

5-6-2007

## Elucidation of Double Bond Position in Unsaturated Lipids by Ozone Electrospray Ionization Mass Spectrometry (OzESI-MS)

M. C. Thomas  
*University of Wollongong*

Todd Mitchell  
*University of Wollongong, toddm@uow.edu.au*

J. M. Deeley  
*University of Wollongong*

D. G. Harman  
*University of Wollongong*

R. C. Murphy  
*University of Colorado Denver Health Sciences Center, USA*

*See next page for additional authors*

Follow this and additional works at: <https://ro.uow.edu.au/scipapers>

 Part of the [Life Sciences Commons](#), [Physical Sciences and Mathematics Commons](#), and the [Social and Behavioral Sciences Commons](#)

---

### Recommended Citation

Thomas, M. C.; Mitchell, Todd; Deeley, J. M.; Harman, D. G.; Murphy, R. C.; and Blanksby, Stephen J.: Elucidation of Double Bond Position in Unsaturated Lipids by Ozone Electrospray Ionization Mass Spectrometry (OzESI-MS) 2007.  
<https://ro.uow.edu.au/scipapers/90>

---

# Elucidation of Double Bond Position in Unsaturated Lipids by Ozone Electrospray Ionization Mass Spectrometry (OzESI-MS)

## Abstract

The position(s) of carbon-carbon double bonds within lipids can dramatically affect their structure and reactivity and thus has a direct bearing on biological function. Commonly employed mass spectrometric approaches to the characterization of complex lipids however, fail to localize sites of unsaturation within the molecular structure and thus cannot distinguish naturally occurring regioisomers. In a recent communication [Thomas, Mitchell, Blanksby in the Journal of the American Chemical Society, 2006, 128, 58-59] we have presented a new technique for the elucidation of double bond position in glycerophospholipids using ozone-induced fragmentation within the source of a conventional electrospray ionization mass spectrometer. Here we report the on-line analysis, using ozone electrospray mass spectrometry (OzESI-MS), of a broad range of common unsaturated lipids including, acidic and neutral glycerophospholipids, sphingomyelins and triacylglycerols. All lipids analyzed are found to form a pair of chemically induced fragment ions diagnostic of the position of each double bond(s) regardless of the polarity, the number of charges or the adduct ion (e.g., [M-H]<sup>-</sup>, [M-2H]<sup>2-</sup>, [M+H]<sup>+</sup>, [M+Na]<sup>+</sup>, [M+NH<sub>4</sub>]<sup>+</sup>). The ability of OzESI-MS to distinguish lipids that differ only in the position of the double bonds is demonstrated using the glycerophosphocholine standards, GPCho(9Z-18:1/9Z-18:1) and GPCho(6Z-18:1/6Z-18:1). While these regioisomers cannot be differentiated by their conventional tandem mass spectra, the OzESI-MS spectra reveal abundant fragment ions of distinctive mass-to-charge ratio. The approach is found to be sufficiently robust to be used in conjunction with the m/z 184 precursor ion scans commonly employed for the identification of phosphocholine-containing lipids in shotgun lipidomic analyses. This tandem OzESI-MS approach was used - in conjunction with conventional tandem mass spectral analysis - for the structural characterization of an unknown sphingolipid in a crude lipid extract obtained from a human lens. The OzESI-MS data confirm the presence of two regioisomers, namely SM(d18:0/15Z-24:1) and SM(d18:0/17Z-24:1) and suggest the possible presence of a third isomer, SM(d18:0/19Z-24:1), in lower abundance. The data presented herein demonstrate that OzESI-MS is a broadly applicable, on-line approach for structure determination and when used in conjunction with established tandem mass spectrometric methods, can provide near complete structural characterization of a range of important lipid classes. As such, OzESI-MS may provide important new insight into the molecular diversity of naturally occurring lipids.

## Keywords

lipids, lipidomics, ozone, electrospray ionization, mass spectrometry, double bond position, ozonolysis, CMMB

## Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

## Publication Details

This article was originally published as Thomas, MC, Mitchell, TW, Deeley, JM, Harman, DG, Murphy, RC and Blanksby, SJ, Elucidation of Double Bond Position in Unsaturated Lipids by Ozone Electrospray Ionization Mass Spectrometry (OzESI-MS), *Analytical Chemistry*, 79(13), 2007, 5013-5022. Original article available [here](#).

## Authors

M. C. Thomas, Todd Mitchell, J. M. Deeley, D. G. Harman, R. C. Murphy, and Stephen J. Blanksby

Elucidation of Double Bond Position in Unsaturated Lipids by Ozone Electrospray  
Ionization Mass Spectrometry (OzESI-MS)

Michael C. Thomas,<sup>†</sup> Todd W. Mitchell,<sup>‡</sup> David G. Harman,<sup>†</sup> Jane M. Deeley,<sup>†</sup> Robert C. Murphy,<sup>§</sup>  
Stephen J. Blanksby<sup>\*,†</sup>

<sup>†</sup> Department of Chemistry, University of Wollongong, Wollongong NSW 2522, Australia.

