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Stability of Ethyl Glucuronide, Ethyl Sulfate, Phosphatidylethanols and Fatty Acid Ethyl Esters in Postmortem Human Blood

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Abstract

The lack of systematic studies on the stability of ethanol's non-oxidative metabolites in postmortem specimens restricts their use in forensic cases. This study aimed to compare the stability of ethyl glucuronide (EtG), ethyl sulfate (EtS), phosphatidylethanols (PEths) and fatty acid ethyl esters (FAEEs) in postmortem human blood. Three groups were established based on the level and source of ethanol: the blank group, the ethanol-spiked group and the ethanol-positive group. Each group contained six blood samples from different corpses. The samples in each group were placed at 37, 25, 4 and −20°C. Every 24 h for 7 days, 50 μL was collected from each sample. The levels of EtG, EtS, PEths and FAEEs were determined by liquid chromatography-mass spectrometry, and their stability was evaluated. EtG was not detected in the blank group, but it was found in samples in the ethanol-spiked group placed at 37°C, and it was degraded in the ethanol-positive group at 37 and 25°C. EtS showed no change in any of the groups. PEths were not detected in the blank group, but formation was found in the ethanol-spiked group at all temperatures. In the ethanol-positive group, PEth levels fluctuated at 37°C, decreased at 25°C and increased at −20°C. FAEEs were generated in the blank group and in the ethanol-spiked group at all temperatures. In the ethanol-positive group, FAEEs were degraded at 37 and 25°C but were generated at 4 and −20°C. EtS is a reliable biomarker of ethanol consumption, and EtG could be used as a biomarker at low temperatures (4 and −20°C), but PEths and FAEEs are not appropriate biomarkers of ethanol consumption.

Introduction

In forensic cases, identifying the source of ethanol in postmortem blood remains a problem. The origin of the ethanol, whether it is from antemortem intake or postmortem production by microorganisms (1, 2), directly impacts liability determinations.

Three classes of biomarkers—ethanol-related toxic effect markers (3), indirect markers (4) and direct markers—have been used to attempt to solve this problem. Ethyl glucuronide (EtG), ethyl sulfate (EtS), phosphatidylethanols (PEths) and fatty acid ethyl esters (FAEEs), which are all non-oxidative metabolites of ethanol, belong

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to the group of direct markers and have been regarded as ideal biomarkers for ethanol intake (5). Compared with ethanol-related toxic effect markers and indirect markers, their high specificity and sensitivity *in vivo* were preliminarily demonstrated by the fact that they were not affected by other biochemical processes (6, 7). Nevertheless, the main concern regarding their use in determining the source of ethanol in postmortem blood is the uncertainty of their stability in specimens.

Recent studies on the stability of these metabolites in postmortem blood have focused on only one or two substances. Among these four metabolites, EtG was most commonly studied because it is most widely used (8–10), and its stability was frequently compared with that of EtS, which appears to have properties similar to EtG (11–13). Comparisons between EtG and PEths have been carried out in hair and blood (14), while the stability of PEths and FAEEs has not been directly compared (15–17). Systematic comparison of the stability of these four ethanol metabolites simultaneously could more comprehensively characterize their stability and allow us to define the applicability of each metabolite in postmortem analyses. To our knowledge, no such study has been performed thus far.

In addition, in previous studies, positive samples containing ethanol metabolites were not obtained from alcohol consumers, as samples spiked with standards were used instead (15, 18, 19), which may not reflect reality. Moreover, few studies have taken into account one crucial scenario, in which ethanol was artificially added after death. Schloegl *et al.* (9) tested ethanol-spiked samples, but this study only focused on EtG, and other metabolites and temperatures were not examined.

The factor of temperature has been systemically studied only in analyses of PEths (15, 20). However, the effects of temperature on the other three types of ethanol metabolites have not been studied extensively thus far.

Therefore, the main purpose of the present study was to simultaneously investigate the stability of these four types of ethanol metabolites in human blood after postmortem incubation at different temperatures.

Material and Methods

Samples

Postmortem blood samples without preservatives or anticoagulant agents were provided by the Department of Forensic Medicine, Fudan University, and were collected from the cardiac chambers of individuals who died in traffic accidents. Blood alcohol concentration (BAC) was quantified by headspace gas chromatography (21).

