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Electromembrane extraction of streptomycin from biological fluids

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ABSTRACT

In this fundamental study, streptomycin was extracted successfully from urine and plasma using electromembrane extraction (EME). Streptomycin is an aminoglycoside with log P -7.6 and was selected as an extremely polar model analyte. EME is a microextraction technique, where charged analytes are extracted under the influence of an electrical field, from sample, through a supported liquid membrane (SLM), and into an acceptor solution. The SLM comprised 2-nitrophenyl pentyl ether (NPPE) mixed with bis(2-ethylhexyl) phosphate (DEHP). DEHP served as ionic carrier and facilitated transfer of streptomycin across the SLM. For EME from urine and protein precipitated plasma, the optimal DEHP content in the SLM was 45–50% w/w. From untreated plasma, the content of DEHP was increased to 75% w/w in order to suppress interference from plasma proteins. Most endogenous substances with UV absorbance were not extracted into the acceptor. Proteins and phospholipids were also discriminated, with <0.6% of proteins and <0.02% of phospholipids found in the acceptor after EME. Thus, despite the fact that the SLM was permeable to more polar molecules, the EME still provided very efficient sample cleanup. Extraction process efficiencies of 98% and 61% were achieved from urine and plasma, respectively, with linear calibration (R^2 > 0.9929), absence of significant matrix effects (94–112%), accuracy of 94–125%, and RSD ≤ 15% except at LLOQ. The average current during extractions was 67 µA or less. The findings of this paper demonstrated that EME is feasible for extraction of basic analytes of extreme polarity.

1. Introduction

For more than two decades, microextraction has been a major point of research focus within analytical chemistry. Electromembrane extraction (EME) is an example, suited for selective extraction of ionizable compounds from complex biological and environmental samples [1]. In EME, the sample solution is separated from a clean acceptor solution by means of a supported liquid membrane (SLM), comprising a water-immiscible (hydrophobic) solvent immobilized in the pores of a supporting polymeric membrane. The aqueous solutions are pH adjusted to favor ionization of the target analytes, and extraction is based on application of an electric field across the SLM. The process may thus be considered as electrophoresis across an oil membrane. EME is an attractive technique for selective extraction, since selectivity is based on analyte charge and partitioning.

Since the introduction of EME in 2006 [2], focus has been diverted to successful extraction of non-polar (log P 2 to 5) [3-6] and moderately polar (log P 0 to 2) [7-10] analytes. Due to effective discrimination of polar matrix constituents by the SLM, excellent sample cleanup has been obtained. In recent years, increasing attention has been drawn to the application of EME for extraction of polar ($-2 < \log P < 0$) and highly polar analytes ($-5 < \log P < -2$). Polar and highly polar analytes are challenging with most extraction techniques due to poor partition, and this is also the case with EME. Even with a high electrical field across the sample-SLM interface, transfer of polar and highly polar cationic or anionic analytes across the interface may be limited. Recently, bases with log P −5.7 to +1.5 were extracted successfully [11-13], and based on this EME was recently mentioned as one of the more promising microextraction techniques for polar and highly polar compounds [14]. Development of suitable EME methods for polar, highly polar, extremely polar ($\log P < -5$) compounds may be very attractive for metabolomics [15], peptidomics [16], and for environmental sciences [17]. However, for such research and development to be successful, more experiences are required. Especially, we need to understand (a) how to design the SLMs, (b) if extremely polar analytes can be extracted, and (c) if such EME systems provide acceptable selectivity and cleanup from complex real samples.

In this work, we selected streptomycin as a model analyte of extreme polarity, to address the research questions above. Strepto-

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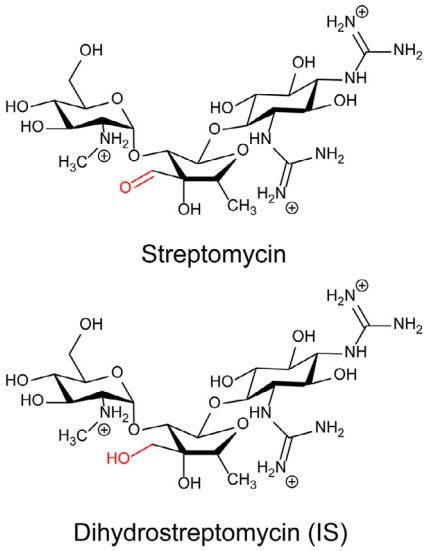


Fig. 1. Chemical structure of streptomycin and dihydrostreptomycin (IS). The red-colored groups indicates the difference of the structures.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mycin is an aminoglycoside. Aminoglycosides are antibiotics used against gram-negative bacteria causing diseases like tuberculosis, and act by inhibiting the bacteria protein synthesis. Chemically, the structures are based on glycosides with amine-based modifications that make the molecules very hydrophilic, and thus very challenging to extract with equilibrium-based microextraction techniques [15]. Streptomycin is listed by the World Health Organization as critically important for human medicine [18]. It is therefore of high importance to have efficient analytical-scale sample preparation methods that can provide good cleanup of streptomycin from complex matrices. The chemical structure of streptomycin is shown in Fig. 1. The log P value is −7.6, but since streptomycin carries three positive charges at pH < 7.4, the effective distribution coefficient (log D) is rather −12.0 under physiological conditions [19]. Additionally, the pH window where streptomycin is uncharged is very narrow. The typical strategy for enhanced extraction of polar bases into a hydrophobic phase by raising the pH above the pKa value is therefore not feasible. Historically, extraction of streptomycin and other aminoglycosides has been performed by solid-phase extraction, using either cation-exchangers, C18 sorbents with added ion-pair reagent, and hydrophilic-lipophilic-balance sorbents [20].

The current paper contributes with new experiences and knowledge related to EME of extremely polar substances. EME was combined with hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry (MS/MS). Initially, different solvents were screened to identify a suitable SLM. Subsequently, the effect of sample pH and SLM composition for urine and plasma samples was studied by means of a design-of-experiments (DOE) approach. Finally, the data reliability of the proposed EME system was evaluated. The paper is a fundamental study, and the conclusions are of high importance for further development of EME.

