High Throughput Removal of Both Phospholipids and Proteins in Bioanalytical Sample Preparation

Charles Mi, Craig Aurand, An Trinh, and Michael Ye
Supelco, Division of Sigma-Aldrich, Bellefonte, PA 16823 USA

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Introduction

Sample preparation is critical in pharmaceutical bioanalysis due to the complexity of biological matrices. Common sample preparation techniques include 96-well protein precipitation, liquid-liquid extraction, and solid phase extraction.

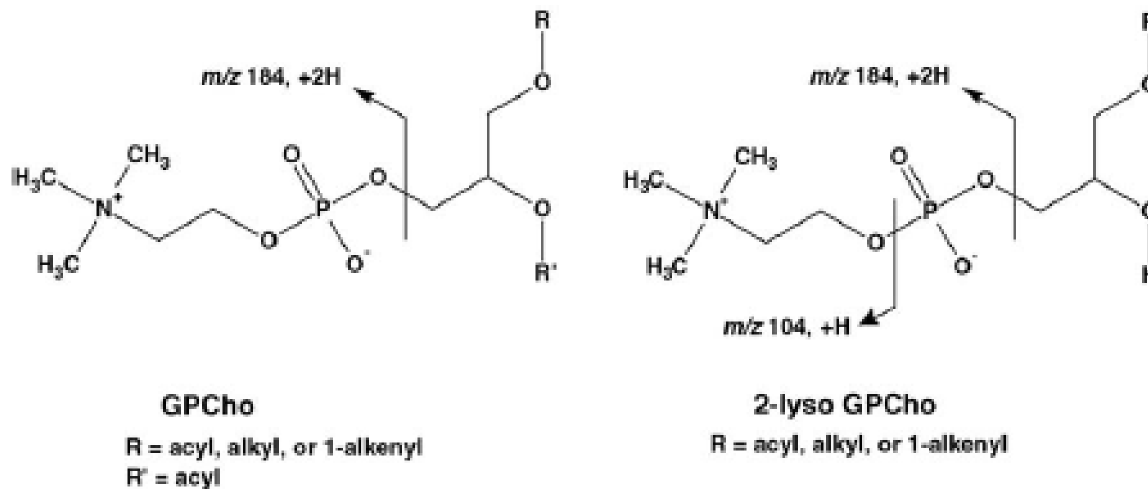
SPE provides superior selectivity relative to simpler techniques; however, it is the most time consuming requiring multiple steps and method development time. Protein precipitation is highly generic with few processing steps. As a result, it is widely adopted for analyzing plasma samples. However it removes only proteins. Other critical endogenous interferences, such as phospholipids, will remain in the sample. It is well-known that phospholipids cause ion-suppression in mass spectrometry analysis, leading to low recovery and high variation of analytical results.

Introduction (contd.)

In this presentation, we discuss a new sample prep platform that combines the speed and simplicity of protein precipitation plate and the selectivity of SPE through the selective removal of proteins and phospholipids. The technology utilizes zirconia-coated particle technology; and exhibits a selective binding towards phospholipids while remaining non-selective towards other compounds.

Monitoring of Phospholipids

- "In the case of LC-MS/MS-based procedures, appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method…" – Guidance for Industry Bioanalytical Method Validation, FDA, 2001.
- Bioanalytical method developers routinely monitor for phospholipid fragment ions m/z 184 & m/z 104 during method development/validation.
- Used as a marker as ion-suppression risk & assessment during LC-MS/MS (co-elution of analytes of interest & matrix-laden regions).
- Determine selectivity effectiveness of sample prep technique.



Little et al., *J. Chromatogr. B 833 (2006) 219–230*

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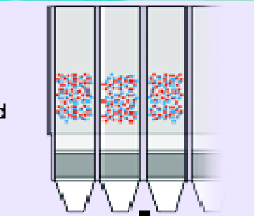
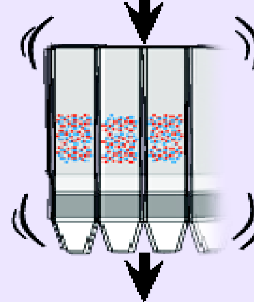
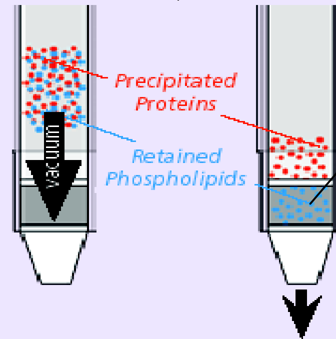
How does HybridSPE™-PPT Work?



