Phospholipid Removal:- A Comparison between Traditional Liquid-liquid Extraction (LLE) and Supported Liquid Extraction (SLE) using LC-MS/MS Analysis

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Introduction

Endogenous phospholipids (outline structure shown in **Figure 1**.) present in biological fluids are a major problem in LC-MS/MS analysis. Due to their strong retention characteristics in reversed phase chromatography they tend not to elute as discrete peaks and are often very difficult to separate from analytes of interest. This co-elution often leads to areas of suppression or enhancement in the chromatogram which in turn can cause quantitation issues. It is well known that traditional liquid-liquid extraction (LLE) provides very clean extracts. Supported liquid extraction (SLE) is analogous to liquid-liquid extraction, however, as a solid supported technique provides subtle differences. This poster compares phospholipid removal between traditional liquid-liquid extraction and supported liquid extraction using a variety of sample pre-treatment and extraction solvent combinations.

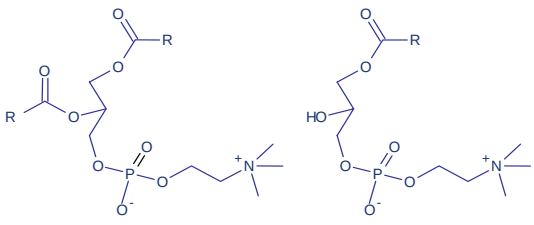

Figure 1. Phospholipid structures

Experimental Procedure

Reagents

Formic acid, 3-methyl-1-butanol and ammonium hydroxide were purchased from Sigma Chemical Co. (Poole, UK). Human plasma was obtained through the Welsh Blood Service (Pontyclun, UK). All solvents were HPLC grade from Fisher Scientific (Loughborough, UK).

Sample Preparation

Supported Liquid Extraction Procedure

Plate: ISOLUTE SLE+ Supported Liquid Extraction Plate 200 mg, part number 820-0200-P01.

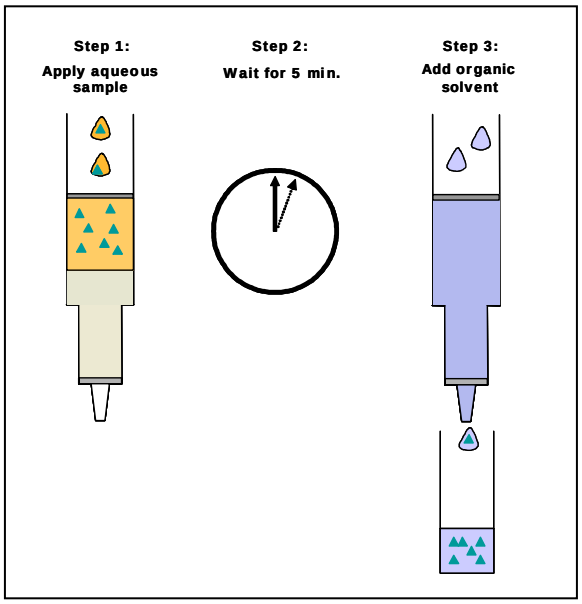

***Figure 1**. Schematic of ISOLUTE SLE+ Supported Liquid Extraction procedure. A single well of the 96-well plate is shown*

Sample pre-treatment: Plasma (100 µL) was diluted (1:1 (v/v)) with either 1% formic acid aq, 0.1% formic acid aq, H_2O or 0.5M NH_4OH.

Sample Application: The pre-treated plasma (200 µL) was loaded onto the plate, a pulse of vacuum applied to initiate flow and the samples left to absorb for 5 minutes.

Analyte Elution: Addition of 1 mL of various water immiscible extraction solvents. The extraction solvents evaluated were 98:2 Hexane:3-methyl-1-butanol, MTBE, EtOAc, DCM, 98:2, 95:5 and 90:10 (v/v) DCM/IPA.

Liquid-liquid Extraction Procedure
Pre-treated plasma (200 µL) was mixed with various extraction solvents (1 mL). The layers were left to separate and the organic aliquot removed.

Post Extraction: The eluate was evaporated to dryness and reconstituted in 1 mL of 70:30 (v/v) H_2O/MeOH prior to analysis.

