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RESEARCH ARTICLE

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Interlaboratory comparison of phosphatidylethanol in dried blood spots using different sampling devices

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Abstract

Phosphatidylethanol (PEth) has become an important marker to assess drinking behaviour and monitor abstinence. Despite its increasing use, knowledge on robustness and standardization and comparability of methods and results are still limited. In 2022, the first international consensus for the use of PEth and its interpretation was published. To establish an experience-based foundation for further harmonization, three rounds of interlaboratory comparison using microsamples were conducted. Participating laboratories sent their sampling devices to the laboratory of Forensic Toxicology at the University of Bern, where for each round, four different authentic blood samples were applied to the devices and sent back. The PEth 16:0/18:1 target concentrations covered a range between 16 and 474 ng/mL (0.023 and 0.676 μmol/L, respectively) and included sample concentrations close to the decision limits of 20 and 200 ng/mL (0.025 and 0.28 μmol/L, respectively). Evaluation of the results based on guidelines by Horwitz and the Society of Toxicological and Forensic Chemistry (GTFCh) showed that 73% of all participating laboratories quantified and reported all samples ($N = 4$ for each round) within the acceptable limits. More than 90% quantified and reported at least one sample within the acceptable limits.

KEYWORDS
alcohol biomarkers, interlaboratory comparison, PEth, phosphatidylethanol

1 | INTRODUCTION

Phosphatidylethanols (PEths) are a group of abnormal phospholipids that are formed enzymatically when ethanol is present in the body.[1] Due to its slow elimination kinetics with a terminal half-life of 8–13 days, PEth can be used as a direct biomarker to monitor alcohol consumption behaviour of up to 4 weeks prior to sample collection.[1,2] In contrast to indirect biomarkers such as carbohydrate-deficient transferrin (CDT) and gamma-glutamyltransferase (GGT), PEth exhibits enhanced sensitivity and selectivity. Unlike ethyl glucuronide in hair (hEtG), PEth has advantages in terms of a reduced risk of pre-sampling manipulation as well as ease of sample preparation in the laboratory.[1,3–5] PEths have routinely been analysed by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) in laboratories in Europe and the United States for driving aptitude assessment (DAA) and abstinence monitoring in the clinical and forensic field.[2,6] Usually, only the most abundant species (PEth 16:0/18:1) is determined, but some laboratories also report other PEth analogues such as PEth 16:0/18:2 and 16:0/20:4.[7,8] As PEth accumulates in the erythrocytes, whole blood has to be analysed.

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Alternatively, dried microsamples such as dried blood spots (DBS) prepared from whole blood can be used. They provide long-term stability and PEth concentrations comparable to those detected in venous blood samples.[9–13]

Standardization and knowledge of the robustness and comparability of the analytical methods that are currently in use for the quantification of PEth are still limited. A first paper on the topic of harmonization was recently published by Luginbühl et al.[7] In addition, White et al. presented results of an interlaboratory comparison of PEth in whole blood and erythrocytes.[14] Van Uytfanghe et al. presented results of a comparison involving two Belgian laboratories using VAMS® devices.[15] Quality control samples are commercially available from different manufacturers, including Equalis (Sweden) and ACQ Science (Germany). However, some of the samples are a mixture of artificially prepared and authentic samples.[14] Helander and Hansson described the results of 10 years of external quality assessment (EQA) samples for PEth that achieved a coefficient of variation (CV) of less than 15%. It should be noted that haemolyzed blood was used in this study.[16] De Sá e Silva et al. recently described that the regioisomeric ratio (16:0/18:1 to 18:1/16:0) in their artificially prepared sample differed from the naturally occurring ratio.[17] Thereby, the different fragmentation efficiencies for the respective side chains, as earlier described by Luginbühl et al., could be the relevant cause.[18] Consequently, for proficiency testing, authentic whole blood samples should be used in addition to synthetic samples.

