Analysis of Nonvolatile Lipids by Mass Spectrometry

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Contents

I. Introduction

Lipids are defined as substances derived from living tissues that can be extracted or solubilized in organic solvents. This rather loose definition encompasses a quite diverse array of important biochemical molecules ranging from relatively low molecular weight fatty acids to complex glycosphingolipids and peptidolipids of high molecular weight. Mass spectrometry has played an important role over the past 50 years in the analysis of lipids, although the types and classes of molecules which could be successfully analyzed were somewhat limited in the early years due to a requirement of thermostability and volatility for this gas-phase analytical technique. As lipids occur in nature, they are largely nonvolatile. Therefore, substantial effort was devoted to developing methods to convert lipids such as fatty acids into derivatives that could pass into the gas phase and even into a gas chromatograph. With the advent of newer ionization techniques, specifically electrospray ionization (ESI) and matrix-assisted laser desorption (MALDI), this situation has profoundly changed so that it is now possible to produce directly gas-phase molecular ions for most lipids whether they are simple fatty acids or the most complex lipid substances. A major evolution of the analytical power of mass spectrometry for nonvolatile lipid analysis has been the concurrent development of collision-induced decomposition (CID) of molecular ions which allowed elucidation of structural details in addition to molecular weight information. Nonetheless, molecular information alone has been of great value when analyzing lipids, and this information has opened new levels of understanding in lipid biochemistry.

One feature of complex lipids is that many subtypes of these molecules exist within their biological venue as a diverse mixture of closely related molecules. Perhaps one of the more relevant examples is that of the glycerophospholipids which make up the defining membrane of mammalian cells. Such membrane phospholipids are a mixture of glycerol esters comprised of at least four major classes which are differentiated by the polar headgroup esterified to the sn-3 (systematic nomenclature) position of glycerol. These include the major classes glycerophosphocholine (GPC), glycerophosphoethanolamine (GPE), glycerophosphoserine (GPS), and glycerophosphoinositol (GPI) lipid species in addition to at least five additional less abundant classes. In addition to the phospholipids being mixtures of these classes, each individual class can have subclasses depending upon the type of oxygen chemical bond at the first carbon atom of glycerol (sn-1). The sn-1 substituent may be an alkyl chain linked via an ether bond, a long-chain alkyl group linked via a vinyl ether bond,

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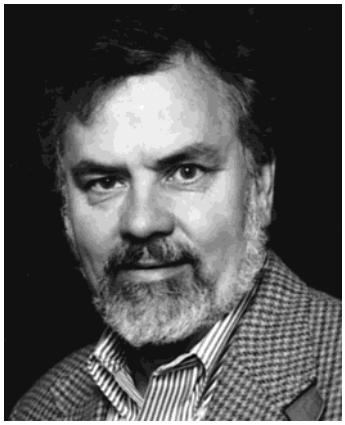

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or a long-chain alkyl ester. Generally, these are referred to as radyl substituents. With up to 15−20 different fatty acid esters in typical cells, the precise speciation of phospholipids (molecular species) is defined by the sn-2 fatty acyl ester and the radyl substituent at sn-1. Thus, phospholipids extracted from a cell may be a mixture of several hundreds of unique molecular species. With electron ionization mass spectrometry it was necessary to degrade intact phospholipids and perform an analysis of the fatty acids that had been present esterified to the glycerol backbone and thereby lose information relative to the native structure of the glycerophospholipid. Using ESI and MALDI, it is possible to deal with the complexity of the mixture of individual glycerophospholipids as intact molecules and obtain precise information about each molecular species. In part,

John Hevko was born in 1972 in Adelaide, South Australia. He received his B.A. degree in Organic Chemistry and Pharmacology from the University of Adelaide in 1994 and pursued synthetic chemistry during his Honours degree, which he completed in 1995. He received his Ph.D. degree in 1998 under the supervision of Professor Hohn Bowie studying gas-phase rearrangements and fragmentations of small organic anions utilizing mass spectrometry. In 1999, he began postdoctoral studies in Professor Robert C. Murphy's laboratory at the National Jewish Medical and Research Center, where he is currently studying mass spectrometric techniques of lipid biochemistry. His research interests are focused on the use of mass spectrometry to study the biosynthesis and metabolism of biological lipids and the identification and characterization of target enzymes.

this can be facilitated by the power of on-line HPLC to separate many components in naturally occurring complex mixtures combined with the power of tandem mass spectrometry to identify and structurally define isobaric molecular species which differ in each sn-radyl substituent.

This article will attempt to review recent advances in the analysis of lipid substances as nonvolatile entities by mass spectrometry largely during the past 10 years. A number of reviews[1,2] have appeared over the course of the past decades covering the development and application of electron ionization and chemical ionization techniques with the gas-phase analysis of lipid substances as volatile derivatives. While advances continue to illuminate our understanding of the electron ionization of lipids, the developments of desorption ionization techniques and spray ionization techniques have only recently emerged[3,4] and this article will focus on these recent modes of ionization used for lipid substances. This review will begin with the analysis of low molecular weight fatty acids, steroids, fat-soluble vitamins, and antioxidant lipids. Subsequently, complex lipids will be discussed first considering the glycerides (diacylglycerols, triacylglycerols, and glycerophospholipids), then proceeding to those lipids which have a sphingosine base at their core structure. Finally, a brief consideration of glycolipids and lipid-transferring biochemical agents such as acyl carnitine and fatty acyl CoA derivatives will be presented. While our broad definition of lipids could include a wide variety of natural products present in plant as well as bacteria including flavanoids and alkaloids, these will not be discussed in this review.

II. Fatty Acids

Fatty acid represents one of the fundamental classes of lipid substances in biology. This class of

lipids ranges from relatively simple long-chain saturated monocarboxylic acids to quite complex cyclic structures with oxygen substituents adjacent to carbon−carbon double-bond sites which are derived from the biochemical oxidation of polyunsaturated fatty acids. The structures of fatty acids have been studied extensively by mass spectrometry using gas-phase analytical techniques, and several excellent reviews of the use of gas chromatography/mass spectrometry of fatty acid derivatives including various esters and trimethylsilyl ether derivatives have appeared.[1,2,5] The major structural issues that emerge in the study of fatty acids concern alkyl branching sites, positions of unsaturation along the alkyl chain of the carboxylic acid, and the nature and position of oxygen substituents. Electron ionization and chemical ionization techniques have been developed which facilitate the assessment of each of these structural issues through a combination of derivatization, chromatographic behavior, and in some cases the need for degradation to previously known molecules. An excellent example is the use of picolinyl esters to assess the position of double bonds in unsaturated fatty acid esters.[6] With the advent of desorption ionization and electrospray ionization, additional methods have emerged which facilitate analysis of fatty acids to localize double bonds, alkyl branching, and oxygen-containing functional groups without derivatization.

A. Saturated, Unsaturated, and Branched Fatty Acids

The facile formation of carboxylate anions from saturated, unsaturated, and polyunsaturated fatty acids during electrospray has focused a considerable amount of attention on the negative-ion electrospray MS/MS behavior.[7] Saturated fatty acids have the tendency to decompose with the only major ion corresponding to the loss of neutral water from the carboxyl moiety observed as $[M - H - 18]^-$.[7] However, the inclusion of a single double bond in the molecule radically alters the situation. Product ion spectra from these molecules have in addition to a predominate ion corresponding to the loss of water, ions derived from bonds cleaved α or β to the site of unsaturation. Polyunsaturated fatty acids decompose in a much more complex manner, which has been revealed using stable isotope-labeled analogues. One example is the negative-ion electrospray tandem mass spectrum of arachidonic acid (four homoconjugated double bonds) and the deuterium-labeled analogues in which each double bond is labeled with deuterium (d_8-arachidonic acid) or the terminal methyl and methylene position (d_4-arachidonic acid).[7] One of the more abundant product ions was observed at *m*/*z* 205 (Figure 1A), which could originate either from cleavage of the double bond between carbon-5 and carbon-6 with charge retention on the alkyl portion of the molecule or cleavage of the vinylic bond between carbon-13 and carbon-14 with charge retention on the carboxyl end of the molecule. The d_4-labeled molecule has this ion shifted to *m*/*z* 209 for 90% of the total ion abundant, but 10% remains at *m*/*z* 205, suggesting a complex origin of *m*/*z* 205 with the majority being derived from cleavage of the C5−C6 double bond. This unlikely fragmentation must be proceeded by double-bond rearrangement. This was supported through the analysis of the d_8-labeled arachidonic acid (Figure 1B) with the shift of this ion to a doublet at *m*/*z* 212 and 213. However, extension of these observations is problematic since rearrangement of double bonds makes unambiguous interpretation of unknown structures somewhat tenuous.

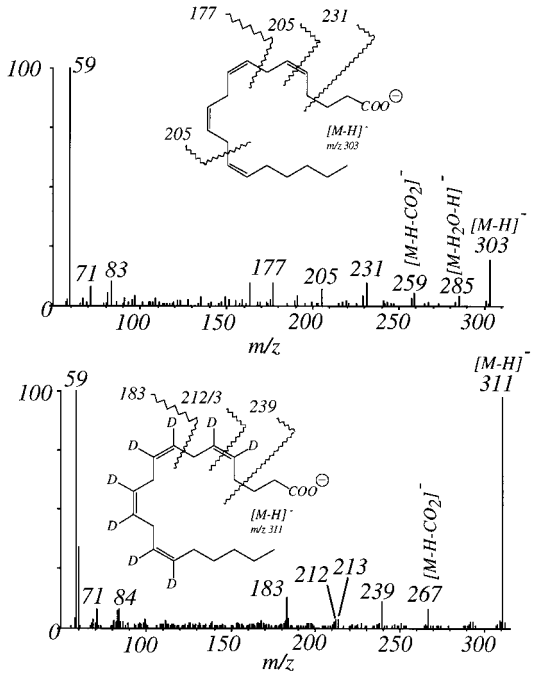
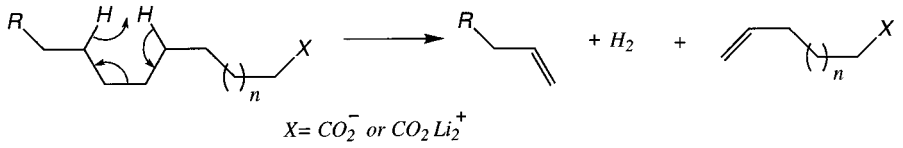


Figure 1. Negative-ion electrospray tandem mass spectrometry of (A) arachidonic acid and (B) arachidonic acid-d_8. Collision-induced decomposition of $[M - H]^-$ carried out essentially as described in ref 7 in a tandem quadrupole mass spectrometer.

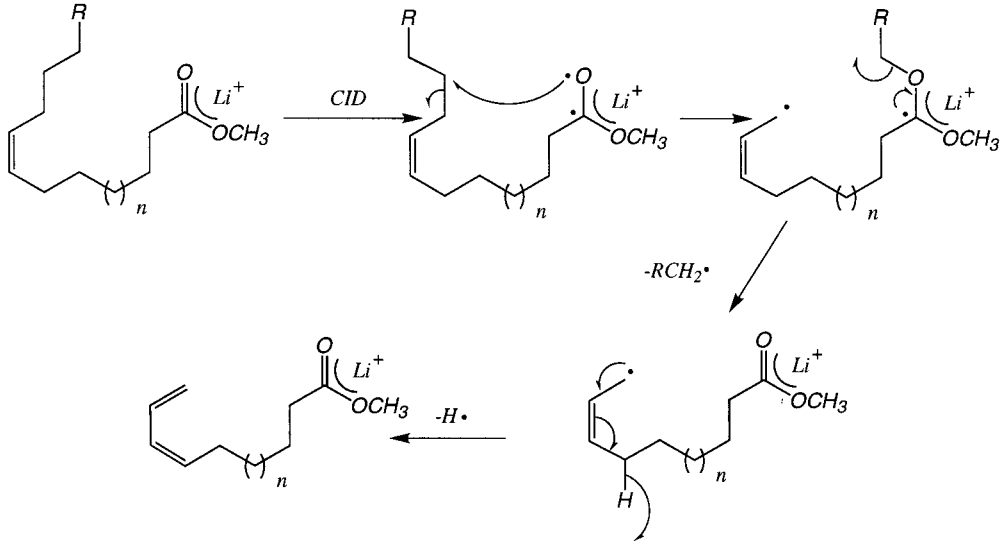
The observation of a series of homologous product ions induced by collisional activation of closed shell, even electron ions has been a major development in the analysis of fatty acid species. These ions arise from charge-remote fragmentation mechanisms.[8] Numerous examples of this interesting phenomenon will be presented throughout this review. Support for the nature of the remote site charge fragmentation of the fatty acid ions was supplied by Cordello and Wesdemiotis[9] using neutralization−reionization mass spectrometry to detect the major neutral species lost during CID. These investigators were able to provide data to suggest that major neutral losses were in fact alkenes or dienes when monounsaturated fatty acids were collisionally activated and not alkanes or alkyl radicals. These studies supported the mechanism as a paracyclic 1,4-elimination of H_2 as shown in Scheme 1.

There have been other suggestions for the mechanism responsible for the fragmentation of cationized fatty acid esters under high-energy conditions as suggested by Claeys et al.[10] Clearly high-energy CID of monounsaturated fatty acids of lithiated or sodiated esters can yield relevant information concerning the localization of a single site of unsaturation in the alkyl chain through a gap in a series of ion fragments. The alternative mechanism (Scheme 2) proposed involved the metalated carboxylate cation directing

Scheme 1



Scheme 2



homolytic cleavage of the carbon−carbon bond allylic to the site of unsaturation with formation of a transient biradical cation. This radical species would then decompose in two steps, leading to the metalated diene ion indicative of the position of the double bond. Support for this mechanism was obtained through quantum chemical calculations. It is also common to find fragmentation processes taking place in functionalized fatty acyl chains that are remote to a fixed charge site such as a closed-shell ion.

Isomeric unsaturated fatty acids have been studied as lithiated adducts using electrospray ionization mass spectrometry followed by low-energy collisional activation in a tandem quadrupole instrument.[11] As illustrated in Figure 2, the CID of two isomeric octadecatrienoic acids (9,12,15-octadecatrienoic acid and 6,9,12-octadecatrienoic acid; Figure 2B and C) are readily distinguished[11] by fragment ions at *m/z* 195 and 193, respectively, which are one type of charge-remote product ions. The proported mechanism for the formation of these specific ions is outlined in Scheme 3 as an allylic carbon−carbon single bond cleavage distal to the carbon−carbon double bond.[9]

The identification of alkyl-substituted fatty acids including methyl-branched variants has been one of the traditional difficulties of electron ionization (EI) mass spectrometry. Specifically, EI does not yield definitive information in the high-mass region of methyl ester derivatives of branch-chained fatty acids so that isomethyl and antisomethyl branched acids could be distinguished. Zirrolli and Murphy[12] found that CID of the molecular ions of fatty acid methyl esters in the tandem quadrupole instrument yielded a wealth of information concerning the position of methyl substituents along the fatty alkyl chain. This was observed at very low collision energies as shown

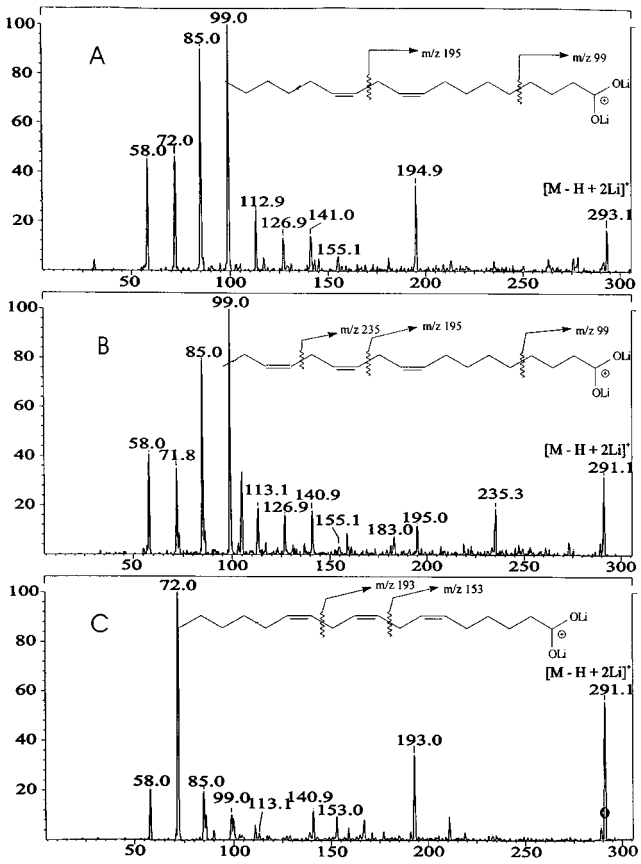
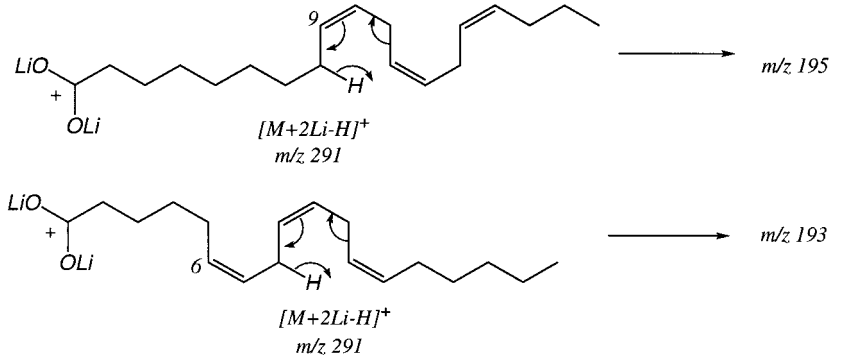


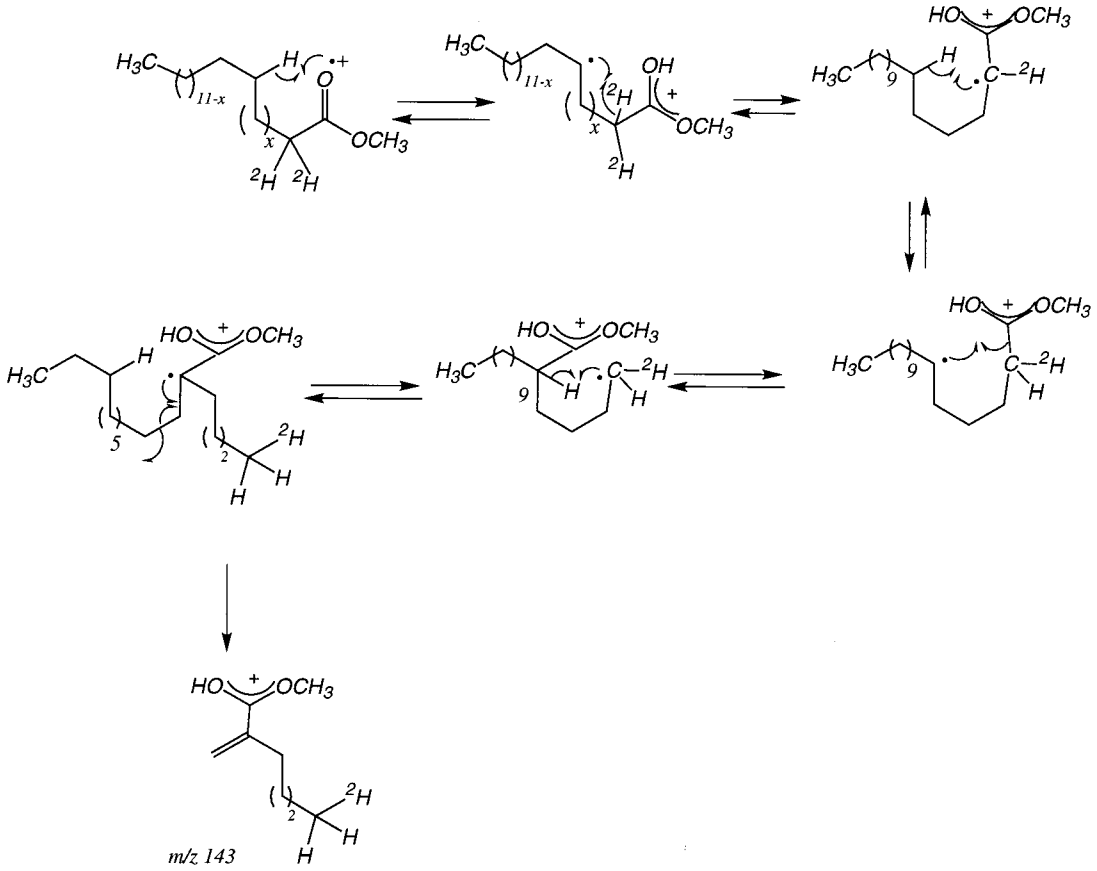
Figure 2. Tandem mass spectra of dilithiated adducts [M − H + 2Li]$^+$ of the polyunsaturated fatty acids: (A) 9,12-octadecatrienoic acid, (B) 9,12,15-octadecatrienoic acid, and (C) 6,9,12-octadecatrienoic acid. (Reprinted with permission from ref 9. Copyright 1994 American Chemical Society.)

in Figure 3 for the electron ionization, tandem mass spectrum of the $M^{+\bullet}$ from methyl 3,7,11,15-methyl-

Scheme 3



Scheme 4



hexadecanoate (methyl phytanate) and with several deuterated fatty acid methyl esters yielded an unexpected series of ions as isotope doublets. McLafferty and co-workers[13] carried out sophisticated collision-induced decomposition studies to further delineate the mechanism of the formation of fatty acid methyl esters product ions and movement of deuterium atoms originally present at C-2. They suggested the mechanism outlined in Scheme 4 for the radical-induced formation of these product ions.

Analysis of derivatized fatty acids remains a mainstay of general fatty acid analysis. Most all applications involve the analysis of volatile derivatives using GC/MS and predominantly capillary gas chromatographic separations.[14] The fatty acid profile following hydrolysis of more complex lipids remains a valuable set of information, for example, in bacterial speciation[15] and pathological tissues,[16] as do pyrolysis techniques that involve gas-phase methylation.[17]

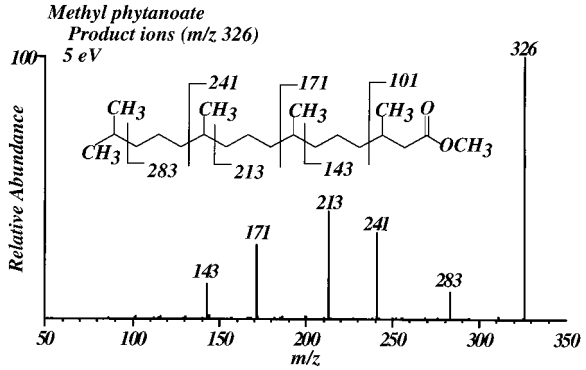


Figure 3. Collision-induced decomposition of the $M^{+\bullet}$ from methyl phytanoate (*m/z* 326) at low energies in a tandem quadrupole mass spectrometer. (Reprinted with permission from ref 12. Copyright 1993 Elsevier Science.)

Double-bond geometry is still an important question in the characterization of fatty acids which requires

more than mass spectrometry to be fully characterized. For example, the analysis of eight geometric isomers of 8,10-, 9,11-, 10,12-, and 11,13-octadecadienoic acid methyl esters was carried out using argentation HPLC followed by analysis by GC/MS with high-resolution selected ion recording.[18] Saturated dicarboxylic acids are also commonly analyzed by GC/MS as their dimethyl esters.[19] An alternative derivative of potential value for very long chain saturated and dicarboxylic acids was recently reported as the dimethylaminoethyl ester which yields abundant $[M + H]^+$ by electrospray MS.[20]

B. Hydroxy Fatty Acids

Functionalization of fatty acyl chains is an important event taking place within virtually all biological systems from the highest evolved animal and plant to the oldest living organisms such as the archaebacteria. Few biochemical mechanisms occur within cells to carry out oxygenation of saturated or monounsaturated fatty acid; however, polyunsaturated fatty acids are common substrates for enzymatic processes that result in oxygen functionality along the fatty acyl chain. Structural elucidation of these

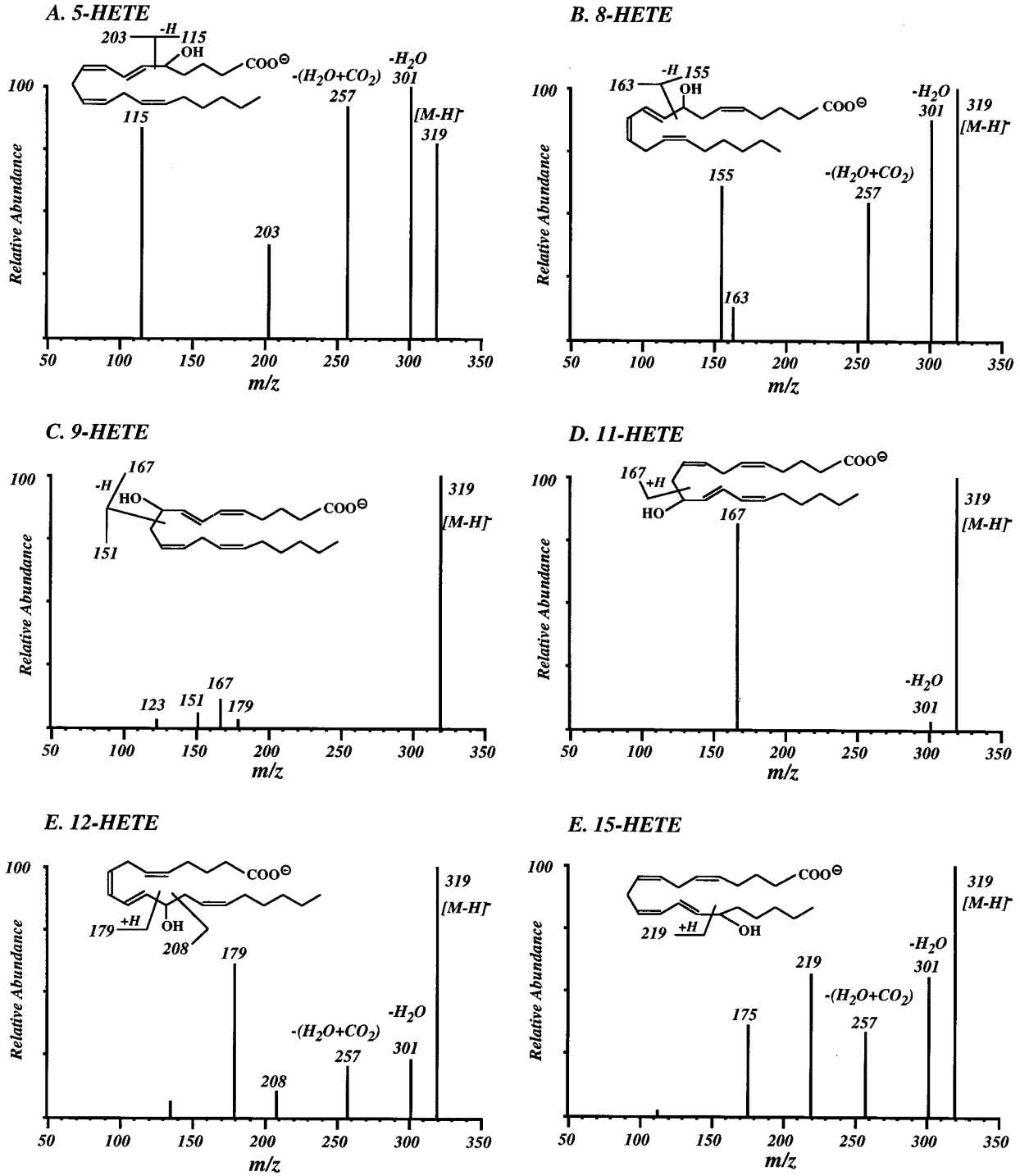
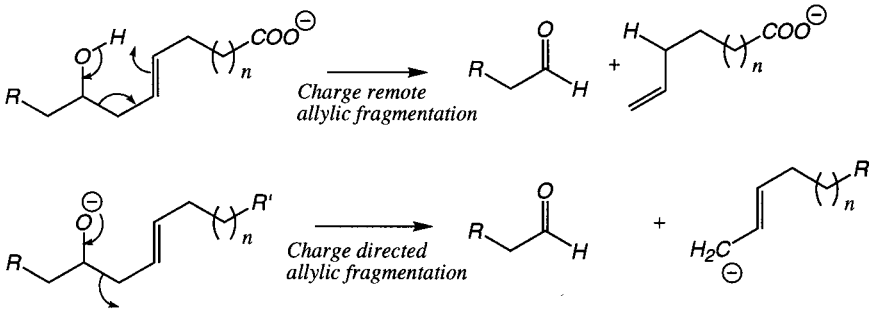


Figure 4. Collision-induced decomposition of $[M - H]^-$ carboxylate anions derived from six regioisomeric monohydroxyeicosatetraenoic acids in a tandem quadrupole mass spectrometer. (Reprinted with permission from ref 22. Copyright 1993 John Wiley & Sons.)

