Electrospray Ionization Tandem Mass Spectrometry of Glycerophosphoethanolamine Plasmalogen Phospholipids

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Collision-induced dissociation (CID) of the $[M + H]^+$ of glycerophospholipids typically results in abundant fragment ions that are related to the polar head group or loss of the polar head group. An exception to this general rule occurs for glycerophosphoethanolamines (GPEtn), which are a class of phospholipids that can have an acyl, 1-O-alkyl, or 1-O-alk-1′-enyl group as a substituent at the *sn*-1 position. The CID of the $[M + H]^+$ of diacyl-GPEtn typically results in the expected loss of the phosphoethanolamine head group (141 Da). Therefore, constant neutral loss of 141 Da has been used as a diagnostic tool for the determination of GPEtn species in complex lipid mixtures. One disadvantage in using constant neutral loss of 141 Da in order to determine GPEtn content in lipid mixtures is that plasmalogen GPEtn does not undergo neutral loss of phosphoethanolamine to the same extent as diacyl-GPEtn. The current studies have used positive ion mode electrospray tandem mass spectrometry to study the collision-induced dissociation of various GPEtn plasmalogens present in the phospholipid membranes of human neutrophils. The CID of the $[M + H]^+$ of plasmalogen GPEtn resulted in two prominent fragment ions; one that was characteristic of the *sn*-1 position and one that was characteristic of the *sn*-2 position. These two ions were used to detect specific molecular species of GPEtn containing esterified arachidonate (precursors of *m/z* 361) present in the human neutrophil. (J Am Soc Mass Spectrom 2004, 15, 1499–1508) © 2004 American Society for Mass Spectrometry

Phospholipids are building blocks of cellular membranes, but their role in biochemistry is only partially understood. Membrane phospholipids are storage sites of arachidonic acid, which is the precursor to many biologically active lipid mediators [1]. These molecules are also precursors for platelet activating factor [2] and for signaling molecules such as inositol triphosphate [3]. Phospholipids have also been recognized as important factors in signal transduction and membrane transport [4]. Additionally, alterations in phospholipid composition have been reported in certain disease states such as atherosclerosis, carcinogenesis, and Alzheimer's disease [5–7]. These findings have catalyzed the need for a detailed analysis of lipid structure and lipid species present in certain tissues and cells.

One way to achieve detailed structural analysis of glycerophospholipids is by using electrospray tandem mass spectrometry [8]. Glycerophospholipids consist of a glycerol backbone with a fatty acyl or 1-O-alkyl group at the *sn*-1 position, an acyl group at the *sn*-2 position, and a phosphate ester group with a polar head group at the *sn*-3 position. Collision-induced dissociation of the $[M + H]^+$ or $[M - H]^-$ of glycerophospholipids results in fragment ions that are related to the polar head group and the fatty acyl substituents esterified to the glycerol backbone [8]. One of the principal fragmentation pathways during collision-induced dissociation (CID) in the positive ion mode is cleavage of the phosphate-glycerol bond, which results in the elimination of the polar head group as a neutral or charged species. These characteristic fragmentations of the polar head group of phospholipids during CID allow the specific detection of a phospholipid class by precursor ion or neutral loss scanning [9].

Glycerophosphoethanolamines are a class of phospholipids that can have an acyl, 1-O-alkyl, or 1-O-alk-1′-enyl group as a substituent at the *sn*-1 position. Collision-induced dissociation of the $[M + H]^+$ of diacyl- or 1-O-alkyl-2-acyl-GPEtn typically shows a strong neutral loss of 141 Da, which is the phosphoethanolamine head group. Therefore, constant neutral loss of 141 Da scanning has been used as a diagnostic tool for the determination of diacyl- or 1-O-alkyl-2-acyl-GPEtn species in complex lipid mixtures. One disadvantage in using constant neutral loss of 141 Da in order

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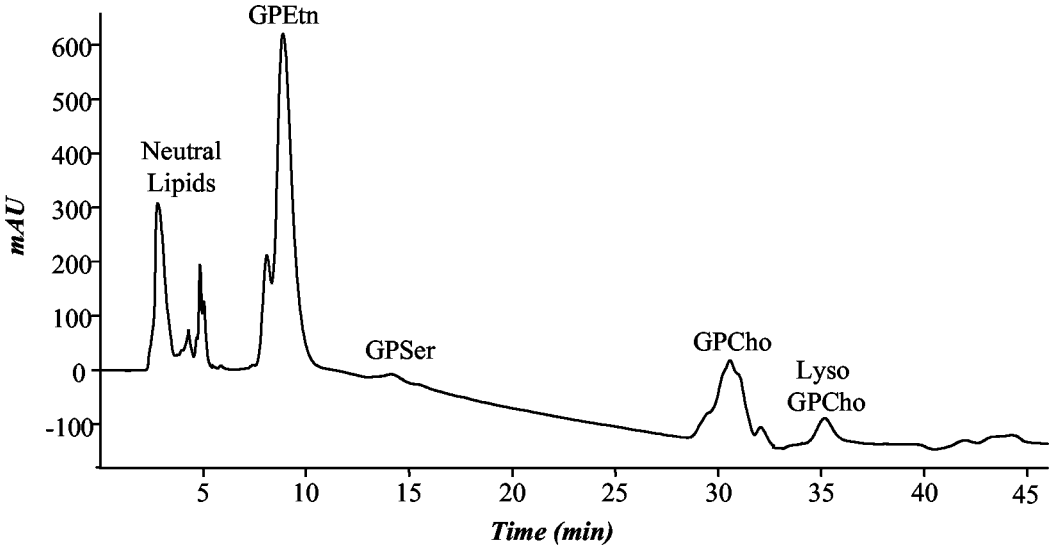


Figure 1. Normal phase HPLC separation of the different phospholipid classes extracted from human neutrophils as described in methods. The phospholipids were detected using a photodiode array detector and the signal corresponding to 206 nm was used to detect the phospholipids containing unsaturated fatty acyl substituents. The elution of each phospholipid class is indicated by the abbreviations GPEtn, glycerophosphoethanolamine lipids; GPSer, glycerophosphoserine lipids; GPCho, glycerophosphocholine lipids; lysoGPCho, lysoglycerophosphocholine lipids.

to determine GPEtn content in lipid mixtures is that plasmalogen GPEtn do not undergo neutral loss of phosphoethanolamine to the same extent as diacyl-GPEtn [10]. This is most likely attributed to the unique gas phase ion chemistry imparted by the vinyl ether substituent which alters the favorable CID mechanism of the neutral loss of 141 Da from GPEtn species. The current studies employed electrospray tandem mass spectrometry to study the positive ion mode collision-induced dissociation of various GPEtn plasmalogens present in the phospholipid membranes of human neutrophils. The CID of the $[M + H]^+$ of plasmalogen GPEtn results in two prominent fragment ions; one that is characteristic of the *sn*-1 position and one that is characteristic of the *sn*-2 position. Additionally, a small neutral loss of 141 Da is observed, but this is not the major fragment ion of plasmalogen GPEtn species.

