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Increase of PEth after Single Consumption of Alcohol and Evaluation of a Volumetric DBS Filter Paper Device

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Abstract

Direct alcohol biomarkers are of growing interest for the assessment of alcohol consumption, with particular interest in phosphatidylethanol (PEth) in recent years. PEth is only formed when alcohol is present in the body. However, there is no statement about how much the PEth concentration increases after single moderate alcohol consumption. This study was conducted to determine the increase in PEth concentrations after a single drinking event. Additionally, a new volumetric sampling device (volumetric dried blood spot cards (DBSV)) was evaluated, which was designed to simplify further sampling processes and to allow for easy self-sampling. Dried blood samples from 31 volunteers were collected before and after single alcohol consumption with a mean maximum breath alcohol concentration of 0.4 mg/L (range: 0.30–0.55 mg/L). PEth concentrations were determined after automated extraction by liquid chromatography-tandem mass spectrometry. PEth 16:0/18:1 and PEth 16:0/18:2 concentrations increased to an average of 45 ng/mL each in patients starting below 20 ng/mL (range: 25.0–57.0 ng/mL for PEth 16:0/18:1; range 26.8–62.3 ng/mL for PEth 16:0/18:2). PEth concentrations in patients starting above 20 ng/mL increased by a mean of 30 ng/mL (range: 6.2–71.3 ng/mL for PEth 16:0/18:1; range 8.8–65.3 ng/mL for PEth 16:0/18:2). In addition, the comparison of the new sampling device DBSV with a standard filter paper card (with volumetrically applied 20 µL of blood samples) yielded a close agreement for the determined PEth concentrations in 24 forensic samples and three external controls. Therefore, the sampling device DBSV proved to be suitable for the determination of PEth concentrations in blood.

Introduction

Alcohol consumption is a global health problem that has been known for centuries. Progress in the research of the direct alcohol biomarkers phosphatidylethanol (PEth) and ethyl glucuronide (EtG) for the assessment of individual alcohol consumption behavior as well as for the proof of abstinence has greatly increased the interest in these biomarkers in clinical and forensic settings. In addition to the long-established alcohol biomarkers EtG, detected in hair and urine, PEth in blood has gained interest and acceptance for differentiating among abstinence, moderate alcohol consumption, and chronic excessive alcohol consumption (1, 2).

PEth is a group of phospholipids that is only formed in the presence of ethanol via the action of phospholipase D (3, 4). PEth accumulates in the cell membranes of erythrocytes, and thus, whole blood samples are required for its determination (5). Alcohol use assessment is based on PEth 16:0/18:1 as this is the most abundant analog (1, 2, 6). The first PEth analyses were therefore performed from venous liquid blood until it was shown by Faller et al. that PEth determination from dried blood spots (DBSs) is feasible (7). DBSs provide increased stability due to enzyme inactivation through the drying process, resulting in easier shipment compared to cooled or frozen liquid blood. DBSs for PEth analysis were initially collected by pipetting venous blood onto filter paper cards. Meanwhile, DBS sampling has evolved to sampling of capillary blood directly from the fingertip, as the PEth concentrations in venous and capillary blood are comparable (7–9). Volumetric blood sampling can be performed with end-to-end micro-capillaries or with the help of special devices such as volumetric absorptive microsampling or Capitainer® (10, 11).

We conducted a drinking study with 31 volunteers for the determination of the increase in PEth concentrations after a single drinking event with a target blood alcohol concentration (BAC) of 0.8 g/kg. In this study, capillary blood samples were collected by trained personnel using volumetric capillaries and applied to filter paper cards.

In addition, we performed a method comparison between a validated method for the determination of PEth using 20 µL of DBS and a new volumetric sampling device using blood from forensic cases. For further drinking studies with PEth determination, we wanted to use a self-sampling DBS device, either to be more flexible in the design of studies or to allow patients to collect samples remotely without having to visit trained personnel. Nowadays, there are several types of filter paper cards that can be filled either volumetrically or non-volumetrically. With the latter, the blood can be spotted directly onto filter paper cards and then extracted by taking a subpunch or by fully automated extraction using a fixed diameter extraction head (8, 12). Thereby, the self-sampling can be challenging, since in our experience, some participants tend to dip drops of blood onto the filter paper cards by repeatedly

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touching the card. In this case, only the surface of the card may be covered with blood, and an insufficient amount may be contained in a subpunch or extraction area with a fixed diameter.

