Efficiency of Biological Fluid Matrix Removal Using Agilent Captiva EMR—Lipid Cleanup

Application Note

Clinical Research

Authors

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Abstract

The Agilent Captiva Enhanced Matrix Removal—Lipid (Captiva EMR—Lipid) is the next generation of EMR product, and is formatted in SPE cartridges or 96-well plates. Phospholipids are widely recognized as the prominent interferences in biological fluids. They not only affect the MS response of many analytes negatively, but are also difficult to remove from samples without analyte loss. This study demonstrates the application of Captiva EMR—Lipid cartridges and plates for phospholipid removal in various biological fluids. The phospholipid removal capabilities of Captiva EMR—Lipid were evaluated for many biological fluids from human and animal sources, with or without the addition of different anticoagulants. The procedure involves an *in situ* protein precipitation step followed by pass-through cleanup by Captiva EMR—Lipid. The efficiency of matrix removal was determined by the weight of residual matrix and the chromatographic profile of phospholipids through a precursor ion scan for product ion 184 *m/z*. A thorough comparison study of currently available products was evaluated for phospholipid removal based on the recommended product protocols. The results demonstrated that Captiva EMR—Lipid provides >99 % phospholipid removal, superior eluent clarity, easier flow, and substantially less clogging when compared to other products performance.

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Introduction

Sample pretreatment before liquid chromatographic separation and quantification using LC/MS/MS is routine within the field of bioanalysis. Sample matrices are normally biological fluids such as plasma and serum. Sample preparation is used to reduce system contamination, improve data integrity, improve method selectivity, and enhance analytical sensitivity. The common sample preparation techniques include protein precipitation (PPT), solid phase extraction (SPE), liquid-liquid extraction (LLE), and supported liquid extraction (SLE). Each technique has advantages and disadvantages in terms of speed of analysis, cost, and quality of data generated. Within these techniques, PPT is widely accepted. In PPT, proteins are efficiently removed by crashing the proteins in the biological fluid sample with an organic solvent such as acetonitrile at ratios of 3:1 to 5:1 (organic solvent/sample). The precipitates are then removed by centrifugation or filtration. However, the phospholipids remain since they are soluble in the organic crash solvent, and are in high concentration in the final extracts to be injected into LC/MS/MS for analysis.

Phospholipids (PPLs) are major constituents of cell membranes and are therefore abundant in serum and plasma. They consist of a hydrophilic head group composed of phosphate and choline units, and a hydrophobic tail made up of long alkyl chains. The most abundant phospholipids are glycerophosphocholines (70 % of total PPL) and lysophosphatidylcholines (10 % of total PPL), which are shown in Figure 1[1]. Phospholipids are identified as a major cause of matrix effects in LC/MS/MS bioanalysis, through competition for space on the surface of droplets formed during the ESI process[2].

Agilent Captiva Enhanced Matrix Removal—Lipid (Captiva EMR—Lipid) cartridges and plates use a novel sorbent material that selectively removes major lipid classes from sample matrix without unwanted analyte loss. The lipid removal mechanism is a combination of size exclusion and hydrophobic interaction between the long aliphatic chain of the lipid substances and the EMR—Lipid sorbent. Because of the specific interaction mechanism, Captiva EMR—Lipid provides highly selective and efficient phospholipid removal from biological fluids after PPT. Captiva EMR—Lipid will also remove lipids other than phospholipids. The pass-through version of Captiva EMR—Lipid produces purified eluents ready for analysis. The 96-well plate is compatible with automation, enabling high-throughput applications in bioanalysis. The frit design provides easy and efficient elution for *in situ* PPT in cartridges/plates without clogging. This study demonstrates that EMR—Lipid cleanup after PPT provides exceptional phospholipid removal from common biological fluids. This cleanup generally exceeds, or is equivalent to, any current available products for lipid cleanup. In addition, ease-of-use and clog-free elution are demonstrated in various biological matrices.

