Technical note | 000625

Toxicology

Direct quantitation of phosphatidylethanol (PEth) in volume-controlled dried whole blood spots using the fully automated Transcend DSX-1 system

Authors

Jingshu Guo, Richard J. Gibson, I-Lin Wu, Stephanie Samra, Kerry Hassell, Tanis Correa, Edward Goucher

Thermo Fisher Scientific, San Jose, CA

Keywords

Transcend DSX-1 system, TSQ Altis mass spectrometer, TraceFinder software, Aria MX software, Hypersil GOLD column, dried blood spot analysis (DBS), phosphatidylethanol (PEth), TurboFlow technology, on-line SPE, 2D-LC/MS, alcohol-specific biomarkers

Application benefits

- A complete and fully automated workflow for dried blood spot analysis with a Thermo Scientific™ Transcend™ DSX-1 UHPLC system and integrated software for straightforward instrument control
- Flow-through desorption (FTD™) technology for direct analyte extraction without manual punching of dried spot discs
- Thermo Scientific™ TurboFlow™ technology provides 2-dimensional LC separation for fast matrix cleanup and analyte separation
- The volume-controlled blood sample collection implemented by HemaXis™ technology ensures accurate and reproducible analyte quantitation

Goal

Phosphatidylethanols (PEth) are mid-term, alcohol-specific biomarkers that have gained rapid interest in forensic testing and clinical research as indicators for alcohol consumption. We demonstrate a complete workflow from sample to results to accurately quantitate PEth in volume-controlled dried blood spots procured by HemaXis precise sampling technology with a Transcend DSX-1 system that combines direct analyte extraction, 2-dimensional sample preparation, and UHPLC separation.

Introduction

Phosphatidylethanols (PEths) are a species of phospholipids that have emerged as attractive biomarkers for monitoring alcohol consumption due to their high specificity and relatively long half-life. PEths are formed from a phospholipase D-catalyzed reaction of phosphatidylcholine in the presence of ethanol and are accumulated on the membrane of erythrocytes.[1] Unlike other ethanol markers, such as fatty acid ethyl esters, ethyl glucuronide, and ethyl sulfate, the half-life of PEth is approximately four days, with some reports of up to 12 days.[2] Thus, PEth can be used as a mid-term biomarker to monitor repeated ethanol exposure.

Among over 50 known homologs of PEth, the most abundant form is PEth 16:0/18:1 (Figure 1), accounting for 40% of total PEth, and may be constantly monitored in whole blood via liquid chromatography-tandem mass spectrometry (LC-MS/MS) for forensic applications.[3-5] Two concentrations of PEth 16:0/18:1 are often used to classify alcohol consumption behavior: 20 ng/mL for no to light alcohol consumption, and >200 ng/mL for substantial and heavy alcohol consumption.[4] It is important to note that PEth can degrade rapidly upon collection if the blood sample is not stored appropriately in cold storage conditions.[6] Hence, sample storage and transportation adds additional complexity and expense to the large-scale implementation of liquid blood for PEth analysis. In contrast, PEth is stable once prepared as dried blood spots (DBS) as the drying process stops any further enzymatic degradation of PEth post sample collection. DBS has advantages over liquid blood as it is minimally invasive to collect and requires less than 100 µL of blood volume per spot. While dried spot analysis has gained popularity over the years, the traditional workflow using manual disc-punching followed by a combination of sample preparation and extraction procedures can take over an hour to complete and is prone to human error.

This technical note develops an automated workflow to extract and quantitate PEth 16:0/18:1 from DBS samples using a Transcend DSX-1 system (Figure 2). This instrument configuration combines a dried matrix spot module (DMS) with innovative flow-through desorption (FTD™) technology and a Transcend DSX-1 UHPLC system capable of 2-dimensional sample preparation via TurboFlow technology. The workflow allows for the rapid sample preparation and quantification of PEth 16:0/18:1 in dried blood spots, with linearity from 20 to 2,000 ng/mL, meeting the cut-off needs of PEth monitoring. The accurate quantitation of PEth is facilitated by the volume-controlled DBS cards using HemaXis technology that ensure collection of precise sample volumes (Figure 3).

