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Enantioselective determination of representative profens in wastewater by a single-step sample treatment and chiral liquid chromatography–tandem mass spectrometry

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ABSTRACT

This manuscript describes, for the first time, the simultaneous enantioselective determination of ibuprofen, naproxen and ketoprofen in wastewater based on liquid chromatography tandem mass spectrometry (LC–MS/MS). The method uses a single-step sample treatment based on microextraction with a supramolecular solvent made up of hexagonal inverted aggregates of decanoic acid, formed in situ in the wastewater sample through a spontaneous self-assembly process. Microextraction of profens was optimized and the analytical method validated. Isotopically labeled internal standards were used to compensate for both matrix interferences and recoveries. Apparent recoveries for the six enantiomers in influent and effluent wastewater samples were in the interval 97–103%. Low method detection limits (MDLs) were obtained (0.5–1.2 ng L^{-1}) as a result of the high concentration factors achieved in the microextraction process (i.e. actual concentration factors 469–736). No analyte derivatization or evaporation of extracts, as it is required with GC–MS, was necessary. Relative standard deviations for enantiomers in wastewater were always below 8%. The method was applied to the determination of the concentrations and enantiomeric fractions of the targeted analytes in influents and effluents from three wastewater treatment plants. All the values found for profen enantiomers were consistent with those previously reported and confirmed again the suitability of using the enantiomeric fraction of ibuprofen as an indicator of the discharge of untreated or poorly treated wastewaters. Both the analytical and operational features of this method make it applicable to the assessment of the enantiomeric fate of profens in the environment.

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1. Introduction

Enantioselective environmental analysis of pharmaceuticals is an emerging area with important gaps in knowledge [1,2]. The importance of enantiomeric analysis of pharmaceuticals for proper environmental risk assessment is easy to understand if we consider that (a) approximately 56% of the drugs currently in use for human and veterinary treatment are chiral, (b) many of them reach the environment owing to inefficient removal in wastewater treatment plants, and (c) enantiomers usually differ in their biological properties and consequently their toxicity [3]. More novel applications of enantioselective analysis of chiral drugs include the use of enantiomeric ratios as markers of biologically-mediated degradation during wastewater treatment, water contamination with sewage, environmental biotransformation and sewage epidemiology [1,4].

Among chiral pharmaceuticals, profens are non-steroidal anti-inflammatory drugs prescribed in high quantities over the world (e.g. ibuprofen is one of the top-ten drugs sold worldwide [5]). Because of their insufficient removal rates during wastewater treatment, profens such as ibuprofen, naproxen and ketoprofen (Table 1) are frequently detected at the ng L^{-1} level in both treated wastewater and surface water [2]. They have scarce tendency to adsorb on sludge and sediments because they are present in the ionized form in the environment (pK_a 3–5). Ibuprofen and ketoprofen are marketed as racemic mixtures, although their therapeutic effect resides almost exclusively in the *S*-enantiomers [6], whereas naproxen is only distributed as *S*-enantiomer since *R*-naproxen is hepatotoxic.

Despite the frequent occurrence of profens in water bodies and significantly different pharmacological activity of their enantiomers, only limited research has been undertaken on their enantioselective fate in the environment [7–9]. Enantiomeric composition of profens can be altered after their administration owing to human or animal metabolism, biological wastewater

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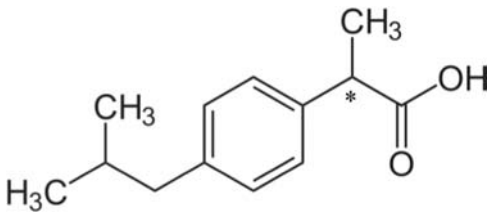
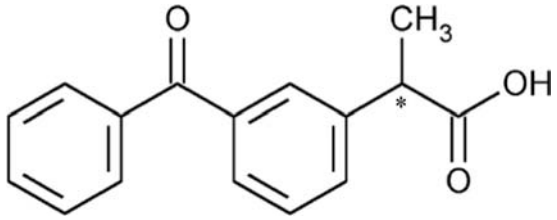
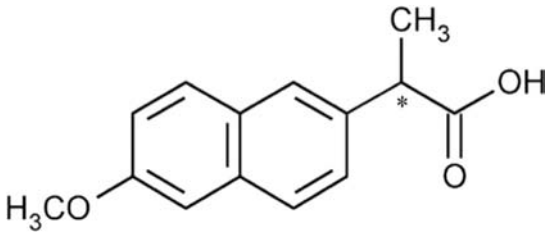
Table 1
Chemical structures, octanol–water partition coefficients (log $K_{o/w}$) and ionization constants (pK_a) for profens.

Profens	Chemical structure[a]	Log $K_{o/w}$[b]	pK_a[b]
(*R*)/(*S*)-Ibuprofen		3.97	4.91
(*R*)/(*S*)-Ketoprofen		3.12	4.45
(*R*)/(*S*)-Naproxen		3.18	4.15

[a] The asterisks denote the chiral center.
[b] *Source*: http://toxnet.nlm.nih.gov.

treatment and biological degradation processes in the environment. Results available to date reveal that the principal enantiomer for ibuprofen in the environment is *S*-ibuprofen, which is around 110 times more potent than *R*-ibuprofen to humans and probably to other vertebrates and invertebrates [2]. Thus determination of enantiomeric composition of profens in wastewater treatment plants (WWTPs) and the environment is essential to understand and predict the mechanisms governing their fate, toxicity and environmental risk.