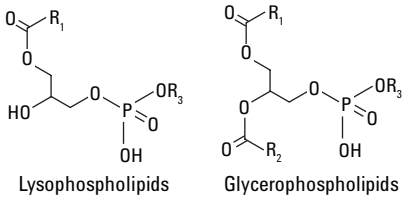


Figure 1. Chemical structures of the two most important groups of phospholipid.

Experimental

Reagent and chemicals

All reagents and solvents were HPLC or analytical grade. Acetonitrile (ACN) was from Honeywell (Muskegon, MI, USA). Reagent grade formic acid (FA) was from Agilent (p/n G2453-85060). The biological matrices were bought from Biological Specialty Corporation (Colmar, PA, USA).

Solution and biological fluids

A solution of 1 % FA in ACN was prepared by adding 200 µL of formic acid to 20 mL of acetonitrile. This solution was used for protein precipitation.

The bulk biological fluids were stored in a –80 °C freezer, and aliquots were thawed the day of testing.

Equipment and Material

Equipment used for sample preparation included:

- Centra CL3R centrifuge (Thermo IEC, MA, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Multitube vortexer and oven (VWR, Radnor, PA, USA)
- Eppendorf pipettes and repeater
- ViaFlo 96 Liquid Handler (Integra, Hudson, NH, USA)
- Captiva vacuum collar (p/n A796)
- CentriVap concentrator, cold trap, and vacuum gauge (Labconco, Kansas City, MO, USA)
- TurboVap Concentration Workstation (Biotage, Charlotte, NC, USA)
- Agilent Captiva EMR—Lipid 1 mL cartridge (p/n 5190–1002)
- Agilent Captiva EMR—Lipid 96-well plate (p/n 5190–1001)
- Captiva 96-well 1 mL collection plate (p/n A696001000)
- Captiva 96-well plate cover, 10/pk (p/n A8961007)

Instrument conditions

The samples were run on an Agilent 1290 Infinity UHPLC system consisting of an:

- Agilent 1290 Infinity binary pump (G4220A)
- Agilent 1290 Infinity high performance autosampler (G4226A)
- Agilent 1290 Infinity thermostatted column compartment (G131C)

The UHPLC system was coupled to an Agilent G6490 Triple Quadrupole LC/MS system equipped with an Agilent JetStream iFunnel electrospray ionization source. Agilent MassHunter workstation software was used for data acquisition and analysis.

Biological fluids

The following biological fluids were used for phospholipid removal evaluation:

- Human plasma Na EDTA
- Human plasma K_3 EDTA
- Human plasma Li Heparin
- Human plasma Na citrate
- Human plasma Na Heparin
- Human serum
- Human CSF (cerebral spinal fluid)
- Horse plasma Na citrate
- Porcine plasma K_3 EDTA
- Canine plasma K_3 EDTA
- Rat plasma Na EDTA

The biological fluids were received and aliquoted into 10-mL samples upon arrival. The small aliquots of matrix samples were stored at –20 °C for tests. On the test day, the frozen sample was thawed in warm water, then centrifuged at 4,000 rpm for 3 minutes before use.

Comparison products/tests

Five currently available products that claim to remove phospholipids were tested for a performance comparison. Samples prepared by PPT were used to evaluate total PPLs. The manufacturer's recommended protocols were followed for the comparison. All competitor products and Agilent Captiva EMR—Lipid used *in-situ* PPT, except for Product 1, which required separate PPT and transfer. To make the comparison more representative, the sample and protein crashing solvent volume were kept consistent for various products and matrices.

HPLC Conditions

Parameter	Value
Column	Agilent InfinityLab Poroshell 120 LC column, EC-C18, 50 × 2.1 mm, 2.7 µm (p/n 699775-902) Agilent InfinityLab Poroshell 120 guard column, EC-C18, 5 × 2.1 mm, 2.7 µm (p/n 821725-911)
Flow rate	0.3 mL/min
Column temperature	60 °C
Autosampler temperature	Ambient
Injection volume	2 µL
Mobile phase	A) 0.1 % FA in water B) 0.1 % FA in Acetonitrile
Needle wash	1:1:1:1 ACN/MeOH/IPA/H_2O with 0.2 % FA
Gradient	Time (min) %B Flow rate (mL/min) 0 5 0.3 18 95 0.3 30 100 0.3
Stop time	40 minutes
Post time	3 minutes

