Recovery of Pharmaceutical Drugs From Small Volume Biological Sample Using HybridSPE-PPT 96-well Plate

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Presentation Outline

- Introduction
- HybridSPE-PPT for Protein Precipitation and Phospholipid Removal
- Elution Volume and Reproducibility
- Applications
- Conclusion

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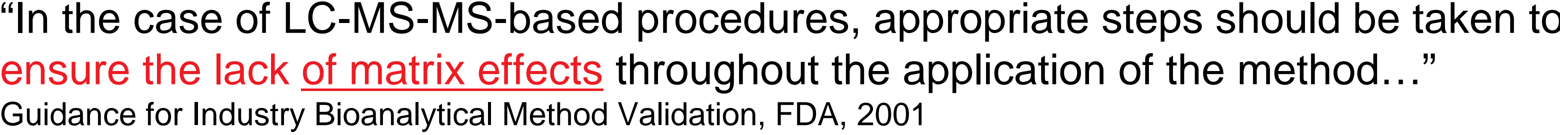
Phospholipid In Biological Sample



"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 chemists 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 with matrix-laden regions)
- Determine selectivity effectiveness of sample prep technique

m/z 184, +2H

m/z 184, +2H

m/z 104, +H

GPCho

R = acyl, alkyl, or 1-alkenyl
R' = acyl

2-lyso GPCho

R = acyl, alkyl, or 1-alkenyl

J.L. Little et al. / J. Chromatogr. B 833 (2006) 219–230

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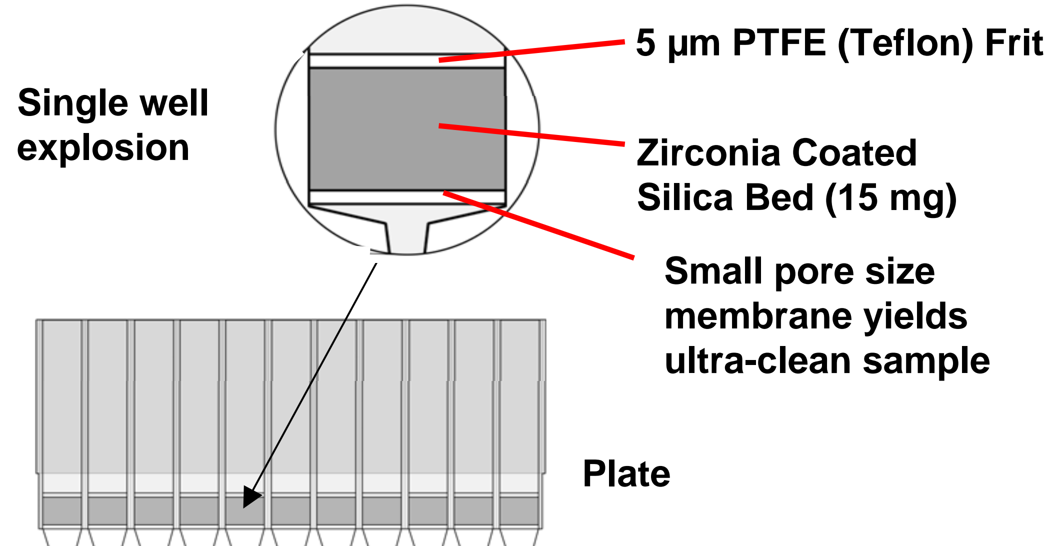
HybridSPE-PPT 96-Well Schematic Diagram

- Removes both phospholipids and precipitated proteins.
- Couples the simplicity of protein precipitation with SPE formats designed for highly selective removal of interfering phospholipids.
- Simple 2-3 step generic procedure- virtually no method development.
- Ideal for high throughput pre-clinical and clinical applications where sample prep speed, selectivity, and reduced ion-suppression is of great importance.
- Unique (patent-pending) technology developed by Supelco.



96-well format employs special frits at the top and bottom of the same selective bed; proteins *can be removed on-line* for added speed and convenience.

