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Electromembrane extraction of peptides

Marte Balchen, Léon Reubsaet, Stig Pedersen-Bjergaard*

School of Pharmacy, University of Oslo, P.O. Box 1068 Blindern, 0316 Oslo, Norway

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ABSTRACT

Rapid extraction of eight different peptides using electromembrane extraction (EME) was demonstrated for the first time. During an extraction time of 5 min, the model peptides migrated from a 500 μL aqueous acidic sample solution, through a thin supported liquid membrane (SLM) of an organic liquid sustained in the pores in the wall of a porous hollow fiber, and into a 25 μL aqueous acidic acceptor solution present inside the lumen of the hollow fiber. The driving force of the extraction was a 50 V potential sustained across the SLM, with the positive electrode in the sample and the negative electrode in the acceptor solution. The nature and the composition of the SLM were highly important for the EME process, and a mixture of 1-octanol and 15% di(2-ethylhexyl) phosphate was found to work properly. Using 1 mM HCl as background electrolyte in the sample and 100 mM HCl in the acceptor solution, and agitation at 1050 rpm, enrichment up to 11 times was achieved. Recoveries were found to be dependent on the structure of the peptide, indicating that the polarity and the number of ionized groups were important parameters affecting the extraction efficiency. The experimental findings suggested that electromembrane extraction of peptides is possible and may be a valuable tool for future extraction of peptides.

1. Introduction

Twenty years ago, the first application of LLE with supported liquid membranes (SLMs) was presented [1]. The SLM was an organic solvent of relatively low polarity, which was held by capillary forces in the pores of a hydrophobic porous membrane (support). The substances of interest were extracted from a flowing aqueous sample (donor solution) on one side of the SLM, through the SLM, and into a stagnant aqueous solution (acceptor solution) on the other side. Since its introduction, there has been a growing interest in the use of SLM extractions in chemical and biochemical analyses [2–7]. Due to the low consumption of hazardous organic solvents, easy automation, and the great potential for extraction and pre-concentration of substances from complex matrices, SLM extractions represent an interesting alternative for sample preparation.

Miniturization of SLM extraction has received substantial attention during the last 10 years through the development of liquid-phase microextraction (LPME). In LPME, a porous hollow fiber impregnated with an organic liquid served as the SLM [8–14]. Like most SLM extractions, LPME was based on passive diffusion, and the process was relatively time-consuming, with extraction times typically between 45 and 60 min. This was also the case for LPME based on carrier-mediated transport [15–17], where the addition of ion–pair reagents to the donor solution or to the SLM enhanced the extraction of polar substances, such as hydrophilic drugs and peptides [18–22], due to the formation of hydrophobic ion–pair complexes.

Recently, a totally new concept based on electrokinetic migration of charged analytes was introduced [23,24]. In this concept, called electromembrane extraction (EME), mass transfer across the SLM was accomplished by application of an electrical potential difference as the driving force. Ionized basic drugs were extracted from an acidic aqueous donor solution (positive electrode), through the SLM, and into an acidic aqueous acceptor solution (negative electrode) with typical extraction times of 5 min. Compared to LPME, EME was found to be a much faster process. Since the introduction of EME in 2006, several factors influencing the extraction and enrichment of basic and acidic drugs have been investigated and optimized. Knowledge about parameters affecting the extraction efficiency has been improved, and a comprehensive theoretical description of the extraction process has been developed [25–28].

The preliminary experiences with using EME on basic and acidic drugs are promising, indicating that this entirely new concept has a strong potential for extraction and enrichment in chemical and biochemical analyses. The applicability of EME for peptides has not been reported yet. Due to the increasing comprehension of the potential of peptides, which can act as diagnostic tools (biomarkers), hormones, neorutransmitters, drugs and antibiotics, there is a need for new and specific peptide enrichment methods, in addition to the traditional methods widely used [29–31]. This paper

* Corresponding author. Tel.: +47 22856576; fax: +47 22854402.
E-mail address: stigpe@farmasi.uio.no (S. Pedersen-Bjergaard).

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shows the first fundamental results of extraction of peptides based on electrokinetic migration across a SLM, and may initiate a new possibility and direction in peptide analysis.

2. Experimental

2.1. Electromembrane extraction

The equipment used for EME of peptides is illustrated in formerly published articles on EME [23,25–28]. The dc power supply used was a model ES 0300-0.45 from Delta Power Supplies (Delta Electronika, Zierikzee, The Netherlands) with programmable voltage in the range of 0–300 V, providing currents in the range of 0–450 mA. Platinum wires with a diameter of 0.5 mm (K. Rasmussen, Hamar, Norway) were placed in the sample and acceptor solutions and used as electrodes. They were both connected to the power supply. The sample compartment was a Brand Snap-Cap container of LDPE (low density polyethylene), with a volume of 0.8 mL, an internal diameter of 6 mm, and a height of 31 mm (Sigma–Aldrich, Steinheim, Germany). The porous hollow fiber used for immobilization of the SLM and for housing the acceptor solution was a PP Q3/2 polypropylene hollow fiber (Membrana, Wuppertal, Germany) with an internal diameter of 1.2 mm, wall thickness of 200 μm, and with 0.2 μm pores. During the experiments, the extraction unit was agitated on a Vibramax 100 agitator (Heidolph, Kelheim, Germany).

