Quantification of phosphatidylethanol 16:0/18:1, 18:1/18:1, and 16:0/16:0 in venous blood and venous and capillary dried blood spots from patients in alcohol withdrawal and control volunteers

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Abstract Phosphatidylethanol species (PEths) are promising biomarkers of alcohol consumption. Here we report on the set-up, validation and application of a novel UHPLC-ESI-MS/MS method for the quantification of PEth 16:0/18:1, PEth 18:1/18:1, and PEth 16:0/16:0 in whole blood (30 µL) and in venous (V, 30 µL) or capillary (C, 3 punches (3 mm)) dried blood spots (DBS). The methods were linear from 10 (LLOQ) to 2000 ng/mL for PEth 16:0/18:1, from 10 (LLOQ) to 1940 ng/mL for PEth 18:1/18:1, and from 19 (LLOQ) to 3872 ng/mL for PEth 16:0/16:0. Extraction efficiencies were higher than 55% (RSD<18%) and matrix effects compensated by IS were between 77 and 125% (RSD<10%). Accuracy, repeatability and intermediate precision fulfilled acceptance criteria (bias and RSD below 13%). Validity of the procedure for determination of PEth 16:0/18:1 in blood was demonstrated by the successful participation to a proficiency test. The quantification of PEths in C-DBS was not significantly influenced by the hematocrit, punch localization or spot volume. The stability of PEths in V-DBS stored at room temperature was demonstrated up to 6 months. The method was applied to authentic samples (whole blood, V-DBS and C-DBS) from 50 inpatients in alcohol withdrawal and 50 control volunteers. Applying a cut-off value to detect inpatients at 221 ng/mL for PEth 16:0/18:1 provided no false positive results and a good sensitivity (86%). Comparison of quantitative results (Bland-Altman plot, Passing-Bablok regression and Wilcoxon signed rank test) revealed that V-DBS and C-DBS are valid alternatives to venous blood for the detection of alcohol consumption.

Keywords Biological samples; Drug monitoring/drug screening; Forensic toxicology; Sampling

Contents

1 Introduction

Phosphatidylethanols (PEths) are a group of abnormal phospholipids formed by the presence of ethanol in cell membranes [1]. They are biomarkers of alcohol consumption [2] present in blood, mainly located in erythrocytes [3], and in different organs [4]. Up to forty-eight different PEths have been detected in blood collected in autopsy cases of heavy drinkers [5]. All PEths have a common phosphoethanol head on which two fatty acid chains of variable length and degree of saturation are attached. Although blood analysis from heavy drinkers shows inter-individual variations of the distribution of the different PEths [6], the predominant species in blood after alcohol consumption are PEth 16:0/18:1 (30-46%) and PEth 16:0/18:2 (16-28%) [5-9]. Other PEths detected are PEth 18:1/18:1 and PEth 18:0/18:2 (identical molecular masses), together accounting for about 11-12% of total PEths [6, 7] while PEth 16:0/16:0 accounts for about 5% [6]. The half-life of PEths in whole blood was calculated to be 4.0 ± 0.7 days [3]. In case of chronic/excessive alcohol consumption, PEths are detectable in blood up to 28 days after sobriety [10]. Moreover, quantification of PEths can be used to detect the degree of alcohol consumption as a significant correlation between the PEths concentrations in blood and the amount of consumed ethanol has been demonstrated [11].

Numerous studies have been published on the quantification of PEths in blood and these have been reviewed in 2012 [10]. The most used extraction technique is a liquid-liquid extraction (LLE) with hexane [5-7, 11-17] (or heptane [8]) after stepwise addition of blood to isopropanol and the internal standard (IS) solution. Some methods added water [6], borate buffer pH 9 [5] or sodium acetate buffer pH 5 [15] to dilute the blood. Some publications reported other types of sample preparation, such as protein precipitation with methanol [18] or protein precipitation followed by an online-solid phase extraction [19]. A number of detection methods is based on HPLC with normal phase columns coupled to light-scattering detection (ELSD); chromatography has been carried out with hexane and propanol-based gradients containing acetic acid and triethylamine [3, 4, 11, 12]. Quantification limits (LLOQ) obtained with these methods ranged between 100–500 ng/mL [4, 11], analysing 250 to 300 µL of whole blood. PEths have also been analysed with non-aqueous capillary electrophoresis (CE) coupled to UV [13] detection. Both HPLC-ELSD and CE-UV [13] methods measure the total amount of PEths. However, LC methods coupled to MS/MS detection allow to obtain much lower LLOQs (between 0.7 and 83 ng/mL, based on the analysis of between 100 and 300 µL of whole blood) and are able to identify and quantify individual molecular species [6-8, 15, 18-20].

To improve the stability of compounds in whole blood and to facilitate the storage and transportation of samples [21], DBS methods have been developed. Numerous DBS-based methods have been published for a wide variety of applications, including therapeutic drug monitoring and toxicology [22]. Also alcohol markers such as ethyl glucuronide, ethyl sulphate and PEths have been determined, starting from DBS ([15, 18, 21, 23-25]; reviewed by Sadones et al, 2014 [26]). Since 2011, two publications have reported on the quantification of PEth 16:0/18:1 [15, 18] and PEth 18:1/18:1 [15] in V-DBS samples, while only one [21] reported on the analysis of C-DBS samples (detection of PEth 16:0/18:1 in newborns to detect prenatal alcohol exposure). V-DBS are prepared by spotting a fixed volume of venous blood onto a filter paper, whereas C-DBS are generated by direct collection of blood drops appearing after a finger or heel prick onto a filter paper. C-DBS offer the advantage compared to venipuncture of being less invasive and not requiring the service of nurses or physicians. Since these are typically collected in a non-volumetric way, these samples are mostly processed by excising punches with a fixed diameter from the global spot. This partial-spot approach requires the assessment of the impact of variables such as hematocrit, punch localization and spot volume on the quantitative result [27, 28].

In this paper, we present the validation of UHPLC-ESI-MS/MS methods for the quantification of the 3 PEths (PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0) in whole blood, V-DBS and C-DBS according to international guidelines [29] and published recommendations [27]. To our knowledge, this is the first report on the rigorous validation of the differences between capillary and venous DBS including the impact of specific parameters such as the influence of hematocrit, punch localization and spot volume on PEths. In addition, a sensitive method for PEth 16:0/16:0 in DBSs was developed and stability of the three species in V-DBS was evaluated over a period of 6 months. Moreover, successful participation to a proficiency test demonstrated the validity of the method for blood (no proficiency tests for DBS are available). Finally, the developed methods were applied to evaluate the agreement between the quantitative results from the analysis of whole blood, V-DBS and C-DBS obtained from 100 volunteers (inpatients in alcohol withdrawal and control volunteers). Receiver operating characteristic (ROC) curves performed on these results allow us to propose a possible cut-off value to detect chronic and excessive alcohol consumption. It was our main objective to investigate whether C-DBS could be a reliable alternative for the detection of PEths in whole blood, as this could lead to a more user friendly and practical approach to detect excessive and chronic alcohol consumption.

2 Materials and Methods

2.1 Chemicals

1,2-dioleoyl-sn-glycero-3-phosphoethanol (sodium salt; PEth 18:1/18:1), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanol (sodium salt; PEth 16:0/16:0) were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). Palmitoyl-2-oleoyl-sn-glycero-3 phosphoethanol (PEth 16:0/18:1) was purchased from Enzo Life Sciences (Antwerp, Belgium). As deuterated analogues have not been commercialised yet, four different internal standards from Avanti Polar Lipids were evaluated during validation: 1,2-dipalmitoyl-sn-glycero-3-phosphomethanol (sodium salt; PMeth 16:0/16:0; IS), 1,2-dioleoyl-sn-glycero-3-phosphomethanol (sodium salt; PMeth 18:1/18:1; IS), 1,2-dipalmitoyl-sn-glycero-3-phosphopropanol (sodium salt; PProp 16:0/16:0; IS) and 1,2-dioleoyl-sn-glycero-3-phosphopropanol (sodium salt; PProp 18:1/18:1; IS).

Isopropanol (ULC/MS), tetrahydrofuran (ULC/MS), ammonium acetate (ULC/MS), water (HPLC) and methanol (ULC/MS) were purchased from Biosolve (Valkenswaard, The Netherlands). Isopropanol and n-hexane, gradient grade for liquid chromatography, were purchased from Merck KGaA (Darmstadt, Germany). Formic acid for mass spectrometry (~98%) was purchased from Sigma-Aldrich (Steinheim, Germany).

2.2 Standard solutions, calibrators and quality control (QC) samples

Stock solutions of PEths (PEth 16:0/18:1 (1.000 mg/mL), PEth 18:1/18:1 (0.970 mg/mL) and PEth 16:0/16:0 (0.968 mg/mL)) and stock solutions of the 4 evaluated ISs (PMeth 16:0/16:0 (0.968 mg/mL), PMeth 18:1/18:1 (0.971 mg/mL), PProp 16:0/16:0 (0.969 mg/mL) and PProp 18:1/18:1 (0.971 mg/mL)) were prepared in methanol. A calibrator working solution (100 µg/mL), a QC working solution (50 µg/mL) and an IS working solution (5 µg/mL) for the blood and V-DBS methods were prepared by diluting the stock solutions in methanol. For the C-DBS method, a calibrator working solution of 250 µg/mL, a QC working solution of 250 µg/mL and an IS working solution of 0.25 µg/mL were prepared in methanol. All working solutions were stored at -18°C.

Daily dilutions of IS working solutions were performed in solution A, consisting of isopropanol, 10 mM ammonium acetate buffer and formic acid (6:4:0.2, v/v), to reach a concentration of 100 ng/mL (used for the whole blood and V-DBS method) and 10 ng/mL (used for the C-DBS method).

