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Mass Spectrometric Analysis of Long-Chain Lipids

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Introduction

Lipids are a diverse array of biomolecules that are essential for all living and replicating cells. Since comprehensive discussion of the mass spectrometry of all known lipid species is not practical, this review will focus on lipid families that are most commonly the target of mass spectrometric analysis. These species tend to have long fatty acyl chains and result from thioester carbanion condensations catalyzed by fatty acid synthase.

Analysis of these molecules by mass spectrometry can be challenging from many different points of view depending upon the type of lipid and its abundance within a biological extract. While modern mass spectrometers and the ionization methods of electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) are capable of generating molecular ion species from all known lipids, the biochemistry that leads to their production often results in the generation of many closely related molecules. This is especially true for those lipids that incorporate long hydrocarbon chains derived from fatty acid biosynthesis. Successful analysis of these molecules requires considerable attention to isolation and purification in addition to consideration of mass spectrometric parameters.

Long-chain lipid substances have been recently categorized into four different families (Table 1) depending upon their mechanism of biosynthesis as well as the number of species within each family (Fahy et al., 2005). Even though this division is somewhat arbitrary, it has been useful because mass spectrometric methods developed for lipid analysis have centered around these divisions. The glycerolipids (triacylglycerols and diacylglycerols) could logically be placed in the same group as the phospholipids (phosphatidylcholine, phosphatidylethanolamine, etc.) since both family members share a common glycerol backbone in their structure. However, these two types of lipids have entirely different chemical properties (neutral versus polar) and biochemical functions (energy storage versus membrane bilayer structure). In addition living systems have vast numbers of molecular species in each of these lipid families.

The complexity alluded to above is due to the number of closely related species, often termed molecular species, which are synthesized by cells. However, not all lipids are present in cells as families of molecular species. For example, mammalian cells produce cholesterol as a single, very abundant sterol making both quantitative and qualitative analysis fairly

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straightforward. In contrast, the phospholipids, glycerolipids, and sphingolipids that are the subject of this review exist as hundreds if not thousands of different molecular species within a single mammalian cell. These molecular species differ by fatty acyl or fatty alkyl ether substituents covalently linked to a common backbone moiety such as a long chain base (sphinganine) or glycerol as well as the positions of double bonds and acylation position of each fatty acid. This chemical diversity greatly complicates both their qualitative as well as quantitative analysis.

Another challenging aspect to lipid analysis is that some exceedingly important lipid species are only present in very low concentrations. For example, several lipid species serve either as signaling molecules that are recognized by specific proteins, or as intermediates in enzymatic transformations. These lipids are typically present at at picomolar or lower concentrations within cells or biological fluids. Another example is lipids that have been the target of reactive oxygen attack. These oxidized lipids often have important functions, but are minor in abundance relative to their precursor pool. When these specific lipids are the target of mass spectrometric analysis, they present a considerable challenge because of their trace concentrations, the presence of isomeric forms, and the chromatographic separation techniques that are required.

Many types of mass spectrometric instrumentation have been employed with success in the analysis of lipids. ESI is useful for continuous analysis of liquid chromatographic effluents. It readily generates positive ions, although negative ion abundance is higher for many different lipids. Electron ionization (EI) is typically used in combination with on-line gas chromatography and prior hydrolysis and derivatization. It is a generally applicable ionization method that yields positive ions with rich fragmentation patterns (Murphy, 1993). Chemical ionization (CI) has also been used in lipid analysis in tandem with gas chromatographic separation. Atmospheric pressure chemical ionization (APCI) is similar to ESI in concept, except that charge is applied to the analyte by ion-molecule reactions during evaporation of the carrier solvent. This is a more energetic form of ionization that frequently induces fragmentation "in-source" through collisions with gas at atmospheric pressure. The fragmentation of ions in-source can be desirable and useful for lipid analysis, as described below. Fast atom bombardment (FAB) has been used effectively in the past (Griffiths, 2003), but has largely been supplanted by ESI, MALDI, and APCI.

The most widely used mass analyzer for lipid studies has been the tandem quadrupole "MS/MS" or MS^2 mass spectrometer. This design is popular because it facilitates fragmentation by collisional activation or "collision induced decomposition" (CID) of molecular ions in conjunction with precursor ion scanning, product ion scanning, neutral loss scanning, and multiple reaction monitoring (MRM). Each of these operating modes has valuable applications in lipid analysis. Ion traps, whether an ion cyclotron resonance instrument, octupole, or linear quadrupole design, have also been used with success for lipid analysis but only for molecular ion detection or product ion analysis. This same limitation also applies to time-of-flight (TOF) instruments that are often coupled to MALDI sources. More complex TOF instruments may be configured with an ESI source and quadrupoles for ion selection and CID (QqTOF). These instruments offer enhanced sensitivity and mass determinations that are sufficiently accurate, for example, to distinguish between lipids containing a vinyl ether group (–CH=CH–O–, 42.0111 Da) and an alkyl chain ($–CH_2–CH_2–CH_2$, 42.0481 Da). However, they are not as versatile as the tandem quadrupole mass spectrometer for the scanning and MRM analyses mentioned above.

Throughout this review, M will be used to designate the mass of an uncharged molecule, with the loss of a proton to create an anion denoted by $[M-H]^-$ and the gain of a proton to create a cation denoted by $[M+H]^+$. Integral masses or element symbols will be included

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within the brackets to indicate various other losses and gains (e.g. $[M+H-18]^+$ to indicate the loss of water or $[M+Li]^+$ to indicate the gain of a lithium ion). The quantity actually measured by a mass spectrometer will be denoted by the mass-to-charge ratio, or *m/z*. Fatty acyl chains, individually and collectively within a lipid molecule, may be described with TC:DB notation in which TC is the total number of carbon atoms and DB is the total number of double bonds in the chains.

Lipid extraction and ionization

Textbooks generally suggest that lipids are soluble in organic solvents. This statement is true for some but not for all lipids. There are very polar lipids that have little or no solubility in organic solvents and therefore cannot be extracted out of an aqueous environment by solvent systems such as chloroform/methanol. Nonetheless, solvent extractions such as the Folch extraction (Folch et al., 1957) and the Bligh/Dyer extraction (Bligh and Dyer, 1959) are quite effective in isolation of most phospholipids, neutral lipids and sphingolipids. Newer extraction solvents systems continue to be reported (Matyash et al., 2008) and one should never underestimate the importance of this first extraction step to isolate relatively pure lipids for subsequent analysis.

There is also value in selective solvent extraction strategies to simplify lipid mixtures prior to chromatographic separation. Triglycerides and cholesterol esters can be selectively extracted with organic solvents such as isooctane/ethyl acetate (Hutchins et al., 2008). Careful consideration and development of a specific extraction strategy can profoundly affect the rapidity of analysis and the robustness of the overall protocol. For example an advantageous aspect of the Bligh/Dyer (Bligh and Dyer, 1959) procedure is that formation of a single organic/aqueous phase (monophase) is an intermediate step in this extraction protocol, prior to phase separation. At this monophase step, equilibrium mixing of internal standards with the endogenous lipid substances is greatly facilitated. Some lipids are best recovered in the monophase rather than separating into two phases, for example certain sphingolipids rather equally distribute in the upper and lower phase.

Other methods to isolate target lipids include solid phase extraction approaches that capitalize on the hydrophobic character of the target lipid to associate with the covalently bonded hydrophobic moiety, such as an 18-carbon alkyl chain on a silica bead. An advantage of solid phase extraction is that a polar functionality, which may prevent organic solvent extraction by reducing the partition of the entire lipid into the immiscible organic phase, does not interfere with the hydrophobic interaction of the nonpolar region of the lipid structure with the solid phase material. This interaction permits facile separation of lipophilic material from non-lipophilic material.

While not the subject of this tutorial, separation techniques play a critical role in lipid analysis. Both normal phase and reversed phase liquid chromatography can be directly coupled to mass spectrometry through an ESI source (commonly denoted as “LC/MS” or “ESI-LC/MS”). Much of the classical research in lipid biochemistry involved normal phase separation of lipids using thin layer chromatography (Touchstone, 1995) and the fundamental principles of this separation technique may be implemented with the coupling of normal phase HPLC (NP-HPLC) to the mass spectrometer. In general, normal phase chromatography separates lipids according to their polar features, while reversed phase HPLC (RP-HPLC) separates according to their hydrophobic properties. Either way, an important aspect of separation by HPLC prior to mass spectrometry is the removal of materials that have the potential for ion suppression during ESI.

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Mass Spectral Characteristics

A. Fatty Acids and Eicosanoids (Figure 1)

Fatty acids (FA) are a basic building block of many complex lipids and are found in biological extracts not only as free carboxylic acids, but also esterified as in wax esters, phospholipids, diacylglycerols, and triacylglycerols. They are also found as simple amides and as complex amides N-linked to the long chain bases of sphingolipids. There is great structural diversity in this class including unsaturated, branched, hydroxyl, keto, and di-carboxylic acids. A rich history exists for the use of mass spectrometry applied to the analysis of free carboxylic acids and their volatile derivatives using EI (Murphy, 1993). As an example, the EI mass spectrum of methyl arachidate (methyl-20:0) (Figure 1A) and methyl arachidonate (methyl-20:4) (Figure 1B) reveal both the power and weakness of this ionization technique. For saturated fatty acids a great deal of structural information is generated by EI including an abundant and characteristic rearrangement ion (*m/z* 74, the McLafferty rearrangement ion), a molecular ion at *m/z* 326 and various fragment ions. The ion chemistry of saturated fatty acid derivatives has been extensively studied for decades and summarized in monographs (Murphy, 1993; Pittenauer and Welz, 2006). Polyunsaturated fatty acids, in contrast, are extensively fragmented by EI, suggesting multiple sites of radical cation formation. Typically, very little specific information is obtained from the EI mass spectrum including little or no molecular ion abundance.

Fatty acid analysis by EI is typically carried out by GC/MS and where GC retention time contributes important information to assign structural features such as methyl group branching site, *cis/trans* double bond geometry, double bond location, and other structural features by comparison with known fatty acids and their gas chromatographic elution order. Only a little information about these structural features can be obtained by interpreting the mass spectrum itself. Specific ion chemistry after chemical ionization with reactive reagent ions (Lawrence and Brenna, 2006) as well as specialized derivatization techniques, which lead to well behaved rearrangement ions during the ionization process (Harvey, 1998), have also been successful in revealing double bond location.

One approach to the analysis of complex lipids is to chemically degrade them, releasing the acyl-ester linked, N-acyl linked, or O-alkyl-ether linked residues that confer species identity for collective analysis. The goal of this analysis may be simple quantitation, but may also include establishing the number and position of double bonds, the number and position of oxygen-containing functional groups, and alkyl branch points. However it is important to note that a lot of information about the chemical nature of a complex lipid is lost when it is degraded in this way.

As a class, alkyl carboxylic acids have pKa values in water between 4-5. In aqueous solution they tend to form micelles or bind to membranes or proteins in biological systems, and these circumstances will alter their pKa. Nevertheless, in most cases they are anions at neutral or physiological pH. Positive mode ESI is greatly facilitated by the presence of metal ions and the formation of cationic “metalated” adducts. These adducts can be made to decompose by collisional activation in the mass spectrometer with alkyl chain C–C bond cleavage at high collision energies. The position of the double bond in monounsaturated species, for example, may be revealed by a relatively small number of fragmentation products involving cleavage at the double bond or either of the two adjacent single bonds. A better strategy has been to collisionally activate $[M + metal]^+$ ions in a high energy instrument, which can induce site remote fragmentation and reveal double bond positions (Cheng and Gross, 2000).

Fatty acids are more typically analyzed by ESI as negative ions. As with most all $[M - H]^-$ anions of carboxylic acids, collisional activation causes free fatty acids to decompose with a

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neutral loss of water to yield an $[M - H - 18]^-$ ion. Somewhat less frequently, decomposition occurs via neutral loss of CO_2 to yield an $[M - H - 44]^-$ ion. Abundant carboxylate anions are formed in ESI due to the solution stability of this ionic form and these ions can be used to unambiguously establish molecular weight of the fatty acid (Figure 1C and 1D). While RP-HPLC retention times are useful in establishing certain structural features, this chromatographic technique is somewhat less powerful when compared to GC, due to the reduced number of theoretical plates that HPLC separation can achieve. Collisional activation of $[M - H]^-$ ions generated as fatty acid carboxylate anions does yield some product ions from polyunsaturated FA (Figure 1D), but little decomposition is observed from saturated FA (Figure 1C). The combined tools of GC/MS with EI and tandem mass spectrometry of ESI generated $[M - H]^-$ ions and $[M + \text{metal}]^+$ ions present a powerful approach for structural characterization of FA even when present in complex mixtures.

FA quantitation has been successfully performed with a variety of approaches, including EI-GC/MS, ESI-LC/MS, and ESI-LC/MS/MS. For such studies, stable isotope dilution is the preferred method for quantitative assessment of a target FA (Watson and Sparkman, 2007). Alternatively, a homologous internal standard may be used for those instances that do not require a high level of quantitative certainty or when isotope labeled internal standards are not available. Quantitation is based on an empirically determined standard curve that converts the signal derived from a target molecule into an absolute quantity. This involves performing a linear regression analysis of the analyte:standard ion yield ratio versus the analyte:standard molar concentration ratio (Figure 2). This example presents the quantitation of a metabolite of arachidonic acid generated by 5-lipoxygenase (Murphy and Gijon, 2007) and is typical of an eicosanoid standard curve used to measure such lipid mediators.

Perhaps the widest application of FA analysis by mass spectrometry has been in the study of biologically active arachidonic acid metabolites. These 20-carbon “eicosanoid” metabolites include the prostaglandins, leukotrienes, and other complex lipids derived from cyclooxygenase, lipoxygenase, cytochrome P_{450}, as well as those derived from other enzymatic reactions that generate bioactive molecules from this unique polyunsaturated fatty acid (Smith and Murphy, 2008). In addition to the enzymatically formed eicosanoids, there are also products formed by free radical oxidation of polyunsaturated fatty acids esterified to phospholipids. When arachidonate is the target of a nonenzymatic free radical reaction, a family of compounds called “isoprostanes” are formed. These compounds are isomers of the prostaglandins formed enzymatically, and some have prostaglandin-like biological activity. Isoprostanes have been widely used as biomarkers of oxidative stress (Milne et al., 2008).

In order to use mass spectrometry for the quantitative analysis of eicosanoids, some unique parameter must also be included in the analytical protocol because isobaric molecules exist in many cases and molecular weight information alone is not sufficient to distinguish among them. This additional parameter could involve chromatographic elution at a specific retention time, or unique ion chemistry following collisional activation (a specific mass loss from the molecular ion) or often both parameters. In order to achieve requisite sensitivity for GC/MS analysis, negative ion chemical ionization is employed after derivatization to the pentafluorobenzyl ester (PFB). Negative ion chemical ionization of this electronegative derivative yields a highly abundant $[M - PFB]^-$ carboxylate anion (Blair, 1990). Gas chromatography of these esters can separate many of the isomeric isoprostanes and all of the primary prostaglandins from each other. Many deuterium-labeled internal standards are commercially available for prostaglandin and leukotriene analysis.

A second approach is to use LC/MS/MS technology where carboxylate $[M - H]^-$ anions are directly formed by ESI, then collisionally activated to yield specific product ions. These

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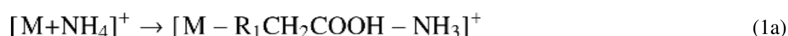
product ions and the precursor molecular ion species can be used as a highly specific MRM experiment that is well suited for quantitative analysis. Since modern instrumentation permits the MRM experiment to be performed on numerous selected ion transitions, often as many as 50 different MRMs in a single analytical protocol, the analysis of eicosanoids in a biological sample may be carried out for a large number of both cyclooxygenase and lipoxygenase metabolites in a single run, greatly reducing the cost per analyte. These assays typically have limits of detection in picograms injected on column (Masoodi et al., 2008). A recent review has summarized our current understanding of the mechanistic origin of product ions from most of the known metabolites of arachidonate acid following collisional activation. Such knowledge is essential in developing either a qualitative or quantitative assay for these types of fatty acids (Murphy et al., 2005).

B. Glycerolipids (GL) (Figure 3)

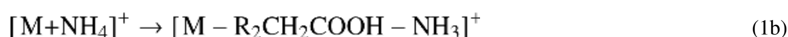
The analysis of glycerolipids, including triacylglycerols (TAGs) and diacylglycerols (DAGs) that have no electrostatic charge in solution, can nevertheless be carried out with APCI, ESI, and in many cases, EI-GC/MS. Most glycerol lipids yield a molecular ion of very low abundance when EI is employed. Abundant fragment ions corresponding to loss of each fatty acyl group as carboxyl radical and abundant low mass ions specific for each fatty acyl chain are observed (Murphy, 1993). Thus it is difficult to determine their mass directly using EI alone, especially when mixtures of TAGs are present. When APCI conditions are employed, fairly weak $[M + H]^+$ ions are observed, but ions corresponding to the CID loss of fatty acyl groups esterified to the glycerol backbone as a neutral carboxylic acid $[M + H\text{-}R_{1,2,3}COOH]^+$ are abundant (Byrdwell, 2005). Since APCI is a relatively energetic ionization technique, the molecular ion species formed in the atmospheric pressure region of the ion source undergo CID prior to entering the low pressure of the mass spectrometer.

