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Enantioselective analysis of ibuprofen, ketoprofen and naproxen in wastewater and environmental water samples

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ABSTRACT

A highly sensitive and reliable method for the enantioselective analysis of ibuprofen, ketoprofen and naproxen in wastewater and environmental water samples has been developed. These three pharmaceuticals are chiral molecules and the variable presence of their individual (*R*)- and (*S*)-enantiomers is of increasing interest for environmental analysis. An indirect method for enantioseparation was achieved by the derivatization of the (*R*)- and (*S*)-enantiomers to amide diastereomers using (*R*)-1-phenylethylamine ((*R*)-1-PEA). After initial solid phase extraction from aqueous samples, derivatization was undertaken at room temperature in less than 5 min. Optimum recovery and clean-up of the amide diastereomers from the derivatization solution was achieved by a second solid phase extraction step. Separation and detection of the individual diastereomers was undertaken by gas chromatography–tandem mass spectrometry (GC–MS/MS). Excellent analyte separation and peak shapes were achieved for the derivatized (*R*)- and (*S*)-enantiomers for all three pharmaceuticals with peak resolution, R_s is in the range of 2.87–4.02 for all diastereomer pairs. Furthermore, the calibration curves developed for the (*S*)-enantiomers revealed excellent linearity ($r^2 \geq 0.99$) for all three compounds. Method detection limits were shown to be within the range of 0.2–3.3 ng L^{-1} for individual enantiomers in ultrapure water, drinking water, surface water and a synthetic wastewater. Finally, the method was shown to perform well on a real tertiary treated wastewater sample, revealing measurable concentrations of both (*R*)- and (*S*)-enantiomers of ibuprofen, naproxen and ketoprofen. Isotope dilution using racemic D_3-ibuprofen, racemic D_3-ketoprofen and racemic D_3-naproxen was shown to be an essential aspect of this method for accurate quantification and enantiomeric fraction (EF) determination. This approach produced excellent reproducibility for EF determination of triplicate tertiary treated wastewater samples.

1. Introduction

2-Arylpropionic acids (2-APAs) are a group of non-steroidal anti-inflammatory drugs (NSAIDs) including ibuprofen, ketoprofen and naproxen, also commonly known as 'profens'. These commonly used drugs possess anti-inflammatory and analgesic activities due to their ability to inhibit cyclooxygenase enzymes that promote inflammation [1]. Their therapeutic effect has been reported to reside almost exclusively in their *S* enantiomers (eutomers) rather than their *R* enantiomers (distomers) [2]. For example, (*S*)-ibuprofen has been reported to be 160 times more active that its antipode [3]. Accordingly, it is necessary to know the enantiomeric compositions of these drugs in order to accurately determine their potencies. Such knowledge generally requires enantioselective chemical analysis.

In addition to understanding variable pharmacological characteristics of chiral profens, there is emerging interest in enantioselective analysis of these chemicals in wastewater and environmental water samples [4,5]. It has been proposed that accurate determination of enantiomeric compositions in these matrices may provide insights to environmental fate and degradation processes of these chemicals, as well as information pointing to the nature and sources of environmental pollution [6].

A number of techniques have been used to describe enantiomeric compositions of chiral substances in aqueous samples (for a review see Hashim et al. [6]). However, the most appropriate of these is generally considered to be the enantiomeric fraction (EF), commonly defined as shown in Eq. (1) [7,8].

$$\mathrm{EF} = \frac{[S\text{-enantiomer}]}{[S\text{-enantiomer}] + [R\text{-enantiomer}]} \quad (1)$$

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Enantioselective analysis of chiral chemicals is hindered by the fact that enantiomer pairs have the same physical properties and thus cannot generally be distinguished or separated by physical separation techniques such as gas chromatography or liquid chromatography. To achieve enantioselective separation, the most common approaches involve transformation of enantiomers to diastereomers (with two chiral centers), which tend to have different physical properties to each other. Such chromatographic separations of chiral chemicals are generally classified as being either 'direct' or 'indirect' enantioseparation methods.

Direct enantioseparation methods are based on the temporary formation of diastereomers, by the interaction of the chiral analyte and a second chiral species, either in a stationary or mobile chromatographic phase. For example, enantiomers can be separated by temporary formation of diastereomers on a chiral stationary phase with an achiral mobile phase [9,10]. Alternatively, diastereomers may be temporarily formed by the addition of chiral mobile phase additives and separated on an achiral stationary phase [11,12].

Indirect enantioseparation is achieved by chemical derivatization of chiral analytes with enantiomerically pure chiral derivatizing reagents to form diastereomers, which may then be physically separated using traditional achiral chromatographic techniques [13,14]. The strengths and weaknesses for each of these direct and indirect approaches tend to be in terms of the time required, final product purity and chemical processing [15,16].

This paper presents the adaptation of a chiral derivatization process for the indirect enantioseparation of three important profens (ibuprofen, naproxen and ketoprofen) in wastewater and environmental water samples. The chiral derivatization reaction was originally described by Bjorkman [17] and the derivatized enantiomers of ibuprofen, naproxen and ketoprofen were separated by gas chromatography by Carlson and Gyllenhaal [18]. While this procedure has been successfully used for the analysis of these chemicals in urine samples [19], the trace quantities expected in wastewater and the environment have required improved sample extraction and clean-up, more sensitive mass-spectral determination, improved control of EF variabilities introduced during the derivatization reactions and more robust techniques for quantification.

The chiral derivatizing reagent (CDR) selected for this method was enantiomerically pure 1-phenylethylamine ((*R*)-1-PEA) [18,20–23], since it leads to the formation of amide derivatives, known for their stability due to the rigidity of the amide bond [19]. Further reported advantages of this CDR include short reaction times (less than 3 min) at room temperature for the production of volatile derivatives suitable for gas chromatographic analysis [19]. However, in order to efficiently derivatize profens with (*R*)-1-PEA, it is necessary to undertake a two-step process with initial formation of an activated dione species (a mixed anhydride) by reaction with ethylchloroformate (ECF). This mixed anhydride may then undergo amidation with (*R*)-1-PEA to form the diastereomeric derivatives. This overall two-step derivatization process has been shown not to be enantiospecific [18], however, previous studies have reported interconversions between (*R*)- and (*S*)-profen enantiomers to occur during this process, thus disrupting the enantiomeric composition or EF [22,24]. A similar difficulty encountered with many chiral derivatization methods is the possibility of racemization of the CDR, which may occur during synthesis, storage or the derivatization reaction [24,25].

