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An accurate and precise liquid chromatography–tandem mass spectrometry method for the determination of six phosphatidylethanol homologues in whole blood with phospholipid interferences minimized

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ABSTRACT

Alcohol consumption is associated with a wide risk of different diseases, injury and death, and has significant social and economic consequences worldwide. Phosphatidylethanol (PEth) is a group of promising direct alcohol biomarkers, with a significantly longer half-life in blood than ethanol, which can be measured to predict different drinking patterns, such as heavy- and social drinking. This study aimed to develop and validate an accurate and precise LC-MS/MS method for the determination of six PEth homologues in whole blood with minimal interference from unwanted phospholipids. Different organic solvent mixtures for liquid-liquid extraction were investigated to obtain satisfactory recovery of PEth homologues and removal of the lyso-phospholipids and other early eluting phospholipids. The mixture of heptane/2-propanol (80:20, *v:v*) gave lower phospholipid background and better signal/noise values for the PEth peaks. An LC-MS/MS TQ-S system from Waters was used for the instrumental analysis. The main part of unwanted phospholipids were separated from the PEth homologues on an Acquity BEH C_{18} column (50 × 2.1 mm ID, 1.7 μm particles) using a buffer-free mobile phase of 0.025 % ammonia in Type 1 water, pH 10.7, as solvent A and methanol as solvent B. Validation and quantification of 22 authentic blood samples showed that the developed LC-MS/MS method is sensitive, precise and accurate for the determination of the six PEth homologues in whole blood. Lower limit of quantification was 10 nM for all compounds. No matrix effects were observed, possibly due to the successful strategies incorporated to avoid the influence of unwanted phospholipids.

1. Introduction

Alcohol consumption is a worldwide health problem that causes an increased risk for different diseases, injury and death, and also has a significant social and economic impact [1–4]. The pharmacological effects of alcohol are associated with the blood alcohol concentration (BAC) and provides information related to the amount of alcohol consumed over a longer time. However, the rapid elimination and short detection window for ethanol of maximum 8–12 h, possess a great limitation [5,6]. For instance, ethanol concentration in the blood will not give us information about alcohol consumed over time [7,8]. Instead, other alcohol biomarkers can be better suited to measure the levels of alcohol consumed, including both indirect and direct biomarkers. Indirect markers compromise cells and enzymes that go through changes due to alcohol use, whereas direct markers are created when ethanol reacts with substances in the human body [9]. Some biomarkers have a detection window of several days up to weeks and are simultaneously selective and sensitive [8,10–12].

A promising direct biomarker is phosphatidylethanol (PEth), a group of abnormal glycerophospholipids, created only in the presence of alcohol [12–15]. The formation of PEth occurs in the cell membranes through the reaction between phosphatidylcholine and ethanol by the catalysis of phospholipase D [16–18]. The most abundant PEth homologue in human blood is normally PEth 16:0/18:1 [19]. Blood

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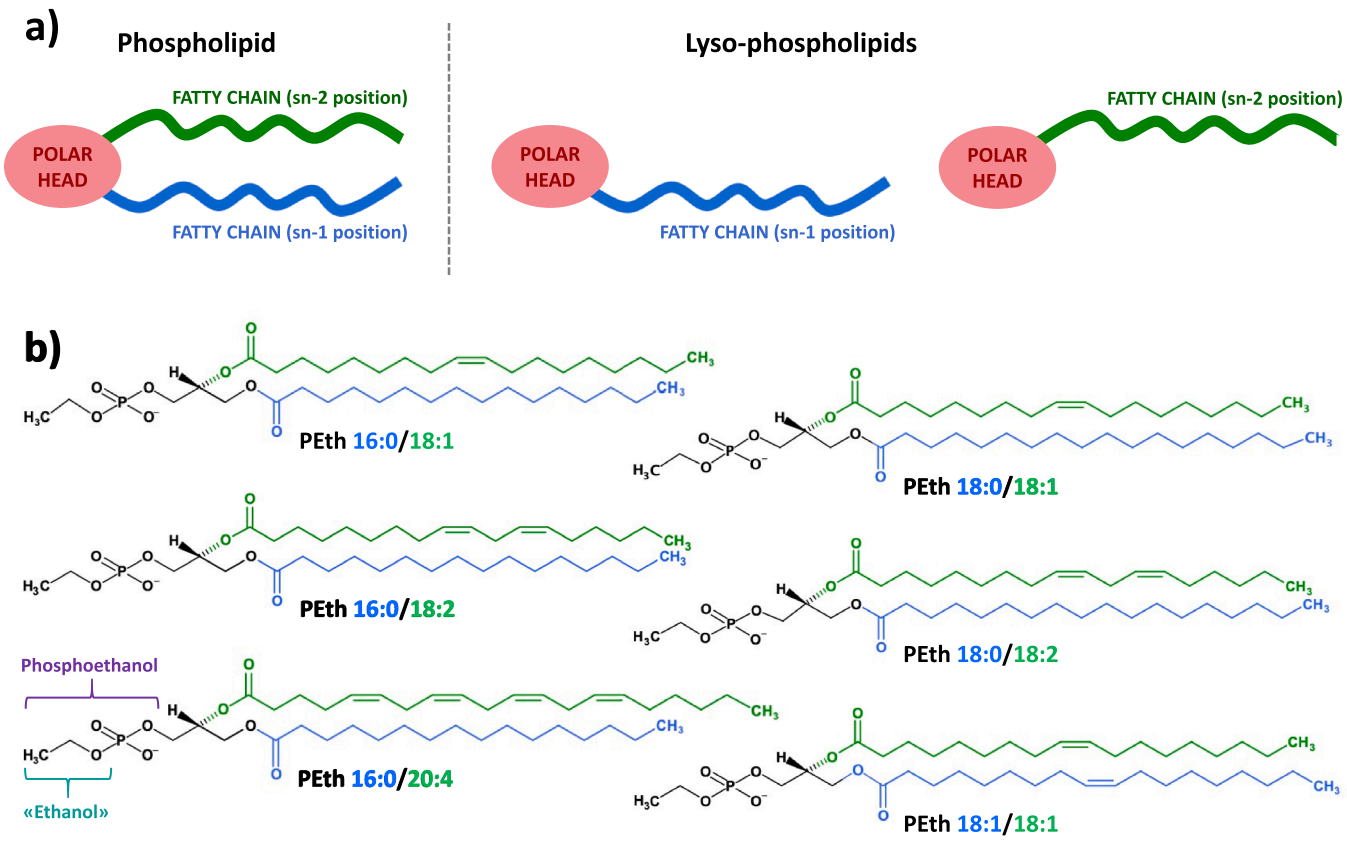

Fig. 1. Simplified structures of phospholipids (a) and molecular structures of PEth homologues included in the developed LC-MS/MS method (b).

concentrations of PEth 16:0/18:1 can be used to estimate the level of alcohol consumed into no/low consumption (PEth < 30 nM), moderate consumption (PEth 30–300 nM) and alcohol abuse (PEth > 300 nM) [20]. PEth 16:0/18:1 got a long half-life of a few days for heavy drinkers and within 5–12 days for social drinkers [12–14,21,22]. As to other homologues, little is known about how they are absorbed, distributed, and excreted. However, the concentration of different PEth homologues varies from person to person, and the elimination rates are not the same for all PEth homologues [23]. Lopez-Cruzan et al. found in a study the half-life of PEth 16:0/20:4 was only 2.1 ± 3 days, while the half-life for PEth 16:0/18:1 was 7.6 ± 3 days and PEth 16:0/18:2 was 6.8 ± 4 days [24]. Knowledge about the blood concentrations of several PEth homologues instead of just one can be used to improve on the estimation of alcohol consumption (time and amount). Therefore, new and better methodologies for the determination of several PEth homologues may be beneficial and contribute to progress in clinical, forensic, and epidemiological studies associated with alcohol use [8,10–12].

