RESEARCH ARTICLE

WILEY

Determination of amphetamine enantiomers in urine by conductive vial electromembrane extraction and ultra-high performance supercritical fluid chromatography tandem mass spectrometry

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Abstract

Separation and quantification of amphetamine enantiomers are commonly used to distinguish between consumption of prescription amphetamine (mostly *S*-amphetamine) and illicit forms of the drug (racemate). In this study, electromembrane extraction with prototype conductive vials was combined with ultra-high performance supercritical fluid chromatography (UHPSFC-MS/MS) to quantify *R*- and *S*-amphetamine in urine. Amphetamine was extracted from 100 μL urine, diluted with 25 μL internal standard solution and 175 μL 130 mM formic acid, across a supported liquid membrane (SLM) consisting of 9 μL of a 1:1(w/w) mixture of 2-nitrophenyloctyl ether (NPOE) and bis(2-ethylhexyl)phosphite (DEHPi) into an acceptor phase containing 300 μL 130 mM formic acid. The extraction was facilitated by the application of 30 V for 15 min. Enantiomeric separation was achieved using UHPSFC-MS/MS with a chiral stationary phase. The calibration range was 50–10,000 ng/mL for each enantiomer. The between-assay CV was ≤5%, within-assay CV ≤ 1.5%, and bias within ±2%. Recoveries were 83%–90% (CV ≤ 6%), and internal standard corrected matrix effects were 99–105 (CV ≤ 2%). The matrix effects ranged from 96% to 98% (CV ≤ 8%) when not corrected by the internal standard. The EME method was compared with a chiral routine method that employed liquid–liquid extraction (LLE) for sample preparation. Assay results were in agreement with the routine method, and the mean deviation between methods was 3%, ranging from −21% to 31%. Finally, sample preparation greenness was assessed using the AGREE-prep tool, which resulted in a greenness score of 0.54 for conductive vial EME, opposed to 0.47 for semi-automated 96-well LLE.

KEYWORDS
amphetamine, chiral chromatography, electromembrane extraction, sample preparation, supercritical fluid chromatography

1 | INTRODUCTION

Although being one of the most common drugs of abuse world-wide, amphetamine is also used therapeutically in treatment of ADHD and narcolepsy. The chirality of the molecular structure enables distinction between licit and illicit forms of the drug. Prescription amphetamine licensed in Norway contains the most pharmacologically active S-enantiomer, either as pure dextroamphetamine or lisdextroamphetamine (prodrug).[1] On the other hand, amphetamine on the illicit market mostly contains a racemic mixture.[2,3] In cases where patients treated with amphetamine are subjected to drug testing, the concentration ratio of R- and S-amphetamine is used to elucidate the origin of the drug.[4] Enantioselective analysis of biological samples is herein necessary to distinguish between an illegal and a legitimate amphetamine intake.[5,6]

Supercritical fluid chromatography (SFC) is a well-established method for chiral separation.[7] Using large fractions of supercritical CO_2 as the mobile phase allows high flow rates without loss of efficiency and resolution, which reduces analysis time. Modern ultra-high performance SFC (UHPSFC) systems coupled to mass spectrometry enables robust, sensitive, and rapid routine analysis of amphetamine enantiomers in urine and serum.[5,6]

Sample preparation remains a critical step in bioanalysis. In analysis of urine, a simple dilution step is often sufficient.[8] In some cases, extraction techniques such as liquid–liquid extraction (LLE) or solid phase extraction (SPE) are required to preserve the chromatographic column and the mass spectrometer. In this paper, we investigated electromembrane extraction (EME) as sample preparation alternative to a routine LLE method.

EME is a microextraction technique in which an electric field is applied to facilitate transfer of ionized analytes from an aqueous donor phase (e.g., blood and urine) into a hydrophobic supported liquid membrane (SLM) and further into a clean aqueous acceptor phase (pH adjusted water). The SLM consists of a small volume ($\sim$10 μL) of organic solvent immobilized in a porous membrane. With EME, extraction and clean-up of the sample can be performed in one step, with minimal consumption of organic solvents. EME has been carried out in a number of technical formats based on hollow-fibres, 96-well plates,[9–11] and microchip technology.[12–14] A commercial EME device based on conductive vials is under development, and prototype equipment has been used in extraction of basic drugs from serum[15] and plasma.[16] Validation data associated with the prototype device were accordance with recommended guidelines for bioanalytical measurements.[17,18]

EME selectivity depends mainly on the polarity and magnitude of the electric field and the physiochemical properties of the SLM.[19] Additionally, the mass transfer of analytes is affected by the sample composition, through the donor pH, presence of complexing agents,[20] nature of background electrolytes,[21] and ionic strength.[22] In urine samples, the aforementioned parameters can vary depending on the health, diet, and the hydration state of individuals. The natural variations in urine constituents are expected to cause matrix-related variations in recovery, which could ultimately affect the analytical results.

The objective of this research was to determine whether conductive vial EME is a viable sample preparation alternative for determination of amphetamine enantiomers in urine when combined with UHPSFC-MS/MS. An EME-UHPSFC-MS/MS method was developed, validated, and compared with a routine LLE-UHPSFC-MS/MS method at the Department of Clinical pharmacology at St. Olav University Hospital (Trondheim, Norway). We also investigated fundamental aspects of the EME process by studying to what extent natural variations in urine pH and creatinine concentrations affect mass transfer of amphetamine.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Certified reference material of racemic (50%:50%) R-/S-amphetamine was purchased from Lipomed (Arlesheim, Switzerland) and Chiron (Trondheim, Norway) to prepare calibrators and quality control samples, respectively. S-amphetamine (purity 99.97%) and racemic R-/S-amphetamine-d_3 were purchased from Lipomed. bis(2-Ethylhexyl) phosphate (DEHP), bis(2-ethylhexyl)phosphite (DEHPi), 2-nitrophenyl octyl ether (NPOE), 2-nitrophenyl pentyl ether (NPPE), and ammonium formate (≥99.995%, trace metal basis) were purchased from Sigma Aldrich (Schnelldorf, Germany). Formic acid (99.0% HCOOH, Optima LC–MS grade) was from Fischer Scientific (Leicestershire, UK). Methanol (MeOH, LC–MS grade), ammonium hydroxide, and ammonia solution (25% NH_3, LiChropur for LC–MS) were from Merck (Darmstadt, Germany). Isopropyl alcohol (IPA, HiPerSolv Chromanorm) was obtained from VWR Chemicals (Leuven, Belgium). Type I water was obtained with an in-house Milli-Q purification system from Millipore (Molsheim, France). Carbon dioxide (grade 5.2/99.9992%) was obtained from Aga (Oslo, Norway).