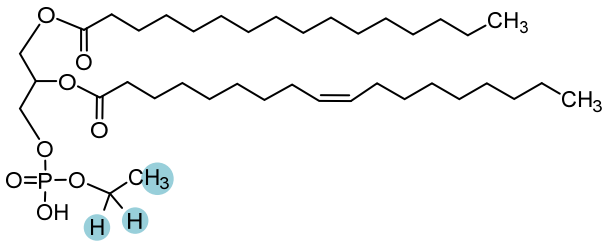

Figure 1. Structures of PEth 16:0/18:1 and its internal standard (IS) PEth 16:0/18:1-D_5 (heavy isotope highlighted in blue circles)

Figure 2. Thermo Scientific Transcend DSX-1 system and TSQ Altis triple quadrupole mass spectrometer (as pictured above)

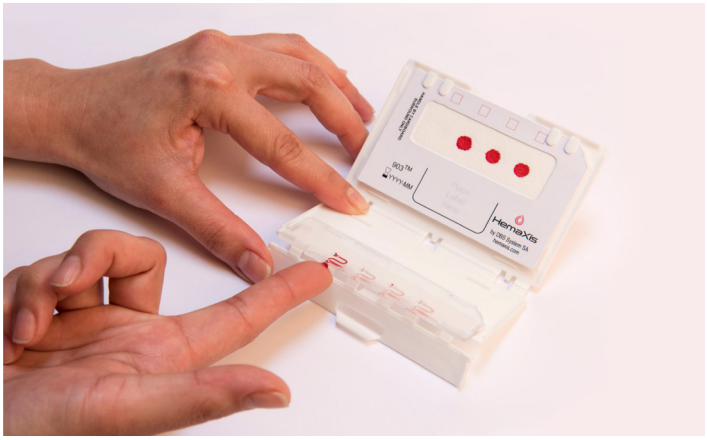

Figure 3. HemaXis DB10 blood collection device precisely collects 10 µL blood via capillary action (courtesy of DBS System SA).

Experimental

Sample preparation

PEth 16:0/18:1 and its internal standard (IS) PEth 16:0/18:1-D_5 were purchased from Cerilliant. All samples were prepared by DBS System SA. PEth-free whole blood was collected from volunteers in citrate tubes and checked for PEth before usage. For calibration samples, PEth 16:0/18:1 was spiked into PEth-free blood at final concentrations of 20, 50, 100, 250, 500, 1,000, and 2,000 ng/mL. All samples were agitated on a thermomixer at room temperature for 1 hr for homogenous mixing. Lyophilized QC samples were obtained from ACQ Science (Germany) and reconstituted to the final concentrations of 50 and 200 ng/mL according to the instructions. DBS samples were prepared using HemaXis DB10 (Switzerland) blood collection devices. A drop of approximately 50 µL of sample was deposited onto paraffin film to mimic a drop of blood at the patient's fingertip. Sampling was then performed following the HemaXis DB10 instructions, where 10 µL of blood was precisely loaded onto the DBS cards. The DBS samples were air-dried at room temperature and placed directly onto the sample cardholder in the DMS module or stored at room temperature in the dark until ready for analysis.

Fully automated sample extraction

The dried matrix spot module (DMS) is equipped with a 6 mm clamp for sample extraction. The internal standard was introduced on-line using the built-in IS pump in the DMS module that overfilled an IS loop to ensure robust and reproducible IS addition. PEth extraction was performed using 10 mM ammonium formate, 0.05% formic acid in water/acetonitrile, 3/7 (v/v) for 1 min with HotCap™ enabled at 100 °C, followed by 20 s of drying using an internal air compressor to flush residual mobile phase. Every sample spot was photographed with the Intelligent Vision Camera (IVC™) prior to and after each analytical run to record the DBS card sample information, check the absence or presence of a spot, and verify the occurrence of extraction. After the desorption process, the clamp head was rinsed with rapid aqueous/organic washing steps using 0.1% formic acid in water (v/v) (wash 1), 30% acetonitrile/30% isopropanol/40% acetone (v/v/v) (wash 2), and isopropanol (wash 3).

Online sample preparation

Sample cleanup and chromatographic separation were performed on the Transcend DSX-1 UHPLC system equipped with TurboFlow technology and configured in "Focus mode." The analysis process and flow path are shown in Figure 3. After loading the extracted samples onto the TurboFlow column (Figure 4A), the analytes were eluted using the high organic eluant stored in the "transfer loop" and refocused on the analytical column (Figure 4B). During the analyte separation on the analytical column, the TurboFlow column was washed (Figure 4C). To prepare for the subsequent analysis, the TurboFlow transfer loop was filled with eluant while the analytical column was washed and equilibrated (Figure 4D). The gradient, mobile phases, and columns used in this technical note are described in Table 1.

