Selective Depletion of Phospholipids Interference Utilizing HybridSPE™-Precipitation Technology

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Ion-Suppression in LC-MS

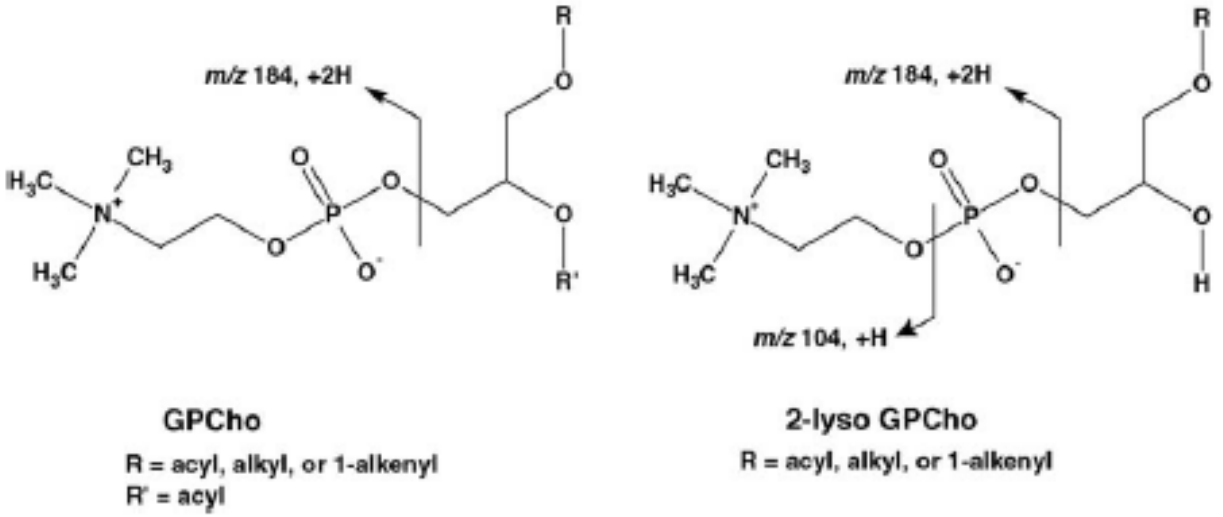
- Analysis of biological samples is often hindered due to interferences carried through the sample preparation technique.
- Protein precipitation is a widely accepted sample preparation method for biological plasma samples due to simplicity and gross level removal of proteins.
- Though widely used, protein 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.

Phospholipids and LC-MS Ion-Suppression

- Phospholipids are a primary constituent of cell membranes and documented to be a major cause of ion-suppression in the positive ion electrospray mode (+ESI)
- Present in extremely high concentrations in biological matrixes and represent the second largest lipid component next to triglycerides and fats
- Glycerophosphocolines (phosphatidylcholine) is the most abundant phospholipid (~50% of total phospholipids in plasma)
- Other less abundant phospholipids include
  phosphatidylethonalmine - dominant in microbrial membranes
  phosphatidylinositol - abundant in brain and liver tissue
  Phosphatidylserine - only amino acid containing phospholipid

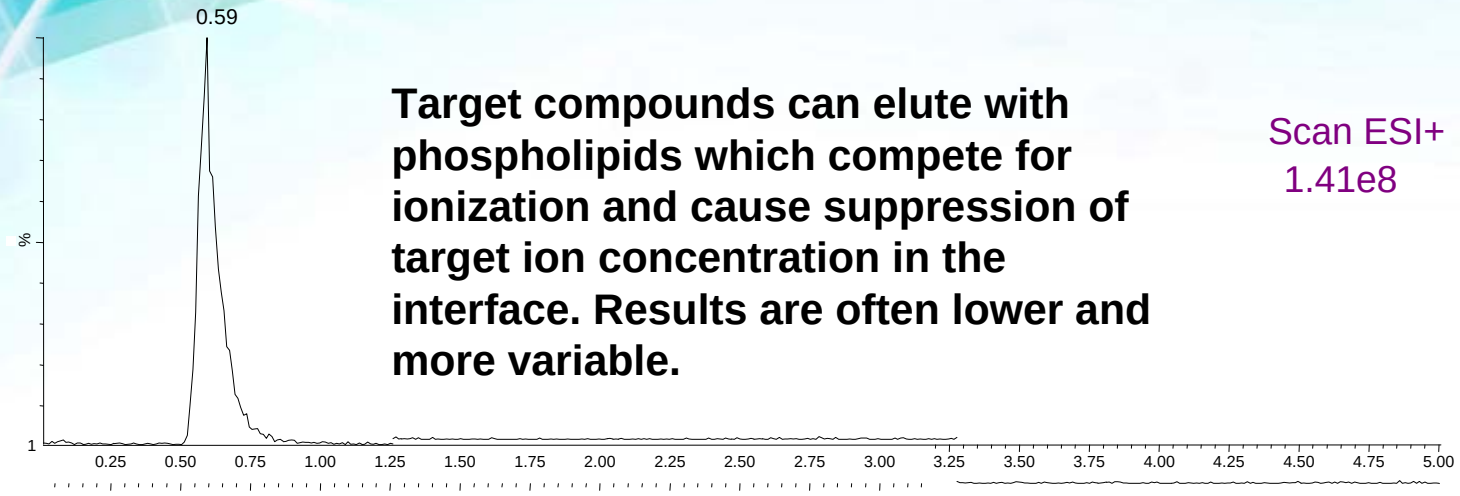
- **“In the case of LC-MS-MS-based procedures, appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method…” – Guidance for Industry Bioanalytical Method Validation, FDA, 2001**

- **Bioanalytical method developers routinely monitor for phospholipid fragment ions m/z 184 & m/z 104 during method development/validation**
  - **Used as a marker for 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*

Phospholipid Free Rat Plasma injection of Clonidine – After Protein PPT

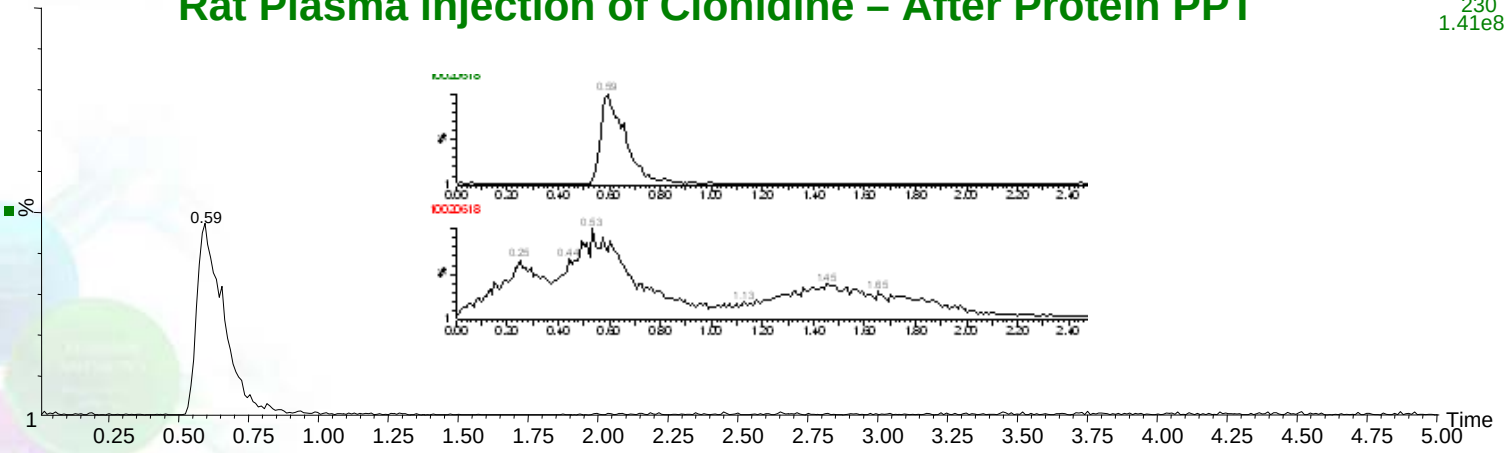


Target compounds can elute with phospholipids which compete for ionization and cause suppression of target ion concentration in the interface. Results are often lower and more variable.

Scan ESI+
1.41e8

Rat Plasma injection of Clonidine – After Protein PPT

1: Scan ES+
230
1.41e8



Difficulties of Phospholipid Removal in Sample Preparation

Solid Phase Extraction:

- **Mixed-Mode & IOX** – often co-extract with basic or acidic analytes of interest
- **Reversed-Phase** – will result in co-extraction with analytes of interest

Protein Precipitation:

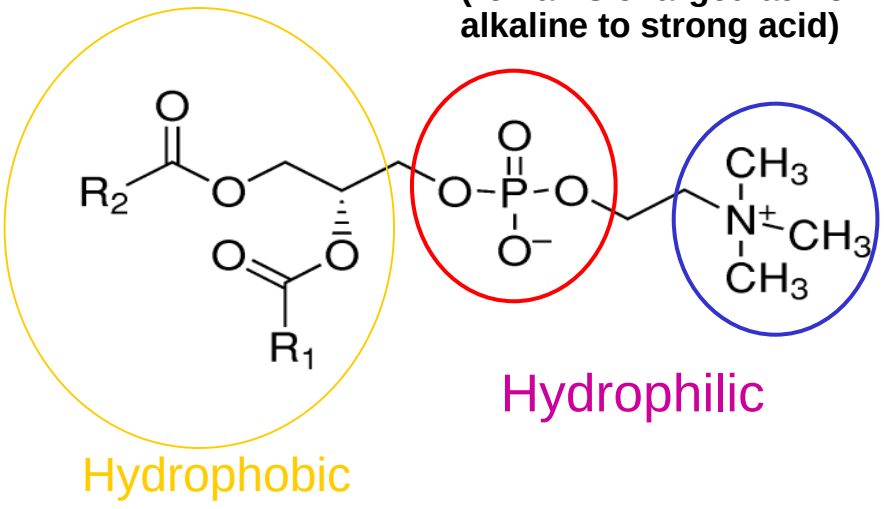
- Will only remove gross levels of protein (albumin)

Liquid Liquid Extraction:

- Hydrophobic tail allows for co-extraction with analytes of interest

Hybrophobic tail – two fatty acyl groups that are hydrophobic

Polar head group – zwitterionic phosphonate (remains charged at from strong alkaline to strong acid)



Difficulties with Phospholipids During HPLC

- Many method developers adjust their LC conditions to ensure that peaks of interest elute in matrix (phospholipid) free zone.
  More difficult with increasing trend to reduce LC run time (< 5 min.).
- Inadequate sample prep can lead to an accumulation of phospholipids on the LC-column.
  Results in build-up and selectivity change with successive injections.
  May elute uncontrollably in subsequent injections and cause variability.
  Use of fast/ballistic gradients often inadequate to purge the column of phospholipids during a run sequence.
  Gradual column pressure increase is another byproduct of inadequate sample prep that can be controlled by HybridSPE.

Development of HybridSPE Platform

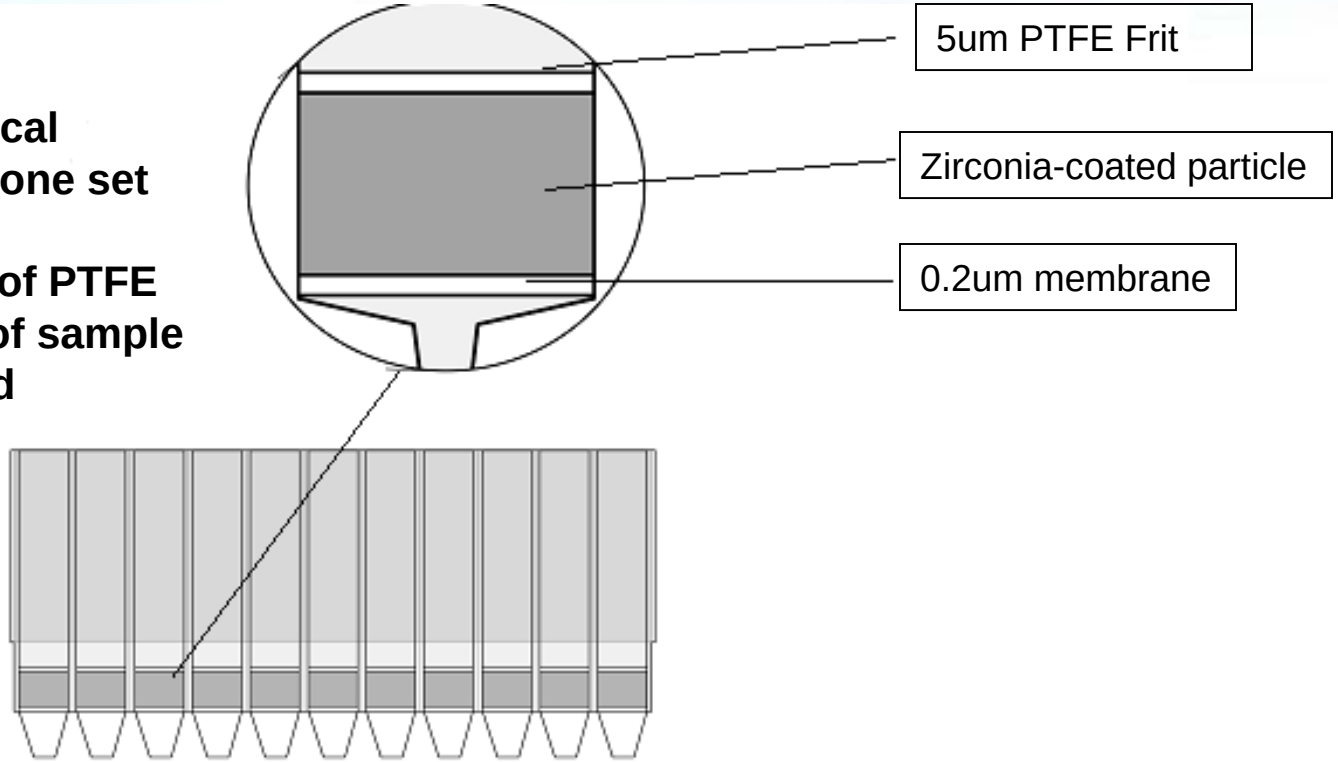
Concept of of this new platform was developed to process various plasma samples using a simplified two-step procedure to produce biological samples depleted of phospholipids prior to LC-MS/MS analysis.

