Application Note

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Lipidomic Analysis of Human Plasma Using Bond Elut Lipid Extraction with the Agilent 6545 LC/Q-TOF

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Abstract

This application note presents a novel solid-phase extraction (SPE) method using the Agilent Bond Elut Lipid Extraction 1 mL cartridge for human plasma lipidomics sample preparation. Following protein precipitation, the plasma sample homogenate was loaded into the cartridge for gravity elution. After a wash with 90:10 ACN/water, the lipids were eluted with 1:2 dichloromethane/methanol (DCM/MeOH). The extract sample was run on an Agilent 6545 LC/Q-TOF for lipid profiling using the Agilent MassHunter Lipid Annotator. In total, 595 lipid compounds were detected and identified in the human plasma sample, including 347 lipid compounds from 13 classes with a positive mode method, and 248 lipid compounds from nine classes with a negative mode method. Method reproducibility was excellent, with <10% RSD for more than three replicates in our different tests. Comparing to traditional liquid-liquid extraction (LLE) methods, the Bond Elut Lipid Extraction SPE method provides time and effort savings, reduced solvent use, fewer sample transferring steps, improved reproducibility, and automation feasibility.

Introduction

Lipidomics is a branch of analytical biochemistry that deals with the large-scale study of lipid molecules in biological systems. Lipidomics research has gained more attention over the past decade because of new insights into functional lipid-lipid and lipid-protein interactions in biochemical systems. Lipidomics research development has rapidly been facilitated by advances in analytical technology, including liquid chromatography (LC), mass spectrometry (MS), and informatics. In return, new research motivations and advanced analytical capabilities have led to an increased demand for fast, convenient, accurate, and precise high-throughput lipidomics sample preparation workflow. This will allow larger cohorts in biological research.

The traditional sample preparation workflow used for lipidomics analysis has been based on LLE, including the Bligh-Dyer method,[1] the Folch method,[2] the Matyash method,[3] and the BUME method.[4] Although these methods have widely been used for lipidomics sample preparation, they are time-consuming, labor-intensive, difficult to automate, and suffer from poor reproducibility.

In this study, a novel SPE method using Bond Elut Lipid Extraction SPE cartridges was introduced for lipidomics analysis in human plasma. Bond Elut Lipid Extraction SPE cartridges and plates feature the use of Enhanced Matrix Removal (EMR)—Lipid sorbent, which demonstrates selective and efficient interaction with lipid molecules. The EMR—Lipid sorbent interaction with lipid molecules is based on the combined mechanism of size exclusion and hydrophobic interaction. Long unbranched hydrocarbon chains on most lipid molecules are selectively retained into pores of the EMR—Lipid sorbent, while other molecules lacking linear acyl chains stay in solution. The current applications of EMR—Lipid sorbent focus on matrix removal, where lipids from the matrix are removed by the interactions with the EMR—Lipid sorbent, while the target analytes are separated for further treatment or analysis.

Bond Elut Lipid Extraction SPE cartridges allow the use of the EMR—Lipid sorbent for lipidomics study sample preparation. This explores a new application area, where lipids become the analytes of interest to be extracted and isolated from other sample matrix components. Like traditional SPE, the sorbent's "catch and release" function is used in this application. Lipid compounds are retained on the EMR—Lipid sorbent first, then eluted with an organic solvent. This study demonstrates a workflow using Bond Elut Lipid Extraction 1 mL cartridges for human plasma lipidomics analysis.

Experimental

Chemicals and reagents

LC/MS grade acetonitrile (ACN), methanol (MeOH), and chloroform were purchased from Honeywell Research Chemicals (Muskegon, MI, USA). High-performance liquid chromatography (HPLC) grade *n*-butanol (*n*-BuOH) and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). SRM 1950 metabolites in human plasma NIST standard were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Solutions and standards

The crashing solvent ACN/MeOH (99:1, v/v) was prepared by mixing 99 mL of ACN with 1 mL of MeOH. The washing solvent ACN/H_2O (9:1, v/v) was prepared by mixing 90 mL of ACN with 10 mL of H_2O. The elution solvent chloroform/MeOH (1:1, v/v) was prepared by mixing 50 mL of chloroform with 50 mL of MeOH. The reconstitution solvent *n*-BuOH/MeOH (1:1, v/v) was prepared by mixing 50 mL of *n*-BuOH with 50 mL of MeOH.

Equipment and material

The study was performed using an Agilent 6545 LC/Q-TOF equipped with an Agilent Jet Stream Electrospray ionization source, and an Agilent 1290 Infinity II LC consisting of a binary pump, a thermostatted multiwell autosampler, and a thermostatted column compartment.

