Poster Reprint

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Message in a Bottle: Analysis of Phosphatidylethanols in Whole Blood by Novel Solid Phase Extraction and LC/TQ

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Introduction

Phosphatidylethanols (PEth) are phospholipids containing a phosphoethanol linked to two fatty acids. They form non-oxidatively in the presence of ethanol and are used as specific biomarkers of recent alcohol consumption or as an indicator of alcohol abstinence. PEth has an average half-life in circulation around 4 days and can be detected weeks after imbibing. There are more than 40 known forms of PEth, but this method focused on two of the most predominant forms found in blood, POPEth (16:0-18:1) and PLPEth (16:0-18:2) (structures in Figure 1).

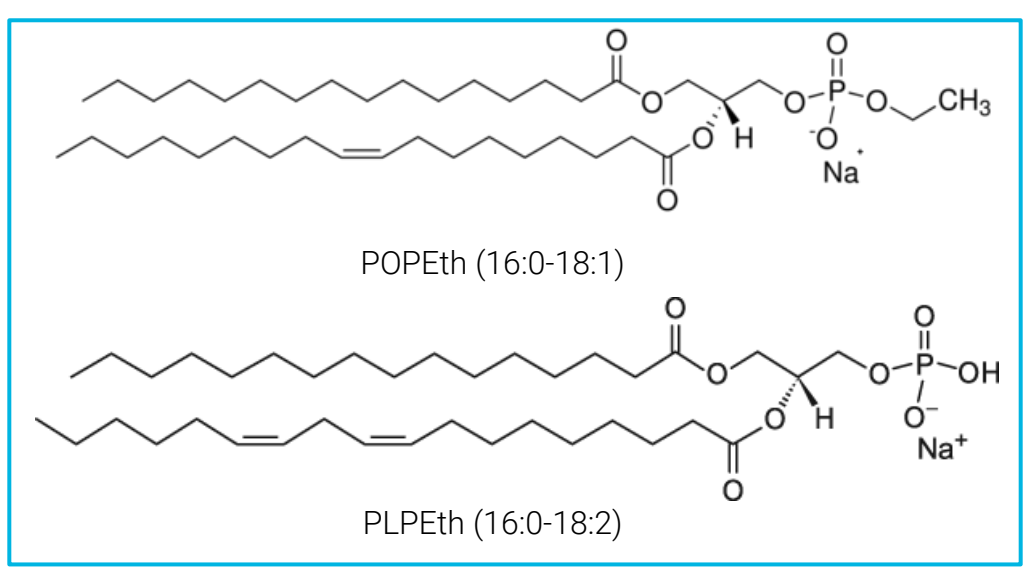

Figure 1. Chemical structures of PEth.

While the analysis of PEth in whole blood is well-established, the matrix can cause significant suppression. Many analyses utilize a simple extraction that, while straightforward, can result in samples with high background and potential interferences. A unique solid phase extraction technique was implemented in this study to reduce sample background and suppression without significantly increasing workflow complexity. Bond Elut Lipid Extraction uniquely combines size exclusion and hydrophobic interactions to selectively bind with fatty acid chains, allowing the lipids of interest to be selectively removed from other matrix components, resulting in cleaner samples.

Agilent Ultivo LC/TQ and 6470 LC/TQ

Experimental

Sample Prep

Whole blood was spiked with drug standards made from a working stock solution. Each sample was combined with an internal standard solution, vortexed, and centrifuged to precipitate out proteins. The sample was loaded onto the SPE cartridge, then rinsed before elution with a DCM:MeOH solvent mixture (Figure 2). The eluate was dried under nitrogen at 40°C for 30 minutes before reconstitution in mobile phase starting conditions. Samples were injected onto an Agilent Poroshell 120 Phenyl-Hexyl column and analyzed in negative mode via LC-MS/MS.

