papers on methodology

Identification of molecular species of glycerophospholipids and sphingomyelin using electrospray mass spectrometry

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Abstract This paper describes the use of positive and negative ion electrospray mass spectrometry (MS) and MS/MS (tandem mass spectrometry) to identify glycerophospholipid and ceramide headgroups and their alkyl, alkenyl and acyl constituents. Molecular ion adducts were the primary products formed by positive ionization, occurring as $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$, $[M + formate]^+$, or $[M + acetate]^+$, depending upon the class of glycerophospholipid and the presence or absence of these ionization-promoting species. Similar (negatively charged) ions corresponding to the loss of the groups listed above were formed in negative ion MS. Positive ion electrospray MS/MS provides information on the nature of the headgroup, with the formation of an ion corresponding to the headgroup itself, or the loss of the headgroup from the molecular ion H^+ or Na^+ adduct. Acyl constituents are identified during negative ion MS/MS from the formation of their $RCOO^-$ ions. The nature of alkyl or alkenyl substituents in glycerophosphoethanolamine (PE) molecular species can be identified from residual ions following the loss of ethanolamine plus loss of the acyl moiety in the *sn*-2 position, and cyclization of a phosphate oxygen with C-2 of glycerol. In glycerophosphoinositol (PI) species, it appears that an RCO^- ion is formed during negative ion MS/MS, possibly to steric interference from the bulky phosphoinositol headgroup that prevents cyclization (and subsequent stabilization) of the ion described for PE species. Positive and negative ion electrospray MS spectra for molecular species of commercial preparations of PE, PI, phosphatidylserine (PS), glycerophosphocholine (PC) and sphingomyelin (SM) produced similar profiles. For phospholipids occurring as Na^+ adducts, concentrations above ca. 1 ng/μl produced significant quantities of both $[M + H]^+$ and $[M + Na]^+$ ions for those molecular species present in the largest quantities, complicating interpretation of the spectra. Complete profiles of molecular species were obtained from as little as 10 picograms of material. Major components of PE were identified from 0.1 picogram total lipid. Using single ion monitoring of the Na^+ adduct of β-acetyl-γ-O-hexadecyl L-α-phosphatidylcholine, 10 femtograms of material was detected. A mixture of 1 nanogram each of PE, PI, PS, and PC was readily resolved into individual molecular species, with little apparent loss of resolution or preferential ionization. Electrospray MS did not provide information on the position (*sn*-1 or *sn*-2) of fatty acids, and was not capable of differentiating in all instances between alkyl-acyl and alkenyl-acyl substituents without prior separation of these lipid subclasses. Although not reported on in detail, molecular species of other classes of lipids and phospholipids including glycolipids, phosphatidylglycerol, and mono- and dimethylphosphatidylethanolamine can also be analyzed using these techniques. Electrospray MS and MS/MS provides a rapid, sensitive and quantitative alternative to previously published methods for analyses of molecular species.—**Kerwin, J. L., A. R. Tuininga, and L. H. Ericsson.** Identification of molecular species of glycerophospholipids and sphingomyelin using electrospray mass spectrometry. *J. Lipid Res.* 1994. **35:** 1102–1114.

Supplementary key words glycerophosphocholine • glycerophosphoethanolamine • phosphatidylserine • glycerophosphoinositol • ceramides

Membranes from plant, animal, and fungal organisms contain diverse glycerophospholipids as major constituents. Numerous molecular species are found in a single class of phospholipid, and these occur in characteristic proportions in different species, organs, subcellular organelles, and developmental stages (1–6). The composition of molecular species can affect membrane fluidity, which in turn will alter the activity of many membrane-bound proteins (7–9). Phospholipids also serve as sources of arachidonic acid and other polyunsaturated fatty acids which can be metabolized by various oxygenase enzymes. This leads to the formation of prostaglandins, hydroxy- or epoxy-fatty acids, jasmonic acids, and related metabolites that regulate diverse biological functions (10–12).

There have been many studies of glycerophospholipid molecular species because of their importance in the development and function of biological systems. Characterization by traditional techniques is difficult, however, due to the great number of individual molecular species that

Abbreviations: MS, mass spectrometry; MS/MS, tandem mass spectrometry; PS, phosphatidylserine; PE, glycerophosphoethanolamine; PI, glycerophosphoinositol; PC, glycerophosphocholine; SM, sphingomyelin; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; FAME, fatty acid methyl esters; GC–MS, gas chromatography–mass spectrometry; CI, electron capture negative chemical ionization; FAB, fast atom bombardment; HAPC, β-acetyl-γ-O-hexadecyl phosphatidylcholine.

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can occur, e.g., the 69 diacyl species identified in various organs of the cod, *Gadus morhua* (4). A variety of approaches to identification of molecular species have been taken, usually requiring one or more preliminary separations of glycerophospholipid classes and subclasses using thin-layer chromatography (TLC) and/or normal phase high pressure liquid chromatography (HPLC) of intact lipids. Most techniques then require separation of diacyl-, 1-alkyl-2-acyl-, and 1-alkenyl-2-acylglycerol fractions, often requiring phospholipase C removal of the headgroups and derivatization of the free hydroxyl moiety (13–16). Identification of molecular species can then be made using a variety of techniques: HPLC of intact glycerophospholipids, followed by transmethylation of individual peaks and analysis of fatty acid methyl esters (FAME) by gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS) (13); HPLC of benzoyl, 3,5-dinitrobenzoyl, or 7-[(chlorocarbonyl)-methoxy]-4-methylcoumarin or other diglyceride derivatives (17–20); electron capture negative chemical ionization (CI) mass spectrometry (21); HPLC in tandem with CI mass spectrometry (22); fast atom bombardment (FAB) (23); and GC-MS of trimethylsilyl or *t*-butyldimethylsilyl derivatives (24–28).

Electrospray mass spectrometry is a relatively new method for soft ionization of biological molecules. In electrospray mass spectrometry, ions dissolved in a suitable solvent are charged and droplets are formed at atmospheric pressure and room temperature by electrostatic nebulization. The solvent is evaporated by a curtain gas until only the charged solute remains, which is subsequently drawn into the low vacuum of a mass analyzer. Although used extensively in protein chemistry (29), this technique has been used sparingly in structure determination of lipids (30–32). In this report, we show that positive and negative ion electrospray MS and MS-MS is a rapid and sensitive method for structure determination of molecular species of intact glycerophospholipids and sphingomyelin.

MATERIALS AND METHODS

Glycerophospholipid standards used in this investigation were: β-acetyl-γ-O-hexadecyl L-α-phosphatidylcholine, synthetic, cat. no. P-4904, lot 91H0444; L-α-phosphatidylethanolamine, type I from bovine brain, cat. no. P-9137, lot 82H83511; L-α-phosphatidylcholine, type III H from bovine heart, lot 40H8356; sphingomyelin from bovine brain, cat. no. S-7004, lot 41H8356 all from Sigma Chemical Co., St. Louis, MI. From Avanti Polar Lipids, Inc., Alabaster, AL, we obtained L-α-phosphatidylserine, Na^+ salt, from brain, cat. no. BPS-493A, lot 840032; and L-α-phosphatidylinositol, Na^+ salt, from liver, cat. no. LPI-170. Phospholipids were at least 98% pure and unoxidized as monitored using diode array detection (200–240 nm) of samples analyzed by normal phase high pressure liquid chromatography. Initially, samples were dissolved in methanol–acetonitrile–formic acid 49:49:2 (v/v/v) for positive ion electrospray, and in methanol with 5 mM ammonium acetate plus 0.1% formic acid for negative ion electrospray. The final series of experiments used 5 mM ammonium acetate in methanol. Unless otherwise noted, the sample concentration was 1–50 ng/μl per phospholipid class.

