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Investigation of endogenous blood plasma phospholipids, cholesterol and glycerides that contribute to matrix effects in bioanalysis by liquid chromatography/mass spectrometry

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ABSTRACT

Matrix effects caused by compounds endogenous to the biological sample are a primary challenge in quantitative LC/MS/MS bioanalysis. Many approaches have been developed to minimize matrix effects such as optimization of sample extraction procedures and use of isotopically labeled internal standards. Unexpected matrix components may still remain undetected, however, because of the selective mass transitions monitored during MS/MS analysis. Glycerophosphocholines are the major phospholipids in plasma that have been widely shown to cause significant matrix effects on electrospray ionization efficiencies for target analytes. The purpose of this work was to investigate potential matrix effects resulting from different endogenous lipid classes, including phospholipids, acylglycerols and cholesterols, in order to establish a library for the relative presence of these components in biological sample extracts obtained by commonly used sample preparation techniques. Thirteen compounds were selected which were representatives of eight phospholipids classes, mono, di, triacylglycerols, cholesterol and cholesterol esters. Post-column infusion experiments were carried out to compare relative ion suppression effects of these compounds. Chlorpheniramine and loratadine were selected as model test analytes. A Concentration Normalized Suppression Factor (%CNSF) was defined to allow comparison of ion suppression effects resulting from different endogenous lipids according to their typical concentrations in human plasma and erythrocytes. A simple LC/MS/MS method was developed to monitor these endogenous components in sample extracts and their extraction recoveries from a plasma pool were compared using protein precipitation, liquid–liquid extraction, supported-liquid extraction, solid phase extraction and Hybrid SPE-precipitation methods. Endogenous lipid components other than GPChos, such as cholesterols and triacylglycerols, may result in significant matrix effects and should be monitored during method development. No single extraction procedure was efficient in removing all of the various lipid components. Use of the results presented here, along with a consideration of analyte chemical structure, the type of matrix and the type of sample preparation procedure, may help a bioanalytical scientist to better anticipate and minimize matrix effects in developing LC/MS/MS-based methods.

1. Introduction

A major challenge in LC/MS/MS method development is variable selectivity due to the effect of the sample matrix on ionization efficiency. Matrix effects resulting from co-elution of organic and inorganic matrix components with the target analytes are often undetected at the selected mass transitions monitored for analysis, but can significantly affect the efficiency and reproducibility of the ionization process [1,2] and thus impact the sensitivity and accuracy of the assay. Matrix effects can also cause analytical problems due to variability in the concentrations of matrix components from one biofluid source (i.e. donor) to another. Matrix ions that compete with the analyte for ionization or disrupt the electrospray processes (e.g. droplet evaporation) constitute the main causes of these matrix effects [3]. Matrix effects may also result in retention time shifts, elevated baselines and divergent calibration curves [4]. These problems may be due to accumulation of strongly retained endogenous matrix components on the analytical column, and over time these components may slowly elute from the column causing increasing background signals and ionization sensitivity drift.

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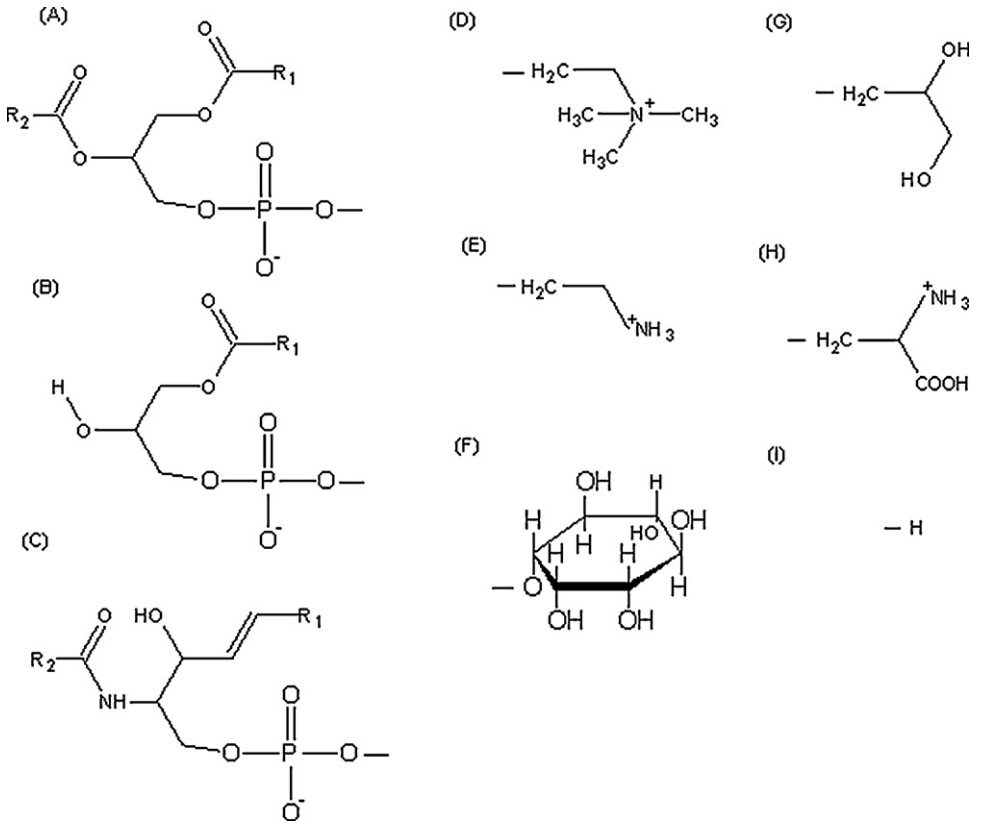

Fig. 1.

Matrix effects are biofluid dependent; matrix components of each biofluid may cause ion suppression or enhancement effects at different times and to a different degree [5].

Matrix effects are also analyte dependant with the degree of matrix effects dependent on the chemical nature of the compound. Generally polar compounds are more susceptible to ion suppression than less polar compounds. Post-extraction addition samples (vs. neat solutions) and post-column infusion techniques are commonly used to evaluate matrix effects. Matrix effects should be investigated even when using stable isotope-labeled internal standards (SIL-ISs) because they may not completely compensate for all matrix effects [6,1]. Phospholipids constitute the majority of the lipid bi-layer of cell membranes, such as erythrocytes, and are considered the main group of endogenous components that are known to cause matrix effects [7–9]. The type and quantity of lipids vary from one plasma source to another according to individual diets and metabolic rates [3]. Phospholipid molecules (e.g. GPChos) are generally characterized by two functional group regions; the first is the polar head group, which includes an ionizable negatively charged phosphate group and a positively charged quaternary amine group that are responsible for the strong ionic character of the phospholipids. The second functional group region is comprised of one or two long chain fatty acid esters that are responsible for the hydrophobicity of phospholipids [9,10]. Phospholipids are composed of ester or amide derivatives of glycerol or sphingosine with fatty acids and phosphoric acid. The phosphate moiety may be esterified with choline, serine, inositol, glycerol or ethanolamine [7,10] (Fig. 1). Glycerophosphocholines (GPChos), e.g. phosphatidylcholine, are considered the major phospholipids in plasma and are known to cause significant matrix ionization effects during LC/MS/MS analysis [7]. GPChos constitute up to 70% of total plasma phospholipids and lysophospholipids, containing a single fatty acid ester, constitute up to 10% of total phospholipids [11].

The presence of phospholipids at significant concentrations in biological samples can be problematic in both positive and negative electrospray ionization modes. Mono- and disubstituted GPChos have been shown to fragment to form trimethylammonium-ethyl phosphate ions at *m*/*z* 184 and 1-mono (2-lyso) glycerophosphocholines (2-Lyso GPChos) fragment to form mainly *m*/*z* 104 ions in the positive ion mode [7]. Other phospholipids that are present at lower concentrations in plasma, such as sphingomyelins (SMs), also fragment to form *m*/*z* 184 ions. Glycerophosphoserines (GPSs) and glycerophosphoethanolamines (GPEs) lose their polar head groups as neutral loss fragments at *m*/*z* 185 and *m*/*z* 141, respectively [7]. Phospholipids have strong ionic character due to their polar head groups and high hydrophobicity due to the alkyl chains. Complete elution of phospholipids from a reversed phase column typically requires a significant amount of time with high organic modifier concentrations. Adjustment of the chromatographic conditions to resolve analyte peaks from phospholipids is considered a reasonable approach to minimize matrix effects, however, late elution of phospholipids in the same or even a subsequent sample injection presents a problem whenever the analytes of interest co-elute. Accumulation or incomplete elution of phospholipids also reduces column life and may lead to unexpected matrix effects such as shifting retention in subsequent sample injections [11]. Little et al. [7] developed an in-source multiple reaction monitoring (IS-MRM) approach for detection of all GPChos during LC/MS/MS analysis using only one SRM channel. High-energy in-source collision induced dissociation (CID) is used to yield *m*/*z* 184 ions as a common ion fragment of the GPChos and similar conditions were used for monitoring 2-Lyso GPChos (*m*/*z* 104) [7]. Optimization of the sample extraction procedure is one of the most effective approaches for minimizing matrix effects and is an important aspect of method development. Protein precipitation (PPT) using organic solvents has been shown to yield the highest degree of matrix effects in comparison with solid phase extraction [SPE] and liquid–liquid extraction

Table 1
Multiple reaction monitoring (MRM) parameters.

