Fully automated electric field-driven liquid phase microextraction system with renewable organic membrane as a front end to high performance liquid chromatography

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ABSTRACT

This article reports for the first time a programmable-flow based mesofluidic platform that accommodates an electric field-driven liquid phase microextraction (μ-EME) in a fully automated mode. The miniaturized system is composed of a computer-controlled microsyringe pump and a multi-position rotary valve for handling aqueous and organic solutions at the low microliter volume, and acts as a front-end to on-line liquid chromatographic separation. The organic phase is automatically renewed and disposed of in every analytical cycle, thus minimizing analyte carry-over effects while avoiding analyst intervention. The proof of concept applicability of the automated mesofluidic device is demonstrated by the liquid chromatographic determination of nonsteriodal anti-inflammatory drugs in μ-EME processed complex samples (such as urine and influent wastewater) using on-line heart-cut approaches. Using 5 μL of 1-octanol, 7.5 μL of untreated sample and 7.5 μL of acceptor solution (25 mM NaOH), and 250 V for only 10 min in a stopped-flow mode, the extraction recoveries for the μ-EME of ibuprofen, ketoprofen, naproxen and diclofenac exceed 40% in real samples. The flow-through system features moderately selective extraction regardless of the sample matrix constituents with repeatability values better than 13%.

The incorporation of novel (micro)extraction approaches in the analytical workflow has attracted a great deal of attention over the past two decades[1] so as to cope with most of the twelve principles of Green Chemistry[2]. Sorbent microextraction in a variety of modalities has already been consolidated as a compelling sample preparation tool for simplification of the analytical procedure in the handling of complex samples and effecting *in-vivo* assays[3,4]. Likewise, efforts have been directed to downscale liquid-phase extraction that enable the use of microliter volumes of organic phase in dispersive or droplet-based approaches, and in supported liquid membrane (SLM) extraction using single or bundled hollow fibers or flat membranes[5]. The adaptation of SLM in a stopped-flow or continuous (micro)flow-injection format[6-10] for further downscaling and enabling of dynamic rather than static/batchwise liquid-phase microextraction (LPME) has been demonstrated lately. Moreover, dynamic LPME resulted in amelioration of the enrichment factors and shortening of the extraction times. Electric field-driven LPME has been also recently seen as a viable option for expedient SLM extraction of ionizable or polar species[11-15] and in micro/mesofluidic platforms[16-18]. Additional advantages of using electric field were (i) improved selectivity for transfer of ionized species, (ii) efficient matrix clean-up and (iii) modulation of the sample preparation process for potentially exhaustive extraction of target species[19,20].

Notwithstanding the development of miniaturized SLM approaches has made tremendous strides over the past decade, there is still a quest for the development of fully automated flow set-ups hyphenated to modern analytical separation and detection techniques. Despite ingenious strategies based on (i) the use of robotic stations to manipulate SLM containing autosampler syringes[21], (ii) the accommodation of multiple membranes in bespoke micromachined modules for simultaneous assays of cationic and anionic species[22] or effecting electrically- and diffusively-driven SLM extractions in a single device[23], (iii) the integration of nano-extraction in separation capillary of an electrophoretic system[24], and (iv) the incorporation of polymer inclusion membranes (PIM) in flow systems[25], only mechanized or semi-automatic methods

have been reported to date. It should be noted that these methods still needed manual impregnation of the polymeric membrane with the organic phase[6,8,9,18,19,21,23,26] or off-line synthesis of the PIM[25]. The organic phase was reused for just a few extractions and was regularly re-impregnated/regenerated or even replaced depending on the complexity of the sample matrix. In fact, several researchers indicated that three-phase hollow fiber or flat membrane incorporating microfluidic devices could only be used once because the SLM had better be replaced in every individual assay[10,22,27,28], thus revealing the lack of reliability and applicability of the previously reported fluidic platforms for unattended operation. Also the frequent leakage of the organic phase-laden SLM and insufficient capacity for repetitive analysis of samples with high organic load[29] might account for the fact that dynamic flow-through SLM analytical methods have not received the attention that they might have deserved. In terms of automation, the use of a single or several uni-directional microsyringe pumps or HPLC pumps in flow-through SLM extraction[9,10,16-18,20,22,23,26,28,30] does not endow the micro/mesofluidic platforms with sufficient versatility for unsupervised execution of the various operations of the analytical procedure, including sampling, system calibration, pH or ionic strength adjustment, halting of the acceptor solution or post-extraction treatment and analysis of the acceptor solution.

