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Hollow fiber liquid phase microextraction as a preconcentration and clean-up step after pressurized hot water extraction for the determination of non-steroidal anti-inflammatory drugs in sewage sludge

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ABSTRACT

A method for the quantitative determination of non-steroidal anti-inflammatory drugs (NSAIDs) in sewage sludge was developed and validated. The target compounds were extracted using pressurized hot water extraction (PHWE) and then purified and preconcentrated by three-phase hollow fiber liquid phase microextraction (HF-LPME) followed by LC–ESI-MS analysis. The PHWE was optimized with regard to the pH of solvent as well as other operational parameters. The optimum conditions were 0.01 M NaOH as the extraction solvent, temperature of 120 °C, pressure of 100 bar, static time 5 min, 5 cycles, flush volume 90% and purge time 60 s. Spike recoveries for sludge samples spiked at 200 ng g^{-1} 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 ketoprofen, naproxen, diclofenac and ibuprofen, respectively. Donor phase pH, ionic strength and extraction time were optimized for HF-LPME after PHWE. The optimum conditions were 2 h extraction at pH 1.5 without salt addition. Enrichment factors in the range of 947–1213 times were achieved (extraction recoveries were 23.6–30.3%) for HF-LPME after PHWE. The matrix effect on the ionization of drugs in LC–ESI-MS was also investigated. The results show that there is a smaller matrix effect (−8.9% to +14.6%) in comparison with other published values obtained using solid phase extraction (SPE) for clean-up after pressurized liquid extraction (PLE). Method detection limits (MDLs) and method quantification limits (MQLs) for different drugs were in the range of 0.4–3.7 ng g^{-1} and 1.5–12.2 ng g^{-1} in dried sludge samples, respectively. The characteristics of the proposed method were compared with those of other published works. The considerably lower ion suppression/enhancement and minimum use of organic solvents (a few microliters of di-n-hexyl ether) in the sample preparation step are two highlighted advantages of the proposed method in comparison with previously published works. The method was applied to determine NSAIDs in sewage sludge from Källby wastewater treatment plant (Lund, Sweden) in April, June, August and October 2010. The highest concentration level was recorded for ibuprofen in the April sewage sludge sample (588 ng g^{-1}) and all of the selected NSAIDs were detected in all the samples analyzed.

1. Introduction

Pharmaceutical compounds are a source of increasing environmental concern since they are used in large quantities and their physical and chemical properties make them likely to be transported into aquatic systems, where their effects on human health and aquatic ecosystems are almost unknown. The principal cause of their presence in the environment is excreta and disposal of unused or expired products, but also the result of manufacturing processes [1–4]. Several pharmaceuticals can therefore reach wastewater treatments plants (WWTPs) in substantial amounts. Many WWTPs in Europe include only three treatment steps (physical, biological and chemical) for removal of organic matter, particulate matter and nutrients. Because of their high cost, further treatments such as ultrafiltration, flocculation, ozonation, advanced oxidation and osmosis are seldom used which leads to an incomplete removal of pharmaceuticals in effluent wastewater and sewage sludge [5]. Several studies carried out in different countries show that one of the most common groups of pharmaceuticals found in effluents and treated sludge from WWTPs are non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. ketoprofen, naproxen, diclofenac and ibuprofen) which are commonly employed in a large scale to reduce ongoing inflammation, pain and fever [6–10]. During wastewater treatment, NSAIDs are to a considerable extent eliminated from

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the water stream and their fate is likely to be associated with sewage sludge because of their strong sorption properties, especially of ibuprofen [5,11–13], but also microbial processes and photodegradation are considered to constitute important removal mechanisms. Application of sewage sludge to soils as a fertilizer may therefore be a potential route for these pharmaceuticals to enter the terrestrial environment continuously. Some studies show the toxicity of NSAIDs to numerous animal species [14]. It is important to determine the occurrence of pharmaceuticals in sewage sludge for two reasons. The first is to evaluate the elimination power of WWTPs and the second is to determine whether sewage sludge can be used as fertilizer.

Most of the studies on the fate of pharmaceuticals in WWTPs are focused on liquid samples from sewage treatment because of the complexity of the sludge matrix. To extract NSAIDs from sludge samples, ultrasonic solvent extraction (USE) [8,15], microwave assisted extraction (MAE) [16] and pressurized liquid extraction (PLE) [6,7,17,18] using organic solvents or their mixture with an aqueous solvent have been employed. Using these methods, a high number of possible interferences can be co-extracted and it is necessary to remove them by applying clean-up techniques, even if there are a few reports in which no clean-up steps are applied [6]. Pressurized hot water extraction (PHWE) is an environmentally friendly organic solvent free technique that seems to be a good alternative to these techniques. In PHWE, water is used as the extraction solvent at elevated temperature and under pressure to keep it in a liquid state. This method has been used to extract organic pollutants from solid matrices such as contaminated soils and sewage sludge [19,20]. There is no specific commercially available device for PHWE but an automatic PLE machine (e.g. ASE-300 or 200, Dionex, Sunnyvale, CA, USA) which uses water as the solvent has been described in some studies [20,21]. The PHWE extract is a relatively diluted aqueous solution which can be easily cleaned up and concentrated in comparison to the PLE extract which always contains a considerable amount of organic solvent (e.g. methanol).

Solid phase extraction (SPE) [7,8,15–18], and other sorptive extraction techniques [16] have been reported to be used for clean-up and preconcentration steps after extraction from solid matrices for NSAIDs. In the case of NSAIDs, being acidic drugs, adjusting pH to acidic values before SPE to obtain higher retention efficiency leads to the formation of some colloidal precipitation which makes it difficult to perform SPE in a reasonable loading time and it is necessary to filter the extract before SPE. Also, considerable matrix effects for NSAIDs (ion suppression/enhancement in MS detection) have been reported after clean-up using SPE [7,17,18]. Two-phase HF-LPME has already been utilized for the enrichment and clean-up of some organic and inorganic pollutants after MAE [22] and USE [23] on solid samples. This technique also has been used as a trapping device for the extracted compounds after PHWE of polycyclic aromatic hydrocarbons (PAHs) from soil and sediments [24]. Recently, three-phase hollow fiber liquid phase microextraction (HF-LPME) has been used for the analysis of pharmaceuticals including NSAIDs providing good clean up efficiency and high enrichment factors [9]. Using a pH gradient for the extraction of acidic compounds in three-phase HF-LPME leads to preconcentration of acidic compounds which have suitable p*K*a values depending on the pH values of the donor and the acceptor phases. Neutral compounds can be partitioned between two aqueous phases without being enriched and basic compounds cannot be extracted due to their positive charge in acidic pH. Therefore, the acceptor phase will contain a narrow range of compounds including target analytes in comparison with the sample matrix which makes it suitable for injection into LC–ESI-MS. It is also possible to utilize three-phase HF-LPME for extraction of acidic drugs directly from sewage sludge slurry, as was recently successfully presented [25].

