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Suitability of hollow fibre liquid-phase microextraction for the determination of acidic pharmaceuticals in wastewater by liquid chromatography–electrospray tandem mass spectrometry without matrix effects

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Abstract

The applicability of hollow fibre liquid-phase microextraction (LPME), as an alternative to solid-phase extraction (SPE), for the extraction/enrichment of acidic drugs (e.g. ibuprofen, clofibric acid, bezafibrate, etc.) from water samples prior to the determination by LC–ESI-MS–MS has been evaluated. After LPME method optimisation, it was found that this technique can provide very clean extracts, which do not lead to signal suppression during LC–ESI-MS–MS analysis of the analytes. The limits of quantification (0.5–42 ng/L) are suitable for the analysis of these drugs in wastewater. However repeatability needs to been improved (intra-day R.S.D. = 3.4–32%), which may be expected by automation and the development of commercially available devices and fibres specially prepared for analytical purposes. The method was finally applied to wastewater samples (treated and untreated) and results comparable to SPE were obtained.

Keywords: Liquid-phase microextraction; LC–MS; Matrix effects; Wastewater; Pharmaceuticals

1. Introduction

The fate of pharmaceuticals residues in the environment and specially during wastewater treatment is a matter that has attracted the attention of the scientific community during the last decade [1,2]. Among these pharmaceuticals, non-steroidal anti-inflammatory drugs (e.g. ibuprofen or diclofenac) and lipid regulators (e.g. bezafibrate) are some of the most commonly detected in concentrations ranging from the low ng/L up to the μg/L level [3–5].

Determination of these compounds can be accomplished by GC–MS [6–9] or, alternatively, they can be analysed by LC–MS–MS [10–14]. This last technique has the advantage that derivatisation of analytes is not required and furthermore has proved to provide better detection limits [12–14].

However, the major drawback in quantitative analysis by LC–MS is the occurrence of matrix effects, mainly signal suppression, during the ionisation of analytes. This can seriously compromise the quantitative data and may increase detection limits when real samples are analysed [13–16]. Signal suppression may be compensated by using the appropriate internal standards, if possible, or by the standard addition procedure, but this leads to increased analysis time and does not improve sensitivity [13,17].

Recently it has been shown that lowering the flow rate directed to the ESI (to about 50 μL/min) can substantially decrease signal suppression in the determination of acidic pharmaceuticals [18]. Anyhow, an average 20% of signal suppression in SPE extracts of untreated wastewater remained

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and the complete elimination of matrix effects may require additional clean-up of the SPE extracts.

Liquid-phase microextraction (LPME) is a relatively recent technique. Normally, this technique is carried out by using a membrane as interface between the sample (donor) and the organic solvent (acceptor), which avoids mixing of the two phases and other problems encountered in classical liquid–liquid extraction [19,20]. The main advantages of LPME are very low organic solvent consumption and low cost.

LPME can also be performed in a three-phase system in which the analyte is first extracted into an organic solvent that impregnates the walls of the membrane, and then back-extracted into an aqueous acceptor solution adjusted to the adequate pH, depending on the acidic properties of the analytes [21–25]. This three-phase system has been shown to provide high selectivity and clean extracts.

Despite the selectivity of this technique, only two papers were found in the literature that combined LPME with LC–MS [22,24] and a qualitative evaluation of matrix effects was performed only once [24]. A systematic quantitative evaluation of matrix effects occurring in LC–MS analysis after LPME has not been performed yet.

Thus, the aim of this work was to test the suitability of LPME as a single step enrichment/clean-up technique, which could allow the extraction of acidic drugs from wastewater samples, possibly eliminating the matrix effects normally encountered by LC–ESI-MS–MS when SPE is employed.

2. Experimental

2.1. Reagents and chemicals

Pharmaceuticals (piroxicam, ketorolac, clofibric acid, naproxen, bezafibrate, fenoprofen, ibuprofen, diclofenac and indomethacin), the internal standard (fenoprop) and sodium chloride were obtained from Sigma–Aldrich (Milwaukee, WI, USA). Stock solutions of 2 mg/mL were prepared in methanol, stored in the dark at 4 °C, and diluted to the desired concentration with ultrapure water.

Ultrapure water was obtained by an ELGA Maxima HPLC ultrapure water system (ELGA, Ubstadt-Weiher, Germany). Methanol, 1-octanol and acetic acid were supplied by J.T. Baker (Deventer, The Netherlands) and tri-*n*-butylamine (TrBA) was purchased from Fluka (Steinheim, Switzerland).

