In-line carbon nanofiber reinforced hollow fiber-mediated liquid phase microextraction using a 3D printed extraction platform as a front end to liquid chromatography for automatic sample preparation and analysis: A proof of concept study

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Abstract

A novel concept for automation of nanostructured hollow-fiber supported microextraction, combining the principles of liquid-phase microextraction (LPME) and sorbent microextraction synergically, using mesofluidic platforms is proposed herein for the first time, and demonstrated with the determination of acidic drugs (namely, ketoprofen, ibuprofen, diclofenac and naproxen) in urine as a proof-of-concept applicability. Dispersed carbon nanofibers (CNF) are immobilized in the pores of a single-stranded polypropylene hollow fiber (CNF@HF) membrane, which is thereafter accommodated in a stereolithographic 3D-printed extraction chamber without glued components for ease of assembly. The analytical method involves continuous-flow extraction of the acidic drugs from a flowing stream donor (pH 1.7) into an alkaline stagnant acceptor (20 mmol L^{-1} NaOH) containing 10% MeOH (v/v) across a dihexyl ether impregnated CNF@HF membrane. The flow setup features entire automation of the microextraction process including regeneration of the organic film and on-line injection of the analyte-laden acceptor phase after downstream neutralization into a liquid chromatograph (LC) for reversed-phase core-shell column-based separation. Using a 12-cm long CNF@HF and a sample volume of 6.4 mL, linear dynamic ranges of ketoprofen, naproxen, diclofenac and ibuprofen, taken as models of non-steroidal anti-inflammatory drugs, spanned from ca. 5-15 μg L^{-1} to 500 μg L^{-1} with enhancement factors of 43-97 (against a direct injection of 10 μL standards into LC), and limits of detection from 1.6 to 4.3 μg L^{-1}. Relative recoveries in real urine samples ranged from 97-105 %, thus demonstrating the reliability of the automatic CNF@HF-LPME method for in-line matrix clean-up and determination of drugs in urine at therapeutically relevant concentrations.

Keywords: Carbon nanomaterial; liquid phase microextraction; flow system; automation; acidic drugs; liquid chromatography; 3D-printing

1. INTRODUCTION

Liquid phase microextraction (LPME) is a miniaturized sample preparation approach that has made tremendous strides over the past decade in exploiting its operational green chemical credentials. A vast amount of effort has been also dedicated to harness (i) advanced water-immiscible green solvents (e.g., deep eutectic solvents, supramolecular solvents and ionic liquids) against conventional organic phases; (ii) innovative external energy sources to aid in dispersive microextractions and expedite mass transfer (e.g., electromembrane extraction), (iii) novel modalities for fast separation of phases (e.g., solvent demulsification-assisted LPME) and (iv) high-throughput analysis (e.g., 96-well plate format) as signaled by a number of review articles published in 2017 [1–6]. Two associated research fields that continue to garner increasing attention by LPME practitioners are those based on resorting to (i) nanoparticle-decorated or nanostructured supported-liquid membranes [7–10] and (ii) micro/meso-fluidic platforms for miniaturization and mechanization/automation of the LPME process [11–15]. Nanostructured membranes composed of carbon nanomaterials or metallic nanoparticles (NP), e.g., AgNP, AuNP and TiO_2 NP, are aimed at improving the stiffness of the polymeric phase and the reliability of LPME. The NP endowed the membranes with unique features for amelioration of the selectivity of the LPME assays and decrease of mass transfer resistance by chemical modification or dynamically coating of the embedded nanosorbent [7,16,17]. Carbon-reinforced LPME capitalizing upon the immobilization in the membrane pores of carbon nanostructures, e.g., carbon nanotubes [18], and graphene/graphene oxide [19] that operate concomitantly as a substrate for the organic solvent offers an extra degree of freedom for enhancing the extraction efficiency of target organic species as compared to diffusion-controlled partitioning by the choice of the adsorption mechanism. However, to the best of our knowledge, the reinforcement of membranes by carbon nanofibers (CNF) [20] consisting of graphite layers that are piled up parallelly or at a certain angle from the fiber axis in a cup-stacked form with large surface area has not been described for analytical applications as of yet.

The last decade has also witnessed cutting-edge advances in employing microscale (fluidic) platforms for simplification of LPME operational and detection sequences as exemplified with recent contributions by Pedersen-Bjergaard's and Petersen's research groups in Oslo and Copenhagen, respectively [12,21–23], Kuban in Brno [24–26], Lee in Singapore [27] and Horstkotte in Hradec Kralové [28–30]. The distinct generations of flow analysis, namely, flow injection analysis, sequential injection analysis and variants thereof [31,32], have been harnessed to either confining the LPME process in the syringe barrel of a microsyringe pump [11,33,34] or within the manifold tubing or external units in a mechanized/automatic format [35–38]. Alternatively semi-automatic microfluidic setups have been devised for continuous-flow supported-liquid membrane microextraction [12,13,21,22,39,40], or membrane-less segmented phase

microextraction [41] as a front end to capillary electrophoresis or mass spectrometric-based detection systems.

Notwithstanding the fact that microfluidic systems served for partial automation of supported-liquid membrane microextrations, usually in a planar membrane format [12,13,21,25], the soaking of the polymeric membrane in the organic solvent is still done in a manual format [22,25,36]. Further, analyst intervention throughout the handling of the donor/acceptor solutions or sample extracts [24–26,40] along with the use of manually-operated unidirectional liquid drivers for continuous LPME [12,13,21–23,36,39] is often reported, making those miniaturized/fluidic platforms inappropriate for fully automated versatile operation.