2.1.1 | Preparation of solutions and storage conditions

Two separate stock solutions of racemic amphetamine (5 mg/mL) for calibrators and quality control (QC) were prepared in type I water. Stock solutions were diluted with MeOH to prepare working solutions for each calibration and QC level. The stock and working solutions were stored at 4°C. Urine calibrators of 50, 250, 750, 2000, and 10,000 ng/mL of each enantiomer were prepared by spiking blank urine with working solution (1% v/v) and were stored at −20°C. Similarly, QC samples were prepared at four concentrations; 50 (LLOQ), 75, 1000, and 8000 ng/mL. The internal standard solution (2.5 μg/mL R-/S-amphetamine-d_3) was prepared in 20% MeOH in H_2O and stored at 4°C.

2.1.2 | Urine samples

A healthy volunteer supplied pooled amphetamine-free urine for method development, calibration, and quality control. The urine had a pH of 6.2 and creatinine concentration of 68.7 mg/dL. External quality

control urine samples were obtained from LGC Standards Proficiency Testing (Bury, UK).

Anonymized urine samples that had been to our laboratory for drug testing were analyzed for method comparison. The samples were stored for 4 weeks at 4°C, then freeze stored (-20°C) for 1 to 3 weeks. In some experiments, anonymized samples free of amphetamine were spiked. According to the Regional Committee of Research Ethics, no formal approval is needed for a brief presentation of routine results as part of a methodological article.

2.2 | Conductive vial EME

EME was performed with a prototype device from Extraction Technologies Norway (ETN, Ski, Norway). The prototype (Figure 1) held 10 EME cells that could operate simultaneously. One EME cell consisted of a donor vial and an acceptor vial connected by a leak tight union that held a flat porous polypropylene (PP) membrane (168 μm, Accurel PP2E, Membrana GmbH; Wuppertal, Germany). The vials were made from a proprietary conductive material. The donor vials held acidified urine samples whereas the acceptor vials held the acceptor solutions. Each vial had a total volume of 600 μL, with working volumes of 200–400 μL. The SLM was prepared by pipetting 9 μL organic solvent onto the PP support membrane. EME cells were placed horizontally on the agitation device to ensure contact between

(a)

(b)

FIGURE 1 (a) Prototype EME device holding 10 EME cells (left) and a dissembled EME cell consisting of conductive vials, union, and polypropylene membrane (right). (b) EME of protonated bases in conductive vials. BH$^+$ represents the protonated base, a$^-$ a deprotonated acid, and N a neutral molecule

the aqueous solutions and the SLM. The electric field was applied to each EME cell by a power supply (ES 0300-0.45, Delta Elektronika BV, Zierikzee, the Netherlands) that connected to the vial surfaces via electrodes in the device lid. To extract cations, the acceptor and donor vial were made cathodic and anodic, respectively. The system current was monitored with a Fluke287 multi-meter (Everett, WA, USA).

Parameters optimized in method development were the composition of the organic phase, sample diluent and acceptor, extraction potential and time. Recovery experiments during method development were carried out using spiked urine (2000 ng/mL racemic mixture), applying 50 V for 15 min unless stated otherwise.
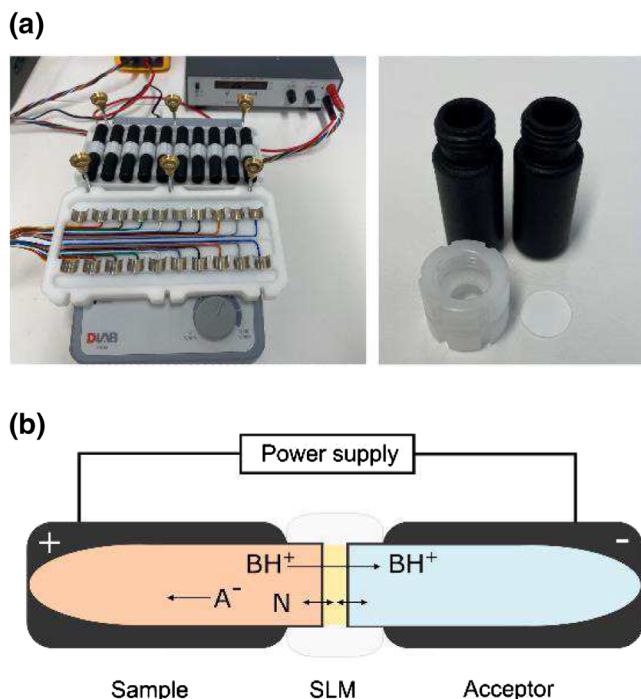
The final extraction protocol was as follows. For each EME cell, the donor vial was filled with urine (100 μL), internal standard solution (25 μL, R-/S-amphetamine-d_3), and sample diluent (175 μL, 130 mM HCOOH). The acceptor vial contained 130 mM HCOOH (300 μL). The leak–tight union was screwed onto the acceptor vial, and 9 μL membrane solvent (mixture of DEHPi and NPOE [1:1, w/w]) was pipetted onto the membrane. EME cells were assembled and placed in the prototype device. Extraction was carried out by applying 30 V for 15 min with agitation (875 rpm). After EME, the acceptor vials were capped and placed on the auto sampler for UHPSFC-MS/MS analysis.

Due to a limited number of available prototype conducting vials, used vials were washed and reused during method development. To avoid carry-over, vials were washed using the following procedure: (1) empty vials were filled with 1% HCOOH in MeOH overnight, (2) vials were rinsed with type I water, and (3) MeOH. During validation, concentrated calibrators and quality controls (Std 3–5, QC2–3) were extracted with reused vials, whereas low concentrated samples (Blanks, Std1–2, LLOQ samples, QC1), external controls, and anonymized patient samples were extracted with new vials.

2.3 | Liquid–liquid extraction: Routine sample preparation method

The current routine method for the determination of amphetamine enantiomers in urine is a modification of the corresponding method for serum.[5] Semi-automated LLE was performed using the Hamilton Microlab Star pipetting robot (Hamilton Company, Bonaduz, Switzerland). Urine (100 μL), internal standard solution (25 μL, R-/S-amphetamine-d_3) and buffer (100 μL, 0.2 M Na$_2$CO$_3$) were pipetted into a 2 mL 96-well collection plate (Porvair Sciences, Norfolk, UK) and mixed at 700 rpm for 20 s. Ethyl acetate (EtOAc, 600 μL) was added to each well, and the collection plate was sealed with foil and mixed (Multi-format Plates & Tubes, Porvair Sciences) at 2100 rpm for 1 min before centrifugation at 4235 RCF (Rotana 460, Hettich Zentrifugen, Tuttlingen, Germany) for 5 min. Aliquots of supernatant (200 μL) were transferred to a new 2 mL 96-well collection plate and acidified with 10 μL 0.3 M HCl in MeOH. The samples evaporated to dryness under ambient air at 40°C for 10 min (UltraVap, Porvair Sciences, North Wales, UK) and were reconstituted in 300 μL IPA.

