

# **Development of Electromembrane Extraction Configurations and Applications**

Thesis for the degree of Philosophiae Doctor

by

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Oslo, March 2014

A handwritten signature in black ink that reads "Lars Erik Eng Eibak". The signature is written in a cursive style with some capital letters.

Lars Erik Eng Eibak

## LIST OF PAPERS

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

**I** L.E.E Eibak, A. Gjelstad, K.E. Rasmussen, S Pedersen-Bjergaard “*Kinetic electro membrane extraction under stagnant conditions-Fast isolation of drugs from untreated human plasma*”, Journal of Chromatography A, (2010) 1217, 5050-5056.

**II** L.E.E Eibak, A. Gjelstad, K.E. Rasmussen, S Pedersen-Bjergaard “*Exhaustive electromembrane extraction of some basic drugs from human plasma followed by liquid chromatography-mass spectrometry*“, Journal of Pharmaceutical and Biomedical Analysis, (2012), 57, 33-38.

**III** R.E.G Jamt, A. Gjelstad, L.E.E. Eibak, E.L Øiestad, A.S Christophersen, K.E. Rasmussen, S. Pedersen-Bjergaard “*Electromembrane extraction of stimulating drugs from undiluted whole blood*”, Journal of Chromatography A (2012) , 1232, 27-36.

**IV** L.E.E Eibak, A.B. Hegge, K.E. Rasmussen, S Pedersen-Bjergaard, A. Gjelstad “*Alginate and chitosan foam combined with electromembrane extraction for dried blood spot analysis*”, Analytical Chemistry (2013), 84, 8783-8789.

**V** C.P. Lødøen, L.E.E. Eibak, K.E. Rasmussen, S. Pedersen-Bjergaard, T. Andersen, A. Gjelstad “*Storage of oral fluid as dried spots on alginate and chitosan foam - A new concept for oral fluid collection*”, Bioanalysis (2013), 5, 317-325.

**VI** L.E.E. Eibak, M.P. Parmer, K.E. Rasmussen, S. Pedersen-Bjergaard, A. Gjelstad “*Parallel Electromembrane Extraction in a Multiwell Plate*“, Analytical and Bioanalytical Chemistry (2013), 406, 431-440.

**VII** L.E.E. Eibak, K.E. Rasmussen, S. Pedersen-Bjergaard, A. Gjelstad. “*Exhaustive Electromembrane Extraction in 96-well Format*“, manuscript submitted to Analytica Chimica Acta 12.february 2014.

**ABBREVIATIONS**

CE-UV	Capillary electrophoresis with ultraviolet detection
DBS	Dried blood spot
DEHP	Di-(2-ethylhexyl) phosphate
DMS	Dried matrix spot
DOI	2,5-Dimethoxy-4-iodoamphetamine
EME	Electromembrane extraction
EMA	European medicines agency
ENB	1-ethyl-4-nitrobenzene
ESI	Electrospray ionization
FDA	Food and drug administration
GC	Gas chromatography
HCl	Hydrochloric acid
HCOOH	Formic acid
HF-EME	Hollow fiber electromembrane extraction
HF-LPME	Hollow fiber liquid phase microextraction
HPLC	High performance liquid chromatography
IPNB	1-Isopropyl-4-nitrobenzene
LC	Liquid chromatography
LLOQ	Lower limit of quantification
MDA	3,4-Methylenedioxy-amphetamine
MDMA	3,4-Methylenedioxy-methamphetamine
NIPH	Norwegian Institute of Public Health
NPOE	2-Nitrophenyl octyl ether
NPPE	2-Nitrophenyl pentyl ether
Pa-EME	Parallel electromembrane extraction
PALME	Parallel artificial liquid membrane extraction
PMA	Para-methoxy-amphetamine
PMMA	Para-methoxy-metamphetamine

PP	Protein precipitation
QbD	Quality by design
R <sup>2</sup>	Regression coefficient
RPM	Revolutions per minute
RSD	Relative standard deviation
SDME	Single drop microextraction
SLE	Supported liquid extraction
SLM	Supported liquid membrane
SPME	Solid-phase microextraction
SPE	Solid phase extraction
TFA	Trifluoroacetic acid
TEHP	Tris (2-ethylhexyl) phosphate
UPLC	Ultra performance liquid chromatography



## ABSTRACT

Electromembrane extraction (EME) was described for the first time in 2006 and demonstrated electrokinetic migration of charged substances from an aqueous sample solution through a supported liquid membrane (SLM) and into an aqueous acceptor solution. EME was reported as a selective and fast extraction technique providing clean extracts from biological samples that are directly compatible with analytical instrumentation. The intention of this thesis was to investigate fundamental aspects of EME and to further develop technical EME configurations. Major focus was on extraction of small molecular substances from human matrices; extraction at low voltages (<10 V), short extraction times (<10 min), exhaustive extraction, and parallel extractions of multiple samples.

In **Paper I** a portable EME device was developed, this device was operated under stagnant conditions with a 9 V battery as power supply. Amitriptyline, citalopram, fluoxetine, and fluvoxamine were isolated electrokinetically from 70  $\mu\text{L}$  undiluted human plasma and into 30  $\mu\text{L}$  of 10 mM HCOOH. The final extract was analyzed by liquid chromatography coupled to mass spectrometry (LC-MS). Extraction time, extraction voltage, SLM, and acceptor solution were optimized in terms of extraction recovery. This EME setup provided extraction recoveries in the range 12-22% after 1 min of extraction. Although the extraction recoveries were relatively low, the combination of EME with LC-MS provided lower limit of quantification (LLOQ) below the therapeutic range. The  $r^2$  in the therapeutic range (1-1000  $\text{ng mL}^{-1}$ ) was above 0.998 for all analytes. In addition, the sample throughput was increased by operating three sample compartments in parallel with a single 9 V battery.

In **Paper II** the extraction recovery with EME was investigated by introducing three separate hollow fibers into a single sample compartment. This setup provided exhaustive EME of citalopram, loperamide, methadone, paroxetine, pethidine, and sertraline from pH adjusted water samples. The three-fiber configuration was also utilized to isolate the same basic analytes from untreated human plasma samples. However, the extraction recoveries were reduced to some extent regarding some of the analytes; probably due to proteinbinding in human plasma and thus limited transportation of the analytes through the SLM. The setup was evaluated regarding linearity, reproducibility, and LLOQ, and the results were considered as acceptable in terms of regulatory guidelines. The three fiber approach in combination with LC-MS was used to isolate citalopram from two patient plasma samples; the reported plasma concentration deviated less than 14% compared to data obtained by a reference laboratory.

In **Paper III** EME was evaluated with samples of human whole blood spiked with the following drugs of abuse: Cathinone, 2,5-dimethoxy-4-iodoamphetamine (DOI), methamphetamine, 3,4-methylenedioxy-amphetamine (MDA), 3,4-methylenedioxy-metamphetamine (MDMA), and ketamine. Extraction time, SLM composition, acceptor solution, and extraction voltage were all optimized regarding extraction recovery. The final setup isolated the analytes of interest from 80  $\mu$ L sample, through an SLM consisting of 1-ethyl-4-nitrobenzene (ENB), and further into 10  $\mu$ L of acetic acid, the applied voltage was 15 V, and the extraction was performed for 5 min. The final EME-configuration with a 15 V battery as power supply was utilized to isolate cathinone, amphetamine, methamphetamine, ketamine, MDA, and MDMA from authentic forensic samples. Although the extraction recovery was in the range 10-30%, the combination of EME with ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) provided LLOQ below the concentrations associated with drug abuse.

In **Paper IV** the approach from paper II was combined with solid sampling of biological samples on sheets of alginate- and chitosan foam. Samples of 10  $\mu$ L whole blood spiked with citalopram, loperamide, methadone, and sertraline were spotted on sheets of either alginate- and chitosan foam and thereafter dried for 2 hours. Subsequently, the dried blood spots (DBS) were punched out and disintegrated in 300  $\mu$ L HCl for 3 min. From this solution the analytes of interest were isolated by EME into 20  $\mu$ L of 20 mM HCOOH, and provided extracts directly compatible with LC-MS analysis within 10 min. In addition, the novel approach was compared with DBS sampling on well-established sampling media like Whatman FTA DMPK-A and Agilent Bond Elut DMS. The elution recoveries reported with those media was inferior compared to sampling on alginate- and chitosan foam and subsequent extraction with EME. The absence of interfering matrix components in the final EME extract was also demonstrated in Paper IV

In **Paper V** samples of oral fluid was spotted on alginate- and chitosan foam. 10  $\mu$ L of oral fluid spiked with buprenorphine, methadone, methamphetamine, para-methoxy-amphetamine (PMA), and para-methoxy-metamphetamine (PMMA) was applied to alginate- and chitosan foam; subsequently the dried spots were punched out and disintegrated in 300  $\mu$ L 1 mM HCl for 5 min. The analytes were subsequently isolated from this solution by EME and into 0.1% trifluoroacetic acid (TFA) within 5 min. The experimental conditions were optimized. The final setup was evaluated regarding linearity in the relevant concentration range, RSD, extraction recovery, and storage-stability for 30 days. The evaluation results were considered as

acceptable with regards to regulatory guidelines. Post-column infusion experiments demonstrated that the final EME extract was free from interfering matrix components and underlined the sample cleanup provided with EME.

In **Paper VI** EME was introduced into the multi-well platform with flat membranes to enable parallel extractions with EME; this approach increased the overall sample throughput with EME. The idea of parallel electromembrane extraction (Pa-EME) with the hollow fiber configuration was briefly investigated in Paper I; however this setup was highly challenging to operate. In Paper VI amitriptyline, fluoxetine, quetiapine, and sertraline were isolated from eight plasma samples into eight separate extracts of 70  $\mu\text{L}$  20 mM HCOOH. The experimental conditions were optimized with a Quality by Design (QbD), and were as follows: Extraction time 8 min, extraction voltage 200 V, sample volume 240  $\mu\text{L}$ , acceptor volume 70  $\mu\text{L}$ , and agitation rate 1040 rpm. The linear calibration curves in the therapeutic range were above 0.9974; this calibration curve was used to quantify plasma concentrations of quetiapine and sertraline in patient samples. Additionally, post-column infusion experiments demonstrated the sample cleanup provided with Pa-EME.

In **Paper VII** the Pa-EME performance was investigated with different matrices to establish how the Pa-EME was affected by small, but deliberate variations in method parameters during normal use. Urine, human plasma, and pH adjusted water samples were utilized as sample matrices. The Pa-EME system was also stressed by perforating one or several of the wells in order to investigate how this influenced the remaining intact wells in the multi-well plate. The total number of samples processed simultaneously was increased from eight in Paper VI to 68 and finally 96 samples in Paper VII. Paper VII demonstrated for the first time extraction of 96 samples simultaneously with EME. The combination of a robust high-throughput sample preparation technique with UPLC-MS/MS provided a powerful platform for analysis of small molecular drug substances from biological matrices.

## 1. INTRODUCTION

The overall objective of analytical chemistry is to accurately detect and quantitate the analyte of interest from a sample; however the majority of the sample matrices available for this purpose are not directly compatible with analytical instrumentation. Biological, environmental, and food samples include several contaminants which normally are present in high concentrations and could interfere with the final analysis. Consequently, a sample preparation procedure is vital to isolate the analytes from the sample and into a solution compatible with the analytical instrumentation. This sample preparation step is considered as the “bottleneck” of the bioanalytical workflow, and the time consumption is estimated to around 75% of the total time [1-3]. Sample preparation is often a compromise between time, sample cleanup, and complexity of the procedure. The search for novel, more environmental- and user friendly sample preparation techniques to solve existing and novel analytical challenges is dynamic and continuous.

### 1.1 Sample preparation in bioanalysis

Traditionally, whole blood, plasma, serum, and urine have been the key matrices for determination of the concentration of small molecular substances in bioanalysis [4]. The abovementioned matrices are incompatible with immediate analysis and a sample preparation step prior to analysis is considered as mandatory [1, 2, 5, 6]. This is mainly due to the numerous interfering endogenous compounds like proteins, salts, lipids, and cells found at high concentrations in such biological matrices. An optimal sample preparation should provide extracts compatible with analytical instrumentation with low consumption of hazardous chemicals and contribute to a reliable detection and quantitation.

Traditionally, liquid-liquid extraction (LLE), protein precipitation (PP), and solid-phase extraction (SPE) have been the selected sample preparation techniques in bioanalytical routine laboratories [7]. The aforementioned sample preparation techniques are based on well-known principles and numerous applications have been published. However, those techniques are associated with large consumption of sample volume, extraction phase, and organic solvents. A large ratio between extraction phase and sample increase the extraction recovery and this approach has been mandatory with the analytical instrumentation available in the past. However, a large extraction phase dilutes the analyte to a significant degree [1, 7]. Consequently, the final extract has to be evaporated to dryness and reconstituted in a proper solvent in order to limit both the dilution of the analyte and reduce the solvent strength prior

to injection into the analytical instrumentation. The evaporation step could be time consuming, especially if the final extract consists of a low-volatile solvent.

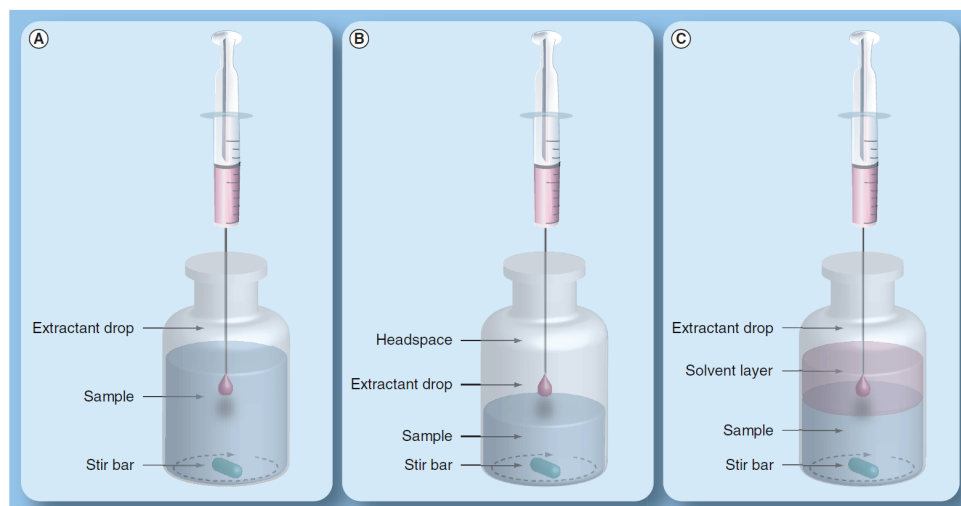
## 1.2 Microextraction techniques

In the early 1990s a shift was observed in the sample preparation field; the sample volume required to quantitate small molecular substances was reduced from milliliter to microliter [8, 9]. The primary contributor to this development was the increased sensitivity provided with the analytical instrumentation; the improved sensitivity also eliminated the requirement for exhaustive extractions. Solid-phase microextraction (SPME) was introduced as the first microextraction technique in 1990 [10]. SPME is considered as a “soft” extraction technique, which means that only a small fraction of the analyte is isolated from the sample. The commercially available device introduced by Supelco [11, 12] has contributed to the interest and the number of papers concerning SPME in the literature. At the same time existing sample preparation techniques were refined in the same direction inspired by the development of SPME [7].

### 1.2.1 Single drop microextraction (SDME)

Single drop microextraction was introduced in 1996 by Liu and Dasgupta [13]; in this approach a drop of a water immiscible organic solvent was suspended in a larger aqueous drop. The outer aqueous drop contained the analytes of interest and diffused into the organic drop. After extraction the organic drop was aspirated with a syringe and the analyte concentration was related to absorbance with a light-emitting-diode-based absorbance detector. Jeannot and Cantwell [14] presented another SDME approach at the same time; in this setup the water immiscible organic solvent was attached to a microsyringe and immersed into an aqueous sample solution as presented in Fig. 1-1a [15]. The analytes of interest diffused from the aqueous sample and into the organic drop; thereafter the organic droplet was aspirated into the microsyringe and injected directly into a gas chromatograph (GC). The idea of both SDME-approaches was to reduce the consumption of hazardous organic solvents to a minimum compared to conventional LLE. The pH value in the sample was strictly controlled to ensure that the analytes present in the aqueous sample solution were unionized in order to facilitate the partition into the organic liquid. A magnetic stirrer induced mixing of the aqueous sample solution and facilitated diffusion of the analyte into the organic drop.

In headspace SDME the organic drop is located just above the sample solution [16-20] as presented in Fig. 1-1b [15]. The direct compatibility of the organic drop with GC in the 2-phase SDME led to the investigation of a 3-phase SDME approach with an aqueous sample solution, an organic liquid, and an aqueous droplet as presented in Fig. 1-1c [15]. The analytes of interest in the sample are unionized in order to facilitate diffusion into the organic liquid, and the aqueous drop is pH-adjusted to facilitate diffusion in the interface from the organic liquid and into this aqueous receiver drop. Subsequently, the drop is aspirated and analyzed with high performance liquid chromatography (HPLC).



**Fig. 1-1.** Single drop microextraction in 2-phase mode (a), head-space mode (b), and 3-phase mode (c), respectively. (Reprinted with permission from [15] © 2009 Future Science Group)

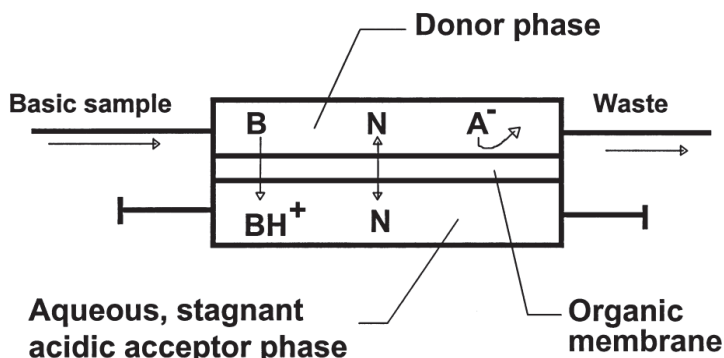
SDME provides high enrichment factors due to the ratio between sample (1-5 mL) and the organic drop (1-50  $\mu\text{L}$ ); enrichment factors up to 500 have been reported with 3-phase SDME [21] within a relatively short period of time (1-20 min) [22]. SDME is also considered as an environmental-friendly sample preparation technique due to the low consumption of organic liquid (1-50  $\mu\text{L}$ ).

The consumption of organic solvent is reduced substantially compared to conventional LLE. The simple equipment needed for SDME, low cost per extraction, ease of operation, and the compatibility with liquid- or gas chromatography is considered as advantages compared to other extraction techniques [9, 23]. However, extensive stirring in the sample solution promotes dislodging of the drop from the microsyringe tip and into the sample solution.

Another issue is the emulsifying properties of biological fluids like blood and plasma; this emulsification could reduce the drop-stability. Consequently, SDME is considered as less compatible sample preparation technique in combination with biological fluids compared to pH adjusted water samples.

