Development and validation of seven phosphatidylethanol-homologues in dried blood spots including preliminary results after excessive use of an ethanol-based hand sanitizer

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Abstract

Phosphatidylethanol (PEth) has become a widespread marker offering an up to four weeks retrospective window to detect alcohol use. Due to the COVID 19 pandemic, ethanol-based hand sanitizers are frequently used. The aim of this study was to develop and validate a method for determination of up to seven different homologues of PEth from dried blood spots (DBS) after use of an ethanol-based hand sanitizer. Objectives of its preliminary application were to prove whether a threshold of 20 ng/mL for PEth 16:0/18:1 is reached and whether other homologues are formed as well as if positive findings of urinary ethyl glucuronide (U\textsubscript{EtG}) can be observed with respect to assess monitoring of abstinence control programs. Ten volunteers (8 occasional, 2 regular drinkers) were recruited to excessively use an ethanol-based hand sanitizer on 5 successive days. Dried blood spots and urine samples were collected daily. PEth and U\textsubscript{EtG} were determined by LC-MS/MS. In total, two volunteers with initial PEth 16:0/18:1-concentrations of 19.3 and 14.6 ng/mL exceeded the threshold of 20 ng/mL six times. Regular drinkers had starting PEth 16:0/18:1-concentrations of 242 and 354 ng/mL, showing a decline of PEth-concentrations in six out of the seven homologues over five days. In teetotalers, formation of PEth species could not be observed. Thus, not satisfying requirements in an alcohol monitoring program with initial PEth-negative blood cannot be explained by a frequent use of ethanol-based hand sanitizer only. In cases of regular alcohol consumption, PEth-homologues are not likely to be further influenced. However, results indicated that individuals with a PEth-concentration close to 20 ng/mL are at risk of exceeding the threshold by using ethanol-based hand sanitizer.

Keywords: PEth, neoformation, ethyl glucuronide, ethanol-based hand sanitizer, fitness-to-drive
Introduction

With the upcoming revision of the German criteria for the driving aptitude assessment, another direct alcohol marker – phosphatidylethanol (PEth) - will be included to evaluate a person’s drinking behavior (1).

Catalyzed by phospholipase D (EC-number 3.1.4.4), PEth is formed from phosphatidylcholine and ethanol. It is incorporated in cell membranes and especially accumulates in erythrocytes, which are devoid of phosphocholine-phospholipase (EC 3.1.3.75) (2–4). This abnormal cell membrane lipid contains two fatty acid chains originating from phosphatidylcholine. Its chains can differ in length and degree of saturation. PEth-homologues are named according to their fatty acid chain located at the sn1- and sn2-positions. To date, 48 different PEth-homologues have been described (5). The most and second most dominant homologues are PEth 16:0/18:1 and 16:0/18:2, respectively. The first PEth species is used to evaluate any drinking behavior and the latter can be used to confirm alcohol consumption. Concentrations of PEth 16:0/18:1 below 20 ng/mL, between 20 and 210 ng/mL, and above 210 ng/mL have been assigned to teetotalers, socially acceptable and risky drinking behavior, respectively (6, 7). A previously published consensus even stated that a PEth 16:0/18:1-concentration of 200 ng/mL is “strongly suggestive of chronic excessive alcohol consumption” (8).

PEth 16:0/18:1 has a half-time of up to 12 days and reportedly, quickly adapt to changes in drinking behavior (9). Analysis is performed on either whole or capillary blood. The latter is commonly used to make up dried blood spots (DBS) for further analysis (10).

Currently, ethyl glucuronide (EtG) is the favored direct alcohol marker to prove alcohol abstinence for the fitness to drive in Germany. In this context, hair and urine are the preferred matrices. Due to different hair growth stages, the EtG-concentration cannot be influenced by
recent changes in drinking behavior. Participants enrolled in a urine-controlled program are advised to deliver a sample on the day following notice under supervision. Musshoff et al. showed that various at first sight non-alcoholic beverages and foods may lead to positive $U_{EtG}$ results (11). Arndt et al. investigated the excessive use of an ethanol-based hand sanitizer, resulting in a positive concentration of $U_{EtG}$ above the commonly applied threshold of 100 ng/mL only (12). Based on these study results, participants are referred to hidden ethanol in everyday products and are advised to keep away from ethanol-based products, paints, or fuels when enrolled in a urine-controlled program.