Only a few methods are available for the determination of the enantiomeric composition of profens, mainly ibuprofen and naproxen, in WWTPs and surface waters. All of these methods are based on the solid-phase extraction (SPE) of the aqueous samples, derivatization of the polar enantiomers, cleanup by a second SPE, and determination by gas chromatography coupled to mass spectrometry (GC–MS) [7–12] or tandem mass spectrometry (CG–MS/MS) [13–15]. The main drawback of these GC-based methods is the need for complex and time-consuming steps that may lead to analyte losses and reduced sample throughput [4]. To the best of our knowledge, only a few methods have been reported to analyze polar chiral drugs in the environment by LC–MS (e.g. β-blockers and drugs of abuse [16–21]) but none of them included profens. Recently, an interesting method for multi-residue enantiomeric separation of chiral drugs on a Chirobiotic V stationary phase has been reported, however because of the acidity of profens ($pK_a < 5$, Table 1), this column failed in their enantiomeric resolution [22].

This paper presents the development of a method for the determination of the enantiomeric composition of ibuprofen, ketoprofen and naproxen in wastewater based on supramolecular solvent (SUPRAS) microextraction and chiral LC–MS/MS on a (*R*)-1-naphthylglycine 3,5-dinitrobenzoic acid stationary phase. Selection of these profens was based on their occurrence and ubiquity in the environment as well as their high human consumption. To the authors' knowledge, this is the first reported LC–MS method for the determination of enantiomers of profens in wastewater.

A SUPRAS made up of nanostructured aggregates of decanoic acid, spontaneously formed in wastewater samples under addition of the amphiphile and THF [23], was used for isolating and concentrating profens. Fig. 1 shows a scheme of the structure of this solvent. It consists of aqueous cavities surrounded by the carboxylic groups with the hydrocarbon chains dissolved in THF and packed in a hexagonal arrangement. Since profens contain donor (OH) and acceptor (O) groups in their structures (see chemical structures of profens in Table 1), it is expected that their solubilisation in the decanoic acid-based nanostructures occurs through a mixed-mode mechanism (viz hydrogen bonding in the surfactant polar group and hydration water, and dispersion forces in the surfactant hydrocarbon chain). On the other hand, the size of the aqueous cavities can be tailored by controlling the THF: water ratio in the wastewater where decanoic acid self-assembles and, consequently, this solvent has the potential to exclude the extraction of macromolecules such as proteins, humic acids, etc. So the SUPRAS can simplify sample treatment by combining both isolation and concentration of profens and sample cleanup.

The final objective of this research was to develop a straightforward, simple and rapid method for the enantiomeric analysis of profens in order to help to understand their environmental fate and risk. Below, the main results of this study are described and discussed.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent-grade and were used as supplied. Decanoic acid (DeA) and ammonium acetate were obtained

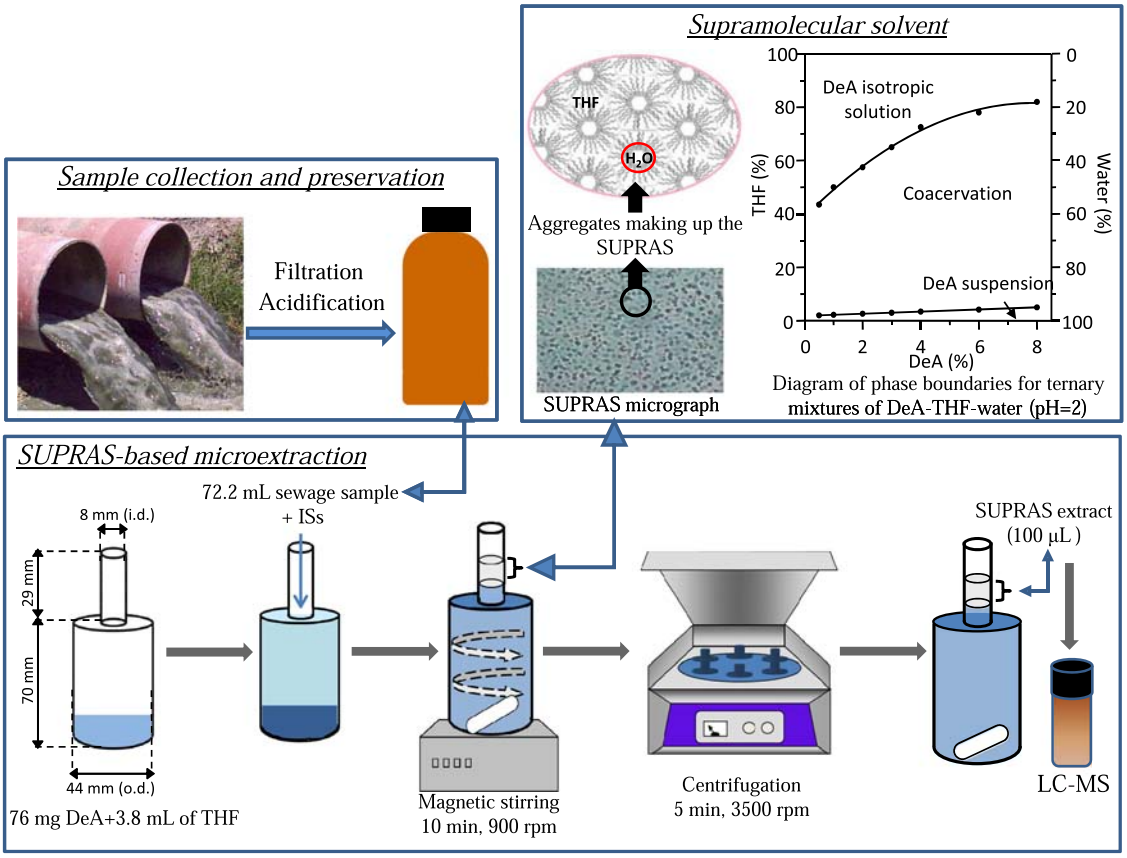


Fig. 1. Scheme of the generalized sample treatment proposed for the microextraction of (*R*)/(*S*)-ibuprofen, (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen in sewage and structure and phase diagram for the supramolecular solvent used for microextration.

