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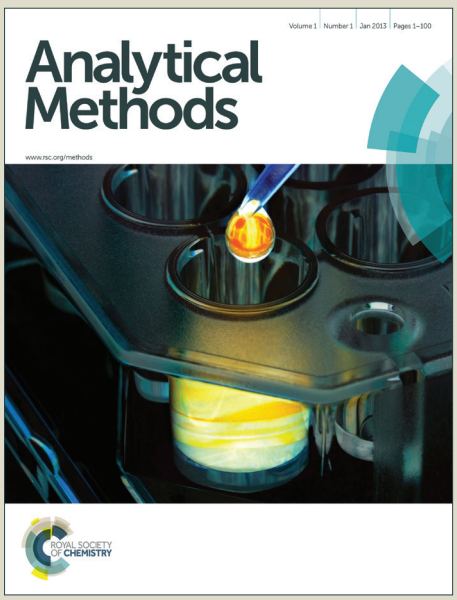

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Rapid analysis of non-steroidal anti-inflammatory drugs in tap water and drinks by ionic liquid dispersive liquid-liquid microextraction coupled to ultra-high performance supercritical fluid chromatography

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Abstract:

A novel rapid analytical method for the determination of four non-steroidal anti-inflammatorys (NSAIDs)-nabumetone, ibuprofen, naproxen and diclofenac in tap water and drinks is presented. The method is based on ultrasound-assisted ionic liquid dispersive liquid-liquid microextraction (US-IL-DLLME) followed by ultra-high performance supercritical fluid chromatography (UHPSFC) coupled to photo-diode array detector (PDA). The ionic liquid 1-octyl-3-methylimidazolium hexafluorophosphate ($[C_8MIM][PF_6]$) and methanol were used as the extraction and dispersion solvent for the DLLME procedure other than using toxic chlorinated solvent. Plackett-Burman and Box-Behnken designs were applied as the experimental design strategies to screen and optimize the experimental variables such as volume of ionic liquid, volume of disperser solvent, sample pH, ionic strength, ultrasonic time and centrifugation time which affected the extraction efficiency. Separation conditions of UHPSFC, such as columns screening, modifiers, column temperature, back pressure and flow rate were also optimized in this study. 4 NSAIDs were simultaneously separated and determined in 2.1 minutes. The optimized US-IL-DLLME-UHPSFC-PDA method showed good enrichment factors (126-132), recoveries (81.37-107.47%) for the rapid extraction of nabumetone, ibuprofen, naproxen and diclofenac in tap water and drinks. The method limits of detection for nabumetone, ibuprofen, naproxen and diclofenac were 1.56, 7.69, 0.62, 7.37 ng mL^{-1} with excellent linearity ($R > 0.9957$).

Keywords: tap water and drinks; non-steroidal anti-inflammatory drugs; ionic liquid dispersive liquid-liquid microextraction; Plackett-Burman design and Box Behnken design; ultra-high performance supercritical fluid chromatography.

1. Introduction

In recent years more and more attention has been paid toward the discharge, presence and potential effects of pharmaceuticals in the water. Thousands of tons of pharmaceuticals are consumed yearly to treat or prevent illnesses, or to help people relieve the stresses of modern life. The discharge of pharmaceuticals from pharmaceutical factory, hospitals and private household effluent produce a big burden on the environment, especially for water resource since the traditional wastewater treatment plants can not eliminate them [1]. Pharmaceuticals are released into the environment either as the parent compound or as active/inactive metabolites. These substances have several characteristics, such as bioaccumulation which can trigger changes in biological sex ratio, biogeochemical cycle and long-time exposures to the active substances can cause potential risks for human health [2-8]. Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of pharmaceutical compounds which have analgesic, antipyretic and platelet-inhibitory actions [9]. A wide range of NSAIDs has been detected in surface water, ground water, sewage treatment plants all over the world [10-19]. In a previous study conducted in Greece, high concentrations of naproxen in Veroia with mean levels of 1.58 ng mL^{-1} were presented [17]. Gracia-Lor et al. found that in the influents of three wastewater treatment plants of Castellon province in Spain, the concentrations of naproxen, were ranged 0.270-3.58 ng mL^{-1} [18]. They also conducted an analysis of around forty water samples (river waters and effluent wastewaters) from the Spanish Mediterranean region. Almost all 47 pharmaceuticals selected in this work were detected, such as ibuprofen with the mean level of 15.1 ng mL^{-1} [11]. Recently, a variety of NSAIDs were investigated in typical aquatic environments in the vicinity of two municipal landfills in a metropolitan area of South China and ibuprofen, salicylic acid, diclofenac and

indomethacin were widely present [20]. Along the Yangtze River, higher total NSAIDs concentrations were found downstream the large cities [21]. Evaluation and monitoring of traces of NSAIDs in different environmental matrices are imperative for human health protection and environmental control.

Most of the methods for analyzing trace level NSAIDs are chromatography coupled with a sensitive detection technique MS (GC-MS or LC-MS) [22-30]. Recently, the use of supercritical fluid chromatography (SFC) for analyze NSAIDs has attracted a renewed interest. Compared to liquid chromatography, there are several advantages offered by SFC, including : (a) low viscosity of mobile phase consisted of a mixture of CO_2 and co-solvent enhancing analyte diffusion and resulting in five times higher flow rate while the pressure drop remains always low; (b) the possibility to perform green separations by reducing the dosage of toxic solvents since CO_2 is non-poisonous, inexpensive and easy to control; (c) providing a large choice of stationary phases and better kinetic performance. Therefore, SFC is characterized as a high resolution, short retention time, and green separation technology, and offers a wide range of separation modes with variable modifiers [31]. With the above mentioned advantages, SFC can meet the demand of the high throughput analysis.

