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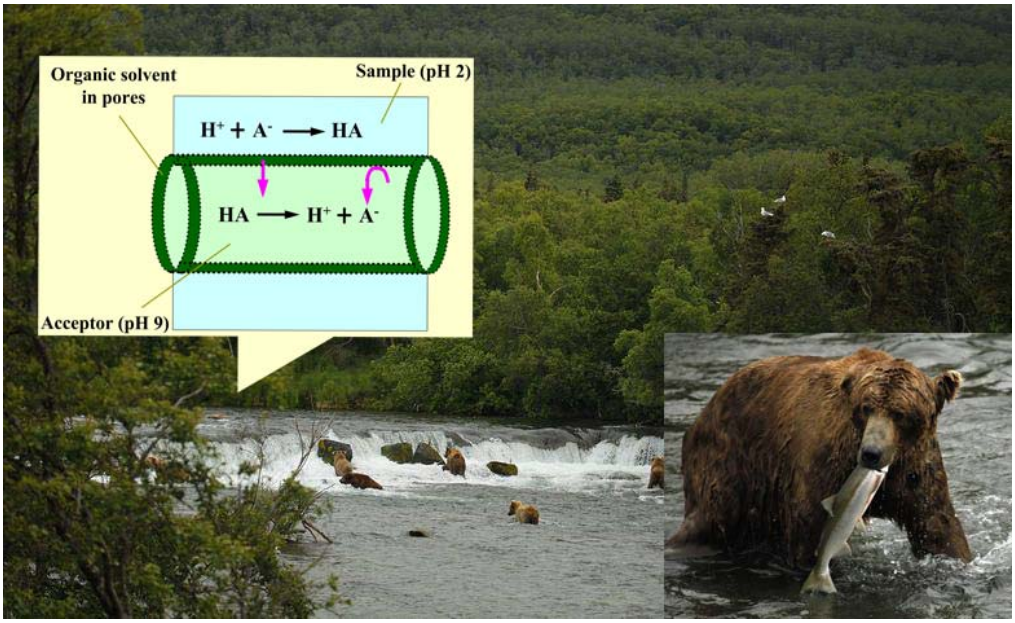

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Abbreviation

NSAID	Non-Steroidal Anti-Inflammatory Drug
KET	Ketoprofen
NAP	Naproxen
IBU	Ibuprofen
DIC	Diclofenac
PPCP	Pharmaceuticals and personal care product
SSRI	Selective Serotonin Reuptake Inhibitor
WWTP	Waste water treatment plants
LLE	Liquid–liquid extraction
PLE	Pressurized liquid extraction
SPE	Solid-phase extraction
SLME	Supported Liquid Membrane Extraction
HF-LPME	Hollow fiber liquid-phase microextraction
Q-TOF	Quadrupole time of flight mass spectrometer
LC-MS/MS	Liquid chromatography tandem mass spectrometry
GC	Gas chromatography
DHE	Di-*n*-hexyl ether
MDL	Method detection limit
USEPA	US Environmental Protection Agency
RSD	Relative standard deviation
SD	Standard deviation
Ee	Enrichment factor
E	Extraction efficiency

Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) have widely been found in STP effluents and surface water with different concentrations. However, the information concerning ecotoxicological risks, surface water and water living organisms is rather scarce. In this thesis, ketoprofen (KET), naproxen (NAP), diclofenac (DIC) and ibuprofen (IBU) were chosen as four model substances to study the bioaccumulation factor, the distribution, and the fate of the NSAIDs. A combination of hollow fiber liquid-phase membrane extraction (HF-LPME) and LC-MS/MS was used to simultaneously extract and detect the NSAIDs in fish. For the freeze-dried fish tissue, this new analytical method showed an average enrichment factor of 3000 times. For water, the average enrichment factor was increased to 3700 times. The average R^2 of the linearity were 0.9902, 0.9945, 0.9802 and 0.9890 for KET, NAP, DIC and IBU, respectively. Method detection limits for KET, DIC, NAP and IBU in the range of 1-3 ng/L. Moreover, this method could be successfully applied to detect the analytes in the real samples. For the wild cod fish, only NAP was found with a concentration of 26 ng/g (dry fish). For the wild rudd fish, two NSAIDs (78 ng/g of KET and 40 ng/g of NAP) were found. When the rudd fish was exposed to the NSAIDs, the four NSAIDs were determined in the range of 6-83 ng/g in the dry fish. Interestingly, for the exposed rudd, the concentrations of KET and DIC in dead rudd fishes were almost twice as that in the alive fish. The developed analytical methodology might be extended to be used to estimate the distribution or the fate of the four NSAIDs in other biota, animals or human beings.

Key words: HF-LPME, LC-MS/MS, selective, NSAID, fish

Contents

1. Introduction

The discharge, presence and potential effects of pharmaceuticals in the environment have attracted increasing attention in recent years. Among these pharmaceuticals, non-steroidal anti-inflammatory drugs (NSAIDs) are one of most prescribed drug groups.[1] NSAIDs and their metabolites reach waste water treatment plants (WWTP) as a fact, and studies of effluent show that elimination of these NSAIDs in the WWTP is incomplete.[2-7] This leads to levels of NSAIDs in aquatic environments reaching at least ng/L concentrations at multiple locations world-wide.[8] Extraction methods such as SPE[9,10] and PLE[11] are commonly used for the enrichment of NSAIDs in waster samples at this level.

Recently, a big concern about the environmental impact of NSAIDs has emerged because little is known about their possible negative effects and they are continuously introduced into the living organisms. To be able to measure if these NSAIDs enter into water living organisms, more efficient extraction methods and selective enrichment techniques are needed for biotic matrixes, because analysis of biological tissues is heavily affected by artifacts and interferences during extraction procedures and quantification compared to that of water samples.

NSAIDs are typical ionisable pharmaceuticals, and they have been extracted from water samples with high enrichment by using hollow fiber liquid-phase membrane extraction (HF-LPME). Moreover, HF-LPME has successfully been used for other ionisable pharmaceuticals in tissues matrixes.[12,13] Therefore, HF-LPME is a promising extraction method for biotic samples. In this project, we will compare the efficiency of different extraction techniques. Under the optimized conditions, HF-LPME and LC-MS/MS will be used for determination of NSAIDs in biota. Fish is selected as the modal biota because fish is an important source of nutrients for humans.

1.1 NSAIDs in environment

For treating conditions such as arthritis, NSAIDs are selected as the most prescribed medications. Six types of NSAIDs are familiar to most people as over-the-counter drugs, which are listed as salicylic acid, ketorolac, KET, NAP, DIC and IBU. NSAIDs are pain relievers, which can help reduce inflammation and lower fevers. Moreover, they are used to reduce the clotting action in some cases and thus have a protective effect against heart disease.

Since NSAIDs are designed to be highly active to the receptors in humans and animals or to be toxic for many infectious organisms, they might also have unintended influences on

bio-organisms in the environment, especially on biota in water systems. Therefore, the occurrence of NSAIDs compounds in the waster environment and their potential effects on environmental biota has become one of the key environmental problems facing humanity.

Although there are some data regarding pollution with NSAIDs residues in water samples, the NSAIDs residues in biota are still a blank. Therefore, the development of an analytical procedure, which allows the determination of NSAIDs in biota at the ng/L level or lower is needed.

1.2 Sample preparation for NSAIDs

As the target NSAIDs are presented in a complex matrix, sample preparation is a crucial procedure in the analysis. The aim of sample preparation is to remove the coexisting substances and pre-concentrate the NSAIDs. The analyte recovery in this reproducible step should be sufficiently high.