A wide variety of internal standards have been previously employed for enhanced quantification of enantioselective analysis of environmental samples. These include achiral internal standards such as triphenylamine [26] and hexachlorocyclobenzene [5] and single enantiomer internal standards such as (+)-levabunolol [27,28]. In some cases, racemic mixtures of chiral internal standards have been used, but the individual enantiomers have not been used to directly quantify corresponding analyte enantiomers [4,29]. However, in the current study, it is shown that the use of enantiomerically specific internal standard calibration is necessary for accurate quantification accounting for enantiomeric disruptions during derivatization. For this purpose we have used racemic isotopically labeled ibuprofen, naproxen and ketoprofen standards. The molecular structures of the three chiral profens and their corresponding isotope standards are presented in Table 1.

2. Experimental

2.1. Chemicals and consumables

Racemic ibuprofen, racemic ketoprofen, enantiomerically pure (*S*)-ibuprofen (99%), (*S*)-naproxen (99%), (*S*)-ketoprofen (98%), mirex, (*R*)-1-phenylethylamine (PEA) (99.5%), triethylamine (TEA) and ethyl chloroformate (ECF) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Racemic (α-methyl-D_3)-ibuprofen (D_3-ibuprofen), (α-methyl-D_3)-naproxen (D_3-naproxen) and (α-methyl-D_3)-ketoprofen (D_3-ketoprofen) were purchased from CDN Isotopes Inc., Canada. HPLC grade acetonitrile and methanol were purchased from Ajax Finechem (Tarron Point, NSW, Australia). Analytical grade ethyl acetate was purchased from Fisher Scientific, Australia. Kimble culture tubes (13 mm i.d. × 100 mm) were purchased from Biolab (Clayton, Vic., Australia). Two sizes of Oasis hydrophilic–lipophilic balance (HLB) solid phase extraction cartridges (3 cc, 60 mg and 1 cc, 10 mg) were purchased from Waters (Rydalmere, NSW, Australia). Whatman filter papers (0.75 μm) were purchased from Millipore, Australia. 1 L Wheaton amber narrow mouth bottles with polypropylene caps were purchased from Sigma–Aldrich (Sydney, Australia).

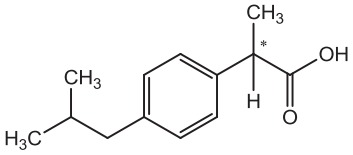
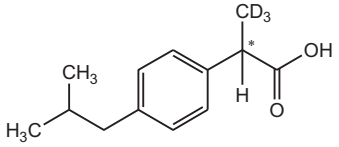
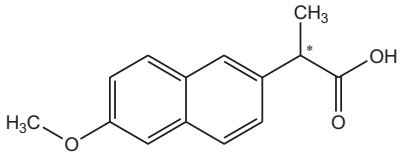
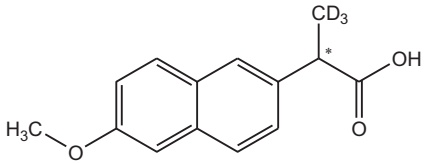
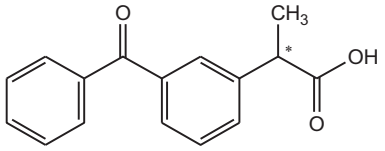
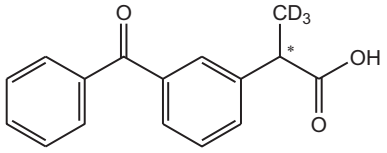
2.2. Preparation of standards, reagents and calibration solutions

Stock solutions of racemic ibuprofen, racemic ketoprofen, (*S*)-ibuprofen, (*S*)-naproxen and (*S*)-ketoprofen were made up at 2 mg mL^{-1} in acetonitrile. Working solutions of (*S*)-ibuprofen, (*S*)-naproxen and (*S*)-ketoprofen were prepared from the stock solutions at appropriate final concentrations of 0.001, 0.01, 0.1, 1 and 10 μg mL^{-1} with acetonitrile. Racemic D_3-ibuprofen, D_3-naproxen and D_3-ketoprofen were used as internal standards and were prepared at concentrations of 1 and 10 μg mL^{-1} in acetonitrile. Racemic ibuprofen and racemic ketoprofen were also prepared at 0.1, 1 and 10 μg mL^{-1} concentrations in acetonitrile. Mirex standard solutions, to be used as a second internal standard for some experiments, were prepared by dissolving 20 mg of mirex in 10 mL of toluene. (*R*)-1-PEA solution was prepared at 0.5 M in methanol. TEA (50 mM) and ECF (60 mM) solutions were prepared in acetonitrile. All prepared reagents were stored at 4 °C until use.

2.3. Sample preparation

All water samples were collected or prepared in 1 L amber narrow mouth bottles with polypropylene caps. Water samples used in these experiments included ultra pure laboratory-grade water, drinking water (from a regular drinking water tap), and surface water (from a local pond). Furthermore, we wished to include wastewater samples in the method validation process. However, since it was not possible to obtain municipal wastewater samples that could be assured to have no background concentrations of profens (even if they be below analytical detection limits), synthetic wastewater samples were obtained from a laboratory-scale membrane bioreactor (MBR) operating on a synthetic feed solution as previously described [30]. Thus the validation samples included synthetic MBR effluent and synthetic MBR mixed liquor. A real

Table 1
Molecular structures of analytes and their isotope labeled standards.

Name	Molecular weight	Molecular structure
(*R*)/(*S*)-ibuprofen	206	
(*R*)/(*S*)-D_3-ibuprofen	209	
(*R*)/(*S*)-naproxen	230	
(*R*)/(*S*)-D_3-naproxen	233	
(*R*)/(*S*)-ketoprofen	254	
(*R*)/(*S*)-D_3-ketoprofen	257	

*Chiral center.

treated wastewater sample was used to finally demonstrate the applicability of the method.

