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Journal of Chromatography B, 852 (2007) 22–34
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

# Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses

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Received 28 September 2006; accepted 27 December 2006
Available online 3 January 2007

**Abstract**

A systematic, comprehensive strategy that optimizes sample preparation and chromatography to minimize matrix effects in bioanalytical LC/MS/MS assays was developed. Comparisons were made among several sample preparation methods, including protein precipitation (PPT), liquid–liquid extraction (LLE), pure cation exchange solid-phase extraction (SPE), reversed-phase SPE and mixed-mode SPE. The influence of mobile phase pH and gradient duration on the selectivity and sensitivity for both matrix components and basic analytes was investigated. Matrix effects and overall sensitivity and resolution between UPLC® technology and HPLC were compared. The amount of specific matrix components, or class of matrix components, was measured in the sample preparation extracts by LC/MS/MS with electrospray ionization (ESI) using both precursor ion scanning mode and multiple reaction monitoring (MRM). PPT is the least effective sample preparation technique, often resulting in significant matrix effects due to the presence of many residual matrix components. Reversed-phase and pure cation exchange SPE methods resulted in cleaner extracts and reduced matrix effects compared to PPT. The cleanest extracts, however, were produced with polymeric mixed-mode SPE (both reversed-phase and ion exchange retention mechanisms). These mixed-mode sorbents dramatically reduced the levels of residual matrix components from biological samples, leading to significant reduction in matrix effects. LLE also provided clean final extracts. However, analyte recovery, particularly for polar analytes, was very low. Mobile phase pH was manipulated to alter the retention of basic compounds relative to phospholipids, whose retention tends to be relatively independent of pH. In addition to the expected resolution, speed and sensitivity benefits of UPLC® technology, a paired *t*-test demonstrated a statistically significant improvement with respect to matrix effects when this technology was chosen over traditional HPLC. The combination of polymeric mixed-mode SPE, the appropriate mobile phase pH and UPLC® technology provides significant advantages for reducing matrix effects resulting from plasma matrix components and in improving the ruggedness and sensitivity of bioanalytical methods.


*Keywords:* Matrix effects; Quantitative bioanalysis; Mass spectrometry; Phospholipids; UPLC; HPLC; Sample preparation

## 1. Introduction

LC/MS/MS is a powerful analytical technique for quantitative bioanalysis due to its inherent high sensitivity and selectivity. It is susceptible, however, to matrix effects. The impact of matrix effects on the accuracy, precision and robustness of bioanalytical methods is of growing concern in the pharmaceutical industry [1–11,13]. Residual matrix components, endogenous phospholipids in particular, are a significant source of imprecision in quantitative analyses commonly conducted by LC/MS/MS. Matrix effects, originally discussed by Kebarle and Tang [1] in the early 1990s, can be described as the difference between the mass spectrometric response for an analyte in standard solution and the response for the same analyte in a biological matrix, such as plasma. Matrix effects result from co-eluting matrix components that affect the ionization of the target analyte, resulting either in ion suppression, or, in some cases, ion enhancement. Matrix effects can be highly variable and can be difficult to control or predict. They are caused by numerous factors, including, but not limited to endogenous phospholipids, dosing media, formulation agents and mobile phase modifiers [14–17]. Furthermore, different sources of plasma from the same species can yield different validation results, such as standard curve slope and precision [5]. The severity and nature of suppression or enhancement may be a function of the concentration of the co-eluting matrix components [17]. Matrix effects can be compounded by co-eluting metabolites, impurities or degradation products. Furthermore, matrix effects are analyte specific.

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doi:10.1016/j.jchromb.2006.12.030

All of the above factors can cause significant errors in the accuracy and precision of bioanalytical methods. Current FDA guidance documents now require that these effects be evaluated as a part of quantitative LC/MS/MS method development, validation and routine use [18]. Consequently, most current papers describing the quantitation of drugs in biofluids discuss matrix effects to some degree.

Several papers describing the evaluation of matrix effects have been published, providing the guidance and techniques necessary for researchers. There are two common methods to assess matrix effects: the post-column infusion method [4,21,30,34,42] and the post-extraction spike method [6,10,12,19,22,28,29]. The post-column infusion method provides a qualitative assessment of matrix effects, identifying chromatographic regions most likely to experience matrix effects. This is carried out by monitoring the instrument response of a constantly infused analyte after injecting an extract from a sample into the LC/MS/MS system. This approach is limited in that it does not provide a quantitative understanding of the level of matrix effect observed by specific analytes, but merely identifies chromatographic regions where an analyte would be most susceptible to suppression or enhancement. This technique allows one to intelligently modify the elution time of an analyte so that it does not fall into suppression zones. However, this process can be quite time consuming and require significant optimization, particularly if quantitation of multiple analytes in a single run is desired. In contrast, the post-extraction spike method quantitatively assesses matrix effects by comparing the response of an analyte in neat solution to the response of the analyte spiked into a blank matrix sample that has been carried through the sample preparation process. In this manner, quantitative effects on ion suppression or enhancement experienced by all analytes in the sample can be measured each time a change is made to the analytical method.

While most researchers now include an evaluation of matrix effects as part of method development, only some attempt to actually reduce or eliminate matrix effects. Some researchers have focused on optimizing sample preparation to reduce matrix effects [20–22], while others have focused on manipulating chromatographic parameters [23,24]. Others assess the level of matrix effects and compensate for the alteration in signal through the use of an internal standard, often a stable isotope labeled (SIL) analog of the drug [25]. Still other papers describe the use of flow splitting to reduce matrix effects [26,27] or the need to resort to standard addition [28,29]. In some cases, researchers opt to use an ionization source, such as APCI, that is less sensitive for their compound, simply because the matrix effects experienced with the more sensitive source are too great [30]. APCI has shown, for certain compounds, less ion suppression and can be a better choice for some assays based on sensitivity and accuracy/precision, but it is not immune to matrix effects [5,6,34].

We believe there is a need for a more complete and thorough evaluation and solution to the problem of matrix effects. This solution should address the bioanalytical method as a whole, breaking it down into its major components and providing recommendations based on logical experimentation. Researchers need an approach that systematically and critically assesses both sample preparation and chromatographic techniques for their effectiveness in minimizing or eliminating matrix effects, leading to the most robust, sensitive assay possible. We endeavour to provide a comprehensive, systematic evaluation of both sample preparation methods and chromatographic optimization aimed at reducing or eliminating matrix effects. In our studies, we evaluated several sample preparation methods, including protein precipitation (PPT), liquid–liquid extraction (LLE), silica-based solid-phase extraction (SPE) and polymeric SPE. Because endogenous phospholipids have been identified as a major source of matrix effects by multiple researchers, we monitor the levels of the various phospholipids in the samples to compare relative cleanliness of final plasma extracts. Additionally, it is important to consider the overall cleanliness of sample extracts as it relates to the concentration of endogenous material being deposited onto an analytical column, which may negatively impact assay robustness, reduce column lifetime and result in increased MS maintenance. We also compared results from fast and slow gradients, low and high pH mobile phases, and HPLC and UPLC® chromatographic systems. The most robust and sensitive analytical methods are developed when each of these elements is considered. Based on these experimental results, we define a comprehensive approach to reducing matrix effects in bioanalytical methods. For this work, the focus was on the removal of phospholipids and the scope is limited to method development from a pharmaceutical quantitative bioanalysis standpoint.

## 2. Experimental

### 2.1. Materials

Propranolol, atenolol, chlorpheniramine maleate, amitriptyline, pseudoephedrine, terfenadine, imipramine, clozapine, formic acid (FA) and ammonium hydroxide were purchased from Sigma Chemical (St. Louis, MO.) Risperidone and 9-OH risperidone were purchased from Research Diagnostics, Inc. (Flanders, NJ.) All SPE sorbents were from Waters Corp. (Milford, MA 01757). Both 96-well 10 mg plate and 1 cc cartridge formats were used. Acetonitrile (ACN) and methanol (MeOH) were from J.T. Baker (Phillipsburg, NJ). Multiple lots of rat plasma were purchased from Equitech Bio, Inc. (Kerrville, TX 78028) and pooled for analysis. Each experiment was carried out with eight replicates.

### 2.2. LC/MS/MS conditions

Tandem mass spectrometry was performed using a Waters Quattro Premier™ tandem quadrupole MS system with positive ion electrospray ionization (ESI). MassLynx® software (versions 4.0 and 4.1) was used for data acquisition. The LC system was either a Waters 1525μ binary HPLC pump with a Waters 2777 Sample Manager or an ACQUITY UPLC® system. Columns used were a 3.5 μm, 50 × 2.1 mm XTerra™ MS $C_{18}$ or a 1.7 μm, 50 × 2.1 mm ACQUITY UPLC® BEH $C_{18}$ column (from Waters Corp., Milford, MA) thermostated at 50 °C. Flow rates were between 0.4 and 0.6 mL/min. Solvent A was

either 0.1% HCOOH in water (pH 2.7), 10 mM ammonium acetate (pH 9, adjusted with ammonium hydroxide) or 10 mM ammonium bicarbonate (pH 10). Solvent B was MeOH. Gradient separations were performed from 5% B to 95% B, $t_g = 2$ min (slow gradient) or $t_g = 1$ min (fast gradient). Injection volume was 2–5 μL, depending on the sample loop installed. The LC flow was directly introduced into the MS source without flow splitting. The MS source and desolvation temperatures were maintained at 120 and 350 °C, respectively. The desolvation and cone gas flows were set at 700 and 50 L/h. The MS capillary voltage was 3.0 or 3.5 kV. MS/MS experiments were carried out with an argon pressure of approximately $2.6 \times 10^{-3}$ mbar in the collision cell (Q2). The cone voltage and collision energy were optimized for each analyte. The dwell time for each MRM transition was 0.05 s.

