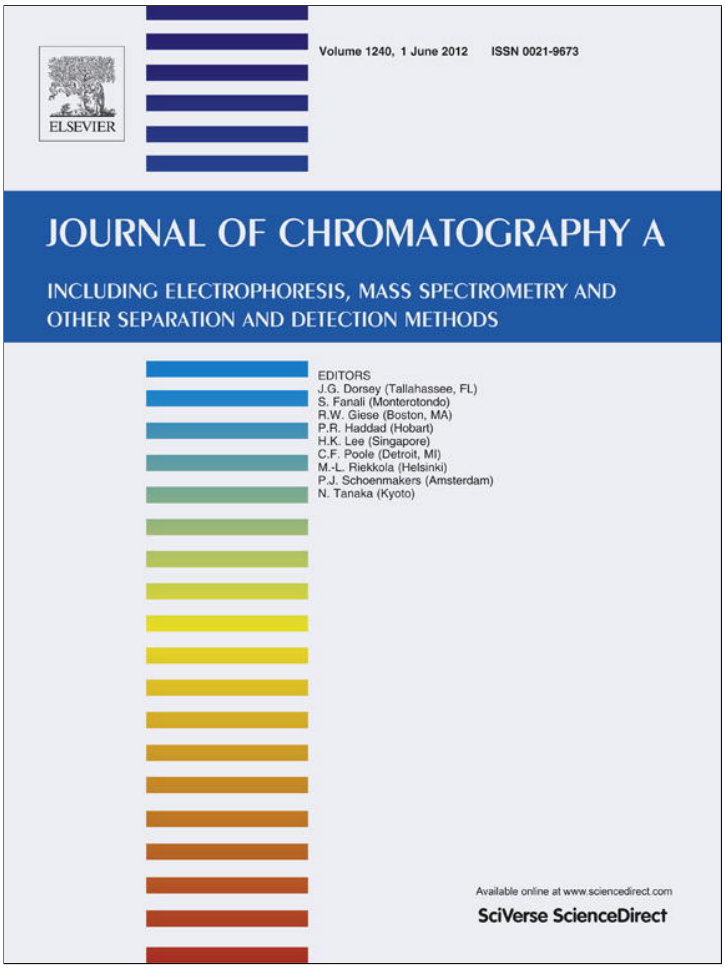

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Effect of ionization modifiers on the simultaneous analysis of all classes of phospholipids by nanoflow liquid chromatography/tandem mass spectrometry in negative ion mode

Dae Young Bang[a,b], Sangsoo Lim[a], Myeong Hee Moon[a,*]

[a] Department of Chemistry, Yonsei University, Seoul 120-749, South Korea
[b] Lotte R&D Center, 23 Yangpyoung-Dong, Seoul 150-194, South Korea

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ABSTRACT

The effect of ionization modifiers added to the mobile phase of nanoflow liquid chromatography–tandem mass spectrometry (nLC-ESI–MS^3) on the simultaneous analysis of all phospholipid (PL) classes in negative ion mode has been investigated. While MS analysis of most PL classes is carried out in negative ion mode, analysis of neutral polar (polar but electrically neutral) lipids like phosphatidylcholine (PC) and sphingomyelin (SM) is highly efficient in positive ion mode. Therefore, analysis of PL mixture samples often requires two separate runs in both positive and negative ion mode. In order to establish run conditions to carry out a single nLC-ESI–MS–MS for all PLs, the ionization efficiency of 13 different types of PL molecules in nLC-ESI–MS has been evaluated in negative ion mode by varying the modifiers and their concentrations. Experiments demonstrated that a mixture of 0.05% ammonium hydroxide and 1 mM ammonium formate added to the mobile phase provided effective ionization for all classes of PLs. The optimized conditions were applied to the analysis of a phospholipid mixture extracted from a human urine sample, yielding the identification of a total of 85 PL species. Analysis of the same sample with dual nLC-ESI–MS^2 runs in both positive and negative ion mode confirmed that nLC-ESI-MS^3 with the mixed modifier run only in negative ion mode gave comparable results.

1. Introduction

Along with the growth of proteomics, lipidomics is a rapidly expanding research field that analyzes lipid composition and distribution changes to understand lipid metabolism in relation to the discovery of biomarkers for various types of human adult diseases such as diabetes and cancer [1–5]. Phospholipids (PLs) are an important class in the lipidome because they are the major components of cellular membranes and they play crucial roles in signaling mechanisms for cell proliferation and cell death [6,7]. The composition of PLs in biological systems is very diverse due to the combination of different polar head groups and different acyl chain lengths, which contain various degrees of unsaturation.

Recent advances in electrospray ionization–mass spectrometry (ESI–MS) [8–10] and the integration of liquid chromatography with ESI-tandem MS (LC–ESI-MS-MS) [11–13] have facilitated the analysis of intact PLs since the separation of complicated PL mixtures reduces spectral congestion and tandem MS provides structural identification of PL species. Recently, capillary LC under the microflow or nanoflow regime has been directly utilized with ESI-MS–MS and has excelled in the simultaneous separation and identification of PLs from human plasma, cerebrospinal fluid, and urine samples from breast cancer or prostate cancer patients [14–20].