Sample preparation equipment included:

- Pipettes and repeater (Eppendorf, NY, USA)
- Agilent positive pressure manifold 48 processor (PPM-48) (part number 5191–4101)
- 1 mL cartridge rack for the PPM-48 (part number 5191–4102)
- Waste rack and waste bin for the PPM-48 (part number 5191–4112)
- Vortexer (VWR, USA)
- Sonicator (VWR, USA)
- Agilent Bond Elut Lipid Extraction cartridge, 1 mL (part number 5610-2041)
- Agilent InfinityLab Poroshell HPH-C18, 2.0 × 150 mm, 2.7 µm column (part number 693775-702)
- Agilent InfinityLab Poroshell HPH-C18, 3.0 mm column, ultra-high-performance liquid chromatography (UHPLC) guard column (part number 823750-928)

Instrument conditions

The LC/Q-TOF instrument conditions are listed in Table 1.

LC/MS/MS data from the extracted SRM 1950 standard plasma sample were annotated to specific lipids using MS/MS *in silico* spectral matching with the MassHunter Lipid Annotator software. The software uses an algorithm that combines probability density, Bayesian scoring, and a non-negative least square fit to search a theoretical lipid library. Positive- and negative-ion mode datasets were treated separately throughout data analysis.

Individual features in the LC/MS data were identified using the Agilent MassHunter Profinder version 10.0 software. They were identified in a batch-targeted feature extraction (BTFE) mode compared against the personal compound database and library (PCDL) generated in the Lipid Annotator, requiring both mass and retention time (RT) criteria. The resulting features were exported to Mass Profiler Professional Version 15.0 for lipid class and statistical analysis. Figure 1 shows the general scheme for data collection and processing for lipid compound identification and confirmation.

Table 1. Analytical method conditions for LC/Q-TOF used for lipidomics analysis.

Parameter	Value
Agilent 6545 LC/Q-TOF with Dual Agilent Jet Stream Ionization Source	
Instrument Mode	2 GHz, extended dynamic range, *m/z* 1,700
Polarity	Positive and negative
Gas Temperature	250 °C
Drying Gas (Nitrogen)	11 L/min
Nebulizer Gas	35 psi
Sheath Gas	300 °C at 12 L/min
Capillary Voltage	3,500 V (+), 3,000 V (–)
Nozzle Voltage	500 V
Fragmentor	160 V
Oct 1 Rf Vpp	750 V
Acquisition Speed	MS-only: 3 spectra/sec (MS)
	Auto MS/MS: 3 spectra/second (MS), 4 spectra/second (MS/MS)
Auto MS/MS Parameters	Isolation width: Narrow (~1.3 amu)
	Collision energy: 20 eV, 35 eV
Reference Correction	Two points at *m/z* 121.050873(+), 922.009798(+)
	Two points at *m/z* 119.036320(–), 980.016375(–)
Agilent 1290 Infinity II LC	
Analytical Column	Agilent InfinityLab Poroshell HPH-C18, 2.0 × 150 mm, 2.7 µm
Guard Column	Agilent Poroshell HPH-C18, 3.0 mm, UHPLC guard
Column Temperature	60 °C
Injection Volume	1 µL
Autosampler Temperature	5 °C
Needle Wash	15 seconds in wash port MeOH/isopropyl alcohol (1:1, v/v)
Mobile Phase	A) Water/MeOH (9:1, v/v) with 10 mM ammonium acetate and 10 µM medronic acid
	B) ACN/MeOH/IPA (2:2:6, v/v/v) with 10 mM ammonium acetate
Flow Rate	0.6 mL/min
Gradient Program	Time (min) %B 0.0 55 5.0 57 25.0 100 27.0 100 28.0 55
Stop Time	30 minutes
Post Time	5 minutes
Observed Column Pressure	300 to 600 bar

Sample preparation

Human plasma was prepared using protein precipitation (PPT) followed by processing through Bond Elut Lipid Extraction SPE cartridges for lipids extraction, isolation, and purification. Figure 2 shows a general procedure for human plasma sample preparation for the lipidomics study.

An aliquot of 100 µL of SRM 1950 plasma was transferred into a 2 mL polypropylene snap cap vial, followed by the addition of 900 µL of ice-cold ACN/MeOH (99:1, v/v) for PPT. The tube was capped tightly, vortexed for 30 seconds, then sonicated for 10 minutes on ice. Sonication on ice was used to release the trapped lipids from PPT, improving lipid extraction efficiency.