To obtain more accurate, reliable and sensitive results, a sample preparation is required prior to detection. Extraction of NSAIDs from water samples has usually been performed by solid-phase extraction (SPE) [22]. In recent years, liquid-phase microextraction (LPME) has been developed and upgraded with a number of advantages: the minimum use of solvents, low cost, simplicity, effectiveness and the excellent sample clean-up ability [32-33]. LPME can be divided into single-drop microextraction (SDME), hollow-fiber liquid phase microextraction (HF-LPME)

and dispersive liquid-liquid microextraction (DLLME) [32]. Much shorter extraction time was required to obtain good extraction efficiencies by DLLME [34-39]. Organic solvents denser than water are most frequently used as extractants because they can carry out sample phase separation by the use of simple centrifugation. However, the number of such solvents is limited and most of them are toxic solvents. Ionic liquids (ILs), known as "green solvents", are a group of non-molecular solvents that can be defined as organic salts that remain in a liquid state at room temperature. These solvents possess several unique physicochemical properties, such as high density, low volatility and high thermal stability [40-43]. The ultrasound treatment is also used to aid the process of dispersion [44], which enhances the formation of the fine cloudy solution, speeds up the mass transfer between sample and extraction phases, and reduces the equilibrium time. Due to the advantages based on the above-mentioned extraction technique, some applications of IL-US-DLLME (ultrasound-assisted ionic liquid dispersive liquid-liquid microextraction) have been reported [45-46].

Several experimental designs have been very popular in the development and the optimization of the process variables on sample preparations [47-51]. Among the experimental design methodologies, Plackett-Burman design (PBD) employs a design which allows testing the largest number of effective factors with the least number of observations [47-49]. Quadratic polynomial models have been considered as the most appropriate solution for building response surface to predict the optimized [50]. The prime advantage of response surface methodology is the ability to acquire useful information about the system by conducting a minimal number of experiments without prior knowledge of the composition or physicochemical properties of the tested sample. Box-Behnken design (BBD) is a second-order model correlating the response

function with the independent factors with three replicates at the central points to estimate the pure error. The response variable was fitted by a second-order model in the form of quadratic polynomial equation:

$$y = b_o + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i + \sum_{i}^{i<j} \sum_{j} b_{ij} X_i X_j$$

Where k is the number of variables, b_o is the constant term, b_i, b_{ii} and b_{ij} represent the coefficient of the first order terms, quadratic terms and interaction terms, respectively. Box-Behnken design as a quadratic response surface was useful in modeling and optimizing the effective parameters on extraction procedure.

Much attention has been paid to NSAIDs studied in studying wastewater treatment plants and surface water which have already shown quite high concentration levels. In the previous work conducted by our lab [52], salicylic acid with the detection levels of 2.85 ng mL^{-1} was detected in tap water and 61.22 ng mL^{-1} in soda. So it is really necessary to investigate other NSAIDs in drinking water. The present study reports the use of IL 1-octyl-3-methylimidazolium hexafluoro phosphate as the solvent to extract four NSAIDs (Naproxen, diclofenac, ibuprofen and nabumetone) by US-IL-DLLME in tap water and beverage. We have applied Plackett-Burman design (PBD) and Box-Behnken design (BBD) for optimizing different experimental conditions on extraction, such as volume of ionic liquid and dispersive solvent, sample pH, salt effect and extraction time. The ultimate aim of this new inspection was to verify the main factors and their interaction effects. Therefore, the effects of the major factors such as volume of ionic liquid, volume of disperser solvent, sample pH were investigated and optimized. Meanwhile, the ultra-high performance supercritical fluid chromatography (UHPSFC) system coupled with photo-diode array detector (PDA) was applied to the quantification of the four drugs. The

chromatographic conditions of UHPSFC had also optimized. Subsequently, the optimized US-IL-DLLME-UHSFC-PDA method was applied to detect the targets in tap water and drinks.

2. Experimental

2.1. Materials and reagents

Naproxen and nabumetone were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Diclofenac sodium was obtained from The China drugs and Biological Products Inspection Institute. Ibuprofen was from Xinhua Pharmaceutical Co., Ltd. (Shandong, China). Properties of the studied analytes are shown in Table 1.

Acetone was purchased from Beijing modern Oriental Fine Chemicals Co., Ltd. and isopropanol was from Beijing Chemical Plant. HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, USA). HPLC-grade water was purified by a Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA, USA). 1-Butyl-3-methylimidazolium hexafluoro phosphate ($[C_4MIM][PF_6]$) (99%), 1-Octyl-3-methylimidazolium hexafluoro phosphate $[C_8MIM][PF_6]$ (99%) were obtained from J&K Chemical Ltd. (Beijing, China). The structures of the evaluated ILs are shown in Fig. 1. Potassium di-hydrogen phosphate was from Beijing Hongxin Chemical Plant.

Purified water, lemon juice, soda and green tea drink were purchased from supermarket in Beijing, China.

2.2. Instrumentation

The Waters Acquity UPC^2 system which stands for Ultra-Performance Convergence Chromatography^{TM} was equipped with a binary solvent delivery pump, an autosampler, a column oven and a back pressure regulator, a UPC^2 PDA detector set at 227 nm for Naproxen, 224 nm for nabumetone, 220nm for ibuprofen, 275 nm for diclofenac. We have calculated the extinction coefficients of 4 NSAIDs using Lambert-Beer law at each lambda max values (Nabumetone: 9.16×10^{-3}, ibuprofen: 2.84×10^{-3}, napxen: 1.30×10^{-4}, diclofenac: 2.77×10^{-3}). Data acquisition and control of the UHPSFC systems were performed using the Waters MassLynx 4.1 Software. The sample was separated at 30°C by an Acquity UPC^2 BEH 2-Ethylpyridine (2-EP) column (100 mm×3.0 mm, 1.7 µm; Waters, Milford, MA, USA) with gradient elution using carbon dioxide (A) and methanol (B) at the flow rate of 1.6 mL min^{-1}. The gradient program started with 1% of component B, constant for 0.6 min, then, a linear gradient was programmed from 1% to 18% for 0.4 min, followed by a linear gradient up to 22% B in 2 min, finally it was held for 2.0 min which allowed ionic liquids to elute out the. The injection volume was 1 µL and 1500 psi and the back pressure was controlled with a back pressure regulator.

A 100 µL Hamilton microsyringe (Bonaduz, Switzerland) was used for the injection of the extraction solvent into the sample solution. The centrifuge instrument model TGL-16G (Anke, China) was used for making centrifugation. A KH2200DB (He Chuang, China) ultrasonic water bath (100 W and 50 kHz) was applied to accelerate the extraction process.

