Available online at www.sciencedirect.com
SCIENCE DIRECT
International Journal of Mass Spectrometry 252 (2006) 242–255
International Journal of Mass Spectrometry
www.elsevier.com/locate/ijms

Multistage tandem mass spectrometry of anionic phosphatidylcholine lipid adducts reveals novel dissociation pathways

Xi Zhang [a], Gavin E. Reid [a,b,*]

[a] *Department of Chemistry, Michigan State University, 234 Chemistry Building, East Lansing, MI 48824, USA*
[b] *Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA*

Received 19 December 2005; received in revised form 2 April 2006; accepted 3 April 2006
Available online 2 May 2006

Abstract

The gas-phase fragmentation reactions of various anionic adducts of phosphatidylcholine lipids have been examined by electrospray ionization and multistage tandem mass spectrometry (MS^n) in a linear quadrupole ion trap mass spectrometer. The MS/MS and MS^n fragmentation reactions of the anionic adducts were found to be highly dependent on the identity of the anions (A). Consistent with previous reports, MS/MS of phosphatidylcholine lipid ions formed by anionic adduction with formate, acetate and chloride resulted in a dominant product ion corresponding to the loss of $(A + CH_3)$ from the $[M + A]^-$ precursor ions. In contrast, MS/MS of other anion adducted precursor ions gave rise to multiple products, including $[M + A{-}(A + CH_3)]^-$, $[M + A{-}(AH)]^-$, $[M + A{-}(AH + CH_3)]^-$, $[M + A{-}(AH + (CH_3)_3N)]^-$, $[M + A{-}(A + (CH_3)_3NCHCH_2)]^-$ and $[M + A{-}(CH_3)_3N)]^-$. In order to gain evidence for the structures of these product ions, and the mechanisms by which they were formed, each of the products formed by MS/MS were subjected to further analysis by MS^3 and MS^4. The MS/MS and MS^n fragmentation reactions of several deuterium isotope labeled phosphatidylcholine lipid standards, as well as authentic or structurally analogous lipid standards corresponding to the proposed product ion structures were also examined to gain evidence to support the proposed mechanisms. The results from this study indicate that great care must be taken to control the identity of the anions employed during lipid isolation, purification and separation, in order to allow the development and application of sensitive and robust neutral loss scan methods for mass spectrometry based lipid identification and quantitative analysis.

Keywords: Phosphatidylcholine; Anionic adducts; Mechanisms

1. Introduction

The identification, characterization and quantitative analysis of biologically important lipids is an important step toward the development of an improved understanding of the functional role of integral phospholipids in large multi-subunit membrane protein complexes [1–6] and lipid micro domains [7–10]. In previous studies employing mass spectrometry, the observation of neutral and acidic lipid species in either positive or negative ionization mode has been found to be highly dependant on the identity of the lipid head group [11–16]. For example, abundant precursor ions of phosphatidylcholine (PC) [17] and sphingomyelin (SM) [18] lipids are readily observed in positive ion mode, while phosphatidic acid (PA) [19], phosphatidyl glycerol (PG) [20], phosphatidyl serine (PS) [21], phosphatidylinositol (PI) [22,23] and cardiolipin (CL) [24] are observed in negative ion mode; phosphatidyl ethanolamine (PE) lipids can be observed in both positive and negative ion modes, albeit at significantly lower abundance when in the presence of other lipid species [14,16,25]. This variability in ion yield as a function of lipid structure may result in poor identification and quantitative analysis capabilities for MS methods applied to complex lipid samples, and often requires two separate analyses with different optimal sample preparation methods for each ionization mode [11–16]. Thus, the ability to observe both neutral and acidic lipids in a single ionization mode, with high sensitivity and reproducibility, would facilitate the development of more comprehensive methods for lipid identification and quantification, with a reduced requirement for extensive sample extraction or purification prior to analysis.

Previously, cationic adduction (e.g., Li^+, Na^+) [12,13,17,18,26–28] has been applied to the analyses of lipid extracts con-

* Corresponding author. Tel.: +1 517 355 9715x198; fax: +1 517 353 1793.
E-mail address: reid@chemistry.msu.edu (G.E. Reid).

1387-3806/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2006.04.001

taining PE, SM, PE, PA, PG, PS, PI and CL in order to enhance the sensitivity of the PE, PA, PG, PS, PI and CL lipid species in positive ionization mode. However, difficulties in reproducibly forming cationic adducts, due to competition between each of the phospholipid species for adduct formation, and the possibility of di-cationic adduction, in addition to mono-cationic adduction, potentially limits the applicability of this approach. In contrast, adduction with formate (HCO_2^-), acetate ($CH_3CO_2^-$) and chloride (Cl^-) anions has previously been observed to result in the selective adduction of PC and SM lipid species in negative ionization mode [16,27,29,30]. Dissociation of these adducts (A) by either in-source dissociation [11,31–33], or by CID MS/MS [11,12,27,29,30,34–38] has resulted in the exclusive loss of (A + 15) from these ions, enabling the use of neutral loss CID MS/MS scan methods in triple quadrupole mass spectrometers for the selective identification of PC and SM lipid species from within complex lipid mixtures [12,29,30,37,38].

Despite the great potential of employing anionic adduction for the selective analysis of PC or SM lipids in negative ion mode, together with most other lipids species, the negative ion fragmentation behavior of anionic adducts of PC lipids have not been explored in detail, in contrast to the detailed studies previously undertaken to elucidate the mechanisms responsible for the fragmentation reactions of protonated or cationic lipid ions in positive ion mode [11,17,18,21,27,39], or for deprotonated lipid ions in negative ion mode [11,19–22,24,25,40–44]. Here, in order to develop an improved understanding of the characteristic ionization and fragmentation behaviors of anionic PC adducts, and to take advantage of this anionic adduction for developing improved methods for lipid identification and quantification in complex mixtures, we have evaluated a broad range of anions for the formation of anionic adducts of PC by electrospray ionization, and characterized their fragmentation reactions by multistage tandem mass spectrometry (MS^n) in a linear quadrupole ion trap mass spectrometer. Based on these results, together with those obtained by dissociation of ^{2}H-labeled lipid standards and authentic or structurally analogous lipids, mechanisms for each of the observed fragmentation reactions are proposed.

2. Materials and methods

2.1. Materials

Synthetic lipid standards PC 16:0, 16:0, PC 16:0, 18:1, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine-1,1,2,2-d_4 (d_4-PC 16:0, 16:0), 1-d_{31},2-d_{31}-dipalmitoyl-*sn*-glycero-3-phosphocholine (d_{62}-PC 16:0, 16:0), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine-1,1,2,2-d_4-*N*,*N*,*N*-trimethyl-d_9 (d_{13}-PC 16:0, 16:0), PA 16:0, 18:1, PE 16:0, 16:0, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine *N*,*N*-dimethyl (diMe-PE16:0, 16:0) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Methanol, benzenesulfonic acid and ammonium dihydrogen phosphate were obtained from Sigma–Aldrich (St. Louis, MO). Ethanol was purchased from the AAPER Alcohol and Chemical Co. (Shelbyville, KY). Chloroform and ammonium acetate were from Mallinckrodt (Paris, KY). Ammonium fluoride, ammonium chloride and ammonium bicarbonate were obtained from Baker (Phillipsburg, NJ). Ammonium iodide, formic acid and hydrobromic acid (48%) were from Spectrum Quality Products (Gardena, CA). Ammonium thiocyanate was purchased from Fisher Scientific (Fairlawn, NJ). Ammonium nitrate, ammonium hydroxide (28.0–30.0%) and sulfuric acid were from Columbus Chemical Industries (Columbus, WI). Trifluoroacetic acid (Sequanal grade) was obtained from Pierce (Rockford, IL). Solvents were filtered with a sintered glass funnel (pore size 4–8 μm) prior to use.

2.2. Mass spectrometry

Mass spectrometry experiments were performed using a Thermo model LTQ linear quadrupole ion trap mass spectrometer (San Jose, CA), equipped with a nanospray ionization (nESI) source. Samples (5 μM) in 50:50 (v/v) $CH_3OH/CHCl_3$ containing 0.1–10 mM ammonium salts were introduced to the mass spectrometer at a flow rate of 0.2 μL/min. The nESI conditions were optimized to maximize the intensity of the adduct ion of interest and to minimize the extent of in-source fragmentation. Spectra were acquired in negative ionization mode. Typical nESI conditions were: spray voltage 1.5 kV, heated capillary temperature 200 °C, capillary voltage −20 V, tube lens voltage −75 V. Unless otherwise specified, CID-MS/MS and MS^n spectra were acquired at an activation q value of 0.2 using an isolation width of 2 Da, a normalized collision energy of 30% and an activation time of 30 ms. Spectra shown are the average of 80 scans.

3. Results and discussion

3.1. Negative ion CID MS/MS of anionic adducts of PC16:0, 16:0 and PC16:0, 18:1

In order to characterize the CID MS/MS and MS^n fragmentation behavior of various anionic (A) adducts of phosphatidylcholine lipids, ammonium salts (0.1–10 mM) of acetate ($CH_3CO_2^-$), formate (HCO_2^-), chloride (Cl^-), bromide (Br^-), iodide (I^-), fluoride (F^-), thiocyanate (SCN^-), nitrate (NO_3^-), phosphate ($H_2PO_4^-$), hydroxide (OH^-), bicarbonate (HCO_3^-), trifluoroacetate ($CF_3CO_2^-$), benzenesulfate ($C_6H_5SO_3^-$) or sulfate (HSO_4^-) salts were added to 5 μM solutions of PC 16:0, 16:0 (molecular weight 733) or PC16:0, 18:1 (molecular weight 759), then introduced to the mass spectrometer by nESI. In positive ionization mode, protonated precursor ions $[M+H]^+$ for the PC 16:0, 16:0 and PC 16:0, 18:1 lipid species were readily observed at *m*/*z* 734 and *m*/*z* 760, respectively. As expected, in negative ionization mode, deprotonated precursor ions $[M-H]^-$ were not observed, consistent with the presence of the 'fixed positive charge' quaternary alkyl ammonium ion within the phosphocholine head group. Instead, abundant anionic $[M+A]^-$ adducts were observed as the dominant precursor ions. The *m*/*z* values observed by adduction of PC 16:0, 16:0 and PC 16:0, 18:1 with each of the anions indicated above are listed in Table 1.