The HybridSPE-PPT platform

- Employs the simplicity of standard protein precipitation with the added selectivity of SPE.
- Exhibits a high affinity towards phospholipids
- Remains non-selective towards a broad range of basic, neutral and acidic compounds

HybridSPE Protein Precipitation Configuration

- **Stacked configuration of frits acts as depth filter enabling faster filtration**
- **Particulate and chemical filtration performed in one set**
- **Hydrophobic surface of PTFE frit prevents seepage of sample until vacuum is applied**



Bed weight of stationary phase is 50mg

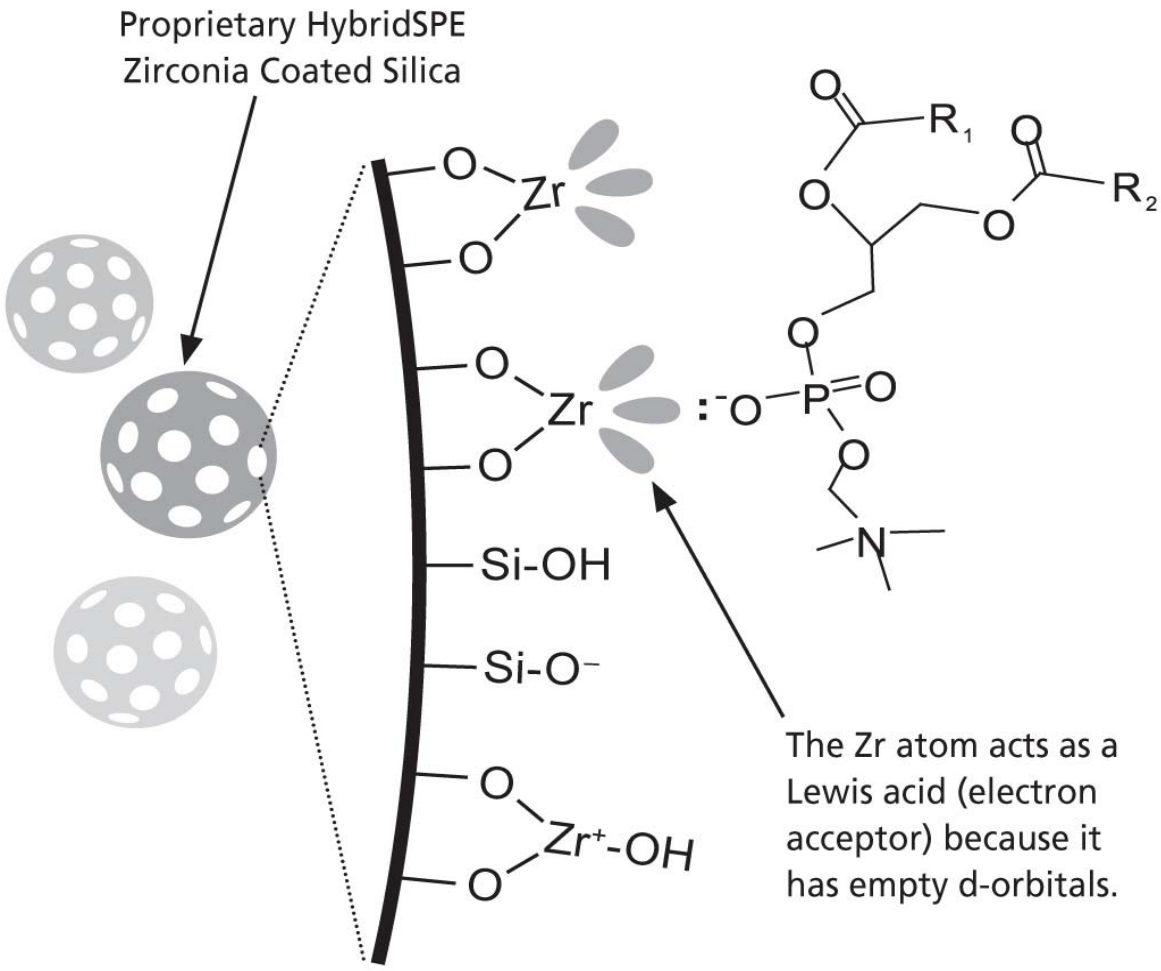
Also available in 1.0mL, 30mg cartridge (no 0.2um membrane

HybridSPE Particle Design

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 is not as Lewis "acidic" as pure zirconium oxide, thus enabling highly efficient extraction of phospholipids while remaining non-selective towards a broad range of basic, neutral and acidic compounds.



Retention Mechanism for HybridSPE

Base on Lewis acid-base interaction between zirconia surface and electron rich compounds

Primary approach:

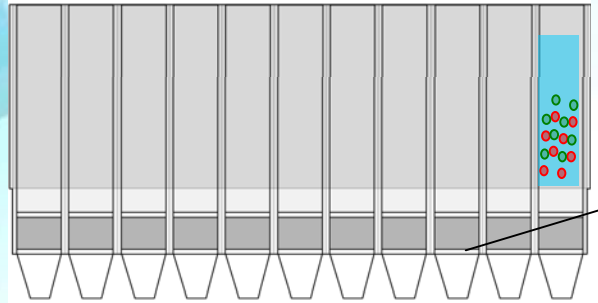
1% formic acid in ACN (primary procedure – optimal recovery for ~80% of all analytes)

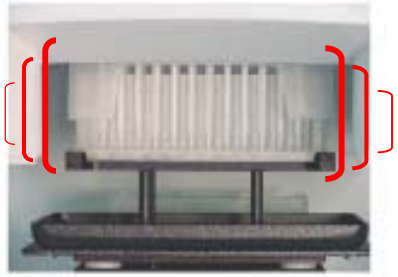
Lewis Base	rel. Strength on Zirconia
Hydroxide	**Strongest**
Phosphate	
Fluoride	
Citrate	
Sulfate	
Acetate	
Formate	
Chloride	**Weakest**

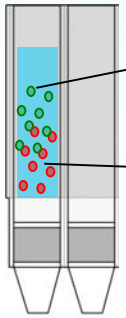
Secondary procedures for low recovery substances:

1. **Basic Compounds**
   1% formic acid in MeOH– *MeOH disrupts any secondary H-bonding interactions b/w basic compounds and zirconia-silica surface*
2. **Acidic Chelator Compounds**
   0.5% citric acid in ACN (Condition before sample addition with 400µL)
   – *citric acid is a stronger Lewis base than formic acid.*

“In-well Precipitation” for HybridSPE 96-well format only





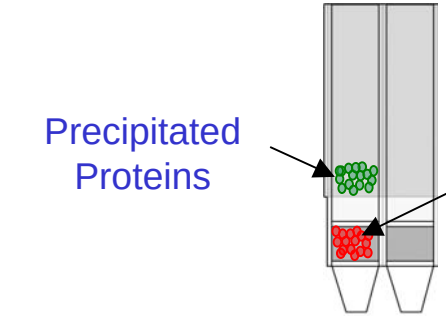


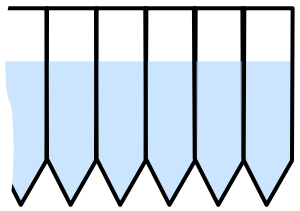
1a) Precipitate Proteins:
100 µL plasma/serum + 300 µL 1% formic acid in ACN (& I.S.)

! PTFE frit keeps plasma from dripping through packed-bed prematurely.

1b) Mix by vortexing HybridSPE plate or by aspirating/dispensing with 0.5-1 mL pipette tip

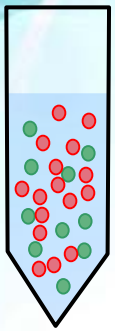
2) Apply vacuum. Packed-bed filter/frit assembly acts as a depth filter





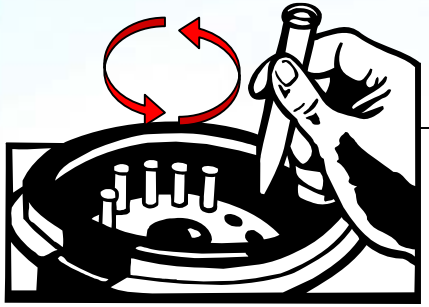
3) Resulting filtrate/eluate
free of proteins and phospholipids
Ready fro ananlysis

Off-Line Precipitation Method for HybridSPE 1 mL cartridge (or 96-well format)



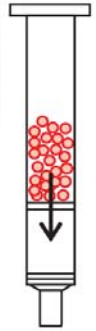
1a) Precipitate Proteins:

100 µL plasma/serum + 300 µL 1% formic acid in ACN (& I.S.)



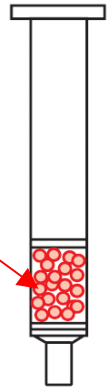
1b) Centrifuge

Separate precipitated proteins from solution



2) Transfer supernatant

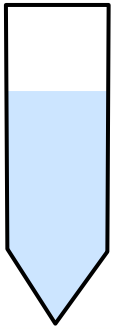
to HybridSPE cartridge or 96-well plate



3) Apply vacuuum.

Phospholipids stay on HybridSPE sorbent

Retained Phospholipids



4) Resulting filtrate/eluate

free of proteins and phospholipids

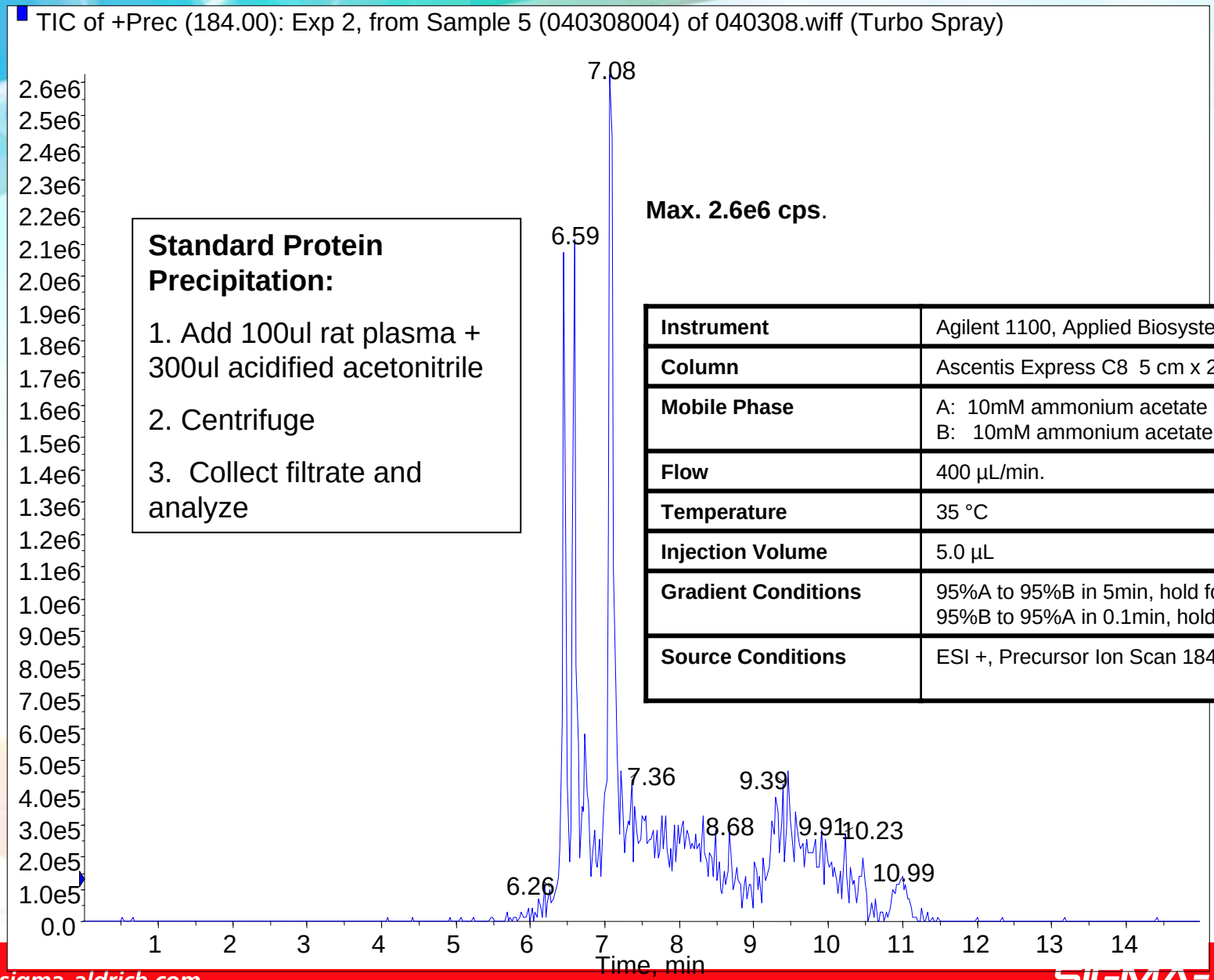
small molecules (e.g., pharma compounds & metabolites) pass through un-retained

Phospholipid Monitoring Method:

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, 100ul aliquots of rat plasma were added directly to the HybridSPE-PPT plate, 300ul 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.