### 1.2.2 Supported liquid membranes (SLM)

The instability of the single organic drop and furthermore lack of robustness reported with the SDME configuration increased the interest for supported liquid membranes in the field of sample preparation. The idea of using SLM for sample preparation in bioanalysis was introduced by Audunsson already in 1986 [24], and the interest for SLM reemerged in the late 1990s [25-30]. In SLM extractions the sample- (donor) and extraction (acceptor) solution is separated by an organic liquid sustained by capillary forces in the pores of a hydrophobic supporting material as presented in Fig. 1-2 [24, 31]. The aim of the SLM extraction is to isolate the analytes of interest from the donor solution, through the SLM, and into an aqueous acceptor solution. The diffusion is facilitated by pH-gradients. Unionized analytes diffuse passively from the donor solution and into the organic liquid sustained in the pores of the SLM. Subsequently, the analytes are ionized upon contact with the pH-adjusted acceptor solution. Sufficient contact in the interfaces between the donor solution, the SLM, and the acceptor solution are mandatory to isolate the analytes efficiently from the donor solution and



**Fig. 1-2.** Principle of supported liquid membrane extraction (SLM). (Reprinted with permission from [25]. ©1999 Elsevier)

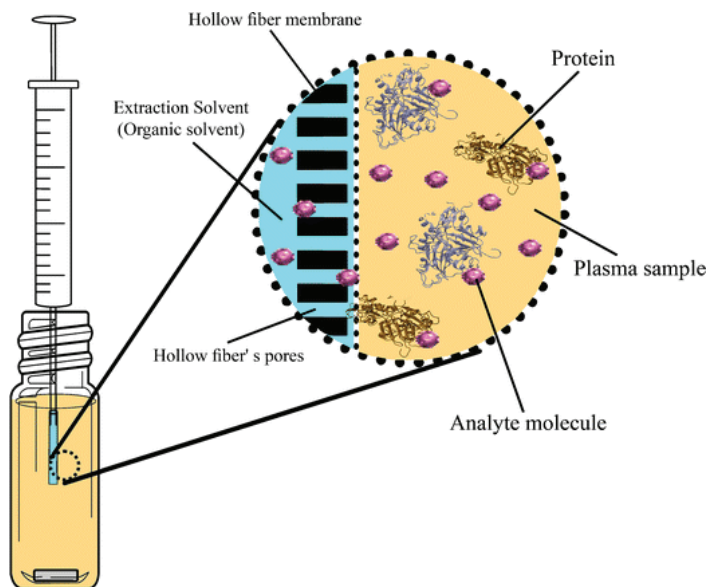
into the acceptor solution. The donor solution is pumped continuously with a syringe pump through the donor chamber and the analytes diffuse passively into the SLM and further into to the stagnant acceptor solution. Typical extraction time for SLM extractions with the flat membrane configuration is 10-20 min. SLM extractions provides decent sample cleanup from biological matrices like plasma, whole blood, and urine [24-26, 28, 32, 33].

Another configuration is the device with two chambers to continuously deliver both donor and acceptor solution to increase the extraction efficiency [26, 28, 33]. The need for syringe pumps to continuously deliver both donor- and acceptor solution requires a relatively complex apparatus and is considered as a drawback. Also the long-term stability of the reusable supporting material is a potential problem.

### **1.2.3 Liquid-phase microextraction (LPME) and parallel artificial liquid membrane extraction (PALME)**

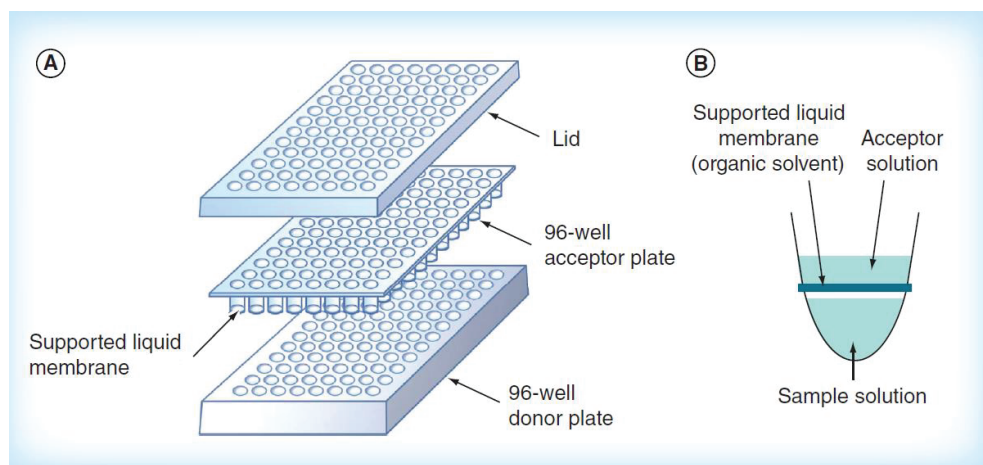
The instability of the single drop reported with SDME and the complex instrumentation with SLM was addressed in 1999 [34]. In this approach the organic liquid was applied to a supporting material comparable to SLM extractions; however in this setup the supporting material was a hollow polypropylene fiber [34]. The hollow fiber based liquid phase microextraction (HF-LPME) was described for the first time by Pedersen-Bjergaard and Rasmussen. The idea of LPME is diffusion of neutral analytes from a sample, into an organic liquid sustained in the pores of a supporting material, and further back-extracted as ionized species into an acceptor solution inside the hollow fiber. The acceptor solution is directly compatible with analytical instrumentation without the need for evaporation and reconstitution. LPME could be operated either in 2- or 3-phase mode. In the 2-phase mode the donor solution is aqueous and both the SLM and the acceptor solution inside the hollow fiber is an organic liquid. The 2-phase mode provides extracts directly compatible with GC [35, 36]. In the 3-phase mode the donor solution is aqueous, the SLM consists of an organic liquid, and the acceptor solution is aqueous and directly compatible with HPLC [37, 38]. This technique has obtained a widespread interest worldwide although HF-LPME has been difficult to incorporate into fully automated liquid handling systems. The HF-LPME configuration is presented in Fig. 1-3 [39].





**Fig. 1-3.** Illustration of liquid phase microextraction (LPME). (Reprinted with permission from [39]. © 2013 Future Science Group)

In order to enable the incorporation of LPME into liquid handling systems a novel LPME setup with flat membranes was introduced by Gjelstad *et.al* in 2013 [40]. In this setup, named PALME, the supporting material was a flat polypropylene membrane. The organic liquid was sustained in the pores of this flat membrane, and the acceptor solution was filled in the multi-well plate. The donor solution was added to a 96 well plate. This setup is presented in Fig. 1-4. The analytes diffused passively from the sample solution into the SLM and further into the acceptor solution under agitation [40]. Thereafter the acceptor solution was analyzed with liquid chromatography combined with mass spectrometry (LC-MS). The altered geometry in the flat membrane configuration compared to hollow fiber LPME increased the acceptor volume reservoirs. This setup provided simultaneous isolation, and enrichment of analytes from a multiple number of samples within 30 min of extraction [40]. This setup could most probably be incorporated into liquid handling systems for increased throughput in the near future.



**Fig. 1-4.** Schematic illustration of a Parallel artificial liquid membrane extraction (PALME) setup. (Reprinted with permission from [40]. © 2013 Future Science Group)

### 1.3 Electro enhanced extractions

The migration of charged species across a liquid-liquid interface under the application of an electrical field is well-known and provides an interesting tool for tuning the extraction selectivity [41-44]. The rationale for applying an electrical field is to increase the extraction kinetics and thus reduce the extraction time.

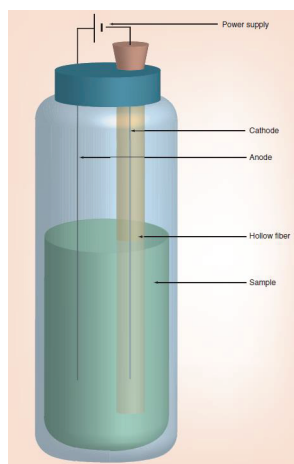
Van der Vlies *et.al* introduced an idea of combining liquid-liquid extraction with isotachopheresis and capillary zone electrophoresis [45-47]. This approach was conducted in a capillary electrophoresis system. A leading buffer was filled in the fused-silica capillary, thereafter an acceptor phase was introduced into the capillary inlet, the capillary inlet and the anode was located in an organic solvent containing cationic analytes. The capillary outlet and the cathode were placed in a vial with leading buffer. The application of an electrical field in such a system facilitated the migration of cationic species from the organic solvent and into the capillary inlet. In another approach the application of an electrical field (dc) in the interface between two immiscible electrolyte solutions (ITIES) was investigated by Berduque *et.al* in 2005 [48]. The application of an electrical field in ITIES extractions created an increased positive donor solution relative to the acceptor solution; this facilitated the transfer of cationic species from the donor- and into the acceptor solution.

Very recently Raterink *et.al* described another electroextraction approach [49]. A single drop of acceptor solution was attached to a conductive pipette tip and immersed into a donor

solution where the electrode with opposite charge was located. After immersion into the donor solution an electrical field was applied and enabled electrokinetic migration of seven different carnitines from the donor solution and into the hanging drop. After extraction the drop was injected directly into a nano ESI-MS system for analysis [49].

#### 1.4 Electromembrane extraction (EME)

Electromembrane extraction was described by Pedersen Bjergaard and Rasmussen in 2006 [50]; EME was a combination of LPME and more traditional electro enhanced extractions [41, 42, 44-48, 51-54]. By applying an electrical field (dc) in the LPME setup it was shown that the time needed to reach steady-state was reduced [55]; this time reduction has been assumed to be a result of the applied electrical field. In EME the sample solution is filled into the sample compartment and an electrode is placed in this solution; a porous polypropylene hollow fiber is impregnated for a given time with an organic liquid, the excess organic liquid is gently removed by a medical wipe. This impregnated hollow fiber serves as the SLM; and is subsequently filled with an acceptor solution before another electrode is placed in the lumen of the hollow fiber. Both the anode and cathode are



**Fig. 1-5.** Schematic illustration of an electromembrane extraction (EME) setup. (Reprinted with permission from Paper V. © 2013 Future Science Group)

connected to an adjustable power supply and an electrical field (dc) is applied for a certain period of time [50]. An EME setup is presented in Fig. 1-5. The direction of the electrical field is dependent on whether anions or cations should be isolated from the sample solution and into the acceptor solution. The extraction compartment is placed on a shaking board, and

the extraction is typically continued for 5 min [56-59]. After extraction the acceptor solution is collected by a microsyringe and transferred to an analytical instrumentation (LC or CE) for the final analysis.

### 1.4.1 Principle

The theoretical description of EME is complex due to both diffusion- and electrokinetic components of mass transfer. Firstly, the analytes diffuse into the SLM; secondly the analytes are transported electrokinetically as ionized species from the SLM and into the acceptor solution. The pH of the acceptor solution prevents the ionized analytes to be back-extracted into the sample solution. The time reduction observed with EME compared to LPME is mainly due to the electrokinetic transportation from the SLM and into the acceptor solution [60]. The theoretical considerations are based upon the Nernst-Planck equation and assume that the SLM is electrically neutral and is valid for thick membranes (200  $\mu\text{m}$ ) [61].

$$J_j = -D_j \frac{dc_j}{dx} + \frac{D_j z_j e E c_j}{kT} \quad (1)$$

Eq. (1) describes the steady-state flux ( $J_j$ ) of an ionic substance across the SLM and the flux is dependent on the diffusion coefficient ( $D_j$ ), its charge ( $z_j$ ), the concentration of the analyte in the SLM ( $c_j$ ), the distance between the analyte and the SLM/acceptor solution interface ( $x$ ), the Boltzmann's constant ( $k$ ), the elementary charge ( $e$ ), the electrical field ( $E$ ), and the absolute temperature ( $T$ ). The first term in Eq. (1) describes the diffusion of the analyte from the sample and into the SLM, and the latter term describes the electro-migration. However, the assumptions in Eq. (1) are not valid for thinner membranes and when there is a difference between the ion concentrations in the donor solution compared to the acceptor solution, which is the most frequent situation in EME. The steady-state flux of a singly charged cationic substance ( $J_i$ ) across an uncharged membrane can therefore be described by Eq. (2).

$$J_i = \frac{-D_i}{h} \left( 1 + \frac{v}{\ln \chi} \right) \left( \frac{\chi - 1}{\chi - \exp(-v)} \right) (c_{ih} - c_{io} \exp(-v)) \quad (2)$$

The steady-state flux is dependent on the diffusion coefficient ( $D_i$ ), the thickness of the membrane ( $h$ ), a dimensionless driving force ( $v$ ) defined by Eq. (3) including the potential difference across the SLM ( $\Delta\phi$ ), the ratio of the total ion concentration on the sample side to that on the acceptor solution ( $\chi$ ) is described by Eq. (4), the ratio of the total ionic concentration in the sample ( $c_{ih}$ ) to that in the acceptor solution ( $c_{io}$ ).

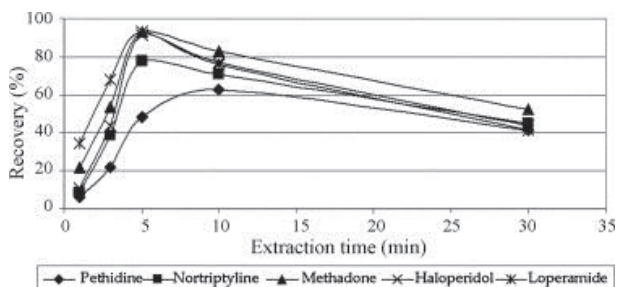
$$v = \frac{z_i e \Delta \phi}{kT} \quad (3)$$

$$\chi = \frac{\Sigma_i C_{ih} + \Sigma_k C^*_{kh}}{\Sigma_i C_{i0} + \Sigma_k C^*_{k0}} \quad (4)$$

For a given EME setup the thickness of the SLM and the diffusion coefficient for a given analyte would be constant. Thus, the flux across the SLM can be improved either by increasing the potential difference across the SLM or by reducing the ion balance over the SLM.

### 1.4.2 Extraction time

Electromembrane extraction is a refined LPME-approach with an electrical field and the extraction time is typically reduced from 45 min in LPME to 5 min with EME [62]. This time reduction is ascribed to the applied electrical field (dc) and the short time to reach steady-state is considered as an important advantage with EME compared to other extraction techniques. In EME it is desirable to continue the extraction until steady-state has been reached; which is typically 5 min as presented in Fig. 1-6 [63]. In the kinetic region of the EME a relatively small difference in extraction time could potentially affect the extraction recovery significantly. The final EME-extract is directly compatible with final analysis, for instance by HPLC, LC-MS, or capillary electrophoresis (CE).



**Fig. 1-6.** Extraction recovery (%) as function of extraction time for pethidine, nortriptyline, methadone, haloperidol, and loperamide. (Reprinted with permission from [63]. © 2008 Elsevier)

### 1.4.3 Supported liquid membrane

The supported liquid membrane in EME is made by impregnating the pores of the supporting material with an organic liquid. The objective of the SLM is to separate the sample solution from the acceptor solution. Another feature of the SLM is to serve as the resistance in the electrical circuit in the EME setup, and an adequate SLM-stability of the organic liquid in the pores of the supporting material is mandatory to prevent electrolysis [55, 62, 64-66]. It is well

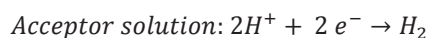
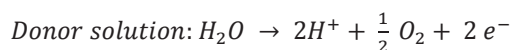
known that different organic liquids exert different stability in the supporting material. EME setups with organic liquids which are to soluble in either the the sample- or acceptor solution would reduce the resistance and consequently increase the flow of current in the system [66]. The loss of resistance in the SLM will further promote electrolysis in both the sample- and acceptor solution. An optimal organic liquid in EME should be non-volatile, low viscous, and immiscible with both sample- and acceptor solution [64]. A wide range of organic liquids with various physical-chemical properties have been investigated as SLM in EME [66]. 2-Nitrophenyl octylether (NPOE) is the most thorough investigated organic liquid and extraction recoveries up to 80% have been reported with non-polar basic drugs as analytes by combining NPOE as SLM with extraction voltages up to 300 V [64]. 1-Ethyl-4-nitrobenzene (ENB) and 1-Isopropyl-4-nitrobenzene (IPNB) provides similiar extraction recoveries with only 20 V as applied voltage [57, 58, 67]. Increased extraction voltage with ENB and IPNB would increase the flow of current due to their higher aqueous solubility compared to NPOE.

In order to facilitate the distribution of more hydrophilic ( $\log P < 1$ ) analytes into the SLM a carrier molecule has been added to the organic liquid prior to impregnation of the supporting material. This approach increased the hydrophobicity of the analyte and thus the affinity for the SLM; the analyte is released from the carrier molecule upon contact with the acceptor solution. This approach has been utilized for isolation of peptides with SLM extractions [68-70]. The same idea was used to increase the extraction recoveries of more hydrophilic basic drugs [64] and peptides in EME [71-73]. It was shown that the isolation of more hydrophilic basic drugs was more efficient by addition of carrier molecules. Cimetidine, a drug with  $\log P$  of 0.4, was not detected in the acceptor solution with pure NPOE as SLM; however by adding 25% (w/w) di (2-ethylhexyl) phosphate (DEHP) to NPOE prior to impregnation of the supporting material the extraction recovery was increased up to 75% [64].

#### 1.4.4 Extraction voltage

The flexibility provided with an external power supply in EME provides a unique opportunity to selectively isolate the analyte of interest from the sample- and into the acceptor solution. Both the direction- and the magnitude of the applied electrical field in the EME-setup are easily altered. The direction of the electrical field is dependent on the type of ionic substances to be isolated into the acceptor solution; for the isolation of anions the anode is located in acceptor solution and for the isolation of cations the cathode is placed in the acceptor solution [50, 74]. The magnitude is easily adjusted with the power supply; however the magnitude of the applied electrical field is highly dependent on the stability of the SLM in the supporting

material [66, 75]. Unstable SLMs would dissolve into the sample- and/or acceptor solution. Consequently, an extensive flow of current would be observed. This flow of current would promote electrolysis and alter the pH-value both in the sample- and acceptor solution according to the following reactions:



In a paper from 2008 the adjustable power supply, which has been utilized in the majority of the EME papers [50, 55, 61, 62, 64, 71-74, 76-83], was replaced by a 9 V battery [63]. The flexibility regarding tuning the extraction voltage was lost; however this paper demonstrated a simple and portable EME setup.

#### 1.4.5 Sample solution

According to Eq. (2) a reduction of the ionic strength in the sample solution to the ionic strength in the acceptor solution (low  $\chi$ ) increase the flux across the SLM [61]. Increased ionic strength in the sample solution provides increased current flow and electrolysis both in the sample- and acceptor solution. The electrolysis, especially in the acceptor solution would increase the pH-value and consequently reduce the back-extraction into the acceptor solution.

