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Electromembrane extraction of stimulating drugs from undiluted whole blood

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ABSTRACT

For the first time, electromembrane extraction (EME) of six basic drugs of abuse from undiluted whole blood and post mortem blood in a totally stagnant system is reported. Cathinone, methamphetamine, 3,4-methylenedioxy-amphetamine (MDA), 3,4-methylenedioxy-methamphet-amine (MDMA), ketamine and 2,5-dimethoxy-4-iodoamphetamine (DOI) were extracted from the whole blood sample, through a supported liquid membrane (SLM) consisting of 1-ethyl-2-nitrobenzene (ENB) immobilized in the pores of a hollow fiber, and into an aqueous acceptor solution inside the lumen of the hollow fiber. The SLM acts as a barrier with efficient exclusion of all macromolecules and acidic substances in the sample. Due to the application of the electrical field, only the cationic compounds of interest are extracted efficiently across the membrane, thus providing extremely clean extracts for analysis with liquid chromatography–mass spectrometry, LC–MS. Recoveries in the range 10–30% were obtained from 80 μl whole blood within 5 min extraction time and an applied voltage of 15 V across the SLM. The optimized technique was tested on real forensic whole blood samples taken from three forensic autopsy cases and on five forensic whole blood samples from living persons. The results were in agreement with the analysis using standard sample preparation methods (liquid–liquid extraction) performed on the same samples by Norwegian Institute of Public Health (NIPH), Division of Forensic Toxicology and Drug Abuse Research. Evaluation data were acceptable, with limit of detections (LODs) in the range 40–2610 pg/mL, well below concentrations associated with drug abuse; linearites in the range between 10 and 250 ng/mL with r^2 values above 0.9939, and with repeatability (RSD) of 7–32%.

1. Introduction

Effective sample preparation of whole blood samples prior to chromatographic or electrophoretic analytical methods is of profound importance due to the complicated nature of the matrix and often low concentrations of the endogenous or exogenous substance of interest. Commonly used sample preparation methods for the isolation and enrichment of drugs from whole blood include protein precipitation (PPT), liquid–liquid extraction (LLE) and solid-phase extraction (SPE) [1]. During the last decades, focus has been directed towards miniaturization of these techniques, amongst other hollow fiber liquid-phase microextraction (HF-LPME) [2–11] or solid-phase microextraction (SPME) [12–16]. HF-LPME has found widespread use particularly in environmental analysis and in the analysis of drugs in biological fluids [5,17]. Numerous reports of high enrichment factors, excellent sample cleanup, a great reduction in the consumption of organic solvents, reduction of sample volumes and the possibility of automation have contributed to the success of HF-LPME. The extraction times are in the range of 20–60 min. Particularly for the analysis of drugs in biological fluids short extraction times which contribute to high sample throughput are essential, and extraction times in the 20–60 min range are therefore considered as a drawback. The reason for investigating electro-assisted extraction systems was therefore based on the hypothesis that charged molecules can be transferred faster across a liquid membrane by the force of an electrical potential than by passive diffusion as in HF-LPME.

In 2006 a new extraction principle, termed electromembrane extraction (EME) was presented [18]. This principle was found to reduce the extraction times 6–17 times compared to HF-LPME [19] and to provide very efficient sample cleanup [18]. The presented method built upon the technical set-up known from HF-LPME, with a hollow fiber with porous walls impregnated with an organic liquid as the central extraction unit. However, application of an electrical field across the supported liquid membrane (SLM) embedded in the fiber walls made a totally new way of forcing ionic compounds across the membrane. Due to the volume difference between the

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sample solution and the acceptor solution, a high degree of analyte enrichment was obtainable. However, EME could also efficiently be performed on sample volumes in the μl scale [19], in a drop-to-drop microextraction system [20,21] or in a chip system [22]. EME has successfully been used to extract basic drugs [23–30], acidic drugs [31], chlorophenols [32], nerve agent degradation products [33], metals [34–36] and zwitterionic peptides [37–40]. Due to the excellent cleanup properties of the technique, it has shown to be extremely useful in the sample preparation of biological matrices [23,25,26,29,37,41–43]. Recently, some stimulating drugs were extracted from urine samples by EME using NPOE with 15% tris-(2-ethylhexyl)phosphate (TEHP) as the SLM gaining recoveries in the range 57–70% [44].

Although the analytes in the sample should be charged in order to migrate in the applied electrical field, studies have shown that EME directly from undiluted plasma and whole blood is highly achievable [23,25]. Due to the sometimes limited amounts of matrix available in forensic science, the method presented in this paper was optimized for small sample volumes (80 μl). Earlier experiments have shown some stability problems of the SLM during extraction caused by emulsification of the organic solvent into the biological matrix [25]. This stability issue is almost eliminated by doing the extractions in a totally stagnant system [20,21,23]. To the best of our knowledge, EME is the only extraction method that does not require any addition of reagents prior to extraction. The sample handling is hence simplified and any possibility of sample contamination is greatly reduced. The major focus in this work was to directly explore the ability of EME to extract drugs of abuse selectively from real hemolysed whole blood samples such as post mortem blood and other forensic blood samples. Very selective sample preparation is especially beneficial for post mortem samples, which represents a difficult matrix due to possible putrefaction [45].

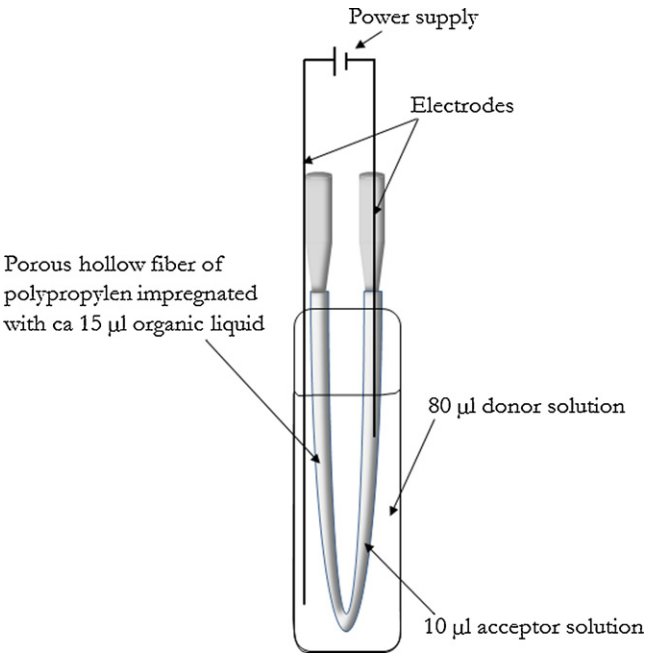


Fig. 1. Illustration of the equipment used in EME.

