Electromembrane extraction of immunosuppressants

Master thesis in pharmacy

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Abstract

Tofacitinib (TFB) and cyclosporine A (CsA) are two immunosuppressants that can cause toxic adverse effects or treatment failure if not dosed properly. Both these drugs have the potential to be affected by individual variability in pharmacokinetics, and interactions with other drugs and nutrients. Therapeutic drug monitoring (TDM), based on serum concentration measurements, could benefit patients in need of these drugs, to ensure safe treatment. Effective sample preparation techniques are essential to precisely determine the concentration of a drug within pharmaceutical bioanalysis. This is to avoid interference from matrix components and prevent contamination or damage of the analytical instrument.

Electromembrane extraction (EME) is a sample preparation technique that was developed in 2006. Over the years, this technique has proven to be efficient for both acidic, basic, polar and non-polar analytes. The concept of the extraction is electrokinetic migration of the analyte by an external power supply over a three-phase system consisting of two aqueous solutions (donor and acceptor), separated by a supported liquid membrane (SLM) comprised of an organic solvent. EME has several advantages, including high sample clean-up and selectivity, enrichment with the possibility of pre-concentration, and low consumption of organic solvents.

In this study, EME was for the first time investigated as a sample preparation technique for the immunosuppressants CsA and TFB.

The EME method development for CsA was obstructed by the lower limit of detection with HPLC-UV, at 2 µg/mL, and the solubility of the analyte. CsA was attempted to be extracted as an anion with highly alkaline conditions using a 10 mM NaOH solution with pH 10 in both donor and acceptor, and 1-octanol as SLM. CsA was not detected in the acceptor solution, and the highest mean recovery of analyte from the donor solution and SLM was 24% at 20 V.

For the method development of TFB, a range of different conditions, i.e. pH, SLMs, voltage and extraction time, were tested and optimized in order to yield high recoveries of the analyte. The aqueous samples without plasma were analyzed by HPLC-UV at 40˚C with an isocratic mobile phase of 10 mM ammonium acetate pH 5 and acetonitrile (60:40). An absolute

recovery (100%) of TFB was obtained after 45 minutes of extraction as a cation from diluted plasma with pH 2.1 at 30 V, using an acceptor solution of 100 mM formic acid with pH 2.4 and a SLM comprised of 6-methylcoumarin and thymol mixed in a weight to weight ratio (1:2). A similar method yielded 79% recovery after 15 minutes extraction from a donor solution of 100 mM formic acid with pH 2.4.

In conclusion, EME extraction appears as a promising sample preparation technique for TFB, but further optimization is needed to conclude if it is viable also for CsA.

Preface

My supervisors Lars Herfindal and Silje Skrede deserves a special thanks for the great guidance given throughout the writing prosses of this thesis. The help from Lars in the laboratory at the beginning of the year encouraged me to continue working independently, and if I ever needed help, you found the time to assist me. During this project there was a lot of failing and feelings of frustration, especially in experimentation with cyclosporin A. When I felt the urge to give up, you guided me in the right direction and motivated me to continue working. The prosses of writing this thesis has taught me to have more faith in myself, and I appreciate that you also gave the impression of having faith in me.

I really need to express my gratitude to Anne Oldeide Hay and Frederik André Hansen for leading me on the right track after visiting you at the University of Oslo. I particularly cherish Anne for the introduction to this field and for answering all my weird questions at any time. You really inspired me!

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Last, but not least, I must thank my family and friends. You know who you are, and I sincerely appreciate all the late night conversations, hugs, phone calls and comforting words in times of need. Thank you so much for believing in me and showing your support throughout the past year.

Table of contents

Abbreviations

6MC:Thy	6-Methylcoumarin:Thymol
ACN	Acetonitrile
bDMARD	Biological disease modifying antirheumatic drugs
CEDIA	Cloned enzyme donor immunoassay
Cmax	Maximum blood concentration
CsA	Cyclosporine A
csDMARD	Conventional synthetic disease modifying antirheumatic drugs
DEHP	Di(2-ethylhexyl) phosphate
DES	Deep eutectic solvents
DMARD	Disease modifying antirheumatic drugs
EMA	European Medicines Agency
EME	Electromembrane extraction
FA	Formic acid
GC	Gas chromatography
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HPLC	High performance liquid chromatography
HPLC-UV	High performance liquid chromatography-ultraviolet detection
IC	Ionic carrier
IL-2	Interleukin-2
JAK	Janus Kinase
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LPME	Liquid-phase microextraction
MeOH	Methanol
MQ	Milli-Q water
MS	Mass spectrometry
MTX	Methotrexate
NHAc	Ammonium acetate
NPOE	2-Nitrophenyl octyl ether
NPPE	2-Nitrophenyl pentyl ether

PP	Polypropylene
PPT	Protein precipitation
PRAC	Pharmacovigilance Risk Assessment Committee
RA	Rheumatoid arthritis
RPM	Revolutions per minute
RSD	Relative standard deviation
SD	Standard deviation
SLM	Supported liquid membrane
SPC	Summary of product characteristics
SPE	Solid-phase extraction
TDM	Therapeutic drug monitoring
TFB	Tofacitinib
TFBc	Tofacitinib citrate
tsDMARD	Targeted synthetic disease modifying antirheumatic drugs

1 Introduction

1.1 Immunosuppressants

An autoimmune disease is caused by the immune system attacking normal cells or tissues in the body. It is estimated that 0.24 to 1% of the population in the developed world are suffering from the autoimmune disease rheumatoid arthritis (RA) alone (1). Over the years, numerous immunosuppressants have been developed to treat patients with autoimmune diseases and for use after organ transplantation. As described by the name, these drugs suppress or inhibit immune responses, and are grouped based on their mechanism or site of action. The majority are so called disease modifying antirheumatic drugs (DMARDs). These drugs are initiated early in patients with RA. Since DMARDs are used as treatment for several different conditions, there is an overall high consumption of them, some cases demanding therapeutic drug monitoring (TDM), i.e. serum drug measurements to ensure therapeutic effect and prevent toxic side effects.

1.1-1 Cyclosporine A

One of the first immunosuppressants, Cyclosporine A (CsA), revolutionized organ transplantation in the early 1980s (2) and is still important in the treatment of various diseases. Lifelong treatment with immunosuppressants is required after organ transplantation to prevent rejection of the new organ. CsA is a calcineurin inhibitor, preventing T-cell activation by blocking the signal transduction that stimulates the transcription of cytokines (3). Cytokines, like interleukin-2 (IL-2), are essential in the inflammatory response by transferring information between cells. CsA belong to the group termed conventional synthetic DMARDs (4). Calcineurin facilitates dephosphorylation of a nuclear factor in activated T-cells, generating the activation of genes encoding cytokines. IL-2 is a signal molecule that activates the T-cell cycle, and with lack of such cytokines, the activation, and immune response, is impaired.

CsA can be administered intravenously or orally, but absorption after oral administration varies between individuals, and bioavailability is usually in the range of 20-50% (5). There are several factors contributing to this wide gap in bioavailability (2). For instance,

interactions with other substances (drugs or nutrients) can interfere with the pharmacokinetics of CsA, and lead to a higher risk of adverse. This is partially due to CsA being a substrate of CYP3A and P-glycoprotein, expressed in the liver and gastrointestinal tractus (3). Substances that inhibit or stimulate these will affect the plasma concentration of CsA, as the main result of drug-drug or nutrient-drug interaction. CsA is a lipophilic compound, and its metabolites are mainly excreted in the bile (2). In addition, CsA has a narrow therapeutic range of 75-400 µg/mL (6). As a result, it is difficult to predict the patient's response to CsA and their optimal dosage.

After solid organ transplantation, the initial oral dose of CsA is quite high (10-15 mg/kg daily), and then slowly reduced to a lower maintenance dose (2-6 mg/kg daily). In the treatment of autoimmune diseases, doses are in the lower range. (5) One of the main concerns when treating a patient with CsA is the risk of nephrotoxicity, followed by renal failure. Other commonly adverse effects are hypertension, hyperlipidemia, tremors and hirsutism. These are usually dose related and can be prevented by adjusting the dose based on monitoring the blood concentration of CsA. The patients should also be evaluated in terms of renal and liver function, blood pressure, blood status and other current medications prior to initiation of treatment. This is to ensure that the patient is suited to be treated with CsA. Frequent follow-up appointments with an experienced doctor in this field is also recommended.

1.1-2 Tofacitinib

Tofacitinib (TFB) is a relatively new drug approved by the European Medicines Agency (EMA), receiving marketing authorization March 2017 in Europe (7). It is a targeted synthetic DMARD used to treat patients with RA, among other autoimmune diseases, if prior DMARDs have given inadequate effect or are not tolerated. TFB inhibits intracellular Janus kinase (JAK) enzymes, which are involved in the downstream signaling process of cytokines (3). Proteins that activate gene transcription are impaired due to lack of phosphorylation by JAK. Consequently, the inflammatory response is compromised (8).

TFB can be administered both orally and intravenously, and shows rapid absorption after oral administration, with a bioavailability of 74% (7). Most of the drug (70%) is metabolized in the liver, primarily by the CYP3A4 enzyme, and to some extent by CYP2C19. TFB is a

relatively small, polar compound, and the remaining 30% is eliminated unchanged in the urine. As a result, both renal and hepatic impairment will reduce the clearance of TFB. In addition, interactions with inhibitors of CYP3A4 can increase the plasma concentration of TFB, and inducers of the enzyme can lead to treatment failure. Adjustment of the dose may be required if such interactions are expected (3).

In RA patients receiving TFB, the dosage is either 5 mg twice daily or an 11 mg modified release tablet once daily (7). One of the main concerns in treatment with TFB is the increased risk of major cardiovascular problems. EMA have started a safety review of JAK inhibitors due to this risk (9), which will be carried out by the Pharmacovigilance Risk Assessment Committee (PRAC). The drug is a subject of additional monitoring (▼), and earlier reviews have led to establishment of safety measures. Today, TFB is not recommended for patients with known cardiovascular disease, with high risk of thromboembolic diseases, or over 65 years of age (7). Other adverse effects are risk of infection, hypertension and anemia. Consequently, hemoglobin is monitored before and during treatment, and with a value less than 9 g/dL, the treatment should not be initiated. As elaborated below, unlike CsA, TDM is not established for TFB, and the analysis is not available in Norway at present (10, 11).

1.2 Therapeutic drug monitoring

Individual differences in patients' pharmacodynamic and pharmacokinetic aspects can impact the response of medicinal therapy (12). In order to obtain the desired therapeutic effect and reduce the risk of adverse effects, the dose can be individually adjusted in accordance with the serum concentration of the respective drug. This practice is known as TDM. Four criteria for a drug to be suited for TDM is listed below (12):

1. The correlation between drug concentration and therapeutic response is identified.
2. The therapeutic range is narrow, which represents the concentration window between therapeutic and toxic effects. This may put a patient at risk for failure of treatment or irreversible adverse effects.
3. The connection between drug concentration and dose is related to pharmacokinetic variability between patients, or risk of poor compliance.

4. The pharmacological response cannot be explained by other factors, e.g. blood glucose, or is difficult to separate from adverse events.

1.2-1 Therapeutic drug monitoring in patients receiving Cyclosporine A

CsA fulfills all the criteria listed above and have been an object of TDM for over 20 years. As already mentioned, CsA has a narrow therapeutic range and is frequently affected by interactions, in addition to the individual differences between patients. It is therefore necessary to monitor the blood concentration of CsA to establish the appropriate dose for the patient and avoid adverse effects or organ rejection.

In Norway, TDM of CsA is practiced at many hospitals, but with different analytical methods (13). Helse Bergen performs a method termed cloned enzyme donor immunoassay (CEDIA) CsA PLUS (6). In an email correspondence with Gro Helen Dale from Haukeland University Hospital (May 2022), I was informed that Helse Bergen analyzes approximately 130 patient samples with CsA each month. The patients' blood is drawn either predose (C0) or at 2 hours postdose (C2). The C0 sample is taken 12 or 24 hours after the last administered dose, depending on the dosing regimen, thus right before the next dose. The C2 sample reflects the maximum blood concentration (Cmax) of CsA after an oral dose. The level of Cmax is correlated to the incidence of organ rejection (12), and is therefore an important predictor of therapeutic failure, hence the latter sample collection is preferred.

The CEDIA CsA PLUS assay determines the CsA concentration in human whole blood by using recombinant DNA technology (14). Prior to analysis, the specimens (EDTA whole blood) are mixed with a lysing reagent to deplete the samples from red blood cells. Two reagents are then added to the samples; one contains mouse monoclonal anti-CsA antibodies and an enzyme acceptor, the other one contains an enzyme donor conjugated to CsA and a β-galactosidase substrate with chlorophenol red. CsA from the hemolyzed patient sample will compete with the conjugated CsA for the antibody binding site. An active enzyme (β-galactosidase) is formed by the two enzyme fragments if CsA from the hemolysate sample binds, leading to cleavage of the substrate that generates a color change. This is shown in Figure 1. The absorbance is measured spectrophotometrically at 570/660 nm, and is directly

proportional to the amount of CsA present in the sample. No active enzyme is formed in the absence of CsA in the sample.

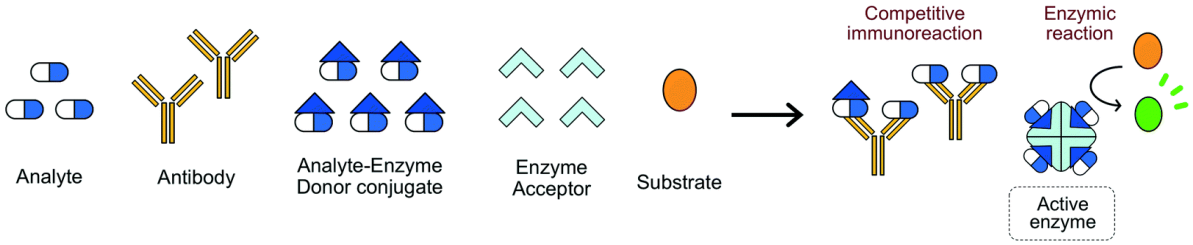


Figure 1. Principle of CEDIA with CsA as the analyte. Adapted and modified from (15).

The measurement range for the «Low Assay» is 25-450 ng/mL and the «High Assay» has a reportable range of 450-2000 ng/mL (14). Higher concentrations demand dilution prior to analysis. The method displays linearity within the measurement range (16), and the recovery is ±10% for concentrations above 150 ng/mL, but ±15 ng/mL for concentrations below this value (14). The assay is relatively fast and have the capacity of more than 400 samples an hour. The therapeutic concentration range of CsA is 75-400 ng/mL (6) so, the low range assay is satisfactory for routine TDM. Despite these advantages, the method has certain limitations in addition to the deviation of the recovery. Factors that may cause low quantitation are high levels of protein, cholesterol, triglycerides and hematocrit (14), i.e. the blood volume ratio of red blood cells.

1.2-2 Therapeutic drug monitoring in patients receiving Tofacitinib

TFB is currently not an object of TDM (10), despite the severe side effects sometimes observed in patients treated with this drug, the potential for interactions and interindividual variation of pharmacokinetics. In a phase II study in patients with active RA treated with methotrexate (MTX) (17), TFB doses ≥ 3 mg twice daily resulted in significant improvement compared to placebo. A similar study (18) showed that TFB 1 mg twice a day was sufficient to achieve improvement. There were 140 and 509 patients participating in these studies respectively, not fully representative for the individual variation of a population. As mentioned, the recommended dosage of TFB in treatment of patients with RA is 5 mg twice daily, and no dose adjustment is required in combination with MTX (7). However, in clinical studies on RA, there was a higher incidence of adverse effects in combination with MTX compared to monotherapy of TFB. This may be explained by the side effect profile of MTX.

Based on the criteria for TDM and individual factors between patients, e.g. age, gender, race, weight, organ function: can we expect that the same dosage will give satisfactory therapeutic response and few adverse effects in RA patients? A series of studies to establish a connection between the serum concentration of TFB and therapeutic response may be necessary to answer this question. To be able to execute such studies, an effective and accurate analysis method is required.

1.3 Sample preparation

Bioanalysis is performed in different fields, but usually with the same aim: To identify and quantify drug substances (19). The purpose varies from TDM and clinical testing to drug abuse in sport or criminal cases. Prior to detection of an analyte's presence in a biological sample, the analyte is commonly isolated through the process of sample preparation. The three main goals of sample preparation are listed below (20):

1. Remove interfering substances.
2. Reduce the impairment of the detector (i.e. accuracy, response, selectivity).
3. Improve the sensitivity of the analysis method by pre-concentrating the target analytes (i.e. extraction into a smaller volume).

Examples of biological samples are whole blood, plasma, serum, urine and saliva (19). Substances that can interfere with the analysis are components in the biological sample, e.g. organic compounds, proteins and salts (20). This interference is termed the matrix effect (21). Without sufficient sample preparation, injection into an analytical instrument can cause contamination and unreliable measurements (19). In order to optimize bioanalysis in means of precision, time consumed, economic and environmental factors, the development of additional sample preparation methods are called for (20).

The analysis method of choice for bioanalysis is often liquid chromatography (LC) or gas chromatography (GC) combined with mass spectrometry (MS) due to the high selectivity and sensitivity of these methods (20). In this study, high performance liquid chromatography-ultraviolet detection (HPLC-UV) have been used for practical reasons. Nevertheless, matrix

effect is a serious drawback that affects the performance of these analysis methods, but the degree of influence greatly depends on the sample preparation technique (21).

The most frequently used sample preparation techniques are protein precipitation (PPT), solid-phase extraction (SPE) and liquid-liquid extraction (LLE) (19). These techniques have a common denominator: they require large volumes of organic solvents (22). PPT removes the proteins in the sample by adding a precipitant followed by centrifugation. This is a fast and simple procedure, but the sample is diluted, and a subsequent filtration step is usually required. In SPE, the analyte adsorbs to a solid phase while other components are washed away. The analyte is next eluted in an appropriate liquid. LLE is based on two immiscible solvents, where the analyte is soluble in only one of them. It often requires repeated extraction steps, which is time-consuming. In addition, the process can lead to inconsistent recovery due to emulsion and inadequate phase separation. To reduce the large consumption of organic solvents, research of liquid-phase microextraction (LPME) techniques have been a very active field over the last decades (20).

1.4 Development of electromembrane extraction

Electromembrane extraction (EME), an LPME sample preparation technique, have been extensively explored since its introduction in 2006 (20). Unlike traditional LLE, where the analyte is extracted by diffusion, mass transfer in EME is facilitated by electrokinetic migration. The process involves a three-phase system consisting of two aqueous solutions separated by an organic supported liquid membrane (SLM) (23). The aqueous donor (sample) and acceptor solutions are coupled to an external power supply with an electrode placed in each solution (20). The positively charged electrode (anode) is in the donor solution and the negatively charged electrode (cathode) is in the acceptor solution for extraction of basic analytes. When extracting acidic analytes, the electrical field is reversed. For EME to be efficient, the target analyte has to be ionized and carry a charge (23). This is accomplished by adjusting the pH in the aqueous solutions. A more detailed description of EME will be covered in section 2.1.

EME provides several advantages in relation to sample preparation of biological fluids (24). As mentioned, EME is a microextraction method and the volumes of solutions are low. If the

acceptor volume is significantly lower than the donor volume, it will result in an enrichment (pre-concentration) of the analyte in the acceptor solution. The volume of organic solvent required for the SLM is just a few microliters per sample, which is relevant in terms of a «green chemistry» approach (25). EME results in excellent sample clean-up by efficiently eliminating matrix components, and high selectively is generally achieved, even with a short extraction time (24). This is due to the effect of the SLM and electric field, but the extraction conditions must be fine-tuned. Unlike traditional LLE, the aqueous acceptor solution containing the analyte is directly compatible with injection into LC instruments. Further sample preparation steps are consequently not necessary. In addition, the EME units are cheap and can be disposed after a single extraction.

1.5 Aim of the study

Several immunosuppressive drugs require routine TDM to ensure safety of patient treatment. Bioanalytical methods that are precise, have high throughput and low costs are called for (20), and research to optimize and fulfill such requirements are the aim of this study.

In this study, EME will be used in an attempt to establish a new method of sample preparation technique that reduces the impact of matrix components seen in the CEDIA method, presently used at Haukeland University Hospital. CsA is a highly hydrophobic, acidic analyte (26), and the investigation of optimal conditions for this exact analyte can also contribute to further application of EME for the extraction of other acidic analytes.

TFB is a relatively polar and basic analyte (27), and EME protocols for analytes with such chemical characteristics have already proven to be efficient (28). This study will be an attempt to establish a sample preparation method for the detection of TFB from plasma, with the long-term aim of providing an effective and accurate method facilitating the identification of correlation between drug concentration and therapeutic response.

