Increased Bioanalytical Throughput Utilizing Fused-Core™ Particles with Selective Phospholipid Depletion

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Introduction

Often the major concern in developing bioanalytical methods is addressing the affect of biofluid matrix on the detection of desired analytes. The impact of matrix affects in bioanalysis has been well documented (1,2). In the majority of cases, co-extracted interferences directly affect the quantitation of analytes due to ionization affects induced by the extracted matrix. This extracted matrix can impact the concurrent chromatographic analysis, but more often is a result of chromatographic buildup that leads to irregularities in both retention and quantitation (5,6). To address these issues, organic gradient elution is often utilized to 'wash' adsorbed contaminants from the column. In most cases gradient elution is not required for resolution of desired analytes, but instead required only to elute extracted matrix from the analytical column.

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Performing a more thorough sample cleanup enables faster chromatographic analysis and thus increases the overall sample throughput. Using the HybridSPE™ platform for selective phospholipid depletion eliminates the need for gradient elution of adsorbed matrix from the analytical column, resulting in the ability to perform isocratic chromato-graphic separation with dramatic increase in throughput. Utilizing the Fused-Core particle technology with this novel sample prep technique enables faster analysis time due to high chromatographic efficiency.

This study evaluates the performance of the newly developed HybridSPE Small Volume 96-well plate for preparation of small volumes of rat plasma samples. The HybridSPE Small Volume plate accommodates plasma volumes of 20-40 µL, ideally suited for bioanalytical testing of mouse plasma. The HybridSPE Small Volume plate makes use of the zirconia-coated silica stationary phase; same as used in the standard HybridSPE plate for phospholipid depletion. The HybridSPE Small Volume plate is a scaled down version with a 1 mL well volume and a 15 mg packed stationary bed. A 0.45 µm polishing filter is used for fine particle removal.

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Introduction (contd.)

In this study, rat plasma samples spiked with methadone and metabolites EDDP and EMDP were processed with the HybridSPE Small Volume 96-well plate and compared against standard protein precipitation methods. 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.

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Figure 1. HybridSPE Small Volume 96-well Schematic Diagram