2. Materials and methods

2.1. Chemicals and biological samples

Cathinone, ketamine, di(2-ethylhexyl)phosphate (DEHP), 2-nitrophenyl octylether (NPOE), methanol, and sodium acetate were obtained from Sigma–Aldrich GmbH (Steinheim,

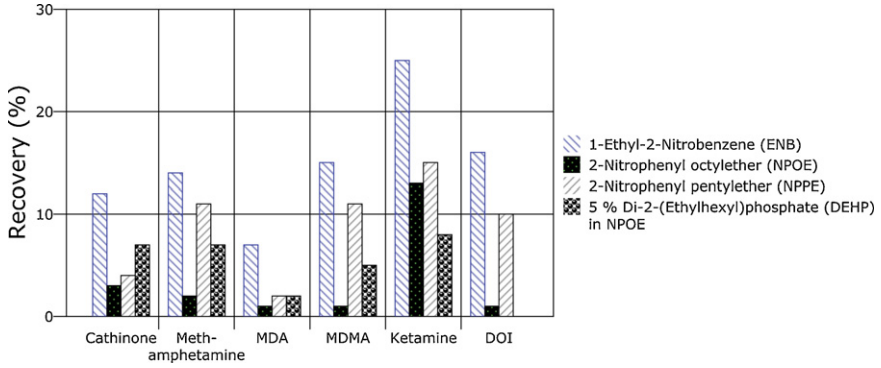


Fig. 2. Recoveries from whole blood with different organic solvents in the SLM (5 μg/ml in 80 μl whole blood, 10 μl 10 mM hydrochloric acid as acceptor phase, 9 V battery, and 5 min extraction time).

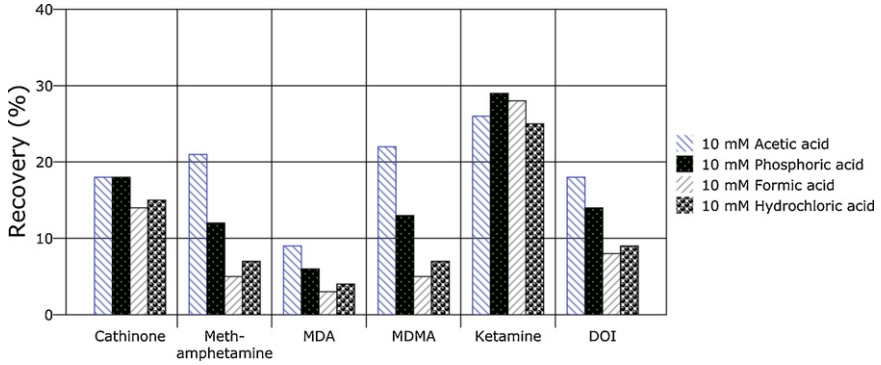


Fig. 3. Recoveries from whole blood with different acceptor solutions (5 μg/ml in 80 μl whole blood, ENB as organic phase, 9 V battery, and 5 min extraction time).

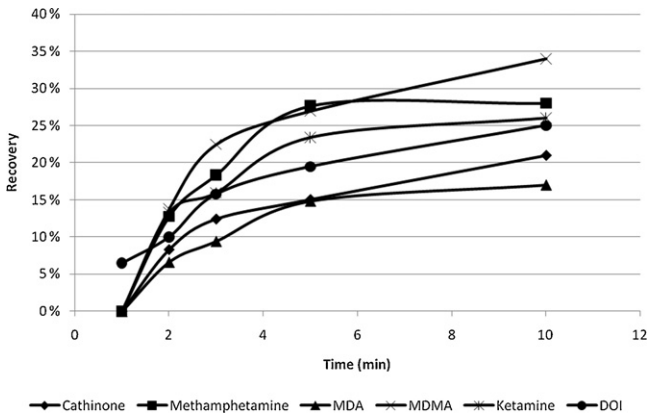


Fig. 4. Extraction recovery as a function of extraction time (2.5 μg/ml in 80 μl whole blood, ENB as organic phase, 10 μl 10 mM acetic acid as acceptor phase, 15 V).

Germany). Methamphetamine was from Lipomed GmbH (Weil am Rhein, Germany), 3,4-methylenedioxy-amphethamine (MDA) was from Alltech (Deerfield, IL, USA), 3,4-methylenedioxy-methamphethamine (MDMA), MDMA-d5, amphetamine-d11 and methampehtamine-d11 was purchased from Cerilliant (Round Rock, TX, USA), while 2,5-dimethoxy-4-iodoamphetamine (DOI) was bought from RBI (Natick, MA, USA). Disodium hydrogenphosphate, sodium dihydrogenphosphate, acetic acid, hydrochloric acid, formic acid, and phosphoric acid were from Merck KGaA (Darmstadt, Germany). 1-Ethyl-2-nitrobenzene (ENB), 2-nitrophenyl pentylether (NPPE), and tris(2-ethylhexyl)phosphate (TEHP) were produced by FlukaChemie GmbH (Buchs, Switzerland). Drug free whole blood was donated by healthy volunteers at School of Pharmacy, University of Oslo, Norway or purchased from The Blood Bank, Ullevaal University Hospital, Oslo, Norway.

Routine samples of whole blood at the Norwegian Institute of Public Health (NIPH), Division of Forensic Toxicology and Drug Abuse are received in 4 mL BD Vacutainer® Plus Plastic Blood Collection Tubes (BD Vacutainer Systems, Franklin Lake, NJ, USA) containing 10 mg sodium fluoride and 8 mg potassium oxalate or for autopsy samples 25 mL Sterilin (Sterilin Limited, Newport, UK) tubes containing 200 mg potassium fluoride. The samples are stored at 4 °C prior to processing. Aliquots of 0.1–0.5 mL whole blood, depending on analysis method, are subsequently transferred to separate 5 mL polypropylene tubes (Sarstedt AG & Co., Nümbrecht, Germany), and stored at 4 °C until the time of analysis. After analysis the whole blood is stored at −20 °C. For the samples used in this study, which had previously been analysed with routine methods, frozen samples were thawed and aliquoted for EME.