Therefore, we evaluated a new commercial device for DBS self-sampling of capillary blood, based on a defined paper area for each blood spot with a paper-comb design. This design guarantees a reproducible self-sampling by filling a restricted area of a paper tooth from its trapeze-shaped end. After blood sampling and drying, a single paper tooth can then be detached easily for the analysis in total, or even subpunches can be used for subsequent analysis. PEth was used as analyte, as it is a good indicator of blood distribution due to its adhesion to red blood cells.

Methods

Drinking experiment

We evaluated the increase in PEth concentrations after controlled alcohol consumption with a target BAC of 0.8 g/kg. Persons with a PEth concentration exceeding 210 ng/mL (as a cut-off for excessive alcohol consumption) were excluded from the study. Abstinence was not required prior to the drinking experiment (except 24 h before the start of drinking—sobriety on the study day was ensured by a breath alcohol test at the beginning of the study), and thus, no "negative" PEth concentration was expected on the day of the drinking experiment. The data were collected as part of a study that examined driving in a simulator under the influence of alcohol and the effect of a single drinking event on PEth concentrations (ClinicalTrails.gov NCT04980846) (13). Approval was granted by the local ethics committee in Bern, Switzerland (ID 2021–00759). The study followed the ethical standards of the institutional research committee and the 1964 Declaration of Helsinki and its later amendments.

Only PEth concentrations are discussed in detail. Complete data sets for PEth were obtained from 31 participants (mean age of 37.6 ± 9.7 years, 15 women and 16 men). Exclusion criteria included, among others, health concerns incompatible with alcohol consumption, pregnancy or breastfeeding, teetotalers and excessive alcohol consumption habits with PEth concentrations > 210 ng/mL. About 1 to 2 weeks prior to the study day, a telephone interview and an initial screening on site were conducted. In the latter, a first capillary blood sample was taken to verify a participant's general alcohol consumption behavior (abstinence or drinking of low amounts/moderate drinking/excessive drinking, with cut-offs of 20 and 210 ng/mL, respectively (2)). On the day of the drinking experiment, three blood samples were taken: prior to drinking, after ethanol resorption and after the elimination of ethanol (for the time schedule, see Supplementary Figure S1).

Capillary blood samples were collected by trained personnel using 20 μL sodium-heparinized end-to-end capillaries from Hirschmann (Eberstadt, Germany) and applied to AutoCollect DBS filter paper cards (CAMAG, Muttenz, Switzerland). The Widmark formula, commonly used for the calculation of maximum BACs after certain drinking amounts, was used to calculate the individual amount of alcohol required to reach a BAC of 0.8 g/kg body weight (14, 15). However, the alcohol concentration was determined by measuring breath alcohol concentrations (BrACs), as this allows real-time monitoring. The target BrAC for the first driving test was 0.35 mg/L. Therefore, the BrAC was monitored in 5–15 min time intervals. If it was foreseeable from the course of the BrAC that the participants would not reach the maximum BrAC required for the driving test, they were given a further dose of alcohol. BrAC was determined using an alcohol breath analyzer Alcotest 6510 (Drägerwerk AG & Co. KGaA, Lübeck, Germany).

The first PEth sample on the study day was collected after the participants arrived (S1), the second about 1 h after the end of drinking with a BrAC of 0.35 mg/L or the highest BrAC reached (S2) and the last sample after the BrAC was below 0.1 mg/L (S3). PEth concentrations were determined after automated extraction using a DBS-MS 500 HCT autosampler (CAMAG, Switzerland) followed by liquid chromatography-tandem mass spectrometry (LC–MS-MS) analysis using a 5500 QTRAP (Sciex, Toronto, Canada) using a fully validated method, which has been described recently (16). The lower limit of quantification (LLOQ) of this method was 20 ng/mL. Calibrators were prepared by spiking blank blood from a teetotaler with PEth 16:0/18:1 and PEth 16:0/18:2 purchased from Cerilliant (Round Rock, TX, USA), which has the highest isomeric purity, and then applying it to the filter paper cards (17).