Rat Plasma Processed Using Protein Precipitation, Precursor Ion Scan of m/z 184 for Phospholipids



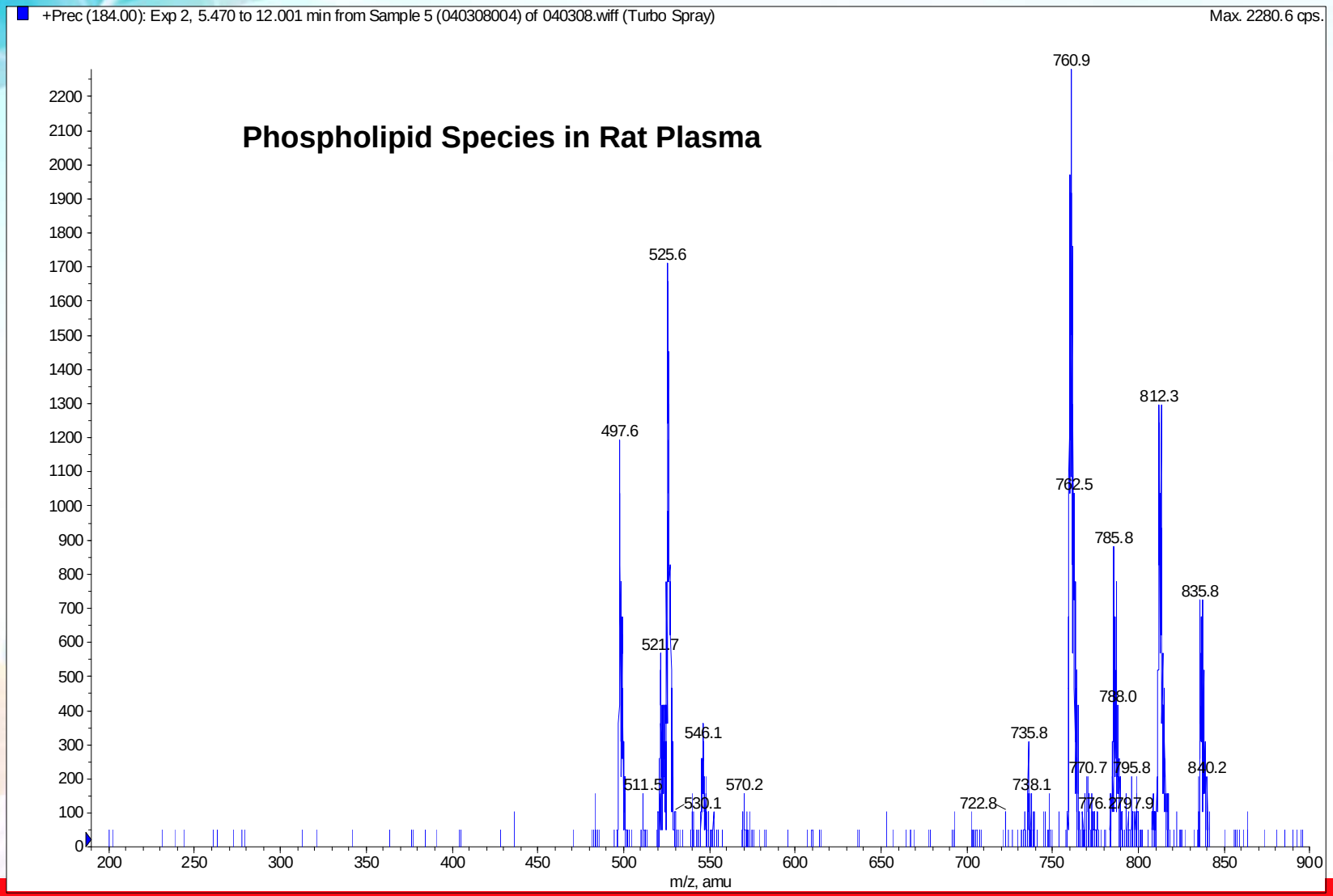
Max. 2.6e6 cps.

Standard Protein Precipitation:

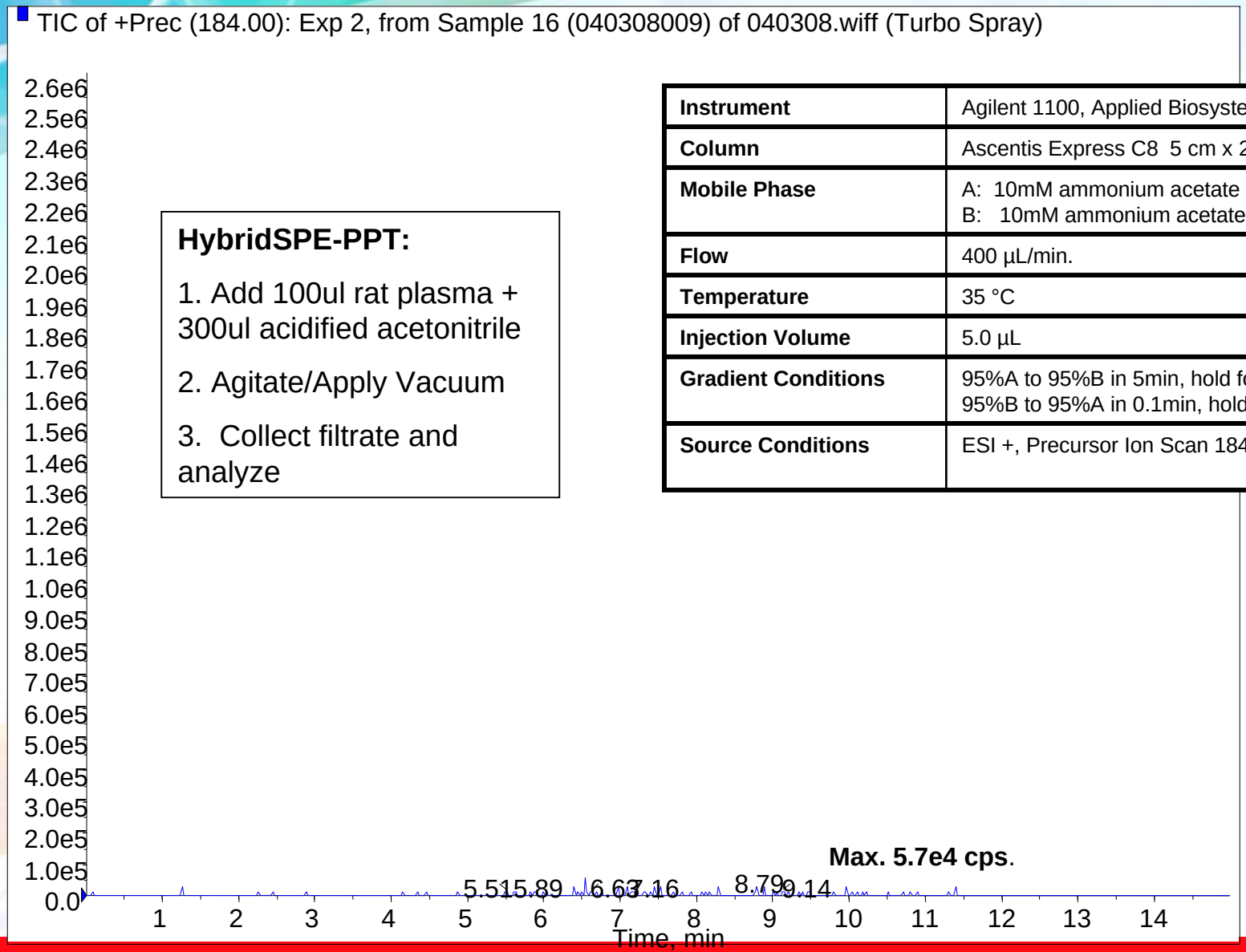
1. Add 100ul rat plasma + 300ul acidified acetonitrile
2. Centrifuge
3. Collect filtrate and analyze

Instrument	Agilent 1100, Applied Biosystems 3200QT
Column	Ascentis Express C8 5 cm x 2.1 mm, 2.7 µm
Mobile Phase	A: 10mM ammonium acetate in water B: 10mM ammonium acetate in acetonitrile
Flow	400 µL/min.
Temperature	35 °C
Injection Volume	5.0 µL
Gradient Conditions	95%A to 95%B in 5min, hold for 3min then 95%B to 95%A in 0.1min, hold for 7min
Source Conditions	ESI +, Precursor Ion Scan 184m/z

Phospholipid Precursor Ion Scan, Protein Precipitated Rat Plasma



Rat plasma processed Using Hybrid SPE, Precursor Ion Scan of m/z 184 for Phospholipids



HybridSPE-PPT:

1. Add 100ul rat plasma + 300ul acidified acetonitrile
2. Agitate/Apply Vacuum
3. Collect filtrate and analyze

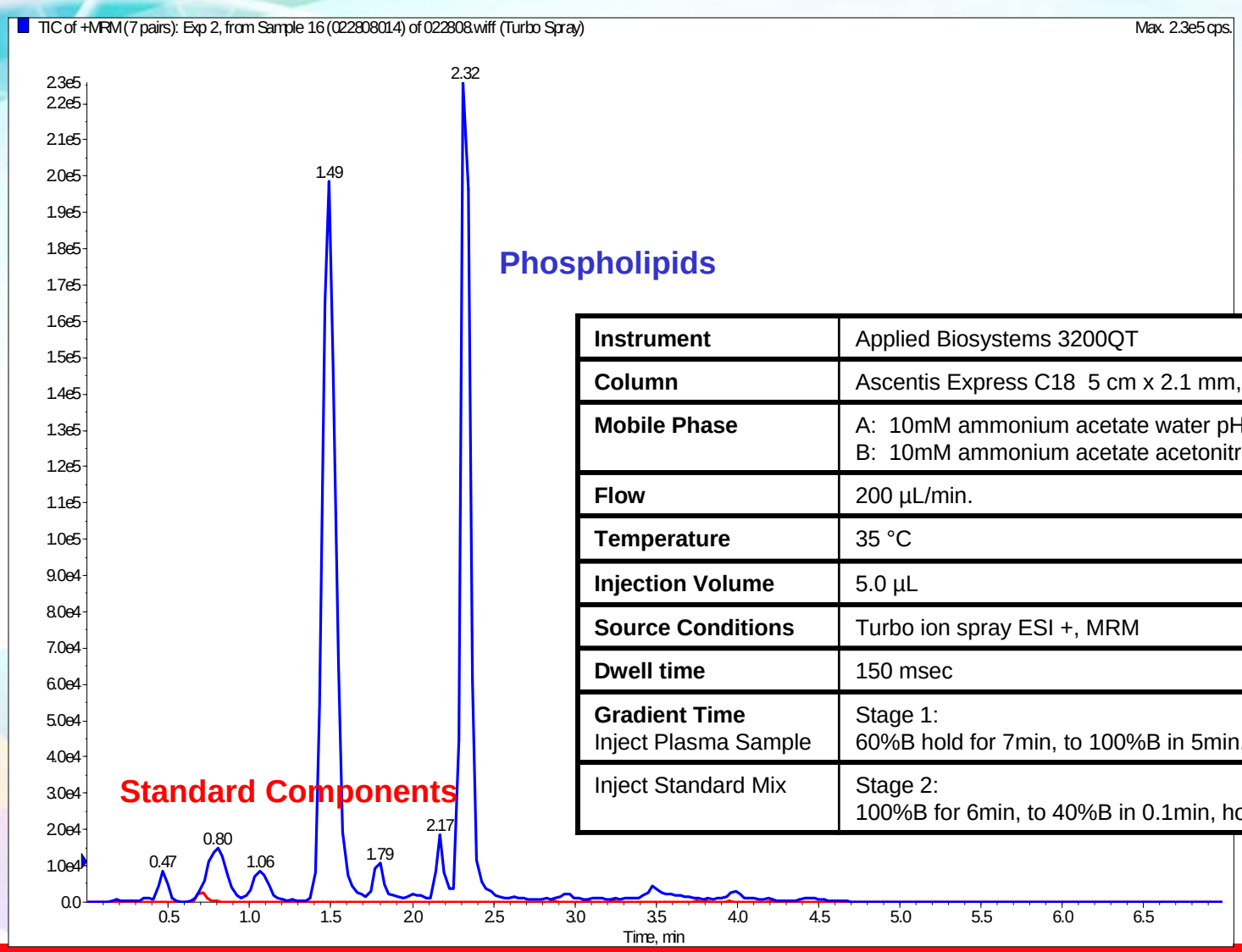
Instrument	Agilent 1100, Applied Biosystems 3200QT
Column	Ascentis Express C8 5 cm x 2.1 mm, 2.7 µm
Mobile Phase	A: 10mM ammonium acetate in water B: 10mM ammonium acetate in acetonitrile
Flow	400 µL/min.
Temperature	35 °C
Injection Volume	5.0 µL
Gradient Conditions	95%A to 95%B in 5min, hold for 3min then 95%B to 95%A in 0.1min, hold for 7min
Source Conditions	ESI +, Precursor Ion Scan 184m/z

- 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.

Ionization Effect Due to Phospholipid Interference:

- A two stage gradient method was develop to enable the overlap of phospholipids with the test analytes.
- Stage one consisted of injecting plasma samples subjected to either protein precipitation or the HybridSPE-PPT platform.
- Plasma sample was injected onto the column and a partial gradient was run.
- Stage two consisted of injecting a 10ng/ml standard solution of analytes onto the column.
- 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.

Overlay of Co-retained Analytes with Phospholipids from Standard Protein Precipitation



Instrument	Applied Biosystems 3200QT
Column	Ascentis Express C18 5 cm x 2.1 mm, 2.7 µm
Mobile Phase	A: 10mM ammonium acetate water pH 6.9 B: 10mM ammonium acetate 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 7min, to 100%B in 5min, hold for 5min
Inject Standard Mix	Stage 2: 100%B for 6min, to 40%B in 0.1min, hold for 5min

Ionization Effect Due to Phospholipid Interference:

Phosholipids Effect on Ionizaion of Basic Test Components

400ul of PPT Rat Plasma STD 10ug/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

- Injection the analytes into the phospholipid retention window eliminated misinterpretation of ionization effect that may have been due to salt in the matrix.
- Two step gradient system enabled a direct determination of ionization effect of phospholipids on test analytes.

- Samples process with only protein precipitation resulted in sever ionization suppression of both clonidine and protryptiline with more than 50% signal loss.
- Ion-suppression was not as sever for desmethyldiazapam 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.

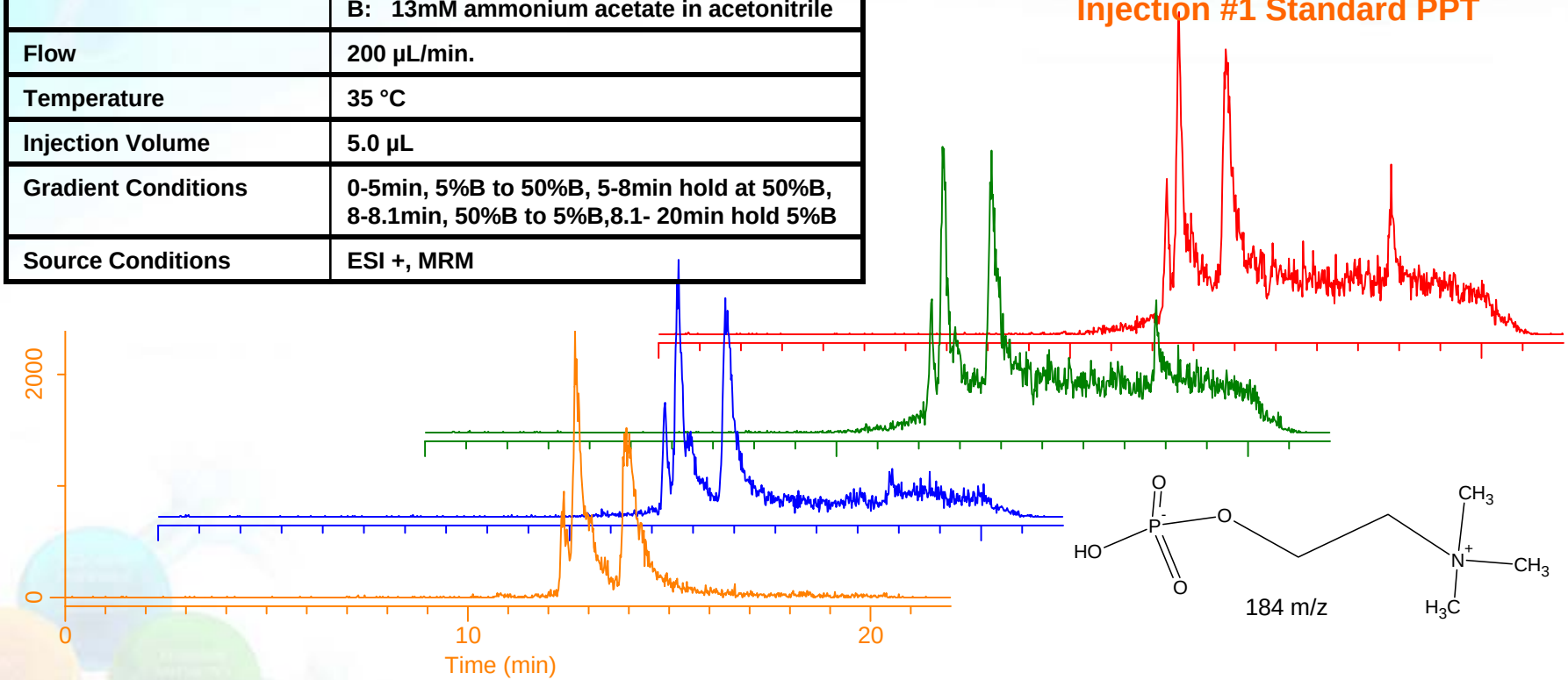
Analyte Recovery and Phospholipids Buildup:

- Chromatographic impact experiments were designed to demonstrate the affect of phospholipid build-up on a typical reversed-phase gradient separation.
- In these experiment rat plasma samples were spiked with a mixture of acidic and basic compounds.
- One set of samples were prepared using standard protein precipitation
- Another set were prepared using the HybridSPE-PPT platform.
- The samples were then analyzed for absolute recovery of test analytes and phospholipid content.

