Electromembrane Extraction of Biologically Active Substances – Investigation of Fundamental Aspects

Thesis for the degree Philosophiae Doctor

by

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CONTENT

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Oslo, February 2014

Knut Fredrik Seip

LIST OF PAPERS

This thesis is based on the following papers which will be referred to by their roman numerals in the text:

I. **K.F. Seip**, J. Stigsson, A. Gjelstad, M. Balchen, S. Pedersen-Bjergaard *"Electromembrane extraction of peptides - Fundamental studies on the supported liquid membrane"*, Journal of Separation Science 2011, 34 (23): 3410-3417

II. **K.F. Seip**, H. Jensen, M.H. Sønsteby, A. Gjelstad, S. Pedersen-Bjergaard ***"****Electromembrane extraction: Distribution or electrophoresis?"*, Electrophoresis 2013, 34 (5): 792-799

III. **K.F. Seip**, A. Gjelstad, S. Pedersen-Bjergaard *"Electromembrane extraction from aqueous samples containing polar organic solvents"*, Journal of Chromatography A 2013, 1308: 37-44

IV. **K.F. Seip**, M. Faizi, C. Vergel, A. Gjelstad, S. Pedersen-Bjergaard *"Stability and efficiency of supported liquid membranes in electromembrane extraction - a link to solvent properties"*, Analytical and Bioanalytical Chemistry 2014, in press

V. **K.F. Seip**, H. Jensen, T.E. Kieu, A. Gjelstad, S. Pedersen-Bjergaard *"Salt effects in electromembrane extraction"*, manuscript submitted to Journal of Chromatography A

Publications not included in the dissertation:

VI. **K.F. Seip**, K.C. Bjerknes, H.T. Johansen, E.W. Nielsen, L. Landrø, L. Reubsaet *"Bradykinin analysis revived - A validated method for determination of its stable metabolite in whole blood by LC-MS/MS"*, Journal of Chromatography B 2014, 947-948: 139-144

VII. A. Gjelstad, **K.F. Seip**, S. Pedersen-Bjergaard *"Electromembrane extraction as a rapid and selective miniaturized sample preparation technique for biological fluids"*, in Sample extraction techniques for biological samples: recent advances and novel applications (e-book), Future Science Ltd. 2014, in press

VIII. **K.F. Seip**, A. Gjelstad, S. Pedersen-Bjergaard *"The potential application of electromembrane extraction for the analysis of peptides in biological fluids"* (editorial), Bioanalysis 2012; 4 (16): 1971-1973

IX. A. Barratt-Due, H.T. Johansen, A. Sokolov, E.B. Thorgersen, B.C. Hellerud, L. Reubsaet, **K.F. Seip**, T.I. Tønnessen, J.K. Lindstad, A.M. Pharo, A. Castellheim, T.E. Mollnes, E.W. Nielsen *"The Role of Bradykinin and the Effect of the Bradykinin Receptor Antagonist Icatibant in Porcine Sepsis"*, Shock 2011, 36 (5): 517-523

ABBREVIATIONS

CE	*Capillary electrophoresis*
DBS	*Dried blood spots*
DEHP	*Di-(2-ethylhexyl) phosphate*
EE	*Liquid-liquid electroextraction*
EME	*Electromembrane extraction*
ENB	*1-Ethyl-2-nitrobenzene*
GC	*Gas chromatography*
HF-LPME	*Hollow fiber liquid phase microextraction*
HPLC	*High-performance liquid chromatography*
HS-SDME	*Headspace single drop microextraction*
IPNB	*1-isopropyl-4-nitrobenzene*
ITIES	*Interface between two immiscible electrolyte solutions*
LC-MS	*Liquid chromatography – mass spectrometry*
LLE	*Liquid-liquid extraction*
LPME	*Liquid phase microextraction*
NPOE	*2-Nitrophenyl octyl ether*
PALME	*Parallel artificial liquid membrane extraction*
pI	*Isoelectric point*
PLS	*Partial least square regression*
PME	*Polymeric membrane extraction*
RP-HPLC	*Reverse phase high-performance liquid chromatography*
RSD	*Relative standard deviation*
SDME	*Single drop microextraction*
SLM	*Supported liquid membrane*
SPE	*Solid phase extraction*
SPME	*Solid phase microextraction*
TDP	*Tridecyl phosphate*
UPLC	*Ultra-performance liquid chromatography*
UV	*Ultraviolet (as a detection principle)*

ABSTRACT

Electromembrane extraction (EME) was introduced in 2006 as a fast and selective microextraction technique that offered good recoveries for basic analytes. Since then, more than 90 publications have been presented on the technique in various applications and technical setups. The principle is based on extraction of analytes across a thin supported liquid membrane (SLM) by the use of an electric field. Several key parameters for an efficient EME setup has been described previously, but systematic knowledge about the extraction process and the importance of the SLM was lacking at the beginning of this PhD project. The main objective of the work with this thesis was to further develop the theoretical understanding of EME on biologically active substances and to build systematic knowledge about the extraction process. Special attention has been given to the SLM, distribution of analytes throughout the EME system over time, stability of the EME system, and how the extraction process was affected by high amounts of either salts or organic solvents in the sample solution.

In **paper I**, a screening of different SLM compositions for the extraction of eight model peptides with EME was performed. The model peptides were selected to represent a broad range of physical chemical parameters. This paper confirmed previous findings on the importance of combining an organic solvent with a carrier for efficient extractions, as well as identifying several new compositions of carriers and solvents that were effective as SLMs. The effective compositions comprised a mono- or dialkylated phosphate acting as a carrier and a primary alcohol or ketone acting as a solvent. Especially the combination 2-octanone and tridecyl phosphate (9:1 w/w) was shown to give higher extraction recoveries and lower standard deviations than previously reported SLMs.

In **paper II**, a phenomenological theoretical model for the time dependent distribution of analytes in EME was presented and experimentally verified on several unpolar basic drugs and peptides, representing a broad range of physical chemical properties. Distribution profiles were made, where the amount of analytes in the sample, SLM, and acceptor solution at different extraction times were investigated. The distribution profiles were in good accordance with the theoretical model, but a deviation was seen for some of the peptides where a relatively high amount became trapped in the membrane. The resulting observations demonstrated that the mass transfer across the SLM in EME had elements of both a

distributive and electrophoretic process. This can be seen from the theoretical model by the inclusion of a voltage dependent distribution coefficient.

In **paper III**, EME was performed on samples containing a substantial amount of the organic solvents ethanol, methanol, dimethyl sulfoxide, or acetonitrile together with five unpolar basic drugs as model analytes. The main purpose was to investigate the stability and efficiency of EME when organic solvents were present in the sample. When nitrophenyl octyl ether (NPOE) was used as SLM, stable extractions were achieved from samples containing up to 50 % (v/v) ethanol or methanol, and up to 75 % (v/v) dimethyl sulfoxide. Acetonitrile partially dissolved the SLM solvent, and samples containing acetonitrile were unsuitable for EME. The maximum recovery was unaffected by the presence of organic solvent in the sample, but the time to reach this level increased from 5-10 minutes to 15-25 minutes. A practical example of these discoveries was successfully performed on the highly organic eluate from a commercial dried blood spot card.

In **paper IV**, a large systematic screening of 61 potential SLM solvents in EME was performed and evaluated according to stability during extractions and their ability to give high extraction recoveries for five unpolar basic model drugs. Several relevant solvent properties were correlated to these parameters through partial least square regression (PLS) analysis. The efficient EME solvents were characterized with a low water solubility (<0.5 g/L), high dipole moments, high proton acceptor properties, and low proton donor properties. Especially some nitroaromatics and ketones belonged to this group, and several efficient solvents that had not been previously described were identified from these criteria. Some solvents were classified as unsuitable because they gave a high extraction current, often combined with an electroosmotic flow of water through the SLM. This was solvents with a low log P value and high water solubility. Finally, some solvents were inefficient and provided no extraction recovery. These were solvents with a high log P value (log P > 4).

In **paper V**, EME was performed on samples containing different concentrations of NaCl. The presence of NaCl in the sample solution and its effect on extraction recovery, repeatability, and membrane current in EME was thoroughly investigated on 17 unpolar basic drugs with various physical chemical properties. For eight drugs, a substantial reduction in recovery was seen when more than 1 % (w/v) of NaCl was present in the sample solution and NPOE was used in a hollow fiber membrane setup. No correlation was seen between this recovery loss and the physical chemical properties of these analytes. With a NaCl content of

5 % (w/v) the repeatability of the extractions was compromised. The reduction in recovery was hypothesized to be caused by ion pairing in the SLM, and a mathematical model was made according to this hypothesis and the experimental data. Changing the SLM solvent from NPOE to 6-undecanone, or reducing the SLM to acceptor solution volume ratio by using a thinner membrane, reduced the observed recovery loss, which was consistent with the ion pair hypothesis.

1 INTRODUCTION

1.1 Importance of sample preparation in bioanalysis

An analytical process is usually divided into five consecutive steps: Sampling; sample preparation; separation; detection; and interpretation of the acquired data. When working with complicated matrices, each of these steps becomes highly important for reliable and reproducible results. Technological achievements in the fields of separation and detection have introduced sensitive and selective analytical instruments, and combined with powerful software for data interpretation, selective data acquisition of low-abundance analytes is possible. However, complex sample matrices can reduce the quality of the results by interfering with the sensitivity and selectivity of the method in both the separation and the detection step. In addition, some matrix components might not be compatible with the analytical instrumentation. Based on these criteria, inefficient sample preparation can easily become a bottle-neck for the quality of an analytical method.

In modern chemical analysis there is a high demand for accurate quantification of small amounts of analytes such as biomarkers, pollutants, toxic substances, and drugs from complex sample matrices. Many bioanalytical methods rely on the ability to detect endogenous compounds that can be found in limited amounts in a biological fluid containing a high abundance of matrix substances. One example is the determination of low-abundance biomarkers in blood, plasma, or serum, where the protein content is dominated by proteins such as albumin and immunoglobulins [1,2]. This high dynamic range in protein concentration can easily overshadow the more limited compounds, making high selectivity crucial. The low amounts of analytes also make high sensitivity an important factor. Recent developments in instrumentation have introduced analytical instruments capable of reaching detection limits in the low attomol levels or lower, as well as having a sufficient degree of selectivity [3]. However, to achieve this in practice, the amount of interfering compounds must be kept to a minimum to avoid loss of resolution in chromatography systems and severe matrix effects [4-7]. In addition, there is often also a demand for preconcentration of analytes to reach lower concentration limits for sufficient detection [8]. This can only be achieved through proper sample preparation, where analytes of interest are selectively extracted and isolated from the original sample matrix, into a compatible solvent.

The incompatibility of certain matrices and matrix compounds with the available instrumentation also remains an important reason to do sample preparation. Biological

samples often contain compounds such as salts, proteins, lipids, and various acids and bases that are not compatible with the instrumentation used for separation or detection. This incompatibility can not only interfere with the quality of the results, but also severely reduce the life time of instrument components such as analytical columns and increase the maintenance requirements for instruments such as mass spectrometers [5,9].

Thorough sample preparation for achieving high quality results is time consuming, and it can take up to 80 % of the total time for an analytical procedure [4]. Combined with increasing demands of analytical methods for high-throughput screening of samples, a focus on how to reduce the time and labor consumption has been established. Integrated and automated systems, that often take advantage of miniaturized and hyphenated techniques, have been increasingly popular to reduce the need for long analysis time and labor [4,6,9,10]. However, the demand for highly time-efficient systems serves as an extra challenge to the sample preparation in addition to efficient isolation of analytes from matrix components. The development of new techniques for sample preparation that fulfill these criteria, without using environmentally or physiologically hazardous chemicals, can greatly improve the quality of analytical methods.

1.2 Supported liquid membranes in sample preparation

A supported liquid membrane (SLM) consists of a small film of liquid, supported by an inert, hydrophobic, and porous material. The liquid is kept inside the pores of the membrane material by capillary forces, and can thus serve as a liquid barrier between aqueous phases on each side [11-13]. In 1986, Audunsson presented the first utilization of SLMs for sample cleanup and enrichment, where the analytes migrate from the sample solution, across the SLM, and into an acceptor solution on the other side where they are trapped [14]. The principle is well suited to give high selectivity and high enrichment factors, and the possibilities for automation are good.

The main principle of extractions through an SLM is depicted in Figure 1.1 and can be seen as an extraction process into an organic solvent with an immediate back-extraction to a different aqueous solvent in a single and efficient step [15]. A donor and an acceptor chamber are divided by an organic liquid based SLM. The pH in the aqueous sample residing in the donor chamber is adjusted to a value that leaves the analytes of interest uncharged. This allows the analytes to be extracted into the SLM. After diffusion through the SLM, the analytes are trapped in an aqueous acceptor solution on the other side of the SLM by using a pH value that

ionizes the analytes. This prevents the analytes from reentering the SLM and causes the transport to be unidirectional, which results in high enrichment factors [16-18]. The acceptor solution can then be analyzed by an analytical instrument either manually or through an on-line system [11]. This principle, with some modifications, appears in most SLM based sample preparation techniques. However, some techniques, such as electromembrane extraction (EME), require ionized analytes in the sample to achieve effective extraction across the SLM (see section 1.5.1) [19].

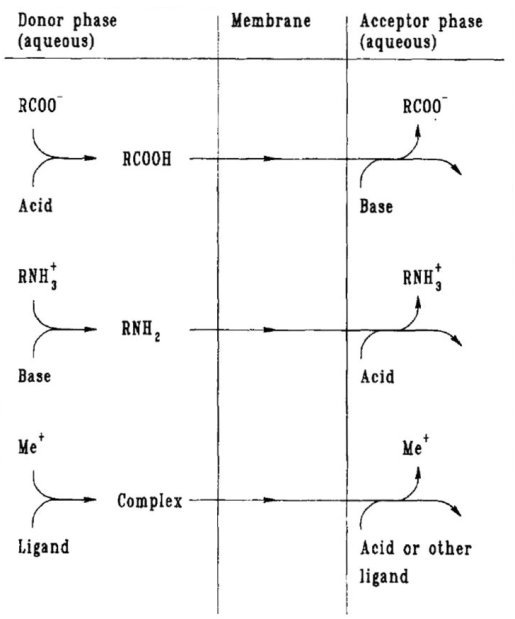


Figure 1.1: *The main principle of SLM extractions. All analytes get transported through the SLM as a neutral complex. Negatively charged analytes complex with acids (upper), positively charged analytes complex with bases (middle), and metal ions complex with a ligand/carrier (lower). Reprinted with permission from [12] © Elsevier B.V. (1992).*

The selectivity of the system can effectively be tuned towards extraction of certain analytes. In the example above, a basic pH in the sample solution and an acidic pH in the acceptor solution will effectively enrich basic analytes. Acidic analytes will remain in the sample solution because of their inability to enter the SLM, and neutral analytes will be mostly trapped in the SLM with a distribution in the two aqueous phases according to partition coefficients. By using opposite pH values, acids will be enriched instead [16-18]. Further enhancement of the selectivity towards certain compounds can be achieved by the addition of trapping reagents in the acceptor phase to prevent back extraction [20] or by the addition of additives to the SLM, such as carrier molecules or ion complexation agents [21-24]. The use of SLM additives can also increase the efficiency of the extraction system for poorly extracting compounds [25]. SLM based extractions have commonly been used on small acids or bases, but the use of additives has even allowed extraction of larger molecules such as peptides [26,27].

Several extraction systems have used the principle of SLM extraction in various setups and with various modifications, such as microporous membrane liquid-liquid extraction (MMLLE) [11], hollow fiber liquid phase microextraction (HF-LPME) [28], polymeric membrane

extraction (PME) [11], parallel artificial liquid membrane extraction (PALME) [29], and electromembrane extraction (EME) [19]. Some of these will be discussed further in section 1.4 and 1.5. In the different configurations, the membrane support material usually consists of polypropylene (PP) or polytetrafluoroethylene (PTFE or Teflon) and is used either as a flat membrane or a hollow fiber membrane [15,18,28,30,31]. The liquids impregnating the membrane are water insoluble and non-viscous, and they are classically non-polar organic liquids such as *n*-undecane, kerosene, dioctyl phosphate, and di-*n*-hexyl ether [11,15,30]. However, later applications and modifications have introduced other SLM solvents, such as toluene, nitroaromatics, 1-octanol, and ionic liquids [19,28,30,32-34].

In the classic SLM extraction setup, the sample is continuously pumped through the donor chamber while the liquid in the acceptor channel is kept stagnant, and the extraction efficiency is highly dependent on the flow rate through the donor chamber [11,16]. In another SLM based system, both the acceptor and donor solution has been pumped through their respective chambers to monitor real time metabolism of certain drugs [35]. SLM based extraction techniques have also been performed from systems where the entire sample volume is located within the donor chamber (HF-LPME, PALME, EME). This setup has been performed from small sample volumes with and without agitation or stirring [28,36].

1.3 The use of electric fields as a driving force in sample preparation

The introduction of an electric field in sample preparation techniques will affect the movement of charged substances according to the electrical force exerted on them. If the electric field is constant, this force (F) is determined by the equation:

$$F = qE \tag{1}$$

where q is the charge of the substance and E is the electric field strength. In addition, an electric field can have several other effects such as affecting the orientation of molecules according to their dipole moments, electroosmosis, and electrochemical reactions [37,38]. The effect of electroosmosis is for instance utilized in capillary electrophoresis (CE), where migration of neutral substances is achieved by an electroosmotic flow [39]. Molecular orientation can be important for passage through membrane systems and reduce frictional forces between molecules, and electrochemical reactions can be utilized as a driving force for molecules across boundaries [37,38,40,41]. All these effects can potentially be used to facilitate selective extraction of analytes during sample preparation.

The use of electric fields as a driving force in sample preparation was first presented with the introduction of electrodialysis in the end of the 19th century, and although the main principles have stayed the same, the technique has been developed substantially up to this date [42]. In electrodialysis, the concept of dialysis is combined with an electric field across the permeable dialysis membrane. This introduces an electrical component to the mass transfer in addition to the diffusion process of conventional dialysis, thus increasing the speed, preventing back-diffusion, and increasing the selectivity by excluding substances with the opposite charge [37,38,43,44]. The same main principles are also used by similar techniques, such as using ionic solvents in combination with ionic interchange membranes to generate an electroosmotic flow across the membrane or using an electric field in combination with a pressure-driven system (electrofiltration) [37].

Electric fields have also been used in combination with liquid-liquid extractions (LLE). One of these techniques is termed liquid-liquid electroextraction (EE) and was presented in 1992 [45]. The main principle is shown in Figure 1.2 for extraction of fuchsine acid from an organic to an aqueous phase, based on the original presentation of the technique. Electrodes are placed in two different liquid phases, generating an electric field between them. This electric field causes ions in the sample to migrate towards the electrode of opposite charge. In the original work, EE was performed both with the sample solution in between these two liquid phases (three-phase system) and with one of the two liquid phases as the sample solution (two-phase system) [45]. An on-line coupling of this technique was presented by combining EE with isotachophoresis and CE [46,47].

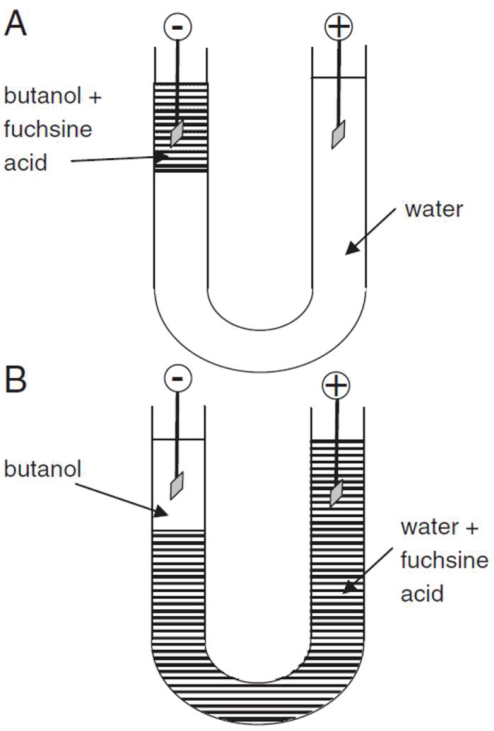


Figure 1.2: *A schematic illustration of EE of fuchsine acid. Before extraction, the fuchsine acid is located in the organic solvent (A), whereas after extraction the fuchsine acid has been extracted to the aqueous solvent. Reprinted with permission from [45] © Wiley-VCH Verlag (2010).*

In this technique, a small amount of terminating buffer is introduced in the end of the capillary. By placing the end of the capillary in the sample solution while applying a voltage and a counterpressure at the same time, analytes get focused between the terminating buffer and leading buffer through isotachophoresis. Since the electric field and resulting electroosmotic flow can destabilize the liquid-liquid interface between the sample and the buffer, the counterpressure is maintained to stop the sample solution from entering the capillary. After this step, the analytes can be determined by conventional CE. An on-line technique was also presented for liquid chromatography by performing electroextraction through a needle device directly in the autosampler [48]. Although electroextraction introduced the concept of electric fields in combination with liquid-liquid extractions, few articles on this technique have been presented during recent years. This has been suggested to be the result of poor compatibility with aqueous samples, limited extraction efficiency due to a small area of liquid-liquid interface where extraction occurs, and practical inconvenience [49].