Spiked ultra pure water, drinking water and synthetic MBR effluent were extracted without any further treatment or processing. Surface water and synthetic MBR mixed liquor were first filtered by 0.75 μm Whatman filter paper before extraction. Prior to filtration, synthetic MBR mixed liquor was centrifuged (3500 rpm, 20 min) to minimize clogging during filtration. All samples were acidified to pH 2.5 (using 1 M H_2SO_4), which is significantly below the acid dissociation constants of ibuprofen (p*K*a = 4.4), naproxen (p*K*a = 4.2) and ketoprofen (p*K*a = 4.4), in order to minimize ionization for optimum solid phase extraction conditions.

2.3.1. Solid phase extraction procedure 1 (SPE 1)

The first SPE procedure (SPE1) was used for extraction and preconcentration of the profens from the water samples. Extractions of analytes and internal standards in prepared water samples were performed on Oasis HLB (3 mL, 60 mg) cartridges. Prior to sample loading, the cartridges were conditioned with acetonitrile (3 mL), methanol (3 mL) and finally ultrapure water (3 mL) adjusted to pH 2.5 with 1 M H_2SO_4. All water samples, prepared with various analyte compositions were prepared in 500 mL solutions for extraction. These were then spiked with a standard solution of D_3-ibuprofen, D_3-naproxen and D_3-ketoprofen in acetonitrile (1 μg mL^{-1}, 300 μL). All samples were then loaded onto the SPE cartridges under vacuum and maintained with a constant flow rate of less than 5 mL min^{-1}. All sample bottles were then rinsed twice with 10 mL ultrapure water, which was also drawn through the SPE tube. The cartridges were then dried under a gentle flow of nitrogen gas for an hour. Analytes were eluted from the SPE cartridges with 4 mL of acetonitrile into Kimble culture tubes. These extracts then were centrifuged under vacuum at 35 °C using a Thermo Speedvac concentrator to reduce the solvent volumes to approximately 300 μL.

2.3.2. Diastereomeric (R)-1-phenylethylamide formation

The 300 μL eluents from SPE 1 were subjected to derivatization by the addition of 1.5 μmol TEA (50 mM, 30 μL) and 2.4 μmol ECF (60 mM, 40 μL). This mixture was sonicated for 2 min. Subsequently, 20 μmol of (*R*)-1-PEA (0.5 M, 40 μL) was added and sonication was repeated for a further 2 min. Finally, sulfuric acid (100 μL, 0.1 M) and ultrapure water (3 mL) were added to stop the reaction, lower the pH to around 9.5 (from approximately 10.3) and prepare the sample for further extraction by SPE 2.

2.3.3. Solid phase extraction procedure 2 (SPE 2)

The second SPE procedure (SPE 2) was used to extract the derivatized profens from the derivatization reaction solution prior to GC–MS/MS analysis. SPE 2 was undertaken on Oasis HLB cartridges (1 mL, 10 mg). The cartridges were initially conditioned with ethyl acetate (1 mL), methanol (1 mL) and ultrapure water (1 mL) adjusted to pH 9.5 with 1 M NaOH. The 3 mL aqueous solutions

from the derivatization step were passed through the SPE cartridges under gravity, without the assistance of any applied pressure or vacuum. The cartridges were then rinsed twice with ultra pure water (1 mL) adjusted to pH 9.5. The combined processes of drawing the samples and rinsing solutions through the SPE cartridges were completed in approximately 1 h. The SPE cartridges then were dried under a gentle flow of nitrogen for 30 min.

During the method optimization procedure, the derivatized profens were eluted from the cartridges with ethyl acetate (900 μL), directly to 2 mL GC autosampler vials, to which a toluene solution of mirex (100 μL, 0.1 μg mL^{-1}) had been pre-added as second internal standard to characterize SPE recoveries. However, after completion of the method optimization, and during the method validation, mirex was no longer required as an internal standard and the volume of ethyl acetate used for cartridge elution was increased to 1 mL. A syringe was used to apply a small amount of pressure to the SPE cartridges in order to elute a final drop of solvent before the GC autosampler vial was capped. All vials were then placed in a vortex mixer for a few seconds to facilitate complete mixing prior to GC–MS/MS analysis.

2.4. Gas chromatography–tandem mass spectrometry

The separation of amide diastereomers was performed on an Agilent 7890A gas chromatograph (GC) equipped with an Agilent 7693 autosampler and split/splitless injector. Identification of separated analytes was accomplished using an Agilent 7000B triple quadrupole mass spectrometer to execute various tandem mass spectrometry (MS/MS) experiments. The enantioseparations of analytes were performed on a HP5-MS fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness) with 0.8 mL min^{-1} helium flow. The injector, interface and source temperature were 270, 260 and 280 °C, respectively. 1 μL samples were injected in splitless mode with a purge delay of 1 min. GC oven temperatures were programmed initially at 120 °C for 1 min then increased by 40 °C min^{-1} to 240 °C and finally by 5 °C min^{-1} to 300 °C and maintained for 4 min. Total run time was 18 min per sample.

Mass spectrometric ionization was undertaken in electron ionization (EI) mode with an EI voltage of 70 eV. Initially, full scan monitoring at MS 1 was performed for all analytes, including their analogue internal standards and mirex, to identify suitable precursor ions and the mass range scanned was 50–600 amu at a rate of 0.99 scans s^{-1}. Full product-ion spectra of selected precursor ions were obtained at 0.99 scans s^{-1}. Optimum collision energies were determined for each identified *m*/*z* transition. Analytes were monitored in the multiple-reaction-monitoring (MRM) mode with the gain set to 100 in all cases. All samples were run with a solvent delay of 6 min and the analytes were separated into four discrete time segments for MRM monitoring with dwell times ranging from 100 to 150 ms, depending on the time segment, to achieve 10–20 cycles across each peak for good quantification.

Three MRM transitions were monitored for all profens and their isotope-labeled internal standards while only two transitions were monitored for mirex. All monitored transitions, as well as the specific dwell times and optimized collision energies, are presented in Table 2. The first transition shown for each molecule was used for quantification while the others were monitored only for molecular identification and confirmation. If necessary, the two qualitative transitions for each of the three isotope-labeled standards could be removed from the method in order to further improve peak definitions.

