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Electromembrane extraction of peptides based on hydrogen bond interactions

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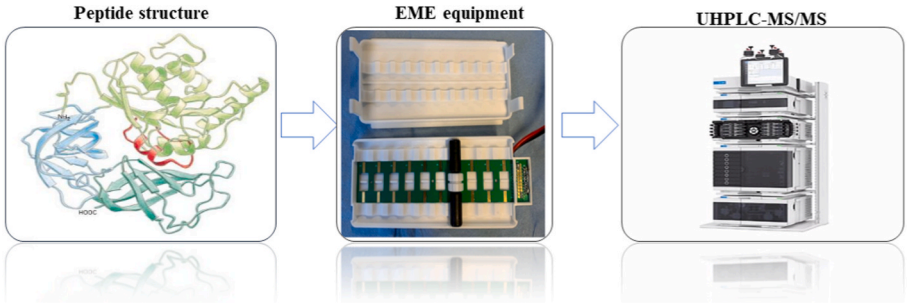
HIGHLIGHTS

- Tri(pentyl) phosphate was used as liquid membrane for electromembrane extraction of peptides for the first time.
- Mass transfer across the liquid membrane was facilitated by hydrogen bond interactions.
- High extraction recoveries were achieved for small peptides of low polarity.
- With tri(pentyl) phosphate, the current across the liquid membrane was low and the system was highly stable.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Electromembrane extraction (EME) of peptides reported in the scientific literature involve transfer of net positively charged peptides from an aqueous sample, through a liquid membrane, and into an aqueous acceptor solution, under the influence of an electrical field. The liquid membrane comprises an organic solvent, containing an ionic carrier. The purpose of the ionic carrier is to facilitate peptide solvation in the organic solvent based on ionic interactions. Unfortunately, ionic carriers increase the conductivity of the liquid membrane; the current in the system increases, the electrolysis in sample and acceptor is accelerated, and the extraction system tend to be unstable and suffers from drifting pH.

Results: In the present work, a broad selection of organic solvents were tested as pure liquid membrane for EME of peptides, without ionic carrier. Several phosphates provided high mass transfer, and tri(pentyl) phosphate was selected since this solvent also provided high operational stability. Among 16 different peptides used as model analytes, tri(pentyl) phosphate extracted those with net charge +1 and with no more than two polar side chains. Tri(pentyl) phosphate served as a very strong hydrogen bond acceptor, while the protonated peptides were hydrogen bond donors. By such, hydrogen bonding served as the primary interactions responsible for mass transfer. Tri(pentyl) phosphate as liquid membrane, could exhaustively extract leu-enkephalin, met-enkephalin, and endomorphin from human blood plasma and detected by LC-MS/MS. Calibration curves were linear (r^2 >

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0.99) within a concentration range from 1 to 500 ng/mL, and a relative standard deviation within 12% was observed for precision studies.
Significance: The current experiments are important because they indicate that small peptides of low polarity may be extracted selectively in EME based on hydrogen bond interactions, in systems not suffering from electrolysis.

1. Introduction

Electromembrane extraction (*EME*) is an extraction technique, where basic or acidic substances are extracted as ionic species from an aqueous sample solution, through a liquid membrane, and into a microliter volume of aqueous acceptor solution (*acceptor*). An external electrical field (*extraction potential*) sustained across the liquid membrane is used as driving force for the mass transfer, and controls the extraction efficiency and selectivity (Fig. 1) [1]. The liquid membrane is a microliter volume of organic solvent (*membrane solvent*), immobilized and held by capillary forces in the pores of a porous polymeric membrane (support *membrane*). For the extraction of bases, the sample solution and the acceptor are neutral or acidic, and the negatively charged electrode (*cathode*) is located in the acceptor. For the extraction of acids, the direction of the electrical field is reversed, and the sample solution and the acceptor are neutral or alkaline.

Since the first research paper on EME in 2006 [1], the interest for the concept has increased, and about 450 research papers have been published. Recently, commercial equipment for EME was launched [2,3], and this is expected to increase EME activity further. The interest of using EME and its future development may be justified in different ways. First, the consumption of chemicals and organic solvents are extremely low as compared to traditional sample preparation techniques. Therefore, EME represents an interesting green and sustainable alternative for the future. Second, acceptors are aqueous, and can be injected directly into instrumental separation and detection systems such as liquid chromatography-mass spectrometry and electrophoresis. Thus, the need for solvent evaporation and reconstitution prior to the instrumental analysis is eliminated. Third, the majority of matrix components in biological fluids, environmental waters, foods, and beverages, which are typical samples, are not transferred into the acceptor, thanks to the liquid membrane and the electrical field. For this reason, EME provides very efficient sample cleanup.

Beyond above justifications, two additional features of EME may be important in future analytical chemistry: namely selectivity and downscaling to microchip technology. The selectivity in EME is controlled by multiple parameters. The direction of the electrical field controls if the system is selective for negatively or positively charged compounds [4]. Furthermore, the selectivity is controlled by the magnitude of the electrical field [5], the chemical composition of the liquid membrane [6], and pH in the sample solution and acceptor [7]. Selectivity is thus a function of multiple parameters, although the fundamental understanding is currently still somewhat limited. To this end, EME has been implemented successfully in microchip systems; the concept is very well suited for downscaling [8–11], and combinations with smartphone detection show great potential [12,13].

A large number of EME applications have been published, and an overview may be obtained from recent reviews on the topic [14–16]. Applications include among others, extraction of pharmaceuticals from biological fluids [17–19], environmental pollutants [20,21], and contaminants from food and beverage samples [22–24]. Target analytes have mainly been organic bases or acids below 1000 Da, and small inorganic ions. Solvation in the liquid membrane is a critical factor to achieve efficient mass transfer of charged solutes in EME. Therefore, solute interactions with the liquid membrane play a key role, and they include hydrogen bond, cation-π, and ionic interactions [25]. The latter type of interactions is strong and requires addition of ion-pair reagent (carrier) to the membrane solvent.