Scheme 5



products remains an important challenge for mass spectrometry, and several advances have been made in the identification of such polyunsaturated, oxygenated fatty acids using electrospray and tandem mass spectrometry. Saturated hydroxy fatty acids have been found to be readily detected as their carboxylate molecular anion but do not yield major fragment ions unless there is a substituent within three carbon atoms of the carboxylate anion or near the methyl terminus.[21] For these special hydroxyl fatty acids, an ion corresponding to $[M - H - 46]^-$ was observed as an abundant product ion that was found to correspond to the loss of water from the carboxylate anion and carbon monoxide from the carboxyl group rather than C_2H_5OH as determined by oxygen-18-labeled analogues.

When a double bond is adjacent to the hydroxy group as typically encountered for oxygenated polyunsaturated fatty acids, CID of the carboxylate anion was remarkably altered. An example of monooxygenated polyunsaturated fatty acids (Figure 4) is the six regioisomeric monohydroxy metabolites of arachidonic acid derived from lipoxygenase reactions or free radical oxidation of arachidonic acid.[22] The mechanism of formation of many abundant product ions either in electrospray or fast atom bombardment ionization[22] was suggested to involve a charge-remote allylic fragmentation which ultimately resulted in an α-hydroxyl fragment ion most abundant when the hydroxyl substituent was allylic to a double bond (Scheme 5). Other proposed mechanisms, supported by stable isotope-labeling experiments, suggested alkoxide anion formation during the electrospray ionization event which facilitated adjacent carbon−carbon bond cleavage. A charge-remote vinylic fragmentation was also observed with bond cleavage adjacent to the hydroxyl group when proton rearrangement processes could take place, leading to a stabilized conjugated system. Both allylic and vinylic fragmentation adjacent to the hydroxyl substituent could also arise by charge-driven processes to account for the most abundant ions.

The unique behavior of the hydroxy fatty acid during CID has permitted development of sensitive quantitative assays used to assess the occurrence of these unique products of polyunsaturated fatty acids in biological systems by using multiple reaction monitoring (MRM) techniques.[23] Quantitative LC/MS/MS assays have been used to measure the formation of various monohydroxy isomers of arachidonic acid formed during free radical oxidation events in biological tissues[24] as well as in vivo in atherosclerotic plaques.[25]

Low-energy tandem mass spectrometry has also been shown to be quite useful for the analysis of dihydroxy- and polyhydroxy-substituted polyunsaturated fatty acids.[26] Abundant anions were readily formed by electrospray ionization, and CID of these ions generally lead to carbon−carbon bond cleavage immediately adjacent to the hydroxyl substituent but was directed by the positions of the double bonds present in the molecules,[26] again supporting the concept of a central role of the alkoxide anion in the fragmentation process. Both charge-driven and charge-remote fragmentation mechanisms have been proposed for the CID of specific dihydroxy metabolites of arachidonic acid (Figure 5). Some of the more abundant CID product ions are derived from either charge-remote allylic fragmentation (*m*/*z* 145 from the CID of *m*/*z* 337 of 5,6-diHETE) or a charge-directed allylic fragmentation (*m*/*z* 191 from the CID of *m*/*z* 337 from the same molecule).

The electrospray ionization of mono- and dihydroxy metabolites of linoleic acid has also been studied using an ion-trap mass spectrometer.[27] Collisional activation of $[M - H]^-$ of these oxygenated lipids resulted exclusively in $[M - H - H_2O]^-$. Subsequent collisional activation of this ion resulted in a rich yield of product ions in the MS/MS/MS (MS^3) experiment. Again, the major products appeared to arise from fragmentation adjacent to hydroxyl substituents by mechanisms previously suggested in studies with fast atom bombardment[22] and electrospray ionization[26] in the tandem quadrupole instrument. It is interesting to note the differences that were displayed between the ion-trap experiment and the tandem quadrupole experiment in terms of the collisional activation of similar hydroxy polyunsaturated fatty acids.[26,27] It would appear that one of the initial events which occurred during collisional activation of the $[M - H]^-$ ion was indeed the loss of water. In the ion-trap experiment, this product ion was then collapsed to the center of the ion trap and did not undergo further collisional activation. However, in the tandem quadrupole, the ion corresponding to the loss of water from the carboxylate anion underwent further collisions as it traversed the second quadrupole collision cell, ultimately leading to the product ions which were observed in the ion-trap mass spectrometer during the MS^3 experiment.

Various bacteria are a rich source of hydroxy fatty acids typically esterified to the peptidoglycan of the

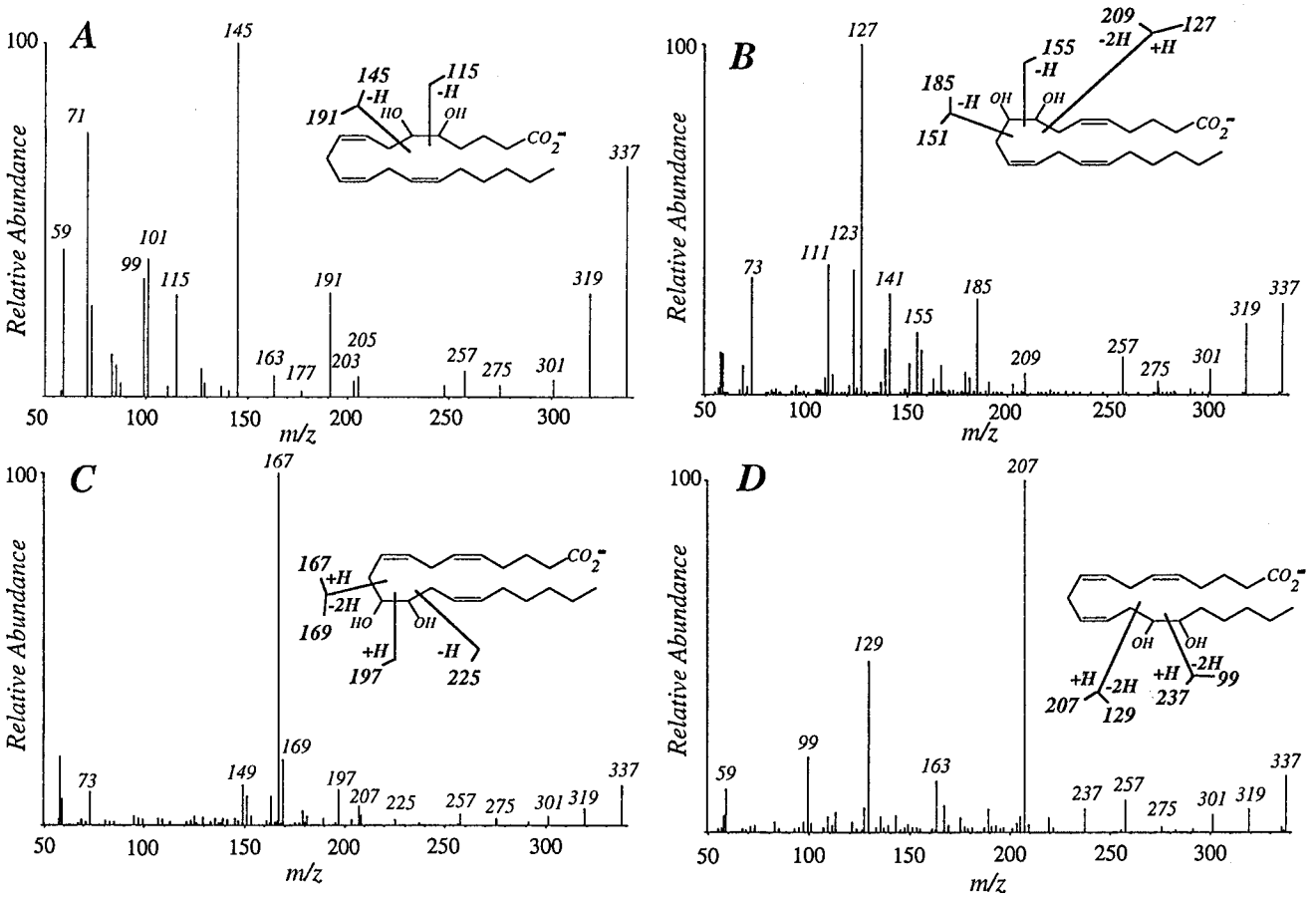
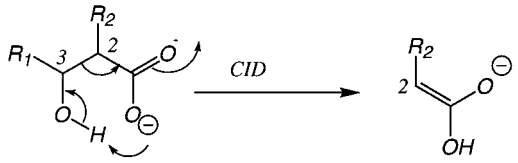


Figure 5. Collision-induced decomposition of carboxylate anions derived from four regioisomeric dihydroxyeicosatetraenoic acids in a tandem quadrupole mass spectrometer. (Reprinted with permission from ref 26. Copyright 1996 Elsevier Science.)

Scheme 6



cell wall. Mycolic acids are very long chain α-alkyl-β-hydroxy fatty acids (Scheme 6) that have challenged the capability of GC/MS as volatile derivatives. Fast atom bombardment and collision activation of the resultant carboxylate anions generated abundant ions corresponding to cleavage between C2 and C3, thus facilitating characterization of the α-alkyl group.[28]

Fast atom bombardment,[29] thermospray,[30] and, more recently, electrospray ionization[31] have been employed to structurally characterize mycolic acids from various mycobacteria. Fast atom bombardment and high-energy MS^3 have also been promoted as a valuable technique to characterize the fatty acyl components in liposidomycins isolated from streptomyces.[32]

C. Other Oxygenated Fatty Acids

1. Oxy-Fatty Acid

Fast atom bombardment of saturated keto acids has been found to yield abundant $[M - H]^-$ carboxylate anions and charge-driven $[M - H - H_2O]^-$ and $[M - H - CO_2]^-$ anions.[33] High-energy collisional activation of these anions yielded a large population of ions derived from charge-remote fragmentation reactions corresponding to cleavage α, β, and γ to the carbon−carbon bonds adjacent to the keto group. The position of the keto group was readily ascertained from the CID product ions by a gap in the series of ions corresponding to the remote site fragmentation reactions (Figure 6). Long-chain keto acids that are cationized with alkali metal ions (e.g., Li^+ or Na^+) also underwent charge-remote fragmentation reactions (high energy) that yield the characteristic gap indicating the position of the keto group from the two abundant ions.[34] This gap corresponded to the carbon−carbon bond β-cleavage (cleavage between the keto group and the carboxyl moiety) and carbon−carbon bond γ′-cleavage (between the keto group and the methyl terminus) (Scheme 7).

Isomeric keto acids derived from arachidonic acid have been studied by electrospray ionization[35] and found to yield abundant $[M - H]^-$ carboxylate anions. Low-energy CID produced ions corresponding to the loss of H_2O, loss of CO_2, and loss of H_2O and CO_2 for all species studied. However, abundant fragment ions were also observed that were quite specific for each isomer of the keto-eicosatetraenoic acids. Again, mechanisms of ion formation likely involved alkoxide anions after ketol−enol tautomerization and fragmentation mechanisms largely driven by the anionic site on this structural moiety.

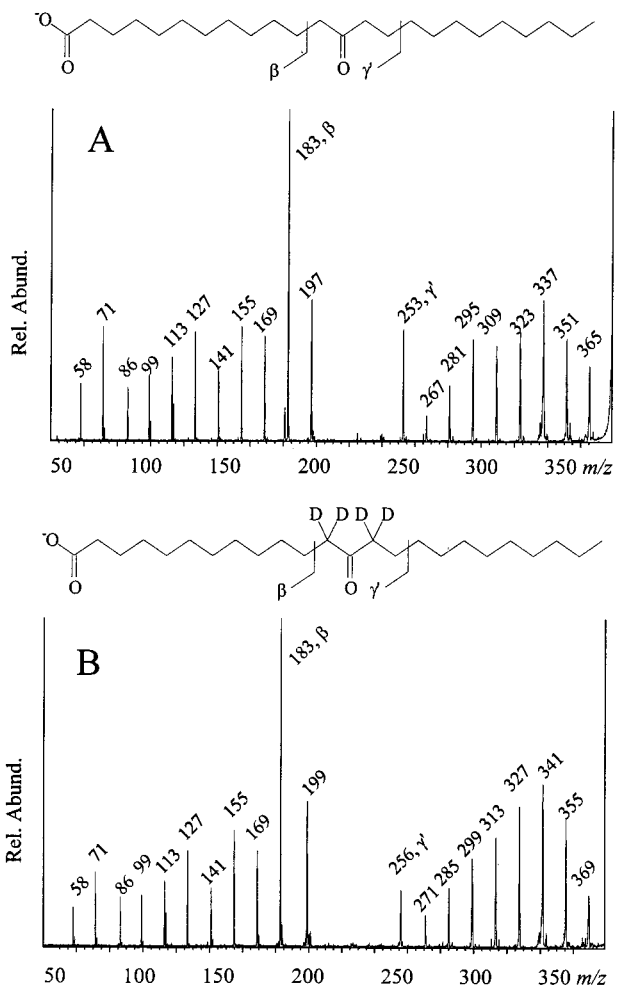


Figure 6. Collision-induced decomposition of carboxylate anions derived from (A) 13-oxatetracosanoate (*m/z* 381) and (B) 13-oxotetracosanoate-12,12,14,14-d_4 (*m/z* 385). (Reprinted with permission from ref 33. Copyright 1998 Elsevier Science.)

2. Hydroperoxy Fatty Acids

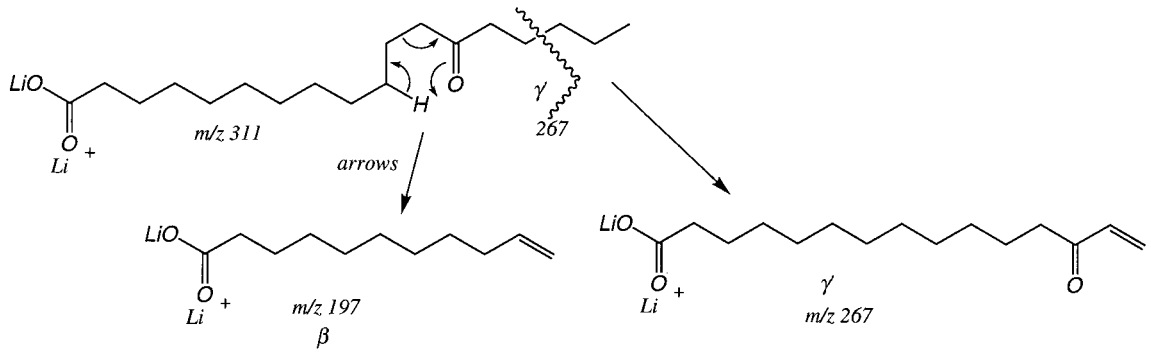
Enzymatic as well as free radical oxidation of polyunsaturated fatty acids leads to the formation of hydroperoxy acids. Despite the central biochemical importance of this class of oxidized lipids, the hydroperoxy fatty acids have been intractable to analysis by mass spectrometry prior to development of electrospray ionization. Abundant $[M - H]^-$ carboxylate anions were observed for three isomeric hydroperoxyeicosatetraenoic acids[34] that decomposed by an initial loss of neutral H_2O to a series of product ions. This $[M - H - H_2O]^-$ carboxylate anion was found to behave identically to the $[M - H]^-$ carboxylate anion derived from the corresponding keto-eicosatetraenoic acids[35] following collisional activation. Stable isotope-labeling studies supported the formation of an oxo intermediate which underwent identical decomposition reactions observed for the keto acids.[35]

Fatty acid hydroperoxides from linoleic acids (9- and 13-hydroperoxyoctadecadienoic acid) formed abundant $[M + NH_4]^+$ when electrosprayed in the presence of 5 mM NH_4OAc.[36] Collisional activation of these cations yielded characteristic ions for each regioisomer (Scheme 8).

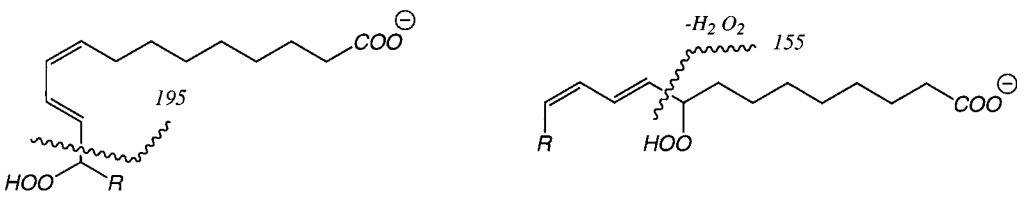
3. Expoxy Fatty Acids

Epoxyeicosatrienoic acids (EETs) derived from the reaction of arachidonic acid with specific cytochrome P-450 isozymes[37] are isobaric to hydroxyeicosatetraenoic acids and display many of the general CID reactions observed for these latter compounds. Interestingly, the most abundant product ions formed during low-energy CID of $[M - H]^-$ carboxylate anions were similar to those ions derived from the two corresponding HETEs. For example, CID of 11,-12-EET results in two abundant ions at *m/z* 167 and 179, which were abundant product ions from 11-HETE and 12-HETE.[38] These similar product ions make it necessary to employ some chromatographic separation to unambiguously HETE and EET species within biological tissues.[38] Simple flow injection and multiple reaction monitoring would detect both HETE and EET as the same species.

Scheme 7



Scheme 8



4. Nitrated Fatty Acids

The analysis of the products of the reaction of polyunsaturated fatty acids with reactive oxides of nitrogen has been carried out with electrospray ionization. Peroxynitrite and NO_2 were found to nitrate methyl linoleate to form a covalent adduct as characterized by a $[M - H]^-$ anion at *m/z* 324 (39). Collisional activation of this carboxylate anion yielded an abundant *m/z* 46 ($[NO_2]^-$), which is quite characteristic of the nitrated fatty acids. The facile formation of *m/z* 46 from nitrated fatty acids sets up a unique precursor−product relationship and a powerful tool to detect formation of such species within biological samples. The products obtained from the reaction of nitrogen dioxide with methyl linoleate (18:2) and linolenate (18:3) have been characterized using negative-ion chemical ionization because of the facile formation of the molecular anion by this ionization process.[40,41]

D. Eicosanoids

Arachidonic acid plays a unique role in eukaryotic biochemistry. This 20-carbon polyunsaturated fatty acid is the substrate for several enzymatic oxidative reactions that convert arachidonic acid into a diverse family of biologically active metabolites including prostaglandins, leukotrienes, lipoxins, EETs, and thromboxanes. These molecules are synthesized within cells in response to various stimuli, released, and then activate nearby cells to initiate other biochemical reactions. Thus, these molecules serve an important role as chemical communicators of cellular activation, and as such they have been the focus of numerous studies of biosynthesis, metabolism, and action. To measure eicosanoids, mass spectrometry has emerged as the *sine qua non* technique for the analysis of these molecules. Much of the work has in the past and continues to involve derivatization of the eicosanoid to a volatile compound that can be analyzed by gas chromatography/mass spectrometry. Recent reviews provide excellent insight into the techniques employed in mass spectrometry to analyze volatile derivatives of eicosanoids.[42,43]

1. Prostaglandins

Prostaglandins are arachidonate metabolites derived from cyclooxygenase-dependent oxidation leading to the formation of a cyclopentane fatty acid with three oxygen substituents and two double bonds. The designation of the prostaglandin (PG) as $PGF_2\alpha$, PGE_2, and PGD_2 refer to the exact arrangement of oxygen atoms at carbons-9, -11, and -15 and are the major biologically active prostaglandins. In addition to this, there are numerous secondary metabolites derived from these initially formed prostaglandins. As hydroxylated, unsaturated carboxylic acids, prostanoids yield abundant carboxylate anion during electrospray ionization. While there have been no detailed studies of the mechanisms involved in CID of these carboxylate anions generated by electrospray ionization, there has been a study of CID of the carboxylate anion as well as the positive ions generated from barium carboxylate salts using fast atom bombardment ionization.[44] Comparison of fast atom bombardment to electrospray ionization did reveal quantitative differences in product ion yields, suggesting differences in ion internal energy and, therefore, pathways available for unimolecular decompositions. Nonetheless, the major product ion species observed in fast atom bombardment ionization were also observed as major ions in the CID of these closed-shell ions generated by electrospray ionization. Margalot, Duffen, and Isaacson[45] used the unique product ions derived from CID of the carboxylate anion from 12 different eicosanoids as the basis for MRM analysis of these eicosanoids in biological fluids. When a deuterium-labeled eicosanoid was used as the internal standard, as little as 1.6 ng/mL injected into the electrospray ionization source by flow injection (corresponding to a 5 μL injection containing 8 pg) could be quantitated. To adequately measure closely related prostaglandins such as PGE_2 and PGD_2, chromatography (e.g., reversed-phase HPLC) is necessary and typically employed for most biological samples. Separation of the prostaglandins permits use of common product ions for MRM analysis with retention time as the important identifying parameter. Tandem mass spectrometry has also been used for the analysis of thromboxane B_2 and PGE_2 by monitoring the specific decompositions *m/z* 369 → 169 and *m/z* 339 → 197, the latter corresponding to the transition observed for the deuterated internal standard.[46]

Analysis of the abundance of the carboxylate anion $[M - H]^-$ has also been used as the basis of a quantitative assay PGE_2 in biological extracts.[47] An important feature of these quantitative assays was the use of a deuterium-labeled internal standard in order to identify the eluting eicosanoid as well as permit accurate quantitative analysis through isotope dilution strategies. While some eicosanoids are isobaric, e.g., PGE_2 and PGD_2, the use of only $[M - H]^-$ abundance rather than a more specific precursor−product ion transition can be appropriate in defined biological systems when only one eicosanoid is produced. In fact, for many isolated cell studies, this is the case.

Prostaglandin E_2 has been the most extensively studied prostanoid by tandem mass spectrometry. Various deuterium- and oxygen-18-labeled analogues were investigated in order to gain insight into unimolecular reaction pathways of decomposition. The MS/MS mass spectrum of PGE_2 is shown in Figure 7 .[48] The major loss of water from the carboxylate anion was consistent with dehydration to form a cyclopentenone (PGA_2) followed by a 1,4-proton rearrangement prior to loss of the methyl terminus as $C_6H_{11}O$ generating the ion at *m/z* 233. The final loss of CO_2 most likely resulted in a stabilized carbon-centered anion *m/z* 189. The CID of the carboxylate anion of PGE_2 generated by fast atom bombardment showed a very similar behavior with the most abundant carbon−carbon cleavage ion corresponding to *m/z* 233.[44] Similar behavior of PGE_2 has also been observed with in-source ion decomposition during the ionization process through elevation of the cone voltage of the electrospray ionizer. This technique

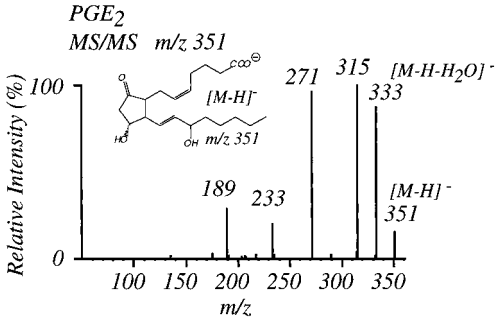


Figure 7. Collision-induced decomposition of the carboxylate anion derived from prostaglandin E_2 (PGE_2) in a tandem quadrupole mass spectrometer. (Adapted with permission from ref 48. Copyright 1997 Academic Press.)

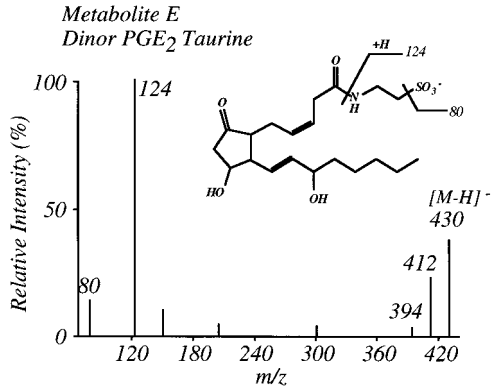


Figure 8. Collision-induced decomposition of the dinor-PGE_2 taurine conjugate (*m*/*z* 430) in a tandem quadrupole mass spectrometer. (Reprinted with permission from ref 48. Copyright 1997 Academic Press.)

was used to identify PGE_2 as a product of red algae in gracaleria asiatica.[49] An identical mass spectrum was obtained through CID of the $[M - H]^-$ ion in the tandem quadrupole instrument to that obtained through in-source decomposition.

The effect of altering functional groups on the PGE_2 structure is seen with the metabolite dinor-PGE_2 taurine[48] (Figure 8). Very different decomposition pathways now become prominent with the ions largely derived from decompositions of taurine in formation of the aminoethane sulfinic acid anion (*m*/*z* 124).

An alternative approach to the analysis of PGE_2 was the formation of the 4-aminobenzenesulfonate derivative (ABS) in order to direct CID of the eicosanoid in unique ways.[50] As shown in Figure 9, formation of the ABS derivatives of PGE_2 and $PGF_{2\alpha}$

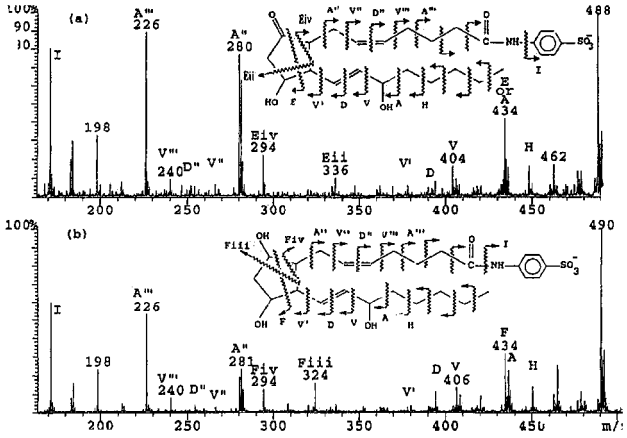


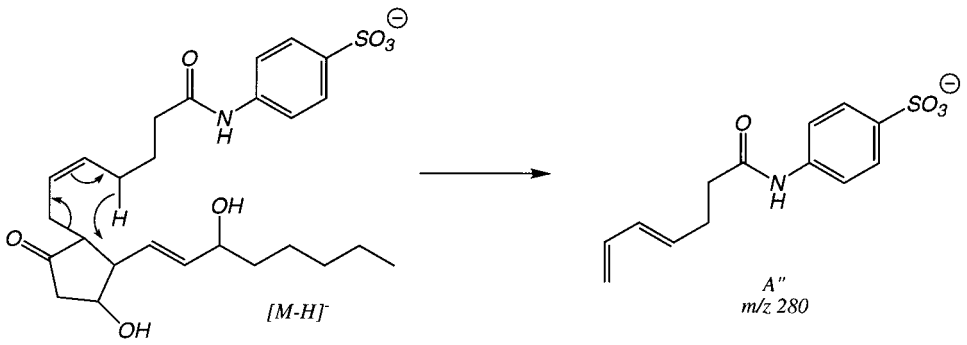
Figure 9. Product ions formed following the collisional activation of $[M - H]^-$ from the 4-aminobenzenesulfonate derivative of (A) PGE_2 (*m*/*z* 506) and (B) prostaglandin $F_{2\alpha}$ (*m*/*z* 508). The stereochemistry of the parent ions is not shown. (Reprinted with permission from ref 50. Copyright 1995 John Wiley & Sons.)

followed by continuous flow negative-ion fast atom bombardment mass spectrometry and CID yielded a rich population of product ions useful for the structural analysis of these eicosanoids. Yang et al.[50] suggested that many of the product ions were derived from charge-remote fragmentations since the sulfonate anion would be quite stable and sterically hindered from interacting with the side chains of the prostanoid. A mechanism proposed for the formation of the abundant ion at *m*/*z* 280 from PGE_2 aminobenzenesulfonate derivative is shown in Scheme 9 as a specific example of a charge-remote fragmentation mechanism. Unfortunately, other prostaglandins were either not investigated or not reported as to whether isomers such as PGD_2 or PGE_2 could be distinguished by this derivatization approach.

