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Simultaneous determination of three phosphatidylethanol homologues, 12 drugs and metabolites in whole blood by LC–MS/MS

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ABSTRACT

The use of alcohol, legal and illicit substances poses great negative consequences on health and economy worldwide. LC-MS/MS allow simultaneous determination of multiple compounds in biological matrices. The aim of this study was to develop a LC-MS/MS method for the determination of the alcohol biomarker phosphatidylethanol (PEth) – including three homologues (PEth 16:0/18:1, PEth 16:0/18:2, PEth 18:0/18:1) - cocaine and three metabolites, and 8 other drugs in whole blood. Whole blood in K2EDTA tubes was prepared by liquid-liquid extraction using heptane/ethyl acetate/2-propanol (16:64:20, v:v:v). Chromatographic separation was achieved on an Acquity BEH C_{18} column (50 × 2.1 mm I.D., 1.7 µm particles). Mobile phase was 0.025 % ammonia, pH 10.7 (Solvent A) and methanol (Solvent B). The method was fully validated with isotope-labelled internal standards for 10 compounds. Inter-assay precision and accuracy were within ± 16 % for all analytes at five to seven tested concentrations. Recovery was within 42–79 % for 14 compounds and 11 % for benzoylecgonine. Matrix effects were within ± 25 % for most analytes. Internal standards compensated for matrix effects for compounds that had their own internal standards. A robust, precise, and accurate LC-MS/MS method for the determinations of three PEth homologues and 12 drugs and metabolites was, developed and validated. The method is valuable, especially for detecting polydrug use and alcohol consumption. To the best of our knowledge, this is the first LC-MS/MS method for the simultaneous determination of three PEth homologues and different drugs and metabolites.

1. Introduction

The use of alcohol, legal and illicit substances pose huge negative consequences on health and the economy worldwide [1,2]. Alcohol is the most used recreational substance in worldwide, whereas cocaine is the second most used illicit drug after cannabis [3]. A growing concern is polydrug use, particularly the combination of alcohol with illicit drugs, such as cocaine or opioids [1,4], as this contributes to higher overdose risks and treatment challenges [1,2]. According to WHO and EUDA 2024 reports, the consumption of alcohol with other psychoactive substances, such as opioids, cannabis or new benzodiazepines, was responsible for nearly 600,000 deaths worldwide [2,3]. Alcohol alone is responsible for over 3 million deaths each year [2]. Therefore, identifying combined use of substances can help develop better treatments and address health and social risks [5].

This study focuses on phosphatidylethanol (PEth), a group of selective and sensitive alcohol biomarkers, cocaine and its metabolites, and other substances of interest. PEth 16:0/18:1, the most abundant homologue, has a half-life in social drinkers that ranges between 5 and 12 days [6–8]. The concentration of PEth 16:0/18:1 in blood can be used to estimate the levels of alcohol consumption into no or low consumption (< 20 ng/mL), moderate consumption (20–200 ng/mL), and alcohol abuse (> 200 ng/mL) [9,10]. A review by Ulwelling and Smith shows that individuals in the no or low consumption category are drinking within approximately 0–2 drinks per day, moderate drinkers consume about 2–4 drinks a day, whereas those in the high consumers category are more likely to drink approximately 4 drinks or more per day [11]. Inclusion of several PEth homologues is particularly useful for the

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identification of patients with hazardous and harmful alcohol use, as well as assessing their patterns of consumption. Hill-Kapturczak et al. and Helander et al. have shown that the ratio of PEth 16:0/18:1 to PEth 16:0/18:2 may be useful for estimating the time since the last alcohol intake [12–14]. Additionally, findings suggest that PEth 16:0/18:2 could serve as a more reliable marker for recent drinking episodes due to its rapid synthesis and elimination [12,13].

Cocaine is a potent addictive central nervous system stimulant drug with a half-life of approximately 0.7 h. When cocaine and ethanol are co-used they produce cocaethylene, an active metabolite with a half-life 4 times longer than cocaine [15]. Cocaethylene results in amplified and prolonged psychoactive effects, and increased heart rate and blood pressure. Additionally, it enhances dependency, exhibits higher cardiotoxicity, and poses a higher risk of intoxication [15,16]. Two main metabolites of cocaine are benzoylecgonine and ecgonine methyl ester, whereas anhydroecgonine methyl ester (AEME) is a product of crack cocaine smoking [15,17].

Opioids and local anaesthetics are medications used for pain management, but can also be associated with misuse, overdose, or adulteration of illicit drugs. For example, local anaesthetics such as lidocaine are frequently used to adulterate street cocaine due to their numbing properties. Opioid use can increase the risk overdose, particularly due to respiratory depression, and this effect is amplified when combined with other psychoactive drugs such as alcohol or benzodiazepines. In fact, 78 % of fatal opioid overdoses involve other substances. In Portugal, tramadol, tapentadol, and codeine are the most used opioids [18,19]. Norway displays a similar pattern, but with a notable rise in fentanyl use [20,21].

For assessing impairment and/or intoxication, blood is the preferred matrix, as it reflects the pharmacological effects of substances on the body, making it the most relevant sample type for forensic and clinical toxicology [22,23].

A technique that allows the simultaneous determination of multiple drug classes is liquid chromatography-tandem mass spectrometry (LC-MS/MS). This analytical technique is commonly used in bioanalysis, due to its ability to detect trace amounts of target analytes with high selectivity, making it well-suited for comprehensive drug analysis [24]. Before LC-MS/MS analysis, sample preparation is needed. However, extracting various analytes and optimizing recovery of compounds from different classes in blood can be challenging, especially when their chemical properties vary significantly. In this study, liquid-liquid extraction (LLE) was selected for its simplicity compared to solid-phase extraction (SPE), its ability to produce cleaner extracts than protein precipitation (PPT), and since it is cheaper than supported liquid extraction (SLE) [25]. Several studies have successfully employed LLE for screening and quantification of multiple drugs and metabolites in whole blood [26,27]. Furthermore, studies have shown that LLE of PEth homologues and/or more polar compounds like pregabalin and amphoteric compounds like benzoylecgonine requires addition of a more polar solvent to achieve adequate recovery [15,28]. PEth analysis in blood is usually performed by LLE, using hexane or heptane together with the addition of 2-propanol, to improve recovery [10,28].

In a recent study we used a mixture of heptane/ethyl acetate/2-propanol (16:64:20, v:v:v) for 96-well SLE to extract PEth 16:0/18:1 and 20 drugs and metabolites from whole blood [29]. Recoveries were above 62 %, except for benzoylecgonine (10 %). All compounds were simultaneously analysed by LC-MS/MS using a basic mobile phase [29]. In another study, a mixture of methyl tert-butyl ether (MTBE)/2-propanol (80:20, v:v) was used for 96-well SLE of PEth 16:0/18:1 and 33 different drugs and metabolites. However, this LC-MS/MS method, where an acidic mobile phase was used, was more time consuming and required two separate injections of each sample for analysis of all compounds [30].