To establish a foundation of experience for further harmonization, this paper reports on the results of three rounds of dried blood based microsampling device interlaboratory comparisons. Participating laboratories have been invited to send their devices, such as Mitra® VAMS, Capitainer®, different DBS filter paper cards and a combination of a volumetric capillary with extraction solvent and vial (of one participant), to the laboratory of Forensic Toxicology at the University of Bern, where authentic fresh blood samples from routine forensic and clinical cases as well as a lyophilized sample were applied to the different sampling devices and sent back to the participants. The results of the participants' laboratories had to be reported latest after 4 weeks and were then evaluated according to standards proposed by Horwitz and the Society of Toxicological and Forensic Chemistry (GTFCh).[19]

2 | MATERIAL AND METHODS

2.1 | Participating laboratories

Three rounds of interlaboratory comparison were conducted. For each round of participation, laboratories could register on the webpage of The Society of PEth Research (PEth-NET). They were asked to send their choice of DBS sampling device to the Forensic Toxicology Laboratory at the Institute of Forensic Medicine, University of Bern.

2.2 | Sampling devices

Different types of capillary blood sampling systems, DBS filter paper cards (e.g., Whatman™ 903 Protein Saver Card and RDA spot®), Capitainer® B Vanadate, and Mitra® VAMS, and a combination of volumetric capillary with extraction solvent and vial (of one participant) were provided by the participants. The requested sample volume ranged from 10 to 50 µL.

2.2.1 | DBS cards

The classic DBS cards usually consist of a filter paper card with printed circles on it. Therefore, these devices are not volumetric themselves. Blood can be applied volumetrically using a pipette or a capillary. However, there are also volumetric filter paper devices as described by Stöth et al.[20] Some participants had the technical capability to correct for the haematocrit concentration by means of spectrophotometric reflectance measurement at 589 nm as described by Luginbühl et al.[21] In this interlaboratory comparison, blood was applied onto the DBS cards using positive displacement pipets (Gilson, Villiers le bel, France).

2.2.2 | Mitra® VAMS

Mitra® VAMS consist of a porous polymeric tip that will accept a predefined volume of blood, that is, they are considered as volumetric sampling devices.[15,22] In this interlaboratory comparison, blood was loaded onto these devices by placing the tip onto the surface of the blood as described by Denniff and Spooner.[22]

2.2.3 | Capitainer®

Capitainer® is another device for volumetric sampling. By the action of a microfluidic system, the transport of a volumetric amount of blood is guaranteed. To ensure the required volume, an excess amount of blood (15 µL) was pipetted onto the sampling area using positive displacement pipets (Gilson, Villiers le bel, France). An inhibitor ($NaVO_3$) is added by the manufacturer for inhibition of enzymatic processes to avoid post-sampling PEth formation.[23]

2.2.4 | Other systems

The combination of a volumetric capillary/extraction solvent/vial (used by one participant) is a 'volumetric device' that does not use drying of the blood, but a direct extraction into a solvent after sampling from the finger-tip into a capillary. For the interlaboratory comparison, the end-to-end capillary was filled with liquid blood and directly placed into the vial containing organic extraction solvent for

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liquid–liquid extraction and sent to the participant's laboratory. This was the only device, where blood samples were not dried during microsampling.

2.3 | Blood samples

The 12 blood samples used within this comparison were collected from case work samples (10 samples), volunteers (one sample) and from commercial lyophilized authentic quality control (one sample). The case work samples and the volunteer sample were collected in lithium-heparin tubes (Sarstedt, Nürnberg, Germany) or ethylenediaminetetraacetic acid (EDTA) tubes (one sample) and stored at 4°C. Sample preparation was conducted within a span of up to 3 weeks from the initial blood collection. The concentrations were chosen to cover both lower and upper decision limit (20 and 200 ng/mL, respectively) according to the 2022 consensus of Basel.[7] In the third round, two of the four samples were pooled by mixing two blood samples. Of each sample, 750 µL was pipetted into a 2 mL plastic tube (Sarstedt, Nümbrecht, Germany). Prior to each sample application onto a collection device, the samples were thoroughly mixed. In each round, four samples were applied volumetrically in duplicate to the sampling devices provided by the participating laboratories. The respective sample volume was chosen by the participating laboratory. After preparation and drying of the samples, the devices were mailed to the participating laboratories without cooling.