<sup>‡</sup> School of Health Sciences, University of Wollongong, Wollongong NSW 2522, Australia.

<sup>§</sup> Department of Pharmacology, University of Colorado at Denver and Health Sciences Center, Aurora  
CO 80045, USA

\*Address reprint requests to Dr Stephen J. Blanksby, Department of Chemistry, University of  
Wollongong, Wollongong NSW, 2522, Australia. E-mail [blanksby@uow.edu.au](mailto:blanksby@uow.edu.au)

## Abstract

The position(s) of carbon-carbon double bonds within lipids can dramatically affect their structure and reactivity and thus has a direct bearing on biological function. Commonly employed mass spectrometric approaches to the characterization of complex lipids however, fail to localize sites of unsaturation within the molecular structure and thus cannot distinguish naturally occurring regioisomers. In a recent communication [Thomas, Mitchell, Blanksby in the Journal of the American Chemical Society, **2006**, *128*, 58-59] we have presented a new technique for the elucidation of double bond position in glycerophospholipids using ozone-induced fragmentation within the source of a conventional electrospray ionization mass spectrometer. Here we report the on-line analysis, using ozone electrospray mass spectrometry (OzESI-MS), of a broad range of common unsaturated lipids including, acidic and neutral glycerophospholipids, sphingomyelins and triacylglycerols. All lipids analyzed are found to form a pair of chemically induced fragment ions diagnostic of the position of each double bond(s) regardless of the polarity, the number of charges or the adduct ion (*e.g.*,  $[M-H]^-$ ,  $[M-2H]^{2-}$ ,  $[M+H]^+$ ,  $[M+Na]^+$ ,  $[M+NH_4]^+$ ). The ability of OzESI-MS to distinguish lipids that differ only in the position of the double bonds is demonstrated using the glycerophosphocholine standards, GPCho(9Z-18:1/9Z-18:1) and GPCho(6Z-18:1/6Z-18:1). While these regioisomers cannot be differentiated by their conventional tandem mass spectra, the OzESI-MS spectra reveal abundant fragment ions of distinctive mass-to-charge ratio. The approach is found to be sufficiently robust to be used in conjunction with the  $m/z$  184 precursor ion scans commonly employed for the identification of phosphocholine-containing lipids in shotgun lipidomic analyses. This *tandem* OzESI-MS approach was used - in conjunction with conventional tandem mass spectral analysis - for the structural characterization of an unknown sphingolipid in a crude lipid extract obtained from a human lens. The OzESI-MS data confirm the presence of two regioisomers, namely SM(d18:0/15Z-24:1) and SM(d18:0/17Z-24:1) and suggest the possible presence of a third isomer, SM(d18:0/19Z-24:1), in

lower abundance. The data presented herein demonstrate that OzESI-MS is a broadly applicable, on-line approach for structure determination and when used in conjunction with established tandem mass spectrometric methods, can provide near complete structural characterization of a range of important lipid classes. As such, OzESI-MS may provide important new insight into the molecular diversity of naturally occurring lipids.

**Keywords:** lipids, lipidomics, ozone, electrospray ionization, mass spectrometry, double bond position, ozonolysis

## Introduction

The application of electrospray ionization tandem mass spectrometry (ESI-MS/MS) to lipid analysis in recent years has demonstrated several key advantages over the more traditional chromatographic methods.<sup>1-4</sup> ESI-MS/MS spectra of an individual lipid can often be obtained from a crude lipid extract without the need for prior separation, saponification or derivatization thus making the analyses faster and more structurally specific.<sup>5, 6</sup> Furthermore, the tandem mass spectra of most major classes of lipids, including glycerophospholipids, sphingomyelins and triacylglycerols, clearly identify the carbon chain length and degree of unsaturation of the fatty acids bound to the parent molecule.<sup>7-14</sup> As a consequence, this is an extremely powerful methodology for the structural characterization of lipids present in biological extracts and is largely responsible for the current renaissance in lipid mass spectrometry.

Despite the advantages that ESI-MS/MS offers, several limitations remain in its use as a tool for the unambiguous structural identification of lipids. In particular, conventional ESI-MS/MS of most lipid classes cannot uniquely assign carbon-carbon double bond position. For example, Figure 1 shows the negative ion ESI-MS/MS spectra of two glycerophospholipids and is typical of data utilized in modern lipid analysis. Significantly, the spectra of regioisomers GPCho(9Z-18:1/9Z-18:1) and GPCho(6Z-18:1/6Z-18:1) show no fragment ions resulting from C-C bond cleavage processes with the result that the regioisomeric fatty acids 6Z-18:1 and 9Z-18:1 and thus the parent glycerophospholipids are indistinguishable. Similar observations have been reported for the tandem mass spectra of other isomeric phospholipids.<sup>15</sup> The inability of conventional ESI-MS/MS to readily identify double bond position represents a significant impediment to the development of rapid and structurally specific lipidomic technologies. Isomeric lipids that differ only in double bond position, can have distinct biochemical and biophysical properties. For example, several naturally occurring isomers of linoleic acid (18:2) have been identified and found to have differing biological activities.<sup>16</sup> Thus the

categorization of lipids purely on their degree of unsaturation belies the inherent diversity in lipid biology.

