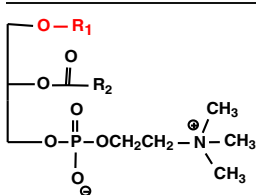


RESEARCH ARTICLE

Structural Distinction of Diacyl-, Alkylacyl, and Alk-1-Enylacyl Glycerophosphocholines as $[M - 15]^-$ Ions by Multiple-Stage Linear Ion-Trap Mass Spectrometry with Electrospray Ionization

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R₁ = COR'; diacyl-PC
CH=CH-R₁'; plasmeryl-PC
CH₂CH₂R₁'; plasmanyl-PC

Abstract. We describe a linear ion-trap (LIT) multiple-stage (MS^n) mass spectrometric approach towards differentiation of alkylacyl, alk-1-enylacyl- and diacyl-glycerophosphocholines (PCs) as the $[M - 15]^-$ ions desorbed by electrospray ionization (ESI)-in the negative-ion mode. The MS^n mass spectra of the $[M - 15 - R^2, CH = CO]^-$ ions originated from the three PC subfamilies are readily distinguishable, resulting in unambiguous distinction of the lipid classes. This method is applied to two alkyl ether rich PC mixtures isolated from murine bone marrow neutrophils and kidney, respectively, to explore its utility in the characterization of complex PC mixture of biological origin, resulting in the realization of the detailed structures of the PC species, including various classes

and many minor isobaric isomers.

Key words: Phosphatidylcholine, Plasmerylcholine, Plasmanylcholine, Linear ion-trap mass spectrometry, Bone marrow neutrophil, Kidney

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Introduction

Ether-linked glycerophosphocholines consist of plasmanylcholines and plasmerylcholines, which have a 1-O-alkyl or a 1-O-alk-1'-enyl group bonded to the *sn*-1 position of the glycerol backbone, respectively, and a fatty acyl group attached to the *sn*-2 position of the glycerol backbone by an ester bond. Alkyl ether glycerophosphocholines are thought to play important roles in cellular signaling. For example, platelet-activating factor (PAF) is a potent phospholipid activator and mediator of many leukocyte functions, including platelet aggregation and degranulation, inflammation, and anaphylaxis [1]. Recently, fatty acid synthase (FAS)-dependent alkyl ether glycerophosphocholine species were shown to be associated with PPAR γ and treatment of 3T3-L1 cells with one such ether lipid increased PPAR γ transcriptional activity [2]. PexRAP, a

protein required for alkyl ether lipid synthesis, was associated with peroxisomes and induced during adipogenesis, and inhibiting PexRAP appears to improve diabetes in mice [2].

The differentiation of a plasmeryl glycerophospholipid (GPL) (plasmalogen) from a plasmanyl GPL by tandem mass spectrometry has been a difficult task because of the structural similarity between the two classes of compounds. Several mass spectrometric approaches have been employed. For examples, Murphy et al. described the confirmation of the plasmalogen glycerophosphoethanolamine (PE) (plasmylethanolamine) molecules in lipid mixture by comparison of the mass spectra of the lipid mixtures before and after the removal of plasmalogen PE by acid treatment [3, 4]. The approach is simple but destructive and resulted in a substantial sample loss. A reaction strategy involving selective modification of the *O*-alkenyl-ether double bond within plasmalogen lipids to separate the isobaric plasmeryl- and plasmanyl-ether GPLs by the mass shifts resulting from derivatization was recently reported for differentiation the two lipid classes [5].

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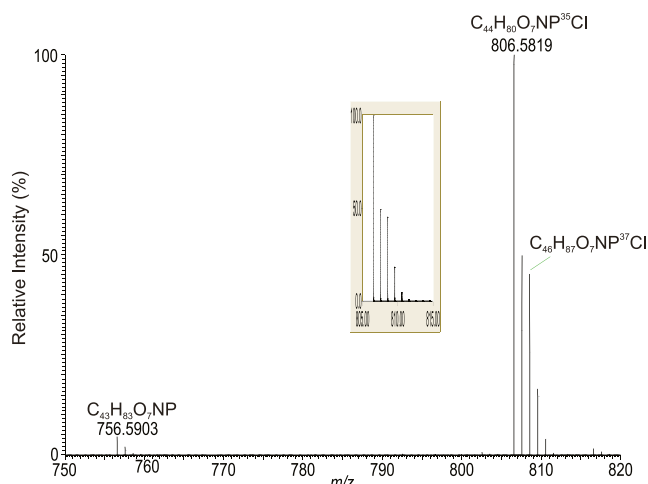


Figure 1. The high resolution ($R = 100,000$ at m/z 400) ESI-MS spectrum of the $[M + Cl]^-$ ions of $p18:0/18:1$ -PC. The theoretical plot (using the software downloaded from <http://www.alchemismatt.com/>) from the elemental composition ($C_{44}H_{86}O_7NPCl$) is shown in “inset,” which gives an identical profile

We previously described tandem quadrupole mass spectrometric approaches for differentiation of plasmanyl and plasmenyl PC as lithiated adduct ions [6]. We also described linear ion-trap mass spectrometric approaches applying MS^3 and MS^4 toward structural characterization of 1-O-alk-1'-enyl-2-acyl-, 1-O-alkyl-2-acyl-, and diacyl-glycerolphospholipids (GPL) as the $[M - H]^-$ ions desorbed by ESI in negative-ion mode [7]. These LIT MS^n mass spectrometric approaches for structural differentiation are applicable to all the GPL classes that readily form the $[M - H]^-$ ions excepting glycerophosphocholines, which consist of a choline head group that precludes the formation of the $[M - H]^-$ ion.