Daily dilutions of calibrator working solutions and QC working solutions were performed in water to obtain 8 different concentrations for calibrators and 3 for QCs. A second dilution was performed in EDTA blank whole blood (Supplementary Tables 1 and 2). Final calibrator concentrations in blood were between 10 and 2000 ng/mL for PEth 16:0/18:1, 10 and 1940 ng/mL for PEth 18:1/18:1 and between 19 and 3872 ng/mL for PEth 16:0/16:0. For the two DBS methods, 30 µL of calibrators and QCs in blood were spotted onto Whatman 903 filter paper (GE Healthcare). Spots were dried for minimum 2 hours at room temperature. The complete DBS was used for the V-DBS method and 3 punches (3 mm) were used for the C-DBS method, unless indicated otherwise. Here, we typically used 3 punches from the same DBS, except in the application study, where not from all C-DBS three 3-mm punches could be obtained. The hematocrit of the blood used to prepare the DBS calibrators was 0.48 ± 0.02, as measured using a Sysmex XP-300™ automated hematology analyzer (Sysmex America, Inc.).

2.3 Sample preparation

PEths were extracted by LLE with n-hexane. For the whole blood method, 30 µL of the sample was added to a 5 mL disposable glass tube containing 250 µL of solution A (consisting of isopropanol, 10 mM ammonium acetate buffer and formic acid (6:4:0.2, v/v)) and 50 µL of the IS solution (100 ng/mL). After a quick mixing (vortex), 1 mL n-hexane was added and the sample was gently mixed for 10 minutes. The tubes were centrifuged (10 min, 14'000 rpm (20'800 x g), 4°C) and the clear supernatant was transferred to a total recovery glass vial (Waters, Zellik, Belgium) and evaporated to dryness during 30 minutes in a rotational vacuum concentrator (RVC 2-33 IR, Martin Christ, Osterode am Harz, Germany). The final dried extract was dissolved in 250 µL of a solution B (50% of mobile phase A and 50% of mobile phase B, see below).

For the V-DBS method, the complete DBS (30 µL) was excised and placed in a 5 mL disposable glass tube containing 250 µL of solution A and 50 µL of the IS solution (100 ng/mL). For the C-DBS method, three (or one, where indicated) punches (3 mm) were excised from the DBS and placed in a 5 mL disposable glass tube containing 250 µL of solution A and 50 µL of the IS solution (10 ng/mL). For both DBS methods, the tubes were gently mixed for 1 hour. After adding 1 mL of n-hexane, the samples were mixed for another 10 minutes. After centrifugation, the clear supernatant was transferred in total recovery glass vials and evaporated to dryness. The final dried extract was dissolved in 250 µL of solution B for the V-DBS and in 100 µL of solution B for the C-DBS.

For the whole blood method and the V-DBS method, 5 µL was injected in partial loop with needle overfill mode. For the C-DBS method, 10 µL was injected in full loop mode.

2.4 Liquid chromatographic and mass spectrometric conditions

Analyses were performed on an Aquity UPLC® system coupled to a Xevo TQ S tandem mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization source operated in negative mode. The compounds were separated on an Acquity UPLC® BEH C8 (2.1 x 50 mm, 1.7 µm) column (Waters) using as mobile phase A 10 mM ammonium acetate buffer with 0.05% formic acid (pH 2) and as mobile phase B isopropanol with 10% of tetrahydrofuran at a flow rate of 400 µL/min. The gradient elution started with 40% of mobile phase A and decreased to 0% of mobile phase A at 1.5 minutes. The washing step, containing 100% of solution B, was held for 1 minute and was followed by 1 minute re-equilibration with the starting condition, resulting in a total run time of 3.5 minutes. The column temperature was set at 60°C.

For the MS/MS detection, the following parameters were used: temperature of source gas (nitrogen) was 150°C, desolvatation gas (nitrogen) flow was 1000 L/h at 650°C, capillary voltage was 3 KV, cone voltage was 10 V with a cone gas flow at 150 L/h and collision gas (argon) flow was 0.15 mL/min. Detection was performed in the multiple reaction monitoring mode (MRM). Two transitions were measured for PEths, one for the quantification (underlined in the text) and one for the qualification. For ISs only one MRM transition was used. The dwell time was fixed at 17 msec and the following precursor/product ion transitions (cone voltage, collision energy) were selected: <u>701.53/255.33</u> (10 V, 35 V), 701.53/124.98 (10 V, 40 V) for PEth 16:0/18:1, <u>727.62/281.22</u> (10 V, 35 V), 727.62/463.22 (10 V, 25 V) for PEth 18:1/18:1, <u>675.56/255.19</u> (10 V, 30 V), 675.56/124.97 (10 V, 35 V) for PEth 16:0/16:0, 741.63/281.27 (20 V, 35 V) for PProp 18:1/18:1, 689.55/255.15 (20 V, 30 V) for PProp 16:0/16:0, 713.57/281.27 (20 V, 30 V) for PMeth 18:1/18:1 and 661.50/255.27 (10 V, 30 V) for PMeth 16:0/16:0.

2.5 Method validation

Selectivity, sensitivity, matrix effect, extraction efficiency, limit of quantification, linearity, accuracy and stability were evaluated based upon international guidelines [29]. The influence of hematocrit, punch localization and spot volume were evaluated for the C-DBS method [27, 28].

To study endogenous interferences, six blank whole blood samples from different teetotallers were analysed. To verify that IS compounds do not interact with PEths, two zero samples (blank samples spiked with IS solution) were analysed. According to the EMA guideline, in our method interferences are acceptable as long as the signal was lower than 20% of the response at the LLOQ [30].

Matrix effect was quantified and evaluated by the post-extraction addition technique using six different blank bloods from teetotallers [31]. Whole blood (30 µL), V-DBS (complete 30 µL DBS) and C-DBS (3 filter paper punches spiked each with 3.5 µL of whole blood) were extracted. The reference standards and IS (diluted in the mobile phase) were added in the total recovery vial before the injection. These samples were compared with control samples spiked at the same theoretical concentration in the mobile phase. Extraction efficiency was evaluated by comparing responses of six blank samples spiked before sample preparation with responses of six blank samples, where the reference standards were spiked after the sample preparation in the mobile phase. Matrix effect and extraction efficiency were evaluated at low, medium and high concentrations. For the C-DBS method, blood samples with varying hematocrit levels (measured from 0.31 to 0.58) were used, to study the influence of the hematocrit variation on the extraction efficiency and on the matrix effect.

The lower limit of quantification (LLOQ) is the lowest concentration of analyte with a signal-to-noise ratio greater than 10/1 for both transitions and for which the bias and precision deviation is less than 20%.

Calibration model and weighting factor were evaluated for each compound and each method. The linearity was tested by performing F-Tests (α=0.05). Homoscedasticity was tested visually by plotting residuals vs. fitted value. In case of heteroscedasticity, a weighted regression ($1/x$ and $1/x^2$) was applied (slope and intercept). The sum of relative errors (difference between the calculated concentration and its nominal concentration) for each model was calculated and plotted against the nominal concentrations. The model with a $R^2 \geq 0.99$ with the lowest sum of relative errors was selected. The goodness of fit of the selected model was tested, calculating the relative errors for calibrators and QCs. The relative errors should be lower than 15% except for the LLOQ (< 20%) [30].

Three internal QCs spiked at low, medium and high concentration, were analysed in duplicate on 8 different days to assess accuracy (bias) and precision (repeatability and intermediate precision). A single factor ANOVA test with significance level (α) of 0.05 allows calculating bias, repeatability and intermediate

precision with these data, with acceptance criteria of 15% (20% for the LLOQ). The measurement uncertainty was also calculated ($2.12*RSD_t$) and used to interpret quantitative results close to the LLOQ or close to the cut-off value.

The validity of the PEth 16:0/18:1 quantification in blood was tested by participation to a proficiency test organized by Equalis (Uppsala, Sweden).

Processed sample stability and long term storage stability were evaluated at low and high concentrations for the whole blood method and for the V-DBS method. The mean response of the stability samples should be within 90 – 110% of the mean response of the control samples and the 90% confidence interval of the stability sample responses should be within ± 20% of the control sample responses.

The influence of the hematocrit on the response was evaluated for five hematocrit values at low and high concentrations. Blank blood samples with variable hematocrit level were prepared by adding or removing plasma to EDTA blank blood samples. The measured hematocrit values were 0.39, 0.42, 0.48, 0.50 and 0.57. Six spots per concentration and per hematocrit level were prepared and single centrally located punches were analysed. Measured responses were compared with a One-way ANOVA test (α=0.05). To evaluate whether no artefactual results were obtained because spiked samples might behave differently from real samples (where PEth species are presumably located in erythrocytes), we set up an experiment in which blood with different hematocrit was prepared from blood of two inpatients. More specifically, 200 µL of blood of an inpatient was diluted with plasma (between 25 and 200 µL) and erythrocytes (between 0 and 175 µL) of an alcohol abstainer to generate 6 blood samples of 400 µL with a different hematocrit (with measured hematocrits between 0.20 and 0.60) but with the same PEths concentrations (PEths virtually exclusively being derived from the 200 µL of inpatient blood). This blood was used to generate DBS, which were processed as real samples (see sample preparation section). The DBS analysis was performed in quadruplicate at each hematocrit level. Also the blood PEths concentrations were determined and served as a reference.

The influence of the punch localization (peripherally or centrally) was evaluated at low and high concentrations and at low (0.39), intermediate (0.48) and high (0.57) hematocrit levels. Six spots per concentration and per hematocrit level were prepared and the responses measured in peripherally and centrally located punches (one central and one peripheral punch were analyzed per DBS) were compared using a One-way ANOVA test (α=0.05).

Three blood spot volumes (20, 35, 50 µL) were tested at low (0.32), intermediate (0.48) and high (0.67) hematocrit levels and at 2 concentrations; low and high. Six spots per concentration and per hematocrit level were prepared and centrally located 3 mm punches (1/DBS) were analysed. Responses were compared using a One-way ANOVA test (α=0.05) to detect significant differences.