In a limited number of papers, EME has been tested for extraction of peptides [26–36]. Recently this literature was reviewed [37]. EME of peptides reported up to date has been anchored in ionic interactions, and it has been accomplished using liquid membranes with ionic carrier. The purpose of the ionic carrier has been to facilitate the solvation of the peptides in the organic solvent, and by such increase their mass transfer across the liquid membrane. Unfortunately, ionic carriers increases the extraction current in the EME system, and this may cause excessive electrolysis in the sample and in the acceptor. Excessive electrolysis is undesirable because the pH may drift on both sides of the liquid membrane, and because gas bubbles are formed. Moreover, addition of ionic carrier may reduce the selectivity of the extraction system due to increased transfer of sample matrix components.

EME has potential for extraction of peptides, but the extraction systems should be stable and provide high selectivity. Therefore, extraction systems without ionic carrier should be investigated and this recall on fundamental research. The current paper conducted for the first time a systematic investigation of EME of peptides in the absence of ionic carrier. A large number of organic solvents, with different functional groups and polarity, were tested as pure liquid membranes to

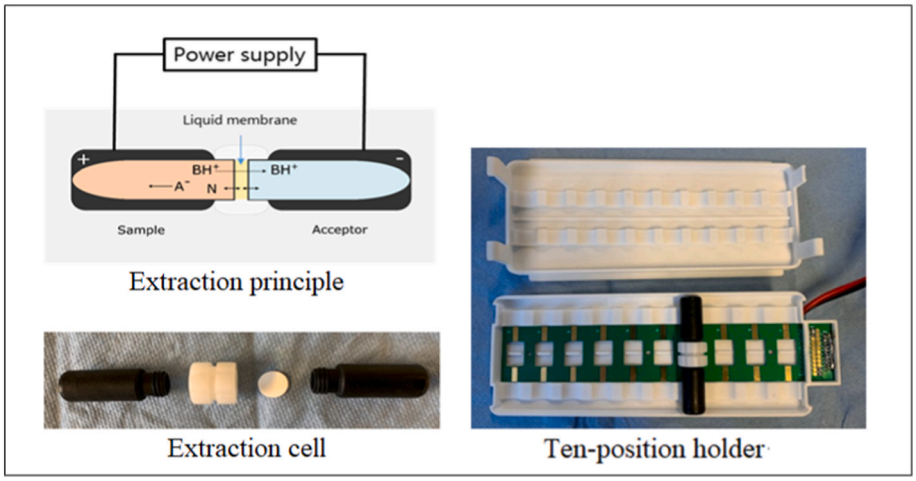

Fig. 1. Principle (for basic analytes) and photo of equipment for electromembrane extraction.

investigate the potential for peptides extraction primarily by hydrogen bond interactions. The research described is preliminary and fundamental.

2. Materials and methods

2.1. Chemical and reagents

Standards of the model peptides such as angiotensin I trifluoroacetate, angiotensin II acetate, angiotensin III, angiotensin IV, neurotensin acetate, neurotensin 1-6 trifluoroacetate, neurotensin 1-8, endomorphin trifluoroacetate, bradykinin acetate, (arg8)-vasopressin trifluoroacetate, oxytocin acetate, leu-enkephalin acetate, met-enkephalin acetate, diprotin A (Ile-Pro-Ile), diprotin B (Val-Pro-Leu), and Glu-Glu-Leu were all supplied by Bachem (Bubendorf, Switzerland). Formic acid, phosphoric acid, coumarin, thymol, di(2-ethylhexyl) phosphate, 1-undecanol, pentyl benzene, 2-nitrophenyl pentyl ether, 2-nitrophenyl octyl ether, dodecyl acetate, dihexyl ether, 2-nonanone, 2-undecanone, tris(2-butoxyethyl) phosphate, tri(butyl) phosphate, tri (pentyl) phosphate, tri(tolyl) phosphate, tris(2-ethylhexyl) phosphate, acetonitrile (LC-MS grade), and methanol (LC-MS grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions and working solutions of the peptides were prepared based on our previous study [35]. A Milipak® (0.22 μm filter) Milli-Q water purification system (Molsheim, France) was used to obtain deionized water. Circular polypropylene membranes with a thickness of 110 μm and a diameter of 9 mm were obtained from Extraction Technologies Norway (Ski, Norway).

2.2. Plasma samples

Human plasma was obtained from Oslo University Hospital (Oslo, Norway), and stored at −28 °C. Plasma was thawed rapidly at room temperature, then spiked with the model peptides to a concentration of 2.0 ng/mL, and finally diluted 1:1 with 50 mM phosphate-citrate buffer (pH = 3.0) to adjust pH.

2.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Analysis was carried out using an Agilent 1290 Infinity II UHPLC system (Agilent Technologies, Santa Clara, CA, USA), consisting of a binary pump, an auto-sampler, and a column compartment with controllable temperature. An Eclipse Plus C18 column (2.1 mm × 50 mm, 1.8 μm, Agilent Technologies) maintained at 40 °C was used for separation of the peptides. Gradient elution was performed using mobile phase A (95:5 v/v deionized water and acetonitrile containing 0.1% formic acid) and mobile phase B (5:95 v/v deionized water and acetonitrile containing 0.1% formic acid). The gradient was as follows; 3% B at 0.00 min, increased linearly to 5% B from 0.00 to 0.50 min, then increased linearly to 30% B from 0.50 to 2.50 min, then 100% B from 2.51 to 3.00 min, and finally to 3% B from 3.00 to 3.01 min. The flow rate was 0.6 mL min^{-1}. Detection was performed by an Agilent 6495 LC/TQ (Agilent technologies) mass spectrometer with positive electrospray ionization at 3 kV. The system was operated in dynamic MRM mode as previously reported [35]. Representative mass spectra and chromatogram after extraction from plasma is found in Fig. S1 in supplementary information.

2.4. EME equipment

In this study, a prototype device for conductive vial electromembrane extraction from Extraction Technologies Norway (Ski, Norway) was utilized to perform the EME experiments (Fig. 1). In this technical format of EME (*conducting vial EME*), vials were used to hold sample solutions and acceptors. The vials were produced in conducting polymer, and in this way they also served as electrodes. A circular porous polypropylene membrane (Extraction Technologies Norway) was used as support membrane, and this was placed in a tailor-made support *membrane union*. The latter served to assemble the entire *extraction cell*, where the sample vial, the liquid membrane, and the acceptor vial were connected. The extraction cell was placed in a *ten-position holder* where the conducting sample and acceptor vials became in contact with electrodes coupled to the external power supply. In such way, the vials served as electrodes, and ten positions were available for simultaneous extraction of multiple samples. The ten-position holder was mounted on a shaker, and by such arrangement the samples were agitated during extraction. The ten-position holder was electrically connected to a power supply Model ES 0300e0.45 from Delta Elektronika BV (Zierikzee, Netherlands). Extraction current was monitored with a Fluke 287 multi-meter (Everett, WA, USA).