Experimental

Materials

1-O-Hexadec-1′-enyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (16:0p/22:6-GPEtn) [abbreviations for individual GPEtn molecular species used in this paper: n:jk/s:t-GPEtn (e.g., 16:0p/20:4-GPEtn), where n is the number of carbon atoms in the *sn*-1 substituent and j is the number of double bonds in the *sn*-1 hydrocarbon chain; k represents the type of *sn*-1 linkage, where p refers to plasmalogen (1-O-alk-1′-enyl), e refers to ether (1-O-alkyl), and a refers to acyl; s is the number of carbons and t is the number of double bonds in the *sn*-2 substituent] was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). HPLC solvents and hydrochloric acid were purchased from Fisher Scientific (Fair Lawn, NJ) and used for HPLC, extraction, and hydrolysis. The ethyl alcohol-*d* (99.5 atom % d_1) used for the deuterium exchange experiments was purchased from Aldrich (Milwaukee, WI).

Electrospray Ionization Tandem Quadrupole Mass Spectrometry and Ion Trap Mass Spectrometry

The glycerophosphoethanolamine lipids of interest were infused into a Sciex API III$^+$ triple quadrupole mass spectrometer (PE Sciex, Toronto, Canada) through a 50 μm fused silica capillary at a flow rate of 5 μL/min. The relevant experimental parameters in the positive ion mode for both full scan and collision-induced dissociation experiments were a spray voltage of 4800 V and an orifice voltage of 65 V. The full mass spectra were obtained by scanning from *m/z* 700 to 850. Additionally, the constant neutral loss of 141 Da scan was obtained over the same *m/z* range described above at a collisional offset of 10 V. The collision-induced dissociation mass spectra of GPEtn plasmalogens were acquired in the positive ion mode at 3 s/scan over the mass range *m/z* 250 to 800 with a collisional offset of 15 V. The pressure of argon used to obtain the CID and constant neutral loss mass spectra was equivalent to 190 $\times 10^{12}$ molecules/cm^2. Exchange of the active hydrogen atoms on GPEtn plasmalogens (1 μg) was performed by drying the samples under a stream of nitrogen and resuspending in ethyl alcohol-*d* (1 ml) with a small amount of ammonium acetate.

Further structural elucidation of GPEtn plasmalogens was achieved using a Finnigan LCQ ion trap

(ThermoFinnigan, San Jose, CA) in the positive ion mode. The capillary temperature was set at 200 °C with an electrospray voltage of 4000 V. The sheath gas flow and the auxiliary gas were set at 70 and 10 units, respectively. GPEtn plasmalogens of interest were infused through a 50 μm fused silica capillary at a flow rate of 10 μL/min and MS^2 was performed at a normalized collision energy of 32%. The two product ions present in the MS^2 spectrum were collisionally activated (MS^3) at a normalized collision energy of 40% to obtain additional structural information about the fragment ions observed during the MS^2 experiments. The data were acquired and averaged over 1 min.

Phospholipid Isolation

Human polymorphonuclear leukocytes (neutrophils) were obtained from the whole blood of volunteers using the Percoll gradient centrifugation technique as previously described [11]. Lipids were extracted from 100×10^6 neutrophils by addition of chloroform-methanol according to the method of Bligh and Dyer [12]. The different lipid classes present in the chloroform layer were separated using normal phase HPLC with an Ultremex 5μ Si (4.6 × 250 mm) column (Phenomenex, Torrance, CA). The normal phase solvents used for the separation of phospholipid classes were 30:40 hexane/2-propanol (Solvent A) and 30:40:7 hexane/2-propanol/water (Solvent B). The initial mobile phase was 47% Solvent B at a flow rate of 1 mL/min. This initial mobile phase was held for 6 min and then a linear gradient was started to 100% Solvent B in 20 min. This was followed by isocratic elution at 100% Solvent B for 20 min. The column effluent was monitored by a photodiode array (HP 1040M, Hewlett Packard, Palo Alto, CA) using absorption at 206 nm to detect unsaturated phospholipids and 1 min fractions were collected.

Acid Hydrolysis

Glycerophosphoethanolamine plasmalogens, which are acid labile, were hydrolyzed by exposure to 12N HCl fumes [13]. This was achieved by drying down normal phase fractions containing GPEtn plasmalogens under a stream of nitrogen and exposing the GPEtn lipids to hydrochloric acid fumes for 1 h. This caused hydrolysis of the labile alkenyl ether bond of the plasmalogens while the 1-O-alkyl-2-acyl- and diacyl-GPEtn remained intact. The lipids were then reconstituted in 60:20:20 methanol/acetonitrile/water with 1 mM ammonium acetate for mass spectrometric analysis.