Due to the COVID-19-pandemic, ethanol-based hand sanitizers being extremely efficient against the virus are often used in excess (13).

However, literature is scarce regarding a possible influence of excessive use on PEth-homologues in individuals. The aim of this study was to establish and determine up to seven PEth-homologues (16:0/18:1; 16:0/18:2; 16:0/20:4; 17:0/18:1; 18:0/18:1; 18:1/18:1; 18:0/18:2) following development and validation of a method using volumetric DBS samples suitable for routine work in an abstinence control program. Moreover, this method was used to investigate a possible (neo-)formation of PEth-homologues after excessive use of an ethanol-based hand sanitizer. $U_{EtG}$ being an established marker in abstinence control programs was monitored for comparison.

Experimental

Chemicals

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol (PEth 16:0/18:1; 1 mg/mL in methanol), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanol (PEth 16:0/18:2; 1 mg/mL in methanol) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol-d5 (PEth 16:0/18:1-D5; 0.1 mg/mL in methanol) were purchased from Cerilliant (Round Rock, Texas, USA). 1,2-Dioleoyl-sn-glycero-3-phosphoethanol (sodium salt; PEth 18:1/18:1; 1 mg solved in 1 mL methanol), 1-
margitoyl-2-dioleoyl-sn-glycero-3-phosphoethanol (sodium salt; PEth 17:0/18:1; 100 µg solved in 0.1 mL methanol), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoethanol (sodium salt; PEth 16:0/20:4; 1 mg solved in 1 mL methanol), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoethanol-d5 (sodium salt; PEth 16:0/20:4-D5; 1 mg solved in 1 mL methanol), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanol-d5 (sodium salt; PEth 16:0/18:2-D5; 1 mg solved in 1 mL methanol), 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphoethanol (sodium salt; PEth 18:0/18:2; 1 mg solved in 1 mL methanol), 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphoethanol-d5 (sodium salt; PEth 18:0/18:2-D5; 1 mg solved in 1 mL methanol), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoethanol (sodium salt; PEth 18:0/18:1; 1 mg solved in 1 mL methanol) and 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoethanol-d5 (sodium salt; PEth 18:0/18:1-D5; 1 mg solved in 1 mL methanol) were obtained from Echelon Bioscience (Salt Lake City, Utah, USA). Ethyl-β-D-glucuronide and ethyl-β-D-glucuronide-D5 were purchased from Cerilliant Cooperation (Round Rock, Texas, USA; 1 mg/mL in methanol) and Merck (Darmstadt, Germany; 1 mg/mL in methanol).

Both external quality controls PEth A 318 WH and PEth S 300 ng/ml WH PEth 16:0/18:1 were purchased from ACQ Science GmbH (Rottenburg-Hailfingen, Germany). For external quality control of EtG analysis, level 1 and 2 controls were purchased from Recipe Chemicals (Munich, Germany).

LC-gradient acetonitrile, methanol, 2-propanol were obtained from Merck (Darmstadt, Germany). Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Instrumentation

The LC-MS/MS system consisted of a Q-trap 5500+ (Sciex, Framingham, Massachusetts, USA) coupled to a HPLC 1290 Infinity II system (Agilent, Waldbronn, Germany). Separation was achieved by using a HyPurity C4 column (50x3mm, 5µm; FisherScientific, Waltham,
Massachusetts, USA) coupled to a C4 precolumn (Phenomenex, Aschaffenburg, Germany). Mobile phase A was 80% acetonitrile with 4 mM ammonium acetate buffer and mobile phase B was 100% 2-propanol. Gradient elution started with 0% B for 3 min, then switched to 100% B at 3 min, 50% B from 5 to 5.5 min and 0% B at 6 min. Total run time was 6 min. Data acquisition was carried out by Analyst (Version 1.7, AB Sciex, Darmstadt, Germany).