Lipid samples were analyzed by electrospray ionization MS on a triple-quadrapole Sciex API III instrument (PE/SCIEX Thornhill, Ontario, Canada). Samples were infused into the electrospray source via a 50 μM i.d. fused silica transfer line using a Harvard Apparatus pump at 3 μl/min. Spectra from samples containing 5 ng/μl of lipid or more were acquired in less than 10 sec total acquisition time. Glycerophospholipids prepared at concentrations lower than this required 10 to ca. 60 sec acquisition time. Positive ion MS or MS/MS was run with an orifice voltage from 50 to 80 volts. Negative ion MS or MS/MS was run with an orifice voltage from −60 to −80 volts. The interface temperature was maintained at 52°C. For tandem mass spectrometry (MS/MS), precursor ions were selected with the first of three quadrapoles (Q1) for collision-induced dissociation with argon in the second quadrapole (Q2). The third quadrapole (Q3) was scanned with a mass step of 0.20 Da and 1 ms/step. Parent ion transmission was maximized by reducing the resolution of Q1 to transmit a 2 to 3 *m/z* window about the selected parent ion, and Q3 resolution was adjusted to approximately 50% valley between peaks 3 Da apart. Spectra were collected and analyzed using proprietary software from Sciex Corporation.

RESULTS

β-Acetyl-γ-O-hexadecyl phosphatidylcholine

Initial electrospray MS used a single compound of known composition, synthetic β-acetyl-γ-O-hexadecyl phosphatidylcholine (HAPC) to determine solvents and operating conditions under which structurally useful information could be obtained from phospholipids. The parent ion at *m/z* 524 was readily obtained in positive ion electrospray MS. Addition of formic acid was not necessary to see the parent ion from choline-containing species due to the presence of the quaternary nitrogen, which is not found in other common phospholipids of biological origin. Also present in this spectrum was the sodium adduct (*m/z* 546), the sodium adduct minus acetate (*m/z* 487) and the phosphocholine headgroup at *m/z* 184.

When both formic acid and ammonium acetate were present, the major ion for HAPC in negative ion electrospray was the formate (*m/z* 568) adduct, with a daugh-

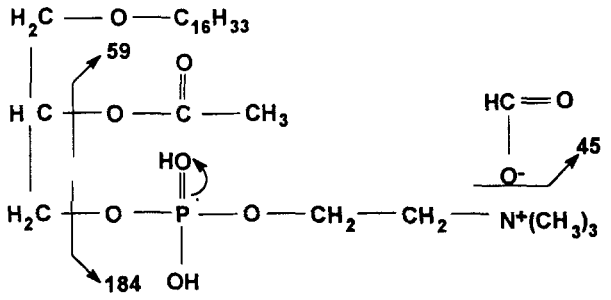

Fig. 1. Fragmentation patterns for HAPC.

ter ion at *m/z* 508, corresponding to loss of acetate from C-2 of glycerol, and abstraction of one of the two equally acidic protons from C-1 or C-2 after cyclization with the phosphate group (**Fig. 1**). There were several less intense ions of unidentified origin. HAPC was unique among the choline-containing compounds in that the formate rather than the acetate adduct was formed, as described below.

When HAPC was dissolved in 5 mM ammonium acetate in methanol, the [acetate adduct – H^+] (*m/z* 582) and [582 – acetate – CH_3] (*m/z* 508) were the major ions in negative ion MS. The positive ion spectrum was comparable to that found when both formate and acetate were present in the methanol used to dissolve the sample.

Positive ion MS/MS of *m/z* 524 from HAPC in formate-supplemented methanol resulted in a major ion from the phosphocholine headgroup at *m/z* 184. MS/MS of the parent ion also produced peaks due to the loss of acetate anion (*m/z* 481), loss of phosphocholine (*m/z* 341), *m/z* 104 [$HO\text{-}CH_2\text{-}CH_2\text{-}N(CH_3)_3^+$], acetate, and several unidentified ions. Negative ion MS/MS of *m/z* 568 produced the 508 ion discussed above.

Bovine brain glycerophosphoethanolamine

Over 50 molecular species were identified from bovine brain phosphatidylethanolamine (PE), which has at least 60% alkyl-acyl or alkenyl-acyl species, using positive and negative ion electrospray (**Table 1, Fig. 2**). Greater than 95% of the total ion intensity was due to [M + H^+] in positive ion and [M – H^+] in negative ion spectra. Note that in this and subsequent tables, only some of the species were examined using MS/MS to precisely determine fatty acid moieties. If the parent ions we examined are any indication, complete analysis of these mixtures would

TABLE 1. Molecular species of bovine brain glycerophosphoethanolamine

	% Composition[a]			% Composition	
Molecular Species	Positive	Negative	Molecular Species	Positive	Negative
16:0/18:0 alkenyl	0.94	1.29	18:0/22:6 alkyl	2.52	4.08
16:0/18:1	1.15	1.59	18:0/22:6 alkenyl	2.66	4.61
16:0/18:1 alkenyl	3.15	4.45	18:1/16:0 alkenyl	0.46	0.65
16:0/18:2	0.81	1.01	18:1/20:3 alkenyl	0.25	0.40
16:0/18:3	0.25	0.22	18:1/20:4	1.39	1.69
16:0/20:4	0.51	0.62	18:1/20:4 alkenyl	1.92	2.61
16:0/20:4 alkyl	0.64	0.88	18:1/22:0 alkenyl	2.01	0.65
16:0/20:4 alkenyl	0.87	1.33	18:1/22:6	1.68	0.45
16:0/22:4	0.47	0.52	18:2/20:4	1.38	1.48
16:0/22:4 alkyl	0.83	1.27	18:2/22:0 alkenyl	1.92	1.32
16:0/22:4 alkenyl	1.46	2.31	18:2/22:6	1.09	0.30
16:1/20:4	0.29	0.44	18:3/20:4	0.35	0.41
16:1/22:3 alkenyl	0.18	0.29	18:3/22:0	2.04	1.30
18:0/18:0	1.14	1.80	20:0/18:1	1.00	1.75
18:0/18:0 alkenyl	1.24	1.61	20:0/18:1 alkenyl	2.41	2.52
18:0/18:1	2.14	3.34	20:0/18:2	1.64	1.34
18:0/18:1 alkenyl	3.94	5.37	20:0/18:2 alkenyl	2.64	2.99
18:0/18:2	2.33	2.63	20:0/18:3	3.88	1.87
18:0/18:2 alkenyl	4.27	6.08	20:0/20:0	0.81	0.36
18:0/18:3	3.28	0.66	20:0/22:0 alkenyl	2.54	0.45
18:0/20:0 alkenyl	0.80	0.80	20:0/22:1	0.36	0.23
18:0/20:4	3.91	3.84	20:0/22:4	1.15	0.45
18:0/20:4 alkyl	1.33	2.03	20:0/22:4 alkyl	1.20	0.51
18:0/20:4 alkenyl	1.65	2.61	20:0/22:4 alkenyl	0.87	0.37
18:0/22:4	3.08	2.77	20:0/22:5	1.74	0.35
18:0/22:4 alkyl	1.90	2.54	20:0/22:5 alkyl	2.65	0.62
18:0/22:5	3.09	3.19	20:0/22:6	3.04	0.34
18:0/22:5 alkyl	3.12	4.94	20:0/22:6 alkyl	1.11	0.63
18:0/22:6	4.49	4.83			

[a]The listed percent composition was based on the relative intensities of each molecular ion from a single representative spectrum. Positive and negative ion spectra were run at least three times each, with less than 1% variation in relative peak intensity.