Table 1
Negative ion mode CID MS/MS of anionic adducts of phosphatidylcholine lipids PC 16:0,16:0 and PC 16:0,18:1

PC lipid	Anion (A)	Gas-phase acidity (kJ/mol)[a]	$[M+A]^-$ precursor ion (*m/z*)	Product ions (% relative abundance)					
				−AH	Intermolecular S_N2 −(A + CH_3)	−(AH + CH_3)	Intermolecular E2 −(AH + $(CH_3)_3N$)	Intramolecular E2 −(A + $(CH_3)_3$ $NCHCH_2$)	Other[b]
				732	718	717	673	647	
16:0, 16:0	$CH_3CO_2^-$	1427	792	–	100	–	–	–	–
	HCO_2^-	1419	778	–	100	–	–	–	–
	I^-	1293.7	860	–	100	–	–	–	–
	Br^-	1331.8	812/814	–	100	–	–	–	–
	Cl^-	1372.8	768	–	100	–	–	–	–
	F^-	1529.3	752	–	100	–	2	–	–
	SCN^-	1329	791	–	100	–	–	1	–
	NO_3^-	1329.7	795	–	100	–	1	11	−(A + 86 + $C_{15}H_{31}CO_2H$), (391), 1; $C_{15}H_{31}COO^-$, (255), 1
	$H_2PO_4^-$	1351	830	–	100	–	3	21	−(**$CH_3)_3$N**, (**771**), **1**; (391), 6; $C_{15}H_{31}COO^-$, (255), 5
	OH^-/CH_3CH_2OH	ND[c]	822	64	49	2	100	40	−(AH + 59 + $C_{15}H_{31}CO_2H$ −2H), (419), 1
	HCO_3^-/CH_3CH_2OH	ND[c]	822	65	33	5	100	41	−(AH + 59 + $C_{15}H_{31}CO_2H$−2H), (419), 1
	OH^-/CH_3OH	ND[c]	808	62	1	6	100	36	−(AH + 59 + $C_{15}H_{31}CO_2H$ −2H), (419), 60
	HCO_3^-/CH_3OH	ND[c]	808	61	1	7	100	34	−(AH + 59 + $C_{15}H_{31}CO_2H$ −2H), (419), 3
	$CF_3CO_2^-$	1328	846	81	1	47	100	18	−(AH + 59 + $C_{15}H_{31}CO_2H$ −2H), (419), 58; $C_{15}H_{31}COO^-$, (255), 5
	$C_6H_5SO_3^-$	ND[c]	890	–	3	–	1	100	−(A + 86 + $C_{15}H_{31}CO_2H$), (391), 1; $C_{15}H_{31}COO^-$, (255), 1

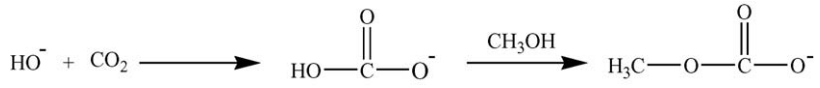
	$CH_3CO_2^-$	818	–	100	–	–	–	–	
	HCO_2^-	804	–	100	–	–	–	–	
16:0,18:1	Cl^-	794	–	100	–	–	–	–	
	$H_2PO_4^-$	856	–	100	–	2	19	**$-(CH_3)_3N$**, **(797)**, **6**; (417), 2; (391), 2; $C_{15}H_{31}COO^-$, (255), 2; $C_{17}H_{33}COO^-$, (281), 2	
	HCO_3^-/CH_3OH	834	55	1	6	100	35	$-(AH+(CH_3)_3N+C_{17}H_{33}CO_2H-2H)$, (419), 2	
	$CF_3CO_2^-$	872	79	45	57	100	18	$-(AH+(CH_3)_3N+C_{17}H_{33}CO_2H-2H)$, (419), 24; $C_{15}H_{31}COO^-$, (255), 1; $C_{17}H_{33}COO^-$, (281), 2	

[a] Gas-phase acidity data taken from the NIST database (http://webbook.nist.gov).
[b] Product ion (*m/z*), % abundance.
[c] Not determined.

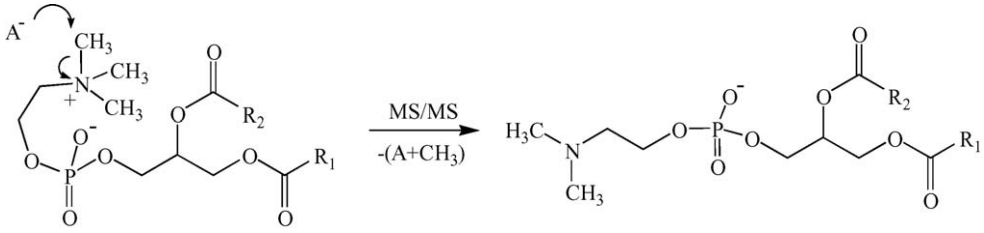
Fig. 1. Negative ionization mode CID MS/MS product ion spectrum of the $[M+A]^-$ precursor ions of PC 16:0, 16:0 formed by anionic adduction with (A) acetate (*m/z* 792) and (B) methylcarbonate (*m/z* 808).

The product ion spectrum obtained by CID MS/MS of the $[M+A]^-$ acetate adduct ion of PC 16:0, 16:0 is shown in Fig. 1A. It can be seen that a single product ion at *m/z* 718, formed by the neutral loss of 74 Da and corresponding to the loss of (A + 15), was observed as the exclusive product, consistent with previously reported in-source fragmentation [11,31–33] and CID MS/MS [11,12,27,29,30,34–38] results using triple quadrupole and quadrupole ion trap mass spectrometry instrumentation. Identical product ion spectra were observed by CID MS/MS of the formate, chloride, bromide and iodide anionic adducts of PC 16:0, 16:0 (neutral losses of 60 Da, 50 Da, 94/96 Da and 142 Da, respectively), and for the acetate, formate and chloride adducts of PC 16:0, 18:1.

In contrast, when 10 mM ammonium hydroxide or ammonium bicarbonate was added to the PC 16:0, 16:0 lipid containing solutions, an abundant precursor ion at *m/z* 808 (+75 Da) was observed, which did not correspond to either of the expected hydroxide (+17 Da, *m/z* 750) or bicarbonate (+61 Da, *m/z* 794) adduct ions (Table 1). Furthermore, in contrast to that observed from the acetate, formate and chloride adducts of PC 16:0, 16:0, CID MS/MS of the *m/z* 808 adduct ions (Fig. 1B) resulted in formation of the −(A + 15) product ion at a relative abundance of only 1%. Instead, the major product ions corresponded to neutral losses of (A + 1), (A + 16), (A + 60) and (A + 86) from the precursor ion. Interestingly, precursor ions corresponding to $[M-15]^-$, $[M-60]^-$ and $[M-86]^-$ ions have also been observed previously for PC lipids formed by fast atom bombardment (FAB) in a sector or triple quadrupole mass spectrometer under negative ionization mode conditions when $CH_3OH/CHCl_3$ was used as the solvent and triethylamine [45–48] or diethylamine [49] was used as the matrix. The structures of these ions were assigned to be $[M-CH_3]^-$, $[M-(CH_3)_3N]^-$, and $[M-(CH_3)_3NCHCH_2]^-$, respectively, using sector FAB CAD MS/MS [48].



Scheme 1.



Scheme 2.

Given that ammonium hydroxide and ammonium bicarbonate are commonly employed as a pH modifier in negative ionization mode MS analysis to promote the formation of abundant $[M-H]^-$ precursor ions from neutral and acidic phospholipids such as PE, PA, PG, PS, PI and CL [14,15], it is important that the identity of the anionic species responsible for adduction of the PC phospholipids in the presence of ammonium hydroxide and ammonium bicarbonate is determined. Furthermore, as the characteristic loss of (A + 15) by CID MS/MS from acetate, formate and chloride adducts of PC and SM have been used previously for the selective identification of these lipid species by neutral loss scan mode analysis, the formation of this product ion at only low relative abundance due to competitive adduction of PC or SM with other anionic species could severely limit the sensitivity of such approaches. Therefore, in order to determine the identity of the +75 Da anionic adduct of PC in the presence of ammonium hydroxide and ammonium bicarbonate, and to characterize the mechanisms and resultant product ion structures formed by CID MS/MS, we have examined the formation and fragmentation reactions of a range of anionic adducts of PC 16:0, 16:0 and PC 16:0, 18:1 by MS/MS and multistage (up to MS^4) tandem mass spectrometry.