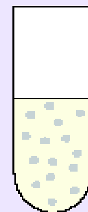
1) **Precipitate Proteins** by adding 100 µL plasma or serum to the HybridSPE-PPT plate followed by 300 µL 1% formic acid in acetonitrile. Add I.S. as necessary.



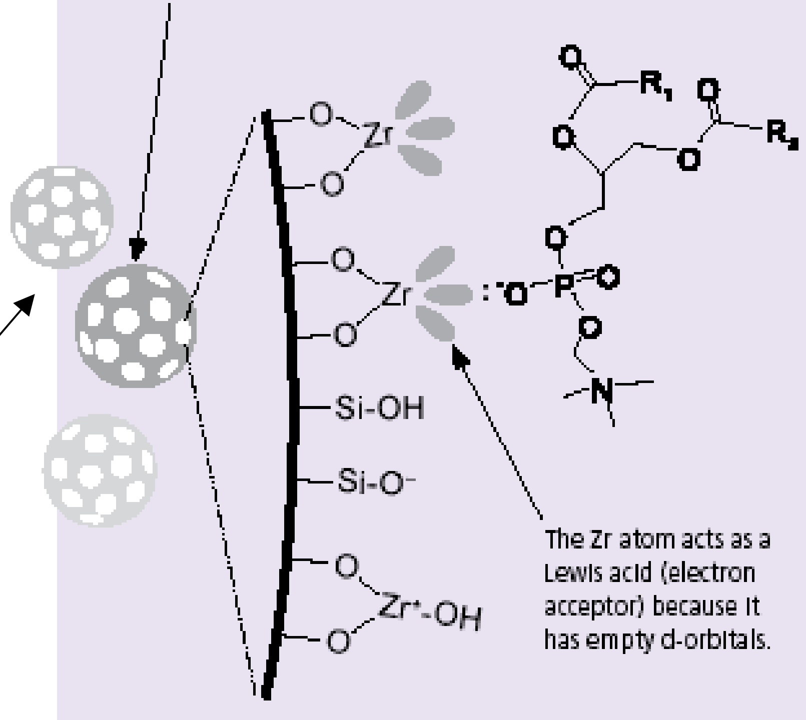
2) **Mix** by vortexing/shaking HybridSPE-PPT plate or by aspirating/dispensing with 0.5-1 mL pipette tip (e.g., TOMTEC Quadra liquid handler)



3) **Apply vacuum.** The packed-bed filter/frit assembly acts as a depth filter for the concurrent physical removal of precipitated proteins and chemical removal phospholipids. Small molecules (e.g., pharma compounds and metabolites) pass through unretained.



4) **Resulting filtrate/eluate** is free of proteins and phospholipids and ready for immediate LC-MS-MS analysis; or it can be evaporated and reconstituted as necessary prior to analysis

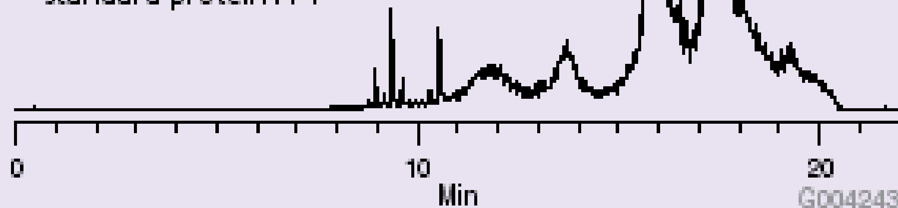


Monitoring of Phospholipid Removal from Rat Plasma

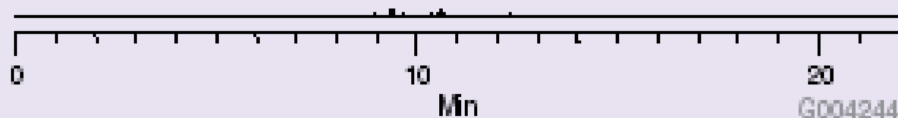
column: Ascentis Express C18, 5 cm x 2.1 mm ID (53822-U)
instrument: Agilent 1100
mobile phase: (A) 10 mM ammonium acetate
(B) 10 mM ammonium acetate in acetonitrile
temperature: 35 °C
flow rate: 0.5 mL/min.
detection: ABI 3200 QT; ESI(+), MRM (184/104 m/z)
inj. vol.: 5 µL
gradient:

Min.	%A	%B
0	95	5
10	50	50
18	50	50
18.1	95	5
22	95	5

No phospholipid removal after standard protein PPT



Complete removal of phospholipids after HybridSPE-PPT



Experiment 1

Comparison of Phospholipid Removal by SPE, Protein Precipitation and HybridSPE

a. Sample Preparation Methods

HybridSPE Techniques:	Protein Precipitation (PPT):
• Apply 100 µL rat or dog plasma to HybridSPE-PPT 96-well plate. Add 300 µL acetonitrile with 1% FA • Vortex 1 min. Apply vacuum and collect eluent • Analyze the eluent via LC-MS	• Combine100 µL rat or dog plasma with 300 µL acetonitrile with 1% FA • Mix for 1 min. and centrifuge at 5K RPM for 3 min. • Analyze the sup via LC-MS

Generic polymeric SPE (60 mg/3 mL):

- Condition with 1 mL methanol and 1 mL water
- Load 500 µL rat or dog plasma
- Wash with 5% methanol in water
- Elute with 1 mL methanol
- Evaporate and reconstitute with 2 mL of water/acetonitrile with 1% FA (1:3)

Note that the SPE method includes condition, load, wash and elution steps while both PPT and HybridSPE methods are basically one-step load and filter through.

Experiment 1

Comparison of Phospholipid Removal by SPE, Protein Precipitation and HybridSPE (contd.)

b. LC-MS condition

LC-MS: Agilent 1100/ABI Q-trap 3200, Turbo Ion Spray ESI+
column: Ascentis® Express C18, 5 cm x 2.1 mm I.D.
mobile phase A: 65% acetonitrile with 0.1% ammonium formate
mobile phase B: 35% water with 0.1% ammonium formate
flow rate: 200 µL/min.
temp.: 30 °C
injection: 5 µL dog plasma sample prepared by different sample preparation methods

Mass Parameters:

CUR	IS	TEM	GS1	GS2	ihe	CAD
20	3500	500	40	55	ON	Medium

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	DP	EP	CEP	CE	CXP
184	184	50	100	10	10	29	4

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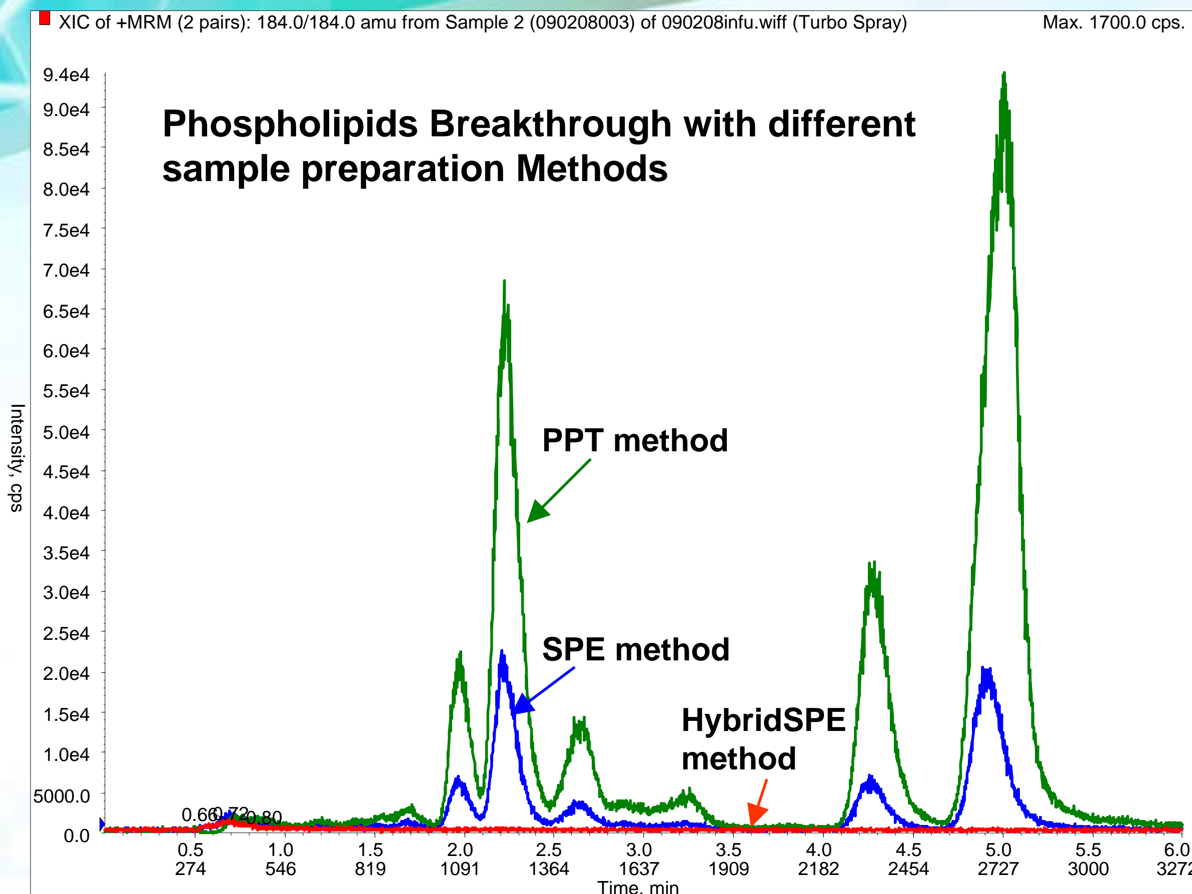


