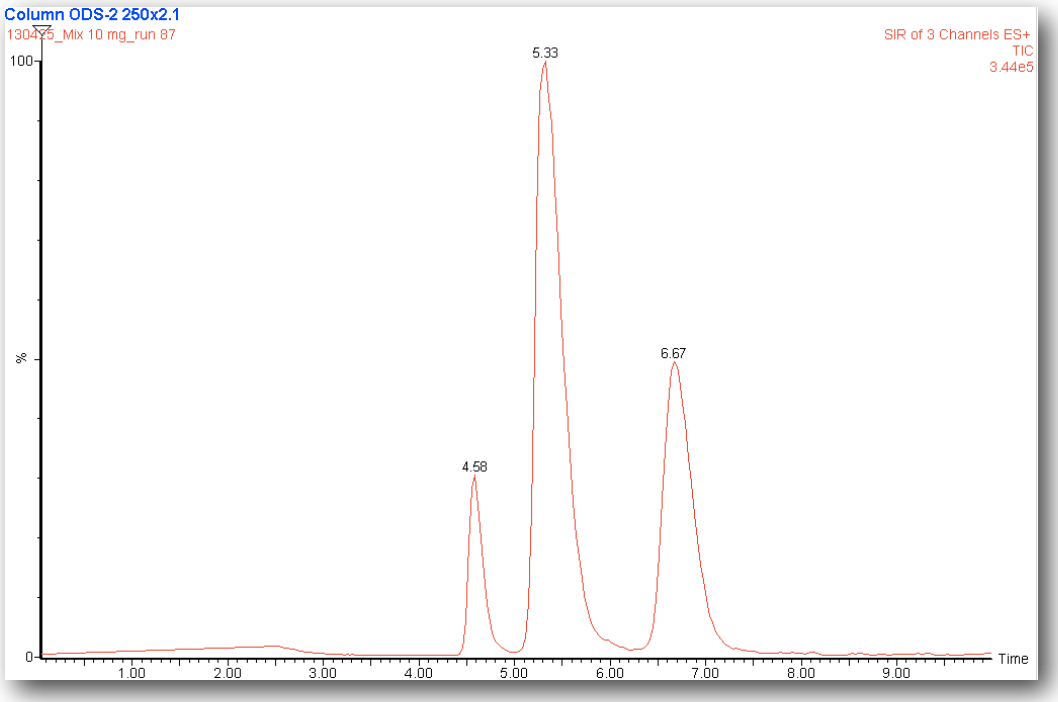


Figure 6. The Alliance Waters 2695 Separations Module with the Thermo Scientific ODS-2 Hypersil column, in connection with the Micromass ZMD achieved the best chromatographic separation. TIC of 3 channels (296, 306 and 310) of a calibration solution containing 10 mgL^{-1} each of norFLU (t_R = 4.58 min), FLU (t_R = 5.33 min) and SER (t_R = 6.67 min).

Figure 7. The Waters 2695 Separations module needle is constructed so that it picks up sample on the side, some 5 mm up from the needlepoint, rather than at the point itself, from [46].

Two different Agilent Hewlett Packard Series 1100 HPLC systems, both with the Thermo Scientific ODS-2 Hypersil column, in connection with Hewlett Packard Series 1100 MSD, were discarded because of being old and out of order. An advantage with this setup, however, was the compatibility with the ChemStation Rev. B.01.01 software (Agilent Technologies, Santa Clara, CA, USA).

In the end a third Agilent Hewlett Packard Series 1100 HPLC system was connected to the Micromass ZMD, along with the Thermo Scientific ODS-2 Hypersil column. The HPLC system would not communicate with the MassLynx 4.0 software, and therefore MassLynx was used to control the ZMD and the HP 1100 remote to control the HPLC. The same separation as with the Waters 2695 was never achieved with the HP 1100, but with the LC-MS setup this is not necessary as long as the masses are separated in the MS (Figure 8). In the end the total runtime for the separation was nine minutes.