2.5. Optimization of diastereomer formation and recovery

A systematic optimization program was undertaken to achieve optimum and efficient amide formation and recovery. All of these optimization processes were performed using the three profens, (*S*)-ibuprofen, (*S*)-naproxen and (*S*)-ketoprofen. In order to minimize the range of possible experimental variabilities during these optimization processes, profen samples were prepared directly in acetonitrile (1 μg in 300 μL), rather than extracting them from aqueous samples, prior to diastereomer formation. A toluene solution of mirex (100 μL, 0.1 μg mL^{-1}) was added to all samples subsequent to SPE 2 elution in order to normalize individual runs for minor variabilities arising as a result of elution volume, solvent evaporation or sample injection. Isotope-labeled standards were not required for this optimization program.

Optimization of diastereomer formation involved experiments to test the effect of various reaction solvents (methanol, acetonitrile, acetone), solvent volumes (200 μL, 300 μL, 400 μL, 600 μL, 800 μL, 1000 μL,), and reaction times (2 min, 5 min, 10 min, 18 min). Optimization of diastereomer recovery (SPE 2) included experiments to test the effect of extraction volume (1 mL, 3 mL, 5 mL, 7 mL, 9 mL), solution pH (3, 6, 9, 10, 11, 12), elution solvents (methanol, acetonitrile, ethyl acetate) and elution volume (500 μL, 800 μL, 1000 μL, 1200 μL, 1500 μL). SPE 'piggy-backing' experiments (extraction through two SPE cartridges in series) were also undertaken to confirm that the diastereomers were fully adsorbed to the first SPE cartridge, even when masses of up to 2 μg of each of three profens and three D_3-profens were used.

2.6. Method detection limit (MDL) in aqueous matrices

Method detection limit (MDL) determination was conducted in five types of water samples; ultra pure water, tap water, surface water, synthetic MBR effluent and synthetic MBR mixed liquor.

For the MDL experiments, quantitative measurements of (*S*)-profens were conducted using at least seven points out of 15 calibration points (0.08, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 5, 10, 20, 50, 100, 200 and 300 ng). These calibration points are reported in units of mass, rather than concentration since they refer to the masses of analytical standards prepared for derivatization and SPE 2 extraction. All calibration samples also contained racemic D_3-ibuprofen, racemic D_3-ketoprofen and racemic D_3-naproxen (each added at 300 ng).

MDLs were determined according to Method 1030C from Standard Methods for the Examination of Water and Wastewater [31]. For each of the tested matrices, seven replicates of 500 mL volume were spiked with the target profens at concentrations close to the estimated MDL (preliminarily determined as the concentrations required to a achieve a signal-to-noise ratio (S/N) of 5–15). All samples were then spiked with racemic isotope labeled internal standards (600 ng L^{-1}) and processed through all method steps including SPE 1, diastereomer formation, SPE 2 and GC–MS/MS analysis. The seven replicates were prepared and analyzed in two batches over a period of at least three days to ensure that MDL determination incorporated any inter-day variabilities. MDLs were calculated by multiplying the standard deviation of the seven replicates by the Student's *t*-test value of 3.14 (one-sided *T* distribution for six degrees of freedom at the 99% level of confidence). Where a calculated MDL was greater than the actually spiked concentration of any target analyte, a further seven replicates spiked with higher concentrations were analyzed to calculate a revised MDL for that analyte. Alternatively, where the calculated MDL was 5-times smaller than the actual spiked concentration, a further seven replicates spiked with lower concentrations were analyzed to calculate a revised MDL. This procedure was repeated until MDLs of all target analytes were determined with a signal-to-variability ratio within the bounds of the above criteria.

Table 2
Optimal analyte dependent parameters for tandem mass spectrometry.

Segment start time	Analyte	Retention time (min)	MRM transition	Dwell time (ms)	Collision energy (CE)
6.00	(*R*)-ibuprofen PEA	7.74	309.2 → 161.2	100	10
	(*S*)-ibuprofen PEA	7.95	309.2 → 119.1	100	20
			309.2 → 105.0	100	35
	(*R*)-D_3-ibuprofen PEA	7.72	312.0 → 164.0	100	15
	(*S*)-D_3-ibuprofen PEA	7.92	312.0 → 122.1	100	30
			312.0 → 105.0	100	30
9.60	Mirex	9.98	271.8 → 236.9	150	15
			271.8 → 234.9	150	15
11.80	(*R*)-naproxen PEA	12.21	333.2 → 185.1	120	10
	(*S*)-naproxen PEA	12.69	333.2 → 171.1	120	35
			333.2 → 105.0	120	35
	(*R*)-D_3-naproxen PEA	12.23	336.0 → 188.1	120	15
	(*S*)-D_3-naproxen PEA	12.67	336.0 → 171.1	120	30
			336.0 → 105.0	120	30
13.20	(*R*)-ketoprofen PEA	13.66	357.3 → 210.2	120	5
	(*S*)-ketoprofen PEA	14.06	357.3 → 120.1	120	10
			357.3 → 105.0	120	25
	(*R*)-D_3-ketoprofen PEA	13.64	360.0 → 213.2	120	5
	(*S*)-D_3-ketoprofen PEA	14.04	360.0 → 120.1	120	15
			360.0 → 105.0	120	30

2.6.1. Effect of derivatization reactions on EF

The possibility of reaction condition-specific alterations to the EF of the profen analytes occurring during the derivatization process was investigated. To undertake these experiments, stock solutions containing racemic ibuprofen, racemic ketoprofen, racemic D_3-ibuprofen and racemic D_3-ketoprofen (10 μg mL^{-1} of each) were prepared in acetonitrile. Naproxen was not included in these experiments since non-labeled naproxen was available only as enantiomerically pure (*S*)-naproxen. Standard solutions of 0.5 μg and 2 μg were prepared (without having processed them through the SPE 1 step) from the stock solutions and all standards were subjected to the derivatization procedure under a variety of reaction conditions. The reaction conditions tested included different reaction times (2 and 20 min) and temperatures (17, 27 and 37 °C). Samples were also reanalyzed after 24 h to observe any potential changes in relative peak areas of the various enantiomers. Observations of EF (as previously described in Eq. (1)) for each compound could be determined directly by the integration of chromatographic peak areas and no calibration curve was involved in this investigation since EF is a relative measure.

