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Dynamic-electromembrane extraction (d-EME): A new technical development for the extraction of neuropeptides

Nicolas Drouin, Serge Rudaz, and Julie Schappler

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Dynamic-electromembrane extraction (d-EME): A new technical development for the extraction of neuropeptides

Nicolas DROUIN, Serge RUDAZ, Julie SCHAPPLER*.

School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland

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ABSTRACT: In this work, a dynamic-electromembrane extraction (d-EME) device was developed for the extraction of neuropeptides. Based on a thin polypropylene hollow fiber (50 µm of wall-thickness and 280 µm i.d.), this set-up allowed for a continual renewal of the acceptor compartment. Due to the reduced size of the device, high preconcentration factors were obtained (up to 50-fold). The extraction remained constant regardless of the extraction time (from 15 to 45 min); accordingly, this new set-up minimized the effect of electrolysis on extraction performance while enabling high extraction yield (up to 72%) for most lipophilic neuropeptides.

INTRODUCTION

The analysis of scarce biological fluids (e.g., sweat, saliva, tears, cerebrospinal fluid) is an important analytical challenge due to both their low available volume and analyte concentration. In this context, microextraction (ME) methods appear to be an excellent strategy for sample preparation prior to the analysis of numerous complex biological matrices. ME methods are defined as non-exhaustive procedures that use very small volumes of the extracting phase and for which the sample volume is relatively large compared with the volume of the extracting phase [1]. ME methods reduce (i) solvent consumption ("green analytical chemistry"), (ii) sample volume, (iii) analysis time, and (iv) operating costs [2] and are thus perfectly adapted to scarce biofluids, for which minute amounts of sample are available. These methods are also relevant for more conventional biological matrices such as urine, plasma, and serum, for which high preconcentration factors (PF) can be attained. ME methods are subdivided into two subfamilies: solid-based and liquid-based ME methods.[3]

The operating principle of liquid-based ME methods is similar to that of conventional liquid-liquid extraction (LLE), with the lipophilic partition of analytes between two immiscible phases, typically an aqueous one (sample) and an organic one. LLE has witnessed resurgence over the past two decades due to the significant miniaturization of the set-up. To improve the recovery and reduce the extraction time, improvements such as the incorporation of microemulsions have been implemented, increasing the contact surface between the two phases as in the dispersive liquid-liquid microextraction approach.[4] Another type of liquid-phase ME method is liquid-liquid-liquid microextraction (LLLME), which is based on the succession of three successive immiscible phases, in which the intermediate organic phase plays the role of a free membrane to improve the selectivity of the system and collect the analyte in a final aqueous phase. High recovery rates can be obtained by appropriately adjusting the pH of both the donor and the acceptor phases to avoid back-extraction. Consequently, LLLME has mainly been used for ionizable compounds.[5] In the first set-ups reported, the acceptor phase was inserted in or on the organic phase with a syringe, making the stability of the two interfaces a problematic issue. To circumvent this issue, fat membrane [6] and later hollow fibers (HF) [7] were introduced by Johnson and coworkers to support the liquid organic membrane. More recently, Pedersen-Bjergaard and Rasmussen introduced hollow fiber-liquid phase microextraction (HF-LPME) under static conditions for the donor and acceptor compartments.[8] To improve the HF-LPME extraction speed, the same authors generated an electric field between two platinum electrodes: one placed in the sample and the other in the HF lumen.[9] This improved design was termed electromembrane extraction (EME) and led to a reduction in the extraction time to 5 min with an extraction recovery of up to 80% for small basic molecules. With this approach, passive diffusion is not the main mass transfer mechanism; instead, active transfer from the sample to the lumen of the fiber dominates due to the migration of the analytes under an electric field.[10] The principal issue of EME concerns water electrolysis, which induces bubble formation and the production of OH- and H+ ions at the cathodic and anodic electrodes, respectively. Kuban and Bocek investigated the effect of electrolysis on the acceptor compartment with a free liquid membrane system termed a micro-supported liquid membrane (µ-SLM).[11-13] The authors concluded that drastic pH changes and bubble formation occurred a few minutes after the beginning of the extraction process, leading to modifications of the ionization state of the molecules and current instability, respectively. This pH alteration during the process could be responsible for analytes' back-extraction and therefore a decrease in the extraction recovery.

EME has been widely used for hydrophobic basic low molecular weight (LMW) compounds, achieving high PF (up to 100-

fold) and high recovery (up to 100%).[14,15] Based on the composition of the SLM, the selectivity of the system can be easily tuned. N-nitrophenyl-octyl ether is the most common organic solvent used for hydrophobic basic drug extraction, whereas acidic compounds cannot be extracted; instead, alcohols are preferred.[16] Basic and acidic LMW drugs can also be extracted in a single step using simultaneously EME and LPME, respectively.[17] To improve the extraction recovery of polar compounds, a carrier can be added to the SLM, facilitating mass transfer through the SLM via ion pairing interactions.[18] In order to improve recovery and/or enrichment, Pedersen-Bjergaard and coworkers introduced on-chip dynamic-EME (on-chip d-EME), which enabled the continuous delivery of the donor and acceptor solutions. The device used an HF of 25 µm thickness implemented in a microchip that was online coupled to ESI-MS for real-time measurements of LMW basic drugs' metabolism.[19,20] More recently, the same group developed a d-EME probe using a conventional HF (wall thickness of 150 µm and i.d. of 330 µm) and a flow of both donor and acceptor solutions at 10 µL/min for LMW basic drugs extraction.[21] Asl *et al.* also introduced a d-EME device including a simultaneous renewal of the acceptor and donor solutions for the extraction of amitriptyline and its primary metabolite from plasma and urine samples. [22]