EME was performed according to the following procedure: 500 μL acidified sample solution was filled into the sample compartment. The lower end of a 2.5 cm piece of polypropylene hollow fiber was closed by mechanical pressure, whereas the upper end was connected to a 2.2 cm length pipette tip of polypropylene (Finntip 200 Ext from Thermo Electron, Vantaa, Finland) as a guiding tube. The hollow fiber was dipped for 5 s in the organic solvent serving as the SLM (typically a mixture of 1-octanol and di(2-ethylhexyl) phosphate (DEHP)). Excess of solvent in the SLM was then removed with a medical wipe. Via the guiding tube, 25 μL of acidified acceptor solution was filled into the lumen of the hollow fiber with a micro syringe. The hollow fiber and the guiding tube were inserted through the vial cap before being placed in the sample solution. Finally, the negative electrode was placed in the acceptor solution, and the positive electrode was inserted through the vial cap and into the sample solution. The electrodes were connected to the power supply, and voltage (50 V dc) as well as agitation (1050 rpm) was applied for EME. After EME, 20 μL of the acceptor solution was collected with a micro syringe and diluted with 20 μL 100 mM phosphate buffer in a small vial of polypropylene. 30 μL of this mixture was injected directly onto the HPLC column.

2.2. Chromatographic system conditions

The chromatographic system, an Ultimate 3000-system, consisted of a WPS-3000TSL Autosampler, a HPGM-3200 pump, and a VWD-3400 UV/vis detector operated at 214 nm, all from Dionex (Sunnyvale, CA, USA). Data acquisition was carried out using Chromeleon software (Version 6.70, Dionex).

Separation was carried out on a Jupiter HPLC 150 mm × 2.0 mm, 4 μm, 90 Å, Proteo column (Phenomenex, Torrance, CA, USA). The injection volume was 30 μL, and the flow was set at 0.2 mL min^{-1}. Gradient elution was used to separate the various peptides. Mobile phase A consisted of 5% acetonitrile in 0.1% trifluoracetic acid (TFA). Mobile phase B consisted of 5% 0.1% TFA in acetonitrile. The system was equilibrated at the starting conditions: 100% mobile phase A. After sample injection the linear gradient started from 100% mobile phase A/0% mobile phase B and increased its elution strength to 72% mobile phase A/28% mobile phase B within 30 min. The mobile phase composition was held constant at 72% mobile phase A/28% mobile phase B for 2 min. Within 2 min after this isocratic part of the run, the mobile phase composition was returned to the starting conditions. The re-equilibration time was 20 min.

2.3. Chemicals

Angiotensin 1, angiotensin 2, angiotensin 3, Glu-Glu-Leu, Ile-Pro-Ile, neurotensin, neurotensin 1–6 and neurotensin 1–8 were purchased via Sigma–Aldrich (Oslo, Norway). DEHP, dihexyl ether, 2-nitrophenyl octyl ether (NPOE), 1-octanoic acid, 2-octanone, nitrobenzene, nitropentane, sodium-2-ethylhexylsulphuric acid, tris(2-ethylhexyl) phosphate (TEHP), tridecyl phosphate (TDP), trihexylamine, and trixylyl phosphate of analytical grade were all obtained from Fluka (Buchs, Switzerland). The supplier of analytical grade 1-heptanol, 1-nonanol, 1-octanol, trifluoracetic acid, and triisopropylnaphtalenesulfonic acid were Sigma (St. Louis, MO, USA). Diphenylphosphate of analytical grade was from Aldrich (Steinheim, Germany). All inorganic chemicals were purchased from Merck (Darmstadt, Germany).

2.4. Standard solutions

Stock solutions containing 1 mg/mL of each peptide were prepared in water and stored at −20 °C protected from light. Sample solutions were prepared daily by dilution of the stock solutions (typically with HCl).

2.5. Calculation of recovery and enrichment

Recovery (R) during EME was calculated according to the following equation for each peptide:

$$R = \frac{n_{a,final}}{n_{d,initial}} 100\% = \left(\frac{V_a}{V_d}\right)\left(\frac{C_{a,final}}{C_{d,initial}}\right) 100\% \quad (1)$$

where $n_{d,initial}$ and $n_{a,final}$ are the number of moles of analyte originally present in the donor solution and the number of moles of analyte finally colleted in the acceptor solution, respectively. V_a is the volume of acceptor solution, V_d is the volume of donor solution, $C_{a,final}$ is the final concentration of analyte in the acceptor solution, and $C_{d,initial}$ is the initial analyte concentration within the donor solution.