Extracts were analyzed with the UHPSFC-MS/MS conditions described below. The method was validated prior to its

implementation to the routine laboratory, and the following results were then obtained: within-assay CVs ≤ 1.8%, between-assay CVs ≤ 1.8%, bias within ±4.7%, matrix effects 83%–111% (CV ≤ 4%), IS-normalized matrix effects 96–100% (CV ≤ 3%), and extraction recoveries 87%–94% (CV ≤ 3%).

2.4 | Ultra high performance supercritical fluid chromatography tandem mass spectrometry

A Waters Acquity ultra performance convergence chromatography (UPC^2) (Waters, Milford, MA, USA) system equipped with sample manager, binary solvent manager, column manager, convergence manager, and isocratic solvent manager was used. Amphetamine enantiomers were separated on an AD-3 [amylose tris (3,5-dimethylphenylcarbamate), 2.1 × 150 mm, 3.0 μm particles] column from Chiralpak Technologies (Danciel group, Illkirch, France). Partial loop injection in needle overfill mode was used with a 10 μL loop and injection volume of 1 μL. The isocratic elution profile (A: CO_2, B: 0.1% NH_4OH in IPA:MeOH [1:1, v/v]) was 6.5% B for 4.0 min. The flow rate was 1 mL/min, and the column temperature was 10°C. The automatic back-pressure regulator was set to 2500 psi. The make-up solvent was 0.1% NH_4OH in IPA delivered with a flow rate of 0.3 mL/min.

A Xevo TQ-S micro tandem quadrupole mass spectrometer (Waters, Manchester, UK) equipped with a Z-spray electrospray interface was used for detection. Positive electrospray ionization (ESI+) was performed in the multiple reaction monitoring (MRM) mode. The capillary voltage was 1.0 kV, ion source temperature 120°C, and cone gas flow rate 20 L/h. The desolvation gas (nitrogen) was heated to 600°C and delivered with a flow rate of 1000 L/h. MRM transitions for R/S amphetamine were m/z 136.1 > 119.0 (quantifying ion, cone voltage: 10 V, collision energy 8 eV) and m/z 136.1 > 91.0 (qualifying ion, cone voltage: 10 V, collision energy 8 eV), and m/z 139.1 > 122.0 (cone voltage: 10 V, collision energy: 8 eV) was monitored for R-/S-amphetamine-d_3.

2.5 | Creatinine and pH measurements

Urine creatinine and pH measurements were performed on an AU680 Chemistry Analyzer (Beckman Coulter, Brea, CA, USA) with DRI® Creatinine-Detect Test and DRI® pH-detect Test reagents (Microgenics Thermo Fisher, Passau, Germany), respectively.

2.6 | Validation

The EME-UHPSFC-MS/MS method was validated based on guidelines given by Peters et al.[23] and the European Medicines Agency (EMA).[18] Validation parameters were linearity, limit of detection (LOD), lower limit of quantification (LLOQ), within- and between-assay precision, accuracy, extraction recovery, matrix effects, selectivity, carry-over, and stability of extracted samples.

Quantification was performed applying weighted (1/x) quadratic calibration based on analyte peak area normalized to IS area on a 5-point calibration curve in the concentration range 50–10,000 ng/mL of each enantiomer. The linearity was evaluated by assessing the correlation coefficient (R) with linear calibration including three replicates of each calibrator. The lower limit of quantification (LLOQ) was set to the lowest calibrator level. Precision and accuracy at LLOQ were assessed by analyzing blank urine spiked with 50 ng/mL of each enantiomer on 10 days. The LOD was estimated through a series of scalar dilutions of LLOQ. The LOD was the concentration corresponding to a signal-to-noise ratio (S/N) above 3 for both the quantifier and qualifier ion.

Between-assay precision and accuracy was evaluated at three QC levels (75, 1000, and 8000 ng/mL) with 10 replicates. Within-assay precision was studied with six replicates. Extraction recoveries were determined at the highest and lowest QC concentration ($n = 6$). Recovery was calculated by comparing IS normalized analyte peak areas in urine samples spiked with R-/S-amphetamine pre- and post-extraction, with the internal standard added post-extraction. Matrix effects (ME) were assessed at the highest and lowest QC concentration by comparing analyte peak areas in spiked blank extracts ($n = 6$, different individuals) and neat acceptor solutions (130 mM HCOOH, [$n = 3$]). Selectivity was assessed by analyzing amphetamine-free urine from six individuals. Instrument carry-over was investigated by inspecting chromatograms of extracted blank urine injected after a sample spiked to a concentration of 20,000 ng/mL. Analyte stability in extracts was evaluated by reinjecting QC samples ($n = 4$ per concentration) and calibrators left on the auto sampler (10°C) for 1, 3, and 7 days.

2.7 | Application: Comparison with routine method at St. Olav University Hospital

Assay results of anonymized patient samples were compared between EME-UHPSFC-MS/MS and the pre-established routine method. Anonymized urine samples ($n = 31$) analyzed with the routine method were reanalyzed with EME after they had been stored for 4 weeks at 4°C and 3 weeks at −20°C. Assay results were compared using Passing and Bablok regression using MedCalc Statistical Software version 20 (MedCalc Software Ltd, Ostend Belgium). Concentrations outside the calibration range ($n = 2$) were excluded from the regression analysis.

2.8 | Evaluation of greenness

Greenness of sample preparation methods was assessed using the AGREEprep tool proposed by Wojnowski et al.[24] A greenness score from 0 (not fulfilling) to 1 (fulfilling) was given based on 10 principles of green sample preparation. Each category was weighted based on importance. Scores were calculated using available software[25] and input guidelines given by Pena-Pereira et al.[26] Conductive vials were regarded as disposable. Details regarding AGREEprep input are included in Supporting Information.

3 | RESULTS AND DISCUSSION

3.1 | The supported liquid membrane

EME can be regarded as an electro-assisted partitioning process, and the chemical properties of the membrane solvent play a key role in selectivity. The following SLM compositions were evaluated for EME of amphetamine from urine ($n = 3$): 2-nitrophenyl octyl ether (NPOE), 2-nitrophenyl pentyl ether (NPPE), bis(2-ethylhexyl) phosphite (DEHPi), 5% w/w bis(2-ethylhexyl) phosphate added to NPOE (DEHP +NPOE [5:95]), and a 1:1 (w/w) mixture of DEHPi and NPOE (DEHPi +NPOE [1:1]). Amphetamine recoveries are shown in Figure 2a. The use of the pure nitroaromatic solvents (NPOE and NPPE) resulted in low recoveries of amphetamine. NPOE is the most widely used solvent in EME, but efficient mass transfer is limited to hydrophobic analytes,[27] whereas amphetamine is a moderately polar drug (log P = 1.8). Common strategies to increase partitioning of polar basic analytes are to add ionic carriers (DEHP) to NPOE or to select a solvent that either is less hydrophobic (NPPE) or interacts through other mechanisms than that of NPOE.[28] Although mass transfer of amphetamine was increased with NPPE compared with NPOE, the extraction was still inefficient with both nitroaromatic solvents. DEHPi was the only efficient single-component membrane solvent, resulting in a recovery of 53%.