### [Figure 1]

Several approaches have been trialed to identify double bond position in intact lipids using mass spectrometry. Conceptually, the simplest of these methods is the collision induced dissociation (CID) of the carboxylate anions formed upon fragmentation of the parent phospholipid anion in an MS<sup>3</sup> experiment. Comparison of the resultant MS<sup>3</sup> spectrum with the MS/MS spectrum of the deprotonated free fatty acid can, in some instances, elucidate the double bond position in the bound fatty acid.<sup>17</sup> In practice however, there are several difficulties with such an experiment; (i) it requires an MS<sup>3</sup> capable mass spectrometer, (ii) the low energy CID of deprotonated fatty acids are often not structurally diagnostic, *e.g.*, often only dehydration and/or decarboxylation is observed,<sup>18</sup> and (iii) the alternative high energy CID can produce excessive fragmentation making for complex interpretation in the absence of comparative standards.<sup>19, 20</sup> As an alternative to MS<sup>3</sup>, several groups have trialed chemical derivatization of the double bond prior to ESI-MS/MS analysis by analogy to earlier chemical ionization studies of unsaturated fatty acids.<sup>21</sup> In two notable examples, (i) Moe *et al.* pretreated phospholipids and free fatty acids with osmium tetroxide prior to ESI-MS/MS analysis.<sup>15</sup> The dihydroxylated lipids formed by the derivatization method were shown to initiate characteristic cleavages upon CID thus identifying the position of the initial double bond.<sup>22, 23</sup> (ii) Harrison and Murphy have shown that ozonolysis of glycerophospholipids in a thin film can produce near quantitative conversion of olefinic bonds to ozonides. ESI-MS/MS of these adducts in either positive or negative ion mode leads to dissociation of the ozonide moiety and thus yields fragment ions uniquely identifying the double bond position.<sup>24</sup> While ESI-MS/MS of derivatized lipids can be used for locating double bond position, it has the undesirable requirement of additional sample preparation prior to

analysis. More recently, Brenna and co-workers have demonstrated an on-line method for the elucidation of double bond position in unsaturated lipids that might be considered as gas phase derivatization.<sup>25-28</sup> In this method chemical ionization of unsaturated lipids in the presence of acetonitrile results in covalent adduct ions that upon collision induced dissociation yield fragment ions indicative of the position of the double bond(s) within the molecule. While this methodology offers much promise, most modern lipid analyses utilize electrospray ionization rather than chemical ionization and as such further complementary technologies must be explored.

In a previous communication,<sup>29</sup> we introduced a novel method of in-source ozonolysis of glycerophospholipids during negative ion electrospray ionization: we now refer to this method as ozone electrospray ionization mass spectrometry (OzESI-MS). It was found that ozone, formed either from a corona discharge at the ESI capillary in an oxygen atmosphere or from an ozone generator, cleaved double bonds in the ionized lipid with the resulting chemically induced fragment ions revealing the position of the double bond. Similar observations have been subsequently reported by Beauchamp and co-workers using field-induced droplet ionization.<sup>30</sup> In the present article, we outline the application of OzESI-MS to several major classes of naturally occurring lipids including glycerophospholipids, sphingomyelins and triacylglycerols. In addition, OzESI-MS is also applied to a lipid extract from a human lens for the purpose of determining double bond assignment in the most abundant unsaturated phospholipid present within the extract.

## **Experimental**

### *Materials and Sample Preparation*

All phospholipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and were used without further purification. 1,2-dipalmitoyl-3-oleoylglycerol and ammonium acetate were purchased from Sigma-Aldrich (Castle Hill, Australia). HPLC grade methanol and chloroform were purchased from Crown Scientific (Sydney, Australia). Industrial grade compressed oxygen (purity 99.5%) was

obtained from BOC gases (Cringila, Australia). Standard solutions of phospholipids were prepared in methanol at concentrations between 1-10  $\mu\text{M}$ . Standard solutions of triacylglycerols were prepared at 1  $\mu\text{M}$  concentrations in methanol in the presence of 5 mM ammonium acetate. A 50-year old cataractous human lens was obtained from the Save Sight Institute (Sydney, Australia), homogenized under liquid nitrogen using a mortar and pestle and extracted using chloroform:methanol (2:1 v/v with 0.01% butylated hydroxytoluene) as previously described.<sup>31</sup> The extracts were diluted to an estimated total phospholipid concentration of 40  $\mu\text{M}$  in 2:1 methanol: chloroform prior to mass spectrometric analysis.

