Maximizing Analyte Recoveries using Phree™ Phospholipid Removal Plates

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Phospholipids have been known to cause a handful of negative affects in LC/MS/MS analysis including ion suppression, reduced column lifetime, and reduced analyte sensitivity. Removing phospholipids from samples can instantly improve LC/MS/MS analysis however it is important to ensure that analyte recovery is not negatively affected. This work focuses on maximizing recovery of target analytes using Phree Phospholipid Removal Plates.

Introduction

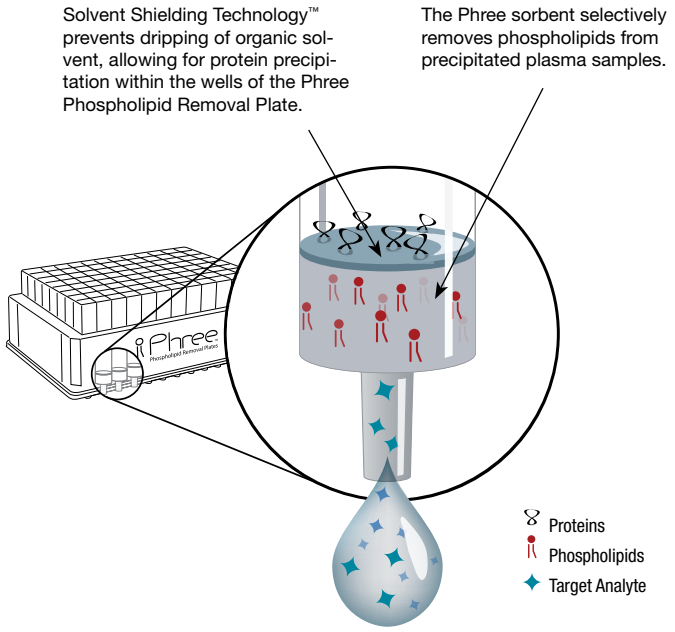
Phospholipids are lipids found in cell membranes and are present in all plasma, serum, and blood samples. They are known to cause problems in LC/MS/MS such as method variability, ion suppression, reduced method sensitivity, and contamination of LC columns and instrumentation. Protein precipitation, which is a popular means of sample cleanup, does not remove phospholipids.

When studying the affects of phospholipids in protein precipitated samples, decreased analyte sensitivity was observed after only 50 injections. Removing phospholipids using Phree Phospholipid Removal Plates reverses these affects. Phree Phospholipid Removal Plates remove both proteins and phospholipids simultaneously. The plates contain a unique frit system which holds up solvent and plasma until pressure is applied, allowing for protein precipitation to be performed within the wells of the plate. After precipitation, sample can be pulled through the Phree sorbent by vacuum, centrifugation, or positive pressure, leaving the precipitated proteins behind on the frit. As sample passes through the Phree sorbent, phospholipids are selectively removed and clean eluent is collected in a collection plate (**Figure 1**).

Although Phree selectively removes phospholipids without interacting with target analytes, some target compounds may exhibit lower analyte recoveries relative to protein precipitation.

In this technical note, two simple and effective ways to increase analyte recoveries are introduced to reinforce the many benefits of using Phree for phospholipid removal.

Figure 1. Protein and Phospholipid Removal using Phree.



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Experimental Conditions

Analyte recoveries were compared using the Phree™ generic procedure and an optimized Phree procedure.

Sample

Human plasma (Bioreclamation, Inc) was prepared with analytical standards obtained from (Sigma Aldrich, CDN Isotopes)

Generic Phree Phospholipid Removal Procedure

1. Dispense†

Acetonitrile with 1 % Formic acid was added to the wells of the Phree Phospholipid Removal Plate in a volume of 3-4x the volume of the intended plasma sample.

2. Add†

Plasma was added directly into the organic solvent in each well of the Phree Phospholipid Removal Plate. A final ratio of 3:1 Acetonitrile/plasma was maintained.

3. Vortex*

The plate was vortexed for 2 minutes at maximum possible speed, taking care not to allow cross contamination.

4. Filter

The Phree Phospholipid Removal Plate was placed on a 96-well manifold with a 96-well collection plate positioned inside the manifold chamber. Vacuum was applied at 2-7 inch Hg until filtrate was collected.

Optimized Phree Phospholipid Removal Procedure

1. Dispense†

Acetonitrile with 1 % Formic acid was added to the wells of the Phree Phospholipid Removal Plate in a volume of 3-13x the volume of the intended plasma sample.

2. Add†

Plasma (100 µL) was added directly into the organic solvent in each well of the Phree Phospholipid Removal Plate. The intended organic solvent to sample ratio (3-13x) was maintained.