The most effective SLM compositions were the mixed SLMs that contained NPOE and an organophosphorus component (DEHP +NPOE (5:95) and DEHPi+NPOE (1:1)). Amphetamine recovery increased from 0% to 82% upon addition of 5% DEHP to NPOE. DEHP is an anionic (pKa = 2) carrier, and analyte transport across the SLM is facilitated through formation of ion pairs.[29] Contrarily, DEHPi is a neutral compound and analyte interaction occurs mainly through strong hydrogen bond interactions.[30] The DEHPi+NPOE (1:1) mixture was recently found to be effective in EME of amphetamines from breast milk[31] and resulted in a recovery of 74% in the current experiment. The presented results are coherent with previous EME of amphetamine from plasma, urine, and breast milk, where amphetamine was better extracted when NPOE was mixed with other compounds[31,32] or accompanied by modifications of the acceptor.[33]

The extraction current was recorded (Figure 2b) and used as a diagnostic tool to evaluate SLM integrity and system stability.[27,34] Systems were regarded as stable when the current was below 50 μA per extraction cell and did not increase over time.[27] Excessive current can affect reproducibility and result in low recoveries due to pH shifts and bubble formation caused by water electrolysis.[35,36] As seen from Figure 2b, all five SLM compositions resulted in stable systems. Interestingly, mixing DEHPi with NPOE resulted in half the extraction current of DEHPi as well as improving amphetamine recovery.

3.2 | Effect of urine pH and creatinine concentration

Previous experiments and theoretical models suggest that EME mass transfer can be affected by pH, salt content,[37,38] ionic strength, and ion balance.[39] In drug testing of urine, the pH and creatinine concentration are determined to detect adulteration of samples. In the following experiments, the effect of urine pH and creatinine on EME mass transfer was assessed to investigate whether these parameters could also affect recovery. Recovery experiments were carried out with seven urine samples with pH 5.3–8.9 and creatinine concentrations 20–317 mg/dL. Amphetamine recovery was determined in each sample after 15-min extractions with both DEHP+NPOE (5:95) and DEHPi+NPOE (1:1) as membrane solvents. The applied voltage was 50 V in extractions with the former SLM and 30 V with the latter. The extraction current was found to increase with increased creatinine concentration (Figure 3c), and the voltage was therefore lowered in extractions with DEHPi+NPOE (1:1) to maintain I < 50 μA in samples high in creatinine. The calculated recoveries were in the range from 58% to 90% (CV 14%) and 66% to 99% (CV 14%) with DEHP+NPOE (5:95) and DEHPi+NPOE (1:1), respectively. As expected, there were variations in amphetamine mass transfer between individual urine
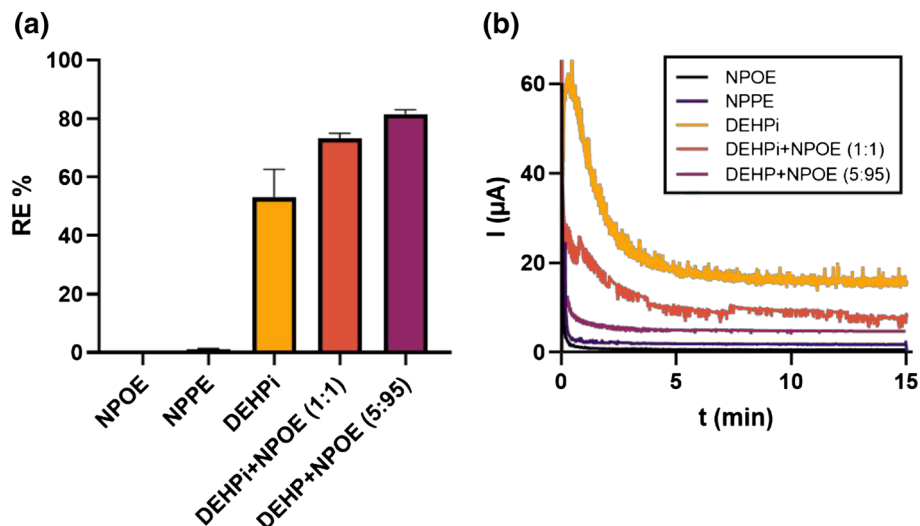
FIGURE 2 Extraction of amphetamine (2000 ng/mL racemic mix) from urine (50 V, 15 min) using five different membrane solvents. (a) Recoveries ($n = 3$) and (b) measured current (I) per EME cell
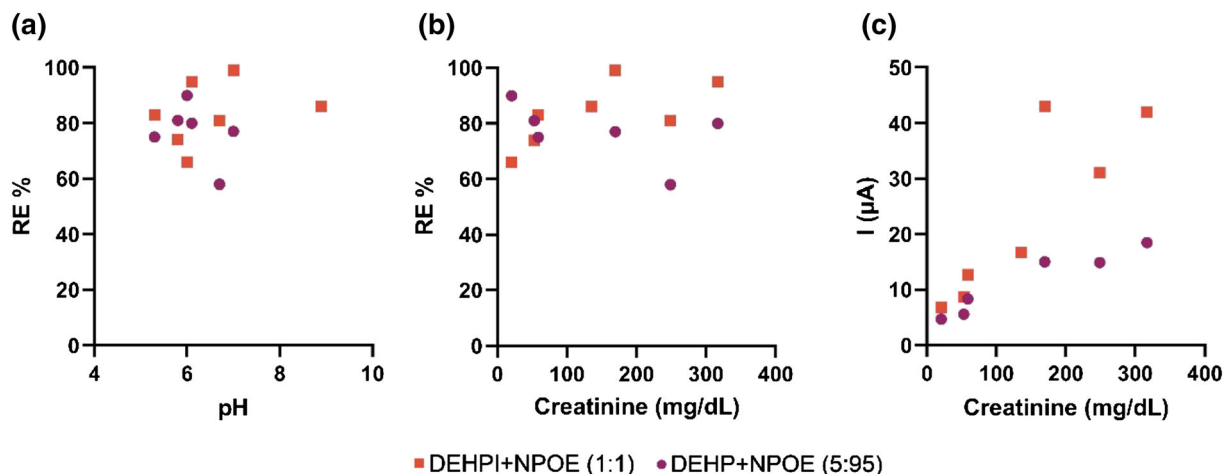
FIGURE 3 Amphetamine recovery from urine samples varying in (a) pH and (b) creatinine concentration. (c) The measured current (I) as a function of creatinine using two different membrane solvents; DEHP+NPOE (5:95) (50 V) and DEHPi+NPOE (1:1) (30 V). $T = 15$ min, spike conc. $= 2000$ ng/mL racemic amphetamine

samples, and the extraction was exhaustive in some samples, but not in others.