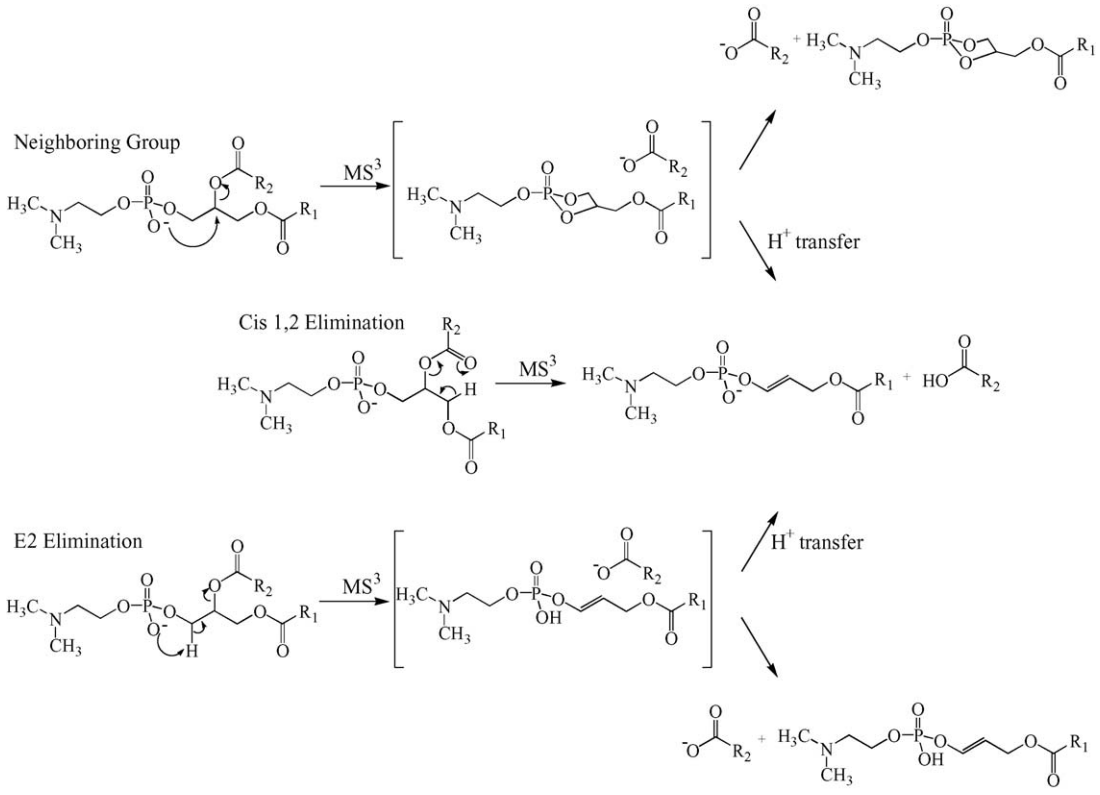
The product ions observed by CID MS/MS of the various anionic adducts of PC 16:0, 16:0 and PC 16:0, 18:1 are shown in Table 1. The appearance and relative abundance of these product ions were observed to vary considerably, depending on the identity of the anion.

When ethanol was employed in the lipid containing solutions, the $[M+A]^-$ adduct ions from PC 16:0, 16:0 and PC 16:0, 18:1 in the presence of ammonium hydroxide or ammonium bicarbonate were observed at *m/z* values 14 Da higher, i.e., an adduct mass of 89 Da, compared to +75 Da when methanol was used, indicating that the alcohol was involved in formation of the adduct. Thus, the +75 and +89 Da adduct ions observed in the presence of ammonium hydroxide or ammonium bicarbonate are proposed to be formed by the process shown in Scheme 1, involving reaction of the hydroxide anion with atmospheric CO_2 to initially form the bicarbonate anion, followed by reaction with methanol or ethanol in the solvent to yield a methylcarbonate (75 Da) or ethylcarbonate (89 Da) anion, respectively.

3.2. CID MS^3 experiments to probe the structure of the product ions formed by neutral loss of (A + 15) from the anionic adducts of PC

MS^3 of all the product ions formed by the loss of (A + 15) from each of the anionic adducts were found to yield identical product ion spectra, indicating the formation of a common product ion in each case. Previous studies have proposed that the loss of (A + 15) from the acetate, formate or chloride adducts of PC occurs via methyl group transfer from the quaternary alky ammonium ion on the phosphatidylcholine head group of the PC lipid to the anion, resulting in the formation of an *N*,*N*-dimethylphosphatidylethanolamine lipid species [11,12,27,29–31,34–37] (Scheme 2). To confirm this here, the MS^3 spectra of the −(A + 15) neutral loss product ion obtained by MS/MS of the PC 16:0, 16:0 acetate anionic adduct (Fig. 2A) was compared with the MS/MS product ion spectrum of an authentic *N*,*N*-dimethyl phosphatidylethanolamine 16:0, 16:0 lipid ion prepared in solution and introduced to the mass spectrometer by nESI (Fig. 2B). It can be seen from Fig. 2 that the product ion spectra are identical, indicating that the loss of (A + 15) does correspond to formation of an $[M+A-(A+CH_3)]^-$ product ion as a result of methyl group transfer from the phosphocholine head group to the anion.

The major product ion (*m/z* 255) observed in Fig. 2 corresponds to the formation of $RCOO^-$, by dissociation of one of the two ester linkages of the fatty acid chains within the 16:0, 16:0 phospholipid ion. A product ion corresponding to the loss of the neutral fatty acid chain (−RCOOH, *m/z* 462) was also observed at low abundance. The R group is used here to indicate the fatty acid chain without specifying its origin from either the *sn*-1 or *sn*-2 position of the glycerol backbone, i.e., the $RCOO^-$ *m/z* 255 product ion in Fig. 2 represents $C_{15}H_{31}COO^-$. For the 16:0, 18:1 phospholipids, the origin of the fatty acid chain is specified by a subscript notation, i.e., R_1COO^- represents a fatty acid product ion formed by dissociation at the *sn*-1 position of the glycerol backbone. A potential mechanism for formation of the $RCOO^-$ and −(RCOOH) product ions, via a 'charge-directed' neighboring group participation reaction mechanism involving nucleophilic attack from the phosphate oxygen anion at an electrophilic carbon adjacent to the



Scheme 3.

fatty acid ester linkage on the glycerol backbone, resulting in the formation of an initial ion-neutral complex, is shown in Scheme 3 [11,19,22,25,41,42,44,50]. Simple dissociation of this ion-neutral complex would result in formation of the $RCOO^-$ product ion, and loss of an RCOOH neutral would be observed if intermolecular proton transfer within the complex occurs prior to its separation (note that only one of the possible pathways for proton transfer is shown). The relative abundances of the two product ions would therefore be expected to be dependent on the lifetime of the ion-neutral complex, and the relative gas-phase acidities of the two neutral species that may be formed. Note that only the pathway for the elimination of the *sn*-2 fatty acid chain, via a 5-membered transition state is shown in Scheme 3. Fragmentation of the *sn*-1 fatty acid chain would proceed via a similar mechanism, involving a 6-membered transition state. An alternative mechanism involving the formation of an ion-neutral complex following a 'charge directed' E2 elimination reaction could also be used to explain the formation of these product ions (Scheme 3). A 'charge-remote' mechanism involving a *cis* 1, 4 elimination pathway has also been proposed previously for formation of the −(RCOOH) product ions from the dissociation of deprotonated phospholipids (Scheme 3) [25]. It is not possible from the studies performed here to distinguish between the neighboring group participation and E2 elimination reaction pathways, as the product ion structures in each case would be identical. We note that a possible way of resolving this issue would be to examine the fragmentation reactions of a multiply charged precursor ion of the PC adduct, that contains an inert charge on the neutral species lost from the singly charged PC adduct, resulting in the formation of structurally distinct product ions amenable to further analysis [51]. The presence of the *cis* 1, 4 elimination pathway could potentially be determined via deuterium labeling of the glycerol backbone. Although beyond the scope of the work described here, experiments are currently underway to obtain further insights into these remaining mechanistic questions.

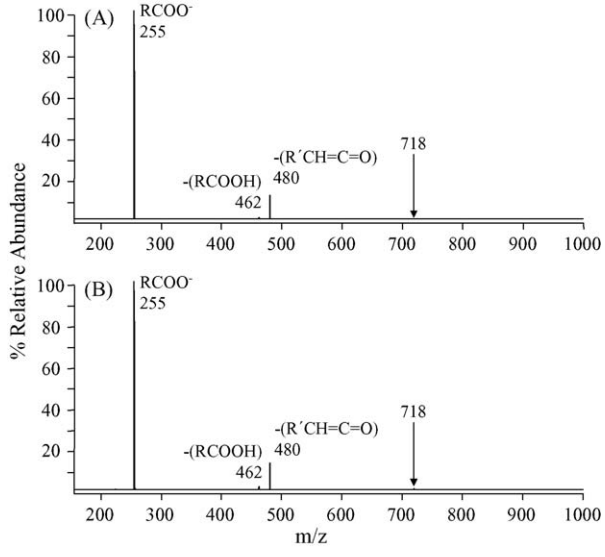
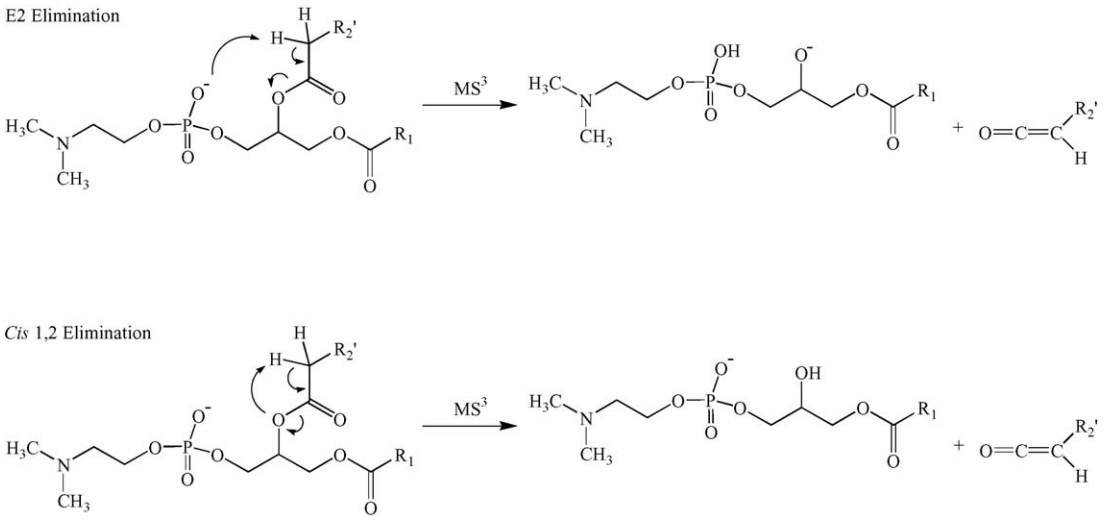


Fig. 2. Negative ionization mode (A) CID MS^3 product ion spectrum of the *m/z* 718 product ion formed via the neutral loss of $(A + CH_3)$ from the acetate adduct of PC 16:0, 16:0 in Fig. 1A; (B) CID MS/MS product ion spectrum of the $[M - H]^-$ precursor ion of *N*,*N*-dimethylphosphatidylethanolamine 16:0, 16:0.