For both drugs, I will do a systematic approach, where different parameters in EME will be changed to find the optimal extraction conditions. Finally, the best methods will be evaluated in human plasma spiked with either CsA of TFB.

2 Theory

2.1 Electromembrane extraction

Electromembrane extraction (EME) is a microextraction technique that extract ionized compounds from an aqueous donor solution across a supported liquid membrane (SLM) and into a clean aqueous acceptor solution (24). The extraction is facilitated by an electric field established by the application of an external power supply. Electrodes are coupled to both the acceptor and donor solution, and ionized analytes will move towards the electrode with opposite charge. This is termed electrokinetic migration. The direction and magnitude of the electrical field can be used to manipulate the extraction selectivity (29). When extracting cations, the negatively charged cathode is in the acceptor solution and the anode is in the donor solution. An illustration of EME for basic compounds is presented in Figure 2. Basic compounds are usually ionized in acidic conditions due to protonation, and a low pH would preserve the positive charge on the analyte. For extraction of anions, this is reversed. The composition of the SLM is critical to form interactions with the target analyte, which will affect the efficiency of the extraction. To increase the possibility of interaction, a sufficient contact between the aqueous solutions and SLM is crucial. This is achieved by agitation.

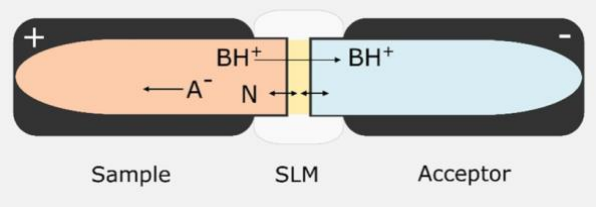


Figure 2. Principle of electrokinetic migration for protonated basic analytes. Adapted from (25).

As mentioned, the pH of the aqueous solutions must ensure ionization of the analyte, otherwise it would not be affected by the electrical field. The solubility of analytes usually increases with an increased charge, which can cause problems in concern of interactions with the SLM. On the other hand, the pH in the acceptor solution should be 3-4 units below the pKa-value of an basic analyte (23) to increase the release from the SLM and caption the analyte in the acceptor.

2.1-1 The electrical double layer

Due to the application of an electric field, there is expected that a charge will form up in the SLM and generate an electrical layer at each side of the membrane, with the opposite charge of that in the aqueous solution (23). The positive charge will accumulate towards the cathode and the negative charge will accumulate in the direction of the anode. This forms a double electric layer, a capacitor, which affects the pH in the boundary layer between the aqueous solutions and the SLM. This has a major impact on performance and mass transfer, since the pH can affect the ionization of the analyte. The formation of this double layer is thought to be established within the first two minutes of extraction, which can be observed by a drastic decrease in current. This is associated to an increase of resistance in the system, possibly due to the formation of the electrical double layer. The relation between current (I), voltage (U) and resistance (R) is described by Ohm's law:

$$U = R \times I \qquad \text{(Equation 1)}$$

2.1-2 General requirements for the supported liquid membrane

When choosing the SLM, some physiochemical characteristics should be taken under consideration (24), e.g. the solubility in water, viscosity, vapor pressure, conductivity and purity. These characteristics will impact the efficiency of the extraction and sample clean-up. A low solubility in water will reduce the leakage of SLM into the aqueous solutions and the viscosity should be low to maintain high permeability of target analytes. The typical volume of organic solution is 5-25 µL (29), and evaporative loss would impact the extraction. The choice of SLM is also depending on the chemical characteristics of the target analyte.

2.1-3 Supported liquid membrane for basic analytes

Upon 2018, 2-Nitrophenyl octyl ether (NPOE) (Figure 3a) was the most commonly used SLM for extraction of basic analytes (30), but in the study by Drouin et al, 2-Nitrophenyl pentyl ether (NPPE) (Figure 3b) appeared to be more efficient by a high extraction current strategy. Compared to NPOE, NPPE carry a shorter alkyl chain of five hydrogenated carbons instead

of eight. A compound with less carbon atoms is accompanied by a lower log P value, that is 3.5 for NPPE, opposed to 4.9 for NPOE (31, 32). Both compounds involve a nitrogen dioxide group and an ether group. The three oxygen atoms in these functional groups provide hydrogen bond acceptor (HBA) sites capable of hydrogen bond interactions.

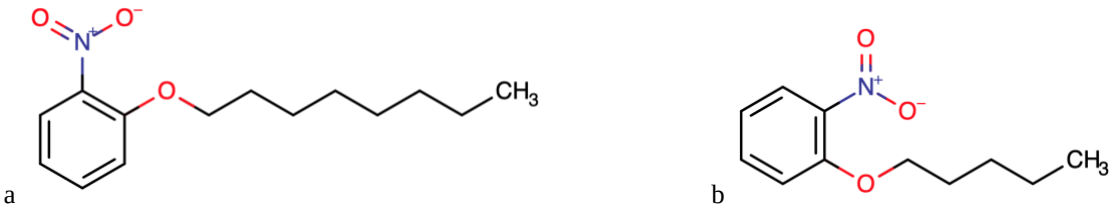


Figure 3. The chemical structures of (a) NPOE and (b) NPPE. Retrieved from Chemicalize (31, 32).

Recent studies of deep eutectic solvents (DES) have proven to be efficient for extraction of polar bases from plasma (28, 33). DES are usually composed of two solid components that form hydrogen bonds with each other when mixed (34). This results in a depression of the melting point and a liquid is formed. Mixtures of coumarin and thymol (Thy), appear to be the first SLM that is efficient for both non-polar bases and acids. Dipole and π-type interactions are dominating for the mass transfer of bases. For more hydrophobic analytes, a substitution of coumarin with 6-methylcoumarin (6MC) could possibly yield more efficient extractions. The addition of the methyl group increases the log P from 2.2 for coumarin, to 2.7 for 6MC (35, 36). The structures of these compounds are presented in Figure 4. No articles, of my knowledge, have yet been published with an SLM of 6MC and Thy, but it is thought to be suitable for both basic and acidic analytes.

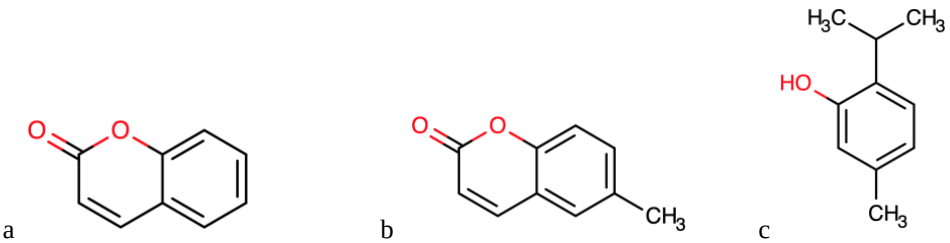


Figure 4. The chemical structures of (a) coumarin, (b) 6-MC and (c) Thy. Retrieved from Chemicalize (35-37).

2.1-4 Supported liquid membrane for acidic analytes

The selection of SLMs for acidic analytes is limited, even though there have been performed several studies with extraction of acidic compounds (24). To form hydrogen bond interactions with acidic analytes, the SLM should have strong hydrogen bond acidity. Higher alcohols,

such as 1-octanol (Figure 5) have this ability, and is probably the most frequently used SLM for extraction of acidic analytes.

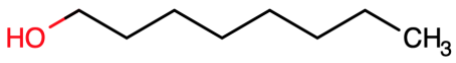


Figure 5. The chemical structure of 1-octanol. Retrieved from Chemicalize (38).

Newer research have shown that DES can yield near-exhaustive extractions of non-polar acidic compounds (34). Hydrogen bond interactions are thought to be the dominating interaction for acids, and DES have strong hydrogen bond donating (HBD) properties.

2.1-5 Ionic carriers

The extraction of highly polar compounds is very difficult, due to the high solubility in aqueous media and limited partitioning into the SLM (39). The addition of an ionic carrier (IC) is the best known strategy to improve mass transfer. Di(2-ethylhexyl) phosphate (DEHP) is the most used anionic IC for extraction of polar basic analytes. The structure is viewed in Figure 6. The negatively charged phosphate group is assumed to attach to protonated bases at the membrane interface. DEHP have a log P of 1.94 (40) and the fraction of ionized compound increases with increased pH. This results in a higher water solubility, and DEHP is leaked into the aqueous solutions with pH above 4 (39). This transfer the ion-pair formation between DEHP an analyte to the bulk of the sample solution, which provided higher analyte mass transfer for polar basic compounds with log P less than 0.7 (39)

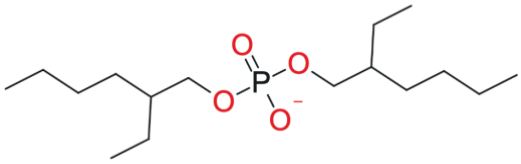


Figure 6. The chemical structure of DEHP. Retrieved from Chemicalize (40).

2.2 Chemical properties of an analyte

The theoretical information known of an analyte's chemical properties is fundamental to choosing the most optimal conditions for EME. This provides basic information regarding the most favorable pH for ionization of the analyte in the aqueous solutions, as well as the composition of the SLM, in concern of organic solvents and potential ionic carriers. To yield adequate recovery rates of an analyte, the conditions must be adjusted accordingly.

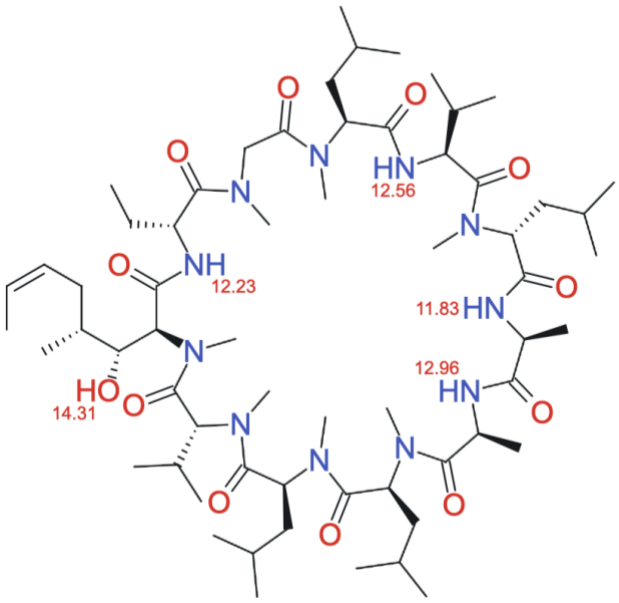
In this study, the chemical properties were collected from the online platform Chemicalize, an application by ChemAxon Ltd. This platform provides calculations and predictions based on the structure of a molecule, e.g. pKa, ionization, solubility and log P. In the past, chemical properties had to be calculated by performing a line of experiments, which was both time consuming and difficult if the molecule had several ionizable groups. Consequently, an application like this is a valuable tool. Although the predictions may slightly deviate from what is found experimentally, they can be used to guide the choice of aqueous solutions and SLM.

The analytes CsA and TFB are quite different regarding their chemical properties. CsA is an acidic and non-polar compound with considerable challenges when it comes to establishing both an EME protocol and analysis method with HPLC. TFB on the other hand, can be imposed to be either an acid or base, and is considered a relatively polar compound. The chemical properties of the analytes will be presented in more detail in the following chapters.

2.2-1 Chemical properties of cyclosporine A

The main chemical properties of CsA, such as the chemical formula, molecular weight, log P and structure are listed in Table 1. The compound is a cyclic peptide consisting of eleven amino acids with mainly hydrophobic side chains.

Table 1. Chemical properties of Cyclosporine A. Retrieved from Chemicalize (26).

Chemical formula	$C_{62}H_{111}N_{11}O_{12}$
Molecular weight	1202.635 g/mol
Log P	3.638
Chemical structure[1] with predicted pKa-values[2]	

[1] The chemical structure is made with a drawing program called Marvin Pro available from Chemicalize (41).

[2] The red numbers are predicted pKa-values for ionization at the given location. It indicates deprotonation of an acid, resulting in a negative charge. Multiple charges can appear simultaneously, depending on the pH.

CsA has five locations for deprotonation, depicted in the figure. The lowest predicted acidic pKa-value is 11.83. As the pH increases beyond 11.83, the fraction of CsA as an anion will increase by loss of hydrogen at multiple locations. This results in a negative sum of charge that continues to decrease with increasing pH. Figure 7 displays the same characteristics by distribution of the different ions in percentage. The blue curve represents the hydrophobic, electrical neutral compound, which is dominating from pH 0-10. At pH 12, 72.45% of the molecules are in a negatively charged form, with a total charge below -1.7. The brown slope that dominates above pH 12.4 is deprotonated at 4 locations, giving a charge of -4.

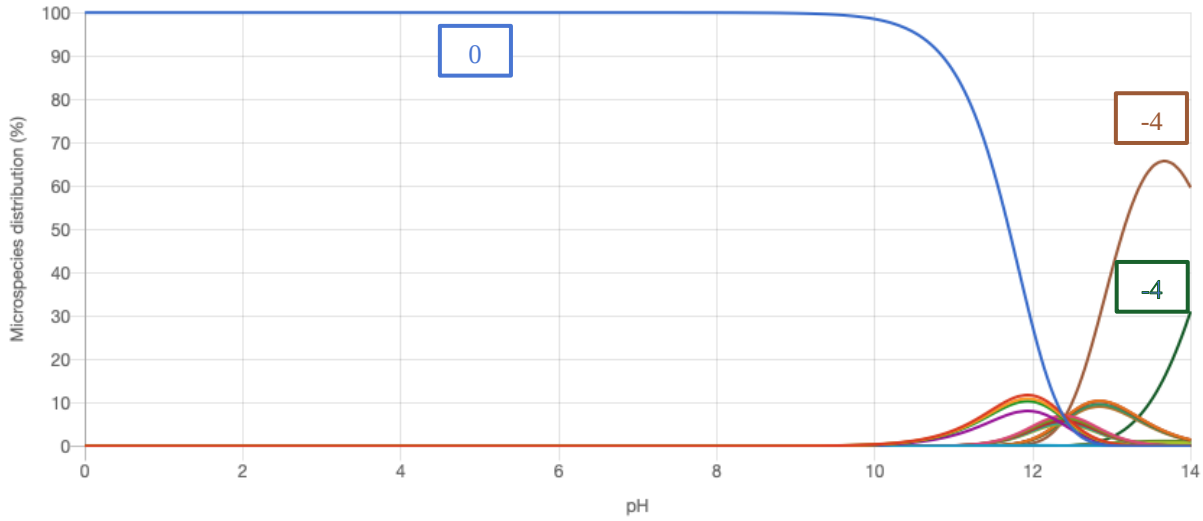


Figure 7. Predicted distribution (%) of CsA at pH 0-14. Retrieved from (26).

The lack of polar groups as side chains in the amino acids contributes to the chemical properties discussed above and emanates a predicted log P-value of 3.638. Log P represents the distribution of the electrical neutral compound in a two-phase system consisting of water and octanol, while log D represent the distribution of both the neutral compound and its ionized forms at any given pH (26). Figure 8 shows that the log D-value decreases with increasing pH, due to the enlarged negative charge, resulting in a more polar compound. This is favorable with regard to EME, but the conditions acquired are still strongly alkaline.

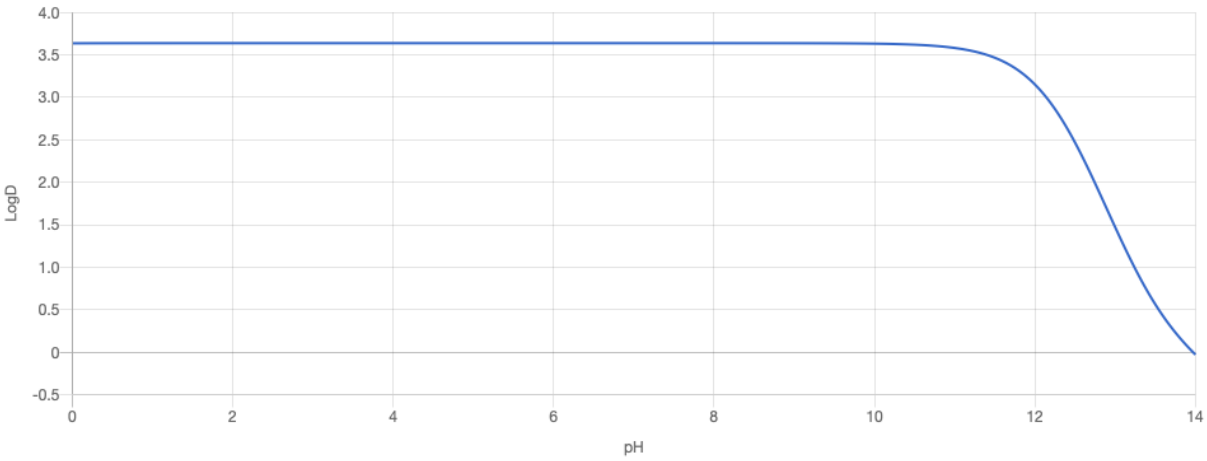


Figure 8. Log D of CsA at pH 0-14. Retrieved from (26).

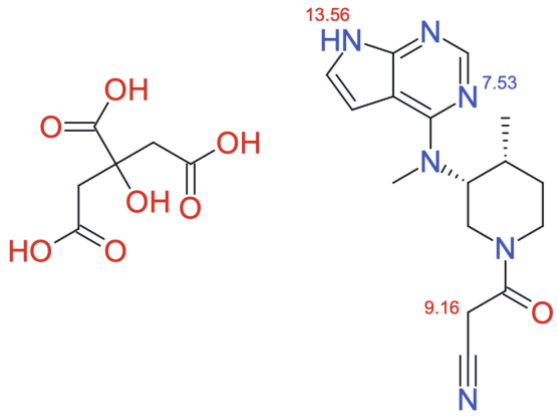
CsA has a predicted solubility of 28.6 µg/mL in water (42), which is very low and may cause complications with EME with regard to the use of aqueous solutions. As discussed above, the

polarity of the compound is strengthened with increasing pH above 12. Nevertheless, it would be favorable to operate with standard solutions of CsA at concentration less than 25 µg/mL to ensure that the molecules are dissolved at any given pH.

2.2-2 Chemical properties of tofacitinib

The main chemical properties of TFB, such as the chemical formula, molecular weight, log P and structure are listed in Table 2. This compound has an aromatic ring system, called pyrrolopyrimidine, with both acidic and basic character on two different nitrogen atoms. In addition, there is a carbon atom with acidic characteristics located between the ketone and nitrile group. Consequently, this analyte has three different sites for ionization. Depending on the pH-value, TFB will exist as a mixture of the neutral and charged forms. In this study, solutions were prepared from powder of tofacitinib citrate (TFBc), but the chemical properties are presumed not to be inflicted by the present of citrate.

Table 2. Chemical properties of Tofacitinib and Tofacitinib citrate. Retrieved from (27, 43) .

	Tofacitinib (TFB)	**Tofacitinib citrate (TFBc)**
Chemical formula	$C_{16}H_{20}N_6O$	$C_{16}H_{20}N_6O \cdot C_6H_8O_7$
Molecular weight	312.377 g/mol	504.49 g/mol
Log P	1.088	
Chemical structure[1] with predicted pKa-values[2]		

[1] The chemical structure is made with a drawing program called Marvin Pro available from Chemicalize (41).

[2] The numbers are predicted pKa-values for ionization at the given location. The blue number indicates ionization by a base being protonated (cation) and red numbers indicates ionization of an acid being deprotonated (anion). Ionization occurs at different pH; opposite and double charges can appear simultaneously.

The predicted pKa-value for the base is 7.53 (blue number in the structure), located at the proximal nitrogen atom in the pyrimidine ring. With decreasing pH-level below 7.53, the amount of protonated TFB will increase, as described by the red curve in Figure 9. The two acidic sites have predicted pKa-values of 9.16 at the carbon and 13.56 at the nitrogen in the pyrrole ring. This is due to deprotonation of TFB with increasing pH.

