



# ***Identification of the Major Endogenous and Persistent Compounds in Plasma, Serum and Tissue That Cause Matrix Effects with Electrospray LC/MS Techniques***

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## PURPOSE

A primary need in bioanalysis is to increase assay throughput and sensitivity. To achieve these goals, an increase in the organic content of the eluting solvent or normal phase chromatography is often employed. However, a common chromatographic result of this can be co-elution of the analyte(s) with matrix components. This co-elution can result in ionization suppression (or enhancement) and may result in matrix effects. We have determined that primary components causing matrix effects are endogenous phospholipids (e.g., phosphatidylcholines) and lysophospholipids. We also identified persistent dioctyl-phthalate as an additional suppressor, but did not elucidate this further in the present work.

## INTRODUCTION

Many factors can interfere with proper quantitation in bioanalytical LC/MS/MS<sup>1</sup>. 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 (e.g., 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.

Table 1 shows an example of matrix effects<sup>2</sup> from four individual human plasma donors.

**C2 SPE & Luna Phenyl-Hexyl HPLC**

Analyte	OPC-P			
Lot Number	1	2	3	4
Replicate				
1	1.556	0.9545	1.692	2.145
2	1.453	1.138	1.666	2.057
3	1.639	1.104	1.765	1.869
<b>Overall Mean</b>	<b>1.682</b>			
Average	1.550	1.065	1.708	2.024
Standard Deviation	0.0930	0.0975	0.0515	0.1409
% Deviation from Overall Mean	-7.9%	<b>-36.7%</b>	1.5%	<b>20.3%</b>
% RSD	6.0%	9.1%	3.0%	7.0%

**C2 SPE & Asahipak ODP HPLC**

Analyte	OPC-P			
Lot Number	1	2	3	4
Replicate				
1	0.848	0.8629	0.944	0.902
2	0.929	0.969	0.842	0.764
3	0.928	0.909	0.856	0.850
<b>Overall Mean</b>	<b>0.884</b>			
Average	0.902	0.914	0.881	0.839
Standard Deviation	0.0467	0.0535	0.0552	0.0697
% Deviation from Overall Mean	2.0%	3.4%	-0.3%	-5.1%
% RSD	5.2%	5.9%	6.3%	8.3%

**C2 SPE & Lightning C8 HPLC**

Analyte	OPC-P			
Lot Number	1	2	3	4
Replicate				
1	0.512	0.5463	0.542	0.628
2	0.576	0.577	0.543	0.507
3	0.596	0.536	0.542	0.561
<b>Overall Mean</b>	<b>0.555</b>			
Average	0.561	0.553	0.542	0.565
Standard Deviation	0.0442	0.0212	0.0007	0.0605
% Deviation from Overall Mean	1.1%	-0.4%	-2.4%	1.8%
% RSD	7.9%	3.8%	0.1%	10.7%

Top Chromatogram:

Illustrates very large deviations from the overall mean for samples 2 and 4 due to co-elution of ion-suppressors with the target analyte (i.e., large matrix effect).

Middle and Bottom Chromatograms:

Using different HPLC columns, show significantly reduced deviations due to better chromatographic selectivity, and separation of the target analyte from the matrix effectors (reduced matrix effects).

## **Standard and Samples**

Phospholipid analytical reference standards, including phosphatidylcholine and lysophosphatidylcholine, were purchased from Avanti Polar 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.

Samples were either pooled or single lot Na-EDTA human plasma or mouse plasma obtained from BioChemed Pharmacologicals, Inc., Winchester, Virginia, USA.