Measurement of PEth applied to conventional DBS filter paper cards compared to a volumetric device

A comparison was performed between routinely used filter paper cards with imprinted circles (GreenCheck DBSC, Protzek, Lörrach, Germany) and a new design of volumetric filter paper cards (GreenCheck DBSV, Protzek, Lörrach, Germany) including a paper comb with teeth with a defined area of 48 mm^2 per tooth as shown in Figure 1. The DBSV has been developed for volumetric sampling of capillary blood without the use of volumetric capillaries. The design of the ecological cardboard cartridge, with the filter paper teeth pointing upward when placed on a table, allows not only assisted sampling but also self-sampling.

Lithium-heparin venous or capillary blood samples from forensic cases were used for the analysis of PEth. After preparation of the DBS on both filter paper types, DBSV and dried blood spot cards with imprinted circles (DBSC), and subsequent extraction with whole spot punches (DBSC) or detached teeth (DBSV) using 1,000 μL of MeOH, the samples were evaporated to dryness and reconstituted in 100 μL of MeOH. The analysis was performed by LC–MS-MS using a 5500 QTRAP (Sciex, Toronto, Canada) with a previously published method (9). The LLOQ of the manual extraction method was 7.5 ng/mL.

A total of 24 samples from forensic cases that tested positive for PEth were applied to both the DBSC and DBSV cards, of which 18 were venous and 6 were capillary blood samples. To ensure that the teeth of the DBSV cards were fully saturated with blood, 23 μL of the venous blood samples were applied with a pipette per spot, resulting in a slight overflow over the perforation of the teeth (see Figure 1). The capillary blood was absorbed directly into the filter paper tooth. For the analysis, a tooth was detached at the perforation. DBSC samples were prepared by pipetting 20 μL of venous blood to the filter paper cards per spot or by using 20 μL sodium-heparinized end-to-end capillary (Hirschmann, Eberstadt, Germany) for capillary blood collected directly from the

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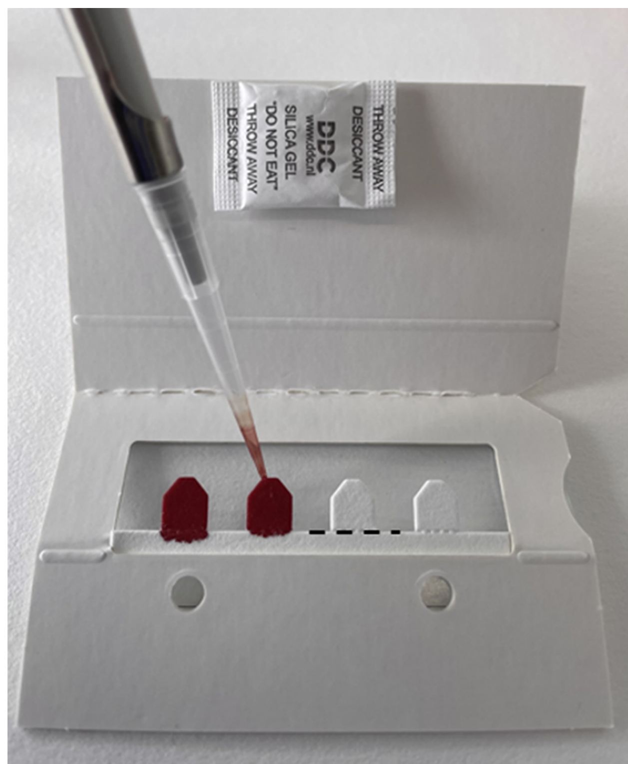

Figure 1. Design of the new volumetric filter paper cards (DBSV) with a paper comb with teeth and a perforation for detaching the teeth. The folding mechanism and the cover with a silica desiccant gel packet provide faster drying and protection of the samples during transport (a colored figure is provided online). The dashed line shows the perforation line where the teeth can be detached for the analysis.

fingertip. Additionally, external accuracy controls from ACQ Science (Rottenburg-Hailfingen, Germany) were added, two samples spiked with PEth 16:0/18:1 only and one authentic sample containing PEth 16:0/18:1 and PEth 16:0/18:2. The lyophilized samples were reconstituted according to instructions and pipetted to both types of cards in the same manner as the venous whole blood samples.

