Extraction of Three Phosphatidylethanol (PEth) Biomarkers in Human Whole Blood Using ISOLUTE® SLE+ Prior to LC/MS-MS Analysis

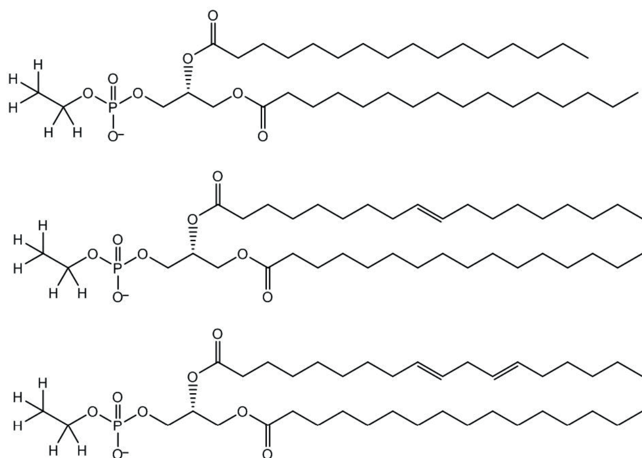

Figure 1. Structures of PEth 16:0, Peth 16:0/18:1 and PEth 16:0/18:2.

Introduction

This application note describes the extraction of a panel of three phosphatidylethanol (PEth) alcohol biomarkers from human whole blood using ISOLUTE® SLE+ Supported Liquid Extraction plates prior to LC/MS-MS analysis. The simple sample preparation procedure delivers clean extracts and analyte recoveries greater than 60% with RSDs lower than 10% for all analytes, with reduced levels of phospholipids to improve extract cleanliness and analyte sensitivity. Linearity of greater than 0.999 is achieved for all analytes from 0.5–5000 ng/mL.

ISOLUTE® SLE+ Supported Liquid Extraction plates offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation.

Analytes

Phosphatidylethanol 16:0, Phosphatidylethanol 16:0/18:1 and Phosphatidylethanol 16:0/18:2

Internal Standards

Phosphatidylethanol 16:0/18:1 – D_5

Sample Preparation Procedure

Format

ISOLUTE® SLE+ 400 µL Sample Capacity Plate, Part Number 820-0400-P01

Sample Pre-Treatment

Add 10 µL of a 250 pg/µL ISTD solution (in H_2O:isopropanol, 1:1, v/v) to 100 µL of human whole blood (to give a final concentration of 25 ng/mL).

Add a further 100 µL of H_2O:MeCN (80:20 v/v). Mix thoroughly.

Sample Loading

Load 200 µL of pretreated whole blood onto each ISOLUTE® SLE+ well. Ensure that the sample covers the entire area of the ISOLUTE SLE+ top frit. Using a Biotage® PRESSURE+96 Positive Pressure Manifold, apply a pulse of pressure to load samples onto the sorbent. Wait 5 minutes for the sample to equilibrate on the sorbent.

Analyte Extraction

Apply an aliquot of ethyl acetate:isopropanol (95:5, v/v, 700 µL) and allow to flow under gravity for 5 minutes. Apply a second aliquot of ethyl acetate: acetate:isopropanol (95:5, v/v, 700 µL) and allow to flow under gravity for 5 minutes. Apply a final aliquot of ethyl acetate:isopropanol (95:5, v/v, 700 µL). For complete solvent recovery apply a pulse of positive pressure at 10 psi (10–20 seconds).

Collection Vessels

Collect the eluent in 2 mL volume, 96-well collection plate.

Post Elution

Evaporate extracts at 40 °C, for 30 mins at a flow rate of 20–40 L/min using the Biotage® SPE Dry-96.

Reconstitute

Reconstitute in isopropanol (200 µL). Vortex mix. Cover plate with a sealing mat prior to injection.

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UHPLC Conditions

Instrument
Shimadzu Nexera x 2 UHPLC

Column
Agilent Poroshell 120, EC-C8, 2.7 µm 2.1 x 50 mm with a guard column of the same chemistry

Mobile Phase
A: 5 mM ammonium formate (aq)

B: Isopropanol

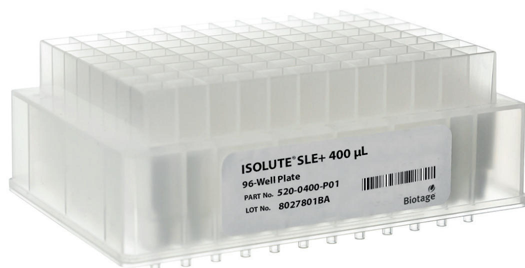
Flow Rate
0.3 mL/min

Column Temperature
40 °C

Injection Volume
5 µL

Table 1. UPLC Gradient.

