

(Excerpt from Reporter 28.1)

Increased Bioanalytical Throughput and Recovery Utilizing HybridSPE-PPT Small Volume Plates

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Often a 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. In the majority of cases, co-extracted interferences directly affect the quantitation of analytes due to ionization effects induced by the extracted matrix. This extracted matrix can impact the chromatographic analysis, but more often results in a chromatographic build-up that leads to irregularities in both retention and quantitation. 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.

Performing a more thorough sample cleanup enables faster chromatographic analysis and thus increases the overall sample throughput. Using the HybridSPE-PPT 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 chromatographic separation with a dramatic increase in throughput.

HybridSPE-Small Volume 96-Well Plate and Closeup of Tips



E001087

Figure 1. HybridSPE-Small Volume 96-Well Schematic Diagram

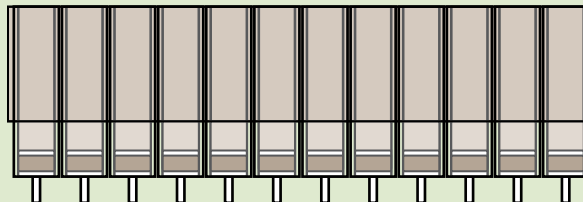
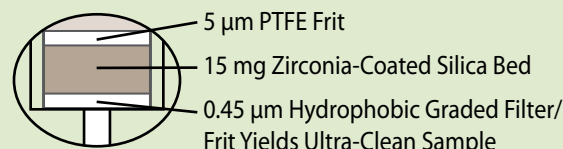


Plate: 96-well format employs special frits at the top and bottom of the same selective bed; proteins **can be removed on-line** for added speed and convenience.

Single Well Explosion:



Frit Yields Ultra-Clean Sample

This study evaluates the performance of the newly developed HybridSPE-PPT Small Volume 96-well plate for preparation of small volumes of rat plasma. The HybridSPE-PPT Small Volume plate accommodates plasma volumes of 20-40 μL , ideally suited for bioanalytical testing of mouse plasma. It makes use of the zirconia-coated silica stationary phase as is also used in the standard HybridSPE-PPT plate for phospholipid depletion. The HybridSPE-PPT 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 also used for fine particle removal. The narrow internal well diameter of the plate, along with small packed bed, results in minimal holdup volume (typically 20-40 μL). This enables sufficient volume recovery when handling small volume plasma samples.

In this study, rat plasma samples spiked with methadone and metabolites 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrrolidine (EMDP) were processed with the HybridSPE-PPT 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.

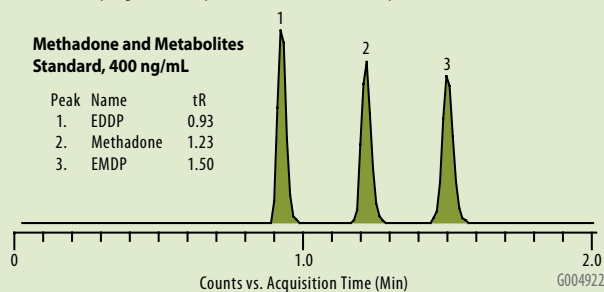
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Figure 2. Chromatographic Conditions and Conditions for Sample Preparation

column: Ascentis Express RP-Amide, 10 cm x 2.1 mm I.D., 2.7 μ m, (53913-U)
 mobile phase: 10mM ammonium formate (65:35 water:acetonitrile) pH 3.6
 flow rate: 0.4 mL/min
 system pressure: 275 bar
 temp: 35 °C
 inj vol: 0.5 μ L
 instrument: Agilent 1200SL Rapid Resolution, Agilent 6210 TOF LC/MS
 detection: ESI+
 sampling rate: 1.5 spectra/sec, 6309 transients/spectra

Methadone and Metabolites Standard, 400 ng/mL

Peak	Name	tR
1.	EDDP	0.93
2.	Methadone	1.23
3.	EMDP	1.50



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 K₂EDTA 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 μ L of plasma to plate, followed by 60 μ L 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 μ L 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 μ L of plasma to centrifuge vial, followed by 300 μ L 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

Each spiked level sample was prepared n=8 for both the HybridSPE-PPT Small Volume technique and the standard protein precipitation method. Samples processed using the HybridSPE-PPT 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 that sufficient sample was drawn into 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.