Phospholipid HPLC Column Contamination

Standard Protein PPT + Sub 3 µm LC Column + MS

Instrument	**Agilent 1100, Applied Biosystems 3200QT**
Column	**Ascentis Express C18 5 cm x 2.1 mm, 2.7 µm**
Mobile Phase	**A: 13mM ammonium acetate in water B: 13mM ammonium acetate in acetonitrile**
Flow	**200 µL/min.**
Temperature	**35 °C**
Injection Volume	**5.0 µL**
Gradient Conditions	**0-5min, 5%B to 50%B, 5-8min hold at 50%B, 8-8.1min, 50%B to 5%B,8.1- 20min hold 5%B**
Source Conditions	**ESI +, MRM**



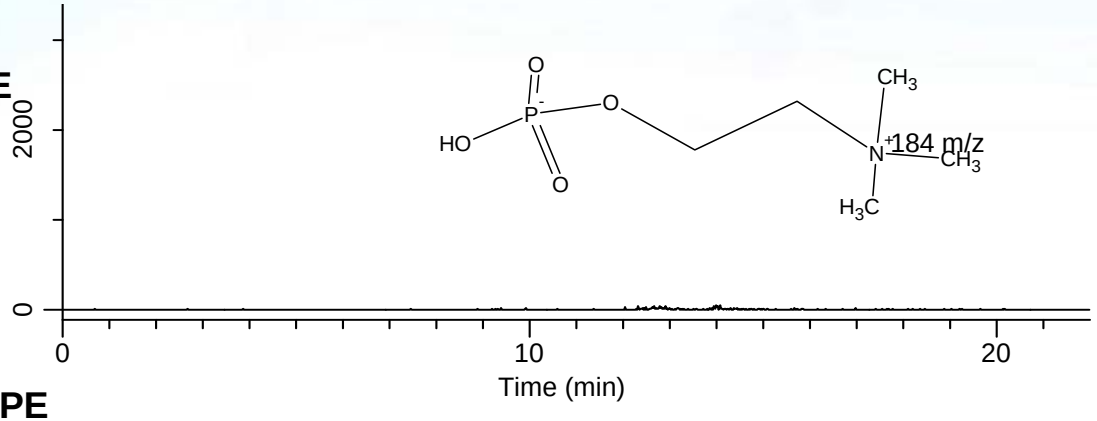
Sample Preparation: Protein precipitation of 100 uL blank rat plasma with 300 uL 1% formic acid in MeCN. Vortex 1 min. followed by centrifuged for 3 min. at 15k RPM. Injection of supernatant.

No Phospholipid Accumulation

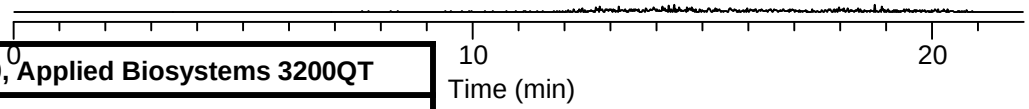
HybridSPE-PPT + Sub 3 µm LC Column + MS

Sample Preparation: 100 uL blank rat plasma added to a HybridSPE – 96-well plate followed by addition of 300 uL 1% formic acid in MeCN. Vortex 1 min. followed by agitation for 1 min. Vacuum was applied and the resulting eluate was analyzed directly via LC-MS.

Injection #1 Hybrid SPE Technology



Injection #20 Hybrid SPE Technology



Instrument	**Agilent 1100, Applied Biosystems 3200QT**
Column	**Ascentis Express C18 5 cm x 2.1 mm, 2.7 µm**
Mobile Phase	**A: 13mM ammonium acetate in water** **B: 13mM ammonium acetate in acetonitrile**
Flow	**200 µL/min.**
Temperature	**35 °C**
Injection Volume	**5.0 µL**
Gradient Conditions	**0-5min, 5%B to 50%B, 5-8min hold at 50%B, 8-8.1min, 50%B to 5%B,8.1- 20min hold 5%B**
Source Conditions	**ESI +, MRM**

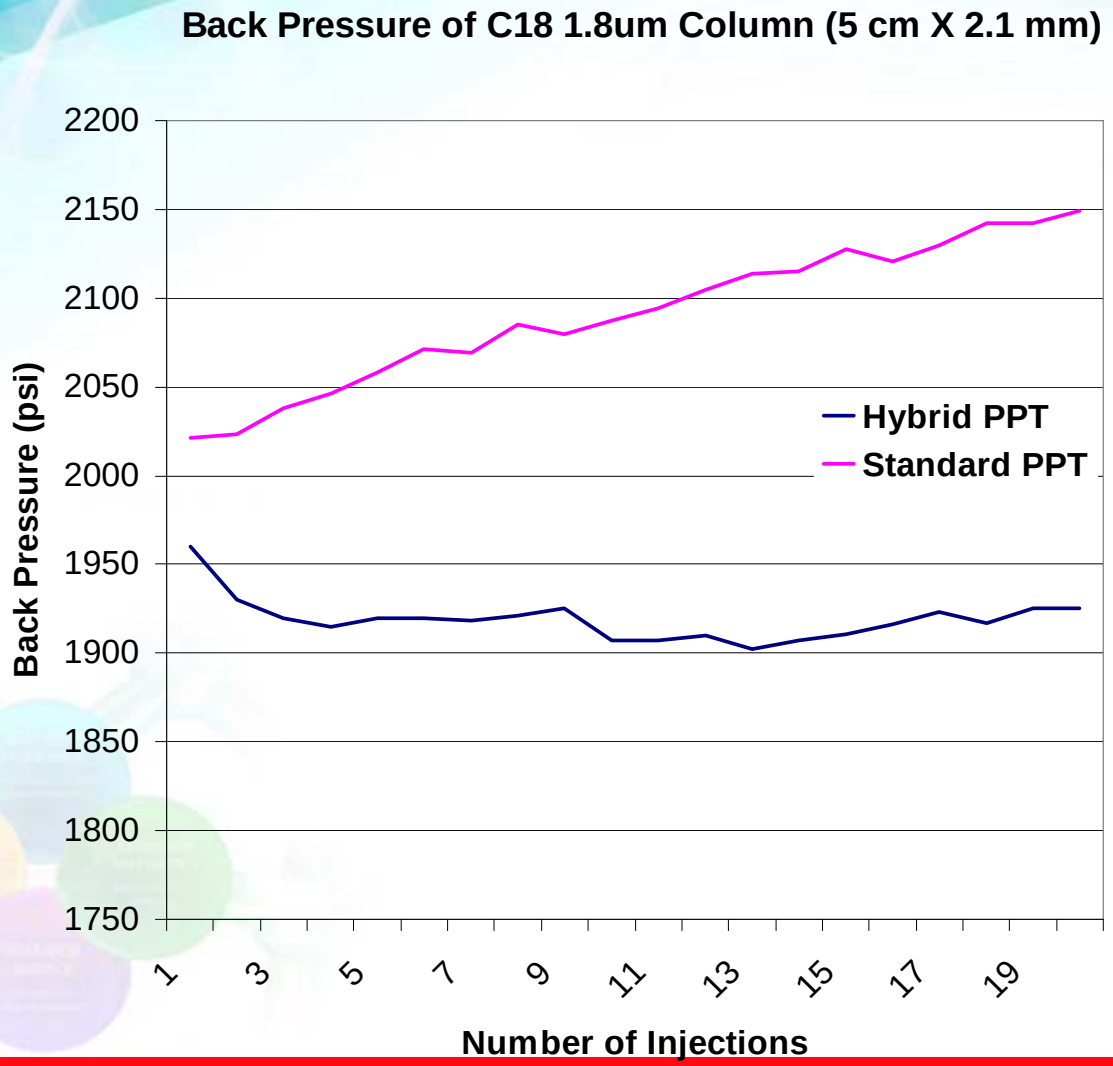
- Chromatographic build-up of phospholipids from protein precipitated samples was clearly evident.
- After 20 injections of protein precipitated plasma samples, build up of phospholipids continued to increase.
- The 45% increase in organic modifier over 5min on the small column dimension was not sufficient to elute the phospholipids form the column.
- Plasma samples prepared using the HybridSPE-PPT platform were depleted of phospholipids resulting in to chromatographic build up,
- eliminating the need to perform timely high organic elution of the column to remove retained matrix.

Absolute Recovery of analytes from generic gradient system

Compound	Hybrid SPE Recovery	Protein Precipatation
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.
- Low response was observed for the protein precipitation samples on procainamide, buspirone and tamoxifen due to interference from co-retained phospholipids.
- A slightly lower recover was observed for phenylboronic acid due to some binding with the phases.

Effect of HybridSPE-PPT vs. Protein PPT Only on Back Pressure of Sub *2* µm C18 Column



Pressure build-up caused by accumulation of small particle aggregates not removed by centrifugation, but removed by HybridSPE 96-well plate.

Capacity of HybridSPE (50mg Bed)

Sample Preparation:

Standard Protein Precipitation (control) performed in centrifuge tube by placing 100ul of spiked rat plasma, 300ul of 1% formic acid acetonitrile into tube. Tube was agitated for 1 minute then spun on centrifuge for 3 minutes to remove solids.

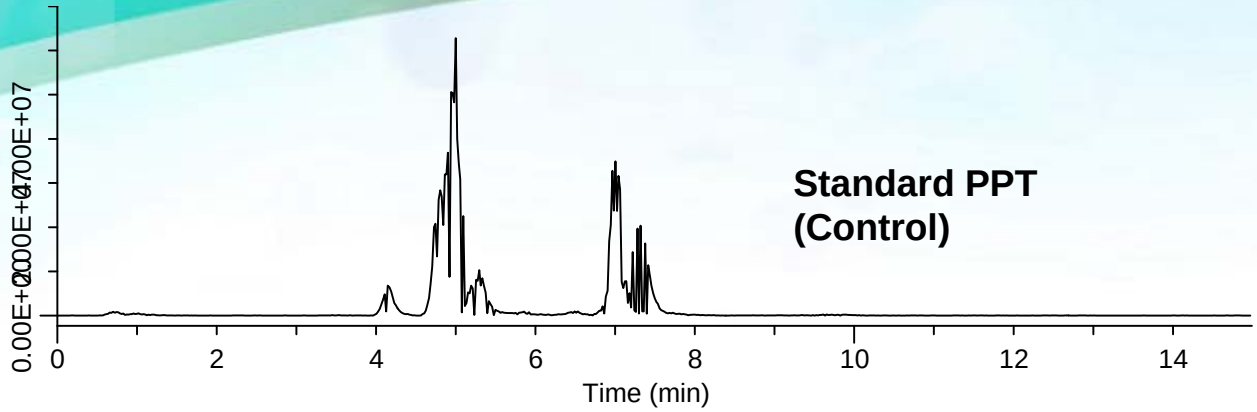
Rat plasma (1ml lyophilized) was reconstituted with 1ml of water.

Protein precipitation performed in 96-well plate by placing a 1:3 ratio of rat plasma and 1% formic acid acetonitrile into well using increasing volumes of sample. Plate was agitated for 1 minute then vacuum applied. Sample collected and analyzed directly.

