HybridSPE™ - Precipitation Technology

Bridging the Gap between Simplicity & Selectivity in Sample Prep

Updated: Mar 13 ‘09

T408104

Agenda

- Introduction
- HybridSPE-PPT (How does it work?)
- LC Column Phospholipid Accumulation
- Comparative Studies
- Phospholipid Removal Relative to Conditions
- Development of Conditions
- FAQs / Troubleshooting

Pharmaceutical Bioanalysis

- Develop & run assays to quantitate pharma candidates, and metabolites in biological fluids (plasma, serum, urine) and tissues
- Data used to determine pharmacodynamic/ pharmacokinetic properties and toxilogical/ therapeutic concentrations in living cells, tissues, and animals
- Advances in Combichem, Genomics, Metabolomics, and Proteomics results in drug designs structurally catered to endogenous biomolecules => Results in drugs more potent allowing for smaller concentration dosages => Requires more sensitivity
- LC-MS/MS technology offers overwhelming benefits in terms of throughput and sensitivity; but good sample prep is still required

Sample Prep Determined by Assay Needs

Characteristic	Preclinical	Clinical
Sample Type:	Animal plasma, serum, urine, tissue, bile	Human plasma/serum and urine
Sensitivity Requirements:	Moderate sensitivity requirements (low-mid ng/mL) due to high dosage levels	Drugs more potent so dosed at lower levels. Very high sensitivity req'd (pg-low ng/mL)
Target LC-MS-MS run times:	1-5 min. (~1 min. is goal)	1-5 min. (~5 min. typically required)
Sample prep MD time allowance:	< 1 day	Up to 5 days
Sample prep platform:	80% 96-well; 20% 384-well	80% 96-well; 20% cartridge/tube format
Preferred Sample Prep Technique:	Protein ppt. (70%); SPE & LLE (30%)	SPE (70%); LLE (20%); Protein ppt. (10%)
Comments:	Constantly getting new samples (~10 x greater than clinical). Avg. = 5k samples per week	Selectivity & method ruggedness are critical.

Comparison of Sample Prep Techniques

Characteristic:	Protein PPT	LLE	SPE
MD Req'd:	Generic/Minimal	Moderate	Moderate/Extensive
No. of Steps:	2-3	3-4	5-8
Amenable to Automation:	Yes	Yes, but contains several disjointed steps	Yes
Selectivity	Low	Moderate	Moderate to High dependent on phase selection and skill of method developer
Analyte Recovery	High (but dilution effect decrease sensitivity)	Moderate to High	Moderate to High
Phospholipid Removal:	No	No	No to Minimal
Drug Development Stage	Dominant in Preclinical	Dominant in Clinical	Dominant in Clinical

Red = Disadvantage ; Green = Benefit/Advantage

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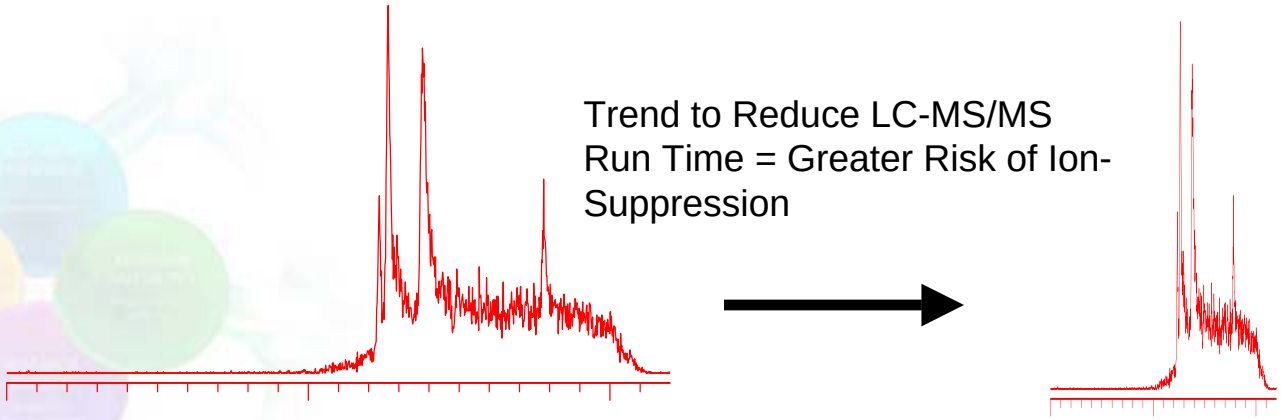
Ion-Suppression

- **Ion-suppression caused by one or more interfering components or species that co-elute with the analyte(s) of interest during LC-MS analysis**
- **Affect droplet formation or ionize concurrently resulting in an erroneous decrease (suppression) or increase (enhancement) in signal response**
- **Leads to poor assay reproducibility, accuracy, and sensitivity, and such - most notable at the lower limits of quantitation (LLOQ).**
- **Often manifests early and later regions of the chromatogram; however, the trend today is to minimize analytical run times (< 5 min.)**
- **Results in greater risk of co-eluting analytes of interest with matrix-suppression causing species**

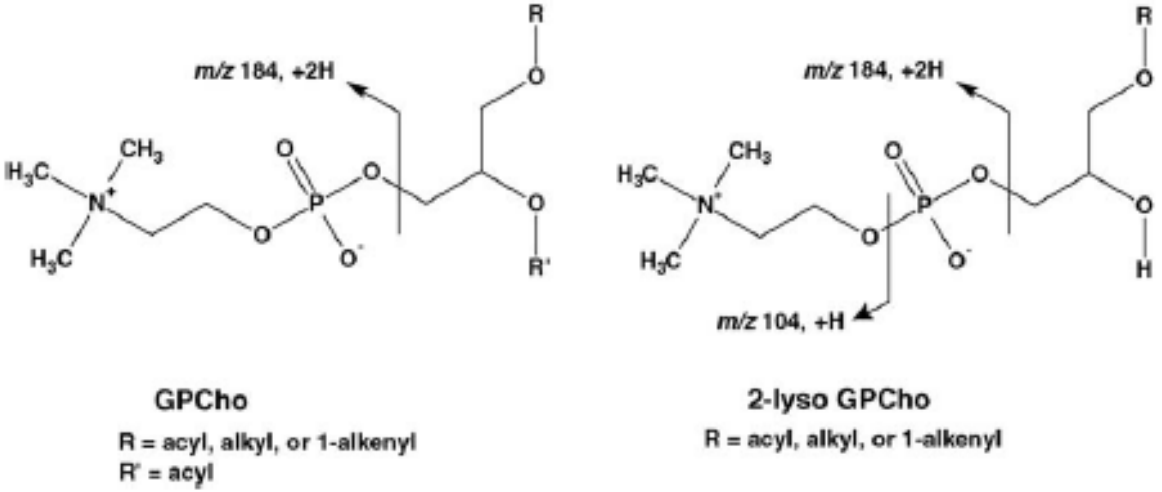


Ion-Suppression (cont.)

- Ion-Suppression potentially caused by a number of contaminating non-volatile species:
  - Phthalates and plasticizers from plastic ware
  - Common buffers/salts used in the life sciences (e.g. TFA, MOPS, Tris) and reducing agents (e.g., DTT and BME)
  - Stabilizers (glycerol) and dosing agents (PEG)
  - Plasma anti-coagulants (lithium heparin and sodium citrate)
- All of which can be moderated at varying degrees with proper sample preparation and chromatographic resolution.
- One of the principal causes of ion-suppression when analyzing biological samples are phospholipids

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**
- **~ 1 mg/mL phospholipids in plasma => very high amounts**
- **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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Difficulties of Phospholipid Removal in Sample Preparation

Phospholipid Structure:

- **Polar head group – zwitterionic phosphonate moiety (remains charged at from strong alkaline to strong acid)**
- **Hybrophobic tail – two fatty acyl groups that are hydrophobic**

Solid Phase Extraction:

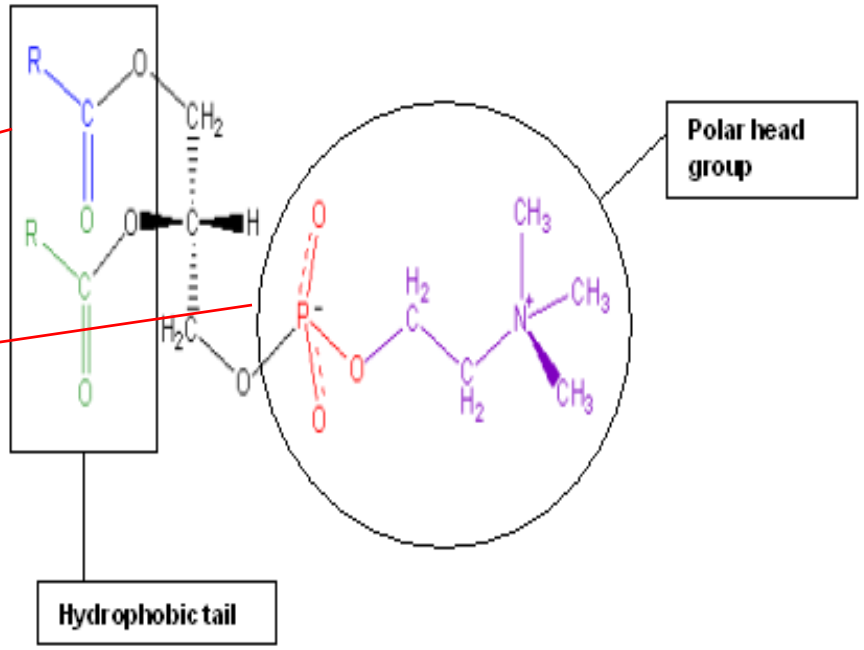
- **Reversed-Phase – hydrophobic tail will result in co-extraction with analytes of interest**
- **Mixed-Mode & IOX – zwitterionic polar head group will often result in co-extraction with basic or acidic analytes of interest.**

Protein Precipitation:

- **Will only remove gross levels of protein (albumin)**

Liquid Liquid Extraction:

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



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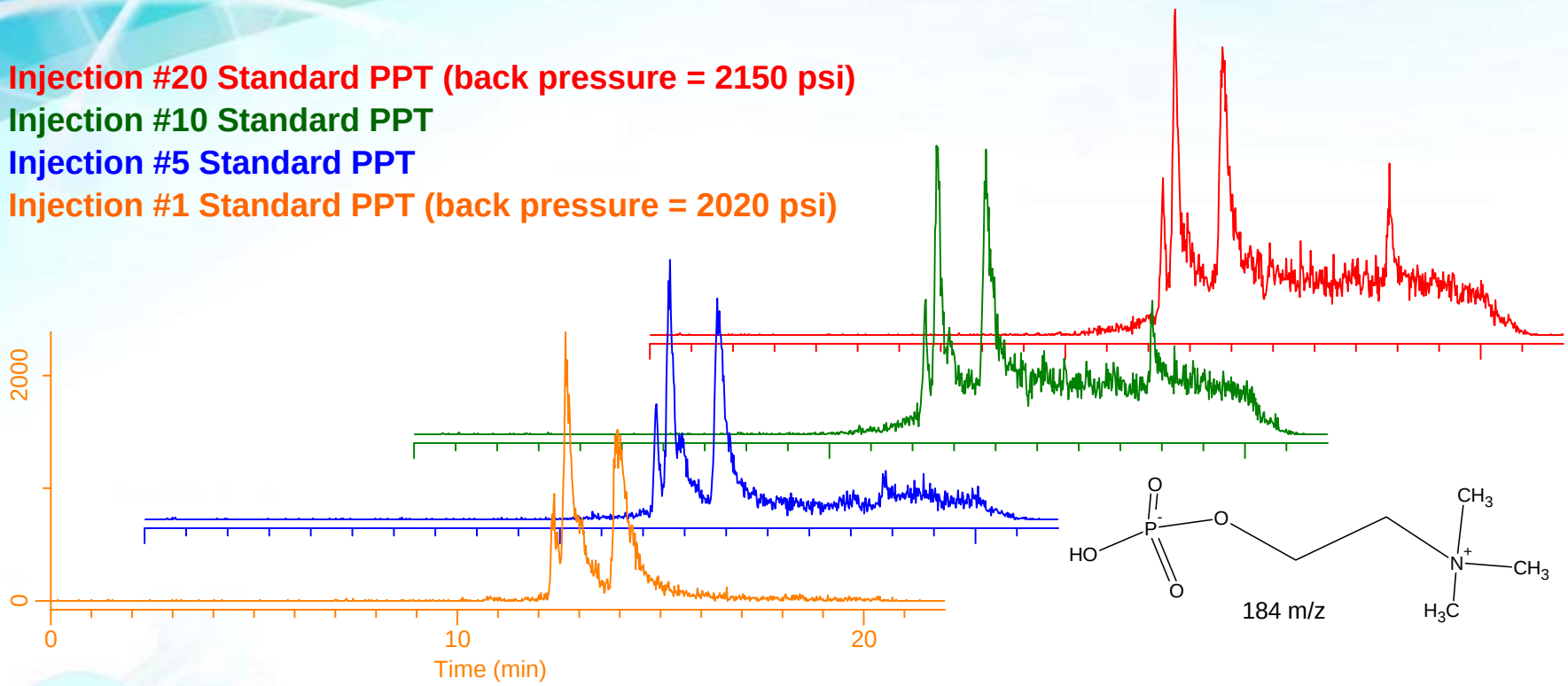
Difficulties with Phospholipid during Liquid Chromatography

- Many method developers develop 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 increase back pressure/column fouling with successive injections
  - May elute uncontrollably in subsequent injections
  - Use of fast/ballistic gradients often inadequate to purge the column of phospholipids during run sequence

Example of Phospholipid Accumulation

Injection #20 Standard PPT (back pressure = 2150 psi)
Injection #10 Standard PPT
Injection #5 Standard PPT
Injection #1 Standard PPT (back pressure = 2020 psi)



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.

Analysis: Agilent C18, 1.8 um, 5 cm x 2.1 mm; Mobile Phase: 13mM ammonium acetate in water (A) and 13mM ammonium acetate in MeCN (B); Gradient: 0-5 min, 95% to 50% A, 5-8 min. hold 50% A, 8-8.1 min. 50% to 95% A, 8.1-15 min. hold 95% A; Temp. 35 °C; Flow rate 0.2 mL/min.; Inj. Vol. 10 uL

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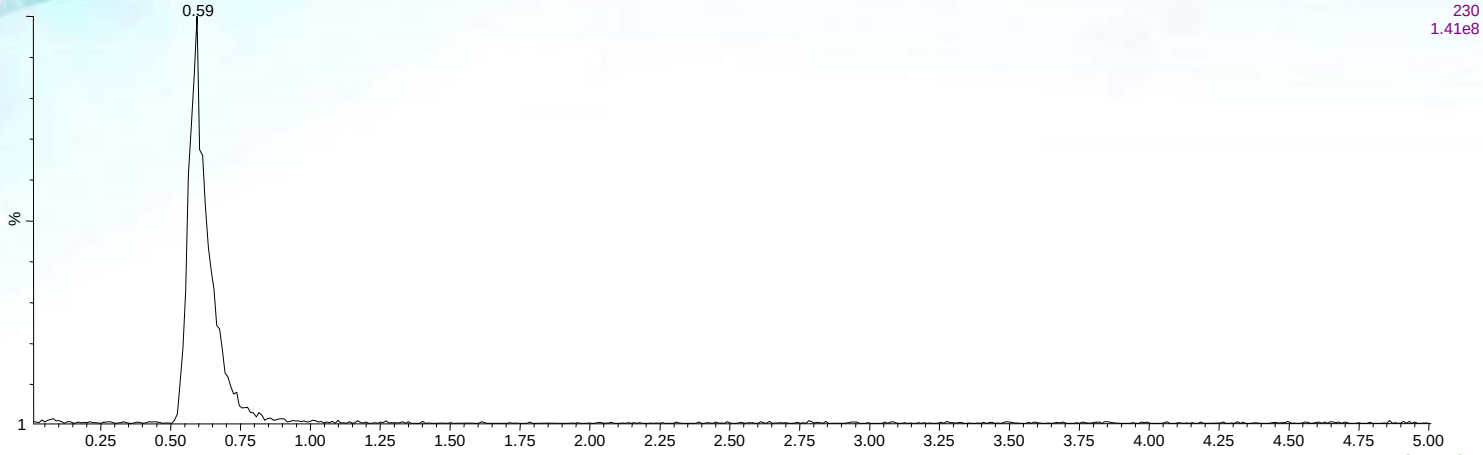
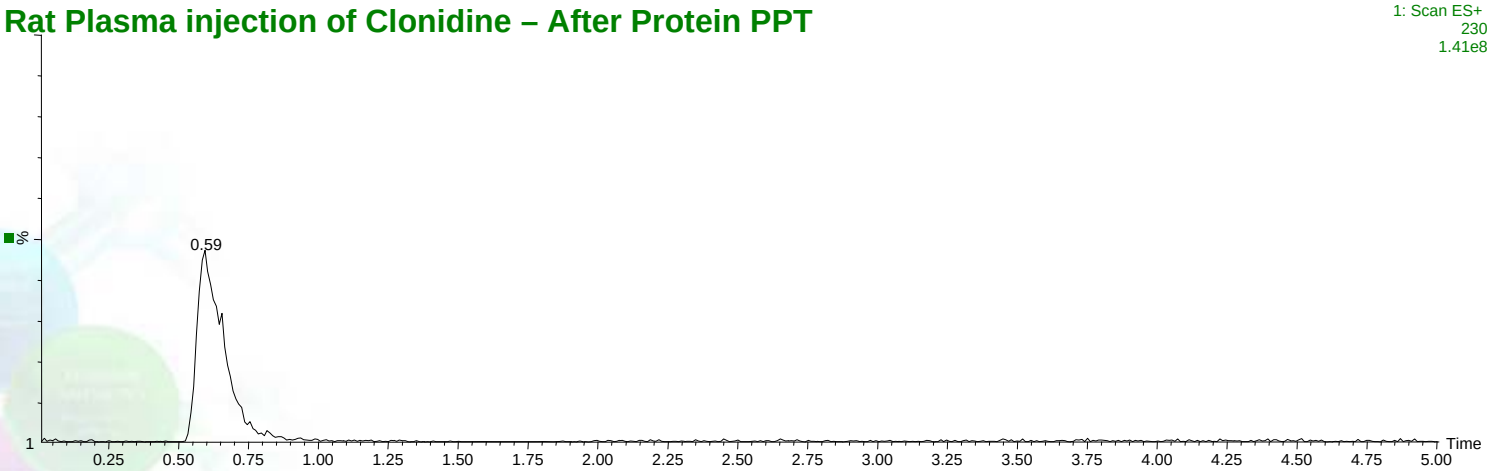
Example Phospholipid Effect on Ionization of Clonidine

Phospholipid Free Rat Plasma injection of Clonidine – After Protein PPT



Rat Plasma injection of Clonidine – After Protein PPT



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HybridSPE – Precipitation Technology
How does it work?