from Fluka (Buchs, Switzerland), (*R*)/(*S*)-ibuprofen (≥ 98% purity) and (*R*)/(*S*)-ketoprofen (≥ 98% purity) were supplied by Sigma (Saint Louis, MO, USA) whereas (*R*)/(*S*)-naproxen (≤ 100% purity) was obtained from European Pharmacopoeia Reference Standard (Strasbourg, France). The isotopically labelled compounds (*R*)/(*S*)-ibuprofen (U-Ring-$^{13}C_6$, 99% purity), (*R*)/(*S*)-ketoprofen ($^{13}C_6$, 99% purity) and (*R*)/(*S*)-naproxen (^{13}C, $^{2}H_3$, 98% purity), acquired from ^{ALSA}CHIM (Strasbourg, France), were used as internal standards (IS). All profen standard contained *R*- and *S*-enantiomers in a 50:50 ratio, which was confirmed by LC–UV. Both individual stock standard (1 g L^{-1}) and isotopically labelled internal standard (40 mg L^{-1}) solutions of racemic profens were prepared on a weight basis in methanol and stored under dark conditions at 4 °C. They were stable for at least 2 months Working solutions containing mixtures of profens (5 mg L^{-1} of each enantiomer) or ISs (0.75 mg L^{-1} of each enantiomer of (*R*)/(*S*)-ibuprofen and 0.15 mg L^{-1} of each enantiomer of (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen) were prepared in methanol and stored at 4 °C. Tetrahydrofuran (THF) and hydrochloric acid were purchased from Panreac (Barcelona, Spain) and LC-grade methanol and acetonitrile from HiperSolv Chromanorm (Fontenay-Sous-Bois, France). Ultra-high-quality water was obtained from a Milli-Q water purification system (Millipore, Madrid, Spain).

2.2. Apparatus

The LC–MS system used was ahybrid triple quadrupole/linear ion trap Applied Biosystems MSD Sciex 4000QTRAP (Applied Biosystems, Foster City, CA, USA) coupled to a liquid chromatograph Agilent HP 1200 Series (Agilent Technologies, Palo Alto, CA, USA) with a TurboIonSpray (TIS) interface. All data were acquired and processed using the Analyst 1.5.1 Software. The analytical column used for separation of profen enantiomers was a Sumichiral OA-2500 (stationary phase: (*R*)-1-naphthylglycine and 3.5-dinitrobenzoic acid, particle size 5 µm, i.d. 4.6 mm, length 250 mm) from Sumika Chemical Analysis Service (Osaka, Japan). It was preceded by a guard column (Chirex 3005 from Phenomenex, Torrance, California) with the same chiral selector, particle size and internal diameter to those of the analytical column and 30 mm length.

A multi-position magnetic stirrer RO 10 power IKAMAG® from IKA®-Werke GmbH & Co. KG. (Staufen, Germany) and a digitally regulated centrifuge Mixtasel equipped with an angle rotor 4 × 100 mL (cat. 7001326) from JP-Selecta (Abrera, Spain) were used for sample treatment. The volume of supramolecular solvent was measured with a digital calliper from Medid Precision, S.A. (Barcelona). Centrifuge tubes with narrow necks were designed by authors in order to make easier the measurement and collection of the solvent after extraction. Pobel S.A. (Madrid, Spain, web page: www.pobel.com) constructed them from commercial heavy-duty glass cylindrical centrifuge tubes with round-bottom (cat. 159080) but reducing the diameter from a specified height (Fig. 1 shows a scheme picture and dimensions of these tubes).

2.3. Determination of the enantiomeric composition of profens in wastewater

2.3.1. Sample collection and preservation

Grab influent and effluent wastewater samples were collected from three different sewage treatment plants in Córdoba (South of Spain) in May 2014. All of them utilize an activated sludge technology and mainly receive domestic wastewater. Samples were collected in dark glass containers and were transported to the lab and immediately filtered through 7–9 µm paper filter (Filter-lab Barcelona, Spain) and 0.45 µm nylon membranes (Millipore HNWP, Bedford, MA, USA) for removing suspended

solids. The pH of the filtrated samples was adjusted to 2 by the addition of concentrated hydrochloric acid, and they were stored at 4 °C until analysis.

2.3.2. SUPRAS-based microextraction

Decanoic acid (76 mg) was dissolved in THF (3.8 mL) into a 100 mL glass centrifugation tube. Then, a sewage sample (72.2 mL) spiked with 26 μL of the working solution of ISs (see Section 2.1) was added. After sealing the tube with parafilm to avoid THF evaporation, the mixture was magnetically stirred for 10 min at 900 rpm to favor analytes extraction and then, centrifuged at 3500 rpm for 5 min to accelerate phase separation. The supramolecular extract obtained after centrifugation (about 100 μL) was standing at the top of the solution in the narrow neck of the tube. An aliquot of 50 μL of the extract was withdrawn using a microsyringe and transferred to an autosampler amber glass vial with insert and it was subject to LC–MS. Fig. 1 shows a scheme of the sample treatment and microextraction procedure.