HPLC Conditons

Instrument: Waters 2795 Liquid Handling System (Waters Assoc., Milford, MA, USA).
Column: Luna Phenyl-Hexyl 5 µm analytical column (50 x 2.0 mm id) (Phenomenex, Cheshire UK).
Guard Column: Luna Phenyl-Hexyl security guard column (Phenomenex, Cheshire, UK).
Mobile Phase: 0.1% formic acid aq and 0.1% formic acid/MeCN at a flow rate of 0.3 mL/min.
Gradient: The gradient conditions were set to 60%, 0.1% (v/v) formic acid aq and 40% MeCN increasing to 100% MeCN over 6 minutes. The high organic mobile phase was held for 7 minutes and initial starting conditions resumed at 13.1 minutes.
Injection Volume: 5 µL
Temperature: Ambient

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Mass Spectrometry

Instrument: Ultima Pt triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface for mass analysis. Positive ions were acquired in the multiple reaction monitoring (MRM) mode using the 184 Da product ion. Previous phosholipid experiments (full scan and SIR) identified the most abundant phospholipid ions (shown in Table 1.) subsequently used in these MRM experiments.

Desolvation Temperature: 350 °C
Ion Source Temperature: 100 °C
Collision Gas Pressure: 2.6 x 10^{-3} mbar
Collision Energy: 16 eV

Table 1. MRM transitions for the various PL's

Lyso-PL's	PL's	PL's
494.4 > 184	701.7 > 184	784.6 > 184
496.4 > 184	703.7 > 184	786.6 > 184
520.4 > 184	732.8 > 184	806.6 > 184
522.4 >184	756.5 > 184	808.7 > 184
524.4 > 184	758.5 > 184	810.9 > 184
	760.5 > 184	

Results

The combinations of sample pre-treatment and extraction solvents, comparing extracts for LLE and SLE+ techniques are shown in **Figures 2-5**. Lyso-phospholipids (494-524) and larger molecular weight phospholipids (701-811) are contained in separate data sets for both techniques. All extracts are compared to protein precipitated plasma, assuming no phospholipid removal.

