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Systematic evaluation of supported liquid extraction in reducing matrix effect and improving extraction efficiency in LC–MS/MS based bioanalysis for 10 model pharmaceutical compounds

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ABSTRACT

In past a few years, there has been a large increase in the application of supported liquid extraction (SLE) for LC–MS/MS based bioanalysis due to its distinct practical advantage in reduced time cost, ease of operation and the feasibility for automation. The main purpose of this study was to systematically evaluate supported liquid extraction in reducing matrix effect and improving extraction efficiency/recovery under various extraction conditions with 10 model pharmaceutical compounds in liquid chromatography coupled to electrospray tandem mass spectrometry (LC–ESI-MS/MS) analysis. Selected compounds have diverse physicochemical properties where log *P* ranges from 0.1 to 6.24 and pK_a ranges from 4.0 to 11.1. The factors that may have the impact on the recovery of analytes and phospholipids (PL) were assessed. Over 75% recovery was achieved for every analyte under its respectively optimized extraction conditions where the selection of the polarity of extraction solvent and buffered pH can be critical for efficient recovery. Furthermore, the matrix effect was assessed by postextraction spike and postcolumn infusion method. The matrix effect was considerably reduced for all analytes under most extraction conditions evaluated for SLE, compared with protein precipitation (PPT) method. The correlation between matrix effect and residual phospholipids in sample extract was clearly shown. Although analyte-dependent matrix effect was observed prominently in sample extract prepared by PPT, it was minimized by SLE sample preparation process that effectively removes the majority of phospholipids. Sample extracted by ethyl acetate contained more phospholipids and demonstrated stronger matrix effect than by other organic solvents. Water-miscible organic content, such as methanol and acetonitrile in samples prior to loading has significant impact on PL recovery when eluting with methyl tert-butyl ether. However, isopropanol does not enhance the recovery of PL when adding to dichloromethane for elution. In addition, the compromise between improved extraction efficiency by SLE and reduced matrix effect is sometimes necessary to yield clean extract with acceptable recovery. The effective removal of phospholipids and reduction of matrix effect, while achieving good recovery for all pharmaceutical compounds with diverse physicochemical properties, demonstrated that SLE is a valuable alternative technique to liquid–liquid extraction (LLE) in high throughput LC–MS/MS based bioanalysis.

1. Introduction

Liquid chromatography coupled to atmospheric pressure ionization tandem mass spectrometry (LC–API-MS/MS) is currently the most widely used technique in high throughput bioanalysis of small molecule due to its high selectivity and sensitivity, and is increasingly applied for the larger molecules, such as peptides and oligonucleotides. Although it rarely shows interference peaks at the selected mass transitions monitored for the analytes of interest, LC–MS/MS does not guarantee the effective removal of high levels of co-eluting endogenous matrix components without adequate sample preparation and/or good chromatography. These co-eluting matrix components including salts, surfactants, and various organic molecules such as uric acid, carbohydrates, amines, lipids, and peptides may cause significant matrix effect via affecting the ionization efficiency and reproducibility of analytes [1,2]. The degree of ion suppression or enhancement depends on biofluids [3], extraction methods, ionization mode and analytes [4,5]. Matrix effect can substantially compromise the reproducibility, linearity, precision,

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accuracy, sensitivity and selectivity of a bioanalytical assay and potentially lead to erroneous quantification [2,6]. Stable isotope-labeled internal standard (SIL-IS), not readily available in many cases for metabolites, can compensate for much matrix effect, especially at high and middle concentration. However, matrix effect could still impact bioanalytical assays' performance, when SIL-IS were used, since the matrix effect may be concentration dependant and nonuniform in different lots of matrices [7]. As a consequence, the caution and effort should be taken to resolve this concern during bioanalytical method development.

Phospholipids, extremely abundant in plasma, have been identified as a major source of matrix effect in LC–MS/MS analysis [8,9]. Based on their core structures, plasma phospholipids are categorized into different classes, i.e. phosphatidylcholine (PC), lysophosphatidylcholine (Lyso-PC), sphingomyelin (SM), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (Lyso-PE), phosphatidylserine (PS), lysophosphatidylserine (Lyso-PS), phosphatidylglycerol (PG), lysophosphatidylglycerol (Lyso-PG), phosphatidylinositol (PI), and phosphatidic acid (PA) [10–13], among which PC and lyso-PC accounts for up to 70% and 10% of total phospholipids (Total-PL), respectively. Previous studies have demonstrated phospholipids could elute at a wide range of retention time and led to significant matrix effect when coeluting with the analyte of interest [14,15]. Therefore, it is not always possible to separate the analyte from interferences in the chromatogram in order to avoid ion suppression. Herein, the removal of phospholipids through sample preparation becomes a critical alternative step towards overcoming matrix effect.

Protein precipitation (PPT), liquid–liquid extraction (LLE), and solid phase extraction (SPE), are most commonly used techniques to prepare biological samples for LC–MS analysis while supported liquid extraction (SLE) and hybrid-SPE precipitation (H-SPE) are gaining the popularity as the alternatives with unique features and benefits. Protein precipitation by organic solvents, a very simple sample preparation technique, has been shown to yield the most significant matrix effect when compared to SPE and LLE [16,17]. Although SPE generally provides clean extract, the selection of appropriate sorbents and washing and elution conditions is critical for the elimination of matrix effect due to phospholipids [18]. SPE is a time-consuming extraction for method development and sample analysis, even though it is feasible for automation. H-SPE was recently developed and applied to minimize matrix effect via the effective removal of phospholipids and proteins [13,19]. Fewer amounts of phospholipids were recovered from H-SPE when compared to several other SPE techniques [13]. LLE also can provide clean extract. However, it is not easily adapted for automation and can present phase separation and emulsion issue. In contrast, SLE, as a flow-through technique, can be used as an alternative to traditional liquid–liquid extraction (LLE) to provide faster extraction and clean samples, while it also has the advantage of easy adaptation to automation and reduction of other liquid handling issues [20–22]. Compared to traditional LLE, SLE also demonstrated higher analyte recovery when loading buffers and extraction solvents were optimized [23–25]. In terms of phospholipids' removal, SLE provided the cleanest extract when compared with PPT, LLE and SPE under the experimental conditions evaluated by Ismaiel et al. [13].

Although studies on optimization of SLE in extraction efficiency [23] and phospholipids' removal [20] have been conducted, comprehensive investigation of SLE in improving recovery, reducing matrix effect, and, particularly, correlating matrix effect with phospholipids removal under various extraction conditions has never been undertaken. Therefore, the main objective of our present study was to investigate the impact, resulted from varying extraction conditions, on the extraction efficiency and matrix effect of SLE extraction method, using 10 model compounds with a wide range of pK_a (4–11) and log$P$ (0.1–6.2) values (Table 1).

Table 1
Properties of the compounds.

