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Solid-phase microextraction with on-fiber derivatization for the analysis of anti-inflammatory drugs in water samples☆

I. Rodríguez*, J. Carpinteiro, J.B. Quintana, A.M. Carro, R.A. Lorenzo, R. Cela

Departamento de Química Analítica, Nutrición y Bromatología, Universidad de Santiago de Compostela, Santiago de Compostela 15782, Spain

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Abstract

A sensitive and solvent-free procedure for the determination of non-steroidal acidic anti-inflammatory drugs in water samples was optimized using solid-phase microextraction (SPME) followed by on-fiber silylation of the acidic compounds and gas chromatography–mass spectrometry (GC–MS) determination. Microextraction was carried out directly over the filtered water samples using a polyacrylate fiber. Derivatization was performed placing the SPME fiber, loaded with the extracted analytes, in the headspace of a vial containing 50 μl of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA). Derivatives were desorbed for 3 min in the GC injector. Influence of several parameters in the efficiency of microextraction (volume of sample, time, pH, type of fiber coating, etc.) and derivatization steps (time, temperature and volume of MTBSTFA) was systematically investigated. In the optimal conditions an excellent linearity over three orders of magnitude and quantification limits at the ng/l level (from 12 to 40 ng/l) were achieved. The proposed method was applied to the determination of acidic compounds in sewage water and results compared to those obtained using solid-phase extraction (SPE) followed by the derivatization of the compounds in the organic extract of the solid-phase extraction cartridge.
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Keywords: Solid-phase microextraction; Derivatization, SPME; On-fiber derivatization; Nonsteroidal anti-inflammatory drugs

1. Introduction

Non-steroidal acidic anti-inflammatory drugs (NSAIDs) are among the group of pharmaceutical compounds most often used in human health care and in veterinary applications. Their annual prescription in developed countries achieve several hundreds of tons [1]. Urinary elimination of the free drugs and of their metabolites, together with their incomplete removal in sewage water treatment plants, have caused the presence of several acidic anti-inflammatory drugs (e.g. ibuprofen, naproxen, diclofenac) in the effluent of water treatment plants at the low ng/ml level and even in river and groundwater in the ng/l range [2–5].

Procedures for the analysis of acidic pharmaceuticals in water samples consist on a preconcentration step, usually using solid-phase extraction (SPE) sorbents [5–7], followed by their chromatographic separation and mass spectrometric detection. HPLC–MS has been proposed for the determination of these species in water samples [8,9]; however, better detection limits are achieved with GC–MS after derivatization of the native drugs to less polar compounds [4,5,10–12]. SPE minimize the use of organic solvents but requires large sample volumes (between 0.5 and 2 l), which previously need to be filtered in order to avoid clogging of the SPE cartridge or membrane. Obviously, filtration is a time-consuming operation specially in the case of sewage water samples containing high levels of suspended particles. Moreover, some derivatization reagents, e.g. diazomethane, are not compatible with the organic solvent used to elute the acidic compounds from the SPE sorbent. Therefore this extract should be evaporated to dryness before derivatization, and once this reaction had been completed the excess of derivatization reagent removed [4]. As a consequence a multi-step sample preparation scheme is obtained.

Solid-phase microextraction (SPME) requires less sample volume than SPE; furthermore, it is a completely solvent free, easily automated technique which allows high enrichment factors in the concentration of organic compounds

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* Corresponding author. Tel.: +34-981-563100x14387; fax: +34-981-595012.
E-mail address: qnisaac@usc.es (I. Rodríguez).

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in aqueous matrices. This technique is specially attractive when the extracted analytes can be thermally desorbed in the hot injector of a gas chromatograph. In fact, SPME fibers with polar coatings have been already evaluated for the determination of ibuprofen and naproxen in water samples using GC–MS detection [11,13]; however, the polarity of these compounds, mainly due to the presence of a carboxylic group, led to wide peaks and therefore relatively high detection limits. Obviously, this is a common problem in the GC analysis of polar species.

The detectability problem of polar species using SPME and GC analysis can be overcome with on-fiber derivatization of the native species [14,15]. On-fiber derivatization can be performed simultaneously with the extraction step, exposing the SPME fiber previously loaded with the derivatization reagent to the sample [16], or after concentration of the analytes in the coating, placing the SPME fiber in the headspace of a vial containing the derivatization reagent [17–19]. The first approach improves not only the detectability of polar compounds in gas chromatography, but also influences the distribution of the analytes between the sample and the fiber coating. However, the second is preferable when water sensitive derivatization reagents are employed and polar non-volatile species, with low affinity for the headspace phase, are determined in aqueous matrices.