Compound	MS (*m*/*z*)	MS2 (*m*/*z*)	Cone Volts	CE (eV)
Phosphatidylcholine (PC)	184	184 $[H_2PO_4\text{-}(CH_2)_2\text{-}N(CH_3)_3]^+$	90	7
Lysophosphatidylcholine (LPC)	104	104 $[HO\text{-}CH\text{-}CH_2\text{-}N+\text{-}(CH_3)_3]$	70	7
Sphingomyeline (SM)	184	184	90	7
Phosphatidylethanolamnie (PE)	746.75 $[M+H]^+$	605.6 $[M-171]^+$	40	25
Phosphatidylserine (PS)	790.5 $[M+H]^+$	605.8 $[M+H\ -185]^+$	40	25
Phosphatidylinositol (PI)	904.06 $[M+NH_4]^+$	627.8 $[M-259]^+$	20	25
Phosphatidylglycerol (PG)	794.77 $[M+NH_4]^+$	605.7 $[M-171]^+$	25	25
Phosphatidic acid (PA)	720.5 $[M+NH_4]^+$	605.8 $[M-97]^+$	30	25
1-Stearyl-rac-l glycerol (MAG)	341.5	341.5 $[M+NH_4\text{-}NH_3\text{-}H_2O]^+$	30	10
1,3-Dilinoleoyl-rac-glycerol (DAG)	634.67 $[M+NH_4]^+$	599.5 $[M+NH_4\text{-}NH_3\text{-}H_2O]^+$	25	20
1,3-Dipalmitoyl,2oleoyl- -glycerol [PPO] (TAG)	850.73 $[M+NH_4]^+$	577.83 $[OP]^+$	40	25
Cholesterol (C)	369.2	369.2 $[M+H\text{-}H_2O]^+$	40	10
Cholestryl oleate (CE)	369.2	369.2 [M+H-Oliec acid]$^+$	40	10

[LLE] [12,13]. For example, SPE extracts, using either polymeric sorbents or silica-based strong cation exchange sorbents, contained only 14% of the phospholipid levels of PPT extracts [11].

The main lipid classes and the lipid composition of human plasma are: phospholipids (PL, 31.6 mole%), cholesterol esters (CE, 39.7 mole%), free cholesterol (C, 19.0 mole%) and triacylglycerols (TAG, 9.7 mole%) [14]. The major classes of PL are phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI); the relative proportions of these classes vary in different blood components; PC constitutes up to 70–72% of total human plasma phospholipids, however, it comprises only 30–36% and 35–40% of the total phospholipids in human erythrocytes and platelets, respectively. On the other hand, PE constitutes up to 2–4%, 25–29% and 19–26% of the total phospholipids in human plasma, erythrocytes and platelets, respectively [15]. Most reported investigations of matrix effects have focused only on phospholipids (mainly monitoring phosphatidylcholine and lysophosphatidylcholine) and there are no published results that have investigated matrix effects resulting from other phospholipid classes, cholesterols or acylglycerols. The purpose of this work was to investigate matrix effects resulting from a broad range of endogenous lipids, including phospholipids, acylglycerols and cholesterols and to compile a library for the relative recovery of these components in sample extracts obtained from commonly used sample preparation techniques such as liquid–liquid extraction, supported-liquid extraction, solid phase extraction and protein precipitation.

2. Experimental

2.1. Materials

Chlorpheniramine maleate (99.8%) and loratadine (99.9%) were purchased from USP (Rockville, MA, USA). 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (>99.0%) (PC), 1-palmitoyl-2-hydroxy-sn-glycerol-3-phosphocholine (>99.0%) (LPC), 1-stearoyl-2-oleoyl-sn-glycerol-3- phosphoethanolamine (>99.0%) (PE), 1-stearoyl-2-oleoyl-sn-glycerol-3-[phospho-rac-(1-glycerol)](Na salt) (>99.0%) (PG), 1-stearoyl-2-oleoyl-sn-glycerol-3-phosphate (monosodium salt) (>99.0%) (PA), L-α-phosphatidylinositol (Liver, Bovine) (Na salt) (>99.0%) (PI) were purchased from Avanti Polar lipids, Inc. (Alabaster, AL, USA). Sphingomyelin (from bovine brain, 98.0%) (SM), 1,2-diacyl-sn-glycerol-3-phospho-L-serine (from bovine brain, 98.0%) (PS), cholesterol (≥99.0%) (C), cholesteryl oleate (≥98.0%) (CE), 1-stearyl-rac-l-glycerol (≥99.0%) (MAG), 1,3-dilinoleoyl-rac-glycerol (≥95.0%) (DAG), 1,3-dipalmitoyl-2-oleoylglycerol (99.0%) (TAG), formic acid (FA), ammonium formate, phosphate buffered saline (PBS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), methanol (MeOH), isopropyl alcohol (IPA), methyl tertiary butyl ether (MTBE), ethyl ether (E), dichloromethane (DCM), ethyl acetate (EAC), n-hexane (HEX) and acetone were from Burdicks & Jackson (B&J) (Muskegon, MI, USA), ammonium hydroxide were from J.T. Baker (Philipsburg, NJ, USA). Oasis® MCX 96-well Plate 30 μm (10 mg), Oasis® MAX 96-well Plate 30 μm (10 mg), Oasis® WCX 96-well Plate 30 μm (10 mg), Oasis® WAX 96-well Plate 30 μm (10 mg), Oasis® HLB 96-well Plate 30 μm (10 mg) were from Waters Corp. (Milford, MA, USA). ISOLUTE® SLE + 200 mg was from Biotage (Charlotte, NC, USA). HybridSPE™-Precipitation (H-PPT) 96-well plate (50 mg) was from SUPELCO (Bellefonte, PA, USA). Human plasma with K_2EDTA was from Biological Specialty Corp. (Colmar, PA, USA).

2.2. Apparatus

The HPLC system consisted of a Shimadzu, System Controller, SCL-10A Vp, Pump, LC 10AD Vp, Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA), Solvent Degasser, DGU14A and autosampler CTC PAL (Zwingen, Switzerland). The Mass Spectrometer was a Micromass Quattro API Micro, Waters Corp., with Masslynx version 4.0 and 4.1 data acquisition software installed on an HP computer (Waters Corp., Milford, MA, USA), that was operated in the electrospray ionization (ESI) positive multiple reaction monitoring (MRM) mode.

2.3. Gradient method for the analysis of endogenous components

Samples were analyzed using a Luna Silica 50 mm × 2.0 mm, 5 μm particle size, analytical column, Phenomenex (Torrance, CA) along with a Gemini C_{18} 4.0 mm × 2.0 mm guard column, Phenomenex (Torrance, CA). Mobile phase A was composed of (20:80) methanol (MeOH):H_2O (v/v) with 10 mM ammonium format and 0.3% formic acid. Mobile phase B was (5:25:70) H_2O:acetonitrile (ACN):MeOH (v/v/v) with 10 mM ammonium format and 0.3% formic acid, and mobile phase C was (5:55:40) H_2O:MeOH:isopropyl alcohol (IPA) (v/v/v) with 10 mM ammonium format and 0.3% formic acid. The autosampler utilized two rinse solutions (5:35:30:30) H_2O:MeOH:ACN:IPA (v/v/v/v), with 0.3% formic acid and (5:25:70) H_2O:ACN:MeOH (v/v/v), with 0.3% formic acid, with a total run time of 10 min, and injection loop volume (30 μL). The MS/MS System parameters were: capillary (4.00 kV), extractor (2.00 V), source temperature (120 °C), desolvation temperature (350 °C), desolvation gas flows (600 L/h and 100 L/h). Collision gas (Argon), collision energy, mass transitions and MRM parameters were as shown in Table 1.

2.4. Post-column infusion

Post-column infusion experiments were conducted such that a 100 ng/ml solution of chlorpheniramine or loratadine in mobile phase (50:475:475) 10 mM ammonium formate:MeOH:ACN (v/v/v) was constantly infused at 10 μL/min into

a tee connecting the analytical column to the MS ion source. A 25-μg/ml solution of each endogenous lipid standard compound was prepared individually in (50:475:475) 10 mM ammonium formate:MeOH:ACN (v/v/v) and a 25 μL of each lipid solution was injected pre-column ($n = 3$) to observe any ion suppression effects on the infused analyte signal during elution.

2.5. Sample preparation methods

2.5.1. Protein precipitation

2.5.1.1. Dry and reconstitute method. 400 μL of cold ACN, 2% HCOOH in ACN, MeOH or acetone was added to a 100-μL aliquot of blank human plasma. The sample was vortex mixed for 10 min and centrifuged at 4000 rpm for 10 min. 200 μL of the supernatant was removed and evaporated under a dry nitrogen stream at approximately 40 °C. The residue was reconstituted with 100 μL of (75:25) MeOH:ACN (v/v), vortex mixed for 5 min, and 25 μL of the resulting solution was injected into the LC/MS/MS.

2.5.1.2. Dilute and inject method. 400 μL of cold ACN, 2% HCOOH in ACN, MeOH or acetone was added to a 100-μL aliquot of blank human plasma. The sample was vortex mixed for 10 min and centrifuged at 4000 rpm for 10 min. 100 μL of the supernatant was transferred into a 96 well plate, diluted with 400 μL of (75:25) MeOH:ACN (v/v), and vortex mixed for 5 min. 25 μL of the resulting solution was injected into the LC/MS/MS.

2.5.2. Liquid–liquid extraction

Liquid–liquid extraction (LLE) was carried out using four different solvents: methyl tertiary butyl ether (MTBE), diethyl ether, (30:70) ethyl acetate:n-hexane (v/v) and dichloromethane (DCM). A 100-μL aliquot of blank human plasma was diluted (1:1) with 100 μL of 1.0% formic acid, 1× phosphate buffer saline (PBS), or 0.5 M NH_4OH and mixed briefly. The final pHs of the blank plasma samples after dilution were 3.32, 7.34 and 10.81, respectively. 2.0 ml of extraction solvent was added, the sample tube was vortex mixed for approximately 5 min, centrifuged at approximately 3000 rpm for 10 min (except for DCM), then placed in a freeze bath at −40 °C. The organic layer was transferred to another tube and evaporated to dryness under a dry nitrogen stream at approximately 40 °C. The residue was reconstituted with 100 μL of (75:25) MeOH:ACN (v/v), vortex mixed for 5 min, and 25 μL of the resulting solution was injected into the LC/MS/MS.

2.5.3. Supported liquid extraction (ISOLUTE® SLE + 200 mg)

Supported-liquid extraction (SLE) was carried out in 96-well plate format using four different elution solvents; methyl tertiary butyl ether (MTBE), diethyl ether, (30:70) ethyl acetate:n-hexane (v/v) and dichloromethane (DCM). A 100-μL aliquot of blank human plasma was diluted (1:1) with 100 μL of 1.0% formic acid, 1× phosphate buffer saline (PBS), or 0.5 M NH_4OH and mixed briefly prior to loading onto the sorbent. Following equilibration for a minimum of 5 min., the sample well was eluted with 3× 350-μL volumes of the extraction solvent into a collection plate. Gravity elution was allowed to occur for 5 min after each solvent addition. The SLE/collection plate assembly was centrifuged at low speed (approximately 300 rpm) for 5 min to completely recover the extract, which was then evaporated to dryness under a dry nitrogen stream at approximately 40 °C. The residue was reconstituted with 100 μL of (75:25) MeOH:ACN (v/v/), vortex mixed for 5 min, and 25 μL of the resulting solution was injected into the LC/MS/MS.