2.2. Standard solutions and sample solutions

Stock solutions of cathinone, ketamine, MDA, MDMA, methamphetamine, and DOI were prepared by solving the substance in methanol to concentrations of 0.929 mg/ml, 1.764 mg/ml, 0.182 mg/ml, 0.176 mg/ml, 1.008 mg/ml and 1.269 mg/ml, respectively. All solutions were stored at 4 °C protected from light. The stock solutions were found to be stable throughout the study. Standard solutions were prepared daily by diluting the stock solutions

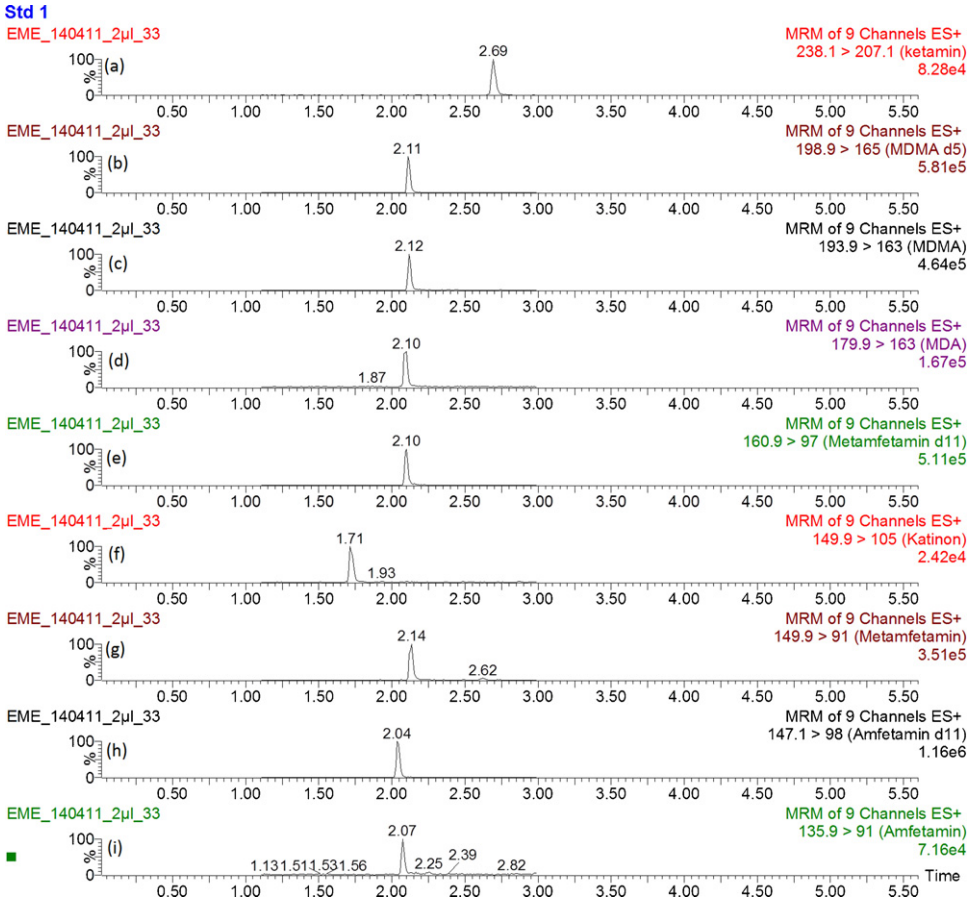


Fig. 5. Chromatograms obtained from EME of the lowest concentration level in the calibration curve for (a) ketamine, (b) internal standard, MDMA-d5, (c) MDMA, (d) MDA, (e) internal standard, methamphetamine-d11, (f) cathinone, (g) methamphetamine, (h) internal standard, amphetamine-d11 and (i) amphetamine (10 ng/mL in whole blood, ENB as organic phase, 10 μl 10 mM acetic acid as acceptor phase, 15 V battery, and 5 min extraction time).

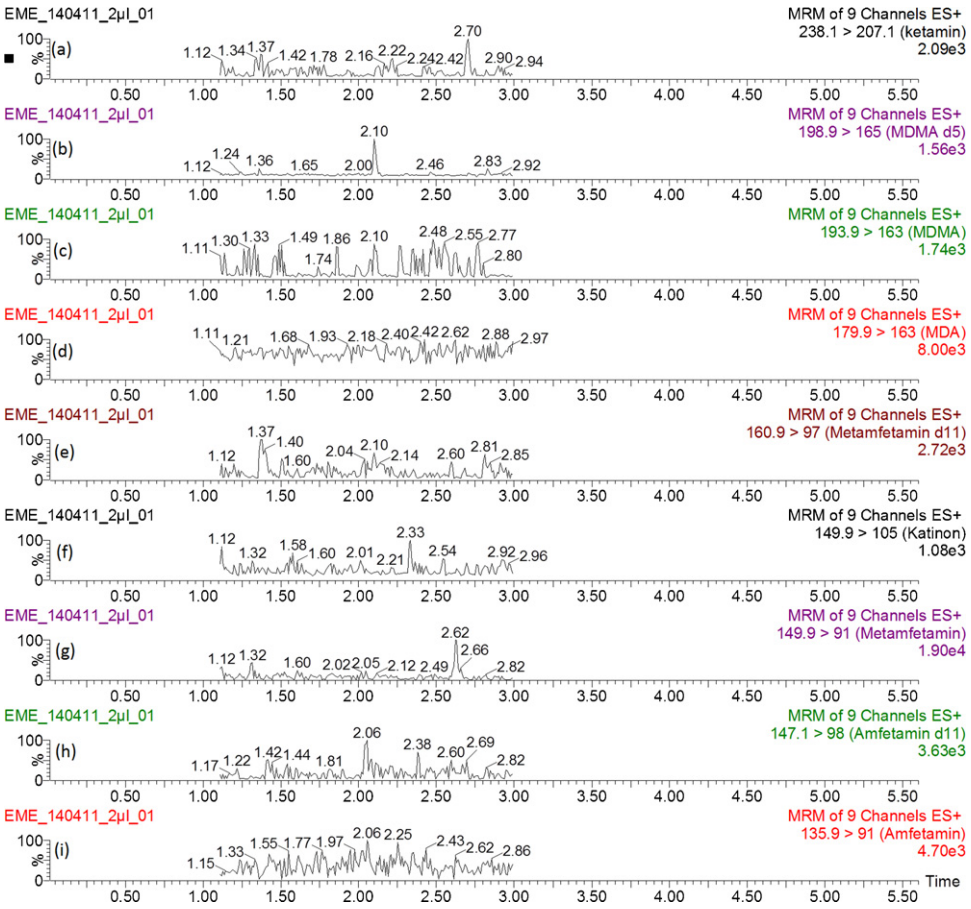


Fig. 6. Chromatograms obtained from EME of a blank whole blood sample for (a) ketamine, (b) internal standard, MDMA-d5, (c) MDMA, (d) MDA, (e) internal standard, methamphetamine-d11, (f) cathinone, (g) methamphetamine, (h) internal standard amphetamine-d11 and (i) amphetamine, ENB as organic phase, 10 μl 10 mM acetic acid as acceptor phase, 15 V battery, and 5 min extraction time).

