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Overcoming ionization effects through chromatography: A case study for the ESI-LC–MS/MS quantitation of a hydrophobic therapeutic agent in human serum using a stable-label internal standard

Guodong Zhang[a], Chad E. Wujcik[b,*]

[a] Bioanalytical Research, Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research and Development, Pfizer Inc., Groton, CT 06340, USA
[b] Environmental Science and Technology Center, Monsanto Company, St. Louis, MO 63167, USA

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ABSTRACT

Significant electrospray ionization effects were observed for a hydrophobic analyte and its stable isotopically labeled internal standard (SIL IS) in human plasma extracts. Analyte and SIL IS were slightly offset in retention within the suppression region resulting in a differential suppression that biased calculated concentrations. Six abundant endogenous phospholipids were identified as potential contributors to ionization suppression. Chromatographic conditions were optimized using pH (relative to the log *D* of the analytes), to better resolve nearby phospholipids from the analyte and SIL IS and minimize ionization suppression. The successful validation of this method demonstrates the value of investigating minor chromatographic changes to remediate detrimental ionization effects without further altering extraction procedures.

1. Introduction

The value and utility of LC–ESI-MS/MS offers several advantages over other approaches including improved sensitivity, selectivity and specificity, all of which can result in lower sample volume requirements, faster run times and minimal mobile phase use [1–3]. However, with the ever-increasing demand for high throughput procedures, analytical methodologies can be pushed to the limit where detrimental ionization effects occur more frequently. Ionization or matrix effects are defined as the impact of ionization efficiency on the target analyte as a result of co-eluting endogenous components from a given matrix [4–8]. The levels of these effects are of great concern in the pharmaceutical industry and FDA guidance since they are directly related the quality and integrity of the data [9–19].

Matrix effects are commonly evaluated using post-column infusion and post-extraction addition methods [17,20,21]. The proposed mechanism for electrospray ionization effects is likely due to competitive ionization between an analyte and any number of co-eluting matrix components [22–24]. They are mostly reported as resulting from endogenous materials in biological samples (i.e., phospholipids, salts and amines) [25–29].

Phosphatidylcholine is the principal phospholipid circulating in human plasma where it resides as a surface component of human plasma lipoproteins at relatively high concentrations [30]. Bennett et al. recently reported phosphatidylcholine and lyso-phosphatidylcholine as the primary class of endogenous components causing significant matrix effects [31] with high concentrations in plasma at total concentration of 1–3 mg/mL.

Overcoming ionization effects associated with electrospray mass spectrometry resulting from endogenous matrix components has proven to be one of the most challenging and time consuming hurdles facing bioanalytical scientists. The most popular published methods to remediate these effects are optimizing sample preparation (such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE)) or chromatographic techniques [9,18,32–35]. Recently, stable isotopically labeled internal standards have become increasingly popular as a tool for compensating potential and known matrix effects due to their nearly identical chemical and physical properties to the target analyte [12,36–38]. However, in certain cases a deuterium labeled IS has demonstrated differing ionization potential compared to the analyte which has compromised the accuracy of analyte concentration due to the slight shift

* Corresponding author at: Monsanto, 800 North Lindbergh Boulevard, Mail Code V1B, St. Louis, MO 63167, United States. Fax: +1 314 694 8774.
E-mail address: chad.e.wujcik@monsanto.com (C.E. Wujcik).

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in retention time between analyte and SIL IS and the retention relationship with the co-eluting endogenous material [38].

In this paper, we have identified the key phospholipids impacting the ionization of our target analyte and its corresponding SIL IS in human serum and identified two simple chromatographic modifications to minimize this effect. Resolving matrix effects through chromatography can allow the analyst to maintain and utilize uncomplicated and cost-effective extraction procedures while ensuring the accuracy and reliability of the data. The modifications described herein resulted in the development and validation of a highly sensitive, rapid and robust ESI-LC–MS/MS assay for the determination of a relatively hydrophobic drug candidate for treatment of obesity, in human serum.

2. Experimental

2.1. Chemicals and reagents

The analyte and [$^2H_4$]-analyte (Fig. 1) were synthesized by Pfizer, Inc. (New York, NY). Reagent grade ammonium hydroxide, HPLC grade water and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Reagent grade formic acid was from Sigma–Aldrich Chemical Co. (St. Louis, MO).

2.2. Instrumentation

The chromatographic system was comprised of a Shimadzu SCL-10A controller, Shimadzu LC-10AD pumps, and a CTC Analytics (LEAP) HTLS PAL autosampler. The autosampler was equipped with L-Mark syringe from Leap Technologies. An Applied Biosystems API 4000 tandem quadrupole mass spectrometer with a TurboIonspray™ source was employed for detection. The source was operated in the positive-ion electrospray mode. Data acquisition and chromatographic review was performed using Applied Biosystems/MDS SCIEX Analyst, version 1.4.1.