3. Vortex*

The plate was vortexed for 2 minutes at maximum possible speed, taking care not to allow cross contamination.

4. Filter

The Phree Phospholipid Removal Plate was placed on a 96-well manifold with a 96-well collection plate positioned inside the manifold chamber. Vacuum was applied at 2-7 inch Hg until filtrate was collected.

Optional steps to optimize analyte recoveries

5. Add

A second elution of up to 1300 µL (using an organic/aqueous mixture in the same solvent to sample ratio used in Steps 1 and 2), was added into each well of the Phree Phospholipid Removal Plate.

6. Filter

The Phree Phospholipid Removal Plate was immediately filtered as described in Step 4 using the same 96-well collection plate or a new collection plate if desired. Filtrates from Steps 4 and 5 are combined, evaporated and reconstituted in mobile phase for LC/MS/MS analysis.

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

* When used with a liquid-handling instrument or automation, aspirate/dispense cycles may be used to promote in-tip mixing and precipitation. This will ensure complete precipitation and filtration. Vortexing is not necessary when in-tip mixing is performed.

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The resulting filtrate was then analyzed using m/z 184-184 to confirm the absence of phospholipids (**Table 1**). Analyte recovery was then observed by injecting the filtrate onto a Kinetex® 2.6 µm C18 core-shell HPLC/UHPLC column coupled with an API 3000™ mass spectrometer (AB SCIEX, Framingham, MA, USA) (**Table 2**).

Table 1. Phospholipid Profile Method

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	
Gradient:	**Time (min)**	**% B**
	0.00	60
	0.50	95
	15.50	95
	15.51	60
	19.50	60
Flow Rate:	400 µL/min	
Detection:	Mass Spectrometer (MS) @ 425 °C; 184 amu	
Temperature:	22 °C	
NEB:	8.00	
CUR:	12.00	
CAD:	10.00	
IS:	3500.00	
TEM:	500.00	
EP:	10.00	

Table 2. Analyte Recovery Method

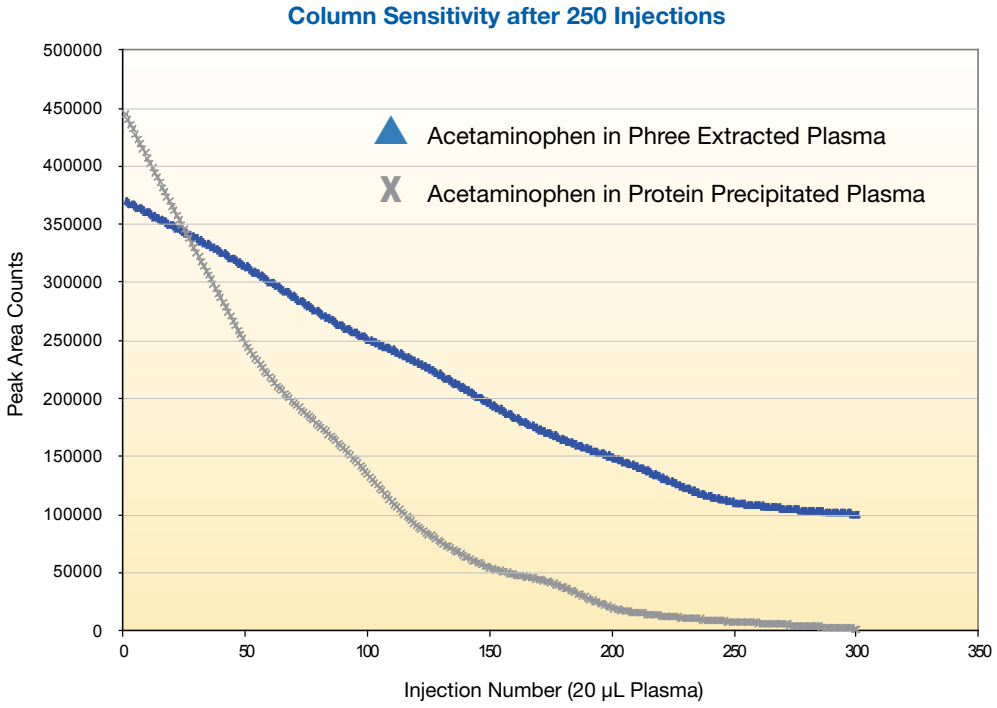
Column:	Kinetex 2.6 µm C18 100 Å	
Dimensions:	50 x 2.1 mm	
Part No.:	00B-4462-AN	
Mobile Phase:	A: 0.1 % Formic acid in Water B: 0.1 % Formic acid in Acetonitrile	
Gradient:	**Time (min)**	**% B**
	0.00	5
	2.00	95
	2.75	95
	2.76	5
	5.76	5
Flow Rate:	400 µL/min	
Detection:	API 3000	
NEB:	8.00	
CUR:	12.00	
CAD:	10.00	
IS:	3500.00	
TEM:	500.00	
EP:	10.00	