The phospholipids monitored were 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (*m/z* 496.35), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (*m/z* 524.37), 1-hexadecanoyl-2-(9*Z*,12*Z*-octadecadienoyl)-sn-glycero-3-phosphocholine (*m/z* 758.57) and 1-(9*Z*,12*Z*-octadecadienoyl)-2-(5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoyl)-sn-glycero-3-phosphocholine (*m/z* 806.57). A fifth glycerophosphocholine lipid of molecular weight 703.57 was also monitored. A preliminary quadrupole-TOF experiment yielded data that were inconclusive with respect to the placement of double bonds and the alkyl chain lengths. We have identified the chemical formula as $C_{38}H_{74}NO_8P$. For each lipid, the transition monitored corresponded to the protonated mass of the precursor fragmented to *m/z* 184.3, the mass of the polar head group, a trimethylammonium-ethyl phosphate cation. An alternate experiment, described by Little et al. [31] employs in-source collision induced dissociation (CID) with a high cone voltage (90 V) and a low collision energy (3 eV) to monitor the 184.3 ions only, resulting in total ion chromatograms (TIC) representing all compounds containing the phosphocholine head group. Both methods were used in this work.

## 3. Sample preparation methods

### 3.1. Protein precipitation

#### 3.1.1. Dry and reconstitute method

Blank rat plasma samples were extracted as follows. A 750 μL aliquot of ACN, MeOH or 2% HCOOH in ACN was added to 250 μL rat plasma; this was vortexed and centrifuged at 13,000 rpm for 5 min. The supernatant was removed, dried down and reconstituted in 250 μL of 50:50 MeOH:$H_2O$ containing 0.1–500 ng/mL of one or several of the 10 analytes listed in Section 2.1. The particular concentration used was dependent on the experiment, but the overall concentration range used in this work encompassed typical analyte concentrations for bioanalytical assays in a pharmaceutical environment.

For recovery calculations, 5 or 10 mL of plasma were fortified with analytes in 50:50 MeOH:$H_2O$, the spike volume being 5% of the total plasma volume. The same volume of unfortified plasma was spiked with 5% by volume of 50:50 MeOH:$H_2O$. The post-spiked reconstitution solvent was prepared in 50:50 MeOH:$H_2O$, using the same volumes as for the plasma samples. Dried-down fortified samples were reconstituted in 50:50 MeOH:$H_2O$. Dried-down blank plasma samples were reconstituted in the post-spike reconstitution solvent.

#### 3.1.2. Dilute and inject method

A 300 μL aliquot of ACN or MeOH was added to 100 μL rat plasma; this was vortexed and centrifuged at 13,000 rpm for 5 min. One hundred microliters of the supernatant was removed and diluted with 100 μL of 50:50 MeOH:$H_2O$ containing 0.1, 1 or 5 ng/mL each of terfenadine, amitriptyline and/or propranolol.

### 3.2. Liquid–liquid extraction

Liquid–liquid extraction (LLE) was carried out using three methods. Method 1 employed a 3:1 ratio of MTBE to plasma. Method 2 employed a 3:1 ratio of basified MTBE (5% $NH_4OH$ in MTBE) to plasma. For Method 3, the extraction with basified MTBE was carried out as a two step extraction. In each case, the sample was vortexed and centrifuged at 13,000 rpm for 5 min. The supernatant was removed, dried down and reconstituted in 250 μL of 50:50 MeOH:$H_2O$. For Methods 1 and 2, 750 μL of MTBE was added to 250 μL rat plasma. For Method 3, 750 μL of basified MTBE was added to the plasma, this was vortexed, centrifuged and the supernatant removed and set aside to be combined with the second extract. The pellet was then extracted a second time with 750 μL of basified MTBE, vortexed, centrifuged and the supernatant removed and added to the first extract. The combined extracts were dried down and reconstituted in 250 μL of 50:50 MeOH:$H_2O$. Recovery calculation samples were carried out as described for protein precipitation.

### 3.3. Solid-phase extraction

#### 3.3.1. Polymeric mixed-mode strong cation exchange SPE

Oasis® MCX 10-mg (1 meq/g) 96-well plate.

*3.3.1.1. Dry and reconstitute method.* The sorbent was conditioned with 500 μL of MeOH followed by equilibration with 500 μL of $H_2O$. A 250 μL aliquot of rat plasma was diluted 1:1 with 4% $H_3PO_4$ and then loaded onto the sorbent. The sorbent was washed with 500 μL of 2% HCOOH in $H_2O$ and followed by 500 μL of MeOH. The latter step (100% MeOH) can serve either as a "wash", to remove hydrophobic interferences or as an elution step for acidic or neutral analytes, which have bound by reversed-phase. The final elution with 250 μL of 5% $NH_4OH$ in 90:10 MeOH:$H_2O$, 100% MeOH, 100% ACN or various ratios of MeOH:ACN, was carried out in two steps of 125 μL each. The eluate was dried down and reconstituted in 250 μL of 50:50 MeOH:$H_2O$ containing 0.1–500 ng/mL of one or several of the analytes specified in Section 2.1.

*3.3.1.2. Dilute and inject method.* The sorbent was conditioned with 500 μL of MeOH followed by equilibration with 500 μL of $H_2O$. A 100 μL aliquot of rat plasma was diluted 1:1 with 4% $H_3PO_4$ and loaded onto the sorbent. The sorbent was washed with 500 μL of 2% HCOOH in $H_2O$, and followed by 500 μL of

MeOH. The final elution with 300 μL of 5% $NH_4OH$ in MeOH was carried out in two steps of 150 μL each. A 100 μL aliquot of the eluate was removed and diluted with 100 μL of 50:50 MeOH:$H_2O$ containing 0.1, 1 or 5 ng/mL each of terfenadine, amitriptyline and propranolol.

For comparison with the silica-based pure ion exchange material, 10-mg 1-cc cartridges of Oasis® MCX were used, and the protocol followed was identical to that described for the pure ion exchange material.

*3.3.2. Polymeric mixed-mode strong anion exchange SPE*

Oasis® MAX 10-mg (0.25 meq/g) 96-well plate. The sorbent was conditioned with 500 μL of MeOH followed by equilibration with 500 μL of $H_2O$. A 250 μL aliquot of rat plasma was diluted 1:1 with 4% $H_3PO_4$ and loaded onto the sorbent. The sorbent was washed with 500 μL of 5% $NH_4OH$ in $H_2O$, and followed by 500 μL of MeOH. The final elution with 250 μL of 2% HCOOH in MeOH was carried out in two steps of 125 μL each. The eluate was dried down and reconstituted in 250 μL of 50:50 MeOH:$H_2O$.

*3.3.3. Polymeric mixed-mode weak cation exchange SPE*

Oasis® WCX 10-mg (0.75 meq/g) 96-well plate. The sorbent was conditioned with 500 μL of MeOH followed by equilibration with 500 μL of $H_2O$. A 250 μL aliquot of rat plasma was diluted 1:1 with 4% $H_3PO_4$ and loaded onto the sorbent. The sorbent was washed with 500 μL of 5% $NH_4OH$ in $H_2O$, and followed by 500 μL of MeOH. The final elution with 250 μL of 2% HCOOH in MeOH was carried out in two steps of 125 μL each. The eluate was dried down and reconstituted in 250 μL of 50:50 MeOH:$H_2O$.

*3.3.4. Polymeric mixed-mode weak anion exchange SPE*

Oasis® WAX, 10 mg (0.6 meq/g) 96-well plate. The sorbent was conditioned with 500 μL of MeOH followed by equilibration with 500 μL of $H_2O$. A 250 μL aliquot of rat plasma was diluted 1:1 with 4% $H_3PO_4$ and loaded onto the sorbent. The sorbent was washed with 500 μL of 2% HCOOH in $H_2O$, and followed by 500 μL of MeOH. The final elution with 250 μL of 5% $NH_4OH$ in MeOH was carried out in two steps of 125 μL each. The eluate was dried down and reconstituted in 250 μL of 50:50 MeOH:$H_2O$.

*3.3.5. Silica-based pure cation exchange SPE*

Experimental silica-based strong cation exchange SPE material (0.2 meq/g), 1-cc 50 mg cartridge. The sorbent was conditioned with 1 mL of MeOH followed by equilibration with 1 mL of $H_2O$. A 250 μL of rat plasma was diluted 1:1 with 4% $H_3PO_4$ and loaded onto the sorbent. The sorbent was washed with 1 mL of 2% HCOOH in $H_2O$, and followed by 1 mL of MeOH. The final elution with 500 μL of 5% $NH_4OH$ in MeOH was carried out in two steps of 250 μL each. The eluate was dried down and reconstituted in 250 μL of 50:50 MeOH:$H_2O$.

