A new microchip design. A versatile combination of Electromembrane Extraction and Liquid Phase Microextraction in a single chip device.

María Ramos Payán†*, Elia Santigosa[x], Rut Fernández Torres †, Miguel Ángel Bello López †

† Department of Analytical Chemistry, Faculty of Chemistry, University of Seville, c/Prof. García González s/n, 41012, Seville, Spain

[x] Department of Analytical Chemistry, Universitat Autónoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

KEYWORDS: microextraction, microchip, sample preparation, simultaneous extraction, drugs, urine sample.

ABSTRACT: For the first time, a novel and versatile microfluidic device was developed to achieve the possibility of combining different extraction principles using a miniaturized approach for the extraction of different classes of analytes. This novel microchip is composed by a sandwich of three poly(methyl methacrylate) (PMMA) layers. Four channels allowed the combination of electromembrane extraction (EME) and liquid phase microextraction (LPME) in three different ways: (I) EME and LPME, (II) EME and EME, or (III) LPME and LPME. The microchip can be used either: a) using a common acceptor phase (for both extractions) for the simultaneous extraction of drugs from different nature in a single step, or b) a common sample solution (for both extractions) and two acceptor solutions for simultaneous drug separation. In this work, the performance of this novel microchip was demonstrated by simultaneous integration of EME and LPME using a common acceptor phase for both extractions. This configuration reduces the time of analysis allowing direct analysis in a single step. The microchip was tested for extracting two different classes of analytes, being five fluoroquinolones and four parabens as model analytes. All effective variables were optimized for EME and LPME. Under the optimized conditions, the reusable microchip enables simultaneous µ-EME/LPME with extraction efficiencies over 77% in only 8 min extraction and sample volume consumption lower than 40 µL. The optimized procedure was successfully applied to urine samples obtaining recoveries over 90 % for all analytes.

Over the last decade, the sample treatment has become one of the most important issue. An important number of analytical studies have been focused on new analytical procedures for improving selectivity, sensitivity and decreasing the time of analysis. In this way, miniaturization of analytical extraction procedures has been developed to accomplished those requirements and additionally, to reduce solvents and chemicals and sample volume consumption. Liquid Phase microextraction (LPME) and lately Electromembrane extraction (EME) (introduced in 2006) are two very well-known and popular techniques used for the extraction of drugs based on passive diffusion or electrokinetic migration, respectively, to extract the analytes from the sample solution to an acceptor solution through a membrane (which supports an organic solvent into its porous). These techniques offer an excellent clean up due to the high selectivity of the support liquid membrane (SLM) and sufficient enrichment factors due to the tunable ratio between the sample and acceptor volume. LPME and EME have been applied to many different fields, especially biological, pharmaceutical, environmental, food, toxicology analysis, among others[1-6]. As known, extraction can be completed in shorter time of analysis when the transport phenomena get faster on the micro-scale. In this concept, miniaturized systems on chip have gained importance in recent years compared to traditional ones due to the advantages they have, such as the fact that they require much lower sample volume and reagents for the analysis and reduce the extraction time[7-18]. This way, many efforts have been focused on the design of microfluidic devices in the field of analytical chemistry. Most works reported in the bibliography have been developed in order to determine substances from similar nature and properties[15,16,18-23], however, in the last years EME and LPME have also been applied for simultaneous group separation of substances from very different nature[24-30].

Different set-ups have been published to overcome the limitations presented by previously reported traditional techniques for simultaneous group separation of acidic and basic compounds by using two acceptor phases and a common donor phase[27-30] without resulting in very high extraction efficiencies. In 2015, Pedersen-Bjergaard et al, used a traditional set-up where both techniques (EME and LPME) were simultaneously implemented, however, LPME process was slightly affected by the EME process[30]. That set-up allowed the simultaneous extraction of acid and basic drugs with recoveries between 22-43 % after 15 min extraction under stagnant conditions (in both phases). Later, Nojavan et al, developed a two-step dual electromembrane obtaining extraction efficiencies from 38 to 68 % after 25 minutes extraction and a sample consumption of 4000 µL[28]. This method showed recoveries lower than 54 % in real samples (wastewater, milk and plasma) improving a previous dual electromembrane method[27]. Most recently (2016), Ymini

et al, presented a miniaturization for the simultaneous extraction of acid and basic drugs via on-chip EME/EME by joining two individual microchip devices using an external peak tube for one sample solution and two acceptor phases[29]. This method showed enrichment factors between 15-17, requiring 1000 µL of sample volume after 33 min extraction. The proposed method used a stagnant acceptor phase while the sample flow rate was 30 µL min^{-1}. However, the methods previously described, required long extraction times, offered extraction efficiencies from 5 to 43 %, did not allow more than one consecutive extraction and the mix of both acceptor solutions was necessary before injection into HPLC since it used two different acceptor solutions for group separation, which consequently diluted the final acceptor extract.

Microfluidic chip devices are very attractive due to the many advantages that it presents; therefore, inspired by the limitations of the methodologies presented above, we herein propose a new chip design that allows combining several or different techniques (as for example LPME and EME) offering much higher extraction efficiencies, shorter extraction times and lower sample consumption. This device can also work with either using a common acceptor phase and two sample solutions, or vice versa. The performance of the proposed device was demonstrated combining the advantages of both EME and LPME using one common acceptor phase to allow direct analysis in one single step for different classes of analytes: Fluoroquinolones and parabens.