### *Instrumentation*

Experiments were performed a Micromass QuattroMicro (Manchester, UK) fitted with a z-spray<sup>®</sup> ion source. Typical settings for negative ion mode were; sample flow rate of 5-10  $\mu\text{L}/\text{min}$ , capillary voltage of 3.5 kV, cone voltage 50 V with a source temperature of 80 °C. For positive ion mode similar settings were used however a lower cone voltage was applied, typically either 30 or 40 V. Precursor ion scans for phosphatidylcholine and sphingomyelin lipids were performed by setting Q3 to  $m/z$  184.1 and scanning Q1 with a fixed collision energy of 35 eV (*e.g.*, Figures 3 and 7). The precursor ion spectrum for TG(16:0/9Z-18:1/16:0) was acquired by setting Q3 to  $m/z$  551.3 and scanning Q1 with a fixed collision energy of 25 eV (Figure 8a). MS/MS spectra were obtained by setting Q1 to the required  $m/z$  and scanning Q3 with a set potential across the collision cell. Argon was used as the collision gas for both the precursor ion and MS/MS scans and was set at a pressure of approximately  $3 \times 10^{-3}$  Torr. Spectra were background subtracted (40% background subtract with a first order polynomial) and smoothed (two Savitzky Golay smoothes using a peak width of 0.75 Th) using the MassLynx software. In several of the MS/MS spectra a median smooth was also used to reduce noise. Ozone was produced using an ozone generator from a NO<sub>x</sub> analyzer (Model 8840, Monitor Labs, CO) and was introduced directly into the desolvation gas inlet of the ESI probe at 160 mL/min via silicone

tubing. Reaction of a known volume of NO<sub>x</sub> analyzer effluent with acidified aqueous potassium iodide, then back titration against sodium thiosulfate in the presence of vitex indicator determined the ozone concentration to be *ca.* 0.3 % (v/v) in oxygen. *CAUTION:* Care was taken in all experiments to ensure that ozone did not vent into the laboratory.

## Results and Discussion

### *Ozone generation for OzESI-MS*

In-source ozonolysis of unsaturated ions can be achieved either by using high ESI voltages in an oxygen rich environment or by substituting nitrogen with an ozone/oxygen gas mixture (produced by an external generator) as the ESI desolvation gas.<sup>29</sup> Applying high voltages to the ESI capillary results in a corona discharge, which in the presence of oxygen introduced as a desolvation gas, produces ozone. The main advantage of this method is that since ozone is being produced *in situ* a dedicated ozone generator is not required and thus the experiment can be carried out on any commercial instrument. A major drawback however, is that increasing the voltage applied to the ESI capillary can result in a decrease in ion abundance of several orders of magnitude. In addition, a stable ozone producing corona discharge was only observed in negative ion mode suggesting that this method is not applicable to positive ions and thus restricting its potential application for lipid analysis. In contrast, introducing ozone produced externally from a generator into the ESI source as the desolvation gas allows the experiment to be performed with voltages optimized for maximum ion abundance. Indeed, under these conditions we have observed ion abundances not significantly lower than those obtained under conventional operating conditions with nitrogen as the desolvation gas. This method can also be performed in both negative and positive ion modes and generates higher working concentrations of ozone, leading to greater reaction efficiency and thus more abundant chemically induced fragment ions. For these reasons OzESI-MS with an external ozone generator is the preferred method and was used for all the experiments described herein.

### *OzESI-MS of glycerophosphocholine cations*

Figure 2(a) shows the OzESI-MS spectrum obtained from a methanolic solution of the monounsaturated glycerophosphatidylcholine, GPCho(16:0/9Z-18:1), obtained on a conventional ESI triple quadrupole mass spectrometer with the desolvation gas inlet connected to a commercial ozone generator. The spectrum obtained in positive ion mode reveals both protonated  $[M+H]^+$  and sodiated  $[M+Na]^+$  molecular ions at  $m/z$  760 and 782 respectively, and additional abundant ions at  $m/z$  650, 672, 698 and 720 that are not observed in the absence of ozone (data not shown). Interestingly,  $[M+H+48]^+$  and  $[M+Na+48]^+$  ions that might be expected to appear at  $m/z$  808 and 830 respectively, were not observed in this experiment (data not shown) suggesting that stable ozonides do not form readily under these conditions. The ions to lower mass however, can be considered as chemically induced fragments arising from ozonolysis of the precursor ions with the neutral losses of 62 Da ( $[M+H]^+ \rightarrow 698$  and  $[M+Na]^+ \rightarrow 720$ ) and 110 Da ( $[M+H]^+ \rightarrow 650$  and  $[M+Na]^+ \rightarrow 672$ ). Analogous losses were previously identified<sup>29</sup> from the negative ion OzESI-MS spectrum of GPA(16:0/9Z-18:1) suggesting that the  $m/z$  698 and 720 fragment ions in Figure 2(a) arise from the protonated and sodiated  $\alpha$ -methoxyhydroperoxide, 2-(9-hydroperoxy-9-methoxynonanoyl)-1-palmitoyl-*sn*-glycero-3-phosphocholine while the  $m/z$  650 and 672 ions, result from the aldehyde, 2-(9-oxonanoyl)-1-palmitoyl-*sn*-glycero-3-phosphocholine (Scheme 1). Further evidence for these structural assignments is provided by the MS/MS spectrum of the  $m/z$  698 ion (Figure 2c) that reveals a base peak at  $m/z$  664, corresponding to an indicative neutral loss of hydrogen peroxide (-34 Da). While the precise mechanism for this rearrangement is unknown, the analogous process has previously been shown to be characteristic of protonated glycerophosphocholine cations containing a hydroperoxide moiety.<sup>32, 33</sup> The production of identical neutral losses characteristic of the n-9 double bond position<sup>34</sup> but independent of the mode of ionization (*i.e.*,  $[M-H]^-$ ,  $[M+H]^+$  or  $[M+Na]^+$ ) suggests that OzESI-MS is a