*3.3.6. Polymeric reversed-phase SPE*

Oasis® HLB, 10-mg 96-well plate. The sorbent was conditioned with 500 μL of MeOH followed by equilibration with 500 μL of $H_2O$. A 250 μL aliquot of rat plasma was diluted 1:1 with 4% $H_3PO_4$ and loaded onto the sorbent. The sorbent was washed with 500 μL of 5% MeOH in $H_2O$. The final elution with 250 μL of MeOH was carried out in two steps of 125 μL each. The eluate was dried down and reconstituted in 250 μL of 50:50 MeOH:$H_2O$.

## 4. Results and discussion

### *4.1. Phospholipids in LC/MS/MS analysis*

Endogenous phospholipids are present in high concentrations in biological matrices, such as plasma [32,33] and have been implicated in causing ion suppression or enhancement in LC/MS/MS analyses. Researchers have described this phenomenon as being due to the effect they have on desolvation of the LC effluent droplets in electrospray MS analysis [34] or as a result of competition for excess charges on the droplet surface [35]. Bradamante et al. [32] characterized the phospholipid composition of plasma using NMR. Glycerophosphocholines make up almost 70% (expressed in mol% of phosphorus) of the total plasma phospholipids. Lysophospholipids make up almost 10% of total phospholipids. These findings confirmed earlier, similar results obtained by TLC and GC [33]. Current matrix effects research [14,15] indicated that these residual plasma phospholipids are a major source of ion suppression and identified specific phospholipids that cause matrix effects. The individual phospholipid transitions we used as surrogates for monitoring the presence of phospholipids are the same ones identified by these researchers as the *major* plasma phospholipids. Phosphatidylcholine containing phospholipids consist of both a polar head group, which contains a negatively charged phosphate group and a positively charged quaternary amine group, and one or two long alkyl chain(s). The polar head group imparts strong ionic character to the phospholipids, while the long alkyl chains make them extremely hydrophobic, often requiring a hold at nearly 100% organic solvent to elute them from a reversed-phase chromatographic column. The late elution time may not present a problem for polar analytes, as they are not expected to co-elute with the phospholipids. Non-polar analytes, however, run a significant risk of co-elution with these phospholipids. In addition, residual phospholipids, if not fully eluted from the analytical column, can build up on the column and significantly reduce column lifetime. James Little demonstrated further risks associated with incomplete removal of phospholipids (from the column), such as their unexpected elution in subsequent analytical runs.

### *4.2. Mass spectrometry*

Several MS methods were used for detection of the various types of phospholipids, depending on the goal of the experiment. Individual MRM traces for specific phospholipids [36] were recorded to better understand the mechanism of removal of specific types of phospholipids. Precursor ion or neutral loss scanning was used to monitor overall cleanliness of the extracts with respect to certain classes of phospholipids [37] and a single

MRM method which takes advantage of the structural commonalities among classes of phospholipids was employed to provide the maximum amount of information within a single MRM trace [31]. The majority of the work presented here focuses on five specific lipids believed to be representative of the lipid classes most strongly implicated in matrix effects.

It was found that the scanning experiments were not practical with respect to efficient use of duty cycle. The method described by Little et al. uses high energy in-source CID of the lipids, which yields a characteristic fragment ion at *m/z* 184, corresponding to the trimethylammonium-ethyl phosphate cation [31]. This ion allows monitoring of multiple phospholipids and lysophospholipids with a single MRM transition, thus maximizing the duty cycle in the mass spectrometer. This approach eliminates the need for scanning experiments, except where required to monitor the presence of distinct classes of phospholipids, specifically phosphatidylethanolamines (neutral loss scanning of 141 Da, positive ion mode) or phosphatidylserines (neutral loss scanning of 185 Da, positive ion mode.)

### 4.3. Control of variables

In this work, variables were controlled to insure a valid and accurate comparison of results. Final solvent composition of reconstituted extracts, ion exchange capacity of sorbents, and volume of plasma were among the variables strictly controlled. For each extraction procedure, the same volume of plasma was used (250 μL) to ensure that the starting lipid levels were consistent among all methods. The final eluates, extracts or supernatants dried down completely and reconstituted in a constant volume of 250 μL. The dry down and reconstitute step is important for several reasons: to keep the volumes and lipid concentrations consistent across different sample prep techniques, and to ensure that all samples are dissolved in identical solvents. The dried down extracts were always reconstituted in the same volume (250 μL) and with the same solvent (50:50 MeOH:$H_2O$). The sample dilution solvent itself can be a source of ion suppression or enhancement [12,17] and may affect the solubility of various plasma components. The final reconstitution solvent was chosen to be as "universal" as possible, meant to solubilize both highly polar and non-polar compounds. We recognize that optimal sensitivity for each compound may be achieved using a specific dilution solvent, but we aimed to keep all steps of the various protocols generic in their approach, including choice of dilution solvent, to avoid any bias due to optimization. For the comparison between polymeric mixed-mode cation exchange and pure ion exchange SPE, particular attention was paid to ensure equivalency of ion exchange capacity. The Oasis® MCX sorbent has an ion exchange capacity of 1 meq/g and a 10-mg cartridge was used. The experimental pure cation exchange sorbent has an ion exchange capacity of 0.2 meq/g. To provide equivalent ion exchange capacity for an accurate comparison, a sorbent bed mass of 50 mg of this material was used. While the masses of the sorbents were different, the volumes of the chromatographic beds were similar due to the different densities of the sorbents.

### 4.4. Calculations

Three criteria were used for comparing different sample preparation methods: analyte recovery, % matrix effect and efficiency of phospholipid removal. Recovery calculations were always carried out against post-extracted spiked standards. This de-couples recovery from matrix effects, which are evaluated separately. Analyte SPE recovery was calculated as follows:

$$\%\mathrm{RE} = \frac{\mathrm{Response}_{\text{extracted sample}}}{\mathrm{Response}_{\text{post-extracted spiked sample}}} \times 100 \quad (1)$$

where $\mathrm{Response}_{\text{extracted sample}}$ is the average area count for the analyte, in matrix, which has been through the extraction process. $\mathrm{Response}_{\text{post-extracted spiked sample}}$ is the average area count for the same quantity of analyte, spiked into extracted matrix after the extraction procedure.

Matrix effects were calculated using a modified version of the equation described by Matuszewki et al. [6]:

$$\%\text{Matrix Effects} = \left(\frac{\mathrm{Response}_{\text{post-extracted spiked sample}}}{\mathrm{Response}_{\text{non-extracted neat sample}}} - 1\right) \times 100 \quad (2)$$

where $\mathrm{Response}_{\text{post-extracted spiked sample}}$ is the average area count for the analyte, spiked into extracted matrix after the extraction procedure and $\mathrm{Response}_{\text{non-extracted neat sample}}$ is the average area count for the same concentration of analyte in neat solution. This neat solution should be the same solvent composition as the reconstitution solution used for the post-extracted spiked sample. The original equation in reference [6] was modified by applying the subtraction of 1 to the quotient so that a negative result indicates suppression, and a positive result indicates enhancement of the analyte signal.

## 5. Optimizing sample preparation

### 5.1. Protein precipitation

The increasing focus on high throughput sample analysis has led to the common practice of preparing samples by the simplest, fastest method possible, which often means using protein precipitation (PPT). Although PPT is quick and easy, it does not result in a very clean final extract. This method fails to sufficiently remove enough of the plasma components, specifically phospholipids, known to cause variability in analyte signal intensity in a mass spectrometer [38–41]. The specific organic solvent used in protein precipitation has a dramatic effect on the overall cleanliness of the final extract. Replicates of both blank plasma and plasma containing either 50 or 100 ng/mL of each of eight analytes (all bases) were prepared. Analyte recovery was determined as well as the % matrix effect. In addition to monitoring the five specific phospholipids transitions, each extract was subjected to the experiment described by Little et al. [31] in order to compare relative amounts of all residual phosphatidylcholine containing lipids. Fig. 1 shows the relative amounts of all phosphatidylcholine containing lipids remaining in plasma extracts prepared by protein precipitation with (A) MeOH and

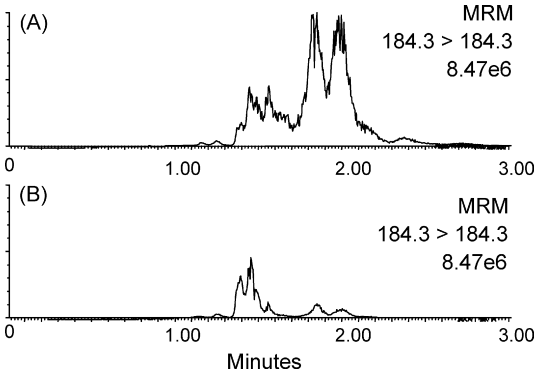


Fig. 1. MRM transition (184.3 > 184.3) detecting all phosphatidylcholine containing phospholipids from PPT samples using either (A) methanol or (B) acetonitrile as the precipitation solvent. HPLC/MS/MS with pH 2.7 mobile phase.