In this work, a fully automatic hollow-fiber (HF) assisted LPME method combining the positive attributes of carbon nanofiber/polymer composite membranes (CNF@HF-LPME) for improved mass flux of target species, and of mesofluidic platforms based on the second generation of flow analysis [29] for unmanned handling of the organic solvent, samples, and donor/acceptor solutions at will, is proposed for the first time. The flow setup composed of software-controlled bi-directional pumps and multi-position selection valves also permits automatic post-processing of the analyte-laden acceptor phase followed by direct liquid chromatographic (LC) analysis using on-line heart-cut injection. Hereto, sample preparation is seamlessly integrated with LC with the automatic mesofluidic system as interface. Advantage is also taken herein of the resilience of 3D printing for prototyping flow-through chambers of varied dimensions for accommodating single-strand hybrid CNF@HF aimed at sample clean-up and analyte concentration. Additive manufacturing, conventionally termed 3D printing, has been recognized as a cutting-edge technology in diversified disciplines and revolutionized the microfluidics and flow injection arenas [42–44] making them available to the novice. In fact, stereolithographic 3D-printing has emergent as a cost-effective and user-friendly approach for fabrication of tailor-made integrated fluidic platforms [42,45] and modular devices for flow-through unit operations in flow injection manifolds including flow-through membrane separation units [46,47] and sorptive preconcentration columns [48,49]. However, to the best of our knowledge 3D printing has not been used as of yet for construction of scaffolds amenable to hollow-fiber liquid-phase microextraction approaches in a continuous-flow mode.

The feasibility of the hyphenated CNF@HF-LPME-LC setup for real-life applications is herein demonstrated with the determination of nonsteroidal anti-inflammatory drugs (NSAIDs), *viz.*, diclofenac (DIC), ibuprofen (IBU), naproxen (NAP) and ketoprofen (KTP), as a model of acidic drugs in urine as a non-invasive biological matrix. NSAIDs are frequently used domestic drugs that can be purchased without medical prescription. Misuse and intake without medical supervision could pose a risk to individuals because of potential side effects, such as stomach ulcers, aplastic anemia, gastrointestinal disorders, renal failure, stroke or myocardial infarction [50,51]. Therefore, novel methods simplifying sample preparation and

possessing appropriate detectability with affordable fluidic components and LC instrumentation are called for.

2. EXPERIMENTAL

2.1. Reagents, samples and materials

All reagents were of analytical grade and obtained from Sigma-Aldrich (Madrid, Spain) unless otherwise stated and used as received without further purification. Carbon nanofibers (diameter: 70-150 nm, length: 20 μm, surface area: 40 m^2 g^{-1} and bulk density: 0.09 g mL^{-1}) were kindly gifted by Dr. Reinhard Kriegbaum from Electrovac AG (Klosterneuburg, Austria). Graphite (particle size: 3-4 nm, surface area: 540-650 m^2 g^{-1} and bulk density: 1.2-2.8g mL^{-1}) and graphene powder (diameter: 2μm, surface area: 750 m^2 g^{-1}) were obtained from SkySpring Nanomaterials, Inc (Houston, USA). Multi-walled carbon nanotubes (inside diameter: 5-10 nm, length: 5-20 μm, surface area > 60 m^2 g^{-1}) were purchased from Nanogeneration (Chiang Mai, Thailand). Multi-walled carbon nanotubes functionalised with 5% of carboxylic acid moieties (diameter: 10 nm and average length: 1-2 μm) were purchased from DropSens (Spain). A mixed stock solution of 100 mg L^{-1} each of ketoprofen, ibuprofen, naproxen and diclofenac was prepared in methanol. The stock was stored in the dark at 4 ºC pending use and stepwise diluted with Milli-Q water (resistivity > 18 MΩ cm; Millipore, Bedford, USA) to the desired concentration for preparation of the working standard solutions, with a final pH adjustment of 1.7 using dilute HCl. The acceptor solution for the three-phase LPME protocol is composed of 20 mmol L^{-1} NaOH in 10% (v/v) methanol. 2-(2,4,5-trichlorophenoxy)propionic acid (also known as silvex or fenoprop) was used as Internal Standard (IS) at the 40 mg L^{-1} level throughout the in-line HF-LPME tests. Buffer solutions of pH 8 and 10 at the 10 mmol L^{-1} level were prepared from sodium phosphate dibasic/sodium phosphate monobasic and boric acid, respectively, using appropriate amounts of sodium hydroxide for pH adjustment.

Urine samples were collected from healthy volunteers in this project aged > 18 year. All participants provided informed consent before participating and the project was approved by the Research Committee of the University of the Balearic Islands (no. FITRACE 1/2018). The volunteers had to receive a medical prescription (oral administration) of an NAIS so as to participate in this research project. A time-course collection of midstream urine, usually fixed at 60 and 240 min after drug administration, was resorted as a proof of concept investigation of drug clearance. All urines were coded, anonymized and stored in a sealed plastic bag at 4 ºC before analysis. A pooled blank urine sample was obtained from the same volunteers before oral administration. The urine samples were filtered through a membrane filter (Nylon membrane filter, 47 mm, 0.45 μm, Merck-Millipore, Darmstadt Germany) to remove suspended matter. The pH was

adjusted to 1.7 akin the standards to ensure that the analytes were in neutral (carboxylic acid) form. After analysis, all of the samples were discarded.

2.2. Preparation of the carbon nanofiber-reinforced hollow fiber (CNF@HF)

Polypropylene hollow fiber membranes (Accurel PP Q3/2, 600 ± 90 µm ID, 200 ± 35 µm thickness, 0.2 µm pore size) obtained from Membrana (Wuppertal, Germany) were cut manually with a scalpel into pieces of 12.0 cm each. A dispersion of 3 mg mL^{-1} carbon nanofiber (CNF) in 1-octanol were injected into the lumen and outer shell of hollow fiber membrane in a 10 mL vial and sonicated at room temperature for 1 h for immobilization of the CNF into the HF wall pores by the joint action of sonication and capillary forces. The surplus of CNF in the lumen was carefully removed by positive pressure with air and 1-octanol using a 1 mL medical syringe. The as-obtained hybrid CNF@HF were air-dried for 48 h for solvent evaporation prior use.