The analysis of triacylglycerol molecular species analysis can be challenging if information about all unique molecular species is desired. This is because of the sheer number of these species arising from the seemingly random assembly of the fatty acyl groups on the glycerol framework. For example, more than 10 different TAG species contain 52 carbon atoms and four double bonds in their fatty acyl chains (i.e. 52:4), and are all observed at the same mass, namely *m/z* 854.7364. In addition there are positional isomers and variations in double bond geometry or location (McAnoy et al., 2005). ESI generates abundant $[M + NH_4]^+$ or $[M + metal]^+$ for TAG species when ammonium ions or alkali metal ions are present in the solvent system being sprayed (Hsu and Turk, 1999; Han and Gross, 2001).

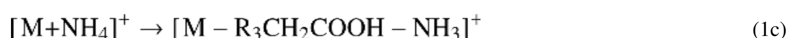
In order to determine the nature of the three groups in each TAG molecular species, a second phase of CID (MS/MS/MS or MS^3) may be employed (McAnoy et al., 2005). MS/MS of $[M + NH_4]^+$ ions generates three "diglyceride type ions" for each isobaric TAG molecular species (for reactions in this section, R_1, R_2, and R_3 correspond to long-chain groups at the sn-1, sn-2, and sn-3 positions of glycerol.)



$$[M{+}NH_4]^+ \rightarrow [M - R_1CH_2COOH - NH_3]^+ \quad (1a)$$



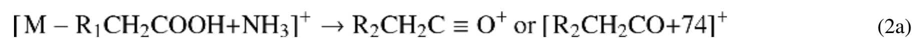
$$[M{+}NH_4]^+ \rightarrow [M - R_2CH_2COOH - NH_3]^+ \quad (1b)$$



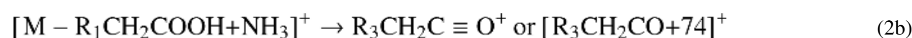
$$[M{+}NH_4]^+ \rightarrow [M - R_3CH_2COOH - NH_3]^+ \quad (1c)$$

These ions correspond to the loss of one esterified fatty acyl group as the free carboxylic acid as well as ammonia (reactions 1a-c) from the molecular ion, and they provide

information about the fatty acyl groups of the TAG molecular species. A mechanism responsible for the formation of these ions has been suggested (Scheme 1) (McAnoy et al., 2005). Each of these diglyceride ions can then be further decomposed in a second stage of CID to create "monoglyceride" ions. The observed mass-to-charge ratio of this ion reveals the nature of a second fatty acyl group. The difference in mass (neutral loss) between the "diglyceride ion" and the "monoglyceride ion" reveals the third fatty acyl group (reactions 2a,b).



$$[M - R_1CH_2COOH+NH_3]^+ \rightarrow R_2CH_2C \equiv O^+ \text{ or } [R_2CH_2CO+74]^+ \quad (2a)$$



$$[M - R_1CH_2COOH+NH_3]^+ \rightarrow R_3CH_2C \equiv O^+ \text{ or } [R_3CH_2CO+74]^+ \quad (2b)$$

The structure of the $[R_3CH_2CO + 74]^+$ and $[R_3CH_2CO + 74]^+$ ions are indicated in Scheme 2. Chromatographic separation of TAG molecular species has been described for simple mixtures from biological sources that have relatively few common fatty acids in their lipidome (Dugo et al., 2006). For mammalian cells, complete separation of all TAG species has not been possible with either RP-HPLC or even the more powerful capillary GC columns. One is often forced to compromise and quantify the isobaric molecular species (Hutchins et al., 2008).

At the level of molecular ion analysis, it is possible to eliminate the systematic error due to naturally abundant carbon-13 through simple calculations (Han and Gross, 2001). Nevertheless, important assumptions are made because of the influence of double bonds and ion stability of each of the different molecular species, and their influence on absolute quantitation relative to molecular ion abundance (Li and Evans, 2005). However, if the data is used in comparative experiments where the same cell population or tissue sample is differently treated, then these errors largely cancel out. Even without absolute concentrations, relative changes in the signals arising from isobaric ion species (after isotope correction) can reveal a wealth of information about changes in this class of lipids as the result of pharmacological intervention. When an interesting change in the abundance of an ion is observed, MS/MS and MS^3 may be employed to drill down and provide more specific information about the molecular species that comprise a set of isobaric ions.

One note of caution regarding assigning TAG isobaric species identity: while it is possible to calculate TC:DB for an observed mass-to-charge ratio (several computer programs do this), built into this calculation is an assumption that the observed mass-to-charge ratio is derived from a triacylglycerol. However, it is now known that in mammalian systems, monoalkyldiacylglycerols exist, which have the same nominal mass as TAGs containing one odd chain fatty acyl group. Fortunately, these unique triradylglycerol molecular species can be separated by normal phase HPLC because of the difference in polarity between an ether group and an ester group, and separation removes the assumption concerning the nature of the triradylglycerol (Hutchins et al., 2008).

When ESI is employed, abundant and specific ions can be generated for DAGs if the free hydroxyl group is derivatized. Derivatization may be implemented easily on the microchemistry scale, and can yield excellent results for DAG analysis. Several reports have appeared using this strategy (Li et al, 2007; Callender et al., 2007).

A similar derivatization strategy has been used to analyze DAGs by GC/MS where thermostability and volatility of the lipid is an absolute requirement. This approach can be readily implemented and used to effect chromatographic separation of diverse molecular species (Hubbard et al., 1996).

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C. Phospholipids (PL) (Figure 4)

The bilayer membranes in and around all living cells are composed in large part of glycerophospholipids (PL). The generic structure of these molecules is that of a glycerol backbone where the carbinol oxygen atoms corresponding to carbon-1 and carbon-2 (commonly named according to the stereospecific numbering convention as the *sn*-1 and *sn*-2 positions) are linked to hydrophobic groups, typically by esterification to long chain fatty acids (Figure 4), although in some cases via alkyl ether linkages to position *sn*-1. The third (*sn*-3) carbinol is linked to a phosphate group either as a monoester (phosphatidic acid, PA) or as a phosphodiester which in turn is linked to a polar alcohol including choline (phosphatidylcholines, PC), ethanolamine (phosphatidylethanolamines, PE), serine (phosphatidylserines, PS), glycerol (phosphatidylglycerols, PG) and myoinositol (phosphatidylinositols, PI). PA, PC, PE, PS, PG, and PI comprise the six major PL headgroup classes. Within a cell the diversity of this family of lipids can include a thousand or more molecular species that differ in hydrophobic groups linked to the *sn*-1 and *sn*-2 positions as well as their polar head groups. This diversity in molecular species arises from *de novo* PL biosynthesis (Kennedy pathway; Kennedy and Weiss, 1956;Weiss et al., 1958) as well as the active remodeling of fatty acyl groups (Lands pathway; Lands, 1960).

Both ESI and MALDI generate $[M + H]^+$ and $[M - H]^-$ ions for PLs, although the polar head group generally drives a preference for either positive ion (PC) or negative ion formation (PS, PI, PA, and PG). PE yields both abundant positive and negative molecular ion species by either ionization method. The abundance of these molecular ion species is often used to indicate the relative quantity of each component in a mixture.

There are important differences between the nominal mass of a molecular ion (calculated with integer mass for the most abundant stable isotopes), its exact mass (calculated with the exact mass on the physical atomic scale of the most abundant stable isotopes), and its chemical molecular weight. For example, 16:0/18:1-PC has a nominal mass of 759 Da, whereas its exact mass is 759.578 Da, and its chemical molecular weight is 760.076 gm/mole. The term “mass defect” refers to the difference between the nominal mass and the exact mass, and it is due to the non-integral masses of elements other than carbon. The number of hydrogen atoms in a molecule is largely responsible for the mass defect because the exact mass of a hydrogen atom is 1.00783 Da. Chemical molecular weight differs from exact mass due to the presence of naturally abundant isotopes, chiefly carbon-13. The natural abundance of the carbon-13 isotope changes the quantity of total ionization carried by the monoisotopic $[M+H]^+$ ion (i.e. the ion in which all carbon atoms are carbon-12). This leads to a natural trend in which the fractional abundance of a molecular species appearing as the monoisotopic ion decreases as molecular weight increases. For example, 16:0/18:1-PC, which has 42 carbons, yields a monoisotopic ion at *m/z* 760.586 that represents 61.7% of the ions created when counting all stable isotopes. The remaining 38.3% appears in carbon-13 and oxygen-18 containing ions at M+1, M+2, etc. This monoisotopic abundance mole fraction decreases to 48.7% for 18:2/18:2/18:2/18:2-cardiolipin observed at *m/z* 1447.965. Correction for this isotope abundance variation is often made to correct the mole fraction of molecular species in a complex mixture of PL isolated from biological systems when the data corresponds to the abundance of the monoisotopic molecular ion species (Han and Gross, 2001).

Another complication in the analysis of PLs is that $[M + H]^+$ or $[M - H]^-$ ions may represent isobaric molecular species with different fatty acyl groups esterified to the glycerol carbinols. The identity of the fatty acyl groups can be determined by collisional activation of the $[M - H]^-$ ions to generate diagnostic fatty acyl ions (see below). For example, lipid anions at a single *m/z* commonly fragment to yield two carboxylate anions at relatively high abundance and additional carboxylate anions of lower abundance derived from isomeric PLs

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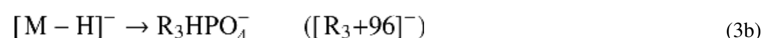
also present. One way to circumvent confusion with this issue is to report the number of carbons double bonds in the fatty acyl chains, without stipulating which fatty acyl groups are actually present.

C.1 MS identification and analysis of PLs—Qualitative and quantitative analysis of specific PLs requires some insight into their patterns of collision induced decomposition (CID). A general pattern is that collisional activation of $[M + H]^+$ ions tends to yield information about their polar head groups, while collisional activation of $[M - H]^-$ ions reveals information about specific fatty acyl or alkylether chains present on the glycerol backbone. PLs can also form adducts with a variety of metal ions to yield both cations and anions. In many cases, these adducts increase the information content of positive ion experiments concerning acyl/alkylether chains, and of negative ion experiments concerning polar headgroups (Han and Gross, 2005).

Protonated PL cations undergo relatively few informative decompositions under normal circumstances. Aside from the headgroup-specific ions described below, the only ion consistently observed in positive mode experiments results from the concerted neutral losses of the phospho-headgroup and the sn-2 chain as a ketene (reaction 3a; Scheme 3; figure 5A). Deprotonated PL anions, in contrast, undergo several common and informative CID for all six PL headgroup classes. The simplest decompositions are charged and neutral losses of the phospho-headgroup (reactions 3b,c). The relative yield of these reactions is chiefly determined by the ability of the headgroup to stabilize a negative charge.

$$[M+H]^+ \rightarrow [R_1+116]^+ \tag{3a}$$



$$[M-H]^- \rightarrow R_3HPO_4^- \qquad ([R_3+96]^-) \tag{3b}$$

$$[M-H]^- \rightarrow [M-R_3H_2PO_4]^- \qquad ([M-R_3-97]^-) \tag{3c}$$

Another common negative ion decomposition produces fatty acid anions from the *sn-1* and *sn-2* chains (reactions 4a,b; scheme 3). The mechanism of reactions 4a,b appears deceptively simple, however the relative yields from these two reactions vary widely among the various PL classes. These differences in yield constitute evidence that the CID behavior involves some degree of interaction between headgroups and acyl chains. At minimum, this interaction involves migration of charge from the phosphate group, although other interactions are likely to be involved and will be discussed below in separate discussions of each PL class. The relative yields of reactions 4a,b may also vary with collision energy, possibly due to the effect of this parameter on rates of secondary ion decomposition rather than on rates of ion production.

$$[M-H]^- \rightarrow R_1CH_2COO^- \tag{4a}$$

$$[M-H]^- \rightarrow R_2CH_2COO^- \tag{4b}$$

Either of the acyl chains may also leave as a neutral fatty acid with the retention of charge on the lyso-PL remnant (reactions 5a,b; Scheme 4). These reactions provide an indirect means to ascertain the chemical composition of the *sn-1* and *sn-2* chains. The remnants may undergo additional concerted decomposition reactions involving the headgroup and release the other chain as a fatty acid anion (reactions 5c,d; Scheme 4). Analysis of such remnants is

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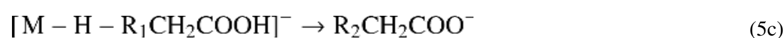
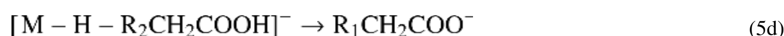
also the most direct way to determine the composition of ether-linked *sn-1* substituents, because these chains cannot be released as carboxylate anions.

(5a)

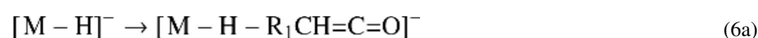
(5b)

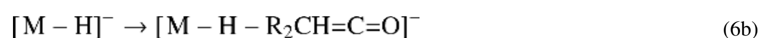
 (5c)

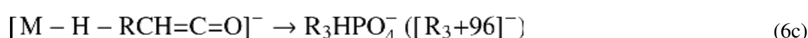
 (5d)

Finally, the neutral loss of acyl chains may occur via ketene formation (reactions 6a,b). Compared to the neutral loss of a fatty acid, the neutral loss of a ketene leaves an –OH group on the charged lyso-PL remnant (Scheme 5).

Oftentimes, collisional activation results in a single decomposition reaction, and the product ion does not retain sufficient internal energy for additional reactions. The –OH group remaining after the loss of a ketene, however, may facilitate concerted decomposition reactions in an MS^2 experiment that produce a phospho-headgroup ion (reaction 6c), or charged cyclic phosphoglycerol remnants (reaction 6d,e; Scheme 6). The loss of both chains as ketenes leaves –OH groups on both the *sn-1* and *sn-2* positions and an ion at $[R_3 + 170]^-$ that is often observed in abundance. MS^3 experiments have shown that this ion decomposes into an $[R_3 + 96]^-$ ion (reaction 6f; Scheme 7), but since it may also arise via reactions 3b or 6c (scheme 6), its origin in an MS^2 experiment is not certain.

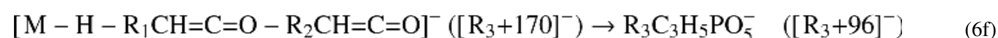
 (6a)

 (6b)

 (6c)

(6d)

(6e)

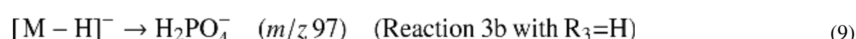
 (6f)

C.2 Patterns characteristic of PA lipids (Figure 5)—PA lipids have the simplest structures among phospholipid classes, but exchangeable protons on the terminal phosphate group in these lipids facilitate several complex reactions that are uncommon in other PL classes (Hsu and Turk, 2000c). In positive mode analyses, the most important decomposition is heterolytic cleavage of a polar C–O bond with neutral loss of H_3PO_4 and the formation of an $[M - 97]^+$ glyceride cation that is diagnostic of PA lipids (reaction 7, Scheme 8; Figure 5A). An $[R_1 + 116]^+$ ion is also formed according to reaction 3a.

$$[M+H]^+ \rightarrow [M-97]^+ \quad (7)$$

The intrinsic negative charge of PA lipids facilitates detection of the parent $[M - H]^-$ ion in negative mode analyses, usually making it much more abundant than the $[M + H]^+$ ion in positive mode analyses. In addition, the array of ions produced by CID is more diverse and informative in negative mode analyses. For example, cleavage of a polar O–P bond produces charged loss of the phosphate group as PO_3^- (*m/z* 79) (reaction 8). Less commonly, the C–O bond is cleaved and the phosphate is lost as $H_2PO_4^-$ (*m/z* = 97) (reaction 9).

