A microfluidic liquid phase microextraction method for drugs and parabens monitoring in human urine

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ABSTRACT

Frequent consumption of pharmaceuticals and personal care products (PPCPs) have emerged as a current problem that highlights the pressing need for new multi-residue analytical methods that allow their simultaneous determination to assess their overall effect on human health. In this regard and for the first time, a versatile microfluidic based- liquid phase microextraction (LPME) method was developed for simultaneous monitoring of ten compounds from six different classes: amoxicillin, sulfadiazine, sulfamerazine, tiamphenicol, ethyl 4-hydroxybenzoate, flumequine, propyl 4-hydroxybenzoate, 5-hydroxydiclofenac, 3-hydroxydiclofenac and diclofenac. The microfluidic device was combined with a HPLC-UV system for the separation and determination of the model analytes in the sample. Optimal conditions were reached using 2-nitrophenyl octyl ether as supported liquid membrane, pH 3.5 as donor phase, pH 11.5 as acceptor phase, 0.5 μL min^{-1} as donor flow rate and 1 μL min^{-1} as acceptor flow rate. Under optimal method conditions, the extraction efficiency was between 85 and 100% for most compounds after 10 min extraction, and it was successfully applied in non-diluted human urine, with recoveries between 70 and 100% for all analytes except for sulfamerazine (52% recovery). In addition, the extraction of metabolites (3-hydroxydiclofenac and 5-hydroxydiclofenac) was also demonstrated in microfluidic systems with recoveries between 71 and 100% in human urine. The proposed method allowed consecutive extraction and only requires 5 μL of organic solvent and less than 15 μL of sample volume.

1. Introduction

The analysis of drugs and parabens in biological samples has gained special importance over the years due to the adverse effects that their accumulation can cause in human health. The presence of parabens in urine may be associated, on the one hand, with accidental exposure to paraben-containing products and, to a lesser extent, with environmental exposure, as their systemic absorption has been previously described in both cases [1,2]. On the other hand, these well-known ubiquitous preservatives, have been linked to adverse health outcomes in humans, specially they may act as weak endocrine disrupters, however, controversy still surrounds this fact, what evidence the need for further studies that include them among other compounds [3]. Due to their frequent co-administration with principal active ingredients, as excipients, in many pharmaceutical formulations, they are expected to be present in samples from individuals undergoing drug treatment. This fact highlights the need to develop analytical methods that allow simultaneous determination of different nature compounds to carry out more complex studies,

even including the evaluation of possible synergistic or antagonistic effects between them.

Liquid phase microextraction (LPME) in different configurations has been proved to be a powerful tool [4] for extracting compound with the same acidic or basic nature [5,6] or from the same family of compounds, such as for example, non-steroidal anti-inflammatories [7,8], parabens [9–10], sulfonamides [11–13], fluoroquinolones [14,15], amphetamines [16], etc. On another hand, other components of the matrix can make analysis difficult. Therefore, finding an appropriate strategy which can simultaneously extract different class of analytes with excellent clean-up is of great importance. Various procedures have been presented to overcome this shortcoming for instance using different sorbents with various polarities, sorbents with different functional groups, combination of polymers and reduction the polar compounds. Nevertheless, they are faced with the challenge of improving greener and faster procedures disable to ensure efficient, accurate and facilitated extraction methods for simultaneous monitoring different types of analytes. To fulfil green analytical chemistry requirements, LPME has been miniaturized and this