general method for structure determination and could be applied to a range of lipids. Furthermore, the MS/MS spectra of both the protonated  $\alpha$ -methoxyhydroperoxide (Figure 2c) and aldehyde (data not shown) show significant fragmentation to the phosphocholine specific  $m/z$  184 product ion<sup>4,9</sup> allowing for the precursor ion scan shown in Figure 2(b). The latter spectrum, reveals exclusively the protonated precursor ion and its chemically induced fragments thus significantly simplifying analysis of the data and suggesting that OzESI-MS may be successfully employed in complex mixture analysis (see later).

### Scheme 1.

### [Figure 2]

The most abundant 18:1 fatty acid in animal tissues is oleic acid (9Z-18:1), however, several other positional isomers of 18:1 are known to occur in biological extracts including petroselinic (6Z-18:1) and *cis*-vaccenic (11Z-18:1).<sup>35</sup> The ESI-MS/MS spectra of GPCho(9Z-18:1/9Z-18:1) and GPCho(6Z-18:1/6Z-18:1) shown in Figure 1(a) and (b) demonstrate that conventional tandem mass spectra produce identical *collision* induced fragment ions and thus cannot differentiate between oleyl and petroselinylyl radicals. In contrast, the positive ion OzESI-MS spectra of the same isomers are distinctive with *chemically* induced fragment ions showing neutral losses of 62 Da ( $m/z$  724) and 110 Da ( $m/z$  676) in Figure 3(a) identifying the n-9 double bonds while the 104 Da ( $m/z$  682) and 152 Da ( $m/z$  634) losses in Figure 3(b) are characteristic of an n-12 olefin.

### [Figure 3]

The OzESI-MS spectrum of the polyunsaturated phosphatidylcholine, GPCho(16:0/9Z,12Z-18:2) is shown in Figure 4 and reveals four major chemically induced fragment ions at  $m/z$  650, 690,

698, and 738. The fragment ions at  $m/z$  650 and 698 are the same as those observed for monounsaturated GPCho(16:0/9Z-18:1) shown in Figure 2(b) thus confirming the n-9 double bond position in the polyunsaturated lipid. The additional pair of fragment ions at  $m/z$  690 and 738 appear 40 Da higher than the n-9 fragments, localizing the additional double bond a further 3-carbons toward the methyl end and confirming that it is an n-6 fatty acyl chain.

An interesting case might arise if two different unsaturated fatty acids are present within the same parent molecule. While regiospecific phosphatidylcholines of this type are not currently available, we predict that unambiguous assignment of double bond position should also be possible within such mixed systems. For example, consider the OzESI-MS spectrum that might result from GPCho(9Z-18:1/9Z,12Z-18:2). As previously shown the 9Z-18:1 acyl chain would be expected to yield neutral losses of 62 and 110 Da while the 9Z,12Z-18:2 acyl chain would be expected to give rise to neutral losses of 20, 60, 68 and 108 Da. These three unique sets of fragment ions would thus allow the positions of all three double bonds to be unambiguously determined.

#### **[Figure 4]**

##### *OzESI-MS of glycerophospholipid anions*

The negative ion OzESI-MS spectra of GPA(16:0/9Z-18:1) and GPSer(5Z,8Z,11Z,14Z-20:4/5Z,8Z,11Z,14Z-20:4) have previously been reported<sup>29</sup> and show the same characteristic fragmentation behaviour as the positive ion spectra discussed thus far. In order to establish the generality of the method for analysis of glycerophospholipids in negative ion mode a range of lipids including phosphatidylethanolamine (GPEtn), phosphatidic acid (GPA), phosphatidylglycerol (GPGro), phosphatidylserine (GPSer), phosphatidylinositol (GPIIns) and cardiolipin (CL) were investigated. In these examples, all glycerophospholipids contain the oleoyl radical (9Z-18:1) and thus the OzESI-MS spectra in Figure 5(a-f) all show two chemically induced fragments with characteristic neutral losses of

62 and 110 Da in addition to the  $[M-H]^-$  or  $[M-2H]^{2-}$  precursor ion. Significantly, despite the structural diversity of this range of lipids all OzESI-MS spectra show abundant fragment ions with no major competing reactions. These observations point to a general method for structure elucidation of unsaturated glycerophospholipids in either positive or negative ion mode.

