Rapid Improvements for LC/MS/MS Analysis without Additional Method Development Using Phree™ Phospholipid Removal Plates

Dr. Stuart Kushon and Erica Pike
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

Protein precipitation is compared to a phospholipid removal product, Phree Phospholipid Removal Plates, to assess the cleanup capabilities of each technique. By measuring for total phospholipids using the 184 → 184 mass transition, LC/MS/MS analysis indicates that Phree Phospholipid Removal Plates result in significantly cleaner samples, reduced ion suppression, and extended HPLC/UHPLC column lifetime. The technique followed a simple procedure that is similar to a traditional protein precipitation, required no method development, and can be automated to provide higher throughput.

Introduction

Many different classes of phospholipids exist including phosphatidyl cholines and lysophosphatidyl cholines. When injected onto an LC/MS/MS, phospholipids have been shown to reduce HPLC/UHPLC column lifetime and sensitivity and are also responsible for ion suppression. Phospholipids are present in a majority of bioanalytical samples including whole blood and plasma. With an increasing number of clinical applications being developed from bioanalytical samples, the removal of phospholipids from the sample prior to LC/MS/MS analysis has become an important step for accurate analysis.

Traditionally, protein precipitation has been used as a cleanup step because it is rapid and does not require method development. We compared the cleanliness of protein precipitation to a cleanup using Phree Phospholipid Removal Plates which remove both protein and phospholipids from samples by monitoring for the presence of total phospholipids using the 184 → 184 mass transition, monitoring for ion suppression, and by performing a column lifetime study.

Experimental Conditions

Plasma samples were subjected to cleanup by both protein precipitation and cleanup using Phree Phospholipid Removal Plates. In order to ensure a fair comparison, plasma samples from the same lot were prepared using both techniques (**Table 1**).

Table 1. Sample Preparation Protocols

	Protein Precipitation	Phree Phospholipid Removal
1.	Add 300 µL Acetonitrile to the wells of a collection plate	Add 300 µL Acetonitrile with 1 % Formic acid to the wells of the Phree plate†
2.	Add 100 µL of plasma directly into the Acetonitrile	Add 100 µL of plasma directly into the Acetonitrile†
3.	Vortex for 2 minutes at maximum possible speed	Vortex for 2 minutes at maximum possible speed
4.	Pellet precipitated proteins by centrifuge	Filter using vacuum at 2-7 in Hg for up to 5 minutes*
5.	Collect supernatant, taking care not to disrupt pellet	

† If preferred, plasma may be added to the Phree plate first, followed by Acetonitrile with 1 % Formic acid.

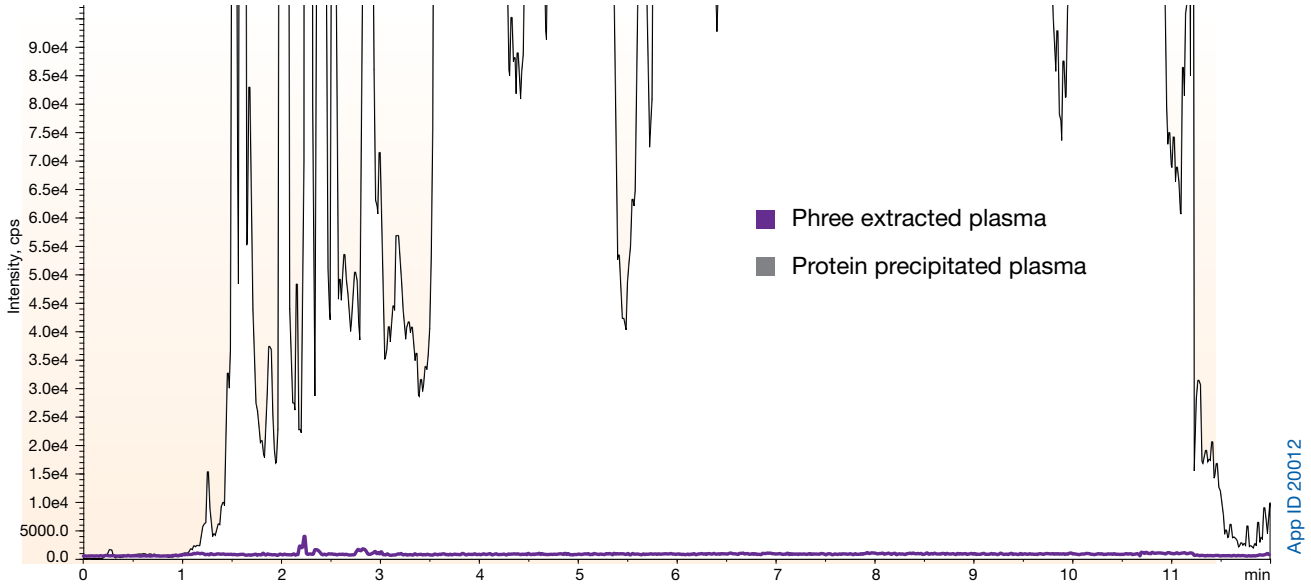
*Centrifugation and positive pressure may also be used.