Notably, PC forms $[M - 15]^-$ (together with $[M - 86]^-$ and $[M - 60]^-$) ions, when subjected to FAB ionization in the negative ion mode [8]. The $[M - 15]^-$ ions are also

formed during electrospray ionization in the negative-ion mode in the presence of anionic species (X^-) that give rise to $[M + X]^-$ adduct ions ($X = Cl, HCO_2, CH_3CO_2, \text{etc.}$) and sequentially undergo demethylation to remove a CH_3X residue upon CAD [9–12]. Here, we exploited the utility of $[M - 15]^-$ ions of PC to characterize the structures, employing high resolution linear ion-trap (LIT) multiple-stage (MS^n) mass spectrometry. This LIT MS^n mass spectrometric approach utilizing the $[M - 15]^-$ ions of PCs provides a powerful tool to readily distinguish the diacyl-, plasmanyl-, and plasmenyl-PC molecules. The application of this mass spectrometric approach to characterize two biological PC mixtures with rich alkyl ether PCs is also presented.

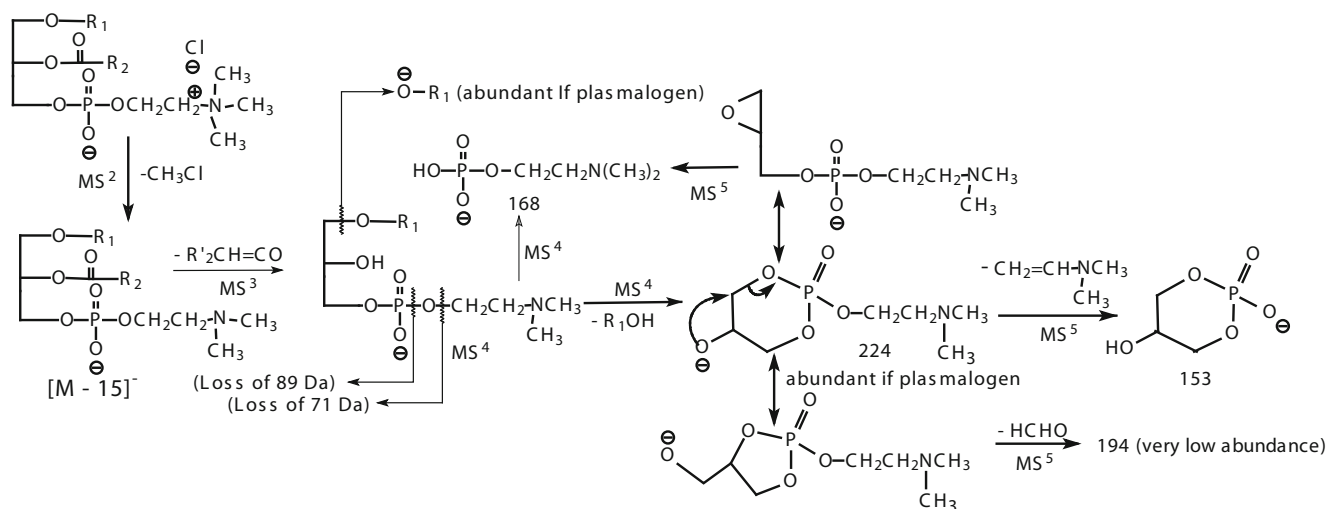
Materials and Methods

Materials

Standard GPC species with defined fatty acid substituents were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Solvents and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation of Bone Marrow Neutrophils and Lipid Extraction

Bone marrow was harvested from the femurs and tibias of 12-wk-old mice by flushing bones with cold phosphate-buffered saline. Marrow was washed, triturated using a 24-gauge needle, collected by centrifugation at 1250 rpm for 4 min, and diluted with phosphate-buffered saline. After lysis of erythrocytes using $1 \times$ Red Blood Cell Lysis Buffer (eBioscience, San Diego, CA, USA), neutrophils (1×10^6) were isolated by negative selection using the EasySep Mouse Neutrophil Enrichment kit (Stemcell Technology, Vancouver, Canada) as described by the manufacturer. Lipid from neutrophils was extracted by



Scheme 1. Fragmentation pathways (MS^n) proposed for alkyl ether PC ($[M - 15]^-$ ions)