2.5.4. Solid phase extraction

2.5.4.1. Polymeric mixed-mode strong cation exchange SPE (Oasis® MCX) and polymeric mixed-mode weak anion exchange (Oasis® WAX) SPE. The sorbent was conditioned with 500 μL of MeOH and equilibrated with 500 μL of H_2O. 500 μL of diluted blank human plasma (diluted 1:1 with 4% H_3PO_4) was loaded onto the sorbent. The sorbent was washed with 2× 250 μL of 2% formic acid in H_2O then by 2× 250 μL of MeOH. Samples were eluted with 2× 125 μL of 5% NH_4OH in MeOH. The eluate was evaporated to dryness under a nitrogen stream at approximately 40 °C. The residue was reconstituted with 250 μL of (75:25) MeOH:ACN (v/v/), vortex mixed for 5 min, and 25 μL of the resulting solution was injected into LC/MS/MS.

2.5.4.2. Polymeric mixed-mode strong anion exchange SPE (Oasis® MAX) polymeric mixed-mode weak cation exchange SPE (Oasis® WCX). The sorbent was conditioned with 50 μL of MeOH and equilibrated with 500 μL of H_2O. 500-μL aliquots of diluted blank human plasma (diluted 1:1 with 4% H_3PO_4) were loaded onto the sorbent. The sorbent was washed with 2× 250 μL of 5% NH_4OH in water then by 2× 250 μL of MeOH. Samples were eluted with 2× 125 μL of 2% formic acid in methanol. The eluate was evaporated under a nitrogen stream at approximately 40 °C to dryness. The residue was reconstituted with 250 μL of (75:25) MeOH:ACN (v/v/), vortex mixed for 5 min, and 25 μL of the resulting solution was injected into the LC/MS/MS.

2.5.4.3. Hydrophilic–lipophilic balance polymeric SPE (Oasis® HLB). The sorbent was conditioned with 500 μL of MeOH and equilibrated with 500 μL of H_2O. 500 μL of diluted blank human plasma (diluted 1:1 with 4% H_3PO_4) was loaded onto the sorbent. The sorbent was washed with 2× 250 μL of 5% methanol in water. Samples were eluted with 2× 125 μL of methanol, and the eluate was evaporated to dryness under a dry nitrogen stream at approximately 40 °C. The residue was reconstituted with 250 μL of (75:25) MeOH:ACN (v/v/), vortex mixed for 5 min, and 25 μL of the resulting solution was injected into the LC/MS/MS.

2.5.5. Hybrid SPE-Precipitation (HybridSPE™-Precipitation (H-PPT) 96-well Plate 50 mg)

100-μL samples of blank human plasma were loaded onto a 96-well (H-PPT) plate, followed by 300 μL of 1% formic acid in ACN. Samples were precipitated by vortexing the plate for 1 min. The 96-well plate was transferred to a 96-well vacuum manifold, vacuum was applied and the eluates were collected. 100 μL of eluate was diluted with 300 μL MeOH and vortex mixed for 5 min. 25 μL of the resulting solution was injected into the LC/MS/MS.

3. Results and discussions

3.1. Post-column infusion

Post-column infusion experiments were carried out to determine and compare ionization effects from injections of standard solutions of different phospholipids classes, acylglycerols and cholesterols on the MS signal produced by constant infusion of two test compounds (Fig. 2). Chlorpheniramine (2-[p-chloro-_-[2-(dimethylamino)ethyl]benzyl]pyridine) is a strongly basic compound (p*K*a 9.1) with an aliphatic amine group (proton acceptor site) and a pyridine ring with a lone pair of electrons (active site for charge transfer) [16,17]. Chlorpheniramine has been shown to be susceptible to matrix effects that resulted in imprecise and inaccurate LC/MS/MS results [17]. Chlorpheniramine has both a proton acceptor site and a electron donor site and represents a good example compound for possible analyte-endogenous matrix component ion suppression interactions and was selected in this study as a test compound. Loratadine, ethyl-4-(8-chloro-5,6 dihydro-11H-benzo[5,6] cyclohepta[1,2*b*]pyridin-11-ylidene)-1-piperidine

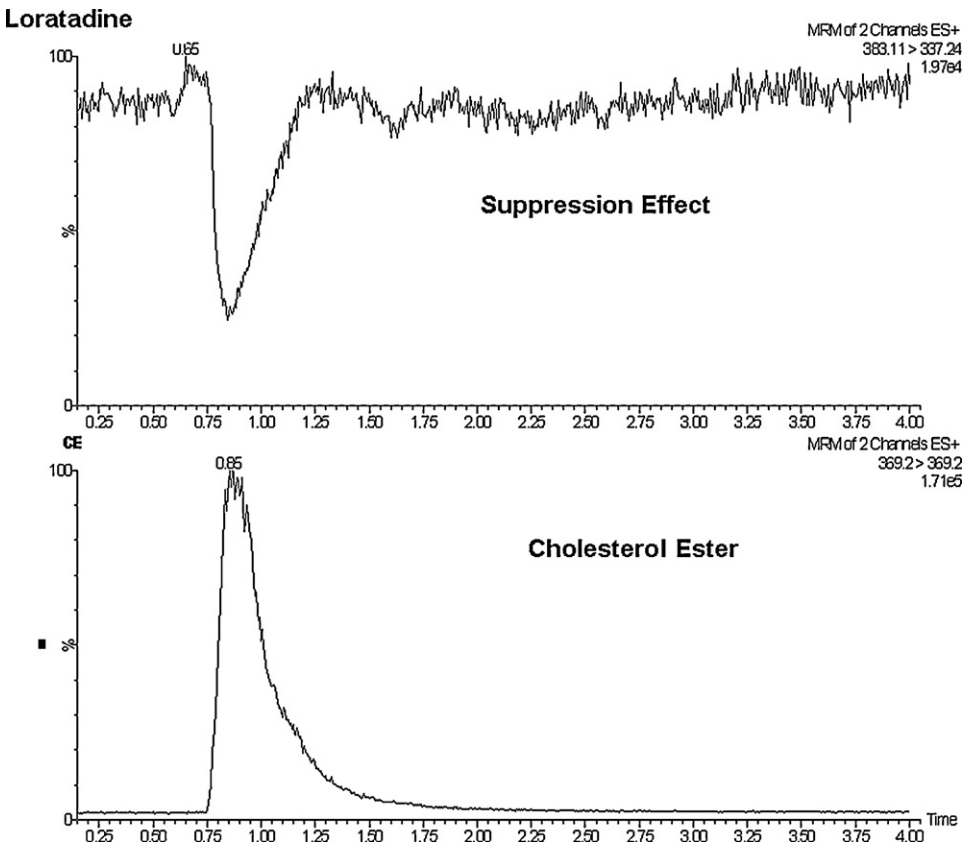

Fig. 2.

carboxylate (p*K*a 5.0) [18] has a pyridine nitrogen atom, a different p*K*a and was chosen as a second confirmatory test compound.

Post-column infusion experiments demonstrated that all phospholipids classes (at equal concentrations) resulted in significant ion suppression effects ranging from 54% to 76% and 60% to 92% for chlorpheniramine and loratadine, respectively (Fig. 3). Some phospholipids such as phosphatidylinositol and phosphatidylglycerol produced greater suppression for chlorpheniramine than did phosphatidylcholine. Glycerides and cholesterols also demonstrated ion suppression effects for both test compounds that ranged from 29% to 78%. Endogenous concentrations of different lipid classes should be considered and a Concentration Normalized Suppression Factor was calculated in order to more effectively compare endogenous lipids accounting for their expected concentrations in biological matrices.

3.2. Concentration Normalized Suppression Factor (%CNSF) calculations

The Concentration Normalized Suppression Factor (%CNSF) was created to compare relative potential ion suppression effects directly. The %CNSF was calculated for both human blood plasma and human erythrocytes using chlorpheniramine and loratadine as test compounds. Since phosphatidylcholine (PC) is considered the main cause of matrix ionization effects in LC/MS/MS, various endogenous lipid components were compared relative to PC according to their % ion suppression effects (obtained from post-column infusion experiments) and their approximate expected concentrations in the biological matrices studied (Table 2).

Calculations of %CNSF were carried out using the following formula:

$$\%\mathrm{CNSF} = \mathrm{A} \times \mathrm{B} \times 100$$

where A for (Target Lipid) = Target Lipid ion suppression/PC ion suppression B for (Target Lipid) = Target Lipid conc. /PC conc. For example, calculation of %CNSF for cholesterol ester (CE) using loratadine as a test compound. The PC ion suppression for loratadine was 85%. The PC concentration in human plasma is approximately 22.5% of total lipids [14]. The CE ion suppression for loratadine was 71%. The CE concentration in human plasma is approximately 40.4% of total lipids [14,19]. A for (CE) = CE ion suppression/PC ion suppression = 0.835. B for (CE) = CE conc./PC conc. = 1.8.

$$\%\mathrm{CNSF} = \mathrm{A} \times \mathrm{B} \times 100 = 150\%.$$

Table 2
Lipid content of human plasma and erythrocytes.

	Human plasma	Human erythrocytes	Ref.
Lipid class		% of Total lipids (% TL)	
PC	22.5	19.0	[14,21]
C	7–19 (13)	25.2	[14,20]
CE	39.7–41(40.8)	–	[14,20]
TAG	10–15 (12.5)	–	[14,20]
DAG	19.6	–	[35]
MAG	0.80	–	[35]
PL class		% of Total phospholipids (% TPL)	
PC	70.8	30.4	[14,19,20]
SM	11.3–18.1 (14.8)	25.6	[14,19,20]
LPC	10.0	1.62	[14,20]
PE	3.4	27.2	[14,19,20]
PI	4.4	2.0	[14,19,20]
PS	2.3	12.9	[19,20]
PA	–	1.78	[20]
PG	–	–	

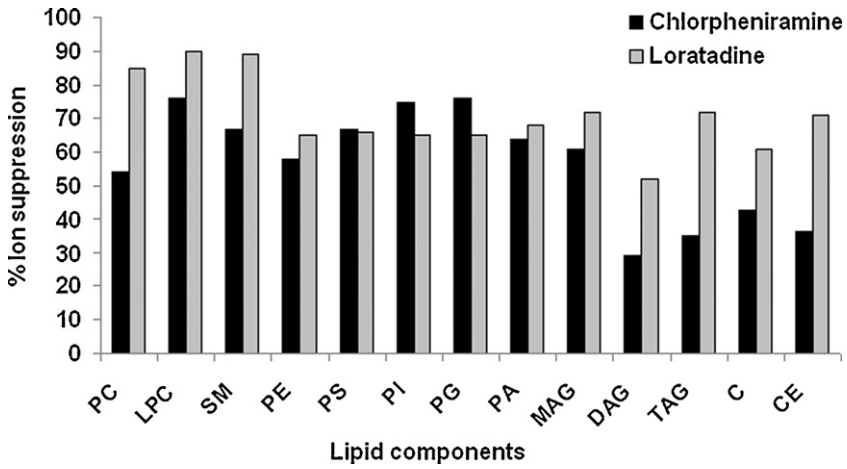

Fig. 3.

