Advances in Sample Preparation 2 (2022) 100011

Contents lists available at ScienceDirect

Advances in Sample Preparation

journal homepage: www.elsevier.com/locate/sampre

Determination of methotrexate and its metabolites in human plasma by electromembrane extraction in conductive vials followed by LC-MS/MS

Anne Oldeide Hay[a,b], Roger Trones[a], Lars Herfindal[c], Silje Skrede[d,e], Frederik André Hansen[b,*]

[a] G&T Septech AS, Verkstedveien 29, 1424 Ski, Norway
[b] Department of Pharmacy, University of Oslo, P.O. Box 1068 Blindern, Oslo 0316, Norway
[c] Centre for Pharmacy, Department of Clinical Science, University of Bergen, Bergen, Norway
[d] Department of Clinical Science, University of Bergen, Bergen, Norway
[e] Section of Clinical Pharmacology, Department of Medical Biochemistry and Pharmacology, Haukeland University Hospital, Bergen, Norway

ARTICLE INFO

Keywords:
Methotrexate
Elelectromembrane extraction
Plasma, LC-MS/MS
Deep eutectic solvents
Green chemistry

ABSTRACT

In the current study, an electromembrane extraction (EME) method for the simultaneous determination of methotrexate (MTX) and its metabolites, 7-hydroxymethotrexate (7-OH-MTX) and 4-amino-4-deoxy-N(10)-methylpteroic acid (DAMPA), was developed for human plasma samples. MTX and its metabolites are characterized as polar and zwitterionic, and the extraction mode (anionic/cationic) together with type of supported liquid membrane (SLM) was therefore initially studied. Extraction efficiency in anionic mode was found to be entirely suppressed by matrix components in plasma, whereas high efficiency could be achieved in cationic mode using an deep eutectic solvent-based supported liquid membrane of 6-methylcoumarin:thymol (1:2 molar ratio) with 0.5% (w/w) di(2-ethylhexyl) phosphate. EME was performed in prototype equipment based on conductive vials, and parameters of extraction time, voltage, shaking rate, sample and acceptor volumes, and plasma dilution factor, were optimized to get the highest possible sensitivity in subsequent LC-MS/MS determination. In the final method, plasma was diluted 2-fold with phosphoric acid to a volume of 500 µL, and extracted for 30 min at 10 V using 10 µL SLM, 200 µL 100 mM formic acid as acceptor solution, and a shaking rate of 900 RPM. Under these conditions, the average current was 10 µA per extraction cell. Recoveries of 23–53% were achieved, with linear ($R^2 \geq 0.9952$) calibration curves, accuracy in the range 98–121%, relative standard deviation (RSD) of 2.8–27.6%, and matrix effects of 103–111%. The greenness of the proposed method was also evaluated by the AGREEprep metric, and compared to other methods from literature. The findings of this paper demonstrate that EME can provide selective and robust extraction of polar and zwitterionic substances in a complex biological fluid, using very green extraction chemistry.

Introduction

The analysis of complex biological samples has historically been one of the most challenging tasks within analytical chemistry. The main challenge is often to separate analytes of interest from the matrix components in such a way that the determination can be selective, sensitive, and reliable. In bioanalysis, such determination is often performed using liquid chromatography – mass spectrometry (LC-MS). Samples are frequently whole blood or plasma that contains high levels of cells and protein, which makes them incompatible with direct LC-MS analysis. Simple sample pretreatment such as centrifugation or protein precipitation may be performed to improve compatibility, but low clean-up efficiency makes ion suppression effects from co-elution of analytes and matrix components a serious disadvantage [1]. Extraction-based sample preparation can provide much more selective analyte clean-up, and put less emphasis on LC-MS method development. Selective and efficient extraction based on partitioning, in solid- or liquid-phase, however often becomes more difficult when analytes are polar [2]. Zwitterionic substances may additionally be particularly challenging because they often have narrow isoelectric points (neutrality) [1,2].

Methotrexate (MTX) is an example of a pharmaceutical drug that is both polar and zwitterionic. MTX is an antifolate drug used for several diseases, including autoimmune like rheumatoid arthritis, or some cancers like acute lymphoblastic leukemia, malignant lymphoma and osteosarcoma [3]. The therapeutic window of MTX is narrow, and overdosing can lead to serious side effects [4]. Therapeutic drug monitor-

Abbreviations: 6-MC, 6-methylcoumarin; Thy, Thymol; DEHP, Di(2-ethylhexyl) phosphate; MTX, Methotrexate; 7-OH-MTX, 7-hydroxymethotrexate; DAMPA, 4-amino-4-deoxy-N(10)-methylpteroic acid.

* Corresponding author.
E-mail address: f.a.hansen@farmasi.uio.no (F.A. Hansen).

https://doi.org/10.1016/j.sampre.2022.100011
Received 30 January 2022; Received in revised form 20 February 2022; Accepted 20 February 2022

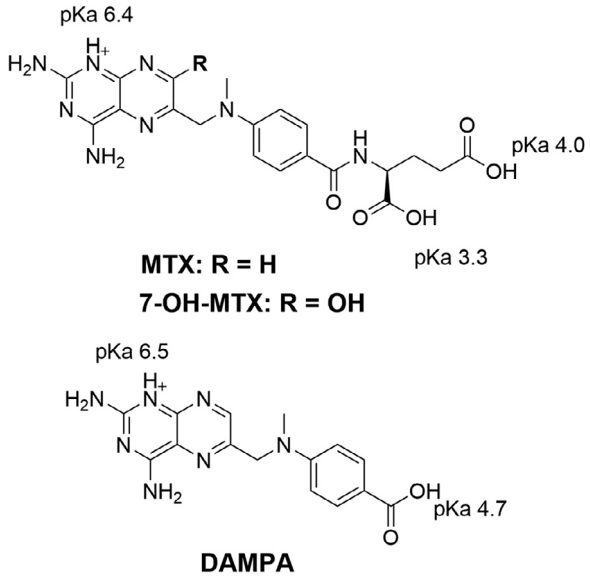

Fig. 1. Chemical structures of methotrexate (MTX), 7-hydroxy methotrexate (7-OH-MTX), and 4-amino-4-deoxy-N(10)-methylpteroic acid (DAMPA) with corresponding pKa values (calculated by MarvinSketch 21.19, ChemAxon). Log *P* values are -0.24, -2.29, and 0.89 (calculated by MarvinSketch 21.19, ChemAxon).

ing (TDM) may therefore be used to ensure the efficiency and safety of the treatment. In the liver, MTX is converted to 7-hydroxymethotrexate (7-OH-MTX) that may contribute to nephrotoxicity at high doses [5]. In cases of renal failure or when a patient has delayed elimination of MTX, an antidote called glucarpidase may be administered, which cleaves MTX and 7-OH-MTX into the non-toxic derivate 4-amino-4-deoxy-N(10)-methylpteroic acid (DAMPA) and glutamate [5]. DAMPA is further a product of intestinal metabolism of MTX. The structures of MTX, 7-OH-MTX, and DAMPA, with corresponding log P and pKa values, are given in Fig. 1.