The aim of this work is to optimize a sample preparation procedure for the analysis of five non steroidal anti-inflammatory species (ibuprofen, naproxen, ketoprofen, tolfenamic acid and diclofenac) in aqueous samples using SPME, on-fiber silylation of the native species with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) and GC–MS analysis. This compound was selected as the derivatization reagent since it has been reported that SPME fibers are more stable when exposed to the vapours of MTBSTFA than to other silylation reagents, e.g. *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), also usually employed in the derivatization of polar drugs, [21]. As MTBSTFA is sensitive to moisture, derivatization was performed in the SPME fiber after extraction. Influence of fiber coating, together with microextraction and derivatization conditions in the performance of the analytical procedure were systematically investigated. Results were compared to those obtained using SPE followed by the derivatization of the eluted species with MTBSTFA.

2. Experimental

2.1. Reagents, materials and samples

HPLC-grade methanol and ethyl acetate were supplied by Merck (Darmstadt, Germany). Ibuprofen, naproxen, ketoprofen, tolfenamic acid, diclofenac and meclofenamic acid (I.S. in SPE experiments) were purchased from Aldrich (Milwaukee, WI, USA). MTBSTFA was also obtained from Aldrich in 1 ml ampoules. Individual stock solutions of NSAI drugs were prepared in methanol. Diluted standards and mixtures of acidic drugs, used to spike water samples, were prepared in methanol.

A manual SPME fiber holder and SPME fibers with different coatings: poly(dimethylsiloxane) (PDMS, 100 μm film thickness), polyacrylate (PA, 85 μm film thickness), poly(dimethylsiloxane-divinilbenzene) (PDMS-DVB, 65 μm film thickness), carboxen-PDMS (CAR-PDMS, 75 μm film thickness) and carbowax-DVB (CW-DVB, 65 μm film thickness), were obtained from Supelco (Bellefonte, PA, USA). Before their first use each fiber was conditioned according to manufacturer instructions. Furthermore, fibers were additionally desorbed for 5 min between injections, in a heated point, at the same temperature than that of the GC injector.

SPE cartridges containing 60 mg of Oasis HLB were obtained from Waters (Milford, MA, USA) and used as received.

Spiked and non-spiked sewage and Milli-Q water samples were used along this study. Sewage water samples (influent and effluent) were taken in a plant equipped with primary and biological treatments. Samples were stored refrigerated at 4 °C and filtered using cellulose filters (0.45 μm pore size) before extraction of the analytes.

2.2. Equipment

Derivatized drugs were determined by GC–MS. The system consisted of a Varian Start 3400 CX gas chromatograph (Walnut Creek, CA, USA) equipped with a split-splitless injector and connected to an ion-trap mass spectrometer (Varian Saturn 4). Separations were carried out using a BP5 type capillary column (30 m × 0.25 mm i.d., d_f: 0.25 μm) purchased from Varian. Helium (99.999%) was used as carrier gas at a constant head pressure of 55 kPa. The GC oven was programmed as follows: 3 min at 50 °C, first ramp at 10 °C/min to 180 °C (held for 7 min), second ramp at 10 °C/min to 230 °C (held for 25 min), third ramp at 20 °C/min to 250 °C (held for 5 min). The GC–MS interface and the ion trap temperature were set at 250 and 200 °C, respectively. Mass spectra were obtained in the electron impact mode (70 eV). Two segment of mass acquisition were set: from *m*/*z* 100 to 330 between 10 and 25 min, and from *m*/*z* 140 to 420 for the rest of the chromatogram. SPME fibers were desorbed during 3 min, in the splitless mode, using the following temperatures: 250 °C for PDMS and PDMS-DVB, 280 °C for PA and CAR-PDMS, and 220 °C for CW-DVB coated fibers. Standards in ethyl acetate and extracts from SPE cartridges were injected (1 μl), after being derivatized, in the splitless mode using the above chromatographic program, with the only exception of the oven initial time (1 min at 50 °C) and the injector temperature (250 °C). Signals at the *m*/*z* ratios of 263 (ibuprofen), 287 (naproxen), 311 (ketoprofen), 318 + 320 (tolfenamic acid) and 352 + 354 + 356 (diclofenac and meclofenamic acid) were used for quantification of the derivatized acidic compounds.