Therefore, in a hypothetical plasma sample extract that contained *total* lipids, the endogenous CE would be expected to produce approximately 1.5 times the ion suppression effects expected for endogenous PC with the loratadine test analyte. %CNSF calculations were carried out for most of the endogenous lipids investigated using either their approximate % of total lipid (%TLP) or % of phospholipids (%PL) in human plasma and human erythrocytes (Fig. 4). Most of the phospholipid classes are found in the literature as the percent of total phospholipids and these percentages are generally consistent through various articles. However, not all of the phospholipids could be found as the percent of total lipids and therefore TAG, CE and C are reported as the percentage of total lipids (Table 2). Lipids for which %CNSF were not reported were either present at negligible concentrations in human plasma and erythrocytes or their concentrations were not found in the literature (Table 2).

3.2.1. *Concentration Normalized Suppression Factor (%CNSF) results for human plasma*

The cholesterol ester showed approximately 1.5 and 1.2 times greater ion suppression than PC for loratadine and chlorpheniramine, respectively. TAG showed 47% and 36% of PC ion

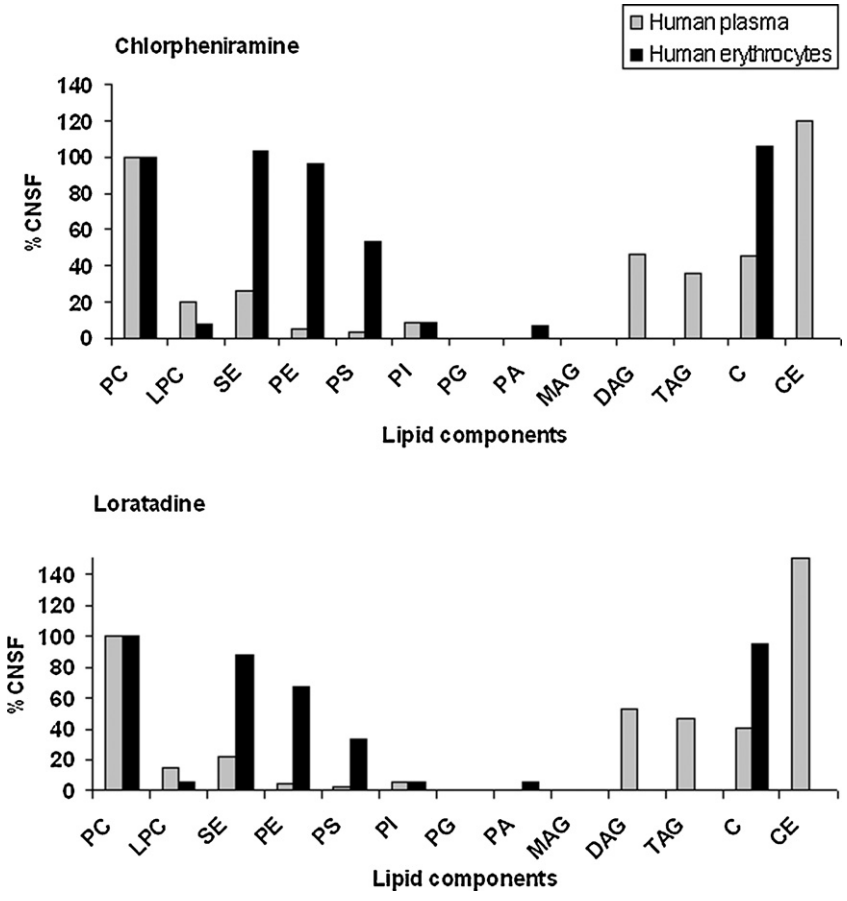

Fig. 4.

suppression effects for loratadine and chlorpheniramine, respectively. LPC, SE, C and DAG resulted in 20%, 26%, 46% and 46% of PC ion suppression for chlorpheniramine, respectively, and 15%, 22%, 41% and 53% of PC ion suppression for loratadine, respectively. Other investigated lipids demonstrated less than 10% of the PC ion suppression effects for both compounds (Fig. 4). These results indicate that, in addition to PC, CE, C and TAG may result in significant matrix effects in LC/MS/MS analysis of human plasma samples. Therefore, monitoring the presence and abundance of CE, C and TAG during method development and removing them during sample clean up, as is now commonly done for phospholipids, are important for human plasma quantitative analysis.

3.2.2. Concentration Normalized Suppression Factor (%CNSF) results for human erythrocytes

Various biological matrices will demonstrate different %CNSF values for each of the endogenous lipids according to their relative concentrations. The whole blood pool is composed of approximately 54% plasma and 46% circulating cells (mainly erythrocytes) [15]. The %CNSF was calculated in human erythrocytes according to the % of total lipids or % of total phospholipids in comparison to PC for most of the investigated endogenous components. PC constitutes approximately 30% of total phospholipids in human erythrocytes (19% of total lipids). Other phospholipids classes such as SE, PE and PS constitute 26%, 27% and 13% of total phospholipids in human erythrocytes, respectively [14,19,20]. Neutral lipids (primarily cholesterol) constitute approximately 25% of human red cells [20,22]. %CNSF calculations showed that SE, PE and C (as the main neutral lipids in human erythrocytes) provide 104%, 96% and 106% of the PC ion suppression for chlorpheniramine, respectively, and 88%, 68% and 95% of the PC ion suppression for loratadine, respectively. These three components in addition to PC may contribute to matrix effects in human whole blood and red blood cell quantitative analysis. PS demonstrated 53% and 33% of the PC ion suppression for chlorpheniramine and loratadine, respectively. LPC, PI and PA showed less than 10% of PC ion suppression for both compounds.

The %CNSF results from human plasma and erythrocytes indicate, some abundant lipid classes such as cholesterol, cholesterol esters and triacylglycerols, and other phospholipids classes such as PE and SM (in addition to the commonly appreciated PC class) can contribute significant matrix effects. The extent of matrix effects from the different endogenous lipids encountered in the analysis of human plasma, whole blood, and red blood cell sample extracts will vary according to a number of factors, including their endogenous concentrations, extraction method recovery, as discussed below, as well as the concentration of the final extract relative to the original sample aliquot and the amount of extracted injected on the instrument system. These results also suggest that hemolysis of a blood sample during collection and processing may cause increased lipid-related matrix effects when analyzing the resulting human plasma sample.

In human saliva, phospholipids constitute a minor fraction of total lipids, however, neutral lipids (CE, C, TAG, DAG, MAG and free fatty acids) constitute 96.4% of the total lipids (32.8%, 9.9%, 22.1%, 19.8%, and 7.6%, respectively) [23]. Matrices from other animal species may also show differences in matrix lipid composition. PL, CE, C and TAG constitute 53.4%, 14.9%, 6.1% and 25.6% of total mouse plasma lipid, respectively and constitute 53.6%, 32.9%, 8.4% and 5.1% of total dog plasma lipid content, respectively [24]. PC constitutes 80.7% and 75.5% of total phospholipids in dog and rabbit plasma, respectively, and constitutes 43.9% and 43.5% of total phospholipids in dog and rabbit erythrocyte membranes, respectively [25].

The above results, based on the approximate lipid concentrations in healthy individuals obtained from the literature, also vary from one plasma source to another according to individual diets and metabolic rates [3]. TAG levels in plasma or serum have been shown to vary with food intake and age; also TAG levels have been reported to be higher in men than women [20]. Individuals with different lipid disorders such as hypercholesterolemia and hypertriglyceridemia would be expected to show large differences in lipid composition and in %CNSF.

3.3. Method development for monitoring of endogenous lipid components

Normal phase HPLC would be expected to separate lipids by class according to the polarity of the head group, however, reversed phase HPLC separates individual molecular species of phospholipids according to their lipophilicity [10]. Normal phase chromatography has been widely used for phospholipid separation; normal phase (silica) and pseudo-normal phase (hydrophilic interaction chromatography, HILIC) were compared in this study.

3.3.1. Chromatographic conditions

Ultra performance liquid chromatography (UPLC) using a sub-2 μm particle size stationary phase provides high separation efficiency of complex samples, and the Waters ethylene-bridged hybrid (BEH) stationary phases are stable over a wide pH range (1–12). The ACQUITY UPLC BEH HILIC Column, 1.7 μm, 2.1 mm × 50 mm was used initially with an isocratic mobile phase consisting of (5:95) A:B (v/v), mobile phase A was 10 mM ammonium formate and mobile phase B was (1:1) methanol:acetonitrile (v/v) at 0.2 ml/min flow rate. Good peak shape was obtained for all components, although the preliminary results with the protein precipitation method demonstrated unexpectedly lower phospholipid responses in comparison to liquid–liquid extraction. We suspected that high concentrations of inorganic salts recovered in the protein precipitation method might have suppressed the responses of the endogenous lipids. Various columns such as (Phenomenex Luna Silica, Thermo Betasil Silica and Atlantis® HILIC silica) with different column length (50 and 100 mm), with different internal diameters (2.0, 3.0 and 4.6 mm) and different particle sizes (3.0 and 5.0 μm) have been compared using different mobile phases at different flow rates. A modified method was developed to overcome potential ion suppression effects from inorganic matrix components as follows: A Phenomenx Gemini C18 4.0 mm × 2.0 mm guard column with a high aqueous loading mobile phase consisting of (20:80) MeOH:H_2O (v/v), with 10 mM ammonium formate and 0.3% formic acid (at a 0.5 ml/min flow rate for 1 min), was used to trap the lipid analytes and remove endogenous salts present in the sample extract. A Valco 6-port switching valve was used to direct the guard column effluent to waste during the desalting period. A concentration of 20% methanol in the loading mobile phase was found to be the maximum that does not elute any of the lipids studied from the guard column. Endogenous lipids were then transferred and separated on a Phenomenex Luna Silica, 5 μm, 2.0 mm× 50 mm column using mobile phases B and C. Mobile phase B was (5:25:70) H_2O:ACN:MeOH (v/v/v) with 10 mM ammonium formate and 0.3% formic acid, and was used to elute all phospholipids, mono- and diacylglycerides. Mobile phase C consisted of (5:55:40) H_2O:MeOH:IPA (v/v/v) with 10 mM ammonium formate and 0.3% formic acid, and was used to elute non-polar lipids such as cholesterol esters and triacylglycerols. The isopropyl alcohol percentage in the mobile phase (at least 35%) was found to be essential to elute cholesterol esters and triacylglycerol from the C_{18} guard column. Use of IPA in the syringe wash solution and in the column flushing mobile phase was also highly beneficial for removing residual non-polar lipids during LC/MS/MS bioanalysis.