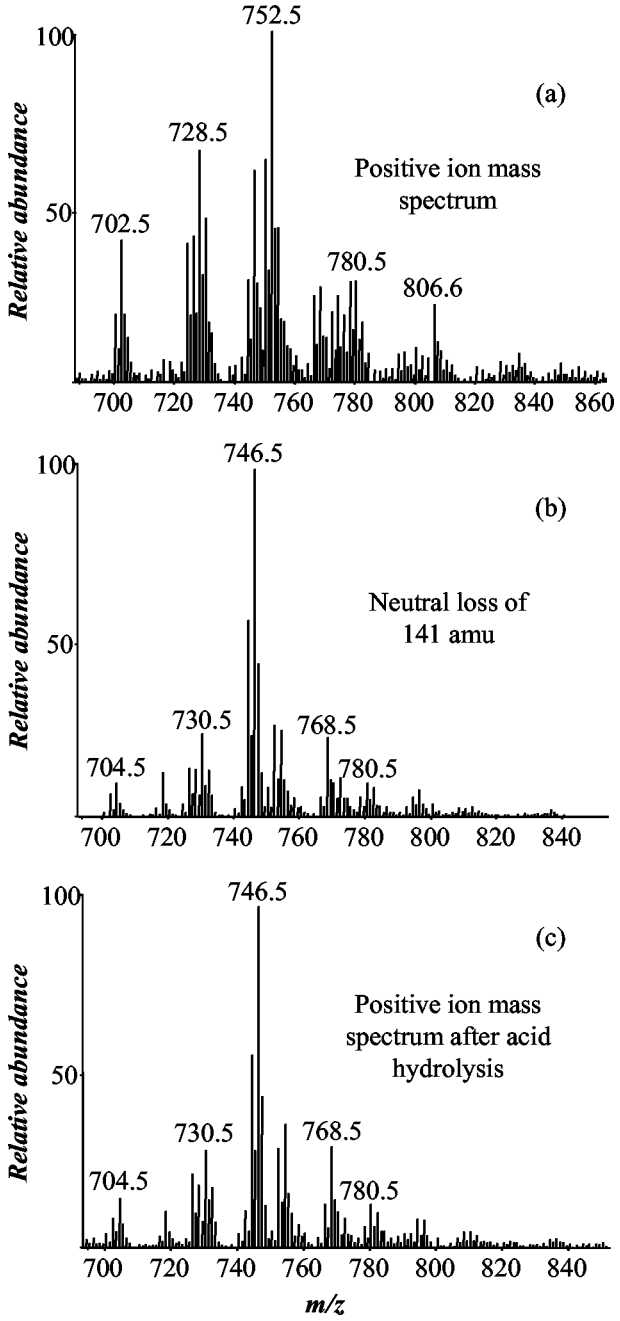


Figure 2. (**a**) Positive ion electrospray mass spectrum of the GPEtn species present in human neutrophils. The ions correspond to $[M + H]^+$ of each GPEtn present. (**b**) Neutral loss of 141 Da scan to analyze GPEtn present in human neutrophils. This neutral loss of 141 Da corresponds to the loss of phosphoethanolamine from $[M + H]^+$ and is typically used as a diagnostic loss to determine the presence of GPEtn species in biological samples. (**c**) Positive ion electrospray mass spectrum of the GPEtn species present in human neutrophils after acid hydrolysis of the vinyl ether bond of plasmalogens.

Results and Discussion

In order to study the collision-induced dissociation of GPEtn plasmalogens in the positive ion mode, the phospholipids of human neutrophils were extracted. Normal phase chromatography was used to separate the glycerophosphoethanolamine lipids, which eluted between 8 and 10 min (Figure 1), from the other phospholipid classes present in the Bligh Dyer extract. The human neutrophil was used for this study because

Table 1. Summary of the CID fragmentation of glycerophosphoethanolamine plasmalogens found in human neutrophils

GPEtn species	$[M + H]^+$	CID fragment ion containing headgroup	CID fragment ion without headgroup	Neutral loss of 141 Da
16:0p/18:2-GPEtn	700	364	337	559
16:0p/18:1-GPEtn	702	364	339	561
16:0p/20:4-GPEtn	724	364	361	583
18:1p/18:2-GPEtn	726	390	337	585
18:0p/18:2-GPEtn	728	392	337	587
18:1p/18:1-GPEtn	728	390	339	587
18:0p/18:1-GPEtn	730	392	339	589
16:0p/22:6-GPEtn	748	364	385	607
18:1p/20:4-GPEtn	750	390	361	609
16:0p/22:4-GPEtn	752	364	389	611
18:0p/20:4-GPEtn	752	392	361	611
18:0p/22:6-GPEtn	776	392	385	635
18:0p/22:5-GPEtn	778	392	387	637
20:1p/20:4-GPEtn	778	418	361	637
18:0p/22:4-GPEtn	780	392	389	639
20:0p/20:4-GPEtn	780	420	361	639

GPEtn plasmalogens represent 64–76% of the total ethanolamine phospholipids present in neutrophil membranes [14]. Additionally, within this subclass present in human neutrophils there is a wide variety of GPEtn plasmalogens present in terms of the substituents at the *sn*-1 and *sn*-2 positions of the glycerol backbone. This diverse sampling of GPEtn plasmalogens was beneficial in terms of determining the structure of the CID products and the mechanisms of the fragmentation process.

The different GPEtn species present in human neutrophils were examined by electrospray mass spectrometry in the positive ion mode (Figure 2). A full scan mass spectrum in the positive ion mode of the GPEtn species present in human neutrophils is shown in Figure 1a. The ions present in this figure represent the $[M + H]^+$ of the ethanolamine phospholipids present in the membranes of human neutrophils. Additionally, a neutral loss scan of 141 Da, which corresponds to loss of phosphoethanolamine, was performed (Figure 2b). Upon comparison of Figure 2a and b, a striking difference between the relative abundances of the $[M + H]^+$ present in the two different spectra was observed. The most abundant $[M + H]^+$ in the full scan (Figure 2a) was *m/z* 752; however this ion was only 10% of the most abundant ion, *m/z* 746, in the neutral loss of 141 Da scan (Figure 2b). It is thought that the discrepancy between Figure 2a and b was due to the fact that GPEtn plasmalogens do not show strong neutral loss of 141 Da during CID [10]. In order to determine if the difference in relative ion abundance observed in Figure 2a and b was due to unique GPEtn plasmalogen CID behavior, the ethanolamine phospholipids extracted from human neutrophils were subjected to acid hydrolysis. Upon acid hydrolysis, the vinyl ether bond of the plasmalogens was hydrolyzed but the 1-O-alkyl-2-acyl- or diacyl-GPEtn species were not affected. The full scan of the GPEtn species present after acid hydrolysis (Figure 2c) was remarkably similar to the neutral loss of 141 Da (Figure 2b), which confirmed that GPEtn plasmalogens do not show a strong loss of 141 Da during CID. This data also demonstrated that a constant neutral loss of 141 Da scan does not accurately reflect the GPEtn species present in a biological sample.

The loss of phosphoethanolamine (141 Da) from GPEtn during CID in the positive ion mode can occur through a 5- or 6-membered ring transition state where the carbonyl oxygen from the *sn*-1 or *sn*-2 position attacks the *sn*-3 methylene carbon with loss of neutral phosphoethanolamine [10]. Both the diacyl and the GPEtn plasmalogens would be able to undergo loss of 141 Da through the 5-membered ring transition state, but the formation of the 6-membered ring transition state would only apply to a diacyl-GPEtn. It has been thought that the 6-membered ring mechanism would be a more facile process than the formation of the 5-membered ring, which is most likely why the loss of 141 Da is abundant for diacyl GPEtn and not for plasmalogen GPEtn.