	Diclofenac (Dic)	Progesterone (Pro)	Ambrisentan (Amb)	Carbamazepine (Car)	Atenolol (Ate)	Metoprolol (Met)	Daunorubicin (Dau)	Paclitaxel (Pac)	Celexocib (Cel)	Fexofenidine (Fex)
log P	4.98	3.58	6.24	2.1	0.16	1.72	0.73	3.96	3.5	5.02 or 0.49[a]
pK_a	4.15	NA[c]	4.0	7.0	9.6	9.7	8.3	NA	11.1	4.25, 9.53[b]
Parent ion (m/z)	296.2	315.1	379.1	237.2	267.3	268.2	528.3	854.6	382.2	502.5
Daughter ion (m/z)	215.2	109.1	303.1	194.2	145.1	116.1	321.4	569.4	362.2	466.5
Retention time (min)	1.39	1.47	1.34	1.11	0.65	0.84	1.02	1.38	1.44	1.07

[a] 0.49 in HCl salt form.
[b] Zwitterionic compound with two pK_a values.
[c] Not ionized at physiological pH.

In general, electrospray ionization (ESI) is much more susceptible to matrix effect than atmospheric chemical ionization (APCI) [26] and positive ESI is the most applicable ionization method for bioanalysis, therefore, this study focuses on the evaluation of matrix effect in positive-ion mode electrospray ionization. We believe our systematic approach critically assesses this particular sample preparation technique for bioanalytical method development, identifies the factors for minimizing or eliminating matrix effect and improving recovery, and provides sensible recommendations based on logical experimentation. It is our hope that this endeavor leads to the most robust and sensitive assay possible, accompanied by accelerated method development time.

In our study, the results by SLE extraction were compared with those obtained from PPT. The extraction conditions were varied by changing the pH and/or organic content of sample loading buffer or by changing the composition of eluting organic solvent to reflect real-world scenario in bioanalysis. The matrix effect was assessed by postextraction addition, phospholipids monitoring, and postcolumn infusion method, and correlated to the recovery of phospholipids which was monitored by specific MRM transitions of phospholipids.

2. Experimental

2.1. Chemical, reagents, materials, and apparatus

Diclofenac sodium (Dic), metoprolol tartrate (Met), and fexofenadine (Fex) were purchased from USP (Rockville, MA, USA). Progesterone (Pro), carbamazepine (Car), atenolol (Ate), daunorubicin (Dau), and paclitaxel (Pac) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ambrisentan (Amb) and Celecoxib (Cel) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). HPLC grade acetonitrile (ACN), methanol (MeOH), formic acid (FA), ethyl acetate (EtOAc), dichloromethane (DCM), 2-propanol (IPA), and methyl tert-butyl ether (MTBE) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Ammonium formate was purchased from Fisher Scientific (Fair Lawn, NJ, USA). A PURELAB Ultra system from ELGA (Marlow, UK) was used to produce deionized water. Human plasma with K_3 EDTA anticoagulant was obtained from Biochemed (Winchester, VA, USA). ISOLUTE supported liquid extraction (SLE) 96-well plates containing 200 mg diacetomaceous earth as support media per well were obtained from Biotage (Charlotte, NC, USA).

An automated SPE system (Quadra 96 model 96-320) for transferring liquid during sample preparation was obtained from Tomtec (Hamden, CT, USA). HPLC system consisted of two LC-20AD binary pumps, a SIL-20AC autosampler, a CTO-20AC column oven, a DGU-20A_3 degasser, and a CBM-20A system controller from Shimadzu (Columbia, MD, USA). An Applied Biosystems API 4000 triple quadrupole mass spectrometer (Foster City, CA, USA) equipped with an ESI turboionspray (TIS) interface was used for the analysis.

2.2. Chromatographic conditions

Chromatographic separations of selected compounds and phospholipids were conducted on a Gemini C18 column (50 mm × 2.0 mm, 5 μm) from Phenomenex (Torrance, CA, USA) at 25 °C. Mobile phase A (MA) consisted of 10 mM ammonium formate and 0.1% formic acid in water, and mobile phase B (MB) consisted of 0.1% formic acid in acetonitrile. The HPLC program for gradient elution was as follows: 2% of MB (0–0.2 min), from 2% to 95% of MB (0.2–1.6 min), 95% of MB (1.6–3.5 min), from 95% to 2% of MB (3.5–3.6 min), and 2% of MB (3.6–4.2 min). The separation was performed at a flow rate of 1.5 mL/min. The sample injection volume was 10 μL for determining recovery and matrix effect while the injection volume was reduced to 1 μL and 2 μL for monitoring phospholipids in plasma sample obtained from PPT and SLE, respectively.

Table 2
Phospholipids and their MRM transitions.

Name	Abbreviation	MS transitions
Lysophosphatidylcholine	Lyso-PC	496 → 184, 524 → 184
Phosphatidylcholine	PC	704 → 184, 758 → 184
		760 → 184, 772 → 184
		784 → 184, 786 → 184
		806 → 184, 808 → 184
Sphingomyeline	SM	731.6 → 184
Lysophosphatidylethanolamine	Lyso-PE	454.3 → 313.3
Phosphatidylethanolamine	PE	768.6 → 627.5
Lysophosphatidylglycerol	Lyso-PG	513.2 → 341.2
Lysophosphatidylserine	Lyso-PS	524.3 → 339.3
Total-phospholipids	Total-PL	184 → 184

2.3. Mass spectrometric conditions

An API 4000 triple quadrupole mass spectrometer was operated in ESI positive ionization mode with multiple reactions monitoring (MRM) for LC–MS/MS analyses. A unit mass resolution was applied for both Q1 and Q3 quadrupoles.

To monitor the 10 model compounds, the following instrument parameters were employed: TIS temperature, 650 °C; TIS voltage, 4500 V; curtain gas, 25; nebulizing gas, 50; TIS gas, 50; collision gas, nitrogen, 8; declustering potential (DP), 50 V; entrance potential, 10 V; dwell time, 8 ms. Collision energy (CE) and collision cell exit potential (CXP) were optimized for individual compound. MRM transition to monitor each compound is shown in Table 1.

For monitoring a specific class of phospholipids, TIS voltage, CXP, DP and CE were set at 5000 V, 10 V, 50 V and 40 eV, respectively. DP of 170 V and CE of 15 eV were employed to enhance in source fragmentation and parent-ion transmission in collision cell when monitoring total phospholipids (Total-PL) with mass transition of m/z 184 → 184. The MRM transitions for quantifying these classes of phospholipids are shown in Table 2. All other instrument parameters for phospholipids were the same as those used for 10 pharmaceutical compounds.

2.4. Preparation of standard solutions and spiked plasma samples

Stock solutions of the 10 model compound were prepared individually in methanol. Intermediate standard solution contains all compounds in methanol/water (50/50, v/v) at 4000, 2000, 400, 10, 500, 200, 1000, 5000, 8000, and 500 ng/mL for Dic, Pro, Amb, Car, Ate, Met, Dau, Pac, Cel, and Fex, respectively, and stored in glass vials in a refrigerator. Spiked plasma samples were freshly prepared by spiking intermediate standard solution into blank human plasma at a ratio of 1:19 before sample extraction.