We found that the hybrid silica ACQUITY UPLC BEH HILIC column did not provide an advantage over the Phenomenex Luna Silica in

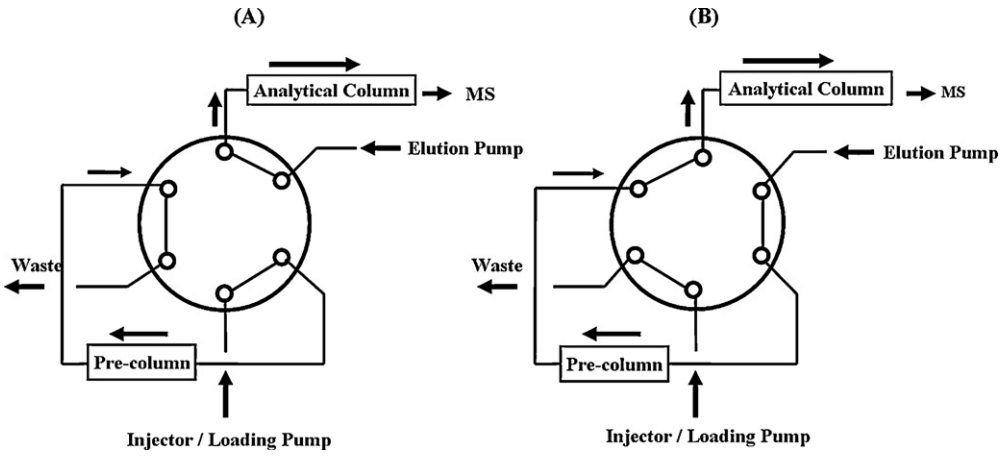

Fig. 5.

terms of peak separation and peak shape. On the other hand, use of the pure silica column allowed use of a lower percentage of ACN and higher percentages of either methanol or IPA to obtain complete and rapid elution of the different lipid components from the C_{18} guard column and silica column. The silica column also provided good peak shape for all components along with lower column back-pressure in comparison to UPLC column. The elution pumps ran a step gradient at 0.5 ml/min flow rate as follows; (0–2.9) min 100% B, (3.0–4.9) min 100% C, (5.0–8.9) min 50/50 B/C at 0.75 ml/min flow rate and (9.0–10) min 100% B at 0.5 ml/min flow rate. A switching valve was used to direct the flow either to the MS or to waste as shown in Fig. 5; (0–1) min position A, (1–9) min position B and (9–10) min position A. Chromatograms of endogenous components are shown in Figs. 6 and 7.

3.3.2. Mass spectrometry

In-source multiple reaction monitoring (IS-MRM) was used for detection of all phosphatidylcholines and sphingomyelin using the *m*/*z* 184/184 mass transition. All lysophosphatidylcholines were detected using the *m*/*z* 104/104 mass transition using a high cone voltage and low collision energy [7]. The characteristic ion fragment *m*/*z* 369 $[M+H-H_2O]^+$of cholesterol was used without further fragmentation to monitor all cholesterols and cholesterol esters using the mass transition *m*/*z* 369/369. The characteristic mass transition for each component was used for the other tested endogenous components as shown in Table 1.

3.4. Carryover

Endogenous lipids have a tendency to build up on analytical and guard columns, which was observed in our experiments. A 4-min flush step for both analytical and guard columns using 50:50 mobile phase B/mobile phase C (v/v) at 0.75 ml/min was employed. Carryover may also result from adsorption of lipids on tubing connections especially in the case of loading with a highly aqueous mobile phase. The loading connection tube was flushed during the run with a mobile phase containing (5:30:35:30) H_2O/ACN/MeOH/IPA (v/v/v/v). This significantly reduced lipid carryover. Four reagent blank samples (25:75 MeOH:ACN (v/v)) were injected between subsequent batches comparing sample preparation techniques to ensure that the results would not be affected by carryover.

3.5. Matrix effects library compilation experiments

A blank human plasma pool consisting of 10 different donors (male and female) treated with K_2EDTA was used for all extractions. Protein precipitation (PPT), liquid–liquid extraction (LLE), supported liquid extraction (SLE), solid phase extraction (SPE) and hybride SPE-PPT (H-PPT) methods were used and the relative presence of lipids in the final extracts was compared.

The protein precipitation (PPT) method using organic solvents is a simple and fast sample preparation technique, however, it is known to yield a high degree of matrix effects in LC/MS/MS. It has been reported that residual phospholipids in MeOH extracts were significantly higher than ACN extracts [11]. Protein precipitation methods using four different organic solvents (acetone, ACN, MeOH and 2% FA in ACN) were applied using either the dry and reconstitute method or dilute and inject method. The latter method showed significant increases in response for most of endogenous lipids especially the components that are present at high concentrations such as phosphatidylcholine, cholesterols and triacylglycerols. This could be due to one or more of the following reasons; source response saturation, lipid adsorption and instability during sample drying or decreased solubility upon reconstitution.

Protein precipitation using either acetone or MeOH produced the highest responses for most of the endogenous lipids. Comparison of area responses of endogenous lipids in the final extracts resulting form the various extraction procedures to the area responses in the final extracts resulting form the protein precipitation method using ACN (dilute and inject method) was employed to compile the matrix effect library data. ACN is a commonly used precipitating agent, and results from most of other extraction procedures demonstrated very low responses in comparison to the PPT-acetone results. All results in this study are presented as the mean percentage of PPT-ACN ($n = 3$).

LLE methods are known to provide cleaner extracts than PPT methods, LLE using either MTBE or (1:1) diethyl ether/ethyl acetate (v/v) solvents has been shown to produce less ion suppression than PPT extracts for different compounds and from different matrices. LLE at different pH values has also been shown to demonstrate different degrees of matrix effects [11,12,26]. The solubility of pure lipids in organic solvent depends on the non-polar fatty acid chain and the polar functional group; non-polar lipids such as TAG and CE are soluble in hydrocarbon solvents like hexane and in slightly polar solvents such as chloroform and diethyl ether. They are insoluble in polar solvents such as methanol. The solubility of non-polar lipids in alcohol increases as the chain length of the alcohol is increased or the chain length of the fatty acid is decreased. Polar lipids are soluble in polar solvents such as methanol. IPA–hexane and chloroform–methanol mixtures are recommended for extraction of lipids from animal tissue. Acetone is a poor solvent for phospholipids and used to precipitate phospholipids from other solvents. However, considering the presence of

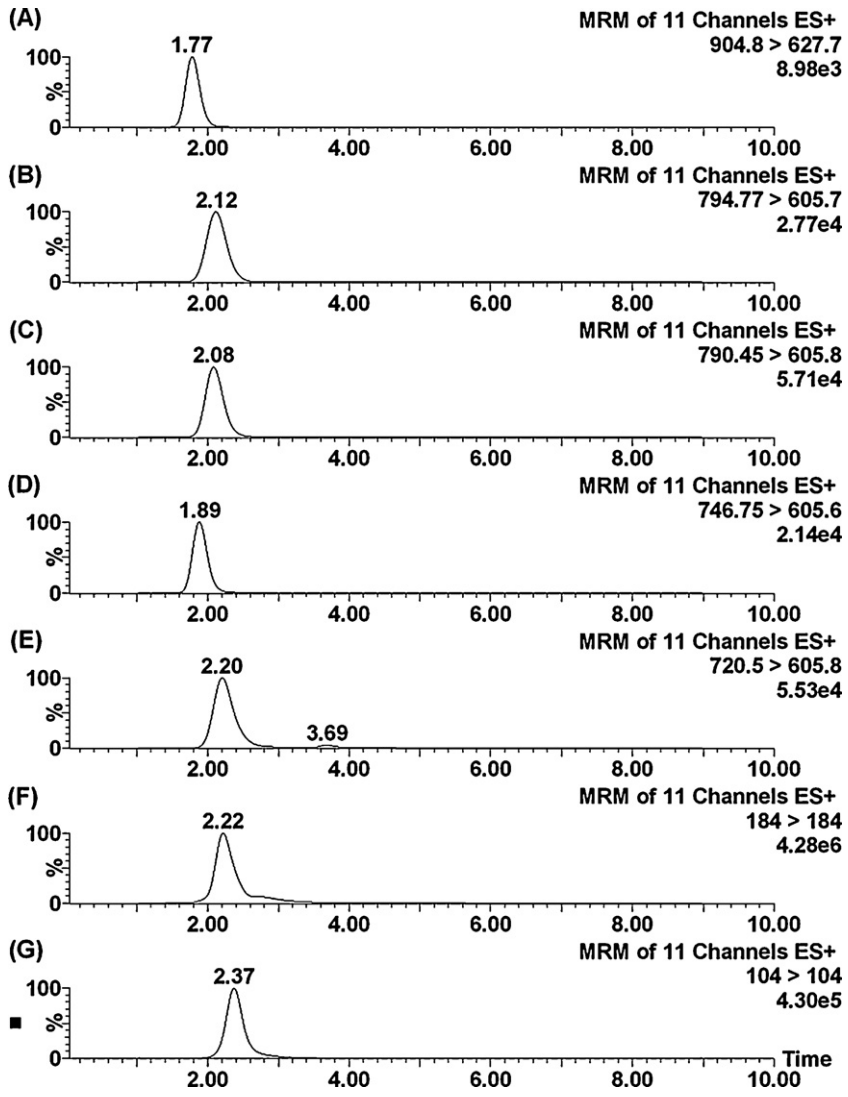

Fig. 6.

different lipids together in a biological sample, in addition to the presence of water, the observed solubility of lipids in a particular organic solvent may be altered. Lipids may also affect the solubility of each other and permit some solvents to extract more lipids than expected. There is no single pure solvent that would be ideal for extracting all lipids, and it is difficult to predict the extractability of various lipid components from biomatrices using different organic solvents according to the solubility of the pure lipid in pure solvents [27]. A mixture of chloroform–MeOH has been widely used to extract lipids from different tissues and biological fluids, DCM–MeOH and MTBE–MeOH solvent mixtures have also been reported as good solvents for lipid extraction that provide equivalent lipid recoveries [28,29]. Isooctane–ethyl acetate (75:25) (v/v) was found to be a successful solvent for the extraction of neutral lipids (cholesterols, acylglycerols) with comparable recoveries to the chloroform–MeOH method [30]. In this study, LLE was carried out using four different solvents based on these considerations, at acidic, basic and neutral pH.