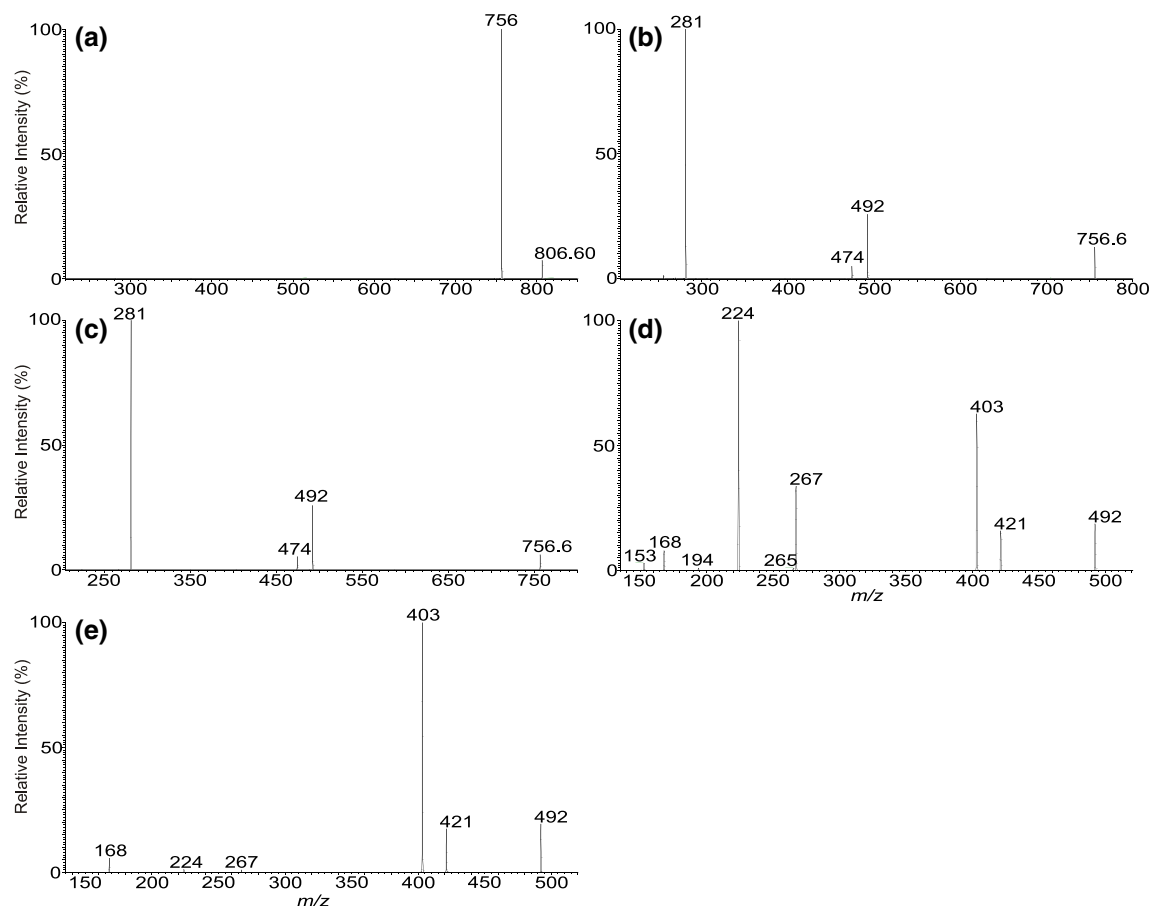


Figure 2. The MS² spectrum of the [M + Cl]⁻ ions of p18:0/18:1-PC at m/z 806.6 (a), its MS³ spectrum of the ion of m/z 756.6 (806→756) (b), the MS³ spectrum of the [M - 15]⁻ ion of a18:1/18:1-PC at m/z 756.6 (806→756) (c), the MS⁴ spectra of the ions of m/z 492 (806→756→492) arising from p18:0/18:1-PC (d), and from a18:1/18:1-PC (e) isomers

the method of Bligh and Dyer with modification [13]. In brief, neutrophils were suspended in a 3 mL of 1/1/1 (v/v/v) chloroform/methanol/water, vortexed for 1 min, and centrifuged at 800×g for 5 min. Total lipid in organic layer was transferred to a 0.5 dram vial and dried under a stream of nitrogen. The dried sample was reconstituted in 200 μL of chloroform/methanol (1:1) for analysis.

Isolation of Lipids from Mouse Kidney

Fifty milligram of kidneys harvested from 6-wk-old C57BL/6J mice were homogenized using ice-cold non-detergent hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 100 mM DTT, protease, and phosphatase inhibitor cocktail). Lipids were extracted from the homogenate using the Bligh and Dyer method with modification as described above.

Mass Spectrometry

Both high-resolution (R=100,000 at m/z 400) and low-energy CID tandem mass spectrometry experiments were conducted on a Thermo Scientific (San Jose, CA,

USA) LTQ Orbitrap Velos mass spectrometer (MS) with Xcalibur operating system. Lipid extracts and PC standards (10 pmol/μL) in chloroform/methanol (2/1) were infused (1.5 μL/min) to the ESI source, where the skimmer was set at ground potential, the electrospray needle was set at 4.0 kV, and temperature of the heated capillary was 300°C. The automatic gain control of the ion trap was set to 5×10⁴, with a maximum injection time of 50 ms. Helium was used as the buffer and collision gas at a pressure of 1×10⁻³ mbar (0.75 mTorr). The MSⁿ experiments were carried out with an optimized relative collision energy ranging from 25% to 45% with an activation q value at 0.25, and the activation time at 10 ms to leave a minimal residual abundance of precursor ion (around 20%). The mass selection window for the precursor ions was set at 1 Da wide to admit the monoisotopic ion to the ion-trap for collision-induced dissociation (CID) for unit resolution detection in the ion-trap or high resolution accurate mass detection in the Orbitrap mass analyzer. Mass spectra were accumulated in the profile mode, typically for 3–10 min for MSⁿ spectra (n=2, 3, 4).