The Bond Elut Lipid Extraction 1 mL cartridges were placed on the PPM-48 with a waste reservoir under the cartridges. Each sample was vortexed again for 10 seconds, and the entire homogenate was pipette-transferred onto the cartridge using a wide-bore pipette tip. It is essential to transfer the entire sample homogenate to prevent potential lipid loss due to temporary binding with protein precipitates.

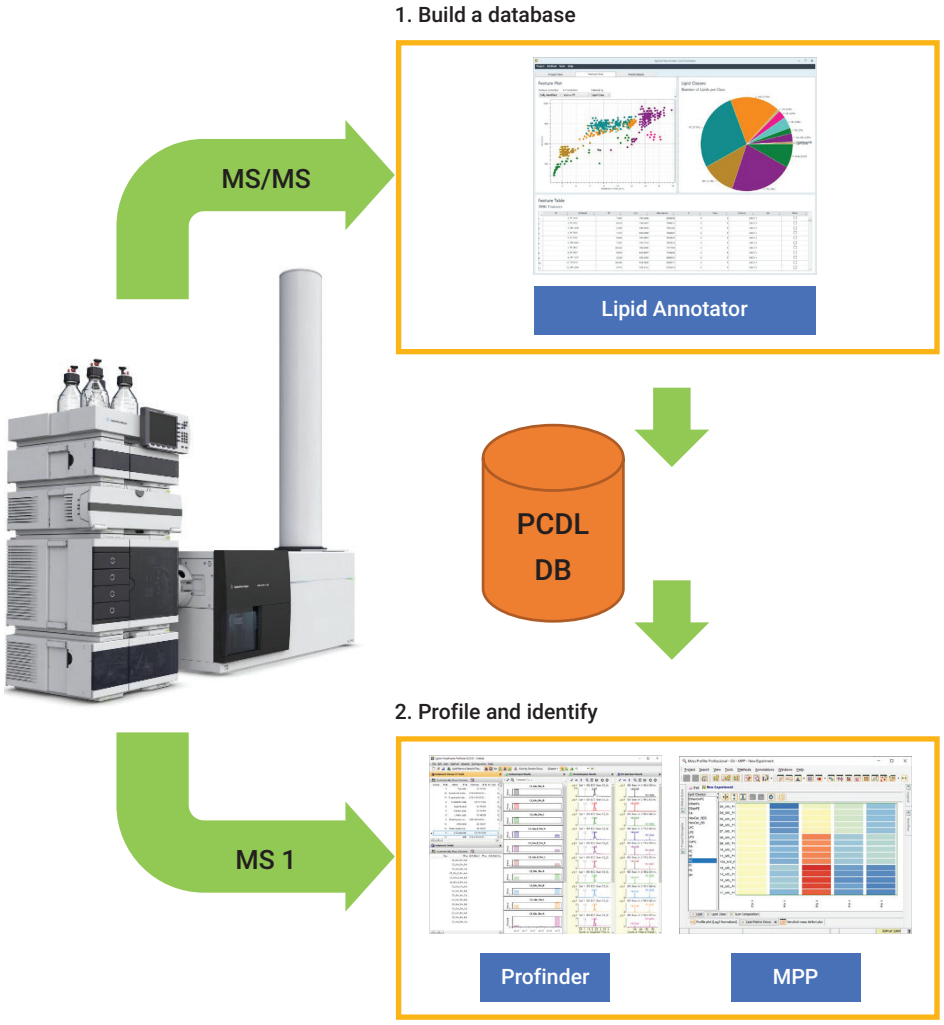

Figure 1. Data collection and processing for lipidomics analysis on an Agilent 6450 LC/Q-TOF.

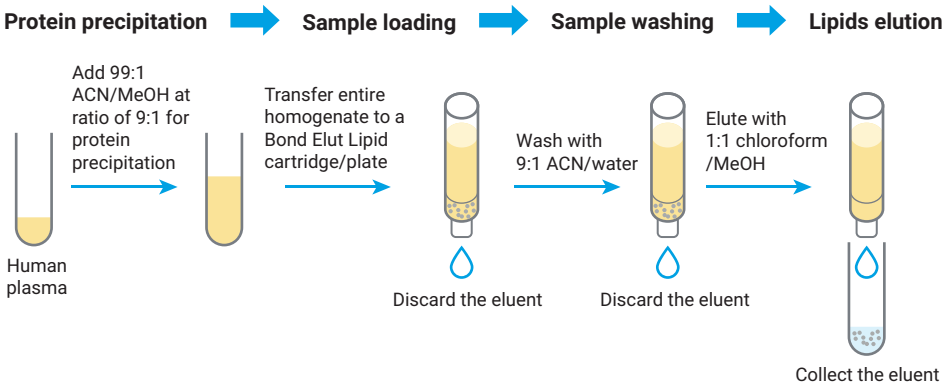

Figure 2. Workflow of human plasma sample preparation for lipidomics study using the Agilent Bond Elut Lipid Extraction 1 mL cartridge.