Peptides, however, remain a challenge to EME because of their low diffusion coefficient and their low lipophilicity. To the best of our knowledge, only a few studies on peptide EME have been conducted. Alcohols and ketones appear to be the most effective SLMs with the addition of di-(2-ethylhexyl) phosphate (DEHP) as a carrier. Results show that a longer extraction time (up to 45 min) and lower recovery (up to 50%) are achieved compared to those obtained for LMW hydrophobic drugs.[23-25]. To minimize the effect of electrolysis and to maximize the mass transfer of peptides through the SLM, Pedersen-Bjergaard and coworkers have used an acceptor compartment with a larger volume (500 µL instead of 20 µL) and a flat membrane with a reduced wall thickness (50 µm instead of 200 µm), respectively.[26] Consequently, the contact surface between the membrane and the compartments increases, leading to an improvement in the extraction speed and a decrease in the effects of electrolysis on acceptor compartment. However, the principal drawback of this approach is the absence of a preconcentration capacity caused by the high volume of the acceptor compartment.

In this study, a new d-EME device was developed for the extraction of various neuropeptides (NPs). This set-up allows for a continuous renewal of the acceptor solution and uses a polypropylene microporous HF with a wall thickness of 50 µm and an i.d. of 280 µm. The main improvements compared to the previous d-EME devices relies on: (i) no renewal of the donor solution, (ii) low renewal flow rate of the acceptor solution, (iii) reduced dimensions of the HF, and (iv) use of disposable parts. These properties enable high enrichment and enhanced recovery due to the improvement in mass transfer and the elimination of the electrolysis effect, leading to a reduction in the extraction current and the analytes' back-extraction. Cross-contamination between extractions is also circumvented.

EXPERIMENTAL

Chemical and reagents. Des-Tyr-Leu-enkephalin (Des), α-neoendorphin (α-NE), β-neoendorphin (β-NE), dynorphin A (1-7) (DynA(1-7)), dynorphin A (1-8) (DynA(1-8)), Met-enkephalin-Arg-Phe (Meap), and β-casorphin (β-caso) were purchased from Phoenix Europe GmbH (Karlsruhe, Germany). Leu-enkephalin (Leu), Met-enkephalin (Met), Met-enkephalinamide (Met-NH2), dynorphin B (20-32) (DynB(20-32)), hemorphin 7 (Hem7), endomorphin 1 (End1), endomorphin 1-COOH (End1-COOH), and endomorphin 2 (End2) were purchased from Genscript (Piscataway, NJ, USA). Angiotensine II (Angio II) was purchased from Bachem (Bubendorf, Switzerland). Nonanone, decanone, octanol and DEHP were purchased from Sigma Aldrich (Steinheim, Germany). Water, formic acid, and acetonitrile were of ULC-MS grade and purchased from Biosolve (Valkenswaald, Netherlands).

Preparation of sample solutions. The individual stock solutions were prepared by dissolving each model peptide in a solution of formic acid 0.1% in water-acetonitrile (95:5) at a concentration of 1 mg/mL. The peptide mixture was prepared by mixing individual stock solutions in a solution of formic acid 0.1% in water-acetonitrile (95:5) at a concentration of 5 µg/mL. Model peptide stock solutions and the peptide mixture were aliquoted in 0.5 mL Protein LoBind Eppendorf vials and stored at -80 °C. Aliquots were diluted with a solution of formic acid 50 mM to a final concentration of 50 ng/mL daily and stored at 4 °C prior to use.

Biological samples. Cerebrospinal fluid (CSF) samples were kindly received from the Geneva University Hospitals (Geneva, Switzerland) and collected from hydrocephalus patients (n=3). Samples were stored at -80 °C until use. Before analysis, the three samples were defrosted at ambient temperature and pooled. Blank pooled CSF was spiked with NPs at desired concentration (n=3) and acidified until pH 2-3 by addition of 187 µL of formic acid. 250 µL of acidified CSF was then extracted with the developed d-EME method.

Materiel. An Accurel PP 50/280 polypropylene hollow fiber (HF) with an i.d. of 280 µm, a wall thickness of 50 µm and a nominal pore size of 10 nm was purchased from Membrana (Wuppertal, Germany). Platinum wire (99.95%) with a diameter of 0.5 mm was purchased from Advent Research Materials Ltd (Oxford, England). Fused-silica capillaries with an i.d. of 50 µm and an o.d. of 150 µm and capillaries with an i.d. of 25 µm and an o.d. 365 µm were purchased from Polymicro Technologies (Phoenix, AZ, USA). P-770 union ferrule was purchased from UpChurch Scientific (Oak Harbor, WA, USA) and 250 µL flat bottom insert vial from BGB Technologies (Boeckten, Switzerland).

d-EME set-up. The d-EME set-up based on a cylindrical membrane is shown in Figure 1. The acceptor compartment consisted of a piece of Accurel PP 50/280 measuring 3.3 cm in length and two fused silica capillaries with an i.d. of 50 µm and an o.d. of 150 µm and lengths of 10.5 cm and 11 cm, termed the "inlet capillary" and "outlet capillary", respectively. The two capillaries were sealed together with a shift of 2.8 cm. After inserting the inlet and outlet capillaries, the HF was welded to them by fusing a polypropylene clog with a soldering iron.