To obtain a sensitive and reliable LC-MS/MS method, optimization of different parameters is important. The use of acidic mobile phases is the most common, partially due to the reduced availability of LC columns suited for basic mobile phases. However, basic mobile phase can increase retention and peak responses, reduce column equilibration time, and improve peak shapes, especially for hydrophilic basic compounds [26,31]. At the same time, changing the mobile phase buffer concentration normally has less influence. In a previous study we observed that the buffer concentration of the mobile phase had no or only minor effect on the retention of different drugs and metabolites analysed but had a huge influence on the retention of the PEth homologues [32]. By reducing the buffer concentration an increased separation between PEths and other unwanted phospholipids was obtained, thus reducing matrix effects [32].

In this study, an LC-MS/MS method using LLE was developed and validated for the determination of three PEth homologues (PEth 16:0/18:1, PEth 16:0/18:2 and PEth 18:0/18:1), cocaine, cocaethylene, AEME, benzoylecgonine, alongside eight additional drugs/metabolites (Supplementary Fig. S1). While, many bioanalytical LC-MS/MS methods focus on one or two drug classes, for example, cocaine and its metabolites [15,33], benzodiazepines [34], amphetamines [35], opioids [36], antidepressants [37], anti-psychotic [38] and PEths [10,32]. Only a few studies have addressed the inclusion of PEth with other licit and illicit drugs in one single analytical method [29,30].

This method represents a significant advancement by combining the simultaneous detection of three PEth homologues—key biomarkers of alcohol consumption, together with cocaine and three metabolites, local anaesthetics, opioids, analgesics, and lysergic acid diethylamide (LSD). To the best of our knowledge, this is the first LC-MS/MS method developed for the simultaneous determination of these three PEth homologues together with cocaine, metabolites and other drugs, monitoring polydrug intake.

2. Materials and methods

2.1. Chemicals and materials

2-Propanol, ethyl acetate, *n*-heptane and nitric acid (p.a.) were purchased from Merck (Darmstadt, Germany). Formic acid (98 %) was acquired from VWR International AS (Oslo, Norway). Type 1 water (18.2 MΩ) purified with a Synthesis A 10 milli-Q system from Millipore (Billerica, MA, USA) was used. Aqueous ammonia (> 25 %) and ammonium carbonate were obtained from VWR Chemicals, Prolabo (Leuven, Belgium). Methanol (MeOH) of LC-MS grade was acquired from Honeywell (Seelze, Germany). Acetonitrile (ACN) was purchased from JT. Baker (Deventer, The Netherlands). Cocaine and benzoylecgonine were purchased from Lipomed (Arlesheim, Switzerland). Compounds obtained from Chiron AS (Trondheim, Norway) included cocaethylene, AEME, $^{13}C_6$-cocaine, $^{13}C_6$-benzoylecgonine, cocaethylene-D_3, PEth 16:0/18:1, buprenorphine-D_5, tramadol-^{13}C-D_3, and PEth 16:0/18:1-D_5. Echelon Biosciences (Salt Lake City, USA) supplied PEth 16:0/18:2, PEth 18:0/18:1, PEth 16:0/18:2-D_5, and PEth 18:0/18:1-D_5. LSD, hydromorphone-D_6, and LSD-D_3 were obtained from Cerilliant (Round Rock, TX, USA), while articaine chloride and bupivacaine-HCl were purchased from Sigma-Aldrich (Darmstadt, Germany). In addition, stock solutions from four pharmaceutical tablets available on the Portuguese market were prepared: Tramal capsules containing 50 mg Tramadol hydrochloride produced by Grünenthal, S.A. (Algés, Portugal); PALEXIA containing 50 mg of Tapentadol produced by Grünenthal, S.A. (Algés, Portugal); Buprenorfina Azevedos produced by Azevedos (Alcabideche, Portugal) containing 8 mg of buprenorphine; Hidromorfona Edunix containing 4 mg of hydromorphone hydrochloride produced by Aristo Pharma (Cacém, Portugal).

2.2. Blank blood

Blood samples were voluntarily donated by employees at the Department of Forensic Sciences at Oslo University Hospital, who reported no use of psychoactive medications, illicit drugs, or alcohol. The

samples were collected in 6 mL Vacuette® K2E K2EDTA tubes from Greiner bio-one (Kremsmünster, Austria).

2.3. Preparation of calibrators, quality control samples and internal standards

Stock solutions of the analytes and internal standards were prepared in MeOH, apart from PEth 16:0/18:1 and PEth 16:0/18:1-D_5 using 2-propanol/ACN (1:1, v:v). The preparation of the stock solution from the four pharmaceuticals tablets was performed according to previously described protocols [39]. Each tablet was individually meshed and dissolved in MeOH, giving final concentrations of 800 mg/L (buprenorphine and hydromorphone) and 5000 mg/L (tramadol and tapentadol). Then, the solutions were sonicated (42 +/- 2.5 kHz, 100 W) on a Elmasonic – S 100 (H) sonicator (Singen, Germany) for 15 min, centrifuged at 3000 rpm (2095 g) for 10 min and filtered (0.45 µm nylon filters). Individual stock solutions were stored at −20 °C in amber glass flasks. Regarding articaine and bupivacaine, the stock solutions were prepared at 100 mg/L by proper dilution with MeOH and stored at 4 °C in amber glass flasks. Calibrator working solutions prepared from tablets were tested and compared to standards prepared from reference materials and deviations within +/- 20 % were obtained for all these compounds.

Working solutions for calibrators, quality control (QC) samples and internal standards were prepared in MeOH by appropriate dilution of the stock solutions. All glassware was pre-washed with nitric acid to prevent degradation and ensure the stability of cocaine. This was performed by adding 1 drop of concentrated nitric acid to the glassware, then approximately 2 mL MeOH were added with a pipette. The mixture was mixed thoroughly, and the MeOH was discarded. This step was repeated with another 2 mL of MeOH, leaving a small amount of MeOH in the glassware. The calibrators and QC samples working solution concentrations were prepared considering the concentration expected to be found in the blood of individuals consuming therapeutic or recreational doses.

2.4. Sample preparation

Calibrators and QC samples were prepared by adding 100 µL of whole blood to 4.5 mL polypropylene tubes. Then, 100 µL type 1 water was added to each sample, followed by vortexing. Type 1 water was added to avoid precipitation before the next steps that include adding organic solvents. Next, 25 µL of the calibrators and QC sample working solutions were added. For authentic and blank blood samples, 25 µL of MeOH was added to ensure equal sample composition in all samples. Then 25 µL of the internal standards working solution was added to all vials. After a brief vortex (1–2 sec), 75 µL of carbonate buffer pH 9.3 was added to all samples, followed by the addition of 800 µL of the organic solvent mixture of heptane/ethyl acetate/2-propanol (16:64:20, v:v:v). The samples were vortexed for 1 min and then centrifuged at 4500 rpm (*4713 g*) for 5 min at 4 °C. From the organic (upper) phase, 500 µL was transferred to new 5 mL plastic tubes by using a pipette. A volume of 10 µL of 0.01 % nitric acid in MeOH was added to all tubes before the samples were evaporated for approximately 15 min at 35 °C using a gas flow (N_2) of 1.2 L/min. The dried samples were reconstituted in 100 µL MeOH, transferred to 300 µL polypropylene plastic autosampler vials (Waters), and analysed using the developed LC-MS/MS method.