MS Conditions

Parameter	Value
Gas temperature	120 °C
Gas flow	14 L/min
Nebulizer	40 psi
Sheath gas heater	400 °C
Sheath gas flow	12 L/min
Capillary	3,000 V
Data acquisition	Precursor ion scan mode under positive polarity
Product ion	184,
MS1 scan	100–1,400

Sample preparation

For *in situ* protein precipitation, 600 µL of ACN with 1 % FA was added into the cartridge or plate well, followed by a 200 µL sample aliquot.

For SPE cartridges, insert cartridges directly into the vacuum chamber with collection the tube beneath.

For SPE 96-well plates, put the plate on the collection plate with the vacuum collar inserted in the middle.

The sample mixture in the cartridge or well was mixed by pipetting 3–5 times. Large bore pipette tips are recommended for mixing to prevent precipitates from clogging and splashing. Appropriate vacuum was applied for gradual elution. The flow was controlled to 1 drop/3–5 seconds. Higher vacuum was applied at the end to drain the cartridge or plate sorbent bed. The collected eluent was dried with N_2 flow or CentriVap at 40 °C.

Samples used for LC/MS/MS evaluation were dried and reconstituted with 200 µL of 10:90 ACN/water with 0.1 % FA, vortexed, sonicated, and centrifuged before instrument analysis. For samples used in residual matrix evaluation, the eluent was collected in preweighed glass test tubes. After complete drying, the test tube was baked in the oven at 110 °C for 30 minutes. The tubes were cooled to room temperature, and weighed to determine the final sample residue mass.

When offline PPT was required, 600 µL of ACN with 1 % FA was added into a 1.5-mL snap cap tube, followed by a sample aliquot of 200 µL. The tube was capped and vortexed for 2 minutes, and centrifuged for 3 minutes at 13,000 rpm on the microcentrifuge. The entire supernatant was transferred into the Product 1 cartridge for cleanup, or directly into a clean glass tube for drying.

Matrix cleanup assessment

A simple method to evaluate PPL levels in a sample is to monitor a common product ion's abundance, trimethylammonium-ethyl phosphate, at *m/z* 184, during the chromatographic run[3].

The biological fluid matrix blank samples obtained by different cleanup methods and PPT without extra cleanup were run on LC/MS/MS to collect 184 precursor ion scan chromatograms. Chromatograms were overlaid to compare PPL removal. To quantitatively evaluate PPL removal efficiency, the chromatogram was manually integrated across the entire window, and the phospholipid removal efficiency was calculated according to Equation 1.

Results and Discussion

The study focused on the evaluation of products based on ease-of-use and matrix removal. Many biological fluids from both human and animal sources, with or without various anticoagulants, were investigated to study the cleanup achieved on currently available and Agilent Captiva EMR—Lipid products.

Ease-of-use

Ease-of-use is the first impression when using the product for sample preparation. Users should see the instructions provided by the manufacturer. All products except Product 1 recommend *in situ* PPT, where the biological fluid is combined with a crashing solvent inside the cartridge or plate wells. The normal recommendation is to add the solvent first, followed by sample. This is to prevent precipitates from clogging frits/membrane, and improve homogeneity. However, the precipitates generated from various biological fluids vary in the amount that forms and in the particle size, and could disrupt filtration for *in situ* PPT. In our study, we tested Captiva EMR—Lipid and other available products for precipitates filtration after *in situ* PPT by recording the flow observations and eluent clarity. Table 1 and Figure 2 show the results. Since *in situ* protein precipitation is not applicable to Product 1, this product is not included.

$$\% \text{ Phospholipids Removal} = \frac{\text{Total Peak Area}_{\text{Sample w/o cleanup}} - \text{Total Peak Area}_{\text{Sample w/ cleanup}}}{\text{Total Peak Area}_{\text{Sample w/o cleanup}}} \times 100$$

Equation 1.

