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Evaluation of Supported Liquid Extraction and Protein Precipitation Clean-up Through Post Column Infusion

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Introduction

Post column infusion is a valuable tool for assessing matrix cleanliness and can help identify potential regions of ion suppression within an analysis. In this application, a post column infusion study is performed to compare the suppression zones associated with an extracted plasma sample. Both Protein Precipitation (PPT) and Supported Liquid Extraction (SLE) were used as clean-up techniques to compare the overall cleanliness of the methods. We selected a common assay, 25-OH Vitamin D3, to study ion suppression and to compare these two sample preparation techniques.

Materials and Methods

25-OH Vitamin D2 and D3 standards were purchased from Cerilliant® (Round Rock, TX). Human plasma was purchased from BioreclamationIVT® (Westbury, NY). All other reagents and chemicals were obtained from Sigma-Aldrich®.

Experimental Conditions

Sample Pre-treatment

Dilute 100 µL of spiked plasma (100 ng/mL) with 100 µL of IPA/Water (1:1) and add 10 µL of 1 µg/mL standard in Ethanol.

Supported Liquid Extraction (SLE)

96-Well Plate:	Strata® DE 200 µL 96-Well Plate
Part No.:	8E-S325-FGB
Load:	200 µL Pre-treated sample on to the Strata DE plate (apply vacuum or positive pressure to pull/push sample into sorbent)
Wait:	5 minutes
Elute:	2x 0.6 mL Heptane. Vacuum or apply positive pressure at 5-10" Hg for 10 seconds
Dry down:	Evaporate eluent to dryness at 30 °C under a gentle stream of Nitrogen
Reconstitute:	100 µL of Methanol/Water (80:20) with internal standard

Protein Precipitation (PPT) Protocol

96-Well Plate:	Impact™ Protein Precipitation
Part No.:	CE0-7565
Add:	800 µL of Acetonitrile/Methanol (1:1) with 0.1% Formic acid
Dispense:	200 µL of plasma with spiked 25-OH Vitamin D2/D3 Internal Standard
Vortex:	2 minutes
Wait:	5 minutes
Elute:	Apply vacuum or positive pressure at 5-10" Hg to pull sample through the Impact PPT 96-Well Plate and into a collection plate

LC Conditions

After sample preparation, both post column infusions were run using the same LC cconditions with an injection volume of 2 µL of blank matrix. Using a 50 x 2.1 Kinetex® 2.6 µm C18 LC Column (00B-4426-AN), and a mobile phase of 0.1 % Formic acid in water (Mobile Phase A) and 0.1 % Formic acid in Acetonitrile (Mobile Phase B), we ran a ballistic gradient from 60 % →100 % of Mobile Phase B over one minute and then held at 100 % organic for 5 minutes at a flow rate of 0.3 mL/min. During the LC run, neat 25-OH Vitamin D3 was continuously infused at 10 µL/min directly into the mass spectrometer resulting in a continuous trace and response for this analyte over the course of the run. By injecting a methanol sample through the LC column, a system blank was determined as the basis for comparison to each of the clean-up procedures.

Results and Discussion

In order to investigate clean-up and ion suppression, we first injected a methanol blank to give us a trace for the system and to serve as the basis for comparison of PPT and SLE sample preparation procedures (**Figure 1**). **Figure 2** shows an overlay of the methanol blank (blue trace) along with the PPT extract sample (red trace), and the SLE extract (green trace). There are many ion suppression zones, or valleys, in the PPT extract between one and three minutes. By contrast, the SLE extract shows a response that more closely mimics the methanol blank, showing a constant response and no suppression zones from one to three minutes in the run.