Nowadays, bioanalysis of PEth are performed mostly by LC-MS and LC-MS/MS, and with chromatographic separation using acidic mobile phases [7,14,25–39]. However, since the pKa value for PEths is to the best of our knowledge, around 1.5–2.0, therefore changing mobile phase pH above pH ≈ 4, will not have much effect on their retention [40]. The LC-MS/(MS) methods are usually developed for the determination of the PEth 16:0/18:1 only, and/or a few other PEth homologues [26,30,31,33,37,38,41,42]. Recently, Aboutara et al. and Josefine Herzog et al. have developed LC-MS/MS methods for the determination of six and seven PEth homologues in dried blood spots (DBS), respectively [25,43]. Sample preparation of PEths from the blood prior to LC-MS/MS analysis has commonly been performed by LLE using 2-propanol and hexane or heptane [23,24,33,34,44–47]. The addition of 2-propanol has been performed to increase analyte recovery [48]. Other sample preparation techniques used are protein precipitation (PPT), solid phase extraction (SPE), supported liquid extraction (SLE), and dispersive liquid–liquid microextraction (DLLME) [14,25,26,31,39,42].

Although a major part of PEth bioanalysis is performed by LC-MS/MS, the determination of PEth in the blood can be challenging for several reasons. First, PEths are phospholipids and got similar molecular structures and physicochemical properties as other phospholipids [40,49]. Therefore, it can be difficult to remove unwanted phospholipids meanwhile as the PEths are extracted during sample preparation. Secondly, it can be difficult to avoid co-elution between PEths and the phospholipid background during LC-MS/MS analysis, which may give matrix effects. In addition, the highly lipophilic PEths may require extra blank injections to avoid carry-over, and the choice of washing solvents suitable is limited [31,49–52].

In a previous study, we investigated how PEths and other phospholipids can be separated by reversed-phase LC-MS/MS to reduce possibilities for matrix effects [40]. It was found that reducing the mobile phase buffer concentrations had a huge effect on the separation between PEths and unwanted phospholipids, whereas changes in the mobile phase pH did not have much effect within pH 5 to pH 10. The best separation between PEths and unwanted phospholipids was obtained using a buffer-free high pH mobile phase consisting of 0.025 % ammonia in Type 1 water as aqueous solvent and methanol (MeOH) as organic solvent. The aim of this study was to use the same buffer-free high pH mobile phase composition and then further develop, optimize and validate an accurate and precise LC-MS/MS method for the determination of six PEth homologues in whole blood. Another aim was to investigate if it was possible to remove lyso-phospholipids, those with only one fatty acid chain (Fig. 1), and other early eluting phospholipids during sample preparation.

2. Materials and methods

2.1. Chemicals and materials

PEth 16:0/18:1, PEth 16:0/18:2, PEth 16:0/20:4, PEth 18:0/18:1, PEth 18:0/18:2 and PEth 18:1/18:1 were purchased from Echelon Biosciences (Salt Lake City, USA). The internal standards PEth 16:0/18:2-D_5, PEth 16:0/18:1-D_5, PEth 16:0/20:4-D_5, PEth 18:0/18:2-D_5, and PEth 18:0/18:1-D_5 were also purchased from Echelon Biosciences (Salt Lake City, USA).

MeOH of LC-MS hyper grade and MeOH of LC-MS Ultra grade was acquired from Honeywell (Seelze, Germany), and acetonitrile of HPLC Far UV grade was purchased from JT. Baker (Deventer, the Netherlands). 2-propanol, ethyl acetate, and n-heptane were purchased from Merck (Darmstadt, Germany). Type 1 water (18.2 MΩ) purified with a Synthesis A 10 milli-Q system from Millipore (Billerica, MA, USA) was used. Formic acid (98 %) was acquired from VWR International AS (Oslo, Norway). Aqueous ammonia (> 25 %), was obtained from VWR Chemicals, Prolabo (Leuven, Belgium).

2.2. Blank blood

Blank whole blood used for calibrators, quality control (QC) samples and test samples were obtained from volunteers employed at the Department of Forensic Sciences at Oslo University Hospital (OUS) were collected in 4 mL Vacuette® K2E K2EDTA tubes from Greiner bio-one (Kremsmünster, Austria).

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

Stock solutions for the six PEths homologues were prepared in MeOH. Internal standards stock solutions for each internal standard were prepared in 2-propanol/ACN (50:50, *v:v*), whereas the internal standards working solution were prepared in methanol by appropriate dilution of the stock solutions. Calibrator and QC working solutions were prepared in MeOH by appropriate dilution of the stock solutions. Calibrators were prepared by spiking blank blood to concentrations of 10, 30, 60, 300, and 800 nM and the QCs to 5, 10, 20, 30, 100, 300 and 600 nM.

2.4. Sample preparation

Calibrators and QC samples were prepared by first adding 100 µL whole blood to 4.5 mL polypropylene tubes. Then 100 µL Type 1 water was added to all samples and then vortexed. Then calibrators and QC samples were prepared by adding 25 µL of the calibrators and QC sample working solutions. For authentic blood samples and blank samples, 25 µL MeOH was added to make sure that the composition of all samples was the same. Then 25 µL of the internal standards working solution was added to all vials. After mixing, by vortexing for 1–2 s, 75 µL of carbonate buffer pH 9.3 was added. Then 1600 µL of the organic solvent mixture of n-heptane/2-propanol (80:20, *v:v*) was added to all samples, and the samples were vortexed for 1 min. The samples were then centrifuged at 4500 rpm for 5 min. From the organic (upper) phase 900 µL was transferred to new 5 mL glass tubes by using a pipette, and then the samples were evaporated for approximately 20 min at 45 °C using a gas flow (N_2) of 1.2 L/min. After evaporation, the samples were reconstituted in 100 µL MeOH and transferred to single 300 µL polypropylene plastic autosampler vials (Waters) and analyzed by LC-MS/MS.

