# Effective Strategies for Phospholipid Removal using Supported Liquid Extraction (SLE) with 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 phospholipids 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. Supported liquid extraction (SLE) is an analogous technique to traditional liquid-liquid extraction. This poster compares phospholipid removal using a wide variety of solvent combinations, pH control and polar extraction solvents on supported liquid extraction plates.

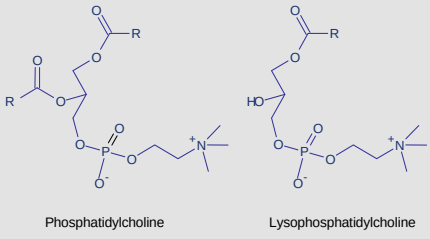


*Figure 1. Phospholipid structures*

## Experimental Procedure

### Reagents

Hydrochloric acid, formic acid, ammonium acetate, dibutylammonium acetate, heptafluorobutyric acid (both Fluka) and ammonium hydroxide were purchased from Sigma Chemical Co. (Poole, UK). Human plasma was obtained through the Welsh Blood Service (Pontyclun, UK). Tetrabutylammonium hydroxide (Acros) was purchased from Fisher Scientific (Loughborough, UK). All solvents were HPLC grade and purchased from Fisher Scientific or Sigma-Aldrich.

### Sample Preparation

**Supported Liquid Extraction:**

ISOLUTE SLE+ Supported Liquid Extraction Plates 200 µL and 400 µL.

**Sample pre-treatment:** Plasma (100-200 µL) was diluted (1:1 (v/v)) with various buffers for pH control.

**Sample Application:** The pre-treated plasma (200-400 µL) was loaded onto the plate, a pulse of vacuum applied to initiate flow and the samples left to absorb for 5 minutes.

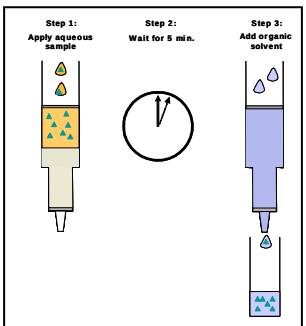


***Figure 2**. Schematic of ISOLUTE SLE+ Supported Liquid Extraction procedure.*

**Analyte Elution:** Addition of 1 mL (200 µL plate) or 2 x 900 µL (400 µL plate) of various water immiscible extraction solvents. The extraction solvents evaluated were 98:2 Hexane:3-methyl-1-butanol, heptane, MTBE, diethylether, chlorobutane, DCM, 98:2, 95:5, 90:10 and 80:20 (v/v) DCM/IPA, butyl acetate and ethyl acetate.
**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 0.4 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 1.2 minutes and initial starting conditions resumed at 7.3 minutes.
**Injection Volume:** 5 µL
**Temperature:** Ambient

## 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:** 2.7 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

All extracts are compared to protein precipitated plasma, assuming no phospholipid removal. Lyso-phospholipids (494-524) and larger molecular weight phospholipids (701-811) are contained in separate data sets***. Figures 3-4***. demonstrate PL content in numerous extraction solvents using a variety of pH pre-treatments from approximately pH 1.7-10.5.

