Is Poor Sample Prep Impacting Your Bioanalytical Methods?

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Introduction

Sample preparation is crucial for bioanalytical analysis using chromatography. Without performing any sample preparation such as protein precipitation, phospholipid removal (PLR), liquid-liquid extraction (LLE) or solid phase extraction (SPE), system performance will be negatively affected. Common issues include blockages within the High Performance Liquid Chromatography (HPLC) tubing, column fouling, dirtying of the mass spectrometer source and reduced sensitivity.

Sample preparation methods vary in clean up performance with protein precipitation being one of the most popular sample preparation methods for preparing biological samples, such as plasma or serum, for chromatographic analysis. It is a quick and simple procedure - samples are added, followed by solvent causing protein to crash out and finally liquid is drawn through a filter leaving the sample free from protein.

While protein precipitation removes protein, it does not remove other matrix components such as phospholipids. These can cause several problems when performing LC-MS/MS analysis:

1. Phospholipids are known to impact ionisation within the mass spectrometer source. This will either cause ion enhancement, or more frequently ion suppression,[1] reducing robustness of analysis.

2. They contaminate the mass spectrometer source which leads to increased maintenance costs, as well as longer downtime of instruments.

3. Phospholipids can build up on an HPLC column resulting in higher backpressures over time and reducing column life.[2]

Phospholipid removal (PLR) is another sample preparation technique which can be used to prepare a biological sample for analysis. PLR uses the same protocol as a protein precipitation plate however, an active component is incorporated into the product which captures phospholipids without retaining analytes of interest (figure 1). Therefore, PLR provides a more complete solution for preparation of biological samples.

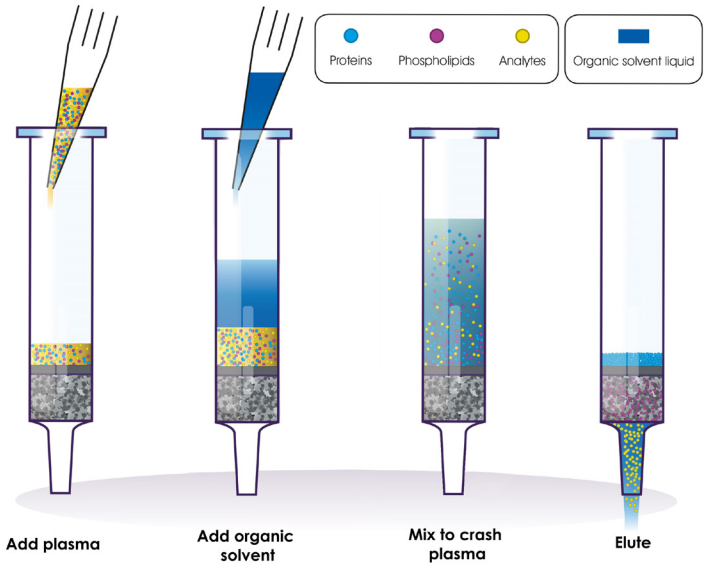

Figure 1 - The simple PLR protocol for preparing plasma for LC-MS analysis

The work in this application note compares the phospholipid removal effectiveness and recovery of analytes between two different commonly used sample preparation methods (PLR and protein precipitation) for LC-MS/MS analysis, using bovine plasma. The Microlute® PLR plate was used to prepare the PLR samples.

The Microlute® range uses a unique composite technology (figure 2) which combines the active material to capture the phospholipids with an inert structure of polyethylene. This offers advantages in reproducibility of sample preparation, caused by improved consistency of sample flow through into a collection plate.