2.3. Preparation and extraction procedure

Four individual pharmaceutical analytical standard solutions (1mg mL^{-1}) were prepared by exactly weighing and dissolving them in methanol. Furthermore, the standard solutions were

protected from light and stored at -20 °C in a freezer and kept stable for at least 3 months. Working standard solutions of the analytes were prepared daily in methanol.

10 mL spiked water sample (adjusted to pH 1.53 with formic acid) was put into a 15 mL centrifuge tube and then 165 μL methanol(disperser solvent) mixed with 74 μL [C_8MIM][PF_6] (extraction solvent) was rapidly injected into the sample solution by microsyringe. The injection of the extraction mixture led to a cloudy sample solution which contains tiny drops of [C_8MIM][PF_6] distributed in the sample solution. Then the tube was subsequently put in the ultrasonic bath system at the ambient temperature for 15 min to enhance the extraction of pharmaceuticals from the sample solution into the tiny droplets of [C_8MIM][PF_6]. After that, it was subjected to centrifugation for 10 min at 8000 rpm. Finally, the sediment phase (60±2 μL) was taken out by using a 100 μL microsyringe and then injected into the chromatographic system.

2.4. Preparation of real water samples

Soda sample was put in an ultrasonic bath for 20 min to remove air bubbles. Then tap water, purified water, treated soda, juice and tea drink samples were filtered with 0.22 μm PES filters (MEMBRANA, Germany) to remove the suspended particles, and the filtered samples were finally adjusted to pH 1.53 with formic acid.

2.5. Experimental design

Plackett-Burman design (PBD) was used to variable screening to define the significant experimental variables in DLLME for the extraction of drugs from the water samples. After determining the variables that mainly affect the extraction process, the Box-Behnken design (BBD) was performed to identify the optimum conditions in the separation process. The software package Design-Expert 8.0.5 (Minneapolis, USA) was employed to analyze the data and the experimental

design.

3. Result and discussion

3.1. The optimization of the instrument conditions

As a matter of fact, due to the uncertainties, the initial choice of a chromatographic system (mobile phase and stationary phase) in SFC is pretty complex. Indeed, all stationary phases available for HPLC, and any solvent that is miscible with carbon dioxide (and be not too soluble for the stationary phase) could be used. Unfortunately, a large diversification has been found out due to the lack of knowledge for the interactions established between the analytes and the chromatographic system globally. As there are fewer clear guidelines for the choice of a stationary phase for a particular analyte, it is often that more than one phase may need to be examined in order to obtain a suitable resolution. In addition, modifiers, gradients, temperature and back pressure are needed to be evaluated because these parameters also affect the resolution and the sensitivity of analysis method.

3.1.1 Column screening

Different chromatographic columns including Waters Acquity UPC^2 BEH (100 mm×3 mm, 1.7 μm), Waters Acquity UPC^2 BEH 2-EP (100 mm×3 mm, 1.7 μm) were tested for separating nabumetone, ibuprofen, naproxen and diclofenac. A mixture of CO_2 and methanol was employed as the mobile phase. The UPC^2 BEH 2-EP column resulted in a better resolution (Fig. 2(a), (b)). For BEH and BEH 2-EP columns, the stationary phases are polar, hydrogen bonding exits for both two stationary phases, and there are π-π inter-actions, dipole-induced dipole interactions on BEH

2-EP. Compared with BEH 2-EP column, there is only one interaction of polar functional groups with Si-OH on BEH column, which resulted in short retention time (Fig. 2 (a)). Meanwhile, naproxen and diclofenac are co-eluting on BEH, while they are separated on BEH 2-EP by π-π inter-actions (Fig. 2 (b)). So the BEH 2-EP column can show better selectivity for the targets than BEH column. Hence, BEH 2-EP was selected as the column to perform the next optimization. We can also conclude from the above phenomenon that if the analytes just own functional group differences, BEH column is a good choice; if the analytes still own benzene Skeleton differences, BEH 2-EP column could behave much better.

Chromatographic elution order on BEH 2-EP was nabumetone, ibuprofen, naproxen and diclofenac (Fig. 2(a)). Considering the structure and properties of the target compounds (Table 1), the order is reasonable. Nabumetone has no strong polar functional groups, so it eluted out firstly. Compared with ibuprofen, naproxen showed much powerful π-π inter-actions with BEH 2-EP column, thus, the retention time was longer. The imino group and hydroxyl in diclofenac can form hydrogen bonding with BEH 2-EP which leads to much stronger interaction.

3.1.2 Optimization of modifiers, column temperature, back pressure and flow rate

In order to improve separation of the four drugs, different modifiers including methanol, acetonitrile, acetonitrile: methanol (1:1, v/v) were evaluated. The best result was obtained by using the solvent mixture CO_2/methanol, with a linear gradient elution mode. Peak tailing (peak 2, 3, 4) also can be viewed from Fig. 2(a). Lower value of pKa faced more serious peak tailing. CO_2/methanol containing 20 mM formic acid, CO_2/methanol containing 20 mM Ammonium acetate was also tested.

Four different column temperatures (30°C, 40°C, 50°C, 60°C) were tested. Higher temperature results in worse resolution and longer retention time. The reason for this phenomenon was that with the increasing temperature the density of CO_2 decreased and the elution capacity of the mobile phase dropped down. Selectivity was attenuated with increasing temperature for all analytes (except nabumetone), which could be caused by the different molecular structure. So the best choice is 30°C for the temperature.

It is well known that the setting of back pressure is an important factor on the density of the supercritical CO_2. Four different pressures (1500psi, 1800psi, 2000psi, 2200psi) are tested. The pressure obviously influences the eluotropic strength of the supercritical fluid. An optimal back pressure of 1500 psi was selected for the UPC^2 analysis. The most suitable flow rate was chosen as 1.6 mL min^{-1}, respectively. The chromatographic conditions were optimized to separate each individual drugs compound with good resolution within a reasonable analysis time (Fig. 3).

3.2. Optimization of experimental conditions for IL-DLLME

3.2.1 Preliminary experiments

In order to achieve high enrichment factors and recoveries for the four NSAIDs from water and drinks, a stepwise optimization procedure was chosen by using the purified water spiked with analytes (100 ng mL^{-1}). The effects of extraction solvents, dispersive solvent, sample pH, ionic strength, ultrasonication time and centrifugation time were investigated and each result was obtained from the mean value of the triplicate extraction.