Previously, the majority of reported methods for determination of MTX and its metabolites in blood or plasma have used protein-precipitation or solid-phase extraction (SPE) for sample preparation [3,6]. These techniques are respectively associated with poor clean-up or large consumption of organic solvents [7]. Electromembrane extraction (EME), on the other hand, is a microextraction technique that may be suitable for selective extraction of polar and charged analytes from biological samples. In EME, a thin liquid membrane of organic solvent (few microliters) held inside the pores of a polymeric membrane (supported liquid membrane or SLM) is used to separate the sample from a clean acceptor solution. The aqueous solutions are typically in a volume of a few hundred microliters. Extraction is performed by applying an electric field across the SLM, which stimulates charged substances to migrate from the sample, through the SLM, and into the acceptor solution. Since very low volumes of organic solvent and sample are used, EME may be considered as a green extraction technique. Extraction selectivity is based on voltage-assisted partitioning, and is therefore governed by the properties of the SLM solvent, as well as the polarity and magnitude of the electric field [8,9]. Because of this, polar and charged substances may principally be extracted by EME [10]. Since the introduction in 2006 [11], EME has been performed in various technical formats, for example hollow-fiber [11,12], on-chip [13–15], and 96-well [16–18]. None of these have been commercially available, and direct transfer of methods between laboratories has therefore been challenging. Currently, a prototype EME device based on conductive vials is however in development, which eventually will enable easy transfer of standardized and validated methods. Recently, the quality of validation data using this prototype device was evaluated by Skaalvik et al [19]. for a small section of non-polar basic pharmaceuticals, using 2-nitrophenyl octyl ether (NPOE) as an SLM that has been extensively used in EME literature [9].

Regarding zwitterions, one fundamental study has previously investigated how zwitterionic analytes could be extracted with EME as acids or bases [20]. The study was performed using clean water samples, and its application for complex samples is therefore unclear. The aim of the present work was therefore to develop and test an EME method for polar and zwitterionic substances in human plasma, using the prototype device. MTX and its metabolites were selected as representative model analytes. After EME, the analyte-enriched acceptor phase was analyzed by LC-MS/MS. A secondary aim was to study how the prototype device should be optimized to gain the highest possible sensitivity from human plasma samples. This was different from Skaalvik et al. who optimized equipment parameters to maximize extraction recovery. The work provides a green alternative to sample preparation for MTX and its metabolites, but may also be considered a fundamental study to provide general starting conditions for future EME of substances with similar properties, in complex biological samples.

Materials and methods

Chemicals and reagents

MTX, MTX-D_3, DAMPA, 1-octanol, 2-nitrophenyl octyl ether (NPOE), cetyltrimethylammonium bromide (CTAB), Aliquat®336, di(2-ethylhexyl) phosphite (DEHPi), di(2-ethylhexyl) phosphate (DEHP), 6-methylcoumarin, coumarin, thymol, formic acid, sodium hydroxide, sodium diphosphate, disodium phosphate, and sodium chloride were all purchased from Merck (Darmstad, Germany). 7-OH-MTX was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). LC-MS grade methanol was purchased from VWR (Radnor, PA, USA), and Milli-Q grade water was produced by a purification system (Molsheim, France). Drug-free plasma was obtained from Oslo University Hospital (Oslo, Norway) and stored at −28 °C

Preparation of solutions

Standards and spiking solution

MTX, 7-OH-MTX, DAMPA, and MTX-D_3 were dissolved individually in DMSO at 1 mg mL^{-1}, and stored at −28 °C. From these, a spiking solution was prepared by mixing and diluting the drugs to 10 μg mL^{-1}. The spiking solution was added to either buffer or plasma samples, depending on the experiments, in a final concentration of 1 μg mL^{-1}.

Preparation of deep eutectic solvents

Deep eutectic solvents were prepared by mixing appropriate amounts of eutectic components into a 5 mL Eppendorf-tube. The exact amount was adjusted to get the desired molar ratio of components. Melting of the mixture was assisted by heating in an oven for approximately 20 min at 80 °C, after which the solvent was vortexed for 10 s. The latter step ensured a homogenous liquid.

Electromembrane extraction equipment and procedure

Electromembrane extraction was performed using a prototype device developed and provided by Extraction Technologies Norway AS (ETN, Ski, Norway). The device (Fig. 2) was based on vials made from a proprietary conductive material to house sample and acceptor solutions. For extraction, the vials were screwed into a leak-tight union holding a flat porous membrane of polypropylene (Accurel PP2E, Membrana GmbH, Wuppertal, Germany). The vials had a total volume of 600 μL. In the current report, we define the vials as donor and acceptor vials, respectively holding sample and acceptor solutions. Prior to extractions, the SLM was prepared by pipetting 10 μL solvent onto the membrane. The solvent immediately penetrated the pores and became immobilized by capillary forces. The vials were screwed into the union, and this EME

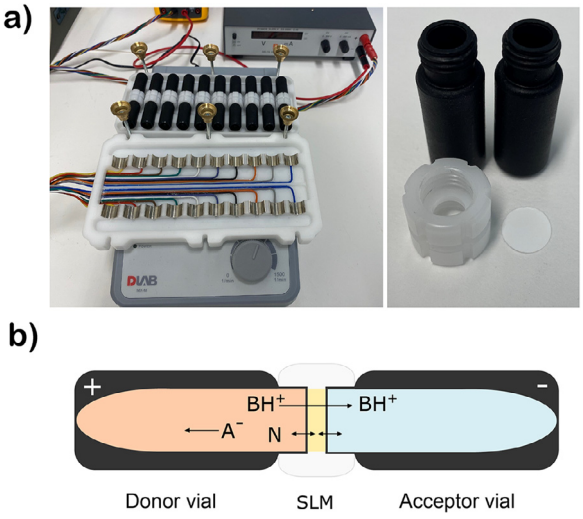

Fig. 2. (a) Photo of prototype EME device (left) holding ten EME units, and one disassembled EME unit (right). (b) Illustration of the EME of protonated based inside the EME unit. The figure was adapted from Skaalvik et al [19]. with permission.

unit was placed horizontally in a shaking device. In total, the prototype could hold up to ten EME units. During operation, horizontal agitation was applied to ensure contact between the aqueous solutions and the SLM, and to enhance the mass transfer be removing diffusion layers. An electric field was applied to each EME unit by a power supply (model ES 0300-0.45, Delta Elektronika BV, Zierikzee, Netherlands) connected to half ring-electrodes held in the lid of the device. For extraction of cations (bases), the cathode was placed on the acceptor vials, and conversely, on the donor vial when anions (acids) were extracted. The flow of current was monitored at 8 Hz acquisition rate using a Fluke287 multi-meter (Everett, WA, USA). Following EME, acceptor solutions were collected and analyzed by LC-MS/MS.