2.5. EME procedure

First, 300 μL of sample solution and 300 μL of acceptor solution was filled into conductive vials. Second, the support membrane was placed in the support membrane union. Third, 8.0 μL of membrane solvent was pipetted onto the support membrane. Finally, the sample vial and the acceptor vial were connected to the support membrane union, and the entire extraction cell was established. The extraction cell was placed in the ten-position holder of the EME device; up to ten extraction cells were processed simultaneously. The sample vial was connected to the positive electrode (anode) while the acceptor vial was connected to the negative electrode (cathode). The extraction potential was set to 20 V and the agitation was set to 900 rpm. The acceptor was collected after 30 min of extraction and subjected to LC-MS/MS for its analysis. The extraction current was measured continuously during extraction.

2.6. Determination of Kamlet-Taft parameters

Kamlet-Taft solvatochromic parameters were determined experimentally according to previously published procedure [38].

2.7. Calculations

For each model peptide, recovery (R) was calculated by Equation (1):

$$R = \frac{n_a}{n_s} x100\% = \frac{V_a C_{a,final}}{V_s C_{s,initial}}\ x\ 100\% \quad (1)$$

Here, n_a and n_s denote the number of moles of peptide finally collected in the acceptor, and originally present in the sample solution, respectively. $C_{a,\ final}$ is the final concentration of peptide in the acceptor, and $C_{s,\ initial}$ is the original concentration of peptide in the sample solution. The terms V_a and V_s are the volumes of acceptor and sample solution, respectively.

Matrix effect (*ME%*) expressed the effect of ion suppression in the electrospray ionization process, and was calculated according to Equation (2):

$$ME\ (\%) = \frac{peak\ area\ of\ post - extraction\ spiked\ matrix}{peak\ area\ of\ unextracted\ standard}\ x\ 100\% \quad (2)$$

3. Results and discussion

In EME, charged solutes are extracted from aqueous sample solution, through a liquid membrane (organic solvent), and into aqueous acceptor solution under the influence of an electrical field. The solvation in the liquid membrane is considered a critical factor to achieve efficient mass transfer of charged solutes. Therefore, solute-(membrane) solvent interactions play a key role, and they include hydrogen bond, cation-π, and ionic interactions. The latter type of interactions is strong, and requires the addition of an ion-pair reagent (carrier) to the membrane solvent. EME of peptides reported up to date, has been anchored in ionic interactions, and has been accomplished using liquid membranes with ionic carrier [37].

In the experiments reported below, the potential of EME of peptides was investigated, based on hydrogen bond and π-type interactions only. Solvents with different functional groups and polarity were tested, using a mixture of 16 different peptides as model solutes (Table 1). The peptides ranged from three to 13 amino acids in size and were dissolved in 50 mM phosphate-citrate buffer pH 3.0 as sample solution. The acceptor was 50 mM phosphoric acid (pH 1.8), and due to the acidic conditions, the peptides were extracted as net cationic species.

3.1. Initial experiments

In a first set of experiments, pentyl benzene was tested as liquid membrane with an aromatic ring as the only functional group. The intention of this was to study the isolated effect of π-type interactions on mass transfer. With this liquid membrane, however, none of the model peptides were detected in the acceptor after EME. The sample solution was analyzed after EME, and the model peptides were found in the sample solution. Thus, although pentyl benzene theoretically can interact with the peptides by different π-type interactions, including cation-π, π-π, and polar-π interactions, these were not sufficiently strong to facilitate mass transfer.

In a next set of experiments, solvents with hydrogen bond acceptor (HBA) and donor (HBD) properties were tested. The intention of this was to study the effect of hydrogen bond interactions on mass transfer. The HBA solvents included organic phosphates, ketones, ethers, esters, and nitro aromatics, while the HBD solvents were higher alcohols. All the solvents are summarized in Table 2, along with selected physiochemical properties obtained computationally (chemicalize.com). Kamlet-Taft values were measured experimentally for all the solvents as described in the experimental section.

A couple of the phosphates, including tri(pentyl) phosphate, tris(2-butoxyethyl) phosphate, and tri(butyl) phosphate extracted several of the model peptides with net charge +1. As shown in Table 2, those phosphates provided strong hydrogen bond basicity, with β-values between 0.80 and 0.88, respectively, while hydrogen bond acidity was zero (α = 0.0). Ketones, ethers, esters, and nitro aromatics were not working as liquid membrane for the model peptides, and most probably, with these membrane solvents hydrogen bond acceptor properties were not sufficiently strong. Higher alcohols were also tested, but in spite of their strong hydrogen bond basicity, these were inefficient for extraction of peptides. Most probably, this was due to their duality as hydrogen bond donor and acceptor. During EME, the most efficient liquid membranes served as hydrogen bond acceptor, while the protonated peptides were hydrogen bond donors. In this way, several of the model peptides were solvated in the liquid membrane and transferred to the acceptor.

In total, five different phosphates were tested, with high β-values and with log P values in the range from 3.94 to 9.18. The extraction efficiency decreased with increasing log P of the membrane solvent. Tris (2-butoxyethyl) phosphate (log P = 3.94) was the most efficient liquid membrane, and extracted six of the model peptides with recovery exceeding 40%. High mass transfer with this particular solvent was attributed to strong hydrogen bond basicity from multiple HBA sites, and due to relatively low log P. Unfortunately, the liquid membrane was unstable. When operated at 3 V, which is a very low extraction potential in EME, the current was initially about 40 μA, but increased during extraction. This indicated that membrane solvent leaked to the sample solution and acceptor, and that the liquid membrane became saturated with traces of water. Electrolysis thus increased during the extraction process, and the entire system became unstable due to H_2/O_2 bubble formation and drifting pH.