Scheme 4.

The product ion observed at *m/z* 480 in Fig. 2 corresponds to the loss of one of the 16:0 fatty acid chains as a neutral ketene $C_{14}H_{29}CHCO$ (labeled as $-(R'CH=C=O)$). Note that the R (or R_1 or R_2 for *sn*-1 and *sn*-2 specific fatty acid chain linkages) group is used here to indicate the presence of the alkyl chain. Formation of this ketene neutral loss product ion may potentially occur via a 'charge-directed' E2 elimination reaction, initiated by nucleophilic attack from the phosphate oxygen anion to one of the α-hydrogens adjacent to the carboxyl group on the fatty acid chains, or by a 'charge-remote' 4-membered *cis* 1,2 elimination reaction mechanism (Scheme 4) [19,20,40,42,43]. We note however, that it is difficult to distinguish between these mechanistic possibilities, for example by the use of deuterium isotope labeling or further MS^n dissociation, due to the likelihood of intramolecular proton transfer occurring within the resultant product ions.

3.3. Deuterium isotope labeling and CID MS^3 experiments to probe the structure of the product ions formed by neutral loss of (A + 60) from the anionic adducts of PC

In order to determine the structure of the product ion formed by loss of (A + 60) from the anionic adducts of PC 16:0, 16:0, we first examined the CID MS/MS fragmentation behavior of a d_4-labeled derivative of PC 16:0, 16:0, where the deuterium labels are incorporated into the CH_2CH_2 positions of the phosphocholine head group. From the product ion spectrum obtained by CID MS/MS of the methylcarbonate adduct of d_4-PC 16:0, 16:0 (*m/z* 812, Fig. 3A) formed by addition of 10 mM ammonium hydroxide to the lipid containing solution of $CH_3OH/CHCl_3$, the loss of (A + 61) was observed at *m/z* 676, in contrast to the loss of (A + 60) observed at *m/z* 673 for the PC 16:0, 16:0 ion in Fig. 1B, indicating that one of the deuterium labels in the phosphocholine head group of the d_4-PC 16:0, 16:0 lipid was lost from the resultant product ion during the fragmentation reaction. Based on this, a mechanism for formation of the $[M + A-(AH + (CH_3)_3N)]^-$ vinyl phosphatidic acid product ion, by deprotonation of the methylene group adjacent to the phosphate via an E2 elimination reaction initiated by the anion, is proposed in Scheme 5.

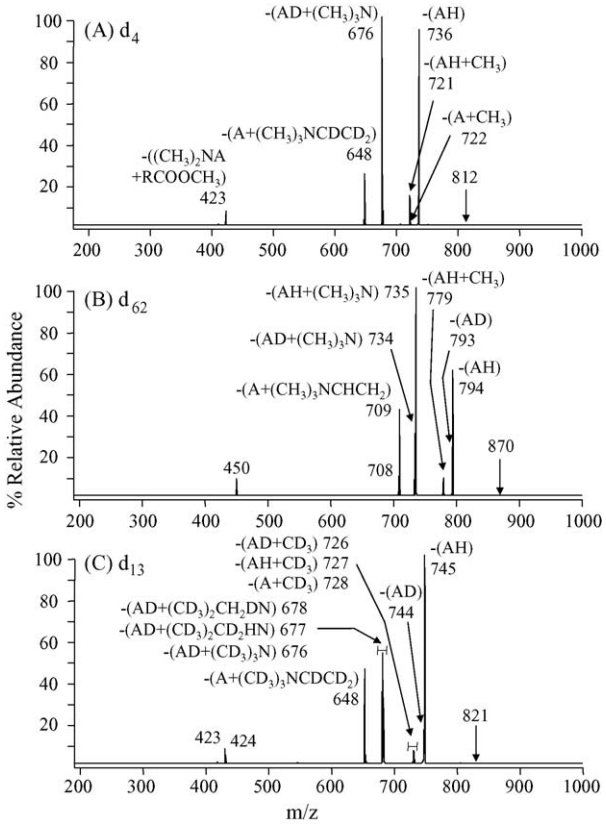
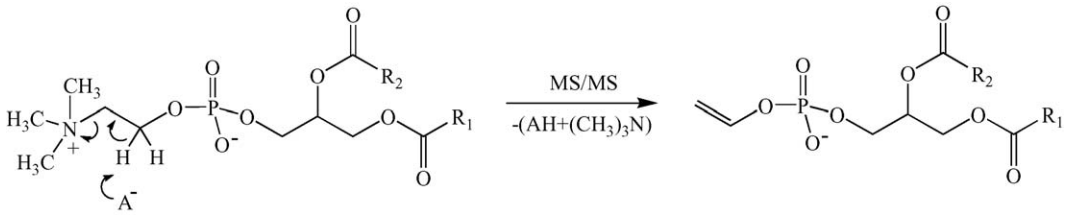


Fig. 3. Negative ionization mode CID MS/MS product ion spectra of the methylcarbonate adducts of (A) d_4-PC 16:0, 16:0 (*m/z* 812); (B) PC d_{31}-16:0, d_{31}-16:0 (*m/z* 870) and (C) d_{13}-PC 16:0, 16:0 (*m/z* 821).

MS^3 of all the product ions formed by the loss of $(AH + (CH_3)_3N)$ from each of the anionic adducts were found to yield identical product ion spectra, indicating the formation of a common product ion. To obtain further evidence to support the proposed vinyl phosphatidic acid product ion structure, the MS^3



Scheme 5.

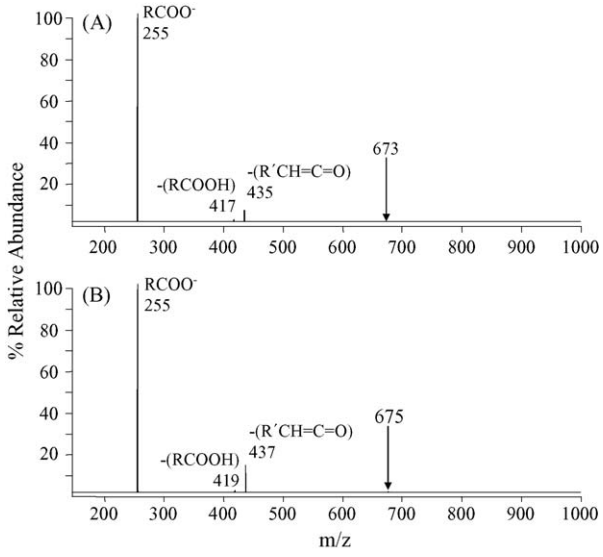


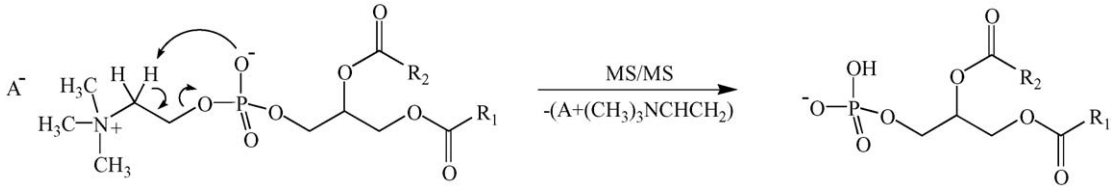
Fig. 4. Negative ionization mode (A) CID MS^3 product ion spectrum of the *m/z* 673 product ion formed via the neutral loss of $(AH + (CH_3)_3N)$ from the methylcarbonate adduct of PC 16:0, 16:0 in Fig. 1B; (B) CID MS/MS product ion spectrum of the $[M - H]^-$ precursor ion of phosphatidylethanol 16:0, 16:0 (*m/z* 675).

spectrum obtained from the *m/z* 673 product ion of the PC 16:0, 16:0 methylcarbonate adduct in Fig. 1B (Fig. 4A) was compared to the MS/MS fragmentation behavior of a structurally related lipid analog, phosphatidylethanol 16:0, 16:0 (*m/z* 675) (Fig. 4B). The difference of one double bond in the head group did not affect the fragmentation reactions observed for these structurally analogous ions. Indeed, aside from a 2 Da mass shift observed between the product ions containing the head group, the same fragmentation pathways and essentially identical product ion abundances were observed for both spectra, consistent with the proposed product ion structure. Furthermore, the product ions observed in Fig. 4 correspond to the same fragmentation pathways, and similar product ion abundances, to those observed in Fig. 2, indicating that neither of the head groups of the proposed $-(A + CH_3)$ and $-(AH + (CH_3)_3N)$ neutral loss product ion structures are involved in their subsequent MS^3 fragmentation reactions.

3.4. Deuterium isotope labeling and CID MS^3 experiments to probe the structure of the product ions formed by neutral loss of (A + 86) from the anionic adducts of PC

To elucidate the structure of the *m/z* 647 product ions formed by neutral loss of (A + 86) from the $[M + A]^-$ anionic adducts of PC 16:0, 16:0, we first examined the CID MS/MS product ion spectrum obtained from the d_4-PC 16:0, 16:0 methylcarbonate lipid adduct (Fig. 3). It can be seen from Fig. 3A that a product ion corresponding to the loss of (A + 89) at *m/z* 648 was formed, in contrast to the loss of (A + 86) observed at *m/z* 647 for the PC 16:0, 16:0 ion in Fig. 1B, indicating that three deuteriums were lost as part of the neutral species upon dissociation of the adduct. Based on this behavior, a mechanism for formation of the $[M + A–(A + (CH_3)_3NCHCH_2)]^-$ product ion, via a charge-directed E2 elimination reaction initiated by the deprotonated phosphate oxygen and resulting in loss of the choline head group to yield phosphatidic acid, is proposed in Scheme 6.