The least complex sample solution in EME is a pH adjusted water sample spiked with the analytes of interest; most of the EME publications in the literature have used pH adjusted water samples as sample solution. Nevertheless, in bioanalysis the used sample matrices to detect and quantiate drug substances are whole blood, plasma, serum, and urine, and oral fluid. The pH value of those matrices is strictly controlled endogenously; the pH of whole blood, serum, and plasma is 7.4. The pH-value of urine and oral fluid fluctuate more, and is highly dependent on food- and beverage intake [84, 85]. In order to ensure sufficient ionization of the drug substances in any sample solution the pH-value should be two units below and above the pKa-value for basic- and acidic substances, respectively. The physiological pH-value of 7.4 provides a sufficient ionization of strong bases without manipulation of the sample and EME can thus be performed directly from those matrices [78].

Another important feature of the analytes is the log P value; this should be in the range 1-4 and in the case of higher lipophilicity (>4) the analytes could be trapped in the SLM and consequently challenging to back-extract into the aqueous acceptor solution [55, 64]. On the

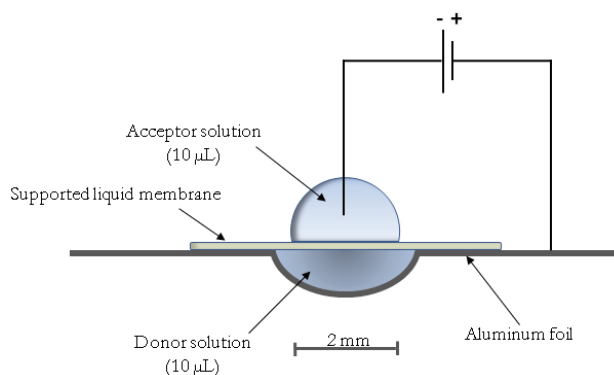
other hand a log P value  $< 1$  would impede the distribution from the sample solution and into the SLM; however this could be solved by addition of carrier molecules in the SLM [64].

#### 1.4.6 Acceptor solution

According to Eq. (2) an increase in the ion balance on the acceptor side to the sample side (low  $\chi$ ) is favourable to increase the flux over the SLM, and this was confirmed by experimental data [61]. The acceptor solution should preferably be directly compatible with LC-MS in order to circumvent the need for evaporation and reconstitution after extraction.

#### 1.4.7 Different EME configurations

Electromembrane extraction was originally performed with the hollow fiber configuration presented in Fig. 1-5; however in recent years several alternative configurations have been developed. The drop-to-drop EME was investigated with flat polypropylene membranes as supporting material [80]; this setup is presented in Fig. 1-7.



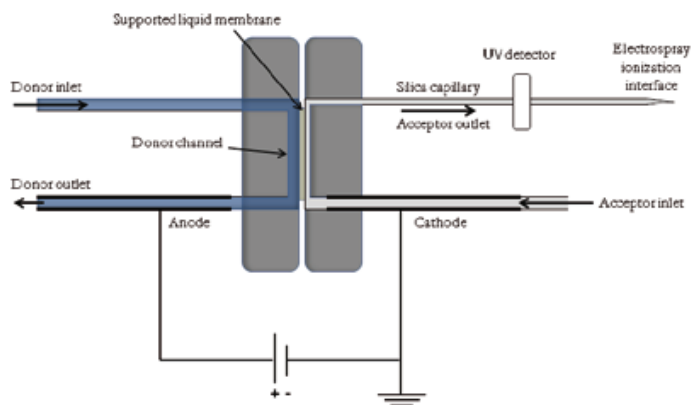
**Fig. 1-7.** Drop to drop EME. (Reprinted with permission from [58]. © 2011 Future Science Group)

The sample solution was added to a sample well made by aluminium foil, a sheet of flat membrane was impregnated with an organic liquid and located in contact with the sample solution; thereafter 10 µL of acceptor solution was added on top of this SLM. This setup was totally stagnant and an electrical field in the range 3-20 V was applied. This approach provided selective isolation of pethidine, nortriptyline, methadone, haloperidol, and loperamide from pH adjusted water samples with extraction recoveries in the range 33-47% after 5 min of extraction [80].

The drop-to-drop EME approach was relatively restricted in terms volume flexibility and thus limited the enrichment factor compared with the hollow fiber EME configuration. Another EME setup introduced in 2010 was the micro-chip configuration; this setup is presented in Fig.



1-8 [58, 83, 86]. In this setup the sample solution was infused with a flow rate in the range 1 to 12  $\mu\text{L min}^{-1}$  and upon contact with the SLM the analytes distributed into the SLM; subsequently the analytes migrated electrokinetically from the SLM and into the stagnant acceptor solution. The applied voltage was varied in the range 5-15 V and the extraction time was investigated up to 60 min; and the enrichment factor was reported to be proportional to the extraction time up to 60 min [83]. In another micro-chip EME approach also the acceptor solution was infused at a rate of 1-3  $\mu\text{L min}^{-1}$  [86]; this setup reported extraction recoveries in the range 65-86% for pethidine, nortriptyline, methadone, haloperidol, loperamide, and amitriptyline. This paper demonstrated that micro-chip EME could be online coupled directly to the mass spectrometer for monitoring real time metabolism of amitriptyline by rat liver microsomes. Another EME approach was to use bags for housing the acceptor solution. This approach increased the acceptor volume which is considered as beneficial regarding extraction recovery [77, 79, 81].



**Fig. 1-8.** Online EME. (Reprinted with permission from [58]. © 2011 Future Science Group)

#### 1.4.8 Extraction recovery, enrichment, and sample cleanup

EME has been considered a non-exhaustive extraction technique; normally the analyte recoveries from aqueous samples are up to 80% [67]. Despite that EME is a non-exhaustive extraction technique, the combination of relatively high extraction recoveries with sensitive analytical instrumentation has provided sufficiently low lower limit of quantification (LLOQ) to quantitate concentrations in the lower  $\text{ng mL}^{-1}$  range. The extraction recoveries are highly dependent on the combination of organic liquid in the SLM, the applied voltage, and the extraction time [57-59] as discussed in previous sections.

The ratio between the sample and acceptor solution in addition to the extraction recovery determines the analyte enrichment. Typical acceptor solution volumes are in the range 10-30  $\mu\text{L}$ , and sample volumes in the range 100-500  $\mu\text{L}$ . Enrichment factor up to 2198 has been reported [87]. The selective isolation of the analyte with EME and the exclusion of matrix components contribute to the efficient sample cleanup. The sample cleanup, often synonymous with absence of matrix components, should be investigated in the method validation protocol with the particular matrix, especially when HPLC is interfaced with electrospray ionization (ESI) in the mass spectrometer. Matrix effects occurs when matrix components retained on the analytical column co-elutes with the analytes; those interfering matrix components are often non-volatile and alters the droplet evaporation and subsequently affects the amount of charged ions eventually reaching the detector [88-90]. Consequently, a comprehensive sample preparation technique is often requested to circumvent the co-extraction of such interfering matrix components.

#### 1.4.9 Calculation of extraction recovery (%) and enrichment

The extraction recovery was calculated for each analyte according to Eq. (5):

$$R = \frac{n_{a,final}}{n_{d,initial}} \times 100\% = \left(\frac{v_a}{v_d}\right) \left(\frac{c_{a,final}}{c_{d,initial}}\right) \times 100\% \quad (5)$$

where  $n_{d,initial}$  and  $n_{a,final}$  are number of moles of the analyte in the the sample solution initially and in the final extract, respectively.  $v_d$  and  $v_a$  are the volume of the sample- and acceptor solution, respectively.  $c_{d,initial}$  and  $c_{a,final}$  are the concentration of the analyte in the sample- and acceptor solution, respectively. The enrichment factor (E) is calculated in accordance with Eq. (6).

$$E = \left(\frac{c_{a,final}}{c_{d,initial}}\right) \quad (6)$$

### 1.5 Dried blood spot (DBS)

The idea of DBS sampling was introduced by Robert Guthrie already in 1963 [91], and this technique has obtained a widespread use in screening of phenylketonuria in newborns. Sampling by DBS was considered as a simplified sampling procedure compared to traditional sampling of blood samples from humans by venipuncture. Although the DBS approach was introduced already in 1963, the applications within the bioanalytical field have been limited. The limited use is mainly due to the fact that low sample volumes (10  $\mu\text{L}$ ) were insufficient to quantify endogenous drug concentrations with the contemporary analytical instrumentation. However, the interest for blood sampling of low sample volumes experienced a renaissance in the late 90s when the sensitivity of the analytical instrumentation was improved [92]. The DBS sampling procedure includes a heel- or finger prick; subsequently the capillary blood is applied to a solid material. Eventually, the blood spot is dried at room temperature for a certain time and stored in an air-tight container and transported to the laboratory. The storage of solid samples excludes the need for storage in a refrigerator or a freezer; dried biological samples are considered as non-hazardous and consequently simplify the transportation to the laboratory prior to analysis. Upon arrival at the laboratory a circular portion of the DBS is punched out and the analytes are eluted with a proper solvent prior to analysis by LC-MS [93-95].

The application of DBS has obtained a widespread use particularly in the preclinical phases in drug development in the pharmaceutical industry; the DBS approach has reduced the need for animals due to the reduction in sampling volume. DBS as sampling technique provides a number of advantages, like simple sampling procedures and low sample volumes, compared to blood collection by venipuncture [93, 96]. A wide range of sampling media is commercially available; however the clinical application of the DBS technique has been limited due to the concern of the blood spot diffusion in those media [94]. The blood spot diffusion is highly inter-individual and the area occupied with the same blood volume withdrawn from different subjects is inversely proportional to the blood viscosity [97]. The concern about the blood diffusion in the commercially available sampling materials has led to the investigation of alternative materials for DBS sampling. Nowadays, the interest for solid sampling of other biological matrices (e.g. urine, oral fluid, and plasma) has increased; the incorporation of other matrices into solid sampling introduced a new abbreviation into the bioanalytical field, Dried Matrix Spot (DMS).

## 1.6 High throughput sample preparation

The interest for automation in the bioanalytical field increased in the 90s when faster LC-MS instrumentation became affordable for most routine laboratories. Consequently, the analysis time per sample was reduced and the sample preparation became the rate-limiting step of the total time consumption per sample [98]. High-throughput sample preparation was especially requested in the pharmaceutical industry to screen drug candidates. The introduction of multi-well plates enabled sample preparation of multiple samples and is currently available in a wide range of dimensions and compartments. Multi-well plates could easily be operated by sophisticated liquid handling systems and the major sample preparation techniques (e.g. SPE, PP, supported liquid extraction (SLE), and filtration) have been incorporated into the multi-well plate format and are now available in 96-, 384-, and 1536 well formats [12, 99, 100]. The combination of high-throughput sample preparation with fast analytical instrumentation provides an impressive sample throughput [98].

## 2. AIM OF THE STUDY

The overall aim of the present study was to further exploit electromembrane extraction (EME) as a sample preparation technique; and develop new technical configurations and applications in line with the recent trends reported in the bioanalytical field. The attention was particularly focused on sample volume, sample cleanup, contemporary sampling techniques, and high throughput sample preparation. A focus throughout the entire project was to reduce the number of steps in the EME procedures to an absolute minimum, and perform EME under physiological conditions to not disturb the fine-tuned endogenous equilibria. The questions we wanted to address in this thesis were as follows:

Is it possible to use a battery to facilitate migration through a supported liquid membrane?

Is it possible to isolate analytes with EME from post mortem blood?

Is it possible to perform exhaustive EME from human plasma?

Is it possible to combine EME with dried matrix spotting?

Is it possible to increase the overall sample throughput with EME?

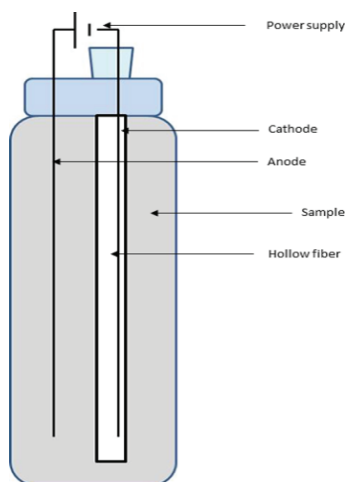
Is the high-throughput EME platform robust?

### 3. RESULTS AND DISCUSSION

The results reported in this section are presented in **Paper I-VII**; and only the key results are included in this context.

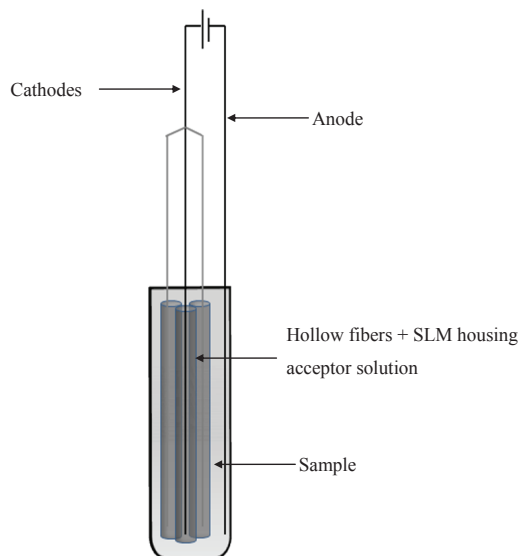
#### 3.1 Electromembrane extraction (EME) configurations

A piece of polypropylene hollow fiber was utilized as supporting material and the lower end of the fiber was closed mechanically with a pincer. The supported liquid membrane (SLM) was made by impregnating the pores of the hollow fiber with an organic liquid for 5 s; the excess organic liquid was gently removed with a medical wipe. The acceptor solution was filled in the lumen of this hollow fiber with a microsyringe. The volume of the sample compartments were varied in the range 50-1000  $\mu\text{L}$ . A pair of inert platinum wires was utilized as electrodes, the cathode and anode was placed in the acceptor- and the sample solution, respectively. The electrodes were connected to an adjustable power supply and the extraction compartment was placed on a shaking board (only for the compartments containing more than 300  $\mu\text{L}$  of sample). This setup was used in **Paper I, III, and V**; a schematic drawing of the setup is presented in Fig. 3-1.



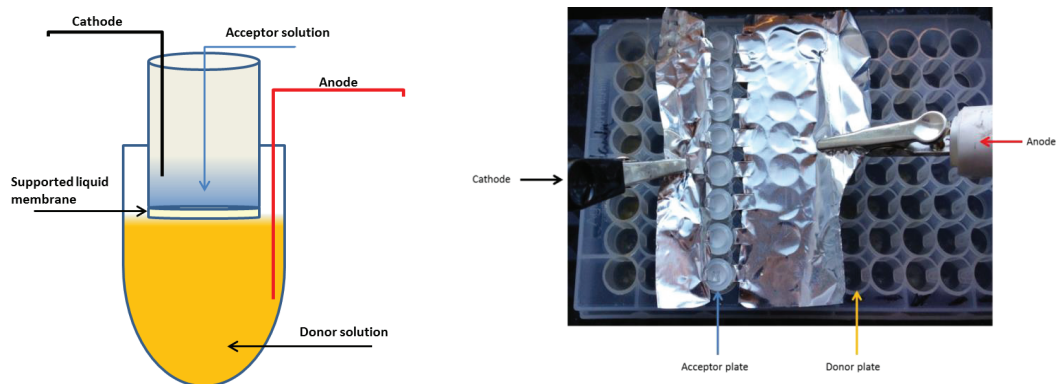
**Fig. 3-1.** *The single fiber EME setup.*

In **Paper II** and **IV** the total number of hollow fibers was increased to three; this approach was selected in order to increase the surface area available for electrokinetic migration and thus improve the extraction recovery. In this configuration the acceptor solution was distributed into three separate hollow fibers, one cathode was placed in each of the three fibers as presented in Fig. 3-2.



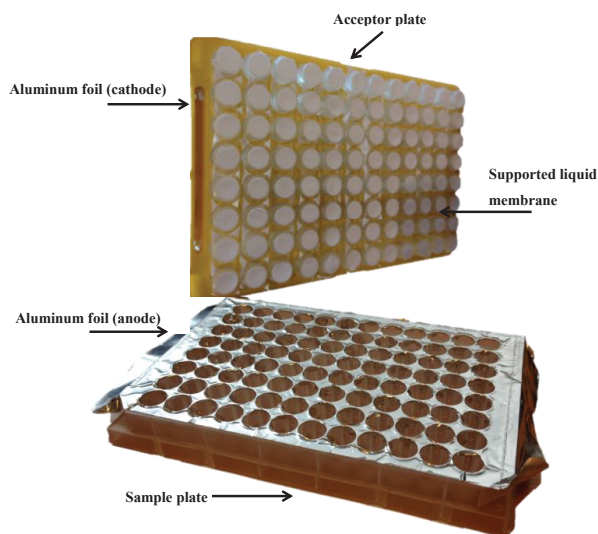
**Fig. 3-2.** *The three fiber EME setup. (Reprinted with permission from Paper II. © 2012 Elsevier)*

In **Paper VI** the hollow fiber configuration was replaced by a flat polypropylene membrane as supporting material. This configuration was made by sealing sheets of flat membranes to strips of plastic vials. Thereafter the lower end of the vials was cut and the polypropylene membrane was impregnated with an organic liquid; the excess organic liquid was gently removed with a medical wipe. Acceptor solution was filled into each of those wells. Aluminum foil coated with inert glue was attached to the walls of each well. A 96-well collection plate was used as sample reservoirs; each well in the 96-well collection plate was also coated with the above mentioned aluminum foil. The aluminum foils were connected with clips to an adjustable power supply, and the extraction compartment was placed on a shaking board. This setup provided a parallel coupled multi-well EME plate as presented in Fig. 3-3.



**Fig. 3-3.** The Pa-EME setup with a schematic drawing of an extraction well. (Reprinted with permission from Paper VI. © 2014 Springer)

In **Paper VII** the total number of parallel extractions in the parallel electromembrane extraction (Pa-EME) setup was increased to 96. The acceptor solution compartments were made with the same technique as described in **Paper VI**; however in **Paper VII** the strips were attached with glue to a pipette-tip rack to provide a 96-well plate. This approach demonstrated the throughput provided with a single power supply as driving force of the 96-wells. A picture of the home-made 96-well Pa-EME configuration is presented in Fig. 3-4.



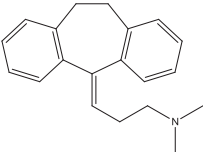
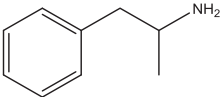
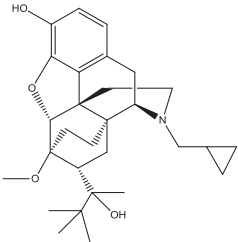
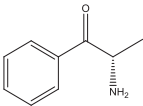
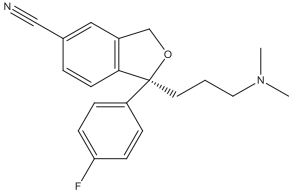
**Fig. 3-4.** A picture of the 96-well Pa-EME setup.



