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Electromembrane extraction of anthracyclines from plasma: Comparison with conventional extraction techniques

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ABSTRACT

Electromembrane extraction (EME) of the polar zwitterionic drugs, anthracyclines (ANT, doxorubicin, daunorubicin and its metabolite daunorubicinol), from rabbit plasma was investigated. The optimized EME was compared to conventional sample pretreatment techniques such as protein precipitation (PP) and liquid-liquid extraction (LLE), mainly in terms of extraction reliability, recovery and matrix effect. In addition, phospholipids profile in the individual extracts was evaluated. The extracted samples were analyzed using UHPLC-MS/MS with electrospray ionization in positive ion mode. The method was validated within the concentration range of 0.25–1000 ng/mL for all tested ANT. Compared with PP and LLE, the EME provided high extraction recovery (more than 80% for all ANT) and excellent sample clean-up (matrix effect were 100 ± 10% with RSD values lower than 4% for all ANT). Furthermore, only negligible amounts of phospholipids were detected in the EME samples. Finally, practical applicability of EME was proved by analysis of plasma samples taken from a pilot *in vivo* study in rabbits. Consistent results were obtained when using both EME and LLE to extract the plasma prior to the analysis, which further confirmed high reliability of EME. This study clearly showed that EME is a simple, rapid, repeatable technique for extraction of ANT from plasma and it is an up to date alternative to routine conventional extraction techniques.

1. Introduction

Electromembrane extraction (EME) is a hybrid microextraction technique combining liquid-liquid extraction (LLE) and electrophoresis, and was firstly introduced in 2006 for extraction of non-polar basic drugs [1]. This technique has been originally developed from hollow fiber liquid-phase microextraction [1], it has been optimized over the years and finally adapted to 96-well format, allowing high throughput operation and automation [2]. EME is performed in a three-phase system where the analyte is transported from an aqueous donor phase through the water immiscible organic supported liquid membrane (SLM) to the aqueous acceptor phase. The electrical potential is applied across the SLM, and this facilitates electrokinetic migration of charged compounds. To ensure ionic character of the analytes, pH of donor and acceptor phases has to be adjusted accordingly. Basic analytes are extracted as cations with the anode located in donor (sample) phase and with the cathode placed in the acceptor phase. Contrary, acidic analytes are extracted as anions with reversed polarity. The SLM, immobilized in the pores of a hydrophobic polymeric membrane, form an efficient barrier for many matrix components and background ions from biological samples [3].

EME has gained attention over the last decade, due to the unique extraction properties and high selectivity and efficiency. Selectivity of the extraction is determined by the direction and magnitude of electrical potential, character of SLM and pH of donor and acceptor phases [4,5]. Efficient sample clean up obtained by EME allow direct injection of

Abbreviations: EME, electromembrane extraction; SLM, supported liquid membrane; ANT, anthracyclines; DOX, doxorubicin; DAU, daunorubicin; DAUol, daunorubicinol; PP, protein precipitation; LLE, liquid-liquid extraction; NPOE, 2-nitrophenyl octyl ether; DEHP, Bis(2-ethylhexyl) phosphate; DEHPi, Bis(2-ethylhexyl) phosphite; ME, matrix effect; EMA, European Medicine Agency; PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine.

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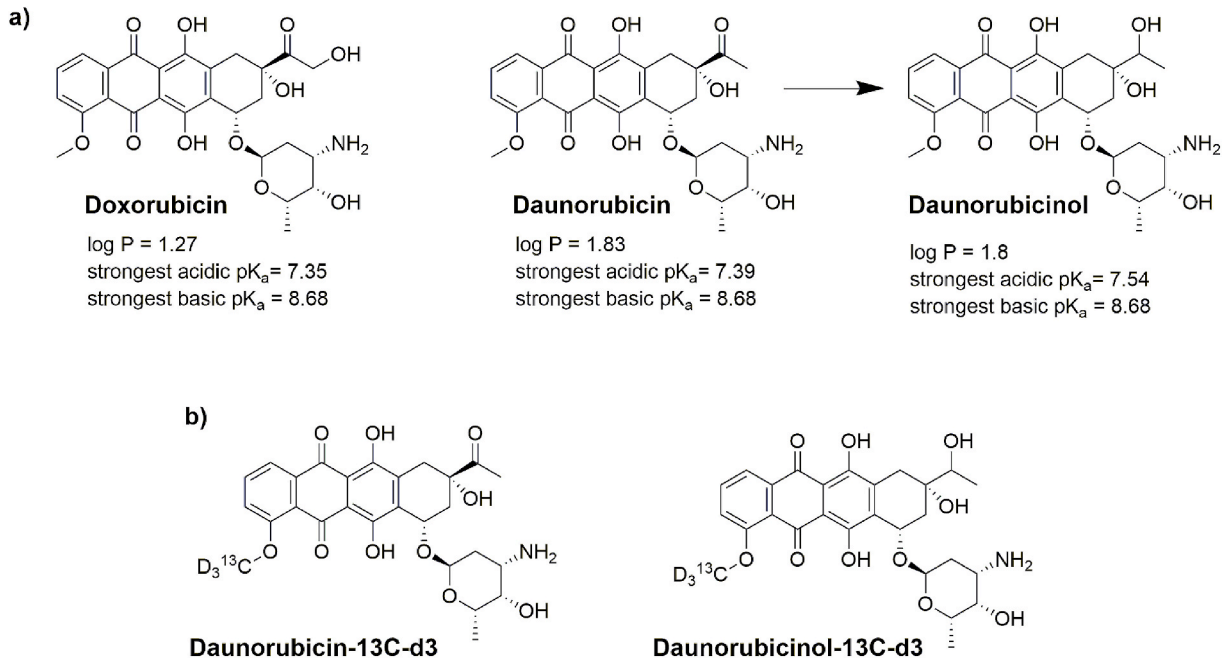

Fig. 1. Chemical structures and the selected chemical constants of (a) the tested anthracyclines and (b) internal standards. The log P values were obtained from the database pubchem.com and pKa values were calculated by chemicalize.com.

extract to a wide range of analytical instruments. Furthermore, EME provides direct enrichment of the sample according to the volume difference between the sample and acceptor phase. At the end, the SLM consists of only few microliters (<5 μL) of an organic solvent, which makes EME a green sample treatment technique.