2.5. Sample preparation

In each experiment, six replicates of each type of samples were extracted and analyzed. The results from three independent extractions and sample analysis were calculated and summarized.

2.5.1. Protein precipitation (PPT)

Blank plasma, spiked plasma sample or water (100 μL) was mixed with 300 μL of acetonitrile or methanol in a 96-well plate (Table 3) and vortexed for 5 min. After centrifugation at 1640 × g for 5 min, 300 μL of clear supernatant was transferred and evaporated to dryness at approximately 45 °C. The residue was, then, reconstituted with 400 μL of acetonitrile/water (25/75, v/v) or standard

Table 3
Sample preparation conditions using PPT and SLE.[a]

Dilution solution	Extraction/elution solvent[b]					
	MeOH	ACN	EtOAc	MTBE	DCM:IPA (95:5, v/v)	DCM
NA	P-A	P-B	–	–	–	–
100 μL 1% FA in H_2O	–	–	E1	M1	DP1	D1
100 μL 0.1% FA in H_2O	–	–	E2	M2	DP2	D2
100 μL H_2O	–	–	E3	M3	DP3	D3
100 μL 0.5 M NH_4OH	–	–	E4	M4	DP4	D4
150 μL H_2O	–	–	–	M5	–	–
100 μL H_2O and 50 μL ACN	–	–	–	M6	–	–
100 μL H_2O and 25 μL ACN	–	–	–	M7	–	–
100 μL H_2O and 12.5 μL ACN	–	–	–	M8	–	–
100 μL H_2O and 50 μL MeOH	–	–	–	M9	–	–
100 μL H_2O and 25 μL MeOH	–	–	–	M10	–	–
100 μL H_2O and 12.5 μL MeOH	–	–	–	M11	–	–

[a] Plasma sample volume is 100 μL.
[b] 300 μL of ACN or MeOH was used for PPT and 1 mL of organic solvent was used for SLE elution.

solution of analyte in acetonitrile/water (25/75, v/v) for LC–MS/MS analysis.

2.5.2. Supported liquid extraction (SLE)

SLE extraction was carried out with different combinations of sample loading buffer and eluting solvents as shown in Table 3. In each case, blank plasma, spiked plasma sample, or water (100 μL) was diluted with 100–150 μL of dilution solution in a 96-well plate. After mixing briefly, the total volume of diluted samples (200–250 μL) was loaded onto a 96-well SLE+ plate with an automated liquid handling system. A minimum positive pressure was applied to facilitate the sample absorption into the cartridge in less than 10 s. After the analytes were allowed to equilibrate with the sorbent for a minimum of 5 min, the compounds were eluted with an aliquot of 1 mL of extraction solvent. The eluate was evaporated to dryness under nitrogen at 45 °C. The residue from fortified samples and blank samples were reconstituted with 400 μl of acetonitrile/water (25/75, v/v) and standard solution of analytes in acetonitrile/water (25/75, v/v), respectively, and vortex-mixed for 2 min. The resulting solution was directly subject to LC–MS/MS analysis.

3. Results and discussion

3.1. LC–MS conditions

Simultaneous separation of 10 model pharmaceutical compounds was conducted on a Gemini C18 column with HPLC gradient program that was optimized to start with low organic content to ensure the sufficient retention of very polar compound such as atenolol and metoprolol. Adequate retention and good peak shape were obtained for all 10 model compounds (Fig. 1). The same HPLC gradient program was used to monitor phospholipids in a separate injection with lower injection volume (see Section 2.3) to avoid saturating MS detection as a result of the large amount of phospholipids in samples, especially in protein precipitation extract. The typical phospholipids elution profiles are shown in Fig. 2.

All 10 model compounds were simultaneously monitored in positive ionization mode at the sacrifice of the sensitivity for a few acidic analytes, such as Dic and Amb. However, the concentration of drugs in plasma was carefully chosen such that the ample signal was obtained for all compounds to get the reproducible data after sample extraction. Two techniques were used to monitor phospholipids. The first method targeted specific MRM transitions of major phospholipids [12,13,27] from different classes as shown in Table 2. These individual MRM transitions were selected based on their abundance in plasma and signal strength in mass spectrometer under current experimental conditions. The second approach utilized in-source collision-induced dissociation MRM (IS-CID-MRM) of m/z 184 → 184 [12,15], which is the common daughter ions, trimethylammonium-ethyl phosphate ions (m/z 184), originated from the fragmentation of the polar head group of all glycerophosphatidylcholines (PC and Lyso-PC) and sphingomyelins. Thus, this mass transition represents the phospholipids profile for a wide variety of glycerophosphocholines and other phospholipids. To this end, a high declustering energy (DP) was applied in the source region of the mass spectrometer to induce the dissociation of phospholipids to generate the ion of m/z 184, and low collision energy (CE) in the collision cell was set to prevent further fragmentation in IS-CID-MRM approach as described in Section 2.3.

Plasma contains a large amount of phospholipids that retains well and potentially can build up on a reverse phase column due to their hydrophobicity arising from their one or two extremely hydrophobic long alkyl chain(s). To acquire reliable quantitative data of phospholipids in different sample extracts, the injection volume was lowered to obtain sufficient signal for each phospholipid species without overloading the column or saturate the detector. A 2-min flushing step with 95% acetonitrile after eluting all the analytes of interest was applied to eliminate the carryover of phospholipids and other matrix component that may cause inconsistent matrix effect measurements and inaccurate phospholipids quantitations in subsequent injections.

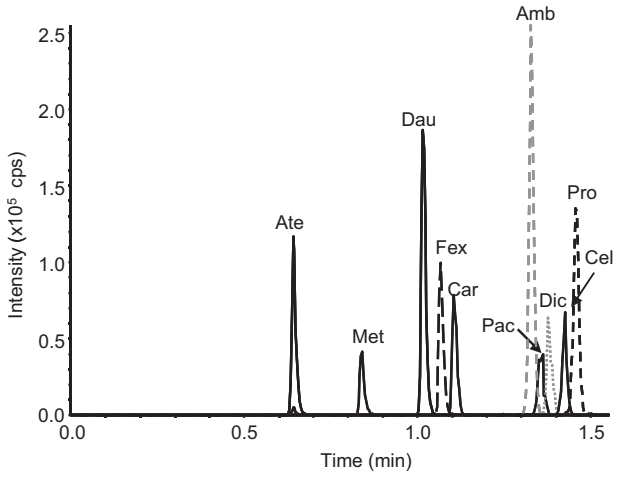

Fig. 1. Representative total ion chromatograms (TIC) of 10 model compounds under optimized LC–ESI-MS/MS conditions.