2. Experimental

2.1. Chemicals and reagents

Milli-Q (MQ) water was provided by an Integral 3 purification system (Milli-Q, Molsheim, France). LC-grade methanol (MeOH), LC-MS grade acetonitrile (ACN), 2-nitrophenyl octyl ether (NPOE), 2-nitrophenyl pentyl ether (NPPE), bis(2-ethylhexyl) phosphate (DEHP), ammonium acetate, formic acid, citric acid, disodium hydrogen phosphate, phosphoric acid (85%), streptomycin sulfate, and dihydrostreptomycin sesquisulfate were purchased from Merck (Darmstadt, Germany). Bovine serum albumin (Pierce™) was purchased from Thermo Scientific (Waltham, MA, USA), and Coomassie dye reagent was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Plasma samples were obtained from Oslo University Hospital (Oslo, Norway) and stored at −28 °C. Urine samples were obtained from a healthy volunteer and stored at −28 °C.

2.2. Preparation of solutions

2.2.1. Standards

Streptomycin sulfate and dihydrostreptomycin sesquisulfate (internal standard - IS) were dissolved individually in MQ water at 2 mg mL^{-1} and frozen in aliquots at −28 °C. From these, working solutions were prepared daily and used for spiking of standards and biological samples.

2.2.2. Buffer solutions for sample adjustment

Buffers in the pH range 2.5–7.3 were prepared as 50 mM citrate/hydrogen phosphate solutions, also known as the McIlvaine buffer [21]. Solutions were prepared by titrating 50 mM citric acid with 50 mM disodium hydrogen phosphate to the desired pH, verified by a pH-meter.

2.2.3. Deep eutectic solvent preparation

Deep eutectic solvents composed of coumarin and thymol were prepared by weighing appropriate amounts of each component into a 5 mL Eppendorf-tube. The amounts weighed depended on the desired molar ratio. The melting of the mixture was assisted by heating in an 80 °C oven for 15 min. After complete melting, the solvent was vortexed for 10 s to ensure homogeneity.

2.2.4. Protein precipitation procedure

500 µL thawed plasma was added to 1000 µL ACN, vortexed at 1800 RPM for 1 min, and centrifuged at 10,000 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 McIlvaine buffer with variable pH. This was equal to a 2-fold dilution of the original plasma concentration.

2.3. EME procedure

EME was performed in 96-well format with potential for high throughput extraction. The setup is illustrated in Figure S1. It comprised a sandwich of three plates. The bottom one was a laboratory-built steel plate with 96 wells, each holding 100 µL of sample. The second one was a commercially available 96-well Multiscreen-IP filter plate (polyvinylidene fluoride (PVDF) membranes, 0.45 µm pores) from Merck Millipore Ltd (Carrigtwohill, Ireland). The third one was a laboratory-built lid in aluminum with 96 rod electrodes. For each sample, 4 µL SLM solvent was pipetted onto the bottom of the PVDF filter, which resulted in immobilization of the solvent to yield the SLM. 100 µL sample solution was loaded into the corresponding well of the steel plate, and the filter plate was clamped onto the steel plate, which put the sample in contact with the SLM. 100 µL acceptor solution (50 mM phosphoric acid pH 2.0) was loaded into the filter plate well, and the aluminum lid was attached. Phosphoric acid was chosen because it has buffer capacity at pH 2. The whole device was placed on a shaking board (Vibramax 100 Heidolph, Kellheim, Germany), which was set for 900 RPM based on previous optimization [13]. The steel plate and aluminum lid were then connected to the anode and cathode, respectively, of a power supply (model

ES 0300e0.45, Delta Elektronika BV, Zierikzee, Netherlands). Extractions were initiated by simultaneous application of shaking and voltage. A Fluke 287 multi-meter (Everett, WA, USA) monitored the extraction current with an 8 Hz acquisition rate. When extraction was finished, acceptor solutions were collected quantitatively and mixed 1:1 with 1 M formic acid in acetonitrile. This was to eliminate adsorption of streptomycin and dihydrostreptomycin to the glass walls of the HPLC vials, and to ensure compatibility of the injection solvent with the HILIC-MS method used for quantitation. For recovery studies, the acceptor solution was spiked with IS to 1 μg mL^{-1} after extraction to correct for LC-MS/MS variability.

2.4. LC-MS/MS and LC-UV methods

2.4.1. HILIC-MS/MS method for streptomycin and dihydrostreptomycin (IS) quantitation

Quantitation of streptomycin and dihydrostreptomyin (internal standard) was performed with a Dionex UltiMate 3000 RS UHPLC system comprising a pump, autosampler, and column compartment, with an LTQ XL linear ion-trap as detector (all from Thermo Scientific, San Jose, CA, USA). The column was an Acquity UPLC® BEH Amide column (150 × 2.1 mm ID, 1.7 μm) from Waters (Wexford, Ireland), and was maintained at 40 °C. The mobile phases consisted of (A) 95:5 v/v ACN:MQ-water with 5 mM ammonium acetate and 0.5% formic acid, and (B) 20:80 v/v ACN:MQ-water with 5 mM ammonium acetate and 0.5% formic acid. Addition of formic acid was important to maintain peak shapes. The flow was kept at 0.3 mL min^{-1}, and elution was performed according the following gradient: 55–75% B for 0–2 min, 75% B for 2–3 min, returned to 55% B in 0.1 min, and finally 4.9 min of re-equilibration. The total run time was thus 8 min, and the injection volume was 5 μL. Detection was performed in positive electrospray ionization mode by selected reaction monitoring (SRM) of streptomycin (transition *m/z* 582 → 540), and dihydrostreptomycin (transition *m/z* 584 → 542), both using 20% normalized collision energy. The capillary temperature was set at 350°C.

