A comprehensive study of a new versatile microchip device based liquid phase microextraction for stopped-flow and double-flow conditions.

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Abstract

A new geometry for a versatile microfluidic-chip device based liquid phase microextraction was developed in order to enhance the preconcentration in microfluidic chips and also to enable double-flow and stopped-flow working modes. The microchip device was combined with a HPLC procedure for the simultaneous determination of two different families as model analytes, which were parabens and non-steroidal anti-inflammatories (NSAIDs): Ethyl 4-hydroxybenzoate (Et-P), Propyl 4-hydroxybenzoate (Pr-P), Butyl 4-hydroxybenzoate (Bu-P), IsoButyl 4-hydroxybenzoate (iBu-P), salycilic acid (SAC), ketoprofen (KET), naproxen (NAX), diclofenac (DIC) and ibuprofen (IBU) in urine samples. The new miniaturized microchip proposed in this work allows not only the possibility of working in double-flow conditions, but also under stagnant conditions (stopped-flow) (SF-µLPME). The sample (pH 1.5) was delivered to the SF-µLPME at 20 µL min^{-1} while keeping the acceptor phase (pH 11.75) under stagnant conditions during 20 minutes. The highest enrichment factors (between 16 and 47) were obtained under stopped-flow conditions at 20 µL min^{-1} (sample flow rate) after 20 min extraction; whereas the extraction efficiencies were within the range of 27-81% for all compounds. The procedure provided very low detection limits between 0.7 and 8.5 µg L^{-1} with a sample volume consumption of 400 µL. Parabens and NSAIDs have successfully been extracted from urine samples with excellent clean up and recoveries over 90 % for all compounds. In parallel, the new device was also tested under double flow conditions, obtaining good but lower enrichment factors (between 9 and

20) and higher extraction efficiencies (between 45 and 95) after 7 min extraction, consuming a volume sample of 140 µL .

The versatile device offered very high extraction efficiencies and good enrichment factor for double flow and stopped-flow conditions, respectively. In addition, this new miniaturized SF-µLPME device significantly reduced costs compared to the existing analytical techniques for sample preparation since this microchip require few microliters of sample and reagents and it is reusable.

Keywords: microextraction, miniaturization, sample preparation, microfluidic, drugs, urine sample.

1. Introduction

Liquid Phase microextraction (LPME) is a very well-known and popular technique used for the extraction of acid and basic drugs based on the passive diffusion of the analytes from the sample (donor solution) into an acceptor solution, through a membrane (which support an organic solvent into its porous). LPME has been applied to many different fields, considering biological, pharmaceutical, environmental, food, toxicology analysis, among others [1–7]. The transport phenomena based on passive diffusion depends not only on the nature of the analytes, and the optimal parameters (as phase's composition, organic solvent, stirring speed, flow rate, etc), but also on the geometry of the system used for LPME. Another popular technique based liquid phase microextraction, named electromembrane extraction (EME), has also been frequently used since it improves the extraction of compounds in many cases due to an external electrical field created to both sides of the support liquid membrane [8–16]. However, EME also offers some limitations since its requirement is the use of a suitable and conductor organic solvent for carrying out the extractions. Both techniques have been widely used for the determination of pharmaceutical drugs either in biological samples (urine) or water samples [17-23] due to the great concern that exists regarding their contribution as emergent pollutants in the environment. Also, parabens have been studied due to the concern about their endocrine disrupting potential [24-29]. This has required the use of powerful, fast and sensitive techniques that offer better limits of quantification.

Up to date, parabens and non-steroidal antiinflamatories haven been determined by traditional LPME and EME procedures resulting in very good enrichment factors [1-4,17,19, 20, 30-33]. Those procedures allowed good enrichment factors but low extraction efficiencies. In the last years, liquid-liquid extraction has been miniaturized into microfluidic devices in order to address the limitations from traditional procedures and these chip devices are becoming an attractive alternative due to the many advantages that it presents [34-42]. The microchip devices for sample treatment have two channels that allow working in two different ways based on the flow rate of each phase: double-flow or stopped-flow conditions. In double-flow conditions, both phases (sample and acceptor) are moving at some flow rate. However, in stopped-flow conditions, the acceptor phase keep stagnant while the sample solution is used at some flow rate. Recent microchip devices based LPME, have been demonstrated to work only under double-flow conditions but not under stopped-flow conditions in a single step since the latter required to collect several extracts for its direct injection into HPLC. This was due to the low sample volume available in the acceptor channel (~ 2 µL) [41,42] and consequently, the analysis time increased and the reproducibility decreased when an enrichment factor was necessary prior to the sample analysis. On the other hand, the devices did not allowed high preconcentration factors although the sample flow rate was significantly increased under double-flow conditions.

Based on the current limitations of microfluidic devices for microextraction procedures, the aim of this work was to develop a new versatile and effective microfluidic device in order to overcome the limitations from previous microfluidic devices, increasing the preconcentration and allowing working under stopped-flow conditions compatible with direct analysis.

Based on geometry aspects, an increase of the depth channel would increase the volume capacity contained in the channel but it could decrease the transport phenomena by passive diffusion since the analytes are farther away from the membrane. Microfluidic systems that follow a laminar regimen do not carry agitation, so diffusion can be slow if the distance between the analytes and extraction solvent is increased. Moreover, an increase of the channel´s width would increase the contact surface between

the sample and the analytes, however, very wide channels could destabilize the laminar flow and affect the membrane stability.

In this work, we present for the first time a new versatile and effective microfluidic chip based LPME which allow the possibility of working under two different working modes (double-flow or stopped-flow conditions). The microchip was applied to the simultaneous determination of two different families in urine samples. This way, a comprehensive study between both different working conditions was carried out. The microchip decreased the sample volume and time of analysis since no collecting samples were needed for direct injection. The proposed stopped-flow device (SF- μLPME) is the easiest microfluidic chip for the simultaneous extractions of different drugs resulting in higher enrichment factors with lower cost instrumentation, simple handling, reusability and is still considered a "green method" by keeping low organic solvent (< 5μL) consumption. The proposed device has been successfully applied to urine samples.