Ammonium carbonate (>99%) was purchased from Roth (Kalsruhe, Germany).

2.2. Samples

Grab samples of the influent and the effluent of a municipal wastewater treatment plant were collected in August 2004. All samples were filtered through 0.45 μm membrane filters (cellulose acetate; Sartorius, Goettingen, Germany) and adjusted to the appropriate pH with 1 M HCl prior to their extraction.

2.3. Instrumentation

A HP1100 (Agilent Technologies, San Jose, CA, USA) liquid chromatographic system consisting of a membrane degasser, binary high-pressure gradient pump, autosampler and column thermostat was used. The system was interfaced to a Quattro LC triple-stage quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray electrospray interface. Nitrogen was provided by a nitrogen generator (Model 75-72, Whatman, Haberville, USA) and used as drying and nebulising gas. Argon (99.999%) was used as collision gas. The system was controlled with Masslynx 3.3 software.

2.4. LC–MS–MS analysis

The pharmaceuticals were separated on a 150 mm × 2.0 mm Luna Phenyl-Hexyl 3 μm column (Phenomenex, Eschborn, Germany) by ion-pair chromatography with a MeOH/water gradient: eluent A (20% MeOH) and eluent B (95% MeOH), both containing 10 mM TrBA and 0.5% acetic acid, and detected by negative electrospray ionisation-tandem mass spectrometry (ESI-MS–MS) in the multiple reaction monitoring (MRM) mode using two transitions for each analyte, if posible. The method was described in detail elsewhere [13]. During the first 4.5 and the last 5 min of the chromatographic run, the column effluent was diverted to waste by a post-column switching valve and additionally, a post column tee allowed the column flow entering the ESI to be reduced to approximately 50 μL/min [18].

2.5. Liquid-phase microextraction

Accurel Q3/2 polypropylene tubular membranes (Membrana, Wuppertal, Germany) with a wall thickness of 200 μm (0.2 μm pore size) and an internal diameter of 600 μm were cut in pieces of 8 cm length for LPME experiments. Each piece of fibre was employed only once to avoid any possibility of carryover.

In the optimised method, the fibre pores were impregnated in 1-octanol for 5 s and the excess of this solvent was removed by sonicating the fibre in ultrapure water for 15 s. After this, the fibre was supported in the U-shape configuration [20] by two medical syringe needles (0.6 cm outer diameter) and it was filled with 20 μL of acceptor solution (aqueous 10 mM ammonium carbonate). The fibre was immersed into the sample (22 mL adjusted to pH 2 and spiked with the IS at the 0.2 μg/L level) in a 24 mL vial (diameter: 2.5 cm, height: 5.0 cm) and stirred for 45 min at approximately 500 rpm.

After extraction, the acceptor solution was removed from the fibre directly to a LC autosampler vial equipped with a 150 μL insert by applying a gentle pressure with a 2 mL

medical syringe. Finally, 80 μL of eluent A were added to this extract in order to adjust the pH of the sample to that of chromatography and to obtain a larger extract volume. Of these 100 μL of extract, a 90 μL volume was injected into the LC system.

For the optimisation of the method, ultrapure water spiked with the analytes (but no IS) at the 5 μg/L level was employed. After each experiment was completed, 70 μL of eluent A were added to the extract and 10 μL of a solution containing 2 μg/L of the IS, in order to compensate for variations in the extract volume and in instrumental sensitivity.

Estimation of matrix effects during the LC–ESI-MS–MS determination was done by LPME (in the optimised procedure) of five replicates of a raw wastewater sample. The five extracts obtained were combined and aliquots of 20 μL of this pooled extract were taken, spiked with 10 μL of a standard solution containing 5 μg/mL of each of the analytes and made to 100 μL with eluent A. Another 20 μL aliquot was taken and made to 100 μL with eluent A, without spiking.

2.6. Solid-phase extraction

Solid-phase extraction of pharmaceutical compounds has been previously described [13]. In brief, Oasis HLB cartridges (3 mL, 60 mg, Waters, Mildford, MA, USA) were sequentially conditioned with 5 mL MeOH and 5 mL ultrapure water (pH 2–2.5). Samples (50 ml, adjusted to pH 2–2.5) were then passed through, the cartridge dried for 30 min and finally eluted with three fractions of 2 mL of MeOH. The combined extracts were finally concentrated down to ca. 0.3 mL, spiked with 100 μL of IS solution (1 μg/mL) and diluted to a final volume of 1 mL with ultrapure water. The standard addition procedure over the extracts was employed for quantification [13].

