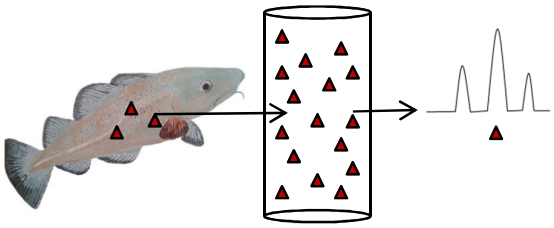
Analytical Methods

A specific, highly enriching and "green" method for hollow fiber liquid phase microextraction of ionizable pharmaceuticals from fish tissue

Journal:	*Analytical Methods*
Manuscript ID:	AY-ART-02-2014-000470.R3
Article Type:	Paper
Date Submitted by the Author:	16-May-2014
Complete List of Authors:	Boström, Marja; Lund University, Biology Huang, Chuixiu; Lund University, Chemistry Engström, Henrik; Lund University, Chemistry Larsson, Estelle; Lund University, Chemistry Berglund, Olof; Lund University, Biology Jonsson, Jan; Lund University, Chemistry

Graphical and Textural Abstract for our manuscript ”A specific, highly enriching and “green” method for hollow fiber liquid phase microextraction of ionizable pharmaceuticals from fish tissue”:



This study presents a new application for the HF-LPME technique; extracting ionizable pharmaceuticals from fish tissue. The thousand fold enrichment achieved with HF-LPME makes low environmental concentrations analytically measurable.

A specific, highly enriching and "green" method for hollow fiber liquid phase microextraction of ionizable pharmaceuticals from fish tissue

Marja Lena Boström[a], Chuixiu Huang[b], Henrik Engström[b], Estelle Larsson[b], Olof Berglund[a], Jan Åke Jönsson[b]

[a] Aquatic Ecology, Dept. Biology, Lund University, Lund, Sweden

[b] Center for Analysis and Synthesis, Dept. Chemistry, Lund University, Lund, Sweden

Abstract

Ionizable pharmaceuticals are a class of emerging contaminants that pose a challenge to analytical chemistry due to low environmental concentrations. To measure such low concentrations in organism tissue, e.g. fish muscle, specific extraction techniques minimizing co-extraction and interference alongside providing high enrichment of the compounds is needed. In this study we present a technique using hollow fiber liquid phase microextraction which is selective and highly enriching due to a pH gradient across a selective membrane, trapping ions in the extract. Microextraction minimizes the use of organic solvents, thereby making the technique "green". We used high volume pharmaceuticals for method development, specifically, the weak acids ketoprofen, naproxen, diclofenac and ibuprofen, and the weak bases fluoxetine and sertraline. Lyophilized tissue extraction gave higher enrichment than fresh tissue extraction and concentration enrichment factors ranged from 1900 to 3000 times. Method detection limits with the analysis instruments used in this study were for ketoprofen, 0.23 ng/g fish tissue; naproxen, 0.32 ng/g fish tissue; diclofenac, 0.12 ng/g fish tissue; ibuprofen, 0.34 ng/g fish tissue; fluoxetine, 13 ng/g fish tissue and sertraline, 23 ng/g fish tissue. All analytes were successfully detected in tissue from fish exposed live via spiked water. The resulting extraction parameters shown in this study suggests the developed technique to be a useful work up method for extensive environmental data collection as well as for toxicokinetic studies.

Introduction

Pharmaceuticals are emerging organic contaminants, which potentially impact organisms especially in aquatic systems influenced by municipal wastewater[1-4]. Aquatic organisms such as fish living in wastewater recipients are under long-term exposure to pharmaceuticals, which could lead to bioaccumulation[5, 6] thus raising internal concentrations to possible toxic levels. To measure chemical concentrations in tissue samples in order to for instance address toxicokinetic questions, methods for chemical extraction are needed. Organism tissue, containing multiple possible analytically interfering

compounds, is a complex matrix and extraction techniques need to be specific to reduce unwanted co-extraction and interference. Furthermore, environmental concentrations of pharmaceuticals in organisms such as fish are low, ng/g levels[5-7], which calls for selective extraction techniques that also enrich the analyte. For extensive data collection, such as in longitudinal monitoring, the technique should furthermore be easy to use, environmentally friendly and cheap.