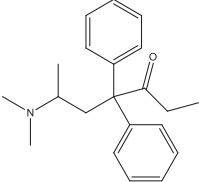
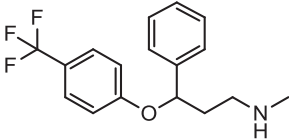
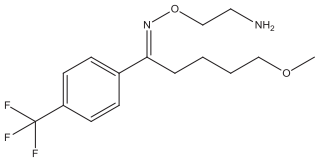
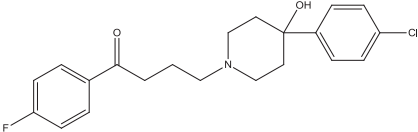
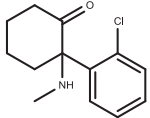
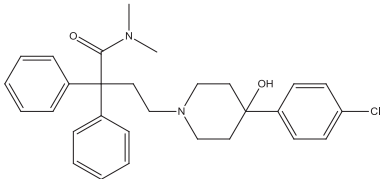
### 3.2 EME of drugs

The focus of this thesis was extraction of small molecular drug substances; their names, chemical structures, pKa values, and log P values are included in Table 3-1.

**Table 3-1.** An overview over the model analytes, their chemical structure, pKa value, and log P.

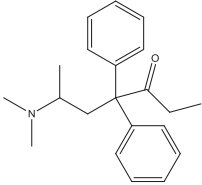
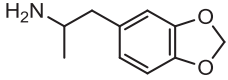
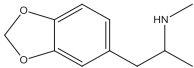
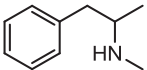
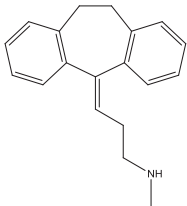
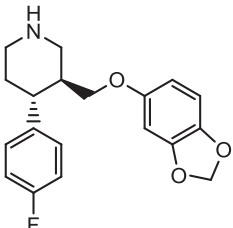
Analyte	Structure	pKa <sup>a</sup>	log P <sup>a</sup>
Amitriptyline		9.18	4.41
Amphetamine		9.94	1.79
Buprenorphine		9.47	2.83
Cathinone		7.97	0.92
Citalopram		9.57	3.48

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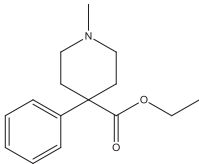
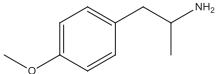
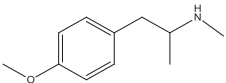
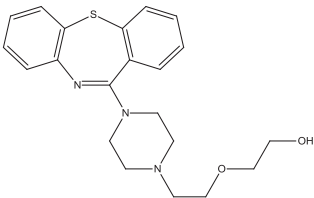
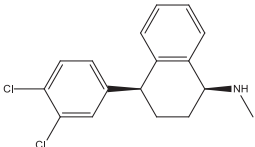
2,5-dimethoxy-4-iodoamphetamine (DOI)		9.46	3.19
Fluoxetine		10.05	3.93
Fluvoxamine		9.39	3.71
Haloperidol		8.04	3.76
Ketamine		6.46	3.01
Loperamide		7.76	4.15

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Methadone		9.05	3.93
3,4-Methylenedioxy-amphetamine (MDA)		9.94	1.64
3,4-Methylenedioxy-methamphetamine (MDMA)		10.32	2.05
Methamphetamine		10.38	2.20
Nortriptyline		10.0	3.97
Paroxetine		9.68	3.70

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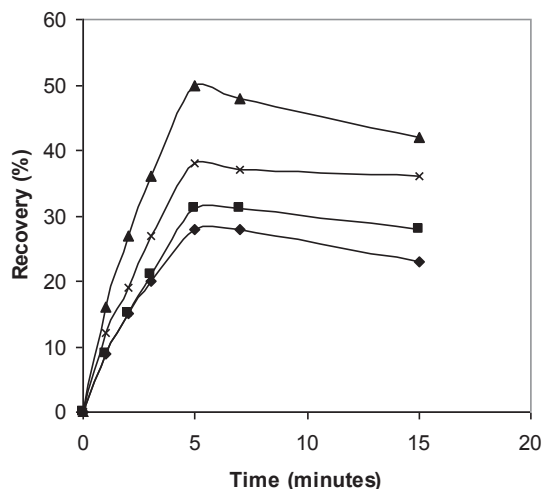
Pethidine		7.84	2.19
Para-methoxy-amphetamine (PMA)		10.00	1.68
Para-methoxy-methamphetamine (PMMA)		10.53	1.86
Quetiapine		6.74	2.60
Sertraline		9.47	5.08

<sup>a</sup> SciFinder (<https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf>, accessed 24.02.2014)

### 3.2.1 Extraction time

The major advantage of EME compared to other sample preparation techniques is the relatively short extraction time (1-10 min), and the fact that the analytes of interest are isolated and enriched into aqueous extracts directly compatible with analytical instrumentation in a single step. There is no need for evaporation to dryness and reconstitution in a proper solvent prior to analysis.

In **Paper I** a stagnant system was operated with a single 9 V battery as power supply. The extraction time in the evaluation protocol was set to 1 min. Despite the extraction time was only 1 min, the extraction recovery was adequate to quantify endogenous concentrations of the model substances. The extraction recoveries increased linearly from time zero up to 5 min and reached steady-state. A prolongation of the extraction time from 5 min did not increase the extraction recovery. Consequently, it is mandatory to have strict time control within the first 5 min of extraction because a slight increase in extraction time will significantly affect the extraction recovery. The extraction recovery (%) as function of extraction time is presented in Fig. 3-5. Extraction recoveries were in the range 12-22% for four basic drugs after 1 min of extraction; the extraction recoveries after 5 min were in the range 28-49%.



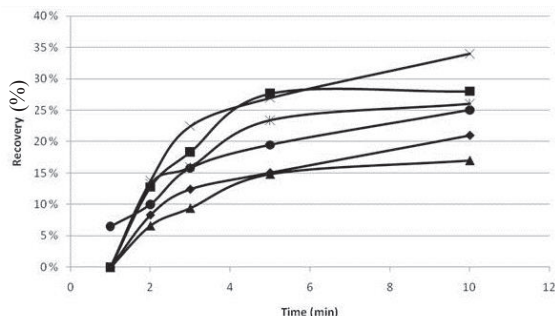
**Fig. 3-5.** Extraction recovery of fluoxetine (◆), amitriptyline (■), citalopram (▲), and fluvoxamine (×) from untreated human plasma. (Reprinted with permission from Paper I. © 2010 Elsevier)

In the theoretical considerations of EME it is suggested that the surface area is an important parameter regarding extraction recovery. Also convection is important to reduce the stagnant boundary layer and to promote electrokinetic migration from the sample solution and into the acceptor solution. In **Paper II** the surface area was investigated by increasing the number of hollow fibers in the sample from one to three. With this three fiber setup, exhaustive extractions were obtained of citalopram, loperamide, methadone, paroxetine, pethidine, and sertraline from samples of pH adjusted water within 10 min of extraction; however by

switching to spiked human plasma as sample matrix the extraction recoveries were reduced to some extent, especially for the highly protein bounded drugs (loperamide, paroxetine, and sertraline).

In **Paper IV** the three fiber configuration investigated in **Paper II** was used to isolate the analytes of interest from 10  $\mu$ L of whole blood spotted on a water soluble biopolymer. The punched out dried blood spot (DBS) was disintegrated in 1 mM HCl for 3 min and the analytes were subsequently isolated with the three-fiber EME setup within 10 min of extraction. This approach provided extraction recoveries similar to those reported in **Paper II**. The extraction recoveries obtained by combining water soluble bipolymers for DBS with EME were compared to commercially available sampling cards (Agilent Bond Elut DMS and Whatman FTA DMPK-A) and procedures for DBS analysis. Those experiments demonstrated that the combination of water soluble bipolymers with EME was superior to commercially available sampling cards both in terms of extraction recovery and time consumption per sample. However, the sample throughput with EME was inferior compared to both Agilent Bond Elut DMS and Whatman FTA DMPK-A, because until then only a single sample could be processed per time with EME.

In **Paper III** the extraction time profile was investigated in the range 1 to 10 min with the optimized setup; the extraction recovery increased as function of the extraction time up to 5 min as presented in Fig. 3-6 and reached steady-state. Consequently, the extraction was terminated after 5 min of extraction in the final setup. Although the extraction time was only 5 min per sample, and considered as superior compared to conventional sample preparation techniques, a concern regarding the sample throughput with EME was emphasized by the collaborators at Norwegian Institute of Public Health (NIPH).



**Fig. 3-6.** Extraction recovery (%) of cathinone (♦), methamphetamine (▪), MDA (♣), MDMA (×), ketamine (\*), and DOI (•) from untreated whole blood. (Reprinted with permission from Paper III. © 2012 Elsevier)

Sampling of oral fluid as dried spots on alginate- and chitosan foam was combined with EME in **Paper V**; in this paper 10  $\mu$ L of oral fluid spiked with model analytes was spotted on sheets of alginate- and chitosan foam. This spot of oral fluid was dried for three hours and subsequently disintegrated for 5 min in 1 mM HCl (pH 3) under strong agitation (3000 rpm), thereafter the analytes were isolated with EME from this solution and into 0.1% trifluoroacetic acid (TFA). The extraction time was optimized and 5 min of extraction was considered as the optimal extraction time and thus utilized in the evaluation protocol.

Pa-EME with flat membranes was described for the first time in **Paper VI**. Due to the altered geometry with flat membranes compared to the hollow fiber EME setup, the extraction time was optimized with a Quality by Design (QbD) in the range of 1 to 10 min. The highest extraction recovery was obtained after 8 min and was in the range 15 to 33%. Consequently, the extraction time was set to 8 min in the evaluation protocol and provided simultaneous isolation of the analytes from eight human plasma samples into extracts compatible with liquid chromatography combined with mass spectrometry (LC-MS). In **Paper VII** the extraction times were varied in the range 5 to 10 min. The combination of 2-nitrophenyl octyl ether (NPOE) as SLM with extraction time of 10 min and extraction voltage of 300 V provided exhaustive extractions of amitriptyline, fluoxetine, and haloperidol. **Paper VI** and **VII** addressed the concern about sample throughput with the hollow fiber EME configuration and is considered as the initial step to in the future incorporate EME into the liquid handling platform.

### 3.2.2 Supported liquid membrane

The optimal organic liquid in EME should be low-viscous in order to effectively impregnate the supporting material and facilitate diffusion of the analytes of interest, non-volatile to prevent evaporation during the extraction process, and water-immiscible to prevent dissolution into the acceptor- and/or sample solution. The organic liquids and carrier molecules utilized in this thesis are presented in Table 3-2.

In **Paper I** 1-ethyl-4-nitrobenzene (ENB), 1-isopropyl-4-nitrobenzene (IPNB), NPOE, and 2-nitrophenyl pentyl ether (NPPE) were examined as SLM to isolate four hydrophobic drugs from human plasma samples; at extraction voltage below 20 V it was clear that ENB and NPPE were superior to NPOE. However, an increase in the extraction voltage provided extensive electrolysis due to a reduction in the SLM stability with both ENB and NPPE. This electrolysis resulted in a pH increase in the acceptor solution and consequently a reduction in

extraction recovery, also the RSD values increased. The observed RSD value increase was probably due to a substantial bubble formation and variations in the acceptor volume withdrawn after extraction. In the evaluation protocol ENB was selected as the SLM.

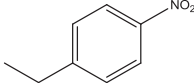
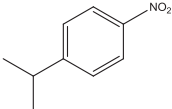
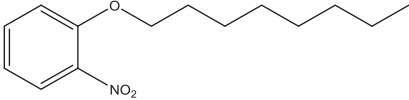
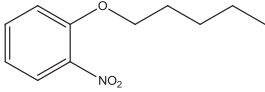
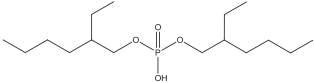
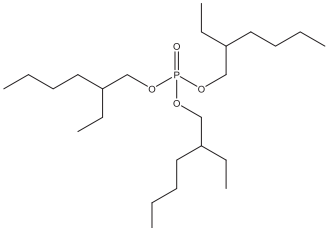
In **Paper II** NPOE was selected as the SLM based on former experience [64] and the aim was to increase the extraction recovery of six hydrophobic drug substances with EME and the total number of hollow fibers was increased three-fold compared to conventional hollow fiber EME. Each of the three hollow-fibers was impregnated with NPOE and an extraction voltage of 200 V was applied and provided exhaustive extraction. The three fiber setup developed in **Paper II** with the same SLM was utilized in **Paper IV** to isolate four hydrophobic drugs from a disintegrated DBS.

In **Paper III** six basic drugs of abuse were used as model analytes, the aim was to isolate those analytes from human whole blood. ENB, NPOE, NPPE, and 5% Di (2-ethylhexyl) phosphate (DEHP) in NPOE (w/w) were investigated in order to select the most efficient SLM, and from those experiments ENB was selected as the organic liquid in the evaluation protocol. In **Paper V** EME was utilized to isolate buprenorphine, methadone, methamphetamine, PMA, and PMMA from samples of dried oral fluid disintegrated in hydrochloric acid. The SLM consisted of 10% tris (2-ethylhexyl) phosphate (TEHP) in IPNB (w/w); TEHP was added to the IPNB as a carrier molecule in order to increase the analytes affinity, particularly the more polar analytes (methamphetamine, PMA, and PMMA), for the SLM.

The parallel EME configuration investigated in **Paper VI** was utilized to isolate amitriptyline, fluoxetine, quetiapine, and sertraline from human plasma samples. Based on former experience with the hollow fiber EME setup NPOE was selected as SLM [101, 102]. In **Paper VII** both NPOE and IPNB were utilized as organic liquids. NPOE was used in combination with high extraction voltage (up to 300 V) in an attempt to improve the extraction recovery. In the 68- and 96-well experiments IPNB was combined with low extraction voltage (20 V) due to safety considerations.



**Table 3-2.** Structure formulas of the organic solvents and carrier molecules in this thesis.

Organic solvent	Structure
1-Ethyl-4-nitrobenzene	
1-Isopropyl-4-nitrobenzene	
2-Nitrophenyl octyl ether	
2-Nitrophenyl pentyl ether	
Di (2-ethylhexyl)phosphate	
Tris (2-ethylhexyl) phosphate	

### 3.2.3 Extraction voltage

Extraction voltage is another decisive parameter regarding extraction recovery in EME; however it is known that the stability of the SLM is highly dependent of the magnitude of the applied electrical field [66]. NPOE is considered as a stable SLM in EME, and the highest extraction recoveries are reported above 100 V with this particular organic liquid as SLM. In the case of IPNB and ENB the optimal extraction recoveries are reported with applied electrical fields below 25 V [63]. In EME there is a non-linear relationship between the magnitude of the applied electrical field and the extraction recovery for a given organic liquid sustained in the SLM. The stability of the organic liquid in the supporting material is mandatory in EME. If the applied voltage is increased up to certain point excessive electrolysis could occur. The more soluble the organic liquid is in the sample- or the acceptor solution the more electrolysis will occur. The electrolysis promotes excessive H<sub>2</sub> gas formation in the acceptor solution, and consequently an increase in the pH-value [66].

In **Paper I** ENB was selected as organic liquid. ENB provides decent extraction recoveries even at low extraction voltages. The extraction recoveries were investigated as function of the magnitude of the applied electrical field in the range 9 to 300 V, and the extraction recovery was considered as independent of extraction voltage in this setup. Since the idea of **Paper I** was to develop a simple and portable EME device, a 9 V battery was utilized as power supply in the final setup. The aim in **Paper II** was to isolate the analytes exhaustively from the sample solution and into the acceptor solution, in this setup three separate hollow fibers were located in the sample solution. NPOE was selected as SLM and the applied electrical field was investigated in the range 50 to 300 V. In the evaluation protocol 200 V was selected as the extraction voltage.

In **Paper III** the extraction voltage was varied in the range 0 to 50 V with ENB as SLM; only a slight difference in extraction recovery was observed with 50 V compared to 5 V and 15 V. Consequently, a 15 V battery was selected as power supply in the final configuration. The three-fiber configuration presented in **Paper II** was utilized in **Paper IV** to isolate four basic drugs from a disintegrated dried blood spot sample, similar extraction recoveries were obtained from the DBS samples compared to human plasma as sample matrix. In **Paper V** the SLM consisted of 10% TEHP in IPNB (w/w) to increase the analytes affinity for the SLM; the extraction voltage was investigated in the range of 5 to 100 V and based on those experiments 25 V was selected as extraction voltage in the evaluation protocol.

In **Paper VI** the porous hollow fiber was replaced with a flat polypropylene membrane and this approach enabled operation of eight separate extraction compartments in parallel. The electrical circuits were connected to a single power supply and thus enabled the isolation of amitriptyline, fluoxetine, quetiapine, and sertraline from eight plasma samples simultaneously. The magnitude of the electrical field was optimized with a QbD in the range 20 to 200 V, and 200 V was selected as the extraction voltage. The relationship between extraction recovery and extraction voltage was non-linear. In **Paper VII** the extraction voltage was varied from 20 to 300 V depending on which organic liquid impregnated in the pores of the supporting material.

### 3.2.4 Acceptor solution

According to the modified Nernst-Planck equation the ionic strength of the acceptor solution would affect the extraction efficiency in the EME system. HCl has been utilized as acceptor solution in the majority of the research concerning EME. However, due to the non-volatility of HCl and incompatibility with LC-MS analysis, HCOOH was investigated as acceptor solution in **Paper I**. The replacement of HCl with HCOOH provided comparable extraction recoveries, and was utilized as acceptor solution in **Paper I, II, IV, VI, and VII**. In **Paper III** acetic acid, phosphoric acid, formic acid, and hydrochloric acid were investigated as acceptor solutions. Due to both MS compatibility and providing the highest extraction recoveries 10 mM acetic acid was selected as the acceptor solution in the final setup. In **Paper V** 0.1% TFA was utilized as acceptor solution due to superior extraction recoveries compared to HCOOH.

## 3.3 Evaluation parameters

The final setup in **Paper I-VI** was evaluated regarding linearity, repeatability, extraction recovery, and sample cleanup as specified in regulatory guidelines [103, 104]. Satisfactory linearity and repeatability in the therapeutic range contributes to reliable detection and quantitation. The concentration range investigated was based upon either the therapeutic range (**Paper I, II, IV, and VI**) for the model substances, or concentrations associated with drug abuse (**Paper III and V**). Another important parameter to evaluate is the sample cleanup; any sample preparation technique should demonstrate its ability to selectively isolate the analytes of interest from the sample solution.

### 3.3.1 Linearity

The aim in **Paper I** was to develop a simple and stagnant EME-setup to isolate amitriptyline, citalopram, fluoxetine, and fluvoxamine from undiluted human plasma samples with relevant

therapeutic concentrations. The linearity of the aforementioned analytes was investigated in the range 1 to 1000 ng mL<sup>-1</sup>. This setup reported  $r^2$ -values above 0.998 for all four analytes and was considered as satisfactory.