(B) ACN. Clearly, the MeOH extract contained significantly more residual phospholipids of this class than the ACN extract. All samples were plotted on the same *y*-axis scale and were run by HPLC/MS/MS using a low pH mobile phase. We compared the total area counts for the five specific lipids monitored in the three extracts. There were approximately 40% more of these specific lipids in the MeOH extract than in the ACN. Recoveries and matrix effects for both methods are summarized in Table 1. Recoveries for the eight analytes ranged from 76% to 114% and from 79% to 108% for the ACN and MeOH extracts, respectively. Significant matrix effects, from 67% to 77% ion suppression, were observed for all eight analytes, regardless of precipitation solvent. Even with ACN, the level of phospholipids remaining and the degree of matrix effects observed were significant enough to negatively impact the assay with respect to both robustness and ultimate sensitivity.

### *5.2. Comparison of protein precipitation with single retention mechanism SPE*

It is widely accepted that SPE offers cleaner extracts than PPT. However, with all of the available SPE sorbents to choose from, it made sense to systematically compare ACN PPT with several common single-mode SPE sorbents for cleanliness of the final extracts as well as to try to eliminate the significant matrix effects observed with PPT. We chose an experimental silica-based strong cation exchange sorbent and the Waters Oasis® HLB polymeric sorbent to represent SPE by cation exchange only and reversed-phase mechanisms, respectively. Fig. 2 contains representative TICs of the five individual phospholipid MRM transitions from (A) PPT with ACN, (B) reversed-phase polymeric SPE and (C) experimental strong cation exchange-only material. All chromatograms were plotted on the same *y*-axis for appropriate comparison. The samples were run by HPLC/MS/MS using a pH 10 mobile phase. It is clear that both SPE methods provide a significantly cleaner extract than PPT. The summed average area counts for the five phospholipids were compared for a more quantitative assessment of the effectiveness of single retention mechanism SPE for phospholipid removal. The final basified MeOH extract resulting from SPE with the silica-based cation exchange material contained on average 14% of the lipid levels present in the PPT extract. Results were nearly identical from the final reversed-phase polymeric SPE extract, which also contained on average 14% of the lipids present in the PPT extract. In addition to 100% MeOH, acidified and basified

Table 1
Calculated % extraction recoveries and % matrix effects for various sample preparation techniques

| | ACN PPT | RP SPE | MeOH PPT | LLE | Basified LLE | 2 step Basified LLE | MCX SPE Basified MeOH | MCX SPE Basified ACN |
|---|---|---|---|---|---|---|---|---|
| % Recovery | | | | | | | | |
| Atenolol (p) | **92** | 14[a] | **89** | 1 | 7 | 12 | **108** | 9 |
| Pseudoephedrine (p) | 76 | 10[a] | **81** | 7 | 43 | 54 | **93** | 17 |
| 9-OH Risperidone (np) | **114** | 78 | **108** | 47 | 47 | **109** | **96** | **96** |
| Risperidone (np) | **91** | **80** | **90** | 53 | 50 | **111** | **96** | **101** |
| Clozapine (np) | **91** | **84** | **95** | 53 | 48 | **99** | **94** | **92** |
| Chlorpheniramine (np) | **87** | **86** | 79 | 66 | 47 | **91** | **92** | **94** |
| Imipramine (np) | **85** | **85** | 79 | 59 | 34 | **117** | **93** | **97** |
| Amitriptyline (np) | **97** | **81** | **96** | 59 | 34 | **104** | **85** | **95** |
| Matrix effects | | | | | | | | |
| Atenolol (p) | −75 | −50 | −73 | **−7** | **<5** | −19 | **−6** | **11** |
| Pseudoephedrine (p) | −68 | −54 | −67 | **<5** | **−9** | **−7** | **<5** | **<5** |
| 9-OH Risperidone (np) | −74 | −60 | −72 | **<5** | **−8** | −27 | **<5** | **7** |
| Risperidone (np) | −75 | −61 | −74 | **<5** | **−11** | −18 | **<5** | **7** |
| Clozapine (np) | −75 | −59 | −71 | **<5** | **−11** | −17 | **<5** | **11** |
| Chlorpheniramine (np) | −74 | −59 | −70 | **<5** | **−7** | −16 | **<5** | **14** |
| Imipramine (np) | −77 | −58 | −72 | **<5** | **−7** | −26 | **12** | **13** |
| Amitriptyline (np) | −77 | −47 | −71 | **−7** | **−6** | −27 | **<5** | **10** |

p = Polar analyte; np = non-polar analyte. Values in bold-face type indicate acceptable results.

[a] *Note*: All samples containing this mixture of analytes were diluted and acidified prior to SPE to ensure disruption of protein binding, particularly for the more hydrophobic analytes. The basic analytes are then in their ionized state, which further decreases reversed-phase retention. Recovery of the polar analytes improves significantly when the plasma is neutral or basic.

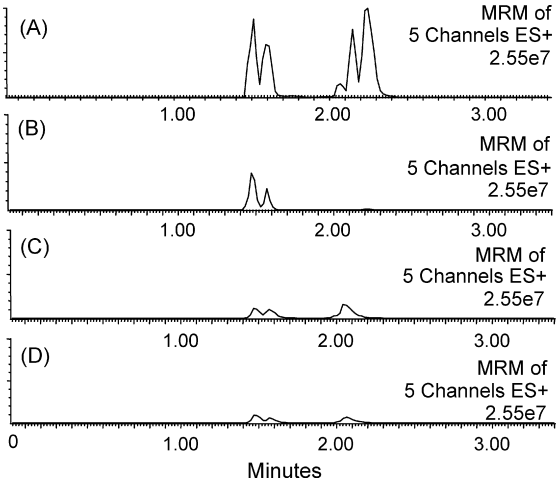


Fig. 2. TICs of MRM transitions for five phospholipids remaining in final extracts after sample preparation by (A) acetonitrile PPT, (B) reversed-phase polymeric SPE, (C) silica-based pure cation exchange and (D) mixed-mode cation exchange SPE. HPLC/MS/MS with pH 10 mobile phase.

MeOH solutions were also evaluated for use as the final eluant in the reversed-phase SPE protocol. The resulting levels of residual phospholipids in these final reversed-phase SPE eluates were not statistically different from the 100% MeOH eluate. Analyte recoveries and matrix effects observed in the reversed-phase SPE extracts are summarized in Table 1. Matrix effects were reduced by an average of almost 20% when reversed-phase SPE was used instead of PPT. Results from the experimental pure cation exchange material were not fully understood. The structure of a typical phosphatidylcholine suggests that it could bind to a sorbent either by reversed-phase (primarily through interaction with the long hydrophobic tail) or potentially by ion exchange. Ion exchange retention may occur either through interaction with the quaternary amine (cation exchange) or through interaction with the phosphate group (anion exchange.) There has been some discussion that since the net charge on the molecule would be zero, ion exchange should not occur at all. At this time, we do not have a good explanation for this observation and continue to carry out experiments intended to further elucidate these mechanisms.

### 5.3. Comparison of single mode and mixed-mode SPE

A recent paper by Shen et al. [42] suggests that pure cation exchange SPE sorbents result in cleaner final extracts than mixed-mode cation exchange SPE sorbents. This conclusion is contrary to previous results obtained in our laboratories, which led us to develop more extensive and carefully controlled experiments to further evaluate this claim. We compared the results from final eluates from Oasis® MCX (polymeric mixed-mode cation exchange), PPT, reversed-phase polymeric SPE and the experimental pure cation exchange material with respect to analyte recovery, matrix effects and presence of residual phospholipids.

Using the generic starting SPE protocol, recoveries for all eight analytes on mixed-mode strong cation exchange were acceptable, ranging from 85% to 108%. Data are listed in Table 1 under the column labeled MCX MeOH. For most analytes, calculated matrix effects were less than or equal to 5%. Atenolol and imipramine exhibited 6% suppression and 12% enhancement, respectively. These results are significant improvements over the PPT results. As shown in Fig. 2D, with respect to residual phospholipid levels, the final polymeric mixed-mode cation exchange eluate is almost twice as clean as either (B) polymeric reversed-phase or (C) cation exchange only SPE. Looking at the five specific phospholipid MRM transitions, we observed that it is primarily the very hydrophobic phospholipids containing two alkyl chains that are more effectively removed by the mixed-mode cation exchange SPE. The lysophospholipids are more effectively removed by mixed-mode cation exchange SPE as well, but not to the extent of their more hydrophobic counterparts. The 100% organic wash step employed with the mixed-mode cation exchange protocol removes 2.5–4 times as much of the very hydrophobic phospholipids than the same step does on the pure cation exchange material. This same wash step removes 1.5–2 times as much of the two lysophospholipids that we monitor. These data indicate that PPT is the least effective sample preparation technique. While pure strong cation exchange SPE or reversed-phase SPE only results in significantly cleaner final extracts compared to PPT, the most effective sample clean up is achieved using mixed-mode cation exchange SPE. During mixed-mode strong cation exchange SPE, significant clean-up is achieved during the 100% organic wash step, where phospholipids bound by reversed-phase are removed as well as any other neutral hydrophobic interferences. This same step, on reversed-phase only SPE, functions as the final elution step, containing significant levels of residual phospholipids and other hydrophobic interferences.

