Improved Phospholipid Removal using a New Mixed-mode Resin-based SPE Sorbent

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Introduction

Sample preparation is an essential technique prior to LC-MS/MS analysis of drugs in biological fluid samples. Interfering matrix components such as salts, proteins and phospholipids can mask or otherwise interfere with the quantitation of the compound(s) of interest. Polymer-based mixed-mode cation exchange SPE sorbents provide a dual retention mechanism, allowing a rigorous interference elution regime, therefore significantly improving extract cleanliness. Co-extracted matrix components are greatly reduced leading to lower overall ion suppression as shown in other work[1]. The problems associated with protein retention are significantly reduced using this new sorbent. However, phospholipids present a far more difficult challenge. Due to their retentive nature, phospholipids (outline structure shown in **Figure 1**) can be very difficult to remove using SPE and other sample preparation techniques.

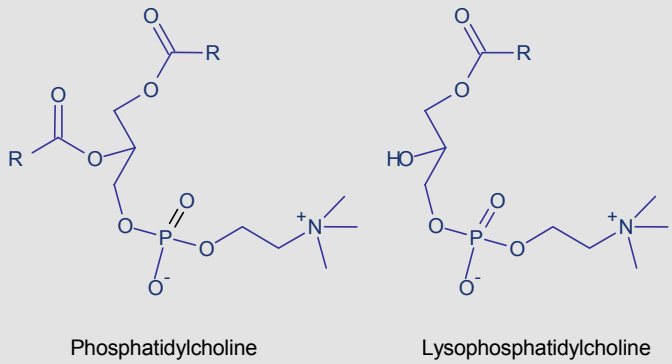

Figure 1. Phospholipid structures

This poster demonstrates the use of a new EVOLUTE® mixed-mode SPE sorbent targeting the specific problems associated with phospholipid removal and investigating various strategies to help reduce their content in the final extract.

Experimental Procedure

Reagents

Formic acid was 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).

Solid Phase Extraction Procedure

EVOLUTE® Mixed-mode Generic SPE Method
SPE Column: EVOLUTE Mixed-mode 25 mg 96-well plate
Sample Pre-treatment: Plasma sample diluted 1:3 (v/v) with aqueous formic acid (1 %, v/v)
Conditioning: Methanol (1 mL)
Equilibration: Aqueous formic acid (0.1%, v/v, 1 mL)
Sample Load: Sample loaded (400 µL diluted plasma)
Interference Wash 1: 0.1M aqueous formic acid (1 mL)
Interference Wash 2: Methanol (1 mL)
Elution: 5% NH_4OH/Methanol (v/v, 1 mL)

Post Extraction: The eluate was evaporated to dryness and the analytes reconstituted in 0.5 mL of 70:30 (v/v) H_2O/MeOH prior to analysis.

All experiments were based on the above generic method varying one specific aspect of the method at a time.

Experiment 1: Sample Load Investigation
Loading Conditions: Sample pre-treatment involved 1:3 plasma dilutions with 1% (v/v) formic acid, H_2O and 0.5% (v/v) aqueous NH_4OH. Column equilibration was also adjusted accordingly with the same buffers.

Experiment 2: Interference Elution Step 2 Investigation
Interference Wash: Methanol, acetonitrile, hexane and isopropyl alcohol were all investigated.

Experiment 3a: Elution Solvent Investigation
Elution: Various concentrations of NH_4OH ranging from 0.1-10% (v/v) were added to MeOH.

Experiment 3b: Elution Solvent Investigation
Elution: Various concentrations of H_2O ranging from 5-20% (v/v) were added to the 5% (v/v) NH_4OH/MeOH elution solvent.

Experiment 3c: Elution Solvent Investigation
Elution: Methanol was replaced as the elution solvent by acetonitrile. In addition to this various concentrations of H_2O ranging from 5-20% (v/v) were added to the 5% (v/v) NH_4OH/MeCN elution solvent.

HPLC Conditons
Instrument: Waters 2795 Liquid Handling System (Waters Assoc., 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/minutes
Gradient: 70:30 (v/v) 0.1% aqueous formic acid / acetonitrile increasing to 90% (v/v) acetonitrile 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 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

Figure 2. shows the phospholipid TIC's comparing a protein precipitated sample with various elution conditions using the EVOLUTE mixed-mode resin.