Results

Determination of the increase in PEth concentrations after a single drinking event

All 31 participants had PEth concentrations below 210 ng/mL on the day of the initial screening 1 to 2 weeks before the study day. The initial PEth concentrations ranged from < 20 to 188 ng/mL on the study day. After participants consumed their calculated amount of ethanol (mean = 60.1 g, range: 44.2–83.2 g) within 29–103 minutes (mean = 43 minutes), the mean maximum BrAC of 0.40 mg/L (range: 0.30–0.55 mg/L) was reached.

The maximum increase in PEth 16:0/18:1 concentrations was found in 80.6% ($n = 25$) in S3 (last sample when BrAC ≤ 0.1 mg/L was reached) and in 19.4% of the participants ($n = 6$) in S2 (sample taken approximately 1 h after the end of drinking). For PEth 16:0/18:2, the highest concentrations were detected in 84% ($n = 26$) of the subjects in S3. One of the participants showed the highest PEth 16:0/18:1 concentration in S3, but the highest PEth 16:0/18:2 concentration in S2. In contrast, two participants reached the highest PEth 16:0/18:2 concentration in S3, but the highest PEth 16:0/18:1 concentration in S2. The concentration–time curves for both PEth analogs are shown in Figure 2.

In the first group, only participants with an initial concentration of 20 ng/mL or above were included for the evaluation of the increase in total PEth concentrations ($n = 24$ for PEth 16:0/18:1; $n = 22$ for PEth 16:0/18:2). The remaining participants started with initial PEth concentrations below the LLOQ of 20 ng/mL ($n = 7$ for PEth 16:0/18:1; $n = 9$ for PEth 16:0/18:2). To calculate the maximum increase in PEth concentrations for each volunteer (> 20 ng/mL), the initial PEth concentration in S1 was subtracted from the highest PEth concentration reached, regardless of the sampling time (S2 or S3). During the observation period (mean = 6.26 h, range: 5.27–7.55 h), an average increase of 31.7 ng/mL (range: 6.2–71.3 ng/mL) was observed for PEth 16:0/18:1. PEth 16:0/18:2 increased by an average of 30.1 ng/mL (range: 8.8–65.3 ng/mL). Thus, concentrations of both PEth analogs increased by an average of approximately 30 ng/mL at a mean maximum BrAC of 0.40 mg/L for a single drinking event without the prior prolonged abstinence period. Nevertheless, wide interindividual differences in PEth concentrations occurred for both analogs.

Participants in the second group, who started with PEth concentrations below the LLOQ of 20 ng/mL ($n = 7$ for PEth 16:0/18:1; $n = 9$ for PEth 16:0/18:2), all reached their maximum concentrations in S3 for both PEth analogs analyzed. The maximum mean PEth 16:0/18:1 and PEth 16:0/18:2 concentrations reached were 44.1 ng/mL (range: 25.0–57.0 ng/mL) and 45.6 ng/mL (range: 26.8–62.3 ng/mL), respectively.

Comparison of PEth measurement using conventional DBS filter paper cards and a volumetric device

Concentrations ranged from 12.7 to 294 ng/mL for PEth 16:0/18:1 and from 7.9 to 122 ng/mL for PEth 16:0/18:2. The scatter plots of the two PEth analogs, with the concentrations resulting from the DBSC and DBSV analyses plotted against each other, are shown in Figure 3. The results show a good linear agreement among the devices, with a slight underestimation of PEth concentrations with DBSV. Figure 3 also depicts the results of the method comparison in Bland–Altman plots for PEth 16:0/18:1 and PEth 16:0/18:2 regarding the mean percentage differences (%difference) = 100 × (difference between DBSC and DBSV/mean) and the 95% confidence intervals (limits of agreement as 1.96 times the SD of the %difference). Deviations of more than 20% of their mean were found in 3.7% of the PEth 16:0/18:1 results and in 8.0% of the PEth 16:0/18:2 results. The mean value of the deviations was 9.8% and 8.7% for all analyzed PEth 16:0/18:1 and PEth 16:0/18:2 samples, respectively.