2.7. Method validation in tertiary treated wastewater

The performance of the enantioselective analytical method was tested on a series of treated effluent samples collected from a tertiary wastewater treatment plant in western Sydney over seven separate sampling dates during June–August 2010. These samples had undergone aerobic and anoxic biological treatment followed by phosphorus precipitation, dual media filtration and chlorination/dechlorination.

Triplicate 1 L grab samples were collected in amber glass bottles and were preserved by the addition of 1 g L^{-1} sodium azide to prevent biodegradation. Sample bottles were kept cool, brought back to the laboratory and extracted within 12 h of collection. Prior to SPE, 50 ng of each racemic D_3-ibuprofen, racemic D_3-naproxen and racemic D_3-ketoprofen were added and pH adjustment was made to the samples. All samples were then extracted, derivatized and analyzed following the methods described in the manuscript.

Concentration of (*R*)- and (*S*)-enantiomers of profens was determined from 6-point standard calibration curves of (*S*)-naproxen (1, 10, 50, 100, 500 and 1000 ng) and racemic ibuprofen and ketoprofen (2, 20, 100, 200, 1000 and 2000 ng). In all cases, these calibration solutions also contained 50 ng of racemic D3-ibuprofen, D3-ketoprofen and D3-naproxen for isotope dilution quantification.

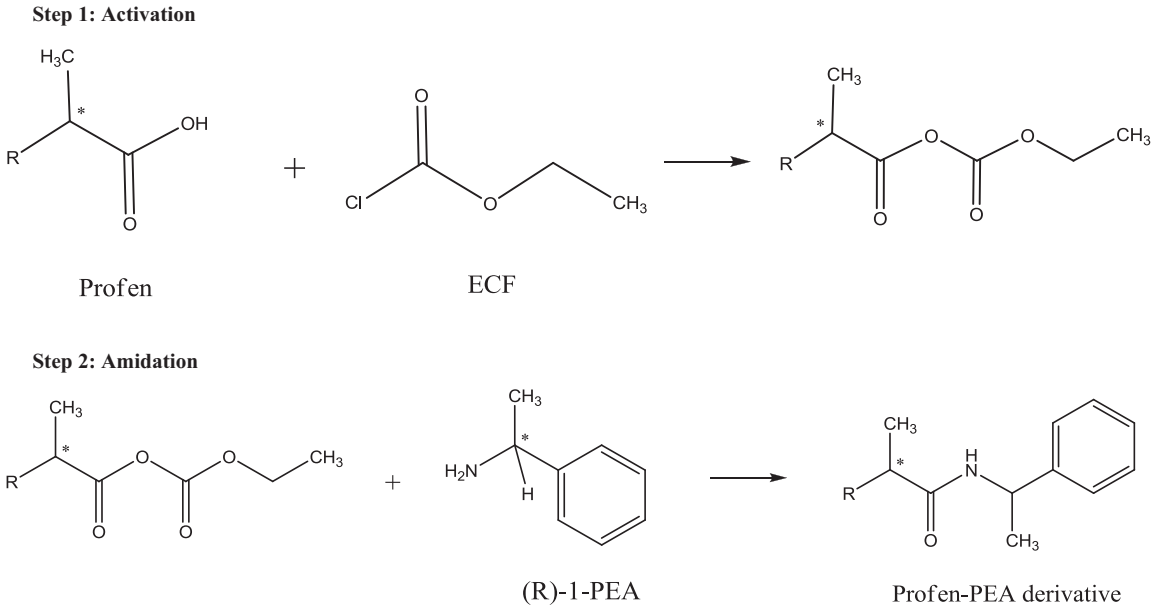
Due to the unavailability of racemic naproxen, (*R*)-naproxen was quantified against the calibration curve developed for (*S*)-naproxen. The validity of using an (*S*)-enantiomer calibration curve (with isotope dilution) to quantify an (*R*)-enantiomer was tested for both (*R*)-ibuprofen and (*R*)-ketoprofen by comparison of the results determined against both (*R*)- and (*S*)-calibration curves. A statistical analysis, using the paired *t*-test for the calculated sample concentrations from both calibration curves revealed no significant difference of EF values regardless of which calibration curve was used to determine (*R*)-ibuprofen or (*R*)-ketoprofen. Accordingly, the same was assumed to apply for (*R*)-naproxen.

3. Results and discussions

3.1. Identification and quantification of diastereomeric (R)-1-PEA derivatives

The two-step reaction mechanism for the formation of diastereomers by (*R*)-1-PEA derivatization is shown in Scheme 1 (adapted from [18]). The first step involves activation of the profen carboxylic acid group by reaction with ECF in the presence of triethylamine to form a mixed anhydride. This mixed anhydride is then subject to further reaction with (*R*)-1-PEA to form the diastereomeric amide derivative.

Derivatives of profens and D_3-profens were successfully separated by gas chromatography with a run time of less than 20 min as shown in Fig. 1. Excellent peak shape and peak resolution was achieved for all analytes and standards. The identification of the (*S*)-enantiomers was undertaken by running enantiomerically pure (*S*)-enantiomer standards. In all cases, the (*R*)-enantiomer of each profen and D_3-profen eluted earlier than the (*S*)-enantiomer. Approximate peak resolutions (R_s) for (*R*/*S*)-ibuprofen, (*R*/*S*)-naproxen, and (*R*/*S*)-ketoprofen derivatives were determined to be 4.02, 3.31 and 2.87, respectively. The GC retention times of the D_3-profens were typically around 0.02 min shorter than their corresponding unlabeled profen enantiomers. This is believed to be is due to the well-known reverse-isotope effect in vapor phase separations [32]. Similarly, the mass spectral fragmentation patterns of the profens and corresponding D_3-profens were the same except for the expected mass differences arising from the deuterium atoms.



Scheme 1. Activation of profens by ECF (Step 1) and amidation by (*R*)-1-PEA (Step 2).

3.2. Extraction and derivatization optimization

The gas chromatographic enantioseparation of profens after amidation with (*R*)-1-PEA has previously been reported for human urine samples [19]. However, the adaptation of this method to wastewater and environmental water samples imposes a number of difficulties due to the significantly lower concentrations to be analyzed and the complexity of the matrices.