Anthracyclines (ANT) are one of the most effective anticancer drugs clinically used for the treatment of hematologic malignancies and several solid tumors (e.g. breast, ovarian and gastric carcinoma, bone sarcoma, etc.) [6,7]. Despite the widespread use in the clinical practice, the ANT therapy is in addition to the common side effects of anticancer treatment, burdened with dose-related cardiotoxicity [8]. The reduction of serious side effects is still focus of a number of experimental investigations [9–12].

Doxorubicin (DOX) and daunorubicin (DAU) belongs to the most clinically important ANT. Their chemical structure is made up of an aglycone ring coupled to an amino sugar (Fig. 1a) [13]. Thanks to their amphoteric character, ANT can bear both positive charge by protonation of the only amino group (basic pKa 8.68) and single or multiple negative charges by deprotonation of the hydroxy groups (most acidic pKa ~7.4, all pKa values were calculated by ACD/Labs Software V11.02). These compounds are polar, as log P is 1.27 and 1.83 for DOX and DAU, respectively (www.pubchem.com). The major metabolite - C13-dihydro derivative is formed by aldoketoreductases (doxorubicinol – DOXol, daunorubicinol – DAUol, Fig. 1a). Moreover, these hydroxy-metabolites are particularly important due to their potential association with cardiotoxicity [13,14].

Thanks to the high clinical significance of ANT, a number of analytical methods (including spectrophotometry, chromatography and electrophoresis) has been published so far for their assay in different biological materials [15–17]. However, the vast majority of the analytical protocols utilized conventional extraction techniques including protein precipitation (PP) [16,18,19], LLE [13,17,20,21] and solid-phase extraction [22–24]. These techniques have certain disadvantages e.g a large consumption of both sample and organic solvents, dilution of sample, multiple operating steps, long duration time, etc. Despite many possible advantages of microextractions, to the best of our knowledge, only two studies were published so far utilizing a miniaturized technique for extraction of ANT. Souza et al. focused on dispersive liquid-liquid microextraction of ANT from hospital effluent [25], and Roszkowska et al. applied a solid-phase microextraction technique for *in vivo* quantification of DOX in lung tissue [26].

The aim of the current research was to test EME for extraction of zwitterionic anthracyclines from rabbit plasma, and based on this experience to develop and optimize a 96-well EME method in combination with UHPLC-MS/MS. Furthermore, the entire method was validated according to EMA guidelines, and compared with standard methods based on LLE and PP. This work represents novelty on two different levels; (1) this is the first time EME is reported for anthracyclines, and (2) this is the first time EME is fully validated according to EMA guidelines and compared with fully validated methods based on LLE and PP under comparable experimental conditions. The reported data are relevant for scientists interested in bioanalysis of anthracyclines, but they also give important general information on how to handle zwitterionic analytes in EME, and general information on data quality when EME is operated with 96-well technology of industrial standard.

2. Materials and methods

2.1. Chemicals

Methanol, acetonitrile, formic acid (98–100%), acetic acid, ammonium acetate, ammonium hydroxide solution (≥25% in H_2O) (all LC-MS grade); hydrochloric acid (36.5–38%), sodium hydroxide (97%), 2-nitrophenyl octyl ether (NPOE), bis(2-ethylhexyl) phosphate (DEHP), and bis(2-ethylhexyl) phosphite (DEHPi) were purchased from Merck (Darmstadt, Germany). Chloroform (stabilized with ~1% ethanol) was obtained from Penta Chemicals (Prague, Czech Republic). Doxorubicin hydrochloride (DOX), daunorubicin hydrochloride (DAU) and daunorubicinol hydrochloride (mixture of diastereomers) (DAUol) were purchased from LGC standards (Lomianki, Poland). The isotopic labeled internal standards daunorubicin-13C-d3 (>85%) (13C-d3-DAU) and daunorubicinol-13C-d3 (mixture of diastereomers) (13C-d3-DAUol) were obtained from Toronto Research Chemicals (North York, Canada). Milli-Q water was provided by a Millipore purification system (Merck-Millipore, Darmstadt, Germany). Blank rabbit plasma (with heparin as

an anti-coagulant) was purchased from a commercial source (ITEST plus s.r.o., Hradec Králové, Czech Republic). Real plasma samples were provided by the Faculty of Medicine in Hradec Králové from an *in vivo* pharmacokinetic study in rabbits approved and supervised by the Animal Welfare Committee of the Faculty of Medicine in Hradec Králové (Charles University, Czech Republic). Blank and real plasma samples were stored at −20 and −80 °C, respectively.

2.2. Preparation of solutions and buffers

The stock solutions of the analytes (DAU, DOX, DAUol) and internal standards (13C-d3-DAU and 13C-d3-DAUol) were prepared at concentrations of 2, 1, 0.5, 0.25 and 0.1 mg/mL, respectively. Relevant amount of substance was dissolved in methanol (DOX, DAU, 13C-d3- DAU) or in a methanol/water mixture (1:1 v/v, DAUol, 13C-d3- DAUol). The stock solutions were stable up to three months when stored at −20 °C, data not shown. Working solutions of analytes (0.00625–25.0 μg/mL) and internal standards (2.5 μg/mL) were prepared by appropriate dilution of the stock solutions with the same solvent, stored at −20 °C and used up to one month.

To adjust pH of donor phase different buffers were prepared as follows: 50 mM formic acid was mixed with 0.2 M sodium hydroxide (pH 3.0 – buffer-I); formic acid was mixed with ammonium acetate, both 0.2 M (pH 3.0 – buffer-II) and 0.2 M acetic acid was neutralized with 0.2 M ammonium acetate (pH 4.0 – buffer-III).