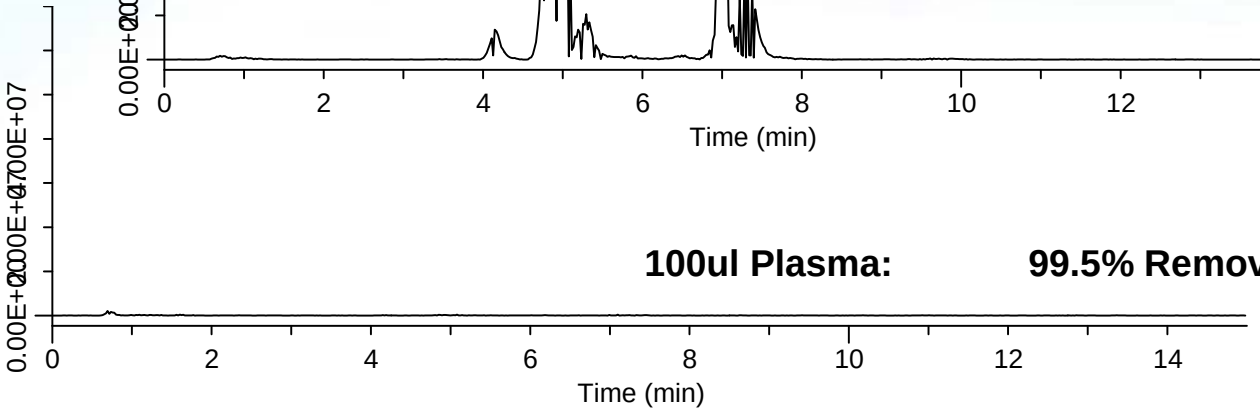
Instrument	Thermo LCQ Ion Trap
Column	Ascentis Express C18 5 cm x 2.1 mm, 2.7 µm
Mobile Phase	10mM ammonium acetate in (95:5 methanol:water)
Flow	200 µL/min.
Temperature	50 °C
Injection Volume	5.0 µL
Sample	100ul Rat Plasma (with citrated anticoagulant)
Source Conditions	ESI +, XIC 184, 496, 758,782, 786

100ul plasma : 300ul 1% Formic ACN	**99.5% PL Removal**
200ul plasma : 600ul 1% Formic ACN	**94.9% PL Removal**
300ul plasma : 900ul 1% Formic ACN	**88.0% PL Removal**

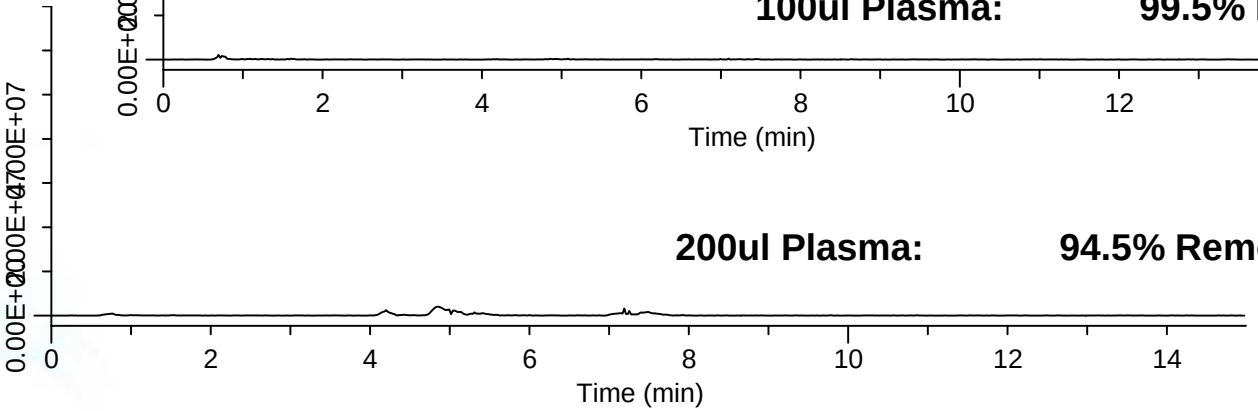
Capacity of HybridSPE for Phospholipid Removal



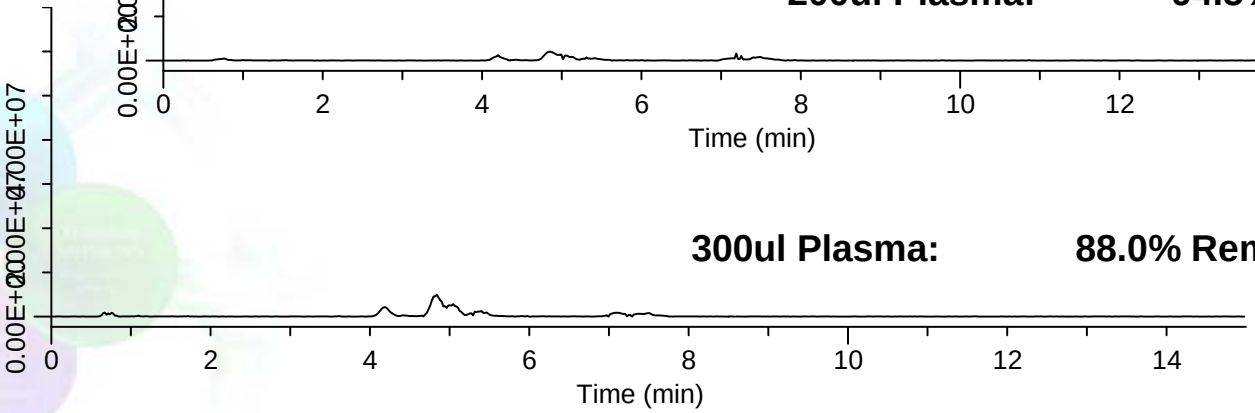
Standard PPT (Control)



100ul Plasma: 99.5% Removal



200ul Plasma: 94.5% Removal

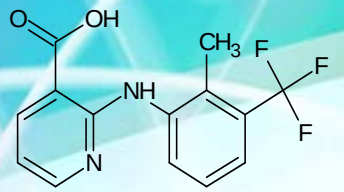


300ul Plasma: 88.0% Removal

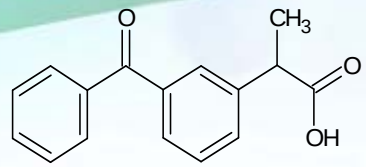
Chelation on Zirconia-Silica Surface

- Weak acidic compounds along with chelating compounds can be retained strongly on the HybridSPE zirconia-coated silica surface.
- Addition of lewis base such as formate, citrate, acetate is normally necessary for good recovery of chelators
- Next study examines the impact of Lewis base modifier on the recovery of acidic compounds.

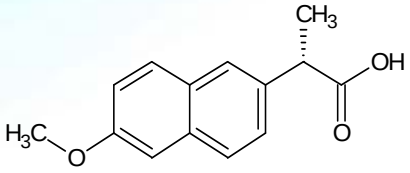
Analysis of NSAIDS from Rat Plasma Using HybridSPE



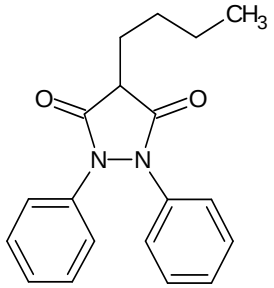
Flunixin



Ketoprofen

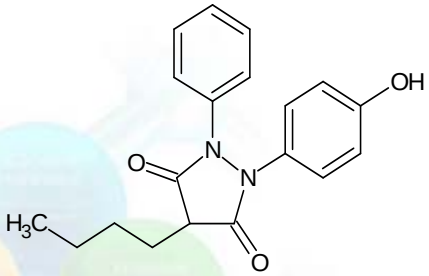


Naproxen



Monoisotopic Mass = 308.152478 Da

Phenylbutazone

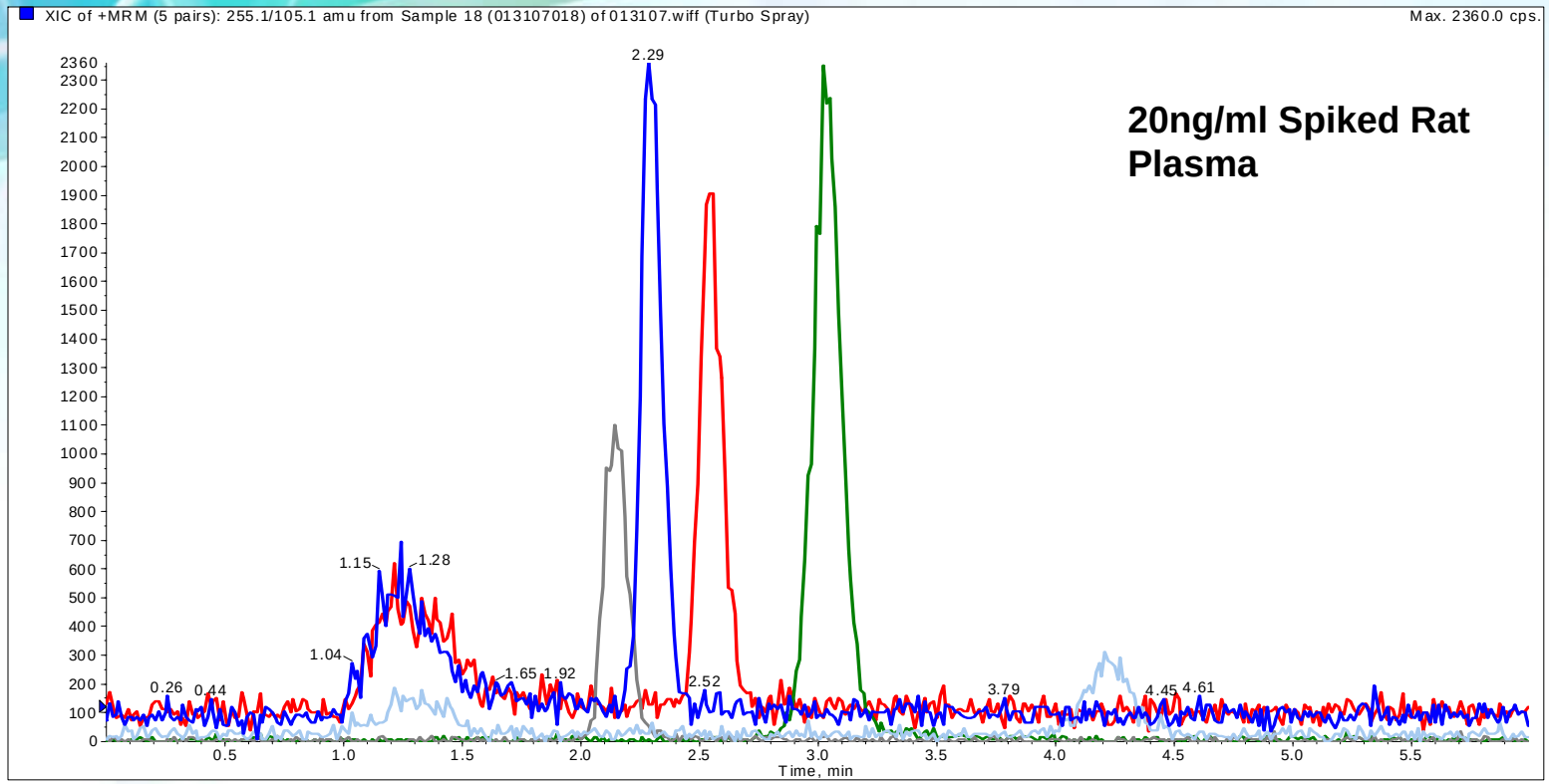


Monoisotopic Mass = 324.147393 Da

Oxyphenbutazone

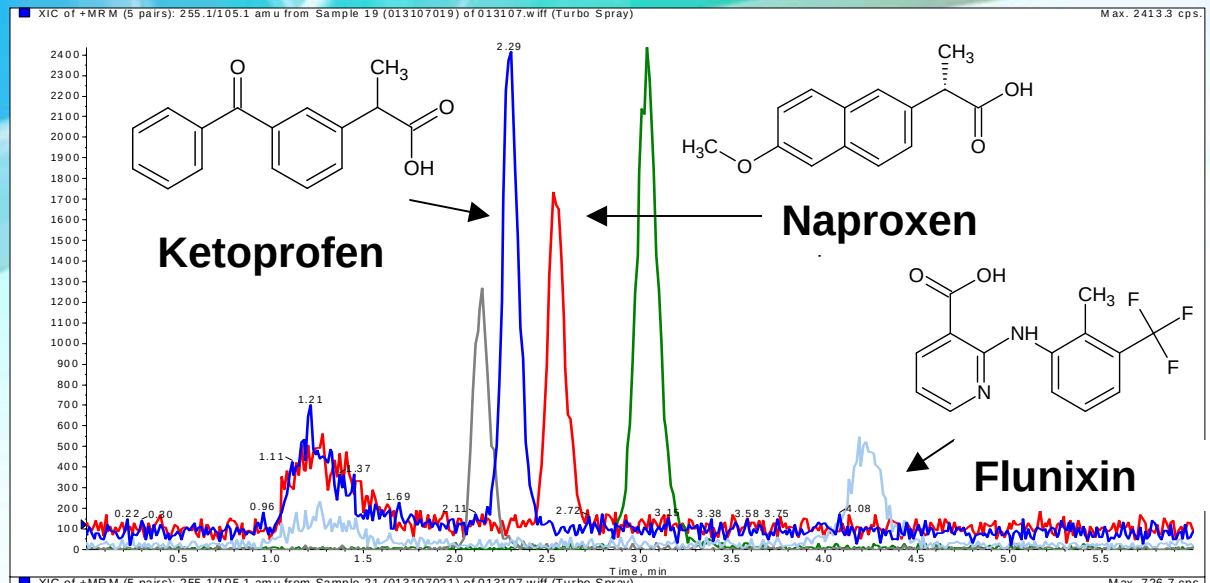
Instrument	Agilent 1100, Applied Biosystems 3200QT
Column	Ascentis RP Amide 5 cm x 2.1 mm, 5 µm
Mobile Phase	10mM ammonium acetate pH 5.5 : methanol (40:60)
Flow	200 µL/min.
Temperature	35 °C
Injection Volume	5.0 µL
Sample:	Rat Plasma (PPT & centrifuged off-line), (1ml rat plasma, 3ml acetonitrile 1% formic acid)
Cartridge	1ml 30mg HybridSPE cartridge

Analysis of NSAIDS from Rat Plasma Using HybridSPE



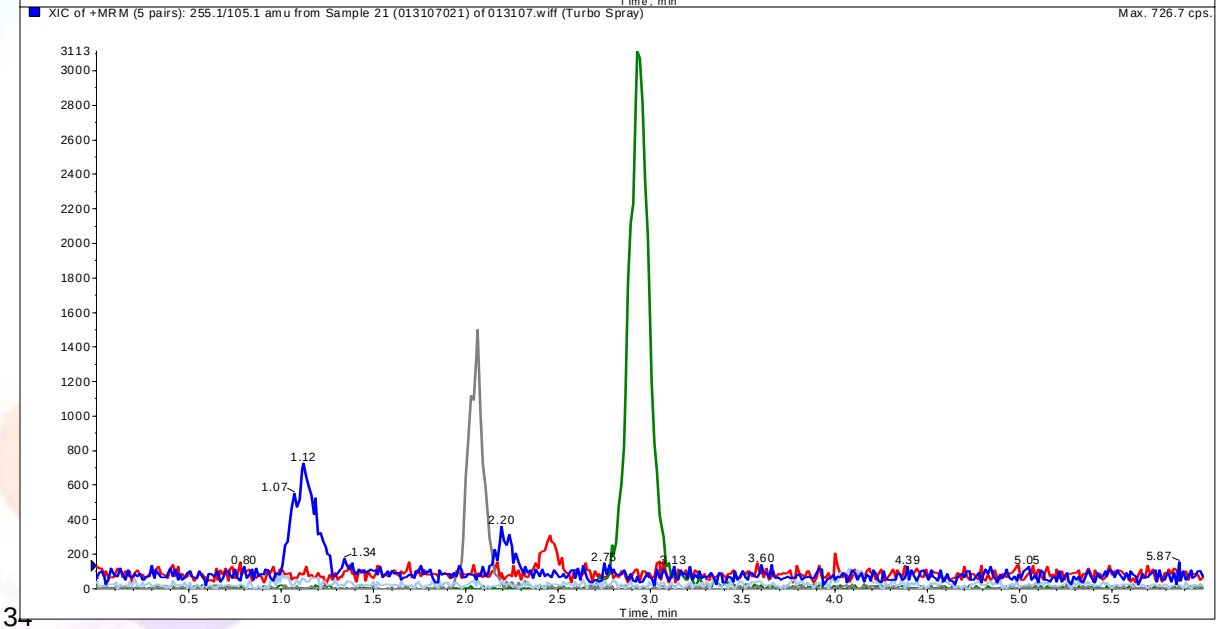
		Retention Time	Absolute Recovery
1.	Oxyphenbutazone	2.14	95.0
2.	Ketoprofen	2.29	88.0
3.	Naproxen	2.54	104
4.	Phenylbutazone	3.04	110
5.	Flunixin	4.22	135