For MS/MS detection, electrospray ionization in negative mode with an ion spray voltage of -4500 V, and additional settings for curtain gas (25.0 arbitrary units (au)), collision gas (9 au) and gas temperature (650°C) were applied.

Data were acquired in the multiple reaction monitoring mode. Two and single mass transitions were monitored for PEth-homologues and their internal standards, respectively (Table I).

Software
For quantification, the Analyst software MultiQuant version 3.0.3 (AB Sciex, Darmstadt, Germany) was used. Validation parameters were calculated using Valistat 2.0 (Arvecon, Walldorf, Germany).

Methods
Sampling devices
For preparing DBS, Whatman 903 paper (Ahlström, Bernstein, Germany) was used. For volumetric collection of the capillary blood specimens, single-use lancets and 20 µL capillaries (both Sarstedt, Nümbrecht, Germany) were used.

Study design
To investigate a possible influence of an ethanol-based hand sanitizer on PEth and U_EtG, ten volunteers were advised to use a sanitizer containing 70 vol% ethanol 30 times over the period of 6 hours on five successive days. This is equivalent of using approximately 3 mL
ethanol-based hand sanitizer every twelve minutes. All participants were supervised to ensure using the sanitizer as scheduled. At the beginning and the end of every 6-hour period, volumetric DBS in duplicates for PEth analysis were collected by the participants themselves after a training period beforehand. Additionally, urine samples for EtG analysis were collected at the very beginning and the end of every 6-hour period without supervision.

Eight out of the ten volunteers (7 female, one male, age 21 to 33 years old) referred to their alcohol consumption as occasionally, and a PEth 16:0/18:1-concentration below 20 ng/mL was observed before the start of the study. They were asked to remain abstinent one week prior to the study. The other two volunteers (both 21 years old, one female, one male) referred to their drinking behavior as socially acceptable and were allowed to drink until the day before the study. All test subjects were not allowed to consume any ethanol-based beverages or food during the study period.

Sample preparation
For DBS collection, fingertips were disinfected with an ethanol-free sanitizer. After puncturing the fingertip with a single-use lancet, the first drop of blood was discarded. The following capillary blood was collected with a 20 µL capillary (Minivette® POCT K3 EDTA, 20 µL, Sarstedt, Nümbrecht, Germany). This sample was transferred to the filter paper and completely dried for at least 3 hours. Samples were stored at room temperature in a dry and dark place following DBS preparation for up to two weeks.

For calibration (10-1000 ng/mL) and internal quality controls (QC1 55 ng/mL and QC2 550 ng/mL), whole blood previously tested to be PEth-free was used. To prepare spiked PEth blood spots, two approaches were tested. For the first approach, 20 µL of blank whole blood was spotted, the analytes were spiked onto the blood spots while still being wet. For the second approach, the blank matrix was spiked with analytes, homogenized, 20 µL aliquoted onto the filter paper. For the external quality control 20 µL of PEth A 318 WH and PEth S
300 ng/ml WH were spotted onto the filter paper as well. Both calibrators and QCs were dried for at least 3 hours and stored at room temperature in the dark.

For PEth analysis, DBS were punched and placed into a tube. Extraction was performed by adding 1 mL of methanol and 5 µL internal standards (n=5, 200 ng/mL in methanol), and shaking on a vortex mixer at speed 4-5 for one hour. Afterwards, samples were centrifuged for 5 min at 18,600 g, 800 µL of clear supernatant was transferred to new tubes and evaporated at 37°C under a gentle stream of nitrogen. Residues were reconstituted with 100 µL of eluent (80% A and 20% B) and transferred to HPLC glass vials. Prior to LC-MS/MS analysis, vials were centrifuged for 10 min at 1,200 g, and 10 µL of each sample was injected into the LC-MS/MS system.

The laboratory also participated in proficiency tests PEth 2/21 and PEth 1/22 (Arvecon, Walldorf, Germany) using the present method.