2. Leukotrienes

Conjugated triene eicosanoid metabolites of the 5-lipoxygenase pathway of arachidonate metabolism include the leukotrienes (LT) which are formed by enzymatic processing of the chemically reactive intermediate leukotriene A_4 (LTA_4). Two important leukotriene family members are 5(*S*),12(*R*)-dihyroxy-6,8,10,14(*Z*,*E*,*E*,*Z*)-eicosatetraenoic acid (LTB_4) and 5(*S*)-hydroxy-6(*R*)-glutathionyl-7,9,11,14(*E*,*E*,*Z*,*Z*)-eicosatetraenoic acid (LTC_4). Related to the leukotrienes are lipoxins which are derived from LTA_4 by further 15-lipoxygenase processing. Gas chromatog-

Scheme 9



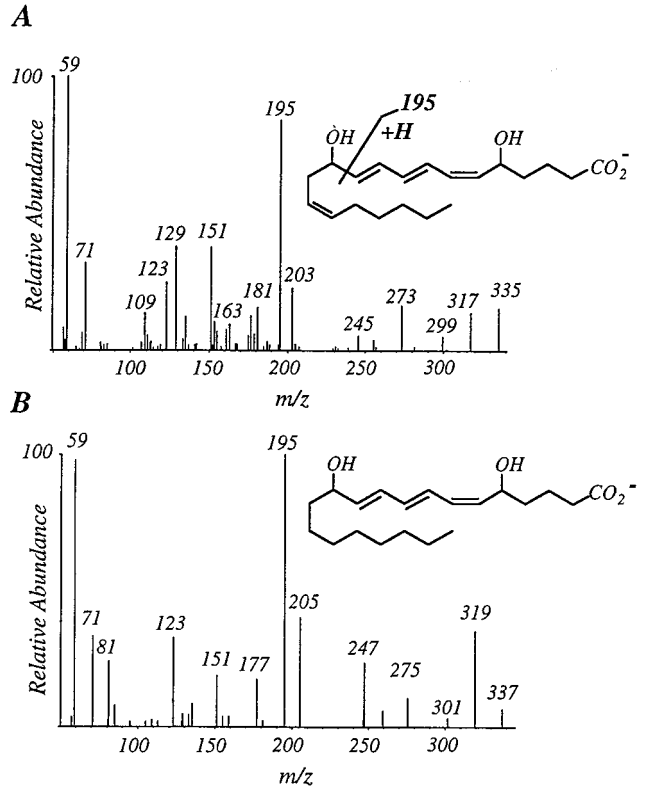


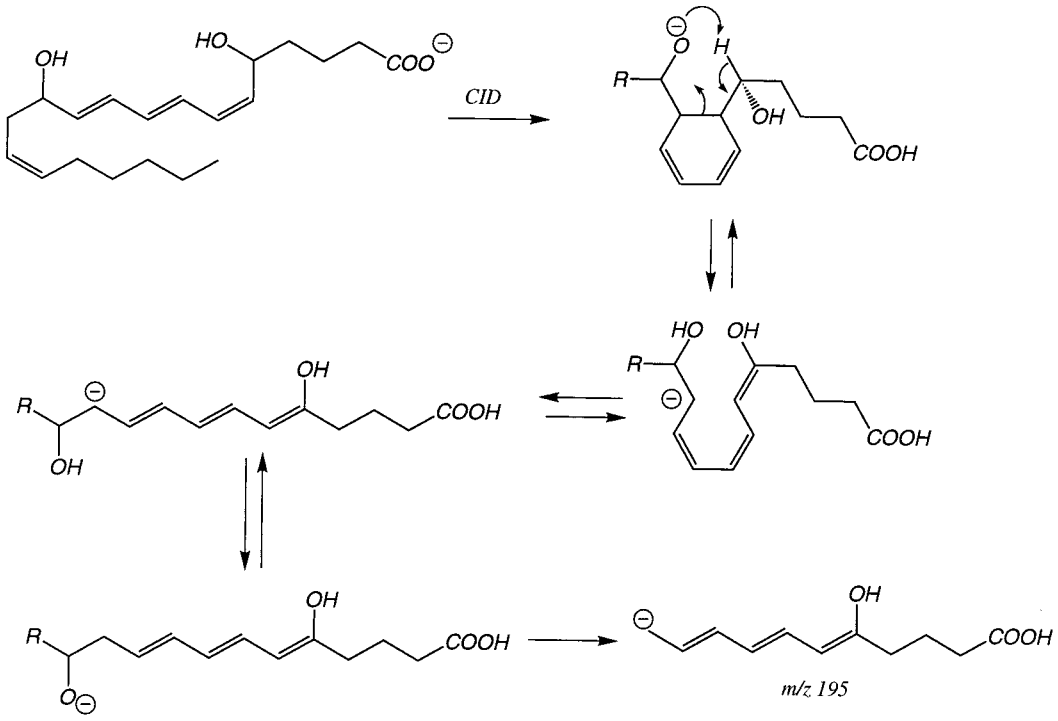
Figure 10. Collision-induced decomposition of the carboxylate anions derived from (A) LTB_4 and (B) 14,15-dihydro-LTB_4 in a tandem quadrupole mass spectrometer. (Reprinted with permission from ref 53. Copyright 1996 Elsevier Science.)

raphy/mass spectrometry continues to be a valued analytical approach for LTB_4, the lipoxins, and their metabolites because of the relative ease in formation of volatile derivatives.[42] However, neither LTC_4 nor the peptide cleavage products LTD_4 and LTE_4, can be derivatized for direct GC/MS analysis. Therefore, these cysteinyl leukotrienes have only been analyzed as intact, nonvolatile molecules by fast atom bombardment[51] and electrospray ionization.[52] Electrospray ionization yielded both abundant positive and negative ions from LTC_4, D_4, and E_4. LTB_4, lipoxins, and their metabolites yielded carboxylate anions by electrospray.[26,53]

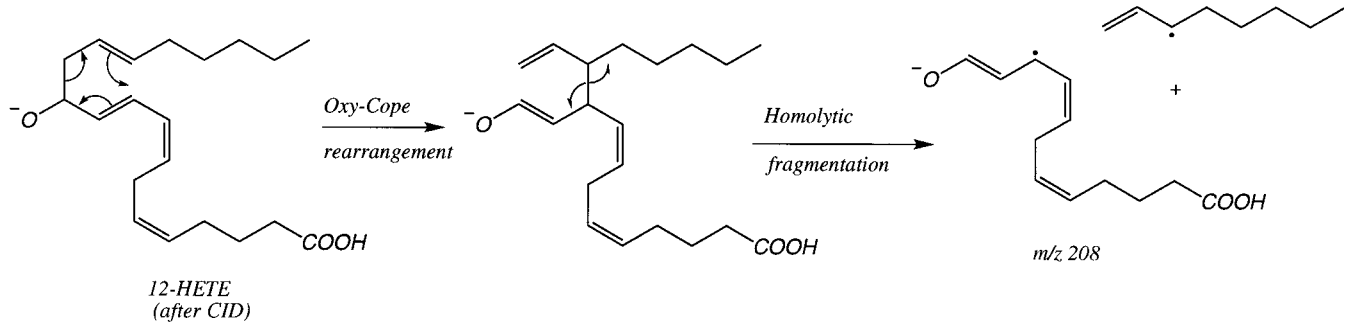
Collision-induced dissociation of the carboxylate anions derived from LTB_4 and other dihydroxy isomers yielded a large array of product ions (Figure 10). The most abundant energy product ions from low-energy CID appeared at *m/z* 195,[53] whose formation has been suggested to involve a complex double bond and proton rearrangement after a cyclization reaction (Scheme 10).

The mechanism responsible for the formation of this ion was supported by collisional activation experiments using deuterium- and oxygen-18-labeled isotopimers of LTB_4.[53] In general, the formation of major ions from collisional activation of the carboxylate anions of LTB_4 were consistent with α-hydroxy charge-remote as well as charge-driven α-hydroxy fragmentation mechanisms. Interestingly, the conjugated triene played an important role in decomposition reaction pathways, and the mass spectra of those metabolites of LTB_4 which were reduced to conjugated dienes were readily distinguished from normal conjugated triene structures as present in LTB_4. The formation of *m/z* 195 from *m/z* 335 was used to characterize 5,12-dihydroxyeicosatetraenoic acids isolated from the seaweed *G. asiatica*.[49] Although proposed to be LTB_4 in this report, the double-bond geometry of the products was not investigated and the appearance of two separate metabolites might suggest these products were derived from free radical oxidation of arachidonic acid present in the seaweed.

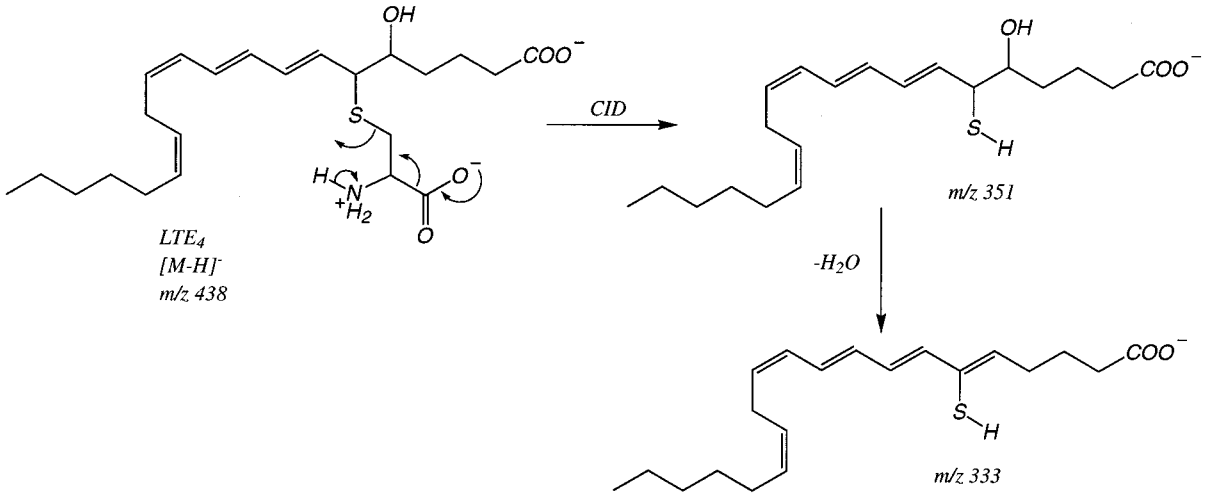
Scheme 10



Scheme 11



Scheme 12



Interestingly, high-energy CID (400 eV) of LTB_4 induced many of the same unimolecular decomposition pathways found in the low-energy CID experiment.[54] For example, the product ion *m/z* 195 was observed after collisional activation of *m/z* 335 derived from LTB_4 at both high and low energies, but the abundance of this ion compared to other decomposition product ions was dependent on the applied collisional energy. One interesting major difference in the higher energy CID for LTB_4 was the formation of abundant distonic ions, likely a result of an oxy-Cope rearrangement process which had been previously described for 12-HETE.[22] The overall mechanism proposed for 12-HETE involved localization of the negative charge as a carbon-12 alkoxide anion (Scheme 11) formed from the initial formed carboxylate anion. The corresponding radical ion at *m/z* 206 in the high-energy CID spectrum (*m/z* 224 − H_2O)[54] was also observed in the low-energy CID product ion spectrum of LTB_4 but at very low abundance.

The sulfur-atom-containing leukotrienes yield both abundant positive as well as negative ions because of the presence of free carboxylic acid moiety and a primary amino group. Most studies of the electrospray ionization of the sulfidopeptide leukotrienes have focused on CID of carboxylate anions. Leukotriene C_4 yields an abundant $[M - H]^-$ at *m/z* 624 under FAB ionization, which decomposes to *m/z* 272 in the tandem quadrupole MS/MS experiment.[51] This latter ion has been clearly shown to involve cleavage of the cysteinyl C−S bond with charge retention on the glutathione (peptide) portion of this eicosanoid in studies of CID of *m/z* 624 obtained by fast atom bombardment ionization.[51]

Leukotriene E_4 (5(*S*)-hydroxy-6(*R*)-cysteinyl-7,9,-11,14(*E*,*E*,*Z*,*Z*)-eicosatetraenoic acid) is both a biologically active leukotriene and urinary metabolite in man.[55] Electrospray ionization followed by low-energy CID tandem mass spectrometry has been used to measure the quantity of LTE_4 with a detection limit of 50 pg/mL in the urine of human subjects.[56] This stable isotope MS/MS assay was based upon the CID of *m/z* 438 $[M - H]^-$ (Figure 11) to form *m/z* 333.[52] Again, the mechanism of formation of this ion species was previously examined in the CID of the carboxylate anion of LTE_4 generated by fast atom bombardment ionization.[57] The formation of *m/z* 333 appeared to involve a charge-driven elimination of the cysteinyl side chain through a β-fragmentation-type mechanism[57] (Scheme 12). Mizukaki et al.[58] described use of this same approach to analyze LTE_4 in human subjects also using stable isotope dilution techniques.

3. Lipoxins

Collisional activation of conjugated tetraene lipoxins also yielded abundant fragment ions in the tandem quadrupole instrument. Many of the fragmentation processes likely involved α-hydroxy cleavage with a prior double-bond rearrangement event as previously described for LTB_4.[26,54] Since lipoxins contain a vicinal diol, abundant ions likely resulted from cleavage between the two carbinol groups with charge retention on the fragment retaining the carboxylate moiety.[54]

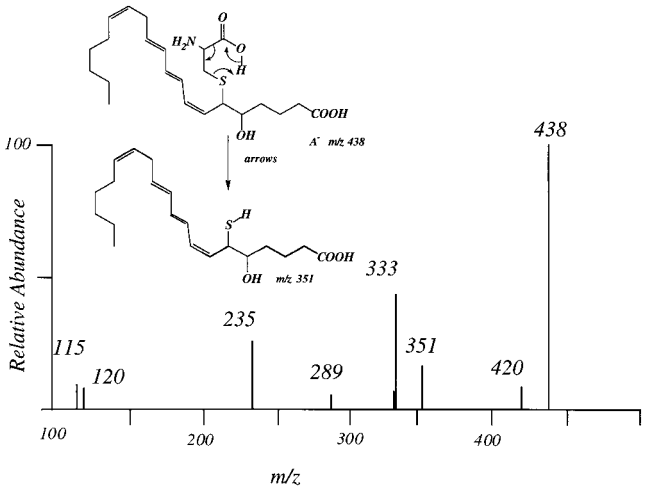


Figure 11. Product ions obtained following the collisional activation of the $[M - H]^-$ from leukotriene E_4 in a tandem quadrupole mass spectrometer. Precise stereochemistry of leukotriene E_4 is not indicated in the structure. (Reprinted with permission from ref 57. Copyright 1991 Elsevier Science.)

4. Isoprostanes

In the early 1990s, it became apparent that prostaglandin-like hydroxy polyunsaturated fatty acids were present in biological tissues that were not formed by enzymatic processes but rather via free radical propagation through arachidonoyl acyl groups esterified to membrane glycerophospholipids.[59,60] A surprising high yield of oxidized arachidonate isomers, which were structurally similar to the prostaglandin $F_{2\alpha}$ in that they contain a cyclopentane ring with two hydroxyl substituents, resulted from the reaction of bisallylic carbon-centered radical intermediates with two molecules of molecular oxygen (Scheme 13). A total of 64 possible F_2-isoprostane isomers may result from such reactions. These products remain esterified to glycerophospholipids[60] and can be liberated either after ester hydrolysis in vivo (phospholipase A_2) or in vitro after saponification.

Interest in these molecules has been severalfold, from an understanding of free radical processes in biology, to the measurement of markers of lipid peroxidation, and to studies concerning the formation of biologically active products from free radical processes. Free radical metabolism of arachidonic acid also resulted in the formation of D-ring and E-ring regioisomers of prostaglandins.[61] Mass spectrometry has been virtually the only technique available for the analysis of these molecules, largely because isoeicosanoids exist in biological extracts as complex mixtures of closely related isomeric molecules. Many of the investigations of these molecules have involved the use of gas chromatography/mass spectrometry after the formation of volatile derivatives.[62,63] Negative-ion chemical ionization mass spectrometry has been particularly valuable because of the inherent sensitivity of this technique, and quantitative assays have been developed with limits of detection of a few picograms (fmols) injected on column.

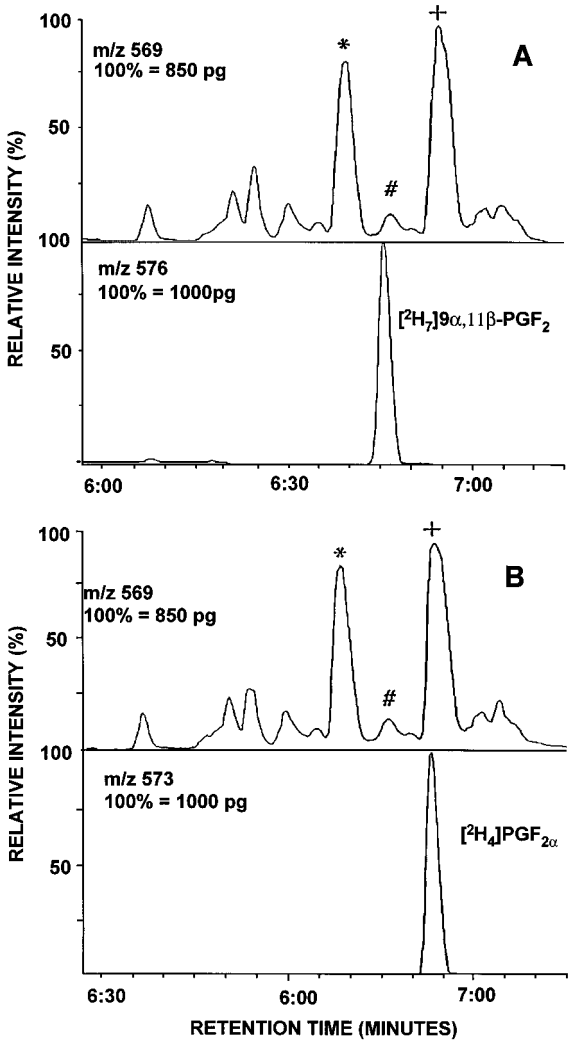
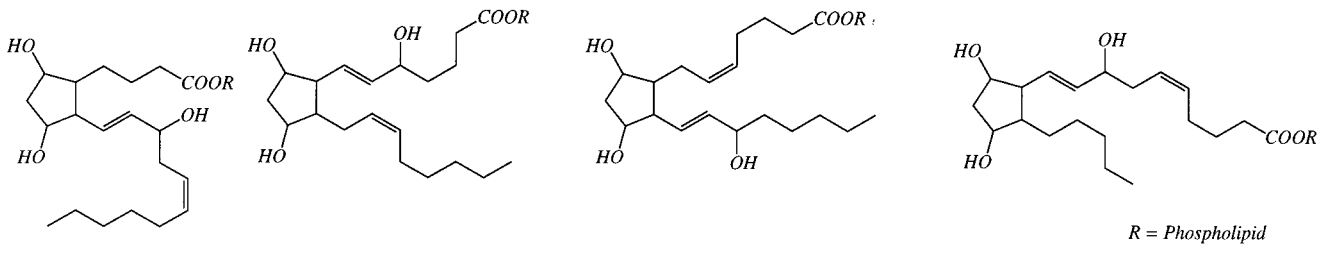


Figure 12. GC/MS analysis of F2-isoprostanes isolated from rat plasma following derivatization to the pentafluorobenzyl ester trimethylsilyl ether derivative followed by selected ion recording (SIM). Analysis of F_2-isoprostanes in plasma obtained from a rat following a 2 h treatment with CCl_4 (2 mL/kg oral gastrically) to produce endogenous lipid peroxidation. (A) The SIM for *m/z* 569 represents the elution of endogenous F_2-isoprostanes, and the SIM for *m/z* 576 corresponds to $[^2H_7]9\alpha,11\beta$-$PGF_{2\alpha}$. (B) Analysis of endogenous F_2-isoprostane (*m/z* 569) using $[^2H_4]PGF_{2\alpha}$ as internal standard (*m/z* 573). (Reprinted and adapted with permission from ref 64. Copyright 1994 Academic Press.)

Scheme 13



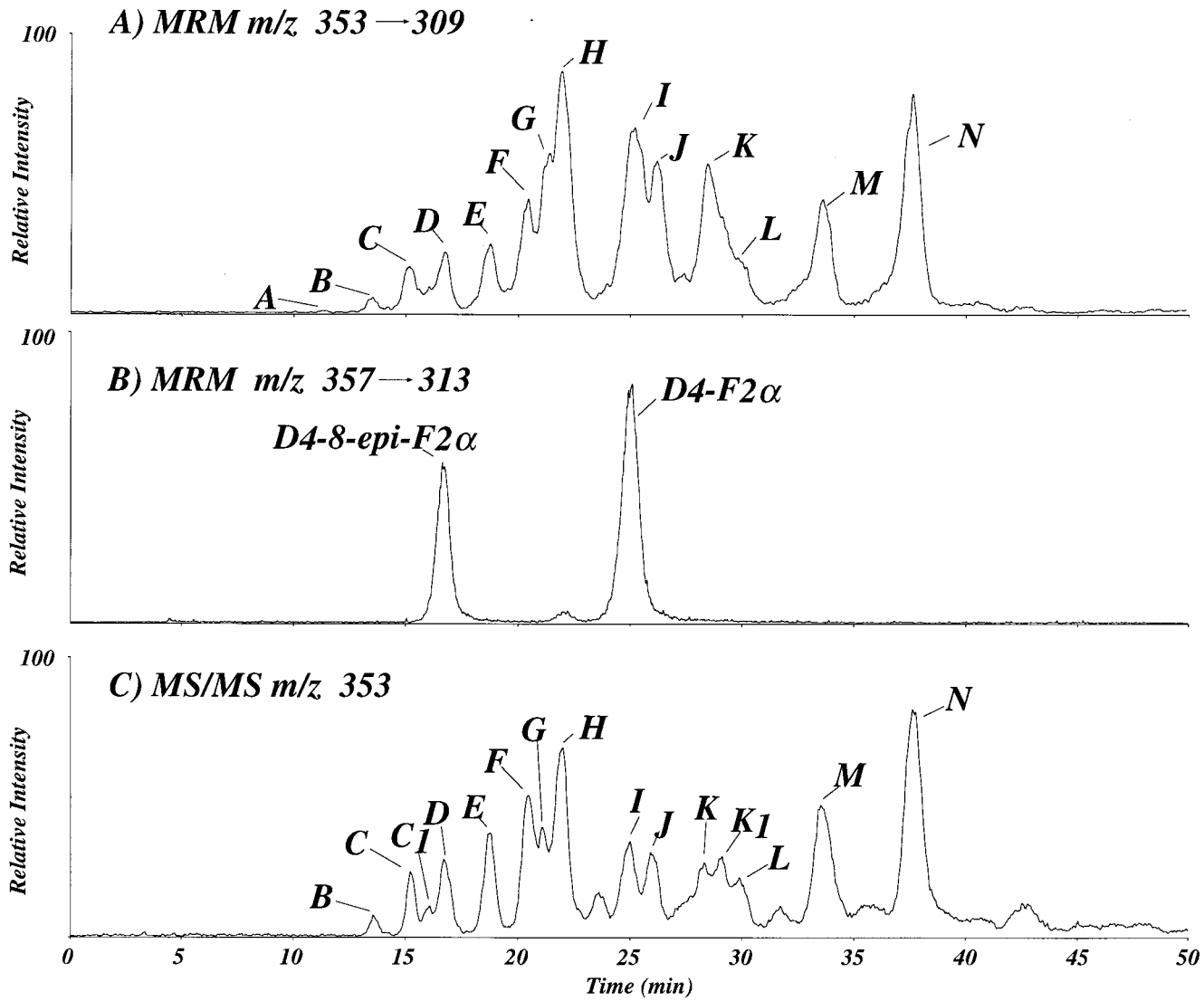


Figure 13. LC/MS/MS assay for isoprostanes present in an extract of liver from rats treated identical to the method in Figure 12. (A) Multiple reaction monitoring (MRM) for the transition 353 (carboxylate anion of F_2-isoprostanes) to *m/z* 309, corresponding to the unique loss of C_2H_4O (44 u). (B) MRM for the corresponding deuterated internal standards, *m/z* 357 → 313 for d_4-8-epi-$PGF_{2\alpha}$ and d_4−$F_{2\alpha}$. (C) Total ionization obtained in the collisional activation of *m/z* 353. (Reprinted with permission from ref 66. Copyright 1997 Elsevier Science.)

Of equal importance is the fact that capillary gas chromatography plays a critical role in partial separation of the closely related molecules. As shown in Figure 12, the derivatized isoprostanes eluted from the capillary gas chromatographic column over a wide range but not with clear separation of each isomer.[64] Nonetheless, quantitative analysis has successfully been carried out using deuterium-labeled isoprostane 8-epi-$PGF_{2\alpha}$ or deuterium-labeled $PGF_{2\alpha}$ added as internal standard (Figure 12). Negative-ion chemical ionization mass spectrometry of the pentafluorobenzyl ester trimethylsilyl ether derivatives resulted in the formation of essentially a single carboxylate anion of high abundance which was used as a measure of the quantity of the isoprostanes. Two alternative strategies have been employed: one specifically identified only 8-epi-$PGF_{2\alpha}$ from coelution with the deuterium-labeled internal standard, and a second involved integration of the entire envelope of isoprostanes eluting from the gas chromatographic column. Both techniques appear to be valid and useful.

Detailed studies of the electron ionization mass spectra of the pentafluorobenzyl ester trimethylsilyl ether derivative were able to clearly detect and differentiate three of the four regioisomeric F_2-isoprostane family members eluting from the GC based upon cleavage adjacent to the trimethylsilyl ether substituent.[65]

Electrospray ionization mass spectrometry has also been of value for the quantitative and qualitative analysis of F_2-isoprostanes. Numerous regioisomers and F_2-isoprostanes were separated by reverse-phase HPLC as shown in Figure 13.[66] Tandem mass spectrometry of individual components present in separated HPLC fractions revealed the elution of specific F_2-isoprostanes having unique CID mass spectra consistent with the four expected regioisomeric family members as shown in Figure 14. Specific cleavages were largely driven by α-cleavage to the hydroxyl

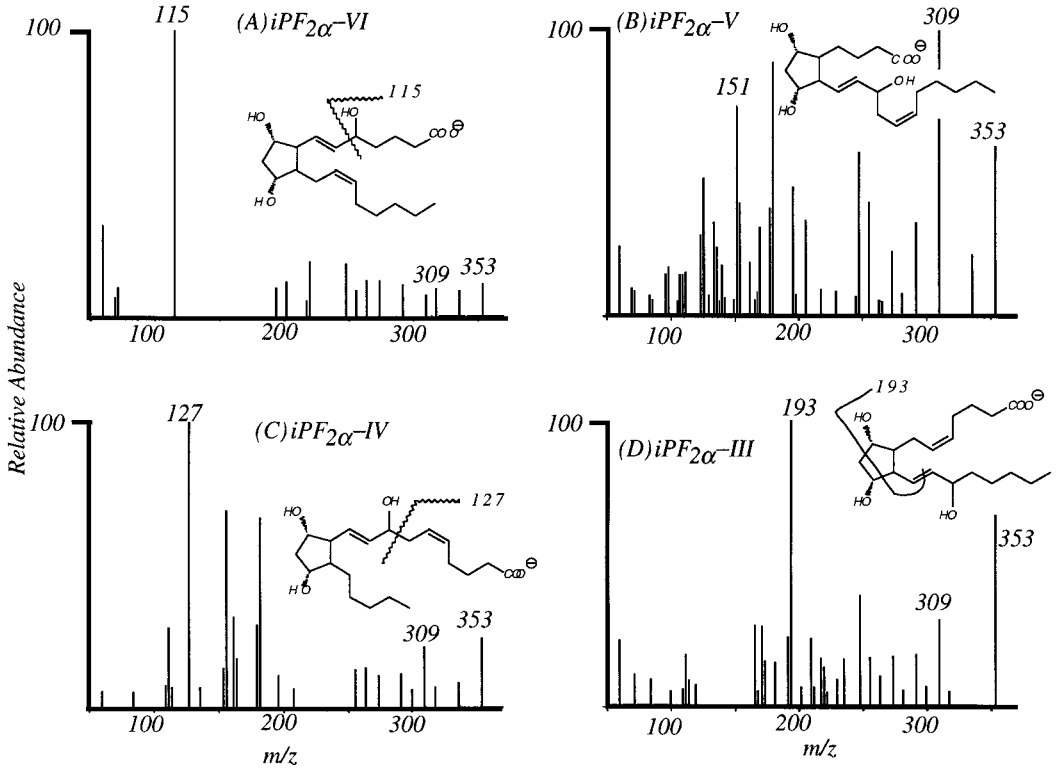


Figure 14. Product ion spectrum of representative members of the four classes of F_2-isoprostane regioisomers. Each regioisomer is indicated in the tandem mass spectrum of the collisional activation of *m*/*z* 353. (Adapted with permission from ref 67. Copyright 1998 American Society for Biochemistry and Molecular Biology.)

group closest to the carboxylic acid moiety. Characteristic product ions such as *m*/*z* 193, 115, 127, and 151 were used to specifically identify regioisomeric family members present in the complex mixture of isoprostanes formed by free radical reactions.[66,67] A specific assay for the regioisomeric F_2-isoprostanes based on these observations has recently been reported and compared to an enzyme immunoassay.[68,69]

The metabolism of one isoprostane, viz., 8-epi-$PGF_{2\alpha}$ has been studied in animals and human subjects.[70] Several metabolites have also been identified, including 2,3-dinor-8-epi-$PGF_{2\alpha}$ as a major endogenous F_2-isoprostane metabolite as well as 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. The structures of these metabolites were determined by GC/MS using the pentafluorobenzyl ester trimethylsilyl ether derivative.[71] One F_2-isoprostane metabolite excreted in human urine in high yield was characterized by mass spectrometry as one derived from the regioisomers with a 5-hydroxyl moiety as a unique structural feature.[72] The approximately 5- to 20-fold higher abundance of this F_2-isoprostane metabolite suggested that the 5-hydroxy group imparted a degree of metabolic stability not inherent in the other isoprostane regioisomers.