2.4 | Evaluation of reported results

All results were reported in ng/mL (conversion to µmol/L possible by dividing the result by the molar mass of PEth 16:0/18:1, 701 g/mol). The results reported by the participating laboratories were analysed and processed according to the recommendations for proficiency testing, based on standards proposed by Horwitz and the GTFCh.[19] First, target concentrations were calculated as the mean of the reported results after exclusion of outliers (Grubbs' test [$\alpha = 5\%$]).[24] Then, conformity was tested according to Horwitz with C being the target concentration in kg/L.[25,26]

$$SD_{Horwitz} = 0.02 \cdot C^{0.8495}$$

The reported result was considered to be compliant if the result was within the acceptable range[19]:

$$Mean \pm 2 \cdot SD_{Horwitz}.$$

The deviation of a measured result relative to the target concentration was then calculated as z-score:

$$z = \frac{result - target\ concentration}{SD_{Horwitz}}.$$

An absolute z-score of less or equal to two was considered as satisfactory, whereas any larger deviations were considered as unsatisfactory.[27]

The reported concentrations were further analysed with respect to the guidelines of the European Medicine Agency (EMA). According to these guidelines, at least 66% of the reported values should be within ±20% of the mean for reanalysis of samples using chromatographic methods.[28] This evaluation was carried out applying the principle of a Bland–Altman plot using the corresponding target value as reference method: For each value, the relative deviation with respect to the corresponding target value was calculated. This was then compared to the mean of the standard deviation for all values.[29,30] A 95% confidence interval (CI) was defined as mean ± 1.96 standard deviation assuming a normal distribution of the reported concentrations.

3 | RESULTS

3.1 | Round 1

In the first round, 16 laboratories participated: 11 (68.8%) laboratories used DBS cards, two (12.5%) Mitra® and one each (6.3%) used HemaXis®, Capitainer® and the end-to-end capillary-based system. Based on the results reported, the target concentrations of the four samples were determined as 16.0 ± 4.77 ng/mL (Horwitz standard deviation), 44.1 ± 11.3 ng/mL, 183 ± 37.8 ng/mL and 362 ± 67.4 ng/mL. In each of the last three samples, one result was classified as outlier, and 87%–93% of the results were within the 2z-limit. For all four samples, the standard deviation of the results (after removal of outliers) was within the limits of two Horwitz standard deviations (Figures 1a and S1). Data evaluation using the EMA criteria showed that 69.0% of the reported values were within 20% of the respective mean. In the Bland–Altman evaluation, 94.8% of the values were within the 95% CI. The mean deviation was found as 0 ± 23.3%. The CV was 22% (range: 15%–34%).

Large deviations were mainly observed for outliers (200%–300% of the target value, one value for each of the last three samples) and reported concentrations reaching the lower limit of quantification (LLOQ) of the participating laboratories.

3.2 | Round 2

In the second round, 19 laboratories reported results: 10 (52.6%) laboratories used DBS cards, four (21.1%) each used Mitra® and Capitainer®, and one (5.3%) an end-to-end capillary-based system. Most of the laboratories ($n = 14$, 78%) used reference material from Cerilliant, followed by Supelco ($n = 2$, 11%), Chiron and Sigma-Aldrich (one each, 5.5%). The target concentrations were calculated as 32.5 ± 8.70 ng/mL, 94.7 ± 21.6 ng/mL, 290 ± 56.0 ng/mL and 478 ± 85.5 ng/mL. For each of samples No 1, 2 and 4, one result was classified as outlier, and 78%–89% of the results were within the 2z-limit. For sample No 3, the standard deviation of the participants

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FIGURE 1 Left: comparison of all received values (full circles) versus target value (asterisk) with respect to the Horwitz limits (dotted lines) for PEth 16:0/18:1. The decision limits of 20 ng/mL (solid line) and 200 ng/mL (dotted line) are shown. (a) First round, (b) second round, (c) third round. Right: corresponding representation of the relative deviation for each value (full circles) with respect to the target value. Upper and lower Horwitz limits are shown (dotted lines).

was larger than the Horwitz limits (Figures 1b and S2). The larger deviations in the second round arose mainly from a few outliers (two values per sample), for which the reported results were a multiple (50% or >200%) of the corresponding target values. Applying the EMA criteria, 72.9% of the values were within the 20% range. In the Bland–Altman evaluation, 91.4% of the values were within the 95% CI. The mean deviation was found as 0 ± 22.5%. The CV was 22% (range: 17%–30%).