With the advent of the new generations of flow injection analysis (FIA), that is, sequential injection analysis (SIA) and Lab-on-Valve approaches[31-33] along with the recently launched 3D-printed µFIA (3D-µFIA)[34,35], reliable and fully computer controlled mesofluidic platforms have been devised for automatic/automated sample preparation. By combining (i) bi-directional microsyringe or diaphragm pumps as liquid drivers, (ii) rotary valves (e.g., multi-position selection valves or solenoid valves) for liquid diversion, and (iii) autosamplers for sample selection, the unattended manipulation of microliter (with option to submicroliter) volumes of samples, standards and reagents and execution of unit operations (e.g., liquid-phase microextraction[29,36]) has been proven feasible. However, as identified by a series of

comprehensive reviews by Horstkotte and co-workers[33,37,38], efforts dedicated by FIA practitioners to automation of LPME approaches have been preferentially directed to accommodate semi-automatic dispersive LPME[37-39]. Little interest has been registered in the field of SLM automation using advanced flow approaches[7,25,29,36] apart from the plethora of miniaturized SLM platforms for EME reported recently in the literature[8-10,14,16-18,20-23].

To the best of our knowledge, no fully automatic/automated flow-through SLM extraction or EME system with renewable organic membrane has been reported to date. To tackle this research gap, the concept of micro-EME (μ-EME) in a free liquid membrane (FLM) configuration as described by Kubáň and Boček[40,41] seems to pave the way for potential automation of the entire analytical procedure and running EME unsupervised. μ-EME using FLM is based on formation of adjacent plugs of immiscible solutions in a transparent polymeric tubing and the number of the plugs is virtually unlimited. Moreover, each plug can have unique composition and/or volume and the set-up is perfectly suited to systems enabling automated handling of minute liquid volumes. Due to the unique characteristics of μ-EME, liquid handling and extraction process can be controlled visually[42], which is important for optimization of the automated filling/extraction protocols. The μ-EME set-up also offers excellent variability of extraction modes since multiple immiscible plugs can be used for extractions[43] or removal[44] of cationic and anionic species. The fact that all phases are formed as free liquids in μ-EME gives an additional feature to the set-up, i.e. to experimentally examine principles of μ-EME by collecting and analyzing each phase separately[45].

In this work, we report for the first time an automated flow set-up based on the coupling of SIA and μ-EME that enable unattended handling of the overall solutions in the conduits of the flow platform as well as the control of the analytical equipment using user-friendly freeware. The flow platform is also configured to allow for on-line ‘heart-cut’ injection of a minute, well-defined volume of acceptor solution into an HPLC for the determination of non-steroidal anti-

inflammatory drugs (NSAIDs) in biological and industrial samples that served as a proof-of-concept for applicability of the hyphenated SIA–μ-EME set-up. Rinsing of the entire flow manifold along with the aspiration of a new plug of organic membrane in every analytical cycle helped minimize analyte carry-over while enabling the unattended handling of complex sample matrices, such as wastewater or urine.

EXPERIMENTAL SECTION

Reagents, standard solutions and real samples. All chemicals were of analytical reagent grade. Milli-Q water with resistivity > 18 MΩ·cm was prepared by a Milli-Q® Advantage A10 Water Purification System (Merck-Millipore, Darmstadt, Germany). Ibuprofen, naproxen, ketoprofen, diclofenac sodium salt and meclofenamic acid (Sigma, Steinheim, Germany) were used as model acidic drugs, and stock solutions of 1 mg/mL were prepared in methanol and stored at −20 °C. Standard solutions of acidic drugs were prepared by dissolving appropriate volumes of the stock solutions in Milli-Q water or sodium hydroxide (Fluka, Buchs, Switzerland). 1-Octanol (≥ 99.5%, Fluka) was used as the organic membrane. Operational working solutions for μ-EME were prepared from Milli-Q water and sodium hydroxide. Methanol (Optima MS-grade, Fisher Scientific SL, Hampton, NH, USA), formic acid (Sigma) and Milli-Q water were used for preparation of the HPLC mobile phase.

A saline solution of 100 mM NaCl mimicking the content of chloride in real samples (i.e. around 100 mM Cl^- in human body fluids[46]) was prepared from crystalline NaCl (Sigma). Urine samples were collected from a volunteer aged > 18 years who signed a written informed consent. This research project was approved by the Research Ethics Committee of the Balearic Islands (ID no. IB 3776/18 PI). The blank urine sample was obtained by 24-hour collection, stored in a sealed bag at 4 °C for one day and then deep-frozen and stored at −20 °C for one month. An influent wastewater sample was collected at the wastewater treatment plant EDAR 1 in Palma de

Mallorca (Balearic Islands, Spain) in April 2018 and stored at –20ºC prior to use. The analytical parameters of the influent wastewater are summarized as follows: pH 6.2; chemical oxygen demand (COD) 1119 mg/L O_2, biochemical oxygen demand (BOD) 460 mg/L O_2, 16.5 mg/L total P and 76 mg/L total N. Both types of samples were filtrated through Nylon syringe filters (0.45 µm, Scharlab, Barcelona, Spain) and analysed by the hyphenated SIA-µ-EME-HPLC system.

