

Depletion of Phospholipid Matrix Interference when Dealing with Small Volume Plasma Samples

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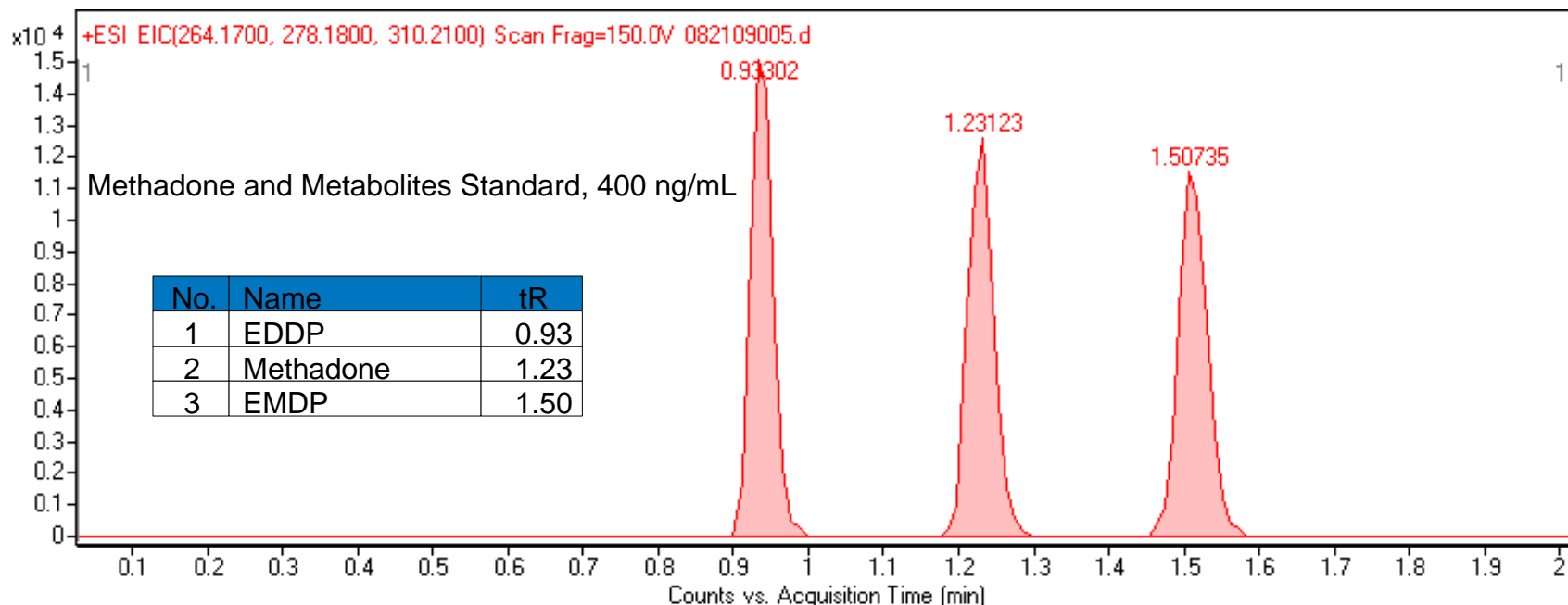
Introduction

- Sample preparation of small volume plasma samples can be restrictive due to the limited volume of available plasma.
- Mouse studies, plasma samples are limited to 30 μL or less.
- Traditional solid phase extraction techniques limited due to large hold up volumes.
- Often limited to liquid-liquid protein precipitation resulting in a high degree of matrix interference.
- Major concern in developing bioanalytical methods is addressing matrix effects.
- Co-extracted interferences directly affect the quantitation of analytes.
- Matrix build up that leads to irregularities in quantitation.
- Gradient elution often required for matrix elution...time consuming.

Experimental

- In this study, comparisons are made between standard protein precipitation methods along with sample preparation by HybridSPE[®]-PPT small volume technique for the recovery of methadone and metabolites from plasma samples along with effective matrix removal.
- Spiked rat plasma samples with methadone and metabolites EDDP and EMDP processed by standard protein precipitation compared against HybridSPE-Small Volume 96-well plate.
- The analysis was conducted on an Agilent 1200SL Rapid Resolution system coupled to an Agilent 6210 TOF LC/MS. Chromatographic separation was performed on the Ascentis[®] Express RP-Amide.
- The high sensitivity of methadone and metabolites enable for direct small volume injection of the processed sample without the need for evaporation or reconstitution.

Figure 1. Chromatographic Conditions



column: Ascentis® Express RP Amide, 10 cm x 2.1 mm I.D., 2.7 µm particles, (53913-U)

mobile phase: 10 mM ammonium formate (65:35 water:acetonitrile) pH 3.6

flow rate: 0.4 mL/min.

system pressure: 275 bar

temp.: 35 °C

det.: ESI+, 1.5 spectra/sec, 6309 transients/spectra

inj.: 0.5 µL

instrument: Agilent 1200SL Rapid Resolution, Agilent 6210 TOF LC/MS

Phospholipid Monitoring

Lysophosphatidylcholines:

m/z

1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine 496.3

1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine 524.3

Glycerophosphocholines:

m/z

1-hexadecanoyl-2-(9Z,12Z-octadecadienoyl)-sn-glycero-3-phosphocholine 758.5

glycerophosphocholine 36:2 786.5

1-(9Z,12Z-octadecadienoyl)-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphocholine 806.5

1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine 810.5

Sample Preparation

Standard Solutions: Standard solutions were prepared from a stock standard in (3:1) 1% formic acid acetonitrile:water at a level of 1, 10, 20, 50, 100, 200, 500 ng/mL.

