

# Selective Depletion of Phospholipids Interference Utilizing HybridSPE-PPT Technology

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## Abstract

Analysis of biological samples is often hindered due to interferences carried through the sample preparation technique. Protein precipitation is a widely accepted sample preparation method for biological plasma samples due to simplicity and gross level removal of proteins. Though widely used, protein precipitation methods often result in chromatographic irregularities due to co-extracted endogenous species such as phospholipids that negatively affect chromatographic analysis. A more thorough sample clean up can be achieved using solid phase extraction (SPE), but at a cost of time and method complexity.

## Introduction

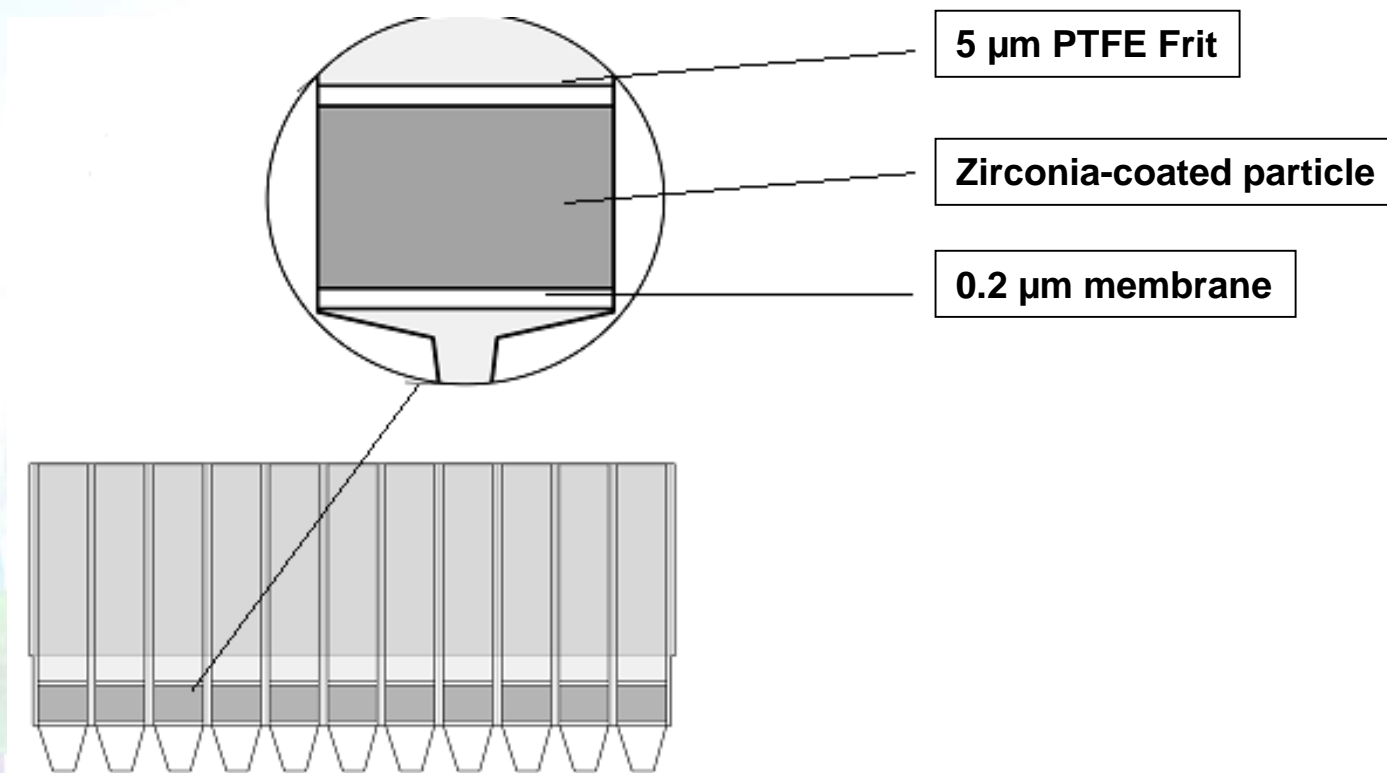
In this presentation a new platform was developed to process various plasma samples using a simplified two-step procedure to produce biological samples depleted of phospholipids prior to LC-MS-MS analysis. The HybridSPE-PPT platform employs the simplicity of standard protein precipitation with the added selectivity of SPE. The platform exhibits a high affinity towards phospholipids while remaining non-selective towards a broad range of basic, neutral and acidic compounds.


## Introduction (contd.)

The concept is to combine the simplicity of performing protein precipitation with the selectivity of solid phase extraction to remove phospholipids. The phase chemistry needed be selective toward retention of phospholipids but non-selective towards a range of neutral, acidic and basic compounds. A simplified sample preparation technique enables the development of more reproducible bioanalytical methods and greatly increase their sensitivity with minimal method development. This configuration combines the particulate removal of a filtration plate with the added chemical filtering of solid phase extraction.