Importance of Formic Acid (Formate Weak Lewis Base)



Acidic Drug Mixture 20ng/ml
75/25 Acetonitrile/H2O
1.0%Formic Acid

High relative concentration of Formic Acid enables recovery of carboxylic acid

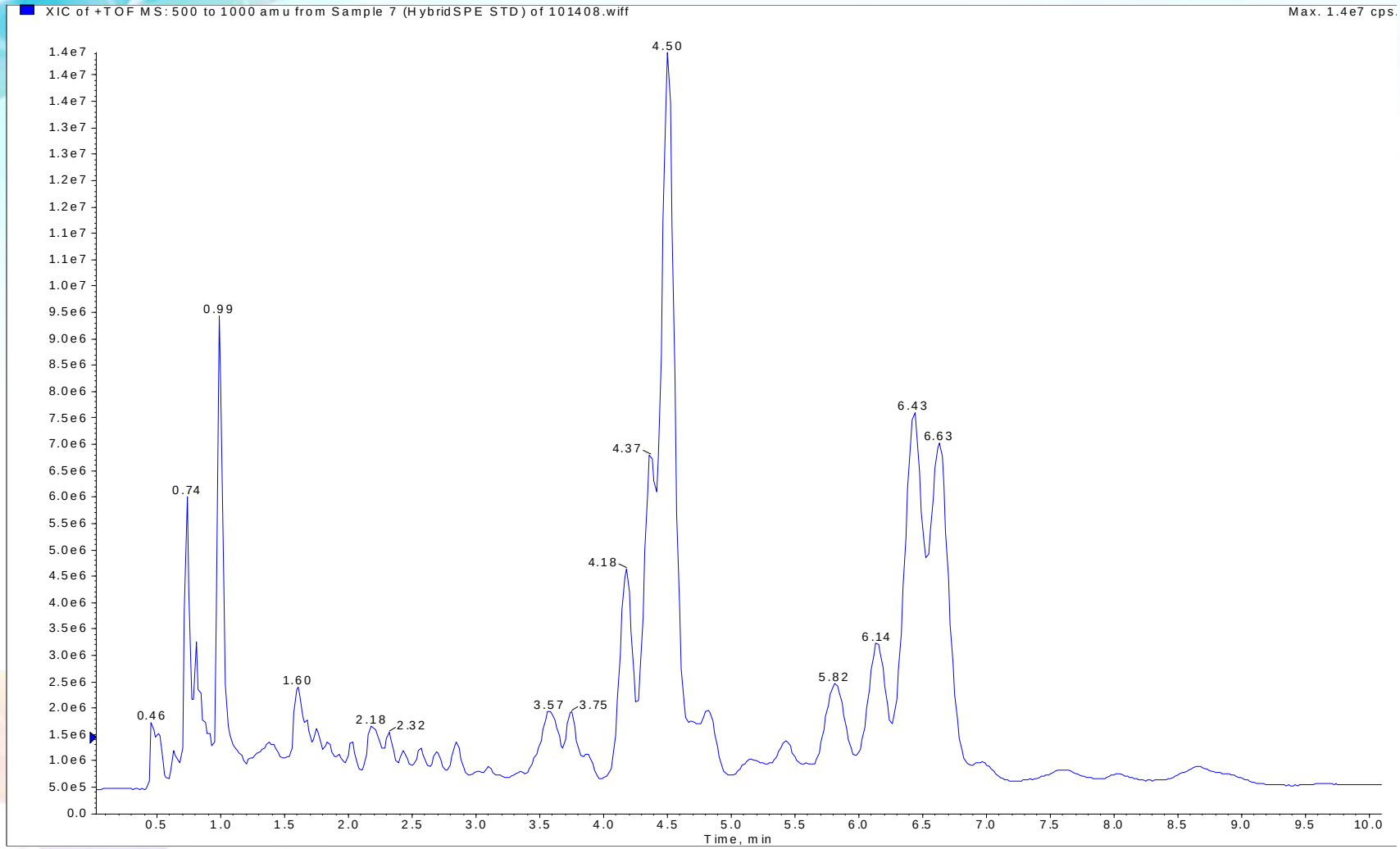


Acidic Drug Mixture 20ng/ml
75/25 Acetonitrile/H2O

PPT performed with out Formic Acid causes carboxylic acids to retain on Zirconia-Si phase.

Non-acidic drugs elute normally

Effect of Organic Ratio, Acidic Modifier and Organic Modifier on Phospholipid Removal with HybridSPE

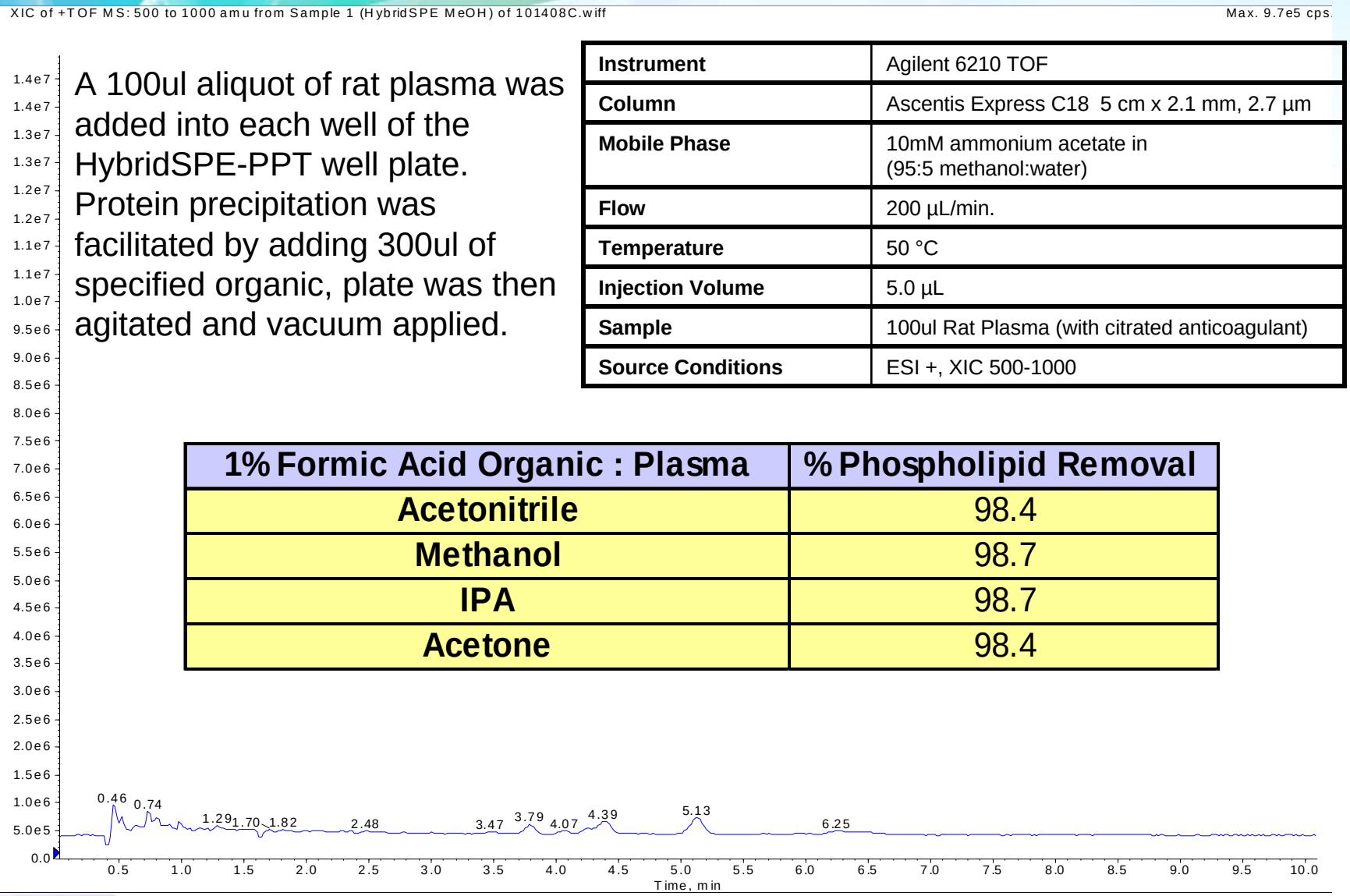


Phospholipid Removal Organic Modifier Impact

A 100ul aliquot of rat plasma was added into each well of the HybridSPE-PPT well plate. Protein precipitation was facilitated by adding 300ul of specified organic, plate was then agitated and vacuum applied.

Instrument	Agilent 6210 TOF
Column	Ascentis Express C18 5 cm x 2.1 mm, 2.7 µm
Mobile Phase	10mM ammonium acetate in (95:5 methanol:water)
Flow	200 µL/min.
Temperature	50 °C
Injection Volume	5.0 µL
Sample	100ul Rat Plasma (with citrated anticoagulant)
Source Conditions	ESI +, XIC 500-1000

1% Formic Acid Organic : Plasma	% Phospholipid Removal
Acetonitrile	98.4
Methanol	98.7
IPA	98.7
Acetone	98.4

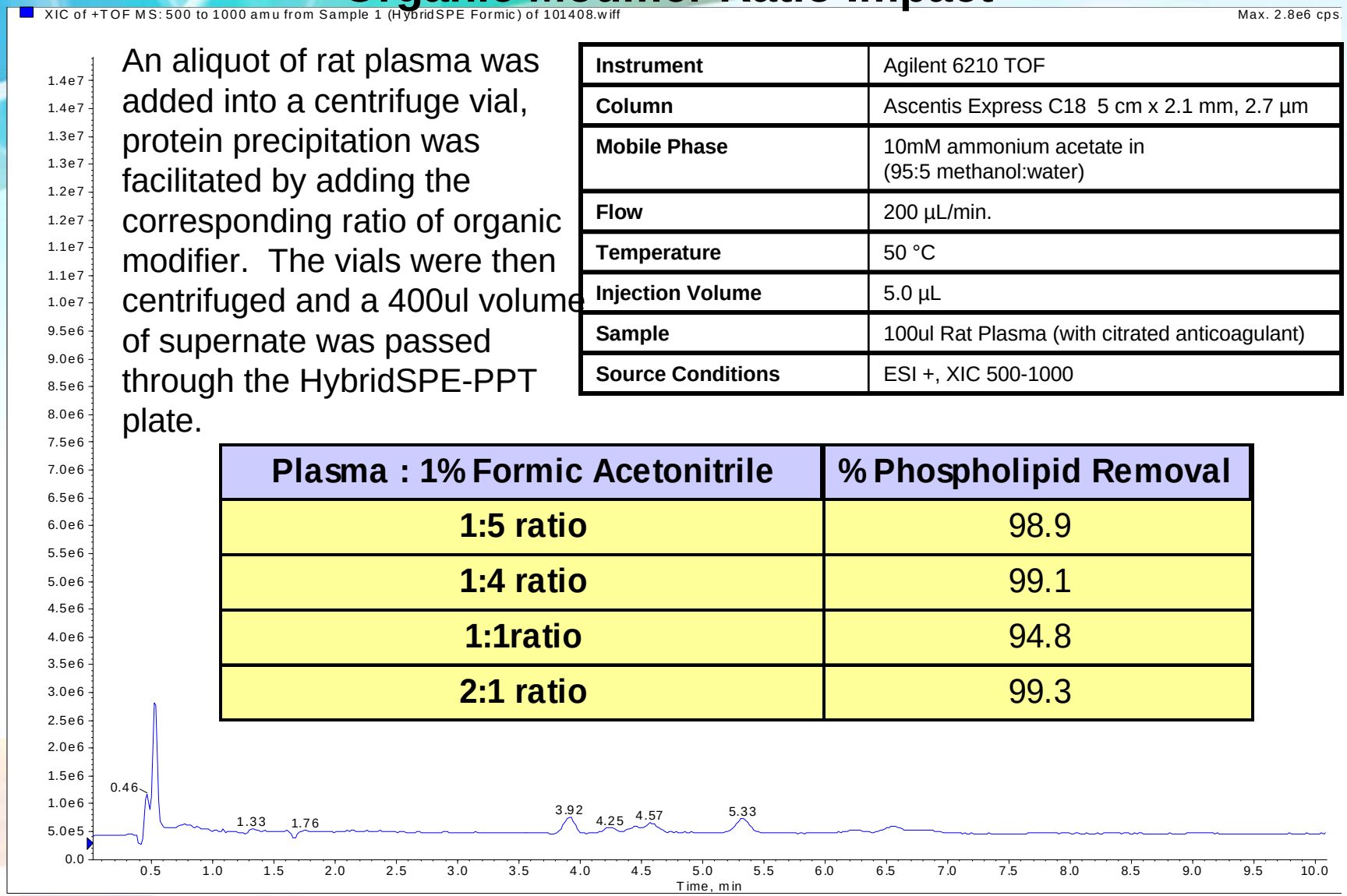


Phospholipid Removal
Organic Modifier Ratio Impact

An aliquot of rat plasma was added into a centrifuge vial, protein precipitation was facilitated by adding the corresponding ratio of organic modifier. The vials were then centrifuged and a 400ul volume of supernate was passed through the HybridSPE-PPT plate.