1.2.1 Sample preparation for NSAIDs in water samples

Extraction of NSAIDs from water samples has been studied by different pre-concentration techniques. For example, T. Kosjek et al. employed commercial SPE to determine common NSAIDs (KET, NAP, DIC, IBU) and mecoprop residues in wastewaters and analyzed by GC-MS.[14] Ali Sarafraz-Yazdi et al. used SPE for the extraction of IBU, NAP and DIC in real water samples.[15] Recently, functional SPE such as magnetic SPE,[16] molecularly imprinted SPE,[17-22] and stir bar sorption extraction (SBSE)[23] has also been used to recognize NSAIDs in water media by many research groups.

LLE can be also used for the extraction of trace levels of drugs in water samples. Examples of this method include ion-pair LLE for the extraction of trace levels of NSAIDs (clofibric acid, IBU, NAP and DIC) in water samples as described by G. G. Noche et al.[24] The analytical procedure involves in situ aqueous derivatization of the NSAIDs and salting-out LLE.

1.2.2 Sample preparation for NSAIDs in semi-solid and solid sample

Extraction of NSAIDs in semi-solid and solid samples can be also investigated by the SPE and LLE methods. Other sample preparation techniques such as hot water extraction, supercritical fluid extraction and membrane extraction have also been used for pre-concentration of NSAIDs.

Pressurized hot water extraction (PHWE) is an environmentally friendly organic solvent free technique. In PHWE, water at high temperature and under pressure is used as the extraction solvent.[25] The PHWE extract is a relatively diluted aqueous solution, which can

be easily cleaned up and analyzed. In PHWE, temperature and vapor pressure are the main factors that affect extraction efficiency of the targets. However, the limitation of the PHWE is the possible destroying of the real samples, because degradation, hydrolysis or oxidation of the target compounds can also occur at high temperatures. In spite of this, PHWE has been successfully used for the analysis of NSAIDs (KET, NAP, DIC and IBU) from sewage sludge by A. Saleh et al.[26] Under the optimum conditions, spiking recoveries for sludge samples spiked at 200 ng/g were in the range of 101~109% but for the native drugs in non-spiked sludge samples, recoveries were 38.9%, 59.8%, 90.3% and 47.8% for KET, NAP, DIC and IBU, respectively.

Supercritical fluid (SF) technology, recognized as a green process, is applied in diversified fields of extraction, reaction, particle formation and material processing.[27] In the 1980s, supercritical fluid extraction (SFE) was introduced as an alternative extraction technique with the advantages of reduced solvent consumption and extraction time compared with the classical extraction techniques.[28] Commonly, CO_2 with methanol as organic modifier is used as fluid for extraction of polar compounds.[29] Although SFE is a useful technique for the pre-concentration of organic analytes from sewage sludge, there are only a few applications to the analysis of NSAIDs.[30] Recently, SFE was employed to analyze selected NSAIDs (ibuprofen, indomethacin, and flufenamic acid) in plasma. The extraction efficiency by SFE was compared with the results of SPE carried out on extraction columns with the identical packing and at the identical adjustment of pH. An advantage of SFE over SPE was extractability of a smaller amount of endogenous substances from plasma.[31]

1.3 Membrane extraction

The main advantages of membrane extraction over other extraction techniques in analytical chemistry are that it can selectively extract target analytes from complex samples, which can be liquid, semi-solid or solid with potentially very high enrichment factors (Ee). Another advantage could be that the extracted analytes can be transferred to the final analytical instrument easier in a quantitative way. The negligible consumption of organic solvent is also an advantage. In few words, the advantages of membrane techniques focus on selectivity, enrichment power, as well as economy and environment friendliness.[32-39] This technique has been used by Sagrista et al. in 2010 [13] to determine NSAIDs in sewage sludge and in 2012[12] to measure SSRI in sewage sludge. Saleh et al. in 2011[32] also employed HF-LPME to determine NSAIDs in sewage sludge after PHWE.

The general principle of membrane techniques is that the target analytes in the sample (donor) pass through the membrane to the acceptor and the membrane acts as a barrier to separate the donor from the acceptor. The classification of membrane extraction techniques

could be porous and nonporous or one-, two-, or three-phase membrane extraction techniques. Filtration and dialysis are one-phase techniques and the membrane is porous, while nonporous membrane techniques include two or three phases.[40] In two-phase membrane systems, the membrane phase is the same as the donor or the acceptor phase, so only one phase boundary exists. In three-phase membrane systems, the donor and acceptor liquid phase surround the membrane to form a two phase-boundaries system and thus two different partition steps. The selectivity can be improved by chemical reactions. The membrane phase can be a liquid, a polymer, or a gas, and the donor and acceptor phases can be either gas or liquid (aqueous or organic). Liquid membrane phases are often arranged in the pores of a porous hydrophobic membrane support material, which leads to a convenient experimental system, termed supported liquid membrane (SLM).

1.3.1 Principle of Supported Liquid Membrane Extraction (SLME)

SLME is the most popular type of a three-phase membrane based extraction in analytical chemistry. The target analytes, which are neutral, first pass through the membrane, then enter into the stagnant aqueous acceptor phase. Finally, the analytes will be ionized in the acceptor phase by acid-base reactions and thus become trapped in a nonextractable form. After trapping, the extract is transferred to an analytical apparatus, either manually or online. Trapping in the acceptor is crucial for three-phase liquid membrane extraction and it is important to optimize the acceptor conditions and then to obtain the desired selectivity and high Ee.

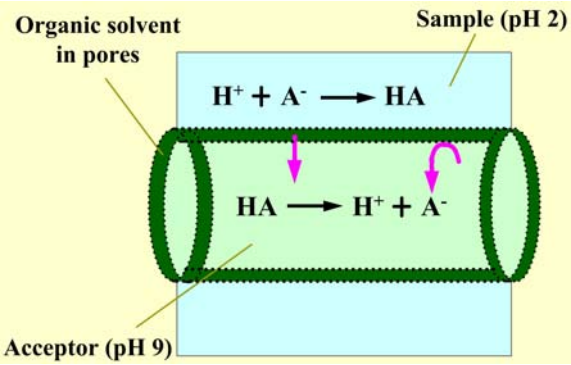

Fig. 1 Principle of HF-LPME.

In an SLM extraction, the barrier between donor and acceptor phases is the immobilized organic solvent in the pores wall of the hollow fiber (Fig.1) and normally used solvents are long-chain hydrocarbons like *n*-undecane or kerosene and more polar compounds like dihexyl ether (DHE).

The analytes must be able to exist in a nonionic and an ionic form on the donor side and

the acceptor side, respectively. Thus, SLM extraction is well suitable for ionizable compounds, providing very selective enrichment. The selectivity can be fine-tuned by adjustment, if the conditions in the three phases as seen in Fig.1.

Ee in HF-LPME is defined as:

$$Ee=C_{Ae}/C_{Di} \quad (1)$$

The extraction efficiency (E) of HF-LPME can also be defined in the same way:

$$E= (m_{Ae}/m_{Di})*100\% = (C_{Ae}*V_A/(C_{Di}*V_D))*100\% = Ee* (V_A/V_D)*100\% \quad (2)$$

Here, C_{Ae} and m_{Ae} are the concentration and mass of the analyte at equilibrium in the acceptor phase, respectively. C_{Di} and m_{Di} represent the concentration and amount of the analyte at initial stage in the donor phase, respectively. v_A and v_D are the volume of acceptor and donor, respectively.

1.3.2 Theoretical basis for HF-LPME

1.3.2.1 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 porous wall of the hall fiber. When the equilibrium of the analytes between the three phases, donor, acceptor and organic phase is achieved, then the partition coefficient of acceptor- donor, K_{AD}, depends on the condition of the donor and the acceptor.[41]

$$K_{AD}=C_{Ae}/C_{De}=(m_{Ae}*v_D)/(m_{De}*v_A) \quad (3)$$

C_{Ae}, C_{De} – the concentration of the analyte (at equilibrium) in the acceptor and the donor phase, respectively.

m_{Ae}, m_{De}- the amount of the analyte (at equilibrium) in the acceptor and the donor phase, respectively.