# Figure 1. Configuration of the 96 Well Plate Hybrid-SPE Platform

## Hybrid Protein Precipitation Configuration

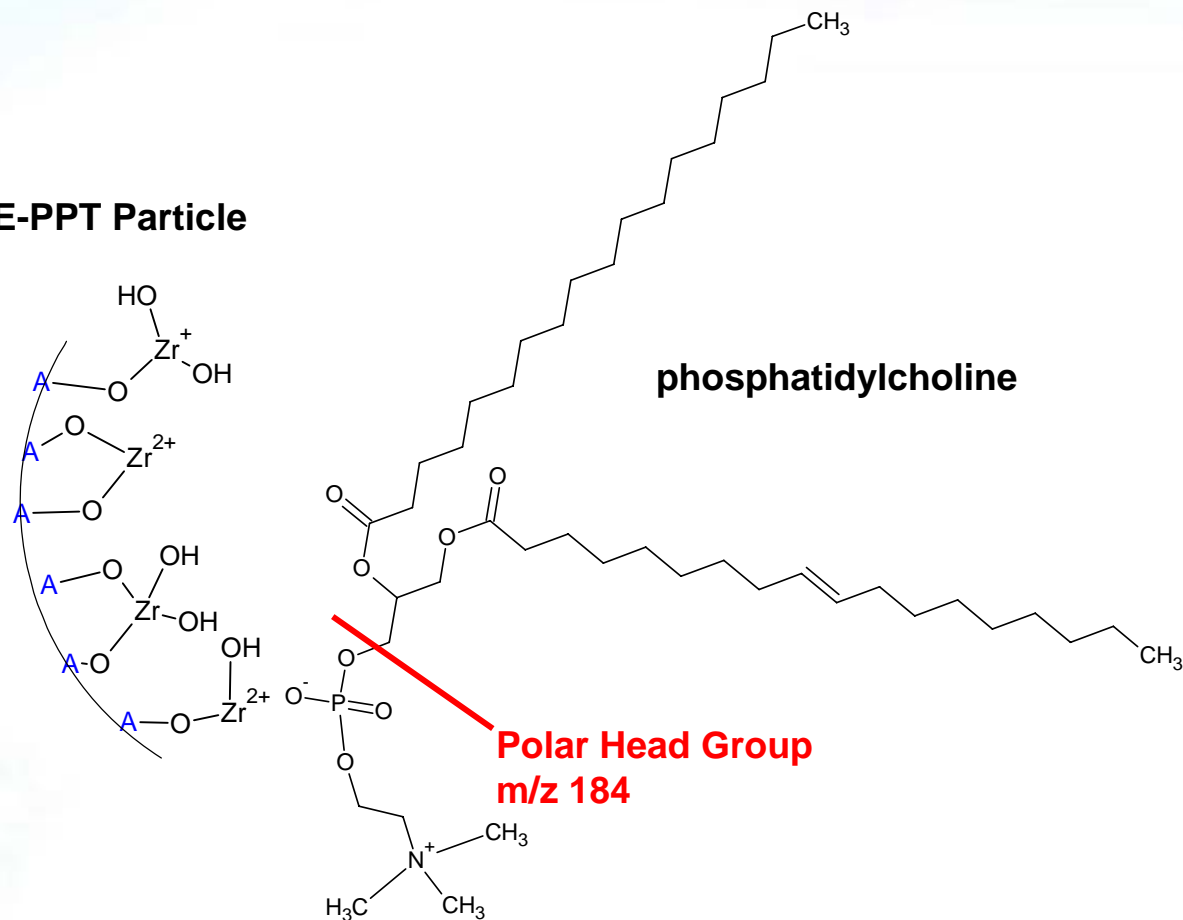




The selective extraction of phospholipids is achieved using a novel zirconia-coated particle technology. The high selectivity towards phospholipids is achieved utilizing Lewis acid/base interaction between the phosphate group of the phospholipids and the zirconia surface. The zirconia-coated particle does not exhibit as strong a Lewis “acidic” strength as pure zirconium oxide, but does enable highly efficient extraction of phospholipids while remaining non-selective towards a broad range of basic, neutral and acidic compounds.

# Figure 2. Interaction between a Representative Phospholipid and the Zirconium Surface of the HybridSPE-PPT Particle via Lewis acid-base Interaction