The selection of extraction solvent which plays a significant role in DLLME procedure tends to satisfy some important condition: (A) density higher than water and low solubility in water; (B)

favorable extraction ability for the target compounds; (C) good chromatographic behavior and no interference with the quantitative and qualitative analysis of the target matter. In this work, considering the upper factors, $[C_4MIM][PF_6]$ and $[C_8MIM][PF_6]$ were tested. $[C_4MIM][PF_6]$ and $[C_8MIM][PF_6]$ are hydrophobic, and can form the sediment phase in the water sample. The results indicated that $[C_8MIM][PF_6]$ can achieve higher recoveries than $[C_4MIM][PF_6]$, illustrating the length of the alkyl chain of the cation increase the hydrophobicity of the $[PF_6]^-$ ionic liquid, See Fig. 4(a). $[C_8MIM][PF_6]$ was selected as extraction solvent for the following experiments.

The effect of different volumes of $[C_8MIM][PF_6]$ (10, 30, 50,70 and 90μL) was investigated when dispersive solvent methanol was 300 μL. As shown in Fig.4(b), the area of drugs increased for volumes from 10 to 70 μl and decreased sharply when the volume is increased to 90 μL. Nevertheless the case of the 70 μL of the IL provided the best results. Higher amounts of the IL do not improve the extraction efficiencywhile increase background signals.

Disperser solvent aids the IL to steadily disperse into the water samples and rapidly reach the extraction equilibrium. It markedly increases the contact surface between the extraction phase and aqueous samples. So, it requires that the disperse solvent should have a good miscibility in both the extraction solvent and the aqueous phase. For this purpose, methanol, acetonitrile, acetone and isopropanol were evaluated as the disperser solvents. 300 μL of each one was mixed with 70 μL of $[C_8MIM][PF_6]$ as an extraction solvent. The results (Fig.4(c)) show that naproxen, ibuprofen obtained better extraction efficiency in methanol, while nabumetone got a little better extraction in acetone. Meanwhile, diclofenac had slightly better extraction in acetonitrile. Therefore, Methanol was chosen as extraction solvent for further experiments.

The influence of Methanol volume was tested for 100, 200, 300, 400 and 500μL. With the

increasing volume, the areas of 4 drugs first increased then decreased (Fig.4(d)). The reason for this is that methanol cannot disperse [C_8MIM][PF_6] effectively at low volume, therefore the cloudy solution is not completely formed; At high volumes, the solubility of the four NSAIDs in water was increased, and led to the lower extraction efficiencies because of a diminution in the distribution coefficient.

The pH value of the solution can affect the ionization status and solubility of the analytes. The pKa values of naproxen, diclofenac and ibuprofen are 4.15, 4.50, 5.20 respectively. The lower the pH value, the more inhibited the ionization of the drugs. Five pH values (ranges from 1.5 to 3.5) were investigated to study their influence on the extraction efficiency. Fig. 5(a) shows that the extraction efficiency is the highest when the pH value is 1.5. The results show that all drugs will be in the neutral form at low pH value, which facilitates the extraction from donor phase.

The salting-out effect on the extraction efficiency of drugs was examined by adding different amounts of KH_2PO_4 (0, 0.2, 0.4, 0.6, 0.8 (w/v %)) to 10mL aqueous samples at pH 1.5. As shown in Fig.5(b), the extraction efficiency increase with enhancing the salt concentration up to 0.4 and then it decreases with further increase in salt concentration. At the beginning, the salting out process plays the predominant role, a higher ionic strength in the sample and decreases the solubility of four drugs in the aqueous solution. However, by increasing the salt concentration, electrostatic interaction will resist organic solvent extraction and decrease the extraction efficiencies.

The extraction time is an important factor which affects the extraction efficiency. When the extraction time is longer, the transferring of the analytes from aqueous into IL phase is more complete. The extraction time was evaluated in the range of 5-25 min. From results in Fig. 5(c),

the extraction efficiencies increased rapidly by increasing the extraction time up to 15min and longer extraction time does not significantly affect the extraction efficiency. It is possible that the extraction equilibrium could be achieved at 15min. The mixture was centrifuged to break down the cloudy solution and formed the phase separation. In order to investigate the effect of centrifugation time, experiments were performed by centrifuging for 2, 5, 10, 15 and 20 min respectively at 5000 rpm after extraction (Fig. 5(d)). The extraction efficiency for the analytes was lower when the centrifugation time was too short because the IL could not be completely collected at the bottom of the glass test tube. However, longer centrifugation had no significant effect on the extraction efficiency for IL-DLLME.

3.2.2 Experimental sceening using PBD

Plackett-Burman design was used to screen the main factors which affect the efficiency during the extraction step rapidly among variables from a multivariate system. To evaluate the main effects of the above six factors (volume of $[C_8MIM][PF_6]$, volume of methanol, pH, ionic strength, ultrasound time, and centrifugation time), a matrix of the P-B design consist of 12 experiments was performed. Each factors were considered at 2 levels, i.e. low (−1) and high (+1). And the experiments were carried out in three replicates in a random manner in order to reduce the experimental error. The sum of the mean peak areas were treated as responses. Based on the single factor experiment, the values corresponding to the each factor level are reported in Table 2.The results obtained were evaluated by the analysis of variance (ANOVA) based on the t-test with 95% probability for determining the main effects. The effects of the studied variables in the screening experiment were expressed by Pareto-chart (Fig. 6). The red line on the plot judges the effects that are statistically significant at the 95% confidence level. According to the Pareto chart, pH was the

most significant factor, subsequently volume of dispersive solvent and volume of extraction solvent were the next most significant factors. Ionic strength and extraction time were less significant in comparison to the above factors. Moreover, as shown in Fig. 6, Centrifugation time revealed no significant effect on extraction efficiency. Out of these, the first three significant factors, i.e., volume of disperser solvent, pH and volume of extraction solvent were chosen for further optimization using BBD. The ionic strength and extraction time, centrifugation time, 0.4% and 15, 10 min respectively were selected for further experiments.