2.5. Instrumental analysis

The samples were analysed on an LC-MS/MS system with an Acquity UPLC I-class FTN comprised of a binary solvent manager, sample manager with sample organizer, and a column oven, coupled to a Xevo TQ-S MS/MS, all from Waters (Milford, MA, USA). Chromatographic separations were performed on an Acquity BEH C_{18} column (50 ×2.1 mm ID, 1.7 µm particles) from Waters (Milford, MA, USA) at a column temperature of 60 °C. The flow rate was set to 0.5 mL/min with a mobile phase of 0.025 % ammonium pH 10.7 (solvent A) and MeOH (solvent B). The injection volume for each sample was 1.5 µL. Gradient profile was: 3 % B in 0–0.25 min; 3–25 % B in 0.25–0.3 min; 25–42 % B in 0.3–1.9 min; 42 %-50 % in 1.9–2.0 min; 50–70 % B in 2–4.0 min; 70–75 % B in 4.0–4.10 min; 75–90 % B in 4.1–5.4 min; 90–100 % B in 5.4–5.5 min; 100 % B in 5.5–8.2 min; 100–3 % B in 8.2–8.3 min; 3 % B in 8.3–9.0 min. Electrospray ionization (ESI)-MS/MS detection was conducted in multiple reaction monitoring (MRM) mode, with argon as collision gas. Positive ESI (ESI^+) was used for all compounds, except for the three PEth homologues analysed using negative ESI (ESI^-). MS/MS settings were as follow: capillary voltage 2 kV (ESI^+) and 2.6 kV (ESI^-), source temperature 150 °C, desolvation gas temperature 600 °C, cone gas flow 300 L/h and desolvation gas flow 1000 L/hr. Data acquisition and processing were carried out using MassLynx™ software (version 4.1, Waters, Milford, MA, USA). Table 1 provides the MRM transition ions, cone voltages, collision energies and dwell times for each compound and internal standard.

2.6. Method validation

The method validation was carried out using blank whole blood as the matrix for calibrators and QC samples. The parameters assessed in the validation included: inter-assay precision and accuracy, limit of detection (LOD), lower limit of quantification (LLOQ), recovery, matrix effects, carry-over (%), selectivity, linear range, intra-assay precision and accuracy, and stability in autosampler vials. Calibration curves were either quadratic or linear (weighted 1/x). Calibration curves used in each assay used for determination of inter-assay precision and accuracy were quadratic for 10 compounds (PEth 16:0/18:1, PEth 16:0/18:2, PEth 18:0/18:1, hydromorphone, benzoylecgonine, cocaethylene, AEME, lidocaine, tramadol and tapentadol). For the remaining 5 compounds both quadratic and linear curves were used. The curves were constructed by plotting the ratio of the peak height of the analyte's quantifier ion against the peak height of the internal standard quantifier ion.

Inter-assay precision values were calculated by the relative standard deviation (RSD) of QC values. Inter-assay accuracy was evaluated by calculating the %-deviation between the mean measured concentrations and the theoretical concentrations of QC samples. QC samples at seven different concentrations were analysed on ten separate assays using the extraction method developed. LOD and LLOQ were determined based on the standard deviation values from QC 2 (the QC sample with second lowest concentration), blank samples and signal/noise (S/N) values (See 3.3).

Recovery was calculated by comparing the mean analyte concentration of samples spiked with the working solution pre-extraction to the samples spiked post-extraction. For the samples spiked with QC sample working solution after extraction, 25 µL MeOH was added before extraction to compensate. For the samples that were added QC sample working solution before extraction, 25 µL of MeOH was added after extraction. The remaining samples not spiked before extraction received 25 µL QC working solution post-extraction. Internal standard working solution (25 µL) was added to all samples after extraction. Samples were then evaporated and reconstituted in 100 µL MeOH.

Matrix effects were investigated as described by Matuszewski et al. [40] and in 3.3.

Carry-over (%) was assessed by analysing an extracted blank sample once before and three times after the highest calibrator.

More detailed information about selectivity, linear range and stability is described in 3.3.

Table 1
MRM transitions, cone voltages, collision energies, and dwell times.

Analytes						Internal standards					
Analytes	**MRM transitions[b]**			**Cone voltage (V)**	**Collision energy (eV)**	**Dwell time (ms)**	**Internal standard**	**MRM transitions[b]**		**Cone voltage (V)**	**Collision energy (eV)**	**Dwell time (ms)**
PEth 16:0/18:1[a]	**701.5**	**255.2**		60	40	3	PEth 16:0/18:1-D_5[a]	**706.5**	**255.2**	60	40	3
	701.5	281.2		60	30	3		706.5	281.2	60	30	3
PEth 16:0/18:2[a]	699.5	**255.2**		55	40	3	PEth 16:0/18:2-D_5[a]	**704.5**	**255.2**	55	40	3
	699.5	279.2		55	30	3		704.5	279.2	55	30	3
PEth 18:0/18:1[a]	729.5	**281.2**		65	40	3	PEth 18:0/18:1-D_5[a]	**734.5**	**281.2**	65	40	3
	729.5	283.2		65	40	3		734.5	283.2	65	40	3
Cocaine	**304.2**	**182.2**		30	20	8	$^{13}C_6$-Cocaine	**310.2**	**182.1**	30	20	8
	304.2	82.0		30	35	8		310.2	150.1	30	25	8
Cocaethylene	**318.2**	**196.1**		25	20	3	Cocaethylene-D_3	**322.2**	**85.0**	25	30	3
	318.2	82.0		25	30	3		322.2	199.1	25	20	3
AEME	**182.1**	**122.1**		20	20	20	$^{13}C_6$-Cocaine	**310.2**	**182.1**	30	20	8
	182.1	118.1		20	20	20		310.2	150.1	30	25	8
Benzoylecognine	**290.2**	**168.1**		30	20	29	$^{13}C_6$-Benzoylecognine	**296.2**	**168.1**	30	20	29
	290.2	150.1		30	25	29		296.2	150.1	30	25	29
Articaine	**285.2**	**58.0**		30	30	8	$^{13}C_6$-Benzoylecognine	**296.2**	**168.1**	30	20	29
	285.2	86.0		30	10	8		296.2	150.1	30	25	29
Bupivacaine	**289.3**	**84.1**		40	50	3	Cocaethylene-D_3	**322.2**	**85.0**	25	30	3
	289.3	140.1		40	30	3		322.2	199.1	25	20	3
Lidocaine	**235.2**	**58.0**		50	48	3	$^{13}C_6$-Cocaine	**310.2**	**182.1**	30	20	8
	235.2	86.0		50	28	3		310.2	150.1	30	25	8
Tramadol	**264.2**	**58.1**		20	14	3	Tramadol-D_4	**268.2**	**58.1**	20	14	3
	264.2	246.1		20	12	3		268.2	250.1	20	12	3
Tapentadol	**222.1**	**107.1**		50	30	3	Tramadol-D_4	**268.2**	**58.1**	20	14	3
	222.1	121.1		50	28	3		268.2	250.1	20	12	3
Buprenorphine	**468.3**	**396.2**		60	35	20	Buprenorphine-D_4	**472.3**	**400.2**	60	35	20
	468.3	414.2		60	35	20		472.3	414.2	60	35	20
Hydromorphone	**286.1**	**185.1**		40	30	24	Hydromorphone-D_6	**292.2**	**185.1**	40	30	24
	286.1	157.1		40	40	24		292.2	157.1	40	40	24
LSD	**324.2**	**223.1**		35	30	9	LSD-D_3	**327.2**	**226.1**	35	30	9
	324.2	208.1		35	35	9		327.2	211.1	35	35	9