HybridSPE-PPT Particle



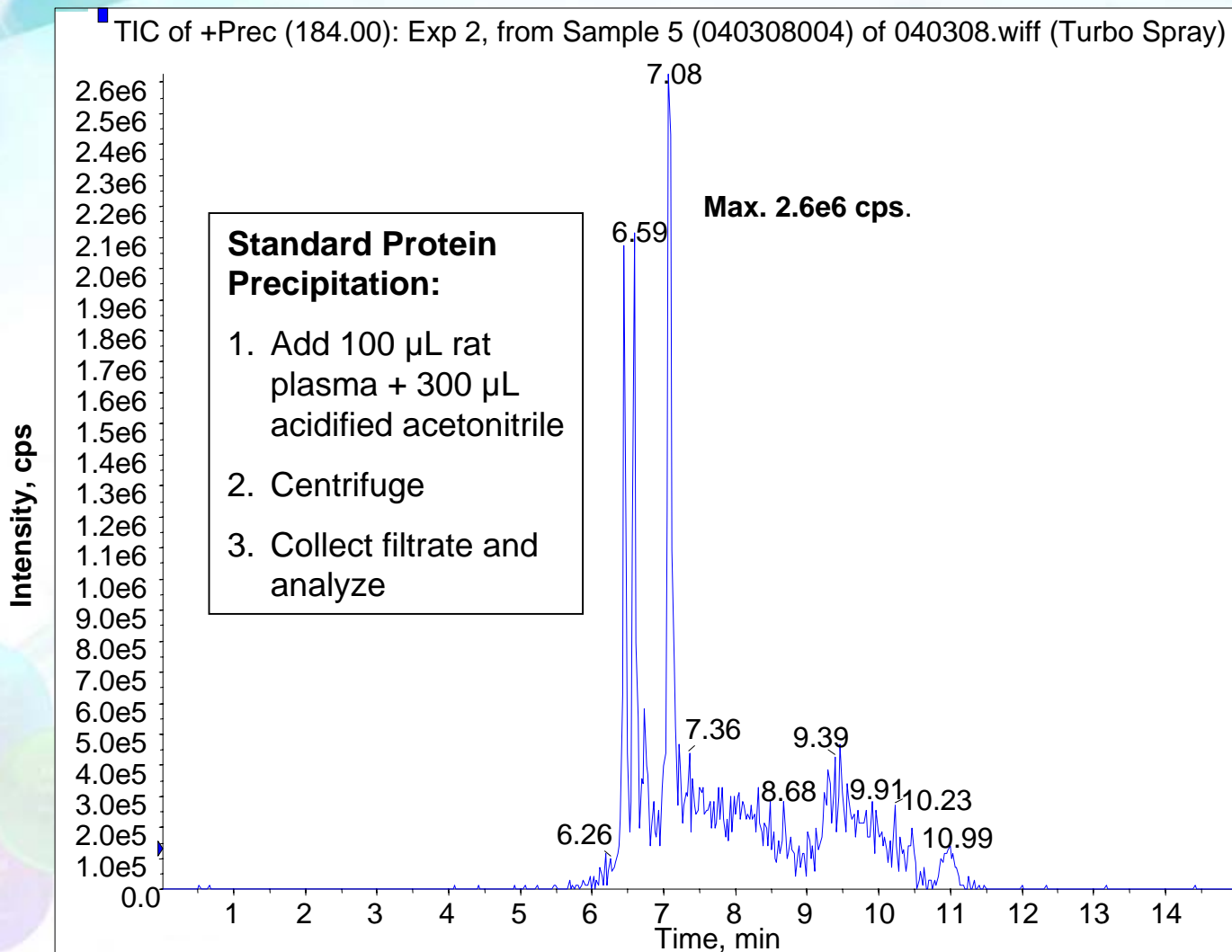
## Experimental

### **Phospholipid Monitoring:**

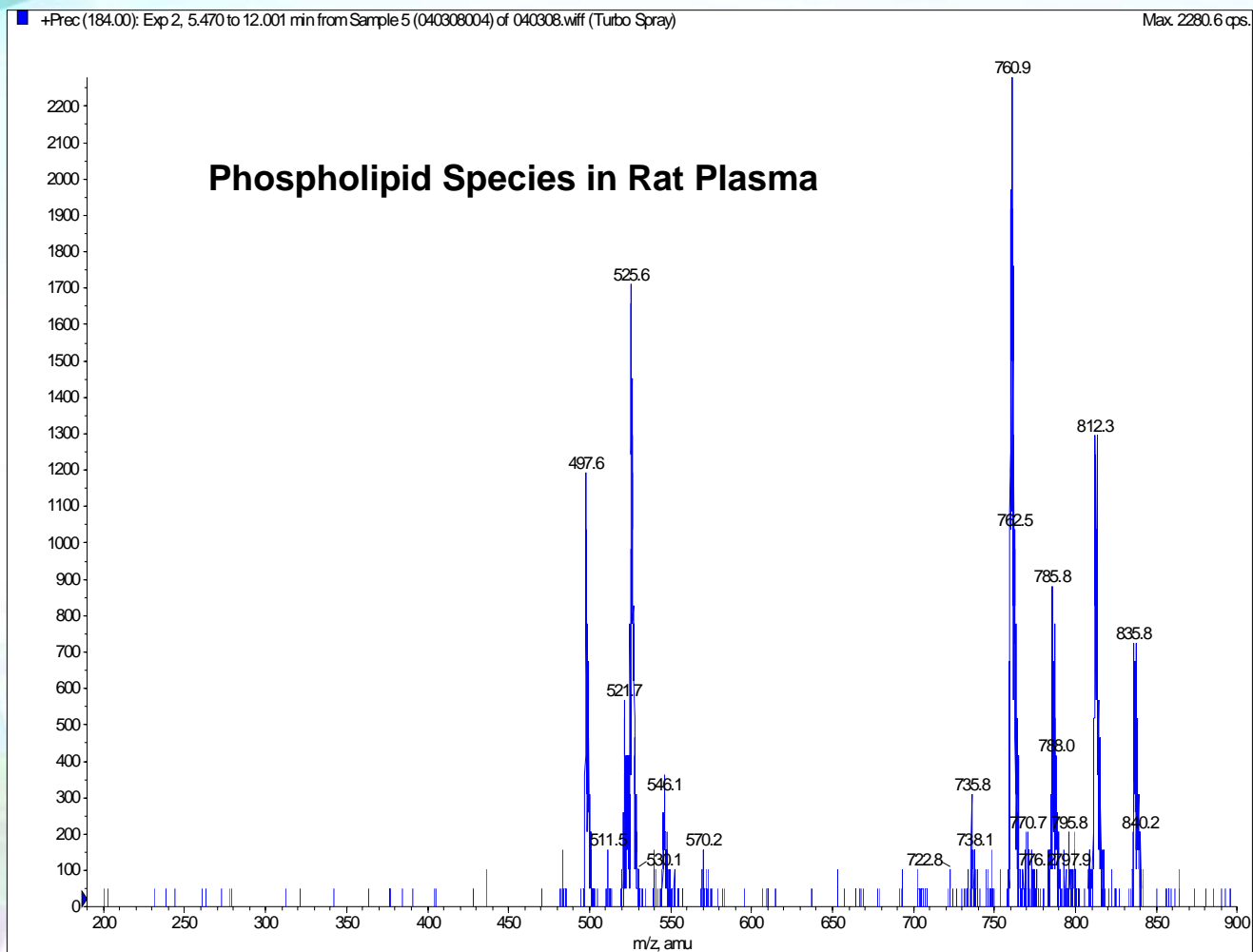
The first experiment consist of monitoring the extracted phospholipids from a rat plasma sample. The plasma was subject to protein precipitation using a 3:1 ratio of acidified acetonitrile to plasma. Sample was centrifuged then analyzed using a generic gradient method. To compare the effectiveness of the HybridSPE-PPT technique, 100  $\mu\text{L}$  aliquots of rat plasma were added directly to the HybridSPE-PPT plate, 300  $\mu\text{L}$  of 1% formic acid acetonitrile were added and agitated to facilitate precipitation. Vacuum was applied to pull the sample through the HybridSPE-PPT. The filtrate was collected and analyzed directly using generic gradient method.

<b>Instrument</b>	Applied Biosystems 3200QT
<b>Column</b>	Ascentis Express C8 5 cm x 3.0 mm, 2.7 $\mu$ m
<b>Mobile Phase</b>	A: 10 mM ammonium acetate water pH 6.9 B: 10 mM ammonium acetate acetonitrile
<b>Flow</b>	400 $\mu$ L/min.
<b>Temperature</b>	35 $^{\circ}$ C
<b>Injection Volume</b>	5.0 $\mu$ L
<b>Sample</b>	100 $\mu$ L Rat Plasma (citrated anticoagulant)
<b>Injection Volume</b>	5.0 $\mu$ L
<b>Source Conditions</b>	Turbo ion spray ESI +, Precursor Ion Scan 184m/z
<b>Dwell time</b>	150 msec
<b>Gradient Time</b>	95% A to 95% B in 5 min., hold for 3 min. then 95% B to 95% A in 0.1 min., hold for 7 min.