Almost 100% phospholipids in dog plasma were removed by HybridSPE technique. Moderate removal of phospholipids was observed using generic polymeric SPE method. No phospholipids were removed by traditional PPT.

Experiment 2

Recovery Study of Probe Compounds

LC-MS: Agilent 1100/ABI Q-trap 3200, Turbo Ion Spray ESI+
column: Discovery® HS F5, 10 cm x 2.1 mm I.D.
mobile phase A: acetonitrile with 10 mM ammonium formate
mobile phase B: water with 10 mM ammonium formate
flow rate: 200 µL/min.
temp.: 30 °C
injection: 5 µL of the rat plasma samples cleanup with different sample preparation methods

Step	Total Time (min.)	Flow Rate (µL/min.)	A (%)	B (%)
0	0.0	200	75	25
1	2.0	200	95	5
2	4.5	200	95	5
3	5.0	200	75	25
4	7.0	200	75	25

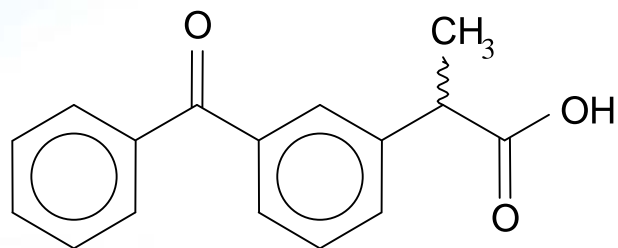
Mass Parameters

CUR	IS	TEM	GS1	GS2	ihe	CAD
25	4500	450	35	20	ON	Medium

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP	EP	CEP	CE	CXP
255.2	209.10	150	36	12	18	17	4
260.30	116.10	150	41	9.5	14	25	4