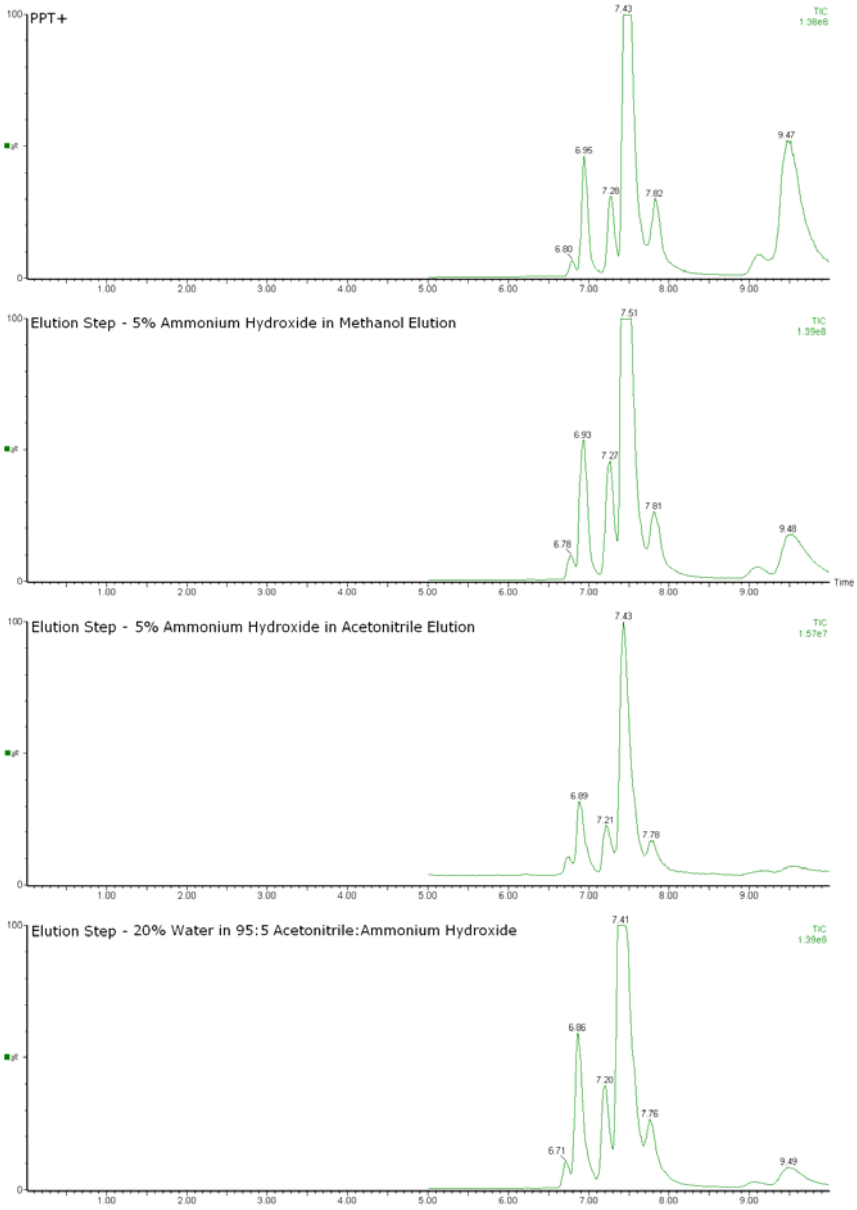

Figure 2. *TIC for selected phospholipid ions comparing human plasma extracted with protein precipitation, EVOLUTE mixed-mode resin with the generic method, 5% NH_4OH/MeCN and 20% H_2O in 5% NH_4OH/MeCN.*

Experiment 1: Load Investigation

The strong cation exchange sorbent is ionized under all pH conditions so it is possible to load using various conditions. **Figure 3**. shows the phospholipid peak areas for the various loading conditions investigated. Changing the load conditions from acidic to neutral or basic loading did not have a significant effect on phospholipid content in the final extract.