LLE with an electrical potential has also been performed in an electrochemically driven extraction procedure. This system is named electrochemically modulated LLE, or ITIES extraction, and is based on extraction across the "interface between two immiscible electrolyte solutions" (ITIES) [40]. An overview of the technique is presented in Figure 1.3, where an electric potential is applied over a phase boundary between two immiscible electrolyte solutions. In this example, an aqueous donor phase is flowing over a phase boundary to another electrolyte solution made into an organo-gel for stability. Relative distribution of ions between the two phases can be adjusted by varying the potential difference between them, where cations migrate towards the phase that is relatively less positive [50]. The opposite is true for anions. Based on specific transfer potentials for the different analytes, selective extractions can be performed by varying the potential difference [49-51]. Analyte ions can thus be trapped in the organo-gel phase, while the ions remaining in the aqueous solution are flushed out [51]. A miniaturized version of the technique has also been presented where ITIES extraction was performed in a microfluidic chip [52]. The technique has been successfully applied on real samples, such as determination of drugs in biological matrices [53] and the determination of food additives [54]. The mass transfer in electrochemically modulated extractions appears to be more efficient than in EE, partially because the interface

between the two phases is larger [49].

Several other techniques utilizing electric fields have been presented, inspired by the pioneering work with EE and ITIES extractions. These involve electric field assisted elution from SPE [55], as well as electrically enhanced microextraction techniques such as electromembrane extraction (EME) [19], single drop microextraction combined with an electric field [56], electric field driven extractions across polymer inclusion membranes [57], and electrochemically enhanced solid phase microextraction (EE-SPME) [58]. Some of these techniques will be described further in section 1.4 and 1.5.

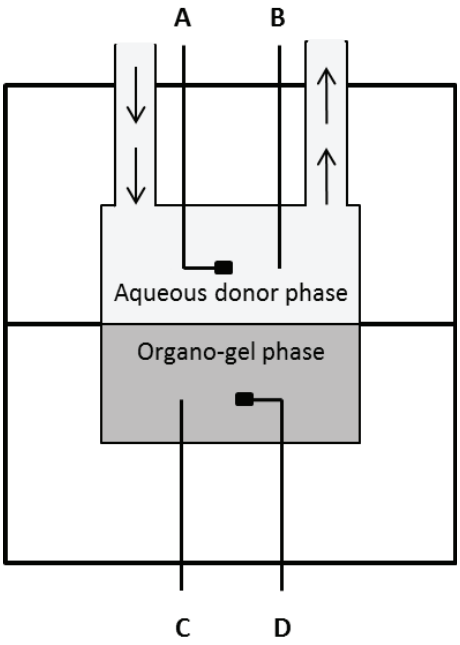


Figure 1.3: *A schematic illustration of an ITIES system. A and D represent platinum mesh counter electrodes for the two phases. B is a Ag/AgCl or AG/AgSO$_4$ reference electrode for the aqueous phase, and C is a pseudo-reference electrode of the same composition in the organic phase.*

1.4 Microextraction techniques

Analytical microextractions can be defined as non-exhaustive sample preparation techniques where small volumes of extracting phase is used (microliters or lower) compared to the volume of sample [59]. The use of microextraction techniques can offer several advantages to conventional sample preparation techniques such as protein precipitation, liquid-liquid extractions (LLE), or solid phase extractions (SPE). These advantages include miniaturization, ease of automation, high-throughput, online coupling, low operation costs, low solvent consumption, and possibilities for tailor made systems for particular applications [60,61]. A minimized sample preparation step also enables measurements of trace levels of analytes in various complex matrices [59,60]. In addition, the miniaturized system can reduce both extraction time and operation cost, and the reduced consumption of organic solvents makes these systems less hazardous to both the environment and the operator. These advantages offered by microextraction techniques fits well into current trends, with the need for automated and advanced high-throughput analytical systems that are highly sensitive and selective [4,10,62]. High enrichment factors and the possibilities of operating with small amounts of sample make these

techniques well suited for both trace level analysis from larger sample volumes and analysis of substances in limited amounts of biological samples.

1.4.1 Microextractions into a solid phase

The principles of microextractions became commercially available with solid phase microextraction (SPME) [63]. In this technique, rapid sample preparation was made possible both in the laboratory and at the site of investigation by a small and relatively simple extraction device that integrates sampling, analyte isolation, and enrichment into a single step [59,64-66]. The extracting phase is attached to a solid support material and then exposed to the sample for a certain time. This causes partitioning of analytes between the sample matrix and the extracting phase until a concentration equilibrium is reached, with no or minimal use of organic solvents [59,67]. The concentrated extracts adsorbed to the extracting phase can then be desorbed in the interface of an analytical instrument and analyzed [63,67]. Several devices using the SPME principle have been presented, such as coated fibers, stir bars, vessel walls, tubes, suspended particles, and membranes [59,60,63,65,68-70]. However, the commercialized format of coated fibers as shown in Figure 1.4 is mostly used. In this format a fused silica fiber is commonly coated with a coating material such as polydimethylsiloxane (PDMS), divinylbenzene (DVB), or carboxen (CAR), but various materials have been used for more specialized extractions [71-73]. This coated fiber is attached to the end of a metal tube that acts as a needle to push the fiber in or out of a larger septum so it can be used as a syringe [59]. Extraction occurs when the fiber is pushed out and exposed to the outside environment. Likewise, desorption will occur when the fiber with adsorbed analytes are placed in the interface of an analytical system that can handle SPME fibers. This is typically a GC system, where the analytes can be thermally desorbed by placing the fiber in the GC injector [63,67]. The simplicity and mobility of the device has made SPME a popular choice for extraction of volatile and semi-volatile substances in environmental [74,75], food [76,77],

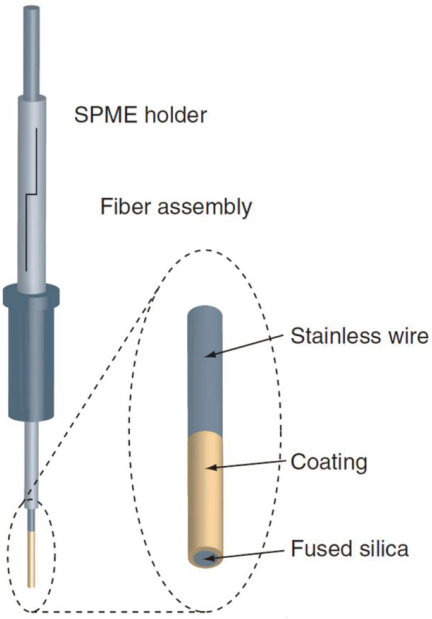


Figure 1.4: *An illustration of a commercial SPME device. Reprinted with permission from [66] © Future Science Ltd. (2013).*

forensic [78-80], and pharmaceutical [81,82] samples; even direct *in vivo* sampling [83].

1.4.2 Microextractions into a liquid phase

In addition to extractions into solid media as in SPME, microextractions into liquid media have also become a popular approach. This principle was introduced in 1996 with the invention of single-drop microextraction (SDME) [84,85]. The technique is easy to operate, possible to automate, fast, inexpensive, and require very low amounts of organic solvents (a single microdrop of 1-8 μL) [86,87]. An example of an SDME extraction procedure is presented in Figure 1.5. It is based on the principle that analytes get extracted in a two phase system based on their distribution constants. Analytes will thus migrate from the aqueous sample and be concentrated in a small amount of a water immiscible solvent, leading to high enrichment factors [66,84,85,88]. The microdrop is either hanging from the end of a Teflon rod [85], from the tip of a microsyringe [89] or as a drop suspended inside a flowing aqueous drop [90]. After extraction, the drop can be injected into an analytical system such as a gas chromatograph [88]. The system has, however, received some critique for its need for careful handling to ensure stability of the drop, especially when it is compromised by complex matrices [86,88]. Later modifications to the original principle has permitted simultaneous back-extraction to another aqueous phase by allowing the organic drop to serve as a liquid membrane between the two aqueous phases [91], online extraction procedures where the microdrop is hanging from the end of a CE capillary (SDME-CE) [92], and extractions from the headspace of samples (HS-SDME) [93]. An electric field has also been applied to a three phase SDME system, where an aqueous drop is hanging from a pipette tip into an organic solvent phase above the aqueous sample solution [56]. The electric field was applied between the sample solution and the pipette tip as a way to reduce extraction time. However, the stability of the drop was still an issue with this technique, especially at higher voltages.

Both microextractions into liquid (SDME) and solid (SPME) media have served as fundaments for newer microextraction techniques that use the same principles with other configurations and modifications. Combinations of these techniques with either SLMs, electric fields, or both have started new trends in the field of microextractions. Some of these techniques will be discussed in more detail below.

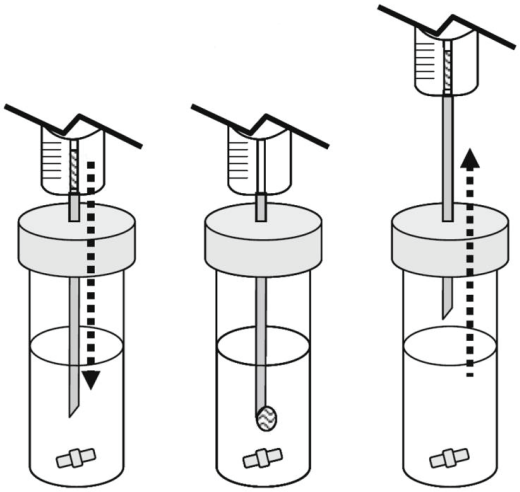


Figure 1.5: *The general procedure for SDME. A droplet is pushed out of a syringe (left) so it is suspended in the sample solution, hanging from the syringe tip (middle). After the extraction the droplet is withdrawn into the syringe and can be injected into for example a GC instrument (right). Reprinted with permission from [87] © Springer Verlag (2009).*

1.4.3 Hollow fiber liquid phase microextraction

As an alternative to SDME as a microextraction technique between liquids, three phase hollow fiber liquid phase microextraction was presented in 1999 (HF-LPME) [28]. In this system, an SLM was utilized as a barrier between two aqueous phases, thus limiting the challenges in SDME with unstable droplets of organic solvent and emulsion formation at the aqueous-organic interface [60]. The resulting device is shown in Figure 1.6 A and makes up a three phase system with two aqueous phases and a thin layer of water immiscible organic solvent (SLM), impregnated in the pores in the wall of a porous hollow fiber membrane through capillary forces. The principle of extraction is similar to that of SLM extractions (section 1.2) and is governed by passive diffusion from the aqueous sample solution, through the organic solvent in the SLM, and into a small volume of an aqueous acceptor solution [60,94-96]. To ensure efficient transport across the SLM, the pH is adjusted so that the analytes in the sample solution are uncharged for better migration into the organic solvent. The acceptor solution pH is adjusted to a pH that charges the analytes, thus trapping them in the acceptor solution, making the transport unidirectional [94]. Agitation of the device is performed to increase efficiency. However, HF-LPME is not an exhaustive technique and the final recovery is determined by partition coefficients and volumes of the sample, SLM and acceptor solutions [95]. The analytes in the sample are selectively isolated and enriched in a

small volume of acceptor solution in the lumen of the impregnated hollow fiber. Substances that remain charged in the sample solution will not penetrate the SLM, and neutral substances will not be trapped and enriched in the acceptor solution. Highly hydrophobic substances will be trapped inside the SLM, and large particles will be excluded based on the pore size of the membrane [97]. These factors make HF-LPME a selective technique that can be tuned towards certain analytes of interest.

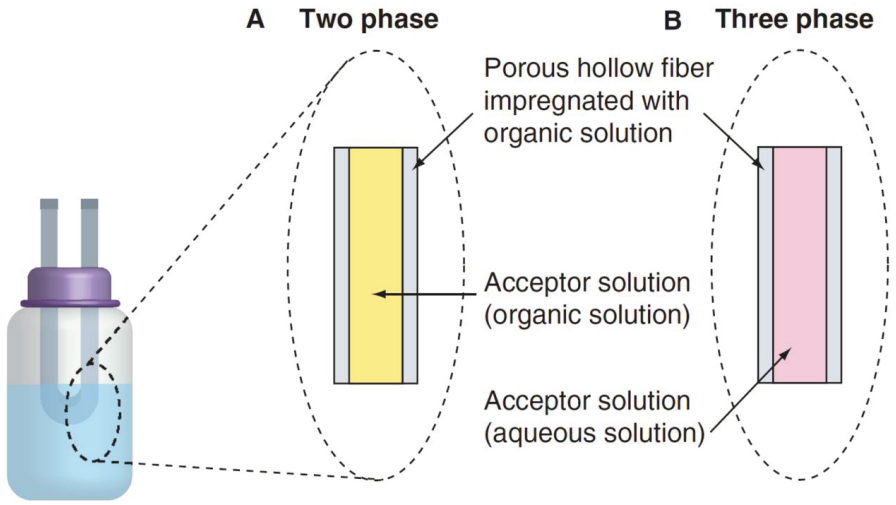


Figure 1.6: *A typical setup for HF-LPME as either a two phase system (A) or three phase system (B). In the two phase system both the SLM and acceptor compartment is filled with an organic solvent, whereas in the three phase system, the acceptor solution is an aqueous solution. Reprinted with permission from [66] © Future Science Ltd. (2013).*

The extraction process is mainly governed by the distribution coefficient between the sample and SLM according to the following mathematical equation, with the assumption of unidirectional transport from the sample to the acceptor phase [98]:

$$J_i(t) = P_i^{D\to A} C_{D_i}(t) = -\frac{V_D}{A_f} \cdot \frac{dC_{D_i}(t)}{dt} \qquad (2)$$

where $J_i(t)$ is the steady state flux of analyte over the SLM at time t after a certain lag time in the system. $C_{D_i}(t)$ is the analyte concentration in the sample at time t, V_D is the volume of the sample solution, and A_f is the exterior surface area of the SLM in contact with the sample. $P_i^{D\to A}$ is the membrane permeability coefficient from the sample to acceptor solution, and can

be expressed in terms of the sample-membrane phase distribution coefficient, K_{di}, the diffusion coefficient of the analyte in the SLM, D_{mi}, and the thickness of the membrane, h.

$$P_i^{D\to A} = \frac{D_{mi} \cdot K_{di}}{h} \qquad (3)$$

The concentration in the acceptor solution can thus be described according to the following equations, where a certain lag time (t_{lag}) for the analytes to enter the acceptor solution is taken into account:

$$C_{A_i}(t) = 0 \qquad t < t_{lag} \qquad (4a)$$

$$C_{A_i}(t) = \frac{V_D C_{D_i}^0 - C_{D_i}(t)\left(V_D + K_{d_i} \cdot V_m\right)}{V_A} \qquad t \geq t_{lag} \qquad (4b)$$

where $C_{A_i}(t)$ is the analyte concentration in the acceptor solution at time t, $C_{D_i}^0$ is the initial analyte concentration in the sample, V_m is the apparent volume of the SLM, and V_A is the volume of the acceptor solution.

In the period following the introduction of HF-LPME, several variations and modifications of the system have been introduced. Several methods take advantage of different additives or specialized solvents in one of the phases to facilitate extraction of certain analytes or improve the technique in other ways, as described in section 1.2 [99-104]. An especially popular modification is two phase LPME, as shown in Figure 1.6 B, where the acceptor solution consists of the same organic solvent as the SLM [105]. In this system the analytes are kept uncharged in the sample to ensure efficient penetration into the extracting phase contained in the SLM and the lumen of the hollow fiber, and it is well suited for extracting hydrophobic analytes. Several automated systems have also been presented [106-109], and recently the use of LPME in a 96 well plate format, where multiple extractions can be performed simultaneously was introduced under the name parallel artificial liquid membrane microextraction (PALME) [29]. Another important modification is the utilization of the HF-LPME principle in combination with an electric field, which has developed into a technique called electromembrane extraction (EME). This technique is discussed in further detail in section 1.5, section 3, and in the articles in this thesis.

HF-LPME offers several benefits, such as being a highly selective sample preparation technique that gives clean extracts, possibilities of high enrichment factors, ease of automation, low costs, low consumption of organic solvents, robust extractions, and low instrumentation requirements [60,95,110-112]. In addition, since the hollow fiber is disposable, the possibilities for carryover are limited, ensuring better reproducibility [97]. HF-LPME is also compatible with several analytical instruments. In three-phase mode, the extract is usually directly compatible with instruments that handle aqueous samples, such as RP-HPLC, UPLC, LC-MS, and CE [28,113,114]. The extract in two-phase mode can usually be directly injected in GC systems [109,113]. Some limitations of the technique have, however, been reported. These are mainly related to difficulties in extraction of very polar analytes, limited recovery, long extraction times due to slow diffusion across the SLM, air bubbles attached to the surface of the hollow fiber, and hydrophobic matrix components blocking the pores in the hollow fiber [66,95,115].

The versatility of HF-LMPE has made it a popular technique in many applications, such as determination of drugs in biological samples [28,60,116,117], metal ions from various matrices [118,119], pollutants in environmental and food samples [120-124], and determination of peptides from aqueous samples [102,103].

1.5 Electromembrane extraction

The concept of electromembrane extraction (EME) was introduced in 2006 under the name electromembrane isolation, and it was presented as a technique that offered high extraction recoveries in a relatively short extraction time [19]. It is based on the extraction system for HF-LPME, but adds an electrokinetic component to the mass transfer of analytes, which effectively overcomes the limitation of long extraction times in HF-LPME. Since its release in 2006, several articles and reviews discussing EME have been published, key extraction parameters have been identified, the theoretical understanding of the technique has been improved, and several applications have been presented [38,49,66,96,120,125-130]. This section will give an overview of theoretical and practical aspects of EME, in addition to a range of applications where the technique has been used up to the time the work on this thesis started. Publications regarding EME after 2010 are thus not included in the overview given in this section. The results and discussion part of this thesis (section 3), and the articles this thesis is based on, go deeper into the theoretical foundation of EME and present new insight into several theoretical aspects of the technique.

1.5.1 Introduction to the EME principle

The principle of EME is similar to that of HF-LPME, and a common EME setup is illustrated in Figure 1.7. A porous hollow fiber is immersed in an organic solvent to make an SLM. The lumen of the SLM is filled with a small volume of aqueous acceptor solution, and the hollow fiber is inserted into the sample solution, resulting in a three phase extraction system. Electrodes are then inserted into the sample and acceptor solution. Extraction is performed by applying an electric field (direct current) between the electrodes, causing mass transfer of analytes from the sample solution, through the organic solvent in the SLM, and into the acceptor solution [19]. Agitation of the system is performed to reduce the thickness of the boundary layer between the sample solution and the SLM [131].

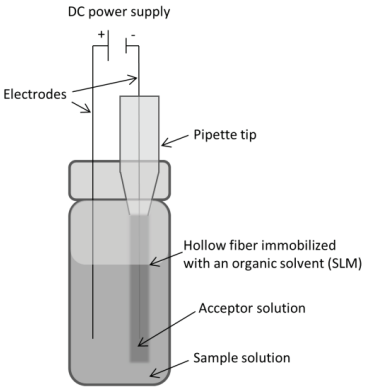


Figure 1.7: *A typical setup for EME. Reprinted with permission from paper III © Elsevier B.V. (2013).*

The main force of mass transfer in EME has been found to be electrokinetic migration, and passive diffusion plays only a minor role, especially for the short extraction times used (5-15 minutes) [19,131]. This is in contrast to HF-LPME where mass transfer is governed by passive diffusion over a pH gradient [95]. For the electric field to have an impact on the mass transfer of an analyte, the analyte have to possess an ionizable group that is charged during extraction. In HF-LPME, the charge of the analytes is suppressed in the sample solution to facilitate penetration of the SLM, and maintained in the acceptor solution to reduce back extraction. However, in EME the pH in both sample and acceptor solutions should maintain the charge on the analytes. Thus, to extract basic substances, an acidic pH is used in both aqueous phases, while the cathode is placed in the acceptor solution and the anode in the sample solution [19]. The opposite is true for acidic substances [132].

The extraction recoveries (R) during EME may be calculated according to the following equation:

$$R = \frac{n_{A,final}}{n_{D,initial}} * 100\ \% = \frac{C_{A,final} * V_A}{C_{D,initial} * V_D} * 100\ \% \qquad (5)$$

where $n_{A,final}$ is the amount of analyte present in the acceptor solution at the end of the extraction, and $n_{D,initial}$ is the amount of analyte initially present in the sample. $C_{A,final}$ is the concentration of analyte in the acceptor solution after extraction, while $C_{D,initial}$ is the initial concentration in the sample. V_A and V_D are the volumes of the acceptor and sample solution respectively. This leads to enrichment factors (E) according to the equation:

$$E = \frac{C_{A,final}}{C_{D,initial}} \quad (6)$$

Because of small volumes of acceptor solution relative to possibly large volumes of sample solution, the enrichment factors can be high in EME [133].

The similarities between HF-LPME and EME cause EME to have the same benefits of being a cheap, robust, simple, and environmentally friendly technique, with good possibilities for automation, low instrumentation requirements, low chance of carryover, and good compatibility with analytical instruments (see section 1.4.3) [37,120,125,128]. However, the introduction of an electric field as the force for mass transfer introduced a new tool for controlling the selectivity, and it significantly reduced the extraction time compared to HF-LPME [120,125,131,134]. In addition, the use of an electric field can break drug-protein bindings, and has been suggested as a way to improve drug extractions from plasma samples [135]. The resulting extracts after EME have proved to be very clean, with minimal interferences from other matrix components [32,126].

1.5.2 Extraction theory and kinetics

In an EME system, the sample solution, SLM, and the acceptor solution act as an electrical circuit, where the SLM is the major source of electrical resistance [19]. The flow of current through an EME system reflects the flow of background ions and analytes across the SLM. Although the transport of substances through the system is increased with higher SLM current, a high current can also affect the stability of the system due to electrolysis at the two electrodes according to the following reactions [19]:

Anode reaction: $H_2O \rightarrow 2H^+ + \frac{1}{2}O_2 + 2e^-$

Cathode reaction: $2H^+ + 2e^- \rightarrow H_2$

A high current will thus generate a substantial amount of bubbles in the sample and acceptor solution, caused by O_2 and H_2 gas formation, as well as affecting the pH. Based on this, a compromise has to be made between transport efficiency and tendency of bubble formation by using optimal organic solvents and applied voltages [19,132,136].