2. Experimental

2.1. Chemicals and solutions

Ethyl 4-hydroxybenzoate (Et-P), Propyl 4-hydroxybenzoate (Pr-P), Butyl 4-hydroxybenzoate (Bu-P), IsoButyl 4-hydroxybenzoate (iBu-P), salicylic acid (SAC), ketoprofen (KTP), naproxen (NPX), diclofenac (DIC), ibuprofen (IBU),1-octanol, dihexyl ether, 2-nitrophenyl octhyl ether (NPOE), formic acid, sodium hydroxide, chloride acid,sodium chloride and methanol were purchased from Fluka–Sigma–Aldrich (Madrid, Spain). 100 mg L^{-1} stocks solutions were prepared in methanol except SAC, DIC and IBU that were prepared in Milli-Q Plus water (Elga, purelab option S-R 7-15 (Madrid, Spain). All working dilutions were prepared using ultrapure water from a Milli-Q Plus by adequate dilutions from stored at 4ºC. A membrane (Celgard 2500) of 25 μm thickness, 55% porosity, and 0.21 μm x 0.05 μm pores was obtained from Celgard (Charlotte, NC, USA).

2.2 Fabrication of the microfluidic-chip device

Figure 1 shows a scheme of the microfluidic device based liquid phase microextraction. This microfluidic device has been re-designed and modified in order to overcome the limitations and disadvantages from previous microfluidic devices. The optimal poly(methyl methacrylate(PMMA) device consisted of two symmetrical patterned plates with one channel of 23 mm length, 120 µm depth and 3 mm width each. Four holes of 3 mm and 1.35 mm diameter were drilled for assembling and fixing in/outlets Teflon tubes, respectively. A flat polypropylene membrane piece of 27 mm length x 5 mm width separated the acceptor phase (channel 1) and the donor phase (channel 2). Firstly, the membrane was placed over one channel and impregnated with 4 µL of dihexyl ether. Once the extracting solvent was immobilized along the membrane by capillary forces, the channels were aligned and the device was closed using four small crews. The final size of a microfluidic device for one single extraction was 47×29×6 mm, however by increasing the size of both PMMA plates, an arbitrarily large number of extraction channels can be implemented and independently addressed. Also, the microchip-device can be opened any time when exchange membrane is needed.

A laser cutter (Epilog Mini 24-30 W) was used to fabricate this chip. The best quality was obtained using a writing speed of 40%, power of 33%, a resolution of 1500 and a frequency of 5000.

Inlets Teflon tubes (acceptor and donor inlets) were connected to two separate micro-syringe pumps (Cetoni GmbH, Korbussen, Germany). The sample (pH 1.5) was pumped into the microfluidic device at 20 $\mu L min^{-1}$ while keeping the acceptor phase (pH 11.75) constant. The microfluidic device was also tested under double-flow conditions as described below, in order to compare different working modes. The acceptor phase was collected using a micropipette and was directly injected into a HPLC for analysis.

2.3. Chromatographic conditions

An Agilent 1100 series (Barcelona, Spain) liquid chromatography equipped with a G1312A Bipump and an autosamplerG1313A for 5 µL of sample injection was used as HPLC system. The column used for the separation of the nine compounds was a LiChroCART® 75-4 Purospher® STAR RP-18e 3 µm

(75 mm x 4.0 mm i.d.) (VWR, Barcelona, Spain) proceeded by a guard column Kromasil1 100 Å, C18, 5 µm (20 mm x 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. Separation was performed at 25ºC. An initial 60% component B was used in isocratic mode for 2 min, and then a linear elution gradient was programmed from 60% to 80% (B) for 3.4 min and from 80 % to 86 % B for another 2.4 minutes. Three minutes were waited between injections which allowed re-equilibration of the column to the initial conditions.

The wavelengths used for DAD were 235, 255, 230, 280 and 225 nm for SAC, KTP, NAX, DIC and IBU, respectively and 255 nm for all parabens. The chromatogram was completed in less than 10 minutes and the retention time was 3.1, 3.3, 4.7, 5.3, 6.3, 6.6, 6.8, 8.9 and 9.1, for SAC, Et-P, Pr-P, KTP, NPX, iBu-P, Bu-P, DIC and IBU, respectively.

2.4. Preparation of biological samples analysis using µLPME extraction

Spiked urine samples were adjusted to pH 1.5 with HCl and filtered through Pall NylafloTM nylon membrane filter 0.45 µm (Pall Corporation, Ann Arbor, Michigan, USA) prior to microextraction procedure.

2.5. Calculations of extraction efficiency and enrichment factor

The enrichment factor (EF_i) for the analyte *i* was calculated according to the following equation (1):

$$EF_i = \frac{C_{f,a,outlet}}{C_{i,s,inlet}} \quad (1)$$

where $C_{f,a,outlet}$ is the concentration of the analyte i at the outlet of the acceptor channel and $C_{i,s,inlet}$ is the initial concentration of the analyte in the sample. $C_{f,a,outlet}$ was determined by HPLC UV-detection using external calibration. The enrichment factor is calculated using the same equation either using double-flow or stopped-flow conditions. The extraction efficiency (*EE*) was defined as the

fraction of analyte transferred to the acceptor phase from the sample. Using a double-flow working mode, the extraction efficiency (EE %) was calculated according to the following equation (2):

$$EE\ (\%) = \frac{C_{f,a,outlet}}{C_{i,s,inlet}}\ x\ \frac{v_a}{v_s}\ x\ 100 = EF_i\ x\ \frac{v_a}{v_s}\ x\ 100 \qquad (2)$$

Where $v_a\ and\ v_s,$ are the acceptor and sample flow rate, respectively.

However, under stopped-flow conditions, the extraction efficiency (EE %) was calculated by substituting the parameter "acceptor and sample flow rate" by the "acceptor and sample volume" corresponding to each phase sample.