In order to probe the unique CID behavior the GPEtn plasmalogens present in the human neutrophil were analyzed by electrospray tandem mass spectrometry. Generally, there were two abundant fragment ions in each CID spectrum with one product ion that was characteristic of the *sn*-1 position and one product ion that was characteristic of the *sn*-2 position (Table 1). For example, all of the GPEtn plasmalogens that had an 18:0p group at the *sn*-1 position, but had different acyl groups at the *sn*-2 position, fragmented into the observed ion at *m/z* 392 (Figure 3). The other GPEtn plasmalogens with a 16:0p, 18:1p, 20:0p, or 20:1p group at the *sn*-1 position decomposed into *m/z* 364, 390, 420, or 418, respectively. Additionally, all of the GPEtn plasmalogens that contained arachidonoyl at the *sn*-2 position, but had different vinyl ether *sn*-1 substituents, yielded *m/z* 361 as a product ion (Figure 4). The GPEtn

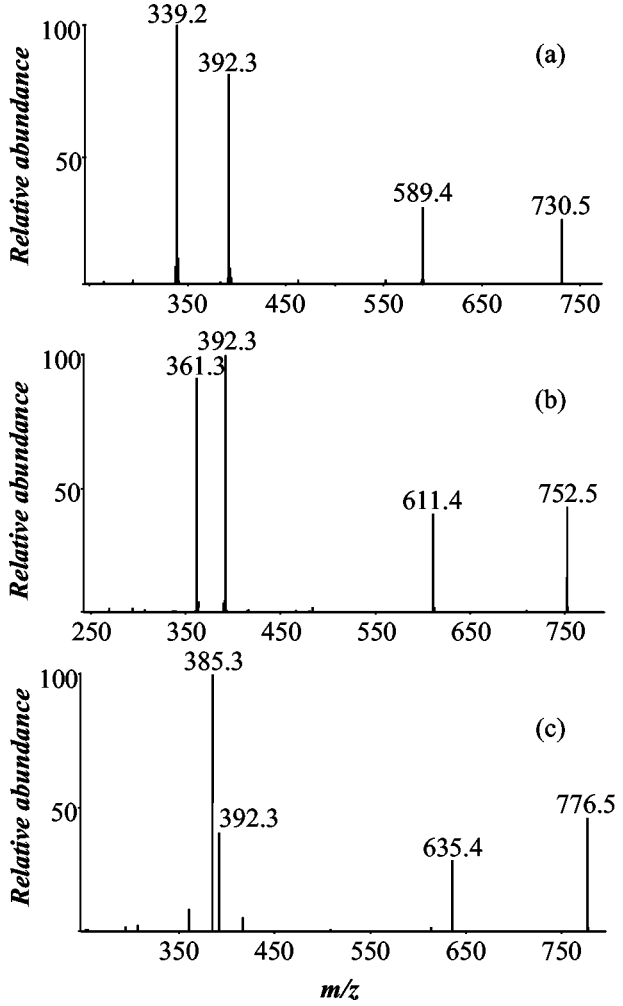


Figure 3. Electrospray tandem mass spectrometry in the positive ion mode of GPEtn plasmalogens with an 18:0p substituent in the *sn*-1 position and different acyl groups at the *sn*-2 position. Collision-induced dissociation spectra of (**a**) 18:0p/18:1-GPEtn, (**b**) 18:0p/20:4-GPEtn, and (**c**) 18:0p/22:6-GPEtn. Note there is a common fragment *m/z* 392 in each of these CID spectra.

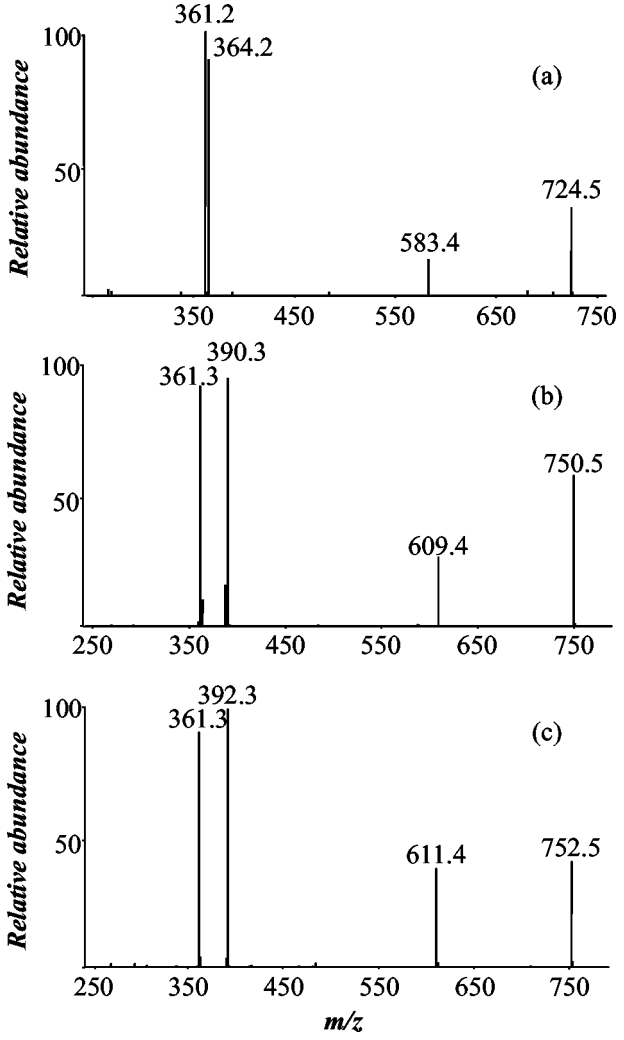


Figure 4. Electrospray tandem mass spectrometry in the positive ion mode of GPEtn plasmalogens with arachidonoyl at the *sn*-2 position and different vinyl ether groups at the *sn*-1 position of the glycerol backbone. Collision-induced dissociation spectra of (**a**) 16:0p/20:4-GPEtn, (**b**) 18:1p/20:4-GPEtn, and (**c**) 18:0p/20:4-GPEtn. Note there is a common fragment *m/z* 361 in each of these CID spectra.

plasmalogens that contained 18:1, 18:2, 22:4, 22:5, or 22:6 at the *sn*-2 position decomposed into *m/z* 339, 337, 389, 387, or 385, respectively. One similar feature in all of the product ion spectra shown in Figures 3 and 4 is that neutral loss of 141 Da was a minor product observed during the CID of GPEtn plasmalogens.