Since the main source of electrical resistance is the SLM, the electrical field strength (V/cm) is very high in this area. However, the effect of transportation through the SLM varies according to the degree of retained charge on the analytes [19]. Analytes that keep their charge are believed to be very effectively transported through the SLM, while analytes that easily lose their charge are discriminated, causing differences in recovery. The pH environment in the sample and acceptor solution is adjusted so that effective analyte migration occurs.

A theoretical model for the flux across the membrane was presented in 2007 and was verified experimentally [137]. The model shows that the steady state flux of an ionic substance (J_i) through the EME system can be described, based on the Nernst-Planck flux equation, according to the following equation:

$$\mathrm{J_i} = -\frac{D_i}{h}\left(1+\frac{\nu}{\ln\chi}\right)\left(\frac{\chi-1}{\chi-\exp(-\nu)}\right)\left(c_i - c_{i0}\exp(-\nu)\right) \qquad (7)$$

In this equation, D_i represents the diffusion coefficient of the ion in question, h is the SLM thickness, χ is the total ion concentration ratio between the donor side and the acceptor side, c_i is the concentration of the analyte at the interface between the sample solution and the SLM, and c_{i0} represents this concentration at the interface between the SLM and the acceptor solution. ν is a dimensionless driving force defined as:

$$\nu = \frac{z_i e \Delta\phi}{kT} \qquad (8)$$

where z_i is the charge of the ion, k is the Boltzmann constant, e the elementary charge, $\Delta\phi$ the electrical potential difference across the SLM, and T the absolute temperature.

Assuming that the system is kept under stable operational parameters, equation 7 and 8 predicts that the flux across the membrane can be increased by decreasing the ion balance (χ) or increasing the potential difference ($\Delta\phi$) by increasing the extraction voltage. These

predictions have been justified experimentally [137]. The effect from adjusting the temperature, however, is difficult to predict, since the diffusion coefficient (D_i) also relies on temperature. Experiments have suggested that the effect of temperature on the dimensionless driving force is insignificant compared to its effect on the diffusion coefficient [137].

Even though a mathematical model for the flux across the membrane and some experimentally verified theories for the extraction process have been presented, a thorough understanding of the distribution of analytes through the system and how the organic solvent in the SLM affects the extraction kinetics has been lacking. This thesis has further investigated these aspects of EME, which is presented in section 3.2 and in the articles this thesis is based on.

1.5.3 Factors of importance for extraction

The mathematical equations for flux across the membrane and the extraction theory discussed in section 1.5.2 present some important and adjustable parameters for optimal extractions. Several articles have been published where parameters such as pH of the sample and acceptor solution, different types of organic solvents in the SLM, extraction voltage, agitation, and extraction time have been investigated and optimized for certain extraction procedures [19,32,131,132,134,136,138,139]. This section will discuss these parameters and their importance for extraction performance.

1.5.3.1 Composition of the sample and acceptor solutions

The pH in the sample and acceptor solutions should be kept at a pH level that ensures ionization of the analytes, so that the electric field has an effect on the mass transfer, as discussed in section 1.5.1. Several experiments have verified this, and commonly 10 mM HCl has been used as both acceptor and sample solution for the extraction of basic analytes [19,32,131,134,136,138], while 10 mM NaOH serves the same function when extracting acidic analytes [132]. However, the pH in the sample solution, in contrast to the pH in the acceptor solution, has been seen to only have a minor effect on extraction recovery and efficiency [19,132,140]. This has also been seen in several articles, where extractions from untreated samples, even from highly complex biological matrices, have been achieved with acceptable recoveries, good validation data, and short extraction times [36,135].

It is possible to obtain high enrichment factors by using a much smaller volume of acceptor solution than sample solution. However, the extraction process itself is more effective when the sample volume is smaller as a result of reduced distance between the electrodes and thus a

stronger electrical field [131,134]. A reduction in efficiency of the extraction process has also been seen from biological matrices such as untreated plasma, where the kinetics became slower compared to diluted samples as a result of high viscosity and protein binding in the sample [135].

According to equation 7 in section 1.5.2, the ion balance between the sample and acceptor solution can affect the ion flux across the SLM negatively [137]. This can happen if the presence of salts is high in the sample solution, or if there is a large difference in background ion concentrations. Practical investigations have shown support to this theory [134,138].

1.5.3.2 Extraction voltage

Increasing the extraction voltage will generally increase the flux of ions over the membrane, as discussed in section 1.5.2. However, to find the optimal extraction voltage, the stability of the system has to be taken into account. An extraction voltage that is too high will cause stability issues such as excessive electrolysis and bubble formation, with a resulting loss in repeatability between samples [19,132,134]. In addition, a high voltage can cause excessive joule heating with a resulting loss of organic solvent in the membrane, thus compromising the integrity of the three phase system [141]. The optimal extraction voltage is also highly dependent on the electrical resistance in the system, and it has to be adjusted according to the choice of organic solvent in the SLM [19,32,139]. However, low voltage extractions have been performed successfully in several cases, for instance by using a common 9 V battery as a power source [32,36]. Extractions with lower voltages have a potential as a method to increase the selectivity towards compounds that migrate more efficiently through the SLM and as a way to extract analytes prone to electrochemical degradation [49,125].

1.5.3.3 Agitation

Agitation of the system during extraction has been described as an important factor for efficient extractions, with an increase in recovery from 8-10 % with no agitation to 70-79 % with optimal agitation when extracting five model drugs [19]. This has been suggested as a result of better convection in the sample compartment, causing the boundary layer between the sample solution and the SLM to be narrower [19,131,132]. A narrow boundary layer will increase the migration efficiency from the sample to the SLM, which has been suggested as a possible rate limiting step in EME [131]. The benefit of agitation is considered insignificant in the small volumes of acceptor solution and organic solvent in the SLM or when extracting from very small volumes of sample solution [36,131].

1.5.3.4 Extraction time

In general, the extraction time in EME has been very short compared to techniques governed by passive diffusion. Compared to HF-LPME, with extraction times of around 45-60 minutes [95], common extraction times for EME have been around 5-15 minutes or even lower [36,131-134,140]. After this time, the system enters a steady state in terms of recovery. The reason for not reaching full recovery has been suggested to be caused by a suppression of the net transfer of analytes, as a consequence of high concentration build up in the acceptor solution, and potential back extraction to the SLM [19,32,132].

1.5.3.5 Supported liquid membrane

The SLM serves as the main boundary between the sample and acceptor solution, the main source of electrical resistance in the extraction system, and as an important factor for controlling the distribution ratios of drugs into the SLM (equation 3) [19,138]. To maintain a three phase system during the extractions and avoid the organic solvent in the SLM to leak out into the aqueous solutions, it is important to choose an organic solvent with low water solubility. However, the solvent should maintain a certain polarity to achieve sufficient electrical conductance [19]. The emulsifying properties of plasma samples can, however, to a certain extent disrupt the SLM by dissolution of the organic solvent [135,142], so the organic solvent should be chosen with care. Adding a small droplet of organic solvent inside the lumen of the hollow fiber has been suggested as a way to stabilize the SLM in these cases [135].

Common solvents that have proved effective in EME include nitroaromatics, such as 2-nitrophenyl octyl ether (NPOE), 1-isopropyl-4-nitrobenzene (IPNB), and 1-ethyl-2-nitrobenzene (ENB) for relatively unpolar basic analytes (log $P > 2$), and aliphatic alcohols such as 1-octanol for acidic analytes [132,134,138,140]. More polar drugs (log $P < 2$) and peptides have been successfully extracted with the introduction of carrier molecules in the SLM, such as di-(2-ethylhexyl) phosphate (DEHP) [136,138,139,142,143]. The carriers effectively reduce the polarity of the analytes by forming analyte-carrier complexes, which facilitate the migration across the SLM [136,139].

The membrane support material has been porous polypropylene in a flat sheet format or hollow fiber format [19,128,134,144]. A small pore size effectively excludes particulate matter, but the support material is in general supposed to be inert in the extraction process.

1.5.4 Modifications to the original EME format

Some modifications to the original EME format have been presented after the first publication in 2006. In all cases the main principle for extraction is the same, but the modifications can solve practical challenges in certain situations. One of these techniques involves extraction from a single drop of sample solution of approximately 10 µL [144]. The extractions were performed through a flat polypropylene membrane, impregnated with NPOE, into an acceptor solution volume of 10 µL (Figure 1.8). In this case, the sample reservoir was a well created in a sheet of aluminum foil, which also served as the anode. The cathode was an electrode placed in the acceptor solution. This setup allowed extractions from very small amounts of sample solution.

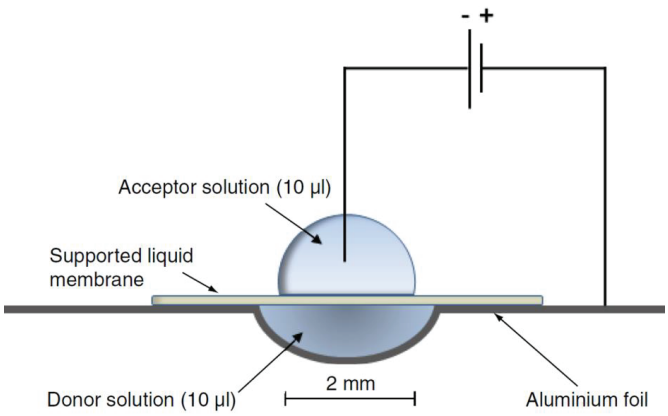


Figure 1.8: *A schematic illustration of an EME setup, where extractions are performed from a single drop of sample/donor solution. Reprinted with permission from [128] © Future Science Ltd. (2011).*

Another format allowed larger acceptor solution volumes (up to 100 µL) by using an envelope of flat polypropylene sheets, impregnated with 1-octanol, as the SLM instead of the original hollow fiber format [134,141]. The use of higher acceptor solution volumes has been suggested as a possible way of improving extraction recoveries [128]. The SLM envelope format has later been used in a setup to simultaneously extract both basic and acidic substances, as shown in Figure 1.9. In this setup, two envelopes are heat-sealed together to make a three compartment envelope. The outer chambers contained solutions of different pH values, while the chamber between them was filled with 1-octanol as an acceptor solution. The SLM was impregnated with toluene, and the electrodes were positioned so that the anode

was in the acidic solution and the cathode in the alkaline solution. When an electric field was applied, the analytes migrated towards the electrode of opposite charge. Because of the pH values in the media around the electrodes, the charge of the analytes were then neutralized, allowing them to be extracted into the organic acceptor solution [145].

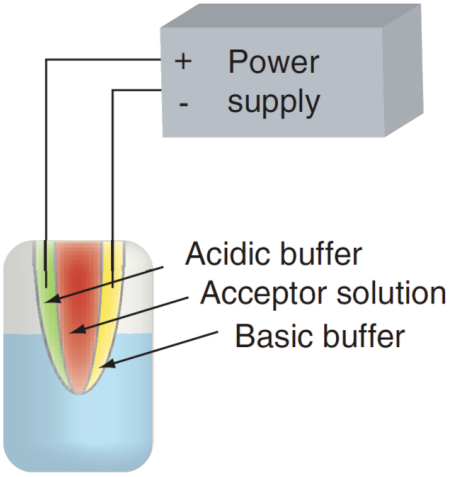


Figure 1.9: *The general setup for EME, using a three compartment envelope for simultaneous extraction of both acidic and basic analytes. Reprinted with permission from [66] © Future Science Ltd. (2013).*

A downscaling of EME to the chip format was presented in 2010 [146]. In this setup, an SLM consisting of a flat sheet of porous polypropylene membrane impregnated with an organic solvent was bonded between two polymethyl methacrylate (PMMA) substrates. A channel with flowing sample solution introduced the sample to the SLM on one side, while an acceptor solution chamber was located on the other side. By adding a potential across this SLM, extraction was performed. The on-chip device has made it possible to use very low volumes of both sample and acceptor solution and to obtain high enrichment of analytes [146].

1.5.5 Applications and performance of EME

The years following the introduction of EME in 2006 have shown a gradual increase in the amount of applications using the technique. The first publication was done in combination with CE-UV on the basic drugs pethidine, nortriptyline, methadone, haloperidol, and loperamide as model analytes [19]. In this setup, 300 V was applied for 5 minutes over an SLM comprised of NPOE, providing recoveries of 70-79 % with less than 16 % RSD from diluted and acidified water, plasma, and urine. A later publication presented a similar EME setup, and it showed that EME can provide reproducible extractions of these analytes from both human plasma, urine, and breast milk, after dilution and acidification of the biological matrix, with recoveries and enrichment factors up to 55 % and 37, respectively [32]. In this case the extractions were performed with a common 9 V battery as power supply. The same analytes have also been used to evaluate the downscaling of EME into the microchip format

and the drop-to-drop EME format from only 10 µL sample solution (see section 1.5.4) [144,146]. These extractions were performed by using distilled water and human urine as sample matrices in the first case, and distilled water, human urine, and plasma in the latter case. CE-UV was used for analyzing the extracts in both cases.

Basic drugs have also been determined from various sample matrices in other applications. It has been shown that it was possible to achieve acceptable recoveries (12-22 %) and low detection limits (0.4-2.3 ng/mL) for parallel extraction of three samples simultaneously, containing the psychiatric drugs amitriptyline, citalopram, fluoxetine, and fluvoxamine, by using very short extraction times (1 minute) [36]. LC-MS was used for analyzing the extracts. The sample matrix was 70 µL untreated human plasma, and the analytes were extracted with reproducibility in the range 3.2-8.9 % RSD through an SLM filled with ENB. A common 9 V battery was used as the power source for extraction in a home-made, small, and mobile EME device. Higher recovery was obtained by using longer extraction times, but this publication showed that acceptable performance can be achieved from untreated biological samples in a very short time. This method was also tested on real patient samples, showing that EME combined with LC-MS was able to detect therapeutic levels of the extracted drugs with results comparable to other methods. EME from untreated biological matrices have also been investigated, where seven basic drugs were extracted from untreated human plasma and whole blood with CE-UV as the method for analysis [135]. In this case, ENB was used as the SLM solvent, and recoveries ranged from 19 to 51 % after 10 minutes of extraction, with reproducibility values showing less than 17 % RSD (n=6). Both this and the previous application showed that EME can be performed at physiological pH from untreated sample matrices.

The use of EME as a sample preparation step in chiral analysis was presented for extractions of the enantiomers of the basic drug amlodipine, where EME was performed from acidified human plasma and urine [140]. NPOE was used as the SLM solvent, and repeatability data in the range 4.4 to 13.4 % RSD and recovery up to 83 % were obtained. This provided limits of detection down to 3 ng/mL and enrichment factors up to 124. The EME extracts were analyzed by cyclodextrin modified CE-UV.

By tuning the extraction parameters towards acidic extractions, it was possible to perform successful and reproducible extractions of a range of eleven acidic drugs from aqueous samples with up to 100 % recovery by using 1-heptanol as the organic solvent in the SLM and

CE-UV for analysis of the extracts [132]. This added to the versatility of EME by showing that both acidic and basic substances can be extracted well by tuning the extraction parameters. The viability of EME in combination with CE-UV for determination of acidic drugs was also shown in a more recent publication [145]. In this case ibuprofen, naproxen, and ketoprofen were used as model analytes together with the basic drugs norephedrine, alprenolol, and propranolol in a modified setup to extract both acidic and basic drugs simultaneously (see section 1.5.4). The extraction system showed a repeatability better than 13 % RSD (n=6), recoveries of up to 80 % and enrichment factors up to 370, and it was able to detect the presence of some of the model analytes in unspiked wastewater samples.

EME has also been applied to some environmental pollutants and investigated in environmental sample matrices. The nerve agent degradation products methylphosphonic acid (MPA), ethyl methylphosphonic acid (EMPA), isopropyl methylphosphonic acid (IMPA), and cyclohexyl methylphosphonic acid (CMPA), were determined in an EME application, in combination with contactless conductivity detection (CE-C4D), from spiked river water samples [141]. In this case the sample solution was untreated, and the anaytes were extracted through an SLM impregnated with 1-octanol. The publication presents recovery data for the analytes ranging from 1.1-56.7 %, with a variation of 2.2-8.8 % RSD. In addition, an application for determination of the chlorophenol pesticides 4-Chlorophenol (4CP), 2,4-dichlorophenol (24DCP), 2,4,6-Trichlorophenol (246TCP), and pentachlorophenol (PCP) in spiked sea water, also with 1-octanol as the organic solvent in the SLM, has been published [134]. Recovery values up to 74 % were reported with an RSD of 6.8 % (n=6), giving a detection limit of 0.1 ng/mL and enrichment factors of up to 23. HPLC was used for analyzing the extracts in this application.

EME as an extraction technique for heavy metals was introduced in 2008 [133], where lead ions were extracted from human amniotic fluid, serum, and urine, as well as in lipsticks, followed by CE-UV analysis. Toluene was used as the SLM solvent, and the method obtained enrichment factors as high as 557 after 15 minutes of extraction. The detection limits were reported as 19 ng/L with repeatability of 4.9-15.6 % RSD (n=3).

A slightly modified EME system, utilizing only two aqueous phases, was presented in 2008, where 1-octanol was used both as the organic solvent in the SLM and as the acceptor solution to ensure compatibility with GC analysis [147]. The system was operated at 60 V for 4

minutes, and it was used to study the mass transfer of the trace compounds nitrobenzene, aniline, and phenol between aqueous media and 1-octanol.

In 2009 the first publication on EME of peptides was presented [139]. In this publication, eight different peptides with amino acid lengths between three and 13 were extracted and analyzed by CE-UV. An SLM containing 15 % DEHP in 1-octanol was used, and recoveries of up to 61 % were obtained after five minutes of extraction. Repeatability data showed less than 21 % RSD (n=6). The concept of peptide extractions and its principal operational parameters were further investigated [143], and it lead to an application for determination of the vasoactive angiotensin peptides angiotensin 1, 2, and 3 from acidified human plasma [142]. In this setup, an SLM containing 8 % DEHP in 1-octanol was used, and the samples were extracted for 10 minutes. The optimized extraction method, combined with LC-MS, generated reproducible data (5.6-11.6 % RSD, n=6), with limits of detection at the pg/mL level and recoveries up to 43 %. These publications showed a potential for the use of EME for peptide extractions. Although shown previously as a good way to improve the extraction of polar basic analytes [136,138], this was the first time an SLM containing DEHP was used in an EME application to improve extraction performance.

During the work with this thesis, the amount of applications on EME and the theoretical understanding of the technique have improved substantially. Applications on several new matrices and analytes have been presented and improvements to the technique have been suggested. Some of this progress is discussed in section 3 and in the publications this thesis is based on.

2 AIM OF THE STUDY

The main goal of this study has been to further develop the theoretical foundation of EME as a sample preparation technique for biologically active substances. Previous publications have identified key parameters for making an efficient EME setup, and some successful SLM solvents have been used. However, systematic knowledge about several aspects of the EME process from a theoretical point of view was limited at the start of this study. The focus has thus been to systematically investigate key aspects of the EME process to establish a better theoretical platform for selecting optimal extraction parameters. The following key areas have been investigated to achieve this goal:

- Investigation of relevant solvent properties for efficient SLM solvents for extraction of:
    - Basic drugs (**Paper IV**)
    - Peptides (**Paper I**)
- Development of a mathematical model for the distribution of analytes throughout the extraction process based on observed extraction kinetics and analyte distribution in EME (**Paper II**)
- Investigations of EME stability:
    - How to identify and measure stability issues? (**Paper III**)
    - Extraction parameters causing stability issues (**Paper III**, **IV**, and **V**)
- Investigations on how samples containing organic solvents affect the extraction recoveries and kinetics, reproducibility, and membrane current (**Paper III**)
- Investigations on how samples containing substantial amounts of salts in the sample solution affect extraction recoveries and kinetics, reproducibility, and membrane current (**Paper V**)

3 RESULTS AND DISCUSSION

This section contains key results from the papers included in this thesis, put into context according to the aim of the study. The results are discussed in more detail in the individual papers.

3.1 Selection of model analytes and extraction conditions

3.1.1 Extraction conditions and technical setup

The extraction conditions and technical setup of the EME system was based on previous publications and experience from this research group, and is depicted in Figure 1.7 [19,131,135,136,138,139,142,143]. A commercially available porous polypropylene hollow fiber with a pore size of 0.2 µm, wall thickness of 200 µm, and internal diameter of 1.2 mm was used as support material for the SLM. This material has been compatible with a broad range of analytes and organic solvents, without signs of degradation [136,138,142,148,149]. The lumen of the hollow fiber served as a compartment for the acceptor solution. A 2.4 cm piece of hollow fiber was used for each extraction, mechanically sealed by a pincer in the lower end, and attached by heat to the 2.1 cm end of a pipette tip in the upper end. The pipette tip served as a guiding tube for the electrodes and the microsyringe that was used to add or remove acceptor solution. To make the SLM, the hollow fiber was immersed in an organic liquid for at least 5 seconds, and excess solvent was wiped off with a medical wipe. Various compositions of organic liquids, with or without carrier molecules, were tested in the different papers. After adding the acceptor solution to the lumen of the hollow fiber by a microsyringe, the hollow fiber was inserted into the sample solution through a punched hole in the lid of its compartment. The sample compartments were glass vials for drug substances (**Paper II-V**) and either polypropylene or polyethylene vials for peptides (**Paper I and II**) to reduce the degree of surface binding. The volume of sample solution was usually 1 mL, except in **Paper I** where 500 µL was used. The volume of acceptor solution was always 25 µL. The sample and acceptor solutions were acidified by 10 mM HCl for all basic drug extractions (**Paper II-V**) and with 1 mM and 50 mM HCl, respectively, for the peptides (**Paper I** and **II**). Platinum electrodes with a thickness of 0.5 mm were placed in the sample (anode) and acceptor solution (cathode), and connected to a DC power supply ranging from 1 to 300 V. This setup resulted in a closed circuit, where the SLM was the main point of electrical resistance. Extraction was performed by applying a voltage (SLM solvent dependent) over this circuit. Agitation of the system during the extractions reduced the thickness of the boundary layer in

the interface between the sample solution and the SLM [19,131,132]. The current in the circuit was monitored and plotted over time throughout the extraction as an indication of system stability. After a predetermined extraction time, the acceptor solution was removed and transferred to vials for CE (**Paper I**), HPLC (**Paper II-V**) or LC-MS (**Paper III**) analysis.

