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Determination of 4′-isobutylacetophenone and other transformation products of anti-inflammatory drugs in water and sludge from five wastewater treatment plants in Sweden by hollow fiber liquid phase microextraction and gas chromatography–mass spectrometry

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ABSTRACT

This work describes the development of a two-phase hollow fiber liquid phase microextraction method for the determination of three hydrophobic transformation products of the nonsteroidal anti-inflammatory drugs ketoprofen, ibuprofen and diclofenac: 3-acetobenzophenone, 4′-isobutylacetophenone and diclofenac amide. The optimized method involved extraction for 180 min at a stirring speed of 440 rpm. Hollow fibers (0.6 mm i.d.) of 6 cm length were employed and the acceptor phase consisted of 1-octanol. 5% Sodium chloride was added to samples to prevent loss of the solvent during extraction. Extracts were analyzed by GC–MS and method detection limits were in the range of 1.6–5.6 ng L^{-1}. The method was applied for the determination of target analytes in influent samples from five Swedish wastewater treatment plants (WWTPs). All three analytes were found in very low or non-detectable concentrations. The most abundant compound was 3-acetobenzophenone found at four of the investigated WWTPs at an average concentration of 62 ng L^{-1}. Diclofenac amide and 4′-isobutylacetophenone were only detected above LOD at one WWTP each at a concentration of 55 and 197 ng L^{-1}, respectively. Samples of water entering and exiting the activated sludge treatment as well as digested sludge were also collected from one of the WWTPs. Only diclofenac amide was detected in these samples. A higher concentration was detected in the effluent from the activated sludge treatment than the influent, thus indicating the formation of this compound during treatment. In the sludge, diclofenac amide was detected at 183 ng g^{-1}wet weight. Based on these results it can be concluded that the amounts of these compounds reaching WWTPs are very small, suggesting negligible risks to the aquatic environment. However, they also indicate the potential formation during the activated sludge process and accumulation into sludge for at least one of the compounds which is why further studies of these processes are needed.

1. Introduction

A wide number of pharmaceutical compounds and their metabolites are introduced into the environment each year threatening the health of aquatic and terrestrial ecosystems. In recent years, interest regarding the occurrence and toxicity of these compounds has grown considerably [1–4]. Nonsteroidal anti-inflammatory drugs (NSAIDs) belong to the most studied pharmaceuticals and their occurrence in the environment has attracted great interest among researchers [5–8]. Today we are aware of their presence, toxicity and damage to the environment. However, these compounds, by human metabolism or by environmental conditions, can give rise to transformation products more persistent and/or more hazardous than the parent compounds [9,10]. Therefore, it is necessary to obtain information on both the parent compounds as well as their possible transformation products. For ibuprofen, more than 10 transformation products have been identified [11–13] of which 4′-isobutylacetophenone (4-IBAP) is one of the most important ones since it causes adverse effects in the central nervous system and presents high dermal absorption [14]. For diclofenac, several transformation products have been identified produced by different mechanisms of oxidation and photo-oxidation [15–17] with diclofenac amide being very common in these transformation pathways. Finally, ketoprofen has

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several known transformation products of which 3-acetobenzophenone (3-AcBP) is one of the most important ones [18–20].

Although there are many studies on the presence of NSAIDs in environmental samples [5–8], to our knowledge, data about the presence of NSAID transformation products in these samples are very scarce. 4-IBAP has been detected in wastewater in Sweden in concentrations between 8–540 ng L^{-1} [21,22] and diclofenac amide in wastewater and river water in Pakistan in concentrations between 30–400 ng L^{-1} [17].