2.7. Software

Experimental design and statistical evaluation of the data obtained were made by means of the software package Statgraphics Plus (Manugistics, Rockville, MD, USA) [26].

3. Results and discussion

3.1. Optimisation of the LPME procedure

1-Octanol was chosen as the organic solvent immobilised in the pores of the fibre wall as previous studies have proven its suitability for three-phase LPME [21,22,25]. A fibre length of 8 cm was selected to provide an inner volume of 20 μL for the acceptor solution and complete immersion of the fibre into the aqueous sample. Ammonium carbonate is a volatile buffer suitable for ESI-MS analysis, as it does not accumulate in the interface.

Another important parameter in LPME is the stirring speed during extraction. Generally, an increasing stirring speed increases the speed of extraction by reducing the thickness of the boundary layer at the outer membrane surface [19,20]. However, in this work the stirring speed was kept constant at 500 rpm as a compromise, because higher speeds led to mechanical stress of the fibre and may cause air bubble formation [25].

3.1.1. Acceptor solution composition and sample volume, pH and ionic strength

The optimisation of five operational parameters (sample pH, salt amendment to the sample, sample volume, buffer concentration and methanol content of the acceptor solution) was carried out in a set of experiments developed by experimental design, which allow a rapid optimisation with a minimal number of experiments [27]. In this case, a 2^{5-1} screening design, with four central points [26] was employed (Table 1). This comprises a total number of 20 experiments, which were performed with ultrapure water spiked at the 5 μg/L level and extraction time fixed as 30 min. The volume of sample was considered as a discontinuous variable, as it was selected to fit the volume of two sizes of vials (24 and 52 mL vials for 22 and 50 mL of sample, respectively) in a manner that the whole fibre could be immersed into the sample.

The results of this experimental design are summarized in Table 2. The sample pH was the only statistically significant variable for piroxicam, ketorolac, clofibric acid, naproxen, bezafibrate and fenoprofen, and it was close to significance for the other compounds. This factor had a negative effect, as higher recoveries by LPME were obtained at low pH, in concordance with the acidity of the analytes. The sample pH was adjusted to 2 in further experiments.

None of the other factors was significant at the 95% confidence level. However, increasing the ionic strength of the sample, increasing the sample volume or the buffer concentration in the acceptor solution tended to reduce the extraction efficiency. Therefore, a low ionic strength (no NaCl added), the lower sample volume (22 mL) and a low buffer concentration (10 mM ammonium carbonate) were used for further extractions.

The influence of the sample volume used for extraction is markedly different in LPME as compared to SPE. In LPME extraction is an equilibration and not an exhaustive process and therefore, the amount of analyte partitioning into the acceptor solution becomes independent of the sample volume when this volume is much higher than the product of the partition constant and the volume of the acceptor solution [28]. Obviously, this partition coefficient depends on the particular three-phase system. Furthermore, a larger sample volume can even be disadvantageous due to poorer mass transfers kinetics, resulting in a worse extraction efficiency (Table 2).

The last factor included in the experimental design, the methanol content in the acceptor solution, showed a positive but non-significant effect. This is assumed to be an erroneous result. In all experiments with methanol added to the acceptor solution, only a volume smaller than the 20 μL could be recovered at the end of the experiments. Obviously,

Table 1
Domain of the experimental screening design for the optimisation of the LPME of acidic drugs

	Sample			Acceptor solution	
	pH	NaCl (g/L)	Volume (mL)[a]	% MeOH	Buffer concentration (mmol/L)
High level	6	300	50	50	100
Low level	2	0	22	0	10

[a] Discontinuous variable (refer to text for further details).