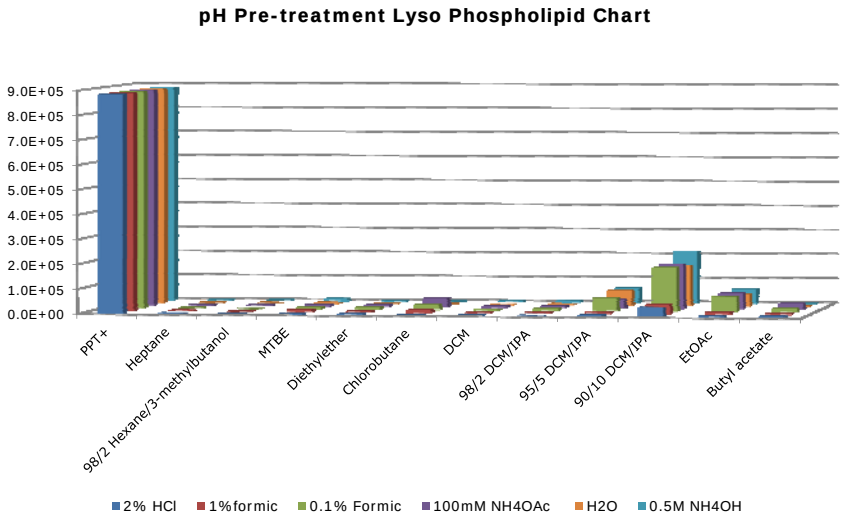


***Figure 3****. Lyso PL comparison using various pH pre-treatment and extraction solvent combinations.*

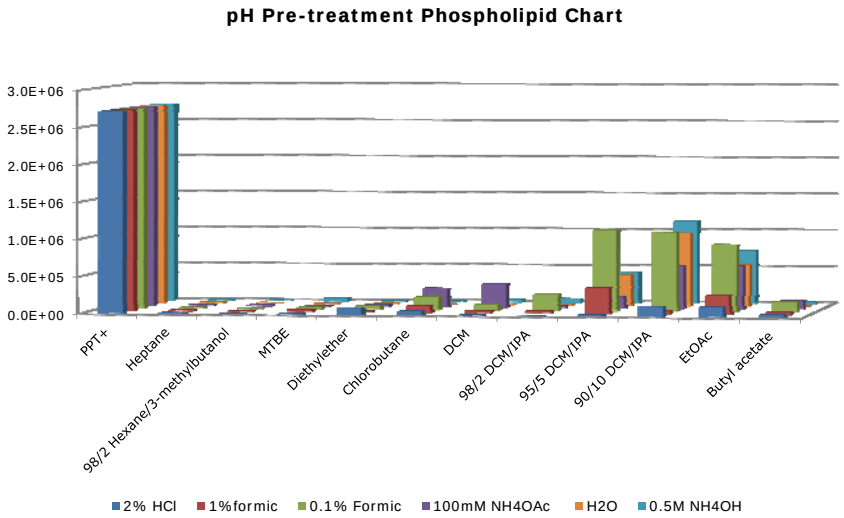


***Figure 4****. PL comparison using various pH pre-treatment and extraction solvent combinations.*

**Figures 5-6**. demonstrate PL content using ion pair reagents as sample pre-treatment.

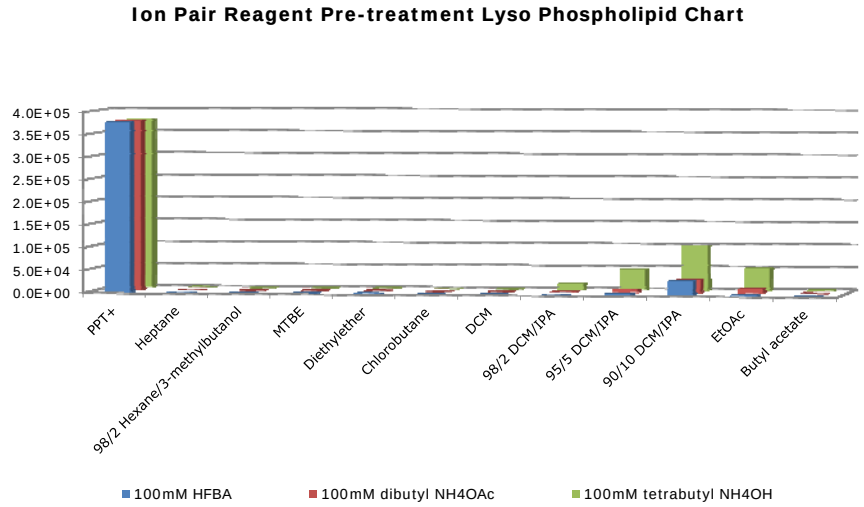


***Figure 5***. *Lyso PL comparison using ion pair pre-treatment.*

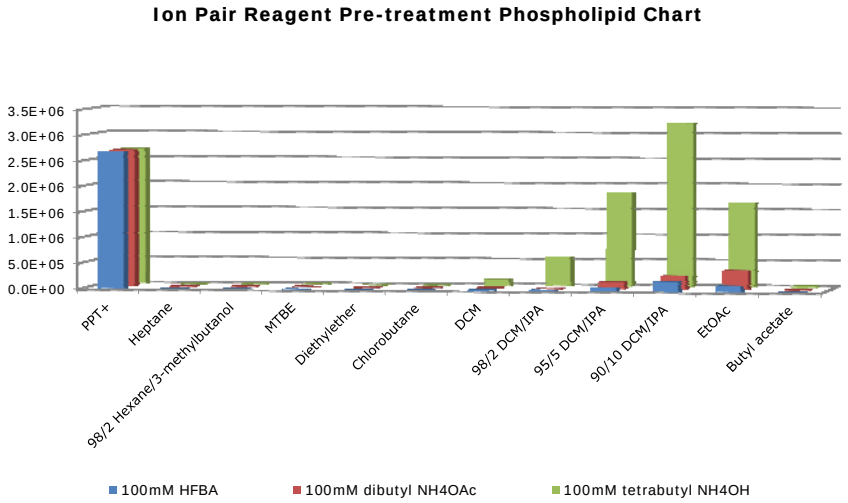