Discussion

The ability to estimate by how much PEth concentrations increase after single consumption of alcohol is particularly important for abstinence control, especially with regard to cut-off concentrations (1, 18). A prerequisite for participation in the presented study was at least occasional alcohol consumption. However, there were no requirements for participants to remain abstinent for a specific period of time prior to the alcohol administration day, other than not drinking

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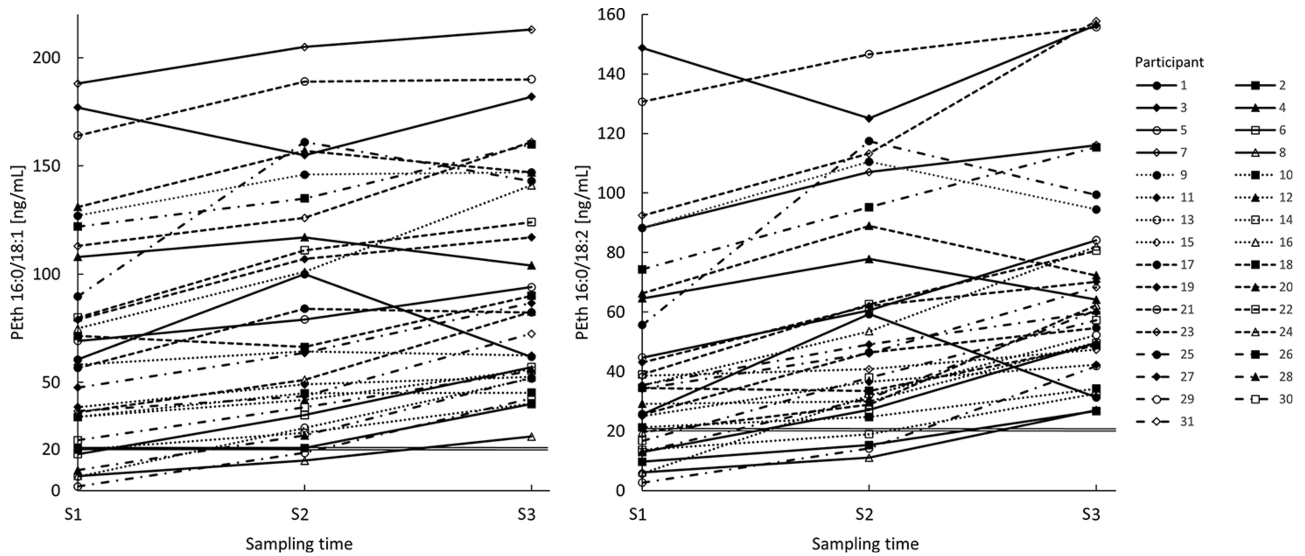

Figure 2. Concentration–time curves for PEth 16:0/18:1 (right) and PEth 16:0/18:2 (left).