2.3. Electromembrane extraction

The EME equipment consisted of conductive stainless steel 96-well donor plate (made in-house), 96-well filter plate (PVDF, 0.45 μm, Millipore Ltd., Carrigtwohill, Ireland) serving as both a support for the liquid membrane and as acceptor reservoir, and aluminum electrodes lid (made in-house). The whole equipment is shown in Supplementary materials Figure S1. Prior to the extraction, 3 μL of the organic solvent were pipetted on the outer part of the filters of the acceptor plate. Then the wells of conductive donor plate were filled in with sample (200–250 μL) and tightly closed by the acceptor plate. Finally, the acceptor phase (50 or 100 μL) was added to the wells of the acceptor plate, and the system was covered by the acceptor electrodes lid. The donor plate and the acceptor lid were connected to the anode and cathode of the power supply (model ES 0300–0.45, Delta Elektronika BV, Zierikzee, The Netherlands), respectively. The extraction process was initiated by application of the voltage (15–75 V) and agitation of the EME equipment (850–1050 rpm) on Vibramax 100 (Heidolph, Kellheim, Germany). The extraction-current in the EME system was monitored using a Fluke 287 multimeter (Fluke, Everett, WA, USA). After extraction, the acceptor phase was transferred to a micro-insert containing vial (0.2 mL, VWR International, Radnor, PA, USA), and analyzed using UHPLC-MS/MS. While each well of the 96-well acceptor plate was for single use only, the conductive donor plate and the top electrodes plate were reused. Therefore, to prevent carry-over between samples, these plates were washed properly immediately after each run with Milli-Q water and ethanol.

Conditions for EME were optimized using either a neat buffer or blank plasma spiked with the analytes at a concentration of 100 ng/mL. The following parameters were used for initial extractions of the tested ANT from a neat buffer: 250 μL of buffer-I (pH 3) as a donor (sample) phase, 100 μL of 500 mM formic acid as an acceptor phase, electrical potential of 75 V, shaking at 1050 rpm and extraction time of 30 min. NPOE, NPOE with addition of DEHP (10–50%), and pure DEHPi were tested as SLM. Optimization of pH of the donor phase was done using buffer-I (pH 3) or buffer-III (pH 4). After setting the basic parameters of EME using a neat buffer, further experimental conditions were optimized using spiked plasma. These were composition (buffer-I or buffer II, both pH 3) and volume (200–250 μL) of the donor phase, composition of the acceptor phase (50 mM HCl, 500 mM formic acid or acetic acid), extraction voltage (20–50 V), agitation (850–1050 rpm), and extraction time (10–60 min). Prior to the extraction, plasma samples (50 μL) were mixed with 150–200 μL of the buffer in the well of the donor plate.

2.4. UHPLC-MS/MS method

The analysis was performed using an Agilent 1290 Infinity II LC with Triple Quad LC/MS (6400 series), a Jet Stream Electrospray and Mass Hunter software (Agilent, Santa Clara, CA, USA). Chromatographic separation was achieved on the column Kinetex C18 (100 × 2.1 mm, 1.7 μm, Phenomenex, Torrance, CA, USA) with the same type of a guard column. Mobile phase A and B consisted of formic acid 0.025% and acetonitrile, respectively. The following gradient was used for elution of the analytes: 0.0–4.0 (20–60% B), 4.1–6.0 (80% B), 6.1–7.5 (20% B). The flow rate of mobile phase was 0.35 mL/min and 10 μL of the sample were injected onto the column. The column and the autosampler thermostat were maintained at 40 and 10 °C, respectively.

Electrospray in positive ion mode was employed for ionization of analytes. The mass spectrometer was tuned automatically, the parameters are summarized in Supplementary materials Table S1. The isotopically labeled internal standards 13C-d3-DAU and 13C-d3-DAUol were used for quantification of DAU and DAUol, respectively (Fig. 1b). DOX was quantified using 13C-d3-DAU as internal standard. Quantification was done using selected reaction monitoring and a dwell time of 20 ms, the transitions and collision energies of the analytes and internal standards are summarized in Table S2. For detection of the phospholipids precursor ion scan in positive ion mode providing fragment m/z 184 was used. The scan time was set at 800 ms and the collision energy was 30 V.

The EME followed by LC-MS/MS for determination of DOX, DAU and DAUol in rabbit plasma was validated according to European Medicine Agency (EMA) guideline for bioanalytical method validation [27]. Selectivity was assessed by analyzing blank rabbit plasma, where detector response at the retention times of the analytes and ISs was evaluated. Linearity was tested within the concentration rage of 0.25–1000 ng/mL (9 calibration levels). For determination of accuracy and precision, five quality control (QC) samples were prepared at four concentration levels (0.25, 1, 500 and 1000 ng/mL, n = 5). Accuracy was expressed as percentage of determined concentrations relative to nominal concentrations and precision as the relative standard deviation (RSD) of the obtained data. The matrix effects were evaluated from six different lots of rabbit plasma at two concentration levels (1 and 1000 ng/mL). This parameter (expressed as percentage) was calculated from the ratio of the peak area of blank plasma sample spiked after extraction and peak area of the neat standard solution. Both individual matrix effects for each compound and IS normalized matrix effects were calculated. Recovery was tested at low (1 ng/mL) and high (1000 ng/mL) concentration levels of the calibration range (n = 5 for each level). It was calculated from the ratio of the peak areas of the blank plasma sample spiked before and after the extraction procedure. To exclude artificial conversion of DOX and DAU to the metabolites during the extraction, the anthracyclines were extracted from spiked plasma individually (1000 ng/mL, n = 3) and the originated DAUol and DOXol were monitored. Postpreparative stability of the analytes was tested at 10 °C for 48 h.

2.5. Protein precipitation and liquid-liquid extraction

LLE and PP were utilized as conventional sample clean-up methods to compare matrix effect (ME), extraction recoveries (at two concentrations 1 and 1000 ng/mL, n = 5) and the presence of phospholipids in the extract (1 ng/mL, n = 3) with that obtained by EME. LLE was also used for quantification of DAU and DAUol in real plasma samples from rabbits as a confirmatory method.