***Figure 6***. *PL comparison using ion pair pre-treatment.*

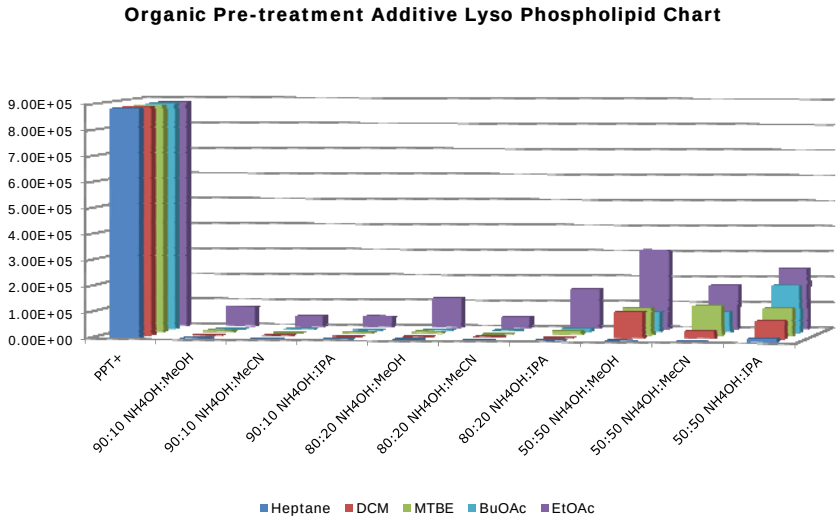


***Figure 7***. *Lyso PL comparison using increasing organic solvent ratio for pre-treatment.*

**Figures 7-8**. details PL content when increasing organic solvent ratio in the pre-treatment.

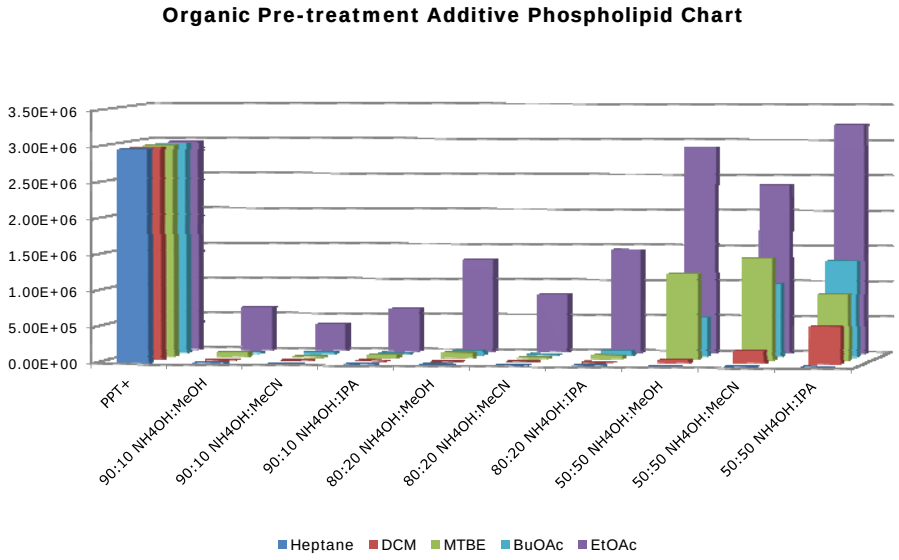


***Figure 8***. *Lyso PL comparison using increasing organic solvent ratio for pre-treatment.*

***Figure 9***. demonstrates PL content when decreasing overall load volume from 400 µL to 200 µL using polar extraction solvents.