2.3. Liquid chromatographic and mass spectrometric conditions

A Hypersil GOLD PFP (50 or 100 mm × 2.1 mm i.d., 5 μm) column was used for chromatographic separations. Mobile phases A and B consisted of 10:90 and 90:10 acetonitrile/water (v/v) and 0.02% ammonium hydroxide, respectively. Both mobile phase systems were adjusted with formic acid to reach either pH 7.0 or 3.4 for the different conditions tested. The flow rate was set to 0.35 mL/min. Injections were made by loading a 20 μL sample loop with 50 μL of the extracted samples. Both pH 7.0 and 3.4 mobile phase systems used the identical gradient for separation (Table 1). From 2.26 to 3.50 min for the 50 mm × 2.1 mm i.d. and from 4.26 to 5.50 min for the 100 mm × 2.1 mm i.d. column, a high concentration of acetonitrile (90%) in mobile phase was used to adequately wash other retained extract components from the column. The column temperature was maintained at room temperature. The mass spectrometer was run in positive-ion ESI mode using multiple-reaction monitoring (MRM) to monitor the mass transitions of analyte, IS and endogenous phospholipids. The MRM transition was established based on the formation of the most abundant product ion. The relevant mass spectrometer MRM parameters are shown in Table 2.

Table 2
Selected mass spectrometer parameters.

Mass spectrometer	Applied biosystems API 4000	
Interface	TurboV™	
Ionization mode	Positive	
MS parameters	Analyte	SIL IS
MRM	347.3/260.1	351.3/262.2
Declustering potential (DP) (V)	61	61
Collision energy (CE) (V)	29	29
Curtain gas (CUR)	15	
Collision gas (CAD)	10	
Entrance potential (EP)	10	
Collision exit potential (CXP) (V)	24	
Ionspray voltage (IS) (V)	2750	
Temperature (TEM) (°C)	400	

2.4. Preparation of standards and quality control (QC) samples

The main stock solution of analyte was prepared at 1.0 mg/mL in 1:1 acetonitrile/water (v/v) and stored at 4 °C in a glass vessel. The calibration standards were prepared by adding appropriate amounts of the stock solution or subsequent calibration standards to blank human serum. Nominal concentrations were 0.025, 0.05, 0.125, 0.5, 5.0, 12.5, 22.5 and 25.0 ng/mL in human serum. QC samples were prepared at 0.075, 1.25 and 20.0 ng/mL following similar procedures as the calibration standards but using a separately weighed and prepared QC stock solution.

2.5. Sample preparation

100 μL aliquots of human serum samples were precipitated with 300 μL of acetonitrile containing 0.2 ng/mL of [$^2H_4$]-analyte (SIL IS). After vortex-mixing and centrifuging for 10 min at 1730 × *g*, 200 μL of supernatant was transferred and diluted with 300 μL of water. This final solution was vortexed and injected into the LC–MS/MS.

2.6. Identification and monitoring of phospholipids and matrix effects evaluation using post-column infusions

Single quadrupole (*m*/*z* 400–800) and precursor ion scans (*m*/*z* 184 product ion) were utilized to assist in phospholipid identification in the human serum extracts. Key phospholipids eluting around the analyte and IS were further monitored during chromatographic runs using the characteristic MRM transitions from the precursor ion of *m*/*z* 496, 520, 522, 524, 544, 758 and 782 to the product ion at *m*/*z* 184.

Post-column infusion experiments were conducted by teeing in a solution 50 ng/mL of both analyte and SIL IS at 10 μL/min during the injection of a double blank matrix extract to identify regions

Table 1
Mobile phase programs for 50 and 100 mm length columns (flow rate: 0.35 mL/min).

Column (50 mm × 2.1 mm) pH 3.4 and 7.0 mobile phase		Column (100 mm × 2.1 mm) pH 7.0 mobile phase	
Time (min)	Mobile phase B (%)	Time (min)	Mobile phase B (%)
0.00	50	0.00	50
2.25	50	4.25	50
2.26	100	4.26	100
3.50	100	5.50	100
3.51	50	5.51	50
5.0	50	7.00	50

Table 3
Stability testing of the analyte in human serum (pH 7.0 mobile phase).

Calibration curve	Stability	Spiked concentration (ng/mL)	Observed concentration ± S.D. (ng/mL)	%RSD	%RE
Peak area ratio	Three freeze–thaw cycle ($n = 3$)	0.075	0.092 ± 0.0013	1.4	22.5
		20.0	24.733 ± 0.4619	1.9	23.7
	LT[a] (5 days) ($n = 6$)	0.075	0.097 ± 0.0017	1.7	28.9
		20.0	24.767 ± 1.0652	4.3	23.8
Peak area	Three freeze–thaw cycle ($n = 3$)	0.075	0.0743 ± 0.0028	3.8	−0.9
		20.0	21.667 ± 0.1155	0.5	8.3
	LT[a] (5 days) ($n = 6$)	0.075	0.0782 ± 0.0023	2.9	4.3
		20.0	21.467 ± 0.3386	1.6	7.3

[a] LT is long-term stability at −20 °C.

of ionization suppression. Additional injections were performed by substituting the double blank extract with an extract fortified with the analyte and IS to ensure proper tracking within the given chromatographic system.