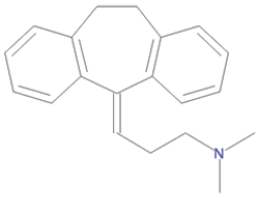
3.1.2 Model analytes

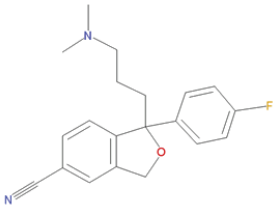
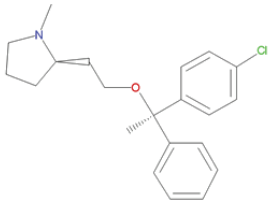
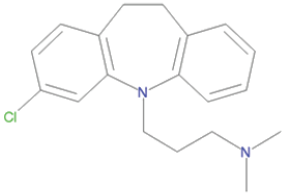
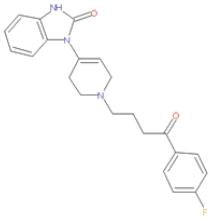
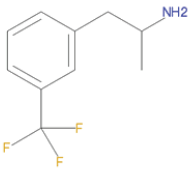
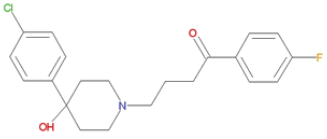
The model analytes were selected to represent a broad range of physiochemical properties in their respective categories; unplolar basic drugs (**Paper II-V**) and peptides (**Paper I and II**). Several of the model analytes used during the work with this thesis had already been used in other fundamental investigations on EME [19,32,35,36,131,135,138,139,142-144,146,150-155]. The reason for choosing several of the same analytes was to make it easier to compare the results to previous observations and because these analytes were already established as good candidates for investigation of the fundamental aspects of EME.

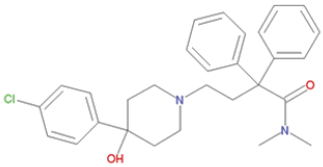
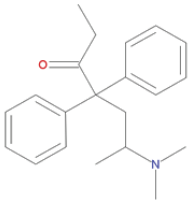
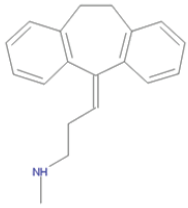
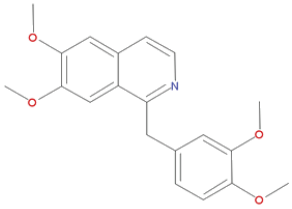
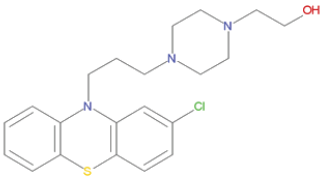
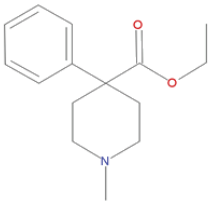
3.1.2.1 Unpolar basic drugs

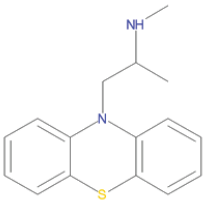
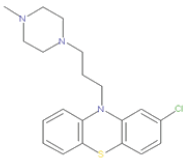
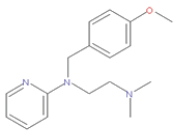
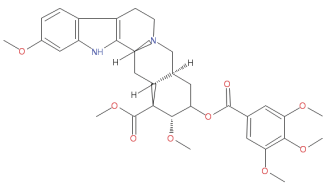
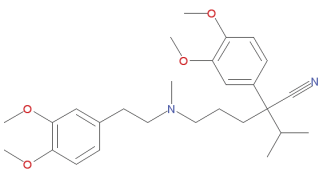
The unpolar basic drugs used in this thesis are shown in Table 3.1, together with their structure, log P values, and pK_a values. The term unpolar was in this case defined as substances with a log P value above 2. More hydrophilic substances have been investigated previously and required the addition of a carrier, such as DEHP, to promote extraction [138]. All the selected basic drugs have log P values between 2.46 to 5.04. Their pK_a values are between 2.68 and 10.47, and all the analytes were thus charged when dissolved in the sample or acceptor solution containing 10 mM HCl (pH 2). All the drugs obtain either a single or double charge distributed on both aliphatic and aromatic amine functional groups. Other criteria for the selection of the final mixture of model analytes were that they should be well separated and give a good signal response from the detector in the analytical method.

Table 3.1: *Structure, log P and pK_a values for the unpolar basic drugs used as model analytes in the work with this thesis. Both pK_a values are shown for substances that can be doubly charged during extractions.*

Drug name	Structure	log P[a]	pK_a[a]	Used in
Amitriptyline		4.81	9.76	**Paper V**

Citalopram		3.76	9.78	**Paper V**
Clemastine		4.92	9.55	**Paper II** and **V**
Clomipramine		4.88	9.20	**Paper II** and **V**
Droperidol		3.01	6.75	**Paper II**
Fenfluramine		3.47	10.22	**Paper V**
Haloperidol		3.66	8.05	**Paper II-V**

Loperamide		4.77	9.41	**Paper III-V**
Methadone		5.01	9.12	**Paper III-V**
Nortriptyline		4.43	10.47	**Paper II-V**
Papaverine		3.08	6.03	**Paper V**
Perphenazine		3.69	2.68/8.21	**Paper V**
Pethidine		2.46	8.16	**Paper III-V**

Promethazine		4.29	9.05	**Paper V**
Prochlorperazine		4.38	2.80/8.39	**Paper V**
Pyrilamine		3.04	5.32/8.76	**Paper V**
Reserpine		3.53	7.30	**Paper V**
Verapamil		5.04	9.68	**Paper V**

[a] pK_a and log P values were found using the web resource www.chemicalize.org (Chemaxon, Hungary).

3.1.2.2 Peptides

A range of commercially available model peptides were chosen based on previous work on EME of peptides [139,142,143] and to represent variety in terms of amino acid composition, molecular weight, hydrophilicity, and isoelectric point (pI). The chosen peptides are shown in Table 3.2. They are all biologically active and function as peptide hormones or mediators *in vivo*, where neurotensin, enkephalin, and endomorphin are neuroactive peptides and the angiotensins, bradykinin, and vasopressin are vasoactive peptides. Angiotensin 2 and 3 are

metabolites from subsequent cleavages of amino acids from angiotensin 1. Angiotensin 2 antipeptide is an angiotensin receptor antagonist.

Table 3.2: *Amino acid sequence, number of amino acid residues, molecular weight, log P, PI and pK_a values for the peptides used as model analytes in the work with this thesis.*

Peptide name	Amino acid sequence	Amino acid residues	Mw[a]	pI[a]	Net charge at pH 1[a]	Net charge at pH 3[a]	Used in
Angiotensin 1	DRVYIHPFHL	10	1296.5	7.91	+4	+3	**Paper I** and **II**
Angiotensin 2	DRVYIHPF	8	1046.2	7.76	+3	+2	**Paper I** and **II**
Angiotensin 2 antipeptide	EGVYVHPV	8	899.0	5.13	+2	+1	**Paper I** and **II**
Angiotensin 3	RVYIHPF	7	931.1	9.84	+3	+2	**Paper I** and **II**
Bradykinin	RPPGFSPFR	9	1060.2	12.4	+3	+2	**Paper I** and **II**
Endomorphin-1	YPWF	4	611.7	5.93	+1	0	**Paper I** and **II**
Leu-enkephalin	YGGFL	5	555.6	5.93	+1	0	**Paper I** and **II**
Neurotensin	pELYENKPRR PYIL	13	1673.0	9.84	+3	+2	**Paper I** and **II**
Vasopressin	CYFQNCPRG	9	1087.3	8.28	+2	+1	**Paper I** and **II**

[a] pI, pK_a, and molecular weight were calculated using the Innovagen peptide property calculator at www.innovagen.se (Innovagen AB, Sweden)

3.2 Investigation of theoretical aspects of EME

One of the main objectives of the work with this thesis has been to investigate theoretical aspects of EME. Before this work, a thorough understanding of the extraction process was lacking, and the number of solvents used as SLMs was strongly limited and selected based on trial and error. This section discusses the discoveries made throughout the work with this thesis related to the fundamentals of EME, with a focus on the theoretical understanding of the extraction process and characteristics of the SLM.

3.2.1 A phenomenological theoretical model for the extraction process

A model for the flux of an analyte in EME has been developed previously [137], as discussed in section 1.5.2, and describes factors that influence the transport of analytes through the SLM. This model, however, is not able to predict how the analytes are distributed in the different aqueous phases of the EME system at a certain time, and it does not describe the final extraction recovery. For HF-LPME, a model describing this distribution was presented in 2012 [98]. Based on this model and practical experiments on different types of analytes, a

similar model for the distribution of analytes in EME was developed (**Paper II**). The model was developed from experimental data on extractions with unpolar (log P > 2) basic drugs and peptides, extracted using different extraction times, and from the assumptions that the transport is uni-directional; the mass transport through the SLM is the rate limiting step; mass transport in the sample is not a limiting factor; and that there is a certain residence time ("lag time") for each analyte in the SLM before it reaches the acceptor solution. Most of these assumptions were verified experimentally (**Paper II** and **III**). If mass is preserved throughout the system, three equations can be derived from differentiating a general flux equation by using the above assumptions and the experimental data. These equations describe the time dependent concentration of an analyte (i) in the sample solution ($C_{D_i}(t)$), SLM ($C_{m_i}(t)$), and acceptor solution ($C_{A_i}(t)$) respectively:

$$C_{D_i}(t) = C_{D_i}^0 \cdot \exp\left(-\frac{A_f \cdot P_i^{D\to A}}{V_D} \cdot t\right) \tag{9}$$

$$C_{m_i}(t) = \frac{V_D\left(C_{D_i}^0 - C_{D_i}(t)\right) - V_A \cdot C_{A_i}(t)}{V_m} \tag{10}$$

$$C_{A_i}(t) = 0 \qquad t < t_{lag} \tag{11a}$$

$$C_{A_i}(t) = \frac{V_D C_{D_i}^0 - C_{D_i}(t)\left(V_D + K_d^* \cdot V_m\right)}{V_A} \qquad t \geq t_{lag} \tag{11b}$$

In these equations, the delay, caused by the residence time in the SLM for the analytes (t_{lag}) before it reach the acceptor solution, has been accounted for in the equation for the time dependent concentration in the acceptor solution (equation 11 a and b). The different parameters represent the initial ($t = 0$) concentration in the sample solution ($C_{D_i}^0$), the active surface area of the hollow fiber (A_f), the volume of the sample solution (V_D), the volume of the acceptor solution (V_A), the volume of the organic solvent in the SLM (V_m), and a distribution coefficient (K_d^*). This distribution coefficient is influenced by the electric field, and can be represented as:

$$K_d^* = \exp\left(\frac{z_i F}{RT}(\Delta_o^w\phi - \Delta_o^w\phi_i^0)\right) \tag{12}$$

where z_i is the charge of the analyte, F the faraday constant, R the gas constant, and T the absolute temperature. The last two terms are related to the hydrophobicity of the analyte ($\Delta_o^w\phi_i^0$) and the Galvani potential difference between the sample solution and the SLM ($\Delta_o^w\phi$).

The major differences between the distribution model for HF-LPME and EME are represented by the inclusion of an electric field in EME, and this is reflected by the voltage dependency of the distribution coefficient. Whereas the only force for mass transfer in HF-LPME was related to the analytes affinity to the SLM in a distribution process, the distribution coefficient in EME has been modeled with terms including both an electrophoretic and distributive component. According to equation 12, the electrical field plays a major role in affecting the distribution of analytes into the SLM, but if the analytes are sufficiently hydrophobic, they may also be extracted by passive diffusion, even in the absence of an electrical force. This was experimentally justified in **paper II**, where the basic drug droperidol showed slower kinetics than the other drugs, similar to a previous publication on the extraction of the same substance by HF-LPME [98]. Since droperidol was less basic than the other model analytes it was hypothesized that it was also less protonated in the aqueous solution, and thus went through the SLM in a deprotonated form, mainly by passive diffusion. The influence from the electric field on the mass transfer in EME was on the other hand justified through the time it took for trace levels of analyte to enter the acceptor solution (break-through time). This break-through time was greatly enhanced for both peptides and basic drugs when an electrical field was present. Additionally, the large contribution from the electric field can be observed through the short extraction time required to reach maximum recovery in EME (**paper II**) compared to HF-LPME [98].

Whereas previous publications on fundamentals of the extraction process has focused on the applied potential as the driving force for mass transfer in EME [19,131,137], the work with this model has shown that there is also a component related to passive diffusion that affects the extraction, especially for hydrophobic analytes.

3.2.2 Characteristics of the SLM

The theoretical model presented in **paper II** and section 3.2.1 introduces a voltage dependent distribution coefficient (equation 12) that controls the distribution of analytes into the acceptor solution. In this equation, the nature of the SLM plays an indirect but very essential role. Since the SLM is the main source of electrical resistance in the system, a change of organic solvent can greatly affect the Galvani potential difference between the sample

solution and the SLM ($\Delta_o^w\phi$). Figure 3.1 illustrates this voltage drop that occurs across the SLM, and the magnitude of this drop will depend on the organic solvent in the SLM. As a second factor, the type of organic solvent will affect the relative affinity of the organic solvent for the analyte ($\Delta_o^w\phi_i^0$), where an analyte can more easily penetrate one type of organic solvent than another. These observations gave insight into the importance and characteristics of the SLM composition in EME, and some of the papers included in this thesis present research on the SLM composition (**Paper I** and **IV**).

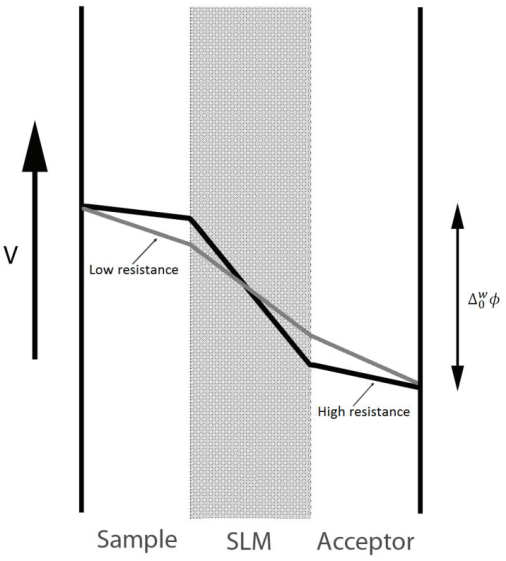


Figure 3.1: *Illustration of the voltage drop that occur across the SLM during EME. The black and grey line represent an SLM with high and low resistance respectively. Reprinted and modified with permission from paper II © Wiley-VCH Verlag (2013).*

Some practical requirements determine what SLM solvents it is possible to use in EME. These requirements are that the solvent has to be a liquid at room temperature, that its boiling point is not so low that the solvent will evaporate during extraction, and that the solvent is sufficiently water-immiscible to avoid dissolution into the aqueous solutions on each side of the SLM. These requirements, as well as availability and reasonable pricing of the solvents, were in mind when selecting organic solvents for the investigation of SLMs for EME (**Paper I** and **IV**).

Previous publications have used organic solvents such as NPOE, ENB, and IPNB for the extraction of unpolar basic analytes, as mentioned in section 1.5.3.5. These organic solvents have offered good stability and efficiency for EME, but the knowledge of why these solvents were effective was lacking. This topic was investigated in **paper IV** where 61 different organic solvents were chosen according to the above criteria, and subjected to a large screening. Through partial least square regression analysis (PLS), physical chemical parameters of the organic solvents that were correlated to recovery and stability of the EME system were identified. According to this screening, the best solvents were found to have low water solubility (< 0.5 g/L), a high

dipole moment, high proton acceptor properties and low proton donor properties. These characteristics, except for the water solubility, are important factors in cluster 2 of a Kamlet-Taft-based solvatochromic classification system [156] which can be a good theoretical starting point for the discovery of new efficient solvents. Previously known organic solvents for extracting hydrophobic basic drugs with EME comply with these characteristics, and especially the nitroaromatics are characterized by high proton acceptor capabilities and high dipole moments. In addition to the previously known effective organic solvents, several ketones and a few new nitroaromatics were also identified as effective. Table 3.3 shows the average recovery, average SLM current, and some important solvent parameters for some of these efficient solvents. The reason for the effectiveness of these compounds was suggested as being an interaction between the proton donor properties of the protonated basic drugs and the acceptor properties of the organic solvent. Based on this, both hydrogen bonding and dipole-dipole interactions probably play important roles during the transport across the SLM.

Table 3.3: *Some of the most successfull EME solvents for extraction of unpolar basic drugs with some relevant physical chemical parameters. Their optimal extraction voltage, with resulting average current and recovery, are also shown.*

SLM	Proton acceptor/donor sites[a]	log P[b]	Water solubility[a] (g/L)	Extraction voltage	Average SLM current	Average recovery
NPOE	4/0	4.86	2.66e-4	250 V	8.6 µA	67 %
2-nonanone	1/0	3.03	0.17	40 V	5,6 µA	61 %
6-undecanone	1/0	4.17	0.05	200 V	3,1 µA	51 %
2,4-dimethyl-1-nitrobenzene	4/0	2.94	0.32	20 V	7,4 µA	71 %

[a] Proton donor/acceptor sites and water solubility values were found using the web resource www.chemspider.com (Royal Society of Chemistry, United Kingdom)
[b] log P values were found using the web resource www.chemicalize.org (Chemaxon, Hungary).

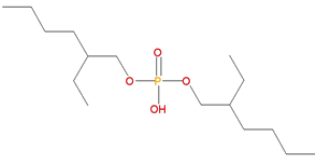
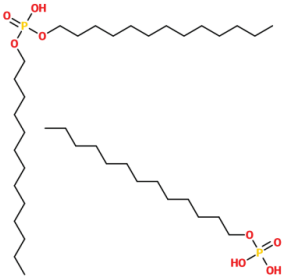
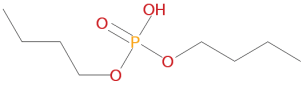
A similar screening was performed in **paper I**, where the analytes were peptides. All pure solvents tested were ineffective for extracting peptides and the addition of a carrier was necessary, as previously described in other publications [139,142,143,157]. Based on previous work on peptide extractions [139,142,143], several new solvent-carrier combinations were investigated. However, this work was done before the screening and investigation of physical chemical parameters for basic unpolar drugs presented in **paper IV**, and the focus for the screening in **paper I** was more related to identification of good solvent and carrier classes. In this aspect both aliphatic alcohols and ketones were identified as good solvents, and the importance of a carrier as an ion pairing reagent for the peptides at the sample/SLM

interface was confirmed. The importance of the interaction with a carrier to get the analytes into the SLM was also seen in **paper II**, where the distribution of peptides into the SLM was highly enhanced when a carrier was present.

In **paper I**, no clear link between the extraction efficiency and physical chemical properties of the organic solvents were seen. However, the importance of proton acceptor and dipole properties were suggested, based on the classification of the successful solvents to group II and VI in Snyder's solvent classification system [158,159]. The proton acceptor and dipole properties of several solvents in these groups are similar to solvents belonging to cluster II in the previously mentioned Kamlet-Taft-based solvatochromic classification system [156] and are thus consistent with the discoveries made in **paper IV**. The aliphatic alcohols also had a slight effect when extracting hydrophobic basic drugs, but even though they have good proton acceptor and dipole properties, they also have relatively high proton donor capabilities, which were suggested as the cause for their limited efficiency. Based on the structural diversity of peptides, however, this might be beneficial for the interaction between the peptides and the organic solvent, and could be a reason why aliphatic alcohols were more successful when extracting peptides in **paper I** than for hydrophobic basic drugs in **paper IV**.

Carriers have previously been seen as an effective additive when extracting both peptides and more polar basic drugs (log P < 2) [136,138,139,143,148,157,160]. Among the carriers tested in **paper I**, only mono- and dialkylated phosphates were effective, and in addition to DEHP that had been used previously, tridecyl phosphate (TDP, a commercial mixture of mono- and di-tridecyl phosphate) and dibutyl phosphate were highly promising. All of these carriers also had pK_a values which allowed them to be charged at the sample SLM interface at pH 3 and uncharged at the SLM/acceptor interface at pH 1.3. This confirms the previously suggested mechanism of complexation between the oppositely charged peptide and carrier at the sample/SLM interface and the release of peptides when the carrier gets protonated at the SLM/acceptor interface [139,148]. Especially the introduction of 2-nonanone and TDP as organic solvent and carrier was superior to the previously used combination of 1-octanol, di-isobutyl ketone, and DEHP, and this knowledge was used when investigating the extraction process for peptides in **paper II**. Table 3.4 shows the structure and some relevant physiochemical parameters for some of the effective carriers.