with 10 mM HCl to a final concentration of 5 μg/ml of each drug. Whole blood samples were prepared by spiking undiluted drug free whole blood with the stock solutions to a final concentration of 5 μg/ml of each drug if nothing else is mentioned.

In the case of real whole blood samples, 20 μl of a solution of internal standards (MDMA-d5, methamphetamine-d11 and amphetamine-d3) were added to 100 μl of the real whole blood samples. 80 μl of this sample was subjected to EME. In the cases were the drug concentration was measured above the highest calibration curve level, the samples were diluted by drug free whole blood from the blood bank prior to extraction.

2.3. EME equipment and performance

The equipment used for EME of whole blood samples is displayed in Fig. 1. The sample compartment was a glass vial of the type 01-CVG (Chromacol, Welwyn Garden City, UK). The porous hollow fiber used for immobilization of the organic solvent and for housing the acceptor solution was a PP Q3/2 polypropylene hollow fiber (Membrana GmbH, Wuppertal, Germany) with an internal diameter of 0.6 mm, with a 200 μm wall thickness, and with 0.2 μm pore size. The guiding tubes connected to each end of the hollow fiber were cut from pipette tips (Microloader 0.5–20 μl, Eppendorf AG, Hamburg, Germany). The electrodes were made by 0.2 mm platinum thread (K.A. Rasmussen, Hamar, Norway) and connected to a 9 V battery from Duracell (Aarschot, Belgium); a 15 V battery from Varta (Hanover, Deutchland) or a d.c. power supply of the model ES 0300-0.45 from Delta PowerSupplies (Delta Elektronika, Zierikzee, The Netherlands) with programmable voltage in the range 0–300 V providing currents in the range 0–450 mA.

The extraction procedure was performed according to the following protocol; 80 μl whole blood sample containing the analytes was transferred to the sample compartment. The hollow fiber was cut into 5 cm pieces and connected to two pipette tips as illustrated in Fig. 1. The fiber unit was then dipped in the organic solvent for 5 s to impregnate the hollow fiber walls with the solvent, thus creating the SLM. The excess of the organic solvent was gently wiped away with a medical wipe. Subsequently, 10 μl acceptor solution was filled into the lumen of the hollow fiber by a microliter syringe. The hollow fiber containing the SLM and the acceptor solution was then placed into the sample in a loop shape showed in Fig. 1. One of the electrodes, the cathode, was introduced into the lumen of the hollow fiber via one of the pipette tips, while the anode was placed directly in the sample solution, thus creating an electrical field across the SLM. The electrodes were coupled to a battery or a power supply where the voltage was set to 15 V if nothing else is mentioned. The extraction was performed in a stagnant system without any convection of the extraction unit. After 5 min extraction time the voltage was turned off and the acceptor solution was recollected by a microsyringe and transferred to a microinsert for capillary electrophoresis (CE) or liquid chromatography–mass spectrometry (LC–MS) or 300 μl polypropylene vials (Waters Corporation, Milford, MA, USA) for ultraperformance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) analysis. The acceptor solution was diluted to 40 μl by 10 mM CH_3COOH prior to the LC–MS analyses, and 10:90 MeOH:H_2O prior to the UPLC–MS/MS analysis.

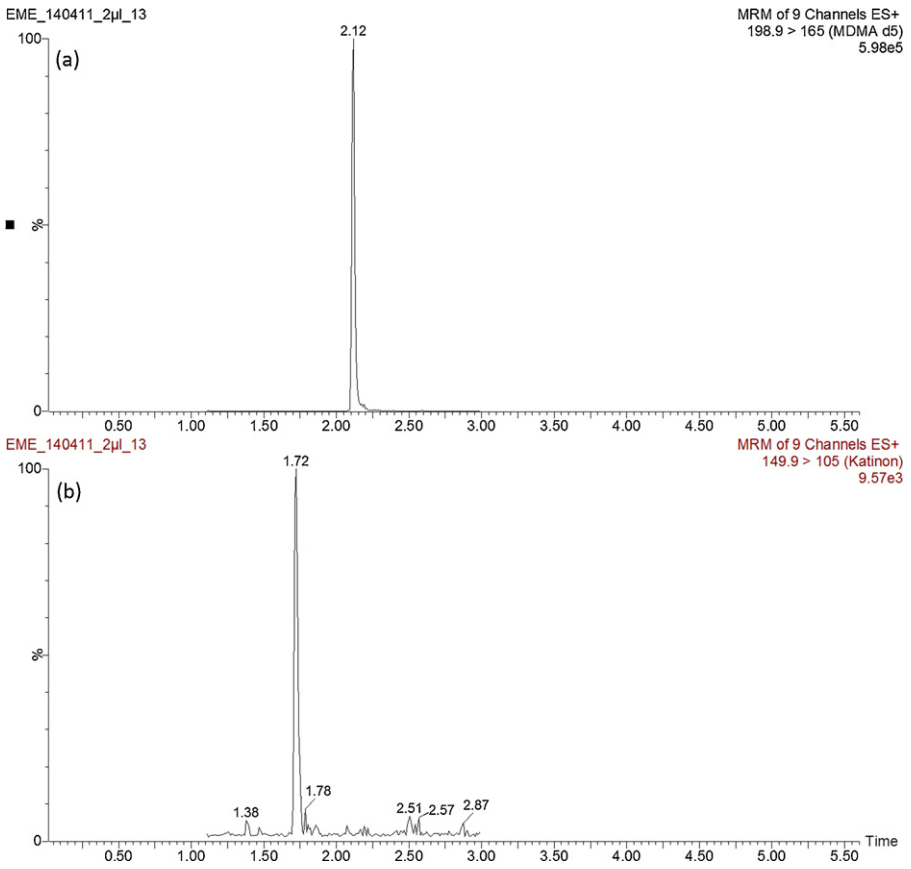


Fig. 7. Chromatograms of (a) internal standard, MDMA-d5 and (b) cathinone derived from EME of a whole blood sample obtained from a living person (Case 1). (ENB as organic phase, 10 μl 10 mM acetic acid as acceptor phase, 15 V battery, and 5 min extraction time.)