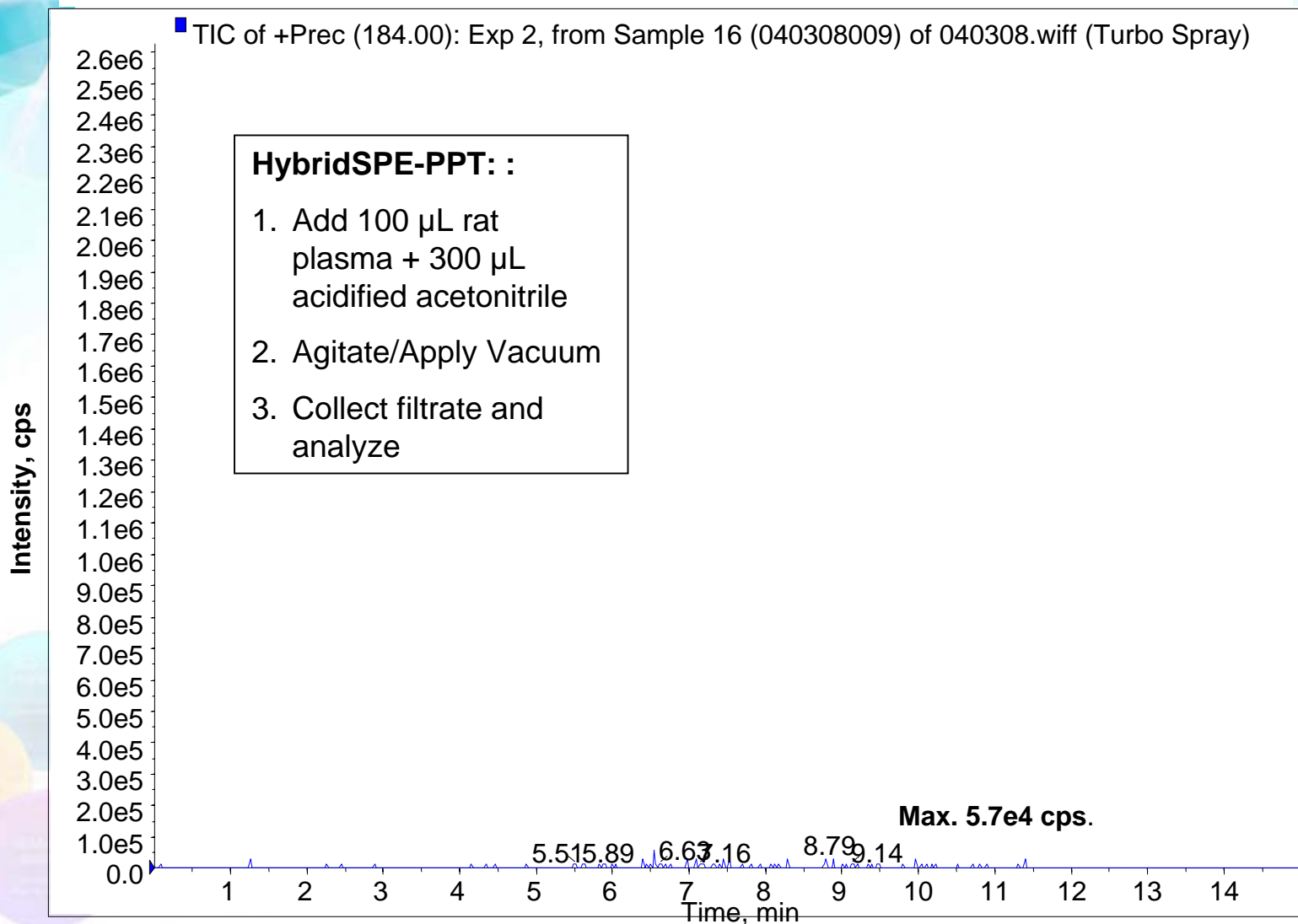
# Figure 3. Rat Plasma Processed using Protein Precipitation, Precursor Ion Scan of m/z 184 for Phospholipids