Table 3.4: *The most successful carriers for peptide extractions from Paper I along with their structure, log P, and pK_a values.*

Carrier	Structure	log P[a]	pK_a[a]
Di-(2-ethylhexyl) phosphate (DEHP)		5.78	1.94
Tridecyl phosphate (TDP)[b]		Monoalkylated: 4.68 Dialkylated: 10.39	Monoalkylated: 1.81 Dialkylated: 1.94
Dibutyl phosphate		2.38	1.94

[a] log P and pK_a values were found using the web resource www.chemspider.com (Royal Society of Chemistry, United Kingdom)
[b] Tridecyl phosphate was used as a commercial mix between mono and dialkylated phosphates.

Another interesting observation from the work with **paper I** was that the hydrophobic moiety of the organic solvent seemed to be important for extraction efficiency for peptides. A large size of the hydrophobic part, as well as the presence of non-aliphatic moieties, reduced the efficiency. This was also partly seen for hydrophobic basic drugs in **paper IV**, where large and highly hydrophobic organic solvents failed to give any extraction recoveries or a measurable current across the SLM during extraction.

An efficient extraction requires the analytes to enter the SLM, but also to be released into the acceptor solution. If the interactions between analytes and the organic solvent are too high, extensive "trapping" in the membrane can occur. For extractions of hydrophobic basic drugs, the degree of "trapping" was generally low (10-20 %), as seen in **paper II** and **III**. This effect

was also seen in **paper II**, where the distribution of these analytes between 10 mM HCl and NPOE completely favored the aqueous phase for most of the analytes. For peptides, however, the inclusion of a carrier in the membrane causes a high affinity for the analytes to the SLM, as seen in **paper I** and **II**. In **paper II** this was seen as the major factor limiting extraction recoveries, and the degree of "trapping" appeared to be reduced with increasing number of positive charges on the peptides. This was somewhat contradictory to the observation that the most highly charged peptide, angiotensin 1, was the most extensively trapped peptide in the work with **paper I**. However, this observation was based on 5 minute extractions, where steady state is not yet reached according to **paper II.** According to the time dependent concentration in the SLM determined in **paper II**, this peptide have a high accumulation in the SLM during the first minutes, before this is gradually reduced, and can thus explain the high concentration of angiotensin 1 in the SLM observed in **paper I**. Possibly, more charges makes it easier for the carrier-peptide complex to penetrate the SLM because of more extensive binding with carriers, but with a lack of carrier molecules to complex with at the SLM/acceptor interface and a higher exerted force from the electrical field, these compounds are more effectively isolated in the acceptor solution as time passes. Based on this there seems to be a compromise between efficient transport into the SLM, and efficient release into the acceptor solution. Thus, the affinity of the SLM towards the analyte has to be high, but not too high, to be efficient. As **paper I-III** and **V** show, the degree of "trapping" varies between analytes, and can thus be a future possibility for creating selective extraction systems in addition to selectivity induced by the applied voltage [155].

The degree of "trapping" was also highly dependent on the organic solvents used in the SLM. In **paper I**, five different compositions of carriers and organic solvents were tested to see the differences in peptide distribution throughout the sample, SLM, and acceptor solutions. The resulting plots are shown in Figure 3.2. For nonanol as an organic solvent, substituting between TDP and DEHP as a carrier caused almost no difference. However, if 2-octanone was used instead, TDP was superior to DEHP when it came to recovery in the acceptor solution, but also when it came to the degree of trapping in the SLM. The distribution into the acceptor solution and SLM was also higher with 2-octanone and TDP than the previously described composition of octanol, DEHP, and di-isobutylketone. This was suggested as being caused by both hydrogen bonding and ionic interactions, because of the strong dipole properties of 2-octanone compared to the strong proton acceptor properties of the alcohols.

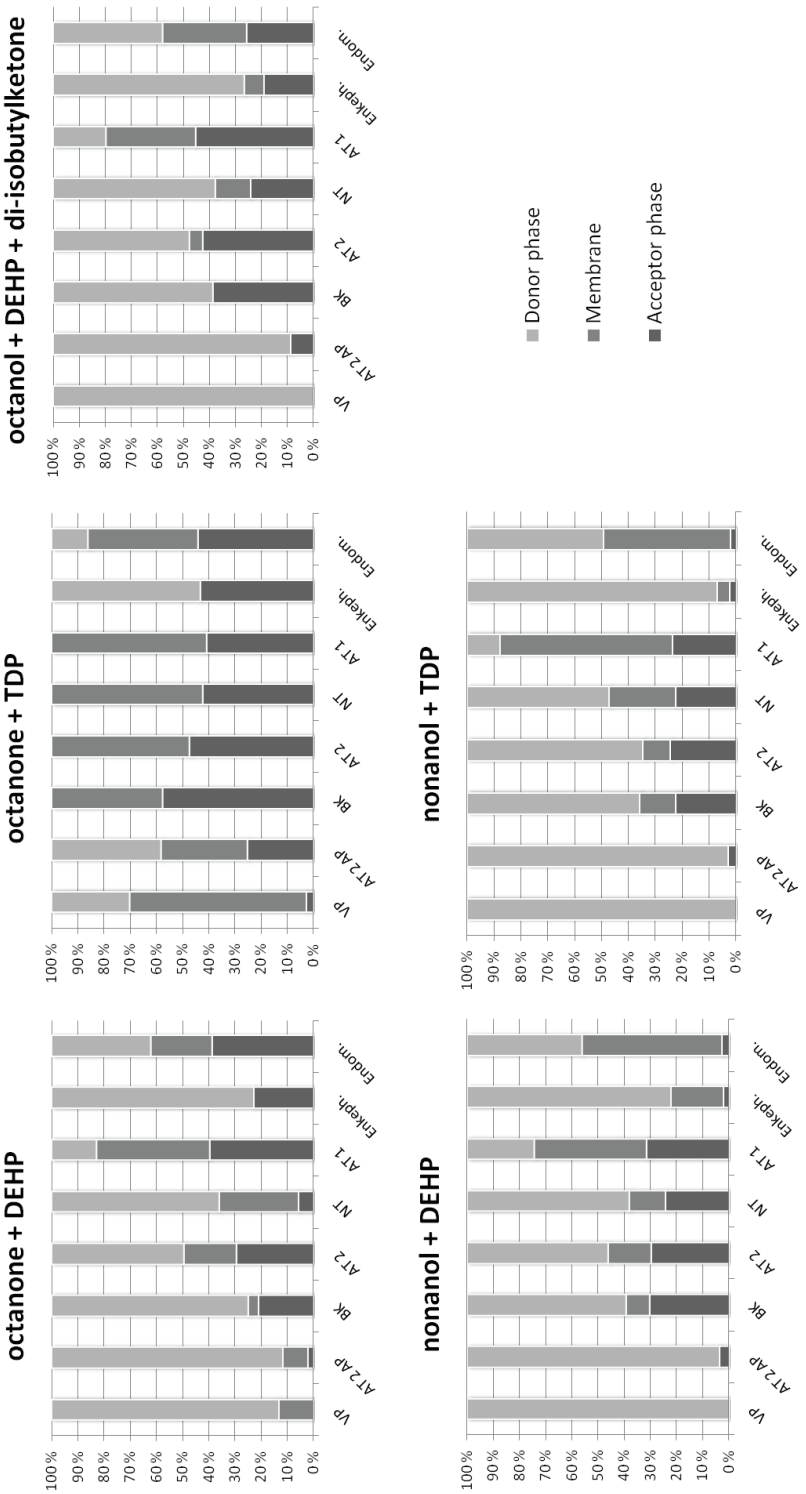


Figure 3.2: *Plots over the distribution of model peptides in the three phases of the EME system (the donor/sample solution, the SLM, and the acceptor solution) for five different SLM compositions, n=6. VP = vasopressin, AT 2 AP = angiotensin 2 antipeptide, BK = bradykinin, AT 2 = angiotensin 2, NT = neurotensin, AT 1 = angiotensin 1, Enkeph. = enkephalin, Endom. = endomorphin. Reprinted with permission from paper I © Wiley-VCH Verlag (2011)*

These differences between organic solvents used in the SLM were also seen in **paper V**, when substantial amounts of NaCl were present in the sample solution. The high NaCl content caused some of the analytes to be extensively trapped in the SLM compared to samples extracted in the absence of salt. This effect was to a large degree eliminated when 6-undecanone was used instead of NPOE in the SLM, except for the two analytes that were most affected by the presence of NaCl; but even for these the degree of "trapping" was reduced.

The contribution to extraction selectivity and clean extracts is also an important factor for the organic solvent in the SLM. This was investigated in **paper IV**, where several organic solvents were considered unsuitable because of the high degree of interferences that entered the acceptor solution after extraction. These interferences were released from the organic solvents and into the acceptor solution during extractions and were mostly experienced with aldehydes, phenols, or phosphates with low log P values. A reasonable theory is that the relatively hydrophilic nature of these solvents causes them to dissolve several water soluble impurities that are released to the aqueous phases during extraction. Even though this type of interferences can be avoided by the use of other analytical techniques such as LC-MS, it shows that these solvents easily dissolve and release several water soluble compounds, which might compromise the selectivity of the SLM.

3.2.3 Recovery and kinetics when extracting from partly organic matrices

Several types of samples can contain substantial amounts of organic solvents, either from an organic matrix or from previous sample preparation steps. In **paper III**, the influence of such partially organic sample solutions in EME was investigated. According to the extraction theory that was investigated in **paper II**, the addition of an organic solvent in the acceptor solution would affect some parameters in the distribution coefficient described in equation 12. First, the conductivity of the sample solution would be affected, influencing the Galvani potential difference ($\Delta_o^w\phi$) between the sample and the SLM. Second, the affinity of the organic solvent in the SLM towards the analyte, relative to the sample solution, also changes, affecting the term related to the distributive behavior of the analyte ($\Delta_o^w\phi_i^0$). The expected net results according to this theory would be slower extraction kinetics. However, equation 9, which describes the time dependent concentration in the sample solution, is unaffected by these changes at steady state when the net flux is zero. According to this, the extraction

recovery at steady state in the sample should be unaffected. The theory from **paper II** thus suggests a slower extraction process, reaching the same end point.

Four different organic solvents, ethanol, methanol, dimethyl sulfoxide, and acetonitrile were added to the sample solution in different concentrations before extraction to investigate this (**paper III**). Because organic solvents in the sample solution could affect the extraction stability by partial dissolution of the SLM, there were some limitations to the maximum concentration and type of organic solvent that could be used (discussed in section 3.3.3). However, extractions from samples with up to 50 % (v/v) ethanol, methanol, or dimethyl sulfoxide in 10 mM HCl were in excellent agreement with the theoretical model in **paper II** when NPOE was used in the SLM. In Figure 3.3, a comparison between extractions with no organic solvent and with 50 % (v/v) methanol in the sample solution is shown. Both samples containing organic solvents and samples containing only 10 mM HCl reached maximum steady state recoveries for the five model analytes in the range 80-90 %. However, the time to reach steady state shifted from between 5 to 10 minutes for the extractions from only 10 mM HCl, to 15-20 minutes when 50 % (v/v) methanol was present. Thus, the experimental data correspond with the predicted behavior according to the theoretical model, with an unaffected maximum recovery and slower kinetics.

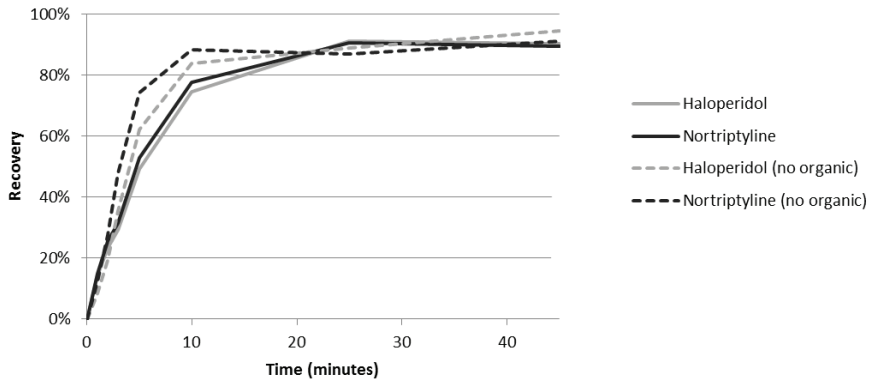


Figure 3.3: *A comparison between extraction recoveries of the model drugs haloperidol and nortriptyline from samples containing no organic solvent (dotted line) and 50 % (v/v) methanol (solid line), n=3. Reprinted with permission from paper III © Elsevier B.V. (2013).*

The same agreement with the theoretical model was seen for 50 % (v/v) ethanol and dimethyl sulfoxide as well. With acetonitrile in the sample, however, the EME system became unstable, and samples containing acetonitrile should be avoided (discussed in section 3.3.3). In the case

of dimethyl sulfoxide, extractions from up to 75 % (v/v) in 10 mM HCl were tested, but in this case the kinetics were slow and steady state was not reached even after 45 minutes of extraction.

The results in **paper III** show that EME can be performed from samples containing organic solvents, but that the extraction time should be increased to account for the slower kinetics. This introduced the possibility for EME from new types of sample matrices and showed that the principles controlling the extractions still behave according to the theory developed for purely aqueous matrices in **paper II**.

3.2.4 Recovery and kinetics when extracting from samples containing substantial amounts of salts

In many cases, the sample solution can contain a substantial amount of salts, such as in blood, urine, or some environmental samples. The influence of high salt content was investigated in **paper V** by varying the NaCl content of the sample solution. Several EME applications have seen a reduction in recovery when salts have been added to the sample solution during optimization [134,161-166]. This was explained according to the general flux equation for EME, where an increasing amount of ions in the sample solution relative to the acceptor solution could affect the flux negatively [137]. The equations for the distribution of analytes over time in the different phases of the EME system presented in **paper II**, however, did not take into account how salts can influence the distribution of analytes. Additionally, the previously presented data on the topic have only been minor observations and no thorough investigation of salt effects in EME. The initial experiments showed that when the salt content was increased to 1 % (w/v) or above, some analytes were highly affected with a large reduction in recovery, whereas others were not. This is shown in Figure 3.4, where extraction recovery from samples extracted without any NaCl in the sample is compared with samples extracted with 2.5 % (w/v) NaCl in the sample. Higher NaCl content (5 % w/v) caused the repeatability of the extractions to be reduced. NPOE was used as the organic solvent in the SLM. In all cases, the remaining concentration in the sample solution after extraction was the same both with and without the presence of NaCl. No apparent link to physical chemical properties was found for the analytes that were influenced by this salt effect, but except for papaverine and verapamil, all the affected substances are tricyclic antidepressants. The salt effect was also observed when the analyte concentration in the sample was reduced by a factor of 10. Interestingly, the amount of salts did not seem to affect the extraction kinetics, as

seen when organic solvents were present in the sample in **paper III**, and all analytes were either at or close to maximum recovery at an extraction time of five minutes.

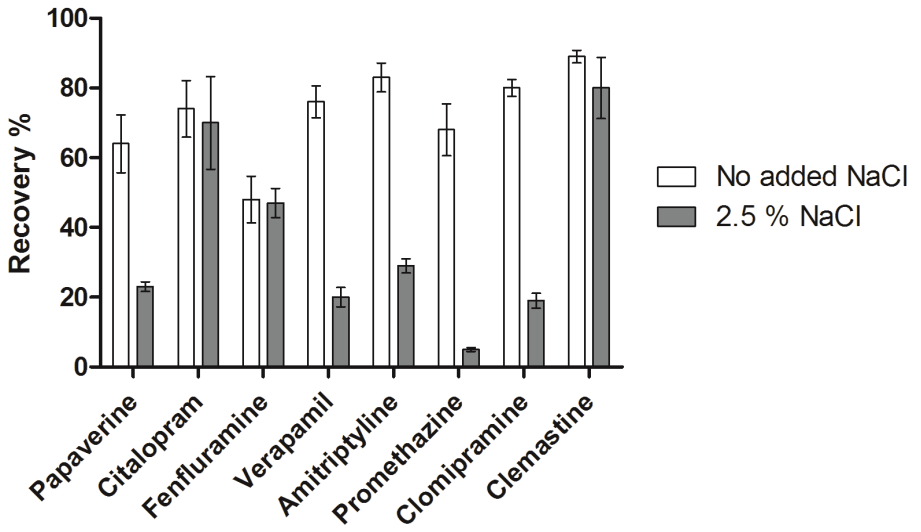


Figure 3.4: *Recovery of basic model analytes after EME performed with and without 2.5 % (w/v) NaCl added to the sample solution, n≥3.*

A possible cause for this salt effect was hypothesized to be ion pairing in the SLM, and some experiments were performed to verify this. One experiment was performed with a different EME setup with a thin membrane [167], creating a lower volume ratio between the SLM and the acceptor solution. If ion pairing was taking place, a smaller volume of the SLM relative to the acceptor solution volume should reduce the effect seen from adding NaCl to the sample solution. This was indeed the case, and the salt effect was no longer significant for most of the analytes. The exceptions were promethazine and clomipramine, which were also the most affected analytes in the original setup, but even for these the salt effect was reduced with a thinner membrane. A similar suppression of salt effects was also seen when the organic solvent in the SLM was changed from NPOE to 6-undecanone in the original EME setup. Also in this case, promethazine and clomipramine showed some reduction in recovery with 2.5 % (w/v) NaCl present in the sample solution, but it was less than in the extraction setup with NPOE. Since the trapping of an ion pair in the SLM should be highly dependent on the nature of the organic solvent used in the SLM, this observation was also in line with the hypothesized theory. A final experiment was performed with addition of K_2SO_4 to the sample

instead of NaCl in the original EME setup with NPOE as the SLM to see if the effect was dependent on the type of counter ion. With doubly charged SO_4^{2-} counter ions in the sample, almost no salt effects were observed. This is also in line with the ion pairing hypothesis, since a complex between a singly charged analyte and a doubly charged counter ion will move in the electric field and will thus not be trapped in the SLM.

Clearly a salt effect is present for some analytes under certain conditions which is not accounted for in the theoretical model presented in **paper II**. However, if the analyte is present as an ion pair, the affinity towards the SLM phase will be higher, thus affecting K_d^* through a change in $\Delta_o^w\phi_i^0$. By using the equation for K_d^* (equation 12) for the distribution between the SLM and the acceptor solution and the ion pair equilibria in the acceptor solution ($K_{BCl(A)}$) and SLM ($K_{BCl(M)}$), an equation for the maximum recovery fraction of an analyte in the acceptor solution ($R_{\max}$) in presence of a large amount of Cl^- ions can be expressed as follows:

$$R_{\max} = \frac{1 + K_{BCl(A)} \cdot C_{Cl^-(A)}}{1 + K_{BCl(A)} \cdot C_{Cl^-(A)} + \frac{V_M}{V_A}\left\{K_{d,A}^* + K_{d,A}^* \cdot K_{BCl(M)} \cdot C_{Cl^-(M)}\right\}} \cdot 100\% \qquad (13)$$

where V_M/V_A is the SLM to acceptor solution volume ratio, $C_{Cl^-(A)}$ the concentration of Cl^- ions in the acceptor solution, and $C_{Cl^-(M)}$ the concentration of Cl^- ions in the SLM. $C_{Cl^-(M)}$ is a parameter that is affected by both the applied extraction potential and the amount of chloride ions present in the sample solution.

According to this equation, the volume ratio between the SLM and acceptor solution V_M/V_A will influence the total recovery, as experimentally justified by extracting with a thinner SLM in **paper V**. If substantial ion pairing occur in the SLM ($K_{BCl(M)} \cdot C_{Cl^-(M)} >> 1$), the recovery will be reduced as seen experimentally for some substances in **paper V**, and since $C_{Cl^-(M)}$ is influenced by the Cl^- concentration in the sample solution, a substantial amount of NaCl in the sample solution is a likely cause.

3.3 Stability considerations in EME

Stability issues can result in poor repeatability and unreliable extractions. To understand what is causing these stability issues and how to avoid them is an important aspect when

investigating the extraction process in EME. The following section presents the discoveries from the work with this thesis related to the stability of the EME system.

3.3.1 Current levels

As discussed in section 1.5.2, a high current can cause stability issues such as electrolysis around the electrodes or perforation of the SLM from joule heating. Visual inspection can detect some of these instabilities through observation of bubble formation, due to electrolysis near the electrodes, or burned or darkened patches on the fiber material. However, these signs only appear when the current levels are relatively high. To be able to identify potential stability issues more accurately, current monitoring during extractions were performed on nearly all of the experiments during the work with this thesis. This enabled detection of possible deviations from a stable current level and could thus detect potential instabilities that would be impossible to detect from visual inspection alone. In **paper III**, this technique of measuring EME stability was presented. Figure 3.5 gives a visual explanation to the difference between a stable (A) and unstable current curve (B). For a stable extraction system (A) the current fluctuates around a stable current level. In Figure 3.5 B the system appears stable for the first two minutes, but then the current increases gradually, which can indicate that the integrity of the SLM is affected.