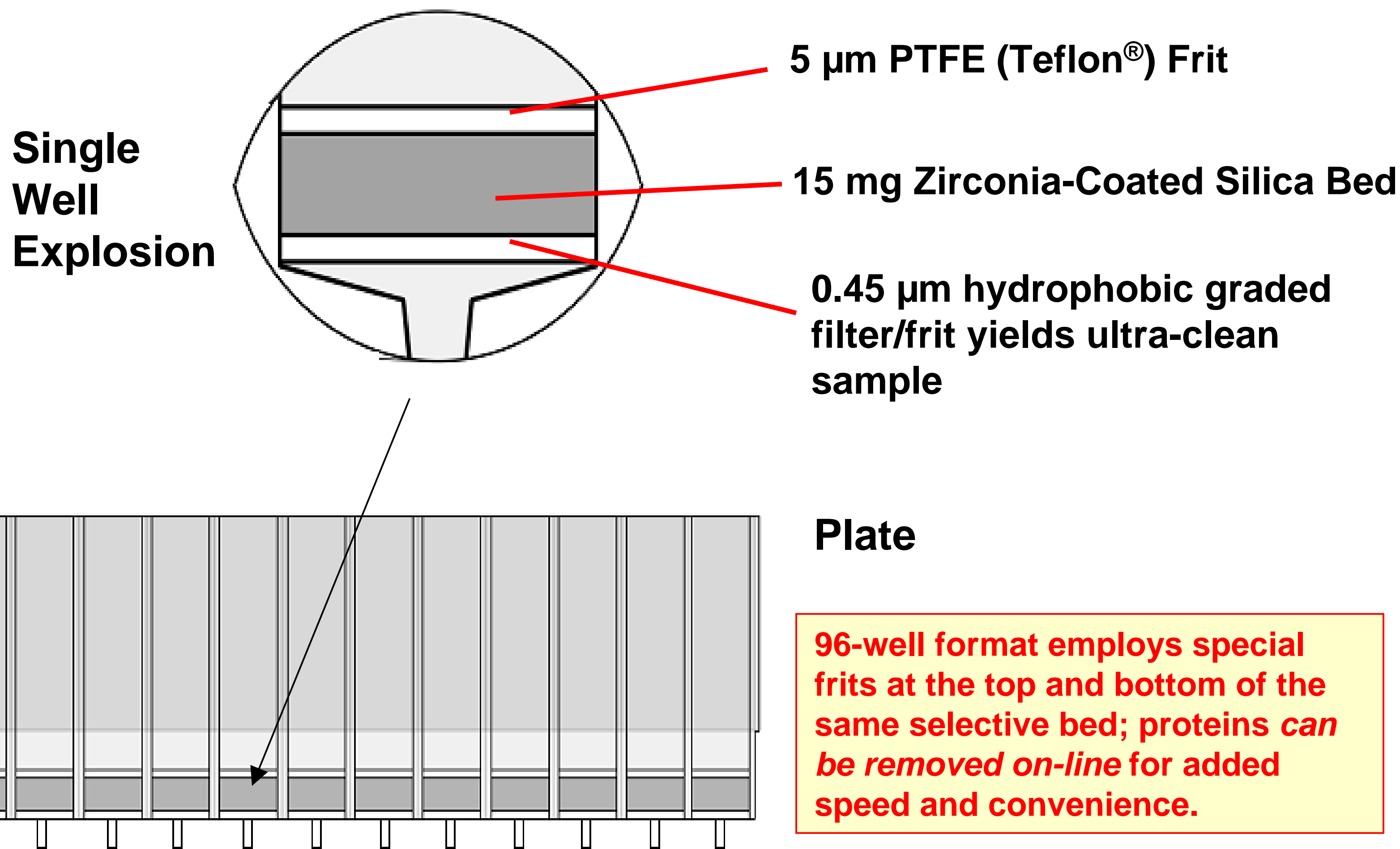

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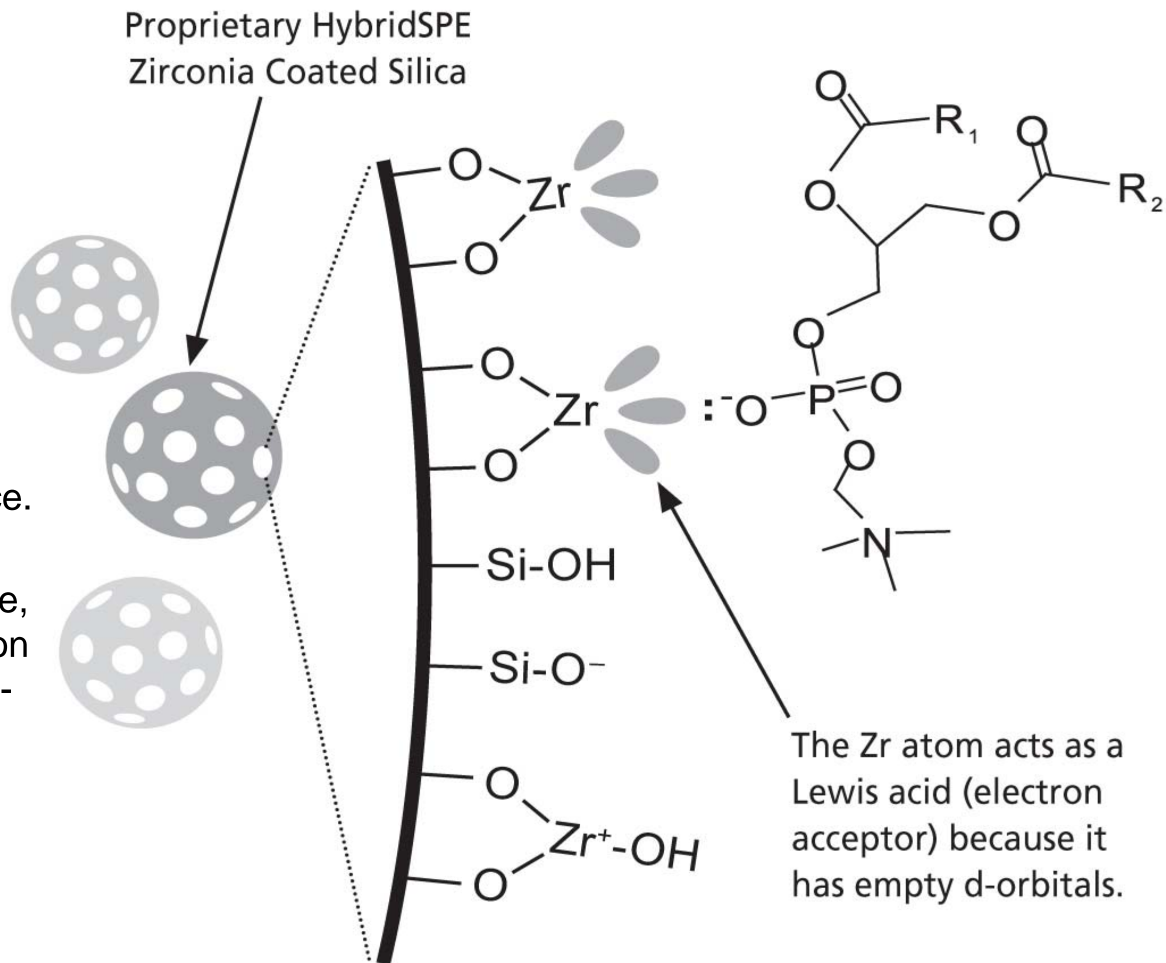
Figure 2. Phospholipid Interaction with Zirconia-Coated Particle

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.



Interaction between a representative phospholipid and the zirconium surface of the HybridSPE-PPT particle via Lewis acid-base interaction (4).

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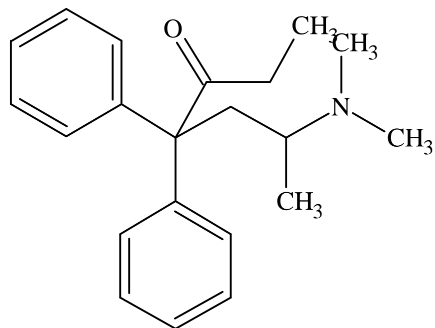
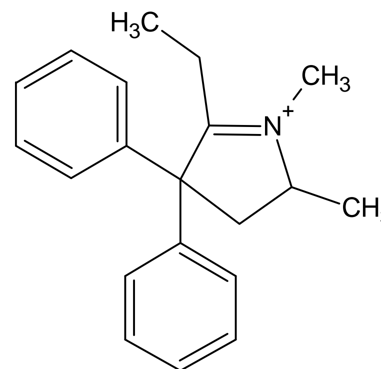
Figure 3. Structures of Methadone and Metabolites



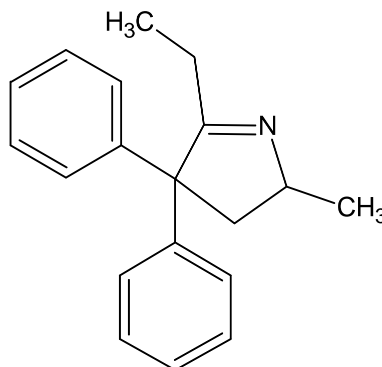
Methadone

Monoisotopic Mass = 309.209264 Da



Monoisotopic Mass = 278.190326 Da

EDDP



Monoisotopic Mass = 263.1674 Da

EMDP

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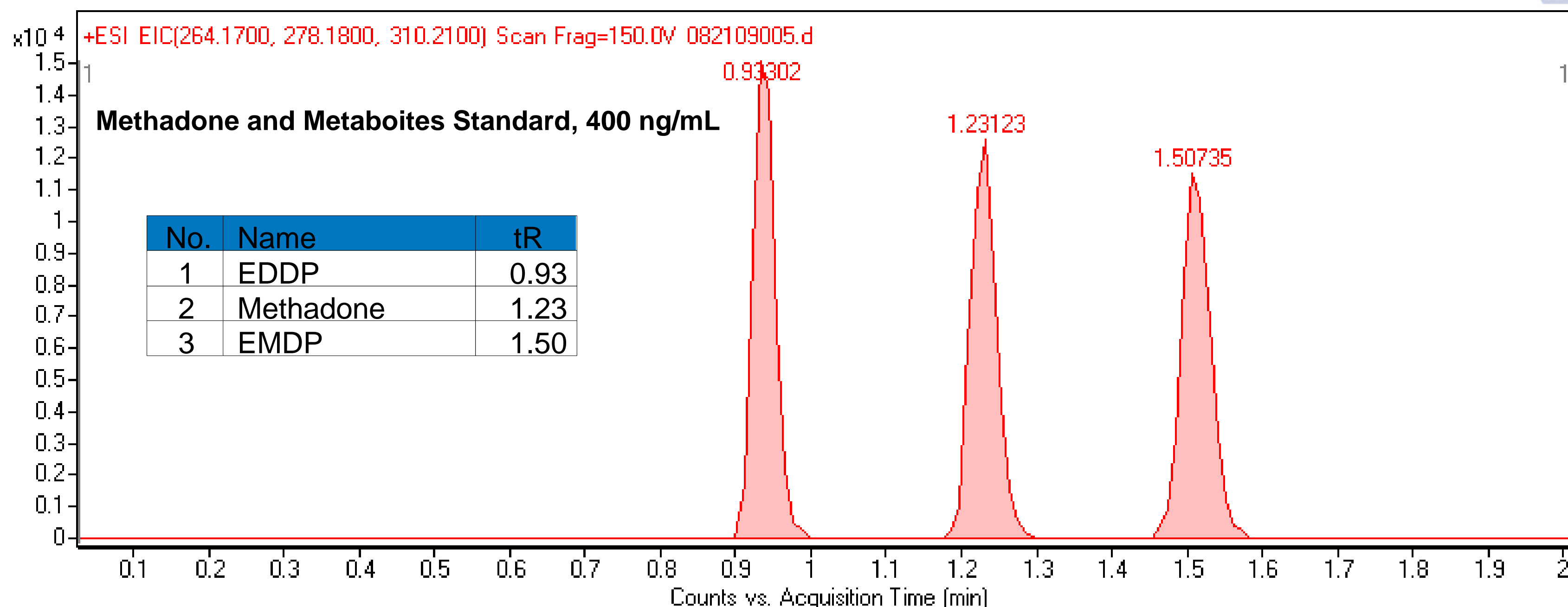
Figure 4. Chromatographic Conditions