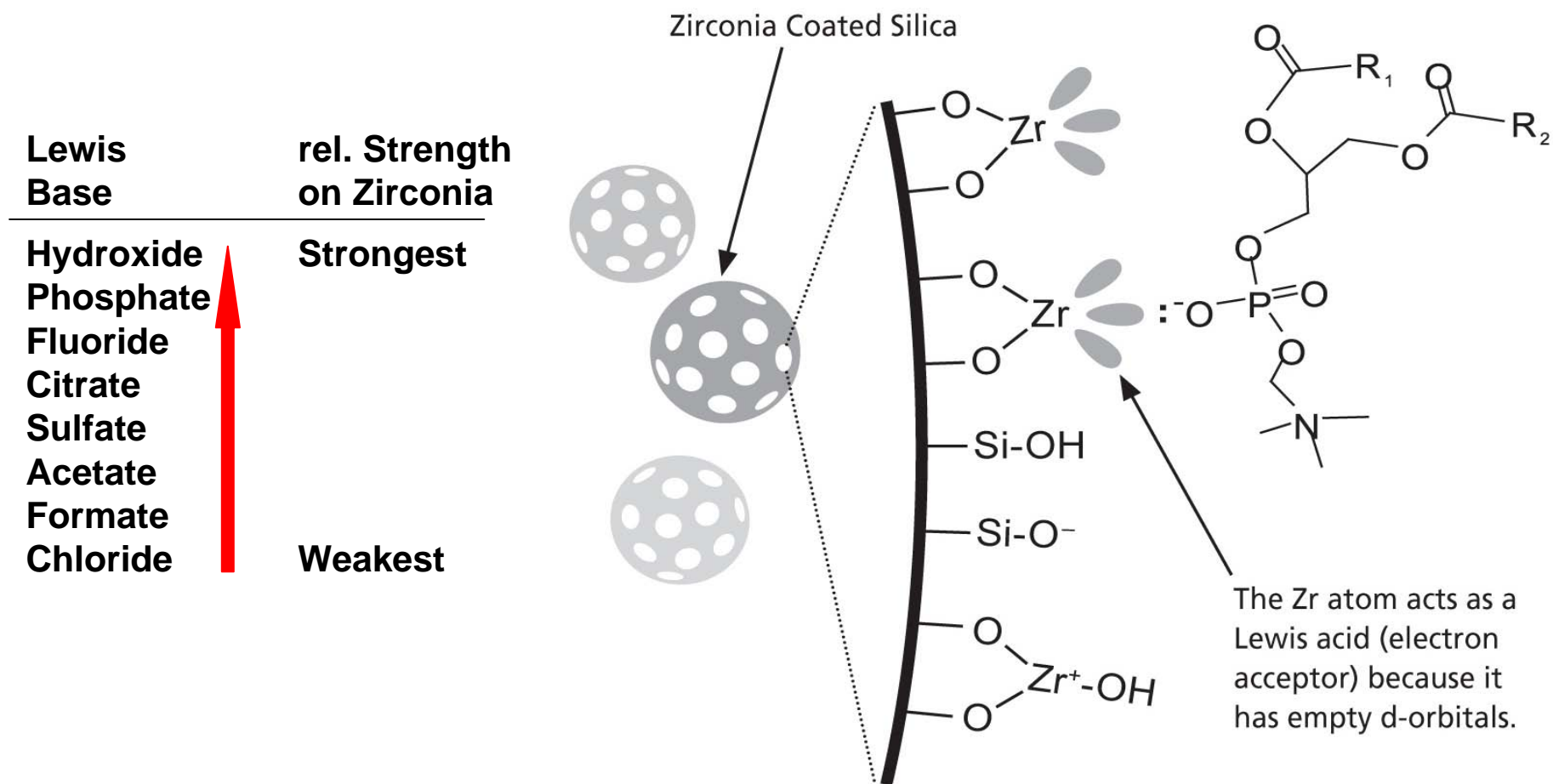
Plasma: Rat plasma stabilized with K₂EDTA was acquired from Lampire Biological Laboratories, (Pipersville PA). Plasma was spiked directly from stock standard to a level of 20, 50, 100, 400, 300 ng/mL.

Standard Protein Precipitation: apply 20 µL of plasma to centrifuge vial, followed by 60 µL of 1% formic acid acetonitrile. Agitate via vortex for 1 minute, place into centrifuge for 2 minutes at 15000 rpm. Collect supernatant and analyze directly.

HybridSPE-small volume Plasma Samples: apply 20 µL of plasma to plate, followed by 60 µL of 1% formic acid acetonitrile. Agitate via vortex for 1 minute, place on vacuum manifold and apply 10”Hg vacuum for 2 minutes. Collect filtrate and analyze directly.

Collection Plate: Eppendorf 150 µL conical with mylar cover.

Figure 2. Lewis Acid Base Strength Scale



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

Figure 3. Crash Solvents and Additives

Precipitated Plasma Sample Composition

- Modifier (1% formic acid) mitigate lewis acid base interaction between chelation/acidic compounds.
- Modifier (formic acid, ammonium formate) acts to disrupt ion-exchange with surface silanols.
- High organic content (75%+) acts as strong solvent when related to hydrophobic retention (acetonitrile, methanol).
- Aqueous content (25%) is strong solvent when related to HILIC retention.