has led to facilitate not only the automation of this technique but also to require a lower volume of extractant and sample volume, the latter being of crucial importance especially when very small volume of biological sample is available. The most used microfluidic system when implementing LPME consists of two channels (one for the donor phase and one for the acceptor phase) that are separated through a flat membrane, which supports an organic solvent. The dimensions of the channels are on the order of millimeters and the volume they contain on the order of microliters. In this way, the analytes present in the sample (in their neutral form) are extracted from the donor to the acceptor phase (ionized form) through the membrane by passive diffusion. Miniaturization in the form of channels accelerates the mass transfer of analytes from one phase to another. To date, microfluidic based liquid phase microextraction procedures has only been mainly optimized for the extraction of compounds belonging to the same class [17–20], or same acidic or basic nature [21,22]. It has been possible to extract acidic and basic compounds simultaneously by using single or double electromembrane (EME) technique using a sample volume consumption of the order of milliliters and stagnant conditions (traditional set-up), [23–26] which has recently been improved in microfluidic systems in order to minimize the organic solvent and sample volume consumption [27–29]. However, electromembrane technique requires a power supply and two electrodes so that the analytes can electromigrated through the membrane due to a potential difference applied between the two electrodes. In addition, the number of EME-compatible conductive solvents is reduced, the optimal voltage can be critical depending on the nature and functional group, and the system as a whole consumes more energy. The simultaneous extraction of compounds from different nature or different families by microfluidic systems based LPME represents still a great challenge. The efficiency and selectivity of the extraction in LPME depends mainly and critically on the type of organic solvent used as the supported liquid membrane (SLM) as well as on the other parameters such as the composition of the donor and acceptor phase, since the compounds (independently of their nature or functional group) must be predominantly in their neutral or ionized form in the donor and acceptor phase, respectively.

In this work, an efficient microfluidic-based liquid phase microextraction method is developed for simultaneously monitoring of ten compounds belonging to six different classes of analytes in human urine. For the first time, not only the simultaneous extraction of compounds of a very different nature has been demonstrated, but the extraction of metabolites in microfluidic systems.

2. Experimental

2.1. Chemicals and materials

Sulfadiazine (SDI), sulfamerazine (SMI), ethyl 4-hydroxybenzoate (EtP), propyl 4-hydroxybenzoate (PrP), diclofenac (DIC), 5-hydroxydiclofenac (5-OH), 3- hydroxydiclofenac (3-OH), amoxicillin (AOX), flumequine (FLU), tiamphenicol (TMF) were provided from Fluka-Sigma-Aldrich (Madrid, Spain). Formic acid, sodium hydroxide, chloric acid, 2-nitrophenyl octyl ether (NPOE), dihexyl ether, and 1-octanol were purchased from Fluka-Sigma-Aldrich (Madrid, Spain). Analytical-reagent grade including methanol, acetonitrile, decanol, and tributyl phosphate (TBP) were supplied from Merck (Darmstadt, Germany). Individual stock solutions of each compound were prepared in methanol (100 mg L^{-1}) and stored at 4 °C in a refrigerator. The stock solution of 5-hydroxydiclofenac, 3- hydroxydiclofenac were prepared in ultrapure water and stored at −70 °C in light resistant before use. Further dilutions and mixtures were daily prepared by dilution from the stock standard solutions in ultrapure water 18,2 MΩ.cm (Millipore, Billerica, MA, USA). Two micro-syringe pumps (Cetoni GmbH, Korbussen, Germany) were used to introduce the donor and acceptor phase solutions into the microchip device.

2.2. Chromatographic conditions

The HPLC system consisted of a VWR-Hitachi (Barcelona, Spain) liquid chromatograph with a quaternary L-2130 pump. The injector was an autosampler L-2200. Separation was performed using a LiChroCART 75–4 Purosphere STAR RP-18e 3 μm (75 mm × 4.0 mm i.d.) (VWR, Germany) proceeded by a guard column Kromasil1 100 Å, C18, 5 μm (20 mm × 4.6 mm i.d.) (Scharlab S.L., Barcelona, Spain). The mobile phase consisted of 0.1% formic acid (pH 2.6) (component A) and methanol (component B) at a flow rate of 0.5 mL min^{-1}. The gradient elution was programmed as follows: a gradient mode from 80% A to 60% A for 3 min and from 60% to 40% A for 2 min and then keep those conditions in isocratic mode for another 2 min. Then, from 40% to 20% A for 4 min and keep it in isocratic mode for 4 min. Finally, from 20% A to 5% A for 5 min. To achieve the reequilibration of the column to the initial conditions, 5 min were waited between injections. The wavelengths selected for diode array detector (DAD) were 230 (for AOX), 240 (for EtP), 249 (for FLU), 255 (for PrP), 267 (for SDI, SMI and TMF) and 275 nm (for 5-OH, 3-OH and DIC). The chromatogram was completed in less than 20 min and the retention time was 3.57, 4.03, 5.81, 8.20, 11.84, 12.72, 13.84, 14.61, 15.17 and 17.76, for AOX, SDI, SMI, TMF, EtP, FLU, PrP, 5-OH, 3-OH and DIC, respectively.