2.7. Method validation

The method was validated for linearity, matrix effects, accuracy and precision in accordance with the U.S. Food and Drug Administration, Guidance for Industry, Bioanalytical Method Validation [19] and Pfizer standard operating procedures. Watson Laboratory Information Management Software (LIMS) version 7.2 was used to perform the required statistical calculations.

Calibration curves were constructed using the peak area ratios of the analyte to that of the corresponding [$^2H_4$]-analyte (SIL IS), and applying a weighted ($1/x^2$) least squares linear regression analysis. Precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % relative error, %RE) were calculated for the four quality control (QC) samples. Six replicates of each QC point were analyzed to determine the intra-run accuracy and precision. To fulfill the requirements for a standard validation, this process was repeated three times over three runs in order to determine the inter-run accuracy and precision. Matrix effect evaluations were performed by comparing area ratios of several independent lots of matrix fortified to the QC Low level to the average ratio of the QC Low samples for the designated validation run. After the completion of the three initial validation runs ionization effects issues were identified from the assessment of the stability samples. A supplemental validation run was performed to accommodate the modification of the chromatographic conditions (change from neutral to acidic mobile phase pH) required to remedy these issues. This run contained both selectivity and ionization assessments. Stock solution stability at room temperature and 4 °C was determined for 27 days, along with the standard bench top, freeze/thaw (3 and 4 cycles), long-term (−20 °C) and autosampler stability assessments.

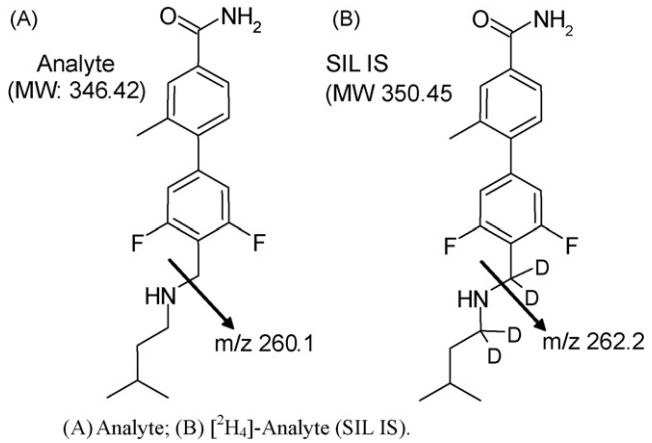

Fig. 1. (A) Analyte; (B) [$^2H_4$]-analyte (SIL IS).

3. Results and discussion

3.1. Observation of matrix effects

The initial chromatographic conditions for this assay were set to pH 7.0 to neutralize the analyte and IS and enhance retention. During the validation a significant bias of over +20% was observed with the freeze/thaw and long-term stability samples at −20 °C for the first measurement at day 6. In an effort to elucidate the cause of these results the data were re-calculated using only analyte peak area instead of the peak area ratio (Table 3). With this approach freeze/thaw and long-term stability fell back within the acceptable limits (±15 %RE). This finding indicated a more substantial degree of ionization suppression was affecting the IS relative to the analyte in the stability matrix lot compared to the lot used for the calibration curve.

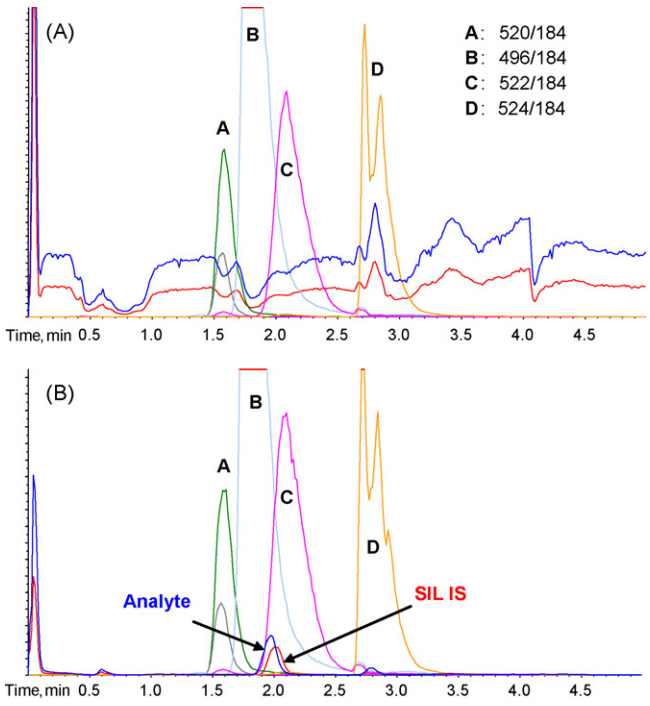

Fig. 2. Chromatographic conditions using pH 7.0 mobile phase with Hypersil GOLD PFP (50 mm × 2.1 mm i.d., 5 μm) analytical column; for other detailed conditions see Section 2.3: (A) post-column infusion of analyte and SIL and injection of blank matrix extract; (B) injection of matrix spiked with analyte and SIL without post-column infusion.