2.3. 3D printed LPME microextraction chamber

The 3D printed extraction chamber was created using the 123D Design Software (Autodesk, San Rafael, California). It consisted of a 12-cm long cylinder with ID of 3 cm and wall thickness of 2 mm (see sketch in Fig. 1). The chamber outlets were thickened for 3D printing of threads. To this end, a ¼ 28" bolt was modelled and subtracted from the cylinder ends so as to accommodate commercially available ¼ 28" nuts that served as a support of the CNF@HF and sealed the flow-through chamber. Two additional connections allowed to perfuse the donor compartment and were placed at 1 cm distant of the cylinder ends, that is, separated 10 cm, serving as donor inlet and outlet, respectively. The 3D model was exported in .stl file format and transferred to the PreForm software (Formlabs, Somerville, Massachusetts, USA) for orientation, and generation of supports prior to 3D printing with a stereolithographic Form 2 printer (Formlabs). The model was tilted 80º from the vertical and fabricated with 503 layers at 100 µm z-resolution. Other features of the 3D print are as follows: automatic supports with density = 1, point size = 0.6 mm, no internal supports, flat spacing = 5 mm, slope multiplier = 1, base thickness = 2 mm and height above base = 5 mm. Four chambers were 3D printed all at once in 220 min, with a FLGPCL02 resin (Formlabs) consumption of 41 mL and a total cost per unit of 2.85 € (1.73 € resin +1.12 € power). After printing, the chambers were separated from the built platform, immersed in isopropyl alcohol for 10 min, followed by removal of the polymeric supports, and curing of the printed platforms overnight in a 16 W low-pressure Hg lamp UV oven (KA-9180, PSKY, China) so as to minimize monomer/polymer leaching as described elsewhere [52].

2.4. Flow setup for automatic carbon-reinforced HF-LPME

The flow setup for dynamic CNF@HF-LPME is composed of an Xcalibur syringe pump (SP1, Cavro, Sunnyvale, USA) furnished with a 1-mL gastight glass syringe (Hamilton, Bonaduz, Switzerland) and a three-way head valve at the top (HV). The valve allowed to either handle the fluidic carrier (MilliQ water, valve position IN) or to connect with the central port of an eight-position selection valve (SV, VICI AG International, Schenkon, Switzerland) through a 2.0 mL holding coil (PTFE, 3.18 mm OD, 1.59 mm ID, IDEX, Oak Harbor, USA). The selection valve allowed to further manipulate the samples, solvent, acceptor phase or air through the holding coil. An AIM3200 autosampler (Aimlab, Virginia, Australia) equipped with two 60-position, 12 mL-sample tube racks was connected to the SV for automatic sample switching and setup conditioning. A diagrammatic representation of the flow manifold and modular components thereof is shown in Fig. 2. The central component of the flow system is a stereolithographic 3-D printed flow-through chamber (see above).

A single-strand 12.0 cm-CNF@HF was inserted into the donor compartment of the 3D-print by fitting the ends into short fluorinated ethylene propylene (FEP) tubing sleeves of 1.07 mm ID (IDEX, Oak Harbor, USA) and sealing them with inverted ferrules and nuts. Short pieces of Tygon tubing were used for connection of the FEP sleeves with the manifold PTFE tubing (1.59 mm OD, 0.76 mm ID). The sample or standard (donor solution) was fed perpendicularly to the CNF@HF and flowed through the fiber shell, whereas the organic solvent or the alkaline acceptor phase were brought through the lumen of the HF as precisely controlled by SP1 (see Fig. 2). The outlet of the fiber lumen was connected to the LC through a 24V solenoid commutation valve (CV, Parker Hannifin Corporation, Mayfield heights, Ohio, USA) that allowed merging the flowing stream with a 20 mmol L^{-1} HCl in methanol as provided by an ancillary XCalibur pump (SP2, furnished with a 500 µL syringe and a three-way head valve, see Fig. 2). This configuration fosters automatic in-line neutralization of the alkaline acceptor with the acidic solvent by flow segmentation prior to LC analyses. The 24V CV was controlled from the XCalibur digital output.

All of the components of the automatic setup in Fig. 2 (viz., two syringe pumps, a selection valve, a solenoid valve, and autosampler) and the synchronization with LC were controlled by the user-friendly CocoSoft 4.3 freeware [53], which serves as an automation suite focused in laboratory instrumentation control and data processing.

2.5. HPLC Instrumentation and on-line interface

The liquid chromatographic (LC) module system (Jasco, Tokyo, Japan) is composed of a quaternary high-pressure pump (PU-4180, Jasco) enduring pressures of ≤ 700 bar; an autosampler (AS-4050) equipped with

a high pressure injection valve furnished with 1/32'' ID stainless steel sample loop of 200 µL; an external GECKO 2000 column heater; a photodiode array detector (MD-4017, Jasco), and a C18 core-shell reversed-phase column (Kinetex 2.6 µm, 150×3 mm, 100 Å, Phenomenex, Torrance, US) preceded by a C18 SecurityGuard™ Standard precolumn (4×2 mm, AJ04286, Phenomenex) for the separation of the target species. Detection of the analytes was performed at 210 nm, yet improved selectivity for ibuprofen was accomplished at 230 nm. Isocratic elution was carried out in ≤ 25 min by a 59.4:39.4:1.2 (v/v/v) methanol/water/formic acid solution flowing at 0.40 mL min^{-1}. The C18 core-shell column underwent a 30-min preconditioning step before starting the chromatographic run, and the temperature was kept at 30°C throughout. The retention times of ketoprofen, naproxen, IS, diclofenac and ibuprofen under the selected experimental conditions were 4.4, 5.4, 11.3, 14.8 and 17.0 min, respectively. Running of the LC analysis sequence, recording of chromatogram peaks and spectra, and data processing using peak area measurements was undertaken by a PC operated under the ChromNAV software.

The sample preparation manifold was on-line hyphenated to the LC separation system for the sake of a complete unsupervised operation. The hyphenation procedure is described as follows: The connections of the high pressure injection valve of the LC autosampler (HPLC IV in Fig. 2) were changed for the valve to remain most of the LC run in the 'load' position. The sample preparation setup was programmed to bring the analyte-enriched acceptor phase into the loop of the HPLC IV whenever the CNF@HF-LPME procedure was finished. When the previous LC run finished, the valve turned to the inject position and waited for a programmed delay of 1.2 min. During this delay, the analyte-laden acceptor was displaced out of the injection coil and flowed toward the analytical column. After this delay, the valve turned to the 'load' position and the LC autosampler emitted a signal. The signal was received by the analogic input of the XCalibur pump (SP1) and transmitted to CocoSoft 4.3, which remained in a 'While' loop while waiting for the LC signal. The sample preparation procedure lasts less than the chromatographic separation, thus ensuring a reliable synchronization of the extraction module and the LC system. The execution of the sample preparation method pursued with the next sample analysis. Further details of the automatic hyphenation scheme are available elsewhere [52].