## **Analytical Conditions**

Mass Spectrometer:	MDS Sciex API 3000
Ionization source:	TurboIonSpray™
HPLC:	Shimadzu 10 ADvP gradient system
Autosampler:	Perkin-Elmer PE200 Series
HPLC Column:	Phenomenex Luna phenyl-hexyl 2mm x 50mm, 5 mm

Analytical Conditions (Figure 1a)

## **HPLC Conditions**

Flow rate:	0.700 mL/min
"A" solvent:	10mM ammonium acetate, pH unadjusted
"B" solvent:	10:90 "A":acetonitrile
Linear gradient:	Starting = 30% "B"; ending = 100% "B"
Gradient time:	18 min
Injection size:	20 µL

Sample conditions

Na-EDTA human plasma, protein-precipitated with ten volumes of acetonitrile

Analytical Conditions (Figures 3a-3d)

## HPLC Conditions

Flow rate:	0.700 mL/min
"A" solvent:	0.1% formic acid in water
"B" solvent:	0.1% formic acid in acetonitrile
Linear gradient:	Starting = 40% "B"; ending = 100% "B"
Gradient time:	6 min
Injection size:	20 µL

### Infusion conditions

- 4-analyte mix, proprietary compounds at 500-1000 ng/mL
- Infusion rate 10 µL/minute

### Sample conditions:

(Figures 3a & 3c): Mouse plasma, protein-precipitated with ten volumes of acetonitrile

(Figures 3b & 3d): Treated with lanthanide column for phospholipid removal

Method	Results	Figures
1. Extracts from plasma and serum from various species were systematically analyzed for endogenous and persistent compounds in sufficient concentrations to likely cause suppression or enhancement	The principal ion-suppressors or enhancers can be seen to elute in two primary regions of the Total Ion Chromatogram using common reverse-phase LC columns	Figure 1a
2. Chromatographic peaks present at high levels were then isolated via fraction collection.	<ul style="list-style-type: none"> <li>• Mid-eluters: 30-50% organic elution m/z 496, 520, 522, 524</li> <li>• Late-eluters: 70-85% organic elution m/z 758, 760, 784, 786, 806, and 808</li> </ul>	Figure 1b Figure 1c
3. MS and MS/MS spectra from these samples were obtained in positive ion mode and compared to pure reference standards.	Product ions m/z 184 and m/z 104 are typical for phospholipids and lysophospholipids <sup>5</sup>	Figure 2a Figure 2b
4. By infusing analytes while injecting reference standards or extracts <sup>3</sup> , the presence of suppression and matrix effects resulting from phospholipids and dioctyl-phthalate were confirmed.	Demonstrates a correlation between suppression regions in the infusion signal and chromatographic elution of the phospholipids	Figure 3a Figure 3b

<b>Method</b>	<b>Results</b>	<b>Figures</b>
5. Once identified, experiments were performed in an attempt to selectively remove these compounds from samples, to promote reduced ion suppression, enhancement and matrix effects using a proprietary lanthanide extraction sorbent <sup>4</sup> .	Demonstrates a reduction of ion suppression effects in the analyte infusion signals and a corresponding > 99% reduction in the phospholipid peak areas	Figure 3c Figure 3d

## **Discussion**

Endogenous phospholipids:

- Are found in plasma at significant concentrations
- Appear at variable levels organism-to-organism
- Are typically not completely removed during extractions
- May be characterized on a molecular level as having two major functional group regions:
  - a) A polar head group substituent, including an ionizable organic phosphate moiety and other various types of polar groups, including groups imposing zwitterionic character, and
  - b) One or two long chain fatty acid ester groups, which impart considerable hydrophobicity to the molecule (Figure 4).
- Are logical candidates to influence ionization in electrospray MS because of their highly ionic nature

The diversity of endogenous phospholipid structure causes widely distributed chromatographic elution, as shown herein. Therefore, it is difficult to move the analyte chromatographically into a zone not exhibiting phospholipid co-elution in order to avoid ionization suppression/ enhancement effects.

Thus, selective pre-analysis removal of the phospholipids may be necessary to allow analyte elution and ionization without interference.

The use of proprietary lanthanide-based extraction chemistries has proven to be effective for this purpose.