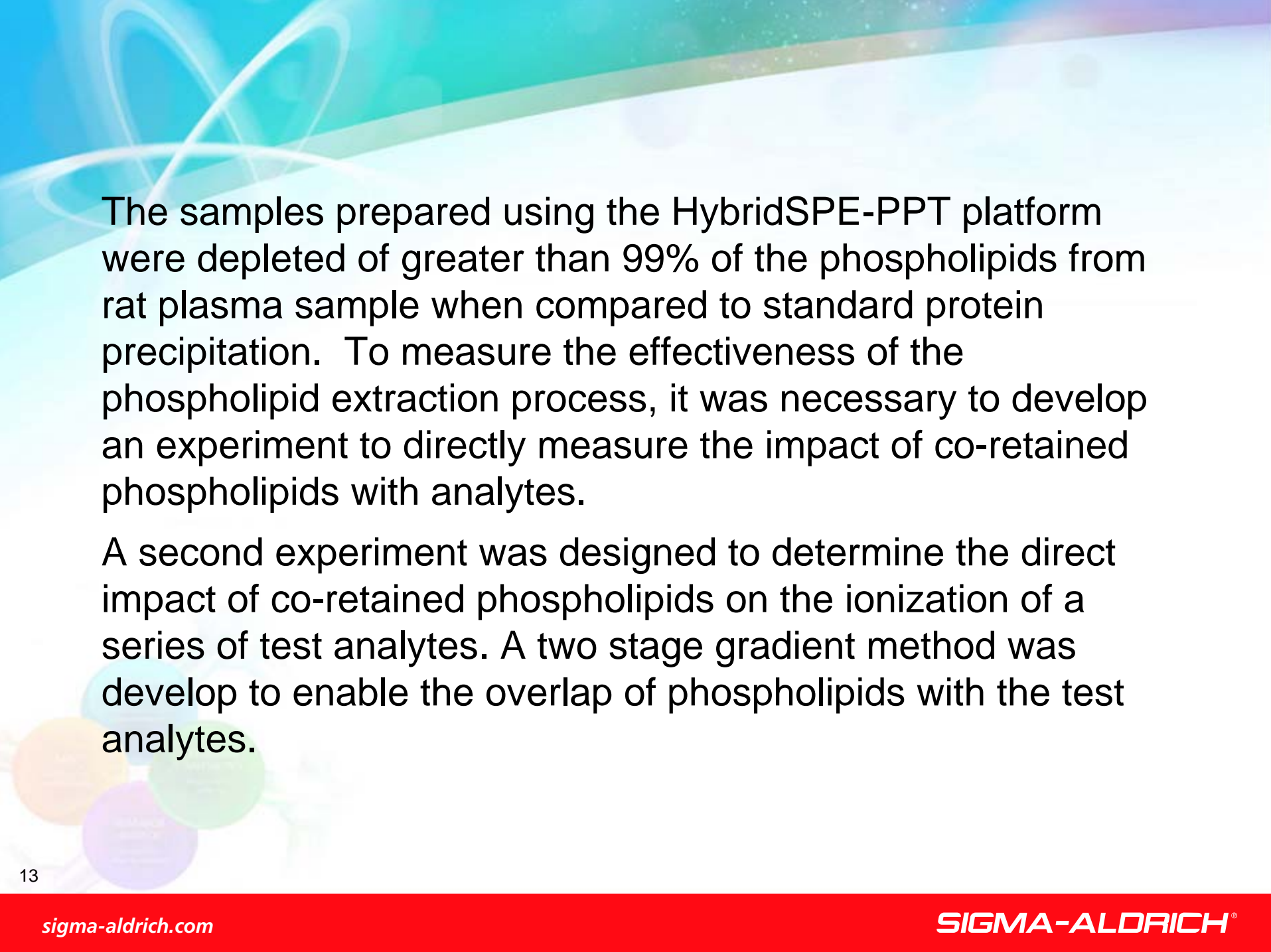


# Figure 4. Mass Spectrum from Precursor Ion Scan, Protein Precipitated Rat Plasma



# Figure 5. Rat Plasma processed using Hybrid SPE, Precursor Ion Scan of m/z 184 for Phospholipids



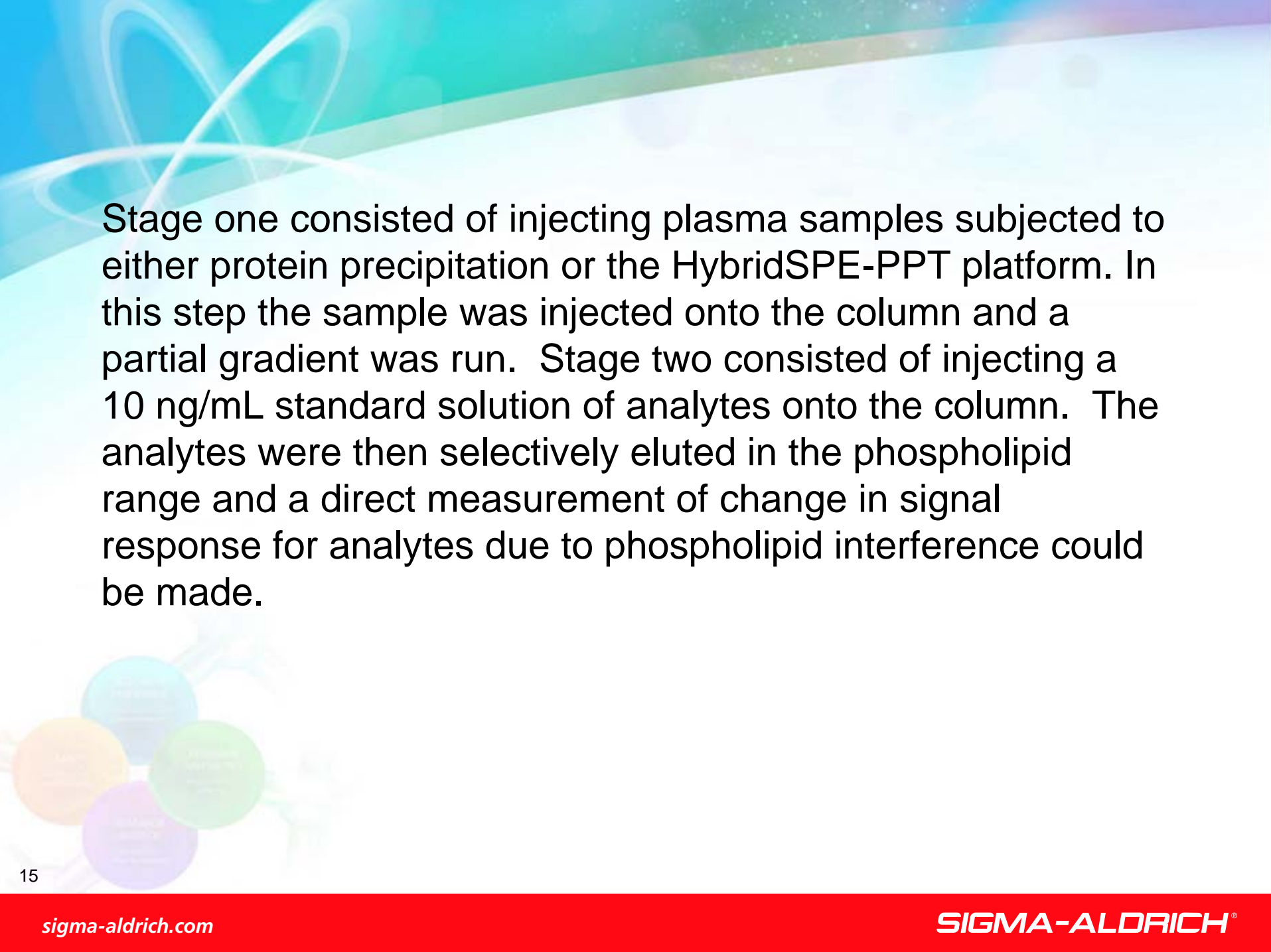


The samples prepared using the HybridSPE-PPT platform were depleted of greater than 99% of the phospholipids from rat plasma sample when compared to standard protein precipitation. To measure the effectiveness of the phospholipid extraction process, it was necessary to develop an experiment to directly measure the impact of co-retained phospholipids with analytes.

A second experiment was designed to determine the direct impact of co-retained phospholipids on the ionization of a series of test analytes. A two stage gradient method was developed to enable the overlap of phospholipids with the test analytes.

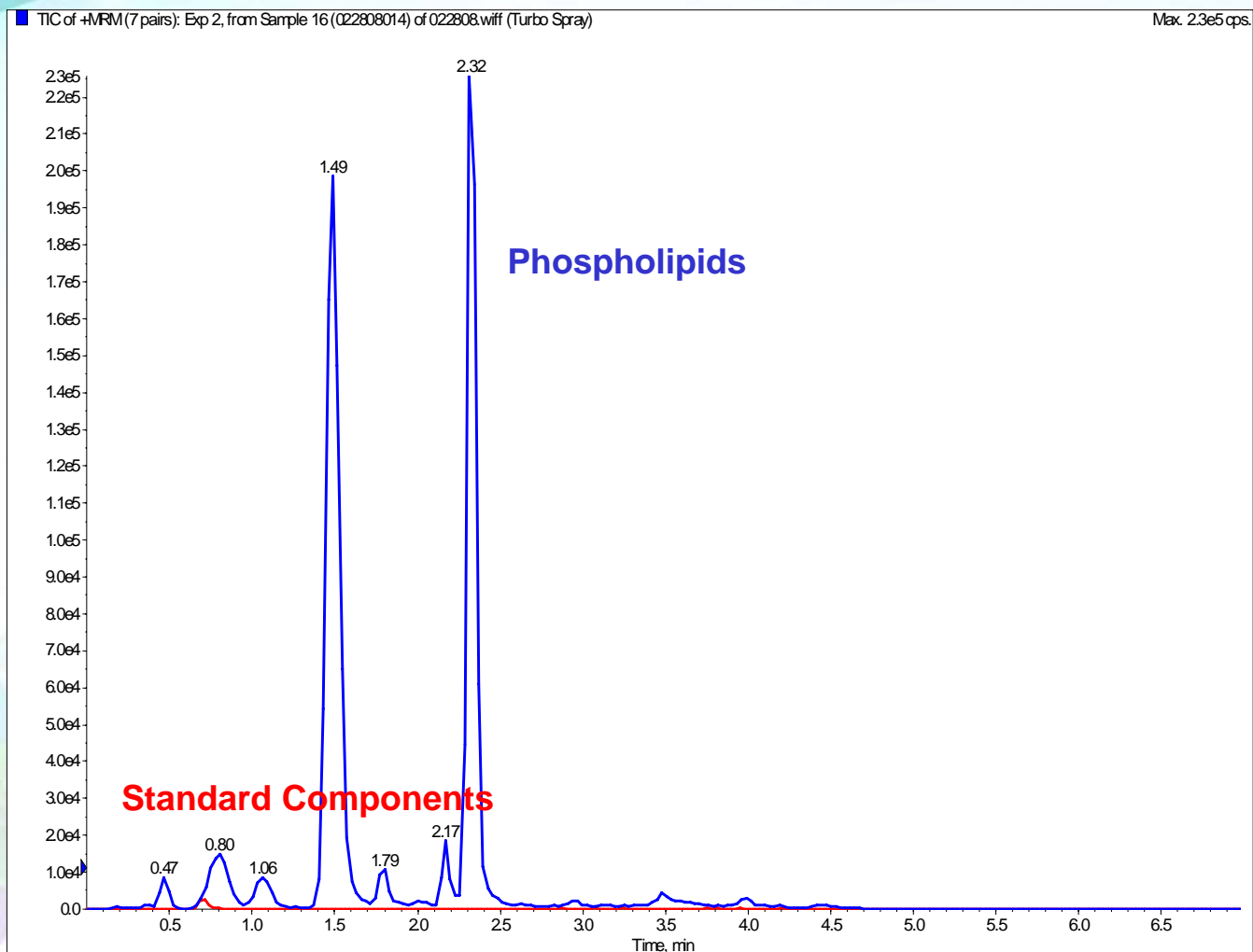
# Ionization Effect Due to Phospholipid Interference