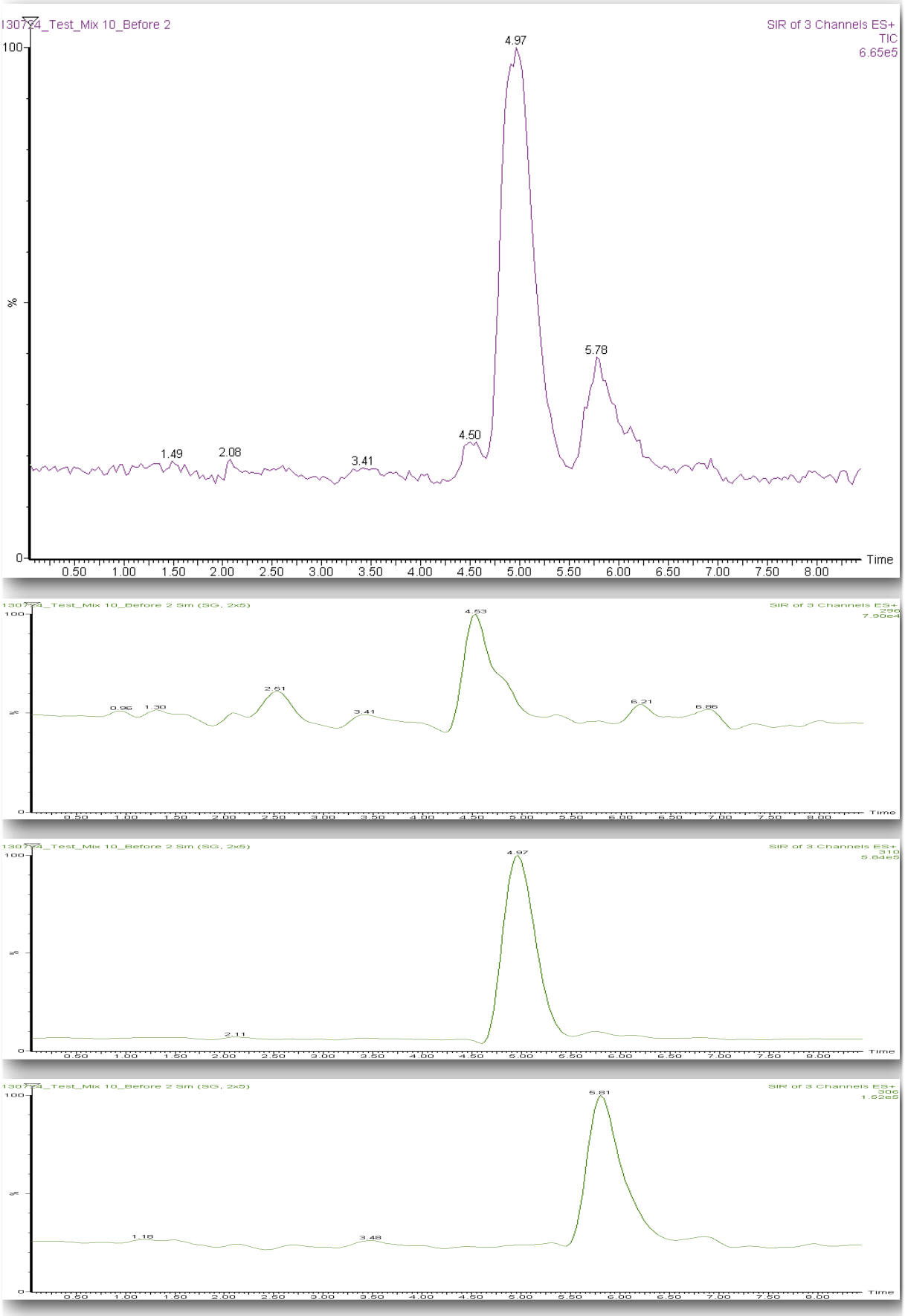


Figure 8. TIC of 3 Channels (296, 306 and 310), top, and SIRs of the individual channels. HP 1100 Series, with Thermo Scientific ODS-2 Hypersil column, in connection with the Micromass ZMD of a calibration solution containing 10 mgL^{-1} each of norFLU (t_R = 4.53 min), FLU (t_R = 4.97 min) and SER (t_R = 5.81 min).

3.1.2 Calibration curves

Calibration data of the LC/MS method (as described in 3.1.1) were processed and calibration curves for each compound were constructed with R^2-values of 0.936, 0.990, 0.966 for norFLU, FLU and SER, respectively.

3.2 Optimisation of extraction

3.2.1 The matrix effect

Biological samples do always present a more complex matrix than do water samples, therefore it is of interest to investigate how much the matrix interacts with the analytes. Spiked water and spiked lyophilised cod were extracted for 5 h with 8 replicates. The overall Ee was in the range of 1507-1805 for the cod samples and 3020-6287 for the water samples, graphically illustrated in Figure 9.