The aim of this work was to apply three-phase HF-LPME as a preconcentration and clean-up step after PHWE for the determination of some NSAID pharmaceuticals (ketoprofen, naproxen, diclofenac and ibuprofen) in sewage sludge samples. A LC–MS with electro-spray ionization (ESI) interface was used to analyze the HF-LPME extracts. Treated sludge samples taken from a WWTP (Lund, Sweden) in different months of the year were analyzed to demonstrate the feasibility of the proposed method.

2. Materials and methods

2.1. Materials and reagents

Ketoprofen, naproxen, diclofenac (sodium salt), ibuprofen, ammonium acetate and NaCl were obtained from Sigma–Aldrich Chemie GmbH (Steinhem, Germany). Gradient grade methanol (Honeywell B&J brand, Sleeze, Germany) was used for preparing standard solutions and also as mobile phase in LC separations. Ammonium carbonate, di-n-hexyl ether (DHE) and concentrated sulfuric acid were supplied by Fluka (Buchs, Switzerland). Acetic acid used for adjusting pH was purchased from Merck (Darmstadt, Germany). Reagent grade sodium hydroxide was obtained from Scharlau Chemie S.A. (Barcelona, Spain). Ultra pure water was obtained from a MilliQ water purification system (Millipore, Billerica, USA).

Individual stock standard solutions of each drug were prepared in methanol at a concentration of 1000 mg L^{-1}. Individual and mixed standard solutions of target drugs with lower concentrations (40, 4 and 0.4 mg L^{-1}) were prepared by appropriate dilution of stock standard solutions in methanol and were used as working standards. All the standard solutions were stored at 4 °C in darkness.

2.2. Sampling

Treated sludge samples were collected from Källby WWTP (Lund, Sweden) in April, June, August and October 2010. This plant receives sewage water from the city as well as surrounding villages. In 2008 the total number of persons connected to the plant was estimated to 84,000 [26]. The plant daily treats about 30,000 m^3 of sewage water. The sewage treatment is made up of screen raking, sand catch, primary sedimentation, secondary (biological) treatment, including anaerobic denitrification and aerobic nitrification, and finally tertiary treatment where phosphate is chemically precipitated. Some activated sludge from the secondary sedimentation unit is returned to the inlet of the primary clarifier. The remaining fraction of secondary sludge is combined with the primary and tertiary sludge and further treated (including thickening, dewatering and anaerobic digestion). The treated sludge is then transferred from the WWTP for storage prior to application onto agricultural fields.

The collected treated sludge samples were transported to the laboratory in plastic buckets, dried at 40 °C, ground by a mortar and sieved (0.5 mm) to obtain particles with the same diameter.

2.3. Pressurized hot water extraction (PHWE)

For the optimized PHWE method, 0.5 g sludge was transferred into a 33 cm^3 stainless steel extraction cell containing 20 g sea sand (washed by acetone and ultra pure water and dried at 200 °C over night). For the spiked samples (200 ng g^{-1}), before mixing with sea sand, 0.5 mL mixed standard solution of drugs with appropriate concentration in methanol was added directly on the sludge sample inside the cell followed by intensive stirring and drying over night. Pressurized hot water extraction was carried out on a Dionex ASE-300 (Sunnyvale, CA, USA). The optimum extraction solvent was 0.01 M NaOH in ultra pure water. The operating conditions were

extraction temperature: 120 °C, extraction pressure: 100 bar, preheating time: 6 min, static extraction time: 5 min, number of cycles: 5, flush volume: 90% of cell volume and nitrogen purge time: 60 s. The final extraction volume was about 90 mL after 5 successive cycles. Extracts were transferred into a 100 mL volumetric flasks and adjusted to pH 1.5 using H_2SO_4 and were diluted up to the mark with ultra pure water prior to three-phase HF-LPME.

To optimize the PHWE conditions, two PHWE extractions were performed for each level of each parameter (non-spiked and 200 ng g^{-1} spiked sludge samples). Thereafter the pH of the extracts was adjusted to 1.5 using H_2SO_4 and they were diluted to 100 mL. Two portions of diluted non-spiked extract (2 × 40 mL) were transferred into HF-LPME vials and one of them was spiked by 40 μL of 2 mg L^{-1} of the mixed standard solution of drugs in methanol. Also, 40 mL of spiked PHWE extract was transferred to another HF-LPME vial. Finally, three samples (non-spiked, spiked after PHWE and spiked before PHWE) were extracted using the HF-LPME method to calculate the recovery of PHWE for each level of target parameter. Using this method, different matrix effects on the HF-LPME due to different PHWE conditions were compensated for.

2.4. Preconcentration and clean-up step

HF-LPME was used for preconcentration and clean-up. All the HF-LPME experiments were done using Accurel Q3/2 polypropylene hollow fiber membranes (600 μm I.D., 200 μm wall thickness and 0.2 μm pore size) purchased from Membrana (Wuppertal, Germany). The hollow fibers were cut into 10 cm pieces (volume of 25 μL) and each was used once to prevent memory effects. A 100 μL syringe (Hamilton, Bonaduz, Switzerland) was applied to introduce the acceptor phase (0.1 M ammonium carbonate) into the lumen of the hollow fiber and also as a support to which the hollow fiber was attached during the extraction period. The hollow fiber, attached to the syringe, was immersed into organic solvent (DHE) for 15 s and then in ultra pure water for 10 s to wash the extra organic solvent from the surface of the fiber. Next, the acceptor phase was introduced into the lumen of the hollow fiber with slow pushing of the microsyringe plunger and the end of the hollow fiber was sealed by a piece of aluminum foil. Then the fiber was bent (U-shape) and was immersed into the sample solution (100 mL PHWE extract adjusted to pH 1.5). A multiple-station magnetic stirrer (IKA, Staufen, Germany) was used for stirring the samples during extraction. At the end of the extraction time (120 min), the hollow fiber was removed from the sample solution, the closed end was opened and the acceptor phase was withdrawn into the syringe. Finally, the extract was transferred into a conical bottom autosampler vial and 10 μL of this sample was injected into the LC–ESI-MS for analysis.