For EtG analysis, spot urine samples were collected daily and frozen at -18°C until further analysis. After thawing, samples were homogenized, and 10 µL of urine was diluted with 90 µL of bi-distilled water in a HPLC vial. The internal standard (2µL of a 10µg/mL solution in bi-distilled water) was added, and the sample was vortexed again for 30 s. Analysis was carried out by an accredited method. To detect a possible dilution, the creatinine concentration was measured on an AU480 Chemistry Analyzer (Beckman Coulter, Krefeld, Germany) as described by Franz et al. (14).

Validation

The method for determination of PEth-homologues was validated according to the guidelines of the German Society of Toxicology and Forensic Chemistry (15). Signals were checked for retention time; two mass transitions were monitored for each analyte and a single transition was monitored for the respective internal standard; both are generally considered as sufficient
identification criteria (Table I). Chromatograms are displayed in the supplementary material (Suppl. Figure I).

Selectivity
This parameter was tested to confirm that a signal in a sample can unambiguously be assigned to any PEth-homologue. Blood samples from six teetotalers were tested. To investigate any interaction of internal standards with PEth-homologues, two additional samples with only internal standards added were analysed as well.

Calibration
To test for linearity, a neat standard solution calibration as well as a matrix calibration was conducted at seven different levels (10, 20, 50, 100, 200, 500 and 1000 ng/mL) and assessed by the Mandel- F-test. Using area ratios of analyte/internal standard, variance homogeneity was proven, and linearity of calibration lines was verified by regression analysis. If necessary, a weighting factor of either 1/x or 1/x² was applied.

To calculate isotopic purity, a highly concentrated solution containing only deuterated standards (1,000 ng/mL) and a blank sample were injected thrice.

Accuracy, precision and repeatability
Accuracy describes the differences caused by systemic and random errors between the actual and the calculated value. Precision describes the degree of scattering of a single value around a median. For repeatability, quality controls were analysed in duplicate to determine the relative standard deviation (RSD, %) (15).

On eight different days, two samples with low (QC1 55 ng/mL) and high (QC2 550 ng/mL) PEth-concentrations were prepared and tested to confirm accuracy in every single measurement. Both bias (%) and the 95%-tolerance intervals were calculated to evaluate accuracy.
**Autosampler stability**

Six samples of QC1 and QC2 were prepared, pooled, and aliquoted into six vials. Over the course of 6 hours, samples were analysed repeatedly to evaluate the stability of the seven analytes in the autosampler.

**Longterm stability**

To investigate the degradation of PEth in authentic samples blood was drawn from eight social drinkers. The samples were aliquoted into four DBS, respectively. Additionally, QCs were prepared on the first day. Both, DBS, and QCs samples were stored at room temperature protected from light for up to 60 days. PEth-concentrations were determined on day 0, 20, 40 and 60.

**Limit of detection**

The limit of detection (LOD) describes the lowest concentration of the analyte in a sample, where identification criteria are fulfilled. The limit of quantification (LOQ) is the lowest concentration of the analyte in the sample with a predefined relative measurement uncertainty (33%, significance: 99%). To detect analytical limits, a calibration consisting of 13 levels with concentrations from 1 to 30 ng/mL were analysed.

**Matrix effects and extraction recovery**

By using deuterated standards (PEth 16:0/18:1-d5; 16:0/18:2-d5; 16:0/20:4-d5; 18:0/18:1-d5 and 18:0/18:2-d5) matrix effects are thought to be resolved. Nevertheless, recovery and matrix effects were checked in the neat solution, matrix and extract using six different sets of PEth-negative whole blood at a median physiological hematocrit.

For matrix effects, PEth-free whole blood from six individuals was used to prepare DBS, as indicated above. After reconstitution, samples resembling extracts from 55 ng/ml and 550 ng/mL were generated. Spiked extracts and spiked neat solutions were compared using the resulting areas after the analysis.
For extraction recovery, two sets of QC1 and QC2 controls using six different blood specimens were prepared. The resulting areas were compared to those of the six spiked extracts.

