Evaluation of a Novel 96-well Filter Plate for the Effective Removal of Serum Proteins and Phospholipids prior to LC-MS/MS Analysis

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Introduction

When analyzing serum or plasma, the very nature of the matrix can cause problems with the analytical methodology. Endogenous components such as salts, proteins and phospholipids are all present and can have a marked effect on instrument response in terms of ion suppression or enhancement effects. This variation in signal can lead to quantitation issues and method reliability problems. In recent years a number of filter plates have been developed to address these specific interferences. This poster evaluates the performance of a novel 96-well filter plate for the simultaneous removal of proteins and phospholipids prior to LC-MS/MS analysis.

Experimental

Reagents

Drug standards and formic acid were purchased from Sigma-Aldrich (Dorset, UK). Plasma was purchased from Sera Labs International (West Sussex, UK.). All solvents were HPLC grade from Fisher Scientific (Loughborough, UK) and Milli-Q (Merck Millipore, Germany) water used throughout.

Sample Preparation

ISOLUTE® PLD+ Optimized Procedure (*Figure 1.*)
96-well Plates: ISOLUTE® PLD+ 50mg; 918-0050-P01.

Solvent first precipitation can be used due to the nature of the proprietary top frit combination.

Apply Solvent: Apply 400 µL of MeCN into the wells. If MeOH is required due to solubility issues use 300 µL.
Sample Application: Apply 100 µL of matrix and mix thoroughly via repeat aspirate/dispense steps or vortex mixing.
Elution: Apply vacuum -0.2 bar or 3 psi positive pressure for approximately 5 minutes. For highly particulate laden samples increased processing conditions may be required.

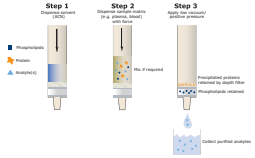

***Figure 1**. Schematic of ISOLUTE® PLD+ Phospholipid Depletion Plate Procedure.*

Post Extraction: Extracts were evaporated to dryness at 40 °C and reconstituted in appropriate mobile phase for analysis.

Gel Electrophoresis

Electrophoresis was performed using a NuPAGE NOVEX 12% Bis/tris mini gel with MOPS SDS running buffer. Gels were run at 200V, 120 mA and 12.5 W for 65 minutes.

HPLC Conditions

Instrument: Waters Acquity UPLC (Waters Assoc., Milford, MA, USA).
Phospholipid HPLC Column: Kinetex Phenyl-Hexyl 5 µm analytical column (50 x 2.0 mm id) (Phenomenex, Cheshire UK).
Analyte HPLC Column: Kinetex Phenyl-Hexyl 2.7 µm analytical column (50 x 2.0 mm id) (Phenomenex, Cheshire UK).

Mass Spectrometry

Instrument: Ultima Pt triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface for mass analysis.
Desolvation Temperature: 350 °C
Ion Source Temperature: 100 °C
Collision Gas Pressure: 2.7 x 10^{-3} mbar
Phospholipid Analysis: The most abundant phospholipid ions were identified and monitored by MRM experiments using the common 184 Da product ion.
Analyte Analysis: Protonated molecular ions were used for all analytes and fragmented to their most abundant product ions for MRM experiments.
Full chromatographic and mass spectrometry details can be obtained from Biotage.

Results

Optimal MeCN crash ratios were investigated for protein removal from the samples using gel electrophoresis experiments. **Figure 2.** shows a comparison of 1:1, 1:2, 1:3 and 1:6 (v/v) serum/MeCN crash ratios. The results demonstrate that 1:3 (v/v) ratios gave more protein removal than either the 1:1 or 1:2 (v/v) ratios. However, there was no improvement when increasing to a 1:6 (v/v) crash ratio.

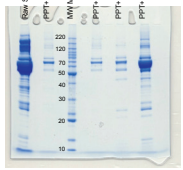

***Figure 2.** Gel electrophoresis protein profile comparing various crash ratios.*

Phospholipid experiments monitoring the common 184 product ion demonstrated excellent removal of lyso and larger molecular weight PLs as demonstrated in ***Figure 3.***

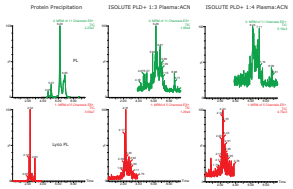

***Figure 3.** Phospholipid profile using 1:3 and 1:4 MeCN crash ratios compared to protein precipitation.*

Figure 4. shows the zoomed overlaid TICs comparing PL removal between protein precipitated plasma and the ISOLUTE® PLD+ using a 1:4 plasma:MeCN crash ratio.