2.4. Separation and detection

The analyses of the extracts were performed on three different instruments due to practical reasons; the optimization experiments were performed on CE because of its short analysis time, the evaluation data were obtained by single-quadrupole LC–MS while the extracts from the real samples were analysed on UPLC–MS/MS at NIPH.

The CE analyses were performed on an Agilent Capillary Electrophoresis System with UV detection (Agilent Technologies, Santa Clara, CA, USA). Data acquisition was performed using ChemStation (Agilent Technologies). The separations were accomplished in a 75 μm I.D. (360 μm O.D.) fused silica capillary with an effective length of 55.0 cm and a total length of 63.5 cm (Polymicro Technologies, Phoenix, AZ, USA). The background electrolyte solution was a 25 mM phosphate buffer adjusted to pH 2.75. The instrument was operated at 30 kV, which generated a current level of approximately 50–80 μA. The samples were introduced by hydrodynamic injection at 50 mbar for 5 s. Detection was accomplished at 200 nm. Total analysis time was 10 min.

The chromatographic system used in the evaluation part consisted of a Shimadzu SIL-10ADvp auto injector, two Shimadzu LC-10ADvp gradient pumps, a Shimadzu DGU-14A degasser, a Shimadzu SCL-10Avp system controller, and a Shimadzu LCMS-2010A single-quadrupole MS detector. Data acquisition and processing were carried out using Shimadzu LCMS solution software Version 2.04-H3 (all from Shimadzu Scientific instruments, Kyoto, Japan). Chromatographic separation was carried out on a 50 mm × 1 mm I.D. Biobasic-C8 column (Thermo Fisher Scientific, Waltham, MA, USA) with average pore size of 300 Å, and particle diameter of 5 μm. The mobile phases consisted of A: 20 mM formic acid and methanol (95:5, v/v) and B: 20 mM formic acid and methanol (5:95, v/v). A linear gradient was run up to 15% mobile phase B in 12 min using 100% mobile phase A/0% mobile phase B as starting point. A linear gradient from 15% to 100% mobile phase B was run from 12 to 20 min. After these 20 min, the mobile phase was kept constant for 2 min. Subsequently, the column was regenerated by 100% mobile phase A for 5 min before next injection. The flow rate was set to 50 μl/min, and the injection volume was 20 μl. An electrospray ionization (ESI) source operated in the positive mode was used to interface the HPLC and the MS. Analysis were performed with selected ion monitoring (SIM), where *m/z* values 150, 180, 194, 238 and 322 represented methamphetamine/cathinone, MDA, MDMA, ketamine and DOI respectively. The MS operating conditions were as follows: Drying gas between 10 and 20 L/min, nebulizer gas of 1.5 L/min, curved desolvation line (CDL) temperature of 200 °C, block temperature of 200 °C, and probe voltage of +4.5 kV.

The analyses of the real samples were carried out on a Waters Acquity UPLC-system, applying an Acquity HSS T3-column 100 mm × 2.1 mm I.D., with average pore size of 100 Å, and particle diameter of 1.8 μm. The mobile phases consisted of A: 10 mM ammonia formate buffer, pH 3.1 and B: methanol. The gradient was run according to the protocol presented in Table 1, with a flow rate of 0.5 mL/min. The column temperature was set to 65 °C and the injection volume was 2 μl, using partial loop injection with a needle overfill flush of 3 μl. Weak wash was performed with 600 μl methanol:water (10:90), and strong wash with 200 μl methanol:water (90:10), for each sample. A Waters Quattro Premier XE tandem mass spectrometer, equipped with a Z-spray electrospray interface, was used for all analyses. Positive ionization was performed in the multiple reaction monitoring (MRM) mode, with one transition for each compound. The capillary voltage was

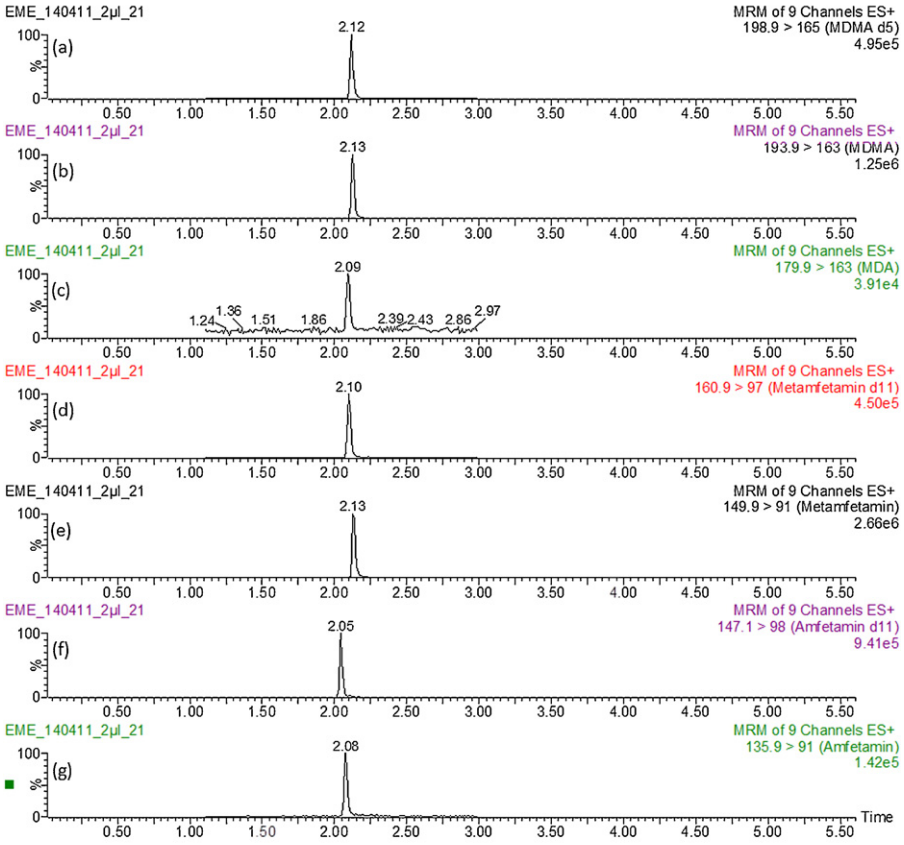


Fig. 8. Chromatograms of (a) internal standard, (b) MDMA, (c) MDA, (d) internal standard, methampethamine-d11, (e) methamphetamine, (f) internal standard, amphetamine-d11 and (g) amphetamine derived from EME of a whole blood sample obtained from a living person (Case 5). (The whole blood sample was diluted 10 times with blank whole blood prior to EME, ENB as organic phase, 10 µl 10 mM acetic acid as acceptor phase, 15 V battery, and 5 min extraction time.)