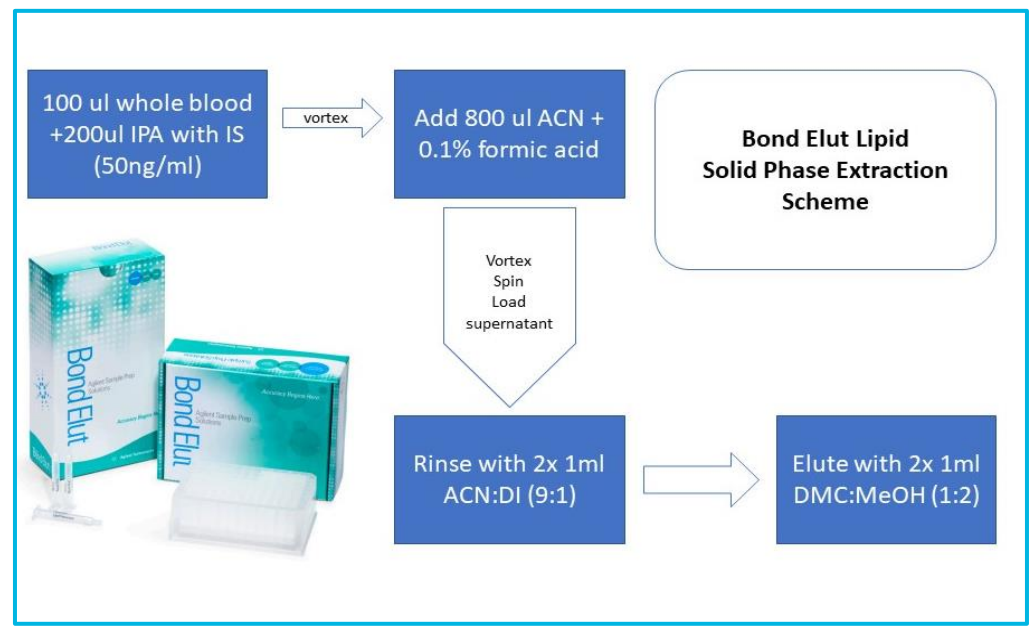

Figure 2. Process schematic for Bond Elut Lipid Extraction.

LC-MS/MS Analytical Method

The LC-MS/MS system consisted of a 1290 binary pump, a thermostatted autosampler, a temperature-controlled column compartment, and either an Ultivo or 6470 triple quadrupole mass spectrometer. Separation conditions are given in Table 1.

Detection of all analytes was undertaken in multiple reaction monitoring (MRM) mode, and the phospholipid transitions were monitored for background levels. MS source conditions for the mass spectrometer are shown in Table 2. The total injection cycle time was approximately 4.5 minutes sample to sample. Data was acquired and analyzed using MassHunter software suite version B.10 for data collection from the 6470 or version 1.2 for data collection from the Ultivo.

Experimental

Analytical Column	Agilent Poroshell 120 Phenyl-Hexyl, 2.1x50mm, 2.7 µm	
Injection Volume	5 µL	
Mobile Phase A	Water + 5 mM ammonium formate	
Mobile Phase B	80:20 Methanol:Isopropanol	
Needle Wash	50:20:20:10 Isopropanol:Methanol:Acetonitrile:Water	
Autosampler Temp	4 °C	
Column Temp	55 °C	
Flow Rate	0.35 mL/min	
Gradient	Time 0.00 2.50 2.51 3.00 3.01	%B 80 90 100 100 80
Stop Time	4 min	
Post Time	Off	

Table 1. LC parameters.

Gas Temp	250 °C
Gas Flow	8 L/min
Nebulizer Pressure	20 psi
Sheath Gas Temp	400 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	3000 V
Nozzle Voltage	1750 V

Table 2. Agilent JetStream ESI source parameters.

Results and Discussion

Chromatography

The two PEth forms monitored were chromatographically resolved from each other and from background phospholipids in 4 minutes (Figure 3).