GPEtn lipids extracted from human neutrophils consist of many different molecular species, which resulted in the presence of several isobaric species during mass spectrometric analysis. For example, the $[M + H]^+$ at *m/z* 780 can be composed of both 18:0p/22:4-GPE and 20:0p/20:4-GPEtn. This is indicated by the two sets of fragment ions observed during CID, which included *m/z* 420 and 361 for 20:0p/20:4-GPEtn and *m/z* 392 and 389 for 18:0p/22:4-GPE (Figure 5). There were also other isobaric GPEtn plasmalogens present in the lipid extract from human neutrophils, which included $[M + H]^+$ at *m/z* 728 and 778. The fragment ions observed in the CID spectrum of *m/z* 728 included *m/z* 337 and 392 and *m/z* 339 and 390, which corresponded to 18:0p/18:2-GPEtn and 18:1p/18:1-GPEtn, respectively. The product ions that were observed after CID of $[M + H]^+$ of *m/z* 778 included *m/z* 361 and 418 and *m/z* 387 and 392, which corresponded to 20:1p/20:4-GPEtn and 18:0p/22:5-GPEtn. Additionally, these GPEtn plasmalogens also exhibited a minor neutral loss of 141 Da.

In order to provide some insight into the structure of the CID products and the mechanisms of the fragmentation process observed during CID of GPEtn plasmalogens, deuterium exchange was employed. For the deuterium exchange experiments a standard sample of 16:0p/22:6-GPEtn was used instead of the GPEtn plasmalogens extracted from neutrophils to ensure that artifacts from an isobaric GPEtn species were not present in the CID spectrum. The $[M + H]^+$ of 16:0p/22:6-GPEtn (*m/z* 748) was collisionally activated and

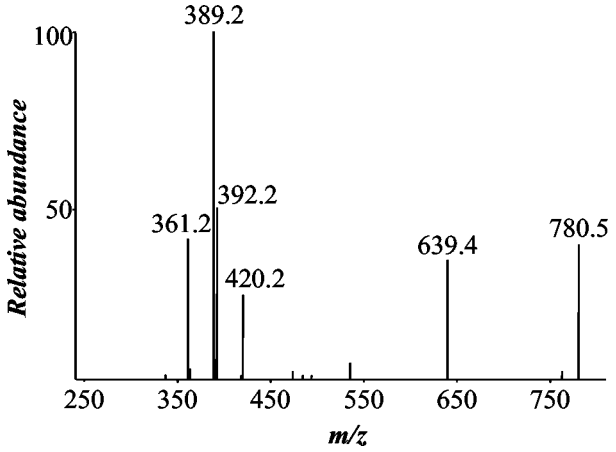


Figure 5. Electrospray tandem mass spectrometry in the positive ion mode of $[M + H]^+$ at *m/z* 780. This $[M + H]^+$ was identified as an isobaric species of 18:0p/22:4-GPEtn and 20:0p/20:4-GPEtn. This is indicated by the two sets of fragment ions observed during CID, which include *m/z* 420 and 361 for 20:0p/20:4-GPEtn and *m/z* 392 and 389 for 18:0p/22:4-GPEtn.

yielded two major fragment ions, *m/z* 364 and 385, along with a small neutral loss of 141 Da (Figure 6a). After reaction with ethyl alcohol-*d*, four protons were exchanged for deuterium atoms and the $[M + H]^+$ of 16:0p/22:6-GPEtn was increased by four Da to *m/z* 752. The CID spectrum of this deuterium exchange ion (*m/z* 752) had two major ions at *m/z* 368 and 386 (Figure 6b). The fragment ion at *m/z* 368 increased by 4 Da from *m/z* 364 in nondeuterated experiments and suggested that

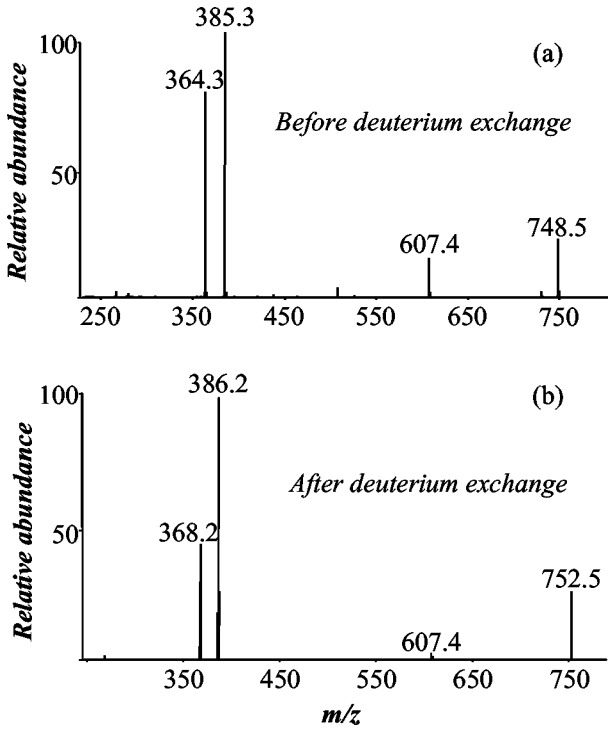


Figure 6. Collision-induced dissociation of $[M + H]^+$ of (**a**) 16:0p/22:6-GPEtn and (**b**) 16:0p/22:6-GPEtn after deuterium exchange with ethyl alcohol-*d*.

Table 2. Summary of the major fragment ion formed during MS^3 of GPEtn plasmalogens with different *sn*-1 substituents

sn-1 substituent	MS^2 product ion	MS^3 product ion
16:0p	364	266
18:1p	390	292
18:0p	392	294
20:1p	418	320
20:0p	420	322

four of the exchangeable hydrogen atoms were involved in this fragmentation process. The ion that was observed at *m/z* 386 was increased by 1 Da from *m/z* 385 in nondeuterated experiments, which suggested that only one of the exchangeable hydrogen atoms was involved in this fragmentation process. Additionally, a small fragment corresponding to a loss of 145 Da (*m/z* 607) was observed, which was loss of neutral phosphoethanolamine-d_4.