Samples prepared using the HybridSPE-PPT Small Volume plate demonstrated high recovery across the concentration range. These samples were not affected by the matrix buildup due to the complete depletion of phospholipids. No signal suppression was observed using the HybridSPE-PPT Small Volume technique. As shown in Tables 1, 2, and 3 nearly equivalent calibration slopes between the standard solution

Table 1. Calibration Table: Methadone

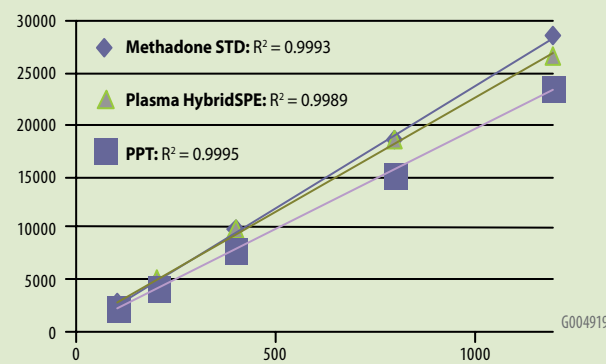


Table 2. Calibration Table: EMDP

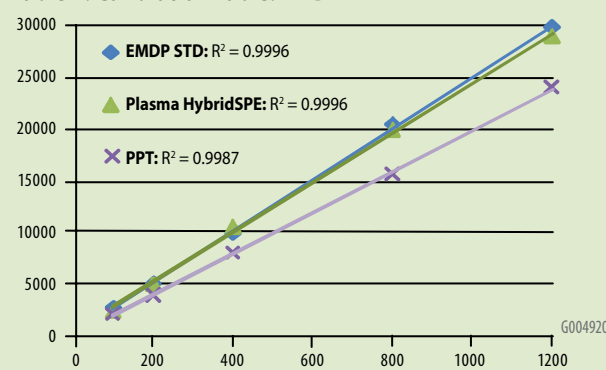
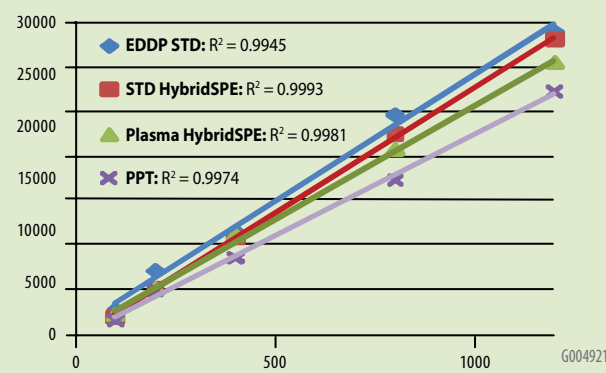


Table 3. Calibration Table: EDDP



and spiked plasma processed with the HybridSPE-PPT small volume technique was achieved. In Table 3, some drug protein binding was observed for the EDDP metabolite, resulting in the slight decrease in response. The recovery of the standard solution (red) was included in this chart to show the high recovery of EDDP from the HybridSPE-PPT Small Volume plate.