set to 1.0 kV, the source block temperature was 120 °C, and the desolvation gas (nitrogen) was heated to 500 °C and delivered at a flow rate of 900 L/h. The cone gas (nitrogen) was set to 60 L/h, and the collision gas (argon) pressure was maintained at 0.004 mbar in the collision cell. The appropriate MRM transitions, cone voltages, and collision energies for the individual analytes were determined by direct infusion into the mass spectrometer.

2.5. UPLC–MS/MS calibration

Calibration curves for each of the drugs were made by spiking drug free whole blood to final concentrations of 10, 20, 60, 180, and 540 ng/mL, respectively. Three parallels were analysed at each level, and a $1/x$ weighting was applied. In addition, one blank whole blood sample and one blank whole blood sample spiked with internal standard were analysed. Amphetamine, methampethamine and MDMA used their own deuterated analogues as internal standards, while cathinone, MDA and ketamine used MDMA-d5.

Table 1
Gradient protocol used in UPLC–MS–MS.

Time (min)	Methanol:ammoniaformate buffer (%)
0.0–0.5	10:90
0.5–1.5	30:70
1.5–2.5	30:70
2.5–2.6	60:40
2.6–3.0	90:10
3.0–4.2	90:10
4.3–5.5	10:90

2.6. Comparison of real samples

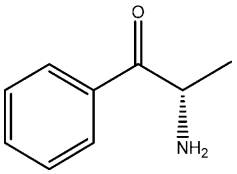
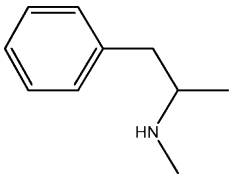
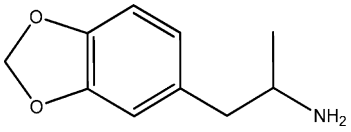
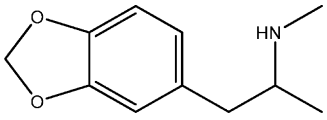
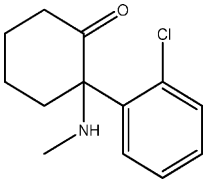
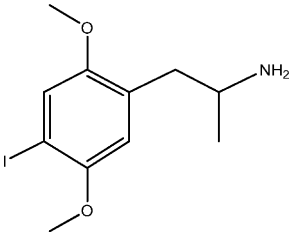
EME-extracted samples were compared to samples analysed by NIPH routine confirmation method [46] where amphetamines are extracted with liquid–liquid extraction with cyclohexane followed by derivatization by pentadecafluorooctanoyl chloride (PFOC) and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) respectively. GC–MS analysis was performed on a 0.25 mm I.D., film thickness 0.4 µm Varian CP-SIL 5 CB capillary column with a length of 12 m. Ketamine is extracted using liquid–liquid extraction with ethyl acetate/heptane (4:1), and analysed with LC–MS on a HICHROM silica-column (2.1 × 150 mm, 3.5-µm particles) with an ammonium formate and acetonitrile gradient.

The recoveries were calculated in the same way as described in earlier works [18].

3. Results and discussion

Six basic drugs of abuse were chosen based on their log P and pK_a values (Table 2). As relatively nonpolar analytes with log P values in the range 0.9–3.0 and pK_a values above 6.5, they were found to be ideal candidates for EME based on earlier experiences [23–26]. In addition, these drugs are drugs of abuse highly relevant in forensic toxicological analysis. Amphetamine/methampethamine are amongst the most frequently misused drugs in Europe [47]. Ecstasy (MDMA) has been a common recreational drug, while DOI represent a model substance for more exotic psychedelic phenethylamines. Ketamine is a dissociative anesthetic, used both for veterinary and human surgery. It is structurally similar to phencyclidine (PCP), and has gained popularity as a "club drug" in certain parts of the world

Table 2
Structures and physiochemical properties of the drugs.

Drug	Structure	pK_a *	log P*
Cathinone		7.97	0.92
Methamphetamine		10.38	2.20
MDA (3,4-methylenedioxy-amphetamine)		9.94	1.64
MDMA (3,4-methylenedioxy-methamphetamine)		10.32	2.05
Ketamine		6.46	3.01
DOI (2,5-dimethoxy-4-iodoamphetamine)		9.46	2.61

* Data from SciFinder (https://scifinder.cas.org) [51].

[48]. Cathinone is a psycoactive compound in the khat plant (*Catha edulis*). Khat has been used as a stimulant in social settings in the Horn of Africa and the Arabian Peninsula for centuries, and is a relevant model for an emerging class of new designer drugs, the substituted cathinones [49].

3.1. Tuning the SLM

As a first series of experiments, the extraction recoveries were studied as a function of the SLM composition. Four different SLMs were chosen based on earlier experiments with low-voltage EME from biological matrices [23,25,26]. Due to the emulsifying properties of biological matrices like plasma and whole blood, the choice of a proper SLM is essential for the stability of the extraction system. 1-Ethyl-2-nitrobenzene (ENB) has earlier been a good SLM in EME operated at low voltages; 2-nitrophenyl octylether (NPOE) has shown to be a well working SLM for a broad range of basic substances while addition of TEHP or the ion pair reagent di(2-ethyl hexyl)phosphate (DEHP) to NPOE is in some cases advantageous for extraction of more polar basic substances [44]. In addition, 2-nitrophenyl pentylether (NPPE) was included in the study as a proper SLM candidate for basic substances. The recoveries obtained with the different SLMs are shown in Fig. 2.