The main difficulties when studying the transformation products of pharmaceuticals in environmental matrices are linked to the complexity of wastewater and sludge samples, and the very low environmentally relevant concentrations (ng L^{-1} ng g^{-1}) which can generate problems in relation to the analytical sensitivity and/or selectivity. For these reasons, sample preparation is necessary in order to obtain sufficient enrichment as well as clean-up. For aqueous samples, generally, solid phase extraction (SPE) is the most common technique applied for NSAID transformation products [16–18]. However, this technique possesses some disadvantages such as using high amounts of organic solvent, being time consuming and providing limited enrichment of the target analytes implying additional pre-concentration steps. Sludge samples are usually lyophilized and extracted using pressurized liquid extraction (PLE) [23–25] although some work has also been performed with microwave assisted or ultrasonic extraction (MAE/USE) [26–28]. All these techniques require additional clean-up of the extract with SPE due to its low selectivity [23–25] thus resulting in a tedious and complex procedure. Hollow fiber liquid-phase microextraction (HF-LPME) is an attractive alternative technique for environmental sample preparation. This technique only consumes negligible amounts of solvents, avoids sample carryover (by the use of disposable membranes), has low cost and has been shown to provide good clean-up efficiency, high selectivity, and high enrichment factors for pharmaceuticals and their transformation products in water and wastewater [5,21,29]. A further advantage is that HF-LPME is also applicable for direct extraction of trace analytes from raw as well as digested sludge, saving large amounts of time and work. This has been demonstrated in several publications [7,8,30,31]. HF-LPME can be applied in different configurations depending on the application [32,33]. In the two-phase mode, a liquid organic acceptor phase fills the lumen of a microporous membrane fiber and is immobilized in membrane pores to extract the analytes from an aqueous donor phase (sample). This mode is preferably used for neutral and/or more hydrophobic organic compounds ($\log K_{ow} \geq 3$).

In a previous work, a two-phase HF-LPME method was developed for the extraction of 4-IBAP from wastewater [21]. In the present paper we present the development of a new method including two other hydrophobic NSAID transformation products (3-AcBP and diclofenac amide) and applying a significantly shorter extraction time (3 h compared to 7 h in the previous study). The method was successfully applied for the determination of target analytes in wastewater as well as in sewage sludge.

2. Material and methods

2.1. Chemicals and solutions

Diclofenac amide (CAS: 15362-40-0) was purchased from TRC Inc. (North York, Canada), 4-isobutylacetophenone (4-IBAP, CAS: 38861-78-8), 3-AcBP (CAS: 66067-44-5) and 4-butylacetophenone (CAS: 37920-25-5) used as internal standard (IS) were purchased from Sigma-Aldrich (Steinheim, Germany). Toluene, n-hexane, isooctane and acetone were purchased from Scharlau Chemie (Barcelona, Spain). Chloroform, 1-octanol and 2-heptanone were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium chloride, sodium hydroxide and sulfuric acid were purchased from Across Organics (Gheel, Belgium). Reagent water was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA). The Q3/2 Accurel PP polypropylene hollow fiber membranes with an inner diameter of 600 μm, 200 μm of wall thickness and 0.2 μm pore size were obtained from Membrana (Wuppertal, Germany).

Standard stock solutions of 4-IBAP, 3-AcBP and IS were prepared in hexane at 1000 mg/L, and diclofenac amide stock solution was prepared in chloroform at 245 mg/L. Working solutions were prepared weekly by appropriate dilution in hexane for calibration and in hexane:acetone (1:50) for samples spiking. Stock solutions were stored in the freezer at −20 °C and working solutions in the fridge at 6 °C.

2.2. Sample collection.

One grab sample of influent water was collected prior to the initial screen at five WWTPs in southern Sweden: Sjölunda, Klagshamn, Källby, Kävlinge and Ellinge at one occasion in June 2013. Klagshamn and Sjölunda WWTPs are situated in the city of Malmö, the third largest city in Sweden. Sjölunda receives wastewater from approximately 420,000 population equivalents (p.e.) from Malmö as well as nearby cities. Klagshamn receives wastewater from approximately 60,000 p.e. from Malmö and close town Vellinge. Källby WWTP is located in the city of Lund and treats the wastewater of approximately 80,000 p.e. from Lund as well as surrounding villages. Kävlinge WWTP is situated in the town of Kävlinge and receives the wastewater of approximately 30,000 p.e. Finally, Ellinge WWTP serves the community of Eslöv and Procordia Food's production plant. Depending on the season, the load varies from approximately 25,000–100,000 p.e. At Ellinge WWTP, one grab sample was also collected from the water entering as well as exiting the conventional activated sludge treatment (CAS) respectively and of digested sludge from the centrifuge providing final dewatering. Samples were collected in glass bottles (wastewater) and a plastic box (sludge), transported to the laboratory, acidified with sulfuric acid to pH 1.8 for preservation and kept at 4 °C in darkness until analysis. All experiments were performed within a few days.

Sludge samples were pre-treated according to the method developed by Sagristà et al. [7]. Aliquots of 2 g (wet weight) of homogenized digested sludge were mixed with 100 mL of reagent water and stirred for 18 h at 660 rpm to reach partitioning equilibrium of the analytes between the solid and aqueous phase. Afterwards, the slurry samples were subjected to the two-phase HF-LPME extraction procedure described below.