Flow set-up for micro-electromembrane extraction. The components of the flow system as a front-end to on-line HPLC analysis are schematically illustrated in Figure 1. The mesofluidic platform, so-called micro-SIA, is built with a low-pressure metal-free-8-port selection valve (MPV, Cheminert C25-3180, VICI Valvo, Schenkon, Switzerland) and a 30 mm-stroke OEM bi-directional syringe pump (SP, Cavro XCalibur, San Jose, CA, USA) in a single portable module. A three ports (In, Up, and Out) head valve (HV) allowed the connection of the SP to the carrier solution (50% ACN/50% MilliQ water containing 0.1% (v/v) HCOOH), air, and the flow set-up via a 15 cm-long holding coil (HC, 1.0 mm ID, 1.6 mm OD fluorinated ethylene polypropylene (FEP) tubing, Idex Health & Science LLC, Oak Harbor, WA, USA), respectively. A 100 µL-borosilicate glass syringe (Cavro XC/XP syringe with PTFE plunger tip seal, Cavro) was used for automatic handling of all the fluids. The peripheral ports (1-8) of the MPV served for the sequential aspiration of the samples, standards, acceptors, cleaning solvents and renewable organic membrane in unsupervised mode through the MPV communication channel (CC) into HC. An AIM3200 autosampler (Aimlab, Virginia, Australia) equipped with two 60-position, 12 mL-sample tube racks was nested to port # 6 of MPV for automatic sample/standard analysis. The in-line µ-EME was performed in a 5 cm long FEP tubing (1.6 mm ID, 3.2 mm OD, Idex Health & Science LLC, port # 7) that was connected with the HPLC injection valve by a 30-cm transfer line (0.76 mm ID, 1.6 mm OD FEP tubing, Idex Health & Science LLC). A 2-

way straight Omnifit connector (Diba Industries Ltd, Cambridge, UK) served for coupling the large-bore µ-EME tubing to the small bore transfer line.

Unattended instrumental control of all the units of the flow system (SP, HV, MPV) and even the µ-EME power supply was accomplished via USB using the open-source software Cocosoft, version 4.3, written in Python programming language (FI-TRACE, University of the Balearic Islands)[47].

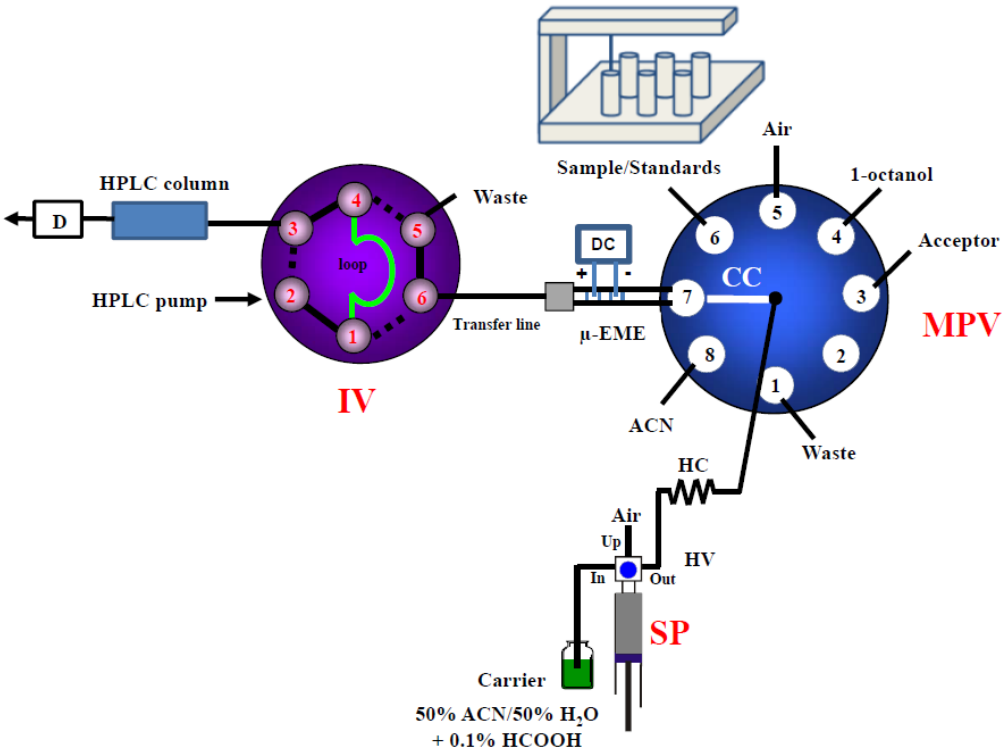

Figure 1. Diagrammatic description of the flow manifold for fully automated µ-EME with on-line hyphenation to HPLC separation. SP: Syringe pump; MPV: Multi-position valve; IV: Injection valve; HV: Head valve; HC: Holding coil; CC: Communication channel; µ-EME: micro-electromembrane extraction; DC: Direct current power supply; ACN: Acetonitrile; HPLC: High performance liquid chromatography