In the typical MS analysis of PLs, neutral polar (polar but electrically neutral) PLs in the zwitter ionic form such as phosphatidylcholine (PC), lyso-PC, and sphingomlyelin (SM) have been widely analyzed in positive ion mode [21,22], while anionic lipids, which include phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidic acid (PA), are readily detected in negative ion mode [23–25]. Therefore, a global analysis of PL species from biological fluids or cells often requires two separate LC–ESI-MS-MS runs in both positive and negative ion mode with each run requiring the addition of a different ionization modifier to the mobile phase, such as formic acid for positive ion mode and ammonium hydroxide or ammonium formate for negative ion mode. In addition, performing two separate LC–ESI-MS-MS runs lengthens the analysis time. Since the background noise of MS signals in negative ion mode is lower than in positive ion mode, it would be advantageous to simultaneously analyze all PLs in negative ion mode if the ionization efficiency of neutral lipids could be maintained at a reasonable level. With

* Corresponding author at: Department of Chemistry, Yonsei University, Yonsei-Ro 50, Seoul 120-749, South Korea. Tel.: +82 2 2123 5634; fax: +82 2 364 7050.
E-mail address: mhmoon@yonsei.ac.kr (M.H. Moon).

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typical mobile phase modifiers such as ammonium hydroxide, the detection limit can be less than 1 fmol for PG; however, under identical conditions, PCs and SMs are not detected at all [17]. In contrast, PCs and SMs can be analyzed in negative ion mode by adding other modifiers to the mobile phase that form adduct ions useful for identification of parent ions. Addition of ammonium acetate as an ionization modifier led to characterization of lysophosphatidylcholines (LPCs) [26], PCs, and SMs through adducts in the form of $[M+CH_3COO]^-$ as the parent ion followed by MS^3 analysis [20]. Ammonium formate has been widely applied to the detection of PCs and SMs through the formation of $[M+HCOO]^-$ ion and for other anionic lipids through the formation of $[M-H]^-$ [27–31]. While neutral lipids can be successfully analyzed in negative ion mode, the limit of detection (LOD) of these species in negative ion mode are not as low as in positive ion mode (2.2 fmol for PC) [16]. Recently, the identification of 721 lipids from rat peritoneal surface layers was reported using 2-dimensional (normal-reversed phase) LC–ESI-MS-MS methods with the addition of 5 mM ammonium formate; however, the process required a long separation time and had a relatively high LOD of 55–65 fmol [32]. Moreover, the addition of 5 mM ammonium acetate in the nLC-ESI–MS analysis in negative ion mode of PLs from cerebrospinal fluid of Alzheimer's disease patients yielded relatively high LODs of 8.44 fmol for PC and 5.79 fmol for SM species [20]. While these studies demonstrated the utility of ionization modifiers for the analysis of neutral lipids in negative ion mode, a systematic study investigating the effect of modifiers on the ionization efficiency of all PL classes during LC–ESI-tandem MS analysis has not been carried out. Moreover in our preliminary experiments, the ionization efficiency of anionic PLs was found to decrease to some degree when ammonium acetate or ammonium formate was utilized.

In this study, an analytical method to analyze PL mixtures with nLC-ESI–MS-MS in negative ion mode in a single run was investigated by examining the effect of modifiers on the ionization efficiency of various PL species. This study compared the ionization efficiency of 13 different PL and LPL standards in nLC-ESI–MS-MS varying the ionization modifiers and their concentrations. These experiments suggested optimum conditions of the modifier mixture, which gave comparable sensitivities for both neutral and anionic PL species. The developed method with a mixed modifier was applied to a human urine sample to compare the identification of urinary PLs from two separate (both negative and positive runs) nLC-ESI–MS^2 runs.

2. Experimental

2.1. Materials and urine sample

Thirty-three PL standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and are listed in Tables 1 and 2. PL standards were dissolved in a solvent mixture (1:3:4 (v/v) H_2O:$CHCl_3$:CH_3OH) and were diluted with 1:1:1 (v/v) H_2O:CH_3OH:CH_3CN to give a concentration of each standard around 1 pmol/μL. All solvents used in this study (CH_3CN, isopropanol, CH_3OH, H_2O, $CHCl_3$) were HPLC grade. Ionization modifiers added to the mobile phase were formic acid for positive ion mode and ammonium hydroxide, ammonium formate, and ammonium acetate for negative ion mode. A urine sample was obtained from a healthy male volunteer (age 25). Extraction of urinary PLs was accomplished by lyophilization followed by solvent extraction. 1 mL of the urine sample was placed in a 15 mL centrifuge tube and was frozen for 5 min in liquid nitrogen. The frozen urine was then transferred to a model Bondiro MCFD 8508 freeze dryer with concentrator from Ilshin Lab Co. (Yangju, Korea) for 12 h to evaporate the liquid phase prior to solvent extraction. The resulting urine powder was dispersed into 0.90 mL of 2:1 (v/v) $CHCl_3$:CH_3OH by vortexing and then left for 1 h at room temperature. After adding 0.18 mL of H_2O to the mixture, the tube was centrifuged at 4,000 rpm for 10 min. The lower phase was retrieved and dried using a SpeedVac. The final extract was dissolved in CH_3OH/CH_3CN (1:1) to give a final volume of 500 μL and was stored at −20 °C. For nLC-ESI-MS-MS analysis, the final lipid solution in storage was diluted to a concentration of 10 μg/μL with 1:1:1 (v/v) CH_3CN/CH_3OH/H_2O. Recovery rate of PLs based on the same extraction method using $CHCl_3$ alone was reported as 94–99% for PC and PE, and 65–100% for PA,PS, PI, and PG [33].