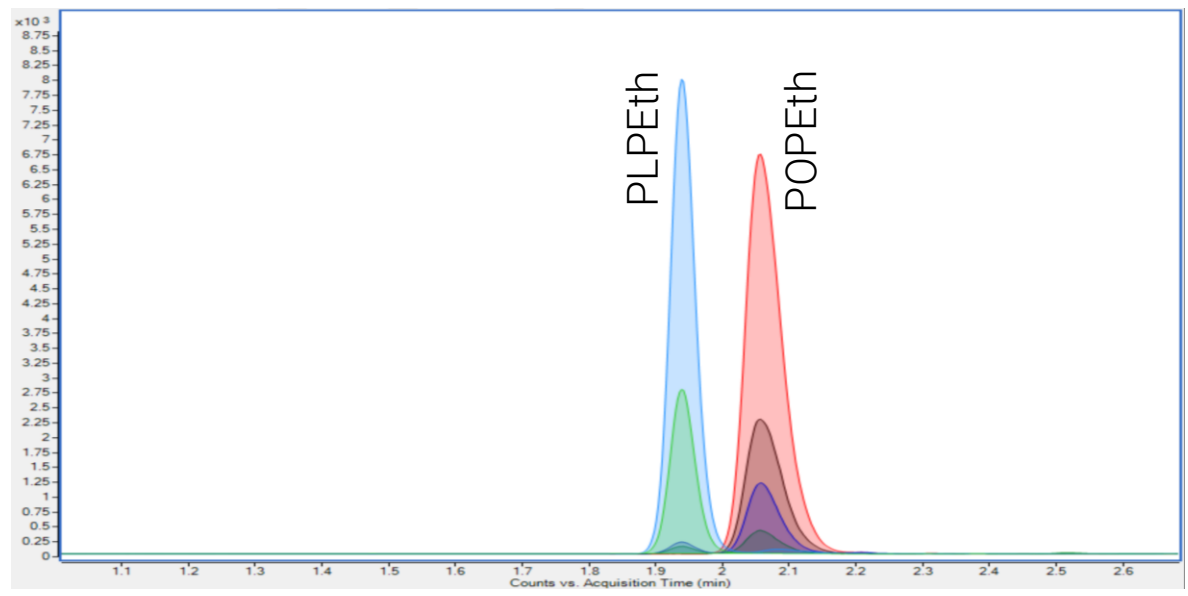

Figure 3. Example overlaid MRM chromatograms showing separation of PEth.

Calibration

The calibration concentrations ranged from 1 ng/mL to 2500 ng/mL, with the linear range spanning 10 ng/mL to 2500 ng/mL. R^2 values were greater than 0.98 over the linear response range (Figure 4). Both calibration curves utilized a 1/x weighting factor.