The inlet capillary was inserted into a Capillary Electrophoresis 7100 system from Agilent Technologies (Waldbronn, Germany) by a fused-silica capillary with an i.d. of 25 µm, an

o.d. of 365 µm, and a length of 25 cm, termed the "CE capillary", allowing for pressure and voltage to build through the CE device. The inlet capillary was connected to the CE capillary with a P-770 union ferrule. This specific union was chosen to allow a connection between capillaries with outer diameters of 365 µm and 150 µm. The outlet capillary was placed on an empty collector LC vial with an insert.

The HF was impregnated by plunging the fiber in the selected solvent for 10 s. After impregnation, the fiber was filled with the acceptor solution by applying a pressure of 5 bar for 120 s. Another impregnation step was carried out afterward.

The donor and acceptor solutions were respectively 250-1000 µL and 500 µL of formic acid 50 mM. A negative voltage was applied to a platinum electrode placed in the acceptor solution and grounded through the CE system. The entire device assembly was set on a homemade plate and connected to a Thermomixer from Vaudaux-Eppendorf AG (Bale, Switzerland) to control the position of the electrode regardless of the agitation.

Final d-EME experiments were performed using a nonanol-decanone (1:1, v/v) SLM, with an applied voltage and flow rate of 20 kV and 280 nL/min, respectively, over a period of 45 min at 20 °C under agitation at 700 rpm. After extraction, the NPs remaining in the outlet capillary were collected by flushing the device with the acceptor solution during 180 s at 3.8 bar (3 µL).

UHPLC-MS/MS. Experiments were carried out with an Infinity 1290 ultra-high performance liquid chromatography (UHPLC) system from Agilent (Waldbronn, Germany). The instrument was equipped with a binary pump with a maximum delivery flow rate of 5 mL/min, an autosampler, a Flexible Cube allowing for the needle seat to be rinsed, and a column compartment thermostated to 50 °C. Separations were performed with a Waters Acquity UPLC™ CSH C18 column (1.7 µm, 2.1 mm × 150 mm, 300 Å). The mobile phases were independently prepared by adding 0.1% formic acid to water and acetonitrile. The injection volume was 5 µL, and the flow rate of the mobile phase was set to 0.4 mL/min. Mobile phase B was increased from 5 to 30% over 10 min. Mobile phase B was further increased to 95% within 0.1 min, and this condition was maintained for 0.5 min before re-equilibration to the initial condition over a period of 7 min. The injector and the needle seat were washed by plunging in methanol 20% for 3 s and in 0.1% formic acid for 15 s at 500 µL/min, successively. A 50 ng/mL standard solution and a blank solution were injected before and after each extracted sample, respectively. The UHPLC system was hyphenated with an Agilent 6490 triple quadrupole mass spectrometer (QqQ/MS) equipped with an Agilent Jet Stream (AJS) ESI source. Electrospray ionization was operated in the positive mode, and spectra were acquired via SRM measurements. The precursor and product ions monitored for each peptide, as well as the collision energies, are reported in Table S1 (Supplementary Material). LC-SRM analyses were acquired in time scheduled mode with a time window set at 2 min. The following source parameters were used: The drying gas temperature was set to 220 °C and 14 L/min. The nebulizer gas was set to 35 psi, and the sheath gas was set to 11 L/min and 250 °C. The capillary and nozzle voltages were adjusted to 3500 V and 1500 V, respectively. The ion funnel voltages were set to 140 V for the high-pressure funnel and 80 V for the low-pressure one. The EMV voltage was set to 300 V, and the cell accelerator voltage was set to 5 V. Data acquisition and instrument control were monitored using MassHunter version B.06.00 (Agilent, Santa Clara, CA, USA). The transition, collision energy, and time schedule were optimized with Skyline version 3.1 (MacCoss Lab, University of Washington, USA); data collection and processing were performed using the same software. The three most intense transitions were selected.

Calculations. The preconcentration factor (PF), extraction yield [27] (EY), and process efficiency (PE) [28] were calculated according to the following equations:

$$PF = \frac{[acceptor]_{final}}{[donnor]_{initial}} = \frac{\sum_{transitions} extract}{\sum_{transitions} standard\ solution}$$

$$EY\ (\%) = PF \times \frac{Va}{Vd_{std}}$$

$$PE\ (\%) = PF \times \frac{Va}{Vd_{CSF}}$$

where Va is the collected volume of the acceptor compartment, Vd_{std} is the volume of the donor compartment (neat standard), and Vd_{CSF} is the volume of the donor compartment (CSF).

RESULTS AND DISCUSSION

Electromembrane extraction (EME) is a liquid-liquid-liquid microextraction technique in which an electric field is generated between aqueous donor and acceptor compartments separated by an organic phase supported by a hollow fiber (HF). With this approach, active transfer from the sample to the lumen of the HF occurs because of the migration of the analytes under the electric field. The principal issue of conventional EME set-ups concerns water electrolysis, which may induce drastic pH changes and bubble formation, leading to modifications of the ionization state of the molecules and current instability, which in turn are responsible for analytes' back-extraction and low extraction recovery.

