



# ***Preventing Matrix Effects By Using New Sorbents to Remove Phospholipids From Biological Samples***

**K.C. Van Horne<sup>1,2</sup> and Patrick K. Bennett<sup>1</sup>**

<sup>1</sup>Tandem Labs, Salt Lake City, UT; <sup>2</sup>Hologent Technologies Inc., Baldwin Park, CA

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**Tandem Labs-Salt Lake City**

1121 East 3900 South  
Salt Lake City, UT 84124  
(801) 293-2400  
(801) 313-6495 Fax

**Tandem Labs-New Jersey**

115 Silvia Street  
West Trenton, NJ 08628  
(609) 434-0044  
(609) 434-0033 Fax

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## **INTRODUCTION**

Many separate factors can interfere with proper quantitation in bioanalytical LC/MS/MS. Among the most significant of these are ionization suppression/enhancement, and matrix effects. Ionization suppression/enhancement is a reduction/increase of detected signal that results when one or more species are ionized concurrently. Species which may fall into this category include eluent modifiers and analytical system contaminants (for example, salts), and both endogenous and exogenous species. Matrix effects are cross-sample differences (i.e., suppression or enhancement) in detected signal that may result from varying sample composition within a particular sample set and a given analytical method. Matrix effects can cause a number of analytical problems, including erroneous quantitative results if stable label internal standard(s) are not used, and can also result in inaccurate determination of a method LLOQ.

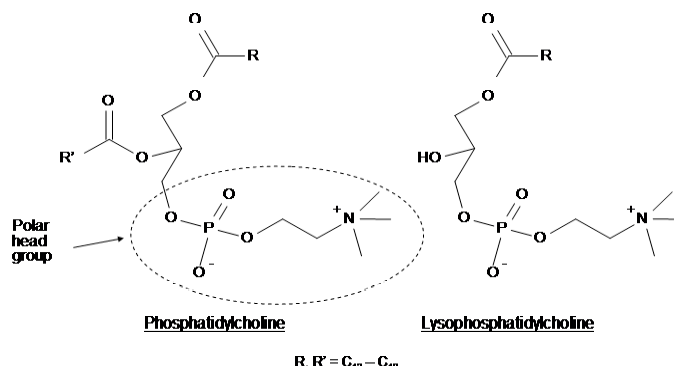
## **RESEARCH GOAL**

In our laboratory we determined that one major class of compounds causing significant ionization suppression/enhancement and matrix effects includes endogenous phospholipids and lysophospholipids. The goal of this research was to develop a generalized approach for removal of phospholipid substances, in order to reduce these effects. This was accomplished through development of new proprietary extraction sorbents with high specificity for phospholipids. These extraction sorbents employ the high oxophilicity of lanthanide metals, as a selective binding mechanism for the phosphate groups within the phospholipid molecules.

## PHOSPHOLIPIDS

Overview: Phospholipids are an important class of biological compounds serving many functions within the organism. Phospholipids may be characterized on a molecular level as having two major functional group regions: a) a polar head group substituent, which includes an ionizable organic phosphate moiety as well as other polar groups of various types, and b) one or two long chain fatty acid ester groups, which impart considerable hydrophobicity to the molecule.

**Figure 1 - Common Phospholipids**



This unique combination of functional groups defines many of the characteristics of phospholipids, and promotes their biological roles, including cell wall composition, transport, and surfactant properties. Moreover, the highly ionic nature of phospholipids makes them logical candidates to influence ionization in electrospray MS sources, since confirmed by this research

## PHOSPHOLIPID REMOVAL

Over the course of our experiments, we evaluated many different sorbent chemistries that might offer facile removal of phospholipids from biological samples and extracts. This goal was complicated by the desire to ultimately identify or create an extraction chemistry that was as selective as possible; i.e., one that would not concurrently remove desirable pharmaceutical analytes. Many different mechanisms were evaluated, including reverse-phase (non-polar), and both anion and cation exchange.

In the end, we identified immobilized lanthanide metal centers as having the highest degree of selective binding for the phosphate groups within the phospholipids. This approach was then implemented via extraction sorbents used alone or in combination with protein precipitation, liquid/liquid extraction ("LLE"), or solid phase extraction ("SPE"). A portion of our results are reported herein.