2.3. Hollow-fiber liquid phase microextraction (HF-LPME)

The HF-LPME assembly designed for the extraction of NSAID transformation products is shown in Fig. 1. Before the extraction, the hollow fibers were cut into 6 cm pieces and cleaned with acetone to remove any possible contaminants whereafter the acetone was allowed to evaporate completely. To carry out the extraction, one end of the cleaned fiber was attached to a medical syringe needle (100 Sterican, inner diameter of 0.7 mm × 50 mm, Scantec lab AB, Gothenburg, Sweden). These needles were found to allow for a quick and tight connection to the hollow fiber. Solvent was passed through the fiber by a 1 mL medical syringe to ensure that the lumen of the membrane was filled with the organic phase. Then, the other end of the fiber was joined to another medical syringe needle. After that, the pores were impregnated with the organic solvent (1-octanol) for around 60 s and the system (U-shaped) was dipped into reagent water to remove the organic solvent excess. The U-shaped fiber was

placed into the aqueous sample (100 mL) for extraction which was performed for 45 min for reagent water and 180 min for wastewater samples. At the end of the extraction, the fiber was removed from the donor solution and was carefully dried with a piece of paper to avoid water traces in the extract. The acceptor phase was collected into a 1.5 mL vial attached to a 250 μL insert by pushing air through the fiber with a 10 mL syringe. A total of 5 μL of the organic extract was transferred to another GC vial containing 45 μL of hexane and internal standard at 10 μg L^{-1}. Finally, 2 μL volume of this solution was injected into the GC–MS.

2.4. Instrumentation

The extracts were analyzed by a 45 × series gas chromatograph equipped with a split/splitless injector, autosampler CP-8400 and a Scion-TQ mass detector (Bruker Corporation, Fremont, CA). Target analytes were separated using a Vf-1MS Factor four column (Varian, Darmstadt, Germany), 30 m × 0.32 mm with a phase thickness of 0.25 μm. Helium carrier gas (purity 99.9999%, Strandmøllen, Klampenborg, Denmark) was maintained at a constant flow rate of 1.5 mL/min. The chromatographic program was from 60 °C, with a hold for 2 min, increasing at 10 °C/min to 130 °C, with a hold for 1 minute and a final rate of 40 °C/min to 280 °C, hold for 3 min. The total chromatographic run time was 18 min. A volume of 2 μL was injected in splitless mode (splitless time: 2 min). The temperature for injector and transfer line was 280 °C and for the ion source 230 °C. The MS was operated in the electron impact ionization (EI) mode (70 eV). Analysis was performed in the selected ion monitoring (SIM) mode using the characteristic ions given in Table 1.

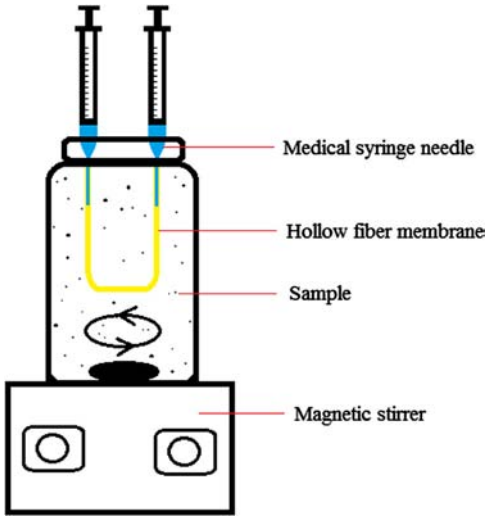


Fig. 1. HF-LPME assembly designed for the extraction of hydrophobic analytes.

3. Results and discussion

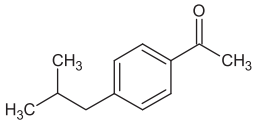
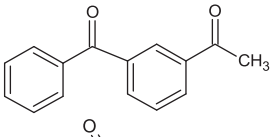
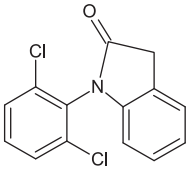
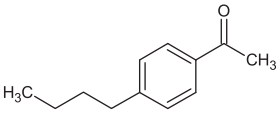
3.1. Method optimization

The analytes of interest are hydrophobic and non-charged compounds and hence are easily extracted from water into an organic solvent. For this type of compounds a two-phase liquid membrane extraction system is the most suitable. To determine the most favorable HF-LPME conditions for the analysis of the samples a univariate optimization approach was performed. The experiments were performed in reagent water and wastewater samples spiked with 1 μg L^{-1} of each analyte. All results were expressed as mean values of three replicates.