2.6. Automatic analytical procedure for in-line CNF@HF-LPME as a 'front end' to LC analysis

The analytical procedure for clean-up and concentration of acidic drugs in urine with further LC analysis of the acceptor extracts starts by activation of the liquid chromatograph, setup which triggers the main micro-syringe pump of the fluidic platform (SP1) to perform: (i) *in-line* CNF@HF pretreatment/regeneration by *in-situ* generation of the organic film, (ii) sample extraction under acidic conditions, (iii) retrieval of the alkaline acceptor phase, (iv) in-line neutralization and injection of the analyte-containing extract into the LC

valve (LC VI), and (v) system cleaning. A short description of the several analytical operations is listed below (the full analytical protocol is available as Table S1):

Membrane pretreatment/regeneration

First, the syringe pump is programmed to fill the donor chamber (step 1) with carrier solution to exert a positive pressure onto the CNF@HF outer shell to overcome solvent leaking during the in-line generation of the wetting liquid organic membrane. Thereafter, 200 µL of acceptor solution (20 mmol L^{-1} NaOH in 10% MeOH) and 60 µL of dihexyl ether were consecutively brought into the holding coil (HC) in air segmented mode (steps 2-4) followed by perfusing the organic solvent into the HF lumen to generate the wetting film over the CNF and impregnate the available pores of the membrane (step 5). A stopped-flow time of 60 s was used for immobilization of the solvent film. The surplus of the organic solvent in the lumen was then flushed off by 160 µL of acceptor solution (step 6), with the remaining volume trapped into the lumen of the CNF@HF to serve as the acceptor plug for the ensuing carbon-reinforced HF-LPME process.

Removal of organic solvent

A 500 µL of acidic methanol (pH 1.7) was aspirated into the external syringe (SP2, see Fig. 2) and pumped toward the LC IV to remove any remnants of the organic solvent in the transfer line (steps 7-8).

Sample processing

A given volume of sample (usually 800 µL) was aspirated into the HC and fed into the donor compartment of the 3D printed chamber by flow reversal at 0.5 mL min^{-1} so as to fill the entire void volume (steps 9-10). A stopped-flow time of 30 s was applied to ameliorate the extraction efficiency. This step was repeated 8 times, thus amounting a total volume of 6.4 mL of urine sample.

Analysis of the extract and system cleaning

The analyte-laden alkaline extract is neutralized with dilute HCl as provided by SP2 prior to reaching the LC IV loop. To this end, the two syringe pumps are programmed to concurrently aspirate 220 µL of acceptor solution into HC and 220 µL of acidic MeOH (pH 1.7) into SP2, respectively (steps 11-12). An iterative segmented-flow protocol consisting of pumping 20 µL of extract/acceptor solution and 20 µL of acidic solution in a tandem-flow configuration downstream was repeated 11 times, thus amounting a total volume of 440 µL (steps 13-14), which is found appropriate to bring the analyte-laden acceptor plug toward the column separation system. The neutralized plug of extract was then injected into the HPLC in a heart-cut

mode. This step also serves to minimize analyte carryover prior to the next analysis and fill the lumen with fresh volume of acceptor. The overall CNF@HF-LPME protocol including membrane regeneration lasts 22.2 min.

3. RESULTS AND DISCUSSION

3.1. Nanomaterials for carbon-reinforced hollow fiber LPME

Preliminary tests were conducted in a batchwise mode to investigate the feasibility of a carbon nanostructured hollow membrane system for extraction of acidic drugs. To study the effect of the type of carbon nanomaterials on the extraction efficiency, mixed standards at the concentration level of 30 μg L^{-1} (25 mL, pH= 1.7) were subjected to microextraction for 10 min at 800 rpm using an 8-cm long HF. The alkaline acceptor (20 mmol L^{-1} NaOH) was diluted 1:1 (v/v) with 20 mmol L^{-1} HCl prior to HPLC analysis. Figure 3 illustrates the dependence of the enrichment factor of the four target drugs upon diverse carbon nanomaterials immobilized in the HF, *viz.*, graphite, graphene, multi-walled carbon nanotubes (MWCNTs), oxidized MWCNTs, and carbon nanofibers (CNFs), using dihexyl ether as a liquid membrane. Initial tests confirmed that the organic phase acts as an efficient barrier between aqueous acceptor and donor phases in a three-phase LPME-type mode. In fact, leaking was rapidly observed in our flow-through configuration using carbon reinforced HF as a sorptive phase in the absence of water immiscible solvent. As compared to unmodified HF-LPME, a statistically significant improvement in extraction efficiencies of the four acidic drugs was observed for the overall carbon reinforced materials (Fig. 3), in particular for graphite and carbon nanofibers, with enhanced enrichment factors by as much as 2.2-fold with the use of CNF@HF-LPME. This demonstrates the positive effect of a nanostructured liquid membrane for isolation of acidic drugs with log P >3 based on selective partitioning in three-phase LPME aided by sorptive microextraction onto carbon nanomaterials. Carbon nanofibers are proven herein to afford the highest enrichment factors regardless of the log P value of the drug within the range of 3.2-4.7 (see Fig. 3) inasmuch as the cup-stacked like configuration of the fibers endows unique surface area by participation of outer and inner reactive edges [20] in the underlying Wan der Waals/π-π stacking mechanism of adsorption. Therefore, carbon nanofibers were chosen for the remained of the work in a hybrid carbon nanomaterial@HF-LPME mode. Scanning electron micrographic images of CNF@HF, which were prepared as described in section 2.2, demonstrated the incorporation of CNF in the pores of the lumen and the shell side of the hollow fibers are shown as Fig. 4.