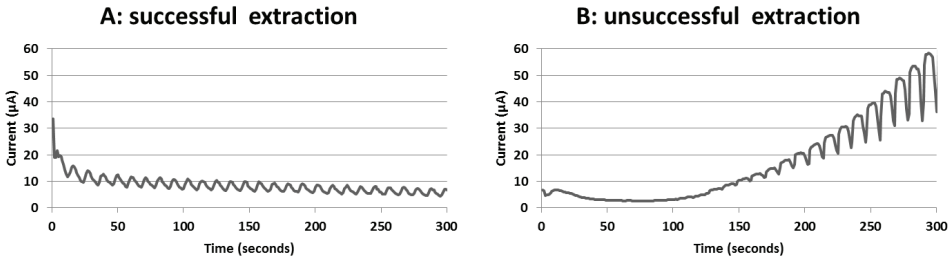


Figure 3.5: *Current curves for a successful extraction (A) and an unsuccessful extraction (B) with EME. Reprinted with permission from paper III © Elsevier B.V. (2013).*

In **paper IV**, a stable current level was defined as stable fluctuations around a current level between 3 and 50 µA. The exact limits were not tested, but extractions where the current was less than 3 µA gave minimal or no extraction recovery and extractions with current levels above 50 µA usually showed signs of instability, as mentioned above.

A high current level can be reduced by decreasing the voltage until a stable current is achieved, and in the work with **paper III** and **IV** this was done. Since the voltage is one of

the main factors controlling the mass transport, both according to the flux equation (equation 7 and 8) for EME [137] and the theoretical model presented in **paper II** (equation 9-12), this reduction could reduce the extraction efficiency. Other recent publications have, however, presented a technique using pulsed voltage and claims that this makes it possible to maintain high extraction voltages with reduced stability issues [160,168].

3.3.2 SLM solvents

The SLM is the major source of resistance in the electrical circuit in an EME system, as discussed in section 1.5.2, which is also the cause of the voltage drop and the high electric field that are generated across the SLM, as discussed in Section 3.2.2, Figure 3.1, and **paper II.** Because of this, the conductivity of the system and thus the measured current will be greatly affected by the organic solvent used in the SLM. **Paper IV** identified a large group of 27 solvents that resulted in a high current during extractions at an applied voltage above 3 V. These solvents were characterized by a low log P value and water solubility exceeding 0.5 g/L. For extraction of basic model analytes, amines were also found unsuitable, even though they did not match the above criteria. This was probably due to their high water solubility caused by protonation in contact with the acidic environment on the interfaces to the aqueous solutions. A reasonable theory is that the organic solvents resulting in a high extraction current dissolves some water, thus affecting the conductivity of the SLM and the transfer of background ions, leading to the gradual increase in current seen in Figure 3.6. This theory was also supported by another observation from **paper IV** that showed that the volume of the acceptor solution increased for many of these solvents, even when extracting at very low voltages (< 3 V). The volume expansion was caused by migration of water through the SLM and was observed to cause a volume expansion of up to 16 % after five minutes of extraction, depending on the applied voltage. This voltage dependency suggests a small electroosmotic flow through the SLM for these relatively polar solvents. A large volume expansion such as this can by itself be a major source for large variations in the measured recoveries, and it was concluded that these types of solvents should be avoided for EME.

The above identification of solvents causing current related instabilities was further supported by discoveries in **paper I**, where most of the solvents categorized as giving too high current share the same criteria of being solvents with a low log P value and water solubility above 0.5 g/L. However, several of the findings in this paper are difficult to compare to the findings in **paper IV** because of the inclusion of carriers, which affects the conductivity of the SLM. In general, the inclusion of a carrier causes the conductivity of the SLM to increase, thus

affecting the optimal extraction parameters and the stability of the SLM [136,143].

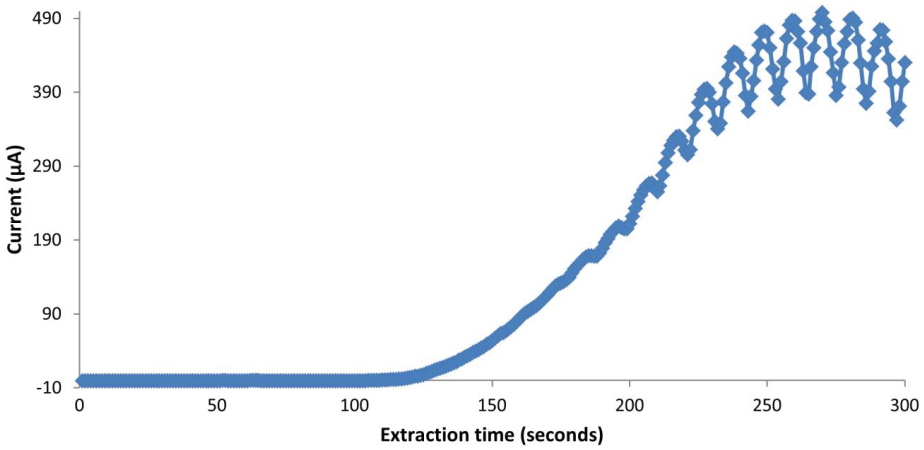


Figure 3.6: *A gradual increase in current over time, seen for some organic solvents in the SLM, which was likely to be caused by increasing conductivity of the SLM throughout the extractions. Reprinted with permission from paper IV © Springer Verlag (2013).*

3.3.3 Stability when extracting from partly organic matrices

In **paper III**, the stability of the EME system was investigated when high amounts of methanol, ethanol, dimethyl sulfoxide, or acetonitrile were present in the sample solution. A high content of polar organic solvents in the sample can result in increased solubilization of the organic solvent used in the SLM, and thus cause a partial dissolution of the SLM. For some SLM solvents this can cause severe stability issues. This was seen in **paper III** where the organic solvents ENB and IPNB were unstable in contact with partially organic matrices. NPOE still provided stable extractions with up to 60 % (v/v) ethanol or methanol in the sample and up to 75 % (v/v) with dimethyl sulfoxide. With acetonitrile, however, the system was unstable, even with 25 % (v/v) in the sample. Table 3.5 shows the observed solubilities of the different organic solvents used in the SLM in 1:1 (v/v) mixtures of water and some of the organic solvents that were added to the sample solution. The lower solubility of NPOE compared to IPNB and ENB, and the increased solubility when acetonitrile was present, points to partial dissolution of the SLM as a cause for the observed stability issues. In the case of ENB in a 1:1 (v/v) mixture of water and acetonitrile, this solubility corresponds to dissolution of about 80 % of the SLM, assuming an SLM volume of 15 µL. These stability issues, caused by an increased solubility of the SLM solvent in the sample medium, share

similarities to the stability issues with certain SLM solvents with high water solubility identified in **paper IV**.

Table 3.5: *Observed solubilities for different organic solvents used as SLM in 1:1 (v/v) mixtures of water and an organic solvent. Reprinted with permission from paper III © Elsevier B.V. (2013).*

Solute	Solvent mixture	Observed solubility (µL/mL)
ENB	ACN : H_2O (1:1 v/v)	**11-13**
	MeOH : H_2O (1:1 v/v)	**1-3**
IPNB	ACN : H_2O (1:1 v/v)	**10-11**
	MeOH : H_2O (1:1 v/v)	**< 1**
NPOE	ACN : H_2O (1:1 v/v)	**2-4**
	MeOH : H_2O (1:1 v/v)	**< 1**
	EtOH : H_2O (1:1 v/v)	**< 1**
	DMSO : H_2O (1:1 v/v)	**< 1**

In **paper IV**, a volume increase in the acceptor solution after the extractions was seen for some of the solvents classified as unsuitable for EME. This was linked to a small electroosmotic flow in the EME system. A similar pattern was noticeable for some of the unstable extractions in **paper III.** This was especially seen during extractions with acetonitrile, causing volume expansions of up to 17 %. In this paper it was hypothesized that this effect was caused by passive diffusion of the organic solvent added to the sample solution, causing some of it to migrate to the acceptor solution. In **paper IV**, when no organic solvent was present in the sample, the concept of a small electroosmotic flow was introduced, and possibly both of these effects could be the cause of the volume expansion seen in **paper III**. During the stable extractions with ethanol, methanol, or dimethyl sulfoxide in the sample, this volume expansion was negligible.

3.4 Tuning the EME system to different applications

For optimal performance of an EME system, certain parameters have to be individually optimized for a certain drug or a group of drugs in a specific sample matrix. The previously published flux equation (equation 7) for EME [137] includes parameters such as applied voltage, ion balance between the sample and acceptor solution, temperature, membrane thickness, and a diffusivity constant that will be related to the analyte ion in question and its diffusivity in the SLM. By optimizing these parameters, a high flux of ions across the membrane is ensured. The theoretical model for distribution of analytes in the EME system presented in **paper II** relates to several of the same parameters through the sample to acceptor

permeability coefficient ($P_i^{D\to A}$), which is linked to the flux at a certain time (t), through the equation:

$$J_{D_i}(t) = P_i^{D\to A} C_{D_i}(t) = -\frac{V_D}{A_f} \cdot \frac{dC_{D_i}(t)}{dt} \qquad (14)$$

According to this equation and equations 9-12, parameters such as the extraction time (t), the active surface area on the SLM (A_f), and the electrically enhanced distribution coefficient (K_d^*) also play essential roles in the mass transport, and could thus be of importance when optimizing the extractions. According to equation 12 and the discussion in section 3.2.1, the electrically enhanced distribution coefficient (K_d^*), is further dependent on variables such as the Galvani potential difference across the membrane ($\Delta_o^w\phi$), the relative hydrophobicity of the analyte in the extraction system ($\Delta_o^w\phi_i^0$), and the absolute temperature. Except for the temperature, these parameters will be affected by the choice of organic solvent in the SLM or from organic additives in the SLM, as studied in **paper I, III** and **IV** and discussed in section 3.2.2-3.2.3. Based on this, the choice of SLM and the applied voltage are important parameters for optimization of an EME system.

Below, some aspects relevant to the optimization of an EME system that were revealed during the work with this thesis are discussed. This discussion is mainly focused on the choice of SLM (**paper I** and **IV**), extraction time (**paper II** and **III**), and stability (**paper I, III, IV** and **V**) for extractions of basic drugs (**paper II-V**) or peptides (**paper I** and **II**) and for extractions from dried blood spots (**paper III**) or samples containing high amounts of salt (**paper V**).

3.4.1 Unpolar basic drugs (log P > 2)

Several publications have thoroughly optimized the principal parameters of the EME system for extractions of a variety of basic drug substances [19,36,135,138,154,155,161,163,164,168-171]. Some of these previously optimized parameters were used for extractions of the same or similar drug substances during the work with this thesis (**paper II-V**). As previously discussed in section 3.2.2, it was seen in **paper IV** that the optimal choice of an SLM for unpolar basic drug substances, when both extraction efficiency and stability was taken into account, was organic solvents with low water solubility (<0.5 g/L), a high dipole moment, high proton acceptor properties and low proton donor properties. Nitroaromatics such as NPOE, IPNB, ENB, and 2-nitrophenyl pentyl ether have been used in several previous

publications and fits well into this category. The work with **paper IV** also revealed some new successful solvents based on the above criteria, including 2-nitrotoluene and 2,4-dimethyl-1-nitrobenzene, as well as the ketones 2-nonanone, 2-decanone, 6-undecanone, and 2,6-dimethyl-4-heptanone. It should be noted, however, that the extractions performed with ketones in the SLM gave slightly lower recovery than the nitroaromatics over the extraction time of five minutes.

The extractions of basic drugs performed in **paper II**, during investigations on distribution profiles for the EME system, revealed an optimal extraction time of 5 to 10 minutes for most of the model drugs. These observations fit well into previously reported optimal extraction times for similar substances [19,98,135,136,138]. In Figure 3.7, this is represented by graphs of recovery over time for five different unpolar basic model drugs. The figure also visualize that some substances might require longer extraction times, as exemplified with the recovery curve for droperidol, (see section 3.2.1). It should be noted that these extraction times were only optimized when using NPOE as the organic solvent in the SLM and might differ when some of the other successful solvents, identified in **paper IV**, are used as SLM. This parameter should thus be optimized for each individual application.

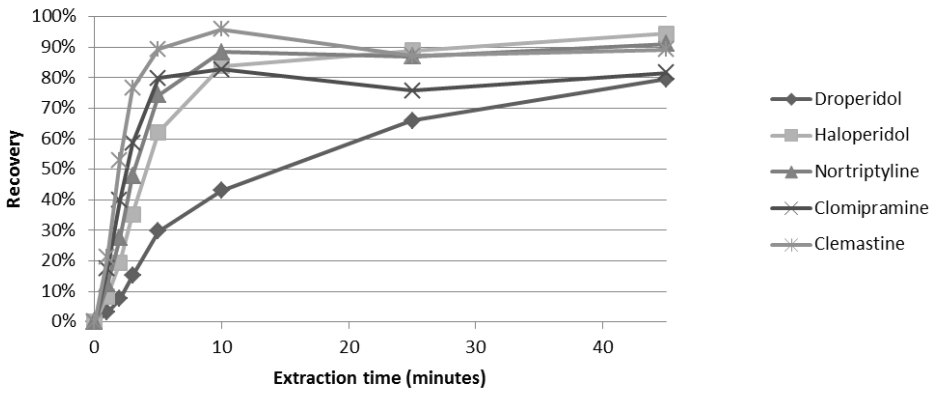


Figure 3.7: *Recovery versus time for five unpolar basic model drugs, n=3. Reprinted with permission from paper II © Wiley-VCH Verlag (2013).*

Optimal extractions are also dependent on the applied voltage, and as discussed in **paper III** and **IV** and section 3.2.2 and 3.3.2, this is highly dependent on the type of SLM used. Too high extraction voltage will give highly unstable extractions, whereas too low voltage will give a suboptimal flux of ions across the SLM. In the work with **paper IV**, the voltage for

each of the SLM solvents was adjusted to give a current of 5-15 μA throughout the extractions. As discussed in section 3.3.2, this was within the current range considered as stable. With this adjustment, the applied voltage used for the various successful SLM solvents varied greatly, from 20 V for 2,4-dimethyl-1-nitrobenzene to 250 V for NPOE. This parameter was, however, not thoroughly optimized for all the different SLM-solvents. A more thorough optimization of extraction voltage is thus needed when making spesific EME applications.

The results from **paper V** show that some of the unpolar basic drugs can be highly affected by the presence of NaCl in the samples. In most cases, this is part of the sample matrix and can thus not easily be avoided. Some of the results from **paper V**, however, indicate that this effect can be reduced by either using a lower SLM to acceptor solution volume ratio, which can be done by using a different EME setup with a thinner SLM [167], or by using a solvent such as 6-undecanone in the SLM.

3.4.2 Dried blood spot eluate

Dried blood spots (DBS) or dried matrix spots (DMS) has increased in popularity as a technique for sample collection and storage. Recently the technique has been combined successfully with EME by using degradable alginate and chitosan foam as storage medium for the matrix spots [151,172]. The conventional method for eluting analytes from commercially available dried blood spot cards includes dissolving the blood spot in a buffered solution containing a high amount of a polar organic solvent such as methanol or acetonitrile. After this, evaporation and resolvation in an appropriate medium is usually required for further sample preparation. In **paper III**, these last steps were avoided by performing EME almost directly on DBS eluate containing pethidine, nortriptyline, and methadone as model analytes. This was made possible by using the combined knowledge of the extraction distribution and kinetics from **paper II** and the knowledge from **paper III** on how organic solvents in the sample solution affects the extraction system, and it was used as an example of a relevant application of EME on samples containing a substantial amount of organic solvents. The cards that were used (Agilent Bond Elut DMS card) needed to be eluted in a mixture of 20 % (v/v) 0.1 % formic acid in either methanol or acetonitrile to dissolve the analytes from the spotting matrix. According to the discussion in section 3.3.3 and **paper III**, acetonitrile is not compatible with the EME system and will partly dissolve the SLM. Additionally the amount of 80 % (v/v) of organic solvent is above the stability limit of the system when using methanol (50 % v/v). By eluting the dried blood spot in methanol and afterwards dilute the

sample 1:1 (v/v) with 10 mM HCl, these challenges were circumvented and the sample was now compatible with EME.

The samples containing 50 % (v/v) methanol was extracted with NPOE as the SLM, an extraction voltage of 200 V, and an acceptor solution acidified with 10 mM HCl. As discussed in section 3.2.3 and in **paper III**, an increased extraction time was needed when extracting samples with a high amount of organic solvent, and the required extraction time in this case was found to be 15-25 minutes, compared to 5-10 minutes for the extractions of basic drugs from aqueous samples in **paper II-V**. An LC-MS/MS chromatogram from an extraction of pethidine, nortriptyline, methadone, and haloperidol by EME of a dried blood spot eluate is shown in Figure 3.8.

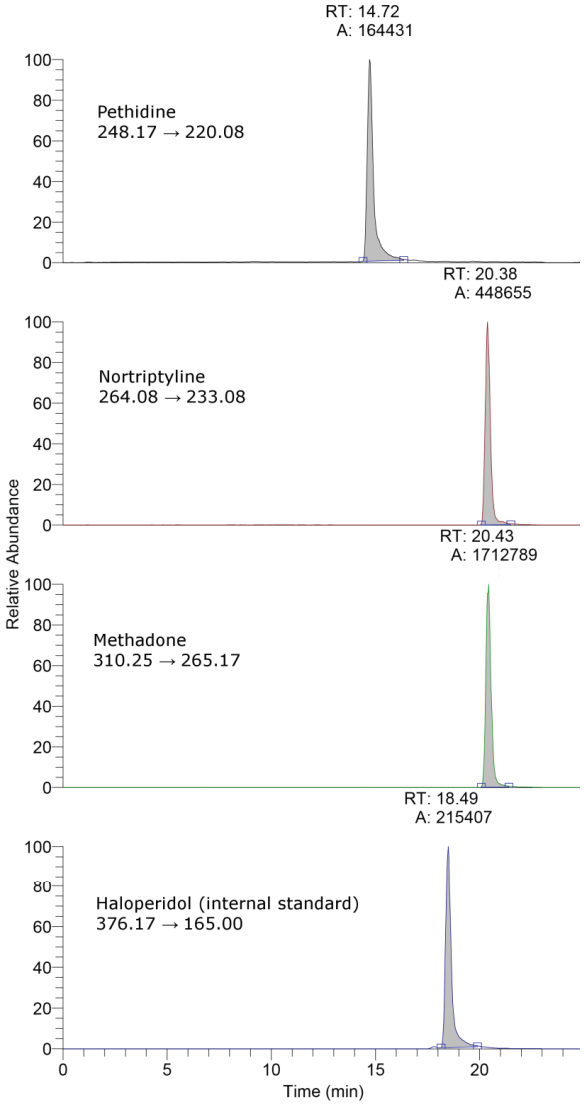


Figure 3.8: *A chromatogram from an extraction of pethidine, nortriptyline, methadone, and haloperidol after EME of dried blood spot eluate. Reprinted with permission from paper III © Elsevier B.V. (2013).*

As discussed in section 3.3.3 and in **paper III**, the choices of organic solvents when extracting from partly organic sample solutions are much more limited than for aqueous samples. Several common SLM solvents for basic drugs, such as ENB or IPNB were too unstable because of their higher solubility in several organic solvents. However, both NPOE and the even more hydrophobic dodecyl-2-nitrophenyl ether were still effective and stable.

The resulting method was comparable to other standard DBS procedures when it came to recovery and repeatability, and no significant matrix effects were detected. Additionally, the time consumption and potential stability issues regarding an evaporation step were avoided when performing EME directly on the DBS eluate.

3.4.3 Peptides

EME of peptides have been less investigated than extractions of basic drugs, but several articles presenting optimized extraction systems for both peptides and amino acids have still been published [139,142,143,148,152,153,157,160]. Many parameters from previously optimized systems for the same set of model peptides were used during the work with **paper I** and **II** [139,143,148]. As discussed in section 3.2.2, it was seen in **paper I** that several alcohols or ketones in combination with a mono- or dialkylated phosphate as a carrier were successful as SLM solvents for peptide extractions. Some of these solvents have been used previously, where the most common SLM have consisted of 1-octanol in combination with DEHP and sometimes also di-isobutyl ketone in various ratios [139,142,143,148]. Some previously unused organic solvents for EME of peptides were revealed during the work with **paper I**. These were ketones such as 2-nonanone and methylacetophenone or alcohols such as 1-nonanol and perillyl alcohol in combination with a carrier such as DEHP. Among the different carriers tested, DEHP, TDP, and dibutyl phosphate were successful. Especially the combination of 10 % (w/w) TDP in 2-octanone was shown to give improved recovery and better repeatability compared to previously described SLMs. Similar effects on recovery, but slightly improved extraction stability, was seen in **paper II** when 2-octanone was substituted with the less water soluble 2-nonanone. Both **paper I** and **II** confirmed the necessity of adding a carrier to the SLM when extracting peptides, as described in previous publications [139,142,143,157,160].

When using an SLM of 2-nonanone with 10 % (w/w) TDP, it was shown in **paper II** that the time to reach maximum recoveries were significantly longer for peptides than for basic drugs. Figure 3.9 shows the recovery of the model peptides over time in this system. Where most of the basic model drugs reached maximum recovery after 5-10 minutes, the model peptides required 10-20 minutes. The time varied somewhat according to the affinity of the model peptide to the organic solvent in the membrane, and it was increased for analytes with less charges and a larger size. These results were consistent with previously optimized extractions for some of the same peptides [139,142]. As for the extractions of basic drugs in **paper II**,

these time curves were also only established for extractions with a single type of SLM, and the required time for each analyte will probably vary when other SLMs are used.