SLE methods have been used recently for analysis of drug components in biological matrices, this technique is based on using a porous adsorbent (diatomaceous earth) as a solid support material, in which, a form of LLE is carried out with the analyte partitioning between a very thin aqueous "stationary film" that is adsorbed on the support particles and a water immiscible organic elution solvent. This technique has been reported to produce less matrix suppression in comparison to PPT and similar matrix effects as compared to LLE [31,32]. SLE methods can be used for extraction of acidic, basic and neutral compounds based on the sample pH. A generic SLE procedure was applied in this study using four different organic solvents at acidic, basic and neutral pH.

SPE methods have been shown to result in clean extracts and to reduce matrix effects [11]. Mixed mode ion exchange and reversed phase SPE sorbents (e.g. Waters Oasis® MCX, MAX, WCX and WAX) have been used for selective retention of basic (p*K*a 2–10), acidic (p*K*a 2–8), strongly basic (p*K*a >10) and strongly acidic compounds (p*K*a <1), respectively. The second wash step (100% MeOH) is used to remove hydrophobic matrix components or to elute weak acids, weak bases and neutral analytes, which are bound by the reversed phase [11]. The balanced hydrophilic–lipophilic SPE sorbent (Oasis® HLB) is used for wide range of acidic, basic and neutral analytes. The extractabilities of lipid components from different SPE sorbents were studied using generic extraction procedures.

HybridSPE™-Precipitation (H-PPT) is a simple sample preparation technique in which selective phospholipid binding has been reported. This stationary phase consists of zirconia-coated silica and provides phospholipid and proteins depleted extracts. Precipitated proteins are removed physically by filtration while phospholipids are removed by interaction with the zirconia–silica

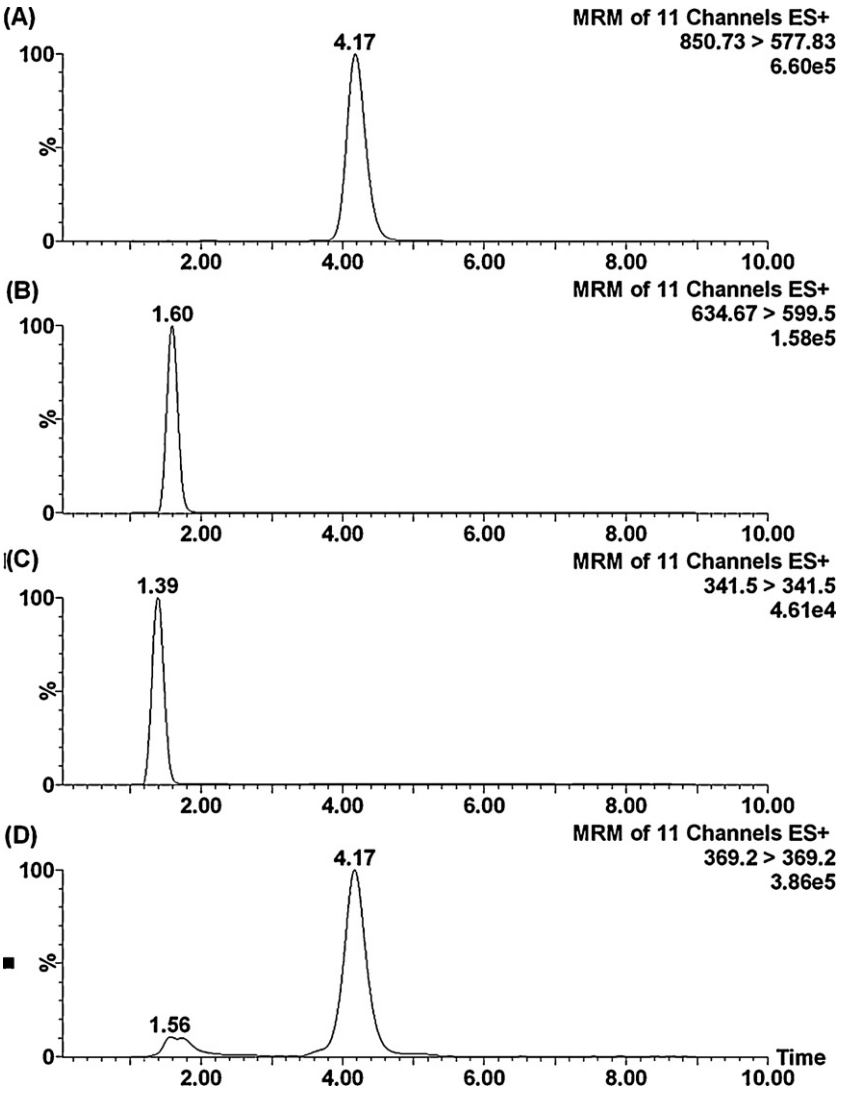

Fig. 7.

stationary phase (Lewis acid–base interaction) [33]. The presence/absence of different lipid components in the H-PPT extracts was also investigated and compared to other extraction procedures.

3.6. Extraction results

3.6.1. Combined responses of glycerophosphocholines (GPChos) and sphingomyelins (SMs)

Phosphatidylcholine, as a zwitterion, can cause matrix ionization effect in both positive and negative ESI modes due to its ability to be ionized in both modes [3]. Other phospholipids that are present at lower concentrations in plasma such as sphingomyelins (SMs) also fragment to form *m*/*z* 184 ion fragments. SMs constitute up to 11–18% of total plasma phospholipids [14,19]. Using in-source multiple reaction monitoring (IS-MRM) *m*/*z* 184/184 enables detection of all GPChos and SMs using only one MRM channel [7]. PC has a polar head group with strong zwitterionic character over the entire pH range, PCs and SMs are considered as neutral phospholipids [21]. Under the proposed chromatographic conditions SMs eluted very close to PCs. By comparing the combined area responses of GPChos and SMs in the final extracts resulting from different extraction procedures, it was found that PPT extracts showed the highest levels of PCs and SMs. PPT using either acetone or methanol showed more than 2.5 times the PC and SM levels that were present in PPT-ACN, while 2% FA in ACN produced approximately 2.0 times (Table 3). PCs either with short side chain fatty acid or long side chain fatty acid are either very soluble or soluble in methanol and acetone [34]. LLE methods using different organic solvents showed 0.2–17% of the PPT-ACN extract levels. The polarity factors of the organic solvents used in this study were 0.1, 2.5, 2.8, 3.1 and 4.4 for n-hexane, MTBE, ethyl ether, DCM and ethyl acetate, respectively [27]. Except for the ethyl acetate/n-hexane solvent mixture, the other three organic solvents used for LLE extraction have similar polarity factors. It was found that the pH of the sample is more important for extraction recovery of PCs and SMs. On the other hand, the combined area response of the PCs and SMs peak include both a long side chain fatty acid (more hydrophobic) and a short side chain fatty acid (less hydrophobic) acidic pH [(1:1) plasma:1% FA (v/v) pH 3.3] was found to be most effective for removing PCs and SMs, basic and neutral pH demonstrated comparable results using different organic solvents except for ether. The 30:70 ethyl acetate:n-hexane (v/v) solvent using acidic pH produced the cleanest extracts in comparison to the other LLE solvents. This may be due to limited solubility of PCs and SMs in n-hexane. Increasing the percentage of ethyl acetate would be expected to extract more PCs and SMs. Ether extracts using either basic or neutral pH showed the highest PC and SM levels in comparison to the other LLE solvents. PCs with a short side chain fatty acid (MW 400–500) are insoluble in ether, however,

Table 3
Calculated % extraction of phospholipids form different sample preparation techniques.

	PCs & SMs	LPCs	PE	PI	PA	PS
PPT						
PPT-ACN	100	100	100	100	100	100
PPT-acetone	257	80.9	1273	922	41.8	1368
PPT-MeOH	262	84.6	1353	1052	63.6	1494
PPT-ACN-FA	196	86.7	364	196	31.6	131
LLE						
MTBE-FA	1.06	0.027	63.6	0.914	44.5	66.4
MTBE-PBS	8.52	0.441	126	24.4	13.4	16.4
MTBE-NH_4OH	6.93	0.316	62.3	19.3	7.21	6.31
Ether-FA	6.76	0.196	183	0.00	6.3	8.42
Ether-PBS	17.0	0.963	291	54.8	2.89	6.52
Ether-NH_4OH	13.8	0.660	137	36.1	2.34	4.45
EthAC-Hex-FA	0.208	0.01	4.30	0.00	1.65	3.89
EthAC-Hex-PBS	6.74	0.299	68.4	15.4	2.38	8.03
EthAC-Hex-NH_4OH	6.34	0.282	39.0	15.6	1.89	5.26
DCM-FA	6.84	0.306	7.00	1.14	1.31	1.68
DCM-PBS	7.45	0.250	4.70	0.684	1.43	1.44
DCM-NH_4OH	5.09	0.194	1.60	0.00	0.80	1.11
SLE						
MTBE-FA	0.006	0.006	14.2	0.00	40.96	15.1
MTBE-PBS	0.003	0.004	0.00	0.00	0.00	0.00
MTBE-NH_4OH	0.004	0.004	0.00	0.00	0.00	0.00
Ether-FA	0.005	0.004	6.70	0.00	5.65	3.27
Ether-PBS	0.034	0.146	18.5	0.00	3.48	1.98
Ether-NH_4OH	0.007	0.005	0.00	0.00	0.00	0.00
EthAC-Hex-FA	0.004	0.004	0.00	0.00	1.87	0.00
EthAC-Hex-PBS	0.002	0.004	0.00	0.00	0.00	0.00
EthAC-Hex-NH_4OH	0.002	0.003	0.00	0.00	0.00	0.00
DCM-FA	10.91	0.158	0.00	0.00	3.00	3.27
DCM-PBS	3.56	0.055	0.00	0.00	3.30	0.00
DCM-NH_4OH	0.850	0.020	0.00	0.00	1.87	0.00
SPE						
MCX	9.76	3.50	157	40.5	2.11	343
WAX	3.65	0.634	38.7	47.9	10.63	325
MAX	0.164	0.07	108	0.00	0.00	187
WCX	0.105	0.112	1.80	0.00	0.00	12.04
HLB	8.27	7.34	88.4	3.43	0.00	44.0
H-PPT	0.018	0.010	0.00	0.00	0.00	0.00

