

# A Simpler, Faster Solution to Bioanalytical Sample Cleanup using Phree™ Phospholipid Removal 96-Well Plates

Xianrong (Jenny) Wei, Erica Pike and Sky Countryman  
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

*Ion suppression/enhancement, a reduction in analyte sensitivity, lowered precision, shifting of analyte retention times, and a decrease in HPLC/UHPLC column lifetime are just a few problems that can arise in LC/MS/MS analysis. These matrix effects are often due to endogenous phospholipids within bioanalytical samples. This technical note explores a common sample preparation technique, solid phase extraction (SPE), and compares it to a simpler, faster technique using a phospholipid removal product, Phree. Our work demonstrates that Phree Phospholipid Removal products can successfully remove more phospholipids as compared to a generic reversed phase SPE procedure without negatively impacting analyte recovery for acidic, basic, and neutral target compounds.*

## Introduction

A primary need in bioanalysis is to increase assay throughput and sensitivity. Common chromatography methods result in co-elution of both analytes and matrix components, forming a matrix effect. The primary cause of these matrix effects is endogenous phospholipids and lysophospholipids which result in ionization suppression or enhancement, thus altering the sensitivity of the LC/MS/MS analysis. Problems associated with the phospholipids include reduced analyte sensitivity, lowered precision, shifting of analyte retention time, decreased column life expectancy and increased mass spectrometry maintenance.

Correcting matrix effects requires an improvement in sample cleanup procedures in order to remove the aforementioned phospholipids. However, this process generally sacrifices sample throughput due to long or complicated extraction procedures, such as solid phase extraction (SPE), contributing to increased cost and time. This technical note demonstrates that Phree Phospholipid Removal 96-well plates provide a simpler and faster set-up to remove phospholipids and minimize matrix effects without compromising analyte recovery when compared to Waters® Oasis® HLB and Biotage® Evolute® ABN SPE.

The Phree plate contains a unique frit system which holds the solvent and plasma until pressure is applied, allowing for a protein precipitation to be performed within the wells of the plate. After precipitation, the sample is pulled through the Phree sorbent by vacuum, centrifugation, or positive pressure, retaining the proteins behind on the frit. As sample passes through the Phree sorbent, phospholipids are selectively removed and clean eluent is collected (**Figure 1**).

## Experimental Conditions

Sample cleanup and analyte recoveries were compared using generic procedures provided by the Phree 96-well plate, the Waters Oasis HLB SPE plate and the Biotage Evolute ABN SPE plate. A combination of acidic, basic, and neutral compounds were analyzed to represent a wide variety of target compounds. To assess the cleanup abilities of each technique, five major phospholipids were monitored during the sample injections and matrix effects were investigated through post column infusion experiments. In addition to SPE, protein precipitation was also performed alongside the Phree phospholipid removal procedure so that we could compare the original concentration of phospholipids to the amount of phospholipids that were depleted using the Phree and SPE procedures.

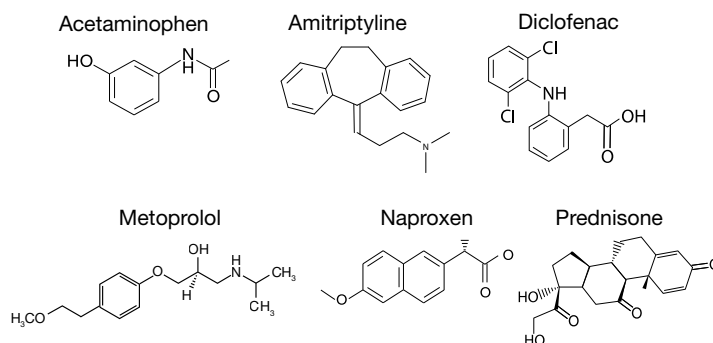
## Materials and Instrumentation

- AB SCIEX API 5000™ triple quadrupole mass spectrometer, TurbolonSpray™ ionization source (AB SCIEX, Framingham, MA)
- Agilent 1260 SL consisting of binary pumps and autosampler (Agilent, Santa Clara, CA)
- TurboVap 96 dryer (Biotage, Charlotte, NC)
- Phree Phospholipid Removal 96-well plates (Phenomenex, Torrance, CA)
- Waters Oasis HLB SPE 96-well plate (Waters, Milford, MA)
- Biotage Evolute ABN Express 96-well plate (Biotage, Charlotte, NC)
- Kinetex C18, 2.6 μm core-shell HPLC/UHPLC column, 50 x 2.1 mm (Phenomenex, Torrance, CA)

