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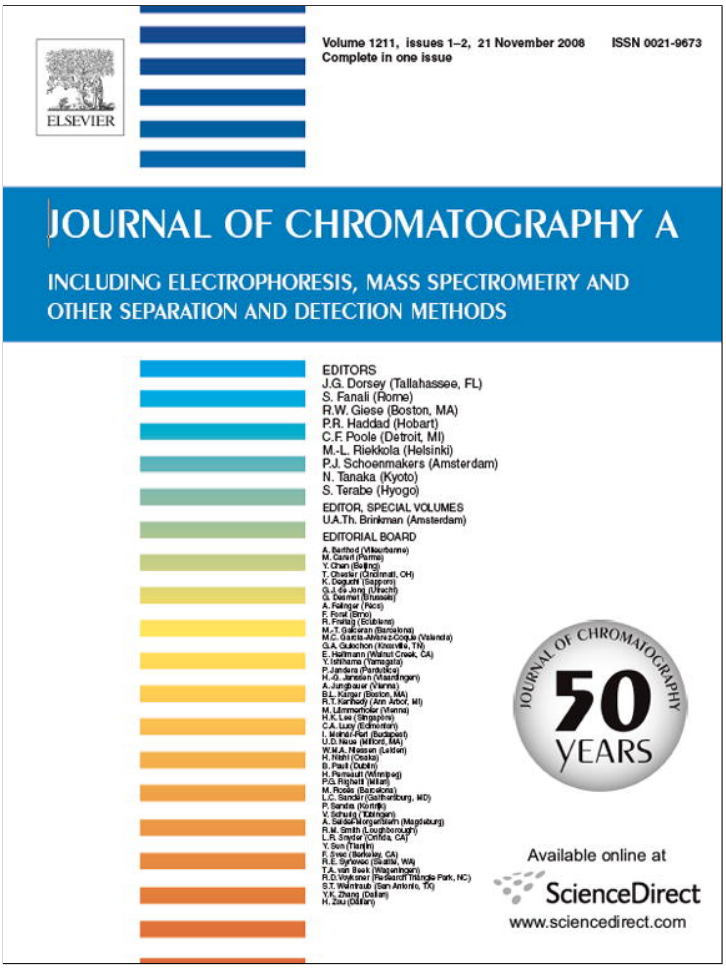

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Chemometric assisted solid-phase microextraction for the determination of anti-inflammatory and antiepileptic drugs in river water by liquid chromatography–diode array detection

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ABSTRACT

In the present work, an analytical method for the simultaneous determination of seven non steroidal anti-inflammatory drugs (naproxen, ketoprofen, diclofenac, piroxicam, indomethacin, sulindac and diflunisal) and the anticonvulsant carbamazepine is reported. The method involves preconcentration and clean-up by solid-phase microextraction using polydimethylsiloxane/divinylbenzene fibers, followed by liquid chromatography with diode array detection analysis. Parameters that affect the efficiency of the solid-phase microextraction step such as soaking solvent, soaking period, desorption period, stirring rate, extraction time, sample pH, ionic strength, organic solvent and temperature were investigated using a Plackett–Burman screening design. Then, the factors presenting significant positive effects on the analytical response (soaking period, stirring rate, stirring time) were considered in a further central composite design to optimize the operational conditions for the solid phase microextraction procedure. Additionally, multiple response simultaneous optimization by using the desirability function was used to find the optimum experimental conditions for the on-line solid-phase microextraction of analytes in river water samples coupled to liquid chromatography and diode array detection. The best results were obtained using a soaking period of 5 min, stirring rate of 1400 rpm and stirring time of 44 min. The use of solid-phase microextraction technique avoided matrix effect and allowed to quantify the analytes in river water samples by using Milli-Q based calibration graphs. Recoveries ranging from 71.6% to 122.8% for all pharmaceuticals proved the accuracy of the proposed method in river water samples. Method detection limits were in the range of 0.5–3.0 $\mu g\,L^{-1}$ and limits of quantitation (LOQs) were between 1.0 and 4.0 $\mu g\,L^{-1}$ for pharmaceutical compounds in river water samples. The expanded uncertainty associated to the measurement of the concentration ranged between 8.5% and 29.0% for 20 $\mu g\,L^{-1}$ of each analyte and between 9.0% and 29.5% for the average of different concentration levels. The main source of uncertainty was the calibration step in both cases.

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1. Introduction

Pharmaceuticals, together with their synthetic precursors and transformation products, are continually released into the environment in enormous quantities as a result of their manufacture, use (via excretion, mainly urine and faeces) and disposals of unused and those that have expired, both directly into the domestic sewage system and via burial in landfills. Thus, the possibility that pharmaceuticals can enter the environment from a number of different routes, and possibly cause untoward effects in biota has been widely noted in the scientific literature [1–5]. Drugs are detected in the environment in the $ng\,L^{-1}$–$\mu g\,L^{-1}$ (ppt–ppb) range, but although parts per billion may not pose much acute risk, other receptors in non target organisms could be sensitive. Moreover, even though individual concentrations of any drug might be low, the combined concentrations from drugs sharing a common mechanism of action could be substantial. Exposures in the aquatic environment are of particular concern, since aquatic organisms (as opposed to those spending at least some time in terrestrial settings) are subjected to continual unabated lifecycle exposures. This is a highly significant consideration for pharmaceuticals (or bioactives metabolites) that are refractory to structural transformations and are continually introduced into surface waters from sewage treatment plants. In

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addition, the polar, non-volatile nature of most drugs prevents their escape from the aquatic realm. Effectively, even pharmaceuticals with relatively short environmental half-lives assume the qualities of highly persistent pollutants because they are continually replenished by infusion to the aquatic environment from wastewater treatment plants (WWTPs).

Stumpf et al. [6] were the first in identifying diclofenac, ibuprofen, acetylsalicylic acid and ketoprofen in sewage and river water and since then, numerous authors reported the presence of non steroidal anti-inflammatory and/or antiepileptic drugs in surface [7–12], ground- [13,14] and waste [11,12,15–18] water. A recent review [19] provides a comprehensive overview about recent scientific research on the sources, occurrence and fate of pharmaceuticals in water and wastewater environments.

Biological environmental sample matrices such as ground superficial and wastewaters (influent and effluent wastewaters) are complex samples, often containing compounds which can interfere with the compounds of interest, so that direct analysis may not be possible and, moreover, pharmaceuticals are generally found in these matrices at trace concentration levels. Therefore, it is necessary to perform an initial sample-preparation step, including purification and concentration of the analytes, to convert the real matrix into a sample suitable for analysis.

Sample preparation may be achieved by a wide range of techniques but all of them show the two above mentioned goals, in addition to provide a robust, reproducible method which is independent of variations in the sample matrix [20]. Even though traditional sample-preparation methods are still in use (e.g. solid-phase extraction, SPE), trends in recent years are focused towards smaller initial simple sizes, small volumes or no organic solvents, greater specificity or greater selectivity in extraction and increased potential for automation [20].

Solid-phase microextraction (SPME) is a modern sampling or sample-preparation technique for isolating and preconcentrating organic compounds, which is in compliance with all these requisites and, additionally, is highly sensitive and can be used for polar and no polar analytes. The most traditional SPME approach uses a fused-silica fiber coated with a polymeric stationary phase placed on a syringe. When the fiber is placed in contact with the sample, the analytes are transported from the matrix into the coating and the process continues until the equilibrium is reached between the sample matrix and the stationary phase. When equilibrium conditions are reached, then exposing the fiber for a longer time does not accumulate more analytes [21]. In the last years SPME is being increasingly used as sample-preparation technique for isolating pharmaceuticals and theirs metabolites from environmental samples [22–27].