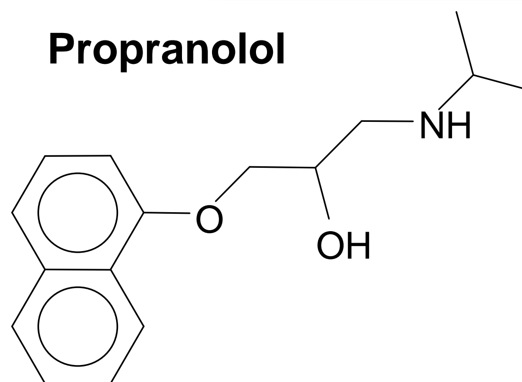
Probe Compounds used in Experiment 2

Ketoprofen



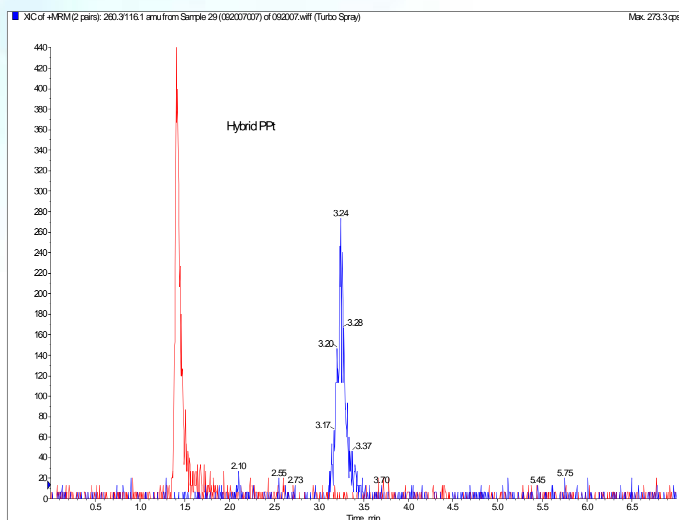
+MRM: 255.20/209.10 amu

Propranolol

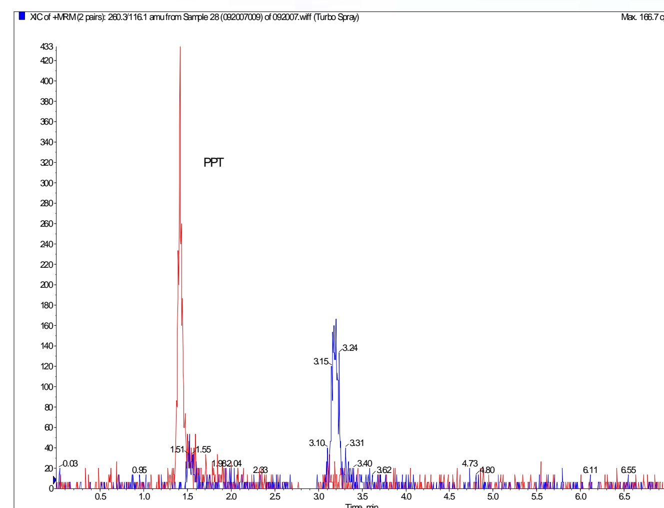


+MRM: 260.30/116.10 amu

Ketoprofen and Propranolol in Rat Plasma cleanup by HybridSPE-PPT



Ketoprofen and Propranolol in Rat Plasma cleanup by Protein-PPT



Lower response was observed for propranolol when using protein precipitation. This was primarily due to endogenous matrix interferences that were not removed during sample preparation, which suppressed the ionization of propranolol in the mass spectrometer, resulting in lower ionization response.

Recovery of Drug Compounds in Rat Plasma by Different Sample Preparation Methods

Sample Preparation Method	Ketoprofen	Propranolol
HybridSPE-PPT	82.0%	68.0%
Protein-PPT	58.8%	37.0%
Generic polymeric SPE1	78.4%	42.0%
Generic polymeric SPE2	76.4%	44.4%

Recoveries of Ketoprofen and Propranolol on HybridSPE were higher than that of comparative techniques. Recovery on Protein-PPT was the lowest due to endogenous matrix interference not removed during sample preparation. Recovery of Propranolol on the Polymer SPE phases (under generic conditions) was also low, due to strong adsorption of the compound on the polymer phases. A stronger solvent is necessary to elute it from the polymer phase.

Recovery of Drug Compounds in Rat Plasma by different Sample Preparation Methods (contd.)

Comparing the results from those three methods, the HybridSPE Technology demonstrated that it is simple and fast, at the meantime it has better selectivity, resulting in better recovery than PPT.

