1 **A comprehensive study of a new versatile microchip device based liquid phase microextraction**

2 **for stopped-flow and double-flow conditions.**

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9 **Abstract**

10 A new geometry for a versatile microfluidic-chip device based liquid phase microextraction

11 was developed in order to enhance the preconcentration in microfluidic chips and also to enable

12 double-flow and stopped-flow working modes. The microchip device was combined with a HPLC

13 procedure for the simultaneous determination of two different families as model analytes, which were

14 parabens and non-steroidal anti-inflammatories (NSAIDs): Ethyl 4-hydroxybenzoate (Et-P), Propyl 4-

15 hydroxybenzoate (Pr-P), Butyl 4-hydroxybenzoate (Bu-P), IsoButyl 4-hydroxybenzoate (iBu-P),

16 salycilic acid (SAC), ketoprofen (KET), naproxen (NAX), diclofenac (DIC) and ibuprofen (IBU) in

17 urine samples. The new miniaturized microchip proposed in this work allows not only the possibility

18 of working in double-flow conditions, but also under stagnant conditions (stopped-flow) (SF-μLPME).

19 The sample (pH 1.5) was delivered to the SF-μLPME at 20 μL min^{-1} while keeping the acceptor phase

20 (pH 11.75) under stagnant conditions during 20 minutes. The highest enrichment factors (between 16

21 and 47) were obtained under stopped-flow conditions at 20 μL min^{-1} (sample flow rate) after 20 min

22 extraction; whereas the extraction efficiencies were within the range of 27-81% for all compounds.

23 The procedure provided very low detection limits between 0.7 and 8.5 μg L^{-1} with a sample volume

24 consumption of 400 μL. Parabens and NSAIDs have successfully been extracted from urine samples

25 with excellent clean up and recoveries over 90 % for all compounds. In parallel, the new device was

26 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.

52 Up to date, parabens and non-steroidal antiinflamatories haven been determined by traditional LPME
53 and EME procedures resulting in very good enrichment factors [1-4,17,19, 20, 30-33]. Those
54 procedures allowed good enrichment factors but low extraction efficiencies. In the last years, liquid-
55 liquid extraction has been miniaturized into microfluidic devices in order to address the limitations
56 from traditional procedures and these chip devices are becoming an attractive alternative due to the
57 many advantages that it presents [34-42]. The microchip devices for sample treatment have two
58 channels that allow working in two different ways based on the flow rate of each phase: double-flow
59 or stopped-flow conditions. In double-flow conditions, both phases (sample and acceptor) are moving
60 at some flow rate. However, in stopped-flow conditions, the acceptor phase keep stagnant while the
61 sample solution is used at some flow rate. Recent microchip devices based LPME, have been
62 demonstrated to work only under double-flow conditions but not under stopped-flow conditions in a
63 single step since the latter required to collect several extracts for its direct injection into HPLC. This
64 was due to the low sample volume available in the acceptor channel (~ 2 µL) [41,42] and consequently,
65 the analysis time increased and the reproducibility decreased when an enrichment factor was necessary
66 prior to the sample analysis. On the other hand, the devices did not allowed high preconcentration
67 factors although the sample flow rate was significantly increased under double-flow conditions.
68 Based on the current limitations of microfluidic devices for microextraction procedures, the aim of this
69 work was to develop a new versatile and effective microfluidic device in order to overcome the
70 limitations from previous microfluidic devices, increasing the preconcentration and allowing working
71 under stopped-flow conditions compatible with direct analysis.
72 Based on geometry aspects, an increase of the depth channel would increase the volume capacity
73 contained in the channel but it could decrease the transport phenomena by passive diffusion since the
74 analytes are farther away from the membrane. Microfluidic systems that follow a laminar regimen do
75 not carry agitation, so diffusion can be slow if the distance between the analytes and extraction solvent
76 is increased. Moreover, an increase of the channel´s width would increase the contact surface between

