COMPARISON OF SPE PROTOCOLS FOR PHOSPHOLIPID REMOVAL IN BASIC ANALYTE BIOANALYTICAL QUANTITATION

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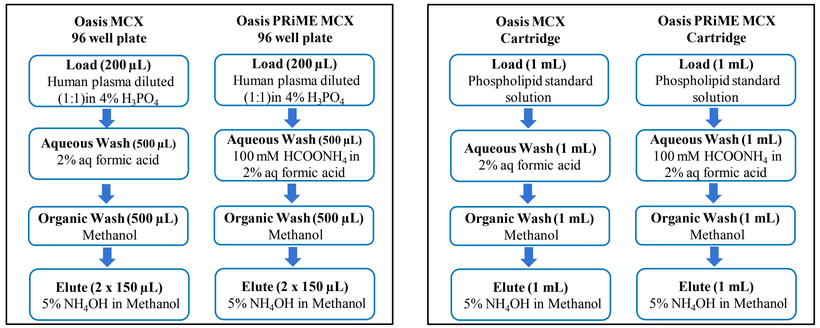
INTRODUCTION

Phospholipids (PLs) are a class of lipids that are known to be important not only as energy source but also as functional molecules in several biological processes. Due to their diverse function and presence as main constituents in cell membrane, their encounter is inevitable in most biological samples, their presence pose a major challenge in small molecule bioanalytical method development and validation. PLs possess complex structures with both non-polar and polar region, due to their complex structure PLs can range in polarity from neutral to polar ionic molecules. For instance over 500 distinct lipid molecular species, categorized into six major PL classes were reported in human plasma [1]. The matrix effect caused by these PLs in bioanalysis is tremendous; their severe interference comes not only from their high concentrations and overall presence, but also, more importantly, from their diversity with regard to classes, subclasses, polarities and molecular weights [2]. For instance in a generic LC-MS run of a protein precipitated plasma sample the PLs could be found to appear throughout the chromatographic run time, imparting ion suppression or enhancement effects on the target analyte eluting at any instance in LC-MS analysis.

Here a direct quantitative comparison of two different mixed-mode reversed-phase/cation-exchange solid phase extraction (SPE) protocols using Oasis mixed mode cation exchange sorbent (Oasis MCX) is made. Oasis MCX sorbent consists of sulfonic acid groups as a strong cation exchange component, N-vinylpyrrolidone as a hydrophilic component and divinylbenzene as a hydrophobic component and hence the analyte retention mechanism could be a combination of ion-exchange, hydrophilic and hydrophobic interactions depending on the charge state and polarity of the analyte and the composition of the solution.

The effectiveness of the protocols in the removal of highly abundant endogenous PLs, phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), sphingomyelins (SM) and cholesterol are compared using human blood plasma sample matrix and commercially available phospholipid standards.

SPE PROTOCOL



SAMPLE PREPARATION

Human Blood Plasma

- 200 µL of replicate human blood plasma (Bioreclamation IVT # HUMANPLK2PN) diluted (1:1) with 4% phosphoric acid.
- The 96 well plate (30 mg sorbent per well) SPE protocol described above for Oasis MCX and Oasis PRiME MCX is performed. A minimum of three replicates were carried out.
- The eluates from each step (Aqueous Wash, Organic Wash and Elute steps) of the protocol were injected into the LC-MS/MS.
- Relative percentage of different PLs and cholesterol measured in each of the eluates.

Phospholipid Standards

- L-α-PC, L-α-LPC, SM, L-α-PI L-α-PI and L-α-PS (Avanti, USA) standard solutions were prepared by dissolving 10 mg of the standards separately in 10 mL of methanol and acetonitrile (1:1, v/v)
- SPE protocol (1 cc Cartridge, 30 mg sorbent /well) described above were performed on standards. Three replicates were carried out.
- The eluates from each step (Load, Aqueous Wash, Organic Wash and Elute Steps) of the protocol were injected into UPLC-PDA.
- Peak areas of PLs in each steps of the protocol were compared to the peak areas in standard solution.