Abbreviations: F: fragment
[a] ESI in negative mode
[b] Transitions used for quantification are written in bold characters

3. Results and discussion

3.1. Sample preparation

Several techniques are used to extract drugs and metabolites from complex biological matrices. Among these techniques, the most common are SPE, PPT, LLE and SLE [25,41]. In this study, LLE was used as a cost-effective alternative to SLE. Although SLE is more suitable for routine analysis due to the reduced sample preparation time and manual interventions, especially in a 96-well format [42]. LLE works under the same extraction principle and can be optimized to extract a broad range of drug classes. However, low recovery can be a challenge when analysing compounds from different drug classes with different chemical properties, such as basic, acidic and amphoteric compounds; within a single method, see pK_a and Log D data in supplementary Table S1 [30,43]. A general rule is to adjust pH of the sample at least two units above pK_a for bases and two units below for acidic compounds, for them to be non-ionized [44]. However, if a compound has several ionizable groups, like the ampholytic benzoylecgonine which is ionized at all pH values, the extraction can be more challenging [15,30].

Sample preparation using LLE or SLE is commonly performed with non-polar solvents such as MTBE, hexane, heptane, ethyl acetate, or mixtures of these solvents [43,45,46]. However, certain analytes in this study, particularly the PEth homologues, benzoylecgonine, buprenorphine and hydromorphone, posed extraction challenges (Fig. 1). A previous study by Takitane et al., 2018, demonstrated that adding 2-propanol to the organic solvent mixture improves benzoylecgonine recovery using LLE [15]. For improved benzoylecgonine recovery, 30–40 % 2-propanol may be added to the organic solvent mixture. This approach also increases the recovery of the PEth, as demonstrated in a previous study [10,28]. However, a higher proportion of 2-propanol also leads to dirtier extracts. Therefore, an organic solvent mixture with 20 % 2-propanol was chosen, as it gave relatively clean extracts while maintaining satisfactory recovery for all compounds [6,10,28]. For compounds with low recovery, such as benzoylecgonine, the inclusion of an isotope labelled internal standard was important to compensate sample-to-sample variations in recovery. Different mixtures of heptane and/or ethyl acetate with 2-propanol were investigated for the LLE of the 15 compounds (Fig. 1). A final solvent mixture of heptane/ethyl acetate/2-propanol (16:64:20, v:v:v) was chosen. This solvent mixture was also used for SLE in a previous method [29]. The presence of ethyl acetate was important for the recovery of hydromorphone and benzoylecgonine (Fig. 1), probably due to ethyl acetate being more polar than the non-polar heptane.

This mixture gave higher recovery for buprenorphine and lower recovery for benzoylecgonine, compared to using ethyl acetate/2-propanol (80:20, v:v). Overall, it provided adequate recovery and S/N ratios for most compounds. An alternative extraction solvent that can be used in further studies is the mixture of ethyl acetate/2-propanol (80:20, v:v), a mixture that also seems to reduce the amount of lysophospholipids compared to using mixtures where heptane also is included [10]

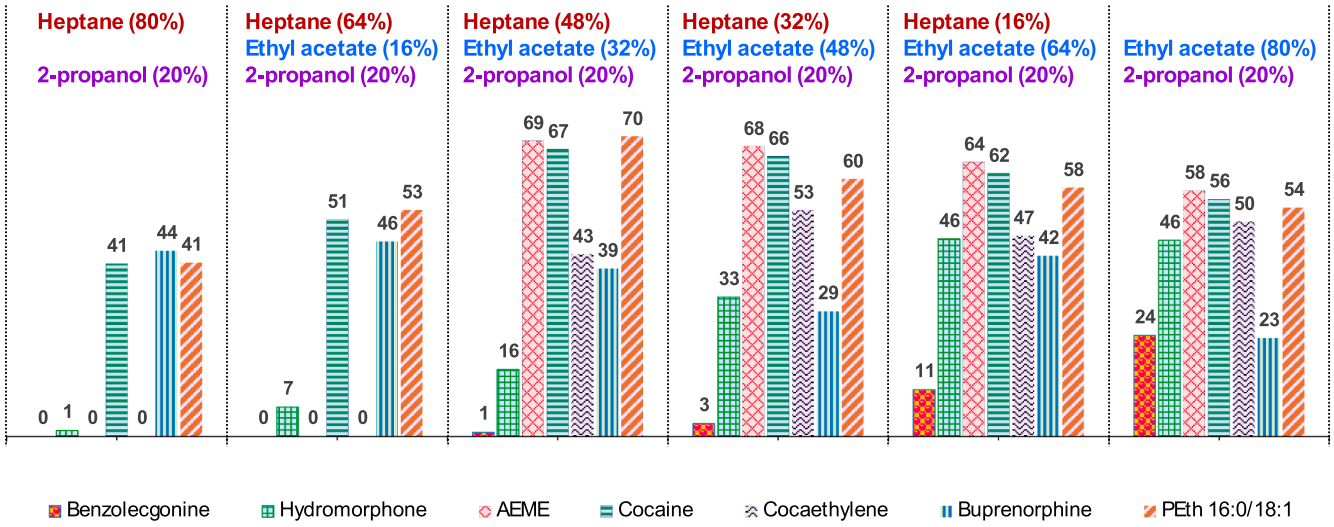

Fig. 1. Recovery (%) of 7 of the 15 compounds using different organic solvent mixtures during LLE. LLE was performed as for the developed method using an organic solvent mixture volume of 800 µL for each sample and transferring of 500 µL to new tubes after mixing and centrifugation. After evaporation and reconstitution in 100 µL MeOH, samples were transferred to autosampler vials and analysed by the developed LC-MS/MS method.

3.2. LC-MS/MS analysis

In LC-MS/MS analysis it is common to use acidic mobile phases, but basic mobile phases have in many studies given increased retention for compounds with basic functional groups, improved peak shapes, and increased sensitivity [31].