### [Figure 5]

#### *OzESI-MS of plasmalogen lipids*

Plasmalogen phospholipids differ from diacyl glycerophospholipids by the unique vinyl ether bond linking the *sn*-1 fatty acid and the glycerol backbone (in place of the conventional ester bond). These molecules are thought to play an important chemical role in biological membranes resulting from the peculiar reactivity of the vinyl ether moiety.<sup>36</sup> The negative ion OzESI-MS spectrum of the phosphoethanolamine plasmalogen, GPEtn(16:0p/9Z-18:1) is shown in Figure 6(a) and differs significantly from the spectrum of the structurally related GPEtn(9Z-18:1/9Z-18:1) shown in Figure 5(b). Notably, despite the presence of the oleyl radyl (9Z-18:1) no neutral losses of 62 or 110 Da are observed in the plasmalogen spectrum and instead a single major fragment ion is observed at  $m/z$  506. The MS/MS spectrum of this ion shows, (i) a major fragment ion at  $m/z$  281 corresponding to the carboxylate anion of oleic acid and confirming that this radyl remains intact within the chemically modified structure and (ii) two minor fragments at  $m/z$  460 and 478 corresponding to losses of formic acid ( $HCO_2H$ ) and carbon monoxide (CO), respectively. The latter fragments are consistent with a formic acid radyl at the *sn*-1 position, that is, an  $m/z$  506 ion structure corresponding to  $[GPEtn(1:0/9Z-18:1)-H]^-$ . This observation suggests that reaction of ozone occurs exclusively at the vinyl ether position and results in a single ionic product in contrast to the pairs of ions observed in previous examples. Interestingly, these observations are consistent with prior ozonolysis research showing, (i)

the gas phase ozonolysis of methyl vinyl ether is some 12 times faster than the analogous reaction of *cis*-2-butene<sup>37</sup> and (ii) the reaction of ozone with methyl vinyl ether in methanol produces only methyl formate and methoxymethyl hydroperoxide, in contrast to the mixture of four reaction products usually observed from the ozonolysis of an asymmetric alkene.<sup>38</sup> Interestingly, the negative ion OzESI-MS spectrum of GPEtn(16:0p/9Z-18:1) also shows an ion 48 Da greater than the  $[M-H]^-$  precursor ion (Figure 6a). This mass corresponds to the addition of ozone and thus perhaps the formation of a relatively stable ozonide. Although structural assignment of this ion could not be confirmed due to insufficient signal, the observation is unique to the plasmalogen and was observed only occasionally for other unsaturated lipids. Overall, reaction of the plasmalogen appears significantly more facile than for other lipids with almost complete conversion of the  $[M-H]^-$  precursor ion to the  $m/z$  506 fragment (Figure 6a). As a consequence a detectable abundance of secondary reaction is observed with ions at  $m/z$  396 (506 - 110 Da) and 444 (506 - 62 Da) arising from ozonolysis of the  $[GPEtn(1:0/9Z-18:1)-H]^-$  fragment ion. The combination of primary and secondary reaction fragments could thus be used to uniquely identify the position of double bonds in an unsaturated plasmalogen.

## [Figure 6]

### *OzESI-MS of sphingomyelins*

Sphingomyelins (SM) are an important class of phospholipids with a sphingosine backbone and an additional fatty acid chain bound *via* an amide linkage. These molecules are found in high levels in the membranes of human lens fibre cells where they are thought to retard oxidation.<sup>39, 40</sup> The positive ion OzESI-MS spectrum of a typical sphingomyelin, SM(d18:1/9Z-18:1), is presented in Figure 7 along with the structure of the precursor ion. These data show two abundant chemically induced fragment ions at  $m/z$  619 and 667, corresponding to the now recognizable neutral losses of 110 and 62 Da thus

locating the double bond at n-9 on the oleic acid radyl. In contrast, no detectable ions resulting from ozone-induced cleavage of the *trans* double bond in the sphingosine backbone are observed. This is a somewhat surprising observation, given that *trans* alkenes are known to be more reactive toward ozone in the gas phase than their respective *cis* isomers and additionally the presence of an allylic hydroxyl group has also been shown to increase the reaction rate in simple olefins.<sup>41</sup> OzESI-MS of a sphingomyelin with a saturated radyl under identical conditions did reveal some chemical induced cleavage of the sphingosine backbone double bond but the resulting ions were only of very low abundance (see Supporting Information, Figure S-1). These observations suggest that the gas phase conformation of the sphingomyelin cation - perhaps induced by hydrogen bonding to the allylic alcohol - may hinder the approach of ozone to the sphingosine double bond. The lack of reaction and thus fragmentation at this position does not, however, limit the utility of OzESI-MS for the structural characterization of sphingolipids. This is because ozone-induced fragmentation of any amide linked unsaturated fatty acid allows the presence or absence (in the case of dihydrosphingomyelin) of the double bond in the sphingosine backbone to be inferred.