Results and Discussion

Figure 2 illustrates the problem of slightly lower recoveries for some compounds by depicting the lower signal observed for acetaminophen after Phree™ extraction relative to protein precipitation. A few modifications to the generic method were examined in search for effective ways to increase overall analyte recoveries. Plasma samples were subjected to cleanup using Phree Phospholipid Removal Plates following the generic Phree procedure and optimized Phree procedures.

The first approach that was evaluated was to increase the organic solvent to sample ratios (8:1 and 13:1 are described). The second approach was to employ a second elution of 500 µL and 1000 µL in the same organic strength as the initial elution. For example, if 100 µL of plasma was added to 400 µL of acetonitrile with 1 % formic acid to perform the initial precipitation, the second elution would be 500 µL (400 µL acetonitrile with 1 % formic acid mixed with 100 µL deionized water). These modifications were compared to the generic Phree procedure to study their affects on analyte recovery. Acidic, basic, and neutral probes were used in order to represent the full spectrum of analyte chemical properties that an assay may have to accommodate.

Figure 2. Sensitivity as a function of column lifetime shows slightly lower sensitivity for acetaminophen in the first 30 injections.

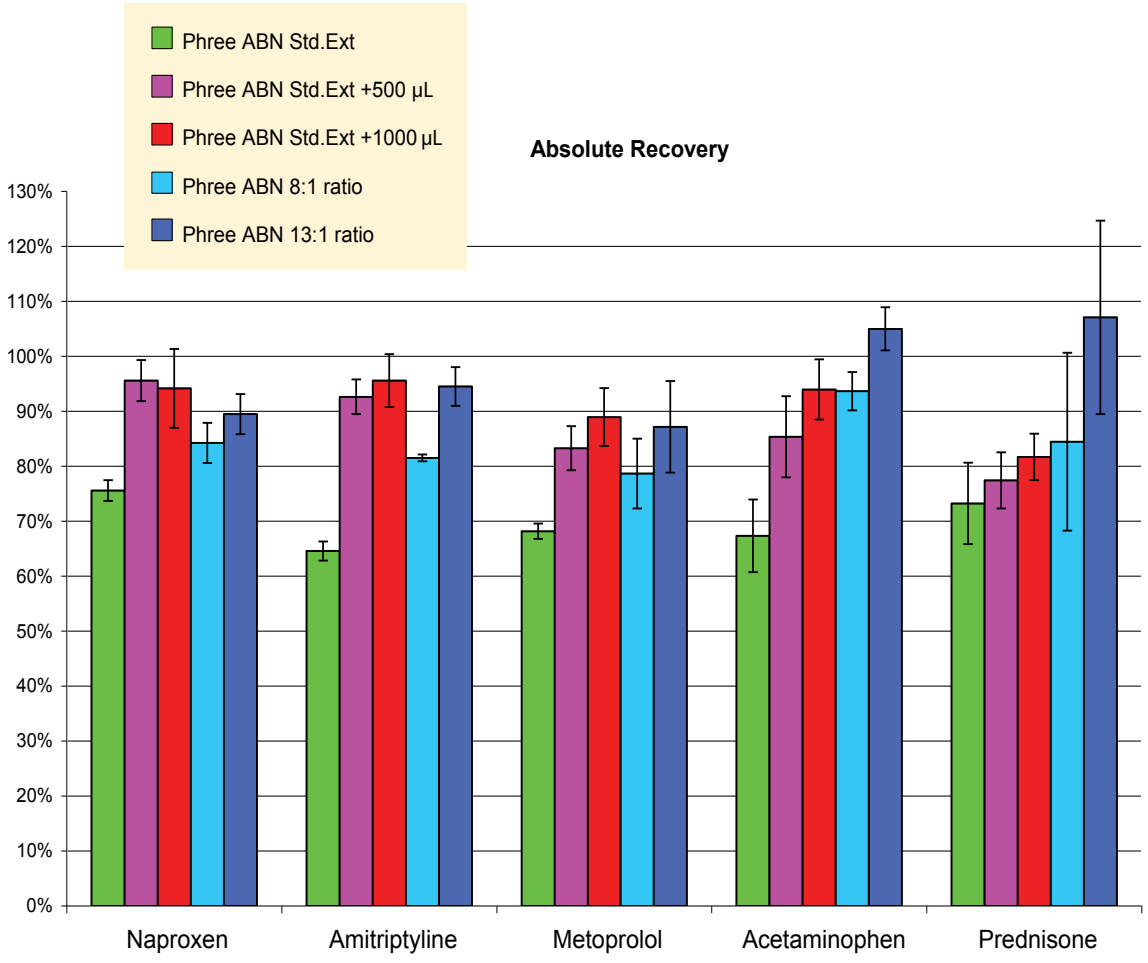


Acetaminophen monitored using m/z 152-110

Resulting analyte recoveries are shown in **Figure 3**. In all cases, higher recoveries were observed when an additional second elution of 500 µL or 1000 µL of the same organic strength as the first elution was applied, or when an increased organic solvent to sample ratio of 8:1 or 13:1 was applied. The data indicates a clear trend that increasing the elution volume will improve analyte recoveries. Phospholipid depletion analysis indicates that removal of phospholipids is excellent regardless of the increased elution volumes (**Table 3**).

Note: Increased elution volumes also require additional time for sample blown down steps. For most applications, an additional elution of 500 µL in the same organic strength as the first elution is recommended to increase overall recoveries while keeping the blow down time to a minimum.

Figure 3. Acidic, basic, and neutral (ABN) analyte recoveries using the generic Phree™ method (green), and a variety of modified methods: An additional elution of 500 µL (pink), an additional elution of 1000 µL (red), an organic solvent to sample ratio of 8:1 (light blue), and an organic solvent to sample ratio of 13:1 (dark blue).