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

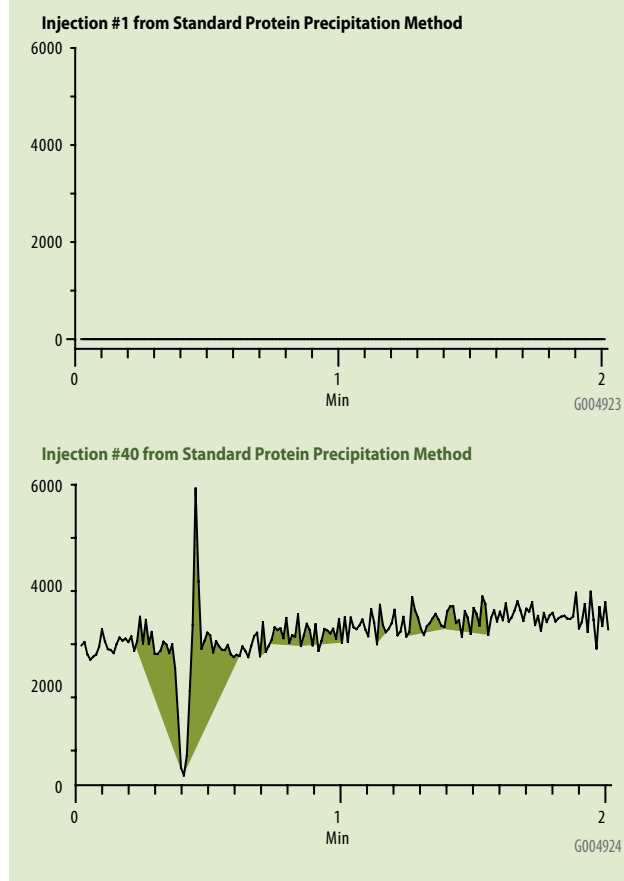
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Sample Handling

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Figure 3. Phospholipid Buildup on Column from Standard Protein Precipitation Method*(continued from page 13)*

methods, phospholipid buildup is continuous and results in an overall background increase, due to phospholipids gradually leaching from the column. As demonstrated in Figure 3, 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 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 chromatographic buildup of phospholipid. Significant signal reduction was observed for the standard protein precipitation method due to the background phospholipids.

This study has demonstrated the detrimental effect of phospholipid buildup and resulting matrix ionization effect when performing standard protein precipitation techniques. By utilizing the HybridSPE-PPT Small Volume plate, excellent recovery of methadone and associated metabolites across the concentration range along with depletion of proteins and phospholipids from the plasma samples was achieved. 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. The ability to perform selective matrix removal enables the use of optimized chromatographic elution conditions without the need for gradient elution of sample matrix, resulting in shorter run times and more rugged bioanalytical methods.

+ Featured Products

Description	Cat. No.
HybridSPE-PPT – Small Volume 96-well plate, 15 mg/well	52794-U
96-Square/Deep Well Collection Plates, 1 mL, PP	575652-U
IKA® MS 3 Digital Orbital Shaker	Z645036-TEA

+ Related Products

Description	Cat. No.
Ascentis Express RP-Amide, 10 cm x 2.1 mm I.D., 2.7 μ m	53913-U
HybridSPE-Precipitation 96-well Plate, 50 mg/well	575656-U
HybridSPE-Precipitation Cartridge, 30 mg/1 mL	55261-U
96-well Protein Precipitation Filter Plate	55263-U
Supelco PlatePrep Vacuum Manifold	57192-U
96-Square/Deep Well Collection Plates, 0.35 mL, PP	575651-U
96-Square/Deep Well Collection Plates, 2 mL, PP	575653-U
96-Square Well Pierceable Cap Mats	575655-U
Methadone, 50 mg and 1 g	M0267
EDDP, 10 mg	E5264

+ Related Information

For more information on the HybridSPE-PPT products, request brochure T409095 (LOP) - *HybridSPE-Precipitation Technology*.

Did you know...?

Supelco's Ascentis Express is a perfect complement to HybridSPE™-PPT sample prep cartridges and 96-well plates for bioanalytical LC-MS-MS assays. If you have an interest in these products, please complete the survey at sigma-aldrich.com/bioanalysis-request.