Capillary tubes with 20, 50, and 75 μm i.d. (all have 360 μm o.d.) were purchased from Polymicro Technology LLC (Phoenix, AZ, USA). Packing materials for the capillary LC column were reversed phase resin Magic C_{18}, 3 μm–100 Å from Michrom Bioresources Inc. (Auburn, CA, USA).

2.2. Nanoflow LC–ESI-MS^n

An LTQ Velos ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA) equipped with a model 1200 capillary pump system and an autosampler from Agilent Technologies (Palo Alto, CA, USA) was utilized to analyze PLs. Capillary LC columns were prepared in our laboratory by packing a methanol slurry of RPLC resin into 75 μm-i.d. capillary tubing under a constant pressure (1000 psi) of He. Before packing, one end of the capillary tube was pulled using a flame to create a sharp needle (tip i.d. ~10 μm) for direct ESI without an emitter. The length of the column was 5 cm. The beginning end of the capillary column was connected with the pump flow via a PEEK microcross from Upchurch Scientific (Oak Harbor, WA, USA) while the other two ports were connected with Pt wire for the ESI electrical source and with a vent capillary tube (20 μm i.d., 360 μm o.d.). The vent capillary

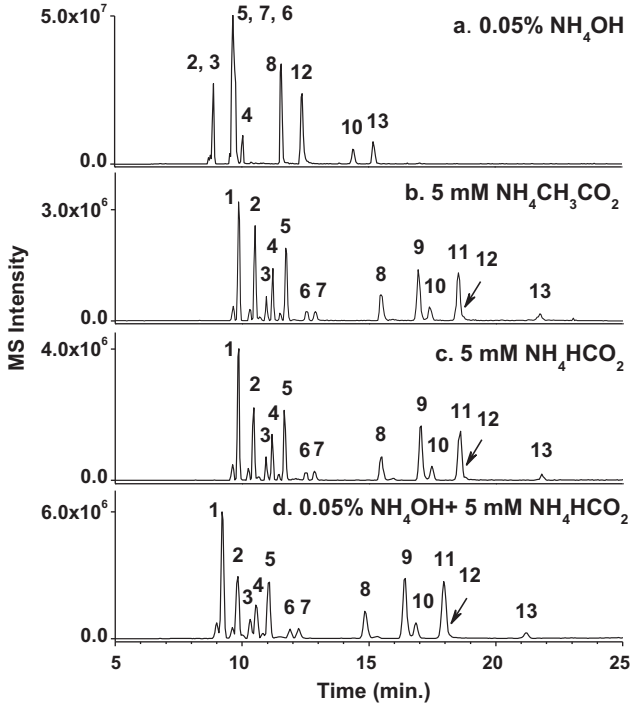

Fig. 1. BPCs (Base peak chromatograms) of 13 different PL standards from nLC-ESI–MS-MS (negative ion mode only) using different modifiers added to the mobile phase under gradient elution program I. Sample No. 1: 12:0-LPC, 2: 14:0-LPG, 3: 18:0-LPA, 4: 14:0-LPE, 5: 18:1-LPI, 6: 12:0/12:0-PA, 7: 18:0-LPS, 8: 12:0/12:0-PG, 9: 12:0/12:0-PC, 10: 12:0/12:0-PE, 11: d18:1/12:0-SM, 12: 14:0/14:0-PS, 13: 16:0/18:2-PI.

Table 1
Observed m/z value of $[M-H]^-$ ion, retention time, and concentration of each standard in Fig. 3 and calculated LOD value of 13 standard species from nLC-ESI-MS in negative ion mode.

No.	Class	Acyl chain	m/z	t_r (min)	Conc. (pmol/μL)	LOD (fmol, $n=3$)	R^2
1	LPA	14:0	381.3	11.24[a], 11.58[b]	1.20		
2	LPG	12:0	455.3	14.32[a], 14.79[b]	1.04	0.25 ± 0.07	0.999
3	LPC	12:0	484.2[c]	14.98[a], 15.67[b]	1.04	2.07 ± 0.24	0.989
4	LPA	18:0	437.3	16.29[a], 16.79[b]	1.12	5.07 ± 0.39	0.998
5	LPI	18:1	597.3	17.26[a], 17.89[b]	1.10	0.32 ± 0.05	0.991
6	LPE	14:0	424.2	17.42[a], 18.19[b]	1.04	0.30 ± 0.05	0.996
7	LPS	18:0	524.4	18.96[a], 19.56[b]	1.10	0.43 ± 0.09	0.997
8	LPG	18:0	511.4	20.16[a], 20.68[b]	1.09		
9	PA	12:0/12:0	535.5	21.00	1.08	9.01 ± 0.30	0.995
10	LPC	16:0	540.3[c]	21.93[a], 22.62[b]	1.09		
11	PG	12:0/12:0	609.5	24.19	1.03	0.70 ± 0.06	0.999
12	LPE	18:0	480.3	24.24[a], 24.97[b]	1.06		
13	PS	14:0/14:0	678.5	28.15	1.08	5.02 ± 0.32	0.993
14	PE	12:0/12:0	578.3	29.14	1.09	0.69 ± 0.01	0.992
15	PC	12:0/12:0	666.2[c]	29.87	1.09	1.73 ± 0.34	0.990
16	PI	16:0/18:2	833.4	32.31	1.01	7.87 ± 0.13	0.998
17	SM	d18:1/12:0	691.3[c]	32.70	1.04	2.59 ± 0.57	0.991
18	PA	16:0/16:0	647.2	33.79	1.09		
19	SM	d18:0/12:0	693.3[c]	33.97	1.08		
20	PG	16:0/16:0	721.6	34.25	1.07		
21	SM	d18:1/16:0	747.4[c]	39.27	1.00		
22	PC	14:0/16:0	750.3[c]	39.42	1.06		
23	PS	18:0/18:0	790.4	39.60	1.09		
24	SM	d18:1/18:1	773.5[c]	39.73	1.06		
25	PG	18:0/18:0	777.7	39.93	1.08		
26	PA	18:0/18:0	703.6	41.10	1.09		
27	PE	16:0/16:0	690.5	42.05	1.08		
28	SM	d18:1/18:0	775.5[c]	42.52	1.07		
29	PC	16:0/16:0	778.5[c]	42.52	1.19		
30	PE	18:0/18:0	746.6	47.89	1.07		
31	PC	18:0/18:0	834.6[c]	48.31	1.14		
32	SM	d18:1/24:0	859.6[c]	51.04	1.03		
33	PC	20:0/20:0	890.7[c]	52.73	1.06		