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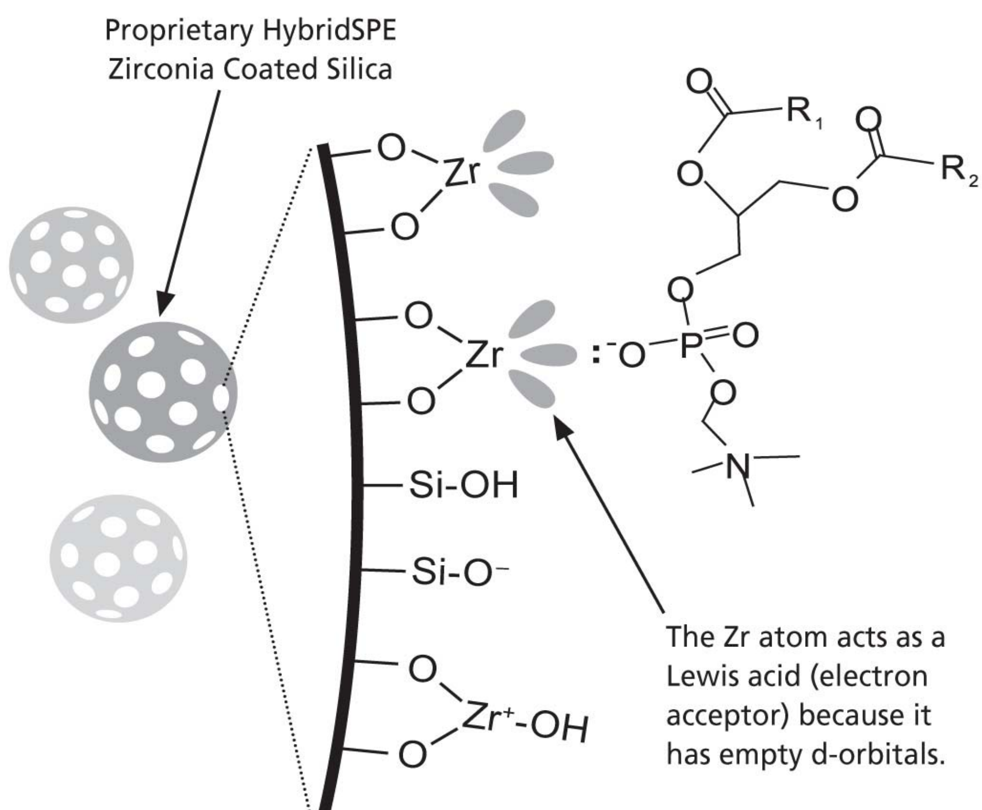
HybridSPE-PPT Interaction

The selective extraction of phospholipids is achieved using a novel zirconia-coated particle technology.

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



Interaction between a representative phospholipid and the zirconium surface of the HybridSPE-PPT particle via Lewis acid-base interaction.

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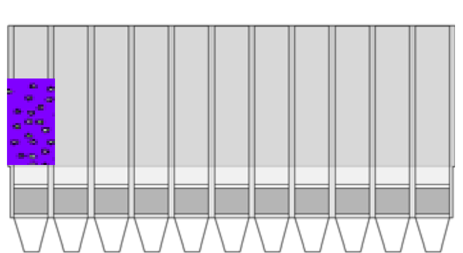
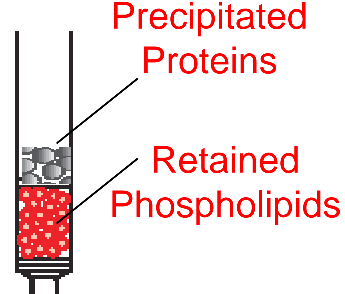
In-Well Precipitation Schematic for HybridSPE-PPT





1) Precipitate Proteins: Add 20 µL plasma/serum to the HybridSPE-PPT Small Volume plate followed by 60 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-PPT Small Volume plate or by aspirating/dispensing with 0.5-1 mL pipette tip. A cover plate may be used to avoid cross-contamination.

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 phospholipids and ready for immediate LC-MS/MS analysis; or it can be evaporated and reconstituted as necessary prior to analysis

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Phospholipid Removal and Capacity

Sample Preparation:

- 20 µL rat plasma + 60 µL MeOH/1%AF (or ACN/1%FA)+cover
- Mix by shaking at 1000/min for two minutes
- Apply Vacuum 10 inHg for two minutes
- Transfer and analyze by LC-MS-MS

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Phospholipid Removal and Capacity (contd.)