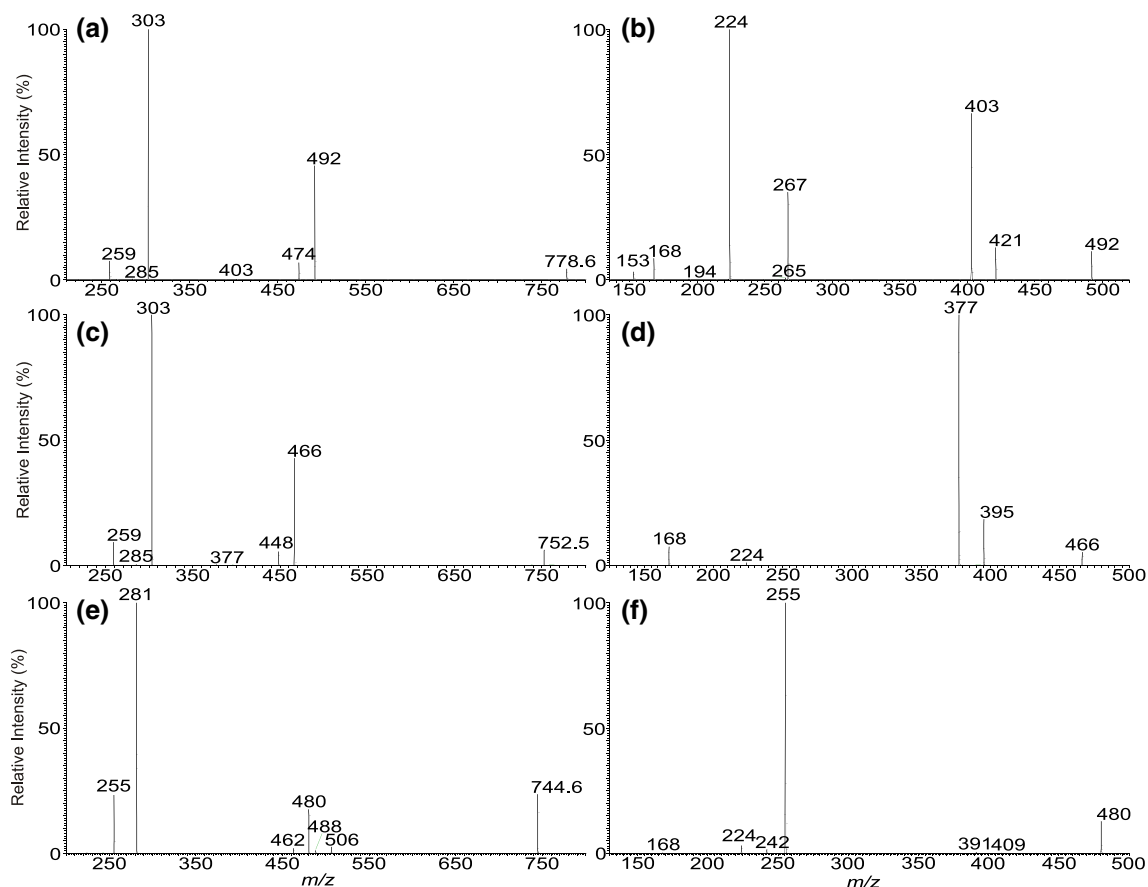


Figure 3. The MS³ spectrum of the [M – 15][–] ions of *p*18:0/20:4-PC standard at *m/z* 778.6 (828→778) (a), its MS⁴ spectrum at *m/z* 492 (828→778→492) (b), the MS³ spectrum of the [M – 15][–] ions of *a*16:0/20:4-PC standard at *m/z* 752.5 (802→752) (c), its MS⁴ spectrum at *m/z* 466 (802→752→466) (d), the MS³ spectrum of the [M – 15][–] ions of 16:0/18:1-PC standard at *m/z* 744.6 (794→744) (e), and its MS⁴ spectra of the ions of *m/z* 480 (794→744→480) (f)

Nomenclature

The abbreviations previously described for diacyl-, plasmanyl-, and plasmenyl (plasmalogen) glycerophospholine (PC) were used [7]. The diacyl PC, for example, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine is abbreviated as 16:0/18:1-PC. The 1-*O*-alk-1'-enyl-2-acyl glycerophospholipids (plasmalogens) and 1-*O*-alkyl-2-acyl glycerophospholipids (plasmanylcholine), for example, the 1-*O*-octadec-1'-enyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1-*O*-octadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine was designated as *p*18:0/18:1-PC and *a*18:0/18:1-PC, respectively. The *N,N*-dimethylphosphatidylethanolamine (DMPE) formed by elimination of CH₃X residue from the [M + X][–] ion of PC, for example, 1-*O*-octadecyl-2-oleoyl-*sn*-glycero-3-phosphoethanol dimethylamine, was designated as *a*18:0/18:1-DMPE.

Results and Discussion

PCs formed [M + X][–] adduct ions (X = Cl, HCO₂, CH₃CO₂, etc.) in the presence of anionic species (X[–]), when subjected to ESI in negative-ion mode. In this

study, all PC standards and extracts were dissolved in 2/1 methanol/chloroform, which provided abundant Cl[–] ions [14] that formed the [M + Cl][–] adduct ions with a unique ³⁵Cl/³⁷Cl pattern facilitating identification. For example, the mass spectrum of the [M + Cl][–] ions of *p*18:0/18:1-PC standard contained ions at *m/z* 806 and 808 (Figure 1) with an isotopic pattern similar to that of the simulated plot (inset, Figure 1). The spectrum also contained the ion at *m/z* 756 arising from loss of CH₃³⁵Cl/³⁷Cl residue (Scheme 1). By contrast, formation of [M + CH₃CO₂][–] adduct ions in the presence of CH₃CO₂[–] often resulted in a spectrum also consisting of the [M + Cl][–] ions that complicate the analysis (data not shown), due to the fact that Cl[–] ions are readily formed.

LIT MS^{*n*} with High Resolution Mass Spectrometry on Plasmenyl-, Plasmanyl-, and Diacyl PC Standards

The MS² spectrum of the [M + Cl][–] ion of *p*18:0/18:1-PC standard at *m/z* 806.6 is dominated by the ions at *m/z*

Table 1. The High Resolution Mass Measurements of the $[M - 15]^-$ and $[M + H]^+$ Ions of the PCs Isolated from Murine Bone Marrow Neutrophils and Their Assigned Structures