*Carryover*

To check for a possible carryover due to highly concentrated samples, a DBS extract at a concentration of 10,000 ng/mL was injected thrice followed by a blank sample, respectively.

**Results**

**Validation**

**Selectivity**

Neither interference signals nor false positive results for any PEth-homologue could be observed.

**Linearity of calibration lines**

Results met requirements for both the Grubbs test for outliers and the Mandel’s linearity test. However, Cochran’s C-test was only passed if a weighting factor was applied. Therefore, a weighting of 1/x of the regression slope was conducted in favor of lower concentrations of all PEth-homologues.

The calibration range for both PEth 16:0/18:1 and 16:0/18:2 was linear from 10 to 1,000 ng/mL with correlation coefficients of 0.994 and 0.999, respectively. The remaining PEth-homologues could be calibrated from 20 ng/mL to 1,000 ng/mL (R 0.993 - 0.996). The amount of undeuterated PEth 16:0/18:1 in deuterated standard solutions was equal to 0.15 ± 0.006%. No interference in the deuterated standard solution was detected in the corresponding undeuterated analyte.

To prepare calibrators and internal QC, two approaches were tested. For the first approach, analytes diluted in methanol were added to wet spotted blood. Using methanol, spreading
over the spot was apparent. For the second approach, the analytes were added to blank blood before spotting. The area ratio of analyte/internal standard for both approaches were calculated and compared, revealing differences of ± 10% (Suppl. Table II).

Accuracy, precision and repeatability

Acceptable results for accuracy, precision and repeatability were achieved for all PEth-homologues. For QC1 and QC2, mean concentration ranged from 52.1 to 58.2 ng/mL (SD= 3.2-5.6; RSD= 5.7 – 10.02 %) and 522 to 571 ng/mL (SD= 22.6-53.5; RSD= 4.2-9.6 %), respectively. Detailed information of PEth-homologues is listed in the supplements (Suppl. Table I).

Stability

Minor deviations for PEth species were observed over a runtime of 6 hours at the autosampler, as well as over 60 days of storage. However, they were still within the tolerance range of 25%.

Limit of detection

For the main PEth-homologue 16:0/18:1, LOD and LOQ were estimated at 1.9 ng/mL and 10.7 ng/mL, respectively. For PEth 16:0/18:2, 16:0/20:4, 17:0/18:1, 18:0/18:1, 18:1/18:1, 18:0/18:2 LOQs were 9.7, 16.0, 15.1, 15.2, 12.0, 18.0 ng/mL, respectively, and LOD ranged between 1.7 and 2.9 ng/mL. Detailed information is listed in the supplements (Suppl. Table I).

Matrix effects and recovery

Recovery of samples at low and high concentrations was calculated using Valistat2.0®. The results are presented in the supplementary materials (Suppl. Table I). Both recovery and matrix effects ranged between 60.7 to 81.2 % and 75.5 to 110.4%, respectively. The standard deviation was always < 22%.
Carryover

No carryover for any PEth-homologue could be detected.

Proficiency testing

As results for the round robin test for PEth 16:0/18:1-concentrations at 208 and 100 ng/mL as at 84.7 and 34.6 ng/mL were determined, and both proficiency tests were passed successfully.

U\textsubscript{EtG} and PEth-concentration

Participants could be divided into three groups based on pre-study concentrations of PEth-homologues and U\textsubscript{EtG}: the 1\textsuperscript{st} group consisted of six persons with negative results for both U\textsubscript{EtG} and any PEth-homologue. The 2\textsuperscript{nd} group included two persons with a negative result for U\textsubscript{EtG} - however, PEth 16:0/18:1-concentrations were between < LOQ and 20 ng/mL. The two regular drinkers comprised the 3\textsuperscript{rd} group; both had U\textsubscript{EtG} -concentrations above the cutoff of 100 ng/mL (3238 and 510 ng/mL), as well as starting PEth 16:0/18:1-concentrations of 354 and 242 ng/mL, respectively. The creatinine concentration in all urine samples was \(\geq\) 20 mg/dL.