Six ethanol-negative blood samples from different sources were selected, in which neither ethanol nor ethanol metabolites were found in a preliminary experiment.

Six authentic ethanol-positive blood samples from different sources with BACs higher than 0.80 mg/mL were selected, since the defined BAC limit for the criminal offense of drunk driving is 0.80 mg/mL according to the law of the People's Republic of China. The official unit for BAC in China is mg/mL. The metabolite concentrations in these samples were determined in a preliminary experiment to ensure that all metabolite levels were high enough to be measured and to observe degradation.

Experimental groups

Three groups were established as follows:

- The blank group consisted of six ethanol-negative blood samples.
- The ethanol-spiked group consisted of six samples from the same source as the blank group that were spiked with ethanol at a final concentration of 1.6 mg/mL. This level was two times higher than the BAC limit for drunk driving and close to the mean BAC (1.51 mg/mL) of alcohol-positive drivers who died in traffic crashes in Shanghai, China, according to our previous study (21). This BAC was supposed to be high enough to generate metabolites.
- The ethanol-positive group consisted of six authentic ethanol-positive blood samples. Information regarding these samples is shown in Table I.

Each sample was divided into four aliquots of 1 mL each that were placed in 2 mL Eppendorf tubes. The tubes were sealed tightly with parafilm and placed at 37, 25, 4 and −20°C. Every 24 h for 7 days, 50 μL of blood was sampled from each tube and stored at −80°C before analysis.

Determination of ethanol metabolites in blood samples

There are several different forms of both PEths and FAEEs. In this study, the targeted PEths were PEth 16:0/18:1 and PEth 18:1/18:1. The targeted FAEEs were ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl stearate (E18:0), ethyl oleate (E18:1), ethyl linoleate (E18:2) and ethyl arachidonate (E20:4). These species were chosen based on their proportions in humans (22, 23) and their commercial availability. In the following experiments, the cumulative concentrations of the two types of PEths were used, as were those of the six types of FAEEs.

EtG, EtS, PEths and FAEEs in postmortem blood were measured by a liquid chromatography–mass spectrometry (LC–MS-MS)-based method developed in our laboratory (24). First, 50 μL of human whole blood was pretreated with 200 μL of organic solvents (acetonitrile:acetone, 1:4), and after centrifugation, 5 μL of the supernatant was injected into the LC–MS-MS system (Ultimate 3000 UHPLC coupled with a TSQ Quantiva mass spectrometer, Thermo Fisher Scientific, Waltham, MA, USA). The analytes were separated at 47°C on a ThermoHypersil Gold C18 column (2.1 mm × 100 mm, 1.9 μm) with gradient elution and detected by a triple

Table I. Characteristics of samples from deceased individuals with positive BACs

No.	BAC (mg/mL)	EtG (ng/mL)	EtS (ng/mL)	Total PEths (ng/mL)	Total FAEEs (ng/mL)
1	0.86	1,047.6	1,240.5	1,363.8	464.3
2	1.02	395.5	293.3	5,694.3	762.3
3	1.48	3,497.0	1,414.3	3,378.3	2,145.5
4	1.66	4,105.5	1,037.0	2,890.5	1,704.6
5	2.62	465.5	343.3	1,185.7	116,189.0
6	3.00	7,312.6	1,537.4	5,980.7	1,797.1

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quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. The LODs of these metabolites ranged from 0.1 to 10 ng/mL. The LOQ was 20 ng/mL for EtG, 0.5 ng/mL for EtS, 2–5 ng/mL for FAEEs (with the exception of 50 ng/mL for E14:0) and 2 ng/mL for PEths (24).

Definition of stability

Stability was defined as the concentration fluctuation within 15% for each analyte in blood, according to the guidance of Bioanalytical Method Validation provided by the US Food & Drug Administration (25).

Results

The two types of PEths exhibited the consistent trends in their variations, and the six FAEE forms also showed consistent trends in individual samples. However, the proportion of each FAEE differed from person to person. For ease of presentation and because we were limited by the number of graphs we could show, the results of the combined subtypes were presented.

Blank group

No newly formed EtG, EtS or PEths were found in any of the blank samples at any temperature studied.