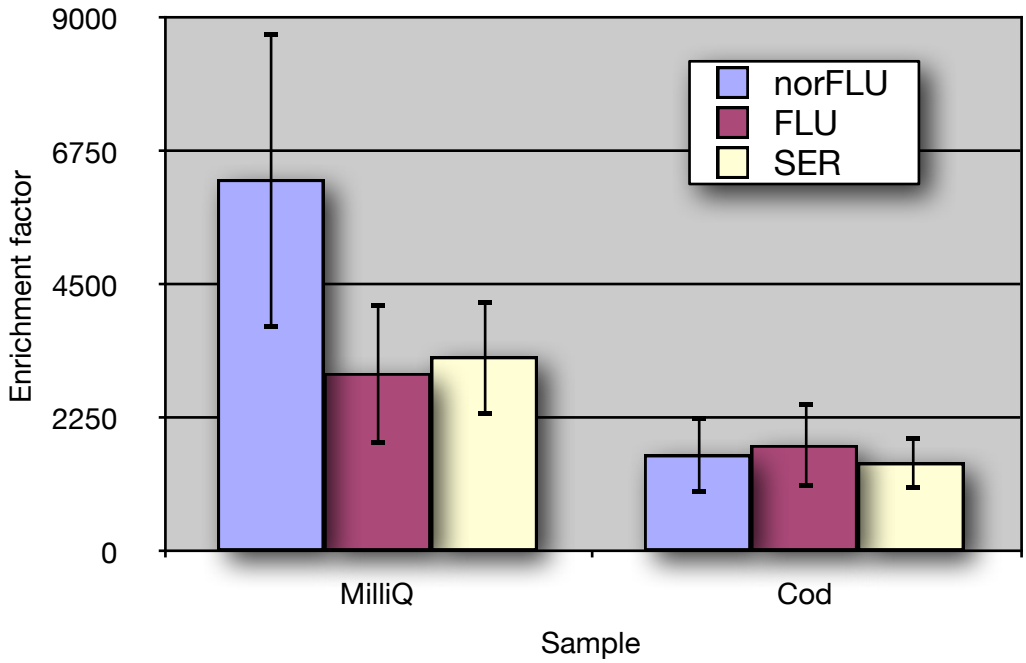


Figure 9. Comparing spiked water samples with spiked cod samples: the matrix effect is significant, possibly because of physical and or chemical binding of the analytes to the proteins in the fish.

The matrix effect is obvious; analytes in water were enriched to a much greater extent, speculatively because of physical and or chemical binding to proteins or lipid tissue in the fish, which would decrease the amount of free analytes in the water phase. Or because of fish tissue physically getting in the way for the analytes diffusing into the fibre, which would only

increase the time for equilibration, i.e. extraction time, and since extraction time was actually not optimised (see 3.1) this cannot completely be ruled out.

3.3 Method validation

3.3.1 Repeatability and reproducibility

To evaluate the validation of the HF-LPME method for norFLU, FLU and SER after 5 h of extraction, the repeatability and reproducibility (intra-day precision) spiked water was tested. The results, expressed as relative standard deviation, are presented in Table 3.

Table 3. Method repeatability and reproducibility (n=3) as relative standard deviations for water with a spiked concentration of 4000 μg L^{-1}.

Analyte	Repeatability (%)	Reproducibility (%)
norFLU	100.9	29.3
FLU	34.8	7.4
SER	45.8	21.1

The range of repeatability were 34.8-100.9% and of reproducibility 7.4-29.3%. The low repeatability in the method shows that the instrument was unreliable in giving the same result for the same concentration from one day to another, especially for norFLU. Even the intra-day precision was rather low, again, especially for norFLU. This may be evidence for norFLU being more difficult to detect, but that hypothesis is outside the scope of this thesis.

3.3.2 Limits of detection and limits of quantification in water samples

Instrumental limits of detection (LOD, and quantification (LOQ) were calculated as 3 and 10 times the signal-to-noise ratio, respectively. The overall method detection limit (MDL) was calculated by dividing LOD by Ee. Moreover, the practical quantitation limits (PQL), defined as 5 times MDL were calculated. All values for 4 μL of injection are presented in Table 4.