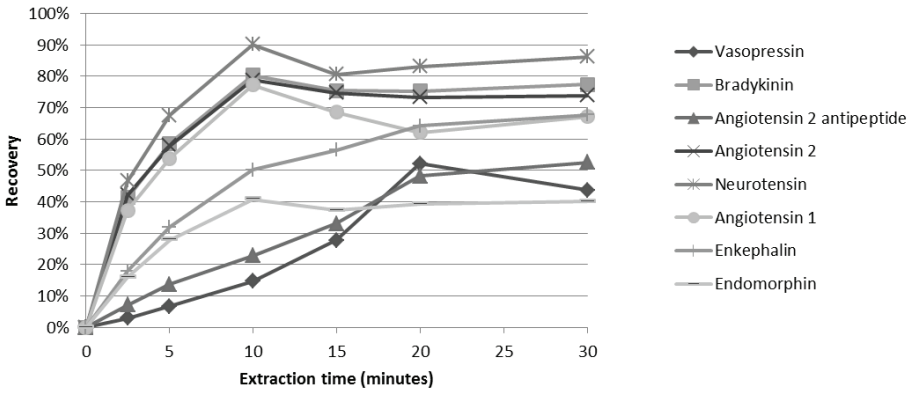


Figure 3.9: *Recovery versus time for eight model peptides, n=3. Reprinted with permission from paper II © Wiley-VCH Verlag (2013).*

In general, the current across the SLM increases when a carrier is added to the organic solvent. In addition, solvents such as aliphatic alcohols, which have been used previously for peptide extractions, have higher water solubility than their counterparts in basic drug extractions, such as NPOE. Because of this, the extractions of peptides in **paper I** and **II** had to be performed at much lower voltage to avoid instabilities with electrolysis or perforation of the SLM, as discussed in section 3.3.2. For the extractions with 2-nonanone and 10 % (w/w) TDP in **paper II**, an extraction voltage of 10 V was used, and this provided stable extractions even at long extraction times.

CONCLUDING REMARKS

In the present thesis, several fundamental aspects of electromembrane extraction (EME) have been investigated. The theoretical understanding of the extraction process in EME as a sample preparation technique has been improved through thorough investigations of key aspects regarding extraction efficiency, stability, and kinetics. These investigations involved efficiency of different types of supported liquid membranes (SLM), extraction stability under various conditions, and extractions from samples containing organic solvents or high amounts of salts. The fundamental aspects investigated in the work with this thesis have been systematically described in five papers. Some of the key discoveries during the work with this thesis are summarized below:

- The distribution of analytes over time in EME for extractions of unpolar basic drugs and peptides was described by a phenomenological theoretical model, showing that the mass transfer across the SLM was governed by both a distributive and electrophoretic component. The time dependent recovery in the acceptor solution was affected by factors such as volume of sample, SLM, acceptor solution, a potential dependent distribution coefficient, effective membrane area, and a permeability coefficient.

- Several organic solvents for the SLM were classified as effective for both extractions of unpolar basic drugs and peptides. For peptides, these solvents included some primary alcohols and ketones combined with a mono- or disubstituted phosphate as a carrier. For unpolar basic drugs, the successful solvents were either nitroaromatics or ketones with a relatively low water solubility (<0.5 g/L), high proton acceptor properties, low proton donor properties, and a high dipole moment.

- The stability of the EME system was highly dependent on the type of SLM, and especially SLM solvents with a relatively high water solubility (>0.5 g/L), small molecular surface area, and low log P were unsuitable for EME because of high current and water migration across the SLM. Current measurements throughout the extractions were seen to be an effective tool to monitor potential stability issues.

- Extractions with organic solvents in the sample were successfully performed in an application where dried blood spot eluate was subjected to EME. These extractions

were possible from samples containing up to 50 % (v/v) ethanol or methanol, or up to 75 % (v/v) DMSO in the sample solution. The total extraction recovery of these samples compared to purely aqueous samples was unaffected, but the extraction kinetics was slower, resulting in longer extraction times.

- Extractions with substantial amounts of NaCl in the sample showed that the recovery was reduced for some unpolar basic model analytes compared to samples without NaCl. This effect was hypothesized to be caused by ion pairing in the SLM, and a mathematical expression for analyte recovery in the presence of salt in the sample solution was developed. It was seen that the reduction in recovery could be reduced or eliminated by changing the organic solvent in the SLM or by reducing the SLM to acceptor solution volume ratio (by using a thinner membrane).

This increased knowledge about the extraction process will hopefully be beneficial for further development of EME as a promising sample preparation technique and as a way to improve optimization of the EME system towards specific applications. Currently, several classes of analytes, such as polar and acidic compounds have been difficult to extract, mainly caused by a lack of efficient SLM materials. The increased understanding of the role of the SLM during the extraction process for unpolar basic drugs and peptides described in this thesis might be a step towards more effective EME systems also for other classes of analytes, and it can thus broaden the applicability of the technique.

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I

II

III

IV

Salt effects in electromembrane extraction

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Highlights:

- Salt in the sample solution can reduce the recovery for some analytes in electromembrane extraction
- Salt effects have been explained by an ion pairing hypothesis
- Salt effects were reduced by changing the solvent in the supported liquid membrane
- Salt effects were reduced by using a lower supported liquid membrane volume

Abstract

Electromembrane extraction (EME) was performed on samples containing substantial amounts of NaCl to investigate how the presence of salts affected the recovery, repeatability, and membrane current in the extraction system. A group of 17 unpolar basic drugs with various physical chemical properties were used as model analytes. When EME was performed in a hollow fiber setup with a supported liquid membrane (SLM) comprised of 2-nitrophenyl octyl ether (NPOE), a substantial reduction in recovery was seen for eight of the substances when 2.5 % (w/v) NaCl was present. No correlation between this loss and the physical chemical properties of these substances was seen. The recovery loss was hypothesized to be caused by ion pairing in the SLM, and a mathematical model for the extraction recovery in the presence of salts was made according to the experimental observations. Some variations to the EME system reduced this recovery loss, such as changing the SLM solvent from NPOE to 6-undecanone, or reducing the membrane thickness from 200 to 100 µm to reduce the SLM to acceptor solution volume ratio. This was in line with the ion pairing hypothesis and the mathematical model. This thorough investigation of how salts affect EME improves the theoretical understanding of the extraction process, and can contribute to the future development and optimization of the technique.

1 Introduction

Biological samples contain substantial amounts of salts. Extracellular fluids and interstitial fluids have an osmolarity that corresponds to a solution of 0.9 % NaCl and the salt content of urine samples can be even higher because of the active salt secretion in the kidneys. Environmental water samples can also contain various amounts of salts from natural sources. The presence of salts in biological and environmental samples may sacrifice the quality of chromatographic measurements, and in some cases removal of salts during the sample preparation step is needed. In other cases, salts are added to samples as an important way to increase extraction recovery from sample preparation procedures such as liquid-liquid extraction (LLE) through the salting out effect [1]. Thus, from a sample preparation point of view, salt contents play an important role for different reasons.

One way to remove much of the salt content in a sample is hollow fiber liquid-phase microextraction (HF-LPME), which has emerged as an interesting alternative to classical sample preparation techniques in recent years. HF-LPME is a supported liquid membrane (SLM) based extraction technique that was introduced in 1999 [2]. The principle is based on extraction of analytes through an SLM comprised of an organic solvent impregnated in the pores of a hollow fiber, and into a small volume of aqueous acceptor solution loaded into the lumen of the hollow fiber. This results in a clean and highly enriched extract. HF-LPME is based on low-price and disposable equipment and each extraction requires only a few µL of organic solvent. Unfortunately, extraction times are typically in the range of 15-60 minutes, and the extractions are normally non-exhaustive [3,4].

To overcome the latter drawbacks, electromembrane extraction (EME) was introduced as an alternative technique in 2006 [5]. The general principle is similar to that of HF-LPME, but utilizes an electric field across the SLM as a way to improve the mass transfer. Several applications using EME have been published, showing the potential for fast and efficient sample cleanup and good recovery from a variety of matrices, including analysis of drug substances or peptides from various biological fluids such as undiluted whole blood, plasma, urine, breast milk, and oral fluids [6-13]. In addition heavy metals and organic micro pollutants have been extracted by EME from environmental water samples [10,14-16].

Even though several EME applications have been published, only a few of these have investigated how the salt content can influence the extractions [11,17-21]. These discussions have mainly been connected to how the salt content can affect the ion balance in the extraction system according to a model describing the flux of analytes in EME [4,22].

A recent review on EME emphasized the need for more thorough investigations on how salts can affect the extraction process [23]. The following article answers this request, and presents a thorough and fundamental approach towards a better understanding of how salts affect the extraction recovery, repeatability, and membrane current in EME. Several experiments on a wide range of analytes have been performed with and without substantial amounts of salt in the sample solution. The results are described theoretically, and a mathematical model for the observed effects have been presented and related to a recently described model for the time dependent distribution of analytes in EME [24]. This is the first time the effect of salts in the sample solution has been thoroughly described for EME and the results can serve as an important contribution towards a better understanding of the extraction process.

2 Experimental

2.1 Chemicals

2.1.1 Model analytes

Amitriptyline hydrochloride, citalopram hydrobromide, clemastine fumarate, clomipramine hydrochloride, fenfluramine hydrochloride, haloperidol hydrochloride, loperamide hydrochloride, methadone hydrochloride, nortriptyline hydrochloride, papaverine hydrochloride, perphenazine, pethidine hydrochloride, promethazine hydrochloride, prochlorperazine dimaleate, pyrilamine maleate, reserpine, and verapamil hydrochloride were all obtained from Sigma-Aldrich (Steinheim, Switzerland).

2.1.2 Other chemicals

Acetonitrile (HPLC-grade) was obtained from VWR International (Leuven, Belgium). Formic acid (> 98 %), 2-nitrophenyl octyl ether (≥99 %), potassium chloride (≥99.9 %), sodium chloride (≥99.5 %), tetradecyltrimethylammonium bromide (99 %), imidazole (≥99 %), and 6-undecanone (97 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium

hydroxide (99 %) was obtained from VWR (Leuven, The Netherlands). Hydrochloric acid (37 %), monosodium dihydrogen phosphate monohydrate (analysis grade), ortho-phosphoric acid (85 %), potassium chromate (≥99 %), and potassium sulfate (≥99 %) were all purchased from Merck (Darmstadt, Germany). A Milli-Q integral 3 water purification system (Millipore, Billerica, MA, USA) supplied deionized water for all experiments.

2.2 Samples

Sample solutions were prepared daily by diluting stock solutions containing 1 mg mL^{-1} of each model drug in methanol; stored at 4°C and protected from light. Dilutions were performed with 10 mM HCl, to achieve the desired concentration of 1 µg mL^{-1} or 100 ng mL^{-1} of each drug substance before extraction.

2.3 Electromembrane extraction

2.3.1 Equipment and setup

Most of the extractions were performed using a hollow fiber setup as depicted previously [7]. The device consisted of three parts: a sample vial containing the sample solution, a porous hollow fiber containing a supported liquid membrane in the pores of the fiber walls, and an acceptor solution located inside the lumen of the hollow fiber. The sample compartment was a glass vial with a volume of 2 mL of the type 2-SV with screw cap (Chromacol, Welwyn Garden City, UK). A 2.4 cm piece of porous hollow fiber of the type PP Q3/2 polypropylene hollow fiber, with a wall thickness of 200 µm, internal diameter of 1.2 mm, and a pore size of 0.2 µm (Membrana, Wuppertal, Germany) was mechanically sealed in the lower end and attached by heat to the 2.1 cm end-piece of a pipette tip (Finntip 200 Ext, Thermo Electron, Vantaa, Finland) in the upper end. Before extraction, this piece of hollow fiber was impregnated by an organic liquid, comprising the SLM in the extraction setup. The lumen of the hollow fiber was filled with an aqueous acceptor solution, making a three phase extraction system when the hollow fiber was placed in the sample solution through a perforated hole in the screw cap of the sample reservoir. Platinum wires with a diameter of 0.5 mm were used as electrodes (K. A. Rasmussen, Hamar, Norway), and placed in the sample solution, through the lid of the sample compartment, and into the acceptor solution in the lumen of the hollow fiber.

The other setup contained a thinner membrane and was used for a few experiments where the membrane thickness had to be reduced, leading to a comparatively low SLM-to-acceptor solution volume ratio. In this previously published extraction setup [25], the extraction system is vertically aligned, with the sample solution in a lower compartment and acceptor solution in an upper compartment, separated by an SLM between them. The sample solution was kept in a 2.0 mL Eppendorf safe-lock PP tube (Eppendorf AG, Hamburg, Germany), and the membrane was of the type Accurel PP 1E (R/P) with a thickness of 100 µm (Membrana, Wuppertal, Germany), sealed by heat on the top of the wide end of a 10-1000 µL pipette tip (Sartorius Biohit Liquid Handling Oy, Helsinki, Finland) by use of a Cotech soldering iron station (Clas Ohlson AB, Insjon, Sweden). The SLM was made by impregnating this membrane with an organic solvent and the acceptor solution reservoir was made up by the remaining volume of the pipette tip. Electrodes of the same type as used in the hollow fiber setup were positioned in the sample and acceptor solution. The piece of pipette tip containing the SLM and the acceptor solution were placed on top of the sample solution in the sample compartment.

In both systems, a power supply of the model ES 0300-0.45 from Delta Power Supplies (Delta Electronika, Zierikzee, The Netherlands), with programmable voltage in the range 0-300 V and current output from 0 to 450 mA, was used to create an electric field between the electrodes. The systems were agitated during the extractions by an Eppendorff thermomixer comfort (Eppendorff, Hamburg, Germany), and the SLM current was measured using a custom-built device for measuring micro-currents. This device was controlled by a computer with LabVIEW 8.2 software (National Instruments, Austin, TX, USA), which resulted in a plot of SLM current over time for each extraction.

2.3.2 Extraction procedure

All extractions were performed according to previously optimized conditions for similar drug substances [26]. A precalculated amount of a standard drug mix, containing the chosen model analytes, was diluted with 10 mM HCl with or without 2.5 % (w/v) amounts of either sodium chloride or potassium sulphate. A final concentration of 1 µg mL^{-1} in the sample solution for each model analyte was typically used.

In the hollow fiber setup, the fiber was immersed in an organic liquid (either 2-nitrophenyl octyl ether (NPOE) or 6-undecanone) for approximately five seconds to make the SLM. Any excess solvent was wiped off by a medical wipe. A volume of 25 µL acceptor solution, comprising 10 mM HCl, was then filled into the lumen of the hollow fiber by the use of a microsyringe. The hollow fiber containing the SLM and acceptor solution was then inserted, through a hole in the lid of the sample reservoir, into 1.0 mL of sample solution. A cathode and anode was placed into the acceptor and sample solution, respectively. The extraction device was then placed on an agitator, and extractions were performed by agitating the system at 900 rpm and applying an electrical potential of 200 V with 6-undecanone and 250 V with NPOE, according to previously described conditions for maintaining a stable SLM during extractions [27]. The extractions lasted for 5 minutes. Immediately after extraction, the hollow fiber containing the SLM and acceptor solution was removed from the sample compartment, and the acceptor solution was transferred to vials for further analysis by HPLC or CE.

In the flat membrane setup, optimized conditions from a previously published article were used [25]. A volume of 600 µL sample solution was added to the sample reservoir and the anode was inserted into the solution. The SLM was then impregnated by adding 6 µL of NPOE to the surface of the membrane, and the acceptor solution reservoir was filled with 600 µL 10 mM HCl. The anode was inserted into the acceptor solution and fixed in position by using a small piece of a pipette tip placed into the acceptor reservoir. The compartment containing the SLM and the acceptor solution was then pushed into the sample reservoir until the two parts became firmly attached and the sample solution and SLM were approximately 1 mm apart. The extraction device was then placed on an agitator, and extractions were performed by agitating the system at 1100 rpm and applying an electrical potential of 250 V. The extractions lasted for 15 minutes. Immediately after extraction, the acceptor solution was transferred to vials for further analysis on HPLC.

2.4 Instrumentation

2.4.1 HPLC

All the extracts were analyzed on a Dionex Ultimate 3000 HPLC-system (Dionex Corporation, Sunnyvale, CA, USA), comprised of a degasser (SRD-3200), pump (HPG-3200M), autosampler

(WPS-3000SL), column oven (FLM-3100), and a UV detector (VWD-3400), running Chromeleon (v. 6.80 SP2 Build 2212) software (Dionex Corporation). Separation was performed on a Gemini C18 (150 mm L x 2.00 mm i.d., 5 µm particle size and 110 Å pore size) column from Phenomenex (Torrance, CA, USA), operated at 60 °C. The mobile phases consisted of 20 mM formic acid and acetonitrile in a 95:5 v/v ratio for mobile phase A and 5:95 v/v for mobile phase B. Gradient elution was used, with a gradient that increased linearly from 0 to 35 % mobile phase B over 20 minutes, followed by a washing procedure at 80 % mobile phase B for three minutes, before re-equilibration with mobile phase A for seven minutes. The flow rate was 0.4 mL min^{-1} and the injection volume 10 µL. Detection was performed with UV at 214 and 235 nm to ensure good signals for all the model analytes. Total analysis time was 30 minutes per sample.

2.4.2 CE

A capillary electrophoresis instrument of the type Agilent Capillary Electrophoresis System with Agilent 3-D-CE ChemStation software (both from Agilent Technologies, Santa Clara, CA, USA) was used for measuring Na^+ and Cl^- ions by indirect UV. Separations were performed in a fused-silica capillary with an inner diameter of 75 µm and effective length of 27 and 57 cm (Polymicro Technologies, Phoenix, AZ, USA) for Na^+ and Cl^- ions respectively. The samples were injected by using an applied hydrodynamic pressure of 50 mbar for five seconds. Other operational parameters consisted of a stable capillary temperature of 25°C and an applied voltage of 5 kV for Na^+ ions and -15 kV for Cl^- ions. Between each sample the capillary was flushed with 100 mM NaOH for two minutes, followed by two minutes flushing with the separation buffer. Detection was performed by indirect UV at a wavelength of 214 nm for Na^+ ions and 254 nm for Cl^-.

For analysis of Na^+ ions, a mixture of 6 mM imidazole and 4 mM formic acid was used as separation buffer, according to a previously published method [28]. The imidazole was used to generate a high background UV signal, which would be reduced in the prescence of Na^+ ions. KCl was used as an internal standard.

When analyzing Cl^- ions, the electroosmotic flow (EOF) was reversed to avoid poor peak shapes, bad reproducibility, and long analysis time according to a previously published method [29]. This was done by using a separation buffer consisting of 0.5 mM

tetradecyltrimethylammonium bromide and 5 mM chromate as well as reversing the polarity of the applied voltage (-15 kV). Chromate was added to the separation buffer to produce a high background UV signal at the detection wavelength (254 nm).

2.5 Calculation of recovery

The extraction recovery after EME was calculated for each analyte using the following equation:

$$R = \frac{n_{A,final}}{n_{D,initial}} \cdot 100\% = \frac{C_{A,final} \cdot V_A}{C_{D,initial} \cdot V_D} \cdot 100\% \quad (1)$$

where $n_{A,final}$ is the amount of analyte in the acceptor solution at the end of the extraction and $n_{D,initial}$ is the amount of analyte that was initially present in the sample. V_A and V_D are the volumes of the acceptor and sample solution respectively. $C_{A,final}$ is the concentration of analyte in the acceptor solution after extraction, while $C_{D,initial}$ is the concentration that was initially present in the sample prior to extraction.

3 Results and discussion

3.1 Experimental observations related to NaCl and K_2SO_4

The focus in this work has been to identify general trends in the data and important observations related to the addition of substantial amounts of salt (NaCl) in the sample matrix. A total of 17 model analytes with log P values between 2.2 and 5.3 were used to see how these salt additions affected recovery, repeatability, and membrane current in EME. The range of model analytes covered the relatively non-polar basic drug substances pethidine, pyrilamine, papaverine, citalopram, fenfluramine, haloperidol, methadone, perphenazine, nortriptyline, verapamil, loperamide, amitriptyline, reserpine, prochlorperazine, promethazine, clomipramine, and clemastine.

Table 1 presents a comparison between extractions performed from aqueous samples containing the model analytes in 10 mM HCl with and without the addition of 2.5 % (w/v) (0.428 M) sodium chloride. All the samples were extracted using NPOE as the organic solvent (SLM) and with an analyte concentration in the sample solution of 1 µg mL^{-1}. For nine of the analytes, recoveries were independent of NaCl in the sample, whereas the remaining eight

compounds clearly were affected by the presence of NaCl. A correlation between these trends and different physical chemical properties of the analytes, such as log P, polar surface area, pK_a, aqueous solubility, polarizability, or molecular surface area was not apparent. It should be noted, however, that several of the analytes were tricyclic antidepressants (TCA) and they were all affected by the addition of NaCl.

Subsequently, additional experiments were performed with eight of the model analytes with both 1 and 5 % (w/v) NaCl added to the sample solution. Even with 1 % NaCl, several of the model analytes demonstrated reduced recovery as compared to samples with no NaCl. With 5 % (w/v) NaCl the RSD values increased substantially, due to poor repeatability of the data. The remaining experiments were thus performed by comparing 2.5 % (w/v) NaCl in the sample solution to no salt addition.

No significant differences in the membrane current were seen when monitoring the extraction current during EME. This indicates that the additional ions from NaCl in the sample do not affect the transfer of current in the system. This was also confirmed by an experiment using CE with indirect UV detection to measure the amount of sodium and chloride ions in the acceptor solution after extraction. This experiment showed no sign of penetration of these ions through the SLM when NPOE was used as the organic solvent, and the EME system with this setup was highly resistant to co-extraction of Na^+ and Cl^- ions.