3.2.3 The optimization of factors by BBD

After these previous experiments, a Box Behnken design was selected to optimize the experimental factors (sample pH, volume of methanol and volume of ILs) since interactions between them may also occur. The BBD was applied with three design factors and three levels. The examined levels of these factors are given in Table 3.The resulting 17 experiments, in which 10 mL of water were spiked with the drugs and submitted to the DLLME procedure were randomly performed. Through ANOVA, the quadratic regression model demonstrated that the model is highly significant. Because responses of *P*-value were lower than 0.05, which are statistically significant. According to the Design Expert 8.0 analysis, in peak area, the model *F*-value of 9.59 implies that the model is significant. There is only a 0.35% chance that as laege as "Model *F*-Value" could occur due to the noise. The Lack of Fit expresses if the model is adequate to describe the observed data or if a more complicated model should be used. As the Lack of Fit of *P*-value (0.1258) was found to be non-significant, it suggests that the model equation was adequate to predict the peak areas under any sets of the variables combination. The

explained variation R^2 is 0.83 which indicated a high significance of the model. Moreover, the Adeq Precision value measures the signal due to noise ratio and a value greater than 4 is desirable. The Peak area has 8.998 Adeq Precision, indicating an adequate signal. Therefore, according to ANOVA results the model fitted the data and it was able to predict and optimize the responses.

The basic strategy for response surfaces methodology had the following four steps: the procedures to move into the optimum region, the behavior of the response in the optimum region, the estimation of the optimal condition and the verification. The visualization of the predicted model equation can also be obtained by response surface plots. Response surfaces estimated for the Box Behnken design are shown in Fig. 4 plotting Volume of methanol vs. Volume of $[C_8MIM][PF_6]$ (Fig. 4a, Fig. 4b), Volume of $[C_8MIM][PF_6]$ vs. pH (Fig. 4c,Fig. 4d) and Volume of methanol vs. pH (Fig. 4e, Fig. 4f). The 3D response surface plots are useful in learning about the main and interaction effects of the independent variables, whereas 2D contour plots give a visual representation of values of the response. In fact, the final optimum DLLME conditions predicted were: 73.53 μL of $[C_8MIM][PF_6]$, 164.98 μL of MeOH and pH 1.53. Several experiments were then developed under these optimum conditions, obtaining the highest peak areas of all previous experiments.

3.3. Method validation

3.3.1. Selectivity

The specificity of the method was evaluated with respect to different water samples by extracting and analyzing the blank samples by using the optimized method. Blank sample has no

interference in drugs detection (Fig. 8(a)). Fig. 8(b) is the chromatogram of a spiked sample (100 ng mL^{-1}).

3.3.2. LOD, LOQ, linearity and enrichment factor (EF)

For the sake of validating the optimized US-IL-DLLME-UPC^2-PDA method to extract drugs from the aqueous solutions, a number of performance parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), enrichment factor (EF), repeatability, reproducibility and extraction recoveries were evaluated.

LOD and LOQ are set up based on the minimum value and the detected concentration of an analyte gives peak value with the signal to noise ratio of at least 3:1 and 10:1. The instrument LODs and LOQs of drugs range from 0.05 to 0.5 ug mL^{-1} and 0.1 to 1 ug mL^{-1}. Method limits (MLOD and MLOQ) are listed in Table 2. External calibration was applied to study the linearity of the method response. Ultrapure water (analytes-free) was spiked with drugs to provide standards with five concentration levels. A calibration curve was constructed and correlation coefficients for all compounds were greater than 0.994 (Table4). EF of analytes during US-IL-DLLME extraction procedure was calculated based on the following equation:

$$EF = Ce/Ca$$

Where *Ce* is the concentration of analyte in extraction solvent, *Ca* is the concentration of analyte in aqueous sample. The developed conditions were used to investigate the enrichment factors of drugs, EFs range from 126 to 132. The results are listed in Table 4.

3.3.3. Recovery and precision

Recoveries of the analytes were measured by spiking blank water samples with four drugs at three different concentrations, nabumetone (2.0, 10.0, 50.0 ng mL^{-1}), ibuprofen (10.0, 50.0, 200.0 ng mL^{-1}), naproxen (2.0, 10.0, 50.0 ng mL^{-1}), diclofenac (10.0, 50.0, 200.0 ng mL^{-1}). Samples were extracted by following the above method. After UHSFC-PDA analysis, recoveries were obtained and the result can be seen in Table 5. The recoveries for nabumetone, ibuprofen, naproxen, diclofenac are 87.43-96.14%, 81.37-104.29%, 81.69-104.29%, 96.05-107.47%, respectively, with the relative standard deviation (RSD) lower than 12.39% based on the peak areas for six replicate runs.

3.4. Analysis of real samples

The developed procedure was applied to real water samples, tap water, soda, lemon juice and green tea drink. A US-IL-DLLME procedure was followed to extract the target analytes. Results are shown in Fig. 9. It showed that ibuprofen with the detection levels of 16.43 ng mL^{-1} (n=6, RSD=3.09%) is detected in soda. Depending on the biological accumulation effect of ibuprofen in long-term, it may produce toxic effects in both human body and environment.

3 Conclusions

This study presents the use of a new US-IL-DLLME method combined with UHPSFC-PDA technique for the accurate determination of pharmaceuticals in tap water and drinks. The advantages over conventional extraction techniques of ionic liquid, ultrasound and DLLME were a simple, low-cost, fast, accurate, sensitive and efficient method for NSAIDs extraction. The proposed extraction procedure has a very low organic solvent consumption (few microliters), and

attain the extraction equilibrium quickly. For the optimization of UHPSFC, NSAIDs were separated by Acquity UPC^2 BEH 2-EP column with a standard elution gradient of methanol in CO_2. The optimized separation program enables NSAIDs separated within 2.1 min. The sensitivity of the proposed method has been successfully demonstrated to be reliable and cost-effective for the determination of NSIADs in water samples. This studied method has a prospective future in different areas.

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Figure captions

Fig. 1. Structures of studied ILs. (a) 1-Butyl-3-methylimidazolium hexafluoro phosphate; (b) 1-Octyl-3-methylimidazolium hexafluoro phosphate.

Fig. 2. Separation of four drugs on (a) BEH 2-EP and (b) BEH. 1: Nabumetone; 2 :Ibuprofen; 3: Naproxen; 4: Diclofenac.

Fig. 3. Chromatogram of four drugs, 0.82: nabumetone; 1.63: ibuprofen; 1.85: naproxen; 2.08: diclofenac.