3.1.1. Selection of organic solvent

The type of organic solvent in the hollow fiber is important to achieve high extraction efficiency. There are several requirements for organic phase selection: the organic solvent should be immobilized in the hollow fiber pores; it should be immiscible with water and non-volatile to avoid solvent loss during extraction. Also, the acceptor phase should be selective towards the analytes of interest. Based on these considerations, five organic solvents were tested to extract the analytes (hexane, isooctane, toluene, 1-octanol and 2-heptanone). As can be seen in Fig. 2, toluene and 1-octanol gave the best results but the experiments showed that toluene was lost during the extraction to a larger extent

Table 1
Names, chemical structures, molecular weights (Mw), octanol/water partition coefficient (log K_{ow}) and SIM target ions.

Compound	Structure	Mw	log K_{ow}[a]	Quantifier ion (m/z)	Qualifier ion (m/z)
4-IBAP		176.24	3.54 ± 0.23	161	176
3-AcBP		224.25	3.02 ± 0.33	105	209
Diclofenac amide		278.13	3.00 ± 0.37	214	242
4-Butylacetophenone (internal standard)		176.25	3.72 ± 0.22	161	176

[a] Values calculated using a computer program: ACDLabs 12.0, Advanced Chemistry Development, Inc., Toronto, Canada.

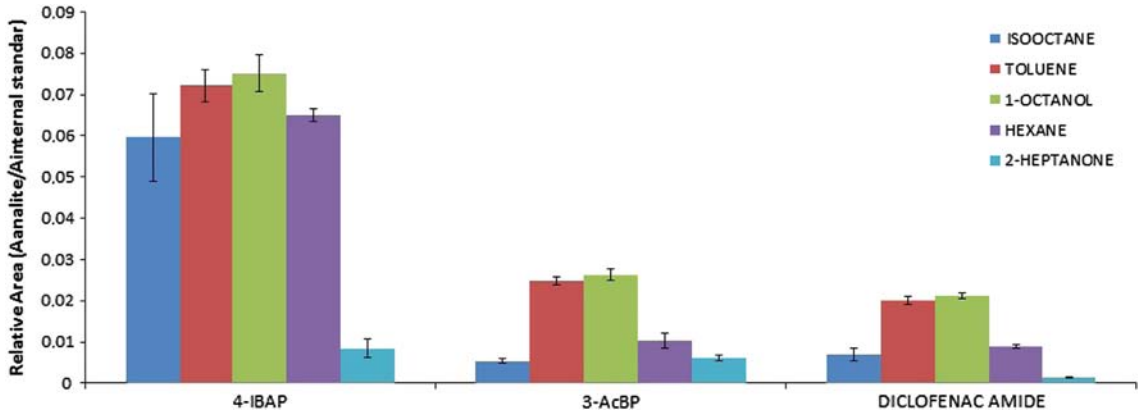


Fig. 2. Effect of the extraction solvents used for HF-LPME on the relative peak area of the analytes. Error bars indicate the standard deviation ($n=3$).

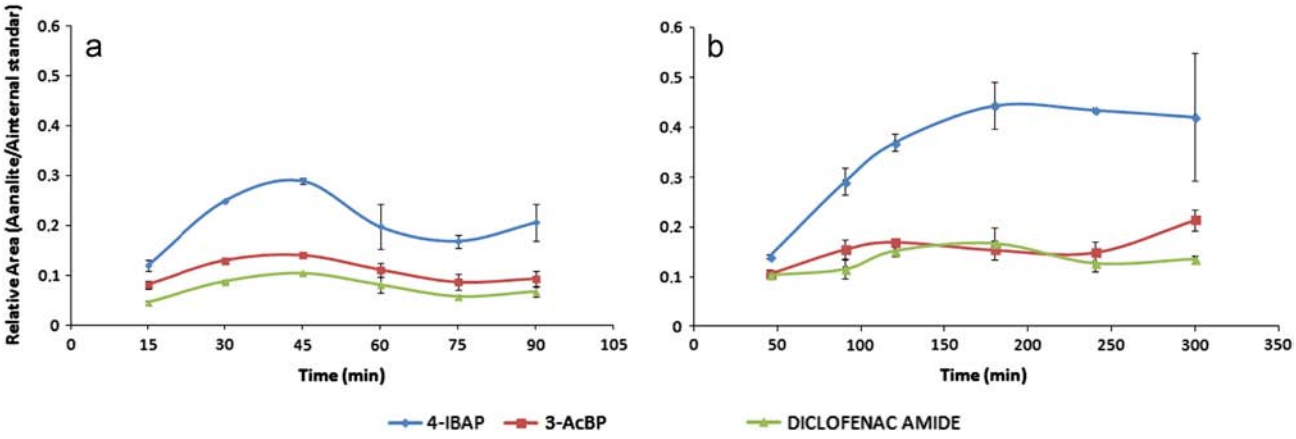


Fig. 3. Effect of the extraction time on the relative peak area of the analytes in: (a) reagent water and (b) wastewater. Error bars indicate the standard deviation ($n=3$).

than 1-octanol. Consequently 1-octanol was chosen for further experiments.