Regarding the aqueous part of the mobile phase, we have seen in a previous study that most compounds are not much affected by changing the buffer concentration, except for the PEth homologues [29,32]. In a recent study, reducing the buffer concentration gave better separation of PEth homologues and the phospholipid background, which can be used to minimizing the presence of matrix effects [32]. Therefore, a buffer-free mobile phase was also employed in this study. As shown in Fig. 2, fine tuning of the gradient profile is important for separation of PEth homologues from the phospholipid background. In this study the gradient profile used for the developed LC-MS/MS method is shown in

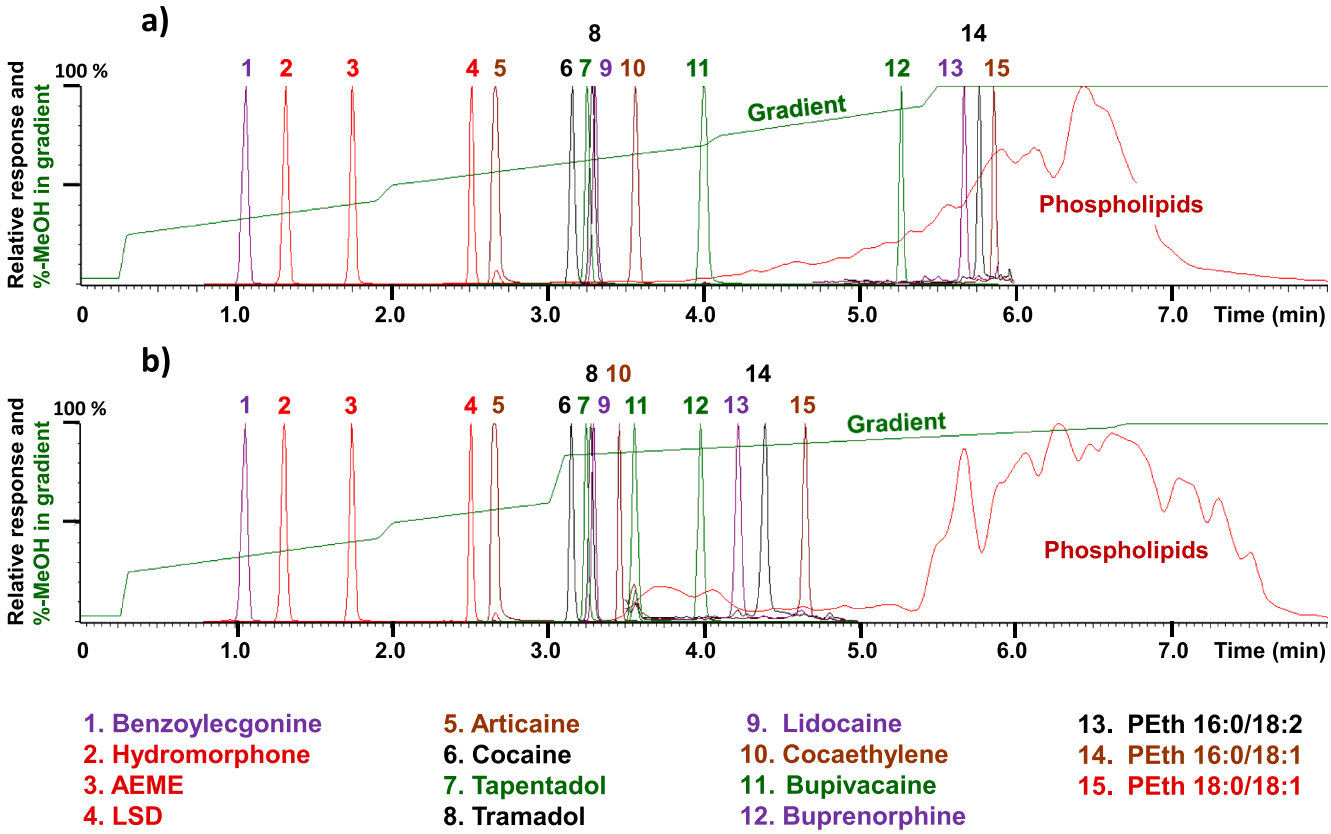

Fig. 2. MRM chromatogram of the 15 compounds of interest and the phospholipid background at two different gradient profiles, the one used in this validated method (a) and a gradient profile with modifications for improved separations of phospholipids and late eluting compounds (b). All peaks are normalized to 100 % height. Phospholipid background was based on Parent ion 184 scan (180–850 amu). Mobile phase composition was 0.025 % ammonia in Type 1 water, pH 10.7 (Solvent A) and MeOH (Solvent B). Gradients used (green) were kept at 100 % MeOH for several minutes to elute all phospholipids. The injected volume was 1.5 µL.

Fig. 2a giving satisfactory separation of most compounds, but clearly co-elution of the four last compounds with the phospholipid background. In retrospect, these four compounds could probably be benefitted by using a gradient profile more like the one in Fig. 2b. This would likely have given less matrix effects and improved sensitivity of the late eluting compounds (buprenorphine and the three PEth homologues). Using isotope labelled internal standards will compensate for matrix effects, and it also provides accuracy and precision. Regarding the organic solvent, MeOH was chosen even though ACN generally gives lower backpressure and less retention of the compounds than MeOH. However, by using the short 5 cm column and a flow rate of 0.5 mL/min, back pressure remained low even when using MeOH. Additionally, MeOH is often considered less toxic than ACN, and the increased retention obtained by MeOH is advantageous for the earliest eluting compounds. In addition, MeOH is used in most LC-MS/MS methods in our laboratory, making MeOH a practical choice for better workflow. Regarding injected volume, some compounds, such as buprenorphine and the three PEth homologues, a higher injection volume could improve S/N values at low concentrations. However, increasing the injection volume resulted in broader peaks of other early eluting compounds. An injection volume of 1.5 μL was chosen as it gave good peak shapes for all compounds (Fig. 2), except for peak broadening occasionally observed for benzoylecgonine (data not shown). A potential solution would be to reduce the injection volume to 1 μL, but this would probably reduce sensitivity for the compounds slightly. An Acquity BEH C_{18} column was used due to its ability to work with a wide pH range, making it suitable for a mobile phase with pH 10.7. A short-length column (50 mm) was used instead of a longer column (100 mm), since it gives low solvent consumption and lower backpressure, and still provides satisfactory separation between compounds. This column is also used in several other LC-MS/MS methods in our laboratory. Altogether, a relatively fast and sensitive method with a satisfactory separation of the compounds was achieved.

3.3. Method validation

3.3.1. Calibration curves and linear range

Calibration curves used for inter-assay precision and accuracy were second-order curves on all assays. The curves were generally highly satisfactory, with average values of the coefficient of determination, R^2 at ≥ 0.996 for all the analytes.

The linear range was determined by LC-MS/MS analysis of 12 extracted standard samples with concentrations within LLOQ and Calibrator 6. A linear calibration curve was applied for all compounds when linearity was tested. The calibrators with the highest concentrations were not used in these curves, to avoid using calibrators outside the linear range. Concentrations within ± 15 % of the theoretical values were considered within the linear range. The upper limit of quantification (ULOQ) was lower for some compounds; lidocaine (ULOQ: 90 ng/mL), articaine (ULOQ: 90 ng/mL), bupivacaine (ULOQ: 90 ng/mL), cocaine (ULOQ: 360 ng/mL); tramadol (ULOQ: 900 ng/mL) and tapentadol (ULOQ: 360 ng/mL).

3.3.2. Precision and accuracy

Table 2 shows the inter-assay precision (% RSD) and accuracy values obtained at concentrations at LOD or higher. For the three PEth homologues the precision and accuracy were below ± 16 %, at five of the six tested concentrations. Buprenorphine and LSD showed precision and accuracy < ± 15 % at six of the seven concentrations. While cocaine and its metabolites, the other opioids and the local anaesthetics got values below ± 16 % across all concentrations. Within assay precision and accuracy was also performed for concentration at LLOQ and above, see results at Supplementary Table S2.