Almost 80% of all pharmaceuticals are ionizable[8] which makes such compounds a prioritized group to study. In the method development described here six high volume pharmaceuticals are considered, four weak acids: the non-steroidal anti-inflammatory drugs (NSAID) ketoprofen, naproxen, diclofenac and ibuprofen, and two weak bases: the selective serotonin reuptake inhibitors (SSRI) fluoxetine and sertraline (Table 1). Both groups have been shown to be taken up by fish downstream of wastewater treatment plants[5-7, 9-12] and to have physiological effects on fish[9, 13-17]. Extraction of these pharmaceuticals from fish samples is usually done from homogenates prepared in water or acetonitrile, followed by clean-up using solid-phase extraction (SPE)[5-7, 14, 18, 19], which is a multistep extraction technique using organic solvents. Methods for extraction used for other biological samples, for instance blood, plasma and urine from other species, are liquid-liquid extraction (LLE), dispersive liquid-liquid microextraction (DLLME), stir membrane liquid-liquid microextraction (SM-LLME), liquid-phase microextraction (LPME), SPE and solid-phase microextraction (SPME)[20-25]. Also among these samples, SPE is the most commonly used technique followed by LLE. To improve extraction by reducing time-consuming steps, use of organic solvents and analyte loss through evaporation and/or centrifugation steps, while keeping or even extending selectivity and high enrichment for the analytes, three-phase hollow fiber liquid phase microextraction (HF-LPME) techniques were developed[26, 27]. In HF-LPME, a hollow fiber is used to separate the sample solution on the outside, termed donor phase, and the acceptor phase inside the fiber. The third phase is an organic phase in the pores of the fiber acting as a selective barrier allowing uncharged molecules to pass while hindering ions. The selective clean-up and enrichment using three-phase HF-LPME is driven by a pH gradient shifting the dissociation equilibrium of the ionisable pharmaceuticals towards the uncharged

form in the donor phase, allowing the molecules to pass into the fiber, and then towards the ionic form in the acceptor phase, trapping and concentrating the ions inside the fiber. The method is thereby very selective, highly enriching and time saving because enrichment and clean-up are done in one single step. Furthermore, because of the miniature scale in which this is performed, the material costs are low and the volume of organic solvent used is very small making this a more environmentally friendly technique compared to for instance SPE.

Three-phase HF-LPME has previously been used for ionizable chemicals in semi-solid samples such as sewage sludge and secum[28-31]. The aim of this study is to develop HF-LPME methods for fish tissue matrix with improved extraction parameters. Analysis following HF-LPME is performed using LC-MS/MS for NSAIDs and LC-MS for SSRIs in accordance with previous studies where similar techniques were applied to other matrices[30-32]. The method is developed and validated using both spiked tissue samples and fish exposed live via water.

Materials and Methods

Chemicals

Diclofenac sodium salt, ibuprofen, ketoprofen and naproxen, fluoxetine hydrochloride, sertraline hydrochloride, ammonium carbonate (30-33% NH_3), di-*n*-hexyl ether (DHE) and NH_4Ac reagent grade were all obtained from Sigma Aldrich Chemie GmbH (Steinheim, Germany). H_2SO_4 trace select (≥95%) was from Sigma Aldrich (Buchs, Switzerland). Methanol HPLC gradient grade and acetonitrile (ACN) gradient grade from Honeywell B&J brand (Seelze, Germany). Glacial acetic acid (HAc, 100%), H_3PO_4 (85%) and $(NH_4)_3PO_4$ (reagent grade) from Merck (Darmstadt, Germany). Reagent NaOH was from Scharlau Chemie S.A. (Barcelona, Spain) and dimethyl sulfoxide (DMSO) from Thermo Scientific (Rockford, IL, USA). Reagent water was produced in a Milli-Q purification system from EMD Millipore Corporation (Billerica, MA, USA).

Stock solutions for the chemical analysis, with concentrations of 1 or 10 mg/L, were prepared in reagent water and stored at 4°C, in amber bottles covered with aluminum foil to prevent potential photodegradation. Stock solutions for the exposure study with the concentration 30 g/L, were prepared in DMSO and stored under the same conditions. A degradation study of water solutions in room temperature and darkness showed no significant change in concentration for any of the chemicals (data not shown).

Hollow fiber liquid phase microextraction

A hollow polypropylene fiber, PP50/280 Accurel, wall thickness 50 μm, 0.1 μm pore size and inner diameter 280 μm (Membrana GmbH, Wuppertal, Germany), was cut to a length of 20 cm and the lumen was filled with approximately 10 μL of acceptor fluid using a 0.5 mL syringe with needle size 0.33×12 mm (Terumo Medical Corporation, Elkton, MD, USA). The NSAID extraction acceptor solution was 0.1 M ammonium carbonate buffer, pH 9.0, and the SSRI extraction acceptor was 0.1 M ammonium phosphate buffer, pH 2.1 as in previous studies[30-32]. The fiber was soaked in DHE for 1 min to fill the pores with organic solvent and any excess was washed off by a short immersion in reagent water. Using DHE as organic phase has previously been optimized for both NSAIDs[28] and SSRIs[33]. Afterwards, fresh acceptor fluid was pushed through the fiber and the ends were sealed using an electric soldering iron (WECP-20, Weller, Besigheim, Germany). Before placing the fiber into the 50 mL donor, *i.e.* fish slurry sample, the fiber was looped twice and weighed down by a small piece of copper wire to ensure complete submersion. Prior to this, the donor was adjusted to pH 2.0 for the NSAID extraction and pH 12.4 for the SSRI extraction as in previous studies[30-32]. During extraction a magnetic stirrer (RO10 Power, IKA, Staufen, Germany) set to 660 rpm was used to mix the donor[30]. After extraction the acceptor was retrieved by opening the ends of the fiber with a scalpel and pushing the fluid out with an air-filled syringe into a 2 mL vial with a μL insert. The acceptor phase was diluted to a concentration within the linear range of the analytical instrument calibration curve, sonicated for complete mixing and stored in darkness at 4°C prior to analysis.