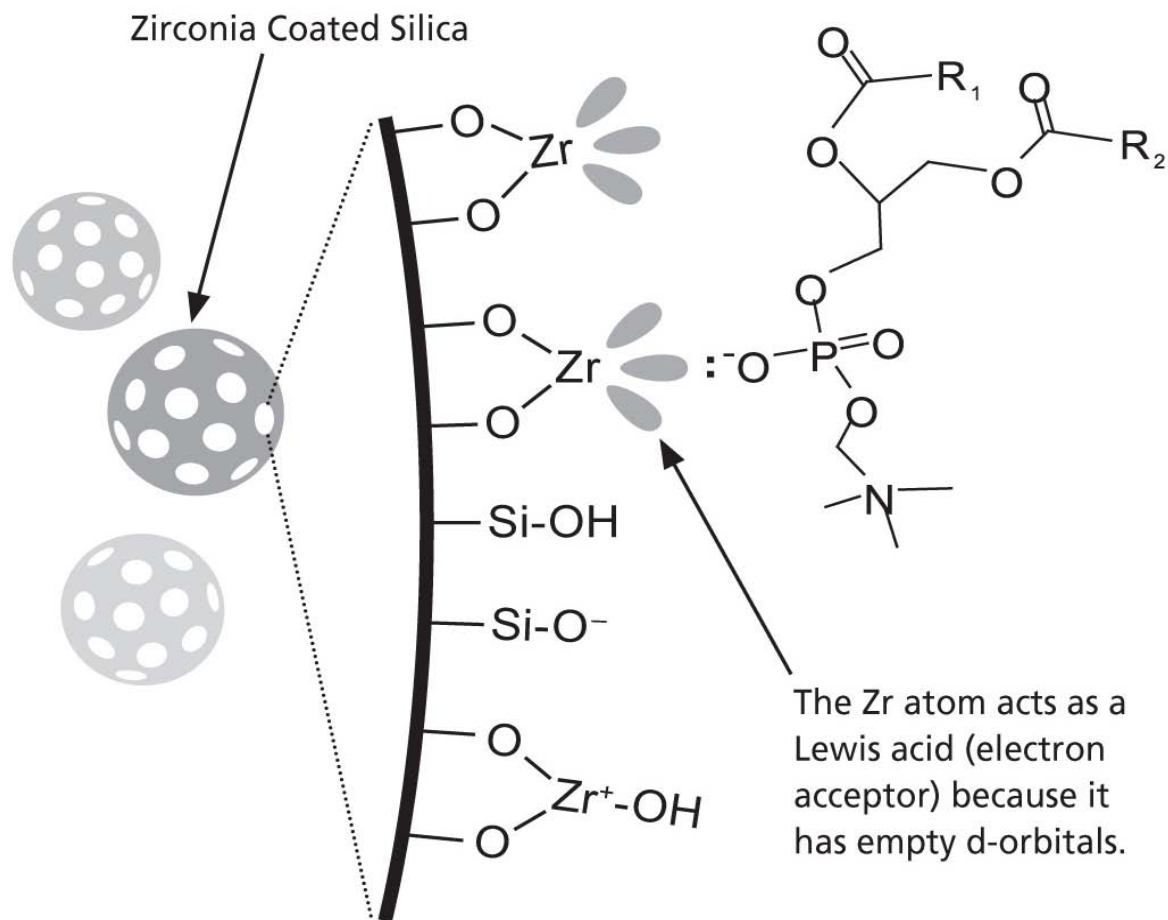
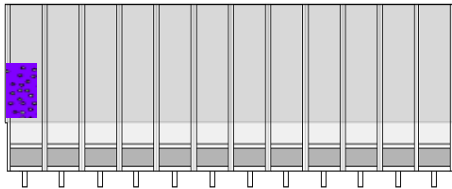
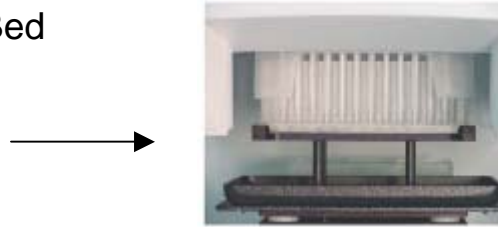


Figure 4. *In-Well* Protein Precipitation Schematic

15 mg Zirconia-Coated Silica Bed



1) **Precipitate Proteins:** Add 20 μL plasma/serum 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.

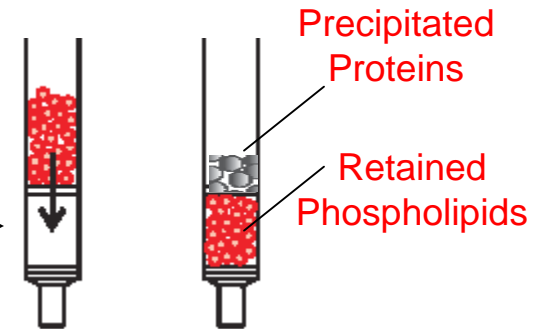


2) **Mix** by vortexing HybridSPE plate or by aspirating/dispensing pipette tip.



Recovery volume 40-50 μL

4) **Resulting filtrate/eluate** is depleted of proteins and phospholipids and ready for immediate LC-MS-MS analysis.



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.

Experimental (contd.)

- Each spiked level sample was prepared n=8 for both sample prep techniques.
- Samples processed using the HybridSPE-Small Volume technique were collected directly into an Agilent low volume 96-well collection plate, average sample volume recovery from the plate was 40 μ L.
- To ensure sufficient sample was drawn up by the injector, the auto sampler was set for bottom well sensing.
- Samples were assayed for content of methadone and metabolites along with matrix monitoring for phospholipids.
- Lyso and glycerophospholipids were monitored as a representative phospholipids matrix ions.

Injection Series

First Series: Calibration Standards

Level 1, 10, 20, 50, 100, 200, 500 ng/mL, n=8 for each level

Second Series: Spiked Plasma Samples HybridSPE

Level of 20, 50, 100, 200, 300 ng/mL, n=8 for each level

Third Series: Spiked Plasma Samples Protein Precipitation

Level of 20, 50, 100, 200, 300 ng/mL, n=8 for each level

Injections made in order of increasing concentration, blank injection performed between sample prep techniques. Severe signal suppression was observed for protein precipitation technique. Conversely, signal response very consistent for samples prepared using HybridSPE technique.