3.3.3. Limit of detection (LOD) and lower limit of quantification (LLOQ)

LOD and LLOQ were determined using the same QC samples that were used for precision and accuracy (Table 2). Equations were applied to calculate LOD and LLOQ. The standard deviation of a low concentration control sample, QC2, was used instead of the blank sample, to avoid unrealistically low LOD and LLOQ values [47,48].

$$\text{LOD} = 3.3 \text{ x standard deviation of QC2} + \text{Blank mean concentration} \quad (1)$$

$$\text{LLOQ} = 10 \text{ x standard deviation of QC2} + \text{Blank mean concentration} \quad (2)$$

Additional criteria for LOD and LLOQ included the S/N values of ≥ 3 and ≥ 10, respectively, for the MRM quantifier transition. In addition, for LLOQ, the inter-assay precision and accuracy values were required to be ≤ 20 %.

Since the method's was optimized for a wide range of chemically and structurally different compounds, achieving high sensitivity for all analytes was challenging, e.g. for the PEth homologues. A previous LC-MS/MS study on six PEth homologues reported an LLOQ of 7 ng/mL, whereas this study obtained LLOQ values that varied from 13 to 19 ng/mL [10]. The main reason for this difference was probably the use of heptane/2-propanol (80:20, v:v) in the previous study, which gave better S/N values due to lower background of early eluting phospholipids. The LOD and LLOQ values for cocaine and benzoylecgonine were also an exception, since the calculated LLOQ values using the defined formula gave values below the QC1 concentration. These values were not considered, and QC3 was used instead of QC2. A recently published study [29], which included some of the same compounds analysed in this method, was used for comparison. The present method achieved lower LLOQ values for cocaine and benzoylecgonine than the previously published study. Tramadol showed similar LLOQ values in both methods, whereas buprenorphine LLOQ was significantly higher in this study (2.9 ng/mL) compared to the previous method (0.5 ng/mL). Possible explanations for the relatively high LLOQ for buprenorphine may be matrix effects by co-eluting phospholipids, alongside slightly lower recovery for buprenorphine obtained by LLE compared to 96-SLE used in the previous published study. From Fig. 2, buprenorphine almost co-eluted with PEth 16:0/18:2 and these compounds are analysed with polarity switching between ESI+ and ESI-. However, this seemed to have no influence on responses for buprenorphine when tested (data not shown). Unfortunately, the LLOQ value for buprenorphine at 2.9 ng/mL is too high for many applications. This result shows the challenges encountered with buprenorphine throughout method development. Fig. 3 presents all compounds at QC2, which corresponds to a concentration near the LLOQ for most compounds, except for the PEth 16:0/18:1, PEth 16:0/18:2 that got LLOQ at QC3 and QC4, respectively.

The four compounds prepared from tablets (hydromorphone, tramadol, tapentadol, and buprenorphine) were tested towards calibrators from another laboratory after the method was validated. Buprenorphine, hydromorphone, tramadol, and tapentadol showed acceptable %-deviations within ±20 %.

3.3.4. Recovery and matrix effects

Recovery was assessed by comparing calculated concentrations in extracted samples spiked with the 15 compounds before and after extraction. Internal standards were added after extraction. As shown in Table 3, the recoveries were between 42 % and 79 % for 14 compounds, while benzoylecgonine had a recovery of 11 %.

Matrix effects were evaluated using the procedure described by Matuszewski et al. at two different concentration levels [40]. Blood samples from six persons were analysed ($n = 6$), the blood was PEth-free. Matrix effects were minimal for eight compounds. LSD, and three of the four opioids exhibited ion enhancement, and ion suppression for the three PEth homologues. However, these effects were compensated standards (Table 3), except for tapentadol with corrected matrix effects values at 123 % and 138 %. For tapentadol, including an isotope labelled internal standard would probably provide better correction of matrix effects and improve accuracy and precision of qualitative and quantitative determinations.

Table 2
Precision, accuracy, LOD, and LLOQ.

Compound, LOD and LLOQ	Theor. conc. (ng/mL)[a]	Calc. conc. (ng/mL)[b]	Prec. RSD (%)	Acc. (%)	Compound, LOD and LLOQ	Theor. conc. (ng/mL)[a]	Calc. conc. (ng/mL)[b]	Prec. RSD (%)	Acc. (%)	Compound, LOD and LLOQ	Theor. conc. (ng/mL)[a]	Calc. conc. (ng/mL)[b]	Prec. RSD (%)	Acc. (%)
AEME	0.90	NA	NA	NA	**Cocaine**	0.90	1.0	4	13	**PEth 16:0/18:1**	1.6	NA	NA	NA
LOD: 1.6 ng/mL	2.9	3.0	16	5	**LOD: 0.9 ng/mL**	2.9	3.0	2	2	**LOD: 5.4 ng/mL**	5.0	5.7	22	13
LLOQ: 4.9 ng/mL	7.5	7.1	7	-5	**LLOQ: 2.3 ng/mL**	8	7.2	3	-4	**LLOQ: 13.7 ng/mL**	13	12.9	6	-4
	11	10.3	8	-5		11	10.4	5	-3		19	18.5	7	-3
	23	20.5	9	-11		23	20.9	6	-9		40	37.5	7	-6
	90	83.7	5	-7		90	86.6	4	-4		160	145.2	5	-9
	360	340.3	6	-5		360	381.2	4	6		640	603.5	7	-6
Articaine	0.90	0.89	NA	NA	**Cocaethylene**	0.90	1.0	8	10	**PEth 16:0/18:2**	1.6	NA	NA	NA
LOD: 0.6 ng/mL	2.9	3.1	22	13	**LOD: 0.7 ng/mL**	2.9	3.0	5	4	**LOD: 7.7 ng/mL**	5.0	NA	NA	NA
LLOQ: 1.7 ng/mL	8	8.1	6	-4	**LLOQ: 1.8 ng/mL**	8	7.2	3	-4	**LLOQ: 19.0 ng/mL**	13	13.5	16	1
	11	11.8	7	-3		11	10.6	4	-2		19	18.0	9	-5
	23	24.1	7	-6		23	21.0	7	-9		40	37.1	9	-7
	90	84.6	5	-9		90	86.6	9	-4		160	146.7	7	-8
	360	277.1	7	-6		360	357.0	8	-1		640	614.1	8	-4
Benzoylecognine	0.90	1.0	5	9	**Hydromorphone**	1.6	1.7	6	5	**PEth 18:0/18:1**	1.6	NA	NA	NA
LOD: 0.8 ng/mL	2.9	3.0	3	5	**LOD: 0.7 ng/mL**	5.0	5.4	3	8	**LOD: 2.7 ng/mL**	5.0	5.4	10	7
LLOQ: 2.2 ng/mL	8	7.5	2	0	**LLOQ: 1.8 ng/mL**	13	13.3	3	-1	**LLOQ: 6.4 ng/mL**	13	13.5	8	0
	11	10.8	3	0		19	19.1	3	1		19	19.7	5	4
	23	22.0	6	-5		40	38.6	5	-4		40	37.5	7	-6
	90	87.0	5	-3		160	154.3	6	-4		160	146.9	5	-8
	360	352.1	5	-2		640	618.1	4	-3		640	599.5	5	-6
Bupivacaine	0.90	0.80	NA	NA	**Licocaine**	0.90	0.87	6	-4	**Tramadol**	3.6	3.9	8	9
LOD: 0.9 ng/mL	2.9	3.2	10	7	**LOD: 0.7 ng/mL**	2.9	3.0	7	4	**LOD: 1.9 ng/mL**	12	11.8	3	-2
LLOQ: 2.8 ng/mL	8	8.3	8	0	**LLOQ: 2.1 ng/mL**	7.5	7.8	6	4	**LLOQ: 4.6 ng/mL**	30	29.3	4	-2
	11	12.2	5	4		11	11.5	5	6		43	42.6	3	-1
	23	23.7	7	-6		23	22.6	4	-2		90	86.0	5	-4
	90	82.1	5	-8		90	83.3	4	-7		360	354.9	5	-1
	360	205.5	5	-6		360	280.8	4	-22		1440	1389.2	10	-4
Buprenorphine	0.60	NA	NA	NA	**LSD**	0.06	NA	NA	NA	**Tapentadol**	3.6	3.8	13	6
LOD: 1.0 ng/mL	2.0	1.9	15	-5	**LOD: 0.08 ng/mL**	0.20	0.21	9	6	**LOD: 3.6 ng/mL**	12	11.6	8	-3
LLOQ: 2.9 ng/mL	5.0	5.2	10	4	**LLOQ: 0.21 ng/mL**	0.50	0.48	5	-3	**LLOQ: 10 ng/mL**	30	28.2	7	-6
	7.2	7.0	13	-3		0.72	0.71	5	-2		43	40.8	8	-6
	15	14.6	7	-3		1.5	1.4	9	-6		90	83.4	11	-7
	60	58.8	5	-2		6.0	5.9	6	-2		360	340.9	6	-5
	240	242.8	14	1		24	23.8	4	-1		1440	1435.1	6	0

