# Electromembrane extraction of methotrexate and its metabolites

Master thesis in pharmacy

*Anne Oldeide Hay*


Centre of pharmacy and Department of Clinical Science

University of Bergen

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# Abstract

Within pharmaceutical analysis, sample preparation is essential to make a sample compatible with the chosen analytical instrument, prevent contamination and damage of the instrument, and to avoid interference from matrix substances in biological samples. Electromembrane extraction (EME) was developed in the mid-2000s, and has proved to be an efficient sample preparation technique for several analytes. It is based on transfer of electrically charged analytes from an aqueous donor solution, across an organic solvent (SLM), and into an aqueous acceptor solution. There are several advantages with EME, including the possibility of rapid extractions, high sample clean-up and enrichment, high selectivity, low consumption of organic solvents, and pre-concentration of the analyte.

To this date, most studies on EME have been performed with non-polar, basic analytes. In the present study, EME was for the first time used for sample preparation of methotrexate (MTX) and its metabolites 7-hydroxymethotrexate (7-OH-MTX) and 2,4-diamino-N10-methylpteroic acid (DAMPA). These are polar, acidic, and zwitterionic analytes, all physicochemical properties that are little explored with EME.

For MTX method development, a range of different conditions were tested and optimized in order to yield high analyte recoveries. The extracted samples were analyzed using HPLC-UV during method development. MTX was extracted as either positively or negatively charged, with subsequent adjustments of pH in the donor/acceptor solutions and composition of the SLM. Due to the polarity of the analyte, an ionic carrier was added to the SLM. The highest recovery (79.6%) was achieved when MTX was extracted as an anion, using a 40 mM phosphate buffer with pH 7.4 as the donor solution, 10 mM NaOH with pH 12 as the acceptor solution, and peppermint oil + 1% aliquat 336 as the SLM. The same method yielded recoveries of 59.0% 7-OH-MTX and 32.4% DAMPA in the acceptor solution.

This method could not be applied to a donor solution containing a physiological concentration of $Cl^-$, due to an interaction between the chloride ions and the cationic carrier aliquat 336. Therefore, MTX was extracted as a positively charged analyte from plasma, using an anionic carrier for transport across the SLM. The extracted plasma samples were analyzed using LC-MS/MS, and the method yielded 5.5% recovery of MTX in the acceptor solution.

Taken together, MTX should be extracted as an anion to achieve high recovery, and the addition of an ionic carrier is essential for transport across the SLM. Of all the conditions tested, results pointed towards the most optimal donor/acceptor solutions, SLM, ionic carrier, and settings for voltage, time, and agitation. However, further experimental work is required in order to improve EME of biological samples, particularly to identify an ionic carrier which has low interference with anionic electrolytes in plasma.

# Preface

First and foremost, I would like to thank my supervisors Lars Herfindal and Silje Skrede. I truly appreciate your help and guidance throughout this year. Whether it was lab related, or theoretical and writing inputs, you always took your time to assist me. I felt motivated by your trust in me, and by the fact that you gave me the opportunity to work independently. There was a lot of trying and failing along the way, but it made me learn much about the process of research, and I am grateful for you letting me be a part of this exciting project.

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Finally, I have to thank my family, friends, and Erlend for moral support and motivation all year. You were always just a phone call away, and it was comforting knowing I could always reach you.

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# Abbreviations

| | |
|---|---|
| 7-OH-MTX | 7-hydroxy-Methotrexate |
| BEA | Bis-(2 ethylhexyl)amine |
| DAD | Diode array detection |
| DAMPA | 2,4-diamino-N10-methylpteroic acid |
| DC | Direct current |
| DEHP | bis(2-ethylhexyl) phosphate |
| DHFR | Dihydrofolate reductase |
| dTMP | Thymidine monophosphate |
| EME | Electromembrane Extraction |
| $FH_4$ | Tetrahydrofolate |
| G6PDH | Glucose-6-phosphate dehydrogenase |
| GC | Gas chromatography |
| HF-LPME | Hollow fibre Liquid phase microextraction |
| HPLC | High performance liquid chromatography |
| IS | Internal standard |
| LC | Liquid chromatography |
| LC-MS | Liquid chromatography-mass spectrometry |
| LLE | Liquid-Liquid extraction |
| LPME | Liquid phase microextraction |
| MEC | Minimum effective concentration |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry (triple quadrupole mass spectrometruy) |
| MTC | Minimum toxic concentration |
| MTX | Methotrexate |
| NPOE | 2-nitrophenyl octyl ether |
| NPPE | 2-Nitrophenyl pentyl ether |
| PBS | Phosphate buffered saline |
| PP | Polypropylene |
| RF | Radio frequency |
| RCF | Relative centrifugal force |

| | |
|---|---|
| RPM | Revolutions per minute |
| RSD | Relative standard deviation |
| SDME | Single-drop microextraction |
| SLM | Supported liquid membrane |
| SPE | Solid Phase extraction |
| TDM | Therapeutic drug monitoring |
| UV | Ultraviolet |

# 1 Introduction

## 1.1 Methotrexate

Methotrexate (MTX) was developed as an anticancer agent in 1940 (1). Today, it is used to treat a range of different diseases (2). In low doses, it is effective against several autoimmune diseases, such as rheumatoid arthritis and psoriatic arthritis. In high doses (> 500 mg/m$^2$), it is applied to treat cancers like adult and childhood acute lymphoblastic leukemia, malignant lymphoma, and osteosarcoma. MTX is an antimetabolite, which means that it works by inhibiting processes involved in synthesis of DNA or its nucleotide building blocks (3). MTX inhibits dihydrofolate reductase (DHFR), an enzyme important to maintain adequate amounts of the enzyme cofactor $FH_4$ (tetrahydrofolate). In cells deprived for $FH_4$, the synthesis of the DNA building block thymidine monophosphate (dTMP) would stop, leading to slower DNA synthesis and cell division. DHFR reduces folic acid into $FH_2$, and further reduces $FH_2$ into $FH_4$. MTX inhibits both processes.

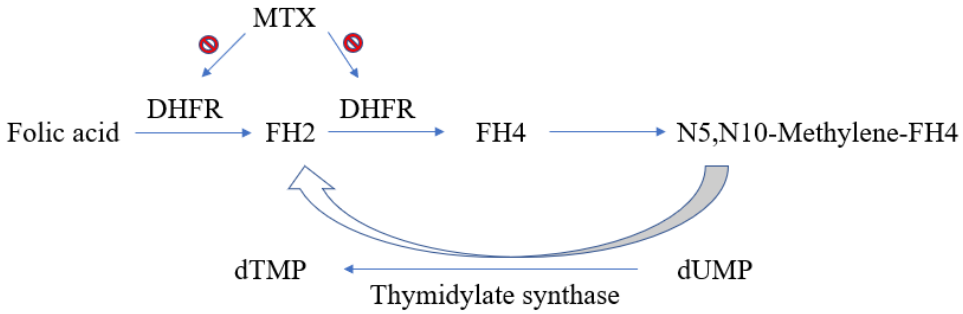



**Figure 1.** Mechanism of action of MTX. Adapted from (3).

Most of the administered dose of MTX (60-90%) is eliminated unchanged in the urine (2). In the liver, MTX can be converted into the metabolite 7-hydroxymethotrexate (7-OH-MTX). This compound is less soluble than MTX and may contribute to nephrotoxicity, which will be covered in the next paragraph. To a lesser extent, MTX is metabolized in the intestine to the non-toxic 2,4-diamino-N10-methypteroic acid (DAMPA).

High dose MTX may cause significant toxicity to some patients (4). Acute kidney injury is a serious condition that may arise due to precipitation of MTX and 7-OH-MTX in the renal tubules. As a result, reduced clearance and resultant accumulation of toxic concentrations of

MTX and 7-OH-MTX may occur. This can further worsen the injury to the kidneys and increase the risk of additional adverse side effects, such as myelosuppression, mucositis, and hepatotoxicity. The incidence of acute kidney injury depends on preventive measures, the dose and schedule of MTX, and individual pharmacokinetic variations among patients. Both MTX and 7-OH-MTX are acidic and poorly soluble at acidic pH. Alkalization of the urine will therefore greatly increase the solubility and elimination of MTX and 7-OH-MTX, and it is recommended to administrate fluids containing sodium bicarbonate during and after administration of high dose MTX. Many patients experience reduced intravascular fluids due to vomiting and diarrhea, leading to reduced urine production. Hydration is therefore an important strategy to prevent concentrated urine and nephrotoxicity. Folinic acid is a derivate of folic acid, administered to protect normal cells from toxicity. It competes with MTX over the binding site on DHFR, allowing the formation of $FH_4$. Since folinic acid neutralizes the effect of MTX, the two agents must not be taken simultaneously, since this will reduce the anticancer effect of MTX.

## 1.2 Therapeutic drug monitoring of MTX

Although the dose of MTX needs to be kept low enough to avoid toxicity, it must be sufficiently high to provide the desired anti-cancer effect (2). MTX has a narrow therapeutic window, which means that the range between minimal effective concentration (MEC) and minimal toxic concentration (MTC) is small. It is therefore necessary to keep the serum concentration within this range. Although MTX is administered in a fixed dose and duration, individual differences between patients can contribute to varying serum concentration of MTX. These differences, or host factors, may be age, gender, renal and hepatic function, and comorbidities. A patient may also be using other medications that can interact with MTX and contribute to delayed elimination. To ensure that the dose of MTX is below the MTC, but still high enough to overcome the MEC, therapeutic drug monitoring (TDM) is performed.

TDM aims to ensure the optimal dosage of a drug to each patient, by analyzing the serum- or plasma drug concentration and comparing it to a target range (5). Most drugs have a relatively large therapeutic window, which means that the range between MEC and MTC is broad. The risk of toxicity is therefore small, and TDM is not a required practice. TDM is neither necessary when the therapeutic effect can be measured by other means. For example, the

blood pressure of a patient gives a clear indication to whether an antihypertensive drug is dosed correctly. Some of the main cases where TDM is performed are listed below (5):

1. When there is an experimentally determined relationship between the plasma drug concentration and the pharmacological effect. TDM is beneficial when individual pharmacokinetic and pharmacodynamic variations between patients can lead to differences in dose-response relationship.
2. To avoid toxicity and lack of effectiveness for drugs with a narrow therapeutic window. Both lack of effect and toxicity may put a patient at risk, and TDM can help prevent this outcome.
3. For patients with problems related to drug compliance. TDM can be performed to examine whether a patient has taken the prescribed dose of drug by analyzing serum values.

As already mentioned, the first two points address the importance of monitoring high dose MTX. However, these aspects, including the third point, are also relevant to patients treated with low dose MTX. Approximately 40% of rheumatoid arthritis patients show no clinical improvement in response to MTX (6). Reasons for this can be individual variations in MTX absorption and metabolism, non-compliance, or prescription of insufficient MTX dose. Increases in dosage due to lack of effectiveness must be performed with caution, because it may in turn lead to increased toxicity. TDM is therefore an option to identify which patients will respond well to the drug, so that the correct drug can be established early. If a patient is non-compliant or experiences no effect from the drug because of low serum levels, TDM can prevent switching from MTX to other, more expensive medications on the incorrect assumption that lack of therapeutic response is due to poor efficacy rather than insufficiently high drug plasma levels.

## 1.3 TDM in patients receiving high dose MTX

TDM of MTX is a common practice in many hospitals in Norway (7). Haukeland University Hospital performs routine TDM in patients receiving high dose MTX. The time after administration and number of measurements differ according to dose, duration of infusion and the clinical status of the patient. Serum samples are analyzed by a homogeneous enzyme immunoassay method called ARK Methotrexate Assay (8). In principle, two reagents are added to the serum sample. One contains methotrexate labeled to the enzyme glucose-6-

phosphate dehydrogenase (G6PDH), and the other contains rabbit polyclonal antibodies to methotrexate. MTX from the serum sample will compete with MTX labeled to G6PDH for binding to the antibodies. If the latter binds, enzyme activity decreases. If the serum MTX binds, the enzyme activity increases, and the activity is directly proportional to the drug concentration. The active enzyme will convert the coenzyme NAD to NADH, which is measured by photometry.

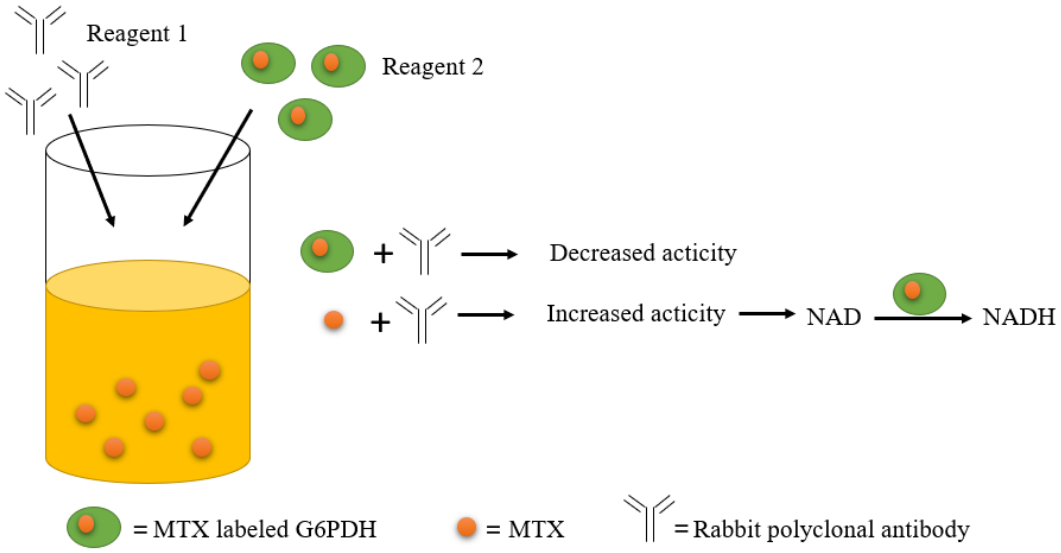



**Figure 2.** Principle of the ARK Methotrexate assay.

The immunoassay method is effective for routine TDM (8). It is fast and does not require sample preparation. The measurement range is 0.04-1.20 µmol/L, and higher concentrations must be diluted prior to analysis. The method shows linearity and high recovery (mean percentage recovery = 104.2%) within the measurement range. Despite the advantages of the ARK Methotrexate Assay, it has one specific limitation that will be discussed in the next section.

Glucarpidase is an antidote administered to patients with delayed MTX clearance due to impaired renal function (9). For example, for a dose of 8-12 g/m$^2$ MTX infused over 6 hours or less, and the 42-hour concentration is above 10 µM, glucarpidase may be indicated. The antidote works by cleaving extracellular MTX into the non-toxic metabolites DAMPA and glutamate (4). The ARK Methotrexate Assay is not able to distinguish MTX from the DAMPA metabolite within 48 hours after glucarpidase administration. This is due to a cross reaction between DAMPA and MTX, leading to a false elevated estimation of the MTX concentration. With the current technology, only a chromatographic method, like high

performance liquid chromatography (HPLC), can separate the two compounds and estimate the true MTX concentration.

At present, samples from patients treated with glucarpidase are sent from Haukeland University Hospital to Rikshospitalet in Oslo for analysis. In a conversation with Anders M. Andersen from Oslo University Hospital (February 2021), it was explained that they receive up to 5 samples yearly, but the number varies. Since 1990, a high-performance liquid chromatography-ultraviolet detection (HPLC-UV) method has been applied for analysis of MTX and DAMPA, but a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method is now fully developed and pre-validated. Mass spectrometry (MS) is a highly valuable detector because of its high selectivity, and ability to detect very low concentrations of analytes (10). The theory behind HPLC-UV and LC-MS/MS will be covered in section 2.2-2.4. Hence, MTX is separated from the DAMPA metabolite prior to detection, resulting in a more reliable measurement of MTX concentration.

## 1.4 TDM in patients receiving low dose MTX

In addition to sample analysis from patients treated with glucarpidase, the new LC-MS/MS method in Oslo will be applied to routine TDM for patients that receive low dose MTX. The measurement range of this method is 0.1 to 24 nmol/L. The therapeutic range is not defined yet, and will depend on the dose, and the time between administered dose and analysis, according to an e-mail from Anders M. Andersen in February 2021. In Haukeland University Hospital, routine TDM is not performed on patients who receive low dose MTX because the serum concentration levels are below the detectable limit of the immunoassay method (11).

Regardless of whether HPLC-UV or LC-MS/MS is the chosen analytical technique used for TDM of MTX, sample preparation is required. According to the e-mail from Andersen, Rikshospitalet in Oslo applies protein precipitation (PP) as their standard sample preparation method prior to HPLC-UV, achieving approximately 70% recovery of the analyte. For the new LC-MS/MS method, PP will also be used, but the degree of recovery is not yet established. The importance of sample preparation, and differences between PP and other methods will be covered in the following sections.

## 1.5 Sample preparation

Bioanalysis involves the identification and quantification of a compound (e.g., drug substance) in a biological sample, such as human blood, saliva or urine (12). As well as being performed in hospitals for TDM, bioanalysis can be applied to different areas. It is significant in drug development and clinical testing in pharmaceutical industry. In forensic and doping laboratories, bioanalysis can reveal recreational drug abuse, or drug abuse in sports. The process of bioanalysis can be divided into three steps: sample preparation, analyte separation, and detection (13).

Sample preparation is the first step in bioanalysis, and it is applied because most biological fluids are too complex to be injected directly into an analytical instrument. There are several reasons for this, and some of them are mentioned below (14):

1. Biological fluids can contain matrix substances that suppress, or falsely elevate the target analyte signal.
2. Biological fluids can contain matrix substances that contaminate the analytical instrument.
3. The biological fluid is incompatible with the analytical instrument because it is aqueous.
4. The concentration of target analyte is too low to be detected by the instrument.

The international Union of Pure and Applied Chemistry (IUPAC) compendium of Chemical Terminology ("Gold book") defines matrix effects as "The combined effect of all components of the sample other than the analyte on the measurement of the quantity. If a specific component can be identified as causing an effect then this is referred to as interference" (15). The cross reaction between DAMPA and MTX in the ARK Methotrexate Assay, where the signal for MTX is falsely elevated due to presence of DAMPA, is an example of interference that may have clinical significance. A way to approach this problem without sending the samples to Oslo for chromatographic analysis would be to apply a sample preparation method which removed DAMPA from the sample prior to analysis. However, there is currently no such technique in use. Nevertheless, it would be of interest to measure both MTX and DAMPA quantitatively, and observe the relationship between them, after administration of glucarpidase.

Matrix effects can also cause drawbacks in LC-MS instruments, where ion suppression is a common problem (16). The mechanism is not fully understood, but it is believed that endogenous compounds (salts, carbohydrates, amines, urea, lipids, peptides, or other organic molecules) enter the mass spectrometer (MS) at the same time as the target analyte. These compounds may increase the viscosity and surface tension of the droplets produced by electrospray ionization (ESI), which will reduce the ability of the target analyte to enter the gas phase. In turn, this will impair its detection.

Another problem related to the complexity of biological fluids, is that the sample may contain components that can damage the instrument or reduce its performance over time (17). For example, serum or plasma samples can usually not be injected directly into a liquid chromatography (LC) system, because the samples contain proteins that can contaminate and clog the columns. LC-MS instruments are susceptible for contamination, for example by non-volatile compounds accumulating in the ion source (18). This requires more frequent instrument maintenance to avoid signal suppression.

The sample must be compatible with the instrument of choice (14). All biological fluids are aqueous, and aqueous solutions are readily compatible with an LC-system. If a target analyte is better suited for gas chromatography (GC) analysis, it should be transferred to an organic solvent prior to injection into the system. This is because the sample must evaporate in order to be separated and detected by GS (19). Typical analytes analyzed by GC are volatile, small, and nonpolar compounds. Most pharmaceuticals are either relatively polar or too large to be evaporated by GC, and LC is therefore more frequently applied in bioanalysis than GC.

The target analyte in biological samples may be present in concentrations that are too low for the instrument to detect (20). To achieve higher concentrations, a sample preparation method can be applied, where the target analyte is extracted from a sample solution into a smaller volume acceptor solution. This causes pre-concentration of the target analyte and will enhance the detection signal. However, today’s LC-MS instruments are highly sensitive and can detect very low concentrations, and pre-concentration has therefore become a less important step in the process of sample preparation (14).

## 1.6 Sample preparation methods

Sample preparation is often the most time consuming and laborious step of bioanalysis, which emphasizes the importance of choosing an optimal protocol (1). The most common sample preparation techniques used prior to LC-MS analysis are protein precipitation (PP), solid phase extraction (SPE) and liquid-liquid extraction (LLE) (12). These methods are continuously optimized and validated to improve the quality of the LC-MS analyses.

Protein precipitation is useful to remove proteins in a sample prior to separation in LC (21). A precipitant, typically methanol, acetonitrile, or trichloroacetic acid, is added to the sample, and the mixture is shaken, leading to precipitation of proteins. The mixture is further centrifuged, and the supernatant is collected for analysis. PP is a rapid procedure, requires minimal equipment, can be automatized, and the method development is relatively simple (14). However, it provides limited sample clean-up. A complex mixture of endogenous compounds will remain. One example is phospholipids, which are particularly known to cause ion suppression in LC-MS, affecting the reliability of quantitative measurements. In addition, the sample is diluted due to addition of precipitant, which will lower the concentration of the target analyte.

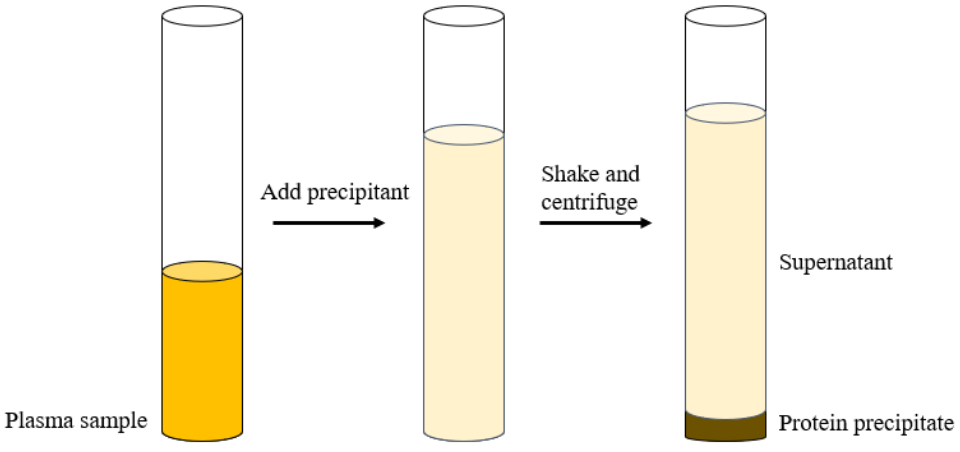



**Figure 3**. Principle of protein precipitation. Adapted from (14).

Alternatively, a drug can be isolated from a sample by extraction, which gives a purer extract (14). In solid-phase extraction (SPE), an extraction column is packed with a stationary phase. When the sample is applied to the column, some analytes interact with the stationary phase

and are retained. A washing step is then applied, where additional matrix substances elute, while the analytes of interest still interact with the stationary phase. Lastly, a suitable liquid is applied to the column to break the interactions between the analyte and stationary phase, and the final solution is collected for further analyses. This extract contains the analyte and is free of major matrix substances.

Liquid-liquid extraction (LLE) is based on the transfer of a target analyte from the aqueous biological sample into an organic solvent (14). The organic solvent is immiscible with water and will form a two-phase system with the aqueous biological sample. The samples are vigorously mixed, and the exchange of analytes occur in the interface between the organic and aqueous phase, see Figure 4. The distribution of analytes between the two liquid phases depends on their partition ratios. To obtain high partition ratios towards the organic phase, the organic solvent must be carefully selected for the particular analyte, facilitating molecular interactions between the analyte and the organic solvent. In general, compounds with low polarity are best suited for LLE. Since many substances of interest are either acidic or basic, the pH value of the biological sample needs to be adjusted to maintain the analyte in a neutral state. This is because ionized analytes are more soluble in water than in an organic solvent. Acidic substances should therefore be extracted from a sample with a pH value at least two units from the pKa-value of the analyte.

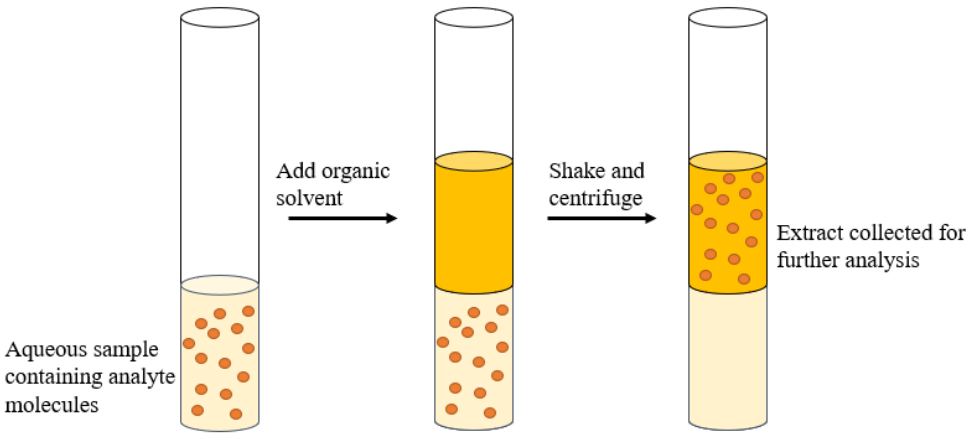



**Figure 4.** Principle of liquid-liquid extraction. Adapted from (14).

## 1.7 Development of Electromembrane extraction

Research on microextraction techniques is a very active field (12). Compared to traditional LLE, liquid phase microextraction (LPME) provides several advantages. The consumption of organic solvent is considerably reduced, leading to a “green chemistry” approach. Effective pre-concentration is possible, due to transfer of analytes into a few microliters of liquid. Different LPME-systems aim to simplify the extraction approach, increase efficiency, selectivity, and sample cleanup (20). Also, the potential of automation is an area of interest. Automation of microextraction techniques will make the analyses more accurate and repeatable, as well as increasing the overall efficiency.

The first LPME technique was single-drop microextraction (SDME), introduced in 1996 (12). In SDME, the organic solvent phase consists of a few microliters and is located at the tip of a micro-syringe needle. Normally, the droplet is lowered into the sample solution. The two phases are immiscible, but stirring the sample will promote mass transfer of the analyte from the sample solution into the droplet. The operation is very simple, but a major drawback is that the droplet can be lost to the sample.

Over more than two decades, other LPME alternatives have evolved, including hollow fiber LPME (HF-LPME) (13). In HF-LPME, the analytes are extracted from the sample solution through an organic solvent, and further into a few microliters of acceptor solution (Figure 5). The organic solvent, named supported liquid membrane (SLM), is immobilized by capillary forces in the pores in the wall of a hollow fiber membrane. It comprises only a few microliters of organic solvent and is immiscible with water. The acceptor solution is located inside the lumen of the hollow fiber membrane (12). It can be either organic (two phase system) or aqueous (three phase system). The aqueous acceptor phase is compatible with LC. This is a notable advantage compared to traditional LLE, where the acceptor phase is an organic solvent and must be reconstituted in another liquid before injection into LC (14). A three-phase system also noticeably enhances method selectivity because the sample solution and acceptor solution is separated by a third layer immiscible with both phases (20).

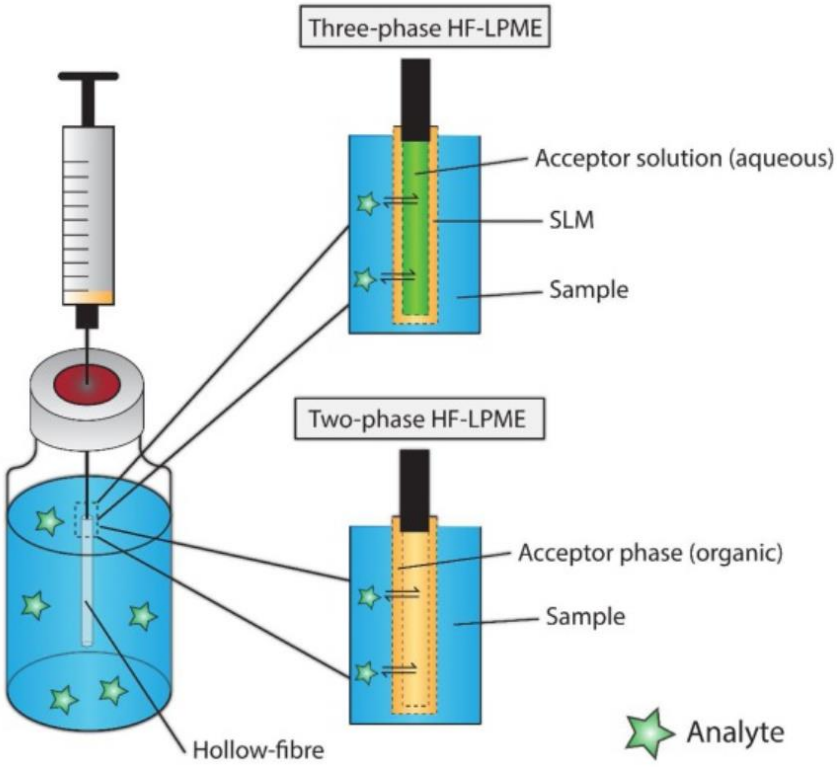



**Figure 5.** Principle of HF-LPME. Downloaded from (12).

In HF-LPME, the driving force for mass transfer through the supported liquid membrane is passive diffusion, achieved by a strong pH-gradient (22). For basic analytes, donor solution must be basic, and the acceptor solution must be acidic. The substance will diffuse into the SLM based on its donor solution-membrane distribution constant, and be further extracted into the acceptor solution. The acidic acceptor solution facilitates ionization of the substance once it reaches the acceptor side of the SLM (23). Since ionized substances are more soluble in aqueous solutions, the substance will be trapped in the acceptor phase.

Electromembrane extraction (EME) was developed in 2006 (13). Like HF-LPME, analytes are extracted from a donor solution across the SLM, into an acceptor solution. However, instead of mass transfer being based on diffusion, an electrical field facilitates electrokinetic migration of the analytes. Electrodes are placed in the donor and acceptor solution, coupled to an external power supply. When extracting acidic analytes, the negatively charged electrode (cathode) is located in the donor solution and the positively charged electrode (anode) in the acceptor solution. The pH in the donor and acceptor solutions is adjusted to a level where the analytes are ionized (24). For acidic analytes, this means that the pH must be above their pKa-value to assure de-protonation of the molecules. This will cause electrokinetic migration

towards the electrode with opposite charge. For basic substances, the electrical field and pH requirements are reversed. The theory of EME will be covered in detail in section 2.1.

There are several advantages with EME (25). If the volume of the acceptor solution is smaller than the donor volume, this will cause pre-concentration of the analytes. EME has the potential to provide high selectivity and sample clean-up. The SLM prevents polar matrix components from entering the acceptor solution, and the electrical field prevents matrix components of opposite charge from entering the acceptor solution. Only a few microliters of SLM are required per sample, which provides a very low consumption of organic solvent. Compared to traditional LLE, the acceptor phase is aqueous and directly compatible with liquid chromatography. Organic solvent evaporation and reconstitution procedures are therefore not necessary. Also, EME can give faster extractions compared to HF-LPME. In HF-LPME, the extraction time is typically 30-60 minutes. In EME it is shorter, usually 5-20 minutes (26).

However, more research into several aspects of EME is needed (27). For instance, the selection of appropriate SLMs is a challenging and crucial part. For non-polar and basic substances, stable SLMs are available. 2-nitrophenyl octyl ether (NPOE) has been the predominant SLM since 2006, and is ideal for basic analytes with $\log P_{OCTANOL/WATER} > 2$. EME becomes more challenging with increasing polarity, due to limited partition into the SLM.

Ionic carriers have been introduced to approach this problem, and they work by providing ionic interactions with the compounds to facilitate partitioning into the SLM (23). For moderately polar cationic analytes ($0 < \log P < 2$), the ionic carrier bis(2-ethylhexyl) phosphate (DEHP) has successfully been added to NPOE to improve the extraction. However, highly polar analytes ($\log P < 0$) have shown to be particularly difficult to extract using EME, and the results have generally been poor to modest.

For acidic analytes, the most successful SLMs have been aliphatic alcohols, but they have shown to be less stable than NPOE in contact with biological fluids (27). Discovering new SLMs, especially for polar acidic analytes, is therefore a high priority in EME research. For EME to become a standard sample preparation method in TDM, it must be applied on

compounds with a variety of chemical properties. To date, most research has been on non-polar, basic substances.

## 1.8 Aim of the study

Methotrexate (MTX) is a highly polar and acidic analyte (28). This study will be an attempt to look deeper into an area of limited research, by applying different SLMs, donor solutions, acceptor solutions, and extraction parameters (voltage, time, agitation) to promote high extraction recoveries of MTX and its metabolites. This can contribute to the future application of EME in the extraction of a wide range of analytes with different physicochemical properties.