2.5. Method validation

To determine the recoveries in non-spiked samples and compare with recoveries obtained using spiked samples, fifteen consecutive extractions (each one including 5 cycles) were performed in triplicate on 0.5 g of the sludge sample which contained the highest amounts of NSAIDs (sampling date: April 2010). The extracts were preconcentrated using HF-LPME followed by LC–ESI-MS analysis under optimum conditions and recoveries for target drugs were calculated using Eq. (1).

$$\text{PHWE\%} = \frac{\text{PA}_1}{\sum_{i=1}^{n}\text{PA}_i} \times 100 \quad (1)$$

where, PHWE% is the recovery for the first extraction (5 cycles); PA_1 is the corrected peak area of drugs after LC–MS analysis of the first extraction and $\sum_{i=1}^{n}PA_i$ is the sum of the corrected peak areas for all the fifteen extractions. Peak areas were corrected for different matrix effects on HF-LPME of NSAIDs from the first PHWE extracts to the sixth one. For correction of matrix effects, six consecutive PHWE were performed on 0.5 g non-spiked sludge sample followed by pH adjustment and dilution up to 100 mL for each extract. Then, two portions of each diluted extract (2 × 40 mL) were transferred into HF-LPME vials and one of them was spiked by 40 μL of 2 mg L^{-1} of the mixed standard solution of drugs in methanol. After HF-LPME and LC–MS analysis of these 12 samples and subtraction of blank signals from spiked ones, the extent of matrix effects in each PHWE extraction was evaluated as a correction factor and used for correction of obtained peak areas. To confirm that no thermal degradation of NSAIDs occurred during PHWE, extraction cells filled with sea sand were spiked with 200 ng NSAIDs, extracted at 120 °C and analyzed using the same protocol including HF-LPME [15].

For the evaluation of enrichment factor (EF) and extraction efficiency (E) of HF-LPME for NSAIDs, five individual PHWE extractions were performed (each sample of 0.5 g). All the extracts were collected and mixed in a 500 mL volume flask and were diluted to the mark. Then, the diluted extract was divided into five extraction bottles and spiked with different concentrations of drugs followed by pH adjustment to 1.5. After performing HF-LPME, the five extracts were analyzed by LC–ESI-MS. Enrichment factors were calculated based on the ratio of the slopes of calibration curves of HF-LPME extracts and those of calibration curves obtained by direct injection of standard solutions of NSAIDs in 0.1 M ammonium carbonate. HF-LPME extraction efficiencies were calculated using Eq. (2):

$$E = \text{EF} \times \frac{V_{\text{ac}}}{V_{\text{do}}} \quad (2)$$

where, V_{ac} and V_{do} are the volumes of acceptor and donor phases, respectively.

The matrix effects on ionization of NSAIDs in ESI-MS were evaluated. For this purpose, five PLE extractions were performed under optimized conditions followed by HF-LPME. Then, the extracts were collected and mixed together in a 1 mL vial. Six portions of this extract (each one 15 μL) were transferred into 6 different conical bottom autosampler vials and spiked with 5 μL of standard solution in the solvent (0.1 M ammonium carbonate) of increasing concentrations of NSAIDs. The samples were analyzed applying LC–ESI-MS. The matrix effects were evaluated by comparing the slopes of the calibration curves from the standard addition experiments with those of calibration curves obtained by the analysis of standards in the solvent (0.1 M ammonium carbonate) (Eq. (3)).

$$\text{Matrix effect} = \frac{\text{Slope}_{(\text{matrix curve})} - \text{Slope}_{(\text{standard curve})}}{\text{Slope}_{(\text{standard curve})}} \quad (3)$$

Method detection limits (MDLs) and method quantification limits (MQLs) were determined as the minimum detectable amount of analyte with a signal to noise ratio (S/N) of 3 and 10, respectively. The concentration corresponding to the defined S/N was determined by dividing the standard deviation of the noise area with the slope of the calibration curve, assuming a linear correlation through zero [7]. Overall method precision studies were carried out by spiking sludge samples with 200 ng g^{-1} of each drug for $n = 5$ replicate extraction, preconcentration and analysis cycles, expressing it as the relative standard deviation (RSD) of these replicate measurements.

2.6. LC–ESI-MS analysis

A LC system composed by a HP 1100 Binary pump, a HP 1100 vacuum degasser, an automatic sample injector (Dynamax, Model AL-1A), a C_{18} column (3 μm, 7.5 mm × 2.1 mm, Supelco) and a single quadrupole mass spectrometer (Waters Micromass ZMD Bench top, Manchester, UK) with ESI interface was used. Chromatographic separation of the four analytes in the PHWE–HF-LPME

Table 1
Calibration parameters of LC–ESI-MS for target drugs.

Analyte	Linearity		RSD%		LOD (ng mL^{-1})
	LR (ng mL^{-1})	R^2	Repeatability (n = 5)	Reproducibility (n = 3)	
Ketoprofen	3.9–4000	0.9999	3.6	8.3	2.0
Naproxen	16–4000	0.9999	6.2	4.3	3.9
Diclofenac	3.9–4000	0.9999	3.0	4.0	2.0
Ibuprofen	2.0–4000	0.9999	3.9	3.8	1.0

extract of spiked sludge samples (including matrix components) was achieved by gradient elution starting from 50% B (methanol) in A (10 mM ammonium acetate buffer at pH 4.0), 50–65% B, 0–15 min; 65–85% B, 15–16 min; 85% B, 20 min; 85–50% B, 20–21 min and 5 min post run time (totally 26 min). The flow rate was 0.25 mL min^{-1}. The μL-pick up method was used for injection of 10 μL of sample without sample loss during injection. The autosampler was equipped with a 250 μL syringe, 100 μL sample loop, 15 μL needle tubing volume and 500 μL buffer tubing.

Data acquisition was performed in negative ion mode and MS parameters for the analysis were the following: capillary voltage 3.5 kV, cone voltage 8, 8, 10 and 10 V for ketoprofen, naproxen, diclofenac and ibuprofen, respectively, ion source block temperature 150 °C, desolvation temperature 350 °C, desolvation gas flow 540 L h^{-1}. Selective ion monitoring was used to detect ions with m/z ratios of 253 (ketoprofen), 229 (naproxen), 294 (diclofenac) and 205 (ibuprofen).