2.3.3. LC–MS/MS analysis

Enantiomers of profens were separated and quantified by using chiral LC coupled with a TurboIonSpray source operating in the negative ion mode and a hybrid triple quadrupole/linear ion trap analyzer operating in the selected reaction monitoring (SRM) mode. The mobile phase consisted of 90% tetrahydrofuran and 10% ammonium acetate (50 mM) in methanol working at a variable flow: 0.5 mL min^{-1} from 0 to 18 min and then 1.2 mL min^{-1}. The injection volume used was 10 μL. The temperature for the analytical and guard column was kept at 25 °C. The eluates from the analytical column were diverted by the switching valve to waste from 0 to 14 min in order to prevent the mass spectrometer from the entrance of other matrix components or decanoic acid. The TIS source and analyzer conditions giving the highest relative intensity were: curtain gas (N_2) 30 psi; nebulizer gas 65 psi; turbo gas 30 psi; temperature of the turbo gas 425 °C; ion spray voltage −4500 V; entrance potential −5 V; collision gas 3.0×10^{-5} Torr. Unit resolution was used for both first and third quadrupoles. Table 2 shows the quantifier and qualifier ions used for each native profen and ISs. Only one SRM transition could be recorded for ibuprofen because of its poor fragmentation. Declustering potential, collision energy and collision cell exit potential parameters were optimized for each analyte (Table 2). Calibration curves were constructed from standard solutions in methanol containing the target enantiomers in the ranges 0.4–4000, 1.0–4000 and 1.2–4000 μg L^{-1} for (*R*)/(*S*)-ibuprofen, (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen respectively, and constant concentrations of ISs (200 μg L^{-1} of each enantiomer of (*R*)/(*S*)-ibuprofen and 40 μg L^{-1} of each enantiomer of (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen).The concentration of the target analytes in the extract were calculated from calibration curves obtained by plotting peak area ratios (A/A_{IS}; A = peak area of individual enantiomers and A_{IS} = peak area of the corresponding IS) versus the concentration of analytes injected.

3. Results and discussion

3.1. Optimization of chiral LC

Some studies were undertaken with the aim of obtaining the best chromatographic resolution for the simultaneous separation of the enantiomers of the three selected profens while keeping reasonable retention times and enough sensitivity in LC–MS/MS. For this purpose, a (*R*)-1-naphthylglycine and 3,5-dinitrobenzoic acid chiral stationary phase was used. The selection of this stationary phase was based on the good resolution that it provides for the enantiomeric separation of individual profens [e.g. (*R*)/(*S*)-ibuprofen, (*R*)/(*S*)-ketoprofen, etc.] in chiral LC/UV detection [24–26] and the compatibility with MS of the mobile phases used (30–80 mM ammonium acetate in methanol). It was checked that ammonium acetate was essential for enantioselectivity but, unfortunately, this additive also caused ionization suppression for profens in the turbo ion spray interface (e.g. the sensitivity for (*R*)/(*S*)-naproxen decreased around 20 fold by increasing 10 times ammonium acetate concentration). Retention times for profens were also dependent on ammonium acetate concentration and unacceptable long retention times for (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen were obtained working at low additive concentrations (e.g. 5 mM).

In order to keep the concentration of ammonium acetate to the minimum required for enantioselectivity (e.g. 5 mM) while obtaining reasonable retention times, stronger mobile phases made up of THF and methanol at different solvent percentage ratios (THF:methanol from 0:100 to 90:10) and flow rates (0.5–1.2 mL min^{-1}) were tested. Enantiomeric resolutions {Rs, calculated from the equation $R_s = (t_{R1} - t_{R2})/[2(w_1 + w_2)]$, where t_{R1} and t_{R2} are the retention times and w_1 and w_2 the peak widths for the *R*- and the *S*-enantiomers, respectively} of 1.4, 1.7 and 2.8 were obtained for (*R*)/(*S*)-ibuprofen, (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen, respectively, by using a mobile phase consisting of 90% tetrahydrofuran and 10% ammonium acetate (50 mM) in methanol, working at a variable flow: 0.5 mL min^{-1} from 0 to 18 min and then 1.2 mL min^{-1}. Fig. 2A shows a representative chromatogram obtained from a standard solution containing racemic profens.

3.2. Microextraction of profens

3.2.1. SUPRAS description

The supramolecular solvent proposed for the microextraction of profens was synthesized in situ in the wastewater sample by addition of decanoic acid (DeA) in THF. Water promoted the self-assembly of DeA and caused the spontaneous formation of oily droplets (i.e. coacervates droplets) that flocculated through the formation of conglomerates of individual droplets and finally led to creaming and separation as a new liquid phase (i.e. coacervate or supramolecular solvent). The term creaming is defined as the macroscopic separation of a dilute emulsion into a highly concentrated emulsion, in which interglobular contact is important, and a continuous phase, under the action of gravity or a centrifugal field [27]. Only the protonated form of decanoic acid (pK_a 4.8 ± 0.1) was able to produce the SUPRAS, so pH values below 4 were required for its synthesis.

Table 2
Quantifier and qualifier ion transitions and MS parameters used to determine profens in wastewater samples.

Profen	Quantifier transition	Qualifier transition	MS Parameters		
			Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)
(*R*)/(*S*)-Ibuprofen	205 > 161	205 > 161	−55	−10	−3
(*R*)/(*S*)-Ketoprofen	253 > 209	253 > 197	−35	−10	−11
(*R*)/(*S*)-Naproxen	229 > 185	229 > 170	−50	−10	−5
(*R*)/(*S*)-$^{13}C_6$ Ibuprofen	211 > 167	211 > 167	−40	−10	−13
(*R*)/(*S*)-$^{13}C_6$ Ketoprofen	259 > 215	259 > 199	−20	−12	−37
(*R*)/(*S*)-$^{13}C_6$, $^2H_3$Naproxen	233 > 189	233 > 170	−35	−10	−9