LLE of ANT was optimized based on the procedure published by Zhang et al. [17]. Briefly, plasma (200 μL) was spiked with 2 μL of the internal standards, subsequently 1.8 mL of chloroform–methanol

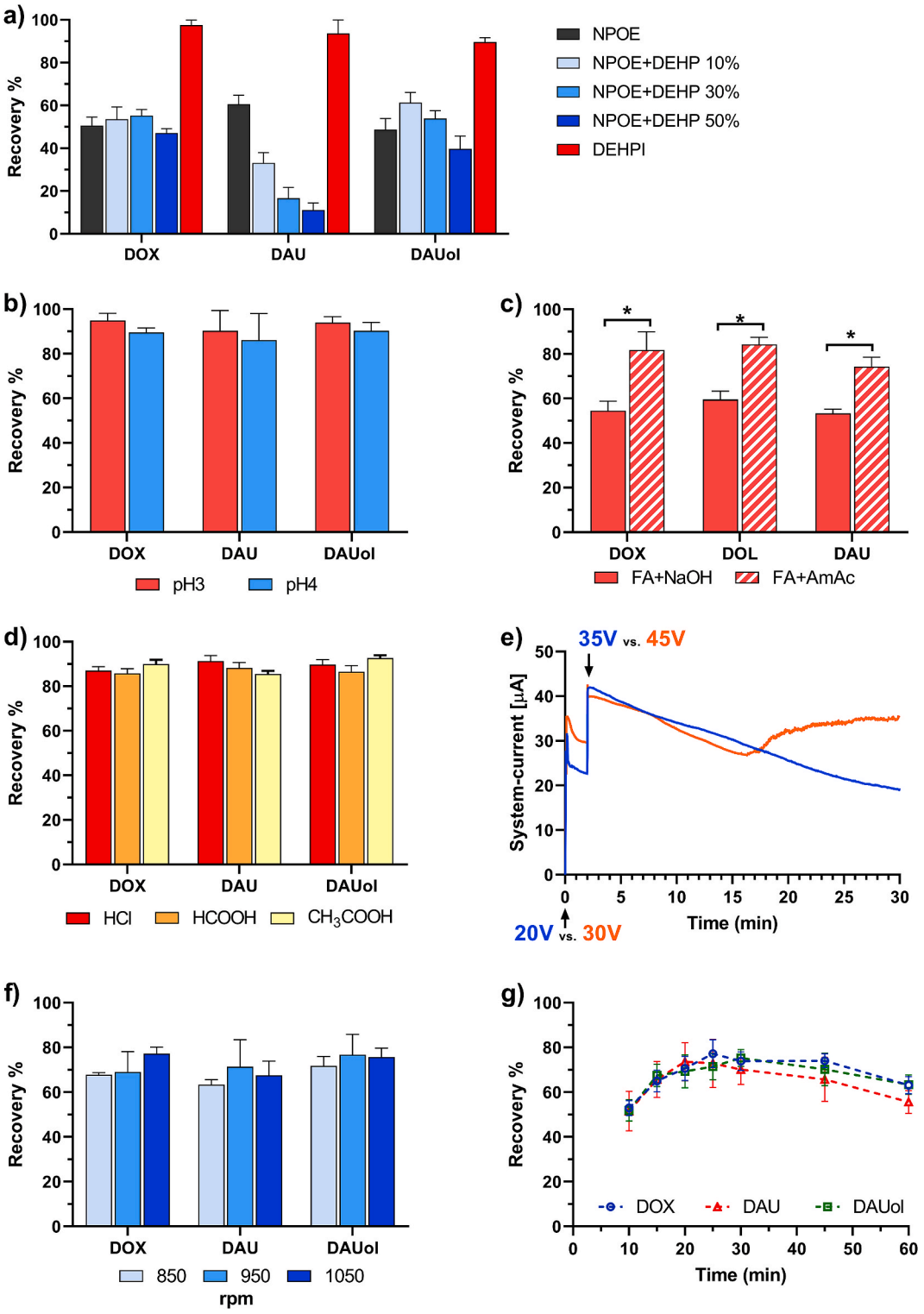

Fig. 2. Optimization of EME parameters. The extraction was conducted from neat donor buffer (a,b) or from spiked plasma samples diluted with donor buffer-II (pH 3) (c–g), data are presented as mean ± SD. (a) Recoveries of ANT with different SLM. The analytes were extracted into 500 mM formic acid (100 μL) across a different SLM (3 μL), (n = 4). (b) Optimization of the donor buffer pH. The buffer-I (pH 3) and buffer-III (pH 4, both 250 μL) were used as a donor phase and the recoveries of tested ANT were evaluated (n = 3). (c) Optimization of donor phase composition. Extraction recovery from plasma samples diluted with buffer-I (FA + NaOH) or buffer-II (FA + AmAc, both pH 3, n = 3). Statistical significance was evaluated using One-way ANOVA, Sidak's multiple comparison test; $p \leq 0.001$. (d) Optimization of the acceptor phase. The extraction recoveries of ANT from plasma (50 μL) diluted with buffer-II (185 μL) into 50 μL of hydrochloric acid (50 mM), formic acid (500 mM) or acetic acid (500 mM) were evaluated (n = 3). (e) Voltage optimization - the system current profile. The extraction voltage was set up at 20 or 30 V and increased to 35 or 40 V after 2 min, respectively. The system-current was recorded every second. Data are shown as the average current from extraction of four plasma samples at the same time. (f) Optimization of agitation speed. The agitation speeds 850, 950 and 1050 rpm were tested, (n = 4). (g) Recovery versus extraction time. The extraction recoveries from plasma samples were evaluated in several time intervals from 10 to 60 min (n = 4).

Table 1
Validation parameters for EME followed by UHPLC-MS/MS assay of ANT from rabbit plasma.

Analyte	Concentration (ng/mL)	Intra-day		Inter-day		Recovery (%)	ME (% ± SD) IS normalized	Linearity (weighted/ R)
		Accuracy (%)	Precision (RSD)	Accuracy (%)	Precision (RSD)			
DOX	0.25	111.6	5.1	97.1	7.4	–	–	$1/x^2$
	1	100.1	5.8	107.9	3.1	82.5 ± 1.6	96.3 ± 3.7	0.9980
	500	95.6	2.9	98.3	1.2	–	–	
	1000	91.5	7.7	96.8	3.1	90.0 ± 1.6	98.1 ± 2.5	
DAU	0.25	103.2	8.5	95.4	8.9	–	–	$1/x^2$
	1	97.1	2.4	95.1	2.0	81.5 ± 3.9	95.5 ± 2.6	0.9982
	500	98.2	1.2	102.8	0.8	–	–	
	1000	99.0	1.8	104.0	0.5	85.5 ± 1.2	102.3 ± 3.7	
DAUol	0.25	105.0	4.2	93.7	8.8	–	–	$1/x^2$
	1	95.0	2.9	105.4	3.1	91.1 ± 2.9	97.7 ± 2.3	0.9983
	500	97.8	1.6	104.7	1.6	–	–	
	1000	98.9	2.0	105.7	0.4	92.6 ± 1.1	103.1 ± 3.5	

ME – matrix effect.