3. Results and discussion

3.1. LC–MS method development

After initial tuning of the ESI-MS, some parameters should be optimized using a sample representative of the analytes to be studied. It will usually be found, with the exception of sample cone voltage (which can be increased to induce in-source fragmentation and give structural information), that settings will vary little from one analyte to another. To achieve the best sensitivity and highest S/N for target drugs in SIM mode the sample cone voltage was optimized for both molecular and product ions using direct injection of a mixed standard solution of NSAIDs and performing chromatographic separation. According to the results, cone voltages of 8, 8, 10 and 10 V were selected as optimum values for molecular ions of ketoprofen (m/z = 253), naproxen (m/z = 229), diclofenac (m/z = 294) and ibuprofen (m/z = 205). For SIM analysis of product ions (m/z = 209, 185, 250 and 161) optimum cone voltages were 20, 20, 40 and 20 V for ketoprofen, naproxen, diclofenac and ibuprofen, respectively. After adjusting the MS detector based on these results, two injections of extracts from spiked sludge were performed. The first one was done under the optimum conditions for the molecular ions and the second one was done under the optimum conditions for the product ions. By considering obtained chromatograms for spiked real extracts, it was concluded that with monitoring of molecular ions in cone voltages of 8, 8, 10 and 10 V, respectively, chromatograms with higher S/N and fewer interferences could be achieved.

Direct calibration of LC–ESI-MS was performed using standard solutions ranging from 1 to 4000 ng mL^{-1}. Table 1 shows the calibration parameters of LC–ESI-MS for the target analytes.

3.2. PHWE optimization

To achieve fast and efficient extraction of NSAIDs from treated sludge samples using PHWE, proper instrumental parameters (temperature, number of cycles and flush volume) and a suitable pH of water as the extraction solvent should be selected. Extraction pressure was fixed to 100 bar for all PHWE experiments since the role of the pressure is to maintain water in its liquid phase at the extraction temperature and often the pressure has little or no effect on the extraction efficiency of PHWE [27–30]. In PHWE, separating the static time into several cycles, rather than using one longer cycle, allows for the introduction of fresh solvent for each cycle. The fresh solvent helps to maintain a favorable solvent-sample equilibrium for samples that are heavily loaded or otherwise difficult to extract [31]. Therefore, according to the optimized values in published reports, the static time of 5 min was selected for all PHWE experiments and only the number of cycles was optimized.

Temperature is the main factor which affects the extraction efficiency and selectivity in PHWE [32]. At elevated temperature, the physical advantages such as high diffusion, low viscosity and low surface tension of solvent are achieved. Also, at higher temperature the vapor pressure of compounds will increase and thermal desorption from matrices could increase which can improve the extraction efficiency of PHWE [33]. However, degradation, hydrolysis or oxidation of target compounds can occur at increased temperature. Three extraction temperatures were investigated: 80, 100 and 120 °C. The initial conditions were extraction solvent: ultra-pure water, extraction pressure: 100 bar, 3 cycles and flush volume: 60%. Results showed that by increasing temperature the extraction recoveries for all compounds increase (Fig. 1). Ketoprofen and naproxen were extracted completely from spiked samples at 100 °C but maximum amount of diclofenac and ibuprofen were extracted at 120 °C, 97.4% and 95.5%, respectively. Although, the extraction recoveries obtained at 120 °C were slightly higher than those obtained at 100 °C, we selected 100 °C as extraction temperature to investigate the effect of other param-

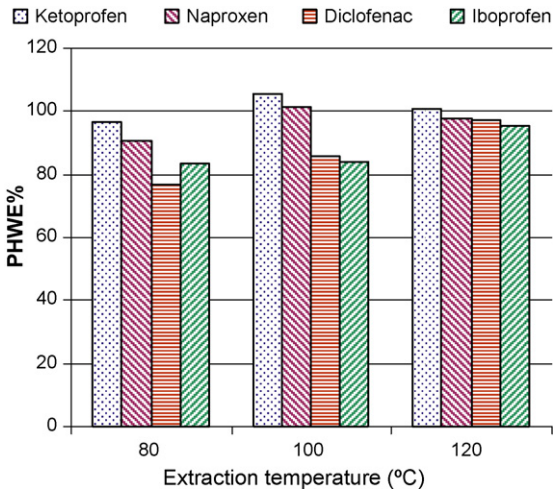

Fig. 1. Effect of extraction temperature on the PHWE spike recoveries of NSAIDs. Conditions: Ultra-pure water as solvent, pressure of 100 bar, static time of 5 min, 3 cycles, 60% flush volume.

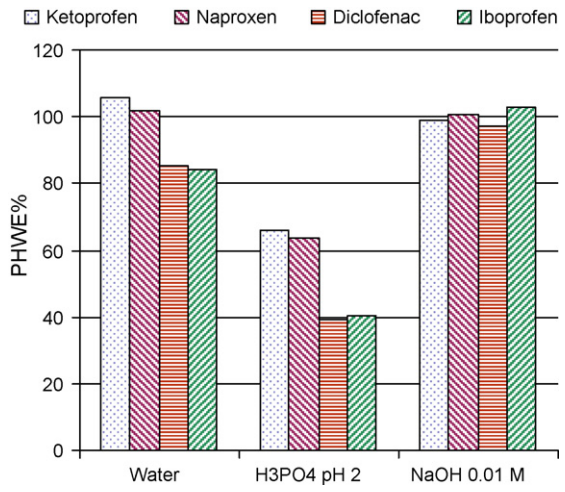

Fig. 2. Effect of extraction pH on the PHWE spike recoveries of NSAIDs. Conditions: temperature of 100 °C, pressure of 100 bar, static time of 5 min, 3 cycles, 60% flush volume.

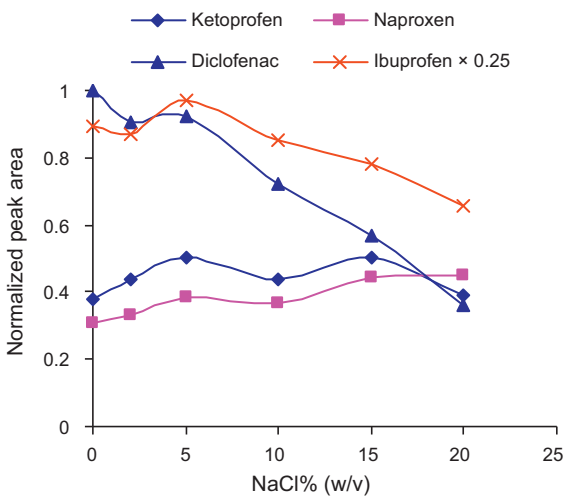

Fig. 3. Effect of NaCl addition on the HF-LPME of NSAIDs in PHWE extract of spiked sludge samples (200 ng g^{-1}). Conditions: 600 rpm stirring rate, sample pH 1.5, 90 min extraction time.

eters to obtain maximum recoveries with less consumption of energy.