Table 4
Matrix effects under various extraction conditions using PPT and SLE.

Extraction conditions	Matrix factor[a,b,c]									
	Dic	Pro	Amb	Car	Ate	Met	Dau	Pac	Cel	Fex
PPT										
P-A	0.86	0.17	0.45	0.29	0.92	0.70	0.53	1.15	0.46	0.59
P-B	0.99	0.26	0.74	0.80	0.89	0.82	1.04	1.46	0.60	1.20
SLE-EtOAc										
E1	1.31	0.81	0.88	0.91	0.95	0.96	1.12	0.94	0.96	1.02
E2	0.93	0.69	0.91	0.98	1.07	1.05	1.06	1.02	1.09	0.94
E3	1.06	0.72	0.89	0.98	0.93	0.93	1.08	1.13	1.29	1.07
E4	1.04	0.88	0.85	0.96	0.98	0.96	1.05	1.09	1.19	1.01
SLE-MTBE										
M1	1.03	0.89	0.87	0.92	0.94	0.94	1.10	1.06	0.96	1.10
M2	0.91	0.87	0.90	0.95	0.97	1.07	1.04	0.98	1.07	0.95
M3	0.96	1.01	0.87	1.03	0.92	1.00	1.03	0.95	1.00	0.98
M4	1.00	0.90	0.79	0.99	1.01	0.97	1.06	1.02	1.14	1.00
M5	1.09	0.90	0.72	0.55	0.86	0.96	2.13	1.18	1.26	0.73
M6	1.20	0.64	0.85	0.62	0.94	0.95	2.35	1.09	0.72	0.98
M7	1.02	0.94	1.01	0.93	0.92	1.01	1.02	1.04	0.97	0.96
M8	1.04	1.08	0.88	0.95	0.99	0.99	0.92	0.83	0.90	1.01
M9	1.04	0.74	0.74	0.38	1.16	1.02	1.59	1.27	0.86	0.61
M10	1.02	0.95	0.91	0.97	1.02	0.99	1.16	1.28	1.07	1.02
M11	1.08	0.90	0.95	0.97	1.04	0.97	1.20	0.89	0.89	1.04
SLE-DCM										
D1	1.04	1.03	0.95	0.94	0.85	0.99	1.16	0.93	1.12	1.06
D2	1.17	1.10	0.84	0.92	0.88	0.91	1.11	0.76	1.11	0.96
D3	1.50	1.01	0.87	0.98	0.95	1.04	0.94	0.93	1.25	0.99
D4	1.30	1.09	0.64	0.96	1.00	0.99	0.99	0.82	0.96	1.04
SLE-DCM/IPA										
DP1	1.06	0.93	0.84	0.89	0.91	1.07	1.28	0.83	0.98	1.15
DP2	0.98	0.93	1.02	0.99	0.97	0.89	1.03	0.86	0.81	0.95
DP3	1.03	1.00	0.81	0.95	0.94	0.97	1.04	1.12	0.96	0.95
DP4	1.09	1.05	0.84	0.98	0.91	0.93	1.05	1.06	0.92	0.92

[a] Ion suppression: MF < 1; no matrix effect: MF = 1; ion enhancement: MF > 1.
[b] Calculated as the ratio of mean peak area of postextraction-spike blank sample extract over postextraction-spiked blank reagent extract.
[c] All values are means of three independent experiments. RSD% is less than 15%.

3.2. Evaluation of recovery and matrix effect

Three types of samples were used to measure recovery and quantitative matrix effect. Samples were defined as: A, samples containing analytes without plasma matrix and extraction (i.e. blank water extract reconstituted in neat solution of model compounds); B, samples containing analytes in blank plasma extract but without extraction (i.e. blank plasma extract reconstituted in neat model compounds solutions); and, C, samples containing analytes extracted from fortified plasma samples. The concentration of compounds in samples A, B, and C would be identical if the recovery was 100%. The extraction recovery is calculated based on the peak area ratio of C/B. The quantitative matrix effect was represented in Matrix Factor (MF) (Table 4), which is defined as a ratio of the analyte peak response in the presence of matrix extract (sample B) to the analyte peak response in the absence of matrix extract (sample A).

Complimentary to matrix factor evaluation, the postcolumn infusion method provided a qualitative assessment of matrix effect [16]. This experiment was carried out by using an infusion pump to deliver a constant amount of analyte into the LC stream between the HPLC column and MS via a "T-mixer". The qualitative matrix effect for 10 model compounds was evaluated by comparing chromatographic response profile obtained from plasma sample extract with that from water extract under the LC–MS/MS conditions for the sample analysis while maintaining the postcolumn infusion set-up. As a main cause of the matrix effect in LC–MS based bioanalysis, the elution traces of phospholipids were also monitored to investigate the correlation between the elution of phospholipids and observed ion suppression/enhancement zone in chromatogram.

3.3. Matrix effect evaluation

3.3.1. Evaluation of matrix factor by postextraction spike method

Protein precipitation with organic solvent is a simple and fast sample preparation technique commonly used in discovery and preclinical studies, where the high throughput sample analysis is a critical factor in method development. However, it fails to effectively remove any endogenous matrix components other than proteins, therefore, resulting in a final extract susceptible to significant matrix effects. The data obtained from PPT experiments (Table 4) revealed that 8 out of 10 model compounds had more severe matrix effect when methanol (condition P-A), instead of acetonitrile (condition P-B), was used for PPT. This suggested that specific organic solvent used in protein precipitation may also have a dramatic effect on the cleanliness of the sample extract. PPT with acetonitrile provided relatively cleaner sample extract and thereby less matrix effect compared to that with methanol. Since phospholipids have been known to cause ion suppression or enhancement in LC/MS/MS analysis due to their effects on the desolvation of LC-effluent droplets in ESI source and competition for excess charge on droplet surface [26,28], this observation is in line with the report that residual phospholipids in MeOH extract are significantly higher than that in ACN extract [14] and with our quantitative results from monitoring specific phospholipids (vide infra). Furthermore, it was known that acetonitrile is a more potent organic solvent to eliminate proteins from plasma samples, which could also contribute to the reduced matrix effect in our studies. Among the compounds, Dic and Ate showed insignificant matrix effect in their PPT extracts while Pro (MF $\leq$ 0.26) and Cel (MF $\leq$ 0.60) showed the most significant matrix effects in both PPT extracts (P-A and P-B). Most late eluted compounds, except for Dic, showed