Figure 3a,b shows amphetamine recovery as a function of pH and creatinine concentration (in raw urine samples). With the selected samples ($n = 7$), the variation in recovery between urine samples could not be attributed to differences in pH or creatinine alone but were likely related to a combination of parameters that vary in spot urine. Although variation in recovery between urine samples was apparent, the internal standard compensated for this. CV percentage of recovery between samples was reduced from 14% (absolute recovery) to <3% when analyte areas were corrected with the deuterated internal standard added prior to EME.

3.3 | EME method development

The final EME protocol for determination of amphetamine enantiomers in urine was developed with attention to the membrane solvent, sample diluent and acceptor phase, extraction potential and extraction time. DEHPi+NPOE (1:1, w/w) was selected as the final membrane solvent due to favorable recoveries and precision. In addition DEHPi was considered less hazardous compared with DEHP.

During initial experiments, the sample diluent and acceptor phase were both 130 mM formic acid. An experiment using 130 mM HCOOH, 200 mM NH$_4$COOH buffer with pH 3.7, and pH 2.8 as sample diluent and acceptor was conducted to evaluate whether the use of a strong buffer could improve between-sample variation in recovery by suppressing differences in pH and ionic strength. No significant difference in CVs of absolute recoveries between urine samples were observed when using a formate buffer vs formic acid; 130 mM HCOOH was thus kept as the final sample diluent and acceptor.

The final extraction potential was 30 V, which allowed sufficient precision, recovery and acceptable current. Figure 4a shows amphetamine recovery after application of 0, 15, and 30 V for 15 min. The experiment verified that the applied potential was essential for mass
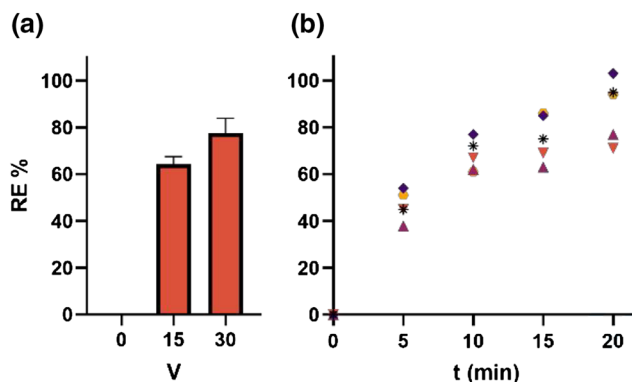
FIGURE 4 Amphetamine recovery with varying (a) voltage ($t = 15$ min) and (b) extraction time (30 V) with DEHPi+NPOE (1:1) as SLM. Symbols represent unique samples

transfer, as amphetamine was not extracted in absence of the electric field. Figure 4b shows amphetamine recovery from five different urine samples after extraction for 5, 10, 15, and 20 min. Extraction kinetics differed among individual samples. In two samples, the mass transfer haltered after 10 min, whereas exhaustive extraction (recovery >85%) could be reached after 20 min for the remaining samples. The final extraction time was selected as 15 min.

In summary, the final extraction protocol comprised application of 30 V for 15 min, using DEHPi+NPOE (1:1) as membrane solvent and 130 mM HCOOH as both sample diluent and acceptor. The selected parameters enabled acceptable recovery and precision with additional benefits of operational simplicity and acceptable sample throughput.

3.4 | Validation

Validation parameters are summarized in Table 1. The calibration curves were linear in the range from 50 ng/mL (LLOQ) to 10,000 ng/

TABLE 1 Validation data for amphetamine enantiomers in urine.

Analyte, concentration (ng/mL)	Calibration range (ng/mL)	Linearity (R)	LOD (ng/mL)	LLOQ (ng/mL)	Within-assay CV (%)	Between-assay CV (%)	Bias (%)	RE (CV %)	ME (CV %)	IS-corrected ME (CV %)
R-amphetamine	50–10,000	0.9989	2.5	50						
75					1.1	3	−0.3	83 (6)	98 (2)	99 (1)
1000					1.2	5	0.1			
8000					1.3	4	−0.7	90 (2)	99 (1)	99 (1)
S-amphetamine	50–10,000	0.9987	5	50						
75					0.9	4	−2	84 (6)	97 (8)	105 (2)
1000					0.8	5	−0.5			
8000					1.5	3	0.3	90 (2)	96 (7)	100 (1)

Note: Calibration range, coefficient of correlation (R), limit of detection (LOD), lower limit of quantification (LLOQ), within- ($n = 6$) and -between assay ($n = 10$) precision, bias ($n = 10$), extraction recovery (RE, $n = 6$), matrix effects (ME, $n = 6$) and matrix effects corrected with internal standard ($n = 6$).
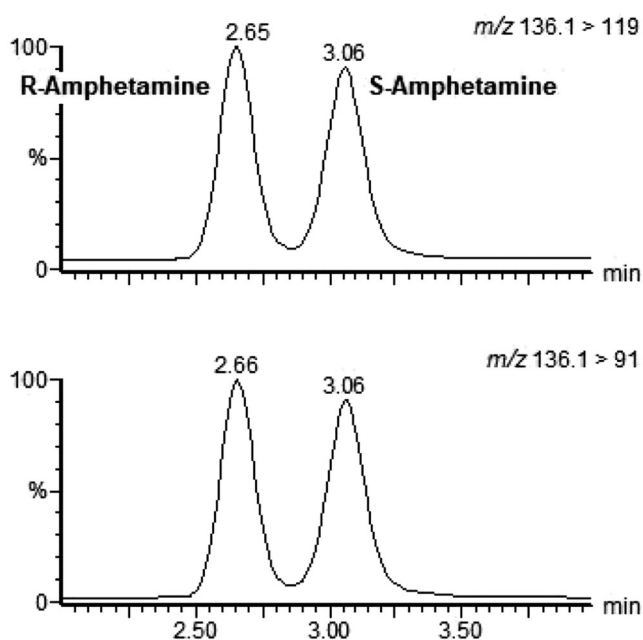
FIGURE 5 MRM chromatograms of R/S-amphetamine in urine at the lowest calibration level (50 ng/mL of each enantiomer)

mL, with $R \geq 0.9987$ for both enantiomers. MRM chromatograms of R-/S-amphetamine at the lowest calibrator level are shown in Figure 5. The elution order was confirmed by analysis of pure S-amphetamine.[5] Estimated LODs (S/N > 3) were 2.5 and 5 ng/mL for R-amphetamine and S-amphetamine, respectively. The LLOQ was set to the lowest calibration concentration (50 ng/mL), and S/N was >30. The between assay ($n = 10$) CV at LLOQ was 7% for both enantiomers and bias was <1.1%. QC samples at low (75 ng/mL), middle (1000 ng/mL), and high (8000 ng/mL) concentrations were quantified with bias within ±2%, within-assay CVs ≤ 1.5%, and between-assay CVs ≤ 5%. External controls ($n = 3$, S-amphetamine) were quantified with acceptable accuracy (|Z| ≤ 1.9).