The clinical approach to this thesis is related to the TDM of MTX. Today, protein precipitation is applied to samples from patients treated with glucarpidase. The method yields approximately 70% recovery, and limited sample cleanup that may result in matrix components remaining in the sample. In this study, EME will be used in an attempt to achieve higher extraction recoveries, and a cleaner extract containing MTX, DAMPA and 7-OH-MTX. During EME method development, a HPLC-UV method will be used to quantify the recovery of MTX and its metabolites in the acceptor solution. For EME of spiked plasma samples, an LC-MS/MS method will be used for separation and quantification of the analytes, due to the high sensitivity and specificity of this method.

# 2 Theory

## 2.1 Electromembrane extraction

Electromembrane extraction (EME) is a microextraction technique based on the transfer of target analytes from a sample, through a supported liquid membrane (SLM), and into an acceptor solution (27). EME is a three-phase system: the sample and acceptor solutions are aqueous, while the SLM is an organic solvent. What distinguishes EME from previous SLM-based extraction techniques, is that the driving force for mass transfer is an external electrical field, which facilitates electrokinetic migration of target analytes across the SLM. For extraction of basic substances (cations), the pH in the aqueous solutions is neutral or acidic, to keep the analytes in a protonated state. A negatively charged electrode (cathode) is located in the acceptor solution, and a positively charged electrode (anode) is located in the sample solution. The charged analytes are prone to electrokinetic migration towards the electrode of opposite charge. For extraction of acidic analytes (anions), the pH in the aqueous solutions is neutral or basic to keep the analytes negatively charged, and the direction of the electrical field is reversed. The choice of pH in the aqueous solutions is crucial, as EME may lead to pH-altering reactions, which in turn may affect extraction efficiency. This will be further discussed in section 2.2.1.

An illustration of electromembrane extraction for acidic compounds is presented in Figure 6. In addition to the application of an electrical field, agitation of the EME system is essential to achieve fast extractions and high analyte recovery (29). This will ensure sufficient contact between the sample solution, SLM and acceptor solution, and promote mass transfer.

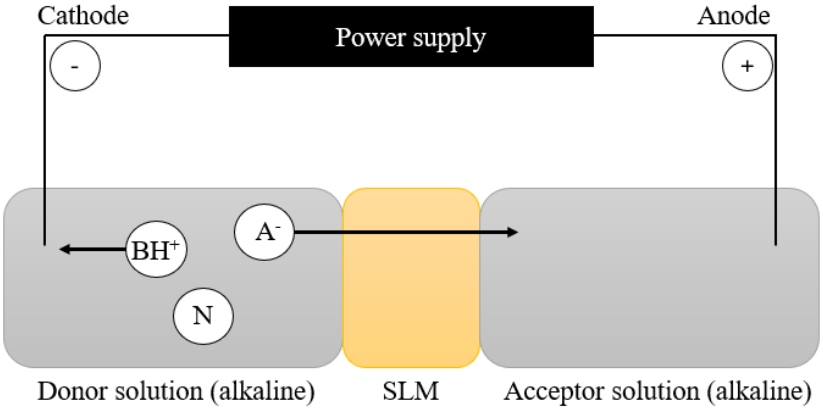



**Figure 6**. Principles of electromembrane extraction of acidic analytes. Adapted from (25).

### 2.1.1 Effects of electrolysis on electromembrane extraction

As already mentioned, the pH must be adjusted to keep the analytes ionized in the donor and acceptor solution, ensuring influence of the electrical field. An important challenge in EME is the occurrence of electrolysis in the system. Cambridge dictionary defines electrolysis as "*the use of an electric current to cause chemical change in a liquid*" (30). The electrical current in EME is the sum of analyte and background ion migration across the SLM. It may influence the pH in the sample and acceptor solutions significantly, based on the following equations (24):

Anode: $H_2O\ (l) \rightarrow \frac{1}{2}O_2(g) + 2H^+\ (aq) + 2e^-$ (Equation 1)

Cathode: $2H^+(aq) + 2e^- \rightarrow H_2(g)$ (Equation 2)

As a result, pH may decrease at the anode (equation 1) and increase at the cathode (equation 2). The level of electrolysis is determined by extraction current and time.

A consequence of electrolysis is reduced extraction recovery (31). When extracting acidic analytes, an increase in pH at the cathode is usually not a challenge because it will keep the analytes deprotonated in the donor solution. However, a gradual decrease of pH at the anode may prevent the analytes from maintaining their negative charge in the acceptor solution. As the analyte becomes neutral and is no longer influenced by the electrical field, diffusive back-

extraction into the SLM may occur.

Typical electrical currents in EME can range from a few units to tens of μA (32). They may even reach levels of hundreds of μA if ion carriers are added to the SLM to transfer polar analytes. The role of ionic carriers will be covered in the section on SLMs. The extraction current can be controlled by the applied voltage (24). It also depends on the chemical composition of the SLM, as the major electrical resistance is located in the SLM, and this is where the significant voltage drop occurs

### 2.1.2 The electrical double layer

Another aspect related to the application of the electrical field, is the formation of electrical double layers at the aqueous solution/SLM interfaces (24). This may have a major impact on mass transfer in EME, and the phenomenon is illustrated below (Figure 7).

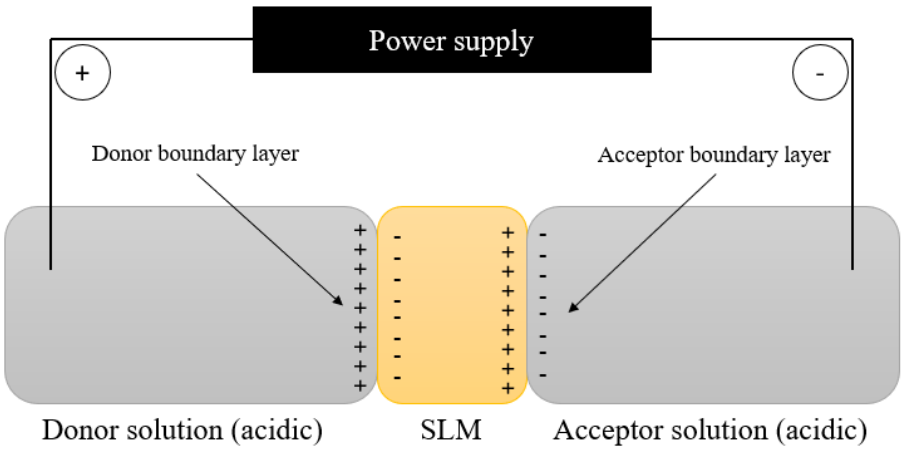



**Figure 7.** Illustration of the electrical double layer in extraction of basic compounds. Adapted from (24).

On application of the electrical field, a charge will build up within the SLM (24). When extracting basic compounds, positive charge will accumulate in the in the direction of the cathode, and negative charge will accumulate in the anode direction. This may, in turn, lead to a layer of elevated pH in the acceptor boundary layer due to the attraction of $OH^-$. Thus, an electrical double layer is formed. The analytes will enter the SLM in a protonated state, but when entering the acceptor/SLM interface, they may become deprotonated and will no longer

be influenced by the electrical field.

### 2.1.3 General requirements for the supported liquid membrane

The SLM is an organic solvent immiscible with water, typically comprising 5-25 microliters (27). It is immobilized by capillary forces in the pores of a porous polymeric membrane, which can be a flat sheet or a hollow fiber membrane. Choosing the optimal SLM for a particular analyte is one of the most important steps of EME. Different target analytes require different SLMs based on the chemical properties of the analyte. There are several physicochemical requirements for the SLM to facilitate efficient extractions.

Ideally, the SLM should be insoluble, or have very low solubility in aqueous solutions (33). This is necessary to avoid leakage into the aqueous donor or acceptor solution. The water solubility of the SLM should not be higher than 1 g/L. The non-polar properties of the SLM allow high selectivity in terms of preventing polar sample matrix components from entering the acceptor solution (25).

Furthermore, the viscosity of the SLM should be as low as possible, to maintain high permeability of the analytes migrating through the membrane (33). Keeping the viscosity low is also favorable for more practical reasons. It will facilitate the pipetting of the same amount of SLM to the porous polypropylene membrane.

Finally, conductivity of the SLM should be low, but not zero (33). It is desirable to have efficient flux of analyte ions, and low flux of background ions and sample matrix ions across the SLM. A current exceeding 50 μA is generally not recommended in EME.

### 2.1.4 SLM for basic analytes

For basic analytes, the main mechanism of solvation in the SLM is thought to be hydrogen bond interactions (33). Preferably, the SLM should have high hydrogen bond basicity, almost zero hydrogen bond acidity, and a logP value between 3 and 5.5. The most used SLM is 2-nitrophenyl octyl ether (NPOE, see Figure 8). It has shown to be efficient in extraction of basic substances with low polarity (logP > 1.5).

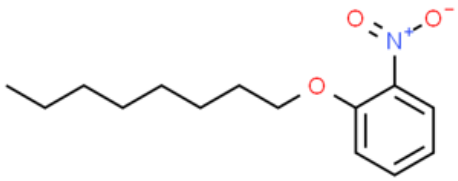


**Figure 8**. Chemical structure of NPOE. Downloaded from (34).

As the structure shows, NPOE has two functional groups capable of hydrogen bond interactions: The nitrogen dioxide group contains two hydrogen accepting oxygen atoms, and the ether group contains one hydrogen accepting oxygen atom.

NPOE is less efficient for extraction of polar basic substances with a logP < 1.5 (33) (Figure 9). To increase extraction of these substances, it is possible to add another component to the SLM that acts as an ionic carrier. Di(2-ethylhexyl) phosphate (DEHP) is an example of such components (35). It is assumed that the negatively charged phosphate groups of DEHP are orientated towards the sample-membrane interface due to the electrical field. Polar basic substances from the sample are attracted to the negatively charged groups on DEHP, forming ion-pairs. This complex is sufficiently soluble in the SLM, which promotes transfer through the interface and into the SLM. The hydrophilic properties of the analytes will allow release to the acceptor solution. Non-polar analytes can form ion-pairs with DEHP and be transferred into the SLM as well, but since the complex is more hydrophobic, it prevents the analyte from being released to the acceptor solution. The addition of 5-25% DEHP to NPOE have proved to be successful for extraction of moderately polar analytes with a logP between 0 and 2 (23). For example, in the study of Hansen et al, EME of ephedrine (logP=1.3) with 10% DEHP in the SLM yielded over 90% recovery of the analyte. A challenge with DEHP is that it increases the current in the system, which makes EME more prone to electrolysis and pH changes (33).

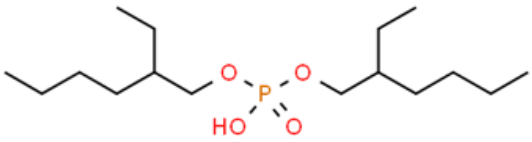


**Figure 9.** Chemical structure of DEHP. Downloaded from (36).

The pKa value of DEHP is approximately 1.94 (23). This suggests that the compound is mostly negatively charged at a pH value above 1.94.

### 2.1.5 SLM for acidic analytes

The choice of SLM for acidic analytes is rather limited (33). One reason for this is that the interaction between acidic analytes and the SLM is not well understood. To be able to form hydrogen bond interactions with the analytes, the SLM should have a strong hydrogen bond acidity. Examples of such solvents are alcohols such as 1-octanol and 1-nonanol. Extraction efficiency usually decreases with increasing hydrocarbon chain length of the SLM ( > C8 ), due to increased viscosity (37). This makes it more difficult for the target analytes, especially hydrophilic, to migrate through the SLM.

Very little research has been done on SLMs for polar and acidic analytes. One candidate ionic carrier, the cationic liquid substance Aliquat 336, was recently introduced for EME (38). It is a quaternary ammonium salt, with the nitrogen atom bound to one methyl group and three hydrocarbon chains of either $C_8$ or $C_{10}$.

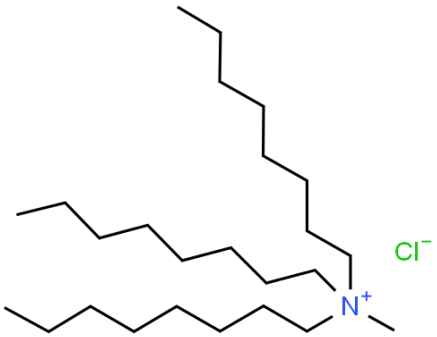


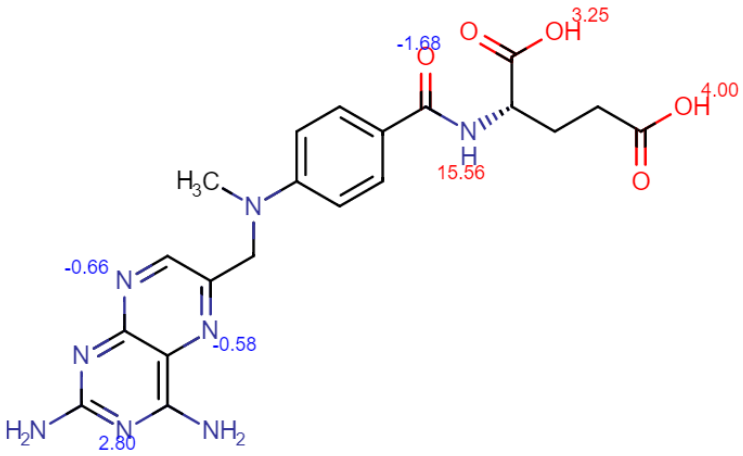

**Figure 10**. Chemical structure of Aliquat 336. Downloaded from (39).

Aliquat 336 has a permanently positive charge on the nitrogen atom. This facilitates ion-pair formation with the negatively charged analytes from the donor solution, and solvation into the SLM.

## 2.2 Chemical properties of methotrexate

In order to succeed with EME, it is important to have a theoretical insight into the chemical properties of the analyte. That way, it becomes easier to facilitate optimal extraction conditions, such as the choice of pH in the aqueous solutions, and the composition of the SLM. MTX is an acidic and polar compound, with chemical properties implying several challenges to developing an EME protocol. The main chemical properties are listed in Table 1.

**Table 1.** Chemical formula, molar mass, logP and structure of Methotrexate (retrieved from Chemicalize (28)).

| | |
|---|---|
| **Chemical formula** | $C_{20}H_{22}N_8O_5$ |
| **Molar mass** | 454.447 g/mol |
| **LogP** | -0,236 |
| **Chemical structure including predicted pKa-values** |  |



One part of the MTX molecule consists of a pteridine ring residue, with two amine groups attached to the distal ring. The nitrogen atom between the two amine groups has a predicted pKa value of 2.8. As pH decreases below 2.8, an increasing fraction of MTX is protonated at this nitrogen atom. The pteridine ring structure is linked to a p-aminobenzoyl part, which is further linked to a glutamic acid residue. The glutamic acid contains two carboxylic acids, with predicted pKa values of 3.25 and 4.00, respectively.

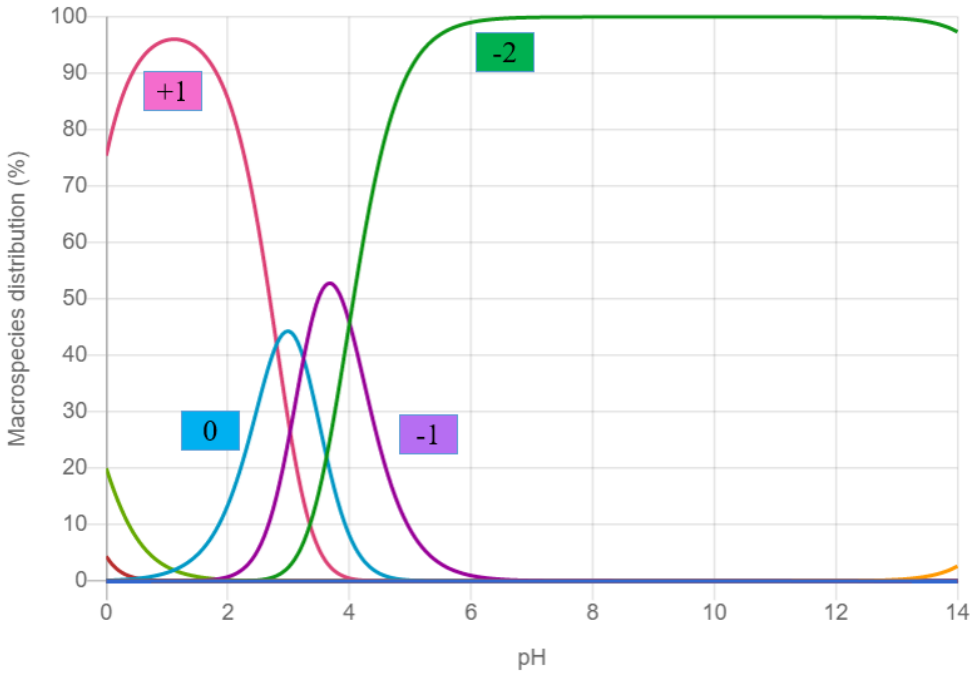



**Figure 11.** Predicted distribution of MTX (%) with different charge at pH 0-14 (retrieved and modified from (28)).

In Figure 11, the green slope represents the percentage of MTX with a charge of -2. At pH 6 and higher, nearly 100% of the molecules hold two negative charges, represented by the loss of a proton at each of the two carboxylic acid groups. At physiological pH (7.4), the amount of MTX carrying two negative charges is 99,96%. The distribution of MTX with a charge of -1 is illustrated by the purple slope. This fraction dominates at pH 3.7. At pH 3, most of the MTX molecules are neutral, represented by the blue slope. Below pH 2.8, the MTX molecules containing one positive charge accounts for the biggest fraction, as shown by the pink line.

MTX has a predicted logP value of -0,236, which is the partition ratio of MTX in octanol-water. However, the log D value may be more relevant for EME, as this represents the distribution of MTX between water and octanol at different pH values (40).

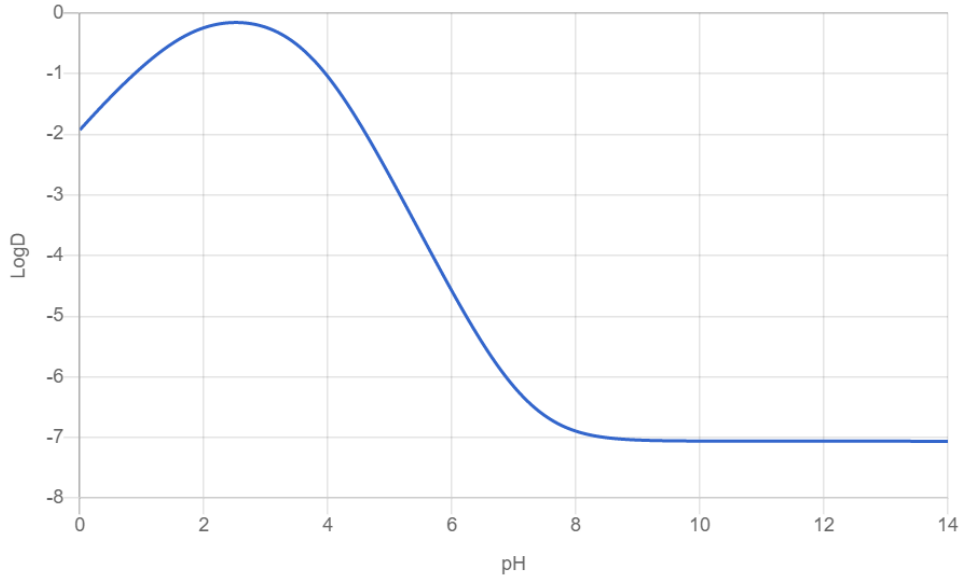



**Figure 12**. Log D of MTX at pH 0-14. Retrieved from (28).

MTX becomes more polar with increasing pH. This may cause difficulties in EME with regards to the partition of the analyte into the hydrophobic SLM.

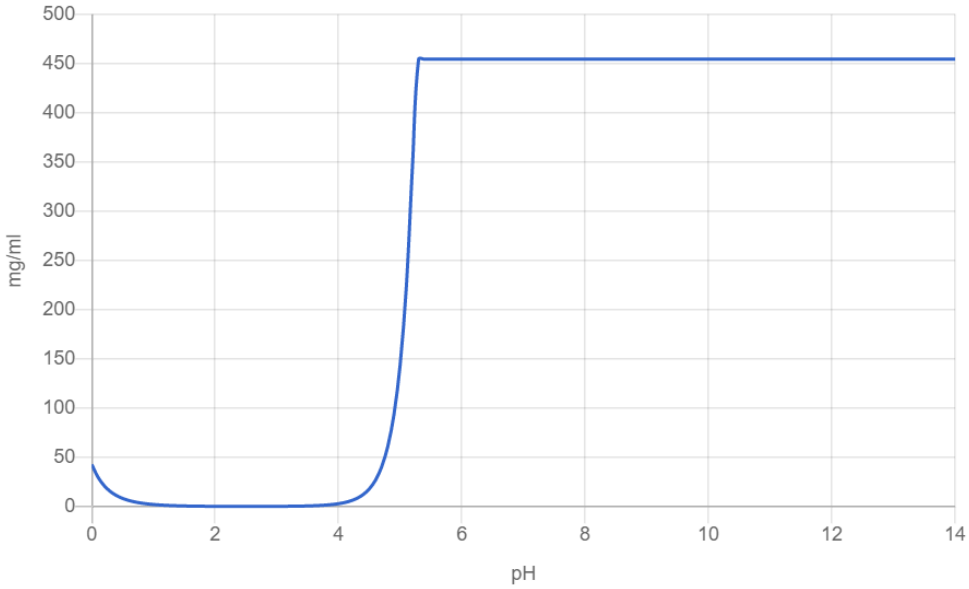



**Figure 13.** Solubility (mg/ml) at pH 0-14. Retrieved from (28).

MTX has a predicted solubility of 0.51 mg/ml at pH 2. As Figure 13 suggests, the solubility of MTX greatly increases from pH 4 to pH 5.3. In the present study, standard solutions with a MTX concentration less than 0.01 mg/ml will be applied in EME, and the analyte will therefore most likely exist as dissolved molecules also in low pH conditions. In EME of MTX

as an anion, solubility will not be an issue, as the pH in solutions are 7.4 or higher.

Nevertheless, the complex chemistry of MTX demands fine-tuned conditions in order to yield satisfactory recovery rates by means of EME extraction, in accordance with the description of the EME technique.

### 2.2.1 Chemical properties of the metabolites 7-OH-MTX and DAMPA

7-OH-MTX is the hydroxylated metabolite of MTX. It highly resembles the parent analyte, differing only by an additional OH-group (Figure 14).

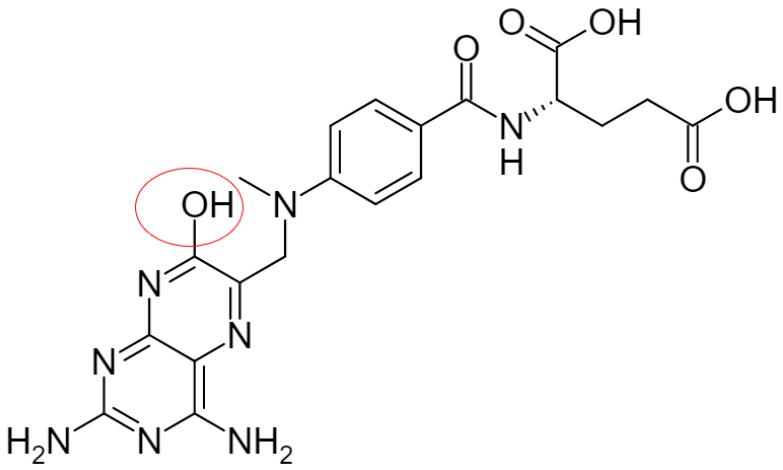



**Figure 14.** Chemical structure of 7-OH-MTX.

The chemical structure of 7-OH-MTX suggests that it behaves similarly to MTX in terms of log D, solubility and pKa values. However, the additional OH-group might increase the polarity of the analyte.

MTX also undergoes metabolism by cleavage of the glutamic acid part from MTX, generating DAMPA.

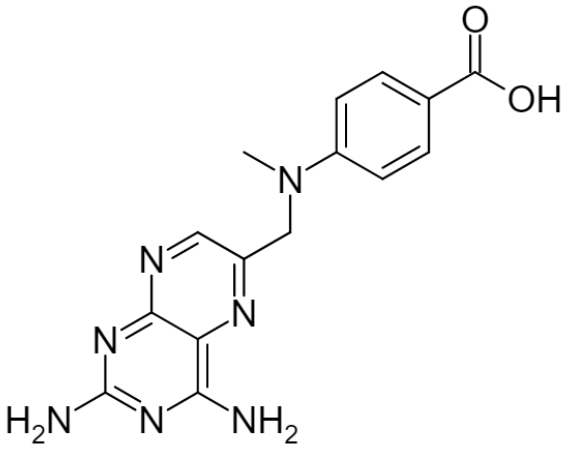



**Figure 15.** Chemical structure of DAMPA.

The loss of the glutamic acid from MTX might alter the chemical properties of DAMPA, making it less polar. Nevertheless, a carboxylic acid remains, and there are reasons to believe that it can be at least as good candidate for EME as MTX and 7-OH-MTX. Due to the presumably reduced polarity of DAMPA, its partition into the SLM could increase.

## 2.3 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is the most common chromatographic technique used to quantify and identify analytes in biological samples (10). All chromatographic methods are based on the separation of different compounds in a sample (41). In HPLC, the sample is injected into the instrument and mixed with a liquid. This liquid is called the *mobile phase*, and it is used to transport the injected sample through a separation column. A pump ensures that the mixture of sample and mobile phase is delivered to a column at a constant flow rate. The column is packed with a non-moving solid, called the stationary phase. Its function is to slow down or retain compounds. If the sample contains several different compounds, the stationary phase may retain these differently, and they will elute from the column at various rates. The time it takes for a compound to elute from the column is called the retention time, which is determined by the compound's distribution between the mobile phase and stationary phase. After elution, a detector can be used for identification and quantification of a compound and express the results as a chromatogram.

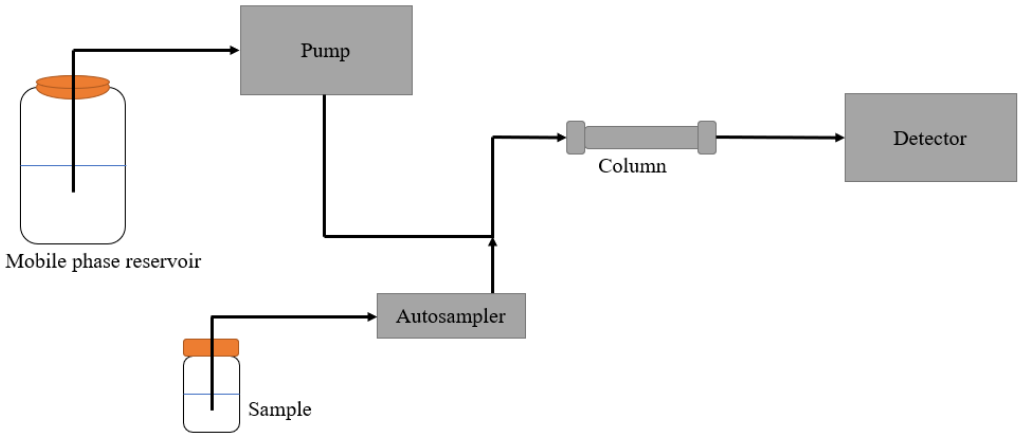



**Figure 16**. Illustration of the main components in HPLC.

### 2.3.1 Reversed phase liquid chromatography

In reversed phase liquid chromatography, the stationary phase is hydrophobic (42). It typically contains hydrocarbon groups bound to silanol groups on silica particles. C18 (octadecyl) is the most commonly used group, but C8 (octyl) and phenyl groups are also common. The retention of compounds is mainly based on van der Waals forces with the hydrocarbon chains on the stationary phase. Thus, hydrophobic compounds will have longer retention times than hydrophilic or polar analytes. The separation efficiency of the column is characterized by high peak resolution and short run time (43). The efficiency increases with decreasing size of the silica particles. Smaller particles provide a more uniform flow through the column, and the eluting peaks will appear narrower in the chromatogram. The optimum flow rate is higher for small particles than for larger particles, which allows shorter run time. However, smaller particles gives higher back pressure in the column. The pump must therefore be able to deliver the mobile phase at a constant rate against the high pressure (10).

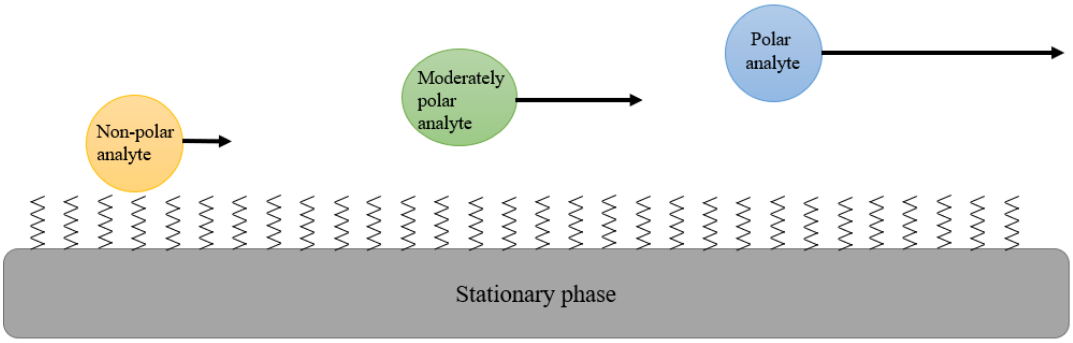



**Figure 17**. Principles of reversed phase liquid chromatography.

The mobile phase in reversed phase HPLC is an aqueous solution, consisting of water and organic solvents that are miscible with water (42). The organic solvents are typically methanol, acetonitrile, or tetrahydrofuran. The elution strength of an organic solvent reflects the ability to compete with the analyte's place on the stationary phase. Shortening the retention time of the analytes, tetrahydrofuran possesses the highest elution strength, followed by acetonitrile and methanol. A mobile phase consisting of 60% methanol in water has the same elution strength as 46% acetonitrile in water, or 37% tetrahydrofuran in water (44). Although methanol is less expensive and toxic than acetonitrile, it can form a more viscous mixture in water, increasing the back pressure in the HPLC system. Acetonitrile forms less viscous mixtures with water. The mobile phase must be chosen carefully to obtain the best results in HPLC (10). The solvents should preferably not give any response in the chosen detector, and must therefore have a high degree of purity.

The pumping system can deliver the mobile phase by combining solvents from up to four different reservoirs (10). The composition may be constant (isocratic) or composed in a way that ensures gradual increase of the eluting strength of the mobile phase during chromatography (gradient elution). Gradient elution can be applied for earlier elution of hydrophobic analytes, resulting in higher and sharper peaks in the chromatogram, and improved detection limits.

The pH in the mobile phase plays an important role when separating acids and bases (42). The retention time is reduced with increasing ionization of the analyte. The pH should be chosen to avoid variation in retention time due to small changes in the composition of the mobile phase. The analyte should be either fully ionized or neutral. To achieve this, the pH value of

the mobile phase should not be too close to the pKa value of the analyte. Also, when using silica packed columns, the pH in the mobile phase must usually be within the range of 2 - 8. With pH higher than 8, the silica can dissolve. With a pH lower than 2, the functional groups can be cleaved off the silica particles.

Liquid chromatography is a separation method that offers many advantages in bioanalysis (45). The sample can be precisely injected into the system by an autosampler, ensuring that the same volume is injected each time. The columns can be changed to adjust to the analyte selectivity. There is less risk of sample degradation since heating is not required, which can be a problem in gas chromatography. There is a high degree of automation of HPLC, because the whole process can be controlled by a computer system (10). The detector will provide an electronic response to the compounds, that can be used by the computer system to calculate the quantity of a compound.

## 2.4 UV detection

After separation in HPLC, analytes can be detected and measured by ultraviolet (UV) spectroscopy (10). This is a technique based on the analytes' absorption of UV light. To be capable of detection, the analyte of interest must contain a chromophore, which is a part of the molecule able to absorb UV radiation in the wavelength range of 190-400 nm. To contain a chromophore, at least one double bond must be present in the molecule. The absorption of radiation energy is achieved if the analyte excites electrons from a ground state to a state of higher energy. The amount of energy that is required to excite the electrons corresponds to a certain wavelength, ranging from 190 to 400 nm. Sigma bond (σ) electrons typically requires energy that corresponds to a wavelength below 200 nm, whereas double bond (π) electrons excite more easily and will result in UV absorbance above 200 nm. According to the molecular structure, analytes will therefore absorb energy at different wavelengths.

The Beer-Lambert law describes the principle of light absorption (46):

$$A = \varepsilon * b * c$$

Where A is the absorbance, which is a measure of the amount of light absorbed by the analyte. A is defined as the logarithm of the intensity of incident radiation divided by the intensity of transmitted radiation. ε is a constant called the molar extinction coefficient, based

on the absorbance of a 1 M solution of the analyte. C is the concentration of the analyte in moles/L. *b* is the pathlength of the flow cell in cm. The flow cell contains the eluent from the LC column, with a typical length of 6-60 mm and volume of 6-10 µl (10).