Enrichment (E) during the EME was calculated according to the following equation for each peptide:

$$E = \frac{C_{a,final}}{C_{d,initial}} \quad (2)$$

2.6. Determination of distribution ratios

The actual peptides were prepared at the 10 μg/mL level in aqueous HCl solutions of different molarities. An aliquot of 150 μL of this solution was mixed with 450 μL of the organic phase of interest in a 1.5 mL micro-centrifuge tube (VWR, Oslo, Norway). The tube was rotated for 45 min by a Rotary Mixer (Cenco, Breda, The Netherlands) at 46 rpm, and kept stagnant at room temperature for 20 h. Subsequently, the micro-centrifuge tube was centrifuged at 8000 rpm for 5 min, the aqueous phase was removed and analyzed by HPLC. The distribution ratio D was determined by the following equation:

$$D = \frac{C_{org}}{C_{HCl}} \quad (3)$$

Table 1
Physio-chemical properties and abbreviations of the peptides used

Peptide	Abbreviation	Amino acid sequence	Hydrophobic amino acids	No. of basic moieties/no. of acidic moieties[a]/actual charge at pH 3[b]
Angiotensin 1	AT 1	NRVYIHPFHL	3	4/2/3.1
Angiotensin 2	AT 2	NRVYIHPF	2	3/2/2.5
Angiotensin 3	AT 3	RVYIHPF	2	3/1/2.3
Glu-Glu-Leu	EEL	EEL	1	1/2/0.08
Ile-Pro-Ile	IPI	IPI	2	1/1/0.16
Neurotensin	NT	ρELYENKPRRPYIL	3	3/2/1.95
Neurotensin 1–6	NT 1–6	ρELYENK	1	1/2/0
Neurotensin 1–8	NT 1–8	ρELYENKPR	1	2/2/1

[a] Negative charge of tyrosine is not taken into account.
[b] Actual charges were calculated from their pK_a values.

where C_{org} is the concentration in the organic phase and C_{HCl} is the concentration in the aqueous phase (containing HCl). C_{HCl} is determined by direct analysis by HPLC, whereas C_{org} is calculated based on C_{HCl} and a total mass balance of the system.

3. Results and discussion

Eight different peptides, three angiotensins, three neurotensins, and two tripeptides, were used as model compounds. Their physiochemical properties are summarized in Table 1. As illustrated here, the model peptides in this study varied with regard to the number of amino acids, amino acid sequence, number of ionized groups, and polarity, and were selected to represent a broad range of physiochemical properties of small peptides. The stability of the eight model peptides in an acidic environment has been tested before [21,22]. Based on these results, it is assumed that the model peptides were stable under the experimental conditions used in this study as well.

3.1. Initial experiments

The basic principle for the EME of peptides is illustrated in formerly published articles on EME [23,25–28]. Since the peptides were intended to migrate in the electric field, efficient ionization of the peptides in the aqueous donor solution and in the aqueous acceptor solution was required. Consequently, these solutions were acidified with 10 mM HCl (pH 2.0). A voltage of 300 V was applied with the positive electrode in the sample and the negative electrode in the acceptor solution. The extraction unit was agitated for 5 min at 1050 rpm and after extraction the acceptor solution was collected with a micro syringe, and its content was determined by HPLC. With a SLM consisting of 10% DEHP in 1-octanol as the SLM, we were for the first time able to extract peptides by EME, and an example is illustrated in Fig. 1. The upper chromatogram (A) demonstrated the presence of three peptides in the acceptor solution after extraction, while the lower chromatogram (B) illustrated the same process without application of the electrical potential across the SLM. Clearly, the peptides were extracted by electrokinetic migration rather than passive diffusion within the time frame of extraction. Based on earlier difficulties with EME of analytes with more than a single charge [32] and the fact of bubble formation during the extraction, the positive initial results with the peptides were unexpected, they were highly promising, and they recalled further studies as reported below.

3.2. Theoretical model

The resulting steady-state flux of the *i*th cationic analyte J_i across the SLM may be calculated by the following equation [28]:

$$J_i = \frac{-D_i}{h}\left(1 + \frac{\nu}{\ln \chi}\right)\left(\frac{\chi - 1}{\chi - \exp(-\nu)}\right)(c_{ih} - c_{i0}\ \exp(-\nu)) \quad (4)$$

where D_i is the diffusion coefficient for the analyte, h is the thickness of the membrane, χ is the ratio of the total ionic concentration in the sample solution to that in the acceptor solution (ion balance), and ν is a dimensionless driving force defined by

$$\nu = \frac{z_i e\,\Delta\phi}{kT} \quad (5)$$

where z_i is the charge of the analyte, e is the elementary charge, $\Delta\phi$ is the electrical potential across the SLM, k is the Boltzmann's constant, and T is the absolute temperature.

In a fixed EME system, where both the diffusion coefficient (D_i) and the SLM thickness (h) are constant, Eq. (4) predicts that the flux of analyte can be improved by increasing the potential difference ($\Delta\phi$) across the SLM, or by lowering the ion balance (χ) over the SLM.