The aim of the current work was to develop a reliable method for the extraction by SPME and determination by liquid chromatography–diode array detection (LC–DAD) of selected acidic and neutral pharmaceuticals: the non steroidal anti-inflammatory drugs (NSAIDs) naproxen, ketoprofen, diclofenac, piroxicam, indomethacin, sulindac and diflunisal and the anticonvulsant carbamazepine in river water samples. Pharmaceuticals were selected among the most frequently detected in surface water samples.

As a considerable number of variables can affect the extraction yield in the SPME procedure, and they may also be correlated, their optimization was carried out through a multivariate approach. Firstly, a Plackett–Burman design was employed as a screening step for the main parameters affecting the extraction and desorption processes and then, a central composite design (CCD) was used to optimize the values of the significant variables in order to obtain the best responses [28]. The multiple response criteria were successfully used to optimize peak areas of analytes and the analysis time using the desirability function.

NSAIDs are commonly analyzed in environmental water samples using hyphenated mass spectrometry techniques. Even though LC–MS and LC–MS–MS have been the detection technique of choice for detection and determination of these polar pharmaceuticals [29,30], in this paper we propose an inexpensive method based on the use of LC–DAD.

2. Experimental

2.1. Chemicals and solvents

Analytical standards (pestanal quality) of naproxen (NAPRO) and ketoprofen (KETO) were available from Riedel de Haën (Seelze, Germany) and diclofenac sodium (DICLO), piroxicam (PIR), sulindac (SUL), diflunisal (DIFLU) and carbamazepine (CBZ) analytical standards were purchased from Sigma–Aldrich (Steinheim, Germany) and indomethacin (INDO) analytical standard was obtained from Fluka (Buchs, Switzerland).

Acetonitrile (ACN) and methanol (MeOH) LC grade were obtained from J.T. Baker (Deventer, The Netherlands). Ultrapure water was obtained from a Milli-Q water purification system from Millipore (Bedford, MA, USA).

Hydrochloric acid (37%) and potassium dihydrogenphosphate (KH_2PO_4) analytical grade were purchased from Merck (Darmstadt, Germany).

LC mobile phases were filtered through a 0.45 μm cellulose acetate (water) or polytetrafluorethylene (PTFE) (organic solvents) and degassed with helium prior and during use.

2.2. Instrumentation and software

The SPME fiber assembly and SPME–LC interface were purchased from Supelco (Bellefonte, PA, USA). The SPME–LC interface consisted of a six-port injection valve and desorption chamber (chamber volume 60 μL) which replaces the injection loop of a six-port injection system. The SPME fibers polydimethylsiloxane/divinylbenzene (PDMS–DVB, 65 μm) and polyacrylate (PA, 85 μm), were purchased from Supelco (Bellefonte, PA, USA).

The LC system consisted of a Waters (Milford, MA, USA) instrument, composed of a gradient Model 600E pump and a Mod 2969 DAD. LC separations were performed with a Discovery RP-Amide C16 (150 mm × 4.6 mm, 5 μm particle size) column from Supelco (Bellefonte, PA, USA).

Data were saved in ASCII format, and transferred to a PC Sempron AMD microcomputer for subsequent manipulation by Design Expert Version 7.1.0 (Stat-Ease Inc., Minneapolis) chemometric program.

2.3. Preparation of standards and spiked samples

Stock individual standard solutions of pharmaceuticals were prepared in MeOH at concentration levels of 400 μg mL^{-1} and maintained under refrigeration at 4 °C in dark. In these conditions they were stable for at least 3 months. Working solutions were prepared daily by dilution of appropriate aliquots in the appropriate solvent and were filtered through Millipore membrane PTFE filters (0.45 μm particle size) before injection into the chromatographic system.

Working standard solutions were prepared in ACN–KH_2PO_4 buffer at pH 3.00 (50:50, v/v) as solvent. For recovery determinations, Milli-Q and river water samples adjusted at pH 3.00 were spiked with mixtures of all analytes at different concentration levels and were analyzed using the proposed SPME–LC–DAD methodology.

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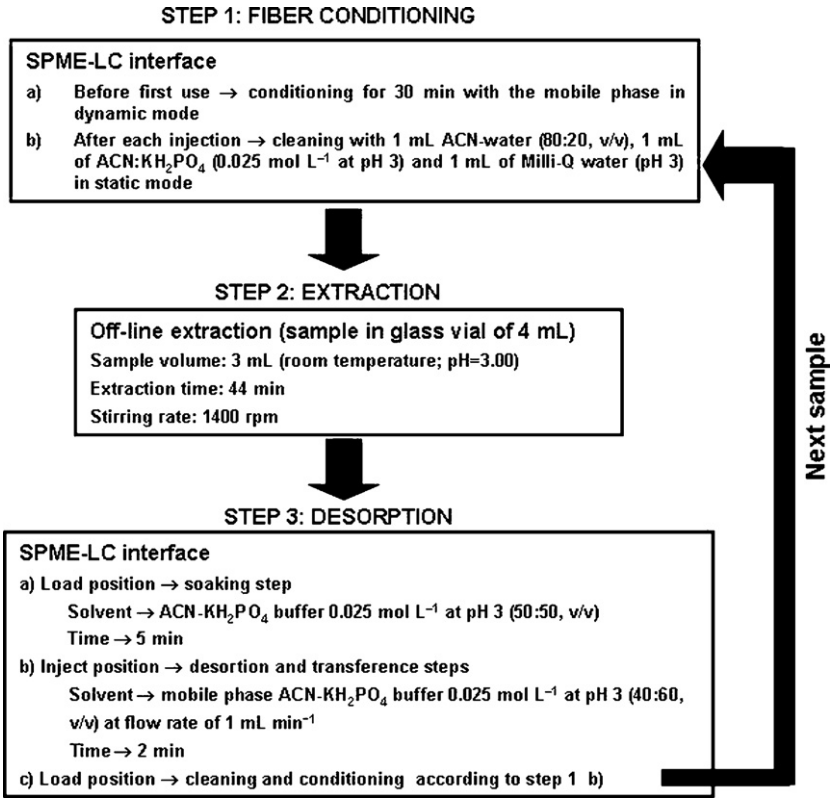

Fig. 1. Schematic diagram of the SPME–LC procedure: (A) conditionation step, (B) extraction step and (C) desorption step.

After collection, the river water samples were vacuum filtered through 0.45 μm acetate cellulose membrane from Millipore to remove suspended matter, acidified to pH 3.00 with hydrochloride acid and stored at 4 °C in dark.

2.4. SPME procedure

SPME experiments were performed using a fused-silica fiber coated with a thin layer of polymeric stationary phase (PDMS–DVB 60-μm film thickness) supplied by Supelco (Bellefonte, CA, USA).

Fig. 1 shows the full SPME procedure for the preconcentration of the analytes in the river water samples. This methodology includes three main steps: fiber conditioning (step 1), extraction of analytes into the SPME fiber from the river water sample (step 2) and, finally, automated desorption and transference of analytes from the fiber to the LC system using the SPME interface (step 3).

2.5. Liquid chromatography analysis

The SPME extracts were chromatographed by a programmed gradient with ACN as solvent A and 0.025 mol L^{-1} KH_2PO_4 buffer pH 3.00 as solvent B for 22 min at a flow rate ranged from 1.0 to 1.5 mL min^{-1}. The solvent program was as follows: initially 8 min isocratic with A:B 40:60 (v/v) at a flow rate of 1.0 mL min^{-1}, then 4 min linear gradient to A:B 50:50 (v/v) at a flow rate of 1.5 mL min^{-1} and 3 min isocratic with A:B 50:50 (v/v) at a flow rate of 1.5 mL min^{-1}, followed by an additional period of 2 min linear gradient to the initial conditions; finally 5 min in the initial conditions was sufficient time before subsequent analysis runs.