The proposed phosphatidic acid structure of the $[M + A–(A + (CH_3)_3NCHCH_2)]^-$ product ion from the PC anionic adducts was further supported by the observation that MS^3 fragmentation of the 16:0, 18:1 lipid $-(A + (CH_3)_3NCHCH_2))$ neutral loss product ion (Fig. 5A) was essentially identical to the MS/MS product ion spectrum obtained from the $[M - H]^-$ ion of the authentic phosphatidic acid 16:0, 18:1 lipid ion prepared in solution and introduced to the mass spectrometer by nESI (Fig. 5B). MS^3 of all the product ions formed by the loss of $(A + (CH_3)_3NCHCH_2))$ from each of the anionic adducts were found to yield identical product ion spectra, indicating the formation of a common phosphatidic acid product ion in each case.



Scheme 6.

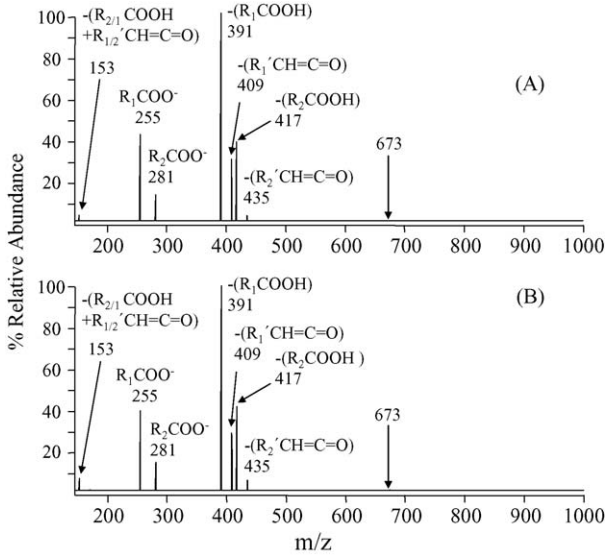


Fig. 5. Negative ionization mode (A) CID MS^3 product ion spectra of the *m/z* 673 product ion formed via the neutral loss of $(A + (CH_3)_3NCHCH_2)$ from the methylcarbonate adduct of PC 16:0, 18:1 in Table 1; (B) CID MS/MS product ion spectrum of the $[M - H]^-$ precursor ion of phosphatidic acid 16:0, 18:1 (*m/z* 673).

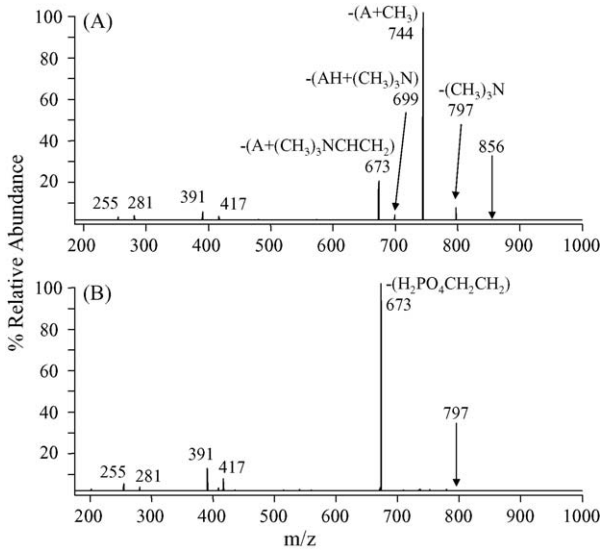
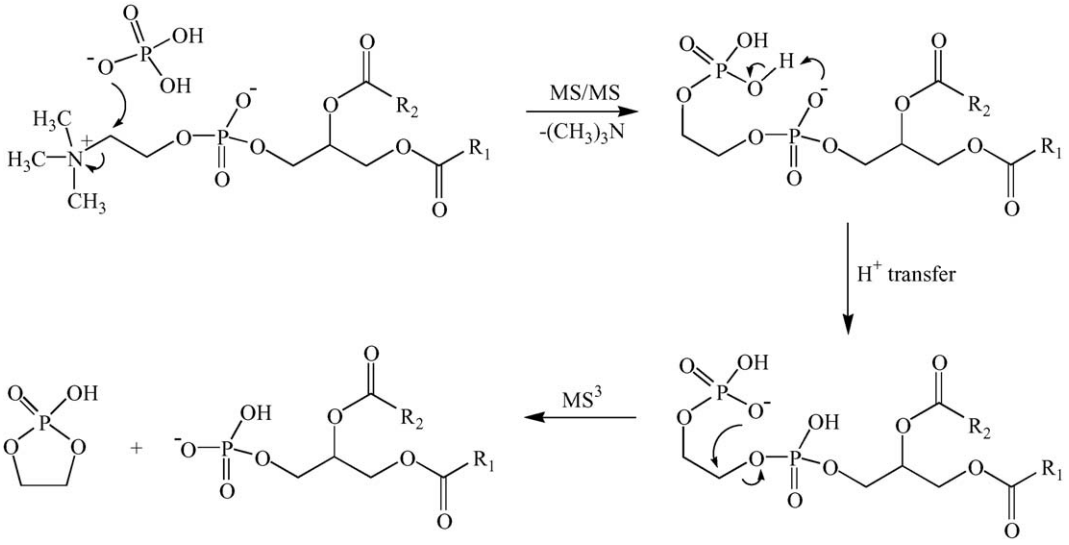


Fig. 6. (A) Negative ionization mode CID MS/MS product ion spectrum of the phosphate ($H_2PO_4^-$) adduct of PC 16:0, 18:1 (*m/z* 856); (B) CID MS^3 product ion spectrum of the *m/z* 797 product ion formed via the neutral loss of $(CH_3)_3N$ (59 Da) from Fig. 6A.

3.5. Deuterium isotope labeling and CID MS^3 and MS^4 experiments to probe the structure of the $[M + A - 59]^-$ product ion formed from the $H_2PO_4^-$ adducts of PC

CID MS/MS of the dihydrogen phosphate $H_2PO_4^-$ anionic adduct of PC 16:0, 18:1 (Fig. 6A) resulted in formation of the $-(A + CH_3)$, $-(AH + (CH_3)_3N)$ and $-(A + (CH_3)_3NCHCH_2)$ neutral loss product ions described above, as well as a unique product ion at *m/z* 797 corresponding to the neutral loss of 59 Da from the $[M + A]^-$ precursor ion. A similar loss was also observed by MS/MS of the dihydrogen phosphate anionic adduct of PC 16:0, 16:0 (Table 1). The formation of this −59 Da neutral loss product ion is consistent with the mechanism shown in Scheme 7, involving 'charge-directed' nucleophilic attack by the phosphate anion on an electrophilic carbon in the choline head group, resulting in elimination of $(CH_3)_3N$ and covalent incorporation of the anion. MS^3 of this product ion resulted in the loss of 124 Da, consistent with a composition of $C_2H_5PO_4$, as the major fragmentation pathway (Fig. 6B). This loss could occur by an E2 elimination reaction pathway similar to that shown in Scheme 6 for the loss of $(A + (CH_3)_3NCHCH_2)$, or by a neighboring group pathway following intramolecular proton transfer between the two phosphates within the product ion, to yield phosphatidic acid (Scheme 7). These two mechanisms can be readily distinguished from each other by examination of



Scheme 7.

the MS^3 spectra obtained from the $[M + A–(CH_3)_3N]^-$ product ion of the d_4-PC 16:0, 16:0 phosphate adduct. Dissociation of the adduct by the E2 elimination pathway would result in the loss of 127 Da ($H_2PO_4CD = CD_2$), whereas dissociation by the neighboring group pathway would result in the loss of 128 Da ($HPO_4CD_2CD_2$). The exclusive loss of 128 Da was observed from the d_4-PC 16:0, 16:0 lipid (data not shown), indicating that the loss of 124 from the $[M + A–(CH_3)_3N]^-$ product ion of the PC 16:0, 18:1 dihydrogen phosphate adduct corresponded to the cyclic $HPO_4CH_2CH_2$ neutral shown in Scheme 7. Finally, MS^4 of the $[M + A–((CH_3)_3N + HPO_4CH_2CH_2)]^-$ product ion at *m/z* 673 in Fig. 6B was observed to yield an identical spectrum (data not shown) to that obtained by MS/MS of the phosphatidic acid 16:0, 18;1 standard in Fig. 5B, further confirming the structure of this product ion.

3.6. Deuterium isotope labeling and CID MS^3 experiments to probe the structure of the $[M + A–(A + 1)]^-$ product ions formed from the anionic adducts of PC and isotope effect

CID MS/MS spectra of negatively charged PC ions formed by anionic adduction with ethyl carbonate, methylcarbonate and trifluoroacetate all resulted in the neutral loss of AH. The formation of these product ions requires either (i) deprotonation of the PC lipid to yield a product ion containing a single negative charge site i.e., neutralization of the quaternary alkyl ammonium ion within the choline head group, or (ii) deprotonation of the PC lipid at a site remote to the choline head group to yield a zwitterionic product ion. To determine the site of deprotonation for this neutral loss, we have examined the MS/MS product ion spectra obtained from the methylcarbonate anionic adducts of d_4-PC 16:0, 16:0, d_{62}-PC 16:0, 16:0, and d_{13}-PC 16:0, 16:0. The exclusive loss of AH (*m/z* 736) from the d_4-PC 16:0, 16:0 methylcarbonate adduct ion (Fig. 3A) indicates that the deprotonation reaction does not involve any of the CH_2CH_2 hydrogens within the phosphocholine head group. In contrast, the loss of AD (*m/z* 793) from the d_{62}-PC 16:0, 16:0 methylcarbonate adduct ion (Fig. 3B) indicates that the deprotonation reaction does involve a hydrogen from within the fatty acid. The loss of AH from the same precursor ion of d_{62}-PC 16:0,16:0 (*m/z* 794 in Fig. 3B) and the loss of AD from the d_{13}-PC 16:0, 16:0 lipid (*m/z* 744 in Fig. 3C) indicated a second site for deprotonation, likely involving a hydrogen from an *N*-methyl group of the phosphocholine head group.