Based on equation (1), the partition coefficient of fish tissue – donor and membrane - donor can be defined as:

$$K_{FD}=C_{Fe}/C_{De}=(m_{Fe}*v_D)/(m_{De}*w_F) \quad (4)$$

$$K_{MD}=C_{Me}/C_{De}=(m_{Me}*v_D)/(m_{De}*v_M) \quad (5)$$

C_{Fe}, C_{De} and C_{Me} are concentrations of the analyte (at equilibrium) in fish, donor and organic phase, respectively. m_{Fe}, m_{De} and m_{Me} are amounts of the analyte (at equilibrium) in fish, donor and organic phase, respectively. w_F, v_D and v_M are mass of fish, volume of donor and organic phase, respectively.

1.3.2.2 Mass balance for standard addition method in HF-LPME

The method of standard addition can be used to determine the amount of analyte in the unknown sample and to avoid the matrix effect problem. To determine the initial concentration of each analyte in the fish tissue, the standard addition method was employed

and the mass balance between the initial stage and the final stage has been taken into account and the relation is shown as follows:

$$m_{Fi} + m_{Sp} = m_{Fe} + m_{De} + m_{Ae} + m_{Me} \quad (6)$$

where m_{Fi}, m_{Sp}, m_{Fe}, m_{De}, m_{Ae} and m_{Me} are the amount of the analyte initially in the fish tissue, initially in the donor, at equilibrium in the fish tissue, at equilibrium in the donor, at equilibrium in the acceptor and at equilibrium in the organic phase, respectively.

Combining all the equations for partition coefficients and equation (6), observing that:

$m_{Ae} = C_{Ae}*v_A$, then the following formula can be obtained as:

$$m_{Fi} + m_{Sp} = K*C_{Ae} \quad (7)$$

$$K = v_A + (K_{FD}*w_F)/ K_{AD} + v_D/K_{AD} + (K_{MD}*v_M) /K_{AD} \quad (8)$$

Thus, the initial amount of analyte in the fish slurry can be abstracted by plotting the spiked amount of the analyte in the donor vs. the measured concentration in the acceptor (Fig. 2).[42]

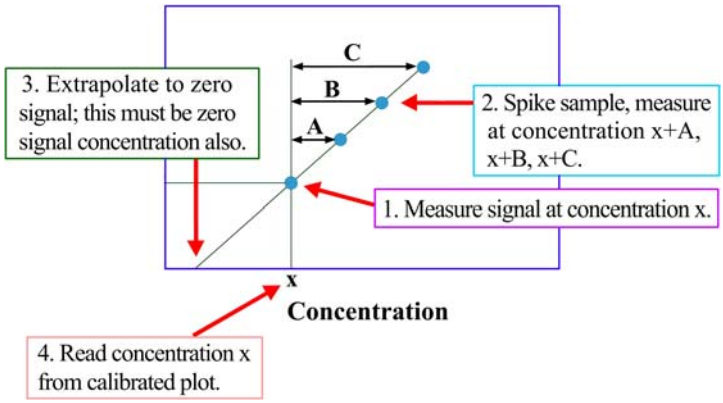

Fig. 2 Principle of the standard addition method. Adapted from ref 42.

1.4 Aim of this study

NSAIDs have widely been found in STP effluents and surface water with different concentrations. For example, four typical parent NSAIDs (e.g. KET, NAP, DIC and IBU) in sewage sludge were extracted by HF-LPME and analyzed by LC-MS/MS by Sagrista et al. in 2010.[13] The determined concentrations for KET, NAP, DIC and IBU were 29±9, 138±2, 39±5 and 122±7 ng/g, respectively, and the repeatability and reproducibility for sludge were 10-18% and 7-15%, respectively. However, the information concerning ecotoxicological risks and the distribution of the drugs in sludge, surface water and water living organisms are rather scarce. Thus, the ultimate aim of the project is to figure out the bioaccumunation factor (e.g. fish), the distribution, even the fate of the NSAIDs.

To the best of our knowledge, only one work was concentrated on the possible toxic effect

of an NSAID, namely DIC in fish after exposure by Schwaiger et al.[9] Using a histopathological method, rainbow trout exposed to a DIC solution with a concentration from 1 μg/L to 500 μg/L for four weeks was studied. The DIC remains in various organs were determined by GC-MS. They found that in the kidney the tubular epithelial cells have hyaline droplet degeneration and interstitial nephritis, in the gills, the capillary wall within the secondary lamellae was damaged, and the lowest observed effect concentration at both renal lesions and alterations of the gills was 5 μg/L. No histopathological alterations were observed in the liver, the gastro-intestinal tract and the spleen in both DIC-exposed and control fish. The accumulation of DIC in all organs was found to be dependent on the DIC concentration. The bioconcentration factor ranged 12-2732 in the liver, 5-971 in the kidney, 3-363 in the gills and 0.3-69 in the muscle, when the concentration of the employed DIC solution was varied from 1 μg/L to 500 μg/L.

Aside from DIC, other NSAIDs such as salicylic acid, ketorolac, KET, NAP and IBU might also have a heavily environmental impact on fish. To investigate this, a new method which can simultaneously extract and detect all these NSAIDs in fish is urgently needed. In this thesis, by combining HF-LPME and LC-MS/MS, the four parent NSAIDs - KET, NAP, IBU and DIC in fish tissue were successfully determined. In comparison with other extraction techniques combining with LC-MS/MS, our method (combining HF-LPME with LC-MS/MS) shows better separation and quantitative ability under the optimized conditions. The achievement of this thesis provides a potential way to establish a library of the NSAIDs in other biota, animals or human beings.

2. Materials and methods

2.1 Chemicals and reagents

DIC sodium salt and di-*n*-hexyl ether (DHE) were obtained from Sigma Aldrich Inc (St Louis, MO, USA). IBU, KET and NAP (purity: 98%), ammonium carbonate (NH_3%: 30-33) and ammonium acetate were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Sulphuric acid (trace select for trace analysis ≥95%) was obtained from Sigma Aldrich (Buchs, Switzerland). Methanol (HPLC gradient grade) was purchased from Honeywell Specialty Chemicals (Seelze, Germany) and acetic acid (100%, glacial) from Merck (Darmstadt, Germany). Ultra pure water was produced by a Milli-Q water purification system (Millipore, Billerica, MA, USA).

Individual NSAID stock solutions were prepared in water and mixed stock solutions containing 1 and 10 mg/L of the four parent NSAIDs diluted with water were stored at 4 ℃

and wrapped with aluminium foil to prevent photodegradation of NSAIDs. The acceptor buffer was 0.1 M ammonium carbonate solution at pH 9.0. The buffer for the mobile phase was 0.01 M ammonium acetate at pH 4. The standard solutions for calibration were mixed stock solutions diluted by acceptor buffer.

2.2 Tissue samples

The cod fish, which was selected for method development, optimization and/or validation was purchased locally. The rudd fish was collected from a pond in Lund in the south of Sweden.

2.3 Sampling

2.3.1 Cod

The meat from the back of the cod was cut into small pieces and mixed in advance. Both fresh and dry fish tissues were studied. The dry fish was obtained using freeze-drying for 16h. Each sample containing 0.5 g (fresh mass) of tissue was first spiked with NSAID standard and homogenized with 50 ml of water.