Under the above-described chromatographic conditions, all the analytes were simultaneously analyzed by DAD using a wavelength range between 200 and 350 nm.

2.6. Statistical methods for optimization

In order to evaluate the main factors affecting the efficiency of extraction, i.e. soaking period, desorption period, stirring rate, extraction time, sample pH, ionic strength, organic solvent and temperature, a Packett–Burman design with 12 experiments was performed. Then, the factors showing significant positive effects such as soaking time, stirring rate and extraction time were considered in a CCD design consisting in 20 experiments to find optimum factor levels for all the response signals by optimizing an objective function. Finally, the multiple response criteria using the desirability function were successfully used to optimize the analysis time through a final adjustment of the soaking period to 5 min.

3. Results and discussion

3.1. LC optimization

In order to achieve a good separation between CBZ, PIR, SUL, KETO, NAPRO, DIFLU, INDO and DICLO in a short analysis time, chromatographic conditions were evaluated and optimized.

According to the supplier recommendations, a Discovery RP-Amide C16 LC column from Supelco was selected for the separation of the target analytes.

The organic solvent content and the pH in the mobile phase are the usual main factors in reversed-phase liquid chromatographic separations, owing to their strong effects on retention and/or selectivity. As most of target pharmaceutical compounds are acidic (Table 1) it seems reasonable to start the LC analysis with a gradient using a low pH mobile phase to obtain sufficient retention into the chromatographic column and reproducible retention times in a first attempt. The benefits of using low pH in the mobile phase

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Table 1
Properties of the pharmaceutical compounds.

Analyte	Used as/effective as	pK_a	PM (g mol^{-1})	Group forming hydrogen bonds
CBZ	Antiepileptic	13.9	236.27	—NH_2
PIR	Anti-inflammatory	1.86/5.46	331.35	—OH
SUL	Anti-inflammatory	4.7	356.42	—COOH
KETO	Anti-inflammatory/analgesic	4.5	230.26	—COOH
NAPRO	Anti-inflammatory/analgesic/antipyretic	4.2	254.28	—COOH
DIFLU	Anti-inflammatory/analgesic	3.3	250.20	—OH
INDO	Anti-inflammatory/analgesic/antipyretic	4.5	357.79	—COOH
DICLO	Anti-inflammatory	4.0	296.15	—NHR

are first to avoid analyte interactions with silanols on the stationary phase and second to protonate the analytes.

To optimize the chromatographic separation, different mobile phases were tested consisting of ACN and KH_2PO_4 buffer (0.025 mol L^{-1}) solutions adjusted at different pH values lower than the pK_a of the target compounds, thus ensuring their complete protonation, and evaluating the resolution between the chromatographic peaks. Fig. 2 shows two chromatograms of a standard solution containing 25 μg L^{-1} of each analyte using ACN–KH_2PO_4 buffer at pH 3.00 and pH 3.50 and a LC gradient as mobile phase, respectively. As expected, acceptable separation for most of analytes was observed in both cases, except for NAPRO and DFLU, which appeared at the same retention time when using buffer at pH 3.50 as mobile phase. Higher pH values, even in the acidic range and at pH lower than the higher pK_a value of analytes, led to severe peak overlapping. Therefore, ACN–KH_2PO_4 buffer (0.025 mol L^{-1}) at pH 3.00 as mobile phase and the gradient elution program detailed above in LC procedure was chosen; these conditions showed a good compromise between adequate separation and short analysis time. Under the above-described chromatographic conditions, all the analytes were simultaneously analyzed by DAD using a wavelength range between 200 and 350 nm, according to the UV spectrum of each analyte.

In order to obtain the higher signal, solvent composition of standards and samples was tested. Thus, stock standard solutions of all analytes prepared using different percentages of ACN–KH_2PO_4 buffer and ACN–water were injected in the LC system. The best results were obtained using ACN–KH_2PO_4 buffer at pH 3.00 (50:50, v/v) as solvent for injection. In general, for the other mixtures assayed the analytical signals were lower and overlapping peaks were found for some analytes.

In addition, the volume of injection (ranged from 20 to 200 μL) was optimized by injecting the same amount of each analyte in the previously established LC conditions. The results obtained showed that the area and shape of peaks were kept constant up to 50 μL, whereas higher volumes yielded broadening and lack of resolution. Therefore, 50 μL of standard in ACN–KH_2PO_4 buffer at pH 3.00 (50:50, v/v) was selected.

Fig. 3 shows LC chromatograms of the eight pharmaceutical compounds at 230 nm for the quantitation of all analytes and at 254 nm for the quantitation of KETO in the established LC conditions.

3.2. Selection of adequate fiber coating

All the analytes considered in this study present low vapour pressure because their molecular mass is ranged between 150 and 450 g mol^{-1} and several compounds have hydrophilic group in their molecule (Table 1). Therefore, the concentration in the headspace is low and the transfer to the fiber is slow at ambient temperature [31]. Consequently, extraction was performed using immersion SPME instead of headspace SPME.

The commercially available SPME fibers PDMS, PDMS–DVB, PA and Carbowax–DVB clearly differ in their ability to extract various substances classes. Selection of the most appropriate fiber should take into account the polarity, volatility, hydrophilicity and molecular size of the analytes. Because all of the selected analytes are polar (they have groups forming hydrogen bonds), we firstly chose the polar fibers PDMS–DVB and PA, according to the rule "like dissolve like" [31–37].

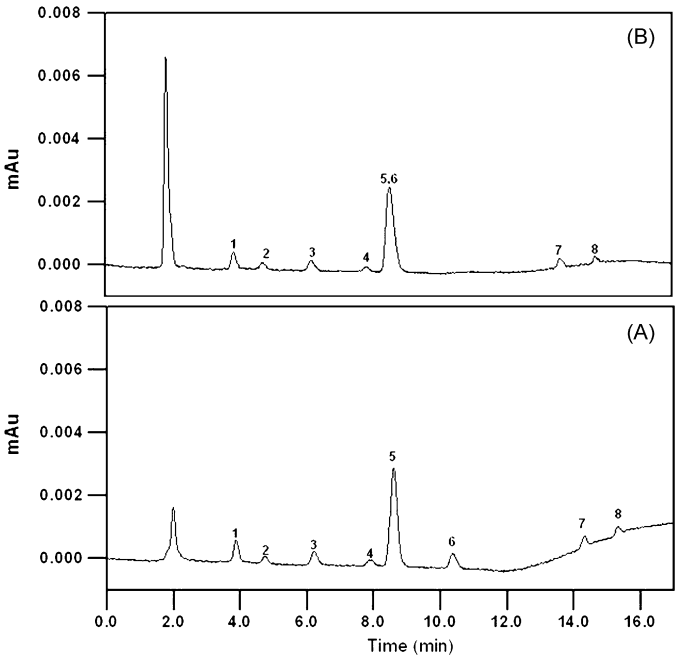

Fig. 2. LC chromatograms of a standard solution containing 25 μg L^{-1} of each analyte using ACN–KH_2PO_4 and 0.025 mol L^{-1} buffer at pH 3.00 (A) and pH 3.50 (B). (1) CBZ, (2) PIR, (3) SUL, (4) KETO, (5) NAPRO, (6) DIFLU, (7) INDO, (8) DICLO.