Commonly, the radiation source in UV spectroscopy is a deuterium lamp, which emits light in the entire UV range (10). This type of radiation is called polychromatic radiation. In a simple wavelength UV detector, a monochromator will ensure that UV radiation with the correct wavelength is directed through the flow cell, containing the eluent from the LC column. A diode array detector (DAD) is another type of UV detector, where the entire polychromatic radiation from the deuterium lamp is passed directly through the flow cell (47). The transmitted light is then spread into separate wavelengths by a fixed grating, and detected by an array of diodes that monitors the intensity of light at each wavelength. This offers several advantages, such as a recording of the full UV-spectrum of the analyte, which is useful in identification if the analyte is unknown (10). Selected wavelengths can also be chosen to detect each analyte in a sample at the wavelengths with highest molar absorption. For optimal detection sensitivity, analytes should be measured at their maximum UV-absorbance.

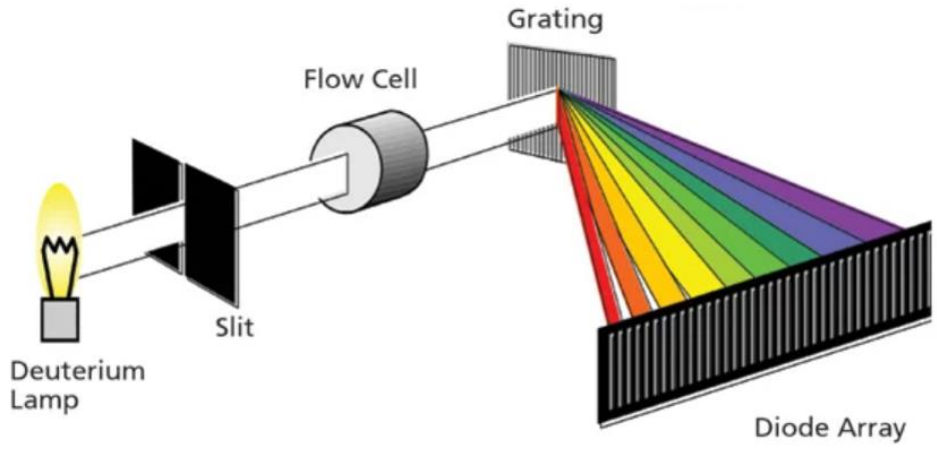



**Figure 18.** Illustration of diode array detection (DAD). Retrieved from (47).

UV spectroscopy is a beneficial detector for many reasons (47). It is easy to use, with a high precision (<0,2% relative standard deviation (RSD)) and provides information on peak identity by using diode array detection (DAD). However, it is not always the best choice in bioanalysis. Sensitivity issues may occur if the analyte exists in very low concentrations, which is typical in biological fluids (10). The lower limit of detection of the UV instrument

may therefore not be low enough to detect the analyte. In other cases, UV detection may not be sufficiently selective if closely related compounds absorb UV radiation at similar wavelengths.

## 2.5 Mass spectroscopy detection

Mass spectroscopy (MS) detection provides more information about the molecular structure of an analyte than UV detection, and it has a high selectivity and sensitivity (10). MS detection has therefore become a method of choice in bioanalysis. Compared to UV-spectroscopy, where detection is based on the analytes' absorption of UV light, MS detection requires information of ionized analytes to provide a signal. Liquid chromatography coupled with mass spectrometry (LC–MS) is currently the preferred instrumental technique for bioanalysis of pharmaceuticals (12).

Mass spectrometry in LC-MS can be divided into three sections (10). First, the analytes are ionized and transferred to a gas phase. This occurs in the coupling between LC and MS, called the interface. Second, a mass analyzer separates the ions based on their mass-to-charge (m/z) ratio, which is the ratio between the exact mass of the analyte and the number of charges of the analyte. Third, the ions are detected based on a generated current.

Electrospray ionization (ESI) is a common ion generator in LC-MS used to ionize polar compounds (10). In ESI, the eluent from the LC column passes through a capillary needle, to which a high electrical potential is applied (48). If the electrical potential is positive, negative ions will be attracted to the needle, and the positive ions will be free to leave it. A flow of nitrogen gas outside of the needle will assist in the evaporation of the positively charged droplets. The droplets disintegrate due to charge-charge repulsion until they exist as gas phase ions. The positively charged ions are attracted to the negatively charged inlet of the mass spectrometer, called a heated capillary. This is a channel that leads into the mass analyzer, where ion-separation under high vacuum pressure occurs.

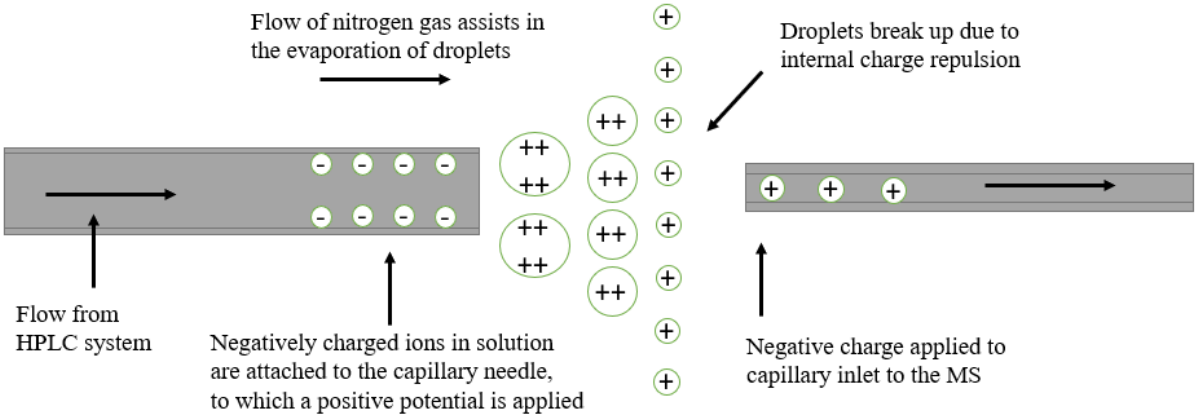



**Figure 19**. Illustration of the electrospray ionization process. Adapted from (48).

There are several types of mass analyzers used for ion separation, and a triple quadrupole mass analyzer is commonly used in bioanalysis (10). It consists of two quadrupole mass analyzers and a collision cell. One single quadrupole consists of four rods placed parallel to each other, where the opposite pairs are electrically connected. Direct current (DC) and a radio frequency (RF) are applied to one pair, and the opposite DC and RF are applied to the other pair. This will create an oscillating, electrical field. The generated ions will move inside this field, and specific combinations of DC and RF will allow the ion of interest to pass the quadrupole stably. Other ions will collide with the quadrupole and be trapped. It is the m/z ratio of the ion that decides what DC and RF combinations that makes the ion move stably. Following the passage through the first quadrupole, the filtered ions will enter another quadrupole called a collision cell. It contains an inert gas that collides with the ions on their way towards the exit of the collision cell. The collision will cause fragmentation of the ions into smaller ions, which will further be transferred to the third quadrupole. Here, the fragmented ions are separated by the same principle as in the first quadrupole. The fragmented, filtered ions will reach a detector at the end of the third quadrupole.

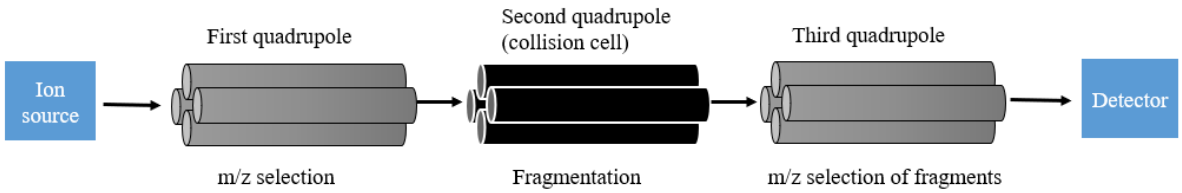



**Figure 20**. Illustration of a triple quadrupole mass analyzer. Adapted from (10).

The function of an ion detector in MS is to measure the presence of an ion (10). In general, detection is based on the impact of an ion on a surface, which will generate a measurable current. Several different ion detectors exist, but an electron multiplier is one of the most common types, and is used for this study. Here, ions will collide with an emissive material that causes the release of electrons. A series of dynodes multiplies the number of electrons by $10^5$, before the electrons arrive at the anode where the current is measured (49).

One of the major challenges in LC-MS analysis of biological samples is so-called matrix effects (10). This refers to the impact of biological components, such as lipids and peptides, that are extracted concomitantly with the analyte of interest during sample preparation. These effects may suppress or elevate the signal intensity of analytes, and occur when matrix components elute at the same retention time as the analytes. The mechanism for signal suppression is not completely understood, but there are two main explanations:

1. During the electrospray ionization process, there is an altered desorption of ions from the droplet surface.
2. Matrix components compete with the analyte for charges.

A common way to check for matrix effects is called post-extraction addition (10). First, a blank biological sample is prepared using the chosen sample preparation method. A fixed amount of analyte is added to the extract, and the sample is analyzed on LC-MS. Then, the same amount of analyte is added to a solvent or buffer that does not contain biological fluids. This sample is analyzed by LC-MS, and the results from the two samples are compared.

The internal standard (IS) method is a valuable technique in bioanalysis (50). An IS is a known quantity of a compound that is added to the unknown analyte in a sample. The signals from the IS and the unknown analyte are compared to find the amount of unknown analyte that is present in a sample. The IS must have similar physicochemical properties as the target analyte, but not to the degree that it cannot be determined accurately (51). In LC-MS, isotopically labeled versions of the analyte are the most ideal candidates, and they can be distinguished from the target analyte by having a higher m/z ratio.

Addition of an IS is useful if the quantity of injected sample, or if the instrument response varies from run to run (50). For example, a change in flow rate may increase the signal from an IS by 5%, but the same increase in signal will also be observed from the analyte. The

relative response of the analyte and the IS is therefore constant. This differs from a calibration curve without IS, which is only accurate for the current set of conditions. An IS can also be applied when sample loss may occur during sample preparation. The ratio of IS and analyte is constant because the same amount is lost from each during the process.

# 3 Experimental

## 3.1 Chemicals

**Table 2.** List of chemicals, their purity, and producer.

| Chemical | Purity | Producer |
|---|---|---|
| Water | Milli-Q quality | Millipore (Burlington, MA, USA) |
| Hydrochloric acid (HCl) | 37% | Sigma-Aldrich (Steinheim, Germany) |
| Potassium hydroxide (KOH) | >85% | |
| Sodium hydroxide (NaOH) | >97% | |
| 2-Nitrophenyl octyl ether $C_{14}H_{21}NO_3$) | >99% | |
| 2-Nitrophenyl pentyl ether ($C_{11}H_{15}NO_3$) | >99% | |
| Bis(2-ethylhexyl) phosphate ($C_{16}H_{35}O_4P$) | 97% | |
| Trifluoroacetic acid ($C_2HF_3O_2$) | >99% | |
| 1-Nonanol ($C_9H_{20}O$) | 98% | |
| L-Menthone ($C_{10}H_{18}O$) | >96% | |
| Menthol ($C_{10}H_{20}O$) | 99% | |
| Phosphate buffered saline tablet | | |
| Sodium phosphate monobasic | 98% | |
| Methanol ($CH_3OH$) | >99.9% | |
| Formic acid ($CH_2O_2$) | >95% | |
| Sodium iodide (NaI) | 99.5% | |

| | | |
|---|---|---|
| Di-Potassium hydrogen phosphate ($K_2HPO_4$) | >99% | Merck KGaA (Darmstadt, Germany) |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | >99.5% | |
| Di-sodium hydrogen phosphate ($Na_2HPO_4$) | 99.5% | |
| 1-Octanol ($C_8H_{18}O$) | >99% | |
| Sodium hydrogen carbonate ($NaHCO_3$) | 99.5% | |
| Potassium nitrate ($KNO_3$) | 99% | |
| Methanol ($CH_3OH$) | Hypergrade for LC-MS | |
| Acetonitrile ($CH_3CN$) | Hypergrade for LC-MS | |
| Acetonitrile ($CH_3CN$) | Gradient grade for liquid chromatography | |
| Aliquat 336 | Unknown | Obtained from the Department of Pharmacy, University in Oslo |
| Bis-(2 ethylhexyl)amine | Unknown | Obtained from the Department of Pharmacy, University in Oslo |
| Ammonium hydroxide ($NH_4OH$) | 74 mM | Stock solution obtained from the lab |
| Peppermint oil | Pharmaceutical grade | Farmagon AS (Oslo, Norway) |
| Silver nitrate ($AgNO_3$) | 99.8% | VWR International AS (Oslo, Norway) |
| Ethanol ($C_2H_5OH$) | Rectified | Antibac AS (Asker, Norway) |

**Table 3.** Analytes extracted with EME.

| Analyte | Formulation | Producer |
|---|---|---|
| Methotrexate | 50 mg/2 ml MTX in sodium chloride, sodium hydroxide (for pH adjustment) and water for injection. | Pfizer Pharma PFE GmbH (Berlin, Germany) |
| 7-hydroxy Methotrexate (sodium salt) | 1 mg 7-OH-MTX sodium salt | Cayman Chemical Company (Ann Arbor, MI, USA) |
| Methotrexate impurity E CRS, Catalogue code: Y0000664 (DAMPA) | 10 mg | European Directorate for the Quality of Medicines & HealthCare (EDQM, (Strasbourg, France) |

## 3.2 Solutions

**Table 4.** Preparation of solutions for EME experiments.

| Solution | Preparation |
|---|---|
| 200 ml 20 mM phosphate buffer, pH 2.6 | 0.544 g $KH_2PO_4$ was weighed and added to 160 ml Milli-Q water. The pH was adjusted to 2.6 with 37% HCl. Milli-Q water was then added to a final volume of 200 ml. |
| 200 ml 40 mM potassium phosphate buffer, pH 7.4 | 969 mg $K_2HPO_4$ and 331 mg $KH_2PO_4$ was weighed and dissolved in 160 ml Milli-Q-water. The pH was adjusted to 7.4 with a 1 M KOH solution. Milli-Q water was then added to a final volume of 200 ml (52). |
| 200 ml 40 mM sodium phosphate buffer, pH 7.4 | 1617 mg $Na_2HPO_4$ and 271 mg $NaH_2PO_4$ was weighed and dissolved in 160 ml Milli-Q-water. The pH was adjusted to 7.4 with a 50 mM NaOH solution. The buffer was diluted with Milli-Q-water until the volume was 200 ml (53). |

| | |
|---|---|
| Phosphate buffered saline | 1 PBS tablet was dissolved in 200 ml Milli-Q-water. |
| 50 mM HCl, pH 1.3 | 37% HCl was gradually added to Milli-Q water until the pH was 1.3. |
| Standard solution of 8 and 5 µg/ml MTX in phosphate buffer pH 7.4 | 6.4 µl 25 mg/ml MTX was added to 20 ml phosphate buffer pH 7.4, generating a 8 µg/ml MTX standard solution.<br><br>4 µl 25 mg/ml MTX was added to 20 ml phosphate buffer pH 7.4, generating a 5 µg/ml MTX standard solution. |
| SLM containing Aliquat 336. | Aliquat 336 is a highly viscous solution. To ensure the right concentration, the amount of aliquat 336 was measured by weight rather than volume. Aliquat 336 has a density of 0.88 g/mL (54). To prepare a 1 ml 1% aliquat solution, 8.8 mg Aliquat was weighed and mixed with 990 µl SLM. |
| Menthol/menthone SLM | Menthol was measured by weight, due to its solid state. The compound has a density of 0.904 g/ml (55).<br><br>For a 75% (w/v) menthol in menthone mixture, 1356 mg menthol was mixed with 0.5 ml menthone.<br><br>For a 50% (w/v) menthol in menthone mixture, 904 mg menthol was mixed with 1 ml menthone. |
| DAMPA stock solution (630.4 µM) | 2.4 mg DAMPA was dissolved in 10 ml methanol, generating a 0.24 mg/ml solution. 0.5 ml was removed from the solution. 1.5 ml 74 mM ammonium hydroxide was added to the remaining 9.5 ml. The new concentration of DAMPA was 0.207 mg/ml, or 630.4 µM. |
| 7-OH-MTX stock solution (1061.1 µM) | 1 mg of the analyte was dissolved in 2 ml methanol and distributed to 10 separate vials, generating 0.5 mg/ml (or 1061.1 µM) stock solutions. |

| | |
|---|---|
| Plasma spiked with 10 µM MTX. | 36 µl of 550.12 µM MTX stock solution in phosphate buffer pH 2.6 was mixed with 1964 µl plasma. The mixture was left in 10 minutes to ensure the establishment of protein binding equilibrium. 2000 µl phosphate buffer 2.6 was then added to the mixture. The pH was 6.89 due to the strong buffer capacity of plasma. The mixture was therefore pH adjusted with HCl to 2.6, and the final volume was 4555 µl. Hence, the new concentration of MTX was 4.39 µM. |
| 4.39 µM MTX standard solution for LC-MS analysis of plasma MTX. | 200 µl plasma was diluted with 200 µl phosphate buffer pH 2.6. The pH was adjusted to 2.6 with 37% HCl. The mixture was extracted according to the relevant procedure, and 192 µl extract was spiked with 7.8 µl of 110 µM MTX stock solution in methanol. |
| Donor solution containing physiological concentration of bicarbonate | Normal concentration range of bicarbonate in serum is 23-30 mM (56). $NaHCO_3$ has a molar mass of 84,01 g/mol. 42 mg $NaHCO_3$ was dissolved in 20 ml Milli-Q water, generating a 0.025 M (25 mM) solution. |
| Donor solution containing 3.4 mg/ml of the cationic carrier bis-(2 ethylhexyl) amine (BEA). | The density of BEA is 0.8 g/ml (57). For preparation of 1 ml solution containing 3.4 mg/ml BEA, 4.2 µl BEA was mixed with 40 mM potassium phosphate buffer. |

## 3.3 Lab equipment

**Table 5.** General lab equipment.

| Equipment | Description | Producer |
|---|---|---|
| pH meter | pH Meter 744 | Metrohm AG (Herisau, Switzerland) |
| pH electrode | LL Biotrode 3 mm | Metrohm AG (Herisau, Switzerland) |
| Concentrator/centrifuge | Concentrator plus | Eppendorf (Hamburg, Germany) |

| | | |
|---|---|---|
| Mixer/heater | Thermomixer Comfort | Eppendorf (Hamburg, Germany) |
| Centrifuge | Allegra™ X-22R Centrifuge | Beckman Coulter (Brea, CA, USA) |
| Weight | AG204 DeltaRange® | Mettler Toledo (Greifensee, Switzerland) |
| Pipettes | Finnpipette® (200-1000 µl, 0.5-10 µl, 20-200 µl, and 5-50 µl) | Thermo Labsystems (Thermo Fisher, (Waltham, MA, USA)) |
| Pipette tips | 1000 µl, 200 µl and 10 µl | VWR (Radnor, PA, USA) |
| Tubes | SafeSeal micro tube (1.5 ml and 2.0 ml) | Sarstedt Ag & Co. KG (Nümbrecht, Germany) |
| Vials for standard solutions | 20 ml LSC vials | PerkinElmer (Waltham, MA, USA) |

**Table 6**. Equipment used for HPLC and LC-MS.

| **Equipment** | **Description** | **Producer** |
|---|---|---|
| Septum for HPLC | 8 mm silicone septum | VWR (Radnor, PA, USA) |
| Screw cap for HPLC | PP black 8 mm centre hole | VWR (Radnor, PA, USA) |
| Vials for HPLC | Screw vial 1.5 ml, 32x11.6 mm clear | VWR (Radnor, PA, USA) |
| Inserts for HPLC | Micro insert 0.1 ml 30x5 mm clear | VWR (Radnor, PA, USA) |
| Screw cap for LC-MS | 9 mm screw caps with septum | Aligent Technologies (Santa Clara, CA, USA) |
| Vials for LC-MS | 2 ml vials | Aligent Technologies (Santa Clara, CA, USA) |
| Inserts for LC-MS | 250 µl polypropylene inserts | Aligent Technologies (Santa Clara, CA, USA) |

## 3.4 EME equipment and setup

The vials containing the aqueous solutions in EME are made of a black, electrically conductive polymer, and can hold volumes up to 600 µl. One of the vials serves as the donor vial, and the other as the acceptor vial, and they are connected by a plastic union (Figure 21). A porous polypropylene (PP) Accurel® flat sheet membrane is positioned in a narrow groove in the middle of the union, with a function of holding the supported liquid membrane (SLM). The vials and unions were obtained from G&T Septech AS (Ski, Norway), and the PP-membranes are produced by 3M (Membrana) (Wuppertal, Germany).

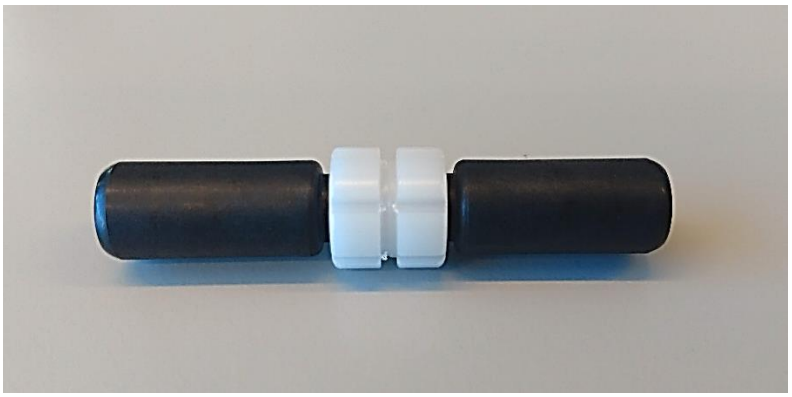


**Figure 21**. Donor and acceptor vial (black) connected by a union (white).

The assembled unit of the vials and the union is placed upon the sample holder, which is attached to a DLAB MX-M agitator (DLAB Science Co. ltd., China), with a programmable agitation in the range of 0-1500 RPM. A top cover with ten pairs of electrodes is positioned over the assembly, and screws are used to secure contact between the electrodes and the assembly. The equipment allows simultaneous extraction of ten samples. The conductive material of the vials maintains the electrical field, delivered by an external DC power supply model ES 0300-0.45 from Delta Power supplies (Delta Elektronika, Zierikzee, the Netherlands) via the electrodes in the top cover. This differs from traditional EME, where electrodes are immersed into the donor/acceptor solutions. The power supply has an adjustable voltage in the range 0-300 V. A Fluke 289 multimeter (Fluke Corporation, Everett, USA) is added to the circuit, to monitor the electrical current across the SLM during EME.

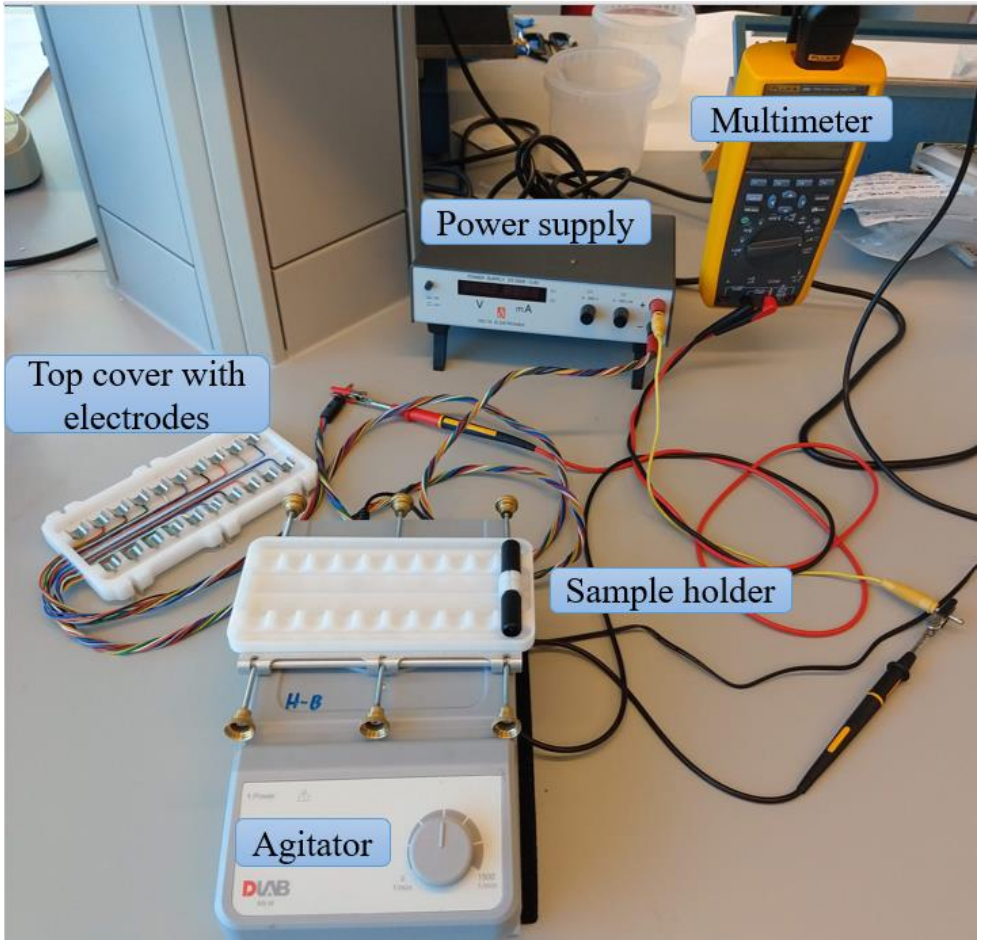



**Figure 22**. EME equipment setup.

## 3.5 EME procedure

A schematic illustration of the EME procedure is showed in Figure 23. Firstly, the precut PP-membrane was placed inside the union. This was achieved by using the bottom of a pipette tip to carefully push the membrane in place. Secondly, acceptor solution was pipetted into the acceptor vial, and donor solution was pipetted into the donor vial. In the present study, all experiments were performed with 250 µl donor and acceptor solutions. Gloves were used when handling the vials, to avoid fingerprints that could impair conductivity. The union, containing the PP-membrane, was attached to the acceptor vial. These two parts were connected as tightly as possible, but without crumpling the PP-membrane. If the assemblance is too loose, the aqueous solutions might leak around the SLM, which could result in poor reproducibility of the extractions. Next, SLM was pipetted onto the PP-membrane. 10 µl SLM was applied throughout the study. The acceptor vial + union complex was attached to the donor vial. When extracting cationic substances, the donor compartment was placed in the

direction of the positive electrode, and the acceptor compartment was placed in the direction of the negative electrode. The direction was reversed when working with anions.

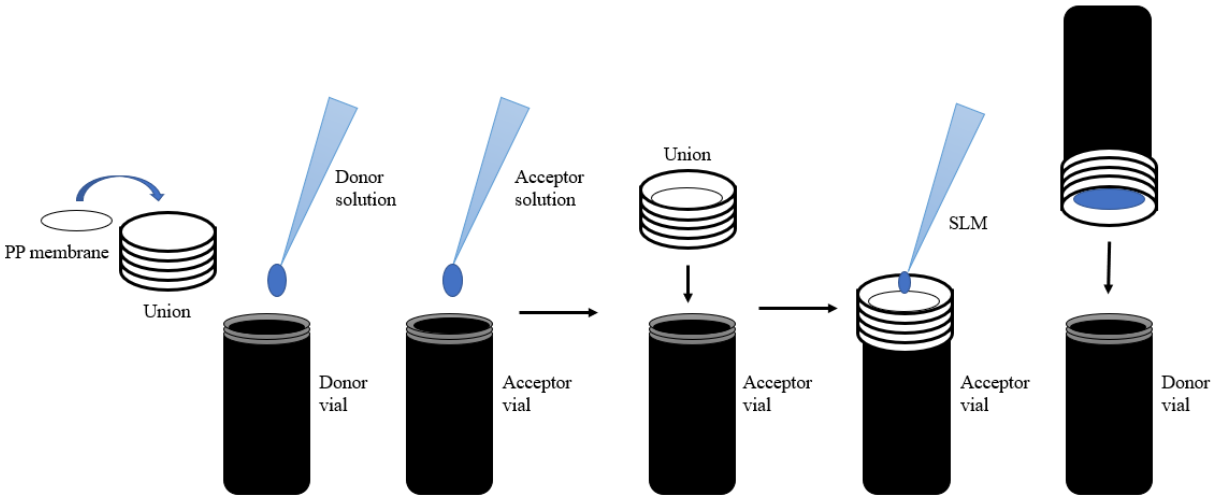



**Figure 23**. Principle of the EME procedure.

Prior to analysis of the extracted samples on HPLC, the donor and acceptor vials were centrifuged on a Concentrator plus 5305 centrifuge (Eppendorf, Hamburg, Germany). This was to remove air bubbles at the bottom at the built-in inserts of the vials.

As mentioned earlier, the extraction current could be observed and recorded using a multimeter during EME. This would give a real time indication of extraction stability and efficacy. If the current was too low, electrokinetic migration of the analyte usually happened to a minimal degree. If the current was too high, it could lead to instability, electrolysis, and subsequent pH changes in the aqueous solutions. Figure 24 shows an example of a stable current curve in EME from experiment 19. A typical curve has a sharp decrease for the first minute, followed by a gradual reduction until the slope flattens out.

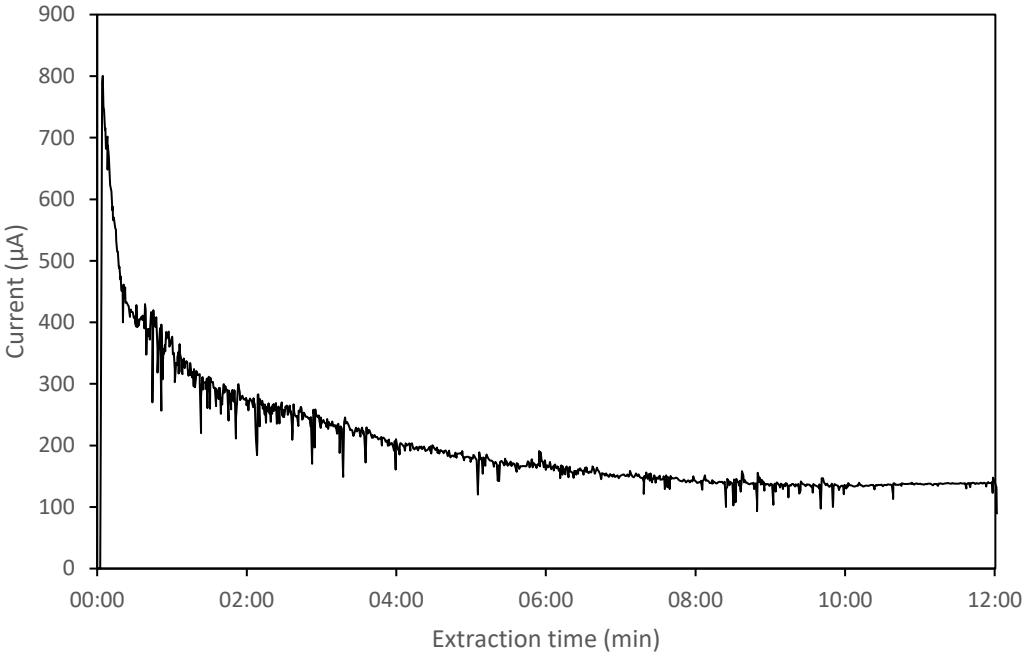



**Figure 24**. Example of an extraction current curve in EME (experiment 19).

## 3.6 HPLC-UV method development

For separation and detection of MTX and its metabolites 7-OH-MTX and DAMPA, a Hitachi Chromaster HPLC instrument was used, produced by Hitachi High-Tech Science Corporation (Tokyo, Japan). HPLC-UV analysis was applied to all experiments involving EME of MTX, 7-OH-MTX and DAMPA from standard buffer solutions. The extracts from spiked plasma samples were analyzed using LC-MS/MS.

**Table 7.** HPLC instrument components overview.

| Component | Description |
|---|---|
| Detector | Hitachi Chromaster 5430 Diode Array Detector |
| Auto Sampler | Hitachi Chromaster 5260 Auto Sampler |
| Pump | Hitachi Chromaster 5160 Pump |
| Degasser | Merck L-7614 (Merck Millipore (Burlington, MA, USA). |

| | |
|---|---|
| Column | Purospher® STAR RP-18 endcapped. 3 μm particle size, 55 mm x 4 mm. LiChroCART® Cartridge set (Supelco (Bellefonte, PA, USA). |
| Software | Chromaster system manager version 2.0 |

### 3.6.1 Establishment of gradient elution of MTX and 7-OH-MTX

To obtain sharp peaks and short retention times, gradient elution was applied to the method with a combination of two mobile phases. Mobile phase A consisted of Milli-Q water with 0.05% trifluoroacetic acid (TFA). Mobile phase B consisted of acetonitrile (ACN) with 0.05% TFA. A sample containing 250 μg/ml of MTX was used for development of the HPLC-method gradient. The flow rate was set to 1.2 ml/min. The initial gradient is listed in Table 8.