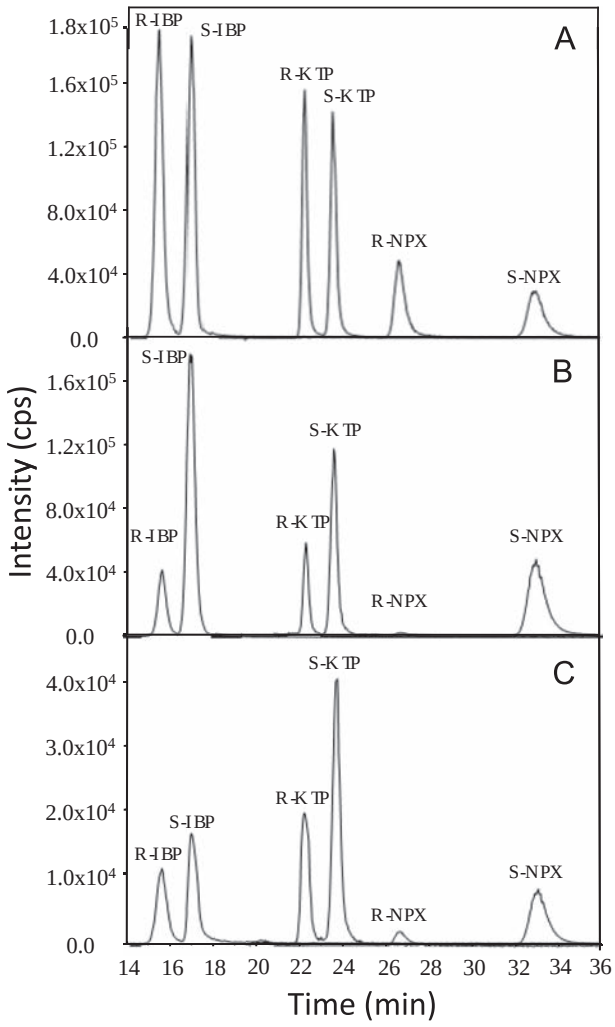


Fig. 2. LC–MS/MS selected ion chromatograms obtained from (A) a standard solution containing 500 μg L^{-1} of (*R*)/(*S*)-ibuprofen (IBP), (*R*)/(*S*)-ketoprofen (KTP) and (*R*)/(*S*)-naproxen (NPX) and the (B) influent and (C) effluent samples collected in the WWTP 2.

Fig. 1 shows a micrograph of the coacervate droplets making up the SUPRAS, obtained by light microscopy, as well as a scheme of the hexagonal packaging of decanoic acid in these droplets. This figure also shows the relative concentrations of THF, water and DeA where the SUPRAS was produced (i.e. the coacervation region). Above this region, the SUPRAS and THF: water bulk solution became miscible, the boundary depending on the DeA concentration. Below the coacervation region, DeA became insoluble. Only concentrations of DeA within the range of analytical interest were investigated (i.e. below 10%).

The volume of SUPRAS obtained increased linearly with the amount of DeA and exponentially with the percentage of THF in the bulk solution. This volume can be accurately predicted from the following, previously derived, equation [28]:

$$y = (1.04 \pm 0.02)\, ae^{(0.0473 \pm 0.0009)\, b} \quad (1)$$

where y is the volume of SUPRAS in μL, a the amount of DeA in mg and b the THF percentage (v/v).

The composition of the SUPRAS was THF dependent; the higher the content of THF in the bulk solution the higher the percentages of THF and water in the SUPRAS, this leading to the synthesis of solvents progressively containing decreased concentrations of DeA. On the other hand, SUPRASs of identical composition were obtained by increasing the amount of DeA in the bulk solution although the volume of SUPRAS obtained linearly increased according to Eq. (1).

3.2.2. Optimization

Optimization of the microextraction process was carried out by extracting wastewater fortified with a racemate concentration of 200 ng L^{-1} for (*R*)/(*S*)-ibuprofen, (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen under a variety of experimental conditions: 0.1–0.5% (w/v) of DeA, 5–20% (v/v) of THF, pH 1–4, NaCl (10^{-3}–1 M), extraction temperature (20–55 °C), stirring time (2–30 min). The final volume for the solution was 76 mL. Unfortified samples were analysed for these profens and the concentration found subtracted from those of fortified samples. Extractions were carried out according to the procedure specified in Section 2.3.2 and varying each variable in turn while keeping the other constant. ISs were added just prior injection in order to correct any potential matrix effects and ensure accurate quantitation during optimization. Experiments were made in triplicate. Selection of the optimal conditions was based on the actual concentration factors (ACFs) obtained for each of the studied profens. They were calculated from the equation: $(0.01 \times R(\%) \times \mathrm{PVR})$ where R is the recovery and PVR is the phase volume ratio (i.e. ratio of waste water volume over the SUPRAS volume). The aim was to get enough sensitivity without the need for evaporation of extracts, thus increasing sample throughput, saving time and cost and reducing analyte losses.

SUPRASs of different compositions were prepared from a constant concentration of DeA (0.1%, w/v) and variable percentages of THF (5–20%, v/v). The minimum concentration of DeA required to get enough volume of SUPRAS (about 100 μL) for profen analysis by LC–MS/MS was tested in order to obtain maximal ACFs. The concentration of DeA in the SUPRASs thus produced varied from 0.76 to 0.38 mg μL^{-1} by increasing the percentage of THF from 5 to 20%. The influence of these SUPRASs on the extraction of racemic profens, along with the respective ACFs, is shown in Table 3. Absolute recoveries were not affected by solvent composition (i.e. % THF), the values being dependent on the polarity of the profen (Table 1). Quantitative extraction was only obtained for ibuprofen ($\log K_{ow} = 3.97$). Actual concentration factors greatly decreased as the THF in the bulk solution increased as a result of the exponential dependence of the volume of SUPRAS produced with THF (Eq. (1)). A percentage of 5% THF was selected for further studies.

The influence of the volume of SUPRAS on the extraction of profens was investigated by increasing the amount of DeA. Table 3 shows the results obtained for percentages of DeA from 0.15 to 0.5% (w/v). Recoveries for the more polar profens gradually increased as the volume of solvent did, but as it was expected, the ACFs progressively decreased as a result of the decrease in the respective phase volume ratios. Because of the possibility to use ISs for correcting extraction recoveries, a SUPRAS made up from DeA and THF percentage of 0.1 and 5% respectively were selected as optimal in order to get the maximal possible ACFs. In this way, ACFs of 736, 469 and 483 were obtained for (*R*)/(*S*)-ibuprofen, (*R*)/(*S*)-ketoprofen and(*R*)/(*S*)-naproxen, respectively.