No.	Name	tR
1	EDDP	0.93
2	Methadone	1.23
3	EMDP	1.50

column:	Ascentis Express RP-Amide, 10 cm x 2.1 mm, 2.7 µm, (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+
injection:	0.5 µL
instrument:	Agilent 1200SL Rapid Resolution, Agilent 6210 TOF LC/MS
sampling rate:	1.5 spectra/sec, 6309 transients/spectra

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Sample Preparation

Standard Solutions: Standard solutions were prepared from a stock standard in (3:1) 1% formic acid acetonitrile:water at a level of 25, 50, 100, 200, 300 ng/mL. These standards were used to establish recovery of methadone and metabolites from the HybridSPE-Small Volume without interference from plasma interactions.

Plasma: Rat plasma stabilized with K_2EDTA was acquired from Lampire Biological Laboratories, (Pipersville PA). Plasma was spiked directly from stock standard to a level of 100, 200, 400, 800, 1200 ng/mL.

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.

HybridSPE Small Volume Standard Solution: apply 80 µL of standard prepared in (3:1) 1% formic acid acetonitrile:water. Agitate via 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 via vortex for 1 minute, place into centrifuge for 2 minutes at 15000 rpm. Collect supernatant and analyze directly.

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Figure 5. HybridSPE Small Volume, Sample Layout

25 STD	50 STD	100 STD	200 STD	300 STD	Spiked Plasma 100	Spiked Plasma 200	Spiked Plasma 400	Spiked Plasma 600	Spiked Plasma 1200		
100 STD	50 STD	100 STD	200 STD	300 STD	Spiked Plasma 100	Spiked Plasma 200	Spiked Plasma 400	Spiked Plasma 600	Spiked Plasma 1200		
100 STD	50 STD	100 STD	200 STD	300 STD	Spiked Plasma 100	Spiked Plasma 200	Spiked Plasma 400	Spiked Plasma 600	Spiked Plasma 1200		
100 STD	50 STD	100 STD	200 STD	300 STD	Spiked Plasma 100	Spiked Plasma 200	Spiked Plasma 400	Spiked Plasma 600	Spiked Plasma 1200		
100 STD	50 STD	100 STD	200 STD	300 STD	Spiked Plasma 100	Spiked Plasma 200	Spiked Plasma 400	Spiked Plasma 600	Spiked Plasma 1200		
100 STD	50 STD	100 STD	200 STD	300 STD	Spiked Plasma 100	Spiked Plasma 200	Spiked Plasma 400	Spiked Plasma 600	Spiked Plasma 1200		
100 STD	50 STD	100 STD	200 STD	300 STD	Spiked Plasma 100	Spiked Plasma 200	Spiked Plasma 400	Spiked Plasma 600	Spiked Plasma 1200		
100 STD	50 STD	100 STD	200 STD	300 STD	Spiked Plasma 100	Spiked Plasma 200	Spiked Plasma 400	Spiked Plasma 600	Spiked Plasma 1200		

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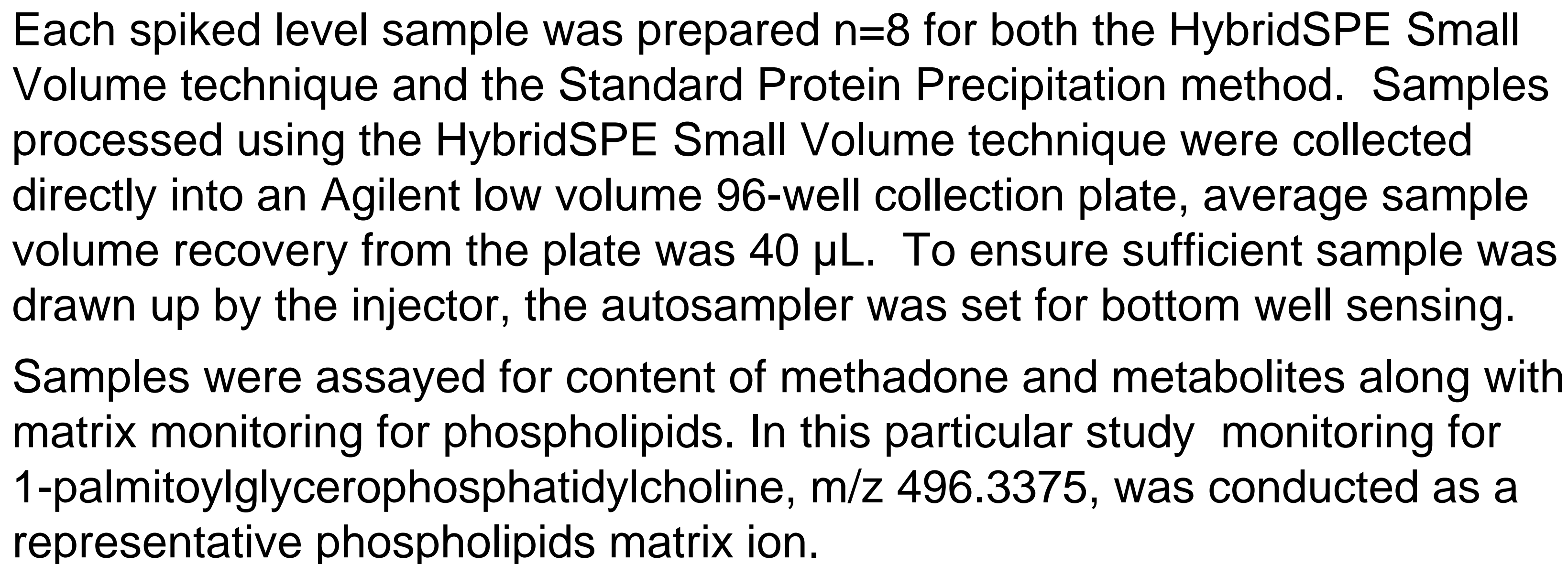

Each spiked level sample was prepared n=8 for both the HybridSPE Small Volume technique and the Standard Protein Precipitation method. Samples processed using the HybridSPE Small Volume technique were collected directly into an Agilent low volume 96-well collection plate, average sample volume recovery from the plate was 40 µL. To ensure sufficient sample was drawn up by the injector, the autosampler was set for bottom well sensing.