3.1.2. Extraction time

The amounts of the analytes transferred into the organic solvent from the donor phase are expected to increase with increasing exposure time in the sample, and reach maximum at the equilibrium state [32]. The effect of extraction time on the extraction efficiency was evaluated from 15 to 90 min in reagent water spiked with the three analytes. The relative peak area of the analytes increased during the first 45 min and decreased for longer extraction times. The reason for the decrease of the extraction efficiency and also larger RSD values may be the loss of the organic solvent from the membrane. However, the mass transfer from the donor phase to the acceptor phase is affected by the sample matrix, so the extraction time was also evaluated from 45 min to 300 min in wastewater. Fig. 3 shows that 180 min is the optimum value. Therefore, 180 min was selected for subsequent experiments.

3.1.3. Stirring speed

Agitation of the sample solution may accelerate the mass transfer of extracted analytes from the sample to the organic solvent and reduce the time to reach equilibrium [32]. To estimate the influence of the stirring speed, four stirring speeds from 330 rpm to 660 rpm were investigated in the experiments. As it can be seen in Fig. 4, the relative area of the analytes increased with increasing stirring speed to 550 rpm, while above this value there was a decrease. However, when the agitation speed was 550 rpm, standard deviation values were too high so 440 rpm was the stirring speed chosen for the following experiments.

3.1.4. Fiber length (acceptor phase volume)

The fiber length is directly related to the acceptor phase volume and to the extraction efficiency. The effect of the organic solvent volume was studied with length values from 4 to 20 cm. One centimeter of hollow fiber membrane can hold approximately 8 μL of organic solvent depending on the density and viscosity of the acceptor phase. Figs. 5 and 6 show that 6 cm of fiber length provides the best results for the analytes. Hence, this length value was chosen for further experiments.

3.1.5. Salt addition

Salt addition to the sample may have different effects. First, the addition of salt can reduce the amount of water available to dissolve the analytes due to the formation of hydration spheres around the ionic salt molecules and can improve the extraction efficiency for the target analytes into the organic phase [34]. On the other hand, the addition of salt can lead to higher ionic strength in the sample ("salting out effect") that can improve the extraction efficiency due to enhancement of the activity coefficients of analytes in organic components in the aqueous solution.

Different amounts of salt (NaCl) from 0% to 15% were studied and the relative areas decreased with increasing salt concentration in reagent water. However, the same amounts of salt in the wastewater did not have any effect on the extraction efficiency and experiments showed that salt prevents loss of solvent from

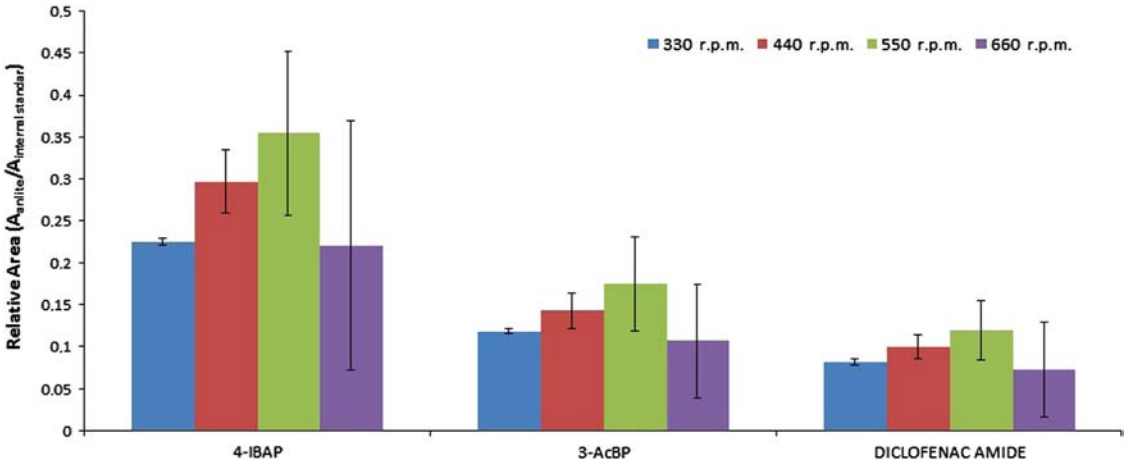


Fig. 4. Effect of the stirring speed on the relative peak area of the analytes. Error bars indicate the standard deviation ($n=3$).