Liquid chromatography – tandem mass spectrometry methods

MTX and its metabolites

Determination of MTX, 7-OH-MTX, DAMPA, and MTX-D_3 as internal standard (IS) was performed by LC-MS/MS using a Dionex UltiMate 3000 RS UHPLC system (Thermo Scientific, San Jose, CA, USA) comprising a pump, an auto-sampler, and a temperature controlled column compartment. The mobile phases consisted of (A) 95:5 v/v purified water and methanol containing 20 mM formic acid, and (B) 5:95 v/v purified water and methanol containing 20 mM formic acid, and the column was an Acquity UPLC® HSS T3 column (100 × 2.1 mm ID, 1.8 mm, Waters, Wexford, Ireland) maintained at 60 °C. The flow was 0.4 mL min^{-1}, and elution was performed by the following gradient: Mobile phase B was ramped from 5 to 50% B from 0 to 2 min, thereafter 50–100% B in 0.1 min, where it was held until 4 min, before returned to 5% B in 0.1 min for 1.9 min of re-equilibration. The total run time was thus 6 min. The injection volume was 5 µL. Detection was performed with an LTQ-XL linear ion-trap mass spectrometer (Thermo Scientific, San Jose, CA, USA), operated in SRM mode with positive electrospray ionization (ESI) at 5 kV and a capillary temperature of 350 °C. SRM transitions were m/z 455 → 308 for MTX, m/z 471 → 324 for 7-OH-MTX, 326 → 175 for DAMPA, and 458 → 311 for MTX-D_3, at normalized collision energies of 51%, 28%, 40%, and 51%, respectively. A representative chromatogram is provided as supplementary material (Fig. S1).

Phospholipids

Determination of phospholipids was performed using the same instrument, column, and mobile phases. The flow was 0.4 mL min^{-1}, and gradient elution was performed by ramping mobile phase B from 10% to 100% in 0.3 min, where it was kept for 12 min, before returning to 10% B in 0.1 min for 2.6 min of re-equilibration. The injection volume was 10 µL. Detection was performed using an in-source fragmentation and SRM method based on a previous report [21]. For this, a source potential of 65 V was applied, and a transition of m/z 184 → 184 at 10% normalized collision energy was monitored. Positive ESI was operated at 5 kV and the capillary temperature was set to 350 °C.

Protein precipitation

500 μL thawed plasma was added to 1000 μL ACN, vortexed at 1800 RPM for 1 min, and centrifuged at 8000 RPM for 3 min. 1000 μL supernatant was collected and evaporated to dryness under a flow of nitrogen gas in a heating block set to 40 °C. The residue was re-suspended in 667 μL acceptor solution, equal to 2-fold dilution of plasma prior to removal of proteins.

Statistical design and analysis of experiments

Experimental design by a design-of-experiments (DOE) approach was set up and analyzed using the software Design-Expert 13 (Stat-Ease Inc., Minneapolis, MN, USA). Multiple linear regression was used to fit quadratic models to the data, and analysis-of-variance (ANOVA) was applied to assess significance of effects.

Linear regression analysis of calibration curves from method validation was performed using Graphpad Prism 9.3.1 (Graphpad Software, San Diego, CA, USA).

Calculations

Process efficiency (PE,%) was used to evaluate extraction efficiency during method development and optimization, and applies for both neat standards and plasma samples. The term does not correct for matrix effects.

$$PE = \frac{AUC_{acceptor,\ final}}{AUC_{unextracted\ standard}} \times \frac{V_{acceptor}}{V_{donor}} \times 100\%$$

AUC is the peak area, $V_{acceptor}$ is the acceptor volume, and V_{donor} is the donor volume.

Recovery (R,%) defined the extraction efficiency with correction for matrix effects, and was calculated relative to the signal from acceptor solutions spiked after extraction of a blank matrix sample.

$$R = \frac{AUC_{acceptor,\ final}}{AUC_{post-extraction\ spiked\ matrix}} \times \frac{V_{acceptor}}{V_{donor}} \times 100\%$$

Enrichment factor was defined as fold increase in analyte concentration in the acceptor solution after extraction, relative to the concentration in raw undiluted plasma samples. EF was calculated from PE or *R*, depending on which was applicable.

$$EF = (PE \text{ or } R) \times \frac{V_{donor}}{V_{acceptor}}$$

Matrix effect (ME,%) defined the effect of ion suppression or enhancement in the electrospray ionization process, and was calculated by:

$$ME = \frac{AUC_{post-extraction\ spiked\ matrix}}{AUC_{unextracted\ standard}} \times 100\%$$

Results and discussion

Selection of extraction mode and SLM screening

MTX and its metabolites are structurally composed of a pteridine-diamine core connected to a p-aminobenzoyl group (Fig. 1). In MTX and 7-OH-MTX, the latter is further linked to a glutamic acid residue with diacidic properties. DAMPA on the other hand is monoacidic. The

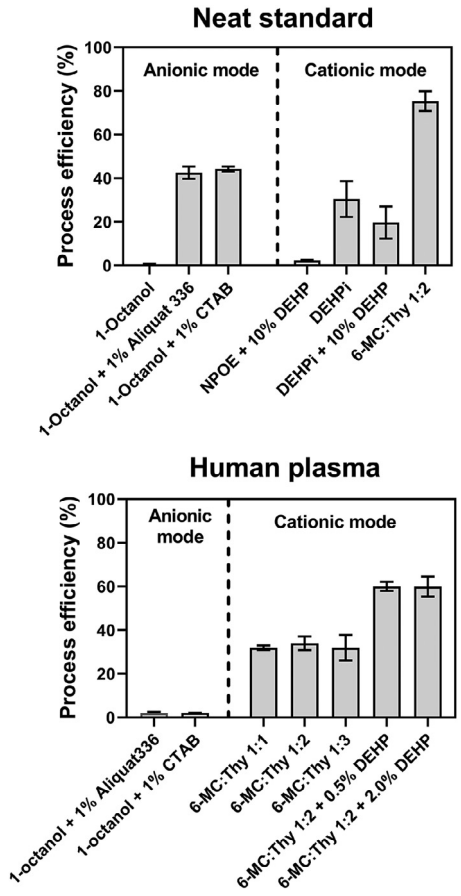

Fig. 3. Extraction process efficiency for MTX using different SLMs in either anionic or cationic extraction mode. The upper panel shows results from neat standard samples, while the lower panel shows results from spiked plasma samples. During spiking, plasma samples were diluted 1:1 with buffer. Extractions from neat standards were performed for 20 min, and 30 min for plasma samples. Error bars represent the standard deviation from triplicate extractions.

pKa values are also given in Fig. 1. The pteridine-diamine core shared by all three compounds has weakly basic properties (pKa 6.4), and the compounds can thus be zwitterionic. This makes them possible to extract as both anions and cations. During initial method development, anionic and cationic extraction modes were therefore evaluated in parallel, first from neat standard solution and subsequently from human plasma. For this screening of conditions, only MTX was extracted. Results are shown in Fig. 3. Initial experimental conditions were chosen based on a previous report using the same prototype equipment [19], with sample/donor and acceptor vials filled to 300 µL, shaking at 750 RPM, and extraction time of 20 min for neat standards and 30 min for plasma samples.