High performance liquid chromatographic apparatus. The chromatographic set-up (LC 4000 series, Jasco, Tokyo, Japan) consisted of (i) quaternary high-pressure pump (PU-4180, Jasco), (ii) autosampler (AS-4050, Jasco), (iii) high-pressure 6-port valve that was furnished with a 25 µL-PEEK injection loop (0.76 mm ID, 1.6 mm OD), (iv) external GECKO 2000 column heater,

and (v) photodiode array detector (MD-4017, Jasco). The separation of the target analytes was performed by the Onyx^{TM} monolithic HD-C18 column (100 × 4.6 mm, Phenomenex, Torrance, CA, USA) preceded by a security guard column Onyx^{TM} monolithic C18 guard cartridge (5 × 3 mm, Phenomenex). Analytes were detected at 210 nm under isocratic elution (60:40 (v/v) methanol/water containing 0.1 % (v/v) HCOOH) at 2.0 mL/min and 30 ºC. The retention times of ketoprofen, naproxen, diclofenac and ibuprofen under the selected experimental conditions were 2.3, 2.8, 6.5, and 7.5 min, respectively. All the chromatographic data were processed with the ChromNAV software version 2.01.06.

In-line micro-electromembrane extraction. μ-EMEs were carried out in chemically inert FEP tubing (1.6 mm ID and 3.2 mm OD) as described above. The highly transparent FEP tubing was selected for the sake of visualization of the different operational steps involved during in-line μ-EME and transfer of the acceptor solution into the HPLC. Two holes with a distance of 6 mm from each other were pierced on top of the large bore tubing using a hypodermic needle (0.45 mm OD, Braun, Melsungen, Germany). Two 5 mm long and 500 μm thick tubular platinum wires (99.95%, Advent RM, Oxford, UK), operating as electrodes, were inserted into the holes and fixed in the position using a drop of a cyanoacrylate glue. The wires protruded by ca. 200 μm into the FEP tubing to avoid disturbance of the laminar flow during liquid handling but to ensure good electric contact with the μ-EME liquid plugs (see scheme in Figure 2). The liquids were provided by the flow system in a way that the anode and the cathode were in contact with the acceptor and the donor solution, respectively. The external voltage for μ-EME was provided by a d.c. power supply ES 0300-0.45 (Delta Elektronika, Zierikzee, The Netherlands) with a programmable voltage in the range of 0 – 300 V and maximum current of 450 mA. Real time monitoring of the electric current during the μ-EME was performed using an UT70B (Uni-Trend

Technology Ltd., Dongguan, China) digital multimeter. Two crocodile/banana plugs allowed connecting the two Pt electrodes to the power supply.

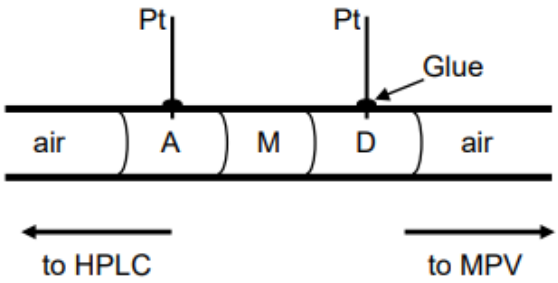

Figure 2. A schematic drawing of the three plugs inside the FEP tubing for in-line µ-EME. D: donor solution (standard or sample, 7.5 µL); M: organic membrane (1-octanol, 5 µL); A: acceptor solution (25 mM NaOH, 7.5 µL); MPV: Multi-position valve.

The analytical procedure for automatic µ-EME included the following steps:

(i) *In-line generation of the three µ-EME phases:* The µ-EME tubing and the transfer line were first filled with air from port # 5 at 20 µL/s. Then, SP and MPV were set to aspirate 3 µL of air (port #5) and 7.5 µL of acceptor solution (port # 3), consecutively, into the HC at 0.5 µL/s (HV was set to Out). Thereafter, the pump flow was reversed and the entire volume of acceptor was dispensed at 0.5 µL/s into the µ-EME tubing (port #7) while keeping the air plug in the HC. The same procedure was repeated for the organic membrane (5 µL, port #4, 0.5 µL/s), yet after delivering the organic membrane into the µ-EME tubing, the HC was rinsed with 20 µL of ACN (port #8) for removal of 1-octanol leftovers, which was then dispensed to waste (port #1, both procedures at 10 µL/s). The last step involved aspiration of 3 µL of air (port #5, 0.5 µL/s) and 7.5 µL of sample/standard from port #6 (autosampler, 0.5 µL/s) into the HC whereupon the sample plug was brought by flow reversal to the µ-EME tubing at 0.5 µL/s for generation of the three-phase µ-EME system.