In order to obtain a global EE value for the 9 analytes, the average extraction efficiency index (avEEi) was defined (3):

$$AvEEi = 1 - \sqrt{\frac{\sum_{i=1}^{n}(EE_i - 100)^2}{n}} \qquad (3)$$

3. Results and discussion

3.1. Principle of the extraction

The model analytes corresponded to two different families: non-steroidal antiinflamatories and parabens. The extraction of the analytes is based on a passive diffusion process due to a strong pH gradient difference between the acceptor and the sample solution. Non-steroidal antiinflamatories contain acid groups within a pK_a range value of 2.5-5, while the paraben are esters of parahydroxybenzoic acid and contain alcohol group which pK_a value are within the range of 5-8.8. A three phases liquid phase microextraction configuration presents two aqueous solutions (acceptor and sample) separated by the support liquid membrane (organic solvent). The analytes of interest were in neutral form in the sample solution and negatively charged in the acceptor solution. This way, a pH value of under 7 (HCl solution) and over 9 (NaOH solution) were used as sample and acceptor solution, respectively.The microfluidic device was tested using two different working modes: double-flow mode and stopped-flow conditions. The membrane was reused for consecutive extractions without observing

memory effects and the acceptor phase collected was analyzed by HPLC once the extraction was completed. Under stopped-flow conditions, the acceptor phase was pumped continuously for at least 2-3 minutes between extractions to clean the SLM avoiding memory effects.

3.2. Optimization of the microchip´s geometry

In LPME, the extraction of the analytes depends on a transport phenomenon based on passive diffusion. The design of a new geometry was focused on the increasement of the channel volume capacity (compatible with direct injection into HPLC) without decelerating the transport phenomena. Different length, wide and depth were tested in order to obtain an adequate and stable laminar flow during the extraction, considering a final channel volume capacity between 7 and 10 µL for its direct analysis by HPLC after stopped-flow conditions. The length was fixed at 23 mm and the wide and depth were tested between 1-3 mm and 50-300 µm, respectively. The depth was the most critical parameter and it was limited to 120 µm since an increased depth significantly decelerated the transport phenomena. In one hand, a less deep channel kept high extraction efficiencies under double-flow conditions (over 90%) but the channel volume capacity was not enough for working under stopped-flow conditions. On the other hand, a depth over 150 µm decreased the extraction efficiency under double-flow conditions (less than 70%) and the enrichment factor decreased 20 % for all compounds under stopped-flow conditions. Additionally, a wide of 2 mm required a deeper channel in order to increase the volume capacity and it decreased the extraction efficiencies and a wide of 4 mm did not offer good reproducibility and stable flow rate. For this reasons, a compromise between depth, length and width was carried out to increase transport phenomena and channel´s volume but still maintaining miniaturization size and simple handling conditions. The best results and the most reproducible and stable flow were obtained with a channel geometry of 23 mm length, 120 µm depth and 3 mm width. Based on the fundamental basis for LPME, this new geometry presents longer and wider channels for increasing the contact area between the analytes and the support liquid membrane compared to

previous one made on PMMA. Additionally, the depth was increased to allow a greater volume capacity compared to the only 2 µL volume capacity from the previous µLPME device [41].

3.3. Optimization and evaluation of experimental conditions

According to data founded in the bibliography for individual microextraction of parabens or non-steroidal antiinflamatories, preliminary experiments were performed to determinate the most suitable organic solvent to be used as SLM for the simultaneous extraction of both families. For the optimization of the organic solvent, a pH 3 (HCl) sample solution, pH 11.75 (NaOH) as acceptor solution, and 1 µL min^{-1} as sample and acceptor flow rate were used. 2-nitrophenil octyl ether (NPOE), 1-octanol, 1-heptanol and dihexylether were tested by opening the microfluidic device for membrane exchange after each organic solvent test. As seen in Table 1, the extraction efficiency (based on 3 replicate experiments) was very different depending on the analyte. Best avEEi was obtained when dihexylether was used as support liquid membrane, which was consequently used as SLM for the rest of the study.

For optimization of sample and acceptor composition, the acceptor and donor phase were tested within the ranges of pH 10-12 (aqueous NaOH solutions) and 1-4 (aqueous HCl solutions), respectively. The donor phase, containing 1 mg mL^{-1} of each analyte, was tested keeping the acceptor phase fixed at pH 11.75. As seen in Figure 2, the highest peak areas were obtained at pH 1.5 after 7 minutes extraction, not observing a significant decrease for the rest of the pH range tested. Then, acceptor phase composition was optimized by keeping the sample solution fixed at pH 1.5 for all experiments. Figure 3 shows that the highest peak areas were obtained at pH 12 and pH 11.75 for five non-steroidal antiinflamatories and four parabens, respectively. On the other hand, parabens were not stable over pH 12 due to a slight degradation during their extraction, so a compromised pH of 11.75 was selected for the extraction of both families. A relative standard deviation (RSDs %) below 4 % for all analytes resulted based on 3 replicate experiments of each experimental point for Figure 2 and 3. Consequently,

a pH of 1.5 and 11.75 were fixed as sample and acceptor composition, respectively, for the study of the flow rate for all compounds.

Next, the device was tested on two different working modes in order to compare the best results obtained related to extraction efficiencies and enrichment factors. This new geometry allows testing both working modes without the necessity of collecting different extracts (from acceptor outlet) under stopped-flow conditions, a limitation that a previous geometry presented [41,42]. First, for double-flow conditions experiments, the acceptor and donor flow rate were optimized within a range of 1-4 μL min^{-1} and 1-20 μL min^{-1}, respectively. The acceptor phase was tested keeping the donor phase flow rate at 1 μL min^{-1}, obtaining the highest extraction efficiencies at 1μL min^{-1} (data not shown). Then, the donor flow rate was tested while the acceptor flow rate was kept constant at 1 μL min^{-1}. Figure 4 shows that the highest extraction efficiencies were obtained at 1 μL min^{-1} flow rate for all compounds, observing a decrease as the donor flow rate increased significantly due to the decrease residence time of the sample. The extraction efficiencies were over 65 % for all compounds, except for Bu-P and SAC being slightly lower. However, as seen in Figure 5, the enrichment factor significantly increased when the donor flow rate increased, resulting in an EF between 8 and 20 at 1 μL min^{-1} (acceptor flow rate) and 20 μL min^{-1} (sample flow rate) for all compounds.