EXPERIMENTAL

Chemicals and solutions

Ethyl 4-hydroxybenzoate (Et-P), Propyl 4-hydroxybenzoate (Pr-P), Butyl 4-hydroxybenzoate (Bu-P), IsoButyl 4-hydroxybenzoate (iBu-P), Marbofloxacin (MRB), Norfloxacin (NRF), Ciprofloxacin (CIP), Danofloxacin (DNF), Flumequine (FLU), 1-heptanol, 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). A flat polypropylene 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).

Preparation of solutions

100 mg L-1 stocks solutions were prepared in methanol except CIP that was prepared in a mix 1:1 Methanol:Milli-Q Plus water (Elga, purelab option S-R 7-15 (Madrid, Spain). The standard solution (STD, 1 µg µL-1 for each analyte) was daily prepared by diluting the stock solutions with Milli-Q water. Each sample solution was adjusted with NaOH and HCl for preparing each sample solution at pH 11 and pH 3.5, respectively. The common acceptor solution was adjusted to pH 11.5 (with NaOH).

Microchip fabrication: Simultaneous µEME/LPME Setup and procedure

The designed microchip for simultaneous µEME/LPME is shown in Figure 1A and 1B. The novel microchip consisted of three symmetrical poly(methyl methacrylate) (PMMA) plates assembled as a sandwich device. A laser cutter (Epilog Mini 24-30 W) was used to fabricate this chip and the best quality was obtained using a writing speed of 40%, power of 33%, a resolution of 1500 and a frequency of 5000. This way, the chip contained one channel (a) below the first layer, two channels (b and d) on top of and below the second layer, respectively and one channel (e) on top of the third layer. The first channel (a) is separated from the second channel (b) by a first membrane (c). The third channel (d) is separated from the fourth channel (e) by a second membrane (f). Firstly, EME was carried out in channel (a) and (b) where the analytes were extracted from the donor solution (channel a) to the acceptor solution (channel b) through the first membrane (c). Secondly, LPME was carried out in channel (d) and channel (e) where the analytes were extracted from the donor solution (channel e) to the common acceptor solution (channel d) through the second membrane (f). In this work, the acceptor solution was common for both extractions (EME and LPME), so channel (b) and (d) were interconnected via a hole (g) (120 µm i.d.) in the second layer. This allowed the acceptor solution to be faced with the first donor solution (EME), and later with the second donor solution (LPME). The channels had a length of 15 mm, depth of 120 µm and a width of 2 mm, and the final size of a microfluidic device for one single extraction was a square of 34 mm and 9 mm thickness (3mm for each layer). Also, the microchip-device could be opened any time when exchange membrane was needed.

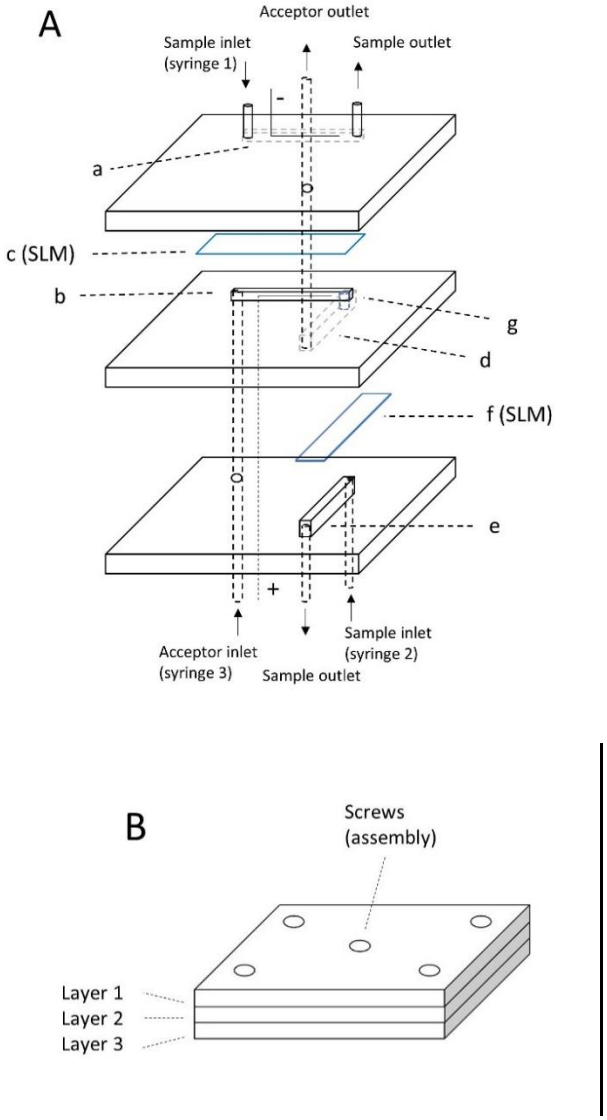


Figure 1. Schematic representation of the microchip device for simultaneous representation of µ-EME/LPME (A) and its assembly (B).