MS^3 of the AH neutral loss product ion from the PC 16:0, 16:0 methylcarbonate adduct at *m/z* 732 in Fig. 1B revealed a unique product ion at *m/z* 269, 14 mass units higher than the $RCOO^-$ ion at *m/z* 255 (Fig. 7A). Likewise, a product ion at *m/z* 272, 17 mass units higher than the $RCOO^-$ ion was observed from the MS^3 of d_{13}-PC 16:0, 16:0 (data not shown). Based on the data obtained from Fig. 3B, which indicated that deprotonation can occur from the fatty acid chains, a mechanism for the formation of this *m/z* 269 product ion is proposed in Scheme 8 (Pathway 2). Deprotonation of a α-CH_2 group adjacent to either of the carbonyl groups in the fatty acid chains would initially yield an enolate anion, which may then undergo

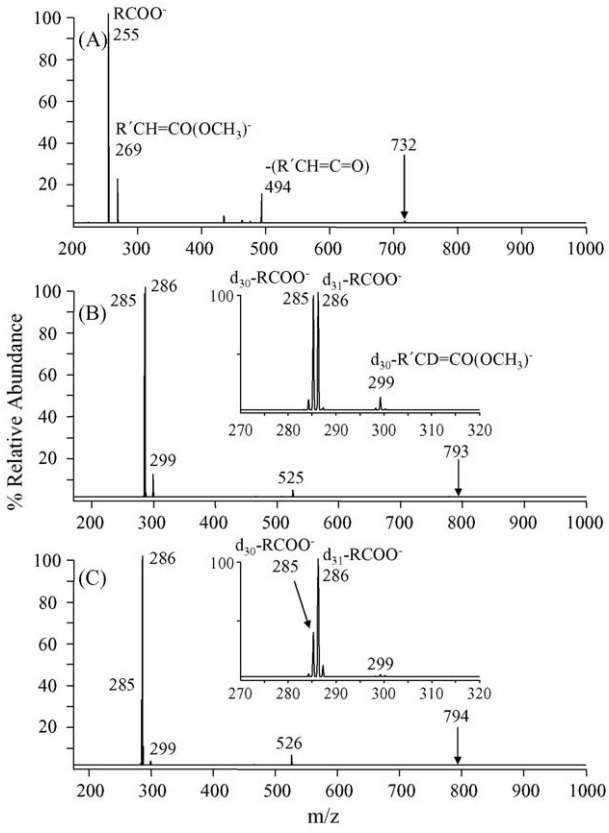
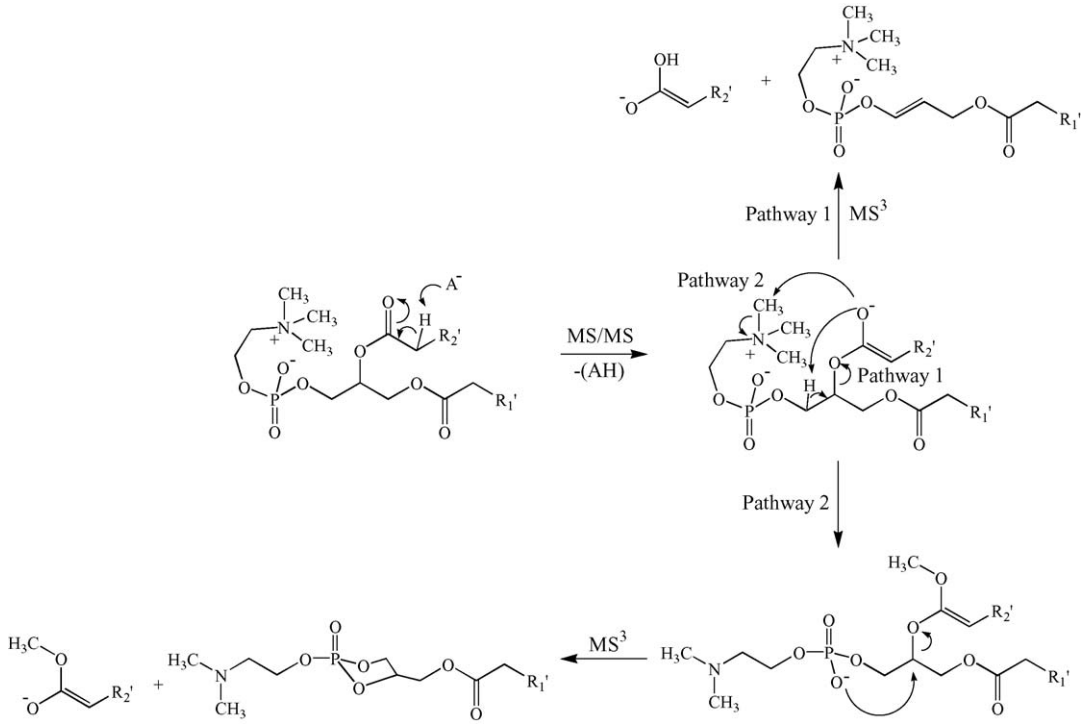


Fig. 7. Negative ionization mode CID MS^3 product ion spectrum of (A) the *m/z* 732 product ion formed via the neutral loss of (AH) from the methylcarbonate adduct of PC 16:0, 16:0 in Fig. 1B; (B) the *m/z* 793 product ion formed via the neutral loss of (AD) from the methylcarbonate adduct of PC d_{31}-16:0, d_{31}-16:0 in Fig. 3B; (C) the *m/z* 794 product ion formed via the neutral loss of (AH) from the methylcarbonate adduct of PC d_{31}-16:0, d_{31}-16:0 in Fig. 3B.

rearrangement by methyl group transfer from the quaternary alkyl ammonium ion of the choline head group. MS^3 of this rearranged product ion would give rise to the product ion at *m/z* 269, corresponding to $R'CH = CO(OCH_3)^-$, via a similar neighboring group elimination reaction mechanism to that proposed earlier for formation of the $RCOO^-$ product ions. Evidence for this mechanism was provided by individually subjecting the –(AD) and –(AH) neutral loss product ions from the d_{62}-PC 16:0, 16:0 lipid adduct, i.e., where the isomeric product ions formed by deprotonation at the two or more different sites within the PC lipid adduct ion are resolved from each other, to further dissociation by MS^3 (Fig. 7B and C, respectively). From these spectra, it can be seen that an equivalent product ion to the one at *m/z* 269 in Fig. 7A is only observed from the –(AD) neutral loss product (*m/z* 299 in Fig. 7B), indicating that this ion is formed by deprotonation of the fatty acid chain.

Interestingly, CID MS/MS of the trifluoroacetate adduct of the d_4-PC 16:0, 16:0 and d_{62}-PC 16:0, 16:0 lipids resulted in exclusive AH loss (*m/z* 736 and *m/z* 794 in Fig. 8A and B, respectively), while that of d_{13}-PC 16:0, 16:0 produced only AD loss (*m/z* 744 in Fig. 8C), indicating that the deprotonation reaction involved a hydrogen (or deuterium) from an *N*-methyl



Scheme 8.

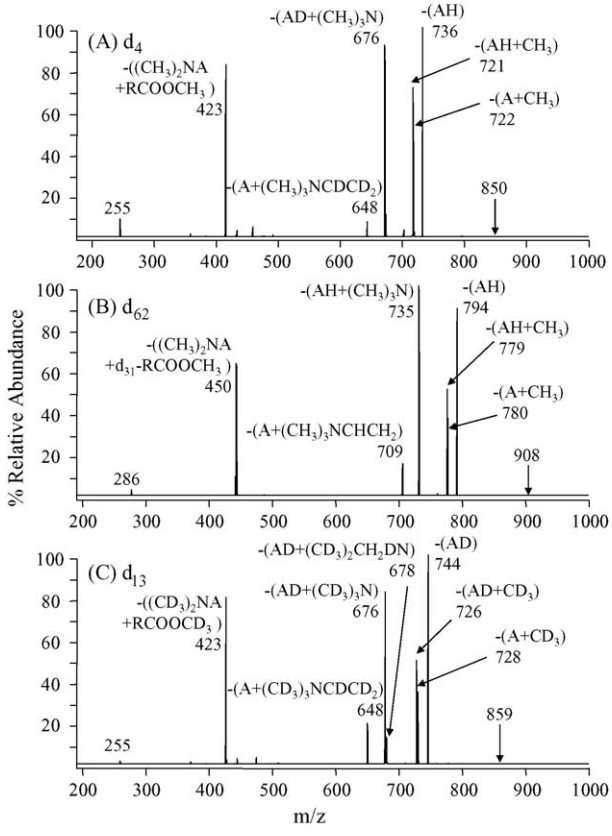
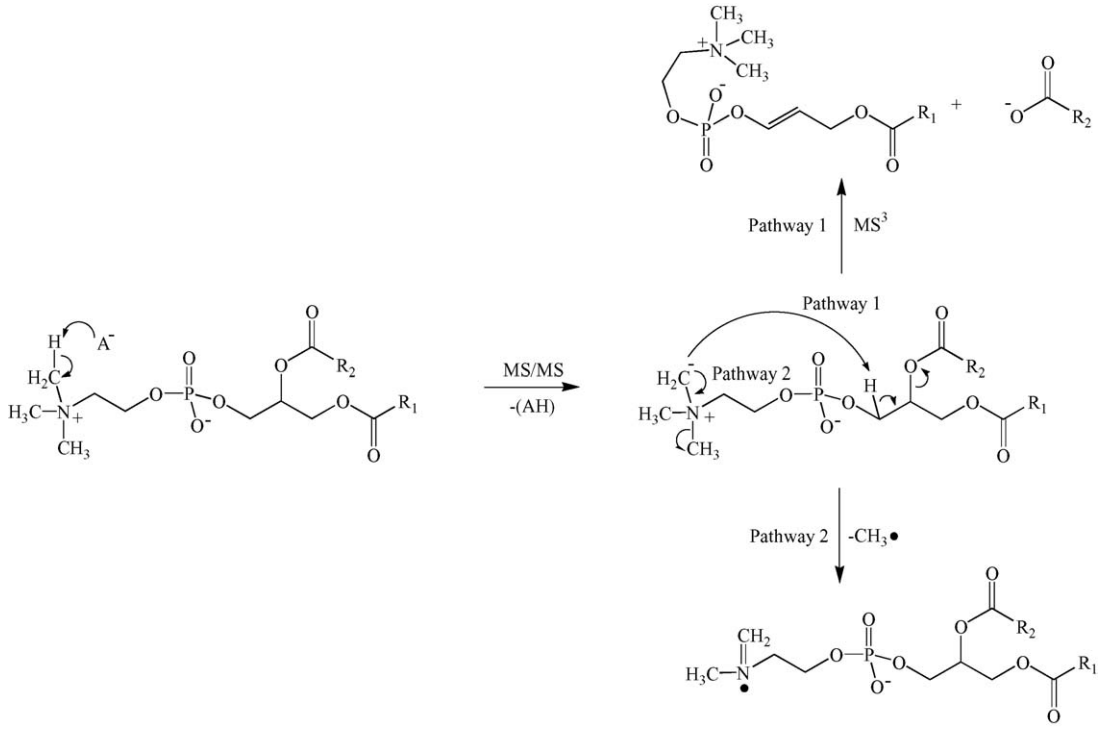