Recovery data from samples using increased organic solvent to sample ratios demonstrated that higher analyte recoveries were attained when a larger organic solvent to sample ratio, such as 8:1, was used. This optimization technique works particularly well for small volume samples <100 µL because recovery losses are attributed to volume remaining in the frits and sorbent bed. When sample volumes are as low as 25 or 50 µL, recovery losses due to dead volume are exacerbated. In these cases, a minimum elution volume of 500 µL is recommended. For example, it is preferable to precipitate a 25 µL sample of plasma with 475 µL of organic.

In terms of phospholipid breakthrough (**Table 3**), only the use of a second elution of 1000 µL showed breakthrough greater than 1 %. It is interesting to point out that drying down the samples followed by reconstitution led to total elimination of any phospholipid signal from this sample.

References

1. General Ion Suppression - T. M. Annesley *Clin Chem*, 2003 v. 49, p 1041-1044
2. ESI suppression general – R. King et al.*J. Am. Soc. Mass*, 2000 v.11, p 942-950
3. General Lipid Ion Suppression – J.L. Little et all, *JChromatogr.*, B 2006 v. 833, p 219-230

Table 3. Percent Removal of Phospholipids from 100 µL Plasma

Phospholipids	Lyso 1	Lyso 2	PC 1	PC 2	PC 4
Phree™ Generic Method	>99	>99	>99	>99	>99
Phree Optimized Method with 500 µL 2nd Elution	>99	>99	>99	>99	>99
Phree Optimized Method with 1000 µL 2nd Elution	98.2	97.6	>99	>99	>99
8:1 Organic to Sample Ratio	>99	>99	>99	>99	>99
13:1 Organic to Sample Ratio	>99	>99	>99	>99	>99

Lyso 1: 1-Palmitoyl-2-OH-sn-glycero-phosphocholine, (16:0) m/z 496-184

Lyso 2: 1-Oleoyl-2-OH-sn-glycero-phosphocholine, (18:1) m/z 522-184

PC 1: 1-Palmitoyl-2-Oleoyl-sn-glycerol-phosphocholine, (16:0, 18:1) m/z 760-184

PC 2: 1-Stearoyl-2-Lindoleoyl-sn-glycerol-phosphocholine, (18:0, 18:2) m/z 786-184

PC 4: 1- Oleoyl-2-Lindoleoyl-sn-glycerol-phosphocholine, (18:1, 18:2) m/z 784-184

Conclusion

This work explored several phospholipid removal procedures using Phree Phospholipid Removal Plates to provide a guide for optimizing analyte recovery. This work concludes that two simple methods can produce significantly improved recoveries without introducing negative affects:

1. An increased organic solvent to sample ratio of up to 8:1
2. A secondary elution in the same organic strength as the initial elution of up to 500 µL

These easy and simple modifications to the generic Phree procedure noticeably increased overall analyte recoveries without additional phospholipid breakthrough, adding to the existing benefits of Phree Phospholipid Removal Plates as a complete and versatile solution for simultaneous protein precipitation and phospholipid removal.

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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)		
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		
AH0-8950	Strata 96-Well Plate Manifold, Universal with Vacuum Gauge	ea

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.

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