2.3.2 Practical sample

Rudd fishes were first cultured in the fish tank for about 2 weeks with tap water. Then they were exposed in 4 L of Milli-Q water containing NSAIDs (approximately 50 μg/L) for three days. The fishes were taken out from the water. After their bodies were dried by a piece of tissue paper, each fish was sliced at one side of the head and was cut into three parts (head, middle and tail) with a similar mass. All these parts were mixed. An approximate mass of 0.5 g of this mixture was added into a vial, then the vial with fish was dried overnight using a freeze-dryer, and the dry tissue was spiked with NSAID standards and homogenized with 50 ml of water.

2.3.3 Water samples

Water samples were diluted or spiked to contain a suitable concentration of NSAIDs. Before membrane extraction, the pH value of the water samples was acidified to 2 using concentrated sulfuric acid prior to extraction.

2.4 LC-MS/MS method

Analysis was performed on an API Q-Star Pulsar I quadrupole time of flight tandem mass spectrometry with a Turboion electrospray interface from Applied Biosystems (Carlsbad, CA, USA) coupled to an Ultimate pump and Famos autosampler originally from LC Packings

(Thermo Scientific, Waltham, MA, USA) and a CSI 6150 vacuum degasser (Cambridge Scientific Instruments, Cambridge, UK). The injection volume was 4 μL for all samples using the pick up mode.

The chromatographic separation was performed on an Agilent Eclipse XDB-C18 column (particle size 5 μm, 4.6×150 mm, Agilent, Waldbronn, Germany). The mobile phase was a mixture of 100% methanol and ammonium acetate buffer (10 mM, pH 4). The employed gradient is shown in Table 1.

In order to shorten the run time, a higher flow rate was employed in the pre-separation process (Seen step 1 and step 3 in Table 1), and a lower flow rate was used during the separation of analytes to avoid overlap and to obtain a good separation. To test the repeatability, a standard solution of four parent mixed NSAIDs (1000 μg/L) was analyzed for several times before real samples analysis. A calibration curve was obtained using the standards with concentrations from 0, 100, 200, 400, 600, 800, 900 to 1000 μg/L, respectively. Before each analysis, the same amount of acceptor solution as each sample was injected to avoid cross contaminations.

Table 1

HPLC separation gradient for the mixture of KET, NAP, DIC and IBU using Agilent Eclipse XDB-C18 column (150×4.6 mm).

Step	Time (min)	Flow rate (ml/min)	Amonium acetate (%)	Methanol (%)
0	2.0	0.3	15.0	85.0
1	4.0	0.6	10.0	90.0
2	5.0	0.3	10.0	90.0
3	6.0	0.6	10.0	90.0
4	7.0	0.3	15.0	85.0

Table 2

Mass spectrometry parameters for the four parent NSAIDs- KET, NAP, DIC and IBU.

Analyte	Collision energy (eV)	Declustering potential (V)	Precursor ion (amu)	Product ion (amu)
KET	-12	-40	253	209.097
NAP	-10	-20	229	185.097
DIC	-10	-20	294	250.019
IBU	-10	-20	205	161.133

For the MS/MS analysis the setting of the ion spray voltage was -4500 V and the ion source temperature was 400℃. The settings of the focusing potential and the declustering potential were -220 V and -10V respectively and the setting of the collision gas was 5 units. Other parameters for each target ion are presented in Table 2.

2.5 HF-LPME methods

PP50/280 accurel polypropylene hollow fiber membranes, with a wall thickness of 50 μm, 0.1 μm pore size and i.d. of 280 μm (Membrana GmbH, Wuppertal, Germany) were cut into 20 cm long pieces, washed in methanol and water, and air dried prior to the extraction. Each fiber was fully filled with fresh acceptor buffer using a syringe (BD Micro-Fine ^{TM} + Demi, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The ends of the fiber were sealed by melting it with an electric soldering iron (Weller, WECP-20) at 400 ℃. Before immersing the fiber into the donor, the sealed fiber was made into a loop and a piece of copper wire was added to increase the weight of the fiber. Extraction was performed under the following optimized conditions: 50 ml of slurry or water samples at pH 2 were stirred at 660 rpm using a magnetic stirrer (RO 10 Power, IKA Werke, Staufen, Germany) for 5 h.

After extraction, the fiber was picked out from the sample solution. Both of the ends were cut open with a scalpel and carefully wiped off with Kleenex tissue, and then an air-filled syringe was attached to the fiber to push the acceptor phase out of the fiber into a 2 mL vial with a μL insert. The volume of the collected acceptor was calculated and determined by the weight difference of the vial before and after filling. The acceptor was diluted with the same amount of water to increase the volume of extract and to dilute the concentration of the extract into the linear range. The extracts were stored in the dark at 4°C before analysis. Before injecting the extracts into LC-MS/MS, sonication of the extracts for several min was needed to completely mix the collected acceptor with water.

During the development of the extraction method, the factors - extraction time, sampling solvent, the spiking order, matrix effect, protector net, and the status of the fish (fresh or freeze-dry fish) were investigated. The processes of the related experiments are presented in the Result and discussion section.

3. Results and discussion

3.1 Optimization of analytical methods

3.1.1 LC separation

First of all, the separation method for the mixed standard NSAIDs containing equal mass of e.g. KET, NAP, DIC and IBU should be optimized. It is not easy to obtain baseline separation for the four NSAIDs in a very short runtime due to their similar physical and chemical properties. Though baseline separation is not necessary for LC combined with tandem Mass Spectrometry, a more efficient separation is better for quantitative analysis. In a previous study, we found the peaks of the four NSAIDs easily overlapped if we wanted to obtain a separation in a short runtime.[13] Using another gradient elution, we could not get a fine separation even in a relatively long runtime (not shown). It is shown in Fig. 3 that adjusting the gradients with suitable mobility solvents, flow rate and columns, a good separation within the shortest runtime (6.5 min) was obtained. The optimized LC conditions with an Agilent Eclipse C18 column were presented in Table 1.