Abbreviations: Theor.: theoretical; Calc: calculated; Conc.: concentration, Acc.: accuracy; Prec.: precision; NA: not analysed

[a] Theoretical concentrations of the extracted QC samples (QC1 - QC7) analyzed by the developed LC-MS/MS method.

[b] Calculated concentrations botained by the developed LC-MS/MS method

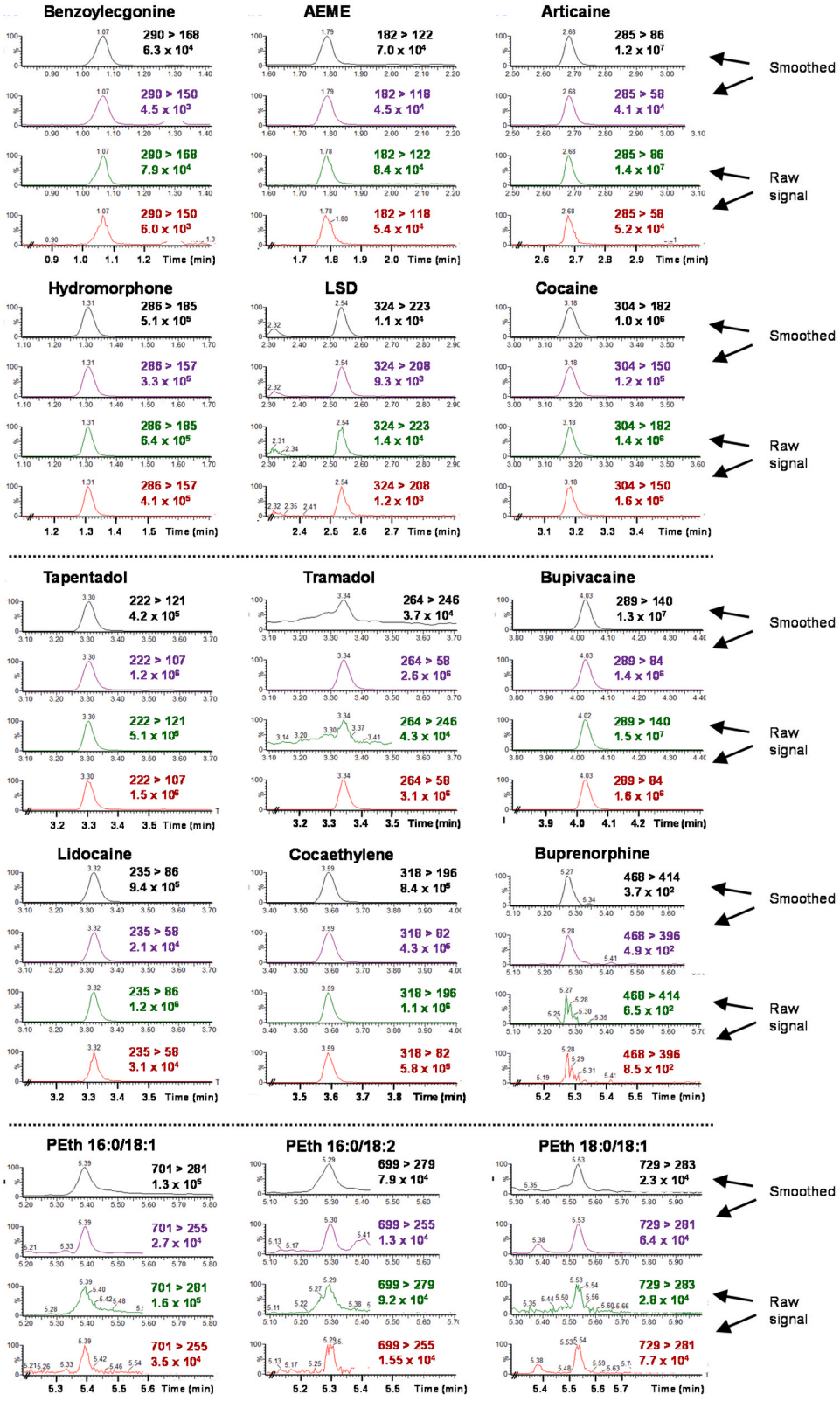

Fig. 3. MRM chromatograms for the 15 compounds obtained by LC-MS/MS analysis of the validated method. The chromatograms shown are both raw and the smoothed signals obtained when 1.5 μL of an extracted QC2 was injected using the validated LC-MS/MS method.

3.3.5. Carry-over and selectivity

The carry-over (%) was determined by comparing both the concentrations and the peak heights obtained for extracted blank samples analysed after an extracted calibrator 6. For all compounds, carry-over in the first blank was $\leq$ 0.09 %, except for the three PEth homologues and LSD, with a carry-over of 0.3 % and 0.2 %, respectively.

Selectivity was investigated by determining the retention times for 50 drugs and metabolites using the developed LC-MS/MS method

Table 3
Recovery and matrix effects.