The resulting filtrate from each cleanup technique was then injected onto a Kinetex® 2.6 µm C18 core-shell column coupled with an API 3000™ mass spectrometer (AB SCIEX, Framingham, MA, USA) (running conditions are listed in **Table 2**). Using m/z 184-184, the presence of phospholipids was monitored (**Figure 1**).

Table 2. LC/MS/MS Running Conditions for All Phospholipid Analyses

Column:	Kinetex® 2.6 µm C18 100 Å
Dimensions:	50 x 2.1 mm
Part No.:	00B-4462-AN
Mobile Phase:	A: 0.1 % Formic acid in Water
	B: 0.1 % Formic acid in Methanol
Flow Rate:	400 µL/min
Gradient:	**Time (min) % B**
	0 60
	0.5 95
	15.5 95
	15.51 60
	19.5 60
Temperature:	22 °C
Detection:	Mass Spectrometer (MS) @ 425 °C; 184 amu

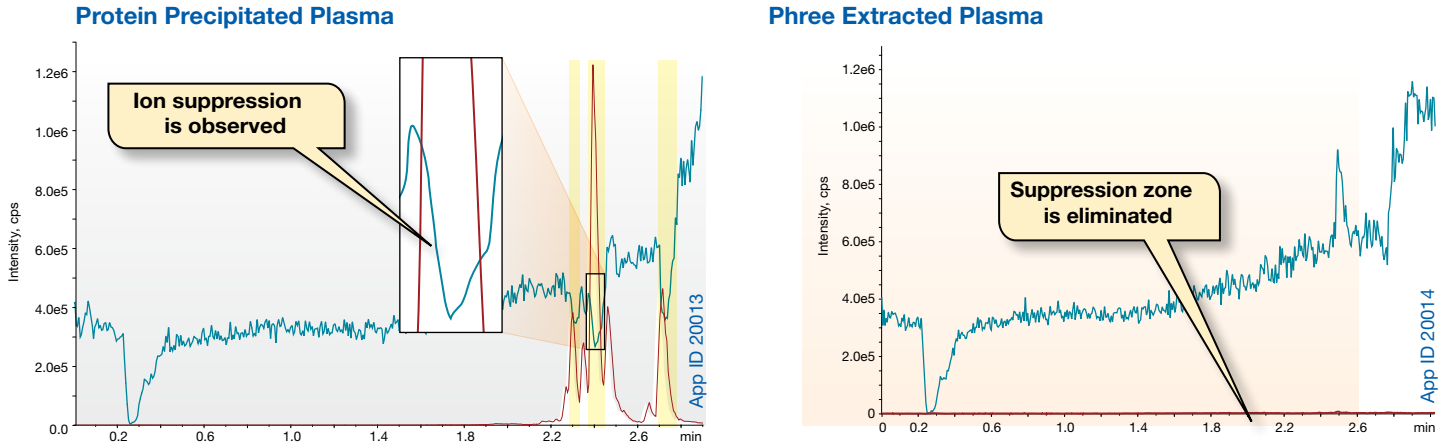
Figure 1. Total Phospholipid Profile of Protein Precipitated Plasma vs. Phree™ Extracted Plasma



See **Table 2** for LC/MS/MS running conditions

After the presence of phospholipids was monitored, ion suppression was studied. Post-column infusions of amoxapine were made to establish an ion suppression/enhancement profile with both protein precipitated plasma and Phree extracted plasma (**Figure 2**).

Figure 2. Ion Suppression/Enhancement Studies of Protein Precipitated Plasma vs. Phree Extracted Plasma