2.3. Sample concentration

2.3.1. SPME with on-fiber derivatization

Water samples (Milli-Q or sewage water) were adjusted at different pH from 2 to 6, with 0.1 M HCl, spiked with the studied compounds when necessary, and placed in glass vials with a PTFE coated silicone rubber septum. In spiked samples, the maximum percentage of methanol was limited to 1% (100 μl of standard per 10 ml of water) to prevent changes in the yield of the extraction, due to high differences in the content of methanol from sample to sample. As a consequence, standard mixtures containing the selected analytes at different concentration levels were employed to prepare the spiked samples, when the linearity of the procedure was investigated in a wide interval of concentrations. Vials (with volumes of 10, 22 and 115 ml) were filled completely with the water sample to minimize the distribution of the analytes in the headspace. Once the sample vessel was closed, the SPME fiber was directly dipped into the aqueous sample which was stirred magnetically. After finishing the extraction step the SPME fiber was placed in the headspace of a 1.5 ml GC autosampler vial containing 50 μl of MTBSTFA.

2.3.2. SPE

SPE of water samples was performed as described previously [12]. In brief, 500 ml of water adjusted to pH 2.0–2.5 were forced to pass through the SPE Oasis HLB cartridge. Cartridges were dried with a stream of nitrogen, analytes eluted with 2 ml of ethyl acetate, and derivatized with MTBSTFA for 1 h at 60 °C.

2.4. Quantification

Levels of NSAIDs in sewage water were quantified using the standard addition procedure in case of SPME. When SPE was used as the concentration technique, the levels of NSAIDs were determined against calibration curves obtained for acidic compounds derivatized in the same conditions than the SPE extracts, using meclofenamic acid as internal surrogate throughout the whole analytical procedure.

3. Results and discussion

3.1. Optimization of the microextraction with on-fiber derivatization

3.1.1. Preliminary experiments

Efficiency of SPME is affected by a number of variables, e.g. pH, temperature, sampling time, position of the fiber, etc. Furthermore, when extraction is followed by on-fiber derivatization, the yield of derivatization also affects the performance of the whole process. Therefore, in order to have a first estimation of the influence of the sample pH, addition of salt (sodium chloride), magnetic stirring and volume of derivatization reagent (MTBSTFA) in the efficiency of the whole extraction, with a minimum of experiments, a fractional experimental design (2^{4-1}) was carried out. Enrichment and derivatization steps were performed at room temperature for 30 min, using Milli-Q water samples with an addition of the considered species at the 30 ng/ml level. Following the literature recommendations for polar species containing hydroxyl and carboxylic groups, a PA coated fiber was initially chosen for the extraction and concentration of the analytes [11,13,20]. Low and high levels for the selected variables, together with the relative importance of the main effect associated to each one are given in Table 1. Magnetic stirring showed a positive influence in the yield of the microextraction for all species; furthermore, for tolfenamic acid and diclofenac it was the most important factor. On the other hand, the effect of the volume of derivatization reagent was negligible, so it was fixed at a medium level (50 μl) for further experiments. The effects of salt and sample pH followed a more complex pattern depending on each specie; therefore, their influence, in the microextraction yield, was studied in more detail after fixing the other two variables.

3.1.2. Effect of pH and sodium chloride

Fig. 1 shows the influence of salt in the peak area of the NSAIDs at four pHs values between 2 and 6. In accordance with the results of the experimental design, the addition of salt affected negatively to the extraction efficiency of tolfenamic acid and diclofenac. Both compounds contain in their structures apart of the carboxylic group an amino moiety; thus, they probably exits as charged species at the four considered pHs. Therefore, the addition of sodium chloride increases the ionic strength of the aqueous solution, decreasing the affinity of both species for the fiber coating. On the other hand, for ibuprofen, naproxen and ketoprofen the yield of the extraction increased when the pH decreased, while the effect of sodium chloride was pH dependent: at values below their pK_a values (4.91 ibuprofen, 4.15 naproxen and 4.45 ketoprofen) the addition of salt showed a positive influence in the extraction because of the salting out effect. However, this effect was negative at pH 6 because they exist mainly as negatively charged species. A similar behaviour to this has been reported for phenol compounds using PA fibers [20]. From these results it was decided to adjust the pH of water samples to 3 and to carry out the extraction without the addition of salt. Obviously, the last was a compromise solution for the analysis of the five selected compounds.