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LC-MS CONDITIONS

Instruments: ACQUITY UPLC I-CLASS coupled to XEVO TQ-S triple quadrupole mass spectrometer
Data Analysis: MassLynx v4.1
LC method conditions:
Column: ACQUITY BEH C18, 1.7 µm, 2.1 x 100 mm
Column temp: 50 ºC, Injection Volume: 1 µL
Flow rate: 0.6 mL/min
Mobile Phase A: 0.1 % (v/v) Formic acid in Water
Mobile Phase B: 0.1 % (v/v) Formic acid in Acetonitrile
Gradient Conditions:

Time (min)	Mobile Phase A %	Mobile Phase B %
0.0	98.0	2.0
1.0	98.0	2.0
4.0	30.0	30.0
4.1	5.0	95.0
10.0	5.0	95.0
10.5	98.0	2.0
12.0	98.0	2.0

MS Conditions: ESI Positive
Capillary voltage: 3.0 V, Desolvation Temp: 500 ºC
Desolvation gas flow: 800 L/hr, Cone gas : 200 L/hr
Nebulizer: 7.0 bar, Cone voltage: 30 V, Collision: 30 V.
LPC, PC and SM were monitored by precursor ion scan of 104.0 and 184.1, respectively.

MRM (Multiple Reaction Monitoring) conditions:

Compound ID	Compound name	Precursor ion (M+H)	Product ion (M+H)
Cholesterol	Cholesterol	369.4	287.4
PG-1	PG(36:1)	301.0	129.0
PG-2	PG(38:5)	797.5	625.5
PE-1	PE(36:2)/ PE(36:3e)/PE(36:2p)	744.6	603.6
PE-2	PE(38:4)	768.6	627.6

LC-PDA CONDITIONS

Instruments: ACQUITY UPLC with PDA detector
Data Analysis: Empower 3
LC method conditions:
Isocratic with either Mobile phase A or B
Column: ACQUITY BEH HILIC 1.7 µm, 2.1 x 100 mm
Column temp: 40 ºC, Sample Temperature: 22 ºC
Injection Volume: 0.5 µL, Flow rate: 0.45 mL/min
Mobile Phase A: 95% Acetonitrile, 4% Water, 1% Phosphoric acid (v/v/v) for all PLs standards except PI
Mobile Phase B: 95% Acetonitrile, 4% Methanol, 1% Phosphoric acid (v/v/v) for PI
PDA conditions:
Channel-1: 200 nm, Absorbance, 4.8 nm Resolution

RESULTS

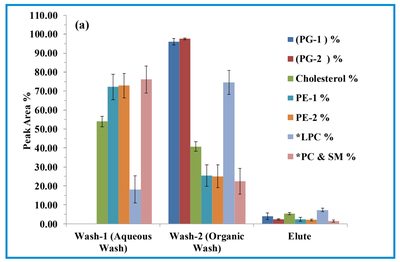


Figure 1. Percentage of different PLs in Human Blood plasma that elute during the WASH and ELUTE steps of (a) Oasis PRiME MCX and (b) Oasis MCX Protocol.

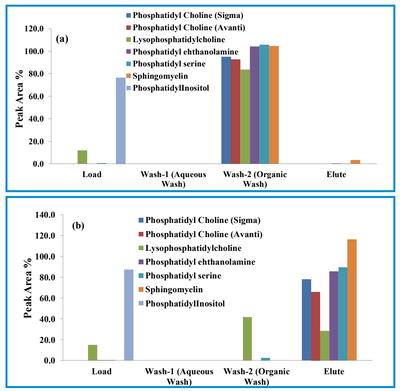
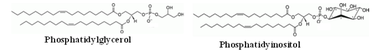


Figure 2. Peak area % observed for different PL standards that elute during LOAD, WASH and ELUTE steps of (a) Oasis PRiME MCX and (b) Oasis MCX protocol.

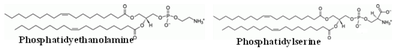
DISCUSSION

Phosphatidylglycerol (PG) and Phosphatidylinositol (PI): Figure 1 shows that PG is mostly removed (>95%) in the organic wash step of the protocols (Oasis PRiME MCX and Oasis MCX), leaving less than 5% in the elute step and Figure 2 shows that PI is unretained and is eluted in the load step. This could be attributed to the charge state of PG and PI under SPE conditions. The phosphate groups in PG and PI have been reported to have pK_a values of 3.5 and 2.5 respectively [4]. Thus under the loading conditions (2% phosphoric acid with pH ≤ 1.0) the hydrophilic head groups of PG and PI are predominantly unionized preventing ion exchange interactions with the sorbent. The interaction of PG with the sorbent is primarily hydrophobic and thus it elutes when washed with organic solvent, while the hydrophilicity of the inositol group in PI weakens any hydrophobic interaction with the sorbent and hence it is unretained under the SPE conditions.