Plasma Vol (µL)	No. of Sample	Average % removal
20	8	98.9
20	4	99.8
30	4	96.4
40	4	77.4
50	4	49.2
60	4	41.6

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Elution Volume

	COL 1	COL 2	COL 3	COL 4	COL 5	COL 6	COL 7	COL 8	COL 9	COL 10	COL 11	COL 12
A	28	39	42	34	31	43	35	37	41	40	40	40
B	38	31	44	35	42	40	35	33	40	37	35	35
C	38	42	43	36	41	41	38	42	34	32	38	40
D	34	44	30	43	37	40	36	35	35	35	41	31
E	28	41	30	43	42	39	38	37	35	40	37	36
F	39	39	36	34	36	41	38	39	35	36	40	31
G	28	32	40	41	38	37	41	42	39	42	35	29
H	33	36	30	40	31	38	40	31	32	28	36	35
AVERAGE	33	38	37	38	37	40	38	37	36	36	38	35
STDEV	4.8	4.7	6.2	3.9	4.5	1.9	2.2	4.0	3.2	4.6	2.4	4.1
%CV	14.4	12.3	16.7	10.2	12.0	4.7	5.8	10.7	8.8	12.7	6.3	11.9
Overall ave	36.9											
overall std	4.1											
%CV	11.2											

Volume measured in µL

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Elution Volume (contd.)

Sample Preparation:

- 20 µL rat plasma + 60 µL ACN/1%AF+cover
- Mix by shaking at 1000/min for two minutes
- Apply Vacuum 10 inHg for two minutes
- Measure volume by 50 µL HPLC syringe

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Recovery of Neutral and Basic Compounds

Sample Preparation:

- Spike compounds into rat plasma at the specific conc.
- Load 20 µL of the spiked rat plasma (make sure the sample was loaded at the bottom of the wells).
- Add 60 µL methanol with 1% ammonium formate
- Mix gently by hand for 1 min
- Cover the plate, and pull the vacuum at 10 inHg for 2 min.
- Transfer 20 µL of the flow-through into a sample vial
- Adjust the sample solvent composite to be compatible with the running LC mobile phase by adding a certain amount of water. For instance, 10 µL water was added to the 20 µL of flow-thru drops sample to adjust the solvent composition to 50% methanol while the running HPLC mobile phase for drops is 60% methanol.

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Recovery of Neutral and Basic Compounds (contd.)

Rec of Neutral & Basic compounds from HybridSPE-Small Volume (20 µL rat plasma)				
	Drospirenone 20 ng/mL spike	Risperidone 8 ng/mL spike	Mirtazapine 8 ng/mL spike	Tiapride 8 ng/mL spike
Class	Neutral compound	Basic	Basic	Basic-chelator
Structrue				
1	103.5	84.3	68.0	25.6
2	98.3	93.5	74.2	26.8
3	109.6	92.7	73.8	24.9
4	115.7	93.3	75.0	28.0
5	104.0	89.3	70.7	24.3
6	107.6	93.8	75.6	28.5
7	101.5	89.9	71.2	26.5
8	112.9	93.2	73.6	25.6
Avg	106.6	91.3	72.8	26.3
STD	5.9	3.3	2.6	1.5
%CV	5.5	3.6	3.5	5.6

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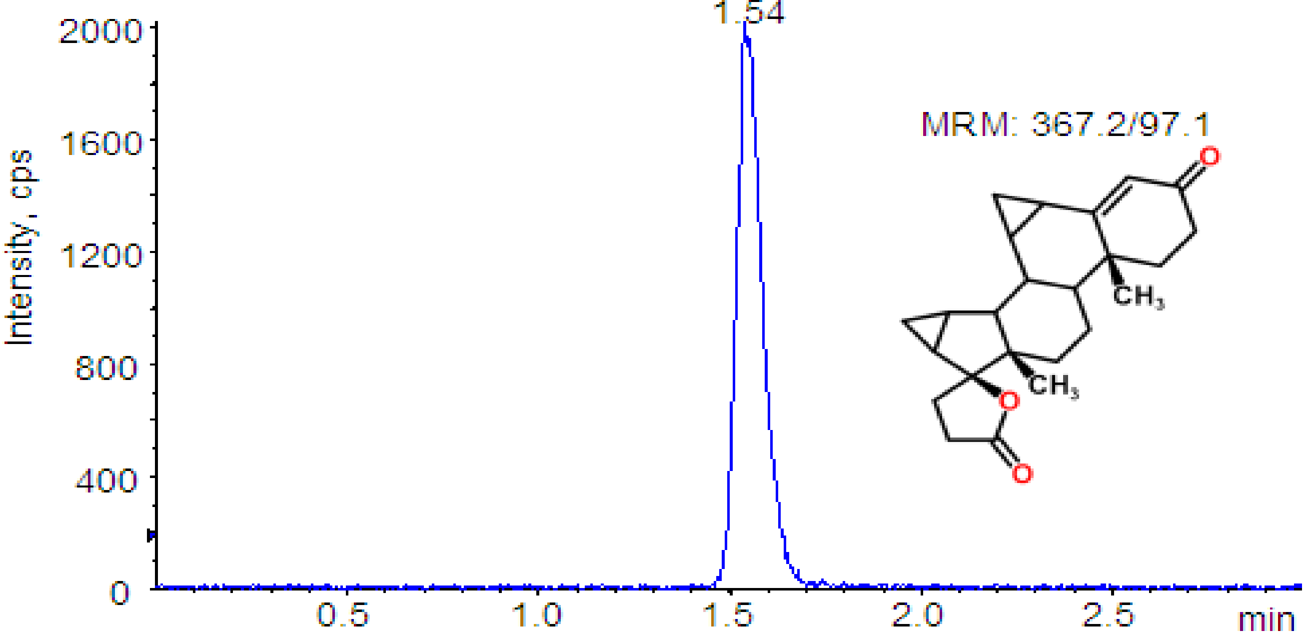
Analysis of Drospirenone