3.1.3. Fiber coating

Influence of the fiber coating in the yield of the microextraction was systematically investigated using the following phases: PDMS, CAR-PDMS, PDMS-DVB, PA and CW-DVB. In all cases fibers were dipped directly into a 10 ml vial filled with Milli-Q water, adjusted at pH 3 and spiked with the selected compounds (30 ng/ml each one). For the CW-DVB fiber, the yield of the extraction could not be tested since most of the coating was stripped from the silica core after a few minutes of exposition to the sample at

Table 1
Experimental domain and relative importance (with their sign) of the main effects associated to each factor in the factorial experimental design for the anti-inflammatory drugs

Factor	Low level	High level	Relative main effects				
			Ibuprofen	Naproxen	Ketoprofen	Tolfenamic acid	Diclofenac
pH	3.4	6	− − − −	− − − −	− − − −	+ +	− − −
NaCl (g/ml)	0	0.32	− −	+	+ +	− − −	− −
MTBSTFA (μl)	20	150	+	− −	−	+	+
Stirring	Without	With	+ + +	+ + +	+ + +	+ + + +	+ + + +

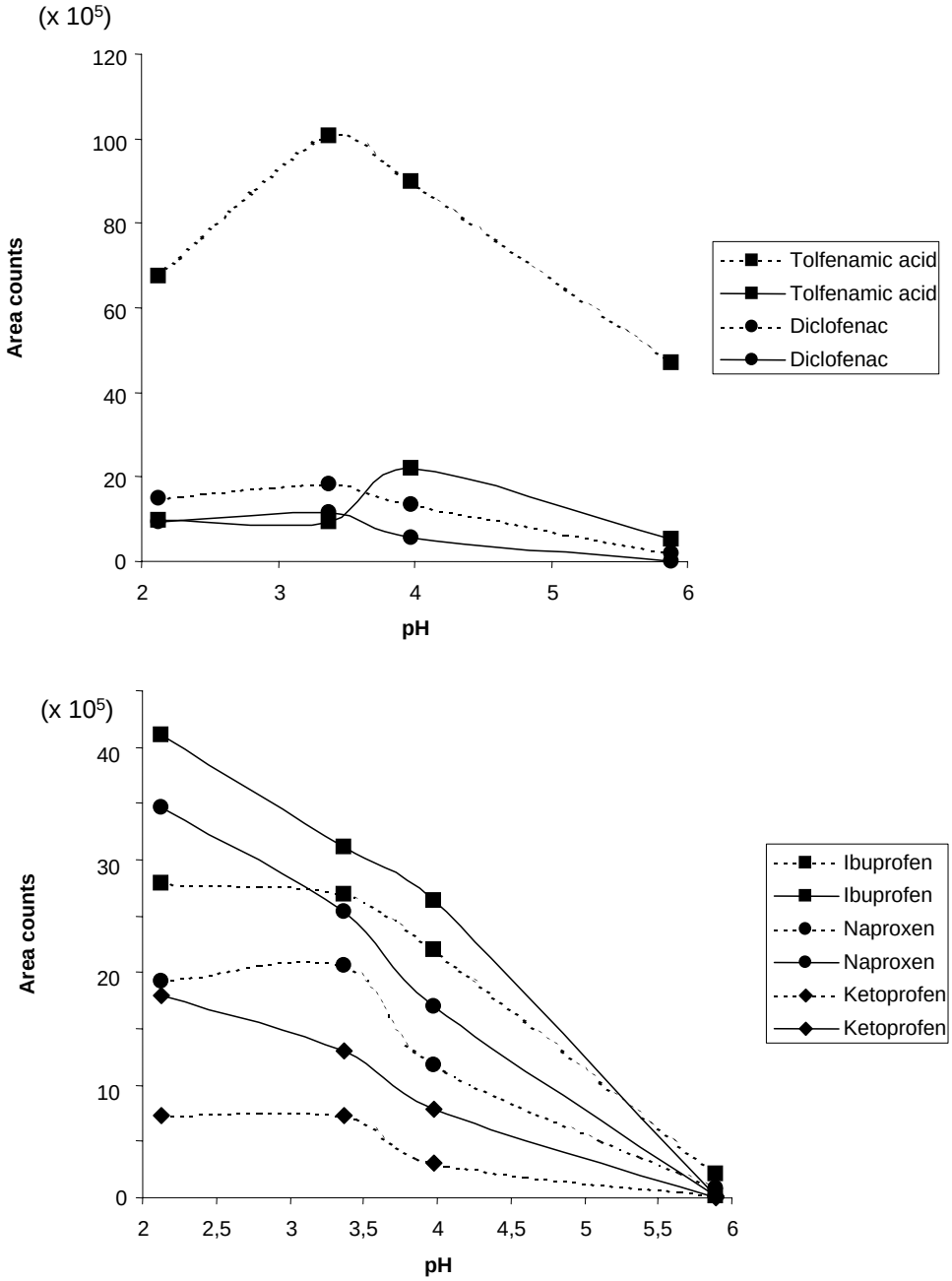

Fig. 1. Influence of sample pH and sodium chloride (0.32 g/ml) in the efficiency of the SPME extraction with on-fiber derivatization using a PA coated fiber. Solid lines samples with salt, dotted lines samples without salt.