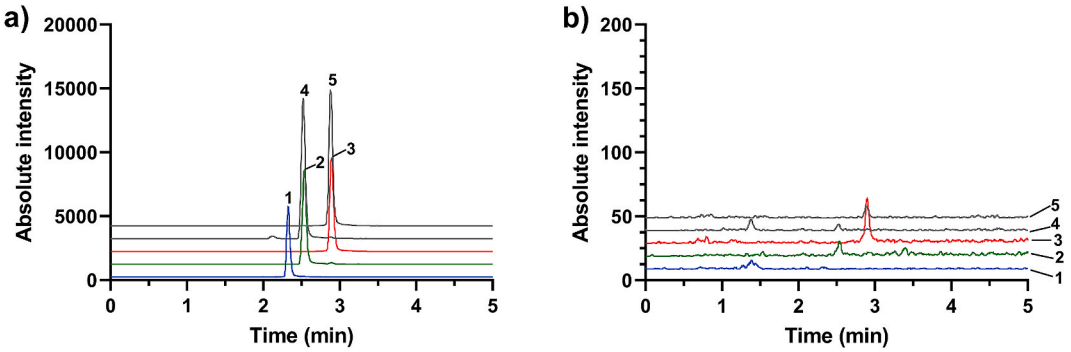

Fig. 3. Representative chromatogram of UHPLC-MS/MS analysis (a) of the ANT and the internal standards (concentration of 50 ng/mL for analytes and 100 ng/mL for internal standards) and (b) the corresponding blank in plasma. *(1) DOX, (2) DAUol, (3) DAU, (4) 13C-d3- DAUol, (5) 13C-d3- DAU.*

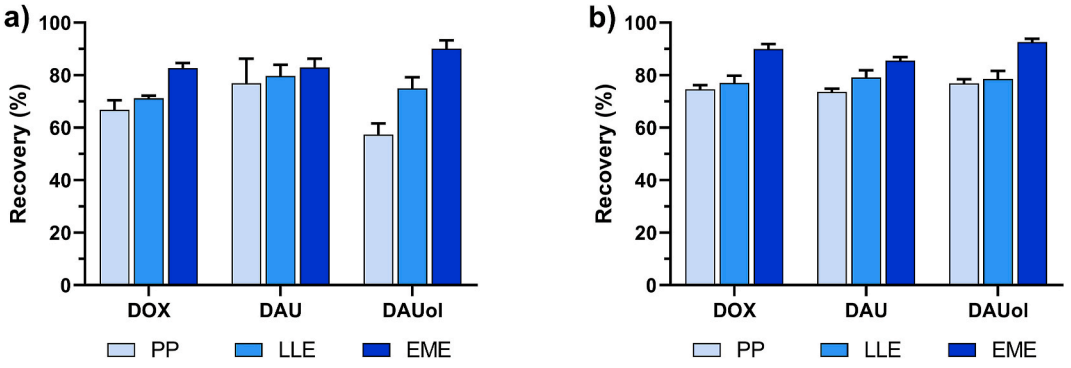

Fig. 4. Comparison of extraction recoveries of ANT from rabbit plasma using different extraction techniques. Protein precipitation (PP), liquid-liquid extraction (LLE) and electromembrane extraction (EME). The ANT were extracted on two concertation levels (a) 1 ng/mL and (b) 1000 ng/mL. Data are presented as mean ± SD, n = 5.

Table 2
Matrix effects (ME) calculated using different extraction techniques. Data are presented as mean±SD, n = 5.

Extraction technique	Concentration (ng/ml)	Absolute ME (% ± SD)			IS normalized ME (% ± SD)		
		DOX	DAU	DAUol	DOX	DAU	DAUol
PP	1	114.9 ± 8.2	133.2 ± 6.2	136.1 ± 14.2	89.1 ± 7.3	103.3 ± 6.4	108.8 ± 13.6
	1000	106.3 ± 7.4	113.7 ± 2.2	112.4 ± 3.0	96.3. ± 7.5	102.9 ± 2.9	102.4 ± 2.6
LLE	1	129.2 ± 10.7	139.2 ± 10.2	125.0 ± 10.4	106.6 ± 5.6	111.2 ± 4.0	104.2 ± 3.5
	1000	139.8 ± 10.7	128.5 ± 13.8	135.5 ± 12.9	110.5 ± 8.6	101.1 ± 3.7	101.5 ± 4.2
EME	1	103.8 ± 0.9	107.2 ± 2.8	102.8 ± 1.7	96.3 ± 3.7	95.5 ± 2.6	97.7 ± 2.3
	1000	107.2 ± 1.9	111.7 ± 2.6	110.4 ± 2.0	98.1 ± 2.5	102.3 ± 3.7	103.1 ± 3.5

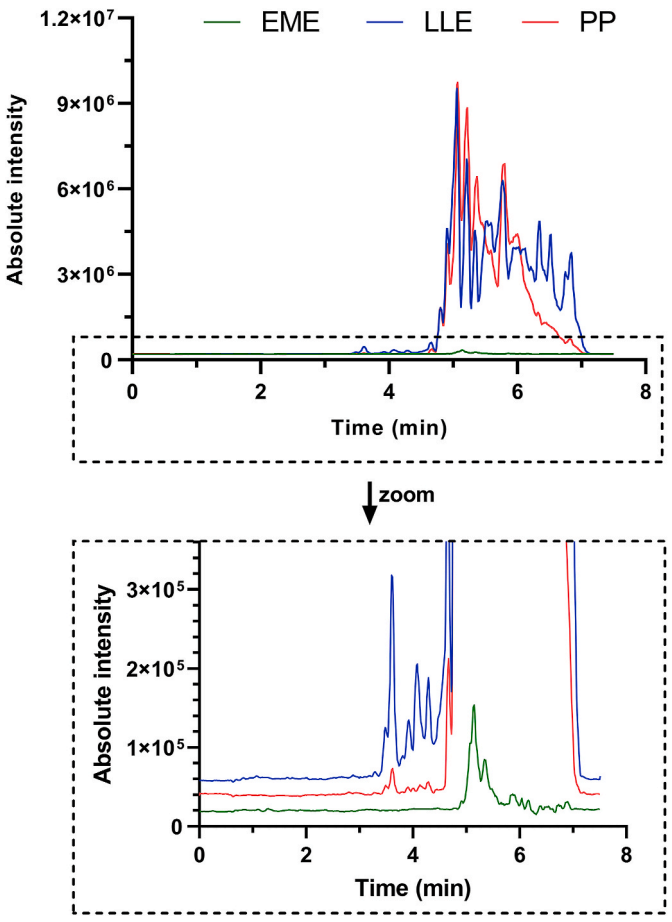

Fig. 5. UHPLC-MS/MS chromatogram to detect phospholipids. Phospholipids were detected in extracted samples using EME, LLE and PP using the precursor ion scan for product ion at m/z 184. The experiment was conducted from three lots of plasma and the obtain chromatograms were comparable.