$[M - 15]^-$				$[M + H]^+$				Rel. Int. (%) ^a	Structures ^{b,c}
<i>m/z</i>	Theo. mass	Deviat. (mDa)	Composition	<i>m/z</i>	Theo. mass	Deviat. (mDa)	Composition		
702.5434	702.5443	-0.87	C39 H77 O7 N P	718.5746	718.5745	0.11	C40 H81 O7 N P	10.06	a16:0/16:1
704.5592	704.5600	-0.77	C39 H79 O7 N P	720.5903	720.5902	0.16	C40 H83 O7 N P	80.78	a16:0/16:0
718.5385	718.5392	-0.77	C39 H77 O8 N P	734.5697	734.5694	0.28	C40 H81 O8 N P	29.64	16:0/16:0
726.5436	726.5443	-0.74	C41 H77 O7 N P	744.5905	744.5902	0.38	C42 H83 O7 N P	100	a16:0/18:2; a18:2/16:0
728.5592	728.5600	-0.76	C41 H79 O7 N P	746.6061	746.6058	0.28	C42 H85 O7 N P	61.47	a18:1/16:0; a16:0/18:1
732.5903	732.5913	-0.98	C41 H83 O7 N P	748.6214	748.6215	-0.07	C42 H87 O7 N P	9.11	a18:0/16:0
740.5229	740.5236	-0.64	C41 H75 O8 N P	756.5536	756.5538	-0.19	C42 H79 O8 N P	3.31	16:1/18:2
742.5385	742.5392	-0.74	C41 H77 O8 N P	758.5693	758.5694	-0.11	C42 H81 O8 N P	81.95	16:0/18:2; 16:1/18:1
744.554	744.5549	-0.85	C41 H79 O8 N P	760.5848	760.5851	-0.27	C42 H83 O8 N P	39.73	16:0/18:1
752.5591	752.5600	-0.86	C43 H79 O7 N P	768.5900	768.5902	-0.2	C44 H83 O7 N P	36.91	a16:0/20:4; a18:2/18:2
754.5746	754.5756	-0.97	C43 H81 O7 N P	770.6056	770.6058	-0.22	C44 H85 O7 N P	36.46	a18:1/18:2; a16:0/20:3
756.5904	756.5913	-0.9	C43 H83 O7 N P	772.6213	772.6215	-0.2	C44 H87 O7 N P	27.28	a18:0/18:2; a18:0/18:1; a16:0/20:2; a20:2/16:0
766.5383	766.5392	-0.91	C43 H77 O8 N P	782.5694	782.5694	-0.07	C44 H81 O8 N P	33	16:0/20:4; 18:2/18:2
768.554	768.5549	-0.9	C43 H79 O8 N P	784.5849	784.5851	-0.17	C44 H83 O8 N P	40.49	18:1/18:2; 16:0/20:3
770.5696	770.5705	-0.89	C43 H81 O8 N P	786.6006	786.6007	-0.13	C44 H85 O8 N P	83.68	18:0/18:2; 18:1/18:1
778.5747	778.5756	-0.93	C45 H81 O7 N P	794.6057	794.6058	-0.14	C46 H85 O7 N P	15.85	a16:0/22:5
780.5903	780.5913	-0.96	C45 H83 O7 N P	796.6213	796.6215	-0.17	C46 H87 O7 N P	10.49	a18:0/20:4
790.5383	790.5392	-0.97	C45 H77 O8 N P	806.5693	806.5694	-0.15	C46 H81 O8 N P	6.33	16:0/22:6
792.5538	792.5549	-1.03	C45 H79 O8 N P	808.5847	808.5851	-0.41	C46 H83 O8 N P	7.32	16:0/22:5
794.5695	794.5705	-1	C45 H81 O8 N P	810.6008	810.6007	0.04	C46 H85 O8 N P	33.83	18:0/20:4
796.5849	796.5862	-1.29	C45 H83 O8 N P	812.6159	812.6164	-0.53	C46 H87 O8 N P	7.5	18:0/20:3

^aRelative intensity (%) based on measurements of $[M + H]^+$ ions^bListed isomers (abundances) are in the descending order^cIsomers are not listed unless identification can be made

756 (Figure 2a), consistent with the notion that the ions arise from cleavage of a CH_2Cl residue, resulting in formation of *N,N*-dimethylphosphatidylethanolamine (DMPE) ions (Scheme 1) [10]. Further dissociation of the ion of m/z 756 ($806 \rightarrow 756$; Figure 2b) yielded prominent ions at m/z 281, representing the 18:1-carboxylate anion, along with ions at m/z 492 and 474 arising from losses of the 18:1-fatty acid substituent as a ketene and as an acid, respectively, indicating the presence of the 18:1-fatty acyl substituent. Similarly, the MS^2 spectrum of the $[M + \text{Cl}]^-$ ion of *a18:1/18:1*-PC standard at m/z 806.6 was also dominated by the ions at m/z 756 (data not shown), which gave rise to a MS^3 spectrum ($806 \rightarrow 756$; Figure 2c) identical to that shown in Figure 2b. The results indicate that differentiation of plasmeyl- and plasmany-PC isomers using MS^2 on the $[M + \text{Cl}]^-$ and MS^3 on the $[M - 15]^-$ ions is not applicable.

Further dissociation of the ions of m/z 492 ($806 \rightarrow 756 \rightarrow 492$; Figure 2d) originating from *p18:0/18:1*-PC gave rise to ions at m/z 224 arising from loss

of alkenol (loss of $\text{HOCH} = \text{CHC}_{16}\text{H}_{33}$) and at m/z 267, representing an alkenoxide anion ($^-\text{OCH} = \text{CHC}_{16}\text{H}_{33}$), indicating the presence of an alkenyl group. The spectrum also contained ions at m/z 421 and 403 arising from losses of a *N,N*-dimethylaziridine and *N,N*-dimethylethanolamine, respectively, along with ion at m/z 168, representing a phosphodimethylethanolamine anion, consistent with the presence of the *N,N*-dimethylethanolamine head group (Scheme 1). The structural assignments of the fragment ions, including ions of m/z 194 and 153, were further confirmed by high resolution MS^n mass spectrometry (see Supplemental Material Table S1 and Figure S1). Similarly, the MS^4 spectrum of the ion of m/z 492 ($806 \rightarrow 756 \rightarrow 492$; Figure 2e) originated from *a18:1/18:1*-PC also contained the identical ions at m/z 421, 403, and 168; however, ions at m/z 224 arising from loss of the 1-*O*-alkyl residue at *sn*-1 as 9-octadecenol and the 9-octadecenoxide anion at m/z 267 are of low abundance. The drastic differences of the two spectra arising from a plasmeyl- and