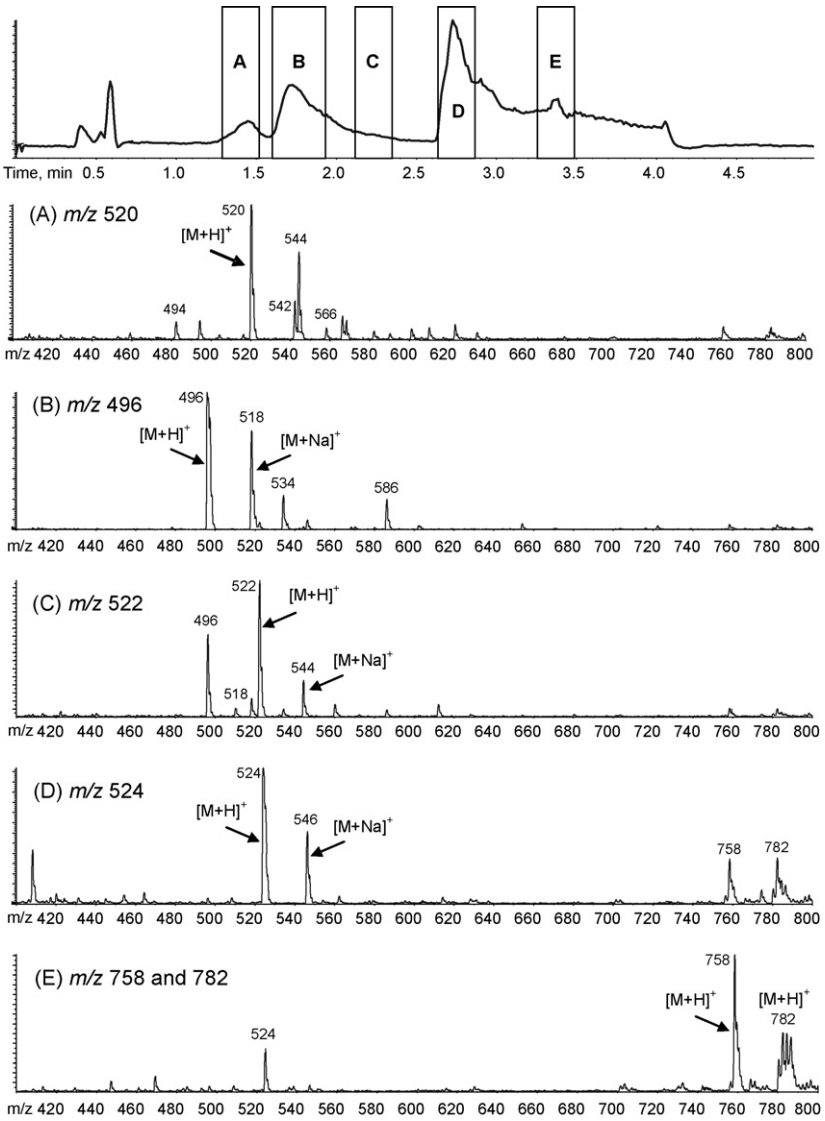

Fig. 3. MS spectra of the most abundant phospholipid class compounds in the ion suppression area from 1.3–3.3 min in blank human serum; significant phospholipid ions are observed as follows: *m*/*z* 520 (A); *m*/*z* 496 (B); *m*/*z* 522 (C); *m*/*z* 524 (D); *m*/*z* 758 and 782 (E); column: Hypersil GOLD PFP (50 mm × 2.1 mm i.d., 5 μm); pH 7.0 mobile phase; for other detailed conditions see Section 2.3.

Additional ionization effects evaluations were performed using multiple matrix lots fortified at the QC Low level to determine if this was a lot-specific phenomenon. A significant range of accuracies was also observed with a range covering 13.5–37.2 %RE for 10 independent lots. The variability and high overall bias from these freshly prepared samples further indicated that ionization effects were present resulting in a differential suppression of the analyte and IS.

Generally the SIL should correct or compensate for changes in the analyte response in the presence of varying matrix component levels. The observed shift in retention between the analyte and SIL IS using the pH 7 chromatographic conditions (Fig. 2B) could potentially provide the situation for differential suppression to occur, depending on the location of matrix interferences. This difference in retention is caused by the replacement of four carbon bound hydrogen with deuterium (Fig. 1B) on the SIL IS. This modification alters the hydrophobic properties and therefore the retention time. This phenomenon was reported as an isotope effect in other examples in the literature [37–39].

The range and magnitude of ionization effects on the analyte and SIL IS were evaluated using post-column infusion. Fig. 2A and B shows the location of the analyte and SIL IS in relation to the regions of ionization suppression. The analyte and SIL IS elute within an area where the ionization effects are rapidly changing as indicated by the sloping responses. Changes in the relative levels of the endogenous components causing these effects may dramatically increase the degree of suppression occurring between the analyte and SIL IS in different lots of matrix. The bias identified for the long-term and freeze thaw stability samples was believed to occur as a result of these factors.