The results stated clearly that amongst the tested candidates, ENB was the best SLM in this case. No stability issues and breakage of the SLM was observed during extraction, probably caused by the absence of convection in the system. The reason why NPOE and NPOE with 5% DEHP worked poorly was probably the low voltage applied in the system. NPOE membranes have earlier showed maximal extraction capacity at voltages above 100 V [50]. Therefore, it was clear that NPOE was not the best choice as SLM in this case. The eminent properties of ENB as a low-voltage SLM demonstrated in earlier works [23,26] was confirmed during the current experiments. ENB was therefore used as the SLM in the rest of this paper.

3.2. Tuning of acceptor solution

To increase the recoveries and to make the acceptor solution compatible with LC–MS analyses, different compositions of the acceptor solution chemistry were tested. Four acidic solutions were chosen based on earlier experience; 10 mM acetic acid, 10 mM

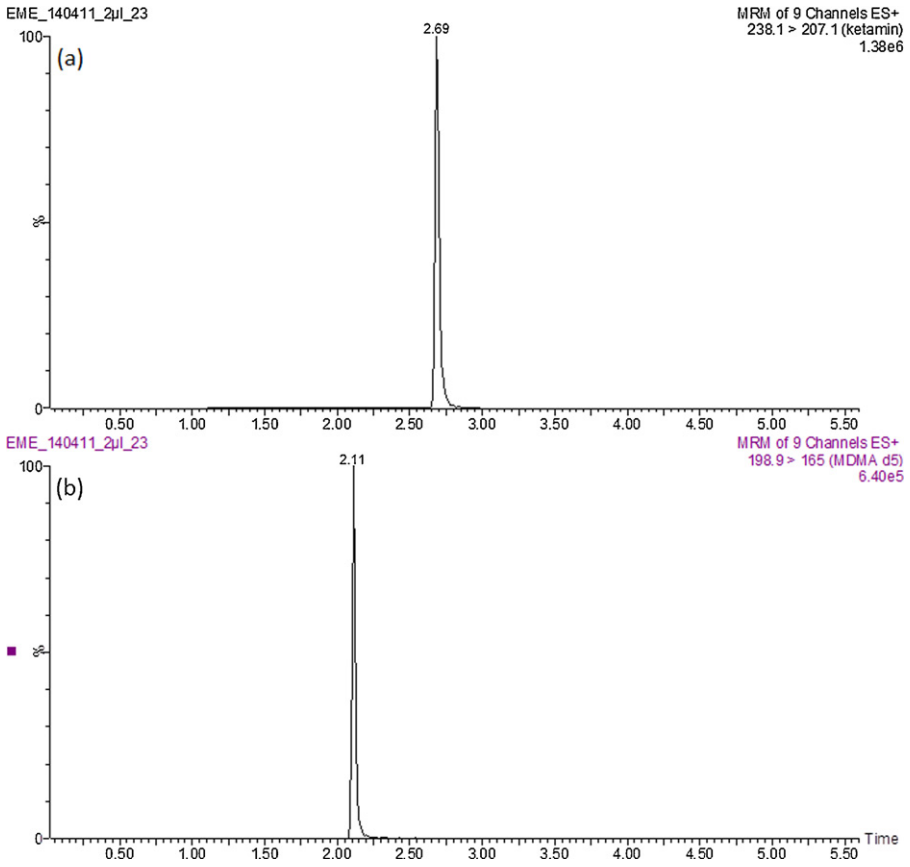


Fig. 9. Chromatograms of (a) ketamine and (b) internal standard (MDMA d5) derived from EME of a post mortem whole blood sample obtained from an autopsy (Autopsy case 1). (The post mortem whole blood sample was diluted 10 times with blank whole blood prior to EME, ENB as organic phase, 10 μl 10 mM acetic acid as acceptor phase, 15 V battery, and 5 min extraction time.)

phosphoric acid, 10 mM formic acid, and 10 mM hydrochloric acid, respectively. The recoveries for some of the substances extracted were clearly dependent on the composition of the acceptor solution as shown in Fig. 3. The reason for this dependency was unclear; however, the acetic acid was picked out as the best acceptor solution candidate for the rest of the study.

In one single experiment, the pH of the whole blood was adjusted with acetate buffer to a final pH of 4. As this experiment did not gain any improvement of the results, pH of the donor solution was not further investigated because earlier results have shown that the pH in the donor solution is not a very critical parameter in EME [18,23].

3.3. Voltage

Another parameter which is shown to influence the extraction recoveries is the applied voltage across the SLM [26,28,50]. Due to safety reasons, voltages in the lower range were tested, and the recoveries obtained with 0, 5,15 and 50 V are given in Table 3. These results confirmed earlier findings that lower voltages are optimal for the present SLM [26]. After 5 min, even the absence of electrical field (0 V) gained some recoveries. At physiological pH (7.4), some of the substances of interest are partially deprotonated. Extraction of cathinone and ketamine (pK_a 7.97 and 6.46, respectively) gave almost similar recoveries at 0 V, 5 V, 15 V and 50 V. Due to the partial

Table 3
Recoveries from whole blood with different voltages (5 μg/ml in 80 μl whole blood, ENB as organic phase, 10 μl 10 mM acetic acid as acceptor phase, 5 min extraction time).

Voltage	Recovery (%)					
	Cathinone	Methamphetamine	MDA	MDMA	Ketamine	DOI
0	11%	4%	2%	4%	24%	10%
5	12%	10%	4%	14%	25%	19%
15	15%	16%	9%	22%	28%	26%
50	16%	20%	13%	19%	20%	14%

Table 4
Evaluation data.

	Cathinone	Methamphetamine	MDA	MDMA	Ketamine	DOI
Limit of detection (pg/ml) (LOD)	161	125	2609	133	39	866
Repeatability (%) 10 ng/mL ($n = 6$)	–	–	–	27	8	12
Repeatability (%) 50 ng/mL ($n = 5$)	16	26	16	26	12	14
Linearity (r^2) 10–250 ng/mL	1.0000	0.9939	0.9975	0.9983	0.9978	0.9975

80 μl whole blood, 15 V, 1-ethyl-2-nitrobenzene immobilized in the SLM, 10 mM acetic acid in the acceptor solution, 5 min extraction time, and extracts analysed by LC–MS.

deprotonation of these compounds at pH 7.4, they were probably extracted across the SLM by passive diffusion. The recoveries of the substances with the highest pK_a values (methamphetamine and MDMA) were significantly improved by the application of voltage. Due to their complete ionization at pH 7.4 they were influenced more strongly by the electrical field and hence more efficiently extracted by EME. Based on these observations, 15 V was applied across the SLM in the rest of the work.