Second, stopped-flow condition was studied. The size and geometry of the new proposed microchip-device allowed the collection of a higher acceptor volume in one step (7 μL) after stagnant conditions extractions. The microfluidic device was tested at different sample flow rates while keeping the acceptor phase constant. Preliminary experiments were tested at different sample flow rate (5,10,20,30, 40 and 50 μL min^{-1}) and extraction times, observing that extraction times depends on the sample flow rate. Hence, extraction time and sample flow rate were investigated together. Flow rates over 60 μL min^{-1} were not tested since it showed not reproducible results due to certain instability of the support liquid membrane. As shown in Figure 6, enrichment factors increased by increasing sample flow rate from 5 to 20 μLmin^{-1}, but it decreased by increasing the sample flow rate over 20 μL min^{-1} since the

target analytes do not have enough time to pass through the SLM into acceptor phase. The highest enrichment factors were reached at 20 µL min^{-1} after 20 minutes extraction. At those conditions, extraction efficiencies and enrichment factors were within the range of 27-81 and 16-47, respectively. Each point from figure 4, 5 and 6 was based on 3 replicate experiments resulting in a relative standard deviation (RSDs %) below 6 % for all analytes.

Therefore, the new geometry of this microfluidic device allowed the possibility of either working under stopped-flow conditions resulting in higher enrichment factors and good extraction efficiencies, or under double-flow conditions obtaining lower enrichment factors but better extraction efficiencies.

Finally, the influence of salt addition was studied under stopped-flow conditions. NaCl and Na_2SO_4 were tested as salting-out reagent within the concentration range of 0–20% (w/v) and 0.5-1.5 M, respectively. It was observed an increase of the recoveries between 5 and 25 % for all compounds when 10 % of NaCl was added, except for DIC that did not show an increase and IBU which offered the highest recovery when 0.5 M of Na_2SO_4 was added. Then, 10 % NaCl was added to each sample experiment.

In order to demonstrate the performance of this new geometry, stopped-flow conditions mode was selected since it offered the highest enrichment factors for its application in urine samples. The optimal experimental conditions were pH 11.75 as acceptor phase, pH 1.5 as sample or donor phase, 20 µL min^{-1} as sample flow rate and 20 minutes extraction.

3.4. Method evaluation

A 10-point calibration curve was constructed using a least-square linear regression analysis of 10 different standard mixtures (in triplicate). Optimal conditions for stopped-flow working mode were applied to find out the linearity, repeatability, LODs, and LOQs of the method that summarized in Table 2. Several aqueous pH 1.5 solutions (containing the nine compounds) were injected into the microfluidic liquid phase microextraction procedure and analyzed according to the described HPLC procedure under stopped-flow conditions. Detection and quantification limits were calculated as three

and ten times the standard deviation of the background signal, respectively. The calibration curves were linear in the range of 6.0-100 μg L^{-1} for SAC, Et-P, Pr-P, NPX, iBu-P and Bu-P; 9.7-100 μg L^{-1} for KTP; 14-100 μg L^{-1} for DIC and 28-200 μg L^{-1} for IBU. The linear range remained up to 10 mg L^{-1} for all compounds. R^2 values exceeded in all cases 0.9990. The repeatability was tested using different membranes in order to evaluate the viability of the device by changing membranes during one month. The relative standard deviation for repeatability (n=4) and interday repeatability (n = 3, 5 days) were below 4% for all analytes. The results obtained after more than 12 consecutive extractions were not reproducible, so the membrane was replaced after 10 extractions. Very low detection and quantitation limits between 0.7-8.5 and 3-28 μg L^{-1} were obtained, respectively.

Under optimized conditions, the SF-μLPME provided high enrichment factors and good extraction efficiencies within the range of 16-47 and 27-81 %, respectively. Compared to our previous optimization for individual extraction of parabens and NSAIDs into a microfluidic chip [41-42], much higher enrichment factors have been obtained with also good extraction efficiencies by using this new microchip compared to double-flow conditions.

3.4. Urine samples analysis using microfluidic SF-μLPME

In order to evaluate the capability of the proposed microchip device in real samples, two urine samples were tested (Table 3).

Urine samples were collected from a 32 and 30 year-old female and male volunteer, respectively. Samples were spiked at three different concentration levels of parabens and NSAIDs and were submitted to the microchip device under stopped-flow conditions. The recovery was studied by comparing the extraction efficiency obtained from aqueous solution (containing the analytes) with the extraction efficiencies obtained from spiked urine samples. The recoveries were over 90 % for all compounds. Compared with previous procedures using HF-LPME for urine sample analysis, this SF-μLPME purposed decrease significantly the sample volume required for the analysis and offers also an excellent clean-up.

Figure 7 shows a representative chromatogram of a spiked urine sample containing 16 µg L^{-1} for all compounds except for IBU that was 30 µg L^{-1}, observing an excellent clean-up with no sample dilution and low sample consumption compared to traditional LPME procedures, which requires much higher sample volume. The results indicated applicability of the proposed SF-µLPME system for simultaneous analysis of parabens and NSAIDs.

Compared to previous methodologies and devices founded in the literature, this microchip based LPME is more versatile and sensitive since it allowed to work under stopped flow conditions in one step, resulting in higher enrichment factors and less sample volume consumption compared to traditional LPME. Table 4 shows a comparison between different techniques based on SPE, LPME, DLLME and EME. Recent microchip devices published in the literature offered advantages like low sample volume and high extraction efficiency [41, 42] with enrichment up to 10 [42]however, the methodology could not be applied to samples where very low LOQ were required. Then, its application was very limited to some real samples where no enrichment factor was required.