2.4.2. Phospholipid determination by reversed-phase LC-MS/MS

For determination of phospholipids, the same instrument described in Section 2.4.1 was used. An Acquity UPLC® HSS T3 column (100 × 2.1 mm ID, 1.8 μm) from Waters (Wexford, Ireland) maintained at 60 °C was used as stationary phase. Mobile phases consisted of (A) 95:5 v/v MQ-water:MeOH with 0.1% formic acid, and (B) 5:95 v/v MQ-water:MeOH with 0.1% formic acid. Elution was performed using a gradient method with 0.4 mL min^{-1} flowrate, where 10% B initially was ramped to 100% in 0.3 min, and kept for 12 min, before returning to 10% B in 0.1 min, for 2.6 min of re-equilibration. The total run time was thus 15 min, and the injection volume was 10 μL. Detection of phospholipids was accomplished using in-source fragmentation with selected reaction monitoring (SRM) in positive electrospray ionization mode, based on a previously reported method [22]. For this, a source potential of 65 V and 10% normalized collision energy was applied, and the transition *m/z* 184 → 184 was monitored. The capillary temperature was set at 350°C.

2.4.3. HILIC-UV and full scan MS method for matrix clean-up assessment

Biological samples were analyzed prior to and after EME to assess the clean-up efficiency of the extraction method. The analysis was performed using a Dionex UltiMate 3000 RS UHPLC system equipped with a variable wavelength detector set at 254 nm, or mass spectrometer detection. An Acquity UPLC® BEH Amide column (150 × 2.1 mm ID, 1.7 μm) from Waters (Wexford, Ireland) maintained at 30 °C was used as stationary phase. The mobile phases consisted of (A) 95:5 v/v ACN:MQ-water with 5 mM ammonium acetate and 0.5% formic acid, and (B) 20:80 v/v ACN:MQ-water with 5 mM ammonium acetate and 0.5% formic acid. Elution was performed at 0.4 mL min^{-1} flowrate, using a gradient with 10% B for 0–1 min, 10–70% B in 1–5 min where it was maintained for 1 min, and then returned to 10% B for 9 min re-equilibration. The total run time was thus 15 min, and the injection volume was 2 μL. Aqueous samples were prior to injection diluted 1:1 with 1 M formic acid in ACN to be compatible with HILIC conditions. For mass spectrometer detection, a full scan (50–600 *m/z*) was formed in both positive and negative electrospray ionization (ESI) mode. The capillary temperature was set to 225°C.

2.5. Total protein determination

The total concentration of protein in plasma samples and acceptor solutions after extraction was determined according to the Bradford assay [23]. For each measurement, 10 μL sample was added to 200 μL Coomassie dye reagent in the wells of a microplate. Presence of protein induced an absorbance shift of the dye from 465 nm to 595 nm. Absorbance measurements of each well at 595 nm were performed after 5 min of incubation, using a microplate reader (Wallac Victor 3, Perkin Elmer, Boston, MA, USA). Calibration was performed with bovine serum albumin standards of 0–400 μg mL^{-1}. Details of calibration are available in Supplementary information 3. Dilutions were made in 50 mM phosphoric acid to match the acceptor solution. pH-related effects of the assay were therefore not present. All measurements were performed in duplicate.

2.6. Statistical design and analysis of experiments

Experimental designs and analysis by means of a design-of-experiments (DOE) approach were handled using the software Design-Expert 12 (Stat-Ease Inc., Minneapolis, MN, USA). Analysis and modeling were performed using multiple linear regression (MLR) and analysis of variance (ANOVA) to assess significance of effects. Regression of calibration curves was performed in GraphPad Prism version 9.0.

2.7. Calculations

For estimation of extraction efficiencies, IS was added to the acceptor solution after extraction, to account for variability in LC-MS quantitation.

Extraction yield (EY,%) was defined as the recovery obtained for extraction from a standard buffer solution, and calculated by the streptomycin/IS peak area ratio of the acceptor ($AUC_{acceptor,final}/AUC_{IS}$) and non-extracted standard ($AUC_{non-extracted\ standard}/AUC_{IS}$), according to (1).

$$EY = \frac{AUC_{acceptor,final}/AUC_{IS}}{AUC_{non-extracted\ standard}/AUC_{IS}} \times \frac{V_{sample}}{V_{acceptor}} \times 100\ \% \quad (1)$$

V_{sample} and $V_{acceptor}$ were the volumes of sample and acceptor solution, respectively. For all extractions, these were both 100 μL.

Process efficiency (PE,%) was similarly defined as the IS-adjusted acceptor signal obtained from a spiked biological matrix ($AUC_{acceptor,matrix}/AUC_{IS}$) relative to that of a non-extracted standard at the same concentration, and was calculated according to (2).

$$PE = \frac{AUC_{acceptor,matrix}/AUC_{IS}}{AUC_{non-extracted\ standard}/AUC_{IS}} \times \frac{V_{sample}}{V_{acceptor}} \times 100\% \quad (2)$$

Matrix effect (ME,%) was defined as the signal change of streptomycin caused by matrix components in the mass spectrometer

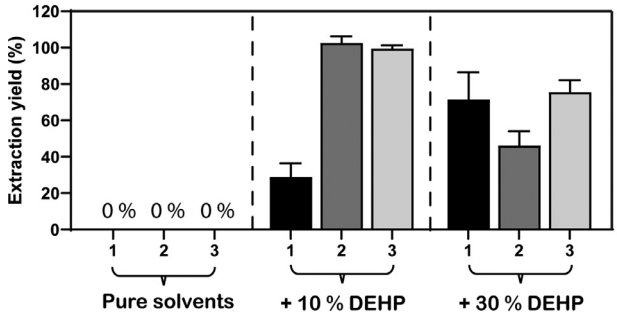


Fig. 2. Extraction yield (%) of streptomycin using 0%, 10%, and 30% DEHP added to 1) NPPE, 2) coumarin:thymol 1.5:1, and 3) coumarin:thymol 1:2. Extraction time: 15 min; sample solution was 1 µg mL^{-1} streptomycin in pH 4.0; acceptor solution was phosphoric acid pH 2.0. Voltage was varied depending on SLM solvent. Error bars represent the standard deviation ($n = 3$).