FAEE formation was observed in five of six samples at 37°C, with the peak concentrations ranging from 213.9 to 485.4 ng/mL. FAEEs were formed at 25°C in all six samples, and the peak concentrations ranged from 33.4 to 556.4 ng/mL. At 4°C, FAEEs were detected in one sample, at a peak concentration of 86.2 ng/mL; FAEEs were found in the same sample at a peak concentration of 78.3 ng/mL at −20°C.

Ethanol-spiked group

Ethyl glucuronide

EtG was present in two of six samples spiked with ethanol at 37°C after 3 days, peaking at 91.0 and 81.4 ng/mL, respectively. No EtG was detected at other temperatures.

Ethyl sulfate

No EtS was found in any of the ethanol-spiked samples at any of the temperatures tested.

Phosphatidylethanols

Formation of PEths was observed in three samples at 37°C, two samples at 25°C, one sample at 4°C and three samples at −20°C. The PEth concentrations ranged from 3.7 to 97.2 ng/mL these samples.

Fatty acid ethyl esters

As shown in Figure 1, the FAEE concentration in the ethanol-spiked group increased substantially, and the levels were correlated with the temperature. The higher the temperature, the more FAEEs were formed. At 37°C, the FAEE levels in all samples increased rapidly from 0 to more than 350 ng/mL within the first day, and subsequently, concentrations of higher than 1 μg/mL were reached. The FAEE levels in two samples peaked at more than 10 μg/mL. Eventually, the FAEE levels began to decrease, but no clear pattern was observed.

At 25°C, FAEE concentrations of more than 500 ng/mL could be found in most samples, and a concentration of more than 10 μg/mL was observed in one sample from the second to the fourth day. At 4°C, formation of FAEEs at concentrations above 200 ng/mL was seen in five of six samples during the experiment. Even at −20°C, FAEE concentrations over 1 μg/mL were detected in two samples.

Notably, FAEE concentrations peaked earlier when the temperature was higher. Peaks at 4 or −20°C were not found.

Ethanol-positive group

To establish and compare more clearly the variations in each metabolite at each temperature, the ratio of the level of the metabolite in each sample on a particular day (C_x, x = 1–7) relative to its initial level on Day 0 (C_0) was calculated, and the ratio at Day 0 was set as 100%, as shown in Figure 2. Each point is the average value of six samples.

Ethyl glucuronide

Figure 2a shows the pattern of EtG stability at all four temperatures. At 37°C, EtG degraded rapidly by ~10% per day, and there was only 26.5% remaining on the seventh day. At 25°C, it degraded at lower rate, with 75.2% remaining on the seventh day. On the other hand, the level of EtG remained nearly constant at 4 and −20°C, with 96.4 and 103.3% of the original amount observed on Day 7, respectively, and standard deviations (SDs) of 5.6 and 5.0%, respectively. These results indicated that the stability of EtG at low temperatures (4 and −20°C) was relatively good.

Ethyl sulfate

Figure 2b clearly shows that EtS was stable at each temperature tested. The average values in samples after 7 days at 37, 25, 4 and −20°C were 102.7, 100.1, 97.5 and 100.2%, respectively, and the corresponding SDs were 4.6, 2.4, 2.7 and 3.0%, respectively, indicating that EtS could be a reliable biomarker of alcohol consumption.

Phosphatidylethanols

As shown in Figure 2c, the levels of PEths at 37°C increased over the first 4 days and then decreased; the average value on Day 7 was 116.4%, with an SD of 14.7%. At 25°C, PEths were degraded, reaching 58.9% on the seventh day. However, in contrast to the trends observed at 37 and 25°C, the PEth levels were relatively constant at 4°C, with an average values at 7 days of 104.7% and an SD of 5.0%. Slight formation of PEths was observed at −20°C, with an average value of ~116.9% and an SD of 9.7%.

Fatty acid ethyl esters

As shown in Figure 2d, in samples placed at relatively high temperatures (37 and 25°C), spontaneous formation of FAEEs was observed, and the levels began to decrease after 5 days in samples placed at 37°C. At 4°C, the mean value at 7 days was 73.9%, with an SD of 16.1%, indicating the instability of these metabolites. At −20°C, FAEEs appeared to be stable: the mean value was 94.7%, with an SD of 4.0%.