Fig. 4. (a) Effect of organic solvents on the extraction of drugs; (b) Effect of $[C_8MIM][PF_6]$ volume on the extraction of drugs; (c) Effect of disperser solvent on the extraction of drugs; (d) Effect of methanol volume on the extraction of drugs.

Fig. 5. (a) Effect of pH values in sample solution on the extraction of drugs; (b)Effect of salt concentration on the extraction of drugs; (c) Effect of extraction time of drugs; (d) Effect of centrifugation time of drugs.

Fig. 6. Pareto chart.

Fig. 7. Response surface plots of BBD. (a) and (b) Volume of extraction solvent-Volume of dispersive solvent; (c) and (d) Volume of extraction solvent-pH; (e) and (f) Volume of dispersive solvent-pH.

Fig. 8. (a) Chromatogram of blank sample; (b) Chromatogram of a spiked sample.

Fig. 9. The chromatogram of ibuprofen in soda.

Fig. 1.

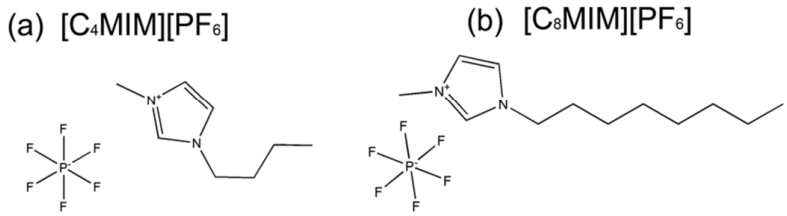


Fig. 2.

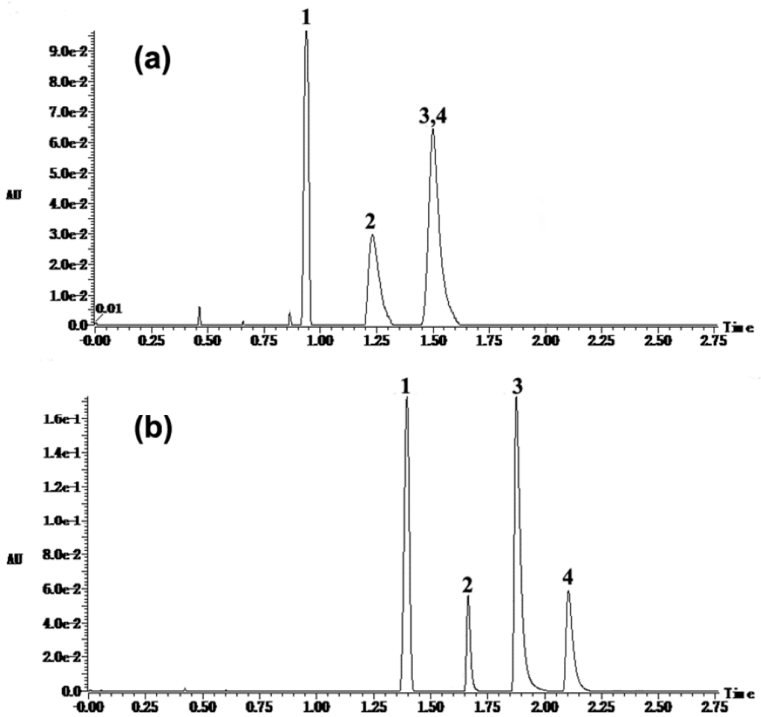
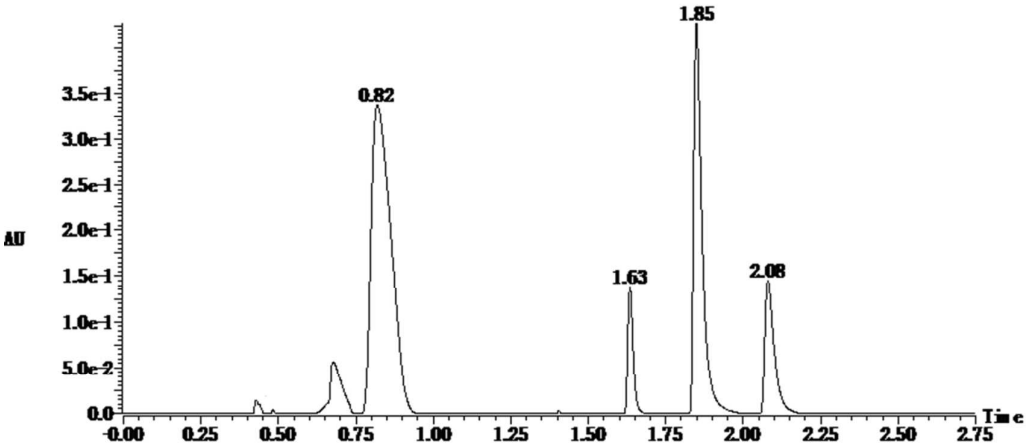


Fig. 3.



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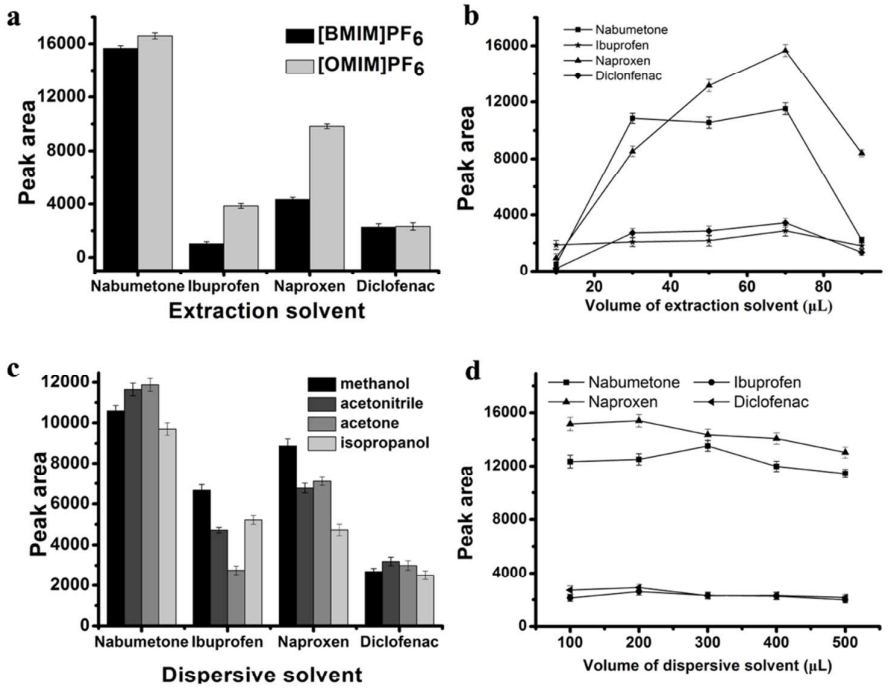