**Table 8**. Initial HPLC gradient for the elution of MTX.

| **Time (min)** | **% A (Milli-Q water + 0.05% TFA)** | **%B (ACN + 0.05% TFA)** |
|---|---|---|
| 0 | 60 | 40 |
| 5 | 20 | 80 |
| 6 | 60 | 40 |
| 7 | 60 | 40 |

After running the sample using the initial gradient, the chromatogram showed the elution of MTX almost immediately after injection. The composition of mobile phases was changed to initially contain more Milli-Q water, resulting in less competition from the mobile phase on the place on the stationary phase. Also, the amount of organic solvent was increased from 70%-80% over the course of a minute, to ensure that possible contaminants would become sufficiently washed from the column.

**Table 9.** Final HPLC gradient for the elution of MTX.

| Time (min) | % A (Milli-Q water + 0.05% TFA) | %B (ACN + 0.05% TFA) |
|---|---|---|
| 0 | 95 | 5 |
| 5 | 30 | 70 |
| 6 | 20 | 80 |
| 7 | 95 | 5 |
| 8 | 95 | 5 |

The peak identifying MTX in this chromatogram was satisfactory, with a retention time of 2.9 minutes. 7-OH-MTX had the same retention time. The metabolite differs from MTX by containing an extra OH-group, making it slightly more polar than MTX. In theory, it would therefore elute faster than MTX if the gradient allowed it. However, the LC-MS method in section 3.7 was developed with the purpose of being able to detect all three metabolites.

A monitoring wavelength at 300 nm was chosen, because MTX and 7-OH-MTX had the strongest absorption of UV-light at this value during method development. Both compounds strongly absorb UV-light, due to the heteroaromatic pterine and p-aminobenzoic chromophores (2).

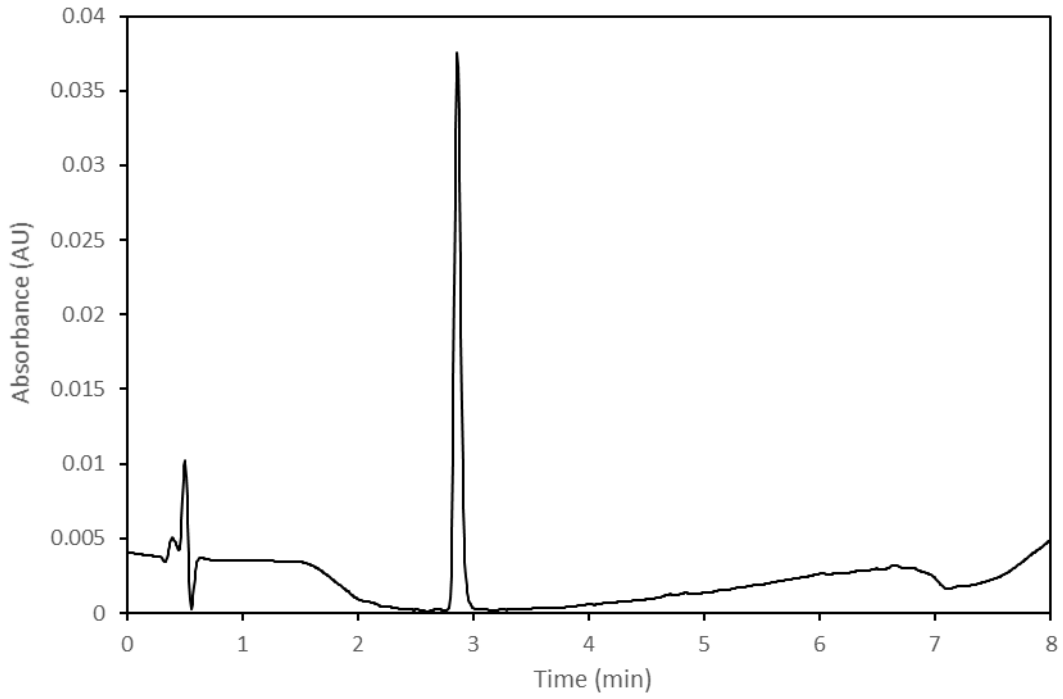


**Figure 25**. Chromatogram showing the elution of 10 µl 5 µg/ml MTX at 2.9 minutes.

A standard curve was developed, to establish the lower limit of detection and linearity. A sample of 250 µg/ml MTX was diluted with a mixture of water and ACN (95:5) 16 times, making the next sample half the concentration of the previous sample.

**Table 10.** Concentrations of MTX and its corresponding absorbance values.

| **Concentration of MTX (µg/ml)** | **Absorbance (AU)** |
|---|---|
| *250* | 3 514 707 |
| *125* | 1 981 605 |
| *63* | 1 031 105 |
| *32* | 527 181 |
| *16* | 265 083 |
| *8* | 132 763 |
| *4* | 66 462 |
| *2* | 33 176 |
| *1* | 16 704 |
| *0.5* | 8753 |
| *0.25* | 4137 |
| *0.125* | 1920 |
| *0.0625* | 1196 |
| *0.0313* | 550 |
| *0.0157* | 417 |
| *0.0078* | 647 |
| *0.0039* | 0 |

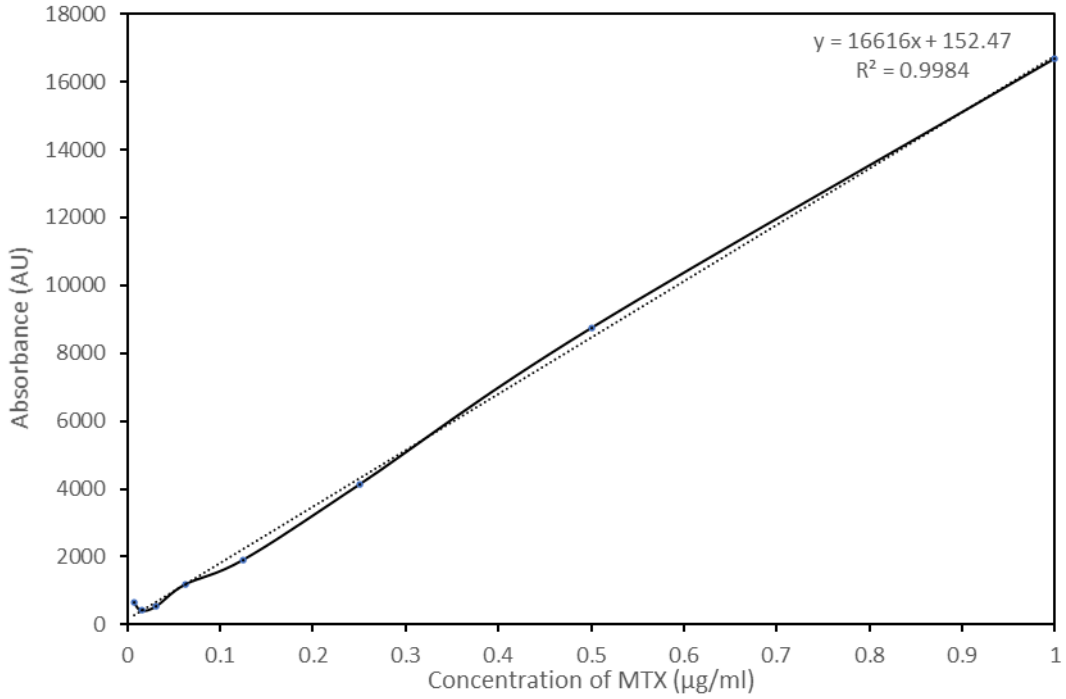



**Figure 26.** Standard curve of 10 µl 0.0078 - 1 µg/ml MTX.

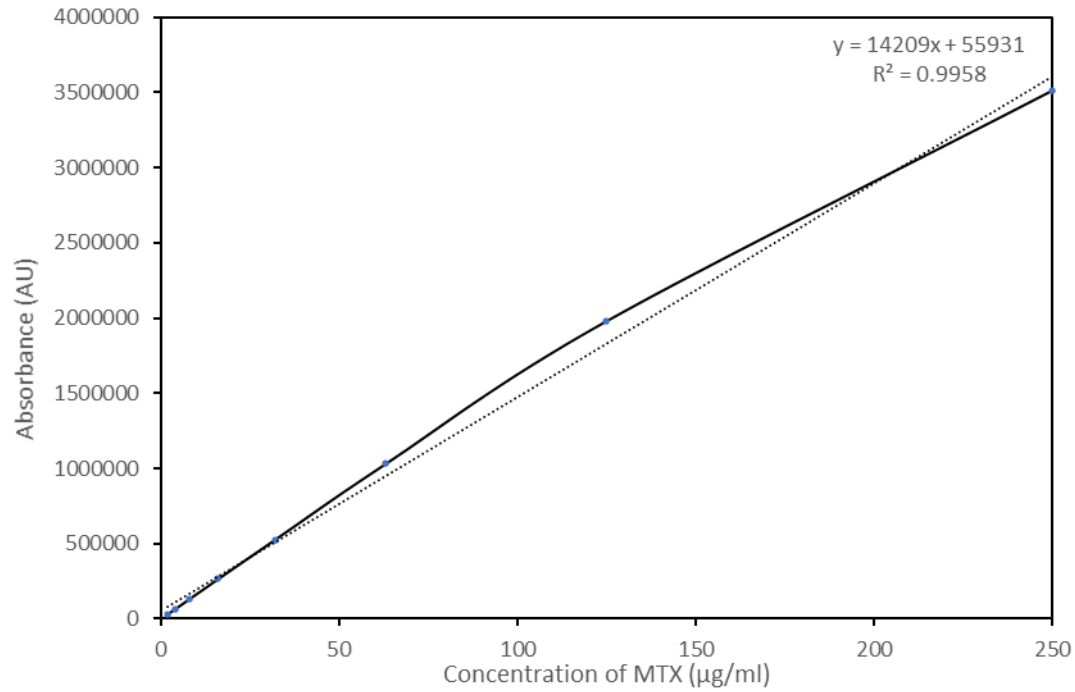



**Figure 27.** Standard curve of 10 µl 2 - 250 µg/ml MTX.

Below a concentration of 0.0313 µg/ml MTX, the standard curve was no longer linear. The chromatograms displayed small and broad peaks, and the area under the peak could not be

calculated accurately. At 0.0039 µg/ml MTX and below, no MTX was detected. Also, the curve flattened out between 125 and 250 µg/ml. In conclusion, the standard curve was linear in the range of 0.0313- 125 µg/ml. The trendline for the linear area has the equation y=15943x + 4119.

A sample of 100 µg/ml 7-OH-MTX was analyzed on HPLC and compared to the standard curve of MTX. When plotting the absorbance value of 10 µl 100 µg/ml 7-OH-MTX (1 278 778) into the equation for the linear trendline of MTX, x became 80 µg/ml. The reason why it was not 100 µg/ml was unclear, but could be that the compound was not sufficiently dissolved in methanol during preparation of the standard solution before distribution into separate vials (Table 4). Nevertheless, the HPLC-UV method was satisfactory for both MTX and 7-OH-MTX for the purpose of the development of an EME protocol.

**Table 11.** HPLC-UV parameters for the separation and quantitation of MTX and 7-OH-MTX.

| Parameter | Value |
|---|---|
| **Flow rate** | 1.2 ml/min |
| **Run time** | 8 minutes |
| **Mobile phase A** | Milli-Q-water + 0,05% trifluoroacetic acid (TFA) |
| **Mobile phase B** | Acetonitrile (ACN) + 0,05% trifluoroacetic acid (TFA) |
| **Injection volume** | 10 µl |
| **Wash solution** | Milli-Q-water + ACN (70:30) |
| **Monitoring wavelength** | 300 nm |

### 3.6.2 HPLC-UV method development for quantitation of DAMPA

The method for quantitation of MTX and 7-OH-MTX in section 3.6.1 did not apply for the metabolite DAMPA. No peaks appeared in the chromatogram when analyzing a sample containing this metabolite. This was somehow unexpected, since the analytes resemble in structure by containing the same ring structures (Figure 14 and 15). However, the loss of the glutamic part may have made DAMPA an unfitting candidate for the method developed for MTX and 7-OH-MTX.

Thus, a different column was applied for the separation of DAMPA. This was a 5 cm x 2.1 mm Ascentis® Express Phenyl-Hexyl column (Supelco (Bellefonte, PA, USA), with a particle size of 2.7 µm. The reason why this column was chosen, was that phenyl hexyl columns are beneficial for the purpose of separating aromatic compounds. Methanol was chosen as mobile phase instead of ACN, because it more easily breaks π-π interactions between aromatic compounds and the stationary phase, facilitating a faster elution of the analyte (58).

The flow rate in this method was adjusted downwards due to the reduction in internal diameter of the column. The C18 column had an internal diameter of 4 mm, while the phenyl hexyl column had an internal diameter of 2.1 mm. Keeping the same flow rate would most likely make the analyte elute too fast. In this method, 0.3 ml/min was applied, consequently leading to increased analysis time.

**Table 12.** HPLC gradient for the elution of DAMPA.

| **Time (min)** | **% A (Milli-Q water + 0.05% TFA)** | **%B (Methanol + 0.05% TFA)** |
|---|---|---|
| **0** | 90 | 10 |
| **8** | 25 | 75 |
| **9** | 90 | 10 |
| **15** | 90 | 10 |

**Table 13.** HPLC-UV parameters for the separation and quantitation of DAMPA.

| **Parameter** | **Value** |
|---|---|
| **Flow rate** | 0.3 ml/min |
| **Run time** | 15 minutes |
| **Mobile phase A** | Milli-Q-water + 0,05% trifluoroacetic acid (TFA) |
| **Mobile phase B** | Methanol + 0,05% trifluoroacetic acid (TFA) |
| **Injection volume** | 5 µl |
| **Wash solution** | Milli-Q-water + ACN (70:30) |
| **Monitoring wavelength** | 310 nm |

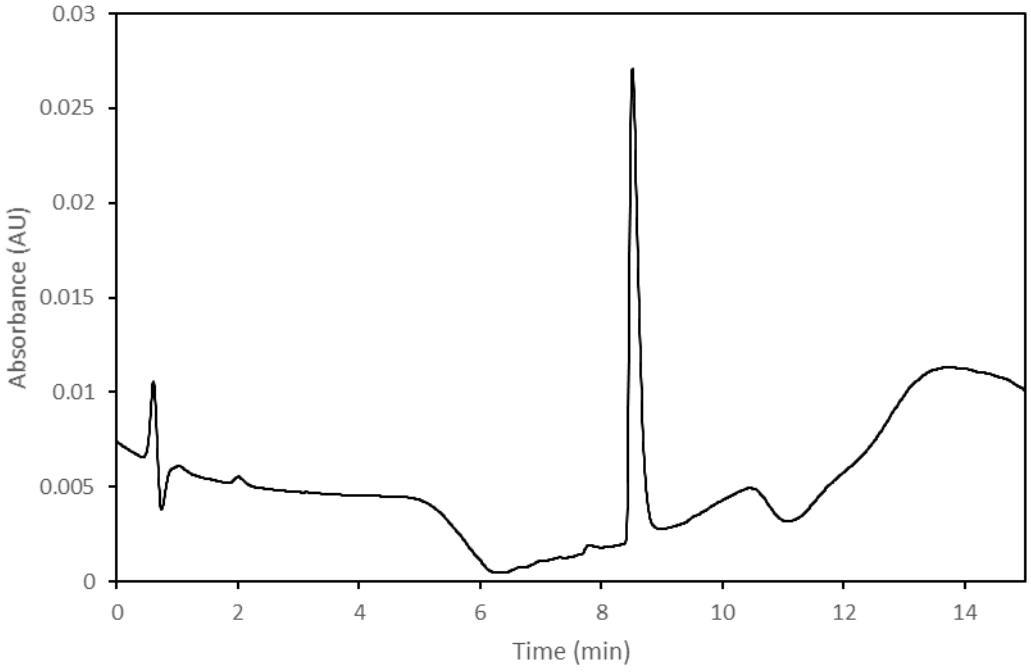



**Figure 28.** Chromatogram showing the elution of DAMPA at 8.3 minutes.

## 3.7 LC-MS/MS method development

An LC-MS/MS method for identification and quantification of MTX, 7-OH-MTX and DAMPA was developed using the instrument described in Table 14. The method was intended for analysis of plasma samples spiked with MTX and extracted by EME.

**Table 14.** LC-MS instrument components overview.

| Component | Description | Producer |
|---|---|---|
| Detector | API 2000 LC/MS/MS system | Applied Biosystems/ MDS Sciex (Framingham, MA, USA) |
| LC binary pump | Aligent 1100 G1312A | Aligent Technologies (Santa Clara, CA, USA) |
| LC autosampler | Aligent 1100 G1367A | |
| LC column oven | Aligent 1100 G1316A | |

| | | |
|---|---|---|
| Column | Kromasil 100-5-C18<br>2.1 × 100 mm | AkzoNobel (Amsterdam, The Netherlands) |
| Software | Analyst® 1.6.3 | Applied Biosystems/ MDS Sciex (Framingham, MA, USA) |

### 3.7.1 LC parameters and gradient for the separation of MTX, 7-OH-MTX and DAMPA

The gradient applied for the elution of the MTX, 7-OH-MTX and DAMPA in the LC-MS/MS method was similar to the one for the HPLC-UV method (section 3.6.1), with an initial high percentage of Milli-Q water followed by an increasing amount of ACN up to 90% (Table 15).

**Table 15.** Composition of mobile phase gradient for the elution of MTX, 7-OH-MTX and DAMPA.

| **Time (min)** | **%A (Milli-Q water + 0.01% formic acid)** | **%B (ACN + 0.01% formic acid)** |
|---|---|---|
| 0 | 90 | 10 |
| 5 | 10 | 90 |
| 6 | 10 | 90 |
| 6.1 | 90 | 10 |
| 10 | 90 | 10 |

**Table 16**. Instrumental conditions for LC separation of MTX, 7-OH-MTX and DAMPA.

| **Parameter** | **Description** |
|---|---|
| Flow rate | 250 µl/min |
| Run time | 10 minutes |
| Column temperature | 35 °C |
| Injection volume | 1 µl |

The retention times for MTX, 7-OH-MTX, and DAMPA were 3.7, 4.2, and 4.2 minutes,

respectively (Figure 29). 7-OH-MTX and DAMPA could not be sufficiently separated by the chromatographic method. However, MS/MS detection made it possible to identify and quantify each metabolite.

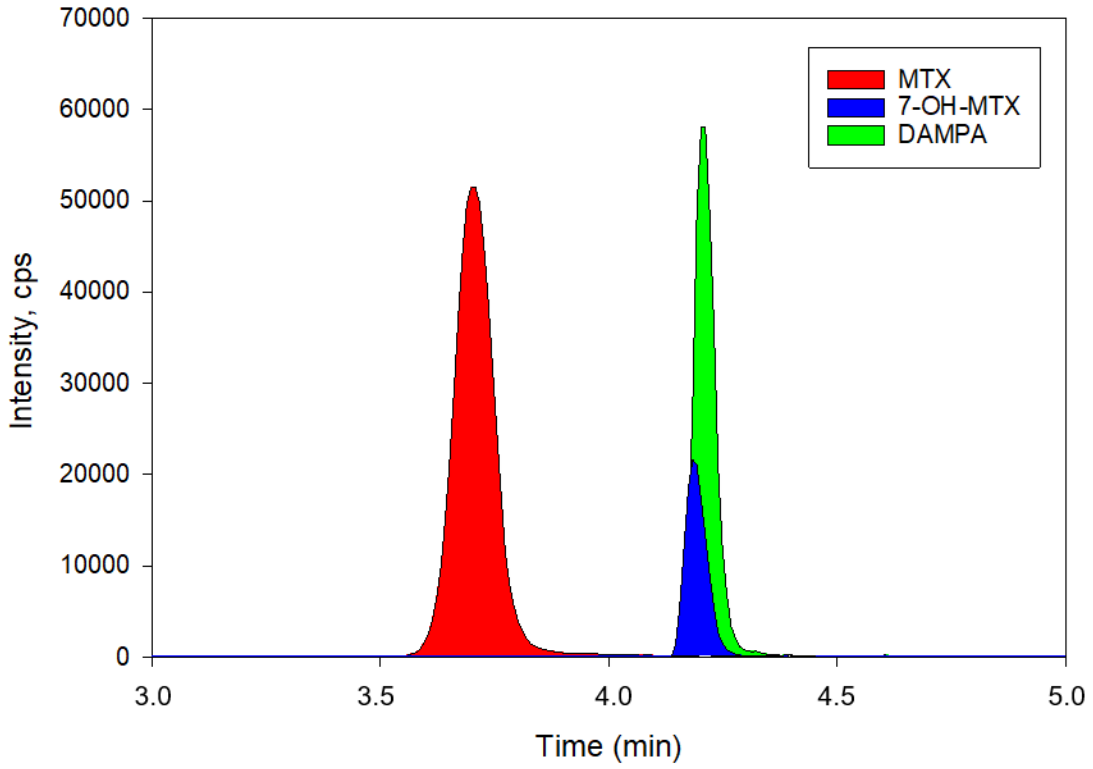



**Figure 29**. Chromatogram showing the elution of 1 µl 0.212 µM MTX, 0.212 µM DAMPA and 1.33 µM 7-OH-MTX.

When analyzing samples containing only MTX, the retention time was approximately four minutes. However, when analyzing MTX in a mixture with the other metabolites, it eluted earlier. This can be explained by instrumental variations such as a small change in mobile phase composition or flow rate, or interference from the other metabolites in the solution or with the stationary phase.

### 3.7.2 MS/MS method development for detection and quantification of MTX, 7-OH-MTX and DAMPA

A multiple reaction monitoring (MRM) scan type was applied, which is a common technique for triple quadrupole MS (59). In the first quadrupole, the precursor ion/target analyte is selected and reaches the collision cell, where it undergoes fragmentation. One (or several) of

the fragmented daughter ions can be selected to flow through the third quadrupole, while other ions are excluded.

The LC-MS/MS method applied electrospray ionization in positive mode for generation of the precursor ions before entering the first quadrupole. The ion spray voltage was 3500 V, and the temperature in the ion source was 450 °C.

The MS/MS conditions in Table 17 were adjusted for MTX only, assuming that the method would be applicable to the other two metabolites. A solution of 1 µM MTX was injected directly into the MS/MS for determination of the best values for each parameter. The values were adjusted based on the signal vs. noise ratio for MTX at different values. The values producing highest signal and lowest amount of noise were chosen.

**Table 17**. MS/MS conditions for the entrance into the MS/MS, and transition of precursor ion to fragment 1 for MTX, 7-OH-MTX and DAMPA.

| Parameter | Value |
|---|---|
| Entrance potential (EP) | 7 V |
| Focusing potential (FP) | 400 V |
| Declustering potential (DP) | 20 V |
| Collision energy (CE) | 30 V |
| Collision cell exit potential (CXP) | 15 V |
| Collision cell entry potential (CEP) | 15 V |

**Table 18**. Mass transitions for MTX, 7-OH-MTX and DAMPA.

| Analyte | Precursor ion (Q1 mass) | Fragment 1 (Q3 mass) | Fragment 2 (Q3 mass) | Fragment 3 (Q3 mass) |
|---|---|---|---|---|
| MTX | 455.33 | 308.10 | 175.00 | 134.00 |
| 7-OH-MTX | 471.20 | 324.10 | 191.10 | 148.20 |
| DAMPA | 326.10 | 175.00 | | |

The precursor ion of MTX and 7-OH-MTX was fragmented into three daughter ions. However, only fragment 1 was used for quantitation.

### 3.7.3 Evaluation of the LC-MS/MS method

Samples analyzed on LC-MS/MS were prepared and marked with µM instead of mg/ml. There were two reasons for this; µM is a more exact unit of measurement, as it presents the number of molecules in a solution. Also, in a clinical context, the µM unit is more frequently used than mg/ml, for denoting concentration in biological fluids.

For establishment of linearity, MTX was prepared in in concentrations ranging from 0.0016 – 51.2 µM and analyzed by LC-MS/MS. The solvent for MTX was methanol.

**Table 19**. Concentrations of MTX and its corresponding analyte signal intensity (cps) peak area.

| **Concentration (µM)** | **Analyte peak area** |
|---|---|
| 0.0016 | 397 |
| 0.0063 | 1160 |
| 0.025 | 4840 |
| 0.1 | 18 500 |
| 0.2 | 36 500 |
| 0.4 | 70 700 |
| 0.8 | 149 000 |
| 1.6 | 289 000 |
| 3.2 | 599 000 |
| 6.4 | 1 140 000 |
| 12.8 | 2 360 000 |
| 51.2 | 7 450 000 |

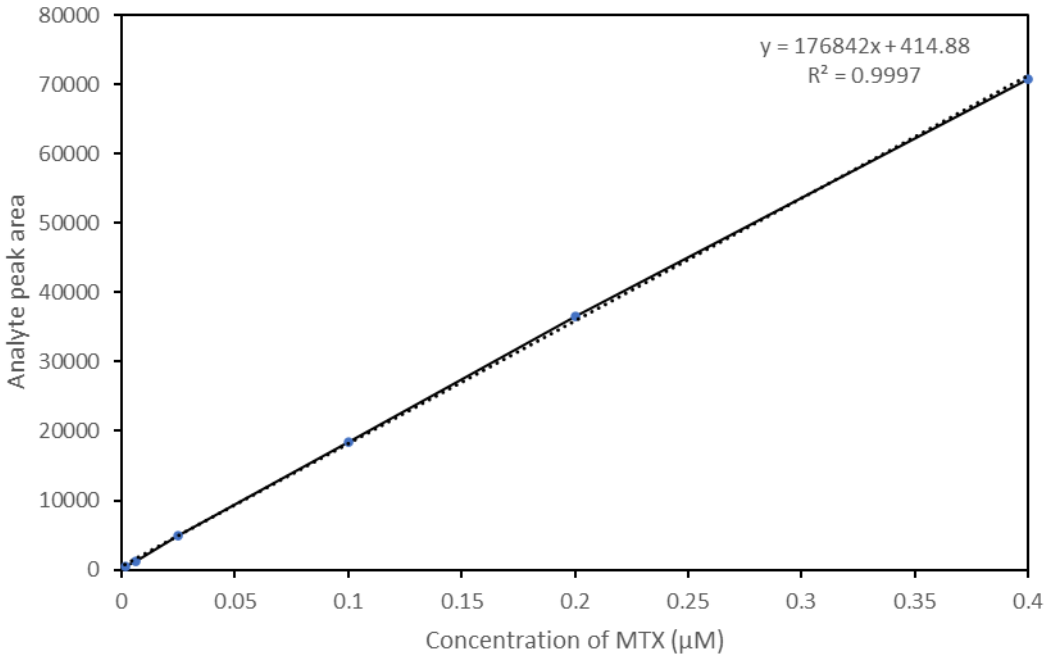



**Figure 30**. Standard curve of 1 µl 0.0016-0.4 µM MTX.

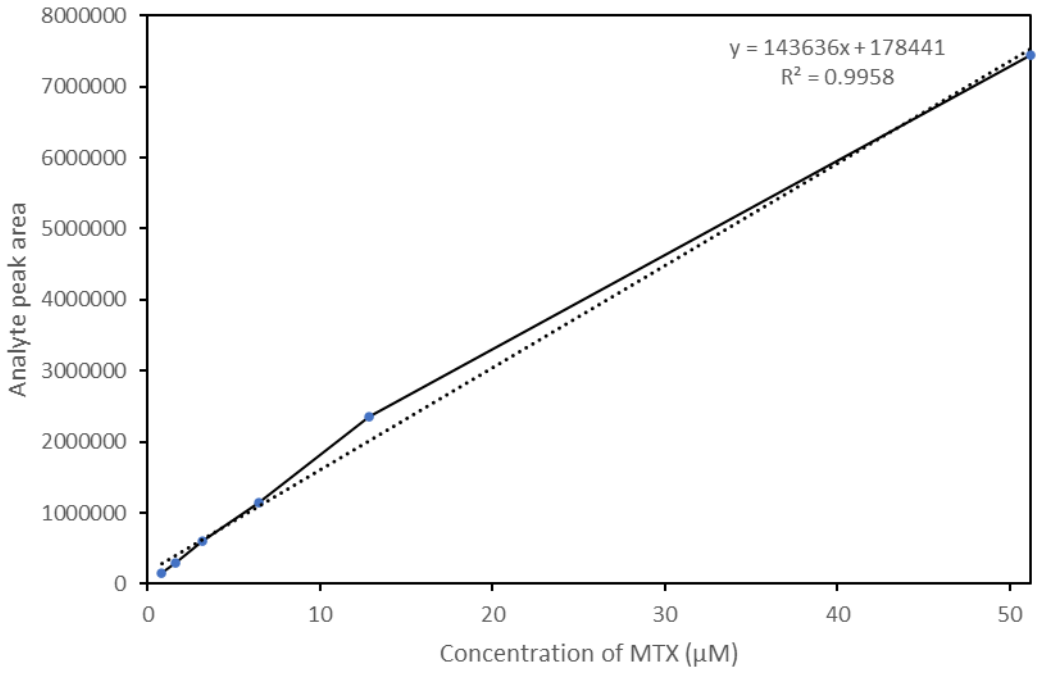



**Figure 31.** Standard curve of 1 µl 0.8-51.2 µM MTX.

A sample of 0.0004 µM MTX was also injected, but it did not produce a detectable peak in the chromatogram. The lower limit of detection was therefore 0.0016 µM MTX, based on the samples analyzed. Above 12.8 µM MTX, the standard curve was no longer linear. Hence, the

LC-MS/MS method for MTX was linear in the range 0.0016-12.8 μM.

To check whether the instrument provided various signals between injections from the same sample, some of the samples from Table 19 were analyzed three times each.

**Table 20**. Investigation of LC-MS/MS instrument precision.

| Concentration (μM) | Analyte peak area (injection 1) | Analyte peak area (injection 2) | Analyte peak area (injection 3) |
|---|---|---|---|
| 0.1 | 18 500 | 18 900 | 17 800 |
| 0.2 | 36 500 | 33 500 | 36 400 |
| 0.4 | 70 700 | 70 900 | 71 200 |
| 0.8 | 149 000 | 146 000 | Not analyzed[1] |
| 1.6 | 289 000 | 285 000 | 288 000 |
| 3.6 | 599 000 | 552 000 | 610 000 |

[1]The needle did not retract from the vial after perforating the septum, and the sample was omitted from the injection queue.

There were minor variations between injections, and there could be several reasons for this. First, the instrument was relatively old. The API 2000 MS/MS model was from 1997, thereby lacking the most recent technology. Second, and perhaps more decisive, the instrument had been idle for two years without regularly maintenance. This could cause variation in the injection volume by the autosampler, or in the elution gradient by the gradient pump. Therefore, it is expected that the precision would be higher using an instrument that had undergone the required maintenance. Nevertheless, the addition of an internal standard (IS) to the samples could adjust for the various signal intensities. This is because the analyte and IS are affected the same way by the instrumental variations. The ratio between them would give a true estimate of the signal intensity of the analyte.

The linearity of 7-OH-MTX was not established, since the LC-MS/MS method did not appear to respond well to this metabolite. In Figure 29, 1 μl 1.33 μM 7-OH-MTX was analyzed in mixture with 0.212 μM MTX and 0.212 μM DAMPA. 7-OH-MTX provided a much lower signal despite the higher concentration. In the HPLC-UV method, the signal of 7-OH-MTX was 80% of the signal produced by MTX. This suggested that the problem lied in the LC-MS/MS method, leading to incomplete ionization of the mother ion to fragment 1. This might

be explained by the additional OH-group of the analyte, interfering with the ring structures due to intramolecular forces and altering the physicochemical properties of the metabolite. Nevertheless, in order to establish an approved method for the analyte, the MS/MS conditions would have to be optimized for 7-OH-MTX specifically.