HybridSPE-PPT Plate and Vacuum Manifold

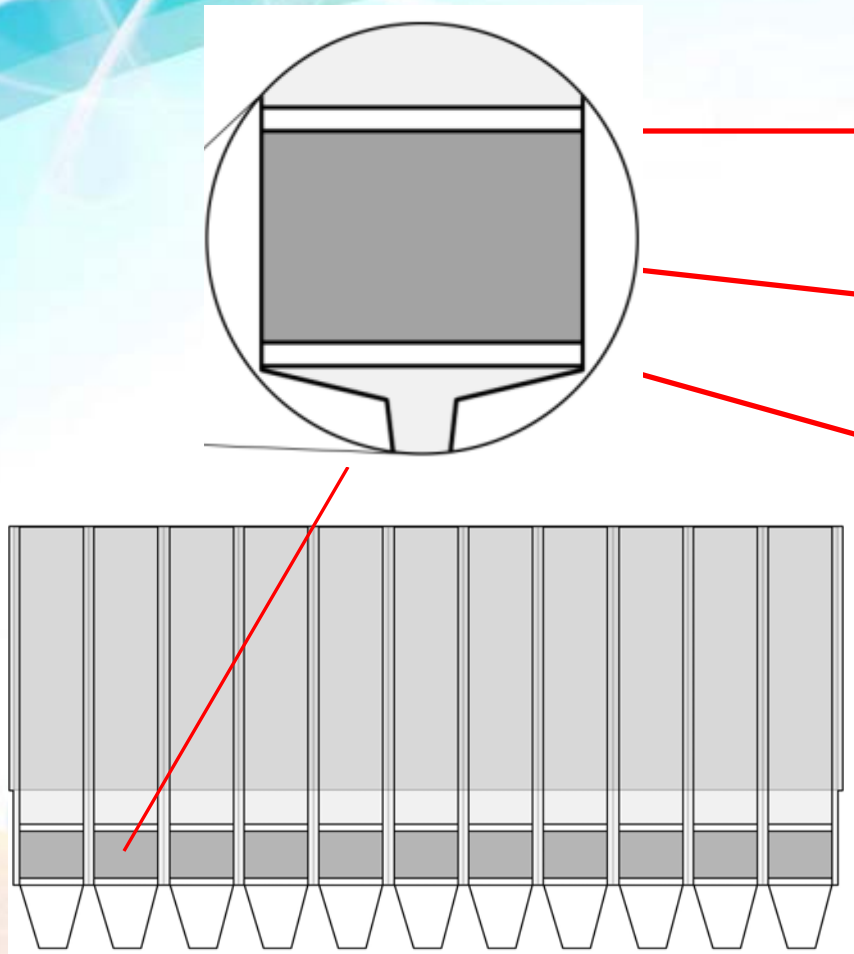
HybridSPE – PPT Summary

- Merges both protein precipitation and solid phase extraction
  - Offers the simplicity and generic nature of protein precipitation

PLUS

  - Selectivity approaching SPE via the targeted and selective removal of phospholipids
- 2-3 step generic procedure
- Minimal to no method development required
- Available in 96-well and 1 mL cartridge dimensions
  - Compatible with TomTec Quadra and MicroLab Microstar
- 100% removal of phospholipids and precipitated proteins
- Fast flow rates
- Ideal for high throughput pre-clinical and clinical applications where sample prep speed, selectivity, and ion-suppression is of concern
- Patent pending technology

HybridSPE – PPT *96-well* Schematics



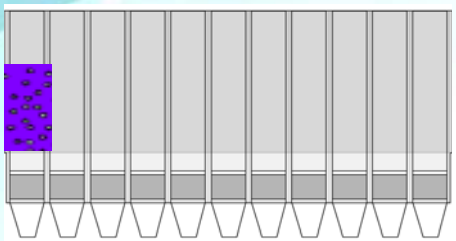
5 µm PTFE (Teflon) Frit

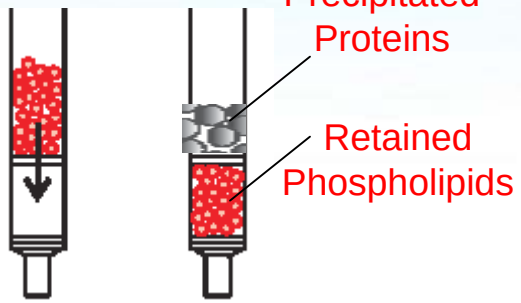
Zirconia Coated Silica

0.2 µm hydrophobic graded filter/frit

Note: The 1 mL cartridge version of the HybridSPE – PPT technology only contains 20 µm PE frits at this time. Therefore, only the "Off-Line HybridSPE-PPT" procedure (see next slide) is recommended for the 1 mL cartridge format at this time.

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

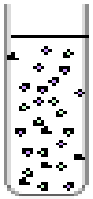




1) Precipitate Proteins: Add 100 µL plasma/serum to the HybridSPE plate followed by 300 µL 1% formic acid in acetonitrile. Add I.S. as necessary. Note: the upper PTFE frit keeps plasma from dripping through packed-bed prematurely.

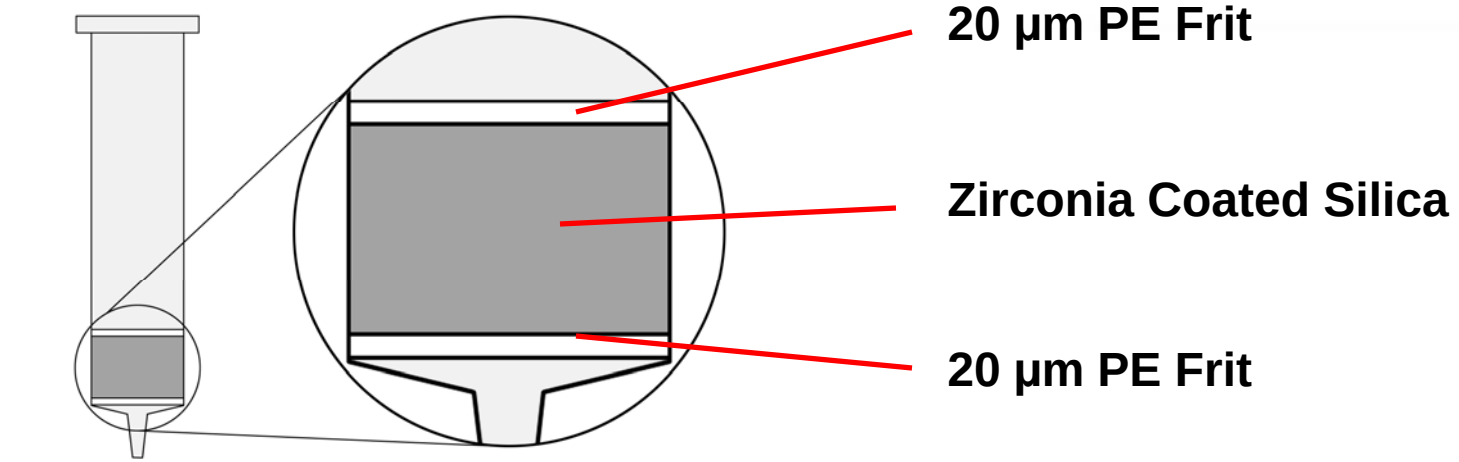
2) Mix by vortexing HybridSPE plate or by aspirating/dispensing with 0.5-1 mL pipette tip

3) Apply vacuum. 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.



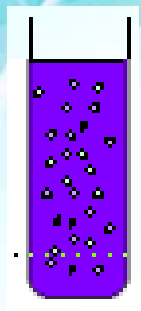
4) Resulting filtrate/eluate is free of proteins and phospholopids and ready for immediate LC-MS/MS analysis; or it can be evaporated and reconstituted as necessary prior to analysis

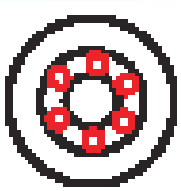
HybridSPE – PPT *1 mL Cartridge* Schematics



Note: The 1 mL cartridge version of the HybridSPE – PPT technology only contains 20 µm PE frits at this time. Therefore, only the “Off-Line HybridSPE-PPT” procedure (see next slide) is recommended for the 1 mL cartridge format at this time.

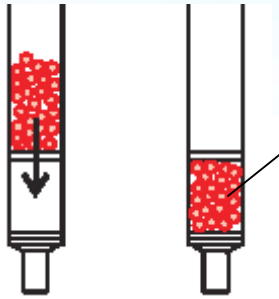
Off-Line Precipitation Method for HybridSPE 1 mL cartridge or 96-well format





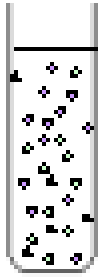
2) Centrifuge

3) Transfer supernatant to HybridSPE cartridge or 96-well plate



1) Precipitate Proteins: Combine 100 µL plasma serum with 300 µL 1% formic acid in acetonitrile. Add I.S. as necessary.

4) Apply vacuuum. Phospholopids are retained on HybridSPE sorbent while small molecules (e.g., pharma compounds & metabolites) pass through un-retained.



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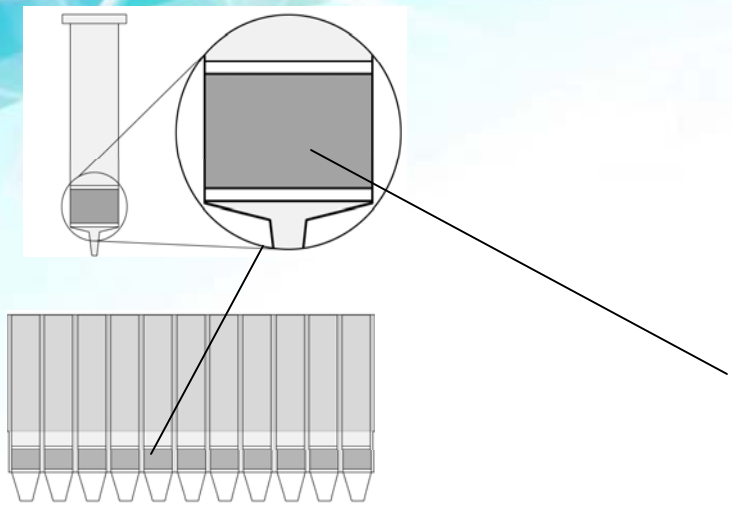
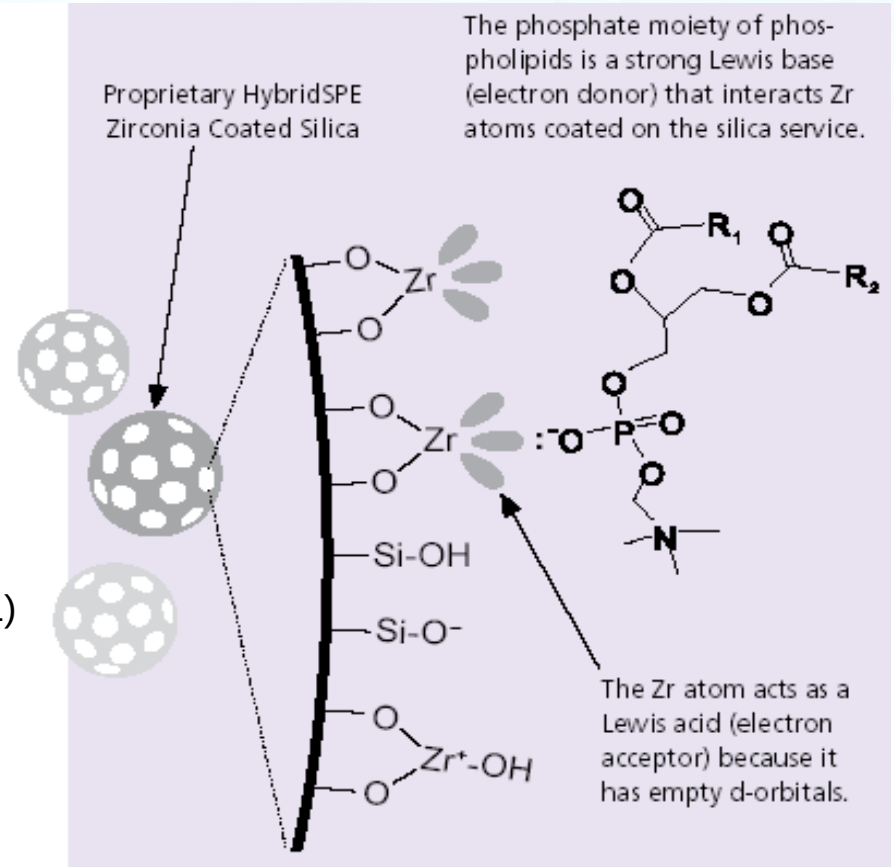
How are Phospholipids Selectively Removed using HybridSPE?





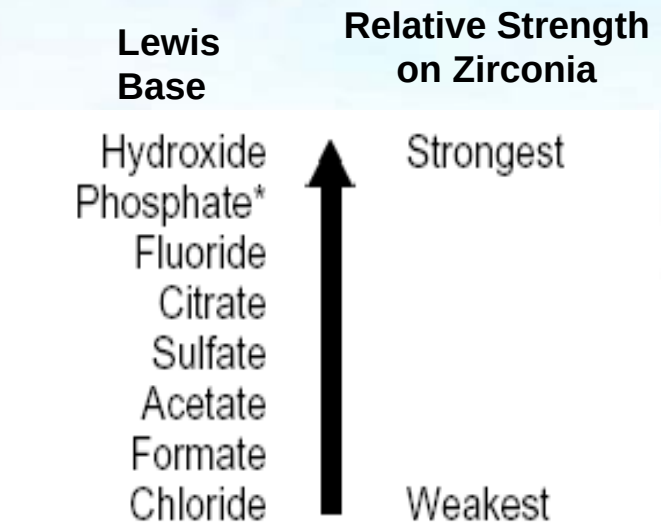
Note: **The presence of ≥ 1% formic acid in the MeCN precipitation agent is critical** because of: 1) formic acid is a stronger Lewis base than most carboxyl (-COOH) groups found in acidic pharmaceutical compounds but not as strong a Lewis base as the phosphate moiety found in phospholipids; and 2) the low pH environment neutralizes residual silanol activity on the silica surface thereby eliminating secondary cation-exchange interaction with basic compounds of interest .

