Evaluation of Inter-Species Phospholipid Removal, using a New Resin-based Mixed-mode SPE Sorbent and LC-MS/MS Analysis

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Introduction

EVOLUTE® CX is a new resin-based mixed-mode SPE sorbent incorporating hydrophobic and strong cation exchange functionalities. The dual retention mechanism used in mixed-mode SPE allows a rigorous interference elution regime resulting in significantly cleaner extracts. Endogenous phospholipids (see examples in **Figure 1**) present in biological fluids are a major problem in LC-MS/MS analysis as they are often very difficult to remove during sample preparation. If they are not removed, they retain very strongly on reversed phase analytical columns. If high organic (end of run) washes are not incorporated into the LC methods these matrix components may elute in subsequent analyses causing regions of suppression/enhancement leading to inaccurate quantitation.

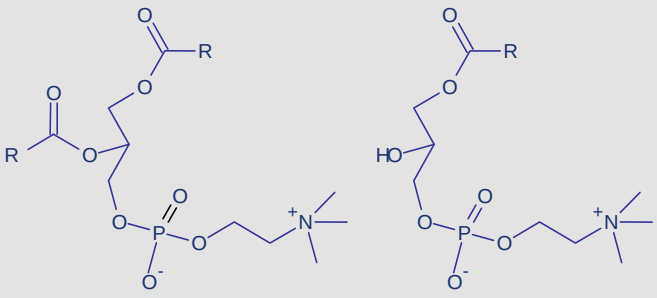

Figure 1. *Phospholipid structures*

This poster describes the use of a new resin-based mixed-mode SPE sorbent to address the specific problems associated with phospholipid removal. An inter-species investigation was performed on dog, rat, mouse and human plasma to evaluate the effectiveness of phospholipid removal when analyzing different species, although an in depth plasma comparison was not the objective.

Experimental Procedure

Reagents

Formic acid, acetic acid and ammonium acetate were purchased from Sigma Chemical Co. (Poole, UK). Blank plasma using lithium heparin as the anti-coagulant was purchased from Sera Laboratories International (West Sussex, UK). All solvents were HPLC grade from Fisher Scientific (Loughborough, UK).

EVOLUTE CX Sample Preparation Procedure

Extractions were performed using the 25 mg 96-well format with blank plasma (100 µL)
Sample Pre-treatment: Plasma sample diluted with 50 mM ammonium acetate buffer at pH 6 (1:3, v/v)
Column Conditioning: Methanol (1 mL)
Column Equilibration: 50 mM ammonium acetate buffer at pH 6 (1 mL)
Sample Loading: Pre-treated plasma sample (400 µL)

Interference Elution 1: 50 mM ammonium acetate buffer at pH 6 (1 mL)
Interference Elution 2: Methanol (1 mL).
Analyte Elution: 5% (v/v) NH_4OH in methanol (1 mL).
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 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

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 full scan, selected ion recording (SIR) and multiple reaction monitoring (MRM) modes. Full scan data was acquired scanning from 250-900 Da and from this SIR ions were selected. Many of these ions were in the $[M+Na]^+$ form and were not subsequently used for the MRM data ($[M+H]^+$ ions only). Full MRM transitions and ionization conditions for the most abundant phospholipids observed are given in **Table 1** below.
Desolvation Temperature: 350 °C
Ion Source Temperature: 100 °C
Collision Gas Pressure: 2.6 x 10^{-3} mbar (MRM only).

***Table 1**. Quattro Ultima Pt mass spectrometer parameters for MRM data.*

Scan Function	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
1	494.4 > 184.0	55	16
	496.4 > 184.0	55	16
	520.4 > 184.0	55	16
	522.4 > 184.0	55	16
	524.4 > 184.0	55	16
2	701.7 > 184.0	55	16
	703.7 > 184.0	55	16
	732.8 > 184.0	55	16
	756.5 > 184.0	55	16
	758.5 > 184.0	55	16
	760.5 > 184.0	55	16
	784.6 > 184.0	55	16
	786.6 > 184.0	55	16
	806.6 > 184.0	55	16
	808.7 > 184.0	55	16
	810.9 > 184.0	55	16

Results

Figure 2 shows the full scan total ion chromatograms obtained with each of the plasma types using ISOLUTE PPT+. Slight differences were observed but the most abundant ions were very similar between the species. A range of ions were subjected to SIR analysis. It was noted that many phospholipids not only gave the $[M+H]^+$ but also gave fairly intense $[M+Na]^+$ ions. Only the most abundant protonated molecular ions were selected for MRM analysis.