To investigate if the effect of NaCl addition was dependent on analyte concentration or a possible saturation in the system caused by high concentrations of the model analytes, another set of experiments was performed on a selection of eight out of the original 17 model analytes, with analyte concentrations in the sample solution of 100 ng mL^{-1}. The results are presented in Figure 1 and show the same trend as the extractions in Table 1 with higher analyte concentrations (1 µg mL^{-1}). This indicated that the effects seen in Table 1 were not due to saturation in the extraction system.

A possible explanation for the observed salt effect could be ion pairing between certain analytes and the substantial amounts of Cl^- ions in the sample solution after addition of NaCl, causing the ion pairs to be trapped in the SLM. To further investigate this hypothesis, three main sets of experiments were performed: 1) the use of a different organic solvent in the

SLM, 2) the use of a lower SLM to acceptor solution volume ratio (thinner membrane), and 3) addition of another salt different from NaCl.

The first of these sets of experiments were done by changing the organic solvent in the SLM to another class of solvents also proven to be effective in EME [27]. 6-undecanone was chosen and the voltage was adjusted to a level compatible with the new SLM (200 V). All other parameters remained the same. A similar comparison as in Table 1 and Figure 1 between NaCl and no NaCl in the sample solution is shown in Figure 2 for the setup with the new SLM. Out of the eight compounds tested, five were originally affected by NaCl in the setup shown in Table 1. However, when using 6-undecanone as the SLM only promethazine and clomipramine showed a substantial reduction in recovery when the amount of NaCl in the samples were increased. These two compounds were also the ones that experienced the greatest recovery loss when NPOE was used as the organic solvent. These observations show that this salt effect is dependent on the type of SLM solvent. The observation is in agreement with the ion pairing hypothesis as trapping of an ion pair in the SLM is expected to be highly dependent on the nature of the organic solvent [30].

The second set of experiments was performed using a recently developed extraction setup utilizing thin (100 µm) membranes [25]. If ion pairing and subsequent trapping in the SLM was the cause of the reduced recovery, a thinner membrane (lower SLM to acceptor solution volume ratio) should reduce this effect due to a relatively smaller volume of the organic solvent (V_M). These extractions were performed according to an optimized procedure for the flat membrane extraction setup [25], and the choice of organic solvent was once again NPOE as in the original experiment in Table 1. The results for these experiments are shown in Figure 3, and show less salt effects than the setup with the thicker hollow fiber membrane. Interestingly, promethazine and clomipramine were, this time also, the only two compounds that were affected by the increased amount of NaCl in the sample solution. These results show that a lower SLM to acceptor solution volume ratio (V_M/V_A) in this setup indeed reduced the recovery loss when NaCl was added to the sample, as compared to the thicker hollow fiber membrane having a higher volume ratio (V_M/V_A).

In the third set of experiments, 2.5 % (w/v) potassium sulphate (K_2SO_4) was used instead of NaCl as the salt added to the sample solution. Extractions were once again performed in the

hollow fiber system, with NPOE as the organic solvent (SLM). The results of these experiments are presented in Figure 4, and show no substantial changes in recovery between the samples with and without K_2SO_4. This suggests that the type of salt play an important role. Since efficient ion pairing with the analytes and trapping of the ion pair complexes are highly dependent on the counter ion, this result also strengthens the hyphotesis of ion pairing as the cause of reduced recovery when NaCl is present in the sample. It may also be noted that a 1 to 1 complex between a singly charged base cation and sulphate will have a charge of -1 and will therefore move across the SLM under the influence of an applied potential rather than being trapped in the SLM as a neutral species.

In all the experiments, a mass balance was established by analyzing both the sample solution and the acceptor solution after EME. Also, the inner surfaces of the sample vials were checked for analyte adsorption or precipitation after the extractions. No measureable adsorption or precipitation was detected on the vial surfaces, so the amounts unaccounted for after measurement of the sample and acceptor solution was believed to be trapped in the SLM. In all the cases of reduced recovery, the additional loss was found to be trapped in the SLM according to the mass balance measurements. Additional experiments were performed with increased extraction times but none of these experiments were able to release any significant amount of the trapped analytes from the SLM.

3.2 Similar experiences from the literature

The reduced recovery for some of the analytes when extracting from samples containing salts are in line with previously reported experiments with NaCl in EME. These experiments have not been quantified and were performed during method development to investigate if large amounts of salts and large changes in the ion balance affect the extraction recovery. The basic substances thebaine [10], ephedrine [18], trimipramine [19], and several amphetamine like stimulants [11], as well as the acidic substances ibuprofen, naproxen [20], and several chlorophenols [21] were extracted with NaCl in the sample commonly ranging from 5 to 15 % (w/v). All the above results were in agreement with the general flux theory in EME, where a high amount of ions in the sample solution reduce the efficiency of the extraction process [22], and concluded that the reason for the lower recovery was because of the unfavorable ion balance in the system. However, for extractions of some haloacetic acids and aromatic acetic acids [16] it was concluded that a concentration of up to 5 % (w/v)

NaCl improved the extraction recoveries because of a salting-out effect, whereas higher concentrations caused the recovery to decrease.

The stability of the EME system when extracting from higher amounts of NaCl in the sample solution was also investigated when extracting uranium (VI) [31], concluding that at higher salt levels (higher than 2 % w/v), the EME system became unstable. This was consistent with the observations reported in section 3.1.

3.3 Theoretical considerations

The experimental observations indicate that formation of ion pairs play a role at least for some of the investigated drug compounds. Ion pair formation in relation partitioning in organic solvents immiscible with water has previously been treated from a theoretical point of view, albeit in a different system [32].

In the present system it was observed that NaCl in the sample solution in some cases has an impact on the recovery. It has also been established, both previously [24] and from the mass balance measurements in this work, that the sample solution is emptied relatively fast. In principle, ion paring may take place in the sample solution and/or a higher chloride concentration in the sample solution may lead to a larger amount of anions (bound and free) in the SLM. In the following we will only consider the recovery after exhaustive extraction, corresponding to the case where the sample solution has been completely depleted of analyte (application of a large extraction potential for a long time). In the mass balance consideration we will therefore assume that there is no analyte present in the sample solution. We shall present the theoretical consideration using the notation B^+ for the base and Cl^- for the anion, and for simplicity we only consider singly charged ions.

In terms of chemical equlibria we may describe the system after exhaustive extraction as follows:

Acceptor solution:

$$B^+(A) + Cl^-(A) \leftrightarrow BCl(A), \qquad K_{BCl(A)} = \frac{C_{BCl(A)}}{C_{B^+(A)} \cdot C_{Cl^-(A)}} \qquad (2)$$

SLM:

$$B^+(M) + Cl^-(M) \leftrightarrow BCl(M), \qquad K_{BCl(M)} = \frac{C_{BCl(M)}}{C_{B^+(M)} \cdot C_{Cl^-(M)}} \tag{3}$$

where $K_{BCl(A)}$ is the ion pair equilibrium constant in the acceptor solution and $K_{BCl(M)}$ is the ion pair equilibrium constant in the SLM.

There are additional partition equilibria involving B^+ and Cl^- which will be dependent on the applied potential. Based on experimental evidence we shall assume that the concentration of Cl^- to a good approximation remains constant over time. This is also to be expected as Cl^- is present in a large excess. The partition equlibrium of the ion pair is potential independent as it is neutral, but the absolute amount of ion pair in the SLM is dependent on potential as it is coupled to the potential dependent concentrations of B^+ and Cl^-. Conversely, the concentration of Cl^- in the SLM is linked not only to the ion pairing equilibrium constant, but also to the concentration of Cl^- in the sample solution and possibly to ion pair formation occurring there.

The expression for the maximum recovery fraction reads:

$$R_{\max} = \frac{n_{A,final(total)}}{n_{D,initial}} = \frac{C_{B^+(A)} \cdot V_A + C_{BCl(A)} \cdot V_A}{C_{D,initial} \cdot V_D} \cdot 100\% \tag{4}$$

where $C_{B^+(A)}$ and $C_{BCl(A)}$ are the final concentrations of B^+ and BCl in the acceptor solution, respectively. V_A is the volume of the acceptor solution.

Assuming that at long extraction times the sample solution has been completely depleted of analyte, the conservation of mass can be expressed as:

$$n_{D,initial} = C_{B^+(A)} \cdot V_A + C_{BCl(A)} \cdot V_A + C_{B^+(M)} \cdot V_M + C_{BCl(M)} \cdot V_M \tag{5}$$

where $C_{B^+(M)}$ and $C_{BCl(M)}$ are the final concentrations of B^+ and BCl in the SLM, respectively. V_M is the volume of the sample solution.

The expression for the maximum recovery fraction can thus be written as:

$$R_{\max} = \frac{n_{A,final(total)}}{n_{D,initial}} = \frac{C_{B^+(A)} \cdot V_A + C_{BCl(A)} \cdot V_A}{C_{B^+(A)} \cdot V_A + C_{BCl(A)} \cdot V_A + C_{B^+(M)} \cdot V_M + C_{BCl(M)} \cdot V_M} \cdot 100\% =$$

$$\frac{V_A + \frac{C_{BCl(A)}}{C_{B^+(A)}} \cdot V_A}{V_A + \frac{C_{BCl(A)}}{C_{B^+(A)}} \cdot V_A + \frac{C_{B^+(M)}}{C_{B^+(A)}} \cdot V_M + \frac{C_{BCl(M)}}{C_{B^+(A)}} \cdot V_M} \cdot 100\% \qquad (6)$$

The partition coefficient of B^+ between the acceptor solution and the SLM is potential dependent according to [24]:

$$K^*_{d,A} = \frac{C_{B^+(M)}}{C_{B^+(A)}} = \exp\left(\frac{F}{RT} (\Delta^w_o \phi - \Delta^w_o \phi^0_{B+}) \right) \qquad (7)$$

Here $\Delta^w_o \phi$ is the Galvani potential difference between the acceptor solution and the SLM. $\Delta^w_o \phi$ is proportional to $-E$ where E is the applied extraction potential. A high positive potential would thus result in a very low K^*_d (typically the case for exhaustive extractions). $\Delta^w_o \phi^0_{B+}$ is a property related to how hydrophobic the analyte B^+ is.

Using the expression for K^*_d , $K_{BCl(M)}$ and $K_{BCl(A)}$ we arrive at:

$$R_{\max} = \frac{V_A + K_{BCl(A)} \cdot C_{Cl^-(A)} \cdot V_A}{V_A + K_{BCl(A)} \cdot C_{Cl^-(A)} \cdot V_A + K^*_d \cdot V_M + K^*_{d,A} \cdot K_{BCl(M)} \cdot C_{Cl^-(M)} \cdot V_M} \cdot 100\% =$$

$$\frac{1 + K_{BCl(A)} \cdot C_{Cl^-(A)}}{1 + K_{BCl(A)} \cdot C_{Cl^-(A)} + K^*_{d,A} \cdot \frac{V_M}{V_A} + \frac{V_M}{V_A} \cdot K^*_{d,A} \cdot K_{BCl(M)} \cdot C_{Cl^-(M)}} \cdot 100\% =$$

$$\frac{1 + K_{BCl(A)} \cdot C_{Cl^-(A)}}{1 + K_{BCl(A)} \cdot C_{Cl^-(A)} + \frac{V_M}{V_A} \left\{ K^*_{d,A} + K^*_{d,A} \cdot K_{BCl(M)} \cdot C_{Cl^-(M)} \right\}} \cdot 100\% \qquad (8)$$

From this expression some limiting situations may be considered:

Case 1: $K_{BCl(A)} \cdot C_{Cl^-(A)} << 1$ (No significant ion pairing in the acceptor solution).

$$R_{\max} = \frac{1}{1+\frac{V_M}{V_A}\left\{K^*_{d,A} + K^*_{d,A}\cdot K_{BCl(M)}\cdot C_{Cl^-(M)}\right\}}\cdot 100\% =$$

$$\frac{1}{1+\frac{V_M}{V_A}\cdot K^*_{d,A}\left\{1+K_{BCl(M)}\cdot C_{Cl^-(M)}\right\}}\cdot 100\% \qquad (9)$$

Here the maximum recovery is dependent on V_M/V_A as also observed experimentally. If substantial ion paring is observed in the SLM ($K_{BCl(M)}\cdot C_{Cl^-(M)} >> 1$), the maximum obtainable recovery may be smaller than 1. As previously stated $C_{Cl^-(M)}$ may be affected if the chloride concentration in the sample solution is increased. This could explain the pronounced dependence of the chloride concentration in the sample solution on the extraction recovery observed for some drug compounds. Additionally, $C_{Cl^-(M)}$ is dependent on the applied extraction potential.

Case 2: $K_{BCl(M)}\cdot C_{Cl^-(M)} << 1$ (No significant ion pairing in the SLM).

$$R_{\max} = \frac{1}{1+\frac{V_M}{V_A}K^*_{d,A}}\cdot 100\% \qquad (10)$$

Since typically $K^*_{d,A} << 1$ at high potentials we simply get a maximum recovery of 100 % in this case.

The behavior seen for the model analytes in Table 1 belong to both of these cases. For the nine unaffected analytes a behavior similar to the second case can be observed, where no significant ion pairing occure in the SLM. The recovery is thus unaffected by the presence of salts. For the eight affected analytes, the behavior is more similar to the first case, and ion pairing in the SLM reduces the recovery. When the SLM to acceptor solution volume ratio V_M/V_A is reduced, this reduction in recovery should also be reduced according to equation 9. This is in line with the experimentally observed results presented in Figure 3. For the two model analytes promethazine and clomipramine, the salt dependency was high, and even though the reduced SLM to acceptor solution volume ratio V_M/V_A somewhat improved the recovery, it was still much lower than without salts present. A substantial ion pairing can

cause the denominator in equation 9 to still be much larger than 1, even after reducing the volume ratio V_M/V_A between the SLM and the acceptor solution, as might be the case for these two substances.

4 Conclusion

The present work has thoroughly investigated how the presence of salt in the sample solution affects EME of various unpolar basic drugs. For eight out of a total of 17 substances, the recoveries were significantly reduced when 2.5 % (w/v) NaCl was added to the sample solution. A correlation between physical chemical properties of the analytes and this observed recovery loss was not apparent. For larger amounts of NaCl (5 % w/v) in the sample solution also the repeatability of the extractions was affected. The observed recovery loss was explained according to a hypothesis of ion pairing in the SLM, and a mathematical model for this effect has been presented. Experimental data showed that the recovery loss could be reduced by changing the organic solvent in the SLM or use a lower SLM to acceptor solution volume ratio, which is in line with the theoretical model. These findings represent the first thorough investigation of how salts in the sample solution can influence EME, and improve the theoretical understanding of the extraction process.

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Figure 1:

Extraction recovery (with RSD) for 8 different basic drugs in a concentration of 100 ng mL^{-1} after 5 minutes of EME with NPOE in the SLM. The extractions were performed with and without 2.5 % (w/v) NaCl added to the sample solution (*n=5*).

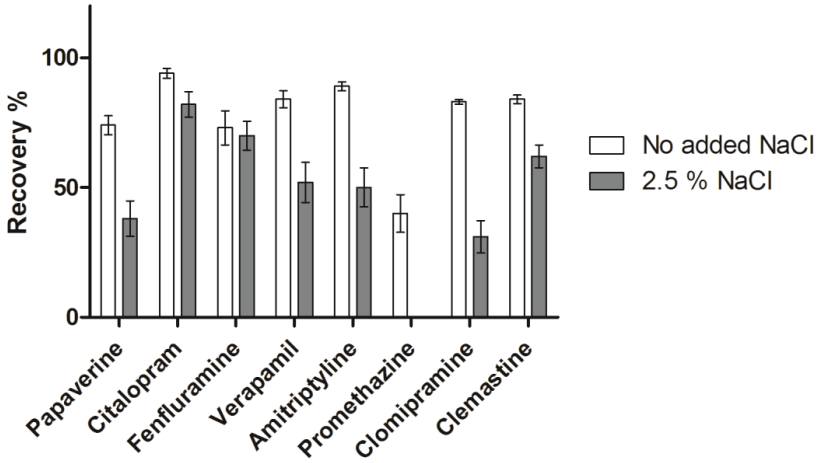

Figure 2:

Extraction recovery (with RSD) for 8 different basic drugs after 5 minutes of EME with 6-undecanone in the SLM. The extractions were performed with and without 2.5 % (w/v) NaCl added to the sample solution (*n=3*).

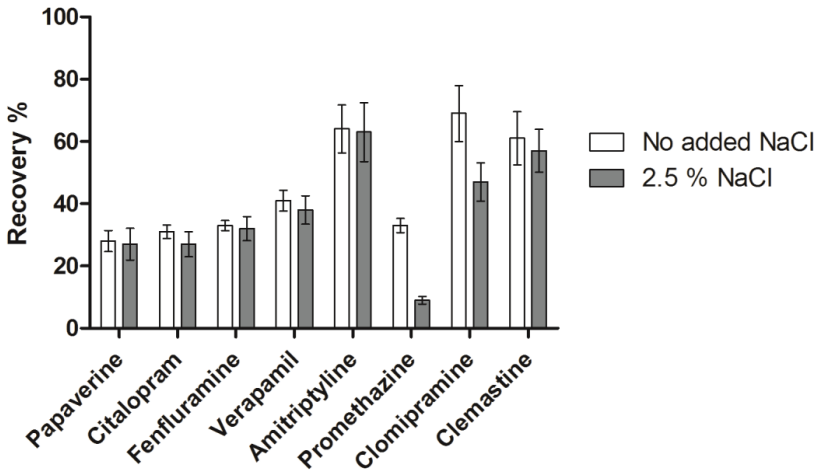

Figure 3:

Extraction recovery (with RSD) for 8 different basic drugs after 15 minutes of EME with a thin membrane setup and NPOE in the SLM. The extractions were performed with and without 2.5 % NaCl (w/v) added to the sample solution (*n=5*).

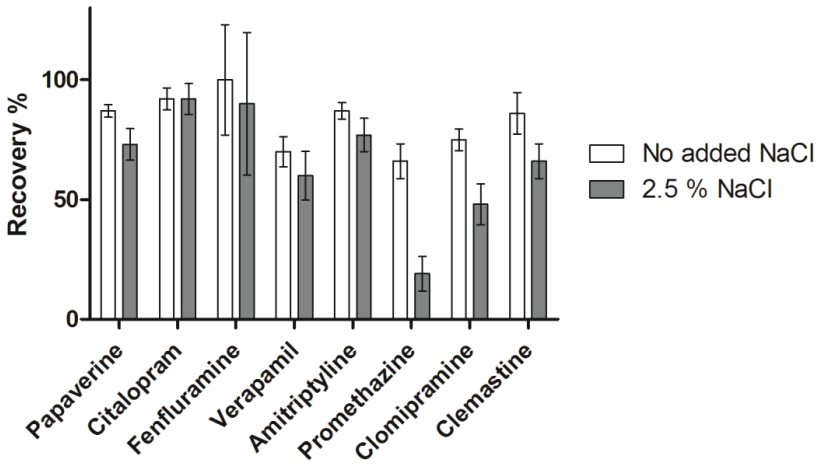

Figure 4:

Extraction recovery (with RSD) for 8 different basic drugs after 5 minutes of EME with NPOE in the SLM. The extractions were performed with and without 2.5 % (w/v) K_2SO_4 added to the sample solution (*n=5*).

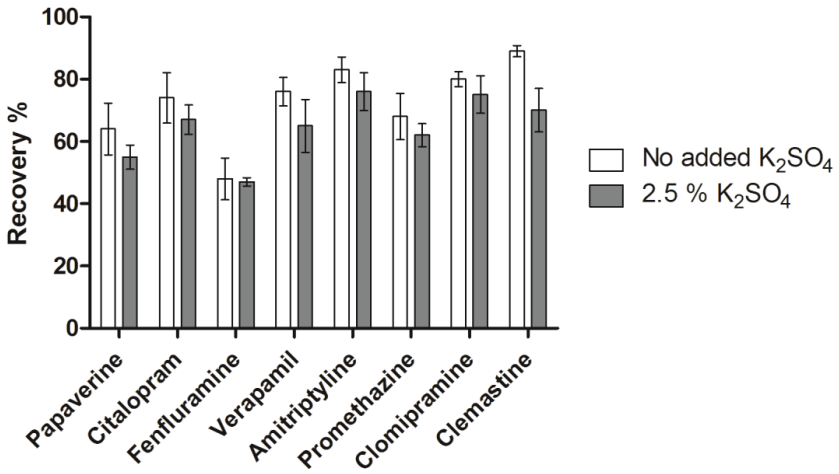

Table 1:

Extraction recovery (with RSD) for 17 different basic drugs in a concentration of 1 µg mL^{-1} after 5 minutes of EME with NPOE in the SLM. The extractions were performed with and without 2.5 % (w/v) NaCl added to the sample solution (*n≥3*).

Drug	*% Recovery (% RSD)*	
	0 % NaCl	***2.5 % NaCl***
Pethidine	62 (11)	70 (11)
Pyrilamine	62 (9)	54 (17)
Papaverine	64 (13)	23 (6)
Citalopram	74 (11)	70 (19)
Fenfluramine	48 (14)	47 (9)
Haloperidol	81 (13)	92 (12)
Methadone	82 (10)	84 (9)
Perphenazine	52 (8)	25 (7)
Nortriptyline	72 (11)	19 (8)
Verapamil	76 (6)	20 (14)
Loperamide	86 (3)	100 (4)
Amitriptyline	83 (5)	29 (7)
Reserpine	55 (14)	55 (12)
Prochlorperazine	86 (7)	51 (7)
Promethazine	68 (11)	5 (11)
Clomipramine	80 (3)	19 (11)
Clemastine	89 (2)	80 (11)