Figure 5. Calibration Table

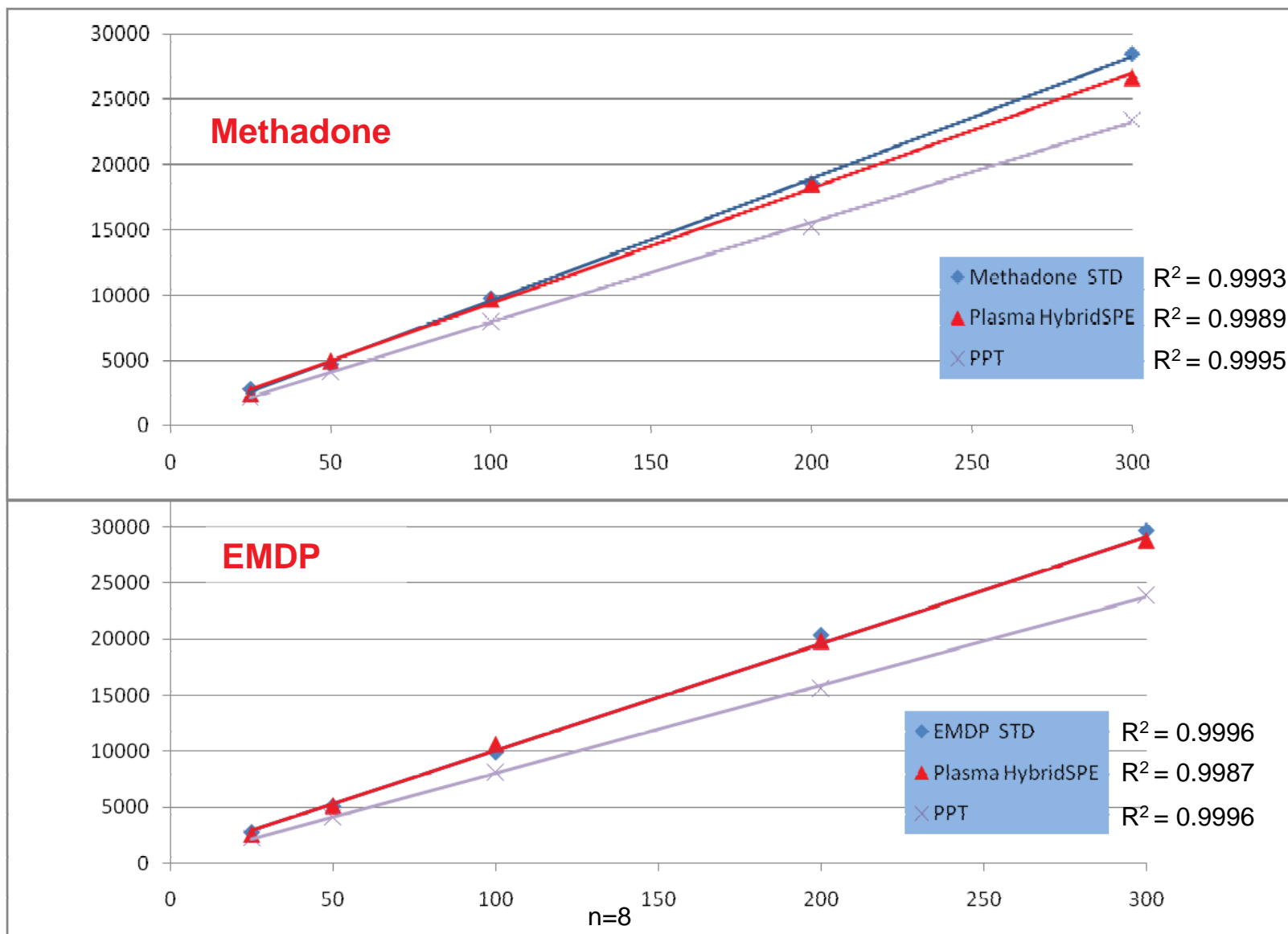


Figure 5. Calibration Table (contd.)