Figure 1.
Methanol Blank for Post Column Infusion

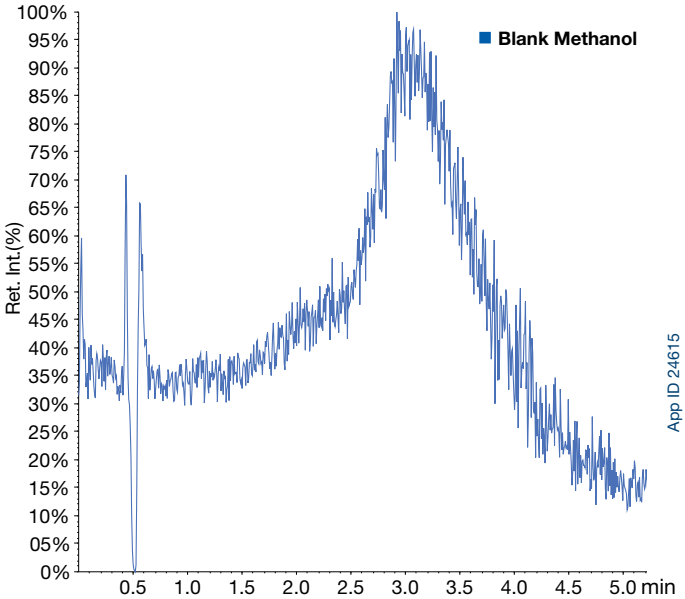

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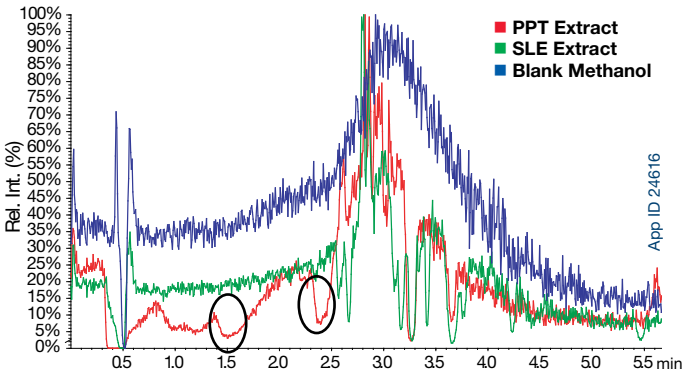
Figure 2.
Normalized Comparison of Blank Methanol, SLE Extract and PPT Extract



PPT does not remove phospholipids and therefore this is a likely source of ion suppression, which will in turn lead to lower response for the analyte of interest if it elutes in one of these suppression zones. **Figure 3** overlays the PPT trace with the chromatogram for 25-OH Vitamin D3 standards. The retention time of this analyte (**black** trace) is about 2.5 minutes, which on the PPT extraction (red trace) is positioned in a large valley of suppression.

In **Figure 4**, the 25-OH Vitamin D3 (**black** trace) is overlaid with the SLE extract (green trace). The SLE extract does not contain the ion suppression zone at around 2.5 minutes that the PPT trace does and the analyte elutes in a constant part of the trace showing, indicating that there is little or no signal suppression.

Figure 3.
Comparison of PPT with 25-OH Vitamin D3 Peak

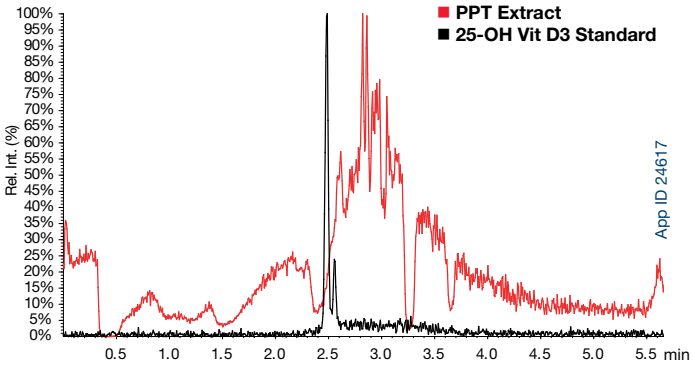


Figure 4.
Comparison of SLE Extract with 25-OH Vitamin D3 Peak

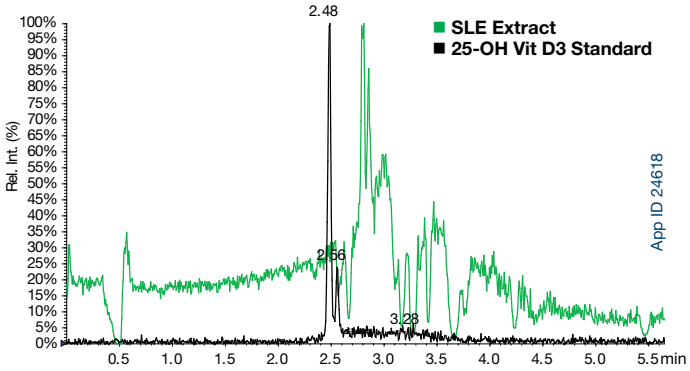
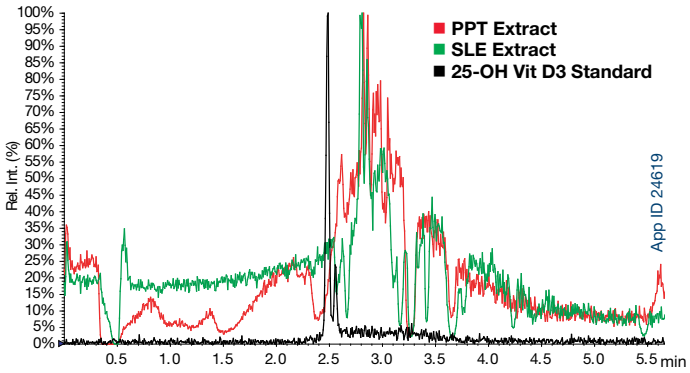


Figure 5 overlays both PPT (red trace) and SLE (green trace) extracts with the chromatogram for 25-OH Vitamin D3 (**black** trace) which distinctly shows that the peak for 25-OH Vitamin D3 elutes in a suppression zone with the PPT extract however, there is no suppression zone at this time period in the SLE extract.

