# Investigation of Phospholipid Removal using 96-well Supported Liquid Extraction

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## Introduction

Endogenous phospholipids present in biological fluids can be a major problem in LC-MS/MS analysis. They tend not to elute as discrete peaks and are often very difficult to separate chromatographically from analytes of interest. This co-elution can lead to areas of suppression or enhancement in the chromatogram and in turn cause analyte quantitation issues. Due to their retentive nature, phospholipids (outline structure shown in **Figure 1**) can be very difficult to remove using solid phase extraction and other sample preparation techniques. Supported Liquid Extraction (SLE) is a 96-well high throughput technique that is analogous to traditional liquid-liquid extraction (LLE). Unlike LLE which is based on the use of two immiscible liquids, the extraction interface occurs between the buffered sample absorbed onto a solid support and a water immiscible solvent. This provides excellent extraction efficiency while alleviating many of the liquid handling issues associated with traditional LLE. The SLE approach is thus far more amenable to high throughput assays than the corresponding LLE technique. Both techniques are known to give very clean extracts showing low ion suppression as shown in previous presentations[1] and good removal of proteins (see SLE+ serum extraction shown in **Figure 2, page 2**).

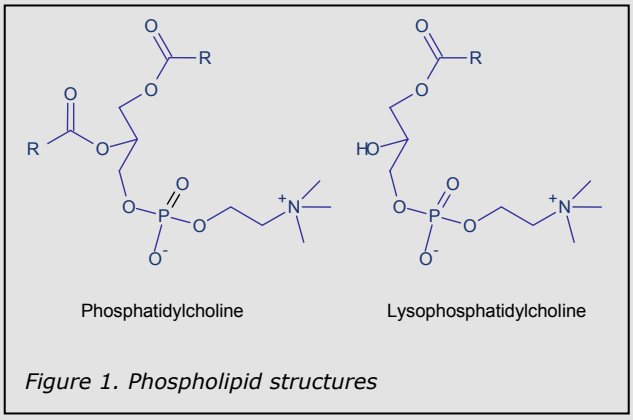


*Figure 1. Phospholipid structures*

This poster will demonstrate the use of SLE, investigating the specific problem of phospholipids and show the effect of loading pH and extraction solvent polarity on their removal.

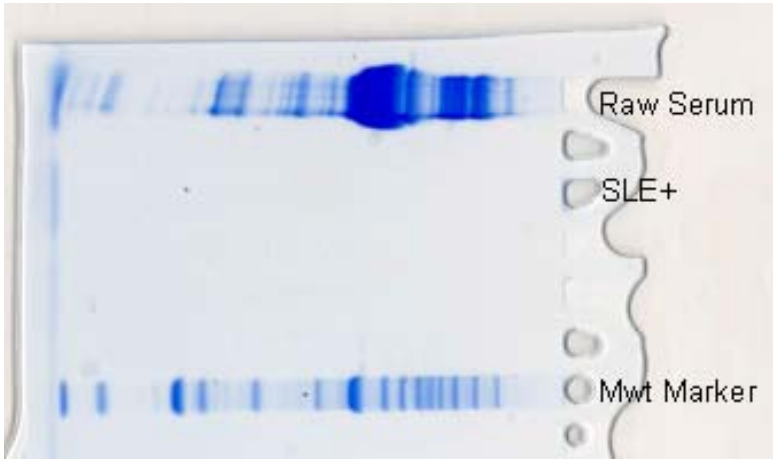


*Figure 2. Gel Electrophoresis image showing the protein profile from a serum sample extracted using ISOLUTE SLE+ with MTBE as the extraction solvent.*

## Experimental Procedure

### Reagents

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

### Supported Liquid Extraction Procedure

Sample: Blank human plasma (100 µL) was diluted 1:1 with various pH buffers prior to loading onto the ISOLUTE SLE+ Supported Liquid Extraction Plate. The buffers included in this study were; 1% (v/v) formic acid aq, 0.1% (v/v) formic acid aq, $H_2O$ and 0.5M $NH_4OH$ aq.

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

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

Post Extraction: The eluate was evaporated to dryness and reconstituted in 0.5 mL of 70:30 (v/v) H2O/MeOH prior to analysis.