Gravity or low pressure (2 to 4 psi) was then used for a gradual and slow elution with a flow rate of 3 to 5 seconds/drop. The steady elution flow rate is to allow sufficient interaction time between lipid compounds and the sorbent so that lipids can be retained on the sorbent efficiently. When there was no visible liquid left in the cartridge, two washing steps using 1 mL of ACN/water (9:1, v/v) (2 × 1 mL) were applied using 2 to 4 psi pressure. Higher pressure (6 to 9 psi) was applied to dry the cartridge completely at the end. The waste rack was removed, and a collection rack with glass tubes was placed under cartridges. Lipids elution was then achieved by 2 × 1 mL of chloroform/MeOH (1:1, v/v) elution with gravity or low pressure (1 to 3 psi), as needed. High pressure (6 to 9 psi) was applied at the end to dry the sorbent completely. The entire eluent was dried with N_2 at 30 °C.

The dried residue was then reconstituted into 100 µL of *n*-BuOH/MeOH (1:1, v/v). The sample was vortexed for two minutes and sonicated for 10 minutes at room temperature. The above reconstitution procedure was important to ensure complete and consistent redissolving of the dried lipids residue. The sample was transferred to a 2 mL sample vial with a glass insert, and was ready for instrument injection. The dried sample can also be stored in the freezer for future instrument analysis.

Total phospholipids (PLs) on cartridge recovery test

The total PLs profile was monitored on an Agilent LC/MS/MS system with the method in Table 2.

The plasma sample after PPT was centrifuged, the supernatant was transferred out, dried, and reconstituted into 100 µL of *n*-BuOH/MeOH (1:1, v/v). This sample was used as the control to compare with the samples after the Bond Elut Lipid Extraction process. The total PLs peak area was obtained by manually integrating through the entire retention window. The comparison of total PLs peak area between plasma with PPT only and PPT followed by the Bond Elut Lipid Extraction SPE process was used for fast on-cartridge recovery evaluation.

Table 2. Method parameters for the total phospholipids profile.

LC Section	
Instrument	Agilent 1290 Infinity II LC
Column	Agilent InfinityLab Poroshell 120, HILIC-Z, 2.1 × 150 mm, 2.7 µm (part number 683775-924)
Mobile Phase A	10 mM ammonium formate buffer with 0.125% formic acid
Mobile Phase B	95:5 ACN/10 mM ammonium formate with 0.125% formic acid
Gradient	Time (min) MPA MPB Flow rate (mL/ min) 0 5 95 0.3 1 5 95 0.3 12 60 40 0.3 15 Stop Stop Stop
MS Section	
Instrument	Agilent 6490 triple quadrupole LC/MS/MS
Ion Source	Agilent Jet Stream Electrospray ionization source
Scan Segments	Positive precursor ion scan for product ion 184 *m/z*, CE 20 V

Results and discussion

SPE method development and considerations

The Bond Elut Lipid Extraction SPE procedure includes three parts: sample loading for lipids retention on sorbent, washing for further removal of unwanted matrix components, and elution for trapped lipids recovery. Protein precipitation was used at the beginning for lipids extraction and protein removal.

A high ratio of ACN/plasma (9:1) was used to improve the extraction efficiency for lipid compounds. A small percentage of MeOH (1 to 5%) was added to prevent the generation of large protein precipitate coagulates that may trap lipids and clog the pipette tip during homogenate transfer. The loading step allowed unwanted matrix co-extractives, including salts and other matrix co-extractives lacking the linear alkyl chain, to pass through the SPE cartridge. Because the sorbent selectively binds lipids, a washing reagent containing high organic (up to 90%) was used, which allowed more efficient washing to remove other matrix interferences. The elution solvent was a mixture of MeOH with either chloroform or dichloromethane, where a minimum 50% of MeOH was important to release the lipids from the sorbent. To prevent the extraction of various plastics leachables with the use of plastic-aggressive solvent, it was important to use a glass tube to collect the eluent.