The new technical development presented in this work relies on the concept of microdialysis. This procedure continuously allows for the renewal of the acceptor compartment during the extraction process to circumvent the electrolysis effect observed in conventional EME devices, hence termed dynamic-EME (d-EME). An impregnated microporous polypropylene HF with reduced dimensions (50 µm wall thickness and 280 µm i.d.) was implemented in this device. The thin wall should improve mass transfer during extraction compared to that of previously reported EME set-ups, while the small i.d. should permit low recovered volumes. The continuous renewal of the acceptor solution and the reduced dimensions of the HF constitute the primary features of this set-up that are anticipated to enable high recovery together with high enrichment. In addition, this new d-EME device is made of disposable parts, avoiding cross-contamination between extractions. The other advantage of conventional EME should also apply, i.e., lipophilic selectivity towards highly polar species such as salts and proteins.

The d-EME device was designed for the extraction of selected polar to moderately polar neuropeptides (NPs). The lipophilicity of such compounds is relatively complex to estimate because NPs can undergo conformational changes depending on the medium conditions. Therefore, a range of experimental log

P values are commonly observed based on the technique used for the determination. In addition, log P values can differ greatly from values estimated *in silico* that have been reported in the literature. For instance, three different log P values were found for Leu-enkephalin (Leu): 1.23 [29], -1.86 [30], and -1.20.[31] In this study, the composition of the mobile phase at elution was used to characterize the NPs' apparent lipophilicity (Figure 2). Polar NPs and moderately NPs were defined as compounds eluting in different parts of the gradient, corresponding to values of an organic solvent ranging between 8-16% and 17-25% acetonitrile, respectively. For clarity, results are hereafter presented for a selection of three NPs *per* polarity range, i.e., DynA(1-7), Des, and Met for polar NPs, and Leu, End2, and End1-COOH for moderately polar NPs.

d-EME set-up and SLM composition. In contrast to conventional microdialysis systems that use a single capillary, two connected capillaries (i.e., CE and inlet capillaries, Figure 1) were used in this device. Consequently, the change of the fiber was facilitated and the flow rate of the acceptor compartment could be controlled by easily adjusting the dimensions of the two capillaries. Different dimensions were tested to optimize the flow rate control and the dead volume. A capillary with an i.d. of 25 µm, an o.d. of 365 µm and a total length of 25 cm was ultimately chosen as the CE capillary because (i) 25 cm was the shortest capillary length that could be loaded into the CE cassette and (ii) 25 µm was the appropriate i.d. for obtaining a sufficient back pressure, enabling a large range of flow rates by applying a pressure to the acceptor solution reaching up to 5 bar. On the other hand, capillaries with an i.d. of 50 µm and an o.d. of 150 µm were chosen for the inlet and outlet capillaries so that both capillaries could be inserted into the 280 µm i.d. HF. Considering this set-up and after inserting the inlet and outlet capillaries, the lumen internal volume of the HF was estimated to be 1.3 µL, which is favorable for achieving a high preconcentration factor (PF).

The flow rate of the acceptor solution was determined by applying a pressure of 4 bar to the acceptor vial for 10 min through the CE system. This experiment was performed in triplicate (n=3) for the same impregnated HF and for three independent devices (k=3). Under these conditions, the obtained flow rate was 1.10 µL/min with a low variability (RSD=1.2%). According to the linear relationship between pressure and flow rate, a pressure of 3.6 bar was applied in further experiments to generate an estimated flow rate of 1 µL/min, and a flow rate of 280 nL/min was obtained by applying a pressure of 1 bar. During the filling of the acceptor solution, air could become trapped inside the fiber and generate bubbles. Experiments were thus performed to evaluate the effect of the filling on the extraction yield (EY). Although bubbles were still observed prior to the extraction at different positions inside the HF lumen, the low variability obtained (RSD<10%, n=10) demonstrated that the presence of residual bubbles had no deleterious effect on the extraction process.

Two different classes of organic solvent, aliphatic alcohols and ketones, and mixtures thereof, were tested because they are known to allow for good peptide migration.[23,26,32] The results obtained for the six selected NPs are shown in Figure 3A. Interestingly, alcohols were the best SLM for peptides, in contrast to results reported in the literature, in which both alcohols and ketones have been found to be equivalent.[26] A mixture of nonanol and decanone (1:1, v/v) yielded an EY similar to that obtained with pure nonanol (EY=2-44% and 1-43%, respectively). A lower extraction variability (e.g. RSD=13% for Des and 9% for Leu) was obtained with the mixture compared to that obtained using pure nonanol (e.g. RSD=21% for Des and 20% for Leu). This effect was particularly pronounced for the moderately polar NPs that were extensively extracted, so the mixed SLM was ultimately selected.

Di-(2-ethylhexyl) phosphate (DEHP), a well-known cationic carrier, was used to improve the mass transfer of polar compounds.[18] Concentrations ranging from 0 to 10% were tested in the SLM. No significant enhancement of the extraction of polar NPs was observed, whereas a significant decrease in the selectivity of the SLM for moderately polar NPs was observed according to the lower EY obtained for those NPs. Thus, no carrier was added to the SLM, ensuring a generic procedure and enabling the simultaneous extraction of polar to moderately polar NPs.