3.2. Selection of the organic extraction solvent for CNFs@HF-LPME

Physicochemical properties of the water-immiscible organic solvent impregnating the modified HF, including viscosity, dielectric constant and adsorption energy onto carbon materials, are expected to affect the extraction efficiency of the target drugs. Strikingly, organic phases in automatic CNF@HF-LPME format with in-line solvent regeneration should possess moderate to low viscosities for facile handling in a programmable-flow mode (aspiration from the vessel and pumping across the CNF@HF under positive pressure aided by a micro-syringe pump) along with expedient mass transfer of target species between extracting phases in a dynamic extraction format. Based on previous works reported in the literature for NSAIDs [4–6] and preliminary physical tests of feasibility of manipulation in a flow-through mode, 1-octanol and dihexyl ether, with contrasting viscosity and polarity, were assayed (see Fig S1). Dihexyl ether bearing the lowest viscosity (1.7 mPa·s vs 7.3 mPa·s for 1-octanol at 20ºC) and the lowest dielectric constant (< 2.7 vs 10.3 for 1-octanol at 20 ºC) afforded better distribution constants of the drugs from the CNFs into the solvent, and fostered a significant increase of EF for the two more hydrophobic species (viz., DIC and IBU) by 31% and 67%, respectively, as compared to 1-octanol. Therefore, dihexyl ether was adopted for in-line CNFs-mediated LPME.

3.3. Investigation of the composition of donor and acceptor phases

On the basis of the acidic behavior of our model analytes (pK_a values of KTP, NAP, DIC and IBU are 4.4, 4.2, 4.2 and 4.1, respectively) and the automatic three-phase HF-LPME mode amenable to on-line hyphenation to LC, the donor solution in both samples and standards was affixed to pH 1.7 with dilute HCl for extraction of neutral species. Notwithstanding the fact that nominal acceptor solutions with pH ≥ 7.0 might be expected to strip quantitatively the species out of the organic phase by formation of carboxylate species, preliminary assays indicated that stronger alkaline conditions are needed for efficient retrieval of species from carbon-decorated supported liquid membranes under non-steady state conditions. To study the dependence of the analyte recoveries and enrichment factors upon the pH of the acceptor phase, mixed standards at the 30 μg L^{-1} level (pH 1.7) were subjected to batchwise CNFs@HF-LPME at distinct acceptor pH values, viz., 8.1, 10.0 and 12.3, using dihydrogen phosphate/hydrogenphosphate buffer, boric acid/borate buffer and 20 mmol L^{-1} NaOH, respectively. The higher the acceptor pH the better were the enrichment factors of the overall target drugs (see Fig. S2). Notwithstanding the distinctness of the underlying principles of the extraction process in carbon-mediated LPME, this trend is in good agreement with previous authors utilizing conventional three-phase HF-LPME for HPLC determination of NAIDS reporting the need of a steep gradient pH across the liquid membrane with acceptor pH values of ca. 12 [13,54]. To circumvent analyte carryover for concentration levels above 300 μg L^{-1} as a result of π-π stacking interactions with the

CNFs, 10% (v/v) MeOH was added to the alkaline acceptor inasmuch as alkaline solutions with methanol percentages up to 20% were proven to be immiscible with dihexyl ether. Hence, the donor and acceptor phases were composed of 10 mmol L^{-1} HCl and 10% (v/v) MeOH in 20 mmol L^{-1} NaOH, respectively.

3.4. Configuration of the 3D-printed microextraction chamber as a front end to LC

A salient asset of 3D printing is the feasibility of rapid prototyping of tailor-made extraction chambers *ad hoc* for expedient optimization of in-line HF-LPME setups as opposed to previous configurations capitalizing on PTFE tubing or glass designs [37,40], yet appropriate curing of the 3D prints (see Experimental) to prevent reactivity of the polymeric scaffolds is called for. To study the effect of the donor chamber size upon the analytical response and system reliability, cylindrical chambers with distinct aspect ratios (H/d) spanning from 27 to 53 with fixed cross-sections (ca.7 mm^2) and variable nominal lengths (*viz*., 8,12 and 16 cm) were 3D printed. Superior enrichment factors for the four drugs were encountered with the middle-sized chamber with increments by 9-31% against the shorter configuration because of the larger amount of extracting phase available across the CNF@HF setup. On the other hand, a further increase in the length of the HF using the 16 cm-long chamber is not recommended in as much as the leaking of the organic solvent and acceptor solution due to the built-up of backpressure has been occasionally observed in the course of the automatic flow-through microextraction process. Therefore, the 12-cm long 3D-printed donor chamber was used throughout. Using this configuration, a sample loading flow rate of 0.5 mL min^{-1} is proven to afford reliable results without jeopardizing the analytical throughput as the length of the automatic microextraction process (ca. 22 min) is synchronized with the HPLC analysis (≤ 25 min) of the preceding sample.

3.5. Analytical figures of merit

The analytical performance of the in-line CNF@HF-LPME method in terms of dynamic linear range, limits of detection (LOD) and quantification (LOQ), enhancement factors and relative recoveries in real-life urine samples was investigated under the experimental conditions indicated in the operational procedure (viz., sample volume of 6.4 mL in a continuous forward-flow mode at 0.5 mL min^{-1} and nominal acceptor volume of 34±10 µL in a stagnant mode followed by heart-cut HPLC injection). The dynamic linear range established by internal standardization at 5 concentration levels spanned from ca. 5-15 µg L^{-1} to 500 µg L^{-1} with determination coefficients (R^2) greater than 0.9949 in all instances. LODs and LOQs, calculated at a peak-to-peak signal-to-noise ratio (S/N) of 3 and 10, respectively, for a standard at the lowest concentration level in the calibration curve, ranged from 1.6 to 4.3 µg L^{-1} and 5.3 to 14.3 µg L^{-1}, respectively. Enhancement factors calculated as the ratio of the sensitivity obtained with the flow-through CNF@HF-LPME concentration