3.2. Identification of the phospholipids producing the matrix effects

The nature and identity of the endogenous compounds producing these ionization effects were determined using a series of LC–MS/MS experiments. First, a precursor ion scan of the 184 product ion and a single quadrupole scan from *m*/*z* 400 to *m*/*z* 800 were run using an extracted matrix double blank. The chromatographic profiles of both experiments were very similar, indicating that the majority of extracted components in the system were endogenous phospholipids (data not shown). Additionally, the most abundant

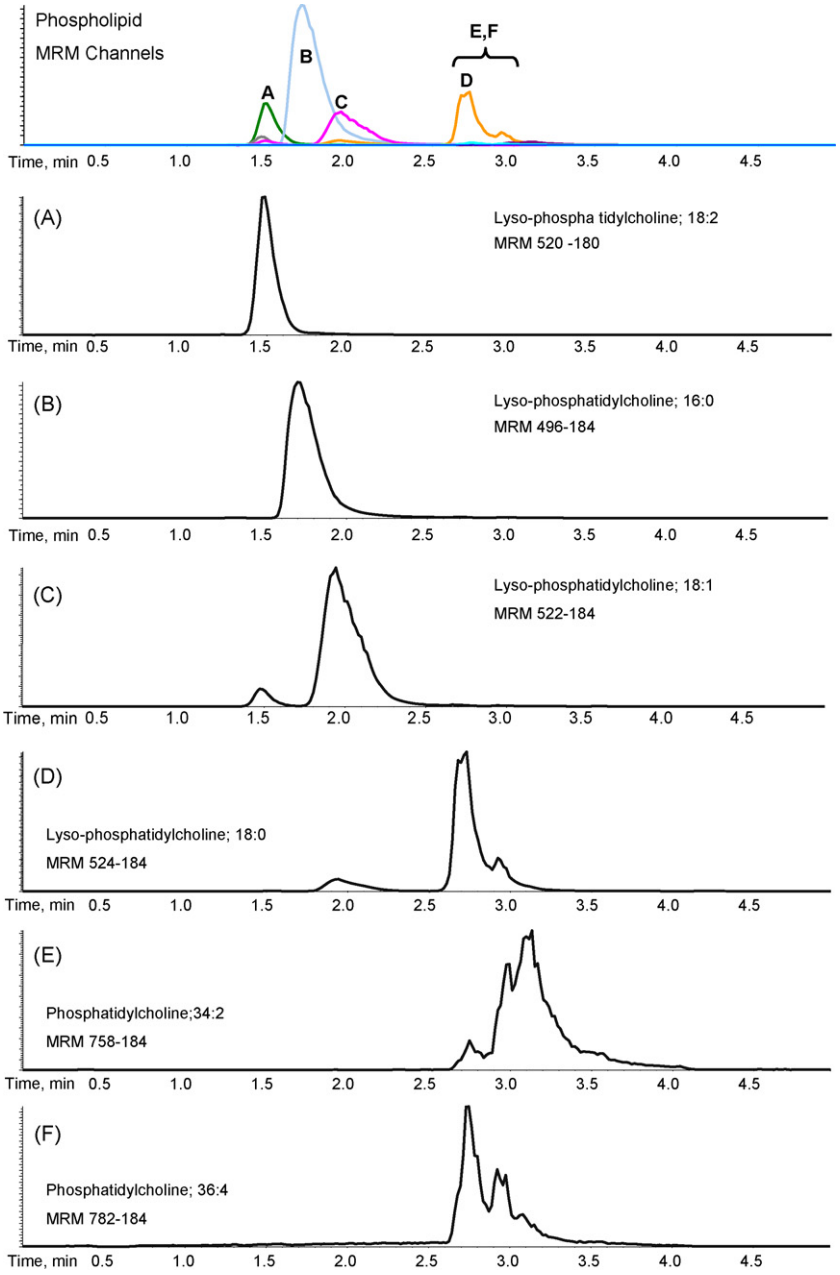

Fig. 4. MRM chromatograms of the most abundant phospholipids in blank human serum (pH 7.0 in mobile phase): (A) lyso-phosphatidylcholine, 18:2, MRM 520.0 → 184.2; (B) lyso-phosphatidylcholine, 16:0, MRM 496.0 → 184.2; (C) lyso-phosphatidylcholine, 18:1, MRM 522.0 → 184.2; (D) lyso-phosphatidylcholine, 18:0, MRM 524.0 → 184.2; (E) phosphatidylcholine, 34:2, MRM 758.0 → 184.2; (F) phosphatidylcholine, 36:4, MRM 782.0 → 184.2; column: Hypersil GOLD PFP (50 mm × 2.1 mm i.d., 5 μm); pH 7.0 mobile phase; for other detailed conditions see Section 2.3.

positive MS spectral ions in these extracts eluting near and within the retention regions of the analyte and SIL IS (1.3–3.3 min) were determined to be *m*/*z* 496, 520, 522, 524, 758 and 782 (Fig. 3). Further MS/MS investigation into these six transitions showed the presence of the characteristic 184 *m*/*z* phospholipid product ion. Among these phospholipids, *m*/*z* 496, 520, 522 and 524 were determined to be in the lyso-phosphatidylcholine class and both 756 and 782 *m*/*z* were from the phosphatidylcholine class. Want et al. had similar findings in determining some the more prominent endogenous components in organic extracts from human serum [40].