2.5. Instrumental analysis

UHPLC-MS/MS analyses were performed on 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 mobile phase flow rate was 0.5 mL/min. The mobile phase composition was 0.025 % ammonia in Type 1 water, pH 10.7, as Solvent A and MeOH as Solvent B. Injection volume was 2 µL. Gradient profile was: 60 % B in 0.0–0.2 min, 60–80 % B in 0.2–0.3 min, 80–98 % B in 0.3–3.8 min, 98–100 % B in 3.8–3.9 min, 100 % B in 3.9–6.4 min, 100–60 %B in 6.4–6.5 min, 60 % B in 6.5–6.8 min.

Electrospray ionization (ESI)-MS/MS detection was performed in negative ESI (ESI−) with multiple reaction monitoring (MRM) using two MRM transitions for each compound and internal standard. Collision gas used was argon. MS/MS settings were as follows; capillary voltage 2.6 kV, source temperature 150 °C, desolvation gas temperature 500 °C, cone gas flow 300 L/h and desolvation gas flow 1000 L/hr. Table 1

Table 1
MRM transitions, cone voltages, collision energies, and dwell times of the six PEth homologues.

Analytes							Internal standards						
	MRM transitions [a]							MRM transitions [a]					
Analytes	$[M+H]^+$		$[F+H]^+$	Cone voltage (V)	Collision energy (eV)	Dwell time (ms)	Internal standard	$[M+H]^+$		$[F+H]^+$	Cone voltage (V)	Collision energy (eV)	Dwell time (ms)
PEth 16:0/18:1	**701.5**	>	**255.2**	60	40	6	PEth 16:0/18:1-D_5	**706.5**	>	**255.2**	60	40	6
	701.5	>	281.2	60	30	6		706.5	>	281.2	60	30	6
PEth 16:0/18:2	**699.5**	>	**279.2**	55	30	6	PEth 16:0/18:2-D_5	**704.5**	>	**279.2**	55	30	6
	699.5	>	255.2	55	40	6		704.5	>	255.2	55	40	6
PEth 16:0/20:4	**723.5**	>	**255.2**	50	35	5	PEth 16:0/20:4-D_5	**728.5**	>	**255.2**	50	35	5
	723.5	>	303.3	50	25	5		728.5	>	303.3	50	25	5
PEth 18:0/18:1	**729.5**	>	**281.2**	65	40	6	PEth 18:0/18:1-D_5	**734.5**	>	**281.2**	65	40	6
	729.5	>	283.2	65	40	6		734.5	>	283.2	65	40	6
PEth 18:0/18:2	**727.5**	>	**279.2**	50	40	5	PEth 18:0/18:2-D_5	**732.5**	>	**279.2**	50	40	5
	727.5	>	283.2	50	40	5		732.5	>	283.2	50	40	5
PEth 18:1/18:1	**727.5**	>	**281.2**	60	40	6	PEth 18:0/18:2-D_5	**732.5**	>	**281.2**	50	40	6
	727.5	>	463.3	60	30	6		732.5	>	463.3	50	40	6

[a] Transitions used for quantification are written in bold characters
Abbreviations: F: fragment.

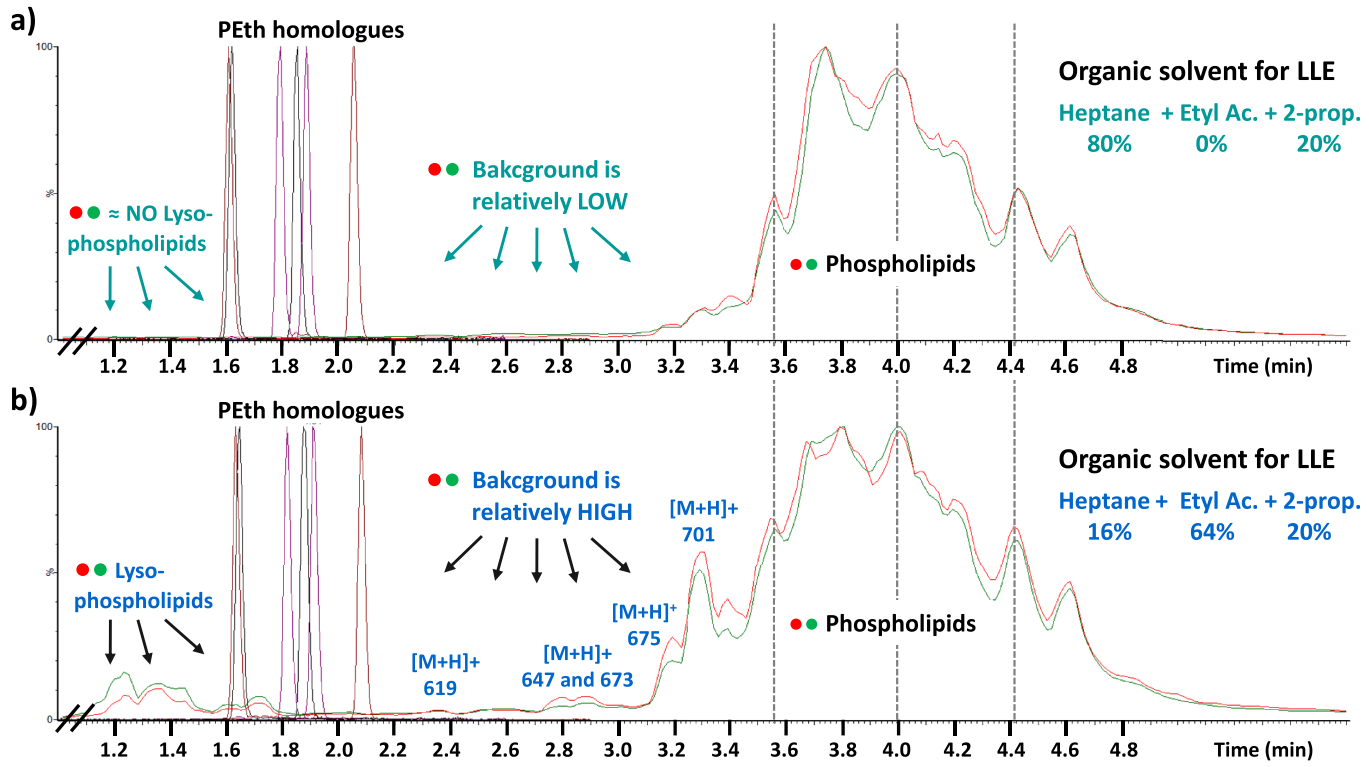

Fig. 2. Background of phospholipids obtained by Parent ion scan of *m/z* 184 [49,51], ESI^+, of extracted blood samples prepared by LLE using heptane/2-propanol (80:20, *v:v*) (a) and heptane/ethyl acetate/2-propanol (16:64:20, *v:v:v*) (b). Two different blank bloods were used for the background scan, shown by the red and the green lines, respectively. Total ion chromatograms for the six PEth homologues were obtained by a separate ESI^- - LC-MS/MS analysis injecting extracted QC sample with PEth homologue concentration 100 nM. The PEth chromatograms are normalized to be equally high as the highest background peak, even though the background peaks (concentrations and responses) were much higher. Gradient profile, mobile phase composition and column were the same as used for the validated method.