DAMPA appeared to respond well to the method, and a standard curve of DAMPA was prepared and analyzed on LC-MS/MS with following concentrations:

**Table 21.** Concentrations of DAMPA and its corresponding analyte signal intensity (cps) peak area.

| Concentration (µM) | Analyte peak area |
|---|---|
| 0.0096 | 1590 |
| 0.039 | 5720 |
| 0.154 | 20 700 |
| 0.616 | 79 100 |
| 2.463 | 279 000 |
| 9.850 | 881 000 |
| 39.40 | 2 520 000 |
| 157.6 | 7 000 000 |

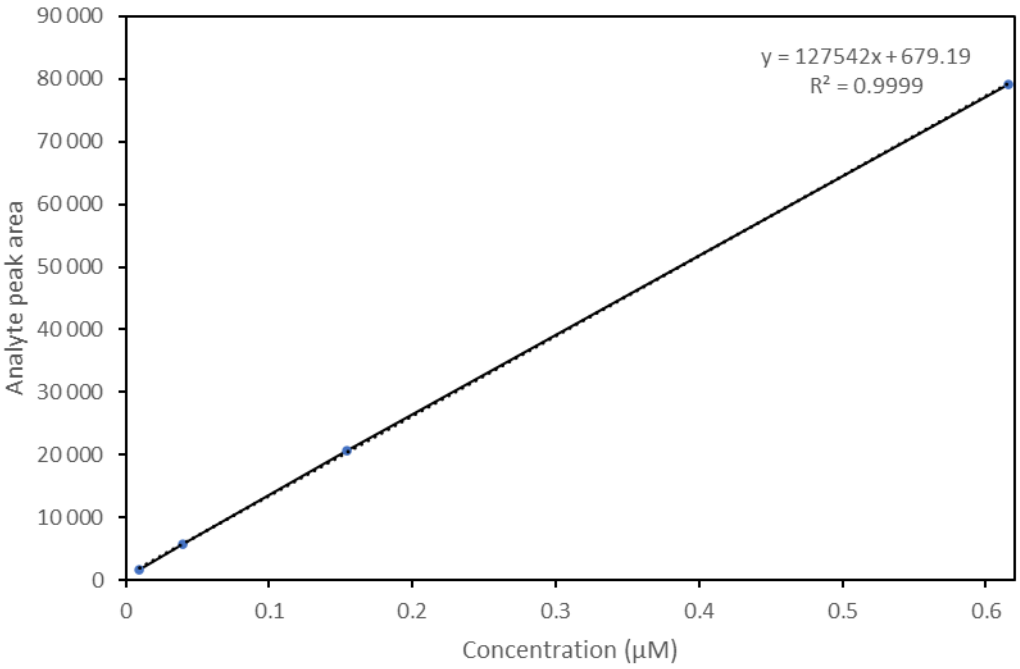



**Figure 32.** Standard curve of 1 µl 0.0096-0.616 µM DAMPA.

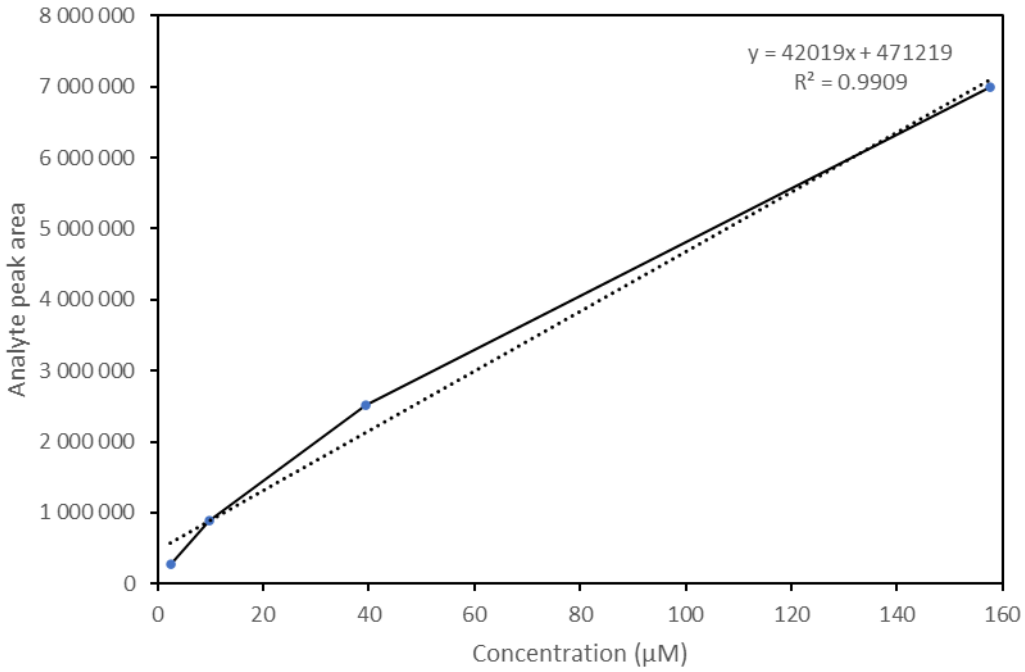



**Figure 33**. Standard curve of 1 µl 2.463-157.6 µM DAMPA.

The standard curve of DAMPA was only linear in the range 0.0096-0.616 µM (Figure 32), which was narrower compared to the linear range for MTX. One reason for this could be that the LC-MS/MS method was developed for MTX, in assumption that it would apply similarly

to the metabolite. Thus, some of the parameters in Table 17 could have been suboptimal for DAMPA. Another explanation could be day-to-day variations of LC-MS/MS instrument performance, or the fact that the instrument is relatively old, as discussed above. Again, an IS could adjust for the reducing signal intensity DAMPA and increase the linearity of the method.

Despite the lack of internal standard for adjustment of precision and linearity, the LC-MS/MS method was more sensitive than the HPLC-UV method for MTX. MTX could be detected at 0.0016 µM by LC-MS/MS, whereas the lower limit of detection of the HPLC-UV method was 0.069 µM. Also, the injection volume of LC-MS/MS was 1 µl, compared to the HPLC-UV method that injected 10 µl. With this taken into account, the LC-MS/MS method was 431 times more sensitive than the HPLC-UV method. It was also more selective, as it could identity and quantitate all three metabolites in a mixture. In the current HPLC-UV method, MTX and 7-OH-MTX could not be separated in the column, and another method had to be used for separation and detection of DAMPA.

## 3.8 Calculations

For calculation of extraction recovery in the acceptor solution, following equation was used.

$$R = \frac{A_A}{A_{Std}} \cdot 100\% \qquad \text{(Equation 3)}$$

Where R is extraction recovery, $A_A$ is peak area for the analyte, and $A_{Std}$ is peak area for the standard solution. The same equation was applied for calculation of percentage of analyte in the donor solution.

For calculation of the standard deviation of samples extracted simultaneously, Equation 4 was applied.

$$S = \sqrt{\frac{\sum(x-\bar{X})^2}{n}} \qquad \text{(Equation 4)}$$

Where S is standard deviation, x is the recovery for each sample, x-bar is the mean recovery and n is the number of samples.

The relative standard deviation was calculated by following equation:

$$RSD = \frac{S}{\bar{X}} * 100\% \qquad \text{(Equation 5)}$$

Where S is the standard deviation, and X-bar is the mean recovery of the samples.

The standard curves were calculated by following equation:

$$Y = ax + b \qquad \text{(Equation 6)}$$

Where a and b are constant numbers, x is the concentration of analyte and Y is the instrument response.

The coefficient of determination ($R^2$) value was calculated to observe how closely the data points fitted the trendline from the standard curves. The closer the $R^2$ value was to 1, the closer the data points fitted the model.

$$R^2 = 1 - \frac{SS\ regression}{SS\ total} \qquad \text{(Equation 7)}$$

Where SS regression is the sum of squares due to regression, and SS total is the total sum of squares (60).

# 4 Results and discussion

## 4.1 Extraction of MTX as a cation through NPOE

The extraction of MTX as a protonated base (cation) was a natural place to start, considering that most studies on EME are done on basic compounds, and successful SLMs and ionic carriers are established. In the initial experiments, MTX was attempted to be extracted through an SLM consisting of the widely used organic solvent, NPOE. Based on information from other users of EME, a supplied power of 100 V for 15 minutes was common for the extraction through NPOE. The agitation rate was set to 750 RPM, based on literature on EME suggesting that agitation rates between 500 and 1000 RPM are optimal (61).

In experiment 1, both the donor and acceptor solution consisted of 50 mM HCl with a pH of 1.3. The low pH value was chosen based on the pKa value of the basic nitrogen atom on MTX, which is 2.8. The Chemicalize database simulation estimates that 96% of MTX molecules are protonated at pH 1.3 (28).

**Table 23.** Experiment 1. Result from extraction of 8 µg/ml MTX through NPOE.

| SLM | Donor solution | Acceptor solution | Voltage (V) | Time (min) | Agitation (RPM) | Recovery acceptor solution (%) |
|---|---|---|---|---|---|---|
| NPOE | 50 mM HCl pH 1.3 | 50 mM HCl pH 1.3 | 100 | 15 | 750 | 0.0 |

MTX was not detected in the acceptor solution. It is well known that the extraction of highly polar analytes (logP < 0) is particularly difficult with EME (23). MTX has multiple oxygen and nitrogen atoms that contribute to its polarity, and Chemicalize predicts that the molecule has a log D value of -0.64 at pH 1.3 (28). The amount of MTX left in the donor solution after extraction was 100%, suggesting no partition into the organic solvent despite applying 100 V to facilitate electrokinetic migration. The electrical current was very low during the extractions (2 µA), indicating minimal flux of ions across the membrane.

### 4.1.1 Extraction through NPOE including the ionic carrier DEHP

The addition of the ionic carrier DEHP in NPOE has shown to be successful for extraction of moderately polar analytes with a logP of 0-2 (23). Experiment 2 was therefore carried out with 10% or 20% DEHP added to NPOE as the SLM, in hopes that it would yield higher transport of MTX into the acceptor solution.

The donor solution consisted of a 20 mM $KH_2PO_4$ solution with a pH of 2.6. A more acidic solution could lead to loss of the negatively charged phosphate groups on DEHP, due to its pKa value of 1.94 (23). A higher pH could have kept the MTX molecules from being sufficiently protonated in the donor solution. Chemicalize predicted that 56% of the MTX molecules carry a positive charge at pH 2.6 (28). In theory, the protonated fraction would be extracted into the acceptor solution, followed by a new established equilibrium in the donor solution, which would ensure a continuous transfer of protonated MTX molecules.

The acceptor solution consisted of a 50 mM HCl solution with a pH of 1.3. The low pH value was chosen to account for the possibility of electrolysis. DEHP has the tendency to increase current in the system, which will make the system more prone to electrolysis and elevated pH at the negatively charged cathode (33).

**Table 24.** Constant parameters for experiment 2-4.

| **Parameter** | **Description** |
|---|---|
| Donor solution | 20 mM $KH_2PO_4$ pH 2.6. |
| Acceptor solution | 50 mM HCl pH 1.3 |
| Time | 15 minutes |
| Agitation | 750 RPM |

**Table 25.** Results from extraction of 8 µg/ml MTX through NPOE + DEHP

| Experiment | SLM | Voltage (V) | Recovery acceptor solution (%)[1] |
|---|---|---|---|
| 2A | NPOE + 10% DEHP | 50 | 0.2 ± 0.1 |
| 2B | NPOE + 10% DEHP | 100 | 0.4 ± 0.4 |
| 2C | NPOE + 20% DEHP | 50 | 1.2 ± 0.9 |

[1]All experiments are based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

The recoveries of MTX were very poor in these experiments (Table 25). The amount of MTX in the acceptor solution was close to 0%. However, it appeared as if a great amount of the molecules had been removed from the donor solution, unlike the extraction through pure NPOE. In experiment 2B, 56.1 % of the added MTX was left in the donor vial, and 0.4% existed in the acceptor vial after extraction. The rest (43.5%) was not detected.

The possibility of degradation of MTX due to high voltage was considered unlikely. In experiment 1, 100% of MTX was detected in the donor vial, despite using 100 V. Chemical degradation or precipitation of MTX due to the low pH neither appeared to be the case, as the analyte remained stable in the donor solution with a pH of 1.3 in experiment 1. Most likely, there was transport of the analyte into the SLM, or to the interfaces between the SLM and the aqueous solutions. This suggested that MTX had an affinity to DEHP, where the positively charged MTX molecules were attracted to the negatively charged phosphate groups on DEHP.

To investigate whether MTX was transported into the SLM, or existed in the aqueous solutions/SLM interfaces, experiment 2C was performed again, with the agitator running for 5 additional minutes after turning off the voltage. If MTX interacted with DEHP in the interfaces, it was hypothesized that the molecules would diffuse back into the donor/acceptor solutions once they were no longer influenced by the electrical field. The results showed a recovery of 46.3% in the donor solution, and 3.1% in the acceptor solution. The additional 2% recovery of MTX in the acceptor solution may be due to release of MTX ions at the acceptor

solution/SLM interface. However, it may also be a coincidence due to variability in the technical or conductive properties of the vials, which will be discussed in section 4.5.1. Nevertheless, a great amount of MTX was still not detected in neither the donor nor acceptor solution.

To check if the MTX molecules were trapped inside the SLM, the PP-membrane was removed from the union and placed inside another vial after extraction. To this vial, 250 µl 10 mM NaOH was added, in which MTX is soluble. The vial was shaken for 10 minutes and its content analyzed on HPLC. 1.2% of MTX was detected, indicating that some of the analyte existed in the SLM, but the rest may not have been released through stirring alone.

The amount of DEHP in NPOE was then reduced to 5%, 2.5%, 0.5% and 0.125% to see if the combined recoveries in the donor and acceptor solutions increased. The theory proposed that a reduced percentage of DEHP in the SLM would lower its ability to bind MTX, and more MTX molecules would exist in the aqueous solutions. The constant parameters for the following experiments are listed in Table 24.

**Table 26.** Results from extraction of 8 µg/ml MTX through NPOE including decreasing amounts of DEHP.

| Experiment | SLM | Voltage (V) | Recovery donor solution (%) | Recovery acceptor solution (%) |
|---|---|---|---|---|
| **3A** | NPOE + 5.0% DEHP | 100 | 54.5% | 1.6 % |
| **3B** | NPOE + 2.5% DEHP | 100 | 51.9 % | 0.5% |
| **3C** | NPOE + 0.5% DEHP | 100 | 50.5 % | 0.6% |
| **3D** | NPOE + 0.125% DEHP | 100 | 75.4 % | 0% |

The results show that with a concentration of 0.5-5% DEHP in the SLM, more than 40% of the MTX molecules were still not detected. A reason for this is that 0.5% DEHP may still be sufficient to interact with MTX and trap the analyte in the membrane. However, at 0.125%

DEHP, 75.4% of MTX was detected in the donor solution. This demonstrates that with a decreasing amount of carrier, an increased fraction of the MTX molecules is left in the donor solution. This also supports the theory that MTX has a strong affinity to DEHP.

Another theory on why such an extensive amount of the MTX molecules was undetectable with a concentration of DEHP from 0.5-20%, is that the positively charged molecules had in fact been extracted into the acceptor solution, but had been adsorbed onto the negatively charged acceptor vial wall during extraction. Due to the low solubility of MTX in acidic conditions, it could be that the molecules were not released from the wall of the acceptor vial after the extraction. To investigate this theory, the acceptor solution was removed from the acceptor vial after extraction, followed by addition of 250 µl phosphate buffer with pH 7.4, in which MTX is soluble, and which should be able to release MTX from the inside of the vials. The vial was shaken for 10 minutes and the contents analyzed on HPLC. No MTX was detected in the phosphate buffer from the acceptor vial, demonstrating that strong interaction between MTX and the negatively charged vials did not occur during EME.

In conclusion, in its cationic state MTX most probably had a strong affinity to DEHP and was immobilized in the SLM. Once the analyte formed ionic bonds with DEHP and diffused into the SLM, it may have lost overall positive charge and was not readily influenced by the electrical field. The reason why the analyte was not released into the acceptor solution could be elevated pH in the acceptor solution during extraction due to electrolysis (equation 2), and/or the formation of an electrical double layer at the acceptor solution/SLM interface. This may have resulted in a lack of protons in the interface, with reduced protonation of DEHP and consequent lack of the ability to release MTX into the acceptor solution. For further investigation, the pH in the acceptor solution could have been measured before and after extraction to draw clearer conclusions. Another reason why MTX was not released to the acceptor solution could be that MTX has a low solubility at low pH values, and its affinity to the carrier may have been greater than to the acidic aqueous acceptor solution.

## 4.2 Extraction of MTX through 2-Nitrophenyl pentyl ether + DEHP

2-Nitrophenyl pentyl ether (NPPE) is another organic solvent used as SLM in EME, and it

differs from NPOE by containing a pentyl hydrocarbon chain instead of octyl hydrocarbon chain.

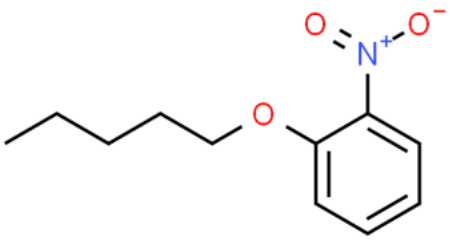


**Figure 34.** Chemical structure of NPPE. Downloaded from (62).

Due to the shorter hydrocarbon chain, NPPE is less hydrophobic than NPOE. This might favor partition of MTX into the SLM and release to the acceptor solution.

Three NPPE experiments were performed, with constant extraction parameters listed in Table 24. The varying parameters in the following experiments were % DEHP in NPPE and voltage.

**Table 27.** Results from extraction of 5 µg/ml MTX through NPPE including DEHP.

| Experiment | DEHP in NPPE (%) | Voltage (V) | Recovery donor solution (%)[1] | Recovery acceptor solution (%)[1] |
|---|---|---|---|---|
| 4A | 20 | 50 | 15.0 ± 7.1 | 7.1 ± 0.4 |
| 4B | 20 | 100 | 5.8 | 4.6 ± 0.7 |
| 4C | 10 | 100 | - | 3.3 ± 0.4 |

[1]All experiments are based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value. In experiment 4B, the chromatogram of one donor solution contained peaks that could not be separated and quantified by HPLC. In experiment 4C, this applied for the chromatogram of both donor solutions.

The extraction recoveries were higher when using NPPE compared to NPOE, with 7.1% as the best result. The enhanced recoveries were most likely due to the reduced hydrophobicity of NPPE, which allowed MTX to partition into the SLM more easily. The amount of MTX removed from the donor solutions was also larger than in the experiments with NPOE, suggesting that more molecules had been transported into the membrane.

The extraction current was observed during extraction of all experiments. In experiment 4A, the current was approximately 20 µA for each sample. In experiment 4B and 4C, the current was 100 and 70 µA, respectively. This confirmed that an increasing amount of DEHP, and higher voltage, increased the current in the system. For experiment 4B-4C, 20% DEHP in the SLM seemed to be favorable compared to 10% DEHP, despite the higher current. However, a high concentration of DEHP (20%) combined with 50 V resulted in the highest recovery.

MTX has a retention time of 2.9 minutes on HPLC. In the experiments with NPPE, another peak appeared in the chromatogram at 2.8 minutes (Figure 35). This peak was visible in all three experiments. However, it only appeared in the donor solutions.

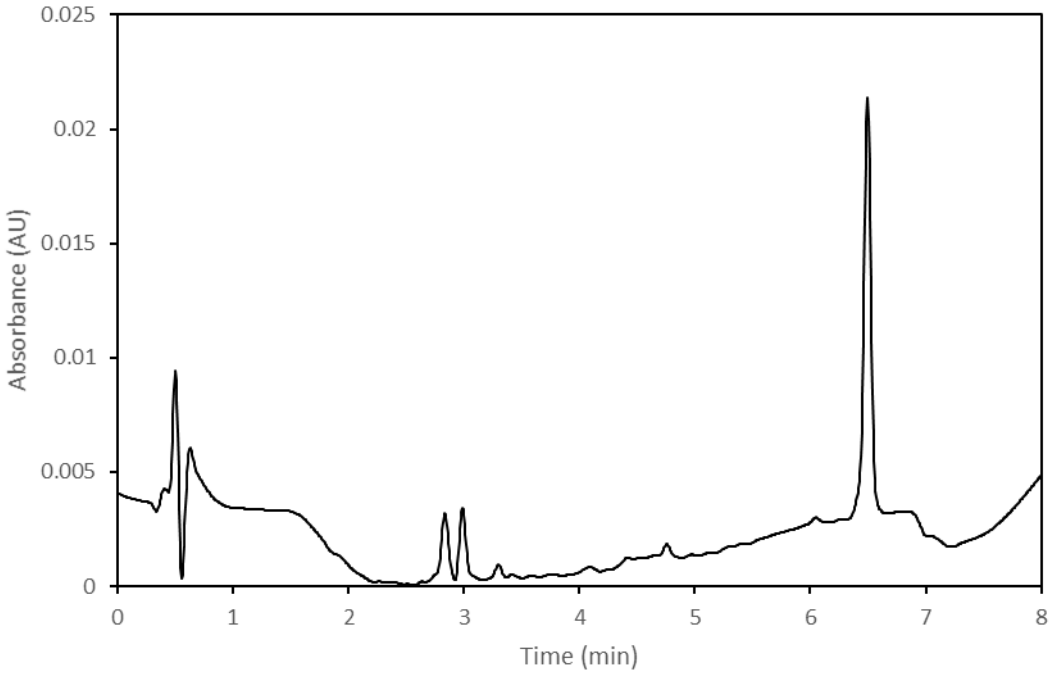



**Figure 35.** Chromatogram of a donor solution from experiment 4A.

At first, it was suggested that this peak could represent DEHP that had leaked into the donor solution. However, after adding 5 µl DEHP to a blank 20 mM $KH_2PO_4$ solution, no peaks appeared on the chromatogram after running the sample on HPLC.

The peak did not represent NPPE, as this compound has a retention time of 6.5 (Figure 35). The identity of the peak was confirmed when running a blank 20 mM $KH_2PO_4$ solution spiked with 5 µl NPPE, providing a strong signal at 6.5 minutes. The same peak also appeared with

the same retention time in the chromatograms from the samples extracted through NPOE. This did not come as a surprise, as the two organic solvents are highly similar in terms of molecular structure.

In two of the experiments (4B-C), the two peaks were not resolved, and the area under the curve was not possible to calculate for each peak. It was hypothesized that the peak at 2.8 minutes represented a derivative of MTX, produced by a chemical degradation during EME. Alternatively, it could represent MTX complexed to DEHP, leaked from the SLM. However, the UV spectrum of the peak at 2.8 minutes showed no similarities to the UV spectrum of MTX, and both theories therefore seemed unlikely. It remains unknown what compound the additional peak represented.

### 4.2.1 Final thoughts on the extraction of MTX as a cation

When extracting MTX as a cation, the positive charge is located at the end of the pteridine ring residue (Table 1). Generally, a charged analyte has a relatively high water solubility, and a relatively poor solubility in organic solvents (14). The other end of the MTX molecule contains two carboxylic acids, which will also contribute to its polarity. Even though the carboxylic acids are neutrally charged in acidic solutions, the oxygen atoms are electronegative and attract bonding pair of electrons, increasing the polarity of the covalent bonds and the molecule (63). Hence, both ends of the MTX molecule can possibly contribute to the polarity of MTX and make its partition into the SLM more demanding. However, when extracting MTX as a deprotonated acid, the pteridine ring residue is neutrally charged and contributes less to the polarity, whereas the deprotonated carboxylic acids are highly polar. Having a relatively non-polar part of the molecule might favor the partition into the SLM, and in the remaining sections, EME of MTX as a deprotonated acid will be discussed.

## 4.3 Extraction of MTX as an anion through aliphatic alcohols

Aliphatic alcohols have proved to be efficient SLMs for the extraction of acidic substances. Long-chain alcohols with strong proton acceptor properties, especially 1-octanol, have shown to yield successful extractions (64).

### 4.3.1 Extraction of MTX through 1-octanol

In the initial experiments, MTX was extracted through 1-octanol. The pH in the donor solution was 7.4, to mimic the physiological pH of plasma, and Chemicalize simulates that almost 100% of the MTX molecules carry two negative charges at this pH (28). The pH in the acceptor solution was set to 12 by using 10 mM NaOH. The reason why the pH was set significantly higher in the acceptor solution than in the donor solution, was to counter for the possible reduction in pH due to electrolysis, and the formation of an electrical double layer.

**Table 28.** Constant parameters for experiment 5-8.

| Parameter | Description |
|---|---|
| Donor solution | 40 mM phosphate buffer, pH 7.4 |
| Acceptor solution | 10 mM NaOH, pH 12 |
| Time | 15 minutes |
| Agitation | 750 RPM |

**Table 29.** Results from extraction of 5 µg/ml MTX through 1-octanol at 50 V and 100 V

| Experiment | SLM | Voltage (V) | Recovery donor solution (%) | Recovery acceptor solution (%) |
|---|---|---|---|---|
| 5A | 1-octanol | 50 | 90.6 | 0.5 |
| 5B | 1-octanol | 100 | 45.4 | 3.3 |

Table 29 shows very poor recovery of MTX in the acceptor solutions. At 50 V, the extraction current was low during the whole extraction (8 µA). This indicates low flux of ions across the membrane. The most likely explanation is that MTX is too polar to partition into the SLM at this voltage. The Chemicalize database simulation estimates that MTX has a log D value of -6.56 at pH 7.4, which indicates that the analyte has very little affinity to the organic phase (28).

At 100 V, the extraction current was significantly higher (150 µA). The recovery in the donor solution was 45.5% (Table 29), suggesting that a great amount of MTX molecules partitioned

into the SLM at this voltage. However, only 3.3% was released into the acceptor solution. The reason why over 50% of MTX molecules were absent from the aqueous solutions is unknown, as one would assume that the analyte had a higher affinity to aqueous conditions than the organic solvent. One possible explanation is that once the analyte partitioned into the SLM at 100 V, intermolecular forces between MTX and 1-octanol hampered the release of MTX into the acceptor solution. 1-octanol has an OH-group capable of hydrogen bond interaction with the deprotonated carboxylic acids on MTX. A more vigorous shaking (>750 RPM) could have been attempted to break the possible interactions between MTX and 1-octanol. However, this would also contribute to higher current in the system, and the current was already high (150 µA). As previously mentioned in section 2.2.3, a current exceeding 50 µA is generally not recommended in EME due to the possibility of adverse electrolysis (33).

Therefore, a more likely explanation for the poor release of MTX into the acceptor solution is that the high current (150 µA) led to electrolysis and reduced pH in the acceptor solution. In addition, if positive charge accumulated at the SLM/acceptor interface due to the formation of an electrical double layer, this might have led to loss of negative charge from MTX once it reaches the acceptor side of the SLM. In this way, MTX would no longer be influenced by the electrical field, and it would accumulate in the SLM.

### 4.3.2 Extraction of MTX through 1-octanol with aliquat 336 as ionic carrier

Aliquat 336 is an ionic carrier used to facilitate extraction of anionic substances. Unlike DEHP, which is a pH dependent carrier of cationic substances, aliquat 336 is characterized by a permanent positive charge. This allows a more flexible choice of pH in the donor and acceptor solutions when extracting MTX as an anion.

5 µg/ml MTX was extracted through 1-octanol containing different amounts of aliquat 336 (0.5%, 1% and 2%). The extraction voltage was 5 or 10 V. A low voltage was chosen because the extraction current increased significantly above 10 V, leading to an unstable system. The pH was measured in the donor and acceptor solutions before and after extraction, to observe the effects of electrolysis. The current was recorded to investigate the relationship between extraction current, voltage, amount of aliquat 336 in the SLM, pH changes and recovery. The

constant parameters listed in table 28 are valid for the experiments described below.

**Table 30.** Results from extraction of 5 µg/ml MTX through 1-octanol including aliquat 336.

| Exp | % Aliquat 336 in 1-octanol | Voltage (V) | Recovery acceptor solution (%)[1] | pH change in donor solution (pH units)[1] | pH change in acceptor solution (pH units)[1] |
|---|---|---|---|---|---|
| 6A | 2 | 5 | 35.2 ± 0.5 | +0.25 ± 0.02 | -3.16 ± 0.07 |
| 6B | 1 | 5 | 43.2 ± 1.2 | +0.16 ± 0.03 | -1.59 ± 0.20 |
| 6C | 1 | 10 | 29.3 ± 5.3 | +0.94 ± 0.16 | -5.87 ± 0.20 |
| 6D | 0.5 | 5 | 33.3 ± 4.1 | +0.13 ± 0.01 | -1.12 ± 0.13 |
| 6E | 0.5 | 10 | 32.8 ± 0.7 | +0.28 ± 0.02 | -3.81 ± 0.59 |

[1]All experiments are based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

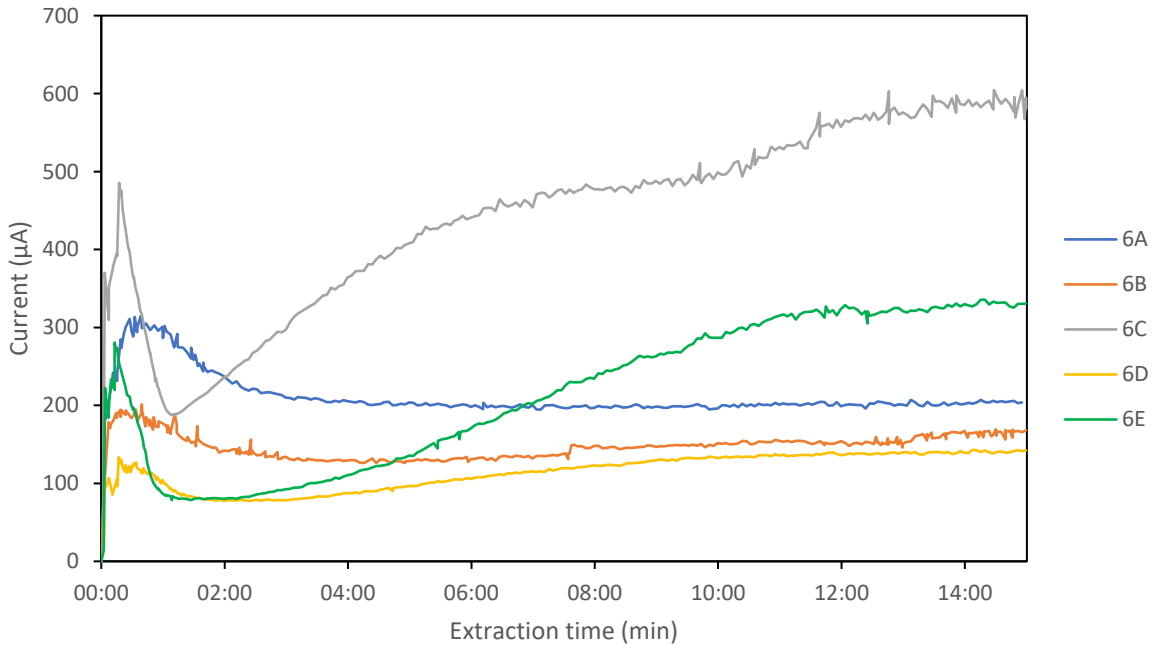



**Figure 36.** Extraction current for experiments 6A-6E. Each trace is from one EME extraction of two samples, representing the sum of the extraction currents of both samples.

The lowest recovery (29.3%) was obtained from experiment 6C. As illustrated in Figure 36, the extraction current greatly increased after approximately 1 minute, and increased throughout the extraction. The pH in the acceptor solution dropped almost 6 units, resulting in a final pH of 6.1. The extraction conditions were most likely too extreme for the system. At

10 V, the concentration of aliquat 336 (1%) in the SLM was too high, and this combination apparently generated an excessive current. The reduced pH in the acceptor solution due to electrolysis might led to a reduced affinity of the MTX molecules to the acceptor solution, and an elevated affinity to the positively charged carrier. A formation of an electrical double layer at the acceptor solution/SLM interface might have led to temporary loss of negative charge of the carboxylic acids on MTX, with resultant back-extraction towards the donor solution. As the MTX molecules reaches the SLM/donor solution interface, the basic electrical double layer might have worked in favor of deprotonation MTX, and re-interaction with aliquat 336.

In experiment 6A, 6D and 6E, the mean recoveries ranged from 32.8% to 35.2%. Hence, the different combinations of the amount of carrier in the SLM and the applied voltage in these experiments yielded almost the same recoveries of MTX. However, experiment 6D stands out compared to experiment 6A and 6E because electrolysis and pH changes occurred to a minimal degree. Despite the relatively stable system conditions, the combination of low voltage (5 V) and low concentration of aliquat 336 (0.5%), might have prevented effective partition of MTX into the SLM and liberation into the acceptor solution. A longer extraction duration might have facilitated higher recoveries, given that the extraction current remained stable over time. A more vigorous shaking (>750 RPM) also might have made faster extraction possible, but this could in turn have generated excessive current.

Experiment 6B yielded the highest recovery (43.2%). It seemed like the combination of 1% aliquat 336 in the SLM and 5 V was beneficial for EME through 1-octanol. The total current for both samples was approximately 150 µA. Ideally, it should be < 50 µA for one sample, but the level of electrolysis was not dramatic. The pH drop in the acceptor solution was 1.59 units. A drawback with this experiment is that the current curve shows a slight increase over time, suggesting extraction instability. Ideally, the curve should initially descend and then stabilize, like the current curve illustrated in Figure 24, or the current curve for experiment 6A. However, in experiment 6A, the overall current was too high.

The experiments show correlation between extraction current and pH changes. As the total current for two samples exceeds 200 µA, the pH reduction in the acceptor solution becomes higher than 3 units, and extraction recovery decreases. The reason why the pH dropped more

dramatically in the acceptor solution compared to the slight increase in the donor solution, is likely to be caused by the donor solution consisting of a 40 mM phosphate buffer. A higher molarity would probably have generated more background ions, and thereby a higher flux of ions over the membrane. A lower concentration could have reduced its buffer capacity and ability to prevent pH changes due to electrolysis.