Time (min)	%A	%B
0	40	60
1	0	100
4	0	100
4.01	40	60
5.5	40	60



Mass Spectrometry Conditions

Equipment
Shimadzu 8060 Triple Quadrupole MS using ES interface

Nebulizing Gas Flow
3 L/min

Drying Gas Flow
5 mL/min

Heating Gas Flow
15 L/min

Interface Temperature
400 °C

DL Temperature
300 °C

Heat Block Temperature
500 °C

CID Gas
270 kPa

Table 2. MS conditions and retention times for target analytes in positive and negative mode.

Analytes	MRM Transition		Collision Energy	Ion Mode
PEth 16:0	675.20 (675.20	255.25 437.20)	32 25	-
PEth 16:0/18:1	701.40 (701.40	281.35 437.30)	34 24	-
PEth 16:0/18:2	699.40 (699.40	279.30 255.30)	32 35	-

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Results

This simple sample preparation method delivers clean extracts and analyte recoveries mostly greater than 60% with RSDs lower than 10% for all analytes (see Figure 2), and LLOQs below 10 ng/mL for all 3 phosphatidylethanol species. Figure 2. below shows recoveries obtained using 400 µL capacity ISOLUTE® SLE+ plates, when loading 200 µL of pre-treated whole blood sample.

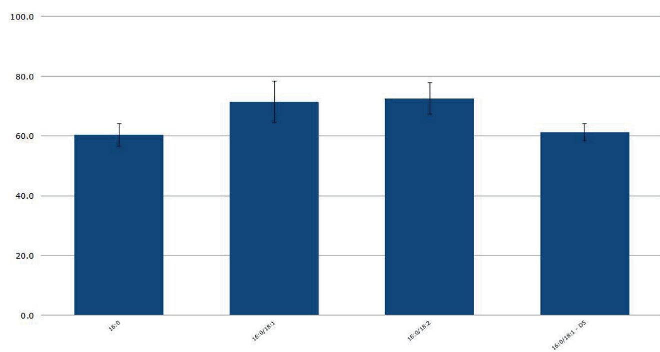

Figure 2. Typical analyte % extraction recoveries (n=7) loading 200 µL of pre-treated whole blood.

Calibration curve performance was investigated from human whole blood spiked between 0.5–5000 ng/mL. Good linearity was observed for all analytes typically delivering r^2 values greater than 0.999. Table 3. details linearity performance and associated LOQ for each analyte loading 200 µL of pre-treated whole blood onto a 400 µL capacity plate. Calibration curves for all analytes are shown in Figure 3.

Table 3. Analyte calibration curve r^2 and LOQ performance.

Analyte	r^2	LLOQ (ng/mL)
PEth 16:0	0.9994	0.5
PEth 16:0/18:1	0.9993	0.5
PEth 16:0/18:2	0.9994	5