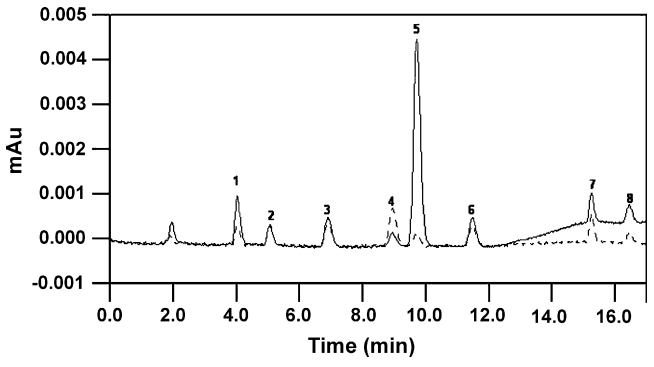

Fig. 3. LC chromatograms of a standard solution containing the eight pharmaceutical compounds (50 μg L^{-1}) at 230 nm (—) and 254 nm (- - - -) in the established LC conditions. (1) CBZ, (2) PIR, (3) SUL, (4) KETO, (5) NAPRO, (6) DIFLU, (7) INDO, (8) DICLO.

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Table 2
Probability values obtained when applying ANOVA to all the nine responses studied with the Plackett–Burman design built for factor selection.

Model	*p*-value[a,b]								
	CBZ	PIR	SUL	KETO	NAPRO	DIFLU	INDO	DICLO	Peak shape
	0.0015	0.0004	0.0019	0.0048	0.0128	0.0099	0.0027	0.0024	<0.0001
A – soaking solvent	0.0044 (+)	0.0135 (+)	0.1411	0.2662	0.1992	0.2173	0.0077 (+)	0.0045 (+)	<0.0001 (+)
B – soaking time	0.0178 (−)	0.2377	0.0076 (+)	0.0795 (+)	0.1569	0.0564 (+)	0.1	0.4407	0.1
C – desorption period	0.0066 (+)	0.0095 (−)	0.0128 (−)	0.0884 (−)	0.1704	0.0274 (−)	0.0140 (−)	0.0053 (−)	0.1
D – stirring rate	0.0039 (−)	0.1691	0.0047 (+)	0.0723 (+)	0.1348	0.0279 (+)	0.0417 (+)	0.0171 (+)	0.1
E – extraction time	0.0027 (+)	0.0486 (+)	0.2016	0.1	0.1713	0.2687	0.0324 (+)	0.0207 (+)	0.1
F – temperature	0.1	0.1774	0.1	0.2307	0.1	0.1	0.1523	0.0130 (−)	0.1
G – sample pH	0.0016 (+)	0.0236 (−)	0.0010 (−)	0.0039 (−)	0.0759 (−)	0.0046 (−)	0.0035 (−)	0.0019 (−)	0.1
H – ionic strength	0.0029 (+)	0.2159	0.0246 (−)	0.1185	0.2309	0.0476 (−)	0.4281	0.1	0.1
J – organic solvent	0.0004 (−)	<0.0001 (−)	0.0005 (−)	0.0016 (−)	0.0097 (−)	0.0029 (−)	0.0002 (−)	0.0004 (−)	0.1
K – dummy	0.0174	0.0105	0.0040	0.0262	0.1152	0.0233	0.0168	0.0063	0.1
L – dummy	0.0020	0.1	0.0177	0.2307	0.2264	0.0470	Aliased	Aliased	0.1

[a] Considered significant when $p < 0.1$.
[b] Signs between parenthesis correspond to the effects on the variables.

However, the PA coating was found to be highly susceptible to damage when it was exposed to the mobile phase gradient flow for 30 min during the conditioning procedure and so, we chose the PDMS–DVB fiber, which showed a robust behaviour.

3.3. Optimization of desorption mode

Two modes of desorption were evaluated for the PDMS–DVB fiber: dynamic and static desorption. With this aim, 3 mL Milli-Q water samples at pH 3.00, containing 1.00 μg mL^{-1} of each analyte were extracted by using stirring speed of 1000 rpm during 20 min, room temperature and without salt and organic solvent.

In the dynamic mode, after the extraction process, the SPME fiber was placed into the desorption chamber and the valve was immediately switched from the load to the inject position and the mobile phase at 1 mL min^{-1} was passed through the desorption chamber for 15 min in order to complete dynamic desorption.

On the other hand, static desorption was implemented using ACN–KH_2PO_4 0.025 mol L^{-1} buffer at pH 3.00 (80:20, v/v) as soaking solvent, soaking and extraction times being 15 and 5 min, respectively.

Better recoveries were obtained when using the static desorption mode vs. the dynamic one. In addition, peak broadening was observed in the dynamic mode, which indicates that the analytes were strongly adsorbed to the PDMS–DVB fiber and slowly desorbed by the mobile phase. Therefore, the static mode was selected and used for desorption in further steps in this study.

3.4. Carry-over study

Changes in the analytical signal may be due to analytes remaining adsorbed on the fiber between experiments, which would lead to erroneous results when optimizing the SPME procedure. In order to avoid this drawback, an evaluation of the carry-over effect was performed by a blank desorption experiment, which was performed after extraction of Milli-Q samples spiked with all the analytes at concentration levels of 1.00 μg mL^{-1} under experimental optimized conditions. Small amounts for all the analytes were detected on the fiber after desorption. Therefore, after each analysis the fiber was flushed with 1 mL ACN:water (80:20, v/v), 1 mL of ACN–KH_2PO_4 [0.025 mol L^{-1} at pH 3.00 (50:50, v/v)] and 1 mL of Milli-Q water at pH 3.00 in order to remove any traces of analytes or residues of crystallized salts.

3.5. Optimization of SPME procedure

3.5.1. Screening phase

The SPME procedure is the result of the combination of two steps (extraction and desorption steps) each one depending on numerous factors, the sequential study of all the potential factors being too complex, and involving a prohibitive long experimental time. Consequently, an experimental Plackett–Burman design was built for the determination of the main factors affecting the extraction efficiency. The analyzed factors were: the stirring rate, extraction time, temperature, ionic strength, organic solvent content and pH in the extraction step and soaking period, soaking solvent and desorption period in the desorption step.

The mentioned factors were evaluated at two levels, selected according to previous experiments. The evaluation consisted in analyzing a Milli-Q water sample, spiked at 1.00 μg mL^{-1} of all analytes, at all the cited conditions and evaluating area and peak shape in each case. Then, an analysis of variance (ANOVA) test was applied to the experimental data, using the effects of dummy variables (K and L in Table 2) to obtain an estimate of standard errors in the

Table 3
Analysis of the effect of each variable on the responses when analyzing the Plackett–Burman design built for factor selection.

Factors	Effect	Causes (observations)	Final conditions	Reference
Soaking solvent	Positive	Increasing in the peak areas but causes peak bordering, peak overlapping and lost of resolution.	ACN–0.025 mol L^{-1} KH_2PO_4 buffer (50:50, v/v)	
Soaking time	Positive	Allows for a full desorption of the analyte from the fiber.	Optimization	
Desorption period	Negative	Diminution of peak area.	2 min	
Stirring rate	Positive	Allows analyte accesses to the coating.	Optimization	[31,32]
Extraction time	Positive	Allows reaching the equilibrium.	Optimization	[31]
Temperature	Negative	Decreases the amount of extracted analyte.	Room temperature	[31]
Sample pH	Negative	Increasing of water analyte solubility, thus decreasing the affinity to the fiber.	pH 3.00	[31,32]
Ionic strength	Negative	Effect on fiber stability due to the crystallization of salt on the fiber coating.	Without salt	[33–36]
Organic solvent	Negative	Increasing in the hydrophobic character of the sample solution, hence decreasing the coefficient of distribution between both phases.	Without organic solvent	[37]

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Table 4
Statistical values obtained when applying ANOVA of lack of fit and regression of the selected models.