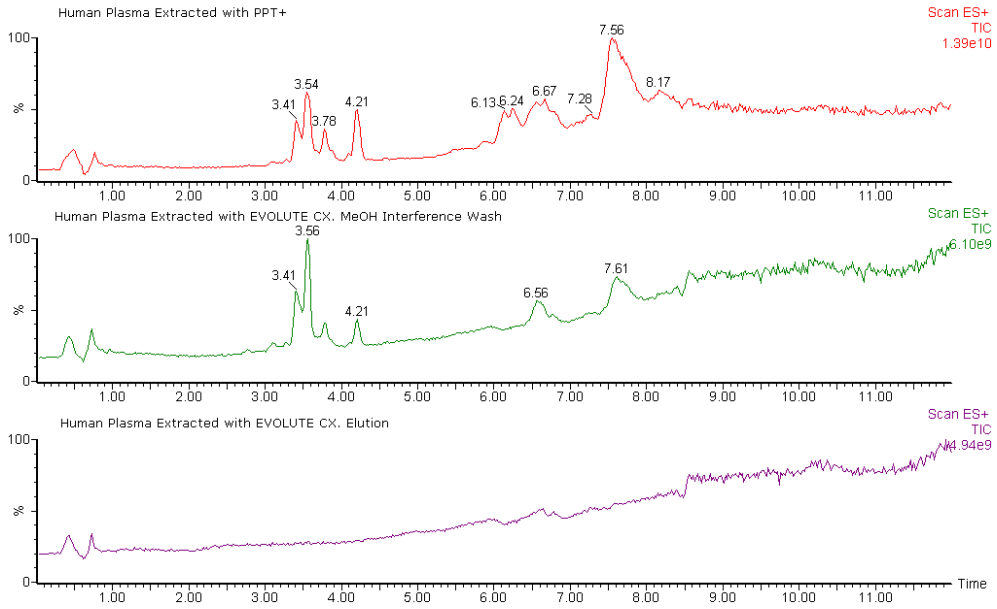

***Figure 2**. Full scan (250-900) TIC for four plasma species extracted using ISOLUTE PPT+.*

Figure 3 shows the full scan TIC's comparing phospholipid removal using human plasma extracted with ISOLUTE PPT+ and EVOLUTE CX. Some phospholipids were contained in the methanol interference wash but very little observed in the final elution fraction.