Effect of d-EME parameters. For all subsequent experiments, the flow rate of the acceptor solution was set at 1 µL/min. According to the high voltage drop occurring between the acceptor solution vial and the sample (where both electrodes are respectively located), voltages up to 30 kV could be applied. This voltage is high compared to previously reported EME, but due to long distance between the electrodes, a substantial part of the voltage drop actually occurs in the aqueous compartment on each side of the membrane. Similar magnitude of electric field is generated. Because the velocity of the compounds is directly proportional to the electric field, higher voltages could reduce the extraction time and improve the NP enrichment. On the other hand, because the generated current is proportional to the applied voltage (according to Ohm's law), higher voltages could be detrimental to the extraction. Voltages ranging from 0 to 30 kV were thus tested, and the results obtained for the six selected NPs are shown in Figure 3B. As expected, the extraction speed increased with the applied electric field until a maximum value of 10 kV (277.7 V/cm) was reached, beyond which the extraction speed was constant. The monitored current was very stable regardless of the applied voltage. In this d-EME configuration, no bubbles appeared inside the HF because the electrode was placed outside of the HF lumen. A voltage of 20 kV was selected for further experiments because the extraction speed was maximized at this value, whereas the generated current was minimized (<5 µA) and the EY variability low (e.g. RSD=5% and 2% for Des and Leu, respectively).

Extraction times ranging from 15 to 45 min were tested, which corresponded to recovered volumes of approximately 18 µL to 48 µL, respectively. As shown in Figure 4, the PFs were independent of the extraction time, indicating that the extraction speed remained constant throughout the entire experimented extraction period. Interestingly, recoveries were found to be directly proportional to the extraction time, with an EY of 104% (RSD=10%) achieved for the most lipophilic NP (i.e., End1-COOH) after 45 min. According to these observations, no saturation occurred in the acceptor compartment, in contrast to previous EME studies in which the system entered a steady state after 10 min of extraction with a limited gain in EY for longer extraction times.[23] These excellent results were obtained because of the original design of the d-EME set-up, in which the working electrode is placed in a large-volume

(500 µL) acceptor solution vial, thereby maintaining a low generated current. Consequently, electrolysis is limited, and no significant effect on the composition of the acceptor solution is observed, as demonstrated by a pH change of less than 0.05 measured in the acceptor compartment after 45 min of d-EME (n=5). The acceptor solution can then be continuously perfused as a fresh solution through the d-EME device, allowing for a constant extraction speed.

The flow rate of the acceptor solution that was continuously flushed through the HF was evaluated by applying a pressure of 1 to 3.6 bar to the acceptor vial. These pressures generated flow rates ranging from 280 nL/min to 1 µL/min, respectively, and recovered volumes of approximately 15 µL to 48 µL, respectively. As shown in Figure 5, the flow rate had a limited effect on the EY, likely due to the saturation of the acceptor compartment at a higher speed than that of the renewal of the compartment by the acceptor solution. On the other hand, the PF was inversely proportional to the flow rate. As previously mentioned, because the extraction speed was constant throughout the entire extraction period, the increase in the flow rate of the acceptor solution induced extract dilution. In addition to higher enrichment, lower variability was also reported at a lower flow rate, particularly for polar NPs. The improved repeatability was attributed to the higher sample concentrations injected into the UHPLC-MS/MS, which exhibited better quantitative performance at higher concentrations.

Under the final d-EME conditions applied (i.e., nonanol and decanone (1:1, v/v), 20 kV, 45 min, 280 nL/min), PFs of up to 50-fold and EYs of up to 72% were obtained for the 16 NPs, with RSDs lower than 29% (Figure 2 and Table S2, Supplementary Material). It should be noted that the d-EME of moderately polar NPs was systematically higher than that obtained for polar NPs, regardless of the conditions tested, in agreement with the lipophilic SLM used in this study.

In order to approach real scarce biofluid conditions, d-EME was performed with 250 µL samples (n=3, Table 1). A vial insert with a flat bottom was used to obtain the same immerged portion of the fiber for 1 mL and 250 µL donor compartments. Interestingly, the EY increased with lower sample volume, especially for moderately polar NPs with EY=92-103% (RSD=1-5%). Lower preconcentration factors were observed and converged to the same value, explained by the rapid depletion of the donor solution for these NPs. Concerning the most polar NPs, because their extraction speed was lower, the donor compartment was not depleted and PF were similar to that obtained with 1 mL donor samples. Due to the lower donor volume, their recovery was improved with EY=10-48% (RSD=7-56%). This d-EME device is thus applicable to small sample volumes, with a total depletion of moderately polar NPs at the expense of their enrichment. For the most polar NPs, recovery can be improved while maintaining their enrichment to an equivalent level.