## [Figure 7]

### *OzESI-MS of triacylglycerols*

Triacylglycerols (TG) are the major component of naturally occurring fats and oils and are at the center of biological energy production and storage.<sup>35</sup> Although a relative simple class of molecules - consisting of three fatty acyl chains esterified to a glycerol backbone - triacylglycerols exhibit considerable structural diversity. This can be understood by considering the different combinations of naturally occurring fatty acids that can be incorporated in the three available positions (*i.e.*, different chain lengths, degrees of unsaturation and position of unsaturation) as well as isomerization arising from the location of these fatty acids on the glycerol backbone. As a consequence, this class of

molecule presents a challenge for analysis and in particular the identification of double bond position. When analyzed by ESI mass spectrometry, triacylglycerols are commonly ionized as ammonium, sodium or lithium adducts as sufficiently basic or acidic sites are not present to allow the formation of protonated or deprotonated ions.<sup>13, 42, 43</sup> While tandem mass spectrometry of such adducts reveals the degree of unsaturation of the fatty acyl chains and provide some information on their position on the glycerol backbone more complex experiments, such as high energy MS<sup>3</sup> or source CID-MS/MS are required to locate the position of double bonds.<sup>42, 44</sup> Figure 8(a) shows the positive ion OzESI-MS spectrum of a 1  $\mu$ M methanolic solution of the monounsaturated triacylglycerol, TG(16:0/9Z-18:1/16:0), in the presence of 5 mM ammonium acetate. In this spectrum, the  $[M+NH_4]^+$  ion from TG(16:0/9Z-18:1/16:0) is observed at  $m/z$  850 along with two ozone-induced fragment ions at  $m/z$  740 and 788. The fragments correspond to neutral losses of 110 and 62 Da suggesting that the structure of these ions correspond to aldehydes and  $\alpha$ -methoxyhydroperoxides analogous to those observed for phospholipids with oleic acid radicals. The MS/MS spectra of these ions shown in Figure 8(b-c) are consistent with these structural proposals. The OzESI-MS product ion at  $m/z$  740 (Figure 8b) exhibits fragmentation characteristic of an ammoniated triacylglycerol ion<sup>13</sup> with the neutral loss of ammonia with palmitic acid ( $m/z$  467) and ammonia with 9-oxononanoic acid ( $m/z$  551). In contrast, MS/MS of the OzESI-MS product ion at  $m/z$  788 (Figure 8c) reveals several ions that are not observed for conventional triacylglycerol including a major neutral loss of 51 Da, which corresponds to the loss of ammonia and hydrogen peroxide and is thus consistent with the putative  $\alpha$ -methoxyhydroperoxide structure shown. The  $m/z$  551 product ion, resulting from the neutral loss ammonia and the *sn*-2 radical, is common to the MS/MS spectra in Figures 8(b) and 8(c) as well as the unreacted precursor ion at  $m/z$  850 (data not shown). As a consequence, the OzESI-MS spectrum can be obtained as a precursor ion scan as shown in Figure 8(a), which significantly simplifies the spectrum and data analysis. No additional features resulting from the different molecular structure of this lipid (compared to

phospholipids) or the use of the ammonium adduct ion were observed in the OzESI-MS analysis of triacylglycerols. It can be concluded therefore, that this technique can be applied to structure elucidation of triacylglycerols with a data interpretation identical to that of glycerophospholipids.

### [Figure 8]

#### *Characterization of an unknown lipid in a biological extract using OzESI-MS*

All lipids examined thus far have been pure compounds of known structure, however, the potential of this technique to characterize the structure of an unknown lipid present within a biological extract remains to be demonstrated. While there is little doubt that OzESI-MS analysis of a purified lipid would prove effective, increasingly lipid analysis is moving towards a shotgun lipidomic approach<sup>6, 45</sup> and thus we examine the application to a crude lipid extract. Currently within our laboratory, the phospholipid composition of the human lens is under investigation using a shotgun approach whereby lenses are homogenized, extracted and the lipid extract infused directly into an electrospray ionization triple quadrupole mass spectrometer for analysis. A typical positive ion mass spectrum of a cataractous human lens is shown in Figure 9(a). The abundant ion at  $m/z$  815 can be identified as the dihydrosphingomyelin, SM(d18:0/24:1) by tandem mass spectrometry<sup>14</sup> and represents the most abundant unsaturated phospholipid thus far identified in lenses of this type. Although the most abundant 24:1 fatty acid present in mammalian tissues is the n-9 nervonic acid (15Z-24:1), the n-7 positional isomer is also present in nature in lower abundance and has been previously identified using covalent adduct ionization of fatty acid methyl esters in conjunction with MS/MS.<sup>27</sup> Given the relatively low abundance of other unsaturated phospholipids in this sample, the elucidation of double bond position within SM(d18:0/24:1) appears feasible by OzESI-MS. When ozone is added to the desolvation gas, significant ions of  $m/z$  753 and 781 are observed (Figure 9b), which are not present in the standard ESI spectrum (Figure 9a). MS/MS of the  $m/z$  753 and 781 ions (data not shown) revealed