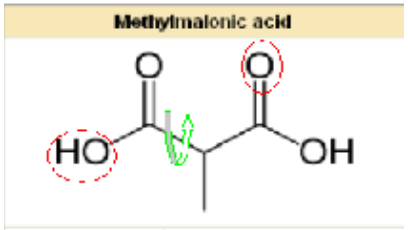
Suggested MD Strategy for HybridSPE

1. **Primary Procedure =** 100 uL plasma + 300 uL 1% formic acid in ACN (**optimal recovery for ~80% of all analytes**)

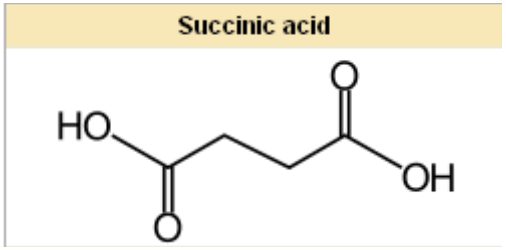
2. **2° Procedure (Some Basic Cmpds) =** 100 uL plasma + 300 uL 1% ammonium formate in MeOH – *Ammonium ions disrupt secondary WCX interactions between silanols (Si-O $^-$) and basic compounds. MeOH disrupts secondary HILIC interactions.*

3. **2° Procedure (Some Acidic Cmpds)** Condition w/ 400 uL 0.5% citric acid in ACN followed by 100 uL plasma + 300 uL 0.5% citric acid in ACN – *citric acid is a stronger Lewis base than formic acid.*





Poor Recovery via 1° Procedure



Excellent Recovery via 1° Procedure

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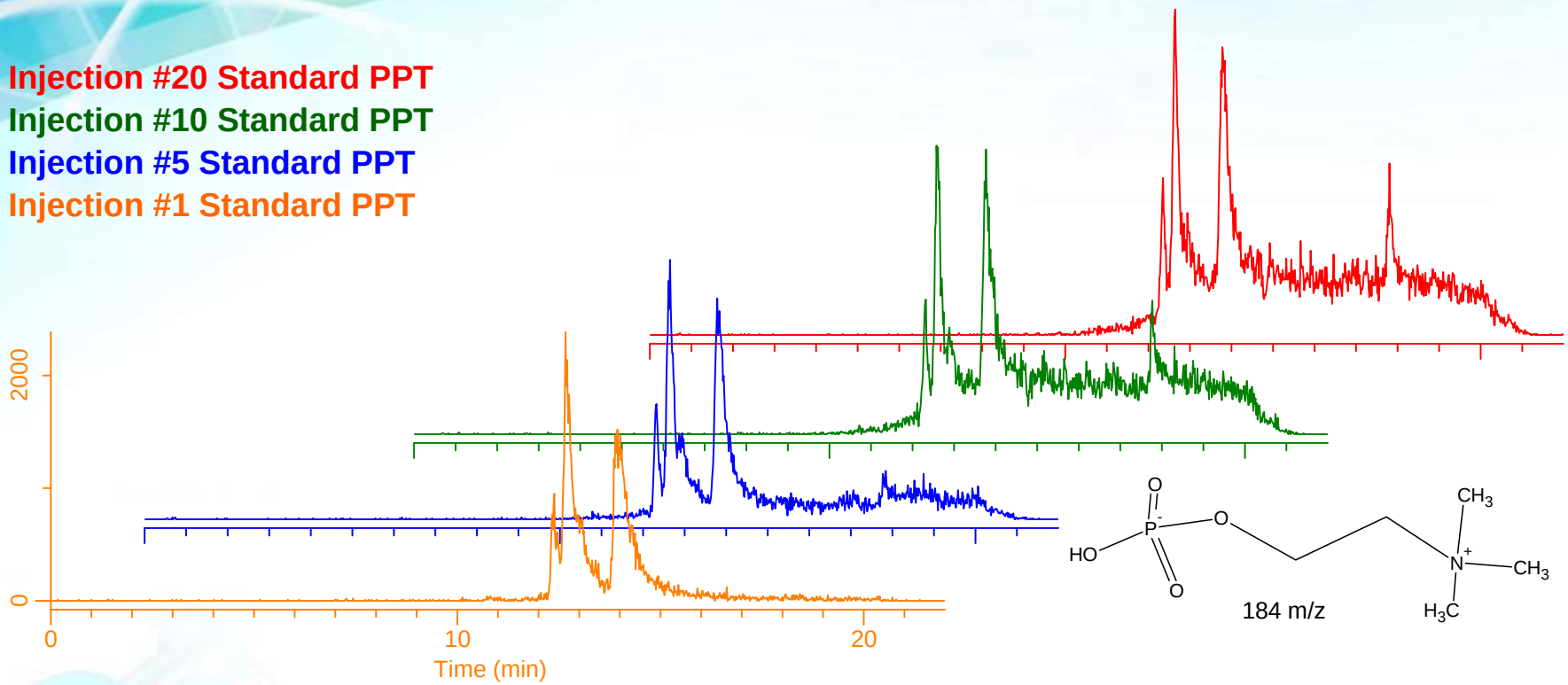
Phosopholipid Accumulation on LC Column HybridSPE-PPT vs. Protein PPT

Example of Phospholipid Accumulation

Standard Protein PPT + Sub 2 µm LC Column

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



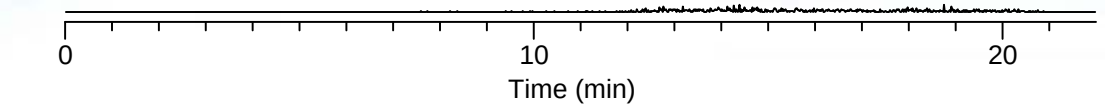
Sample Preparation: Protein precipitation of 100 uL blank rat plasma with 300 uL 1% formic acid in MeCN. Vortex 1 min. followed by centrifuged for 3 min. at 15k RPM. Injection of supernatant.

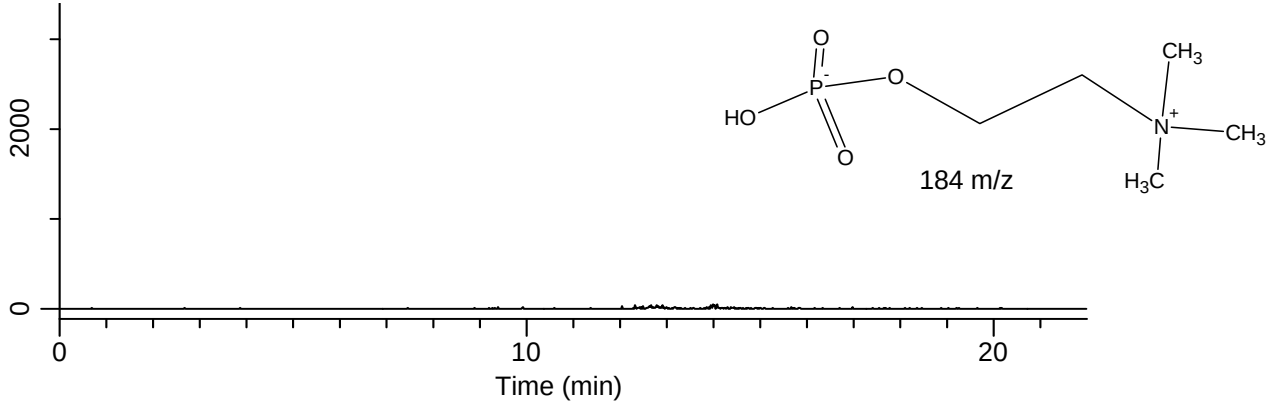
Analysis: Agilent C18, 1.8 um, 5 cm x 2.1 mm; Mobile Phase: 13mM ammonium acetate in water (A) and 13mM ammonium acetate in MeCN (B); Gradient: 0-5 min, 95% to 50% A, 5-8 min. hold 50% A, 8-8.1 min. 50% to 95% A, 8.1-15 min. hold 95% A; Temp. 35 °C; Flow rate 0.2 mL/min.; Inj. Vol. 10 uL

No Phospholipid Accumulation

HybridSPE-PPT + Sub 2 µm LC Column

Injection #20 Hybrid SPE Technology



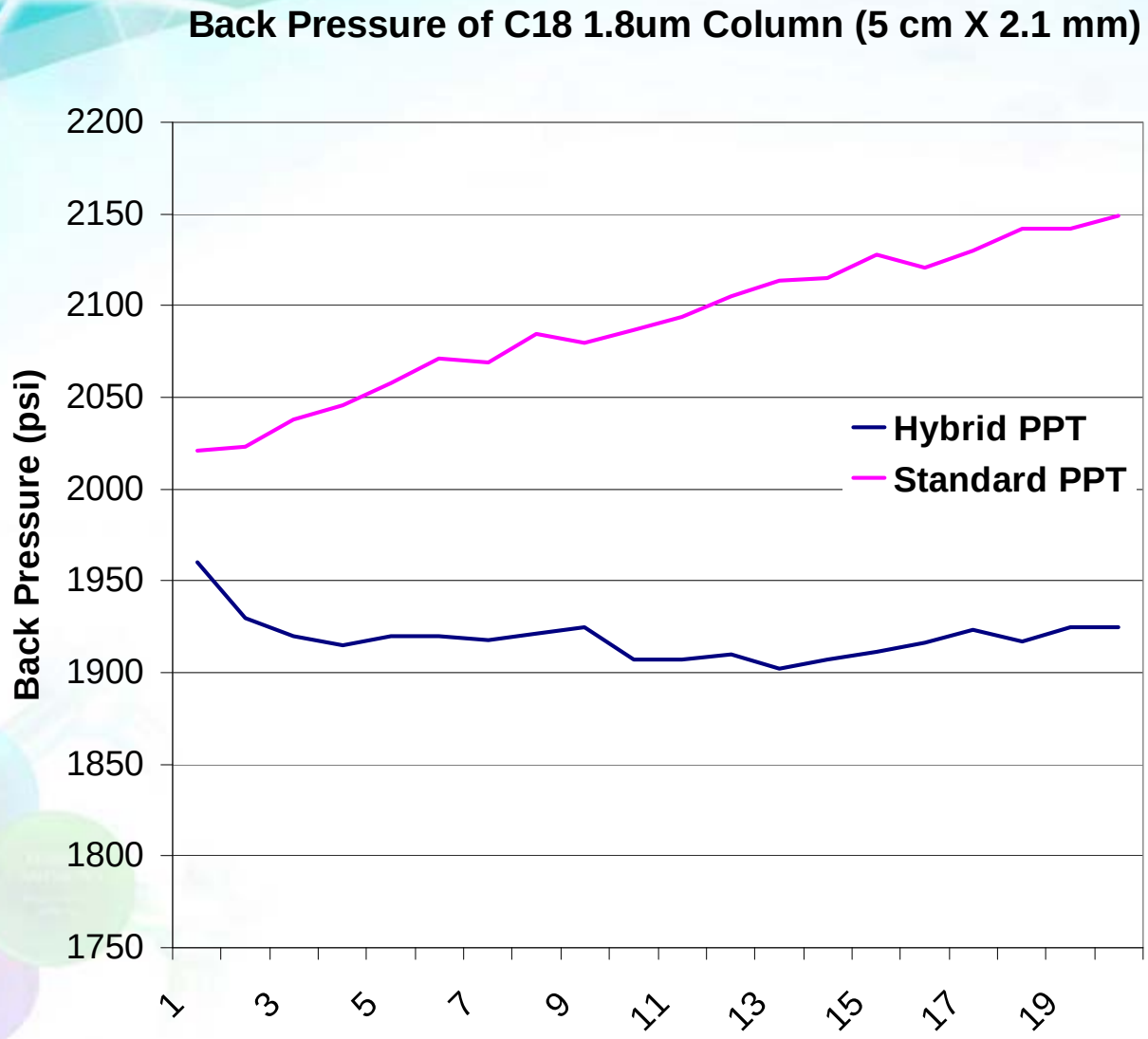


Injection #1 Hybrid SPE Technology

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.

Analysis: see previous slide.

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



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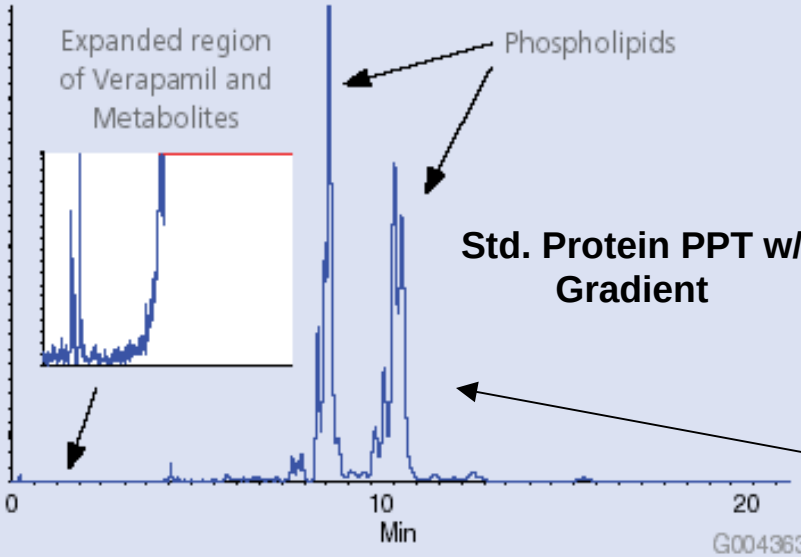
Example 1: Verapamil & Metabolites in Rat Plasma (Std Protein PPT + Gradient) vs. (HybridSPE-PPT + Isocratic)

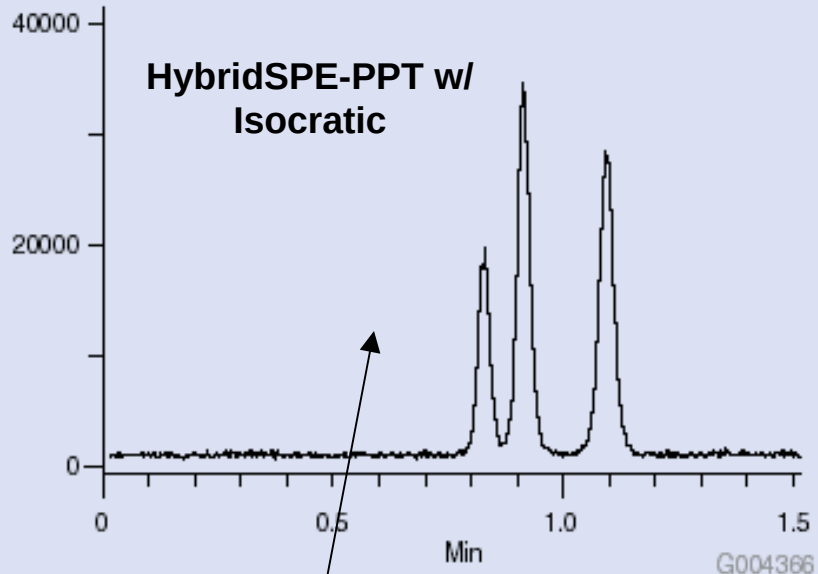
column: Ascentis Express C18, 5 cm x 2.1 mm (53822-U)
instrument: Agilent 1200 RR
mobile phase: (A) water:acetonitrile, pH 2.55 adjust with formic acid (30:70)
(B) 13 mM ammonium formate diluted in 95% methanol

gradient:	Min	%A	%B
	1.0	100	0
	2.5	0	100
	10	0	100

Temp.: 35°C
Flow: 0.6 mL/min.
Inj.: 1 µL
Detection: TOF/MS
Phospholipids (750-850 m/z range)
Verapamil (455.305 m/z)
Normethyl verapamil (441.280 m/z)
Methoxy verapamil (485.323 m/z)



Mobile phase: water:acetonitrile, pH 2.55 adjust with formic acid (30:70) (isocratic)



No risk phospholipid contamination = short isocratic run times

Requires > 10 min. to elute phospholipids

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Example 2: Loratadine & Desloratadine in Rat Plasma – HybridSPE-PPT vs. Std. Protein-PPT

Column: Discovery HS F5, 5 cm x 2.1 mm

Instrument: Agilent 1200RR

Mobile Phase: *Isocratic*

60% 10 mM ammon acetate in MeCN

40% 10 mM ammon acetate in water, pH 3.5

Temp: 35 ºC

Flow: 0.6 mL/min.

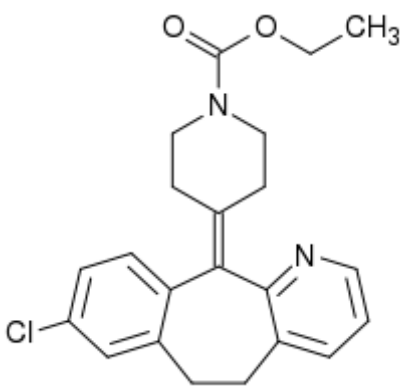
Inj.: 2 µL

Detection: TOF-MS

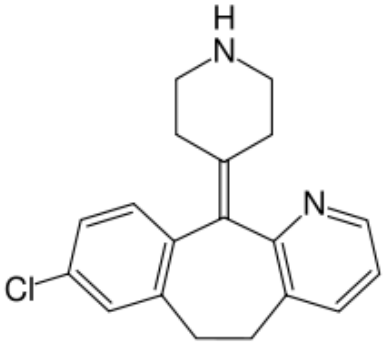
Lortatadine 338.157 m/z

Desloratadine 311.122 m/z

Phospholipids 184 m/z

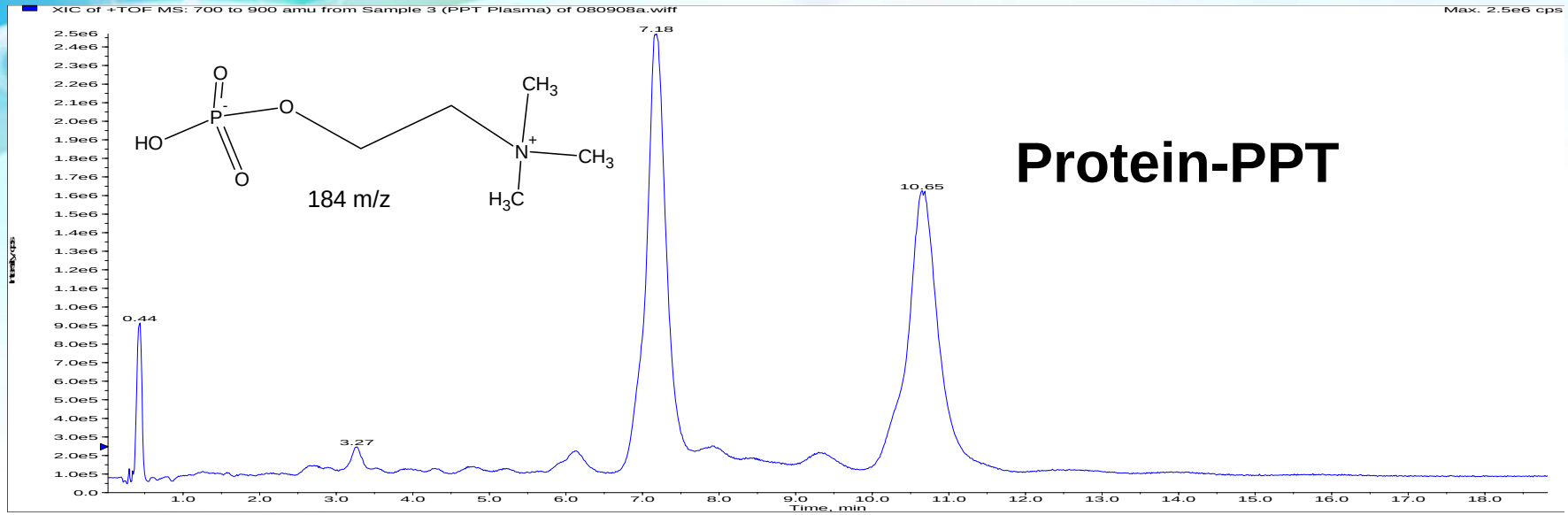


Loratadine

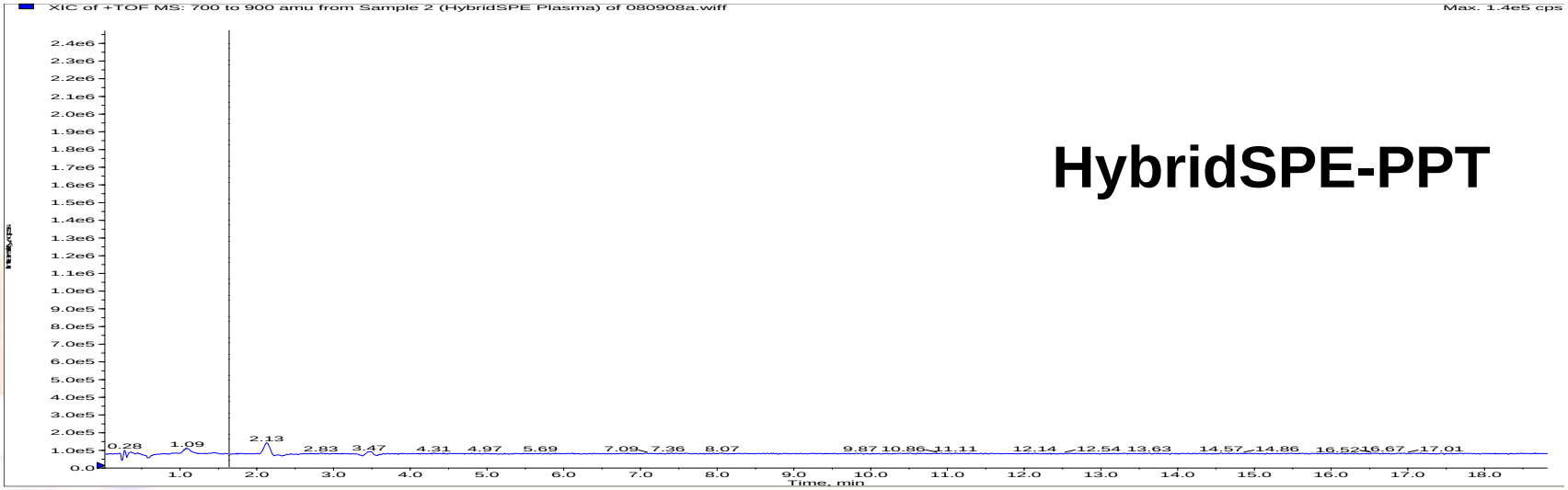


Desoratadine

Example 2 (cont.): Monitoring of Phospholipids of Blank Plasma – HybridSPE-PPT vs. Std. Protein-PPT