Samples were assayed for content of methadone and metabolites along with matrix monitoring for phospholipids. In this particular study monitoring for 1-palmitoylglycerophosphatidylcholine, m/z 496.3375, was conducted as a representative phospholipids matrix ion.

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Figure 6. Calibration Table: Methadone

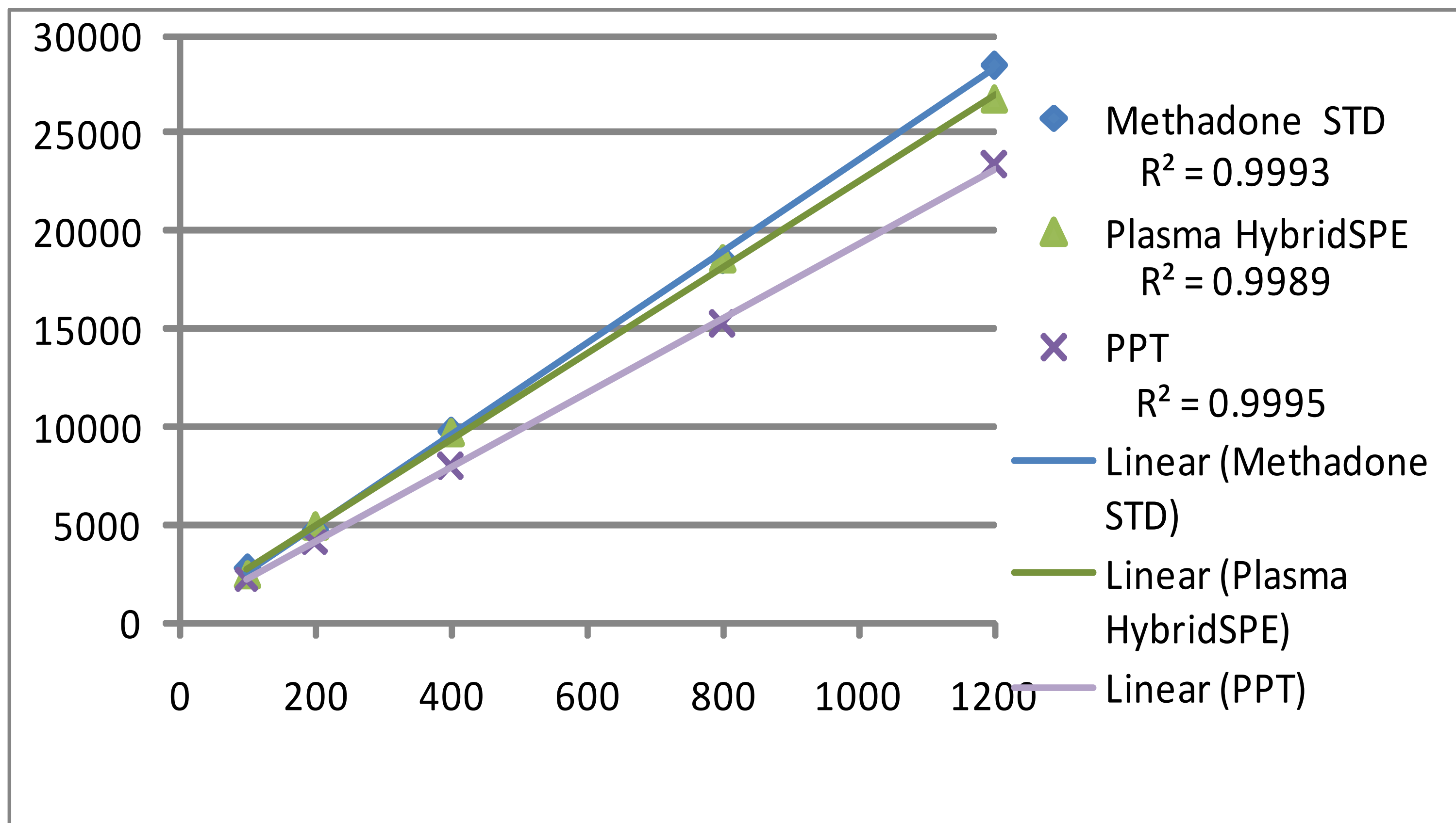

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Figure 7. Calibration Table: EMDP