For anionic extraction, the sample was adjusted to pH 7.4 using 40 mM phosphate buffer. At this pH, the basic functionality was mostly neutralized and the acidic groups were ionized. The net charge was therefore -2. The acceptor solution was 10 mM NaOH at pH 12, which maintained analytes in anionic form once extracted into the acceptor solution. The voltage was set to 30 V. 1-Octanol has previously been suitable as SLM for EME of acidic substances, by acting as a hydrogen bond donor to interact with the negative charge(s) of analytes [10,22]. As seen in Fig. 3 (upper panel), MTX was however not extracted with 1-Octanol. The electric field was thus insufficient to transport the polar MTX through the hydrophobic SLM. Next, cationic carriers Aliquat®336 and cetyltrimethylammonium bromide (CTAB) were therefore added to the SLM in 1%w/w, based on previous experience [22–24]. The addition of carriers increased the PE from neat standard samples to 42.5% and 44.3%, respectively for Aliquat®336 and CTAB, by providing strong ionic interactions between MTX and the SLM. The conductivity of the SLM however also increased substantially, and voltage had to be reduced to 4 V to avoid generating excessive current. When applied for human plasma samples, the extraction efficiency was near-zero (Fig. 3, lower panel). The deterioration was hypothesized to be caused by quenching of the cationic carriers by anionic matrix components. This was confirmed in a subsequent experiment, where as little as 10 mM chloride in a neat standard samples was observed to quench the extraction (data not shown). Focus was therefore directed to extraction in cationic mode.

To extract MTX as a cation, both donor and acceptor solution were acidified with 100 mM formic acid at pH 2.4. At this pH, the weakly basic functionality (pKa 6.4) was protonated and the acidic residues (pKa 3.3 and 4.0) were mostly neutralized. The net charge was therefore approximately +1. Based on previous literature on EME of polar bases [10,25–28], 2-nitrophenyl octyl ether (NPOE), di(2-ethylhexyl) phosphite (DEHPi), and a deep eutectic solvent system based on 6-methylcoumarin (6-MC) and thymol (Thy) were selected as SLMs. The solvents were either tested as pure solvent or with addition of the anionic carrier di(2-ethylhexyl) phosphate (DEHP). The extraction voltage was 75 V. As seen from Fig. 3 upper panel, extractions from neat standards using NPOE and DEHPi, alone or with DEHP added, yielded low efficiency and/or high variability. As an alternative, we recently developed SLM systems for EME of polar bases based on deep eutectic solvents prepared by mixing components coumarin and thymol [28,29]. These solvents are characterized by a low intrinsic hydrophobicity, and offer strong hydrogen bond, cation-π, and π-stacking interactions, which makes them suitable for transfer of protonated polar bases. In the present work, coumarin was however replaced with 6-methylcoumarin (6-MC), as 6-MC is three-fold less soluble in water (MarvinSketch v. 21.19, ChemAxon) and therefore makes a more stable SLM. Initially, an SLM of 6-MC and thymol mixed in a 1:2 molar ratio was tested, which yielded 75.3% PE of MTX. For comparison, an extraction with coumarin:thymol 1:2 as SLM was also performed, and no significant difference in PE was found (*t*-test, $p = 0.10$, $n = 3$). When applied to plasma samples (Fig. 3, lower panel), the extraction efficiency of MTX with 6-MC:Thy 1:2 as SLM was reduced to 34%. This was expected in the presence of high levels of matrix compound. Changing the ratio of 6-MC and thymol was found not to have any impact on extraction efficiency. Instead, adding 0.5%w/w DEHP to 6-MC:Thy 1:2 could increase the efficiency to 60%, with no additional benefit of increasing the amount of DEHP. 6-MC:Thy 1:2 + 0.5% DEHP was therefore considered optimal and applied in further method development.

Optimization of extraction parameters for human plasma samples by response surface modeling

Next, other extraction parameters were optimized for human plasma samples. The goal of the optimization was to gain highest possible sensitivity of the final method. The effective analyte enrichment factor (EF) from raw plasma to final concentration in the acceptor solution was therefore chosen as response variable. With this in mind, extraction time (A), shaking rate (B), donor volume (C), acceptor volume (D), plasma dilution factor (E), extraction voltage, and donor pH were considered for optimization. Design-of-experiments (DOE) methodology was chosen as optimization strategy, since the effect of several extraction parameters were expected to be interconnected. The optimal setting of extraction time and shaking rate may for example depend on the donor and acceptor volumes. Plasma dilution factor (E) was included since greater dilution will reduce the analyte concentration, but on the other hand also may reduce matrix suppression effects. Extraction voltage was not included in the DOE methodology, because high levels of voltage in combination with certain other parameter settings may cause unforeseen problems associated with too high current levels. Instead, the voltage was set to 10 V based on initial tests. Donor pH was found optimal at pH 2.4 prior to optimization by DOE (see supplementary material, Fig. S2), and was therefore not included in the design.

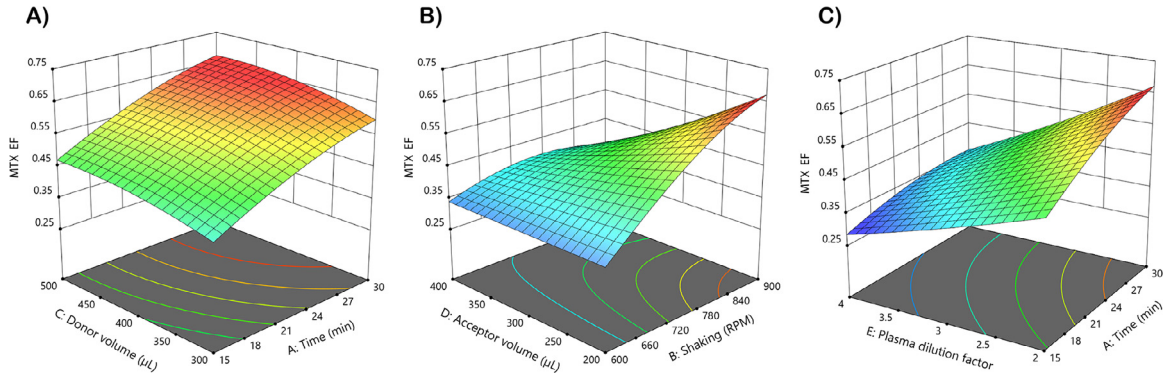

Fig. 4. Surface plots of MTX enrichment factor (EF) as function of different extraction parameters for plasma samples. For each plot, all other parameters were set to their optimal value.

Table 1
Coded and un-coded factor levels used on central composite design.

Coded level	-2	-1	0	+1	+2
A: Extraction time (min)	7.5	15	22.5	30	37.5
B: Shaking rate (RPM)	450	600	750	900	1050
C: Donor volume (µL)	200	300	400	500	600
D: Acceptor volume (µL)	100	200	300	400	500
E: Plasma dilution factor	1	2	3	4	5

A central composite design was chosen for response surface modeling. The design included 32 runs, divided into 16 factorial points ($\frac{1}{2}$ fraction), 10 star points ($\alpha = 2$), and 6 center points. Experimental runs were performed in randomized order with three simultaneous replicates per run. The average enrichment factor of the replicates was used as response variable, and the data was fitted to quadratic models. Details regarding model construction and evaluation are provided as supplementary material (#3). The factor levels used for each parameter, corresponding to the coded levels, are given in Table 1.

Due to lack of availability, 7-OH-MTX was not included in the optimization study. Results for MTX and DAMPA were however highly correlated, and 7-OH-MTX was therefore assumed to have similar response to changes in factor settings. Surface plots for MTX are shown in Fig. 4.