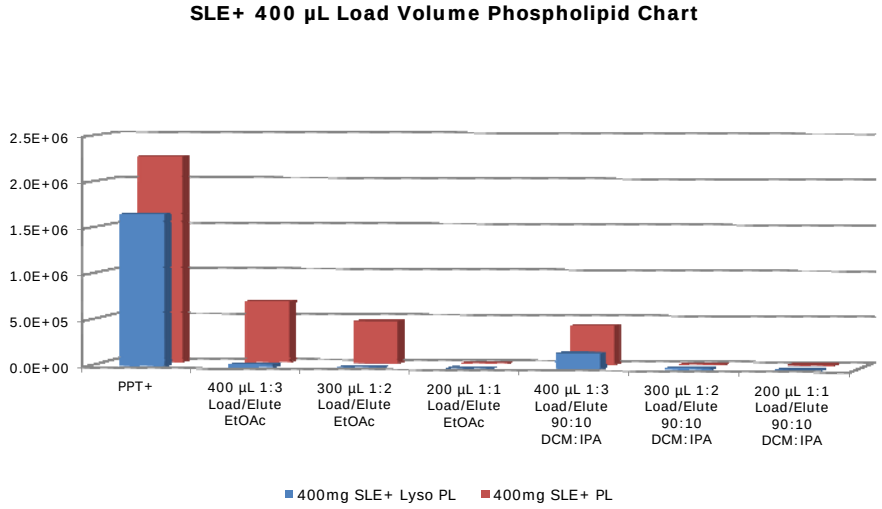


***Figure 9***. *PL comparison of load volume on 400 µL plate with polar extraction solvents*

***Figure 10***. demonstrates the maximum amount of IPA that can be used in the extraction solvent before PL breakthrough when using a 200 µL load on a 400 µL plate.

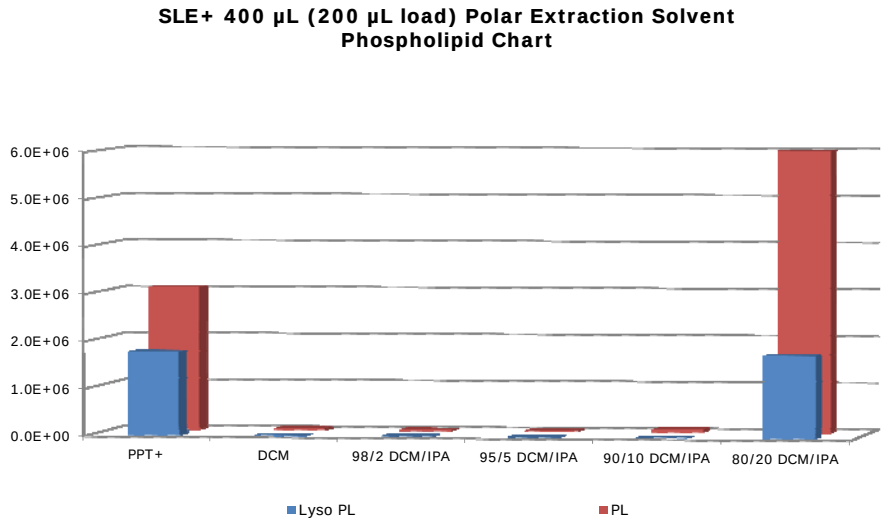


***Figure 10***. *PL comparison of maximum % IPA in extraction solvent.*

## Conclusions

- Low level PL are observed at a variety of pH pre-treatment/extraction solvent combinations.
- The use of ion pair reagents for pre-treatment shows low level PLs for a variety of extraction solvents.
- Up to 50% organic solvent (MeOH, IPA or MeCN) can be used for sample pre-treatment depending on extraction solvent.
- If more polar extraction solvents are required then decreasing the overall load volume demonstrates signifant improvements in PL content.
- 90/10 DCM/IPA can be used as an extraction solvent when combining a 200 µL load on a 400 µL plate.



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