A B

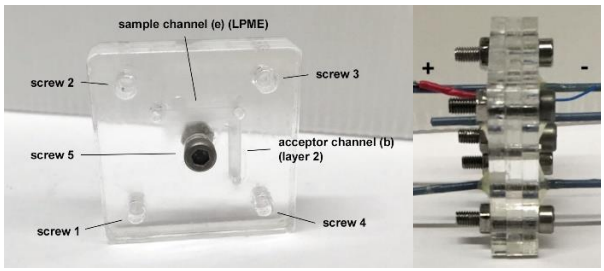


Figure 2. Real picture of the microchip device µ-EME/LPME. A) Frontal picture and B) Side view image with electrodes for EME, five screws and inlets/outlets tubes.

Five holes of 3 mm in each layer and a total of eight holes of 1.35 mm diameter were drilled for assembling (using screws) and fixing in/outlets Teflon tubes, respectively. Finally, two platinum electrodes (100 µm i.d.) were located along the channel (a) and (b) through two 100 µm i.d. holes in the PMMA plates for EME. One electrode was glued to the first layer and the other to the second layer. The third layer had a 200 µm i.d. hole to allow the introduction of the electrode from outside till the second (middle) layer. This way, the microchip can be opened and reused as many times as needed. Figure 2 shows a real picture of the microchip device.

The SLM for EME and LPME were 2 µL of octanol and diexylether, respectively. The membrane was placed over each channel and impregnated with the organic solvent. Once the extracting solvent was immobilized along the membrane by capillary forces, the channels were aligned and the device was closed using five small screws. Inlet Teflon tubes (one acceptor and two donor inlets) were connected to three separate micro-syringe pumps (Cetoni GmbH, Korbussen, Germany). The sample solutions for EME and LPME were 1 mM of NaOH and 0.32 mM of HCl, respectively. The common acceptor solution was 3.1 mM of NaOH. Samples and acceptor were pumped into the microfluidic device at 1 µLmin^{-1}. Finally, the collected acceptor phase was directly analyzed by HPLC-UV. The extraction volume collected from the acceptor phase (acceptor outlet) was only 8 µL which was directly injected into HPLC for analysis. In this new microchip design there are two independent sample channels and a minimum of 16 µL of sample was needed for each sample channel after 8 minutes extraction at 1 µL min^{-1} (8 µL for each sample channel). Additionally, 5 minutes stabilization was waited before starting the first extraction (in each sample channel) to ensure that the channels were completely full without any bubbles. Then, the minimum sample volume consumption required was 26 µL (13 µL for each sample channel). In this work, a total sample volume of 40 µL was used (20 µL for each sample solution), which depend of tubes connection length and syringe volume used to carried out the experiments. Once extraction starts, no stabilization time is needed. Extractions are carried out in continuous by collecting extracts every 8 minutes for its injection into HPLC (with an acceptor phase volume of 8 µL).

High Performance Liquid Chromatography (HPC)-UV Analysis

An Agilent 1100 series 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 0.8 mL min-1. Separation was performed at 25ºC. Mobile phase B was increased from 25 % to 100 % within 15 min; afterward, it was decreased to 75 % within 0.2 min, and this condition was kept for 2 min to re-equilibrate the column to initial conditions.

The wavelengths used for DAD were 255 for all parabens, 280 for all fluoroquinolones, except for FLU that was 315 nm. The chromatogram was completed in less than 12 minutes and the retention time was 2.6, 3.4, 3.6, 3.8, 8.4, 9, 10, 11.3 and 11.5 for MRB, NRF, CPR, DNF, Et-P, FLM, Pr-P, iBu-P and Bu-P, respectively.

Preparation of biological samples analysis using µEME/LPME extraction

Spiked urine samples were adjusted to pH 11 (for EME) and pH 3.5 (for LPME) and filtered through Pall NylafloTM nylon membrane filter 0.45 µm (Pall Corporation, Ann Arbor, Michigan, USA) prior to µEME/LPME.

Calculations of extraction efficiency and enrichment factor

The enrichment factor (EFi) 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):

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

where v_a and v_s, are the acceptor and sample flow rate, respectively.

RESULTS AND DISCUSSION

Proof of concept

The aim of the current work is to offer a novel and new microchip device which allow the possibility of combining either: (I) EME and LPME, (II) EME and EME, and (III) LPME and LPME using a common acceptor phase or a common sample solution, resulting in a potential versatile chip which can be used for either (IV) simultaneous extraction of drugs from different nature or (V) separation of drugs from a common sample solution. This way, the device can combine the advantages of both EME and LPME. For example, in EME, the extraction selectivity can be controlled by the magnitude and direction of the electrical field. On the other hand, LPME does not require the use of a suitable conductor organic solvent for extractions, thus a wider range of SLM can be tested for improving the selectivity or efficiency for certain compounds. Combination of EME and LPME not only combines the ad-vantages of both methods,

but also, provides the opportunity of extraction of analytes with completely different properties and it addresses the main disadvantage related to the EME procedure that in a certain electrical field direction only one class of analytes (acidic or basic) can be extracted. In EME/LPME combination, the electrical field distribution is good (applying only one voltage) and not requiring an exact control of the distribution of the electrical field across the extraction system[30]. For proof of concept, a combination of two different microextraction principles (EME and LPME) were selected using a common acceptor phase (contained in one common chamber) for (IV) simultaneous extraction of different classes of analytes extraction in a single step for its direct analysis.