The normality of the distributions and the homogeneity of variances were tested using the Shapiro-Wilk test and the Levene's test prior to One-way ANOVA tests [32].

2.6 Application to a comparative study

2.6.1 Sample collection

Whole blood and C-DBS from inpatients in alcohol withdrawal were collected at the Brugmann Hospital (Brussels, Belgium) one business day after their admission. Whole blood and C-DBS from control volunteers were collected by the medical staff of the Military Hospital in Brussels (Belgium). The inpatients group was composed of 37 males and 13 females, between 27 and 71 years (mean = 47, median = 47) and with a self-reported number of abstinence days before the sampling between 1 and 21 (mean = 4, median = 2). The control group was composed of 23 males and 27 females, between 22 and 64 years (mean = 40, median = 37) and with a self-reported mean alcohol consumption per week between 0 and 16 units (mean = 5, median = 6). Seven out of the 50 control volunteers were teetotallers.

Venous whole blood samples were collected in a 4 mL EDTA tube and were stored at -80°C until analysis. Five C-DBS were collected onto a Whatman 903 filter paper card after a fingertip prick with a contact-activated lancet (BD Microtainer®, Becton Dickinson). Five V-DBS were prepared from the EDTA tubes by pipetting 30 µL of venous blood onto a filter paper. C-DBS and V-DBS were left to dry for minimum 2 hours at room temperature and were then stored in zip-closure plastic bags containing a desiccant packet (Sigma-Aldrich) at room temperature until the analysis.

The study protocol was approved by the ethics committee of the Brugmann Hospital (Brussels, Belgium) and informed consent was obtained from each subject before enrolment in the study (B077201420445).

2.6.2 Statistical analysis

Bland-Altman plot, Passing-Bablok regression analysis and Wilcoxon signed rank test were used to study the agreement between quantitative results obtained from whole blood, V-DBS and C-DBS samples [33]. A Bland-Altman plot is used to assess the absence of systematic differences between two measurements. The mean of the two measurements is plotted against the difference between these, 95% of the differences are expected to lie within the limits of agreement (mean ± 1.96 SD). The Passing-Bablok regression analysis is a scatter diagram of the concentrations obtained with two different methods. The regression line and equation are used to detect measurement errors. No proportional differences are observed as long as the 95% confidence interval of the slope includes 1 and no systematic differences are observed as long as the 95% confidence interval of the intercept includes the zero value. Wilcoxon signed rank test was performed to detect significant differences (p-value<0.05) between the concentrations obtained from two methods.

ROC curve analyses were performed to determine optimal cut-off values (higher sensitivity with 0 false positive results) to distinguish between inpatients in alcohol withdrawal and control volunteers. The area under the curve (AUROC) was used to quantify the overall ability of the method to discriminate between the two populations. A perfect diagnostic method (0 false positives and 0 false negatives) will have an area of 1, where a method with no diagnostic ability will have an area of 0.5.

3 Results

3.1 Method Validation

Linearity, LLOQ, matrix effects, extraction efficiency, selectivity, sensitivity and accuracy were assessed for the three methods. Stability was tested for the whole blood and V-DBS methods. For the C-DBS method, the impact of hematocrit, punch localization and blood spot volume were evaluated.

The linear (1/x) calibration curves ranged from 10 (LLOQ) to 2000 ng/mL for PEth 16:0/18:1, from 10 (LLOQ) to 1940 ng/mL for PEth 18:1/18:1 and from 19 (LLOQ) to 3872 ng/mL for PEth 16:0/16:0.

The non-IS-compensated matrix effect was between 68 and 137% (RSD<20%) for PEth 16:0/18:1, between 73 and 121% (RSD<12%) for PEth 18:1/18:1 and between 59 and 110% (RSD<20%) for PEth 16:0/16:0. For the three PEths, PMeth 18:1/18:1 was selected as IS, because it better compensated for matrix effect than PMeth

16:0/16:0 and PProp 18:1/18:1 (Table 1) and had a better peak shape compared to PProp 16:0/16:0. The matrix effect compensated by this IS was between 77 and 125% (RSD<10%) for all PEths. Visual inspection of the results indicated no influence of the hematocrit level on matrix effect for the C-DBS method. Results for the extraction efficiency, repeatability, intermediate precision and bias are reported in Table 2.

Table 1: Matrix effect and matrix effect compensated with PMeth 18:1/18:1, PMeth 16:0/16:0, PProp 18:1/18:1 and PProp 16:0/16:0 in whole blood. Matrix effect and matrix effect compensated with PMeth 18:1/18:1 in V-DBS and C-DBS.

PEth 16:0/18:1			**PEth 18:1/18:1**			**PEth 16:0/16:0**		
L	**M**	**H**	**L**	**M**	**H**	**L**	**M**	**H**
Blood			**Blood**			**Blood**		
ME (%) (RSD%)			**ME (%) (RSD%)**			**ME (%) (RSD%)**		
116 (16)	89 (4)	72 (12)	115 (10)	101 (3)	97 (8)	103 (8)	88 (3)	83 (6)
$ME_{PMeth\ 18:1/18:1}$ (RSD%)			**$ME_{PMeth\ 18:1/18:1}$ (RSD%)**			**$ME_{PMeth\ 18:1/18:1}$ (RSD%)**		
116 (10)	102 (5)	88 (3)	115 (6)	117 (6)	118 (5)	103 (4)	102 (7)	101 (5)
$ME_{PMeth\ 16:0/16:0}$ (RSD%)			**$ME_{PMeth\ 16:0/16:0}$ (RSD%)**			**$ME_{PMeth\ 16:0/16:0}$ (RSD%)**		
81 (11)	67 (8)	57 (10)	80 (6)	77 (9)	76 (7)	72 (5)	66 (7)	65 (6)
$ME_{PProp\ 18:1/18:1}$ (RSD%)			**$ME_{PProp\ 18:1/18:1}$ (RSD%)**			**$ME_{PProp\ 18:1/18:1}$ (RSD%)**		
43 (18)	36 (12)	30 (15)	43 (12)	41 (8)	41 (11)	38 (9)	36 (8)	35 (9)
$ME_{PProp\ 16:0/16:0}$ (RSD%)			**$ME_{PProp\ 16:0/16:0}$ (RSD%)**			**$ME_{PProp\ 16:0/16:0}$ (RSD%)**		
118 (10)	101 (5)	91 (3)	118 (6)	116 (6)	122 (4)	105 (4)	100 (7)	104 (3)
V-DBS			**V-DBS**			**V-DBS**		
ME % (RSD%)			**ME % (RSD%)**			**ME % (RSD%)**		
137 (9)	99 (9)	76 (8)	121 (6)	97 (5)	94 (6)	110 (9)	81 (11)	84 (6)
$ME_{PMeth\ 18:1/18:1}$ (RSD%)			**$ME_{PMeth\ 18:1/18:1}$ (RSD%)**			**$ME_{PMeth\ 18:1/18:1}$ (RSD%)**		
125 (6)	108 (4)	87 (3)	110 (5)	107 (4)	108 (2)	102 (8)	89 (9)	96 (5)
C-DBS			**C-DBS**			**C-DBS**		
ME % (RSD%)			**ME % (RSD%)**			**ME % (RSD%)**		
101 (15)	79 (17)	68 (20)	106 (11)	89 (12)	73 (12)	107 (9)	69 (16)	59 (20)
$ME_{PMeth\ 18:1/18:1}$ (RSD%)			**$ME_{PMeth\ 18:1/18:1}$ (RSD%)**			**$ME_{PMeth\ 18:1/18:1}$ (RSD%)**		
108 (6)	89 (4)	89 (3)	114 (4)	101 (3)	97 (8)	116 (7)	78 (3)	77 (5)

Table 2: Validation results for PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 in blood, V-DBS and C-DBS. L = low QC, M = medium QC, H = high QC.

	PEth 16:0/18:1		
	Blood	V-DBS	C-DBS
	Extraction efficiency % (RSD%)		
L	90 (10)	59 (14)	67 (4)
M	80 (9)	61 (5)	65 (8)
H	79 (17)	58 (11)	74 (13)
	Repeatability % (RSDr)		
LLOQ	9	11	11
L	9	7	7
M	6	3	4
H	4	4	3
	Intermediate precision % (RSDt)		
LLOQ	11	12	11
L	9	12	11
M	6	6	6
H	5	5	6
	Bias %		
LLOQ	3	5	0
L	5	3	3
M	2	6	6
H	3	4	1

	PEth 18:1/18:1		
	Blood	V-DBS	C-DBS
	Extraction efficiency % (RSD%)		
L	96 (7)	61 (7)	64 (9)
M	85 (9)	63 (6)	61 (13)
H	81 (18)	59 (11)	77 (12)
	Repeatability % (RSDr)		
LLOQ	7	6	7
L	4	5	5
M	4	3	3
H	4	4	3
	Intermediate precision % (RSDt)		
LLOQ	12	10	11
L	7	8	8
M	3	6	7
H	4	5	5
	Bias %		
LLOQ	-1	2	0
L	3	3	4
M	4	2	3
H	3	1	-1

	PEth 16:0/16:0		
	Blood	V-DBS	C-DBS
	Extraction efficiency % (RSD%)		
L	100 (13)	58 (14)	78 (13)
M	79 (11)	57 (4)	66 (7)
H	66 (15)	55 (9)	74 (15)
	Repeatability % (RSDr)		
LLOQ	11	7	7
L	6	6	4
M	3	4	3
H	4	3	2
	Intermediate precision % (RSDt)		
LLOQ	13	10	9
L	10	9	11
M	8	6	6
H	7	6	6
	Bias %		
LLOQ	-5	0	-2
L	-3	0	-2
M	1	4	4
H	-1	1	0

In summary, no interfering peaks were detected in blank samples and the addition of the IS did not interfere with PEths detection. The recovery was between 66 and 100% (RSD<18%) for the blood method, between 55 and 63% (RSD<14%) for the V-DBS method and between 61 and 78% (RSD<15%) for the C-DBS method. Visual inspection of the results indicated no influence of the hematocrit level on extraction efficiency (Figure 1).