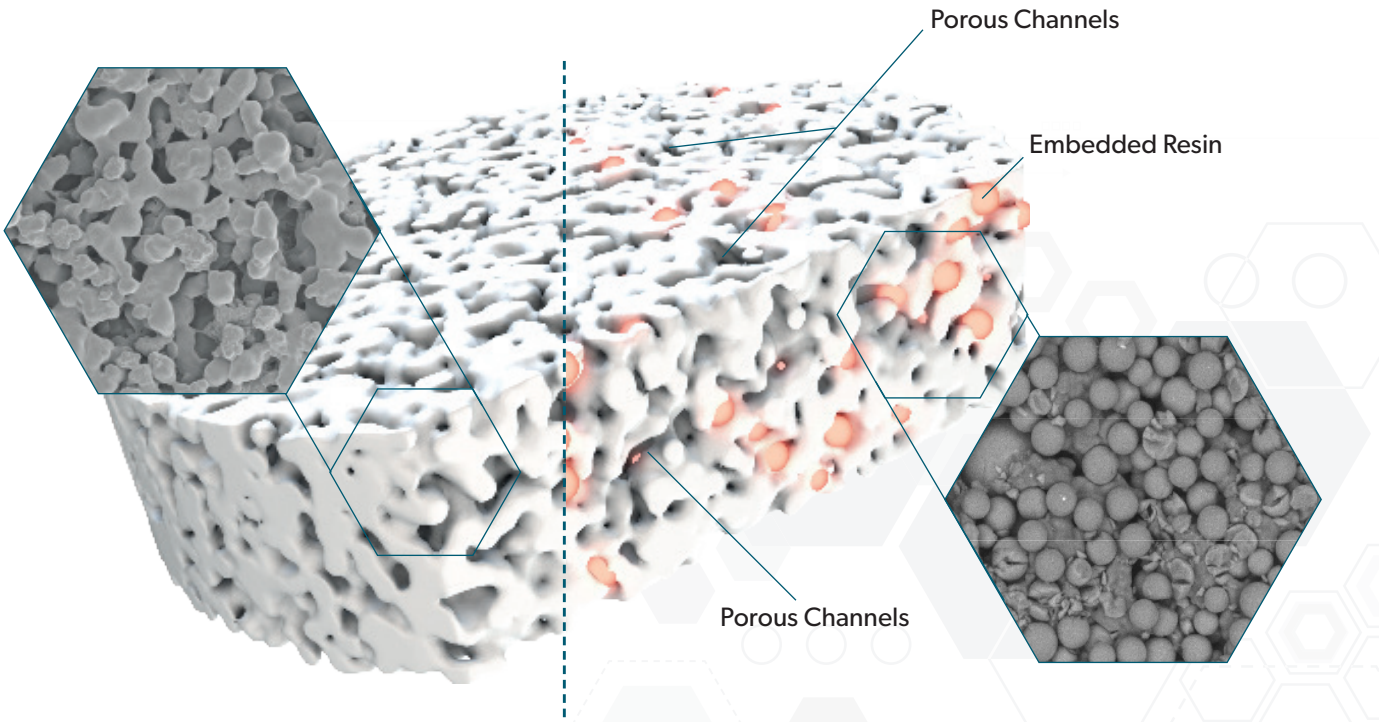

Figure 2 – Diagrammatic representation of the Microlute® composite technology

Experimental

Preparation of spiked plasma

Bovine plasma was spiked with procainamide at three different concentrations – 25 ng/mL, 250 ng/mL and 1250 ng/mL. Solutions were mixed and left to equilibrate for one hour.

Calibration curve standards

100 µL of blank unspiked plasma was added to four wells of a Microlute® PLR plate followed by 300 µL of acetonitrile with 1% formic acid (v/v). Each well was aspirated by pipette five times to ensure the solution was adequately mixed and protein was fully crashed. The crashed solution was eluted into a 1.1 mL collection plate (Porvair Sciences product code: 219250) using positive pressure at a flow rate of approximately 1 drop per second.

All of the processed plasma was pooled into a 1.5 mL microcentrifuge tube and vortexed for 10 seconds. 100 µL of the combined processed plasma was added to six wells to make up one blank and five calibration standards. The five calibration standards were spiked to create the following standards – 10, 100, 200, 500 and 1,500 ng/mL.

Processing spiked plasma

100 µL of each of the different spiked concentrations were added to wells of a Microlute® PLR plate and a Protein Precipitation Plate (Porvair Sciences product code: 240100) in duplicate. To each of these wells, 300 µL of acetonitrile with 1% formic acid (v/v) was added. The liquid in each well was aspirated by pipette five time to ensure the solution was adequately mixed and protein was fully crashed. The crashed solutions were eluted into a 1.1 mL collection plate using positive pressure at a flow rate of approximately 1 drop per second.

Dilution step

To improve the peak shape and robustness of the LC-MS/MS method, the processed spiked plasma and standard solutions were diluted 1:10 with water with 0.1% formic acid (v/v). This prevented poor peak shape which occurs due to the high organic strength of the eluent.