In EME, the analytes were negatively charged in the sample and acceptor solution and the analytes were extracted by electro-kinetic migration through a SLM of 1-octanol with a voltage of 30 V. As shown in Figure 1, the cathode and anode were placed in the acceptor and sample solution, respectively. Marbofloxacin (MRB), Norfloxacin (NRF), Ciprofloxacin (CIP), Danofloxacin (DNF) and Flumequine (FLU) have previously been extracted by traditional EME[20] and were selected as model analytes. On the other hand, in LPME, the analytes were neutral in the sample and charged in the acceptor solution and they were extracted by passive diffusion through an SLM of diexylether. Ethyl 4-hydroxybenzoate (Et-P), Propyl 4-hydroxybenzoate (Pr-P), Butyl 4-hydroxybenzoate (Bu-P) and IsoButyl 4-hydroxybenzoate (iBu-P) have also previously been extracted by LPME[14] and were selected as model analytes for LPME. For preliminary experiments, some of the operational parameters used for µEME and µLPME were based on previous experiences[14, 20], however they have been re-optimized since in this work, only one common acceptor phase was used.

The order of each extraction is extremely important in order to avoid back-extraction of the analytes from the acceptor toward the sample solution. The microchip was performed and tested in two different ways. Initially, the chip was tested when LPME and EME was carried out as first and second extraction. This way, the second sample solution was analyzed after both extractions and parabens were detected. Then, parabens were partially back-extracted from the acceptor to the second sample solution during the subsequent second extraction (by EME). Secondly, the chip was tested in the opposite way where EME was carried out as first and LPME as second extraction. This way, no drugs were detected in the second sample solution, so there was no back-extraction. As expected, the negatively charged analytes (in the acceptor solution) showed no affinity toward the SLM (DHE) during the LPME and remained in the acceptor solution. This was due to the selectivity of the SLM, where only neutral species can pass through the SLM barrier by passive diffusion. Consequently, the selected order of techniques for chip operation was EME first and then LPME.

Optimization of the microchip´s geometry

The geometry of the microchip was strategically designed in order to offer much more versatility, reusability, and lower sample volume compared to previous set-ups while maintaining miniaturization size and simple handling conditions. Methacrylate (PMMA) and polydimethylsiloxane (PDMS) were tested as possible materials for the manufacture of the chip due to its low cost. However, preliminary tests of analyte adsorption were made as a function of time, where it was observed that when using PDMS the analytes adsorbed to the surface of the material, while this did not occur with PMMA. For this reason, PMMA was used to manufacture the device. First, the thickness of each layer of PMMA was tested within a range of 1-5 mm. At the same time, for assembly, different holes were drilled on each layer symmetrically and closed with screws. Different geometries were tested and a final symmetry of 5 holes, one in each corner and one located in the center, resulted in the best distributed pressure. A thickness of 1 mm showed leaks from all layers and 2 mm showed leaks over the EME layer. However, 3 mm showed a good assembly with no leaks and it was selected as the optimal thickness. Secondly, the channels' size were studied. Different length, width and depth were tested in order to obtain an adequate and stable laminar flow during the extraction. The length was tested between 10 and 20 mm, the width was tested between 2 and 4 mm and the depth was tested between 60 and 200 µm. Shorter length but deeper channels showed lower efficiency since the contact surface was smaller per unit of time and the analytes were further away from the SLM. In addition, channels of 4 mm width showed no stable laminar flow when increasing the depth. On the other hand, channels no longer than 20 mm were tested to maintain the miniaturization size. This way, the best proportion size in order to obtain the most stable flow and the higher extraction efficiency was 15 mm length, 120 µm deep and 2 mm wide.

Optimization of Major Operational Parameters

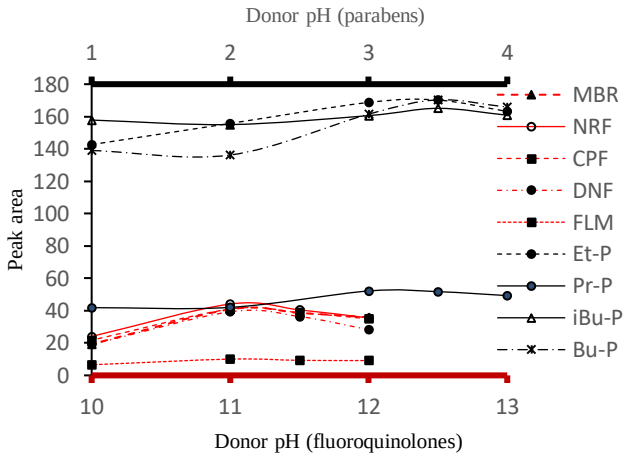
The performance of this novel microchip was studied integrating two different techniques (µEME and µLPME) and keeping only one common acceptor phase for direct analysis. As mentioned before, analytes must be charged in the sample and acceptor solution for EME and in its neutral and charged form in the sample and acceptor solution, respectively, for LPME.

Based on the data founded in the bibliography for individual microextraction of fluoroquinolones (EME) and parabens (LPME), preliminary experiments were performed to determine the most suitable organic solvent to be used as SLM in each extraction. These experiments were carried out containing 1 mg L^{-1} of each analyte in each sample solution, a flow rate of 1 µL min^{-1} for sample and acceptor solution, a pH of 11 as acceptor phase and a pH of 11 (for µEME) and a pH of 3.5 (for µLPME) as sample composition . For the µLPME, DHE was used as optimal organic solvent without further optimization based on previous experience on a chip[14]. For µEME, different organic solvents were selected based on previous experiences20, including 1-octanol, 1-heptanol and NPOE. The recoveries (based on 3 replicate experiments) showed that 1-octanol was optimal for the recovery for fluoroquinolones (data not shown). Therefore, 1-octanol was selected as the SLM for µEME.