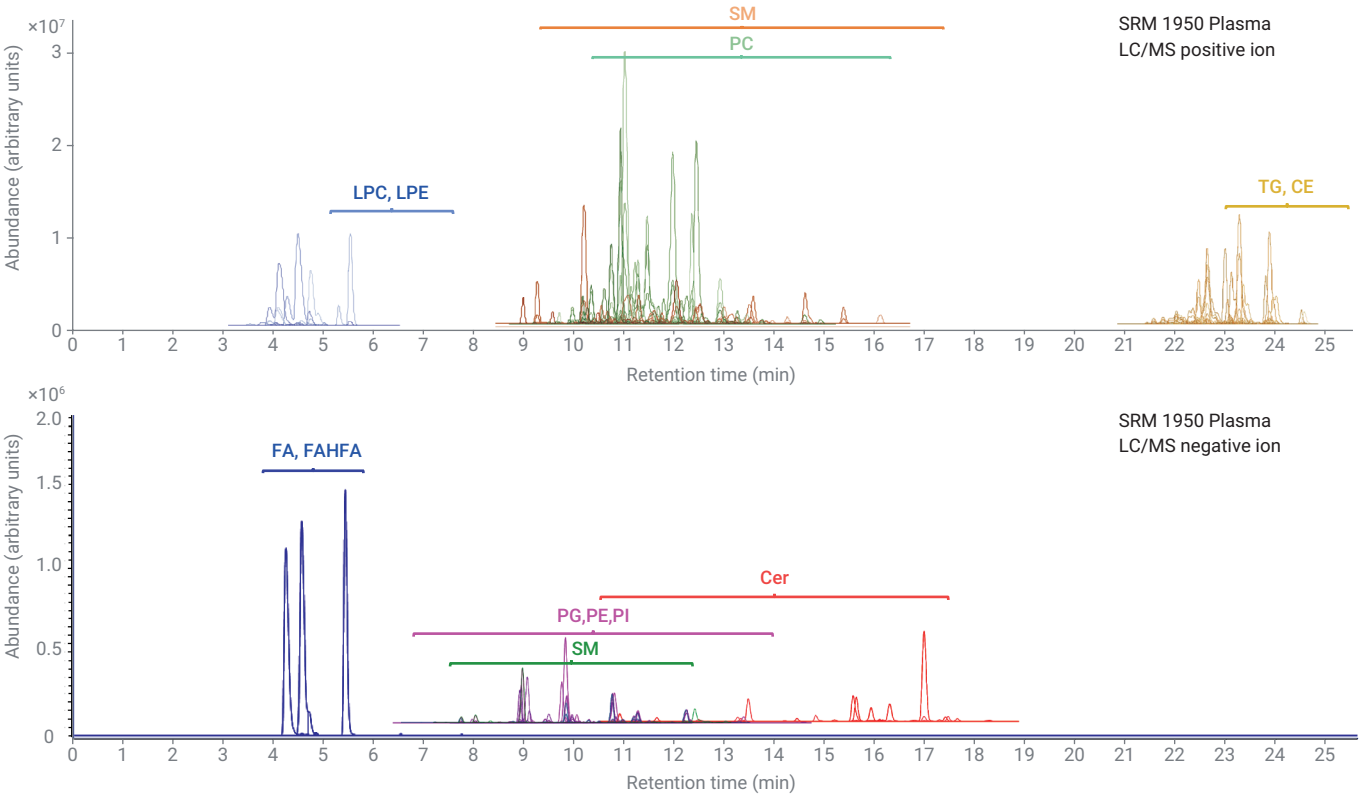

Figure 3. Positive and negative EICs for identified 11 classes of lipid compounds in human plasma using the Agilent Bond Elut Lipid Extraction SPE method.

Human plasma lipidomics analysis

Figure 3 shows the extracted ion chromatograms (EICs) in both positive and negative mode for identified major classes of lipid compounds in human plasma. Figure 4 shows the identified lipid compounds distribution based on lipid class in terms of total peak intensity. The results in positive and negative modes were the averaged results across three LC/MS replicates for each of three preparation replicates based on the identified lipid compounds intensity distribution. The identified lipid compounds are from the following lipid classes:

- Acylcarnitines (Acar)
- Glycerophosphocholines (PC)
- Glycerophosphoethanoloamines (PE)
- Glycerophosphoinositols (PI)
- Glycerophosphoserines (PS)
- Glycerophosphoglycine (PG)
- Lysoglycerophosphocholines (LPC)
- Lysophosphoethanoloamines (LPE)
- Sphingomyelins (SM)
- Ceramides (Cer)
- Cholesterol esters (CE)
- Diacylglycerides (DG)
- Triacylglycerides (TG)
- Fatty acids (FA)
- Fatty acid esters of hydroxy fatty (FAHFA)

Except for free cholesterol, the identified lipid compound class distributions are comparable to the consensus distribution for SRM 1950[5]. Free sterols, such as cholesterol, are not retained efficiently on the EMR—Lipid sorbent due to the lack of a long linear alkyl chain.