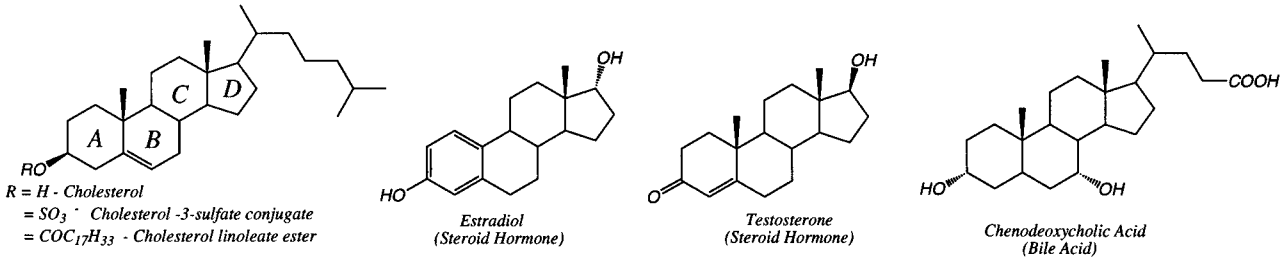
An even more complex distribution of products structurally related to the isoprostanes resulted from the free radical oxidation of docosahexaenoic acid.[73] These molecules, termed neuroprostanes, were logically formed in tissues having a high abundance of docosahexaenoic acid such as the central nervous system. Electrospray ionization mass spectrometry has been used to structurally characterize the D- and E-isoprostanes derived from docosahexaenoic acid.[74]

Another interesting eicosanoid recently identified and structurally characterized by mass spectrometry has been termed anandamide (arachidonoyl ethanolamide). This unique lipid was identified in brain extract by mass spectrometry as an endogenous cannabinoid receptor ligand.[75] Mass spectrometry, either using LC/APCI/MS[76] or electrospray LC/MS/MS,[77] has also been used to measure this eicosanoid in biological extracts.

III. Steroids

The steroids are a diverse class of lipids derived from the cyclization of squalene 2,3-epoxide resulting in a characteristic fused four-membered ring system. Cholesterol is the precursor of a large number of other related molecules including bile acid, steroid hormones (e.g., testosterone and estradiol), steroid esters, and polar steroid conjugates (sulfate esters and glucuronide conjugates) (Scheme 14). Mass spectrometry has been used for over four decades to study the structure of known and unknown steroids, largely because of the innate volatility of a large number of steroids or the ready formation of volatile derivatives suitable for electron ionization GC/MS.[78] In general, such steroids yield a complex distribution of ion fragments during electron ionization; however, some processes are quite prominent, for example, an abundant ring-D cleavage ion.[79] In many cases, complex hydrogen-atom transfer reactions take place that further complicate a clear understanding of the decomposition of these important biologically derived lipids. This ion chemistry involved in the decomposition of steroids during electron ionization has been the subject of several reviews.[80−82] The direct analysis

Scheme 14



of nonvolatile steroids, steroid esters, bile acids, and polar steroid conjugates, which were not amenable to electron ionization mass spectrometry, had to await the emergence of desorption ionization techniques, such as fast atom bombardment, electrospray ionization, and MALDI. These developments have added important new avenues for steroid analysis.

A. Neutral Steroids

Both electrospray and atmospheric pressure chemical ionization (APCI) have been used to qualitatively and quantitatively analyze steroids including both naturally occurring and synthetic analogues as well as their metabolites. For example, the qualitative study of corticosterone metabolism employed LC/MS and detection of metabolite $[M + H]^+$ formed by electrospray ionization.[83] Electrospray ionization yielded a family of additional ions, including those corresponding to the loss of one and two molecules of water as well as alkali adduct ions. APCI does not yield these ions but rather only $[M + H]^+$ or $[M - H]^-$ ions.[84] Picomole quantities of corticosterone in serum were quantitated using microbore RP-HPLC and electrospray ionization with detection of $[M + H]^+$.[85] APCI was successfully employed in an isotope dilution quantitative assay for pregnenolone and its long-chain ester pregnenolone-3-sterate.[86] Studies of the decomposition of ions formed during APCI in response to changes in collision energy in the ion source have suggested energy-resolved CID as a method to distinguish isomeric steroids including digitoxigenin and androstanediols.[87] Decomposition of testosterone in the ion source has also been investigated by ESI.[88] The relatively low ion yield during electrospray ionization of neutral steroids, nonetheless, remains one of the limiting features of this technique; however, the application of capillary electrochromatography and nanospray techniques was demonstrated at the femtomole level with several neutral steroids including androstanenone, hydrocortisone, and dexamethasone.[89]

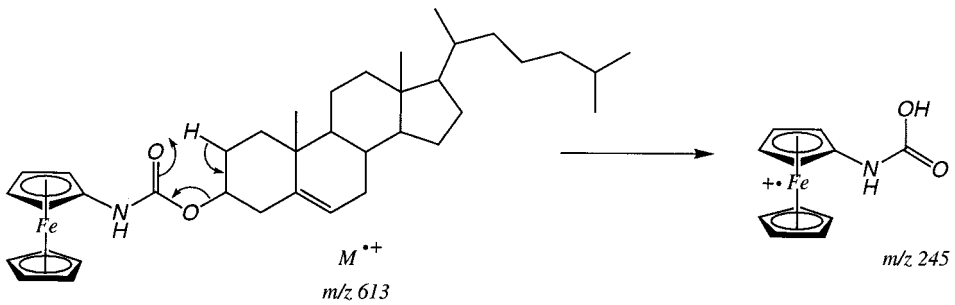
An alternative approach to increase the sensitivity of electrospray ionization for neutral steroids has been to make the ferrocenoyl carbamate ester of the neutral steroid such as cholesterol or stigmasterol (Scheme 15).[90] The ferrocene derivative was readily ionized by oxidation during the electrospray ionization process by electrolysis inherent in this technique. The major ion observed was a radical cation $[M^{+\bullet}]$ which lost cholesterol to form ionized ferrocene carbamic acid at *m/z* 245. Quantities as low as 2 fg could be detected using the transition *m/z* 613 → 245 in flow injection analysis.[90]

B. Steroid Conjugates

In contrast to the relatively low yield of either positive or negative ions during direct electrospray ionization of neutral steroids, the analysis of steroids conjugated to sulfuric acid, taurine, and glucuronic acid has been quite successful.[91] All of these conjugated steroids contain functional groups that are typically ionized in the electrospray buffer solution. While these are quite amenable to ESI-MS, they could not be directly studied by other techniques of mass spectrometry such as electron or chemical ionization. In an interesting reversal of strategy for a quantitative mass spectrometric assay, cholesterol was converted to a nonvolatile cholesterol-3-sulfate using a sulfur trioxide−pyridine complex for an ESI-based assay.[91] Using nanoelectrospray and a tandem quadrupole mass spectrometer, CID of *m/z* 465 $[M - H]^-$ and detection of *m/z* 97 (HSO_4^-) enabled the quantitation of 22.3 ± 1.5 fmol/cell cholesterol from as little as 1000 cells.[91] Other steroids, including ergosterol and stigmasterol, were also detected by this derivatization to sulfate esters.

Detailed studies of the CID of $[M - H]^-$ anions derived from sulfuric acid ester conjugates have been carried out under high-energy collisional activation.[92] Even though this work was done with fast atom bombardment ionization, the decomposition of the $[M - H]^-$ ions were similar to that observed when

Scheme 15



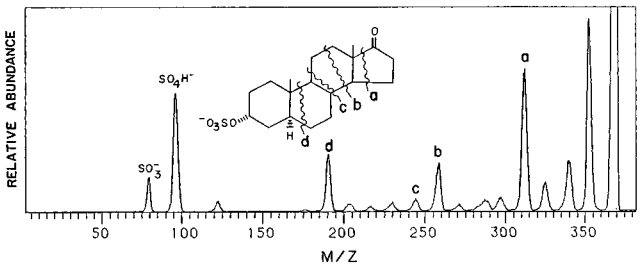
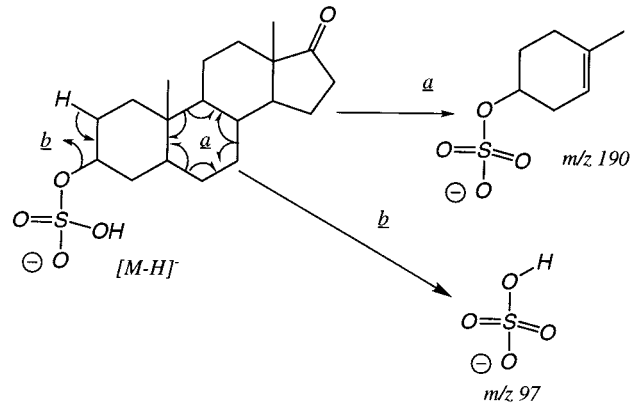


Figure 15. Product ions following collisional activation of $[M - H]^-$ ion of 5α-androstane-3α-al-17-one-sulfate obtained in a high-energy three-sector mass spectrometer. The observed mass-to-charge ratios for a, b, c, and d were *m/z* 191, 245, 259, and 313, respectively. (Reprinted with permission from ref 92. Copyright 1988 John Wiley & Sons.)

Scheme 16

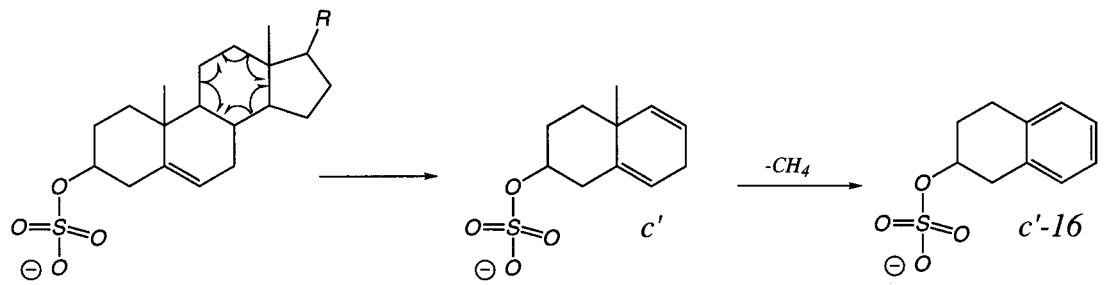


electrospray ionization was used to generate $[M - H]^-$. In general, fragmentation reactions remote to the charge site were observed in these FAB studies by Tomer and Gross.[92] Over 35 different steroid conjugates were investigated with an archetypal product ion spectra seen from 5α-androstan-3α-ol-17-one-sulfate (Figure 15). In this instance, product ions were observed as indicated in the figure as cleavage of the B-ring (*m/z* 190, pathway d) and to a lesser extent cleavage of the C-ring (pathways b and c) and one of the more abundant ions (a) corresponding to cleavage of the D-ring. The predominance of charge-remote fragmentation reactions was likely a result of this highly stable closed-shell sulfate anion which localized the charge at the carbon-3 (substituent) as well as the rigid steroid nucleus preventing this charge from interacting with other portions of the molecule. This being the case, it was not surprising that many of the collisional activation spectra of isomeric steroid sulfate esters were quite similar, making it somewhat difficult to distinguish among isomers such as 5α-cholestan-3α-ol-sulfate, 5α-cholestan-3β-ol-sulfate, and 5β-cholestan-3α-ol-sulfate. These high-energy CIDs also caused the characteristic loss of CH_4 following collisional activation of $[M - H]^-$, most likely as a result of loss of one of the angular methyl substituents after a hydrogen rearrangement reaction.[92] The electrospray-generated $[M - H]^-$ from 5α-androstan-3-ol-17-one sulfate decomposed in the tandem quadrupole to yield *m/z* 97 (HSO_3^-) as the most abundant ion, along with lesser abundant ions including the B-ring cleavage ion at *m/z* 190[93] (Scheme 16).

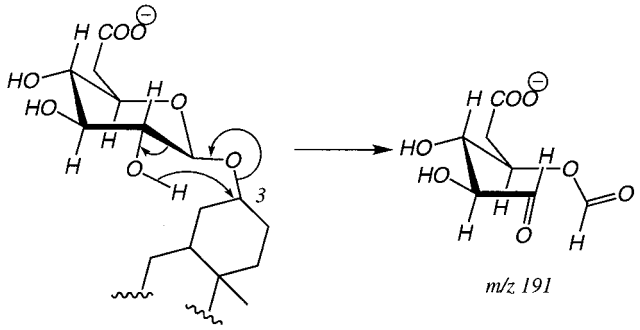
Sjövall and co-workers[94] recently reported an extensive study of the nanoelectrospray and collisional activation of mono- and disulfate esters of several steroids including pregnenolone, hydroxy androstenone, and their reduced metabolites. Using a hybrid magnetic section-orthoganol accelerated time-of-flight tandem mass spectrometer, electrospray-generated $[M - H]^-$ anions could be collisionally activated at 400 eV and product ion masses measured with high accuracy. Steroid ring cleavage reactions similar to those previously reported[92] were observed for cleavage of the B- and D-rings. Mechanisms for formation of these ions were studied using deuterium-labeled analogues. The most abundant product ions were observed at *m/z* 80 (SO_3^-) and *m/z* 97 (HSO_4^-), but abundant ions were also observed corresponding to cleavage of the C-ring. Most interesting was a C-ring cleavage ion with a further loss of 16 u (CH_4) designated by the authors as $'c_1 - 16$. The mechanism responsible for this ion (Scheme 17), where the final product ion formed either in a two-step or a concerted mechanism, was likely stabilized by the aromaticity of the B-ring.[94]

High-energy CID of the carboxylate anions derived from steroid glucuronides were found to be quite similar to that observed for CID of the steroid sulfate anions. Again, charge-remote fragmentations were readily observed. The glucuronic acid moiety did open several additional pathways for anion decomposition, and these ions gave important structural clues as to the site of glucuronidation. For the C-3 glucuronides, a fragment ion was observed at *m/z* 191 (Scheme 18); however, for the C-21 glucuronide this ion was observed at *m/z* 193 (Scheme 19). The charge-driven loss of 176 u is another characteristic of steroid conjugate ions (Scheme 20) and became the most abundant collision-induced product ion for aromatic steroids such as 17β-estradiol-3β-D-glucuronide[92] as well as testosterone glucuronide in the tandem quadrupole instrument.[93]

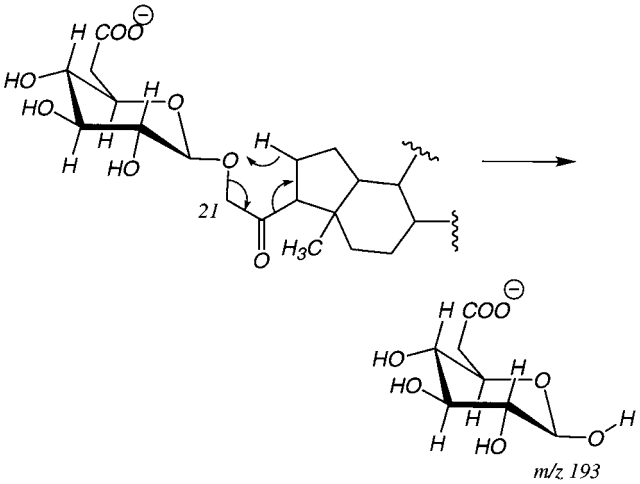
Scheme 17



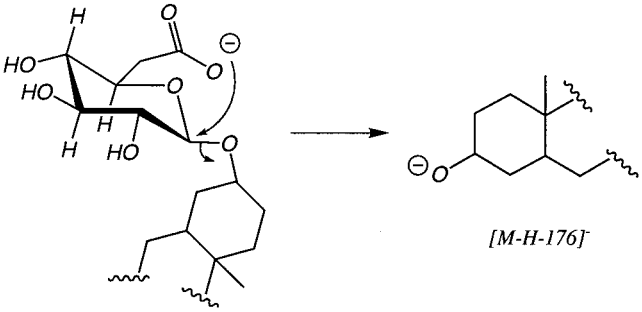
Scheme 18



Scheme 19



Scheme 20



A doubly conjugated cholesterol (24-hydroxy cholesterol-3-sulfate-24-glucuronide) was recently described in the extracts of urine from pediatric patients with cholestatic liver disease.[95] The high-energy tandem mass spectrometry of the $[M - H]^-$ anion formed by fast atom bombardment revealed many fragment ions and pathways both similar to as well as different than that observed for the singly conjugated sulfate esters or glucuronides (Figure 16). The most abundant product ion was a unique $[M - H - SO_3]^-$ observed at *m/z* 577, but the next most abundant ion was loss of 176 u from the glucuronide at *m/z* 481 previously described above as loss of the neutral glucuronide. A D-ring cleavage product ion was observed at *m/z* 311, and a B-ring cleavage ion was shifted to *m/z* 217 as expected for Δ^5-unsaturated steroid nucleus.[92] Again, these latter two decomposition pathways were likely charge site remote (Scheme 21).

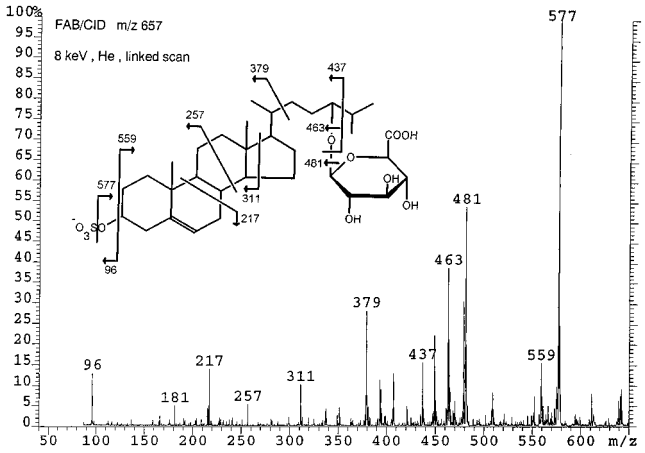
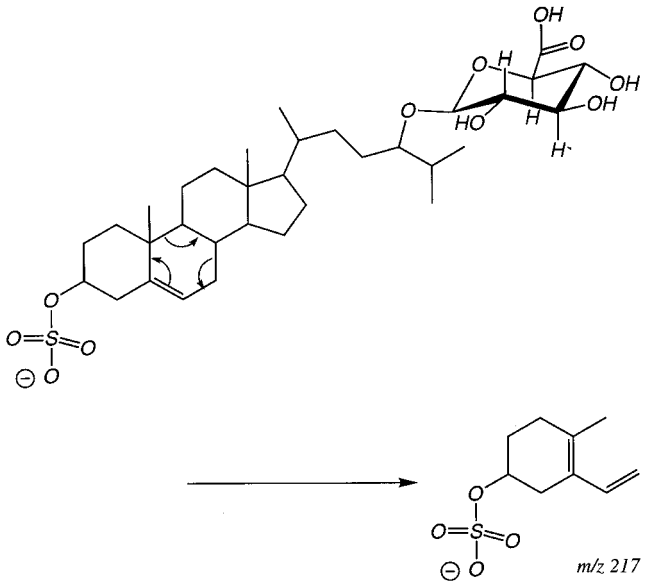


Figure 16. Product ions following collisional activation of $[M - H]^-$ (*m/z* 657) derived from the fast atom bombardment ionization of the sulfated and glucuronidated double conjugate of 24-hydroxy cholesterol. Fragment ions at *m/z* 559 are formed by the loss of H_2SO_4 and those at 481 and 463 by losses of $C_6H_8O_6$ and $C_6H_{10}O_7$, respectively. (Reprinted with permission from ref 95. Copyright 1997 Lipid Research, Inc.)

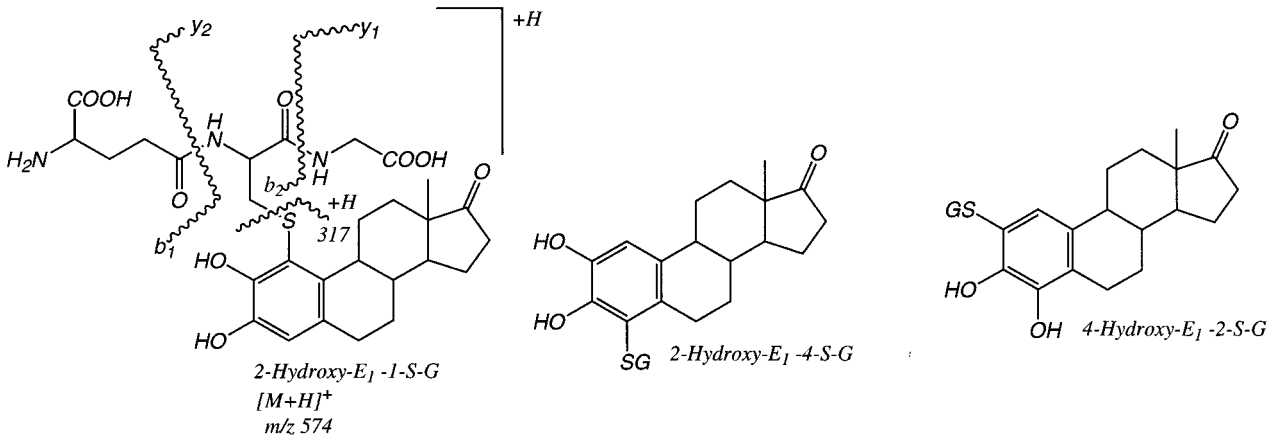
Scheme 21



Gross and co-workers[96] studied the collisional activation of electrospray-generated $[M + H]^+$ ions from three structural isomers of the glutathione (GSH) conjugate of estrone and estradiol under low-energy ion trap collisions, tandem quadrupole collision conditions, and high-energy collisional ion activation in a four-sector magnetic sector instrument. The major product ions were remarkably similar between the three highly different experimental collisional activation conditions; major ions were observed for the expected y_2 (*m/z* 463), b_2 (*m/z* 517), and a major ion species at *m/z* 317 due to cleavage of the glutathionyl C−S bond in the estrone series (Scheme 22).

While product ion spectra for these three isomeric molecules were different, the differences were subtle, making it difficult if not impossible to determine the site of GSH attachment to the aromatic A-ring of estrone if one did not have reference compounds.[96]

Scheme 22



C. Bile Acids

Bile acids are a large family of C_{24} and higher carboxylic acids biosynthesized from cholesterol and formally derivatives of cholanic acid. These compounds are synthesized in liver and released into the bile often after conjugation with either taurine or glycine. The first report concerning the mass spectrometry of nonconjugated bile acids was in 1960,[97] and the electron ionization mass spectrometric behavior of these acids as volatile derivatives has been reviewed.[98−99] Fast atom bombardment,[100,101] thermospray,[102] and, more recently, electrospray ionization[103] have enabled direct analysis of taurine and sulfate esters of bile acids that are typically encountered in vivo. Collision-induced decomposition of chenodeoxychoyltaurine $[M - H]^-$ ion (Figure 17) generated by fast atom bombardment ionization yielded characteristic steroid ring cleavage product ions rich with structural information.[104] Many of the observed fragmentation reactions were found to be charge remote.[101,104] This favorable decomposition behavior of taurine conjugates was the basis of a strategy to convert bile acids that were sulfated, glycine-conjugated, or glucuronidated into taurine esters using chemical esterification with taurine and 1-ethyl-3,3-dimethylaminopropylcarbodiimide in pyridine buffer.[104] Once this semisynthetic taurine ester was made, FAB analysis was greatly facilitated and tandem mass spectrometry used to generate structurally significant ions. Another derivative that facilitated HPLC separation as well as the FAB bombardment ionization process for bile acid was the 4-aminobenzenesulfonic acid amide which could be made in high yield as well.[103] Collisional activation of $[M - H]^-$ anions from this sulfonic acid derivative yielded quite characteristic cleavage reactions of the bile acid side chain as well as the expected steroid ring cleavage reactions.

The development of electrospray ionization significantly improved the analysis of bile acids as well as conjugated bile acids through facile LC/MS and LC/MS/MS techniques.[103,105] Multiply charged bile acid molecular anions were readily observed, and interestingly, the sulfate ester of taurine or glycine conjugates of bile acid had a characteristic high abundance of doubly charged ions under electrospray conditions.[105] Most likely, this doubly charged ion was

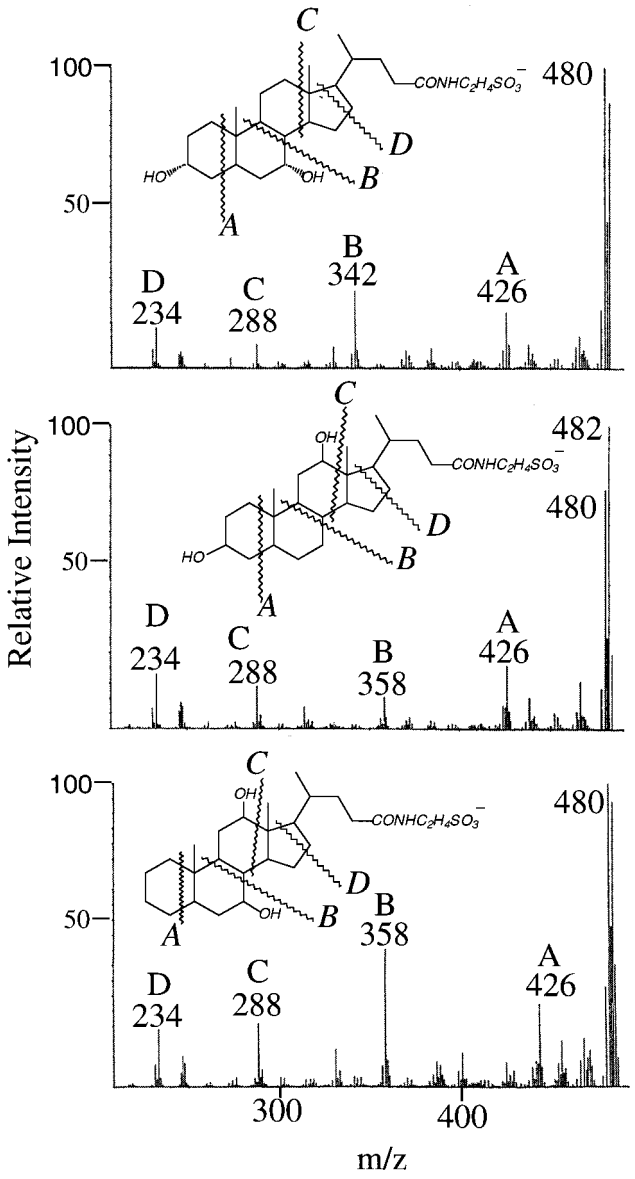
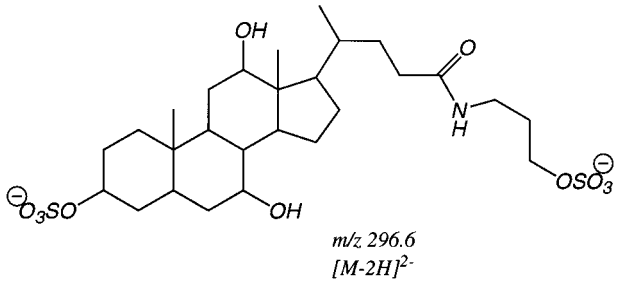


Figure 17. Product ions obtained following collisional activation of $[M - H]^-$ ions from chenodeoxychonoyltaurine: (A) chenodeoxychonoyltaurine, (B) deoxychonoyltaurine, and (C) 7α,12α-dihydroxychonoyltaurine. The $[M - H]^-$ ions were generated by fast atom bombardment. Collisions were carried out at high energy using helium as the collision gas. (Reprinted with permission from ref 104. Copyright 1993 Lipid Research, Inc.)

Scheme 23



formed because the rigid steroid ring system that kept the two charge sites well separated and enabled this relatively small molecule to retain two different charge sites without inordinate charge repulsion (Scheme 23).

As the proliferation of electrospray capability continues in terms of instruments available to investigators in the biological sciences, the use of electrospray and tandem mass spectrometry will undoubtedly find additional applications in the analysis of new steroid-related natural products. Complex lipids related to steroids, including gensingosides,[106] saponins,[107] and dianeackerone steroid esters from the African dwarf crocodile,[108] illustrate just a few applications.

IV. Fat-Soluble Vitamins

Vitamins A, D, E, and K represent a diverse class of lipid substances required in the diet for various critical important biological processes. Each fat-soluble vitamin is unique in terms of both chemistry and biochemistry, and recent advances in mass spectrometry have improved the ability to study these important lipids.

A.Vitamin A

The retinoids consist of a family of closely related polyunsaturated, cyclic lipids (Scheme 24) that have been studied extensively as derivatized entities by electron ionization (for review, see ref 109) and chemical ionization[110] mass spectrometry. These thermally unstable molecules have been most recently examined by electrospray ionization by van Breemen and co-workers.[111] Retinol was found to yield exclusively the dehydrated positive ion $[M + H - H_2O]^+$ during electrospray and the same ion (*m/z* 269) from retinol acetate corresponding to $[M + H - CH_3COOH]^+$. The aldehyde, retinal, yielded an abundant $[M + H]^+$, but retinoic acid produced only low-abundance positive ions. Negative-ion electrospray of retinoic acid yielded an abundant $[M - H]^-$ anion.[111] Atomospheric pressure chemical ionization (APCI) was found to be a better quantitative tool for the analysis of retinal and retinol esters.[112] Using an LC/MS-based assay, APCI was found to produce a linear response over 4 orders of magnitude with a limit of detection between 600 and 800 fmol injected onto the column.

The natural volatility and UV absorbance of retinoids has enabled direct laser desorption as an alternative ionization mode and analysis in a time-of-flight mass spectrometer (nonmatrix assisted) to yield an abundant odd-electron radical cation $[M^{+\bullet}]$ from retinol at *m/z* 286 (113). Some loss of hydroxyl radical was also observed as well as formation of sodium adduct ions $[M + Na]^+$. Retinol acetate yielded abundant $[M^{+\bullet}]$ and additionally lost acetate $[[M - OAc]^+$ and CO_2 observed at *m/z* 284 through a proposed cyclic transition state.[113] Matrix-assisted laser desorption-TOF has also been used to analyze retinoids, and with this ionization technique abundant $[M + H]^+$ ions were observed.[114] The disadvantage of MALDI was the production of abundant matrix ions between *m/z* 150 and 350, a region of interest for the retinoids.