## Analytical Conditions

### Chromatographic Conditions

Column:	Phenomenex 5mm Luna phenyl-hexyl column, 2 mm x 50 mm
Mobile Phase:	A: 10mM ammonium acetate, pH unadjusted B: 10:90 water:acetonitrile containing 10mM ammonium acetate
Injection Volume:	10 - 20 $\mu$ L
Flow rate:	300 $\mu$ L/min
Linear gradient:	Starting at either 30 or 45% (B) Ending at 100% (B) at 5 minutes Followed by 5 additional minutes at 100% (B)

### Mass Spectrometer Conditions

Instruments:	MDS Sciex API 365 API 3000 API 365 with Ionics <i>EP10</i> <sup>+</sup> upgrade
Ionization source:	TurboIonSpray <sup>TM</sup>
HPLC:	Shimadzu 10 ADvP gradient system
Autosampler:	Perkin-Elmer PE200

### Standards/Samples

Phospholipid analytical reference standards, including phosphatidylcholine and lysophosphatidylcholine, were purchased from Avanti Lipids, Alabaster, Alabama, USA. An additional mixed-phospholipid reference sample was prepared from pooled Na-EDTA human plasma using a classical Bligh-Dyer LLE technique.

Triprolidine, quinidine, ketoconazole, and reserpine analytical reference standards were purchased from Sigma Chemical, St. Louis, MO, USA.

Blank samples were either pooled or single lot Na-EDTA human plasma. Spiked plasma samples were prepared by dry-down of standard solutions, followed by reconstitution in the appropriate volume of plasma.

Proprietary SPE columns were supplied by Hologent Technologies, Inc., Baldwin Park, CA, USA.

### Phospholipid/Analyte Measurement

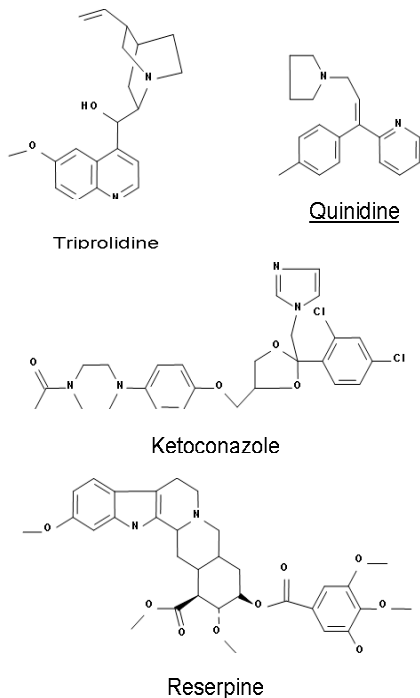
Five naturally-occurring phospholipids in human plasma, including two lysophospholipids, were used to monitor phospholipid removal from biological extracts and samples. These five species exhibit MRM transitions via TurboIonSpray in positive ionization mode:

Lysophospholipids	Phospholipids
m/z 496→m/z 184	m/z 704→m/z 184
m/z 524→m/z 184	m/z 758→m/z 184
	m/z 806→m/z 184

To evaluate the selectivity of our extraction procedures for phospholipids versus desired pharmaceutical analytes, a mixture of four test analytes was employed, at low mg/mL concentrations. The test analytes and their respective MRM transitions via TurboIonSpray in positive ionization mode were:

- Triprolidine (m/z 279→m/z 208)
- Quinidine (m/z 325→m/z 81)
- Ketoconazole (m/z 531→m/z 82)
- Reserpine (m/z 610→m/z 195)

Figure 2 – Analyte mix



- The lanthanide center is an essential element for highly selective phospholipid extraction.
- The new sorbents consistently removed over 90% of endogenous phospholipids from plasma or serum, yet gave high recoveries of the test analytes.
- The sorbents may be readily used in combination with protein precipitation or LLE.

- Infusions from traditional extraction techniques resulted in significantly lower responses for the analyte test mixture than extractions in which endogenous phospholipids were removed by lanthanide sorbent treatment.