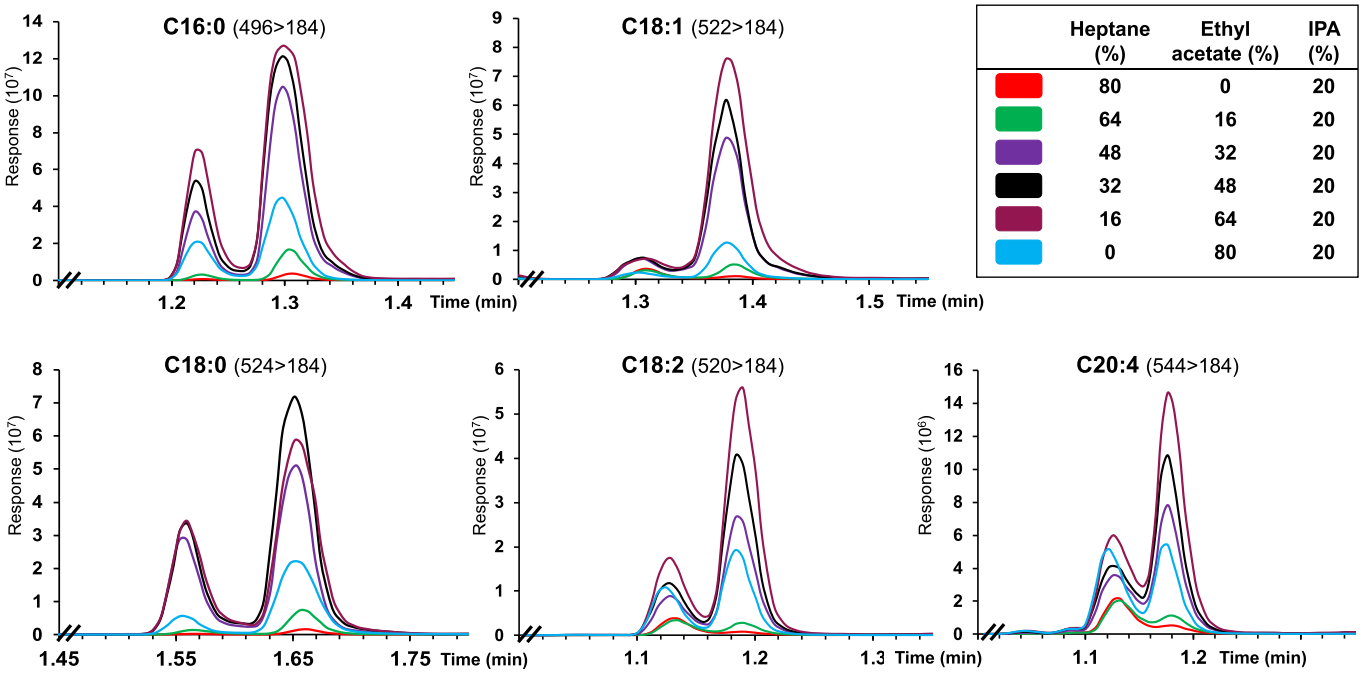

Fig. 3. Relative response for lyso-phospholipids obtained by LC-MS/MS analysis of extracted whole blood prepared by different extraction solvents for LLE. Gradient profile, mobile phase composition and column as used for the validated method. LC-MS/MS analyses were performed by positive ESI and with cone voltage at 40 V and collision energy of 40 eV. Abbreviation: IPA: 2-propanol.

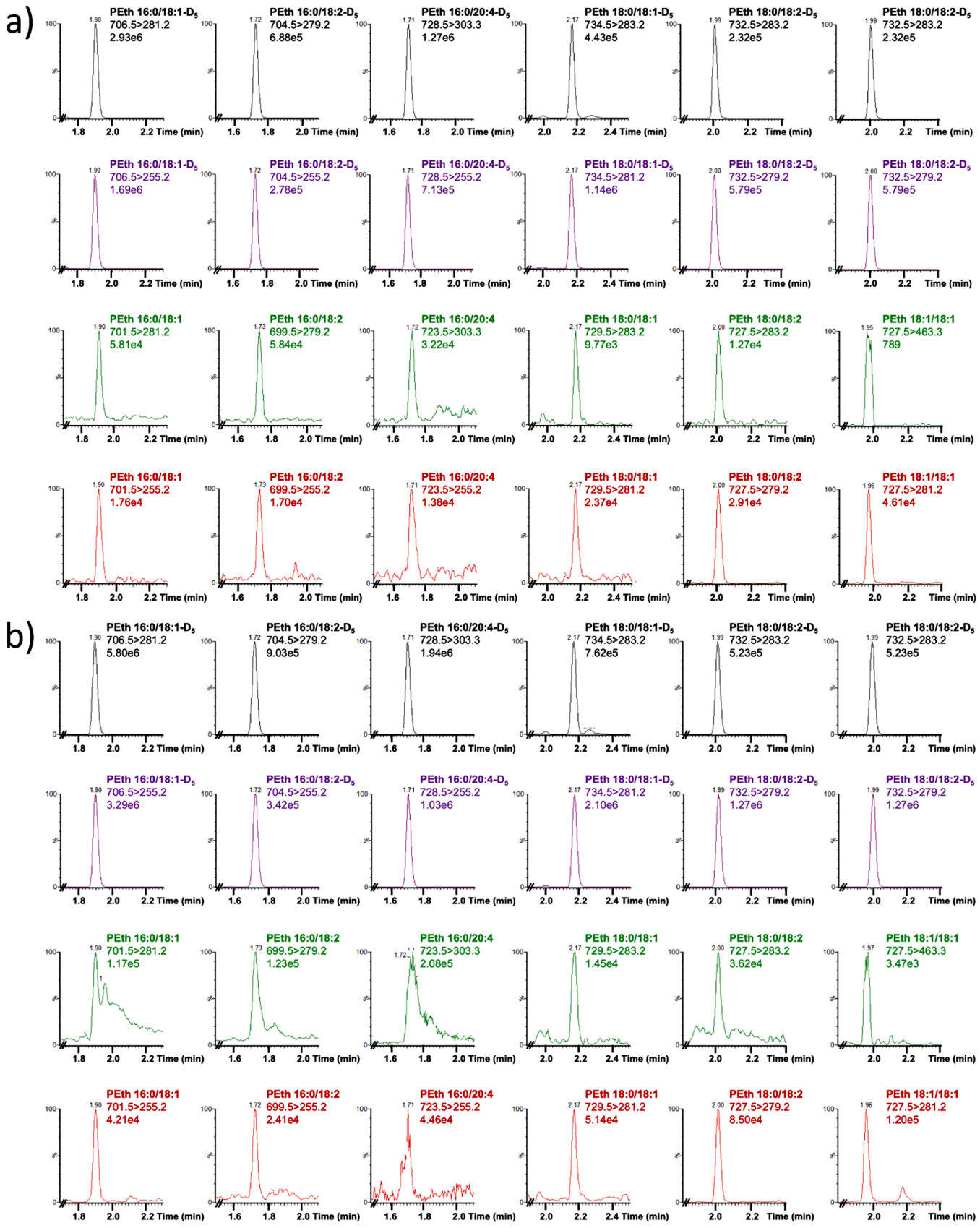

Fig. 4. MRM chromatograms of PEth homologues obtained by LC-MS/MS analysis of a standard sample with PEth homologue concentrations of 10 nM using two different organic solvents during LLE, heptane/2-propanol (80:20, *v:v*) (a), and heptane/ethyl acetate/2-propanol (16:64:20, *v:v:v*) (b). LC-MS/MS conditions same as for the validated method.

shows the MRM transition ions, cone voltages, collision energies and dwell times of each compound and internal standard. Parent ion scan of *m/z* 184 used to determine general phospholipid background was performed by positive ESI with cone voltage at 40 V and collision energy at 40 eV. Data was acquired and processed using Masslynx™ software (version 4.2, Waters, Milford, MA, USA).