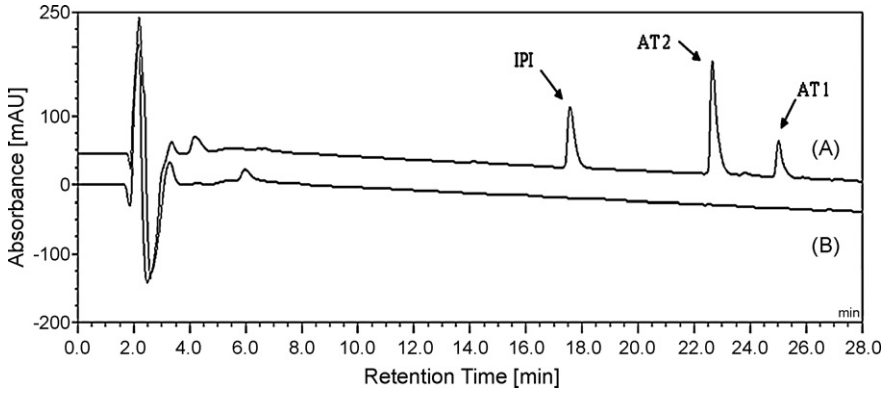

Fig. 1. Chromatograms demonstrating the effect of voltage on EME: (A) acceptor phase after 5 min of EME at 300 V; (B) acceptor phase after 5 min of extraction with no voltage. Supported liquid membrane: 1-octanol + 10% DEHP; pH in donor solution: 2.0; pH in acceptor solution: 2.0.

3.3. Variation in the SLM composition (organic solvent)

According to Eqs. (4) and (5), the flux of analyte is affected by the analyte peptide concentration gradient across the SLM, this is partially determined by the sample-to-SLM distribution ratio, and this in turn is controlled by the type of solvent used as the SLM. In addition, the type of solvent also affects the diffusion coefficient, another parameter affecting the flux. In order to investigate this in practice, ten organic solvents were tested as SLMs in a first experiment: dihexyl ether, NPOE, 1-octanoic acid, 1-heptanol, 1-nonanol, 1-octanol, 2-octanone, nitrobenzene, nitropentane, and trihexylamine. These solvents represented different functional groups, and were selected based on earlier experience from SLM extractions and EME [23,25,26]. Initial experiments indicated that the presence of an anionic carrier in the liquid membrane was important for the extraction of peptides, and consequently DEHP was added to the various organic phases at 10% (w/w) levels prior to extraction. Among all the solvents, the alcohols were superior for EME of the model peptides. Dihexyl ether, NPOE, and trihexylamine (with DEHP) provided no recovery of any of the peptides, whereas 1-octanoic acid, 2-octanone, nitrobenzene, and nitropentane (with DEHP) provided low recovery (<20%) for AT 2 and IPI. Based on these experiments, 1-octanol was selected as the principal SLM constituent for the rest of the study.

3.4. Variation in SLM composition with anionic carrier

In a second experiment, various anionic carriers were added to 1-octanol at 10% levels. Diphenylphosphate, sodium-2-ethylhexylsulphuric acid, TDP, TEHP, triisopropylnaphtalenesulfonic acid, and trixylyl phosphate were examined and compared with DEHP. The overall extraction efficiency was best with DEHP as the carrier, and subsequent experiments were conducted with this substance added to 1-octanol.

Additionally, different amounts of DEHP were added to 1-octanol. As illustrated in Fig. 2, the extraction recoveries increased with an increasing amount of DEHP added to 1-octanol. Due to an increase in the current flowing in the system at 25 and 50% (w/w) levels of DEHP, heat was generated in the donor and acceptor solutions which negatively affected repeatability. As a consequence, a combination of 1-octanol and 15% DEHP was selected as the SLM for the rest of this study.

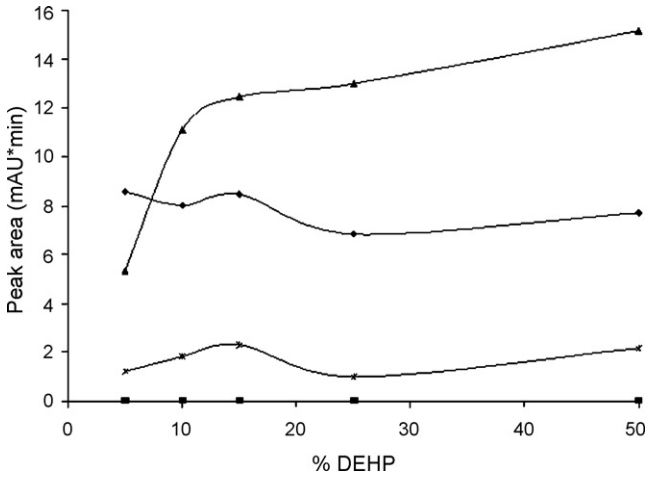

Fig. 2. Recovery with different amounts of DEHP in 1-octanol. Electrical potential: 50 V; extraction time: 5 min; agitation: 1050 rpm; pH in donor solution: 3.0; pH in acceptor solution: 1.0. Each peptide was present at 1 μg/mL in the sample. Symbols in graph: (✳) AT 1; (▲) AT 2; (♦) IPI; (■) NT 1–6.