PCs with a long side chain fatty acid (MW ~ 800) are very soluble in ether [34]. SLE methods resulted in very clean extracts (the lowest area responses) using different solvents except for DCM. The final extracts resulting from the SLE methods using different solvents at different pHs showed (<0.04%) of the PCs and SMs levels that were found in the PPT-ACN extracts. SLE-DCM extracts at different pH values showed <11.0% of that found in the PPT-ACN extracts. SPE methods also resulted in extracts with low levels of PCs and SMs (0.1–10% of PPT-ACN levels). Phospholipids can bind to the SPE sorbent through interaction with the hydrophobic side chain fatty acid, by cation exchange through interaction with quaternary amine group or by anion exchange through interaction with the phosphate group. It has been suggested in the literature that as the net charge on PCs and SMs approaches zero, no ion exchange interaction should take place [11]. In the same article, it was reported that the final extracts of a pure cation exchange sorbent and a reversed phase polymeric SPE contained comparable amounts of phospholipids and the authors did not have an explanation for these results [11]. Our results show that different mixed mode ion exchange SPE extractions resulted in different levels of PCs and SMs which may support the possibility of ion exchange interactions. Also, the absence of a 100% methanol wash step in the polymeric reversed phase SPE extraction should result in high levels of PCs and SMs in the final extracts with these sorbents, in comparison to the extracts of the mixed mode ion exchange SPE, provided the interaction with the latter occurred through a reversed phase interaction only. The main polar heads group of the phospholipids are the phosphate group (p*K*a 0–2), amine group (p*K*a 9–11) and carboxyl group (p*K*a 3–5). These p*K*a values may be changed in some cases due to H-bonding [34]. PCs and SMs contain both the phosphate and the quaternary amine group and the possible ion exchange interactions could be with strong cation exchange and weak anion exchange. Strong anion exchange and weak cation exchange may occur but with smaller possibility. Our results showed that the Oasis® MCX and Oasis® HLB demonstrated comparable results and the highest levels of PCs and SMs. The Oasis® WAX, Oasis® WCX and Oasis® MAX extracts showed the least area responses for PCs and SMs in comparison to other SPE extracts. The Hybrid SPE-PPT procedure produced less than 0.02% of the PPT-ACN area responses. All phospholipids are considered to be strong Lewis bases; the electron pair of the phosphate group strongly interacts with the empty zirconia d-orbital (Lewis acid) [33]. Our results show that SLE (except for DCM) and the Hybrid SPE-PPT methods were efficient procedures for removing GPChos and SMs from human plasma.

3.6.2. *Lysoglycerophosphocholines (LGPChos)*

LGPChos (LPCs) have been shown to elute earlier than GPChos from reversed phase HPLC, accordingly, monitoring LGPChos is important during method development to avoid co-elution with early eluting analytes. LPCs have the same head group although the presence of only one side chain fatty acid and the presence of the hydroxyl group may result in differences in solubility and extractability and LPC may show more solubility in more polar solvents. PPT extracts using different organic solvents showed very similar levels of LPCs (Table 3). LLE methods using different organic solvents showed 0.01–1.0% of the PPT-ACN extract levels; the acidic pH and 30:70 ethyl acetate:n-hexane (v/v) solvent was found to be the most efficient LLE procedure for removing most of the LPCs. The trend in LLE extractability of LPCs was similar to PCs but with lower percentages in comparison to the PPT extracts. This may be due to the higher solubility of the LPCs in the more polar PPT solvents (polarity factors; 5.1, 5.1 and 5.8 for acetone, methanol and acetonitrile, respectively [27]) and less solubility of LPCs in the less polar LLE solvents. The final extracts resulting from the SLE methods showed <0.2% of the LPCs levels found in the PPT-ACN extracts. SPE methods also resulted in 0.1–7.0% of the PPT-ACN LPCs levels with a similar trend as the PCs (the same head group). The Oasis® HLB was the least efficient SPE method for removing LPCs. Approximately two times as much of the LPCs were found in the strong cation exchange extracts. This may be due to higher solubility of the LPCs in the final elution solvent (methanol) used with the Oasis® HLB. The Hybrid SPE-PPT procedure produced only 0.014% of LPCs area responses as compared to the PPT-ACN extracts (Lewis acid–base) (Table 3).

3.6.3. *Other phospholipids*

Glycerophosphoethanolamine (*GPEtn*) (e.g. phosphatidylethanolamine, PE) constitute only 3–4% of total plasma phospholipids, however, the concentration of PE is more abundant in human erythrocytes showing 27% of total phospholipids [14,19,20]. GPEtn have been shown to yield an abundant ion $[M+H-141]^+$ after CID that corresponds to the neutral loss of the polar head group (phosphoethanolamine) [10]. PE is an acidic phospholipid, it is a zwitterion over the pH range of 2–7 and presents in the anionic form in the range of pH 7–10 [21]. Using PE as a marker for GPEtn lipid extraction recovery, we found that PPT using either acetone or methanol produced the highest levels of PE. Approximately 13 times the PE levels in PPT-ACN extracts were demonstrated and 2% FA in ACN produced 3.5 times the PE levels in the PPT-ACN extracts. PE with a saturated side chain fatty acid 1,2-(18:0) is soluble in ethanol and insoluble in acetone, ethyl acetate and ether. PE with a unsaturated side chain fatty acid 1,2-(18:1) is very soluble in acetone and ether [34]. The solubility

of the PE marker 1-(18:0), 2-(18:1) that was used in this study could not be found in the literature, however, it is expected to be between the previous two examples. LLE use of various organic solvents demonstrated different levels of PE. MTBE and ether showed relatively high levels of PE. Neutral pH [(1:1) plasma:1× PBS (v/v) pH 7.3] showed the highest levels of PE using most of the organic solvents. Acidic pH and 30:70 ethyl acetate:n-hexane (v/v) showed only 4.0% of the PE area response in the PPT-ACN extracts, DCM at different pH levels showed relatively low PE area responses 1.9–7% of the PE levels in PPT-ACN. No PE peak was observed in most of SLE extracts and Hybrid SPE-PPT. PE has both a quaternary amine group and a phosphate group, however, the $^{+}NH_3$ group in PE is less basic than the $^{+}N(CH_3)_3$ group in PC due to the presence of the electron donating groups (CH_3) in the latter. This suggests the possibility for strong cation exchange interaction and a very small possibility for weak cation exchange interaction. PE is a weakly acidic compound that has the capability of strong anion exchange interactions. The SPE results demonstrated that the Oasis® WCX extraction produced only 1.8% of PE levels in the PPT-ACN extracts, while the Oasis® MCX extraction produced approximately 1.5 times as much PE area response in the PPT-ACN (Table 3).

Glycerophosphoserine (*GPSer*) (e.g. phosphatidylserine, PS) is a weakly acidic lipid. It is also one of the major classes of lipids and constitutes up to 2% and 13% of total phospholipids in human plasma and erythrocytes, respectively [19,20,27]. GPSer has been shown to yield an abundant ion $[M+H-185]^+$ after CID that corresponds to the loss of the polar head group as a neutral loss (phosphoserine) [10,21]. PPT using either acetone or methanol produced approximately 14 and 15 times as much as the PS levels in PPT-ACN extracts, respectively. 2% FA in ACN produced 1.3 times as much as the PS levels in the PPT-ACN extracts; PS has the same side chain fatty acid as PE and the PPT results for PS showed the same trend as for PE. LLE using different organic solvents produced various levels of PS ranged from 1.0% to 66% of PS level in the PPT-ACN extract; acidic pH showed the highest levels in most cases. No PS peak was observed in most of the SLE and the Hybrid SPE-PPT extracts. The possible ion exchange interactions with PS could be through strong cation exchange with the ($^{+}NH_3$) group, weak anion exchange with the phosphate group and strong anion exchange with the COOH (p*K*a 3–5) group. MCX, WAX, MAX extracts showed high levels of PS. WCX and HLB produced 12% and 44.0% of the PS levels in PPT-ACN extracts, respectively (Table 3).

3.6.4. Combined responses of cholesterol (C) and cholesterol esters (CE)

Cholesterol esters (CE) and free cholesterol constitute up to 40% and 13% of total lipids in human plasma, respectively [14,20]. The characteristic ion fragment at *m/z* 369 $[M+H-H_2O]^+$ was used to monitor all C and CE without further fragmentation in Q3 using one MRM channel. The combined area responses were used to compare different extracts. PPT extracts using either ACN or MeOH showed low responses of C and CE that may be due to limited solubility of non-polar lipids, such as CE and TAG in polar solvents [20]. The solubility of cholesterol (at 26 °C) was found to be 0.161, 0.648, and 1.219 g/100 g of solvent for acetonitrile, methanol and acetone, respectively [35]. PPT using acetone, 2% FA in ACN, and MeOH produced approximately 14, 1.5 and 3.0 times as much as the C and CE levels that were present in PPT-ACN extracts, respectively. The final extracts resulting from LLE and SLE methods using different organic solvents contained 11–38% of C and CE levels present in the PPT-ACN extracts. C and CE are considered neutral lipids, and their interaction with different SPE sorbents may be due to a reversed phase interaction (CE stronger than C). H-bond formation with sorbents may also occur (C stronger than CE). The final extracts resulting from SPE methods contained 34–75% of the C and CE levels that were found in the PPT-ACN extracts. The Hybrid SPE-PPT procedure produced the cleanest extracts in terms of C and CE area responses (4.36% of PPT-ACN area responses) (Table 4). The hydroxyl group is also considered a strong Lewis base [33] and C may be retained by the same mechanism as the phospholipids (Lewis acid–base interaction). CE may also react as a Lewis base, and both C and CE have limited solubility in the polar solvent used for the precipitation (acetonitrile).