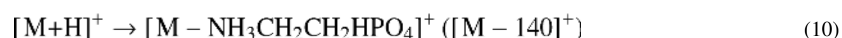
$$[M-H]^- \rightarrow PO_3^- \quad (m/z\ 79) \quad (8)$$



$$[M-H]^- \rightarrow H_2PO_4^- \quad (m/z\ 97) \quad (\text{Reaction 3b with } R_3{=}H) \quad (9)$$

Either fatty acyl chain may leave as a charged species via reactions 4a and 4b, as neutral acids via reactions 5a,b, or as ketenes via reactions 6a,b (Figure 5B). The loss of one chain as a ketene provides a proximate hydroxyl group that facilitates the concerted loss of the other chain as an acid, yielding a major ion with *m/z* 153 (reactions 6d,e; Scheme 6). The neutral loss of one chain as a fatty acid according to reactions 4a,b yields a major $[M - R_1CH_2COOH - H]^-$ ion. In-source CID studies have demonstrated that this ion loses 136 Da to produce a fatty acid anion (reactions 5c,d; Scheme 4 with R_3=H). However, the extent to which these reactions add to the yield of reactions 4a,b in an MS/MS experiment is unclear.

C.3 Patterns characteristic of PE lipids (Figure 6)—Condensation of ethanolamine and glycerophosphate yields a zwitterionic lipid headgroup with distinctive CID patterns. In positive mode analyses, the $[M + H]^+$ ion might be expected to form by protonation of the phosphate, since it bears a formal negative charge, and protonation of the basic amino group to form a cationic site. Fragmentation at the C – O bond by mechanisms analogous to those described above for PA lipids would be expected to then yield a phosphoethanolamine cation appearing at *m/z* 142 (Scheme 9). However, this scenario is not observed (Figure 6A). Instead, there is neutral loss of phosphoethanolamine, and the proton associates with an ester group to form $[M - 140]^+$ ions that are diagnostic of lipids in the PE class (reaction 10), but of lower abundance for ether lipids and plasmalogens (Zemski Berry and Murphy, 2004). As with PA lipids, an $[R_1 + 116]^+$ ion may be observed. A richer array of positive ions yielding information about chain identities may be obtained from lithium ion adducts of PE (Hsu and Turk, 2000a).



$$[M+H]^+ \rightarrow [M - NH_3CH_2CH_2HPO_4]^+ \ ([M-140]^+) \quad (10)$$

Negative mode CID of PE lipids (Figure 6B) produces weak phosphoethanolamine anions at *m/z* 140 according to reaction 3b, and abundant fatty acid anions according to reactions 4a,b (Hsu and Turk, 2000e). Neutral chain losses occur according to reactions 5a,b and 6a,b, with the most abundant observed ion corresponding to loss of a ketene from the sn-2 fatty acyl group in the example shown (Figure 6B).

C.4 Patterns characteristic of PC lipids (Figure 7)—Lipids with the PC headgroup exhibit distinctive CID behavior due to the permanent positive charge of the quaternary nitrogen. All species yield abundant molecular ion species in positive ion mode studies, most likely because the phosphate group readily accepts a proton during ESI. Molecular ion species decompose efficiently to produce phosphocholine ions with *m/z* 184 that are

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diagnostic for the PC headgroup class (reaction 11, Figure 7). However, these ions do not appear to be produced by simple cleavage of the C–O bond at *sn-3* (Haroldsen and Gaskell, 1989). Instead, stable isotope studies indicate that a proton is acquired from the β carbon of the *sn-2* chain (Scheme 10). Because phosphocholine ions are produced with high efficiency, other decomposition products are only produced in relatively low abundance (Figure 7A).

$$[M+H]^+ \rightarrow m/z\ 184 \quad (11)$$

The phosphate group in PC lipids also readily accepts alkali metal ions, and a distribution of species containing Na^+ and K^+ can complicate quantitative analysis in biological samples. Metal ions also induce additional decomposition patterns. For example, sodiated PC lipids produce $[M – 59 + Na]^+$ ions representing the neutral loss of $(CH_3)_3N$, and $[M – 183 + Na]^+$ ions representing the neutral loss of phosphocholine (Ho and Huang, 2002). An ability to detect the PC headgroup by the neutral loss of phosphocholine rather than the production of phosphocholine cations is advantageous when using ion trap instrumentation, because it is easier to trap ions with *m/z* values that are closer to the parent ion. In some cases, metal ions induce enough neutral loss of RCOOH and RCH=C=O to identify the acyl chains (Ho and Huang, 2002).

PC lipids form negative ions by forming adducts with anions such as chloride $[M + 35]^-$ and acetate $[M + 59]^-$ (Figure 7B). The anions are loosely bound, and may be lost before entering the spectrometer at high orifice (declustering) potentials (Harrison and Murphy, 1995). On the other hand, when removed they often result in the neutral loss of a methyl group and the formation of $[M − 15]^-$ ions at the orifice. Thus, by judicious choice of solvent conditions and orifice potentials, anion-adduct as well as $[M − 15]^-$ ions are available for analysis by negative mode mass spectrometry. When subjected to collisional activation, these ions yield decomposition products according to reactions 4-6 (Hsu and Turk, 2003).

C.5 Patterns characteristic of PS lipids (Figure 8)—Despite their negative charge at neutral pH, lipids with the PS headgroup readily form $[M + H]^+$ ions (Hsu and Turk, 2005). They decompose to $[M − 184]^+$ ions by neutral loss of phosphoserine with such efficiency that few other ions are produced (reaction 12a; Scheme 11; Figure 8A). Nevertheless, an $[R_1 + 116]^+$ ion according to reaction 3a may be observed, and secondary fragmentations may occur to produce acylium ions from the fatty acyl chains (scheme 11).

PS lipids may also bind up to 3 alkali cations. $[M + Li]^+$ ions decompose into $[M − 184]^+$ ions as with protonated species, however they also produce lithium–phosphoserine adduct ions with *m/z* 192, and undergo the neutral loss of serine to produce $[M − 87]^+$ ions (Hsu and Turk, 2005). Lithiated-PS may undergo rearrangement during decomposition to produce an $[M + Li − 98]^+$ ion with an apparent triacylglycerol structure. Neutral losses from these ions enable identification of the *sn-1* and *sn-2* chains. By virtue of their two anionic sites, PS lipids may bind 2 or 3 Li^+ ions to form $[M − H + 2Li]^+$ and $[M − 2H + 3Li]^+$. The decomposition products obtained are analogous to those obtained from $[M + Li]^+$ ions, although there are marked differences in yields. In most cases, Na^+ may substitute for Li^+, with altered product ion yields.

$$[M+H]^+ \rightarrow [M - 184]^+ \quad (12a)$$

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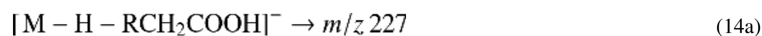
$$[M-H]^- \rightarrow [M-88]^- \quad (12b)$$

$[M - H]^-$ ions readily decompose to $[M - 88]^-$ ions from the neutral loss of serine, often occurring in-source (Figure 8B; reaction 12b; Scheme 12). Isotope exchange studies suggest that the mechanism of this decomposition involves a hydrogen from either the amino or the carboxyl groups of the serine (Hsu and Turk, 2005). The resulting $[M - 88]^-$ ions are equivalent to the $[M - H]^-$ ions of PA, and they decompose accordingly within an MS^2 experiment to yield fatty acid anions and remnant ions following the neutral loss of fatty acid chains or ketenes (Hsu and Turk, 2005). PS lipids may form lithiated $[M - 2H + Li]^-$ ions, or their sodiated analog. These ions follow qualitatively similar decomposition patterns as $[M - H]^-$ ions, but with an increased tendency to decompose by neutral loss of ketenes.

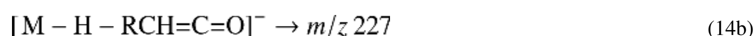
C.6 Patterns characteristic of PG lipids (Figure 9)—PG lipids form $[M + H]^+$ ions that decompose via neutral loss of glycerophosphate to yield $[M - 171]^+$ product ions that are diagnostic of the PG headgroup (Figure 9A; reaction 13; Scheme 13). They also produce an $[R_1 + 116]^+$ ion according to reaction 3a.

$$[M+H]^+ \rightarrow [M-171]^+ \quad (13)$$

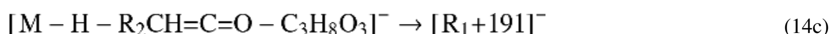
Collision induced negative mode decomposition of PG $[M - H]^-$ ions yields several informative ions via reactions 4-6 (Figure 9B) (Hsu and Turk, 2001). Secondary decompositions within an MS^2 experiment are also common, facilitated and influenced by the free hydroxyl groups of the glycerol. For example, one chain may leave as RCOOH, and the other may leave as a ketene, to yield an intermediate ion with *m/z* 227 (reactions 14a,b; small signal present in figure 9B). The glycerol residue in this ion appears to undergo the neutral loss of $C_3H_6O_2$ to yield a product with *m/z* 153 (reaction 6f; Scheme 14; Figure 9B). PG lipids that have lost an acyl chain as RCOOH or RCH=C=O may also undergo neutral losses of $C_3H_6O_2$ via reaction 6f to yield $[M - RCOOH - 75]^-$ ($[R_1 + 191]^-$, *m/z* 405 in Figure 9B; reaction 14c; Scheme 14)or $[M - RCH{=}C{=}O - 75]^-$ ions (Hsu and Turk, 2001).



$$[M-H-RCH_2COOH]^- \rightarrow m/z\,227 \quad (14a)$$



$$[M-H-RCH{=}C{=}O]^- \rightarrow m/z\,227 \quad (14b)$$



$$[M-H-R_2CH{=}C{=}O-C_3H_8O_3]^- \rightarrow [R_1+191]^- \quad (14c)$$

C.7 Patterns characteristic of PI lipids (Figure 10)—PI lipids can form positive ions (Figure 10A) that decompose upon collisional activation by the loss of phosphoinositol (reaction 15a; Scheme 15). However, negative ions are more abundant and more informative due to the intrinsic negative charge of the PI headgroup (Figure 10B). PI lipid anions decompose according to reactions 3-6 and under some conditions, inositol may be lost as a neutral fragment to yield the corresponding PA species (Hsu and Turk, 2000b).

$$[M+H]^+ \rightarrow [M-259]^+ \quad (15a)$$

$$[M-H]^- \rightarrow m/z\,259 \quad (15b)$$

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The close proximity of hydroxyl groups on the inositol ring to the phosphate facilitates concerted decomposition reactions involving two or more CID reactions in a single MS^2 experiment. For example, reaction 3b yields *m/z* 259 (reaction 15b), but the concomitant loss of H_2O, presumably by the condensation of proximate hydroxyl groups on phosphate and inositol, produces an even more abundant *m/z* 241 ion (Scheme 16; figure 10B). Another neutral loss of the inositol ring in the same MS^2 experiment accounts for a major ion that is observed at *m/z* 153 (Hsu and Turk, 2000b). Yet another example of three concerted reactions in an MS^2 experiment begins with the loss of two neutral fatty acids to produce an *m/z* 297 ion. Adjacent hydroxyl groups then appear to promote cleavage of an O – P bond have $C_3H_5O_2$ to produce an *m/z* 223 ion that is prominent in MS^2 spectra (Scheme 17). PI lipids may have one (PIP) or two (PIP_2) additional phosphate groups on the inositol ring, each of which may add another negative charge to the molecular ion. Because the molecular ion species and their decomposition products may be multiply charged, the mass spectra become quite complex to interpret. Their decomposition mechanisms are similar to those of PI lipids; however, the phosphate groups may also undergo various gas phase rearrangements after CID that produce additional ions such as diphosphate (Hsu and Turk, 2000b).

C.8 Patterns characteristic of Cardiolipins—The general structure of cardiolipins shares all of its motifs with PA lipids, and therefore they undergo the same CID reactions (Hsu et al., 2005). However, their spectrum is complicated by the presence of two phosphate groups, three glycerol backbones, and four fatty acyl chains per molecule, along with the tendency to bear two negative charges and thereby become doubly charged. Therefore, cardiolipins may form various ions that combine two or more of the motifs observed with PA lipids. One such ion is produced following the concerted neutral loss of two fatty acid chains from one glycerol (Scheme 18). The resulting ion may then lose a 136 dalton fragment to produce an ion equivalent to an $[M - H]^-$ PA ion.

D. Sphingolipids (Figure 4)

Sphingolipids are perhaps the most diverse long-chain lipids found in cells in terms of chemical structure and biological function. While these derivatives of long-chain bases such as sphingosine and sphinganine were originally thought to be primarily located at the outer membrane bilayer of cells, it is now known that they have intracellular roles as well as serving as mediators in intracellular signaling (Table 1) (Merrill et al., 2007). The diversity in their structure is also reflected in their chemical properties and approaches used by mass spectrometry to analyze these molecules.

Efficient isolation of sphingolipids and preparation for mass spectrometric analysis can be quite challenging. Some sphingolipids do not favorably distribute in organic extraction systems and can be found in equal concentrations in both the aqueous as well as the organic layers of a chloroform/methanol/water extraction protocol. In fact, one approach has been to analyze the monophase of the Bligh/Dyer extract method to maximize yield of the sphingolipids targeted for analysis (Sullards et al., 2007). One widely used technique to isolate sphingolipids relies on their stability to high pH. Glycerolipids and glycerophospholipids are co-extracted, but their ester groups are saponified to free fatty acids and glycerol at high pH, leaving the sphingolipids concentrated in the organic extract.

The analysis of sphingolipids by mass spectrometry can be exemplified in approaches to quantify signaling ceramides (Merrill et al, 2005), analysis of sphingomyelin (a phospholipid incorporating a long-chain base as well as an N-fatty acyl chain), and glycosphingolipids (Merrill et al, 2005). The diversity in mass spectrometric behavior of the latter group of sphingolipids is best studied in context of complex carbohydrate analysis

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since structural challenges revolve around the carbohydrate subunit identity and linkage analysis.

D.1 Ceramides (Figure 11)—Ceramides are the simplest long-chain sphingolipids, consisting of a fatty acyl chain N-linked to a long chain base known as sphingosine (Figure 4). However, their analysis is complicated by variations in carbon chain length, position and degree of unsaturation, and position and number of hydroxylation sites. The most abundant ceramides have an 18-carbon sphingosine and an acyl chain of variable length. They may or may not have a double bond in the acyl chain and a hydroxyl group on the second carbon of the acyl chain. In positive mode analysis (Figure 11A), common ceramides readily lose H_2O to form $[M - H_2O + H]^+$ ions, and undergo amide bond cleavage with neutral loss of the fatty acid to yield an ion with *m/z* 264 (Scheme 19) (Fillet et al., 2002). This has been a particularly useful product ion for MRM analysis of ceramides (Merrill et al., 2007).

The decomposition pattern of ceramide anions is particularly rich, and varies in an informative way depending on structural variation of the long-chain base and the fatty acyl substituent such as a 2'-hydroxyl ceramide (Han, 2002; Hsu and Turk, 2002). For example, $[M - H - 327]^-$ ions are produced only when the hydroxyl group is present in the N-fatty acyl substituent (Scheme 20). A very abundant CID product ion corresponds to $[M - H - 256]^-$ which most likely is a conjugated anion (Scheme 20). This same decomposition pathway can lead to the abundant ion at m/z 323.7. Another common product ion is $[M - H - 240]^-$ which is abundant in Figure 11B.

D.2 Sphingomyelin (Figure 12)—Sphingomyelin is a ceramide bearing an O-linked phosphocholine group (Figure 4). The efficiency with which collisionally activated sphingomyelin produces phosphocholine ions (in a manner similar to that illustrated in Scheme 10) is sufficiently high that very few other cations are produced with ESI (Figure 12A). Some investigators have produced an array of more informative ions by first producing ceramide cations in-source with APCI (Karlsson et al., 1998). Others have produced a similar array of product ions by ESI of lithium adducts (Hsu and Turk, 2000d). As with sodium–PE adducts, lithiated-sphingomyelin $[M + Li]^+$ ions produce $[M - 59 + Li]^+$ ions representing the neutral loss of $(CH_3)_3N$, $[M - 183 + Li]^+$ ions representing the neutral loss of phosphocholine, and $[M - 429]^+$ representing the neutral losses of phosphocholine and most of the sphingosine chain.

Sphingomyelin produces ions for negative mode mass spectrometry by forming anion adducts in a manner similar to that of PC lipids (Figure 12B). However, sphingomyelin has no acyl-linked chains and therefore, they do not produce informative decomposition products with simple collisional activation. This problem may be solved by selecting anion adducts that produce $[M - 15]^-$ ions by N-demethylation, and subjecting the product ions to further collisional activation in an MS^3 experiment. Under these conditions, neutral loss of the N-acyl chain is observed, permitting one to distinguish SM from PC, and identify the N-acyl and long chain base substituents (Houjou et al., 2004).

D.3 Glycosphingolipids—GSLs are ceramides with one or more O-linked sugar residues that may be modified by phosphate, sulfate, or various other groups. In positive mode analysis, relatively low collision energies cause neutral loss of the glycoside from with retention of the charge on the ceramide. Higher energies in positive mode and negative mode afford insight into the glycoside composition. The nomenclature of GSLs and their decompensation patterns is complex and has been reviewed by others (Levery, 2005).

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Figure 1.