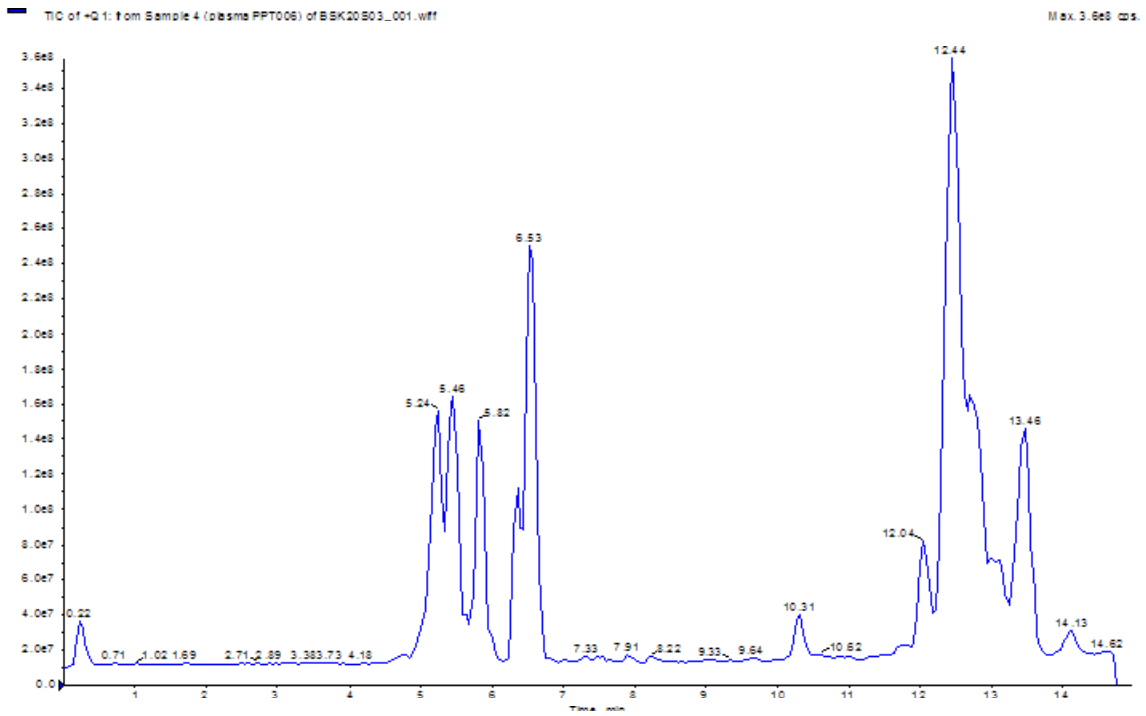
## CONCLUSION

1. Two major sources of ionization suppression and matrix effects in positive ion mode result from endogenous phospholipids and environmentally persistent dioctyl-phthalate.
2. The presence of these compounds may result in divergent calibration curves and assay imprecision.
3. Matrix effects can sometimes be avoided by resolving these components from analytes chromatographically.
4. Because of instability, removal of these compounds prior to injection is optimal.
5. We have demonstrated an effective approach to removal of the phospholipids by utilizing lanthanide-based extraction chemistries.