The following results were obtained during the study. In the 1\textsuperscript{st} group every test subject showed a U\textsubscript{EtG}-concentration above 100 ng/mL after 6 hours of using an ethanol-based hand sanitizer (Suppl. Figure II). On day five, volunteer 3 dropped out of the study. The average U\textsubscript{EtG} concentration was 626 ng/mL (median=529 ng/mL); minimal and maximal concentrations were observed at 175 and 1,988 ng/mL, respectively. For all PEth-homologues, concentrations were below the LOD and were therefore stated as negative throughout the study.

In the 2\textsuperscript{nd} group, the concentration of U\textsubscript{EtG} also exceeded the threshold of 100 ng/mL after 6 hours of using an ethanol-based hand sanitizer (Suppl. Figure II). Initial PEth 16:0/18:1-concentration for volunteers 7 and 8 were 19.3 and 14.6 ng/mL, respectively. During the
study period, the threshold was exceeded five times after using the ethanol-based hand sanitizer (Figure I). On day 2, the initial PEth 16:0/18:1-concentration in volunteer 7 was 20.3 ng/mL; however, it declined over 6 hours. Also, PEth 16:0/18:2 was detected in a range of 10.6 – 34.5 ng/mL. Volunteer 8 showed traces of PEth 18:0/18:1 slightly below the LOQ. Both test subjects tested positive for PEth 18:0/18:2 ranging from 20.1-51.4 ng/mL.

Both regular drinkers in the 3rd group started with initial $U_{EtG}$-concentrations above the threshold of 100 ng/mL. During the five days, both test $U_{EtG}$-concentrations remained above this threshold (Suppl. Figure II). PEth 17:0/18:1 was not detected in any of the test subject. Volunteer 9 was positive for all PEth-homologues during the observation period: PEth 16:0/18:1 (152 – 242 ng/mL), PEth 16:0/18:2 (57.6 – 144 ng/mL), PEth 18:0/18:1 (22.5 – 99.2 ng/mL), PEth 18:1/18:1 (22.2 – 27.8 ng/mL) and PEth 18:0/18:2 (103 – 253 ng/mL). During the study period, PEth 16:0/18:1 and 16:0/18:2 showed a decline in concentration of 25.1 and 34.2 %, respectively. Subject 10 showed positive values for six out of seven PEth-homologues. The concentrations of these PEth-homologues and their decline are shown in Figure II. PEth 16:0/20:4 showed the largest decrease (44.5 %), whereas PEth 18:0/18:1 resulted in the smallest decrease (16.3 %).

**Discussion**

**Validation**

Chromatographic separation was based on the method published by Helander et al. (16). The proportion of ammonium acetate was adjusted, and elution was prolonged to successfully separate the seven homologues.

By preparing volumetric DBS and using the whole blood spot for analysis, the hematocrit will be of negligible influence on the assay performance. For calibration and internal QCs, the hematocrit of the blank matrices was within the normal physiological range which also applies to the hematocrit values of all study subjects.
Both preparation gave consistent results, with the second one being the more common one (17–19). To exclude the fact that the prepared calibrators and internal QCs behave differently from actual samples, the concentration of PEth-positive blood was compared to spiked DBS. The PEth-positive blood concentration was determined, and afterwards, the given concentrations were spiked to blank DBS, revealing a maximal deviation of ± 20% for the respective PEth-homologues.

The development and validation of the LC-MS/MS method to simultaneously determine up to seven PEth-homologues in blood met the acceptance criteria. By passing the proficiency test for routine blood analysis, this method may be considered suitable to produce acceptable quantitation for PEth 16:0/18:1. The calibration covers both thresholds of 20 and 210 ng/mL PEth 16:0/18:1, which have been proposed by different international research teams (7, 10, 20–23), and which will be included in the German criteria for the driving aptitude assessment (1). In the literature, methods cover up to six homologues (19) which, however, did not cover PEth 17:0/18:1.