Table 1. Ease-of-elution for *in situ* PPT, and elution/filtration observations. 1 = Easy flow (2–4 inch Hg) and no clogging; 2 = Moderate flow (5–8 inch Hg) and no clogging; 3 = Difficult flow (>10 inch Hg) and occasional clogging; 4 = Extremely difficult flow, full vacuum needed, and frequent clogging resulting in sample loss.

Biological fluid	Anticoagulant	Agilent Captiva—EMR plate	Product 2	Product 3	Product 4	Product 5
Human serum	–	1	3	2	3	2
Human plasma	K_3EDTA	1	3	3	3	2
	NaEDTA	1	2	2	3	2
	Li Hep	1	2	2	3	2
	Na Citrate	2	3	3	3	3
	Na Hep	1	2	2	3	2
Human CSF	–	1	1	1	1	1
Horse plasma	Na Citrate	2	4	4	4	3
Porcine plasma	K_3EDTA	1	4	3	3	2
Canine plasma	K_3EDTA	1	4	4	3	2
Rat plasma	NaEDTA	1	2	2	2	2

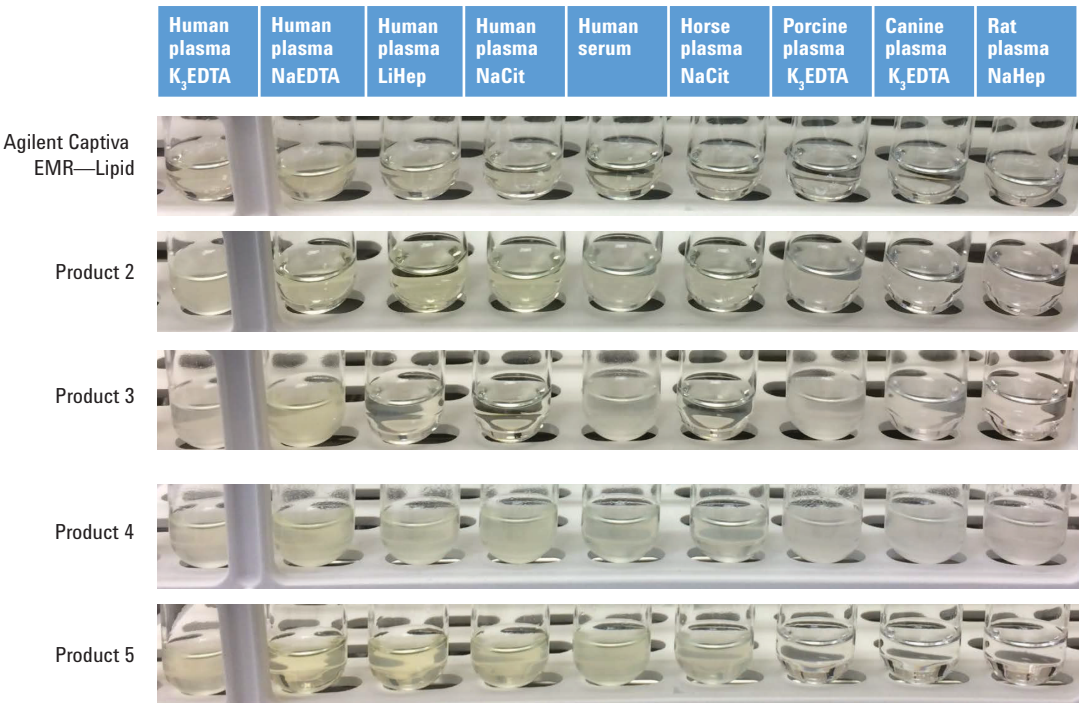


Figure 2. Comparison of sample eluent clarity collected by *in situ* PPT.