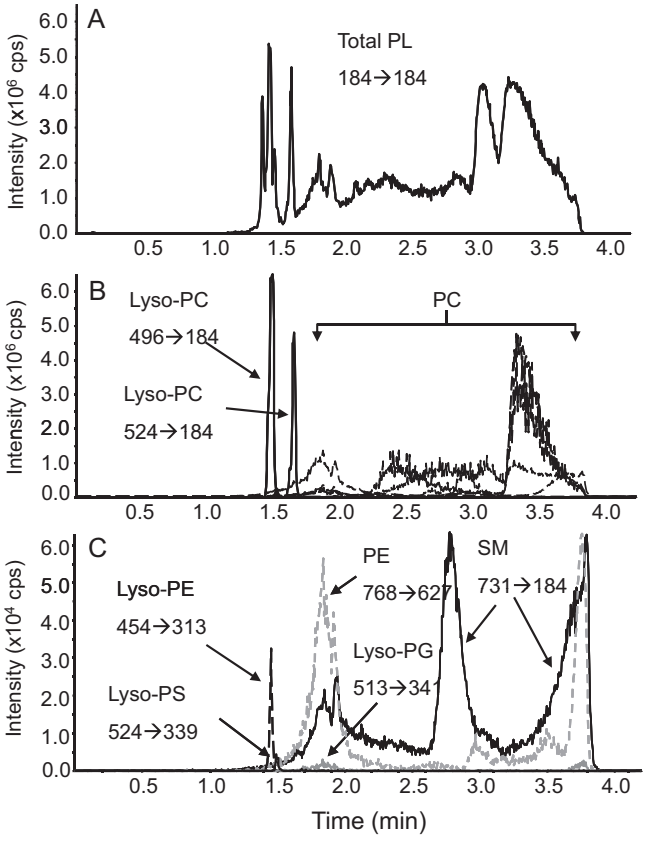

Fig. 2. Elution profiles of phospholipids illustrated with LC–MS/MS chromatograms obtained from human plasma extract from protein precipitation using methanol (condition P-A). (Panel A) MRM transition of total phospholipids; (Panel B) MRM transitions of Lyso-PC and PC; (Panel C) MRM transitions of Lyso-PE, Lyso-PS, SM, PE and Lyso-PG.

more matrix effect than early eluted ones, which coincides with the elution time of major phospholipids in Fig. 2.

When the compounds were extracted on SLE with ethyl acetate under extraction conditions E1–E4 (Table 4), all 10 compounds showed insignificant matrix effect. For 9 out of 10 compounds except for Pro, the loading buffer at different pH for sample dilution did not result in apparent variation in matrix effects (Table 4). Matrix effect for Pro, the most severely suppressed compound in PPT extract with MF of 0.17 and 0.26, was also significantly reduced with SLE extraction under all loading buffer conditions, where its MF increased to the value between 0.69 and 0.88. Minimal matrix effect was observed for Pro with basic loading buffer, while moderate matrix effect was observed for this compound with the loading buffers at other pH values.

Various extraction conditions (M1–M11) using MTBE, which may be encountered in bioanalysis, were evaluated. Little matrix effect was observed with loading buffers at different pH (conditions M1–M4) for all 10 compounds including Pro that demonstrated stronger matrix effect when extracted by ethyl acetate (Table 4). It is common to have a low percentage of water miscible organic solvents, such as acetonitrile or methanol, in diluted plasma prior to loading onto SLE. The organic solvent could be introduced into the plasma either by adding internal standard solution dissolved in organic solvent or occasionally by adding intentionally to facilitate sample loading. The impact of methanol and acetonitrile in diluted sample on matrix effect of different analytes were evaluated under conditions M6–M11. As shown in Table 4, insignificant matrix effect was observed for all 10 compounds when up to 25 μL of acetonitrile or methanol were added to mixture of 100 μL plasma and 100 μL diluting buffer under conditions M7, M8, M10, and M11. However, moderate to significant matrix effect was observed for several compounds, such as Dau, Cel, and Car, when 50 μL of acetonitrile or methanol were added under conditions M6 and M9. Furthermore, moderate to significant matrix effect was also observed for several analytes such as Dau and Car when the same amount of additional water (50 μL) was added under condition M5. This indicated possible sample break-through during elution when 250 μL of diluted samples was loaded on the plate even though it was not visually detected. When loading 200 μL, 225 μL, or 250 μL plasma containing 2% whole blood onto SLE plate with 200 mg bed size, no break-through was visually observed during sample loading step for three tested volumes. However, the light red color was observed only in the filtrate of samples loaded with 250 μL when MTBE was applied to elute the compounds, suggesting sample break-through at the loading volume of 250 μL during elution stage. Therefore, the caution should be taken to avoid loading more than 225 μL of diluted sample onto SLE plate with 200 mg bed size to avoid break-through during elution even though it may not affect the quantification and matrix effect of some analytes, e.g. Dic, Ate and Met.

Insignificant matrix effect was achieved for each of these 10 model compounds when plasma samples were diluted with appropriate loading buffer and eluted with DCM or DCM/IPA (95/5, v/v) as shown in Table 4. Comparable results were obtained by using DCM or DCM/IPA for elution under the identical dilution conditions, suggesting that addition of up to 5% IPA in DCM affords the minimal impact on matrix effect for these model compounds.

3.3.2. *Evaluation of phospholipids recovery*

Representative LC–MS/MS chromatograms of phospholipids extracted from blank human plasma using PPT with methanol are shown in Fig. 2. MRM transition m/z 184 → 184 was used to monitor Total-PL, including PC, Lyso-PC and SM (Fig. 2A). Generally, Lyso-PC with a single fatty-acyl chain elutes earlier and appears as sharper peaks than PC with two fatty-acyl chains (Fig. 2B) because the hydrophobic interaction with stationary phase dominates under reversed phase chromatographic conditions [15,18]. Several other classes of phospholipids which cannot be monitored with MRM transitions of m/z 184 → 184 are shown in Fig. 2C. The intensity of these phospholipids, i.e. SM, PE, Lyso-PE, Lyso-PS, and Lyso-PG, was much lower when compared to PC and Lyso-PC.

The removal efficiency of several major phospholipids by SLE under various extraction conditions is summarized in Fig. 3. The amount of phospholipids recovered under various conditions was normalized to that of PPT extract with methanol, which was set as a unit value. Over 90% of Lyso-PC, PC, SM, Lyso-PE and Lyso-PS was removed (Fig. 3A1 and B1) when EtOAc was used as eluting solvent. For some low abundant phospholipids, such as PE and Lyso-PG, at least 75% was removed (Fig. 3C1 and D1). Total-PL was removed by at least 85% (Fig. 3E1). The removal of individual phospholipid was impacted by the loading buffers at different pH value (Fig. 3A1–D1). However, no significant change was observed for Total-PL removal with different loading buffers (Fig. 3E1). Correlating data in Table 4, even though SLE using EtOAc is an effective way to reduce matrix effect for all compounds, approximate 15% phospholipids still remains in sample extracts.