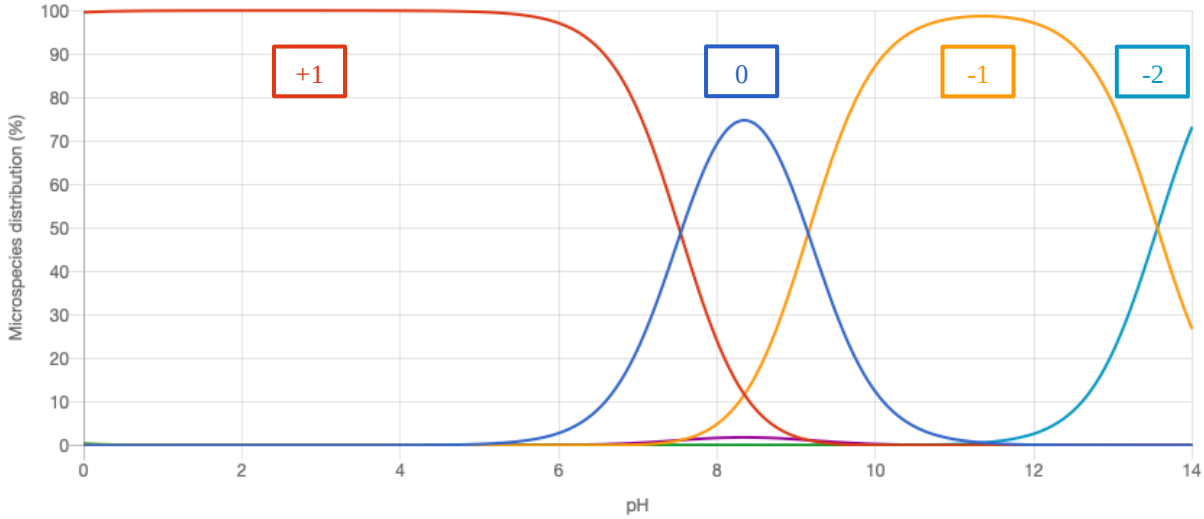


Figure 9. Predicted distribution (%) of TFB at pH 0-14. Retrieved from (43).

The different colors in Figure 9 reflects the fractional amount of a particular ionized form of TFB at different pH-levels. The dark blue slope represents the neutral compound, which dominates at pH 8.3 with 74.67%. At the physiological pH of 7.4, 56.91% of the TFB molecules are estimated to carry a net positive charge of +1. At pH 5 and lower, approximately 100% are protonated with one positive charge, as illustrated by the red curve. The yellow slope represents one negative charge on the carbon atom, and the light blue slope represents deprotonation at both acidic locations, giving a charge of -2. The isoelectric point, where the net sum of charge is zero, is at pH 8.8. The TFB molecules carry a net positive charge below this pH and negative charge above (43).

The log D as a function of pH is presented by Figure 10. TFB is a rather small compound with five hydrogen bond acceptor sites, making it relatively polar with an estimated log P of 1.088 (27). When put in context with the ionization of the molecules, this can lead to difficulties in concern of EME. A charge on the analyte is necessary for it to be affected by the electrical field. In addition, the negative log D and polarity may reduce the partition of TFB into the hydrophobic SLM.

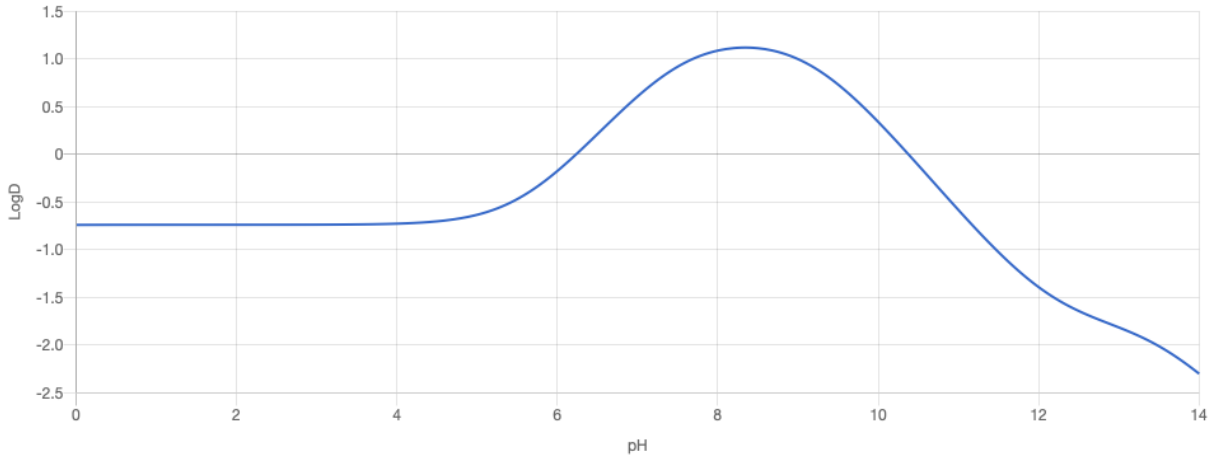


Figure 10. Log D of TFB at pH 0-14. Retrieved from (43).

The solubility of TFB in water is also explained by the degree of ionization and polarity, and is categorized as moderate, 120 µg/mL at pH 7.4 (27). By an increase of the fractional amount of charged molecules, as presented in Figure 9, the solubility of TFB greatly increases with a pH below 5 or above 11. In this study, the TFB concentration will not exceed 5 µg/mL, and the solubility will accordingly not be a concern.

3 Materials and methods

3.1 Chemicals

Table 3. List of chemicals, their purity and producer

Chemical	Purity	Producer
Ethanol, CH_3CH_2OH (EtOH)	Rectified	Antibac AS (Asker, Norway)
Dimethyl sulfoxide (DMSO), C_2H_6OS	≥ 99.5%	Honeywell (Muskegon, MI, USA)
Ortho-phosphoric acid, H_3PO_4	85%	Merck KGaA (Darmstadt, Germany)
Thymol (Thy), $C_{10}H_{14}O$	≥ 98.5%	Merck KGaA (Darmstadt, Germany)
1-Octanol, $C_8H_{18}O$	> 99%	Merck KGaA (Darmstadt, Germany)
Acetonitrile (ACN), CH_3CN	Gradient grade for liquid chromatography	Merck KGaA (Darmstadt, Germany)
Acetonitrile, CH_3CN	Hypergrade for LC-MS	Merck KGaA (Darmstadt, Germany)
Ammonium acetate (NHAc), CH_3COONH_4	> 98%	Merck KGaA (Darmstadt, Germany)
Di-potassium hydrogen phosphate trihydrate, $K_2HPO4 \cdot 3H_2O$	> 99%	Merck KGaA (Darmstadt, Germany)
Methanol (MeOH), CH_3OH	Hypergrade for LC-MS	Merck KGaA (Darmstadt, Germany)
Potassium dihydrogen phosphate, KH_2PO_4	> 99.5%	Merck KGaA (Darmstadt, Germany)
Deionized water (MQ)	Milli-Q quality	Millipore (Burlington, MA, USA)
Sodium hydroxide, NaOH	1 M stock solution	Obtained from the lab
2-Nitrophenyl octyl ether (NPOE), $C_{14}H_{21}NO_3$	> 99%	Sigma-Aldrich (Steinheim, Germany)

2-Nitrophenyl pentyl ether (NPPE), $C_{11}H_{15}NO_3$	> 99%	Sigma-Aldrich (Steinheim, Germany)
6-Methylcoumarin (6MC), $C_{10}H_8O_2$	> 99%	Sigma-Aldrich (Steinheim, Germany)
Bis(2-ethylhexyl) phosphate (DEHP), $C_{16}H_{35}PO_4$	97%	Sigma-Aldrich (Steinheim, Germany)
Formic acid (FA), CH_2O_2	> 95%	Sigma-Aldrich (Steinheim, Germany)
Hydrochloric acid, HCl	37%	Sigma-Aldrich (Steinheim, Germany)
Methanol (MeOH), CH_3OH	> 99.9%	Sigma-Aldrich (Steinheim, Germany)
Sodium hydroxide, NaOH	> 97%	Sigma-Aldrich (Steinheim, Germany)
Trifluoroacetic acid (TFA), $C_2HF_3O_2$	> 99%	Sigma-Aldrich (Steinheim, Germany)

Table 4. Analytes for extraction with EME

Analyte	**Formulation**	**Producer**
Cyclosporine A (CsA)	25 mg white powder of crystals, purity ≥ 98.5% (HPLC)	Sigma-Aldrich (St. Louis, MO, USA)
Tofacitinib citrate (TFBc)	25 mg white powder purity > 98% (HPLC)	Sigma-Aldrich (St. Louis, MO, USA)

3.2 Solutions

Table 5. Preparation of solutions used in experiments with EME

Solution	Preparation
10 mM HCl, pH 2.0	While continually measuring the pH, a 37% HCl solution was gradually added to MQ until a pH of 2.0.
10 mM NaOH, pH 12.0	While continually measuring the pH, a 1 M NaOH solution was gradually added to MQ until a pH of 12.0.
100 mM formic acid, pH 2.4 **(44)**	795 µL FA was added to 180 mL MQ. The pH was adjusted to 2.4 with 37% HCl. The final volume of the solution was then adjusted to 200 mL with MQ.
40 mM ammonium acetate, pH 4.0	308.3 mg NHAc was dissolved in 80 mL MQ. The pH was adjusted to 4.0 with 37% HCl. The final volume of the solution was then adjusted to 100 mL with MQ.
40 mM potassium phosphate buffer, pH 7.4 **(45)**	1269.6 mg $K_2HPO_4 \cdot 3H_2O$ was dissolved in 160 mL MQ. Adjusted the pH to 7.4 with a 1 M NaOH solution. The final volume was adjusted to 200 mL by adding MQ.
5 M phosphoric acid **(44)**	3421 µL 85% phosphoric acid was slowly added to 2.5 mL MQ. The final volume was then adjusted to 10 mL with MQ.
Stock solution of CsA, 1 mg/mL in MeOH	1.0 mg of the analyte CsA was dissolved in 1000 µL of methanol.
Donor solution of CsA, 20 µg/mL in a 10 mM NaOH solution, pH 12	60 µL of the 1 mg/mL CsA stock solution was added to 2940 µL of 10 mM NaOH solution with pH 12, generating a 3 mL donor solution.
Stock solution TFBc, 1 mg/mL in DMSO	1.0 mg of the analyte TFBc was dissolved in 1000 µL of DMSO.
Stock solution TFBc, 10.5 ug/mL in a 100 mM FA solution, pH 2.4	210.5 µL of 1 mg/mL TFBc stock solution was added to a 20 mL solution of 100 mM FA with the pH 2.4.

Donor solution of 5 µg/mL TFBc	100 µL of 1 mg/mL TFBc stock solution was added to the desired pH-solution with a final volume of 20 mL, resulting in donor solutions with pH 2.4, 4.0 and 7.4.
Donor solution, diluted plasma spiked with 5 µg/mL TFBc	2000 µL plasma and 100 µL 5 M phosphoric acid was mixed to break the buffer capacity of the plasma. 1900 µL of 10.5 µg/mL TFBc stock solution in 100 mM formic acid pH 2.4 was then added to the solution. The mixture was left for at least 10 minutes to ensure equilibrium of protein binding. The pH in the solution was measured to be 2.1.
SLM of 6-methylcoumarin and thymol (6MC:Thy)	Due to the chemicals solid state, they were measured by weight. 200 mg of 6MC and 400 mg of Thy were heated at 70˚C for 5 minutes with aggitation. The melted solution was then vortexed, making a 1:2 w/w mixture of 6MC and Thy.
SLM containing DEHP	The SLMs with DEHP were made by weighing, resulting in a w/w fraction of the ionic carrier. This was done with NPOE, NPPE and 6MC:Thy in various amounts, from 0.125 to 25% DEHP. Example of preparation: 20 mg DEHP was weighed and added to 380 mg of 6MC:Thy and then vortexed, resulting in a solution with 5% DEHP in 6MC:Thy.

Table 6. Preparation of solutions used in analysis with HPLC

Solution	**Preparation**
Mobile phase **A** (CsA): 2 mM ammonium acetate and 0.1 % formic acid in MQ	30.8 mg NHAc was dissolved in 180 mL MQ. 200 µL of formic acid was then added to the solution. The final volume of the solution was adjusted to 200 mL with MQ.

Mobile phase **B** (CsA): 2 mM ammonium acetate and 0.1 % formic acid in MeOH	77.1 mg NHAc was dissolved in 450 mL of MeOH. 500 µL of formic acid was then added to the solution. The final volume of the solution was adjusted to 500 mL with MeOH.
Wash solution for CsA-method: MeOH and MQ 80:20 (v/v)	For 200 mL washing solution, 160 mL MeOH and 40 mL MQ were mixed, and then degassed in an ultrasonic bath for 30 minutes.
Stock solution of CsA, 1 mg/mL in MeOH	1.0 mg of the analyte CsA was dissolved in 1000 µL of MeOH.
Standard solution of CsA, 100 µg/mL	300 µL of the 1 mg/mL CsA stock solution was diluted in a mixture of 2100 µL MeOH and 600 µL MQ, making a 3 mL solution of MeOH and MQ 80:20 (v/v).
Standard solutions of CsA, 1-50 µg/mL	The required amounts of the 100 µg/mL CsA standard solution were diluted with an 80:20 (v/v) mixture of MeOH and MQ to a total volume of 1000 µL.
Mobile phase **Iso** (TFB): 60% 10 mM NHAc, pH 5 40% ACN (v/v)	462.5 mg NHAc was dissolved in 550 mL of MQ. The pH was then adjusted to 5.0 with 37% HCl, and MQ was added to a total volume of 600 mL. 400 mL of ACN was then added to the solution.
Wash solution for TFB-method: MQ and ACN 60:40 (v/v)	For a 200 mL wash solution was 120 mL Milli-Q water and 80 mL acetonitrile mixed, and then degassed in an ultrasonic bath for 30 minutes.
Stock solution of TFBc, 100 µg/mL in MeOH	1.0 mg of the analyte TFBc was dissolved in 10.00 mL of MeOH.
Standard solution of TFBc, 10 µg/mL	500 µL of the 1 mg/mL TFBc stock solution was diluted in a mixture of 1500 µL ACN and 3000 µL MQ. Making a 5 mL solution of MQ and organic solvent with the ratio 60:40 (v/v).
Standard solutions of TFBc, 0.01-5 µg/mL	0.1-5 µg/mL standard solutions were made with the required amount of the 10 µL/mL TFBc standard solution and diluted with a 60:40 (v/v) mixture of MQ and ACN. 0.01-0.05 µg/mL were made from the 1 µg/mL TFBc standard solution in the same way.

3.3 Laboratory equipment

Table 7. General equipment in the laboratory

Equipment	Description	Producer
Ultrasonic cleaner	Bransonic® ultrasonic cleaner, 3510E-MT	Branson Ultrasonics (Danbury, CT, USA)
Concentrator/centrifuge	Concentrator plus	Eppendorf (Hamburg, Germany)
Thermoregulated mixer	Thermomixer Comfort	Eppendorf (Hamburg, Germany)
Magnetic stirrer	IKA® magnetic stirrer, big squid	IKA®-Werke (Staufen, Germany)
Balance	AG204 DeltaRange®	Mettler Toledo (Greifensee, Switzerland)
Milli-Q dispenser	Milli-Q® IQ Advantage A10 Water Purification System, Millipak® Express Filter, 0.22 µm	Millipore, Merck (Burlington, MA, USA)
pH meter	pH 6+ Meter	Oakton® Instruments (Vernon Hills, IL, USA)
Vials for different solutions	20 mL high performance glass vial with foil-lined urea screw cap	PerkinElmer® (Waltham, MA, USA)
Vortexer	Vortex-Genie 2, Model G560E	Scientific Industries (Bohemia, NY, USA)
Pipettes	Finnpipette® 0.5-10 µL, 5-50 µL, 20-200 µL, 200-1000 µL	Thermo Labsystems, Thermo Fisher Scientific (Waltham, MA, USA)
Pipette tips	10 µL, 200 µL, 1000 µL	VWR Corporate (Radnor, PA, USA)
Vials for standard solutions	1.5 mL HPLC vials with screw cap and septum	VWR Corporate (Radnor, PA, USA)

Table 8. Equipment and components used for EME

Equipment	Description	Producer
Agitator	DLAB MX-M agitator, Agitation in the range 0-1500 RPM	DLAB Science CO ltd. (China)
Multimeter	Fluke 289 multimeter	Fluke corporation (Everett WA, USA)
Power supply	DC power supply, model ES 0300-0.45	Delta Elektronika, (Zierikzee, The Netherlands)
Flat sheet membrane	Porus polypropylene (PP) Accurel® Flat, white sheet membrane	3M, Membrana (Wuppertal, Germany)
Sample holder	Room for 10 sample units, attached to the agitator	G&T Septech AS (Ski, Norway)
Top cover	Top cover with 10 pairs of electrodes	G&T Septech AS (Ski, Norway)
Union	White plastic, connection between two vials, keeps the SLM in place	G&T Septech AS (Ski, Norway)
Vials for EME	Black conductive polymer, volumes up to 600 µL	G&T Septech AS (Ski, Norway)

Table 9. Equipment and components used for HPLC

Equipment	Description	Producer
Software	Chromaster system manager version 2.0	VWR Hitachi
Auto sampler	Hitaci Chromaster 5260 Auto sampler	Hitachi High-Tech Science Corporation (Tokyo, Japan)
Detector	Hitachi Chromaster 5430 Diode Array Detector	Hitachi High-Tech Science Corporation (Tokyo, Japan)
Pump	Hitachi Chromaster 5160 Pump	Hitachi High-Tech Science Corporation (Tokyo, Japan)
Degasser	Merck L-7614	Merck Millipore (Burlington, MA, USA)
Column	Kromasil® KR100-5-C18, 5 µm particle size, 4.6 × 150 mm	Nouryon (Bohus, Sweden)

Column oven	HPLC column heater CO20, Version 4.0	Torrey Pines Scientific (Carlsbad, CA, USA)
Inserts	Micro-insert ND8, clear glass, conical, 0.1 mL, 5×31 mm, 15 mm top	VWR Corporate (Radnor, PA, USA)
Screw caps	Skrew cap ND8, black PP, central hole 5.5 mm	VWR Corporate (Radnor, PA, USA)
Septa	Septum ND8, white silicone / blue PTFE, slitted, 8 mm Ø, 0.9 mm thickness	VWR Corporate (Radnor, PA, USA)
Vials	Screw neck vial ND8, clear glass, 1.5 mL, 11.6×32 mm	VWR Corporate (Radnor, PA, USA)

3.4 EME setup and procedure

The equipment described in Table 8 was used for EME of analytes, and the components for a sample unit is depicted in Figure 11a. A sample unit consists of two conductive vials connected by a union. One vial serves as the donor and the other as the acceptor. A porous polypropylene (PP) flat sheet membrane is positioned inside the union. An organic solvent immersed in the PP membrane is termed the supported liquid membrane (SLM).

Figure 11. (a) Sample equipment prior to assembly, from the left: acceptor vial, flat sheet PP membrane, union and acceptor vial. (b) The assembly of a sample unit.

The full EME equipment setup is shown in Figure 12a. The assembled sample unit (Figure 11b) is placed in the sample holder, which has ten pairs of grooves for the placement of up to ten sample units, as shown in Figure 12b. The sample holder is attached to an agitator. A top cover with ten electrode pairs is positioned over the assembled sample units in the sample holder, and the top cover is tightened by six screws and nuts to ensure contact between the electrodes and conductive vials.The electric field is delivered by a power supply, which is connected to the electrodes and a multimeter. The power supply can deliver direct voltage in

the range from 0 to 300 V, equal for all electrodes, and the desired voltage level is set prior to positioning the top cover.