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

89 **2. Experimental**

90 *2.1. Chemicals and solutions*

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

100 *2.2 Fabrication of the microfluidic-chip device*

101 Figure 1 shows a scheme of the microfluidic device based liquid phase microextraction. This
102 microfluidic device has been re-designed and modified in order to overcome the limitations and
103 disadvantages from previous microfluidic devices. The optimal poly(methyl methacrylate(PMMA)
104 device consisted of two symmetrical patterned plates with one channel of 23 mm length, 120 µm depth
105 and 3 mm width each. Four holes of 3 mm and 1.35 mm diameter were drilled for assembling and
106 fixing in/outlets Teflon tubes, respectively. A flat polypropylene membrane piece of 27 mm length x
107 5 mm width separated the acceptor phase (channel 1) and the donor phase (channel 2). Firstly, the
108 membrane was placed over one channel and impregnated with 4 µL of dihexyl ether. Once the
109 extracting solvent was immobilized along the membrane by capillary forces, the channels were aligned
110 and the device was closed using four small crews. The final size of a microfluidic device for one single
111 extraction was 47×29×6 mm, however by increasing the size of both PMMA plates, an arbitrarily large
112 number of extraction channels can be implemented and independently addressed. Also, the microchip-
113 device can be opened any time when exchange membrane is needed.
114 A laser cutter (Epilog Mini 24-30 W) was used to fabricate this chip. The best quality was obtained
115 using a writing speed of 40%, power of 33%, a resolution of 1500 and a frequency of 5000.
116 Inlets Teflon tubes (acceptor and donor inlets) were connected to two separate micro-syringe pumps
117 (Cetoni GmbH, Korbussen, Germany). The sample (pH 1.5) was pumped into the microfluidic device
118 at 20 µLmin^{-1} while keeping the acceptor phase (pH 11.75) constant. The microfluidic device was also
119 tested under double-flow conditions as described below, in order to compare different working modes.
120 The acceptor phase was collected using a micropipette and was directly injected into a HPLC for
121 analysis.
122 *2.3. Chromatographic conditions*
123 An Agilent 1100 series (Barcelona, Spain) liquid chromatography equipped with a G1312A Bipump
124 and an autosamplerG1313A for 5 µL of sample injection was used as HPLC system. The column used
125 for the separation of the nine compounds was a LiChroCART® 75-4 Purospher® STAR RP-18e 3 µm

126 (75 mm x 4.0 mm i.d.) (VWR, Barcelona, Spain) proceeded by a guard column Kromasil1 100 Å, C18,
127 5 µm (20 mm x 4.6 mm i.d.) (Scharlab S.L., Barcelona, Spain).
128 The mobile phase consisted of 0.1% formic acid (pH 2.6) (component A) and methanol (component
129 B) at a flow rate of 0.5 mL min-1. Separation was performed at 25ºC. An initial 60% component B
130 was used in isocratic mode for 2 min, and then a linear elution gradient was programmed from 60% to
131 80% (B) for 3.4 min and from 80 % to 86 % B for another 2.4 minutes. Three minutes were waited
132 between injections which allowed re-equilibration of the column to the initial conditions.
133 The wavelengths used for DAD were 235, 255, 230, 280 and 225 nm for SAC, KTP, NAX, DIC and
134 IBU, respectively and 255 nm for all parabens. The chromatogram was completed in less than 10
135 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,
136 KTP, NPX, iBu-P, Bu-P, DIC and IBU, respectively.
137 *2.4. Preparation of biological samples analysis using µLPME extraction*
138 Spiked urine samples were adjusted to pH 1.5 with HCl and filtered through Pall NylafloTM nylon
139 membrane filter 0.45 µm (Pall Corporation, Ann Arbor, Michigan, USA) prior to microextraction
140 procedure.
141 *2.5. Calculations of extraction efficiency and enrichment factor*
142 The enrichment factor (EF_i) for the analyte *i* was calculated according to the following equation (1):
143
144 $$EF_i = \frac{C_{f,a,outlet}}{C_{i,s,inlet}} \quad (1)$$
145
146 where$C_{f,a,outlet}$ is the concentration of the analyte i at the outlet of the acceptor channel and $C_{i,s,inlet}$
147 is the initial concentration of the analyte in the sample. $C_{f,a,outlet}$was determined by HPLC UV-
148 detection using external calibration. The enrichment factor is calculated using the same equation either
149 using double-flow or stopped-flow conditions. The extraction efficiency (*EE*) was defined as the