2.6. Method validation

The method validation was performed by using blank whole blood as matrix in calibrators and QC samples and included: inter-assay precision and accuracy, the limit of detection (LOD), the lower limit of quantification (LLOQ), recovery, matrix effects, carry-over, stability in autosampler vial, and selectivity. There are many guidelines and recommendations for the validation of analytical methods that are published [53,54]. The validation in this study is based on internal guidelines used at OUS for bioanalytical LC-MS/MS methods, which are partly based on previously published papers by Magnusson and Örnemark (2014), Rivier et al. (2003) and by Peters et al. (2007) [53,55,56]. Experimental details from the different tests performed during method validation can be found in the Section 3.2.

3. Results and discussion

The present study aims to introduce a precise and accurate LC-MS/MS method that can determine six PEth homologues. For that, we optimized the sample preparation and certain LC-MS/MS parameters.

3.1. Sample preparation and LC-MS/MS analysis

In a previous study we succeeded to separate eight PEth homologues and most of the unwanted phospholipids by LC-MS/MS using a mobile phase of 0.025 % ammonia in Type 1 water (Solvent A) and MeOH (Solvent B) [40]. Therefore, the same mobile phase composition and the same UHPLC column were used in this study. However, only six of the eight PEths were included this time. PEth 16:0/16:0 and PEth 17:0/18:1 were not included due to poor signal/noise values and the absence of available isotope-labeled internal standards. In addition to the knowledge from the previous study, we also wanted to investigate if lyso-phospholipids (those with one fatty chain) and other early eluting phospholipids could be removed during sample preparation. Here, LLE was chosen, since it is an easy and economical technique to perform, and it has been used successfully in several LC-MS/MS methods for the preparation of PEth from blood [34,57,58]. Usually, the organic solvent used for LLE of PEth from blood has been a mixture of 2-propanol and heptane [34]. In this study, we investigated LLE using different mixtures of heptane/ethyl acetate, all combined with 20 % of 2-propanol. The lyso-phospholipids selected had similar carbon chains regarding length and number of double bonds as in the fatty acid chains of the PEth homologues in the study; C16:0; C18:0; C18:1; C18:2 and C20:4. By using heptane/2-propanol (80:20, *v:v*) for the LLE, the lyso-phospholipids were almost completely removed, in contrast to when ethyl acetate was included in the mixture (Figs. 2 and 3). In addition to removing lyso-phospholipids, other phospholipids were also removed by using the heptane/2-propanol mixture (specified in Fig. 2). Regarding recovery, slightly lower recoveries were observed using heptane and 2-propanol, compared to using the mixtures containing ethyl acetate (Supplementary Table 1). However, average recoveries for the PEth homologues within 38–51 % were considered acceptable, especially since PEth signal/noise values and better removal of early eluting phospholipids were observed when the heptane/2-propanol mixture was used. The benefit of removing the lyso-phopholipids and other early eluting phospholipids by proper choice of organic solvent was clearly illustrated by LC-MS/MS analysis of extracted standard samples with PEth concentrations of 10 nM (Fig. 4). Better signal/noise values, improved peak shape and less disturbing peaks, especially for PEth 16:0/18:1 (701>281) and PEth 16:0/20:4 (both MRM transitions), were observed when heptane/isopropanol (80:20, *v:v*) was used as extraction solvent.

3.2. Method validation

Method validation included inter-assay precision and accuracy, LOD, LLOQ, recovery, matrix effects, stability, selectivity and carry-over.

3.2.1. Calibration curve and linear range

Calibration curves used for determination of inter-assay precision and accuracy were linear for all curves on all assays, except for one assay where a second order curve was used for PEth 16:0/18:1, since it gave better curve fit. The curves were generally very good shown by the coefficient of determination, R^2-values, that were on average $\geq$ 0.997 for all six PEth homologues.

Linear range was tested for five of the six PEth homologues (PEth 16:0/18:1, PEth 16:0/18:2; PEth 18:0/18:1; PEth 18:0/18:2; PEth 18:1/18:1) within concentrations of 30–2500 nM. Calculated concentrations were within ± 15 % from theoretical values considered to be within the linear range. All five compounds had linear curves within the tested concentration range of 30–2500 nM. Preferably, lower concentrations could have been included in the linearity test. However, precision and accuracy data shows the curves are linear within the range of 10 to 30 nM. PEth 16:0/20:4 was not included in the linearity test as the stock solution of this compound was too low for preparation of the highest concentrations. However, inter-assay precision and accuracy values from Table 2 shows that PEth 16:0/20:4 is linear within the concentration range of 10–800 nM.

3.2.2. Inter-assay precision and accuracy

Inter-assay precision and accuracy were determined based on calculated concentrations of extracted QC samples analyzed on 10 different assays. The 10 assays were extracted and analyzed over a period of two weeks with one replicate for each QC sample. Precision was determined as the relative standard deviation (%-RSD) of the calculated concentrations. The accuracy (bias) values were calculated as %-deviation between calculated concentrations and theoretical values of the QC samples. Table 2 shows that inter-assay precision and accuracy at the six concentration levels, within 10–600 nM, were $\leq$ 15 % and $\leq \pm$ 14 %, respectively.