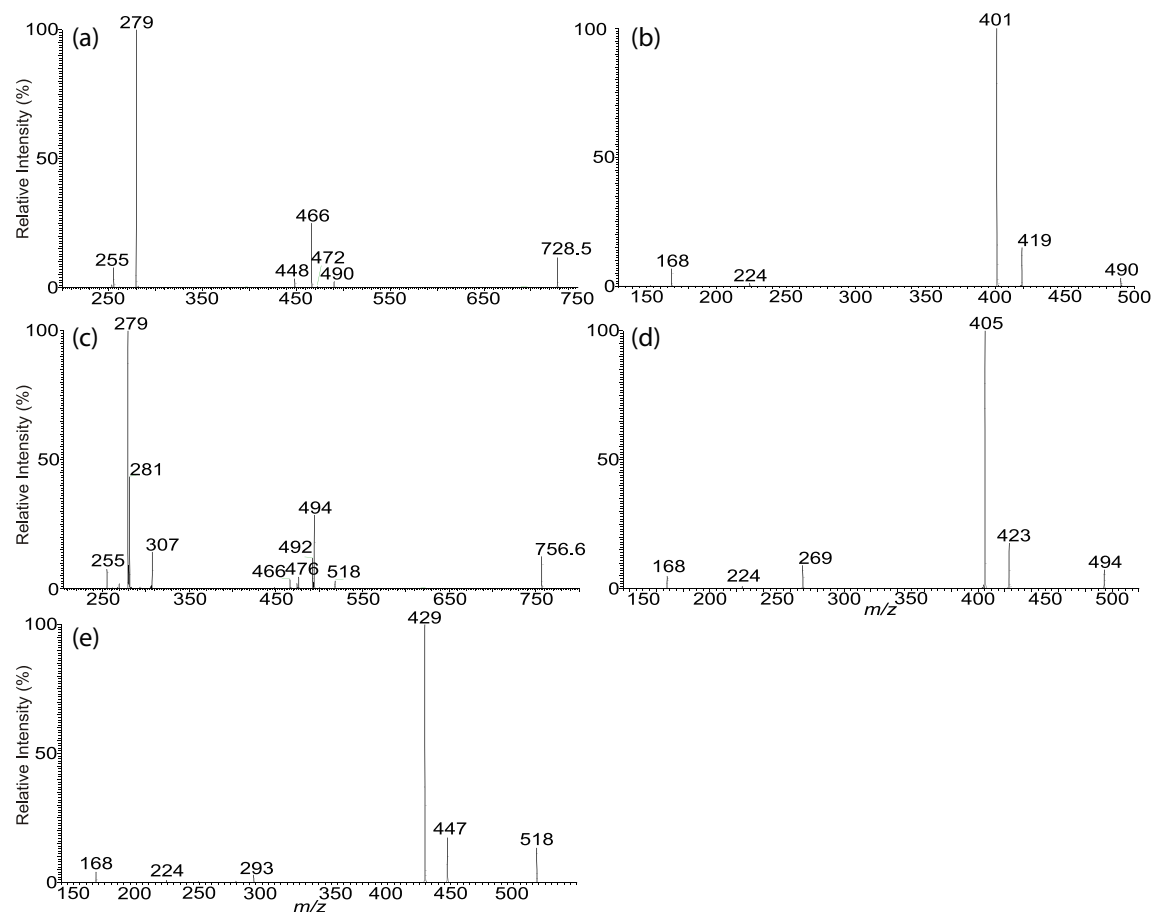


Figure 4. The MS³ spectrum of the [M – 15][–] ions of *m/z* 728.5 (a), its MS⁴ spectra of the ions of *m/z* 490 (778→728→490) (b), MS³ spectrum of the [M – 15][–] ions of 756.6 (c), its MS⁴ spectra of the ions of *m/z* 494 (806→756→494) (d), and 518 (806→756→518) (e) arising from the PC extract of bone marrow neutrophils

plasmanyl-PCs provide a simple method for differentiation between the two isomers.

The utility of LIT MSⁿ on the [M – 15][–] ions in the differentiation of various PC subfamilies were further tested using the *p*18:0/20:4-PC, *a*16:0/20:4-PC, and 16:0/18:1-PC standards. The MS² spectrum of *p*18:0/20:4-PC at *m/z* 828 is dominated by the [M – 15][–] ion at *m/z* 778 (data not shown), which gave rise to ions at *m/z* 492 and 474 (828→778; Figure 3a), arising from losses of 20:4-fatty acid substituent as a ketene and as an acid, respectively, along with 20:4-carboxylate anions at *m/z* 303 and ions at *m/z* 259 (303 – CO₂) [10, 15]. The MS⁴ spectrum of the ion at *m/z* 492 (828→778→492; Figure 3b) is identical to that shown in Figure 2d, indicating the presence of 1-*O*-alk-1'-enyl substituent, consistent with the *p*18:0/20:4-PC structure. The profile of the MS³ spectrum of the [M – 15][–] ion of *a*16:0/20:4-PC at *m/z* 752 (802→752; Figure 3c) is similar to that shown in Figure 3a, and contained ions at *m/z* 466 and 448 arising from similar losses of the 20:4-FA substituent, and ions at *m/z* 303

(20:4-carboxylate anion) and 259 (303 – CO₂). However, the profile of the MS⁴ spectrum of the ion of *m/z* 466 (802→752→466; Figure 3d) is readily distinguishable from that of Figure 2d, and identical to that of Figure 2e, consistent with the *a*16:0/20:4-PC structure.

In contrast, the MS³ spectrum of the [M – 15][–] ion of diacyl PCs, for example, the 16:0/18:1-PC standard at *m/z* 744 (794→744; Figure 3e), contains two ion pairs at *m/z* 480 and 462 arising from losses of the 18:1-fatty acid substituent at *sn*-2 as ketene and as fatty acid, respectively, and at *m/z* 506 and 488 arising from the analogous losses of 16:0-fatty acid at *sn*-1. The former ion pairs are respectively more abundant than the latter, indicating that the 16:0- and 18:1-fatty acyl substituents are located at *sn*-1 and *sn*-2 of the glycerol backbone, respectively. This assignment is consistent with the observation of the carboxylate anions at *m/z* 281 (18:1-carboxylate anion), which is more prominent than the ion at *m/z* 255 (16:0-carboxylate anion) [10]. The MS⁴ spectrum of the ion of *m/z* 480 (794→744→480; Figure 3f), analogous to the ions of *m/z*