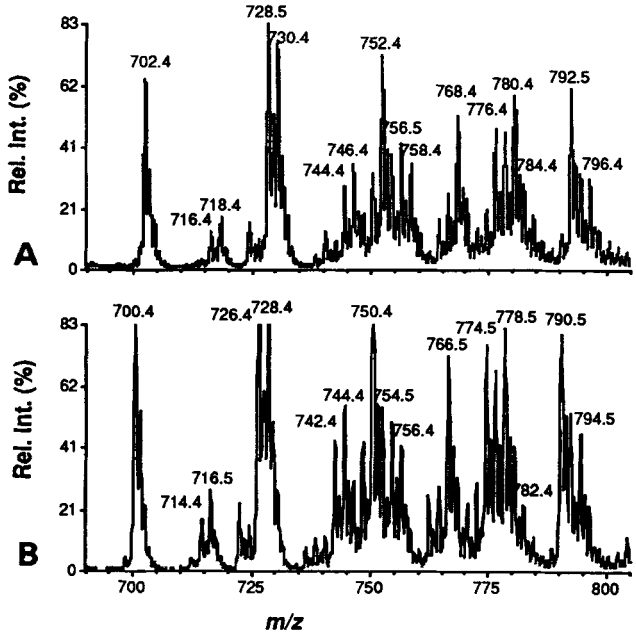

Fig. 2. Positive (A) and negative (B) ion spectra of bovine brain phosphatidylethanolamine.

result in the identification of at least twice the number of species listed in these tables. In those instances where more than one combination of fatty acyl or alkyl moieties could generate the same parent ion, listed molecular species are those most likely to occur based on fatty acid content of the preparations and molecular species data for these or similar phospholipids in the literature. Unlike the choline-containing lipids, molecular ion adducts were found at [M + 1] in positive ion and [M − 1] in negative ion MS. Interpretation of MS and MS/MS spectra for diacyl species was straightforward. For instance in the positive ion mode, MS/MS of *m/z* 792 showed a major ion at 651, corresponding to the loss of phosphoethanolamine. Also found was a peak at [M − 181] and a corresponding *m/z* 181 peak, probably from the PE headgroup plus glycerol. The negative ion MS/MS spectrum for this peak had two major peaks at *m/z* 283 and 327, corresponding to $RCOO^-$ for 18:0 and 22:6, respectively, showing this species to be 18:0/22:6 diacyl PE (**Fig. 3**).

A second example of diacyl PE spectra will now be presented to demonstrate the potential for quantitation using electrospray MS/MS. When the peak corresponding to M = 767 was examined, it was found that two species were present. Positive ion MS/MS showed the characteristic loss of the PE headgroup, with a major ion at *m/z* 627. The negative ion MS/MS spectrum had peaks at *m/z* 255, 283, 303, and 331, corresponding to $RCOO^-$ for 16:0, 18:0, 20:4, and 22:4, respectively, supporting the presence of both 16:0/22:4 and 18:0/20:4 diacyl PE. When the peak intensities for the complementary fatty acids were summed, it was found that ca. 88% of the signal was due to 18:0/20:4. Moreover, when the molecular weights of the respective pairs of fatty acids were divided, and compared with their corresponding peak intensities, these ratios were in very close agreement. The ratio of the molecular weight of 20:4 to 18:0 is 1.07 and the signal intensity ratio was 1.05. For 22:4 and 16:0 these figures were 1.29 and 1.36, respectively. This shows that there does not seem to be preferential loss of the fatty acid moiety from either the *sn*-1 or *sn*-2 carbon, and that signal intensities, certainly in MS/MS, can be used for quantitation.

MS/MS of alkyl-acyl and alkenyl-acyl PE species produced spectra significantly different from diacyl PE species in the positive ion mode. Using M = 751 as an example, positive ion MS/MS showed a peak at 611 corresponding to loss of the headgroup, and two major peaks at 361 and 389. The corresponding negative ion MS/MS spectrum had two major ions at *m/z* 303 and 331, due to $RCOO^-$ of 20:4 and 22:4 (**Fig. 4**). This data is best interpreted by assuming that there are two species present, 18:0/20:4 alkenyl-acyl and 16:0/22:4 alkenyl-acyl PE. The *m/z* 361 and 389 peaks seen in positive ion MS/MS resulted from loss of the acyl groups from the *sn*-2 of carbon of glycerol, cyclization of phosphate oxygen with C-2, loss of ethanolamine, and formation of a phosphonium ion (**Fig. 5**). Comparable fragmentation was found for M = 741, where we documented the occurrence of 16:0/18:2 alkenyl-acyl plus 18:2/16:0 alkenyl-acyl species, and for M = 753 where both 18:0/20:3 and 16:0/22:3 were found. As with PC, the alkyl or alkenyl fragment is not charged, (and perhaps not even formed), so is not seen in either mode of electrospray MS/MS; also, without subclass

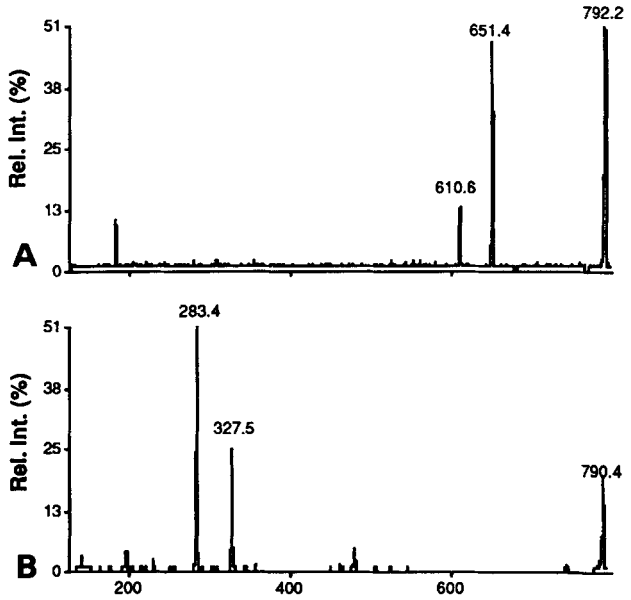

Fig. 3. Positive (A) and negative (B) ion MS/MS of 18:0/22:6 diacyl phosphatidylethanolamine.