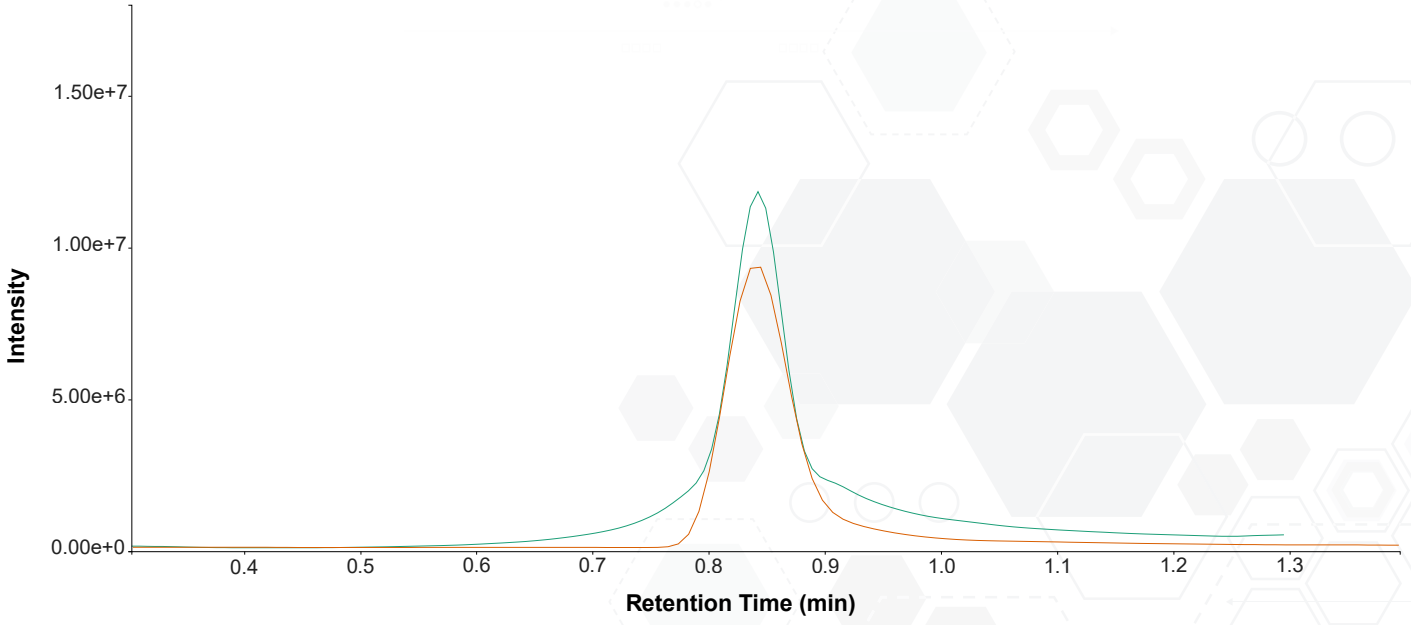

Figure 3 – Overlaid comparison of procainamide peak shape – undiluted (green trace) versus 1:10 diluted (orange trace)

LC-MS/MS Method for Post Column Infusion

The following method was used to screen for common phospholipids[3] present within the protein precipitated and Microlute® PLR samples, as well as monitor for any matrix effects (ion enhancement/ suppression) through post-column infusion. The solution being infused was a 100 ng/mL solution of procainamide in mobile phase A, infused at 10 µL/min.

HPLC conditions:

System: ACQUITY Premier BSM/FTN	**Column:** Thermo Fisher Hypersil GOLD™ C18 (2.1 mm x 50 mm, 1.9 µm)
Solvent A_1 H_2O + 0.1% formic acid	**Injection Volume** 2 µL
Solvent B_1 MeOH + 0.1% formic acid	**Column Temperature** 40 °C
Gradient	**Sample Temperature** 10 °C
	Flow rate 400 µL/min

Time	A_1	B_1
0.00	20	80
0.50	20	80
1.00	0	100
3.00	0	100
3.01	20	80
4.00	20	80

Mass Spectrometer Conditions

System: Xeno TQ-S micro
Ionisation mode: Positive ESI
MRM transitions: See table below
Capilary voltage: 2.5 kV
Source temperature: 150 °C
Desolvation temperature: 550 °C
Procainamide post column infusion rate: 10 µL/min
Infusions concentration: 100 ng/mL