### 4.3.3 Extraction of MTX through 1-nonanol and 1-nonanol/1-octanol added aliquat 336

The chemical structure of 1-nonanol is one carbon unit longer than 1-octanol, making it more hydrophobic and viscous. It was therefore introduced as an SLM due to its potential to counteract high current and electrolysis in EME, while keeping the properties that are suitable for solvation of deprotonated analytes.

Since 1% aliquat 336 in the SLM appeared to facilitate the highest recoveries in the extraction through 1-octanol, the same concentration of carrier was applied to the experiments with 1-nonanol, and 1-octanol/1-nonanol (v/v) mixture. The parameters listed in Table 28 are constant in the following experiments (7A-7E).

**Table 31.** Results from extraction of 5 µg/ml MTX through 1-nonanol and 1-nonanol/1-octanol including 1 % aliquat 336.

| Exp | SLM | Voltage (V) | Recovery acceptor solution (%)[1] | pH change in the donor solution (pH units)[1] | pH change in the acceptor solution (pH units)[1] |
|---|---|---|---|---|---|
| 7A | 1-nonanol | 20 | 10.7 ± 2.2 | Not measured | Not measured |
| 7B | 1-octanol/ 1-nonanol (1:1) | 10 | 27.6 ± 6.1 | +0.16 ± 0.01 | -1.77 ± 0.06 |
| 7C | 1-octanol/ 1-nonanol (1:1) | 20 | 21.5 ± 0.1 | +0.52 ± 0.02 | -4.80 ± 0.04 |

| | | | | | |
|---|---|---|---|---|---|
| 7D | 1-octanol/ 1-nonanol (2:1) | 10 | 31.2 ± 1.1 | +0.25 ± 0.05 | -2.13 ± 0.37 |
| 7E | 1-octanol/ 1-nonanol (2:1) | 20 | 17.8 ± 1.3 | +0.48 ± 0.03 | -4.56 ± 0.04 |

[1]All experiments are based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

The increased hydrophobicity and viscosity of 1-nonanol made the partition of MTX into the SLM more demanding. The voltage therefore had to be increased, to be able to force the analyte through the membrane. At 20 V, the extraction current in experiment 1 was around 60 µA for two samples (Figure 37).

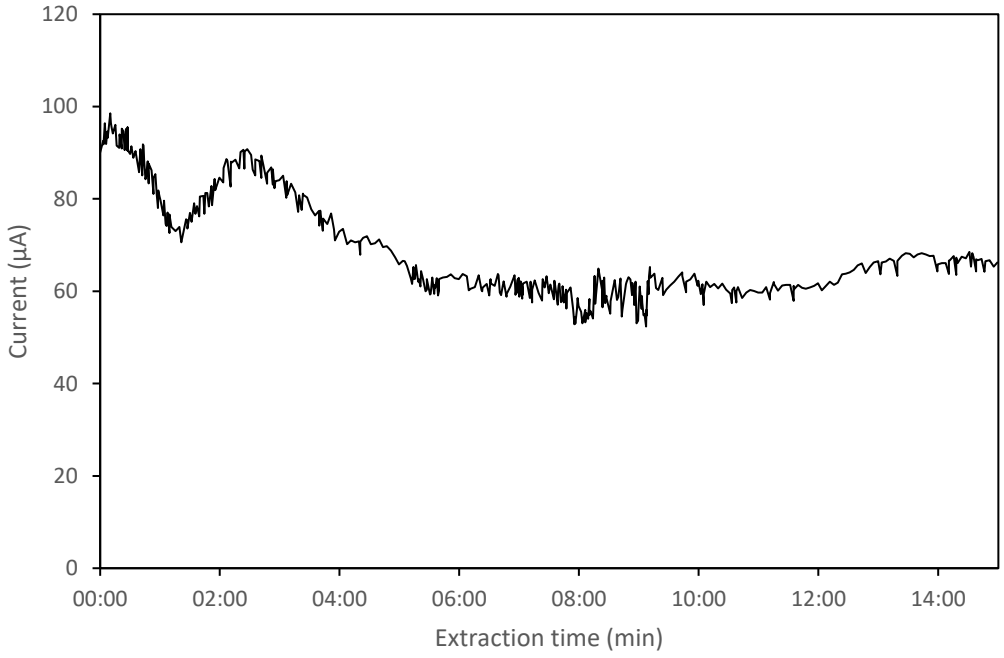



**Figure 37.** Extraction current for experiment 7A. The trace is from one EME extraction of two samples, representing the sum of the extraction currents of both samples.

A recovery of 10.7% was achieved in experiment 7A. It is likely that a higher voltage, such as 30 or 40 V, could have facilitated higher recoveries. Based on the experiments 6A-E, it is reasonable to think that minimal pH changes have occurred in experiment 7A due to the

relatively low current, and that the system could have handled a higher voltage. A prolonged extraction duration and more forceful agitation could also have contributed to increase the recovery in the acceptor solution.

When 1-octanol was added to 1-nonanol in the SLM, the extraction current greatly increased. Current curves were not recorded during these experiments, because experiment 7C+7E and 7B+7D were performed simultaneously, and the curves would be based on a mixture of the conditions in these two experiments. However, the pH changes in Table 31 strongly suggest that electrolysis occurred. Apparently, 20 V was too powerful for the system, even with 50% 1-nonanol in the SLM, and the pH dropped more than 4 units in the acceptor solution.

The highest recoveries were achieved in experiment 7B and 7D (27.6 and 31.2%, respectively). In both experiments, 10 V was applied, and the pH was not dramatically altered (around 2 units). However, compared to the experiments with only 1-octanol added aliquat 336, recoveries in the acceptor solution generally decreased when introducing 1-nonanol to the SLM.

To achieve higher extraction recoveries, different combinations of 1-octanol/1-nonanol ratio, amount of ionic carrier and extraction voltage would have to be investigated further. Also, the other parameters (e.g., time and agitation) would have to be adjusted to optimize the extraction of MTX through aliphatic alcohols added aliquat 336. Still, it is unlikely that adjustment of these parameters would lead to the desired level of recovery in the acceptor vial. In this study, the aim is to reach higher than 70% recovery of MTX in the acceptor solution, as ≈ 70% is the level of recovery of MTX obtained by protein precipitation in Rikshospitalet in Oslo (section 1.4).

## 4.4 Extraction of MTX as an anion through NPOE with aliquat 336 as an ionic carrier

Since the ionic carrier aliquat 336 seemed to be the key to achieve higher extraction recoveries in the experiments with aliphatic alcohols, it was added to several different oils, including NPOE. Even though NPOE has mostly been used in extraction of cationic analytes, it holds qualities that represents a successful SLM, such as low solubility in aqueous solutions

and relatively low viscosity (33).

A combination of different amounts of aliquat 336 in the SLM (1% and 0.5%), and different voltages (5, 10 and 20 V) were applied to get an insight into favorable conditions for EME of MTX through NPOE added aliquat 336. The pH was measured before and after extraction, but only the reduction of pH in the acceptor solution is listed in Table 32. This is because the pH was relatively stable in the donor solution due to the presence of the phosphate buffer. The extraction parameters listed in Table 28 were applied to the following experiments.

**Table 32.** Results from extraction of 5 µg/ml MTX through NPOE added aliquat 336.

| Exp | % aliquat 336 in NPOE | Voltage (V) | Recovery donor solution (%)[1] | Recovery acceptor solution (%)[1] | pH change in the acceptor solution (pH units)[1] |
|---|---|---|---|---|---|
| 8A | 1 | 5 | 18.1% ± 3.5 | 30.6 % ± 2.9 | -1.4 ± 0.1 |
| 8B | 1 | 10 | 8.5% ± 0.9 | 30.3% ± 4.7 | -3.2 ± 0.4 |
| 8C | 1 | 20 | 7.3% ± 0.1 | 23.7 % ± 2.2 | -4.5 ± 0.0 |
| 8D | 0.5 | 5 | 89.3% ± 4 | 5.0 % ± 1.6 | -0.7 ± 0.1 |
| 8E | 0.5 | 10 | 100.3% ± 2.8 | 0.3 % ± 0.3 | -0.8 ± 0.1 |
| 8F | 0.5 | 20 | 43.7% | 32.7% | -1.22 |

[1]All experiments except from 8F are based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

The results show that a low concentration of carrier (0.5%) combined with low voltages (5 and 10 V) was disadvantageous for EME, and most of the MTX was left in the donor solution (Table 32). However, a higher concentration of carrier (1%) combined with low voltages (5 and 10 V) seemed to dramatically increase the recoveries. The best parameters for these experiments appeared to be the combination of low concentration of carrier (0.5%) and high voltage (20 V). Experiment 8E showed peculiar results, because it was expected that the donor/acceptor recoveries would be between the values of experiment 8D and 8F. Instead, the recovery in the acceptor solution was 0.3%. The reason is unclear, but might be poor conductive properties of one or two of the vials used during extraction, which will be further

discussed in section 4.5.1.

Apparently, there is a correlation between the proportion of aliquat 336 in the SLM and voltage, and the combination must be fine-tuned to obtain the best results. The reason is most likely that both parameters increase the current in the system. The voltage as well as the proportion of aliquat 336 must be high enough to facilitate electrokinetic migration and partition into the SLM, but not so high that the current exceeds the level where adverse electrolytic reactions affects the extraction, like what occurred in experiment 8C (Table 32).

Compared to the experiments 6B-E with 1-octanol, where the same parameters were applied, NPOE was generally a less efficient SLM than 1-octanol. This did not come as a surprise, since long-chained alcohols are more known for successful extractions of anionic analytes (33).

## 4.5 Extraction of MTX as an anion through peppermint oil with aliquat 336 as an ionic carrier

The extraction of MTX through peppermint oil was attempted at a relatively early stage, in a period of experimenting with different SLMs. Peppermint oil is not a frequently used SLM for EME, but has shown promising results in a study of EME of non-polar, basic analytes (65). Even though the analytes in the study by Pedersen-Bjergaard and Rasmusen had physicochemical properties highly differing from MTX, peppermint oil was considered worth trying. The product was easily obtained from a local pharmacy, it was cheap and represented a "green-chemistry" alternative to other solvents. Although not suitable as an ingredient in a routine lab for drug analyses due to incomplete characterization, experiments using peppermint oil could give valuable information on how to extract negatively charged molecules by EME.

At first, MTX was extracted through pure peppermint oil, and peppermint oil with 1% aliquat 336. The experiments involved 40 mM phosphate buffer pH 7.4 as donor solution and 10 mM NaOH pH 12 as acceptor solution.

**Table 33.** Initial results from extraction of 8 µg/ml MTX through peppermint oil.

| Experiment | SLM | Voltage (V) | Time (min) | Agitation (RPM) | Recovery acceptor solution (%)[1] |
|---|---|---|---|---|---|
| 9A | Peppermint oil | 100 | 15 | 750 | 6.9% ± 0.8 |
| 9B | Peppermint oil + 1% aliquat 336 | 10 | 20 | 750 | 61.4% ± 2.2 |

[1]Experiments are based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

The extraction through pure peppermint oil yielded poor recoveries. The extraction current was approximately 7 µA, indicating a stable, but low flux of ions across the SLM. However, when 1% aliquat 336 was added to the SLM, the recovery was higher than those obtained from previous experiments using the same carrier together with 1-octanol, 1-nonanol, or NPOE.

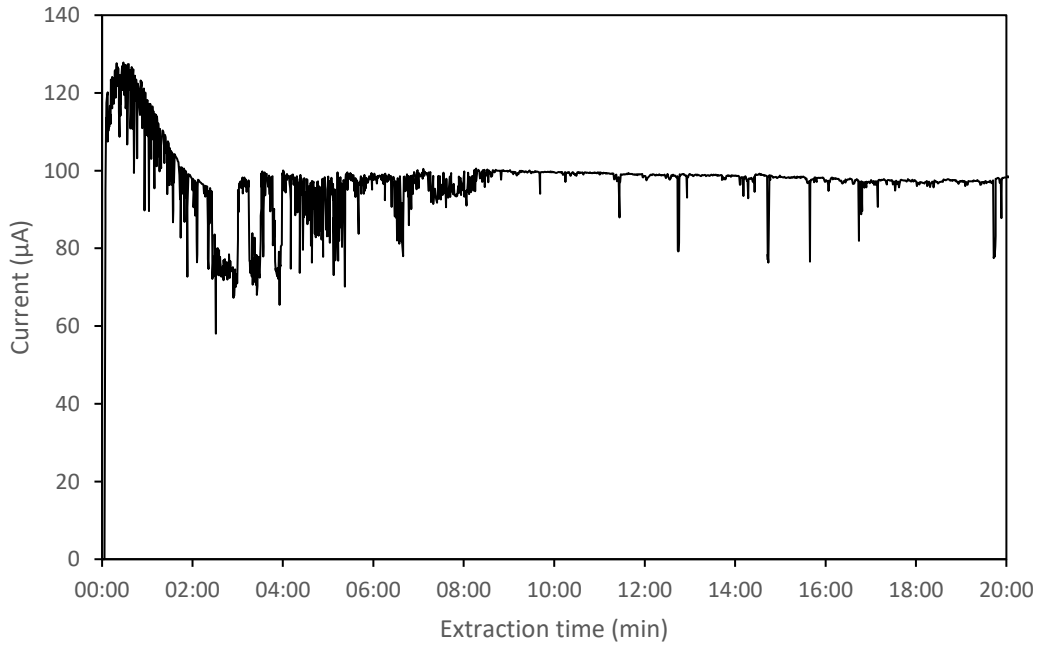



**Figure 38**. Extraction current for experiment 9B. The trace is from one EME extraction of two samples, representing the sum of the extraction currents of both samples.

The extraction current for experiment 9B was 100 µA distributed to 2 samples, resulting in an individual current of approximately 50 µA per sample (Figure 38).

Based on the promising initial results using peppermint oil, further conditions were tested. However, during the experimentation with EME of MTX through peppermint oil added aliquat 336, it was discovered that the recoveries often varied highly among parallels. For example, 3 samples of 5 µg/ml MTX were extracted through peppermint oil added 2% aliquat 336 at 5 V in 15 minutes. The recoveries were 22.9%, 19.9% and 8.0%. Results like these were obtained regularly, and they were difficult to draw conclusions from. Given that only one of the three samples were extracted, 8% recovery could have been the result, whereas the extraction conditions were in fact optimized for over 20% recovery. The problem with varying results applied to other SLMs (NPOE, 1-octanol, 1-nonanol) as well. The relative standard deviation was often unexpectedly high, even though the samples were prepared similarly and extracted under the same conditions.

### 4.5.1 Investigation of intra-experimental variation with peppermint oil as SLM.

The variation in the extractions could be due to the operational technique, various supply of voltage from the electrodes, poor contact between the electrodes and the vials, or differences in vial performance. To get a better impression of exactly how much the recoveries varied among parallels, 8 samples were extracted simultaneously under following conditions:

**Table 34.** Constant parameters for experiment 10-12.

| Parameter | Description |
|---|---|
| Time | 20 minutes |
| Agitation | 750 RPM |
| Voltage | 10 V |
| SLM | Peppermint oil + 1 % aliquat 336 |
| Donor solution | 40 mM phosphate buffer |
| Acceptor solution | 10 mM NaOH |

**Table 35.** Experiment 10. Results from the investigation of the RSD.

| Sample | Recovery acceptor solution (%) |
|---|---|
| 1 | 79.6 |
| 2 | 75.2 |
| 3 | 70.4 |
| 4 | 52.5 |
| 5 | 66.2 |
| 6 | 59.6 |
| 7 | 60.8 |
| 8 | 45.6 |
| Mean | 63.7 |
| Standard deviation, σ | 10.69 |
| RSD (%) | 16.8 |

The results varied from 45.6% to 79.6%. The extraction of multiple parallels was therefore crucial to see the trend in recovery, given that the causes for the large RSD shown in Table 35 was not yet revealed.

Different explanations on why the results varied by such a degree were considered. The reason could have been practical; the EME procedure involves multiple operational steps, with the potential of operational variance. Examples could be different amounts of SLM pipetted onto the PP membrane due to the viscosity of the SLM, or the PP-membranes positioned differently inside the unions. If these were the main reasons for the large RSD, it would have been difficult to correct, since the procedure already was performed with utmost care.

Another possible explanation was variable or poor contact between electrodes and the conducting vials, or that the respective electrodes supplied different amounts of voltage. This could have facilitated a high degree of electrokinetic migration in some samples, and poor

transport in others. However, the multimeter was used to measure the performance of each pair of electrodes by adding a conducting object across each pair of electrodes, measuring the electrical current. The current was identical for all ten pairs of electrodes, demonstrating that the cause of variation was not due to the electrodes.

A third reason for the considerably large RSD, was that the conductive vials lost their performance over time. This could have been due to a coating of unwashed substances inside or outside of the vials, altering their conductive properties. Even though the vials were washed with ethanol 3-4 times, followed by a 3-4 times wash with Milli-Q water, some of the more hydrophobic substances leaked from the SLM could have remained inside the vials. It could also be that the vials were produced with slight differences, and that some were technically more suited for reproducible results. For example, the upper vial surface that connects to the PP membrane could vary in diameter, possibly securing some samples better than others and prevented leaking.

To investigate whether the elevated RSD was due to loss of vial performance over time, the experiment in Table 35 was performed again, but with new and unused vials.

**Table 36.** Experiment 11. Results from the investigation of the RSD by using new vials.

| **Sample** | **Recovery acceptor solution (%)** |
|---|---|
| 1 | 68.8 |
| 2 | 72.8 |
| 3 | 70.8 |
| 4 | 71.7 |
| 5 | 72.8 |
| 6 | 70.1 |
| 7 | 73.7 |
| 8 | 68.0 |
| Mean | 71.1 |
| Standard deviation, σ | 1.9 |
| RSD (%) | 2.7 |

Here, the RSD was only 2.7%, compared to 16.8% with the older vials that had been frequently used. With this new insight in mind, multiple experiments were performed again with new vials to obtain more reliable results.

### 4.5.2 Extraction of MTX without the application of voltage

To confirm the advantage of the power supply in EME, 5 µg/ml MTX was extracted through an SLM consisting of peppermint oil + 1% Aliquat 336 in 20, 30, 40, 50, 60 and 90 minutes, but without applying voltage. The agitation was 750 RPM.

**Table 37.** Experiment 12. Results from extraction of 5 µg/ml MTX without the application of voltage.

| Sample | Extraction time (min) | Recovery donor solution (%) | Recovery acceptor solution (%) |
|---|---|---|---|
| **1** | 20 | 76.2 | 18.0 |
| **2** | 30 | 78.2 | 21.3 |
| **3** | 40 | 76.4 | 25.5 |
| **4** | 50 | 75.4 | 22.0 |
| **5** | 60 | 63.6 | 33.9 |
| **6** | 90 | 46.51 | 18.9 |

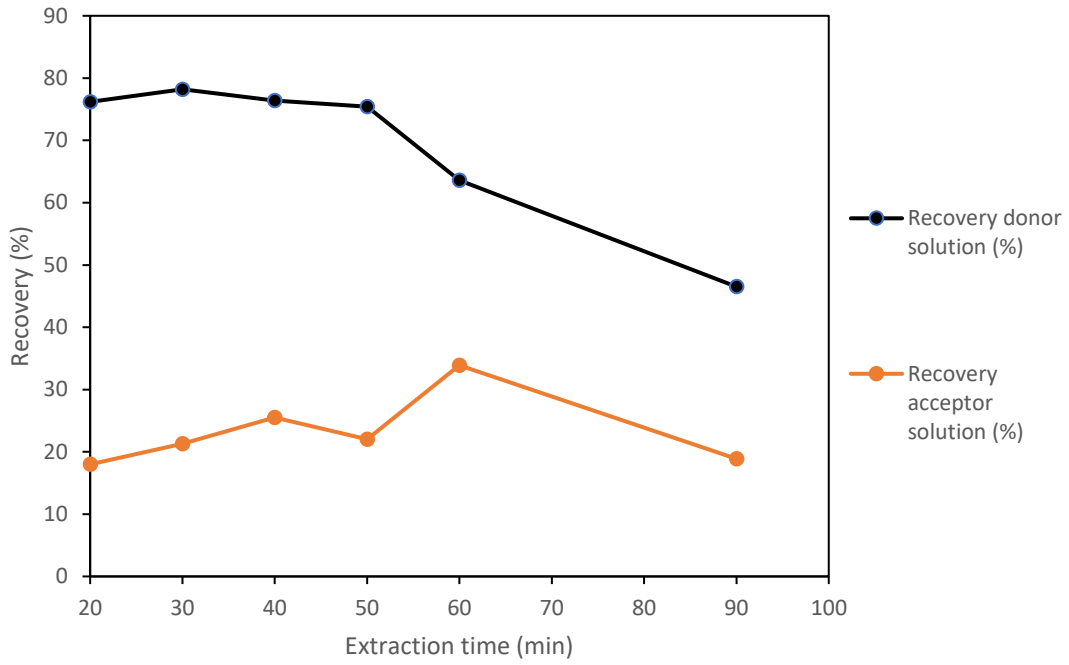



**Figure 39**. Recovery (%) of donor and acceptor solution plotted against time from experiment 12.

The results in Table 37 shows that MTX could, to a lesser degree, be transported through the SLM without being influenced by an electrical field. This means that the migration of the analyte was based on passive diffusion across the SLM. Most likely, the ionized MTX molecules from the donor solution formed ionic bonds with the positively charged carrier and diffused into the SLM. Since MTX is polar, and more soluble in basic conditions, it was liberated into the more basic acceptor solution.

Above 60 minutes, the recovery in the acceptor vial presumably reached its maximum. It is unclear why the recovery decreased in the acceptor solution at 90 minutes. An interesting observation from Table 37 was that almost all of the originally added MTX molecules were detected either in the donor or the acceptor solution at 20-60 minutes. This was unexpected, as most extractions including an ionic carrier resulted in some loss of the analyte to the SLM. For sample 3 and 5, the total recovery even slightly exceeded 100%, likely to be explained by the precision of the HPLC-UV method. In sample 6, however, the total recovery was only 65%. This sample was shaken for 30 minutes after sample 5, but there is no explanation to why the sample would behave differently from the rest.

In conclusion, the highest recovery without applying voltage was obtained after 60 minutes, with 33.9% MTX detected in the acceptor solution. This demonstrates the importance of the power supply in order to get high recoveries and faster extractions with EME.

### 4.5.3 Investigation of optimal parameters in extraction of MTX through peppermint oil with 1 % aliquat 336

New vials were used to observe the relationship between extraction time, voltage, agitation, and recovery in the extraction of MTX through peppermint oil + 1% aliquat 336. At first, 8 samples of 5 µg/ml MTX were extracted at 10 V. Samples were removed at different times, to observe the time where the highest recoveries were achieved.

**Table 38.** Constant parameters for experiment 13-17.

| Parameter | Description |
|---|---|
| Donor solution | 40 mM phosphate buffer, pH 7.4 |
| Acceptor solution | 10 mM NaOH, pH 12 |
| SLM | Peppermint oil + 1% aliquat 336 |

**Table 39.** Experiment 13. Results from extraction of 5 µg/ml MTX at 10 V and 750 RPM.

| Sample | Extraction time (min) | Recovery acceptor solution (%) |
|---|---|---|
| 1 | 10 | 51.4 |
| 2 | 12 | 58.4 |
| 3 | 14 | 59.9 |
| 4 | 16 | 67.7 |
| 5 | 18 | 68.1 |
| 6 | 20 | 60.7 |
| 7 | 22 | 71.3 |
| 8 | 24 | 77.3 |

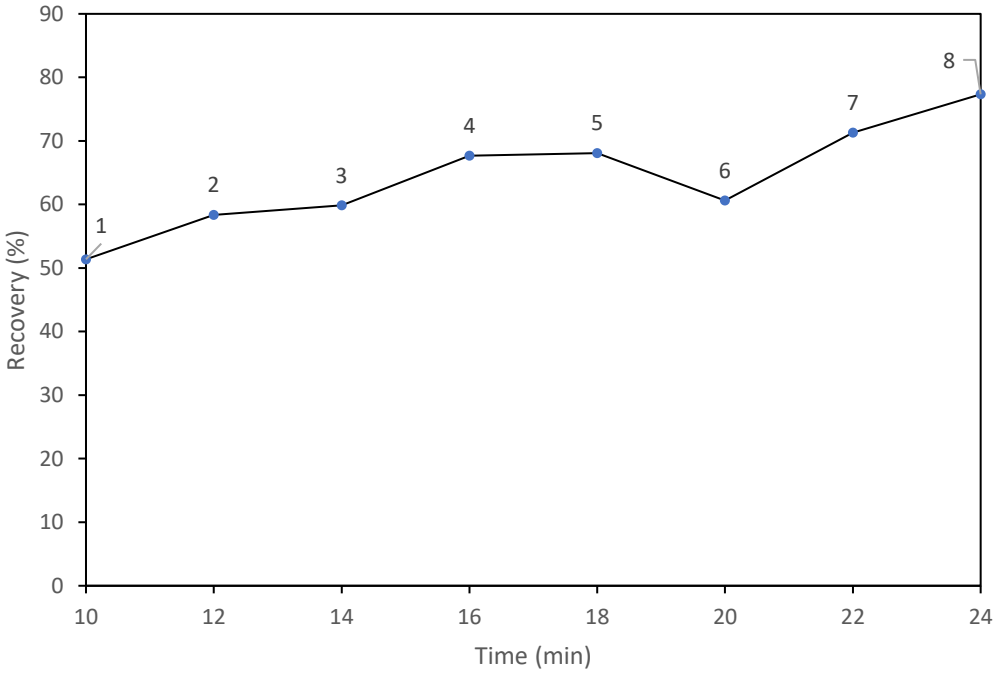



**Figure 40.** Recovery plotted against time (experiment 13).

The recovery increased with time, with sample 6 being an outlier. The trend clearly demonstrates that longer extraction times facilitated higher yields in experiment 13. To investigate at what point the recovery in the acceptor solution reached its threshold, more samples would have to be extracted for a longer time. During the investigation of the RSD of new vials in experiment 11, the pH in the donor and acceptor solutions was measured before and after extraction. After 20 minutes of extraction, the mean pH reduction in the acceptor solution was 1.4 units. The pH would most likely have dropped further with longer extractions, and based on the experiments with 1-octanol, a pH reduction larger than approximately 2 units appeared to be unfavorable for EME. However, the current curve for the eight samples extracted simultaneously in experiment 11 seemed to be stable for 20 minutes (Figure 41), and it is reasonable to assume that the EME system could tolerate extractions at 10 V for longer than 24 minutes, possibly yielding higher recoveries (>77.3%).

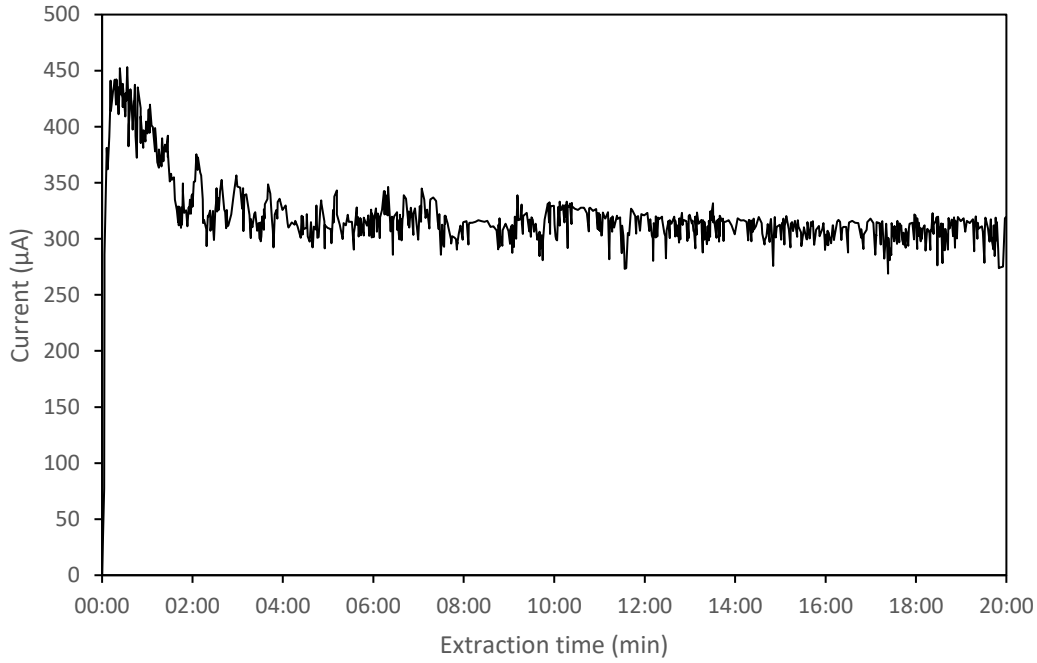



**Figure 41.** Extraction current for 8 samples extracted with new vials to investigate the RSD (experiment 11). The trace represents the sum of the extraction currents of all samples. Conditions for the experiment is given in Table 34.

However, for EME to be a routine sample preparation technique, it is desirable with fast extractions. Extractions longer than 24 minutes are therefore not ideal. Considering this, 8 new samples were extracted at 15 V instead of 10 V, to find if high recoveries in the acceptor solution could be obtained faster. Also, additional 8 samples were extracted at 10 V with 800 RPM instead of 750 RPM, to find if stronger agitation could increase extraction.

**Table 40.** Experiment 14. Results from extraction of 5 µg/ml MTX at 15 V and 750 RPM.

| Sample | Extraction time (min) | Recovery acceptor solution (%) |
|---|---|---|
| 1 | 8 | 40.5 |
| 2 | 10 | 48.4 |
| 3 | 12 | 48.6 |
| 4 | 14 | 60.4 |
| 5 | 16 | 48.2 |
| 6 | 18 | 53.7 |

| | | |
|---|---|---|
| 7 | 20 | 63.0 |
| 8 | 22 | 59.1 |

**Table 41.** Experiment 15. Results from extraction of 5 µg/ml MTX at 10 V and 800 RPM.

| Sample | Extraction time (min) | Recovery acceptor solution (%) |
|---|---|---|
| 1 | 8 | 44.5 |
| 2 | 10 | 52.4 |
| 3 | 12 | 61.0 |
| 4 | 14 | 68.5 |
| 5 | 16 | 72.3 |
| 6 | 18 | 54.8 |
| 7 | 20 | 59.2 |
| 8 | 22 | 68.4 |

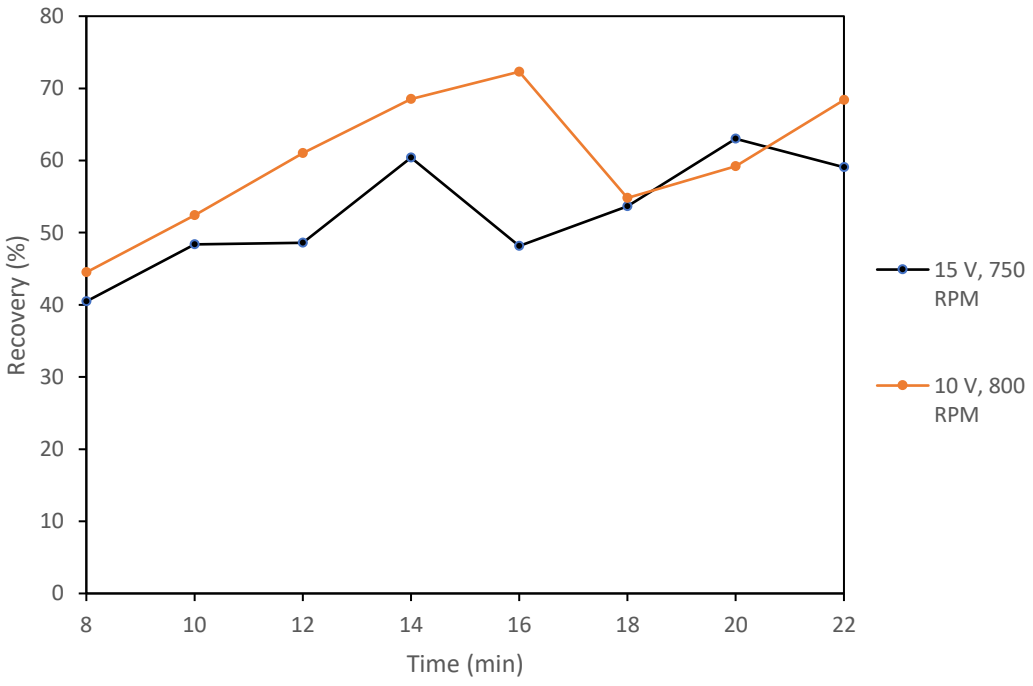



**Figure 42.** Recovery plotted against time at two different voltages and agitation speeds (experiment 14 and 15).

The results suggest that applying higher voltage (15 V) was not a favorable step for EME, in terms of achieving high recoveries faster. The amount of MTX detected in the acceptor

solutions were generally less than in experiment 13. However, when increasing the agitation from 750 RPM to 800 RPM at 10 V, 72.3% recovery was achieved at 16 minutes, compared to 67.7% in experiment 13.