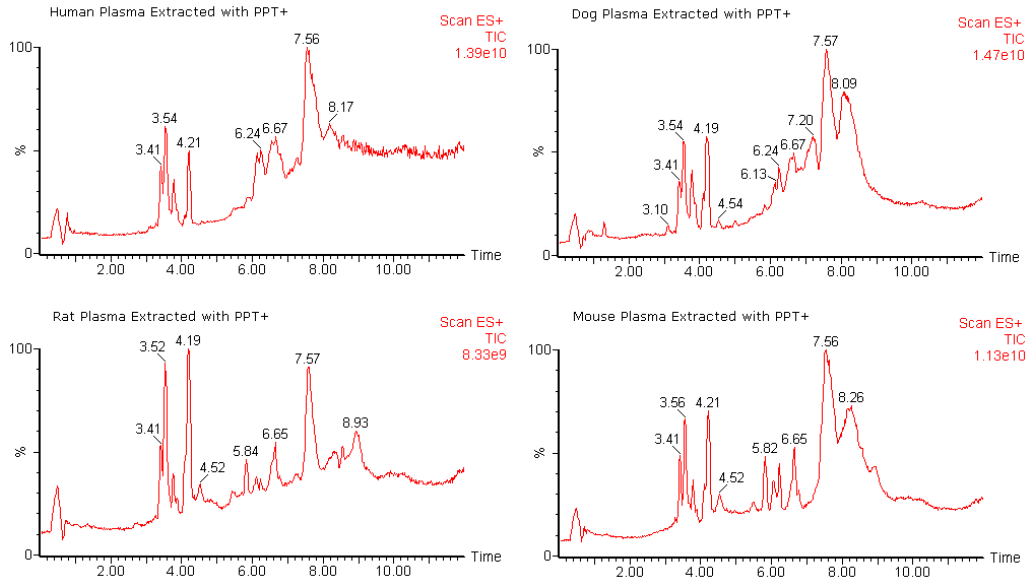

***Figure 3**. Full scan (250-900) TIC comparison of human plasma extracted with ISOLUTE PPT+ and EVOLUTE CX.*

Figure 4 shows the SIR traces observed for dog plasma extraction using both ISOLUTE PPT+ and EVOLUTE CX. Some of the phospholipids are retained until the MeOH interference wash step but very little retained until the elution fraction.

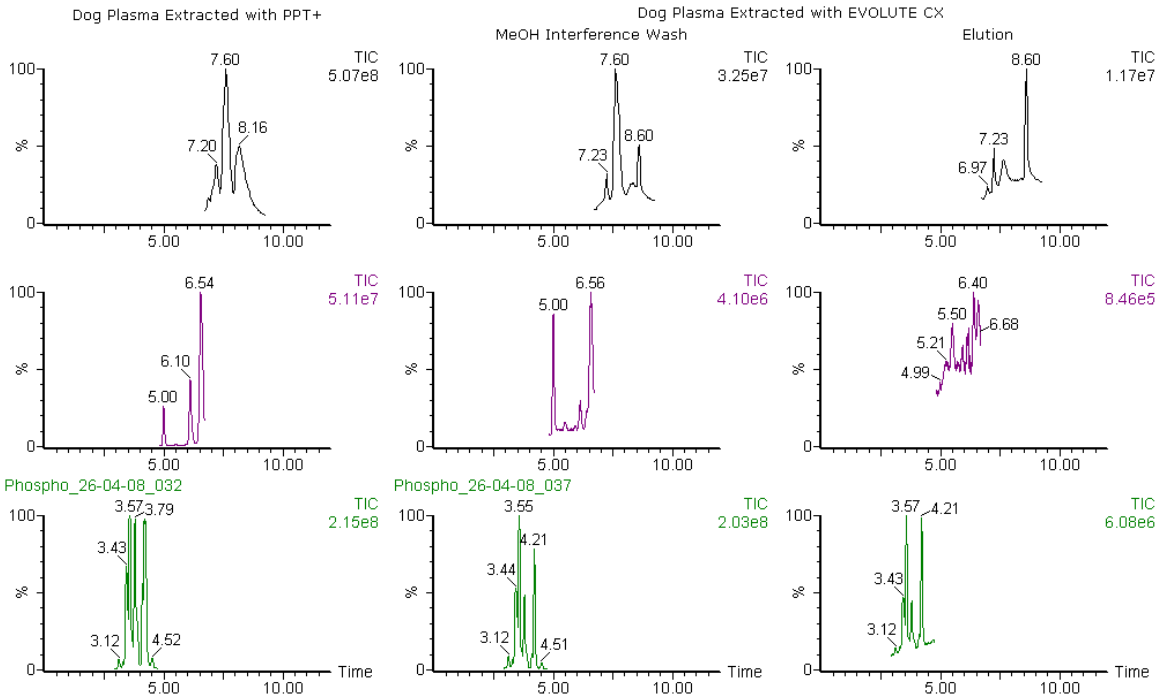

***Figure 4**. SIR TIC comparison of dog plasma extracted with ISOLUTE PPT+ and EVOLUTE CX.*

Figures 5 and 6 show the MRM traces obtained from rat and mouse plasma, respectively. As can be seen most of the phospholipids are removed prior to the elution fraction.