mixture (4:1, v/v) was added. The mixture was vigorously mixed (10 s) and the extraction was conducted under continuous stirring (900 rpm) for 5 min followed by centrifugation (10,000 rpm, 10 min, 4 °C). The organic phase (700 μL) was collected and evaporated to dryness at 40 °C under nitrogen flow. The dry residue was reconstituted with 200 μL of methanol, filtered (0.22 μm, PVDF) and analyzed.

PP was done using following procedure: spiked plasma (50 μL) was mixed with internal standards (2 μL) and precipitated with ice-cold methanol (1:4, v/v). It was vigorously mixed (20 s), centrifuged (10 min, 10,000 rpm, 4 °C), supernatant was collected, filtered (0.22 μm, PVDF) and analyzed.

2.6. The practical application of EME for extraction of plasma samples

The practical utility of EME to extract DAU and DAUol from plasma were confirmed by analysis of samples taken from a pilot *in vivo* experiment. The plasma samples were taken at 3 min, 12 and 24 h post *i. v.* administration of DAU (3 mg/kg) to rabbits. The dose, route of administration and experimental setting of the *in vivo* experiment are in agreement with that used and described previously to investigate chronic cardiotoxicity of DAU [28]. These real samples were kindly provided by dr. Štěrba (Faculty of Medicine in Hradec Králové, Charles University, Czech Republic). The *in vivo* experiment was a part of the investigations approved by the Animal Welfare Committees of Faculty of Medicine in Hradec Králové, Charles University. The plasma samples were extracted using both EME and LLE, analyzed with UHPLC-MS/MS method in a single assay and the results were compared.

3. Results and discussion

3.1. Optimization of EME parameters

In order to develop a reliable and robust method for extraction of ANT the following parameters were optimized: selection of organic solvent as SLM, composition of acceptor and donor phases, electrical potential, agitation speed, and extraction time. Initial experiments were performed with neat buffer spiked with the analytes, while final optimization was done with spiked plasma samples. Since ANT are polar zwitterionic drugs, they can be extracted as either cations or anions. Considering the higher number of SLMs suitable for extraction of basic drugs compared with that for acids, as well as the number of successful extractions of polar bases [29–32], extraction of ANT with net positive charge was preferred in this study.

3.1.1. Organic solvent for SLM

First set of experiments aimed at selection of optimal organic solvent used as SLM. The extractions were done from a neat buffer-I (pH 3) spiked with the analytes. The following solvents were tested: NPOE, NPOE with addition of ion-pair carrier DEHP, and DEHPi. NPOE is a commonly used SLM for extraction of non-polar basic drugs (log P > 2) [3,33,34]. Although log P of ANT are less than two, the extractions with NPOE showed surprisingly high extraction efficiency. Recoveries were 51, 61, and 49% for DOX, DAU, and DAUol, respectively (Fig. 2a).

To increase the recoveries of polar basic drugs (log P < 2), NPOE has been mixed with hydrophobic ion-pair reagents [35–39]. As DEHP has been frequently used as a hydrophobic carrier [36,38,39], the effect of an addition of 10, 30 and 50% (v/v) of DEHP into NPOE was investigated. Nevertheless, as seen in Fig. 2a, DEHP did not improve the recoveries of ANT. With increasing amount of DEHP the recoveries decreased, especially for DAU. This phenomenon could be explained by trapping of the analytes inside the SLM by the ionic interactions: DEHP is negatively charged and thus it can act as an ion-pair reagent for

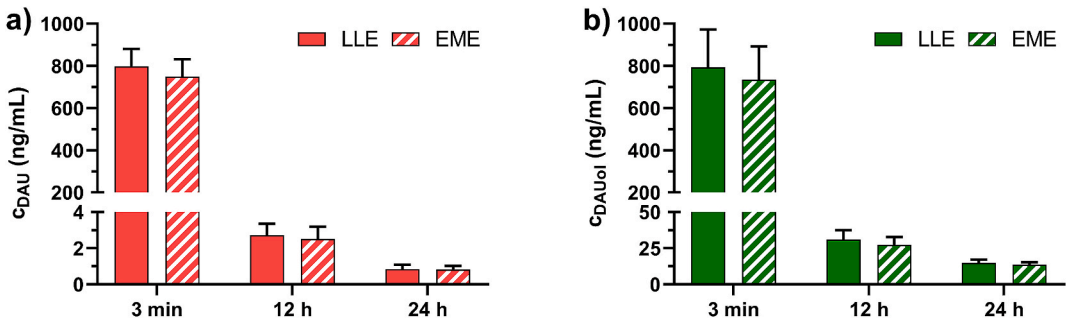

Fig. 6. Concentrations of ANT assayed in real plasma samples. Concentration of (a) DAU and (b) DAUol determined in plasma taken after administration of DAU (3 mg/kg, i.v.) to rabbits. Samples were threated using two extraction techniques (EME and LLE). Data are expressed as mean ± SD, n = 4.

positively charged ANT. Furthermore, slightly higher system-current was detected when using DEHP as carrier which is in line with previous findings [31,35]. Significant increase of the current with time was observed for NPOE with 10% of DEHP while the system with addition of 30 and 50% of DEHP was stable. This observation is rather unusual in EME.