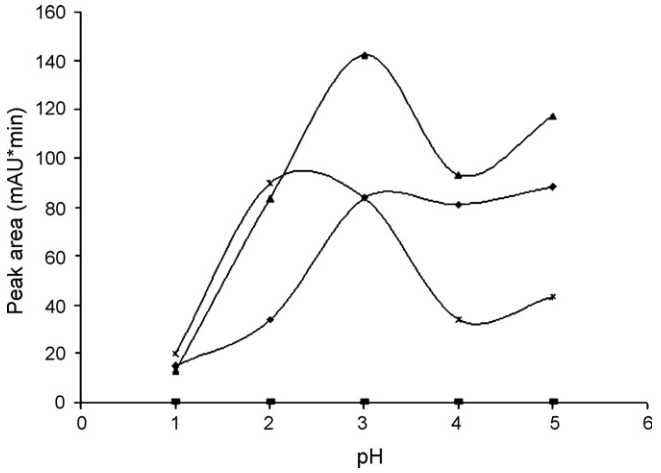

Fig. 3. Recovery with varying pH in the donor solution. Electrical potential: 50 V; extraction time: 5 min; agitation: 1050 rpm; supported liquid membrane: 1-octanol + 15% DEHP; pH in acceptor solution: 1.0. Each peptide was present at 10 μg/mL in the sample. Symbols in graph: (✳) AT 1; (▲) AT 2; (♦) IPI; (■) NT 1–6.

DEHP was adsorbed at the donor phase/organic phase interface. Here, the molecules were orientated with the hydrophobic chains into the organic phase, and the partly ionized phosphoric acid moieties directed towards the aqueous phase (pK_a 1.47 ± 0.50) [33]. During extraction, the dissociated molecules of DEHP formed a neutral and hydrophobic complex with positively charged peptides moieties, thereby enhancing their transport into the organic phase. At the organic phase/acceptor phase interface, the peptides were exchanged with a proton and released into the acceptor phase. The pH in the acceptor phase was lower than the pK_a-value of DEHP in order to protonate the molecule (pH 1.0). A high concentration of protons in the acceptor solution was therefore favorable.

3.5. Variation of pH in the donor solution

The effect of different pH values in the donor solution was investigated. The acceptor phase pH was kept constant at 1.0. The results are summarized in Fig. 3. The highest overall recoveries were obtained at pH 3.0. This was expected, since too high a pH in the donor solution caused an increase of the negatively charged moieties on the peptides, and a subsequent decrease in the positive net charge. In order to form a strong ion–pair complex with DEHP, and to migrate effectively towards the negative electrode in the acceptor solution, the peptides should have a high positive net charge. At pH 1.0, the peptides have the highest positive charge possible. Nevertheless, the recoveries were low at this pH value (<12%) which might be due to protonation of DEHP at the donor phase/organic phase interface (see below). This suppressed the peptide–DEHP complex formation and therewith the uptake of peptides in the organic phase.

3.6. Variation of pH in the acceptor solution

The effect of different pH values in the acceptor solution was investigated. The results are shown in Fig. 4. Keeping the donor phase composition constant (pH 3.0), the extraction recoveries increased with an increased concentration of HCl in the acceptor phase. There were two possible reasons for this result. With a low pH in the acceptor phase (below the pK_a of DEHP), the peptide was easily released from DEHP into the acceptor phase, as a result of protonation of DEHP at the organic phase/acceptor phase interface. In addition, the proton gradient formed across the membrane with

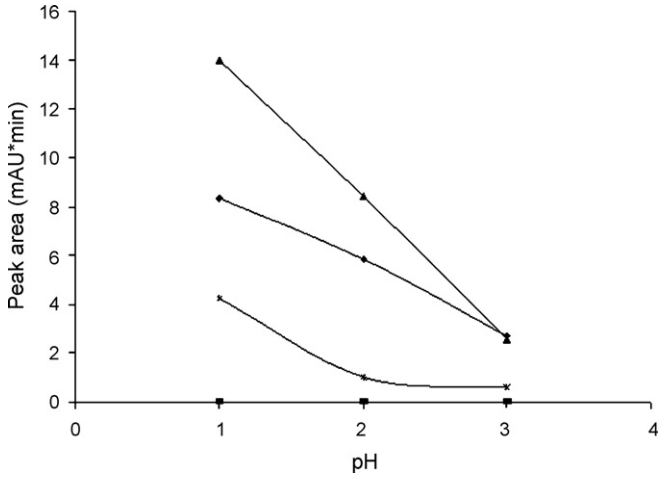

Fig. 4. Recovery with varying pH in the acceptor solution. Electrical potential: 50 V; extraction time: 5 min; agitation: 1050 rpm; supported liquid membrane: 1-octanol + 10% DEHP; pH in donor solution: 3.0. Each peptide was present at 1 μg/mL in the sample. Symbols in graph: (✳) AT 1; (▲) AT 2; (♦) IPI; (■) NT 1–6.