Compared to extraction by EtOAc (conditions E1–E4), sample preparation using MTBE as the eluting solvent (conditions M1–M4), provided much cleaner extract with all four loading buffers (Fig. 4A2–E2). Over 99.999% of Lyso-PC and PC were removed as shown in Fig. 4A2–B2, which is negligible in these figures. Over 99% of Lyso-PE, PE, Lyso-PG and Lyso-PS were also eliminated from plasma samples (Fig. 4C2 and D2). Less than 0.1% of Total-PL remains in sample extract (Fig. 4E2). Contrary to ethyl acetate, the pH value of buffered sample does not play a significant

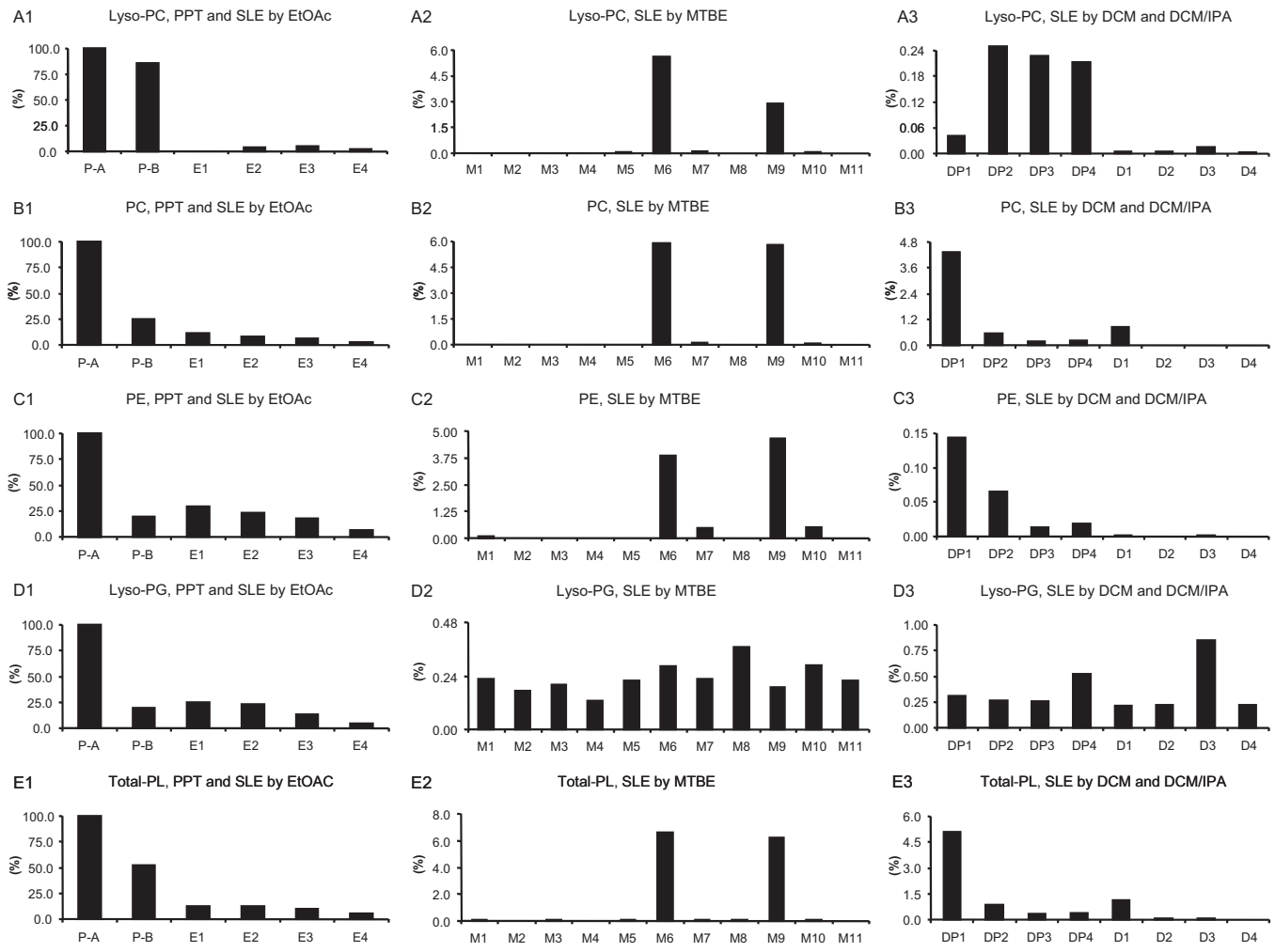

Fig. 3. Phospholipids recovery by PPT and SLE extraction under various conditions (Table 3). The value is normalized to phospholipids recovered from protein precipitation extract by methanol (condition P-A). Lyso-PC: A1–A3; PC: B1–B3; PE: C1–C3; Lyso-PG: D1–D3; Total-PL: E1–E3.

role in the recovery of phospholipids when MTBE is used as elution solvent. When a small amount (12.5 μL or 25 μL) of acetonitrile or methanol (conditions M7, M8, M10, and M11) was added to the diluted sample prior to loading onto SLE plate, the recovery of some phospholipids (Lyso-PC, PC, Lyso-PE, PE) increased dramatically by more than a hundred folds compared with the sample diluted with 100 μL or 150 μL water (conditions M3 and M5) (Fig. 4A2–E2). The other types of phospholipids (Lyso-PG and Lyso-PS) did not vary much when the small amount of organic solvent was added (Fig. 4D2). The addition of 50 μL of acetonitrile or methanol to the diluted sample (condition M6 and M9) resulted in dramatically increased phospholipids recovery efficiency. Even though over 90% removal efficiency was still achieved for most of phospholipids monitored, the recovery of Lyso-PC, PC, SM, Lyso-PE and PE phospholipids in this case increased by at least 10 folds than the recovery with the addition of 25 μL of ACN or methanol (conditions M7 and M10). This observation can be attributed to the sample breakthrough during elution step as described earlier in Section 3.3.1. Interestingly, the phospholipids removal efficiency under conditions M6 and M9 are 5–20 folds lower than that under condition M5 even though they have the identical sample loading volume of 250 μL. This suggested that water-miscible organic content in loading buffer or diluted sample drastically impacts the phospholipids removal and should be carefully controlled to reduce phospholipids-related matrix effect. Furthermore, matrix effect for many compounds, i.e. Amb, Car, Dau, Pac, Cel, Pro and Fex, is more severe under condition M6 and/or M9 when compared to condition E1–E4 (Table 4) while the remaining phospholipids in the extracted sample are approximately in the same level (Fig. 4A1–E2), which implicated that matrix components other than phospholipids breaking through during elution step may also contributed to stronger matrix effect in this case.

We also investigated phospholipids recovery by DCM and DCM/IPA mixture. When DCM was used as elution solvent to extract the sample at different pH (condition D1–D4), over 99.5% phospholipids were removed with three out of four different loading buffers (Fig. 4A3–E3). However, the removal efficiency of PC and Total-PL significantly decreased when 1% formic acid in H_2O was used as the loading buffer (Fig. 4B3 and E3). Similar observation was obtained when DCM/IPA was used as elution solvent under conditions DP1–DP4. Although the addition of IPA in DCM decreased the phospholipids removal, over 95% phospholipids were still eliminated when eluted with DCM/IPA mixture. Correspondingly, insignificant matrix effect was observed for all 10 model compounds under most extraction conditions using DCM or DCM/IPA (95/5, v/v) (Table 4).