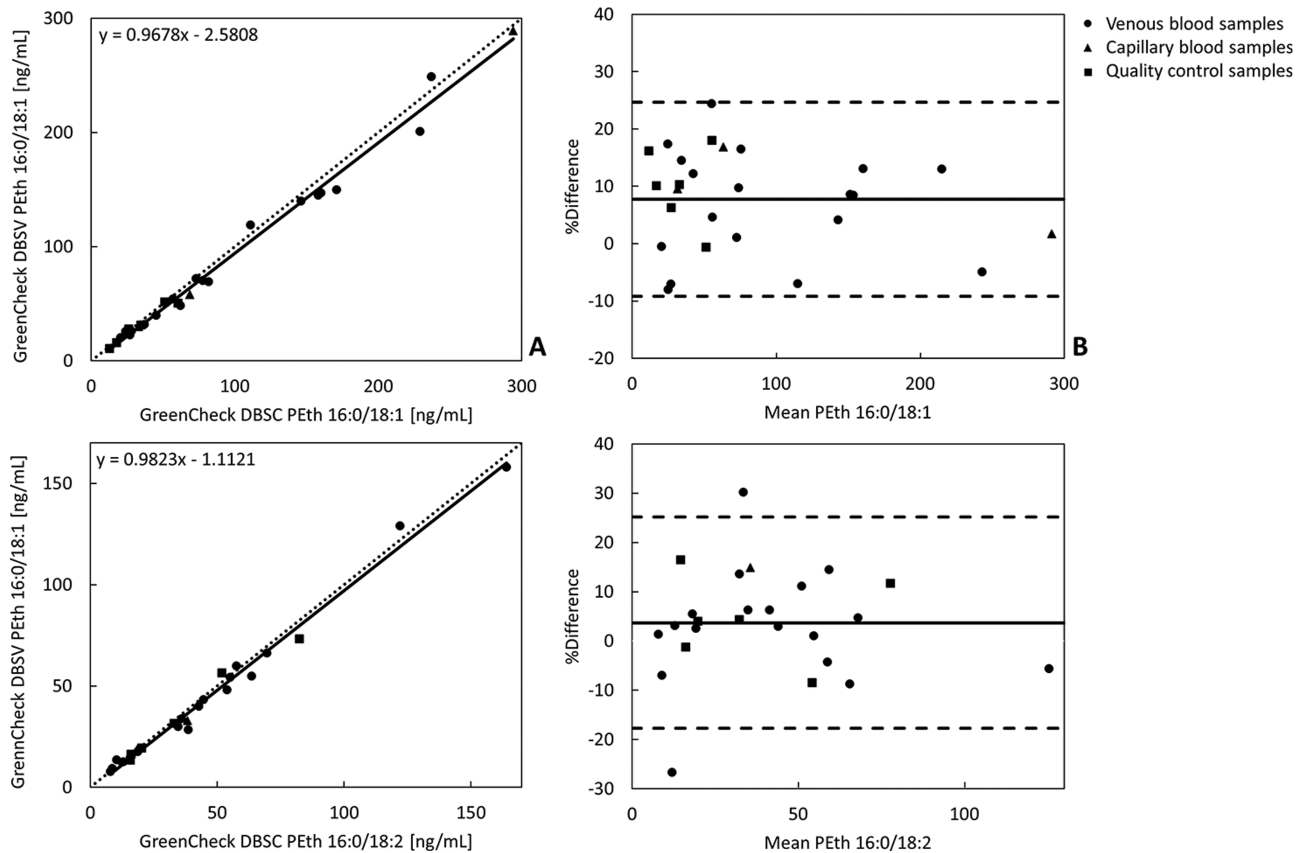

Figure 3. Comparison of PEth data, analyzed using DBSV and DBSC with PEth 16:0/18:1 at the top and PEth 16:0/18:2 at the bottom. A, Scatter plots showing the correlation of PEth measurements: the solid line is the regression line, and the dashed line is the line of equality. B, Bland–Altman plots with the solid lines representing the mean %difference and the dashed lines indicating the limits of agreement (1.96SD of the %differences).

alcoholic beverages for 24 h before the visit and not eating for at least 4 h prior to alcohol administration. Therefore, initial PEth concentrations were highly variable (< 20–188 ng/mL). Thus, they provide a good overview of PEth concentrations when alcohol consumption is not excessive (1, 2). The administration of alcohol on an empty stomach ensured a uniform absorption of alcohol. However, the time during which alcohol was consumed increased to more than 1 h for six participants when additional doses (mean = 13.2 g, range = 11.2–16 g) were administered to reach the target concentration. Five participants, two of whom received additional doses, did not reach the target BrAC of 0.35 mg/L for

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the first driving test. The remaining three participants did not receive a further dose, since it was not possible to predict during BrAC measurements whether the target concentration might be reached. Nevertheless, a mean maximum BrAC of 0.40 mg/L was achieved by the participants.

PEth is formed as long as ethanol is present in the body, and the formation curve of PEth is shifted relative to the BrAC concentration–time curve. This indicates that the PEth concentration can still increase when BrAC is already decreasing (19, 20). Most of the study participants showed the highest PEth concentrations in S3, which was taken after the BrAC was below 0.1 mg/L. We found that the mean increase in PEth concentrations in patients with initial PEth concentrations above 20 ng/mL was 31.7 and 30.1 ng/mL for PEth 16:0/18:1 and PEth 16:0/18:2, respectively.

In this study, the focus was put on a BrAC of 0.04 mg/L and on DBS, which provide the best possible stability of PEth. Javors et al. performed a similar study using lower doses of alcohol, but independent of demographic factors, and using liquid blood for PEth analysis. Both can lead to greater variability in the resulting PEth concentrations. The authors do not report in detail the increase in PEth concentrations. Other studies on alcohol consumption focused on higher doses of alcohol consumed on one or consecutive days or on exceeding a cut-off concentration after prolonged abstinence or on the influence of repeated consumption of small amounts of alcohol (18, 19, 21, 22). Regioisomerically pure PEth reference material was not used in all these studies. However, the highest reliability of PEth results, and therefore the best possible comparability, is achieved by using regioisomerically pure PEth reference material (17).