Discussion

Blank group

The blank samples in our experiment showed that no EtG, EtS or PEths were formed if there was no ethanol in postmortem blood,

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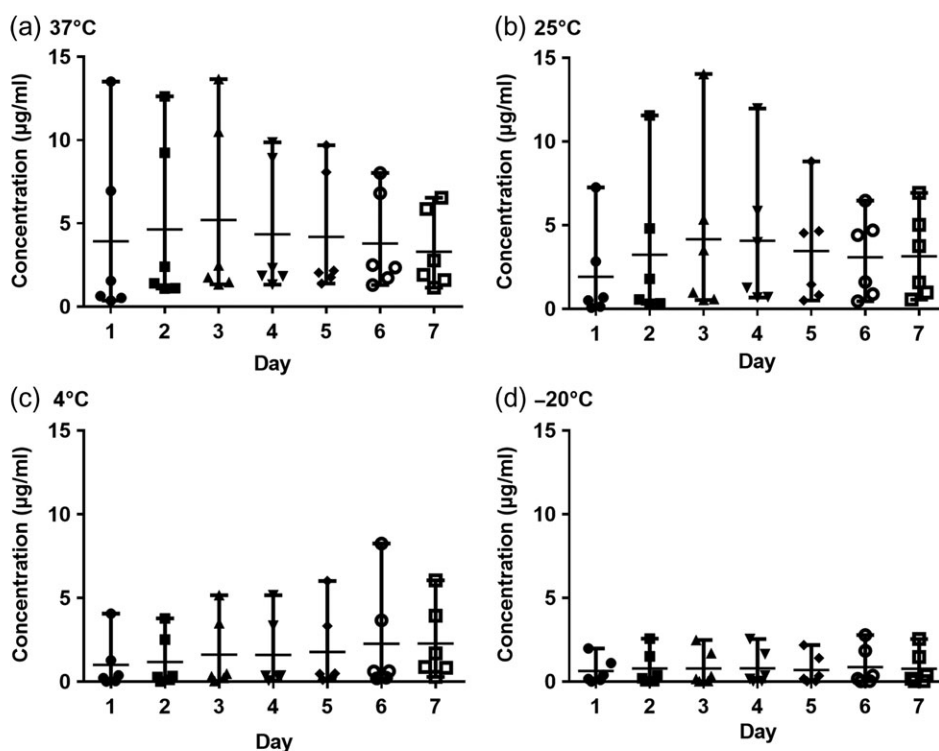

Figure 1. Concentrations of FAEEs in samples from the ethanol-spiked group incubated at four temperatures: (a) 37, (b) 25, (c) 4 and (d) −20°C. One symbol represents the total concentrations of six forms of FAEEs. Six samples were included in each temperature group and were monitored for a total of 7 days.