3.3 | Round 3

In the third round, 20 laboratories reported results: 10 (50%) laboratories used DBS cards, five (25%) Mitra®, four (20%) Capitainer® and one (5%) end-to-end capillary-based system. Most of the laboratories used reference material from Cerilliant (13, 68%), followed by Supelco (3, 16%), ACQ, Echelon and Tebubio (each one, 5.3%). The target concentrations were calculated as 26.1 ± 6.39 ng/mL, 40.3 ± 11.6 ng/mL,

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232 ± 57.5 ng/mL and 473 ± 95 ng/mL. For each of the samples, one result was considered as outlier. Overall, 83%–95% of the results were within the 2z-range. For samples No 2 and 3, the standard deviation of the participants was larger than the Horwitz limits (Figures 1c and S3). Applying EMA criteria, 58.3% of the values were within 20% of the respective mean. In the Bland–Altman evaluation, 95.8% of the values were within the 95% CI. The mean deviation was found as 0 ± 24.9%. The CV was 25% (range: 20%–29%).

The large deviations arose mainly from a few extreme outliers. The outliers above the accepted range were in a linear correlation to each other, suggesting a constant offset in the calibration of the laboratories involved. Some of those values were a multiple (50%–400%) of the corresponding target value (one to two values per sample).

Samples No 2 and 3 were pooled from each two samples. The standard deviation for these two samples was not significantly different from the other two samples. Sample No 1 consisted of lyophilized quality control sample provided by ACQ Science. The standard deviation was in a similar range as other samples from this interlaboratory comparison in a similar concentration range.

4 | DISCUSSION

In contrast to other proficiency testing schemes for PEth, the samples were not subjected to any pre-treatment such as haemolysis or lyophilization. In a survey conducted prior to the first round, it was found that samples provided on a standard filter paper card could not be analysed by all interested participants readily. This is due to the fact that the laboratories are specialized in different DBS collection systems. Therefore, the participants were asked to send their DBS collection devices to the central laboratory.

The samples used for this series of interlaboratory comparisons were not analysed for EtOH. The presence of EtOH in blood could lead to post-sampling formation of PEth. However, the drying of the blood on DBS devices and/or the addition of an inhibitor such as $NaVO_3$ lead to deactivation of the involved enzyme, phospholipase D. Nevertheless, post-sampling formation on some devices such as Mitra® or HemaXis® cannot be completely ruled out. This would lead to significantly higher PEth concentrations that were not observed in this study.[23] Extraction with organic solvent used in the capillary collection system (by one of the participants) effectively inhibits enzymatic formation of PEth.[31]

In summary, 73% of all laboratories quantified and reported all four samples in the acceptable range (target value ± 2 Horwitz deviations), 13% three of four samples and 4% each two and one samples. None of the four samples were correctly quantified and reported by 7% of the laboratories. Laboratories participating in different rounds were counted multiple times.

In the course of the three rounds, an increasing number of participants joined using different sampling systems other than the classic DBS card. Overall, results of the classic DBS cards showed the smallest deviation from the target value, whilst samples collected on VAMS devices tend to yield higher concentrations (median 6.96%) and samples spotted on Capitainer devices lower concentrations (median −6.35%). However, due to the low number of participants for the alternative sampling systems, no further conclusion can be drawn in terms of differences in determined concentrations.

Method validation in the forensic laboratory requires the participation in interlaboratory comparisons to determine the combined measurement uncertainty u(x) and the expanded measurement uncertainty U. They can be calculated based on the results of multiple interlaboratory samples and precision data obtained by quality control samples as described by Magnusson et al. (Nordtest Technical Report) and applied by the GTFCh.[32,33] Exemplary, these calculations have been performed for the analysis of PEth at the Institute of Forensic Medicine at the University of Bern using nine results from this series of interlaboratory comparison and data from 10 precision control measurements from 2023/2024. The combined measurement uncertainty was found as 14.6%, resulting in an expanded measurement uncertainty (coverage factor k = 2) of 29.2%, which is comparable to what has been found by Van Uytfanghe et al. with 38%.[15] As the decision limits of 20 and 200 ng/mL are based on experimental research, these values already include a measurement uncertainty.[34,35] Therefore, it is not necessary to add a CI.