EI mass spectra of fatty acid methyl esters and collision induced decomposition (CID) of carboxylate anions from free carboxylic acids. (A) EI mass spectrum of methyl arachididate (70 eV); (B) EI mass spectrum of methyl arachidonate (70 eV); (C) CID mass spectrum of the carboxylate anion derived from arachidondic acid by ESI in the negative ion mode; (D) CID mass spectrum of the carboxylate anion derived from arachidonic acid by ESI in the negative ion mode. The CID mass spectra for this figure and subsequent figures were obtained using a tandem quadrupole (ABI 4000 Qtrap).

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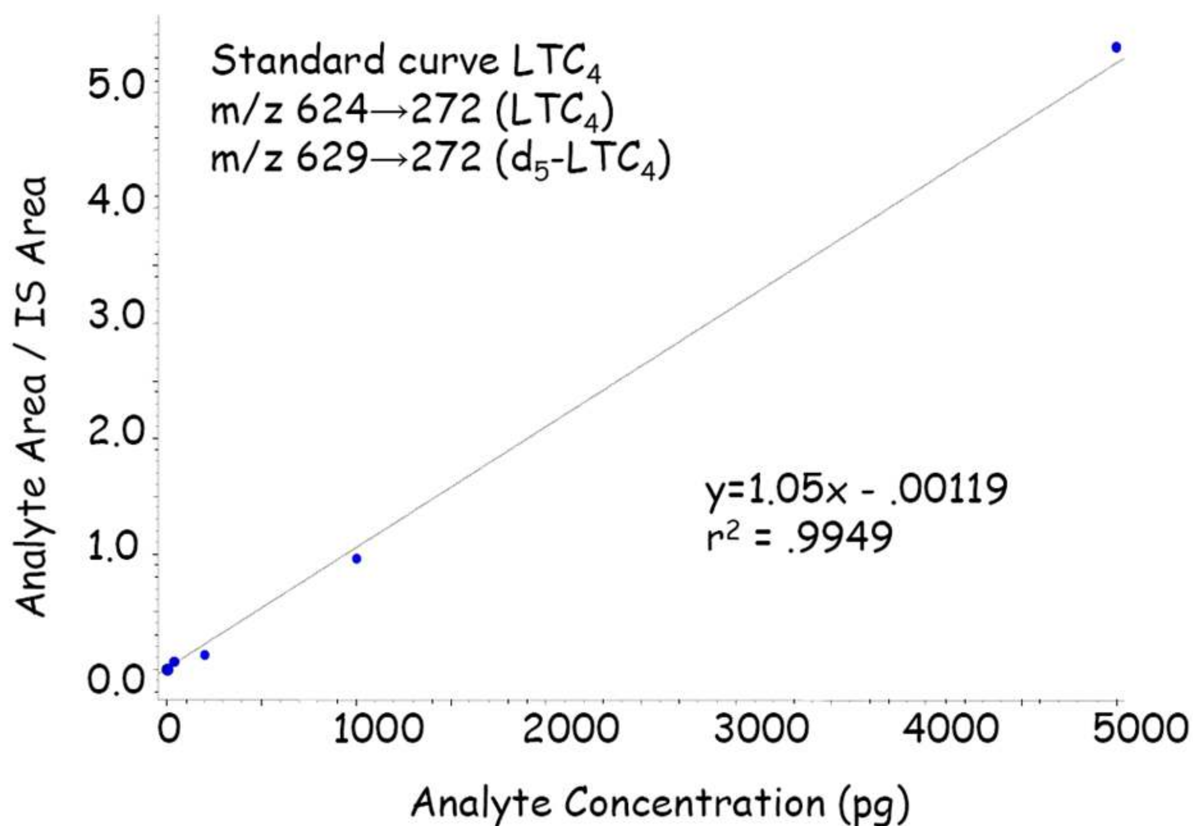

Figure 2.

Quantitation by stable isotope dilution of lipids typically involves establishing a standard curve where a fixed amount of a deuterium labeled internal standard (in this case d_5-LTC_4) is added to increasing quantities of unlabeled LTC_4. When the area for the MRM transition for LTC_4 (*m/z* 624 → 272) is divided by the area for the corresponding internal standard (*m/z* 629 → 272 for d_5-LTC_4), a linear relationship is found with an excellent correlation coefficient.

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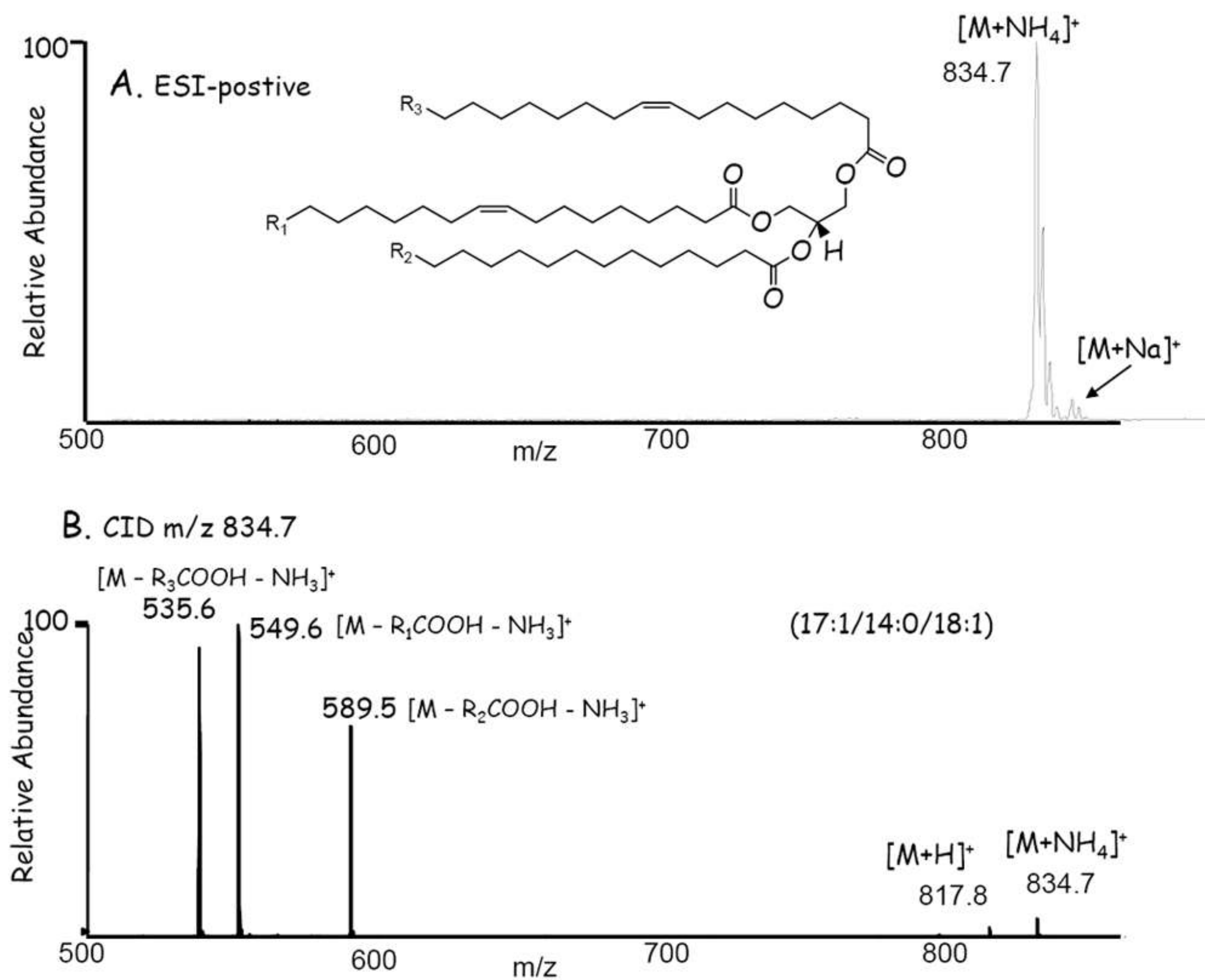

Figure 3.

(A) ESI in the positive ion mode of synthetic 17:1/14:0/18:1 triacylglycerol in the presence of electrospray buffer containing 5 mM ammonium acetate; (B) CID of $[M + NH_4]^+$ at *m/z* 834 resulting in indicated product ions corresponding to loss of each of the fatty acyl substituents as a free carboxylate anion.

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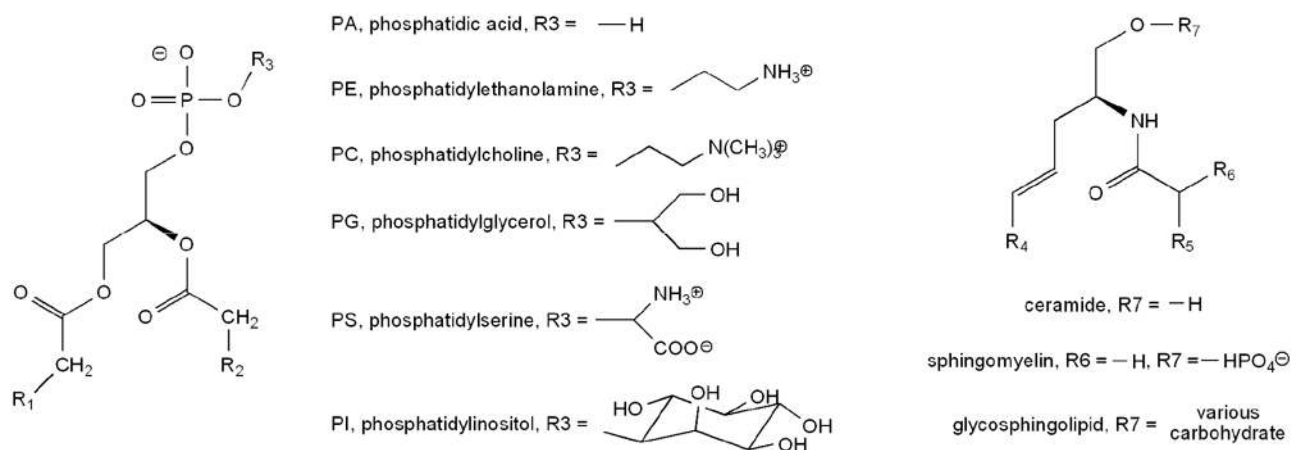

Figure 4.

General structures of glycerophospholipids (left) and sphingolipids (right). For the glycerophospholipids, stereospecific sites on the glycerol backbone are numbered (“sn” numbering). R_1 and R_2 represent alkyl chains that may be saturated or unsaturated. Together with an attached -CH_2CO-group, they constitute the “fatty acyl” chains at positions sn-1 and sn-2 of the glycerol backbone. The α methylene groups of these chains are depicted apart from the R groups because their hydrogen atoms participate in some of the decomposition reactions discussed in the text. The -CH_2CO- group at sn-1 position is replaced with -O- in ether lipids, and with -CH=CH-O- in vinyl ether lipids or plasmalogens. The R_3 group defines the six major phospholipid headgroup classes. For sphingolipids, R_4 is usually -$C_{13}H_{27}$ but may be -$C_{12}H_{25}$. The R_5 is an alkyl group ranging in length from 2-20 carbon atoms. R_6 may be -H or -OH.

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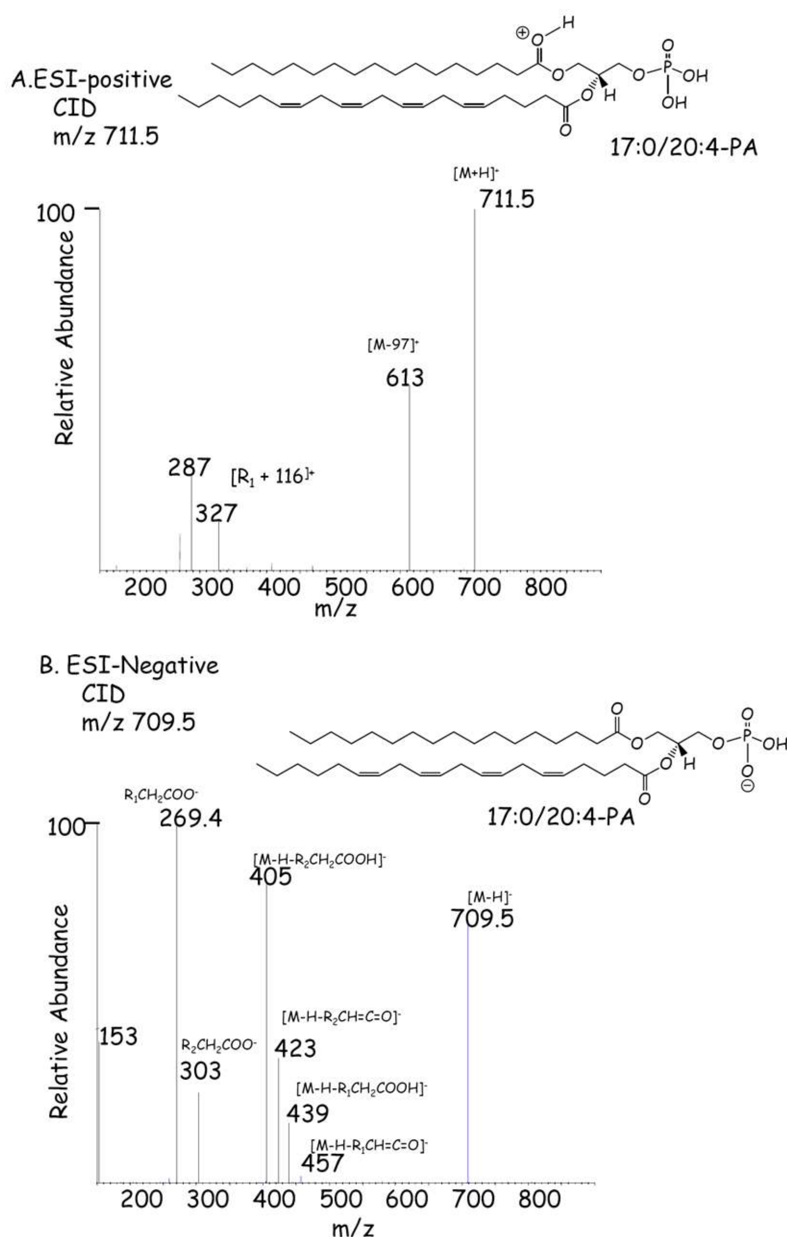

Figure 5.

Positive and negative ion CID mass spectra from 1-heptadecanoyl-2-arachidonoyl-3-glycerophosphate (17:0/20:4-PA). Molecular ion species generated by ESI. (A) Collisional activation of the $[M + H]^+$ ion at *m/z* 711.5; (B) collisional activation of the $[M - H]^-$ ion at *m/z* 709.5.

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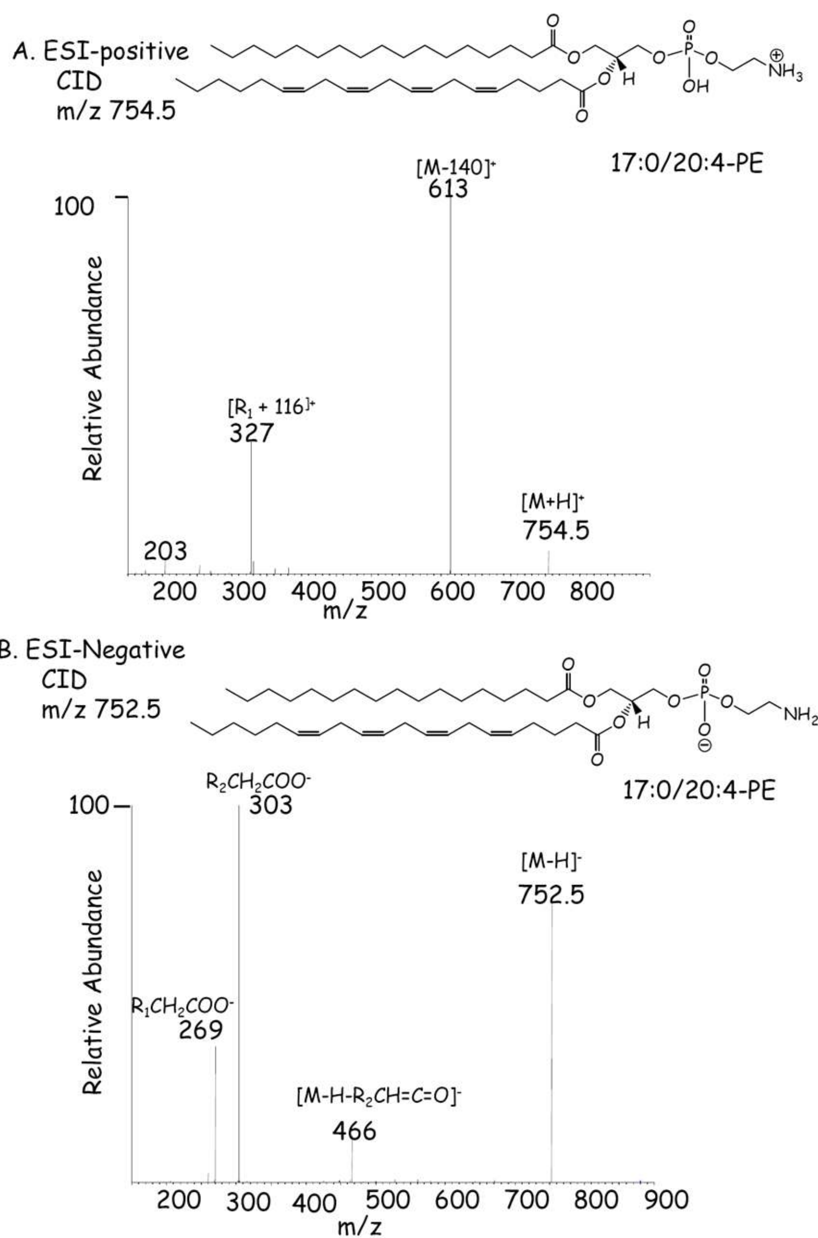

Figure 6.