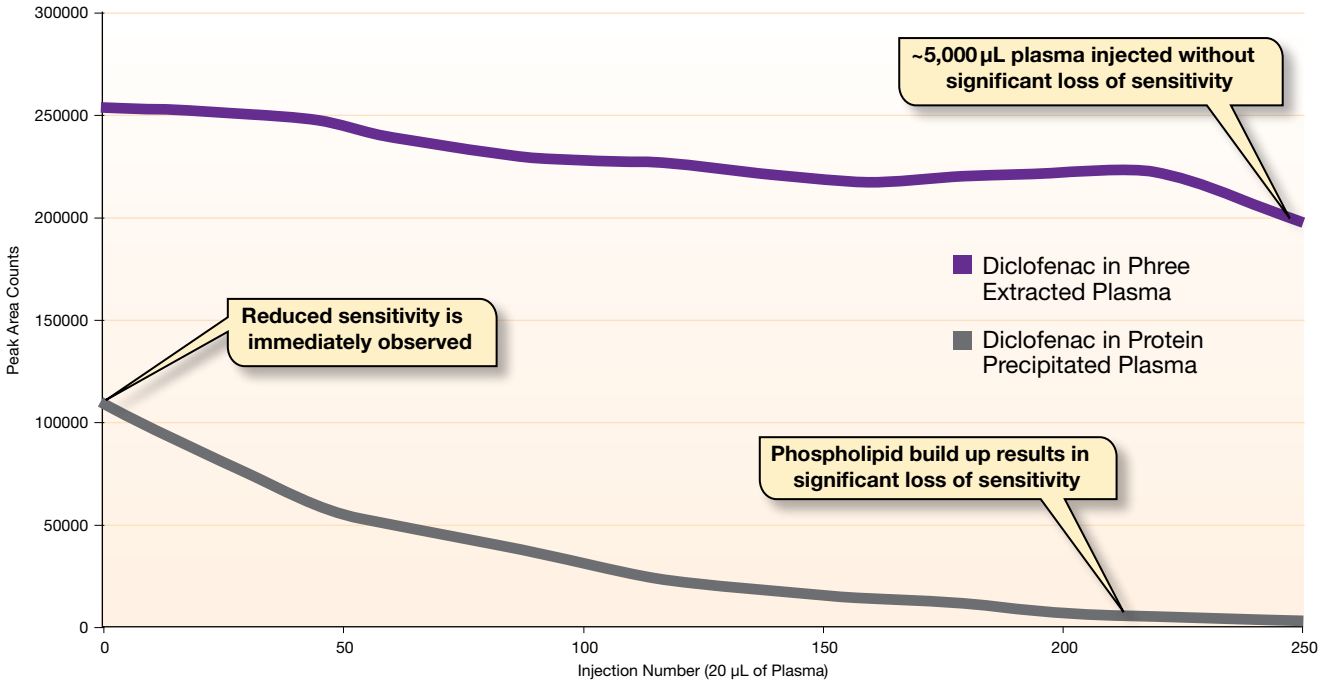


Amoxapine was infused post-column to establish an ion suppression/enhancement profile with both protein precipitated plasma (left) and Phree extracted plasma (right), showing that Phree can successfully reduce ion suppression.

See **Table 2** for LC/MS/MS running conditions

A final comparison was then performed by making repetitive 20 µL injections of diclofenac in each prepared plasma sample to study the effects of phospholipids on HPLC/UHPLC column lifetime (**Figure 3**).

Figure 3. Column Lifetime and Sensitivity after 250 Injections of Protein Precipitated Plasma vs. Phree™ Extracted Plasma



See **Table 2** for LC/MS/MS running conditions

Results and Discussion

By monitoring for the presence of phospholipids using the 184 →184 mass transition, it was clear that protein precipitation did not remove phospholipids (**Figure 1**). By following a nearly identical protein precipitation method on the Phree Phospholipid Removal Plates, the presence of phospholipids was greatly reduced. This confirmed that the Phree extracted plasma was virtually free of phospholipids while the protein precipitated plasma contained many phospholipids.

Once the presence/absence of phospholipids was confirmed in each of our prepared plasma samples, ion suppression was studied. The phospholipid rich protein precipitated plasma displayed ion suppression effects in the elution profile of amoxapine, creating a large dip in the amoxapine signal. The same amoxapine infusion study using Phree extracted plasma did not result in ion suppression (**Figure 2**), confirming that the ion suppression seen in the protein precipitated sample was due to the presence of phospholipids.

Our final study measured analyte response and column lifetime in diclofenac spiked in each of the prepared plasma samples. The protein precipitated sample immediately displayed a lower signal for diclofenac as compared to the Phree extracted plasma (about 100,000 peak area counts as compared to about 250,000 peak area counts that was observed in the Phree extracted plasma). This is attributed to an increase in ion suppression effects that occurred due to the presence of phospholipids in the protein precipitated samples. As repetitive 20 µL injections are made, the protein precipitated sample completely loses sensitivity after 200 injections while the Phree extracted plasma displays only a slight loss in sensitivity at 250 injections (**Figure 3**). This confirms that the removal of phospholipids in the Phree extracted samples greatly increased analyte sensitivity and column lifetime, allowing for more accurate quantitation and higher throughput due to the longer column lifetime. It can also be inferred that the reduction in phospholipids injected onto the HPLC/UHPLC column reduces the amount of phospholipids that can build up on the MS source, reducing the amount of mass spec maintenance that will be required.