(ion suppression or enhancement), and was calculated according to (3).

$$ME = \frac{AUC_{\text{post extraction spiked matrix}}}{AUC_{\text{non-extracted standard}}} \times 100\% \quad (3)$$

$AUC_{\text{post extraction spiked matrix}}$ is the signal of streptomycin in a post extraction spiked acceptor solution, after extraction of a blank matrix sample.

3. Results and discussion

3.1. Selection of SLM solvent

In EME, the extraction selectivity is largely determined by the SLM solvent. Highly polar analytes are easily discriminated from extraction, unless the SLM solvent offers sufficiently strong interactions to overcome the hydrophobic discrimination by the non-polar SLM solvent. In two recent reports, Drouin et al. successfully used 2-nitrophenyl pentyl ether (NPPE) as SLM solvent for extraction of a variety of basic analytes in the range $-5.7 < \log P < 1.5$ [11, 12]. The extraction system however operated under high current (~300 µA), and most analytes suffered from poor extraction efficiency in complex samples. In another recent report, we investigated the use of deep eutectic solvents (DESs) as SLM [24]. DESs based on mixtures of coumarin (hydrogen bond acceptor - HBA) and thymol (hydrogen bond donor - HBD) provided exhaustive extraction of various moderately polar bases (log P −0.4 to +1.8). The DESs provided strong hydrogen bonding and aromatic interactions with the analytes.

These two solvent systems were tested for EME of streptomycin. Two different DESs were prepared, namely coumarin mixed with thymol in molar ratios 1.5:1 and 1:2 (coumarin:thymol molar ratio). The former with excess HBA and the latter with excess HBD properties. The DESs and NPPE were initially tested as pure solvents at 50 V. Extraction conditions were based on previous experience. These are given in Fig. 2 along with recovery data. As seen, recoveries were zero with the pure solvents. However, analysis of sample solutions after extraction (data not shown) revealed that 40% of streptomycin was retained in the coumarin and thymol 1.5:1 SLM, and 20% was in the coumarin and thymol 1:2 SLM. With pure NPPE, the entire content of streptomycin was found in the sample after extraction. Streptomycin showed highest affinity for the DES with excess HBA properties, and this is in agreement with previous observations for basic analytes [25].

From experiments above, extraction of streptomycin was not efficient based on hydrogen bonding interactions alone. The ionic carrier, bis(2-ethylhexyl) phosphate (DEHP), was therefore added to the three solvents at 10% and 30% w/w level, to introduce ionic interactions. This resulted in exhaustive extraction with both DESs containing 10% w/w DEHP and approximately 30% extraction yield with NPPE containing 10% DEHP. The DESs containing 30% DEHP were less efficient, and substantial amounts of streptomycin were retained in the SLM. The DESs with 30% DEHP also provided excessive current during extraction, and therefore they were operated at 3–5 V. In contrast, NPPE with 30% DEHP provided an extraction yield of 70%, while the extraction current was maintained low even at 50 V. This solvent system was therefore chosen for further study and optimization.

3.2. Effect of matrix type on optimal extraction conditions

The screening of different SLM solvents revealed that DEHP mixed with NPPE was suitable as SLM solvent, and the tested levels of DEHP indicated that increasing the amount of DEHP was favorable to the extraction yield. However, the ion-paring mechanism of DEHP with analyte cations is dependent on sample pH [13]. Under strongly acidic conditions (pH < 2.5) DEHP stays entirely in the SLM and acts by interfacial ion-pair formation with analytes. At neutral and basic conditions (pH > 6.5), DEHP leaks to a large extent into the sample solution, and ion-pairing thus take place in bulk sample. In between these extremes, ion paring occurs by a combination of the two modes (mixed-mode complexation). The effects of sample pH and DEHP concentration in the SLM are thus highly interconnected, and a central composite design was utilized for further optimization. In these experiments, the effect of (A) sample pH and (B)% DEHP were studied for EME from samples of pure buffer, urine, protein precipitated (PP) plasma, and untreated plasma. Details about the central composite design and the statistical analysis are given in Supplementary information 2. Briefly, the design included four factorial point, four center points, and four axial points ($\alpha = 1.41$) for a total of 12 runs performed in randomized order. Each run was performed in triplicate, and the average extraction process efficiency (PE,%) was used as the response. For pH adjustment, urine and protein precipitated plasma were diluted 1:1 in buffer and simultaneously spiked with streptomycin. Untreated plasma was, based on a few preliminary experiments, diluted 1:4 in buffer and spiked simultaneously. All other experimental parameters such as extraction time (15 min) and voltage (30 V) were kept constant, as the purpose of this initial set of experiments was to investigate the interrelationship between sample pH and% DEHP from different sample matrices. The factor level settings for each matrix type are indicated in Table 1. Following experimentation, the data were analyzed and fitted to quadratic regression models. Fig. 3 shows the 2D contour plots obtained for each sample matrix. As seen, the optimal settings in pure buffer were found at sample pH 4.5 and at 34% w/w DEHP in NPPE. At higher pH, and at higher% DEHP, recovery decreased due to retention in the SLM. Conversely, at lower levels of pH and DEHP, streptomycin remained in the sample.

When EME was conducted from urine and PP plasma (Fig. 3), optimal conditions shifted towards higher sample pH and% DEHP. Clearly, matrix components interacted with DEHP and caused interference. However, with increased sample pH and% DEHP, release of DEHP into the sample increased for ion-paring with streptomycin, and EME provided exhaustive extraction. For urine, optimal sample pH was 5.5 and optimal amount of DEHP in the SLM was 50%. For PP plasma, optimal conditions were found at sample pH 5.0 and 45% DEHP. In both cases, the EME system operated according to the principle of mixed-mode complexation.