(ii) *Automatic µ-EME:* A metered volume of 54 µL of air (from port #5, 0.5 µL/s) was employed to push the three µ-EME phases toward the anode and the cathode until the organic phase was

positioned between the two electrodes. Thereafter, the power supply was automatically activated and set to 250 V for 10 min.

(iii) *On-line heart-cut injection of the acceptor solution into HPLC:* After μ-EME, the power supply was automatically switched off and the SP was programmed to aspirate the plugs of donor and organic membrane along with about 2 μL of acceptor (from port # 7 at 0.5 μL/s) back to the HC, which were forwarded to waste (port # 1 at 10 μL/s). The analyte-laden remaining acceptor plug was dispensed toward and trapped in the injection loop of the HPLC valve by a stream consisting of 25 μL of 50% ACN/MilliQ water + 0.1% HCOOH (carrier from SP), 20 μL of ACN (from port #8) and 2 × 100 μL of air (from port #5) at 10 μL/s.

(iv) *Cleaning of the fluidic system:* After on-line HPLC analysis of the acceptor, the HC and the μ-EME tubing were washed with 3 × 90 μL ACN (from port #8 at 10 μL/s) in air-segmented mode. The HC was finally emptied and rinsed with 50 μL of air (HV in Up position) and 150 μL of carrier stream (HV in In position, all at 20 μL/s) to avoid analyte carry-over and to eliminate potential remnants of the organic membrane.

On-line coupling of μ-EME to HPLC. The hyphenation of the μ-EME to the chromatographic system for analysis of the acceptor solution was carried out following the approach described elsewhere[34]. In brief, the default connections of the stator of the 6-port HPLC injection valve were modified clockwise for the valve to remain in the 'load' position, that is, available for the flow system, throughout the analysis, except during the 'injection delay' instruction. The connection to the autosampler's syringe was replaced by the 0.76-mm ID FEP tubing (transfer line) that is also attached to port #7 of the MPV. The first chromatographic run was started manually and served for recording a chromatographic blank, because the injection loop was empty at that time. The signal provided by the autosampler after the injection, when turning the HPLC valve back to the load position and starting data acquisition, was captured by one of the

digital inputs of the SP through the CocoSoft software. The automatic μ-EME procedure was then triggered and ended by filling the injection loop with the acceptor solution. The μ-EME procedure lasted 12 min more than the HPLC run. After recording the chromatogram, the HPLC pump was stopped until the acceptor solution was brought to the injection loop. An injection delay of 5 s was set for the HPLC valve so as to enable the content of the loop (25 μL) to be completely displaced by the mobile phase towards the chromatographic column. The main advantage of this synchronization protocol is that both instruments operate simultaneously and thus the sample throughput is maximized. A diagrammatic depiction of the analytical workflow and synchronization of the flow-through μ-EME with HPLC analysis is shown in Figure S1 of the Supporting Information.

RESULTS AND DISCUSSION

Configuration of the flow system and interface to HPLC. Initial experiments were carried out with aqueous and organic solutions, which were successively brought in a fully automatic mode into the μ-EME tubing (with embedded Pt electrodes), but the high voltage of the μ-EME power supply was switched off and the μ-EME tubing was disconnected from the HPLC valve. 1-octanol, 25 mM NaOH and Milli-Q water were used as the organic membrane, the acceptor solution and the donor solution, respectively. The minimum volumes which formed stable plugs inside the μ-EME tubing were examined first to determine the suitable volumes of μ-EME operational solutions. The minimum length of each plug for the three-phase in-line μ-EME system was around 2 mm (due to a pronounced curvature of the phase interfaces in tubing with larger ID than used previously[40]), which corresponded to volumes of approx. 4 μL. In order to ensure safe separation of the donor and the acceptor solution, the volume of 1-octanol was set to 5 μL (plug length ~ 2.5 mm) and was used as the organic membrane volume in all subsequent experiments. Volumes of acceptor and donor solutions were optimized based on our previous

study on exhaustive μ-EME, which afforded maximum extraction recoveries for the shortest donor/acceptor plugs[20], but also with respect to subsequent in-line processing of the plugs with the SP and the automated transfer of the acceptor solution into the HPLC injection loop. Because approx. 2 μL of the acceptor solution were discarded together with the donor and the organic membrane for a reliable on-line heart-cutting procedure, the minimum volume of the acceptor solution was set at 7.5 μL and the same volume was also used for the donor solution. The three-phase μ-EME system was approx. 10 mm in length.