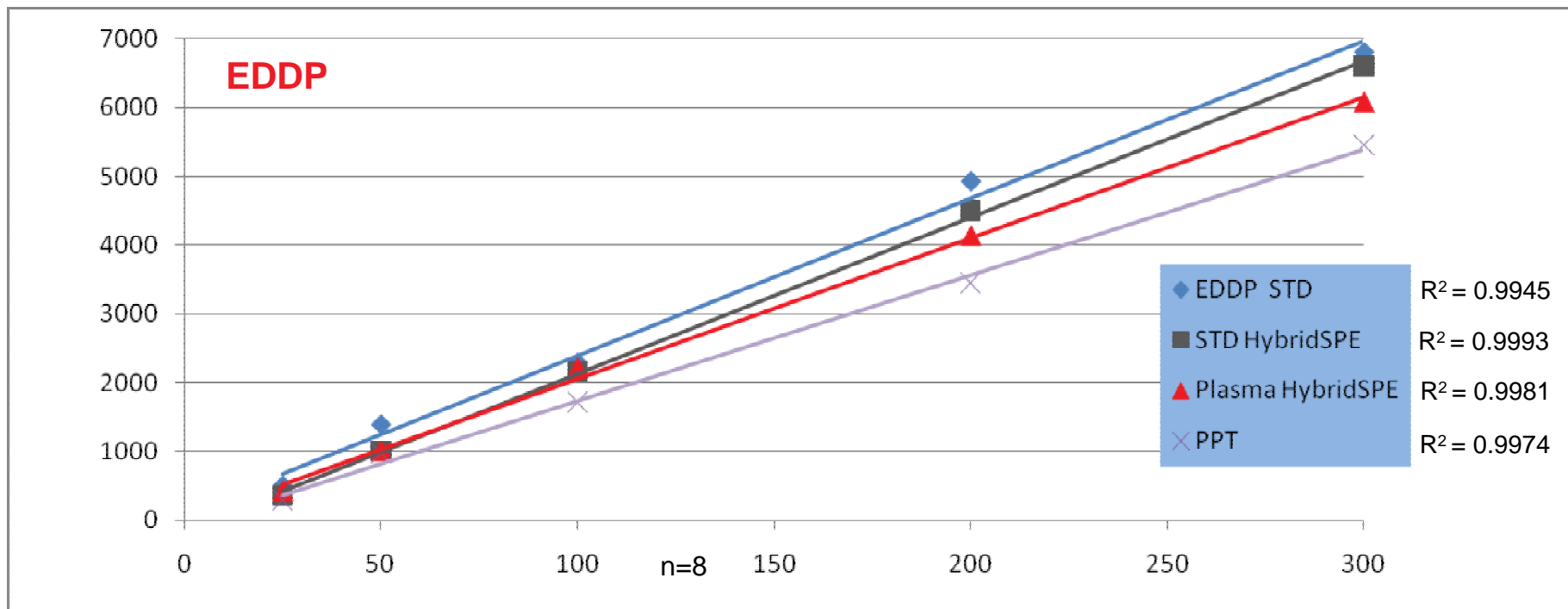


Figure 6. Phospholipid Buildup on Column from Protein