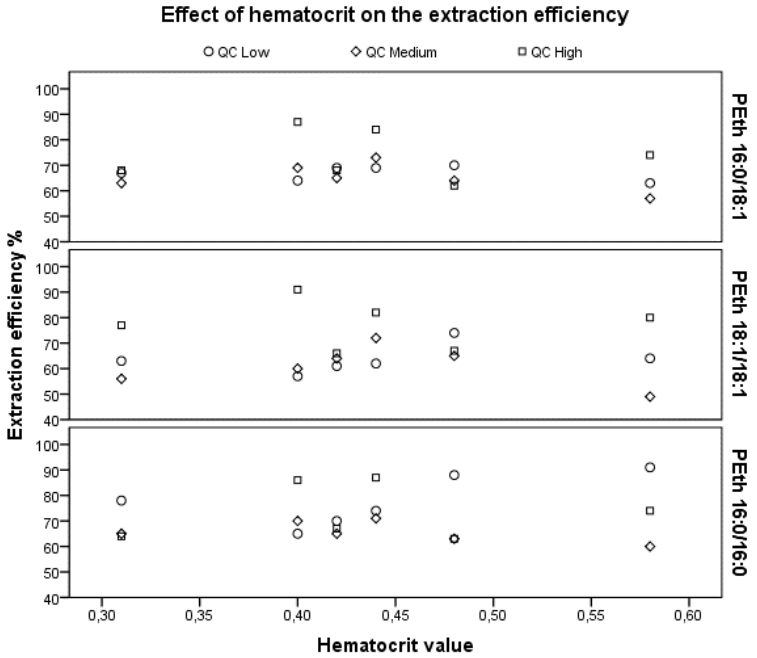

Figure 1: Extraction efficiency % for PEths from DBS at three concentrations (low, medium and high) and prepared from 6 whole blood samples with varying hematocrit levels.

The bias (%), repeatability (%RSD_r) and intermediate precision (%RSD_t) were less than 13% for whole blood, V-DBS and C-DBS methods. The maximal uncertainties of measurement (2.12*RSD_t) were 23% (blood), 25% (V-DBS) and 24% (C-DBS) for PEth 18:1/16:0; 25% (blood), 22% (V-DBS) and 23% (C-DBS) for PEth 18:1/18:1 and 22% (blood, C-DBS) and 21% (V-DBS) for PEth 16:0/16:0.

The validity of the quantification of PEth 16:0/18:1 in blood was demonstrated by the successful participation (z-score<2) to a proficiency test. The z-score obtained ((reported value – target value) / SD) was 0.11 for sample B (reported value = 2.52 µmol/L, target value = 2.50 µmol/L, SD=0.23, N=8) and 1.43 for sample C (reported value = 0.17 µmol/L, target value = 0.16 µmol/L, SD=0.01, N=8). The samples were used to create V-DBS and z-scores of 0.38 for sample B (measured value = 2.59 µmol/L) and 0.22 for sample C (measured value = 0.16 µmol/L) were calculated ((measured value – target value) / SD). PEths were not detected in sample A (reported value < LLOQ). Sample A was whole blood from a teetotaller.

All samples were stable in the autosampler for 72 h. PEths were stable up to 6 months when stored at -80° C in EDTA tubes. PEth 16:0/18:1 and PEth 18:1/18:1 were stable during 6 months in V-DBS samples stored at room temperature in zip-closure plastic bags containing a desiccant packet. The 90% confidence interval of the stability sample responses for PEth 16:0/16:0 in V-DBS were within ± 20% of the control sample responses, although the mean response of the high stability samples was 119% of the mean response of the control samples.

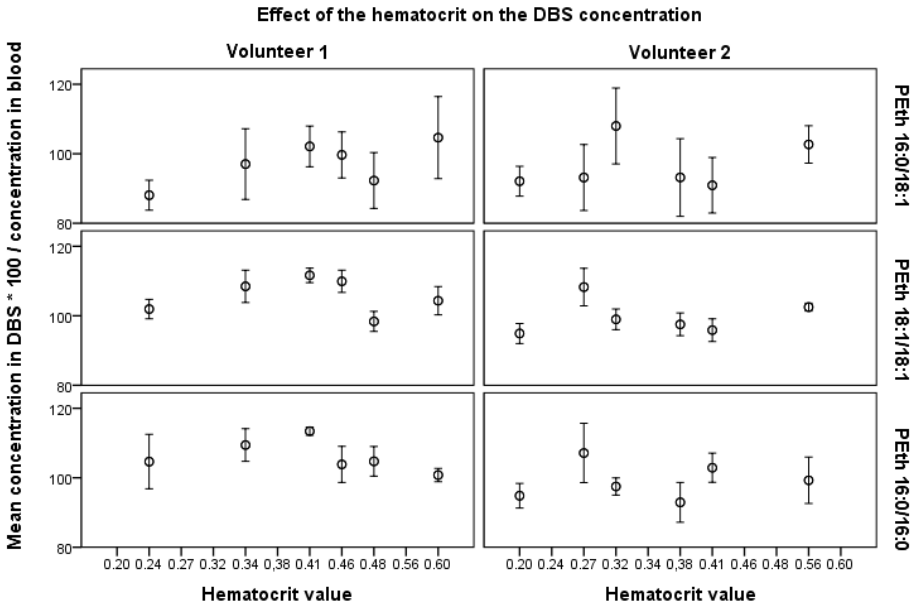

Figure 2: Influence of the hematocrit on PEths quantification in real positive samples (3 punches excised from DBS created from the blood of inpatients in alcohol withdrawal). Results, with standard deviation (RSD%), are presented for each haematocrit level as mean % bias (N=4) compared with the reference value measured in whole blood. The mean measured blood concentrations for PEth 16:0:18:1, PEth 18:1/18:1 and PEth 16:0/16:0 were 728, 52 and 89 ng/mL for inpatient 1 and 659, 46 and 100 ng/mL for inpatient 2, respectively.

No significant differences ($p>0.05$) were observed between the mean responses obtained for the analysis of V-DBS samples spiked with PEths reference standard, prepared from blood with hematocrit levels spanning a normal to high range (0.39, 0.42, 0.48, 0.50, 0.57). In addition, varying the hematocrit level (between 0.20 and 0.60) of real inpatients' blood samples (by adding blank plasma and red blood cells) did not adversely affect quantification, as demonstrated in Figure 2, which depicts the bias when comparing results obtained from DBS with those obtained from blood. Similar mean responses were obtained from 20, 35, 50 µL V-DBS samples. Finally, a one-way ANOVA test showed no significant differences between mean responses obtained with peripherally and centrally located punches. The influence of hematocrit and volume spotted on PEths responses are presented in Supplementary Figure 1.

3.2 Comparative study

Whole blood, V-DBS and C-DBS from inpatients in alcohol withdrawal (N=50) and control volunteers (N=50) were analysed to quantify PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0. For the C-DBS method, the 3 punches analysed

were excised either from the same spot (15% of the cases), from two different spots (30% of the cases) or from three different spots (55% of the cases).

Concentrations measured in whole blood, V-DBS and C-DBS from all study participants were compared using Bland-Altman plot, Passing-Bablok regression analysis and Wilcoxon signed rank test.

PEth 16:0/18:1 was quantified (>LLOQ) in 50/50 inpatients and in 18/50 control volunteers. Concentrations measured in blood ranged from 16 to more than 2000 ng/mL (mean = 1232, median = 1087) in alcoholics and were between 13 and 220 ng/mL (mean = 59, median = 49) in control volunteers with a quantifiable result. PEth 18:1/18:1, with blood concentrations ranging from 17 to 307 ng/mL (mean = 101, median = 78), was measured in 47/50 inpatients and in 1/50 control volunteers (17 ng/mL). PEth 16:0/16:0 was quantified only in some inpatient samples (34/50) with concentrations varying from 25 to 203 ng/mL (mean = 97, median = 89). An overview is given in Figs. 4 and 5.

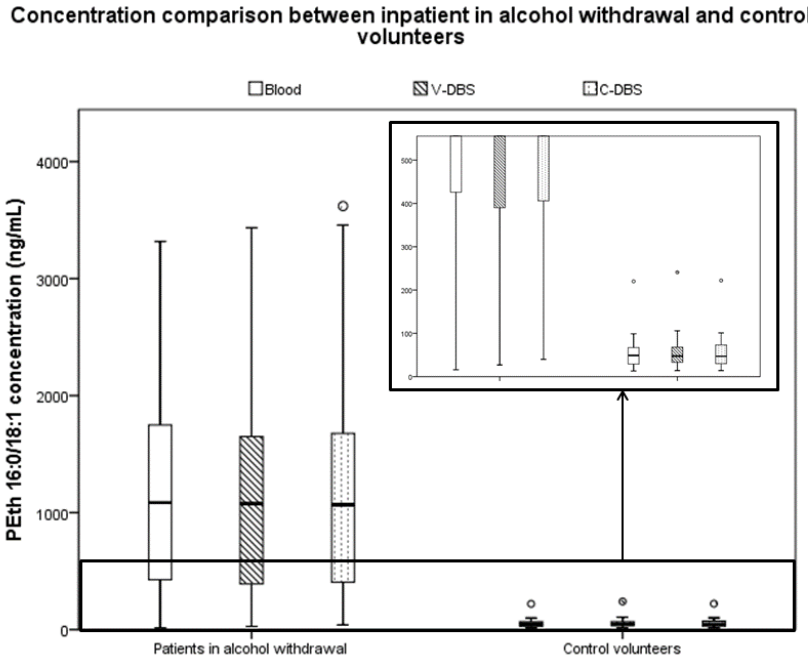

Figure 3: Distribution of the PEth 16:0/18:1 concentrations measured in blood, V-DBS and C-DBS of patients in alcohol withdrawal (n=50) and in control volunteers (n=18). The box represents the values between the lower and upper quartile and the middle line represents the median. The whiskers represent the extremes value, excluding outliers (represented by dots). The indicated area (below) is enlarged (right above).