Mass spectrometry

PEth quantification was performed using a Thermo Scientific™ TSQ Altis™ mass spectrometer equipped with a Thermo Scientific™ OptaMax™ NG ion source with a heated electrospray ionization probe in the negative mode. The capillary voltage was set at -3,500 V, and other MS parameters are listed in Table 2. Selected reaction monitoring (SRM) transitions of compounds, optimized collision energy, and RF Lens settings are shown in Table 3.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software.

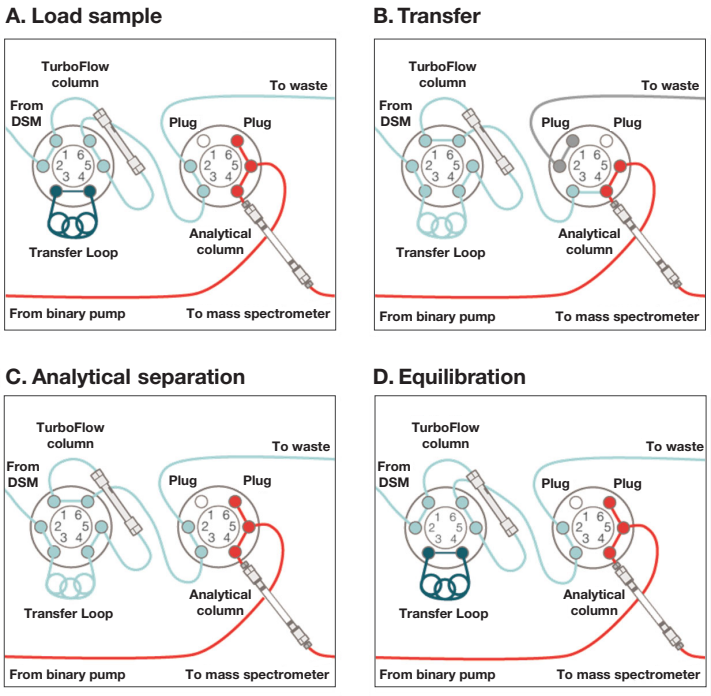

Figure 4. TurboFlow technology for sample cleanup (Focus mode)

Table 1. Liquid chromatography conditions

TurboFlow column								Analytical column		
Time (min)	Flow rate (mL/min)	%A	%B	%C	%D	Tee	Loop	Flow rate (mL/min)	%A	%B
0	0.2	100	-	-	-	====	out	0.5	100	-
0.2	0.0	100	-	-	-	====	out	0.5	100	-
0.3	0.2	100	-	-	-	====	out	0.5	100	-
0.5	0.6	100	-	-	-	====	out	0.5	100	-
1.1	0.1	100	-	-	-	T	in	0.4	100	-
2.1	4	100	-	-	-	====	in	0.5	36	64
2.2	4	-	100	-	-	====	in	0.5	24	76
2.95	3	-	-	100	-	====	in	0.5	12	88
3.7	1	-	-	-	100	====	in	0.5	-	100
4.45	2.5	100	-	-	-	====	in	0.5	-	100
4.7	4	-	100	-	-	====	in	1.0	100	-
4.95	3	-	-	100	-	====	in	1.0	-	100
6.45	2	100	-	-	-	====	out	0.5	-	100
6.7	2	100	-	-	-	====	out	0.5	100	-
Mobile phases	A: 10 mM ammonium formate, 0.05% formic acid in water/acetonitrile, 3/7 (v/v) B: 10 mM ammonium formate, 0.05% formic acid in methanol C: acetonitrile/isopropanol/acetone, 2/2/1 (v/v/v) D: isopropanol							A: 10 mM ammonium formate, 0.05% formic acid in water/acetonitrile, 3/7 (v/v) B: acetonitrile/isopropanol, 1/1 (v/v)		
Columns	TurboFlow XL C8 column, 50 × 0.5 mm at room temperature							Hypersil GOLD C8, 50 × 2.1 mm, 5 µm at 23 °C		