Analysis of the surface plots provided several important insights. For example, increasing the donor volume from 300 µL to 500 µL only gave a small, and not proportional, increase in EF. This was independent of extraction time (Fig. 4A). The process efficiency (though not EF) thus decreased with increasing donor volume. This may be caused by a greater absolute amount of plasma in the donor solution Fig. 4.B shows the effect of acceptor volume and shaking rate. In the current vial-based EME format, proper shaking is highly important to ensure an optimal contact between the aqueous solutions and the SLM. At an acceptor volume of 400 µL the optimal shaking rate was 750 RPM. With 200 µL in the acceptor, the optimum however shifted towards a higher shaking rate of approximately 900 RPM. The optimal shaking rate, on the other hand, was independent of the donor volume. From the models, it was predicted that a higher EF should be obtained by reducing the acceptor volume below 200 µL. When tested, the contact between SLM and acceptor solution however became inadequate, and extractions became less efficient and less repeatable.

Fig. 4C shows the combined effect of extraction time and plasma dilution factor. As seen, the highest EF was obtained with a 2-fold dilution. Greater dilution did thus not reduce matrix suppression effects proportionally to the dilution, though some improvement was observed. The dilution factor also affected the extraction kinetics. As such, with 4-fold dilution the system came to steady-state after approximately 25 min, whereas steady-state for 2-fold dilution could be estimated to 35 min. It

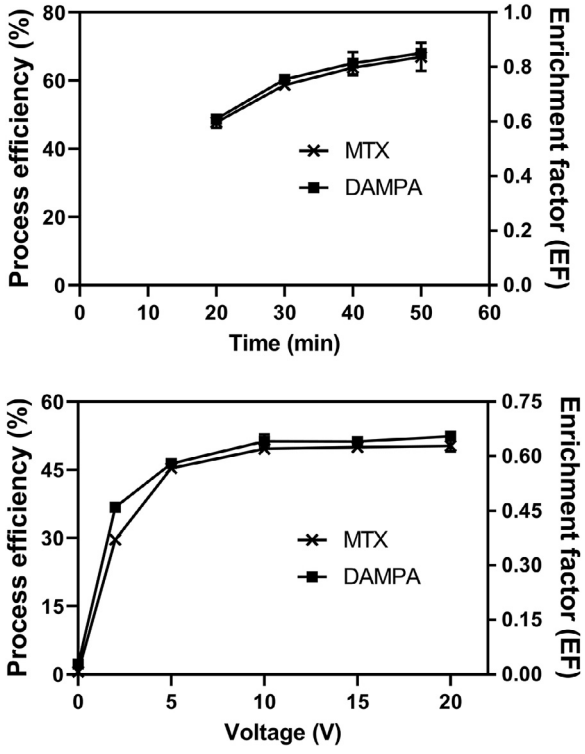

Fig. 5. Upper panel) Process efficiency and EF of MTX and DAMPA as function of time. Voltage was set to 10 V. Lower panel) Process efficiency and EF of MTX and DAMPA as function of voltage. Extraction time was 20 min. Error bars represent the standard deviation for triplicate experiments. Missing error bars are due to very low variability of replicates.

may be noted that decreasing the dilution factor to less than 2-fold was predicted to give higher EFs. This was however considered impractical since acidification of plasma to pH 2.4 required some dilution.

Based on these interpretations, the parameters of the final method were set to 900 RPM shaking, 500 µL donor solution, 200 µL acceptor solution, and 2-fold dilution of plasma. Acidification at this dilution factor was performed by 1:1 mixing of plasma with 250 mM phosphoric acid. Prior to defining the extraction time, the effect of extending the extraction beyond that of the optimization experiment was evaluated. As seen in Fig. 5 (upper panel), a minor increase in efficiency could be gained from 30 min to 50 min. However, to maintain throughput the extraction time was set to 30 min in the final method. Lastly, the extraction voltage was optimized for the final method. As seen in Fig. 5 (lower panel), efficiency was improved from 0 to 10 V, after which no further gains were

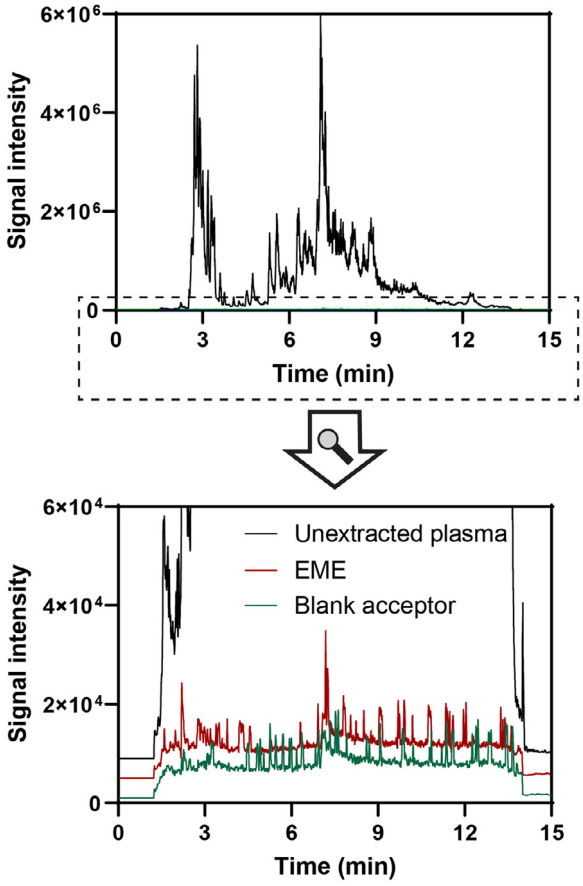

Fig. 6. Representative LC-MS/MS chromatograms of phospholipids (m/z 184 → 184) determined in unextracted plasma (black trace), in the acceptor solution after EME (red trace), and in a blank acceptor solution prior to extraction (green trace). The unextracted plasma was protein-precipitated prior to extraction, as described in Section "*Protein precipitation*".

made. 10 V was therefore used in the final method. At these conditions, the process operated robustly with a low extraction current of 10 μA in average during 30 min. A representative profile of the current during extraction is provided as supplementary material (Fig. S4).

Method evaluation

Phospholipid cleanup

Phospholipids are matrix components in plasma that are abundantly present, and are notorious for causing ion suppression if co-eluting with analytes in LC-MS. If not removed from samples, their high hydrophobicity further leads to build-up and fouling on reversed-phase columns. Previously, other SLM solvents for EME, such as NPOE, have been found to provide excellent clean-up of phospholipids [18,19,30]. Deep eutectic solvents based on 6-MC, coumarin, and thymol have however not previously been evaluated in the same manner, until now. To determine phospholipids, an in-source fragmentation LC-MS/MS method was utilized (Section "*Phospholipids*"). For this, phosphatidylcholines, lyso-phosphatidylcholines, and sphingomyelins were fragmented into a common fragment at m/z 184, which accounted for the majority of phospholipids in plasma [21]. As control sample, plasma was protein-precipitated (Section "*Protein precipitation*") and diluted in acceptor solution to match the plasma concentration used for EME. The obtained signals could thus be compared directly Fig. 6. shows the resulting chromatographic traces. As seen, the chromatogram of the acceptor solution after EME was very similar to that of a blank acceptor solution, which represented >100-fold reduction in signal intensity compared to unextracted plasma. The EME method based on a deep eutectic solvent SLM thus provided similar high clean-up efficiency of phospholipids as previously reported SLMs.