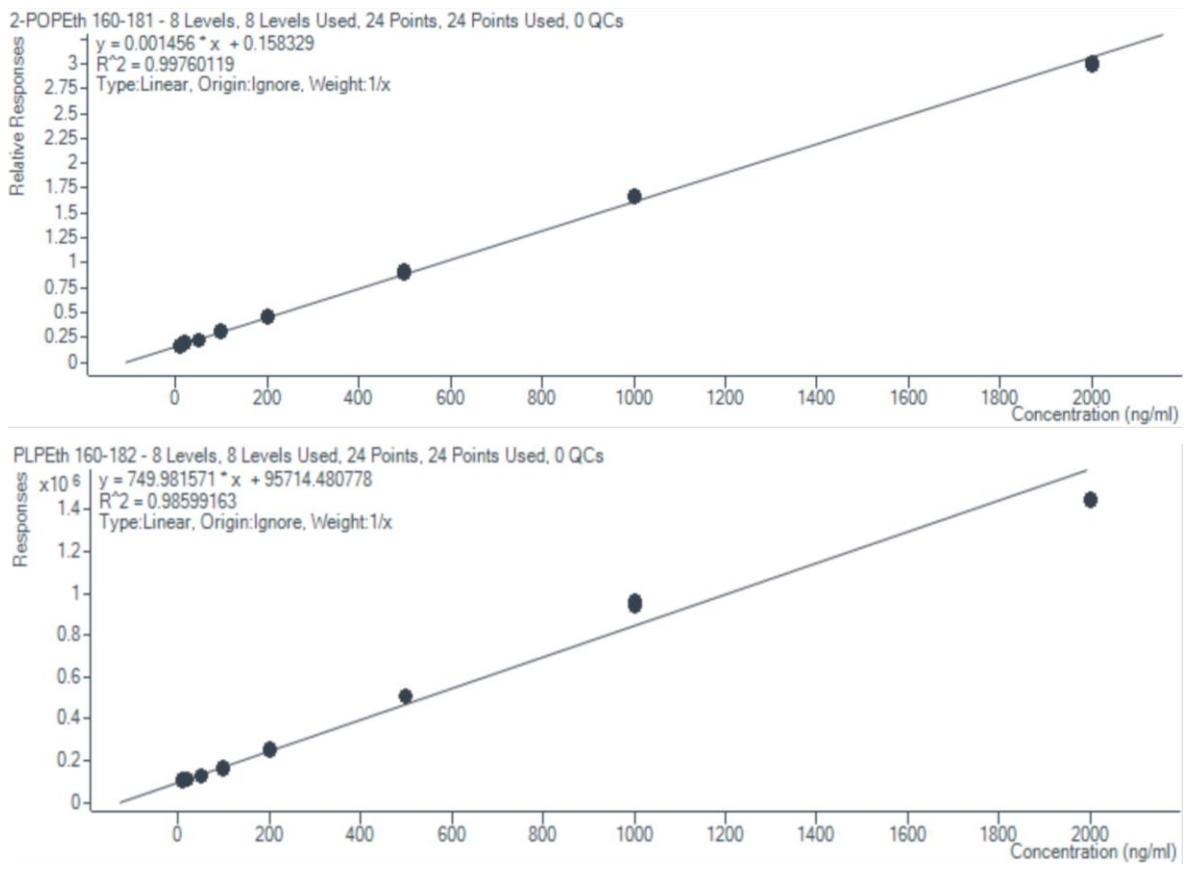

Figure 4. Calibration curve for POPEth (top) and PLPEth (bottom).

Results and Discussion

During the method development stage of this experiment, it was found that using the supernatant instead of the whole extract (pellet and supernatant) gave a cleaner baseline with comparable recoveries, and that the SPE cleanup minimized interfering background phospholipids better than a simple IPA extraction (Figure 5). The inset highlights the chromatographic region of interest where the PEth compounds elute, with the black peak coeluting over, and potentially suppressing, one of the analytes of interest.

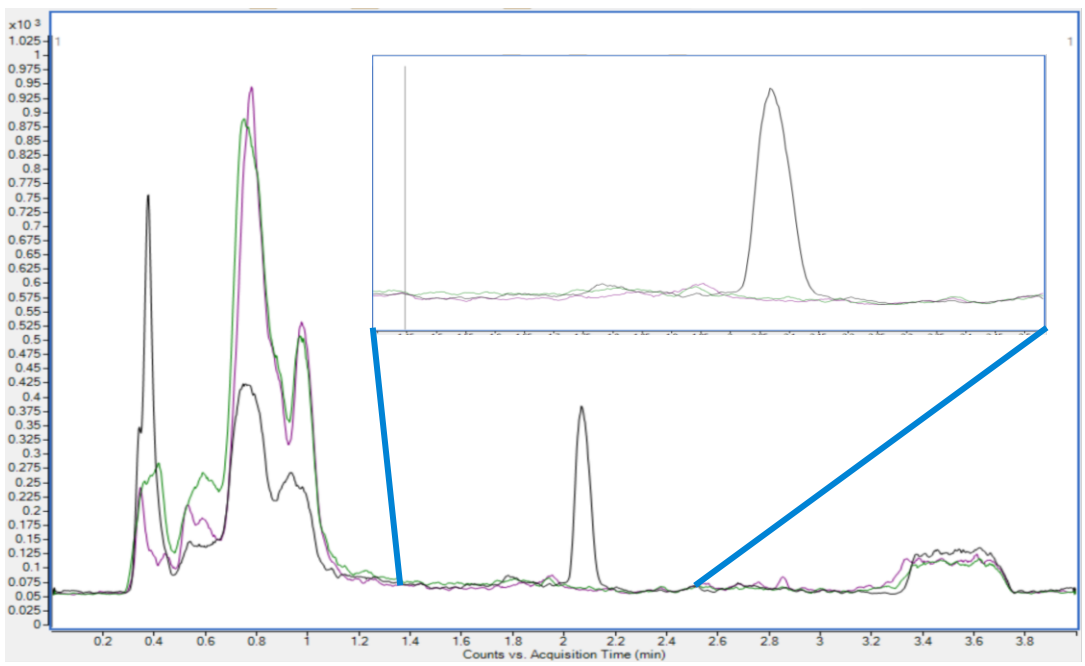

Figure 5. Overlaid chromatograms showing background of a simple IPA extraction (black), Bond Elut pellet (purple), and Bond Elut supernatant (green). The inset shows that both methods utilizing Bond Elut Lipid Extraction had much less background over the RTs of interest (1.94 and 2.08) when compared to IPA.