Table 2
Normalized peak areas for the considered anti-inflammatory compounds using different SPME fiber coatings

Compound	Normalized peak area (%)			
	PA	PDMS-DVB	CAR-PDMS	PDMS
Ibuprofen	61.0	100.0	83.4	10.3
Naproxen	100.0	46.1	5.1	3.0
Ketoprofen	100.0	48.1	3.1	3.6
Tolfenamic acid	100.0	89.0	6.5	43.8
Diclofenac	100.0	44.4	4.2	4.8

Values obtained for 10 ml Milli-Q water samples, adjusted at pH 3, stirred and spiked with the selected species at 30 ng/ml.
Sampling and derivatization steps were performed at room temperature during 30 min each one.

acid pH; however, according with the available information from the manufacturer this phase should be stable between pH 2 and 9. In agreement with the polar nature of the considered analytes the maximum efficiency in the extraction, with the only exception of ibuprofen, was achieving using the PA coated fiber, Table 2. PDMS-DVB was the best phase for ibuprofen and in case of tolfenamic acid it also led to a similar response (peak area) than the PA fiber, for the rest of species peak areas obtained with this fiber were half of those corresponding to the PA one. Efficiency of extraction using PDMS and CAR-PDMS coated fibers was very low for all species, with the exception of ibuprofen using CAR-PDMS.

3.1.4. Sampling time and position of the fiber

Effect of sampling time (direct immersion) in the peak area of each compound is shown in Fig. 2. In accordance with previously published results [11,13], the kinetic of the microextraction is quite slow and the equilibrium was achieved only after a period of 2 h (except for diclofenac, which has even a slower kinetic). In further experiments a compromise sampling time of 40 min was used, assuming the corresponding decrease of the detection limits in view of the slow extraction kinetic, Fig. 2. The yield of microextraction, when the PA fiber was placed in the headspace over the sample, was also evaluated with a sampling time of 40 min at room temperature and 100 °C; however, in the best case (100 °C), peak areas were <2% of those corresponding to direct immersion during the same time (figure not given).

3.1.5. Sample volume

Influence of sample volume in the peak area for each selected compound was evaluated using vials with capacities of 10, 22 and 115 ml. Vessels were completely filled with a spiked sample of Milli-Q water (5 ng/ml for each specie) adjusted at pH 3. As shown in Table 3, a slight improvement in the yield of the extraction was obtained when the sample volume was increased from 10 to 22 ml but not between 22 and 115 ml, therefore, 22 ml vials were used in further experiments.