Precipitation Method

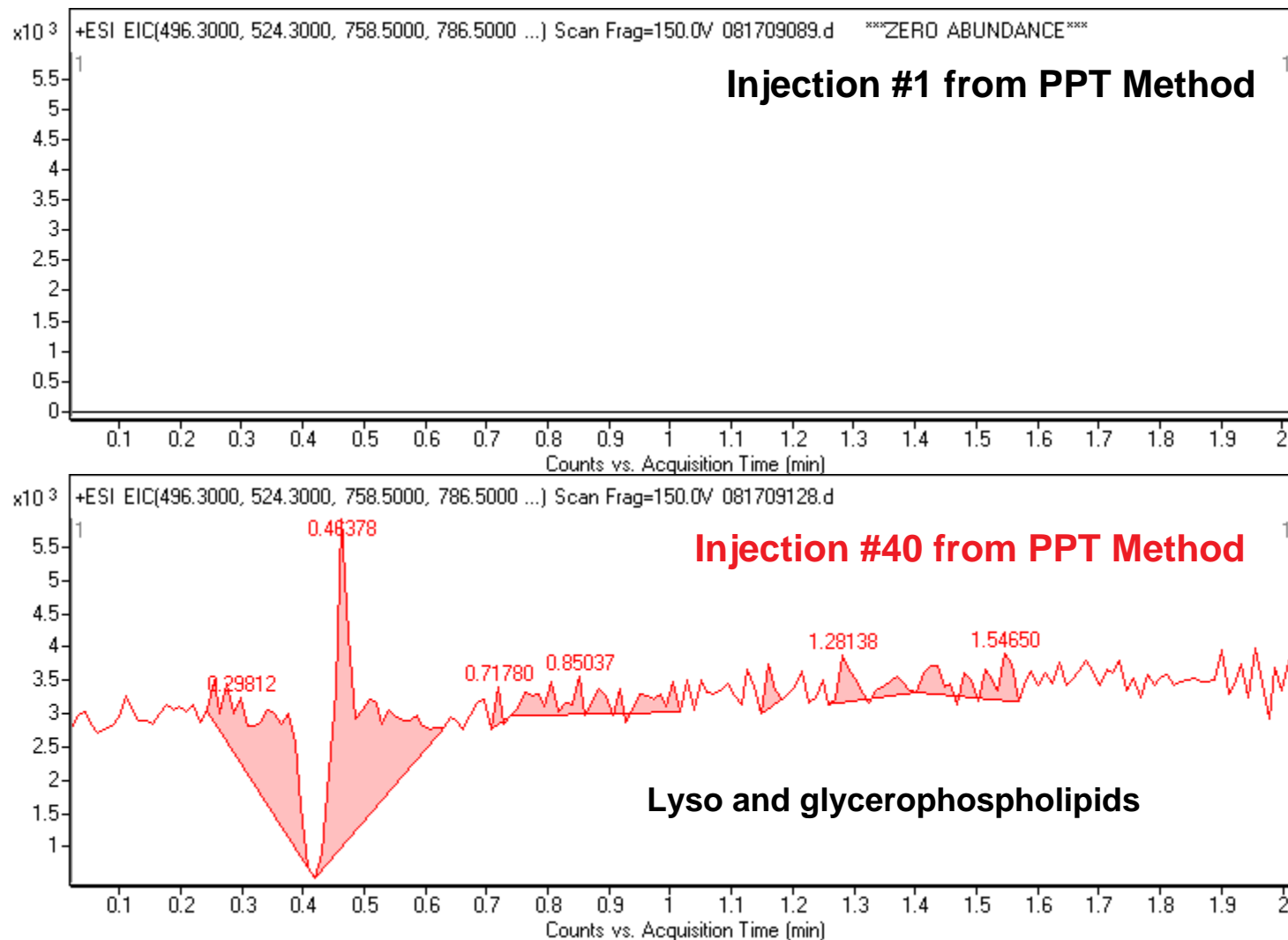
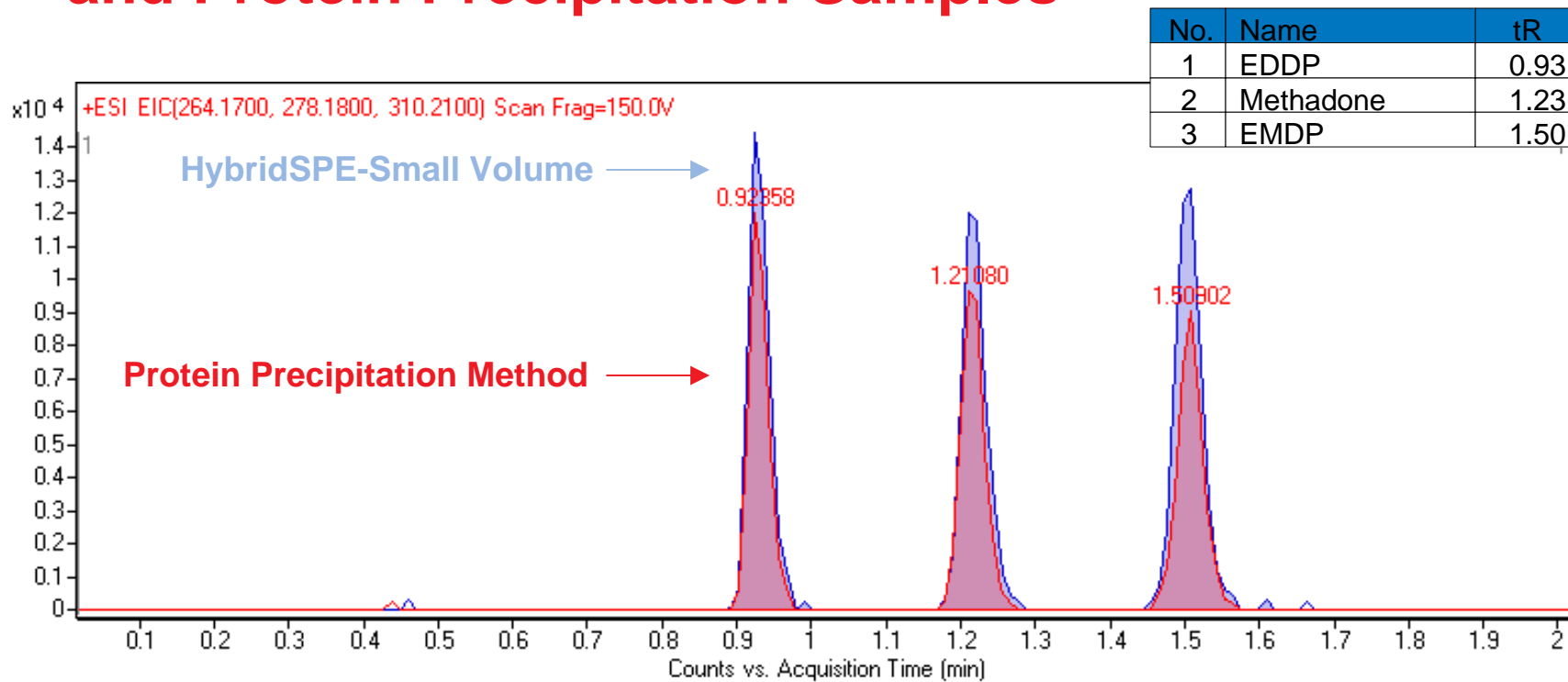