MRM transitions

Analyte name	Precursor (m/z)	Product (m/z)	Core voltage (V)	Collision energy (V)
Procainamide	235.92	163	23	15
18:0 LPC	524.3	148.1	80	90
34.2 PC	758.6	184.1	80	90
38.4 PC	810.7	184.1	80	90
36.2 PC	786.5	184.1	80	90
34.1 SM	703.5	184.1	80	90
16:0 LPC	496.3	184.1	80	90

LPC = Lysophophatidylcholines, PC = Phosphatidylcholine, SM = Sphingomyelin

LC-MS/MS Method for Procainamide

To analyse the spiked samples and calibration curve, the following LC-MS/MS method was used:

System: ACQUITY Premier BSM/FTN	**Column:** Restek Biphenyl (2.1 mm x 30 mm, 1.8 µm)
Solvent A$_1$ H_2O + 0.1% formic acid	**Injection Volume** 2 µL
Solvent B$_1$ MeOH + 0.1% formic acid	**Column Temperature** 45 °C
Gradient	**Sample Temperature** 10 °C
	Flow rate 400 µL/min

Time	A_1	B_1
0.00	90	10
1.20	90	10
1.21	0	100
5.00	0	100
5.01	90	10
10.00	90	10

Mass Spectrometer Conditions

System: Xeno TQ-S micro
Ionisation Mode: Positive ESI
MRM Transitions: Procainamide 235.92 -> 163
Capilary Voltage: 0.5 kV
Source Temperature: 150 °C
Desolvation Temperature: 550 °C
Desolvation Gas Flow: 1,000 L/h
Cone Voltage: 23V
Collision Energy: 15V

Results and Discussion

Phospholipid removal

To analyse the presence of phospholipids remaining in a plasma sample prepared by protein precipitation and by composite PLR technology (Microlute® PLR), an MRM LC-MS/MS method was used to scan for the common phospholipids in plasma.

The phospholipid removal plate had very little signal for the phospholipids, with the chromatogram showing primarily baseline noise. This shows that all phospholipids which interfere with the analysis were removed from the sample (figure 4). Whereas, the protein precipitated sample had larger peak areas, showing they remained in the sample.