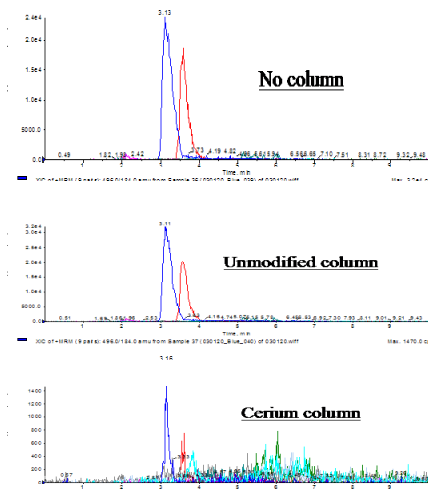
## Experiment 1

This experiment demonstrates the significance of lanthanide modification of an extraction sorbent for lysophospholipid retention.

A commercial silica-based carboxylic acid sorbent column was modified with a suitable inorganic cerium salt. A non-modified column was used as a control. 200  $\mu$ L of Bligh-Dyer phospholipid extracts of human Na-EDTA plasma, reconstituted in methanol, were used a) non-processed, b) processed through the non-modified column, c) processed through the cerium-modified column. Recoveries of lysophospholipids having molecular ions of 496.0 and 524.0, were calculated for both column-processed samples as compared to the non-processed samples.

Sample	496.0	%	524.0	%
No column	2.4E+04	100%	1.9E+04	100%
Unmodified column	3.2E+04	133%	2.0E+04	105%
Cerium-modified column	1.5E+03	6%	7.0E+02	4%

Typical chromatograms are shown.



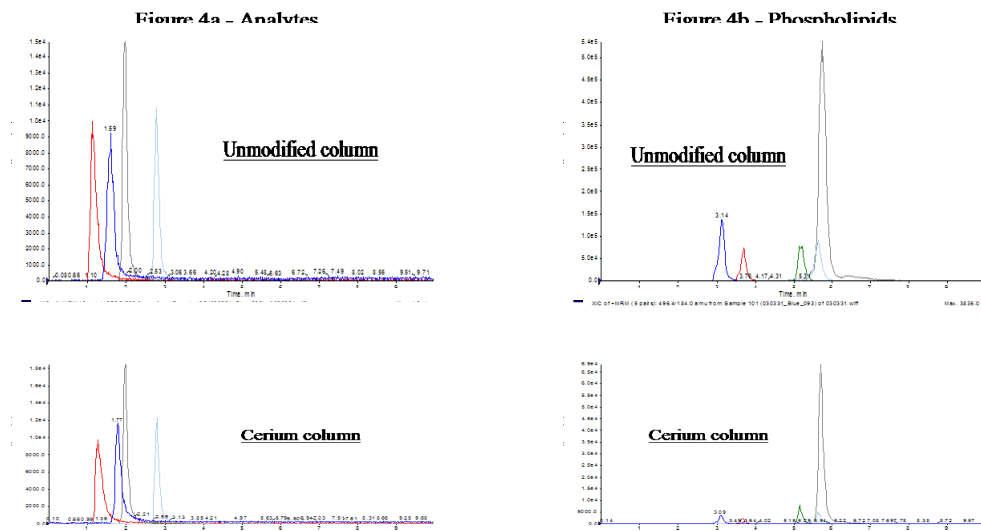
## Experiment 2

This experiment demonstrates the use of a lanthanide sorbent column for selective removal of phospholipids after a protein precipitation clean-up procedure.

A commercial silica-based propyl sulfonic acid sorbent column was modified with a suitable inorganic cerium salt. A non-modified column was used as a control. To 400  $\mu$ L of spiked human Na-EDTA plasma was added 2 mL of methanol, followed by vortexing, centrifugation, supernatant removal, dry-down, and reconstitution in methanol. The final methanol extracts were passed through, respectively, a) the non-modified column and b) the cerium-modified column. Relative recoveries of the analyte spiking compounds, as well as endogenous phospholipids, from the modified versus non-modified column are shown. (Table 2)

Sample	Triprolidine		Quinidine		Ketoconazole		Reserpine			
Unmodified column	1.23E+05	100.0%	1.17E+05	100.0%	1.37E+05	100.0%	9.17E+04	100.0%		
Cerium-modified column	1.36E+05	110.6%	1.27E+05	108.5%	1.56E+05	113.9%	106000	115.6%		
	<b>496</b>		<b>524</b>		<b>704</b>		<b>758</b>		<b>806</b>	
Unmodified column	1.53E+06	100.0%	7.01E+05	100.0%	9.62E+05	100.0%	7.12E+06	100.0%	1.27E+06	100.0%
Cerium-modified column	3.24E+04	2.1%	1.80E+04	2.6%	6.04E+04	6.3%	6.01E+05	8.4%	5.49E+04	4.3%

Representative chromatograms for analytes (Figure 4a), and for phospholipids (Figure 4b) are shown.