DEHPi was tested based on its ability to achieve high extraction efficiency of polar basic drugs without causing excessive system-current [31,35]. Furthermore, DEHPi is a non-ionic solvent and thus extraction is mediated mainly by hydrogen-bond interactions [35]. With this solvent, no ion pairing occurred: DHEPi provided the highest extraction efficiency and was therefore selected for further optimization of operational parameters (Fig. 2a). Even though the system-current was higher compared to pure NPOE, it did not exceed the limit 50 μA/well, which is recommended to avoid bubble formation or pH changes in acceptor and donor phases due to excessive electrolysis [3,31,35].

3.1.2. Donor and acceptor phase

In a next series of experiments, the optimal pH, composition and volume of acceptor and donor phases were examined. The pH of the donor phase was optimized based on extraction of ANT from neat buffer. To ensure sufficient ionization of ANT, buffers of pH 3 (buffer-I) and pH 4 (buffer-III) were tested as donor phases. Similar recoveries were observed for both tested buffers (Fig. 2b) which is in line with the pK_a values of tested analytes as they are all fully ionized bellow pH 6 (www.chemicalize.org). The buffer of pH 3 was preferred as the donor phase since it mediated lower system-current in comparison with the buffer of pH 4.

Further optimization was done using spiked plasma samples. At first, it was necessary to reduce the extraction voltage to keep the system-current bellow 50 μA/well during the extraction of ANT from plasma. Furthermore, we revealed that the application of the current extraction protocol on plasma samples resulted in significantly lower recoveries (app. 55% for all compounds) compared to those obtained from the neat buffer. This discrepancy was solved by adjusting the composition of the donor buffer. We found that replacement of sodium hydroxide with ammonium acetate significantly improved extraction recoveries from plasma (Fig. 2c). We have currently no clear explanation for this observation. Based on this finding, optimal donor buffer consisted of formic acid and ammonium acetate, adjusted to pH 3 (buffer-II).

The experiments with plasma also showed that the volume of the donor phase affects extraction in 96-well format. To optimize this parameter, spiked plasma (50 μL) was mixed in the extraction well with different volumes of buffer-II (from 150 to 200 μL) and the recoveries were evaluated. It was found that a total sample volume (plasma + buffer) of 200 μL was not sufficient for appropriate contact with the SLM. This led to poor precision with RSD values > 15% (Figure S2). On the other hand, when plasma sample was diluted with donor buffer to a total volume 250 μL, we observed leakage of sample due to overloading of the well. Hence, the total sample volume was optimized to 235 μL (50 μL of plasma +185 μL of buffer-II).

As a next step, the composition of the acceptor phase was optimized. Hydrochloric acid 50 mM (pH approx. 1.5), formic acid 500 mM (pH app. 2.0), and acetic acid 500 mM (pH app. 2.5) were tested as acceptor phases. Different voltages (15–50 V) were used to keep the system-current below 50 μA/well. Recoveries higher than 70% were observed with all tested acceptor phases (Fig. 1d). The hydrochloric acid (50 mM) was evaluated as an inappropriate acceptor phase as the pH 1.5 is incompatible with the chromatographic column, therefore the sample required neutralization with ammonium hydroxide (150 mM). This resulted in both an additional step in extraction process and sample dilution. Acetic acid was selected as the acceptor phase, due to high compatibility with LC-MS/MS, slightly higher recoveries and lower system-current compared to formic acid.

3.1.3. Extraction voltage

Since it is known from literature that extraction efficiency is increasing with applied voltage, the electrical potential was optimized [31]. Extractions from plasma using different voltages (ranging from 20 to 50 V) were performed and the system-current was measured during all the experiments. As illustrated in Fig. 1e, the system was stable up to 35 V and above this level, the system-current increased gradually during extraction. Based on these experiments, start on 20 V and instantaneous increase to 35 V after 2 min was set up as the optimal extraction voltage. Under these conditions, the system was stable for at least 60 min (Figure S3).

3.1.4. Extraction time and stirring

Last parameters optimized were agitation speed and extraction time. Agitation was tested within the range of 850–1050 rpm. As apparent from Fig. 1f, only minor variation in recoveries and no difference in system-current was observed. Agitation at 1050 rpm was selected, which fulfilled both high recoveries and reproducibility of extraction represented by low RSD values. The extraction time was examined from 10 to 60 min. Recoveries of all tested analytes increased with increasing time of extraction, and steady-state was achieved at about 30 min. After that, recoveries slightly decreased (Fig. 1g). A similar observation was described previously by Huang et al., when DEHPi was used as SLM [31]. This observation is explained by pH changes in the acceptor phase due to electrolysis. Based on these findings, the optimal extraction time was set at 25 min.

3.2. Method validation

EME of ANT from rabbit plasma was performed based on the systematic optimization experiments described above. Analytes were extracted from 50 μL of plasma mixed with 185 μL of buffer-II (pH 3) through the SLM covered with DEHPi (3 μL) and into 50 μL of acetic acid (500 mM) as acceptor phase. The extraction was conducted for 25 min, and the electrical potential was initially set at 20 V and increased to 35 V after 2 min. The system was agitated at 1050 rpm.

The optimized EME procedure followed by UHPLC-MS/MS was fully validated according to EMA guideline for bioanalytical method validation [27]. The validation parameters are summarized in Table 1 and the representative chromatograms are shown in Fig. 3. Appropriate selectivity was proved by no significant coelutions at the retention times of either analytes or internal standards in blank plasma sample (Fig. 3). The method was linear within the concentration range of 0.25–1000 ng/mL using weighted standard curves ($1/x^2$) with correlation coefficients (R) above 0.998 for all tested analytes. The lowest calibration level represents the lower limit of quantification (LLOQ). The inter- and intra-day accuracy and precision were within ±15% (Table 1). The mean extraction recoveries were higher than 81% for all tested ANT. These recoveries obtain from plasma were only slightly lower compared to those obtain from neat buffer-I (Figure S4). The IS normalized matrix effects on two calibration levels ranged from 89.5 to 108.3 with RSD values within ±15%, as recommended by EMA. The extraction process did not affect the stability of the analytes as no artificial conversion of ANT to the metabolite was observed after EME. The processed sample were stable prior to analysis in an autosampler set at 10 °C for at least 48 h.