The sequence of the three phases for μ-EME was separated at both ends by air plugs (5 μL) to avoid mixing with the carrier solution (see Figure 2). The transfer speed of the three phases through the μ-EME tubing was then examined. The μ-EME plugs were distorted during their transfer at flow rates > 0.5 μL/s and partial penetration of the organic phase into the donor solution was observed. This was due to the gradual formation of a wetting-film of 1-octanol on the inner tubing wall (small droplets of 1-octanol were visible inside the tubing by the naked eye), which has been reported previously in the literature[48], and subsequent re-collection of 1-octanol at the rear end of the donor solution. In fact, 1-octanol was detached from the tubing by the front end of the air plug and was re-collected as a fourth phase at the rear end of the donor solution. In order to avoid such distortions, all of the liquid handling procedures involving displacement of the three μ-EME phases were carried out at a flow rate of 0.5 μL/s.

With regard to the order of the three phases that are brought into the EME tubing, the final configuration described in Experimental section, namely, acceptor/organic membrane/donor will help in trapping the acceptor solution after the μ-EME and removal of the other two phases to waste. Pumping the sample first will most likely contribute to potential analyte/matrix cross-contamination of the μ-EME and delivery tubing. Due to the possible contamination of the internal components of the flow network (viz., tubing, MPV and connecting lines) with remnants of 1-octanol, the entire flow system was properly flushed with ACN after each μ-EME. This

ACN flushing and other liquid handling procedures (i.e. filling the HC and transfer of acceptor solution to HPLC) were performed at a flow rate of 10 µL/s in order to minimize the total processing time.

Coupling of the µ-EME to HPLC was performed in on-line heart-cut injection mode by automated trapping of the acceptor solution in the injection loop of the HPLC valve. Transfer of off-line methods to on-line format needs revisiting of some of the experimental parameters (e.g., position of the plugs inside the µ-EME tubing) because of the backpressure caused by the HPLC valve. Using a forward-flow pumping mode that entails the pushing of the three phases towards the injection valve of the HPLC set-up resulted in poorly repeatable readouts. This was attributed to the unwanted injection of droplets of the organic membrane into the chromatographic system. To tackle this shortcoming, a backward-forward flow injection mode was used instead. To this end, the volumes of 1-octanol and donor solution were retrieved into the HC and eliminated to waste while the analyte-containing acceptor plug was whereupon brought to the HPLC injection loop by 45 µL of an ACN-water mixture followed by an air plug that ensured the quantitative injection of the extracted analytes and displacement of the solutions. Using the above protocol – adapted to off-line analysis mode with the addition of meclofenamic acid as internal standard to the acceptor collected in an HPLC vial – the maximum volume that could be retrieved from the acceptor solution while ensuring withdrawal of the entire volume of the organic membrane was 5.1 ± 0.5 µL. By optimization of the on-line injection of the acceptor plug into the HPLC, no traces of the organic membrane were observed in the recorded chromatograms (see Figure 3 inset).

Because of the compressibility of the air and dead volumes of the two valves in series connecting the SP with the HPLC injection loop, the volume of the air stream for the delivery of the acceptor plug towards the HPLC set-up should be explored. Volumes ranging from 180 to 250 µL were

assayed and efficient trapping of the ca. 5 µL-acceptor as identified by the on-line heart-cut HPLC readouts was achieved with 200 µL of air.

Selection of operational parameters for µ-EME of acidic drugs. The composition of the acceptor and the donor solution, the extraction voltage and the extraction time were evaluated in a manual (off-line) µ-EME mode as described in the Supporting Information. Three acceptor solutions (Milli-Q water, 10 mM NaOH and 25 mM NaOH) were examined for µ-EME of a donor solution containing 100 mM NaCl and 10 µg/mL of the four acidic drugs in Milli-Q water. The µ-EMEs were carried out at 200 V for 10 min and poor transfers of the four drugs (extraction recoveries < 5%) were observed for acceptors consisting of Milli-Q water. The unavoidable electrolysis of water in electric field-driven extractions is connected with the formation of H^+ and OH^- at the anode (acceptor) and the cathode (donor), respectively, which leads to the gradual acidification of the acceptor phase[42]. In fact, the pH value of the Milli-Q water as acceptor phase changed from ~ 6 to less than 3 during the µ-EME process. At acidic acceptor conditions, the target drugs become neutral at the organic membrane/acceptor interface. Therefore, they are not released into the acceptor solution whereby their µ-EME transfer is hindered. On the other hand, the extraction recoveries increased significantly with the use of alkaline acceptor solutions, which promote the release of the acidic drugs from the organic membrane. In fact, similar recoveries (~ 40 – 60%) were obtained for the target drugs extracted into 10 mM and 25 mM NaOH. Nevertheless, pH measurements of the resulting acceptor solutions after µ-EME revealed slight decrease in the pH value of the 10 mM NaOH solution (pH dropped from 12 to 11.5), whereas no measurable pH changes were observed for the 25 mM NaOH solution. Therefore, 25 mM NaOH was selected as the most appropriate acceptor solution for all subsequent experiments due to its better pH stability.