3.2.3. Limit of detection (LOD), lower limit of quantification (LLOQ) and cut-off

LOD and LLOQ were determined based on data from the 10 assays used for the determination of inter-assay accuracy and precision. LOD values were calculated by Eq. (1), giving; 3.3 nM for PEth 16:0/18:1; 2.1 nM for PEth 16:0/18:2; 4.8 nM for PEth 16:0/20:4; 3.1 nM for PEth 18:0/18:1 and 2.4 nM for PEth 18:0/18:2. For PEth 18:1/18:1 a considerable difference in response and signal/noise values were observed between quantifier and qualifier MRM transition. Therefore, even though the calculated LOD was 2 nM for PEth 18:1/18:1, LOD was determined to be 5 nM for this compound. LLOQ was determined to be 10 nM for all six PEth homologues, based on that signal/noise values were $\geq$ 10/1 and that inter-assay precision and accuracy values were $\leq \pm$ 15 %. Fig. 4a shows MRM chromatograms of extracted QC sample with PEth homologue concentrations of LLOQ, 10 nM.

$$LOD = 3,3 \times SD_{QC5nM} + Average\ concentration\ of\ Blank \quad (1)$$

The developed LC-MS/MS method will be used to distinguish between different levels of alcohol consumed. The PEth 16:0/18:1 concentration cut-off limit used to separate between low/no-drinkers and medium alcohol consumption in Norway is normally 30 nM (21.1 ng/mL) [20,59].. However, outside Norway, the cut-off limit often used is slightly different; 20 ng/mL (28.5 nM) [60,61]. In a paper recently published by Luginbühl et al., it was concluded that the limits of 20 ng/mL (28.5 nM) and 200 ng/mL (285 nM) should be used as limits to

Table 2
Inter-assay accuracy and precision of the six PEth homologues.

		PEth 16:0/18:1			PEth 16:0/18:2			PEth 16:0/20:4		
Theor. conc. (nM)	n	Average calc. conc. (nM)	Precision (% - RSD)	Accuracy (%)	Average calc. conc. (nM)	Precision (% - RSD)	Accuracy (%)	Average calc. conc. (nM)	Precision (% - RSD)	Accuracy (%)
5	10	4.8	17	−4	4.7	12	−6	5.0	26	−1
10	10	10.0	15	1	9.9	8	−1	8.6	10	−14
20	10	20.6	8	4	20.6	5	3	18.2	11	−9
30	10	29.6	14	0	31.3	6	4	30.2	11	1
100	10	103.0	10	4	112.4	5	12	107.0	7	7
300	10	315.8	10	6	328.0	7	9	316.3	7	5
600	10	602.7	5	0	621.5	5	4	601.0	7	0

		PEth 18:0/18:1			PEth 18:0/18:2			PEth 18:1/18:1		
Theor. conc. (nM)	**n**	**Average calc. conc. (nM)**	**Precision (% - RSD)**	**Accuracy (%)**	**Average calc. conc. (nM)**	**Precision (% - RSD)**	**Accuracy (%)**	**Average calc. conc. (nM)**	**Precision (% - RSD)**	**Accuracy (%)**
5	10	5.0	13	0	4.8	12	−4	4.5	10	−10
10	10	9.8	14	−2	9.9	7.6	−1	9.6	8	−4
20	10	19.6	8	−2	19.1	9.1	−4	19.4	8	−3
30	10	30.0	5	0	30.1	5.6	0	31.0	7	3
100	10	107.3	6	7	102.7	7.0	3	107.2	7	7
300	10	318.8	8	6	309.1	10	3	312.0	9	4
600	10	598.5	6	0	579.6	5.6	−3	597.7	5	0

Abbreviations: Theor: theoretical; conc.: concentrations; calc.: calculated.

distinguish different drinking patterns [60]. The developed method has shown to be well suited to be used with a cut-off level at a concentrations of both 20 ng/mL (28.5 nM) and 30 nM (21.1 ng/mL). Fig. 5 shows MRM chromatograms of extracted QC sample with concentration 30 nM and an extracted blank whole blood sample.

3.2.4. Recovery

Recovery was determined at two concentrations, 30 nM and 300 nM, in PEth-free blood from four different persons ($n = 4$ per level). The %-recovery was determined by comparing the concentrations of the samples spiked with the six PEth homologues working solution before LLE with the samples of the PEth homologues added after LLE. The internal standards were added after the extraction of all samples. Table 3 shows that %-recovery of the PEth homologues was within 37 −51 %.

3.2.5. Matrix effects

Matrix effects were studied at two concentration levels as described by Matuszewski et al. [62]. At both concentration levels two sets of samples were analyzed. In the first set ($n = 6$), PEth-free blood samples from six persons were extracted, transferred to new vials, evaporated, and reconstituted in 60 µL MeOH. Then, 50 µL of the extracts were transferred to a 5 mL plastic tube and spiked with the analytes and the internal standards to a total volume of 100 µL. In the second set ($n = 6$), in 5 mL plastic tube was added 50 µL of MeOH, and then added the analytes and internal standards. The sample solvent and total sample volume of both sets were the same. The samples were then transferred to polypropylene autosampler vials and analyzed by the developed LC-MS/MS method. No significant matrix effects were observed (Table 3).

$$\text{Matrix effect} = [\text{Peak height}_{\text{Set1}}/\text{Peak height}_{\text{Set2}}] \times 100 \quad (2)$$

Matrix effect = 100 indicates no matrix effects, whereas values > 100 indicates possible matrix enhancement, and values < 100 indicates possible matrix suppression.

3.2.6. Carry-over (%)

Carry-over in the first and second blanks analyzed behind a sample with high PEth homologue concentrations was ≤ 0.07 % and ≤ 0.04 %, respectively. Although carry-over values are low, precautions should be taken and when analyzing authentic samples should be considered including blank injections between samples to reduce the possibility for carry-over.

3.2.7. Selectivity

Blank blood from five different persons was analyzed without the PEth homologues and internal standards. No interfering peaks were observed at the retention times of the different PEth homologues.

3.4.8. Sample stability

Stability was tested by LC-MS/MS analysis of 22 authentic blood samples prepared by the developed LLE. These samples were extracted and prepared by the developed method. Then the samples were analyzed twice, first once on the day of extraction, then once again after storage for 7 days at 4 °C in the fridge. Deviations within ± 20 % were considered acceptable regarding sample stability. Table 4 shows that the calculated PEth concentrations, at concentrations ≥ LLOQ, were within ± 17 % for five of the six PEth homologues. For PEth 16:0/20:4 two samples had concentrations that deviated more than ± 20 % after 7 days, 21 % and 29 %, respectively.

3.4.9. PEth homologues concentrations in 22 authentic blood samples

Among the 22 authentic blood samples analyzed by the developed LC-MS/MS method, 17 samples were positive for one or more PEth homologues (Table 4). As expected from previously published studies the concentration of PEth 16:0/18:1 was generally the highest detected in whole blood [19,63]. Concentrations of PEth 18:0/18:1, PEth