## Chemicals and Reagents

- Human plasma EDTA (Bioreclamation Inc, Hicksville, NY)
- Milli-Q Water (In-house)
- Acetonitrile (Fisher Scientific, Pittsburgh, PA)
- Methanol (Fisher Scientific, Pittsburgh, PA)
- Formic Acid (Fisher Scientific, Pittsburgh, PA)
- Amitriptyline, m.w. 277 (Sigma Aldrich, St. Louis, MO)
- Acetaminophen, m.w. 151 (Sigma Aldrich, St. Louis, MO)
- Diclofenac, m.w. 296 (Sigma Aldrich, St. Louis, MO)
- Metoprolol, m.w. 267 (Sigma Aldrich, St. Louis, MO)
- Naproxen, m.w. 230 (Sigma Aldrich, St. Louis, MO)
- Prednisone, m.w. 358 (Sigma Aldrich, St. Louis, MO)
- Amitriptyline-D6, m.w. 283 (CDN Isotopes, Pointe-Claire, QC)
- Acetaminophen-D7, m.w. 158 (CDN Isotopes, Pointe-Claire, QC)
- Diclofenac-D4, m.w. 300 (CDN Isotopes, Pointe-Claire, QC)
- Metoprolol-D7, m.w. 274 (CDN Isotopes, Pointe-Claire, QC)
- Naproxen-D3, m.w. 233 (CDN Isotopes, Pointe-Claire, QC)
- Prednisolone, m.w. 360 (CDN Isotopes, Pointe-Claire, QC)

## Analyte Structures



## SPE and Phree™ Extraction Procedures

SPE Method using  
Waters® Oasis® HLB6-10 minutes  
per samplePlus 10 Minutes  
to Prepare Solvents**Condition**  
1 mL Methanol**Equilibrate**  
1 mL Water**Load**  
100 µL Plasma diluted with 300 µL Water**Wash**  
1 mL 5 % Methanol in Water**Elute**  
500 µL MethanolSPE Method using  
Biotage® Evolute® ABN6-10 minutes  
per samplePlus 15 Minutes  
to Prepare Solvents**Condition**  
1 mL Methanol**Equilibrate**  
1 mL 0.1 % Formic Acid in Water**Load**  
100 µL Plasma diluted with 300 µL 2 % Formic  
Acid in Water**Wash**  
1 mL of 5 % Methanol in Water**Elute**  
500 µL MethanolPhree™ Phospholipid  
Removal Method3 minutes  
per samplePlus 5 Minutes  
to Prepare Solvents**Dispense**  
100 µL Plasma**Dispense**  
300 µL 1 % Formic Acid in Acetonitrile**Mix****Filter**  
via vacuum, positive pressure, or centrifugation

## Chromatographic Conditions

Column:	Kinetex® 2.6 µm C18 100 Å	Gradient:	Time (min)	B (%)
Dimensions:	50 x 2.1 mm		0.00	5
Part No.:	00B-4462-AN		2.00	95
Mobile Phase:	A: 0.1 % Formic Acid in Water		3.00	95
	B: 0.1 % Formic Acid in Acetonitrile		3.01	5
			5.00	5
Flow Rate:	400 µL			
Temperature:	50 °C			
Detection:	API 5000™, AB SCIEX			

## Mass Transitions

ID	Q1	Q3	Dwell	DP	CE	CXP
Acetaminophen	152.1	110.1	25	73	25	11
Acetaminophen-D7	159.1	115.1	25	76	62	11
Amitriptyline	278.3	233.3	25	90	70	13
Amitriptyline-D6	284.3	233.3	25	90	70	13
Diclofenac	296	278.1	25	100	50	14
Diclofenac-d4	300	255.3	25	100	50	13
Metoprolol	268.3	116.3	25	100	45	11
Metoprolol-D7	275.8	123.2	25	100	60	15
Naproxen	231.1	185.1	25	100	30	13
Naproxen-D3	234.1	188.2	25	100	30	13
Prednisone	359.3	341.2	25	120	68	13
Prednisolone	361.2	343.2	25	100	50	13