Conclusion

By confirming the presence/absence of phospholipids in both protein precipitated and Phree extracted plasma samples, it was determined that phospholipids were directly responsible for negative chromatographic effects including ion suppression, reduced analyte sensitivity, and decreased column lifetime as well as an increase in the amount of mass spec maintenance required. By preparing samples with Phree, analysts can remove proteins and phospholipids in the same amount of time as a traditional protein precipitation, resulting in immediate improvements to their chromatography work.

APPLICATIONS

Ordering Infomation

Phree Phospholipid Removal Products

Part No.	Description	Unit
8B-S133-TAK	Phree Phospholipid Removal 1 mL Tube	100/box
8E-S133-TGB	Phree Phospholipid Removal 96-Well Plates	2/box

Accessories

Collection Plates (deep well, polypropylene)		
AH0-7192	Strata® 96-Well Collection Plate 350 µL/well	50/pk
AH0-7193	Strata 96-Well Collection Plate 1 mL/well	50/pk
AH0-7194	Strata 96-Well Collection Plate 2 mL/well	50/pk
AH0-8635	Strata 96-Well Collection Plate, 2 mL Square/Round-Conical	50/pk
AH0-8636	Strata 96-Well Collection Plate, 2 mL Round/Round, 8 mm	50/pk
AH0-7279	Strata 96-Well Collection Plate, 1 mL/well Round, 7 mm	50/pk
Sealing Mats		
AH0-8597	Sealing Mats, Pierceable, 96-Square Well, Silicone	50/pk
AH0-8598	Sealing Mats, Pre-Slit, 96-Square Well, Silicone	50/pk
AH0-8631	Sealing Mats, Pierceable, 96-Round Well 7 mm, Silicone	50/pk
AH0-8632	Sealing Mats, Pre-Slit, 96-Round Well 7 mm, Silicone	50/pk
AH0-8633	Sealing Mats, Pierceable, 96-Round Well 8 mm, Silicone	50/pk
AH0-8634	Sealing Mats, Pre-Slit, 96-Round Well 8 mm, Silicone	50/pk
AH0-7362	Sealing Tape Pad	10/pk
Vacuum Manifolds		
AH0-6023*	SPE 12-Position Vacuum Manifold Set, for tubes	ea
AH0-6024*	SPE 24-Position Vacuum Manifold Set, for tubes	ea
AH0-8950	Strata 96-Well Plate Manifold, Universal with Vacuum Gauge	ea

*Manifolds include: Vacuum-tight glass chamber, vacuum gauge assembly, polypropylene lid with gasket, male and female luers and yellow end plugs, stopcock valves, collection rack assemblies, polypropylene needles, lid support legs. Waste container included with 12-positive manifold.

guarantee

If Phree Phospholipid Removal products do not perform as well or better than your current phospholipid removal product, return the product with your comparative data within 45 days for a FULL REFUND.

Australia
t: 02-9428-6444
f: 02-9428-6445
auinfo@phenomenex.com

Austria
t: 01-319-1301
f: 01-319-1300
anfrage@phenomenex.com

Belgium
t: 02 503 4015 (French)
t: 02 511 8666 (Dutch)
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: (800) 543-3681
f: (310) 328-7768
info@phenomenex.com

Denmark
t: 4824 8048
f: +45 4810 6265
nordicinfo@phenomenex.com

Finland
t: 09 4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: 01 30 09 21 10
f: 01 30 09 21 11
franceinfo@phenomenex.com

Germany
t: 06021-58830-0
f: 06021-58830-11
anfrage@phenomenex.com

India
t: 040-3012 2400
f: 040-3012 2411
indiainfo@phenomenex.com

Ireland
t: 01 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: 051 6327511
f: 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: 001-800-844-5226
f: 001-310-328-7768
tecnicomx@phenomenex.com

The Netherlands
t: 030-2418700
f: 030-2383749
nlinfo@phenomenex.com

New Zealand
t: 09-4780951
f: 09-4780952
nzinfo@phenomenex.com

Norway
t: 810 02 005
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: (800) 541-HPLC
f: (310) 328-7768
info@phenomenex.com

Sweden
t: 08 611 6950
f: +45 4810 6265
nordicinfo@phenomenex.com

United Kingdom
t: 01625-501367
f: 01625-501796
ukinfo@phenomenex.com

United States
t: (310) 212-0555
f: (310) 328-7768
info@phenomenex.com

All other countries: Corporate Office USA
t: (310) 212-0555
f: (310) 328-7768
info@phenomenex.com

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