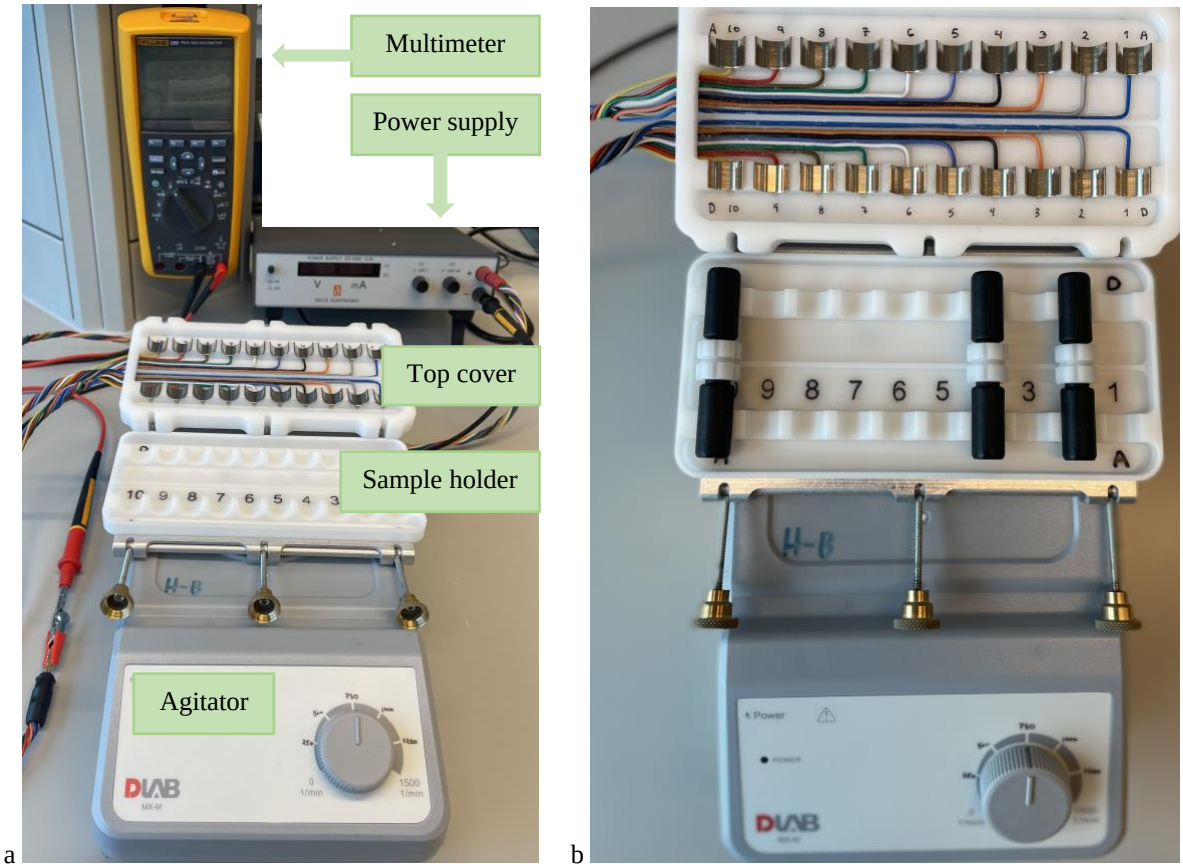


Figure 12. (a) EME equipment setup. (b) Top cover with electrodes and sample holder with 3 samples.

The principle of the EME procedure is schematically illustrated in Figure 13. The first step is placing the PP membrane inside the union. This was done by using the back end of a pipette tip and carefully push the membrane in place without damaging it. Next, the acceptor solution was pipetted to the acceptor vial and the union-membrane complex was carefully, but tightly, attached to the acceptor vial without crumbling the membrane. This is to ensure no leakage of the aqueous solutions around the SLM. Then, 10 µL of the chosen organic solvent was immersed in the flat sheet membrane by capillary forces, making the SLM. Next, the donor solution was pipetted to the donor vial. Lastly, the acceptor-union complex was attached to the donor vial. When extracting TFB (cationic analyte), the donor vial was coupled with the positively charged electrode (anode), and the acceptor vial was coupled with the negatively charged electrode (cathode). The direction was reversed when working with CsA, since it is an anionic analyte. Prior to analysis with HPLC-UV, the vials were centrifuged for 10 seconds to remove air bubbles in the solution.

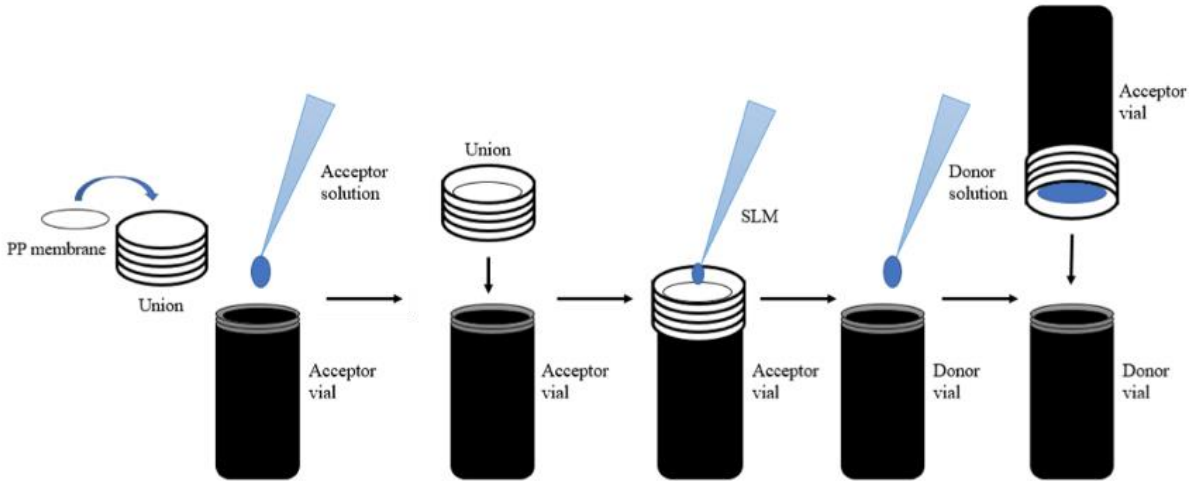


Figure 13. Schematic view of the EME procedure. Adapted and modified from (46).

The multimeter monitors the electrical current in the system during EME, which can be recorded and converted to an extraction current curve. The curve will give an indication of the stability and efficiency of the extraction. A low current is not necessarily correlated to a low recovery of analyte. The composition of the SLM will impact the observed current. On the other hand, a high current (> 50 µA/sample) could indicate instability or electrolysis in the extraction system, and subsequent pH changes in the aqueous solutions. Figure 14 display an example of a stable current curve in EME, with a simultaneous extraction of 9 samples. The sharp decrease in current during the first two minutes is attributed to the establishment of the electrical double layer (47).

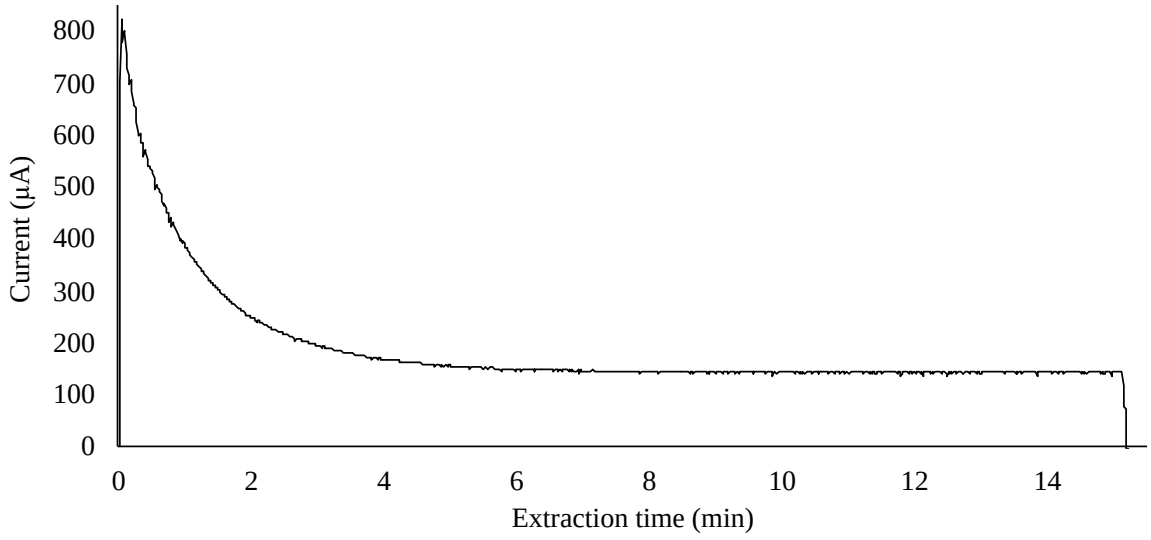


Figure 14. Example of a stable EME current curve with nine samples extracted simultaneously at 30 V with a variation of extraction conditions. This is taken from an experiment performed at the University of Oslo.

The conductive vials are disposable, but to be able to use them more than once, a washing procedure was develped. The aqueous solutions were disposed, and the vials were placed in a 70% ethanol bath. The inside of the vials was first flushed three times with an alcohol solution and then three times with Milli-Q water. The vials were then airdried over night or in an oven at 75˚C for approximately two hours.

3.4-1 Processing of CsA samples after extraction

After EME of CsA, the aqueous solutions were transferred to separate HPLC-vials. 250 µL of 10 mM HCl (pH 2.0) was added to reduce the pH in the solutions. The mixture was vortexed for 10 seconds. The flat sheet membrane was engaged in a HPLC-vial with 500 µL methanol for one hour, to extract the analyte trapped in the SLM. Next, the PP-filter was removed. The solutions were placed in a centrifugal concentrator to evaporate the liquid by vacuum. After drying, 100 µL of a methanol and MQ solution (80:20) was added to each vial. The salt was then attempted dissolved by vortexing for one minute with 10 seconds intervals and pipette mixing times ten. The sample solutions were then transferred to inserts and analyzed by HPLC-UV.

3.5 HPLC-UV analysis

A Hitachi Chromaster HPLC instrument, with components as described in Table 9, was used for the separation and detection of the analytes CsA and TFB. HPLC-UV analysis was performed on all aqueous solutions after extraction, except from the donor solution with diluted plasma.

3.5-1 HPLC-UV analysis method for CsA

The analysis method with HPLC-UV for CsA was based on the study by Antunes et al (48), with some modifications. Mobile phase A consisted of 2 mM ammonium acetate (NHAc) in Milli-Q water (MQ) with 0.1 % formic acid (FA). Mobile phase B consisted of 2 mM NHAc in methanol with 0.1% FA. The HPLC gradient is listed in Table 10. The flow rate was set to

1.0 mL/min and the column was kept at 55˚C. The sample injection volume was 20 µL and the monitoring wavelength was 210 nm.

Table 10. HPLC gradient for the elution of CsA.

Time (min)	% Mobile phase A	% Mobile phase B
0.0	20	80
1.0	20	80
6.0	0	100
8.5	0	100
10.0	20	80
13.0	20	80

As seen in the chromatogram in Figure 15, CsA eluted as a wide peak with retention time around 7.3 minutes. A high temperature and a large fraction of organic solvent was needed in order to obtain a chromatogram where CsA could be quantified.

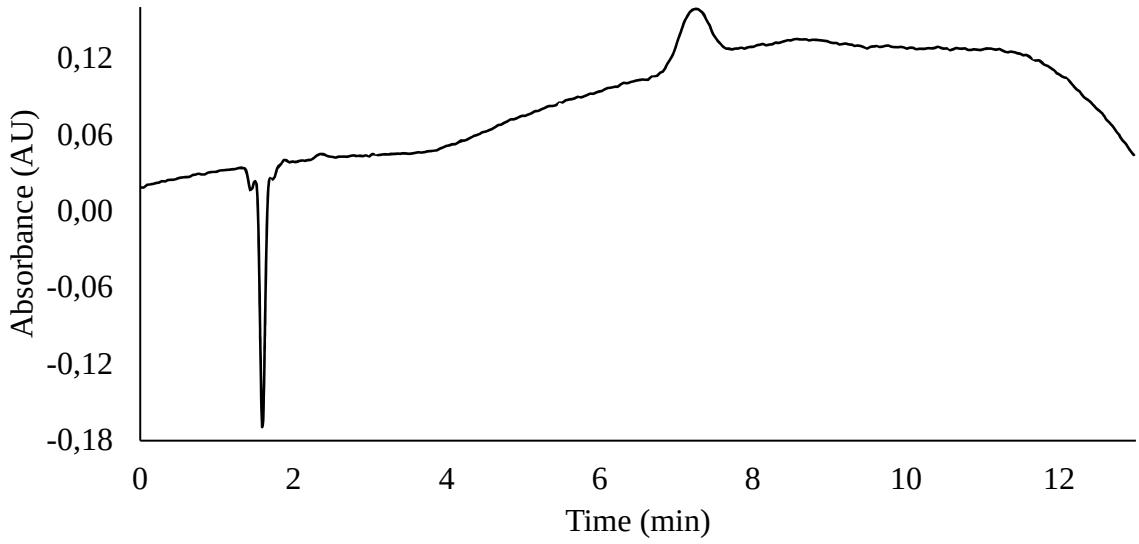


Figure 15. Chromatogram showing the elution of 20 µL 20 µg/mL CsA at 7.3 minutes.

A standard curve using the method described in Table 10 was developed to establish the lower limit of detection and linearity. Standard solutions of 1-50 µg/mL CsA in a mixture of methanol and MQ (80:20) was prepared for this purpose. At 1 µg/mL CsA, no analyte was detected, and the lower limit of detection was therefore determined to be 2 µg/mL. The standard curve is presented in Figure 16 and shows linearity in the range of 0.2-50 µg/mL.

The trend line has the equation y = 30220x + 3621.6, which was used to calculate the amount of CsA in the EME-samples.

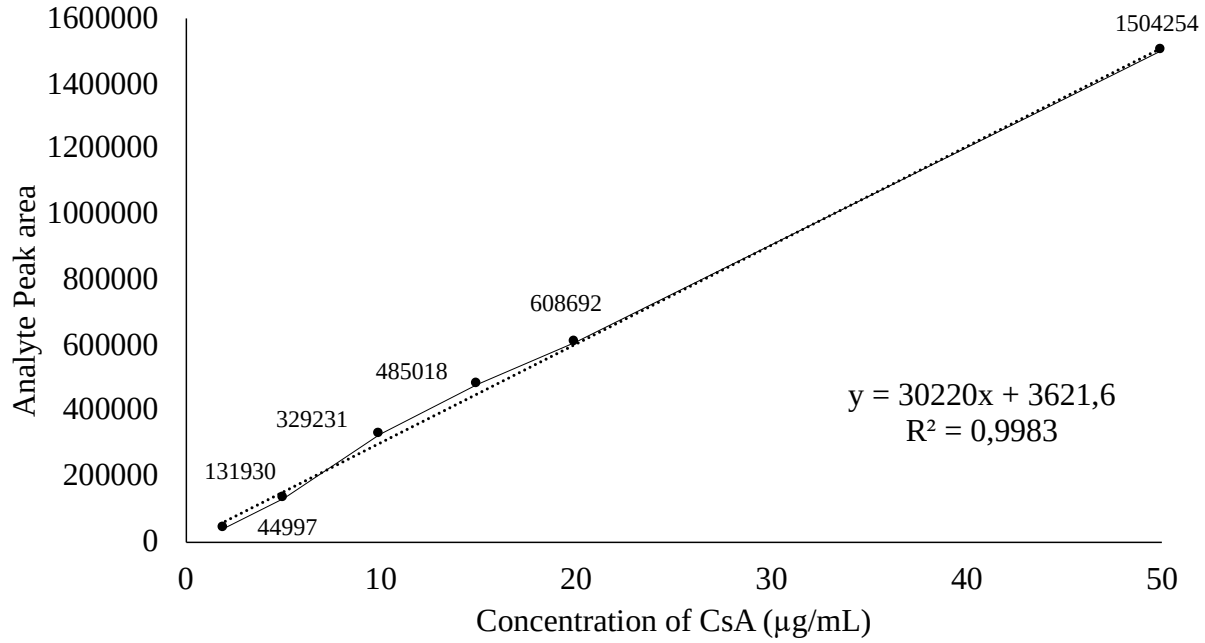


Figure 16. Standard curve of CsA obtained from 20 µL injections of 2-50 µg/mL CsA.

3.5-2 HPLC-UV analysis for TFB

The HPLC-UV analysis method for TFB was based on the study by Kim et al (49), with some modifications. An isocratic mobile phase consisting of 10 mM NHAc in MQ with pH 5 and acetonitrile (60:40) was used. The flow rate was set to 1.4 mL/min and the column was kept at 40˚C. The sample injection volume was 10 µL and the monitoring wavelength was 287 nm. A chromatogram for the elution of 5 µg/mL TFB is presented in Figure 17, and shows that TFB eluted as a sharp peak with a retention time of 1.6 minutes.

Standard solutions from 0.01-5 µg/mL TFB citrate was prepared for the standard curve presented in Figure 18. Concentrations below 0.05 µg/mL gave small and broad peaks, and the area under the peak could not be calculated accurately. The lower detection limit was 0.05 µg/mL TFB citrate, and the trend line shows linearity in the range of 0.05-5 µg/mL. The trend line has the following equation: y = 4990.5x + 148.89. This is a satisfactory analysis method for EME of TFB.

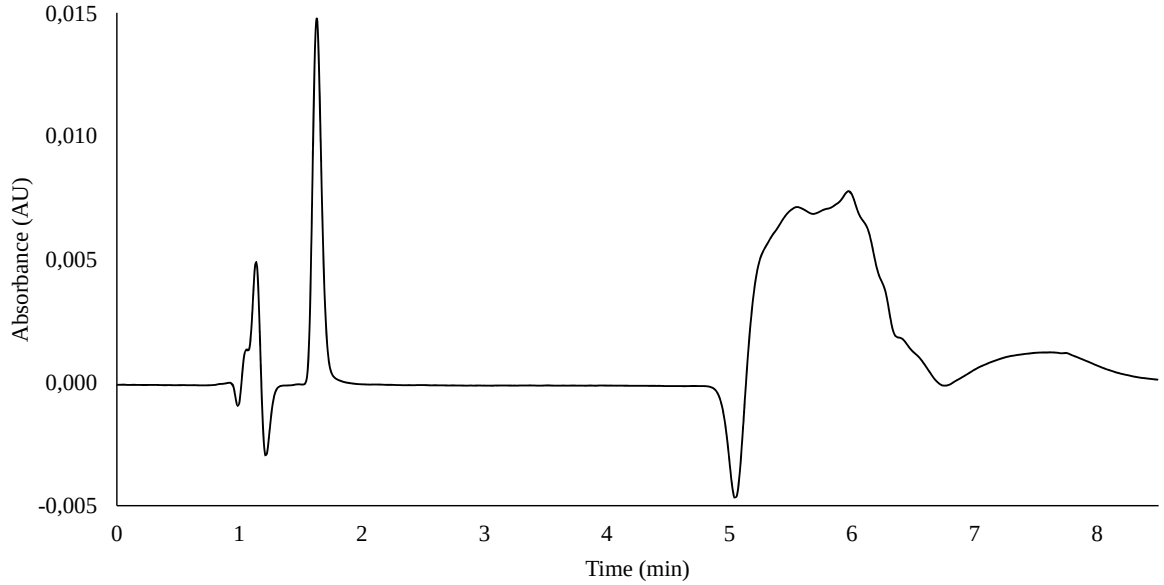


Figure 17. Chromatogram of the elution of 10 µL 5 µg/mL TFBc at 1.6 minutes.