Donor solutions containing 100 mM NaCl and 10 μg/mL of the four acidic drugs were prepared in Milli-Q water, 1 mM NaOH and 10 mM NaOH. Extraction recoveries were examined using 25 mM NaOH as acceptor solution at 200 V for 10 min. Comparable results were obtained for μ-EMEs of the three donor solutions. The four acidic drugs have pK_a values between 4.0 and 4.6[49] and are almost fully charged at pH ~ 6.0, which is the pH of the donor solution containing NaCl and the acidic drugs. The extraction performance was not improved by addition of NaOH to the donor solutions and thus donor solutions at near neutral or slightly acidic conditions do not need pH adjustment. Because the cathode is placed in the donor solution, hydroxyl ions are electrolytically generated during the μ-EME process and thus the donor solution is gradually alkalinized, thereby maintaining suitable ionization of the acidic drugs.

Extraction voltage was examined within the 100 – 300 V range at the above optimized acceptor and donor conditions. A gradual increase in the extraction recovery of μ-EMEs performed for 10 min was observed for 100 – 200 V, the recovery peaked at 250 V and remained nearly constant until 300 V. Best extraction recoveries (40 – 60%) were obtained between 250 and 300 V. Electric currents increased at higher extraction voltages and reached a maximum value at 300 V. The voltage was finally set to 250 V because of the low electric currents generated during μ-EME ($i < 25$ μA, see Figure S2) that served as a quality control of the μ-EME stability, and of the selective transfer of the target species.

The effect of the extraction time on the extraction recovery was investigated by μ-EMEs at 250 V from the standard donor solution (drugs at 10 μg/mL in 100 mM NaCl) into the alkaline acceptor solution (25 mM NaOH). A gradual increase in the extraction recoveries was observed from 0 to 7.5 min with a further improvement until 10 min. Based on the above observations, the following experimental conditions were selected, which were subsequently used for on-line coupling of the three-phase μ-EME to HPLC: acceptor solution, 25 mM NaOH; donor solution, untreated; extraction voltage, 250 V; and extraction time, 10 min.

Analytical performance and application to real matrices. Under the experimental conditions selected in the foregoing section, the clean-up effect of in-line μ-EME processing of complex samples was studied by on-line HPLC and by continuous recording of the electric current throughout the microextraction process. To this end, three different donor solutions (100 mM NaCl, untreated urine and untreated influent wastewater) spiked with the four analytes at the 50 μg/mL level were subjected to in-line μ-EME. Virtually identical trends were observed for the three temporal profiles (see Figure S2 in the Supporting Information) and electric currents recorded with the high matrix samples were in all instances lower than that of the standard with 100 mM NaCl (i peaked at 10, 15, and 24 μA). This indicated that the untreated urine and influent wastewaters did not jeopardise the stability of the organic membrane and that small ions from the matrix were not transferred to the acceptor solution to a significant extent. Slight changes in the electric current were attributed to differences of ionic strengths and pH of the analysed matrices; the higher the acidity (as it was the case with the urine sample, pH: 5.5) the lower the current was detected.

The analytical performance of the in-line μ-EME fluidic set-up as a front-end to HPLC is summarized in Table 1. Table 1 includes: (i) repeatability values (n = 5, 5 μg/mL level), (ii) dynamic linear range and coefficients of determination for standard solutions containing 100 mM NaCl, (iii) limits of detection (LODs) for urine, defined as S/N=3, with N calculated as the average peak to peak signals of HPLC runs of unspiked urine at the retention times of the four target analytes, and (iv) extraction recoveries. Relative standard deviation (RSD) values for untreated urine and influent wastewater were much alike those of saline standards except for ibuprofen in wastewater and usually were < 7%. Remarkably, the extraction recoveries for ketoprofen, ibuprofen and diclofenac in untreated urine were not statistically different at the 0.05 significance level to those of untreated wastewater indicating again that the matrix constituents do not alter the μ-EME process significantly and that the method of the standard additions is not

necessary. Further, absolute extraction recoveries of the NSAIDs in urine (52 – 77%) were improved as compared to batchwise EME methods (14 – 43%) for which extraction times similar or higher than those used herein are usually applied[50-52].

Table 1. Analytical parameters of the developed μ-EME-HPLC method for the determination of acidic NSAIDs.