The sample composition was investigated considering one common acceptor solution at pH 11 to ensure the analytes to be in charged form. Based on previous experiments in microfluidic devices for parabens extraction by LPME[14], the optimal donor phase composition was re-optimized within a pH range of 2-4 using this new geometry. For the extraction of fluoroquinolones by EME (considering their pKa range between 5.7-8.9), the sample solution was tested within a pH range of 10-12 in order to get negatively charged analytes in the sample solution. These preliminary experiments were carried out by applying 20 V between the cathode (channel a, sample solution) and the anode (channel d, acceptor solution). The results are summarized in figure 3, which shows that 1 mM NaOH (pH 11) and 0.32 mM HCl (pH 3.5) showed the highest and the best recovery for FQs and parabens, respectively. The optimal donor composition for parabens coincided with our previous optimization using another chip geometry[14]. The voltage was tested within 10-40 V.

Figure 4 shows the highest peak area at 30 V, resulting in a low constant current intensity of 30 µA. Consequently, 30 volts (for EME) and a sample composition of 1 mM NaOH for EME and 0.32 mM HCl for LPME were fixed for the rest of the study. Secondly, the common acceptor phase was investigated within a pH range of 9.5-12 to assure all the analytes to be fully negatively charged. Figure 5 shows the highest efficiency for parabens between pH 11.5 and 12, however a slight degradation was observed over pH 12 for those compounds. On the other hand, the best efficiency for FQs was obtained at pH 10 although the efficiency did not significantly decreased when increasing the pH up to 11.5. Therefore, a pH of 11.5 was selected as compromise donor composition to obtain high efficiency for the parabens without significantly decrease the efficiency for FQs when using pH values closer to pH 12. The microchip was stabilizing during 5 minutes before to start collecting extracts. The extraction was completed after 8 minutes. Under optimal conditions for sample and acceptor composition, the fluoroquinolones (negatively charged) were not affected by the subsequent µLPME, since the analytes were negatively charged and prevented from back-extraction into the second SLM (DHE).

Figure 3. Optimization of the sample phase composition for fluoroquinolones. SLM: octanol, flow rate (donor and acceptor phase): 1 µL min^{-1}, acceptor phase composition: pH 11.5.



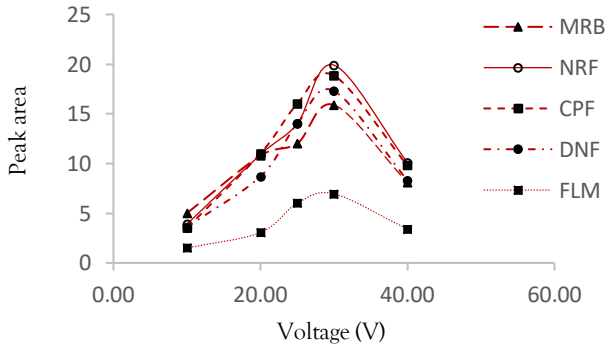


Figure 4. Influence of the voltage for EME. SLM: octanol, flow rate (sample and acceptor phase): 1 µL min^{-1}, sample phase composition: pH 11 and acceptor phase composition: pH 11.5.

Finally, the sample flow rate was tested within a range of 1-4 µL min^{-1}. Figure 6 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 significantly increased due to the decrease residence time of the sample. However, the EF for parabens slightly increased when the donor flow rate increased up to 4 µL/min. For parabens, EF within 2.9 and 3.5 were obtained at 4 µL min^{-1} whereas no EF was obtained for FQs. The fact that no enrichment factor were obtained for fluoroquinolones is in accordance with the low extraction efficiencies obtained at flow 4µL min^{-1} since, unlike parabens, their efficiency decreased significantly at higher flow. Flow rates over 5 µL min^{-1} were not investigated since no-stable current was observed and the extraction efficiencies decreased significantly. The extraction efficiencies were within 77-100 % for all compounds. Each point from figure 3, 4, 5 and 6 was based on 3 replicate experiments resulting in a relative standard deviation (RSDs %) between 1.5-4 % for all analytes. The acceptor flow rate was not investigated since the extraction efficiencies usually decrease when increasing the flow rate, previously investigated in microchip devices[7,14,15]. Then 1 µL min^{-1} was used as samples and acceptor flow rates.

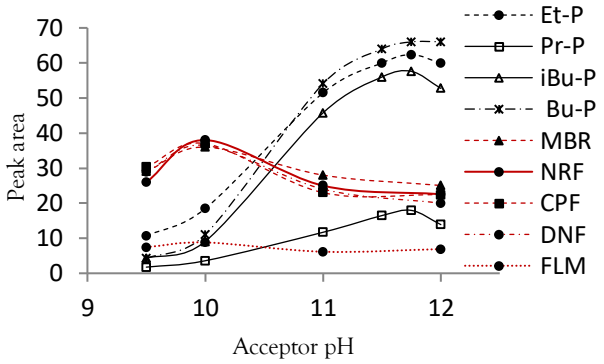


Figure 5. Optimization of the common acceptor phase composition. SLM (EME): octanol, SLM (LPME): dihexylether, flow rate (sample and acceptor phase): 1 µL min^{-1}, sample phase composition: pH 11 (for EME) and pH 3.5 (for LPME).