Table 2
Statistical significance of factors (with their sign) from the factorial screening design (see Table 1) and selected optimal values

Compound	Sample			Acceptor solution	
	pH	NaCl (g/L)	Volume (mL)	% MeOH	Buffer concentration (mmol/L)
Piroxicam	−*	+	−	+	−
Ketorolac	−**	−	−	+	−
Clofibric acid	−**	−	−	+	−
Naproxen	−**	−	−	+	−
Bezafibrate	−*	−	−	+	−
Fenoprofen	−*	−	−	+	−
Ibuprofen	−	−	−	+	−
Diclofenac	−	−	−	+	−
Indomethacin	−	−	−	+	−
Optimal values	2	0	22	0	10

+: Positive effect on the extraction yield, −: negative effect on the extraction yield.
* Statistically significant factor at the 95% confidence level.
** Statistically significant factor at the 99% confidence level.

methanol was lost from the inner fibre volume during the extraction process, by either evaporation or migration through the membrane into the aqueous sample. This effect has been recognised before [22]. Therefore, a pure aqueous acceptor solution (with 10 mM $(NH_4)_2CO_3$) was used in further experiments as this was expected to yield higher precision and selectivity.

3.1.2. Extraction time

A study on the extraction kinetics was performed with ultrapure water spiked at the 5 μg/L level at a constant stirring speed with the extraction time increased from 5 to 90 min.

Fig. 1 shows the influence of the extraction time on the yield of clofibric acid, naproxen, ibuprofen and diclofenac (time profiles for the other analytes are similar). Equilibrium was reached around an extraction time of 45 min (in most cases, extraction times for LMPE are in the range between 30 and 45 min [20]). This extraction time is comparable to SPE considering also conditioning and elution and furthermore, several samples can be extracted in parallel, as only standard laboratory equipment is required.

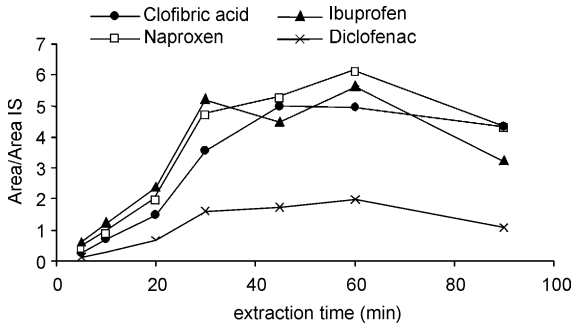

Fig. 1. LPME extraction profile, presented as relative response of clofibric acid, naproxen (×10), ibuprofen (×10) and diclofenac (×10) against the extraction time.

Moreover, the preparation of PP fibres with thinner walls and less mechanical susceptibility (to stirring) will help to speed up kinetics of the process in the future.

3.2. Analytical performance

Using these optimised conditions, the performance of the whole process involving LPME and LC–ESI-MS–MS was tested with spiked ultrapure water samples (Table 3). LPME shows a good linearity over 3 orders of magnitude (20 ng/L to 20 μg/L). Accuracy and precision can be considered only acceptable even when the internal standard was employed. Low repeatability values of LPME have already been reported for the determination of ibuprofen by LC-UV (21%) [25] and even when an isotopically labelled internal standard has been used a relative standard deviation of 21% has been obtained for bisphenol A [29].

This relatively low precision of LPME may be attributed to the fact that fibre preparation, conditioning and arrangement as well as the handling of very small extract volumes (20 μL) have to be done manually. Furthermore, variations in the wall thickness and pore size of the PP membranes may occur [19]. Therefore, improvement of R.S.D.s may require further development of fibres and manifolds especially devoted to LPME and automation and/or better internal standards.

Table 3
Performance of the LPME–LC–MS–MS procedure for acidic pharmaceuticals

Compound	Accuracy[a]	Precision (R.S.D.)		Linearity (R^2)[d]	Enrichment	LOQs (ng/L)[e]		
		Intra-day[b]	Between-day[c]			LPME/LC–MS–MS[f]	SPE/LC–MS–MS[g]	SPME/GC–MS[h]
Piroxicam	106	9.5	16	0.9993	38	33	1.3	–
Ketorolac	108	7.4	12	0.9994	196	15	5.6	–
Clofibric acid	87	3.4	11	0.9973	234	0.5	0.8	–
Naproxen	80	15	16	0.9994	186	10	6.5	15
Bezafibrate	80	8.5	9.2	0.9932	200	1.8	2.1	–
Fenoprofen	82	7.0	23	0.9977	154	6.5	3.2	–
Ibuprofen	86	32	30	0.9979	118	14	2.5	18
Diclofenac	111	25	29	0.9956	70	25	3.9	20
Indomethacin	110	21	31	0.9985	56	42	4.6	–

[a] Ultrapure water spiked at 0.2 μg/L level; accuracy = 100[found concentration/spiked concentration].
[b] Ultrapure water spiked at 0.2 μg/L level ($n = 4$).
[c] Ultrapure water spiked at 0.2 μg/L level ($n = 10$).
[d] Seven point calibration from 0.02 to 20 μg/L.
[e] For wastewater effluent and S/N ≥ 10.
[f] This work, 22 mL sample.
[g] Ref. [13], 50 mL sample.
[h] Ref. [9], 22 mLsample.