Because of the acidic character of NSAIDs, the effect of pH of the extraction solvent on the extraction efficiency was studied. Under elevated temperature, strong acids (e.g. HCl and HNO_3) oxidize the steel components of the extraction cell [34] and are therefore not suitable. Hot phosphoric acid has been reported to enhance the extraction of humic acids and fluoroquinolone antibacterial agents from sewage sludge and soil samples [35]. Thus, a solution of H_3PO_4 at pH 2.0 was selected as an acidic extraction solvent and a 0.01 M NaOH solution as a basic extraction solvent. After PHWE, the pH of the extract for different extraction solvents was measured. The pH for phosphoric acid solution, ultra-pure water and sodium hydroxide were 2.6, 7.0 and 9.7 after extraction, respectively. Fig. 2 shows the results of extraction recoveries for NSAIDs using diluted H_3PO_4 (pH 2.0), ultra-pure water and 0.01 M NaOH as extraction solvents. Ketoprofen and naproxen were extracted completely using ultra-pure water and 0.01 M NaOH as solvent. Results showed that diclofenac and ibuprofen can be extracted up to 100% using 0.01 M NaOH as extraction solvent. In the case of hot phosphoric acid as extraction solvent, low extraction recoveries were obtained. The enhanced extraction efficiencies in 0.01 M NaOH can be explained by the acidic characteristics of NSAIDs (pK_a 4.15–4.91) which are deprotonated and more soluble in aqueous solvents at basic pH. According to the obtained results, 0.01 M NaOH was selected as the optimal extraction solvent.

To investigate the effect of the number of cycles and therefore the total time of each extraction, one, three and five cycles were examined. In our study, we observed a slight raise in extraction efficiency of NSAIDs when the number of cycles was increased from 1 to 5. Obtained extraction efficiencies for ketoprofen, naproxen, diclofenac and ibuprofen after 5 cycles of extraction were 106%, 101%, 104% and 111%, respectively.

The parameter flush volume (flush%) defines the amount of solvent to be used by the ASE for extracting a sample, i.e. the amount of solvent to flush through the cell following the static heating step expressed as a percentage of the cell volume. When more than one cycle is specified, the flush volume is divided among the cycles [31]. Flush volumes of 30%, 60% and 90% of the extraction cell volume were examined. The maximum extraction recoveries were achieved at 60% flush volume (101–111%) and remained constant by increasing the flush volume up to 90% (101–109%).

3.3. HF-LPME optimization

To separate NSAIDs from the matrix components of PHWE extracts in order to reduce the ionization interferences in ESI-MS analysis, and to concentrate analytes increasing the sensitivity of determinations and lowering the limits of quantification, three-phase HF-LPME was applied. The effects of the pH of PHWE extracts before clean-up (donor phase pH), salt addition and extraction time were investigated by the "one variable at a time" method. Ammonium carbonate buffer (0.1 M) was used as acceptor phase because it is a volatile buffer suitable for ESI-MS and provides a suitable pH (9.5) for analytes to become deprotonated and trapped in the acceptor phase. According to the results of our previous work [9], di-n-hexyl ether was used as organic phase.

For the study of the effect of donor phase pH on the extraction efficiencies of target drugs, different pH values in the range of 1–6 were tested. The extractions were performed at 600 rpm for 90 min. The results showed that by decreasing pH from 6 to 4 the extraction efficiencies of NSAIDs increased, remained constant till pH 2 and increased slightly at pH lower than 2. Finally, pH 1.5 was chosen as the optimum value for the rest of the experiments.

For HF-LPME in aqueous samples, the addition of salts (such as NaCl or Na_2SO_4) can decrease the solubility of analytes and enhance their partitioning into the organic phase (salting-out effect). For this aim, the optimization experiments were carried out by dissolving solid NaCl into the PHWE extract in the range of 0–20% (w/v). It was demonstrated that salt addition had a significantly negative effect on the extraction efficiencies of diclofenac and ibuprofen (Fig. 3). Precipitation of some matrix components in PHWE extracts after salt addition can probably lead to co-precipitation of target drugs which decrease the free concentration of these drugs in the aqueous phase. The highest decrease in HF-LPME extraction efficiency was observed for diclofenac which has the highest log P_{ow} (4.51) and lowest water solubility (2.37 mg L^{-1}). In the case of ketoprofen and naproxen no considerable change in extraction efficiencies were observed by adding NaCl. Based on these results, further extractions were carried out without salt addition.

In order to study the influence of the extraction time, several HF-LPME extractions were performed on the PHWE extracts of spiked sludge samples with extraction times in the range of 30–270 min. The results showed that by increasing the extraction time from 30 to 120 min the extraction efficiency of target analytes increased considerably (Fig. 4). At longer extraction times a slight increase in

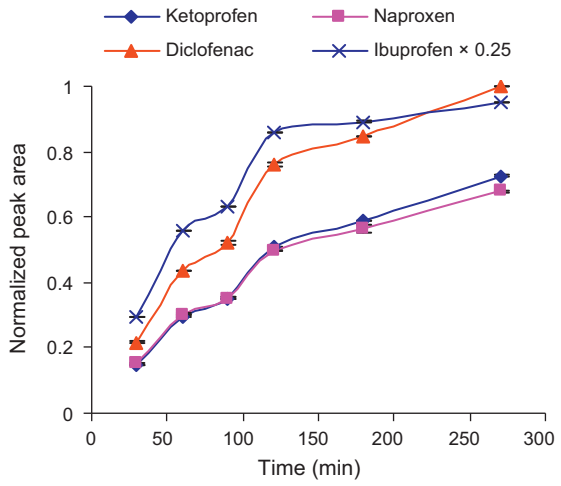

Fig. 4. Effect of extraction time on the HF-LPME of NSAIDs in PHWE extract of spiked sludge samples (200 ng g^{-1}). Conditions: 600 rpm stirring rate, sample pH 1.5.

extraction efficiency could be observed for all NSAIDs. The HF-LPME extraction time chosen for further experiments was 120 min.