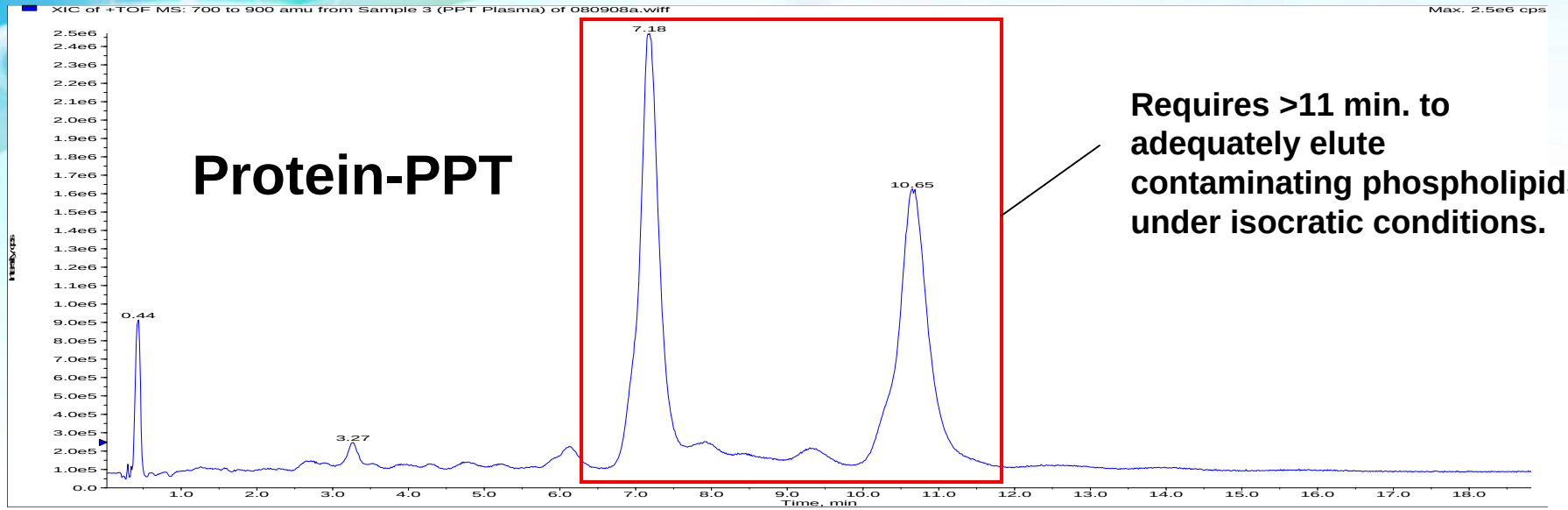


Protein-PPT

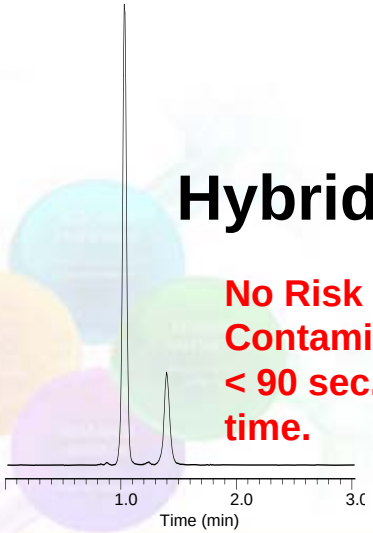


HybridSPE-PPT

Example 2 (cont.): Reduced Analytical Run Time through HybridSPE-PPT Phospholipid Removal



Requires >11 min. to adequately elute contaminating phospholipids under isocratic conditions.



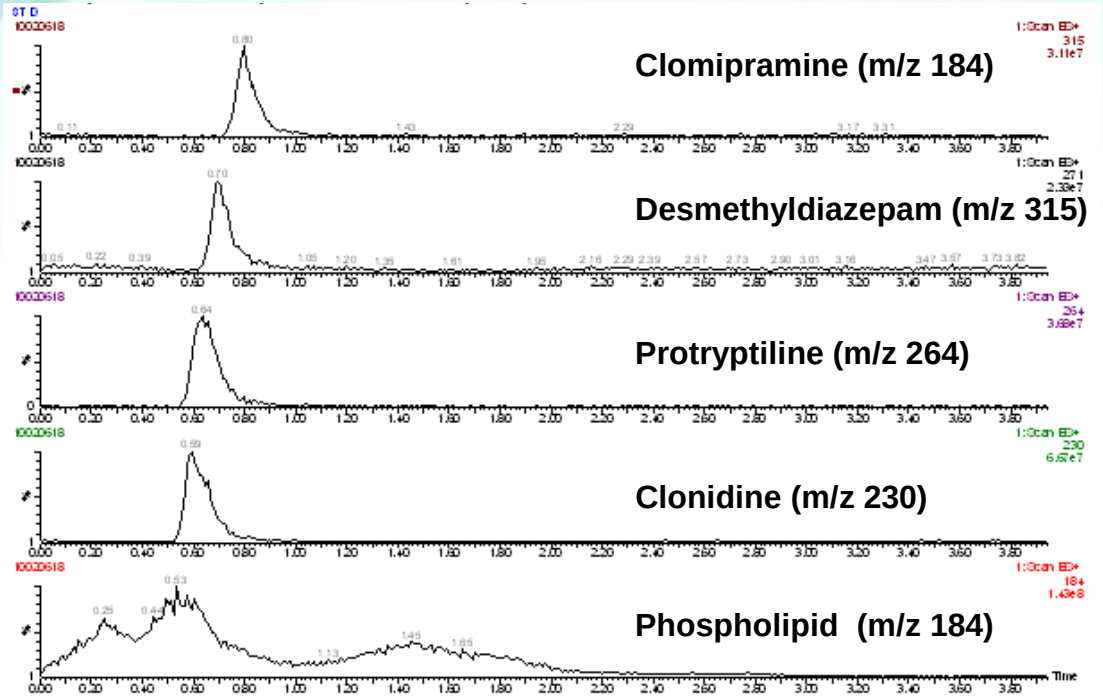
HybridSPE-PPT

No Risk of Phospholipid Contamination allows for < 90 sec. isocratic run time.

Spike Level	Loratadine	Desloratadine
80 ng/mL	98% recovery	80% recovery
200 ng/mL	83% recovery	78% recovery
400 ng/mL	96% recovery	93% recovery

HybridSPE-PPT Comparative Studies

Example 1: Overlap of Basic Compounds with Phospholipids



	% Recovery - phospholipid effect			
100 ng/mL STD	**Clonidine (m/z 230)**	**Protryptiline (m/z 264)**	**Desmethyldiazepam (m/z 315)**	**Clomipramine (m/z 184)**
Hybrid SPE -PPT	**96.7%**	**104.11%**	**102.55%**	**97.81%**
Standard Protein PPT	**54.50%**	**44.72%**	**81.85%**	**110.91%**

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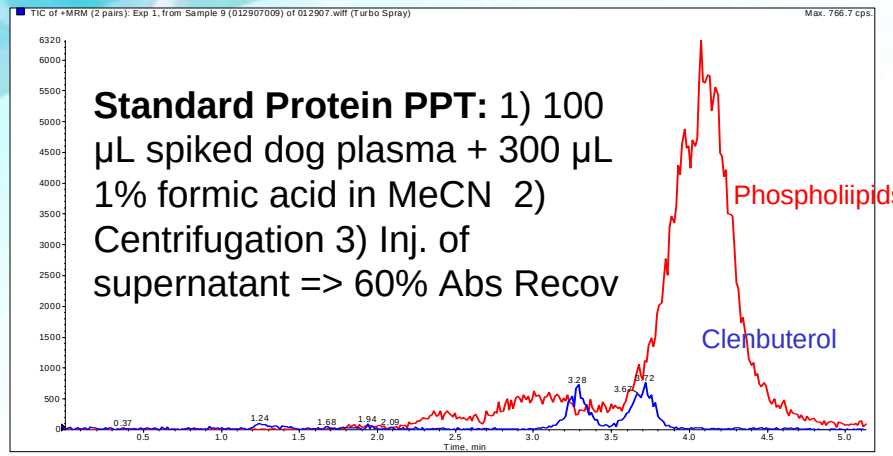
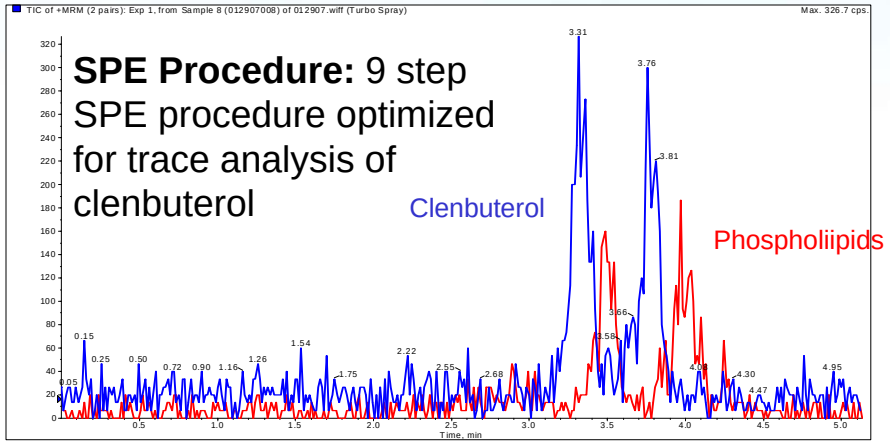
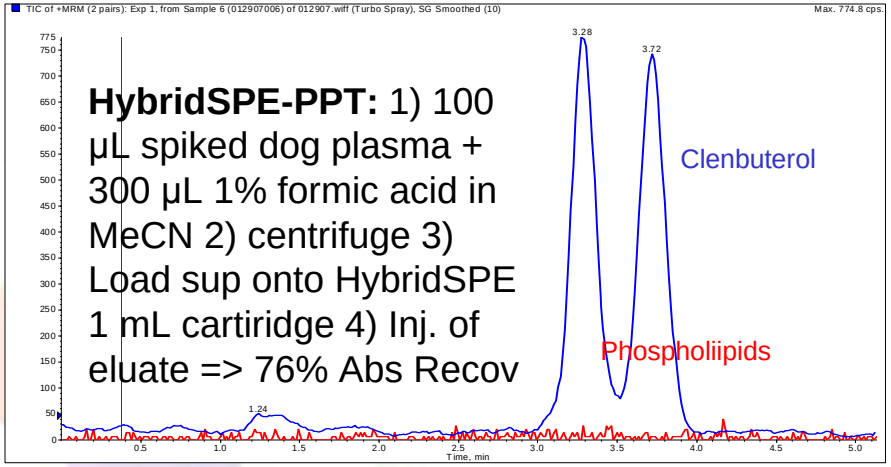
Example 2: Extraction of 10 ng/mL Clenbuterol (R(-) and S(+) Enantiomers) from Dog Plasma







Column: Chirobiotic T 10cm X 2.1mm, 5 um

Mobile Phase: 10mM Ammonium Formate/Methanol

Flow: 300 ul/min

Temp: 30 °C

Detection: MS-MRM

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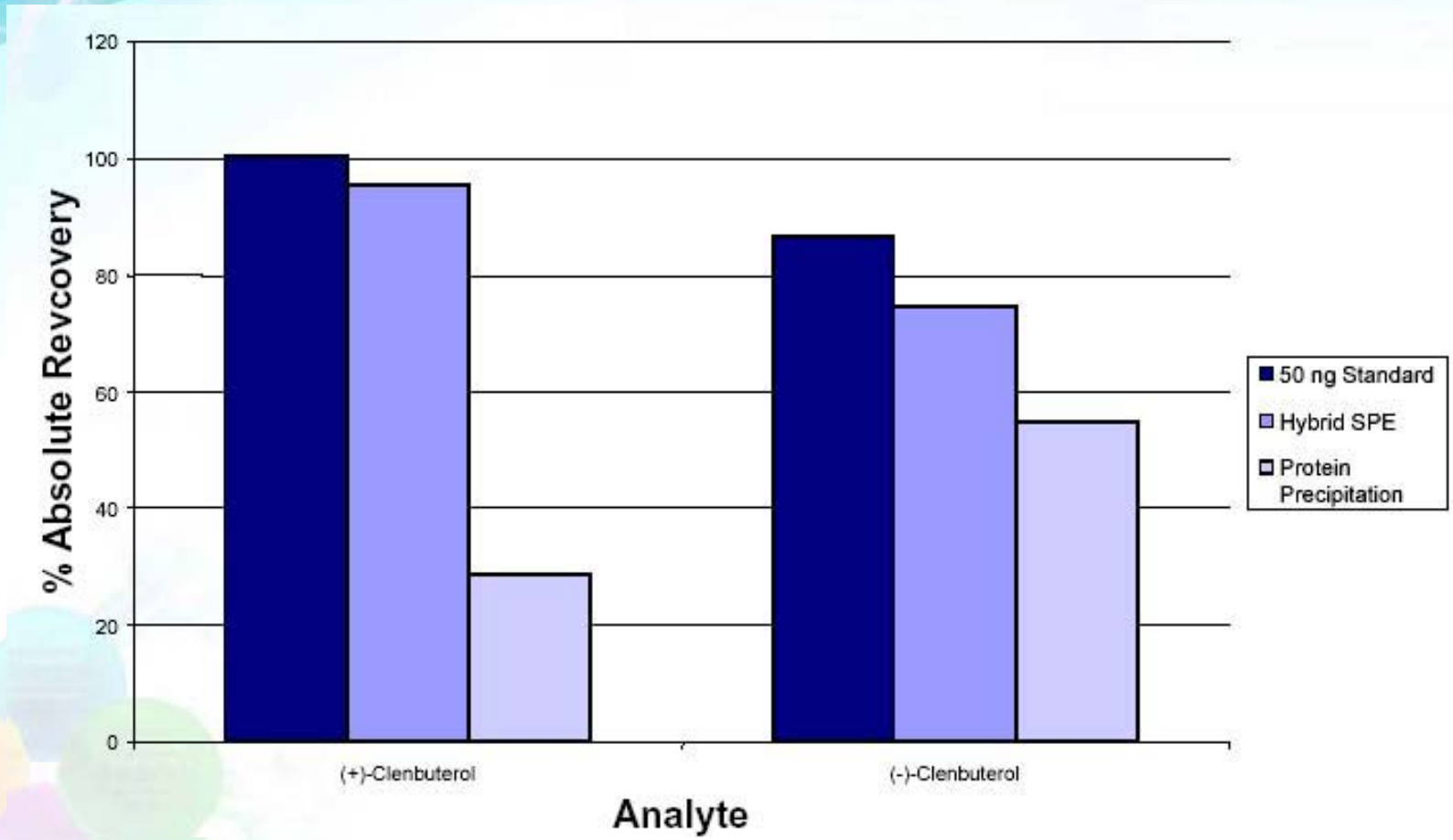
Example 2 (cont.): Abs Recovery Comparison of Clenbuterol (50 ng/mL spike)