Table 4. LOD as 3 times the signal-to-noise ratio; LOQ as 10 times the signal-to-noise ratio; MDL as LOD divided by Ee; PQL as 5 times MDL. All values for water samples.

Analyte	LOD (μg L^{-1})	LOQ (μg L^{-1})	MDL (ng L^{-1})	PQL (ng L^{-1})
norFLU	1755	5851	280	1400
FLU	395	1316	130	650
SER	868	2893	260	1320

High limits of detection are, again, proof of rather poor instrumentation. LOD is especially high for norFLU, again, evidence of norFLU being hard to detect. Because of these poor values, norFLU was excluded from the exposure solution.

3.4 Application of the developed method

3.4.1 Enrichment factors in spiked cod samples and water samples

The developed method was used to determine the enrichment factor, Ee, of norFLU, FLU and SER in spiked cod as 1642, 1805 and 1507, respectively. Enrichment factors in water were determined as 6287, 3020 and 3298 in norFLU, FLU and SER, respectively.

3.4.2 Determination of SSRIs in crucian carp samples

The developed method was used to determine the concentration of norFLU, FLU and SER in exposed crucian carp and in the fish tank water where 51 μg L^{-1} was added to expose the fish. The results are presented in Table 5.

Table 5. Concentration analyte detected in exposed crucian carp and in the fish tank water, expressed both as C_{Ae} and C_{Di} or μg g^{-1}. 95% confidence, n=24 for fish samples, n=1 for water samples.

Analyte		Conc. in fibre, C_{Ae} (μg L^{-1})	Conc. in sample, C_{Di} (μg L^{-1})	Conc. in fish (μg g^{-1})
Fish	norFLU	ND	-	ND
	FLU	31.7±4.9	-	1.70±0.28
	SER	43.8±5.2	-	2.78±0.33
Water, 72 h	norFLU	ND	ND	-
	FLU	46.7	15.5	-
	SER	31.9	20.6	-

In the exposed crucian carp FLU and SER was detected at concentrations 1.70±0.28 μg g^{-1} and 2.78±0.33 μg g^{-1}, respectively. The concentration of the analytes in the fish tank water was measured. At the point of exposure (Water, 0 h) no analytes were detected, and after 3 days (Water, 72 h) concentrations of 15.5 μg L^{-1} and 20.6 μg L^{-1} of FLU and SER, respectively, were measured. This is to be compared with the 51 μg L^{-1} which were added, and signify some adsorption or degradation losses. Unfortunately, no analytes were detected in the water, 0 h-sample. This may well be due to poor mixing in the fish tank. Because of poor repeatability and high LOD (see sections 3.3.1 and 3.3.2) norFLU was not added to the

exposure solution. Nor was norFLU detected in the exposed fish. This may indicate a slow metabolism of FLU in fish.

The developed method was also used to determine the concentration of norFLU, FLU and SER in unexposed crucian carp, no analytes were detected. This indicates a valid method.

4. Conclusion

A direct method to determine norFLU, FLU and SER in fish was developed based on HF-LPME extraction and LC-MS analysis. The obtained enrichment factors for the analytes ranged 1500-1800 and 3000-6300 for exposed cod and water samples, respectively. In crucian carp, exposed to a 51 μg L^{-1} FLU and SER mixture for 72 h, the SSRIs were detected at concentrations 1.70±0.28 μg g^{-1} and 2.78±0.33 μg g^{-1}, respectively. The FLU metabolite norFLU was not detected in the exposed fish. In the unexposed crucian carp, none of the analytes were detected. This method could be successfully applied to detect norFLU, FLU and SER in real fish samples.

5. Further work

Since the potential environmental impacts of PPCPs are not well studied, information concerning ecotoxicological risks and distribution in biota is needed. The elaboration and improvement of HF-LPME with LC-MS methodologies is one step in the determination of trace level and ionisable analytes in environmental samples, especially for samples with complex matrices.

Because of the situation with the instruments, described in section 3.1.1, most of the time allocated was spent on maintenance rather than development and optimisation. For this reason this method is in need of some refining. Primarily the exposure experiment should be carried out again and samples diluted to fit the linear range of the calibration curve. Because of the substantial matrix effect (section 3.2.1) the method of standard addition should be applied. In standard addition known quantities are added to the original sample, in this case a series of samples is spiked with increasing concentrations of standard. The initial concentration of the sample can subsequently be derived from the y-intercept of a spike vs. signal plot [47].

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