method against that of direct HPLC analysis of standards in MeOH at concentration levels > 500 μg L^{-1}, using an injection volume of 10 μL to prevent peak broadening effects, spanned from 43.2 (KET) to 96.8 (DIC). The extraction efficiency under dynamic flow conditions (estimated from the mass of analyte transferred to the accepted phase and injected into HPLC) for the target drugs ranged from 6.8-15.1%. Method repeatabilities, expressed as the intra-day precision (RSD) from five consecutive measurements at the 80 μg L^{-1} level, were ≤ 6.1% (see Table 1). A single fiber was proven to be reusable with negligible cross-contamination effects up to 10 times. Chromatograms of spiked urine samples (see Fig. S3) featured stable baselines and negligible interfering effects from the biological matrices. The feasibility of the automatic microextraction approach for reliable sample clean-up and analysis of urine samples (pH adjusted to 1.7, see Experimental) was done by spike recoveries using both internal standardization and matrix-matched calibration with drug-free urine. The latter rendered better results for low-abundance drugs in urine samples collected from the healthy volunteers and thus was adopted throughout. Experimental results compiled in Table 2 and illustrated in Fig. S4 indicated the feasibility of the fluidic method for reliable analysis of NSAIDs drugs in urine samples collected at varied sampling times as demanded in pharmacokinetic studies, with recoveries spanning from 96.7-105% regardless of the drug and sampling time. This demonstrates that the automatic flow system serves as an efficient clean-up platform for removal of salts and non-ionizable organic matrix components.

The analytical performance of the automatic CNF@HF-LPME as a 'front end' to LC is compared against that of previous articles reporting LPME for assays of acidic drugs using LC or capillary electrophoresis in combination to UV-Vis or photodiode array detection (see Table 3). All of the papers surveyed are based on manual/semi-automatic operation of the LPME setup with off-line analysis of the extracts [13,18,40,54,55]. Miniaturization of supported liquid membrane extraction in a microfluidic format has been also reported [13]. With limits of detection as high as 300 μg L^{-1} the actual applicability of the microfluidic method to real analysis of biological samples, e.g., urine., is however debatable. In contrast to a conventional HF-LPME method [54], the use of CNF afforded better sensitivity and dynamic linear range with LODs more than one decade better. LODs in our work are also on a par of those previously reported for electromembrane extraction [18,56,57], yet with much lower sample volume and with no need of resorting to external energy sources.

4. CONCLUSION

In this paper, a fluidic concept for automation of CNF-reinforced HF-LPME including organic phase regeneration has been presented and validated. Utilizing the features of flow analysis, membrane impregnation with organic phase, acceptor handling, sample loading, and on-line dilution and injection of

the extract into LC have been fully automated. 3D-printed flow chambers have been proven appropriate as affordable flow components in method development for optimization of physicochemical variables. Because of the large surface area and sorption capacity for organic molecules of carbon nanofibers, the use of carbon nanostructured membranes impregnated with dihexyl ether have permitted enhanced mass transfer of acidic drugs (NSAIDs) in a three-phase HF-LPME dynamic extraction mode. The analytical figures of merit (LOD < 4.3 μg L^{-1}, repeatability < 6.1% and enhancement factors > 43), compared to previous works (see Table 3), and the lack of bias in analyzing urine samples within a broad timeframe after drug uptake have demonstrated the suitability of the proposed automatic fluidic setup encompassing sample handling and on-line detection as invaluable tool for toxicokinetic urinalysis.

Current research is underway in our research group in ascertaining the effect of green solvents (deep eutectic solvents and ionic liquids) on the extraction behavior of CNF-HF-LPME and expanding the scope of the miniaturized flow-through sample preparation device by implementation of electric-field driven separation for automatic trace level analysis of ionizable emerging environmental pollutants.

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Table 1. Figures of merit of the on-line CNF@HF-LMPE-LC method for determination of NSAIDs

Compound	Linear range ($\mu g\ L^{-1}$)	R^2	LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)	Enhancement factor*	RSD% (n=5)
KET	7.4-500	0.9998	2.2	7.4	43.2	6.1
NAP	5.3-500	0.9997	1.6	5.3	51.2	4.9
DIC	12.4-500	0.9992	3.7	12.4	96.8	3.7
IBU	14.4-500	0.9993	4.3	14.4	58.9	5.9

* Referred to direct injection of standards in MeOH into HPLC (10 µL)

Table 2. Concentration of NSAIDs in real-life urine samples using in-line CNF@LPME hyphenated to LC

Sample	Administration		Sampling time (min)[b]	Concentration (μg L^{-1})[c]	RSD (%)	Recovery (%)
	NSAIDs	Dosage (mg)[a]				
Urine 1	KTP	50	60	160	0.3	
Urine 2 (sample C in Fig S4)	KTP	50	240	198	3.1	
Urine 3 (sample B in Fig S4)	DIC	100	60	58	1.5	
Urine 4 (sample A in Fig S4)	IBU	600	240	79	4.6	
Urine 2 + spiked KTP (100 μg L^{-1})				298	1.5	100
Urine 3 + spiked DIC (30 μg L^{-1})				87	2.1	96.7
Urine 4 + spiked IBU (40 μg L^{-1})				121	1.7	105

[a] Amount of drug administrated via oral
[b] Sampling time after drug administration
[c] Concentrations of drugs in urine after 1:3 dilution

Table 3. Analytical performance of previous LPME methods reported in the literature for determination of NSAIDs using column separation systems coupled to photometric detection*

Method	Sample preparation	Analytes	Sample volume (mL)	Recovery (%)	Linear range (μg L^{-1})	LOD (μg L^{-1})	Ref.
LC/UV-Vis	HF-LPME	IBU,DIC, SAC	50	83-99	135-10,000	40-53	[54]
LC/UV-Vis	EME	SAC,KTR, KTP,NAP, DIC,IBU	10	58-100	0.29-100	0.08-3.36	[56]
LC/UV-Vis	EME	NAL, DIC	24	90-98	12-500	4.0	[57]
LC/UV-Vis	DLPME	KTP,DIC, MEF	5	96-116	15.5-10,000	4.7-5.2	[55]
LC/UV-Vis	EME	KTP,NAP, DIC, IBU	10	NA	0.18-100	0.06-1.36	[9]
LC/UV-Vis	Microchip-LPME	KTP,NAP, DIC, IBU	5×10^{-3}	85-100	100 or 500-10,000	70-300	[13]
LC/UV-Vis	Semi-automatic HF-LPME	KTP,NAP, DIC, IBU	1000	90-100	0.01-1.0	0.01-0.05	[40]
CE/UV-Vis	CNF-HF-EME	NAP, IBU	4	85-88	5.0-500	1.0-1.5	[18]
LC/PD	Automatic CNF@HF-LPME coupled on-line to LC	KTP,NAP, DIC,IBU	6.4	99-105	5.0-500	1.6-4.3	This work