3.4. Method validation

The recoveries of selected pharmaceuticals obtained for spiked samples were in the range of 101–109% at the optimum conditions of PHWE. Recoveries over 100% could be due to method errors or intrinsic inhomogeneity of solid samples which has been reported in sludge samples [7,18,36]. Recoveries obtained for spiked samples could overestimate the efficiency of the method for the native target analytes. Spiked analytes are not exposed to the same active sites as native contaminants nor are they adsorbed in the same manner. Therefore, the recoveries obtained in this way may not be representative for real samples. Because of the limitations in diffusion and kinetics of the sorption process [37], spiked analytes will always be less retained in environmental matrices than the native ones. Therefore, to obtain the recoveries for the native NSAIDs in sludge samples, fifteen consecutive PHWE extractions were performed at optimum conditions on a 0.5 g sludge sample containing the highest levels of native drugs. The obtained peak areas for target analytes were corrected for different matrix effects in the HF-LPME step. It can be realized by considering the color of the extracts that the number and the concentration of interferences, which co-extract with analytes, decrease from the first extraction to the last one which can affect the HF-LPME efficiency of analytes. The PHWE recoveries were calculated for the first extraction using Eq. (1). Fig. 5 shows typical recovery graphs obtained for diclofenac and ibuprofen. Extraction recoveries with and without matrix effect correction are shown in Table 2. The results showed that there are some errors in the range of −0.7% to −3.7% in the calculated PHWE recoveries without matrix effect correction because of the negative effects of matrix components in earlier PHWE extracts. The extraction recoveries obtained for the native analytes were 38.9, 59.8, 90.3 and 47.8 for ketoprofen, naproxen, diclofenac and ibuprofen, respectively. The large differences in the extraction recoveries of the spiked and native organic compounds in sludge samples are consistent with the idea that the native ones are formed with the sample matrix and are therefore located in less accessible locations throughout the matrix particles. Native recoveries were used to calculate the real amounts of NSAIDs in sludge samples after standard addition calculations.

Enrichment factors for the clean-up step were evaluated for three sludge samples with different sampling dates and the average values were calculated for each compound. The average enrichment

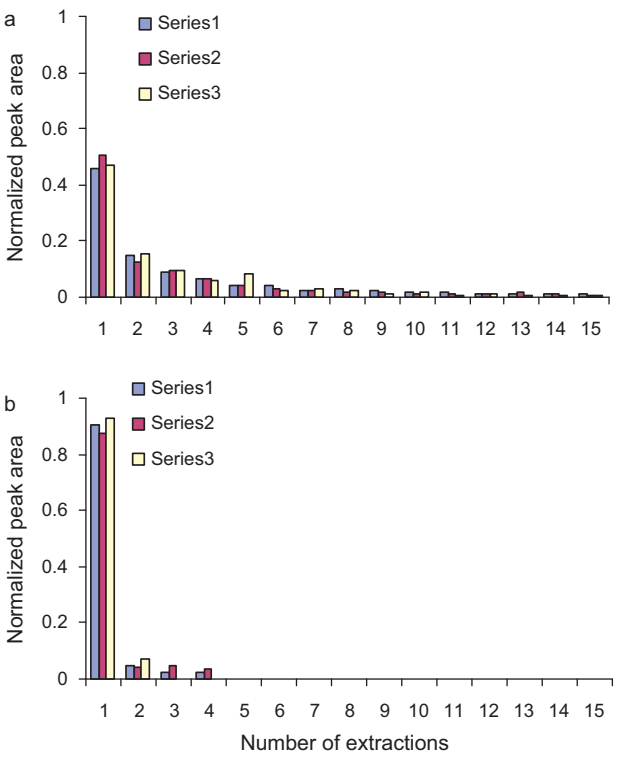

Fig. 5. PHWE recoveries of (a) ibuprofen and (b) diclofenac for the fifteen consecutive extractions of 0.5 g non-spiked sludge samples (in triplicate).

factors were 1213, 947, 1058 and 1212 for ketoprofen, naproxen, diclofenac and ibuprofen, respectively (Table 2). Absolute extraction efficiencies for target compounds in the clean-up step were in the range of 23.6–30.4%. By considering the corresponding standard deviations (see Table 2), it can be concluded that extraction recoveries of less polar compounds in the HF-LPME step are more influenced by sample matrix variations. This observation is similar to the salt addition effect (Section 3.3) in which there was a considerable change in the extraction recoveries of less polar compounds (diclofenac and ibuprofen) during the clean-up step.

The performance of LC–ESI-MS analysis is strongly affected by ionizable impurities originating from the matrix (e.g. natural organic matter, salts, ion-pairing agents, non-target contaminants, etc.) that can interfere with the ionization presses. This may result in a signal suppression or enhancement leading to low sensitivity and inaccurate results. These effects can be more extensive when the matrix is more complex such as e.g. sewage sludge. Therefore, to ensure the reliability of obtained results it is advisable to evaluate the matrix effect as a part of the method validation. The results of the matrix effect experiments are shown in Table 3 and Fig. 6. Changes in calibration sensitivity with the presence of matrix components were considered as matrix effects. The changes were −8.9%, −3.8%, +0.2% and +14.6% for naproxen, ketoprofen, ibuprofen and diclofenac, respectively. Comparison of the observed matrix effects with previously published values [7,17,18] shows that using HF-LPME for the preconcentration and clean-up of NSAIDs after pressurized liquid extraction can be an efficient solution to reduce the ion suppression/enhancement in ESI-MS analysis. Using pressurized hot water as extraction solvent instead of pressurized hot organic solvents (e.g. methanol, acetonitrile, etc.) can also be another reason for the relatively low ion suppression/enhancement in the current method. When using organic solvents, higher amounts of interfering contaminants are dissolved during the extraction period, resulting in a higher matrix effect [16].

Table 2
Recovery results for PHWE and HF-LPME of NSAIDs in sewage sludge.