Using several different homologues might help to further investigate a person’s drinking history. By considering different homologues, single drinking episodes could be differentiated from regular daily drinking. Lopez-Cruzan et al. observed that PEth 16:0/20:4 has a relatively short half-life of only up to 2.1 ± 3 (mean ± SD) days. Therefore, if PEth 16:0/18:1 but not 16:0/20:4 is detected, the last drinking event might have occurred within up to four weeks but not during the last days prior to sample collection (24). It has been reported that PEth-homologues and their distribution origin from phosphatidylcholine, and respective fatty acid chains might depend on individual eating habits, i.e. vegetarian, vegan or low carb diets (2). Further research regarding different nutritional styles and their influence on the different fatty acid chains incorporated in phosphatidylcholine should be conducted.
At present, categorizing a person’s drinking behavior is preferably based on the concentration of the main PEth-homologue, which is very well studied (9, 10, 25).

Use of ethanol-based hand sanitizer
The results of the 1st group confirmed the findings of Reisfeld et al. (26). In subjects with an initial negative PEth-concentration (<LOD), no formation could be observed using an ethanol-based hand sanitizer. This supports the hypothesis that excessive use of an ethanol-based hand sanitizer is too small to initiate PEth formation, leading to a concentration higher than the LOQ.

Two volunteers enrolled in the 2nd group had initial PEth 16:0/18:1-concentrations between 10 and 20 ng/mL. Exceeding the threshold of 20 ng/mL during the study period five times after the 6 hours period indicates that the hand sanitizer might have had an influence. Volunteer 7 had an initial PEth 16:0/18:1-concentration of 19.3 ng/mL. On the second day of the trial, he showed a concentration of 20.3 ng/mL before the six-hour study period. A difference of 5% can be attributed to the method’s inaccuracy itself and may not be solely a consequence of using an ethanol-based hand sanitizer. On day one and five after the six-hour period, volunteer 8 showed a PEth 16:0/18:1-concentration of 36.5 and 35.6 ng/mL, respectively. The concentration almost tripled during the six-hour period. To verify these results, confirmation was done analyzing the second DBS resulting in concentrations of 38.2 and 34.2 ng/mL, respectively. Moreover, DBS collection was conducted in a laboratory where ethanol-containing solvents were prohibited. Therefore, contamination by ethanol of the filter paper before or after sampling can be ruled out. The increase in the PEth-concentration may be caused by leftover ethanol on the hand leading to post-sampling formation while the DBS was still drying. These results highlight the fact that handling ethanol-based sanitizers can influence the PEth-concentration; moreover, post-sampling formation of PEth can increase to an extent, thus exceeding the threshold of 20 ng/mL when the initial PEth-concentration is
between LOQ and 20 ng/mL. Nevertheless, subjects with PEth-concentrations slightly below 20 ng/mL may be at risk of exceeding the hitherto existing threshold using an ethanol-based hand sanitizer.

In the 3rd group, initial PEth 16:0/18:1-concentrations of 242 and 345 ng/mL indicate that the participants` drinking habit is harmful. Group 3 exceeded the threshold for $U_{\text{EtG}}$ before the study started, which indicates very recent drinking (27, 28). The decrease in detectable PEth-homologues over five days supports the observation that PEth quickly adapts to changes in drinking behavior. Moreover, this proves that neither ethanol was consumed nor that using an ethanol-based hand sanitizer influenced the decline.

Every volunteer, regardless of their drinking habit, exceeded the threshold of 100 ng/mL of $U_{\text{EtG}}$ every day of the study. This indicated that ethanol is absorbed and metabolized by the body. However, most ethanol intake may have occurred through inhalation rather than absorption through the skin, as previously presented by Arndt et al. (12).

**Conclusion**

By passing the GTFCh accepted validation criteria, the present method is useful for determining up to seven PEth-homologues in DBS via LC-MS/MS. It can be used to further investigate the decrease in homologues in the blood of regular drinkers after abstinence. Furthermore, it can be applied to understand the distribution of PEth-homologues in a different context, e.g. nutrition habits, medical issues, or controlled drinking. A sudden increase in PEth-concentrations cannot be explained by excessive use of an ethanol-based hand sanitizer if the individual is initially PEth-free. However, individuals close to the threshold should use only ethanol-free sanitizers as a precaution to avoid post-sampling formation.
Data availability

Data available upon request.