Table 2. Mass spectrometer source settings

Polarity	Negative	Cycle time (s)	0.4
Sheath gas (Arb)	50	Q1 Resolution (FWHM)	0.7
Aux gas (Arb)	10	Q3 Resolution (FWHM)	1.2
Sweep gas (Arb)	0	Source fragmentation	0
Ion transfer tube temperature (°C)	325	Chromatographic peak width (s)	6
Vaporizer temperature (°C)	350	CID gas (mTorr)	1.5

Table 3. Optimized SRM transitions, collision energies (CEs), and RF Lens for PEth and its IS

	Precursor (*m/z*)	Product (*m/z*)	CEs (V)	RF Lens (V)
PEth 16:0/18:1	701.5	255.3[a]	33.5	110
	701.5	281.3[b]	31.2	110
PEth 16:0/18:1-D_5	706.5	255.3[a]	33.5	110
	706.5	281.3[b]	31.2	110

[a] Qualifier
[b] Quantifier

Results and discussion

The most abundant PEth homolog, PEth 16:0/18:1, was quantified from DBS cards. Ten-microliter volumes of spiked blood were precisely spotted onto DBS cards using HemaXis technology. The IS was introduced on-line via an IS pump in the DMS module to ensure the accurate IS addition. The DBS samples were analyzed directly without any additional sample treatment in a total run-time of 8 minutes, starting from the analyte extraction to the MS detection. The Thermo Scientific™ Aria™ MX integrated software package was used to control all steps of analyte extraction and separation.

PEths have very high hydrophobicity and similar structures, which make them challenging to separate chromatographically while maintaining a minimum carryover. The analyte separation was achieved on a Thermo Scientific™ Hypersil GOLD™ C8 column, which was selected over a C18 column to mitigate carryover.

In addition, rapid aqueous/organic switching steps were also used to wash the clamp, TurboFlow column, and analytical column to minimize analyte carryover to below 5% at LOQ.

Linear calibration curves for PEth were generated using a weighting factor of 1/× from a lower limit of quantification (LLOQ) of 20 ng/mL to an upper limit of quantification (ULOQ) of 2,000 ng/mL. The R^2 value was > 0.99; the %Diff and %RSD were < 15% (Figure 5).

Accuracy and precision data of QC samples at two levels were compiled over two days, with % accuracy within 100 ± 10% and %RSD below 10% (Table 4). The results were comparable with those from the manual disc-punch method of the same QC samples performed by our collaborators at DBS System SA, Switzerland (Table 3). Representative chromatograms of PEth quantitation in PEth-free blood and the low and high QC samples are shown in Figure 6.