<b>Instrument</b>	Applied Biosystems 3200QT
<b>Column</b>	Ascentis® Express C18 5 cm x 2.1 mm, 2.7 µm
<b>Mobile Phase</b>	A: 10 mM ammonium acetate water pH 6.9 B: 10 mM ammonium acetate acetonitrile
<b>Flow</b>	200 µL/min.
<b>Temperature</b>	35 °C
<b>Injection Volume</b>	5.0 µL
<b>Source Conditions</b>	Turbo ion spray ESI +, MRM
<b>Dwell time</b>	150 msec
<b>Gradient Time</b>	Stage 1:
<b>Inject Plasma Sample</b>	60% B hold for 7 min., to 100% B in 5 min., hold for 5 min.
<b>Inject Standard Mix</b>	Stage 2: 100% B for 6 min., to 40% B in 0.1 min., hold for 5 min.



Stage one consisted of injecting plasma samples subjected to either protein precipitation or the HybridSPE-PPT platform. In this step the sample was injected onto the column and a partial gradient was run. Stage two consisted of injecting a 10 ng/mL standard solution of analytes onto the column. The analytes were then selectively eluted in the phospholipid range and a direct measurement of change in signal response for analytes due to phospholipid interference could be made.

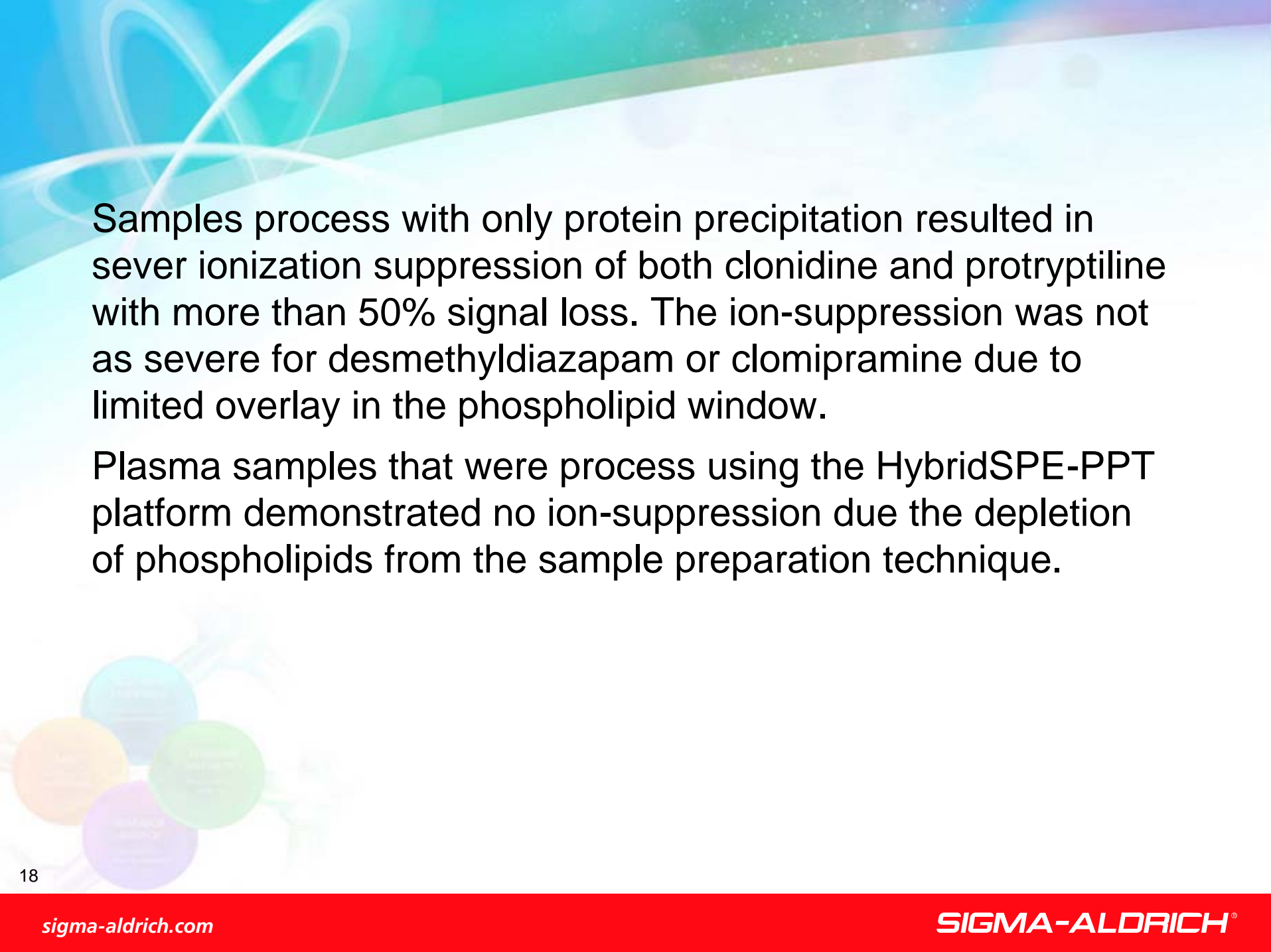
# Figure 6. Overlay of Co-retained Analytes with Phospholipids from Standard Protein Precipitation



## Figure 7. Ionization Effect of Co-Retained Phospholipids on Analytes

The two step gradient system enabled a direct method for determining the impact of co-retained phospholipids. By injecting the analytes into the phospholipid retention window, this eliminates misinterpretation of ionization effect that may have been due to salt in the matrix.