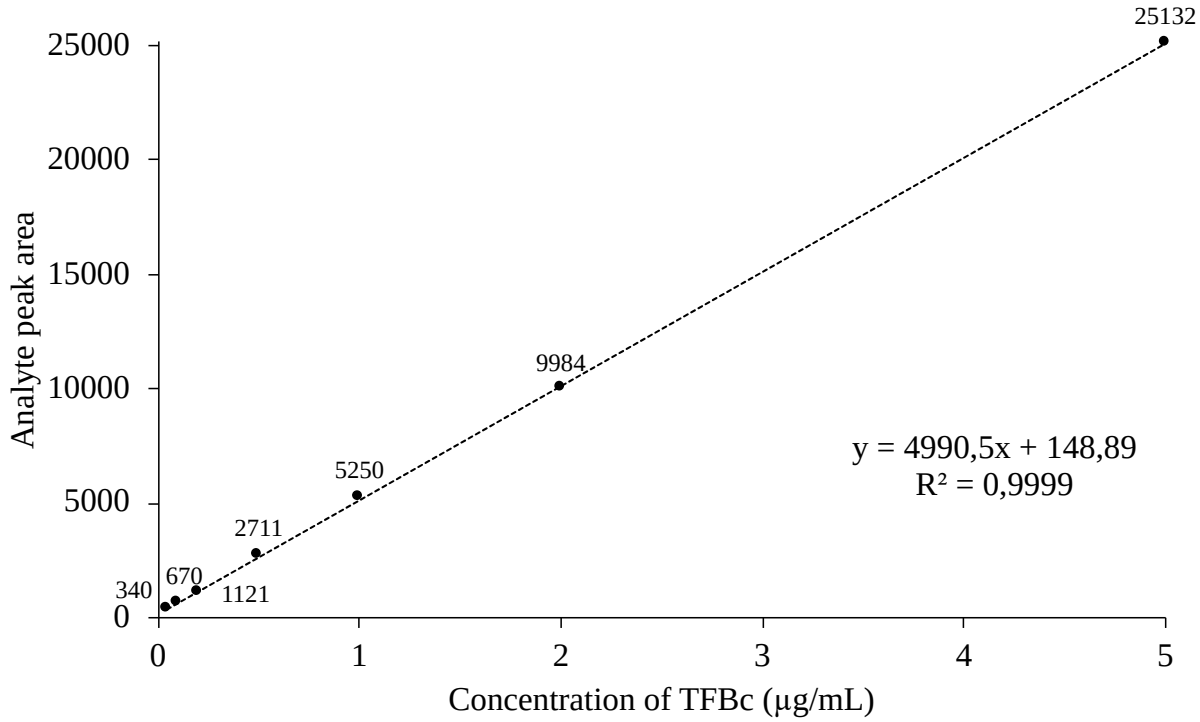


Figure 18. Standard curve of 10 µL 0.05-5 µg/mL TFBc

3.6 Calculations

The standard curves from HPLC-UV were calculated by linear regression, based on the equation below. *y* is the instrument response, i.e. the area under the peak, *a* is the constant increase, *b* is a constant parameter, where the trend line crosses the x-axis, and *x* is the concentration of analyte.

$$y = ax + b \qquad \text{(Equation 2)}$$

The coefficient of determination (R^2) was calculated to observe how the data points fitted the trend line from the standard curve. An R^2 value close 1 indicates a good model for the data points. *SS regression* is the sum of squares due to regression, and *SS total* is the total sum of squares.

$$R^2 = 1 - \frac{SS\,regression}{SS\,total} \qquad \text{(Equation 3)}$$

The extraction recovery of CsA (R_{CsA}) in the concentrated donor solution and extract from the SLM was calculated with use of the standard curve by the following equations, where A_A is the peak area for the analyte, *V* is the volume of the sample solution after concentration (0.1 mL), *a* and *b* are the constant parameters from the standard curve for CsA and C_D is the concentration of the donor solution prior to extraction.

$$mass\ sample = \frac{A_A - b}{a} \times V \qquad \text{(Equation 4)}$$

$$mass\ donor = C_D \times V \qquad \text{(Equation 5)}$$

$$R_{CsA} = \frac{mass\ sample}{mass\ donor} \times 100\% \qquad \text{(Equation 6)}$$

The extraction recovery of TFB was calculated by absorbance using the following equation, where *R* is recovery, A_A is the peak area for the analyte and A_{std} is the peak area for the standard solution.

$$R = \frac{A_A}{A_{Std}} \times 100\% \quad \text{(Equation 7)}$$

The standard deviations (*SD*) were calculated by the following equation, where *x* is the recovery for each sample and *x-bar* is the mean recovery and n is the number of samples.

$$SD = \sqrt{\frac{\sum(x-\bar{x})^2}{n}} \quad \text{(Equation 8)}$$

The relative standard deviation (*RSD*) was calculated by the following equation, where *SD* is the standard deviation and *x-bar* is the mean recovery.

$$RSD = \frac{S}{\bar{x}} \times 100\% \quad \text{(Equation 9)}$$

4 Results

4.1 Investigation of the EME setup

EME equipment with ten electrode pairs, as presented in Figure 12 was utilized in the experiments. To establish an electric field, an external power supply was used. To ensure that the applied voltage was equal for all electrodes, the voltage was measured for each electrode pair on the top cover between experiments. Variations in the direct voltage were observed over the electrode pairs between the measurements. The direct voltage was measured to either the set voltage or zero. Figure 19 illustrates an example of a voltage control at 30 V, where electrode pair number 3 was measured to 0 V. This was the case over a long period of time, probably due to a break in the wiring. Electrode pair number 3 was therefore not used in TFB experiments after this was discovered.

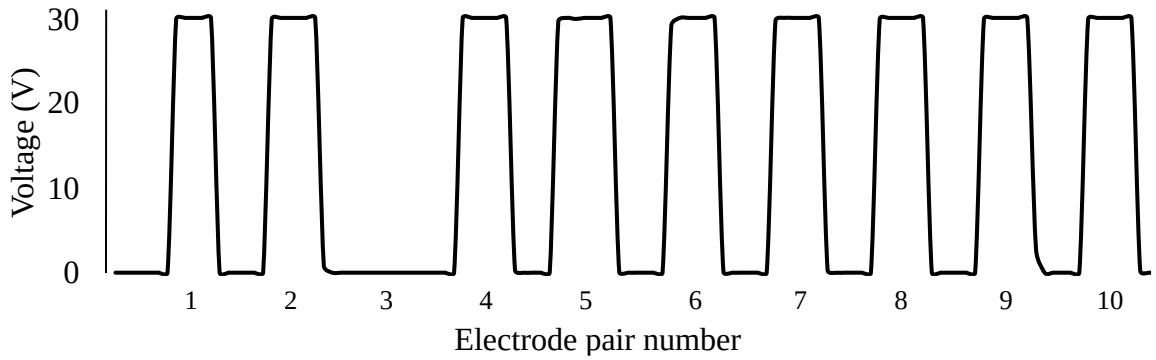


Figure 19. Measured voltage (V) of the electrode pairs at 30 V.

The measurements were performed prior to and after extraction, and are not directly transferable to the conditions during extraction. Electrode pair number 1, 6 and 8 were primarily used for extraction of TFB with three parallels. In the middle of the experiment series, several electrode pairs became unstable, and I decided to change to number 4, 6 and 8. Towards the end of experimentation, the measured voltage was unpredictable for the majority of the electrode pairs, but number 2, 4 and 10 appeared to be stable at 30 V at that time.

4.1-1 Recovery variation between electrode pairs

During experimentation, variation occurred in the recovery of analyte between parallels. One parallel often stood out as a possible outlier compared to the two others. This could be due to unreliable supply of voltage from the electrodes. To test this hypothesis, 10 parallel samples were extracted simultaneously, and this was repeated three times. The extraction conditions used are listed in the table below:

Table 11. Constant parameters for investigation of recovery variation between electrodes.

Parameter	**Description**
Donor solution	300 µL of 5 µg/mL TFBc in 100 mM formic acid (pH 2.4)
SLM	10 µL of 6MC:Thy (1:2)
Acceptor solution	300 µL of 100 mM formic acid (pH 2.4)
Voltage	30 V
Agitation	750 RPM
Extraction time	15 minutes

The results are presented in the three following figures, describing acceptor (Figure 20), donor (Figure 21) and total (Figure 22) recovery plotted against the electrode pair number. Each color represents one extraction with ten samples. The standard deviation and numbers showed in the figures are related to the mean recovery (black circles) for each electrode pair. Extraction «b» and «c» were performed on the same day, and the calculated recovery from extraction «a» diverge from these two for the majority of the electrodes. However, all three extractions are displayed to emphasize the possibility of variation.

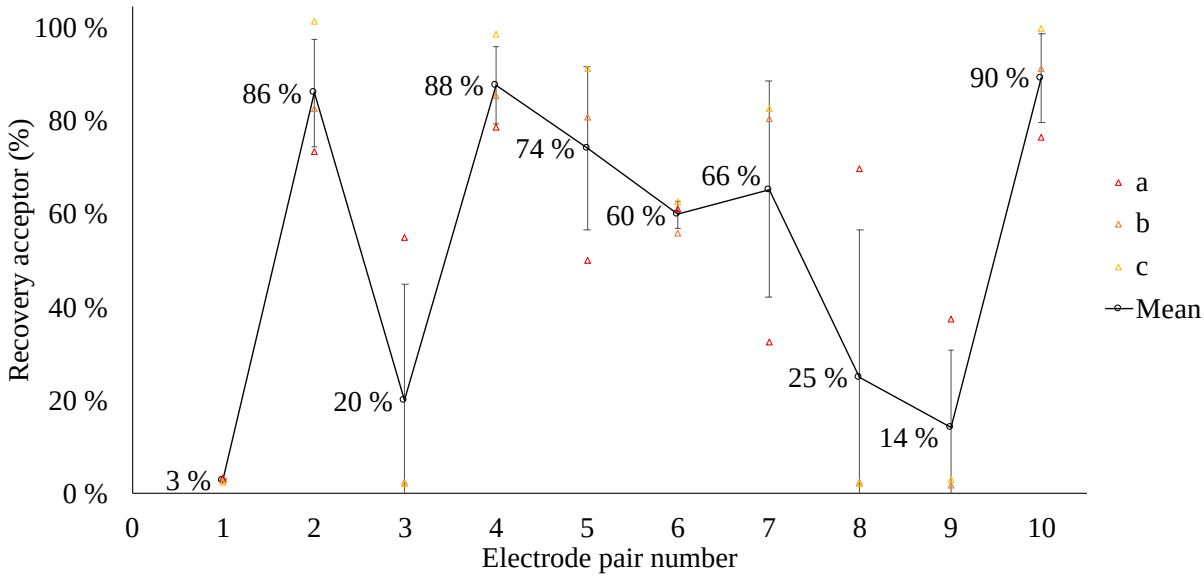


Figure 20. Recovery (%) of TFB from the acceptor solution plotted against the electrode pair number on the top cover of the EME setup.

The recovery of analyte in the **acceptor solutions** varied from 2% to 100%. Four of the electrode pairs had parallels with recovery less than 5%, and pair number 1 was stable at 3% for all three extractions. Three electrode pairs stood out with mean recoveries above 85% and standard deviations below 12%. The mean acceptor recovery for all parallels was 53% with a SD of 32% and a large RSD of 60%.

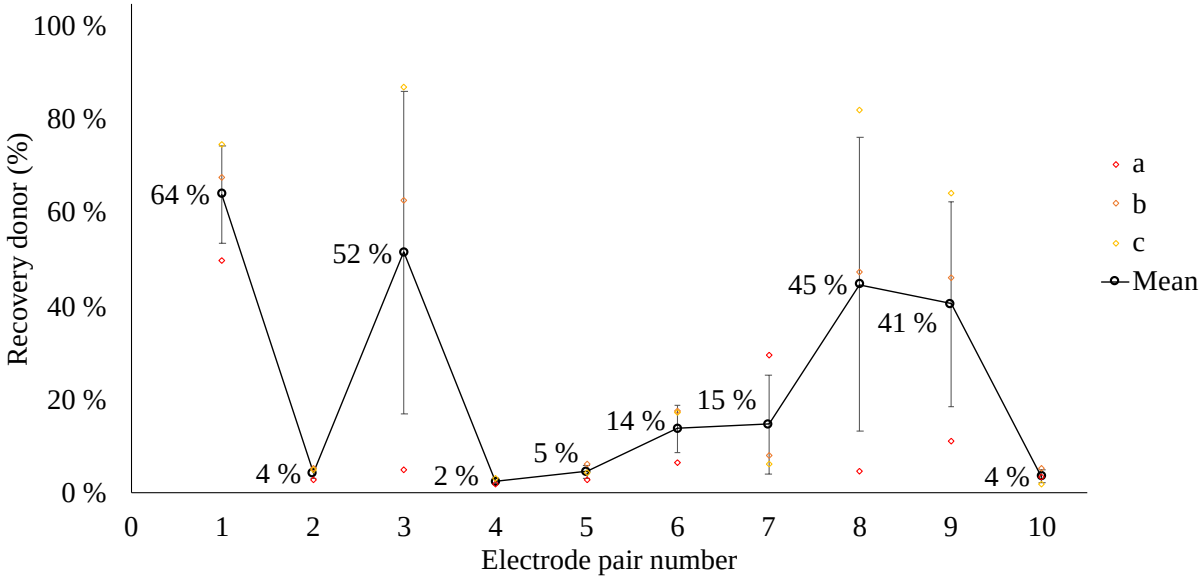


Figure 21. Recovery (%) of TFB from the donor solution plotted against the electrode pair number on the top cover of the EME setup.

The recovery of analyte in the **donor solutions** varied from 2% to 87%. The same three electrode pairs that had large acceptor recoveries (> 85%) showed the lowest mean donor recoveries of 4% or less, with SD of 1%. The two electrode pairs with the greatest intra-experimental variations had SDs above 30%. The mean donor recovery for all parallels was 24% with SD of 22% and a very large RSD of 91%.

It is expected that high recovery in the acceptor vial is correlated to low recovery in the donor, and vice versa. When comparing Figure 20 and 21, we can see that this is the case.

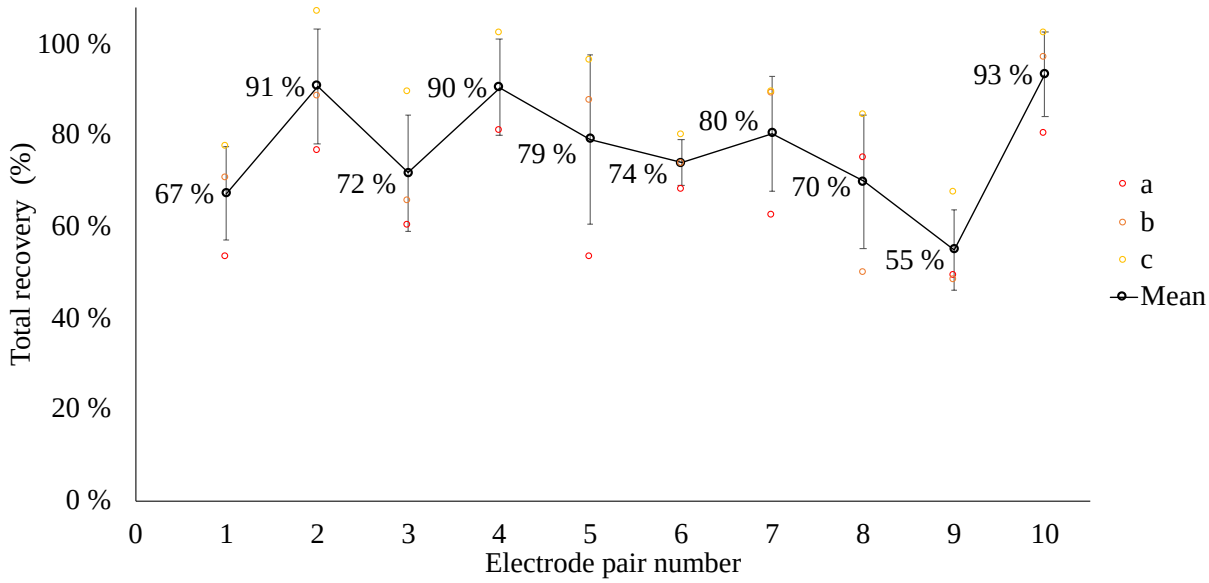


Figure 22. Total recovery (%) of TFB from the donor and acceptor solution plotted against the electrode pair number on the top cover of the EME setup.

The **total recovery** of analyte represents the sum of recovery from the acceptor and donor solutions, and the mean varied from 55-93%. The fraction of analyte that may was trapped in the SLM is not a part of this estimate. The same three electrode pairs as mentioned earlier had mean recoveries above 90%. The lowest mean recovery was 55% for number 9. The overall mean recovery was 77 % with SD of 11% and RSD of 15%.

Electrode pair number 2, 4 and 10 are the ones that stood out from the rest, with the highest recoveries in acceptor solution (> 85%) and lowest recoveries in the donor solution (≤ 4%). Their RSDs for the acceptor recovery were 14%, 9% and 11%, respectively.

The measured sum of extraction currents from experiment «a» and «c» are illustrated in Figure 23 below. The current in experiment «b» was mostly overlapping with «c», and are therefore not included in the figure. The red slope («a») shows a fluctuating current throughout the extraction time. The blue slope («c») shows a more stable extraction current, except from a sudden leap at approximately 7 minutes. Extraction «c» had overall a higher total mean recovery for all electrode pairs, at 90%, compared to «a» at 66%, with RSDs at respectively 13 % and 17%.

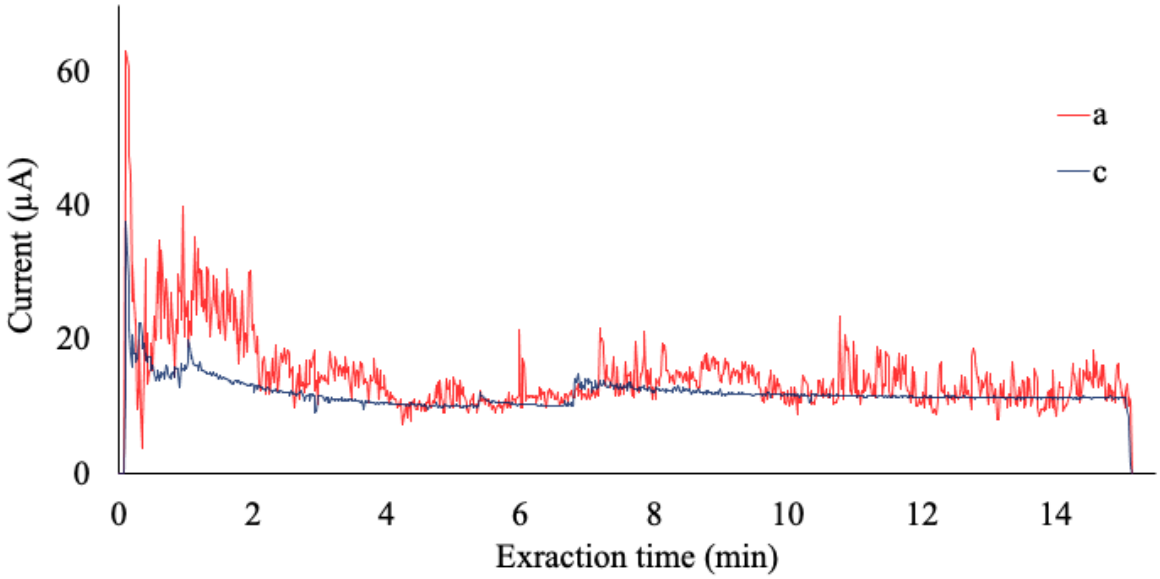


Figure 23. Extrication currents for the investigation of recovery variation between electrode pairs. The red slope is from extraction «a» and the blue slope is from extraction «c». Both curves represent the sum of extraction currents for 10 samples with 1 reading/sec.

4.2 Extraction of cyclosporine A

Due to its chemical properties, described in section 2.2-1, the only option for extraction of CsA is as a deprotonated acid (anion) under highly basic conditions. To ensure the presence of a negative charge on the analyte, the pH in the aqueous solutions was set to 12. Chemicalize estimates that 72% of the CsA molecules carry a negative charge at this pH (26). SLMs suitable for EME of acidic compounds are rather limited, but higher alcohols, like 1-octanol have previously been used (20).

4.2-1 Extraction of CsA through 1-octanol

In this study, extraction of CsA was performed through 1-octanol. The concentration of analyte in the donor solution was 20 µg/mL, since the lower detection limit of the HPLC-UV method was 2 µg/mL. Prior to analysis, the pH in the samples had to be reduced to below 8, to avoid decomposition of the column. A step to concentrate the samples was then applied. Due to the hydrophobic character of CsA, I suspected that the analyte could be trapped in the SLM, and an extraction to recover this amount was completed. The conditions used in the EME of CsA are listed in the table below:

Table 12. Constant parameters for extraction of CsA.

Parameter	Description
Donor solution	250 µL of 20 µg/mL (5 µg) CsA in 10 mM NaOH (pH 12)
SLM	10 µL 1-octanol
Acceptor solution	250 µL of 10 mM NaOH (pH 12)
Agitation	750 RPM
Extraction time	15 minutes

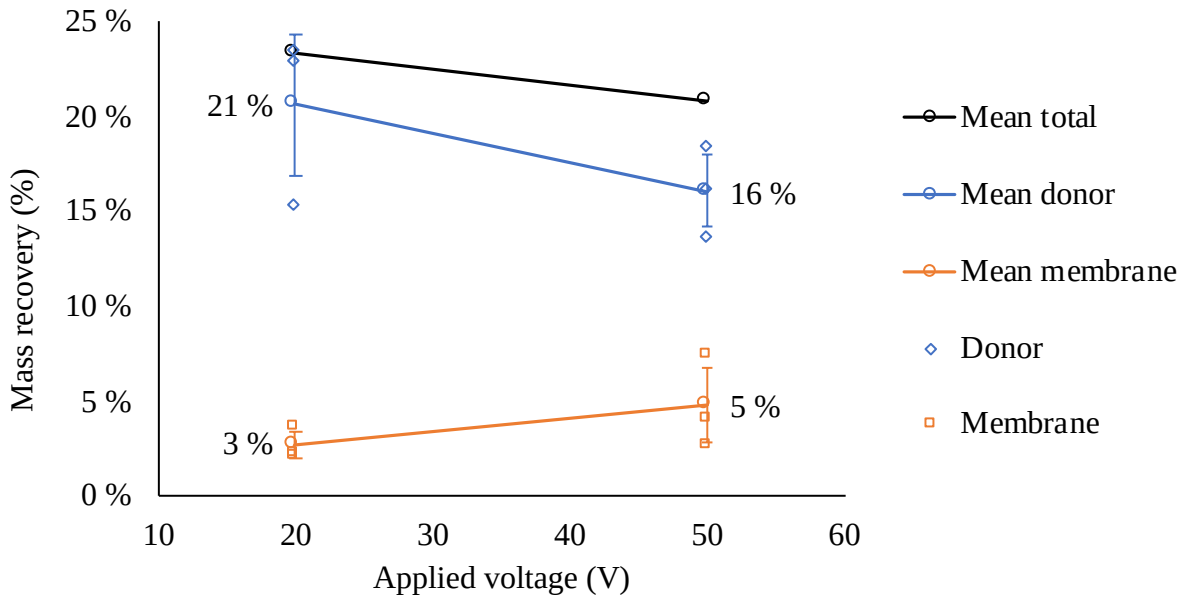


Figure 24. Results for the extraction of CsA. Mass recovery (%) from the donor solution and membrane plotted against the applied voltage (V) after concentrating and extraction. The amount of analyte in the system was 5 µg. The samples were extracted at electrode pair number 3, 6 and 9.