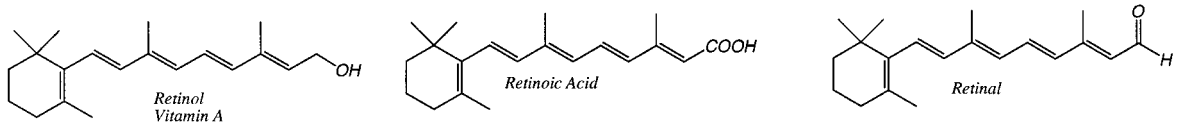
B. Vitamin D

Electron ionization mass spectrometry has been used extensively in studies of vitamin D_3 and its biologically active metabolites,[115] even though these lipids are thermally labile and must be derivatized (Scheme 25).

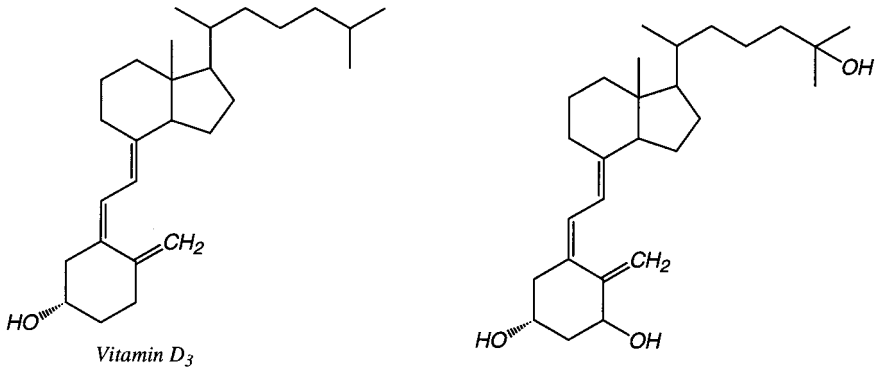
Gas chromatography/mass spectrometry based assays remain a useful approach.[115−117] Continuous flow FAB mass spectrometry after derivatization by cyclization with 4-phenyl-1,2,4-triazoline-3,5-dione yielded abundant $[M + H]^+$, and CID yielded product ions derived from the loss of the steroid-like side chain.[118] Thermospray ionization has also been used to generate $[M + H]^+$ and $[M + NH_4]^+$ as molecular ion species as well as fragment ions.[119] A particle beam interface has also been used for the LC/MS analysis of vitamin D_3 metabolites.[120]

Direct analysis of vitamin D_3 and its hydroxylated metabolites by electrospray ionization has been reported.[121,122] Capillary LC/MS/MS with a multiple reaction monitoring based electrospray ionization assay was found to be 20-fold more sensitive than detection of metabolites by UV absorption techniques.[123] Glucuronides and sulfate esters of vitamin D_3 that contain highly ionized acidic groups have been studied by FAB mass spectrometry with excellent sensitivity.[124] APCI,[125,126] as well as electrospray ionization,[125,127,128] have been used to analyze vitamin D_3 and its metabolites with reasonable sensitivity comparable to that obtained by GC/MS in the analysis of vitamin D related compounds. The LC/MS assay was found to be more sensitive (using abundant $[M + NH_4]^+$ and $[M + H-H_2O]^+$ ions) but had the disadvantage of providing little information concerning specific oxidation sites along the side chain.[129]

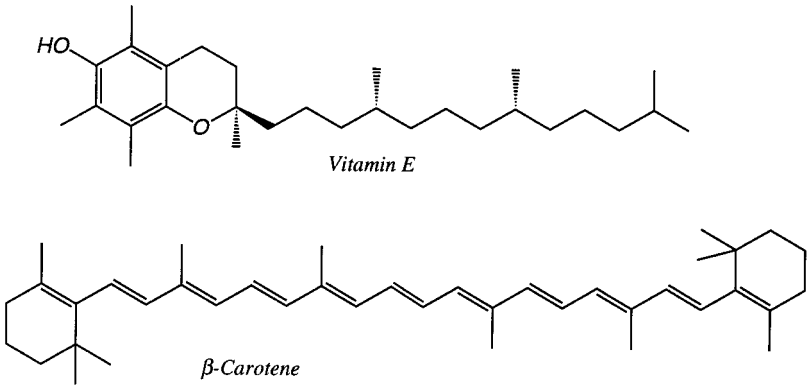
Scheme 24



Scheme 25



Scheme 26



C. Vitamin E and Carotenoids

The analysis of vitamin E_1, vitamin E oxidation products, and other antioxidant hydrocarbon lipids such as the carotenoids has been a particular challenge because of the absence of an ionizable functional group. The inherent nonvolatility and the thermoinstability of these lipids (Scheme 26) also make direct mass spectrometric analysis difficult. Nonetheless, GC/MS and electron ionization remains as a major technique to detect vitamin E_3 and its oxidized metabolites in biological systems.[130,131] Quantitative analysis to levels as low as 180 amol has been reported using stable isotope dilution capillary GC/MS.[132] Analysis of β-carotene by electron ionization and collision-induced loss of neutral toluene (m/z 536 → 444) has also been used as a specific assay for this polyunsaturated lipid.[133]

A novel method to use electrospray ionization to generate positive ions from these neutral lipids involved the addition of 50 μg/mL silver perchlorate, post HPLC column, to effect formation of silver attachment ions during electrospray ionization.[134] The naturally occurring isotopes of silver (^{107}Ag and ^{109}Ag) are of almost equal abundance, resulting in a very characteristic two mass doublet observed for the Ag^+ adduct ions of α-tocopherol acetate (m/z 579 and 581) and lycopene (m/z 643 and 645) (Figure 18). Collision-induced decomposition of these Ag^+ adducts from those lipids resulted in the loss of elemental silver, somewhat characteristic for adducts with low oxidizing potential of the extented π-system.[134]

APCI has also been used in studies of lycopene, α-carotene, and β-carotene. The mass spectrum of β-carotene consisted of essentially one ion at m/z 537 $[M + H]^+$ which could be used in a selected ion monitoring LC/MS assay.[135]

Matrix-assisted laser desorption-generated radical cation molecular ions $[M^{+\bullet}]$ of carotenoids and caro-

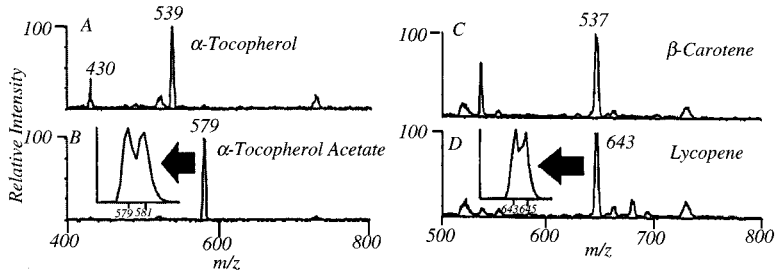
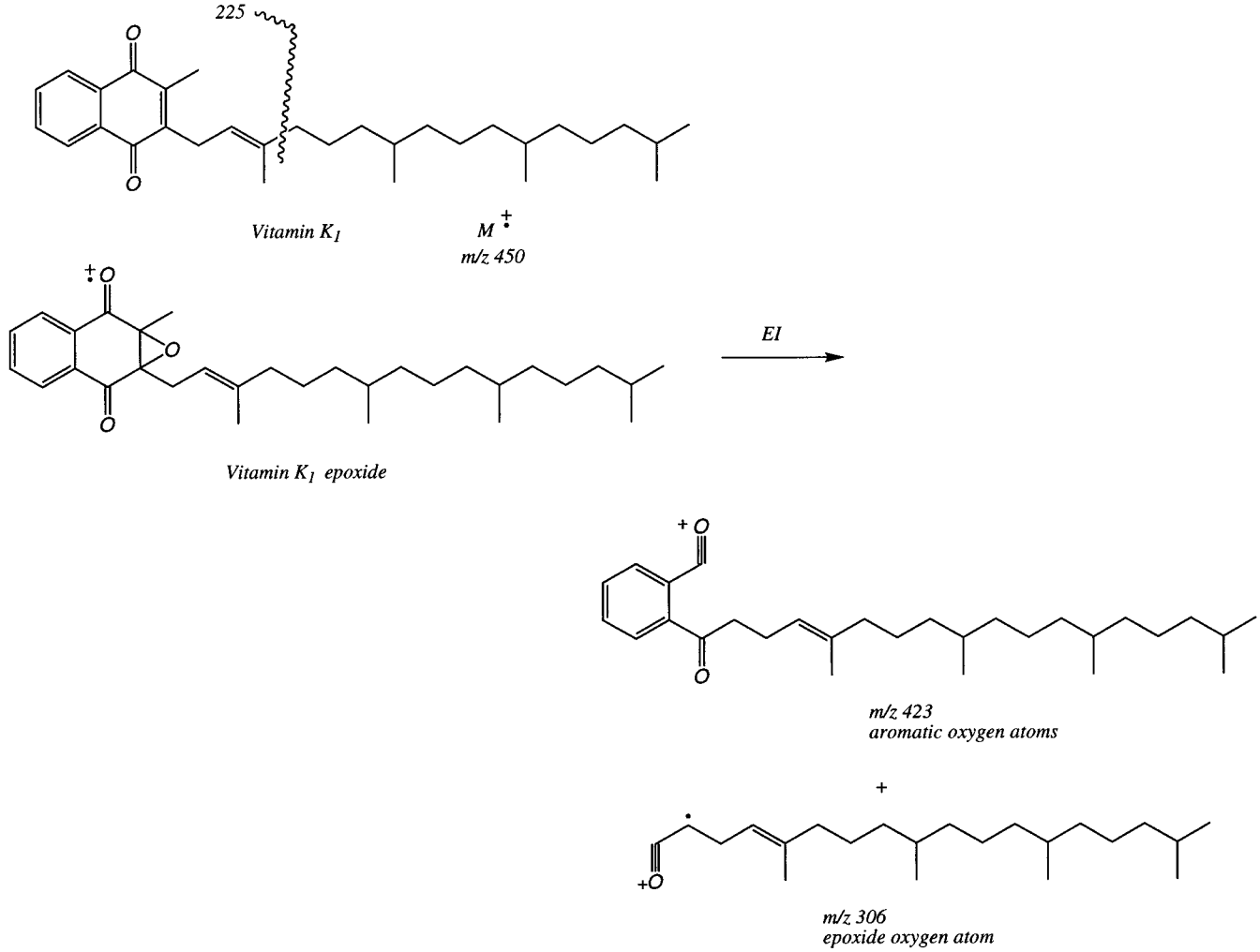


Figure 18. Electrospray ionization of the silver salt of tocopherol and three different carotenoids. Electrospray ionization in a tandem quadrupole mass spectrometer. Each $[M + Ag]^+$ was present as an isotope doublet as shown in the inset. (Adapted with permission from ref 134. Copyright 1998 American Chemical Society.)

Scheme 27



tenoid esters even with subpicomolar quantities using 2,5-dihydroxybenzoic acid as matrix.[136] Post-source decay (PSD) of these [$M^{+\bullet}$] ions was found to yield numerous product ions. For example, under PSD experimental conditions, β-carotene ($M^{+\bullet}$, *m/z* 536) decomposed to yield *m/z* 444 with the loss of toluene[136,137] similar to that seen in the electron ionization MS/MS experiment.[133]

D. Vitamin K

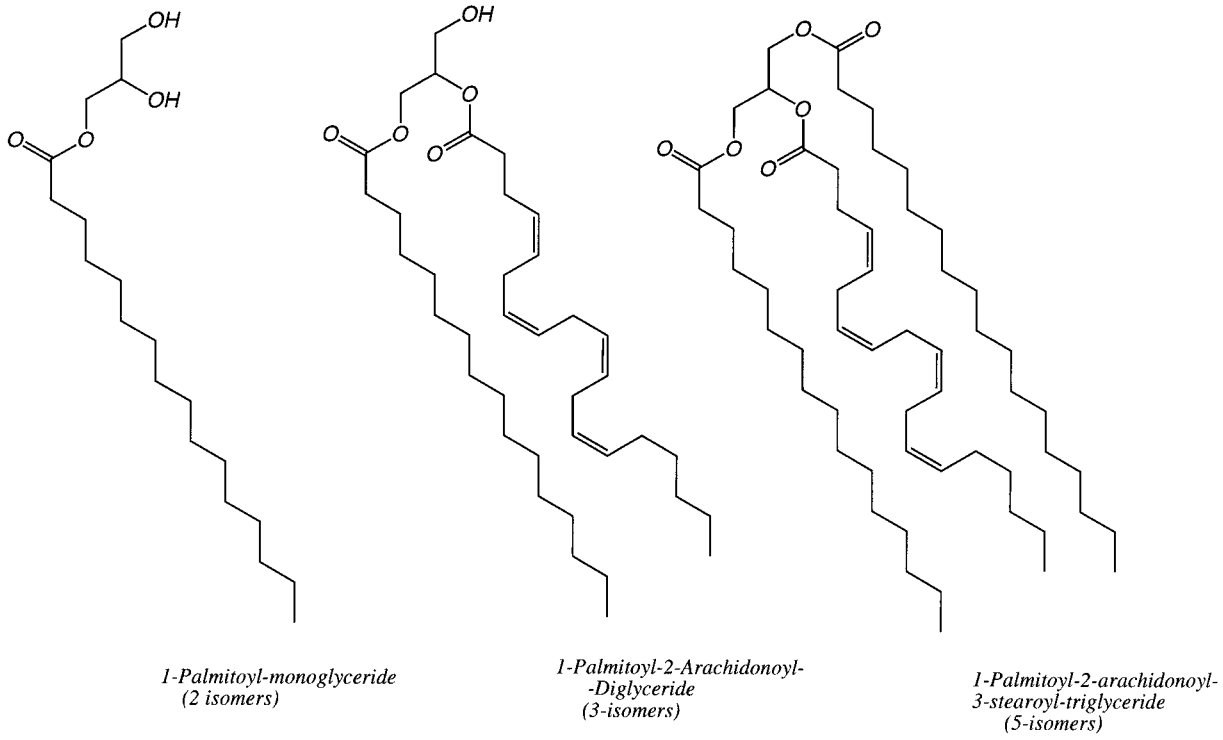
Vitamin K is a required cofactor of the enzymatic carboxylation of glutamate residues in a number of protein coagulation factors in plasma. Very few detailed mass spectrometric investigations have been carried out with this fat-soluble vitamin. Electron ionization yields an abundant [$M^{+\bullet}$] observed at *m/z* 450 for vitamin K_1 (Scheme 27) and a major cleavage ion at *m/z* 225 corresponding to the cleavage adjacent to the side-chain double bond with charge retention on the aromatic portion of the molecule.[138] Most likely, this fragmentation occurs after conjugation of the side chain double bond with the naphthoquinone moiety.

Electron ionization of the important vitamin K epoxide intermediate was shown to yield an abundant [$M^{+\bullet}$] at *m/z* 466. This ion has been used in several studies to follow the specific incorporation of oxygen-18 from molecular oxygen in studies to elucidate the exact biochemical mechanism involved in the carboxylation reaction with glutamate residues from the formation of an epoxide-oxygen-containing fragment ion (*m/z* 306) and a fragment ion containing the aromatic oxygen atoms (*m/z* 423).[139]

V. Complex Glycerolipids

Lipids that contain one or more long-chain fatty acyl groups esterified to glycerol, phosphoglycerol derivatives, and long-chain bases such as sphingosine represent important classes of relatively high molecular weight substances that have been of considerable analytical challenge for mass spectrometry. Even though triglycerides are the most volatile representatives of complex lipids, analysis by mass spectrometry has stretched the limits of electron ionization[140] and chemical ionization[141] techniques. Electrospray ionization and desorption ionization techniques have surmounted the volatility problem, but chromatography remains as an essential component of the analysis strategy in order to identify individual molecular species as well as assignment of acylation positions for even simple mono-, di-, and triglycerides (Scheme 28).

Scheme 28



A. Mono- and Diglycerides

The analysis of monoacyl glycerol esters can be conveniently carried out after derivatization and capillary GC/MS using electron ionization,[142] chemical ionization,[143] or negative-ion chemical ionization as the bispentafluorobenzoyl ester.[144] Electrospray ionization has also been reported following the addition of Na^+ or NH_4^+ ions to the electrospray buffer in order to cationize these neutral lipids and form $[M + Na]^+$ or $[M + NH_4]^+$ species.[145] Little or no fragmentation occurs during the ionization process, but the absolute abundance of either $[M + Na]^+$ or $[M + NH_4]^+$ appears to be related to the total number of double bonds in the long-chain acyl groups. Very poor yields of any adduct ions are observed for saturated mono-, di-, or triglycerides. Matrix-assisted laser desorption (MALDI-MS) has been reported to be of value for the analysis of diacylglycerols[146] using 2,5-dihydroxybenzoic acid as matrix. The best results were obtained with the addition of sodium ions to form $[M + Na]^+$ adduct ions.

Particle beam mass spectrometry and LC/MS analysis had been used to study the nicotinate esters of diacylglycerols. The total number of carbon atoms and double bonds in each fatty acyl group could be readily ascertained from the chemical ionization-like mass spectrum.[147] Some information as to the position of the double bond in each fatty acyl chain could also be gleaned from an abundant ion series.. The charge-remote decomposition mechanisms responsible for this ion series were likely similar to that described for the nicotinate esters of unsaturated and polyunsaturated fatty acids.[6]

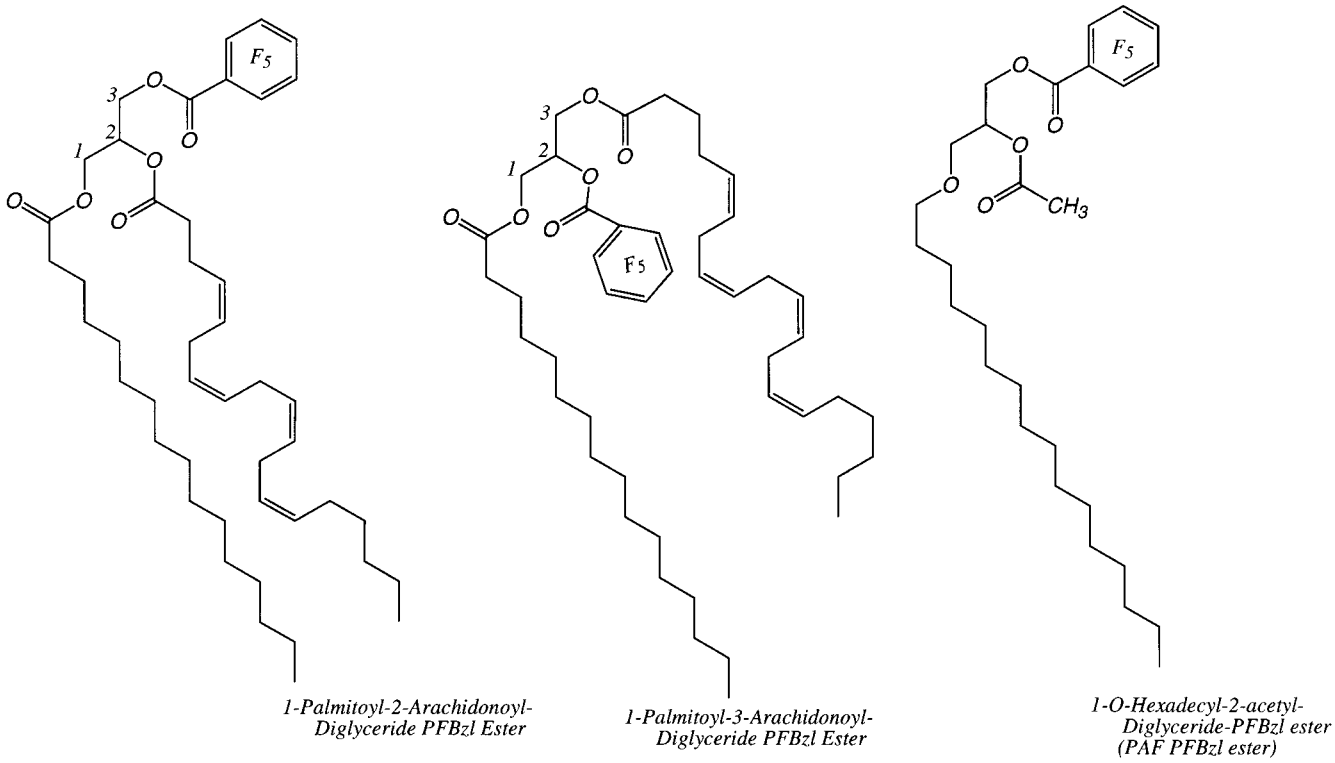
The most sensitive means to detect and quantify diacylglycerols remains to be capillary GC/MS with negative-ion chemical ionization.[144,148−151] 1-Stearoyl-2-arachidonoyl-sn-3-glycerol was quantitated as the pentafluorobenzyl ester molecular anion $[M]^-$ at *m/z* 838. This derivatization procedure does result in some migration of the acyl groups leading to formation of thermodynamically stable 1,3-diacyl pentafluorobenzoyl esters (Scheme 29). As little as 30 fmol of endogenous 1-stearoyl-2-arachidonoyl-sn-3-glycerol could be detected in an extract of human basophils.[149]

The analysis of the biologically active glycerophosphocholine ether lipid called platelet activating factor is typically carried out by analysis of a specific diglyceride, namely, 1-*O*-hexadecyl-2-acetyl-sn-3-glycerol derivatized to the pentafluorobenzoyl ester. Recent advances in the formation of this derivative have appeared.[150,151]

B. Triacylglycerols

This class of complex lipids comprises one of the most diverse mixtures of potentially isomeric molecules in biological systems with three fatty acyl substituents esterified to a glycerol backbone. In the past, GC/MS was used to ascertain total fatty acid content for these triesters by analysis of fatty acids released by saponification. With the development of direct LC/MS techniques and the ability to analyze intact lipid species, a renewed interest has emerged in obtaining direct positional acylation information as well as details concerning double-bond locations within each fatty acyl residue. Previously, direct analysis of triacylglycerols was carried out by electron ionization[143] and chemical ionization,[152,153] fast atom bombardment,[154] thermospray,[155] and APCI.[156] Electrospray typically yields an alkali-metal adduct ion ($[M + Na]^+$ or $[M + K]^+$), while APCI yields primarily $[M + H]^+$ ions.[157] The combination of HPLC

Scheme 29



separation along with the molecular weight information, as provided by direct mass spectrometric analysis, has proven to the be quite valuable; but the abundance of each molecular species present in a complex mixture remains difficult to quantitate with great accuracy. This APCI response for different triacylglycerol molecular species calculated from the integrated area of the $[M + H]^+$ eluting from the HPLC was found to be surprisingly disparate for individual molecular species of triglycerides. This was especially significant for those with widely different numbers of total unsaturations in the fatty acyl chain.[157] The best quantitative results from mass spectrometric analysis in terms of agreement with synthetic mixtures were based upon applying a response factor calculated from the fatty acid compositions.[157] One of the major problems inherent in using mass spectral data for quantitation of these lipids is that fully saturated molecular species often yield very low abundances of $[M + H]^+$ ions and the signal appears to increase as the total number of double bonds increases within the molecular species. The use of silver ion HPLC to improve separation of triacylglycerols combined with APCI detection of each molecular species as the $[M + H]^+$ ion permitted separation and molecular weight determination of 43 different molecular species in cloudberry seed oil, 39 from evening primrose oil, 79 from barrage oil, and 56 different molecular species in black currant seed oil.[158]

MALDI has also been used to analyze triacylglycerols in natural oils such as olive oil[159] and cod liver oil.[160] MALDI-TOF analysis of these oils using an α-cyano-4-hydroxy cinnamic acid matrix yielded predominately $[M + Na]^+$. A ratio of 1 ng of triglyceride dissolved in a solution containing 500 ng of matrix on the target surface (1 μL) was empirically observed as optimal. With this MALDI technique, 64 different molecular species of cod liver oil could be detected[160] without prior separation of the individual molecular species.

One recent report suggested that capillary supercritical fluid (CO_2) chromatography could separate molecular species of triacylglycerol. Detection of the effluent was accomplished using APCI with ammonia in methanol in the ionization chamber to form $[M + NH_4]^+$ adduct ions of the triglycerides.[161] Importantly, this method did generate $[M + NH_4]^+$ adduct ions from saturated triacylglycerols, unlike the generation of $[M + Na]^+$ by the more traditional APCI process.[157,158] The fatty acyl groups from long chain to very short chain (e.g., 4:0) that make up each molecular species in milk fat could be readily ascertained.[161] This APCI process also generated $[M - RCOH]^+$, and these ions provided information concerning each fatty acyl group that made up the individual molecular structures.

Fast atom bombardment as well as electrospray ionization of triacylglycerol produce abundant alkali adduct ions $[M + Na]^+$ from triacylglycerols.[162,163] High-energy CID of the $[M + Na]^+$ were surprisingly dissimilar in terms of decomposition pathways when ions formed by electrospray were compared with ions formed by fast atom bombardment (Figure 19). At high mass a series of ions were observed, corresponding to the loss of C_nH_{2n+1}, from electrospray ionization that were likely distonic. The FAB-generated $[M + Na]^+$ ions, however, were collisionally activated to a series of ions corresponding to the loss of C_nH_{2n+2}, most likely by a charge-remote 1,4-elimination reaction (as seen in Scheme 1) or homolytic bond cleavage in loss of a hydrogen radical species (Scheme 30).

One possible mechanism for the decomposition of the electrospray-generated ion series would proceed

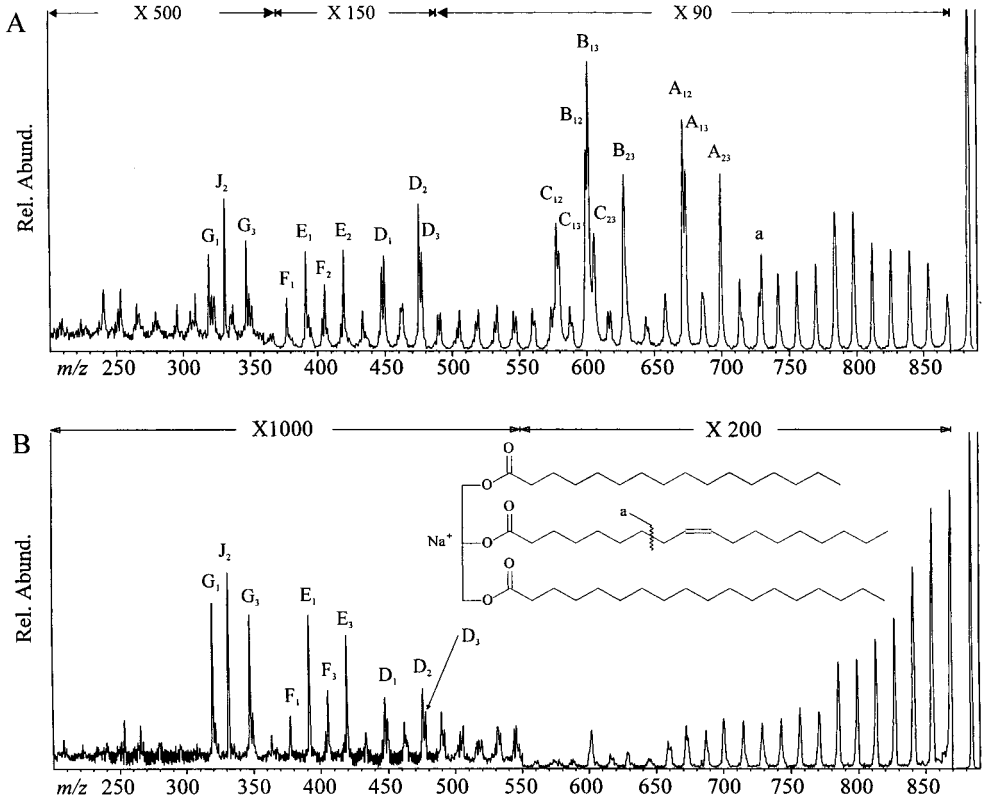
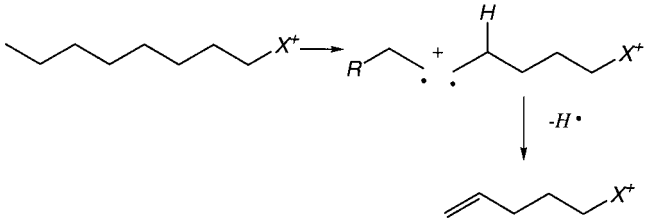
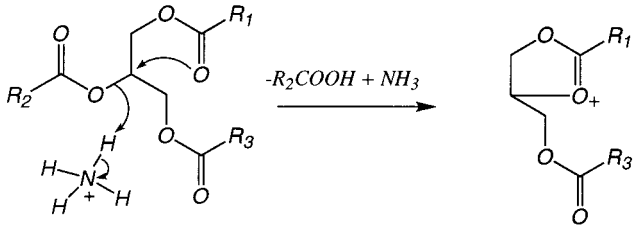


Figure 19. High-energy collision-induced decomposition of $[M + Na]^+$ ions derived from 1-palmitoyl-2-oleoyl-3-stearoylglycerol (16:0/18:1/18:0/MW= 860.8) with (A) FAB ionization and (B) electrospray ionization. (Reprinted with permission from ref 162. Copyright 1998 Elsevier Science.)