With tri(butyl) phosphate (log P = 4.09) as liquid membrane, recoveries were slightly lower than with tris(2-butoxyethyl) phosphate, five peptides were extracted with recovery exceeding 40%, but the EME system was more stable and was operated at 30 V. However, tri(butyl) phosphate is considered carcinogenic, and was therefore not used for further experiments. With tri(pentyl) phosphate (log P = 5.42) the extraction potential was kept at 30 V, and the liquid membrane was robust and stable (section 3.2.1). Tri(pentyl) phosphate was less efficient due to high log P, but still met-enkephalin, leu-enkephalin, and endomorphin were extracted exhaustively (90% recoveries). These peptides are four or five amino acids in size, with no polar/acidic/basic side chains, and with net charge +1. In addition, Glu-Glu-Leu, Val-Pro-Leu, and Ile-Pro-Ile were extracted with recoveries of 30, 15, and 30%, respectively. These peptides are three amino acids in size, and with net charge +1. Glu-Glu-Leu contain two acidic side chains, while the two other tripeptides contain no polar/acidic/basic side chains. The remaining ten peptides were not extracted with tri(pentyl) phosphate. They were all with log P < −5 or net charge > +1. Mass transfer tended to increase with increasing molecular size and with decreasing number of polar/acidic/basic side chains. Peptides with net charge > +1 and with > 2–3 polar/acidic/basic side chains were effectively discriminated by the liquid membrane.

Tri(tolyl) and tris(2-ethylhexyl) phosphate were also tested, but none of the model peptides were extracted with these liquid membranes due to high log P and lower β-value, respectively.

3.2. Optimization of operational parameters

Based on the discussion above, tri(pentyl) phosphate was selected as liquid membrane for further experiments. To ensure compatibility with biological fluids, the following optimization experiments were carried out from diluted human plasma samples (section 2.2): the effect of extraction potential, pH of sample solution and acceptor, and extraction time.

Table 1
Model peptides and selected physicochemical data.

	Amino acids	Sequence	Isoelectric point	Acidic residues	Basic residues	Net-charge pH 3.0	log P
Glu-Glu-Leu	3	EEL	3.59	1	1	+1	−3.71
Neurotensin 1-8	8	pE-LYENKPR	6.71	1	2	+2	−8.57
Vasopressin	9	CYFQNCPRG	10.00	0	1	+2	−7.25
Neurotensin 1-6	6	pE-LYENK	3.62	1	1	+1	−5.54
Val-Pro-Leu	3	VPL	6.05	0	0	+1	−1.50
Bradykinin	9	RPPGFSPFR	10.88	0	2	+3	−6.36
Ile-Pro-Ile	3	IPI	6.05	0	0	+1	−0.98
Angiotensin III	7	RVYIHPF	8.48	0	2	+3	−1.82
Angiotensin II	8	DRVYIHPF	7.45	1	2	+3	−5.27
Met-enkephalin	5	YGGFM	5.82	0	0	+1	−2.47
Angiotensin IV	6	VYIHPF	7.45	0	1	+4	−0.69
Oxytocin	9	CYIQNCPLG	8.57	0	0	+1	−5.00
Neurotensin	13	pE-LYENKPRRPYIL	9.24	1	3	+3	−8.82
Angiotensin I	10	DRVYIHPFHL	7.66	1	3	+4	−5.95
Leu-enkephalin	5	YGGFL	5.86	0	0	+1	−1.86
Endomorphin	4	YPWF	8.61	0	0	+1	1.90

Table 2
Membrane solvents, physicochemical properties, and suitability for peptide extraction.

Solvent	Peptides with R>40%	log P	Water solubility	HBAs	HBDs	Aromatic rings	α	β	π*
Pentyl benzene	0	4.26	0.016	0	0	1	0.23	0.12	0.42
2-Nitrophenyl pentyl ether	0	3.52	0.024	3	0	1	0.00	-[a]	0.82
2-Nitrophenyl octyl ether	0	4.86	0.0008	3	0	1	0.00	-[a]	0.81
Dodecyl acetate	0	4.80	0.0016	1	0	0	0.29	0.58	0.40
Dihexyl ether	0	4.55	0.019	1	0	0	0.00	0.50	0.22
2-Nonanone	0	3.03	0.26	1	0	0	0.35	0.61	0.63
2-Undecanone	0	3.92	0.027	1	0	0	0.34	0.61	0.61
1-Octanol	0	2.58	0.54	1	1	0	0.69	0.82	0.55
1-Nonanol	0	3.03	0.17	1	1	0	0.66	0.79	0.55
1-Undecanol	0	3.91	0.018	1	1	0	0.72	0.81	0.51
Tris(2-butoxyethyl) phosphate	6	3.94	0.053	4	0	0	0.00[b]	0.83	0.69
Tri(butyl) phosphate	5	4.09	0.031	1	0	0	0.00[b]	0.88	0.65
Tri(pentyl) phosphate	3	5.42	0.0011	1	0	0	0.00[b]	0.80	0.63
Tri(tolyl) phosphate	0	6.63	0	1	0	3	0.00[b]	0.66	0.79
Tris(2-ethylhexyl) phosphate	0	9.18	0	1	0	0	0.00[b]	0.84	0.57

[a] Exact values could not be determined due to high background absorbance of the solvents (α and π* from literature have been included [38,40]).
[b] α value for organic phosphates could not be determined correctly due to polar interactions to some extent (α from literature has been included [41]).

3.2.1. Extraction potential

In a first set of optimization experiments, the extraction potential was studied in the range 5–40 V. Extraction was conducted from 300 μL of plasma at pH = 3.0, and into 300 μL 50 mM phosphoric acid as acceptor. The liquid membrane was 8 μL of tri(pentyl) phosphate, and extractions were performed for 30 min. Recoveries are presented in Fig. 2. As can be seen, met-enkephalin (five amino acids), leu-enkephalin (five amino acids), and endomorphin (four amino acids) were extracted when the extraction potential was 10 V, and recoveries increased when the extraction potential was increased from 10 to 25 V. Increased recovery with increasing voltage is generally observed in EME and is in accordance with theory [14–16]. At 25 V, also Glu-Glu-Leu, Val-Pro-Leu, and Ile-Pro-Ile (all peptides with three amino acids) were also extracted, but recoveries were in the range 5–10%.