Fish tissue sample preparation

For extraction time optimization, slurries containing muscle tissue from locally purchased cod (*Gadus morhua*) was spiked to 0.5 µg/L with all four NSAIDs. Previously Sagristà *et al*.[30] found that the optimal extraction time was 3 to 5 hours for sewage sludge, but different matrices may affect the mass transfer processes in different ways so 3, 4, 5 and 6 hours were tested. Batches of fresh tissue homogenate were prepared by homogenizing (Ultra-Turrax T25, IKA Werke) equal amounts by weight of fish tissue and reagent water for 10 min. From the batch 1 g was taken out for each replicate, spiked with analyte and 49 mL of water was added followed by 2 min of additional homogenization. Samples were prepared in 100 mL wide neck brown bottles and left overnight in darkness at 4°C for equilibration prior to HF-LPME extraction.

Pre-extraction conditioning by lyophilization was compared with direct extraction from the tissue. Prior to overnight lyophilization, fresh tissue was weighted into sample sizes of ~0.5 g each and homogenization was done individually for each sample. The lyophilized fish tissue was homogenized in 1 mL reagent water for 10 min and after spiking 49 mL of water was added followed by 2 min of additional homogenization. All samples were left to equilibrate overnight at 4°C before extraction. Extraction solutions were spiked with NSAIDs to a concentration of 0.5 µg/L and with SSRIs in a concentration range from 0.2 to 200 µg/L. Comparisons between extractions were made using the enrichment factor (Ee)

$$Ee = \frac{C_{Ae}}{C_{Di}} \qquad \text{Eq. 1}$$

where C_{Ae} is the concentration for the acceptor phase at equilibrium and C_{Di} the initial donor phase concentration. Final Ee, used for calculating tissue concentrations in fish exposed live via water, was determined using lyophilized spiked tissue.

Method detection limits (MDL) referring to the whole analytical procedure and expressed as ng/g fish tissue were obtained according to

$$MDL = \frac{LOD}{Ee} \times \frac{V_{Di}}{m_{fish}} \qquad \text{Eq. 2}$$

where LOD (ng/L) is the limit of detection of the analytical instrument used (S/N=3), V_{Di} is the volume (L) of the donor phase and m_{fish} is the mass fish tissue (g) in the slurry.

To validate the extraction techniques applicability on environmental samples, live fish were exposed through spiked water (ethical approval no M459-12, Malmö/Lund djurförsöksetiska nämnd, Lund, Sweden). Rudd (*Scardinius erythrophthalmus*) from Lake Krankesjön, Sweden (55° 42′ 29″ N, 13° 28′ 21″ E), weight ~1.5 g, were exposed to NSAIDs and crucian carp (*Carassius carassius*) from a pond on the Revinge fields, Sweden (55° 42′ 38″ N, 13° 27′ 22″ E), weight ~2.5 g, was exposed to SSRIs. The fish were acclimatized in the lab for two to four weeks in a flow-through tap water system prior to exposure. The exposure set-up was designed to keep the water to fish ratio high: Eight rudds were exposed for three days in 4 L of water containing approximately 50 µg/L of each NSAID and nine crucian carps were exposed in the same way but using SSRI (identical concentration) and 40 L of water. After exposure termination the fish were cut into small pieces and approximately 0.5 g of tissue was randomly combined for each replicate from the whole batch of cuttings. The samples were lyophilized prior to HF-LPME.

NSAID analysis using LC-MS/MS

Analysis of NSAIDs was performed on an API Q-Star Pulsar I quadrupole time of flight tandem mass spectrometer with a Turboion electrospray interface from Applied Biosystems (Carlsbad, California, USA) coupled to an Ultimate pump and a Famos autosampler from LC Packings (Thermo Scientific, Waltham, MA, USA) and a CSI 6150 vacuum degasser (Cambridge Scientific Instruments, Cambridge, UK). The system was controlled by Analyst QS 1.1 from Applied Biosystems. The injection volume was 4 µL for all samples using the pick-up mode of the autosampler. Before each analysis, clean acceptor solution was injected to avoid cross contamination.

Chromatographic separation was achieved with an Agilent Eclipse XDB-C18 column (particle size 5 µm, 4.6 x 150 mm). The employed gradient was 85:15 (100% methanol:NH_4Ac buffer 10 mM, pH 4) for the first 2 min at a flow rate of 0.3 mL/min, followed by a linear increase to 90:10 for 2 min at an increased

flow rate, 0.6 mL/min, in order to shorten the runtime. To obtain good separation the flow rate was then lowered to 0.3 mL/min for 1 min to again be raised to 0.6 mL/min for 1 min. The run ended by a linear decrease to 85:15 during 1 min.