Positive and negative ion CID mass spectra from 1-heptadecanoyl-2-arachidonoyl-3-glycerophosphoethanolamine (17:0/20:4-PE). Molecular ion species generated by ESI. (A) Collisional activation of the $[M + H]^+$ ion at *m/z* 754.5; (B) collisional activation of the $[M - H]^-$ ion at *m/z* 752.5.

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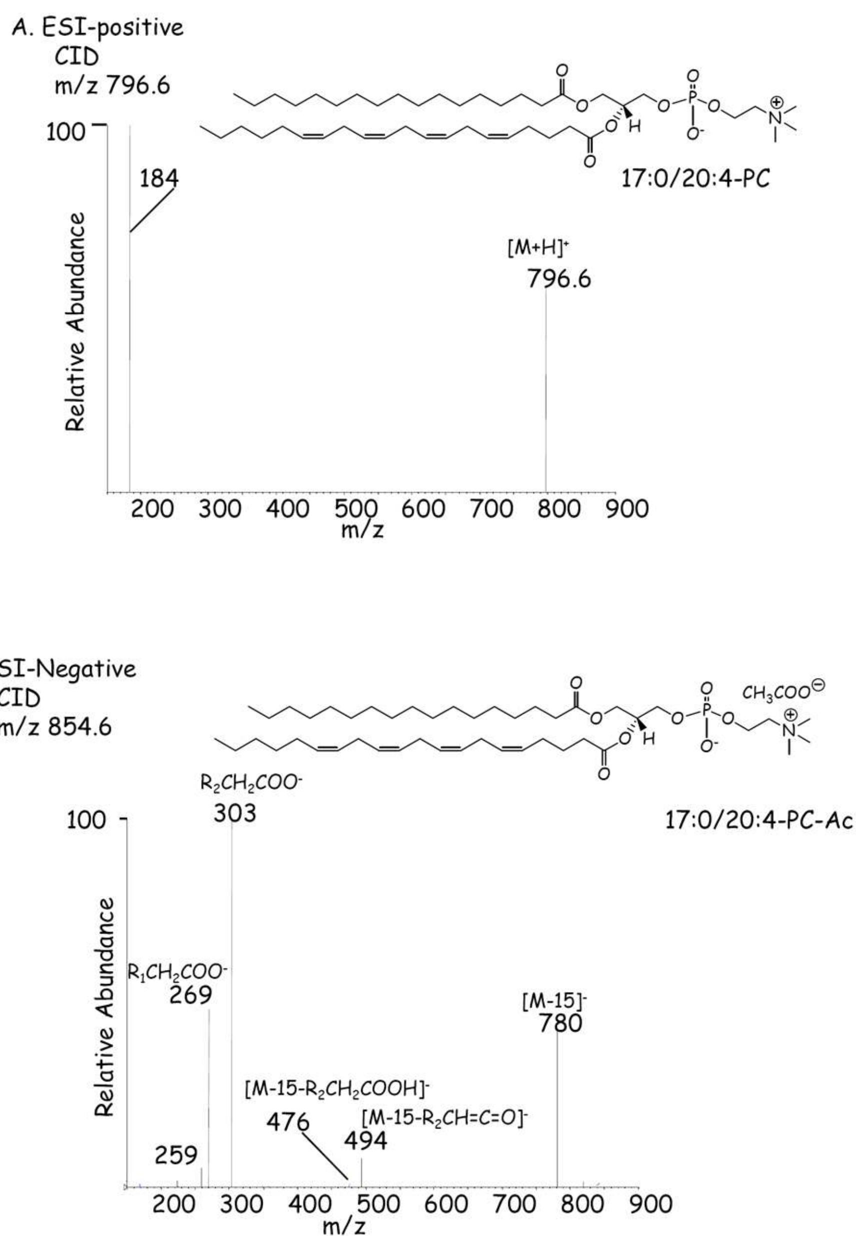

Figure 7.

Positive and negative ion CID mass spectra from 1-heptadecanoyl-2-arachidonoyl-3-glycerophosphocholine (17:0/20:4-PC). Molecular ion species generated by ESI. (A) Collisional activation of the $[M + H]^+$ ion at *m/z* 796.6; (B) collisional activation of the $[M + \text{acetate}]^-$ ion at *m/z* 854.6.

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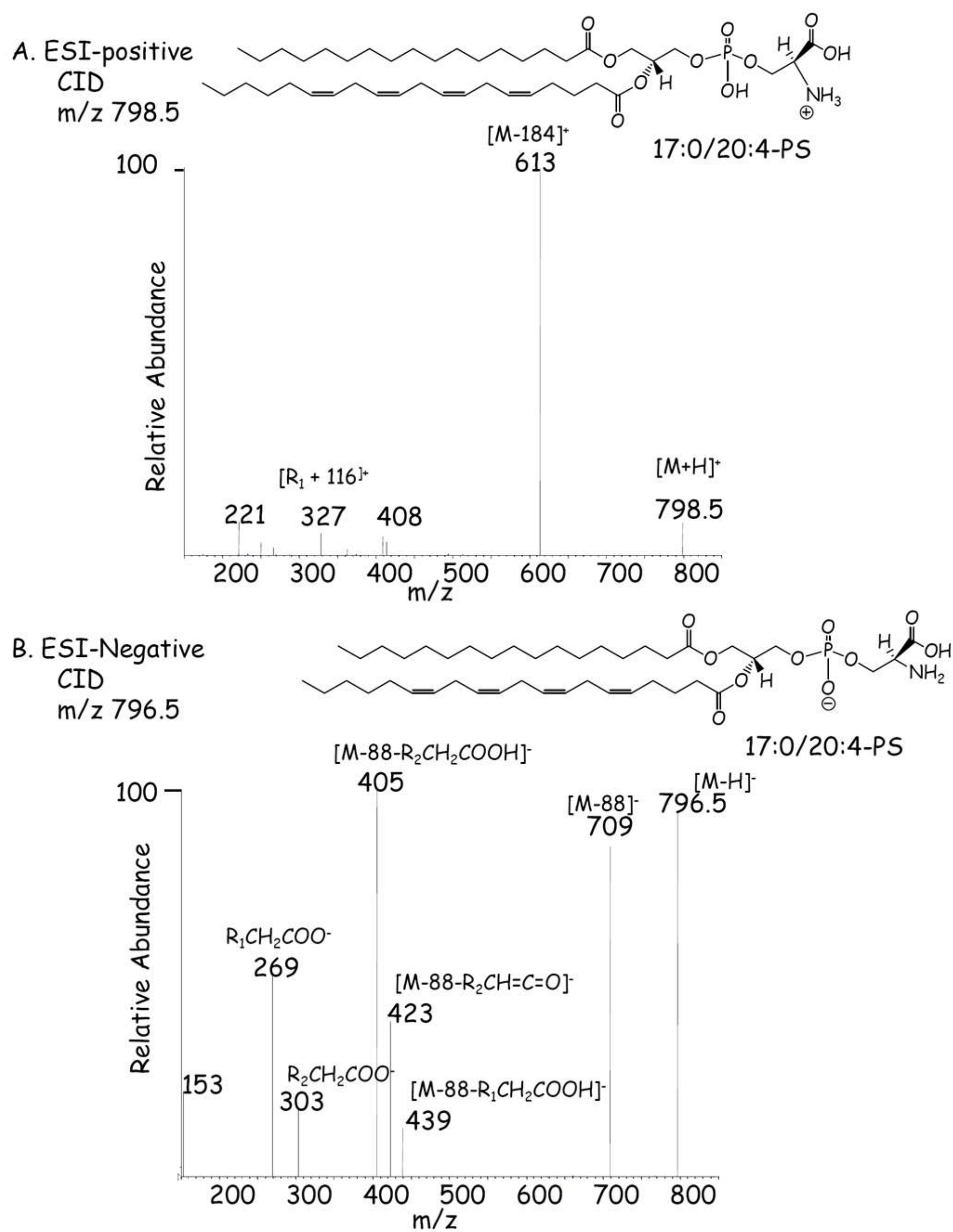

Figure 8.

Positive and negative ion CID mass spectra from 1-heptadecanoyl-2-arachidonoyl-3-glycerophosphoserine (17:0/20:4-PS). Molecular ion species generated by ESI. (A) Collisional activation of the $[M + H]^+$ ion at *m/z* 798.5; (B) collisional activation of the $[M - H]^-$ ion at *m/z* 796.5.

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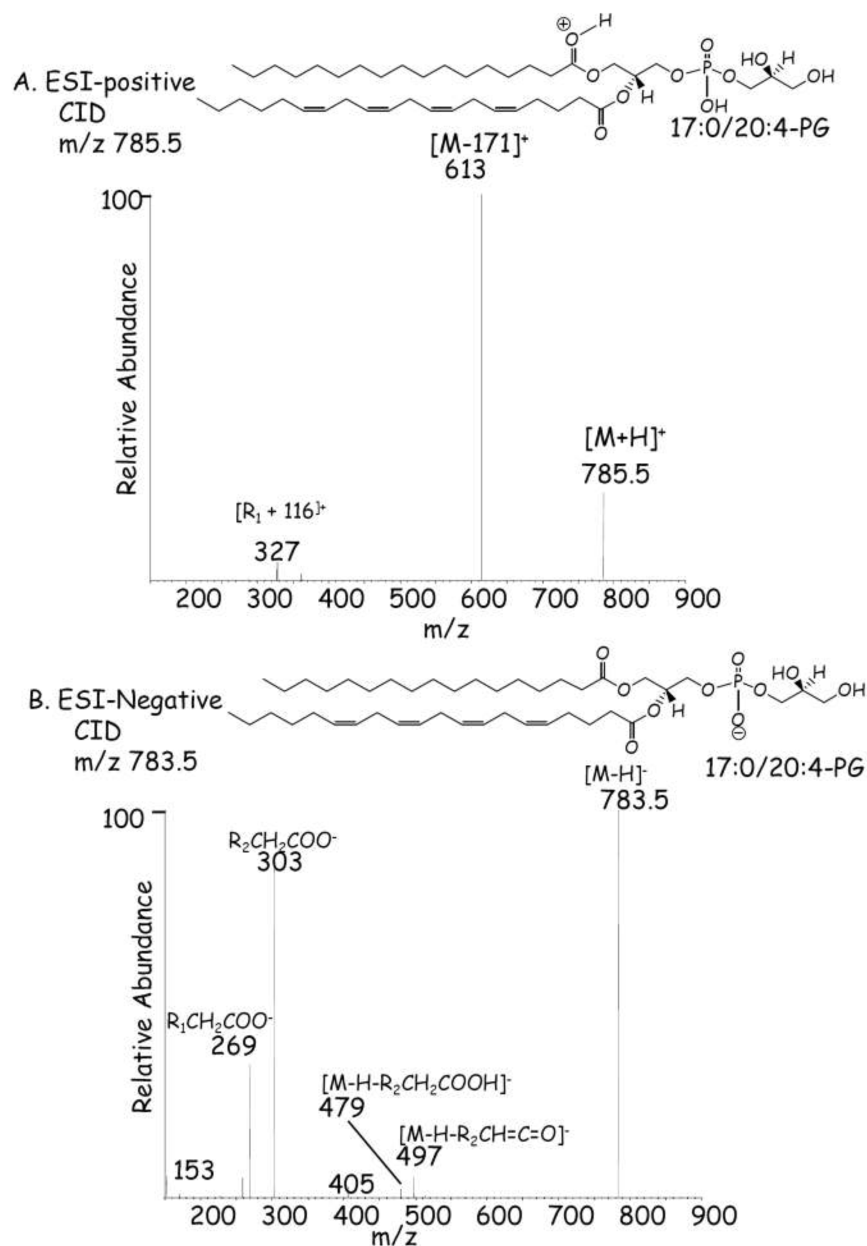

Figure 9.

Positive and negative ion CID mass spectra from 1-heptadecanoyl-2-arachidonoyl-3-glycerophosphoglycerol (17:0/20:4-PG). Molecular ion species generated by ESI. (A) Collisional activation of the $[M + H]^+$ ion at *m/z* 785.5; (B) collisional activation of the $[M - H]^-$ ion at *m/z* 783.5.

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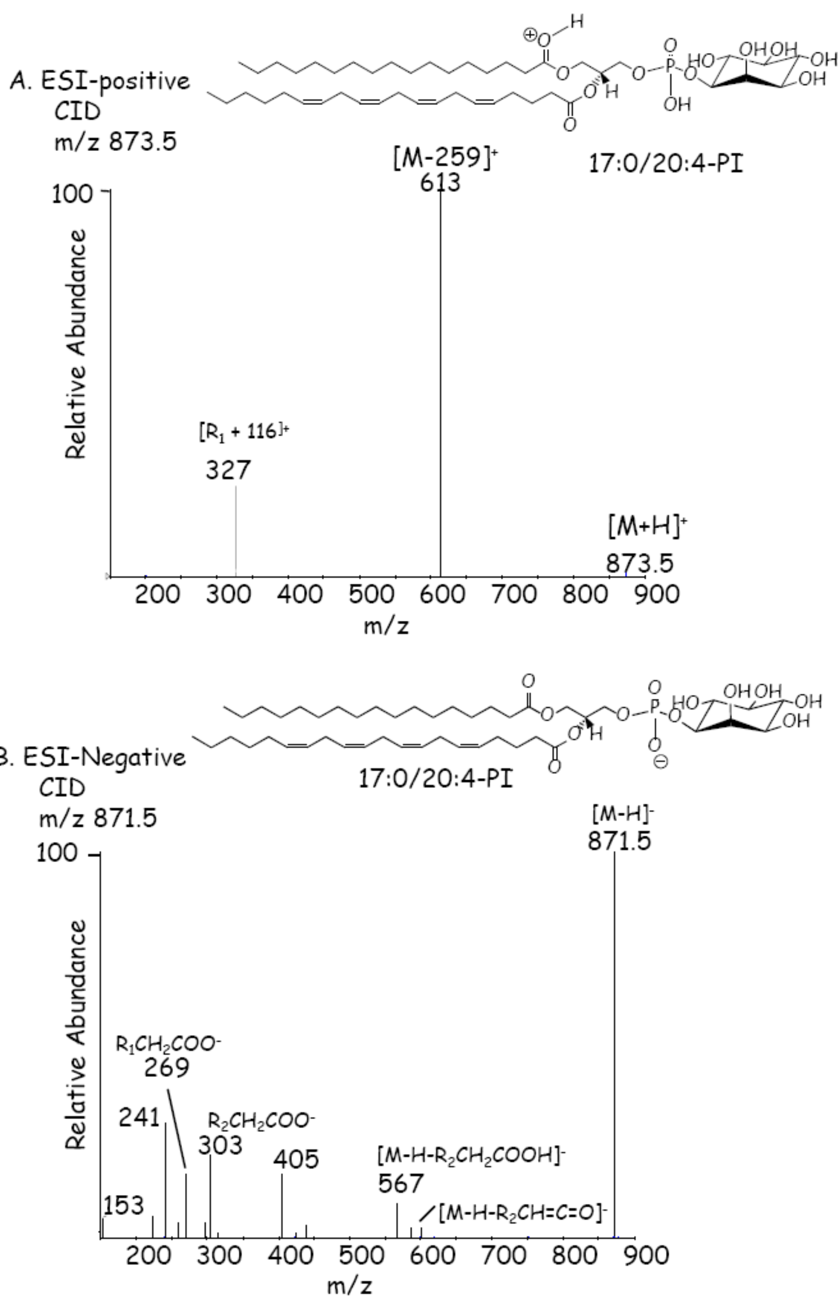

Figure 10.

Positive and negative ion CID mass spectra from 1-heptadecanoyl-2-arachidonoyl-3-glycerophosphoisnositol (17:0/20:4-PI). Molecular ion species generated by ESI. (A) Collisional activation of the $[M + H]^+$ ion at *m/z* 873.5; (B) collisional activation of the $[M - H]^-$ ion at *m/z* 871.5.