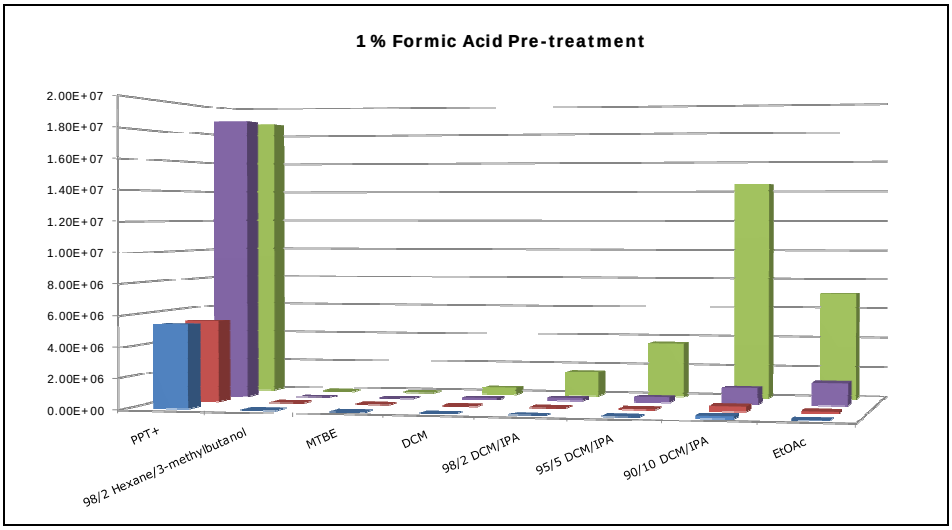

***Figure 2**. Phospholipid comparison using plasma pre-treated with 1% formic acid.*

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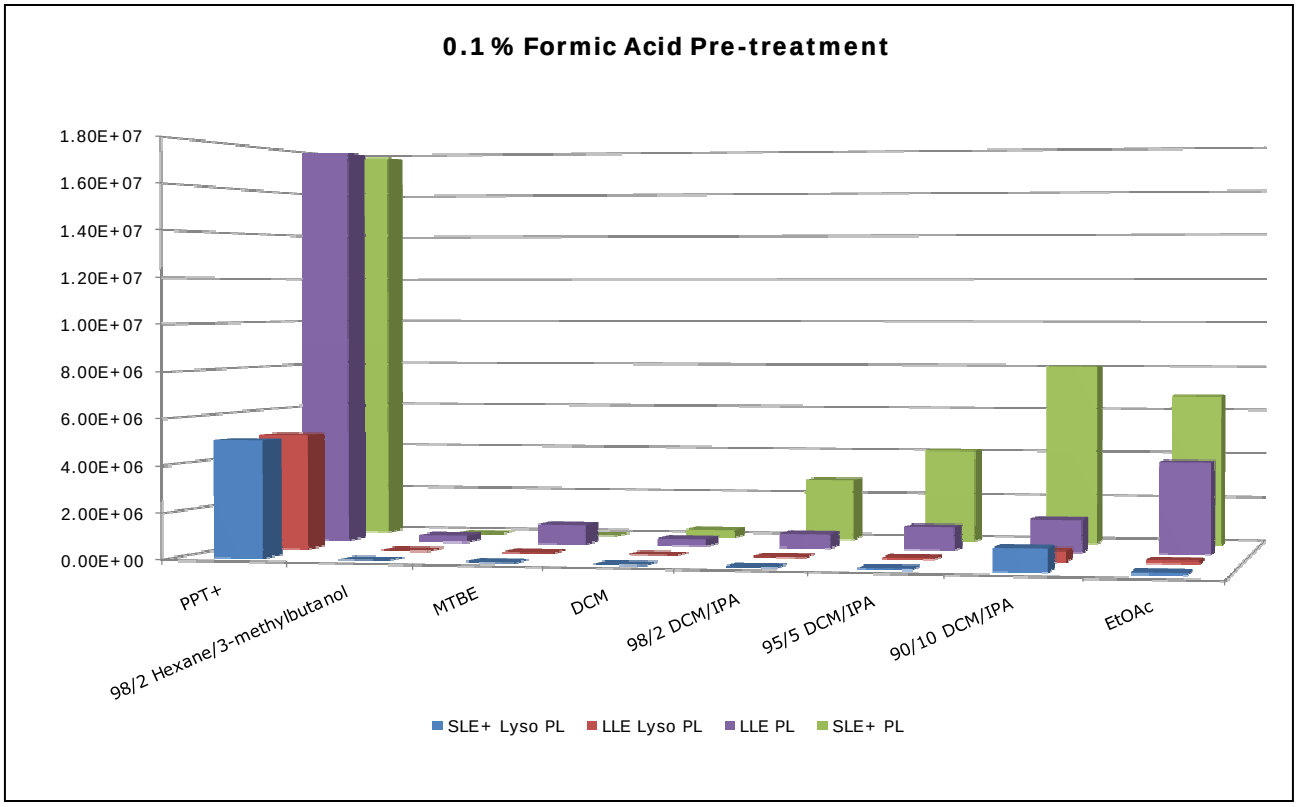

Figure 3. *Phospholipid comparison using plasma pre-treated with 0.1% formic acid*