2.3. Chip design and liquid phase microextraction methodology

A laser ablation cutter was used for microfluidic device microfabrication (Epilog Mini 24–30 W). The optimal poly(methyl methacrylate) (PMMA) device was comprised of two symmetric patterned layers with one channel each (0.070 mm depth × 27 mm width × 23 mm length) along with six holes of 3 mm for assembling and four holes of 1.35 mm diameter as inlets and outlets (Fig. 1). A flat polypropylene membrane piece separated the acceptor and donor phase. The flat polypropylene membrane supported 3 μL NPOE covering the whole channel surface. The microchip device was opened and the membrane was replaced when needed. The acceptor and donor inlets (Inlets Teflon tubes) were connected to two separate micro-syringe pumps to introduce the donor (pH 3.5) and acceptor solutions (pH 11.5) into the microfluidic device. After 10 min for SLM stabilization to ensure both channels are bubble free, the extraction was operated for 12 min at 0.5 and 1 μL min^{-1} as donor and acceptor flow rates, respectively. After extraction, the acceptor phase was collected in a microinsert tube, and 10 μL of the collected solvent was injected into the HPLC-UV port for analysis.

2.4. Real samples

Urine samples were collected from a 30 year old volunteer. Non-diluted urine samples were spiked at three different concentration levels (low, medium and high) within the linear range of each analyte, and then it was adjusted to pH 3.5 with HCl solution. In addition, five urine samples were collected at intervals of 30 min between them after a volunteer took a single oral dose of 50 mg of diclofenac. All samples were filtrated through Pall NylafloTM nylon membrane filter 0.45 μm (Pall Corporation, Ann Arbor, Michigan, USA) and then submitted to the microfluidic device for extraction.

3. Results and discussion

3.1. Optimization and evaluation of experimental parameters

Passive diffusion of the analytes between donor and acceptor phase mainly depends on the composition of the supported liquid membrane and physico-chemical properties of the analytes (log P and pKa values). On the other hand, mass transfer rate between donor and acceptor depends on the surface contact. In this regard, the influence of chip geometry was investigated using an initial device of 23 mm length, 70 μm
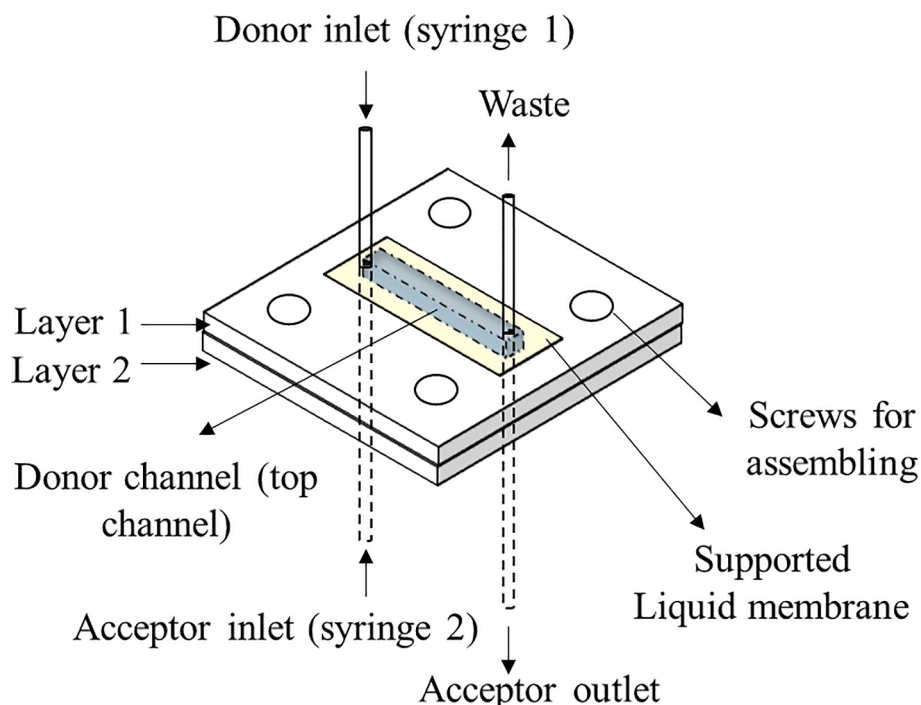
Fig. 1. Scheme of the microfluidic device based liquid phase microextraction.

deep and 3 mm wide based on our previous studies [20,30]. First, the characteristics of the supported liquid membrane was comprehensively studied. Afterward, the influence of donor and acceptor phase composition and the flow rates were optimized.