MRM transitions comprised of the *m*/*z* 496, 520, 522, 524, 758 and 782 precursor ions and 184 *m*/*z* product ion were used for determining the location of ionization suppressing components relative to the analyte and SIL IS in a given chromatographic run (Fig. 4). Along with previously published work [40] lyso-phosphatidylcholine (16:0, *m*/*z* 496) was found to be the most abundant phospholipid in human serum. Using the neutral chromatographic conditions the analyte and SIL IS peaks elute within the retention regions of lyso-phosphatidylcholine (16:0, *m*/*z* 496) and lyso-phosphatidylcholine (18:1, *m*/*z* 522) (Fig. 2). The elevated levels of these two main endogenous components resulted in the bulk of the detrimental ionization effects on the analyte and SIL IS. Variation in phospholipid homology between matrix lots and samples can introduce a significant degree of variability to the expected results.

3.3. Techniques utilized to reduce matrix effects for PPT method

LLE and SPE methods are often used to reduce phospholipid levels and, as a result, their corresponding ionization effects [9].

Other techniques using phospholipids depletion plates and complexing additives have been recently employed to achieve similar results [41,42]. Our efforts focused on remediating ion suppression using chromatography while maintaining the existing PPT method. This approach is more cost-effective and straightforward than SPE, allowing for simplified method transfer/cross-validation.

3.3.1. Improving the chromatographic separation by theoretical plates

The first technique utilized for attaining better separation from the major phospholipid regions was that of increasing column theoretical plates. While mobile phase pH and compositions remained the same, the column was changed from a Hypersil GOLD PFP 50 mm × 2.1 mm i.d., 5 μm to a 100 mm × 2.1 mm i.d., 5 μm column. This modification increased retention of the analyte and SIL IS from 1.9 min to 3.6 min (data not shown here) while moving both out beyond the more abundant phospholipids to a region where ion effects would be expected to have less of an impact. The longer run time that was required as a result of this modification in addition to the fact that the analyte and SIL IS have slightly different retention and are still co-eluting with portions of phospholipids prompted the investigation into other changes that could better ensure the robustness of the methodology.

3.3.2. Changing retention and resolution through pH modification

The distribution coefficient, *D*, is defined as the equilibrium concentration ratio of a given compound in both its ionized and un-ionized forms between an aqueous buffer at a specific pH and octanol [43,44]. Software packages such as ACD/LogP DB Suite (version 9.0, Advanced Chemistry Development, Inc. (ACD Labs)) are capable of calculating theoretical log *D* values [45]. Chromatographic retention has been directly correlated to log *D* for reversed-phase and hydrophilic interaction chromatography (HILIC) systems [46,47]. In these reports, compounds with similar log *D* values will exhibit similar retention. Calculated log *D* profiles from pH 1 to 14 for both the analyte and lyso-phosphatidylcholine (16:0) showed that the log *D* of analyte and SIL IS are significantly different at lower pH (e.g., pH 3) compared to neutral conditions as a result of the ionization state of the amine functional group. Phospholipids such as lyso-phosphatidylcholine (16:0) show a stable log *D* plateau from pH 2.0 to pH 12.5, covering the operating pH range of most analytical columns. Both phospholipids and the analyte/SIL IS at neutral pH have relatively close log *D* values, which explain their similar elution on the PFP system. By modification of the mobile phase pH from 7.0 to 3.4 the ionization state of the analyte and its corresponding log *D* value changed, resulting in a significant shortening of retention time from approximate 1.98 min to 1.26 min (Fig. 5). Additionally, the retention time of analyte and SIL IS now exhibited an identical overlap without the shift observed under neutral conditions. The similarity in chromatographic retention is believed to be a result of spatial proximity of the ionized amine and deuterium with the hydrophobic influence from the deuterium being masked by the stronger local ionic influence from the protonated amine. The phospholipids, as expected from their calculated log *D* readouts, were unaffected by this pH change. This allowed for the now-ionized, lower log *D* analyte and IS to elute prior to the phospholipid retention regions giving adequate resolution to mitigate any detrimental ionization effects (Fig. 5).