To identify the optimal DEHP content for untreated plasma, the design-space had to be extended as shown in Table 1. The corresponding contour plot is shown in Fig. 3. As seen, the op-

Table 1
Coded and un-coded factor levels used for different sample matrices in central composite design.

Coded level	$-\alpha$		-1		0		$+1$		$+\alpha$	
	pH	%DEHP	pH	%DEHP	pH	%DEHP	pH	%DEHP	pH	%DEHP
Buffer	1.7	1.7	2.5	10	4.5	30	6.5	50	7.3	58
Urine	1.7	5.7	2.5	15	4.5	37.5	6.5	60	7.3	69.3
PP plasma	1.7	5.7	2.5	15	4.5	37.5	6.5	60	7.3	69.3
Full plasma	1.7	53.8	2.5	60	4.5	75	6.5	90	7.3	96.2

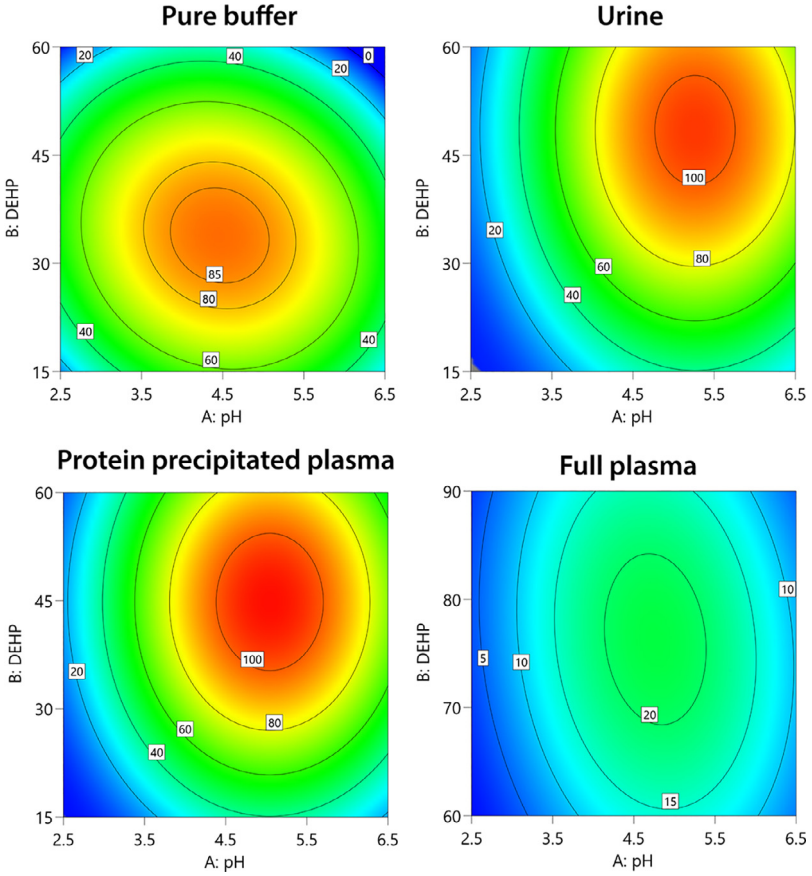


Fig. 3. 2D contour plots of (A) sample pH-value and (B)%DEHP/NPPE effect on process efficiency (%) of streptomycin extraction from different matrix types. The color gradient indicates process efficiency from 0% to 100% for blue and red, respectively. Extraction time: 15 min; extraction voltage: 30 V; sample matrices were spiked to a final concentration of 1 µg mL^{-1} streptomycin; acceptor solution was phosphoric acid pH 2.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

timal amount of DEHP was very high (~75% w/w), and the estimated process efficiency was only 22% under these conditions. Since plasma protein binding of streptomycin is approximately 30% [26, 27], the poor efficiency was not attributed to drug-protein interactions. More likely, DEHP interacted heavily with plasma proteins, and this suppressed mass transfer of streptomycin. Levels of DEHP higher than 75% were tested, but recoveries decreased due to the relative high viscosity of DEHP. Optimal sample pH was found at 5.0.

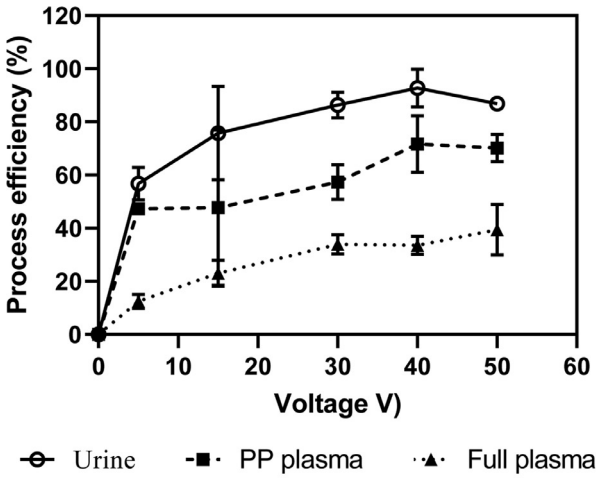
The data discussed above were obtained with 15-minute extractions at 30 V. The effects of voltage and time were investigated subsequently for all biological matrices, as seen in Fig. 4. For this, optimal sample pH and%DEHP was set according to the discussion above. The optimal extraction voltage was for all found between 30 and 50 V. However, 30 V was finally selected to limit the current level and thus ensure a more robust system. At 30 V, both urine and PP plasma reached exhaustive extraction after approximately 20 min. The highest process efficiency with full plasma was obtained after 60 min of extraction. The decrease observed after 90 min was attributed to effects of electrolysis. Poor process efficiency with full plasma could thus partially be compensated for by increasing the extraction time. The final optimal conditions are summarized in Table 2. Fig. 5 shows representative current profiles under optimal extraction conditions for the three matrices. As seen, the curves for urine and PP plasma were quite similar, while the profile for untreated plasma indicated a slower progression of mass transport. The latter was in agreement with the time-curves of Fig. 4. At the optimal extraction conditions, the average current per well was 67, 73, and 64 µA for urine, PP plasma, and full plasma, respectively, which was considered sufficiently low to provide stable and robust systems.