3.1.1. Supported liquid membrane optimization

Initial tests were carried out to select an appropriate supported liquid membrane for all compounds from different nature. Different solvents related to the extraction of acidic or basic compounds were tested based on the reported literature [30–32]: 2-nitrophenyl octyl ether (NPOE), dihexyl ether (DHE), tributyl phosphate (TBP), decanol, octanol and some mixtures among those that offer better results. The preliminary conditions of donor and acceptor phase composition were studied based on their pK_a values. The compounds studied have different pK_a values [33–36]: pK_a of 2.8 and 7.2 for AOX; pK_a of 1.6 and 6.5 for SDI; pK_a of 1.58 and 6.9 for SMI; pK_a of 8.9 for EtP and PrP; pK_a of 4.5 for DIC; pK_a of 7.4 for TFM and pK_a of 6.7 for FLU.A pH below the pK_a value for analytes with acid groups and between the two pK_a values for compounds containing amino groups was selected as preliminary donor phase composition to ensure that the predominant species of each analyte is neutral in that phase. The selected acceptor phase pH value was above the pK_a value of each analyte (regardless of whether they have one or two pK_a values) to ensure that the species formed is ionized. Based on that, donor phase composition, acceptor phase composition and flow rates were preliminary fixed at pH 3.0 (HCl), pH 12 (NaOH) and 1 μL min^{-1}, respectively. A new membrane was placed for each organic solvent test

and three consecutive extractions were carried out for each solvent. As seen in Table 1, NPOE and decanol showed efficiencies between 22 and 97% and 6–60%, respectively, for all compounds. DHE and octanol showed efficiencies between 8 and 54% and 4–54%, respectively, for all compounds except for TMF. Based on the results, two mixtures NPOE: TBP 1:1 and DHE:TBP 1:1 were also tested but as seen in Table 1, only good efficiencies between 40 and 70% were obtained for AOX, SDI, SMI and TMF. As is known, the affinity of each compound for a certain SLM will depend on the physical–chemical characteristics and the structure of each compound. For this reason, some compounds such as TFM are more related to TBP: NPOE 1: 1, while the rest are related to NPOE. For this reason, a compromise is reached for the SLM that is more efficient for most of the compounds. Consequently, NPOE was selected as SLM for further experiments since it showed the highest efficiencies for all compounds.

3.1.2. Donor and acceptor solutions composition

The pH composition of donor and acceptor phase plays a critical role on the diffusion coefficient in liquid based microextraction methods. Due to the variety of functional groups and pK_a values of the analytes, donor phase composition (containing 1 mg L^{-1} of each compound) was tested between 1 and 7 pH. SLM, acceptor phase composition and flow rate were fixed with NPOE, at pH 12 (NaOH) and 1 μL min^{-1}, respectively. As shown in Fig. 2, the optimal extraction efficiencies were obtained at pH 3.5 for all compounds, except for SDI and SMI, which showed a slight increase in extraction at pH 3 due to their pK_a values.

Table 1
Extraction efficiencies (RSD %) of the analytes using different organic solvents as SLM.