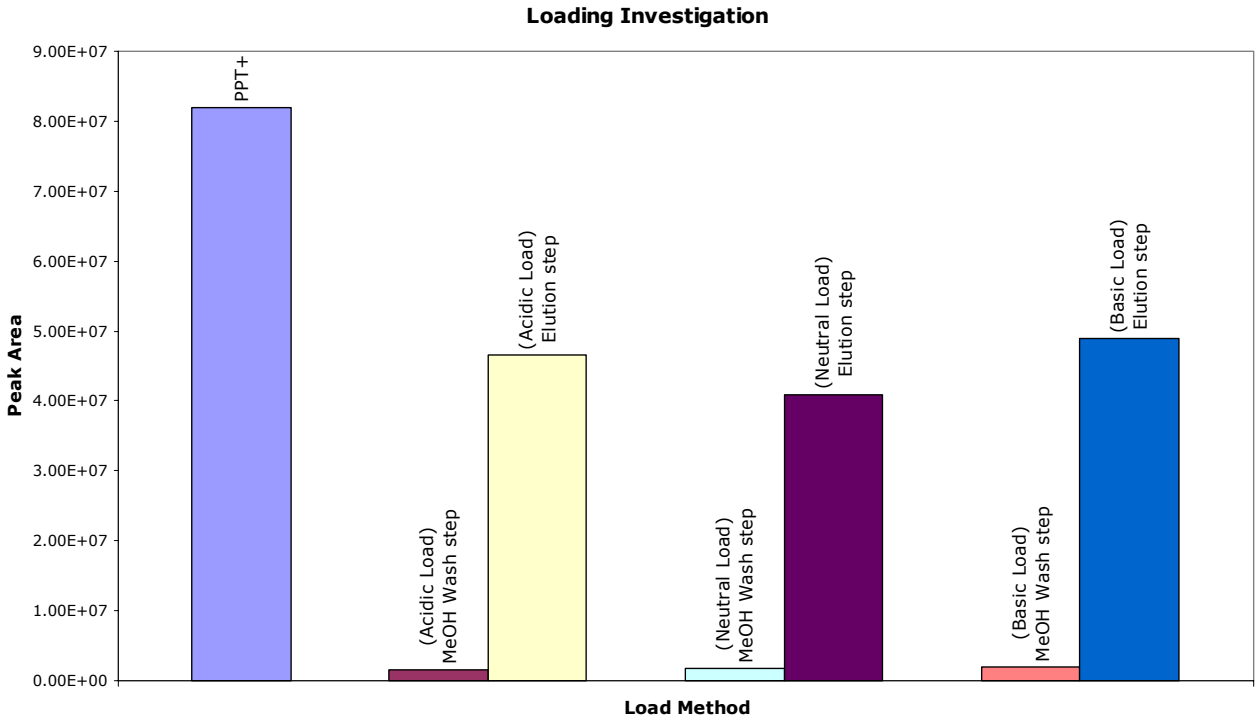

Figure 3. Bar chart showing phospholipid peak areas in wash step 2 and elution steps with various loading protocols compared to ISOLUTE PPT+ protein precipitation.

Experiment 2: Interference Wash 2 Investigation

The effect of varying wash step 2 between methanol, isopropyl alcohol, hexane and acetonitrile is shown in **Figure 4**. No significant difference exists between the various washing protocols.