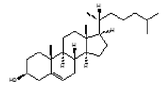
Representative Structures

Phosphatidylethanolamine (PE) and Phosphatidylserine (PS): Figures 1b and 2b show that PE and PS are retained at significant levels until the elution step using the Oasis MCX protocol. However in the case of Oasis PRiME MCX, > 95% of PE and PS are eluted during the aqueous and organic wash steps. This could be attributed to the charge state and polarity of PE and PS under the SPE conditions. The phosphate groups in PE and PS have been reported to have pK_a values of 1.7 and 2.6 respectively and the amine moieties pK_a values of 9.8 and 11.55 respectively [4]. Thus under the loading conditions (2% phosphoric acid with pH < 1.0) the hydrophilic head groups of PE and PS are predominantly positively charged (cationic) resulting in cation exchange interaction with the sorbent, in addition to hydrophobic interactions with the hydrophobic tails. The aqueous wash steps (2% formic acid: pH 2.0 or 100 mM ammonium formate with 2% formic acid: pH 2.7) partially convert PE and PS to zwitterions, weakening the cation exchange interaction with the sorbent thereby causing them to elute in the aqueous and organic wash steps. The greater pH and ionic strength of the ammonium formate buffer over aqueous formic acid improves the



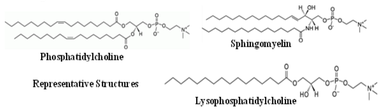
Representative Structures

Cholesterol: Figure 1 shows that cholesterol is removed during both the aqueous and organic wash steps of the protocol. This could be attributed to the charge state and polarity of cholesterol under the SPE conditions. Cholesterol does not have a charged head group for cation exchange interaction with the sorbent. Therefore its interactions with the sorbent is primarily hydrophobic and it thus elutes in the organic wash step. Cholesterol is inherently present in plasma at very high concentrations (115 to 295 mg/dl) [5] and thus requires a large wash volume to remove, which may explain why it elutes in both the wash steps.



Cholesterol

Phosphatidylcholine, Lysophosphatidyl choline and Sphingomyelin (PC, LPC and SM): Figures 1 and 2 show that for Oasis PRiME MCX PC, LPC and SM are largely removed in the aqueous and organic wash steps, while for Oasis MCX a significant portion is found in the final elute step. This could be attributed to the charge state and polarity of PC, LPC and SM under SPE conditions. Under the loading conditions (2% phosphoric acid, pH < 1.0) the polar head groups of PC, LPC and SM are positively charged and retained on the sorbent by cation exchange in addition to hydrophobic interactions. The strong cation exchange binding is weakened due to the higher pH and ionic strength of the aqueous 100 mM ammonium formate with 2% formic acid wash for Oasis PRiME MCX by partially converting PC, LPC and SM to zwitterions, thus causing them to elute in the aqueous and organic wash steps. The lower pH and ionic strength of the 2% aqueous formic acid wash for Oasis MCX is not effective at converting PC, LPC and SM from cations to zwitterions, thus the cation exchange interaction retains these phospholipids until the elute step, wherein they are eluted with a solution of a strong base in an organic solvent.



Representative Structures

CONCLUSIONS

This poster highlights the removal of different PLs using Oasis PRiME MCX and Oasis MCX. Both protocols were effective in the removal (> 95%) of phosphatidylinositol, phosphatidylglycerol and cholesterol from the sample either during the load, aqueous wash or organic wash steps of the protocol. While Oasis MCX was less effective in the removal of phosphatidylcholine, lysophosphatidylcholine, sphingomyelin, and phosphatidylethanolamine, Oasis PRiME MCX effectively removed (>95%) of all the phospholipids either during the aqueous wash or organic wash steps of the protocol, producing a much cleaner sample for LC-MS analysis.

REFERENCES

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