LC-MRM on Q-Trap 3200 w/ Agilent 1100 LC, column Express RP-Amide, 2.1 mm x 5 cm x 2.7 um x 100 A, 20 ng/mL Drospirenone, 3 uL injection

HPLC Isocratic: 60% B

A: 10 mM NH_4FA/0.1%FA in H_2O (pH ~4.5)

B: 10 mM NH_4FA/0.1%FA in MeOH/ACN (1:1)

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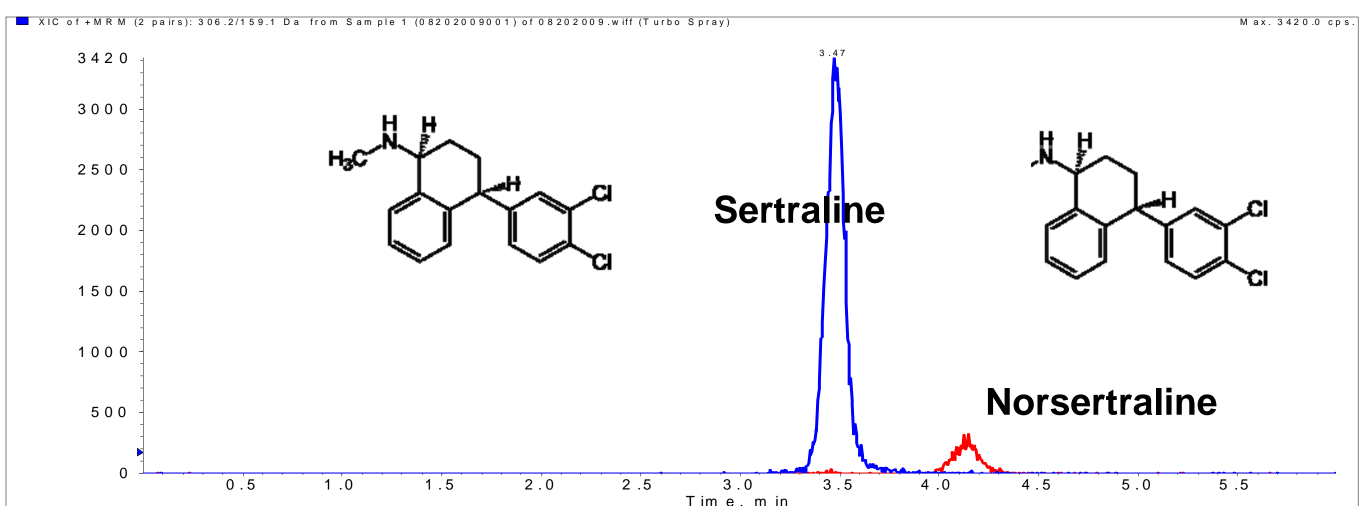
Sertraline and Norsertraline Recovery



column:	Ascentis Express 10 cm x 3 mm, 2.7 µm
mobile phase A:	65% 10 mM ammonium formate pH 4.3
mobile phase B:	35% 10 mM ammonium formate (95:5 acetonitrile-water), pH 4.3
flow rate:	600 µL/min
temp.:	55 °C
det.:	MRM Q-Trap 3200
inj.:	2 µL