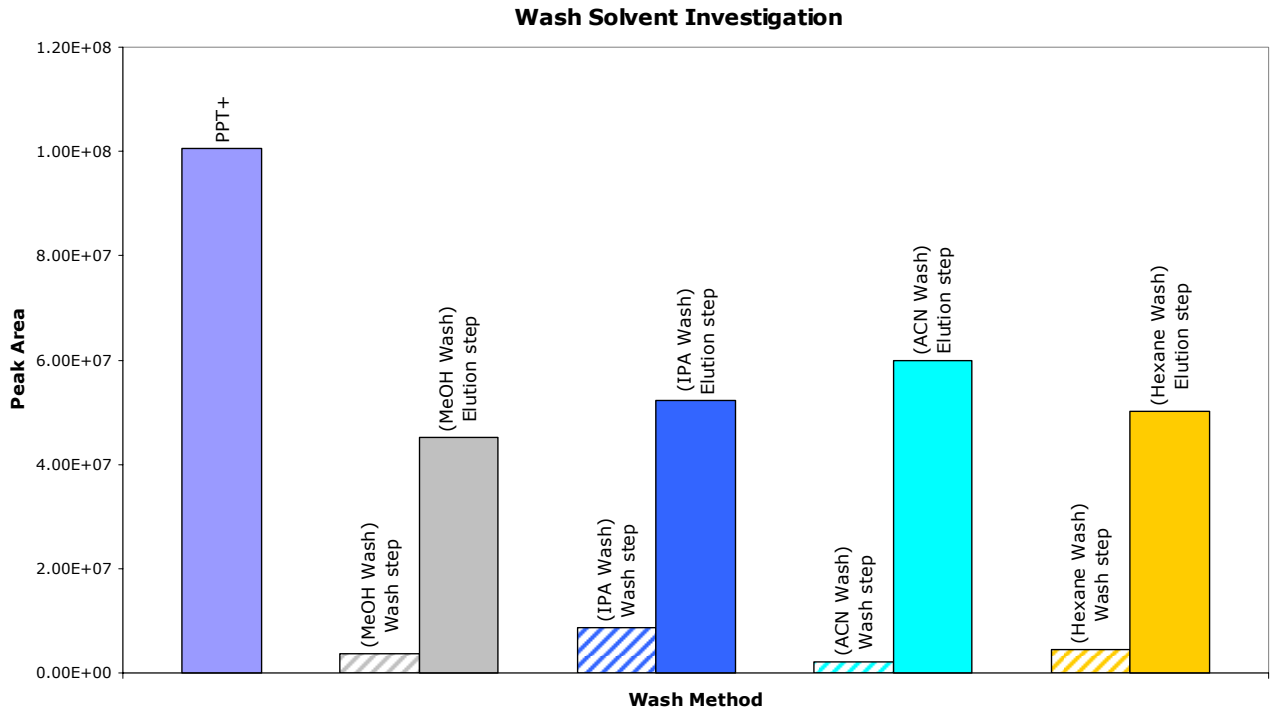

Figure 4. Bar chart showing phospholipid peak areas in wash step 2 and elution steps with various washing protocols compared to ISOLUTE PPT+ protein precipitation.

Experiment 3: Elution Solvent Investigation

Three experiments were conducted in order to determine the most effective elution solvent leading to reduced phospholipid content in the final extracts. The first experiment investigated the elution solvent in terms of the NH_4OH concentration. Most mixed-mode cation exchange methods use at least 2% NH_4OH in the elution solvent so we investigated a range between 0.1 and 10%. At 0.1% NH_4OH in MeOH the phospholipid content in the final extraction is about 50% of all the other levels. The phospholipid content is then relatively consistent between 0.5-10 %, as shown in **Figure 5**.

The second set of experiments investigated the addition of various proportions of H_2O, between 5-20%, in the 5% NH_4OH/MeOH elution solvent. This resulted in lower levels of phospholipids in the final extracts as shown in **Figure 6**. At 20% H_2O the levels were approximately 20% when compared to the generic method.

The final elution investigation involved replacing the 5% NH_4OH/MeOH elution solvent with 5% NH_4OH/MeCN. The addition of H_2O to this elution solvent was also investigated as per the previous investigation. 5% NH_4OH/MeCN showed far lower phospholipid levels than the corresponding 5% NH_4OH/MeOH generic method. Once H_2O was added to the elution solvent the phospholipid levels increased showing the opposite effect previously noted for H_2O/MeOH combinations. **Figure 7** shows the bar chart containing these comparisons.