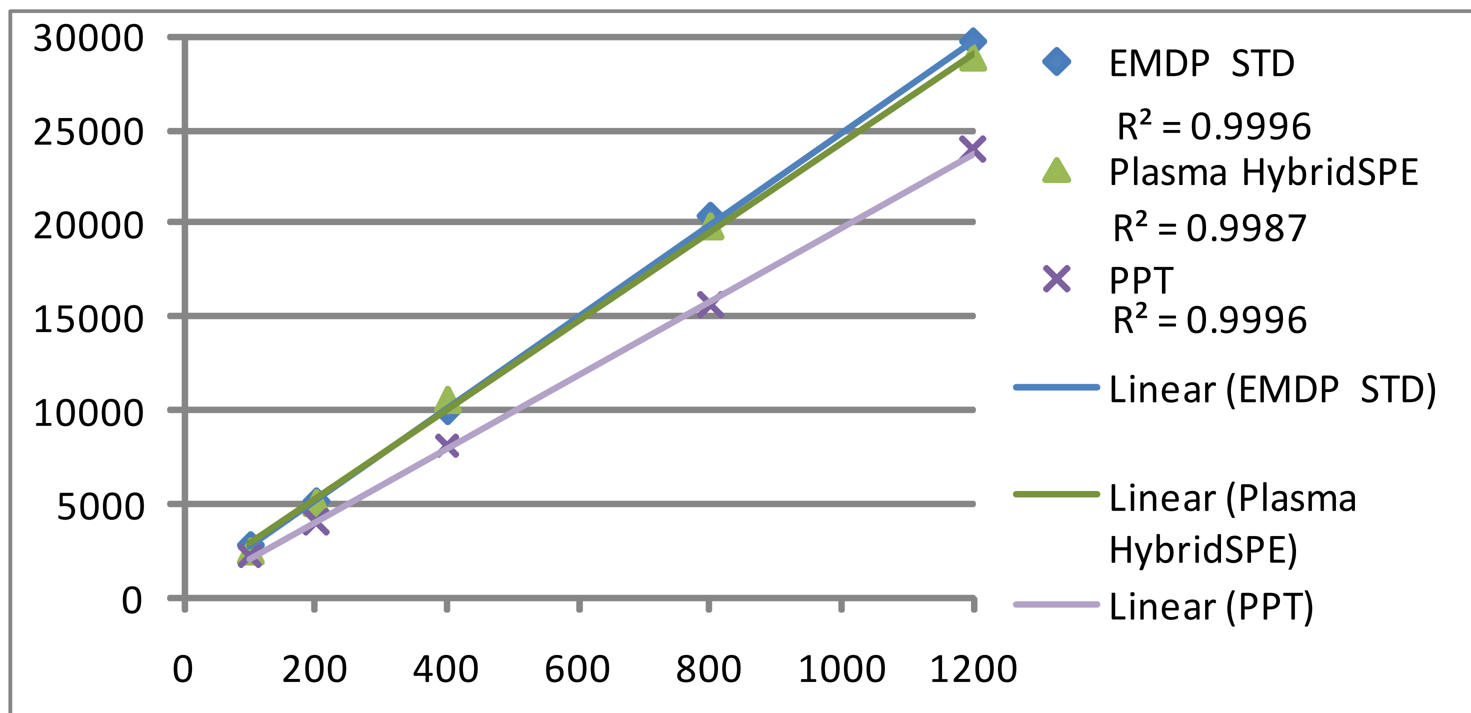

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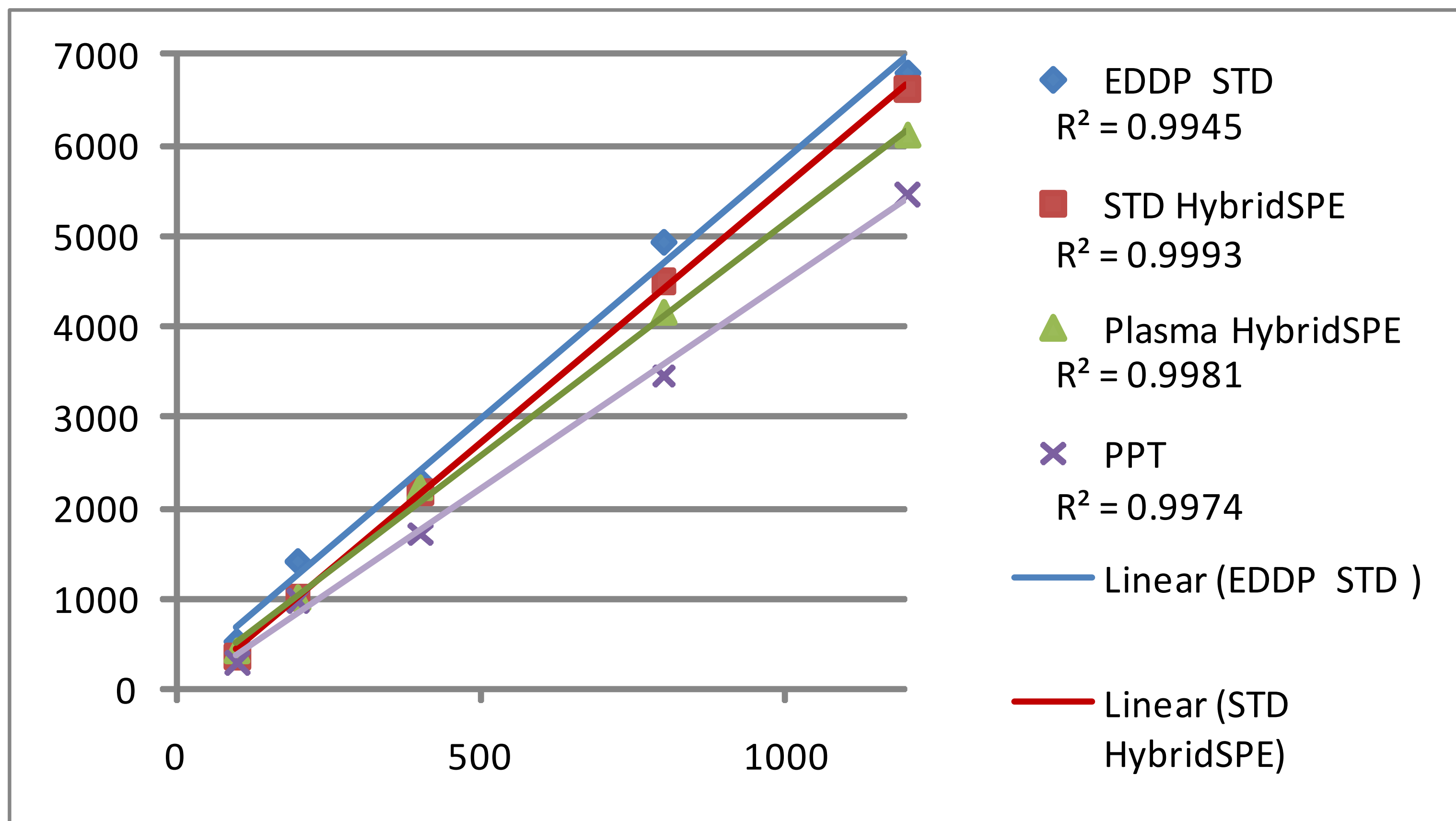
Figure 8. Calibration Table: EDDP