		Recovery			Matrix Effects			
Compounds	**Theor. conc. (ng/mL)**	**Rec. (%)**	**RSD (%)**	**Min. -Max. (%)**	**ME**	**RSD (%)**	**ME Corr.**	**RSD (%)**
AEME	15	58	10	48–65	105	15	98	12
	90	57	8	50–62	99	5	96	3
Articaine	15	70	5	65–75	104	7	96	9
	90	77	3	72–81	102	3	95	3
Benzoylecognine	15	11	4	10–12	107	6	99	4
	90	11	3	11–12	105	4	98	3
Bupivacaine	15	72	3	68–75	104	4	100	4
	90	79	3	74–83	95	3	94	2
Buprenorphine	10	42	12	32–47	178	46	107	7
	60	43	19	30–59	171	31	100	9
Cocaethylene	15	69	3	67–74	99	7	94	6
	90	71	3	67–74	95	3	94	3
Cocaine	15	69	2	66–71	105	5	98	4
	90	68	1	67–70	102	3	99	3
Hydromorphone	27	54	2	51–55	125	9	101	4
	160	54	2	52–57	123	3	97	3
Lidocaine	15	71	4	67–76	105	6	98	5
	90	77	2	74–79	94	2	91	2
LSD	1.0	65	4	62–69	158	11	103	5
	6.0	65	3	62–70	144	4	98	3
PEth 16:0/18:1	27	68	5	63–73	78	21	100	4
	160	70	5	65–76	60	7	101	3
PEth 16:0/18:2	27	71	9	60–82	56	17	97	7
	160	70	3	67–74	52	12	101	3
PEth 18:0/18:1	27	71	4	67–76	97	21	102	6
	160	68	4	65–72	74	6	97	3
Tapentadol	61	68	10	58–79	165	19	138	19
	360	69	7	62–79	142	6	123	5
Tramadol	61	69	3	66–73	115	5	97	4
	360	70	2	68–73	111	2	97	3

Abbreviations: Theor.: Theoretical; Conc.: concentration; Rec.: recovery; ME: matrix effects; Corr.: corrected

(Supplementary Table S3). Among the 50 compounds, 15 were the compounds included in the developed method. Morphine has the same molecular mass and retention time as hydromorphone. However, they can be separated by their differences in fragment ion response ratios [31]. Hydromorphone has, in contrast to morphine, a very high response for the MRM transition 286 > 185. Similarly, articaine and diazepam had the same molecular mass but different fragment ions, and they were also separated chromatographically.

3.3.6. Stability

An extracted assay with calibrators and QC samples was analyzed three times by LC-MS/MS, first on the day of extraction, and again after 48 h and 7 days of storage in the autosampler (Supplementary Table S4). Deviations within ± 20 % were considered acceptable for sample stability. At concentrations ≥ LLOQ, all compounds got deviations within this criterion with a few exceptions. For PEth 16:0/18:2, deviations of 36 % and 26 % were observed respectively for QC3 (48 h) and QC4 (7 days). Deviation for cocaethylene in QC4 (48 h) was 44 % and for cocaine at QC4 (7 days) was 28 %.

3.4. Authentic blood samples

Table 4 shows the results for PEth 16:0/18:1, PEth 16:0/18:2 and PEth 18:0/18:1 from 20 authentic samples analysed using the developed method. For PEth 16:0/18:1, the table also includes a comparison of results obtained with the developed method with those obtained in another laboratory at OUS. This laboratory where PEth 16:0/18:1 is analysed routinely prepared the blood samples was an LC-MS/MS method with an acidic mobile phase [49].

Analysis of PEth 16:0/18:1 in authentic blood samples can also be used as a test of regio-isomeric purity. Luginbühl et al. has observed that in baches of reference material of PEth 16:0/18:1 from different suppliers, contained mixtures of PEth 16:0/18:1 and PEth 18:1/16:0, which will impact on quantitative results [50]. An approach to evaluate the regio-isomeric purity is to evaluate the relative ion ratio between 701 > 255 and 701 > 281 transitions and compare it to the ion ratios observed in authentic samples with positive results. Regarding the 20 samples analysed, the relative ion ratios for all three PEth homologues were similar as the relative ion ratios observed in calibrators and QC samples.

Table 4
PEth 16:0/18:1, PEth 16:0/18:2 and PEth 18:0/18:1 concentrations in authentic samples.

	PEth 16:0/18:1 (Lab. 1 vs Lab. 2)		PEth 16:0/18:2 (Lab.1)		PEth 18:0/18:1 (Lab. 1)	
Sample	Calc. conc. (ng/mL)	%-dev. for calc. conc. (Lab. 1 vs Lab. 2)[a]	Calc. conc. (ng/mL)	Conc. ratio PEth 16:0/18:2 vs PEth 16:0/18:1 (%)	Conc. (ng/mL)	Conc. ratio PEth 18:0/18:1 vs PEth 16:0/18:1 (%)
1	16	58	5	31	21	131
2	19	7	17	89	11	58
3	20	27	2	10	30	150
4	34	3	25	74	20	59
5	48	-18	60	125	21	44
6	61	7	60	98	39	64
7	71	3	29	41	79	111
8	141	-16	170	121	75	53
9	159	-7	22	14	178	112
10	224	-16	159	71	122	54
11	238	-13	150	63	113	47
12	271	-8	258	95	116	43
13	427	-2	413	97	257	60
14	644	-3	618	96	340	53
15	772	-18	556	72	319	41
16	1126	-6	1349	120	527	47
17	1391	-4	1221	88	737	53
18	1582	-22	888	56	473	30
19	2055	2	899	44	883	43
20	2358	-14	3764	160	824	35

Abbreviations: calc: calculated; conc.: concentration; Lab.: laboratory; dev.: deviation;

[a] %-devitaion based on calculated concentrations obtained by the developed method compared to calculated concentrations obtained for the same samples in another laboratory at OUS that are using another LC-MS/MS method with acidic mobile phase and sample preparation by protein precipitation [49]. Using the anonymous 20 blood samples were approved by the Data Protection Officer at OUS.

4. Conclusion

An accurate, precise and sensitive LC-MS/MS method was fully developed and validated for the determination of three PEth homologues and 12 drugs and metabolites. LLE recoveries ranged between 42 % and 79 % for 14 compounds. Benzoylecgonine had a recovery of 11 %, but since S/N values, inter assay accuracy and precision values were good, this was considered satisfactory. Basic mobile phase was chosen for increased retention and sensitivity of basic compounds. A buffer-free mobile phase was chosen as it has been successfully used in a previous study for separation of PEth homologues from the unwanted phospholipids. However, minor adjustments to the gradient profile of this study are needed to separate PEth homologues from the phospholipid background. The method demonstrated good accuracy values. Inter-assay precision values had RSD below 14 % for all concentrations at the LLOQ and above. The use of isotope-labelled internal standards for 10 of the 15 compounds effectively compensated for matrix effects. To the best of our knowledge, this is the first LC-MS/MS method that enables a more comprehensive assessment of alcohol consumption, through the simultaneous quantification of the three PEth homologues together with cocaine and other drugs and metabolites.

CRediT authorship contribution statement

Marisa H. Maria: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Thomas Berg:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Data curation, Conceptualization. **Nuno R. Neng:** Writing – review & editing, Supervision.

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Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2026.117337.

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