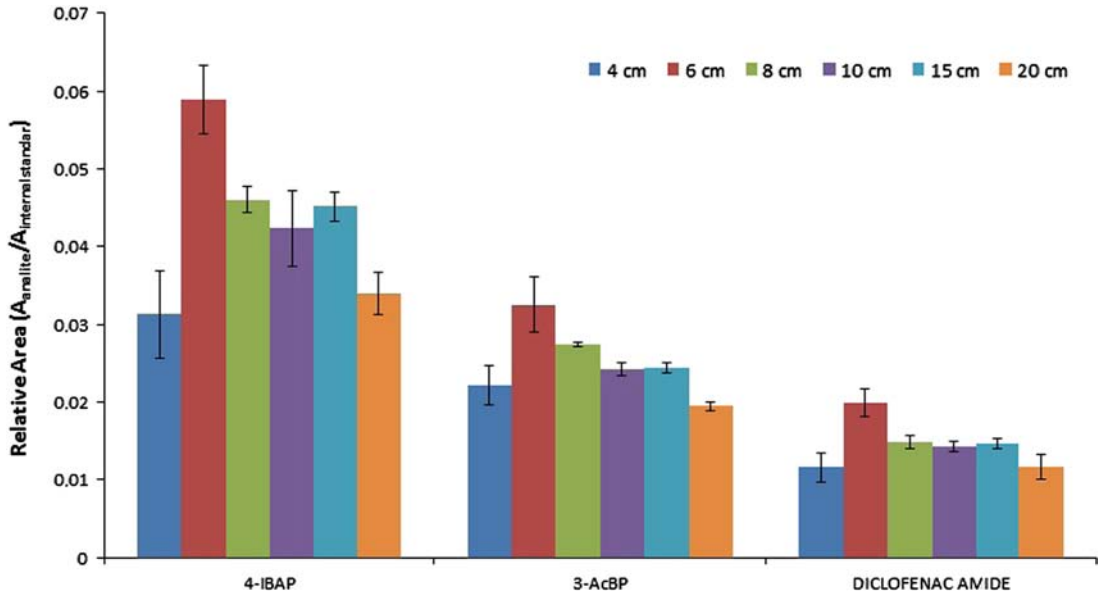


Fig. 5. Effect of the membrane length (organic phase volume) on the relative peak area of the analytes. Error bars indicate the standard deviation ($n=3$).

the membrane. Hence, for subsequent experiments 5% of salt was added to the samples for extractions.

3.1.6. Temperature and pH

The temperature has a significant effect on both the kinetics and thermodynamics of the extraction process. The mass transfer coefficients and rate constants are usually enhanced with increasing temperature; as a result, an increase of the sample temperature may improve extraction efficiency [35]. The effects of temperature were studied from 25 to 50 °C but the relative areas were not affected by this increase so the following analyses were performed at room temperature.

Considering the structure and chemical properties of the analytes it is expected that the pH does not affect the extraction efficiency significantly. The pH studied varied from 2 to 9. As it was expected, no effect of sample pH on the extraction was observed. Therefore, no further pH adjustment was done to the samples.

3.2. Validation of the method

In order to evaluate the practical applicability of the proposed method, enrichment factor (E_e), linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability and reproducibility were studied in wastewater free of target analytes. Results are shown in Table 2.

In two-phase LPME methods, the enrichment factor is used to evaluate the effectiveness of the procedure of extraction, see Eq. (1). This parameter is defined as the ratio of the concentration of analyte in the acceptor phase (c_a) after the extraction divided by the initial concentration in the sample (donor phase) before extraction (c_d).

$$E_e = \frac{C_a}{C_d} \quad (1)$$

The linearity of the method was evaluated over the concentration range 10–500 ng L^{-1}. Overall, linearity was very good along the whole evaluated range with the determination coefficients (R^2) ranging between 0.9968 and 0.9997. The detection and quantification limits (LODs and LOQs, respectively) were calculated with the data generated in the linearity studies. The LODs have been calculated as 3 times the standard deviation for the lowest measured concentration (5 ng L^{-1}) and LOQs as 10 times the standard deviation. The values range from 1.6 to 5.6 ng L^{-1} for LOD and from 5.3 to 18.6 ng L^{-1} for LOQ.