The total number of hollow fibers in **Paper II** was increased to three in order to facilitate exhaustive EME, and the linearity regarding citalopram, loperamide, methadone, paroxetine, pethidine, and sertraline was investigated in the range 1 to 1000 ng mL<sup>-1</sup>. This particular setup reported  $r^2$ -values above 0.990 regarding all six model analytes and was considered as acceptable.

The concentration range selected in **Paper III** to evaluate the linearity was 10 to 250 ng mL<sup>-1</sup> and this was based on concentrations typically associated with drug abuse of cathinone, methamphetamine, MDA, MDMA, ketamine, and DOI [105]. This EME approach combined with UPLC-MS/MS reported  $r^2$ -values above 0.9939 for the selected drugs of abuse.

In **Paper IV** the linearity was examined in the range 17.5 to 560 ng mL<sup>-1</sup>, and the  $r^2$ -values reported were 0.983, 0.986, 0.992, and 0.9952 for citalopram, loperamide, methadone, and sertraline, respectively. Although the  $r^2$ -value for citalopram and loperamide was below 0.990 it was considered as satisfactory due to the homebuilt equipment and the fact that only 10  $\mu$ L of whole blood, which is highly challenging to pipette accurately, was spotted on the sheets of foam.

In **Paper V** the linearity was investigated in the range 25 to 1000 ng mL<sup>-1</sup> for five basic drugs of abuse and the reported  $r^2$ -values were above 0.991 for all analytes except from buprenorphine. The  $r^2$ -value obtained by combining oral fluid spiked with buprenorphine and sampling on chitosan foam was 0.989; nevertheless this was considered as acceptable.

In the Pa-EME approach investigated in **Paper VI**, the linearity for amitriptyline, fluoxetine, quetiapine, and sertraline was examined in the range 1 to 400 ng mL<sup>-1</sup>, and reported  $r^2$ -values above 0.9974 and considered as acceptable. **Paper VII** was an extension of the setup developed in **Paper VI** and the linearity was therefore not investigated with this particular configuration.

### 3.3.2 Repeatability

The repeatability is considered as an important evaluation parameter in the field of bioanalysis, and guidelines often requests RSD-values below 20 and 15% for low- and high concentration, respectively [103, 104]. In this study the repeatability has been utilized as one of the

parameters to rate the success of the investigated EME configurations. Although the EME configurations were homebuilt, an acceptable repeatability was reported throughout this study.

In **Paper I** the RSD-values were in the range 3.2-8.9% at 100 ng mL<sup>-1</sup>. In **Paper II** the RSD-values were below 22% and 21% at 10- and 1000 ng mL<sup>-1</sup>, respectively, for the six basic model substances. In **Paper III** the RSD-values were below 27% and 26% at 10- and 50 ng mL<sup>-1</sup>, respectively. In **Paper IV** the RSD-values were below 20% at 17.5-, 140-, and 1120 ng mL<sup>-1</sup>. In **Paper V** the RSD-values were below 15% at 50 ng mL<sup>-1</sup>. In **Paper VI** the RSD-values were below 15% at 2-, 10-, and 200 ng mL<sup>-1</sup>. In **Paper VII** the RSD-values were below 15%, the 96-well experiment reported RSD values of 4-, 4-, and 6% for amitriptyline, fluoxetine, and haloperidol, respectively. Those small RSD values were probably due to the number of parallels (96) in this experiment.

### 3.3.3 Extraction recovery

The extraction recovery has been investigated in each setup and utilized to grade the success of the extraction process. The extraction recovery is highly dependent on the extraction time, the organic liquid sustained in the pores of the supporting material, the applied voltage, the acceptor solution volume, and the rate of agitation (especially with larger compartments).

A stagnant configuration was utilized in **Paper I** to isolate amitriptyline, citalopram, fluoxetine, and fluvoxamine from 70 µL of untreated human plasma; and the extraction recovery was in the range of 28-49% at the steady-state level. Another approach was investigated in **Paper II**; the total number of hollow fibers introduced into the sample was three and this increased the acceptor solution volume and the total surface area available for electrokinetic migration. According to the modified Nernst-Planck equation this should increase the extraction recovery. The extraction recoveries of citalopram, loperamide, methadone, paroxetine, pethidine, and sertraline were in the range 97-115% from pH adjusted water samples; however from undiluted human plasma the extraction recoveries were in the range 56-102%. This reduction in extraction recovery observed from human plasma, was probably due to proteinbinding of the analytes.

In **Paper III** the six stimulating drugs cathinone, methamphetamine, MDA, MDMA, ketamine, and DOI were isolated from samples of undiluted whole blood. The extraction recoveries after 5 min were in the range 9-28%. In **Paper IV** the setup from **Paper II** was combined with DBS sampling on sheets of alginate foam with citalopram, loperamide, methadone, and sertraline as model substances. After disintegration of the punched out DBS,

the analytes were isolated with EME and provided extraction recoveries in the range 44-105%. Those recoveries were comparable to the results reported in **Paper II**. In **Paper V** sheets of alginate- and chitosan foam were utilized as sampling media for oral fluid spiked with buprenorphine, methadone, methamphetamine, PMA, and PMMA. The punched out dried oral fluid was disintegrated instantly with 1 mM HCl and the analytes were isolated from this solution and into the acceptor solution prior to analysis. The extraction recoveries with EME were 25-65% and 28-64% for sampling on alginate- and chitosan foam, respectively.

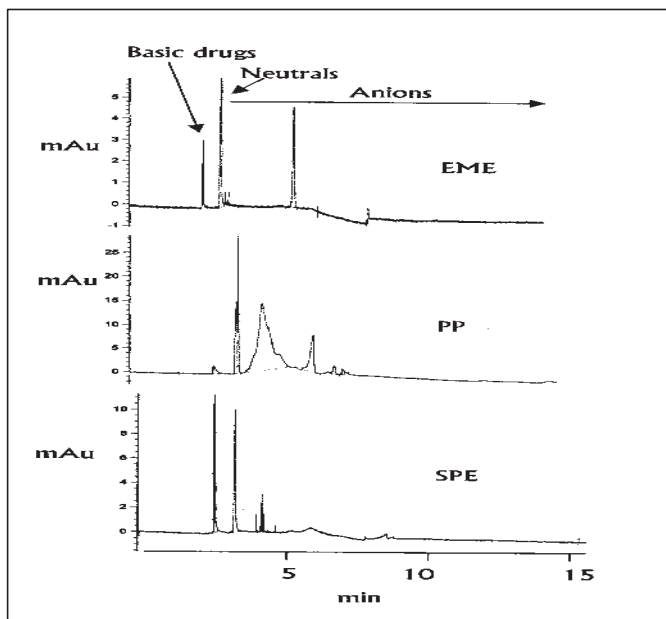
In **Paper VI** the hollow fiber was replaced by a flat membrane, and thus altered the geometry of the extraction unit. The volume of the reservoirs housing acceptor solution was increased and this provided an increased flexibility in terms of acceptor solution volume compared to hollow fiber EME. The Pa-EME configuration provided extraction recoveries in the range 16 to 31% from samples of undiluted human plasma (at 10 ng mL<sup>-1</sup>) after 8 min of extraction.

In **Paper VII** the magnitude of the applied electrical field and extraction time was 250 V and 10 min, respectively, and this configuration provided exhaustive extraction of amitriptyline, fluoxetine, and haloperidol from pH adjusted water samples. In addition, this setup was utilized to isolate the aforementioned analytes from undiluted human plasma and reported extraction recoveries in the range 79-101%.

### 3.3.4 Sample cleanup

Another considerable advantage with EME is the sample cleanup and the compatibility of the extracts with analytical instrumentation without the need for evaporation and reconstitution. The sample cleanup provided with EME is mainly due to the selective character of the SLM. The sample cleanup has been investigated both with blank extractions and post-column infusion experiments throughout this study.

In **Paper I** EME was compared with solid phase extraction (SPE) and protein precipitation (PP) in terms of co-extracted matrix components from human plasma in the final extract. The sample cleanup provided with EME was comparable with the sample cleanup obtained with the SPE procedure; PP provided a massive peak which comprised matrix components as presented in Fig. 3-7 with capillary electrophoresis with ultraviolet detection (CE-UV).

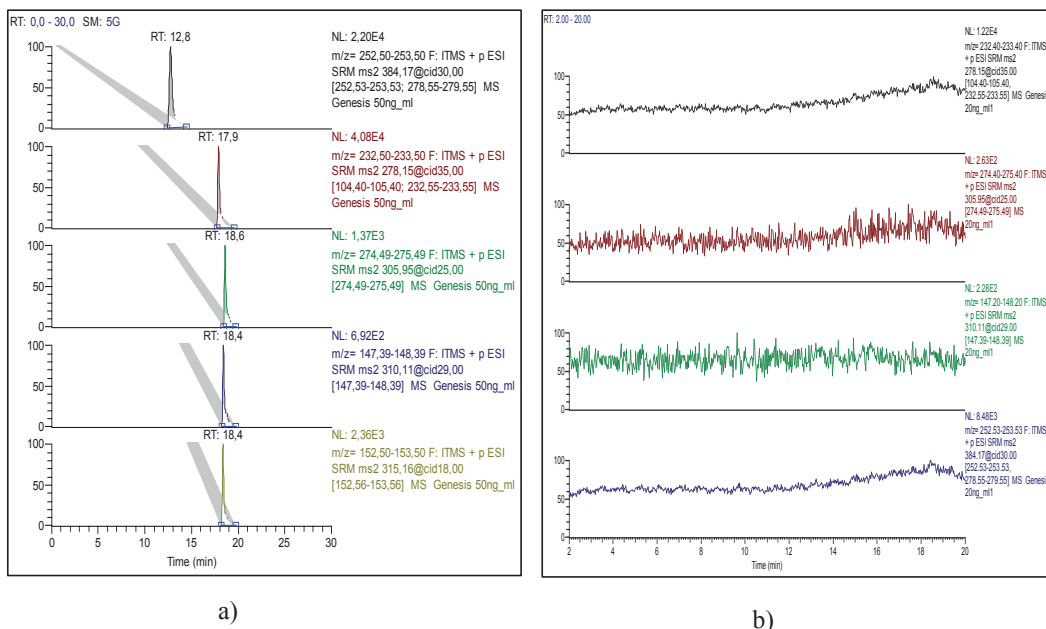


**Fig. 3-7.** CE-UV examination of sample cleanup provided with EME, PP, and SPE, respectively. (Reprinted with permission from Paper I. © 2010 Elsevier)

In **Paper IV** the sample cleanup was examined with a post-column infusion LC-MS experiment in order to detect potential matrix components in the final extract. The sample matrix was highly complex and consisted of whole blood, foam constituents, and the four basic analytes; nevertheless neither an increase nor decrease in the signal intensity in the MS was observed after injection of a blank extract. This underlined the excellent sample cleanup provided with EME from complex matrices. The same setup was utilized in **Paper V**; however in this case the sample matrix was oral fluid. Traditionally sampling of oral fluid is conducted with a sampling pad in the oral cavity; subsequently the pad is stored in a buffer prior to analysis by LC-MS. This buffer has been reported to provide extensive matrix effects in the MS and inaccurate quantitation [106]. In **Paper V** the sampling of oral fluid was conducted on a solid material and the need for a storage buffer was omitted. The post-column infusion experiments in **Paper V** demonstrated the excellent sample cleanup obtained with

EME; in another experiment a blank extract was analyzed to examine any matrix constituents in the final extract. No additional peaks in the chromatogram were detected in this experiment.

In **Paper VI** the sample cleanup from human plasma was investigated with a post-column infusion LC-MS experiment presented in Fig. 3-8; also the flat membrane configuration provided excellent sample cleanup and no matrix components were detected in the final extract. The absence of matrix components from plasma, whole blood, and oral fluid in the EME extracts emphasized the sample cleanup provided with EME.



**Fig. 3-8.** LC-MS/MS chromatogram after injection of a 50 ng mL<sup>-1</sup> solution of amitriptyline, fluoxetine, fluoxetine d5, quetiapine, and sertraline (a). Post-column infusion LC-MS/MS chromatogram (b). (Reprinted with permission from Paper VI. © 2014 Springer)

### 3.4 Real samples

A sample preparation technique should be easy to implement in routine laboratories, be user friendly, and contribute to accurate detection and quantitation in the final analysis. In this section the applicability of the different EME approaches are discussed based on experiences with real samples. The results from the 15 different subjects are summarized in Table 3-3.

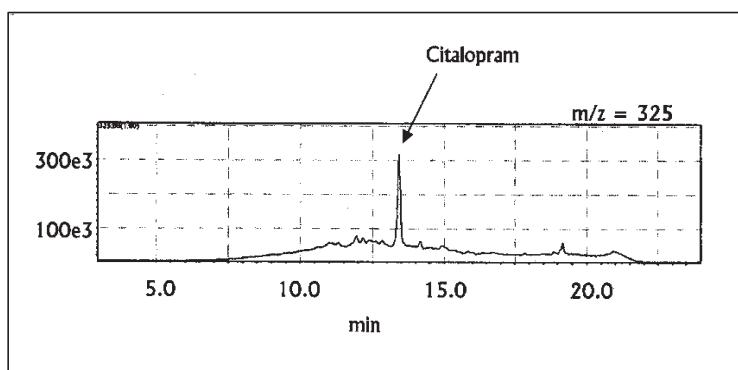


**Table 3-3.** Overview over the endogenous concentrations reported with EME on real samples and the deviation (%) from the concentrations reported by a reference laboratory, paper no. in parentheses.

Subject #	Drug	EME-LC/MS	Deviation
<b>Subject 1 (I)</b>	Citalopram	42 ng mL <sup>-1</sup>	-17%
<b>Subject 2 (I)</b>	Citalopram	67 ng mL <sup>-1</sup>	-23%
<b>Subject 3 (II)</b>	Citalopram	46 ng mL <sup>-1</sup>	-4%
<b>Subject 4 (II)</b>	Citalopram	86.5 ng mL <sup>-1</sup>	-
<b>Subject 5 (II)</b>	Citalopram	58 ng mL <sup>-1</sup>	14%
<b>Subject 6 (III)</b>	Cathinone	2.9 ng mL <sup>-1</sup>	<i>n.m</i>
<b>Subject 7 (III)</b>	Cathinone	1.3 ng mL <sup>-1</sup>	<i>n.m</i>
<b>Subject 8 (III)</b>	Amphetamine	138 ng mL <sup>-1</sup>	28%
	Methamphetamine	558 ng mL <sup>-1</sup>	34%
<b>Subject 9 (III)</b>	Amphetamine	90 ng mL <sup>-1</sup>	32%
	Methamphetamine	226 ng mL <sup>-1</sup>	26%
	Ketamine	379 ng mL <sup>-1</sup>	-6%
<b>Subject 10 (III)</b>	Amphetamine	386 ng mL <sup>-1</sup>	-14%
	Methamphetamine	996 ng mL <sup>-1</sup>	-13%
	MDMA	405 ng mL <sup>-1</sup>	-25%
	MDA	21 ng mL <sup>-1</sup>	<i>n.m</i>
<b>Subject 11 (III)</b>	Ketamine	1952 ng mL <sup>-1</sup>	24%
<b>Subject 12 (III)</b>	MDMA	3005 ng mL <sup>-1</sup>	4%
	MDA	80 ng mL <sup>-1</sup>	<i>n.m</i>
<b>Subject 13 (III)</b>	Amphetamine	141 ng mL <sup>-1</sup>	16%
	Methamphetamine	316 ng mL <sup>-1</sup>	25%
<b>Subject 14 (VI)</b>	Quetiapine	56 ng mL <sup>-1</sup>	-30%
<b>Subject 15 (VI)</b>	Sertraline	6 ng mL <sup>-1</sup>	-

*n.m* = not measured by the reference laboratory

In **Paper I** plasma samples from three subjects treated with the antidepressant drug citalopram were obtained (subject 1-3 in Table 3-3); 70  $\mu\text{L}$  untreated plasma from those subjects were added to the sample vial. Subsequently, the hollow fiber was impregnated with ENB and 30  $\mu\text{L}$  10 mM formic acid was filled in the lumen of this hollow fiber. Citalopram was isolated from each sample ( $n=3$ ) under an applied electrical field of 9 V within 1 min of extraction, and the final extract was analyzed by LC-MS. A chromatogram from one of the subjects is presented in Fig. 3-9. The calibration curve was utilized to determine the plasma concentration in the patient samples; the plasma concentrations found were 42-, 67-, and 46  $\text{ng mL}^{-1}$  and deviated less than 23% compared to the plasma concentration reported by a reference laboratory.

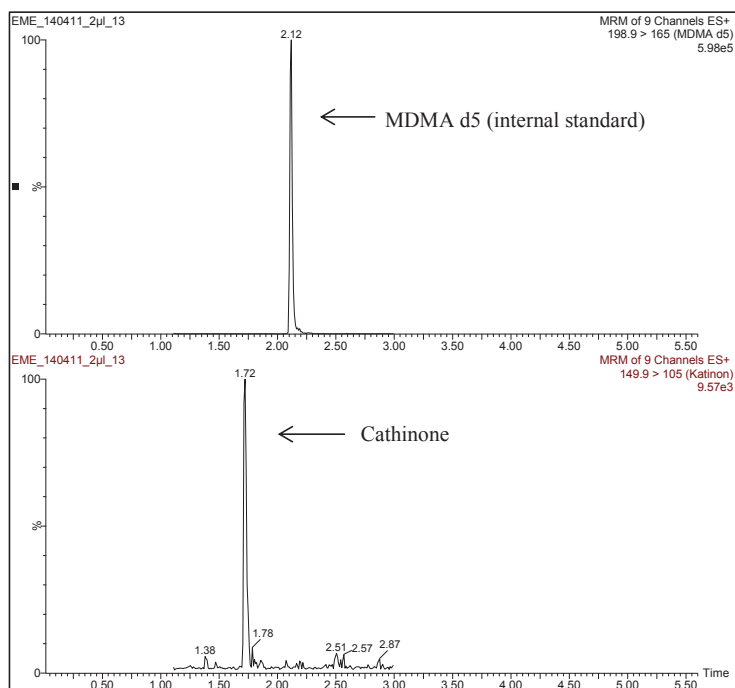


**Fig. 3-9.** EME combined with LC-MS for determination of citalopram in a real sample. (Reprinted with permission from Paper I. © 2010 Elsevier)

Also in **Paper II** the final EME setup was utilized to isolate citalopram from two subjects (subject 4 and 5 in Table 3-3); in this setup 50  $\mu\text{L}$  undiluted plasma was filled in the sample compartments and each of the three hollow fibers were impregnated with NPOE and filled with 7  $\mu\text{L}$  formic acid (21  $\mu\text{L}$  in total). Citalopram was isolated from the samples and into the acceptor solution within 10 min and with 200 V as the magnitude of the applied electrical field. The final extract was analyzed by LC-MS. The plasma concentration reported with EME-LC-MS of subject 4 and 5 was 86.5 and 58  $\text{ng mL}^{-1}$ , respectively; those results deviated less than 14% compared to the plasma concentration reported by a reference laboratory.