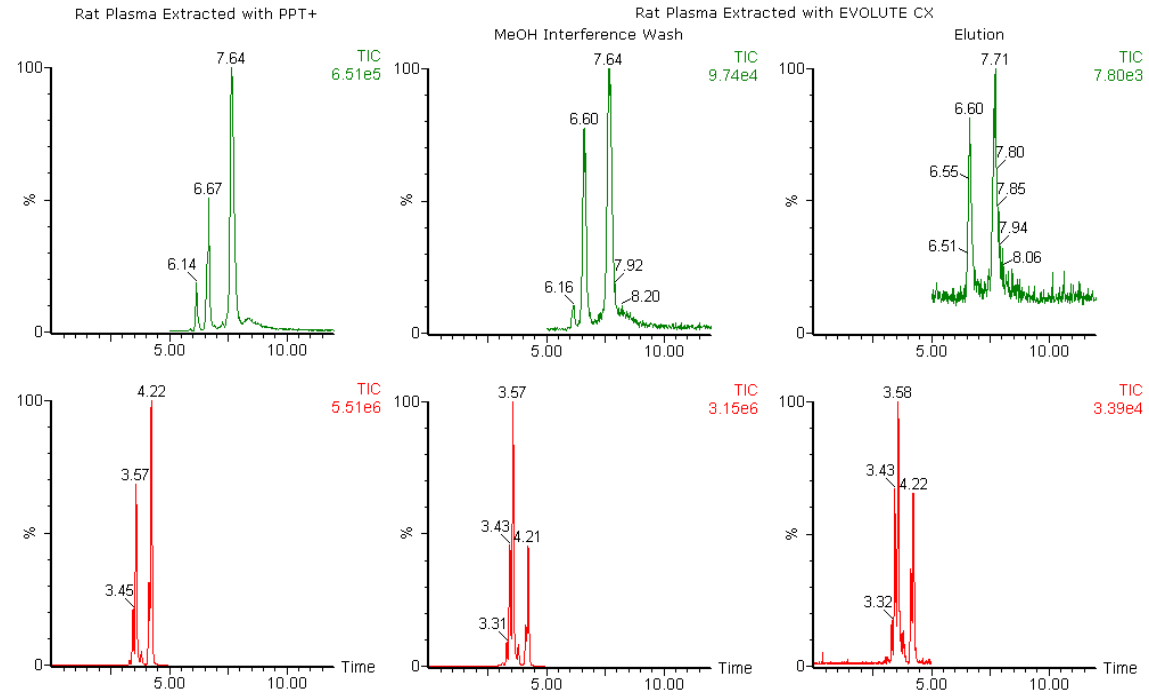

***Figure 5**. MRM TIC comparison of rat plasma extracted with ISOLUTE PPT+ and EVOLUTE CX.*

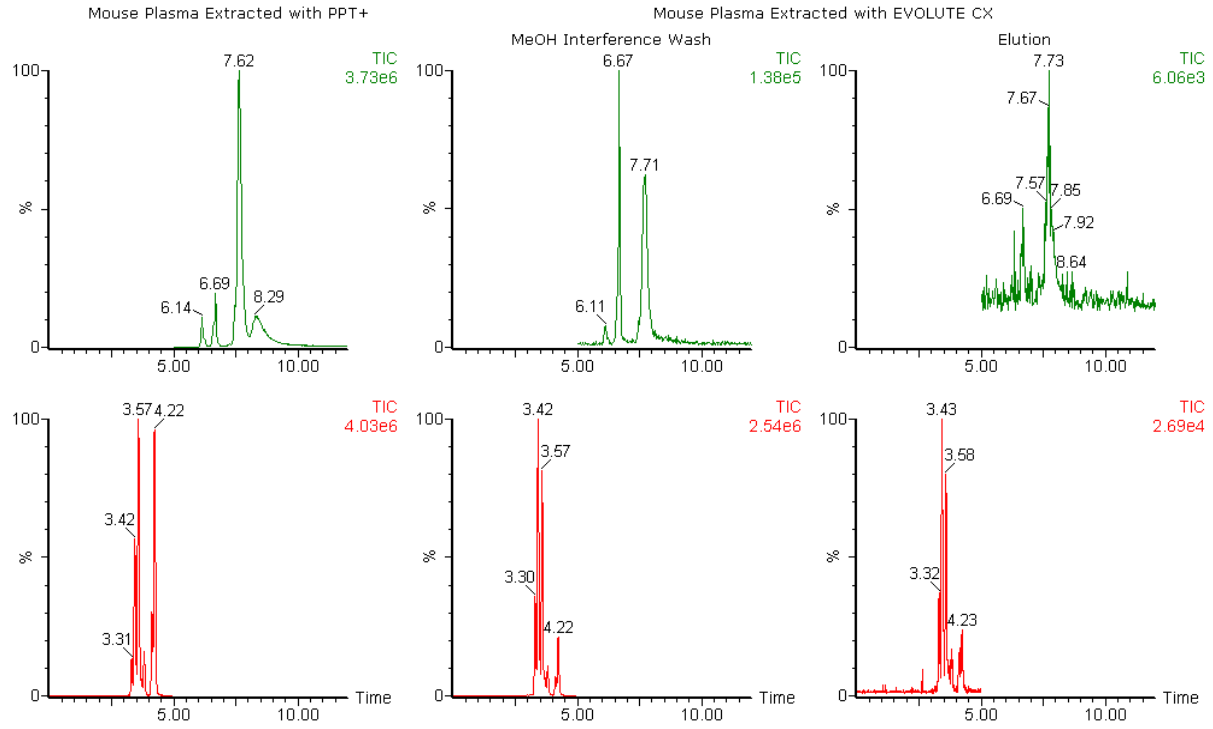

***Figure 6**. MRM TIC comparison of mouse plasma extracted with ISOLUTE PPT+ and EVOLUTE CX.*

Figure 7 shows a bar chart showing the total amount of phospholipids in the elution fractions for each plasma species compared to an ISOLUTE PPT+ extracted sample. The PPT+ phospholipid levels for each species was set to 100% and used as the authentic standard for comparison.