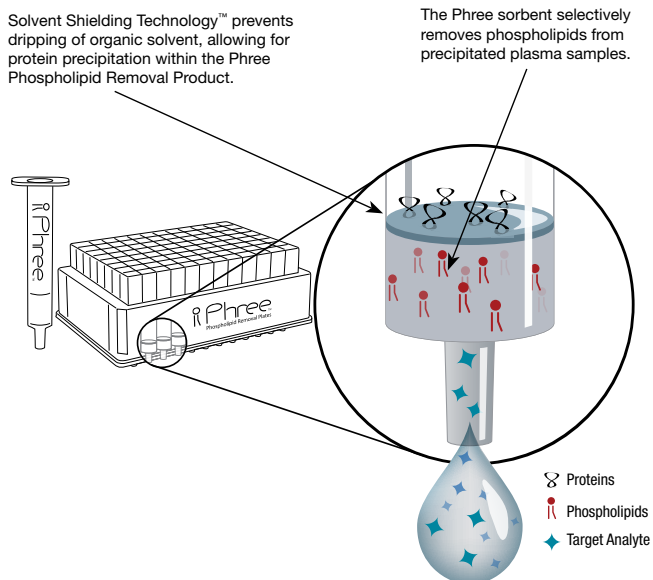
## Results and Discussion

Based upon the data, phospholipid removal using Phree provided a simple and generic method that resulted in consistent recoveries for acids, bases, and neutrals. When extracted using generic reversed phase SPE procedures, the same analytes did not exhibit reproducible recoveries indicating that it may be necessary to develop separate SPE methods for each compound class. Phree allowed us to analyze a broader range of compounds in a single cleanup step without the need to develop new methods for each compound. By reducing or eliminating the time spent developing methods and preparing samples, Phree optimizes cost efficiency

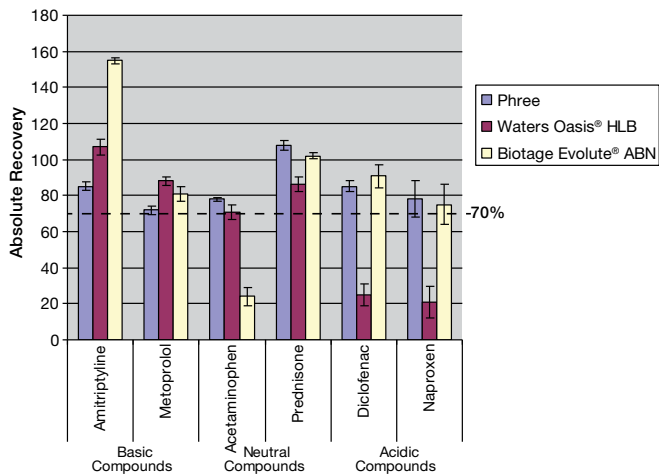
without compromising results. Phree exhibited a 72-108 % range in recovery with an average CV % of only 7.24 % (n=5 for each compound) across all samples tested. (Figure 2)

After analyte recovery was determined, we monitored for the presence of phospholipids to determine the extent of cleanup provided by each technique as well as to monitor for matrix effects due to the endogenous phospholipids. Phree Phospholipid Removal products selectively removed more than 99 % of all phospholipids from the plasma samples including phosphatidyl cholines and lysophosphatidyl cholines. As compared to generic reversed phase SPE procedures, which left a significant amount of phospholipids in the cleaned up sample, Phree was superior at removing the five major phospholipids that we monitored during analysis. This minimized matrix effects while reducing the cost and time required for sample analysis (Figure 3).

**Figure 1.**  
Protein and phospholipid removal using Phree

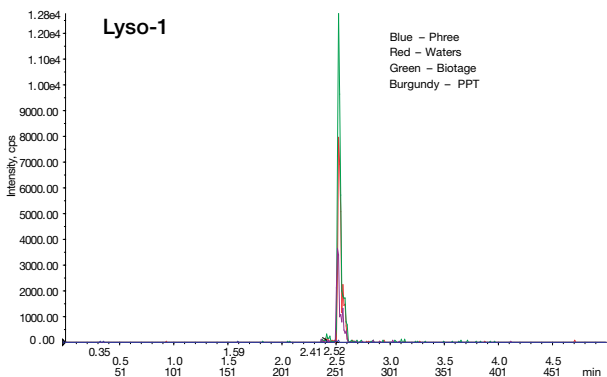


**Figure 2.** Acidic, basic and neutral analyte absolute recoveries using Phree™ Phospholipid Removal (blue), Waters® Oasis® HLB SPE (red) and Biotage® Evolute® ABN SPE (yellow)