### 5.4. Comparison of mixed-mode SPE to liquid–liquid extraction

Liquid–liquid extraction (LLE) is viewed by some researchers as an efficient means of sample preparation that is a cleaner option than PPT, and we therefore included LLE in our experiments. As with the extraction methods previously discussed, we used several generic procedures for comparison. We evaluated the reconstituted LLE extracts for overall cleanliness, matrix effects and analyte recovery. Specific values for matrix effects and recovery from three LLE methods are summarized in Table 1. Matrix effects and overall cleanliness in LLE extracts from Methods 1 and 2 were comparable to cation exchange mixed-mode SPE using basified MeOH. For the analytes tested, which range widely in hydrophobicity, recovery using Method 1 or 2 was not always satisfactory, and is in general lower than that achieved with a generic mixed-mode strong cation exchange SPE method. Recovery values for the eight analytes, using Method 1, ranged from 7% and 1% for the polar analytes pseudoephedrine and atenolol, respectively, to 47–66% for the more non-polar analytes. The extraction was also performed with basified MTBE (Method 2), which puts the analytes in their non-ionized state, and is expected to increase solubility and improve recovery, particularly for polar analytes. Under these conditions, recovery for atenolol and pseudoephedrine increased to 7% and 43%, respectively. The best recovery was

achieved using Method 3, a two step extraction. Recovery for the polar analytes ranged from 12% to 54%. A significant increase in recovery was also observed for the non-polar analytes. Using Method 3, their recovery ranged from 99% to 117%. However, the recovery improvement using Method 3 was accompanied by an increase in matrix effects, which ranged from 7% to 27% suppression. In addition to the potential for lower recoveries and/or increased matrix effects, LLE is less practical for several reasons. The final extraction solvent is not particularly compatible with common initial reversed-phase LC mobile phase compositions and would require time consuming removal of the supernatant, followed by dry down and reconstitution. In addition, a degree of variability is introduced in the supernatant removal step as it is difficult to remove a consistent exact volume if the entire supernatant is to be used. If it is possible to directly inject from the top layer, dilution may occur, depending on the ratio of solvent to sample used. (This is further confounded as multiple extraction steps may be used to improve recovery.) This will result in overall lower sensitivity and thus is not desirable for assays characterized by a challenging LOQ.

### 5.5. Comparison of organic elution solvents used in mixed-mode cation exchange SPE

For ease of discussion, we need to define the various elution steps used in SPE. Protocols for mixed-mode SPE sorbents contain both a 100% organic solvent elution (to elute analytes bound by reversed-phase interactions) and an acidified or basified organic solvent elution (to elute analytes bound by ion exchange interactions). If the 100% organic eluate contains analytes of interest, we refer to this elution as Elute 1. However, if this eluate does not contain analytes of interest, we refer to this elution as Wash 2. (Wash 1 is an acidic or basic aqueous eluant used to ensure ion exchange occurs.) In the remainder of our discussion, we refer to this 100% organic eluate as Wash 2/Elute 1. In general, the acidified or basified organic solvent always contains analytes of interest, and we therefore refer to this as Elute 2. For example, if a mixture of acidic, basic and neutral analytes was loaded onto the mixed-mode cation exchange sorbent, we would expect to find the acidic and neutral analytes in the 100% MeOH Elute 1, and the basic compounds in the final basified organic Elute 2.

It is common practice to use MeOH as the organic solvent for elution of analytes in SPE methods as it is an effective universal solvent, has good compatibility with common LC mobile phases, and readily evaporates if dry down is needed. During the course of our evaluations, we found that many of the phospholipids appeared to be more soluble in MeOH than ACN. Although only negligible matrix effects were observed in the polymeric mixed-mode strong cation exchange extracts, it is important to consider some of the other reasons for sample preparation, specifically extract cleanliness and its impact on method robustness and column lifetime. We wanted to determine if solvents other than MeOH would result in cleaner final eluates with equal or better analyte SPE recoveries. Using the same test mixture of eight analytes, we extracted rat plasma samples by polymeric mixed-mode cation exchange, varying the type and composition of organic solvents used in Wash 2/Elute 1. Both 100% MeOH and 100% ACN were evaluated for Wash 2/Elute 1. Table 2 summarizes the residual area counts for the five phospholipids in these Wash 2/Elute 1 eluates. As Wash 2, MeOH is the best solvent since it removes approximately 64% more residual lipids than ACN, leaving fewer lipids to elute in Elute 2, and thus providing a cleaner final extract. However, since ACN results in a cleaner extract, in some applications, ACN may be used as Elute 1, provided SPE recoveries are acceptable. Additionally, the data in Table 3 indicate that Wash 2/Elute 1 from the mixed-mode strong cation exchange sorbent is significantly cleaner (2% residual phospholipids) than the comparable extract from the reversed-phase only sorbent (13% residual phospholipids.).

Table 2
Average area counts for five phospholipids in mixed-mode cation exchange Wash 2/Elute 1 using either ACN or MeOH as the organic solvent

| Organic solvent | Lipids found in Wash 2/Elute 1 (*m/z*) | | | | | |
|---|---|---|---|---|---|---|
| | 496 | 524 | 704 | 758 | 806 | Totals |
| MeOH | 71, 029 | 58,609 | 21 | 292 | 24 | 129,975 |
| ACN | 39, 282 | 39,143 | 30 | 632 | 59 | 79,145 |

### 5.6. Comparison of acidified or basified elution solvents used in mixed-mode cation exchange SPE

Similar to our discussion in the previous section, we wanted to investigate the overall cleanliness of various solvents used in Elute 2. Fig. 3 shows the TIC of the five specific phospholipid MRM transitions in (A) basified MeOH and (B) basified ACN Elute 2. A comparison of the two extracts clearly shows a significant improvement in overall cleanliness when ACN is substituted for MeOH in Elute 2. This is not entirely unexpected based on the results for ACN and MeOH PPT samples. Both sets of data suggest that phospholipids are more soluble in MeOH. We also compared average area counts for these specific lipids in Elute 2 to obtain a more quantitative assessment of relative cleanliness. For each of the five phospholipid transitions monitored, the basified ACN Elute 2 contained approximately

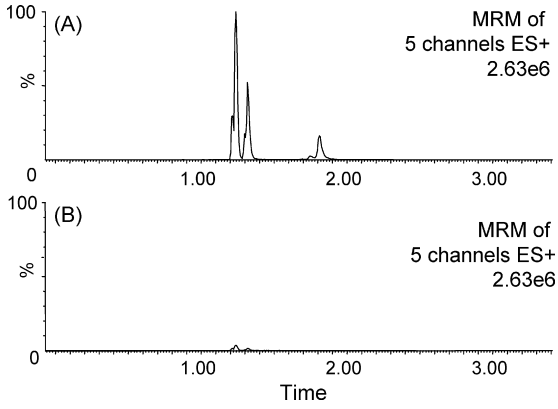


Fig. 3. TICs of MRM transitions for five phospholipids remaining in Elute 2 from mixed-mode cation exchange SPE using (A) basified methanol and (B) basified acetonitrile as the elution solvent. UPLC®/MS/MS with pH 9 mobile phase.

Table 3
Comparison of residual levels of five phospholipids in final eluates of five polymeric SPE sorbents, relative to ACN PPT, which is set at 100%

| Sorbent type | Wash 2/Elute 1 (100% MeOH): % phospholipids relative to ACN PPT | Elute 2: % phospholipids relative to ACN PPT |
|---|---|---|
| Reversed-phase | 13 | n/a |
| Mixed-mode strong cation exchange (Elute 2 is basified MeOH) | 2 | 8 |
| Mixed-mode strong cation exchange (Elute 2 is basified ACN) | 2 | 1 |
| Mixed-mode strong anion exchange (Elute 2 is acidified MeOH) | 9 | <1 |
| Mixed-mode weak cation exchange (Elute 2 is acidified MeOH) | 12 | <1 |
| Mixed-mode weak anion exchange (Elute 2 is basified MeOH) | 11 | <1 |

n/a: Indicates not applicable.

2–5% of the same lipid found in the MeOH Elute 2. The sums of the area counts are listed in Table 4, in the 100% MeOH and 0% MeOH (100% ACN) columns. The improvement due to ACN is quite significant, especially when compared to the levels of these phospholipids remaining in a PPT extract. Our experimental data indicate that the level of residual phospholipids (based on average area counts of the five representative MRM transitions) in basified MeOH Elute 2 from mixed-mode SPE is only 7–8% of the level of those lipids in the ACN PPT extract. In the same comparison to ACN PPT, there are less than 1% of residual phospholipids remaining in the ACN Elute 2 from mixed-mode cation exchange SPE. This represents the cleanest extract we have seen in our studies. However, it is also important to consider the impact of different organic elution solvents on analyte recovery. We have shown MeOH to be a good universal solvent for elution from SPE, with recoveries for the eight analytes being acceptable (>90%) on a first pass. More non-polar solvents, such as the MTBE used in LLE have proven to be less universal in their ability to adequately recover analytes with a wide range of chemical properties and hydrophobicities. Table 1 summarizes the recoveries for all analytes using mixed-mode cation exchange with basified ACN and basified MeOH for Elute 2. While basified ACN produces the cleanest final extract, recovery for polar analytes is lower than when basified MeOH is used as Elute 2. Recovery of the more non-polar analytes is not affected and remains above 92%. Therefore, we investigated using combinations of ACN and MeOH as Elute 2 in hopes of finding an eluate, which produced extracts having the superior cleanliness of the basified ACN while maintaining the excellent overall recoveries of the basified MeOH.