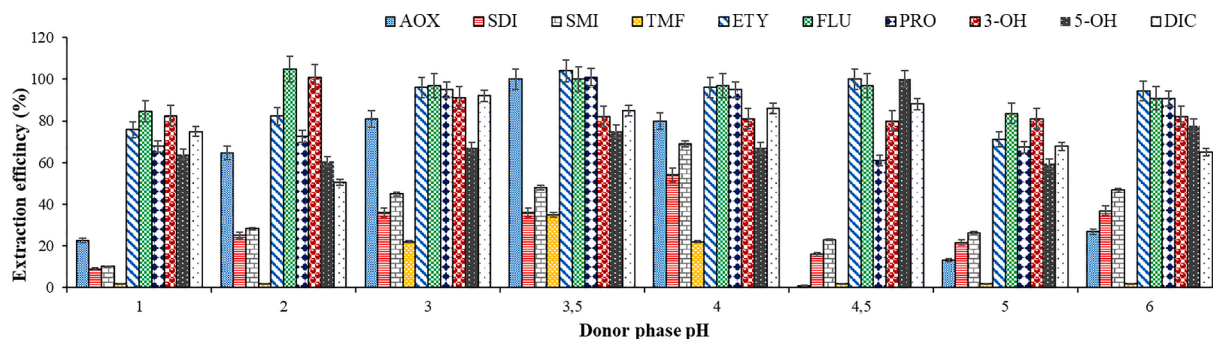| SLM/compounds	AOX	SDI	SMI	TMF	ETY	FLU	PRO	3-OH	5-OH	DIC
Octanol	54 (3)	26 (2)	35 (5)	n.d (3)	17 (4)	34 (2)	4 (2)	10 (3)	13 (2)	12 (4)
NPOE	81 (4)	54 (2)	69 (3)	22 (2)	96 (3)	97 (6)	95 (2)	91 (2)	67 (3)	92 (4)
TBP	72 (2)	44 (3)	41 (2)	13 (2)	n.d	n.d	n.d	10 (3)	n.d	n.d
Decanol	60 (3)	16 (2)	26 (4)	6 (3)	41 (3)	44 (2)	37 (2)	31 (5)	27 (2)	44 (5)
DHE	35 (6)	8 (2)	10 (4)	–	37 (2)	47 (6)	54 (3)	48 (4)	30 (3)	41(2)
TBP:NPOE 1:1	70 (3)	43(6)	51(3)	75 (6)	n.d	n.d	n.d	10 (4)	n.d	n.d
TBP:DHE 1:1	60 (2)	38 (4)	50 (2)	50 (3)	n.d	9 (3)	n.d	8 (2)	n.d	n.d

n.d: non detected.

Fig. 2. Optimization of donor phase composition. SLM: NPOE, acceptor phase pH: 12, flow rate: 1 μL min^{-1} (acceptor and donor phase).

Then, a pH 3.5 was selected showing the highest extraction efficiency for most of the compounds. The acceptor phase pH was optimized within the range 9.0–12.5 by keeping the optimal donor pH (3.5) fixed. Based on the data in Fig. 3, pH 11.5 can be considered as the most appropriate point for the compounds since the highest extraction efficiencies were obtained for most of the compounds. Only SMI, 5-OH and 3-OH showed better efficiencies at pH 12, 11 and 10, respectively. Under these conditions (pH 3.5 and pH 11.5 as donor and acceptor phase composition, respectively), the compounds are predominantly in their neutral form in the donor phase and charged in the acceptor phase.

In addition to initially selecting a suitable SLM for most compounds, the selection of the composition of the acceptor and donor phase is decisive, especially when it comes to different classes of compounds with very different acid constants. The optimal conditions selected correspond to the best efficiencies for most compounds. However, three compounds are more efficiently extracted using selectively other conditions, such as 1: 1 NPOE: TBP solvent for TFM and pH 4 conditions for SDI and SMI.

3.1.3. Donor flow rate and geometry optimization

Sample flow rate was tested between 0.5 and 10 μL min^{-1} while the acceptor flow rate was fixed at 1 μL min^{-1}. Previous studies have shown that the extraction efficiency would significantly decrease with greater acceptor flow since the time that this phase is in contact with the donor phase (containing the analytes) will decrease [17,21]. For this reason, the acceptor flow has been set to 1 μL min^{-1} avoiding loss of extraction efficiency. As seen in Fig. 4, the extraction efficiency decreased with increasing flow rate. This phenomenon might be due to decreasing the residence time of the sample during flow rate enhancement. Thus, 0.5 μL min^{-1} was selected as donor flow rate.