Application to biological samples. The new d-EME device was eventually evaluated for the extraction of NPs from 250 µL of spiked CSF samples (n=3, Table 1). To ensure a similar charge state of the NPs compared to neat standards, the CSF was acidified with formic acid until a pH *ca*. 2-3 prior to d-EME. Process efficiency (PE) was calculated and ranged between 25-69% (RSD=3-7%), corresponding to a decrease of a factor 1.3 to 3.8 (compared to EY) for moderately polar NPs. As expected, PEs were found very low and variable (PE=0-6%, RSD=8-63%) for polar NPs, which already exhibited low EYs because of the lipophilic nature of the SLM used. PE corresponds to the absolute performance of the entire analytical process and is a combination of the matrix effect (i.e., MS signal alterations from interfering substances specific to the biological sample coeluting with the targeted analytes and affecting their ionization) and the extraction recovery (i.e., EY of d-EME when applied to the biological matrix). Both might have been affected by the increase in the sample ionic strength because of the high amount of formic acid added (1.5 M). Nevertheless, most of the NPs could be extracted from CSF and the developed d-EME set-up thus appears compatible with complex and scarce biofluids.

CONCLUSION

A dynamic-electromembrane extraction (d-EME) device, with a renewal system for the acceptor compartment and a small microporous polypropylene hollow fiber (HF) was developed for neuropeptides' extraction.

Several advantages were demonstrated: (i) high enrichment (preconcentration factor of up to 50-fold), (ii) high extraction yield (up to 72%), (iii) constant extraction speed, (iv) low and stable current (lower than 5 µA), (v) no bubble formation, (vi) low cost (< 2 € *per* device), and (vii) disposability (< 5 min *per* device to assemble all parts), making the device particularly suitable for applications involving biological fluids and carry-over issues.

This enhanced performance was afforded by the original design of the d-EME device: the thin wall thickness of the HF facilitates mass transfer, while its small i.d. allows for a low acceptor compartment volume (approximately 1.3 µL) that is favorable for enrichment. The renewal of the acceptor solution at a low flow rate (down to 280 nL/min) is also favorable for enrichment and recovery and circumvents the effects of electrolysis.

This device was eventually applied for the extraction of neuropeptides from cerebrospinal fluid to demonstrate the applicability of d-EME as a high-performance microextraction technique for low-volume and low-concentration biological fluids.

ASSOCIATED CONTENT

Supporting Information

The supporting Information is available free of charge at http://pubs.acs.org.

Tables S1 and S2 (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail:julie.schappler@unige.ch.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no conflict of interest.

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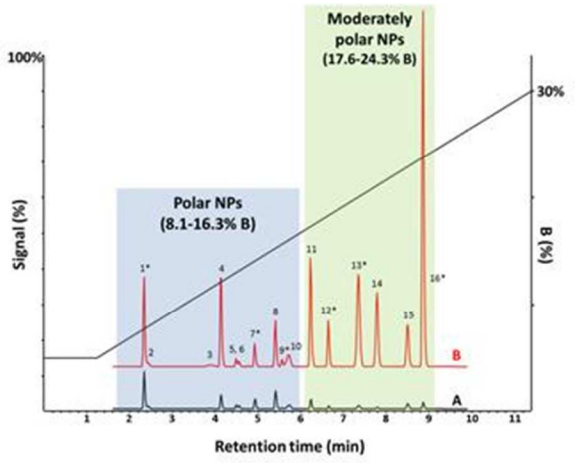
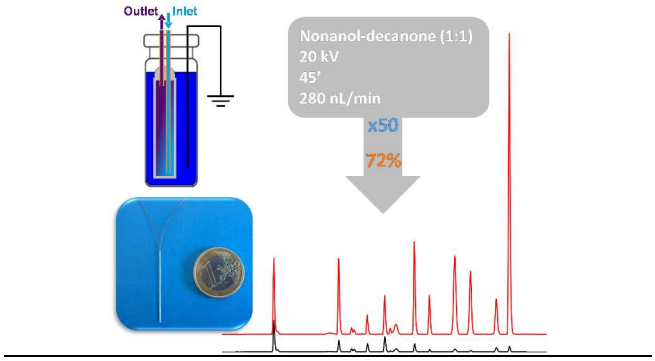


Figure 2. UHPLC-MS/MS chromatograms obtained for the 16 neuropeptides (NPs). A. Neat standard (spiked with each NP at 50 ng/mL) and B. Extracted standard (spiked with each NP at 50 ng/mL). See section 2.4 for experimental conditions. * NPs selected for presenting results.

Table 1. d-EME applied to 250 µL of neat standards spiked with each NP at 50 ng/mL (n=3) and CSF samples spiked with each NP at 50 ng/mL (n=3).

	Neat standards		CSF samples	
	PF (%RSD)	EY (%RSD)	PF (%RSD)	PE (%RSD)
DynA(1-7)	2 (56)	10 (56)	0 (63)	0 (63)
Des	5 (7)	31 (7)	1 (8)	5 (8)
Met	8 (16)	48 (16)	1 (19)	6 (19)
Leu	16 (1)	96 (1)	4 (7)	25 (7)
End2	17 (3)	103 (3)	7 (7)	47 (7)
End1-COOH	16 (5)	92 (5)	12 (3)	69 (3)