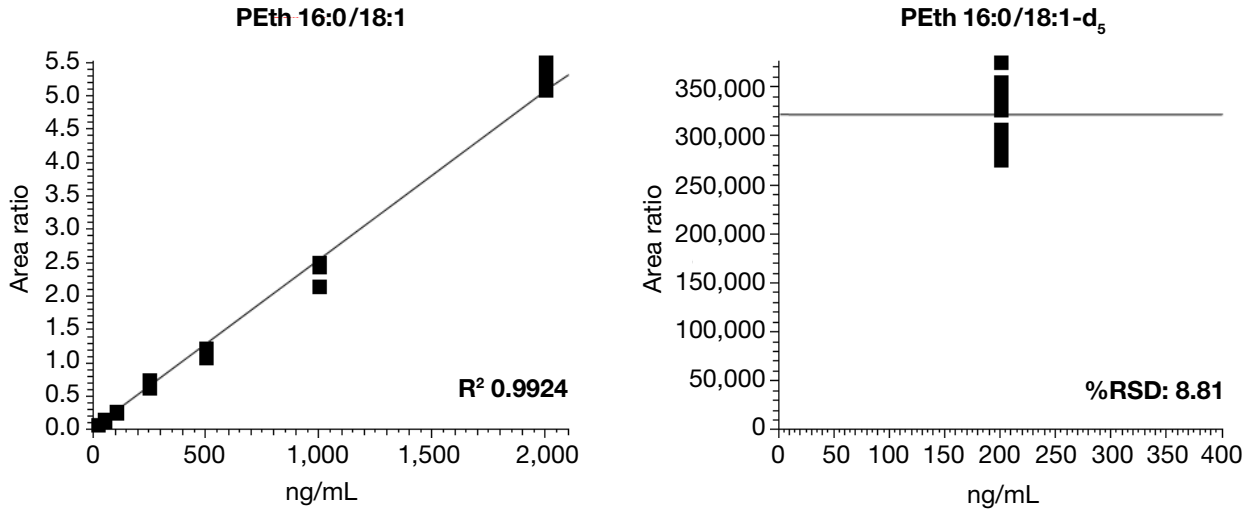

Figure 5. The calibration curve of PEth 16:0/18:1 and the %RSD of IS from day 1

Table 4. Precision and accuracy results in QC samples

Target (ng/mL)	Transcend DSX-1 system							Disc-punch and manual extraction (DBS System SA)
	Rep. (N = 3)	Within-day			Between-day			
		Mean	%Acc.	%RSD	Mean	%Acc.	%RSD	
50	Day 1	48.0	96.0	1.2				47 (%RSD 5.7, N = 19)
	Day 2	48.9	97.8	3.5	48.4	96.8	1.3	
200	Day 1	208.0	104.0	7.4				210 (%RSD 4.2, N = 20)
	Day 2	214.0	107.0	0.5	211.0	105.5	2.0	

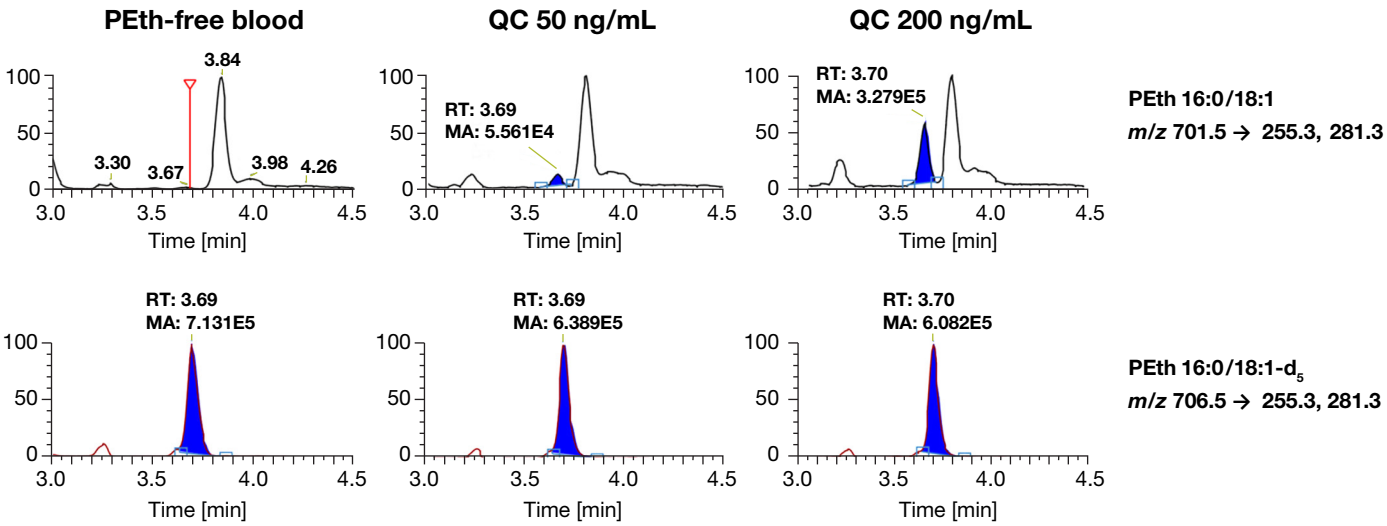

Figure 6. Representative EIC of PEth and IS at RT 3.70 min in PEth-free blood and the two QC samples. The interfering peak at RT 3.84 was separated from PEth 16:0/18:1 on the analytical column.

Conclusion

The analysis of the alcohol-specific biomarker PEth has been frequently used to distinguish heavy, casual, and abstaining alcohol users in recent years. The Transcend DSX-1 system combines a DMS module and the TurboFlow 2-dimensional LC separation to provide a complete workflow for fast and robust quantification of PEth in dried blood spots. The association of a volume-controlled HemaXis blood collection device together with the current Transcend DSX-1 system method provides accurate and efficient quantification of the alcohol-specific biomarker PEth in blood.

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