[a] Lyso/acyl-LPL.
[b] Acyl/lyso-LPL.
[c] m/z of $[M+HCOO]^-$.

tube was connected to an on-off valve, which was turned to the on position to split the pump flow during the LC run. The sample was loaded directly on to the capillary column at a flow rate of 0.6 μL/min with the on-off valve in the off position. After loading, gradient elution was begun with a pump flow rate of 11 μL/min and the on-off valve in the on position. Under these conditions, only 300 nL/min of flow was delivered to the capillary column for separation, and the rest of the flow exited through the vent tube. Using a much higher pump rate (11 μL/min), the dwell time could be minimized.

Mobile phase solutions for binary gradient elution were 9/1 (v/v) dH_2O/CH_3CN for mobile phase A and 2:3:5 (v/v) CH_3OH/CH_3CN/isopropanol for mobile phase B. For positive ion mode, 0.1% formic acid was added to both mobile phases. For negative ion mode, three different modifiers in varying concentrations were used: ammonium formate, ammonium acetate, and ammonium hydroxide. Gradient elution program I began with 100% A, ramped to 50% B for 1 min, increased to 85% B over 15 min, and then to 100% B over 15 min. Gradient program II began with 100% A, ramped to 40% B for 1 min, increased to 80% B over 20 min, to 100% B over 25 min, and then maintained at 100% B for 15 min. The injection volume was fixed at 1 μL. The ESI voltage was 3.0 kV, and the MS capillary temperature was 200 °C. Precursor scans for the MS ranged from 350 to 950 amu. To achieve CID, data-dependent MS^n analysis mode was used at a collision energy of 40%. For most of the PL species, MS–MS analysis was carried out for three prominent ions from each precursor scan. For LPC, PC, and SM species in negative ion mode, MS-MS-MS analysis was carried out. Identification of urinary PLs was performed with the LiPilot software developed in our laboratory and was confirmed by manual examination.

3. Results and discussion

The effect of ionization modifiers on the ionization efficiency and the simultaneous characterization of all phospholipid classes by nLC-ESI–MS-MS in negative ion mode has been examined using four different types of modifiers added to mobile phase solutions. Fig. 1 shows a comparison of the base peak chromatograms (BPCs) of 13 different classes of PL species (six different head groups of PL and LPL along with one SM) obtained by nLC-ESI–MS using different modifiers: (a) 0.05% NH_4OH (AH, pH 10.5), (b) 5 mM $NH_4CH_3CO_2$ (AA, pH 8.1), (c) 5 mM NH_4HCO_2 (AF, pH 7.8), and (d) 0.05% NH_4OH + 5 mM NH_4HCO_2 (pH 8.8). As shown in Fig. 1a, PL and LPL species eluted in increasing order of hydrophobicity depending on the type of head group, acyl chain length, and degree of unsaturation; PL species with shorter acyl chains or more double bonds eluted earlier than those with greater saturation and longer chains. However, neutral polar PLs such as LPC (peak No. 1), PC (No. 9), and SM (No. 11) were not apparent (very weak signals) since formation of negative ions from these neutral lipids with 0.05% NH_4OH was not favorable. When the modifier was changed to the weakly basic salts shown in Fig. 1b–d, 5 mM $NH_4CH_3CO_2$ (AA, pH 8.1), 5 mM NH_4HCO_2 (AF, pH 7.8), and a mixture of 0.05% NH_4OH and 5 mM NH_4HCO_2 (pH 8.8), ionizations of species No. 1, 9, and 11 were successful with the formation of adduct ions ($[M+CH_3CO_2]^-$ with AA or $[M+HCO_2]^-$ with AF) from the phosphocholine moiety. Using weakly basic modifiers, the retention times of all species increased to some degree. In addition, ethanolamine containing lipids such as LPE (No. 4) and PE (No. 10) eluted earlier than peak 5 and 12, respectively(see retention times listed in Supplementary Table 1) because weakly anionic PE and LPE species became electrically neutral in

Table 2
Phospholipids identified from a healthy human urine sample obtained from nLC-ESI–MS^2 (-MS^3 for PC's, LPC's, SM's) in negative ion mode using a mixed modifier compared with results obtained from two separate runs in both positive and negative ion mode.