More information about the structures of the fragment ions that were produced during CID of GPEtn plasmalogens was obtained from MS^3 experiments on the two major product ions present in each CID spectrum (Table 2). For example, the MS^3 spectrum of *m/z*

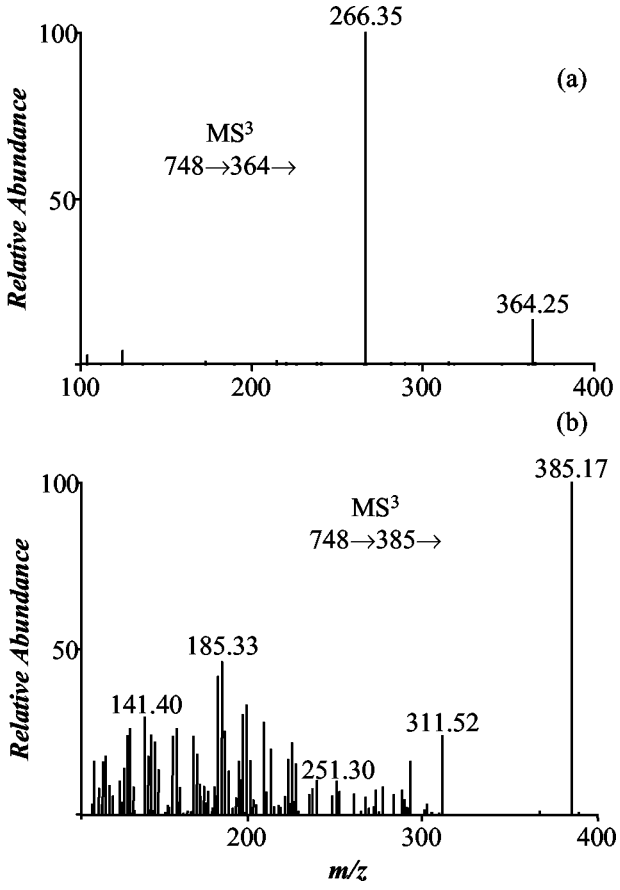
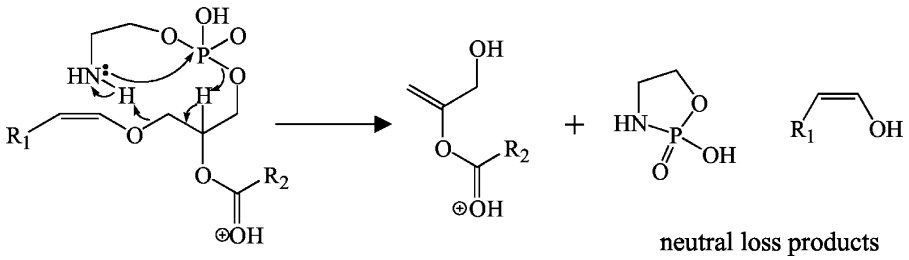


Figure 7. Product ions (MS^3) in an ion trap mass spectrometer following collisional activation of (**a**) *m/z* 364 generated from the $[M + H]^+$ of GPEtn plasmalogens with a 16:0p group at the *sn*-1 position and (**b**) *m/z* 385 generated from the $[M + H]^+$ of GPEtn plasmalogens with a 22:6 group in the *sn*-2 position.

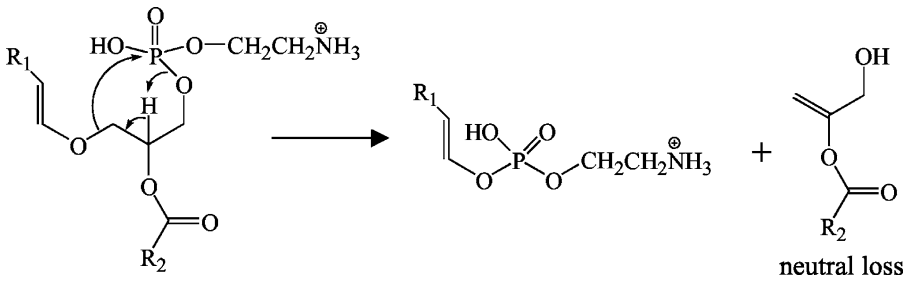


Scheme 1

364, which was obtained from CID of any GPEtn plasmalogen that had 16:0p as a *sn*-1 substituent, indicated one major fragment ion at *m/z* 266 (Figure 7a). Similar MS^3 experiments were performed for the other GPEtn plasmalogens that had 18:0p, 18:1p, 20:0p, or 20:1p at the *sn*-1 position (Table 2). It was apparent from the data (Figure 7a and Table 2) that neutral loss of 98 Da, which corresponded to H_3PO_4, was consistently observed. MS^3 experiments were also performed on the deuterated 16:0p/22:6-GPEtn analog and the *m/z* 368 product ion, which was obtained from CID of *m/z* 752, fragmented into *m/z* 268 with a neutral loss of 100 Da that corresponded to D_2HPO_4. Furthermore, the MS^3 spectrum of *m/z* 385, which was obtained from CID of any GPEtn plasmalogen that had docosahexaenoyl as a *sn*-2 substituent, contained many different fragment ions that corresponded to the loss of consecutive methylene groups (Figure 7b). Additionally, similar MS^3 spectra were obtained from activation of *m/z* 337, 339, 361, 387, and 389. This fragmentation pattern was typically observed during the CID of the $[M - H]^-$ of polyunsaturated acids [15]. From these MS^3 spectra it was thought that an unsaturated fatty acid was present in this fragment ion.

The information obtained from the tandem mass spectrometry experiments described above allowed for the proposal of structures and the mechanisms of formation for the two major product ions (Tables 1 and 2) that were formed upon CID of GPEtn plasmalogens. It is proposed that the major product ion that was characteristic of the *sn*-2 position (*m/z* 337, 339, 361, 385, 387, or 389) contained the glycerol backbone and the fatty acid esterified to the *sn*-2 position of the glycerol backbone (Scheme **1**) consistent with the tandem mass spectrometric result of a characteristic product ion for each GPEtn plasmalogen species with a different *sn*-2 substituent. A proposed mechanism of formation of this fragment ion required a protonation site at the *sn*-2 ester and involved an attack of the nitrogen atom lone pair of electrons on the phosphorous atom, which leads to abstraction of hydrogen from carbon-2 of the glycerol backbone (Scheme **1**). This resulted in double bond formation between carbon-1 and -2 of the glycerol backbone and a hydrogen abstraction from the amine. This mechanism led to a fragment ion that was characteristic of the *sn*-2 position of GPEtn plasmalogens, as well as two neutral products that included the 1-O-alk-1′-enyl as an alcohol ($C_{14}H_{29}CHCHOH$) and 5-membered ring compound, $P(O_2H)O(CH_2)_2NH$ (Scheme **1**). One of the key features of this product ion is that the positive charge is located on the acyl group at the *sn*-2 position, which is consistent with the ethyl alcohol-*d* data (Figure 6b) where the *m/z* of this product ion was increased by only 1 Da. Additionally, it was determined from these studies that this mechanism would occur for all GPEtn plasmalogens independent of the acyl group present at the *sn*-2 position.