PF: preconcentration factor
EY: extraction yield
PE: process efficiency



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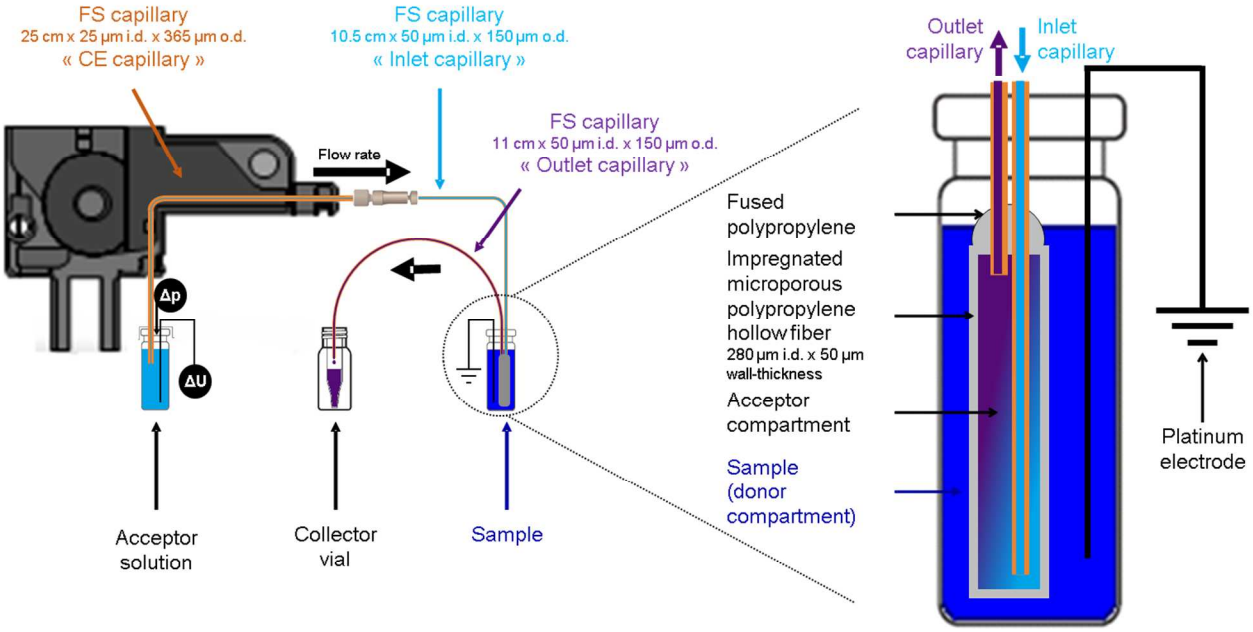

Figure 1. Schematic illustration of the new d-EME set-up.

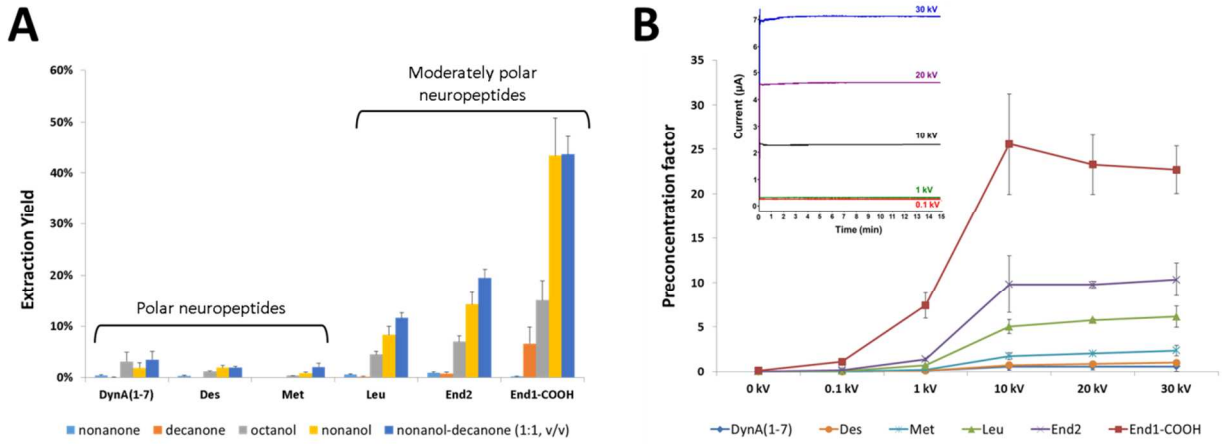

Figure 3. Effect of the experimental parameters on the extraction performance of six selected neuropeptides (NPs). A. SLM composition and B. applied voltage.