Other traditional methods for parabens or NSAIDs extraction (as SPE, HF-LPME or EME) have showed lower [3], similar [33] or higher enrichment factors [4, 32], however, it required 20x and 125x higher sample volume, longer extraction times and did not allow consecutive extractions using the same membrane. On the other hand, other methods previously published offered higher EF between 28-49 [17] and 51-86 [20] and lower extraction time, but required 250x higher sample volume, the membrane could not be reusable and it did not allow working under double-flow conditions which requires lower sample volumes and offer shorter extraction times and very high extraction efficiencies. Finally, this new device presented as SF-µLPME demonstrate to be a very high and fast potential method which is also reusable, allow several consecutive extractions and offer satisfactory EF from very low microliters of sample.

4. Conclusions

In this work, a versatile on-chip liquid phase extraction was successfully designed for enabling working either under double-flow or stopped-flow conditions allowing good enrichment factors. The microchip was optimized and employed for the simultaneous analysis of SAC, Et-P, Pr-P, KTP, NPX, iBu-P, Bu-P, DIC and IBU as model analytes in the low volumes of urine samples.

The new microchip device proposed not only offer advantages over traditional LPME or EME, but it also gives many new important advantages over previous miniaturized LPME since it allows (1) an enhancement of the pre-concentration factors in microfluidic systems, and (2) the possibility of also working under stopped-flow conditions (without the necessity of collecting extracts) resulting in a sample outlet volume for injection (7 µL) that is compatible for direct analysis. Under stopped-flow conditions, low LODs, high sample cleanup, high preconcentration factors (16-47), and good extraction efficiencies (27-81) were achieved by this microchip chip compared to previous microfluidic devices based LPME. On the other hand, very high extraction efficiencies were achieved using double-flow conditions (90-100).

Additionally, this miniaturized device also offer the additional advantages of using miniaturized systems compared to traditional ones: decrease of the organic solvent volume, simple handling, the possibility of being reusable (decreasing cost instrumentation) and small sample volume consumption.

The microchip can be used either in one mode or another depending of the LOQ requirements for real samples and can be coupled online to analytical instruments such as HPLC allowing automation of both the extraction procedure and its consequent analysis. The new geometry proposed in this work (SF-µLPME) could be introduced as an appropriate alternative for the simultaneous analysis of different classes of analytes in complicated matrices, importantly for the analysis of samples with limited available volumes (especially for biofluids).

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Figure 1. Schematic of the microchip device based liquid phase microextraction

Figure 2. Optimization of the donor phase composition. SLM: dihexylether, flow rate (donor and acceptor phase): 1 µL min^{-1}, acceptor phase composition: pH 11.75

Figure 3. Optimization of the acceptor phase composition. SLM: dihexylether, flow rate (donor phase): 1 µL min^{-1}, flow rate (acceptor phase): 1 µL min^{-1}, donor phase composition: pH 1.5

Figure 4. Extraction efficiency versus sample flow rate. SLM: dihexylether, flow rate (acceptor phase): 1 µL min^{-1}, donor phase composition: pH 11.5 and acceptor phase composition: pH 1.5

Figure 5. Extraction enrichment versus sample phase flow rate. SLM: dihexylether, flow rate (acceptor phase): 1 µL min^{-1}, donor phase composition: pH 11.75 and acceptor phase composition: pH 1.5

Figure 6. Optimization of sample solution flow rate and extraction time for SAC, Et-P, Pr-P, NPX, KTP, iBu-P, Bu-P, DIC and IBU.

Figure 7.Chromatogram of a spiked urine sample containing 16 µg L^{-1} for all compounds except for IBU that was 30 µg L^{-1}. Extraction time: 20 minutes. SLM: diexylether; donor phase composition: pH 11.75 and acceptor phase composition: pH 1.5. Sample flow rate: 20 µL min^{-1}. No sample dilution

Table 1. Extraction efficiencies (RSD %) of the model substances using different organic solvents as the SLM for µLPME of acid drugs.

Table 2. µLPME calibration parameters, method detection limit (MLOD), method quantitation limit (MLOQ), extraction efficiencies and enrichment factor for all analytes in a) stopped-flow conditions mode after 20 min extraction and b) in double-flow conditions mode with an extraction time of 7 min.

Table 3. SF-µLPME/HPLC recoveries (average of three determinations ± standard deviation) from non-diluted spiked urine samples.

Table 4. Comparison of figures of merit of µLPME with other analytical techniques for determination of non-steroidal anti-inflammatories and parabens.

Tables

Table 1

Table 1. Extraction efficiencies (RSD %) of the model substances using different organic solvents as the SLM for µLPME of acid drugs.

	% Extraction efficiency (%RSD, n=3)			
	NPOE	**Dihexylether**	**1-heptanol**	**1-octanol**
Salicylic acid	15 (2)	89 (1)	3 (1)	9 (1)
Ethyl 4-hydroxybenzoate	82 (1)	100 (2)	77 (2)	85 (1)
Propyl 4-hydroxybenzoate	99 (1)	99 (1)	79 (1)	88 (1)
Ketoprofen	94 (4)	98 (2)	12 (3)	86 (3)
Naproxen	81 (2)	93 (2)	17 (1)	58 (2)
IsoButyl 4-hydroxybenzoate	100 (2)	100 (1)	82 (2)	94 (2)
Butyl 4-hydroxybenzoate	70 (2)	98 (2)	81 (3)	97 (1)
Diclofenac	54 (1)	88 (2)	10 (1)	33 (1)

Ibuprofen	70 (2)	100 (3)	5 (2)	22 (3)
AvEEi	64	94	31	51

[a] Sample: 1 µL min^{-1} of HCl at pH 3 containing the nine drugs each at 1 µg mL^{-1}; acceptor: 1 µL min^{-1} of NaOH at pH 11.75; extraction time: 7 min. NPOE: 2-Nitrophenyl octyl ether

Table 2. µLPME calibration parameters, method detection limit (MLOD), method quantitation limit (MLOQ), extraction efficiencies and enrichment factor for all analytes in a) stopped-flow conditions mode after 20 min extraction and b) in double-flow conditions mode with an extraction time of 7 min.