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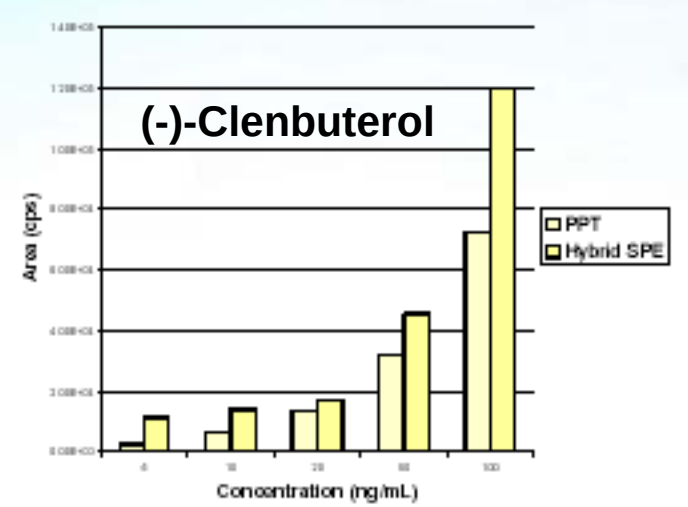
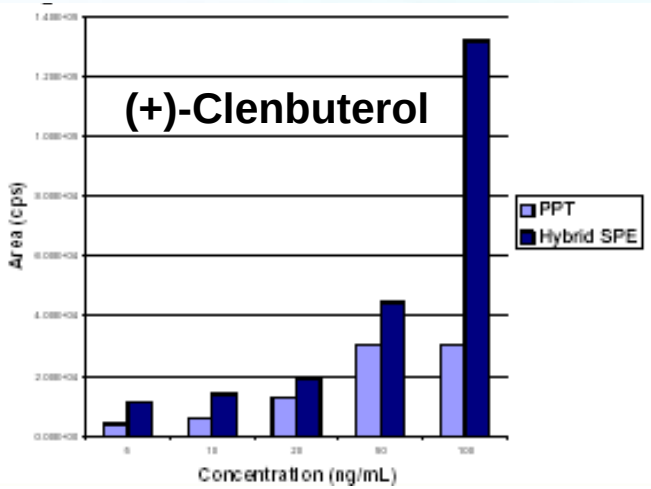
Example 2 (cont.): Peak Areas of Clenbuterol Over Concentration Range (5-100 ng/mL spike)





	(-)-Clenbuterol			(+)-Clenbuterol		
Concentration (ng/mL)	HybridSPE Area	PPT Area	% difference	HybridSPE Area	PPT Area	% difference
5	1.13E+04	2.66E+03	76.5	1.11E+04	4.13E+03	62.8
10	1.41E+04	6.70E+03	52.5	1.39E+04	6.20E+03	55.4
20	1.72E+04	1.38E+04	19.8	1.87E+04	1.29E+04	31
50	4.56E+04	3.18E+04	30.3	4.47E+04	3.06E+04	31.5
100	1.20E+05	7.21E+04	39.9	1.32E+05	3.06E+04	77

Example 3: Comparative Extraction of Propanolol and Ketoprofen

Standard Protein PPT:

1. Combine 100 µL spiked rat plasma (100 ng/mL + 300 µL 1% formic acid in MeCN.
2. Mix for 1 min.and centrifuge at 6k rpm for 3 min.
3. Analyze sup via LC-MS

HybridSPE PPT:

1. Apply 100 µL spiked rat plasma (100 ng/mL to HybridSPE well plate followed 300 µL 1% formic acid in MeCN.
2. Vortex for 1 min. and apply vacuum (-10 inHg) for 2 min.
3. Collect eluate for LC-MS

Conventional SPE: Two commercially available polymer SPE Phases (A) and (B), 60 mg/3 mL

1. Condition with 1 mL MeOH
2. Equilibrate with 1 mL DI water
3. Load 200 µL spiked rat plasma (100 ng/mL)
4. Wash with 2 x 5% MeOH
5. Elute with 1 mL MeOH
6. Evaporate and reconstitutute with 0.8 mL DI water:1% formic acid in MeCN (1:3, v/v)

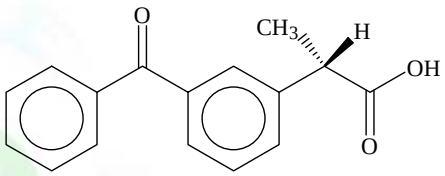
100 ng/mL propanol and ketoprofen were spiked into rat plasma for subsequent LC-MS analysis. Two commercially available polymer SPE phases were compared against standard protein PPT and HybridSPE-PPT

Example 3 (cont.): LC-MS Conditions for Propanolol & Ketoprofen

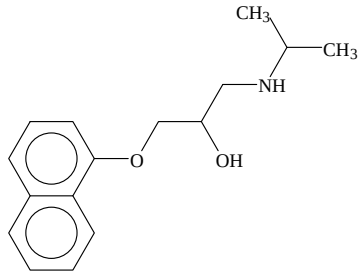
Instrument: Agilent 1100 LC System/3200 Q TRAP (AB Sciex)
Column: Discovery HS F5, 100 x 2.1mm

Mobile phase A: 13mM ammonium acetate in acetonitrile
Mobile phase B: 13mM ammonium acetate in water

Step	Total Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0.00	200	75.0	25.0
1	2.00	200	95.0	5.0
2	4.50	200	95.0	5.0
3	5.00	200	75.0	25.0
4	7.00	200	75.0	25.0



Ketoprofen



Propanolol

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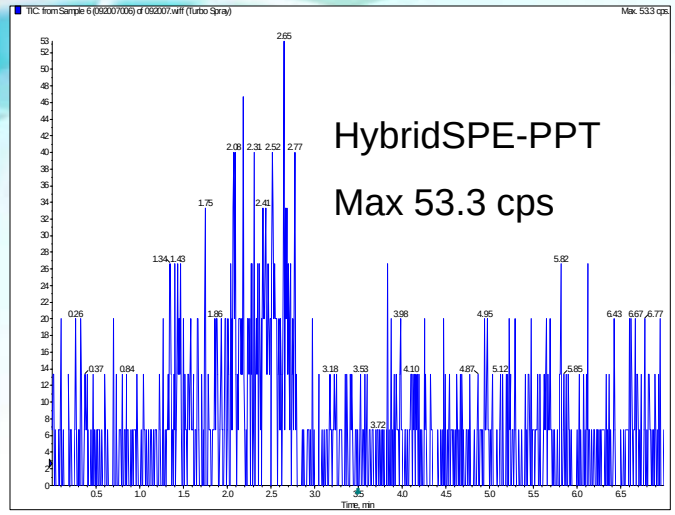
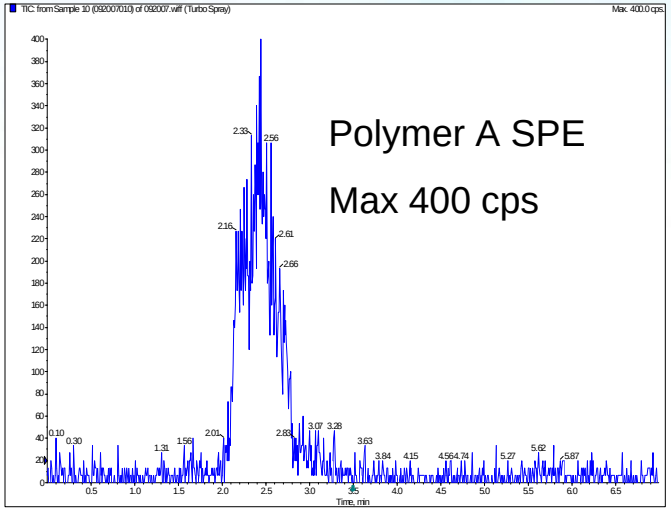
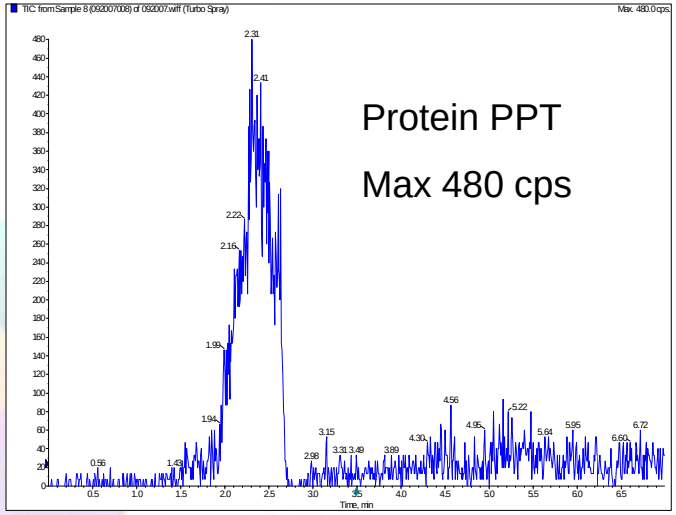
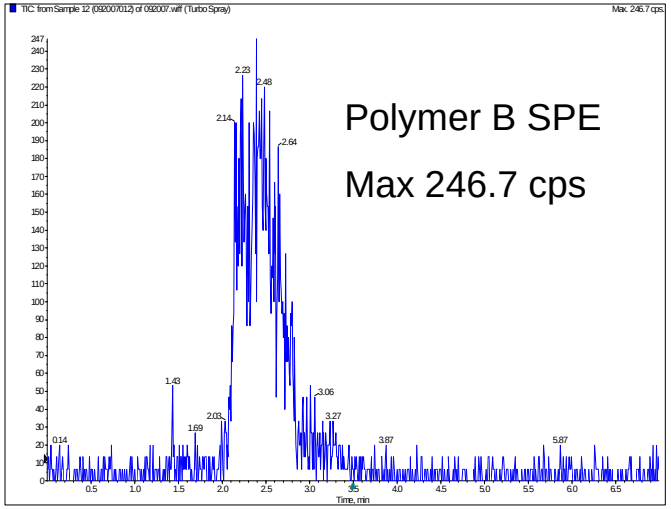
Example 3 (cont.): TIC of Blank Rat Plasma Extracts (Propanolol & Ketoprofen Study)









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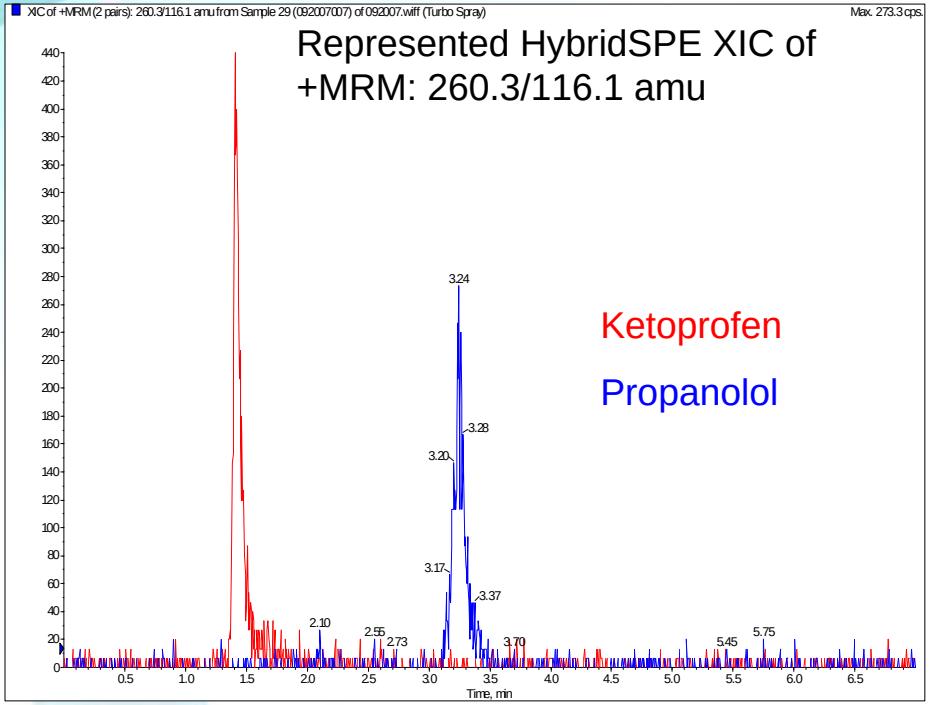
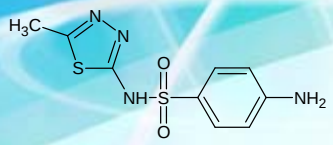
Example 3 (cont.):Absolute Recovery of Propanolol & Ketoprofen from Rat Plasma



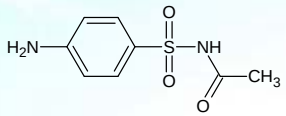
	% Absolute Recovery (100ng/mL spike)	
Extraction Method	**Ketoprofen**	**Propanolol**
HybridSPE PPT	**82.0%**	**68.0%**
Std. Protein PPT	**58.8%**	**37.0%**
Polymer A SPE	**78.4%**	**42.0%**
Polymer B SPE	**76.4%**	**44.4%**

Example 4: Extraction of Sulfa Drugs in Dog Plasma



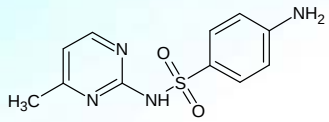
Monoisotopic Mass = 270.024516 Da

Sulfamethizole



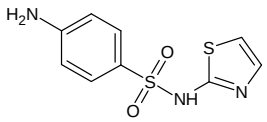
Monoisotopic Mass = 214.041212 Da

Sulfacetamide



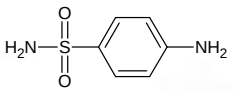
Monoisotopic Mass = 264.068096 Da

Sulfamerazine



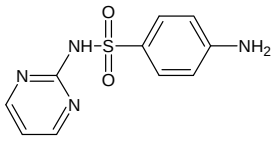
Monoisotopic Mass = 255.013617 Da

Sulfathiazole



Monoisotopic Mass = 172.030648 Da

Sulfanilamide



Monoisotopic Mass = 250.052446 Da

Sulfadiazine

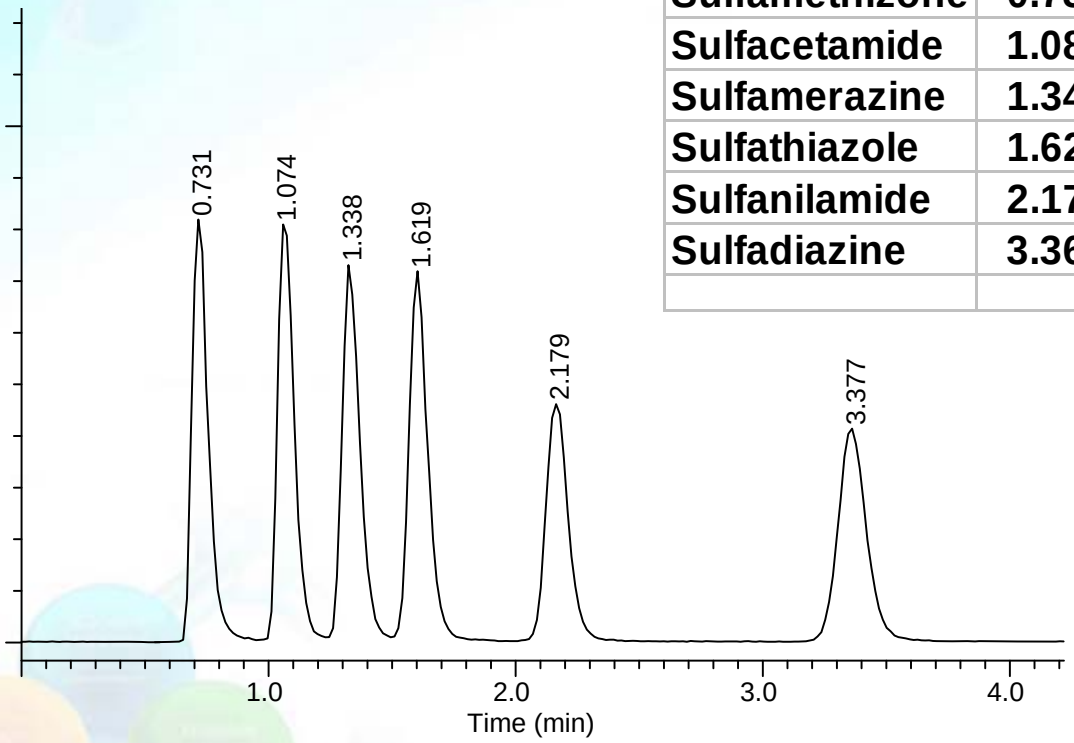
column:	Ascentis Express C18, 5 cm x 2.1 mm 2.7 µm
mobile phase:	85:15, 0.1% acetic acid : methanol
flow rate:	0.2 mL/min
temp.:	35 °C
det.:	MS/MS
injection:	2 µL
MS conditions:	

CUR:	45.00
IS:	4200.00
TEM:	425.00
GS1:	40.00
GS2:	55.00
ihe:	ON
CAD:	Medium
CXP	4.00

Sample Preparation: 10 ng/mL sulfa drug samples were prepared both dog plasma and a solvent mixture containing 1% formic acid in MeCN:Water (75:25, v/v). The 100 µL of the spiked plasma sample was precipitated with 300 µL 1% formic acid in MeCN. The precipitated plasma sample was centrifuged and the resulting supernatant was loaded onto the HybridSPE 1 mL cartridge. The sulfa drug sample prepared in 1% formic acid in MeCN:Water (75:25, v/v) was loaded onto a separate HybridSPE 1 mL cartridge. The eluate was collected for each eluate for LC-MS recovery assessment.

Example 4 (cont.): HybridSPE Results for Sulfa Drugs

	RT	Standard (No Plasma)	Spiked Dog Plasma
Sulfamethizone	0.73	108.33	100.78
Sulfacetamide	1.08	102.46	97.54
Sulfamerazine	1.34	108.41	94.28
Sulfathiazole	1.62	120.83	98.46
Sulfanilamide	2.17	109.79	95.74
Sulfadiazine	3.36	106.02	49.46



High recovery was observed for both standards and spiked dog plasma (except sulfa diazine) indicating low analyte binding retention of sulfa drugs on HybridSPE phase and/or minimizing of matrix effect.

Example 5: Drugs of Abuse in Plasma

Column: Ascentis Express HILIC, 10 cm x 2.1 mm, 2.7 µm

Instrument: Agilent 1200RR

Mobile Phase: *Isocratic*

98% 10 mM ammon acetate in MeCN

2% 10 mM ammon acetate in water, pH 3.5

Temp: 50 ºC

Flow: 0.4 mL/min.