The sample matrix in **Paper III** was whole blood; and EME was utilized to isolate different stimulating drugs from whole blood samples (subject 6-13 in Table 3-3). The optimized setup was utilized to isolate the analytes of interest from five forensic samples from living persons

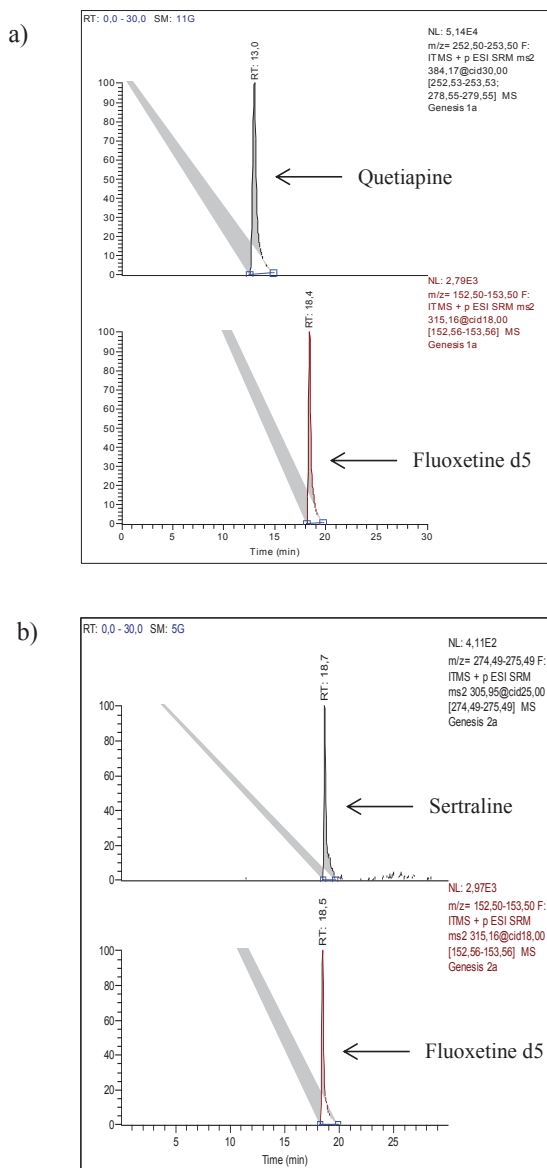
and three forensic autopsy cases. A chromatogram of subject 6 is included in Fig. 3-10. Post-mortem blood from autopsy cases is considered a more complex matrix compared to whole blood; mainly due to the hemolysis of blood cells and increased viscosity. In this setup 80  $\mu\text{L}$  whole blood was filled in the sample vial, the hollow fiber was impregnated with ENB, and subsequently filled with acetic acid; the analytes were isolated from the sample and into the acceptor solution within 5 min of extraction and 15 V as extraction voltage. The final extract was analyzed by UPLC-MS/MS. The results obtained with EME combined with UPLC-MS/MS provided a deviation in the range -13 to 34% compared to the results reported by NIPH.



**Fig. 3-10.** EME combined with UPLC-MS/MS for determination of cathinone in an authentic sample, MDMA d5 was utilized as internal standard. (Reprinted with permission from Paper III. © 2012 Elsevier)

In **Paper VI** plasma samples from two subjects (subject 14 and 15 in Table 3-3) treated with quetiapine and sertraline, respectively, were obtained. The analytes of interest were isolated from 240  $\mu\text{L}$  of plasma sample, across a supported liquid membrane consisting of NPOE, and into 70  $\mu\text{L}$  formic acid. The extraction time was 8 min, the applied voltage was 200 V, and the extraction compartment was subjected to 900 rpm throughout extraction. The final extract

was analyzed by LC-MS/MS. The reported plasma concentration was 56- and 6 ng mL<sup>-1</sup> for quetiapine and sertraline, respectively. The chromatograms of subject 14 and 15 are presented in Fig. 3-11. In **Paper VI** three parallels from each subject was processed simultaneously.



**Fig. 3-11.** EME combined with LC-MS/MS for determination of quetiapine (a) and sertraline (b), respectively. Fluoxetine d5 was utilized as internal standard. (Reprinted with permission from Paper VI. © 2014 Springer)

The concentration reported with EME in combination with LC-MS, LC-MS/MS or UPLC-MS/MS deviated  $\pm 30\%$  compared to the drug concentration stated by the reference laboratories. Taken into consideration that some of the samples have been stored over a year under unknown conditions this deviation is considered as acceptable and demonstrated the applicability of EME on real samples.

### 3.5 EME in combination with dried matrix spot (DMS)

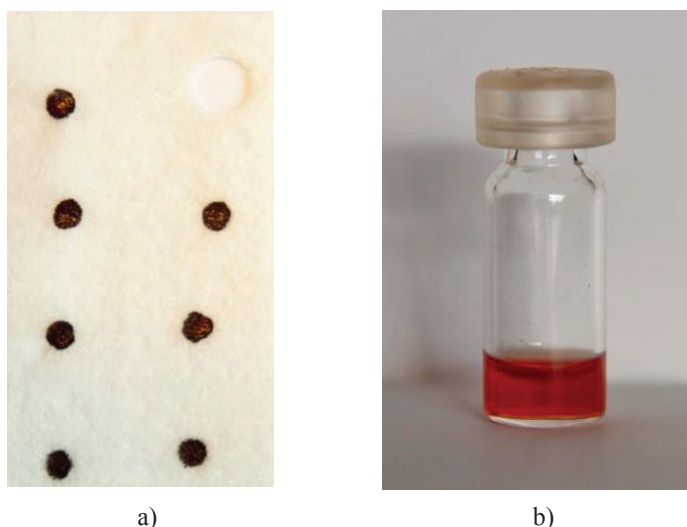
The interest for solid sampling of microliter volumes of biological fluids has increased the last five years; mainly due the increased sensitivity provided by state-of-the-art analytical instrumentation [94, 95, 107-110]. However, the robustness of the current sampling materials for DMS analysis has been extensively debated the last years. The discussions are mainly focused on the homogeneity of the applied blood spot and the influence of hematocrit on the blood spot diffusion [111-113]. The introduction of sampling media with pH-dependent solubility demonstrated a totally new approach to address the concern about blood diffusion in a solid material. The selected media exerted high absorbing capacity and were stable under physiological conditions (pH 7), however upon hydration at pH 3 it fully disintegrated within 5 min. After disintegration, the analytes were isolated with EME into aqueous extracts directly compatible with analytical instrumentation.

#### 3.5.1 Human whole blood

In **Paper IV** EME was combined with solid sampling on a novel DMS medium addressing the challenges reported with the commercially available materials. Two different media were provided as sheets of foam by the Department of Pharmaceutics at School of Pharmacy (University of Oslo). The first one consisted of alginate which is a linear copolymer with homopolymeric blocks of (1-4)-linked  $\beta$ -D-mannuronate and the C-5 epimer  $\alpha$ -L-gulonate. Chitosan was the second medium and this is a cationic copolymer of glucosamine units. Those media should be stable at physiological pH, and disintegrate instantly under acidic conditions. Both media provides high absorbing capacity [114-116].

Human whole blood spiked with citalopram, loperamide, methadone, and sertraline was spotted on the either alginate- or chitosan foam and dried for 3 hours at room temperature; thereafter the entire DBS spot was punched out and disintegrated in 1 mM HCl for 3 min. A picture of the DBS and the solution obtained after disintegration for 3 min is presented in Fig. 3-12. The analytes were isolated from this solution (Fig. 3-12b) and into 21  $\mu$ L formic acid

within 10 min of EME with the three-fiber configuration introduced in **Paper II**. The final extract was analyzed by LC-MS. The extraction recovery with this setup for the four basic analytes was in the range 44-105% and 57-115% for alginate- and chitosan foam, respectively, and comparable to the extraction recoveries reported for the same analytes in **Paper II** from untreated human plasma.

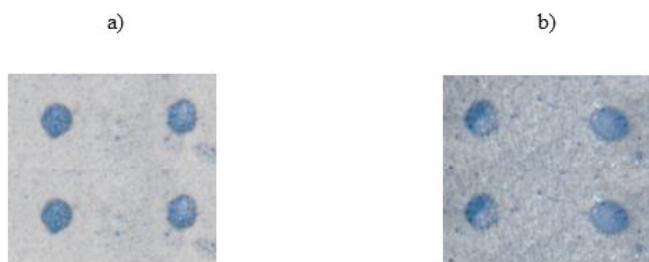


**Fig. 3-12.** Pictures of 10  $\mu\text{L}$  whole blood spotted on sheets of alginate foam (a) and the solution obtained after disintegration of the punched out blood spot for 3 min in 1 mM HCl (b), respectively. (Reprinted with permission from Paper IV. © 2012 American Chemical Society)

Sampling on alginate- and chitosan foam in combination with EME was compared with sampling on Agilent Bond Elut DMS and Whatman FTA DMPK-A in combination with standard elution procedures (typically acidified acetonitrile or methanol). The different procedures were evaluated regarding recovery and time consumption. Agilent Bond Elut DMS provided elution recovery from the punched out DBS in the range of 59 to 69% within 60 min of elution, and Whatman FTA DMPK-A provided recoveries in the range of 11 to 33% within 10 minutes of elution. Additionally, the solution obtained with Agilent Bond Elut DMS and Whatman FTA DMPK-A after elution has to either be evaporated to dryness and reconstituted or centrifuged prior to final analysis. This post-elution step prolong the sample preparation time. With sampling on either alginate- or chitosan foam the sample preparation time is 15 min to provide extracts directly injectable in LC-MS.

### 3.5.2 Human oral fluid

In **Paper V** sheets of alginate- and chitosan foam were used for sampling of oral fluid. 10  $\mu\text{L}$  oral fluid spiked with buprenorphine, methadone, methamphetamine, PMA, and PMMA was spotted on sheets of alginate- and chitosan foam as presented in Fig. 3-13. A fixed circular portion, which included the entire spot, was punched out and disintegrated in 300  $\mu\text{L}$  1 mM HCl for 5 min, the five analytes were subsequently isolated with EME into 25  $\mu\text{L}$  0.1% TFA in 5 min, and analyzed by LC-MS. The selective isolation of the analytes from the complex solution consisting of oral fluid, foam constituents, and hydrochloric acid was provided within 10 min. The reason for sampling oral fluid on solid materials was to investigate an alternative to oral fluid sampling with a sampling pad in the oral cavity which has been reported to provide extensive matrix effects as discussed in section 3.3.4.

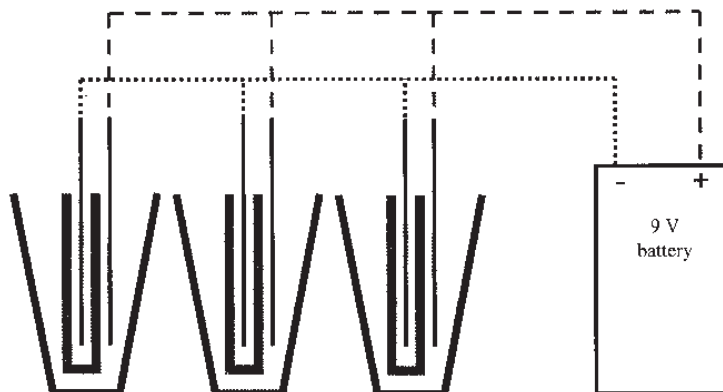


**Fig. 3-13.** Picture of 10  $\mu\text{L}$  oral fluid spotted on sheet of alginate foam (a) and chitosan foam (b). (Reprinted with permission from Paper V. © 2013 Future Science Group)

In **Paper IV** and **V** EME was successfully combined with DMS sampling. A novel DMS concept was introduced as an attempt to address the challenges reported in the literature concerning the commercial DMS media. The novel media in combination with EME as sample preparation technique provided a powerful tool both for sampling and subsequent isolation of the analyte prior to LC-MS analysis.

### 3.6 High throughput EME

The idea of parallel extractions with EME was briefly investigated in **Paper I** by coupling three separate electrical circuits in parallel to a single 9 V battery. This setup isolated amitriptyline, citalopram, fluoxetine, and fluvoxamine from 70  $\mu\text{L}$  human plasma into 30  $\mu\text{L}$  formic acid in 60 seconds of extraction. The extraction recoveries were found to be unaffected by the total number of samples processed at the same time. The hollow fiber configuration presented in Fig. 3-14 was challenging to operate, and the idea of Pa-EME re-emerged after the introduction of parallel artificial liquid membrane extraction (PALME) in 2013 [40].

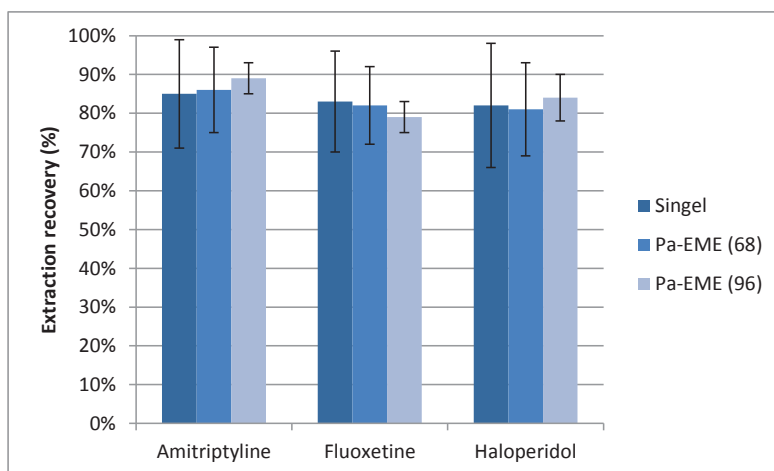


**Fig. 3-14.** Pa-EME with the hollow fiber configuration. (Reprinted with permission from Paper I. © 2010 Elsevier)

In **Paper VI** the Pa-EME configuration with flat membranes was described for the first time. In this setup flat polypropylene membranes were attached with heat to strips of plastic vials. The lower end of those vials were cut off and served as a multi-well plate. A 96-well plate served as sample reservoirs. Sheets of aluminum foil were attached in fixed positions to both the multi-well plate and the sample reservoirs; thus creating an electrical circuit across each of the 96 wells. The four basic drugs amitriptyline, fluoxetine, quetiapine, and sertraline were selected as model substances. NPOE was selected as the SLM based upon experiences with the hollow fiber electromembrane extractions (HF-EME) configuration [101, 102]; undiluted human plasma was the sample matrix and 20 mM formic acid was chosen as acceptor solution due to its LC-MS-compatibility. Since Pa-EME with flat membranes was investigated for the first time: extraction time, extraction voltage, agitation speed, sample solution volume, and acceptor solution volume were optimized with QbD. The final Pa-EME configuration provided isolation of amitriptyline, fluoxetine, quetiapine, and sertraline from eight plasma samples into LC-MS compatible extracts simultaneously by utilizing a single power supply.

The sample throughput with the Pa-EME configuration was increased in **Paper VII**, 68- and 96 samples were processed in parallel with the configuration presented in Fig. 3-4. The extraction recoveries were unaffected by the total number of samples processed in parallel with the 96 well Pa-EME configuration, the results are presented in Fig. 3-15.





**Fig. 3-15.** Extraction recovery (%) with the Pa-EME configuration of amitriptyline, fluoxetine, and haloperidol, respectively.

Also the volume of the acceptor solution compartments were increased in **Paper VII** and thus provided an opportunity to alternate the acceptor solution volume compared to the HF-EME configurations. Typical acceptor solution volume with the hollow fiber setups have been in the range 10-30  $\mu\text{L}$ , in **Paper VII** the volume of acceptor solution was 100-150  $\mu\text{L}$ . High acceptor solution volume in EME has been considered as favourable to increase the extraction recovery and exhaustive extraction was reported from pH adjusted water samples in **Paper VII**.

## CONCLUDING REMARKS

In this thesis the potential of selective isolation with electromembrane extraction (EME) of ionized analytes from biological matrices has been investigated. Major focus was on extraction of small molecular drug substances from human matrices; extraction at low voltages (<10 V), short extraction times (<10 min), exhaustive extraction, and parallel extractions of multiple samples. EME was also combined with dried matrix spotting on sheets of soluble alginate- and chitosan foams.

- The development of a portable and stagnant electromembrane extraction device with a single 9 V battery as the only driving force provided selective isolation in the therapeutic range of four basic drugs within 1 min of extraction. This approach also reported interesting findings concerning the potential of parallel extractions with a single power supply as driving force.
- Exhaustive extractions from undiluted human plasma were reported by increasing the total number of hollow fibers in the sample solution from one to three and thus increase the surface area available for electrokinetic migration.
- The selective isolation of ionized analytes provided with EME was demonstrated from both whole- and post-mortem blood. This approach demonstrated the potential of EME in forensic science.
- Electromembrane extraction was combined with sampling on a novel sampling material for dried blood spot analysis. The combination of EME with sampling on water soluble biopolymers reported a substantial reduction in time consumption compared to commercially available sampling media.
- Sampling of oral fluid on sheets of water soluble biopolymers was combined with electromembrane extraction for isolation and enrichment of five basic drugs of abuse. This approach reported findings regarding sampling of oral fluid as dried spots on water soluble biopolymers and selective isolation of the drugs with EME.

- The sample throughput with EME was increased with the multi-well flat membrane format and demonstrated the potential for incorporation of electromembrane extraction into the liquid handling platform in the future.
- The sample throughput was increased with the 96-well format, and was considered as unaffected by small, but deliberate variations in method parameters in a normal laboratory setting.

In order to be established as a sample preparation technique of significance in the future the homemade configurations has to be omitted and electromembrane extraction has to be commercialized. Hopefully, this will increase the interest from academic as well as routine laboratories worldwide, the theoretical understanding and fundament should be even more thoroughly investigated in order to solve challenges which are not possible with existing sample preparation techniques.

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# 3 Exhaustive Electromembrane Extraction

## 4 In 96-Well Format

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23 **Keywords**

24 Electromembrane extraction

25 96-well format

26 High throughput sample preparation

27 Supported liquid membranes

28 Biological matrices

29 Ultra performance liquid chromatography

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## 42 **Abstract**

43 The robustness of parallel electromembrane extraction (Pa-EME) was thoroughly investigated  
44 in the present project. Amitriptyline, fluoxetine, and haloperidol were isolated from eight  
45 samples of pure water, undiluted human plasma, and undiluted human urine, respectively;  
46 totally 24 samples were processed in parallel. The extraction performance, expressed as  
47 extraction recovery and repeatability, was found to be independent of the different sample  
48 matrices processed in parallel although the respective samples contained different matrix  
49 components. In another experiment seven of the 24 wells were perforated. Even though the  
50 perforation caused the total current level in the Pa-EME setup to increase, the intact circuits  
51 were unaffected by the collapse in seven of the circuits. In another approach, exhaustive  
52 extraction of amitriptyline, fluoxetine, and haloperidol was demonstrated from pure water  
53 samples. Amitriptyline and haloperidol were also isolated exhaustively from undiluted human  
54 plasma samples; the extraction recovery of fluoxetine from undiluted human plasma was 81%.  
55 Finally, the sample throughput was increased with the Pa-EME configuration. The extraction  
56 recoveries were investigated by processing one, eight, 68, or 96 samples in parallel in 10 min;  
57 neither the extraction recoveries nor the repeatability was affected by the total numbers of  
58 samples. Eventually, the Pa-EME was combined with ultra performance liquid  
59 chromatography (UPLC) to combine high-throughput sample preparation with high-  
60 throughput analytical instrumentation.