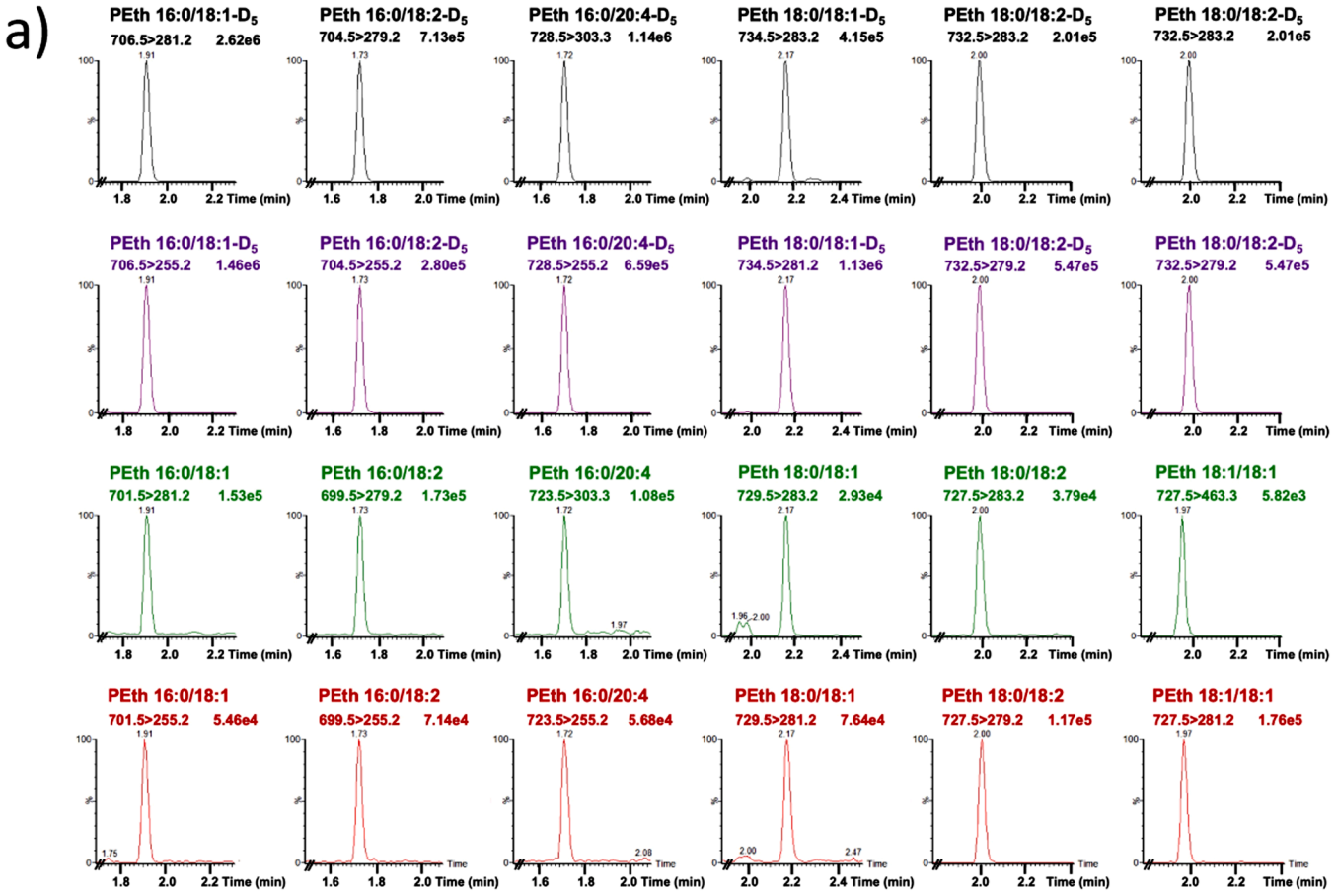

Fig. 5. MRM chromatograms of PEth homologues obtained by LC-MS/MS analysis of an extracted QC sample with PEth homologue concentrations of 30 nM (a), and of an extracted blank blood sample (b).

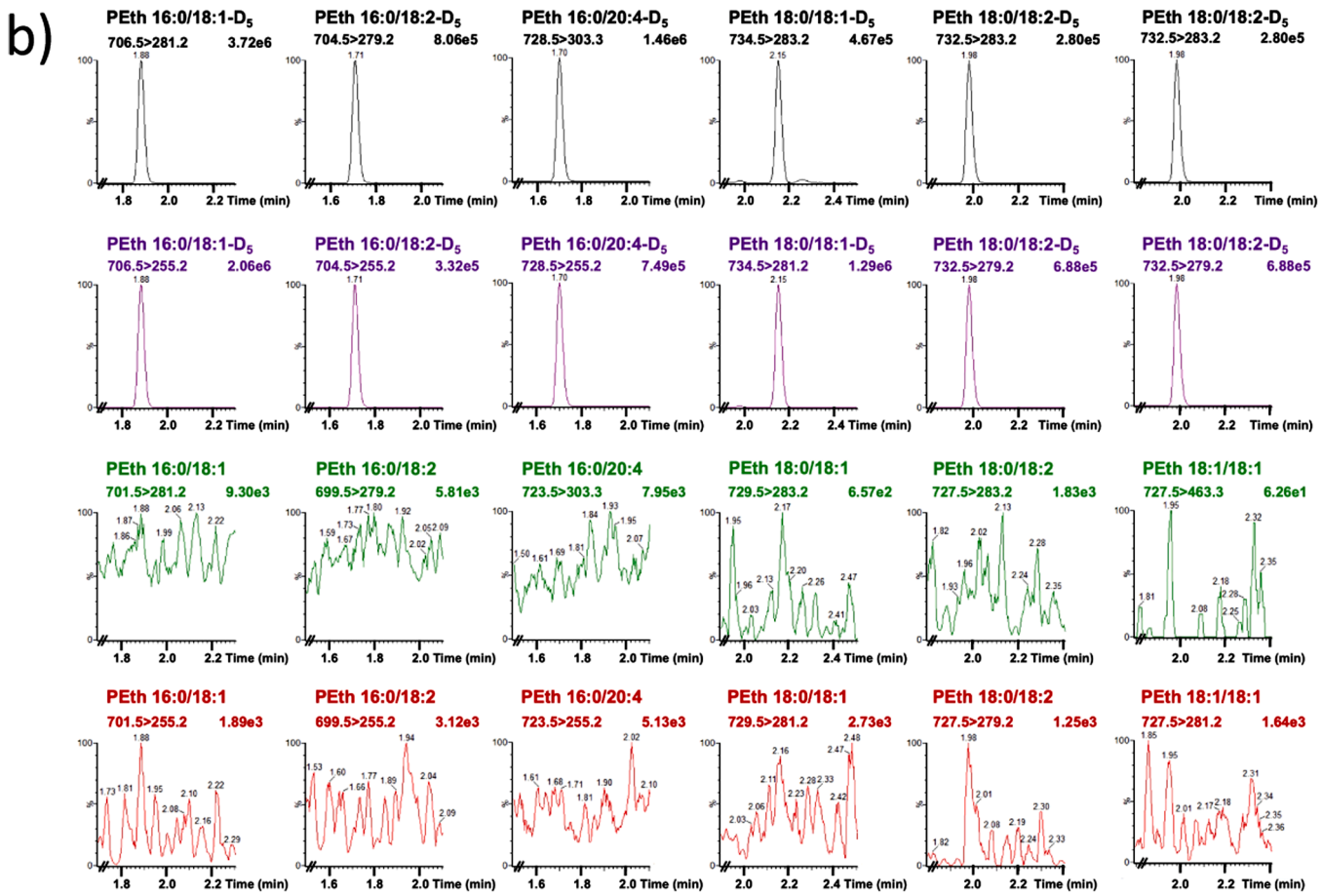

Fig. 5. (*continued*).