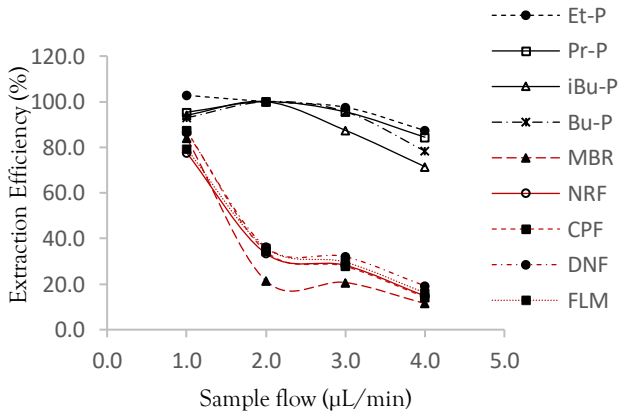


Figure 6. Extraction efficiency versus sample flow rate. SLM (EME): octanol, SLM (LPME): dihexylether, voltage (for EME): 30 V, flow rate (sample and acceptor phase): 1 µL min^{-1}, sample phase composition: pH 11 (for EME) and pH 3.5 (for LPME), acceptor phase composition: pH 11.5.

In conclusion, the optimal experimental conditions were 3.1 mM NaOH as common acceptor phase, 1mM NaOH as sample solution for µEME and 0.32 mM HCl as sample solution for µLPME, 1 µL min^{-1} as sample and acceptor flow rate, 30 volts for EME and 8 minutes extraction.

Evaluation

Figures of merit of the proposed µ-EME/LPME chip was studied on standard aqueous solutions of the target analytes at seven different standard mixtures (in triplicate).Optimal conditions were applied to evaluate the linearity, repeatability, LODs, and LOQs of the method that summarized in Table 1. The linearity range was 0.25-10 µg mL^{-1} for MRB, NRF, CPR, DNF and FLU and 0.06-5 for Et-P, Pr-P, iBu-P and Bu-P. The R^2 values were no less than exceeded in all cases 0.997 within the tested linearity range. The relative standard for repeatability (n=4) and interday repeatability (n = 3, 5 days) were below 3% for all analytes. The reproducibility (n=5) resulted in RSD values all below 4 % and it was tested using different membranes in order to evaluate the viability of the device by changing membranes during one month. The results obtained after more than 10 consecutive extractions were not reproducible, so no more than 10 consecutive extractions were carried out. As seen in table 1, the limit of detection (LOD, S/N=3) and limits of quantification (LOQ, S/N=10) were within 16-75 and 53-250 µg L^{-1} for all compounds. Under optimized conditions, the µEME/LPME chip provided high extraction efficiencies between 77-100 %, for all the target analytes.

Table 1. µ-EME/LPME calibration parameters, method detection limit (MLOD), method quantitation limit (MLOQ) and extraction efficiencies for all analytes.

	Double-flow conditions [a]			
	MLOD (µg mL^{-1})	**MLOQ** (µg mL^{-1})	**R^2**	**EE***
MRB	0.075	0.25	0.999	84
NRF	0.075	0.25	0.999	78
CPF	0.075	0.25	0.999	87
DNF	0.075	0.25	0.997	87
Et-P	0.029	0.096	0.999	100
FLM	0.075	0.25	0.999	79
Pro-P	0.017	0.056	0.997	95
iBu-P	0.016	0.053	0.999	94
Bu-P	0.022	0.073	0.998	93

*% Extraction efficiency (%RSD, n=4)

[a] Acceptor and sample flow rate of 1 µL min^{-1}

Simultaneous µEME/LPME from Urine Samples into a chip

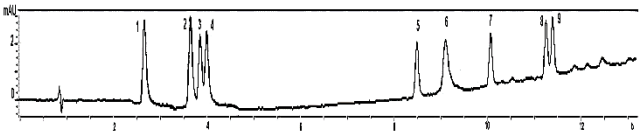
In order to evaluate the capability of the proposed microchip device in real samples, urine samples were tested (Table 2). Urine samples were collected from a 32 year-old female. Samples were spiked at three different concentration levels of the target analytes (parabens and fluoroquinolones) and were submitted to the microchip device. The fact of using urine samples did not affect to the membrane reusability. The extraction efficiencies were 81%, 76%, 84%, 85%, 98%, 75%, 93%, 92% and 91% for MRB, NRF, CPF, DNF, Et-P, FLM, Pro-P, iBu-P and Bu-P, respectively, in urine samples. The recoveries were over 90 % for all compounds and it was studied by comparing the extraction efficiency obtained from aqueous solution (containing the analytes) with the extraction efficiencies obtained from spiked urine samples. Figure 7 shows a representative chromatogram of a spiked urine sample containing 1 µg mL^{-1} for all compounds, observing an excellent clean-up with no sample dilution and very low sample consumption compared to traditional EME and HF-LPME procedures, which required much higher sample volume.