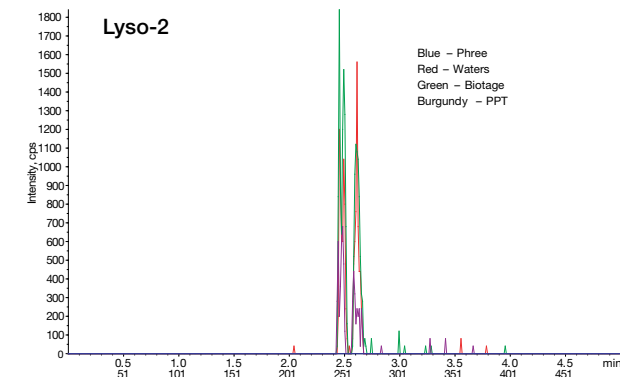


N=5 for all cleanup techniques

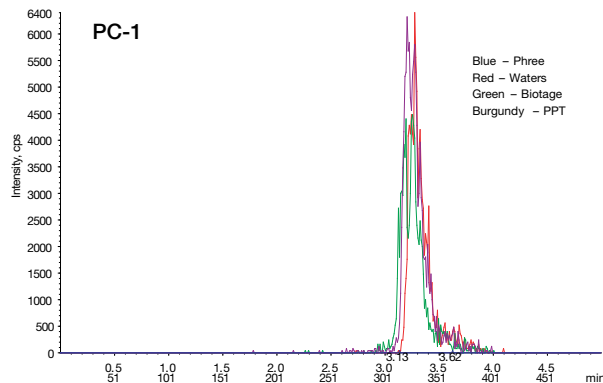
**Figure 3.** Profile of five major phospholipids in Phree extracted plasma, SPE extracted plasma, and protein precipitated plasma



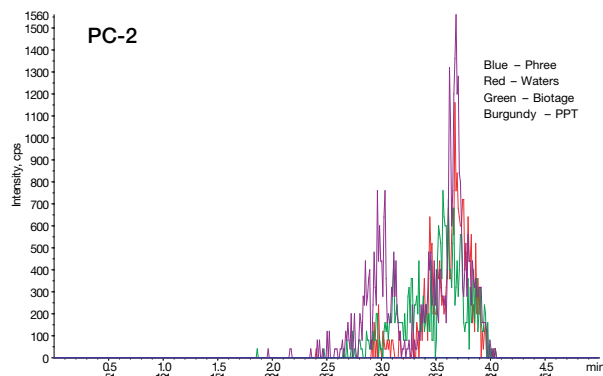
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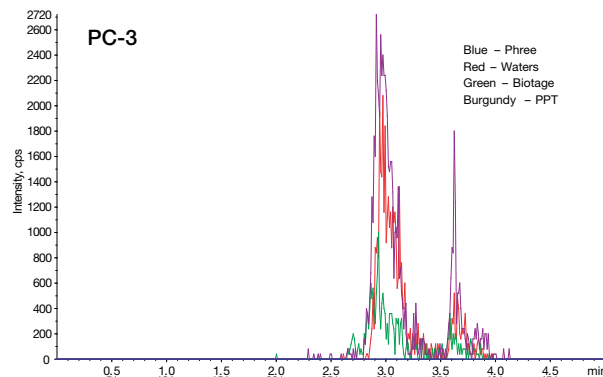
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**Lysophosphatidyl cholines:**

Lyso-1: 1-Palmitoyl-2-OH-sn-glycero-phosphocholine, 496>184 m/z

Lyso-2: 1-Oleoyl-2-OH-glycero-phosphocholine, 522>184 m/z

**Phosphatidyl cholines:**

PC-1: 1-Palmitoyl-2-Oleoyl-sn-glycerol-phosphocholine, 760>184 m/z

PC-2: 1-Stearoyl-2-Linoleoyl-sn-glycerol-phosphocholine, 786>184 m/z

PC-3: 1-Oleoyl-2-Linoleoyl-sn-glycerol-phosphocholine, 784>184 m/z

**Conclusion**

This project demonstrated recoveries of acids, bases and neutrals and their respective matrix effects caused by phospholipids and lysophospholipids using Phree Phospholipid Removal 96-well plates, Waters Oasis HLB and Biotage Evolute ABN SPE 96-well plates. The data concludes that Phree selectively removed both phospholipids and lysophospholipids better than SPE when acids, bases, and neutrals were extracted using a generic reversed phase procedure. The Phree extraction method provided a rapid, simple, and transferable platform to achieve cleaner samples, saving time on method development and sample preparation. In addition, analyte recovery was uncompromised using Phree in all cases yielding the same or better results than SPE.



## Ordering Information

### 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)

AHO-7192	Strata® 96-Well Collection Plate 350 µL/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-6023*	SPE 12-Position Vacuum Manifold Set, for tubes	ea
AHO-6024*	SPE 24-Position Vacuum Manifold Set, for tubes	ea
AHO-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.

#### Australia

t: 02-9428-6444  
f: 02-9428-6445  
auiinfo@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



### www.phenomenex.com

Phenomenex products are available worldwide. For the distributor in your country, contact Phenomenex USA, International Department at international@phenomenex.com



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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