When the extraction potential was increased above 25 V, the recoveries started to decrease. This was attributed to electrolysis and drifting pH in the acceptor. Therefore, 20 V was selected as the optimal extraction potential. This was a compromise between high recovery and low current. With 20 V, the current was acceptable as shown in Fig. 3, where measured total current for three extraction cells coupled in parallel, was plotted as function of time. The extraction potential was ramped initially to avoid excessive peak current; 0 V from 0.0 to 1.0 min, 5 V from 1.0 to 2.0 min, and then 25 V. In such way, the current never exceeded 50 μA, and current was not increasing during extraction.

3.2.2. pH

In a next series of experiments, pH in the sample solution and in the acceptor was optimized. The pH of the sample solution was tested within the range 2.0–4.5, using 50 mM phosphoric acid as acceptor. Data are summarized in Fig. S2 in supporting information. Generally, recoveries were highest around pH 3.0, and decreased at higher pH because the positive ionization of the peptides decreased (except for endomorphin). Recoveries also decreased from pH 3.0 to 2.0. This was considered as an ion balance effect. The ion balance is defined as the ratio of the total amount of ions in the sample and acceptor, respectively, and low ion balance may favor EME under conditions were mass transfer into the liquid membrane is limited [39]. A pH of 3.0 was selected for the remaining experiments. In a following set of experiments, four different concentrations of phosphoric acid were tested (10, 25, 50, and 100 mM) as acceptor where pH ranged from 2.6 to 1.6, respectively. Data are summarized in Fig. S2 in supporting information. Recoveries increased by approximately 8% with increasing molarity up to 50 mM phosphoric acid, and this acceptor was selected for the remaining experiments. The latter observation was attributed to the pH boundary layer at the acceptor-liquid membrane interface [7]. With the negative electrode in the acceptor, pH in the acceptor is higher close to the liquid membrane than in the bulk solution, due to local charge accumulation. Thus, upon transfer from the liquid membrane and into acceptor, the peptides were exposed to somewhat higher pH in a thin layer as compared to the rest of the acceptor. By increasing the molarity of phosphoric acid, the negative of the pH boundary layer was reduced.

3.2.3. Extraction time

In a final set of optimization experiments, recoveries were

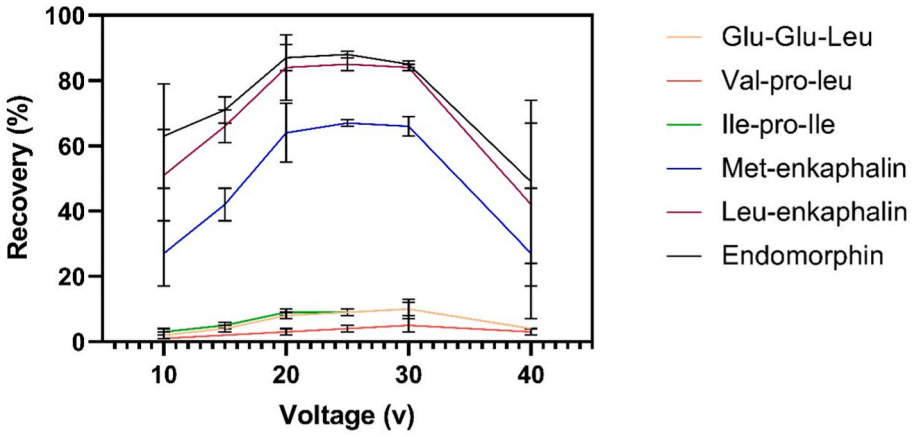

Fig. 2. Extraction recovery as function of extraction potential (donor phase: 300 μL of plasma at pH = 3.0, acceptor phase: 300 μL 50 mM phosphoric acid, liquid membrane: 8 μL of tri(pentyl) phosphate, and extraction time: 30 min).

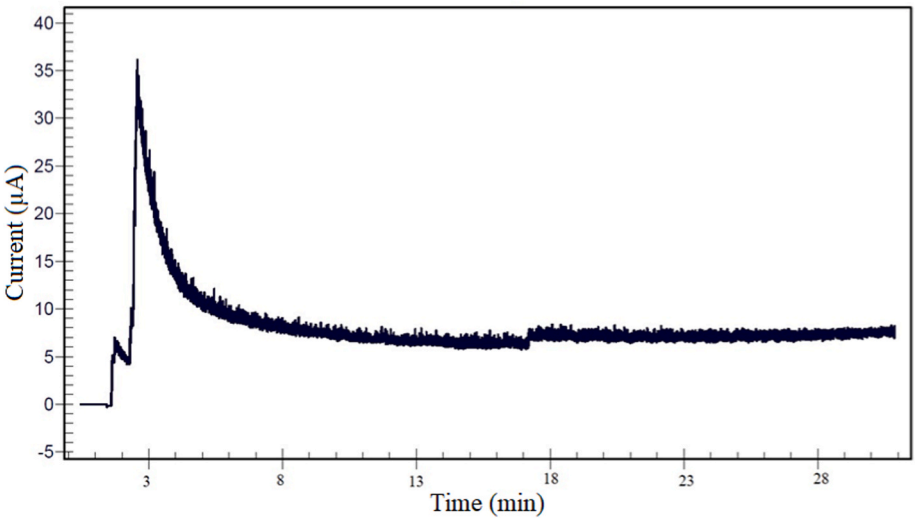

Fig. 3. Extraction current for three extraction cells (three samples simultaneously) coupled in parallel, as function of time (donor phase: 300 μL of plasma at pH = 3.0, acceptor phase: 300 μL 50 mM phosphoric acid, liquid membrane: 8 μL of tri(pentyl) phosphate, extraction time: 30 min, and extraction potential: 20 V).

investigated as function of time. As observed in Fig. 4, recoveries increased with increasing extraction time. This is generally observed in EME, and is in accordance with theory [14–16]. Exhaustive extraction was achieved after 30–45 min for met-enkephalin, leu-enkephalin and endomorphin. For Glu-Glu-Leu, Val-Pro-Leu, and Ile-Pro-Ile, kinetics were much slower and recoveries increased continuously up to 120 min. As discussed above, the latter observation may be related to the number of HBA sites, or to the van der Waals volume. Both parameters are expected to be favored with increasing molecular size of the peptides.