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Sertraline and Norsertraline Sample Preparation

1. **Standard** - Standard samples were analyzed as is.
2. **Standard Hybrid** - 80 µL standard samples were taken in each well and pass through and collected in sample collection plate. Samples were transferred in HPLC vials.
3. **Plasma Hybrid** - Spiked rat plasma (20 µL) samples and crush solvent (60 µL), precipitated in plate, pass through and collected in a sample collection plate. Samples were transferred in HPLC vials.
4. **Protein Precipitation** – Spiked rat plasma (20 µL) and crush solvent (60 µL) were taken in centrifuge tube, vortex and centrifuge. Supernatant liquid was taken in HPLC vial.
5. All final of the samples were same (1, 20, 70, 150 and 300 ng/mL)
6. Minimum three samples were in each concentration.

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Sertraline - Recovery and Reproducibity

Sertraline-std
Sertraline-Hybrid
Sertraline-Pppt
Sertraline-Hybrid-Plasma
Linear (Sertraline-Pppt)
Linear (Sertraline-std)
Linear (Sertraline-Hybrid)
Linear (Sertraline-Hybrid-

Peak Area(counts)
25000
20000
15000
10000
5000
0

0 50 100 150 200 250 300 350

Concentration (ng/mL)

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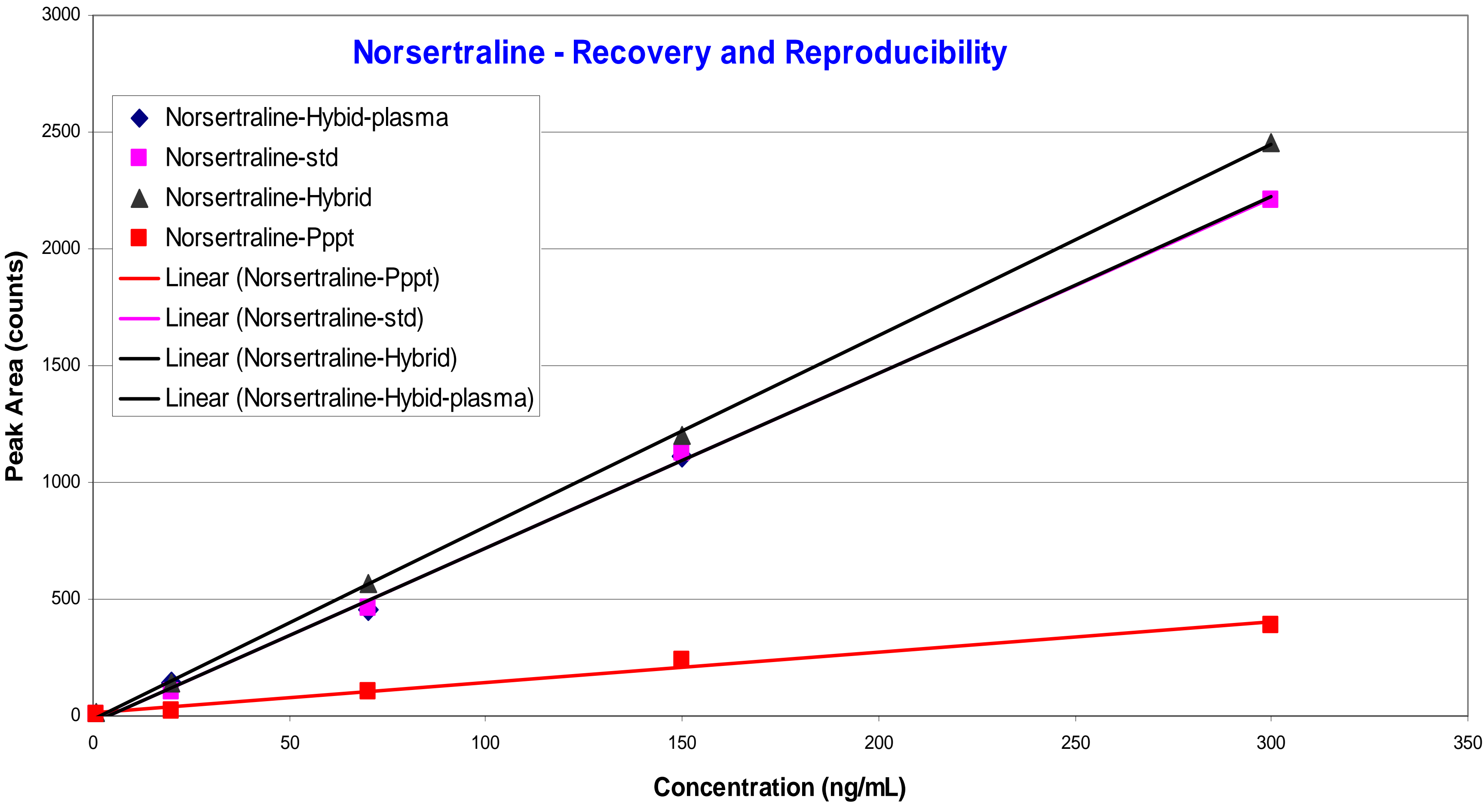
Sertraline and Norsertraline Recovery (contd.)