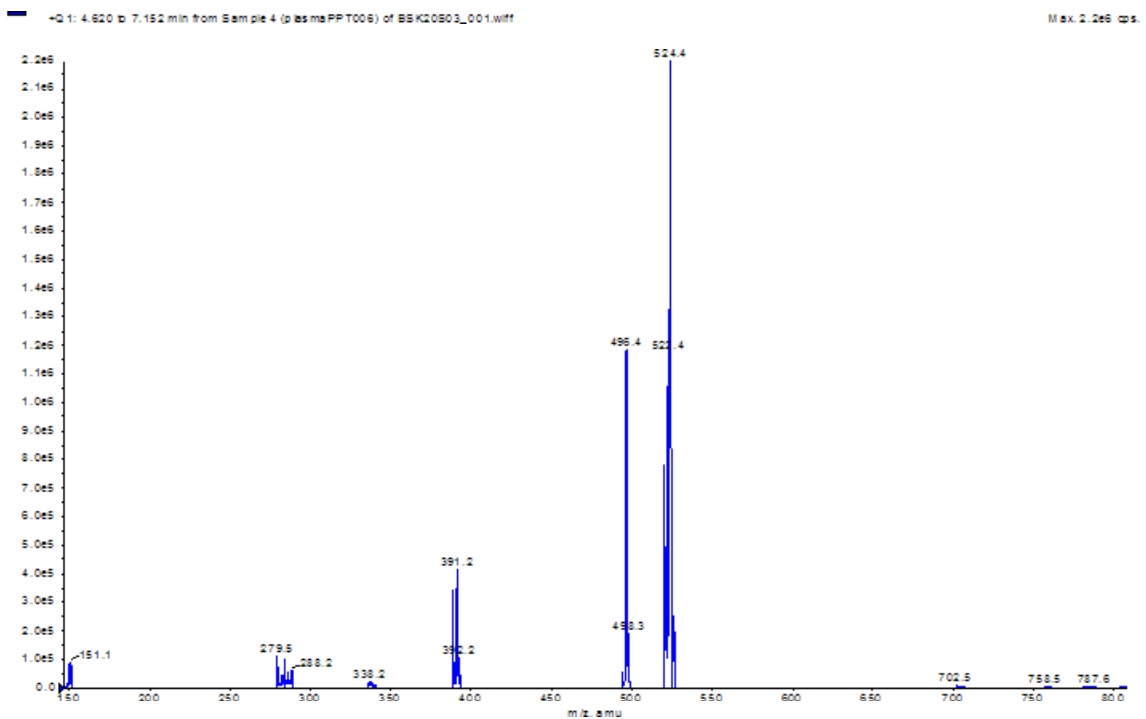
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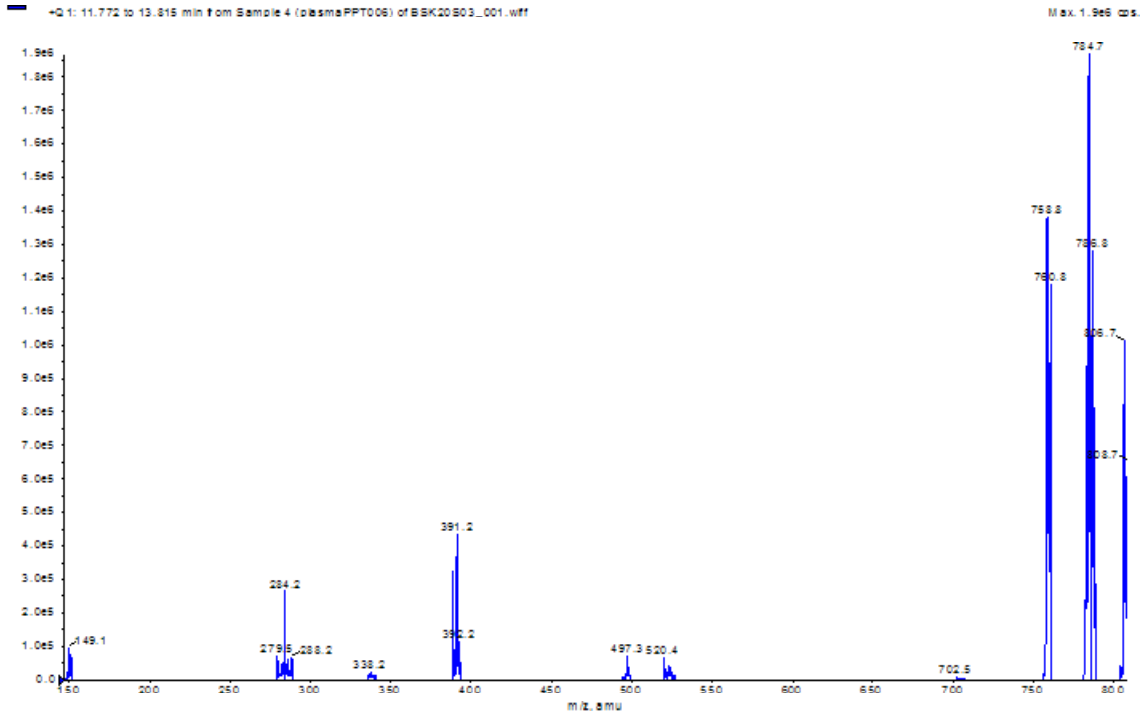
**Figure 1a**