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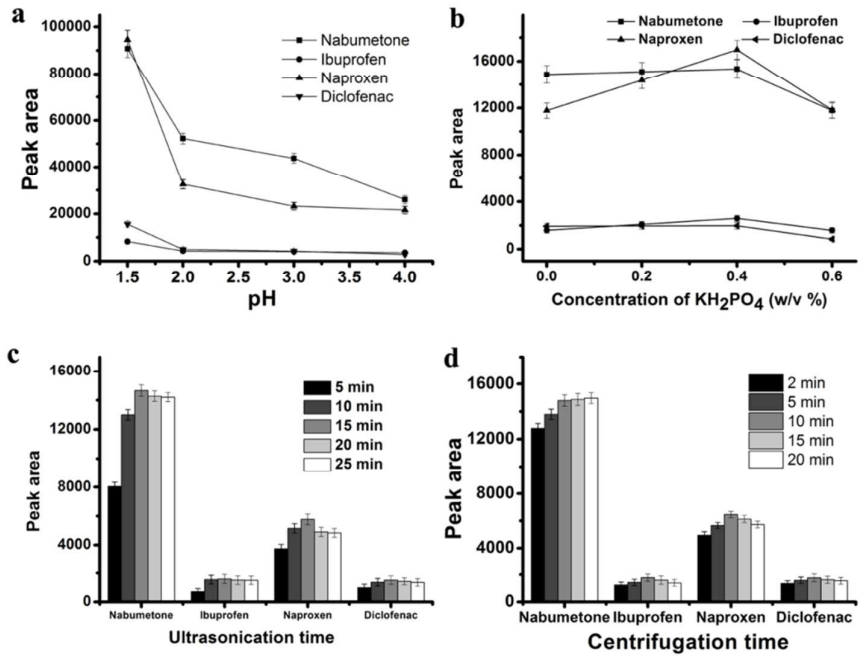

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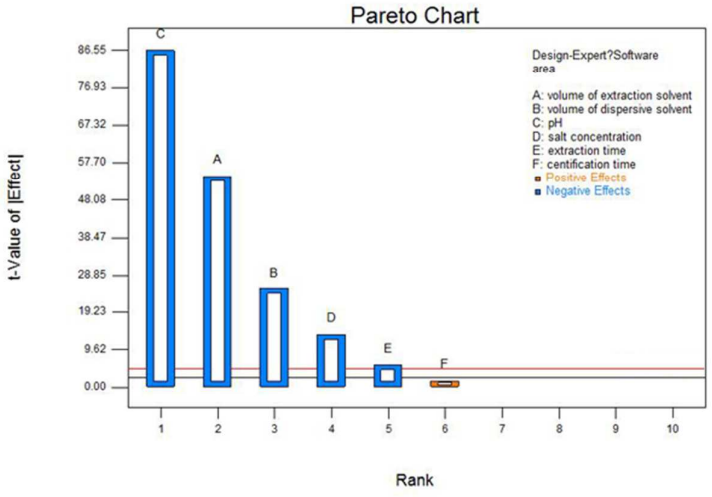
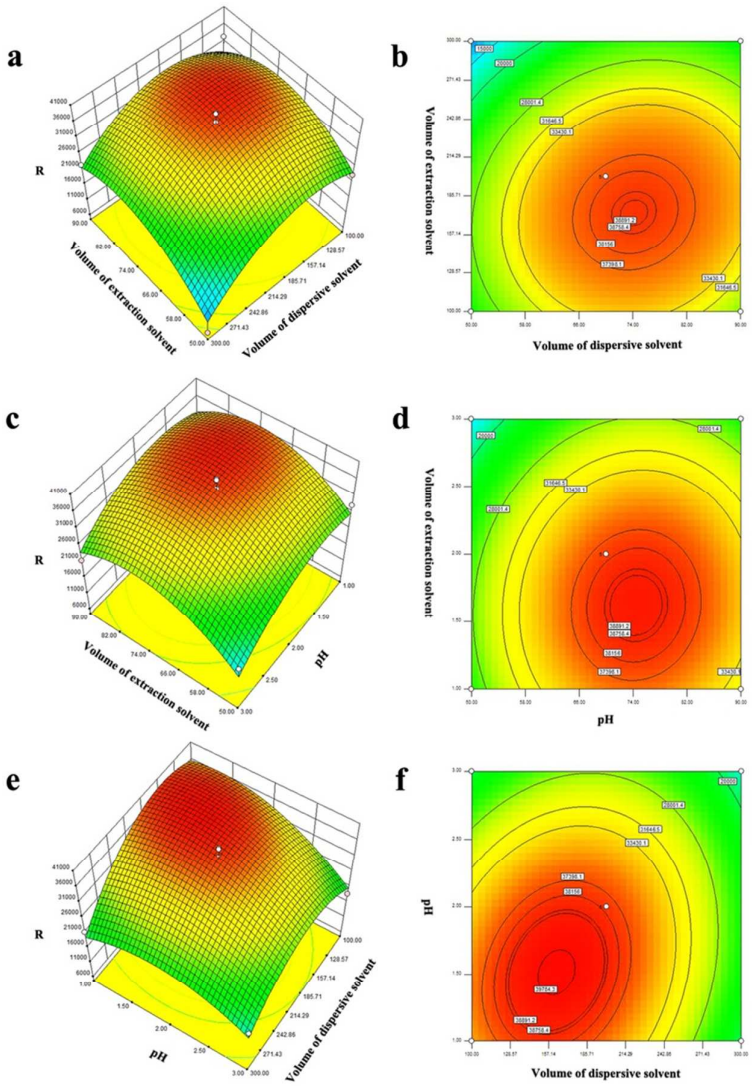


Fig. 7.