The enrichment factors ranged from 38 for piroxicam to 234 for clofibric acid (Table 3), which are suitable for the determination of these analytes in wastewater samples. These enrichment factors refer to the original acceptor solution (20 μL). The final dilution of the extract (to 100 μL) does not compromise the sensitivity, as 90% of the total extract volume is injected for analysis.

A much higher enrichment factor of 15,000 has been obtained for ibuprofen when a larger sample volume (100 mL) was extracted in a laborious and time-consuming two-step procedure [25]. However, the detection limits obtained in that work remained orders of magnitude higher, because LC-UV was used for analysis.

Thus, the limits of quantification obtained by LPME and LC–MS–MS ranged from 0.5 to 42 ng/L (Table 3). These limits are suitable for the determination of acidic pharmaceuticals in wastewater and similar to those obtained by SPME with on-fibre derivatisation and GC–MS detection (15–20 ng/L) [9]. They are higher than those obtained by SPE of 50 mL of sample using the same LC–MS–MS detection as in this work (0.8–6.5 ng/L) [13]. These higher LOQs observed for LPME are due to the low enrichment obtained with equilibrium techniques (Table 3) than by exhaustive SPE. However, this is also one reason for the higher selectivity expected for LPME.

3.3. Matrix effects

3.3.1. LC–ESI-MS–MS

As mentioned above matrix effects are often encountered in the electrospray process that may reduce the instrumental sensitivity and require enhanced effort in quantification, especially with raw wastewaters [13,18]. It was investigated whether three-phases LPME, as an one-step concentration/clean-up technique, could avoid matrix effects in the determination of acidic pharmaceuticals. A raw wastewater sample was selected for this investigation as the worst case sample.

As in previous studies [13,18] the analyte response obtained from analytes spiked into extracts was compared to the response obtained from pure standard solutions (Fig. 2). Significant matrix effects (Student's *t*-test, 99% confidence interval) were observed for clofibric acid, naproxen and diclofenac, but they remained below 5.6%. For the other six analytes matrix effects were insignificant.

These results differ markedly from SPE, where matrix effects between 3.5 and 32% were observed in raw wastewater extracts [18]. Thus, LPME yielded very clean extracts and signal suppression during the ESI process can be considered negligible. Similar results were observed for basic pharmaceuticals in biological samples by three-phases LPME–LC–ESI-MS [24].

This selectivity can be attributed to several factors. Generally, LPME is non-exhaustive, so that the largest portion of matrix components remains in the aqueous sample. Ex-

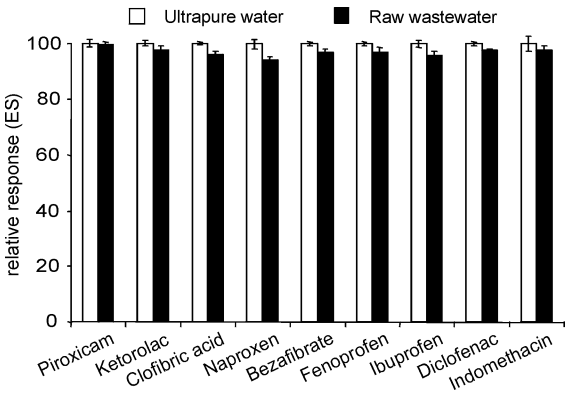

Fig. 2. Matrix effects observed during LC–ESI-MS–MS analysis of LPME extracts of raw wastewater ($n = 3$). Response normalised to ultrapure water (100% means no matrix effects).