Response (peak area)	Model	*p*-value[a]		*R*-squared	C.V. (%)
		Model	Lack of fit		
CBZ	Linear	<0.0001	0.0804	0.771	3.91
PIR	Linear	<0.0001	0.8894	0.906	3.40
SUL	Cubic	<0.0001	0.8546	0.997	0.98
KETO	Linear	<0.0001	0.1115	0.750	6.41
NAPRO	Quadratic	<0.0001	0.0636	0.947	4.57
DIFLU	Linear	<0.0001	0.1717	0.899	4.06
INDO	Linear	<0.0001	0.0929	0.868	7.13
DICLO	Linear	<0.0001	0.0833	0.804	8.23

[a] Considered significant when $p < 0.05$.

coefficients. As a conclusion of this analysis, all the variables were shown to be significant ($p < 0.1$), with positive and negative effects (Table 2).

The extraction efficiency was statistically improved when the soaking solvent, soaking time, stirring rate, and extraction time increased from low to high level for most of the target analytes.

The greatest peak areas were obtained using ACN–KH_2PO_4 [0.025 mol L^{-1} pH 3.00 (80:20, v/v)] as soaking solvent. However, a peak broadening effect was observed when using this soaking solvent, fact that caused peak overlapping and loss of resolution. Consequently, additional studies using different mixtures of ACN–KH_2PO_4 (0.025 mol L^{-1} at pH 3.00) as soaking solvent were performed. The best results were obtained when using a mixture of ACN– KH_2PO_4 [0.025 mol L^{-1} pH 3.00 (50:50, v/v)], since better desorption and peak shape were achieved. Thus, only soaking time, stirring rate, and extraction time were considered in a further optimization analysis.

On the other hand, the desorption period, temperature, sample pH, ionic strength and organic solvent showed negative effects (decreasing the extraction efficiency) when levels changed from low to high value. The anti-inflammatory and antiepileptic drugs used in this work have different pK_a values (see Table 1), and therefore the partitioning of the drugs between the sample and the fiber is strongly affected by pH. The behaviour of each factor is resumed in Table 3. In the light of these results, the following SPME extraction procedure was selected: room temperature, pH 3.00, without salt and organic solvent and using ACN–KH_2PO_4 [0.025 mol L^{-1} pH 3.00 (50:50, v/v)] buffer as soaking solvent.

3.5.2. Response surface design

An optimization procedure was applied in order to find out the exact values of the most important factors to obtain maximum peak areas. Systematic optimization procedures are carried out by selecting an objective function, finding the most important factors and investigating the relationship between responses and factors by the so-called response surface methodology (RSM) [28]. A CCD was used to optimize the three variables (soaking time, stirring rate and extraction time), consisting of 20 experiments: combinations of the selected independent variables in the following ranges: (a) soaking time: 5–17 min, (b) stirring rate: 800–1400 rpm and (c) extraction time: 21–44 min. These ranges were selected based on prior knowledge about the system under study.

On the other hand, other variables such as soaking solvent, desorption period, temperature, sample pH, ionic strength and organic solvent were set fixed according to the results obtained in the screening phase (see above). Their values were: (a) soaking solvent: ACN–KH_2PO_4 [0.025 mol L^{-1} pH 3.00 (50:50, v/v)], (b) desorption period: 2 min, (c) room temperature, (d) without salt, (e) without organic solvent, and (f) sample adjusted to pH 3.00.

Experiments for the CCD were performed in two blocks (two consecutive days) in order to remove the expected variation caused by some change during the course of the experiment [28] by SPME preconcentration of Milli-Q water samples spiked at 1.00 μg mL^{-1} of all analytes. Peak areas for all the experiments were fitted to polynomial models, once outliers were removed by analyzing the difference between fitted values test (DFFITS). This test measures the influence each point has on the predicted value, computing a standardized value, which can be interpreted as the number of standard deviation units owed to experimental data exerts disproportionate influence on the model [28].

The model coefficients were calculated by backward multiple regression [28] and validated by the ANOVA. As can be seen in Table 4, linear and modified quadratic and cubic models are those which better explain the behaviour of peak areas under the studied factors, although irrelevant main terms were maintained in order to fit hierarchical models. As can be appreciated in Table 4, the lack of fit test is not significant ($p > 0.05$) in all cases. Table 4 also shows the statistical parameters corresponding to the fitting for peak areas. The coefficient of variation was ranged between 0.98% and 8.23%. The large adjusted *R*-squared (between 0.773 and 0.997) values indicate a good relationship between the experimental data and the fitted models.

Table 5 shows the probability values for each factor corresponding to the selected model. As can be observed, most model terms are significant ($p < 0.05$). The *p*-values showed that at 95% confidence level, the soaking period affects the areas of PIR, SUL and NAPRO, the stirring rate affects the areas of CBZ, NAPRO, DIFLU, INDO and DICLO and all responses were affected by the extraction time. Besides the interactions (a) between the three factors, (b) between stirring rate and extraction time, and (c) between the squared of soaking period

Table 5
Probability values for each factor corresponding to the selected model when applying ANOVA test.

Source	Prob > *F*[a]							
	CBZ	PIR	SUL	KETO	NAPRO	DIFLU	INDO	DICLO
A – soaking period (min)	0.7158	0.0108	<0.0001	0.6846	0.0023	0.0608	0.0878	0.1752
B – stirring rate (rpm)	0.0292	0.601	0.822	0.7462	0.0096	0.0204	0.0076	0.0315
C – extraction time (min)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
AB	–	–	0.2108	–	0.0877	–	–	–
AC	–	–	0.0842	–	–	–	–	–
BC	–	–	0.0002	–	–	–	–	–
A^2	–	–	0.6163	–	0.0005	–	–	–
B^2	–	–	0.0639	–	0.0203	–	–	–
C^2	–	–	0.796	–	0.0009	–	–	–
ABC	–	–	0.0118	–	–	–	–	–
A^2C	–	–	< 0.0001	–	–	–	–	–
A^2B	–	–	0.5371	–	–	–	–	–

[a] Considered significant when $p < 0.05$.

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Table 6
Criteria for the optimization of the individual responses.

Response (area)	Criteria	Lower limits	Upper limits	Importance
CBZ	Maximize	153,080	205,584	5
PIR	Maximize	163,579	239,736	5
SUL	Maximize	333,539	518,286	3
KETO	Maximize	246,031	434,902	4
NAPRO	Maximize	1186,719	2179,539	1
DIFLU	Maximize	717,205	1185,552	2
INDO	Maximize	447,500	926,924	2
DICLO	Maximize	347,938	689,684	3

and extraction time were significant for SUL area, while the squared of soaking solvent, stirring rate and stirring time were significant for NAPRO area.