Instrument	Agilent 6210 TOF
Column	Ascentis Express C18 5 cm x 2.1 mm, 2.7 µm
Mobile Phase	10mM ammonium acetate in (95:5 methanol:water)
Flow	200 µL/min.
Temperature	50 °C
Injection Volume	5.0 µL
Sample	100ul Rat Plasma (with citrated anticoagulant)
Source Conditions	ESI +, XIC 500-1000

Plasma : 1% Formic Acetonitrile	% Phospholipid Removal
1:5 ratio	98.9
1:4 ratio	99.1
1:1ratio	94.8
2:1 ratio	99.3

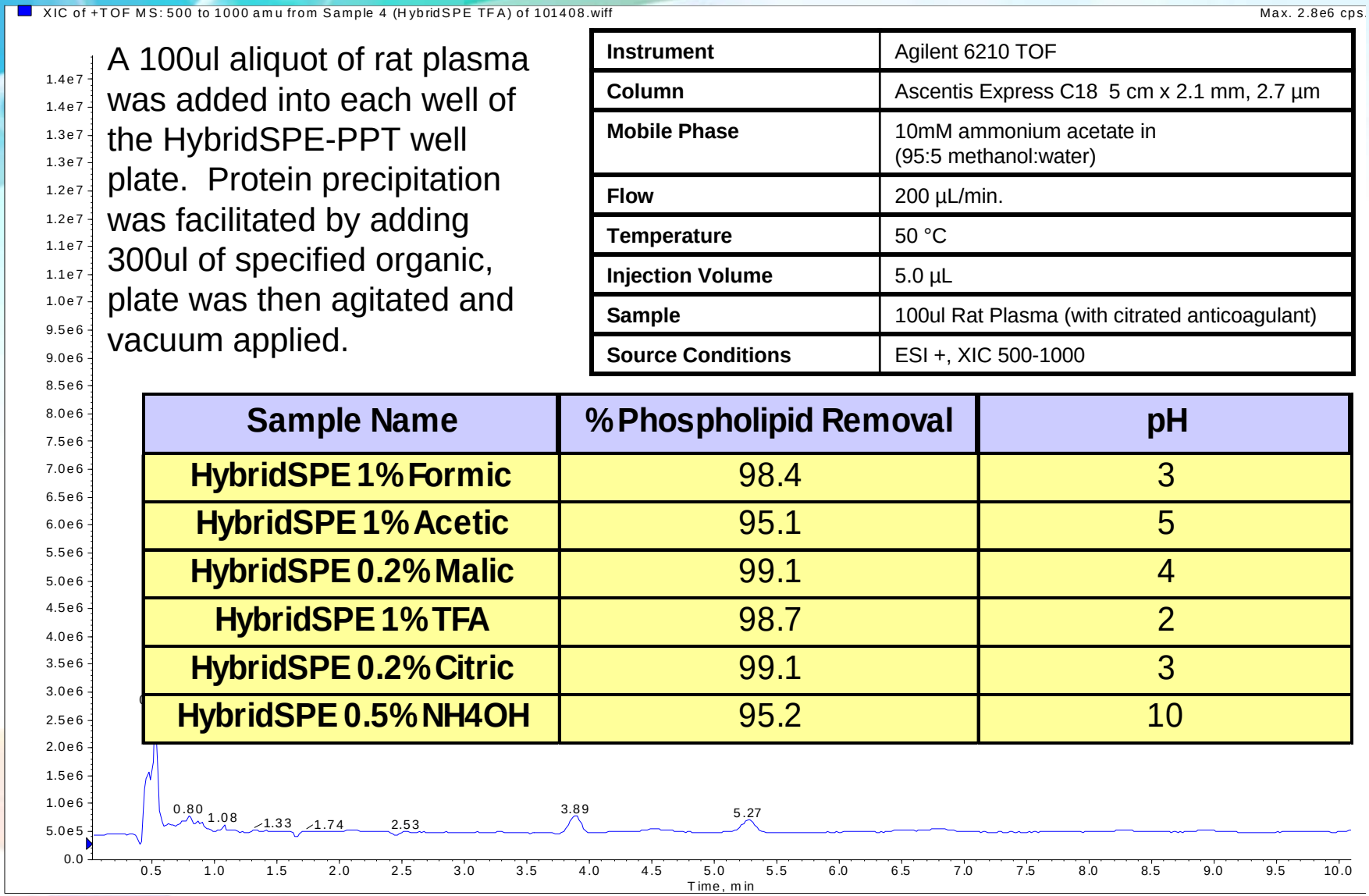


Phospholipid Removal
Acidic Modifier Impact

A 100ul aliquot of rat plasma was added into each well of the HybridSPE-PPT well plate. Protein precipitation was facilitated by adding 300ul of specified organic, plate was then agitated and vacuum applied.

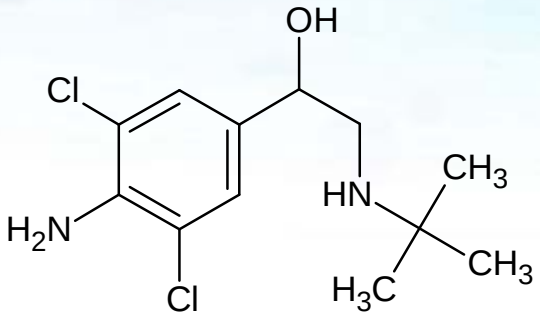
Instrument	Agilent 6210 TOF
Column	Ascentis Express C18 5 cm x 2.1 mm, 2.7 µm
Mobile Phase	10mM ammonium acetate in (95:5 methanol:water)
Flow	200 µL/min.
Temperature	50 °C
Injection Volume	5.0 µL
Sample	100ul Rat Plasma (with citrated anticoagulant)
Source Conditions	ESI +, XIC 500-1000

Sample Name	% Phospholipid Removal	pH
HybridSPE 1% Formic	98.4	3
HybridSPE 1% Acetic	95.1	5
HybridSPE 0.2% Malic	99.1	4
HybridSPE 1% TFA	98.7	2
HybridSPE 0.2% Citric	99.1	3
HybridSPE 0.5% NH4OH	95.2	10



Applications of Phospholipid Impact on Chromatographic Separations

Effect of Phospholipids on Enantiomeric Separation of Clenbuterol

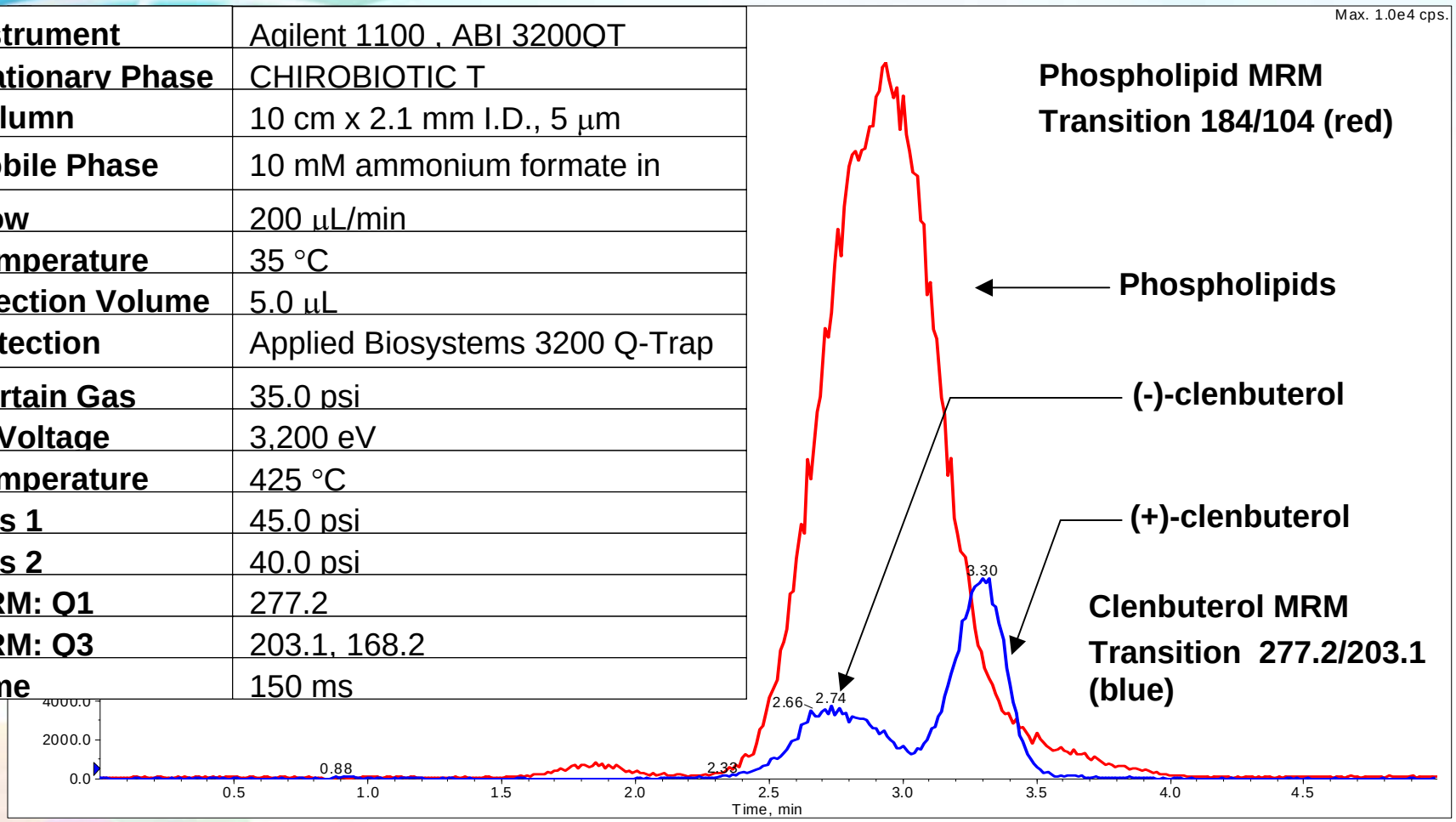


HybridSPE Protocol:

- Rat plasma samples were spiked at a concentration of 50 ng/mL with (+/-) -clenbuterol standard.
- A 1 mL aluquot of spiked plasma was combined with 3 mL of 1% formic acid in acetonitrile and agitated for 30 sec. The mixture was centrifuged for 3 min at 15,000 rpm.
- A 400 µL aliquot of the supernatant was then passed through a 1 mL, 30 mg bed HybridSPE cartridge at a flow rate of 1 drop/sec.
- The eluant was collected and analyzed directly.

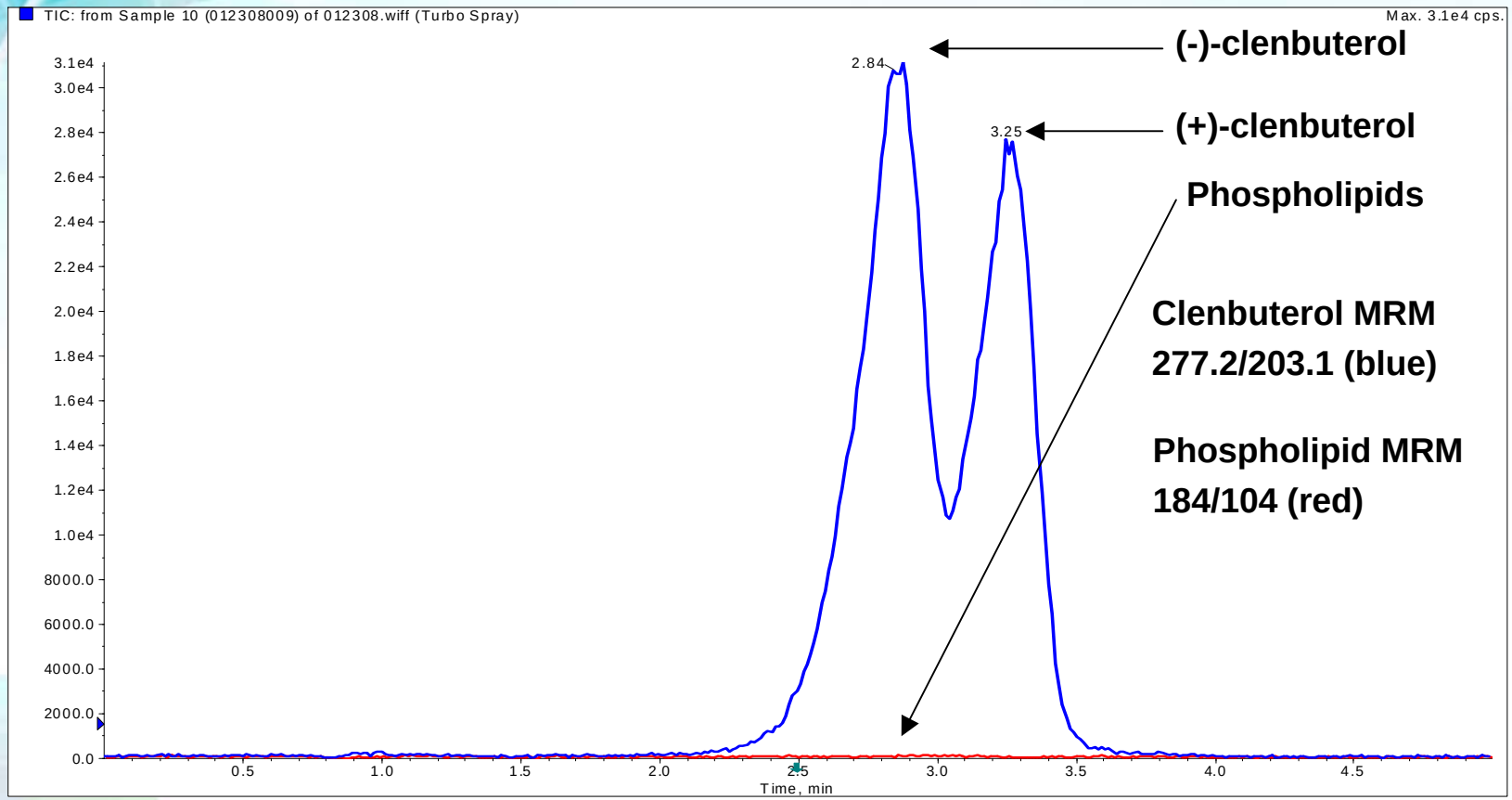
Overlay of Phospholipid and Clenbuterol after standard protein precipitation.