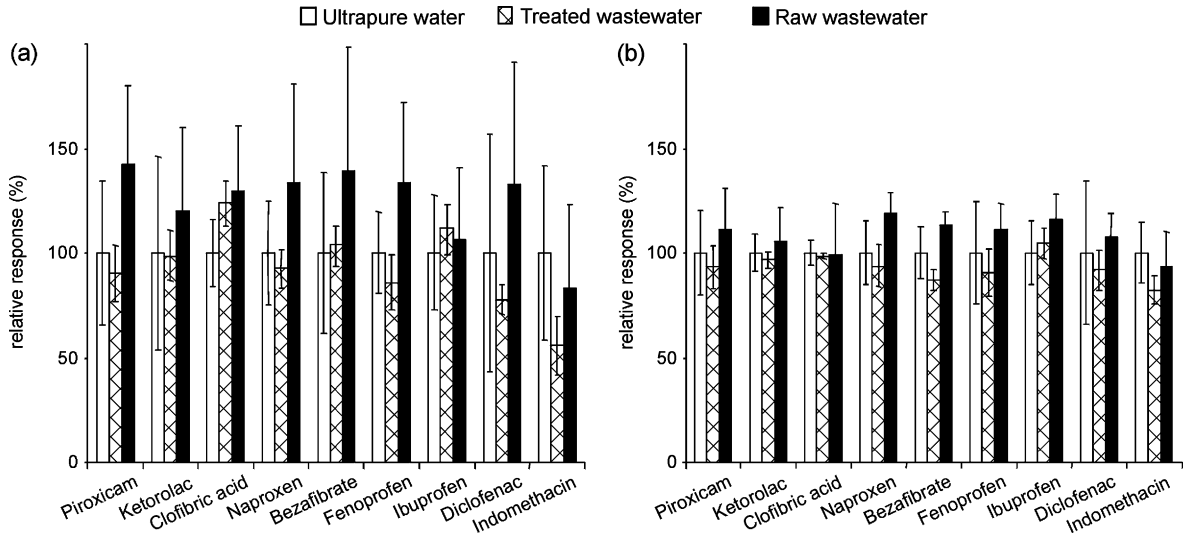

Fig. 3. Matrix effects observed during LPME of wastewater samples ($n = 4$). Response normalised to ultrapure water (100% means no matrix effects). (a) No internal standard employed and (b) after internal standard correction.

traction of organic matrix components of higher molecular weight is hampered by their slower mass transfer kinetics. Also, in three-phase systems such as the one employed in this work selectivity is even higher due to the pH shift from the acidic sample via the organic phase to a basic acceptor solution, which allows only acidic compounds to be extracted.

3.3.2. LPME

Equilibrium-based extraction techniques like SPME [9,28] and LPME are themselves prone to matrix effects. Organic and inorganic sample constituents can influence the partitioning process of an analyte with the acceptor solution by shifting its equilibrium or altering its speed of transfer. In three-phase LPME, surface active matrix components in a sample may also provoke a loss of the organic solvent immobilised within the pores of the membrane. Such matrix effects occurring during extraction, rather than during LC–MS detection, would also cause lower sensitivity and would require quantification by standard addition. In that case, LPME would have no advantage over SPE.

To check for such matrix effects during extraction, ultrapure water, treated wastewater and untreated wastewater was spiked with the analytes at the 5 μg/L level, extracted by LPME and analysed by LC–MS–MS. The results were compared to those of non-spiked samples and pure aqueous solutions (Fig. 3a). The mean recovery of the acidic pharmaceuticals was in the range of $93 \pm 35\%$ for treated wastewater and $123 \pm 45\%$ in the raw wastewater (Fig. 3a). Anyhow, due to the limited precision of the LPME procedure, these differences are not statistically significant and can be compensated adequately by the internal standard (Fig. 3b). Thus, no loss of sensitivity is produced and quantification can be performed without standard addition and just internal standard