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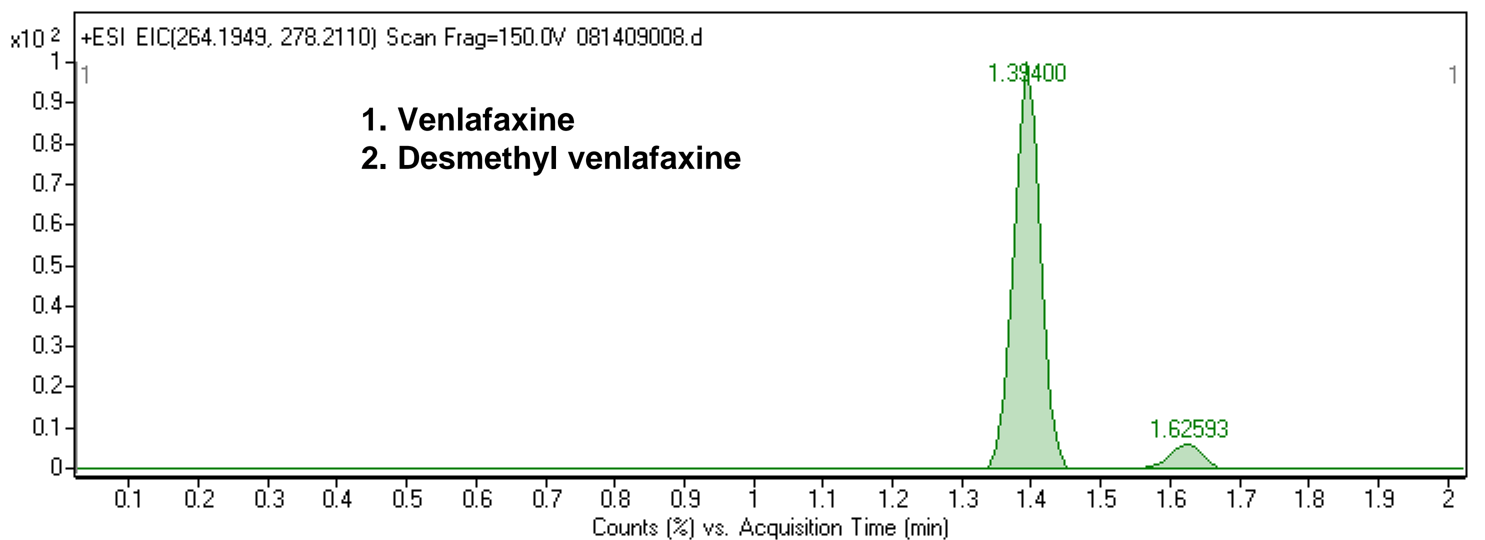
Increasing Throughput for HILIC Applications



column: Ascentis Express HILIC 10 cm x 2.1 mm, 2.7 µm, (53939-U)
mobile phase: 5 mM ammonium formate (10:90 water:acetonitrile) pH 6.87
flow rate: 0.6 mL/min
temp.: 35 °C
det.: Agilent 6210 TOF, ESI+
inj.: 0.6 µL

Venlafaxine [*{BAN}; *{INN}]
Monoisotopic Mass = 277.204179 Da

desmethyl venlafaxine [*{BAN}; *{INN}]
Monoisotopic Mass = 263.188529 Da

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Sample Preparation

Standard Solutions: prepared in (3:1) 1% formic acid acetonitrile:water at a level of 25, 50, 100, 200, 300 ng/mL.