For the MS/MS analysis the setting of ion spray voltage was -4500 V and the ion source temperature was 400°C. The settings of focusing potential and declustering potential were -220 V and -10 V respectively and the setting of collision gas was 5 units. Other parameters for each target ions are presented in Table 2 and Figure 1 show typical chromatograms from live exposed fish. Linear calibration was made up to 1 mg/mL, and R^2-values were 0.990, 0.995, 0.980 and 0.989 for ketoprofen, naproxen, diclofenac and ibuprofen, respectively.

SSRI analysis using LC-MS

Analysis was performed on a Micromass ZMD single quadrupole mass spectrometer (Micromass Ltd, Manchester, UK) with an electrospray interface connected to an Agilent/HP 1100 Series HPLC system, consisting of degasser, pump and autosampler (Agilent Technologies, Waldbronn, Germany). The injection volume was 4 µL for all samples, using needle wash between each pick up, and flow rate was 0.3 mL/min. The ZMD was controlled by MassLynx 4.0 software (Micromass) and the HPLC system was controlled by a Hewlett Packard remote control (Hewlett Packard, Karlsruhe, Germany). The ZMD was run in positive-ion mode, capillary voltage 3.6 kV, cone voltage 15 V. ESI source block temperature was 150 °C, desolvation temperature 350 °C, desolvation gas (N_2) at a flow of 540 L/h, 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 fluoxetine (310 m/z) and sertraline (306 m/z) and Figure 2 show typical chromatograms from live exposed fish.

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). The employed gradient was a linear rise from 50:50 (ACN:NH_4AC buffer) to 60:40 in 0.5 min, holding for 1.5 min, a linear decrease to

50:50 in 0.1 min and finally holding for 6.9 min. The flow rate was 0.3 mL/min. A calibration curve was acquired using standard solution of the two analytes of concentrations up to 10 mg/L, R^2-values were 0.990, 0.966 for fluoxetine and sertraline respectively.

Statistics

To test for increase in the chromatographic signal variation by the extraction technique compared to that produced by the analytical instrument alone, one-tailed F-tests at 95% confidence level were made. Extraction variation was calculated from a mean of replicate spiking at one concentration for NSAIDs (n=5) and from regression lines from multiple concentrations for SSRIs (spiked lyophilized samples, n=4; fresh tissue spiked with sertraline, n=8 and fresh tissue spiked with fluoxetine, n=9). Analytical instrument variation was calculated from regression lines made from standard solutions (NSAIDs, n=5 and SSRIs, n=7).

Results and Discussion

Extraction time

The plateau for optimal time for NSAID extraction is similar to that found by Sagristà *et al*[30] in sewage sludge, which leads to the conclusion that the matrices are reasonably similar in affecting the mass transfer (Figure 3). The optimal extraction time for sewage sludge samples has been determined to 4 h for the NSAIDs[30] and 6 h for the SSRIs[31]. As the differences for Ee of the NSAIDs were similar between 4 h and 5 h both in this study and in the study by Sagristà *et al*[30] and between 5 h and 6 h for the SSRIs in Sagristà *et al*[31], 5 h was determined as the optimal extraction time for both NSAIDs and SSRIs. Prolonging the extraction time to 6 h decreases Ee for the NSAIDs, which could be due to pH changes in the acceptor phase or loss of the organic phase in the hollow fiber pores.

Sample preparation and variance

Lyophilizing samples prior to extraction were compared to extraction from fresh tissue. Ee for the two different sample types was in the same range but relative standard deviation (RSD) for the extractions from the spiked lyophilized tissue was always smaller than fresh tissue extraction RSD: ketoprofen; 7.1% vs 30%, naproxen; 12% vs 26%, diclofenac; 15% vs 28%, ibuprofen; 15% vs 35%, fluoxetine; 17% vs 39% and sertraline; 12% vs 40%. When replicate membrane extractions were compared with standard curve injections the variance was not above that of the analytical equipment except for fresh tissue spiked with ketoprofen (F-tests, 1-tailed, 95% confidence, n=2-7). The smaller variance using lyophilized tissue, possibly due to samples being more homogenous, suggests this being a preferable sample pre-treatment prior to extraction.

Weak acids and bases can be co-extracted using a single SPE but HF-LPME does not have that advantage. This potentially means that twice the amount of tissue and time is needed to extract both groups of chemicals with HF-LPME. Preliminary results from serial extraction of NSAIDs and SSRIs from the same sample show promising results though, which would overcome this disadvantage and reduce the amount of tissue needed for analysis.

Enrichment factors

Ee used for concentration determination in pre-exposed fish was obtained by analyzing spiked lyophilized fish slurry (Table 1). Reported values of Ee from HF-LPME using spiked reagent water is within the range found here for naproxen and ibuprofen but higher for ketoprofen and diclofenac, 15% and 38% respectively[30]. For SSRI, Ee in spiked reagent water was higher for both fluoxetine (30%) and sertraline (43%). The lower Ee in the fish slurry samples could be due to the analyte binding to fat and/or proteins in the matrix lowering partition into the fiber during the extraction process. The explanation is supported by the log-transformed octanol-water partitioning coefficient ($\log K_{OW}$, Table 1), being close to or above 4.0 for diclofenac, fluoxetine and sertraline, the pharmaceuticals strongest affected by the matrix, and below 3.5 for the other three NSAIDs.