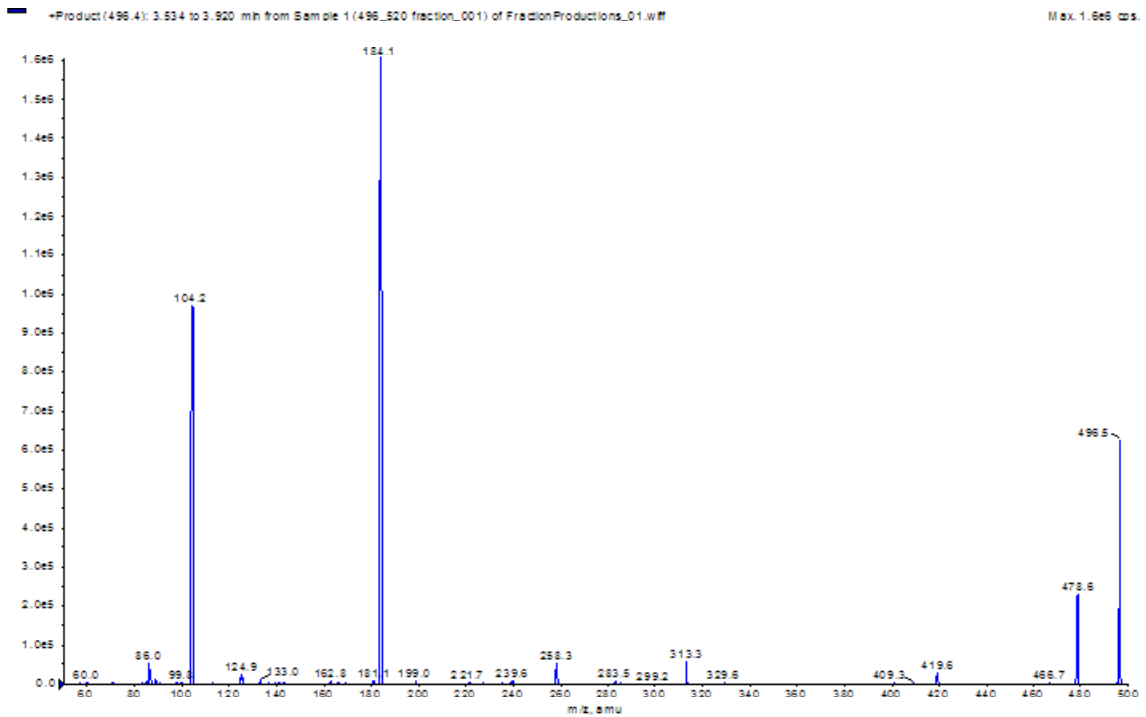
**Figure 1b**



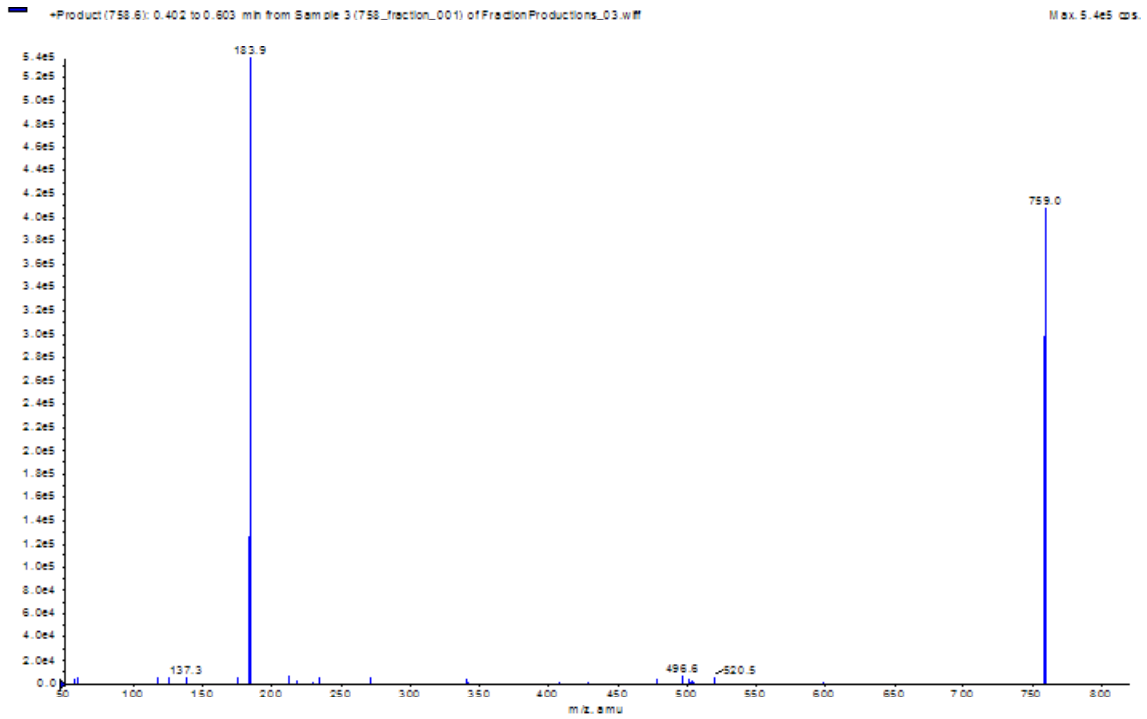