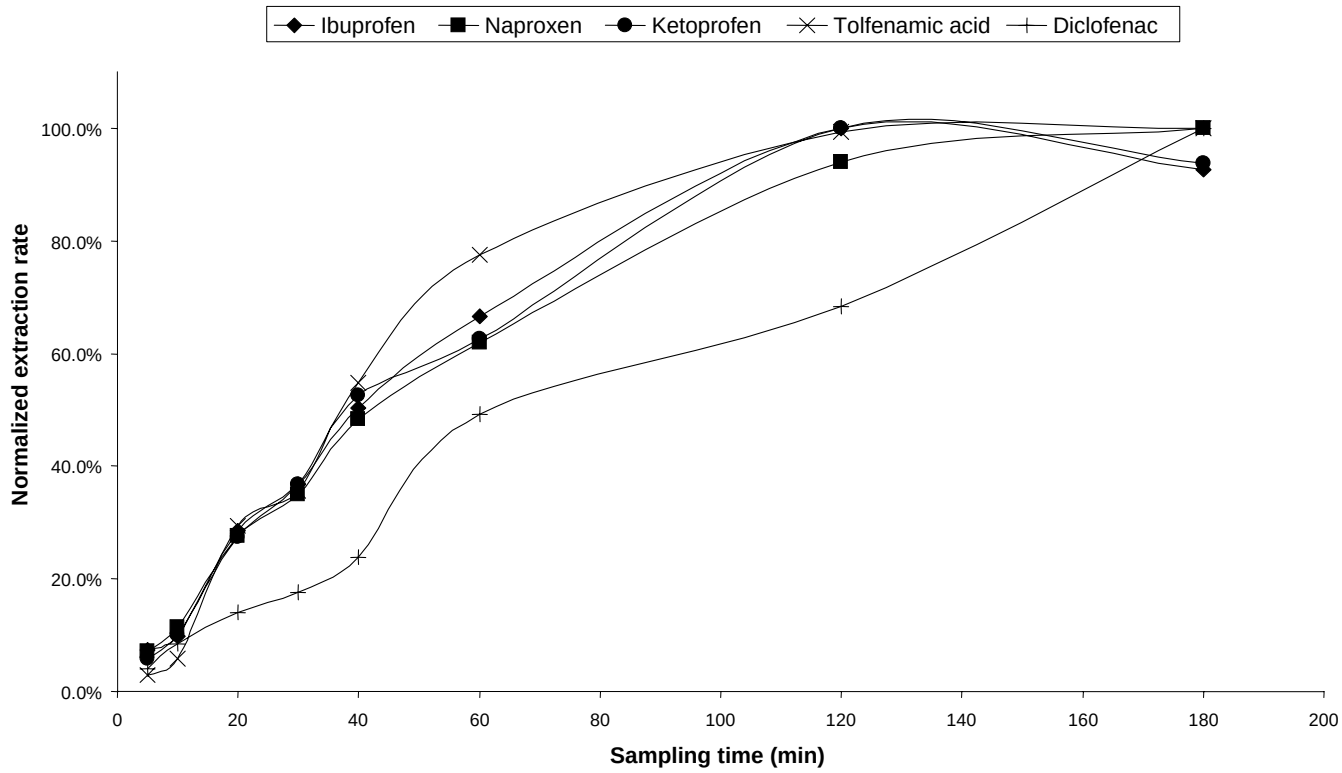

Fig. 2. Kinetic of the extraction over PA fibers, for 10 ml samples, using direct immersion at room temperature. Derivatization was performed also at room temperature for 30 min using 50 μl of MTBSTFA.

Table 3
Effect of sample volume in the efficiency of the extraction

Compound	Normalized peak area at different sample volume		
	10 ml	22 ml	115 ml
Ibuprofen	76	100	93
Naproxen	81	100	99
Ketoprofen	67	100	91
Tolfenamic acid	60	100	98
Diclofenac	70	100	97

Extraction and derivatization steps were carried out at room temperature for 30 min each one.
Samples were adjusted at pH 3 and stirred during the sampling step. Normalized peak area for each compound ($n = 2$ extractions).

Table 4
Effect of the derivatization temperature in the response of the considered anti-inflammatory species

Compound	Normalized mean response (%) ± R.S.D. (%)			
	25 °C	40 °C	60 °C	100 °C ($n = 1$)
Ibuprofen	85 ± 19	100 ± 4	93 ± 7	37
Naproxen	89 ± 19	100 ± 4	97 ± 8	73
Ketoprofen	70 ± 36	100 ± 7	100 ± 6	71
Tolfenamic acid	79 ± 27	86 ± 9	100 ± 13	68
Diclofenac	92 ± 25	100 ± 7	95 ± 14	65

Normalized mean responses with their relative standard deviations ($n = 3$ replicates).

3.1.6. Derivatization time and temperature

Derivatization times of 5, 20, 30, 60 and 120 min, using 50 μl of MTBSTFA and room temperature, did not affect in a significant extension to the average peak areas of the anti-inflammatory drugs, data not shown. A derivatization time of 20 min was fixed, in order to adjust the duration of the sample preparation steps (40 min of extraction plus 20 min of on-fiber derivatization) and gas chromatographic separation of the NSAIDs. The average peak areas were similar at derivatization temperatures of 25, 40 and 60 °C; however, they suffered a significant decrease (specially in case of ibuprofen) at 100 °C, probably due to desorption of analytes out of the fiber, Table 4. As the smaller standard deviation was obtained at 40 °C, this temperature was chosen as the optimum.