Figure 24 displays the recovery of CsA in the donor solution and SLM. The results were calculated by using the standard curve and are presented as mass recovery. No analyte was detected in the acceptor solution, but the lower detection limit was 2 µg/mL, translated to 4% mass recovery. The extractions were performed at two levels of voltage, 20 V and 50 V respectively, with three parallels each.

The mean recovery of analyte in the **donor solution** was 21% at 20 V and 16% at 50 V. Thus, it was observed a reduction in the recovery from the donor solution with increased voltage. The mean recovery from the extracted **membrane** was 3% at 20 V and 5% at 50 V, which is the opposite change of that observed for the donor. The black slope, representing the mean **total** recovery, shows a decrease with increased voltage.

After transferring the concentrate of donor solutions to HPLC inserts, I observed remaining fragments of undissolved salt.

4.2-2 Investigation of affinity to different materials

The summary of product characteristics (SPC) for CsA as a mixture explicitly advise against using plastic containers when diluting it in a beverage. The affinity of CsA to three different materials was therefore investigated, also due to the suspicion of CsA adsorbing to the inside of the EME-vials. A solution of 20 µg/mL CsA in methanol and water (80:20) remained in the respective containers for approximately 24 hours. The HPLC-vial was used as a standard, since it is made of glass. The other two containers were an EME-vial (conductive polymer) and Eppendorf tube (plastic). The results are showed in Figure 25.

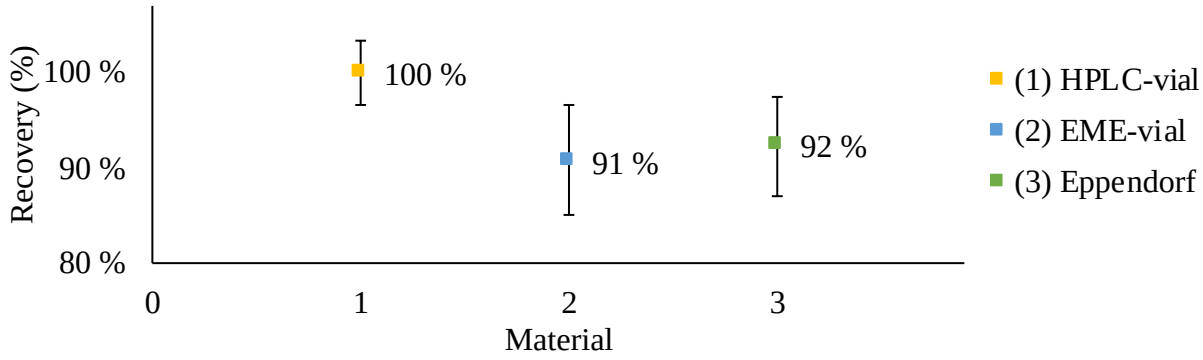


Figure 25. Recovery (%) of a 20 µg/mL CsA solution plotted against three different materials. HPLC-vial (glass) is used as the standard material. The recovery and SD are estimated from two parallel injections of the sample solution on HPLC-UV.

Since glass was chosen as standard material, the mean recovery was set to 100% for the HPLC-vial. The recoveries from EME-vial and Eppendorf tube are presented as a fractional recovery in comparison to glass. The mean loss of analyte was 9% for the EME-vial and 8% for the Eppendorf tube.

4.3 Extraction of tofacitinib

Extraction of TFB can be performed as either a deprotonated acid (anion) or protonated base (cation). The latter was a reasonable place to start, since previous studies of polar basic analytes have been performed, and successful SLMs and ICs are already established (28). To optimize an EME method for the extraction of TFB, I started with a variation of SLM compositions and pH in the donor solution. Experiments with the addition of different fractional amounts of DEHP was then executed. Next, the most promising pH and SLMs were tested with a variety of voltage. The most favorable conditions were applied in the extraction of TFB from plasma, and further in the investigation of optimal extraction time. Every experiment was performed with three parallels. Based on the chemical properties of TFB (pKa at 7.5) and studies of pH effect on EME and DEHP (23, 39), the acceptor solution was maintained at pH 2.4, as a constant parameter.

After EME of TFB, the acceptor and donor solutions were analyzed by HPLC-UV. The external standard solution was the same as the one used as donor solution, and was accordingly replaced with every new preparation of a donor solution. The recoveries of analyte from donor and acceptor solutions were calculated as a fraction of the mean absorbance for the current external standard solution.

4.3-1 Extraction of TFB using different SLMs and pH

Nitroaromatic solvents, such as NPOE and NPPE, have been widely used in EME of polar basic analytes, both with and without the addition of IC (28). In the initial experiments, TFB was attempted extracted through pure NPOE and NPPE, and then with the addition of 10% DEHP, all with the applied voltage set to 50 V. A 6MC:Thy based SLM was tested with an applied voltage of 30 V. A volume of 10 µL SLM was used throughout this study. The SLMs

containing DEHP was not tested at physiological pH (7.4), due to the possibility of leakage of DEHP into the donor solution (39). The extraction through 6MC:Thy at the same pH is not included in the presentation of the results in Figure 26, because all the PP-filters were perforated for unknown reasons. The extraction current for the first seven minutes of that experiment is presented in Figure 7. The common constant parameters and variable parameters are listed in the tables below:

Table 13. Constant parameters for extraction of TFB through diverse SLMs and pH

Parameter	**Description**
Acceptor solution	300 µL of 100 mM FA (pH 2.4)
Agitation	750 RPM
Extraction time	15 minutes

Table 14. Variable parameters for extraction of TFB through diverse SLMs and pH. The concentration of TFBc in the donor solution was overall 5 µg/mL, with a volume of 300 µL.

SLM	**Donor solution**	**Voltage**
NPPE	pH 2.4: 100 mM FA	30 V
NPOE	pH 4.0: 40 mM NHAc	50 V (for 6MC:Thy)
10% DEHP in NPPE	pH 7.4: 40 mM K_2HPO_4	
10% DEHP in NPOE		
6MC:Thy		

As seen in Figure 26, the mean recovery of analyte in the **acceptor solution** was below 10% at all pH levels for SLMs of pure NPPE and NPOE. With addition of 10% DEHP, the recovery increased considerably. The highest recovery was through 6MC:Thy at pH 2.4 with 78%, followed by 10% DEHP in NPOE and NPPE, with acceptor recoveries at 74% and 71% respectively. Increasing the pH to 4.0 resulted in a decrease of recovery from the acceptor solution for all three SLMs, i.e. 6MC:Thy and 10% DEHP in NPPE and NPOE. The biggest decline was observed for the extraction through 10% DEHP in NPOE (pink slope), with a 25% reduction.

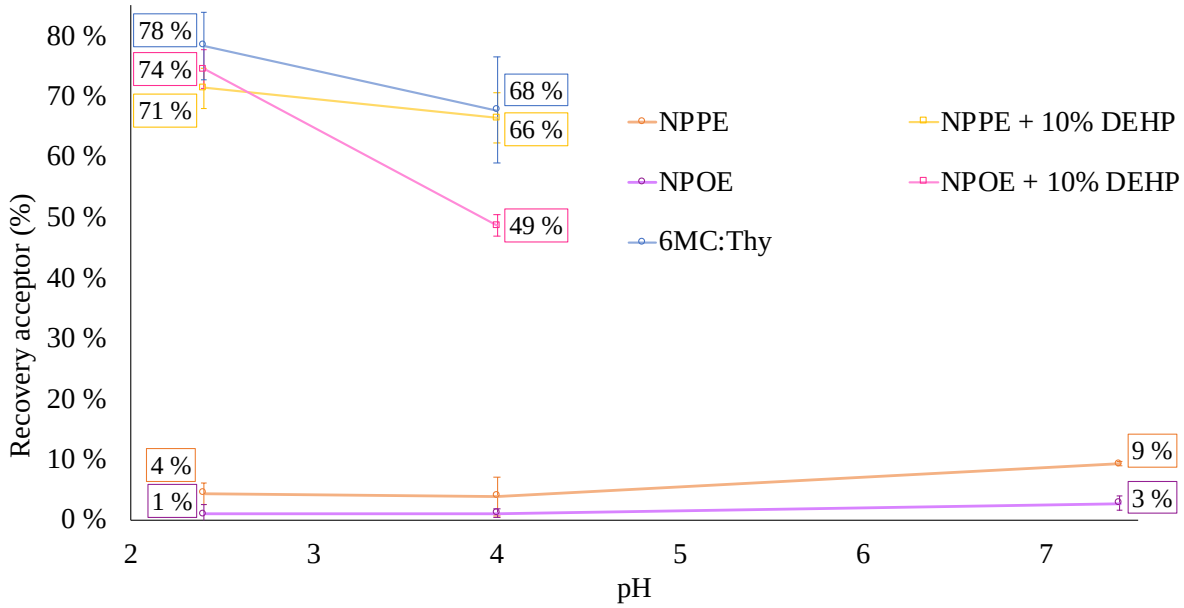


Figure 26. Results for the extraction of TFB through diverse SLMs and pH. Recovery (%) of TFB from the acceptor solution plotted against the pH in the donor solution, respectively 2.4, 4.0 and 7.4.

For the extraction performed at pH 7.4 through 6MC:Thy, all the SLMs appeared to be perforated. This was determined by inspecting the flat sheet membranes after extraction for discoloration, leakage and structural changes, i.e. matted, darkened color, the absent of a dry outer ring or a bump. The extraction current presented in Figure 27 shows an increasing amount of electrokinetic migration (way beyond 300 µA) in the system after approximately 3 minutes, which can be interpreted as perforation of one or more membranes.

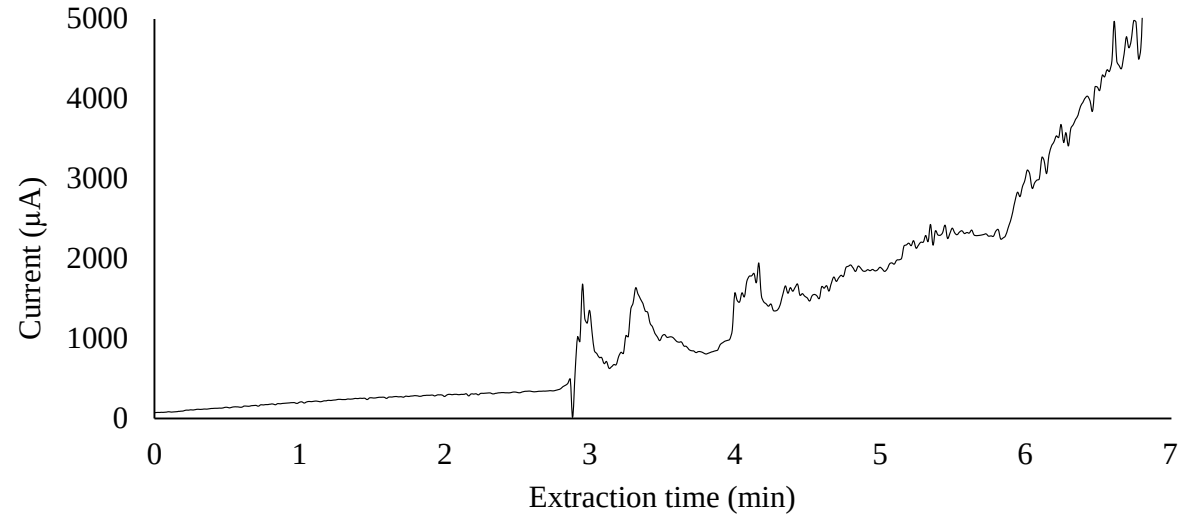


Figure 27. Extraction current for the first seven minutes of extraction through 6MC:Thy at pH 7.4. The trace is from three samples extracted simultaneously, representing the sum of the extraction currents for all three samples.

4.4 Extraction of TFB through 2-nitrophenyl octyl ether

Based on the results presented in the previous section, I wished to explore the possibilities of extraction through NPOE with different fractions of the ionic carrier DEHP. Two series of experiments were executed with the intention of investigating the optimal fraction (v/v) of DEHP in NPOE for the extraction of TFB. The first was performed with 5% intervals in the range from 0% to 25% DEHP, and the second within the range from 0.125% to 5% DEHP in NPOE. The most promising composition was then tested with alterations of applied voltage from 0 V to 50 V.

4.4-1 Extraction of TFB through NPOE with the ionic carrier DEHP

In the initial experiment with TFB, the analyte was extracted through 10% DEHP in NPOE. Based on the results from that experiment, an aqueous solution with pH 2.4 was chosen for the following extractions. The constant parameters are listed in the table below:

Table 15. Constant parameters for extraction of TFB through a SLM comprised of NPOE with the addition of various fractions DEHP.

Parameter	**Description**
Donor solution	300 µL of 5 µg/mL TFBc in 100 mM FA (pH 2.4)
Acceptor solution	300 µL of 100 mM FA (pH 2.4)
Voltage	30 V
Agitation	750 RPM
Extraction time	15 minutes

As already described in section 4.3, the addition of IC resulted in a great increase of recovery in the **acceptor** solution. This is seen in Figure 28 as well, with a mean escalation in recovery of 75% from pure NPOE to 5% DEHP in NPOE. With increasing amount of DEHP in NPOE, both the acceptor recovery (green slope) and total recovery (black slope) were decreasing. The recovery from the **donor** solution was below 10% for all extractions with IC.

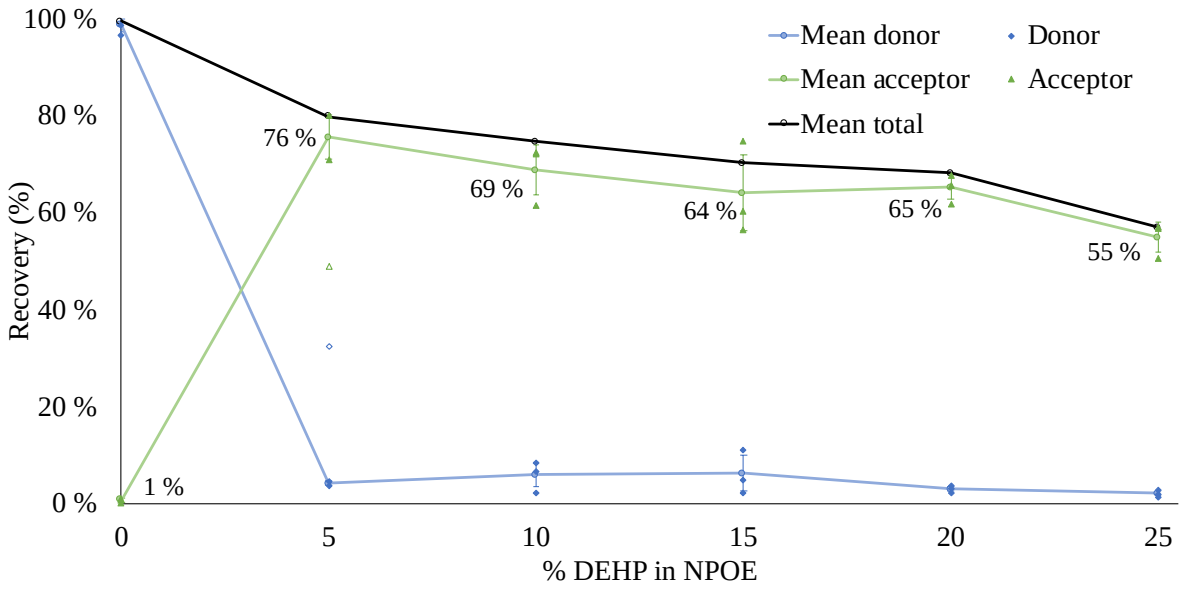


Figure 28. Results for the extraction of TFB through various fractions of DEHP in NPOE. Recovery (%) of TFB in donor and acceptor solution is plotted against the amount of ionic carrier DEHP (0-25%) in NPOE. The samples were extracted at electrode pair number 1, 6 and 8. The two indicators without fill are defined as outliers, and originate from the same sample unit, extracted from electrode pair number 1.

To further explore the optimal SLM composition of DEHP and NPOE, I decided to carry out an experiment with fractions in the range of 0.125-5% DEHP. The constant parameters were the same as in the previous experiment and are listed in Table 15.

As displayed in Figure 29 (below), the recoveries of analyte from the donor and acceptor solutions mirrored each other; an increase in the acceptor (green slope) resulted in a concurrent decrease in the donor (blue slope). The **total recovery** was also decreasing with an increasing amount of DEHP, corresponding with that seen in Figure 28 (above). The mean **acceptor recovery** varied from 28% at 0.125% DEHP to 75% at 2.5% DEHP. The overall acceptor recovery from extraction through 5% DEHP in NPOE was 74% with an RSD of 5%.

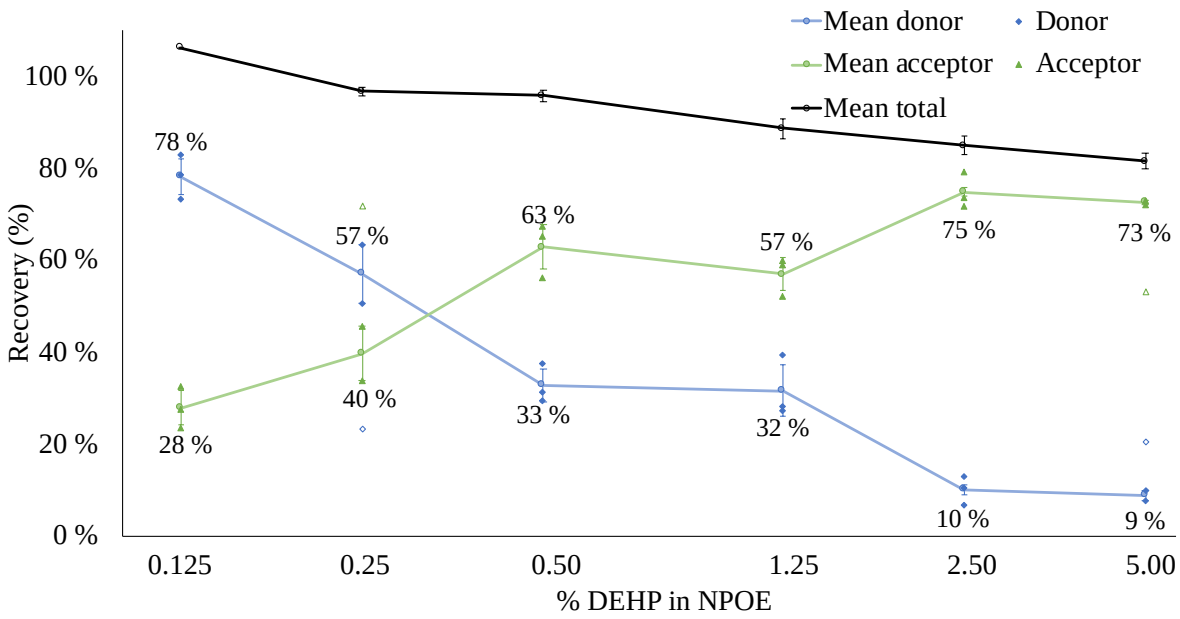


Figure 29. Results for the extraction of TFB through various fractions of DEHP in NPOE. Recovery (%) of TFB in donor and acceptor solutions is plotted against the amount of ionic carrier DEHP (0.125-5%) in NPOE. The samples were extracted at electrode pair number 1, 6 and 8. The four indicators without fill are defined as outliers, and originate from two sample units, both were extracted at electrode pair number 1.

4.4-2 Extraction of TFB through 5% DEHP in NPOE, applied voltage

Even though the extraction of TFB through 2.5% DEHP in NPOE may seemed to be most efficient, according to the results from the section above, it was decided to use a fraction of 5% DEHP for the investigation of applied voltage. The extractions were performed from 0 to 50 V. An extraction without the application of voltage was executed to confirm the advantage of the power supply in EME through the specified SLM.