Table 4
Calculated % extraction of cholesterols and acylglycerols form different sample preparation techniques.

	C & CE	TAG	DAG	MAG
PPT				
PPT-ACN	100	100	100	100
PPT-Acetone	1442	2952	877	106
PPT-MeOH	154	121	871	97.8
PPT-ACN-FA	301	246	587	99.3
LLE				
MTBE-FA	34.9	83.6	66.9	6.66
MTBE-PBS	28.6	74.7	69.0	6.58
MTBE-NH_4OH	23.3	80.1	39.0	6.85
Ether-FA	32.6	63.7	27.2	4.02
Ether-PBS	25.5	50.4	86.3	7.56
Ether-NH_4OH	22.9	56.1	69.9	7.79
EthAC-Hex-FA	26.0	49.7	68.1	6.39
EthAC-Hex-PBS	25.0	42.7	75.6	8.21
EthAC-Hex-NH_4OH	22.2	39.0	57.9	8.15
DCM-FA	20.7	58.2	4.72	11.08
DCM-PBS	21.6	47.3	6.11	10.08
DCM-NH_4OH	17.5	37.5	4.93	11.06
SLE				
MTBE-FA	38.0	130	45.0	5.88
MTBE-PBS	34.5	104	20.6	4.66
MTBE-NH4OH	32.7	103	8.67	4.10
Ether-FA	32.7	103	34.5	5.40
Ether-PBS	31.5	84.9	36.8	5.08
Ether-NH_4OH	25.3	95.8	8.26	3.54
EthAC-Hex-FA	25.6	104	8.32	5.77
EthAC-Hex-PBS	23.1	77.7	6.68	4.37
EthAC-Hex-NH_4OH	11.2	58.0	3.47	3.08
DCM-FA	29.4	85.5	33.0	6.71
DCM-PBS	23.7	74.3	17.7	6.26
DCM-NH_4OH	18.0	75.7	6.31	5.37
SPE				
MCX	74.6	629	38.7	39.7
WAX	38.8	293	12.4	6.29
MAX	54.6	371	4.90	4.59
WCX	72.5	437	0.00	3.49
HLB	34.4	156	10.6	53.5
H-PPT	4.36	0.0	284	48.2

3.6.5. Triacylglycerols

Triacylglycerols (TAGs) are one of the major classes of lipids and constitute up to 10–15% of total lipids in human plasma [14,20]. TAGs have been shown to form ammonium adducts $[M+NH_4]^+$ in mass spectrometry. The MS/MS of TAGs ammonium adducts show ions formed by loss of ammonia $[(M+NH_4)\text{-}NH_3\text{-}FA]^+$, $[(M+NH_4)\text{-}NH_3]^+$ or formed by cleavage of fatty acids [36]. 1,3-Dipalmitoyl-2-oleoylglycerol (TAG) formed $[M+NH_4]^+$ at *m/z* 850.73 and the MS/MS of the ammonium adduct showed an intense ion at *m/z* 577.83 $[OP]^+$. It was found that at least 30% IPA is required to elute TAG (non-polar lipid) from a C_{18} guard column. TAG is a non-polar neutral lipid that may behave similarly to CE although with more hydrophobicity; ACN and MeOH PPT extracts produced very similar TAG levels (low levels of TAG may be due to the limited solubility of TAG in these polar solvents). 2% FA in ACN showed 2.5 times the TAG levels in PPT-ACN. PPT extracts using acetone showed approximately 30-fold the TAG levels demonstrated in PPT-ACN extracts. The final extracts resulting from LLE methods

using the various organic solvents contained 37.5–84% of TAG levels present in the PPT-ACN extracts. The SLE methods produced 58.0–130% of the TAG responses present in PPT-ACN. Comparing to the LLE and SLE results for C and CE, similar trends were observed but with higher percentages for TAG. This may be due to use of the combined area response for C and CE, however, TAG is a more hydrophobic component (three side chain fatty acid) and may show higher solubility than C and CE (one side chain fatty acid) in slightly polar solvents and non-polar solvents. The final extracts resulting from SPE methods showed high levels of TAG approximately 1.5–6 times the TAG levels that were found in the PPT-ACN extracts. This may be due to strong reversed phase interactions. The pH of the final elution solvent (methanol) may affect TAG recovery as different mixed ion exchange sorbents showed higher TAG than the reversed phase SPE. Another type of interaction such as H-bonding may also affect the interaction of TAG with the different sorbents. The hybrid SPE-PPT procedure was efficient in removal of TAG, which may be due to a possible Lewis acid–base interaction and limited solubility of TAG in acetonitrile (Table 4).

3.6.6. Diacylglycerols

Diacylglycerols (DAGs) constitute up to 20% of total lipids in human plasma [37]. DAGs have been shown to also form ammonium adducts $[M+NH_4]^+$. 1,3-Dilinoleoyl-rac-glycerol (DAG) formed $[M+NH_4]^+$ at *m/z* 634.67 and the MS/MS of the ammonium adduct showed an intense ion at *m/z* 599.5 $[M+NH_4\text{-}NH_3\text{-}H_2O]^+$. DAG is a neutral lipid with two side chain fatty acids and one hydroxyl group, PPT extracts using 2% FA in ACN, MeOH and acetone showed approximately 6-, 8- and 8-fold the responses for DAG as in the ACN-PPT extracts, respectively. The final extracts resulting from LLE methods using different organic solvents contained 27–86% of the DAG levels present in the ACN-PPT extracts. LLE incorporating DCM showed less than 7.0% of the DAG levels present in the ACN-PPT extracts. SLE extracts contained 3.5–45% of the DAG levels present in the ACN-PPT extracts. Basic pH (10.8 1:1 Plasma:0.5 M NH_4OH (v/v)) showed lower responses in most cases using different LLE and SLE organic solvents. The final extracts resulting from the SPE methods showed 5–39% of the DAG levels present in the ACN-PPT extracts. No DAG peak was observed in the WCX extracts, however, the Hybrid SPE-PPT procedure demonstrated unexpected higher levels of DAG which were approximately 3.0 times the DAG level in PPT-ACN extracts, possibly through Lewis acid–base interaction with the hydroxyl group of DAG. Other possible mechanisms of interaction between the analytes and zirconia-coated silica are through a chelation interaction. Chelation of compounds with oxygen atoms in the alpha or beta positions may cause interactions with the Zr–Si stationary phase. These secondary interactions may lead to low recovery of some compounds [33].

3.6.7. Minor lipids

Phosphatidylinositols (PI) constitute up to 4.4% and 2.0% of total phospholipids in human plasma and erythrocytes, respectively [14,19,20]. PI is a strongly acidic phospholipid that contains a phosphodiester of a six carbon sugar inositol as a polar head group [10,21,27]. PI forms $[M+NH_4]^+$ at *m/z* 904.06 and $[M-259]^+$ at *m/z* 627.8 in MS and MS/MS, respectively. LLE using acidic pH, SLE, MAX, WCX and H-PPT methods were found to be efficient methods to remove PI from human plasma (Table 3).

Phosphatidic acid (PA) is present in trace amounts and is a strongly acidic lipid. It exists as a salt in the cell and acidic pH is essential for extraction of PA to convert it to the free acid form [21,27]. PA formed $[M+NH_4]^+$ at *m/z* 720.5 and the MS/MS of the ammonium adduct showed an intense ion at *m/z* 605.8 $[M-97]^+$. PPT extracts using different solvents showed (32–64%) of PA in ACN-PPT extracts. Final extracts of the various extraction procedures contained less than 14% of the PA responses found in ACN-PPT extracts except for LLE and SLE using MTBE at acidic pH (40–45%).

Phosphatidylglycerols (PGs) are present in trace amounts and no peak was observed for PG in the final extracts of all proposed extraction procedures.

Monoacylglycerol (MAG) is present in trace amounts and the PPT extracts showed approximately 50% of MAG in the ACN-PPT extracts. The other extraction procedures showed less than 6.0% of the MAG level in ACN-PPT extracts except for the MCX, H-PPT and HLB extracts that were 19%, 23% and 25%, respectively.

4. Conclusions

A matrix effect library has been established for different lipid components in biomatrices including lipids present in trace amounts from commonly employed extraction procedures. It was found that no single extraction procedure was efficient in removing all of the different lipid components. Obtaining high target analyte extraction recovery should not be the only factor to consider in selecting a sample preparation technique. Achieving extracts with lower but acceptable analyte recovery, that are also low in co-extracted components that can cause significant matrix effects, will often result in more rugged analytical method. High levels of endogenous lipid components, other than the commonly appreciated GPChos class, such as cholesterols and triacylglycerols, may also result in major matrix effects. It is impractical to evaluate all lipids during method development, however, it is recommended that GPChos (*m/z* 184/184), LGPChos (*m/z* 104/104) and cholesterols (*m/z* 362/362) should be monitored. In addition, the mass transition of *m/z* 850.7/ 577.8 can be used to monitor TAGs. Results from this study will help bioanalytical scientists to anticipate different matrix effects from various extraction procedures, decide whether or not the absence or presence of various lipid components is important and help ensure avoidance of any co-eluted lipid components with the target analyte. These decisions would be made based on the particular lipid component and the lipid composition of the biomatrix. The specific mass transitions proposed in this study may be used for lipid monitoring and the extraction procedures evaluated are intended as a general guide to which approach may be more effective in lipid removal for a particular assay. The lipid-related matrix effects profile and the extent of the matrix effect were found to vary with the biomatrices studied. SLE methods were found to provide effective procedures for removal most of phospholipids, however, some of non-polar lipids such as TAG may be significantly recovered in the extract. SPE extracts demonstrated low levels of phospholipids in most of the modes evaluated, however, cholesterols and TAG levels were high in some of these extracts. The H-PPT method was an effective method for removal of most of phospholipids, cholesterol and TAG. A consideration of analyte chemical structure, the type of matrix and the type of sample preparation procedure will help the scientist to better anticipate and minimize matrix effects during LC/MS/MS method development.

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