The MDL were for ketoprofen, 0.23 ng/g fish tissue; naproxen, 0.32 ng/g fish tissue; diclofenac, 0.12 ng/g fish tissue; ibuprofen, 0.34 ng/g fish tissue; fluoxetine, 13 ng/g fish tissue and sertraline, 23 ng/g fish tissue. The higher MDL values for the SSRIs are due to the higher LOD of the LS-MS system compared to the LS-MS/MS system used for the NSAIDs. This only shows that the high Ee values shown in this study are of better use if the precision of the analytical instrument is high.

Determining pharmaceuticals in fish exposed via water

The extraction method was applied to extract, detect and quantify the pharmaceuticals in rudd and crucian carp exposed live via spiked water. All the pharmaceuticals could be detected in the fish after exposure. Measured tissue concentrations after a three day exposure to nominal concentrations of 50 µg/L was for ketoprofen 24 (±8) µg/kg fresh weight, naproxen 73 (±23) µg/kg fresh weight, diclofenac 50 (±17) µg/kg fresh weight, ibuprofen 60 (±19) µg/kg fresh weight, fluoxetine 1300 (±400) µg/kg fresh weight and sertraline 2000 (±600) µg/kg fresh weight (95% CI, n=7 for NSAIDs and n=5 for SSRIs). Calculated times to 95% of steady state suggests close to steady state after 3 days for ketoprofen, naproxen and ibuprofen (Table 1). This gives rough estimates of steady state bioconcentration factors (BCF_{SS}), when dividing tissue concentration (using the 95% CI range) and water nominal concentrations (Table 3). Diclofenac, fluoxetine and sertraline were calculated to have reached or be beyond 50% of the time to steady state after 3 days (Table 1). Despite not reaching steady state, thereby not being able to calculate BCF_{SS}, it should be noted that the BCFs found are high for the SSRIs (Table 3). BCFs have been determined in other studies on fish muscle tissue for diclofenac, ibuprofen and fluoxetine, and the NSAIDs have shown lower BCF_{SS} than the SSRIs, which is consistent with our results[34-37] (Table 3). Different fish species potentially having differing lipid and/or protein content were used, both between studies and within this study, which may influence equilibrium partitioning of these substances. BCFs reported in table 3 are all on a wet weight basis, and lipid corrected normalization of the BCFs may reduce variation between species[38]. Also, because of larger matrix effect in tissue samples compared to standard solutions making up the calibration curve absolute values in this study should be treated with caution. To

compensate for the matrix effect when determining tissue concentrations standard addition could be used to create an internal calibration curve[30].

Conclusion

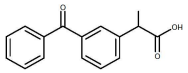
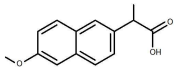
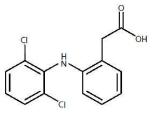
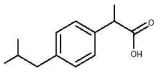
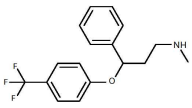
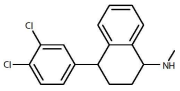
HF-LPME has not previously been used for extraction and workup of ionizable pharmaceuticals in tissue samples. The technique show advantages over the most commonly used work-up technique for fish tissue, SLE, including high enrichment and cleanup in one single step and low solvent use. SPE should instead be used if one wants a more general extraction and if a large volume is needed for injection onto the analytical instrument used. SPME is another common workup technique used sharing many of the advantages over SLE, but it adds extra laboratory work as the acceptor cannot be direct applied to a HPLC-detector system. The conclusion is that HF-LPME is a recommendable workup technique for tissue samples.