Ethical approval

All conducted experiments comply with the current laws of the Federal Republic of Germany. All participants voluntarily agreed to participate in the study.

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Disclosure of potential conflicts of interest

The authors declare no competing interests.

Research involving human participants

Informed consent was obtained from all individual participants included in the study.

Oral Presentation

Results of this study were presented at the 2022 “PEth in Mind” conference in Basel, Switzerland.

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Table I: Mass spectrometric parameters of PEth-homologues and deuterated internal standards, Q1: first mass analyzer, Q3: second mass analyzer, DP: declustering potential, CE: collision energy, CXP: collision cell exit potential, m/z: mass to charge ratio, V: volt, RT: retention time

| Compound transition | Q1 (m/z) | Q3 (m/z) | Dwell Time (msec) | DP (eV) | CE (eV) | CXP (eV) | RT (min) |
|---------------------|---------|---------|------------------|--------|--------|---------|---------|
| PEth 16:0/18:1 1    | 701.4   | 281.2   | 50               | -155   | -46    | -15     | 1.55    |
| PEth 16:0/18:1 2    | 701.4   | 255.2   | 50               | -155   | -48    | -13     | 1.55    |
| PEth 16:0/18:2 1    | 699.4   | 279.2   | 50               | -20    | -48    | -5      | 1.20    |
| PEth 16:0/18:2 2    | 699.4   | 255.2   | 50               | -20    | -44    | -13     | 1.20    |
| PEth 16:0/20:4 1    | 723.4   | 303.2   | 50               | -150   | -38    | -17     | 1.15    |
| PEth 16:0/20:4 2    | 723.4   | 255.2   | 50               | -150   | -46    | -15     | 1.15    |
| PEth 17:0/18:1 1    | 715.4   | 281.2   | 50               | -165   | -44    | -15     | 1.15    |
| PEth 17:0/18:1 2    | 715.4   | 269.2   | 50               | -165   | -48    | -15     | 1.64    |
| PEth 18:0/18:1 1    | 729.5   | 281.2   | 50               | -155   | -44    | -15     | 1.94    |
| PEth 18:0/18:1 2    | 729.5   | 283.2   | 50               | -155   | -48    | -15     | 1.94    |
| PEth 18:1/18:1 1    | 727.4   | 281.2   | 50               | -185   | -46    | -15     | 1.55    |
| PEth 18:1/18:1 2    | 727.4   | 78.9    | 50               | -185   | -138   | -9      | 1.55    |
| PEth 18:0/18:2 1    | 727.5   | 279.2   | 50               | -160   | -44    | -15     | 1.57    |
| PEth 18:0/18:2 2    | 727.5   | 283.3   | 50               | -160   | -48    | -15     | 1.57    |
| PEth 16:0/18:1 -D5 | 706.4 | 281.2 | 50 | -10 | -42 | -15 | 1.55 |
|---------------------|-------|-------|----|-----|-----|-----|------|
| PEth 16:0/18:2 -D5 | 704.4 | 279.5 | 50 | -155| -42 | -15 | 1.20 |
| PEth 16:0/20:4 -D5 | 729.4 | 303.2 | 50 | -150| -38 | -17 | 1.15 |
| PEth 18:0/18:1 -D5 | 734.5 | 281.2 | 50 | -150| -44 | -15 | 1.94 |
| PEth 18:0/18:2 -D5 | 732.5 | 279.2 | 50 | -160| -44 | -15 | 1.57 |

Figure I: 2nd Group: Phosphatidylethanol (PEth) 16:0/18:1-concentration (ng/mL) after 6 hours of using an ethanol-based hand sanitizer

Figure II: Decline of six phosphatidylethanol (PEth)-homologues over five days in subject 10