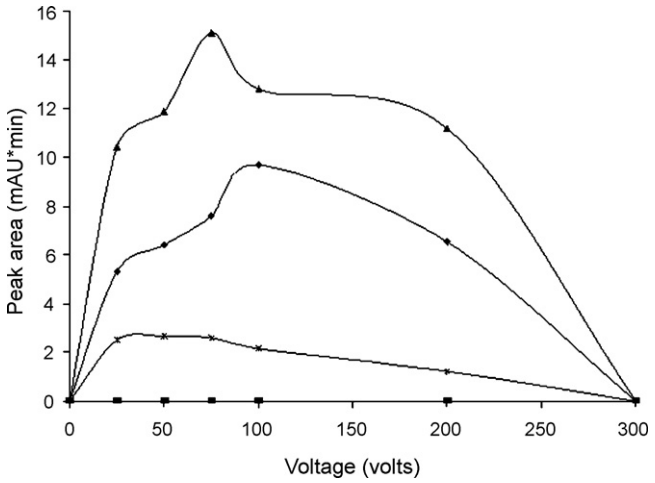

Fig. 5. Effect of voltage on recovery. Extraction time: 5 min; agitation: 1050 rpm; supported liquid membrane: 1-octanol + 15% DEHP; pH in donor solution: 3.0; pH in acceptor solution: 1.0. Each peptide was present at 1 μg/mL in the sample. Symbols in graph: (✳) AT 1; (▲) AT 2; (♦) IPI; (■) NT 1–6.

pH 1.0 in the acceptor phase and pH 3.0 in the donor phase was essential for the extraction process, as it reduced the pH effects of electrolysis. The pH value of the acceptor solution increased due to electrolysis, whereas the pH value of the sample solution decreased [23,26].

3.7. Verification of experimental findings with distribution ratios

In a separate experiment, the distribution behavior of the peptides between donor solution/1-octanol and donor solution/1-octanol + 15% DEHP was investigated by measurement of distribution ratios (see Section 2). The results are summarized in Table 2. Distribution ratios are shown in the range from −1.8 to 0.8. These values were chosen to take into account the method variation and detection limit. With pure 1-octanol, distribution ratios were low, and the peptides were not efficiently transferred into the SLM. This in turn resulted in a low concentration gradient across the SLM, and a low flux of peptides according to Eq. (4). When DEHP was added to 1-octanol, distribution ratios were improved, especially when pH was increased from 1.0 to 3.0 or 5.0. At pH 1.0, the peptides were efficiently charged, but the ionization of DEHP was suppressed, which reduced the ion–pair forming effect. As pH increased however, DEHP became ionized, ion–pair formation with the peptides occurred, and the distribution ratios increased.

3.8. Effect of voltage on the extraction recovery

According to Eqs. (4) and (5), the flux of analyte is affected by the magnitude of the applied potential. Therefore, the voltage applied across the SLM was varied in the range of 0–300 V to investigate the exact behavior of the peptides. As illustrated in Fig. 5, relative high recoveries were already obtained at 25 V. In general, the efficiency of the extraction system increased as the voltage was increased from 25 to 75 V. After that, a slight decrease in recovery was observed to 200 V. With 300 V, no recovery was observed. The deviations from Eqs. (4) and (5) observed above arose from bubble formation at the electrodes, leading to instability problems and low extraction recoveries. With a voltage of 50 V, the current level in the system was below 0.01 mA, and this was found to be appropriate.

3.9. Effect of time on the extraction recovery

In Fig. 6, the recovery is plotted against the extraction time. For AT 2 and IPI, the recoveries increased rapidly as a function of time during the first 5 min of extraction. A further increase was observed from 5 to 10 min. After 10 min, the system entered steady-state conditions, and the gain in recovery became minimal. This observation was consistent with earlier results for basic and acidic drugs [23,26,28], and 5 min was used for the rest of the study in order to minimize extraction time.

3.10. Evaluation of EME of peptides after optimization

Following the experiments reported above, the eight model peptides were extracted under optimal conditions to investigate the performance of the system. As summarized in Table 3, relatively high recoveries (22–56%) were obtained for five of the peptides, two of the peptides were extracted below the 10% level, and the most polar peptide was not extracted at all. The great distinction in

Table 2
Distribution ratios between donor solution/1-octanol and donor solution/1-octanol + 15% DEHP

Organic phase	pH in the donor solution	log D[a]			
		Angiotensin 1	Angiotensin 2	Ile-Pro-Ile	Neurotensin 1–6
1-Octanol	1	<−1.8	<−1.8	−1.2	<−1.8
1-Octanol	3	<−1.8	<−1.8	−1.6	<−1.8
1-Octanol	5	−1.0	<−1.8	−1.3	<−1.8
1-Octanol + 15% DEHP	1	<−1.8	<−1.8	−0.6	<−1.8
1-Octanol + 15% DEHP	3	>0.8	>0.8	>0.8	−1.3
1-Octanol + 15% DEHP	5	>0.8	>0.8	>0.8	−1.4

[a] $\log D = \log[\text{Compound}]_{\text{organic phase}}/[\text{Compound}]_{\text{donor solution}}$.