3.1.7. Memory effects

Memory effects in the PA fiber, after a desorption step of 3 min at 280 °C, were investigated in order to detect the presence of both: derivatized and non-derivatized species in the coating. In the first approach, the same fiber was desorbed twice for 3 min at 280 °C. Peak areas in the second injection were less than 0.1% of those obtained in the first one. The presence of not derivatized species in the PA fiber was tested exposing the fiber to the derivatization reagent after the first desorption. In this case, a percentage around 0.7% of the tolfenamic acid remained in the fiber as non derivatized specie after the first desorption. Memory effects for the rest of species were under 0.3%. To avoid contamination problems during the analysis of samples, containing very different levels of anti-inflammatory species, PA fibers were additionally heated at 280 °C for 5 min after finishing the chromatographic injection.

3.2. Performance of the analytical method

The linearity of the proposed method was investigated using Milli-Q water samples spiked with the NSAIDs at seven concentration levels from 50 ng/l to 30 ng/ml. The linearity at higher concentrations was not investigated, since it is unexpected to found the analytes over this levels in real samples, even in case of non treated sewage water. Correlation coefficients (r^2) from 0.998 to 1.000 were found for all compounds showing an excellent linearity for the proposed method over 3 orders of magnitude, Table 5. Quantification limits were calculated for a ratio S/N of 10 at the *m/z* values given in the experimental section for the determination of each compound. Levels of 40 ng/l were achieved for ketoprofen and around 15–20 ng/l for the rest of investigated species. These values are similar to those obtained for a sampling intake of 500 ml using SPE as concentration technique [12]. Repeatability of the extraction-derivatization was studied using samples of Milli-Q water spiked with the NSAIDs at two different concentration levels. Relative standard deviations from 4 to 8% were obtained for all species.

3.3. Application to real samples

The described method was applied to the determination of anti-inflammatory compounds in the influent and effluent

Table 5
Linearity, repeatability and quantification limits (S/N 10) of the proposed analytical procedure

Compound	Correlation coefficients (r^2)	Repeatability ($n = 4$ samples), R.S.D. (%) spiked level		Quantification limits (ng/l)
		0.1 ng/ml	2 ng/ml	
Ibuprofen	0.998	6.8	5.0	18
Naproxen	0.999	9.3	5.0	15
Ketoprofen	0.998	6.1	7.9	40
Tolfenamic acid	1.000	7.9	4.2	12
Diclofenac	0.998	6.5	6.6	20