Table 16. Constant parameters for extraction of TFB through 5% DEHP in NPOE with diverse applied voltage.

Parameter	**Description**
Donor solution	300 µL of 5 µg/mL TFBc in 100 mM FA (pH 2.4)
SLM	10 µL of 5% DEHP in NPOE
Acceptor solution	300 µL of 100 mM FA (pH 2.4)
Agitation	750 RPM
Extraction time	15 minutes

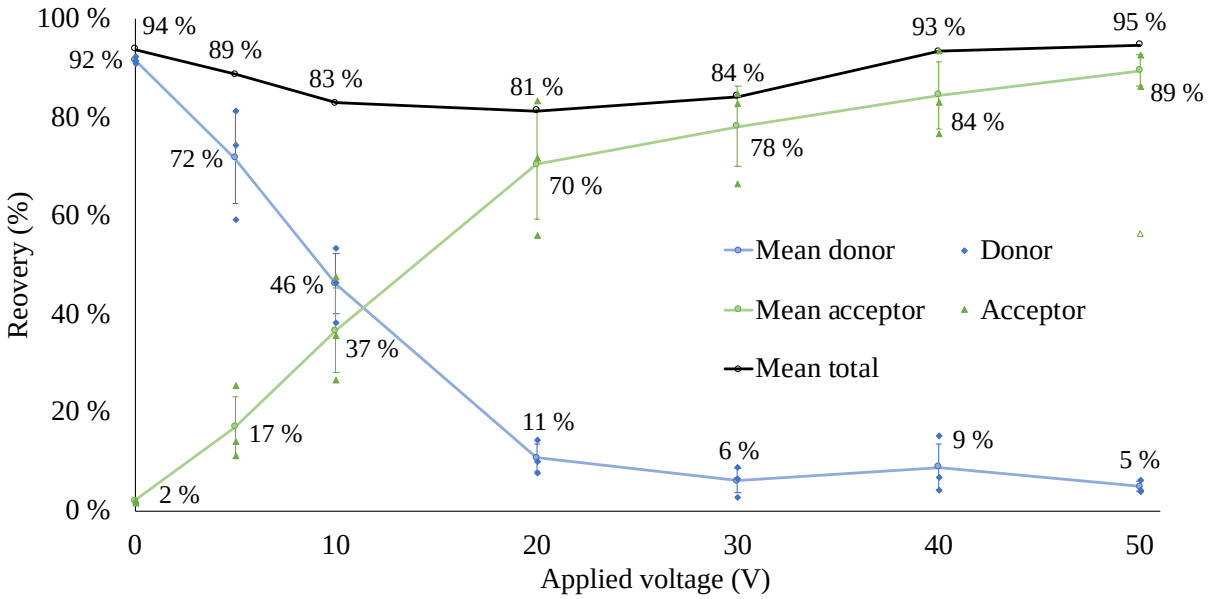


Figure 30. Results for the extraction of TFB through 5% DEHP in NPOE with variation in applied voltage. Recovery (%) of TFB in donor and acceptor solutions is plotted against the applied direct voltage (V). The indicator without fill is defined as an outlier. The samples were extracted at electrode pair number 4, 6 and 8.

The green slope in Figure 30 shows that the application of voltage is necessary to facilitate the migration of analyte into the acceptor solution. Without the application of voltage, 92% was recovered in the donor solution. The fraction of recovered analyte in the **acceptor** increased drastically from 2% without voltage to 70% at 20 V, and continued to increase to 89% at 50V. The recoveries from the donor solution (blue slope) mirrored the acceptor recoveries, like in the previous experiment. The black slope, representing the mean **total** recovery, shows a concave characteristic between 0 and 40 V, with no significant increase from 40 V to 50 V.

4.5 Extraction of TFB through 6-methylcoumarin and thymol (1:2)

Based on the results presented in section 4.3 and 4.4-2, I wanted next to explore the effect of 6MC:Thy with the addition of IC. SLM of coumarin and thymol mixed in a 1:2 ratio with 2% DEHP have proven to be efficient for extraction from plasma (28, 33). In the present study, a fraction of 5 % DEHP in 6MC:Thy (v/v) was diluted five times, making the next SLM half the concentration of the previous. The most promising composition was then tested with alterations of applied voltage from 0 V to 50 V.

4.5-1 Extraction of TFB through 6MC:Thy with the ionic carrier DEHP

In the initial experiment with TFB, the analyte was extracted through pure 6MC:Thy. Based on the results from that experiment, an aqueous solution with pH 2.4 was chosen for the following extractions. The constant parameters are listed in the table below:

Table 17. Constant parameters for extraction of TFB through a SLM comprised of 6MC:Thy with the addition of various fractions DEHP.

Parameter	Description
Donor solution	300 µL of 5 µg/mL TFBc in 100 mM FA (pH 2.4)
Acceptor solution	300 µL of 100 mM FA (pH 2.4)
Voltage	30 V
Agitation	750 RPM
Extraction time	15 minutes

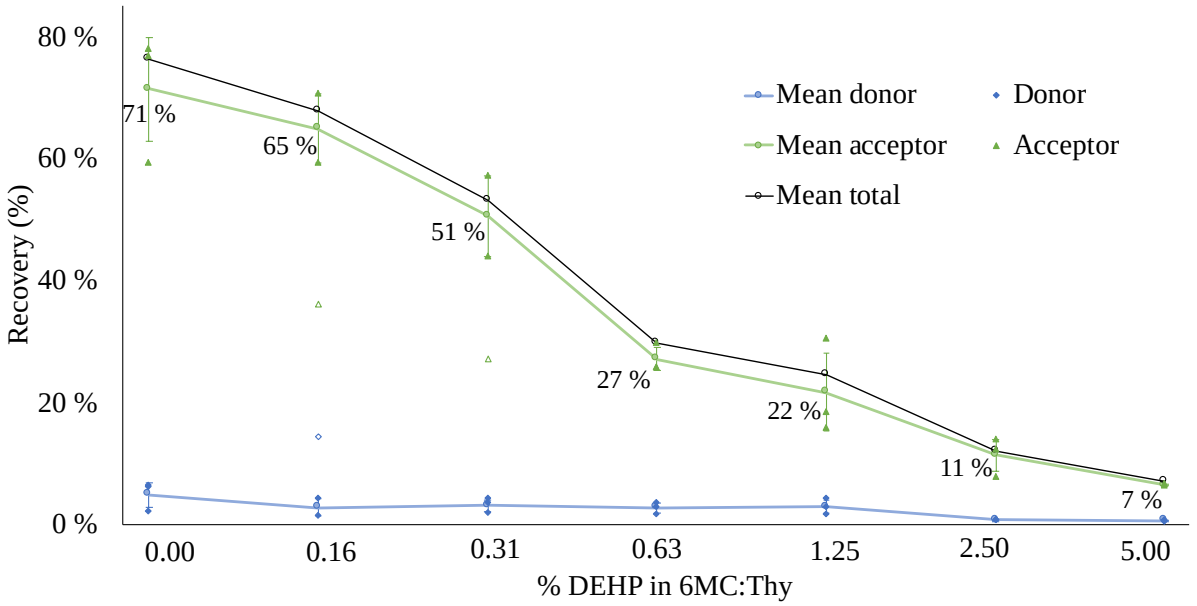


Figure 31. Results for the extraction of TFB through various fractions of DEHP in 6MC:Thy. Recovery (%) of TFB in donor and acceptor solution plotted against the amount of ionic carrier DEHP (0-5%) in 6MC:Thy. The indicators without fill are defined as outliers. The samples were extracted at electrode pair number 4, 6 and 8.

The green slope in Figure 31 shows that the recovery of analyte in the **acceptor** solution decreased with addition of IC, and continued to decrease with an increasing fraction of DEHP. The mean recovery was 78% through pure 6MC:Thy and 7% with the addition of 5%

DEHP. Less than 5% of the analyte was recovered in the **donor** solution throughout the extractions. Thus, the mean **total** recovery more or less followed the acceptor recovery, as described by the black slope. With a SLM consisting of 5% DEHP, 93% of the analyte was unaccounted for.

4.5-2 Extraction of TFB through 6MC:Thy, applied voltage

Since the extraction of pure 6MC:Thy showed the highest acceptor recovery, this SLM was chosen for investigation of the influence of applied voltage. The extractions were performed at 10 V intervals up to 50 V. An extraction without the application of voltage was also executed, to confirm the advantage of the power supply in EME through the specified SLM.

Table 18. Constant parameters for extraction of TFB through 6MC:Thy with seven different levels of applied voltage.

Parameter	Description
Donor solution	300 µL of 5 µg/mL TFBc in 100 mM FA (pH 2.4)
SLM	10 µL of 6MC:Thy
Acceptor solution	300 µL of 100 mM FA (pH 2.4)
Agitation	750 RPM
Extraction time	15 minutes

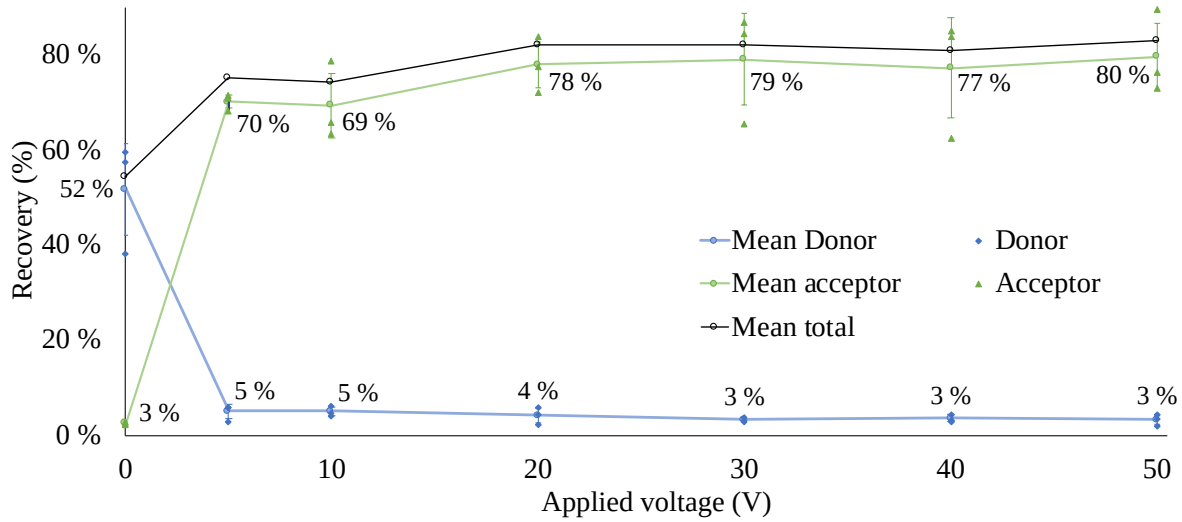


Figure 32. Results for the extraction of TFB through 6MC:Thy with variation in applied voltage. Recovery (%) of TFB in donor and acceptor solution plotted against the applied direct voltage (V). The samples were extracted at electrode pair number 4, 6 and 8.

The results in Figure 32 shows that the application of voltage is necessary to facilitate the migration of analyte into the acceptor solution. Without the application of voltage, 52 % was recovered in the donor solution and only 3% was recovered in the acceptor. The mean recovery of analyte from the **acceptor** solution increased to 70% with the application of 5 V. The green slope shows that a further increase of the applied voltage resulted in a mean recovery around 80% in the range from 20 V to 50 V. The highest single acceptor recovery was 89% at 50V, with a mean recovery of 80%. At 30 V, two of the three parallels had recoveries above 85%. The mean recovery of TFB from the **donor** solution was less than 6% for all extractions through 6MC:Thy with applied voltage.

4.6 Extraction of TFB from plasma

Plasma was obtained from the department of clinical pharmacology at Haukeland University Hospital. The plasma was diluted (1:1) with 5 M phosphoric acid and 10.5 µg/mL TFBc stock solution at pH 2.4. This yielded a donor solution of 5 µg/mL TFBc, and the pH was measured to 2.1. For preparation of the external standard, a 5 µg/mL TFBc solution with pH 2.4 was made from the same stock solution and volumes as the plasma donor solution. After extraction, the acceptor solutions were transferred to HPLC-inserts and analyzed by HPLC-UV. A detailed description of the preparations of solutions are given in Table 5. Three parallel samples were extracted simultaneously in the experiments with EME of TFB from plasma.

4.6-1 Extraction of TFB from plasma through three different SLMs

Based on the results presented in section 4.4 and 4.5, I decided to perform an extraction of TFB from plasma through the three following SLMs: 6MC:Thy, 5% DEHP in NPOE and 2.5% DEHP in NPOE. The applied voltage was set to 30 V for extraction through 6MC:Thy and 50 V for the two latter SLMs, i.e. NPOE with the addition of DEHP. Constant parameters for the extractions are listed in Table 19.

Table 19. Constant parameters for extraction of TFB from plasma through different SLMs.

Parameter	Description
Donor solution	300 µL diluted plasma spiked with 5 µg/mL TFBc (pH 2.1)
Acceptor solution	300 µL of 100 mM FA (pH 2.4)
Agitation	750 RPM
Extraction time	15 minutes

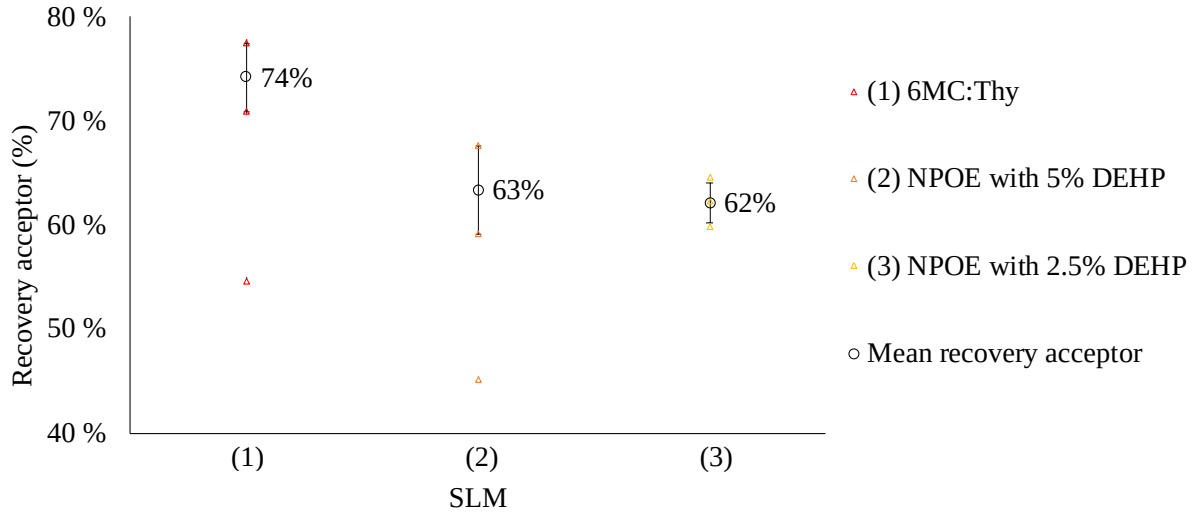


Figure 33. Results from the extraction of TFB from plasma through three different SLMs. Recovery (%) of TFB from the acceptor solution plotted against SLMs, respectively 6MC:Thy, 5% and 2.5% DEHP in NPOE. The two indicators without fill are defined as outliers. The samples were extracted at electrode pair number 4, 6 and 8.

The results presented in Figure 33 shows a mean analyte recovery above 60% in the acceptor solution for all three SLMs. The highest recovery was 77% for a single parallel with **6MC:Thy**, and with a mean recovery at 74% and SD at 3% for two parallels. The lowest recovery was 45% for a single parallel with **5% DEHP in NPOE**, but this was ruled as an outlier. The mean recovery was 63% with a SD at 4% for the other two parallels. Extraction of TFB from plasma through **2.5% DEHP in NPOE** had the lowest SD at 2% for three parallels, with a mean recovery of 62%.

4.6-2 Extraction of TFB from plasma through 6MC:Thy, extraction time

Extraction of TFB from plasma through 6MC:Thy showed the highest acceptor recovery, as presented in the section above. An experiment to investigate how the extraction time inflicted

the recovery in the acceptor solution was performed, with a desire to improve the recovery. Constant parameters for the extraction are listed below:

Table 20. Constant parameters for extraction of TFB from plasma through 6MC:Thy with four different extraction times.

Parameter	Description
Donor solution	300 µL diluted plasma spiked with 5 µg/mL TFBc (pH 2.1)
SLM	10 µL of 6MC:Thy
Acceptor solution	300 µL of 100 mM FA (pH 2.4)
Voltage	30 V
Agitation	750 RPM

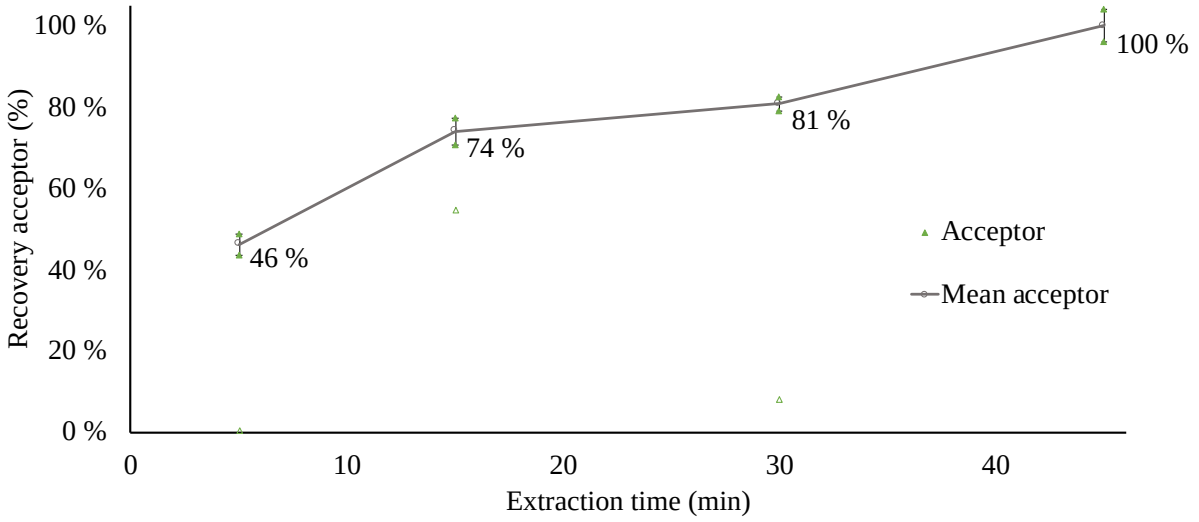


Figure 34. Results for the extraction of TFB from plasma at four different extraction times. Recovery (%) of TFB from the acceptor solution plotted against the extraction time (min) of 5, 15, 30 and 45 minutes. The samples were extracted at electrode pair number 2, 8 and 10. The extraction at 15 minutes is the same as in Figure 33. The three indicators without fill are defined as outliers. The two lowest, at 5 V and 30 V, were extracted at number 8.

The results in Figure 34 shows that an increased extraction time increases the recovery of TFB from the acceptor solution up to 100% after 45 minutes. After only 5 minutes of extraction, the mean recovery was 46%. All mean recoveries are calculated from two parallels, due to outliers and a perforated membrane at 45 minutes. The same plasma donor solution was used for the following extraction times: 5, 30 and 45 minutes. The results for the extraction time of 15 minutes are taken from the previous experiment, Figure 33.

5 Discussion

5.1 The EME setup

The EME **equipment** used in the present study was a prototype, which had already been used extensively for one year, and some parts showed signs of being worn. Loose electrodes had to be reattached to the top cover with glue. In the result section 4.1, an investigation of the EME setup was performed with emphasis on the reproducibility of the electrodes. Figure 20, presenting the acceptor recovery, shows a large inter-variability (RSD 60%) in the recovery between the samples extracted simultaneously. The donor recovery (Figure 21) shows an even greater inter-variability with an RSD at 91%. These results imply that the extraction recovery is affected by which electrode pairs were in use. Number 2, 4 and 10 appeared to yield the highest and most reliable recoveries, based on the results from section 4.1 (Figure 20), and number 1, 3, 8 and 9 appeared to yield low, unstable acceptor recoveries. Unfortunately, the investigative experiment presented in section 4.1-1 was performed at the end of experimentation. If this experiment had been executed earlier, electrode pair number 2, 4 and 10 would preferably have been used for all experiments in this study.