The methodology employed by Paik et al. [19] involved extraction of urine samples by liquid–liquid extraction (diethyl ether) and derivatization in a dichloromethane solution with the addition of TEA and ECF, both as solutions prepared in acetonitrile, and (*R*)-1-PEA in methanol. This reaction was followed by addition of dilute HCl solution and extraction of the amide derivatives by diethyl ether followed by ethyl acetate. Finally, these solvents were evaporated to dryness and reconstituted in a mixture of

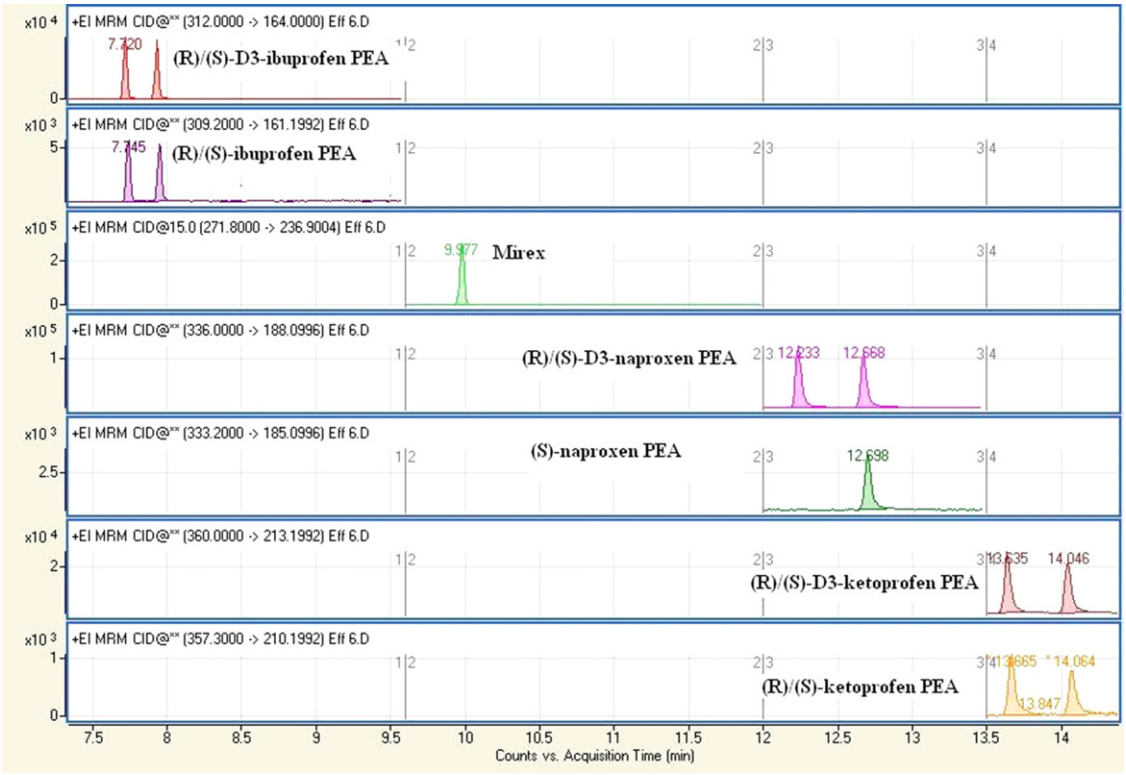
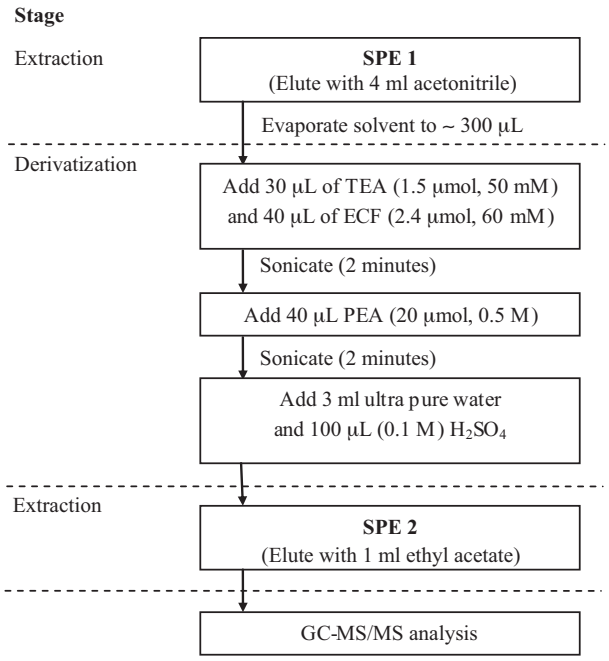


Fig. 1. Example of a MRM chromatogram of extracted synthetic MBR effluent sample containing mirex (10 ng), racemic D_3-profens (all 300 ng) racemic ibuprofen (6 ng), racemic ketoprofen (6 ng) and (*S*)-naproxen (3 ng).



Scheme 2. Illustration of the overall analytical method.

toluene and ethyl acetate. Unfortunately, this procedure resulted in poor recoveries, most likely associated with the observed turbid and non-dissolved materials in final extracts, when tested for wastewater and environmental water samples. Consequently, initial investigations revealed that this procedure did not provide an adequately low level of detection for these types of samples. The methodology was significantly improved (as described in this paper) by amending the process to undertake the derivatization reaction in acetonitrile, followed by acidification and further dilution in water prior to SPE. This modification in procedure resulted in significantly improved sample clean-up and derivatized analyte recovery. The overall analytical method procedure is illustrated in Scheme 2.

Paik et al. [19] reported that an interfering 'artifact' was formed during derivatization and that the formation of this artifact appeared to be dependant on the concentration of TEA in the reaction media. The (single quadrupole) GC–MS peak from this artifact presented problems in that study since it commonly interfered or overlapped with the (*R*)-ibuprofen peak. Accordingly, the optimization of derivatization reaction conditions was necessarily constrained by the need to minimize the formation of this artifact. We also observed this artifact peak when monitoring samples by single quadrupole GC–MS. However, our investigations revealed that this artifact could also be formed in samples not containing any profens (or D_3-profens) and thus would not affect the method quantification if it could be eliminated. Elimination of the artifact peak was achieved by changing the method to use triple quadrupole GC–MS/MS detection with the selection of appropriately exclusive ion transitions. The elimination of interference by the artifact allowed us to further optimize the applied concentration of TEA and other reagents, with final concentrations as summarized in Scheme 2.