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## 64 **Introduction**

65 The interest for less solvent consumption and miniaturized sample preparation techniques  
66 have increased recently due to faster and more sensitive analytical instrumentation. The  
67 sample volume has been reduced substantially, typically from 1-5 mL to 10-50  $\mu\text{L}$ , with  
68 miniaturized extraction techniques in comparison with more traditional sample preparation  
69 techniques like liquid-liquid extraction (LLE), solid-phase extraction (SPE), filtration, and  
70 protein precipitation (PP). Electromembrane extraction (EME) was described for the first time  
71 in 2006 and demonstrated electrokinetic migration of charged substances from an aqueous  
72 sample solution through a supported liquid membrane (SLM) and into an aqueous acceptor  
73 solution [1]. The driving force in EME is an applied electrical field. The acceptor solution is  
74 directly compatible with final analysis for instance by, but not limited to, LC-MS without the  
75 need for evaporation to dryness and reconstitution in an appropriate solvent.

76 Since the introduction of EME in 2006 several papers have described the fundamentals of  
77 electrokinetic migration of charged analytes through an SLM [2-5]. EME is considered as a  
78 robust, fast, and environmental-friendly sample preparation technique due to the acceptable  
79 RSD-values (<20%), the short extraction time (1-10 min), and the low consumption of  
80 organic liquid (<10  $\mu\text{L}$ ); several review articles have been published the recent years [6, 7].

81 The SLM provides a barrier which excludes sample constituents from entering the acceptor  
82 solution; and provides extracts free of interfering matrix constituents [8-10]. Applications  
83 based on EME have been utilized to isolate basic drugs [11, 12], acidic drugs [13], peptides  
84 [14], analytes from dried matrix spotting [8, 10, 15], amino acids [16, 17], and lead ions [18]  
85 from different types of sample matrices. Although numerous benefits have been reported with  
86 EME, the sample throughput has been limited and considered as a drawback compared to  
87 other extraction techniques which have been incorporated into the high-throughput platforms.  
88 High sample throughput is especially requested by the pharmaceutical industry as a tool in the

89 search for novel drug candidates. The incorporation of sample preparation techniques into  
90 multi-well plates and parallel extractions has been considered as an important advance in the  
91 field of sample preparation [19].

92 The idea of parallel electromembrane extraction (Pa-EME) was briefly investigated in 2010  
93 with the hollow fiber configuration; in this setup three extraction units were connected in  
94 parallel to a single 9 V battery [20]. The Pa-EME configuration enabled the isolation of  
95 amitriptyline, citalopram, fluoxetine, and fluvoxamine into formic acid from three different  
96 plasma samples simultaneously with RSD-values in the range 7.8 to 18.1% [20]. The Pa-EME  
97 with the hollow fiber configuration was challenging to operate; however the introduction of  
98 flat membranes [21] simplified the processing of multiple samples with Pa-EME substantially.  
99 Very recently the idea of Pa-EME with the flat membrane configuration was introduced [9];  
100 in this setup amitriptyline, fluoxetine, quetiapine, and sertraline was isolated into formic acid.  
101 Eight plasma samples were processed simultaneously in separate wells within eight minutes  
102 of extraction with RSD-values in the range 5-15% [9]. This setup reported extraction  
103 recoveries in the range 15 to 33% from undiluted human plasma spiked with the  
104 abovementioned model analytes. Although the extraction recoveries were relatively low, the  
105 combination of Pa-EME with sensitive LC-MS/MS instrumentation provided limit of  
106 quantification (LOQ) below the therapeutic levels. Eventually, the Pa-EME setup in  
107 combination with LC-MS analysis was successfully utilized to determine the plasma  
108 concentration from patients treated with quetiapine and sertraline. This was considered as the  
109 first attempt to incorporate EME with flat membranes into the multi-well format [9].

110 In the present investigation the Pa-EME setup was exploited more thoroughly. Different  
111 biological matrices were processed in parallel to investigate if the Pa-EME setup remains  
112 unaffected by small, but deliberate variations in method parameters and is reliable during  
113 normal use. In addition, several of the wells in the Pa-EME setup were perforated during

114 extraction in order to investigate the impact of this interruption on the remaining wells. Also  
115 the total number of samples processed simultaneously was increased from eight to 68 and  
116 finally to 96. The magnitude of the applied electrical field, the acceptor solution volume, and  
117 the extraction time were optimized in order to accomplish exhaustive EME from undiluted  
118 human plasma.

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## 133 **Experimental**

### 134 **Chemicals**

135 Amitriptyline hydrochloride, fluoxetine hydrochloride, and haloperidol were all obtained from  
136 Sigma-Aldrich (St.Louis, MO, USA). Formic acid and 2-nitrophenyl octyl ether were  
137 obtained from Fluka (Buchs, Switzerland). Drug free human plasma was obtained from Oslo  
138 University Hospital (Oslo, Norway) and stored at -32 °C. Drug-free human urine was  
139 obtained from a healthy volunteer. Water was obtained from a Milli-Q water purification  
140 system (Molsheim, France).

### 141 **Preparation of the parallel electromembrane extraction setup**

142 The Pa-EME setup was homemade by heat-sealing of circular sheets of a porous  
143 polypropylene flat membrane Accurel PP 1E (R/P) from Membrana (Wuppertal, Germany)  
144 with 244 °C for 5 sec with a heater from CoTech (Innsjön, Sweden) to nABgene 0.2 mL  
145 strip tubes from Thermo Scientific (Loughborough, UK). Subsequently, the lower part of the  
146 plastic vials were cut off and attached with glue to a pipette tip rack. A 96-well collection  
147 plate from Agilent (Santa Clara, CA, USA) was utilized as the donor solution compartment.  
148 Both the multi-well plate and the donor compartments were coated with Platemax™  
149 aluminum foil (Union City, CA, USA) in a fixed position to serve as electrodes in the Pa-  
150 EME setup. The 96-well Pa-EME setup is presented in Fig.1. The aluminium foils were  
151 connected with clips to an adjustable power supply from Delta Power Supplies (Zierikzee, the  
152 Netherlands). The extraction compartment was placed on a Vibramax 100 from Heidolph  
153 Instruments (Kelheim, Germany) and subjected to a given agitation rate throughout the  
154 extraction.

### 155 **Capillary electrophoresis**

156 The final analysis was performed by capillary electrophoresis in combination with UV-  
157 detection with an HP<sup>3D</sup> CE instrument from Agilent Technologies (Waldbronn, Germany).

158 The three basic drug substances were separated in a 50 cm effective length fused silica  
159 capillary with an internal diameter of 75  $\mu\text{m}$  from Polymicro Technologies (Phoenix, AZ,  
160 USA). The electrolyte solution was a 25 mM phosphate buffer adjusted to pH 2.7 with 25 mM  
161 orto-phosphoric acid. The final extract was injected by hydrodynamic injection of 0.5 psi for  
162 5 sec., and separated at 30 kV. The drug substances were detected at 200 nm.

### 163 **Flow injection analysis coupled with electrospray ionization mass** 164 **spectrometry**

165 The flow injection system consisted of a Dionex UltiMate 3000 WPS 3000 TSL autosampler,  
166 connected to a Thermo Scientific LTQ XL Linear Ion Trap Mass Spectrometer (all from  
167 Thermo Scientific, Sunnyvale, CA, USA). Data acquisition and processing were performed  
168 using Xcalibur version 2.1 software from Thermo Scientific. The flow rate of the running  
169 solution was set to 50  $\mu\text{L min}^{-1}$  and consisted of 20mM HCOOH:acetonitrile (1:1), and the  
170 injection volume was set to 5  $\mu\text{L}$ .

171 An electrospray ionization (ESI) source operated in the positive ionization mode was used to  
172 interface the FIA and the MS. Analyses were performed with selected reaction monitoring  
173 (SRM) using He as a collision gas. The sheath gas was set to 25 units, auxiliary gas 5 units,  
174 capillary temperature 250°C, and the spray voltage to 4 kV. The quantifier SRM transitions  
175 were used to quantify the compounds while the qualifiers were used as confirmatory signals.  
176 The SRM transitions and collision energies are shown in Table 1.

### 177 **Ultra performance liquid chromatography (UPLC) combined with mass** 178 **spectrometry**

179 A Waters Aquity UPLC-system was used for separation, applying an Aquity HSS T3-column  
180 (2.1 x 100 mm, 1.8  $\mu\text{m}$ ). The mobile phase consisted of 100% methanol (mobile phase A) and  
181 25 mmol/L formic acid (mobile phase B). The flow rate was 0.5 mL/min, and the following  
182 gradient was used. The initial composition 70% B and 30% A was held for 0.5 minutes, a



183 linear gradient up to 100% A in 0.5 minutes was then applied followed by 0.5 minutes at 100%  
184 A and return to initial conditions. The total cycle time was 2 minutes. The column  
185 temperature was held at 65°C, and the injection volume was 3 µL. Partial loop injection with a  
186 needle overfill flush of 3 µL was used, with each sample using 600 µL methanol:water (10:90)  
187 for “weak wash” and 200 µL methanol:water (90:10) for “strong wash”.

188 A Waters Quattro Premier XE tandem mass spectrometer, equipped with a Z-spray  
189 electrospray interface, was used for detection. Positive ionization was performed in the  
190 multiple reaction monitoring (MRM) mode. One transition was used for each compound. The  
191 capillary voltage was set to 1.0 kV, the source block temperature was 120°C, and the  
192 desolvation gas (nitrogen) was heated to 450°C and delivered at a flow rate of 900 L/h. The  
193 cone gas (nitrogen) was set to 60 L/h, and the collision gas (argon) pressure was maintained at  
194 0.006 mbar in the collision cell. The UPLC conditions are presented in Table 2. System  
195 operation and data acquisition were controlled using Mass Lynx 4.1 software. Peak area was  
196 used for quantification using TargetLynx soft-ware.

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## 206 **Results and Discussions**

207 In parallel electromembrane extraction (Pa-EME) several electrical circuits are connected in  
208 parallel to a single power supply; from a theoretical point of view the voltage should be  
209 constant across each of the parallel-coupled wells. The eight well Pa-EME recently introduced  
210 was considered as the first attempt to increase the sample throughput with EME [9]. The  
211 robustness against small but deliberate variations in method parameters, focusing on RSD-  
212 values and extraction recovery, was investigated in the present paper. The chemical structure,  
213 log P, and pKa-values of the three basic drug substances are included in Fig.2.

### 214 **Extraction recovery and robustness**

215 The first Pa-EME paper reported extraction recoveries in the range 15 to 33% for the four  
216 basic model analytes from untreated human plasma within 8 min of extraction [9]. An  
217 important observation from the experimental design in this paper was that both increased  
218 extraction voltage- and acceptor solution volume increased the extraction recovery. A recent  
219 publication also indicated that the volume of acceptor solution was an important parameter  
220 toward increased extraction recovery in electromembrane extraction [22]. However, due to  
221 safety considerations and technical challenges the upper limit for applied extraction voltage  
222 was set to 200 V in the first Pa-EME publication [9]. In the present investigation the strips of  
223 vials with the flat membrane was attached with glue to a 96-well rack for pipette tips to obtain  
224 a safer and more user-friendly Pa-EME configuration. This enabled 300 V to be used as  
225 extraction voltage. Additionally, the volume of the wells in the multi-well plate was increased  
226 and thus enabled an increased flexibility regarding acceptor solution volume. The 96-well Pa-  
227 EME configuration is presented in Fig.1.

228 This configuration was utilized to isolate three basic model substances from 20 mM formic  
229 acid. The sample solution volume was 240  $\mu$ L, volume of 2-nitrophenyl octyl ether (NPOE)  
230 applied to the supporting material was 4  $\mu$ L, the excess NPOE was gently removed after 30

231 seconds of drying, the volume of acceptor solution was 100  $\mu$ L 20 mM formic acid, and 300V  
232 was applied for 10 min, and the Pa-EME system was agitated at 1050 rpm throughout  
233 extraction. This approach provided the following extraction recoveries: 99% (2%, RSD), 99%  
234 (2%, RSD), and 97% (3%, RSD), for amitriptyline, fluoxetine, and haloperidol, respectively;  
235 eight samples were processed in parallel and the RSD-values (%) are given in parentheses.  
236 The development of a more robust Pa-EME setup enabled an increased flexibility regarding  
237 magnitude of the applied electrical field and volume of the acceptor solution compared to the  
238 first Pa-EME publication, and this enabled exhaustive extraction.

239 Although the first Pa-EME setup reported interesting findings regarding sample throughput  
240 and reproducibility a question about the robustness was raised after completing that paper.  
241 The purpose of the present investigation was to examine if a collapse in a single or multiple  
242 wells affected the extraction performance, and also how the extraction performance was  
243 affected by small, but deliberate variations in method parameters.

244 In a parallel coupled configuration with direct current, the voltage in each circuit would be  
245 equal; however the current would be dependent on the resistance in each circuit. The total  
246 current in the system would be the sum of the current in each of the circuits. In EME the  
247 resistance is the organic liquid sustained in the pores of the supporting material; this  
248 resistance is controlled by addition of the same volume of organic liquid to each of the flat  
249 polypropylene membranes. If every circuit in the Pa-EME remains intact throughout  
250 extraction, the total current could be calculated by adding the current across each circuit; the  
251 voltage would consequently be constant. In the Pa-EME setup a perforation in one or several  
252 of the circuits would increase the total current in the system due to the elimination of the  
253 resistance in the circuit or circuits. In order to investigate this 240  $\mu$ L 20 mM formic acid  
254 spiked with the three basic model substances was added to each of the eight wells, 4  $\mu$ L  
255 NPOE was added to the supporting material, and the eight acceptor compartments were filled

256 with 100  $\mu$ L 20 mM formic acid, 100 V was applied for 10 min, and the Pa-EME setup was  
257 subjected to an agitation rate at 900 rpm throughout extraction. The same procedure was  
258 utilized in another experiment; however, in the latter experiment the membranes in two of the  
259 wells were punctured. The punctured membranes resulted in an increase in the total current  
260 and subsequent electrolysis in the perforated wells. The extraction performance in both  
261 experiments was compared in terms of extraction recovery and reproducibility; the results are  
262 presented in Table 3. The extraction performance was unaffected in the non-punctured wells  
263 by the collapse in two of the extraction compartments. The same approach was used to  
264 investigate the impact of perforation of two sample compartments with undiluted human  
265 plasma as sample matrix. This experiment reported similar results and demonstrated that the  
266 Pa-EME setup was unaffected by small, but deliberate variations in method parameters also  
267 with human plasma as sample matrix as presented in Table 3.

268 In another experiment the aim was to investigate the robustness of Pa-EME by extraction of  
269 eight human plasma samples, eight human urine samples, and eight water samples  
270 simultaneously. All samples were spiked with amitriptyline, fluoxetine, and haloperidol. In  
271 total 24 samples were processed in parallel, and 100 V was the magnitude of the applied  
272 electrical field with NPOE as organic liquid in the SLM. After 1 min of extraction the power  
273 supply was turned off and the SLM in several wells were perforated with a pipette tip.  
274 Thereafter, the power supply was turned on and the extraction was continued for another 6  
275 min. The perforation of some of the SLMs increased the total current significantly and caused  
276 extensive bubble formation in the associated wells. However, the extraction recoveries and  
277 the repeatability in the intact wells were unaffected by the perforations and demonstrated that  
278 even though some of the extractions failed all the remaining extracts were unaffected. This  
279 experiment demonstrated the robustness of Pa-EME and the data are presented in Table 4.

## 280 **Sample throughput and effect of the electrical field**

281 In a next series of experiments the total number of samples processed in parallel was  
282 increased from 24 and both the extraction recovery and the repeatability were investigated. In  
283 this experiment isopropyl nitrobenzene (IPNB) in combination with low voltage (20 V) was  
284 selected as the organic liquid sustained in the pores of the supporting material and applied  
285 voltage, respectively. The choice of using IPNB in combination with low voltage was made  
286 essentially due to safety considerations regarding the magnitude of the applied electrical field  
287 and experiences with this particular combination in the HF-EME configuration [23]. The  
288 donor- and acceptor solution was 240  $\mu\text{L}$  20 mM formic acid spiked with amitriptyline,  
289 fluoxetine, and haloperidol- and 100  $\mu\text{L}$  20 mM formic acid, respectively. The volume of  
290 IPNB applied to the supporting material was 4  $\mu\text{L}$  and the extraction was continued for 10  
291 min on a shaking board at 1050 rpm.

292 In the first experiment a single sample was processed per time ( $n=4$ ) and provided extraction  
293 recoveries in the range from 82 to 85% with RSD values in the range 13 to 16% as presented  
294 in Fig.3. Both with eight-, 68- and 96-well setup, and the extraction recoveries were similar to  
295 the extraction recoveries reported by processing one sample per time. The results are  
296 presented in Fig.3. The extraction performance was clearly unaffected by the total number of  
297 samples processed in parallel. This series of experiments demonstrated the potential  
298 concerning sample throughput with electromembrane extraction; in the most extreme  
299 experiment amitriptyline, fluoxetine, and haloperidol was isolated from 96 different samples  
300 and into LC-MS compatible extracts within 10 min. Interestingly, RSD-values were low, and  
301 with 96 samples the RSD-values were all below 6%.

302 In a another experiment the impact of the applied electrical field was investigated; the  
303 extraction recoveries were examined with untreated human plasma at 0- and 250 V with  
304 NPOE as organic liquid in the SLM. Also the volume of acceptor solution was increased to

305 150  $\mu\text{L}$  to facilitate increased extraction recovery. This approach reported a two to eight fold  
306 increase in extraction recovery within 10 min of extraction by applying 250 V compared to 0  
307 V. The results are presented in Fig.4 and demonstrated the importance of the applied electrical  
308 field in EME. Although the pKa-values were 9.4, 9.8, and 8.3 for amitriptyline, fluoxetine,  
309 and haloperidol, respectively, and they were partially- or fully ionized at physiological pH  
310 (7.4) still the extraction recoveries were 51-, 10-, and 42% in the absence of an electrical field  
311 for amitriptyline, fluoxetine, and haloperidol, respectively. The acceptor solution volume  
312 could be an essential factor to the relatively high extraction recoveries even without an  
313 applied electrical field.