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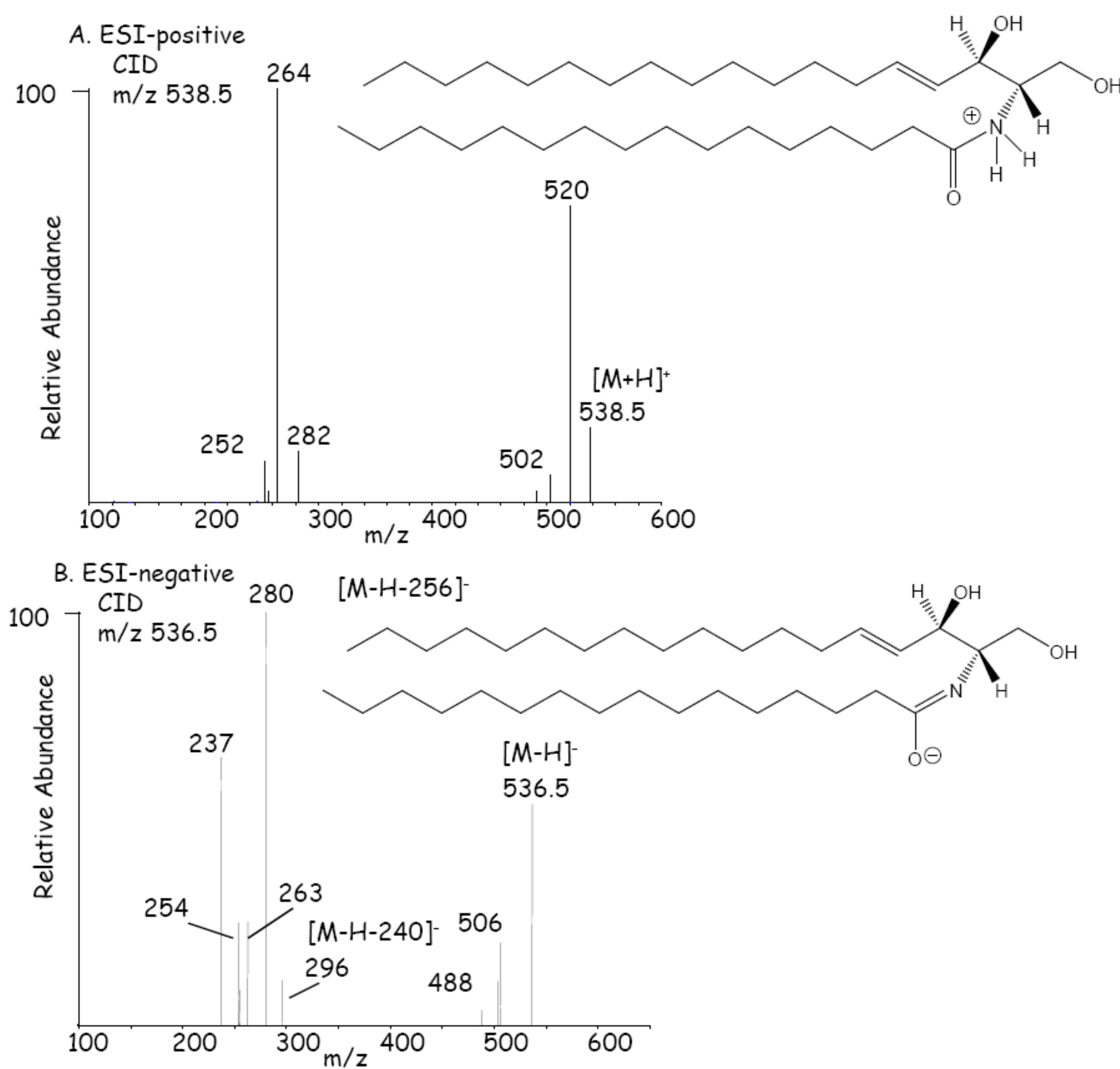

Figure 11.

Positive and negative ion CID mass spectra from N-hexadecanoyl-ceramide (Cer d18/16:0). Molecular ion species generated by ESI. (A) Collisional activation of the $[M + H]^+$ ion at *m/z* 538.5; (B) collisional activation of the $[M - H]^-$ ion at *m/z* 536.5.

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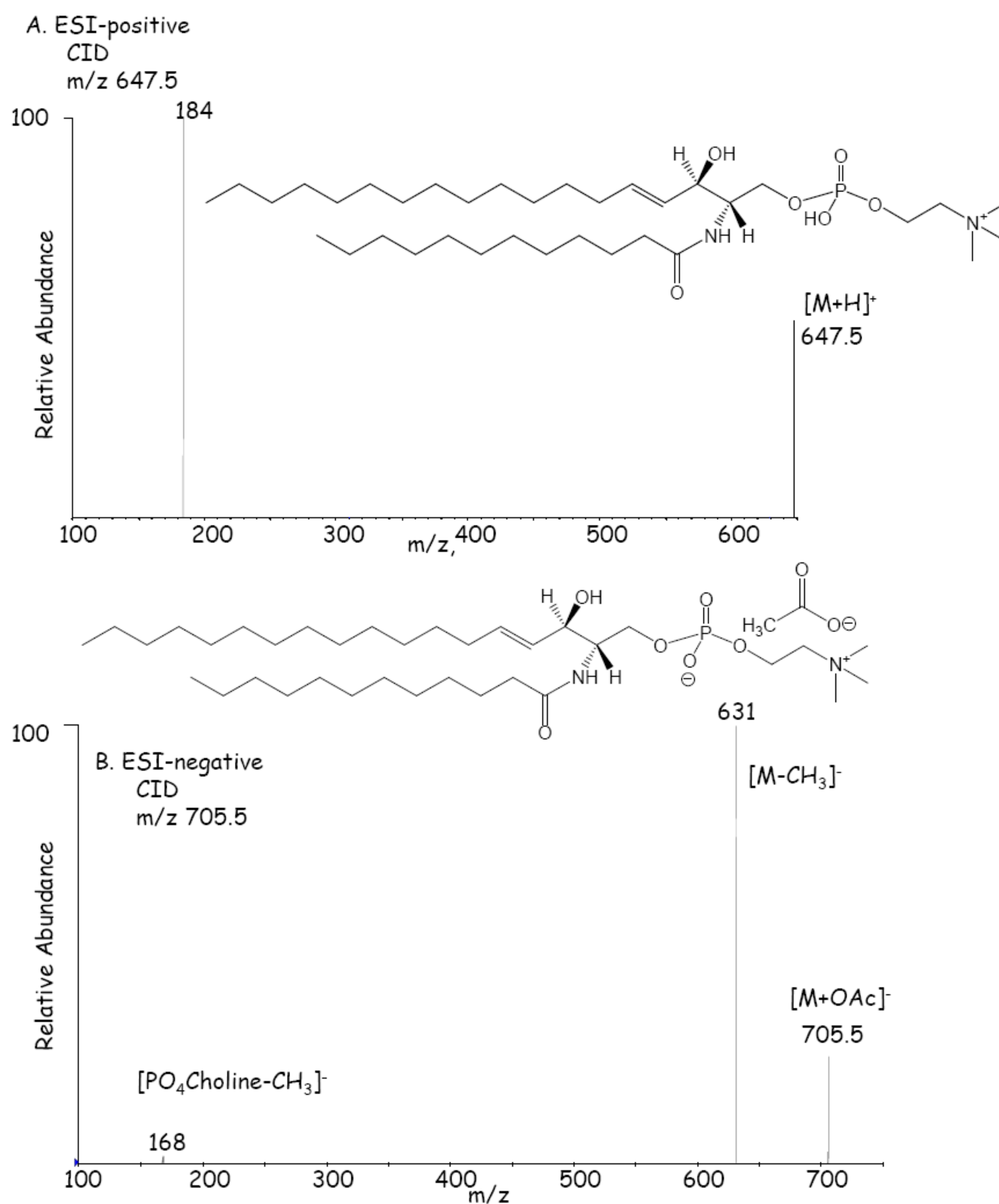

Figure 12.

Positive and negative ion CID mass spectra from N-dodecanoyl-sphyngomyelin (SM d18:1/12:0). Molecular ion species generated by ESI. (A) Collisional activation of the [M + H]$^+$ ion at *m/z* 647.5; (B) collisional activation of the [M + acetate]$^-$ ion at *m/z* 705.5.

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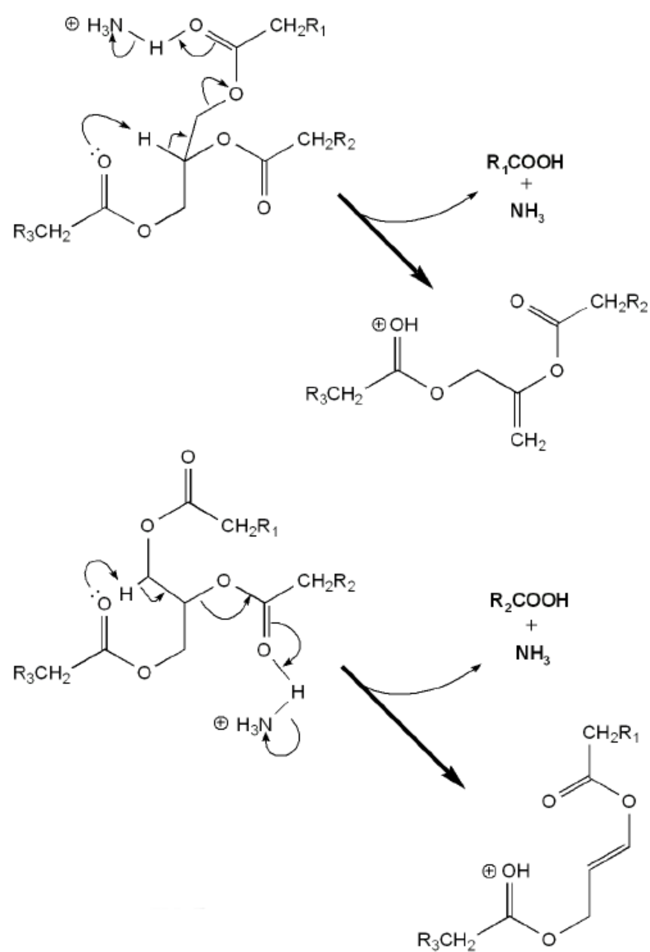

Scheme 1.

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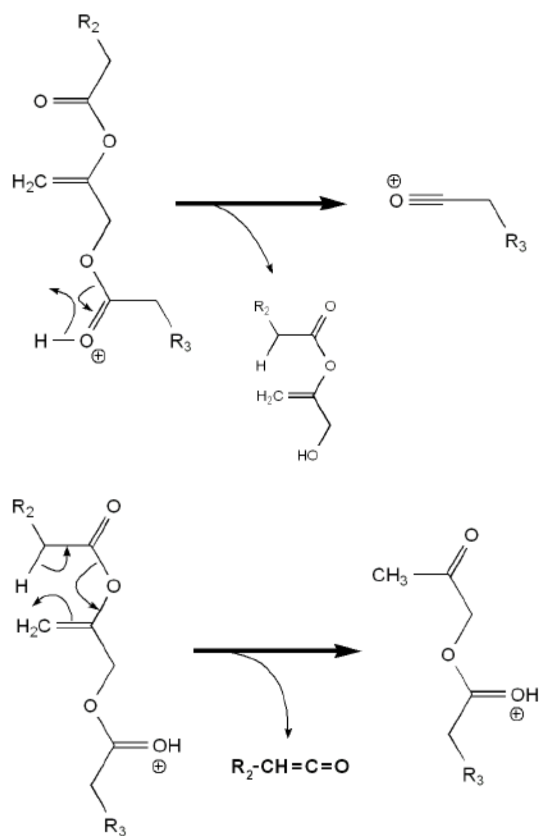

Scheme 2.

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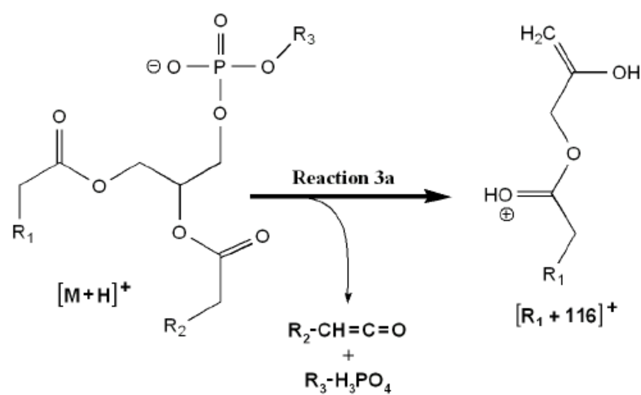

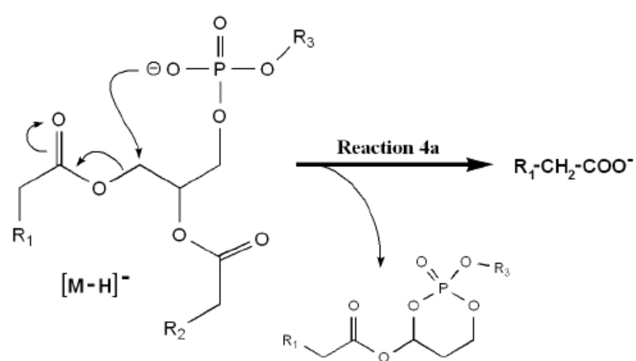

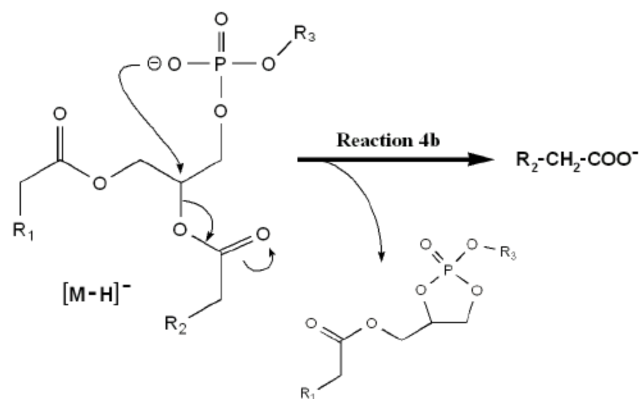

Scheme 3.

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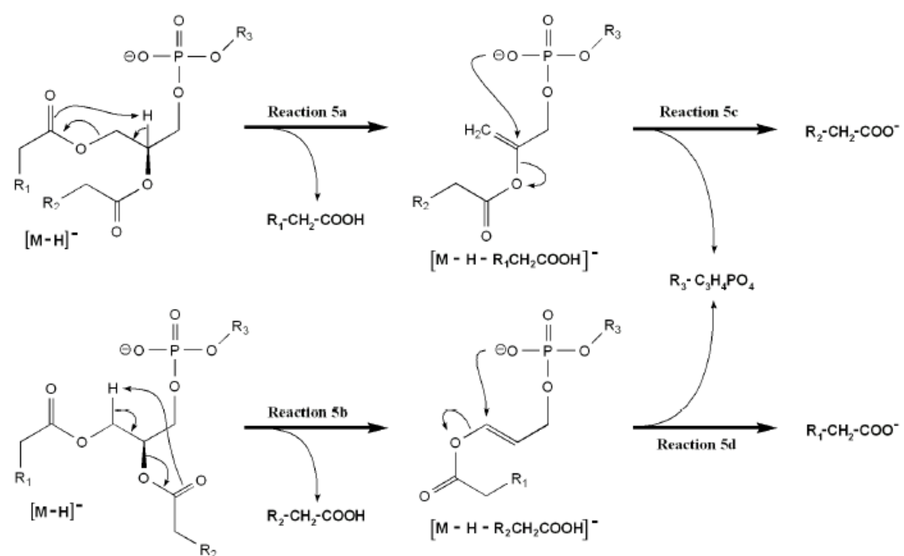

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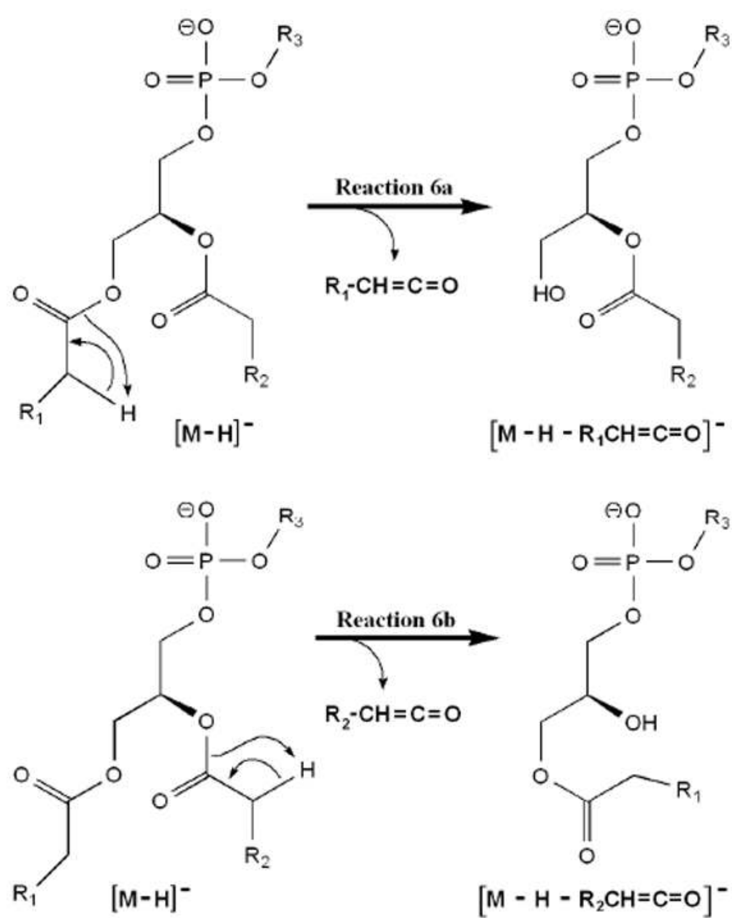

Scheme 5.