	Stooped-flow conditions [a]					**Double-flow conditions[b,c]**		**Double-flow conditions[b,d]**	
	MLOD (µg L^{-1})	**MLOQ** (µg L^{-1})	**R^2**	**EF**	**EE***	**EF**	**EE***	**EF**	**EE***
Salicylic acid	2.0	6.7	0.9997	47	81	9	44	-	98 (1)
Ethyl 4-hydroxybenzoate	1.1	3.7	0.9995	42	73	15	74	-	100 (1)
Propyl 4-hydroxybenzoate	0.7	2.3	0.9992	35	61	17	87	-	98 (1)
Ketoprofen	2.9	9.7	0.9991	34	60	18	89	-	99 (1)
Naproxen	1.8	6.0	0.9994	41	71	13	64	-	100 (1)
IsoButyl 4-hydroxybenzoate	0.9	3.0	0.9990	21	44	14	70	-	100 (1)
Butyl 4-hydroxybenzoate	1.5	5.0	0.9992	16	27	11	55	-	99 (1)
Diclofenac	4.2	14.0	0.9989	19	34	15	76	-	92 (1)
Ibuprofen	8.5	28.3	0.9991	35	61	19	94	-	99 (1)

[*]% Extraction efficiency (%RSD, n=4)
[a] Extraction time: 20 min and sample flow rate 20 µL min^{-1}
[b] Extraction time: 7 min
[c] Acceptor flow rate of 1 µL min^{-1} and sample flow rate of 20 µL min^{-1}
[d] Acceptor and sample flow rate of 1 µL min^{-1}

Table 3. SF-µLPME/HPLC recoveries (average of three determinations ± standard deviation) from non-diluted spiked urine samples.

	Spiked level (µg L^{-1})	SAC	KTP	NAX	DIC	IBU
Urine 1	7	92.5 ± 0.6	N.Q	94.4 ± 0.9	N.Q	N.D
	18	94.2 ± 1.2	93.3 ± 0.8	92.4 ± 1.1	86.6 ± 0.7	89.1 ± 0.3*
	50	95.5 ± 0.6	95.5 ± 0.8	98.2 ± 0.5	85.0 ± 0.5	90.2 ± 0.4
Urine 2	7	90.1 ± 0.5	N.Q	95.2 ± 0.4	N.Q	N.D
	18	92.4 ± 0.8	93.4 ± 0.9	93.3 ± 0.8	86.1 ± 0.7	87.2 ± 1.5*
	50	95.9 ± 1.2	94.0 ± 1.0	100.0 ± 0.8	88.8 ± 0.4	91.7 ± 0.6

*****Spiked concentration: 30 µg L^{-1}

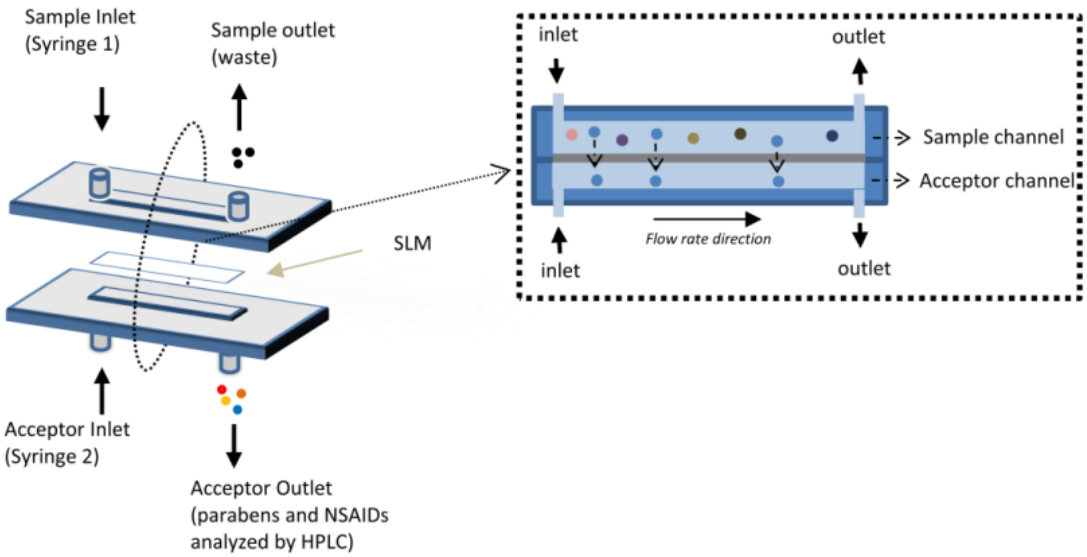
Table 4. Comparison of figures of merit of µLPME with other analytical techniques for determination of non-steroidal anti-inflammatories and parabens.

Analytical method	Analyte	Matrix	Sample Volume (mL)	LOQ (µg L^{-1})	EF	EE %	Extraction time (min)	Multi-extraction	Reference
HF(3)-LPME-HPLC/UV	SAC, DIC, IBU	Urine	50	41-180	70-900	-	15	No	4
HF(3)-LPME-MS/MS	SAC, DIC, IBU	Waste water	50	0.5-5	-	50-100	15	No	1
SPE-LC-MS/MS	SAC, DIC, IBU	Waste Water	500	0.1-3	-	70	> 30	No	21
DLLME-SFO-HPLC/UV	KTP, DIC	Urine	5	4-5	-	95-100	5	No	22
µLPME-HPLC/UV double-flow	SAC, KTP, NAX, DIC, IBU	Urine	0.007	100-500	-	75-100	5	Yes	41
HF-LPME-GC[a]	MeP, EtP, PrP	Water and urine	8	100-300	21-154	-	40	No	32
HF-LPME-[b]	MeP, EtP, PrP, BuP, iPrP iBuP. BzP	Water	3.5	0.5	3-16	24-60	30	No	3