Combinations of basified ACN:MeOH in the following ratios were evaluated for matrix effects, levels of residual phospholipids and analyte recoveries: 80:20, 60:40, 40:60 and 20:80. Table 4 summarizes both the SPE recoveries for eight basic analytes using the different combinations of organic solvents as Elute 2, and the average area counts for the five representative phospholipids in each of the same eluates. We discovered that as little as 20% MeOH in the final extract was enough to elute approximately 77% of the lipid levels found in the 100% MeOH based extract. The 40%, 60% and 80% MeOH combinations yielded lipid levels that were similar to the 100% MeOH. With respect to analyte recovery, 20% MeOH was enough to improve the recovery for atenolol and pseudoephedrine to 86% and 80%, respectively. The question had been raised as to whether it was a property of MeOH specifically that seemed to increase the level of phospholipids in solution, or the fact that it is a protic solvent, while ACN is not. To more fully understand the behavior of phospholipids using MeOH and ACN as Elute 2, an additional experiment was carried out using a basified solution of 20:80 $H_2O$:ACN, which eliminates MeOH, while maintaining methanol's elutropic strength. This eluate still contained a significant amount of residual phospholipids: approximately 60% of the residual phospholipids found in the 20:80 MeOH:ACN eluate. Therefore, the elution of phospholipids from the sorbent is related to the use of protic solvents, not necessarily MeOH itself. In comparison to the 100% MeOH Elute 2, the 20:80 $H_2O$:ACN Elute 2 contained half the amount of phospholipids.

Table 4
Calculated % SPE recoveries and average area counts for five phospholipids using various combinations of basified ACN, MeOH or ACN/$H_2O$ in Elute 2

| | % MeOH in ACN | | | | | | 20:80 $H_2O$:ACN |
|---|---|---|---|---|---|---|---|
| | 0 | 20 | 40 | 60 | 80 | 100 | |
| Atenolol (p) | 6 | 86 | 80 | 89 | 131 | 98 | 101 |
| Pseudoephedrine (p) | 27 | 80 | 91 | 93 | 113 | 97 | 68 |
| 9-OH Risperidone (np) | 92 | 92 | 93 | 93 | 110 | 95 | 95 |
| Risperidone (np) | 97 | 96 | 94 | 95 | 106 | 93 | 102 |
| Clozapine (np) | 89 | 81 | 82 | 90 | 94 | 94 | 100 |
| Chlorpheniramine (np) | 98 | 98 | 95 | 95 | 113 | 98 | 100 |
| Imipramine (np) | 86 | 87 | 90 | 83 | 104 | 85 | 89 |
| Amitriptyline (np) | 93 | 84 | 82 | 78 | 90 | 80 | 90 |
| Total lipids (average area counts for five lipids) | 6568 | 94,510 | 110,857 | 124,366 | 125,457 | 122,605 | 59,300 |

Wash 2/Elute 1 was 100% MeOH in all cases. p = Polar analyte; np = non-polar analyte.

In both cases, the SPE recoveries were similar or improved for all analytes except for pseudoephedrine, whose recovery decreased. In comparison to the 100% ACN Elute 2, the 20:80 $H_2O$:ACN Elute 2 contained approximately ten times the residual phospholipids levels and recovery for atenolol and pseudoephedrine were improved to 101% and 68%, respectively. To summarize, acetonitrile can be used in Elute 2 for moderately polar to non-polar compounds, while MeOH remains the best solvent in Elute 2 for generic methods and polar analytes.

### *5.7. Comparison of four mixed-mode SPE sorbents and additional elution steps*

Up to this point, we have focused our evaluations on the final eluates produced by each method of sample preparation and have discussed mixed-mode strong cation exchange polymeric SPE in detail. We also evaluated Wash 2/Elutes 1 and 2 from three additional polymeric mixed-mode sorbents against the final (100% MeOH) reversed-phase polymeric SPE elution, polymeric mixed-mode strong cation exchange SPE and ACN PPT for the presence of residual lipids. In this case, we monitored the five individual MRM traces for the representative phospholipids. Table 3 summarizes the percentage of phospholipids remaining in Wash 2/Elutes 1 and 2 from five polymeric sorbents relative to those remaining in ACN PPT, which is set at 100%. The percentages are based on summed average area counts of the five phospholipids. The cleanest Wash 2/Elute 1 extract, containing 2% of the five phospholipids relative to PPT, is obtained on mixed-mode strong cation exchange. The levels of lipids remaining in Wash 2/Elute 1 from other mixed-mode sorbents and the reversed-phase polymeric sorbent are similar, containing no more than approximately 13% of the calculated level in the PPT extract. The mixed-mode weak cation and weak and strong anion polymeric sorbents produced the cleanest Elute 2, or final, extracts, containing less than 1% of the residual lipids in the ACN PPT extract. Matrix effects for all sorbents were <5%.

## 6. Optimizing chromatographic conditions

### *6.1. Use of mobile phase pH to manipulate retention and sensitivity*

If the analytes are ionizable, the pH of the mobile phase can significantly impact the retention, selectivity and sensitivity of the separation. We chose to investigate the impact of mobile phase pH on matrix effects at low (pH 2.7) and high (pH 9) pH. Fig. 4 shows the elution profile of phosphatidylcholine containing phospholipids under both low and high pH. The sample was prepared by ACN PPT to represent a typical "quick and dirty" sample preparation and run with a generic gradient from 98% A to 98% B over 2 min on the UPLC® system. The chromatograms clearly show that the elution times of the least hydrophobic phospholipids (earliest eluters) are independent of pH, while the more hydrophobic di-alkyl phospholipids require a longer organic hold at high pH to fully elute them. Fig. 4 also includes MRM transitions for the bases amitriptyline and terfenadine under low and high mobile phase pH. These samples are neat standards run under UPLC® conditions. The retention times of these ionizable analytes are appreciably affected by pH, demonstrating that mobile phase pH can effectively be used to manipulate analyte elution away from those chromatographic regions shown to contain phospholipids and thus susceptibility to ion suppression. In addition, we found that the high pH mobile phase provided better ESI+ sensitivity for many basic analytes. In these examples, sensitivity increased by 2.3× for terfenadine at high pH versus low pH. The sensitivity increase for amitriptyline at high pH was 1.6×. Additionally, the retention time for terfenadine increased from 0.83 to 1.17 min and for amitriptyline from 0.64 to 1.1 min at high pH. Basic analytes will be in their neutral form at high pH, and thus are retained longer on an RP column and eluted at a higher organic concentration during gradient LC. It is believed that droplets with a higher organic content are more efficiently desolvated in the MS source lead-

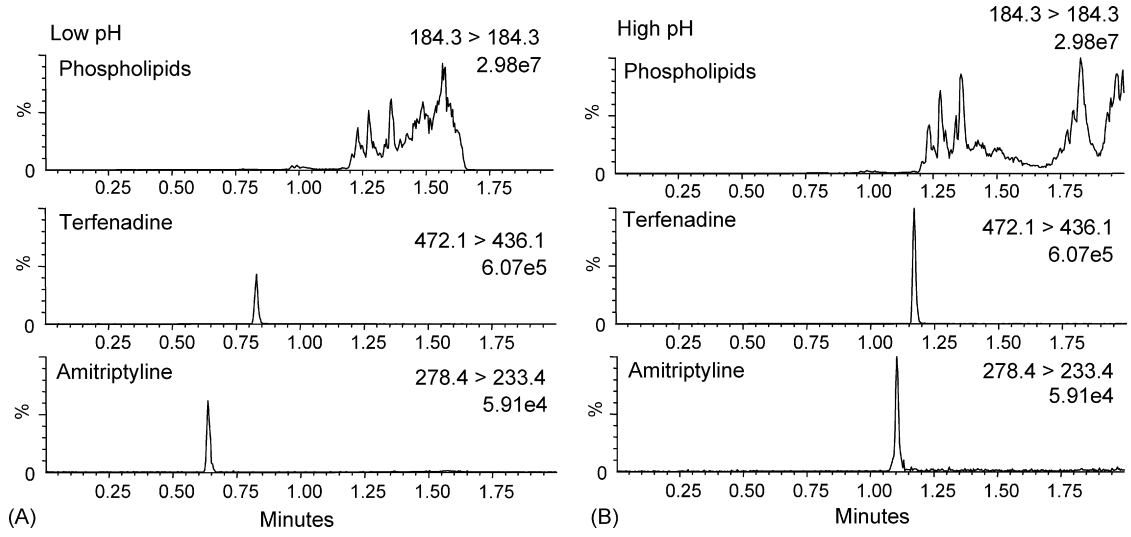


Fig. 4. MRM transitions for all phosphatidylcholine-containing phospholipids (top) from plasma samples prepared by ACN PPT analyzed by UPLC®/MS/MS under (A) pH 2.7 and (B) pH 9 mobile phase conditions. MRM transitions for 1 ng/mL terfenadine (middle) and amitriptyline (bottom) under the same mobile phase conditions.

ing to higher MS sensitivity. No attempt was made to control the $k'$ of the analytes in these experiments. As long as the analytes are adequately separated from residual phospholipids, high pH mobile phases are recommended for basic compounds. As extending analyte retention time may now cause co-elution with residual matrix components, the expected sensitivity gain must be balanced with any matrix effects that may arise.