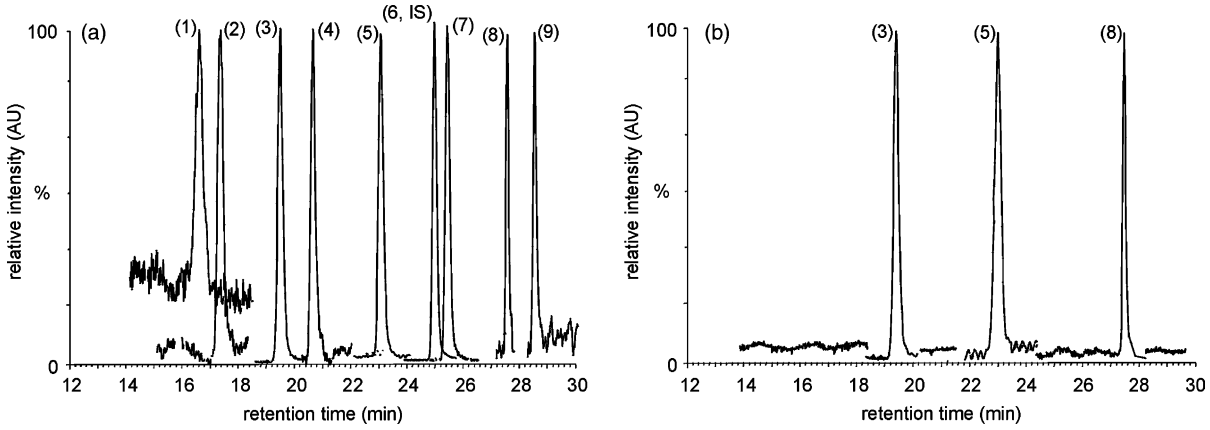

Fig. 4. LC–ESI-MS–MS chromatograms of LPME extracts of (a) an ultrapure water sample spiked at the 50 ng/L level and (b) a (non-spiked) tertiary treated municipal wastewater (found concentrations as in Table 4). Compounds: (1) piroxicam, (2) ketorolac, (3) clofibric acid, (4) naproxen, (5) bezafibrate, (6) fenoprofen, (7) ibuprofen, (8) diclofenac and (9) indomethacin.

Table 4
Concentration (ng/L) of the acidic pharmaceuticals found in raw and tertiary treated municipal wastewater

Compound	Raw wastewater[a]		Treated wastewater[a]	
	LPME ($n = 4$)	SPE ($n = 3$)	LPME ($n = 4$)	SPE ($n = 3$)
Piroxicam	nd	nd	nd	nd
Ketorolac	nd	nd	nd	nd
Clofibric acid	64 (25)	98 (2.2)	24 (27)	23 (11)
Naproxen	682 (14)	806 (2.2)	nd	nd
Bezafibrate	1478 (12)	1738 (0.91)	18 (14)	18 (11)
Fenoprofen	nd	nd	nd	nd
Ibuprofen	4513 (20)	5518 (1.3)	nd	nd
Diclofenac	1708 (19)	1532 (6.6)	476 (33)	437 (5.6)
Indomethacin	nd	nd	nd	nd

[a] Expressed as "mean (R.S.D.)"; nd = below detection limits.

calibration. However, it must be noted, that samples have to be filtered prior extraction as for non-filtered raw wastewater samples, extraction efficiency was almost negligible (data not shown). The reason for this dramatic loss of efficiency is not clear.

3.4. Application to real samples

The LPME method with LC–MS–MS analysis was finally applied to the determination of acidic pharmaceuticals in wastewater samples. Fig. 4 presents the chromatograms obtained for a treated wastewater and for a pure aqueous solution of standards (50 ng/L level). Clear signals were obtained for clofibric acid, bezafibrate and diclofenac in the treated wastewater, whereas no signals were recorded for the other analytes. No other signal was recorded in any of the MRM traces, reflecting the very high added selectivity of three-phase LPME and LC–MS–MS with MRM-detection that effectively avoids false positive findings.

Concentrations found after LPME with internal standard calibration and after SPE with standard addition [13] of these samples are compared in Table 4. The data agree relatively well (correlation coefficient, $R^2 = 0.9893$, $n = 8$) and range from 18 ng/L for bezafibrate in treated wastewater to 4.5 μg/L for ibuprofen in raw wastewater. As expected, SPE exhibited a higher precision than LPME.

4. Conclusions

A new method based on the three-phase hollow fibre liquid phase microextraction for the determination of acidic pharmaceuticals in wastewater by LC–ESI-MS–MS has been developed.

LPME has demonstrated its good selectivity as negligible matrix effects occurred when extract of raw wastewater were analysed for acidic pharmaceuticals by LC–ESI-MS–MS. This is advantageous as compared to SPE. Moreover, it provides acceptable quantification limits and good linearity with lower sample consumption (22 mL). Other advantages of LPME are very low cost and single use of fibres, eliminating possible carry-over problems as compared to SPME.

However, the major drawback in this study was the relatively poor precision of LPME. This was likely due to the completely manual operation from fibre preparation and conditioning to the handling of small extract volumes. The precision of LPME may improve by automation and design of LPME equipments especially devoted to analytical purposes.

In conclusion, three-phase LPME is a technique that may develop into a good alternative to other extraction/clean-up procedures for LC–MS determination of polar pollutants in wastewater samples in the future.

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