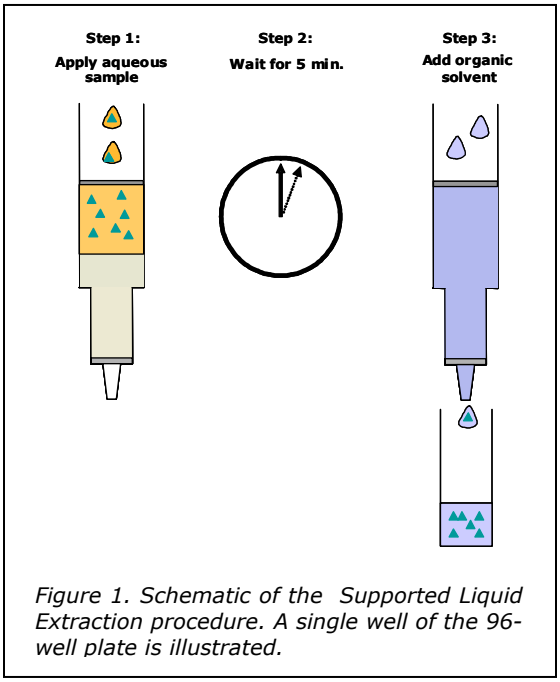


*Figure 1. Schematic of the Supported Liquid Extraction procedure. A single well of the 96-well plate is illustrated.*

HPLC Conditons
Instrument: Waters 2795 Liquid Handling System (Waters Assoc., Milford, MA, USA).
Column: Zorbax Eclipse XDB C18 3.5 µm analytical column (50 x 2.1 mm id) (Agilent Technologies, Berkshire, UK)
Guard Column: C8 guard column (Agilent Technologies, Berkshire, UK)
Mobile Phase: 0.1% formic acid (aq) and MeCN at a flow rate of 0.25 mL/min
Gradient: 70%, 0.1% formic acid (aq) and 30% MeCN increasing to 90% MeCN over 6 minutes. The high organic mobile phase was held for a further 2 minutes then returned to the initial starting conditions
Injection Volume: 10 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 selected ion recording mode (SIR)

Desolvation Temperature: 350 °C
Ion Source Temperature: 100 °C

**Table 1, page 3** shows the phospholipid ions monitored and the respective mass spectrometer parameters.

Table 1. Quattro Ultima Pt mass spectrometer parameters

| | SIR Mass | Dwell Time (s) | Cone Voltage (V) |
|---|---|---|---|
| Phospholipids | 496 | 0.1 | 55 |
| | 520 | 0.1 | 55 |
| | 522 | 0.1 | 55 |
| | 524 | 0.1 | 55 |
| | 760 | 0.1 | 55 |
| | 786 | 0.1 | 55 |
| | 806 | 0.1 | 55 |

## Results

The pH values recorded when mixing pooled human plasma with the various buffers used in this study are shown in **Table 2**.

Table 2. pH conditions when mixing plasma / buffer 1:1.

| **Plasma/buffer 1:1** | **pH** |
|---|---|
| 1% (v/v) Formic acid aq | 3.17 |
| 0.1% (v/v) Formic acid aq | 6.08 |
| $H_2O$ | 8.01 |
| 0.5M $NH_4OH$ | 10.34 |

An example of the phospholipid total ion chromatograms is shown in **Figure 4, page 4**. The top chromatogram shows the TIC from a PPT+ extracted sample. The SLE+ TIC's show the data from plasma extracted at pH 6 with the various extraction solvents.

The comparison of loading pH and extraction solvents in terms of overall peak areas can be seen in **Figure 5, page 4**. The extraction solvents of 98:2 (v/v) hexane/3-methyl-1-butanol, DCM and MTBE show very clean extracts at all loading pHs. At all pH loading conditions the 90:10 (v/v) DCM/IPA gave the highest levels of phospholipids in the final extract compared to the other extraction solvents.

As the pH of the loading conditions increased so did the levels of the phospholipids using the 90:10 DCM/IPA. Ethyl acetate gave some phospholipid content which seemed to be highest between pH 8-10.34.