### 6.2. Effect of gradient duration on matrix effects

We assessed the effect of gradient duration on the level of matrix effects observed by pushing methodologies to more aggressive conditions that were expected to promote suppression. These conditions result in much less or minimal chromatographic retention, less resolution and more susceptibility to ion suppression. Independent of mobile phase pH, matrix effects increased for amitriptyline when gradient time was reduced from 2 to 0.5 min. The samples were prepared by the dilute and inject method of ACN PPT in each case. Under low pH mobile phase conditions, the level of ion suppression increased from 15% to 58% when the gradient time was decreased 4×. Similarly, under high pH conditions, ion suppression increased from 25% to 77% when the gradient time was decreased by the same amount. When shorter gradient times are used, extra care must be taken to optimize sample preparation (i.e. use SPE) and to use the appropriate mobile phase pH.

### 6.3. A comparison of matrix effects in HPLC and UPLC® separations

In this work, we define "HPLC" as liquid chromatography run using 3.5 μm particle size columns. We define "UPLC® technology" as liquid chromatography run using sub-2 μm particle size columns on a chromatographic system specifically designed to run at the optimum linear velocities for these columns (i.e. high pressures and minimal system volume). UPLC® technology has demonstrated significant advantages with respect to speed, sensitivity and resolution, making it an attractive option for trace level quantitative analysis [43–47]. Researchers have demonstrated 5–12× increases in speed [43–45], 3–10× improvements in sensitivity [43,44,47] and close to a 2× improvement in resolution [43,45,46]. It was proposed that the added resolution might provide a benefit with respect to matrix effects, through improved separation from endogenous components. For this reason, many of the previously described experiments have been carried out using both LC techniques. We evaluated multiple sample preparation techniques, several mobile phase pHs, numerous gradient profiles, and ten different analytes using both HPLC and UPLC® systems. Matrix effects were quantitatively determined for each analyte under the various chromatographic conditions and sample preparation methods. The HPLC matrix effects data were paired with their UPLC® counterparts run under identical pH, gradient and sample prep conditions. Forty-six sets of paired data were then subjected to a paired *t*-test to determine if there was a statistically significant difference between the two data populations. The paired *t*-test returned a *p* (probability) value of 0.0006, objectively indicating that the

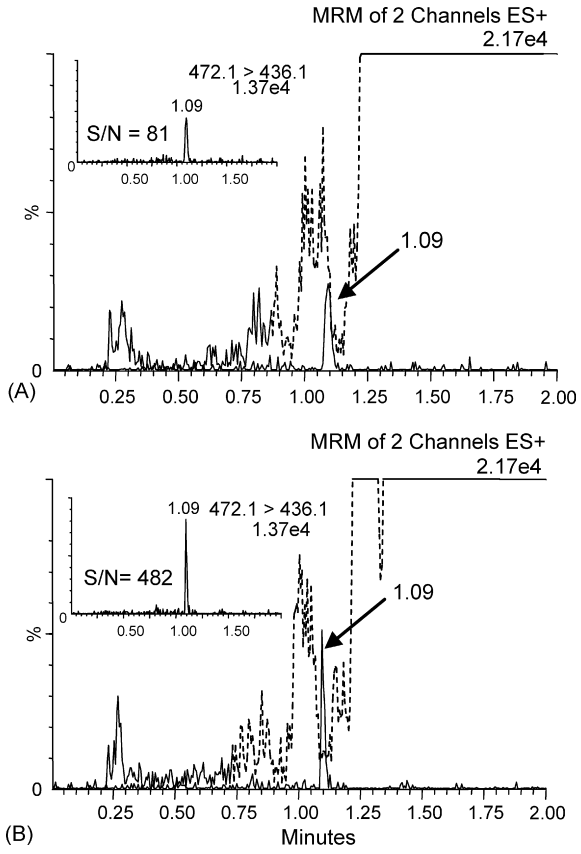


Fig. 5. A plasma sample containing 0.1 ng/mL of terfenadine (MRM transition 472.1 > 436.1) was prepared by ACN PPT and analyzed by (A) HPLC/MS/MS and (B) UPLC®/MS/MS using pH 9 mobile phase. The S/N for terfenadine by HPLC/MS/MS is 81 and by UPLC®/MS/MS is 482, a 6X improvement. Dotted lines (· · ·) are the phospholipids transition (184.3 > 184.3) and solid lines (–) are the terfenadine transition.

reduction in matrix effects observed under UPLC® conditions is indeed statistically significant, using a value of 0.05 as the threshold value. A representative demonstration of this observed phenomenon is depicted in Fig. 5. Fig. 5 shows a 0.1 ng/mL sample of terfenadine prepared by ACN PPT under (A) HPLC and (B) UPLC® conditions. The increase in S/N ratio of 6× is a combination of theoretical increase due to particle size (1.4×) and reduction in matrix effects. The narrower chromatographic peaks, and thus higher peak capacity, generated by the UPLC® system increase the resolution of one chromatographic peak from another. For example, an analyte peak would be expected to be better resolved from an interference.

### 6.4. Cumulative benefit of optimizing sample preparation and chromatographic conditions

Fig. 6 demonstrates the cumulative benefit realized when both sample preparation and chromatographic conditions are optimized for sample cleanliness and sensitivity. The compounds were risperidone, 9-OH risperidone and clozapine (1 ng/mL each). In Fig. 6A, the data were generated using techniques and practices common in analytical labs today: low pH (generic gradient from 98% A to 98% B over 2 min), ACN PPT and HPLC.

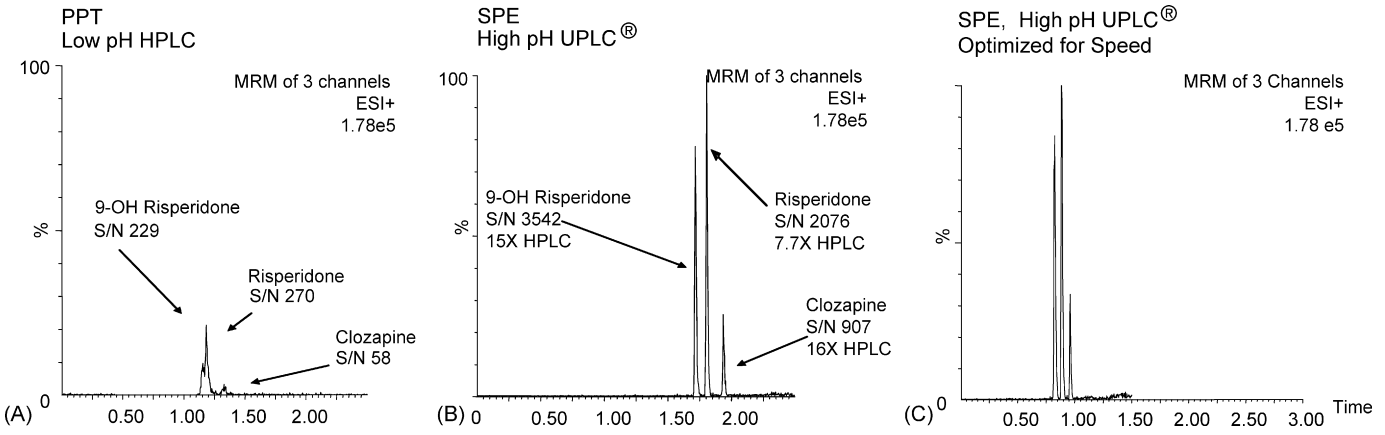


Fig. 6. A plasma sample containing 1 ng/mL each of risperidone, 9-OH risperidone and clozapine was analyzed by either (A) ACN PPT, HPLC/MS/MS using pH 2.7 mobile phase, (B) mixed-mode cation exchange SPE, UPLC®/MS/MS using pH 9 mobile phase while maintaining the gradient duration and slope of A and (C) mixed-mode cation exchange SPE, UPLC®/MS/MS using pH 9 mobile phase with an optimized gradient. The results demonstrate the cumulative benefits achieved by using the best sample preparation methods and chromatographic techniques.

The signal-to-noise ratios for the three compounds range from 58 to 270, and under the generic gradient used here, the API and its metabolite co-elute. In Fig. 6B, the data were generated following each of the recommendations in this paper: high pH for bases (a generic gradient from 98% A to 98% B over 2 min), mixed-mode cation exchange SPE and UPLC® technology. In Fig. 6B, gradient duration and slope were kept consistent with Fig. 6A. The signal to noise ratios for the three compounds range from 907 to 3542, which corresponds to a net increase of 7.7–16× over the accepted practices of using HPLC, low pH for basic compounds and PPT. Fig. 6C, demonstrates the additional time saving benefit afforded by UPLC® technology, without loss of sensitivity.