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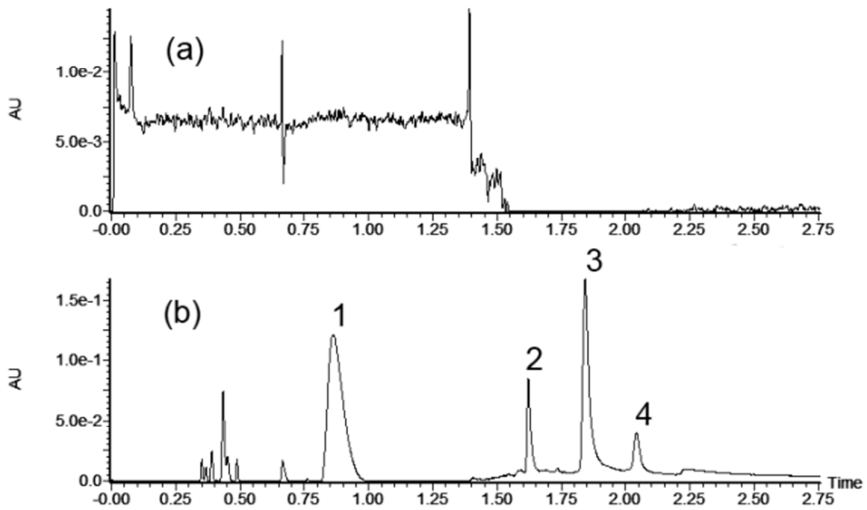

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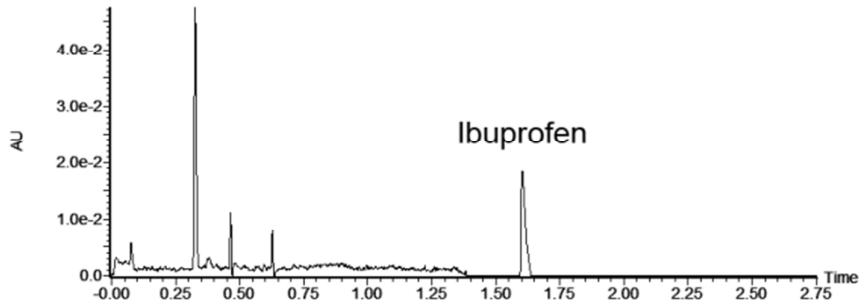
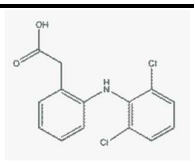
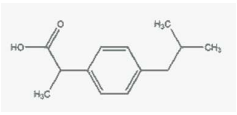
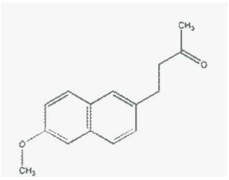
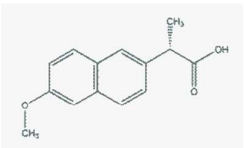


Table 1

492 Structure and properties of the target compounds

Compound	Formula	Relative molecular weight	LogP	pKa	Chemical Structure
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	278.13	4.26	4.50	
Ibuprofen	$C_{13}H_{18}O_2$	206.28	3.84	5.20	
Nabumetone	$C_{15}H_{16}O_2$	228.29	3.22	–	
Naproxen	$C_{14}H_{14}O_3$	230.26	2.99	4.15	

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494 **Table 2**

495 Experimental variables and levels of the Plackett-Burman design.

Factor	Levels	
	Low(-1)	High(+1)
Volume of $[C_8MIM][PF_6]$ (μL)	50.0	90.0
Volume of methanol (μL)	100.0	300.0
pH	1.0	3.0
Ionic strength (% w/v)	0.2	0.6
Ultrasound time (min)	10.0	25.0
Centrifugation time (min)	5.0	20.0

496 **Table 3**

Plan of experiment for Box-Behnken design

Experiment		Factor1 (Volunme of extraction solvent)	Factor2 (Volunme of dispersive solvent)	Factor3 (pH)	Response (Peak area)
13	1	70.00	200.00	2.00	34499.4
2	2	90.00	100.00	2.00	32778.9
9	3	70.00	100.00	1.00	34272.4
7	4	50.00	200.00	3.00	18218.5
14	5	70.00	200.00	2.00	40654.8
8	6	90.00	200.00	3.00	21914.7
11	7	70.00	100.00	3.00	19243.6
17	8	70.00	200.00	2.00	36032.9
6	9	90.00	200.00	1.00	29314.3
15	10	70.00	200.00	2.00	38024.6
12	11	70.00	300.00	3.00	18547.5
3	12	50.00	300.00	2.00	6768.1
16	13	70.00	200.00	2.00	40389.1
5	14	50.00	200.00	1.00	28531.3
1	15	50.00	100.00	2.00	23873.2
10	16	70.00	300.00	1.00	21914.7
4	17	90.00	300.00	2.00	22838.4

Table 4

509 Method linearity, MLOD, MLOQ and enrichment factor (EF).

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Compound	Calibration curve	R	Linear range (ng mL^{-1})	MLOD (ng mL^{-1})	MLOQ (ng mL^{-1})	EF
Nabumetone	Y=216.56 X + 983.651	0.9997	1.56-156.00	0.78	1.56	129
Ibuprofen	Y=12.5776 X + 259.515	0.9985	7.69-192.25	2.56	7.69	130
Naproxen	Y=92.5356 X + 1986.49	0.9967	0.62-64.40	0.31	0.62	126
Diclofenac	Y=35.9567 X + 47.0005	0.9957	7.37-184.25	2.26	7.37	132

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Table 5

Relative recoveries and precision of the compounds

Compound	Spiked level (ng mL^{-1})	Recovery[2] (%)
Nabumetone	2.00	96.14±7.90
	10.00	93.72±5.28
	50.00	87.43±8.69
Ibuprofen	10.00	104.64±4.87
	50.00	85.61±9.52
	200.00	81.37±3.50
Naproxen	2.00	104.29±6.91
	10.00	86.33±9.76
	50.00	81.69±5.77
Diclofenac	10.00	107.47±12.39
	50.00	100.54±6.18
	200.00	96.05±8.79