Instrument	Agilent 1100 , ABI 3200QT
Stationary Phase	CHIROBIOTIC T
Column	10 cm x 2.1 mm I.D., 5 μm
Mobile Phase	10 mM ammonium formate in
Flow	200 μL/min
Temperature	35 °C
Injection Volume	5.0 μL
Detection	Applied Biosystems 3200 Q-Trap
Curtain Gas	35.0 psi
IS Voltage	3,200 eV
Temperature	425 °C
Gas 1	45.0 psi
Gas 2	40.0 psi
MRM: Q1	277.2
MRM: Q3	203.1, 168.2
Time	150 ms



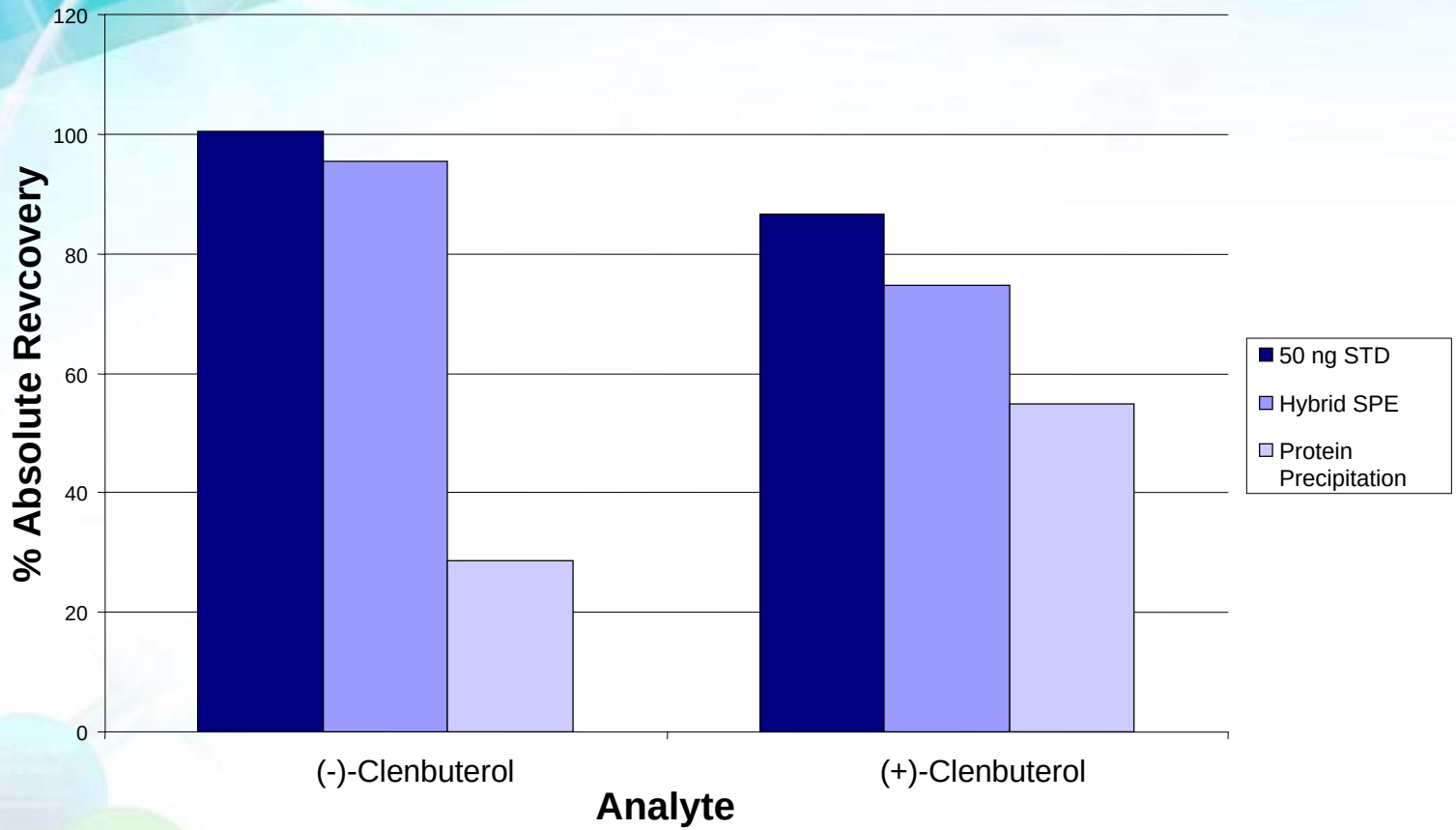
Chiral conditions optimized for overlap of enantiomers with phospholipids, not optimized for enantiomeric resolution

Overlay of Clenbuterol and Phospholipid XICs after HybridSPE



Phospholipids depleted using HybridSPE resulting in increased response of both (+,-) enantiomers of Clenbuterol

Absolute Recovery Comparison of Clenbuterol using standard protein precipitation and HybridSPE Methods



Absolute values calculated using external standard which was not passed through cartridge. Bar graph of STD represents recovery of standard which had been passed through cartridge and compared to external standard.

Decreasing Analysis Time using HybridSPE

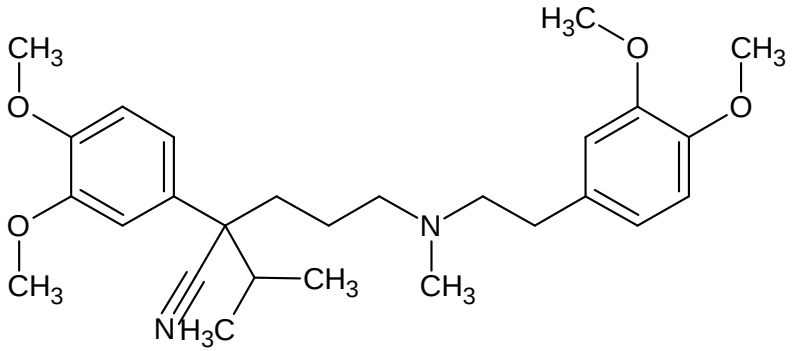
Gradients typically required to remove phospholipids

Column: Ascentis Express C18, 5 cm x 2.1 mm
Chromatograph: Agilent 1200RR
Mobile Phase: A. 30:70 Water : Acetonitrile pH 2.55 with Formic Acid
B. 13mM ammonium formate (5:95 water : methanol)

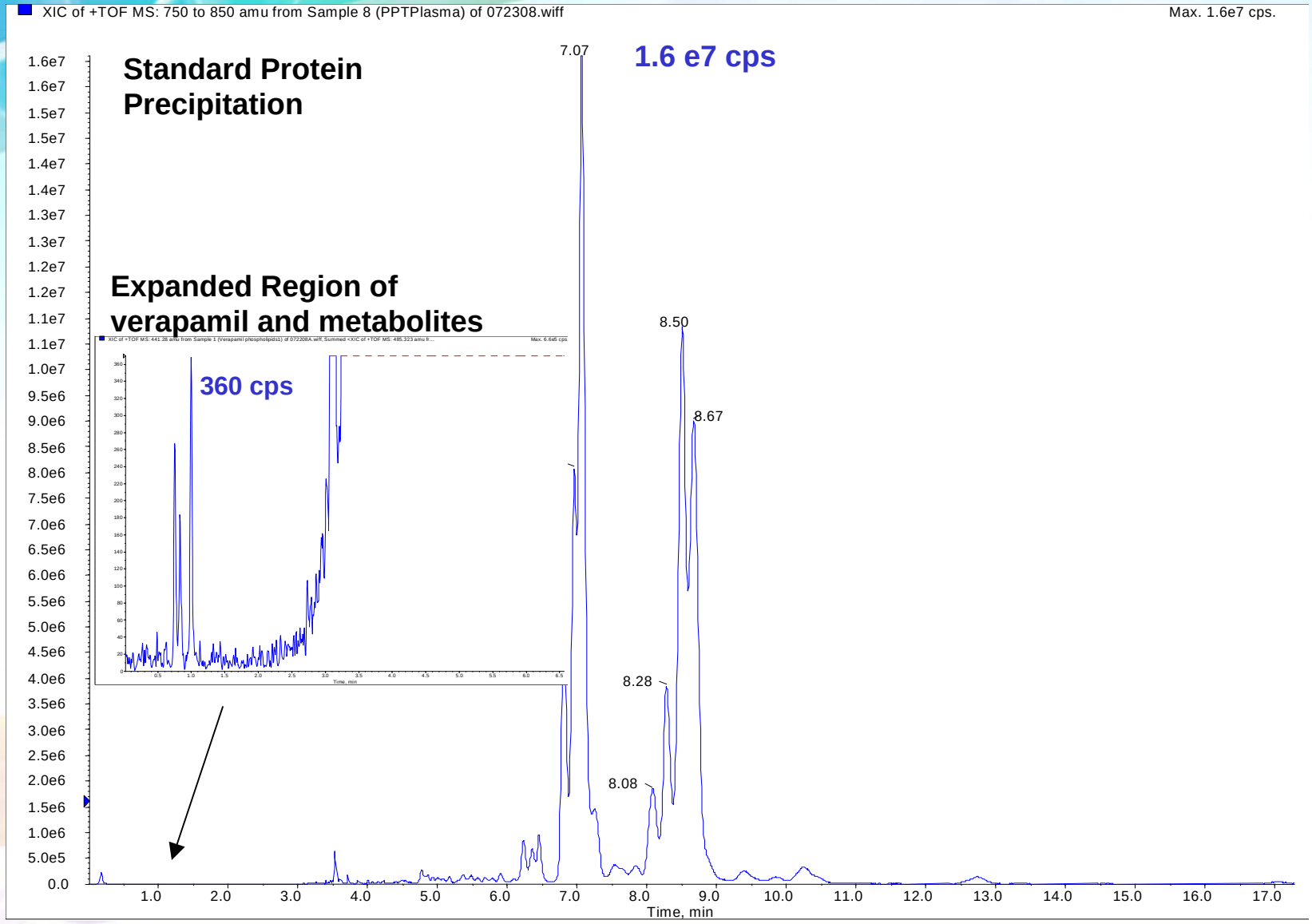
Gradient:

Time	%A	%B
0	100	0
1.0	100	0
2.5	0	100
10	0	100
Post time:	2min	

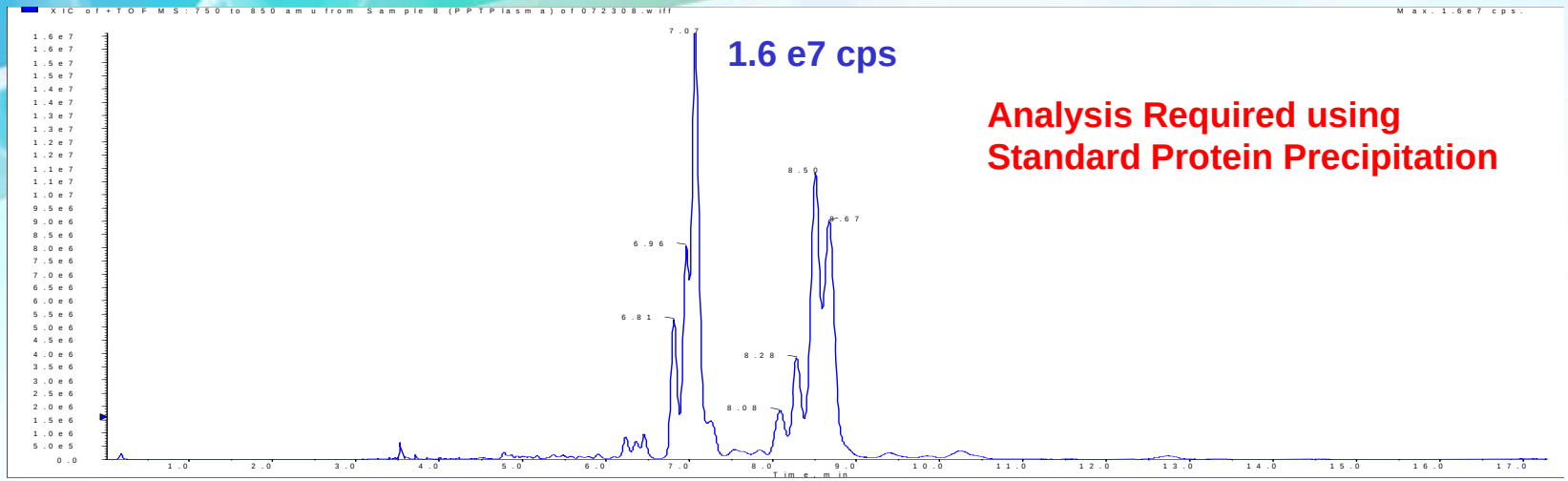
Temp: 35 °C
Flow: 0.6 mL/min
Detection: Agilent TOF
Injection: 1 µL
Components: normethyl verapamil, verapamil, methoxy verapamil
Spiked Concentration: 10ng/mL



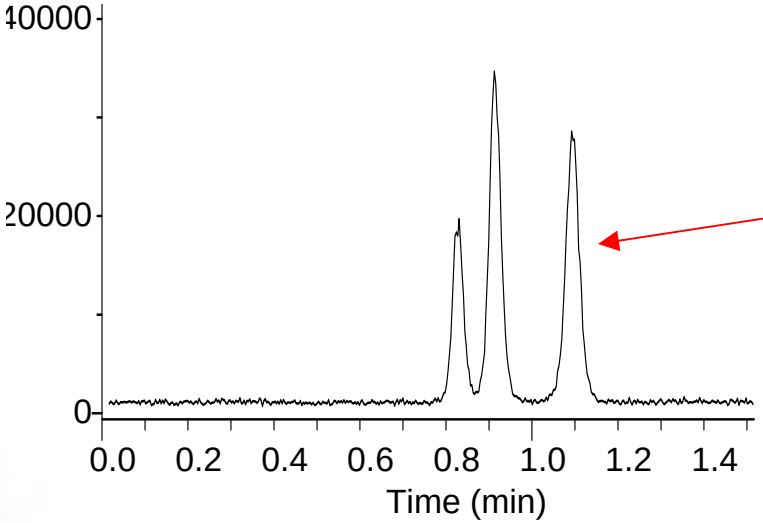
Decreasing Analysis Time using HybridSPE



Decreasing Analysis Time using HybridSPE



Run Time Less Than 90sec!



Increase time savings ….more than 10X fold increase in productivity

Isocratic analysis may be possible with HybridSPE clean-up

Column: Ascentis Express C18, 5 cm x 2.1 mm **Mobile Phase:** A. 30:70 Water : Acetonitrile pH 2.55 with formic acid **Temp:** 35 °C **Flow:** 0.6 mL/min

Standard PPT Method vs HybridSPE

	Accurate Mass m/z	Absolute Recovery (STD) (with HybridSPE)
Verapamil	455.305	117%
Normethyl verapamil	441.280	123%
Methoxy verapamil	485.323	124%

Recovery from Plasma*	Standard PPT	HybridSPE	HybridSPE Relative
Verapamil	26.6%	26.8%	100.6%
Normethyl verapamil	24.1%	28.1%	116.5%
Methoxy verapamil	26.2%	26.2%	100%

*** Possible signal losses due to drug-protein interaction or salt effects.**

Summary

- HybridSPE-PPT platform demonstrated a high selective toward phospholipids while excluding basic compounds.
- Packed well plates can increase processing time by selective filtering.
- 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

Acknowledgements:

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