3.4. Extraction time

Based on the former experiments, a study of the extraction time dependency was performed on the whole blood samples. One of the highlighted advantages with EME is the rapid extraction time [42]. However, it has been shown that the extraction time is somewhat slower from biological samples compared to spiked aqueous samples, probably because of strong protein bindings [25]. Fig. 4 shows that for three of the substances, maximum recoveries in the presented system are reached after 5 min extraction time. The other substances showed slight increase in the recoveries with longer extraction time, probably caused by strong protein binding. However, this increase was considered to be of minimum importance in this study; therefore, 5 min was set as standard extraction time.

3.5. Evaluation

An evaluation was performed to assess the applicability of EME from undiluted whole blood. EME was tested with regards to limit of detection (LOD), linearity and repeatability. The extracts were analysed on a single-quadrupole LC–MS to obtain lower detection limits as compared to CE. To carry out the study, three experiments were set up. First, the LODs for the drugs were estimated according to a signal-to-noise ratio of 3. The values were acceptable in the range 39–2610 pg/mL (Table 4). The linearity was tested at five different concentration levels (10 ng/mL; 25 ng/mL; 50 ng/mL; 100 ng/mL; and 250 ng/mL). The results displayed in Table 4 demonstrated that the proposed method resulted in linearity in the tested concentration range, with r^2 values above 0.9939 for all the drugs.

The repeatability was determined at three different concentration levels (10 ng/mL; 50 ng/mL; and 500 ng/mL, respectively). As it appears from Table 4, the relative standard deviations (RSDs) are in the range between 7% and 32%. These results are found to be satisfactory, taken into consideration the home-build equipment and small sample volumes. However, the data might be improved by using appropriate internal standards like deuterated drugs.

3.6. Real samples

To test the applicability of the extraction method, the optimized EME conditions were tested on real samples obtained from NIPH. Three post mortem whole blood samples and five other forensic samples from living persons were subjected to EME and the results were compared to standard methods run by NIPH as described in Section 2.6.

The chromatograms obtained from EME of the lowest concentration level in the calibration curve (10 ng/mL, Fig. 5) showed clearly defined peaks for all the drugs and the internal standards, with almost no contamination peaks in the present system. To further check for possible interferences, a blank whole blood sample was extracted under the same conditions, whose resulting chromatograms are shown in Fig. 6. No interfering peaks of importance were observed at the retention times of the drugs. The resulting

Table 5
Drug concentrations in real samples measured by EME-UPLC-MS-MS and the deviations from standard measurements performed by NIPH.

Sample	Cathinone		Amphetamine		Methamphetamine		MDMA		MDA		Ketamine	
	Conc. (ng/mL)	Deviation	Conc. (ng/mL)	Deviation	Conc. (ng/mL)	Deviation	Conc. (ng/mL)	Deviation	Conc. (ng/mL)	Deviation	Conc. (ng/mL)	Deviation
Case 1 ($n = 2$)	2.9*	N.M.										
Case 2 ($n = 2$)	1.3*	N.M.										
Case 3 ($n = 2$)			138	28%	558	34%						
Case 4 ($n = 2$)			90	32%	226	26%					379	-6%
Case 5 ($n = 2$)			386	−14%	996	−13%	405	−25%	21	N.M.		
Autopsy case 1 ($n = 2$)											1952	24%
Autopsy case 2 ($n = 2$)							3005	4%	80	N.M.		
Autopsy case 3 ($n = 2$)			141	16%	316	25%						

N.M., not measured.
* Below the lowest level of the calibration curve.

chromatograms demonstrated nicely the selective properties of EME in combination with UPLC–MS/MS and MRM.

The concentrations measured in the real samples are given in Table 5, including % deviation from the concentrations measured with conventional methods by NIPH. In the cases where the drug concentrations were measured by NIPH to be higher than the highest concentration level in the calibration curve, the samples were diluted with blank whole blood prior to EME. Even if amphetamine was not included in the EME optimization work, it was measured in the extracts from the real samples because of the structural similarities with the other drugs. MDA, which is present as a metabolite of MDMA in the analysed samples, is not routinely measured in whole blood at NIPH. The same is the case for DOI, and therefore unfortunately no real samples including DOI were available for comparison. Cathinone is usually analysed in urine at NIPH, but was detected in blood samples, from two living persons with positive cathinone in urine, which was selected for analysis. The concentrations of cathinone were extrapolated from the calibration curve because they were below the lowest level of the calibration curve (10 ng/mL). However, as shown in Fig. 7, the resulting peak height was above a signal-to-noise ratio of 10, which indicates the limit of quantification.

Amphetamine and methamphetamine were detected and quantified in three of the samples from living persons and one of the autopsy cases. The results were within −13% to 34% deviation from the values previously measured by NIPH, and for most of the samples this is within the limits for the accepted deviation between replicates of forensic samples at the NIPH (below ±30%). In case 5, MDMA and its metabolite MDA were detected and quantified in addition to amphetamine and methamphetamine (Fig. 8). The chromatograms are extremely clean, demonstrating the excellent selective properties of EME. The efficient exclusion of most of the blood components by the SLM were also obtained from EME on post mortem whole blood samples, which are exemplified in Fig. 9. Because of the complex nature and the diversity between post mortem whole blood matrices, they are sometimes a challenge during the sample preparation step [45]. Therefore, the presented EME technique has a great potential as an alternative sample preparation technique for complicated matrices.

4. Concluding remarks

The present study has demonstrated that further development of known stagnant EME systems [20,23] in combination with LC–MS or UPLC–MS/MS has potential as a powerful, rapid and selective technique for sample preparation of complicated biological matrices like post mortem whole blood. Extraction times of 5 min reduce the sample preparation time significantly compared to other sample preparation methods used on post mortem samples [45]. Extractions directly from small whole blood aliquots of 80 μl without any pretreatment gained recoveries in the range 10–30% within 5 min. By use of calibration curves, these recoveries were usable for detection and quantification of six misuse drugs in eight real samples. The results were highly comparable with the results from conventional sample preparation methods performed by NIPH. The costs per extraction should also be mentioned. Due to the small amounts of reagent consumption and the low costs of the hollow fibers, the cost price is only a few euro cents per extraction.

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