### Experiment 3

This experiment demonstrates the use of a lanthanide sorbent column for selective removal of phospholipids after a MTBE LLE clean-up procedure.

A proprietary cerium sorbent column was prepared. To 400  $\mu$ L of spiked human Na-EDTA plasma was added 2 mL of MTBE. The samples were vortexed, centrifuged, and the MTBE fraction removed by freeze-pour. One of the MTBE fractions was further treated by passing through the cerium column. The final extracts were then dried down, reconstituted in methanol, then analyzed.

Relative recoveries of the analyte spiking compounds, as well as endogenous phospholipids, from the column-treated sample, versus the non-treated sample are shown (Table 3).

Sample	Triprolidine		Quinidine		Ketoconazole		Reserpine			
No column treatment	1.09E+05	100.0%	8.40E+04	100.0%	1.40E+05	100.0%	9.82E+04	100.0%		
Cerium column treatment	1.10E+05	100.6%	8.54E+04	101.7%	1.43E+05	102.5%	9.57E+04	97.5%		
	<b>496</b>		<b>524</b>		<b>704</b>		<b>758</b>		<b>806</b>	
No column treatment	1.26E+04	100.0%	6.26E+03	100.0%	1.01E+05	100.0%	2.32E+06	100.0%	3.00E+05	100.0%
Cerium column treatment	6.44E+02	5.1%	2.37E+02	3.8%	2.98E+03	3.0%	1.22E+05	5.3%	1.21E+04	4.1%

Representative chromatograms for analytes (Figure 5a), and for phospholipids (Figure 5b) are shown.

Figure 5a - Analytes

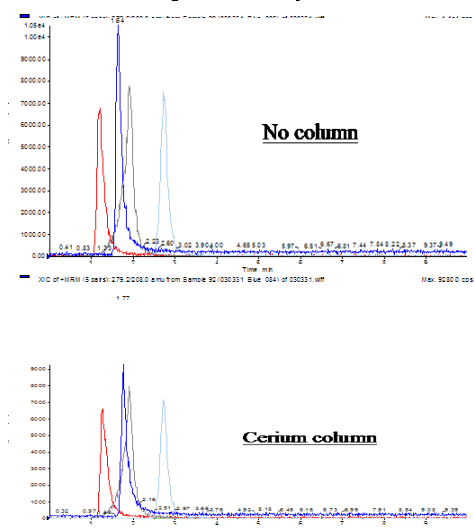
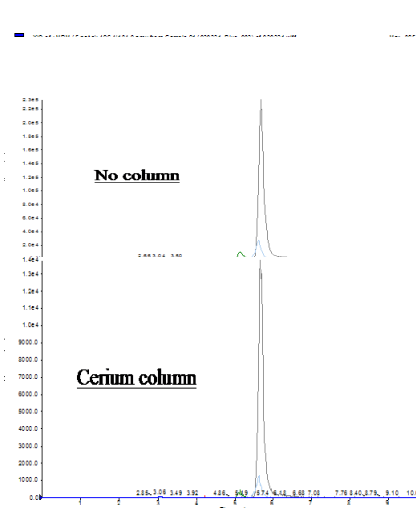


Figure 5b - Phospholipids



## Experiment 4

This experiment demonstrates the effect on analyte signal levels during co-infusion of plasma extracts, with and without treatment by a lanthanide sorbent column.

A proprietary cerium sorbent column was prepared. To 300  $\mu$ L of human Na-EDTA plasma was added 1.5 mL of MTBE. The samples were vortexed, centrifuged, and the MTBE fraction removed by freeze-pour. One of the MTBE fractions was further treated by passing through the cerium column. These extracts were then spiked with the analyte mixture, vortexed, dried down and reconstituted in acetonitrile. The reconstituted samples were then infused at 10  $\mu$ L per minute, against an isocratic mobile phase of 20:80:water:methanol containing 10mM ammonium formate. Comparison of the analyte signal levels for sorbent-treated and non-sorbent treated infusion samples is shown. (Figure 6).