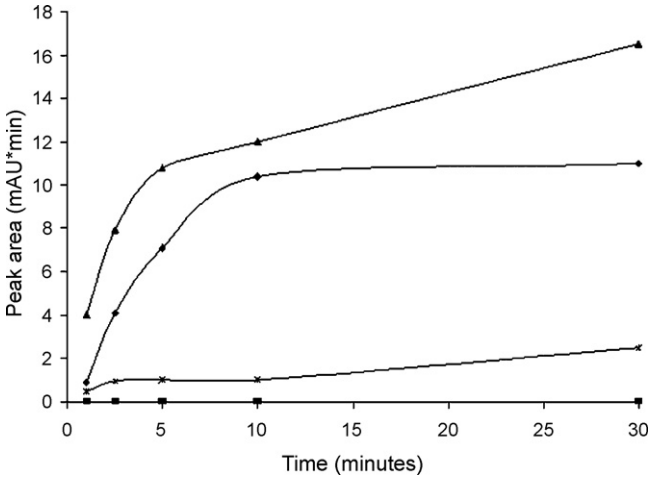

Fig. 6. Effect of time on recovery. Electrical potential: 50 V; agitation: 1050 rpm; supported liquid membrane: 1-octanol + 15% DEHP; pH in donor solution: 3.0; pH in acceptor solution: 1.0. Each peptide was present at 1 μg/mL in the sample. Symbols in graph: (✳) AT 1; (▲) AT 2; (♦) IPI; (■) NT 1–6.

extraction recovery among the peptides was clearly due to structural variations and the variation in the physio-chemical properties of the peptides (Table 1), indicating that the polarity and the number of ionized groups, were important parameters for the extraction efficiency. The three peptides with only a single hydrophobic amino acid were extracted poorly, whereas the peptides with two or three hydrophobic amino acids were the high recovery compounds.

To investigate the effect of peptide structure on phase distribution, both the donor solution and the acceptor solution were analyzed after extraction in a separate experiment. Thus, a mass balance of the system after extraction was established. The results, summarized in Table 4, clearly indicated a correlation between the physio-chemical properties of the peptides and their distribution in the three phases. Only a small fraction of the hydrophobic peptides with high positive charge (AT 1, AT 2, AT 3, and NT) was found in the donor phase. Based on a mass balance of the system, the majority of the unextracted peptides were trapped within the SLM. Due to their high positive charge, these peptides formed a strong complex with DEHP in the organic phase. Hence, the migration from the organic phase and into the acceptor phase was the critical step in the extraction process, limiting the extraction recoveries. As seen in Table 4, a high amount of the hydrophilic peptides with low positive charge (EEL, NT 1–6 and NT 1–8) was still remaining in the donor phase after 5 min of EME, and hardly any recovery was observed in the acceptor phase.

Without the application of voltage, no recovery was observed in the acceptor phase. However, the migration of peptides from the donor phase and into the organic phase was not affected. Probably, the formation of a peptide/DEHP complex was based principally on passive diffusion. Still, the electrical potential difference was the driving force for the migration of peptides into the acceptor phase, and application of voltage was necessary in order to recover the peptides in the acceptor solution. In comparison to LPME of the model peptides [21,22] EME is shown to be a much faster process. Extraction recoveries in the same range were obtained after 5 min of EME compared to 45 min of LPME.

To evaluate the optimized system, repeatability and linearity for three of the model peptides were examined in a final experiment. The results are illustrated in Table 5. Considering the laboratory-built equipment used in these experiments, the repeatability was acceptable. With r^2-values ranging from 0.9972 to 0.9995 in the concentration range of 0.5–10 μg/mL for AT 2 and IPI, and 1.0–10 μg/mL for AT 1 (Table 5), the linearity was definitely satisfactory.

Table 3
Recovery after 5 min of EME

Peptide	Recovery (%)	Enrichment
Angiotensin 1	22	4.4
Angiotensin 2	49	9.8
Angiotensin 3	49	9.8
Glu-Glu-Leu	2	0.4
Ile-Pro-Ile	53	10.6
Neurotensin	42	8.4
Neurotensin 1–6	n.d.	–
Neurotensin 1–8	7	1.4

Electrical potential: 50 V; extraction time: 5 min; agitation: 1050 rpm; supported liquid membrane: 1-octanol + 15% DEHP; pH in donor solution: 3.0; pH in acceptor solution: 1.0. Each peptide was present at 1 μg/mL in the sample.

Table 4
Effect of peptide structure on phase distribution

Peptide	Recovery (%)		
	Donor phase	Organic phase[a]	Acceptor phase
Angiotensin 1	n.d.	75	25
Angiotensin 2	4	59	37
Angiotensin 3	2	37	61
Glu-Glu-Leu	73	27	n.d.
Ile-Pro-Ile	21	44	35
Neurotensin	2	39	59
Neurotensin 1–6	54	46	n.d.
Neurotensin 1–8	85	7	8

Electrical potential: 50 V; extraction time: 5 min; agitation: 1050 rpm; supported liquid membrane: 1-octanol + 15% DEHP; pH in donor solution: 3.0; pH in acceptor solution: 1.0. Each peptide was present at 10 μg/mL in the sample.

[a] Mass balance calculation based on the peptide concentration determined in the sample and acceptor solutions.