3.3. Validation

The optimized EME procedure was then evaluated for quantification of the model peptides in human plasma. The data are only shown for leu-enkephalin, met-enkephalin, and endomorphin, which have been exhaustively extracted. The method evaluation included the limits of detection, the limits of quantification, intra-day and inter-day precisions, extraction recoveries, linearity, matrix effects, accuracy and stability in the acceptor solution. No internal standards were used. The results are summarized in Table 3.

The extraction recoveries were consistent for intra-day and inter-day experiments, and ranged between 59 and 86%. The calibration curves were linear in the range 1–500 ng/mL with correlation coefficients, R^2 values exceeding 0.9985. The limits of detection (S/N = 3) and

Table 3
The analytical characteristics of the method from human plasma.

		Met-enkephalin	Leu-enkephalin	Endomorphin
LDR[a] (ng/mL)		1–500	1–500	1–500
R^2		0.9985	0.9998	0.9998
LOQ[b] (ng/mL)		0.90	0.10	0.28
LOD[c] (ng/mL)		0.27	0.03	0.08
Accuracy (Error %)		2	2	8
Spiked-level	25 (ng/mL)	13	17	15
	75 (ng/mL)			
Repeatability[d]		12	6	3
Spiked-level	25 (ng/mL)	10	5	4
	75 (ng/mL)			
Reproducibility[d]		10	6	6
Spiked-level	25 (ng/mL)	8	4	5
	75 (ng/mL)			
Recovery (%)		59	80	85

[a] Linear dynamic range.
[b] Signal-to-noise ratio 10.
[c] Signal-to-noise ratio 3.
[d] RSD %, n = 3.

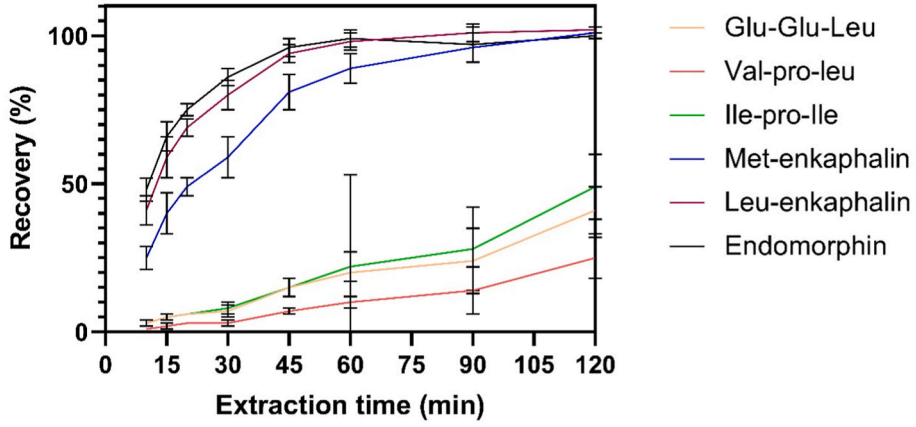

Fig. 4. Extraction recovery as function of extraction time (donor phase: 300 μL of plasma at pH = 3.0, acceptor phase: 300 μL 50 mM phosphoric acid, liquid membrane: 8 μL of tri(pentyl) phosphate, and extraction potential: 20 V).

quantification (S/N = 10) were in the ranges of 0.03–0.27 and 0.10–0.90 ng/mL, respectively. Based on three replicates, the RSD values were between 3% and 12% for intra-day experiments, and between 6% and 10% for the inter-day experiments (Table 3). Accuracy data were obtained at the 25 and 75 ng/mL level, and ranged between 2 and 17%.

Plasma and urine contain multiple components that may cause ion suppression or enhancement in LC-MS/MS analysis. In this context, the matrix effect was calculated according to Equation (2) (section 2.5) and data are shown in Table S1 in supplementary information. For all extracted peptides, the ME (%) values ranged between 105 and 108%. Due to the selectivity of the EME system, the extracted peptides were detected without ion suppression. This support that quantification can be performed without interference from other compounds extracted into the acceptor.

The stability of the peptides in acceptor (1.0 ng/mL) was also investigated. Pure acceptor was spiked with the peptides, and the solution was injected into LC-MS/MS after 1 min, 4 h, 8 h, 12 h, and 24 h. Based on the data, all the 16 model peptides were stable in the acceptor.

3.4. Comparison with system based on DEHP

The performance of the present liquid membrane was finally compared with an alternative liquid membrane based on deep eutectic solvent (coumarin and thymol (1:2) + 2% (v/v) di(2-ethylhexyl) phosphate (DEHP)) [35]. With both liquid membranes, the sample solution was 300 μL diluted plasma (pH = 3.0) spiked with the model peptides 1.0 ng/mL). 300 μL of 50 mM phosphoric acid solution was used as acceptor in both cases. Since the volumes of sample and acceptor were equal, there was no enrichment. Extractions were conducted at 20 V for 15 min. The results are summarized in Table 4. As illustrated, recoveries with the deep eutectic liquid membrane ranged from 21 to 52%, with RSDs below 18%. This membrane extracted based on ionic and hydrogen bond interactions, and the majority of model peptides were extracted. The current liquid membrane principally extracted based on hydrogen bond interactions, and for this reason it was more selective, and extracted only model peptides with net charge +1, and with no more than two polar/acidic/basic side chains. EME of leu-enkephalin, met-enkephalin, and endomorphin have been extracted previously in different laboratory-built equipment, and results are summarized and compared with the current data obtained with commercial equipment in Table S2 in supporting information.

4. Conclusion

The present work has for the first time, demonstrated EME of peptides without ionic carrier in the liquid membrane. With pure tri(pentyl) phosphate (or closely related phosphates) as membrane solvent, several peptides were extracted exhaustively (≥85%) based on hydrogen bond interactions. Tri(pentyl) phosphate served as a very strong hydrogen bond acceptor, with proper log P, while the protonated peptides were hydrogen bond donors. In such way, small and relatively hydrophobic peptides were extracted from plasma. Because EME was conducted without ionic carrier, the extraction current was low, and the EME system was highly stable. In addition, EME with tri(pentyl) phosphate provided higher selectivity than previous systems published in the literature.