**Figure 1c**



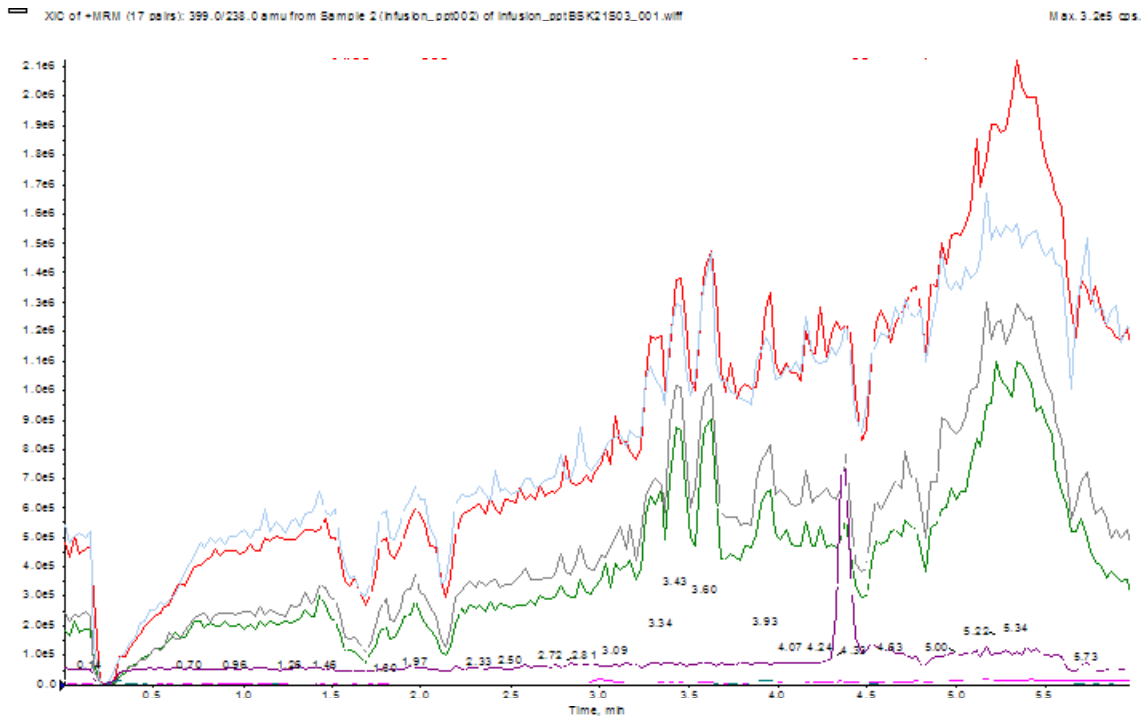
**Figure 2a**



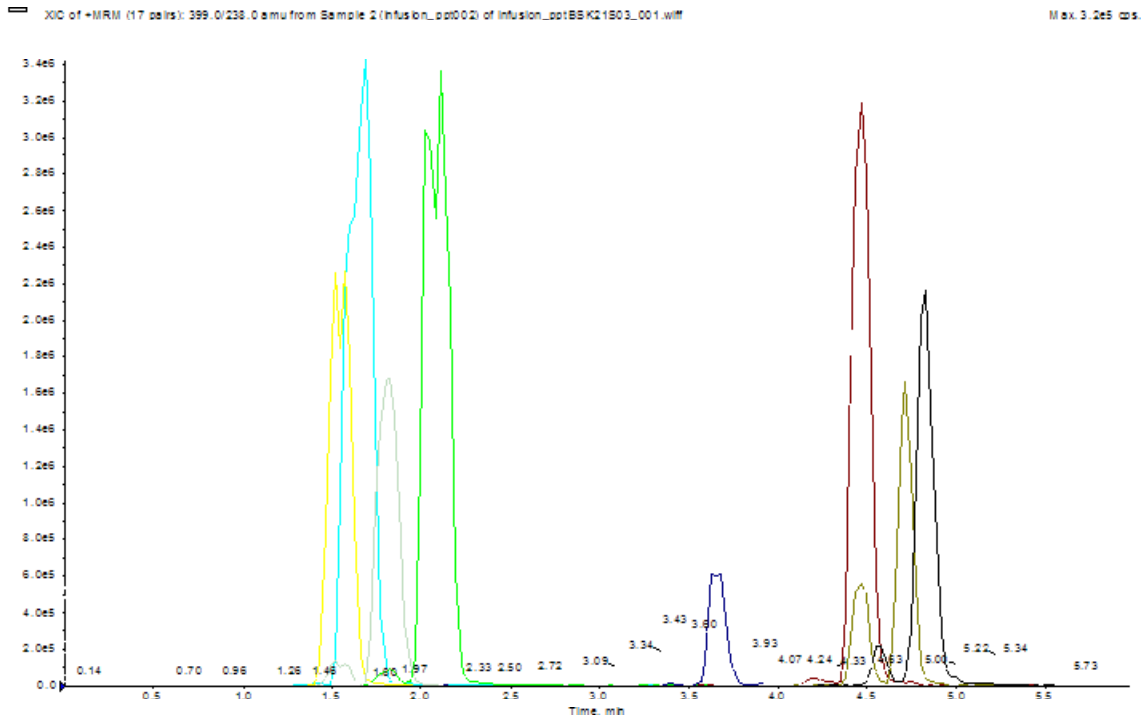