400 $\mu$ L of PPT Rat Plasma STD 10 $\mu$ g/mL concentration	Clonidine (m/z 230)	Protryptiline (m/z 264)	Desmethyldiazepam (m/z 271)	Clomipramine (m/z 315)
HybridSPE-PPT Rat Plasma	102.22	97.76	99.53	101.00
Protein Precipitated Rat Plasma	55.11	43.92	80.19	112.26



Samples process with only protein precipitation resulted in severe ionization suppression of both clonidine and protryptiline with more than 50% signal loss. The ion-suppression was not as severe for desmethyldiazepam or clomipramine due to limited overlay in the phospholipid window.

Plasma samples that were process using the HybridSPE-PPT platform demonstrated no ion-suppression due the depletion of phospholipids from the sample preparation technique.

## **Analyte Recovery and Phospholipids Buildup:**

A third set of experiments were designed to demonstrate the affect of phospholipid build-up on a typical reversed-phase gradient separation. For these experiment rat plasma samples were spiked with a mixture of acidic and basic compound. One set of samples were prepared using protein precipitation, another set were prepared using the HybridSPE-PPT platform. The samples were then analyzed for absolute recovery and phospholipid content.

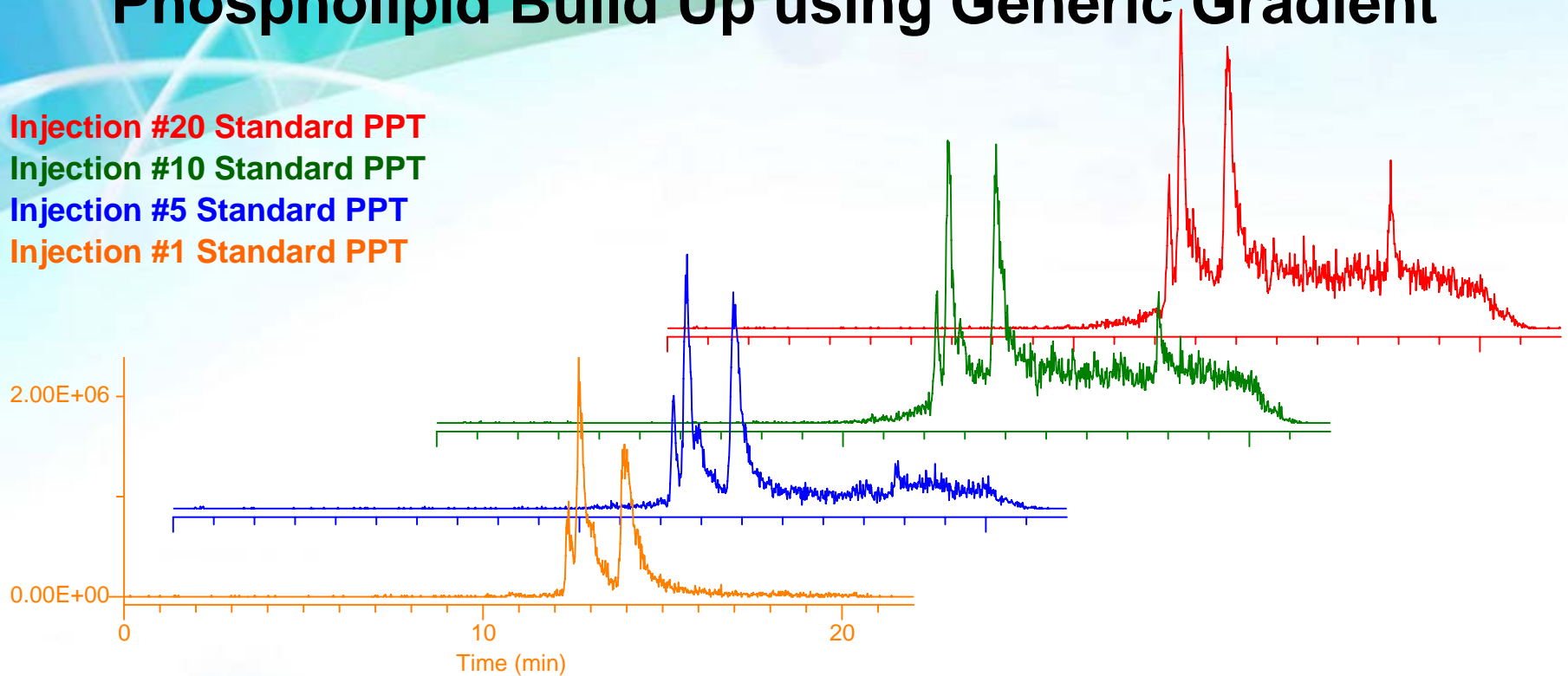
# Figure 8. Standard Components with MRM Conditions

<b>Instrument</b>	Applied Biosystems 3200QT
<b>Column</b>	Ascentis Express C18 5 cm x 2.1 mm, 2.7 $\mu$ m
<b>Mobile Phase</b>	A: 10 mM ammonium acetate water pH 6.9 B: 10 mM ammonium acetate acetonitrile
<b>Flow</b>	200 $\mu$ L/min.
<b>Temperature</b>	35 $^{\circ}$ C
<b>Injection Volume</b>	5.0 $\mu$ L
<b>Source Conditions</b>	Turbo ion spray ESI +, Precursor Ion Scan 184m/z
<b>Dwell time</b>	150 msec
<b>Gradient Time</b>	5% B to 50% B in 5 min., hold for 3 min. then 50% B to 5% B in 0.1 min., hold for 7 min.

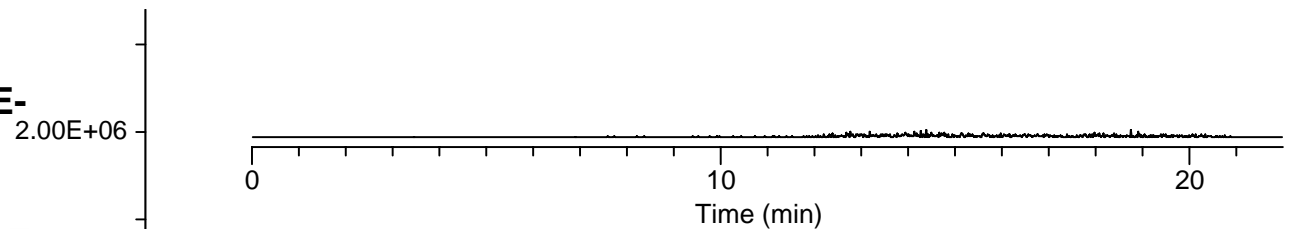
Compound	Q1	Q3	Dwell	DP	EP	CEP	CE
phenylboronic acid	123.9	82.9	75	56	12	10	15
memantine	180.1	163.2	75	41	10	10	19
propazine	230.1	146	75	51	10	14	31
procainamide	236.1	163.1	75	31	10	12	21
dapsone	248.9	92	75	51	10	14	33
protriptyline	264.1	191.1	75	46	19	14	37
tamoxifen	372.1	72.1	75	61	12	18	39
buspirone	386.1	122.1	75	71	12	16	43