Analyte	RSD (%)			LOD (μg/mL) Urine	Linearity (μg/mL)	R^2	Extraction recovery		
	Standard	Urine	Wastewater				Standard	Urine	Wastewater
Ketoprofen	4.7	5.4	4.2	0.5	0.5 – 20	0.999	48 ± 2	60 ± 3	55 ± 2
Naproxen	1.4	6.8	3.1	0.1	0.4 – 20	0.998	48 ± 1	77 ± 5	40 ± 1
Diclofenac	3.5	5.3	3.8	1.2	1.2 – 20	0.999	50 ± 2	67 ± 4	76 ± 4
Ibuprofen	7.3	5.8	13.0	2.3	2.3 – 20	0.999	47 ± 3	52 ± 3	52 ±7

It should be also kept in mind that the small volumes of acceptor solution collected after μ-EME cannot be directly analysed off-line by conventional HPLC equipment unless readjustment of the depth of the autosampler needle is made[53]. In fact, sample volumes of at least 50 μL using microinserts were proven necessary for reliable HPLC injection of microvolumes. Also, the quantitative collection of the acceptor plug into a vial microinsert might be cumbersome for off-line methods. In our case, HPLC signals of the acceptor plug that was injected on-line were up to 6.7 times higher than those recorded by off-line analysis because of the approx. 10-fold dilution of the acceptor solution prior to off-line injection by the HPLC autosampler.

Illustrative chromatograms of a urine sample spiked with 5 μg/mL of each of the four analytes before and following the in-line μ-EME are presented in Figure 3A and Figure 3B, respectively. The differences of retention times in both chromatograms are caused bythe waiting/synchronization times of the automated SIA-HPLC protocol. The matrix clean-up effect of in-line μ-EME is demonstrated by the elimination of polar compounds at the dead time position in the HPLC run (Figure 3, inset). The concentration effect of the on-line heart-cut approach for analysis of ca. 5 μL of the acceptor as compared with the off-line HPLC approach is also observed in Figure 3.

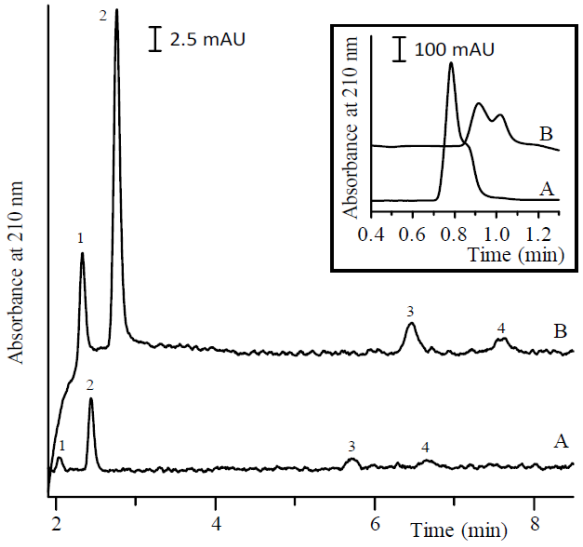

Figure 3. Chromatograms of urine spiked with 5 μg/mL of NSAIDs before (A) and after (B) in-line μ-EME. The inset corresponds to the dead time chromatographic zone, in which the non-retained urine compounds are eluted. The chromatographic conditions are indicated in the Experimental section. Peak identification: ketoprofen (1), naproxen (2), diclofenac (3) and ibuprofen (4). Note: The analytical column was conditioned by the mobile phase for 10 min after analysis of untreated urine.

CONCLUSIONS

We have herein presented a novel concept of a fully automated micropreparative-analytical procedure based on μ-EME with disposable organic membrane, which forms the moderately selective interface between aqueous solutions. The microextraction fluidic system acts as the front end for clean-up and processing of complex samples, and is on-line coupled to HPLC-UV for the determination of the extracted analytes. The organic membrane is formed as a plug of an organic solvent, which is sandwiched between two aqueous solutions (donor and acceptor), and can be automatically renewed after each extraction cycle. This approach thus efficiently avoids analyte/sample carry-over issues and a loss of membrane capacity reported previously in semi-automatic EME procedures. The proof-of-concept application of our computer-controlled flow system is demonstrated for unattended clean-up, extraction and quantification of selected NSAIDs in high matrix samples. The possibility of unattended operation makes this fluidic approach an appealing tool for the routine determination of NSAIDs in quality control

laboratories. Further work might be devoted to on-line coupling of μ-EME to nano-LC-MS for improved selectivity and sensitivity, and the automated determination of various other analytes in complex samples. In addition, the in-line/on-line hyphenation might not be restricted to the use of LC systems, but other separation techniques (e.g., CE) or flow-through detection techniques (e.g., SIA) might be used as the analytical end. The major advantage is the minute consumption of all operational solutions and thus the proposed method might be extended to automated analysis of less conventional biological samples (such as cerebrospinal fluid, dried blood spot, saliva, exhaled breath condensate, etc.), which are usually available in limited volume but are highly attractive for clinical, toxicological and forensic studies[54].

SUPPORTING INFORMATION

Detailed description of (i) the analytical procedure for batchwise μ-EME, (ii) the synchronization of the flow method with the HPLC analysis, and (iii) the electric currents recorded throughout in-line μ-EME of distinct biological and environmental samples.

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