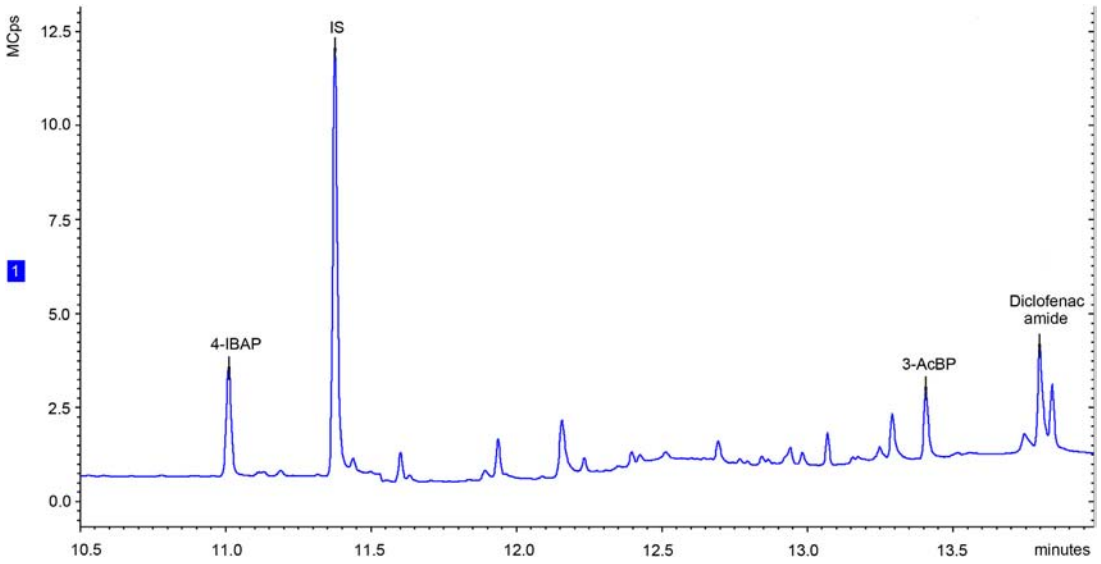


Fig. 6. GC–MS-SIM chromatogram obtained from spiked wastewater at 10 μg L^{-1} after hollow fiber membrane extraction.

Table 2
Analytical performance of the optimized HF-LPME method.

Analyte	E_e	R^2	LOD (ng L^{-1})	LOQ (ng L^{-1})	Repeatability (RSD %)[a,b]		Reproducibility (RSD %)[a,c]	
					Reagent water	Effluent water	Reagent water	Effluent water
4-IBAP	965	0.9997	5.6	18.6	4.7	8.0	7.2	9.0
3-AcBP	519	0.9981	1.6	5.3	10.3	12.4	12.2	14.9
Diclofenac amide	315	0.9968	3.5	11.5	3.4	15.6	9.5	16.1

[a] Concentration 1 μg L^{-1}.
[b] $n=3$.
[c] $n=6$.

The repeatability was studied by analyzing three wastewater samples and reagent water spiked at 1 μg L^{-1}. The RSD values were lower than 10.3% in reagent water and 15.6% in wastewater. The reproducibility within two days was studied by analyzing six wastewater samples and reagent water with a final concentration of 1 μg L^{-1}. The RSD values for the analytes in this case were lower than 12.2 and 16.1%.