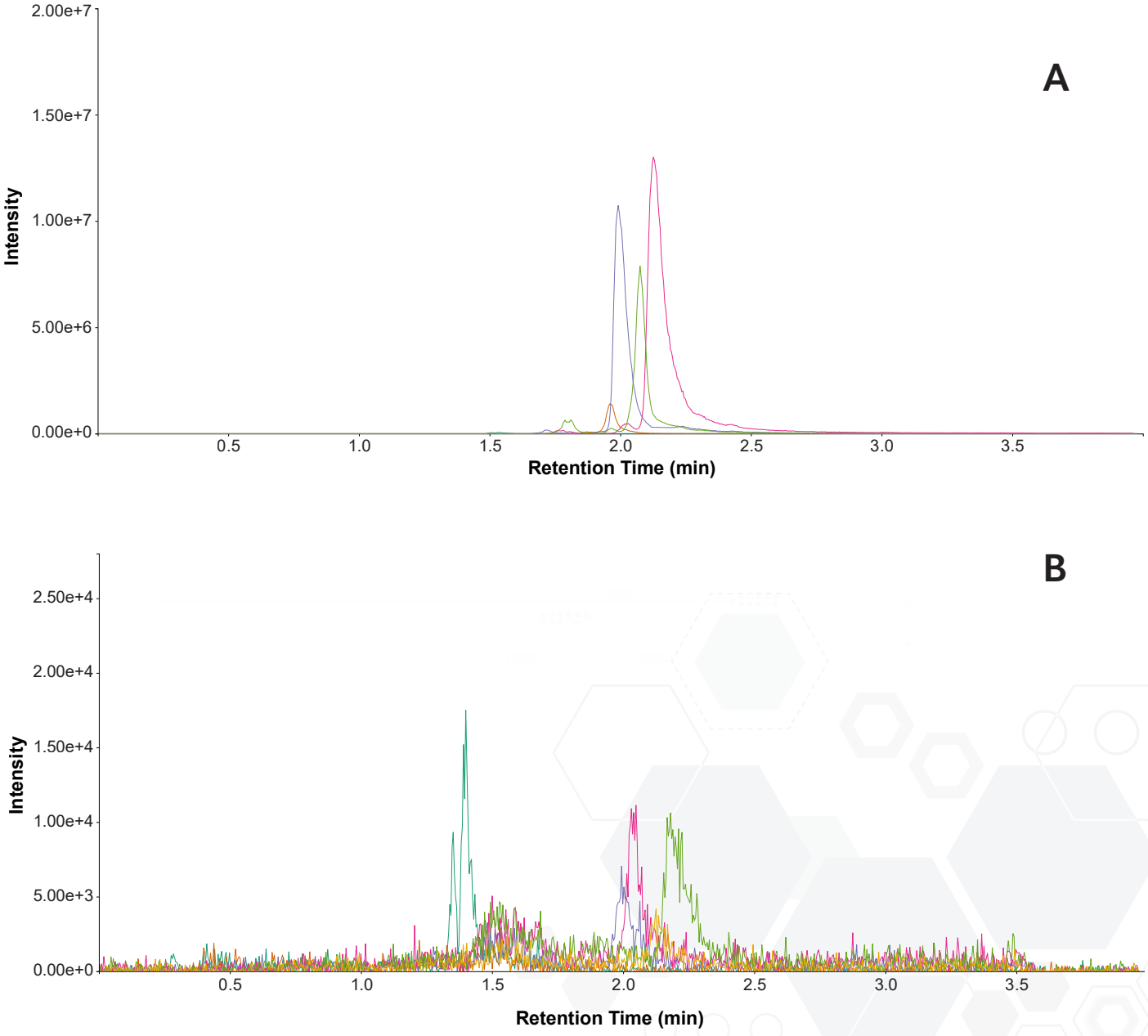

Figure 4 – Overlaid traces of phospholipid MRMs in each sample. A = protein precipitated sample B = phospholipid removal plate

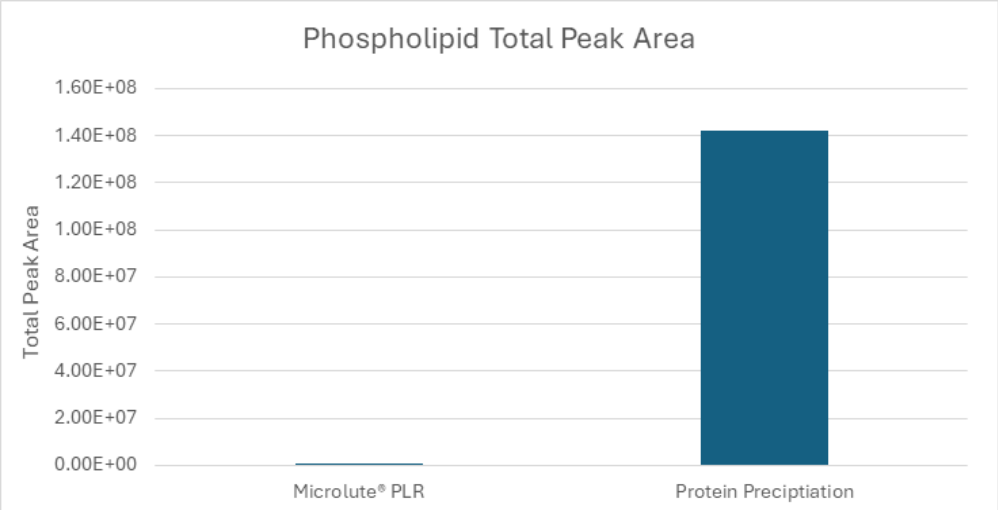

Figure 5 – Comparison of total phospholipid peak areas in samples prepared using a Microlute® PLR plate and a protein precipitation plate. Total peak area for Microlute® PLR = 5.47 x 10^4 and protein precipitation = 1.42 x 10^8

Total peak areas were compared from the phospholipid traces (figure 4) and are presented in a bar chart format (figure 5). Minimal response was observed for the phospholipids in the Microlute® PLR sample (5.47 x 10^4) whereas, the protein precipitation sample had a very large total peak area (1.42 x 10^8).

Matrix effects – ion suppression

To quantify the effect of the phospholipids on ionisation, a post-column infusion of procainamide was performed while injecting a blank sample prepared using both the protein precipitation plate and the Microlute® PLR plate.