Analytes	PHWE recoveries% (RSD%)			HF-LPME	
	Spike	Native U.C.[a]	Native C.[b]	EF[c] (RSD%)	E^d (RSD%)
Ketoprofen	102 (11.4)	36.9 (8.5)	38.9 (9.1)	1213 (3.4)	30.3 (3.4)
Naproxen	103 (11.1)	56.8 (1.4)	59.8 (2.0)	947 (9.9)	23.6 (9.9)
Diclofenac	101 (9.4)	89.7 (3.5)	90.3 (3.1)	1058 (11.1)	26.4 (11.1)
Ibuprofen	109 (7.4)	44.1 (5.6)	47.8 (5.2)	1212 (12.8)	30.3 (12.8)

[a] Uncorrected values.
[b] Corrected values.
[c] Enrichment factor.
[d] Extraction efficiency.

Table 3
Figures of merit of PHWE–HF-LPME–LC–MS analysis of NSAIDs and the concentrations found in real samples.

Analytes	MDL ($ng\,g^{-1}$)	MQL ($ng\,g^{-1}$)	Matrix effect%	Concentration in sludge samples ($ng\,g^{-1}$)				RSD% ($n=5$)	
				April	June	August	October	Repeatability	Reproducibility
Ketoprofen	3.7	12.2	−3.8	89.6	57.7	60.4	51.3	11.4	13.1
Naproxen	1.7	5.8	−8.9	14.1	12.2	7.7	10.5	11.1	11.5
Diclofenac	0.4	1.5	+14.6	21.2	22.9	18.8	13.7	9.4	9.1
Ibuprofen	1.4	4.7	+0.2	588	541	304	313	7.4	9.3

MDLs and MQLs for the NSAIDs were in the range of 0.4–3.7 $ng\,g^{-1}$ and 1.5–12.2 $ng\,g^{-1}$ in dried sludge samples, respectively. The precision of the proposed method was evaluated in terms of repeatability (RSD% <11.4, $n=5$) and reproducibility (RSD% <13.1, $n=5$) for spiked sludge samples (see Table 3). Table 4 shows a comparison between the characteristics of the proposed method with those of the methods described in the literature for the determination of NSAIDs in sewage sludge samples. The considerably lower ion suppression/enhancement and minimum use of organic solvents (a few microliters of DHE) in the sample preparation step are two highlighted advantages of the proposed method in comparison with the previously published works. Other characteristics are comparable.

3.5. Application of the method

The developed method was applied for the determination of NSAID pharmaceuticals in treated sewage sludge samples from Källby WWTP (Lund, Sweden) from different months of the year (April, June, August and October, 2010). Standard addition after

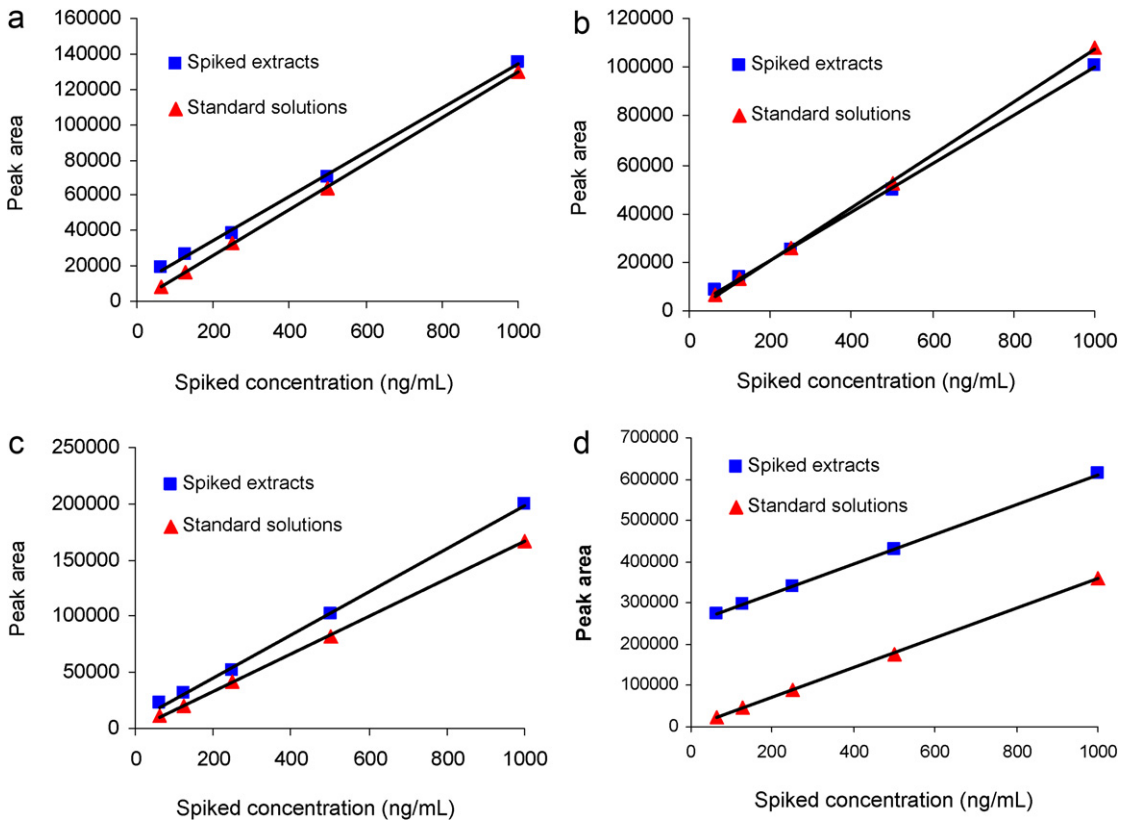

Fig. 6. Comparison of calibration curves for spiked HF-LPME extracts and standard solutions of NSAIDs for the evaluation of matrix effect on the ionization of the target compounds.

Table 4
Comparison of the characteristics for the methods described in the literature with the proposed method for the determination of NSAIDs in sewage sludge samples.

Extraction technique (clean-up technique)	Solvent, volume (mL)	Analytical determination	Sample amount (g)	MQL (ng g^{-1})	Spike recoveries (%)	RSD (%)	Ion suppression/ enhancement (%)	References
PLE (SPE)	Methanol:water (1:2), 22	LC–MS/MS	1.0	51–96	59.9–102.3	1–5.7	−50 to −90	[7]
USE (SPE)	Methanol–acetone (7–2), 9	LC–UV and LC–FLD	1.5	3.69–192	41.6–115	0.1–23	–	[8]
PLE (–)	Phosphoric acid:methanol (1:1), 40	LC–ESI-MS	5.0	22–32	68–82	7	–	[6]
PLE (SPE)	Methanol:water (1:2), 22	LC–MS/MS	1.0	0.24–3.13	81.4–125	1–5	14–79	[17]
USE (SPE)	Methanol–acetone (6–4), 10	LC–MS/MS	0.5	20–50	49–76	–	–	[15]
MAE (DME[a]–SPE)	Water	GC–MS	0.5	15–22	80–105	12–18	–	[16]
PLE (SPE)	Methanol:water (1:1), 53	LC–MS/MS	1.0	7–30[b]	105–120	–	−41 to −56	[18]
PHWE (HF-LPME)	0.01 M NaOH	LC–ESI-MS	0.5	1.5–12.2	38.9–90.3[c]	7.4–11.4	−3.8 to +14.6	This method

[a] Dispersive matrix extraction.
[b] MDLs.
[c] Native recoveries.