Analytical performance

Finally, the analytical performance of the developed method was evaluated. At this point, 7-OH-MTX was included in the method without any additional optimization. An internal standard (IS) of MTX-D_3 was likewise added. Results of the validation are given in

Table 2. The current data provides a basic evaluation of the analytical performance, and is not intended as a full validation. In summary, calibration curves were demonstrated as linear with $R^2 \geq 0.9952$, and accuracy was within 98–121% for all analytes at both LLOQ and ULOQ. No ion suppression effects were found for either analyte; a slight ion enhancement effect (111%) was however observed for DAMPA. Repeatability of the method was <15%, expect at LLOQ where RSD for MTX and DAMPA were 27.6% and 21.1%, respectively. At ULOQ, less than 5% RSD was however obtained for all analytes. Despite the analyte concentration after extraction was slightly decreased (EF = 0.29–0.67), the detection limits were within biologically relevant concentration levels [31,32].

Comparison to other methods and greenness evaluation

The analytical performance of the proposed EME-LC-MS/MS method was compared to previous methods for determination of MTX and its metabolites, as seen in Table 3. Several previous microextraction methods have been applied for MTX, however, to the best of our knowledge none have determined 7-OH-MTX and DAMPA simultaneously. An SPE-based method for simultaneous determination of all analytes was therefore also included in the comparison. As seen, the analytical performance of the EME-LC-MS/MS method was comparable to previous methods regarding sensitivity, precision, and consumed amount of sample. When comparing methods, the environmental impact and safety of methods (or greenness) are however also important to consider. A greenness evaluation was therefore performed based on the very recently introduced metric tool, Analytical Greenness Metric for Sample Preparation (AGREEprep) [33]. AGREEprep was chosen for the comparison because it gives prominence to the sample preparation step of the analysis.

For a detailed discussion on AGREEprep we refer to Wojnowski et al [33].. Briefly outlined, a sample preparation method is assigned a score from 0 to 1 (1 representing the ideal system) for each of the 10 principles of Green Sample Preparation [38]. The categories include, among others, consumption of hazardous reagents, waste generation, sample amount, throughput, and energy consumption. The assessment produces a pictogram summarizing the overall greenness of the method. For comparison, a DLLME-based method [35] was selected as another microextraction method, while an SPE-method [34] was selected to represent a conventional sample preparation strategy. Details on the input used to score each sub-category are provided as Supplementary material (#5) Fig. 7. shows the pictograms for the three methods. The EME method had the highest overall score of 0.61, and excelled in the three impact categories (#2 amount of hazardous substance, #4 waste generation, and #8 energy consumption). It should be mentioned that the conductive vials were reused and thus did not count as waste. If the vials instead are considered as single-use the overall score drops to 0.56. Surprisingly, the DLLME-based method scored lower than a conventional SPE method. This was primarily due to a low throughput (criterion #6) and higher energy consumption (#8) from use of vortexer, magnetic stirrer, and centrifuge. EME was on the other hand superior to SPE because less hazardous substance was used, and less waste was generated. The assessment thus supports the argument that EME is principally a green sample preparation and extraction technique.

Table 2
Validation data for EME-LC-MS/MS method for MTX, 7-OH-MTX, and DAMPA spiked into human plasma. All concentrations are gives as ng mL^{-1}, and all extractions were performed in quadruplicate. Calibration curves were weighed by 1/x. Limit of detection (LOD) and lower limit of quantitation (LLOQ) were defined by the concentrations with a signal-to-noise ratio of 3 and 10, respectively. ULOQ represents the upper limit of quantitation (i.e. upper linear range). MTX-D_3 was spiked to a final concentration of 500 ng mL^{-1} for all samples.

Analyte	Linear range	R^2	EF*	R (%)*	LOD	LLOQ	ME ± SD (%)*	Accuracy (%)		Repeatability (%)	
								LLOQ	ULOQ	LLOQ	ULOQ
MTX	0.4–700	0.9993	0.67	53	0.1	0.4	104 ± 2	108	99	27.6	2.8
7-OH-MTX	2–2000	0.9994	0.29	23	0.7	2.0	111 ± 2	105	101	7.2	4.4
DAMPA	0.4–200	0.9952	0.67	53	0.1	0.4	103 ± 2	121	98	21.1	4.9

* determined at 700 ng mL^{-1}.

Table 3
Comparison of method performance data for determination of MTX and its metabolites from biological fluids. Abbreviations: SERS – surface-enhanced Raman spectroscopy, DLLME – dispersive liquid-liquid microextraction, dSPE – dispersive solid-phase extraction.

Analytical method	Analytes	Matrix	Sample volume	Linear range (ng mL^{-1})	%RSD	*R* (EF)	Refs.
SPE-LC-MS/MS	MTX	Saliva	1.4 mL	2–2000	1.4–5.9%	90% (0.9)	[34]
DLLME-LC-UV	MTX	Whole blood	0.4 mL	0.1–150	2.6%	91% (8.1)	[35]
dSPE-SERS	MTX	Urine	0.02 mL	20–300	11–19%	–	[36]
SPE-LC-MS/MS	MTX	Plasma	0.1 mL	1–2500	1.4–1.8%	90% (1.2)	[37]
	7-OH-MTX			4–9870	6.7–12.3%	84% (1.1)	
	DAMPA			1–2500	0.8–3.9%	90% (1.2)	
EME-LC-MS/MS	MTX	Plasma	0.25 mL	0.4–700	2.8–27.6%	53% (0.7)	This work
	7-OH-MTX			2–2000	4.4–7.2%	23% (0.3)	
	DAMPA			0.4–200	4.9–21.1%	53% (0.7)	

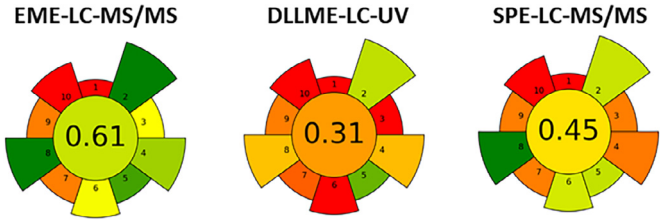

Fig. 7. AGREEprep pictograms for EME-LC-MS/MS (this work), DLLME-LC-UV [35], and SPE-LC-MS/MS [34]. The score in the pictogram center represents the overall greenness, while the color and size of the surrounding boxes represent the score and assigned weight of each category, respectively. The pictograms were generated using software v. 0.9 available from https://mostwiedzy.pl/pl/wojciech-wojnowski,174235-1/agreeprep.

Conclusion

The aim of the present work was to identify suitable EME systems for extraction of polar and zwitterionic substances in human plasma, using MTX and its metabolites as model analytes. Initially, MTX was attempted extracted as an anion, which yielded moderate efficiency from neat standards, using 1-Octanol and cationic carriers. The extraction was however entirely suppressed by matrix components in the plasma. Extracting MTX as a cation was on the other hand successful from plasma, using an SLM composed of the deep eutectic solvent 6-methylcoumarin and thymol (1:2 molar ratio) with 0.5% DEHP added. The success of this solvent was attributed to the high availability of hydrogen bonding, ionic, and π-type interactions, which enabled MTX to overcome the hydrophobicity of the SLM. From these results, we conclude that there currently is a performance gap for EME of polar anions and cations, and more research should be undertaken to improve the matrix tolerance for anionic substances.