Scheme 30



Scheme 31



through a distonic ion intermediate which may have less internal energy than the FAB-generated ion and could subsequently lose hydrogen radical more efficiently.[162] In general, detailed studies of the decomposition of $[M + Na]^+$ from triacylglycerols have added insight into the complexity of reactions termed charge remote. Further detailed studies of the decomposition of $[M + NH_4]^+$ and $[M + Na]^+$ generated by FAB and ESI have revealed charge-remote and charge-driven decomposition mechanisms relevant to precise information about the fatty acyl chains.[163] For example, decomposition of $[M + NH_4]^+$ induced by high-energy collisions produced abundant ions corresponding to loss of R_nCOONH_4 (Scheme 31) (where $n = 1-3$ corresponding to the position esterified on the glycerol backbone) from each fatty acyl group. Additional fatty acyl derived ions were observed as R_nCO^+, $[R_nCO + 74]^+$, and $R_nCO + 128]^+$.[163]

The decomposition of lithiated adduct ions of triacylglycerols generated by electrospray ionization has also been studied at low collision energies within the tandem quadrupole instrument.[164] These lithiated species were formed by the postcolumn addition of lithium acetate to the mobile phase prior to electrospray ionization. Several ion series could be identified, including the abundant $[M + Li - (R_nCO_2H)]^+$, $[M + Li - (R_nCO_2Li)]^+$, and R_nCO^+. The latter acylium ions characterized the fatty acyl substituent in terms of the chain length and total number of double bonds (Figure 20). It was suggested that neutral loss tandem mass spectrometry could be used to determine the fatty acyl group esterified to the glycerol sn-2 position. The abundance of the $[M + Li-R_nCO_2H]^+$ from the neutral loss of R_2COOH (the sn-2 fatty acyl group) was approximately 1.5-fold less abundant than the corresponding ion derived from the neutral loss of the fatty acyl groups from the sn-1 and sn-3 positions. However, only a few triacylglycerols have been examined in detail, and this interpretation must be viewed with some caution. In addition to this, the ions derived from the triglyceride 16:0/18:0/18:1 which corresponded to $[M + Li - (R_1\text{-}CO_2H) - (R_2'CH{=}CHCO_2H)]^+$ and $[M + Li - (R_3\text{-}CO_2H) - (R_2'CH{=}CHCO_2H)]^+$ were observed at m/z 329 and 303, respectively. The algebraic difference between these two ions would correspond to the difference in mass between R_3COOH and R_1COOH (16:0 and 18:1), thus identifying uniquely the sn-2

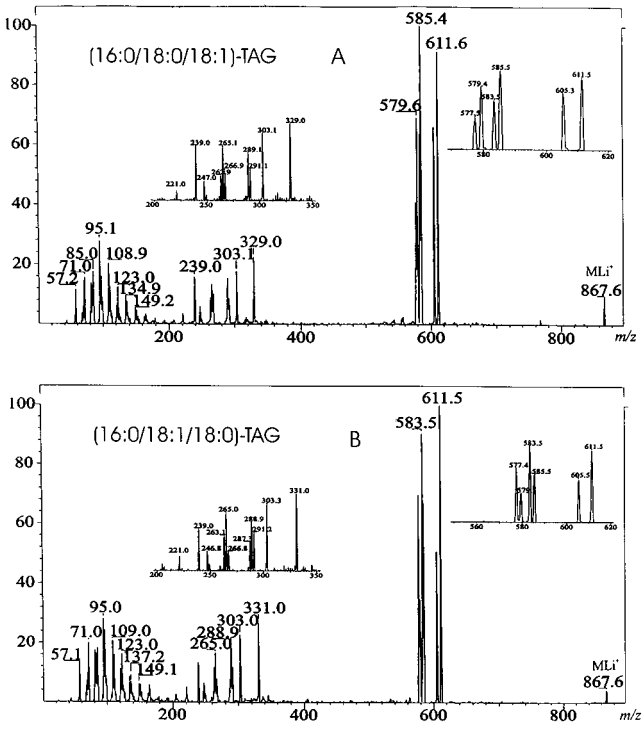


Figure 20. Product ions obtained from the collisional activation of the lithiated adducts of (A) 16:0/18:0/18:1-triacylglycerols and (B) 16:0/18:1/18:0-triacylglycerol in a tandem quadrupole mass spectrometer. (Reprinted with permission from ref 164. Copyright 1999 Elsevier Science.)

fatty acyl position as 18:0. This type of analysis would become a possibility because of the previously described ions ($RnCO^+$) which clearly defined the three different fatty acids present in the triacylglycerol. One of the most interesting observations made by these authors[164] was that an in-source collisional activation process could be used to generate $[R_nCO_2\text{-}Li_2]^+$ during electrospray ionization when the skimmer voltage was 50−60 V. In this case, it was possible to carry out a MS^3 tandem mass spectrometric investigation of the lithiated carboxylate ion selected in the first quadrupole region, and the product ion spectra from this ion yielded abundant ions relevant to the positions of double bonds in polyunsaturated fatty acids (Figure 21).[164] These studies clearly revealed that it was possible to ascertain a significant amount of information concerning the structure and fatty acyl substitutions within triacylglycerol using electrospray ionization mass spectrometry.

Mixtures of triacylglycerols containing oxidized fatty acyl groups add an additional challenge to the analysis because of the potential for elution of normal species during chromatographic separation as well as oxidative species being isobaric with nonoxidized species. Of additional importance is that some oxidized lipid species such as hydroperoxides or epoxides may not be amenable to all types of mass spectrometric ionization techniques. Electrospray analysis of synthetic triacylglycerols that had been previously oxidized resulted in readily detectable hydroperoxy and dihydroperoxy products for 1,2-dilinoleoyl-3-stearoyl-sn-glycerol as the $[M + Na]^+$ adduct ions. These products were detected as they eluted from an HPLC, based upon the appearance of specific $[M + Na]^+$ ions. A linear relationship between theoretical carbon number and HPLC retention time was determined for a series of 22 different oxidized and chain-shortened aldehyde-containing triacylglycerols.[165]

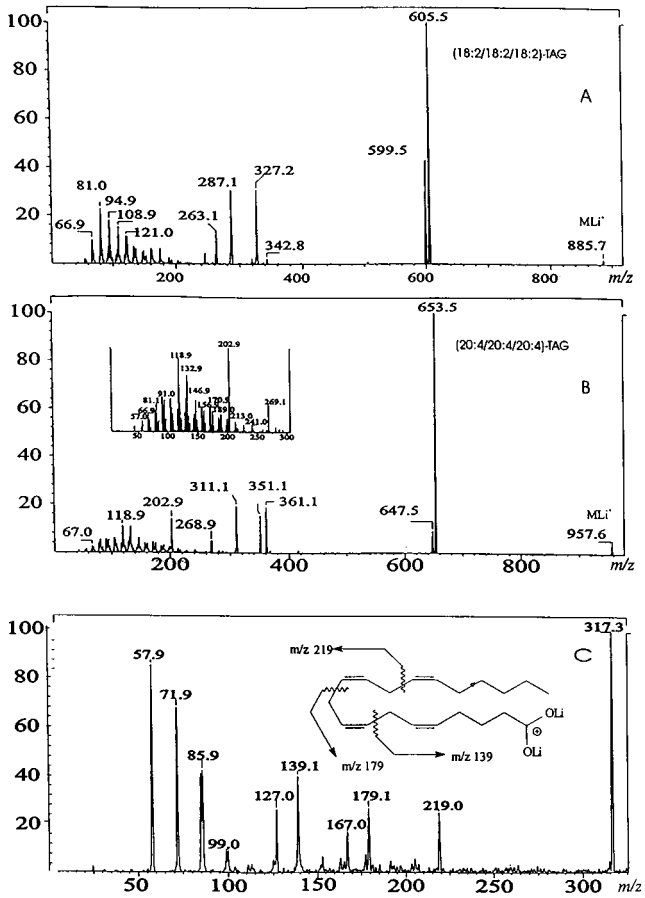
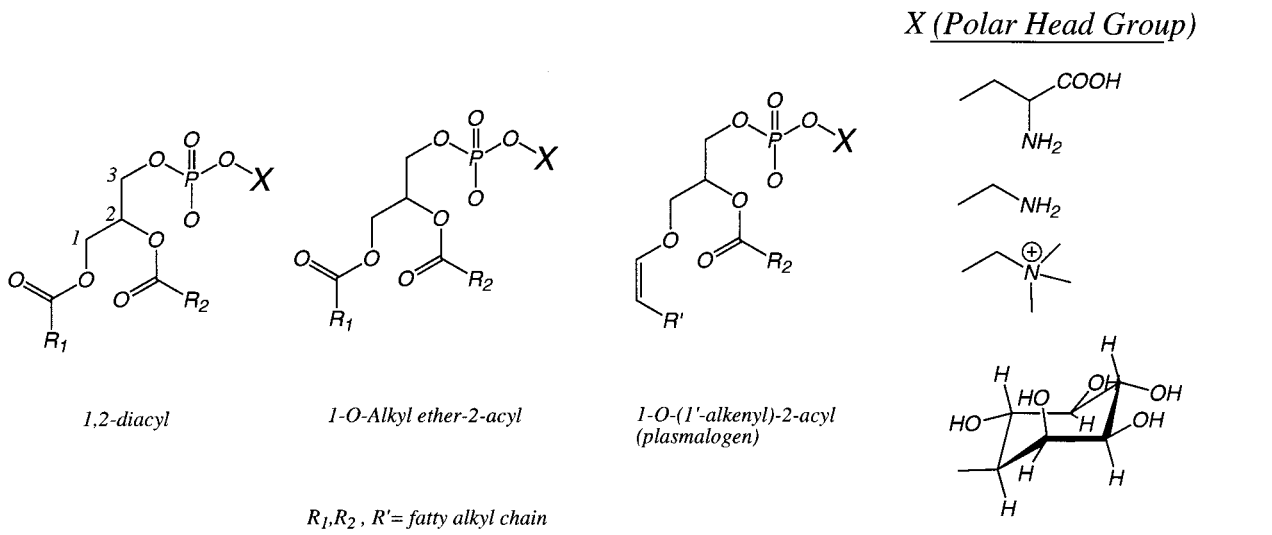


Figure 21. (A) Product ions obtained from the collision-induced decomposition of the lithiated adduct of (A) trilinolein (18:2/18:2)-triacylglycerol and (B) that of triarachidonene (20:4/20:4/20:4)-triacylglycerol in a tandem quadrupole mass spectrometer using argon as collision gas. (C) Collision-induced decomposition of *m/z* 317 derived from the in-source activation of triarachidonene followed by collisional activation in the collision zone of the tandem quadrupole mass spectrometer. (Reprinted with permission from ref 164. Copyright 1999 Elsevier Science.)

C. Glycerophospholipids

The outer membranes of eukaryotic cells are composed of a bilayer of glycerophospholipids which exist as a diverse mixture of closely related glycerolphosphodiesters containing one of several varied polar headgroups, variants in fatty acyl groups, and subspecies with variations at the sn-1 glycerol position (Scheme 32). The ability to analyze intact phospholipids was first realized using field desorption mass spectrometry[166] then more widely employed following the development of FAB-MS.[167,168] Fundamental studies in a number of laboratories contributed to a wealth of information concerning the ion chemistry of FAB-generated molecular ion species derived from glycerophospholipids.[169−171] These studies published in the past decade facilitated the rapid progress experienced in the application of electrospray to phospholipid analysis. Collisional activation of ESI-generated molecular ions species were found to

Scheme 32

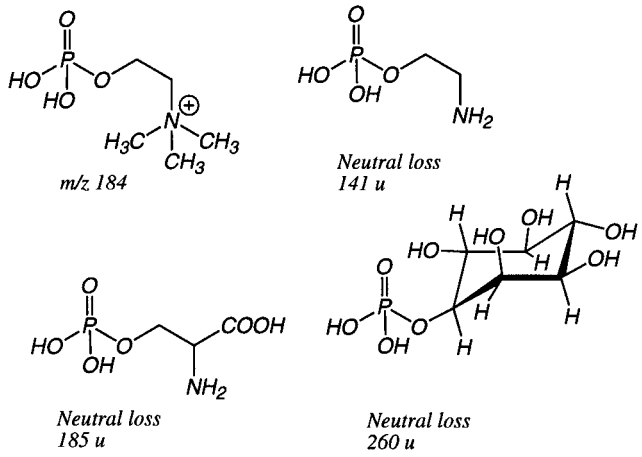


decompose to products identical to those product ions derived from activation of FAB-generated molecular species ions. The critically important product ions were indicative of the polar headgroup and the fatty acyl groups. Thus, the first reports concerning the application of ESI to phospholipid analysis had an established base of information already available. However, since FAB ionization was essentially a surface phenomenon, a considerable variation in ion yields were typically observed for different classes of phospholipids, rendering quantitative analysis in complex mixtures somewhat problematic. Nonetheless, FAB[171−174] and, in particular, continuous flow FAB remains a useful technique.[175] Electrospray ionization was shown to overcome many limitations of FAB ionization in a series of papers published in 1994[176−178] following demonstration of the potential of electrospray ionization for phospholipids analysis with platelet activating factor by Weintraub and coworkers.[179]

Abundant $[M + H]^+$ and $[M + \text{alkali metal}]^+$ were reported in the early studies of glycerophospholipids by electrospray ionization mass spectrometry, depending upon the presence of buffers and alkali metal in the spray solvent system.[176] Tandem mass spectrometry of the $[M + H]^+$ ions yielded unique product ions and neutral losses quite indicative of the polar headgroup. For example, collisional activation of $[M + H]^+$ derived from glycerophosphocholine lipids (GPC) yielded the phosphocholine ion at *m*/*z* 184. For glycerophosphoethanolamine lipids (GPE), CID of $[M + H]^+$ resulted in loss of phosphoethanolamine (neutral loss of 141 u), for glycerophosphoinositol the neutral loss of 259 u, and for GPS lipids collisional activation of $[M + H]^+$ resulted in the loss of phosphoserine (neutral loss of 185 u) (Scheme 33). The abundance of the resulting diglyceride ions that resulted from the neutral loss of the polar headgroup was found to be a function of capillary exit voltage in the electrospray interface.[177]

Most glycerophospholipids and, in particular, acidic phospholipids such as GPS, phosphatidic acid (GPA), GPI, and phosphatidylglycerol (GPG) yield abundant negative ions. The observed mass of each $[M - H]^-$

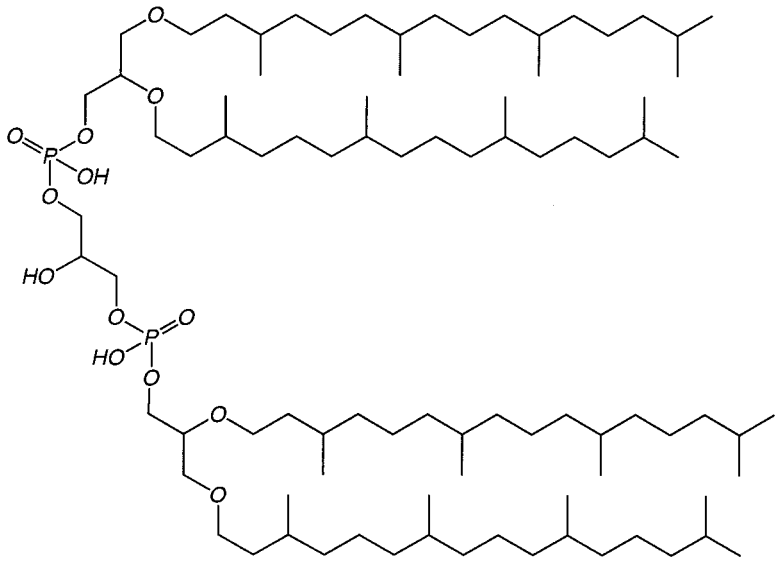
Scheme 33



ion was itself quite useful in the identification of the most prominent phospholipids present in a biological extract as exemplified by the study of rat and human pancreatic islet cells where the major $[M - H]^-$ ions were used to identify individual constituents of each phospholipid class.[180] A quantitative assay for a specific GPS molecular species (1-hexadecanoyl-2-octadecanoyl-GPS) in human blood was developed using 1-hexadecanoyl-2-hexadecanoyl-GPS as internal standard and measurement of the abundance of the corresponding $[M - H]^-$ ions.[181] Glycerophosphocholine lipids also yield abundant negative molecular ion species such as $[M - 15]^-$ and $[M + \text{acetate}]^-$ when acetate buffers are used during electrospray ionization.[182] The abundance of $[M - 15]^-$, which corresponds formally to loss of a choline methyl group but most likely is the result of the loss of a neutral species, was found to be a function of electrospray orifice potential. The acetate adduct ions were found to decompose to $[M - 15]^-$ by a loss of methyl acetate during collisional activation of the acetate adduct ions in the tandem quadrupole mass spectrometer.[182]

Collision-induced decomposition of electrospray-generated negative ions from GPL species produced abundant carboxylate anions from the fatty acyl groups esterified to the sn-1 and sn-2 positions.[176] The yield of carboxylate anions from CID of GPLs in the

Scheme 34



negative-ion mode was found to increase with collision energy for all lipid classes.[183] However, the ratio of the abundance of sn-1/sn-2 carboxylate anions was found to depend on collision energy for GPE and GPC and for acidic phospholipids (GPI, GPA, and GPS). The abundance of the sn-1 carboxylate anion predominated at all CID energies for those molecular species studied.[183]

The importance of the ability to structurally study the fatty acid acyl groups as carboxylate anions cannot be overestimated. The appearance of these ions has been particularly valuable in structural studies of oxidized phospholipids and has made the use of negative-ion analysis of glycerophospholipids an important application. Collisional activation of the negative molecular ion $[M - H]^-$ also yields ions corresponding to the neutral loss of the polar headgroup.[184]

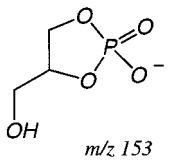
An alternative approach for the identification of fatty acyl substituents present in GPC was proposed to capitalize on the collisional activation of positive lithiated adduct ions.[185] This adduct ion $[M + Li]^+$ was formed in high yield when 2 nmol/μL LiOH was present in the electrospray solution which also contained 1 pmol/μL GPC lipid. The lithium adduct ions could be collisionally activated to yield product ions corresponding to the loss of the fatty acyl group as R_1COOH and R_2COOH neutrals after initial loss of trimethylamine from the choline headgroup.[185]

Electrospray ionization was found to be useful for quantitative analysis of phospholipid molecular species within a class by using both positive- and negative-ion species.[178] Abundance of $[M + Na]^+$ derived from dipalmitoyl−GPC was found to be linear between 0.01 and 10 pmol infused into the electrospray ion source. Using a deuterium-labeled internal standard and LC/MS analysis, quantitation to a limit of 5 fmol (entering the electrospray ionizer) was possible.[177] The use of electrospray ionization to quantitatively assess the abundance (both absolute and relative) of glycerophospholipids by abundance of either the positive or negative molecular ion species was studied in detail and used as a means to characterize the complex mixture of glycerophospholipid molecular species found within CHO cells.[184] This approach was then used to analyze the phospholipids in rat bile[186] and a similar one for the analysis of phospholipids in bronchoalveolar lavage fluid[187] and glycerophosphocholine lipids in bacteria.[188] Glycerophosphoethanolamine lipids in heart have a high plasmalogen content, and individual molecular species were quantitatively assessed using electrospray tandem mass spectrometric techniques.[189] Fourier transform ion cyclotron resonance (FTIR) of electrospray-generated $[M + H]^+$ and $[M - H]^-$ from GPE, GPS, GPI, GPG, and GPA (phosphatidic acid) were used to quantitate the relative abundance of individual molecular species within each phospholipid class isolated from RBL-2H3 mast cells carried in culture.[190]

Two novel phospholipids from the purple membrane of *Halobacterium salinarium* were recently reported as a diether bisphosphatidylglycerol (cardiolipin) and a closely related diether phosphoglycerol phosphate (Scheme 34). Interestingly, this cardiolipin was observed as a doubly charged ion species in the negative-ion electrospray mass spectrum of the purified phospholipid at *m/z* 760.0 and as the singly charged $[M - H]^-$ at *m/z* 1521.3 (191). Cardiolipin and lysocardiolipin from *Streptomyces hygroscopicus* were analyzed using negative-ion ESI and tandem mass spectrometry used to determine the fatty acyl substituents in each molecular species.[192] A rather unusual cardiolipin species having the middle glycerol oxygen atom substituted with D-alanine, L-lysine, or α-D-glucose were studied using FAB MS/MS as both positive and negative ions.[193]

One less commonly studied glycerophospholipid has been GPG, even though this phospholipid is abundant in some tissues. The tandem mass spectrometry of several phosphatidylglycerol species has been reported using FAB ionization.[194] Negative-ion electrospray ionization has been used to detect the elution of specific enantiomers of GPG as the dinitrophenylurethane derivative separated by a chiral phase HPLC column in order to determine exact

Scheme 35



m/z 153

stereochemical configuration of these phospholipids that have two chiral centers.[195]

A detailed investigation into the use of tandem mass spectrometric techniques to analyze membrane phospholipids by nanoelectrospray was recently reported and exemplified in studies with as little as 1000 cells.[184] Both positive- and negative-ion tandem mass spectrometry were used to specifically analyze GPC, GPI, GPS, and GPE lipids as well as sphingomyelin. All glycerophosphocholine lipids could be detected by the formation of the product ion *m/z* 153 (Scheme 35) following collision-induced decomposition of the $[M - H]^-$ or $[M - 15]^-$ ion.

The formation of *m/z* 184 is a common product ion following collisional activation of $[M + H]^+$ from GPC lipids, but a substantial mass-dependent response to electrospray ionization and CID formation of this phosphocholine ion has been demonstrated.[184] This was manifest in the abundance of the precursor ions that lead to the formation of *m/z* 184 (Figure 22) and was most clearly evident when four internal standards were added in equal molar amounts to phos-

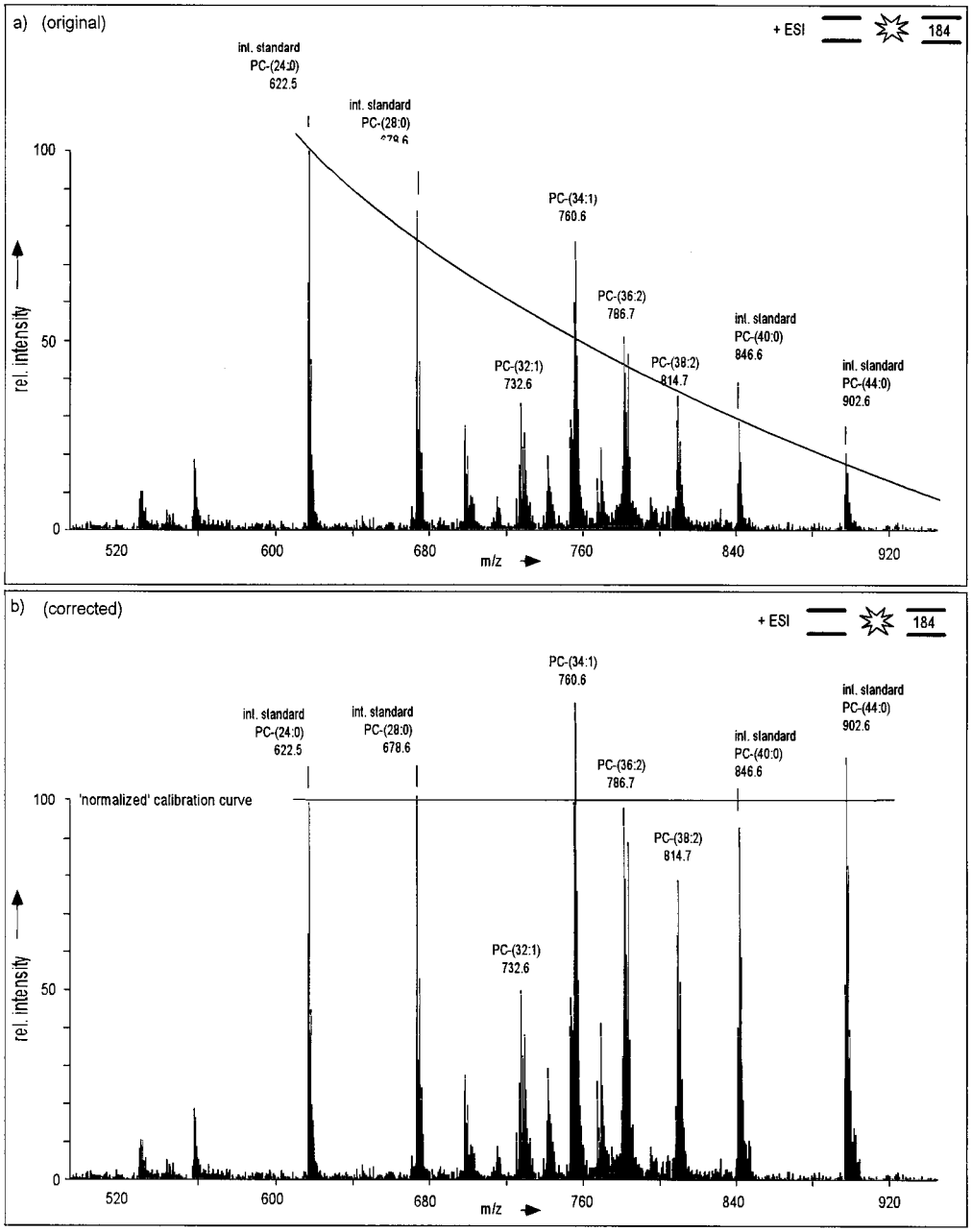
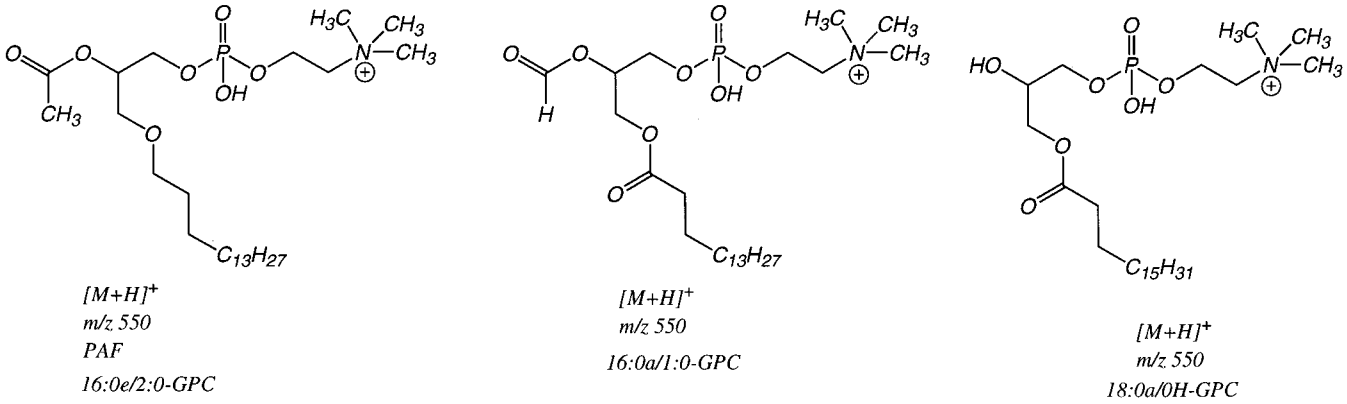


Figure 22. Precursor ions of *m/z* 184 obtained during the electrospray ionization of the total lipid extract of 5000 CHO cells to which had been added equal molar amounts of glycerophosphocholine standards with a total carbon number of 24:0, 28:0, 40:0, and 44:0. (A) Uncorrected ion intensities observed in the nanoelectrospray ionization signal. (B) Ion intensities corrected for the signal intensities derived from the internal standards which were used to generate an intensity calibration curve. (Reprinted with permission from ref 184. Copyright 1997 National Academy of Sciences, USA.)