Extraction recoveries (RE) were 83% (CV = 6%) at low concentration and 90% (CV = 2%) at the high concentration. Matrix effects (ME) were 96%–99% (CV ≤ 8%), which indicated neither signal

suppression nor enhancement. When corrected with IS, the matrix effects were 99%–105% (CV ≤ 2%).

Carry-over in blank samples injected after a concentrated sample (20,000 ng/mL) was 26%–36% of the lowest calibrator, which exceeded recommended requirements (20% of LLOQ[18]). Attention to carry-over should thus be taken in routine analysis. Samples that arrive at our laboratory are first screened with a non-chiral screening method for multiple drugs of abuse.[8] Selected positive amphetamine samples are further analyzed with the enantioselective method. Thus, the total amphetamine concentration is known prior to analysis, and blank samples are injected after concentrated samples. A note should be made that the carry-over seemed to be related to the high water content in EME acceptor phases. This is further discussed in Section 3.5.

No interfering peaks were found in extracted blank urine ($n = 6$). Extracted QC samples were stable at 10°C (auto sampler) for at least 7 days.

3.4.1 | Method comparison with routine lab

The EME-UHPSFC-MS/MS method was compared with the current enantioselective routine method. The two methods differ only in the sample preparation step. In the routine method, amphetamine is extracted from urine using LLE followed by solvent evaporation and reconstitution. The LLE-UHPSFC-MS/MS method has been applied in drug testing for 15 months in analysis of over 6250 samples.

Anonymized urine samples ($n = 31$) previously analyzed in the routine laboratory were reanalyzed with EME. Passing and Bablok regression is presented in Figure 6. Assay results of both enantiomers were included, making a total of 45 concentration pairs. According to the Regional Committee of Research Ethics, no formal approval is needed for brief presentation of routine results as part of a methodological article.

Assay results using EME deviated from the routine assay by an average of 3%, ranging from −21% to 31%. One sample deviated 85% from the original routine assay, and was subjected to re-analysis with the routine method. Re-analysis with the routine method paralleled the EME assay (2% dev.) and deviated from the original LLE assay by

82% suggesting an error in sample handling. The Passing and Bablok regression line between EME and LLE was [EME] = 1.04 [LLE] + 2.94. The 95% confidence intervals of the intercept [−3.38, 11.5] and slope [1.02, 1.05] indicated that there was no constant bias, but a proportional difference of 4%. As the proportional difference between methods was minimal with a narrow confidence interval, the agreement between methods was considered satisfactory.

3.5 | Evaluation of EME as sample preparation

Based on validation data, conductive vial EME allowed precision and accuracy in accordance with recommended guidelines.[17,18] Assay
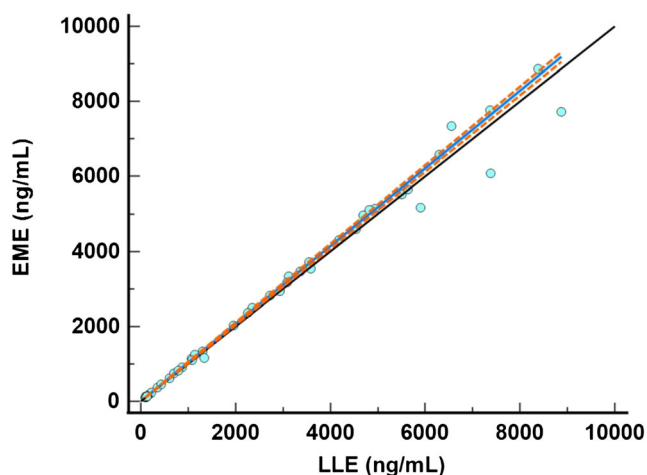

FIGURE 6 Passing and Bablok regression for the determination of R- (n = 16) and S-amphetamine in urine (n = 29) with EME-UHPSFC-MS/MS and LLE-UHPSFC-MS/MS. Y-intercept = 2.94 (95% CI −3.38 to 11.52), slope = 1.04 (95% CI 1.02 to 1.05). The Spearman rank correlation coefficient was 0.997 (P < 0.0001, 95% CI 0.995 to 0.998)

results were in agreement with the routine method using LLE. Thus, the data quality obtained with conductive vial EME was within our requirements for routine methods.

Increased focus on sustainability and environmental impact make discussing new methods in the context of green chemistry relevant. Ultimately, future analytical procedures should promote green chemistry in all steps. The recently presented greenness evaluation tool dedicated to sample preparation, AGREEprep,[24] was used to compare conductive vial EME with LLE. Greenness scores from AGREEprep analysis are presented in Figure 7 (calculations are provided in Supporting Information). In the diagrams, the overall score is given in the center, surrounded by 10 performance criteria. The length of each criteria represents the assigned weight (to final score), whereas the color visualizes performance. AGREEprep scores were 0.54 and 0.47 for EME and LLE, respectively, making EME the greener alternative among the two. The consumption of organic solvent was 9 μL per sample with EME and 900 μL per sample with LLE, which corresponds to a yearly usage of approximately 0.004 L versus 4 L solvents for sample preparation. In addition, EME was a one-step procedure, whereas the routine LLE method included several operations including extraction, evaporation, and reconstitution. Sample throughput was superior with LLE, as the method was semi-automated and performed in 96-well plates. However, 96-well EME is under commercial development and will enable high-throughput and semi-automated operation of EME in the near future.

Comparing the proposed EME method with sample preparation protocols reported in other chromatography-based bioanalysis of amphetamine enantiomers, it is evident that the organic solvent consumption of the majority of reported methods is an order of magnitude higher than EME.[40–44] Notable exceptions are methods proposed by Chermá et al.,[45] where urine was diluted with water, and by Hädener et al.,[46] which employed on-line column switching in combination with LC–MS.
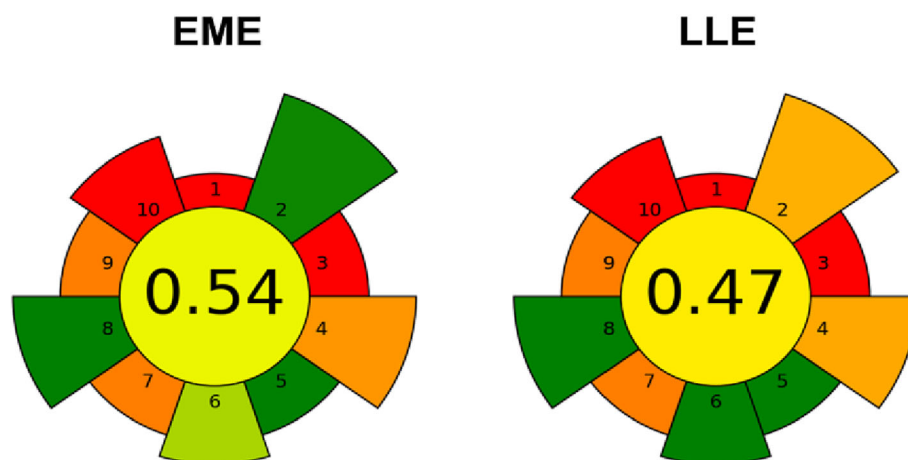
FIGURE 7 Results of AGREEprep assessment of 10-position conductive vial EME, and 96-well LLE. Overall impact score in Centre, surrounded by 10 performance criteria relating to (1) in-situ sample preparation, (2) safe solvents/reagents, (3) sustainable materials, (4) waste, (5) sample, chemical and material amounts, (6) sample throughput, (7) integration of steps and automation, (8) energy consumption, (9) post-sample prep. Configuration and (10) safety for operators. Length of each criteria represents weight (on final score) and color represent performance