Figure 7. Overlay of HybridSPE-Small Volume and Protein Precipitation Samples



- Signal suppression observed even at highest spike level.
- Dramatic signal reduction over short number of samples for protein precipitation technique. Phospholipid buildup causing signal suppression of analytes from protein precipitation technique.

Long Term Injection Series

Continuation of study, this time set up as long term bioanalytical assay.

- Set up as standard bioanalytical method
- Spiked control samples run every 40 sample injection
- Control spiked at 100 ng/mL level
- Experiment conducted over a 250 injection series
- Control response was plotted
- Comparison between sample prep techniques

Figure 8. Phospholipid Buildup on Column from Protein Precipitation Method

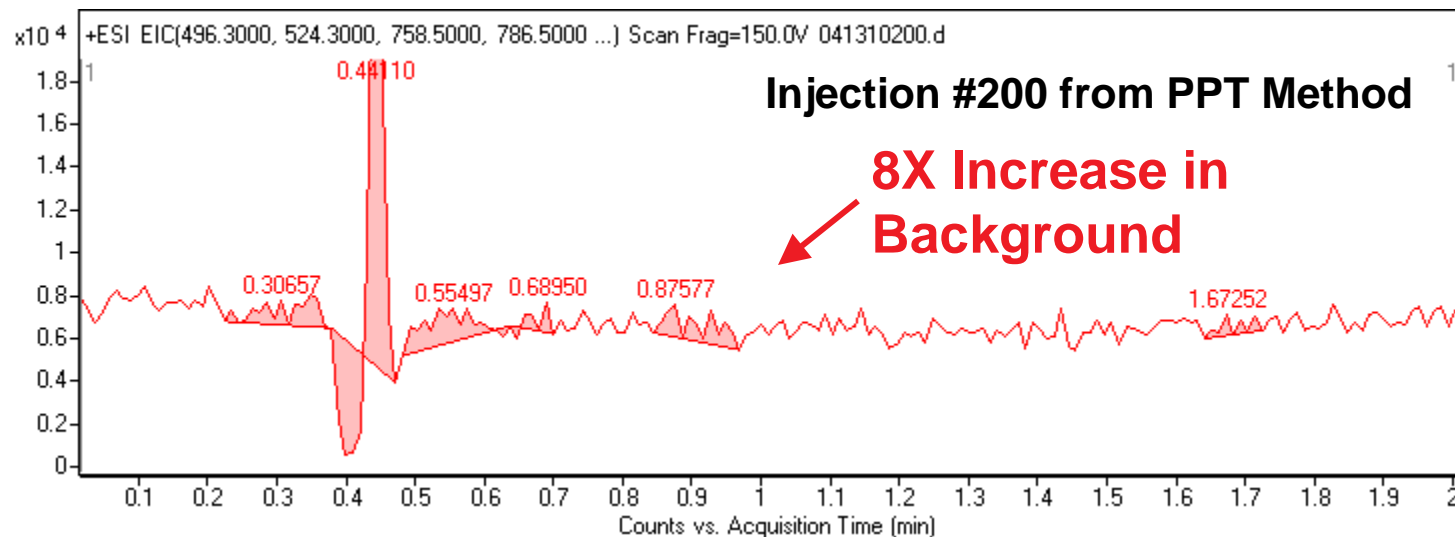
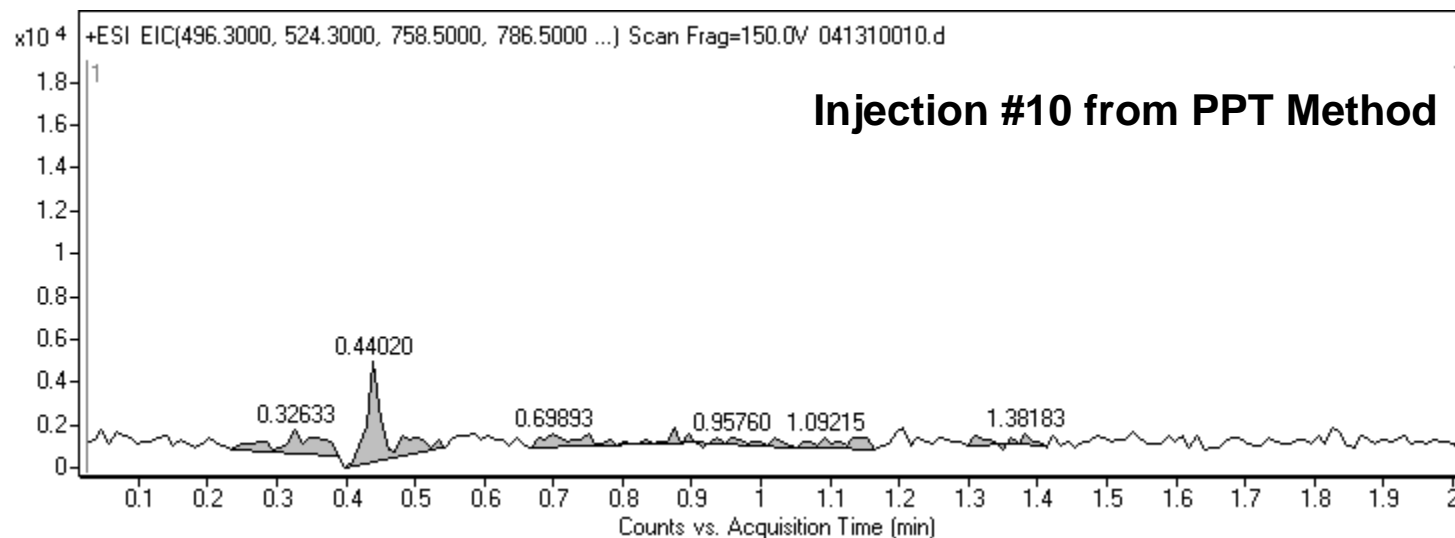