Fig. 8. Negative ionization mode CID MS/MS product ion spectra of the trifluoroacetate adducts of (A) d_4-PC 16:0, 16:0 (*m/z* 850); (B) PC d_{31}-16:0, d_{31}-16:0 (*m/z* 908) and (C) d_{13}-PC 16:0, 16:0 (*m/z* 859).

group of the phosphocholine head group (Scheme 9, pathway 1). Furthermore, MS^3 of each of these product ions indicated that the characteristic methyl group transfer product ion was almost absent in each case (data not shown), further indicating that the deprotonation reaction did not involve a hydrogen from the fatty acid chains. These differences observed by dissociation of the neutral loss product ions from the methylcarbonate and trifluoroacetate adducts indicate that the site of deprotonation, and therefore the structures of the −(AH) neutral loss MS/MS product ion at *m/z* 794, are highly dependent on the identity of the anion.

3.7. Deuterium isotope labeling and CID MS^3 experiments to probe the structure of the $[M + A–(A + 16)]^-$ product ions formed from MS/MS of anionic adducts of PC

The (A + 16) neutral loss CID MS/MS product ion observed at *m/z* 717 from the methylcarbonate adduct of PC 16:0, 16:0 (Fig. 1B), should contain one nitrogen atom but has an odd mass, suggesting that it is an odd electron species generated from the even electron precursor ion $[M + A]^-$ via the combined losses of AH and a $CH_3^\bullet$ radical. This neutral loss was found at particularly high abundance from the trifluoroacetate adduct of both the PC 16:0, 16:0 and PC 16:0, 18:1 lipid species (Table 1). MS/MS analysis of the $CF_3CO_2^-$ adducts of d_4-PC 16:0, 16:0 and d_{62}-PC 16:0, 16:0 (Fig. 8A and B, respectively) both resulted only in the neutral loss of $(AH + CH_3^\bullet)$, while the d_{13}-PC 16:0, 16:0 (Fig. 8C) exclusively lost $(AD + CD_3^\bullet)$, indicating that the proton involved in such a neutral loss originates only from an *N*-methyl group of the phosphocholine head group (Scheme 9, pathway 2).



Scheme 9.

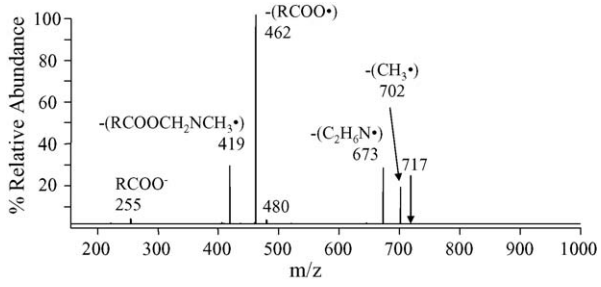


Fig. 9. Negative ionization mode CID MS^3 product ion spectrum of the *m/z* 717 product ion formed via the neutral loss of (A + 16) from the trifluoroacetate adduct of PC 16:0, 16:0 in Table 1.

MS^3 of the *m/z* 717 product ion generated by neutral loss of (A + 16) from the trifluoroacetate adduct of PC 16:0, 16:0 in Table 1 resulted in the spectrum shown in Fig. 9. Neutral losses of 15 Da (*m/z* 702), 43 Da (*m/z* 673), 255 Da (*m/z* 462) and 298 Da (*m/z* 419) were observed, corresponding to even electron species generated via the loss of neutral radicals $CH_3^{\bullet}$, $C_2H_6N^{\bullet}$, $RCOO^{\bullet}$ and $RCOOCH_2NCH_3^{\bullet}$ respectively, in addition to a product ion at *m/z* 255 ($RCOO^-$). Similar losses of neutral radicals $CD_3^{\bullet}$, $C_2D_5HN^{\bullet}$, $RCOO^{\bullet}$ and $RCOOCD_2NCD_3^{\bullet}$ were observed from the MS^3 spectrum of the d_{13}-PC 16:0, 16:0 lipid species.

3.8. Rationalizing the effects of anions on the gas-phase fragmentation behavior of anionic adducts of PC

A previous ab initio computational study on the gas-phase structure of zwitterionic PC has predicted that the phosphocholine head group adopts a cyclic conformation, stabilized by multiple intramolecular electrostatic interactions between the phosphate oxygens and the *N*-methyl groups within the choline head group [52]. Another study to examine the effect of hydration on the gas-phase structure of PC lipid molecules has found a strong interaction between water molecules and the phosphate oxygens, as well as with methyl and methylene moieties of the choline headgroup, resulting in a hydration driven conformational change of the PC headgroup [53]. Extensive computational studies to determine the gas-phase structures of the various anionic PC lipid adducts described above were beyond the scope of this paper. However, it is reasonable to expect that the fragmentation behaviors observed for the various PC lipid anionic adducts are likely to be highly dependent on the ability of the anion to disrupt these intramolecular electrostatic interactions, for example by forming multiple intermolecular electrostatic interactions with the *N*-methyl groups within the choline head group or by forming strong hydrogen bonds [53–55] with the phosphate oxygens of the phosphocholine head group, or due to the ability of the anion to form strong hydrogen bonds with the glycerol backbone or the ester linkage of fatty acid chains, hence opening up alternative dissociation pathways. Here, in order to obtain a rudimentary picture of the major inter- and intra-molecular interactions associated with formation of the anionic PC adducts, we have conducted a preliminary conformational search at the PM3 semi empirical level of theory of the chloride-PC anionic adduct, using the model phosphatidylcholine lipid PC 6:0, 6:0. PM3 level calculations were performed here due to the large size of the lipid structures, thereby making ab initio or density functional theory searches computationally prohibitive. Fig. 10 shows the lowest energy conformation found

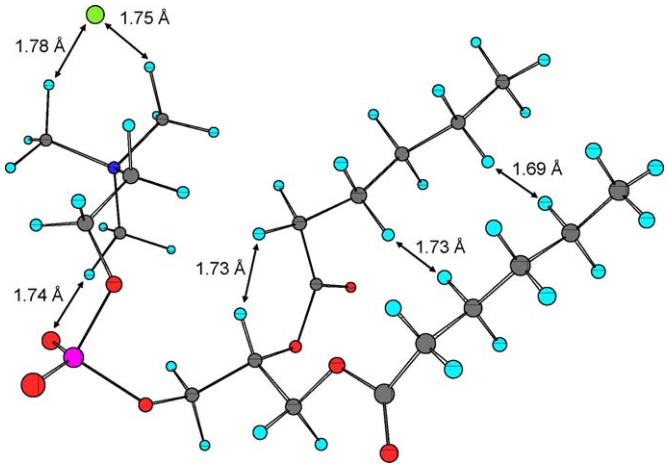


Fig. 10. Most stable structure (calculated at the PM3 level of theory) for the anionic chloride adduct of the model phosphatidylcholine lipid PC 6:0, 6:0.

for the PC 6:0, 6:0 lipid with the chloride anionic adduct. It can be seen from Fig. 10 that the chloride anion is exclusively associated with the lipid species via intermolecular electrostatic interactions with two of the choline methyl groups, while the remaining choline methyl group is involved in an intramolecular electrostatic interaction with the anionic phosphate oxygen. The two acyl fatty acid chains were found to be closely associated with each other by the formation of multiple intramolecular hydrophobic interactions. Overall, these interactions are consistent with the experimentally observed fragmentation behavior for this adduct, involving the exclusive loss of $(A + CH_3)$ via an intermolecular S_N2 reaction.