The addition of the isotope-labeled standards, prior to derivatization, was not undertaken for this optimization process since these standards were assumed to be subject to the same degree of derivatization efficiency and SPE 2 recovery as the analytes. Accordingly, normalization of the analyte peaks to the isotope peaks would not have revealed variabilities occurring during these steps in the analytical method. Similarly, addition of the isotope-labeled standards after derivatization or SPE 2 recovery was not used since profens are generally not amenable to GC–MS analysis without some form of derivatization. Mirex was identified as a suitable alternative internal standard that could be added to all samples subsequent to SPE 2 elution in order to normalize individual runs for minor variabilities arising as a result of elution volume, solvent evaporation or sample injection. Mirex is a polychlorinated pesticide ($C_{10}Cl_{12}$), known to be highly resistant to rapid chemical, thermal or microbiological degradation.

3.3. Method validations

Calibration standards were prepared and run for (*S*)-ibuprofen, (*S*)-naproxen and (*S*)-ketoprofen. A total of 15 calibration points were run for all three analytes over a range of 0.08–300 ng, all of which were taken through derivatization and SPE 2 steps prior to GC–MS/MS analysis. The purpose of running such a large number of calibration points over such a wide range was to test the linearity of calibration over this range. This is particularly important in cases where the applied concentration of isotope-labeled standards may prove (in hindsight) to be significantly greater or smaller than the corresponding analyte. The sensitivity of the method was sufficient to achieve satisfactory peaks for (*S*)-ibuprofen and (*S*)-naproxen throughout this entire calibration range. However, the sensitivity was somewhat less for ketoprofen and only the highest 8 calibration points (3–300 ng) could be used. This reduced sensitivity for ketoprofen compared to ibuprofen and naproxen is also reflected in the calculated MDLs (Table 3). Good linear relationships were obtained with regression coefficients for all three (*S*)-profens above 0.99 in all sample batches.

Experiments undertaken to derivatize relatively large quantities (2 μg) of racemic D_3-profens revealed that they may contain up to a maximum of 0.03% unlabeled profens. Accordingly, the use of 300 ng of D_3-profens may result in the addition of up to 0.09 ng of unlabeled profens. This is below the analytical detection limit in all cases. However, it does indicate that when unlabeled profens are measured at very low concentrations (up to 1 ng L^{-1}), caution should be exercised and experiments may need to be repeated with a lower mass of D_3-profens (e.g. 30 ng) added.

Final determined MDLs for (*S*)-profens in ultrapure water, drinking water, surface water, synthetic MBR effluent, and synthetic MBR mixed liquor (all 500 mL) are presented in Table 3. These MDLs were determined to be in the range of 0.2–1.4 ng L^{-1} for (*S*)-ibuprofen, 0.3–1.2 ng L^{-1} for (*S*)-naproxen and 1.3–3.3 ng L^{-1} for ketoprofen.

3.4. Effect of derivatization reactions on EF

Alteration of EF has previously been shown to occur during activation/amidation of profens [22,25,33]. The causes of such alterations could theoretically include enantiomeric inversion, thermodynamic differences in the equilibrium of the derivatization reactions for (*R*)- and (*S*)-enantiomers, or kinetic differences in the rate of formation of the diastereomers by the (*R*)- and (*S*)-enantiomers. EF alteration has been reported to be dependent upon the selection of reaction solvent [25] and the choice and concentrations of reagents [22,25,33]. Since these factors are kept constant in this analytical method, no further characterization of them was undertaken. However, factors which may potentially be subject to small variations between method runs, including reaction time, temperature and concentration of profens were selected for detailed characterization.

In this study, slightly different values of EF were observed from standard solutions containing different concentrations of racemic profens and racemic D_3-profens, suggesting that some

Table 3
Method detection limits (MDLs) of (*S*)-enantiomer target analytes in various aqueous matrices.

	Ultra pure water ($n=7$) ng L^{-1}	Drinking water ($n=7$) ng L^{-1}	MBR effluent ($n=7$) ng L^{-1}	MBR MLSS ($n=7$) ng L^{-1}	Surface water ($n=7$) ng L^{-1}
(*S*)-ibuprofen PEA	0.4	0.4	0.7	0.2	1.4
(*S*)-naproxen PEA	0.3	1.2	0.7	0.7	0.7
(*S*)-ketoprofen PEA	2.6	2.5	2.2	1.3	3.3

minor, concentration-dependant effect had occurred. For example, racemic ketoprofen was determined to have an EF = 0.55 when derivatized from a solution containing 2 μg ketoprofen and EF = 0.49 from a solution containing 0.5 μg ketoprofen. This variability was observed to be consistent for both unlabelled profens and their isotope labeled counterparts. However, it was shown to be more significant for ketoprofen (and D_3-ketoprofen) compared to ibuprofen (and D_3-ibuprofen). Similarly, slight variations in EF were observed after storage of the derivatized profens for extended periods. An EF = 0.49 was determined for a freshly derivatized racemic ketoprofen sample, while an EF = 0.53 was observed for the same sample after 24 h. Similar variability in EF determination was observed after storage for up to one week. Variations in reaction time and reaction temperature did not have any observable impact on EF for any of these analytes.

The fact that identical changes were observed for racemic D_3-profens as were observed for non-labeled profens enabled this variability to be effectively corrected by the isotope dilution process. The isotope dilution calculation normalizes for effective loss (or gain) of the individual enantiomers. By quantifying each enantiomer individually with isotope dilution against its corresponding enantiomeric D_3-profen, consistent EF values were determined under all tested experimental conditions. In the case of racemic ketoprofen and racemic ibuprofen, these EFs were determined to be 0.48 and 0.49, respectively, in all cases. Accurate correction of any variability in EF would appear to be an essential aspect of any stereoselective analysis of enantiomers from diverse environmental samples. This is because the diversity of sample properties may have an otherwise unknown impact on the analytical conditions and therefore on observed EF.