3.3.3. Evaluation of matrix effect by postcolumn infusion

The quantitative information of matrix effect was evaluated readily by postextraction spike method in Section 3.3.1. Postcolumn infusion of each of these 10 model compounds was conducted to qualitatively identify ion suppression or enhancement region

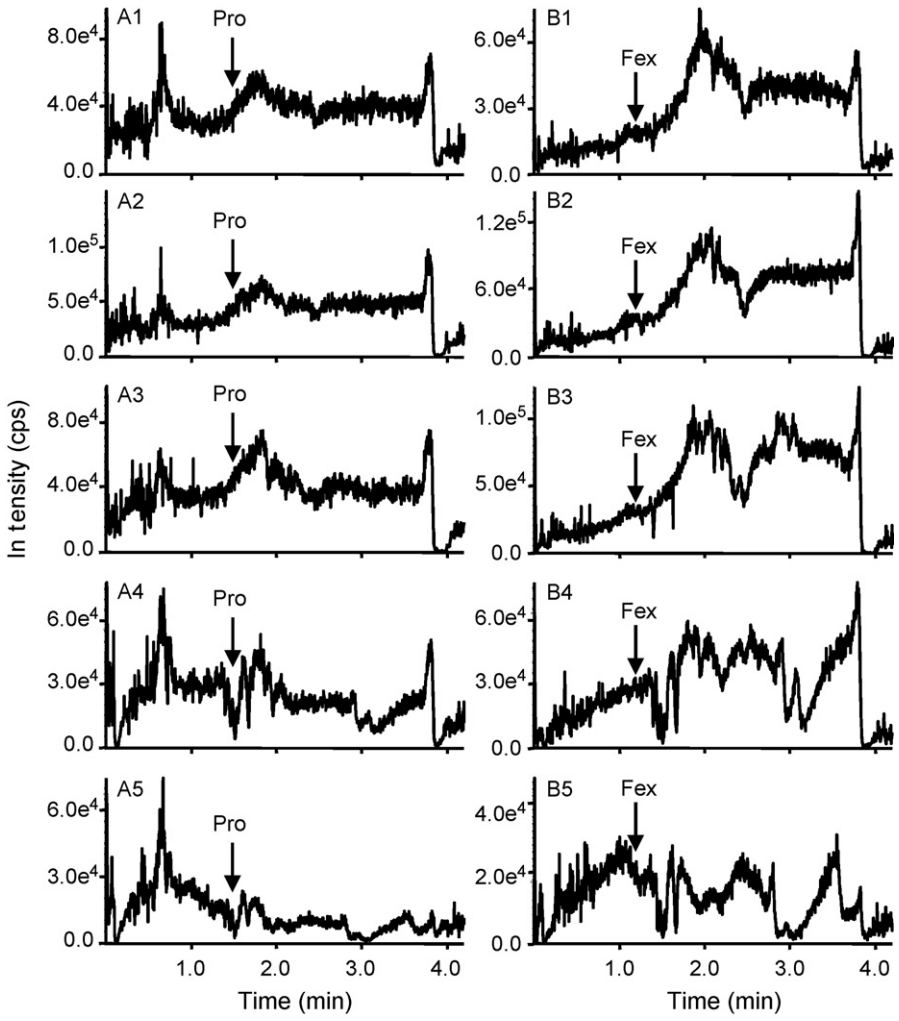

Fig. 4. Comparison of matrix effect by injecting sample extract on column with postcolumn infusion of Pro (A) and Fex (B). Panels 1–5 are MRM chromatogram of each type of sample extract. Blank reagent: A1 and B1; SLE extract by MTBE (condition M3): A2 and B2; SLE extract by EtOAc (condition E4): A3 and B3; PPT extract by ACN (condition P-B): A4 and B4; PPT extract by MeOH (condition P-A): A5 and B5. The arrows point to retention time of Pro and Fex.

in the chromatogram under current chromatographic condition. It involves the continuously introduction of the analytes by the means of a syringe pump connected to the column effluent. Representative chromatograms of compound Pro and Fex from extraction conditions M3, E4, P-B, and P-A were selected to illustrate the postcolumn infusion approach in Fig. 4. Two major ion suppression zones with retention time at around 1.2–1.8 min and 2.8–3.5 min were identified for all 10 compounds when blank matrix extract from PPT was injected (Fig. 4A4, B4, A5 and B5). Two major suppression zones are probably related to the elution of phospholipids that have the same retention time as indicated in Fig. 3A. The signal at suppression zone at around 2.8–3.5 min was more severely quenched than the early one at 1.2–1.8 min because the late-eluted phospholipids (PC, SM, PE) are much more abundant than early-eluted phospholipids (Lyso-PC, Lyso-PS, Lyso-PE). However, the presence of ion suppression or enhancement zone does not necessarily lead to observed matrix effect in bioanalysis if it does not overlap with the retention of analyte of interest. As shown in Fig. 4A4 and A5 the early ion suppression zone at around 1.2–1.8 min overlaps with the retention time of compounds Pro in protein precipitation samples (condition P-A and P-B). Consequently, significant matrix effect was observed for the compound under the same extraction conditions as indicated in Table 4. When the samples were extracted by SLE with MTBE or EtOAc (condition M3 and E4), the majority of phospholipids were removed as shown in Fig. 3H1 and H2, therefore, no ion suppression zone was observed (Fig. 4A2 and A3). This demonstrated that matrix effect can be eliminated by carefully choosing the proper sample preparation techniques and conditions for Pro. For Fex, more severe ion suppression was observed from PPT extraction by methanol (condition P-A) than from PPT extraction by acetonitrile (condition P-B) (Table 4). This was supported by the postcolumn infusion experiments (Fig. 4B4 and B5) since a deeper suppression zone was observed from condition P-A. This result illustrates the effect of different protein precipitation solvent on ion suppression of the analyte. The matrix effect for Fex (RT at 1.07) cannot be directly linked to phospholipids monitored in this study with relatively late retention after 1.4 min. In this case postcolumn infusion became an alternative tool to qualitatively determine matrix effects that are not directly associated with common phospholipids. Along this line, this also suggested that other phospholipids or lipids [13] not monitored in this study or other matrix components may contribute to the matrix effect of Fex. When extracted in neutral pH with MTBE or under basic conditions with ethyl acetate (condition M3 and E4, respectively), however, little matrix effect (Table 4) was obtained and no ion suppression zone around the retention of Fex (Fig. 4B2 and B3) was observed. The fact implies that SLE is an effective sample preparation technique to eliminate

Table 5
Recovery under various extraction conditions using PPT and SLE.