EME-HPLC/UV	EtP, PrP, BuP, iBuP. BzP	Water	10	2.4-5	32-49	< 8	40	No	33
DF-µLPME-HPLC/UV double-flow	EtP, PrP, BuP, iBuP	Water	0.05	5-12	9-10	84-100	5	yes	42
DF-µLPME- HPLC/UV (1 µL min^{-1})	SAC, KTP, NAX, DIC, IBU EtP, PrP, BuP, iBuP	Urine	0.007	55-980	-	92-100	7	Yes	This work
DF-µLPME- HPLC/UV (20 µL min^{-1})	SAC, KTP, NAX, DIC, IBU EtP, PrP, BuP, iBuP	Urine	0.14	4.5-49	9-19	44-94	7	Yes	This work
SF- µLPME- HPLC/UV	SAC, KTP, NAX, DIC, IBU EtP, PrP, BuP, iBuP	Urine	0.4	2.3-28	21-47	27-81	20	Yes	This work

[a] Hollow fiber liquid phase microextraction- 2 phases
[b] Hollow fiber liquid phase microextraction- 3 phases

Fig. 1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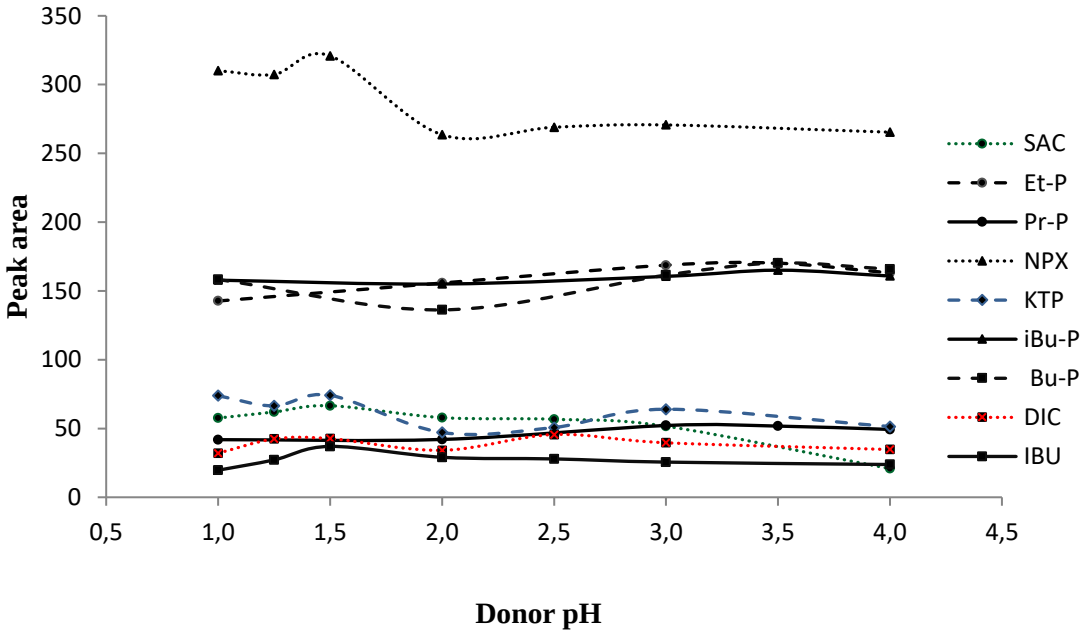
Sample outlet
(waste)
SLM
Acceptor Inlet
(Syringe 2)
Acceptor Outlet
(parabens and NSAIDs
analyzed by HPLC)
inlet
outlet
Sample channel
Acceptor channel
Flow rate direction
inlet
outlet

Fig. 2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3,5
4,0
4,5
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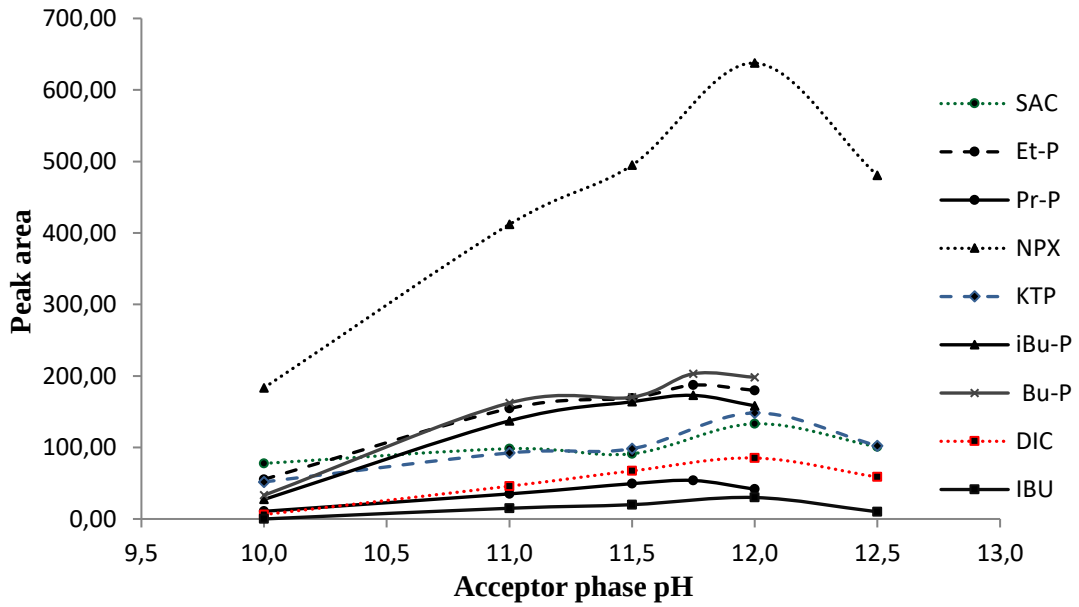
Pr-P
NPX
KTP
iBu-P
Bu-P
DIC
IBU