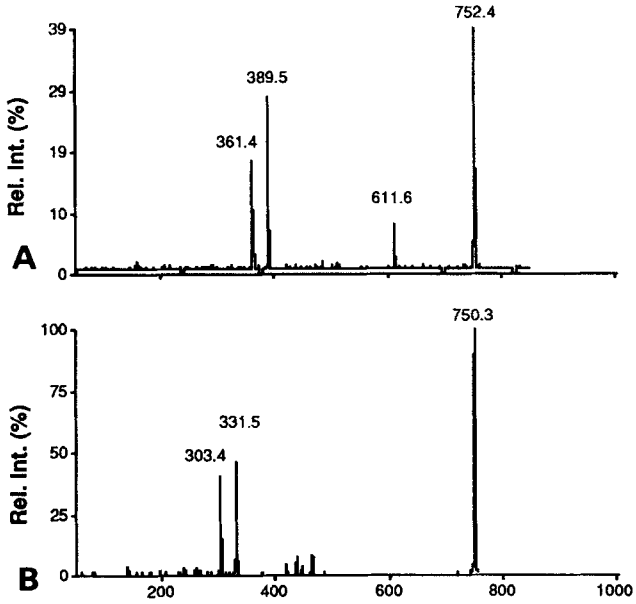

Fig. 4. Positive (A) and negative (B) ion MS/MS of 18:0/20:4 and 16:0/22:4 alkenyl/acyl phosphatidylethanolamine.

separation, it is not possible in some instances to differentiate between alkyl and alkenyl substituents.

Bovine brain glycerophosphocholine

Over 30 molecular species of glycerophosphocholine (PC) were found in bovine brain PC using positive ion electrospray MS (**Table 2**), including diacyl, alkyl-acyl, and alkenyl-acyl moieties. Because of the positively charged quaternary N, parent ions of choline-containing species occur as M^+ rather than $[M + 1]^+$ that is found for other phospholipid classes in positive ion electrospray. A comparable number of PC molecular species were identified using negative ion electrospray MS (Table 2), and the relative percentage composition was similar to that found with positive ion MS. Unlike HAPC, parent ions of PC species with longer chain fatty acids in the *sn*-2 position occurred primarily as acetate adducts in negative ion electrospray MS when both acetate and formate were present to promote ionization, with the acetate presumably quenching the positively charged quaternary N and a hydrogen ion extracted from one of the phosphate oxygens to produce the requisite negative charge. For some species

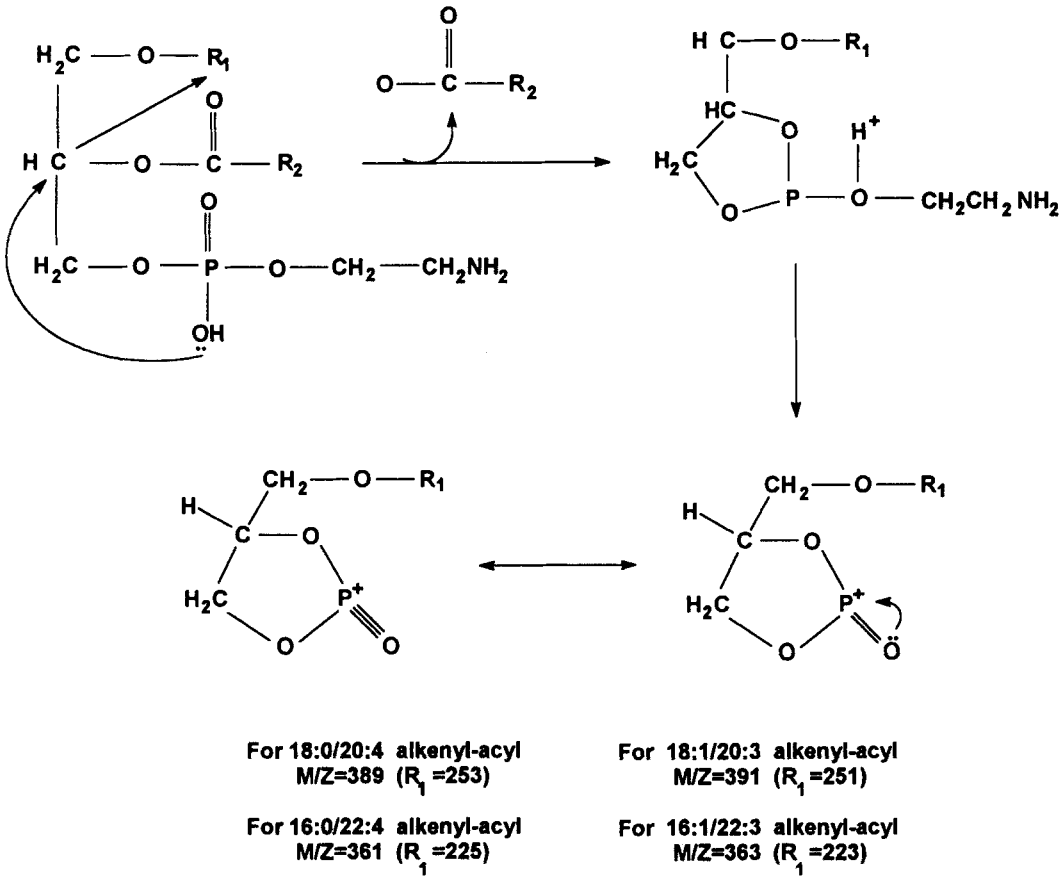

Fig. 5. Fragmentation patterns for PE ether lipids.

TABLE 2. Molecular species of bovine brain glycerophosphocholine

Molecular Species	% Composition[a] Positive	Negative	Molecular Species	% Composition Positive	Negative
14:0/18:0	1.18	1.09	18:0/18:2 alkyl	2.06	2.90
14:0/18:1	1.59	1.06	18:0/18:3	3.06	2.58
14:0/18:2	0.69	1.65	18:0/20:4	2.28	2.14
14:0/18:3	0.92	2.85	18:0/20:4 alkyl	1.43	2.78
14:0/20:4 alkenyl	0.31	0.45	18:0/22:5 alkyl	0.54	1.27
14:0/20:4 alkyl	1.40	0.74	18:0/22:6	0.49	0.98
16:0/18:0	1.57	1.88	18:0/22:6 alkyl	0.53	1.32
16:0/18:1	9.60	7.28	18:1/18:2 alkyl	1.13	1.07
16:0/18:1 alkyl	1.17	3.37	18:1/20:4	1.69	1.25
16:0/18:2	14.02	10.39	18:1/20:4 alkyl	1.28	1.37
16:0/18:2 alkyl	6.60	6.36	18:1/20:4 alkenyl	1.32	0.98
16:0/18:2 alkenyl	2.62	1.95	18:1/22:6	0.68	0.57
16:0/18:3	1.86	1.03	18:2/16:0 alkenyl	6.41	4.78
16:0/20:4	3.77	2.32	18:2/18:1 alkyl	0.82	0.77
16:0/20:4 alkyl	2.81	2.53	18:2/20:4	0.82	0.89
16:1/20:4	2.49	1.16	18:3/18:0 alkyl	0.59	0.56
16:1/20:4 alkyl	2.54	2.53	18:3/20:4	0.52	1.06
16:1/20:4 alkenyl	1.51	1.15	16:0/22:1 alkyl	0.34	1.39
16:2/20:4	1.47	0.97	16:0/22:2	0.46	2.89
18:0/18:0	2.00	1.39	16:0/22:2 alkyl	0.38	1.06
18:0/18:1	3.03	2.71	16:0/22:3	1.06	3.51
18:0/18:1 alkyl	1.15	2.26	16:0/22:3 alkyl	1.61	1.81
18:0/18:2	5.68	4.11	16:0/22:4	0.51	0.80

[a]The listed percent composition was based on the relative intensities of each molecular ion from a single representative spectrum. Positive and negative ion spectra were run at least three times for each sample, with less than 1% variation in relative peak intensity observed.

at higher concentrations (greater than ca. 1 ng/μl total lipid), both the M and M + 60 peaks for a given molecular species were present, somewhat complicating interpretation of the spectra. The same phenomenon occurred when examining phospholipids that were present as Na^+ adducts as described in subsequent sections.