The geometry in terms of length, depth and width of microchannel

was studied. In this way, three devices with different length (mm), width (mm) and depth (mm) sizes were utilized with the following characteristics: (a) 23x3x0.07, (b) 13x3x0.12, (c) 13x3x0.07 and (d) 23x3x0.14. Extraction efficiencies between 53 and 100%, 7–108%, 51–110% and 6–103% were obtained for the device (a), (b), (c) and (d), respectively. The geometry (a) showed the highest efficiencies compared to (b) and (d) and slightly higher efficiencies compared to (c) Geometry 23 × 3 × 0.07 was selected as the optimal one although no significant differences were observed between device (a) and (c).

The method provides good extraction efficiencies after only 10 min extraction, requires 10 μL of sample, 3 μL of organic solvent and allow consecutive extractions.

3.2. Analytical performance

Under optimal conditions, different experiments were performed to evaluate the analytical performances of the proposed microfluidic method. Linearity, repeatability, method detection limits (MLODs), and method quantitation limits (MLOQs) were calculated and summarized in Table 2. A calibration curve was constructed using a least-square linear regression analysis at eight different concentrations within the linear range. The linearity range was 0.3–10 μg mL^{-1} for SMI, EtP and PrP, 0.83–10 μg mL^{-1} for TMF, FLU and 3-OH, 0.1–10 μg mL^{-1} for SDI, 2.6–10 μg mL^{-1} for AOX and 5-OH; and 0.14–10 for DIC. A linear relationship with r^2 values over 0.9996 was obtained in all cases. LODs (S/N = 3) and LOQs (S/N = 10) were between 0.04 and 10 μg mL^{-1} and 0.1–10 μg mL^{-1} for all compounds, respectively. Extraction efficiencies were also shown in Table 2 based on three replicate experiments with relative standard deviation below 6% in all cases. Efficiencies were between 54 and 100% for all analytes except for TMF which was lower (35%). Repeatability (n = 4) and intraday repeatability (n = 4, 15 days)
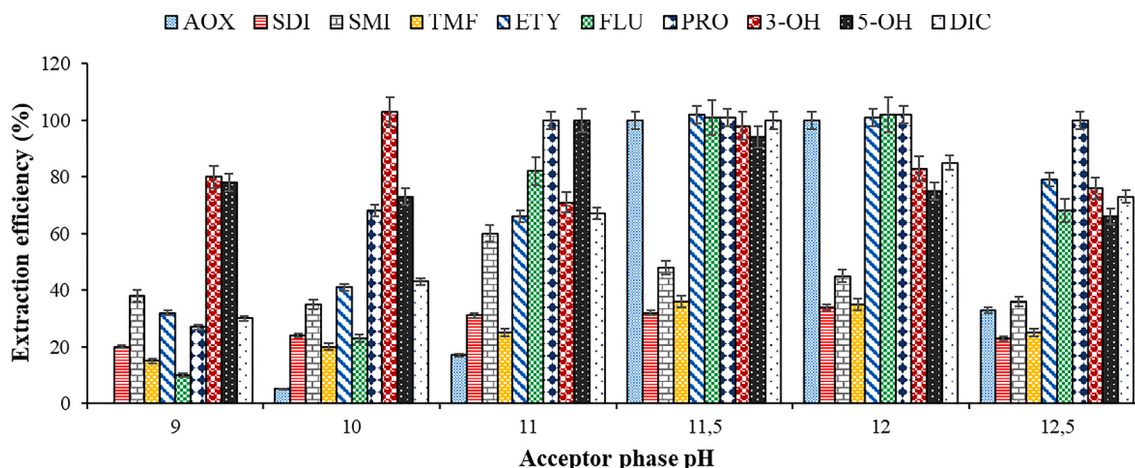