Class	Acyl chain	Two separate runs: positive ion mode		Single run with mixed modifier in negative ion mode		
		0.1% FA		0.05% AH + 1 mM AF		Relative peak area (%)
		m/z of $[M+H]^+$	t_r (min.)	m/z of $[M-H]^-$	t_r (min.)	
LPE	lyso/20:4	502.30	18.27	500.3	19.59	0.97
	20:4/lyso	502.30	18.97	500.3	20.89	1.08
	16:0/lyso	454.40	20.40	452.4	22.16	0.41
	18:1/lyso	480.30	21.19	478.3	22.80	0.65
LPC	14:0/lyso	468.40	18.07		N.D.	-
	lyso/18:2	520.40	18.57	564.4[a]	19.72	0.96
	16:0/lyso	496.40	19.63	540.3[a]	21.91	0.71
SM	d18:1/14:0	675.70	36.56	719.6[a]	36.79	0.37
	d18:1/16:0	703.80	39.43	747.6[a]	39.42	5.35
	d18:1/18:0	731.70	42.56	775.6[a]	42.58	0.56
	d18:1/20:0	759.60	45.28	803.7[a]	45.50	1.21
	d18:1/24:2	811.80	46.09	855.7[a]	46.05	0.50
	d18:1/22:0	813.80	48.23	831.7[a]	48.08	2.69
	d18:1/24:1	787.80	48.46	857.7[a]	48.25	1.84
	d18:1/23:0	801.80	49.89	845.8[a]	49.71	1.34
	d18:1/24:0	815.90	51.16	859.8[a]	50.94	1.97
PE	16:0/22:6	764.70	40.47	762.6	40.13	0.66
	16:0/20:4	740.70	41.05	738.6	40.55	1.98
	18:1/22:6		N.D.	788.6	40.61	0.11
	18:1/20:4	766.60	41.37	764.6	40.71	1.71
	16:0/18:2	716.60	41.46	714.6	40.86	1.36
	18:1/18:2	742.50	42.75	740.6	41.11	1.45
	16:0/18:1	718.60	43.60	716.6	42.76	0.85
	18:1/18:1	744.70	44.02	742.6	43.15	1.25
	18:0/20:4	768.70	44.17	766.6	43.27	3.33
	18:0/18:2	744.70	44.38	742.6	43.61	1.69
	18:0/18:1	746.80	46.55	744.6	45.71	0.96
PC	16:0/22:6	806.60	39.94	850.6[a]	39.30	0.22
	18:2/18:2	782.70	40.49	826.6[a]	40.27	0.20
	16:0/20:4	782.60	40.49	826.6[a]	40.68	0.62
	16:0/18:2	758.70	40.77	802.7[a]	40.91	1.81
	18:1/16:1	758.70	40.85	802.7[a]	40.94	0.52
	18:0/20:5	808.60	41.91	852.6[a]	41.04	0.26
	18:1/18:2	784.70	41.18	828.7[a]	41.21	0.91
	16:0/16:0	734.70	42.57	778.6[a]	42.69	0.47
	16:0/18:1	760.70	42.83	804.6[a]	42.86	1.26
	18:0/20:4	810.60	43.20	854.6[a]	43.33	0.37
	18:1/18:1	786.70	43.60	830.7[a]	43.63	0.68
	18:0/18:2	786.70	43.71	830.7[a]	43.75	0.89
	18:0/18:1	788.60	45.75	832.6[a]	45.86	0.39
	18:0/22:4	830.60	45.97	874.6[a]	45.90	0.26

Class	Acyl chain	Two separate runs: negative ion mode		Single run with mixed modifier in negative ion mode		
		0.05% AH		0.05% AH + 1 mM AF		Relative peak area (%)
		m/z of $[M-H]^-$	t_r (min.)	m/z of $[M-H]^-$	t_r (min.)	
LPA	lyso/16:0	409.5	11.55	Similar to the left	13.99	5.81
	lyso/18:0	437.5	14.42		16.44	0.84
	18:0/lyso	437.5	14.99		16.70	3.88
LPG	22:6/lyso	555.4	10.46		15.91	0.33
	20:4/lyso	531.3	14.14		19.75	0.12
	18:0/lyso	511.3	15.24		20.49	1.25
LPS	lyso/18:0	524.4	17.69		19.26	0.85
LPI	18:0/lyso	599.4	19.24		20.60	0.17
PA	16:0/16:0	647.6	31.19		33.20	0.77
	18:1/18:2	697.3	31.57		34.66	0.81
	18:0/22:6	747.5	33.41		36.43	5.35
	18:1/18:1	699.5	34.30		37.00	0.55
	18:0/18:2	699.5	34.52		37.06	0.61
	20:1/18:1	727.6	40.08		40.08	0.26
PS	16:0/20:4	782.5	25.04		34.11	0.94
	16:0/18:1	760.6	26.38		35.85	3.95
	18:0/20:4	810.6	27.16		36.07	4.06
	18:1/18:1	786.6	27.25		36.12	0.41
	20:4/18:0	810.6	27.32		36.53	0.60

Table 2 (*continued*)