Fig. 3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Peak area
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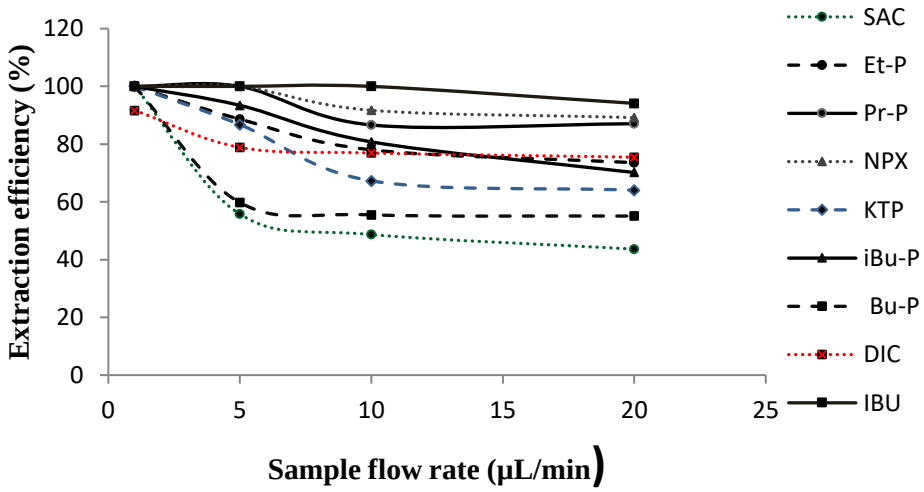
KTP
iBu-P
Bu-P
DIC
IBU

Fig. 4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Extraction efficiency (%)
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5
10
15
20
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Sample flow rate (µL/min)
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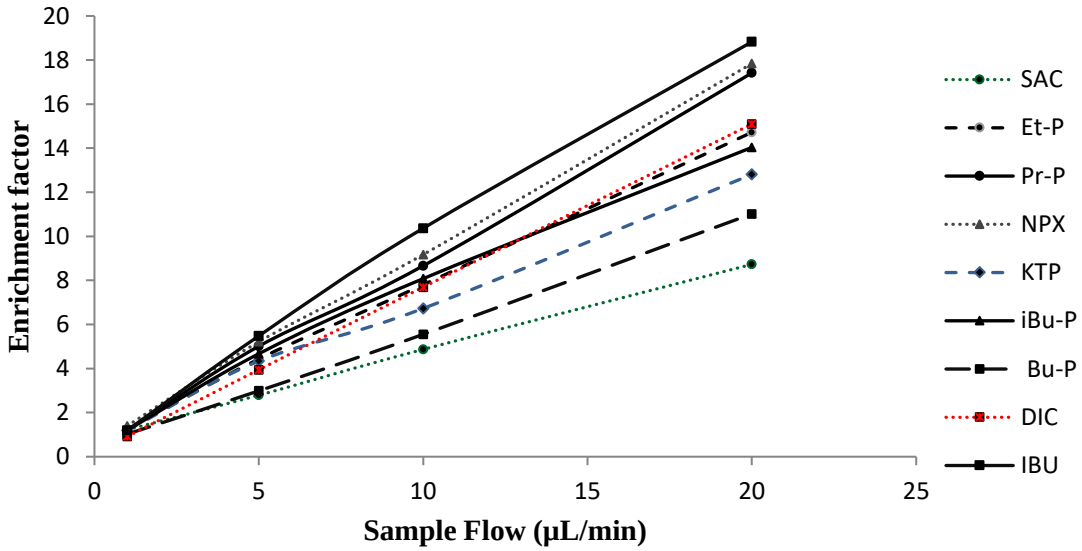
Pr-P
NPX
KTP
iBu-P
Bu-P
DIC
IBU

Fig. 5


Enrichment factor
Sample Flow (µL/min)
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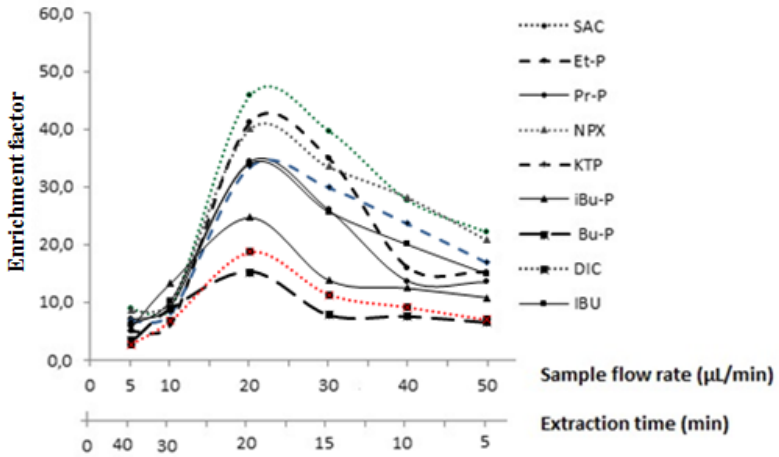
Pr-P
NPX
KTP
iBu-P
Bu-P
DIC
IBU

Fig. 6


Enrichment factor
SAC
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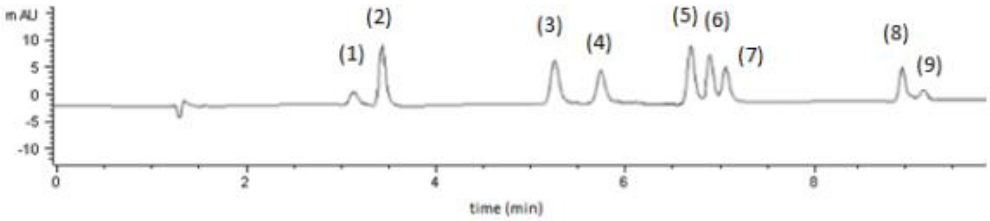
Sample flow rate (µL/min)
Extraction time (min)

Fig. 7



(1) SAC, (2) Et-P, (3) Pr-P, (4) KTP, (5) NPX, (6) iBu-P, (7) Bu-P, (8) DIC and (9) IBU