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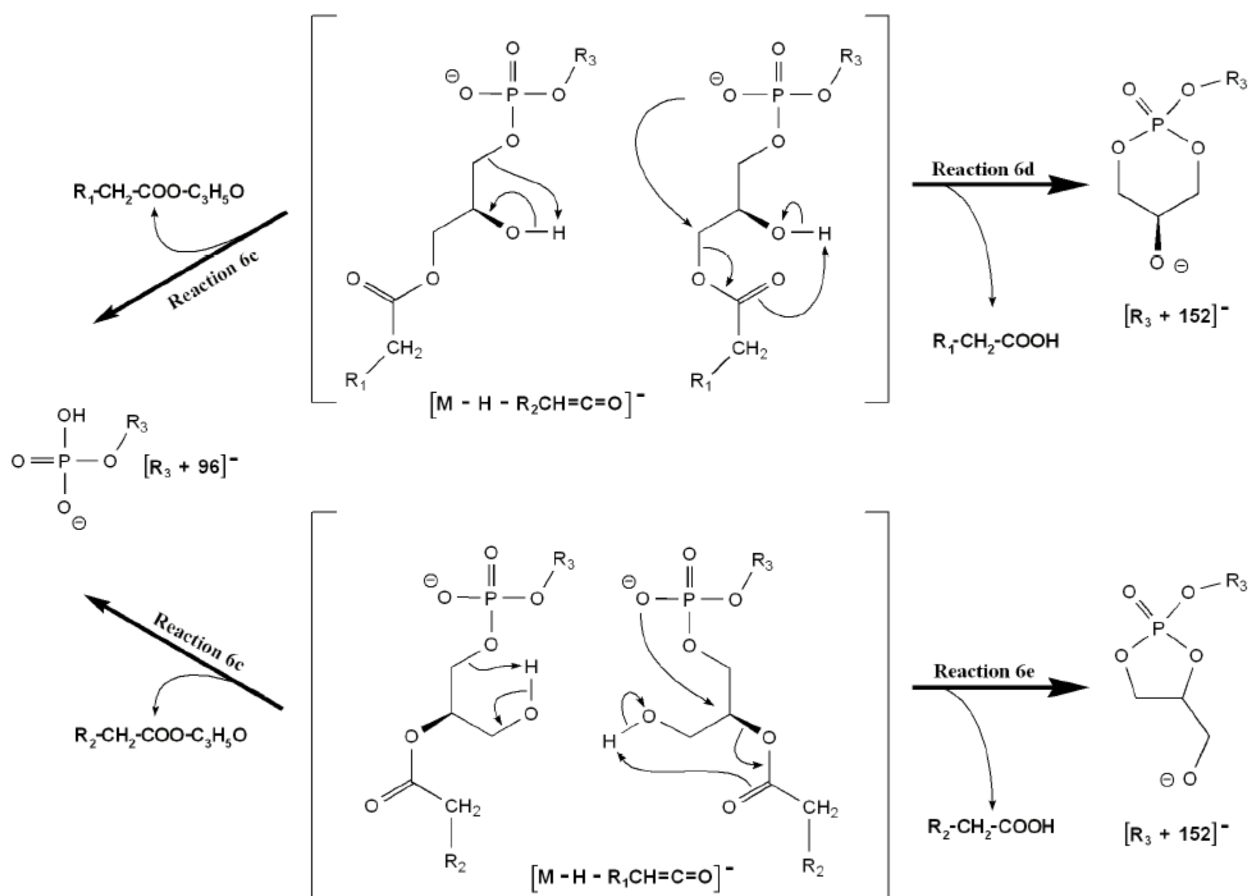

Scheme 6.

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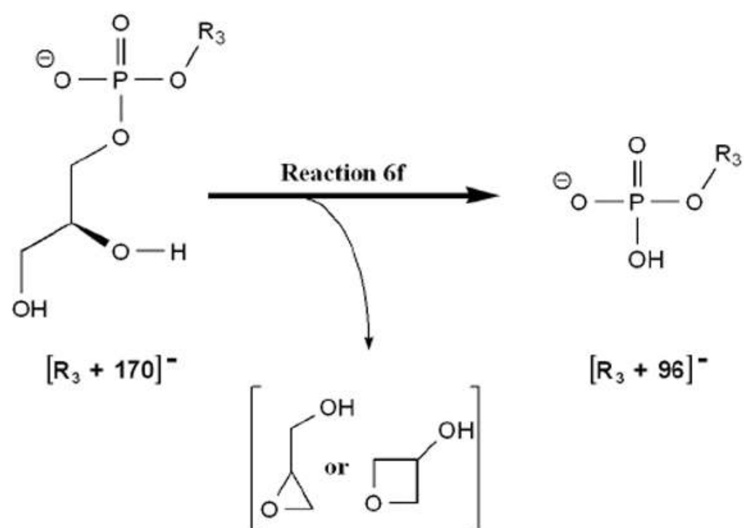

Scheme 7.

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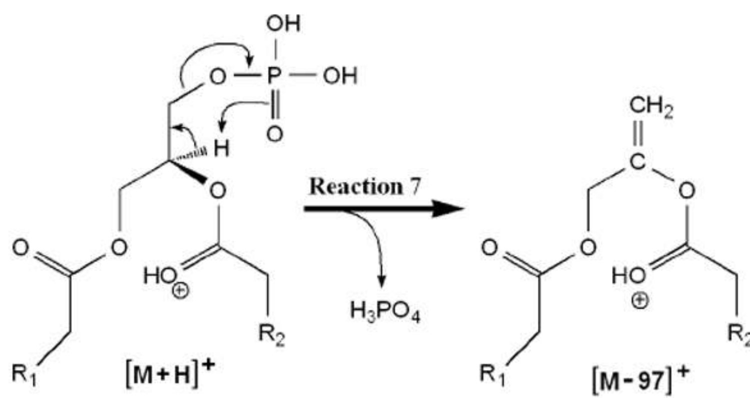

Scheme 8.

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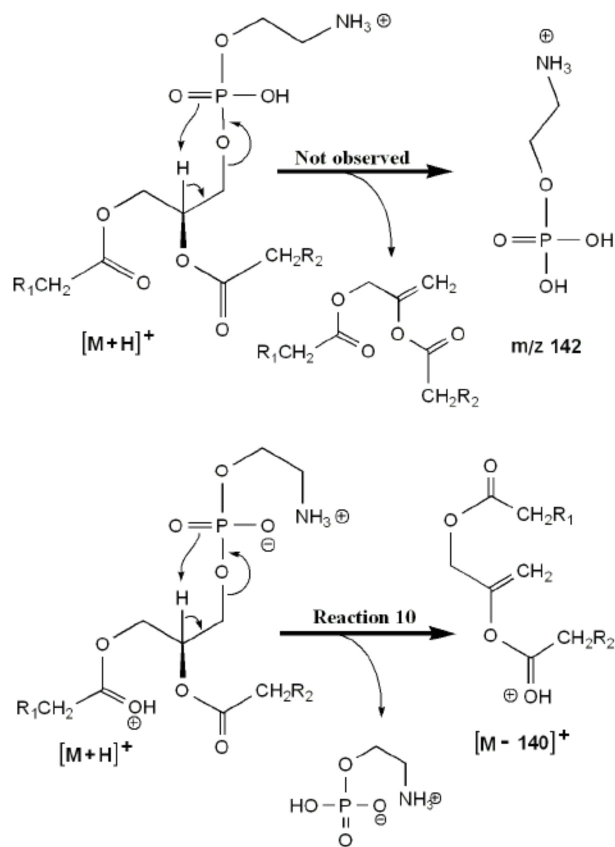

Scheme 9.

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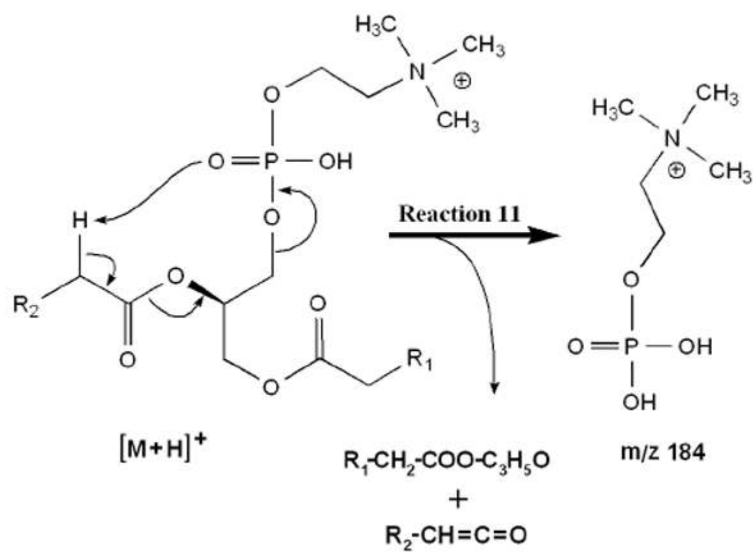

Scheme 10.

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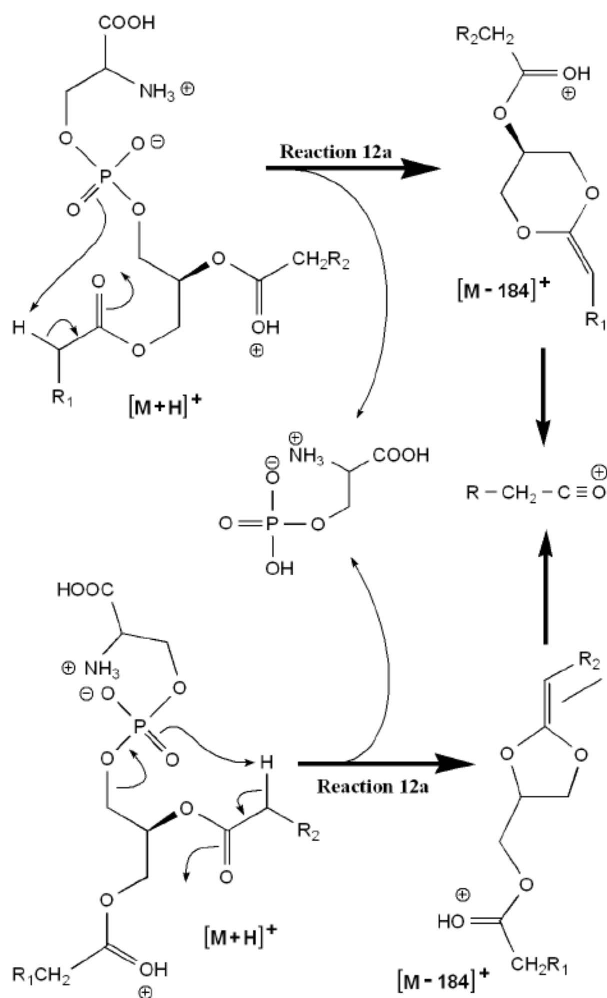

Scheme 11.

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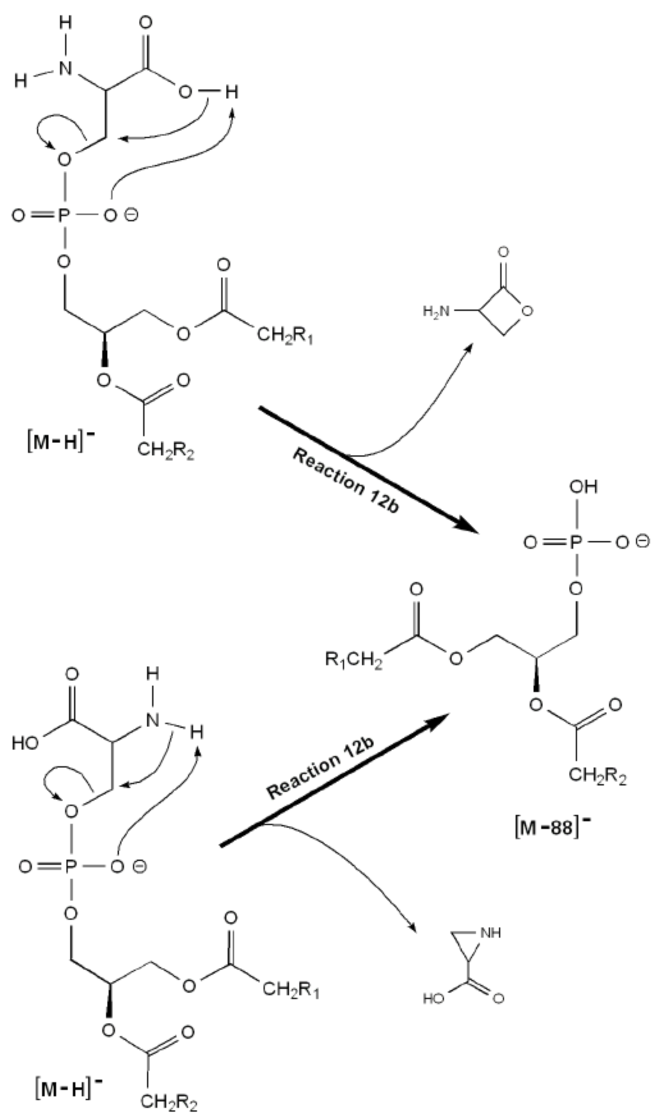

Scheme 12.

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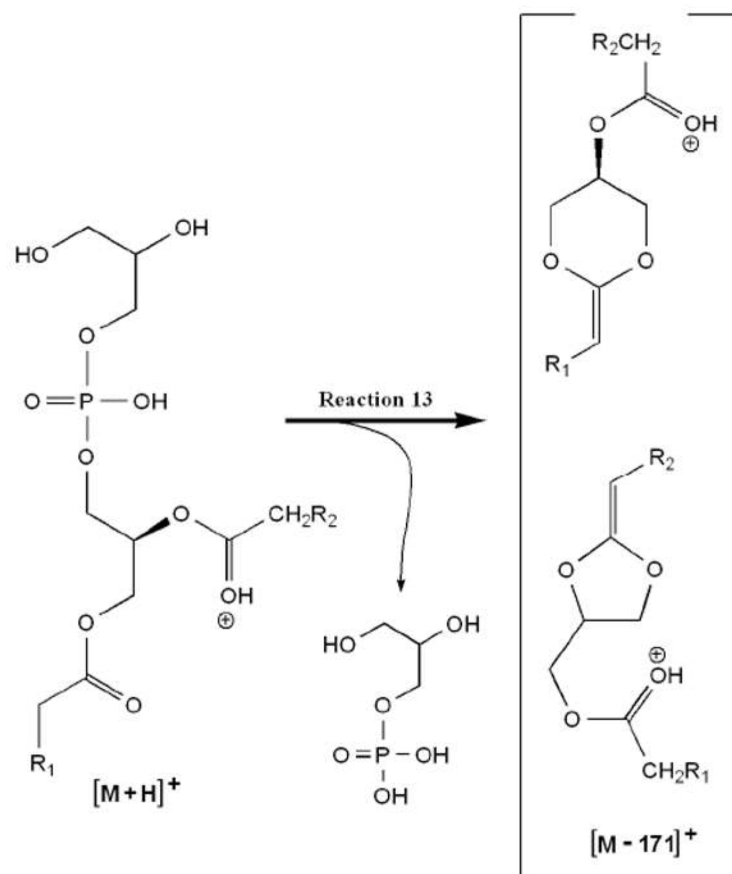

Scheme 13.