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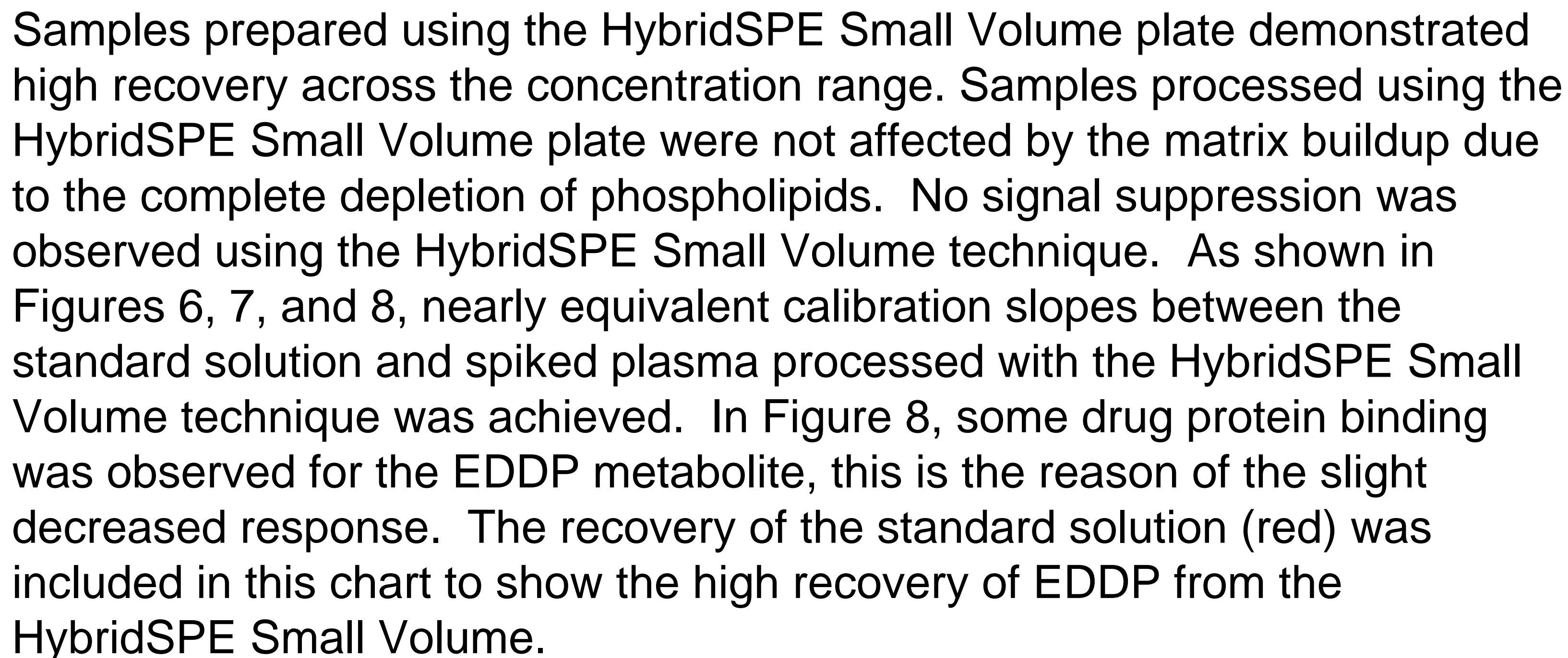

Samples prepared using the HybridSPE Small Volume plate demonstrated high recovery across the concentration range. Samples processed using the HybridSPE Small Volume plate were not affected by the matrix buildup due to the complete depletion of phospholipids. No signal suppression was observed using the HybridSPE Small Volume technique. As shown in Figures 6, 7, and 8, nearly equivalent calibration slopes between the standard solution and spiked plasma processed with the HybridSPE Small Volume technique was achieved. In Figure 8, some drug protein binding was observed for the EDDP metabolite, this is the reason of the slight decreased response. The recovery of the standard solution (red) was included in this chart to show the high recovery of EDDP from the HybridSPE Small Volume.

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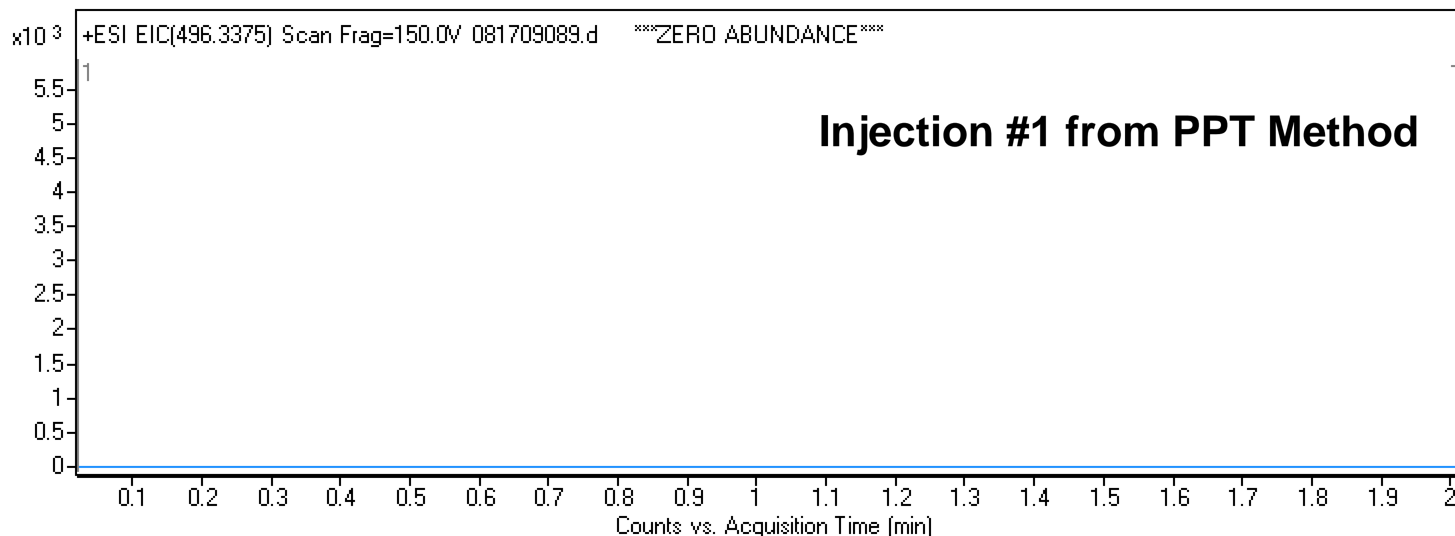
Figure 9. Phospholipid Buildup on Column from Protein Precipitation Method



