

# APPLICATIONS

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

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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, the most abundant being 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	

\*Centrifugation and positive pressure may also be used.

The resulting filtrate from each cleanup technique was then injected onto a Kinetex<sup>®</sup> 2.6 µm C18 core-shell column coupled with an API 3000<sup>™</sup> 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

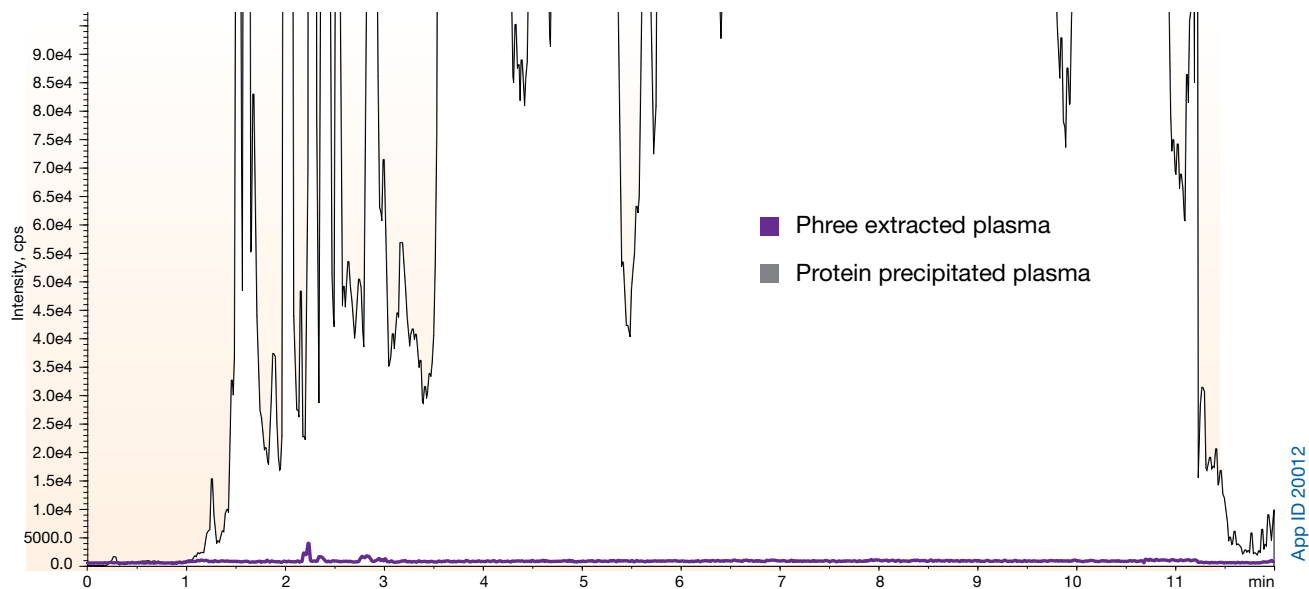
**Column:** Kinetex<sup>®</sup> 2.6 µm C18 100 Å  
**Dimensions:** 50 x 2.1 mm  
**Part No.:** 00B-4462-AN  
**Mobile Phase:** A: 0.1 % Formic acid in Water  
                   B: 0.1 % Formic acid in Methanol  
**Flow Rate:** 400 µL/min  
**Gradient:**

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



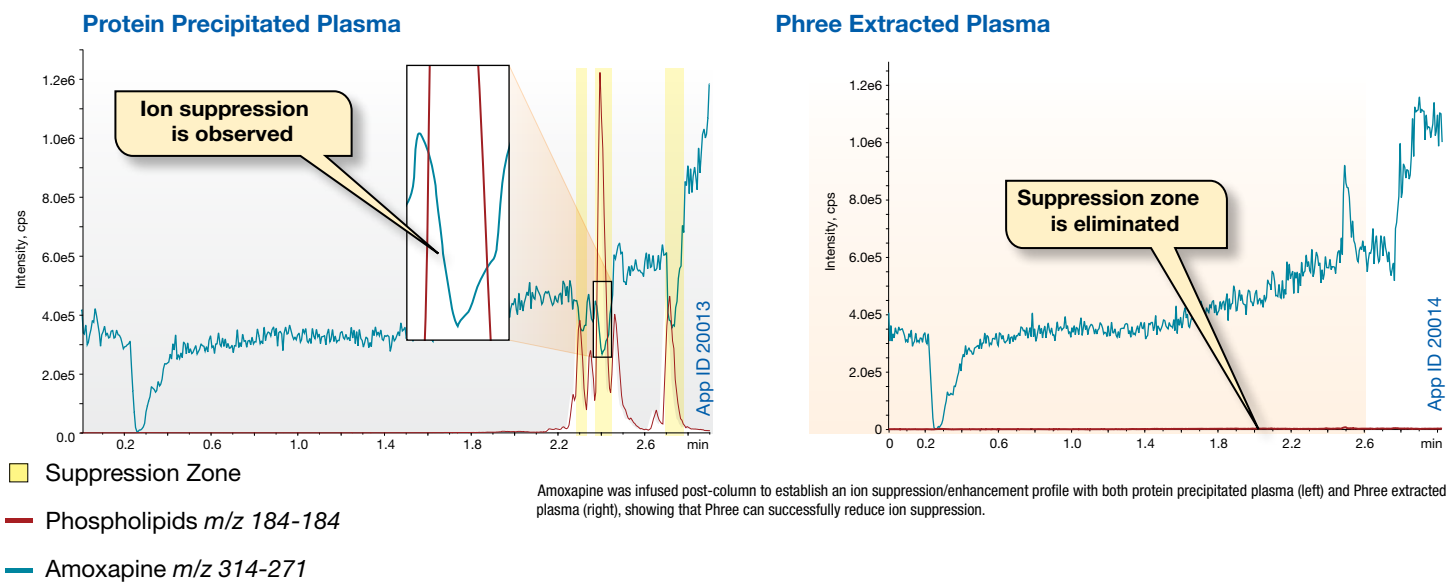
**Figure 1.** Total Phospholipid Profile of Protein Precipitated Plasma vs. Phree™ Extracted Plasma



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**).