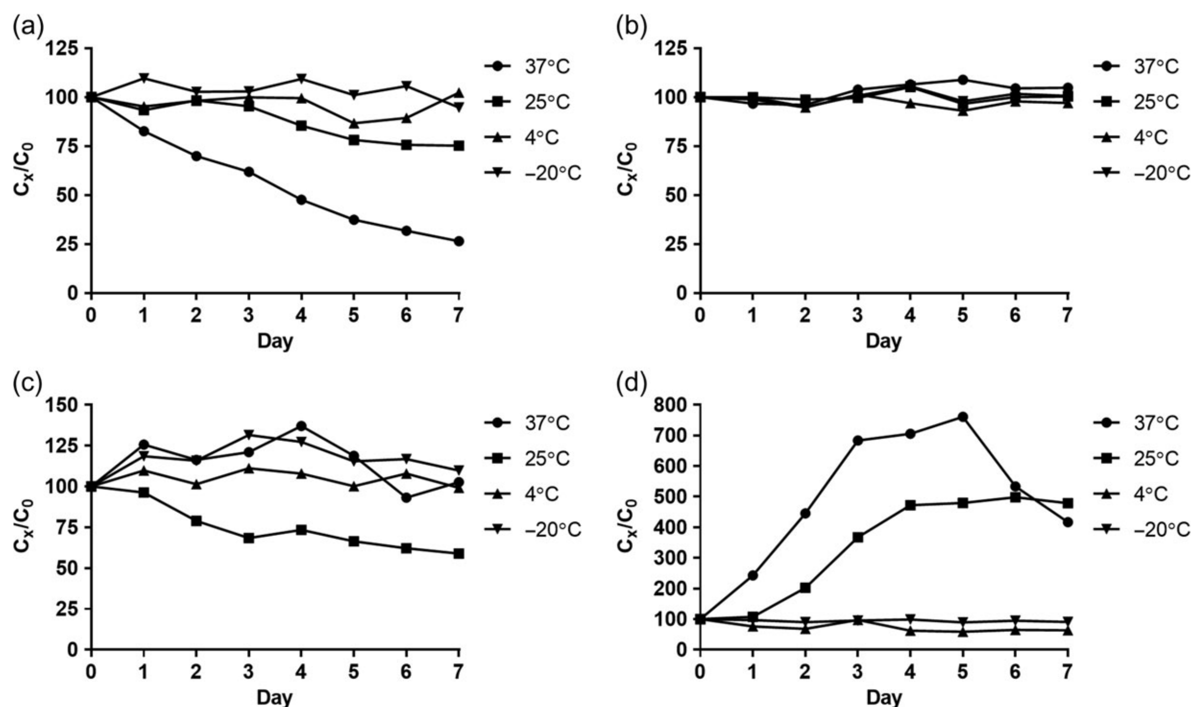

Figure 2. Stability of (a) EtG, (b) EtS, (c) PEths and (d) FAEEs in the ethanol-positive group at four temperatures. The ratio of the metabolite in each sample on a particular day (C_x, x = 1–7) relative to its initial level on Day 0 (C_0) was calculated, and the ratio on Day 0 was set as 100%. Each point is the average ratio of six samples. PEths represent the total concentration of two forms of PEth; FAEEs represent the total concentration of six forms of FAEE.

indicating that the risk of a false positive result was low under ethanol-free conditions when using EtG, EtS and PEths as biomarkers for ethanol intake. The results of Schloegl *et al.* and Aradóttir *et al.* also demonstrated the reliability of EtG and PEths in ethanol-free samples (9, 20, 26). However, FAEEs were formed, and the levels were correlated with the temperature. In blank samples,

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FAEEs reached concentrations as high as those seen in samples selected from corpses with confirmed antemortem ethanol intake tested in this study, as well as those observed in a study of alcohol consumption by volunteers (27). Hence, we advise against using positive findings of FAEEs in postmortem blood as a biomarker for antemortem ethanol intake confirmation. Previous studies have indicated that the production of FAEEs occurs only in the presence of ethanol, but they did not explain why this is the case. Based on the literature, we are unfortunately not able to explain why FAEEs were formed in blank blood samples in our study, whereas PEths were not.

However, it was noticed that, the concentrations of FAEEs formed in the blank group were found to be at least ten times lower than that in the ethanol-spiked group. Meanwhile, in the presence of high level of ethanol, the concentrations of PEths formed were far lower than that of FAEEs in the ethanol-spiked group. Therefore, it was estimated that the reason why no PEths were detected in the blank group, might be due to the fact that their levels were under the limit of detection.

Ethanol-spiked group

The ethanol-spiked group was used to investigate whether a false positive finding of ethanol metabolites could be obtained in cases in which ethanol was added by an individual after death, or those in which ethanol in the sample was derived from microorganism fermentation.

EtG was generated in samples in this group at 37°C. The level (<100 ng/mL) of EtG formed was far lower than that observed in the ethanol-positive group on Day 0, but it was still as high as the level of EtG observed after several days of degradation in the ethanol-positive group. At lower temperatures, no EtG was detected, which was consistent with previous studies. Schloegl *et al.* (9) did not find EtG in ethanol-spiked samples stored at room temperature (22–27°C) and 4°C. By combining our results with those of Schloegl *et al.*, the conclusion could be drawn that forensic practitioners should not use EtG to determine the source of ethanol in postmortem blood if the corpse was found at high temperature (such as 37°C) or when only small amount is detected, especially without any other supporting evidence.

EtS was not detected at any temperature in the ethanol-spiked group, and together with the findings in the blank group, these data suggest that using EtS as a biomarker would not lead to false positive result. The use of EtS is especially practical in situations in which postmortem ethanol administration or postmortem microorganismal production of ethanol may be a concern.