The phospholipid removal plate (blue trace) demonstrated that ionisation was unaffected throughout the run when compared to an infusion with a blank solution (green trace). However, the protein precipitated solution (black trace) had a dip in the baseline due to ion suppression from phospholipids from between 1.5 minutes to 2.5 minutes. This is indicated in figure 6 where there is a large reduction in signal where the phospholipids co-elute (red trace). The largest observed reduction in signal was ~75% reduction in signal for the procainamide at a retention time of ~2 minutes.

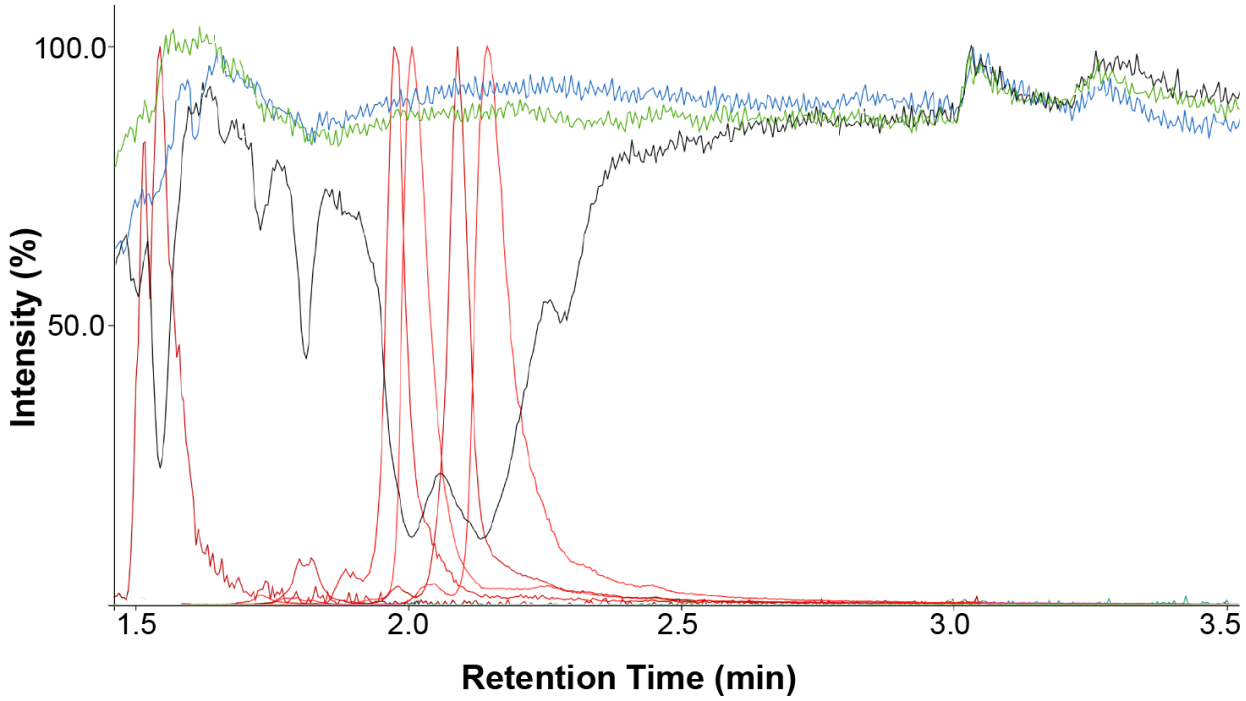

Figure 6 – An overlay of the infusion traces of procainamide with an injection of a solvent blank (green), Microlute® PLR prepared sample (blue) and protein precipitated samples(black). The phospholipid trace for the protein precipitated sample is also overlaid (red).

Procainamide calibration curve

Matrix matched standards were prepared to demonstrate the creation of a calibration curve with the Microlute® PLR (figure 7). The calibration curve was linear and had a correlation coefficient (r^2) of 0.9995. This work showed that the calibration range was linear for procainamide from 10 ng/mL up to 1500 ng/mL.