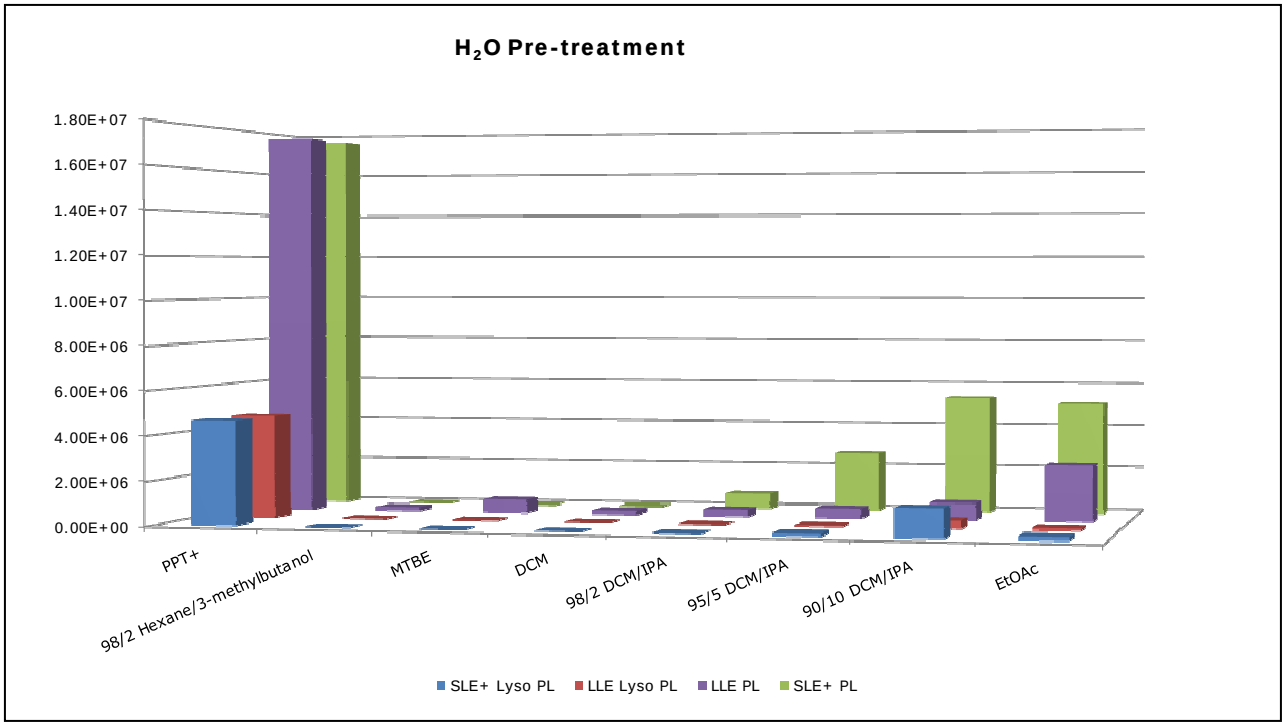

Figure 4. *Phospholipid comparison using plasma pre-treated with H_2O.*

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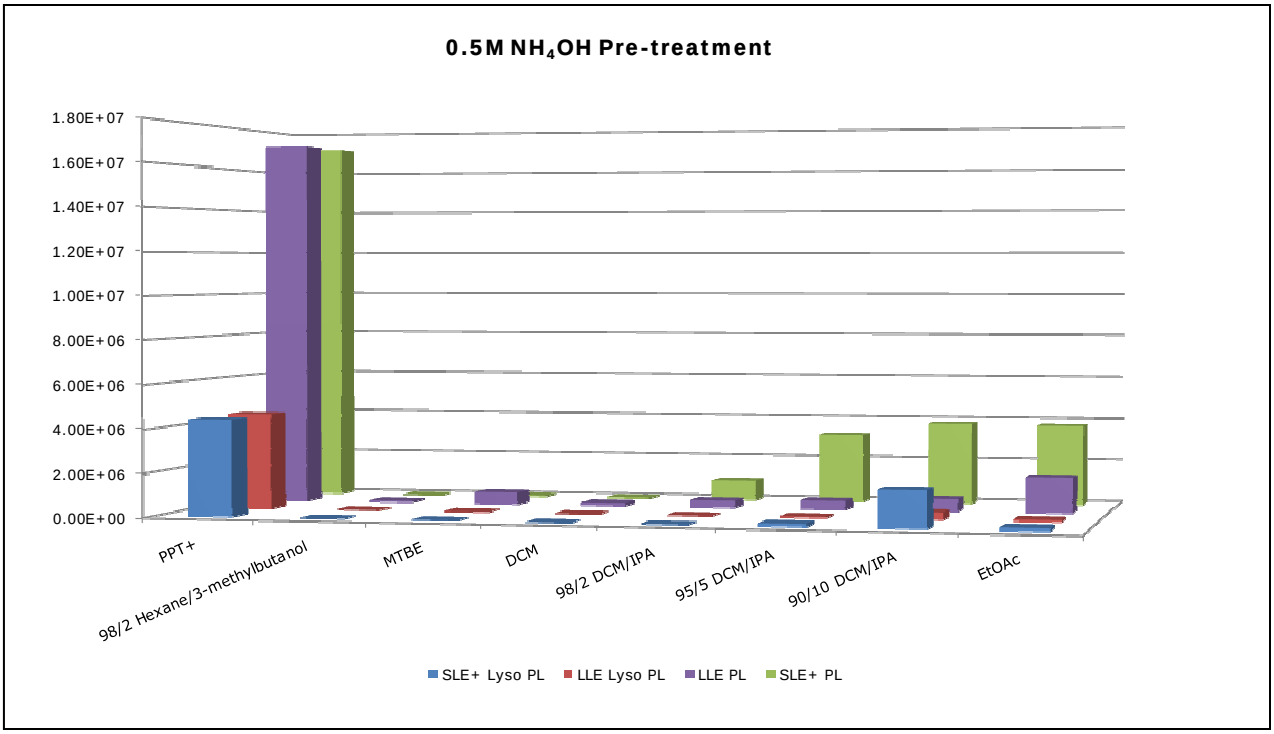

***Figure 5**. Phospholipid comparison using plasma pre-treated with 0.5M NH_4OH.*

Conclusions

- Low levels of lyso-PLs were present in both SLE+ and LLE extracts for most extraction solvents.
- The amount of lyso-PL extracted with 90/10 DCM/IPA increased with pH using SLE+ (to a maximum of 40% of the PPT+ level). This was not observed with LLE.
- Low levels of PL's were seen using extration solvents of 98/2 hexane/3-methyl-1-butanol, MTBE and DCM for both LLE and SLE+.
- As the extraction solvent polarity increased, SLE+ showed higher levels of PL's compared to LLE. Highest levels were seen at acidic pHs.

SLE+ does show increased levels of phospholipids when using more polar extraction solvents. However using traditional extraction conditions SLE+ and LLE provide comparable removal of phospholipids.

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