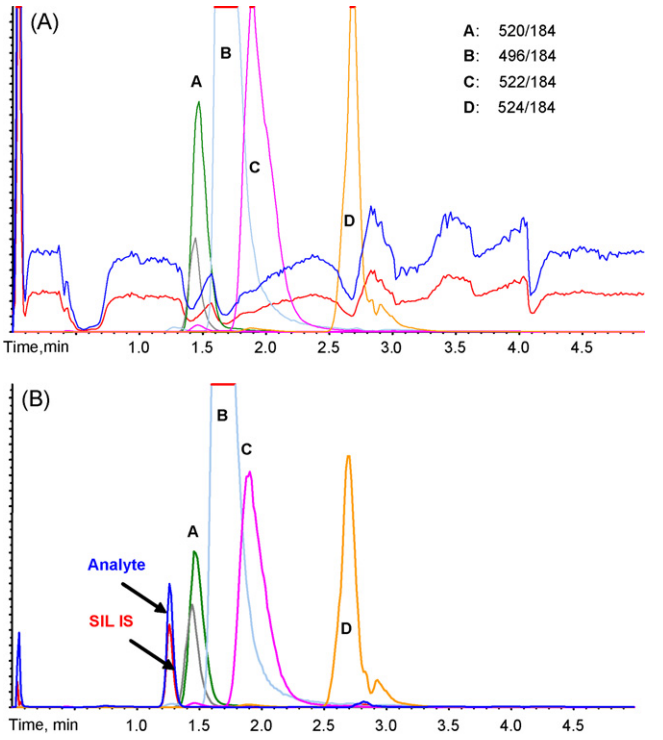

Fig. 5. Chromatographic conditions using pH 3.4 mobile phase with Hypersil GOLD PFP (50 mm × 2.1 mm i.d., 5 μm) analytical column; for other detailed conditions see Section 2.3: (A) post-column infusion of analyte and SIL and injection of blank matrix extract; (B) injection of matrix spiked with analyte and SIL without post-column infusion.

Using the updated pH 3.4 mobile phase conditions, the freeze/thaw and −20 °C long-term stability samples were re-evaluated. The accuracy of these results (Table 4) now fell within the targeted acceptance criteria (%RE was less than ±15.0%). These findings provided further evidence that the ionization effects observed under neutral mobile phase conditions were alleviated by adjusting the pH to 3.4. Additionally, ionization effects evaluations for the same lots failing under neutral mobile phase conditions were now within the acceptable limits.

Table 4
Stability testing of the analyte in human serum (pH 3.4 mobile phase).

Stability	Spiked concentration (ng/mL)	Observed concentration ± S.D. (ng/mL)	%RSD	%RE
Four freeze–thaw cycle (n = 3)	0.075	0.0717 ± 0.0046	6.4	−4.4
	20.0	19.767 ± 0.1155	0.6	−1.2
LT[a] (38 days) (n = 6)	0.075	0.0714 ± 0.0012	1.7	−4.9
	20.0	20.050 ± 0.3271	1.6	0.3
Bench top stability (44 h) (n = 3)	0.075	0.0797 ± 0.0012	1.5	6.3
	20.0	21.433 ± 0.1528	0.7	7.2
Stock solution stability (27 days) (n = 5)	1.0 (mg/mL)[b]	1.021 ± 0.0047	0.5	2.1

[a] LT is long-term stability at −20 °C.
[b] Stock solution in 1:1 acetonitrile/water (v/v) and stored at 4 °C.