Evaluation of sample elution in cartridge/plate were classified into four groups:

- Easy flow
- Intermediate flow
- Difficult flow
- Extremely difficult flow

These classifications are based on the amount of vacuum needed and clogging observations (Table 1). Table 1 lists the definitions of the groups. Observations indicated that anticoagulant can significantly impact the PPT. Plasma using Na citrate and K_3 EDTA usually generates fine precipitates that increase backpressure during sample elution. Plasma using Na Heparin generates large precipitates that can be filtered easily at lower vacuum. Cerebral spinal fluid (CSF) is clear and low in proteins, producing low precipitation after PPT. Serum does not use anticoagulants, but generates large amounts of precipitate, and can make the elution difficult. Overall, Captiva EMR—Lipid provides the smoothest and easiest elution when compared to the other products evaluated. Typically, a 2–4 inch Hg vacuum was required, increasing to 6–8 inch Hg for plasma with Na citrate. There was no clogging observed during the testing of Captiva EMR—Lipid regardless of sample type. Conversely, all four of the other products showed some difficulties with elution after *in situ* PPT, high vacuum was required, clogging was observed, and partial or complete sample loss occurred. This elicits uncertainty and variability during sample preparation, and results in inconsistent results, especially for 96-well plate high-throughput analysis.

The clarity of eluent is another important parameter that indicates efficient particulate filtration. Figure 2 shows the eluent clarity of biological fluids using different products. When using Captiva EMR—Lipid, the eluent was clear regardless of biological fluids, while other products gave cloudy eluents, especially Product 4, where cloudiness was observed for all sample types. Cloudy eluent suggests inefficient filtration for precipitates or break-through. Unremoved precipitates can negatively impact the LC system by increasing backpressure, clogging columns, and ultimately becoming detrimental for method reliability.

Gravimetric determination of residue

Gravimetric determination of residue is a method to evaluate total matrix removal from sample preparation[4]. In this study, the extracted sample was dried completely and the final residue was weighed. The amount of residue weight directly reflects how much matrix was removed from the sample extraction by a cleanup method. Larger residue weight correlates to inefficient cleanup, whereas smaller residue weight represents efficient cleanup.

The matrix residue weight was determined using 200 µL of biological fluid. To make an objective comparison, residue weight data were normalized based on the corresponding dried residue weight from PPT. Seven types of human biological fluids were used in this test, which are commonly used in bioanalytical applications. The fluids included serum, plasma with five types of anticoagulants, and CSF. Figure 3 shows the normalized residue weight data.

The results are grouped based on each matrix, while each column shows the normalized results for this matrix by various sample treatments. As shown in each group, the dried residue weight of Captiva EMR—Lipid cleanup (orange column) is always the lowest or one of lowest in comparison to the results by other methods, indicating less coextractives in the final sample extract. When comparing the final residue mass to PPT for other products, similar or even higher matrix residue weight is occasionally observed, indicating inefficient matrix removal during cleanup, or inefficient protein precipitate removal during elution, or introduction of contaminants during sample elution

Phospholipid removal (PLR)

PPLs are abundant in biological fluids such as plasma and serum, and are widely acknowledged as the major source of matrix effects in LC/MS/MS analysis. The matrix effect caused by phospholipids can vary based on the MS ionization mode. ESI is sensitive to coeluting PPLs because PPLs at the surface of droplets in the MS source can trap analyte ions inside causing suppression in both positive and negative ESI[5].

Chromatographic separation of target analytes from interfering compounds is one approach to reduce the ion suppression effect[6], but it can result in longer run times and add difficulty to methods targeting multiple analytes. PPLs that enter the LC column and MS system can accumulate over time, resulting in column contamination and shorter lifetime, MS contamination, and sensitivity loss. Using isotopically labeled internal standards to correct matrix effect is another approach, however, isotopic internal standards can be expensive or difficult to obtain. This approach only corrects for ion suppression effects but does not reduce the amount of endogenous matrix interferences entering into the detection system. A third approach is to use sample preparation to remove phospholipids. Sample preparation reduces or eliminates matrix ion suppression effects, protects the LC column, improves data integrity, and prevents the MS from being contaminated over time.