***** Ranges listed in the Table are merely related to the target analytes in this work

Acronyms: HF-LPME: Hollow fiber-liquid phase microextraction; EME: Electromembrane extraction; DLPME: Dispersive liquid phase microextraction; CNF: Carbon nsnofibers; LC: Liquid chromatography; CE: Capillary electrophoresis, PD: Photometric detector; KTP: Ketoprofen; NAP: Naproxen; IBU: Ibuprofen; DIC: Diclofenac; SAC: Salicylic acid; KTR: Ketorolac; NAL: Nalmefene; MEF: Mefenamic acid; NA: Not applied to real samples

Figure captions

Fig. 1. Sketch of a 3D flow-through chamber model for in-line HF-LPME as created by the 123D Design software

Fig. 2. Schematic diagram of the fully automatic 3D-printed HF-LPME module using CNF@HF for in-line microextraction of acidic drugs as a front end to LC analysis. SP1&2: Syringe pumps, HV1&2: Head Valves, IV: Injection Valve, AS: AutoSampler, CV: Commutation Valve, A: Air, AC: Acceptor, HC: Holding Coil, OS: Organic Solvent, PDA: PhotoDiode Array, IN, OUT: Positions of the main head valve, W: Waste.

Fig. 3. Enrichment factors for carbon nanomaterials-reinforced hollow fiber membrane microextraction of NSAIDs in a batchwise extraction mode as compared to conventional HF-LPME using raw polypropylene fibers. MWCNTs: Multiwalled carbon nanotubes; CNFs: Carbon nanofibers. Results are the average of three replicates ±SD. Experimental conditions: sample volume: 25 mL, analyte concentration: 30 μg/L (pH 1.7), solvent: dihexyl ether, extraction time: 10 min, stirring speed: 800 rpm, acceptor phase: 20 mM NaOH, acceptor volume: 22 μL. Enrichment factors were calculated as the concentration of drug in the acceptor phase after HF-LPME against the initial concentration in the donor compartment. A five-point calibration graph with standards subjected to 1:1 dilution with dilute acid was used for determination of the concentration of drugs in the alkaline acceptor. In all cases, the injection volume into the LC was 10 μL.

Fig. 4. Scanning electronic micrographs at 10k magnification illustrating the polypropylene hollow fibers prior (a,b) and after (c,d) carbon nanofiber reinforcement. Lumen (a,c) and shell side (b,d).