Scheme 36



pholipid extracts of CHO cells. These internal standards varied in molecular weight from 622 to 902 u. The results of multiple internal standards added to the glycerophosphocholine mixture enabled a precise calibration curve to be established which corrected for the change in precursor ion abundance of *m*/*z* 184. This approach has been used to analyze molecular species of glycerophospholipids in GPC, GPE, GPS, and GPI isolated from yeast (*S. cerevisiae*).[196]

MALDI has also been used for glycerophospholipid analysis. MALDI-TOF was used to analyze GPC and GPI with 2,5-dihydroxybenzoic acid (DHB) containing 1% TFA as matrix. Abundant $[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$ were observed as well as ions most likely the result of DHB adducts being formed.[146] Both positive and negative ions were detected by MALDI-TOF analysis of GPI lipids and phosphorylated forms GPIP and $GPIP_2$. This approach for the analysis of glycerophospholipids was illustrated by analysis of complex glycerophospholipids isolated from human neutrophils.[146] MALDI was also used as an ionization technique in combination with FTICR analysis to study GPC, GPE, GPS, and GPI lipids also using 2,5-dihydroxybenzoic acid as matrix.[197] Both $[M + H]^+$ and $[M + Na]^+$ were observed for all glycerophospholipid classes. Negative ions $[M - H]^-$ were formed for all glycerophospholipids except glycerophosphocholine. For this specific class, $[M - 60]^-$ (corresponding to the loss of protonated trimethylamine) was found to be the predominate molecular ion species. Collisional activation of each negative molecular ion species yielded abundant carboxylate anions as R_1COO^- and R_2COO^-. The most abundant product ions in the series of phospholipids examined were found to be those carboxylate anions derived from the sn-2 position.[197] As little as 40 attomoles of dipalmitoylphosphatidylcholine could be detected as $[M + H]^+$, $[M + Na]^+$, and $[M + Na - 60]^+$ using MALDI/FTICR.[198]

1. Platelet Activating Factor

Analysis of the biologically active glycerophosphocholine ether lipid, platelet activating factor (PAF, 1-*O*-hexadecyl-2-acetylglycerophosphocholine) has also been reported using electrospray ionization. PAF and related acetylated phosphocholine lipids yielded abundant $[M + H]^+$ ions and formed *m*/*z* 184 following collision-induced decomposition.[179,199] When a chemically related internal standard [2-methoxy-3-(octadecylcarbamoyloxy)propyl-2-3−thiazoloethyl phosphate] was used, a LC/MS/MS multiple reaction monitoring assay was able to quantitatively assess the presence of PAF-lipids in human endothelial cells.[199] In biological extracts, the presence of an isobaric lysoglycerophosphocholine lipid and a diacylglycerophosphocholine lipid could lead to a misidentification of PAF since these lipids often elute quite close to PAF in normal phase HPLC (Scheme 36). One alternative method for specific PAF analysis was based on the LC/MS/MS assay of negative ions derived from PAF ($[M - 15]^-$) since the product ions from each of the three isobaric species yielded quite different CID spectra.[200] Although this assay was not as sensitive as the positive-ion assay, both 18:0a/lyso-GPC and PAF could be separately assessed in the same analysis by CID of *m*/*z* 508 $[M - 15]^-$ with formation of the carboxylate anions *m*/*z* 283 (sterate) and 59 (acetate), respectively.

2. Plasmalogen Phospholipids

Plasmalogen phospholipids which have a vinyl ether substituent at the sn-1 position are a major component of GPE lipids and to a lesser extent GPC lipids in certain tissues (Scheme 32). While plasmenyl GPE behave identically to diacyl-GPE lipids in formation of $[M + H]^+$ and $[M - H]^-$ molecular ion species, collisional activation of $[M - H]^-$ yields only one carboxylate anion as expected for ether phospholipids.[201,202] Treatment of the crude mixture of phospholipid extracts with HCl followed by reanalyzis has been used as an effective way to distinguish alkyl ether GPE molecular species from plasmenyl GPE species. This strategy was used to establish that plasmalogen GPE lipids that contain arachidonic acid or other polyunsaturated fatty acids esterified at the sn-2 position were more susceptible to free radical oxidation than those plasmalogen-GPE lipids with saturated fatty acyl groups esterified at the sn-2 position.[201]

3. Lysophospholipids

Lysophospholipids have a single radyl group at either sn-1 or sn-2 and a free hydroxyl group at the alternative position of glycerol, rendering two differ-

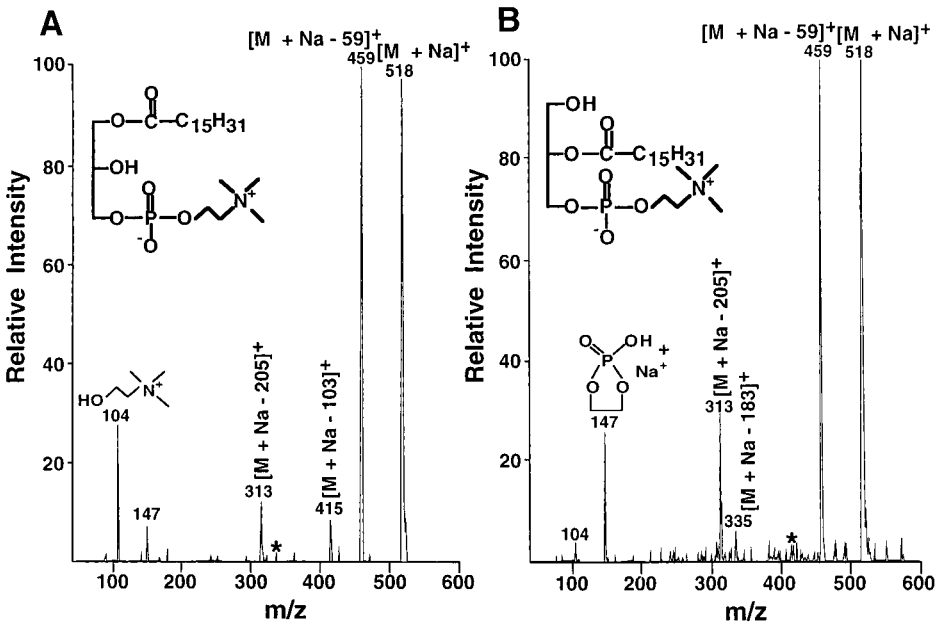
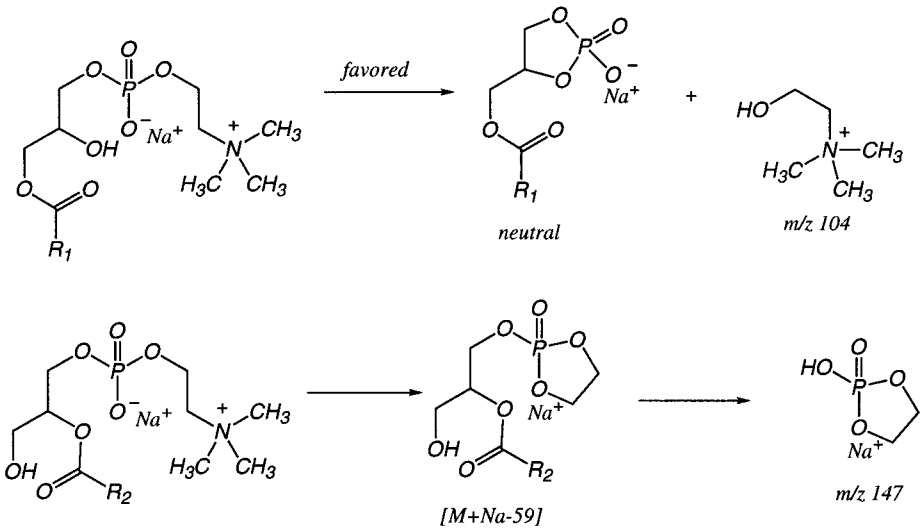


Figure 23. Product ions obtained from the collisional activation of electrospray positive ions for two lysoglycerophosphocholine regioisomers. (A) Positive-ion electrospray tandem mass spectrum of sodiated 1-hexadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine and (B) sodiated 1-hydroxy-2-hexadecanoyl-sn-glycero-3-phosphocholine. Spectra were obtained in a tandem quadrupole mass spectrometer using argon as collision gas. (Reprinted with permission from ref 203. Copyright 1996 American Chemical Society.)

Scheme 37



ent regioisomers. These compounds are important biosynthetic precursors and have biological activity in their own right. The formation of a particular isomer implies specific biochemical processes, which makes differentiation between the sn-1 lyso or sn-2 lyso an important observation. Han and Gross[203] presented an interesting method to distinguish the regioisomers of lyso-GPC using CID of the $[M + Na]^+$ generated by electrospray ionization (Figure 23). These investigators found a more abundant product ion at *m*/*z* 104 relative to the abundance of the ion at *m*/*z* 147 for sn-2 lyso-GPC molecular and the reverse ratio of *m*/*z* 104 to 147 for sn-1 lyso-GPC lipids. This data was consistent with a favored formation and loss of a five-membered phosphodiester neutral species from the sn-2 lyso regioisomers (Scheme 37) leading to the favored production of *m*/*z* 104. With the hydroxyl present at the sn-1 position, the loss of trimethylamine results in a cyclic phosphodiester corresponding to $[M + Na - 59]^+$ which can readily decompose to form *m*/*z* 147, indicated in Scheme 37.

Another variation of lysophospholipid analysis involved consideration of the sn-1 moiety which can exist as a vinyl ether, alkyl ether, or ester radyl substituent. These three possible structural variants of sn-1 lysophospholipids can be distinguished by CID of $[M + H]^+$, which results in an abundant loss of water only when an ester substituent is present at sn-1 or sn-2.[204] Loss of neutral water was not prominent for plasmalogens or sn-1 alkyl ether lyso-GPC species. A likely explanation for this major difference in the behavior of lyso-GPC subtypes was the higher proton affinity of the carbonyl oxygen atom or vinyl ether oxygen atom found in acyl and plasmenyl lyso-GPC lipids, respectively, as compared to the oxygen

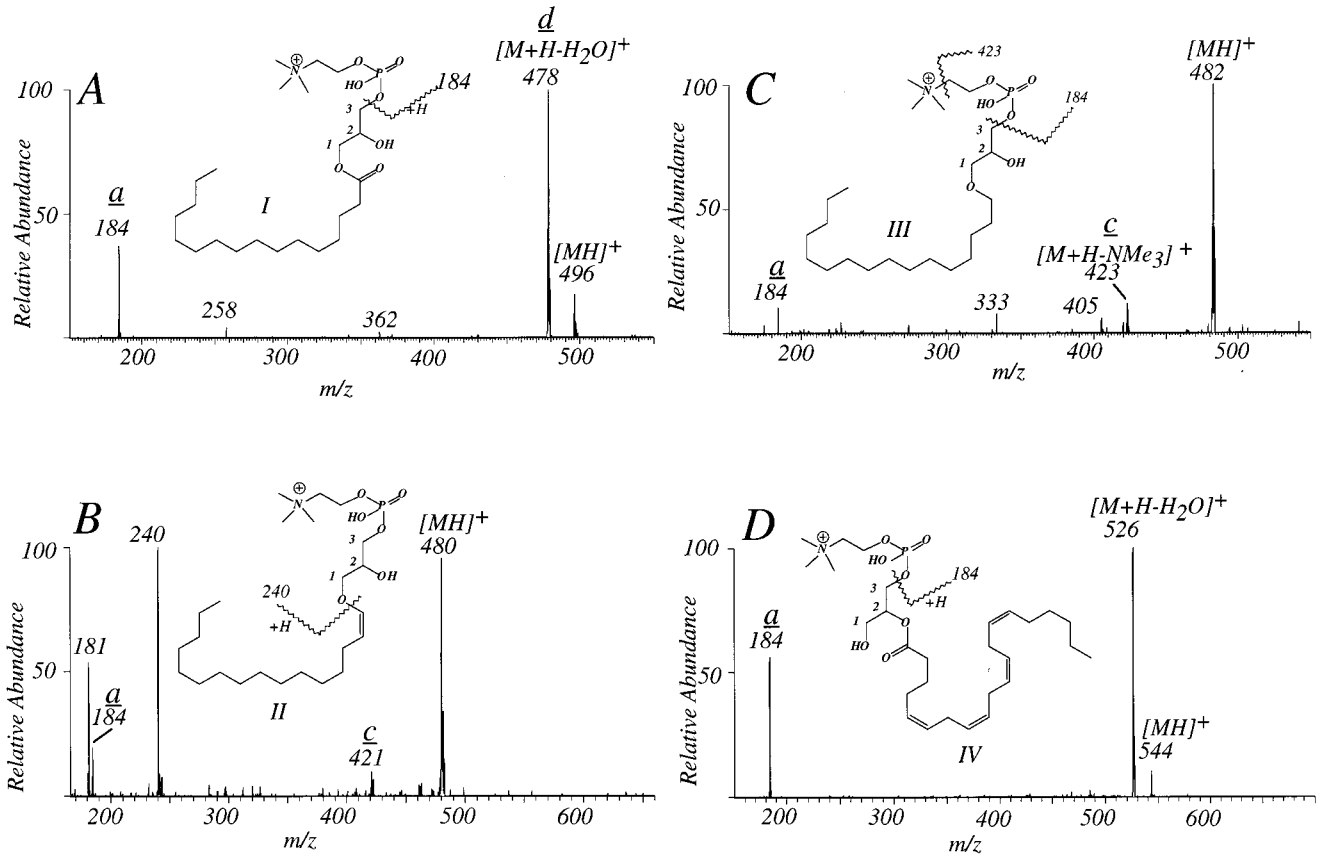


Figure 24. Collision-induced decomposition of electrospray-generated $[M + H]^+$ ions from four lysoglycerophosphocholine lipids. (A) 1-hexadecanoyl-2-lyso-glycerophosphocholine; (B) 1-*O*-(1′-ene-hexadecyl)-2-lyso-GPC. Collision-induced decompositions were carried out on a tandem quadrupole mass spectrometer using argon as collision gas. (Reprinted with permission from ref 204. Copyright 2000 Elsevier Science.)

atom present as a free alcohol. The plasmanyl lysophosphatidylcholines have an abundant ion at *m/z* 240 as a result of protonation of the vinyl ether oxygen atom in a charge-driven mechanism (Figure 24).[204]

4. Oxidized and Covalently Modified Phospholipids

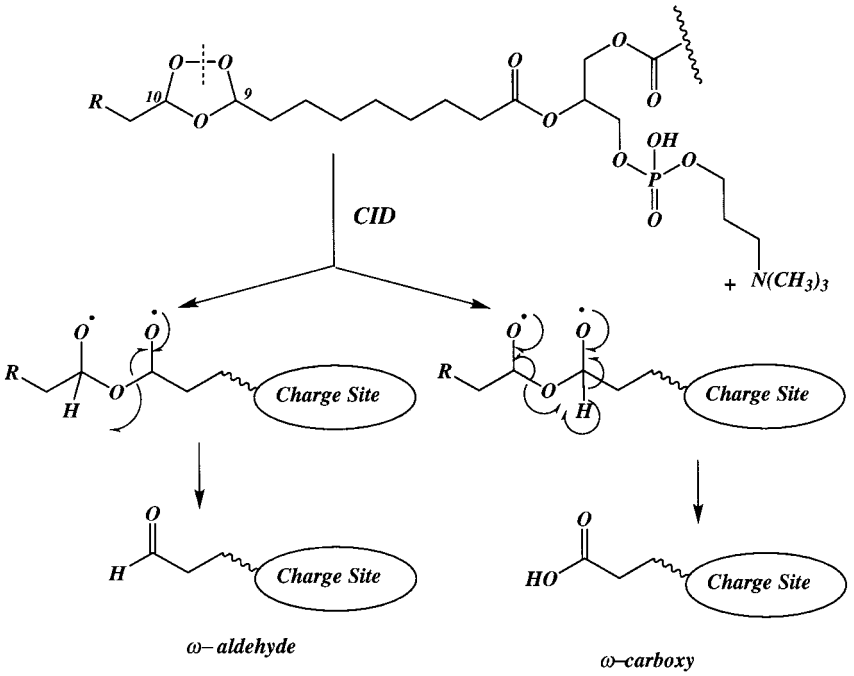
The ability to structurally characterize oxidized phospholipids isolated from biological membranes has emerged within the past few years as a result of developments of electrospray ionization and online HPLC to efficiently separate complex mixtures of molecular species present in cell or tissue extracts. Fast atom bombardment mass spectrometry first enabled studies of oxidized phospholipids such as with the reaction of ozone with 1-hexadecanoyl-2-hexadecenoyl-sn-glycerol-3-phosphocholine to yield the oxidized products 9′-oxononoyl- and 9′-carboxynonoyl acyl esters at the sn-2 position.[205]

Glycerophosphocholine lipids containing oleate, linoleate, or arachidonate esterified to the sn-2 position were reacted with ozone, and intact ozonide products could be detected by ESI-MS.[206] Collision-induced decomposition products resulted in ω-aldehyde and ω-carboxyl product ions (Scheme 38). This method gave information on double-bond positions within the fatty acyl group esterified to the phospholipid back-bond.

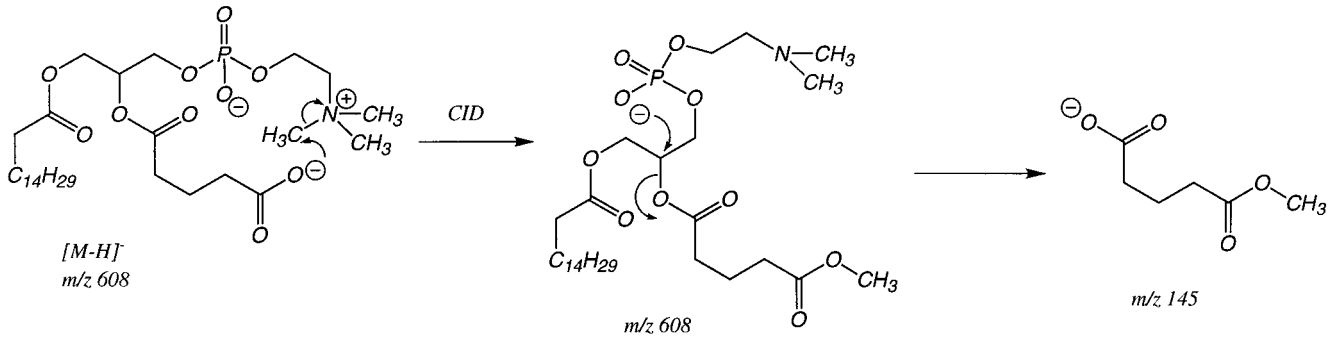
Hydroxyeicosatetraenoic acids esterified to GPC lipids have also been detected using FAB tandem mass spectrometry.[207] Collisional activation of $[M - 15]^-$ was found to yield product ions indicative of the numerous regioisomers of HETEs esterified to the glycerol phospholipid backbone. Tandem mass spectrometric studies have been reported for the collision-induced decomposition of the FAB and ESI molecular ion species derived from 5′-oxopentanoyl and 5′-carboxypentanoyl acyl esters of 1-hexadecanoyl-2-acyl-sn-glycerol-3-phosphocholine derived from the free radical oxidation of the precursor phospholipid which contained arachidonate esterified at the sn-2 position.[208,209] The presence of the chain-shortened, dicarboxylic acid which was esterified at the sn-2 position in 1-hexadecanoyl-2-(5′-carboxypentanoyl)-GPC altered considerably the product negative ions generated during fast atom bombardment ionization with an abundant ion corresponding to $[M - H]^-$ rather than $[M - 15]^-$, the molecular ion species typically observed for GPC lipids.[208] Collision-induced decomposition of $[M - H]^-$ yielded *m/z* 145 corresponding to the sn-2 carboxylate anion of the monomethyl ester of 1,5-pentanedicarboxylic acid. Using deuterium-labeled choline methyl groups, transfer of one choline methyl moiety to the μ-carboxyl moiety to form the methyl ester of the oxidized sn-2 acyl group was found to be responsible for this unexpected $[M - H]^-$ species and carboxylate anion as illustrated in Scheme 39.

Fast atom bombardment has also been used to detect the formation of isoprostanes esterified to

Scheme 38



Scheme 39



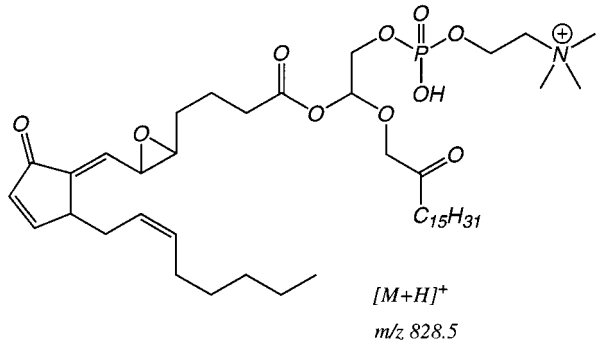
glycerophospholipids as products of oxidized arachidonic acid following initiation of free radical events within cellular membranes.[60,210] For these studies rats were treated with CCl_4, then phospholipids extracted from the livers of treated rats. There was a clear indication of several phospholipid molecular species that yielded the isoprostane carboxylate anion (*m*/*z* 353) during scanning for this specific precursor ion during the analysis of the complex mixture of phospholipids in the extract. One advantage of fast atom bombardment ionization was that carboxylate anions typically are formed during the ionization process, and this enabled collision-induced decomposition of *m*/*z* 353 from GPC-isoprostanes. The product ion spectrum of *m*/*z* 353 was found to be similar to that observed for prostaglandin $F_{2\alpha}$. These spectra were characterized by an abundant loss of C_2H_4O observed at *m*/*z* 309 as well as other characteristic ions, including *m*/*z* 193.[210]

Electrospray ionization has been used to follow the oxidation of polyunsaturated fatty acids esterified to GPC present in egg yolk phospholipids using *tert*-butylhydroperoxide (tBuOOH) as the free radical initiator.[211] The ion 1-stearoyl-2-arachidonoyl-GPC ($[M + Na]^+$, *m*/*z* 832) was found in this mixture of phospholipids to be most susceptible to oxidation. Formation of mono-, di-, and trihydroperoxides was evident from the appearance of $[M + Na]^+$ ions at *m*/*z* 864, 896, and 928, respectively. Tandem mass spectrometry of these suspected hydroperoxides was not employed to further examine the structures of these molecules. Electrospray ionization mass spectrometry was recently used to analyze oxidized polyunsaturated fatty acids present in glycerophospholipids isolated from red blood cell ghosts following treatment with tBuOOH. Those phospholipid molecular species that had one and two oxygen atoms attached to the arachidonate and linoleic portion of the fatty acyl groups in GPC and GPE lipids were investigated. Several different molecular species were identified as hydroperoxide phospholipids.[24,212] Upon elevation of the orifice potential in the negative-ion mode, it was possible to induce formation of carboxylate anions from hydroperoxy fatty acids that readily decomposed with loss of water to the $[M - H - H_2O]^-$ ions at *m*/*z* 317 (arachidonate as precursor) and 293 (linolenate as precurosr), respectively. Collisional activation of *m*/*z* 317 from the dehydrated hydroperoxy carboxylate anion, resulted in the appearance of product ions corresponding to 5-, 8-, 9-, 11-, 12-, and 15-oxo-ETE and a similar set of ions derived from the CID of *m*/*z* 293 supporting the presence of 13-

oxo-ODE and 9-oxo-ODE. Thus, tandem mass spectrometry was able to detect the formation of monohydroxy- and hydroperoxyeicosatetraenoic acids as well as monohydroxy- and hydroperoxyoctadecadienoate glycerophospholipids following oxidation of red blood cell plasma membranes. Electrospray ionization mass spectrometry was also used to detect the presence of hydroperoxy fatty acyl groups (hydroperoxyeicosatetraenoic acid) esterified to intact brain GPE molecular species following initiation of free radical oxidation.[213]

Some interest has been focused on the identification of biologically active phospholipids present in oxidized LDL that may be responsible, in part, for the progression and development of atherosclerosis. Development of electrospray ionization and LC/MS/MS strategies have been central to the advances in this area. LC/MS/MS was used to monitor HPLC effluents for the elution of phospholipid products isolated from oxidized LDL and their abundance correlated with biological activity measured in these LDL extracts.[214−216] Phospholipids isolated from oxidized LDL that induced monocyte−endothelial cell interaction were found to be 1-hexadecanoyl-2-(5′-oxopentanoyl)-GPC and 1-hexadecanoyl-2-(5′-carboxypentanoyl)-GPC.[214] These identifications were based in part on the appearance of $[M + H]^+$ ions at *m/z* 610 and 594 in the biologically active fractions. Additionally, collisional activation of $[M - 15]^-$ derived from the pentafluorobenzyl ester of one active GPC (*m/z* 774) yielded carboxylate anions at *m/z* 255 (16:0) and 311 (5:0 PFB ester). The other biologically active glycerophospholipid in this extract was converted to the methoxime derivative prior to collisional activation of $[M - 15]^-$ ion. Carboxylate anions at *m/z* 255 (16:0) and 144 were then observed, the latter ion corresponding to a methoxime derivative of an *ω*-aldehyde ester at the sn-2 position.[214] The use of derivatization to identify functionalized fatty acyl substituents in oxidized LDL followed by electrospray and tandem mass spectrometry has been employed in additional studies of oxidized phospholipids with trimethylsilylation of free alcohols, methoximation of carbonyl moieties, and ozonolysis of double bonds.[209] The trimethylsilyl ether derivatives of primary and secondary alcohols present in the fatty acyl chains of oxidized phospholipids were found to be completely stable to the aqueous alcohol solvent system used for the LC/MS/MS studies. The identifications of biologically active oxidized phospholipids in LDL with short alkyl chains at the sn-2 (acetate and butanoate) and an alkyl ether substituent at the sn-1 position were also performed by electrospray-tandem mass spectrometry.[215] A structurally diverse phospholipid with the biological activity of inducing monocyte adhesion to endothelial cells was recently described as an epoxyisoprostane GPC.[216] This oxidized GPC product was initially detected using electrospray mass spectrometry, and its structure, in large part, was determined by tandem mass spectrometry and NMR spectroscopy (Scheme 40). Treatment with sodium borohydride ($[M + H]^+$, *m/z* 832) or methoximation ($[M + H]^+$, *m/z* 875) were used to assist in functional group characterization. Tandem mass spectrometry

Scheme 40



of the phospholipase A_2 released fatty acyl substituent in the presence and absence of $H_2{}^{18}O$ led to the production of the carboxylate anion $[M - H]^-$ that was collisionally activated to further characterize this unusual sn-2 fatty acyl substituent.

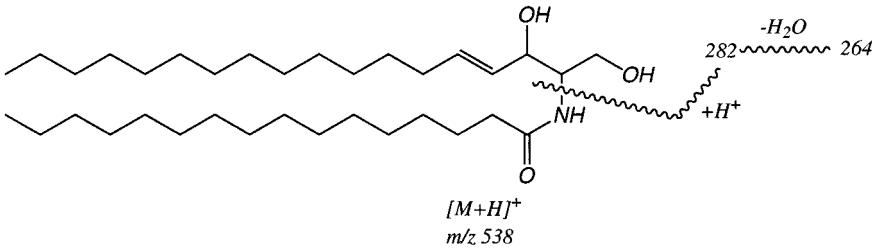
Other oxidatively modified glycerophospholipids have been investigated using electrospray mass spectrometry. One example[217] was the oxidation of 1-octadecanoyl-2-arachidonoyl-GPC ($[M + H]^+$, *m/z* 782) by soybean lipoxygenase in the presence of oxygen and nitric oxide (·NO). The extent of oxidation was followed by electrospray ionization and specific products identified by the appearance of new ions corresponding to the addition of one and two molecules of oxygen and oxygen plus NO. The products were consistent with an identification of monohydroxy (*m/z* 798), monohydroperoxy (*m/z* 846), dihydroperoxy (*m/z* 846), nitrito (*m/z* 827), and nitrosoperoxy (*m/z* 843) derivatives among others. The detection of adducts of nitrogen dioxide and nitric oxide with free radical intermediates of glycerophospholipids was somewhat easier by the fact that the major ions observed at $[M + H]^+$ or $[M - 15]^-$ were at odd mass because of the presence of an additional nitrogen atom in these species.

Other unusual glycerophospholipids which have been covalently modified and then analyzed by electrospray mass spectrometry include glycated ethanolamine phospholipids which were found to make up approximately 10% of the total phosphatidylethanolamine phospholipids in red blood cells of diabetic patients.[218]

VI. Sphingolipids

Sphingolipids are a structurally diverse family of lipid molecules that include ceramides, cerebrosides, sphingomyelin, sulfatides, gangliosides, and quite complex glycosphingolipids. The common feature for this class of lipids is the presence of a long-chain base sphinganine or 4-*E*-sphinginene (sphingosine) to which a fatty acid has been acylated to the free amino group. Sphingolipids play unique and important roles in cell biology and cellular function, for example, ranging from intracellular signaling as a second messenger in cellular apoptosis[219] to membrane structural functions. Several reviews describing the electron ionization mass spectrometry of long-chain bases[220,221] and fast atom bombardment of sphingolipids including glycosphingolipids have appeared.[222]

Scheme 41



A. Ceramides

Acylation of the primary amine of a long-chain base such as sphingosine or sphinganine yields a ceramide which typically exists within cells as a family of molecular species defined by the fatty acyl group (Scheme 41) as well as the long-chain base. While the number of molecular species is somewhat smaller than that encountered for glycerophospholipids, the fatty acyl groups typically encountered are more diverse, including odd chain and very long chain fatty acyl groups such as nervonyl (24:1) and lignoceryl (24:0) and α-hydroxy fatty acyl substituents. Interest in potential biological activity of ceramides has initiated many studies involving the use of electrospray ionization and tandem mass spectrometry for quantitative analyses.