#### 314 **Combination with high throughput analytical instrumentation**

315 The Pa-EME setup could potentially process 96 samples in parallel, and isolate the analytes of  
316 interest into aqueous extracts directly compatible with analytical instrumentation within 10  
317 min of extraction. However, this high throughput requires an analytical instrumentation with  
318 high capacity, meaning short analysis time per sample. An approach could be flow injection  
319 analysis (FIA), to omit the chromatographic separation. However, experiments demonstrated  
320 extensive ion suppression/enhancement due to altered ionization in the electrospray ionization  
321 source (ESI) when the substances were not separated prior to the MS analysis. Those FIA-  
322 experiments are presented in Fig. 5a and emphasized the need for a chromatographic  
323 separation prior to MS-analysis.

324 In another approach UPLC-MS/MS was selected as the analytical instrumentation to  
325 demonstrate the feasibility to analyze 96 EME-extracts within a relatively short time-frame  
326 with run-time of 2 min per extract. Three different samples, each spiked with amitriptyline,  
327 fluoxetine, and haloperidol at 50  $\text{ng mL}^{-1}$ , respectively, were processed with the Pa-EME  
328 configuration (n=4). In the next experiment a single sample spiked with the aforementioned  
329 model analytes at 50  $\text{ng mL}^{-1}$  was processed with the Pa-EME setup (n=4). In the last

330 experiment a sample spiked with the same three basic model substances at 50 ng mL<sup>-1</sup>;  
331 additionally this sample was spiked with ibuprofen, nortriptyline, and paracetamol at high  
332 concentrations (1000 ng mL<sup>-1</sup>) before extraction in the Pa-EME setup (n=4). The aim of those  
333 experiments was to investigate if a chromatographic separation of only 2 min was acceptable  
334 to avoid matrix effects in the MS. The data obtained from those experiments are presented in  
335 Fig. 5b. The signal intensities for amitriptyline, fluoxetine, and haloperidol in the first  
336 experiment were compared to the signal intensities obtained for the same model substances in  
337 the presence of ibuprofen, nortriptyline, and paracetamol in the sample. A chromatogram of  
338 amitriptyline, fluoxetine, fluoxetine d-5, and haloperidol is included in Fig. 6. The signal  
339 intensities regarding amitriptyline, and fluoxetine were considered as unaffected by the  
340 presence of the other model substances. In the case of haloperidol a slight increase in signal  
341 intensity was observed; however taken the homebuilt Pa-EME configuration into  
342 consideration we considered this as acceptable. The combination of high-throughput Pa-EME  
343 with high throughput analytical instrumentation (UPLC-MS) was considered as a success.

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## 352 **Conclusions**

353 In the present investigation the robustness of parallel electromembrane extraction was  
354 investigated. The Pa-EME setup demonstrated to be robust against small but deliberate  
355 variations in method parameters. Different types of sample matrices were added to different  
356 wells and processed in parallel to investigate if the sample composition influenced the  
357 extraction performance. The extraction performance was found to be independent of the  
358 sample composition. Also the extraction recovery with different number of samples loaded  
359 into the multi-well plate was examined and the extraction recovery was found to be  
360 independent of the total number samples processed in parallel. In order to fully demonstrate  
361 the sample throughput, 96-samples were processed successfully in parallel within 10 min of  
362 extraction. Both the excellent sample clean-up provided and the high throughput provided  
363 with Pa-EME requested a very fast analytical technique. In a final series of experiments Pa-  
364 EME was combined with flow injection analysis (FIA); however extensive matrix effects  
365 were observed without a chromatographic separation and consequently ultra performance  
366 liquid chromatography combined with mass spectrometry was selected as the analytical  
367 instrumentation to combine high-throughput sample preparation with high-throughput  
368 analytical instrumentation.

369 The combination of a robust high-throughput electromembrane extraction setup with fast  
370 analytical instrumentation provides a powerful platform for analysis of small molecular drug  
371 substances from undiluted biological fluids.

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376 **Figure captions**

377 Figure 1. Picture of the 96-well Pa-EME setup utilized in this paper.

378 Figure 2. Structure, log P, and pKa-value for amitriptyline, fluoxetine, and haloperidol.

379 Figure 3. Extraction recovery (%) with 1, 68, and 96 samples in the Pa-EME setup.

380 *Donor solution: 240  $\mu$ L, acceptor solution: 100  $\mu$ L 20 mM HCOOH, SLM: 4  $\mu$ L IPNB,*  
381 *extraction voltage: 20 V, extraction time: 10 min, agitation rate: 900 rpm*

382 Figure 4. Comparison of the extraction recovery with 0 V and 250 V.

383 *Donor solution: 240  $\mu$ L, acceptor solution: 150  $\mu$ L 20 mM HCOOH, SLM: 4  $\mu$ L NPOE,*  
384 *extraction voltage: 0 V or 250 V, extraction time: 10 min, agitation rate: 900 rpm*

385 Figure 5. Flow injection analysis- (a) and ultra performance liquid chromatography (b) results.

386 *Donor solution: 240  $\mu$ L, acceptor solution: 150  $\mu$ L 20 mM HCOOH, SLM: 4  $\mu$ L NPOE,*  
387 *extraction voltage: 200 V, extraction time: 10 min, agitation rate: 900 rpm*

388 Figure 6. UPLC-MS/MS chromatogram of haloperidol, fluoxetine d-5, fluoxetine, and  
389 amitriptyline.

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402 Figure 1

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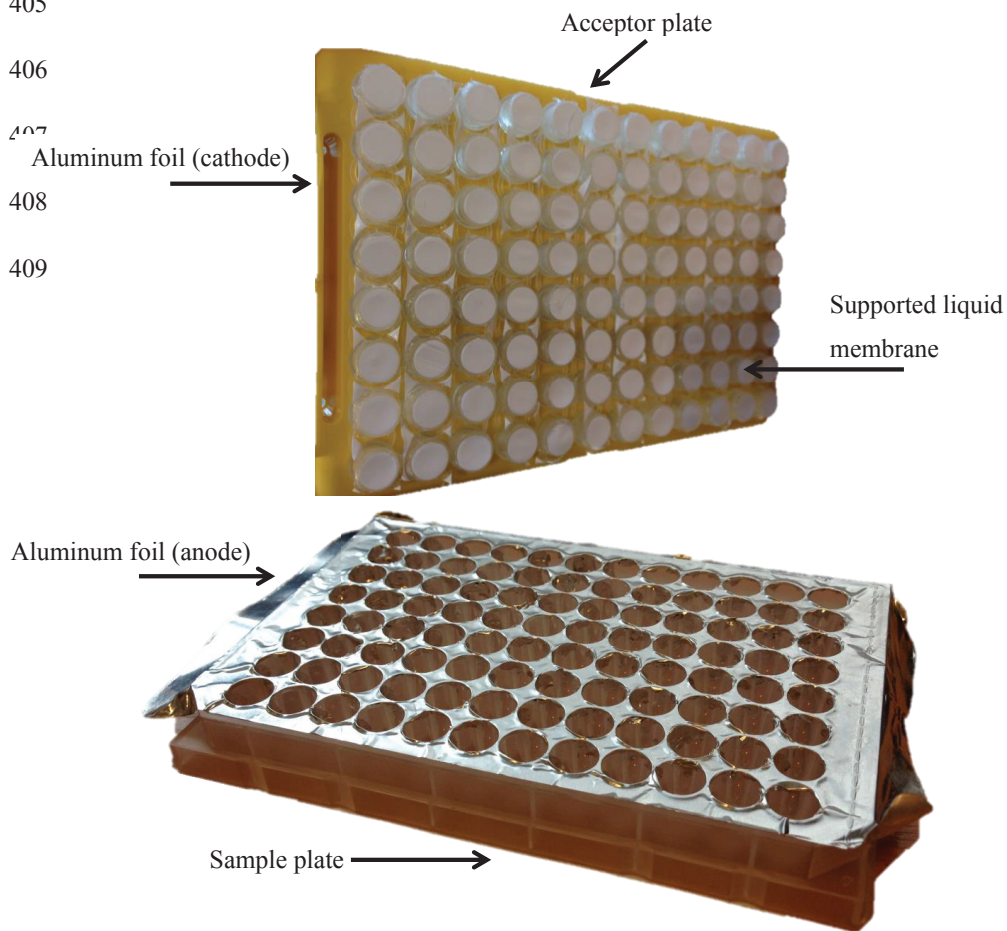
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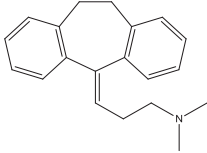
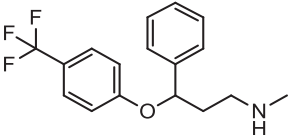
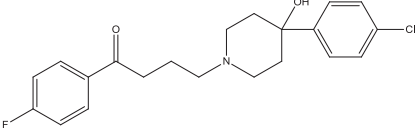
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414 Figure 2

Model substance	Structure	pKa <sup>a</sup>	log P <sup>a</sup>
Amitriptyline		9.8	7.9
Fluoxetine		9.8	4.1
Haloperidol		8.0	3.2

415 <sup>a</sup> <https://scifinder.cas.org>

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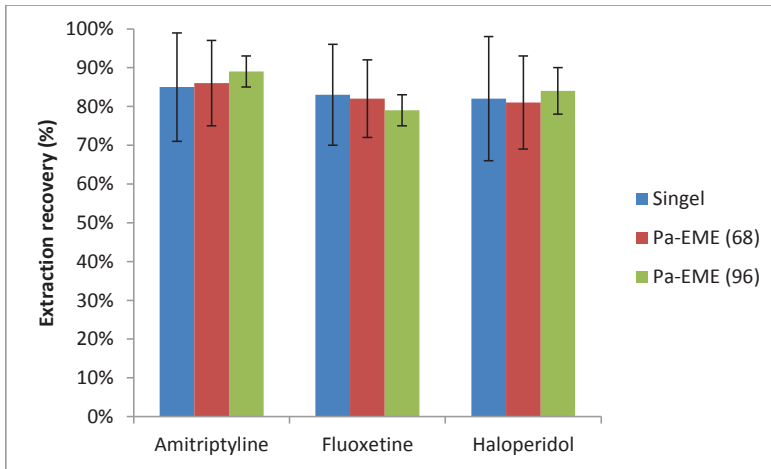
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428 Figure 3



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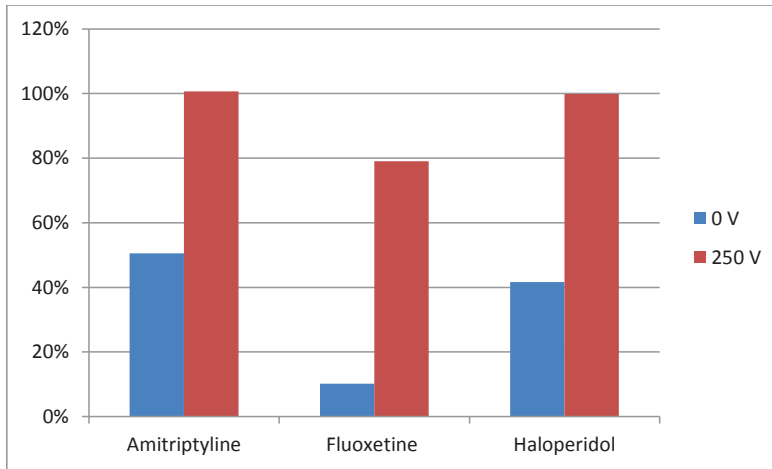
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444 Figure 4



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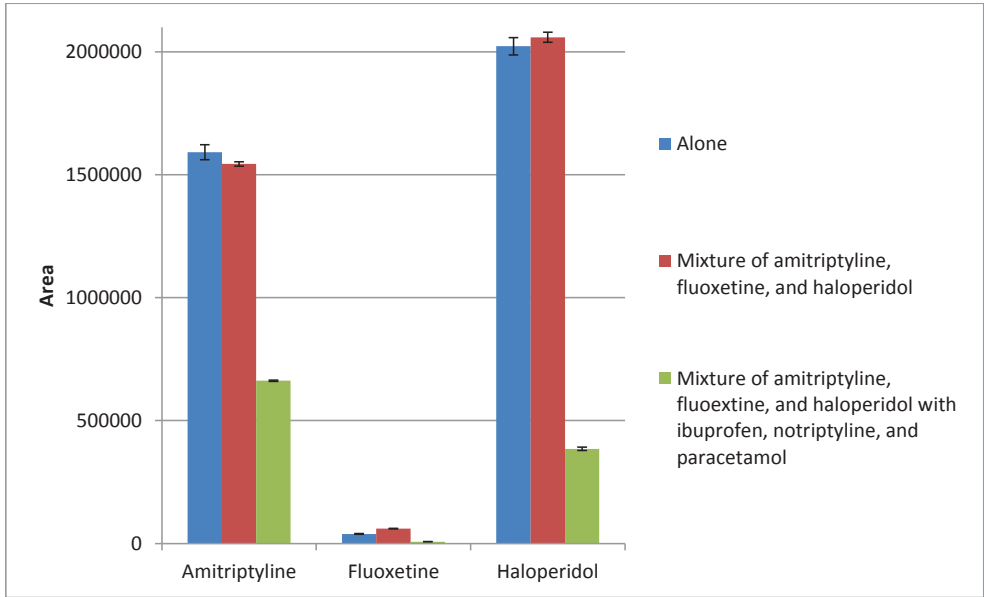
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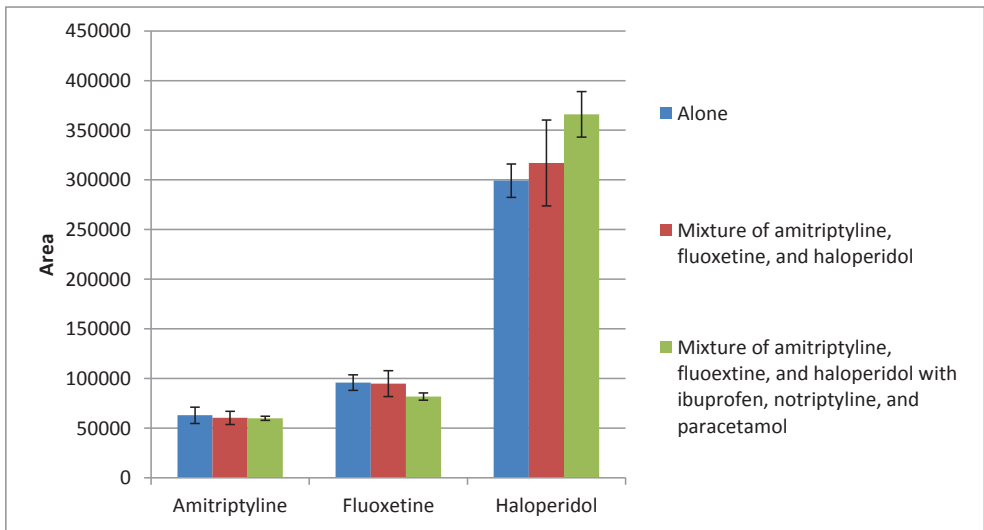
460 Figure 5

461 a)



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463 b)



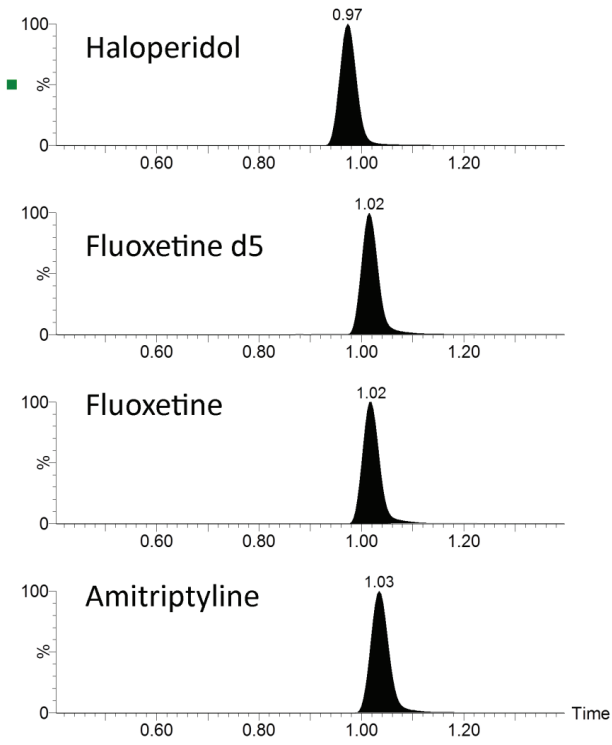
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467 Figure 6

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477 Table 1. Flow injection analysis (FIA) conditions

	SRM transitions			478 Collision energy
	<i>Precursor</i>	<i>Quantifier</i>	<i>Qualifier</i>	
<b>Amitriptyline</b>	278.15	233.1	104.9	35%
<b>Fluoxetine</b>	310.11	147.99	-	29%
<b>Haloperidol</b>	376.19	165.00	123.0, 358.20	29%



479 Table 2. Ultra performance liquid chromatography (UPLC) conditions

	<b>MRM transitions</b>	<b>Cone Voltage (V)</b>	<b>Collision Energy (eV)</b>
	<b>278.2&gt;105.0</b>	<b>32</b>	<b>22</b>
<b>Amitriptyline</b>			
	310.1>148.1	20	8
<b>Fluoxetine</b>			
	376.3>165.0	38	24
<b>Haloperidol</b>			
	315.1>153.1	20	10
<b>Fluoxetine d-5</b>			

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490 Table 3. Perforation of two wells with undiluted human plasma- and pure water as samples, respectively.

491 *Donor solution: 240  $\mu$ L, acceptor solution: 100  $\mu$ L 20 mM HCOOH, SLM: 4  $\mu$ L NPOE, extraction voltage: 100 V, extraction time: 10 min,*  
492 *agitation rate: 900 rpm*

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495

**Undiluted human plasma**

**Pure water samples**

496

**All wells intact**

**Two wells perforated**

**All wells intact**

**Two wells perforated**

497

**Amitriptyline**

48% (10)

49% (11)

94% (11)

94% (11)

497

**Fluoxetine**

38% (12)

38% (11)

75% (8)

76% (9)

497

**Haloperidol**

53% (11)

57% (11)

90% (13)

88% (14)

498 Table 4. Perforation of 7 wells with pure water samples, urine, and human plasma as sample  
499 matrices.

500 *Donor solution: 240  $\mu$ L, acceptor solution: 100  $\mu$ L 20 mM HCOOH, SLM: 4  $\mu$ L NPOE,*  
501 *extraction voltage: 100 V, extraction time: 7 min, agitation rate: 900 rpm*

502

	Pure water samples	Urine	Plasma
<b>Amitryptiline</b>	104% (14)	58% (12)	32% (15)
<b>Fluoxetine</b>	87% (10)	76% (18)	26% (16)
<b>Haloperidol</b>	87% (8)	82% (18)	33% (16)

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