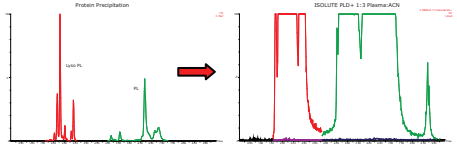

***Figure 4.** Zoomed view showing overlaid phospholipid TICs.*

Figure 5. demonstrates analyte recovery profiles comparing various commercially available PL depletions plates. For the most part recoveries were comparable between all products.

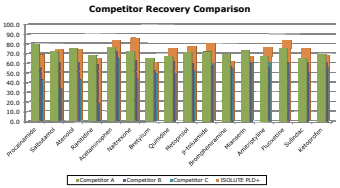

***Figure 5.** Recovery comparison between various PL depletion plates.*

***Figure 6.** demonstrates overall signal response detailing peak areas for extracted samples compared to authentic standards.*

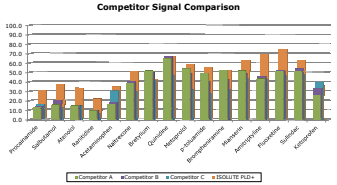

***Figure 6.** Overall signal comparison between various PL depletion plates.*

Table 1.
shows analyte properties with corresponding recovery and RSDs for a typical extraction.

***Table 1.** Analyte properties.*

Analyte	Functionality	pKa	logP	% Recovery	% RSD (n=7)
Ketoprofen	Acidic	4.2	2.8	67.8	6.7
Sulindac	Acidic	4	3.59	74.5	4.1
Atenolol	Basic	9.1	0.16	74.0	5.8
Ranitidine	Basic	8.8	0.27	64.0	11.8
Procainamide	Basic	9.4	0.88	67.8	4.1
Salbutamol	Basic	9.4	1.31	73.9	6.2
Naltrexone	Basic	9.2	1.8	85.6	5.2
Metoprolol	Basic	10.8	1.88	77.3	4.8
Quinidine	Basic	9.28	2.88	75.1	5.0
Amitriptyline	Basic	9.4	3.1	75.6	4.5
Mianserin	Basic	8.3	3.6	67.0	2.2
Brompheniramine	Basic	9.2/3.6	4.06	61.1	3.0
Fluoxetine	Basic	9.5	4.2	83.1	3.5
Bretylium	Quat	N/A	1.17	60.3	7.4
Acetaminophen	Neutral	N/A	0.34	82.6	3.7
p-toluamide	Neutral	N/A	1.18	79.7	5.6

Figure 7. demonstrates signal to noise comparisons between ISOLUTE® PLD+ and competitor products. Better peak shape and S/N was returned resulting in lower achievable LOQs.

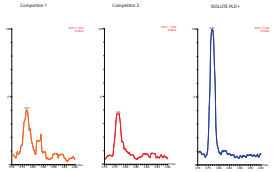

***Figure 7.** Signal to Noise comparison for amitriptyline between various PL depletion plates.*

Monitoring of analyte signal response and UPLC column back pressure was performed using a Waters ACQUITY UPLC and Premier XE triple quadrupole MS system.

Figure 8. shows metoprolol peak area plots for 300 injections. No signal intensity degradation is observed illustrating MS source integrity is maintained.

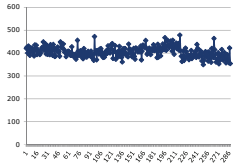

***Figure 8.** Metoprolol peak area plot for 300 injections of ISOLUTE® PLD+ extracted samples.*

Figure 9. demonstrates UPLC back pressure plots comparing 100 injections of protein precipitated samples and 300 injections of ISOLUTE® PLD+ extracted samples. Considerable pressure increases were observed with protein precipitated samples. However, ISOLUTE® PLD+ extracted samples demonstrated minimal impact on backpressure.

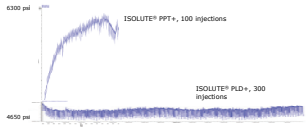

***Figure 9.** UPLC back pressure plots comparing protein precipitation and ISOLUTE® PLD+.*

Conclusion

- ISOLUTE® PLD+ provides very simple processing of various matrices.
- Mixing can be effected via vortex mixing, repeat aspirate/dispense steps or forced pipette crashing
- We demonstrated excellent removal of proteins and phospholipids leading to excellent reproducibility over 300 injections and no HPLC back pressure increase.
- Better signal response and overall signal to noise ratios were returned compared to other commercially available PL depletion plates leading to lower achievable LOQs.

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