Class	Acyl chain	Two separate runs: negative ion mode		Single run with mixed modifier in negative ion mode		
		0.05% AH		0.05% AH + 1 mM AF		Relative peak area (%)
		m/z of $[M-H]^-$	t_r (min.)	m/z of $[M-H]^-$	t_r (min.)	
	18:0/18:2	786.6	27.43		36.66	2.50
	18:0/18:1	788.6	28.67		38.26	10.59
	20:0/18:1	816.6	30.95		41.72	0.55
PI	16:0/20:4	857.6	27.50		34.29	0.32
	18:1/20:4	883.6	27.77		34.56	0.15
	16:0/20:3	859.6	28.54		35.11	0.10
	18:1/20:3	885.5	28.71		35.22	0.04
	16:0/16:0	809.6	28.84		35.47	0.29
	16:0/18:1	835.6	28.76		35.78	0.49
	18:1/16:0	835.6	29.11		36.11	0.15
	18:0/22:6	909.6	29.67		36.22	0.16
	18:0/16:1	835.6	29.56		36.48	0.06
	18:0/20:4	885.6	29.83		36.55	2.78
	18:1/18:1	861.6	29.98		36.65	0.15
	18:0/18:2	861.6	30.08		36.75	0.34
	18:0/20:3	887.6	30.24		37.47	0.75
	16:0/18:0	837.6	30.35		38.11	0.73
	18:0/16:0	837.6	30.44		38.14	0.49
	18:0/18:1	863.6	31.02		38.40	0.96
	18:0/20:2	889.6	31.58		38.78	0.17
PG	22:6/22:6	865.6	30.22		32.09	0.29
	18:2/22:6	817.6	30.53		32.21	0.27
	18:2/20:4	793.6	30.70		32.81	0.09
	18:1/22:6	819.6	31.32		33.46	0.51
	22:6/18:1	819.6	31.64		33.74	0.14
	18:0/18:1	775.6	38.25		39.20	0.56

[a] m/z of $[M+HCOO]^-$.

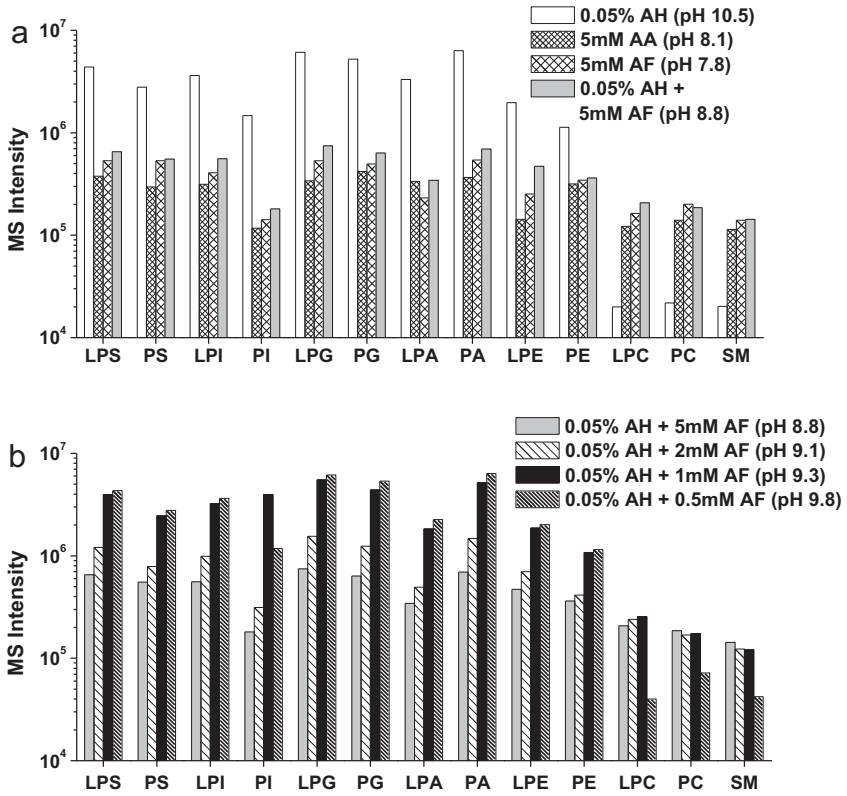

Fig. 2. (a) The effect of modifiers on the ionization efficiency of 13 different classes of phospholipids during ESI-MS (shown in Fig. 1) and (b) the effect of AF concentration in 0.05% AH solution on ionization efficiency. All runs were performed using ESI-MS in negative ion mode. AA, ammonium acetate, AF, ammonium formate and AH, ammonium hydroxide.

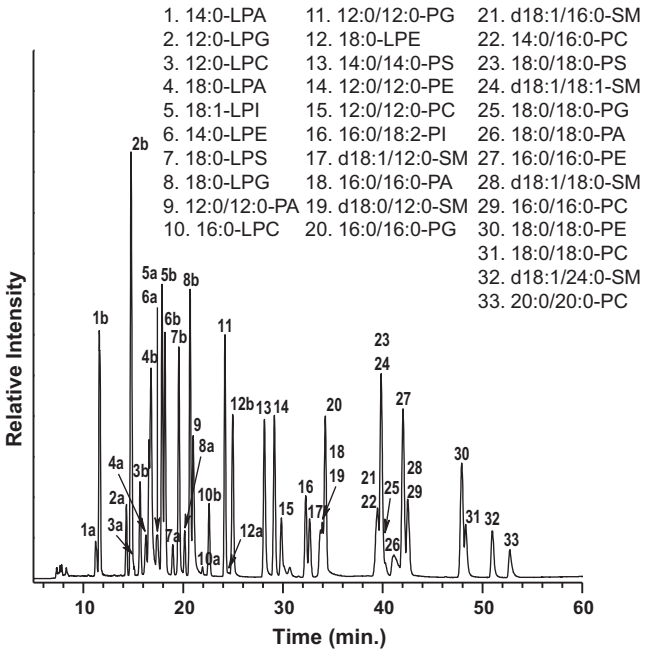

Fig. 3. BPCs of 33 PL standard mixtures from nLC-ESI-MS in negative ion mode.