Positive and negative ion MS/MS of individual molecular species provided different kinds of information. Positive ion MS/MS of *m/z* 742 and its M + 60 adduct at *m/z* 802, yielded a peak at *m/z* 184 corresponding to the phosphocholine headgroup + H^+. The corresponding negative ion spectra yielded two major peaks at *m/z* 255 and 279, corresponding to $RCOO^-$ for 16:0 and 18:2, respectively (**Fig. 6B**). The MS-MS spectrum of *m/z* 802 confirmed that it was the M + 60 derivative of *m/z* 742. By generating tables of molecular weights for combinations of fatty acid moieties, headgroups, and acyl, alkyl or alkenyl linkages, it is possible to evaluate the possible combinations that will generate this parent ion. A parent ion of *m/z* 742 (or of 802) can result from any combination of 28 carbons and 2 or 3 double bonds in the *sn*-1 and *sn*-2 positions, depending upon whether an alkyl-acyl or alkenyl-acyl moiety is present. For instance 16:0/18:3 alkyl-acyl PC or 16:0/18:2 alkenyl-acyl PC will produce a parent ion of *m/z* 742. This molecular weight cannot be generated for a diacyl PC species. Since the alkyl or alkenyl moiety was lost as either a neutral ion, a free radical, or not lost at all, these fragments were not present in either the positive or negative ion spectra; therefore, there were two species contributing to this parent, one with 16:0 and a second with 18:2 in the *sn*-2 (acyl) position. The most likely species are 16:0/18:2 alkenyl-acyl and 18:2/16:0 alkenyl-acyl PC. A drawback to identification of phospholipid molecular species using electrospray MS and MS/MS is that it is not possible to differentiate between alkyl and alkenyl linkages in many instances without prior separation of these different subclasses, since characteristic fragments from these constituents are not found in PC or PE alkyl/alkenyl-acyl species. For instance, the loss of a 16:0 alkenyl fragment produces the same spectrum as a complementary species with a 16:1 alkyl component. For the *m/z* 742 PC species, the presence of 16:1/18:2 alkyl-acyl and 18:3/16:0 alkenyl-acyl PC cannot be definitely ruled out. Further instances of this were presented for bovine brain glycerophosphoethanolamine.

One advantage of electrospray MS and MS/MS is that the relative contribution of individual species can be obtained by monitoring the relative peak intensities. For *m/z* 742, the 16:0/18:2 species contributed 71% of the signal while the 18:2/16:0 component made up 29%. By including an internal standard, quantitative results can also be obtained.

The MS/MS of *m/z* 770 (*m/z* 830 in the negative ion spectrum) demonstrated that even the components of a

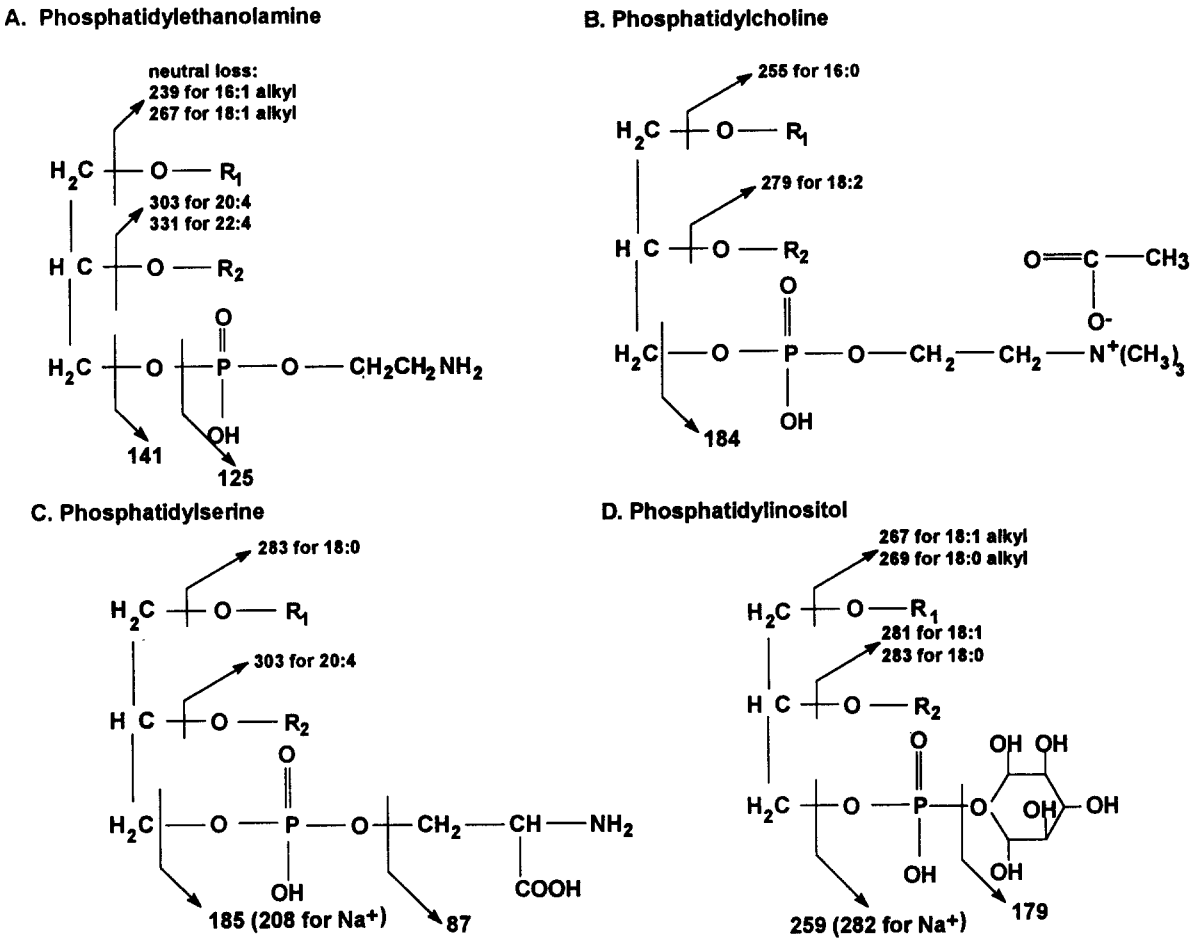

Fig. 6. Fragmentation patterns (composite of positive and negative MS/MS) characteristic of PE, PC, PS, and PI.

parent peak consisting of three components can be resolved. As before, the positive ion electrospray MS/MS confirmed the presence of the phosphocholine headgroup, while the negative ion spectrum revealed peaks at 279, 281, and 283 (relative intensities of 40:29:21), from $RCOO^-$ of 18:2, 18:1 and 18:0, respectively. This data shows the presence of alkyl-acyl and/or alkenyl-acyl species, most likely 18:0/18:3 alkyl-acyl, 18:1/18:1 alkenyl-acyl, and 18:2/18:0 alkenyl-acyl species.