The initial experiments with TFB were performed with **electrode pair** number 1, 6 and 8, based on the measurement of voltage (Figure 19) and no need for reattachment. After observing repeated outliers at electrode pair number 1, number 4, 6 and 8 was used in the experiments described from section 4.4-2 and onwards. The outlier at electrode pair number 8 in Figure 34 was not discovered until the investigation of extraction time. Number 8 appeared to be stable upon that experiment. This can be confirmed by extraction «a» in Figure 20, which were performed one day prior to the extraction time samples. The outliers related to electrode pair number 1 and 8 supports the results from section 4.1-1 «Recovery variation between electrode pairs». The extraction of CsA was performed at electrode pair number 3, 6 and 9, but none of the parallels were identified as outliers.

The observed variation in recovery between parallels, and frequency of outliers that exceeded the expected recovery range can be explained by the **power supply**. The maximal applied voltage is limited by the settings on the DC power supply, but faulty wiring could lead to an on- and off voltage supply, and a breakage in the circuit would be consistent with extractions

deprived of applied voltage. This is consequently thought to be the primary cause of variation between the parallels, and the explanation of outliers. In addition, the rapid revolutions of the sample holder during agitation could also impact the wiring.

The experiments where the application of voltage was tested (section 4.4-2 and 4.5-2), shows the necessity of **direct voltage** to yield near-exhaustive extractions. The acceptor recovery is also affected by **extraction time**, as viewed in Figure 34. These results show how the acceptor recovery is affected by alternating extraction conditions. With that in mind, parallels defined as outliers due to a lower acceptor recovery or higher donor recovery, could be explained by the absence of direct voltage throughout the experiment, or an on- and off voltage supply, as suggested above. This will affect the recovery, since the actual extraction time with applied voltage would be shorter than the performed extraction time. Segments of the extraction are thought to have been performed without the application of voltage. The current curve for extraction «c» in Figure 23 showed a leap at approximately 7 minutes, which can be explained by a reconnection in the wiring.

A typical **extraction current**, like the one in Figure 14, has a sharp decrease in current during the first minute of extraction, followed by a gradual decrease until the slope stabilizes. The current curve for extraction «a» in Figure 23 does not show the same pattern, but rather a fluctuating current, which implies that the system was unstable. This could be due to unreliable voltage supply, but also other factors (e.g. SLM, vials, electrolysis), which will be discussed later. The extraction current in Figure 27 shows how a perforated membrane inflicts the sum of current in the system. When a membrane is perforated, the resistance in the system is drastically reduced. Since the applied voltage is constant, the current will increase accordingly. This relation between voltage, current and resistance is described by Ohm's law (Equation 1). A sudden leap in current, as seen for extraction «c» in Figure 23, can also be explained by this relation. If one assumes that the resistance is constant at the time, an increase in voltage results in a positive correlated increase in current.

As mentioned, perforation of the **membrane** was observed during certain experiments, as detected both by visual inspection and interpretation of the extraction current. It is difficult to pin-point the exact cause for this, but it could be that the PP-filter was broken during assembly into the union, or by the pipette while immersing the organic solution. Leakage due to insufficient assembly of the union-vial complex can also lead to unsatisfactory extractions,

but this is detected by visual inspection of the membranes after extraction. In summary, the abovementioned processes are vulnerable to user mistakes and more user-friendly procedures should bea a focus of future refinement of the equipment.

Deviations in the **SLM volume** can affect the recovery of analyte. Depending on the organic solution and its adsorbance to the pipette tip, deflecting volumes could be immobilized in the flat sheet membrane. Since the volume of organic solution is significant for the extraction efficiency, small deviations can lead to variation in the acceptor recovery. This is due to the amount of analyte that can potentially be trapped in the SLM, and the impact on electrokinetic migration.

As established earlier, a reliable application of voltage is crucial for EME. **Conductive vials** are the connection between electrodes on the top cover and aqueous solutions, which make the electrodes fully integrated. SLM residues, finger prints and other impurities can impact their conductivity. To avoid contamination of the conductive vials, all handling was done while wearing gloves. The vials in this study were new when I started the experiment series with TFB. After extraction, the vials were washed as described in section 3.4 and then reused. The washing procedure exposes the vials to alcohol and heat, but it is uncertain if this could impact the conductivity. Reuse of the vials can be a source of error that results in unsatisfactory extractions and cross contamination. Total recovery above 100% can be explained by analyte residues from previous extractions or contaminants overlapping with the analyte in the chromatogram. The vials are disposable, and it would be ideal to only use them once.

5.2 Cyclosporine A

The EME method development with CsA held several challenges. The chemical properties posed limitations in terms of ionization, pH, log P and solubility, as described in section 2.2-1. Strong basic conditions to ensure the presence of a negative charge of the CsA molecules led to the need of sample dilution to reduce the pH prior to analysis. The HPLC-UV **analysis method** represented a limitation due to a lower detection limit of 2 µg/mL. It was therefore decided to use a donor solution consisting of 20 µg/mL CsA, and attempt to concentrate the sample before analysis with HPLC-UV. A chromatogram for a 20 µg/mL CsA solution is

shown in Figure 15. The peak area of the analyte is wide, which complicates quantification of low amounts of CsA. Several different combinations of mobile phases, gradients, flow and temperature were tested, but the limitations in operating conditions for the column, i.e. pH and temperature, hampered further optimization of the HPLC-UV method. The parameters and gradient used for the separation and quantification of CsA are described in section 3.5-1. The lower detection limit of 2 µg/mL, as well as the low solubility of CsA in aqueous media, were the main limiting factors for not executing further EME experiments with CsA as an analyte.

As mentioned in section 4.2-2, CsA is known to adsorb to plastic materials. An **affinity** experiment on three different materials was performed to investigate if CsA was lost during experiments due to adsorption to the EME-vials. The results in Figure 25 show a mean 9% loss of analyte for the EME-vial compared to the HPLC glass vial. This is considered a possible problem for conduction of CsA with this EME approach and equipment.

There are limited examples on **SLMs** that are successful in EME of acidic analytes. In this study, EME of CsA was performed through 1-octanol, and the analyte trapped in the SLM was presumed to fully be extracted with methanol prior to HPLC-analysis. The results in Figure 24, shows that only 3-5% of the analyte was recovered from the SLM. This is thought to be due to the hydrophobic character of CsA. It has a log P of 3.6, a parameter which describes the distribution between octanol and water. Considering the amount of analyte (5 µg), and the volume of the SLM in comparison to the donor solution (10:250), a 5% mass recovery in the SLM is quite high. It is likely that there would be a considerable loss of CsA in the SLM when analyzing at therapeutic concentrations, and 1-octanol is therefore not an ideal choice for EME of CsA. Extraction through other SLMs, such as 6MC:Thy, could have been tested, but due to issues with high lower limit of detection using HPLC-UV, we decided not to extend the experiments with EME of CsA.

The recovery of analyte in the **donor** solutions after EME of CsA are presented in Figure 24. An observed reduction in the recovery with an increased voltage corresponds with an increase in the recovery from the SLM. The **total** recovery from the extractions shows a slight decrease with increased voltage. This could imply that more of the analyte migrated to the acceptor solution, although no CsA was detected in the **acceptor** solution. With the lower limit of detection using HPLC-UV, any recovery below 4% (0.2 µg) will not be detected. In

addition, CsA could be entangled in the undissolved salt crystals after the concentration step, or adsorbed to the inside of the EME-vial. These are likely explanations to why approximately 75-80% of the analyte was unaccounted for.

To circumvent the problems identified above, EME of CsA could have been performed with a smaller volume in the acceptor solution than in the donor solution. This would act as a concentration step. In addition, the samples could have been analyzed without the concentration and re-disperse step of the aqueous solutions using a more sensitive method for analysis, such as LC-MS.

5.3 Tofacitinib

In this study, TFB was extracted as a cation. The HPLC standard solution was used as a reference when calculating the recovery, and this standard was the exact same as the donor solution without plasma. This was to reduce the possibility of wrongful predictions of recovery due to minor variations in concentration from pipetting errors or possible decomposition of the analyte over time. The donor concentration of 5 µg/mL was prepared with TFB citrate (TFBc), as described in Table 5, and is used in the graphical presentation of the standard curve (Figure 18). That concentration is directly proportional with a 9.9 µM TFB solution, since the molarity of TFBc and TFB is equivalent. The lower detection limit of 0.05 µg/mL is related to TFBc, which can be converted to 99 nM or 31 ng/mL of TFB. The decision to consequently refer to the mass concentration of TFBc does not inflict the prediction of recovery, as that is calculated by the ratio between peak area of analyte for the sample solution and external standard (Equation 7).

In the initial experiment with TFB (Figure 26), the purpose was to investigate suitable SLMs for extraction related to **pH conditions** in the donor solution. At pH 2.4, the SLMs consisting of pure 6MC:Thy, and 10% DEHP in NPPE and NPOE appeared to be efficient, with mean recoveries above 70% in the acceptor solution. These three SLMs showed a concurrent decrease in recovery (5-25%) when pH was increased to 4.0. The TFB molecules are predicted to be fully protonated at pH-levels below 5 (27). In the event of electrolysis, the pH would have decreased in the donor solution (anode) and increased in the acceptor solution (cathode) (23). It is thus not known why the recovery decreased when pH was increased from

2.4 to 4.0 To account for the possibility of electrolysis, a low pH value of 2.4 was chosen for the acceptor solution, but an increase of pH at the cathode could lead to leakage of DEHP. A decrease of pH in the donor solution would not influence the amount of protonated analyte, but it could impact the complexation between DEHP and TFB. Unfortunately, the pH of the aqueous solutions after extraction was not measured. By measuring the pH of the donor and acceptor solutions, potential changes in the pH during extraction could have been detected and further associated with the possibility of electrolysis.

Unlike the three SLMs discussed above, the pure **nitroaromatic SLMs**, i.e. NPPE and NPOE, appeared to yield higher acceptor recoveries with an increased pH of 7.4, but the mean recovery was still below 10 %. As shown in Figure 26, NPPE had overall a higher mean recovery than NPPE. This is thought to be explained by the difference in log P. NPPE has a log P value of 3.5, which is lower than the log P for NPOE of 4.9, and would therefore be more applicable for polar compounds (30). The applied voltage should probably have been higher for the extraction through pure NPPE and NPOE, e.g. 100 V, to increase the recoveries, but extraction of polar substances is known to be difficult without the addition of ionic carrier (IC) (28). DEHP has been the preferred anionic IC in combination with nitroaromatic solvents for the extraction of polar basic analytes. DEHP has also proven to increase the efficiency of extractions with DES of coumarin and thymol (33). The results presented in Figure 26 show how the addition of 10% DEHP in NPOE and NPPE drastically increased the acceptor recovery with approximately 70 % at pH 2.4.

As mentioned earlier, the complexation between **DEHP** and analyte is affected by the pH in the donor solution. Donor pH and fraction of DEHP should therefore have been optimized together (28). DEHP has a predicted pKa of 1.94, and an increase of pH leads to a greater fraction of ionized molecules with a negative charge (40). Consequently, the solubility in water increases and leakage of DEHP into the donor solution can be observed (39). At pH above 4, the leakage of DEHP from the SLM was substantial. This leakage was pH dependent only, and not influenced by changing voltage.

In the study by Hansen et al (39), leakage into the donor solution was shown to be beneficial for extraction of highly polar compounds with log P below 0.7 and pKa above 9, especially for cationic analytes. The leakage of DEHP to the aqueous solutions was highest at pH 7.0, due to the increase of ionized molecules. This made the IC more prone for ion-pairing with

the analyte, which lead to more efficient extractions. The results presented in Figure 26 show the opposite trends for extraction of TFB through 10% DEHP in NPPE and NPOE: an increased pH led to a reduction in acceptor recovery of the analyte. TFB has a log P of 1.1 and a pKa of 7.5 (27), which is outside the conformed range in the study (39), and may explain the opposite conclusion. Unfortunately, extractions through 10% DEHP in NPPE and NPOE at pH 7.4 was not performed, but judging from the results in Figure 26, it is likely that the recovery would have been further reduced with an increase in pH.

The impact of different fractions of **DEHP in NPOE** are viewed in Figure 28 and 29. With the addition of only 0.125% DEHP, the mean acceptor recovery of TFB increased with 27% compared to pure NPOE. The acceptor recovery proceeded to increase with an enlarged amount of DEHP upon 5%. When the fraction of DEHP elevated beyond 5%, the acceptor recovery appeared to decrease. The total recovery of analyte showed a negative correlation to the fraction of DEHP, which implies an increasing amount of TFB being trapped in the SLM due to the formation of ion-analyte complex.

The impact of **DEHP in 6MC:Thy**, shown in Figure 31, exposed a large negative correlation between the amount of DEHP and recovery of TFB from the acceptor solution. The addition of DEHP led to a decrease in both the acceptor and total recovery. 6MC:Thy possesses both HBD and HBA properties, but π-interactions due to the aromatic character are thought to be dominating (50). DEHP leads to an addition of ionic interaction with the protonated analyte. TFB also has recognized HBD and HBA properties, in addition to the aromatic system and carrying a positive charge at low pH levels (27). The sum of all these interactions could lead to extensive entrapment of the analyte in the SLM, as indicated by the results in Figure 31, but prolonged extraction time can reduce the entrapment (34).

When comparing the results discussed in the two previous paragraphs, the impact of small fractions of DEHP (0-5%) on entrapment of the analyte appears to be completely dependent on the composition of the SLM. The addition of IC to NPOE increased the extraction efficiency of TFB, but the addition of IC to 6MC:Thy considerably reduced the efficiency. A common denominator was the entrapment of analyte in the SLM with increasing amount of DEHP.

The results in Figure 30 presents the influence of **applied voltage** for the extraction of TFB through 5% DEHP in NPOE. It indicates that an applied voltage above 40 V is necessary to facilitate the migration of analyte out of the SLM and into to the acceptor solution. This is due to the concave characteristics for the total analyte recovery at 0-40 V, which implies that the residue of undetected analyte trapped in the SLM is following a convex curve in the same range. Nevertheless, it appears to be a positive correlation between the applied voltage and acceptor recovery, as described by the green slope in Figure 30. Yet, the increase in recovery seems to abate with a voltage beyond 40 V. Extractions with applied voltage above 50 V could have been performed to confirm this, but an increase in voltage would lead to a higher current in the system and an increased risk of electrolysis.

The impact of **applied voltage** for the extraction through 6MC:Thy is shown in Figure 32. Unlike the extraction described in the previous paragraph, the amount of analyte trapped in the SLM appeared to be relatively stable at any given voltage in the range from 5-50 V. A slight increase of 10% was observed between 10 V and 20 V. A further increase in the applied voltage beyond 50 V is not expected to increase the recovery, but rather increase the risk of electrolysis, as explained earlier. I decided to continue to use the applied voltage at 30 V for further experimentation with 6MC:Thy, due to two parallels distributed above the mean acceptor recovery of 79%. One could argue if the lowest parallel at 30 V could be ruled as an outlier. In that case, the mean recovery would have been 86%, which is considered as an exhaustive extraction recovery (25).

However, a drastical enrichment of analyte in the acceptor solution was observed when comparing extraction without the application of voltage to the extraction with only 5 V. This confirms the benefits of EME in comparison to original LLE. On that note, the results of inter-variability of acceptor recovery between electrode pairs, as presented in Figure 20, is verified to originate from an alternating rather than direct supply of voltage. Especially the recovery of 3 % at electrode pair number 1 in Figure 20, is directly proportional to the acceptor recovery for an extraction without the application of voltage, as shown in Figure 32.

Prior to the final optimization of extraction conditions, with regard of extraction time, three promising SLMs were tested for the extraction of TFB from diluted plasma, namely 6MC:Thy and NPOE with either 5% or 2.5% DEHP. All three SLMs gave mean acceptor recoveries above 60% (Figure 33). The highest mean recovery was observed for 6MC:Thy with 74%.

When compared to similar extraction conditions from an aqueous solution (Figure 32, 30 V), the mean acceptor recovery reduction was only 5%. For NPOE with 5% and 2.5% DEHP (Figure 33), the reductions were 11% and 12% respectively. The pH in the diluted plasma solution was 2.1 instead of 2.4, which will impact the ionization of DEHP. This could lead to reduced ion-analyte complexation and a reduction in recovery. However, a decrease in acceptor recovery when switching from aqueous solutions to plasma is not uncommon, due to components in the plasma, like lipids and proteins, which can influence for instance the SLM surface in the donor vial.

To gain the highest extraction efficiency, parameters should be optimized upon extraction from the matrix of interest (28). In this study, only the extraction time was optimized with extraction from diluted plasma. Other combinations of extraction parameters could possibly yield exhaustive extractions with a shorter extraction time, but as presented in Figure 34, EME of TFB from plasma through 6MC:Thy with a 45-minute extraction time was successful with a 100% mean recovery of analyte in the acceptor solution, which is considered an acceptable recovery in routine laboratories (28).

Trends in analyte recovery for the extraction of TFB

- Equipment failure: certain electrodes gave unreliable results due to alternating voltage supply.
- The SD for analytes recovered from the acceptor solutions were rarely above 10%.
- The addition of DEHP to NPOE increased the acceptor recovery to a certain level, but higher fractions of DEHP decreased the total recovery due to entrapment of analyte in the SLM. The addition of DEHP to 6MC:Thy lead to a reduction in recovery.
- Increasing the applied voltage increased the recovery up to a certain level.
- The acceptor recoveries after extraction from diluted plasma were reduced compared to the aqueous solutions without matrix components in the donor solution.
- Increased extraction time increases the recovery.

6 Future perspectives

EME has the potential to provide results comparable with hospital routine methods for sample preparation, and data in compliance with regulatory requirements (25). If an EME procedure is to be developed for the extraction of CsA, the loss of analyte due to adsorption to the vial must be taken into consideration. The lower limit of detection for the analysis method by HPLC-UV of CsA was a limiting factor in this study. With the utilization of an analysis method with a lower detection limit of e.g. 20 ng/mL, further experimentation with EME of CsA can be performed, and the solubility of CsA in aqueous media would no longer be an issue. Enrichment of the acceptor solution by adjusting the volumes can also benefit the detection of low analyte concentrations in the sample. Testing other SLMs would be a crucial step towards developing an EME method for the extraction of CsA. DES have given efficient extractions of acidic analytes (33), and SLMs, like 6MC:Thy, with high hydrophobicity and HBD properties, may yield sufficient extractions of CsA. The investigation of candidate ICs would also be of interest. However, strongly alkaline conditions will still be required to ensure ionization.

Optimization of the SLM and other extraction conditions for the EME of TFB would also be of interest in the future. Even though an efficient extraction method for TFB with high recovery was developed in this study, there are some parameters that could be improved. The extraction time of 45 minutes is rather long, and it would be desirable to optimize extraction conditions which gives shorter extraction times. For instance, the impact of the volume of SLM could be investigated.

If EME is to be implemented as a method for drug analysis in routine TDM, the prototype equipment must be commercialized. The possibility of high throughput already exist in a 96-well plate format (24), but atomization of the EME procedure, and development of equipment that retains the advantages of disposable conductive vials and enrichment due to regulation of the volume ratio between donor and acceptor solutions are still needed. New and improved equipment that can easily be adjusted to a variation of conditions, and that matches automated sample handling system would also be of interest. This will reduce the risk for both technical and mechanical errors, as well as human errors.

7 Conclusions

In the experiments conducted in this project, a systematic approach for developing EME methods for the immunosuppressive drugs CsA and TFB were performed for the first time.

No satisfactory EME method for the extraction of CsA was developed in this study. CsA was not detected in the acceptor solution, and the highest mean recovery of analyte from the donor solution and SLM was 24% at 20V. However, the main challenge was not the acidity of the analyte, but rather limitations of the HPLC-UV analysis method and the low solubility of the analyte in aqueous media. Further investigation of optimal extraction conditions and an analysis method with lower detection limit should be the focus for the establishment of an EME procedure that can provide results comparable with hospital routine methods, like CEDIA.

TFB, on the other hand, appears to be suited for EME, and during method development, a protocol for high recovery of the analyte was established. By using an SLM with IC consisting of 5% DEHP in NPOE at 50 V and pH 2.4, a recovery of 89% in the acceptor solution was obtained after 15 minutes of extraction. In addition, extraction through 6MC:Thy gave reliable results of 79% acceptor recovery at 30 V. When TFB was extracted from plasma, the highest recovery was 74% with the latter conditions. By prolonging the extraction time from 15 to 45 minutes, a recovery of analyte in the acceptor solution of 100% was achieved.

The results obtained in this study demonstrates that TFB can be successfully extracted from human plasma with excellent precision using electromembrane extraction at optimal conditions. The method involves low-cost, commercially available ingredients, and represents a promising sample preparation technique for TFB. Further optimization of the extraction parameters is necessary to make it even more effective in terms of extraction time.

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