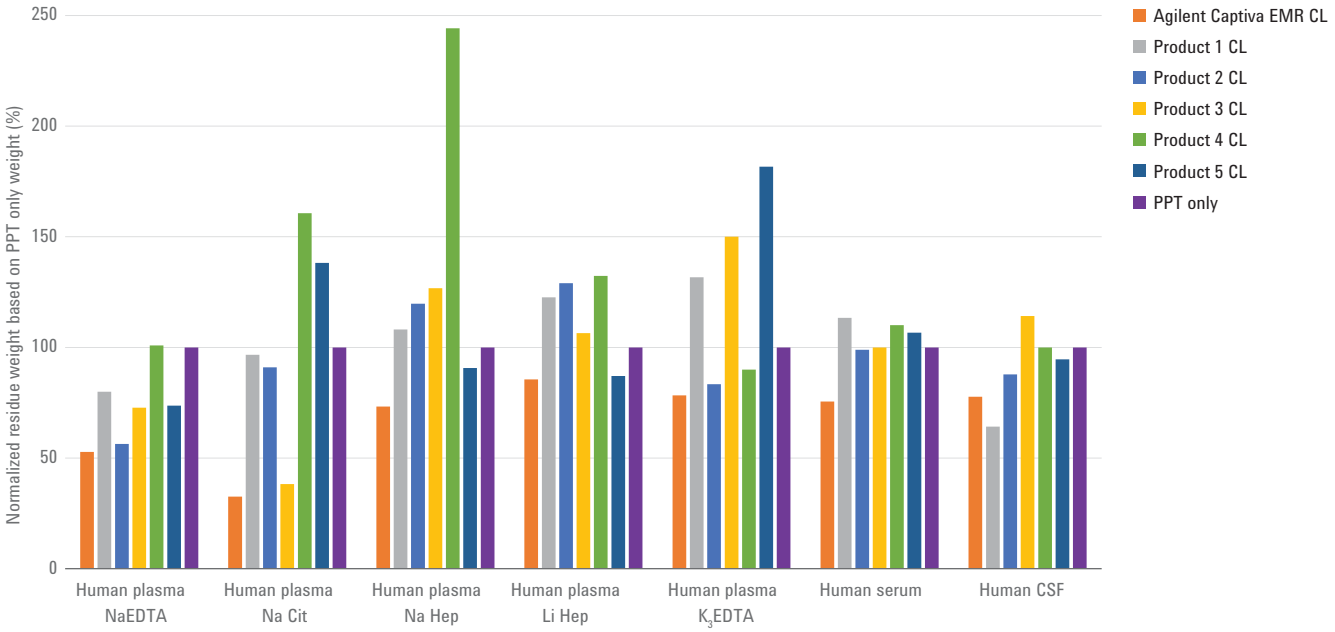


Figure 3. Gravimetric determination of sample residue. Samples of 200 µL of biological fluids were prepared by PPT followed by various cleanup methods (n = 2). All values were normalized with the residue weight of sample prepared by PPT only.

Efficient removal of phospholipids from biological matrices is a prominent feature of Captiva EMR—Lipid. The chromatographic overlay comparison in Figure 4 shows that Captiva EMR—Lipid cleanup delivers >99 % phospholipid removal. To investigate matrix impact on PPL removal, 11 common biological fluids were tested: serum, plasma, and CSF from different sources: human, horse, porcine, canine, and rat, as well as different anticoagulants: Na EDTA, Na citrate, Na Heparin, Li Heparin, and K_3EDTA. These biological fluid samples prepared by protein precipitation followed by Captiva EMR—Lipid plate or cartridge cleanup showed >99 % PPL removal for all matrices tested. An important caution during the operation is the elution speed. It is important to control vacuum/pressure carefully to maintain the flow rate of ~1 drop/3–5 seconds, which allows sufficient interaction between the sample and the EMR—Lipid sorbent.