Figure 5.
Overlay Comparison of PPT, SLE and 25-OH Vitamin D3 Standard.



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To test the assumption that phospholipids are the source of ion suppression in our PPT trace, we examined the 184-184 m/z transition which corresponds to the head group of phospholipids and therefore describes the overall phospholipid content of the sample. **Figure 6** shows that the valleys of suppression correlate to increased response for the 184-184 transition and suggest that phospholipids are causing the ion suppression.

Figure 6.
Phospholipid Profile Overlay Compared to PPT

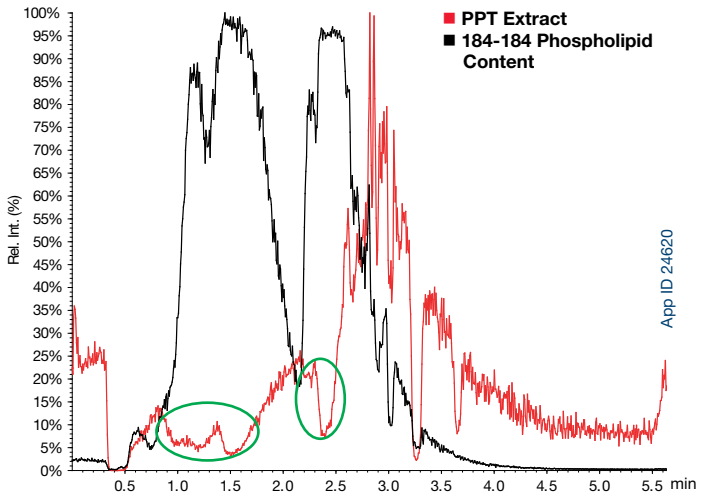
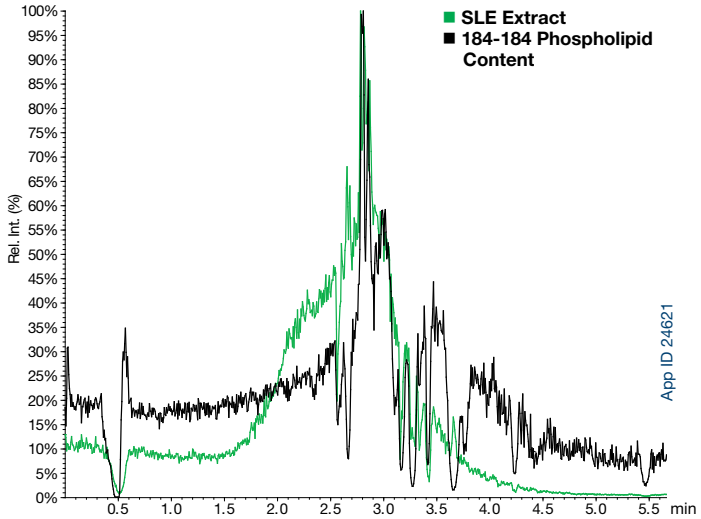


Figure 7.
Phospholipid Profile Overlay Compared to SLE



In contrast, **Figure 7**, shows that there is a significantly lower response for the 184-184 m/z transition and no observable valleys of suppression from one to three minutes as compared to the PPT trace.

Lastly, we compared two SLE extraction solvents: Heptane (green trace) and Ethyl Acetate (orange trace) in **Figure 8**. Ethyl Acetate has several ion suppression zones between 1 and 3 minutes. Not only does selecting the wrong elution solvent cause signal suppression, in this case, using Ethyl Acetate lengthens dry down times.

The recovery values for the SLE method are shown in **Table 1**. The dilution with organic solvent in the pre-treatment is required for achieving high recovery. The Isopropyl Alcohol functions to precipitate out the proteins and to release any protein bound analyte. The spiking standards were made up in ethanol which also contributed to additional protein precipitation, forming a cloudy solution when added. Therefore it is recommended to dilute plasma samples 1:1 with a mixture of 5:5:1 (Water/IPA/Ethanol).