3.5. Enantioselective analysis of profens in tertiary treated wastewater

The enantioselective analysis of ibuprofen, naproxen and ketoprofen was undertaken in order to validate the use of the analytical method for real wastewater samples. This validation was undertaken using a tertiary treated wastewater from a wastewater treatment plant in Sydney. The results from triplicate samples collected over seven separate sampling events (during June–August, 2010) are presented in Table 4.

The total concentrations of each of the three profens were determined as the sum of the two enantiomers and presented as the means and standard deviations of the triplicate concentrations for each sampling date. Similarly, the EF was determined for each grab sample and the means and standard deviations are reported for the triplicate samples from each of the seven sampling dates.

This validation experiment demonstrated that the analytical method is sufficiently sensitive for the detection of the target compounds at their ambient concentrations in this tertiary wastewater. Both enantiomers of all three profens were detected and quantified in all cases except one. The one exception was that (*R*)-naproxen was not observed above the MDL on the final sampling date (4 August, 2010). While considerable variation was observed in the concentrations of the three profens over the seven sampling dates, triplicate samples were highly reproducible, as indicated by the relatively small standard deviations.

The measured concentrations of the three profens were all within an expected range, based on previously published (non-enantioselective) reports of these analytes in treated municipal effluents. For example, mean naproxen concentrations of 32 ng L^{-1} and ibuprofen concentrations of 14 ng L^{-1} have been reported in a treated sewage effluent in Kristianstad, Sweden [34]. A survey of eight sewage treatment plants in Southern Ontario, Canada revealed variable final effluent concentrations of ibuprofen (0.11–2.17 mg L^{-1}), naproxen (0.36–2.54 mg L^{-1}) and ketoprofen (0.04–0.09 mg L^{-1}) [35]. This variability has been partly explained by a study showing that facilities employing longer detention times during treatment (nitrifying and denitrifying plants) achieve significantly lower effluent concentrations for these drugs compared to trickling filter or activated sludge facilities applying shorter detention times [36]. In that study, effluents from four US tertiary treatment facilities were reported with concentrations of ibuprofen (5–2550 ng L^{-1}), naproxen (<5–710 ng L^{-1}) and ketoprofen (<5–35 ng L^{-1}).

Calculated EF values for triplicate grab samples were highly consistent with coefficients of variability ($C_v = \sigma/\mu$) no greater than 0.06 for ibuprofen, 0.02 for naproxen and 0.09 for ketoprofen. However, some greater variability was observed in EF from samples collected on different sampling dates. This suggests suitable sensitivity of the method for identifying relatively subtle variations in EF. The EF values determined for ibuprofen were consistent with those previously reported by Buser et al. [4] (EF = 0.47–0.67) and naproxen by Matamoros et al. [26] (EF = 0.71–0.86) in secondary treated effluents.

Table 4
Total concentration (ng L^{-1}) and EF (mean, μ and standard deviations, σ) of ibuprofen, naproxen and ketoprofen in tertiary treated wastewater samples (triplicate grab samples collected from seven separate sampling dates).

Date	Ibuprofen		Naproxen		Ketoprofen	
	Total concentration (ng L^{-1})	EF ($\mu \pm \sigma$)	Total concentration (ng L^{-1})	EF ($\mu \pm \sigma$)	Total concentration (ng L^{-1})	EF ($\mu \pm \sigma$)
25/6/2010	4.6 ± 0.2	0.62 ± 0.03	3.0 ± 0.1	0.66 ± 0.00	3.1 ± 0.1	0.66 ± 0.05
5/7/2010	11.9 ± 1.3	0.54 ± 0.01	7.0 ± 0.9	0.74 ± 0.02	4.5 ± 0.4	0.54 ± 0.05
11/7/2010	33.7 ± 1.1	0.50 ± 0.01	74.3 ± 1.6	0.83 ± 0.01	12.7 ± 0.3	0.62 ± 0.02
17/7/2010	16.9 ± 0.7	0.52 ± 0.02	31.7 ± 0.4	0.83 ± 0.01	10.3 ± 0.6	0.60 ± 0.02
23/7/2010	16.3 ± 1.8	0.53 ± 0.02	30.5 ± 0.4	0.82 ± 0.01	15.9 ± 0.3	0.63 ± 0.02
29/7/2010	120.0 ± 1.2	0.49 ± 0.01	178.9 ± 8.4	0.86 ± 0.00	20.7 ± 0.1	0.61 ± 0.02
4/8/2010	20.0 ± 1.1	0.54 ± 0.02	1.6 ± 0.9	n.d	7.1 ± 1.5	0.56 ± 0.01

n.d. = not determined due to very low concentration of (*R*)-naproxen (<MDL).

4. Conclusion

A sensitive enantioseparation method was developed for the three 2-arylpropionic acids, ibuprofen, ketoprofen and naproxen in wastewater and environmental water samples. This method employs an initial solid phase extraction from the aqueous matrix, followed by diastereomer formation using the chiral derivatizing reagent (*R*)-1-PEA. The diastereomers so formed were then efficiently extracted from the reaction solution by a second SPE process and analyzed by GC–MS/MS. The improved sample extraction and clean-up provided by the two SPE steps, along with the improved sensitivity of the tandem mass spectrometer, provided the necessary sensitivity for the analysis of wastewater and environmental water samples. Comprehensive method validation revealed that MDLs between 0.2 and 1.4 ng L^{-1} could routinely be achieved for enantioselective analysis of ibuprofen and naproxen in a variety of aqueous matrices. MDLs for enantioselective analysis of ketoprofen were slightly higher at between 1.3 and 3.3 ng L^{-1}.

This work represents the first employment of racemic D_3-ibuprofen, racemic D_3-naproxen and racemic D_3-ketoprofen as their analogue internal standards for the analysis of environmental samples. In addition to the more conventional use of isotope standards to correct for any losses that may occur during sample preparation or analysis, their use was shown to be essential for accurate and reproducible EF determination. This analytical method is now highly suited for further research to investigate the phenomena of EF variability during engineered and environmental biodegradative processes.

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