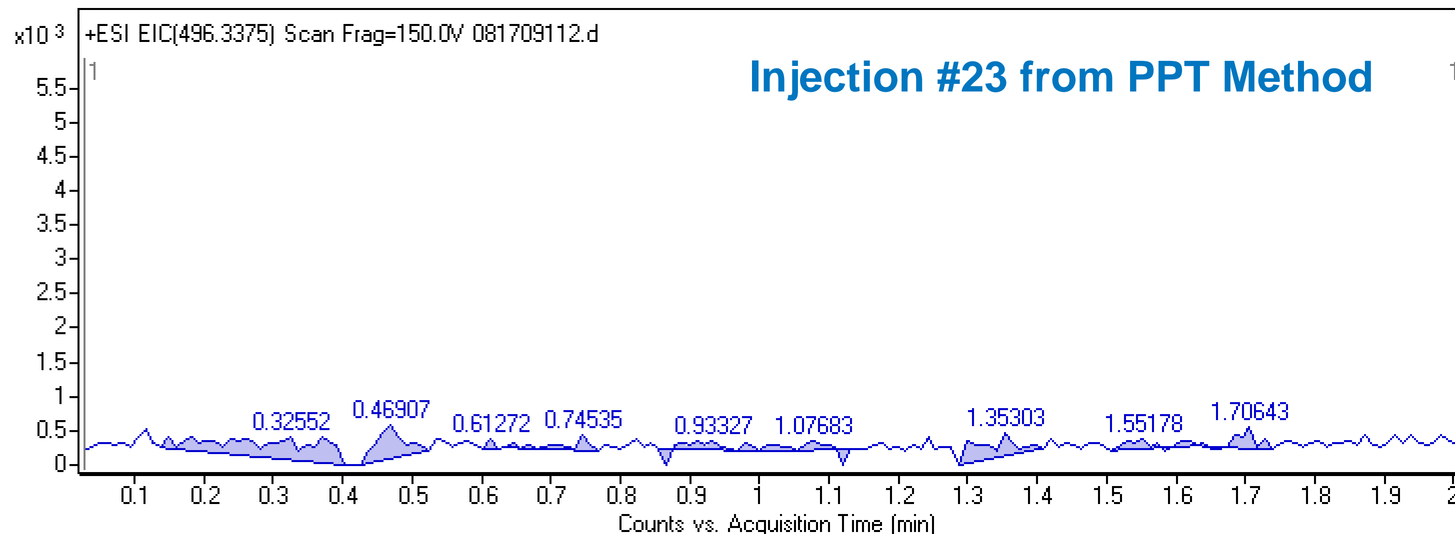


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Figure 9. Phospholipid Buildup on Column from Protein Precipitation Method (contd.)

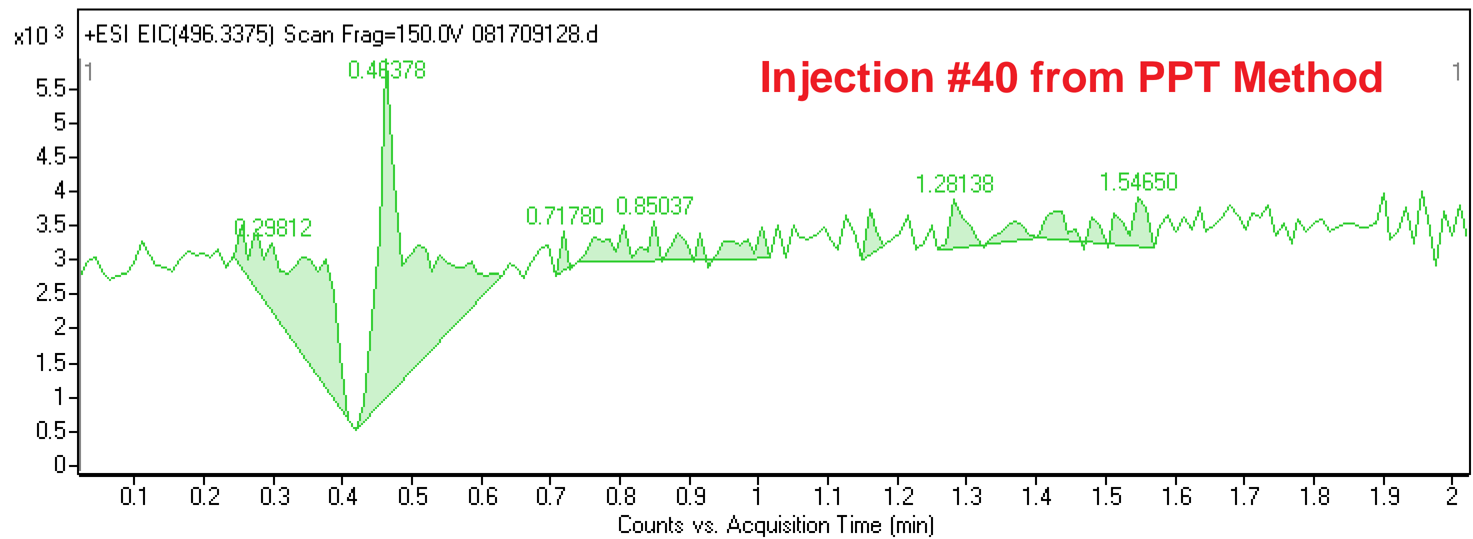

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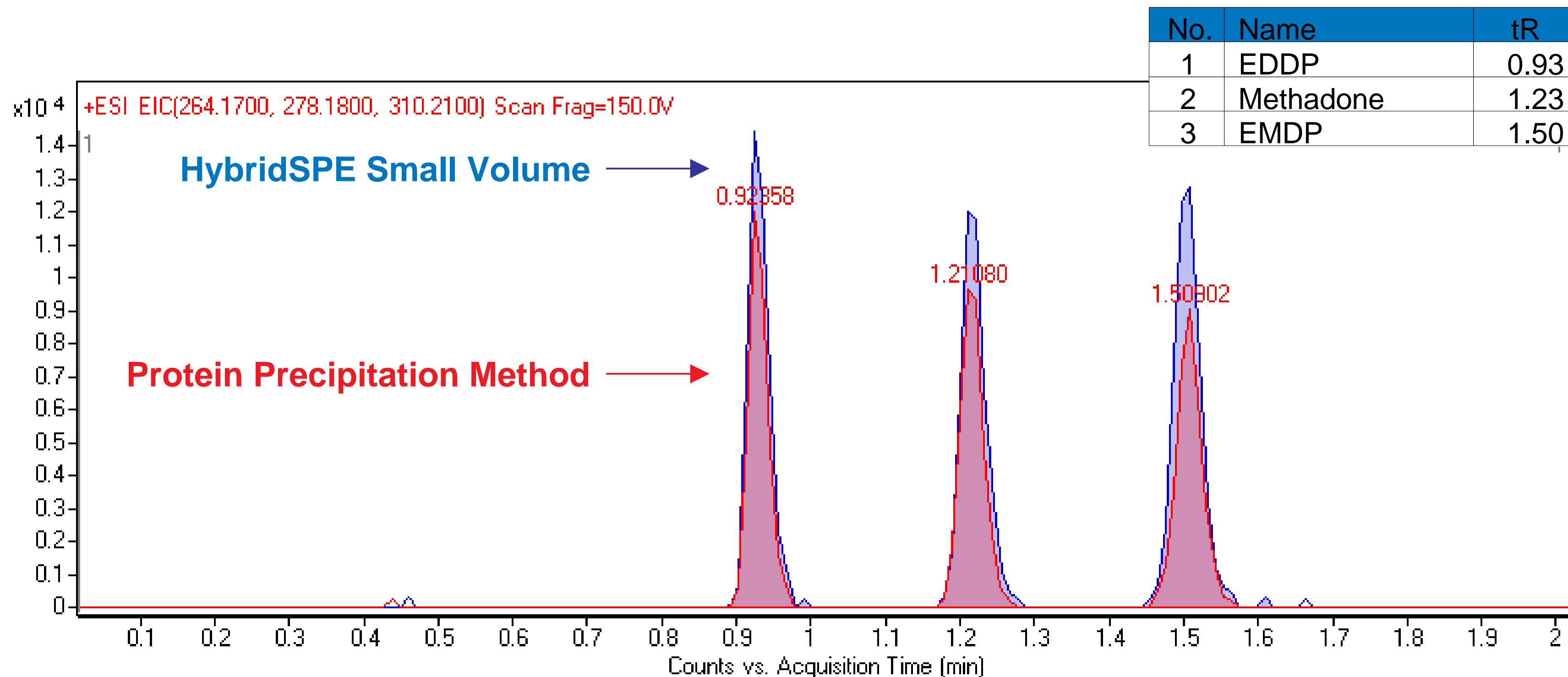
Figure 10. Overlay of HybridSPE Small Volume and Protein Precipitation Samples