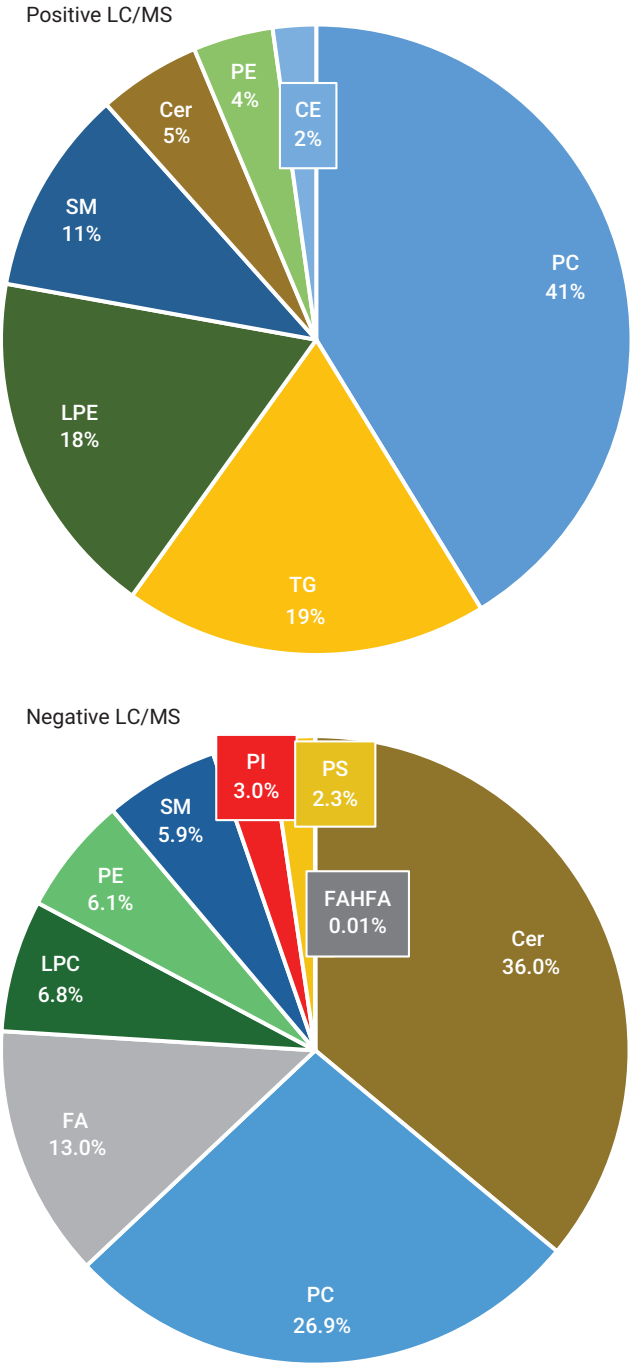

Figure 4. Summary of identified lipid compound distribution by lipid class based on total peak intensity.

Table 3 lists the number of identified lipid compounds in plasma and the average total peak areas within each class, in positive and negative modes, using the Bond Elut Lipid Extraction SPE method. A total of 347 lipid compounds from 13 classes were identified in positive mode, and 248 lipid compounds from nine classes were identified in negative mode. The total peak area intensity was 1.1E+09 in positive mode, and 4.3E+07 in negative mode.

Additionally, the method reproducibility was evaluated by running three replicates of prepared samples with three injections for each prepared sample on instrument. The number of identified lipid compounds and the peak areas for identified lipid compounds were used for evaluation. All identified lipid compounds were filtered to remove low-abundance compounds and outliers using an interquartile statistical test (<first quartile – 1.5 × Interquartile Range, >third quartile + 1.5 × Interquartile Range). The %RSD of the total peak intensities was then calculated for the rest of the identified lipid compounds. The RSD% of the instrument injection replicates indicates the instrument method reproducibility, whereas the RSD% of the sample preparation replicates indicates the entire workflow reproducibility. The instrument method reproducibility was 6.4%, while the entire workflow reproducibility was 9.4%. The results show that the Bond Elut Lipid Extraction method delivers excellent sample preparation consistency, which does not contribute significant workflow variability.