As it has been previously mentioned, a value of pH below the pK_a of DeA (i.e. 4.8) is required in the bulk solution to form the SUPRAS. So, the extraction of profens will be carried out in their neutral form (see pK_a values in Table 1). In order to determine any influence of the pH on recoveries and ACFs for profens, extractions were carried out in the pH interval from 1 to 4. Recoveries kept constant in this interval for ibuprofen and ketoprofen, but decreased for naproxen (around 60%) at pH 4 owing to its stronger acidity (pK_a 4.15). No meaningful influence of the pH on ACFs was observed. So, for experimental convenience, the pH of the samples previously adjusted to 2 for their preservation, was maintained during extraction.

Ionic strength and temperature could affect the aggregation process driving SUPRAS formation and the distribution equilibria of the analytes between the two liquid phases involved in SUPRAS-based microextractions, and therefore both variables could influence on the extraction yield and concentration factors provided by

Table 3
Mean percent recoveries and actual concentration factors obtained for the racemic mixtures of ibuprofen, ketoprofen and naproxen as a function of the percentage of tetrahydrofuran (THF) and decanoic acid (DeA).

Variable	Recovery ± s[a] (%)			Actual concentration factor (ACF) ± s[a]		
	(*R*)/(*S*)-Ibuprofen	(*R*)/(*S*)-Ketoprofen	(*R*)/(*S*)-Naproxen	(*R*)/(*S*)-Ibuprofen	(*R*)/(*S*)-Ketoprofen	(*R*)/(*S*)-Naproxen
THF[b] (%)						
5	102 ± 3	65 ± 2	67 ± 1	740 ± 20	470 ± 20	487 ± 8
10	99 ± 2	70.1 ± 0.4	69 ± 2	530 ± 10	378 ± 2	372 ± 9
15	98 ± 2	65.3 ± 0.2	68.1 ± 0.5	392 ± 7	262 ± 1	273 ± 2
20	96 ± 1	58 ± 2	61 ± 1	287 ± 3	174 ± 5	182 ± 4
DeA[c] (%)						
0.15	102 ± 2	74 ± 1	74 ± 2	490 ± 10	354 ± 7	357 ± 9
0.2	101.7 ± 0.1	79.2 ± 0.7	79.1 ± 0.3	367.1 ± 0.3	286 ± 2	286 ± 1
0.3	99.6 ± 0.7	87.7 ± 0.1	84.8 ± 0.2	242 ± 2	213.3 ± 0.2	206.2 ± 0.5
0.5	101 ± 2	98 ± 2	93 ± 3	147 ± 4	143 ± 3	135 ± 4

pH=2;
[a] Standard deviation (n=6).
[b] DeA=0.1%;
[c] THF=5%.

SUPRASs. Electrolytes have been proved to cause a slight increase in the recoveries and a small decrease in the concentration factors obtained for mecoprop and dichlorprop when they were extracted using a dodecanoic acid-based SUPRAS [29]. Increasing the ionic strength of the wastewater sample by addition of NaCl (10^{-3}–1 M) or varying the temperature for extraction from 20 to 55 °C did not affect profens extraction efficiencies or concentration factors. The time for magnetic stirring during extraction was investigated in the interval 2–30 min at 900 rpm. Equilibrium conditions were reached after 5 min but reproducibility increased about 4-fold as the extraction time increased to 10 min, so this time was selected as optimal.

3.3. Method validation

3.3.1. Recoveries and matrix effects

Three sets of calibration were prepared to evaluate method recovery (R), absence or presence of matrix effects (ME) and overall process efficiency (PE) [30]. The first set (A) consisted of eight standards in methanol at concentrations of each enantiomer in the ranges 0.4–4000, 1.0–4000 and 1.2–4000 μg L^{-1} for (*R*)/(*S*)-ibuprofen, (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen, respectively. The second set (B) was prepared by spiking the profen enantiomers (same concentration range as used for set 1) in SUPRAS aliquots obtained after influent wastewater extraction according to the procedure in Section 2.3.2. In set 3 (C), enantiomers were spiked before extraction into the influent wastewater sample and subjected to the whole procedure. Matrix effects, absolute recoveries and process efficiency were evaluated by comparing the slopes of the calibration curves obtained by sets 1 and 2, sets 2 and 3 and sets 1 and 3, respectively, according to:

$$\mathrm{ME}\ (\%) = \frac{B}{A} \times 100 \quad (2)$$

$$R\ (\%) = \frac{C}{B} \times 100 \quad (3)$$

$$\mathrm{PE}\ (\%) = \frac{(\mathrm{ME} \times R)}{100} = \frac{C}{A} \times 100 \quad (4)$$

The ME calculated in this manner may be referred to as an absolute matrix effect; percentages higher than 100 indicate ion enhancement, while percentages lower than 100 are indicative of ion suppression.

Table 4 shows the values obtained for ME, R and PE for the different profen enantiomers investigated. It was checked that recoveries were quantitative for both *R*- and *S*-Ibuprofen enantiomers and they varied in the range 65–69% for the rest of enantiomers. These values were in agreement with the results obtained during the optimization process under the selected experimental conditions (e.g. ACFs from 469 to 736 in Table 3) and confirmed both the similar behavior of enantiomers from each profen regarding extraction and the independence of recoveries of enantiomer concentration in the range evaluated. Quantitative recoveries could be obtained for (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen in samples requiring lower actual concentration factors (e.g. around 134–141, see Table 3).

Table 4
Matrix effect (ME), recovery (R) and process efficiency (PE) data for profen enantiomers in an influent wastewater sample.