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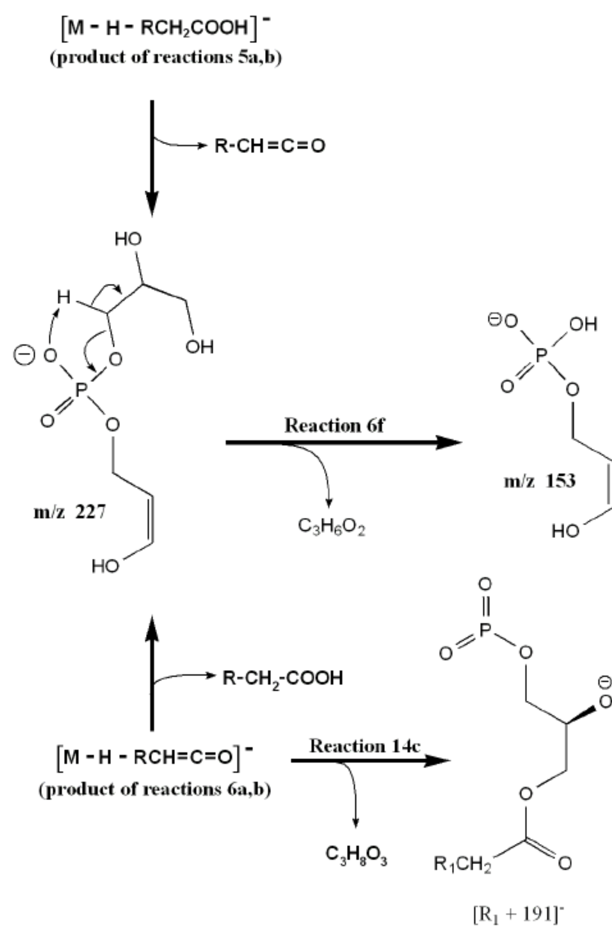

Scheme 14.

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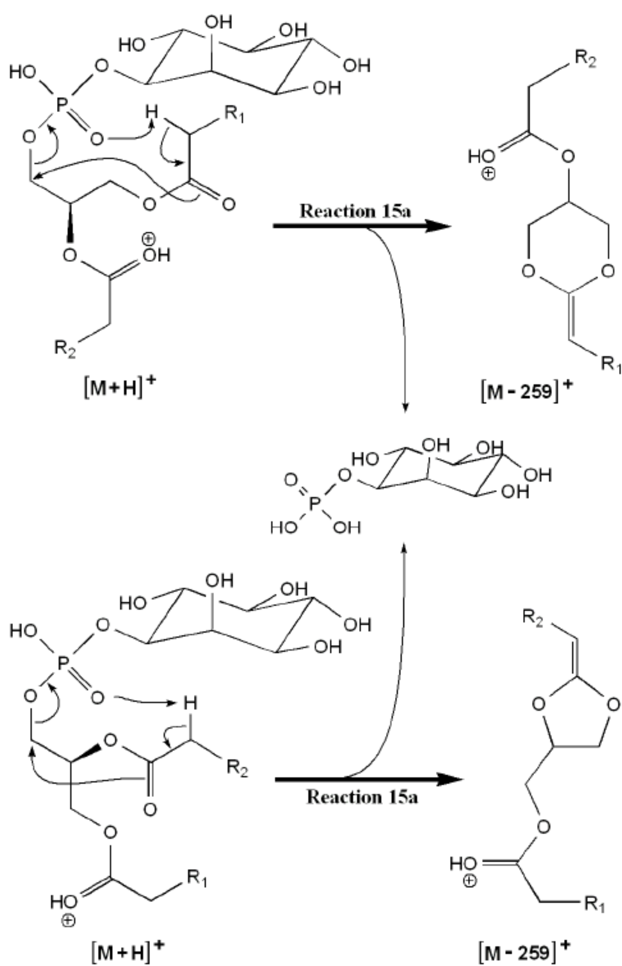

Scheme 15.

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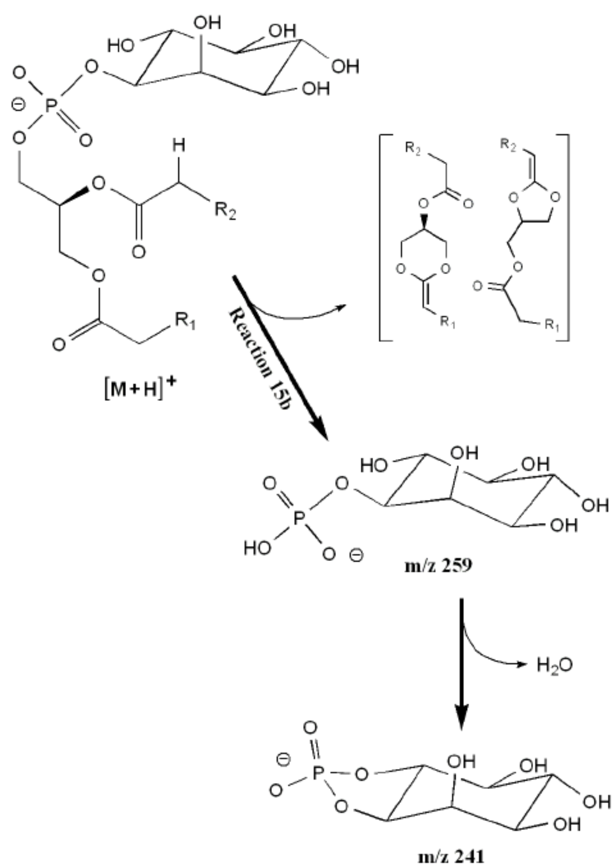

Scheme 16.

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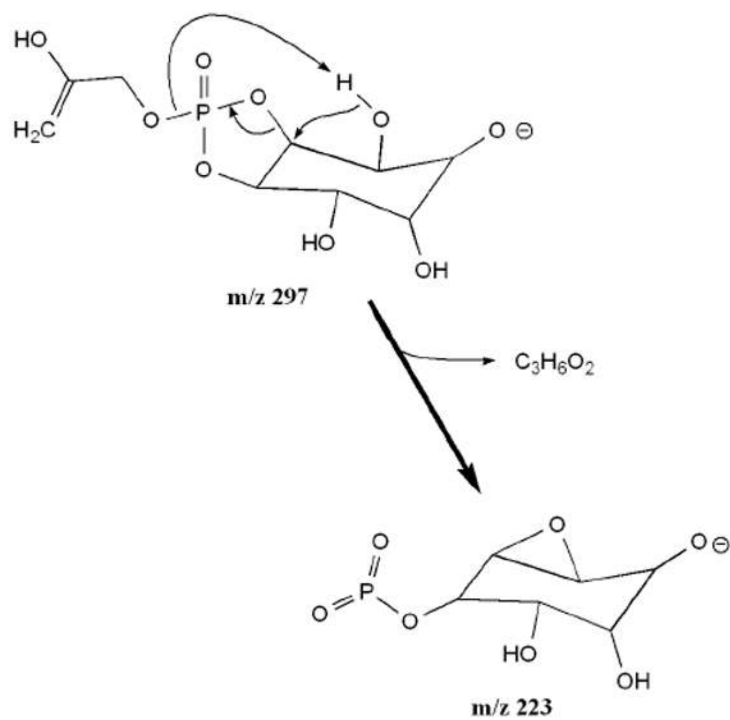

Scheme 17.

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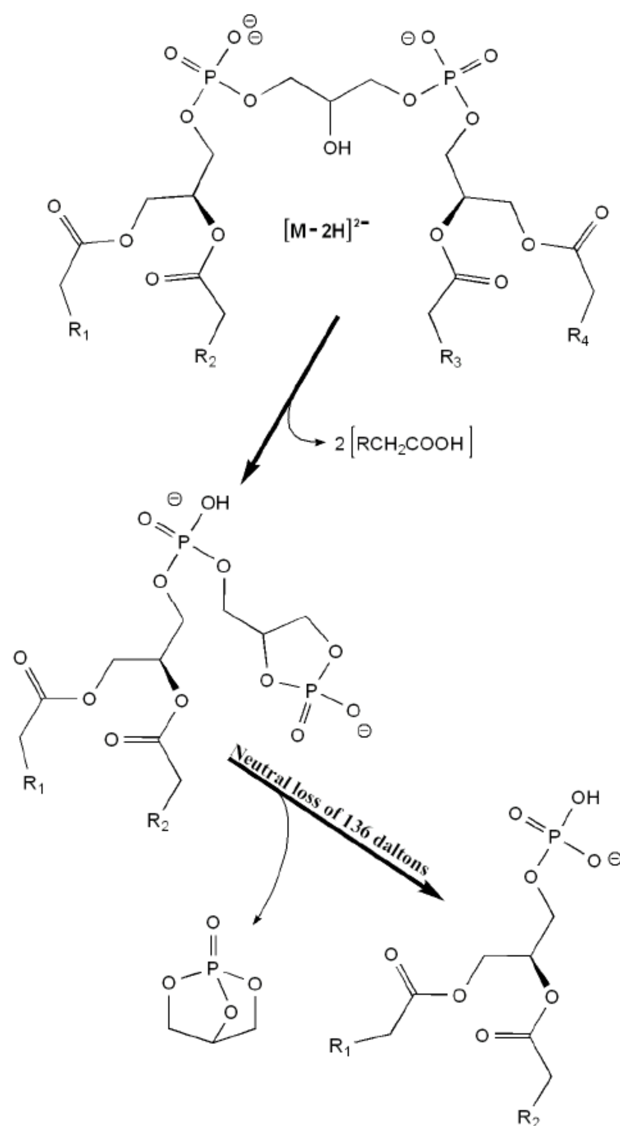

Scheme 18.

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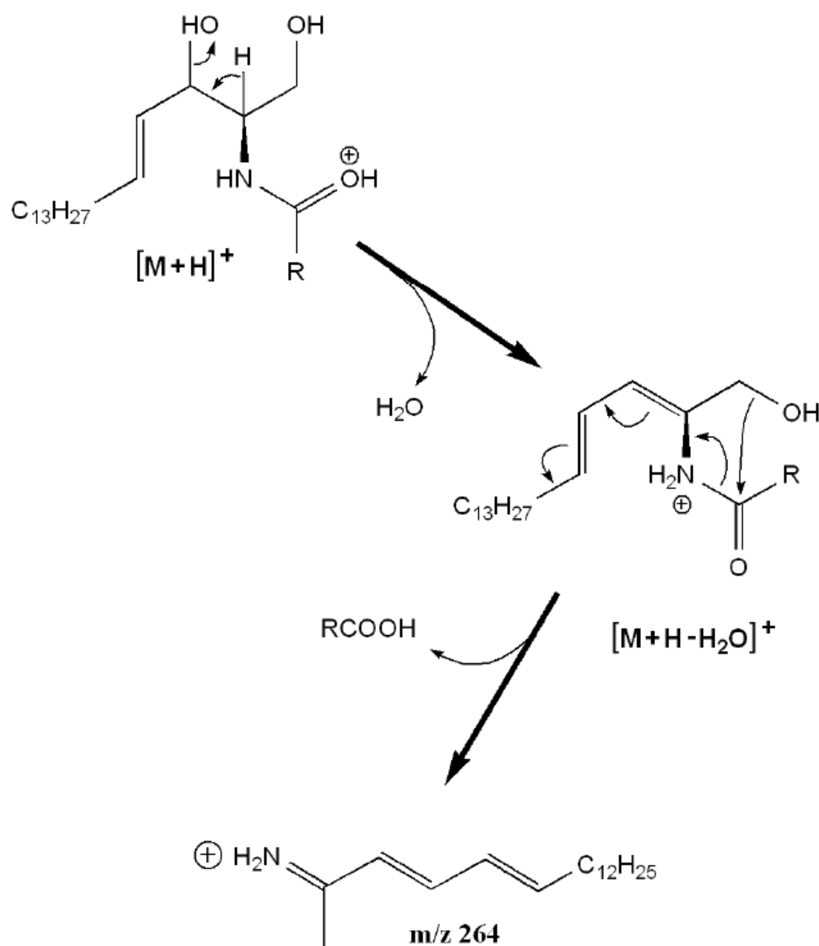

Scheme 19.

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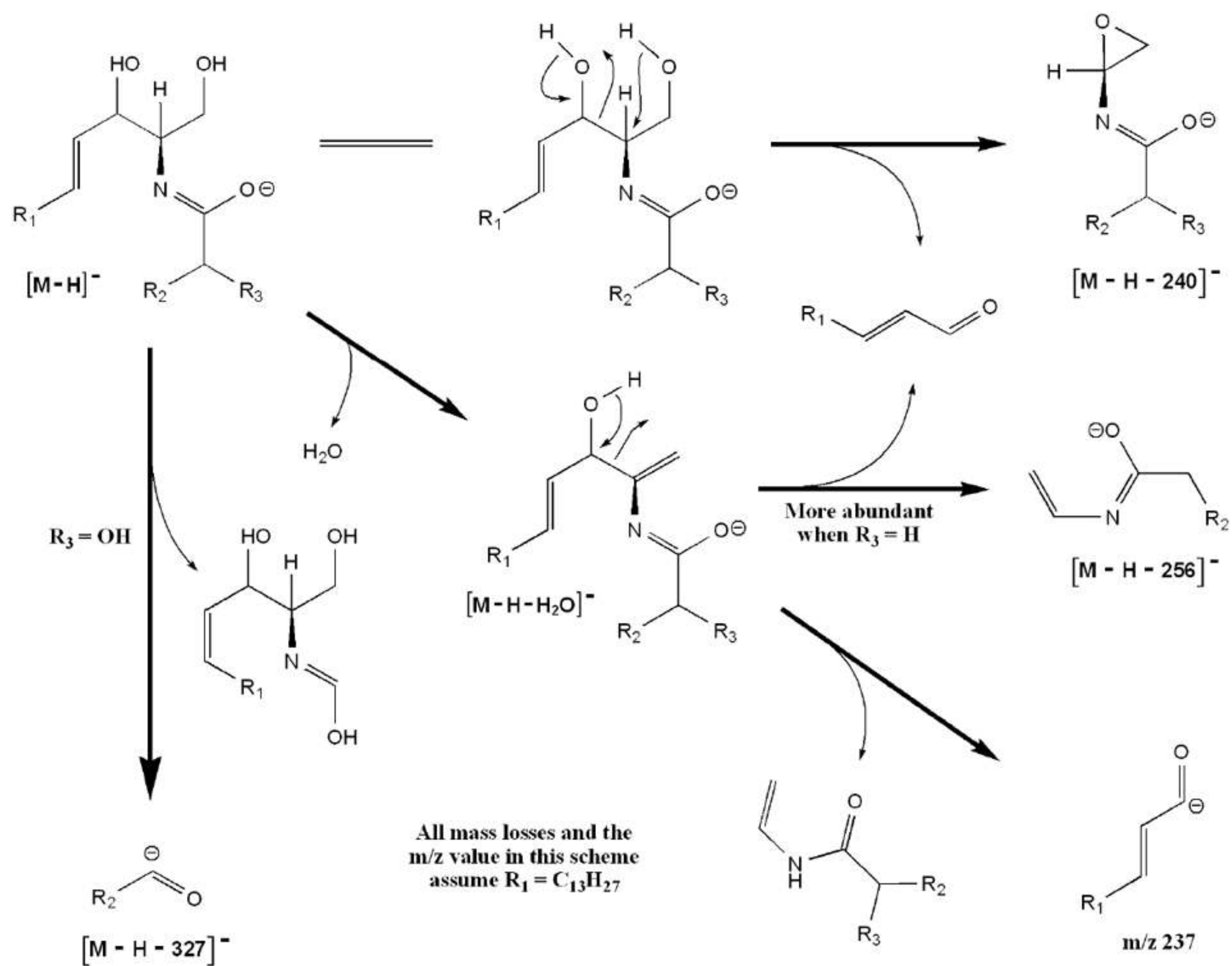

Scheme 20.

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Table 1

Classification of lipids containing long hydrocarbon chains derived from the biosynthesis of fatty acids and examples of common roles played by these lipid species.

		Cellular Function
Fatty Acids		
	Fatty acids	Energy production, intermediates
	Fatty acids amides	Signaling
	Prostaglandins	Signaling
Glycerolipids		
	Triacylglycerols	Energy storage
	Diacylglycerols	Signaling, intermediates
	Monoetherdiacylglycerols	Intermediates
Phospholipids		
	Phosphatidic acid	Intermediates, signaling
	Glycerolphosphoethanolamine lipids	Membrane bilayer
	Glycerolphosphocholine lipids	Membrane bilayer
	Glycerolphosphoserine lipids	Membrane bilayer
	Glycerolphosphoinositol lipids	Membrane bilayer
	Glycerolphosphoglycerol lipids	Membrane bilayer
	Cardiolipin	Mitochondria bilayer
Sphingolipids		
	Sphingomyelin	Membrane bilayer, microdomains
	Ceramides	Signaling
	Gangliosides	Protein modulation
	Sulfatides	Adhesive molecule