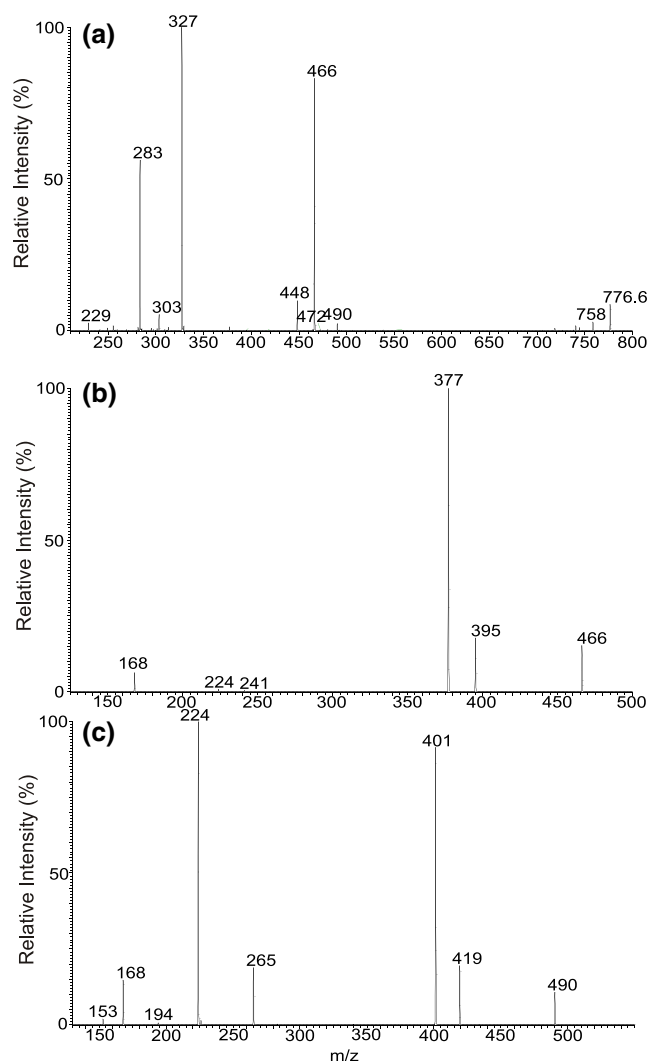


Figure 5. The MS³ spectrum of the [M – 15][–] ions of *m/z* 776.5 (a), its MS⁴ spectra of the ions of *m/z* 466 (826→776→466) (b), and of *m/z* 490 (826→776→490) (c) arising from the PC extract of mouse kidney

492 in Figure 2d and e, arising from loss of the fatty acyl ketene at *sn*-2, is dominated by the carboxylate anion at *m/z* 255, and the ions corresponding to losses of the dimethylamine head group are nearly absent. These results are similar to those seen for the [M – H][–] ions of diacyl PE [16], consistent with the notion that the [M – 15][–] ions of PC are equivalent to DMPEs [17]. The profile of the spectrum is readily distinguishable from those arising from plasmenyl- (Figures 2d and 3b) and plasmanyl-PC (Figures 2e and 3d), providing its distinction from the alkyl ether PC families.

Characterization of Alkyl Ether PCs Isolated from Murine Bone Marrow Neutrophils

The high resolution mass measurements on the [M + H]⁺ and the [M – 15][–] ions of the PC species extracted from mouse bone

marrow neutrophils, and the assigned structures deduced from LIT MSⁿ on the [M – 15][–] revealed that the PC molecules consist of rich plasmanyl-PCs, which comprise 50% of total PC (based on the calculated ratio of the total percent plasmanyl-PC intensities to the total percent PC intensities), and many PCs are composed of several isomeric structures (Table 1).

For example, the MS³ spectrum of the [M – 15][–] ions at *m/z* 728 (778→728; Figure 4a) consists of ions at *m/z* 466 and 448 arising from losses of 18:2-fatty acid substituent, along with the presence of the prominent ion at *m/z* 279. The MS⁴ spectrum of the ion of *m/z* 466 (778→728→466; data not shown) is identical to that shown in Figure 3d, indicating the presence of 1-hexadecanol residue at *sn*-1 and the presence of *a*16:0/18:2-PC isomer. In Figure 4a, the ions at *m/z* 490 and 472, arising from losses of 16:0-fatty acid as a ketene and as an acid is also present, along with the ion at *m/z* 255, indicating the presence of 16:0-fatty acid substituent. The profile of the MS⁴ spectrum of the ion of *m/z* 490 (778→728→490; Figure 4b) is similar to those shown in Figures 2e and 3d, indicating the presence of a minor *a*18:2/16:0-PC isomer.

Plasmanyl PCs containing several isomeric structures are also present (Table 1). For example, the MS³ spectrum of the [M – 15][–] ions of *m/z* 756 (806→756; Figure 4c) contained ions at *m/z* 494 (loss of 18:2-ketene) and 476 (loss of 18:2-acid) arising from cleavage of 18:2-fatty acid substituent, and consistent with the observation of the ion at *m/z* 279, representing a 18:2-carboxylate anion. Further dissociation of the ion of *m/z* 494 gave rise to MS⁴ spectrum (756→494; Figure 4d) with profile identical to that shown in Figure 2e, indicating the presence of *a*18:0/18:2-PC. The spectrum (Figure 4c) also contained ions at *m/z* 492 and 474 arising from loss of 18:1-fatty acid substituent. The MS⁴ spectrum of the ion of *m/z* 492 (806→756→492; data not shown) is identical to Figure 2d, leading to assignment of *a*18:1/18:1-PC. In Figure 4c, ions at *m/z* 466 arising from loss of 20:2-fatty acid substituent as a ketene, together with ions at *m/z* 307, representing a 20:2-carboxylate anion are also present. The MS⁴ spectrum of the ion of *m/z* 466 (data not shown) is identical to that shown in Figure 3c, indicating the presence of *a*16:0/20:2-PC isomer. A minor ion at *m/z* 518 arising from loss of 16:0-fatty acid ketene, along with ion at *m/z* 255 is also present in Figure 4c. The profile of the MS⁴ spectrum of the ion at *m/z* 518 (Figure 4e) represents a plasmanyl-PC, leading to the assignment of *a*20:2/16:0-PC structure.