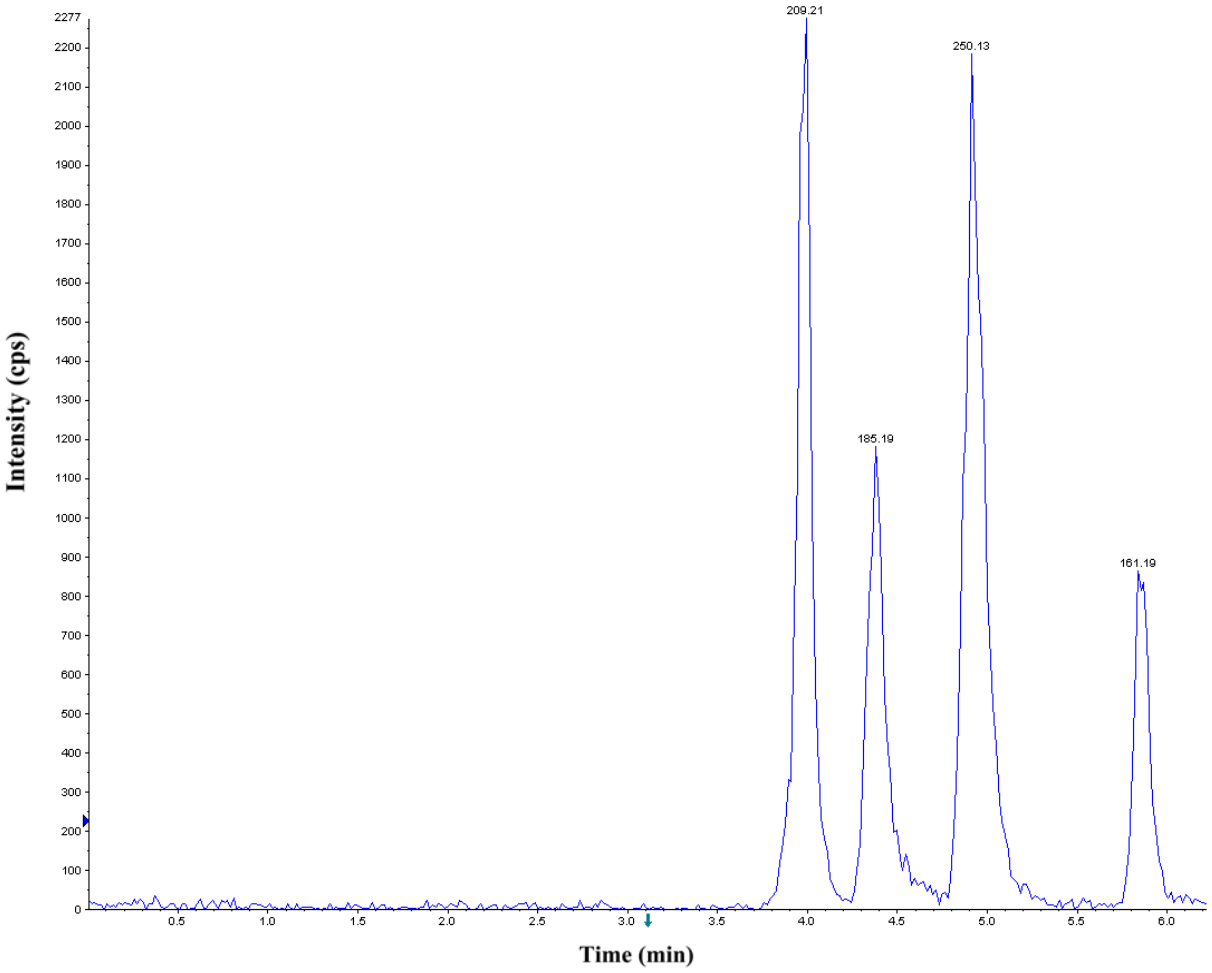

Fig. 3 Separation of KET, NAP, DIC and IBU using Agilent Eclipse C18 column with gradient elution.

3.1.2 Linearity of calibration curve

Based on the above LC separation method, a calibration curve was obtained from several standard solutions. The standard solutions with different concentrations were diluted by the standard stock solution containing the four parent NSAIDs with an equal mass-volume concentration. The linear relationship between peak area and concentration can be used to quantify the concentration in the unknown samples. The linear range for the four drugs was 100-1000 μg/ml, and the linearity as R^2 was 0.9902, 0.9945, 0.9802 and 0.9890 for KET, NAP, DIC and IBU, respectively.

3.2 Sampling optimization of fresh fish

The semi-solid sample is heterogeneous, thus sampling significantly affects the final results. Here, several factors were optimized to obtain a reliable sampling process.

3.2.1 Effect of sampling solvent

3.2.1.1 Effect of sampling solvent on spiked fresh fish

For this study, the meat from the back of the fresh cod (which was supposed to contain no NSAIDs) was cut into pieces and mixed completely in advance. 0.5 g of fresh meat was used for each sample in a brown wide mouth bottle.

When water, ammonium carbonate or ammonium acetate was employed as sampling solvent, the total solvent volume was 50 ml for each sample. First, the sample was spiked with 25 μl of a 1000 μg/L standard NSAIDs solution, then the spiked sample was homogenized for 10 min with 1 ml of solvent, then the rest solvent was added (49 ml) and homogenized for 2 min.

With ACN as sampling solvent, first, the sample was homogenized for 10 min with 5 ml of ACN in the fume hood. After homogenization, the ACN was evaporated by purging with air. Then the tissue without ACN was spiked with 25 μl of a 1000 μg/L standard NSAIDs solution in 50 ml of water and homogenized again for 10 min.

Finally, a fish “soup” was obtained and stored overnight in the dark at 4°C to equilibrate the distribution of the NSAIDs between the fish matrix and the sampling solvent. The homogenizer was washed with acetone and pure water before homogenizing another sample. On the second day, prior to extraction, the equilibrium soup was acidified with concentrated sulfuric acid to pH 2.

All extracts were collected and diluted with the same amount of water after 4 h of extraction using HF-LPME. The diluted extracts were sonicated for 10 min and analyzed following the calibration curve by LC-MS/MS. All the peaks were integrated manually and

handled by Excel with DPX. The final results with error bars are shown in Fig. 4, except the result of NH_4Ac.

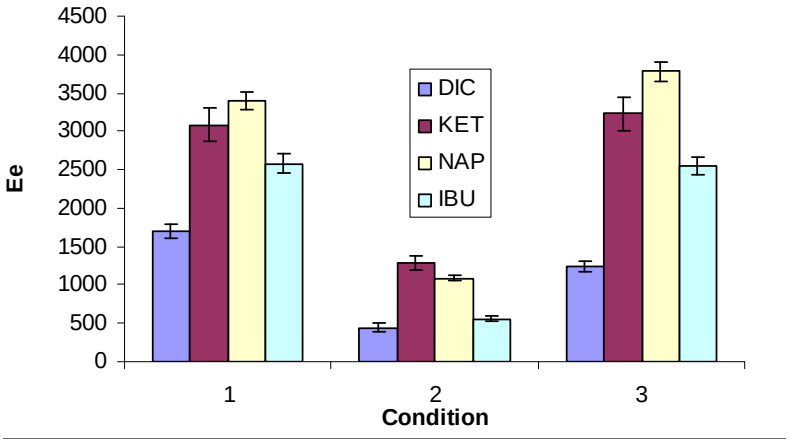

Fig. 4 Comparison of the achieved Ee on spiked fish using different solvents: 1: Water pH 7; 2: 0.1 M $(NH_4)_2CO_3$, pH 9; 3: ACN.

When NH_4Ac was selected as sampling solvent, no NSAIDs were extracted by HF-LPME, which is probably due to the competitive partition between HAc and NSAIDs and the partition of produced HAc suppressed the partition of NSAIDs between donor, organic even acceptor phases.

Fig. 4 clearly shows that the $(NH_4)_2CO_3$ solution resulted in the lowest Ee for four NSAIDs. From this figure, we can also calculate the E and RSD (see Table 3). In comparison with water as sampling solvent, ACN did not change the Ee for IBU and led to a slightly higher Ee for KET and NAP. However, ACN resulted in a lower Ee for DIC. Unfortunately, the Ee of DIC was always the lowest among the four NSAIDs in all the extraction methods. To get the best sensitivity of the extraction method for all the NSAIDs, water was chosen as the sampling solvent for spiked fresh fish.

Table 3

Linearity and extraction efficiency (E) with RSD (n=5) using water, $(NH_4)_2CO_3$ and ACN solutions for a 1% fish slurry with a spiked concentration of 0.5 μg/L.

Analyte	R^2	E_{water} (RSD) %	$E_{(NH4)2CO3}$ (RSD) %	E_{ACN} (RSD) %
KEI	0.9902	61.73 (7.0)	25.85 (6.5)	64.62 (7.2)
NAP	0.9945	68.08 (3.4)	21.77 (2.6)	75.71 (3.7)
DIC	0.9802	33.95 (5.7)	8.90 (10.6)	24.70 (5.7)
IBU	0.9890	51.59 (4.6)	11.14 (5.7)	51.04 (4.8)

3.2.1.2 Effect of sampling solvent on exposed fish

According to the above results, pure water and ACN were chosen as the sampling solvent for the exposed fish. Before exposure, the rudd fishes were fed for 2 weeks to ensure they were active. The exposure was conducted in a 5-liter-beaker with 4 L of mixed standard NSAIDs (50 μg/L) for 72 h. The exposed fish were killed by slicing their head and then cut into three pieces (head, middle and tail). These pieces were mixed randomly for extraction. The sampling steps were the same as described in 3.2.1.1, and the peak area for each analyte is illustrated in Fig. 5.