Extraction conditions	Recovery (%)[a,b]									
	Dic	Pro	Amb	Car	Ate	Met	Dau	Pac	Cel	Fex
PPT										
P-A	120	113	119	120	110	84.2	107	98.7	114	112
P-B	116	82.8	103	108	111	68.8	78.2	95.5	138.0	101
SLE-EtOAc										
E1	69.1	77.6	92.2	84.0	0.6	2.0	4.3	64.4	49.5	86.7
E2	78.5	101.1	3.7	84.0	4.3	58.7	60.7	75.8	65.9	76.6
E3	34.4	93.0	2.7	83.9	7.3	89.8	76.1	72.4	64.3	63.9
E4	14.2	84.2	2.0	81.7	71.7	80.7	63.1	58.9	77.3	14.5
SLE-MTBE										
M1	74.9	73.2	88.0	80.5	0.2	0.2	0.1	71.8	60.6	6.1
M2	64.1	76.3	0.5	79.9	0.2	29.1	0.8	67.8	47.3	21.3
M3	23.7	78.9	0.4	74.3	0.2	79.1	25.3	68.5	61.4	19.3
M4	2.9	68.3	0.1	72.0	8.3	80.7	36.4	54.8	61.2	1.8
SLE-DCM										
D1	73.0	57.9	86.5	86.6	0.5	2.0	0.4	88.2	73.3	12.3
D2	0.7	58.7	1.9	87.7	2.9	82.0	60.0	59.5	71.6	12.0
D3	0.5	63.5	1.4	88.0	4.6	79.7	60.8	63.9	66.6	8.1
D4	0.9	66.1	0.4	90.1	53.1	83.7	48.8	64.4	70.0	0.5
SLE-DCM/IPA										
DP1	79.2	56.8	94.0	90.0	0.7	10.2	4.0	74.2	80.5	83.6
DP2	18.8	65.6	20.3	88.2	6.7	89.5	81.4	67.5	84.4	92.2
DP3	6.0	63.8	6.6	92.5	11.1	94.6	72.7	74.6	81.1	84.1
DP4	0.8	64.5	1.7	87.5	99.4	92.3	53.8	44.8	87.4	8.9

[a] Calculated as the percentage of mean peak area of extracted sample over postextraction-spiked blank extract at the concentration with 100% recovery.
[b] All values are means of three independent extractions and analysis. RSD% is less than 15%.

not only phospholipids but also other matrix components that may introduce significant matrix effect.

3.4. Evaluation of analytes recovery

We also assess the extraction efficiency of acidic, neutral, and basic drugs with very diverse hydrophobicity/polarity using SLE under various extraction conditions (Table 5). The pH of sample was adjusted with loading buffers, i.e. 1% FA in H_2O, 0.1% FA in H_2O, H_2O or 0.5 M NH_4OH, and then the analyte was eluted with MTBE, ethyl acetate, DCM or DCM/IPA (95:5, v/v). The recovery of extraction was obtained by comparing the average peak areas of the analyte extracted from plasma samples prepared at the pre-designated level with those of blank plasma postextraction spiked with neat analytes solution at the same concentration. As anticipated, no significant variations in recovery were observed for neutral compounds with appropriate log *P* value including Pro (3.58), Car (2.10), and Pac (3.96) when different loading buffers were evaluated (Table 5). As a weak base with a pK_a of 11.1, Cel stay as a charged compound at physiological pH [29], as well as under buffered conditions, and gave similar recovery using different loading buffers. The recovery of acidic analytes, such as Dic, Amb, and Fex, increased dramatically under acidic extraction conditions (Table 5). On the contrary, basic analytes, such as Ate (pK_a 9.6), Met (pK_a 9.7), and Dau (pK_a 8.3), affords higher recovery under basic extraction conditions.

log *P* value, other than pK_a, of analyte is also a key factor to consider when exploring appropriate extraction conditions. When selecting the proper solvent to maximize the recovery of compounds, log *P* value can serve as a good guideline. For instance, over 50% recovery was achieved for Ate, a drug with a log *P* value of 0.1, under basic conditions and elution with EtOAc, DCM, or DCM/IPA. On the other hand, less than 10% recovery was obtained in any loading buffer when eluted with MTBE. MTBE is a much less polar solvent than EtOAc, DCM or IPA and, consequently, has relatively poor solubility for very polar compounds; hence the extraction efficiency of polar compounds in MTBE is hampered. Even though SLE elution with MTBE produces extracts with less phospholipids, it is not an efficient solvent for the extraction of very polar drugs, such as Ate, especially when the sensitivity and reproducibility become a concern. Furthermore, the more caution should be taken to select the proper loading buffer when the analyte has a low log *P* value. Three analytes with low log *P*, e.g. Ate, Dau, and Fex, did not provide over 50% recovery when MTBE was used as the eluting solvent (Table 5 condition M1–M4). In contrast, EtOAc provided over 70% recovery for all three compounds, but only when pH of the sample was adjusted with appropriate loading buffers. DCM, with higher polarity than MTBE, also gave the higher recovery for polar analyte, Ate and Dau, while yielding similar recoveries for most of other compounds. The recovery of polar analytes can be further improved by adding certain percentage of polar solvent into less polar eluting solvent. For instance, the recovery of Ate, Dau and Fex was significantly improved when the mixture of DCM:IPA (95:5) was used as eluting solvents under conditions DP1–DP4 (Table 5). Alternatively, the minimal changes in recovery was observed (data not shown) when up to 50 μL acetonitrile or methanol was added to the sample prior to loading and eluting by MTBE (conditions M8–M11), even though, these condition affords much higher recovery of phospholipids (Fig. 3).

4. Conclusion

Over 75% recovery was achieved for all 10 model pharmaceutical compounds with a wide range of pK_a and log *P* values using SLE by optimizing the appropriate loading buffer and elution solvent. Insignificant matrix effect was observed for these 10 compounds under optimized SLE conditions. Phospholipids monitoring demonstrated that SLE is a very effective sample extraction technique in term of the removal of phospholipids when appropriate loading buffers and eluting solvents were applied. Postcolumn infusion further supported that matrix effect largely correlates with the elution of phospholipids in chromatogram and can be minimized or eliminated via SLE extraction. To maximize the recovery of analytes, pK_a and log *P* of the analytes and polarity of the solvent should be carefully evaluated. The amount of water-miscible organic solvent in samples also impacts the phospholipids recovery by MTBE but not

matrix effect. In summary, the present study demonstrated that the minimal matrix effect and effective extraction can be achieved for pharmaceutical compounds with diverse physicochemical properties using SLE when sample loading buffers and eluting solvents are carefully chosen. Together with its facile adaptation to automation and reduced sample preparation time compared to traditional LLE, SLE demonstrated distinct advantages, as an increasingly popular sample preparation method, for high throughput LC–MS/MS based bioanalysis.

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