Extraction parameters for a prototype of a commercial EME device were optimized to gain the highest possible sensitivity of the analysis. Key observations were that the optimal shaking rate was dependent on the volume of acceptor solution, and highest sensitivity was gained with minimal dilution (2-fold) of plasma. The latter indicated that the system had a good tolerance to high levels of matrix components. Analytical performance data of the proposed EME-LC-MS/MS method were acceptable, and demonstrated the feasibility of using EME as selective sample preparation for MTX and its metabolites in human plasma. Once the equipment is commercially available, EME methods may be transferred directly between laboratories without requiring re-optimization. For the current contribution, it may be noted that the deep eutectic solvent mixture represents a limitation in this aspect, since it is not a common commercially available solvent. Research activity with deep eutectic solvent is however strong, and we expect some solvents eventually will become commercially available.

The proposed system also represents green extraction chemistry, which was confirmed by assessment according to the AGREEprep metric tool. The final SLM was based on a deep eutectic solvent with constituents of natural original. Such solvents are generally accepted to be environmentally friendly [39–41], and the very small volume (10 µL) applied to the SLM further highlights the greenness of the extraction chemistry. It may be argued that hyphenation to LC-MS reduces the total greenness of the method. However, though this instrumentation was necessary to distinguish MTX and its metabolites, future methods based on the current conceptual EME system may apply other and more environmentally friendly detection approaches.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Coauthors Anne Oleide Hay and Roger Trones are working in the company Extraction Technologies Norway, and this company is developing EME technology towards commercialization. All other authors declare that they have no known competing financial interests.

Acknowledgment

This work was supported by the Research Council of Norway (NFR 310086).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sampre.2022.100011.

References

[1] N. Drouin, S. Rudaz, J. Schappler, Sample preparation for polar metabolites in bioanalysis, Analyst 143 (1) (2018) 16–20, doi:10.1039/C7AN01333G.

[2] S. Knoll, T. Rösch, C. Huhn, Trends in sample preparation and separation methods for the analysis of very polar and ionic compounds in environmental water and biota samples, Anal. Bioanal. Chem. 412 (24) (2020) 6149–6165, doi:10.1007/s00216-020-02811-5.

[3] M.F. Silva, C. Ribeiro, V.M.F. Gonçalves, M.E. Tiritan, Á. Lima, Liquid chromatographic methods for the therapeutic drug monitoring of methotrexate as clinical decision support for personalized medicine: a brief review, Biomed. Chromatogr. 32 (5) (2018), doi:10.1002/bmc.4159.

[4] A.F. Hawwa, A. AlBawab, M. Rooney, L.R. Wedderburn, M.W. Beresford, J.C. McElnay, Methotrexate polyglutamates as a potential marker of adherence to long-term therapy in children with juvenile idiopathic arthritis and juvenile dermatomyositis: an observational, cross-sectional study, Arthritis Res. Ther. 17 (2015) 295 -295, doi:10.1186/s13075-015-0814-z.

[5] H. Tian, B.N. Cronstein, Understanding the mechanisms of action of methotrexate: implications for the treatment of rheumatoid arthritis, Bull. NYU Hosp. Jt. Dis. 65 (3) (2007) 168–173.

[6] F. Karami, S. Ranjbar, Y. Ghasemi, M. Negahdaripour, Analytical methodologies for determination of methotrexate and its metabolites in pharmaceutical, biological and environmental samples, JPA 9 (6) (2019) 373–391, doi:10.1016/j.jpha.2019.06.001.

[7] Y. Yamini, M. Rezazadeh, S. Seidi, Liquid-phase microextraction – the different principles and configurations, Trends Anal. Chem. 112 (2019) 264–272, doi:10.1016/j.trac.2018.06.010.

[8] N.C. Dominguez, A. Gjelstad, A.M. Nadal, H. Jensen, N.J. Petersen, S.H. Hansen, K.E. Rasmussen, S. Pedersen-Bjergaard, Selective electromembrane extraction at low voltages based on analyte polarity and charge, J. Chromatogr. A 1248 (2012) 48–54, doi:10.1016/j.chroma.2012.05.092.

[9] N. Drouin, P. Kubáň, S. Rudaz, S. Pedersen-Bjergaard, J. Schappler, Electromembrane extraction: overview of the last decade, Trends Anal. Chem. 113 (2019) 357–363, doi:10.1016/j.trac.2018.10.024.

[10] L.V. Eie, S. Pedersen-Bjergaard, F.A. Hansen, Electromembrane extraction of polar substances – status and perspectives, J. Pharm. Biomed. Anal. 207 (2022) 114407, doi:10.1016/j.jpba.2021.114407.

[11] S. Pedersen-Bjergaard, K.E. Rasmussen, Electrokinetic migration across artificial liquid membranes: new concept for rapid sample preparation of biological fluids, J. Chromatogr. A 1109 (2) (2006) 183–190, doi:10.1016/j.chroma.2006.01.025.

[12] A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, Electrokinetic migration across artificial liquid membranes: tuning the membrane chemistry to different types of drug substances, J. Chromatogr. A 1124 (1–2) (2006) 29–34, doi:10.1016/j.chroma.2006.04.039.

[13] N.J. Petersen, H. Jensen, S.H. Hansen, S.T. Foss, D. Snakenborg, S. Pedersen-Bjergaard, On-chip electro membrane extraction, Microfluid. Nanofluid. 9 (4) (2010) 881–888, doi:10.1007/s10404-010-0603-6.

[14] M. Kamankesh, A. Mollahosseini, A. Mohammadi, S. Seidi, Haas in grilled meat: determination using an advanced lab-on-a-chip flat electromembrane extraction coupled with on-line HPLC, Food Chem. 311 (2020) 125876, doi:10.1016/j.foodchem.2019.125876.

[15] F.A. Hansen, N.J. Petersen, J.P. Kutter, S. Pedersen-Bjergaard, Electromembrane extraction in microfluidic formats, J. Sep. Sci. 45 (1) (2022) 246–257, doi:10.1002/jssc.202100603.

[16] L.E.E. Eibak, K.E. Rasmussen, E.L. Øiestad, S. Pedersen-Bjergaard, A. Gjelstad, Parallel electromembrane extraction in the 96-well format, Anal. Chim. Acta 828 (2014) 46–52, doi:10.1016/j.aca.2014.04.038.

[17] N. Drouin, J.F. Mandscheff, S. Rudaz, J. Schappler, Development of a new extraction device based on parallel-electromembrane extraction, Anal. Chem. 89 (12) (2017) 6346–6350, doi:10.1021/acs.analchem.7b01284.

[18] F.A. Hansen, S. Pedersen-Bjergaard, Electromembrane extraction of streptomycin from biological fluids, J. Chromatogr. A 1639 (2021) 461915, doi:10.1016/j.chroma.2021.461915.

[19] T.G. Skaalvik, E.L. Øiestad, R. Trones, S. Pedersen-Bjergaard, S. Hegstad, Determination of psychoactive drugs in serum using conductive vial electromembrane extraction combined with UHPLC-MS/MS, J. Chromatogr. B 1183 (2021) 122926, doi:10.1016/j.jchromb.2021.122926.