Interpretation of components of diacyl PC species was definitive, e.g., *m/z* 808 could be due to either 18:1/20:4 or 18:0/20:5 diacyl PC. The negative ion spectrum showed major ions at *m/z* 281 and 303, corresponding to $RCOO^-$ of 18:1 and 20:4. These spectra do not provide information on which constituents are present in the *sn*-1 or *sn*-2 positions.

While this paper was being reviewed, the spectrometer used for the investigations was upgraded. During a subsequent analysis of bovine brain sphingomyelin, which also contains the phosphocholine headgroup (see below), we discovered that in negative ion electrospray MS, molecular ion adducts were found at [M + 44] rather than the expected [M + 60]. When this was observed, we repeated analysis of PC, and found the same shift in the molecular ion adducts for this lipid class. These ions are most likely due to the loss of a CH_3- moiety from the phosphocholine headgroup, with retention of the acetate group to quench the positively charged N as described above. This was confirmed by MS/MS of selected molecular PC ions, which resulted in the characteristic loss of 60 Da corresponding to loss of acetate.

Brain phosphatidylserine

The Na^+ salts of phosphatidylserine (PS) molecular species, which are commonly encountered in, for example, preparations purified using buffered HPLC systems, were examined using positive and negative electrospray. The molecular ion adducts in positive electrospray MS occurred as both $[M + H^+]$ and $[M + Na^+]$ adducts $[M + 23]^+$. The positively charged sodium ion was not seen in negative ion MS, so the molecular ion adducts were present as $[M - H^+]^-$. The positive ion spectra were more

complex than those seen with PE and PC, due to enhanced fragmentation in the presence of Na^+, as documented for other classes of compounds (32). The negative ion spectrum, lacking the sodium adducts, was more straightforward to interpret. Over 30 different (primarily diacyl) PS molecular species were found using positive and negative ion MS (**Table 3**).

As was also seen in spectra of the Na^+ adducts of glycerophosphoinositol (PI), there were discrepancies in the percent composition of molecular species between positive and negative ion spectra. This is due to the presence of both H^+ and Na^+ adducts. To obtain reliable data on relative composition, samples should be desalted prior to examination by electrospray.

Positive ion MS/MS of several PS molecular ions confirmed the presence of Na^+ adducts and provided confirmation of headgroup composition. For example, MS/MS of *m/z* 834, which corresponds to the Na^+ salt of 18:0/20:4 diacyl PS, had major peaks at *m/z* 747 (loss of CH-C(COOH)H-NH_2 from the headgroup); *m/z* 651 (loss of phosphoserine); *m/z* 627 (loss of phosphoserine + Na^+); and the (phosphoserine + Na^+) cation at *m/z* 208 (Fig. 6C). Comparable fragmentation, albeit with different peak intensities, corresponding to loss of fragments from the headgroup was found when MS/MS was done on *m/z* 812, i.e., the H^+ - adduct rather than the Na^+-adduct.

Negative ion MS/MS of *m/z* 810 confirmed the presence of $RCOO^-$ ions corresponding to 18:0 and 20:4. Also found were ions corresponding to loss of CH-C(COOH)H-NH_2 at *m/z* 723; and *m/z* 419 and 437 corresponding to loss of RCOOH from 18:0 and 20:4, respectively, from *m/z* 723 (Fig. 6C).

Similar fragmentation was found after MS/MS of peaks corresponding to 18:0/18:1 diacyl PS and 18:0/22:6 diacyl PS. Alkyl-acyl and alkenyl-acyl species were not examined for this phospholipid class by MS/MS.

Liver glycerophosphoinositol

As was found with the Na^+ salts of PS molecular species, positive electrospray spectra of the Na^+ salts of liver glycerophosphoinositol (PI) were more complex than phospholipid classes not associated with this cation. The added complexity is due only in part to the presence of both H^+ and Na^+ adducts of the parent ions. The negative electrospray spectra were predictably easier to interpret. Over 30 molecular species were identified using either mode of ionization (**Table 4**).

Electrospray MS/MS of *m/z* 859, the peak corresponding to the Na^+ adduct of 16:0/18:1 and/or 18:0/16:1 diacyl PI produced ions at *m/z* 599 and 577, corresponding to loss of the H^+ and Na^+ adducts, respectively, of phosphoinositol, and at *m/z* 282 from the Na^+ adduct of phosphoinositol. Negative ion MS/MS of this parent ion (*m/z* 835) produced ions at *m/z* 255 and 281 due to $RCOO^-$ from 16:0 and 18:1, respectively, showing the presence of only the 16:0/18:1 molecular species. Also found was an ion at *m/z* 241 due to loss of PO_3^- from the headgroup. Comparable fragmentation was found for the Na^+ adducts of diacyl PI species corresponding to the 18:0/18:1, 18:0/20:4, and 18:1/22:4 species.

Analysis of an alkyl-acyl PI species again demonstrated the sensitivity and resolution of electrospray MS. The positive electrospray MS/MS spectrum of an Na^+ adduct at *m/z* 873 produced two major peaks corresponding to loss of the PI headgroup as described above. The MS/MS spectrum in the negative mode produced *m/z* 241 from the PI headgroup, and four peaks from the acyl and/or alkyl constituents: *m/z* 267, 269, 281, and 283, corresponding to

TABLE 3. Molecular species of phosphatidylserine

	% Composition[a]			% Composition	
Molecular Species	Positive	Negative	Molecular Species	Positive	Negative
16:0/18:1	0.50	1.31	18:0/22:5	7.78	10.10
16:0/22:0	1.60	0.94	18:0/22:5 alkyl	1.06	0.72
16:0/22:1	4.46	2.55	18:0/22:6	9.60	13.95
16:0/22:2	5.88	2.40	18:0/22:6 alkyl	1.06	0.77
16:0/22:3	7.42	2.57	18:1/20:4	1.02	1.45
18:0/18:0	3.92	4.25	18:1/22:6	1.06	1.57
18:0/18:1	17.13	21.44	18:2/20:4	0.55	0.84
18:0/18:2	4.69	5.09	20:0/22:0	7.16	1.40
18:0/20:4	5.68	6.21	20:0/22:1	4.45	1.75
18:0/22:1	1.49	1.23	20:0/22:2	2.12	2.14
18:0/22:2	1.02	1.76	20:0/22:3	1.29	1.31
18:0/22:3	0.88	3.22	20:0/22:4	1.14	1.53
18:0/22:4	5.80	7.93	20:0/22:5	1.24	1.57

[a]The listed percent composition was based on the relative intensities of each molecular ion from a single representative spectrum. Positive and negative ion spectra were run at least three times each, with less than 1% variation in relative peak intensity.