Experimental 3

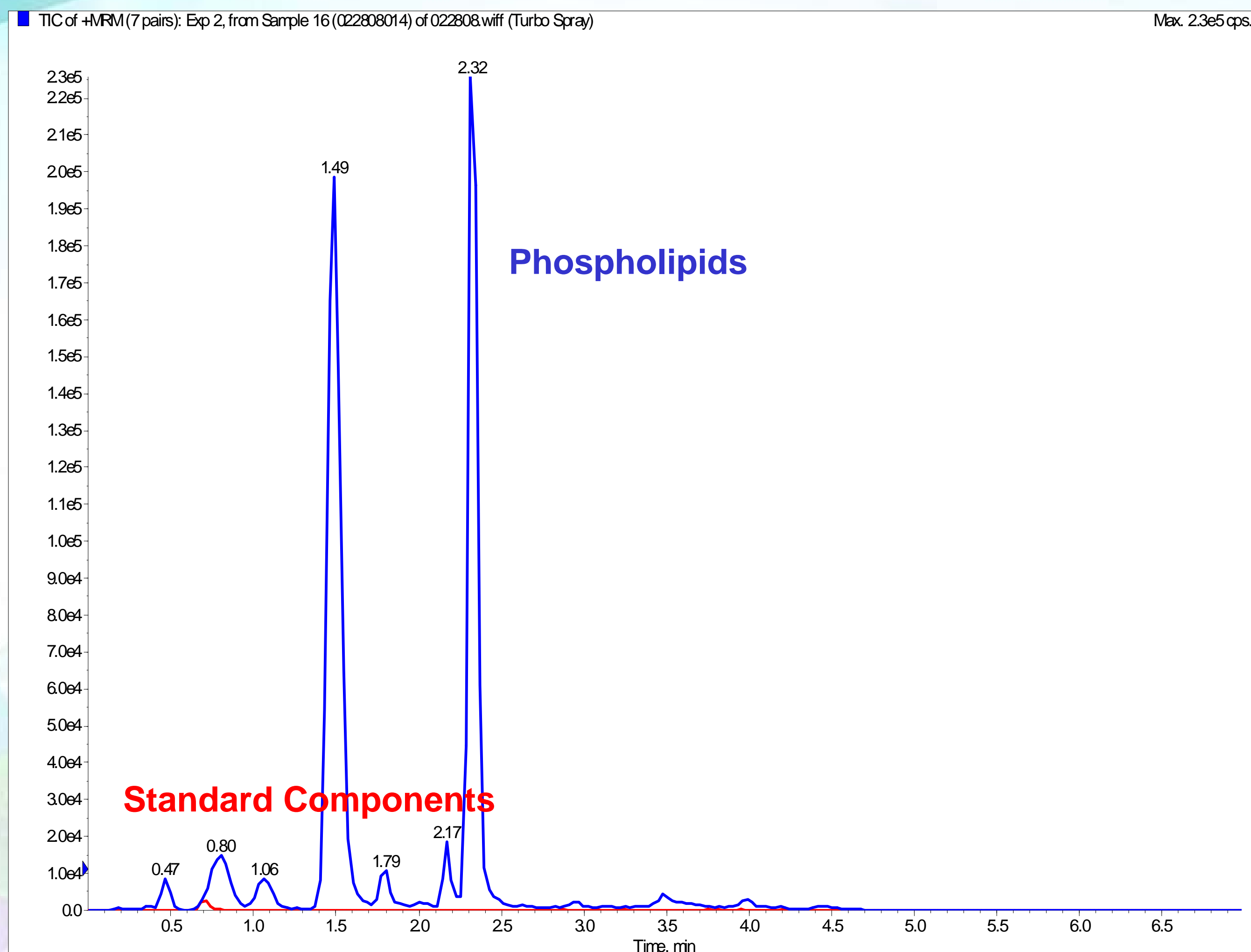
Ionization Effect of Phospholipids on Standard Compounds

A 2-stage LC-MS/MS study was conducted to determine the ionization effect of contaminating phospholipids on 4 representative pharmaceutical compounds. Blank rat plasma was first subjected to either standard Protein-PPT or HybridSPE-PPT. In stage 1, the extracts were injected onto the LC column, and the mobile phase was held until stage 2. In stage 2, the standard test mix was injected onto the column. This approach effectively allowed standard compounds to co-elute with potential phospholipid contaminants present in the sample.

Ionization Effect Due to Phospholipid Interference

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

Overlay of Co-retained Analytes with Phospholipids not Removed Standard Protein-PPT



Ionization Effect of Co-Retained Phospholipids on Analytes

400 µL of PPT Rat Plasma STD 10 µg/mL concentration	Clonidine (m/z 230)	Protryptiline (m/z 264)	Desmethyldiazapam (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

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, it eliminates misinterpretation of ionization effect that may have been due to salt in the matrix. In addition, because the analytes were not subjected to sample prep, a drop in response cannot be due to analyte loss during sample prep.

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Summary

- HybridSPE is a fast and simple method for sample preparation in bioanalysis. It merges the advantages of both protein protein precipitate (fast/simple) and SPE (selectivity/superior cleanup).
- HybridSPE-PPT platform demonstrated a high selectivity toward phospholipids while excluding basic compounds.
- Co-extracted phospholipids from plasma sample using standard protein precipitation results in severe ion-suppression.
- Demonstrated good recovery across a range of analytes.