Plasma: spiked directly to a level of 100, 200, 400, 800, 1200 ng/mL.

HybridSPE-PPT Small Volume Plasma Samples: apply 20 µL of plasma to plate, followed by 80 µL of 1% formic acid acetonitrile. Agitate on vortex for 1 minute, place on vacuum manifold and apply 10”Hg vacuum for 2 minutes. Collect filtrate and analyze directly.

HybridSPE-PPT Small Volume Standard Solution: apply 80 µL of standard prepared in (3:1) 1% formic acid acetonitrile:water. Agitate on vortex for 1 minute, place on vacuum manifold and apply 10”Hg vacuum for 2 minutes. Collect filtrate and analyze directly. Samples were prepared n=8.

Standard Protein Precipitation: apply 100 µL of plasma to centrifuge vial, followed by 300 µL of 1% formic acid acetonitrile. Agitate on vortex for 1 minute, place into centrifuge for 2 minutes at 15000 rpm. Collect supernatant and analyze directly.

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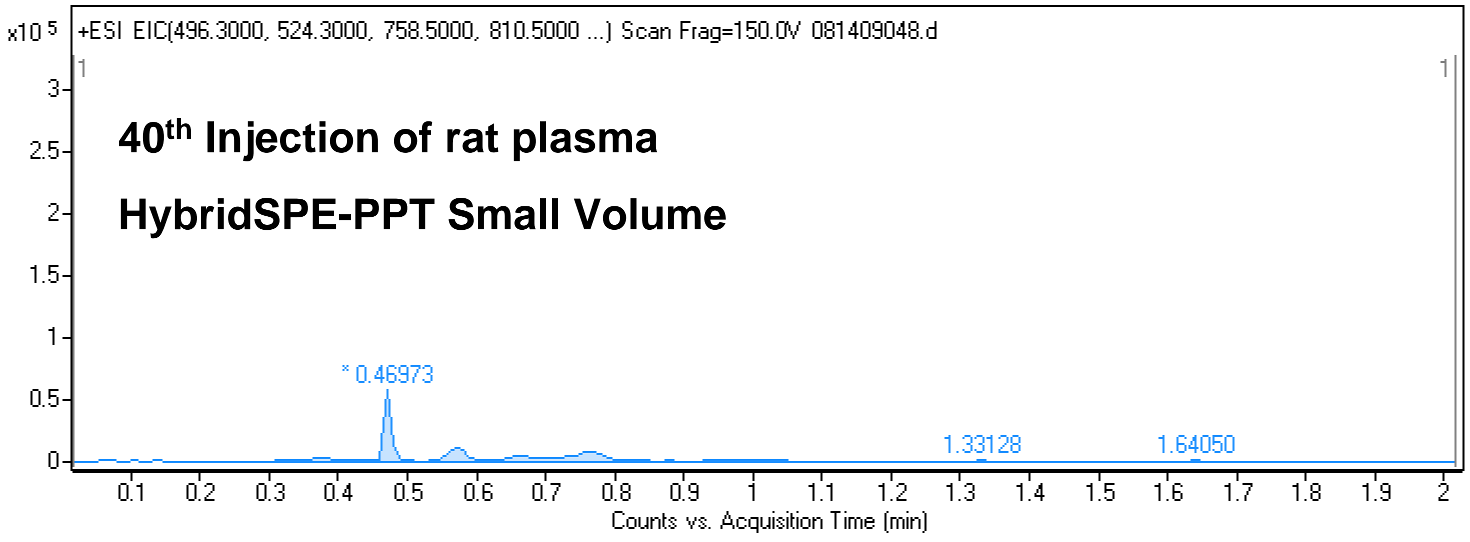
Phosphologies Monitoring Standard Protein Precipitation

Phospholipid Monitoring HybridSPE-PPT Small Volume



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Conclusion

- The HybridSPE 96-well plate combines the benefit of protein precipitation and SPE. It removes both proteins and phospholipids simultaneously. The HybridSPE-Small Volume plate allows processing small biological sample (20 to 30 µL).
- The HybridSPE-Small Volume plate demonstrated excellent recovery of most of compounds across the concentration range along with depletion of proteins and phospholipids from the plasma samples.
- Phospholipid buildup and resulting matrix ionization effect was demonstrated when performing standard protein precipitation techniques.
- The combination of facile protein precipitation/phospholipid depletion and fast analysis using modern chromatographic columns shows great promise in increasing the throughput for bioanalytical methods.

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