[20] S. Nojavan, A. Pourahadi, S.S.H. Davarani, A. Morteza-Najarian, M.B. Abbassi, Electromembrane extraction of zwitterionic compounds as acid or base: comparison of extraction behavior at acidic and basic pHs, Anal. Chim. Acta 745 (2012) 45–52, doi:10.1016/j.aca.2012.07.044.

[21] J.L. Little, M.F. Wempe, C.M. Buchanan, Liquid chromatography–mass spectrometry/mass spectrometry method development for drug metabolism studies: examining lipid matrix ionization effects in plasma, J. Chromatogr. B 833 (2) (2006) 219–230, doi:10.1016/j.jchromb.2006.02.011.

[22] C.X. Huang, A. Gjelstad, S. Pedersen-Bjergaard, Organic solvents in electromembrane extraction: recent insights, Rev. Anal. Chem. 35 (4) (2016) 169–183, doi:10.1515/revac-2016-0008.

[23] M.H. Koruni, H. Tabani, H. Gharari, A.R. Fakhari, An all-in-one electro-membrane extraction: development of an electro-membrane extraction method for the simultaneous extraction of acidic and basic drugs with a wide range of polarities, J. Chromatogr. A 1361 (2014) 95–99, doi:10.1016/j.chroma.2014.07.075.

[24] M.S. Restan, M.E. Pedersen, H. Jensen, S. Pedersen-Bjergaard, Electromembrane extraction of unconjugated fluorescein isothiocyanate from solutions of labeled proteins prior to flow induced dispersion analysis, Anal. Chem. 91 (10) (2019) 6702–6708, doi:10.1021/acs.analchem.9b00730.

[25] C. Huang, K.F. Seip, A. Gjelstad, S. Pedersen-Bjergaard, Electromembrane extraction of polar basic drugs from plasma with pure bis(2-ethylhexyl) phosphite as supported liquid membrane, Anal. Chim. Acta 934 (2016) 80–87, doi:10.1016/j.aca.2016.06.002.

[26] F.A. Hansen, P. Kubáň, E.L. Øiestad, S. Pedersen-Bjergaard, Electromembrane extraction of highly polar bases from biological samples – deeper insight into bis(2-ethylhexyl) phosphate as ionic carrier, Anal. Chim. Acta 1115 (2020) 23–32, doi:10.1016/j.aca.2020.04.027.

[27] H.B. Piskáčková, E. Leere Øiestad, N. Váňová, J. Lengvarská, P. Štěrbová-Kovaříková, S. Pedersen-Bjergaard, Electromembrane extraction of anthracyclines from plasma: comparison with conventional extraction techniques, Talanta 223 (Pt 2) (2020) 121748, doi:10.1016/j.talanta.2020.121748.

[28] F.A. Hansen, E. Santigosa-Murillo, M. Ramos-Payán, M. Muñoz, E.L. Øiestad, S. Pedersen-Bjergaard, Electromembrane extraction using deep eutectic solvents as the liquid membrane, Anal. Chim. Acta 1143 (2021) 109–116, doi:10.1016/j.aca.2020.11.044.

[29] F.A. Hansen, S. Tirandaz, S. Pedersen-Bjergaard, Selectivity and efficiency of electromembrane extraction of polar bases with different liquid membranes—link to analyte properties, J. Sep. Sci. 44 (13) (2021) 2631–2641, doi:10.1002/jssc.202100167.

[30] L. Vårdal, A. Gjelstad, C. Huang, E.L. Øiestad, S. Pedersen-Bjergaard, Efficient discrimination and removal of phospholipids during electromembrane extraction from human plasma samples, Bioanalysis 9 (8) (2017) 631–641, doi:10.4155/bio-2017-0027.

[31] R.D. Hilgers, E.S. Newlands, R. Hoffman, H. Mitchell, K.D. Bagshawe, Correlation of plasma methotrexate concentration with human chorionic gonadotropin and therapeutic response to low-dose methotrexate-citrovorum factor in low-medium-risk gestational trophoblastic tumors, Gynecol. Oncol. 41 (2) (1991) 117–122, doi:10.1016/0090-8258(91)90269-B.

[32] N. Fabresse, B. Devictor, C. Pissier, H. Chambost, B. Lacarelle, G. Michel, C. Solas, Plasma 7-hydroxymethotrexate levels versus methotrexate to predict delayed elimination in children receiving high-dose methotrexate, Ther. Drug Monit. 40 (1) (2018), doi:10.1097/FTD.0000000000000445.

[33] W. Wojnowski, M. Tobiszewski, F. Pena-Pereira, E. Psillakis, AGREEprep – analytical greenness metric for sample preparation, Trends Anal. Chem. 149 (2022) 116553, doi:10.1016/j.trac.2022.116553.

[34] I. Rodin, A. Braun, A. Stavrianidi, O. Shpigun, A validated LC–MS/MS method for rapid determination of methotrexate in human saliva and its application to an excretion evaluation study, J. Chromatogr. B 937 (2013) 1–6, doi:10.1016/j.jchromb.2013.07.026.

[35] R. Akramipour, N. Fattahi, M.R. Golpayegani, Sensitive determination of methotrexate in plasma of children with acute leukemia using double-solvent supramolecular systemas a novel extractant for dispersive liquid-liquid microextraction, J. Chromatogr. B 1171 (2021) 122628, doi:10.1016/j.jchromb.2021.122628.

[36] N.E. Markina, A.M. Zakharevich, A.V. Markin, Determination of methotrexate in spiked human urine using SERS-active sorbent, Anal. Bioanal. Chem. 412 (28) (2020) 7757–7766, doi:10.1007/s00216-020-02932-x.

[37] M.S. Roberts, N.S. Selvo, J.K. Roberts, V.M. Daryani, T.S. Owens, K.E. Harstead, A. Gajjar, C.F. Stewart, Determination of methotrexate, 7-hydroxymethotrexate, and 2,4-diamino-N10-methylpteroic acid by LC–MS/MS in plasma and cerebrospinal fluid and application in a pharmacokinetic analysis of high-dose methotrexate, J. Liq. Chromatogr. Relat. Technol. 39 (16) (2016) 745–751, doi:10.1080/10826076.2016.1243558.

[38] Á.I. López-Lorente, F. Pena-Pereira, S. Pedersen-Bjergaard, V.G. Zuin, S.A. Ozkan, E. Psillakis, The ten principles of green sample preparation, Trends Anal. Chem. 148 (2022) 116530, doi:10.1016/j.trac.2022.116530.

[39] A. Paiva, R. Craveiro, I. Aroso, M. Martins, R.L. Reis, A.R.C. Duarte, Natural deep eutectic solvents – solvents for the 21st century, ACS Sustain. Chem. Eng. 2 (5) (2014) 1063–1071, doi:10.1021/sc500096j.

[40] P. Makoś, E. Słupek, J. Gębicki, Hydrophobic deep eutectic solvents in microextraction techniques–a review, Microchem. J. 152 (2020) 104384, doi:10.1016/j.microc.2019.104384.

[41] E. Carasek, G. Bernardi, D. Morelli, J. Merib, Sustainable green solvents for microextraction techniques: recent developments and applications, J. Chromatogr. A 1640 (2021) 461944, doi:10.1016/j.chroma.2021.461944.