3.5.3. Multi response optimization

When a single response is being analyzed, the model analysis indicates areas in the design region where the process is likely to give desirable results, which is a relatively easy task. However, a function of more than one response can be used: the desirability function, which includes the researcher's priorities and desires on building the optimization procedure. Its application involves creating a function for each individual response d_i and finally obtaining a global function D that should be maximized choosing the best conditions of the designed variables. The latter function varies from 0 (value totally undesirable) to 1 (all responses are in a desirable range simultaneously), and can be defined by the Eq. (1):

$$D = (d_1^{r1} \times d_2^{r2} \times \ldots \times d_m^{rm})^{1/\sum r_j} = \left(\prod_{j=1}^{m} d_j^{rl}\right)^{1/\sum r_j} \quad (1)$$

where $d_1, \ldots, d_m$ correspond to the individual desirability function for each response being optimized, m is the number of responses in the measure and r is the importance relative of each response over the other responses.

Following the mentioned procedure, eight responses were simultaneously optimized by using the desirability function. Table 6 shows the criteria, which were followed to maximize the individual responses (peak area), the lower and upper limits and the importance assigned to each response, giving more importance to the analytes with smaller areas (CBZ and PIR) and in decreasing order of importance to the rest of analytes.

Under the mentioned optimization criteria, the experimental conditions corresponding to one maximum in the desirability function (D = 0.941) are: soaking period 17 min, stirring rate 1372 rpm and stirring time 43.75 min.

A complementary study was performed in order to evaluate the possibility of reducing ever more the analysis time, which consisted in adding a new response to the optimization procedure: the total analysis time.

As a consequence of the new study, the experimental conditions corresponding to one maximum in the desirability function (D = 0.766) are: soaking period 5 min, stirring rate 1400 rpm and stirring time 44 min. A soaking time of 5 min significantly decreases the total time of analysis, compared with the previously selected 12 min.

Following the conditions and restrictions previously discussed, the optimization procedure was carried out and the response surfaces obtained for the global desirability function are presented in Fig. 4A–C. These plots were obtained for a given pair of factors, while maintaining the other fixed at their optimal values. As can be seen in Fig. 4A (soaking period vs. stirring rate) when the soaking period is between 11 and 15 min the desirability is 0 (considering the stirring rate complete range). This fact is caused because the soaking period is too long so increases the analysis time despite that the peak area increased. The desirability increases when soaking period is decreased.

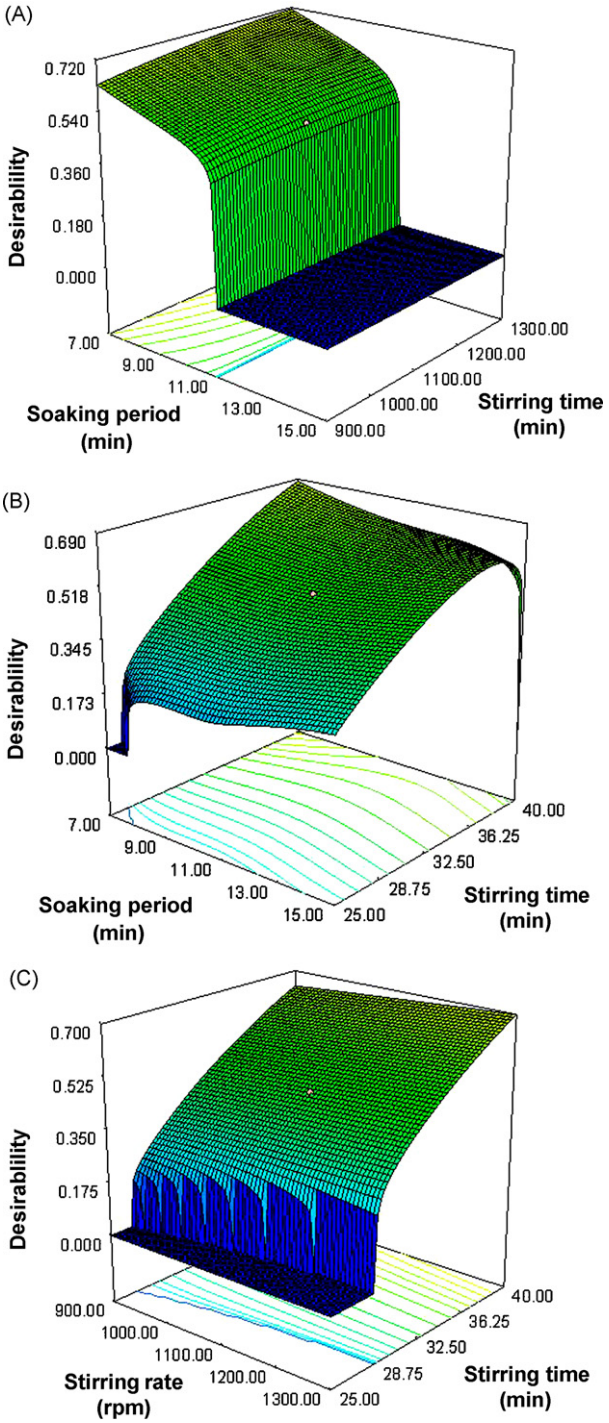

Fig. 4. Response surface plots corresponding to the desirability function when optimizing the following pair of factors, while maintaining constant the remaining one at their optimum values: (A) soaking period–stirring rate; stirring time = 43.75 min, (B) soaking period–stirring time; stirring rate = 1400 rpm and (C) stirring rate–stirring time; soaking period = 5 min.

In Fig. 4B (soaking period vs. stirring time), the desirability is greater than 0 for the entire range analyzed. Although, as the stirring time increases, the desirability becomes higher, probably because the contact time of the analytes with the fiber is increased, fact that produces a larger recovery.

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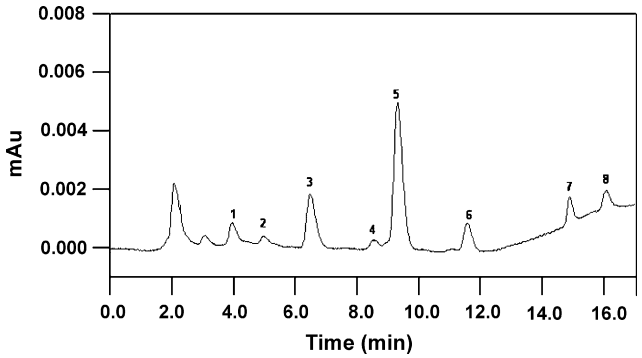

Fig. 5. SPME–LC–DAD chromatogram of a Milli-Q water containing 10 μg L^{-1} of each pharmaceutical. (1) CBZ, (2) PIR, (3) SUL, (4) KETO, (5) NAPRO, (6) DIFLU, (7) INDO, (8) DICLO.

Finally, Fig. 4C (stirring rate vs. stirring time) shows that when the stirring time is less than 25 min, the desirability is 0. This fact can be ascribed to an insufficient contact time of the analytes–fiber to obtain good recoveries. However, as the stirring time increases, the desirability becomes better, because the contact time of the analytes with the fiber is increased, allowing larger recoveries.

The values suggested through the optimization procedure were experimentally corroborated, and the corresponding chromatogram obtained after SPME for a mixture containing 10 μg L^{-1} of each pharmaceutical compound is shown in Fig. 5.

3.6. Validation of the instrumental method

Firstly, the instrumental method was validated by using univariate methodology, based on peak areas at a fixed wavelength (252 nm for KETO and 230 nm for the other analytes). The analytical figures of merit, calculated by using Milli-Q and river water samples fortified with the analytes and undergone to SPME–LC–DAD optimum conditions described in experimental section, are summarized in Table 7.

Method detection limits (MDLs) were calculated as proposed by the U.S. EPA [38] in such way that this parameter takes into account not only matrix effect, but also the variability introduced by all the sample processing steps.