Profen enantiomer	ME ± s[a] (%)	R ± s[a] (%)	PE ± s[a] (%)
(*R*)-Ibuprofen	36 ± 2	103 ± 6	37 ± 2
(*S*)-Ibuprofen	38 ± 1	100 ± 7	38 ± 2
(*R*)-Ketoprofen	98 ± 3	69 ± 4	68 ± 3
(*S*)-Ketoprofen	95 ± 3	67 ± 4	64 ± 4
(*R*)-Naproxen	98 ± 4	68 ± 3	67 ± 2
(*S*)-Naproxen	99 ± 4	65 ± 4	64 ± 3

[a] Standard deviation.

Matrix effects only were observed for ibuprofen, the signal suppression observed being similar for both enantiomers (Table 4). Because of the high mobile phase strength required for enantiomeric separation [i.e. 90% tetrahydrofuran and 10% ammonium acetate (50 mM) in methanol], the most polar and medium polar matrix components were expected to elute quickly and affect mainly ibuprofen. High selectivity was obtained for both (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen. The overall process efficiency was in the range 37–68%.

The use of isotopically labelled standards to compensate for both matrix effects and extraction efficiencies was investigated by comparing the slopes of calibration curves obtained from standards (n=8) of native profen enantiomers (0.4–4000, 1.0–4000 and 1.2–4000 μg L^{-1} for (*R*)/(*S*)-ibuprofen, (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen) in methanol and influent wastewater sample, the latter fortified before extraction. Both, methanol and wastewater samples were spiked with a constant concentration of IS (final concentration of 200 μg L^{-1} of each enantiomer of (*R*)/(*S*)-ibuprofen and 40 μg $L^{-1}$of each enantiomer of (*R*)/(*S*)-ketoprofen and (*R*)/(*S*)-naproxen). The concentration of the target analytes in the extract were calculated from calibration curves obtained by plotting peak area ratios (A/A_{IS}; A=peak area of individual

enantiomers and A_{IS} = peak area of the corresponding IS) versus the concentration of analytes injected.

No statistically significant difference between both slopes was revealed by applying a Student's test [31]. The experimental *t*-values obtained for *R*-ibuprofen, *S*-ibuprofen, *R*-ketoprofen, *S*-ketoprofen, *R*-naproxen and *S*-naproxen were 0.54, 2.94, 1.55, 1.85, 2.26 and 2.84 respectively. These values were below the critical t value (3.05, significant level = 0.01). So, the isotopically labelled standards proposed were able to compensate for both matrix effects and recoveries for the profens investigated.

3.3.2. Linearity and sensitivity

Method detection limits (MDLs) were calculated from six independent complete analyses (experimental procedure in Section 2.3) of both influent and effluent wastewater samples by using a signal-to-noise ratio of 3. Since no typical matrix low-level material could be obtained, an estimate of the background signal was made at a representative part of the readout, adjacent to the analyte signal, in the analyte-containing sample. MDLs were 0.7, 0.7, 09, 0.8, 1.2, 1.1 and 0.5, 0.5, 0.8, 0.7, 1.1, 1.1 ng L^{-1} for *R*-ibuprofen, *S*-ibuprofen, *R*-ketoprofen, *S*-ketoprofen, *R*-naproxen and *S*-naproxen, in influent and effluent wastewater samples, respectively. MDLs for enantiomers were in a narrow range (0.5–1.2 ng L^{-1}) and they were practically independent of the type of sample. High sensitivity was achieved as a result of the high concentration factors provided by the proposed method.

Quantification of profen enantiomers based on peak area was performed from standards in methanol ($n=8$) using the internal standard approach. Isotopically labelled enantiomers were used as internal standards (see Section 2.3.3. for concentration ranges and procedure). The linear range was confirmed by visual inspection of the plot residuals versus analyte amount; the residuals were randomly scattered within a horizontal band and a random sequence of positive and negative residuals was obtained. Correlation between peak areas and enantiomer concentrations was determined by linear regression and $1/x$ weighted calibration. Correlation coefficients were in the range 0.9997–0.99991 for all enantiomers indicating good fits.

3.3.3. Precision

The precision was assessed by applying the whole analytical process to six independent unfortified influent and effluent sewage samples. The values expressed as relative standard deviations were 2.1/1.3% for (*R*)/(*S*)-ibuprofen, 3.3/1.0% for (*R*)/(*S*)-ketoprofen and 5.3/1.2% for (*R*)/(*S*)-naproxen in influent and 4.5/3.2% for (*R*)/(*S*)-ibuprofen, 3.1/2.4% for (*R*)/(*S*)-ketoprofen and 7.8/1.7% for (*R*)/(*S*)-naproxen in effluent. The concentrations found for enantiomers in the selected wastewater samples were in a wide range (i.e. 31 to 2026 ng L^{-1} in influents and 10 to 327 ng L^{-1} in effluents) and were always higher for *S*-enantiomers compared to *R*-enantiomers. The precision was satisfactory, with RSD values below 8% for all the enantiomers.

3.4. Determination of profen content and enantiomeric fraction in wastewater

Unfortified and fortified influent and effluent samples from three WWTPs, all of them located in Córdoba province (South of Spain), were analysed to prove the suitability of the method to determine profen enantiomers. Table 5 shows the concentrations obtained, the enantiomeric fractions calculated and the relative recoveries found for each enantiomer. All these values were expressed as the mean of three independent determinations, besides their corresponding standard deviations. EFs were defined as [32]:

$$EF = [S-\text{enantiomer}]/[S-\text{enantiomer}]+[R-\text{enantiomer}]) \quad (5)$$

EF equals 1 or 0 in the case a single enantiomer form and 0.5 in the case of racemates. Fortification of wastewater with profen enantiomers was made at a different level for influents (300 ng L^{-1}) and effluents (100 ng L^{-1}).

Both enantiomers of all three profens were quantified in the analysed samples. The measured concentrations were all within an expected range based on previously reported non-enantioselective and enantioselective analysis of profens in wastewater [2,4,22]. Removal of profen enantiomers ranged between 65 and 92% during wastewater treatment except for *S*-naproxen with removal percentages varying in the interval 25–51%.