When ACN was selected as sampling solvent, KET and NAP showed higher peak areas than with water, and this result agreed with the conclusions in Fig. 4. ACN lead to a much higher peak area for IBU than water did and the Ee of IBU was the lowest among the four NSAIDs in this extraction process. ACN was selected as sampling solvent for the exposed fresh fish to get the best sensitivity of the extraction method for all the NSAIDs. In comparison with spiked fish, exposed fresh fish needs a different sampling solvent. The reason might be that NSAIDs are physically adsorbed in the tissues of the spiked fish, whereas the NSAIDs are chemically bound to the proteins of the exposed fish.[43] An organic sampling solvent is better for the release of the bound NSAIDs from proteins.

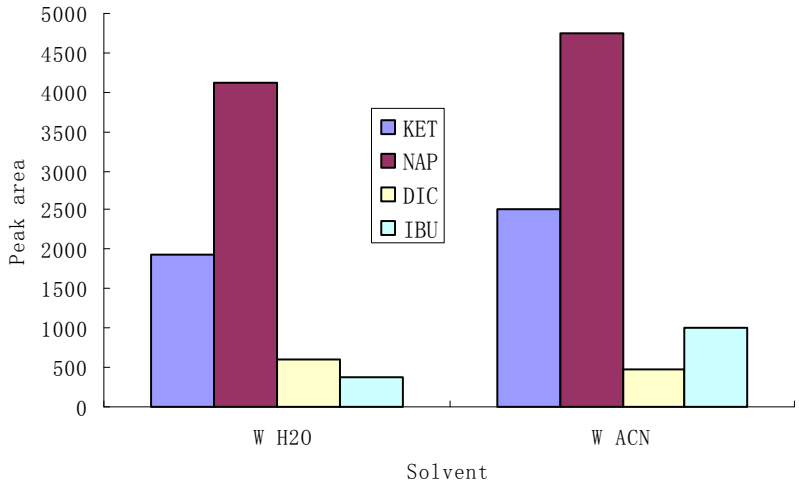

Fig. 5 Peak areas of four NSAIDs extracted from exposed fresh fish using water and ACN.

3.2.2 Effect of spiking order for fresh fish

It has been shown in the above section that water was a better sampling solvent for the spiked fish. Besides sampling solvent, spiking order (spiked before or after the homogenization of the fish) also affects E. To investigate the possible effect of the order of spiking, fresh cod was used as the ideal fish. Typically, 0.5 g of fresh fish meat was spiked

with NSAIDs (0.5 μg/L) before or after homogenization. Then the homogeneous tissues were extracted for 5 h. The experimental results are illustrated in Fig. 6, which shows that the peak areas for the fish spiked before homogenization was higher than that spiked after homogenization. Thus, NSAIDs spiking before homogenization was chosen in this work to yield higher Ee and E.

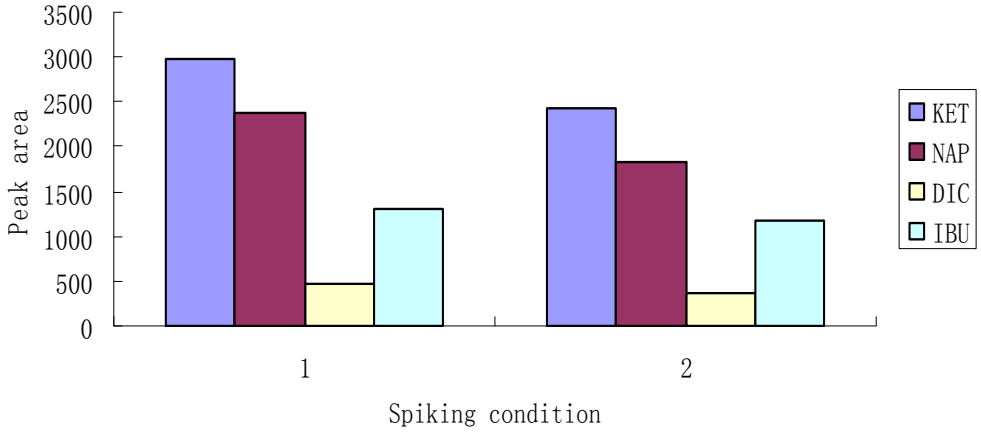

Fig. 6 Effects of spiking order: 1: spiking before homogenization, 2: spiking after homogenization.

3.3 Sampling optimization of dried fish

In the above studies, we have shown that the interaction between the proteins and NSAIDs influenced the extraction of these drugs. Therefore, decreasing the interaction between the proteins and NSAIDs might enhance the extraction efficiency. In the literature, many reports have shown that water molecules played an important role during the binding of the proteins towards the drugs.[44] Without water, this interaction would disappear.[45] Therefore, the water contents in the fish might affect the extraction efficiency. To make it clear, spiked and exposed fresh fish were dried in this study. The dry fish was obtained by keeping the fresh fish meat (0.5 g) in a freeze-dryer for about 16 h.

3.3.1 The effect of water content of spiked dry fish

From the above studies, we concluded that the spiked fish was simpler than the exposed fish. For the spiked fish, water was selected as sampling solvent to study the extraction efficiency. Typically, the dry fish was spiked with 25 μl of standard NSAIDs (1000 μg/L) in a brown wide mouth bottle. After adding 1 ml of water, the sample stood for half an hour before homogenization. This homogeneous mixture was extracted and tested by the same way as that used for fresh sample. The Ee of each analytes with error bars for both fresh cod and freeze-dried cod are shown in Fig. 7. It is seen that a slightly higher Ee and error values

were obtained from freeze dried fish than from fresh fish for all the analytes. The RSD (%) for all analytes were in the range of 4.8-7.7 and 5.4-8.7 for fresh fish and freeze-dried fish, respectively.

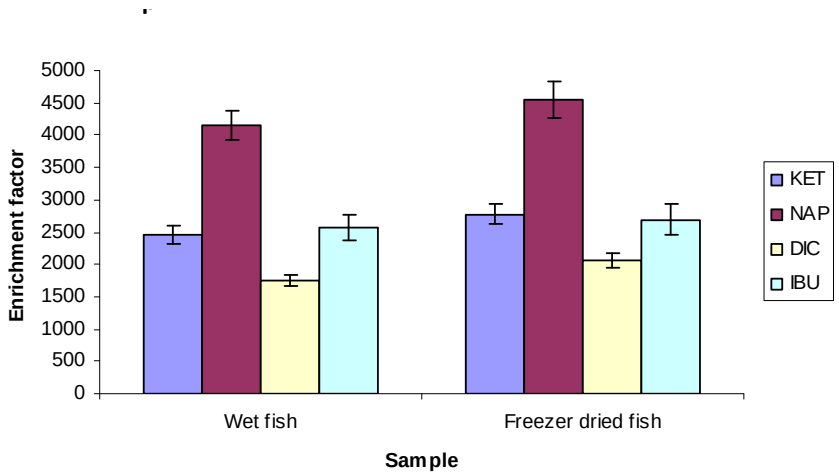

Fig. 7 Effects of water content using fresh and freeze-dried fish.

3.3.2 The effect of water content of exposed fish

According to the results in 3.2.1.2, exposed fish was more complex because the NSAIDs are bound to proteins. To confirm that the drying process can also destroy the interaction between NSAIDs and proteins, ACN as well as water were selected as sampling solvents. The exposed fish was of the same batch and handled in the same way as in 3.1.2.2. When water was used as solvent, all the sampling steps were the same as mentioned in 3.2.3.1. When ACN was employed as sampling solvent, after transferring the dry fish into a brown wide mouth bottle, 5 ml of ACN was added. Before homogenization, the covered bottle was left for 0.5 h. The rest of the procedures were the same as described in 3.2.1.1.