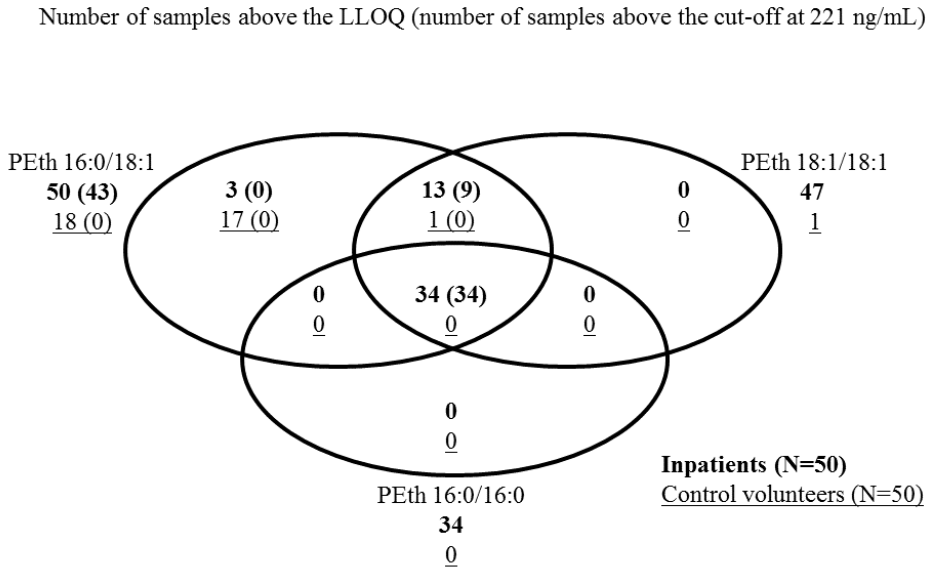

Figure 4: Number of blood samples with a measured concentration (above LLOQ + U%) of PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 and above the cut-off (+ U%) for PEth 16:0/18:1:

In the comparison of the results obtained from blood, C-DBS and V-DBS, correlation coefficients exceeded 0.995 for PEth 16:0/18:1 (N=68), 0.978 for PEth 18:1/18:1 (N=48) and 0.962 for PEth 16:0/16:0 (N=32). As shown in Figure 3 (Right) and reported in Table 3 (presenting the numerical results), the mean % differences in the concentration between venous blood and C-DBS included the 0 value for the three PEths. The 95% confidence intervals of the slope obtained from the Passing-Bablok regression analysis included or were very close to 1 and the 95% confidence intervals of the intercept included the 0 value (Figure 3, Left and Table 3). No significant differences ($p \geq 0.05$) in the mean measured concentrations were detected using Wilcoxon signed rank test. The same comparisons were performed between blood and V-DBS and between V-DBS and C-DBS for the three compounds (Table 3 and Supplementary Figs. 2 and 3), with essentially the same conclusions. Only in 3 cases with measurable (i.e. above LLOQ) PEth 16:0/16:0 in whole blood, V-DBS and C-DBS, a discrepancy was observed, when taking into account the measurement uncertainty at the LLOQ. The blood, V-DBS and C-DBS concentrations in these 3 cases were respectively 32, 23* and 25 ng/mL (case 1), 22*, 31 and 21* ng/mL (case 2) and 27, 29 and 22* ng/mL (case 3). For 4 quantitative results (indicated with an asterisk) in these 3 cases, the results should actually be considered negative when the measurement uncertainty is taken into account (exemplified in Supplementary Figure 4). These three cases were not taken into account for the statistical analysis.

Distributions of the concentrations of PEth 16:0/18:1 measured in whole blood, V-DBS and C-DBS from inpatients in alcohol withdrawal and control volunteers are presented in Figure 4.

ROC analysis was performed to determine a cut-off value to distinguish between control volunteers and inpatients in alcohol withdrawal using the concentration of PEth 16:0/18:1 in blood. A cut-off value at 221 ng/mL (AUROC=0.947) for PEth 16:0/18:1 provided no false positive results (1-specificity=0) and a sensitivity of 0.86 (7 out of 50 inpatients were classified as social drinkers). Application of this cut-off for C-DBS and V-DBS yielded exactly the same result, lending further support to the validity of the approach of using DBS.

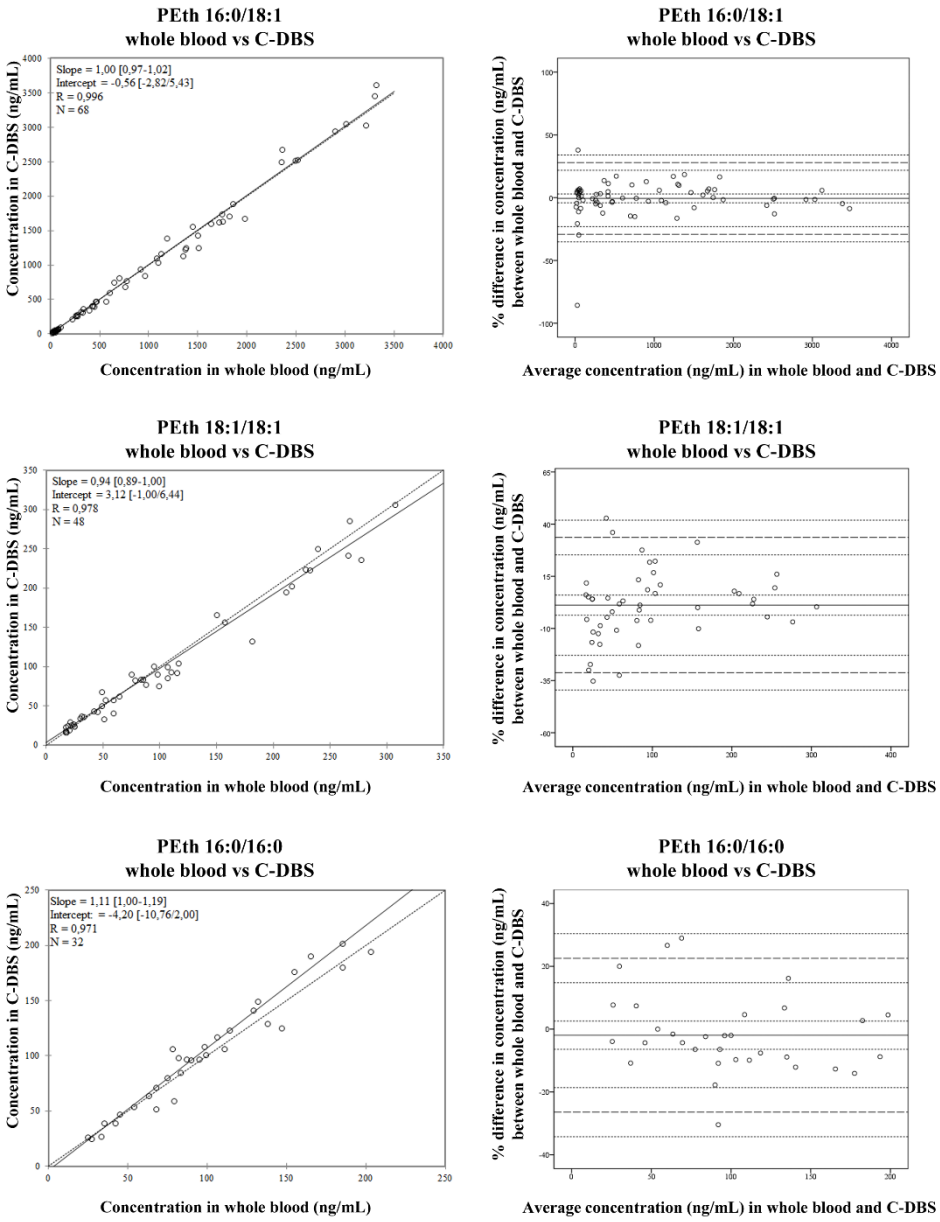

Figure 5: (Left) Passing-Bablok regression analyses of PEths concentrations measured in blood and in C-DBS. The identity line is indicated using a dotted line. **(**Right) Bland-Altman analyses of PEths plotting the % difference between blood and C-DBS concentrations. The average difference is represented by a solid line, the limits of agreement (1.96 SD) by dashed lines. The 95% confidence intervals for the mean and the limits of agreement are shown with dotted lines.

Table 3: Results obtained for the Passing-Bablok analysis, Bland-Altman analysis and Wilcoxon signed rank test performed on PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 to compare Blood vs. C-DBS, Blood vs. V-DBS and V-DBS vs. C-DBS. N = number of positive results (above LLOQ + U%), CI = confidence interval. * 3 samples were in disagreement concerning the detection or no detection of PEth 16:0/16:0 when analysed in blood, V-DBS and C-DBS. These samples were not used in the statistical analysis.