The competition between S_N2 and E2 reactions in the gas-phase, such as those involving the loss of $(A + CH_3)$, $(AH + (CH_3)_3N)$ and $(A + (CH_3)_3NCHCH_2)$ via intermolecular S_N2, intermolecular E2 or intramolecular E2 reactions in the work described here, respectively, have been the subject of several previous studies by Gronert [56]. Notably, Beauchamp and co-workers have recently observed competition between S_N2 and E2 pathways for reaction of the triphosphate dianion with choline, a closely related system to that examined here [57]. Also, O'Hair and co-workers have recently described an S_N2 reaction for the fragmentation a PC dimer in positive ion mode MS/MS, resulting in gas-phase cross-linking via the loss of $N(CH_3)_3$ [58]. However, none of these studies have demonstrated a clear correlation between the observed gas-phase S_N2 or E2 fragmentation behaviors as a function of the gas-phase acidity (Table 1), nucleophilic strength [59], hard/soft acid base properties [60] or steric accessibility of the investigated species. Thus, it is likely that it is a combination of these factors, together with the respective activation barriers, that control the experimentally determined branching ratios for the observed fragmentation reactions. Further studies involving molecular dynamic simulations combined with molecular orbital calculations are currently underway to obtain more detailed insights into the nature of the various anion-PC lipid interactions, and their role in controlling the observed fragmentation reactions.

4. Conclusions

Phosphocholine containing lipids such as PC are readily observed in negative ion mode by adduction with a range of different anionic species. However, the observed MS/MS fragmentation reactions of the $[M + A]^-$ ions formed from these anionic adducts are highly dependent on the identity of the anions. Based on MS^n experiments of each of these adducts, as well as MS/MS and MS^n experiments using deuterium labeled lipids and authentic lipid standards, structures and mechanisms for the formation of each of the product ions have been proposed. The results from this study indicate that great care must be taken to control the identity of the anions employed during lipid isolation, purification and separation, in order to allow the development and application of sensitive and robust neutral loss scan methods for mass spectrometry based lipid identification and quantitative analysis.

Acknowledgements

We gratefully acknowledge Professor Shelagh Ferguson-Miller for providing support for this study to Xi Zhang via NIH GM26916, the Quantitative Biology and Modeling Initiative (QBMI) and the Research Excellence Funds Center for Structural Biology of Membrane Proteins at Michigan State University.

References

[1] M. Svensson-Ek, J. Abramson, G. Larsson, S. Tornroth, P. Brzezinski, S. Iwata, J. Mol. Biol. 321 (2002) 329.
[2] T. Tsukihara, K. Shimokata, Y. Katayama, H. Shimada, K. Muramoto, H. Aoyama, M. Mochizuki, K. Shinzawa-Itoh, E. Yamashita, M. Yao, Y. Ishimura, S. Yoshikawa, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 15304.
[3] A.M. Distler, J. Allison, C. Hiser, L. Qin, Y. Hilmi, S. Ferguson-Miller, Eur. J. Mass Spectrom. 10 (2004) 295.
[4] J.-P. Cartailler, H. Luecke, Annu. Rev. Biophys. Biomol. Struct. 32 (2003) 285.
[5] A. Camara-Artigas, D. Brune, J.P. Allen, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 11055.
[6] R.M. Garavito, S. Ferguson-Miller, J. Biol. Chem. 276 (2001) 32403.
[7] K. Simons, W.L.C. Vaz, Annu. Rev. Biophys. Biomol. Struct. 33 (2004) 269.
[8] L.J. Pike, X. Han, R.W. Gross, J. Biol. Chem. 280 (2005) 26796.
[9] L.J. Pike, X. Han, K.-N. Chung, R.W. Gross, Biochemistry 41 (2002) 2075.
[10] B. Brugger, G. Erben, R. Sandhoff, F.T. Wieland, W.D. Lehmann, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 2339.
[11] M. Pulfer, R.C. Murphy, Mass Spectrom. Rev. 22 (2003) 332.
[12] X. Han, R.W. Gross, Mass Spectrom. Rev. 24 (2005) 367.
[13] X. Han, R.W. Gross, J. Lipid Res. 44 (2003) 1071.
[14] M. Koivusalo, P. Haimi, L. Heikinheimo, R. Kostiainen, P. Somerharju, J. Lipid Res. 42 (2001) 663.
[15] A. Zacarias, D. Bolanowski, A. Bhatnagar, Anal. Biochem. 308 (2002) 152.
[16] R. Taguchi, T. Houjou, H. Nakanishi, T. Yamazaki, M. Ishida, M. Imagawa, T. Shimizu, J. Chromatogr. B 823 (2005) 26.
[17] F.-F. Hsu, A. Bohrer, J. Turk, J. Am. Soc. Mass Spectrom. 9 (1998) 516.
[18] F.-F. Hsu, J. Turk, J. Am. Soc. Mass Spectrom. 11 (2000) 437.
[19] F.-F. Hsu, J. Turk, J. Am. Soc. Mass Spectrom. 11 (2000) 797.

[20] F.-F. Hsu, J. Turk, J. Am. Soc. Mass Spectrom. 12 (2001) 1036.
[21] F.-F. Hsu, J. Turk, J. Am. Soc. Mass Spectrom. 16 (2005) 1510.
[22] F.-F. Hsu, J. Turk, J. Am. Soc. Mass Spectrom. 11 (2000) 986.
[23] S.B. Milne, P.T. Ivanova, D. DeCamp, R.C. Hsueh, H.A. Brown, J. Lipid Res. 46 (2005) 1796.
[24] F.-F. Hsu, J. Turk, E.R. Rhoades, D.G. Russell, Y. Shi, E.A. Groisman, J. Am. Soc. Mass Spectrom. 16 (2004) 491.
[25] F.-F. Hsu, J. Turk, J. Am. Soc. Mass Spectrom. 11 (2000) 892.
[26] X. Han, R.W. Gross, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 10635.
[27] X. Han, R.W. Gross, J. Am. Soc. Mass Spectrom. 6 (1995) 1202.
[28] X. Han, R.A. Gubitosi-Klug, B.J. Collins, R.W. Gross, Biochemistry 35 (1996) 5822.
[29] T. Houjou, K. Yamatani, H. Nakanishi, M. Imagawa, T. Shimizu, R. Taguchi, Rapid Commun. Mass Spectrom. 18 (2004) 3123.
[30] T. Houjou, K. Yamatani, M. Imagawa, T. Shimizu, R. Taguchi, Rapid Commun. Mass Spectrom. 19 (2005) 654.
[31] K.A. Harrison, R.C. Murphy, J. Mass Spectrom. 30 (1995) 1772.
[32] C.J. DeLong, P.R.S. Baker, M. Samuel, Z. Cui, M.J. Thomas, J. Lipid Res. 42 (2001) 1959.
[33] A.A. Karlsson, P. Michelsen, G. Odham, J. Mass Spectrom. 33 (1998) 1192.
[34] W.D. Lehmann, M. Koester, G. Erben, D. Keppler, Anal. Biochem. 246 (1997) 102.
[35] J.L. Kerwin, A.R. Tuininga, L.H. Ericsson, J. Lipid Res. 35 (1994) 1102.
[36] N. Khaselev, R.C. Murphy, J. Lipid Res. 41 (2000) 564.
[37] S. Uran, Å. Larsen, P.B. Jacobsen, T. Skotland, J. Chromatogr. B 758 (2001) 265.
[38] D. Pacetti, M. Malavolta, F. Bocci, E. Boselli, N.G. Frega, Rapid Commun. Mass Spectrom. 18 (2004) 2395.
[39] F.-F. Hsu, J. Turk, J. Mass Spectrom. 35 (2000) 596.
[40] Å. Larsen, S. Uran, P.B. Jacobsen, T. Skotland, Rapid Commun. Mass Spectrom. 15 (2001) 2393.
[41] E. Hvattum, G. Hagelin, Å. Larsen, Rapid Commun. Mass Spectrom. 12 (1998) 1405.
[42] F.-F. Hsu, J. Turk, J. Am. Soc. Mass Spectrom. 15 (2004) 536.
[43] F.-F. Hsu, J. Turk, Y. Shi, E.A. Groisman, J. Am. Soc. Mass Spectrom. 15 (2004) 1.
[44] N. Mazzella, J. Molinet, A.D. Syakti, A. Dodi, P. Doumenq, J. Artaud, J.-C. Bertrand, J. Lipid Res. 45 (2004) 1355.
[45] M.L. Gross, Int. J. Mass Spectrom. 200 (2000) 611.
[46] N.J. Jensen, K.B. Tomer, M.L. Gross, Lipids 21 (1986) 580.
[47] Z.H. Huang, D.A. Gage, C.C. Sweeley, J. Am. Soc. Mass Spectrom. 3 (1992) 71.
[48] A. Hayashi, T. Matsubara, M. Morita, T. Kinoshita, T. Nakamura, J. Biochem. 106 (1989) 264.
[49] K.A. Kayganich-Harrison, D.M. Rose, R.C. Murphy, J.D. Morrow, L.J. Roberts II, J. Lipid Res. 34 (1993) 1229.
[50] C.Y. Lee, A. Lesimple, Å. Larsen, O. Mamer, J. Genest, J. Lipid Res. 46 (2005) 1213.
[51] S. Gronert, Mass Spectrom. Rev. 24 (2005) 100.
[52] J. Landin, I. Pascher, D. Cremer, J. Phys. Chem. A 101 (1997) 2996.
[53] W. Pohle, D.R. Gauger, M. Bohl, E. Mrazkova, P. Hobza, Biopolymers 74 (2004) 27.
[54] Y. Cai, B.C. Richard, Anal. Chem. 74 (2002) 985.
[55] Y. Cai, M.C. Concha, J.S. Murray, R.B. Cole, J. Am. Soc. Mass Spectrom. 13 (2002) 1360.
[56] S. Gronert, Acc. Chem. Res. 36 (2003) 848.
[57] H. Cox, R. Hodyss, J.L. Beauchamp, J. Am. Chem. Soc. 127 (2005) 4084.
[58] P.F. James, M.A. Perugini, R.A.J. O'Hair, J. Am. Soc. Mass Spectrom. 17 (2006) 384.
[59] A. Streitweiser, Chem. Rev. 56 (1956) 571.
[60] T.-L. Ho, Chem. Rev. 75 (1975) 1.