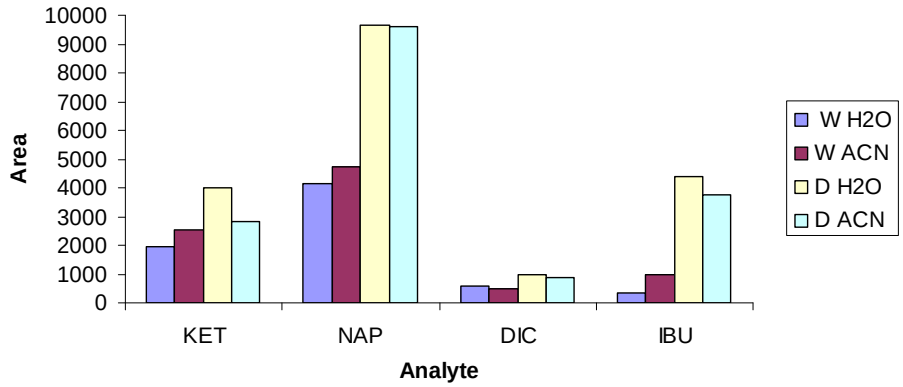

Fig. 8 Effect of water content in fish for exposed fish with different sampling solvent (light blue: fresh fish with water; purple: fresh fish with ACN; light yellow: dried fish with water; light green: dried fish with ACN).

The obtained peak area of the extracts for each analyte for freeze-dried rudd is shown in Fig. 8. As a control, fresh fish was analyzed in the same way. It is seen that much higher peak areas were obtained from freeze-dried fish for all analytes than that from fresh fish, which agrees with the result in section 3.2.3.1. As has been estimated, the extraction efficiency for all analytes from dry fish using water as sampling solvent was almost the same as using ACN.

3.4 Optimization of extraction

3.4.1 Matrix effect

Reagent water and fresh fish meat were selected as samples to investigate the effects of the matrix. For water samples, homogenization was not needed before spiking. After spiking, the water sample was acidified by concentrated sulfuric acid to pH 2 and extracted by HF-LPME. The sampling of fresh fish was the same as in 3.2.1.1.

Spiked water and spiked fish matrix were extracted by HF-LPME for 5 h with 5 replicates. The obtained Ee for spiked reagent water and spiked fish matrix are shown in Fig 9. It is obvious that a much lower Ee was obtained from the fish matrix than from water, which means a significant matrix effect. The Ee for all analytes was in the range of 3904-6118 and 1419-2745 for water and fish, respectively. DIC has an RSD value as high as 27% (R^2 is just 0.83), other NSAIDs showed RSD values below 15%. The lower Ee obtained from fish might be due to the wall of the fiber was covered by the fat or protein, which might result in that a longer equilibrium time was required for the NSAIDs reaching equilibrium in the donor, organic and acceptor phases. Physical or chemical binding between the NSAIDs and the proteins could lead to a lower Ee as well.

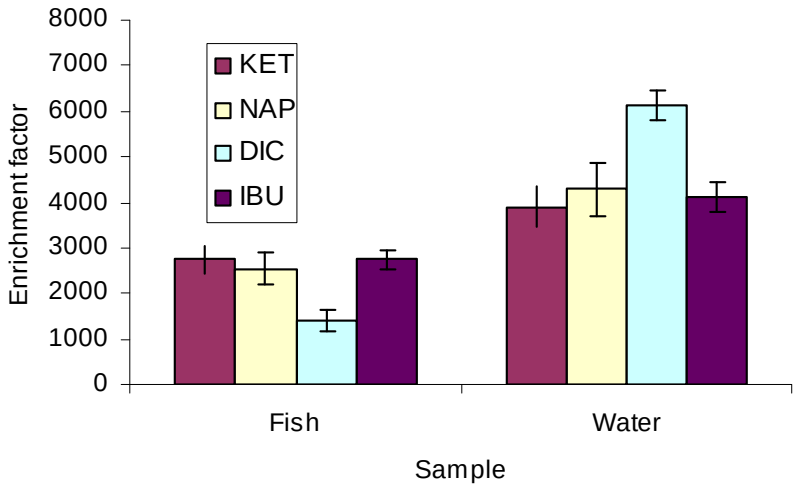

Fig. 9 Results of the matrix effect using fresh fish and reagent water.

3.4.2 Studying the effect of the net

Because the matrix affects the extraction efficiency heavily, a kind of nylon net was introduced to the extraction to reduce the matrix effect by protecting the membrane from being covered with fat or proteins. The effect of the net was investigated first with spiked reagent water, then with spiked fresh fish slurry, and the corresponding results are presented in Table 4. Both water and fish samples with 5 replicates were extracted for 5 h. Unfortunately, an additional net decreased the Ee, which might be due to the strong adsorption of the NSAIDs on the net fibers and the net also affected the partition of the NSAIDs.

Table 4

Effects of net on Ee of water and 1% fish slurry (spiked at 2 μg/L).

Sample	Net	Ee			
		KET	NAP	DIC	IBU
Spiked water	With	3101.4	2565.1	939.7*	2192.1
	Without	4103.3	4136.1	1345*	2721.0
Spiked fish	With	1979.3	1864.3	821.2	1818.1
	Without	2738.1	2539.5	1419.3	2745.4

*: Peak area

3.4.3 Effect of extraction time

In a similar study by Sagrista et al. in 2010 [13], an extraction time of 4 h was selected as the optimal for sewage sludge by HF-LPME. However, different matrices will affect the mass transfer process of NSAIDs. Thus the extraction time for fish matrix needs be optimized again.

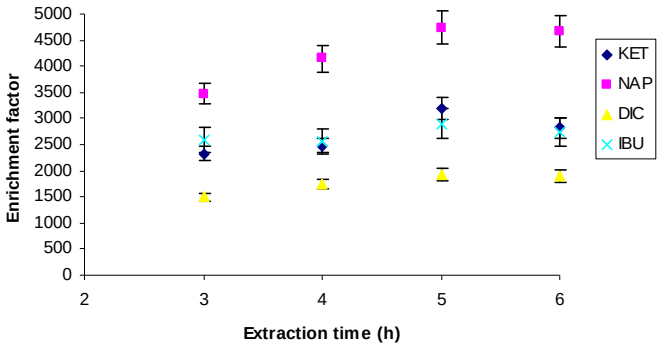

Fig. 10 Effect of extraction time on enrichment factors.

Fig. 10 shows the influence of extraction time on the Ee for all NSAIDs. The extraction was performed using fresh fish spiked by 0.5 μg/L NSAIDs. The investigated extraction times were 3, 4, 5 and 6 h. It is seen that, after 5 h of extraction, the highest Ee for all the analytes was achieved. Prolonging the extraction time to 6 h, Ee decreased for all NSAIDs. Thus, an extraction time of 5 h was selected as the optimum extraction time.

3.5 Method Validation (for both water and fish)

3.5.1 Repeatability and reproducibility

To evaluate the validation of the HF-LPME method for KET, NAP, DIC and IBU after 5 h of extraction, the repeatability and reproducibility (intra-day precision) for both spiked water and spiked fresh fish slurry samples containing 1% fish tissue were tested. The results are presented in Table 5 and Table 6.

Table 5

Method reproducibility and repeatability (n=5) as relative standard deviations for reagent water with a spiked concentration of 0.5 μg/L.