Our results showed that PEths could be generated in the ethanol-spiked group. Thus, the detection of a small amount (<100 ng/mL according to this study) of PEths could not be used to differentiate the source of ethanol intake. Among the four observed temperatures, the sample at −20°C produced the highest level of PEths, the concentration of which was 97.2 ng/mL. One study in which researchers asked healthy volunteers to drink 1 g/kg and then measured the level of PEth 16:0/18:1 reported that the concentration of PEth 16:0/18:1 could reach more than 50 ng/mL within 1 h (28). Thus, the use of PEths is not recommended for distinguishing the source of ethanol.

Compared with the blank group, FAEEs in the spiked group significantly increased in the presence of ethanol at 25 and 37°C and later decreased. The highest concentrations observed reached or even exceeded those seen in the ethanol-positive group. Though most FAEEs are synthesized by enzyme catalysis, spontaneous formation by the conjugation of ethanol to endogenous free fatty acids can also occur (29). It is speculated that the mechanism might involve the integrated effects of esterification reactions and bacterial decomposition, but this hypothesis requires further investigation.

Ethanol-positive group

In the ethanol-positive group, EtG was not a stable ethanol biomarker at high temperatures, including 37 and 25°C, as a clear decreasing trend was observed from the beginning of the experiment, whereas it was stable at low temperatures (4 and −20°C). The instability of EtG at room temperature has been reported previously (9), especially when putrefaction occurred at 30/40°C (10). Some researchers have reported that bacterial β-glucuronidase could account for the degradation of EtG (30, 31).

The stability of EtS has been widely reported (12, 30, 32) and was confirmed in our experiment. As shown in Figure 2b, the lines indicating the concentration of EtS at each temperature are nearly overlapping, and each point is at ~100%. However, it is worth noting that a high concentration of bacteria could still cause EtS degradation in a standardized culture solution (33). Thus, a positive EtS finding could be used to infer antemortem ethanol intake.

No specific pattern was observed for PEths at 37°C. Some samples exhibited an increasing trends, while others showed decreases, and the concentration of PEths even fell zero within 3 days in one sample. Therefore, a fluctuating line is shown in Figure 2c. In our study, PEths were stable at 4°C, but their levels increased at −20°C, consistent with other research (20). The production of PEths at −20°C could be explained by phospholipase D activation (34, 35).

FAEE concentrations fluctuated significantly in the ethanol-positive group at 37 and 25°C. In one sample, the level of FAEEs produced was more than 23 times higher than the original concentration, while in another sample, FAEEs were degraded to one-third of the original concentration. The levels of FAEEs decreased at 4°C and remained unchanged at −20°C. The increased FAEE levels in some samples might be due to spontaneous formation by the conjugation of ethanol to endogenous free fatty acids, as described above.

Limitations

It should be mentioned that due to the limited sample volume (only ~5 mL of blood was available for each sample, which was collected from cardiac chambers of individuals who died in traffic accidents), a 7-day experiment was conducted, and samples for analysis were repeatedly taken from the same tube. This allowed repeated exposures of the sample to the external environment, which can potentially influence the original system of samples. Due to the experimental conditions and ethical issues, we were not able to carry out an *in vivo* experiment using human bodies. In addition, due to the complex internal environment of the human body, the results cannot be directly applied to *in vivo* situations.

Conclusion

This study systematically compared the stability of EtG, EtS, PEths and FAEEs in postmortem human blood stored at 37, 25, 4 and −20°C. EtS was the best biomarker among these four types of ethanol metabolites, because of its high stability in all groups at all temperatures tested. The use of EtG as a biomarker for inferring antemortem ethanol intake is recommended at low temperatures (4 and −20°C). Our PEth results indicated the possibility of obtaining a false negative

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result at high temperatures (37 and 25°C) and a false positive result at both high (37 and 25°C) and low (4 and −20°C) temperatures. Therefore, PEths are not an appropriate metabolite for distinguishing the source of ethanol in postmortem blood. The FAEE findings suggested that both false positive and false negative results were possible; thus, FAEEs should not be used to determine the source of ethanol in a corpse.

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