One major product ion series observed in the CID spectra of GPEtn plasmalogens was quite characteristic of the *sn*-1 position and observed at *m/z* 364, 390, 392, 418, or 420 for 16:0p, 18:1p, 18:0p, 20:1p, and 20:0p molecular species. A proposed mechanism of formation of this fragment ion involved the oxygen atom at the *sn*-1 position attacking the phosphorous atom, which resulted in the formation of a new oxygen-phosphorous bond and concomitant abstraction of the hydrogen from carbon-2 of the glycerol backbone to form a double bond between carbon-1 and -2 of the glycerol backbone (Scheme **2**). This mechanism would yield a fragment ion characteristic of the *sn*-1 position of GPEtn plasmalo-

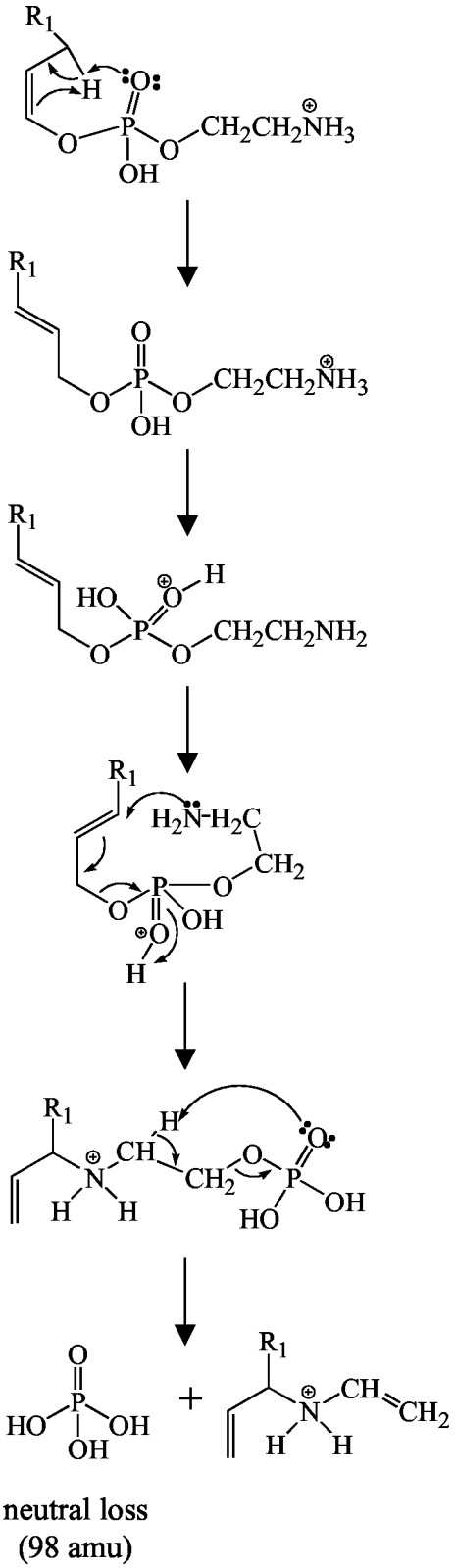


Scheme 2

gens, as well as a neutral product that contained the remaining glycerol backbone and the *sn*-2 substituent (Scheme **2**). The positive charge would be located on the nitrogen atom in this mechanism consistent with the deuterium labeled collisional spectra (Figure 6b) where this product ion was increased by 4 Da corresponding to inclusion of all exchangeable protons. Additionally, it was found that this ion was observed for all GPEtn plasmalogens independent of the length of the chain or the number of double bonds present in the substituent at the *sn*-1 position.

MS^3 experiments were performed with the CID fragment ion indicative of the *sn*-1 substituent and revealed that the only product ion formed upon activation of the resultant ion in Scheme **2** was due to a loss of H_3PO_4 (98 Da). In order to obtain this loss of H_3PO_4 from the fragment ion indicative of the *sn*-1 substituent, rearrangement of the resultant ion in Scheme **2** from a phosphodiester to a phosphomonoester was required. It is proposed that the phosphoryl oxygen catalyzes the sigmatrophic shift to an intermediate where the charge site shifts to the phosphorous oxygen (Scheme **3**). This allowed the lone pair of electrons located on the nitrogen atom to attack the double bond, which resulted in the formation of a new carbon-nitrogen bond and a shift of the double bond. This shifting of the double bond resulted in cleavage of the C–O bond and in the formation of a rearrangement ion as a phosphomonoester, which can undergo facile loss of H_3PO_4 (98 Da). This mechanism is also consistent with the deuterium labeled collisional spectra (data not shown) where this neutral loss was increased to 100 Da.

Diacyl-GPEtn lipids undergo an abundant neutral loss of 141 Da during CID of $[M + H]^+$ [9] and the product ions derived from collisional activation of the $[M + H]^+$ of GPEtn plasmalogens described above are not typically observed in the CID spectra of diacyl-GPEtn. Somewhat less is known about the 1-O-alkyl phosphoethanolamine lipids. In order to establish the importance of the double bond of the vinyl ether at the *sn*-1 position in terms of fragmentation patterns during collisional activation, the human neutrophil derived GPEtn lipids were subjected to acid hydrolysis. This allowed the examination of the CID fragmentation of 1-O-alkyl phosphoethanolamine lipids without interference from an isobaric GPEtn plasmalogen. The CID spectra of the $[M + H]^+$ of *m/z* 726 (16:0e/20:4-GPEtn and 18:1p/18:2-GPEtn) before and after acid hydrolysis illustrate the importance of the vinyl ether double bond at the *sn*-1 position (Figure 8). Before acid hydrolysis there were three fragment ions in the CID spectrum that included *m/z* 337, 390, and 585 (Figure 8a). It was thought that *m/z* 337 and 390 were due to CID of 18:1p/18:2-GPEtn. The neutral loss of 141 Da at *m/z* 585 was more abundant than the neutral loss of 141 Da observed in Figures 3 and 4. Therefore, it was thought that this strong neutral loss of 141 Da was due to 16:0e/20:4-GPEtn. In order to verify this hypothesis, the CID spectrum of $[M + H]^+$ at *m/z* 726 was obtained after acid hydrolysis and it was found that only one product ion, which corresponded to neutral loss of 141 Da, was present in the CID spectrum (Figure 8b). This