3.3. Clean-up efficiency from complex samples under optimal extraction conditions

The clean-up efficiency of EME from biological samples, with conditions suited for non-polar analytes, has in previous reports

Table 2
Summary of optimal extraction parameters for each sample matrix.

Matrix	Matrix:buffer ratio	Sample pH	%DEHP/NPPE in SLM	Voltage (V)	Time (minutes)
Urine	1:1	5.5	50	30	20
PP plasma	1:1	5.0	45	30	20
Full plasma	1:4	5.0	75	30	60



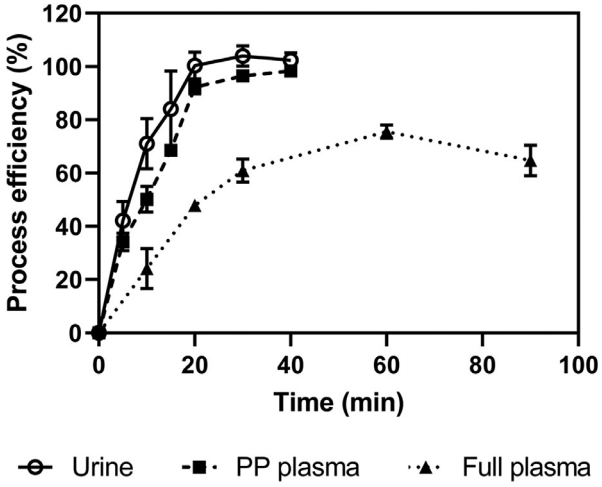


Fig. 4. Effect of extraction voltage (upper graph) and extraction time (lower graph), on process efficiency for urine, PP plasma, and full plasma. 50%, 45%, and 75% DEHP/NPPE was used as optimal SLM composition for the three samples, respectively, and the voltage curve was performed with 15-minute extractions, while the time curve was with 30 V applied. The error bars represent the standard deviation ($n = 3$).

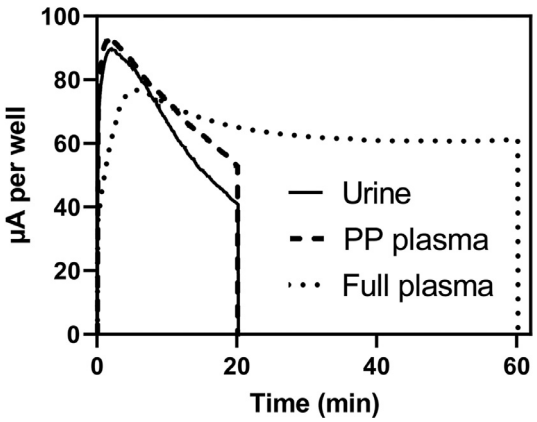


Fig. 5. Representative current profiles obtained from extraction of different matrices under optimal extraction conditions for each. The curves display the average current per well of six wells extracted simultaneously.

been excellent [28, 29]. This is because the hydrophobic SLM efficiently discriminates polar matrix constituents, and thus makes the extraction selective. A typical SLM for non-polar bases is 2-nitrophenyl octyl ether (NPOE). Compared to this, the SLM compositions identified for EME of streptomycin (Section 3.2) were much more permeable to polar bases. The extraction selectivity / clean-up efficiency from urine, PP plasma, and full plasma, under optimal extraction conditions for each matrix, was therefore studied next. Attention was focused on proteins, phospholipids, and endogenous substances with UV absorbance, positive and negative ESI-MS detection.

The total protein content of untreated plasma before and after extraction was determined by the Bradford assay (Section 2.5). For extractions with NPOE as SLM, no protein was detected in the acceptor solution after extraction. This confirms previous assumptions that proteins are completely discriminated by this SLM. With 75% DEHP/NPPE as SLM, optimized for streptomycin extraction, 0.57 ± 0.41% ($n = 3$, ±SD) of original protein in the sample was transferred to the acceptor solution. This was comparable to the clean-up expected from conventional protein-precipitation [30]. However, the Bradford assay is not specific to large proteins, and may also detect smaller peptides. The small number measured with the assay was therefore expected primarily to be peptides, which are extracted by EME [31-33].

Presence or absence of phospholipids in the acceptor solution was determined by LC-MS/MS using in-source fragmentation, as discussed in Section 2.4.2. For this, the SRM transition *m/z* 184 → 184 was monitored. This fragment corresponds to the backbone of phosphatidylcholines, lyso-phosphatidylcholines, and sphingomyelins, which account for the majority of phospholipids in plasma [22]. As reference for the original amount of phospholipid, one volume plasma (previously diluted 5-fold) was protein precipitated with two volumes ACN, centrifuged, and the supernatant was analyzed directly by LC-MS/MS. Non-extracted PP plasma samples were diluted in the same manner to reduce the concentration of phospholipids prior to injection. The chromatograms of the non-extracted references are shown in the top of Fig. 6. EME was performed according to the optimized conditions (Table 2), and acceptor solutions were subsequently diluted 1:2 to allow direct quantitative comparison with the references. EME with NPOE as SLM was also performed to compare with conditions suited for non-polar analytes. With NPOE, no traces of phospholipids were found in the acceptors after EME (green traces, Fig. 6). This confirmed previous data [34]. Under EME conditions optimized for streptomycin, small traces were identified in the acceptor after extraction (R_t 2.9 min, red trace, Fig. 6). For untreated plasma, the peak area after EME was however only 0.02% of the reference (original sample), and for PP plasma the corresponding value was 0.18%. Although traces of phospholipids were detected, the clean-up was thus very good and represented >500-fold improvement compared to a simple protein precipitation strategy.