Inj.: 1 µL

Detection: TOF-MS

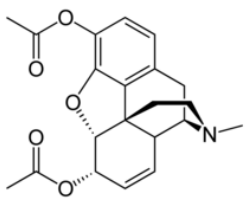
Heroin 370.1655 m/z

Cocaine 304.1550 m/z

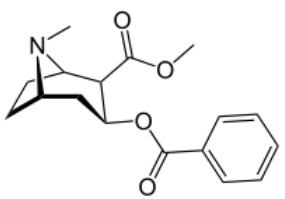
Amphetamine 136.1124 m/z

MDMA 194.1880 m/z

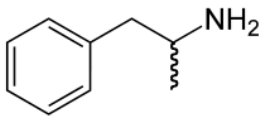
Phospholipids 184 m/z



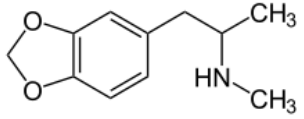
Heroin



Cocaine



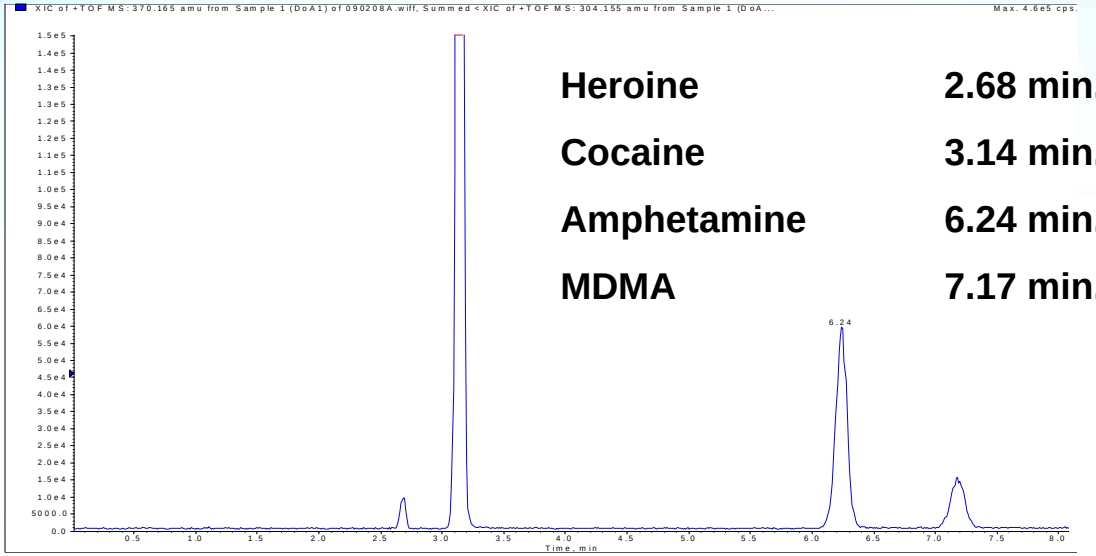
Amphetamine



MDMA

Sample Preparation: Drugs of abuse were spiked in rat plasma at the level of 40 ng/mL. 100 µL of spiked plasma was precipitated with 300 µL 1% formic acid in MeCN. The precipitated plasma sample was centrifuged and the resulting supernatant was loaded onto the HybridSPE 1 mL cartridge. The resulting eluent was analyzed. 10 ng/mL drugs of abuse standards were prepared in solvent mixture containing 1% formic acid in MeCN:Water (75:25, v/v) and passed through a 1 mL HybridSPE cartridge as the plasma sample.

Example 5 (cont.): Drugs of Abuse in Plasma

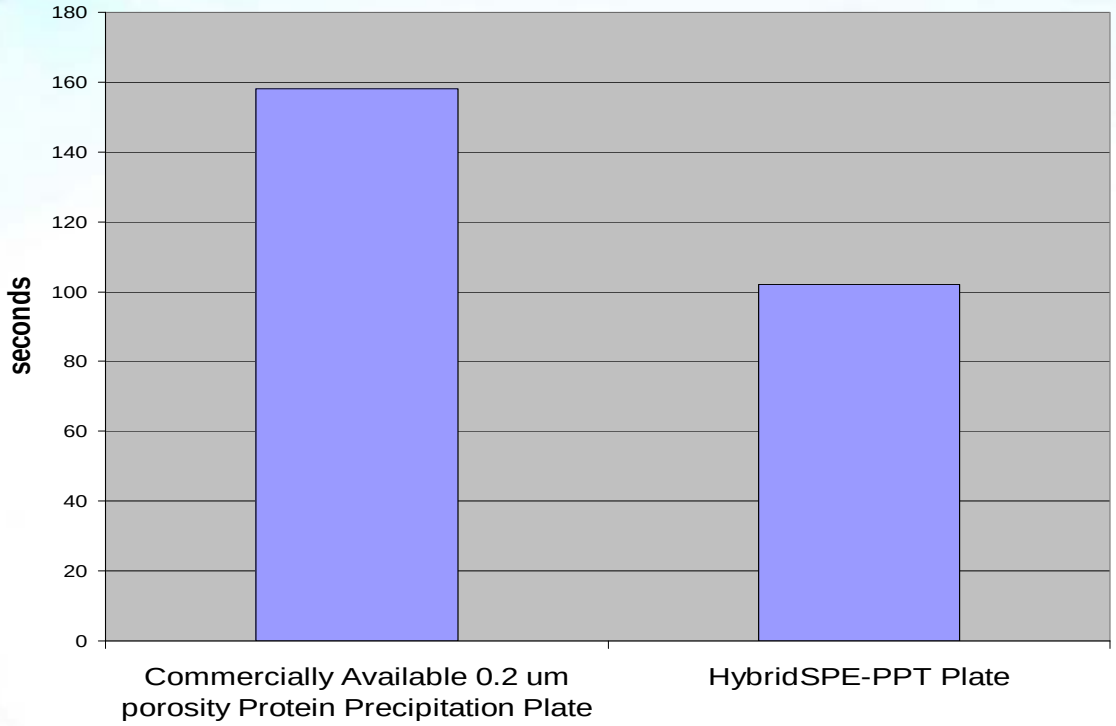


Heroine	2.68 min.
Cocaine	3.14 min.
Amphetamine	6.24 min.
MDMA	7.17 min.

HybridSPE-PPT Absolute Recovery

40 ng/mL spike	DI Water (no matrix)	Plasma
Heroin	88.8%	84.7%
Cocaine	100.5%	80.5%
Amphetamine	101.1%	77.8%
MDMA	109.9%	84.6%

Improved Flow Rate for HybridSPE – PPT Plate vs. Standard Protein Precipitation Filter Plate



250 µL rat plasma was added to each well plate followed by 750 µL 1% formic acid in MeCN. After vortexing, equivalent levels of vacuum were applied. The "depth-filter" like format of the HybridSPE Plate provided faster flow-rate relative to traditional protein precipitation filter plates.

Comparison of Sample Prep Techniques

	Protein PPT	HybridSPE-PPT	SPE
No. of Steps	**2-3**	**2-3**	5-8
Time Req'd	**< 10 min.**	**< 10 min.**	30-45 min.
Method Development	**Generic/Minimal**	**Generic/Minimal**	Moderate to Extensive
Selectivity/Clean-Up	Minimal	**Moderate to High**	**Moderate to High**
Phospholipid Removal	None	**High**	Moderate
Recovery	**High**	Moderate to High	Moderate to High
Price	**$**	**$$**	$$$$

HybridSPE-PPT Phospholipid Removal Relative to Capacity, Organic Modifier, and Acid Modifier

Phospholipid Capacity Test – HybridSPE-PPT 96-well, 50 mg/well

Standard Protein PPT -

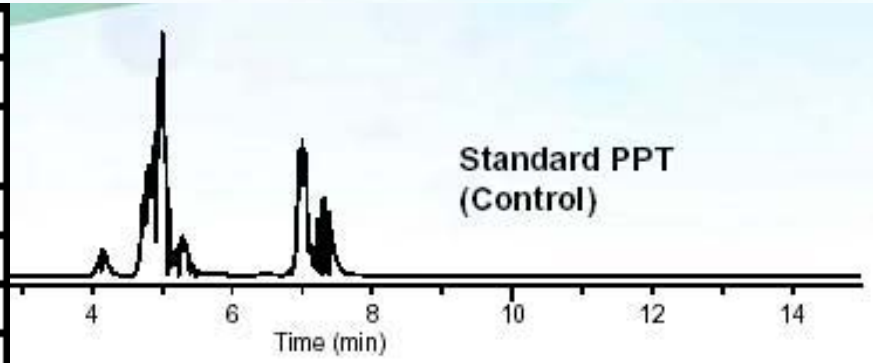
- 100 uL plasma + 300 uL 1% formic acid in MeCN
- Vortex for 1 min. followed by centrifugation for 3 min.
- Analyze supernatant via LC-MS

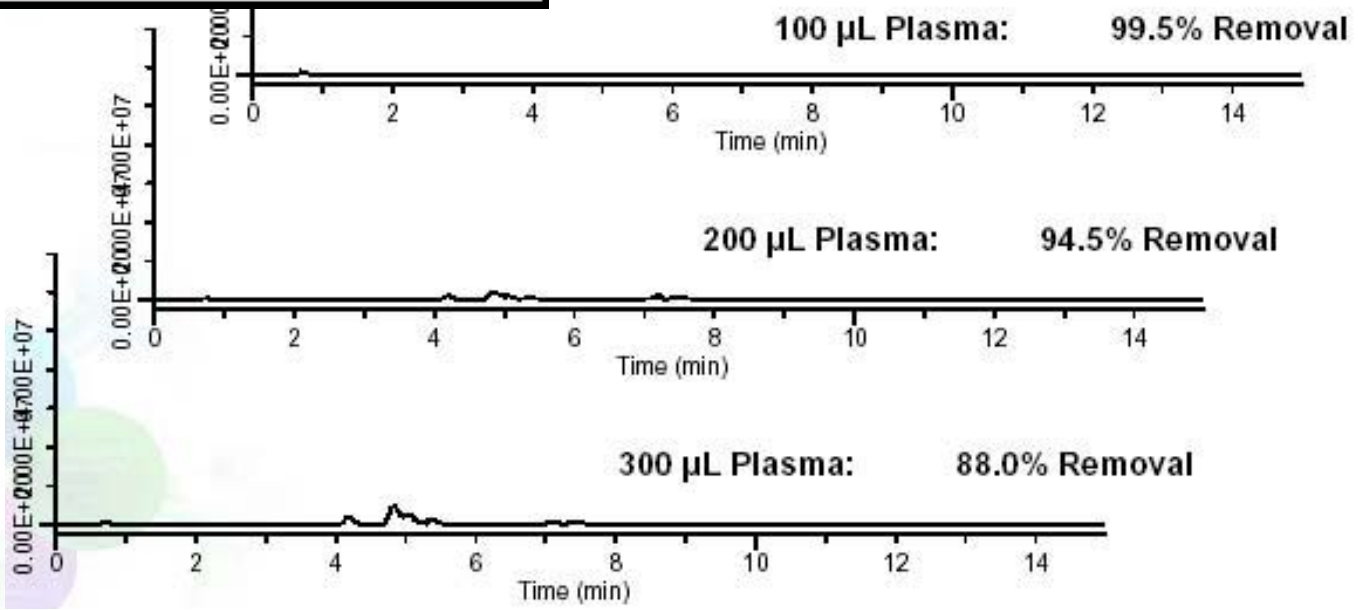
HybridSPE-PPT Capacity Test -

- Apply rat plasma to HybridSPE-PPT Plate followed by 1% formic acid in MeCN (1:3, v/v – plasma:1% formic acid in MeCN)
  - 100 uL plasma + 300 uL 1% formic acid in MeCN
  - 200 uL plasma + 600 uL 1% formic acid in MeCN
  - 300 uL plasma + 900 uL 1% formic acid in MeCN
- Vortex plate for 1 min. Apply vacuum. Analyze resulting eluent via LC-MS

Results – Phospholipid Capacity Test

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





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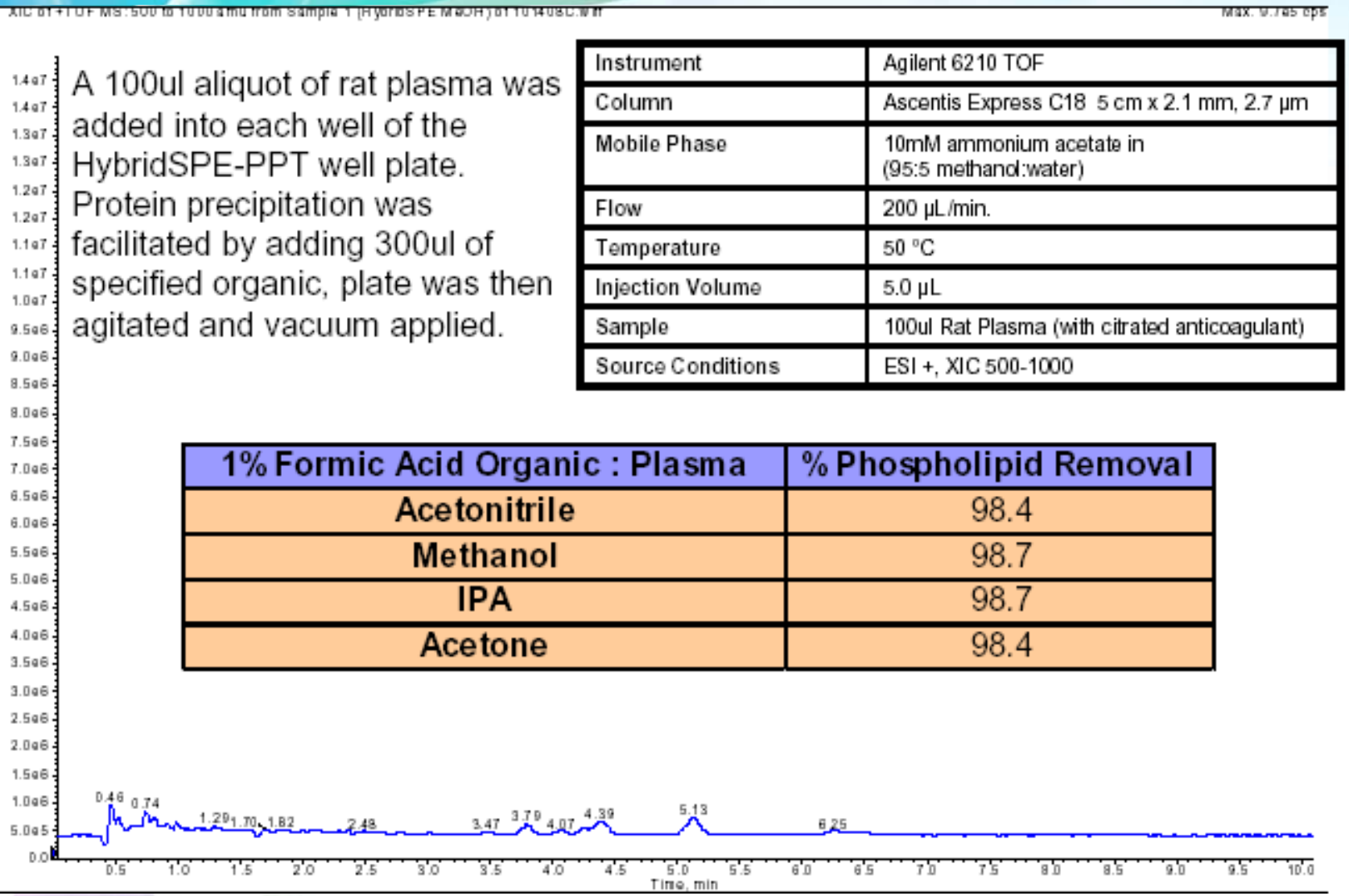
Phospholipid Removal vs. Organic Modifier Type

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

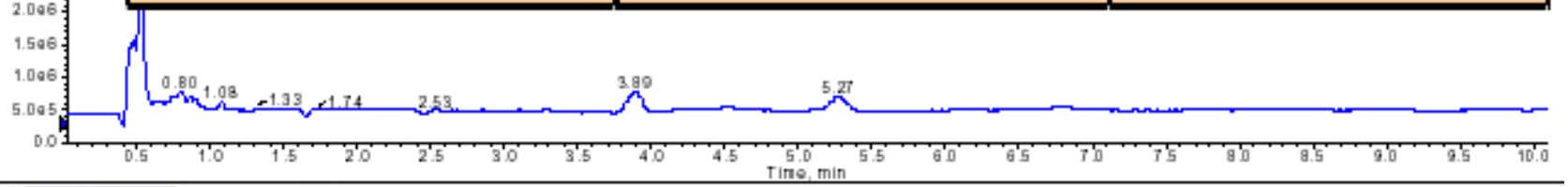


Phospholipid Removal vs. Acid Modifier Type

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



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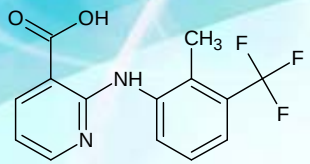
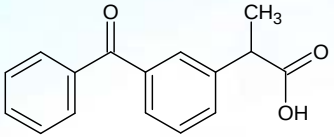
Development of HybridSPE-PPT Conditions, FAQs & Troubleshooting Tips

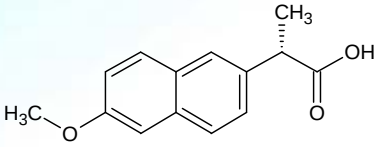
Formic acid is critical for recovery of acidic compounds – Example NSAIDS