Figure 1

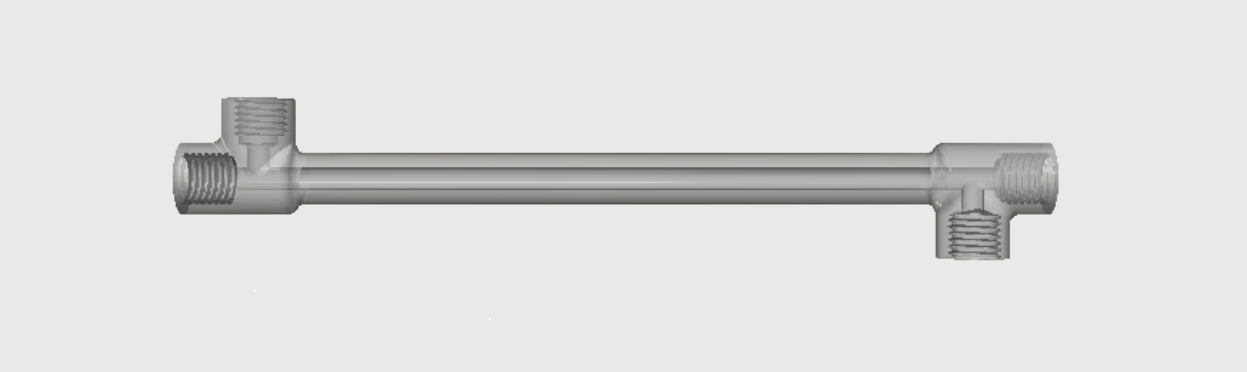


Figure 2

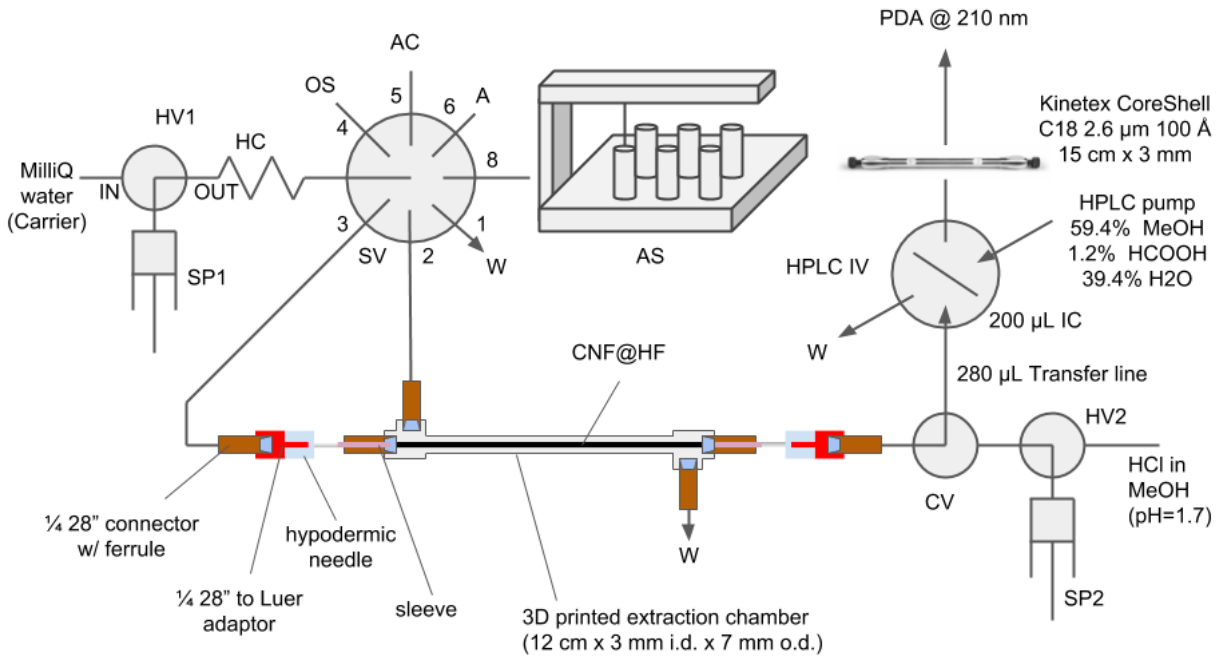
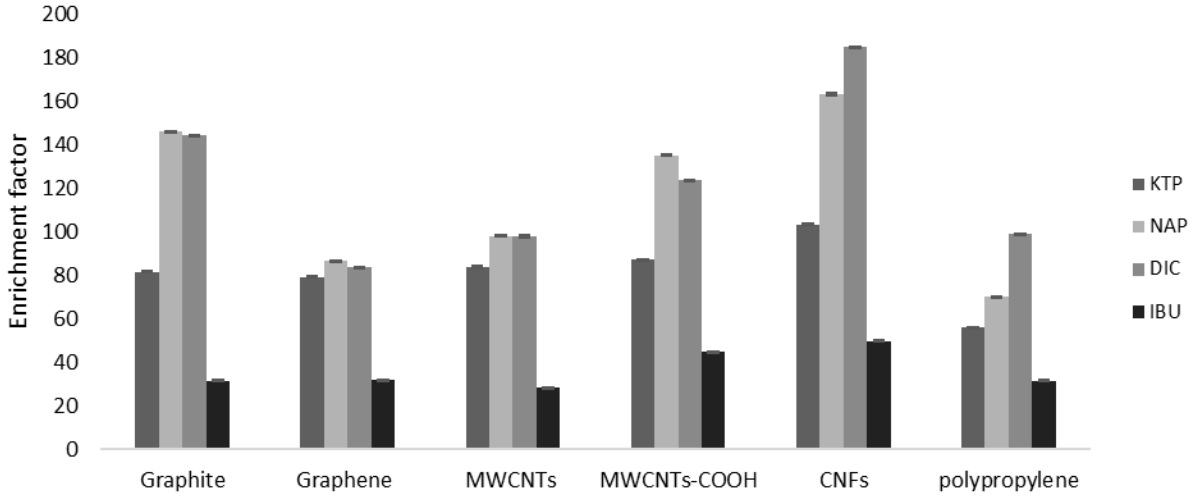

PDA @ 210 nm
AC
OS
5
6
A
4
8
3
SV
2
W
1
HV1
HC
MilliQ
water
(Carrier)
IN
OUT
SP1
AS
Kinetex CoreShell
C18 2.6 µm 100 Å
15 cm x 3 mm
HPLC pump
59.4% MeOH
1.2% HCOOH
39.4% H2O
HPLC IV
200 µL IC
W
280 µL Transfer line
CNF@HF
HV2
HCl in
MeOH
(pH=1.7)
CV
¼ 28" connector
w/ ferrule
hypodermic
needle
¼ 28" to Luer
adaptor
sleeve
W
3D printed extraction chamber
(12 cm x 3 mm i.d. x 7 mm o.d.)
SP2

Figure 3


200
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Enrichment factor
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NAP
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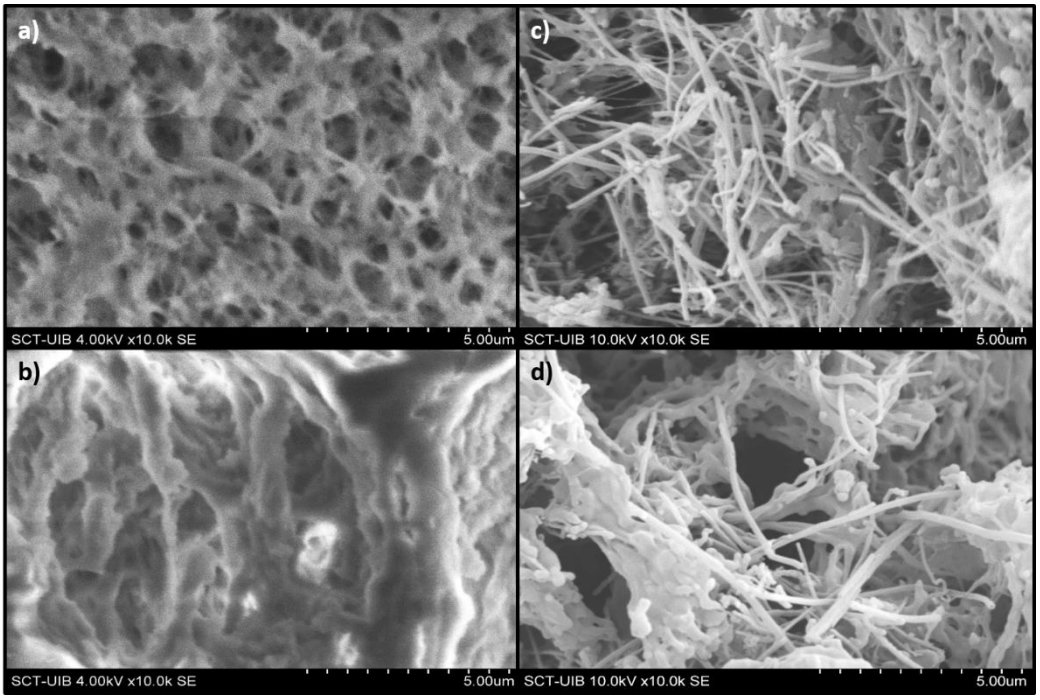
Figure 4


a)
SCT-UIB 4.00kV x10.0k SE
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SCT-UIB 10.0kV x10.0k SE
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b)
SCT-UIB 4.00kV x10.0k SE
5.00um
d)
SCT-UIB 10.0kV x10.0k SE
5.00um

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