these weakly basic solutions. The ionization efficiency of the standard species upon addition of different modifiers was compared by plotting the MS intensity (based on peak area) of each peak as shown in Fig. 2a. Ionization of anionic PL species (from LPS to PE species on the *x*-axis of Fig. 2a) appeared more efficient with 0.05% AH than with the other modifiers, while ionization of neutral polar species (LPC, PC, and SM) was very poor with 0.05% AH. While the use of 0.05% AH provided better ionization of anionic lipids in negative ion mode, high pH condition caused a reduced lifetime of stationary phase of RPLC column. When 5 mM AA or 5 mM AF was utilized as the modifier, the ionizations of neutral polar species were greatly enhanced (>10^5 in intensity), however ionizations of anionic lipids decreased about 1 order of magnitude for most species. In order to increase the ionization efficiency of anionic lipids, 0.05% AH was added to 5 mM AF, resulting in a slight increase in the peak intensities of all species. However, with a decrease in the concentration of AF in the mixed modifier solution, shown in Fig. 2b, ionization efficiencies of anionic lipids were found to significantly increase while those of neutral polar lipids were maintained until the concentration of AF was reduced to 1 mM. Further reduction to 0.5 mM AF with 0.05% AH, significantly decreased the ionization efficiency of neutral polar lipids. From these observations, the mixed modifier of 0.05% AH plus 1 mM AF (pH 9.3) was selected as optimal for the simultaneous analysis of PLs containing any of the six different head groups. For the case of neutral PLs, the molecular structure of each species required identification by a data dependent MS^3 experiment since the MS^2 experiment of the precursor ion ($[M+HCOO]^-$) produced predominantly the $[M-CH_3]^-$ ion, which could be followed by MS^3 yielding characteristic fragment ions. The types of typical fragment ions of each species along with experimental retention time, observed *m*/*z* values for each of the modifier conditions are listed in Supplementary Table 1.

PL separation with the mixed modifier (0.05% AH + 1 mM AF) was further fine-tuned to find more efficient gradient run conditions to accommodate the broad spectrum of PL mixtures typically originating from biological samples. Fig. 3 shows a separation of 33 PL standards, which included fast eluting to long retaining species, with a modified gradient run condition (refer to program II in the experimental section) by nLC-ESI–MS-MS in negative ion mode (total ion chromatogram (TIC) is inserted in Supplementary Fig. 1). Notably, the regioisomers of LPL species, labeled a and b after each species' number, were clearly separated with this run condition. For instance, 2a and 2b are peaks from lyso/12:0-PG and 12:0/lyso-PG, respectively, in which lyso/12:0-PG represents the hydroxyl group attached to the sn-1 carbon of the glycerol backbone. The difference between the retention times of these regioisomers is due to the geometrical differences of the regioisomers with the hydroxyl group at the sn-2 carbon having a more extended structure to interact with the stationary phase, as was reported previously [34]. Table 1 lists the observed *m*/*z*, retention time, and concentration of 33 standard species utilized in Fig. 3 along with the limit of detection (LOD) values calculated for the 13 representative PL species (utilized for Fig. 1). The LOD value of PC in Table 1 was calculated to be 1.73 fmol, which was close to or slightly lower than the previously reported value (2.2 fmol for PC [16]) in positive ion mode. The LOD value of PG in negative ion mode was found to be 0.7 fmol, which was similar to the reported value (1 fmol) [17]. While PA, PI, and PS exhibited relatively high LOD values (9.01–5.02 fmol), some species like LPG, PG, LPE, and PE showed sub fmol levels of LOD. These results support the mixed modifier conditions being utilized for the simultaneous analysis of PL mixtures in negative ion mode.

The run conditions used in Fig. 3 were applied to the separation of a human urinary extract sample, and the results were compared with two separate nLC-ESI–MS-MS runs performed in both positive and negative ion mode using specific modifiers as has been previously utilized. Chromatograms are compared in Supplementary Fig. 2, which shows BPC's of nLC-ESI–MS-MS runs in (a) positive ion mode with 0.1% HCO_2H for detection of PC's, PE's, and SM, (b) negative ion mode with 0.05% NH_4OH for PA's, PG's, PS's, and PI's, and (c) negative ion mode with 0.05% NH_4OH + 1 mM NH_4HCO_2 for all PL species. Identification of the PL molecular structures was readily carried out using the characteristic fragment ion patterns listed in Supplementary Table 1. However for PC and SM species, data dependent MS^3 analysis was carried out to obtain typical fragment ions due to the formation of adduct. The first MS scan at t_r = 42.58 min (labeled with a star in Supplementary Fig. 1c) of the nLC run obtained with the mixed modifier in negative ion mode is shown in Fig. 4a together with other prominent precursor ions. The precursor ion at *m*/*z* 775.6 was found to be the formate adduct ion $[M+HCO_2]^-$ of which the first CID yielded an exclusively intense ion peak at *m*/*z* 715.8 in Fig. 4b. This was found to be $[M-CH_3]^-$ since the *m*/*z* difference between the precursor ion and the first product ion was 60 Da. In this case, the MS system was programmed to carry out the second CID (MS^3) experiment, resulting in the fragment ion spectra in Fig. 4c with characteristic ions to identify the molecular structure. Fragment ions at *m*/*z* 626.6 and 644.5 originated from dissociation of the choline group from $[M-CH_3]^-$ in the form of $-CH_2CHN(CH_3)_2$ with and without loss of the hydroxyl group by dehydration, respectively. These were typical findings of SM molecules as found from literature [29,35], whereas such fragments were not commonly found with PC (see later). While the SM molecule did not produce fragment ions from neutral loss of the N-acyl chain, loss of the acyl chain as ketene and carboxylic acid produced ions of *m*/*z* 449.3 for $[M-CH_3-R'CH{=}C{=}O]^-$ and 431.3 for $[M-CH_3-RCOOH]^-$ along with free carboxylate ion $[RCOO]^-$ at *m*/*z* 283.4 as found in literature [36,37]. From these fragment ions, the ion at *m*/*z* 775.6 was identified as originating from d18:1/18:0-SM. A similar scheme to identify PC molecules in negative ion mode through formate adducts is shown in Fig. 4d and e. PC species produced prominent fragment ions of *m*/*z* 744.6 in Fig. 4d differing by 60 Da from the precursor ions (*m*/*z* 804.6 in Fig. 4a), and the resulting MS^3 experiments of