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

152 $$EE\ (\%) = \frac{C_{f,a,outlet}}{C_{i,s,inlet}}\ x\ \frac{v_a}{v_s}\ x\ 100 = EF_i\ x\ \frac{v_a}{v_s}\ x\ 100 \qquad (2)$$

153 Where $v_a\ and\ v_s,$ are the acceptor and sample flow rate, respectively.
154 However, under stopped-flow conditions, the extraction efficiency (EE %) was calculated by
155 substituting the parameter "acceptor and sample flow rate" by the "acceptor and sample volume"
156 corresponding to each phase sample.
157 In order to obtain a global EE value for the 9 analytes, the average extraction efficiency index (avEEi)
158 was defined (3):

159 $$AvEEi = 1 - \sqrt{\frac{\sum_{i=1}^{n}(EE_i - 100)^2}{n}} \qquad (3)$$

160

161 **3. Results and discussion**

162 *3.1. Principle of the extraction*

163 The model analytes corresponded to two different families: non-steroidal antiinflamatories and
164 parabens. The extraction of the analytes is based on a passive diffusion process due to a strong pH
165 gradient difference between the acceptor and the sample solution. Non-steroidal antiinflamatories
166 contain acid groups within a pK_a range value of 2.5-5, while the paraben are esters of
167 parahydroxybenzoic acid and contain alcohol group which pK_a value are within the range of 5-8.8. A
168 three phases liquid phase microextraction configuration presents two aqueous solutions (acceptor and
169 sample) separated by the support liquid membrane (organic solvent). The analytes of interest were in
170 neutral form in the sample solution and negatively charged in the acceptor solution. This way, a pH
171 value of under 7 (HCl solution) and over 9 (NaOH solution) were used as sample and acceptor solution,
172 respectively.The microfluidic device was tested using two different working modes: double-flow mode
173 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

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

200 *3.3. Optimization and evaluation of experimental conditions*

201 According to data founded in the bibliography for individual microextraction of parabens or non-
202 steroidal antiinflamatories, preliminary experiments were performed to determinate the most suitable
203 organic solvent to be used as SLM for the simultaneous extraction of both families. For the
204 optimization of the organic solvent, a pH 3 (HCl) sample solution, pH 11.75 (NaOH) as acceptor
205 solution, and 1 µL min^{-1} as sample and acceptor flow rate were used. 2-nitrophenil octyl ether (NPOE),
206 1-octanol, 1-heptanol and dihexylether were tested by opening the microfluidic device for membrane
207 exchange after each organic solvent test. As seen in Table 1, the extraction efficiency (based on 3
208 replicate experiments) was very different depending on the analyte. Best avEEi was obtained when
209 dihexylether was used as support liquid membrane, which was consequently used as SLM for the rest
210 of the study.

211 For optimization of sample and acceptor composition, the acceptor and donor phase were tested within
212 the ranges of pH 10-12 (aqueous NaOH solutions) and 1-4 (aqueous HCl solutions), respectively. The
213 donor phase, containing 1 mg mL^{-1} of each analyte, was tested keeping the acceptor phase fixed at pH
214 11.75. As seen in Figure 2, the highest peak areas were obtained at pH 1.5 after 7 minutes extraction,
215 not observing a significant decrease for the rest of the pH range tested. Then, acceptor phase
216 composition was optimized by keeping the sample solution fixed at pH 1.5 for all experiments. Figure
217 3 shows that the highest peak areas were obtained at pH 12 and pH 11.75 for five non-steroidal
218 antiinflamatories and four parabens, respectively. On the other hand, parabens were not stable over pH
219 12 due to a slight degradation during their extraction, so a compromised pH of 11.75 was selected for
220 the extraction of both families. A relative standard deviation (RSDs %) below 4 % for all analytes
221 resulted based on 3 replicate experiments of each experimental point for Figure 2 and 3. Consequently,