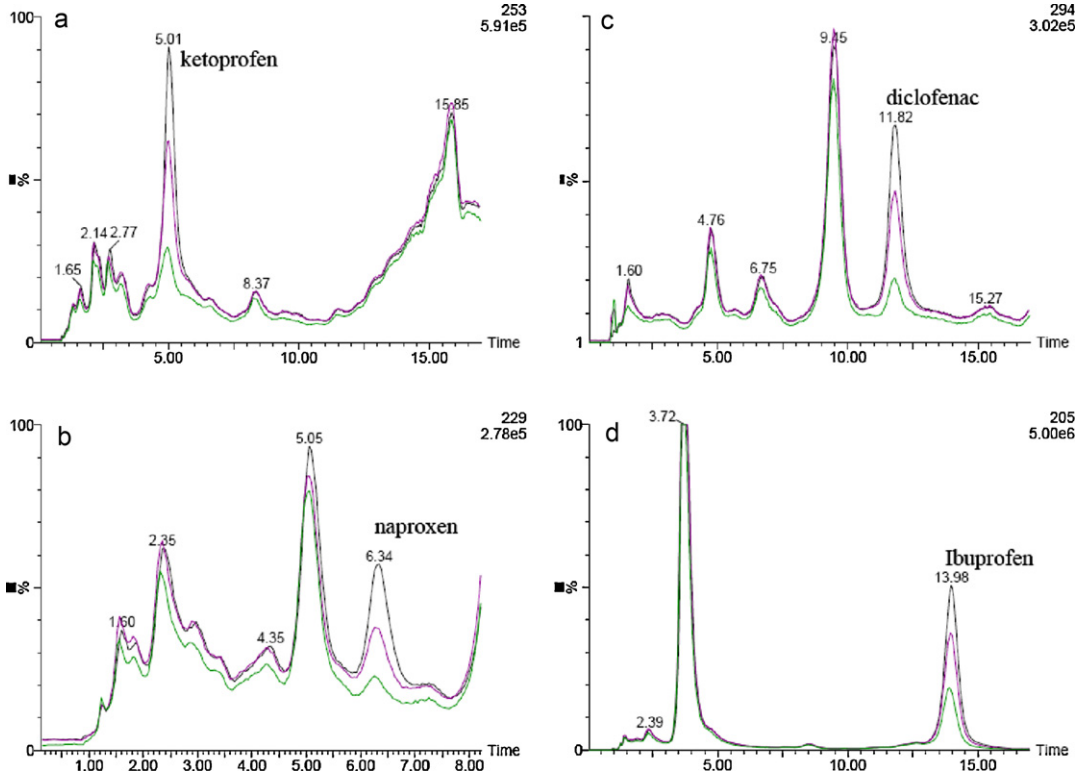

Fig. 7. SIM LC–ESI-MS chromatograms for the standard addition analysis of NSAIDs in PHWE extracts of 0.5 g April dried sludge sample in optimized conditions. Spiked concentrations were: (a) 0.0, 0.4 and 0.8 ng mL^{-1}, (b) 0.0, 0.08 and 0.24 ng mL^{-1}, (c) 0.0, 0.2 and 0.4 ng mL^{-1} and (d) 0.0, 1.0 and 2.0 ng mL^{-1}.

PHWE was applied for the quantification of target analytes in PHWE extracts and the PHWE recoveries obtained for the native NSAIDs in sludge samples were used to calculate the amount of these drugs in dried sludge samples (ng g^{-1}). Fig. 7 shows the LC–ESI-MS chromatograms of NSAIDs in April sludge samples after sample preparation. In our analysis, all of the studied pharmaceuticals were detected in all sludge samples of different sampling dates. The highest concentration levels were found for ibuprofen in the range of 304–588 ng g^{-1} decreasing from April to August. Maximum amounts of NSAIDs (total concentration: 712 ng g^{-1}) were found in April sludge samples and minimum amounts (389 ng g^{-1}) were found in October sludge samples. These changes in the concentration of NSAIDs could be explained by less consumption of NSAIDs in summer due to good weather conditions or a more efficient biodegradation in the biological treatment step during warmer months. The lowest concentration levels in sludge samples were found for naproxen in the range from 7.7 to 14.1 ng g^{-1}.

4. Conclusions

A new sample preparation procedure was developed for the quantification of selected acidic pharmaceuticals (ketoprofen, naproxen, diclofenac and ibuprofen) in treated sludge samples applying pressurized hot water extraction (PHWE) followed by clean-up and LC–ESI-MS analysis. Three-phase hollow fiber liquid-phase microextraction (HF-LPME) as a clean-up method decreased the matrix effect and produced relatively high enrichment factors in the extract of the sewage sludge. Matrix effects and enrichment factors in the range of −8.9% to +14.6% and 947–1213 were obtained, respectively. PHWE recoveries were obtained for both spiked and

native NSAIDs in treated sludge samples. There was a considerable difference between spike recoveries (101–109%) and the recoveries obtained for the native drugs (38.9–90.3%) in sludge samples. Native recoveries were used for the quantification of target compounds in dried sludge samples. The developed sample preparation method was successfully applied to sewage sludge samples from Källby WWTP (Lund, Sweden) during four different months (April, June, August and October 2010) and the total measured concentrations of target drugs were in the range of 7.7–588 ng g^{-1}. These results agree well with a previous study where the content of the four NSAIDs were determined to 29–138 ng g^{-1} in samples from the same WWTP collected in October 2009 [25]. By considering the obtained characteristics, it can be concluded that the developed method is environmentally friendly with efficient clean-up for the determination of NSAIDs in solid samples. This sample preparation technique should be useful for the quantification of other ionizable target molecules from solid matrices such as soil, sediments, sludge and plant materials.

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