Finally, in order to show the applicability of this microchip device for µEME/EME, another two 100 µm i.d holes were added to allow the introduction of a second pair of electrodes placed in parallel in the second EME extraction. The µEME/EME was tested in spiked urine sample by selecting two FQs (norfloxacin and danofloxacin) and two well-known non-steroidal anti-inflamatories (naproxen and ketoprofen) as model analytes. A pH 11 and pH 10.5 (NaOH) were selected as sample solutions for FQs and AINEs, respectively, a 3.1 mM NaOH (pH 11.5) was fixed as acceptor phase, octanol was used as SLM and 25 V was applied for both extractions. The µEME/EME offered efficiencies within 32 and 83 % for all compounds and allowed 4 consecutive extractions with a stable current of 0.22 mA. The middle layer (layer 2) must be of 6 mm thickness if using EME/EME, a common sample solution and the use of different voltage in each sample channel.

Table 2. µ-EME/LPME/HPLC recoveries (average of four determinations ± standard deviation) from non-diluted spiked urine samples.

	Urine sample		
	Low[a]	Medium[a]	High[a]
MRB	96.3±0.8	96.2±1.3	95.1±1.0
NRF	95.4±1.3	96.4±1.3	96.8±1.3
CPF	94.5±1.1	95.8±1.1	98±1.1
DNF	96.7±0.9	97.3±1.1	99.6±1.2
Et-P	98.3±1.4	99.2±1.5	99.8±1.1
FLM	93.3±1.3	94.8±1.3	94.1±1.5
Pro-P	96.9±1.5	97.1±1.0	100.2±0.9
iBu-P	99.7±1.2	97.7±1.4	101.1±1.4
Bu-P	97.4±1.7	98.5±1.2	99.4±0.9

[a]Low, medium and high was 0.3, 0.9 and 3 µg/mL for fluoroquinolones and 0.1, 0.5 and 2.5 µg/mL for parabens.



Retention time (min)

Figure 7. Chromatogram of a spiked urine sample containing 1 µg $µL^{-1}$ for all compounds. Extraction time: 8 minutes. No sample dilution. (1) MRB, (2) NRF, (3) CPF, (4) DNF, (5) Et-P, (6) FLM, (7) Pr-P, (8) iBu-P and (9) Bu-P.

Comparison with other setups

Table 3. Comparison of figures of merit of µ-EME/LPME with other analytical set-up for simultaneous extraction of analytes from different nature.

Analytical method	Analyte	Matrix	Sample Volume (µL)	EF	EE %	Extraction time (min)	Consecutive Extraction	Ref.
EME/LPME	CIT, SER, KET, IBU	Plasma	600	-	22-43	15	No	30
Dual EME	IBU, THB	Urine and plasma	4000	160	17-46	25	No	27
TSV EME	COD, KTM, NPX, IBU	Plasma, wastewater, breast milk	4000	76-136	38-68 (R < 54)	25	No	28
EME/EME (two chips)	BET, DIC, MEF	Urine and Plasma	1000	15-17	-	33	No	29
µEME/LPME (one chip)	MRB, NRF, CPR, DNF, FLM, EtP, PrP, BuP, iBuP	Urine	< 40	-	75-100 (R > 90)	8	Yes	This work

Citalopram (CIT), sertraline (SER), ketoprofen (KET), ibuprofen (IBU), mefenamic (MEF), betaxolol (BET), diclofenac (DIC), Codeine (COD), Naproxen (NPX), Ketamine (KTM), Ibuprofen (IBU), thebaine (THB), R (relative recovery)

The performance of this chip was compared with previous methodologies for simultaneous combination of microextraction procedures. Table 3 shows the comparison of the proposed microchip based µEME/LPME with recent extraction methods for also combination of microextraction procedures. Compared to previous methodologies and devices founded in the literature for simultaneous determination of drugs from different nature, the proposed novel microchip presented in this work is more versatile since it allows

combining either (I) µLPME/LPME, (II) µEME/EME or (III) µLPME/EME by using one common donor phase or one common acceptor phase. The versatility of this novel microchip was tested combining µEME/LPME in a single step using one common acceptor phase, resulting in a decrease of time of analysis by injecting only one extract. Additionally, this microchip presented as µ-EME/LPME demonstrated to be a very high and potential device which is also reusable, allowed consecutive extraction without the necessity of regenerating the membrane after each extraction, decreased the extraction time, offered higher extraction efficiencies (over 77 %), significantly decreased the sample volume consumption by 30x and integrated both extractions in a single device without the necessity of an external tube.

CONCLUSIONS

In this work, a novel versatile microchip which integrate different extraction principles is proposed. This novel microchip allows working in any combination by LPME and EME and also using one common acceptor phase and two sample solutions or vice versa. In addition, it allows the extraction of analytes with very different nature improving the extraction efficiencies, decreasing the extraction time and the sample consumption compared to other techniques for the simultaneous extraction of different classes of analytes. This miniaturized device represents a very significant advance in miniaturized systems for its application to sample pre-treatment and opens up new perspectives to simultaneous extraction of different classes of analytes in a very selective way. Additionally, the device can be directly coupled to different analytical instruments. In conclusion, the evaluation of the new microchip indicate that the new chip-system could be introduced as a high potential and appropriate device for the simultaneous analysis of different classes of analytes in complicated matrices, especially from samples with limited available volume.

AUTHOR INFORMATION

Corresponding Author

* E-mail: ramospayan@us.es

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was supported by the Grant Juan de la Cierva-Incorporación (Grant number JCI-2015-26647) and the Project TEC2006-79367-C2-1-R from the "Dirección General de Investigación y Gestión del PlanNacional de I + D + I" (Ministerio de Educación y Ciencia, Spain).