References

1. H. R. Buser, T. Poiger and M. D. Mueller, *Environ Sci Technol*, 1998, **32**, 3449-3456.
2. A. Daneshvar, J. Svanfelt, L. Kronberg and G. A. Weyhenmeyer, *Environ Sci Pollut Res*, 2010, **17**, 908-916.
3. D. W. Kolpin, E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber and H. T. Buxton, *Environ Sci Technol*, 2002, **36**, 1202-1211.
4. C. Tixier, H. P. Singer, S. Oellers and S. R. Muller, *Environ Sci Technol*, 2003, **37**, 1061-1068.
5. J. N. Brown, N. Paxeus, L. Förlin and D. G. J. Larsson, *Environ Toxicol Pharmacol*, 2007, **24**, 267-274.
6. J. Fick, R. H. Lindberg, J. Parkkonen, B. Arvidsson, M. Tysklind and D. G. Larsson, *Environ Sci Technol*, 2010, **44**, 2661-2666.
7. B. W. Brooks, C. K. Chambliss, J. K. Stanley, A. Ramirez, K. E. Banks, R. D. Johnson and R. J. Lewis, *Environ Toxicol Chem*, 2005, **24**, 464-469.
8. D. T. Manallack, *Perspect Medicin Chem*, 2008, **1**, 25-38.
9. B. W. Brooks, C. M. Foran, S. M. Richards, J. Weston, P. K. Turner, J. K. Stanley, K. R. Solomon, M. Slattery and T. W. La Point, *Toxicol Lett*, 2003, **142**, 169-183.
10. A. J. Ramirez, M. A. Mottaleb, B. W. Brooks and C. K. Chambliss, *Anal Chem*, 2007, **79**, 3155-3163.
11. G. Ouyang, K. D. Oakes, L. Bragg, S. Wang, H. Liu, S. Cui, M. R. Servos, D. G. Dixon and J. Pawliszyn, *Environ Sci Technol*, 2011, **45**, 7792-7798.
12. O. P. Togunde, K. D. Oakes, M. R. Servos and J. Pawliszyn, *J Chromatogr A*, 2012, **1261**, 99-106.
13. M. M. Schultz, M. M. Painter, S. E. Bartell, A. Logue, E. T. Furlong, S. L. Werner and H. L. Schoenfuss, *Aquat Toxicol*, 2011, **104**, 38-47.
14. F. Cuklev, E. Kristiansson, J. Fick, N. Asker, L. Förlin and D. G. J. Larsson, *Environ Toxicol Chem*, 2011, **30**, 2126-2134.
15. B. Hoeger, B. Kollner, D. R. Dietrich and B. Hitzfeld, *Aquat Toxicol*, 2005, **75**, 53-64.
16. J. Schwaiger, H. Ferling, U. Mallow, H. Wintermayr and R. D. Negele, *Aquat Toxicol*, 2004, **68**, 141-150.
17. J. Corcoran, M. J. Winter and C. R. Tyler, *Crit Rev Toxicol*, 2010, **40**, 287-304.
18. J. M. Brozinski, M. Lahti, A. Oikari and L. Kronberg, *Environ Sci Pollut Res Int*, 2011, **18**, 811-818.
19. J. M. Kallio, M. Lahti, A. Oikari and L. Kronberg, *Environ Sci Technol*, 2010, **44**, 7213-7219.
20. S. M. R. Wille, E. A. De Letter, M. H. A. Piette, L. K. Van Overschelde, C. H. Van Peteghem and W. E. Lambert, *Int J Legal Med*, 2009, **123**, 451-458.
21. D. S. Jain, M. Sanyal, G. Subbaiah, U. C. Pande and P. Shrivastav, *J Chromatogr B*, 2005, **829**, 69-74.
22. L. Kristoffersen, A. Bugge, E. Lundanes and L. Slordal, *J Chromatogr B*, 1999, **734**, 229-246.
23. N. Unceta, A. Gomez-Caballero, A. Sanchez, S. Millan, M. C. Sampedro, M. A. Goicolea, J. Salles and R. J. Barrio, *J Pharm Biomed Anal*, 2008, **46**, 763-770.
24. R. E. Winecker, in *Clinical applications of mass spectrometry: Methods and protocols*, eds. U. Garg and C. A. Hammett-Stabler, Humana Press Inc, NJ, USA, 2010, vol. 603, pp. 45-56.
25. A. I. Olives, V. Gonzalez-Ruiz and M. Antonia Martin, *Anti-Inflammatory Anti-Allergy Agents Med Chem*, 2012, **11**, 65-95.
26. K. F. Bårdstu, T. S. Ho, K. E. Rasmussen, S. Pedersen-Bjergaard and J. Å. Jönsson, *J Sep Sci*, 2007, **30**, 1364-1370.
27. K. E. Rasmussen and S. Pedersen-Bjergaard, *Trends Anal Chem*, 2004, **23**, 1-10.
28. N. Larsson, E. Petersson, M. Rylander and J. Å. Jönsson, *Anal Methods*, 2009, **1**, 59-67.
29. T. Ghaffarzadegan, M. Nyman, J. Å. Jönsson and M. Sandahl, *J Chromatogr B*.

30. E. Sagrista, E. Larsson, M. Ezoddin, M. Hidalgo, V. Salvado and J. Å. Jönsson, *J Chromatogr A*, 2010, **1217**, 6153-6158.
31. E. Sagrista, J. M. Cortes, E. Larsson, V. Salvado, M. Hidalgo and J. Å. Jönsson, *J Sep Sci*, 2012, **35**, 2460-2468.
32. E. Larsson, A. Rabyah and J. Å. Jönsson, *J Environ Prot*, 2013, **4**, 946-955.
33. S. Zorita, L. Martensson and L. Mathiasson, *J Sep Sci*, 2007, **30**, 2513-2521.
34. U. Memmert, A. Peither, R. Burri, K. Weber, T. Schmidt, J. P. Sumpter and A. Hartmann, *Environ Toxicol Chem*, 2013, **32**, 442-452.
35. G. C. Nallani, P. M. Paulos, L. A. Constantine, B. J. Venables and D. B. Huggett, *Chemosphere*, 2011, **84**, 1371-1377.
36. Y. Nakamura, H. Yamamoto, J. Sekizawa, T. Kondo, N. Hirai and N. Tatarazako, *Chemosphere*, 2008, **70**, 865-873.
37. G. Paterson and C. D. Metcalfe, *Chemosphere*, 2008, **74**, 125-130.
38. R. M. Seston, D. E. Powell, K. B. Woodburn, G. E. Kozerski, P. W. Bradley and M. J. Zwiernik, *Integr Environ Assess Manag*, 2014, **10**, 142-144.