Analyte	R^2	Repeatability (%)	Reproducibility (%)
KET	0.980	8.6	7.3
NAP	0.981	6.6	6.5
DIC	0.984	5.4	6.8
IBU	0.997	2.2	12.8

Table 6

Method reproducibility and repeatability (n=5) as relative standard deviations for 1% fish slurry with a spiked concentration of 0.5 μg/L.

Analyte	R^2	Repeatability (%)	Reproducibility (%)
KET	0.986	6.2	9.9
NAP	0.996	5.4	10.9
DIC	0.983	6.3	4.1
IBU	0.988	14.0	12.3

It is seen that the values of repeatability were 2.2-8.6% and 5.4-14.0% for spiked reagent water and spiked fish slurry, respectively. The values of reproducibility were 6.5-12.8% and 4.1-12.3% for spiked reagent water and spiked fish slurry, respectively.

3.5.2 Limits of detection

The limits of detection (LOD) of the LC-MS/MS (calculated as 3 times the background noise) were 6.3, 9.7, 2.3 and 8.6 µg/L for KET, NAP, DIC and IBU, respectively. The overall method detection limit (MDL) is calculated using the LOD values divided by the enrichment factor of each analytes, which shows a range from 1 to 3 ng/L and 0.8 to 2.3 ng/L for the four parent NSAIDs in fish slurry and in water samples, respectively. Moreover, the practical quantitation limits (PQL) defined as 5 times of MDL were 5 - 15 ng/L and 4 - 11.5 ng/L for the four parent NSAIDs in fish matrix and in water samples, respectively.

3.6 Application of the developed method

3.6.1 Determination NSAIDs in cod

The developed method involving standard addition calibration was used to determine the concentration of KET, NAP, DIC and IBU in cod. The concentration in the acceptor was a function of the amount of analyte added in the fish slurry samples. The experimental results are shown in Fig. 11, and the related parameters are presented in Table 7.

Table 7

Linearity of determination (R^2), slope and intercept for the regression lines in Fig. 11, m_{fi}, C_{fi} for cod also showing the relative standard deviation.

Analyte	KET	NAP	DIC	IBU
Intercept	-0.0914	0.7464	-0.2328	-0.1879
Slope	0.2545	0.2859	0.2318	0.2461
R2	0.934	0.960	0.996	0.988
Amount in fish (ng)	-0.359	2.610	-1.004	-0.764
Conc in fish (ng/g)	-0.718	5.220	-2.009	-1.527
RSD (%)	6.3	5.5	11.8	6.1

In Fig. 11, for all interesting analytes, the linearity regression between the amount of spiked analytes in the fish slurry and the measured concentration in the acceptor phase was obtained as expected from Equation (7).

The R^2 for the four NSAIDs were higher than 0.934, and the intercept for all analytes was close to zero except for NAP in Table 7. Using equation (7), the initial amount of NAP in the fish slurry can be calculated with a relative standard deviation of 6%. The mass spectrum and chromatogram of the fish slurry showed a peak having the same mass and also the same daughter ion, and at the same retention time as the standard solution.

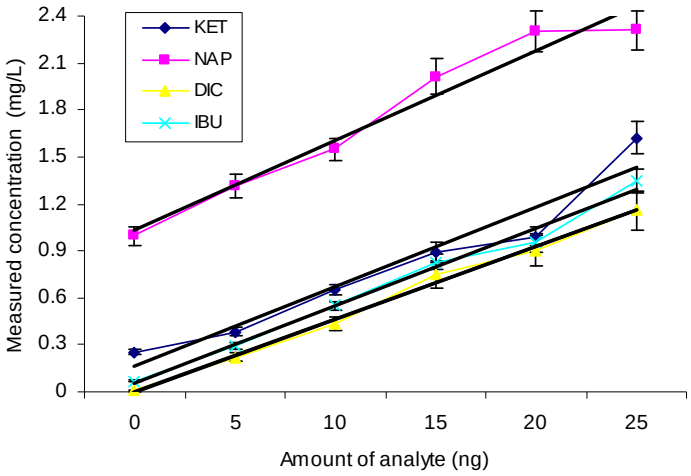

Fig. 11 Concentration of KET, NAP, DIC and IBU obtained in the acceptor phase plotted as a function of the amount of analyte added in the fish slurry samples with standard deviations (n=5).

3.6.2 Determination NSAIDs in exposed fish

The developed method was used to determine the concentration of KET, NAP, DIC and IBU in non-exposed fish, fish exposed but dead before 72 h of exposure and fish exposed for 72 h.

Table 8

m_{fi} and C_{fi} for four analytes with standard deviation found in non-exposed, exposed but dead and exposed fish after freeze-drying (n=2).

Analyte (RSD)	Sample	m_{fi} (ng)	C_{fi} of fresh fish (ng/g)	C_{fi} of dried fish (ng/g)
KET (30%)	Non-exposed	7.8 ± 2.3	15.6 ± 4.7	78 ± 23
	Exposed but dead	16 ± 4.8	32.0 ± 9.6	160 ± 48
	Exposed	8.3 ± 2.5	16.6 ± 5.0	83 ± 25
NAP (14.4%)	Non-exposed	4.0 ± 0.6	8.0 ± 1.2	40 ± 6
	Exposed but dead	2.6 ± 0.4	5.2 ± 0.7	26 ± 4
	Exposed	3.3 ± 0.5	6.5 ± 0.9	32 ± 5
DIC (8.4%)	Non-exposed	Not Detected	Not Detected	Not Detected
	Exposed but dead	3.8 ± 0.3	7.6 ± 0.6	38 ± 3
	Exposed	0.6 ± 0.05	1.2 ± 0.1	6.0 ± 0.5
IBU (6.5%)	Non-exposed	Not Detected	Not Detected	Not Detected
	Exposed but dead	0.4±0.03	0.8 ± 0.1	4.0 ± 0.3
	Exposed	1.9±0.1	3.7 ± 0.2	19 ± 1

By using standard addition method, the NSAIDs in these fish were detected. For exposed fish (dead or living), the obtained concentrations of KET, NAP, DIC and IBU ranged in 78-160, 26-40, 6-38 and 4-19 ng/L, respectively (see Table 8). It is noted that KET and NAP were unfortunately found in non-exposed fish.

The concentrations of the analytes in the reagent water both before exposure and after 3 days of exposure were also measured. Before exposure, the determined concentrations in water were 72±6, 68±3, 80±4 and 57±3 ng/g for KET, NAP, DIC and IBU, respectively. After exposure, the concentrations of these NSAIDs decreased to 57±5, 47±2, 49±3 and 37±2 ng/g for KET, NAP, DIC and IBU, respectively. By estimating the adsorption of NSAIDs in fishes, the obvious decrease of concentration for all analytes mainly was due to the degradation.

4. Conclusion

A new and direct method to determine KET, NAP, DIC and IBU in fish was developed based on HF-LPME extraction and LC-MS/MS analysis. The obtained average enrichment factors for the four NSAIDs were 3700 and 3000 for reagent water and freeze-dried fish tissue, respectively. Acetonitrile has a better performance for extraction than water for exposed fresh fish. However, for the freeze-dried fish, water has a higher extraction efficiency than acetonitrile. This method could be successfully applied to analyze real samples. For wild cod fish, only NAP was found with a concentration of 26 ng/g (dry fish). For wild rudd fish, two NSAIDs (78 ng/g of KET and 40 ng/g of NAP) were found. When rudd fish was exposed to the NSAID solution containing KET, NAP, DIC and IBU, these compounds were determined as 83, 32, 6, 19 ng/g in the alive fish, respectively. Interestingly, the concentrations of KET and DIC in the dead rudd fish were almost twice as that in the alive fish.

The breakthrough and improvement of this analytical method are significant for determination the trace level and ionizable analytes in environmental samples, especially for samples with complex matrices.

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