3.3. Comparison of EME with PP and LLE

Compared to conventional sample clean up techniques, EME presents the advantages typical for microextractions such as low consumption of organic solvent, reduction of the number of extraction steps and extraction time, while facilitating high throughput. In this study, 3 μL of the organic solvent (DEHPi) per sample were used for EME, while LLE required 1600 μL of chloroform per sample. Moreover, LLE involves multiple extraction process steps including also extract evaporation and

reconstitution to get LC-MS compatible sample. The time needed for EME is comparable with PP, however PP suffers from poor sample clean-up and the extract is diluted.

PP and LLE were directly compared with EME in 96-well format in terms of extraction recoveries of tested ANT, ME and phospholipids profile detected in the extract sample. Although 96-well technology is available for both LLE and PP, experiments with these techniques reported in this section were performed manually in individual Eppendorf tubes, and comparison was balanced accordingly. Recovery and ME were evaluated at low (1 ng/mL) and high (1000 ng/mL) levels of calibration range. As seen in Fig. 4, PP demonstrated the lowest recovery. Moreover, in the case of DAUol, relatively high variation (RSD = 14.6%) was observed between recoveries obtained at low and high levels. Compared to PP, LLE showed higher recoveries (approx. 80%) for both concentrations tested. EME provided the highest recoveries for all tested ANT (more than 80%) with coefficient of variations less than 5% between low and high concentrations.

Investigation of ME disclosed signal enhancement for all extraction methods and all tested ANT (Table 2). The highest ME were surprisingly observed for LLE where the mean values variated within the range of 125–140% (Table 2). Nevertheless, the internal standards compensated this enhancement and the IS normalized ME met the recommendations of EMA validation guidelines [27]. Similar trend was observed in the samples treated by PP (Table 2). EME provided lowest enhancement of the MS signal (ME 100 ± 15%, Table 2), as well as showed no significant difference between ME calculated for low and high concentration level.

The method for evaluation of phospholipids was modified from Little et al. [40]. This method assumes that most abundant phospholipids present in plasma (phosphatidilcholines – PC and lyso-phosphatidylchlines – lyso-PC, 70 and 10% of total plasma phospholipids, respectively [41]) give a typical fragment at *m/z* 184. Hence, in our study the precursor ion scan providing fragment at *m/z* 184 in positive ion mode was used for detection of phospholipids. As observed in Fig. 5, phospholipids were detected in all samples. High signals detected after PP and LLE (absolute intensity up to 9×10^6) indicate high abundance of phospholipids in the extracts. While in the EME sample only minor signal of phospholipids was observed (absolute intensity up to 0.15×10^6). Contrary, Vårdal et al. previously reported that the EME of plasma samples using DEHPi as SLM provides totally phospholipids free extracts [42]. This discrepancy could be explained by using different MS instrument as well as more sensitive method in our study, as the fragmentation in collision cell could be more effective compared to in-source fragmentation utilized in the former study.

In addition, our method allows the detection of the precursors providing the fragment at *m/z* 184. The MS spectra of the detected precursor ions were similar for LLE and PP, while a different pattern can be seen for the EME sample (Figure S5). Furthermore, the intensity order of detected precursors does not correlate with that in LLE and PP (Table S3). Surprisingly in the MS spectrum of LLE and PP the four most intense signals (*m/z* 524.5, 469.5, 520.5 and 522.5) belong to phospholipids from the lyso-PC class. Contrary, in the MS spectrum of EME extract a phospholipid from PC class (*m/z* 786.9) was detected as the most intensive one. All precursors found in the EME extract had intensities of less than 1% of the highest intensity precursors found with LLE or PP. Precursors detected in the extracts are listed in Table S3. The amount of phospholipids determined in the samples after different extraction procedures corresponds to ME determined above. All together it suggests that EME provided the most effective sample clean-up with lowest impact of the biological matrix on the assay of ANT compared to PP and LLE.

3.4. Application of EME to real plasma samples

In order to verify the practical applicability of the EME, real plasma samples taken at 3 min, 12 and 24 h from pharmacokinetics study of DAU in rabbits (n = 4) were analyzed. Three minutes is closed to the peak plasma concentration, while 12 and 24 h represent the plasma concentrations in the elimination phase. The samples were treated by both optimized EME and routine LLE and analyzed in a single run. The determined concentrations were compared (Fig. 6). No significant difference between the results obtained using EME and LLE was found (One-way ANOVA, Sidak's multiple comparison test). Thus, these data show that reliability of EME is comparable to conventional extraction techniques.

4. Conclusion

In this work, microextraction of ANT from plasma was demonstrated for the first time using electromembrane extraction in 96-well format. Experimental conditions for EME of ANT were systematically optimized in terms of the selection of proper SLM, composition and pH, of donor and acceptor phases, voltage, stirring and time of extraction. The highest recovery of ANT was achieved employing DEHPi as SLM. The extraction was followed by direct UHPLC-MS/MS analysis of acceptor phases (no evaporation and reconstitution), and the whole process was validated. EME proved to be a rapid and effective sample treatment technique providing higher than 80% extraction recovery of tested ANT from plasma and high sample clean-up. In comparison with conventional PP and LLE, EME provided acceptable ME, and negligible amount of phospholipids was detected in the extract. Furthermore, the analysis of real plasma samples verified that the EME provides reliable results comparable with that obtained with routine conventional LLE. Based on the experiences from the current work, and based on the fact that EME is under commercial development, we expect EME to be implemented for routine applications in the future. Major incentives for this may be (1) to eliminate the use of hazardous organic solvents, (2) to avoid ion suppression in LC-MS, (3) to simplify laboratory workflow, or (4) to take advantage of the selectivity of EME. The latter is controlled by the direction and magnitude of the electrical field, by the chemical composition of the SLM, and by pH. With commercially available equipment, routine laboratories can take advantage of these properties, using equipment of industrial standard.

Credit author statement

Hana Bavlovič Piskáčková: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing review & editing. **Elisabeth Leere Øiestad:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - review & editing. **Nela Váňová:** Methodology, Investigation, Writing - review & editing. **Júlia Lengvarská:** Investigation, Writing - review & editing. **Petra Štěrbová-Kovaříková:** Supervision, Conceptualization, Formal analysis, Funding acquisition, Writing - original draft, Writing review & editing. **Stig Pedersen-Bjergaard:** Supervision, Conceptualization, Methodology, Formal analysis, Writing - original draft, review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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