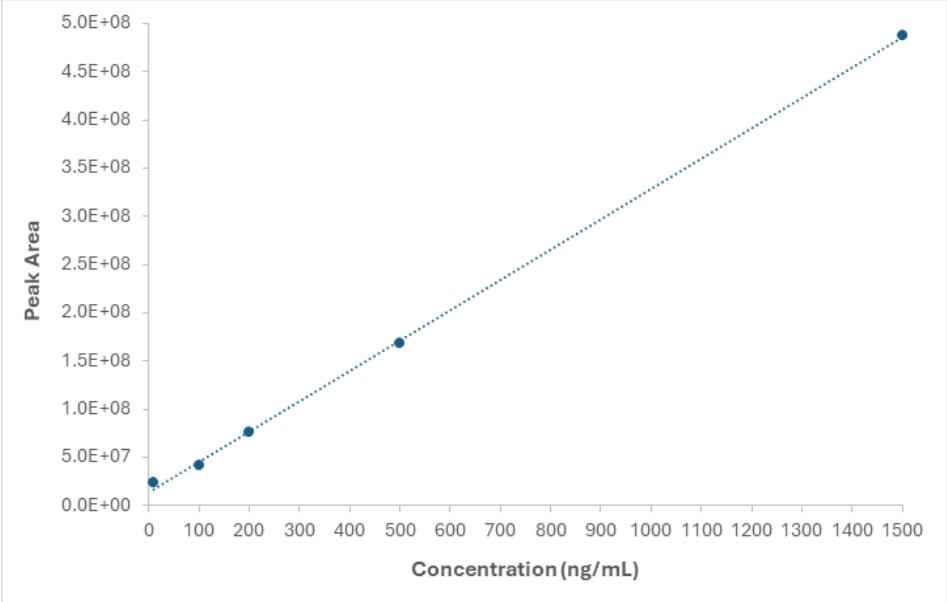

Figure 7 – A matrix matched standard calibration curve ranging from 10 – 1500 ng/mL r^2 value = 0.9995.

Comparison of preparation of procainamide – protein precipitation versus PLR

To simulate quality control (QC) samples used in bioanalytical methods, plasma was prepared in duplicate at three different concentrations – low QC (25 µg/mL), medium QC (250 µg/mL) and high QC (1,250 µg/mL). Each concentration was prepared by protein precipitation and a Microlute® PLR plate.

These samples were analysed by LC-MS/MS and the peak areas were compared to quantify the differences by percentage difference. At the lowest, most challenging concentration, the peak areas differed by 9.6% (table 1). This showed that both preparation methods were equally effective at recovering procainamide from the plasma.

Technique	Concentration (ng/mL)								
	25			250			1250		
	R1	R2	Mean	R1	R2	Mean	R1	R2	Mean
Protein Precipitation	1.0E+07	1.1E+07	1.0E+07	8.2E+07	8.5E+07	8.4E+07	3.8E+08	4.0E+08	3.9E+08
Phospholipid Removal	9.7E+06	9.4E+06	9.5E+06	7.9E+07	7.8E+07	7.9E+07	3.7E+08	3.7E+08	3.7E+08
Percentage Difference	9.6			5.9			4.5		

Table 1 – Peak areas for procainamide in plasma prepared at the three concentrations levels in duplicate. The percentage difference between the peak areas at each concentration for the two techniques.

Conclusion

This short study demonstrates the necessity of carrying out sample preparation prior to chromatographic analysis of biological samples. It prevents ion suppression, which can lead to lower signal and reduced sensitivity, as well as reducing the need for instrument maintenance. Furthermore, when comparing the two techniques (protein precipitation and PLR), recovery was unaffected by the active component removing phospholipids. This showed that the Microlute® PLR plate delivers superior performance, maintaining recovery while reducing ion suppression, leading to more reliable and robust analytical results.

References:

1: O. A. Ismaiel, M. S. Halquist, M. Y. Elmamly, A. Shalaby and H. T. Karnes, "Monitoring phospholipids for assessment of ion enhancement and ion suppression in ESI and APCI LC/MS/MS for chlorpheniramine in human plasma and the importance of multiple source matrix effect evaluations," Journal of Chromatography B, vol. 875, no. 2, pp. 333-343, 2008.

2: J. Carmical and S. Brown, "The impact of phospholipids and phospholipid removal on bioanalytical method performance," Biomedical Chromatography, vol. 30, no. 5, pp. 710-720, 2016.

3: G. B. Phillips and J. T. Dodge, "Composition of phospholipids and of phospholipid fatty acids of human plasma," Journal of Lipid Research, vol. 8, no. 6, pp. 676-681, 1967.