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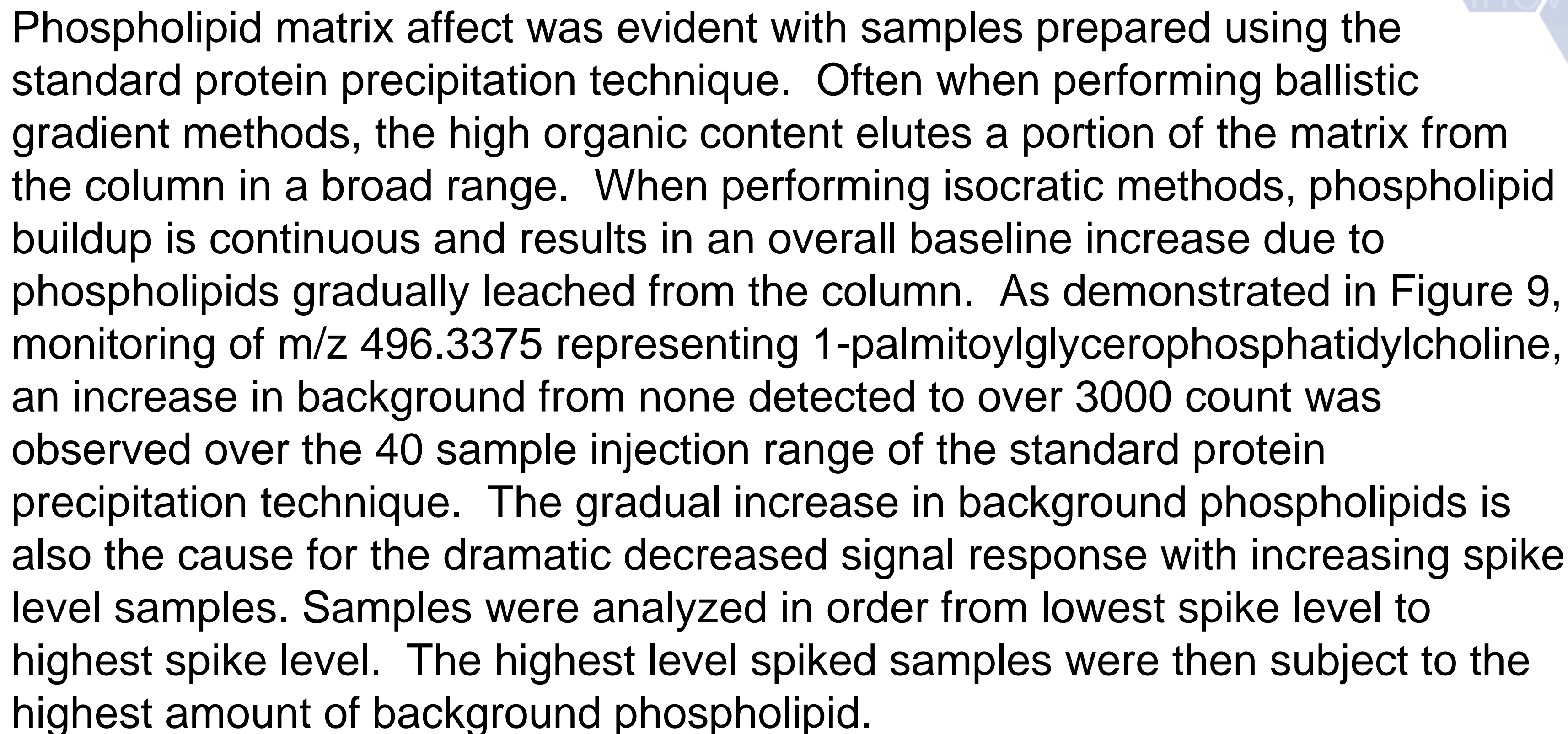

Phospholipid matrix affect was evident with samples prepared using the standard protein precipitation technique. Often when performing ballistic gradient methods, the high organic content elutes a portion of the matrix from the column in a broad range. When performing isocratic methods, phospholipid buildup is continuous and results in an overall baseline increase due to phospholipids gradually leached from the column. As demonstrated in Figure 9, monitoring of m/z 496.3375 representing 1-palmitoylglycerophosphatidylcholine, an increase in background from none detected to over 3000 count was observed over the 40 sample injection range of the standard protein precipitation technique. The gradual increase in background phospholipids is also the cause for the dramatic decreased signal response with increasing spike level samples. Samples were analyzed in order from lowest spike level to highest spike level. The highest level spiked samples were then subject to the highest amount of background phospholipid.

Figure 10 demonstrates the signal difference between the highest spike level sample prepared using the HybridSPE Small Volume technique (blue) and the standard protein precipitation method (red). Significant signal reduction was observed for the standard protein precipitation method due to the background phospholipids.

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Summary

- Phospholipid buildup and resulting matrix ionization affect was demonstrated when performing standard protein precipitation techniques.
- The HybridSPE Small Volume plate demonstrated excellent recovery of methadone and associated metabolites across the concentration range along with depletion of proteins and phospholipids from the plasma samples.
- The combination of facile protein precipitation/phospholipid depletion and fast analysis using modern chromatographic particles shows great promise in increasing the throughput for bioanalytical methods.
- Shorter run times and longer column life are expected to result from such an approach.
- The ability to perform selective matrix removal enables the use of optimized chromatographic elution conditions without the need for gradient elution of sample matrix.
- The Fused-Core particle of the Ascentis Express RP-Amide enabled high efficiency separation at much lower backpressures than compared with sub-2 µm particles.

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