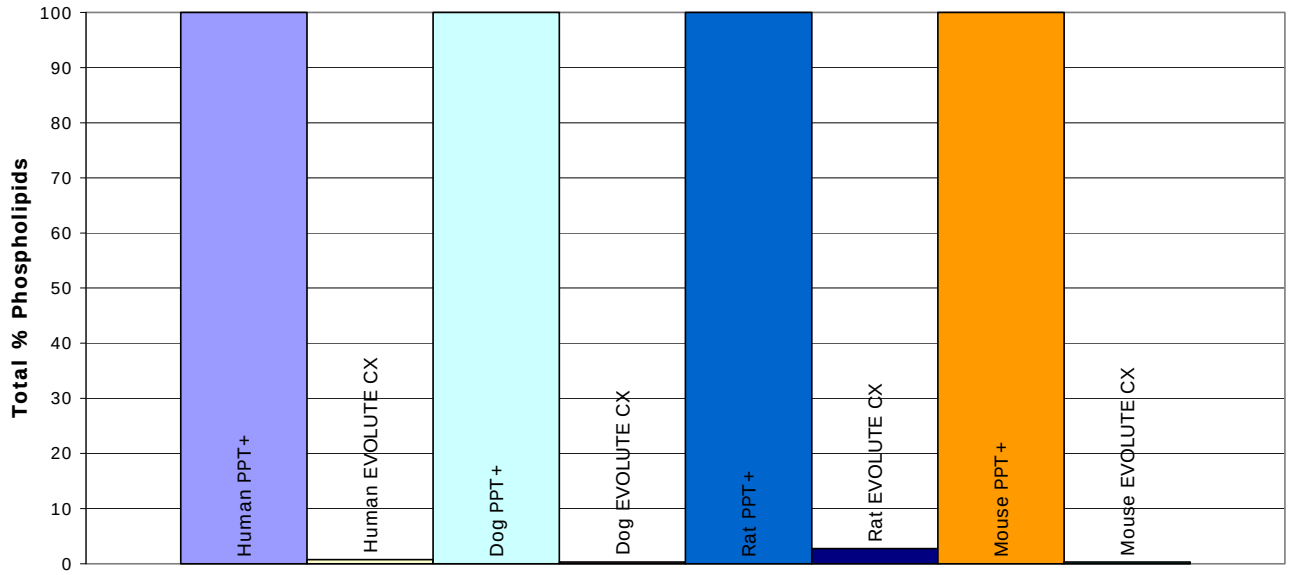

***Figure 7**. Total Phospholipid levels comparing ISOLUTE PPT+ and EVOLUTE CX*

Overall Conclusions

- EVOLUTE CX provides excellent phospholipid removal for all four plasma species as shown by full scan, SIR and MRM data.
- Overall phospholipid removal using EVOLUTE CX compared to ISOLUTE PPT+ extracted samples for all plasma types showed greater than 97%.

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