With this aim, seven river water samples were spiked at concentration levels between 5.0 and 10.0 μg L^{-1}, depending on the compound, and the procedure described by the U.S. EPA was carried out. In this way, each replicate was processed through the entire analytical method and an initial estimate of the MDL was then calculated by multiplying the standard deviation of the results by the appropriate t statistic.

$$\mathrm{MDL} = t_{(n-1,\alpha=0.01)} \times S_A$$

where n is the number of replicate analyses, S_A is the standard deviation of the replicate analyses, and t is the student's t value for $n-1$ degrees of freedom at 99% confidence level.

Thus, the estimated MDLs were between 1.0 and 4.0 μg L^{-1}, according to the compound.

Next, seven aliquots of river water samples, spiked at concentration levels ranging between 8.0 and 20.0 μg L^{-1}, depending on the compound, were analyzed through the entire method and S_B was also calculated for each pharmaceutical. After to verify that S_A and S_B were not statistically significant (based on the F statistic of their ratio), these two variances were pooled to obtain a single estimated S^2 as follows:

$$S^2_{\mathrm{pooled}} = \frac{(n_A-1)S^2_A + (n_B-1)S^2_B}{n_A+n_B-2}$$

where n_A and n_B are the number of samples analyzed in each set.

The MDLs were then calculated using the pooled standard deviation as:

$$\mathrm{MDL} = t_{(n_A+n_B-2,\alpha=0.01)} \times S_{\mathrm{pooled}}$$

The values of MDL obtained in this way for the target analytes ranged between 0.5 and 3.0 μg L^{-1} and they are summarized in Table 7.

Quantitation limits (LOQs) were calculated, according to the EURACHEM Guidance [39] as the lowest concentration of the analyte for which the relative standard deviation (RSD) of the signal is equal to a fixed percentage (10% in our case). River water samples (n = 3) fortified with the analytes were processed through the entire analytical method and the results obtained are summarized in Table 7.

Linear range was established for each pharmaceutical, the lower limit being the LOQ calculated according the above criterion and the upper limit, the concentration for which the signal deviates from the linearity by 3–5% [40].

Calibration curves were obtained with eight standards prepared in Milli-Q water covering the whole linear range (each point in triplicate), processed through the entire analytical method. They showed good linear relationship (r^2 > 0.993) between 1.0 and 50.0 μg L^{-1} for NAPRO, between 2.0 and 50.0 μg L^{-1} for SUL, and between 4.0 and 50.0 μg L^{-1} for the other analytes.

In addition, when calibration graphs obtained using solvent-based and matrix-matched standards (prepared with extracts from Milli-Q and river water samples, respectively) were compared by a t-test [41], no significant differences were found, which demonstrates that the river water matrix does not affect the analytical signal for the target pharmaceuticals. Therefore, calibration graphs built with standards prepared in Milli-Q water samples were used quantitation in further experiments.

The repeatability was tested at an intermediate concentration level (20.0 μg L^{-1} for each analyte in the river water, n = 6) with

Table 7
Analytical figures of merit for the determination of pharmaceutical compounds in water using SPME-LC-DAD.

Analyte	Linear range (μg L^{-1})	r^2	RSD (%)[a]		MDLs (μg L^{-1})	LOQs (μg L^{-1})
			Repeatability	Intermediate precision		
CBZ	4.0–50.0	0.994	8.0	8.5	3.0	4.0
PIR	4.0–50.0	0.994	4.6	6.1	2.6	4.0
SUL	2.0–50.0	0.997	4.9	5.4	0.8	2.0
KETO	4.0–50.0	0.991	7.7	8.9	2.2	4.0
NAPRO	1.0–50.0	0.999	8.2	9.3	0.5	1.0
DIFLU	4.0–50.0	0.992	4.3	5.1	0.7	4.0
INDO	4.0–50.0	0.998	4.4	7.6	2.2	4.0
DICLO	4.0–50.0	0.997	5.9	6.4	1.5	4.0

[a] 20.0 μg L^{-1} (n = 6).

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Table 8
Some chromatographic methods for detecting NSAIDs and CBZ in surface water samples.

Compounds	Analytical technique	Extraction method	LODs or LOQs (ng L^{-1})	Recovery (%)	RSD (%)	Reference
NSAIDs and CBZ	GC–MS	SPME (Carbowax-DVB)	LODs ≤ 50	–	≤18	[22]
NSAIDs	GC–MS	SPE (Oasis HLB)	LODs ≤ 10	71–99	≤7	[11]
NSAIDs	GC–MS	SPE (SDB-XC)	LODs ≤ 3.5	47–88	≤13	[42]
NSAIDs	GC–MS	SPE (Strata X)	LODs ≤ 6	84–157	–	[43]
NSAIDs and CBZ	GC–MS	SPE (Oasis HLB)	LODs ≤ 8	59–100	≤10	[44]
NSAIDs and CBZ	GC–MS	SPE (Oasis HLB)	LOD ≤ 10	47–130	≤17	[9]
NSAIDs and CBZ	LC–MS–MS	SPE (Bondesil ODS)	LODs ≤ 0.6	43–96	–	[45]
NSAIDs	LC–MS	SPE (LiChrolut-EN)	LODs ≤ 1	79–91	≤15	[12]
NSAIDs	LC–MS–MS	SPE (Oasis MCX)	LODs ≤ 1	88–110	≤11	[46]
NSAIDs	LC–QqTOF	SPE (Oasis HLB)	LOQ ≤ 3	88–110	≤10	[47]
NSAIDs and CBZ	LC–MS–MS	SPE (Oasis HLB)	LOD ≤ 10	62–89	≤34	[48]
NSAIDs	LC–DAD–MS	SPE (LiChrolut RP-18)	LOD ≤ 59	80–103	≤8	[49]
NSAIDs and CBZ	LC–MS–MS	SPE (HLB)	LOD ≤ 12	73–100	≤15	[50]
NSAIDs and CBZ	LC–MS–MS	SPE (Oasis HLB)	LOD ≤ 0.1	61–89	≤11	[51]
CBZ	LC–MS–MS	SPE (Oasis HLB)	LOD = 3	99	2.2	[52]

RSDs values lower or equal to 8.2%. The intra-day precision was also tested with six repeated analyses of river water samples containing the analytes at 20.0 μg L^{-1}, the RDSs being lower than or equal to 9.3%.

Comparing the above results of validation with those reported by other authors (Table 8), it was found that the SPME–LC–DAD method provides precision values (between 4.3% and 9.3%) similar or better than most of reported off-line SPE methods using commercially available cartridges [9,11,12,42–52], due to the avoidance of the intermediate steps in manual SPE applications. In addition, the values of intermediate precision are not different from the repeatability values and therefore external factors have no influence on the precision of the results.

Other advantage of the SPME over SPE is its cost-effectiveness as SPME is a solvent-free technique and SPME fibers are re-usable. An additional advantage of this SPME methodology is that the enrichment of the analytes is more easy and rapid than with SPE techniques. The only advantage of SPE is the possibility of improving the LODs and LOQs using large volume samples (Table 8).

3.7. Estimation of the expanded uncertainty

The determination of the global uncertainty associated to the results obtained by applying the proposed methodology was evaluated according to the EURACHEM/CITAC guide [53].

This approach uses the "bottom up" procedure, which assesses each individual uncertainty for every single step of the measurement process, where the combined standard uncertainty results from the sum of each contribution. In this way, a detailed analysis of uncertainty sources can show critical stages of the analytical method where uncertainty should be reduced.

Then, the expanded uncertainty, that provides an interval within which the value of the mesurand is believed to lie with higher level of confidence, was calculated by multiplying the combined standard uncertainty by a coverge factor (k).