Characterization of Alkyl Ether PCs Isolated from Mouse Kidney

High resolution mass measurements on mouse kidney PCs desorbed as [M – 15][–] and [M + H]⁺ ions (Table 2) indicate that the mixture mainly consist of

Table 2. The High Resolution Mass Measurements of the $[M - 15]^-$ and $[M + H]^+$ Ions of the PCs Isolated from Mouse Kidney and Their Assigned Structures

$[M - 15]^-$				$[M + H]^+$				Rel. int. (%) ^a	Structures ^{b,c}
<i>m/z</i>	Theo. mass	Deviat. (mDa)	Composition	<i>m/z</i>	Theo. mass	Deviat. (mDa)	Composition		
718.5395	718.5392	0.25	C39 H77 O8 N P	734.5695	734.5694	0.05	C40 H81 O8 N P	88.69	16:0/16:0
730.5758	730.5756	0.22	C41 H81 O7 N P	746.6059	746.6058	0.11	C42 H85 O7 N P	4.82	a18:1/16:0; a16:0/18:1
742.5394	742.5392	0.17	C41 H77 O8 N P	758.5692	758.5694	-0.18	C42 H81 O8 N P	87.84	16:0/18:2
744.5549	744.5549	0.07	C41 H79 O8 N P	760.5848	760.5851	-0.3	C42 H83 O8 N P	63.72	16:0/18:1
746.5689	746.5705	-1.67	C41 H81 O8 N P	762.6001	762.6007	-0.6	C42 H85 O8 N P	9.84	18:0/16:0
750.5445	750.5443	0.15	C43 H77 O7 N P	766.5743	766.5745	-0.2	C44 H81 O7 N P	3.89	p16:0/20:4; a16:0/20:5
752.5598	752.5600	-0.17	C43 H79 O7 N P	768.5899	768.5902	-0.22	C44 H83 O7 N P	5.83	a16:0/20:4
766.5393	766.5392	0.09	C43 H77 O8 N P	782.5693	782.5694	-0.11	C44 H81 O8 N P	63.34	16:0/20:4; 18:2/18:2
768.5526	768.5549	-2.24	C43 H79 O8 N P	784.5847	784.5851	-0.35	C44 H83 O8 N P	23.53	18:1/18:2; 18:2/18:1; 16:0/20:3
770.5706	770.5705	0.09	C43 H81 O8 N P	786.6006	786.6007	-0.14	C44 H85 O8 N P	63.81	18:0/18:2; 18:1/18:1; 16:0/20:2
772.5862	772.5862	0	C43 H83 O8 N P	788.6160	788.6164	-0.39	C44 H87 O8 N P	23.6	18:0/18:1
776.5599	776.5600	-0.03	C45 H79 O7 N P	792.5900	792.5902	-0.17	C46 H83 O7 N P	43.56	a16:0/22:6; p18:1/20:4
790.5395	790.5392	0.24	C45 H77 O8 N P	806.5693	806.5694	-0.11	C46 H81 O8 N P	100	16:0/22:6
792.5541	792.5549	-0.8	C45 H79 O8 N P	808.5842	808.5851	-0.9	C46 H83 O8 N P	17.11	18:1/20:4; 16:0/20:5; 18:0/20:5
794.5706	794.5705	0.07	C45 H81 O8 N P	810.6006	810.6007	-0.14	C46 H85 O8 N P	55.09	18:0/20:4
802.5756	802.5756	0	C47 H81 O7 N P	818.6056	818.6058	-0.22	C48 H85 O7 N P	11.43	a18:1/22:6
814.5395	814.5392	0.31	C47 H77 O8 N P	830.5692	830.5694	-0.18	C48 H81 O8 N P	6.81	18:2/22:6
816.5549	816.5549	-0.01	C47 H79 O8 N P	832.5848	832.5851	-0.28	C48 H83 O8 N P	5.45	18:1/22:6
818.5706	818.5705	0.04	C47 H81 O8 N P	834.6006	834.6007	-0.15	C48 H85 O8 N P	33.33	18:0/22:6

^aRelative Intensity (%) based on measurements of $[M + H]^+$ ions

^bListed isomers (abundances) are in the descending order

^cIsomers are not listed unless identification can be made

diacyl-PCs, along with a major alkyl ether containing PC species at m/z 776.6 ($[M - 15]^-$ ion) (the corresponding $[M + H]^+$ is seen at m/z 792.6) and several minor alkyl ether PCs at m/z 730.6, 750.5, 752.6, and 802.6 ($[M - 15]^-$ ions).

MS³ on the $[M - 15]^-$ ion at m/z 776.6 (Figure 5a) gave rise to ions at m/z 466 and 448 arising from losses of 22:6-fatty acid substituent as a ketene and as an acid, respectively, consistent with the observation of the ion at m/z 327, representing a 22:6-carboxylate anion, and the ion at m/z 283, arising from loss of CO₂ from ion of m/z 327. The spectrum also contained ions at m/z 490 and 472, arising from the analogous losses of 20:4-fatty acid moiety, along with the ion at m/z 303 (20:4-carboxylate anion), indicating the presence of 20:4-fatty acid substituent. The MS⁴ spectrum of the ion of m/z 466 (826→776→466; Figure 5b) is identical to that shown in Figure 3d, indicating the presence of 1-*O*-hexadecyl residue and the presence of *a*16:0/22:6-PC major isomer. The MS⁴ spectrum of the ion of m/z 490 (826→776→460; Figure 5b) contained the octadecenoxide anion at m/z 265 and the profile of the spectrum is similar

to that shown in Figure 2d, indicating the presence of the 1-*O*-octadecen-1'-enyl group, leading to the assignment of a minor *p*18:1/20:4-PC isomer.

Conclusions

The LIT MSⁿ mass spectrometric approach for differentiation of 1-*O*-alk-1'-enyl-2-acyl-, 1-*O*-alkyl-2-acyl- and diacyl-PC as $[M - 15]^-$ ions is complementary to the existing methods that are used for differentiation of other 1-*O*-alk-1'-enyl-2-acyl-, 1-*O*-alkyl-2-acyl-, and diacyl-glycerolphospholipid classes as the $[M - H]^-$ ions [7], facilitating analysis of the entire glycerolphospholipid classes. Notably, our data showed that 50% of the PCs found in the extract are plasmanyl-PCs (Table 1), consistent with the earlier report that in neutrophils, up to 46% of the PC pool is in the plasmanyl form [18]. The observation of the abundant m/z 776 ion consisting of major *a*16:0/22:6-PC and minor *p*18:1/20:4-PC isobaric isomers in murine kidney is interesting. To the best of our knowledge, this is the first time that such a species in mouse kidney is reported.

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