Table 3
Recovery and matrix effect of six PEth homologues using liquid-liquid extraction[b].

			Recovery [a]				Matrix Effects [c]				
PEth homologue	Theor. Conc. (nM)	Theor. Conc. (ng/mL)	n	Recovery (%)	RSD (%)	Min. - Max.	n[c]	ME	RSD (%)	ME corrected	RSD (%)
PEth 16:0/18:1	30	21	8	42	11	36 - 48	6	102	6	101	5
	300	211	8	42	4	39 - 44	6	102	4	99	6
PEth 16:0/18:2	30	21	8	37	5	34 - 40	6	99	5	99	4
	300	210	8	38	4	36 - 40	6	101	4	98	5
PEth 16:0/20:4	30	22	8	38	21	26 - 54	6	100	5	101	5
	300	217	8	38	4	36 - 40	6	100	5	99	5
PEth 18:0/18:1	30	22	8	51	6	49 - 58	6	92	6	101	4
	300	219	8	49	3	47 - 51	6	89	8	100	6
PEth 18:0/18:2	30	22	8	45	10	40 - 52	6	97	5	100	6
	300	219	8	47	3	45 - 49	6	98	5	99	4
PEth 18:1/18:1	30	22	8	43	9	38 –49	6	97	5	100	6
	300	219	8	45	3	43 - 46	6	99	4	99	5

[a] PEth-free whole blood from four different persons were tested to determine %-recovery.
[b] During LLE 1.6 mL organic solvent was added to each sample, whereas 0.9 mL of the organic solvent was transfered to new vials. Calculated values are not corrected for this loss.
[c] PEth-free whole blood from six different persons, three samples from each, were used.
Abbreviations: Theor: theoretical; Conc: Concentration; ME: Matrix Effect; Min: Minimum; Max: Maximum.

Table 4
PEth homologue concentrations of 22 authentic blood samples analyzed by the developed LC-MS/MS method.

Sample nr.	PEth 16:0/18:1				PEth 16:0/18:2			PEth 16:0/20:4			PEth 18:0/18:1			PEth 18:0/18:2			PEth 18:1/18:1		
	Conc. day 0 (nM)	Conc. day 0 (ng/mL)	%-dev. day 7 [a]	Relative conc. [b] (%)	Conc. day 0 (nM)	%-dev. day 7 [a]	Relative conc. [b] (%)	Conc. day 0 (nM)	%-dev. day 7 [a]	Relative conc. [b] (%)	Conc. day 0 (nM)	%-dev. day 7 [a]	Relative conc. [b] (%)	Conc. day 0 (nM)	%-dev. day 7 [a]	Relative conc. [b] (%)	Conc. day 0 (nM)	%-dev. day 7 [a]	Relative conc. [b] (%)
1																			
2	8	6	12	100							7	6	85	5	37	69			
3																			
4	842	592	4	100	512	2	61	203	0	24	502	6	60	501	−9	60	122	−8	15
5	22	16	−6	100							26	3	116	9	17	40			
6	1552	1091	17	100	647	12	42	257	10	17	1373	6	88	967	0	62	375	−3	24
7	175	123	−4	100	85	12	49	41	4	23	109	16	62	99	2	57	26	0	15
8	218	153	−5	100	106	2	49	48	5	22	123	12	56	107	−7	49	37	−8	17
9																			
10	362	254	5	100	117	−5	32	41	−1	11	260	12	72	178	2	49	47	−12	13
11	120	84	−4	100	90	−2	75	27	21	23	63	6	53	60	−5	50	23	−6	19
12	1842	1295	11	100	1041	14	56	734	−12	40	1536	6	83	951	5	52	413	−7	22
13																			
14	902	634	9	100	462	−1	51	188	1	21	494	3	55	329	−5	36	238	−7	26
15	77	54	6	100	43	−5	56	15	29	19	58	0	75	39	−1	51	12	2	16
16	159	112	−6	100	9	2	6				128	10	80	78	5	49	10	4	6
17	476	335	6	100	153	0	32	61	14	13	314	6	66	222	0	47	66	−7	14
18	37	26	−8	100	11	12	31				40	1	108	25	4	69	5	−21	13
19	41	29	4	100	6	−1	13				49	0	119	33	−7	80	6	−21	13
20	1436	1009	15	100	588	11	41	344	−6	24	863	1	60	581	−9	40	285	−11	20
21	1848	1299	11	100	559	1	30	310	−8	17	1001	8	54	457	2	25	343	−13	19
22																			
Average[c]				**100**			**42**			**21**			**76**			**51**			**17**

[a] Samples were stored 7 days in fridge at 4 °C, then added 100 μL reconstituted solvent (due to evaporation) and reanalyzed by LC-MS/MS.
[b] Relative concentration compared to PEth 16:0/18:1 concentration for each sample. Concentrations ratios were based on LC-MS/MS analysis at day 0.
[c] Average values based on samples with PEth 16:0/18:1 concentrations ≥ 10 nM (7 ng/mL) meanwhile as concentration of PEth homologue was ≥ LOD.
Abbreviations: Conc.: concentration; Dev.: deviation.

18:0:18:2 and PEth 16:0/18:2 were in average 24 %, 49 % and 58 % lower than PEth 16:0/18:1 concentrations.. Measuring concentrations of the PEth homologues in unknown samples without comparison towards "gold standards," can rise uncertainty to which are the correct concentrations. However, regarding PEth 16:0/18:1 and PEth 16:0/18:2, calculated concentrations of calibrators had a deviation of $< \pm 15$ % and $< \pm 10$ %, respectively, compared to values obtained from calibrators used at another department at Oslo University Hospital. This indicates that calibrators and quantifications for these two compounds are reliable using the developed method. As observed in other studies, the PEth homologues proportions in the blood also vary between individuals in our study [15,19,20,23,63–65].

4. Conclusions

A sensitive, precise and accurate LC-MS/MS method for the determination of six PEth homologues in whole blood was developed and fully validated. No matrix effects were observed, possibly due to the successful strategies incorporated to avoid the influence of unwanted phospholipids. Lyso-phospholipids and other early eluting phospholipids were removed by LLE using an organic solvent mixture of heptane/2-propanol (80:20, *v:v*). Analysis of the phospholipid background and the PEth homologues clearly showed the importance of choosing the right organic solvent for LLE. Then, the main part of the phospholipids were separated chromatographically from the six PEth homologues by LC-MS/MS analysis on an Acquity BEH C_{18} column using a buffer-free high pH mobile phase. LLOQ was 10 nM for all six PEth homologues. Analysis and quantification of the six PEth homologues in 22 authentic blood samples showed that the method is well suited for routine analysis and epidemiological studies.

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CRediT authorship contribution statement

Marisa Maria: Data curation, Writing – original draft, Writing – review & editing. **Nuno R. Neng:** Writing – review & editing. **Thomas Berg:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Supplementary materials

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