Table 5
Repeatability and linearity

Peptide	Repeatability (n = 6, 1 μg/mL)		Linearity, r^2
Angiotensin 1	21	1.0–10 μg/mL	0.9978
Angiotensin 2	18	0.5–10 μg/mL	0.9995
Ile-Pro-Ile	15	0.5–10 μg/mL	0.9972

Electrical potential: 50 V; extraction time: 5 min; agitation: 1050 rpm; supported liquid membrane: 1-octanol + 15% DEHP; pH in donor solution: 3.0; pH in acceptor solution: 1.0.

4. Conclusions

The present work has for the first time demonstrated that peptides can be extracted across a thin hydrophobic SLM by the application of an electrical potential difference. The eight model peptides varied significantly with regard to the number of amino acids, amino acid sequence, number of ionized groups, and polarity, which were important parameters for the extraction efficiency. Another critical factor was the nature and the composition of the SLM; a mixture of 1-octanol and DEHP was found to be optimal. DEHP probably acted as an ion-pairing reagent with the ionized peptides at the interface between the sample and the membrane, resulting in migration of the peptides into the organic phase. The process was further optimized by proper selection of pH in the donor solution and acceptor solution, the extraction time, and the voltage applied across the membrane. After strong agitation (1050 rpm) in only 5 min, high recovery values were obtained for several of the peptides, indicating that EME of peptides was a very rapid process. In addition, the consumption of organic solvents was

very low, no more than approximately 15 μL was needed to sustain the SLM. More work is required to fully understand the process of EME of peptides. In the future, EME may have a potential as an innovative sample preparation technique in peptide analysis from different matrices.

References

[1] G. Audunsson, Anal. Chem. 58 (1986) 2714.
[2] J.Å. Jönsson, L. Mathiasson, Trends Anal. Chem. 11 (1992) 106.
[3] J.Å. Jönsson, L. Mathiasson, Trends Anal. Chem. 18 (1999) 318.
[4] J.Å. Jönsson, L. Mathiasson, Trends Anal. Chem. 18 (1999) 325.
[5] J.Å. Jönsson, L. Mathiasson, J. Chromatogr. A 902 (2000) 205.
[6] J.Å. Jönsson, L. Mathiasson, J. Sep. Sci. 24 (2001) 495.
[7] G. Nilve, G. Audunsson, J.Å. Jönsson, J. Chromatogr. 471 (1989) 151.
[8] S. Pedersen-Bjergaard, K.E. Rasmussen, Anal. Chem. 71 (1999) 2650.
[9] K.E. Rasmussen, S. Pedersen-Bjergaard, Trends Anal. Chem. 23 (2004) 1.
[10] K.E. Rasmussen, S. Pedersen-Bjergaard, M. Krogh, H.G. Ugland, T. Gronhaug, J. Chromatogr. A 873 (2000) 3.
[11] D.W. Kou, X.Y. Wang, S. Mitra, J. Chromatogr. A 1055 (2004) 63.
[12] E. Psillakis, D. Mantzavinos, N. Kalogerakis, Anal. Chim. Acta 501 (2004) 3.
[13] J.B. Quintana, R. Rodil, T. Reemtsma, J. Chromatogr. A 1061 (2004) 19.
[14] C. Basheer, H.K. Lee, J. Chromatogr. A 1057 (2004) 163.
[15] T.S. Ho, T.G. Halvorsen, S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. A 998 (2003) 61.
[16] T.S. Ho, J.L.E. Reubsaet, H.S. Anthonsen, S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. A 1072 (2005) 29.
[17] T.S. Ho, S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. Sci. 44 (2006) 308.
[18] A. Drapala, P. Dzygiel, J.Å. Jönsson, P. Wieczorek, Acta Biochim. Pol. 48 (2001) 1113.
[19] A. Drapala, P. Wieczorek, Desalination 148 (2002) 235.
[20] A. Drapala, P. Wieczorek, Desalination 163 (2004) 47.
[21] J.L.E. Reubsaet, H. Loftheim, A. Gjelstad, J. Sep. Sci. 28 (2005) 1204.
[22] J.L.E. Reubsaet, J.V. Paulsen, J. Sep. Sci. 28 (2005) 295.
[23] S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. A 1109 (2006) 183.
[24] S. Pedersen-Bjergaard, K.E. Rasmussen, Anal. Bioanal. Chem. 388 (2007) 521.
[25] A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, J. Chromatogr. A 1124 (2006) 29.
[26] M. Balchen, A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, J. Chromatogr. A 1152 (2007) 220.
[27] A. Gjelstad, T.M. Andersen, K.E. Rasmussen, S. Pedersen-Bjergaard, J. Chromatogr. A 1157 (2007) 38.
[28] A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, J. Chromatogr. A 1174 (2007) 104.
[29] R.C. Causon, R.D. McDowall, J. Control. Release 21 (1992) 37.
[30] T. Herraiz, Anal. Chim. Acta 352 (1997) 119.
[31] T. Herraiz, V. Casal, J. Chromatogr. A 708 (1995) 209.
[32] T.M. Middelthon-Bruer, A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, J. Sep. Sci. 31 (2008) 753.
[33] R.K. Biswas, R.A. Banu, M.N. Islam, Hydrometallurgy 69 (2003) 157.