Table 3. Summary of identified lipid compounds and peak intensity in human plasma using the Agilent Bond Elut Lipid Extraction SPE method.

Mode	Class	No. of Identified Lipids	Total Peak Area
Positive	PC	93	4.5E+08
	TG	72	2.1E+08
	LPC	44	2.0E+08
	SM	50	1.2E+08
	Cer	18	5.8E+07
	PE	6	4.5E+07
	CE	19	2.4E+07
	DG	20	1.4E+06
	ACar	17	8.0E+05
	LPE	2	3.2E+05
	PI	3	4.4E+04
	PS	2	2.1E+04
	PG	1	9.9E+03
Total		347	1.1E+09
Negative	Cer	47	1.5E+07
	PC	89	1.2E+07
	FA	5	5.6E+06
	LPC	7	2.9E+06
	PE	52	2.6E+06
	SM	26	2.5E+06
	PI	16	1.3E+06
	PS	5	9.9E+05
	FAHFA	1	5.3E+03
Total		248	4.3E+07

Total phospholipids recovery on-cartridge

Phospholipids are a group of important lipids in biological matrices such as plasma, and thus PLs in plasma are partly of importance to lipodomics. The total PLs from a plasma extract can easily be monitored by the precursor ion scanning of product ion 184. The method was thus used as a fast and easy assessment for total phospholipids recovery on-cartridge. In addition, since PLs separation was not important for the purpose of this test, HILIC chromatography was used for the fast elution on column using a short LC gradient, and minimal carryover on column.

Figure 5 shows a chromatographic profile comparison of total PLs in sample prepared by PPT and SPE methods. Overall, the two chromatograms show high similarity for profile comparison with some minor differences. For profile intensity within the retention time (RT) window of 0 to 3.5 minutes, the SPE method shows slightly lower abundance than the PPT only method, indicating a slight loss on-cartridge. According to the total peak area integrated within this RT window, the total PLs recovery was 90%. For profile intensity within the RT window of 3.5 to 15 minutes, the SPE method shows higher abundance than the PPT-only method. This indicates that certain lipids trapped in the protein precipitates were recovered by applying SPE extraction to the entire homogenate sample. With the total peak area integrated within this window, the PLs total recovery was 143%. The total PLs recovery within the entire RT window was 102%, based on the total peak area integration of the Bond Elut Lipid Extraction SPE method versus the PPT-only method. This fast comparison demonstrates that there was insignificant PLs loss through the SPE process, and that the SPE procedure can recover some of the lipids trapped by protein precipitates.