REFERENCES

(1) Zhang, H.; Du, Z.; Ji, Y.; Mei, M. *Talanta* 2013, *109*, 177–184.

(2) Krishna Marothu, V.; Gorrepati, M.; Vusa, R. *J. Chromatogr.Sci*. 2013, pp 619–631.

(3) Sharifi, V.; Abbasi, A.; Nosrati, A. *J. Food Drug Anal.* 2015, *24* (2), 264–276.

(4) Hasheminasab, K. S.; Fakhari, A. R.; Shahsavani, A.; Ahmar, H. *J. Chromatogr. A* 2013, *1285*, 1–6.

(5) Peng, J.-F.; Liu, J.-F.; Hu, X.-L.; Jiang, G.-B. *Journal of chromatography A* 2007, *1139* (2), 165–170.

(6) Khajeh, M.; Shakeri, M.; Natavan, Z. B.; Moghaddam, Z. S.; Bohlooli, M. *J. Chromatogr.Sci*. 2015, *18*, 1217–1221.

(7) Ramos Payán, M. D.; Jensen, H.; Petersen, N. J.; Hansen, S. H.; Pedersen-Bjergaard, S. *Anal. Chim. Acta* 2012, *735*, 46–53.

(8) Cai, Z. X.; Fang, Q.; Chen, H. W.; Fang, Z. L. *Anal. Chim. Acta* 2006, *556* (1), 151–156.

(9) Petersen, N. J.; Jensen, H.; Hansen, S. H.; Foss, S. T.; Snakenborg, D.; Pedersen-Bjergaard, S. *Microfluid. Nanofluid.* 2010, *9* (4–5), 881–888.

(10) Li, B.; Petersen, N. J.; Pay??n, M. D. R.; Hansen, S. H.; Pedersen-Bjergaard, S. *Talanta* 2014, *120*, 224–229.

(11) Petersen, N. J.; Foss, S. T.; Jensen, H.; Hansen, S. H.; Skonberg, C.; Snakenborg, D.; Kutter, J. P.; Pedersen-Bjergaard, S. *Anal. Chem.* 2011, *83* (1), 44–51.

(12) Asl, Y. A.; Yamini, Y.; Seidi, S. *Talanta* 2016, *160*, 592–599.

(13) Petersen, N. J.; Pedersen, J. S.; Poulsen, N. N.; Jensen, H.; Skonberg, C.; Hansen, S. H.; Pedersen-Bjergaard, S. *Analyst* 2012, *137* (14), 3321–3327.

(14) Ramos-Payan, M.; Maspoch, S.; Llobera, A. *Talanta* 2017, *165* (November 2016), 496–501.

(15) Ramos-Payan, M.; Maspoch, S.; Llobera, A. *Anal. Chim. Acta* 2016, *946*, 56–63.

(16) Abdossalami Asl, Y.; Yamini, Y.; Seidi, S.; Ebrahimpour, B. *Anal. Chim. Acta* 2015, *898*, 42–49.

(17) Kazarine, A.; Kong, M. C. R.; Templeton, E. J.; Salin, E. D. *Anal. Chem.* 2012, *84* (16), 6939–6943.

(18) Wu, W.I; Rezai, P.; Hsu, H.H.; Selaganapathy, P.R. Microfluidic Devices for Biomedial Applications, Woodhead Publishing, 2013. 3-62

(19) Ramos Payán, M.; López, M. Á. B.; Fernández-Torres, R.; Navarro, M. V.; Mochón, M. C. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2011, *879* (2), 197–204.

(20) Ramos-Payán, M.; Villar-Navarro, M.; Fernández-Torres, R.; Callejón-Mochón, M.; Bello-López, M. Á. *Anal. Bioanal.Chem.* 2013, *405* (8), 2575–2584.

(21) Eibak, L. E. E.; Gjelstad, A.; Rasmussen, K. E.; Pedersen-Bjergaard, S. *J. Pharmaceut. Biomed.* 2012, *57* (1), 33–38.

(22) Huang, C.; Seip, K. F.; Gjelstad, A.; Pedersen-Bjergaard, S. *Anal. Chim. Acta* 2016, *934*, 80–87.

(23) Hasheminasab, K. S.; Fakhari, A. R. *J. Chromatogr. A* 2015, *1378*, 1–7.

(24) Basheer, C.; Lee, J.; Pedersen-Bjergaard, S.; Rasmussen, K. E.; Lee, H. K. *J. Chromatogr. A* 2010, *1217* (43), 6661–6667.

(25) Nojavan, S.; Asadi, S. *Electrophoresis* 2016, *37* (4), 587–594.

(26) Asl, Y. A.; Yamini, Y.; Seidi, S. *Analyst* 2016, *141* (1), 311–318.

(27) Tabani, H.; Fakhari, A. R.; Shahsavani, A. *Electrophoresis* 2013, *34* (2), 269–276.

(28) Asadi, S.; Nojavan, S. *Anal. Chim. Acta* 2016, *923*, 24–32.

(29) Abdossalami, Y.; Yamini, Y.; Seidi, S.; Rezazadeh, M. *Anal. Chim. Acta* 2016, *937*, 1–8.

(30) Huang, C.; Seip, K. F.; Gjelstad, A.; Shen, X.; Pedersen-Bjergaard, S. *Anal. Chem.* 2015, *87* (13), 6951–6957.

Graphical Abstract