Nevertheless, the current in experiment 14 and 15 kept increasing throughout the extraction, despite the removal of a sample every two minutes (Figure 43). This indicates instability and electrolysis, and might explain the overall poorer recoveries in the acceptor solutions. It is possible to see a direct correlation between the current and recovery based on the current curves. When looking at the red trace representing experiment 14 in Figure 43, the current significantly decreased after sample 5 was removed from the sample holder. Sample 5 therefore had an excessive flux of ions across the membrane at 16 minutes (approximately 700 - 200 = 500 µA). The recovery of sample 5 was 48.2 %, which was less than the samples removed before and after.

The trace for experiment 13 indicated much more stable conditions. As expected, the total current generally decreased as samples were removed one by one from the sample holder.

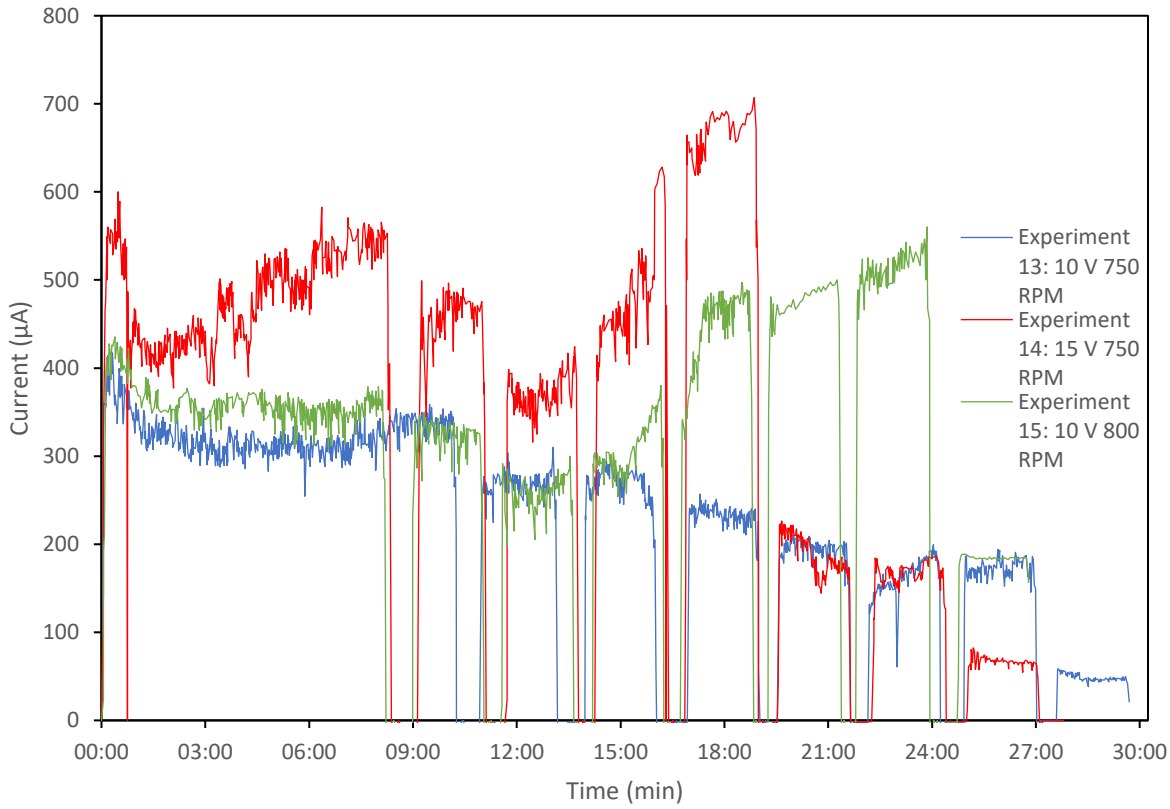



**Figure 43.** Extraction current for experiment 13-15. Each segment indicates removal of one sample from the EME instrument.

Based on the three experiments, the experiment with 10 V and 750 RPM seemed to be the most reliable in terms of extraction current and recovery. However, the extraction duration would have to be more than 20 minutes in order to yield above 70% recovery in the acceptor solution. Multiple samples could have been prepared for the extraction at 10 V and 800 RPM for 16 minutes. If the results were reproducible, and yielded approximately 73.6% recovery each time, this would be convenient in terms of faster extractions.

More experiments could have been attempted to optimize extraction recoveries. For example, 7, 8 or 9 V combined with 800 RPM might have facilitated faster extractions and combated electrolysis. Also 10 V could have been combined with an agitation between 750 and 800.

### 4.5.4 Extraction of 7-OH-MTX and DAMPA through peppermint oil with aliquat 336 as an ionic carrier

Three samples of 5 µg/ml 7-OH-MTX were extracted for 20 minutes using the same parameters as in Table 38. Due to structural resemblance to MTX, it was expected that the metabolite would behave the same way, in terms of interacting with aliquat 336 and be transported across the SLM.

**Table 42**. Experiment 16. Result from extraction of 5 µg/ml 7-OH-MTX.

| **SLM** | **Voltage (V)** | **Time (min)** | **Agitation (RPM)** | **Recovery acceptor solution (%)[1]** |
|---|---|---|---|---|
| Peppermint oil + 1% aliquat 336 | 10 | 20 | 750 | 59.0 |

[1]Experiment is based on three parallels, with an RSD of 5.3%.

The recovery was approximately 10% lower than for MTX. This can be explained by the additional OH-group of the metabolite (Figure 14), which contributes to its increased polarity. Hence, 7-OH-MTX had less affinity to the hydrophobic SLM. In order to gain higher recovery of 7-OH-MTX, the voltage or agitation could have been adjusted upwards. This might have forced the more polar metabolite more efficiently through the SLM.

The metabolite DAMPA was also extracted by the same parameters as in Table 38. Its chemical structure suggested that it was less polar than MTX due to the loss of the glutamic acid part, which could possibly favor its partition into the non-polar SLM. The metabolite still contained a carboxylic acid, which was expected to interact with aliquat 336 the same way as MTX and 7-OH-MTX.

**Table 43**. Experiment 17. Result from extraction of 5 µg/ml DAMPA.

| SLM | Voltage (V) | Time (min) | Agitation (RPM) | Recovery acceptor solution (%)[1] |
|---|---|---|---|---|
| Peppermint oil + 1% aliquat 336 | 10 | 20 | 750 | 32.4 ± 1.0 |

[1]Experiment is based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

The extraction current in experiment 17 was stable, with a total of 100 µA for 2 samples. However, the extraction recovery in the acceptor solution in this experiment was lower than for MTX and 7-OH-MTX. An explanation could be that the single carboxylic acid was neutralized through the interaction with aliquat 336. The analyte was therefore no longer influenced by the electrical field, and due to its reduced polarity, it was not sufficiently released to the aqueous acceptor solution.

### 4.5.5 Thoughts on peppermint oil as an SLM in EME

Peppermint oil is not a defined product. It consists of many different compounds, and it is uncertain what compounds are responsible for the observed effects. When extracting MTX through peppermint oil, some small peaks appeared in the chromatogram in addition to the MTX peak at 2.9 (Figure 44). These most likely represent different compounds from the peppermint oil that have leaked into the aqueous solutions.

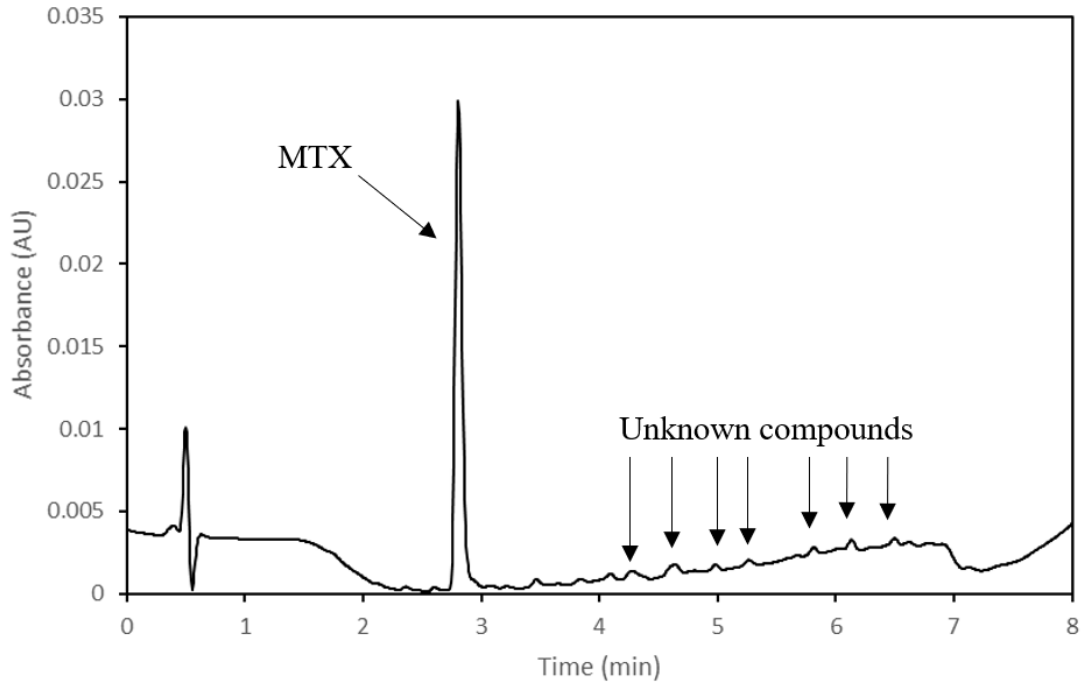



**Figure 44.** Chromatogram of an acceptor solution showing the retention of MTX and unknown compounds leaked from peppermint oil.

The two main components of peppermint oil are menthol and menthone, which accounts for 40.7% and 23.4% of the oil, respectively (66). The structures of the two compounds are shown beneath.

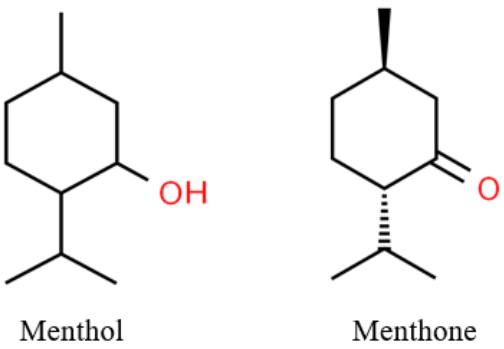



**Figure 45.** The two main components of peppermint oil (downloaded and modified from (67) and (68)).

The only difference between the two structures is that menthol has an alcohol functional group, whereas menthone contains a ketone. Menthol is, like 1-octanol, an aliphatic alcohol, and it is reasonable to think that it can perform well as an SLM for acidic compounds.

In order to obtain the best recoveries in EME with peppermint oil, multiple parameters would have to be optimized. This includes composition of the SLM (peppermint oil/aliquat 336 ratio), volume and pH of acceptor/donor solution, volume of the SLM, time, agitation, and voltage. The best way to do this would be a factorial design of experiments. Due to limited time, and the problems discussed in section 4.7, it was decided not to go ahead with this. Instead, experiments with menthol and menthone were performed to find if it was possible to develop a method of MTX extraction using well-defined SLM ingredients.

## 4.6 Extraction of MTX through menthol and menthone with aliquat 336 as ionic carrier

Since menthol and menthone are the two most prominent compounds of peppermint oil, the substances were obtained and used as SLM. The aim was to find the combination of the two compounds that would yield the same recoveries as obtained with peppermint oil. That way, the identity of the compounds responsible for the high recoveries could be determined.

The water solubility of menthol and menthone was 0.69 g/L and 0.42 g/L, respectively (55, 69), making them both good candidates as SLM in terms of low leakage into the aqueous solutions during extraction. A water solubility higher than 1 g/L is generally not recommended in EME (33). Menthone was obtained in oil form. It was easy to handle during EME, and therefore attempted as an SLM first. Menthol was obtained in solid form and had to be dissolved in another oil and/or heated prior to EME.

8 samples were extracted through menthone at 10 V, 750 RPM, and 1% aliquat 336. The purpose was to observe at what time the recoveries were highest, at the given voltage. New vials were applied to the experiment to exclude the fact that a potentially high RSD was due to poor vial performance.

**Table 44.** Constant parameters for experiment 18 and 19.

| Parameter | Description |
|---|---|
| Donor solution | 40 mM phosphate buffer, pH 7.4 |
| Acceptor solution | 10 mM NaOH, pH 12 |

**Table 45.** Experiment 18. Results from extraction of 5 µg/ml MTX through menthone at 10 V.

| Sample | Extraction time (min) | Recovery acceptor solution (%) |
|---|---|---|
| 1 | 8 | 29.4 |
| 2 | 10 | 22.2 |
| 3 | 12 | 25.5 |
| 4 | 14 | 37.9 |
| 5 | 16 | 57.0 |
| 6 | 18 | 16.1 |
| 7 | 20 | 27.9 |
| 8 | 22 | 52.1 |

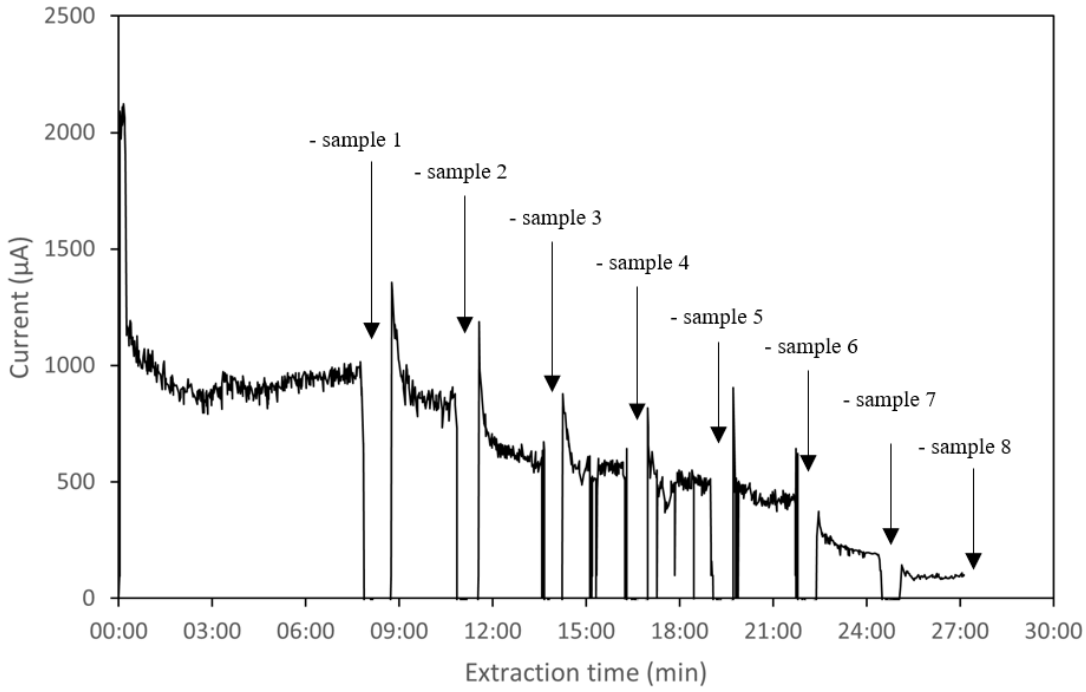



**Figure 46.** Extraction current for experiment 18. Each segment indicates removal of one sample from the EME instrument.

The results from experiment 18 were difficult to interpret, as the recovery vs. time did not show linearity. The reason was most likely the high current and electrolysis during extraction, facilitating an unstable and unpredictable system. Figure 46 shows that the extraction current

was nearly 1000 µA distributed to 8 samples for the first 8 minutes. This means a current of approximately 125 µA running through each sample. However, unlike Figure 43, where two of the graphs show an increasing current with time, the trace in Figure 46 descends despite the high total current. This is usually a good sign in EME, as transport slows down and stabilizes.

In conclusion, 10 V was most likely too high for the system in experiment 18. An experiment was therefore carried out at 8 V for 12 minutes.

**Table 46**. Experiment 19. Result from extraction of 5 µg/ml MTX through menthone at 8 V.

| **SLM** | **Voltage (V)** | **Time (min)** | **Agitation (RPM)** | **Recovery acceptor solution (%)[1]** |
|---|---|---|---|---|
| Menthone + 1% aliquat 336 | 8 | 12 | 750 | 52.3 % ± 3.6 |

The result supports the theory that the voltage in experiment 18 was too high. Compared to experiment 18, where 25.5% recovery was achieved after 12 minutes, 53.3% recovery was achieved after 12 minutes in experiment 19. The extraction current was stable and descending, with approximately 80 µA running through each sample. Figure 24 was used as an example current curve in section 3.5, and represents this experiment. More experiments could have been attempted, such as longer extractions at 8, 7, 6 and 5 V to yield higher recoveries.

However, instead of optimizing parameters for extraction through menthone, menthol was added to the SLM. This compound is an aliphatic alcohol, and was theorized to be the component mainly responsible for the high recoveries obtained with peppermint oil. Since the compound existed in a solid form, it was mixed with menthone and heated for 5 minutes. This generated a more viscous liquid, which was thought to be favorable in terms of lowering the extraction current.

A 75 % (w/v) menthol in menthone mixture was prepared, and 1% aliquat 336 was added. Two parallels were extracted, and the pH was measured before and after the extraction.

**Table 47**. Experiment 20. Result from extraction of 5 µg/ml MTX through menthol/menthone (3:1)

| SLM | Voltage (V) | Time (min) | Agitation (RPM) | Recovery acceptor solution (%)[1] | pH change acceptor solution (pH units)[1] |
|---|---|---|---|---|---|
| **Menthol/ menthone (3:1) + 1% aliquat 336.** | 10 | 15 | 750 | 29.9 ± 0.8 | -2.0 ± 0.2 |

[1]All experiments are based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

Apparently, the inclusion of 75% menthol did not result in improvements in extraction recovery in this experiment. The pH reduction was not extensive, but higher recoveries were yielded with menthone alone, as shown in experiment 18. The proportion of menthol was therefore reduced, and the following experiment applied 50% (w/v) menthol in menthol.

**Table 48**. Experiment 21. Result from extraction of 5 µg/ml MTX through menthol/menthone (1:1)

| SLM | Voltage (V) | Time (min) | Agitation (RPM) | Recovery acceptor solution (%)[1] |
|---|---|---|---|---|
| **Menthol/menthone (1:1) + 1% aliquat 336.** | 10 | 10 | 750 | 36.7 ± 7.5 |

[1]Experiment is based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

Here, the extraction time was only 10 minutes, but recovery in the acceptor solution was still higher than in experiment 20. This suggests that the amount of menthol must not be too high compared to menthone. Experiment 21 yielded higher recoveries after 10 minutes compared to experiment 18. This suggested that the addition of menthol might be beneficial, but the ratio between menthol and menthone must be optimized further to yield recoveries close to the ones obtained with peppermint oil. For example, a menthol/menthone (2:1) ratio could have been experimented with at higher voltage and/or longer extraction time, as this ratio mimics the real composition of menthol/menthone in peppermint oil.

However, further experimentation with the two components were put on hold as an unexpected problem appeared, which will be discussed in the next sections.

## 4.7 Impairment of EME of MTX due to interference between anionic electrolytes and the SLM carrier

### 4.7.1 Discovery of problems related to NaCl in the donor solution

Until now, a 40 mM phosphate buffer with no additional electrolytes had been used as the donor solution for EME of anionic MTX. However, during the experiments with menthone and menthol, phosphate buffered saline (PBS) with pH 7.4 was prepared as the donor solution. The PBS contained 0,01 M phosphate buffer, 0.137 M NaCl and 0.0027 M KCl. Upon the application of this donor solution, the extraction recoveries unexpectedly became 0% (Table 49). In experiment 22, the samples were extracted for 15 minutes with an agitation of 750 RPM. As usual, the acceptor solution consisted of 10 mM NaOH.

**Table 49**. Results from extraction of 5 µg/ml MTX from a PBS donor solution.

| Experiment | SLM | Voltage (V) | Recovery donor solution (%)[1] | Recovery acceptor solution (%)[1] |
|---|---|---|---|---|
| **22A** | Menthol/ menthone (3:1) + 2% aliquat 336. | 10 | 99.7 ± 0.8 | 0.0 ± 0.0 |
| **22B** | Menthol/ menthone (3:1) + 2% aliquat 336. | 20 | 99.8 ± 0.3 | 0.0 ± 0.0 |

[1]Experiments are based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

Experiment 22A and B shows that 0% MTX was detected in the acceptor solution, and 100% was left in the donor solution (Table 49). This was unexpected, as it was reasonable to expect some degree of recovery based on the application of similar parameters in experiment 20. Nevertheless, there were two possible explanations for the absent recoveries: poor extraction

parameters in experiment 22, or the introduction of PBS as donor solvent.

A new sample was extracted using parameters known to achieve reliable recoveries in the acceptor solution. 5 µg/ml MTX was extracted through peppermint oil with 1% aliquat 336 according to the method that yielded 67.7% recovery after 16 minutes in experiment 13. The only difference from experiment 13 was the application of a PBS donor solution instead of 40 mM phosphate buffer.

**Table 50** - Experiment 23. Result from extraction of 5 µg/ml MTX from a PBS donor solution through peppermint oil.

| SLM | Voltage (V) | Time (min) | Agitation (RPM) | Recovery donor solution (%) | Recovery acceptor solution (%) |
|---|---|---|---|---|---|
| **Peppermint oil + 1% aliquat 336.** | 10 | 15 | 750 | 99.3 | 0.0 |

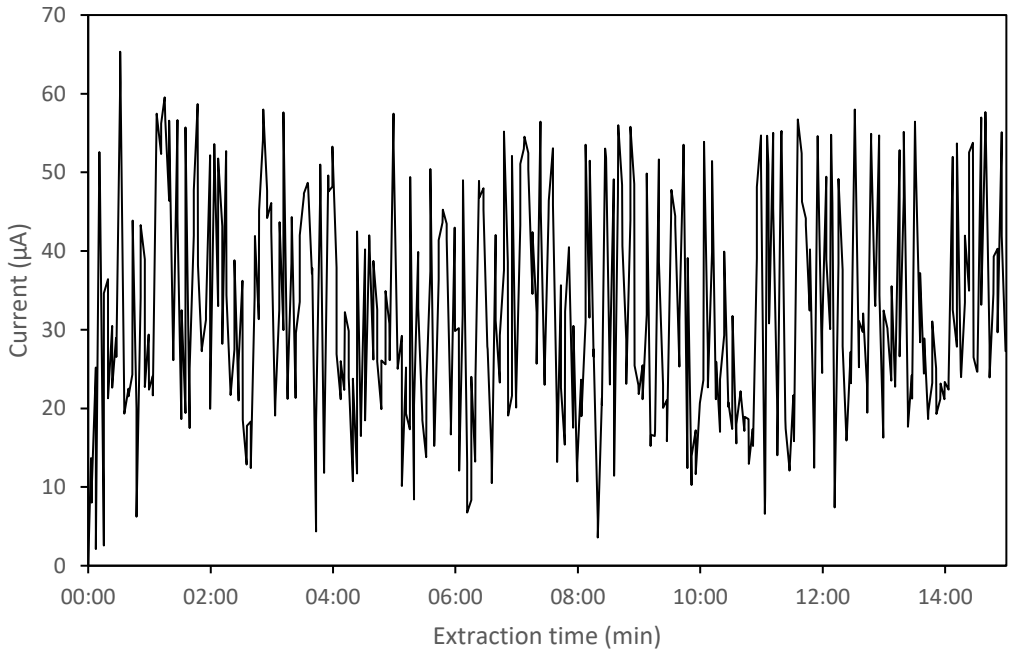



**Figure 47**. Extraction current for experiment 23. The trace is from one EME extraction of one sample.

The absent recoveries from experiment 22 were also applicable to experiment 23. The

extraction current in Figure 47 shows a highly untypical pattern. The current was generally lower than expected, and behaved remarkably unstable.

The first theory on why the recoveries dropped to 0% when using PBS, was that the newly introduced chloride ions from PBS were pulled by the electrical field in the direction of the anode and had a strong affinity to the positively charged carrier, aliquat 336. Due to the high concentration of chloride ions, aliquat 336 would be saturated and lose the ability to interact with, and transport MTX across the SLM. This may explain the lower extraction current in Figure 47.

To investigate whether NaCl in PBS was responsible for the absent recoveries or not, an increasing amount of NaCl was added to a regular 40 mM phosphate buffer. The concentration of added NaCl ranged from 0.0025 M to 0.14 M. The samples were extracted through peppermint oil + 1% aliquat 336, at 10 V in 15 minutes, since this method had proved to yield reliable recoveries (Figure 40).

**Table 51**. Experiment 24. Results from extraction of 5 µg/ml MTX from a donor solution with an increasing concentration of NaCl.

| **Amount of NaCl in a 40 mM potassium phosphate buffer** | **Recovery (%)** |
|---|---|
| 0.0000 M | 52.1% |
| 0.0025 M | 11.5% |
| 0.0050 M | 7.1% |
| 0.0075 M | 8.8% |
| 0.0100 M | 5.7% |
| 0.0200 M | 1.6% |
| 0.0400 M | 0.0% |
| 0.0700 M | 0.0% |

The results in Table 51 clearly show a correlation between increasing concentration of NaCl in the donor solution and decreasing recovery of MTX in the acceptor solution. Already with 0.04 M NaCl in the donor solution, no MTX was detected in the acceptor solution. This indicates that the extraction of MTX from plasma using aliquat 336 as carrier could be

problematic, since the physiologic concentration of $Cl^-$ is 0.098-0.106 M (70). Based on the experiments so far, it was confirmed that either $Na^+$ or $Cl^-$ impaired the extraction of MTX from PBS across an SLM containing aliquat 336.

### 4.7.2 Extraction from a sodium phosphate buffer

The next step was to confirm or disprove whether sodium ions were the source of the problem. Since MTX has two negative charges at physiologic pH (7.4), it was suggested that the analyte would become neutralized by the introduced $Na^+$ ions ($2COO^-Na^+$) in the donor solution. Hence, MTX would no longer be influenced by the electrical field.

A 40 mM sodium phosphate buffer with a pH of 7.4 was prepared (Table 4) and applied as the donor solution in experiment 25.

**Table 52**. Experiment 25. Result from extraction of 5 µg/ml MTX from a sodium phosphate donor solution into a 10 mM NaOH acceptor solution.

| SLM | Voltage (V) | Time (min) | Agitation (RPM) | Recovery acceptor solution (%)[1] |
|---|---|---|---|---|
| **Peppermint oil + 1% aliquat 336.** | 10 | 20 | 750 | 66.2 ± 2.7 |

[1]Experiment is based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

The sodium phosphate buffer did not have an evident impact on the electrokinetic transfer of MTX across the SLM with aliquat 336 as a carrier. Based on Figure 13, MTX has a predicted solubility of 450 mg/ml at pH 7.4. Based on this, it seemed unlikely that the analyte interacted with sodium ions to such a degree that it would lose its negative charges in the donor solution. Furthermore, if the sodium ions were the source of the problem, one would assume that the same problem would apply to the potassium phosphate buffer. This buffer also contains monovalent cations ($K^+$) with similar chemical properties as $Na^+$. In conclusion, the possibility that sodium ions impaired EME of MTX was considered unlikely, and the focus was directed towards the $Cl^-$ ions.

### 4.7.3 Different approaches to overcome the impact of Cl- ions in the donor solution

Different experiments were carried out to determine if it was possible to raise the recoveries from 0%. For example, longer extractions were performed, examining whether this would eventually lead to a dissociation of the interactions between aliquat 336 and $Cl^-$ ions, allowing more transport of MTX. Also, the concentration of aliquat 336 was increased from 1% to 2% in the SLM, to investigate whether this would increase the capacity of analyte transport. In experiment 26C, 1-octanol was applied as SLM instead of peppermint oil, in case that the reason for absent recoveries was due to an interaction between $Cl^-$ ions and peppermint oil.

In experiment 26, PBS was applied as donor solution, and 10 mM NaOH as acceptor solution. The agitation was 750 RPM.

**Table 53**. Results from extraction of 5 µg/ml MTX in order to study the impact of $Cl^-$ ions from a PBS donor solution.

| Experiment | SLM | Voltage (V) | Time (min) | Recovery donor solution (%) | Recovery acceptor solution (%) |
|---|---|---|---|---|---|
| 26A | Peppermint oil + 1% aliquat 336 | 20 | 30 | 98.6 | 2.0 |
| 26B | Peppermint oil + 2% aliquat 336 | 15 | 20 | 99.7 | 0.0 |
| 26C | 1-octanol + 1% aliquat 336 | 5 | 15 | 96.9 | 0.0 |

No MTX was detected in the acceptor solution in experiment 26B and 26C. The reason why 2% was achieved in experiment 26A might have been due to passive transport of MTX across peppermint oil without any interaction with the carrier. Experiment 9A supports this theory, as 6.9% MTX was detected in the acceptor solution after extraction at 100 V in 15 minutes without an ionic carrier in the peppermint oil.

In experiment 26B, 2% aliquat 336 in the SLM was most likely not a sufficiently high

concentration to allow ionic transport of MTX, as the carrier was occupied by $Cl^-$. Also, the voltage and time might have been insufficient to allow passive transport of MTX independent of aliquat 336.

In both experiment 26A and 26B, the extraction current was approximately 80 µA. Based on this observation, the time or voltage was not adjusted further upwards in experiment 26A, and the amount of carrier was not set higher than 2% in experiment 26B. These adjustments would lead to higher current in the system. Based on previous experience, extraction recoveries normally decreased as a result of high current, electrolysis and consequent pH changes.

Experiment 26C confirmed that recovery was 0% regardless of whether 1-octanol or peppermint oil was applied as the SLM, which strengthened the theory that the problem regarded the interaction between chloride ions and aliquat 336.

New experiments were performed by increasing the concentration of chloride ions in the acceptor solution. In theory, the flux of ions across the SLM is partly dependent on ion balance ($\chi$), which is the ratio between the total concentration of ions in the donor and acceptor solutions. A low $\chi$ value favors extraction kinetics (71). Therefore, it was suggested that a higher concentration of NaCl in the acceptor solution would prevent the $Cl^-$ ions in the donor solution from diffusing into the SLM and favor the extraction of MTX.

0.5 M NaCl was added to the acceptor solution, which previously only consisted of 10 mM NaOH. The agitation was set to 800 RPM this time, with hopes that it could more easily break interactions between aliquat 336 and chloride.

**Table 54**. Results from extraction of 5 µg/ml MTX from PBS, investigating the impact of ion balance.

| Exp. | Acceptor solution | SLM | Voltage (V) | Time (min) | Recovery acceptor solution (%)[1] |
|---|---|---|---|---|---|
| 27A | 10 mM NaOH + 0.5 M NaCl | Peppermint oil + 1% aliquat 336 | 20 | 15 | 2.7 |
| 27B | 10 mM NaOH + 0.5 M NaCl | Peppermint oil + 1% aliquat 336 | 10 | 15 | 1.1 |

[1]Experiment 27A and 27B are based on three parallels, with an RSD of 98.7 and 70.7%, respectively.

The high concentration of NaCl in the acceptor solution might have had a slight positive impact on the extraction recoveries in experiment 27. However, the aliquat 336 carrier was presumably still saturated with chloride ions and incapable of sufficient interaction with MTX.

The extraction of MTX from PBS without the application of voltage was attempted, to see if passive diffusion of MTX was possible despite the chloride ions in the donor solution. Three experiments were performed, with parameters altering between acceptor solution composition, amount of aliquat 336 in the SLM, and extracting time.

**Table 55**. Results from extraction of 5 µg/ml MTX from PBS without applying voltage.

| Experiment | Acceptor solution | SLM | Time (min) | Agitation (RPM) | Recovery acceptor solution (%)[1] |
|---|---|---|---|---|---|
| 28A | 10 mM NaOH | Peppermint oil + 1% aliquat 336 | 30 | 800 | 2.2 |
| 28B | 10 mM NaOH | Peppermint oil + 10% aliquat 336 | 45 | 800 | 0.0 ± 0.0 |
| 28C | 10 mM NaOH + 0.5 M NaCl | Peppermint oil + 10% aliquat 336 | 45 | 800 | 1.4 ± 0.4 |

[1]Experiment 28B and 28C are based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value. Experiment 28A is based on the extraction of one sample.

The results in experiment 28 were difficult to interpret, as the recoveries were generally very poor. It is uncertain why experiment 28A yielded higher recovery than in 28B and 28C. It might be due to random variance, and it is unlikely that the result would be statistically significant if experiment 28 was performed multiple times. Nevertheless, it seemed like the chloride ions had a great affinity to the carrier, regardless of influence from the electrical field.

### 4.7.4 Impact of plasma anions on aliquat 336, other than chloride

Since it became increasingly clear that the interaction between $Cl^-$ ions and aliquat 336 was responsible for the poor recoveries of MTX, the extraction of the analyte from plasma through an SLM containing aliquat 336 seemed unpromising. This was unfortunate, since the carrier had been the key component responsible for high extraction recoveries.