Extraction into an aqueous acceptor EME is normally considered an advantage, due to greenness and operational simplicity. However, in the current work, comparison of carry-over in the UHPSFC instrument when amphetamine was spiked in 130 mM HCOOH (EME acceptor phase) and in a mixture of 130 mM HCOOH and IPA (3:2 v/v) showed that carry-over was reduced in the latter injection solvent. In the present application, the total amphetamine concentration was known prior to the enantioselective analysis, and carry-over related issues could be circumvented by injection of blank samples.

4 | CONCLUSION

EME with prototype conductive vials was combined with UHPSFC-MS/MS for determination of amphetamine enantiomers in urine. The developed method showed high precision and accuracy, and assay results were in agreement with the routine method that employed LLE as sample preparation. EME offers lower organic solvent consumption compared with LLE, making it a favorable technique in terms of green chemistry. As the sample throughput of a 10-position vial system is reduced compared with routine methods that employ automated liquid handling and 96-well plates the study serves as a promising link to the next step forward in conductive EME, a conductive 96-well system.

Extraction from samples of different origin showed that the large and natural compositional variations of urine affected the mass transfer. Therefore, an isotopically labelled internal standard was used to correct quantitative data. Under these conditions, performance was in compliance with regulatory requirements for bioanalytical methods. Thus, the quantitative data obtained with EME, based on conductive vial technology, were in accordance with those obtained using a routine method.

CONFLICT OF INTEREST STATEMENT
The authors declare no financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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REFERENCES
1. The Norwegian Prescription Data Base. 2023. https://www.reseptregisteret.no/Prevalens.aspx
2. Losacker M, Zörntlein S, Schwarze B, Staudt S, Röhrich J, Hess C. Determination of the enantiomeric composition of amphetamine, methamphetamine and 3,4-methylendioxy-N-methylamphetamine (MDMA) in seized street drug samples from southern Germany. *Drug Test Anal*. 2022;14(3):557-566. doi:10.1002/dta.3118
3. Dhabbah AM. Determination of chiral amphetamine in seized tablets by indirect enantioseparation using GC–MS. *J King Saud Univ Sci*. 2020;32(5):2622-2628. doi:10.1016/j.jksus.2020.05.003
4. George S, Braithwaite RA. Using amphetamine isomer ratios to determine the compliance of amphetamine abusers prescribed dexedrine. *J Anal Toxicol*. 2000;24(3):223-227. doi:10.1093/jat/24.3.223
5. Havnen H, Hansen M, Spigset O, Hegstad S. Enantiomeric separation and quantification of R/S-amphetamine in serum using semi-automated liquid-liquid extraction and ultra-high performance supercritical fluid chromatography-tandem mass spectrometry. *Drug Test Anal*. 2020;12(9):1344-1353. doi:10.1002/dta.2879
6. Hegstad S, Havnen H, Helland A, Spigset O, Frost J. Enantiomeric separation and quantification of R/S-amphetamine in urine by ultra-high performance supercritical fluid chromatography tandem mass spectrometry. *J Chromatogr B*. 2018;1077-1078:7-12. doi:10.1016/j.jchromb.2018.01.028
7. Harps LC, Joseph JF, Parr MK. SFC for chiral separations in bioanalysis. *J Pharm Biomed Anal*. 2019;162:47-59. doi:10.1016/j.jpba.2018.08.061
8. Hegstad S, Hermansson S, Betner I, Spigset O, Falch BM. Screening and quantitative determination of drugs of abuse in diluted urine by UPLC-MS/MS. *J Chromatogr B*. 2014;947-948:83-95. doi:10.1016/j.jchromb.2013.12.014
9. Schüller M, McQuade TA-P, Bergh MS-S, Pedersen-Bjergaard S, Øiestad EL. Determination of tryptamine analogs in whole blood by 96-well electromembrane extraction and UHPLC-MS/MS. *Talanta Open*. 2023;7:100171. doi:10.1016/j.talo.2022.100171
10. Drouin N, Mandscheff JF, Rudaz S, Schappler J. Development of a new extraction device based on parallel-Electromembrane extraction. *Anal Chem*. 2017;89(12):6346-6350. doi:10.1021/acs.analchem.7b01284
11. Eibak LEE, Rasmussen KE, Øiestad EL, Pedersen-Bjergaard S, Gjelstad A. Parallel electromembrane extraction in the 96-well format. *Anal Chim Acta*. 2014;828:46-52. doi:10.1016/j.aca.2014.04.038
12. Payán MR, Santigosa E, Torres RF, López MAB. A new microchip design. A versatile combination of Electromembrane extraction and liquid-phase microextraction in a single Chip device. *Anal Chem*. 2018;90(17):10417-10424. doi:10.1021/acs.analchem.8b02292
13. Karami M, Yamini Y, Abdossalami Asl Y, Rezazadeh M. On-chip pulsed electromembrane extraction as a new concept for analysis of biological fluids in a small device. *J Chromatogr a*. 2017;1527:1-9. doi:10.1016/j.chroma.2017.10.049
14. Petersen NJ, Jensen H, Hansen SH, Foss ST, Snakenborg D, Pedersen-Bjergaard S. On-chip electro membrane extraction. *Microfluid Nanofluid*. 2010;9(4-5):881-888. doi:10.1007/s10404-010-0603-6
15. Skaalvik TG, Øiestad EL, Trones R, Pedersen-Bjergaard S, Hegstad S. Determination of psychoactive drugs in serum using conductive vial electromembrane extraction combined with UHPLC-MS/MS. *J Chromatogr B*. 2021;1183:122926. doi:10.1016/j.jchromb.2021.122926
16. Hay AO, Trones R, Herfindal L, Skrede S, Hansen FA. Determination of methotrexate and its metabolites in human plasma by electromembrane extraction in conductive vials followed by LC-MS/MS. *Adv Sample Prep*. 2022;2:100011. doi:10.1016/j.sampre.2022.100011
17. U.S Department of Health and Human Services. Food and Drug Administration. Guidance for Industry, Bioanalytical Method Validation 2021. https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf
18. European Medicines Agency. ICH guideline M10 on bioanalytical method validation and study sample analysis 2023. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m10-bioanalytical-method-validation-step-5_en.pdf
19. Huang C, Chen Z, Gjelstad A, Pedersen-Bjergaard S, Shen X. Electromembrane extraction. *Trends Anal Chem*. 2017;95:47-56. doi:10.1016/j.trac.2017.07.027
20. Fernández E, Vårdal L, Vidal L, Canals A, Gjelstad A, Pedersen-Bjergaard S. Complexation-mediated electromembrane extraction of highly polar basic drugs-a fundamental study with catecholamines in

urine as model system. *Anal Bioanal Chem*. 2017;409(17):4215-4223. doi:10.1007/s00216-017-0370-2