Electrospray ionization of ceramide molecular species yielded abundant $[M + H]^+$ ions.[223] Ceramides also formed abundant $[M + H]^+$ ions using APCI with the most abundant ceramide species present in HL60 cells determined to be *N*-palmitoyl-(16:0), *N*-nervonyl (24:1), and *N*-lingnoceryl (24:0) sphingosine using direct APCI analysis.[224] The collisional activation of ceramide-derived $[M + H]^+$ ions yielded abundant product ions corresponding to $[M + H - H_2O]^+$ and somewhat less abundant $[M + H - 2(H_2O)]^+$[223] as well as an ion corresponding to the cleavage between the carbon and nitrogen bond of the sphingosine base (Figure 25). This ion is observed at *m/z* 282 for sphingosine. This cleavage and the product ions resulting from the loss of water were also observed in the collision-induced decomposition of various glycosphingolipid $[M + H]^+$ ions found by FAB ionization.[225] The loss of an additional neutral water molecule resulted in the abundant ion at *m/z* 264 for *N*-palmitoyl sphingosine (Scheme 41). Sphinganine-based ceramides yield the corresponding product ion at *m/z* 266. Generation of this product ion from ceramides has been used as a basis for the sensitive detection of individual ceramide species present in cell extracts.[223,226] Due to the facile loss of water, several precursor ions are detected for each molecular species when using precursor ion scanning; nevertheless, the precursor ion scanning is quite useful for the analysis of ceramide molecular species isolated from tissues. The precursor ion scanning protocol has been used to measure the generation of specific ceramide molecular species in T-cells using C2:0 ceramide added as a quantitative internal standard.[227]

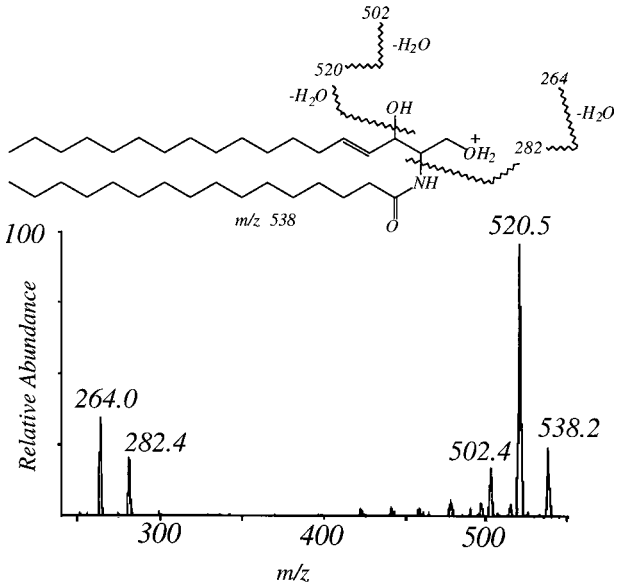


Figure 25. Electrospray tandem mass spectrometry of 16:0 ceramide derived from *Lagenidium giganteum* in a tandem quadrupole mass spectrometer. (Reprinted with permission from ref 223. Copyright 1997 Academic Press.)

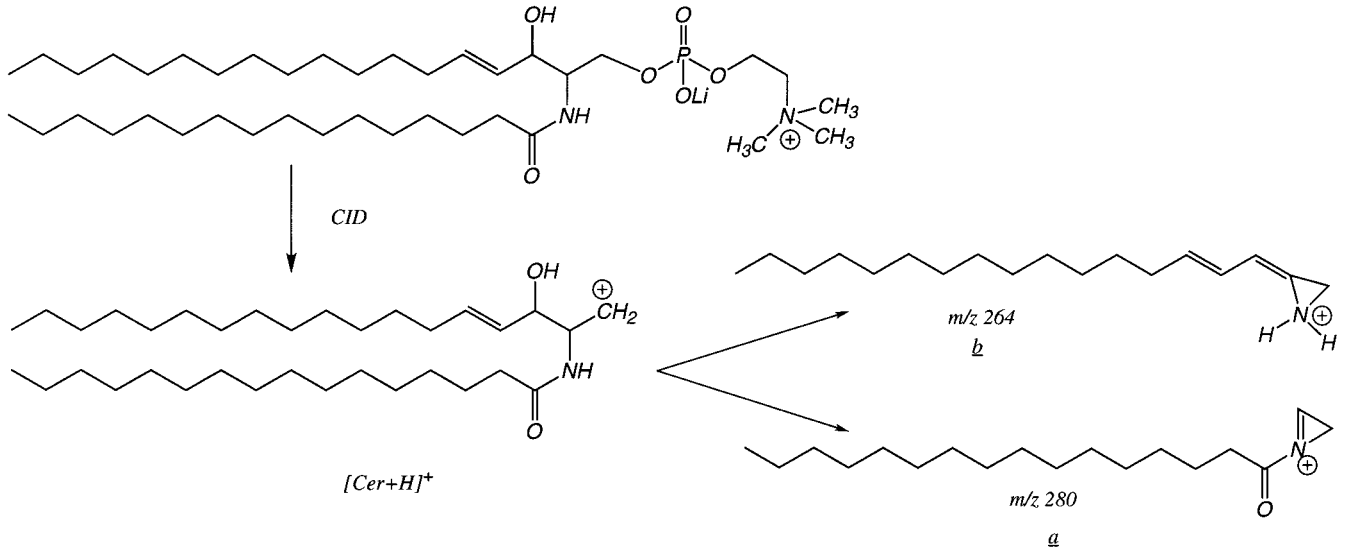
A quantitative assay for ceramides using the MRM assay for *m/z* 264 was also employed for the analysis of a complex mixture of naturally occurring ceramides in other cells.[228] The ratio of ion abundances of the precursor $[M + H]^+$ as well as $[M + H - H_2O]^+$ relative to the corresponding ions derived from a C8:0-ceramide, added as internal standard, was found to be linear with up to 20 nmol ceramide present in lipid extracts. This assay was also found to be quite specific for ceramides and did not detect other sphingolipids.

Negative ions are also generated during electrospray ionization of ceramides as illustrated for *N*-palmitoyl ceramide with the production of $[M + Cl]^-$ observed at *m/z* 572.[229] Collision-induced dissociation of this adduct ion generated essentially only one ion, Cl^- (*m/z* 35). This observation was employed to identify one of the lipid substances that significantly increased in Jurkat cells that were induced to undergo apoptosis.[229]

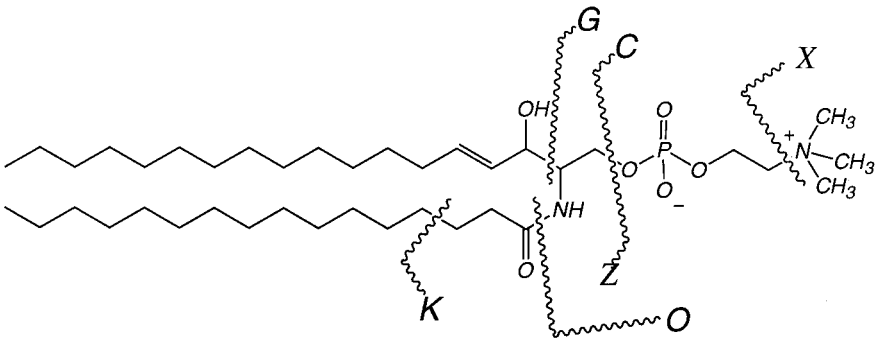
B. Sphingomyelin

Sphingomyelin is an abundant phospholipid found in the cellular membranes of most cells and is a phosphocholine ester of ceramide. This sphingolipid readily generated abundant $[M + H]^+$ ions and $[M - 15]^-$ anions as may be expected for a phosphocholine lipid as previously described for GPC lipids by electrospray ionization.[176] Collisional activation of $[M + H]^+$ ions yielded the abundant phosphocholine ion at *m/z* 184, but CID of the $[M - 15]^-$ did not yield abundant product ions.[176]

Scheme 42



Scheme 43



Sphingomyelins readily form alkali-metal adducts, and Hsu and Turk[230] described the electrospray tandem mass spectrometric behavior of $[M + Li]^+$ derived from sphingomyelin in a tandem quadrupole instrument. Collisional activation of $[M + Li]^+$ yielded abundant fragment ions corresponding to loss of the phosphocholine $[M + Li - 183]^+$ and $[M + Li - 183 - H_2O]^+$. Less abundant product ions were also formed; however, these ions were indicative of the fatty acyl group (a) and long-chain base (b) (Scheme 42).

As in the case with GPC lipids, much of the behavior of the $[M + H]^+$ and $[M + Na]^+$ ions derived from sphingomyelin following low-energy collisional activation was first investigated using FAB ionization[231] with abundant formation of *m/z* 184 as described above. However, CID of $[M - 15]^-$, $[M - 60]^-$, and $[M - 86]^-$ species generated by FAB did yield product ions. Specific negative ions derived from cleavage of these phosphocholine negative ions as well as the ceramide portion were observed at high-energy collisions and are summarized in Scheme 43.

In contrast to the failure to obtain structurally relevant product ions following collisional activation of sphingomyelin negative ions generated by electrospray ionization, APCI yields several abundant positive ions (Figure 26) suggested to arise from in-source fragmentation of this sphingolipid.[232] One of the more abundant APCI ions was suggested to originate from the ceramide portion of the molecule after loss of the phosphocholine with structures suggested for $[M + Li]^+$ product ions (Scheme 41). APCI and ESI were used to identify dihydrosphingomyelin and sphingomyelin in brain tissue and human plasma.[156,233] ESI has been more widely used to determine sphingomyelin molecular species composition by the abundant of $[M + H]^+$ ions as exemplified by the studies of the phospholipid extracted from the horseshoe crab (*L. polyphemus*).[234]

C. Glycosphingolipids

Quite complex lipids occur on the membrane of eucaryotic cells such as ganglioside and sialyl Lewis-type glycosphingolipids. The cell walls of lower organisms contain carbohydrate residues covalently linked to the ceramide that serve, in part, to anchor these structures to the lipid bilayer. The structural characterization of these diverse lipids is exceedingly difficult, often requiring NMR spectroscopy as well as mass spectrometric approaches. Electrospray,[235−243] MALDI,[243−249] and FAB ionization[250−255] have been used to successfully study these molecules as underivatized species. High-performance tandem mass spectrometers (four-sector instruments) were found to be of particular value in the analysis of these structurally complex substances.[256] Chemical ioniza-

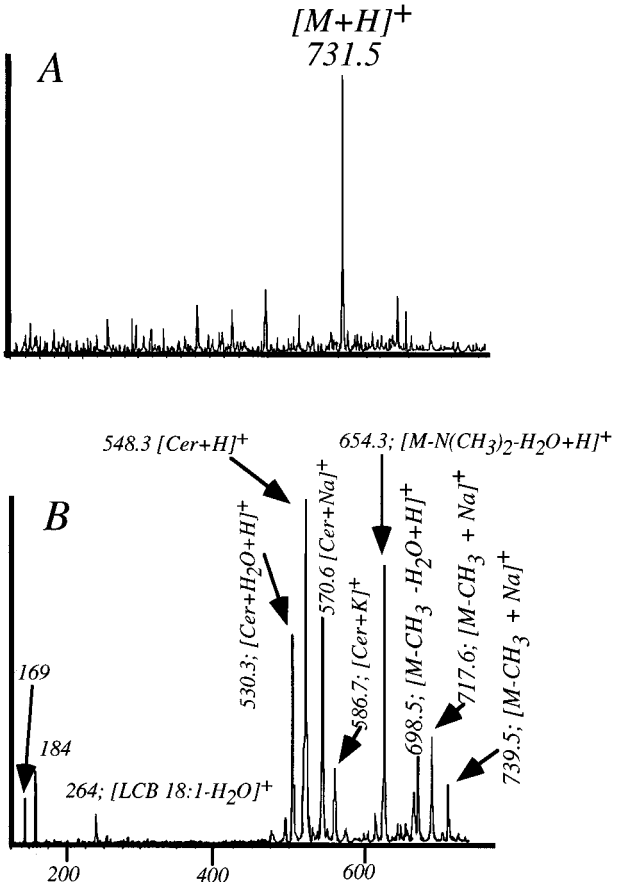


Figure 26. Positive ions from sphingomyelin (d18:1/18:0) obtained by (A) electrospray ionization and (B) atmospheric pressure chemical ionization. (Adapted with permission from ref 232. Copyright 1998 John Wiley & Sons.)

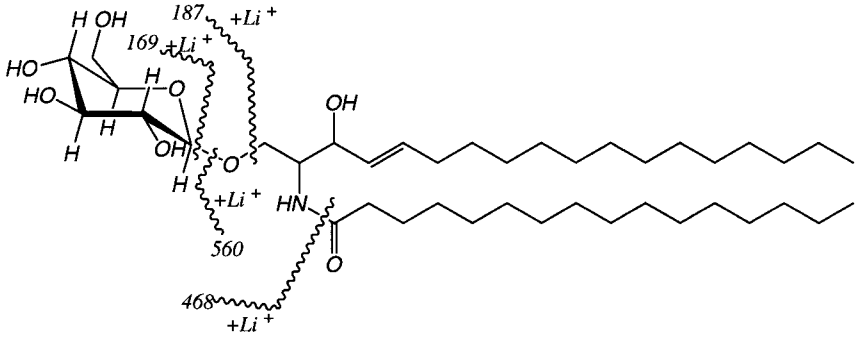
tion using supercritical fluid chromatography has also been used for this class of lipid.[257] Since much of the detailed mass spectrometry is focused on the carbohydrate portion of these molecules, only a few examples will be discussed.

Glucosylceramides (Scheme 44) can also be directly studied by mass spectrometric techniques as exemplified by the characterization of glycosyl ceramide from the spleen of a patient with Gaucher's disease,[258] by blood group glycosphingolipids,[259] and more recently, by the report of the FAB mass spectrometry of this class of sphingolipids present in soybean and wheat extracts using a four-sector tandem mass spectrometer.[260] These latter investigators used 3-nitrobenzyl alcohol containing LiI as matrix to generate abundant $[M + Li]^+$ and $[M - H]^-$ molecular ion species. Studies of the high-energy CID of these molecular ion species yielded a number of structurally relevant ions that could be used to identify not only the long-chain base, but also the acylated fatty acid substituents. FAB has also been used to study unique phosphonosphingolipids in shell fish and structurally identify a series of ceramide aminoethylphosphonate and *N*-methylaminoethylphosphonate molecular species.[261]

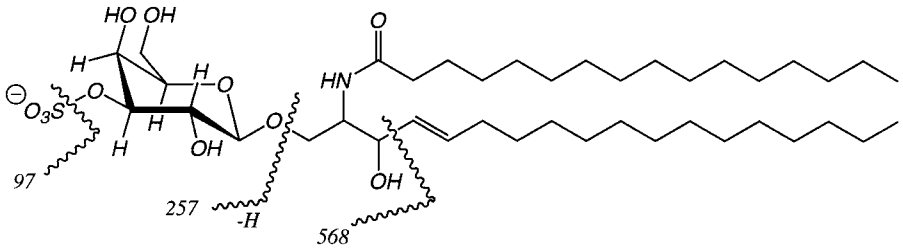
Sulfatides are glycosphingolipids containing a 3-sulfogalactosyl group linked to ceramide (Scheme 45). Abundant negative ions $[M - H]^-$ were found by electrospray ionization[262,263] that readily decomposed in a tandem quadrupole instrument following collisional activation. The most abundant product ion was *m/z* 97 (SO_3^-), and this transition could be used to uniquely detect sulfatides in lipid extracts. Other product ions are also indicative of the fatty acyl group and sulfogalactosyl moiety.[263] MALDI also yielded abundant $[M - H]^-$ ions.[264]

A hybrid magnetic sector-TOF mass spectrometer was used to investigate the collisional activation of glycosphingolipids containing one to six saccharide groups attached to ceramide isolated from human intestine.[265] The CID of $[M + Li]^+$ resulted in an abundant array of structurally relevant ions derived from the Li^+ attachment to the hexose which defined the carbohydrate portion of the molecule. One important product ion corresponded to cleavage of the

Scheme 44



Scheme 45



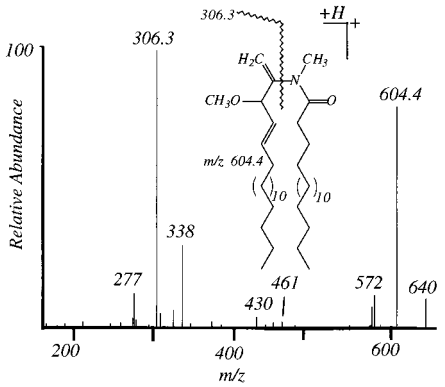
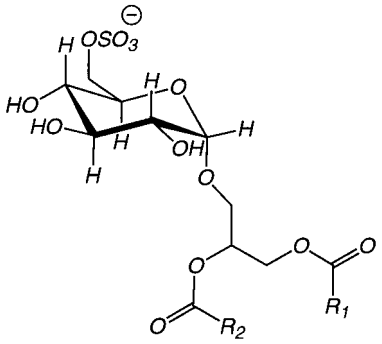


Figure 27. Electrospray ionization of methylated disialylglycerolsphingolipid in an ion trap mass spectrometer. Collisional activation of the doubly charged ion observed at *m/z* 1131.7 ($[M + 2H]^{2+}$) resulted in a product ion at *m/z* 604.5. This ion was subsequently trapped and subjected to collisional activation in an MS^3 experiment with the resultant product ions shown in this mass spectrum. (Reprinted with permission from ref 270. Copyright 1998 Academic Press.)

Scheme 46



fatty acyl substituent, and although somewhat less abundant, this ion enabled the identification of the fatty acyl substituent on the long chain base.[265]

Cerebrosides isolated from mycopathogens have been characterized by ESI-tandem mass spectrometry based on similar fragmentations to those outlined above for glucosylceramides.[266] These fragmentations have been observed for many of the glycosphingolipids using FAB ionization, and a nomenclature for the designation of these ions has been established.[267]

An alternative approach to structurally investigate such complex glycosphingolipids has been to covalently modify the lipid in a way to facilitate subsequent analysis. This was the case in a study of the alkaline oxidation of galactosyl-, lactosyl-, and glucosyl-ceramides along with other glycosphingolipids followed by ESI-tandem mass spectrometry.[268] The alkaline $KMnO_4$ efficiently converted the hydroxyallylic moiety of the sphingolipid long-chain base into a carboxyl group and cleavage of the hydrocarbon chain. The resulting serine acids were found to behave quite well by electrospray tandem mass spectrometry. Glycosphingolipids have also been analyzed by APCI ionization methods.[269]

Electrospray ionization of a methylated disialylglycerosphingolipid was used to generate the dicationic species $[M + 2H]^{2+}$ (*m/z* 1131.7), which was studied in an ion- trap mass spectrometer.[270] Collisional activation of this doubly charged ion (MS^2) resulted in a series of product ions including one observed at *m/z* 604.5 suspected to have been derived from the lipid portion of the molecule. Further isolation of this ion and collisional activation (MS^3) in the ion trap resulted in an abundant product ion at *m/z* 306, indicative of cleavage of the sphingosine base C−N bond (Figure 27A). The ability to generate this MS^3 spectrum (Figure 27B) suggested that the analysis of complex lipids such as glycosphingolipids may be successfully carried out using ion-trap technology or Fourier transform ion cyclotron resonance.

VII. Other Lipids

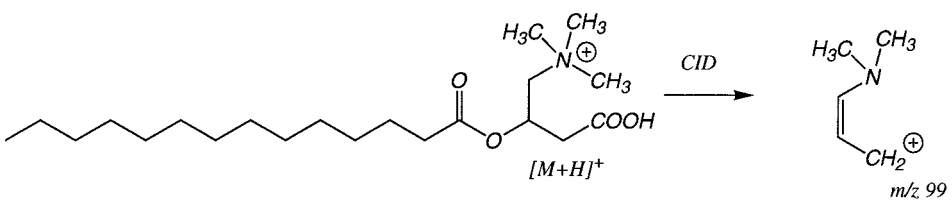
A host of other lipid substances occur in biology. While mass spectrometry has been used to detect and even quantitate these lipids, only a few detailed studies of their gas-phase ion chemical behavior have been carried out. One example is the recent use of FAB[271] and MALDI-TOF with postsource decay[272] to probe the structures of numerous molecular species of sulfoquinovosol diacylglycerols (Scheme 46) isolated from plants.

Another relevant example is the measurement of acylcarnitines to screen for genetic defects in newborn infants (inborn errors of metabolism). Acylcarnitines (Scheme 47) are biosynthetic intermediates within the cell and also present in plasma that assist in the transport of fatty acids as carnitine esters.

A major common ion observed in FAB and ESI tandem mass spectrometry of the positive ions $[MH]^+$ from acylcarnitines was found to be *m/z* 99.[273] Therefore, a precursor ion scan for *m/z* 99 yielded those molecular ions derived from acylcarnitine molecular species present in an extract. This approach has been used to screen samples even from blood dot blots[274] to detect inborn errors of metabolisms in a large number of studies.[275−277]

The coenzyme A esters of fatty acids are the required intracellular intermediates in the biosynthesis of all phospholipids, sphingolipids, glycerides, as well as proteolipids and most glycolipids. They have not been widely studied by mass spectrometry but have been shown to behave quite well by FAB,[278]

Scheme 47



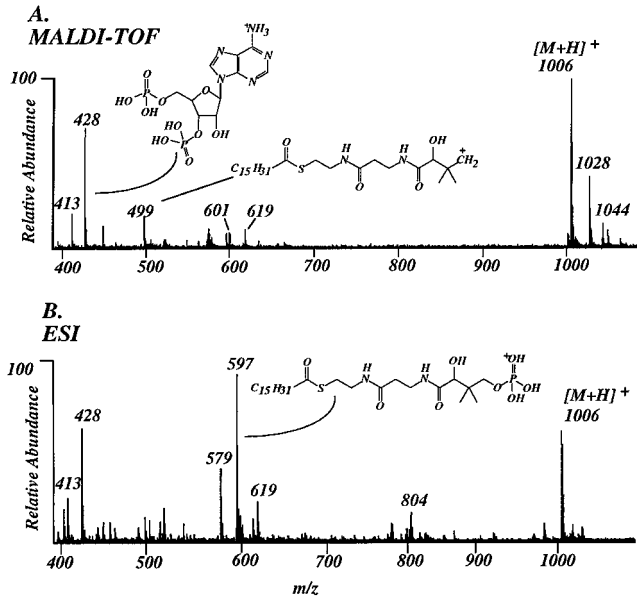


Figure 28. Positive ions obtained for the analysis of hexadecanoyl coenzyme A using (A) postsource decay of $[M + H]^+$ ion (*m*/*z* 1006) obtained by matrix-assisted laser desorption time-of-flight analysis. (B) Collisional activation of $[M + H]^+$ formed by electrospray ionization in a tandem quadrupole mass spectrometer using argon as collision gas. (Reprinted with permission from ref 279. Copyright 1997 John Wiley & Sons.)

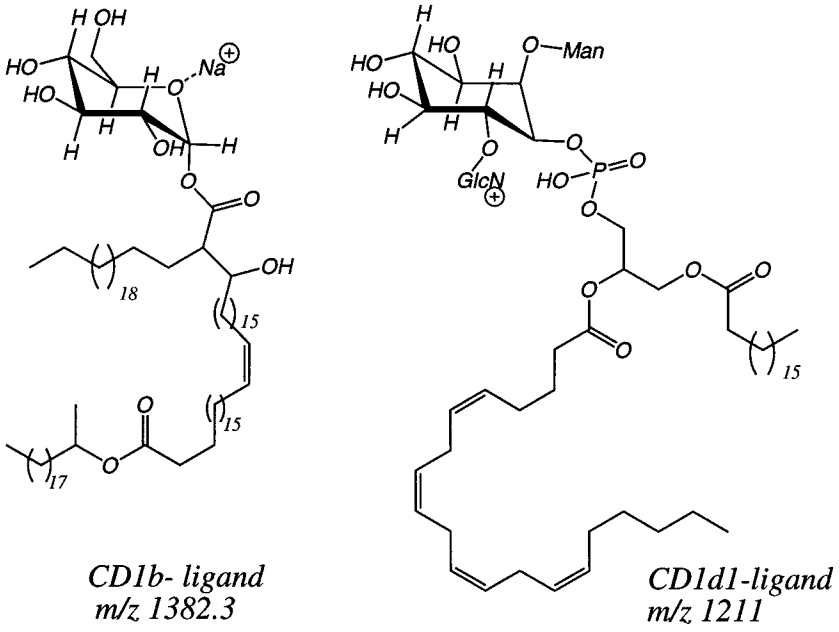
electrospray, or MALDI-TOF[279] to yield the structurally relevant ions (Figure 28).

The complexity of the bacterial cell wall and the constituent lipids including lipopolysaccharides have rendered these molecules difficult to study even with the most sophisticated tools available. Molecular ion species from these complex lipids (Figure 29) can be generated using electrospray mass spectrometry as illustrated for lipid extracted from *P. aeruginosa*.[280] Tandem mass spectrometry can also yield information concerning fatty acyl substituents.[281] At the present time, molecular weight information obtained by electrospray ionization is a critical component of all structural studies of the lipopolysaccharides isolated from various bacterial strains.[282−289]

Additional complex lipids such as glycosylphosphatidyl membrane anchors present in mammalian cells are now being studied using electrospray mass spectrometry,[290] and collisional activation has been found to yield relevant information concerning the fatty acyl groups present in these molecules.[291−292]

Challenges remain in the structure elucidation of complex lipids that play important biological roles as exemplified in the recent advances made in the field of immunology. It is now recognized that certain complex lipids serve a central role in T-lymphocyte recognition of bacteria and foreign antigens.[293] A family of major histocompatibility-like glycoproteins, called CD1, are known to bind specific and complex lipids for subsequent presentation to T-lymphocytes as an important element of adaptive immunity. The structural characterization of lipids recognized by CD1-presenting proteins is a difficult task even with present-day tools. Typically, these molecules are isolated in small quantities as complex mixtures which must be separated prior to analysis. One ligand for human CD1b was recently identified as a glucose monomycolate (GMM) (Scheme 48) isolated from a T-cell line exposed to extracts of *Mycobacterium leprae*. The electrospray mass spectrometric analysis of this ligand revealed a family of molecular species likely differing by alkyl chain length with the most abundant molecular ion species observed at *m*/*z* 1382 in the positive-ion mode corresponding to the sodium salt of GMM containing a monounsaturated C_{80}-ester of mycolic acid.[294] MALDI mass spectrometry was used to characterize another CD1 ligand isolated from CD1d1 as a complex glycosyl phosphatidylinositol.[295] Hydrolysis with ammonium sulfate yielded 1-octadecanoyl-2-arachidonoyl-glycerophosphoinositol backbone (Scheme 48). Negative-ion MALDI of this phospholipid product generated the expected anion at *m*/*z* 885.3

Scheme 48



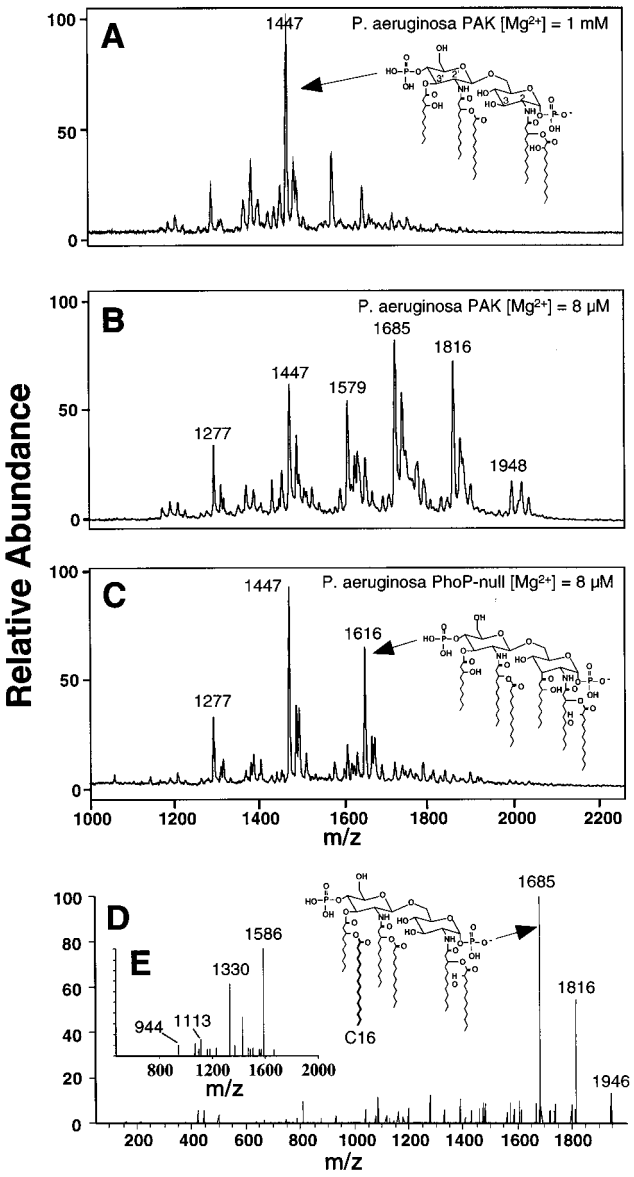


Figure 29. MALDI-TOF mass spectra of lipid A from different strains of *P. aeruginosa*. (A) Lipid A from PAK strain grown under high-magnesium conditions with the dominant penta-acylated from yielding the $[M - H]^-$ at *m/z* 1447. (B) Lipid A from PAK strain under low-magnesium conditions, showing additions of C16:0 (*m/z* 1685), C16:0 with one amino arabinose group (*m/z* 1816) and C16:0 with two amino arabinose groups (*m/z* 1948). (C) MALDI-TOF mass spectrum of lipid A from *P. aeruginosa*, PhoP mutant strain. (D) Electrospray MS^2 (triple quadrupole) mass spectrum of the precursor ions of *m/z* 1948. (E) Ion fragments derived from the C16:0-modified hexa-acylated precursor for *m/z* 1685. (Reprinted with permission from ref 280. Copyright 1999. American Association for the Advancement of Science.)

using α-cyano-4-hydroxycinamic acid as matrix. The carbohydrate portion of the molecule was not fully elucidated but was consistent with the addition of mannose and glucosamine to this backbone structure. Other antigenic lipids were clearly present in the extract of CD1d1 ligands with the addition of more complex oligosaccharides attached to the glycosylphosphatidylinositol. Structural characterization of such antigenic compounds represent the leading edge in the mass spectrometry of nonvolatile lipids.

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