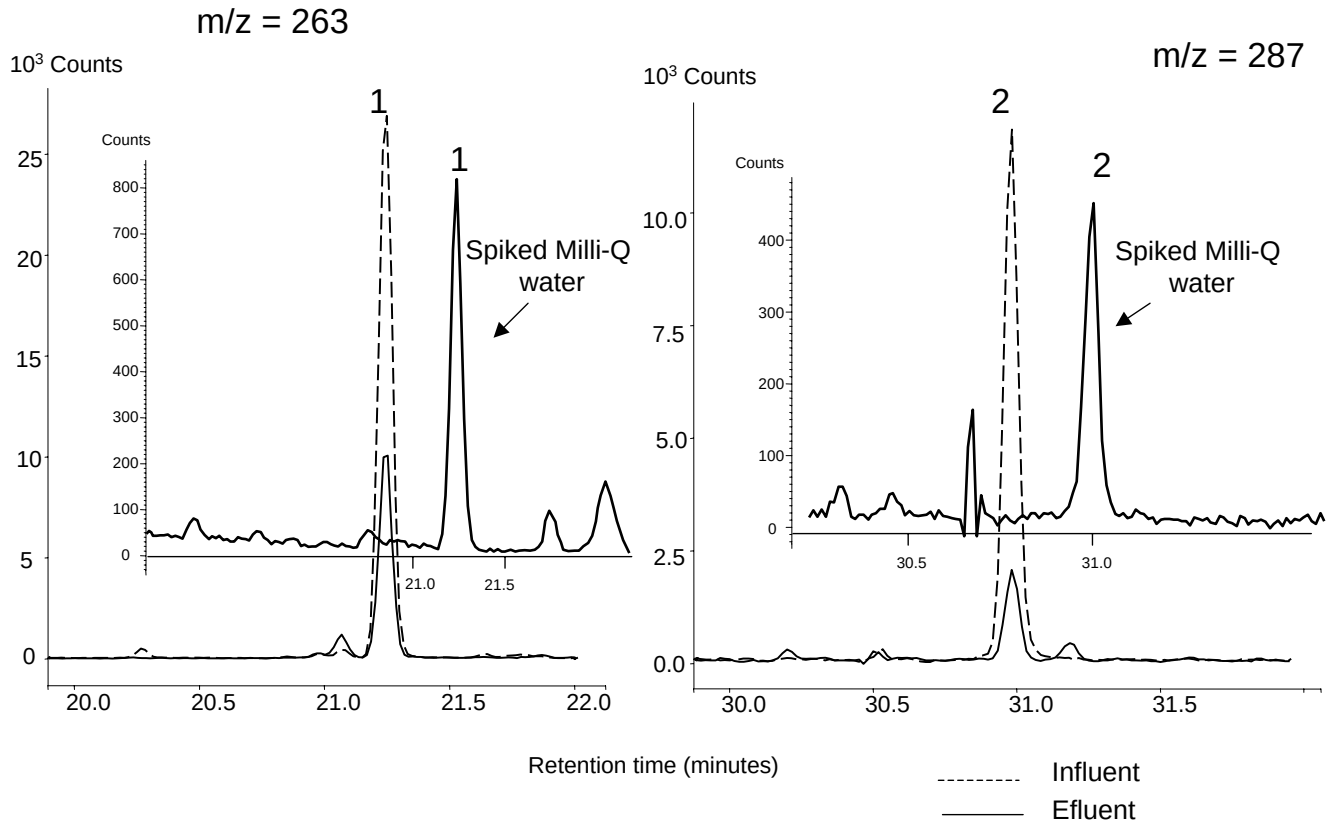

Fig. 3. Overlay of GC–MS chromatograms corresponding to an spiked Milli-Q water sample at low concentration level (thick line), the influent (dotted line) and the effluent (solid line) of a sewage treatment plant. Analytes were extracted using a PA fiber and on-fiber derivatized. Compounds: (1) ibuprofen, (2) naproxen. Concentrations of ibuprofen and naproxen corresponded to mean values given in Table 6 for sewage water samples. Levels of 51 and 48 ng/l, respectively, were added to the Milli-Q water.

of a sewage water treatment plant. Samples were taken in June 2002, stored at 4 °C and filtered before analysis. Fig. 3 shows the chromatographic trace of ibuprofen and naproxen in the influent and effluent of the sewage plant, together with a Milli-Q water sample spiked with both compounds at the 50 ng/l level. Concentrations of NSAIDs in the influent and effluent samples were determined using the procedure developed in this article and quantified with the standard addition method (4 samples without addition and 3 different spiking levels). Results were compared to those obtained using SPE followed by the derivatization of the acidic species in the organic extract of the cartridge, using the same derivatization reagent. Ibuprofen and naproxen were found in both samples using both sample preparation techniques. Globally, a reasonable agreement was found between results obtained with both pre-concentration techniques, Table 6.

Using SPME with on-fiber derivatization, slopes of the standard addition curves in sewage water samples were approximately 80–85% of those corresponding to spiked Milli-Q water, showing a moderate matrix effect on the yield of the whole process. All microextraction experiments included in this article were performed using two PA fibers. The first was mechanically broken after ca. 50 injections, the second one was used in more than 70 extraction-derivatization cycles, without detecting any problem with the stability of the coated phase; however, a progressive darkening of the stationary phase, with none apparent effect on method performance, was noticed.

Table 6
Concentrations of ibuprofen and naproxen in the influent and effluent of a sewage water treatment plant using SPE and SPME with on-fiber derivatization

Compound	Influent (ng/ml)[a]		Effluent (ng/ml)[a]	
	SPME ($n = 4$)	SPE ($n = 3$)	SPME ($n = 4$)	SPE ($n = 3$)
Ibuprofen	2.74 ± 0.16	2.75 ± 0.17	0.55 ± 0.04	0.44 ± 0.03
Naproxen	2.39 ± 0.20	2.18 ± 0.15	0.21 ± 0.02	0.16 ± 0.02

[a] Mean value ± S.D.

4. Conclusions

A simple two steps (microextraction followed by on-fiber derivatization) sample preparation method for the determination of five NSAIDs in water samples by GC–MS has been developed. Quantification limits in the low ng/l level were achieved for all compounds, therefore it can be used for the determination of acidic pharmaceuticals in sewage and groundwater samples. Conversely to classic extraction

strategies based on SPE, which require the concentration of large volumes of sample, only 22 ml of water are necessary to carry out the microextraction; therefore, real samples containing relatively high amounts of suspended particulate matter can be easily filtered. Furthermore, although this possibility was not investigated in this article, because of the simplicity of steps involved in the sample pre-concentration and derivatization procedures, we understand that after the filtration step, the whole analytical procedure could be easily automated just using a SPME autosampler for gas chromatography.

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