Participants with initial PEth concentrations below the LLOQ of 20 ng/mL showed an increase in PEth concentrations to a mean of 45 ng/mL. In contrast, Stöth and Kotzerke et al. recently reported that after long-term abstinence and a single alcohol consumption up to a BrAC of 0.32 mg/L, PEth concentrations did not exceed the cut-off of 20 ng/mL (22). Aboutara et al. recently found that after long-term abstinence and consumption of a single dose of 20 g alcohol, only one of 75 participants showed a PEth 16:0/18:1 concentration above 20 ng/mL on the next day. However, no BrAC or BAC was described in this study. Therefore, the alcohol concentration for exceeding the 20 ng/mL PEth 16:0/18:1 cut-off by single consumption of alcohol appears to be somewhere in the range of 0.3 to 0.4 mg/L BrAC.

However, the reported results show that even with small differences in the maximum BrAC, wide variations in the formation of PEth can occur. Our results are in line with previous studies reporting variability in PEth formation (21, 23, 24).

The appropriate choice of the sampling technique and instrument is a crucial step for optimized capillary blood sampling, data acquisition and data analysis (25). The data from this drinking study demonstrated that using volumetric capillaries is a reliable method to collect defined volumes of capillary blood and spot them on filter paper cards. Therefore, it is recommended that the sampling and spotting is performed by trained personnel, as it is necessary to prick the finger, fill the capillary and empty it to the filter paper, which takes experience and time, and assistance is desired by untrained participants.

As an alternative, self-sampling devices that are easy to handle can be used for studies, where participants are responsible for collecting capillary blood samples by themselves, e.g., if additional samples are to be collected on subsequent days. However, unsupervised self-sampling is critical in forensic cases. Various sampling techniques are used for self-sampling, e.g., polymer tips are filled or blood is transferred directly to the inserted filter paper card via microcapillaries built into plastic cartridges (10, 11). The results presented in this study demonstrate that the GreenCheck DBSV cards—without the use of a capillary—are as suitable for PEth analysis as the conventional DBS cards with the use of a pipette for a 20 μL volume. The exact volume of one tooth was not determined, which entails the risk of deviations. However, the method comparison with an established validated method for PEth determination using forensic samples shows good agreement between the two sampling approaches (see Figure 3).

The blood samples in this study were obtained from healthy individuals. Normal hematocrit reference ranges are from 0.36 to 0.44 for women and from 0.41 to 0.50 for men (26). Kummer et al. demonstrated that hematocrit values between 0.20 and 0.60 have no significant effect on the determination of PEth (8). Therefore, in our study, the hematocrit appears to have a negligible effect on PEth determination in our study, but it cannot be excluded unequivocally.

Considering the results in relation to the criteria proposed by the European Medicines Agency for the reanalysis of samples (at least two-thirds of the samples should not deviate more than 20% from their mean), all samples were within these limits (3.7% and 8.0% of PEth 16:0/18:1 and PEth 16:0/18:2 measurements, respectively) (27). Since capillary blood is the main target for this device, especially with regard to self-sampling and mainly venous blood was available for the comparison, the GreenCheck DBSV cards need to be tested on a larger scale for the use of capillary blood.

Conclusions

The results of this study show that there are relatively large interindividual differences in the formation of PEth. However, at a mean maximum BrAC level of 0.40 mg/L, an increase of 30 ng/mL on average was found for volunteers who started with PEth concentrations above 20 ng/mL, whereas an increase to an average concentration of 45 ng/mL was observed in participants who started with concentrations below 20 ng/mL.

The study also highlights the good comparability of conventional filter paper cards and the volumetric GreenCheck DBSV sampling device. The DBSV cards provide the accuracy and precision required for clinical and forensic analyses and can be used to collect capillary blood, which is a less invasive collection method than venous blood and does not require qualified personnel.

Supplementary data

Supplementary data are available at *Journal of Analytical Toxicology* online.

Data availability

The data underlying this article are available in the article and in its supplementary material.

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