Figure 9. Phospholipid Buildup from HybridSPE

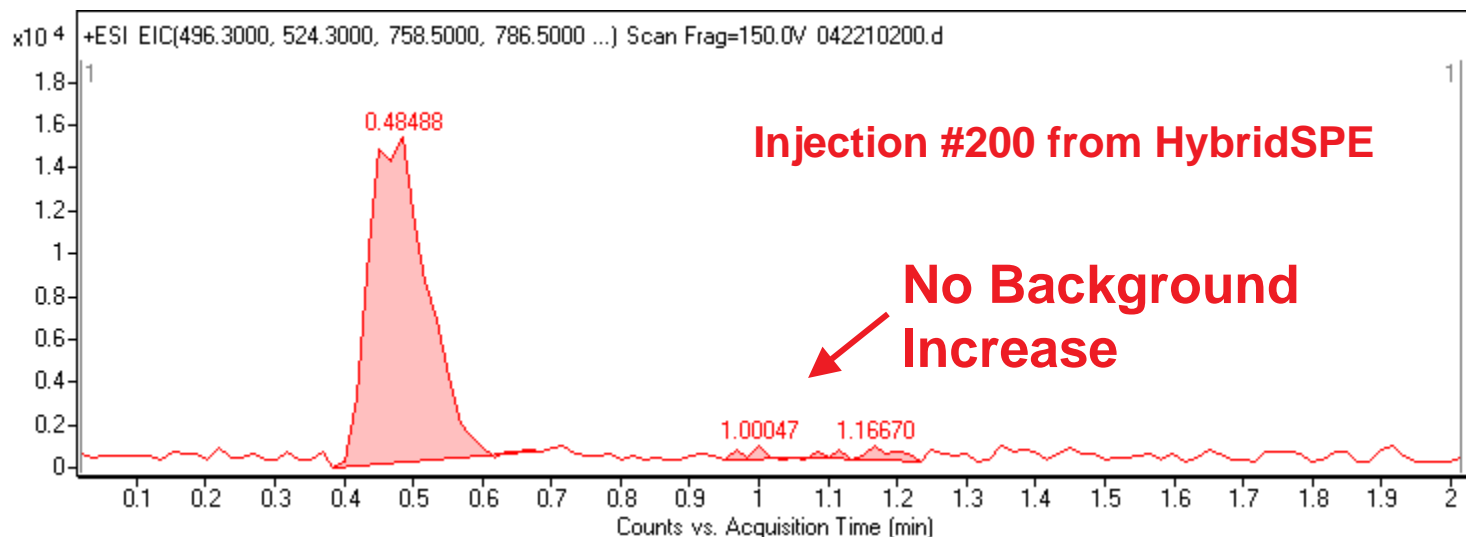
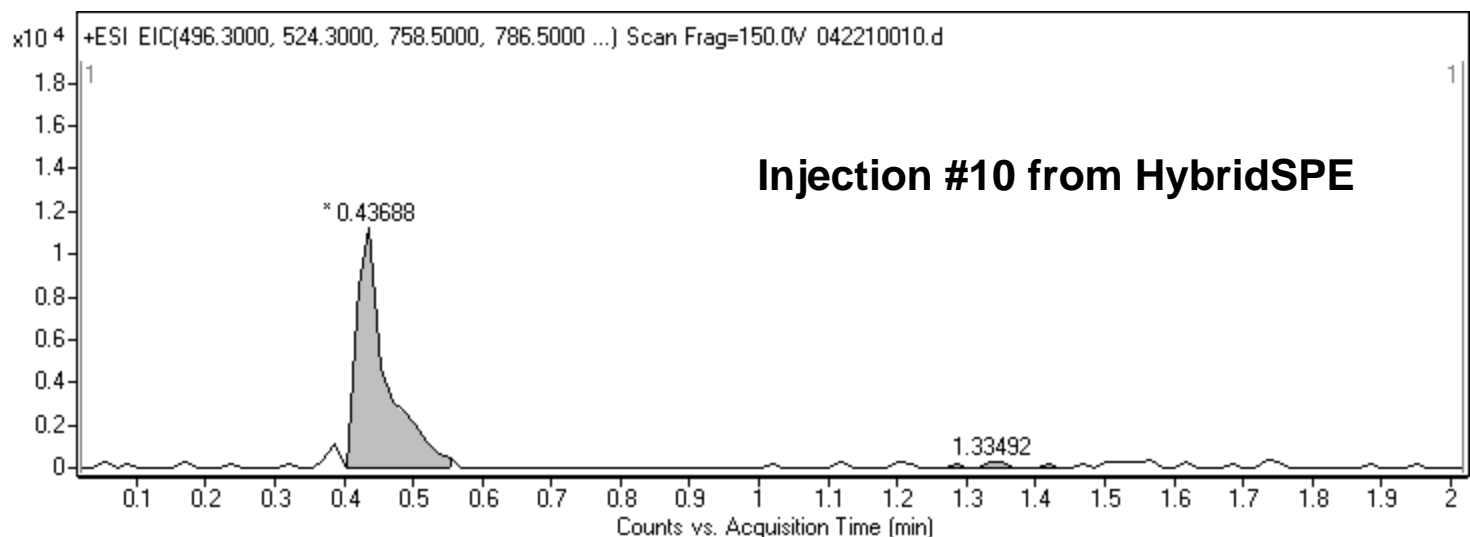
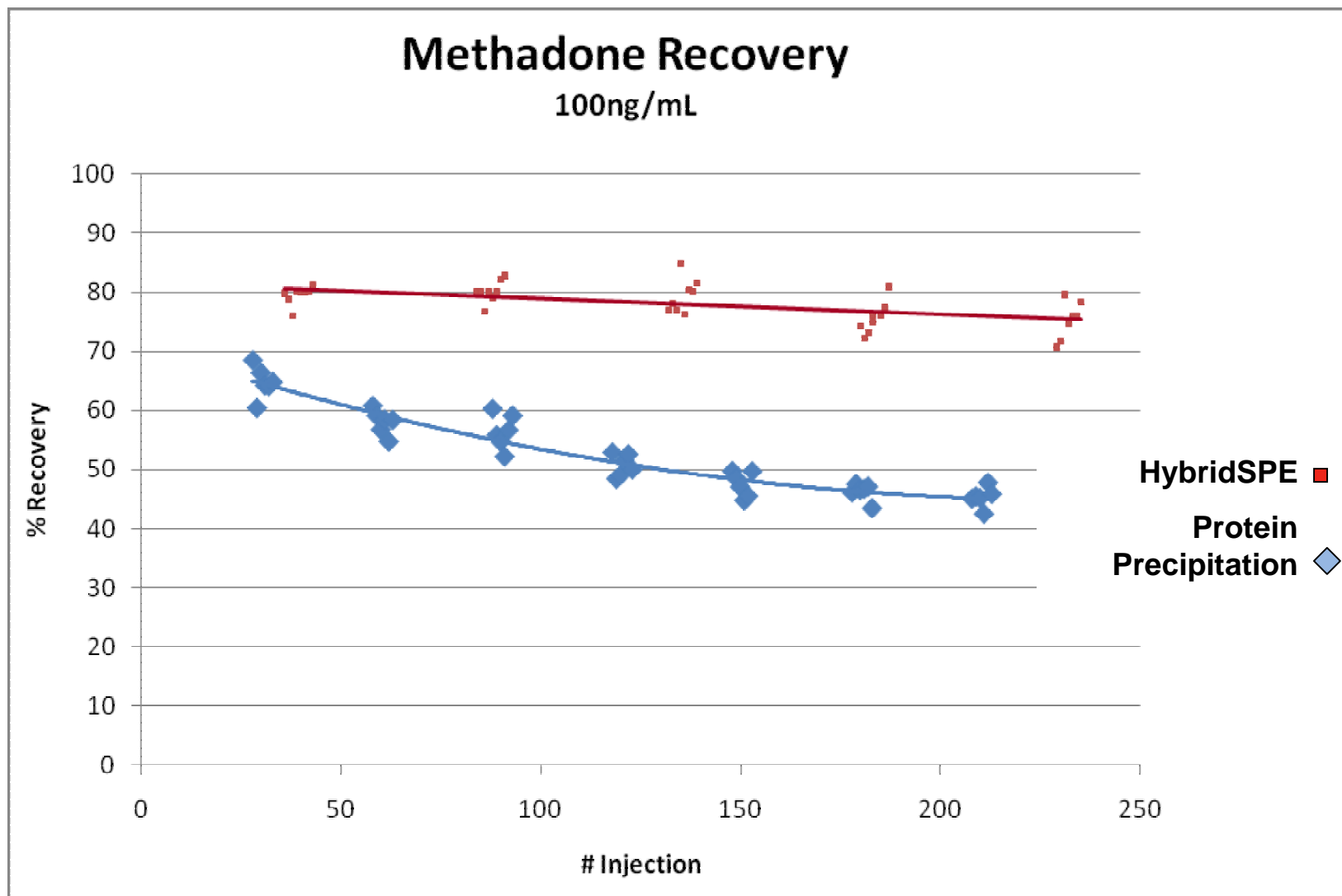


Figure 10. Ionization Effect of Phospholipid Buildup



Conclusions

- Phospholipid buildup and resulting matrix ionization effect was demonstrated when performing standard protein precipitation techniques.
- HybridSPE-Small Volume plate demonstrated high recovery of methadone and associated metabolites across the concentration range along with depletion of proteins and phospholipids from the plasma samples.
- Unique approach to processing small volume samples enable a high degree of matrix removal when dealing with minimal plasma volumes.
- Utilizing the zirconia-Si particles as a sample preparation media, phospholipid matrix interference was selectively bound to the particle via Lewis acid/base interaction resulting in high degree of phospholipid removal.
- Consideration towards sample matrix... fast chromatographic separation do not always translate to fast bioanalytical methods.