222 a pH of 1.5 and 11.75 were fixed as sample and acceptor composition, respectively, for the study of
223 the flow rate for all compounds.
224 Next, the device was tested on two different working modes in order to compare the best results
225 obtained related to extraction efficiencies and enrichment factors. This new geometry allows testing
226 both working modes without the necessity of collecting different extracts (from acceptor outlet) under
227 stopped-flow conditions, a limitation that a previous geometry presented [41,42]. First, for double-
228 flow conditions experiments, the acceptor and donor flow rate were optimized within a range of 1-4
229 µL min^{-1} and 1-20 µL min^{-1}, respectively. The acceptor phase was tested keeping the donor phase flow
230 rate at 1 µL min^{-1}, obtaining the highest extraction efficiencies at 1µL min^{-1} (data not shown). Then,
231 the donor flow rate was tested while the acceptor flow rate was kept constant at 1 µL min^{-1}. Figure 4
232 shows that the highest extraction efficiencies were obtained at 1 µL min^{-1} flow rate for all compounds,
233 observing a decrease as the donor flow rate increased significantly due to the decrease residence time
234 of the sample. The extraction efficiencies were over 65 % for all compounds, except for Bu-P and SAC
235 being slightly lower. However, as seen in Figure 5, the enrichment factor significantly increased when
236 the donor flow rate increased, resulting in an EF between 8 and 20 at 1 µL min^{-1} (acceptor flow rate)
237 and 20 µL min^{-1} (sample flow rate) for all compounds.
238 Second, stopped-flow condition was studied. The size and geometry of the new proposed microchip-
239 device allowed the collection of a higher acceptor volume in one step (7 µL) after stagnant conditions
240 extractions. The microfluidic device was tested at different sample flow rates while keeping the
241 acceptor phase constant. Preliminary experiments were tested at different sample flow rate (5,10,20,30,
242 40 and 50 µL min^{-1}) and extraction times, observing that extraction times depends on the sample flow
243 rate. Hence, extraction time and sample flow rate were investigated together. Flow rates over 60 µL
244 min^{-1} were not tested since it showed not reproducible results due to certain instability of the support
245 liquid membrane. As shown in Figure 6, enrichment factors increased by increasing sample flow rate
246 from 5 to 20 $\mu Lmin^{-1}$, but it decreased by increasing the sample flow rate over 20 µL min^{-1} since the

247 target analytes do not have enough time to pass through the SLM into acceptor phase. The highest
248 enrichment factors were reached at 20 µL min^{-1} after 20 minutes extraction. At those conditions,
249 extraction efficiencies and enrichment factors were within the range of 27-81 and 16-47, respectively.
250 Each point from figure 4, 5 and 6 was based on 3 replicate experiments resulting in a relative standard
251 deviation (RSDs %) below 6 % for all analytes.
252 Therefore, the new geometry of this microfluidic device allowed the possibility of either working under
253 stopped-flow conditions resulting in higher enrichment factors and good extraction efficiencies, or
254 under double-flow conditions obtaining lower enrichment factors but better extraction efficiencies.
255 Finally, the influence of salt addition was studied under stopped-flow conditions. NaCl and Na_2SO_4
256 were tested as salting-out reagent within the concentration range of 0–20% (w/v) and 0.5-1.5 M,
257 respectively. It was observed an increase of the recoveries between 5 and 25 % for all compounds
258 when 10 % of NaCl was added, except for DIC that did not show an increase and IBU which offered
259 the highest recovery when 0.5 M of Na_2SO_4 was added. Then, 10 % NaCl was added to each sample
260 experiment.
261 In order to demonstrate the performance of this new geometry, stopped-flow conditions mode was
262 selected since it offered the highest enrichment factors for its application in urine samples. The optimal
263 experimental conditions were pH 11.75 as acceptor phase, pH 1.5 as sample or donor phase, 20 µL
264 min^{-1} as sample flow rate and 20 minutes extraction.
265 *3.4. Method evaluation*
266 A 10-point calibration curve was constructed using a least-square linear regression analysis of 10
267 different standard mixtures (in triplicate). Optimal conditions for stopped-flow working mode were
268 applied to find out the linearity, repeatability, LODs, and LOQs of the method that summarized in
269 Table 2. Several aqueous pH 1.5 solutions (containing the nine compounds) were injected into the
270 microfluidic liquid phase microextraction procedure and analyzed according to the described HPLC
271 procedure under stopped-flow conditions. Detection and quantification limits were calculated as three