The values calculated for EF were also all consistent with previous reports [4,8,33]. Thus, as usual, ibuprofen was found at very high concentrations in influents with enantiomeric excess of the pharmacologically active (*S*)-enantiomer. It is known that (*R*)-ibuprofen undergoes chiral inversion during human metabolism that resulting in an excess of (*S*)-ibuprofen in urine. EF for ibuprofen in influents ranged between 0.63 and 0.68 that indicating that

Table 5
Concentration found, apparent recoveries and enantiomeric fractions (EF) for (*R*)/(*S*)–IBP, (*R*)/(*S*)–KTP and (*R*)/(*S*)–NPX in wastewaters influents and effluents.

Sample	Concentration found[a] ± s[b] (ng L^{-1}) Recovery[a] ± s[b] (%)						EFa ± sb		
	(*R*)–IBP	(*S*)–IBP	(*R*)–KTP	(*S*)–KTP	(*R*)–NPX	(*S*)–NPX	IBP	KTP	NPX
WWTP 1									
Influent	415 ± 3	1643 ± 50	191 ± 9	220 ± 3	29.4 ± 0.2	3172 ± 37	0.798 ± 0.006	0.54 ± 0.02	0.9908 ± 0.0002
	100.8 ± 0.9[c]	99 ± 2[c]	99 ± 2[c]	99 ± 2[c]	101 ± 1[c]	100 ± 1[c]			
Effluent	83 ± 2	172 ± 8	28 ± 1	60 ± 3	22 ± 1	481 ± 17	0.67 ± 0.02	0.683 ± 0.002	0.9555 ± 0.0002
	101.4 ± 0.7[d]	103 ± 7[d]	98 ± 1[d]	103 ± 3[d]	99 ± 3[d]	103 ± 3[d]			
WWTP 2									
Influent	257 ± 5	957 ± 12	240 ± 8	510 ± 5	21 ± 1	1049 ± 13	0.788 ± 0.003	0.680 ± 0.007	0.9808 ± 0.0008
	101 ± 2[c]	101 ± 1[c]	99 ± 1_c	100 ± 1_c	99 ± 3_c	99,5 ± 0.3[c]			
Effluent	42 ± 2	72 ± 2	86 ± 3	177 ± 4	14 ± 1	175 ± ± 3	0.629 ± 0.008	0.673 ± 0.008	0.927 ± 0.005
	102 ± 2[d]	101 ± 2[d]	100 ± 2[d]	99 ± 2[d]	101 ± 1[d]	100.6 ± 0.5[d]			
WWTP 3									
Influent	256 ± 3	1536 ± 47	87.9 ± 0.9	121 ± 2	18 ± 1	1747 ± 3	0.857 ± 0.002	0.5797 ± 0.0003	0.9899 ± 0.0006
	100 ± 1[c]	99 ± 1[c]	98 ± 2[c]	99 ± 3[c]	99 ± 2[c]	99 ± 1[c]			
Effluent	98 ± 4	210 ± 5	24 ± 2	38 ± 1	8.7 ± 0.4	241 ± 7	0.68 ± 0.02	0.61 ± 0.03	0.96 ± 0.002
	102 ± 3[d]	100 ± 2[d]	104 ± 4[d]	101 ± 2[d]	97 ± 3[d]	100 ± 2[d]			

[a] Mean of three independent determinations.
[b] Standard deviation.
[c] Fortified with 300 ng L^{-1} of each enantiomer.
[d] Fortified with 100 ng L^{-1} of each enantiomer.

(*S*)-ibuprofen was preferentially degraded compared to (*R*)-ibuprofen [4]. Because of the similarity of ibuprofen EF values in effluents and surface waters and the widespread presence of this compound in the environment, ibuprofen EFs have been proposed as indicators of the discharge of untreated or poorly treated wastewater [8].

Values of EFs for ketoprofen were quite near in both influents and effluents because of their similar rate of degradation [33]. The EFs in influents confirmed that minimal enantioselectivity occurs for this drug, administered as racemate, during human metabolism [34]. Regarding naproxen, it is administered as an enantiomerically pure formulation of (*S*)-naproxen due to the known hepatotoxicity of (*R*)-naproxen and, similar to ketoprofen, minor chiral inversion occurs during human metabolism. So, EFs for naproxen in influents were near 1.Although EFs for naproxen in effluents indicated a minor preferential degradation for the (*S*)-enantiomer during wastewater treatment, no significant differences were found for any of the samples analyzed compared to EFs in effluents. As it has been previously reported [33], EF values for each enantiomer in influents and effluents were relatively stable parameters (e.g. independent of drug concentration).

Apparent recoveries for fortified influent and effluent samples were all in the range 97–103% that proving the suitability of the internal standards proposed to compensate for both matrix effects for ibuprofen and recoveries for naproxen and ketoprofen. Relative standard deviations for both the concentrations found and the recoveries obtained were all in the interval 0.1–7%. Fig. 2 depicts, as an example, the chromatograms obtained from the analysis of an (*B*) influent and (*C*) effluent sample collected in the WWTP 2.

4. Conclusions

The need for enantioselective analysis of drugs in order to understand their environmental fate has been widely recognized in the last few years as an important issue [1,2,4]. In addition, enantiomeric fractions of drugs have been proposed as useful indicators in different applications [1,4]. Most drugs are polar and consequently, LC should be the technique of choice over GC for these types of analysis.

In this manuscript, LC–MS/MS has been proposed for the first time for the simultaneous enantioselective analysis of the most ubiquitous profens in wastewater. The method detection limits obtained by LC–MS were in the same range as GC–MS [15]. The sample treatment proposed allows quick and simple microextraction of profen enantiomers while delivering accurate and precise data and extends the range of eco-friendly methods in labs.

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