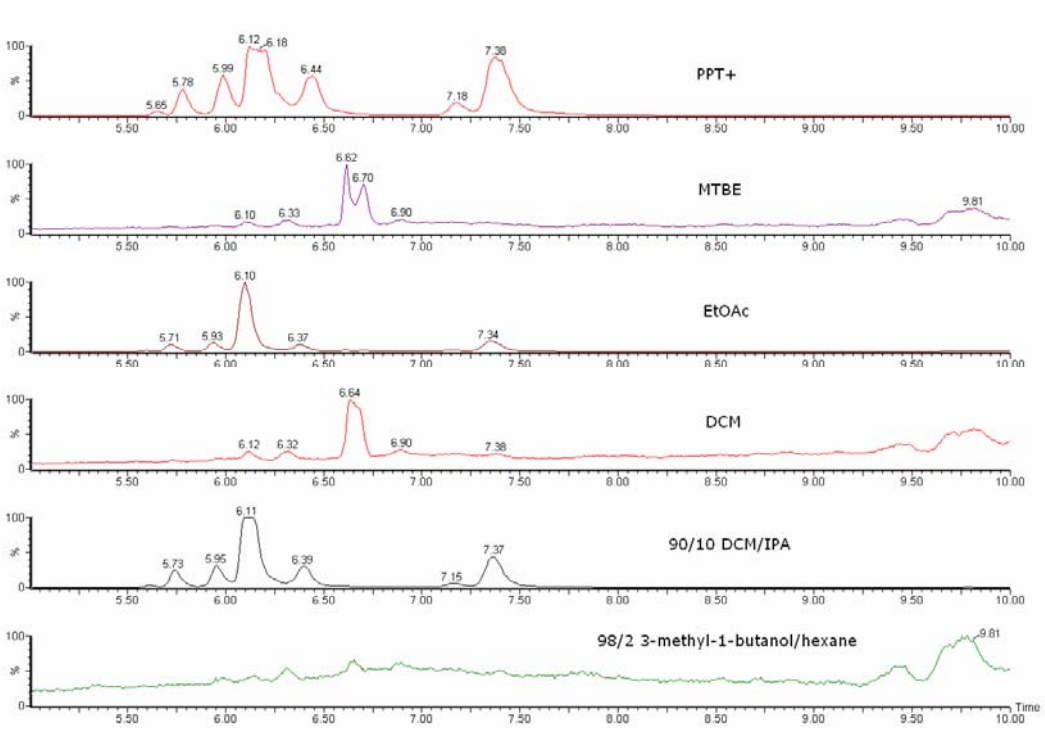


*Figure 4. Total ion chromatograms obtained for all extraction solvents when loading plasma/buffer mixtures at pH 6 compared to ISOLUTE PPT+*

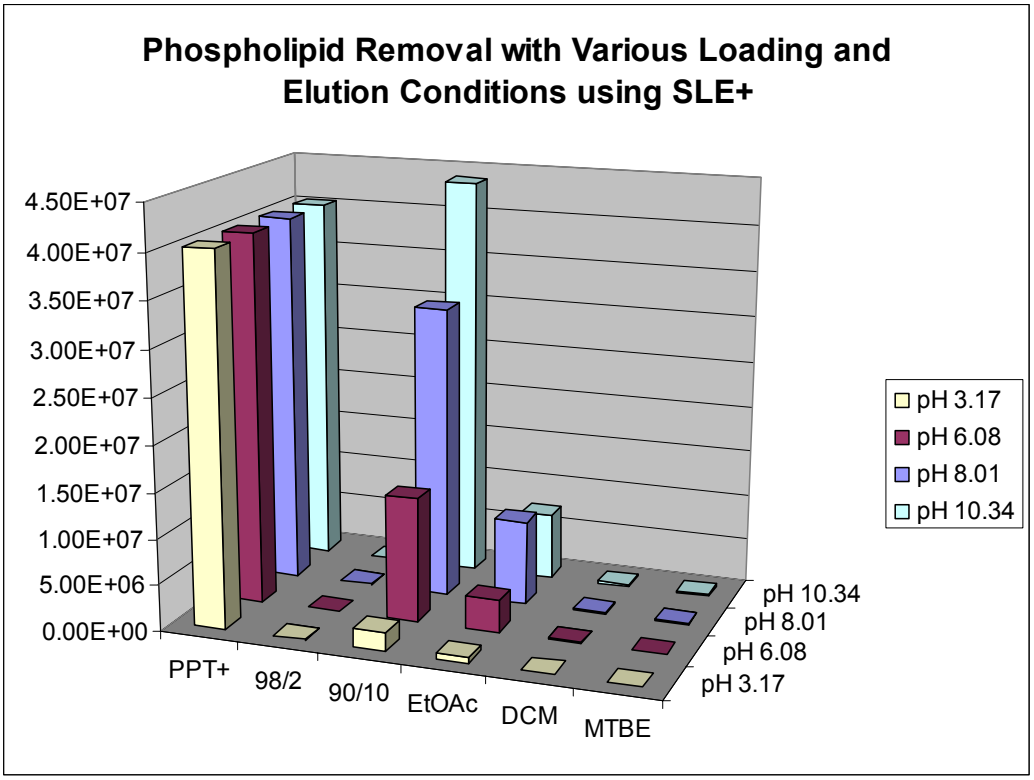


*Figure 5. Chart showing phospholipid peak areas with various loading and extraction solvent protocols compared to PPT+ protein precipitation.*

## Overall Conclusions

1. Excellent phospholipid removal was observed at all loading pH's with 98:2 (v/v) hexane/3-methyl-1-butanol, DCM and MTBE.
2. EtOAc performed better at low pH and showed the highest levels at around pH 8. Since EtOAc is a few percent water soluble then some degree of phospholipids were expected.
3. 90:10 (v/v) DCM/IPA as the most polar extraction solvent gave the highest levels of phospholipids. At acidic pH the phospholipid levels were far lower than when around neutral or basic pH.
4. Overall phospholipid levels were lower at acidic pH 3, even with very polar extraction solvents.

**References**

1. Supported Liquid Extraction: Automate those Tiresome Bioanalytical LLE Protocols Presented at Eastern Analytical Symposium 2005. L. Williams et al

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