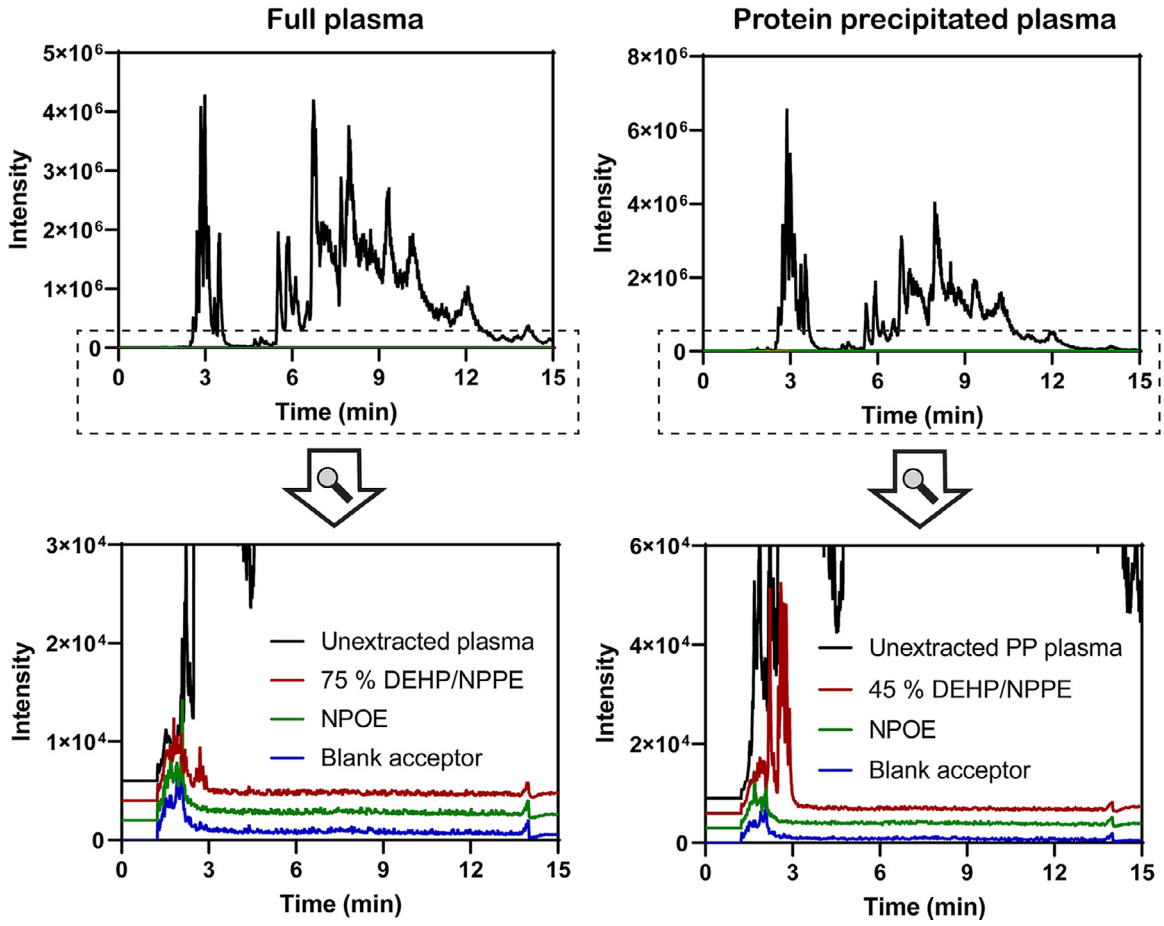


Fig. 6. Representative LC-MS/MS chromatograms of phospholipids (*m/z* 184 → 184) found in the acceptor solution after extraction with optimal conditions (red trace) for full plasma (left panel) and PP plasma (right panel). For both conditions, extractions with NPOE (green trace) as SLM were included for comparison to a system suited for non-polar substances. The black traces are the unextracted samples. Each extraction was performed in triplicate.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Finally, the clean-up of endogenous substances was evaluated by HILIC analysis with UV-254 nm, as well as positive and negative full scan ESI-MS detection. The analysis was thereby capable of detecting a wide range of substances. For UV-detection, 254 nm was found to provide the best visualization of matrix peaks. This was because the mobile phase gradient gave substantial changes in baseline absorption at lower wavelengths. Representative UV-chromatograms are shown in Fig. 7. For urine, the optimal EME system for streptomycin (red trace) provided high clean-up efficiency for the vast majority of matrix components, with only few minor matrix substances present in the acceptor. One major peak was detected at 2.4 min, and this was attributed to creatinine (highly abundant in urine). Total ion chromatograms (Figure S3) obtained from the MS detection showed a similar trend, that some matrix substances were present in the acceptor after extraction. However, acceptable selectivity was achieved, despite that the SLM was optimized for an analyte of extreme polarity.

The high selectivity resulted from discriminative effects of both the electrical field and chemical composition of the SLM. Under the optimal conditions for streptomycin, net anionic substances were retained in the sample due to the direction of the electrical field. Neutral substances were not influenced by the electrical field, and mainly distributed between the sample and the SLM according to hydrophobicity. Net cationic substances were forced towards the SLM/acceptor, but discrimination occurred based on sample pH and% DEHP in the SLM. The latter parameters were specifically optimized for streptomycin (Fig. 3), and because this optimum is very variable for individual substances dependent on their hydrophilicity and charge [13], many net cationic substances were discriminated. These substances either remained in the sample, or were trapped in the SLM. Extraction of an extremely polar substance from a polar matrix could thus be achieved with reasonable selectivity. The same trend was observed with EME from PP plasma and untreated plasma, albeit UV-signals of matrix substances were less.