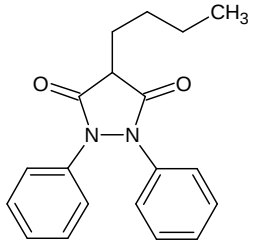
Flunixin



Ketoprofen

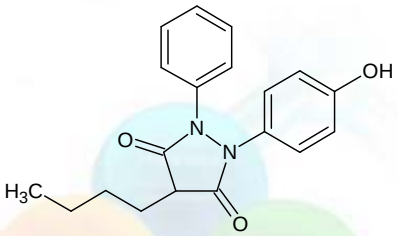


Naproxen



Monoisotopic Mass = 308.152478 Da

Phenylbutazone



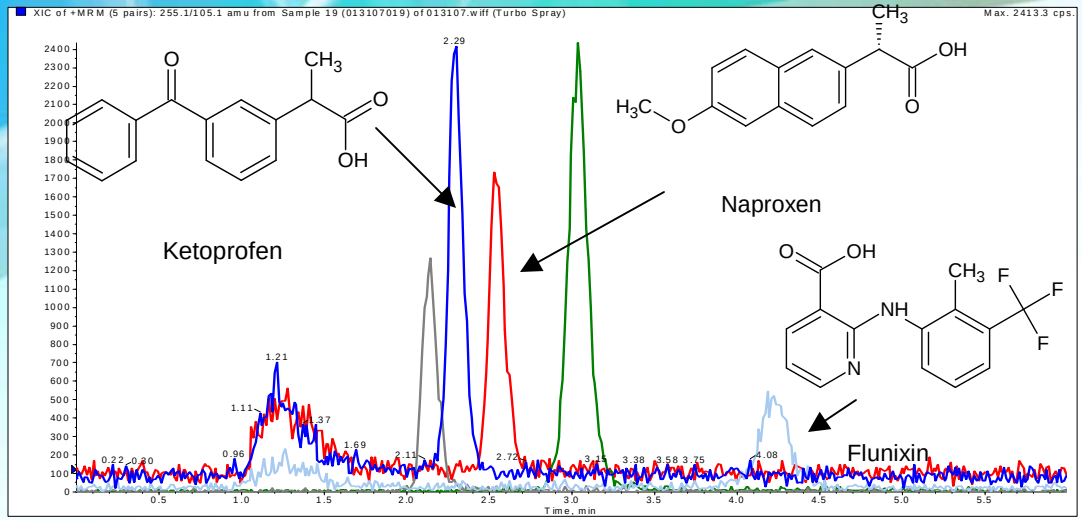
Monoisotopic Mass = 324.147393 Da

Oxyphenbutazone

Sample Preparation: 20 ng/mL drugs spiked into rat plasma. 100 µL plasma samples were precipitated with either 300 µL 1% formic acid in MeCN or 300 µL neat MeCN for comparison The samples were centrifigued and the supernatant was loaded onto a HybridSPE 1 mL cartridge and analyzed via LC-MS

Column:	Ascentis RP-Amide 10 X 2.1, 5um
Mobile Phase:	10mM ammonium acetate pH 5.5: methanol (40:60)
Flow:	200ul/min
Temp:	35°C
Inj Vol:	5ul
MS Conditions:	Multiple MRM

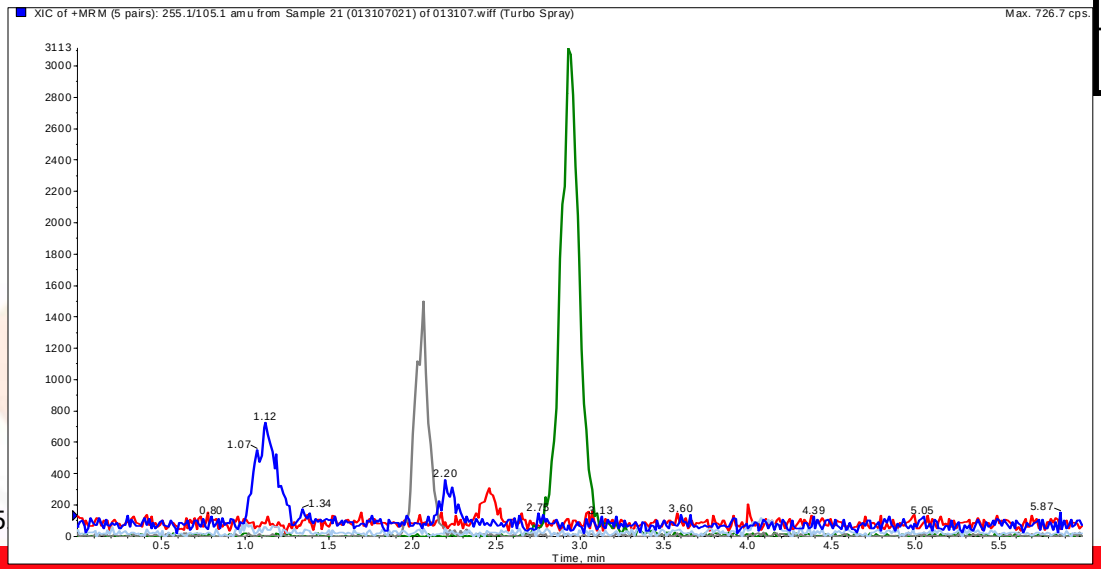
HybridSPE-PPT with 1% formic acid in MeCN



High relative concentration of Formic Acid enables recovery of acidic compounds containing cabonyl groups

HybridSPE-PPT with Formic Acid	RT	Abs. Recov. w/ formic acid
1. Oxyphenylbutazone	2.14	95.5%
2. Ketoprofen	2.29	88.0%
3. Naproxen	3.54	104.0%
4. Phenylbutazone	3.04	110.0%
5. Flunixin	4.22	135.0%

HybridSPE-PPT with MeCN only

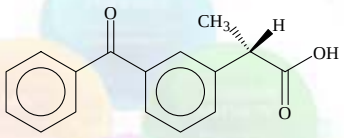


Protein PPT performed without Formic Acid causes analytes with carboxylic acid functionality to retain on HybridSPE Zr-Si phase.

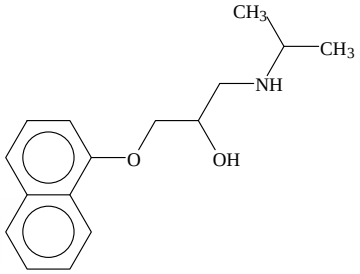
Analyte Recovery Relative to Amount of Formic Acid

In this study, two different acid modifers (Lewis Bases) were compared. The acids tested were formic acid (FA) and acetic acid (HA). The acid concentration was systematically adjusted and measured against recovery using a representative acidic compound (ketoprofen) and basic compound (propanalol) diluted in rat plasma (100 ng/mL).

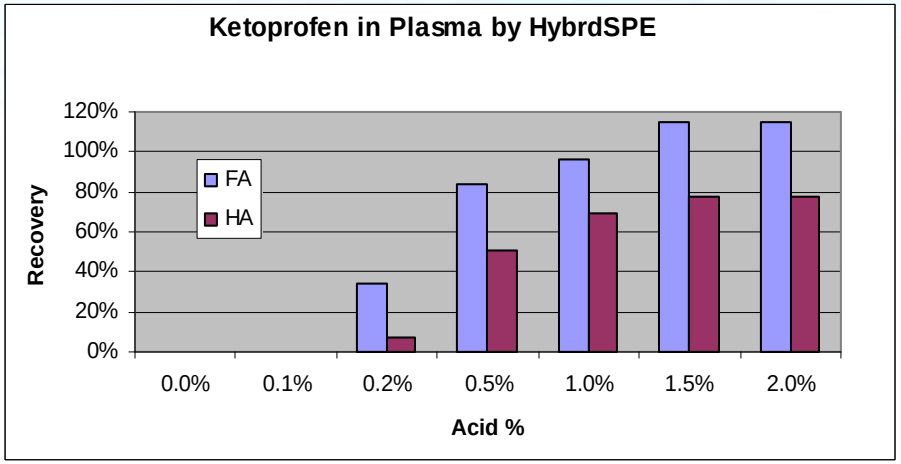
From the results we see that acid type and amount had little affect on the recovery of propanolol (basic compound). However, at least 1-2% formic acid is needed for optimal recovery of ketoprofen (acidic compound).

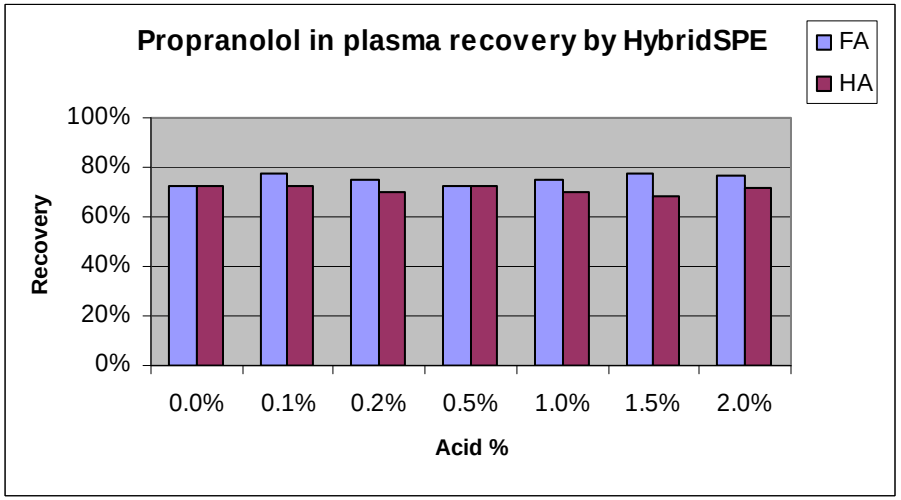


Ketoprofen



Propanolol





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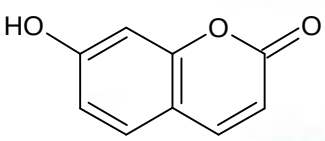
Controlling Secondary HILIC / Normal-Phase Interactions during HybridSPE-PPT

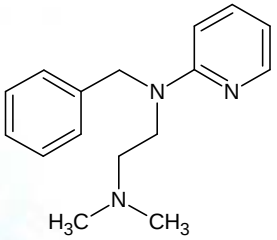
HybridSPE-PPT technology is based on Zr coated silica. The precipitation agent used in this technique comprises of an organic solvent (MeCN or MeOH). As a result, there's risk of analyte retention (low recovery) through secondary HILIC or normal-phase interactions.

In this study, plasma samples spiked with 4 basic drugs were protein precipitated with 1% formic acid in MeOH or 1% formic acid in MeCN at different plasma:precipitation agent ratios (1:19 to 1:4, v/v) prior to HybridSPE-PPT for phospholipid removal.

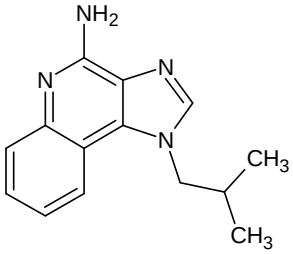
Recovery was accessed by LC-MS.



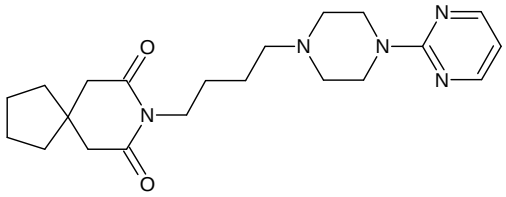
Umbeliferone



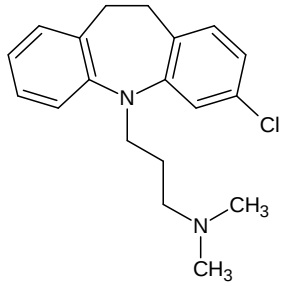
Tripelennamine



Imiquimod



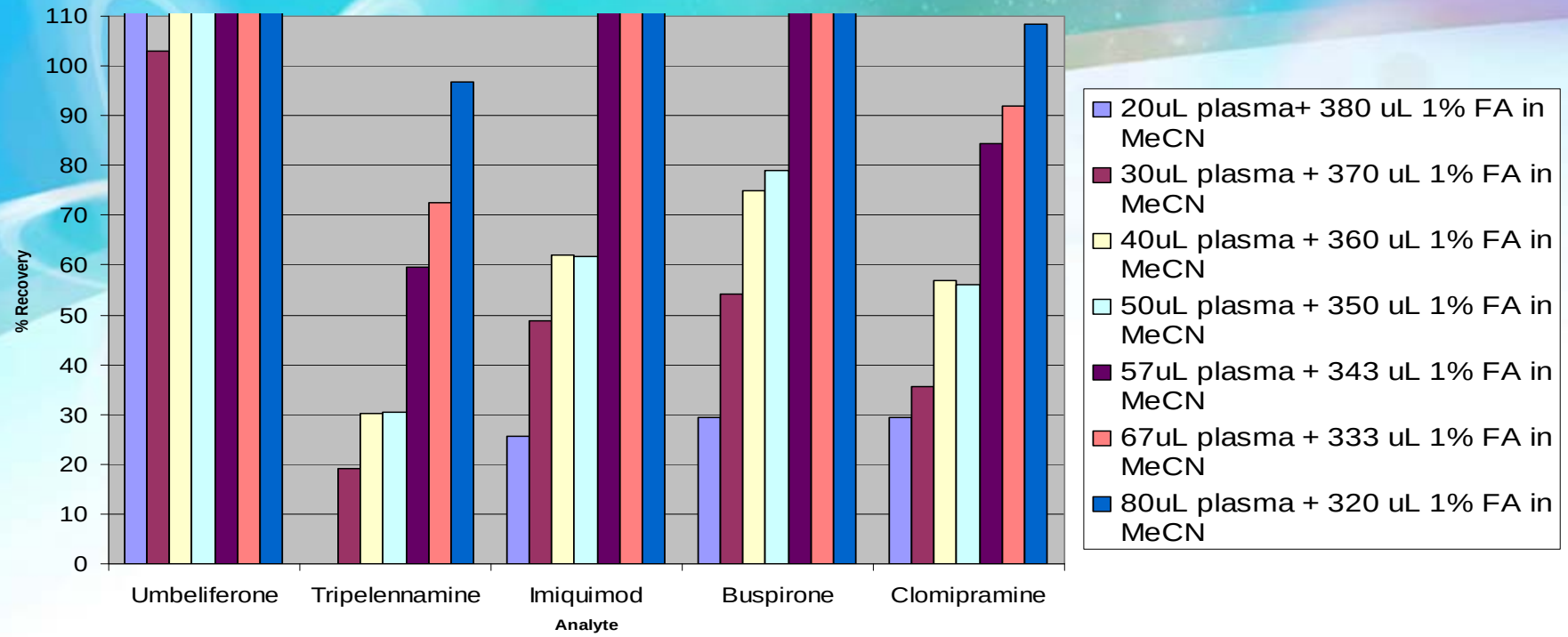
Busiperone

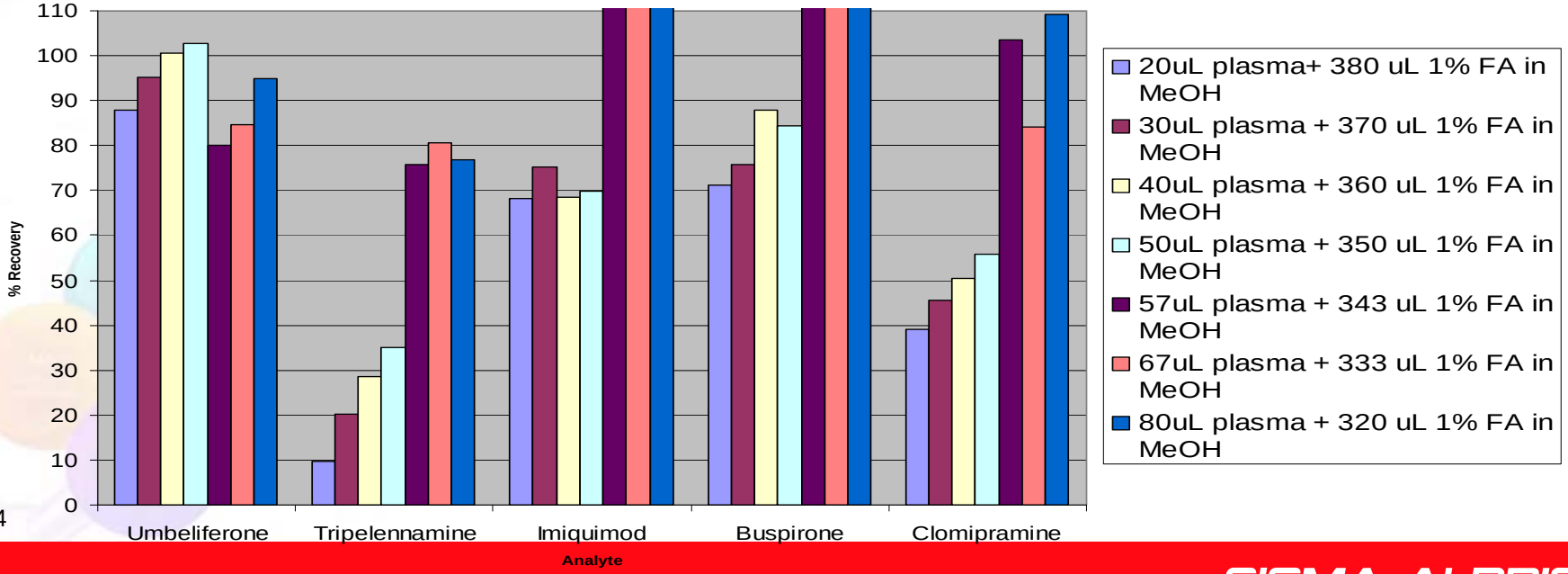


Clomipramine

Results on Next Slide

Note: LC conditions were not fully optimized so peak broadening occurred resulting in higher than actual recovery levels. Nevertheless, trends in analyte retention profiles during HybridSPE-PPT should be accurate.