In this SPME–LC method, the main identified uncertainty contributions were the uncertainty associated to the instrumental calibration (u_{cal}), the uncertainty associated to repeatability (u_{repeat}) and the uncertainty associated to the matrix (u_{matrix}) which includes the uncertainty associated to preparation of the river water sample for SPME extraction and the uncertainty associated to recovery.

The results of these individual uncertainties allowed the calculation of the combined uncertainty (u_{C_0}) and finally the expanded uncertainty (U) using a coverage factor $k = 2$ for a confidence level of 95%.

The combined standard and expanded uncertainties for a concentration level of the analyte in the real sample (C_0) were calculated using the equations:

$$u_{C_0} = \sqrt{u_{cal}^2 + u_{repeat}^2 + u_{matrix}^2}, \quad U = k u_{C_0}$$

The obtained results are presented in Table 9 for a concentration of all analytes in the river water sample at two levels (20 μg L^{-1} and the average for different concentration levels). The expanded uncertainty was lower than 29.5% of the final concentration for all compounds and similar for the two studied levels. As can be seen the uncertainty associated to the calibration curve (u_{cal}) is the main responsible for such variation in all cases while the uncertainties associated to the repeatability (u_{repeat}) and the samples matrix (u_{matrix}) are quite similar, although increasing when using river water samples spiked at different concentration levels.

NSAIDs have also been determined by LC–DAD using SPE in the preconcentration step [49]. In this case, the combined standard uncertainty associated with a concentration measurement was considered dependant on the uncertainty associated with:

Table 9
Detailed evaluation of uncertainty contributions in the determination of NSAIDs in river water samples by SPME–LC–DAD.

Compound	20 μg/L				22.4 μg/L[b]			
	u_{cal}	u_{repeat}	u_{matrix}	U (%)[a]	u_{cal}	u_{repeat}	u_{matrix}	U (%)[a]
CBZ	1.02	0.035	0.079	10.5	1.02	0.162	0.051	10.5
PIR	1.08	0.020	0.045	11.0	1.08	0.227	0.045	11.0
SUL	2.07	0.020	0.043	20.5	2.06	0.225	0.094	21.0
KETO	0.85	0.020	0.056	8.5	0.85	0.208	0.076	9.0
NAPRO	2.46	0.035	0.083	24.5	2.46	0.220	0.063	24.5
DIFLU	2.02	0.020	0.043	20.0	2.04	0.178	0.180	20.5
INDO	1.73	0.023	0.044	17.5	1.74	0.205	0.102	17.5
DICLO	2.91	0.032	0.059	29.0	2.93	0.203	0.117	29.5

[a] 95% confidence level.
[b] Average concentration for five river water samples.

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Table 10
Predictions for the test samples obtained by spiking different analyte amounts on a real river water sample.

Sample	Added ($\mu g\,L^{-1}$)	Found ($\mu g\,L^{-1}$)[a]							
		CBZ	PIR	SUL	KETO	NAPRO	DIFLU	INDO	DICLO
1	12.0	8.9 (74.1)	14.2 (118.6)	10.4 (86.8)	14.7 (122.8)	14.2 (118.1)	13.2 (110.0)	13.4 (111.6)	14.1 (117.4)
2	15.0	11.5 (76.6)	17.8 (119.0)	11.3 (75.2)	16.5 (109.8)	16.1 (107.3)	11.2 (74.9)	12.9 (85.9)	12.3 (82.2)
3	25.0	18.1 (72.4)	29.7 (119.0)	22.0 (88.0)	29.7 (119.0)	28.2 (113.0)	24.7 (99.0)	26.3 (105.1)	27.2 (108.7)
4	25.0	19.8 (79.4)	28.1 (112.3)	24.3 (97.3)	26.6 (106.2)	29.5 (117.9)	25.4 (101.8)	25.3 (101.1)	26.8 (107.1)
5	35.0	25.0 (71.6)	36.5 (104.2)	31.4 (89.7)	33.8 (96.6)	34.2 (97.6)	25.3 (72.5)	31.1 (89.0)	33.3 (95.1)

[a] Recovery (%) between parentheses.

(i) the calibration step, (ii) the final volume of the extract, (iii) the volume of the sample, (iv) the LOD and (v) the recovery of extraction, the major source of uncertainty being the LOD followed by the calibration step.

As for SPME, the main source of uncertainty found in this work was those associated with the calibration step. This finding is in agreement with the results obtained by other authors, who compared the uncertainty associated to the determination of nonylphenol in water by using SPE and SPME [54].

Moreover, it must be pointed out that the expanded uncertainty associated to the proposed SPME–LC–DAD methodology is significantly lower than the values found by other authors in the analysis of NSAIDs in environmental water samples using SPME–LC–DAD [49].

3.8. Application of the analytical method to river water samples

Nacimiento river water samples from a selected location in Almería (Spain) were analyzed by the developed method without detecting the target compounds. Fig. 6 shows two chromatograms corresponding to extracts from a river water sample and a river water sample spiked with 10 $\mu g\,L^{-1}$ of each pharmaceutical. Peaks were well resolved and showed no interferences with the river water matrix.

With the aim of checking if the method is suitable for determining these pharmaceuticals in river water, five river water samples were spiked at different concentration levels of each analyte (between 12.0 and 35.0 $\mu g\,L^{-1}$), extracted and analyzed following the described method. Recoveries for the target pharmaceuticals obtained using calibration graphs built with standards of analytes prepared in Milli-Q water preconcentrated with the SPME procedure were between 71.6% and 122.8% (Table 10) which can be considered satisfactory. These recoveries are quite similar to those obtained by other procedures to determine these compounds in surface waters (Table 8) mainly using SPE–LC–MS techniques.

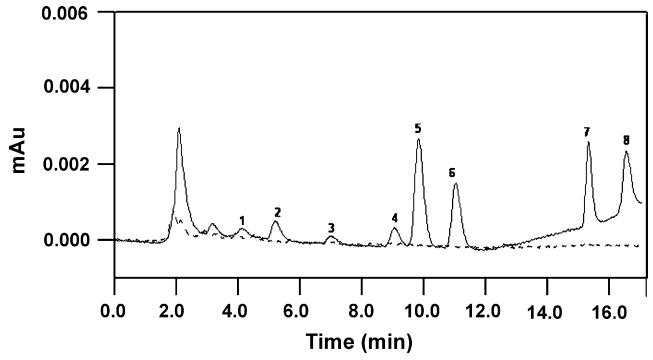

Fig. 6. SPME–LC–DAD chromatograms corresponding to extracts from a river water sample (- - - -) and a river water sample spiked (—) with 10 $\mu g\,L^{-1}$ of each pharmaceutical.

4. Conclusions

The use of solid-phase microextraction and liquid chromatography coupled to diode array detection provides a simple, efficient, selective and low cost methodology for the determination of seven non steroidal anti-inflammatory and the anticonvulsant carbamazepine.

The multivariate optimization strategy used, i.e. experimental design and response surface methodology enhanced by the application of the desirability function, allowed the successful determination of the optimal SPME extraction conditions.

The proposed method showed no matrix effect when calibration graphs built with Milli-Q water-based and river water-matched were compared, which makes it possible to use Milli-Q water based standards for quantitation.

Expanded uncertainties of the results in river water samples were <30% for all pharmaceutical compounds, according to the commonly established requirements for analytical measurements.

The SPME technique coupled to LC–DAD permits reducing the cost of analysis due to the simple equipment and to the low times of analysis. In addition, it decreases the influence of sample handling by the analyst in the final results, thus lowering the uncertainty associated with these steps.

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