## 7. Conclusions

Endogenous phospholipids in plasma are a significant source of matrix effects in LC/MS/MS analyses. In this work, we have provided a thorough, systematic evaluation of various sample preparation techniques and chromatographic methods aiming to reduce or eliminate matrix effects. We have compared results from the various sample preparation methods with respect to extract cleanliness, matrix effects and analyte recovery. We have compared fast and slow gradients, low and high pH mobile phases, as well as HPLC and UPLC® chromatographic systems, with respect to matrix effects and overall method sensitivity. Our results indicate that although ACN is a better choice of organic solvent than MeOH for PPT, PPT is the least effective sample preparation technique and causes significant ion suppression for many compounds. While both reversed-phase only and cation exchange only SPE result in significantly lower levels of phospholipids relative to PPT, mixed-mode strong cation exchange SPE, which combines the retention mechanisms of reversed-phase and ion exchange, is the most effective sample preparation technique, leading to minimal matrix effects from biological samples and excellent recoveries for a range of polar and non-polar analytes on a first pass. Single step LLE with either MTBE or basified MTBE, yields extracts of comparable cleanliness to mixed-mode cation SPE (performed with methanol as the organic solvent in the final elution.) Analyte recovery for the polar compounds used in this study, using either of the single step LLE methods, was not acceptable. Recovery for the less polar analytes, though an improvement over the polar analyte recovery, was still significantly lower than either PPT or mixed-mode strong cation exchange SPE. Recovery for the less polar analytes was significantly improved, and equal to that of mixed-mode strong cation exchange SPE when a two step LLE method with basified MTBE was employed. Recovery for polar analytes was still less than optimal. Furthermore, when ACN is substituted for MeOH in the final mixed-mode strong cation exchange extract, it results in the cleanest extract of all methods evaluated. The most effective sample clean-up and highest recoveries for analytes with a range of chemical properties is achieved using mixed-mode SPE, which can be further optimized to remove up to >99% of phospholipids, relative to PPT. Mobile phase pH was shown to be an effective tool for manipulating the retention of ionizable analytes. Analytes can be moved away from residual phospholipids, whose reversed-phase retention tends to be relatively independent of pH, resulting in a reduction of matrix effects. This benefit should be balanced with the sensitivity gain observed when analytes are chromatographed in their neutral state, thus retaining them longer (and potentially moving them closer to regions of phospholipids elution) on a reversed-phase column. We have shown that fast gradient LC promotes matrix effects by reducing chromatographic separation between analytes and endogenous compounds. If increased sample throughput and ultrafast gradients are desired, effective sample preparation becomes critical, as one can no longer rely on chromatographic separation to reduce matrix effects. Finally, the added resolution of UPLC® technology over HPLC yielded a statistically significant reduction in matrix effects under a variety of chromatographic conditions, and with multiple basic analytes. In summary, a combination of mixed-mode SPE, appropriate mobile phase pH, and UPLC® technology results in the cleanest extracts and most sensitive and robust analytical methods for trace-level determination of drugs in plasma.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2006.12.030.

## References


[1] P. Kebarle, L. Tang, Anal. Chem. 65 (1993) 972A.
[2] D. Buhrman, P. Price, P. Rudewicz, J. Am. Soc. Mass Spectrom. 7 (1996) 1099.
[3] Y. Hsieh, H. Mei, in: W. Korfmacher (Ed.), Using Mass Spectrometry for Drug Metabolism Studies, CRC Press, Boca Raton, FL, 2004.
[4] J. Chen, W. Korfmacher, Y. Hsieh, J. Chromatogr. B 820 (2005) 1.
[5] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[6] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[7] L. Jessome, D. Volmer, LCGC N. Am. 24 (2006) 498.
[8] F. Calbiani, M. Careri, L. Elviri, A. Mangia, I. Zagnoni, J. Mass Spectrom. 41 (2006) 289.
[9] P.J. Taylor, Clin. Biochem. 38 (2005) 328.
[10] I. Fu, E. Woolf, B. Matuszewski, J. Pharm. Biomed. Anal. 18 (1998) 347.
[11] G. Wang, Y. Hsieh, W. Korfmacher, Anal. Chem. 77 (2005) 541.
[12] C.R. Mallet, Z. Lu, J.R. Mazzeo, Rapid Commun. Mass Spectrom. 18 (2004) 49.
[13] M.J. Avery, Rapid Commun. Mass Spectrom. 17 (2003) 197.
[14] P. Bennett, H. Liang, Tandem Capabilities Publications, available at http://www.tandemlabs.com/documents/PatrickASMSPaper.pdf.
[15] M. Meng, P. Bennett, Tandem Capabilities Publications, available at http://www.tandemlabs.com/documents/MinASMSPaper.pdf.
[16] P. Larger, M. Breda, D. Fraier, H. Hughes, C. James, J. Pharm. Biomed. Anal. 39 (2005) 206.
[17] M. Ahnoff, H. Hagelin, Poster MPN211, ASMS, 2004.
[18] FDA 2001. Available at http://www.fda.gov/cder/guidance/4252fnl.pdf.
[19] I. Ferrer, J. Garcia-Reyes, M. Mezcua, E. Thurman, A. Fernandez-Alba, J. Chromatogr. A 1082 (2005) 81.
[20] M. Urpi-Sarda, O. Jauregui, R. Lamuela-Raventos, W. Jaeger, M. Miksits, M. Covas, C. Andres-Lacueva, Anal. Chem. 77 (2005) 3149.
[21] N. Van Hoof, D. Courtheyn, et al., Rapid Commun. Mass Spectrom. 19 (2005) 2801.
[22] B. Guo, C. Li, Z. Deng, et al., Rapid Commun. Mass Spectrom. 19 (2005) 2840.
[23] L. Sabatini, A. Barbieri, M. Tosi, A. Roda, F. Violante, Rapid Commun. Mass Spectrom. 19 (2005) 147.
[24] J. Bhatt, G. Subbaiah, S. Singh, Rapid Commun. Mass Spectrom. 20 (2006) 2109.
[25] E. Stokvis, H. Rosing, J. Beijnen, Rapid Commun. Mass Spectrom. 19 (2005) 401.
[26] A. Kloepfer, J. Quintana, T. Reemtsma, J. Chromatogr. A 1067 (2005) 153.
[27] E. Gangl, M. Annan, N. Spooner, P. Vouros, Anal. Chem. 73 (2001) 5635.
[28] M. Petrovic, et al., J. Chromatogr. A 1067 (2005) 1.
[29] J. Quintana, T. Reemtsma, Rapid Commun. Mass Spectrom. 18 (2004) 765.
[30] H. Liang, T. Takagaki, R. Foltz, P. Bennett, Rapid Commun. Mass Spectrom. 18 (2005) 2284.
[31] J. Little, M. Wempe, C. Buchanan, J. Chromatogr. B 833 (2006) 219.
[32] S. Bradamante, E. Barchiesi, L. Barenghi, F. Zoppi, Anal. Biochem. 185 (1990) 299.
[33] G. Phillips, J. Dodge, J. Lipid Res. 8 (1967) 676.
[34] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.
[35] C. Enke, Anal. Chem. 69 (1997) 4885.
[36] P. Bennett, M. Meng, C. Van Horne, Tandem Capabilities Publications, available at http://www.tandemlabs.com/documents/PatReport.pdf.
[37] R. Taguchi, et al., J. Chromatogr. B 823 (2005) 26.
[38] P. Bennett, K.C. Van Horne, Poster 6006, 2003 AAPS.
[39] V. Capka, S. Carter, Tandem Capabilities Publications, available at http://www.tandemlabs.com/documents/ASMS_06_Vladimir_lowres.pdf.
[40] V. Capka, P. Bennett, Tandem Capabilities Publications, available at http://www.tandemlabs.com/documents/VladimirReport.pdf.
[41] P. Bennett, C. Van Horne, Tandem Capabilities Publications, available at http://www.tandemlabs.com/documents/AAPS03PB.pdf.
[42] J.X. Shen, R.J. Motyka, J.P. Roach, R.N. Hayes, J. Pharm. Biomed. Anal. 37 (2005) 359.
[43] M. Churchwell, N. Twaddle, L. Meeker, D. Doerge, J. Chromatogr. B 825 (2005) 134.
[44] I. Wilson, J. Nicholson, J. Castro-Perez, J. Granger, K. Johnson, B. Smith, R. Plumb, J. Proteome Res. 4 (2005) 591.
[45] I. Lurie, J. Chromatogr. A 1100 (2005) 168.
[46] J. Shen, H. Wang, S. Tadros, R. Hayes, J. Pharm. Biomed. Anal. 40 (2006) 689.
[47] R. Plumb, J. Castro-Perez, J. Granger, I. Beattie, K. Joncour, A. Wright, Rapid Commun. Mass Spectrom. 18 (2004) 2331.