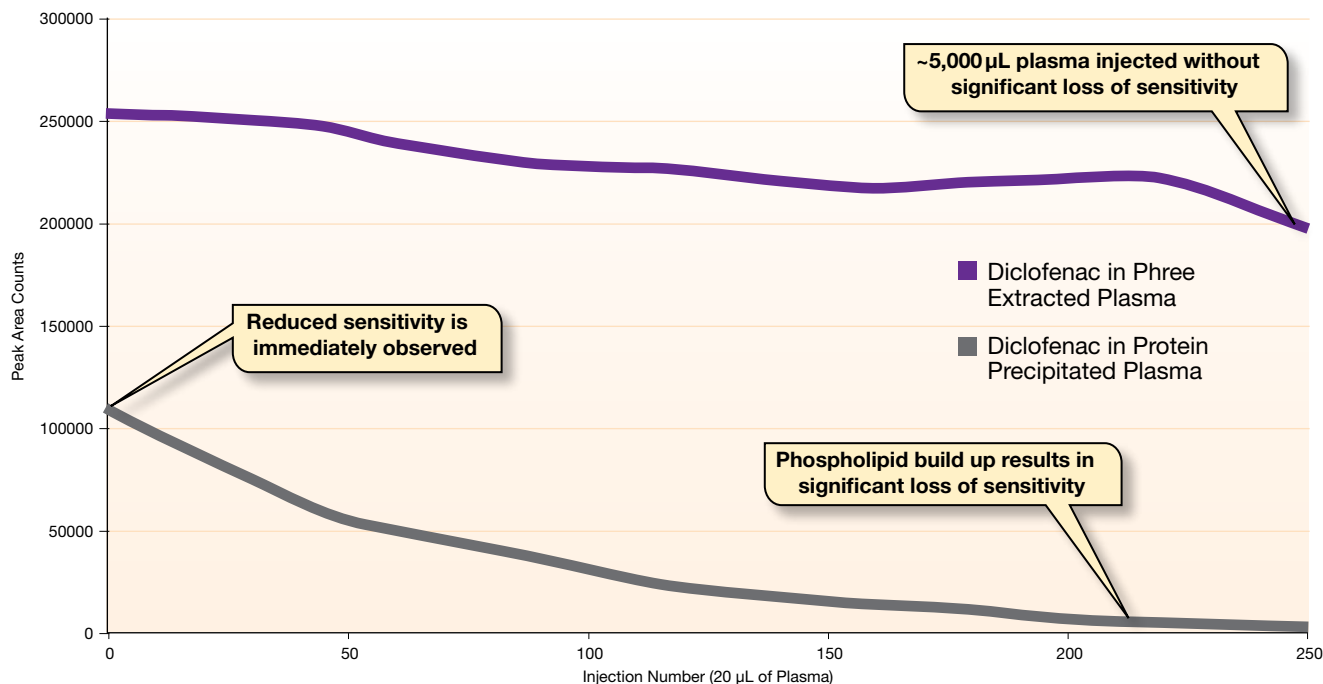
**Figure 2.** Ion Suppression/Enhancement Studies of Protein Precipitated Plasma vs. Phree Extracted Plasma



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

A final comparison was then performed by making repetitive 20  $\mu$ L 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 of 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 pre-

cipitated 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 Information

### Phree Phospholipid Removal Products

Part No.	Description	Unit
8E-S133-TGB	Phree Phospholipid Removal 96-Well Plates	2/box

### Accessories

#### Collection Plates (deep well, polypropylene)

AHO-7192	Strata <sup>®</sup> 96-Well Collection Plate 350 $\mu$ L/well	50/pk
AHO-7193	Strata 96-Well Collection Plate 1 mL/well	50/pk
AHO-7194	Strata 96-Well Collection Plate 2 mL/well	50/pk
AHO-8635	Strata 96-Well Collection Plate, 2 mL Square/Round-Conical	50/pk
AHO-8636	Strata 96-Well Collection Plate, 2 mL Round/Round, 8 mm	50/pk
AHO-7279	Strata 96-Well Collection Plate, 1 mL/well Round, 7 mm	50/pk

#### Sealing Mats

AHO-8597	Sealing Mats, Pierceable, 96-Square Well, Silicone	50/pk
AHO-8598	Sealing Mats, Pre-Slit, 96-Square Well, Silicone	50/pk
AHO-8631	Sealing Mats, Pierceable, 96-Round Well 7 mm, Silicone	50/pk
AHO-8632	Sealing Mats, Pre-Slit, 96-Round Well 7 mm, Silicone	50/pk
AHO-8633	Sealing Mats, Pierceable, 96-Round Well 8 mm, Silicone	50/pk
AHO-8634	Sealing Mats, Pre-Slit, 96-Round Well 8 mm, Silicone	50/pk
AHO-7362	Sealing Tape Pad	10/pk

#### Vacuum Manifolds

AHO-8950	Strata 96-Well Plate Manifold, Universal with Vacuum Gauge	ea
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**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.

#### Terms and Conditions

Subject to Phenomenex Standard Terms and Conditions, which may be viewed at <http://www.phenomenex.com/TermsAndConditions>.

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