3.3. Real sample analysis

The two-phase LPME method developed in this work was applied to the determination of target analytes in influent wastewater from five WWTPs in southern Sweden. The results are shown in Table 3. According to these results, 3-AcBP was the most frequently detected compound, found at concentrations > LOD in the influent to four of the five studied WWTPs. The main pathway of pharmaceuticals into wastewater is human excretion via urine and feces following consumption. 3-AcBP is not a major human metabolite of ketoprofen although it has been shown to form in rats in very low amounts corresponding to approx. 0.03% of the administered ketoprofen dose [36]. It has however been identified as a major phototransformation product of ketoprofen, formed even after very short exposure to light [18]. In a survey by Falås et al. [37], ketoprofen showed median influent concentrations > 1 μg L^{-1} in a screening of data from 162 Swedish WWTPs. There is thus potential for phototransformation processes to take place. However, in all cases, the sampling in our study was performed of the influent water directly after reaching the WWTPs via underground sewage lines which means that the exposure to light has been negligible. We thus consider it more likely that the 3-AcBP found in the influents is the result of human metabolism. The detected concentrations in our study are approx. 60 ng L^{-1} which corresponds to around 0.06% of the median ketoprofen concentration found in WWTP influents. The ibuprofen transformation product 4-IBAP was found in the highest amount of all analytes (197 ng L^{-1}) in the influent to Sjölunda WWTP, although not detected or below LOQ at the four other WWTPs. Previous studies show a similar pattern: in 2007, Zorita et al. [21] only detected 4-IBAP in two out of four samples collected from different parts of the sewage system of Kristianstad city in southern Sweden in rather low concentrations (26.5 and 39.7 ng L^{-1}). In a new study in 2009, the compound was detected in the influent to Kristianstad WWTP at three out of five sampling occasions in significantly higher concentrations (320–540 ng L^{-1}) [22]. The occurrence of this compound thus shows an intermittent pattern. Like 3-AcBP, 4-IBAP is a phototransformation product and not a human metabolite. It is however often found in low amounts in ibuprofen preparations,which could be the reason for its presence in wastewater [22]. Finally, diclofenac amide was only detected in low

Table 3
Concentration of the studied NSAID degradation products in influent water from WWTPs in southern Sweden.

Analyte	Concentration (ng L^{-1})				
	SJÖLUNDA	KÄLLBY	ELLINGE	KLAGSHAMN	KÄVLINGE
4-IBAP	197 ± 11	n.d.	n.d.	< LOQ	n.d.
3-AcBP	55 ± 13	n.d.	89 ± 23	47 ± 13	58 ± 19
Diclofenac amide	n.d.	n.d.	55 ± 20	28 ± 10	25 ± 10

Table 4
Concentration of diclofenac amide in CAS influent, CAS effluent water and sludge from Ellinge WWTP.

Analyte	Concentration (ng L^{-1})		Concentration (ng g^{-1} wet weight)
	CAS in	CAS out	Sludge
Diclofenac amide	109 ± 10	132 ± 6	183 ± 6

concentrations in three of the studied WWTPs. No clear trend between the studied WWTPs can be observed, except that none of the analytes were detected at Källby WWTP. It is hard to provide a satisfactory explanation for this observation since the sewage system of Lund city does not differ in principle from the other cities and we find it highly unlikely that the consumption of the NSAIDs would be significantly lower in Lund than its neighboring cities. Since this study is performed by screening of several WWTPs based on sampling at a single occasion we therefore attribute the absence of the analytes in the Källby influent to the overall intermittent detection of these analytes in wastewater. This behavior is most likely due to these compounds not being main human metabolites, but present in the water either by their occurrence as contaminants in consumed preparations of the NSAIDs or potential microbial transformation processes in the sewage system, which could be highly dependent on the biotic and abiotic conditions changing from day to day.

These results show that non-detectable or very low amounts of these compounds reach the WWTPs via wastewater which would imply that they probably are of minor environmental concern. However, studies have revealed that NSAIDs undergo substantial biological transformation within WWTPs [38,39]. Previous work for instance has shown the transformation of diclofenac into diclofenac amide during activated sludge batch experiments [40]. Due to the hydrophobicity of the investigated analytes they could also have a potential to accumulate in the sludge, which would provide another pathway into the environment via the use of sludge as a fertilizer in agriculture. To investigate this, samples of water entering as well as exiting the activated sludge treatment and samples of dewatered, digested sludge were collected from Ellinge WWTP and analyzed. On this occasion, only diclofenac amide was detected in the samples. As can be seen in Table 4, the concentration increased during the activated sludge treatment resulting in 132 ng L^{-1} in the secondary effluent, thus implying a formation of this compound during the treatment. In the digested sludge, also, only this compound was detected at a concentration of 183 ng g^{-1}, showing its potential to accumulate in the sludge.

4. Conclusions

In this work, a two-phase HF-LPME method was developed and validated for the direct extraction of three hydrophobic NSAID transformation products from wastewater and sludge. Analysis of influent water from five Swedish WWTPs revealed the presence of these compounds in most samples, although in very low amounts. It can hence be concluded that the input of these compounds to WWTPs is of minor concern. However, analysis of water from the activated sludge treatment and digested sludge showed that at least one of the analytes, diclofenac amide, has a potential to form during wastewater treatment and accumulate in sludge. Significant environmental release via WWTPs can thus not be totally excluded which is why further studies regarding the formation of these compounds and their partitioning into sludge are motivated.

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