**Figure 2b**



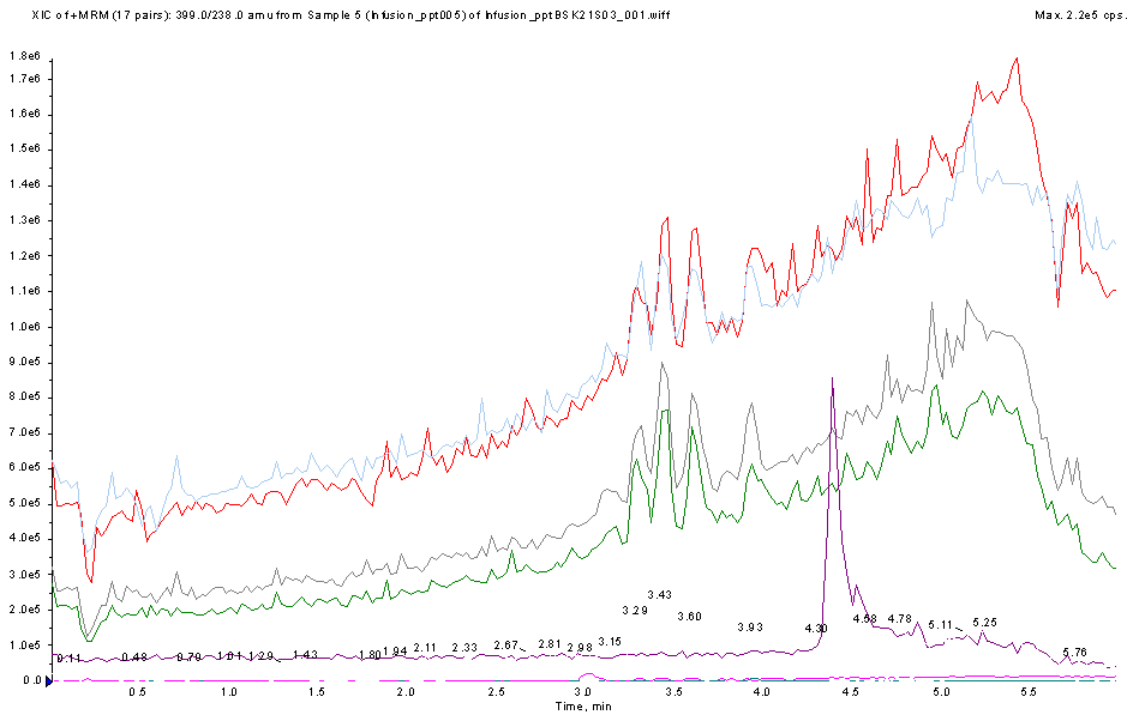
**Figure 3a**



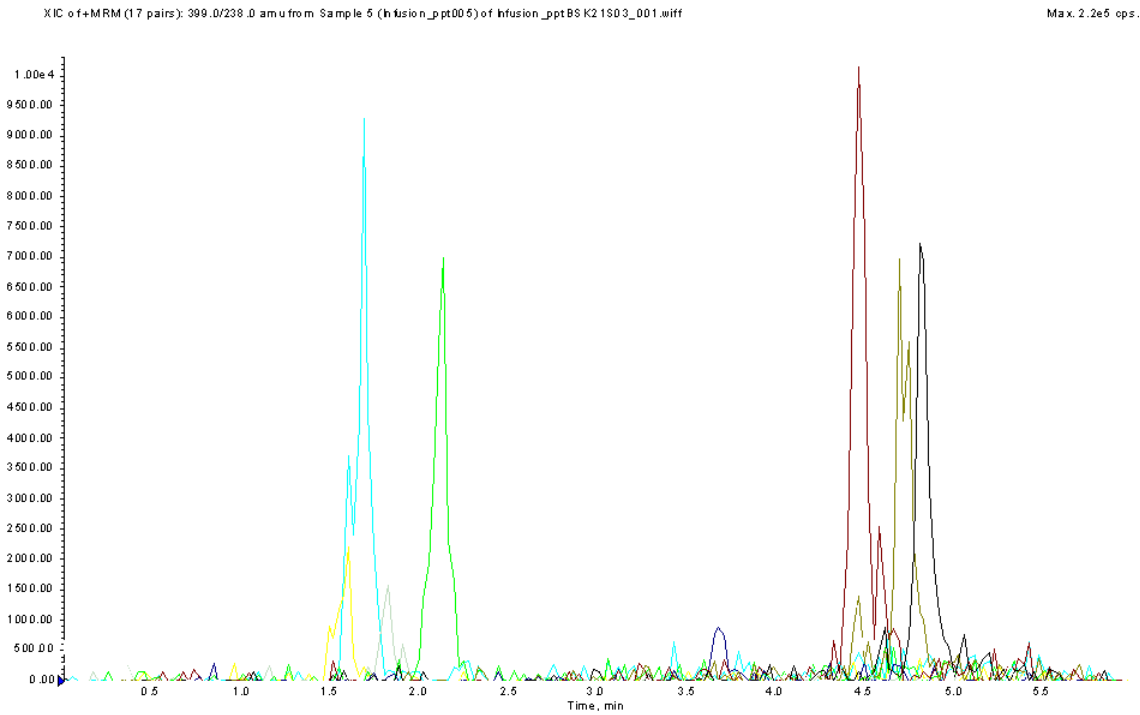
**Figure 3b**



**Figure 3c**



**Figure 3d**



**Figure 4. Common Phospholipids**