110
100
90
80
70
60
50
40
30
20
10
0
% Recovery
Umbeliferone
Tripelennamine
Imiquimod
Buspirone
Clomipramine
Analyte
20uL plasma+ 380 uL 1% FA in MeCN
30uL plasma + 370 uL 1% FA in MeCN
40uL plasma + 360 uL 1% FA in MeCN
50uL plasma + 350 uL 1% FA in MeCN
57uL plasma + 343 uL 1% FA in MeCN
67uL plasma + 333 uL 1% FA in MeCN
80uL plasma + 320 uL 1% FA in MeCN


110
100
90
80
70
60
50
40
30
20
10
0
% Recovery
Umbeliferone
Tripelennamine
Imiquimod
Buspirone
Clomipramine
Analyte
20uL plasma+ 380 uL 1% FA in MeOH
30uL plasma + 370 uL 1% FA in MeOH
40uL plasma + 360 uL 1% FA in MeOH
50uL plasma + 350 uL 1% FA in MeOH
57uL plasma + 343 uL 1% FA in MeOH
67uL plasma + 333 uL 1% FA in MeOH
80uL plasma + 320 uL 1% FA in MeOH

Discussion: Controlling Secondary HILIC / Normal-Phase Interactions during HybridSPE-PPT

- To minimize secondary HILIC / Normal-Phase interactions, during HybridSPE-PPT, the sample (after protein ppt with organic) needs to contain a high enough percentage of aqueous to counteract these polar interactions.
- Precipitated samples containing $\leq$ 1:7 plasma:organic (50 uL plasma + 350 uL precipitation agent) will often result in low recovery. Therefore, 1:3 plasma:precipitation agent (100 uL plasma + 300 uL precipitation agent) is recommended.
- If 1% formic acid in MeCN does not provide adequate recovery for basic compounds, replacing MeCN with MeOH will often increase recovery. MeOH is more polar than MeCN which will further inhibit secondary polar interactions.
- Note that MeCN is a better precipitation agent than MeOH for protein removal (protein particles easier to centrifuge and filter out). Therefore MeCN should be the first choice when developing a new assay.

HybridSPE Recovery of Representative Pharma Compounds

Summary of Abs. Recovery from Plasma:

Compound Name	Spike Conc.	Matrix	Abs. Recovery
Propranolol	100ng/ml	Rat plasma	62%
Ketoprofen	100ng/ml	Rat plasma	82%
Doxepin	7.5ng/ml	rat plasma	87%
Imipramine	7.5ng/ml	rat plasma	92%
Amitriptyline	7.5ng/ml	rat plasma	90%
Oxyphenbutazone	20ng/ml	rat plasma	104%
Ketoprofen	20ng/ml	rat plasma	92%
Naproxen	20ng/ml	rat plasma	93%
Phenylbutazone	20ng/ml	rat plasma	99%
Flunixin	20ng/ml	rat plasma	76%
Procainamide	12.5ng/ml	rat plasma	42%
Doxepin	12.5ng/ml	rat plasma	89%
Mirtazapine	12.5ng/ml	rat plasma	56%
Dextromethorphan	12.5ng/ml	rat plasma	88%
p-Coumaric Acid	12.5ng/ml	rat plasma	54%
Clenbuterol	10ng/ml	dog plasma	75%
Sulfamethizone	10ng/ml	dog plasma	101%
Sulfacetamide	10ng/ml	dog plasma	98%
Sulfamerazine	10ng/ml	dog plasma	94%
Sulfathiazole	10ng/ml	dog plasma	98%
Sulfanilamide	10ng/ml	dog plasma	96%
Sulfadiazine	10ng/ml	dog plasma	49%

Summary of Abs. Recovery Solvent (no plasma):

During the course of development, 12 mixes containing 5-8 unique/representative pharma compounds were prepared at a concentration of 600 ng/mL (diluted in 1% formic acid in MeCN:Water (75:25, v/v)) – total of 66 compounds.

Each test mix was passed through a 1 mL HybridSPE cartridge and the eluate was collected for LC-MS/MS analysis. Recovery was assessed by measuring the analyte response of the sample before and after HybridSPE processing.

An average absolute recovery of 83% observed for was observed for the 66 compounds tested. Of the 66 compounds tested, 7 of which observed recoveries > 100%; and 7 had recoveries <60%. The majority of the analytes tested achieved recoveries > 80% (data not shown).

FAQs & Troubleshooting Tips

Are the well plates amenable to automation?

- Yes, the well plates are compatible with the TomTec Quadra, Tecan Genesis, and Hamilton Microlab Star (or Microstar)
- The well plates can also be used for negative pressure and positive pressure.

Tomtec Quadra

Tecan Genesis

FAQs & Troubleshooting Tips

- **How to increasing Assay Sensitivity**
  - Increase plasma volume
    - 98-100% phospholipid removal for ≤ 300 µL plasma
    - Must increase precipitating agent volume accordingly
  - Evaporate & Reconstitute (takes ~10 min. w/ acetonitrile)
- **Dealing with more polar compounds not soluble in MeCN.**
  - Use 1% formic acid in MeOH
  - If MeOH used, recommend “Off-Line” approach
- **Why is ion-suppression still evident during LC-MS analysis after HybridSPE-PPT?**
  - HybridSPE-PPT will only remove phospholipids and gross levels of precipitated proteins from biological samples. Other chemical entities common in biological samples can lead to ion-suppression. Examples include:
    - Sodium citrate (plasma anti-coagulant)
    - Phthalates, plasticizers and other mold release agents found in plastic ware
    - Polyethylene glycol (common dosing vehicle)
    - Extractables from o-rings, plastic ware, and seals used to store biological samples

FAQs & Troubleshooting Tips

- **Why am I experiencing absolute recovery > 100%?**
  - Acetonitrile is volatile. Disengage vacuum immediately.
  - Further vacuum application can evapoarate the eluent therby erroneously giving misleadingly high analyte response during LC-MS Analysis
- **Why is the HybridSPE-PPT Eluate Volume < 400 uL?**
  - HybridSPE Dead Volume (80 uL loss) & Evaporation effect (20 uL loss)
  - Therefore, 400 uL applied results in 300 uL eluent volume.
  - If evaporation/reconstitution is desired, evaporate a known volume.
  - Use of I.S. strongly recommend to account for volume reduction
- **Should the HybridSPE phase be conditioned?**
  - In general, conditioning the HybridSPE is not required nor recommended. If ≥ 80 µL is used to condition the phase prior to sample addition, there could be a dilution effect in which is the final HybridSPE eluent is ~80 µL greater than it should be. As a result, absolute recovery will appear lower than it actually is.

FAQs & Troubleshooting Tips

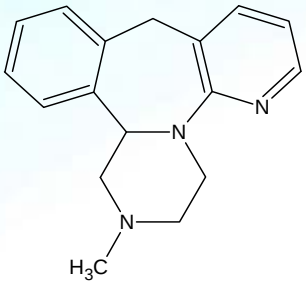
- **Can HybridSPE-PPT be used with smaller plasma volumes (e.g. 20 uL mouse plasma)?**
  - Yes. If smaller plasma volumes are employed, the plasma sample needs to be diluted with DI water so that the final volume is = 100 uL.
  - For example, first dilute 20 uL plasma with 80 uL DI water. Second, combine diluted plasma sample with 300 uL 1% formic acid in ACN.
  - To increase assay sensitivity, evaporate the resulting HybridSPE eluent and reconstitute in a smaller volume of LC mobile phase.
  - A minimum volume of 400 uL (plasma + precipitation agent) is necessary to minimize the impact of volume loss during HybridSPE processing (see previous slide.
  - The precipitated sample (prior to HybridSPE processing) needs to contain at least 1 part aqueous to 3 parts organic to minimize secondary HILIC / normal-phase interactions.

FAQs & Troubleshooting Tips

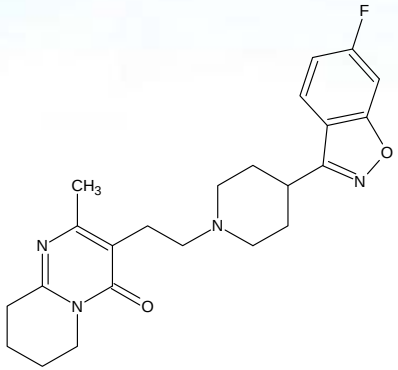
- **Can HybridSPE-PPT be used for whole blood?**
  - **Yes and No.** If the researcher does NOT need to lyse blood cells, then the "Off-Line Procedure" is recommended.
  - If the researcher needs to lyse blood cells prior to protein ppt, then they will add 1:1 (v/v) 0.1 M zinc sulfate. The sulfate ions will most likely interfere with phospholipid retention.
- **Why am I experiencing low absolute recovery (≤ 50%)?**
  - *The primary "In-well Precipitation" and "Off-Line Precipitation" procedures using formic acid in MeCN as a precipitating agent will work well for ~80% of the applications encountered.*
  - However, ~20% of the analytes encountered will co-retain with phospholipids under these conditions resulting in absolute recoveries of ≤ 50%.
  - Please refer to the Instruction Sheet (T708008) for detailed secondary procedures or next slide(s).

Low Abs Recovery – Basic Compounds

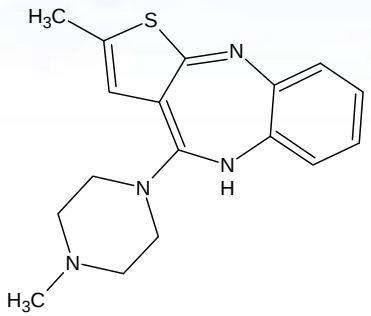
- Some basic compounds will experience low (< 40%) using the primary method (100 uL plasma + 1% formic acid in ACN)
- Example compounds include:



Mirtazapine



Risperidone

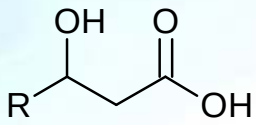


Olanzapine

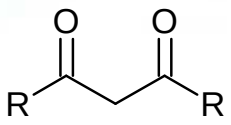
- These compounds co-retain w/ phospholipids due to:
  1. Secondary WCX interactions b/w HybridSPE silanol groups ($Si\text{-}O^-$) and Basic Cmpds.
  2. Secondary HILIC interactions b/w HybridSPE silica surface and Analyte(s)
- **Recommendation:** Combine 100 uL plasma + 300 uL 1% ammonium formate in methanol followed by HybridSPE-PPT processing.
  - Recovery of basic and neutral compounds can improve from < 40% to > 89%
  - NH_4^+ (ammonium formate) is a stronger counter-ion than H^+ (formic acid) inhibiting most basic compounds from interacting with HybridSPE silanol groups ($Si\text{-}O^-$).
  - Methanol is a more polar solvent than acetonitrile further inhibiting any potential secondary HILIC interactions between the analyte and HybridSPE silica surface.

Low Abs Recovery – Acidic Chelator & Chelator Cmpds

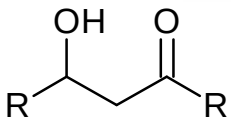
The below analyte functional groups will often form chelation complexes with HybridSPE Zr ions resulting in low recovery.



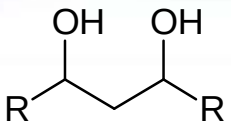
Beta-hydroxy caroboxylic acids



1,3-Diketone type compounds



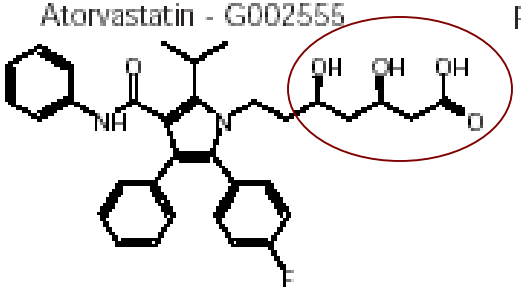
Beta-hydroxy ketones



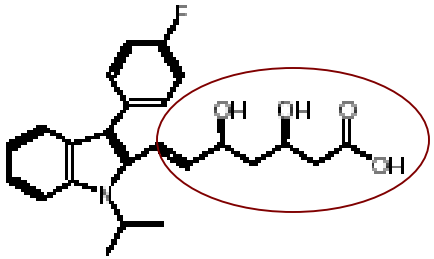
1,3-Diol type compounds

Example Compounds:

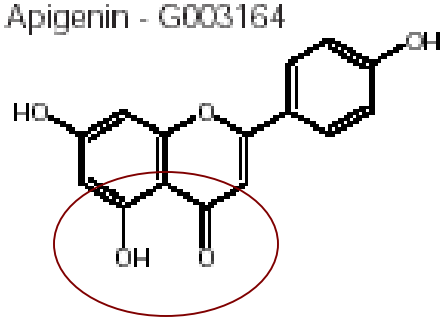
Atorvastatin - G002555



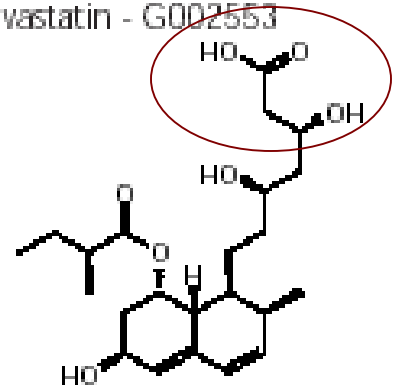
Fluvastatin - G002554



Apigenin - G003164

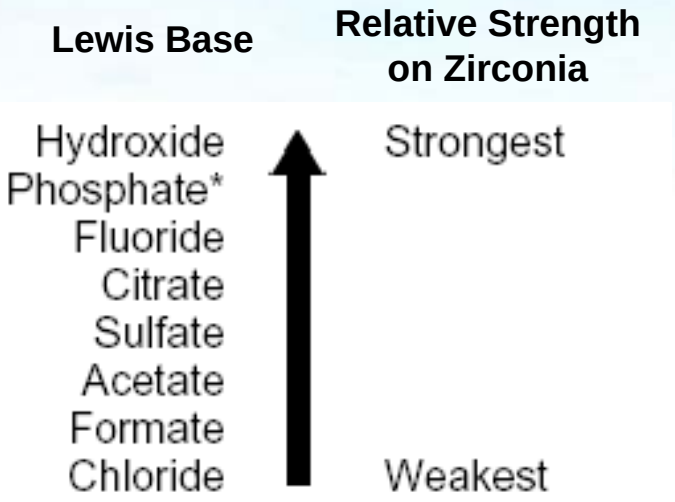


Pravastatin - G002553



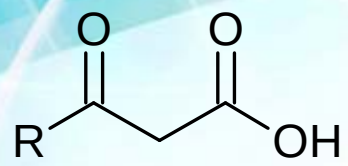
Low Abs Recovery – Acidic Chelator & Chelator Cmpds

- **Example Functional Groups:**
  - **Beta-hydroxy carboxylic acids**
  - **1,3-diketone type compounds**
  - **Beta-hydroxy ketones**
  - **1,3-diol type compounds**
- **<u>Recommendation:</u> substitute formic acid with citric acid and add a conditioning step**
  1. **Condition w/ 400 uL 0.5% citric acid in ACN**
  2. **Combine 100 uL plasma w/ 300 uL 0.5% citric acid in ACN**
  3. **Continue with HybridSPE-PPT processing**

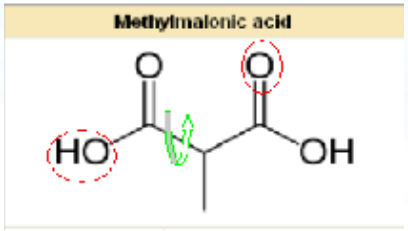


- **Recovery of chelator compounds can improve from < 40% to 65-90%**
- **Citric acid is a stronger Lewis base than formic acid inhibiting the retention of chelator compounds.**
- **Citric acid is not a strong enough Lewis base to inhibit phosphates (phospholipids) from retaining on the HybridSPE phase.**

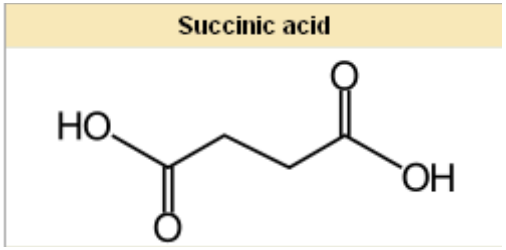
Very Difficult Chelators – Retained more strongly than phospholipids



Beta-ketone caroboxylic acids

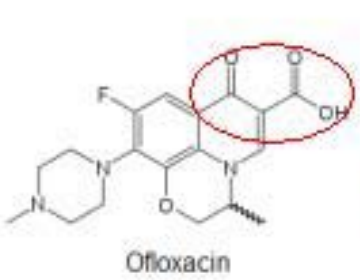


Poor Recovery via 1° Procedure



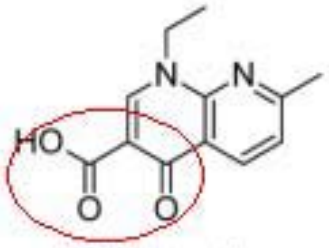
Excellent Recovery via 1° Procedure

Example Compounds:

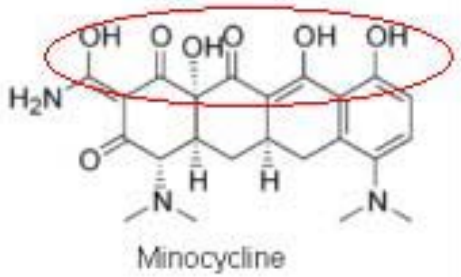


Ofloxacin

Mesalazine



Nalidixic acid



Minocycline

Good Recovery?

SIGMA-ALDRICH®

Acknowledgements

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  - Michael Ye, R&D Manager, Sample Prep
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  - Shane Needham, Alturas Analytics

Ordering Information

Featured Products

Description	Pkg. Qty.	Cat. No.
HybridSPE – Precipitation 96-well Plate, 50 mg/well	1	575656-U
HybridSPE – Precipitation Cartridge, 30 mg/1 mL	100	55261-U

Related Products

Description	Pkg. Qty.	Cat. No.
96-well Protein Precipitation Filter Plate	1	55263-U
Supelco PlatePrep Vacuum Manifold	1	57192-U
96 Square/Deep Well Collection Plates, 0.35 mL, PP	50	575651-U
96 Square/Deep Well Collection Plates, 1 mL, PP	50	575652-U
96 Square/Deep Well Collection Plates, 2 mL, PP	50	575653-U
96 Square Well Pierceable Cap Mats	25	575655-U

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