TABLE 4. Molecular species of liver glycerophosphoinositol

Molecular Species	% Composition[a] Positive	Negative	Molecular Species	% Composition Positive	Negative
16:0/18:0	1.36	0.95	18:0/18:2 alkyl	0.00	0.79
16:0/18:1	1.13	1.96	18:0/20:4	11.34	20.03
16:0/18:2	0.96	1.37	18:0/20:4 alkyl	0.73	0.61
16:0/20:4	9.09	0.78	18:0/22:0 alkyl	2.26	0.41
16:0/22:0 alkenyl	1.72	0.47	18:0/22:1	0.37	0.40
16:0/22:1	1.67	1.09	18:0/22:1 alkyl	1.32	0.45
16:0/22:1 alkenyl	0.94	0.46	18:0/22:2	0.42	0.60
16:0/22:2	3.16	5.48	18:0/22:2 alkyl	1.30	0.39
16:0/22:3	7.66	17.17	18:0/22:3	0.57	0.98
16:0/22:3 alkyl	1.15	0.66	18:0/22:3 alkyl	1.31	0.51
18:0/16:1	0.46	0.80	18:0/22:4	1.68	2.96
18:0/18:0	7.94	2.30	18:0/22:4 alkyl	0.00	0.39
18:0/18:1	13.55	13.32	18:0/22:5	1.83	2.32
18:0/18:1 alkyl	0.00	0.83	18:0/22:6	2.27	0.68
18:0/18:2	7.35	15.68	18:1/18:0 alkyl	0.00	0.46
18:0/18:3	6.70	2.40	18:1/20:4	9.76	2.26

[a]The listed percent composition was based on the relative intensities of each molecular ion from a single representative spectrum. Positive and negative ion spectra were run at least three times each, with less than 1% variation in relative peak intensity observed.

RCOO- of 17:1, 17:0, 18:1 and 18:0. If it is assumed that the alkyl constituents do not contribute to the spectrum, as seen for PE and PC alkyl-acyl species, this would imply that there are four species contributing to *m/z* 849: 19:0/17:1, 19:1/17:0, 18:0/18:1, and 18:1/18:0 alkyl-acyl PI. However, it is unlikely that there are appreciable quantities of odd-chain-length fatty acids in a preparation of mammalian origin. As *m/z* 267 and 269 could also be due to the alkyl [RCH_2O-] substituents of 18:1 and 18:0, it is possible that there are only 18:0/18:1 and 18:1/18:0 alkyl-acyl species present (Fig. 6D).

These results can perhaps best be rationalized using steric arguments. The results with alkyl-acyl PE species were explained by stabilization of the ion generated by loss of the fatty acid in the *sn*-2 position by formation of a 5-membered ring that included a phosphonium ion (Fig. 5). It is possible that the relatively bulky inositol group interfered with formation of this ring, but allowed formation of a 6-membered ring at *sn*-1 that promotes loss of RCO- at that position. Additional research is required to establish mechanistic details of fragmentation.

Sphingomyelin

Sphingomyelin (SM, ceramide phosphocholines), which are major constituents of many mammalian membranes, consist of an N-acyl-fatty acid linked to a long-chain hydrocarbon and phosphorylcholine (**Fig. 7**). Note that these spectra were collected after the mass spectrometer upgrade described previously for bovine brain PC. Bovine brain SM yielded 17 molecular species (**Table 5**). In

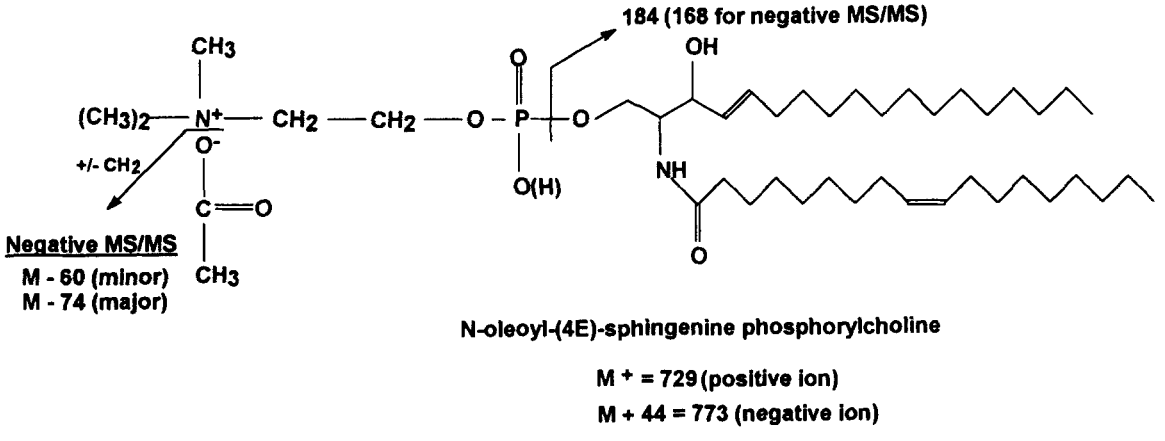

Fig. 7. Structure and fragmentation patterns (composite of positive and negative MS/MS) characteristic of SM.

TABLE 5. Molecular species of bovine brain sphingomyelin

Molecular Species[a]	% Composition[b] Positive	Negative
14:1/SP	0.33	0.23
16:1/SP	0.53	0.23
16:0/SP	4.01	3.09
16:0/DISP	1.35	0.60
18:1/SP	2.55	1.36
18:0/SP	16.65	23.81
18:0/DISP	4.89	2.86
20:0/SP	1.78	1.73
22:3/SP	0.33	0.23
22:2/SP	0.16	0.83
22:1/SP	2.52	1.58
22:0/SP	4.08	2.03
24:2/SP	1.26	1.66
24:1/SP	44.83	48.00
24:0/SP	12.02	7.23
26:1/SP	1.51	3.69
26:0/SP	1.19	0.83

[a]SP, sphingenine; DISP, sphinganine (dihydrosphingosine).
[b]The listed percent composition was based on the relative intensities of each molecular ion from a single representative spectrum. Positive and negative ion spectra were run at least three times each, with less than 1% variation in relative peak intensity.

positive ion MS, molecular ions were found at M^+ as described for PC. In negative ion MS, molecular ion adducts were found as $[(M + \text{acetate})\text{-}CH_4]^-$, i.e., $[M + 44]^-$ due to loss of one methyl group from the quaternary nitrogen, with acetate complexed to the choline headgroup. Positive ion MS/MS of $[M + H^+]$ ions yielded a strong peak at *m/z* 184, corresponding to the phosphorylcholine headgroup, as was found for glycerophosphocholine. In negative ion MS/MS spectra, the major ion found was due to loss of acetate from the parent ion described above, with less intense peaks due to loss of acetate plus an additional $-CH_3$ from the headgroup, and at *m/z* 168 from the phosphorylcholine headgroup minus one methyl moiety (Fig. 7). Attempts to increase fragmentation to generate peaks characteristic of the N-acyl and sphingenine/sphinganine constituents using 1% methanolic formate were not successful. This should not seriously limit the usefulness of electrospray MS analysis of SM, as there are many fewer combinations of sphingoid base and N-acyl moieties in this lipid class compared to glycerophospholipids.

Other lipid classes and biological samples

Detailed data are not presented here, but other classes of lipid and phospholipid have been examined using positive and negative ion electrospray MS and MS/MS (J. L. Kerwin, unpublished observations), including monomethylphosphatidylethanolamine, dimethylphosphatidylethanolamine, phosphatidylglycerol, and galactolipids. Electrospray MS is easily adapted to the examination of complex mixtures encountered in biological samples. We have identified glycerophospholipid molecular species from mosquito larvae and a fungal parasite of mosquitoes using lipid extracts prepared in three different ways. First, using purified nuclei from the pathogenic fungus *Lagenidium giganteum* (Oomycetes : Lagenidiales), Folch extraction of the nuclei followed by evaporation of chloroform, and resuspension in methanolic 5 mM ammonium acetate, allowed direct examination of molecular species. This approach can only be used in samples such as some types of purified organelles that have minimal amounts of other classes of compounds that would interfere with the analysis. Second, after Folch extraction, we have separated glycerophospholipids from other lipid classes using Sep-Paks or TLC, and used these lipids without further fractionation for electrospray analysis. Finally, phospholipid classes separated using normal phase HPLC have been examined following evaporation of the mobile phase.