Compounds	Methods	Correlation coefficient	Passing-Bablok regression		Bland-Altman analysis		Wilcoxon signed rank
		R (N)	Slope [95% CI]	Intercept [95% CI]	% Mean diff. [95% CI]	Limits of agreement	p
PEth 16:0/18:1	Blood C-DBS	0.996 (68)	1.00 [0.97/1.02]	-0.56 [-2.82/5.43]	-0.61 [-4.13/2.91]	-29.11/27.89	0.94
	Blood V-DBS	0.995 (68)	0.98 [0.96/0.99]	1.96 [-3.32/6.90]	0.22 [-2.82/3.26]	-24.39/24.82	0.07
	V-DBS C-DBS	0.996 (68)	1.02 [1.00/1.05]	-1.25 [-5.64/2.54]	-0.89 [-3.89/2.12]	-25.19/23.42	0.18
PEth 18:1/18:1	Blood C-DBS	0.978 (48)	0.94 [0.89/1.00]	3.12 [-1.00/6.44]	1.19 [-3.62/5.99]	-31.25/33.62	0.19
	Blood V-DBS	0.990 (48)	0.95 [0.92/0.98]	1.20 [-0.41/3.38]	1.47 [-1.67/4.61]	-19.72/22.66	0.05
	V-DBS C-DBS	0.985 (48)	0.97 [0.93/1.01]	1.01 [-1.60/3.55]	-0.26 [-4.15/3.62]	-26.50/25.97	0.41
PEth 16:0/16:0	Blood C-DBS	0.971 (32*)	1.11 [1.00/1.19]	-4.20 [-10.76/2.00]	-1.92 [-6.43/2.58]	-26.43/22.58	0.11
	Blood V-DBS	0.976 (32*)	1.09 [1.01/1.18]	-4.68 [-11.14/3.36]	-2.82 [-7.25/1.61]	-26.90/21.26	0.05
	V-DBS C-DBS	0.962 (32*)	0.97 [0.89/1.06]	-0.58 [-5.55/6.13]	0.93 [-3.92/5.78]	-25.44/27.30	0.41

4 Discussion

UHPLC-ESI-MS/MS methods for the quantification of PEths in whole blood, V-DBS and C-DBS have been developed and validated using international guidelines [29] and published recommendations [27]. PEth 16:0/18:1 and PEth 16:0/18:2 are the two predominant PEths detected in blood after alcohol consumption. Taking into account the commercial availability of the PEths standards at the moment, the methods presented in this paper have been developed for PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0. As deuterated analogues have not been commercialised yet, four different ISs (PMeth 16:0/16:0, PMeth 18:1/18:1, PProp 16:0/16:0 and PProp 18:1/18:1) were evaluated during validation. In this study, PMeth 18:1/18:1 compensated best for matrix effect for each compound and was therefore chosen as IS for all 3 methods.

The detection of PEths requires highly sensitive techniques, due to the low amount of a certain PEth present in the sample (e.g. 16:0/16:0) and/or due to a low amount of sample (e.g. C-DBS). Therefore, special attention was paid to decrease possible ion-suppression by optimizing both the extraction and the chromatographic separation.

Variable recoveries, ranging from 33% (PEth 16:0/16:0 and PEth 16:0/18:1) [20] to 80% (PEth 16:0/16:0, PEth 18:1/16:0, PEth 18:1/18) [7], have been reported in past publications using a LLE with a mixture of isopropanol and hexane (2:3, v/v). We have optimised the LLE procedure to extract PEth from venous blood. Therefore, the pH during extraction was adjusted to 2 by adding 2% formic acid in a mixture of 10 mM ammonium acetate buffer and isopropanol before extraction with hexane. This resulted in a mean extraction efficiency from venous blood of 83% (RSD=13%) for PEth 16:0/18:1, 87% (RSD=13%) for PEth 18:1/18:1 and 82% (RSD=20%) for PEth 16:0/16:0. Somewhat lower percentages were observed (between 55 and 78%) for the V-DBS and the C-DBS methods. Similar percentages, ranging from 68 to 91% [15] and 56 to 76% for PEth 16:0/18:1 [18] and from 27 and 43% for PEth 18:1/18:1) [15], were reported earlier for other DBS-based methods. The basis for this somewhat lower extraction efficiency is not known. Interaction with the filter paper might be a possibility, as recently suggested by Koster et al. for immunosuppressants [34].

Reversed phase LC separation is the method of choice for the identification and quantification of phosphatidylethanol species. The retention is based on the lipophilicity, determined by the length and number of double bonds present in the fatty acid side chains [35]. Because the nonpolar part of PEths tends to interact

very strongly with the nonpolar hydrocarbon phase of a reversed phase column, the use of a more polar phase (i.e. C8 [14, 18, 20], C4 [7, 15] or phenyl [16]) instead of a C18 phase allows to decrease the retention of PEths [14]. The use of less polar solvents, such as tetrahydrofuran (index polarity = 4.0), isopropanol (index polarity = 3.9) or methanol (index polarity = 5.1) instead of acetonitrile (index polarity = 5.8) also improved the elution of PEths using a reversed phase column. In our methods, gradient elution based on an ammonium acetate buffer and a mixture of 10% tetrahydrofuran in isopropanol on a 50-mm C8 column was chosen.

Our three methods (blood, V-DBS and C-DBS) have a LLOQ of 10 ng/mL for PEth 16:0/18:1 and PEth 18:1/18:1 and of 19 ng/mL for PEth 16:0/16:0. In literature, LC-MS/MS methods for PEths quantification in blood have reported LLOQs between 8 to 83 ng/mL for PEth 16:0/18:1, between 0.7 to 73 ng/mL for PEth 18:1/18:1 and between 0.7 to 68 ng/mL for PEth 16:0/16:0. While it seems at first sight that our method is less sensitive, the most sensitive method published [6] required 300 µL of blood while our methods use 30 µL of sample. Using a low sample volume, such as 30 µL, was necessary for development of the C-DBS method. Published methods about the validation of PEths in DBS have reported LLOQs of 8 ng/mL (3x3mm punches from a 30 µL DBS) [18] and 87 ng/mL (100 µL DBS) [15] for PEth 16:0/18:1 and of 23 ng/mL (100 µL DBS) for PEth 18:1/18:1 [15]. The DBS methods presented here provide comparable or lower LLOQs for PEth 16:0/18:1 and 18:1/18:1, and have included PEth 16:0/16:0. Furthermore, to our knowledge, no publication has already evaluated the influence of hematocrit, punch localization and spot volume on the quantification of PEths in DBS. One-way ANOVA tests did not reveal a significant influence ($p>0.05$) of these parameters on quantification of the evaluated PEths (Supplementary Figure 1). In addition, no influence of the hematocrit on matrix effect and extraction efficiency was observed (Figure 1) and quantification was not affected when comparing DBS and blood concentrations in real samples with a wide hematocrit range (Figure 2).

An important advantage of DBS compared with venous blood is the improvement of analyte stability, avoiding the degradation of PEths in venous blood not stored at -80°C [24] and the post-collection synthesis of PEths in samples exposed to ethanol [18]. Helander et al. have demonstrated that PEths were stable in venous blood, if stored at − 80 °C, and this up to 14 months [7]. A decrease of the concentration of PEth 18:1/18:1 (18%) and PEth 16:0/18:1 (25%) has been described for EDTA whole blood samples stored at -20°C for 30 days [24]. Stability of PEth in DBS (at -20°C and 20°C) has been assessed up to 30 days by Faller et al. [24]. Our results confirm the stability of PEths in blood stored at -80°C and, more

importantly, demonstrate that PEths were stable in DBS samples stored in zip-closure plastic bags containing a desiccant packet at room temperature for up to 6 months, although a slight bias (119%) was observed for PEth16:0/16:0 in the QC high.

Finally, the successful participation (z-scores<1.43) to an international proficiency test organised by Equalis (Uppsala, Sweden) proved that the venous blood method for the quantification of PEth 16:0/18:1 is accurate.

Hundred authentic samples (50 inpatients in alcohol withdrawal and 50 control volunteers) were analysed using our 3 methods. To ensure C-DBS method validity, the hematocrit level of inpatients in withdrawal therapy (N=48) was measured and ranged between 0.33 and 0.49 (mean = 0.43, median = 0.44), with 83% (40/48) of the inpatient hematocrit levels lying within the reference range [28, 36] (0.41-0.50 for men and 0.36-0.44 for women).

Comparisons of the PEths concentrations measured using the three assays (Table 3) have shown limits of agreement of less than 33.62%, with no significant differences using Wilcoxon signed rank test analyses ($p \geq 0.05$) and Bland-Altman analyses (mean differences < 2.82%, with the zero value included in the 95% CI). Passing-Bablok regressions indicated a good overall correlation ($R > 0.962$), no systematic differences (95% CI of the intercept values include the zero value) and no proportional differences, although 1 was just not included in the 95% CI of the slope in 3 out of 9 comparisons. In literature, agreement between venous blood and V-DBS concentrations has been assessed using Bland-Altman analysis for PEth 18:1/18:1 and PEth 16:0/18:1 [15, 18]. One study showed good agreement, with a mean difference of 95.8 ng/mL (RSD=3.0%) and -4.3 ng/mL (RSD=2.9%) for PEth 16:0/18:1 and PEth 18:1/18:1, respectively [15]. Another study, despite a limit of agreement of more than 50%, reported no significant bias (mean -4.5%; RSD=33.8%) for PEth 16:0/18:1 and a good correlation (R=0.94) when comparing 281 results obtained from the analysis of venous blood and of 3 punches excised from V-DBS [18]. Both studies concluded that PEth 16:0/18:1 and 18:1/18:1 in V-DBS were a useful tool to monitor alcohol misuse. Our population study not only confirms these conclusions, but also extends these to PEth 16:0/16:0, and, importantly, demonstrates the agreement between blood and C-DBS. The latter is the most relevant comparison, as in real practice, C-DBS will be collected from a fingertip. Thus, the results presented here strongly suggest that C-DBS analysis is a valid alternative to venous blood analysis for the quantification of PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0. In addition, we studied the

distribution of PEths within the two groups (inpatients in alcohol withdrawal and control volunteers). In 50, 47 and 34 out of the 50 inpatients in alcohol withdrawal, PEth 16:0/18:1 (from 16 to more than 2000 ng/mL), PEth 18:1/18:1 (17-307 ng/mL) and PEth 16:0/16:0 (25-203 ng/mL), respectively, were quantified. PEth 16:0/18:1 was quantified in 18 out of the 50 control volunteers (13-220 ng/mL), while PEth 18:1/18:1 (17 ng/mL) was quantifiable in only one. PEth 16:0/16:0 was not present above LLOQ in control volunteers. These results suggest that, using the methods presented in this publication, only PEth 16:0/18:1 could be used to distinguish inpatients in alcohol withdrawal from control volunteers. More sensitive methods are required to search for a cut-off value for PEth 18:1/18:1 and PEth 16:0/16:0.