Fig. 3. Optimization of acceptor phase composition. SLM: NPOE, donor phase pH: 3.5, flow rate: 1 μL min^{-1} (acceptor and donor phase).
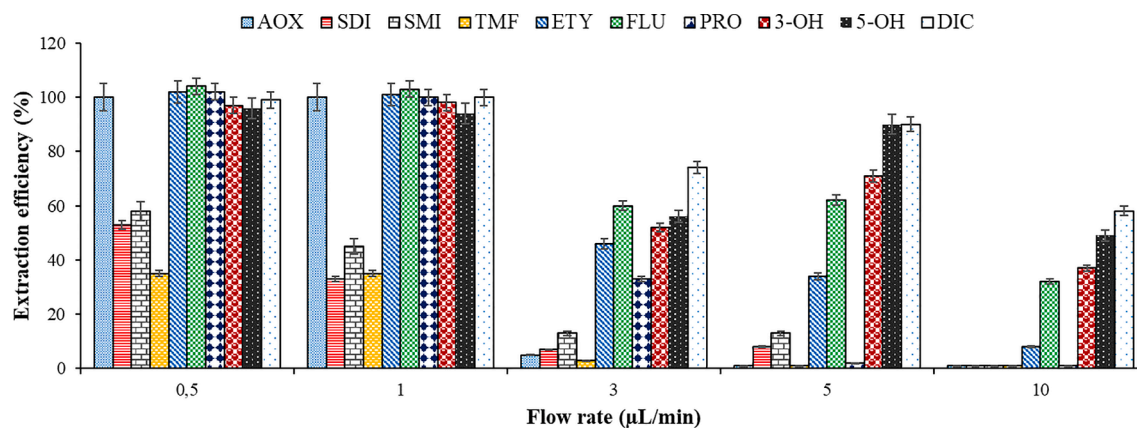
Fig. 4. Extraction efficiency versus donor flow rate. SLM: NPOE, donor phase pH: 3.5, acceptor phase pH: 11.5, acceptor flow rate: 1 μL min^{-1}.

Table 2
Method detection limit (MLOD), method quantitation limit (MLOQ) and extraction efficiencies at optimal conditions.

Analyte	AOX	SDI	SMI	TMF	EtP	FLU	PrP	3-OH	5-OH	DIC
% EE [a]	98 (3)	54 (3)	58 (2)	35 (1)	102 (2)	110 (1)	102 (3)	97 (3)	85 (2)	99 (1)
MLOD (μg mL^{-1})	0.8	0.04	0.1	0.25	0.1	0.25	0.1	0.25	0.8	0.04
MLOQ (μg mL^{-1})	2.7	0.1	0.3	0.83	0.3	0.83	0.3	0.83	2.6	0.14

[a] % Extraction efficiency (%RSD, n = 4).

were studied at three different levels (low, medium, high) of the calibration curve of each analyte, obtaining a relative standard deviation below 6% and below 5% for repeatability and intraday repeatability, respectively. Reproducibility of the device was tested replacing three times the membrane, observing a relative standard deviation below 5% for all analytes.

3.3. Real samples

Human urine samples were collected from a 30 year-old healthy adult female volunteer (staff working in the laboratory) to show applicability. Non-diluted urine sample was directly spiked at two different concentration levels and each concentration was tested in triplicate. After the pH adjustment of the sample, it was submitted to the microfluidic device and the acceptor phase collected was analyzed by HPLC. As seen in Table 3, the spiking recoveries (n = 3) were between 70 and 100% for all analytes (AOX, SDI, TMF, EtP, FLU, PrP, 3-OH, 5-OH and DIC) except for SMI (52% recovery) with a relative standard deviation below 6% for all analytes. Recovery was not influenced by the level of spiking in the urine samples. The same volunteer took a single oral dose of 50 mg of diclofenac and urine samples were collected at intervals of 30 min observing 1.6 mg L^{-1}, 2 mg L^{-1}, 2.5 mg L^{-1}, 3.1 mg L^{-1} of the metabolite 5-OH after 30, 60, 90 and 120 min, respectively. Membrane was stable for at least 10 consecutive extractions in urine samples without carry over effect. Fig. 5 shows the corresponding DAD chromatogram from (A) spiked human urine and (B) blank urine sample.

4. Conclusions

In this work, an effective downscaled microfluidic device was proposed for simultaneous monitoring of ten compounds from six different families including two metabolites in human urine. NPOE proved to be a compatible solvent for the extraction of compounds of a very different nature. In most compounds, the efficiency is higher than 85%, and in those in which the efficiency is slightly lower (SDI, SMI and TMF), it is due to the commitment in which the extraction conditions have been set and can be modified to benefit the extraction of other compounds. As explained above, the physical–chemical and structural characteristics of

Table 3
Recoveries (average of three determinations ± standard deviation) from spiked urine samples.