Six laboratories stated that they could not quantify all samples, as the samples were above or below the calibrated concentration range. These values could therefore not be taken into any consideration for the statistical evaluation. The LLOQ of the laboratories ranged between 2 and 21 ng/mL. Especially, the highest LLOQ of 21 ng/mL provides difficulties when considering the 2022 consensus of Basel that states 20 ng/mL with an accuracy and precision of 15% as the decision limit to distinguish between abstinence/low alcohol consumption and alcohol consumption.[7] However, there is also another guideline that proposes 35 ng/mL as lower decision limit.[36] The lowest upper limits of quantitation were reported between 200 and 500 ng/mL. When analysing samples of patients undergoing withdrawal treatment, PEth concentrations may decrease over several weeks and therefore, a higher upper limit is recommended.[12]

Furthermore, we observed that in some cases, concentrations below 20 ng/mL, but above the LLOQ, were only reported as being below the reporting limit. Whilst this is in accordance with procedures for reporting case work results, it is crucial to report quantitative results for interlaboratory comparisons. This is of particular importance for interpretation of samples that are close to the lower decision limit of 20 ng/mL, such as sample No 1 in Round 1 and Round 3. For practical reasons, we recommend to use methods with a LOQ no more than 50% of the lower decision level—as suggested in the field of workplace testing for drugs of abuse.[37] This is in analogy to the determination of EtG in hair, for which methods can quantify as low as 3 pg/mg (or even lower) with a decision limit of 5 pg/mg.[38,39]

The haematocrit level of the individual samples was not analysed. This could be a potential cause for deviating results when a sub-punch/partial punch is taken from a DBS filter paper card. Samples measured from Capitainer® and Mitra® devices were described in literature to be independent of the haematocrit value.[22,40]

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For one of all samples (sample No 4 in the third round), EDTA-blood was used, whilst all other case samples used consisted of lithium-heparinized blood. This was due to availability of blood samples in the desired concentration range. With regard to stability, literature is ambiguous: Faller et al. state that EDTA-blood should be preferred over heparinized blood, Skråstad et al. state that the decrease in PEth concentration of time was larger for the blood with EDTA as additive compared to blood with heparin.[10,41]

All microsampling devices were prepared at the same time and shipped under the same conditions to minimize influences on analyte stability. However, the shipping duration could not be completely controlled due to customs issues in different countries. It is worth to mention that PEth 16:0/18:1 on DBS is described to be stable at room temperature for up to 60 days.[8,42]

In the third round, two samples were produced by pooling and thoroughly mixing two case samples each for reasons of sample availability and target concentrations. The observed deviations of two laboratories were mostly of extreme nature exceeding the concentrations of the respective unpooled samples. Therefore, a potential sample inhomogeneity does not seem to be the reason for these outliers. For one sample (sample No 1 in the third round), a lyophilized quality control blood sample was used to compare its performance with fresh blood samples. Except for one extreme outlier, all remaining results were within the Horwitz range.

In the third round, the evaluation applying EMA criteria revealed a lower success score compared to the first and second round. This could hint to a reduced precision that would also explain the range of the participants' standard deviation for two of four samples being larger than the Horwitz limits.

Most of the participating laboratories reported using reference material from Cerilliant (>66%) for the calibration that produces PEth 16:0/18:1 with a high regioisomeric purity.[18] Other producers were Supelco, Chiron, Echelon and others (each used by three or less laboratories). Due to the low number of participants using reference material from another provider, no further conclusion can be drawn in terms of differences in determined concentrations.

Overall, the CV was found to be 23% (range: 15%–34%), after removal of outliers. This is larger than what was found by Helander and Hansson.[16] However, the samples used for this series of interlaboratory comparison were authentic samples and did not undergo sample pre-treatment such as haemolysis. Furthermore, the samples were not provided in a standardized collection system but pipetted onto the devices provided by the participants. Even though standardized pre-treated samples allow a certain standardization in terms of sample quality, the effect of different sample collection devices and the differences in the nature of authentic blood samples add additional complexity to the testing scheme.

5 | CONCLUSION

The three rounds of interlaboratory comparison for PEth 16:0/18:1 using authentic fresh blood samples as well as a lyophilized quality control sample showed that there is already a good comparability of the results in a large concentration range covering both lower and upper decision limit according to the 2022 consensus of Basel for most laboratories. More than 90% of the participating laboratories reported at least one sample within the acceptable ranges, and 73% quantified and reported all samples correctly. The higher CV compared to PEth proficiency testing schemes using standardized samples indicates that pre-treatment of samples, such as lyophilization or haemolysis, can improve the apparent comparability but may not reflect the authentic blood samples from routine analysis. There are still challenges in terms of calibration range and reporting limits, whereby PEth-NET will continue its interlaboratory comparison in the future.

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CONFLICT OF INTEREST STATEMENT

None.

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