Figure 8.
Normalized Overlay with Ethyl Acetate and Heptane

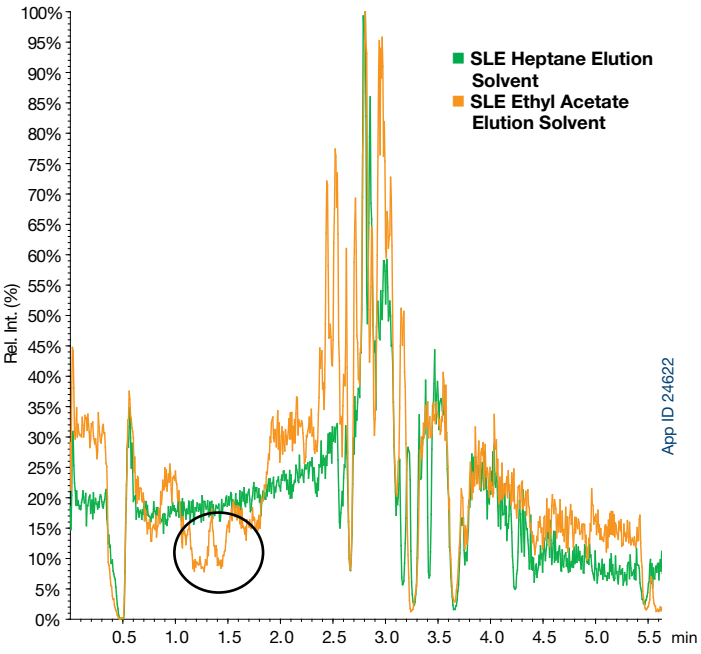


Table 1.
Recovery Values of 25-OH Vitamin D2/D3 Using a Heptane Elution Solvent

	25-OH Vitamin D2	25-OH Vitamin D3
Recovery	96 %	84 %
RSD (n=8)	9 %	9 %

Conclusion

In conclusion we have shown that performing an SLE extraction, using Strata® DE SLE with heptane as the extraction solvent can minimize ion suppression regions associated with phospholipids that are not removed when performing a simple protein precipitation.

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Ordering Information
Kinetex® Core-Shell LC Columns

2.6 µm Columns (mm)			SecurityGuard™ ULTRA Cartridges‡ (mm)			SecurityGuard ULTRA Cartridges‡ (mm)				SecurityGuard ULTRA Cartridges‡ (mm)
Phases	50 x 2.1	150 x 2.1	3/pk	50 x 3.0	100 x 3.0	3/pk	50 x 4.6	100 x 4.6	150 x 34.6	3/pk
C18	00B-4462-AN	00F-4462-AN	AJ0-8782	00B-4462-Y0	00D-4462-Y0	AJ0-8775	00B-4462-E0	00D-4462-E0	00F-4462-E0	AJ0-87768
			for 2.1 mm ID			for 3.0 mm ID				for 4.6 mm ID

‡SecurityGuard ULTRA Cartridges required holder, Part No.: AJ0-9000.

Other particle sizes and dimensions are available. Visit www.phenomenex.com/Kinetex for a complete list of available columns.

Strata® DE Supported Liquid Extraction

Part No.	Description	Unit
8E-S325-FGB	Strata DE SLE 200 µL 96-Well Plate	2/pk
8E-S325-5GB	Strata DE SLE 400 µL 96-Well Plate	2/pk
8B-S325-KDG	Strata DE SLE 12 cc Tube	20/pk
8B-S325-VFF	Strata DE SLE 60 cc Tube	16/pk

Presston™ 100 Positive Pressure Manifold

Part No.	Description
AH0-9334	Presston 100 Positive Pressure Manifold, 96-Well Plate
AH0-9342	Presston 100 Positive Pressure Manifold, 1 mL Tube Complete Assembly
AH0-9347	Presston 100 Positive Pressure Manifold, 3 mL Tube Complete Assembly
AH0-9343	Presston 100 Positive Pressure Manifold, 6 mL Tube Complete Assembly

Presston 100 Tube Adapter Kits (for AH0-9334)

Part No.	Description
AH0-9344	1 mL Tube Adapter Kit
AH0-9345	3 mL Tube Adapter Kit
AH0-9346	6 mL Tube Adapter Kit

WARRANTY Phenomenex warrants that for a period of 12 months following delivery, the Presston 100 Positive Pressure Manifold you have purchased will perform in accordance with the published specifications and will be free from defects in materials or workmanship. In the event that the Presston 100 Positive Pressure Manifold does not meet this warranty, Phenomenex will repair or replace defective parts. Please visit www.phenomenex.com/Presston for complete warranty information.

guarantee

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