EME of peptides are still in early phase, and the current fundamental research is an important step forward in terms of understanding and applicability. The work is fundamental and should not be considered as a finished analytical method. However, the procedure has potential as a green method for quantification of peptides in plasma, in combination with LC-MS/MS. In such cases, an internal standard is recommended to correct for variations due to the liquid handling and pipetting, which are considered to be the major error sources of the current procedure.

Table 4
Comparison of the present EME method with DEHP based method for extraction of the peptides.

	present study		DEHP based method	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Glu-Glu-Leu	–	–	–	–
Neurotensin 1-8	–	–	–	–
Vasopressin	–	–	–	–
Neurotensin 1-6	–	–	–	–
Val-Pro-Leu	–	–	32	3
Bradykinin	–	–	47	1
Ile-Pro-Ile	–	–	45	1
Angiotensin III	–	–	44	3
Angiotensin II	–	–	21	5
Met-enkephalin	40	17	23	4
Angiotensin IV	–	–	52	2
Oxytocin	–	–	–	3
Neurotensin	–	–	26	2
Angiotensin I			35	5
Leu-enkephalin	59	11	36	3
Endomorphin	66	8	46	18

Extraction conditions; donor phase: 300 μL of plasma at pH = 3.0, acceptor phase: 300 μL 50 mM phosphoric acid, extraction time: 15 min, and extraction potential: 20 V.

CRediT authorship contribution statement

Samira Dowlatshah: Formal analysis, Validation, Writing – original draft, Methodology, Writing – review & editing, and, Conceptualization. **Frederik André Hansen:** Writing – review & editing, Supervision. **Chen Zhou:** Formal analysis, Writing – review & editing. **María Ramos-Payán:** Writing – review & editing. **Trine Grønhaug Halvorsen:** Writing – review & editing, Supervision. **Stig Pedersen-Bjergaard:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.341610.

References

[1] S. Pedersen-Bjergaard, K.E. Rasmussen, Electrokinetic migration across artificial liquid membranes: new concept for rapid sample preparation of biological fluids, J. Chromatogr., A 1109 (2) (2006) 183–190.
[2] M. Drobnjak, F. Hansen, E.L. Øiestad, T. Løvli, R. Trones, Ø.G. Martinsen, S. Pedersen-Bjergaard, Electromembrane extraction with vials of conducting polymer, LCGC North Am. 38 (8) (2020) 435–439.
[3] T.G. Skaalvik, E.L. Øiestad, R. Trones, S. Pedersen-Bjergaard, S. Hegstad, Determination of psychoactive drugs in serum using conductive vial electromembrane extraction combined with UHPLC-MS/MS, J. Chromatogr., B 1183 (2021), 122926.