In literature, HPLC-ELSD methods analysing total PEths in blood generally used cut-off values between 0.2 and 1 µmol/L [7, 10, 11, 17, 19] to detect alcohol consumption. In Sweden, 0.7 µmol/L of total PEths is used as the clinical threshold [7]. These values were fixed by the LLOQ of the methods used and are limited to the detection of relatively high alcohol consumption (i.e. more than 50 g ethanol per day at an LLOQ of 0.7 µmol/L total PEths [17]). For PEth 16:0/18:1, an upper reference value for blood donors (N=200) of 141 ng/mL (0.2 µmol/L) has been proposed, which provided 5% false positive results and 17 samples detected as outliers [8]. In addition, two cut-off values for PEth 16:0/18:1 have been proposed, one of 700 ng/mL to detect problematic drinking [19] and another of 80 ng/mL to detect alcohol consumption (4 drinks daily during 30 days) in patients with liver disease (N=222) [37]. This second proposed cut-off value was selected to improve the sensitivity of the test (91%), and so provides a lower specificity (77%), which nevertheless can be improved up to 90% using a cut-off value of 300 ng/mL [32].

In our case, we have calculated a cut-off value of 221 ng/mL in blood to detect chronic and excessive alcohol consumption (inpatients on alcohol withdrawal), based on the highest sensitivity (86%) which was associated with the absence of false positive results (specificity=100%). It is of interest to add that 3 out of the 7 inpatients with PEth 16:0/18:1 concentrations lower than the chosen cut-off value declared to have ceased their alcohol consumption 2-3 weeks before the sampling. Importantly, and lending further support to the validity of using C-DBS, is that application of the blood cut-off to the C-DBS and V-DBS data yielded the same sensitivity and specificity.

5 Conclusion

This report describes the validation of three UHPLC-ESI-MS/MS methods for the quantification of PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 in 30 µL venous blood, 30 µL V-DBS and 3 punches (3mm) from C-DBS. The calibration curves ranged from 10 (LLOQ) to 2000 ng/mL for PEth 16:0/18:1, from 10 (LLOQ) to 1940 ng/mL for PEth 18:1/18:1 and from 19 (LLOQ) to 3872 ng/mL for PEth 16:0/16:0. Our results have confirmed the stability of PEths in blood stored at -80°C and have demonstrated that PEth 16:0/18:1 and PEth 18:1/18:1 were stable in V-DBS at room temperature for up to 6 months. The quantification of PEths via the C-DBS method was not significantly influenced by the hematocrit, the punch localization or the spot volume. Statistical comparisons (Bland-Altman plot, Passing-Bablok regression analysis and Wilcoxon signed rank test) of the measured concentrations obtained from venous blood, V-DBS and C-DBS from 100 volunteers (alcoholic inpatients and control volunteers) showed good agreement. Furthermore, application of a cut-off value of 221 ng/mL for PEth 16:0/18:1 to distinguish between inpatients in alcohol withdrawal and control volunteers provided a sensitivity of 86% and no false positive results (specificity=100%). To conclude, the developed method for C-DBS can be of interest to detect high and chronic alcohol consumption, as it offers distinct advantages such as a less invasive blood sample collection, stability during storage and transportation and a relatively simple sample preparation before analysis.

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Supplementary information

Number of pages: 6

Number of tables: 2

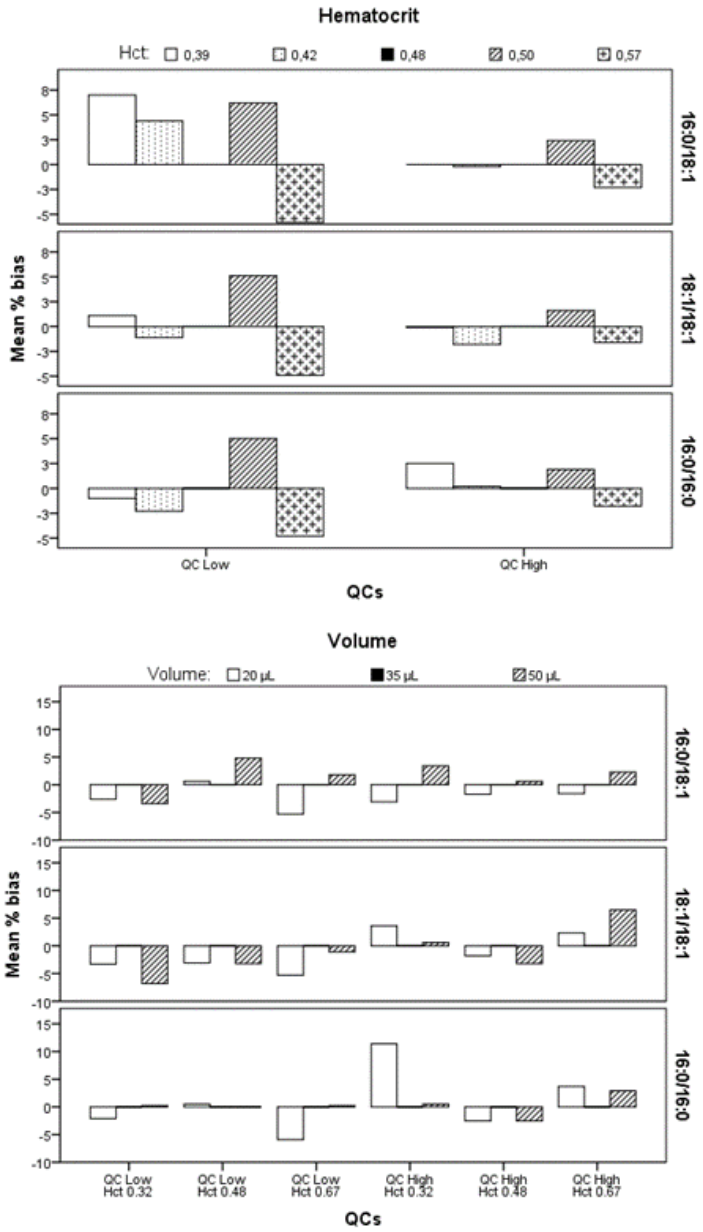
Number of figures: 4

Suppl. Table 4: Dilution table for the whole blood method and for the V-DBS method.

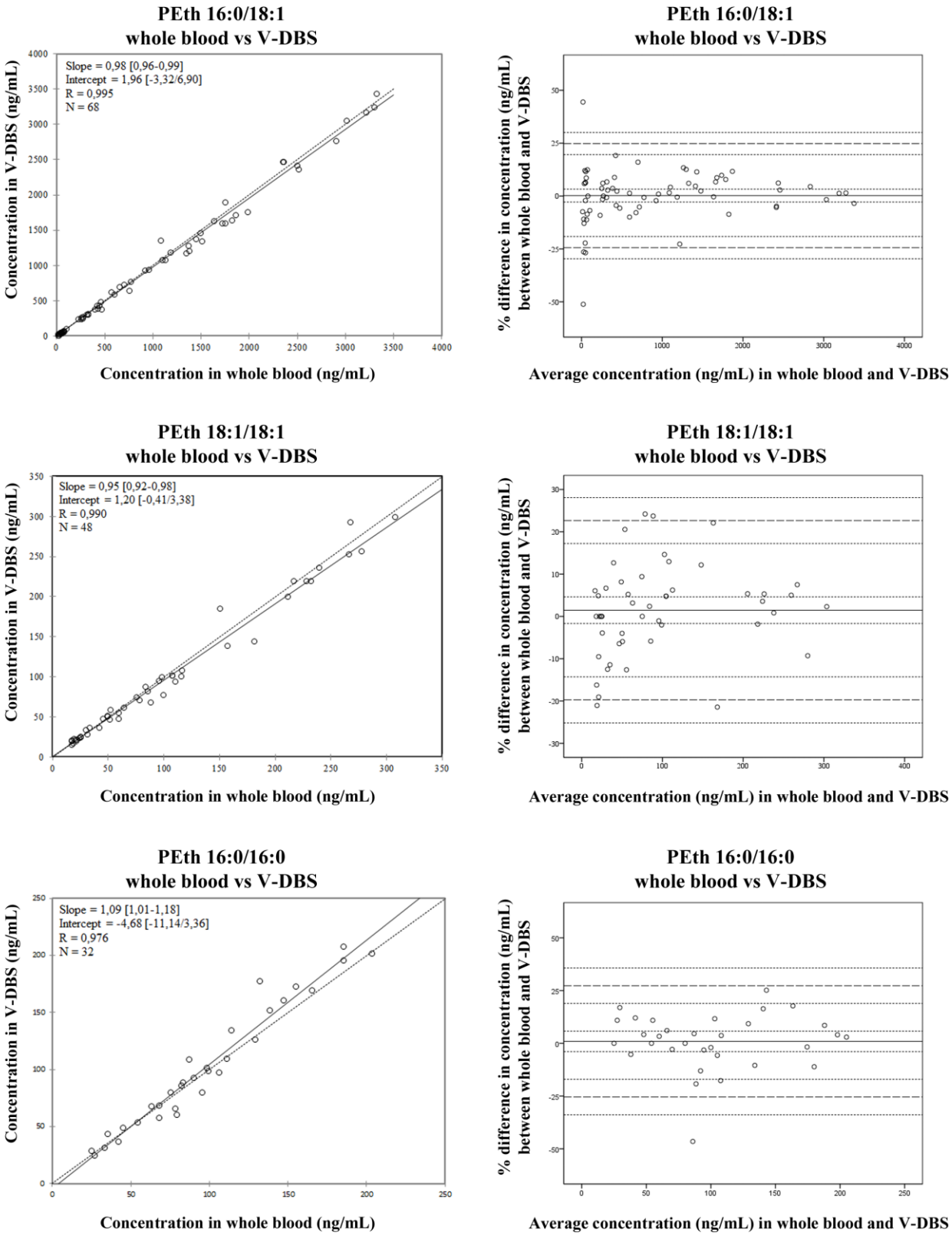
Blood and V-DBS methods						
	Dilution 1			**Dilution 2**		
Calibration curve				(50 µL Dilution 1 in 200 µL Blood)		
Cal-WS (100 µg/mL)				**PEth species concentration (ng/ml)**		
	STD (µL)		H_2O (µL)	**16:0/18:1**	**18:1/18:1**	**16:0/16:0**
Cal 1	50	Cal-WS	450	2000	1940	3872
Cal 2	200	Cal 1	200	1000	970	1936
Cal 3	200	Cal 2	200	500	485	968
Cal 4	100	Cal 3	100	250	243	484
Cal 5	100	Cal 3	400	100	97	194
Cal 6	100	Cal 5	100	50	49	97
Cal 7	100	Cal 5	400	20	19	39
Cal 8	100	Cal 7	100	10	10	19
QCs						
Cal-QC (50 µg/mL)						
QC H	50	Cal-QC	350	1250	1213	2420
QC M	40	Cal-QC	960	400	388	774
QC L	100	QC M	700	27	26	52