# Phospholipid Build Up using Generic Gradient

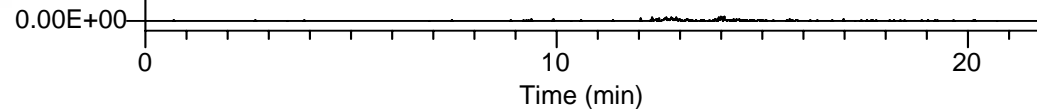
- Injection #20 Standard PPT
- Injection #10 Standard PPT
- Injection #5 Standard PPT
- Injection #1 Standard PPT

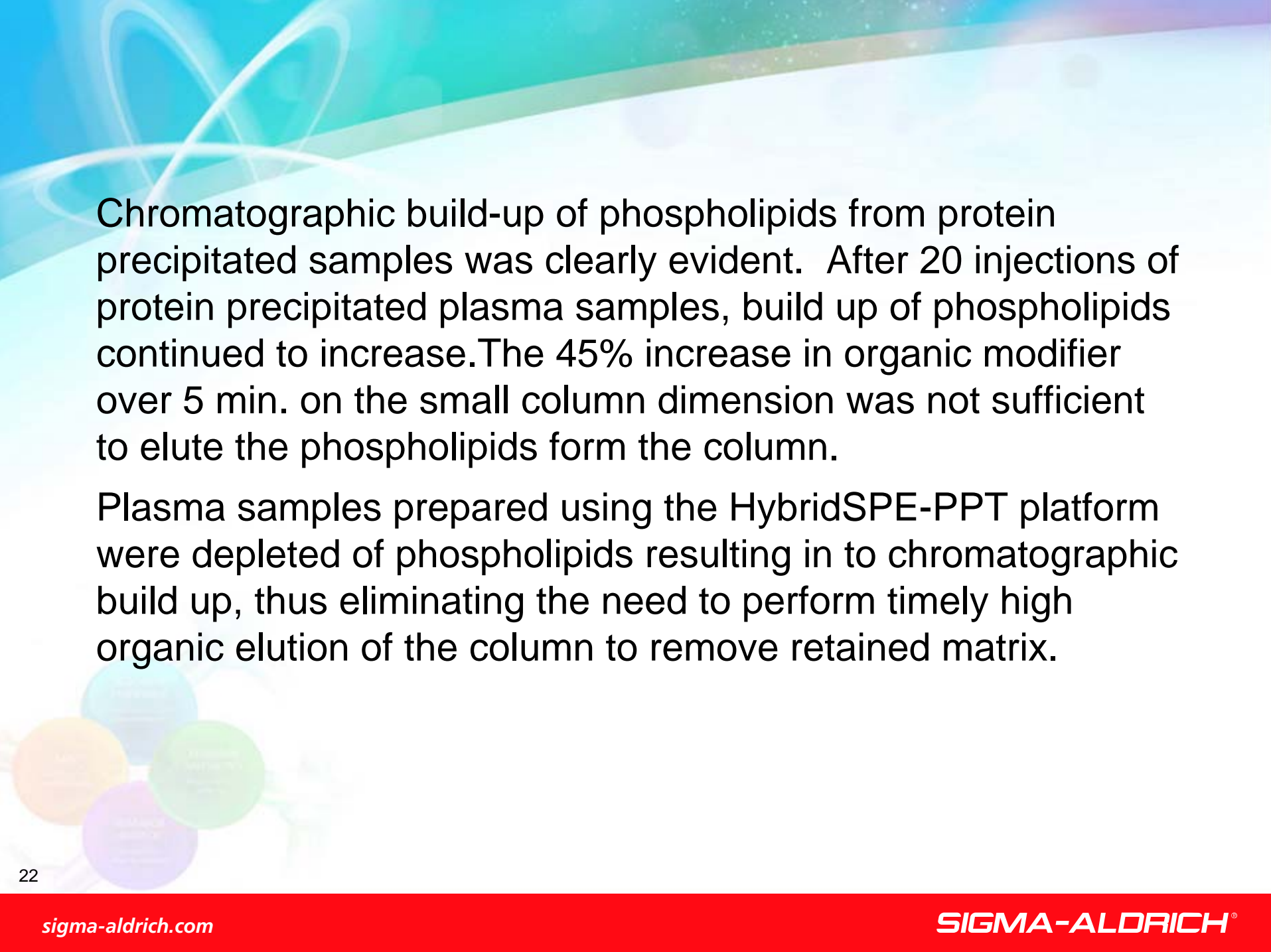


Injection #20 HybridSPE-  
PPT Technology



Injection #1 HybridSPE-  
PPT Technology





Chromatographic build-up of phospholipids from protein precipitated samples was clearly evident. After 20 injections of protein precipitated plasma samples, build up of phospholipids continued to increase. The 45% increase in organic modifier over 5 min. on the small column dimension was not sufficient to elute the phospholipids from the column.

Plasma samples prepared using the HybridSPE-PPT platform were depleted of phospholipids resulting in to chromatographic build up, thus eliminating the need to perform timely high organic elution of the column to remove retained matrix.

## Figure 9. Absolute Recovery of Analytes from Generic Gradient System

Compound	Hybrid SPE Recovery	Protein Precipitation
phenylboronic acid	68	31
memantine	102	90
propazine	113	77
procainamide	70	40
dapsone	110	97
protriptyline	95	82
tamoxifen	102	81
buspirone	98	75

High absolute recovery was observed from the HybridSPE-PPT platform. A slightly lower recover was observed for phenylboronic acid due to some binding with the phases. Low response was observed for the protein precipitation samples on procainamide, buspirone and tamoxifen due to interference from co-retained phospholipids.

## Summary

- HybridSPE-PPT platform demonstrated a high selective toward phospholipids while excluding basic compounds.
- Co-extracted phospholipids from rat plasma sample using standard protein precipitation result in severe ion-suppression.
- HybridSPE-PPT enable one step sample preparation with reduced processing time.
- Remove interfering matrix due to co-extracted phospholipids from protein precipitation.
- Decrease variability due to phospholipid interference.
- Increase reproducibility and sensitivity of bioanalytical methods.
- Demonstrated good recovery across a range of analytes.