3.4. Evaluation of data reliability

Finally, the analytical reliability of the proposed extraction method was evaluated, with urine and untreated plasma samples extracted according to Table 2. The validation data are provided in Table 3. The linear range of calibration was from 20–500 ng mL^{-1} and 100–5000 ng mL^{-1} for urine and plasma, respectively, with an $R^2 \geq 0.9929$. Accuracy was within 94–107%, except for plasma at LLOQ (125%). Similarly, repeatability was within 15%, except at LLOQ. Matrix effects were insignificant for urine, while slight ion enhancement was observed for full plasma samples.

The current data were not intended to be a full validation of the proposed extraction method; the purpose was rather to demonstrate that reliable extraction performance could by achieved for an extremely polar substance like streptomycin from complex matrices with EME.

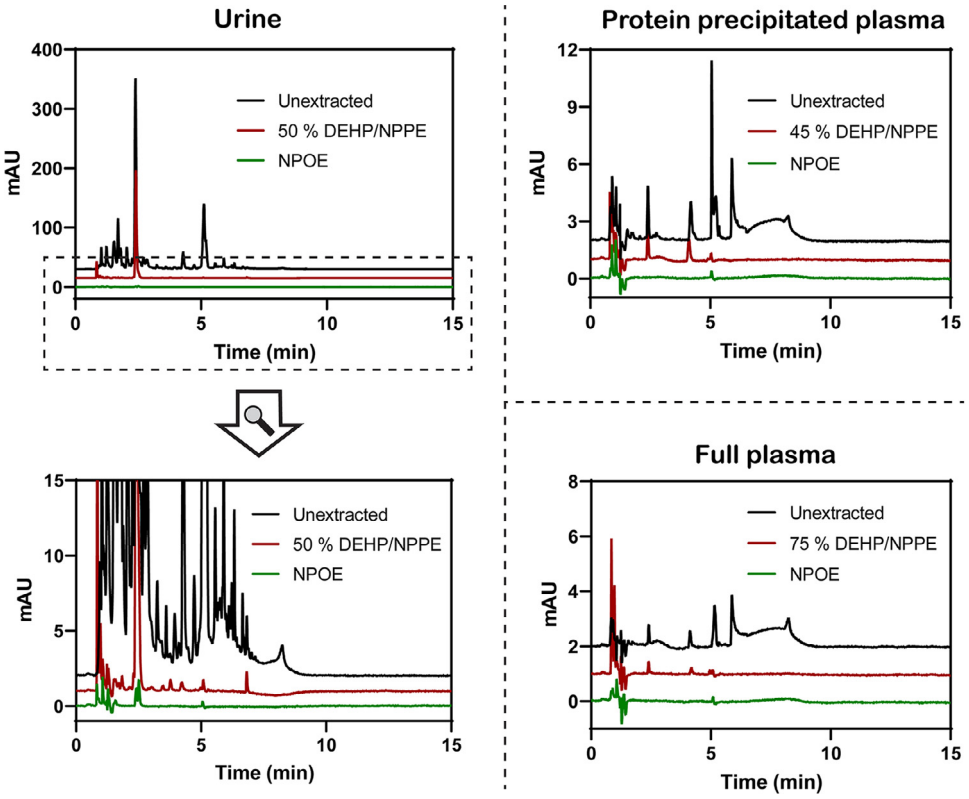


Fig. 7. Representative HILIC-UV chromatograms at 254 nm of acceptor solutions after extraction of urine, PP plasma, and full plasma, under optimal conditions (red trace) for each sample. Similar extractions with NPOE as SLM were included for comparison with a system suited for non-polar substances. The black traces are the unextracted samples. All extractions were performed in triplicate. Prior to injection, all samples were mixed 1:1 with ACN for compatibility with HILIC conditions.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Validation data for EME of streptomycin from urine and plasma. Process efficiency (PE) and matrix effects (ME) were determined at 200 ng mL^{-1} and 1000 ng mL^{-1} for urine and plasma, respectively. Calibration curves were weighted by 1/x. Internal standard was added prior to extraction, except for determination of PE where it was added post extraction. 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 represent the upper limit of quantitation (i.e. upper linear range). All concentrations are in ng mL^{-1}.

Matrix	Linear range ($n = 4$)	R^2	PE (%, $n = 4$)	LOD	LLOQ	ME (%) ± SD ($n = 4$)	Accuracy (%, $n = 4$)			Repeatability (%, $n = 4$)		
							LLOQ	Within range	ULOQ	LLOQ	Within range	ULOQ
Urine	20–500	0.9991	98	8	20	97 ± 9	107	94*	101	21	4*	9
Full plasma	100–5000	0.9929	61	40	100	112 ± 9	125	100**	104	36	11**	15

* at 100 ng mL^{-1},.
** at 500 ng mL^{-1}.

4. Conclusion

The present study has reported successful EME of streptomycin (log $P = -7.6$) for the first time. The extraction was enabled by addition of bis(2-ethylhexyl) phosphate (DEHP) as ionic carrier to the SLM comprising 2-nitrophenyl pentyl ether (NPPE). The interrelationship between carrier content, pH, co-solvent, recovery, repeatability, and extraction current (i.e. system stability), was studied carefully during method development. This was done based on design-of-experiments (DOE) using urine and plasma samples. The data obtained demonstrated that the optimal amount of DEHP in the SLM, pH in the sample, and the mechanism of complexation, were different with water, urine, and plasma samples. This was because matrix components partly interacted and interfered with DEHP.

Using optimized conditions for urine and plasma, the selectivity (i.e. clean-up) of streptomycin extraction was evaluated. Proteins and phospholipids were almost entirely discriminated by the SLM, and this was the case also for the majority of endogenous substances with UV absorbance. Lastly, the proposed EME method was demonstrated to give reliable analytical data with exhaustive extraction from urine and 61% extracted from full plasma.

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.

CRediT authorship contribution statement

Frederik André Hansen: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - re-

view & editing, Visualization. **Stig Pedersen-Bjergaard:** Methodology, Formal analysis, Writing - review & editing, Supervision.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.461915.

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