A major complication arises when the HPLC mobile phase includes phosphate, which interferes with electrospray analyses. By partitioning individual phospholipid classes isolated in this way using chloroform–0.75% aqueous KCl 1:2 (v/v), we have been able to remove phosphate sufficiently to permit electrospray MS of individual fungal and mosquito phospholipid classes separated using phosphate-containing solvent systems. Trace amounts of detergent also seriously interfere with generation of electrospray spectra, and will quench the signal from compounds of interest when present in minute quantities.

Sensitivity

As there are occasions when a minimal amount of biological material is available for phospholipid analyses, e.g., when investigating the composition of individual organelles, we investigated the sensitivity of electrospray MS for molecular species determination. Using bovine brain PE, and scanning from *m/z* 600 to 900 in either the positive or negative ion mode, we were able to obtain spectra with over 50 peaks corresponding to different molecular species of PE using as little as 10 picograms of material (data not shown). Over 20 parent ions were obtained using 0.1 picogram of PE, but minor species were lost. Scanning over a range of 500 to 550, the parent ion and the Na^+ adduct of HAPC were identified using 10 femtograms of total lipid. As electrospray MS is a relatively new technique, there is a great potential for significant increases in sensitivity. For instance, the Sciex mass spectrometer used in these investigations was upgraded at the conclusion of this study, and is now 10 to 20 times more sensitive than reported above. Positive ion MS was consistently 10 times more sensitive than negative ion MS using the operating conditions described.

DISCUSSION

Data presented in this paper demonstrate the sensitivity, resolution, and speed at which simple and complex mixtures of glycerophospholipid molecular species can be examined using electrospray MS and MS/MS. Most alternative methods require more material, take much longer to complete, and/or are less definitive in identification (3–6, 13–28). As an example, we have initiated the investigation of sterol-mediated changes in phospholipid molecular species of purified fungal nuclei. By using electrospray MS and MS/MS of total phospholipid extracts, we have reduced the sample preparation and analysis time by at least a factor of 25.

We have not rigorously examined the relationship between peak intensity and sample concentration, but for a given phospholipid class, increasing lipid concentration (over ca. 2–3 orders of magnitude) using a standard acquisition time, or a standard lipid concentration using an increasing acquisition time (5 to 120 sec) generally produced a linear relationship. The major problem encountered, due to the sensitivity of the technique, is "folding over" of parent ion peaks upon reaching a critical concentration, which will vary according to the spectrometer used and the configuration of the injection port. For instance, direct sample injection into the Sciex machine used in these investigations is at least 50 times more sensitive and has much better resolution than a VG single quadrapole instrument we have used, in which the sample is diluted by a carrier solvent.

Relative concentrations of individual molecular species, in the absence of interfering Na^+, K^+, or other salts, is very reproducible, even if more than one phospholipid class is examined simultaneously. By having the option of both positive and negative ionization modes, it is possible to check the reliability of the data generated. If the relative compositions do not closely match, preferential ionization of individual species would be suspected.

Fragmentation patterns of intact phospholipid molecular species that were observed after negative and positive ion electrospray MS/MS were, with the exception of the PE ether lipids, similar to those found by others using a variety of mass spectrometric techniques. Jungalwala, Evans, and McCluer (22) were able to identify both headgroup and fatty acyl composition of phospholipids and lysophospholipids using a combination of HPLC and positive and negative ion chemical ionization mass spectrometry, but their technique required 5 μg of individual lipids. Fast atom bombardment (FAB) mass spectrometry has been used to examine intact PC, PE, and PI molecular species (23, 33), but problems with matrix interference evidently precluded identification of minor species without prior HPLC purification. More recent studies using FAB tandem mass spectrometry have refined this methodology such that, after mild acid hydrolysis to selectively cleave 1-*O*-alk-1'-enyl bonds, this fraction of glycerophosphoethanolamines can be distinguished from diacyl and alkylacyl species (34). As in electrospray MS/MS, in the absence of hydrolysis it was not possible to differentiate between isobaric compounds with unsaturation at the vinyl position (1-*O*-alk-1'-enyl) and those with one unsaturated bond at any other position in the alkyl chain. It was not clear how much material is required for these analyses, or the utility of the technique without separating individual phospholipid classes.

Electron capture negative chemical ionization mass spectrometry of dinitrobenzoate derivatives of diglycerides was used to identify as little as 0.5 pmol of individual PE and PC molecular species, but required preliminary separation using TLC, phospholipase C hydrolysis of the headgroup, derivatization, and subsequent separation using reversed phase HPLC, all of which require substantially more starting material (35).

More recently, negative ion fast atom bombardment combined with mass-analyzed ion kinetic energy analysis has been used to characterize headgroups as well as fatty acyl chain position due to the high intensity peak generated by $[M-headgroup-R_2COOH]^-$ anions. This technique was successfully used only following separation of individual molecular species within each class as parent ions were not easy to select (36).

Perhaps the most widely used method of molecular species analysis involves various preliminary chromatographic separations, enzymatic removal of the headgroup, derivatization (using a UV-absorbing moiety) of the free hydroxyl, and ending with reverse phase HPLC (15–20, 37–39). If done with sufficient concurrent controls to insure quantitative hydrolysis, extraction, and derivatization, these methods are accurate and in some cases as little as 100 pmol of individual species can be detected (38); however, much more starting material is needed for the preliminary chromatographic steps. Many of these protocols rely on log retention times and/or comigration with standards for species identification, although some techniques can be adapted to continuous flow mass spectrometric analyses (22, 38).

There have been a number of techniques developed for analysis of molecular species using gas chromatography–mass spectrometry (GC–MS) (24–28). These require preliminary separation of phospholipid classes, enzymatic hydrolysis of the headgroup, and usually derivatization of the resulting free hydroxyl moiety. Although the GC–MS analyses per se are relatively rapid, the preliminary steps are time consuming, and require running concurrent controls to assure that preferential hydrolysis or extraction of individual classes or species does not occur.

Fast atom bombardment has been used fairly extensively to characterize sphingolipids (40–44). A recent study of sphingomyelins using FAB and collision-induced decomposition (45) demonstrated that this technique

could be used to characterize the sphingoid base, the fatty acyl chain, and confirm the phosphocholine headgroup; however, when a sample of bovine brain SM was analyzed, only two main species, compared to 17 found in this study, were identified. Minor species in the FAB analysis may have been obscured by background from matrix ions.

With the increasing availability of electrospray mass spectrometers at many institutions, this method should be considered as a possible alternative to many protocols used in previous investigations of phospholipid molecular species.

This work was supported in part by a grant from the National Institutes of Health (R01 AI22993-06). We are grateful to P. Davis, W. Howald, R. Johnson, and K. Walsh for their comments on this manuscript.

Manuscript received 27 July 1993 and in revised form 13 December 1993.

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