Suppl. Table 5: Dilution table for the C-DBS method.

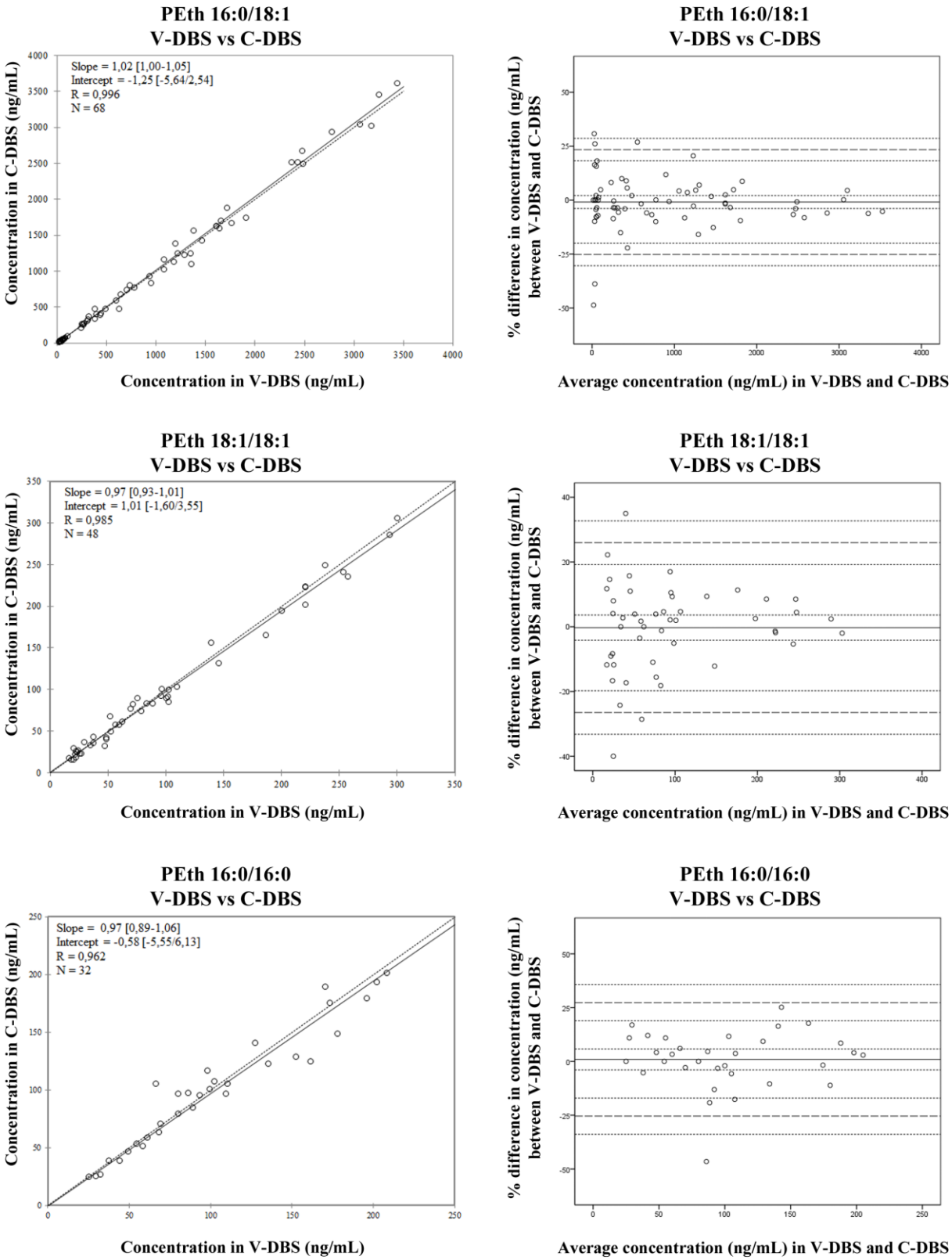
C-DBS methods						
	Dilution 1			**Dilution 2**		
Calibration curve				(10 µL Dilution 1 in 240 µL Blood)		
Cal-WS (250 µg/mL)				**PEth species concentration (ng/ml)**		
	STD (µL)		H_2O (µL)	**16:0/18:1**	**18:1/18:1**	**16:0/16:0**
Cal 1	50	Cal-WS	200	2000	1940	3872
Cal 2	100	Cal 1	100	1000	970	1936
Cal 3	100	Cal 2	100	500	485	968
Cal 4	50	Cal 3	50	250	243	484
Cal 5	50	Cal 3	200	100	97	194
Cal 6	100	Cal 5	100	50	49	97
Cal 7	50	Cal 5	200	20	19	39
Cal 8	100	Cal 7	100	10	10	19
QCs						
Cal-QC (250 µg/mL)						
QC H	30	Cal-QC	210	1250	1213	2420
QC M	30	Cal-QC	720	400	388	774
QC L	30	Cal M	420	27	26	52



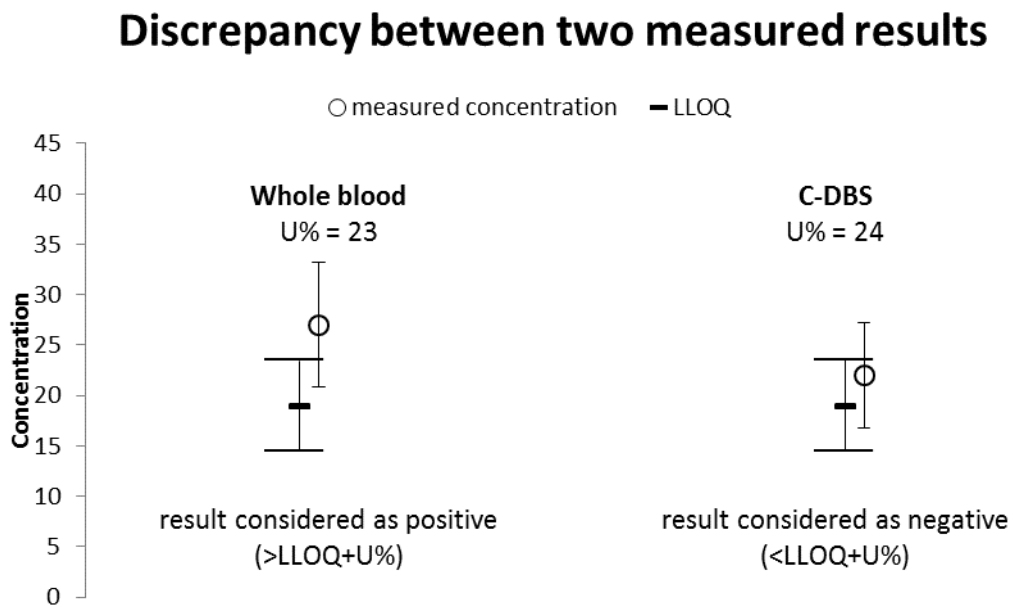
Suppl. Figure 6: (Above) Influence of the hematocrit on PEths responses measured in low and high QCs. Results are presented as mean % bias compared with the reference value (Hct = 0.40). (Below) Influence of the volume on PEths responses measured in low and high QCs at three Hct levels. Results are presented as a mean % bias compared with the reference value (volume = 35 µL).



Suppl. Figure 2: (Left) Passing-Bablok regression analyses of PEth species concentrations measured in blood and in V-DBS. The identity line is indicated using a dotted line. (Right) Bland-Altman analyses of PEth species plotting the % difference between blood and V-DBS concentration. The average difference is represented by a solid line, the limits of agreement (1.96 SD) by dashed lines and the 95% confidence intervals for the mean and the limits of agreement by dotted lines.



Suppl. Figure 3: (Left) Passing-Bablok regression analyses of PEth species concentrations measured in V-DBS and in C-DBS. The identity line is indicated using a dotted line. (Right) Bland-Altman analyses of PEth species plotting the % difference between V-DBS and C-DBS concentration. The average difference is represented by a solid line, the limits of agreement (1.96 SD) by dashed lines and the 95% confidence intervals for the mean and the limits of agreement by dotted lines.



Suppl. Figure 4: Concentrations measured (case 3) and LLOQ in whole blood and C-DBS presented with the measurement uncertainty (U). Taking into account the measurement uncertainty for the LLOQ, whole blood result is considered as positive while C-DBS results is considered as negative.