272 and ten times the standard deviation of the background signal, respectively. The calibration curves
273 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}
274 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}
275 for all compounds. R^2 values exceeded in all cases 0.9990. The repeatability was tested using different
276 membranes in order to evaluate the viability of the device by changing membranes during one month.
277 The relative standard deviation for repeatability (n=4) and interday repeatability (n = 3, 5 days) were
278 below 4% for all analytes. The results obtained after more than 12 consecutive extractions were not
279 reproducible, so the membrane was replaced after 10 extractions. Very low detection and quantitation
280 limits between 0.7-8.5 and 3-28 µg L^{-1} were obtained, respectively.
281 Under optimized conditions, the SF-µLPME provided high enrichment factors and good extraction
282 efficiencies within the range of 16-47 and 27-81 %, respectively. Compared to our previous
283 optimization for individual extraction of parabens and NSAIDs into a microfluidic chip [41-42], much
284 higher enrichment factors have been obtained with also good extraction efficiencies by using this new
285 microchip compared to double-flow conditions.
286 *3.4. Urine samples analysis using microfluidic SF-µLPME*
287 In order to evaluate the capability of the proposed microchip device in real samples, two urine samples
288 were tested (Table 3).
289 Urine samples were collected from a 32 and 30 year-old female and male volunteer, respectively.
290 Samples were spiked at three different concentration levels of parabens and NSAIDs and were
291 submitted to the microchip device under stopped-flow conditions. The recovery was studied by
292 comparing the extraction efficiency obtained from aqueous solution (containing the analytes) with the
293 extraction efficiencies obtained from spiked urine samples. The recoveries were over 90 % for all
294 compounds. Compared with previous procedures using HF-LPME for urine sample analysis, this SF-
295 µLPME purposed decrease significantly the sample volume required for the analysis and offers also
296 an excellent clean-up.

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

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

310 Other traditional methods for parabens or NSAIDs extraction (as SPE, HF-LPME or EME) have
311 showed lower [3], similar [33] or higher enrichment factors [4, 32], however, it required 20x and 125x
312 higher sample volume, longer extraction times and did not allow consecutive extractions using the
313 same membrane. On the other hand, other methods previously published offered higher EF between
314 28-49 [17] and 51-86 [20] and lower extraction time, but required 250x higher sample volume, the
315 membrane could not be reusable and it did not allow working under double-flow conditions which
316 requires lower sample volumes and offer shorter extraction times and very high extraction efficiencies.
317 Finally, this new device presented as SF-µLPME demonstrate to be a very high and fast potential
318 method which is also reusable, allow several consecutive extractions and offer satisfactory EF from
319 very low microliters of sample.