It was also found that the supernatant-only approach (SPEs) enhanced the response for one of the PEth forms, while using the whole extract (SPEp) enhanced the other form (Figure 6), suggesting that one of the forms of PEth is more heavily membrane bound than the other; however, this did not impact the linear range observed.

Recovery experiments demonstrated excellent extraction efficiency of Bond Elut Lipid Extraction cartridge when compared to the IPA extraction process. Recoveries were between 40 and 50% for both analytes when implementing the SPE protocol.

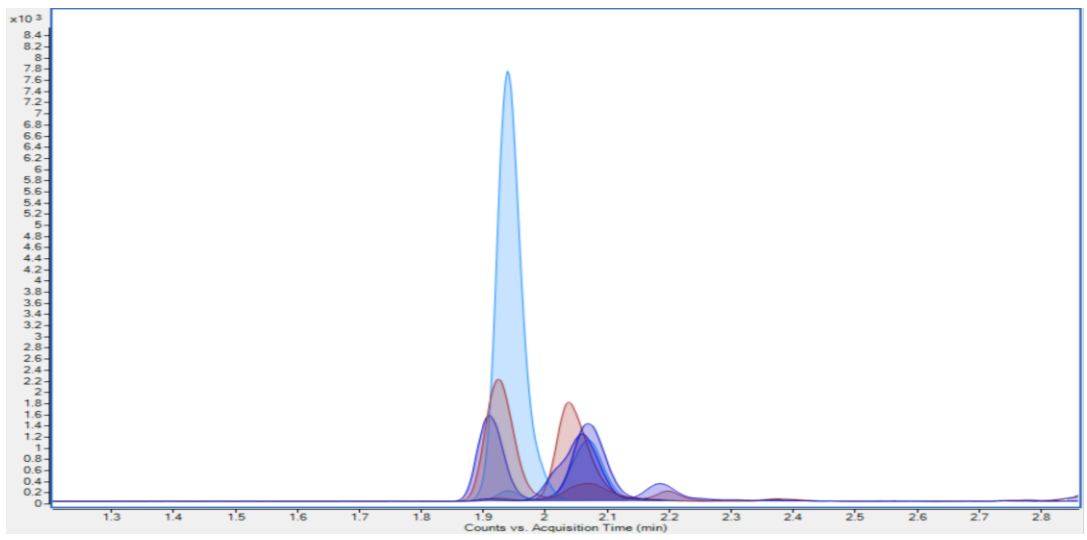

Figure 6. Comparison of responses from SPEp (light blue), SPEs (maroon), and IPA (dark blue).

One issue that arose during experimentation was the lack of a suitable blank. The purchased whole blood used in this series of experiments had detectable baseline levels of PEth, highlighting the importance of finding a suitable source for blank whole blood. Despite the presence of detectable levels of PEth in the blood matrix, they were found to be below the LOQ established by the linear range. Future experiments would examine a variety of sources for whole blood to determine if it is in fact possible to find blood that is blank for PEth.

Conclusions

The novel workflow utilizing the Bond Elut Lipid Extraction SPE cartridges showed significantly reduced background over the chromatographically relevant range when compared to a simple IPA extraction procedure. This workflow demonstrated great recoveries, and it lends itself well to automation both on the sample preparation side, as well as the detection side. Future work is needed to determine whether dried blood spots are a suitable alternative sample type for this analysis.

References

Applications and Challenges for the Use of Phosphatidylethanol Testing in Liver Disease Patients (Mini Review)- Van Long Nguyen, et al., Alcoholism: Clinical and Experimental Research, Feb. 2018.

https://explore.agilent.com/asms

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