Scheme 3

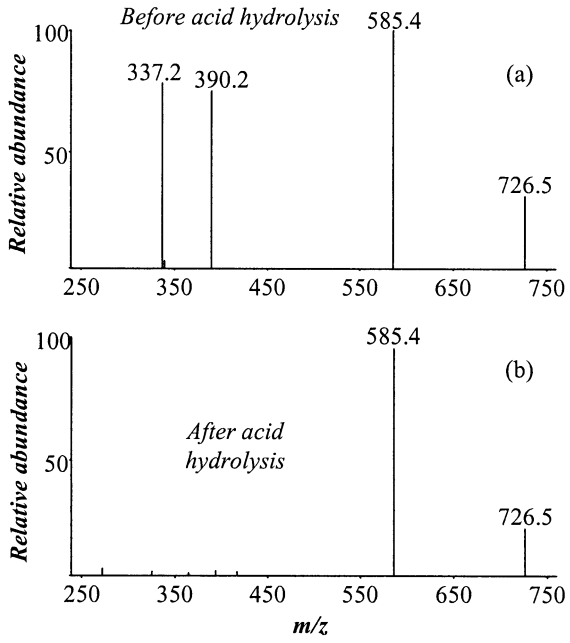


Figure 8. Electrospray tandem mass spectra in the positive ion mode of the isobaric $[M + H]^+$ at *m/z* 726 (16:0e/20:4-GPEtn and 18:1p/18:2-GPEtn) (**a**) before and (**b**) after acid hydrolysis.

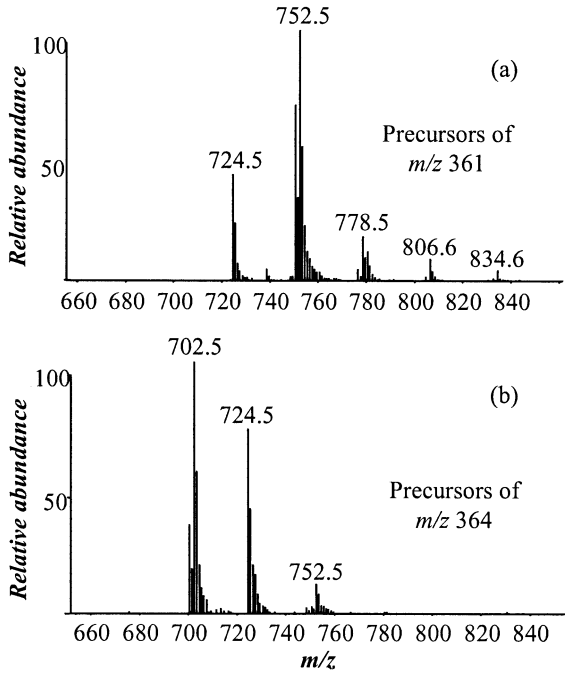


Figure 9. Precursor ion scans used to determine the identities of the fatty acid esterified at the *sn*-2 position of the glycerol backbone and the 1-O-alk-1′-enyl ether at the *sn*-1 position. (**a**) Precursor ion scan of *m/z* 361 allows for specific detection of GPEtn plasmalogens with arachidonate esterified at the *sn*-2 position. (**b**) Precursor ion scan of *m/z* 364 allows for specific detection of GPEtn plasmalogens a hexadecyl vinyl ether moiety at the *sn*-1 position.

indicated that 1-O-alkyl-2-acyl-GPEtn was only collisionally activated with a neutral loss of 141 Da during CID in the positive ion mode and that the presence of a vinyl ether at the *sn*-1 position was a key driving force in the product ions that are observed in the CID spectra of GPEtn plasmalogens. It is possible that the vinyl ether double bond present at the *sn*-1 position of plasmalogens withdrew electron density away from the C–O bond that attaches the *sn*-1 substituent to the glycerol backbone consistent with the proposed mechanisms described above.

While the results of these studies revealed that the use of the neutral loss of 141 Da can be problematic in the detection of plasmalogen glycerophosphoethanolamine lipids, there were ions observed common to specific plasmalogen molecular species which could be used as a powerful means to detect such molecular entities. For example, there has been considerable interest in the distribution of arachidonic acid within glycerophospholipids [16] and precursor ion scan of *m/z* 361 would be anticipated to detect those phospholipids in a complex mixture which contain this polyunsaturated fatty acid. When this experiment was carried out with the neutrophil phospholipids (Figure 9a), indeed the individual molecular species which contained arachidonate were detected with the most abundant molecular species identified in Figure 2a by positive ion electrospray ionization also detected at *m/z* 752.5, indicating this component contained arachidonate (18:0p/20:4-GPEtn). Another ion derived from the vinyl ether substituent at the *sn*-1 position could readily also be used to detect hexadecyl containing molecular species by carrying out a precursor ion scan for *m/z* 364 (Figure 9b). Three quite abundant molecular species were observed at *m/z* 702.5, 724.5, and 752.5, corresponding to 16:0p/18:1-GPEtn, 16:0p/20:4-GPEtn, and 16:0p/22:4-GPEtn. A combination of these two precursor ion scanning experiments clearly revealed that the ion at *m/z* 752.5 (Figure 2a) actually contained two isomeric components.

In summary, the fragmentation processes observed following collisional activation of GPEtn plasmalogens generate unique determinants of the identities of the 1-O-alk-1′-enyl at the *sn*-1 position and the fatty acid esterified at the *sn*-2 position of the glycerol backbone. For example, it is possible to uniquely detect arachidonate at the *sn*-2 position of GPEtn plasmalogen lipids using a precursor ion scan of *m/z* 361 (Figure 9a). Additionally, it is also possible to detect all of the GPEtn plasmalogens with a hexadecyl vinyl ether substituent at the *sn*-1 position by a precursor ion scan of *m/z* 364 (Figure 9b). Such characteristic product ions found in the CID spectra of GPEtn plasmalogens should be useful in identifying unique plasmalogen molecular species in biological samples.

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