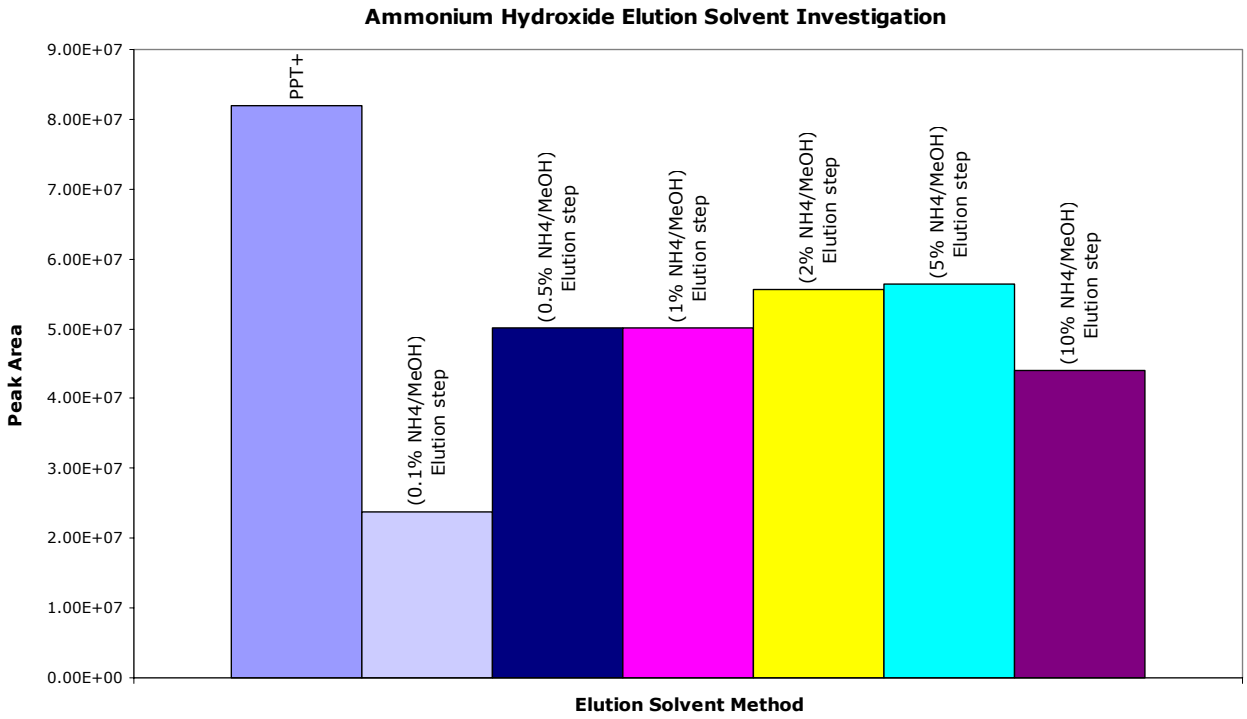

Figure 5. Bar chart showing phospholipid peak areas using various NH_4OH elution protocols compared to ISOLUTE PPT+ protein precipitation.

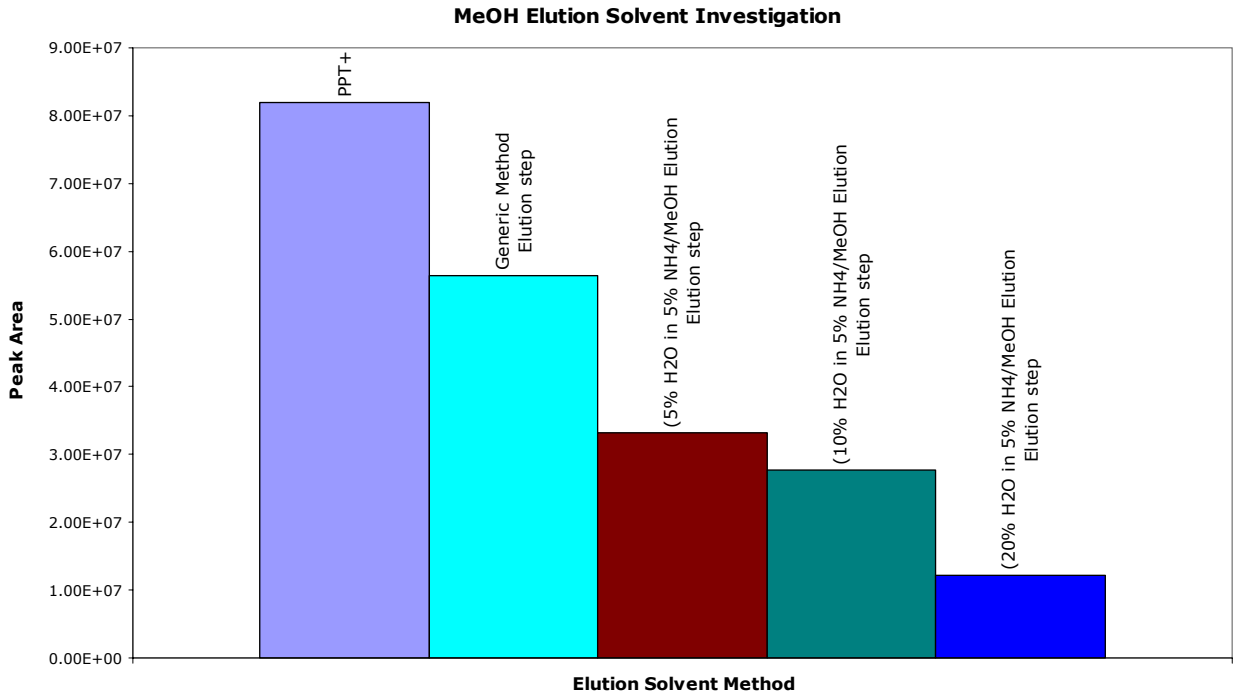

Figure 6. Bar chart showing phospholipid peak areas using various MeOH/H_2O elution protocols compared to ISOLUTE PPT+ protein precipitation.