Table 1. General information, chemical structure, molecular weight (MW), pK_a and logP, for the six pharmaceuticals used in the study. Enrichment factors (Ee with 95% confidence interval) determined for lyophilized fish tissue with number of replicates (n) are reported alongside calculations of 50% and 95% of time to uptake steady state.

Substance		MW	pK_a [a]	logK_{OW} [a]	Ee (95% CI)	n	$t_{50\%}$ [b] (days)	$t_{95\%}$ [b] (days)
Ketoprofen		254	4.23	2.9	2700 (±200)	5	0.37	1.6
Naproxen		230	4.84	2.9	3000 (±300)	5	0.37	1.6
Diclofenac		296	4.18	4.5	2000 (±300)	5	1.7	7.4
Ibuprofen		206	4.41	3.5	2500 (±400)	5	0.66	2.9
Fluoxetine		309	10.1	3.9	2100 (±600)	6	0.96	4.2
Sertraline		306	9.47	5.1	1900 (±600)	6	3.0	13

[a]Scifinder database (© 2013 American Chemical Society), calculated values

[b]Calculated time to 50% or 95% of uptake steady state ([38] OECD guideline 305, Annex 5)

Table 2. Mass spectrometry parameters and monitoring ions used for detection of ketoprofen, naproxen, diclofenac and ibuprofen, respectively.

Analyte	Collision energy (V)	Declustering potential (V)	Precursor ion mass (m/z)	Product ion mass (m/z)
Ketoprofen	-12	-40	253	209.10
Naproxen	-10	-20	229	185.10
Diclofenac	-10	-20	294	250.02
Ibuprofen	-10	-20	205	161.13

Table 3. Reported BCF values (kg/L) for the investigated substances. Values reported from other studies are assumed steady state values (BCF_{SS}) accompanied by comments on type of tissue analyzed and experimental conditions.

Species	Ketoprofen	Naproxen	Diclofenac	Ibuprofen	Fluoxetine	Sertraline	Comments	Source
Rainbow trout (*Oncorhynchus mykis*)			3-5				BCF_{SS}, whole fish, 28 day exposure	34
Fathead minnow (*Pimephales promelas*)				0.7			BCF_{SS}, muscle tissue, 28 day exposure	35
Channel catfish (*Ictalurus punctatus*)				0.08			BCF_{SS}, muscle tissue, 7 day exposure	35
Rudd (*S. erythrophthalmus*)	0.3-0.6	1-2		0.8-2			Possible BCF_{SS}, muscle tissue, 3 day exposure	This study
Rudd (*S. erythrophthalmus*)			0.7-1				$BCF_{>50\% \text{ to SS}}$, muscle tissue, 3 day exposure	This study
Crusian carp (*C. carassius)*					20-30	30-50	$BCF_{>50\% \text{ to SS}}$, muscle tissue, 3 day exposure	This study
Japanese medaka (*Oryzias latipes*)					7-50		BCF_{SS}, whole fish, pH 7 and 8, 30 day exposure	36
Japanese medaka (*O. latipes*)					74		BCF_{SS}, whole fish, 7 day exposure	37