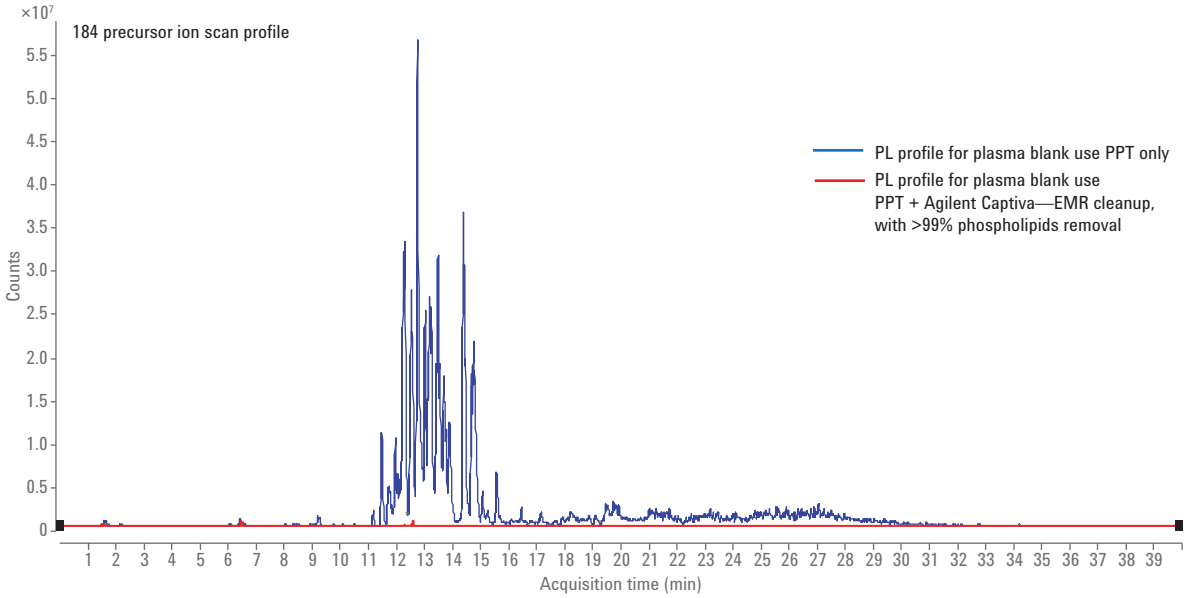
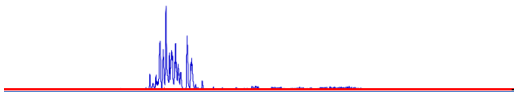
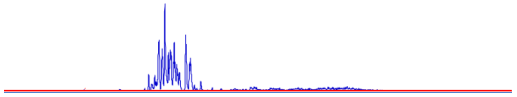


Figure 4. Overlapped chromatograms for phospholipids profile by monitoring a precursor ion scan for 184 *m/z*.

Table 2 shows the results of a comparison with other products on phospholipids removal using human plasma with Na Heparin. The results demonstrate that Captiva EMR—Lipid provides superior or equivalent phospholipid removal efficiency to other products used for lipid removal. Three of these products can provide >99 % PLR, and one product provides barely 99 % PLR due to some interference peaks observed in the early chromatogram window, seemingly from contamination introduced by the product. The final two products give less than 99 % PLR, as shown in the red trace.

Table 2. Phospholipids removal efficiency comparison among various cleanup methods after PPT of human plasma Na Heparin.

Matrix cleanup after PPT	Calculated PLR (%)	Chromatogram comparison with and without cleanup
Agilent Captiva EMR—Lipid	>99	
Product 1	>99	
Product 2	99	
Product 3	>99	
Product 4	>99	
Product 5	93	
Product 6	82	

Conclusions

Agilent Captiva EMR—Lipid cleanup after PPT provides excellent phospholipid removal (>99 %) from biological fluids, and is superior or equivalent to other currently available lipid removal products. The function of the Captiva EMR—Lipid sorbent for phospholipids removal is not impacted by biological matrix variations such as different matrix types, sources, or anticoagulants. The gravimetric determination of the residue study also demonstrates excellent total matrix removal efficiency provided by Captiva EMR—Lipid cleanup. The cartridge assembly design ensures clogging-free sample elution under low vacuum/pressure, and efficient participates filtration during elution with *in situ* PPT. The unique Captiva EMR—Lipid sorbent demonstrates selective lipid removal without unwanted analyte retention, and future applications will explore the cleanup of fatty foods for multiclass, multiresidue analysis.

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