[4] E. Santigosa-Murillo, S. Maspoch, M. Muñoz, M. Ramos-Payán, An efficient microfluidic device based on electromembrane extraction for the simultaneous extraction of acidic and basic drugs, Anal. Chim. Acta 1160 (2021), 338448.
[5] N.C. Dominguez, A. Gjelstad, A.M. Nadal, H. Jensen, N.J. Petersen, S.H. Hansen, K. E. Rasmussen, S. Pedersen-Bjergaard, Selective electromembrane extraction at low voltages based on analyte polarity and charge, J. Chromatogr. A. 1248 (2012) 48–54.
[6] C.X. Huang, A. Gjelstad, S. Pedersen-Bjergaard, Organic solvents in electromembrane extraction: recent insights, Rev. Anal. Chem. 35 (4) (2016) 169–183.
[7] M.S. Restan, H. Jensen, X.T. Shen, C.X. Huang, O.G. Martinsen, P. Kuban, A. Gjelstad, S. Pedersen-Bjergaard, Comprehensive study of buffer systems and local pH effects in electromembrane extraction, Anal. Chim. Acta 984 (2017) 116–123.
[8] M. Ramos Payán, E. Santigosa, R. Fernández Torres, M.Á. Bello López, A new microchip design. A versatile combination of electromembrane extraction and liquid-phase microextraction in a single chip device, Anal. Chem. 90 (17) (2018) 10417–10424.
[9] S. Seidi, M.H. Ranjbar, M. Baharfar, M. Shanehsaz, M. Tajik, A promising design of microfluidic electromembrane extraction coupled with sensitive colorimetric detection for colorless compounds based on quantum dots fluorescence, Talanta 194 (2019) 298–307.
[10] F. Zarghampour, Y. Yamini, M. Baharfar, G. Javadian, M. Faraji, On-chip electromembrane extraction followed by sensitive digital image-based colorimetry for determination of trace amounts of Cr(vi), Anal. Methods. 12 (4) (2020) 483–490.
[11] F.A. Hansen, N.J. Petersen, J.P. Kutter, S. Pedersen-Bjergaard, Electromembrane extraction in microfluidic formats, J. Separ. Sci. 45 (1) (2022) 246–257.
[12] Y. Ma, Y. Luo, X. Feng, C. Huang, X. Shen, Smartphone-controlled biosensor for viral respiratory infectious diseases: screening and response, Talanta 254 (2023), 124167.
[13] F. Zarghampour, Y. Yamini, E. Alipanahpour Dil, A. Shokrollahi, G. Javadian, A new microfluidic-chip device followed by sensitive image analysis of smart phone for simultaneous determination of dyes with different acidic–basic properties, Talanta 254 (2023).
[14] Q. Shang, H. Mei, C. Huang, X. Shen, Fundamentals, operations and applications of electromembrane extraction: an overview of reviews, Microchem. J. 181 (2022), 107751.
[15] L.V. Eie, S. Pedersen-Bjergaard, F.A. Hansen, Electromembrane extraction of polar substances – status and perspectives, J. Pharm. Biomed. Anal. 207 (2022), 114407.
[16] N. Drouin, P. Kubáň, S. Rudaz, S. Pedersen-Bjergaard, J. Schappler, Electromembrane extraction: overview of the last decade, Trends Anal. Chem. 113 (2019) 357–363.
[17] A. Šlampová, P. Kubáň, Micro-electromembrane extraction through volatile free liquid membrane for the determination of β-lactam antibiotics in biological and environmental samples, Talanta 252 (2023).
[18] H. Rahbarian, S. Nojavan, M. Maghsoudi, H. Tabani, In-tube gel electromembrane extraction: a green strategy for the extraction of narcotic drugs from biological samples, J. Chromatogr., A 1688 (2023).
[19] R. Hu, Y. Yan, L. Jiang, C. Huang, X. Shen, Determination of total cathinones with a single molecularly imprinted fluorescent sensor assisted by electromembrane microextraction, Microchim. Acta 189 (9) (2022) 324.
[20] G.L. de Araújo, G.F. dos Santos, R.O. Martins, G. da Silva Lima, I. Medeiros, R.M. de Carvalho, R.C. Simas, L.F. Sgobbi, A.R. Chaves, B.G. Vaz, Electromembrane extraction of naphthenic acids in produced water followed by ultra-high-resolution mass spectrometry analysis, J. Am. Soc. Mass Spectrom. 33 (8) (2022) 1510–1517.
[21] S. Yousefi, S. Makarem, W. Alahmad, F.D. Zare, H. Tabani, Evaluation of complexing agents in the gel electro-membrane extraction: an efficient approach for the quantification of zinc (II) ions in water samples, Talanta 238 (2022), 123031.
[22] A. Sahragard, W. Alahmad, P. Varanusupakul, Application of electrocolorimetric extraction for the determination of Ni(II) ions in chocolate samples: a green methodology for food analysis, Food Chem. 382 (2022), 132344.
[23] L. Wan, H. Gao, H. Gao, R. Du, F. Wang, Y. Wang, M. Chen, Selective extraction and determination of steroidal glycoalkaloids in potato tissues by electromembrane extraction combined with LC-MS/MS, Food Chem. 367 (2022).
[24] C. Román-Hidalgo, G. López-Pérez, M. Villar-Navarro, M.J. Martín-Valero, Green electromembrane extraction procedure based on biodegradable chitosan films for determination of polyphenolic compounds in food samples: greenness assessment of the sample preparation approach, Talanta 253 (2023).
[25] F.A. Hansen, S. Pedersen-Bjergaard, Electromembrane extraction – looking closer into the liquid membrane, Adv. Sample Prepar. (2022), 100020.
[26] M. Balchen, L. Reubsaet, S. Pedersen-Bjergaard, Electromembrane extraction of peptides, J. Chromatogr., A 1194 (2) (2008) 143–149.
[27] M. Balchen, T.G. Halvorsen, L. Reubsaet, S. Pedersen-Bjergaard, Rapid isolation of angiotensin peptides from plasma by electromembrane extraction, J. Chromatogr., A 1216 (41) (2009) 6900–6905.
[28] M. Balchen, H. Jensen, L. Reubsaet, S. Pedersen-Bjergaard, Potential-driven peptide extractions across supported liquid membranes: investigation of principal operational parameters, J. Separ. Sci. 33 (11) (2010) 1665–1672.
[29] M. Balchen, A.G. Hatterud, L. Reubsaet, S. Pedersen-Bjergaard, Fundamental studies on the electrokinetic transfer of net cationic peptides across supported liquid membranes, J. Separ. Sci. 34 (2) (2011) 186–195.
[30] M. Balchen, H. Lund, L. Reubsaet, S. Pedersen-Bjergaard, Fast, selective, and sensitive analysis of low-abundance peptides in human plasma by electromembrane extraction, Anal. Chim. Acta 716 (2012) 16–23.
[31] N. Drouin, S. Rudaz, J. Schappler, Dynamic-electromembrane extraction: a technical development for the extraction of neuropeptides, Anal. Chem. 88 (10) (2016) 5308–5315.
[32] C.X. Huang, A. Gjelstad, S. Pedersen-Bjergaard, Exhaustive extraction of peptides by electromembrane extraction, Anal. Chim. Acta 853 (2015) 328–334.
[33] C.X. Huang, A. Gjelstad, S. Pedersen-Bjergaard, Selective electromembrane extraction based on isoelectric point: fundamental studies with angiotensin II antipeptide as model analyte, J. Membr. Sci. 481 (2015) 115–123.
[34] A. Pourahadi, S. Nojavan, S.S. Hosseiny Davarani, Gel-electromembrane extraction of peptides: determination of five hypothalamic agents in human plasma samples, Talanta 217 (2020), 121025.
[35] T.K. Rye, G. Martinovic, L.V. Eie, F.A. Hansen, T.G. Halvorsen, S. Pedersen-Bjergaard, Electromembrane extraction of peptides using deep eutectic solvents as liquid membrane, Anal. Chim. Acta 1175 (2021), 338717.
[36] K.F. Seip, J. Stigsson, A. Gjelstad, M. Balchen, S. Pedersen-Bjergaard, Electromembrane extraction of peptides – fundamental studies on the supported liquid membrane, J. Separ. Sci. 34 (23) (2011) 3410–3417.
[37] L.V. Eie, T.K. Rye, F. Hansen, T.G. Halvorsen, S. Pedersen-Bjergaard, Electromembrane extraction of peptides and amino acids – status and perspectives, Bioanalysis 13 (4) (2021) 277–289.
[38] F.A. Hansen, E. Santigosa-Murillo, M. Ramos-Payán, M. Muñoz, E. Leere Øiestad, S. Pedersen-Bjergaard, Electromembrane extraction using deep eutectic solvents as the liquid membrane, Anal. Chim. Acta 1143 (2021) 109–116.
[39] A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, Simulation of flux during electro-membrane extraction based on the Nernst–Planck equation, J. Chromatogr., A 1174 (2007) 104–111.
[40] L. Sun, K. Jiao, S.G. Weber, Charge transport through osmium-containing redox polymers in nitrophenyl-based solvents: effect of solvent size, J. Phys. Chem. B 102 (11) (1998) 1945–1950.
[41] Y. Marcus, The use of chemical probes for the characterization of solvent mixtures. Part 2. Aqueous mixtures, J. Chem. Soc., Perkin Trans. 2 (8) (1994) 1751–1758.