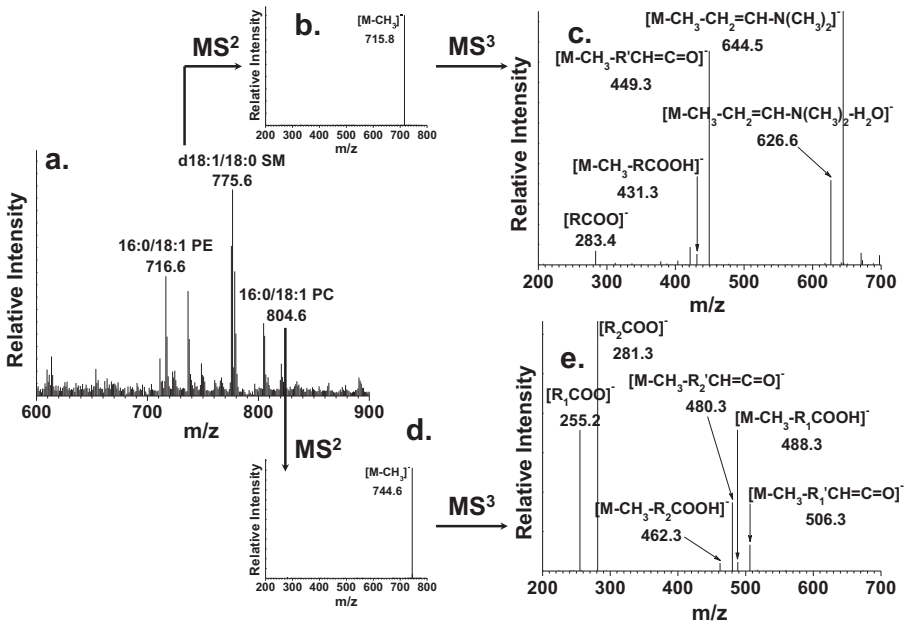

Fig. 4. (a) MS scan of t_r = 42.58 min from the nLC-ESI–MS-MS run of a human urine sample in Supplementary Fig. 1c showing few parent ion spectra in negative ion mode, (b) the first CID (MS^2) spectra of the parent ion m/z 775.6 in the MS scan showing a prominent fragment ion, $[M-CH_3]^-$, (c) MS^3 spectra of m/z 715.8 showing the typical fragment ion spectra, resulting in the identification of d18:1/18:0-SM, (d) MS^2 spectra of m/z 804.6 from the precursor MS scan, and (e). MS^3 spectra of m/z 744.6 leading to the identification of 16:0/18:1-PC.

the first product ion yielded the neutral loss of acyl chains from $[M-CH_3]^-$ in the form of ketene and carboxylic acid. In addition, the relative abundance of the fragment ions from the loss of acyl chain as a form of ketene ($[M\text{-}CH_3\text{-}R_1'CH{=}C{=}O]^- < [M\text{-}CH_3\text{-}R_2'CH{=}C{=}O]^-$) supported the location of the acyl chains. Structural identification from the raw MS spectra yielded a total of 85 PL species under the mixed modifier conditions, which was the same number of species identified by the conventional method using two runs in both positive and negative ion mode. While PC and SM produced formate adducts which required MS^3 experiments, anionic PLs did not generate adduct and showed fragment ions enough to determine molecular structures with MS^2 in which structural identification was similarly achieved as reported in literature [17,18,34]. Table 2 lists the experimental retention times and the observed m/z values of identified urinary PL species obtained by the two different methods: two separate runs in both positive and negative ion mode vs. a single run in negative ion mode with the mixed modifier. PL species identified from the single run with the mixed modifier were almost identical to those identified from the two separate runs with the exception of 14:0/lyso-PC, which was not identified.

4. Conclusions

This study describes the utility of a mixed modifier in nLC-ESI–MS^n for the simultaneous analysis of all classes of PLs in negative ion mode. Since neutral PLs (PC's and SM's) are not readily ionized in negative ion mode with ammonium hydroxide alone and the ionization efficiency of anionic PLs with ammonium formate alone was not as high as with ammonium hydroxide, a mixed modifier (0.05% ammonium hydroxide plus 1 mM ammonium formate) was found to be effective in the analysis of PL mixtures without losing ionization efficiency. With the selection of effective modifier conditions, analysis of a PL mixture can be operated in a single ionization mode, which reduces the analysis time and obviates the need for solvent exchange in nLC-ESI–MS^n.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2012.03.073.

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