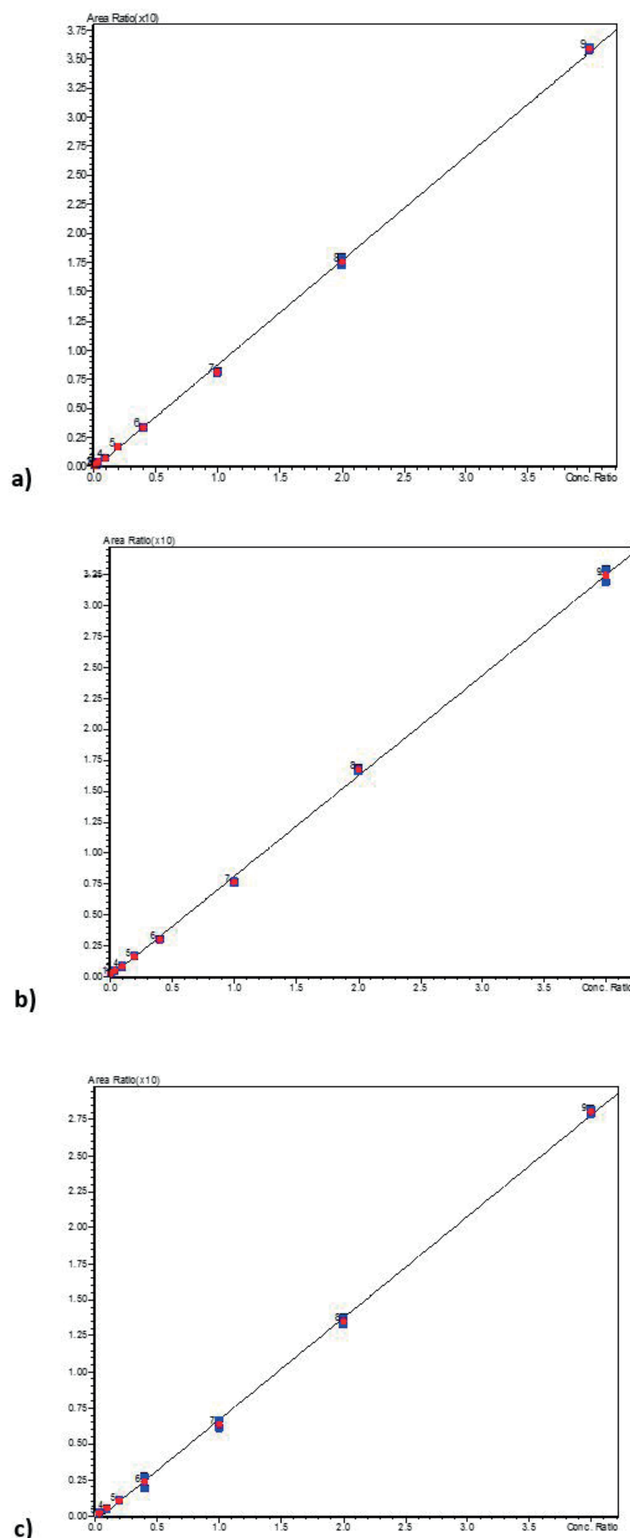

Figure 3. Calibration curves for PEth 16:0 (a), PEth 16:0/18:1 (b), and PEth 16:0/18:2 (c) in whole blood.

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Discussion

When analyzing blood products, it is important to eliminate interferences caused by phospholipids. Unfortunately, phosphatidylethanol is very similar in structure to phospholipids that can reduce sensitivity and quickly lead to the degradation of the LC column and MS system. One of the main aims of this study was to develop a sample preparation method that would allow for good recovery of the phosphatidylethanol species without significant levels of phospholipids being present in the extract. The optimum extraction solvent system identified to achieve this leads to slightly reduced recovery of the three phosphatidylethanol species, however the method sensitivity is such that the required detection limits are exceeded.

In this extraction, underloading of the sample was utilized to further clean up the extraction of phospholipids. Underloading (i.e. loading a lower sample volume than the theoretical capacity of the extraction product) when using whole blood also helps to eliminate possible breakthrough of red blood cells, as well as allowing for the use of more polar elution solvents.

Conclusion

This method provides clean extracts and highly sensitive detection for the analysis of three phosphatidylethanol biomarkers. This method has LOQs of at least 5 ng/mL with the usual cut off being 20 ng/mL.

Ordering Information

Part Number	Description	Quantity
820-0400-P01	ISOLUTE® SLE+ 400 µL 96-Well Plate	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS-EU	Biotage® SPE Dry 96 Sample Evaporator 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry 96 Sample Evaporator 100/120 V	1
121-5203	Collection Plate, 2 mL Square	50
121-5204	Pierceable Sealing Mat	50

Chemicals and Reagents

- Acetonitrile (LC-MS grade), isopropanol (Gradient MS) and ethyl acetate were purchased from Honeywell Research Chemicals (Bucharest, Romania).
- All analyte standards, deuterated internal standards and ammonium formate were purchased from Sigma- Aldrich Company Ltd. (Gillingham, UK).
- Water used was 18.2 MOhm-cm, drawn daily from a Direct-Q5 water purifier.
- Mobile phase A (5 mM ammonium formate (aq)) was prepared by adding 315.3 mg of ammonium formate to 1 L purified water.
- Internal standards (250 pg/µL) were prepared from a 10 ng/µL stock solution by adding 25 µL of each of to 955 µL of H_2O:isopropanol (50:50 v/v). 10 µL of this solution was then added to each calibration solution.
- Pre-treatment solution was prepared by adding 20 mL of MeCN to 80 mL of purified water.
- Elution solvent was prepared by mixing 95 mL ethyl acetate with 5 mL isopropanol.

Additional Information

- All data shown in this application note was generated using human whole blood from Golden West (California, US) with LiHep anti-coagulant.
- For increased sensitivity:
  - Decrease reconstitution solvent volume below 200 µL

EUROPE
Main Office: +46 18 565900
Toll Free: +800 18 565710
Fax: +46 18 591922
Order Tel: +46 18 565710
Order Fax: +46 18 565705
order@biotage.com
Support Tel: +46 18 56 59 11
Support Fax: + 46 18 56 57 11
eu-1-pointsupport@biotage.com

NORTH & LATIN AMERICA
Main Office: +1 704 654 4900
Toll Free: +1 800 446 4752
Fax: +1 704 654 4917
Order Tel: +1 704 654 4900
Order Fax: +1 434 296 8217
ordermailbox@biotage.com
Support Tel: +1 800 446 4752
Outside US: +1 704 654 4900
us-1-pointsupport@biotage.com

JAPAN
Tel: +81 3 5627 3123
Fax: +81 3 5627 3121
jp_order@biotage.com
jp-1-pointsupport@biotage.com

CHINA
Tel: +86 21 68162810
Fax: +86 21 68162829
cn_order@biotage.com
cn-1-pointsupport@biotage.com

KOREA
Tel: +82 31 706 8500
Fax: +82 31 706 8510
korea_info@biotage.com
kr-1-pointsupport@biotage.com

INDIA
Tel: +91 22 4005 3712
india@biotage.com

Distributors in other regions are listed on www.biotage.com

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