320 **4. Conclusions**

321 In this work, a versatile on-chip liquid phase extraction was successfully designed for enabling
322 working either under double-flow or stopped-flow conditions allowing good enrichment factors. The
323 microchip was optimized and employed for the simultaneous analysis of SAC, Et-P, Pr-P, KTP, NPX,
324 iBu-P, Bu-P, DIC and IBU as model analytes in the low volumes of urine samples.
325 The new microchip device proposed not only offer advantages over traditional LPME or EME, but it
326 also gives many new important advantages over previous miniaturized LPME since it allows (1) an
327 enhancement of the pre-concentration factors in microfluidic systems, and (2) the possibility of also
328 working under stopped-flow conditions (without the necessity of collecting extracts) resulting in a
329 sample outlet volume for injection (7 µL) that is compatible for direct analysis. Under stopped-flow
330 conditions, low LODs, high sample cleanup, high preconcentration factors (16-47), and good
331 extraction efficiencies (27-81) were achieved by this microchip chip compared to previous
332 microfluidic devices based LPME. On the other hand, very high extraction efficiencies were achieved
333 using double-flow conditions (90-100).
334 Additionally, this miniaturized device also offer the additional advantages of using miniaturized
335 systems compared to traditional ones: decrease of the organic solvent volume, simple handling, the
336 possibility of being reusable (decreasing cost instrumentation) and small sample volume consumption.
337 The microchip can be used either in one mode or another depending of the LOQ requirements for real
338 samples and can be coupled online to analytical instruments such as HPLC allowing automation of
339 both the extraction procedure and its consequent analysis. The new geometry proposed in this work
340 (SF-µLPME) could be introduced as an appropriate alternative for the simultaneous analysis of
341 different classes of analytes in complicated matrices, importantly for the analysis of samples with
342 limited available volumes (especially for biofluids).
343
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479 Figure 1. Schematic of the microchip device based liquid phase microextraction

480 Figure 2. Optimization of the donor phase composition. SLM: dihexylether, flow rate (donor and

481 acceptor phase): 1 µL min^{-1}, acceptor phase composition: pH 11.75

482 Figure 3. Optimization of the acceptor phase composition. SLM: dihexylether, flow rate (donor phase):

483 1 µL min^{-1}, flow rate (acceptor phase): 1 µL min^{-1}, donor phase composition: pH 1.5

484 Figure 4. Extraction efficiency versus sample flow rate. SLM: dihexylether, flow rate (acceptor phase):

485 1 µL min^{-1}, donor phase composition: pH 11.5 and acceptor phase composition: pH 1.5

486 Figure 5. Extraction enrichment versus sample phase flow rate. SLM: dihexylether, flow rate (acceptor

487 phase): 1 µL min^{-1}, donor phase composition: pH 11.75 and acceptor phase composition: pH 1.5

488 Figure 6. Optimization of sample solution flow rate and extraction time for SAC, Et-P, Pr-P, NPX,

489 KTP, iBu-P, Bu-P, DIC and IBU.

490 Figure 7.Chromatogram of a spiked urine sample containing 16 µg L^{-1} for all compounds except for

491 IBU that was 30 µg L^{-1}. Extraction time: 20 minutes. SLM: diexylether; donor phase composition: pH

492 11.75 and acceptor phase composition: pH 1.5. Sample flow rate: 20 µL min^{-1}. No sample dilution

493 Table 1. Extraction efficiencies (RSD %) of the model substances using different organic solvents as

494 the SLM for µLPME of acid drugs.

495 Table 2. μLPME calibration parameters, method detection limit (MLOD), method quantitation limit
496 (MLOQ), extraction efficiencies and enrichment factor for all analytes in a) stopped-flow conditions
497 mode after 20 min extraction and b) in double-flow conditions mode with an extraction time of 7 min.
498 Table 3. SF-μLPME/HPLC recoveries (average of three determinations ± standard deviation) from
499 non-diluted spiked urine samples.
500 Table 4. Comparison of figures of merit of μLPME with other analytical techniques for determination
501 of non-steroidal anti-inflammatories and parabens.
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511 **Tables**
512 **Table 1**
513 Table 1. Extraction efficiencies (RSD %) of the model substances using different organic solvents as
514 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)

537 [*]% Extraction efficiency (%RSD, n=4)
538 [a] Extraction time: 20 min and sample flow rate 20 μL min^{-1}
539 [b] Extraction time: 7 min
540 [c] Acceptor flow rate of 1 μL min^{-1} and sample flow rate of 20 μL min^{-1}
541 [d] Acceptor and sample flow rate of 1 μL min^{-1}
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568 **Table 3.** SF-μLPME/HPLC recoveries (average of three determinations ± standard deviation) from
569 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

570 *Spiked concentration: 30 μg L^{-1}
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590 Table 4. Comparison of figures of merit of µLPME with other analytical techniques for determination

591 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