21. Nasrollahi SS, Davarani SSH, Moazami HR. Impedometric investigation of salt effects on electromembrane extraction: practical hints for pH adjustment. *Electrochim Acta*. 2019;296:355-363. doi:10.1016/j.electacta.2018.11.037

22. Seip KF, Jensen H, Sønsteby MH, Gjelstad A, Pedersen-Bjergaard S. Electromembrane extraction: distribution or electrophoresis? *Electrophoresis*. 2013;34(5):792-799. doi:10.1002/elps.201200587

23. Peters FT, Drummer OH, Musshoff F. Validation of new methods. *Forensic Sci Int*. 2007;165(2-3):216-224. doi:10.1016/j.forsciint.2006.05.021

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

25. Wojnowski W. AGREEprep Analytical Greenness Metric for Sample Preparation. mostwiedzy.pl/AGREEprep. Accessed January 2023

26. Pena-Pereira F, Tobiszewski M, Wojnowski W, Psillakis E. A tutorial on AGREEprep an analytical greenness metric for sample preparation. *Adv Sample Prep*. 2022;3:100025. doi:10.1016/j.sampre.2022.100025

27. Hansen FA, Pedersen-Bjergaard S. Electromembrane extraction – looking closer into the liquid membrane. *Adv Sample Prep*. 2022;2:100020. doi:10.1016/j.sampre.2022.100020

28. Eie LV, Pedersen-Bjergaard S, Hansen FA. Electromembrane extraction of polar substances – status and perspectives. *J Pharm Biomed Anal*. 2022;207:114407. doi:10.1016/j.jpba.2021.114407

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

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

31. Bavlovič Piskáčková H, Nemeškalová A, Kučera R, et al. Advanced microextraction techniques for the analysis of amphetamines in human breast milk and their comparison with conventional methods. *J Pharm Biomed Anal*. 2022;210:114549. doi:10.1016/j.jpba.2021.114549

32. Seidi S, Yamini Y, Baheri T, Feizbakhsh R. Electrokinetic extraction on artificial liquid membranes of amphetamine-type stimulants from urine samples followed by high performance liquid chromatography analysis. *J Chromatogr a*. 2011;1218(26):3958-3965. doi:10.1016/j.chroma.2011.05.002

33. Vårdal L, Øiestad EL, Gjelstad A, Jensen H, Pedersen-Bjergaard S. Electromembrane extraction with solvent modification of the acceptor solution: improved mass transfer of drugs of abuse from human plasma. *Bioanalysis*. 2019;11(8):755-771. doi:10.4155/bio-2018-0308

34. Seip KF, Gjelstad A, Pedersen-Bjergaard S. Electromembrane extraction from aqueous samples containing polar organic solvents. *J Chromatogr a*. 2013;1308:37-44. doi:10.1016/j.chroma.2013.07.105

35. Šlampová A, Kubáň P, Boček P. Quantitative aspects of electrolysis in electromembrane extractions of acidic and basic analytes. *Anal Chim Acta*. 2015;887:92-100. doi:10.1016/j.aca.2015.06.040

36. Kubáň P, Boček P. The effects of electrolysis on operational solutions in electromembrane extraction: the role of acceptor solution. *J Chromatogr a*. 2015;1398:11-19. doi:10.1016/j.chroma.2015.04.024

37. Abbasi S, Haeri SA. Enrichment of psychotropic drugs using rhamnolipid bioaggregates after electromembrane extraction based on an agarose gel using a rotating electrode as a green and organic solvent-free strategy. *J Chromatogr a*. 2021;1655:462500. doi:10.1016/j.chroma.2021.462500

38. Seip KF, Jensen H, Kieu TE, Gjelstad A, Pedersen-Bjergaard S. Salt effects in electromembrane extraction. *J Chromatogr a*. 2014;1347:1-7. doi:10.1016/j.chroma.2014.04.053

39. Middelthon-Bruer TM, Gjelstad A, Rasmussen KE, Pedersen-Bjergaard S. Parameters affecting electro membrane extraction of basic drugs. *J Sep Sci*. 2008;31(4):753-759. doi:10.1002/jssc.200700502

40. Hess C, Losacker M, Maas A. Chromatographic separation of R-(−)/S-(+)-enantiomers of amphetamine and methamphetamine: differentiation between single methamphetamine consumption and co-consumption with amphetamine using enantioselective quantitative LC-MS/MS analysis. *Int J Leg Med*. 2019;133(2):467-473. doi:10.1007/s00414-018-1979-1

41. Losacker M, Kraemer M, Philipsen A, et al. Enantioselective quantification of amphetamine and metabolites in serum samples: forensic evaluation and estimation of consumption time. *Metabolites*. 2021;11(8):521. doi:10.3390/metabo11080521

42. Nielsen MKK, Andersen NZ, Rasmussen BS, Johansen SS. Driving under the influence of amphetamine: analytical evaluation of illegal or prescription drug intake using chiral UHPLC-MS-MS. *J Anal Toxicol*. 2023;47(3):280-286. doi:10.1093/jat/bkac103

43. Robbins B, Carpenter RE, Long M, Perry J. A human Oral fluid assay for D- and L- isomer detection of amphetamine and methamphetamine using liquid-liquid extraction. *J Anal Methods Chem*. 2022;2022:4819599. doi:10.1155/2022/4819599

44. Wang T, Shen B, Shi Y, Xiang P, Yu Z. Chiral separation and determination of R/S-methamphetamine and its metabolite R/S-amphetamine in urine using LC-MS/MS. *Forensic Sci Int*. 2015;246:72-78. doi:10.1016/j.forsciint.2014.11.009

45. Chermá MD, Nilsson GH, Johansson A, Jönsson AK, Ahlner J. Use of Lisdexamfetamine or amphetamine? Interpretation of chiral amphetamine analyses. *J Anal Toxicol*. 2022;46(1):10-16. doi:10.1093/jat/bkaa170

46. Hädener M, Bruni PS, Weinmann W, Frübis M, König S. Accelerated quantification of amphetamine enantiomers in human urine using chiral liquid chromatography and on-line column-switching coupled with tandem mass spectrometry. *Anal Bioanal Chem*. 2017;409(5):1291-1300. doi:10.1007/s00216-016-0056-1

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