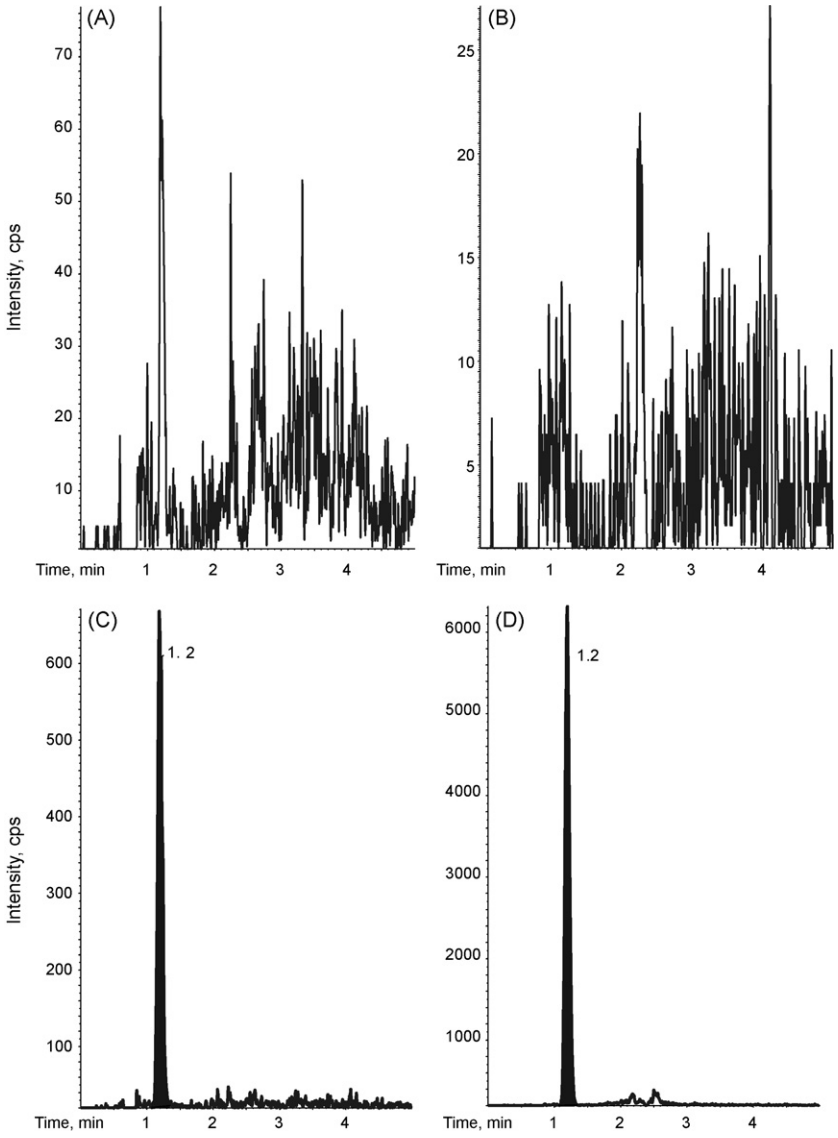

Fig. 6. Chromatographic conditions using pH 3.4 mobile phase with a Hypersil GOLD PFP (50 mm × 2.1 mm i.d., 5 μm) analytical column; representative chromatograms obtained from human serum: (A) blank human serum for the analyte; (B) blank human serum for SIL IS; (C) human serum spiked at 0.025 ng/mL LLOQ level for the analyte; (D) SIL IS at 0.6 ng/mL in human serum.

3.4. Validation results

Calibration curves for the updated methodology (mobile phase pH 3.4) showed good linear response ($R^2 > 0.99$) over the range of 0.025–25.0 ng/mL for human serum. A $1/x^2$-weighting scheme was used consistently throughout all batch and stability runs for the validation. The LLOQ, defined as the lowest concentration of analyte with accuracy within ±20% and a precision of ≤20%, was 0.025 ng/mL. A signal-to-noise ratio (S/N) greater than 35 was attained and reproducible at this level. Representative chromatograms obtained from double blank, blank with SIL IS and LLOQ samples are shown in Fig. 6. No interfering peaks from endogenous compounds were observed at the retention times of either the analyte or SIL IS.

Precision and accuracy for the additional validation batch run utilizing the low pH conditions as well as the combined inter-run results for the original three neutral pH batch runs are shown in Table 5. Current laboratory and SOP procedures required an additional validation batch run including selectivity assessments for a

Table 5
The intra-run ($n = 6$) and inter-run precision (%RSD) and accuracy (%RE of the LC–MS/MS method used to quantitate the analyte in human serum ($n = 24$).[a].

Matrix	Concentration added (ng/mL)	Intra-day			Inter-day		
		Observed concentration (ng/mL)	%RSD	%RE	Observed concentration (ng/mL)	%RSD	%RE
Serum	0.025	0.023 ± 0.002	7.62	9.29	0.027 ± 0.003	9.47	10.49
	0.075	0.070 ± 0.002	2.99	6.94	0.074 ± 0.004	5.91	4.77
	1.25	1.196 ± 0.048	4.00	4.32	1.272 ± 0.063	4.93	4.40
	20	19.196 ± 0.493	2.57	4.02	20.206 ± 0.838	4.15	3.75

[a] pH in mobile phase of Intra-day was 3.4 for one run and pH in mobile phase of Inter-day was 7.0 for three runs and 3.4 for one run.

Table 6
The processed samples stability (4 days at ambient) ($n = 6$).[a].

Matrix	Concentration added (ng/mL)	Processed samples		
		Observed concentration (ng/mL)	%RSD	%RE
Serum	0.025	0.025 ± 0.001	4.91	3.93
	0.075	0.071 ± 0.003	3.69	5.43
	1.25	1.186 ± 0.036	3.03	5.08
	20	18.977 ± 0.360	1.90	5.12

[a] pH in mobile phase was 3.4; column: Hypersil GOLD PFP (50 mm × 2.1 mm i.d., 5 μm).

chromatographic modification. The values for the intra- and inter-run precision and accuracy were well within the acceptable limits (Table 5).

Stock solutions were determined to be stable at the storage conditions (4 °C) for 27 days. Fortified and processed samples were stable at room temperature for a minimum of 24 h (Table 4 and Table 6). The freeze/thaw cycle and long-term stability were discussed above (see Section 3.3).

4. Conclusions

SIL IS are commonly used to compensate for these effects by having theoretically equivalent ionization tracking allowing for the ratio of analyte-to-SIL IS to be maintained. When chromatography is adequately selective to reveal the slight difference in physiochemical properties from certain types of labeling, the retention of the analyte and SIL IS may shift relative to each other. Variation in phospholipid levels observed between numerous lots can introduce a relatively high degree of variability when samples are analyzed against calibration standards prepared in different matrix lots. These impacts were demonstrated in our case with the slight retention time shift between the analyte and its corresponding SIL IS at neutral mobile phase pH where the accuracy of freeze/thaw and long-term stability samples in addition to supplemental ionization effects assessments were affected.

Two chromatographic strategies including increased column length and lower pH in mobile phase were developed to minimize ionization effects from their co-elution. While longer column length did move the analyte and SIL IS out of the main region of phospholipid impact, it did not completely isolate them from other identified phospholipids and apparent regions of suppression. However, pH (3.4) adjusted in mobile phase lead to complete chromatographic separation of the tracked phospholipids and the associated regions of ionization suppression without any other procedural modifications. These results highlight the importance of optimizing chromatographic conditions to achieve separation to allow the ability to continue using simple and cost-effective extraction techniques like protein precipitation. The final validation results for the analyte demonstrate the effectiveness of this approach in producing an accurate and precise bioanalytical assay.

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