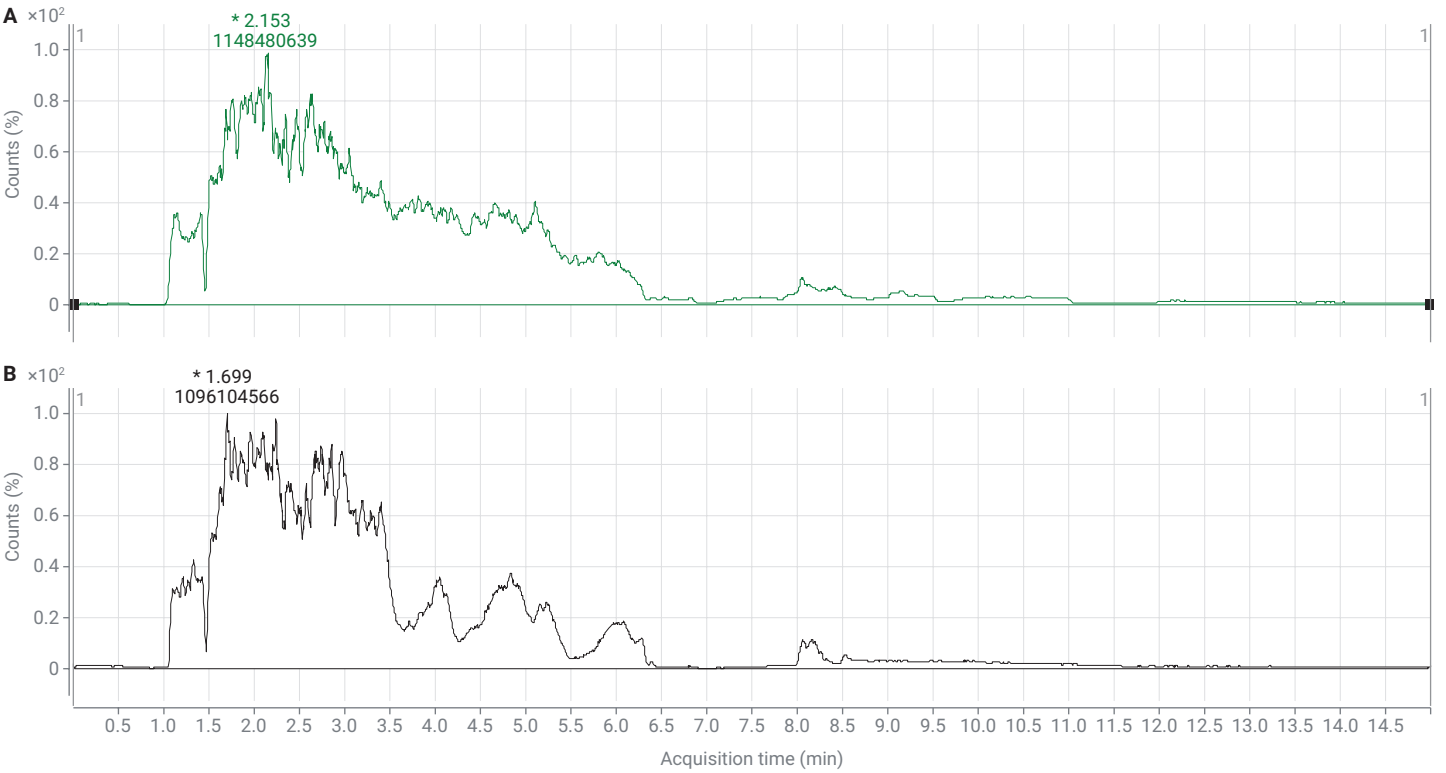

Figure 5. Total phospholipids profile comparison using precursor ion scan for product ion 184. (A) Plasma sample prepared by PPT followed by the Bond Elut Lipid Extraction SPE method. (B) Plasma sample prepared by PPT only.

Simplified workflow and matrix cleanup

Compared to traditional LLE methods used for lipidomics sample preparation, the Bond Elut Lipid Extraction SPE method significantly simplifies the entire workflow with time and labor savings. In LLE methods, repeated extractions are usually used to improve the lipid extraction efficiency, and to remove unwanted sample matrix co-extractives such as salts. This procedure could be time-consuming and labor-intensive, as it involves multiple steps of phase separation, organic phase transfer, and sample drying. This also introduces more room for errors and analyte loss. For the Bond Elut Lipid Extraction method, the EMR—Lipid sorbent provides selective interaction with lipid compounds, which allows the use of a relatively strong wash using 90:10 ACN/water. With the loading and washing steps, sample matrix can be cleaned efficiently and conveniently, especially for unwanted salts and other interferences that do not have long aliphatic chains. Figure 6 shows the sample dried residue for sample prepared by the Bond Elut Lipid Extraction method and PPT extraction only. It clearly shows that the SPE method provides significant matrix cleanup after PPT extraction.

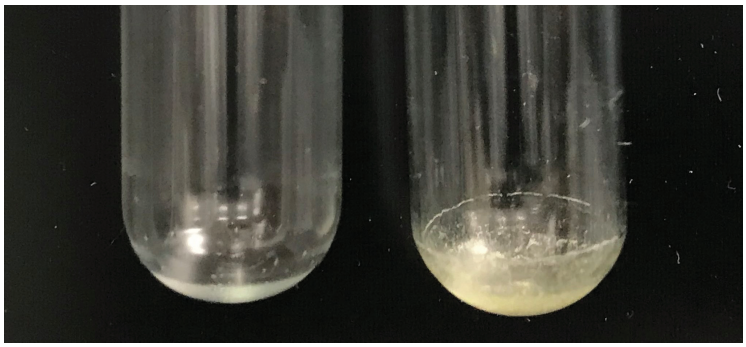

Figure 6. Sample dried residue appearance for samples prepared by Bond Elut Lipid Extraction method and PPT extraction only.

Conclusion

A simple, rugged, and reliable SPE method using Bond Elut Lipid Extraction cartridges was developed for human plasma lipidomics analysis. The SPE method was demonstrated to provide phenomenal lipids identification for untargeted lipidomics analysis in human plasma, for both identified lipid compounds number and peak intensity. The quick evaluation for total phospholipids recovery also demonstrated insignificant loss for phospholipids on the cartridge. Compared to traditional LLE methods, the SPE method provided significant time and effort savings, and improved reproducibility. The method also shows the potential for high-throughput sample preparation using the 96-well plate.

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