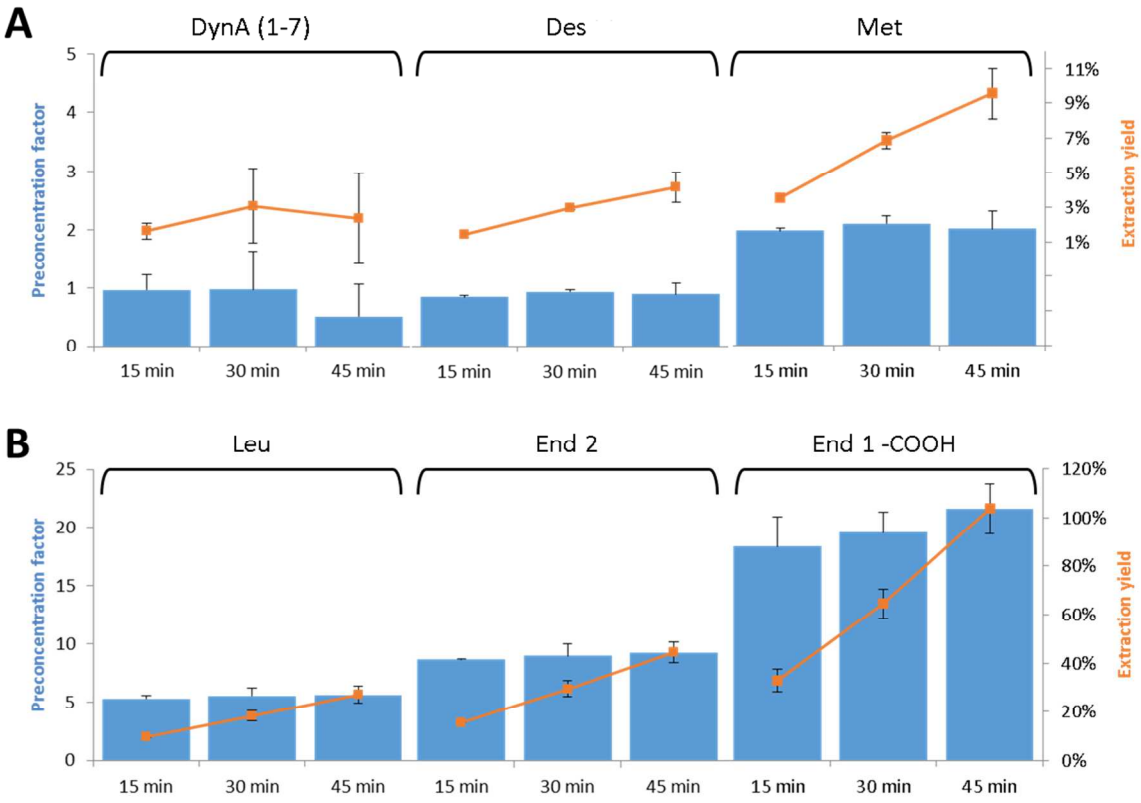

Figure 4. Effect of the extraction time on the extraction performance of six selected neuropeptides (NPs). A: polar NPs and B: moderately polar NPs.

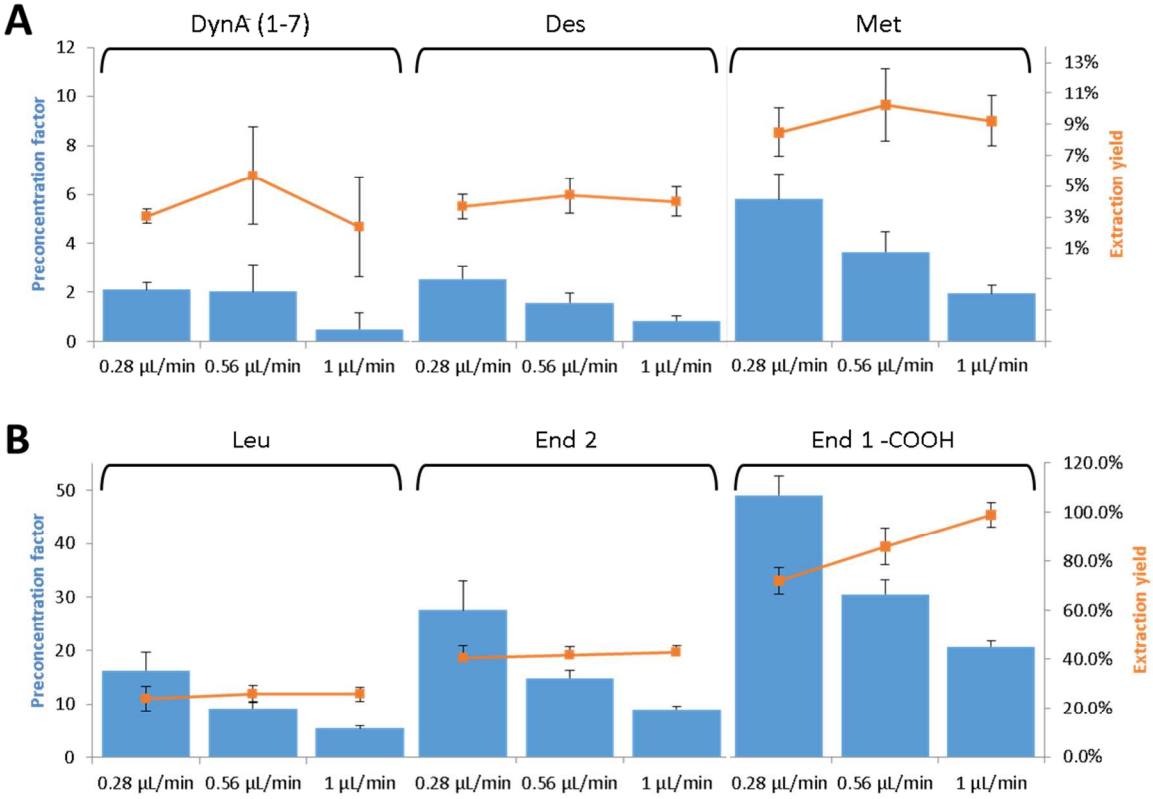

Figure 5 Effect of the acceptor solution flow rate on the extraction performance of six selected neuropeptides (NPs). A: polar NPs and B: moderately polar NPs.