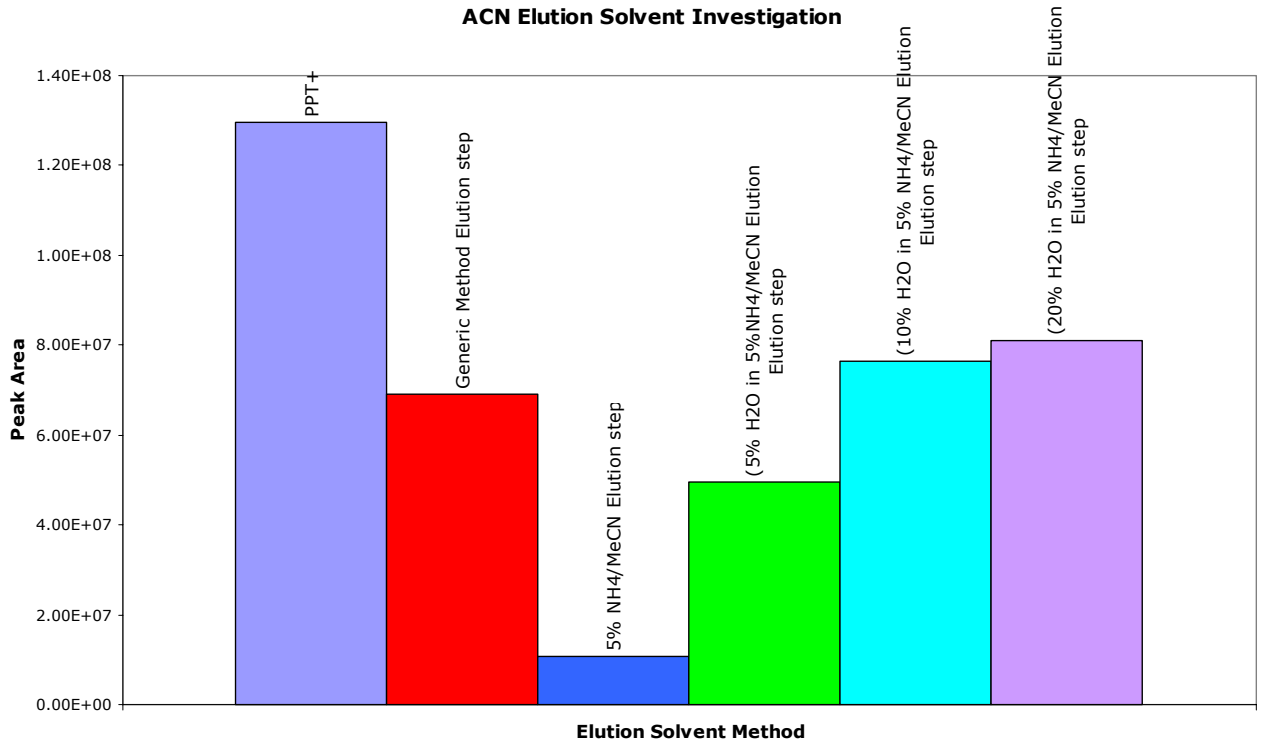

Figure 7. Bar chart showing phospholipid peak areas using various ACN/H_2O elution protocols compared to ISOLUTE PPT+ protein precipitation.

Conclusions

- Changing the load conditions did not significantly reduce the phospholipid content in the final extracts.
- Modifying the organic interference wash did not significantly affect the phospholipid content in the final extracts.
- Reducing the NH_4OH content to 0.1% (v/v) in the MeOH elution solvent resulted in an approximate 50% reduction in phospholipid content. However, depending on analyte retention this may not be sufficient to yield good recoveries.
- The addition of up to 20% (v/v) H_2O in the 5% (v/v) NH_4OH/MeOH elution solvent reduces phospholipids by up to 80%.
- Using 5% (v/v) NH_4OH/MeCN shows far lower phospholipid levels than the corresponding 5% (v/v) NH_4OH/MeOH elution solvent. However, increasing the H_2O content increases the phospholipid levels.
- Overall, modification of the generic method, depending on analyte properties can give significantly enhanced removal of phospholipids and result in cleaner extracts and more reliable quantitation.

Reference:
1. A Generic Approach to the Extraction of Multi-functional Drugs using Mixed-mode SPE with LC-MS/MS Analysis.
Matthew Cleeve, Scott Merriman, Lee Williams et al.
Presented at EAS 2007 12-15 November 2007, Somerset, NJ.

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