Analyte	Spiked level (μg/mL)	Recovery (%) (n = 4)
AOX	3	68.1 ± 2.5
	5	70.2 ± 5.5
SDI	0.2	69.4 ± 4.5
	2	71.7 ± 3.3
SMI	0.5	52.4 ± 4.7
	2	55.8 ± 6.6
TMF	2	91.9 ± 1.8
	5	93.1 ± 2.2
EtP	0.5	98.7 ± 3.9
	2	99.0 ± 2.6
FLU	2	95.1 ± 1.8
	5	97.4 ± 2.0
PrP	0.5	100.2 ± 3.3
	2	98.4 ± 4.2
3-OH	0.7	101.1 ± 1.7
	2	96.6 ± 2.4
5-OH	2	71.4 ± 6.1
	5	73.9 ± 5.5
DIC	0.2	99.7 ± 1.9
	1	99.3 ± 4.6

the compounds analyzed are very different and the optimal operational parameters vary for the case of these three compounds. This method also benefits from the low sample volumes required (15 μL), low organic solvent volume (3 μL) and a short extraction time (15 min). The method has been successfully applied in non-diluted human urine, with recoveries between 70 and 100% for most analytes except for SMI (52% recovery). For the first time, the extraction of metabolites (3-hydroxydiclofenac and 5-hydroxydiclofenac) has been satisfactorily demonstrated in microfluidic systems with recoveries between 71 and 100% in human urine. Despite the high selectivity and specificity that has been demonstrated so far by LPME, this work opens new application strategies with LPME for the selective extraction of compounds of a very different nature.
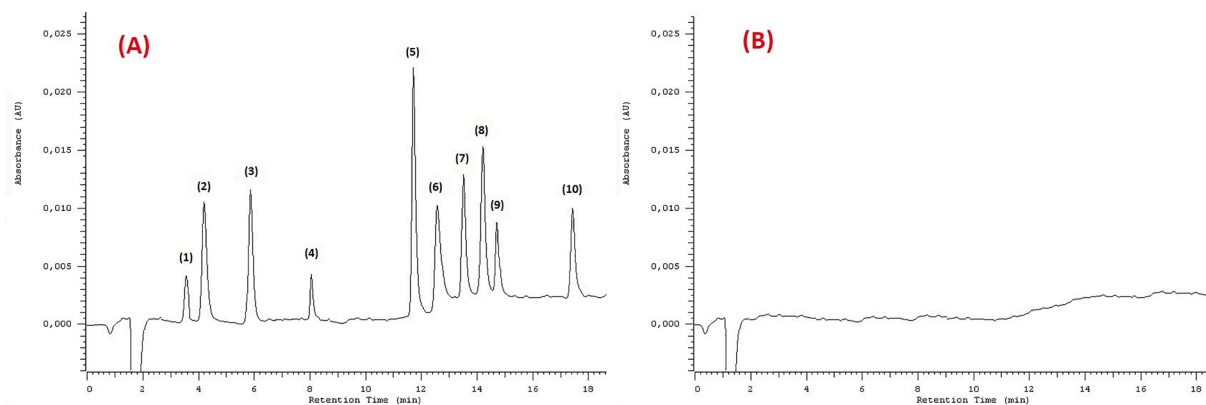
Fig. 5. Chromatogram of a (A) spiked human urine at 1 µg mL^{-1} and (B) blank human urine sample: (1) amoxicillin, (2) sulfadiazine, (3) sulfamerazine, (4) tiamphenicol, (5) ethyl 4-hydroxybenzoate, (6) flumequine, (7) propyl 4-hydroxybenzoate, (8) 5-hydroxydiclofenac, (9) 3-hydroxydiclofenac, (10) diclofenac.

5. Ethics declarations

The sample was provided voluntarily and with informed consent.

CRediT authorship contribution statement

Samira Dowlatshah: Formal analysis, Investigation, Data curation. **Mohammad Saraji:** Writing - original draft. **Rut Fernández-Torres:** Writing - original draft. **María Ramos-Payán:** Methodology, Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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