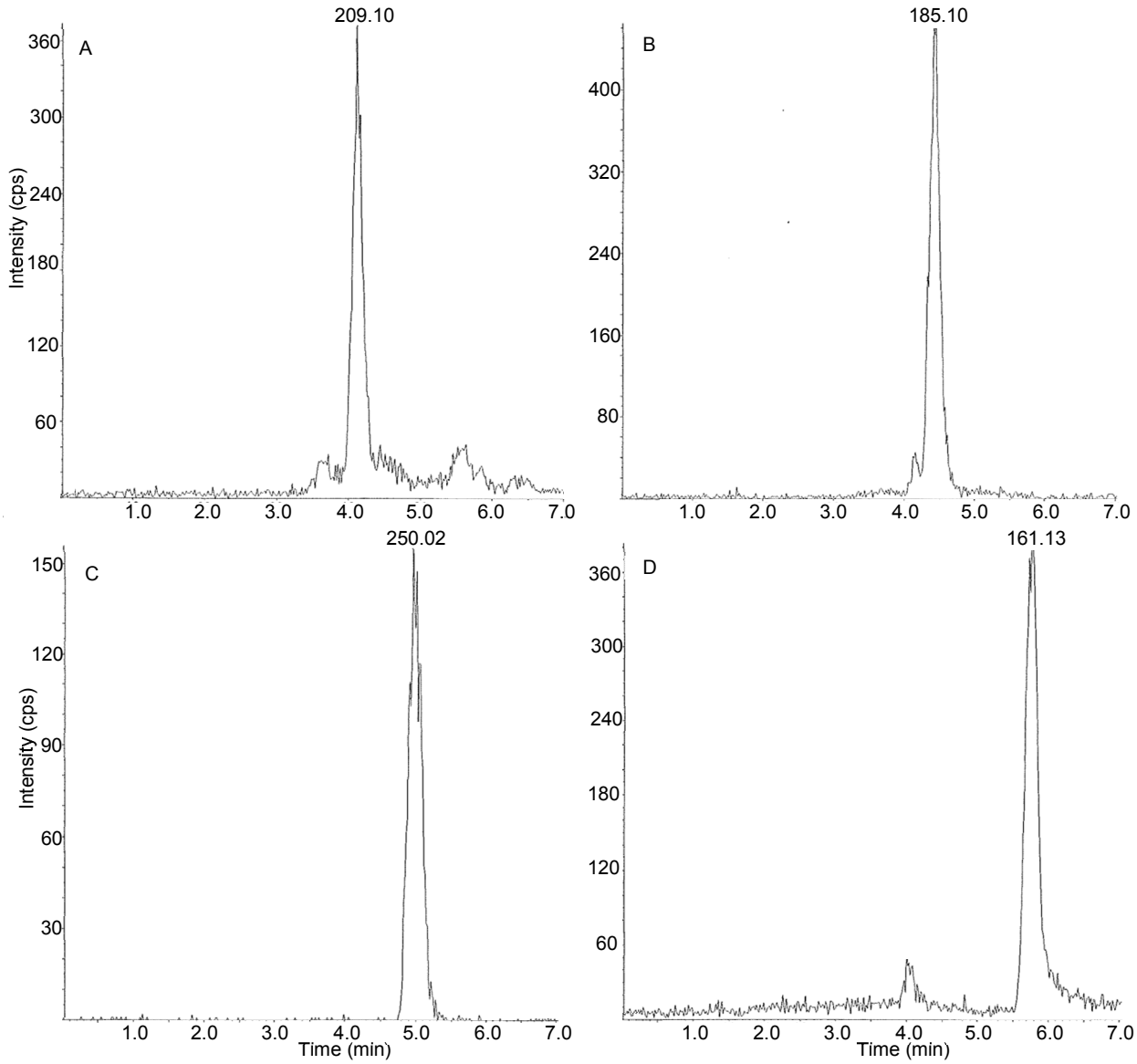


Figure 1. Typical specific ion chromatograms from live exposed fish for A. ketoprofen, B. naproxen, C. diclofenac and D. ibuprofen. Total MS run time was 7 minutes and signal intensity was measured as counts per second (cps).

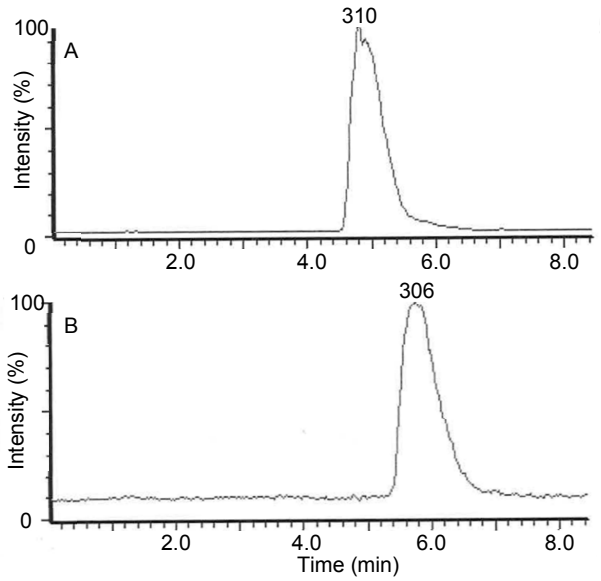


Figure 2. Typical specific ion chromatograms from live exposed fish for A. fluoxetine and B. sertraline. Total MS run time was 8.4 minutes and signal intensity was measured as percentage of highest signal.

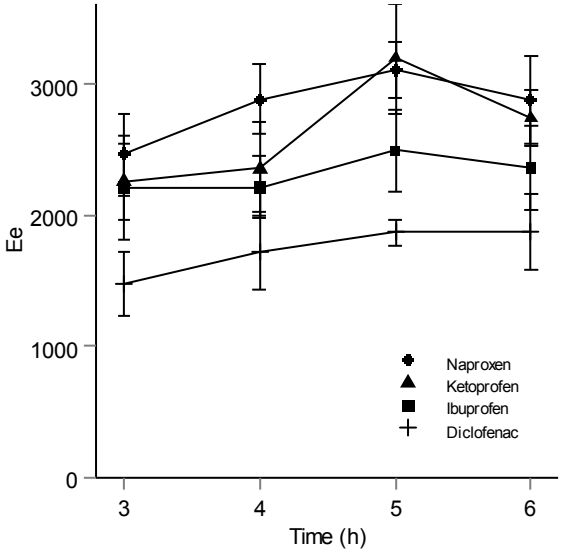


Figure 3. Enrichment factors (Ee) with 95% confidence intervals (n = 3 to 5) for the NSAIDs versus extraction time in hours.