The idea of precipitation of chloride ions came up early in the phase of problem solving. However, before doing this, experiments were performed to assure that other anions in the plasma did not affect extraction recoveries like $Cl^-$ did. Electrolytes present in the plasma are sodium, chloride, magnesium, potassium, bicarbonate, phosphorous and calcium (56). Hence, bicarbonate ($HCO_3^-$) was the only anionic compound, in addition to chloride, that could be problematical to EME.

A donor solution containing Milli-Q water and $NaHCO_3$ in a concentration that mimicked the concentration of bicarbonate in blood was prepared (Table 4), and spiked with 5 µg/ml MTX. The sample was extracted by parameters listed in Table 56. Additionally, 5 µg/ml MTX was extracted from pure Milli-Q-water to compare the result with the $NaHCO_3$ experiment. The reason why Milli-Q water was chosen as a solvent instead of 40 mM phosphate buffer was to avoid unnecessary interference from other ions during the experiment.

**Table 56**. Constant parameters for experiment 29 and 30.

| Parameter | Description |
|---|---|
| *SLM* | Peppermint oil + 1% aliquat 336 |
| *Acceptor solution* | 10 mM NaOH, pH 12 |
| *Time* | 15 minutes |
| *Voltage* | 5 V |
| *Agitation* | 750 RPM |

**Table 57**. Results from extraction of 5 µg/ml MTX from a $NaHCO_3$ containing donor solution, and a Milli-Q water containing donor solution.

| Experiment | Donor solution | Recovery acceptor solution (%)[1] |
|---|---|---|
| 29A | 0.025 M $NaHCO_3$ in Milli-Q water | 22.2 ± 1.8 |
| 29B | Milli-Q water | 23.3 ± 3.7 |

[1]Experiments are based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

$NaHCO_3$ presumably did not have a negative influence on the carrier aliquat 336, since experiment 29A yielded almost the same recovery as experiment 29B. Theories regarding why the $HCO_3^-$ anion did not have a negative impact on the carrier will be discussed in section 4.7.6. Nevertheless, the possibility of negative interference from plasma bicarbonate ions in EME was excluded.

### 4.7.5 Precipitation of Cl- ions

Since $Cl^-$ was identified as the main problem in EME of MTX using aliquat 336 as carrier, an attempt to remove $Cl^-$ from the solution was made. Silver nitrate was obtained for the precipitation of $Cl^-$ ions based on the following reaction:

$$AgNO_3\,(aq) + NaCl\,(aq) \rightarrow AgCl\,(s) + NaNO_3\,(aq) \qquad \text{Equation 8}$$

However, by precipitating chloride, negatively charged nitrate ions would remain in the donor solution. Considering this, another experiment was carried out to see the impact of nitrate ions on aliquat 336. Based on the stoichiometry of equation 8, the concentration of $AgNO_3$ needed to precipitate the physiological concentration of NaCl in plasma (0.154 M) would be 0.154 M. Also, the amount of remaining nitrate ions after precipitation of 0.154 M NaCl would be 0.154 M, because of the molar ratio of 1:1.

A donor solution containing Milli-Q water and 0.154 M $KNO_3$ was prepared and spiked with 5 µg/ml MTX. The sample was extracted by the parameters listed in Table 56.

**Table 58.** Experiment 30. Result from extraction of 5 µg/ml MTX from a $KNO_3$ containing donor solution.

| **Donor solution** | **Recovery acceptor solution (%)[1]** |
|---|---|
| **0.154 M $KNO_3$ in Milli-Q water** | 3.4% ± 0.2 |

[1]Experiment is based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

The reduced recovery in experiment 30 compared to experiment 29 suggests that the nitrate ions act similarly as chloride ions, in terms of blocking transport of MTX across the SLM. This could therefore pose a problem with the precipitation of $Cl^-$ with $AgNO_3$.

Nevertheless, an experiment of precipitation was attempted. Since a PBS solution contained 0.137 M NaCl and 0.0027 M KCl, a concentration of 0.140 M $AgNO_3$ was used to ensure precipitation of all chloride ions from the PBS solution. However, to avoid excessive dilution of the sample, the $AgNO_3$ solution was prepared with twice as high concentration, 0.28 M. That way, 1 ml 7.5 µg/ml MTX in PBS was mixed with 0.5 ml 0.28 M $AgNO_3$. The tube was vortexed for one minute, prior to centrifugation for five minutes on an Allegra™ X-22R Centrifuge (Beckman Coulter (Brea, CA, USA)), with a relative centrifugal force (RCF) of 2800 for five minutes.

Before EME, 200 µl of the supernatant was analyzed on HPLC to observe whether MTX had recovered the precipitation and centrifugation process. The theoretical amount of MTX in the

supernatant was 5 µg/ml. However, the remaining amount of MTX was only 4.0% when comparing the supernatant with a standard solution of 5 µg/ml MTX in PBS, suggesting that most of the MTX had coprecipitated/sedimented with the AgCl.

To confirm or disprove whether it was the centrifugation process itself that removed MTX from the solution or not, 5 µg/ml MTX in PBS was centrifuged without addition of precipitant. This time, the recovery was 100% after centrifugation. This suggested that MTX might have precipitated from the addition of $AgNO_3$. Even though MTX has a high solubility in PBS at pH 7.4, the combination of high concentration of $Ag^+$ and centrifugation might have led to the formation of an insoluble complex between MTX and $Ag^+$ ($-COO^-Ag^+$).

Since MTX most likely co-precipitated with AgCl, the supernatant was spiked with new 5 µg/ml MTX after precipitation. This sample was extracted to check if the extraction recovery was similar to the one obtained when extracting MTX from a nitrate containing donor solution in experiment 30, which was 3.4% (Table 58).

**Table 59**. Experiment 31. Result from extraction of 5 µg/ml MTX from supernatant after precipitation of AgCl.

| SLM | Voltage (V) | Time (min) | Agitation (RPM) | Recovery acceptor solution (%) |
|---|---|---|---|---|
| **Peppermint oil + 1% aliquat 336** | 10 | 20 | 750 | 1.1 ± 0.1 |

[1]Experiment is based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

Based on the precipitate, much of the chloride ions had reacted with silver nitrate. However, the presence of nitrate ions and possibly chloride ions in the donor solution prevented high extraction recovery.

In conclusion, $AgNO_3$ was not a good choice for the precipitation of chloride ions in PBS. There were two reasons for this. The first reason was that only a small amount of MTX was present in the supernatant after precipitation and centrifugation. Secondly, nitrate ions remained in the sample after precipitation of $Cl^-$, which also appeared to interact with aliquat

336 and hinder MTX extraction. A way to combat these problems could be to use another Ag-salt for precipitation, such as $Ag_2SO_4$. This compound would generate $SO4^{-2}$ ions in the donor solution after precipitation, which could be favorable for EME, as discussed in section 4.7.6. However, $Ag_2SO_4$ has very low solubility in water ($1.4 \times 10^{-5}$ M at 25 °C) (72). Also, for EME of MTX to be a routine technique in the future, it is undesirable with a time-consuming process including several operational steps.

### 4.7.6 Monovalent vs. divalent anion effect on aliquat 336

At this point, it was established that chloride and nitrate ions strongly impaired EME of MTX. Both anions had the common property of being small and carrying one negative charge. To investigate whether other monovalent anions acted in a similar fashion, 0.14 M sodium iodide was added to a donor solution containing Milli-Q water.

**Table 60**. Experiment 32. Result from extraction of MTX from a sodium iodide containing donor solution.

| SLM | Donor solution | Voltage (V) | Time (min) | Agitation (RPM) | Recovery acceptor solution (%) |
|---|---|---|---|---|---|
| **Peppermint oil + 1% aliquat 336** | 0.14 M NaI in Milli-Q water | 5 | 15 | 750 | 0.1 |

Experiment 32, 30, and the experiments with chloride containing donor solutions supports the theory that monovalent anions interact with aliquat 336 and prevents MTX from entering the SLM.

However, the monovalent theory did not seem to apply for $NaHCO_3$ in experiment 29. The pKa value for bicarbonate ↔ carbonic acid is 6.4. The pKa value for bicarbonate ↔ carbonate is 10.3 (73). This means that $NaHCO_3$ mostly exist in monovalent state from pH 6.4-10.3.

One explanation to why the bicarbonate ions did not affect the function of aliquat 336, can be that it was added at a lower concentration; 0.025 M $NaHCO_3$ was added to Milli-Q water, compared to 0.154 M $KNO_3$. Also, bicarbonate might to some degree have equilibrated

between bicarbonate and carbonate in the donor solution. The donor solution in experiment 29A had a pH of 8.48, and the pH was presumably higher in the donor solution/SLM interface due to the electrical double layer. Since some of the molecules might have existed in divalent state and kept a net negative charge in the SLM, they could be pulled towards the positively charged anode by the electrical field. In experiment 29, the pH drop in the acceptor solution was only 1 unit, and was therefore 11 at the end of extraction, in which the divalent carbonate ions are the dominant species. Extraction of the divalent carbonate ions into the acceptor solution might therefore have made room for MTX to interact with aliquat 336.

Divalent ions in the donor solution have seemed unproblematic for EME. For example, in most experiments, a 40 mM phosphate buffer was used. Phosphoric acid has three dissociation constants, with pKa 2.16, 7.21 and 12.32 (74). At pH 7.4, the dominating form is $HPO_4^{2-}$, accounting for 74% of the molecules. (Figure 48). The divalent anions might have been extracted across the SLM due to the net negative charge. Alternatively, the buffer ions were too bulky in structure to interact strongly with aliquat 336. Also, they existed in relatively low concentration (0.04 M) which might have prevented excessive saturation of aliquat 336.

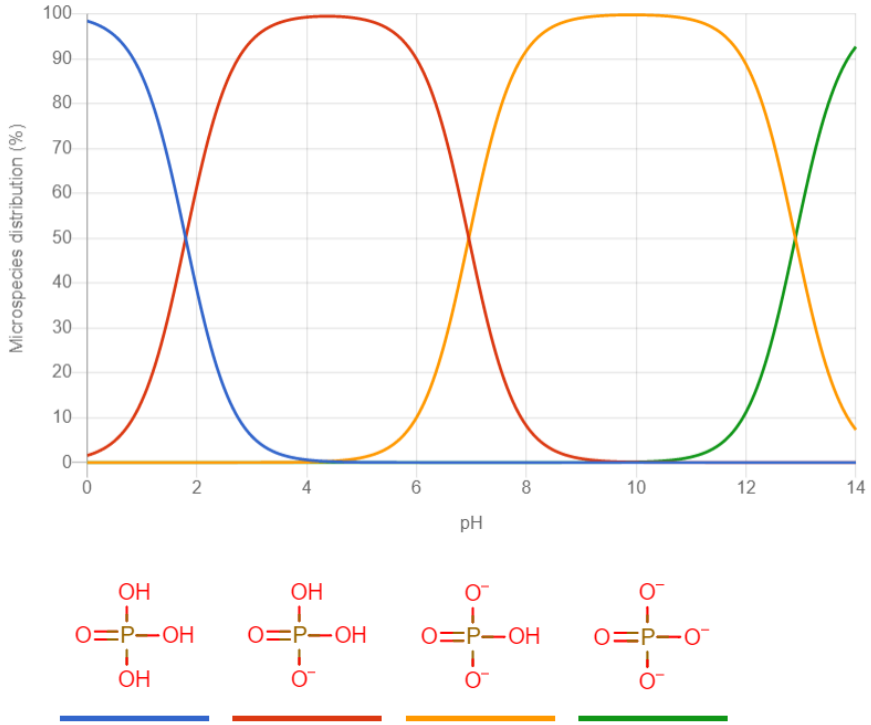



**Figure 48**. Predicted distribution of Phosphoric acid (%) with different charge at pH 0-14. Retrieved from (75).

MTX is also a divalent anion, which supports the theory on why it was released from the carrier aliquat 336. The net negative charge of the molecule within the SLM might have contributed to its pull against the anode in the acceptor solution. However, more factors could have been responsible for the high recoveries of MTX. For example, its high polarity was responsible for low affinity to the SLM. Also, its relatively large molecular size might have prevented the analyte from strongly interacting with aliquat 336.

In experiment 17, DAMPA was extracted through an SLM containing aliquat 336. The recovery in this experiment was lower than for the same experiment applying the analytes MTX and 7-OH-MTX. The fact that DAMPA is monovalent, and MTX and 7-OH-MTX are divalent ions might explain the poor recovery of DAMPA.

In conclusion, it was established that the small and monovalent anions chloride, nitrate, and iodide strongly impaired the extraction of MTX, most likely due to strong interactions between the anions and the positively charged carrier aliquat 336. To further investigate the function of aliquat 336, more anions (monovalent and divalent) would have to be tested systematically in order to understand the relationship between number of positive or negative charges and extraction efficiency.

### 4.7.7 Experiments with a new ionic carrier bis-(2 ethylhexyl)amine (BEA)

Based on the experiments so far, the extraction of MTX from a donor solution with a physiological concentration of NaCl through an SLM containing aliquat 336 was not possible. Also, the nitrate ions left in solution after precipitation of chloride ions would equally impair the extraction. Thus, a new carrier was needed to extract MTX from plasma. A cationic carrier, bis-(2 ethylhexyl)amine (BEA), was obtained from Department of Pharmacology, University of Oslo. The compound has structural similarities to DEHP (Figure 9), except that BEA is a cationic carrier.

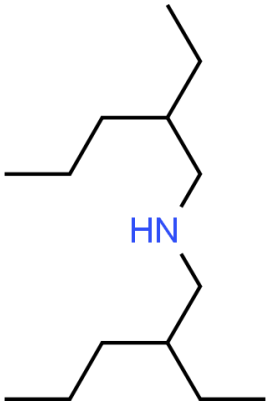



**Figure 49**. Chemical structure of bis-(2 ethylhexyl)amine (BEA). Downloaded from (57).

Unlike aliquat 336, this carrier does not keep a permanent positive charge. Its predicted pKa value is 11.1, meaning that below this pH, an increasing fraction of the molecule exists with a +1 charge at the nitrogen atom (76). Above pH 11.1, an increasing fraction of the molecule would be neutral.

Before using the new carrier in extraction of MTX from PBS, a regular 40 mM phosphate buffer was chosen as the donor solution. The intention was to see if the carrier functioned as well as aliquat 336 under conditions without chloride, which could pose a problem to extraction.

10 mM NaOH was chosen as the acceptor solution. The voltage was set to 100 V, based on a test extraction from a blank donor solution to see how much current the new carrier generated. The extraction current was approximately 10 µA with 1% BEA, and slightly higher (15-20 µA) with 10% BEA in the SLM.

**Table 61**. Results from extraction of 5 µg/ml MTX through peppermint oil + BEA from a 40 mM phosphate buffer.

| Experiment | SLM | Voltage (V) | Time (min) | Agitation (RPM) | Recovery acceptor solution (%)[1] |
|---|---|---|---|---|---|
| 33A | Peppermint oil + 1% BEA | 100 | 15 | 750 | 0.7 ± 0.7 |
| 33B | Peppermint oil + 10% BEA | 100 | 15 | 750 | 0.0 ± 0.0 |

[1]Experiments are based on two parallels, where the deviation represents the difference between the mean value and the highest/lowest value.

The extraction recoveries in the acceptor solutions were practically absent. The cause of this is unclear, but the low extraction current indicated a poor flux of ions across the SLM, despite the application of 100 V. In experiment 33, the donor solution still contained all MTX after the extraction.

The BEA carrier was added to 1-octanol instead of peppermint oil, since 1-octanol is more frequently applied as SLM in EME. Based on a test extraction from a blank donor sample, the voltage was set to 50 V, generating approximately 40 µA.

**Table 62**. Experiment 34. Results from extraction of 5 µg/ml MTX through 1-octanol + BEA.

| SLM | Voltage (V) | Time (min) | Agitation (RPM) | Recovery donor solution (%) | Recovery acceptor solution (%) |
|---|---|---|---|---|---|
| **1-Octanol + 1% BEA** | 50 | 15 | 750 | 33.2 | 23.7 |

Compared to experiment 33, where the extraction current was approximately 10 µA, the extraction current in experiment 34 was initially 40 µA, increasing to 160 µA at the end of extraction. This also explained why the recovery was significantly higher in experiment 34 than experiment 33. The cause of the improved function of the carrier with 1-octanol compared to peppermint oil remains unknown.

It was hypothesized that BEA could function even better if added to the donor solution, in addition to being present in the SLM. That way, more carrier molecules would be able to interact with MTX in donor solution, form ion complexes and more easily partition into the SLM. According to Chemicalize, BEA has a solubility of 3,4 mg/ml at pH 7.4 (76). A donor solution consisting of 3.4 mg/ml BEA in 40 mM phosphate buffer was prepared.

**Table 63**. Experiment 35. Result from extraction of 5 µg/ml MTX from a donor solution containing BEA.

| Donor solution | SLM | Voltage (V) | Time (min) | Agitation (RPM) | Recovery donor solution (%) | Recovery acceptor solution (%) |
|---|---|---|---|---|---|---|
| **40 mM phosphate buffer + 3.4 mg/ml BEA** | 1-Octanol + 1% BEA | 50 | 15 | 750 | 11.4 % | 30.9 |

The addition of carrier to the donor solution enhanced the recovery in experiment 35. Also, more of the MTX molecules had presumably been transported from the donor solution and into the SLM. However, the current gradually increased during the extraction, from 20 to 400 uA by 10 minutes. The current stabilized at 400 uA for the remaining 5 minutes, but this was still much higher than recommended in EME. For additional experiments, the voltage could be reduced, but the purpose with experiment 34 and 35 was to see if BEA could function as an ionic carrier to MTX.

Since BEA appeared to be promising for the extraction of MTX from a 40 mM phosphate buffer, the carrier was tested in extraction from PBS. Experiment 34 and 35 were performed again, but with PBS as donor solution, in hopes that chloride ions would not interact as strongly with BEA as with aliquat 336. Like earlier, the voltage was 50 V, the extraction time was 15 minutes, the agitation was 750 RPM, and the SLM was composed of 1-octanol + 1% BEA.

**Table 64**. Results from extraction of 5 µg/ml MTX from PBS using the ionic carrier BEA.

| Experiment | Donor solution | Recovery donor solution (%) | Recovery acceptor solution (%) |
|---|---|---|---|
| **36A** | PBS | 99.4 | 0 |
| **36B** | PBS + 3.4 mg/ml BEA | 98.3 | 1.1 |

In experiment 36A and 36B, almost all MTX was found in the donor solution after EME. The application of PBS lowered the recoveries from 23.7% and 30.9% to 0% and 1.1%. The current in experiment 36 was high (200 µA for 36A and up to 400 µA for 36B) However, it was buffer ions that accounted for the high current. All the added MTX was left in the donor solution and had apparently no interaction with the SLM.

In conclusion, it was likely that the new cationic carrier BEA got saturated with chloride ions the same way as aliquat 336. Hence, there was no good solution to the problem related to chloride ions in the donor solution in combination with cationic carrier.

## 4.8 Extraction of MTX from plasma

Due to all the problems with the extraction of anionic MTX from a chloride containing donor solution, attempts were made to extract MTX as a cation. The high concentration of NaCl in plasma would most likely make extraction of anionic MTX through aliquat 336 impossible. EME of spiked plasma was therefore performed using the method that yielded 7.1% in section 4.2.

Approximately 4 ml blood was obtained in EDTA tubes and centrifuged on a Universal Refrigerated Centrifuge Model 5930 (Kubota, Japan) with a relative centrifugal force (RCF) of 2100 in 10 minutes. The plasma was spiked with 10 µM MTX. After dilution with phosphate buffer and pH adjustment to 2.6 with HCl, the final concentration of MTX in the donor solution was 4.39 µM. Tree identical samples were extracted simultaneously, by the extraction conditions in Table 65. The acceptor solutions were analyzed on LC-MS/MS after EME.

For preparation of the external standard solution, a blank plasma sample was preprocessed the same way as the spiked sample (diluted with phosphate buffer and pH adjusted to 2.6) and extracted by same procedure. After EME, the acceptor solution was spiked with 4.39 µM MTX and analyzed by LC-MS/MS. See Table 4 for detailed information concerning the preparation of the spiked donor solution and external standard solution.

**Table 65.** Extraction conditions for experiment 37.

| Parameter | Value |
|---|---|
| Donor solution | Plasma spiked with 4.39 µM MTX |
| Acceptor solution | 50 mM HCl pH 1.3 |
| SLM | NPPE + 20% DEHP |
| Voltage | 50 V |
| Time | 15 minutes |
| Agitation | 750 RPM |

**Table 66.** Experiment 37. Results from extraction of 4.39 µM MTX from plasma.

| Sample | Analyte peak area[1] | Recovery (%) |
|---|---|---|
| 1 | 44 700 ± 4 700 | 5.8 |
| 2 | 47 200 ± 2 600 | 6.2 |
| 3 | 34 700 ± 300 | 4.5 |
| External standard | 766 500 ± 4 500 | |

[1]All results are based two injections from the same sample, where the deviation represents the difference between the mean and the highest/lowest analyte peak area.

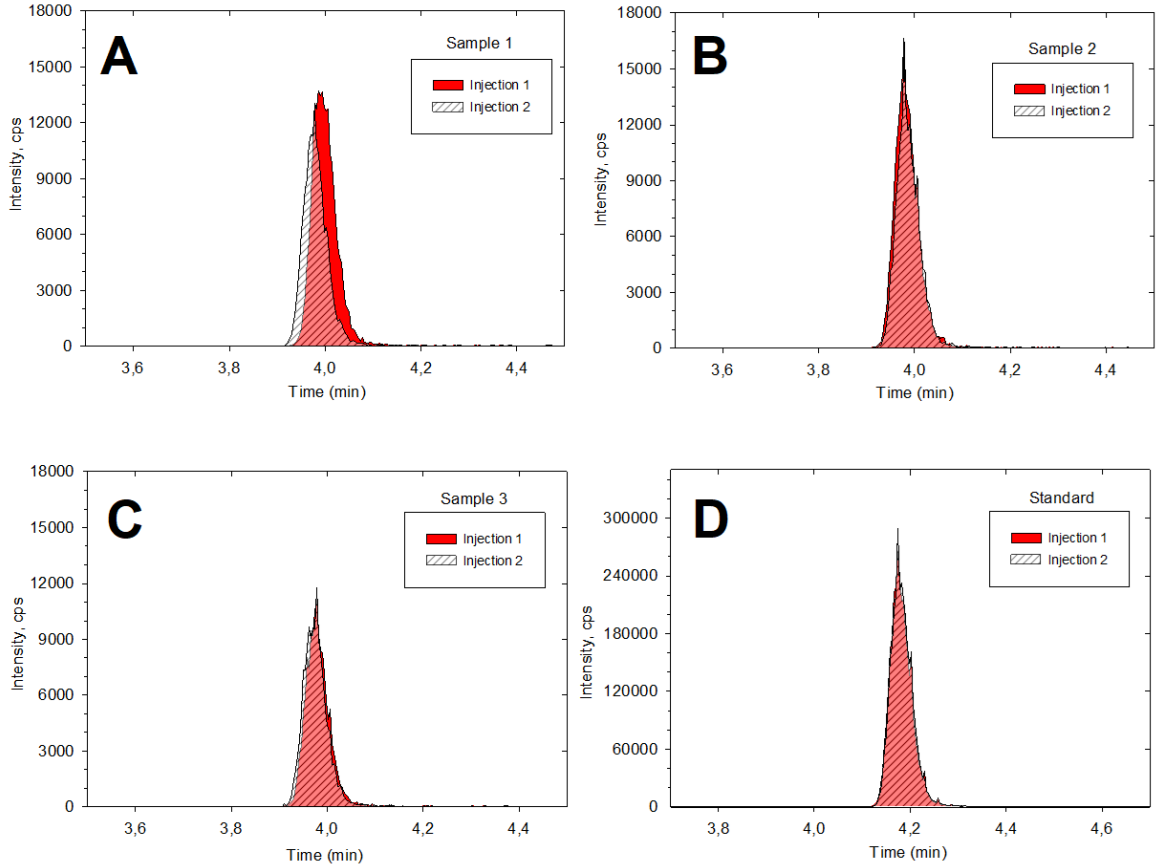



**Figure 50**. LC-MS/MS chromatograms showing the elution and intensity of sample 1 (A), 2 (B) and 3 (C) from experiment 37. From all the samples, including the standard (D), 1 µl was injected into the LC-MS/MS twice.

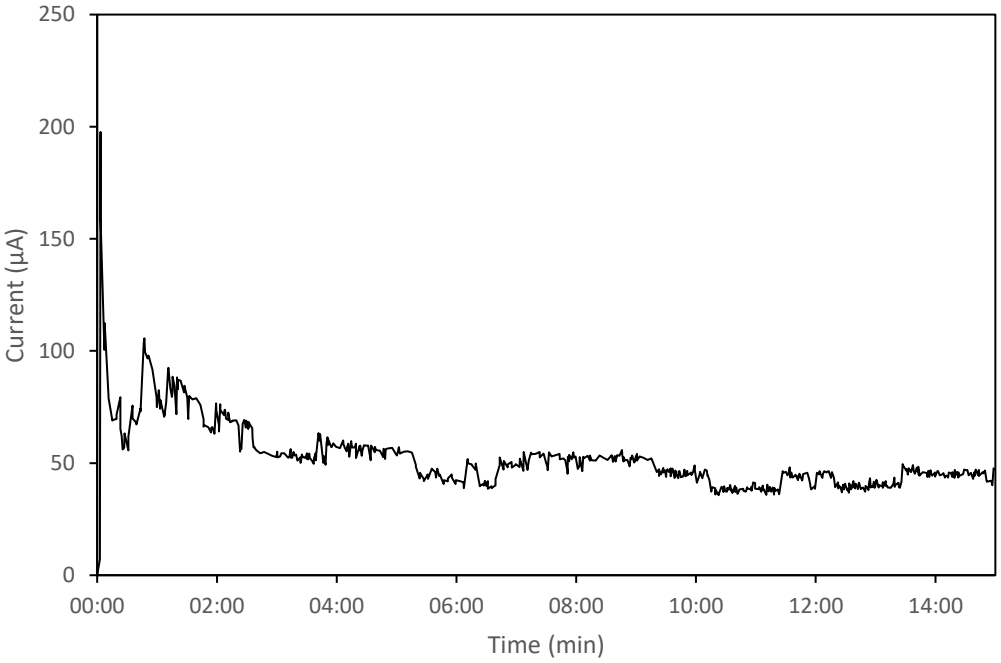



**Figure 51**. Extraction current for experiment 37. The trace is from one EME extraction of three samples, representing the sum of the extraction currents of all samples.

The recoveries in experiment 37 were poor, as expected based on the results in section 4.2. The mean recovery was 5.5% with an RSD of 13.1%. However, this was not far from the recovery achieved by extraction of MTX from phosphate buffer (7.1%), which is promising for future development of the method, although it is uncertain whether it is possible to extract protonated MTX with high levels of recovery. The extraction current was low and descending for the three samples (Figure 51), indicating a stable system with no adverse electrolysis. It thus appears that the different constituents in plasma did not interfere substantially with the extraction of MTX over an SLM consisting of NPPE and 20% DEHP. For future experiments, the voltage could have been adjusted upwards, which might have facilitated greater transport of MTX across the SLM.

The difference between the highest and lowest analyte peak area was relatively high between injections from the same sample (Table 65). This was especially evident for sample 1 (Figure 50A). Differences in signal between injections were reoccurring, and occurred in standard samples dissolved in methanol, as discussed in section 3.7.3. To compensate for this, a fixed concentration of an IS could be added to the sample. That way, the analyte and IS would be affected by the instrumental deviations and possible matrix effects similarly, and the ratio

between them could be measured for a better estimate of the concentration of MTX.

The EME extract appeared clear and visibly free from contaminants, which is beneficial for injection into the LC-MS instrument. A high sample clean-up enhances the performance of the LC-MS instrument, since matrix effects and subsequent signal suppression of the analyte is avoided to a greater extent (16). Also, the need for maintenance will be reduced, as contamination is avoided to a greater extent.

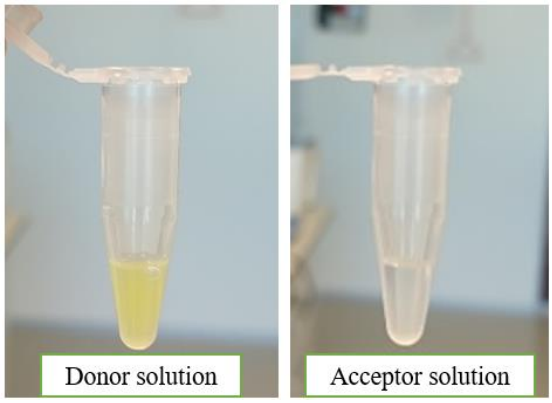



**Figure 52.** Donor and acceptor solution after EME of MTX from plasma.

# 5 Limitations

Based on all the experiments performed, several aspects made the extraction of MTX particularly challenging. The extraction of MTX as a cation resulted in poor recoveries. This might be explained by an elevated pH in the acceptor solution or the SLM/acceptor solution interface, with consequent reduction in the release of MTX from the ionic carrier DEHP.

The conducive vials appeared to lose their performance over time. Most likely, this was due to substances accumulating in the vials over time, which altered their conductive properties. This resulted in varying extraction recoveries and high RSD values, confounding the process of EME method development. New and unused vials provided more reproducible results, and for EME to become a routine method, disposable vials must be developed to avoid variance in conductivity.

The ionic carriers used to transport anionic MTX across the SLM (aliquat 336 and BEA) both

interacted with chloride, impairing the extraction of MTX from PBS. Even though the extraction of anionic MTX from plasma was not practically performed, the physiological concentration of chloride in plasma would in theory be excessive and hinder the function of the ionic carriers. The ideal carrier for MTX has obviously not yet been identified.

Considering that a range of different parameters were tested (composition of SLM, donor/acceptor solutions, voltage, time, and agitation), a systematic approach was required to draw meaningful conclusions from the extraction results. This included a consistent change of one parameter at a time to observe the relationship between different extraction conditions and recovery in the acceptor solution. Unnecessary time was spent on changing multiple parameters at the same time, hoping it would facilitate high recoveries faster. The most efficient approach for EME would be to perform a fractional factorial design of experiments. This would give a clear indication on the relationship between extraction parameters and results, but without spending too much time, since multiple parameters are changed simultaneously in a systematic matter.

# 6 Conclusions

In the experiments conducted in this thesis, EME of the analytes MTX, 7-OH-MTX and DAMPA have been performed for the first time. A range of different SLMs were applied, including NPOE, NPPE, 1-octanol, 1-nonanol, peppermint oil, menthone, and a mixture of menthone and menthol. Also, ionic carriers, such as DEHP, aliquat 336 and BEA were added to the SLM to facilitate migration of the polar analyte. The combination of SLM and ionic carrier that yielded the highest recovery of MTX throughout the study was peppermint oil with 1% aliquat 336. By adjusting other extraction parameters like voltage, time, and agitation, a recovery of 77.3% MTX, 59.0% 7-OH-MTX and 32.4% DAMPA, was achieved in the acceptor solution. Since peppermint oil is not a defined product, the future focus should be directed towards its main components, menthol and menthone, by finding the combination that would facilitate the best extraction of MTX.

At Rikshospitalet in Oslo, ≈ 70% recovery of MTX and its metabolites from samples of patients treated with glucarpidase is achieved by protein precipitation. In the present study, the recovery of MTX was higher, fulfilling a main aim of the study. However, the SLM used

to achieve this recovery (1% aliquat 336 in the SLM), was not applicable to EME of MTX from plasma samples. Anionic chloride ions strongly impaired the extraction of MTX by interacting with aliquat 336 and blocking transport of the analyte. Hence, the physiological concentration of $Cl^-$ in plasma would not allow EME of MTX by the method developed using buffer without monovalent anions in the donor vial. Therefore, EME of MTX from plasma was performed independent of aliquat 336, by extracting MTX as a protonated base. This experiment yielded a mean recovery of 5.5% (RSD = 13.2%) in the acceptor solution.

Thus, no satisfactory EME method for the extraction of MTX and its metabolites from plasma was achieved in this study. The main challenge was not the acidity of the analytes. There are several studies on EME of acidic analytes, such as ibuprofen, diclofenac, and ketoprofen, where successful extraction from plasma was achieved (37, 77, 78). The challenge with MTX and its metabolites was their high polarity, resulting in a definite requirement for an ionic carrier in order to achieve transport across the SLM. Both aliquat 336 and BEA lost their ability to transport MTX in the presence of $Cl^-$, and it thus appears as if another ionic carrier with less interference with $Cl^-$ must be found.

In conclusion, the large body of results significantly contribute to a theoretical and practical understanding of conditions facilitating EME extraction of acidic and polar compounds like MTX and its metabolites. The multiple observations noted throughout the study, such as the importance of controlling the extraction current to avoid electrolysis, the composition of the SLM to facilitate partition of the analytes into the SLM, and the significance of an ionic carrier to transport the analytes across the SLM and into the acceptor solution, provide an improved general understanding of EME of acidic and polar compounds. While forming a base for establishment of an applicable method for MTX and its metabolites, the work also paves the way for methodological development of other TDM candidates with similar chemical properties, generally regarded as challenging in an EME perspective.

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