the neutral losses of 34 and 49 Da from the parent ions, which are characteristic of the protonated  $\alpha$ -methoxyhydroperoxide as discussed above. The  $\alpha$ -methoxyhydroperoxide ions of  $m/z$  753 and 781 correspond to neutral losses of 62 and 34 Da, respectively and can thus be assigned to the dihydrosphingomyelins, SM(d18:0/15Z-24:1) and SM(d18:0/17Z-24:1), where the stereochemistry about the double bond is assumed to be Z. The abundance of the  $m/z$  753 ion is greater than the  $m/z$  781 ion (Figure 9b) suggesting that SM(d18:0/15Z-24:1) is, as expected, the more abundant of the two lipids. Unfortunately, the expected aldehydic product ions from the OzESI-MS of SM(d18:0/15Z-24:1) and SM(d18:0/17Z-24:1) are predicted at  $m/z$  705 and 733 and are thus isobaric with phospholipids already present in the extract. Interestingly, the OzESI-MS spectrum (Figure 9b) also shows minor ions present at  $m/z$  761 and 809 that do not appear to be present in the absence of ozone. This fragment ion pair could be assigned to the n-5 dihydrosphingomyelin, SM(d18:0/19Z-24:1) but the  $m/z$  809 ion abundance was too low to obtain structural confirmation by MS/MS and thus this structural assignment remains ambiguous.

## Conclusion

OzESI-MS has been applied to a range of unsaturated lipids of known structure including the major classes of glycerophospholipids, GPCCho, GPEtn, GPA, GPGro, GPSer, GPIIns and CL, sphingomyelins and triacylglycerols. In almost all these cases, in-source ozonolysis of unsaturated lipids results in the formation of two distinctive chemically induced fragment ions that localize the position of the double bond within the molecule. These diagnostic reactions are observed in both positive and negative ion modes and, for the most part, occurred independent of the lipid headgroup or backbone or the adduct ion employed for ionization. The exceptions appear to be plasmalogen and sphingomyelin lipids, with the vinyl ether double bond of plasmalogens showing enhanced reactivity and the backbone double bond of sphingomyelin showing reduced reactivity. Further investigation into the in-source ozonolysis of these unique phospholipids is required to determine if these effects reflect

the intrinsic reactivity of the functionalized olefin moieties and/or the gas phase conformation of the lipids. Significantly, the OzESI-MS method was found to be suitable for use in conjunction with precursor ion scans of the type typically employed in lipid mass spectrometry and thus the technique shows some promise in shotgun lipidomic applications in certain cases.

OzESI-MS applied to a crude lipid extract obtained produced diagnostic fragment ions identifying the presence of at least two regioisomeric sphingomyelins with double bonds at both the n-9 and n-7 positions, with the tentative assignment of a third, n-5, isomer. This example serves to illustrate the surprising diversity of lipids present in nature that can be easily overlooked by conventional lipidomic analyses. Isobaric interferences in the analysis of the crude extract did not prove prohibitive to structure elucidation in this instance but are illustrative of the potential difficulties that may arise in more complex lipid mixtures. This represents a limitation in the application of OzESI-MS for shotgun-type analyses, however, development of hyphenated techniques may provide on-line structure assignment of chromatographically isolated, or at least simplified mixtures of lipids. Despite these limitations, OzESI-MS provides a powerful new adjunct to existing lipidomic technologies and appears to be sufficiently general that it may also find application in more broad ranging problems in the structure elucidation of unsaturated organic molecules.

### **Acknowledgements**

S.J.B., D.G.H. and T.W.M. acknowledge the financial support of the University of Wollongong and the Australian Research Council (DP0452849 and LP0455472). M.C.T. and J.M.D. are supported by Australian Postgraduate Awards. M.C.T and R.C.M. acknowledge the financial support from the Lipid Maps Large Scale Collaborative Grant (GM069338) from the National Institutes of Health (USA). R.C.M. also acknowledges support by the National Institutes of Health (GM069338). We gratefully acknowledge the technical assistance of Mr. Larry Hick, Prof. Roger Truscott and Dr Sudha Awasthi-

Patney for providing the human lens and Prof. David Griffith for provision of the ozone generator. We acknowledge Dr Kim Ekroos for helpful discussions.

**Supplementary Material Available:** Additional OzESI-MS spectra are available as Supporting Information.

## References

- (1) Kerwin, J. L.; Tuininga, A. R.; Ericsson, L. H. *J. Lipid Res.* **1994**, *35*, 1102-1114.
- (2) Han, X.; Gross, R. W. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 10635-10639.
- (3) Murphy, R. C.; Fiedler, J.; Hevko, J. *Chem. Rev.* **2001**, *101*, 479-526.
- (4) Pulfer, M.; Murphy, R. C. *Mass Spectrom. Rev.* **2003**, *22*, 332-364.
- (5) Ekroos, K.; Chernushevich, I. V.; Simons, K.; Shevchenko, A. *Anal. Chem.* **2002**, *74*, 941-949.
- (6) Han, X.; Gross, R. W. *Mass Spectrom. Rev.* **2005**, *24*, 367-412.
- (7) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 1036-1043.
- (8) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 986-999.
- (9) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 352-363.
- (10) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 892-899.
- (11) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 797-803.
- (12) Hsu, F. F.; Turk, J.; Rhoades, E. R.; Russell, D. G.; Shi, Y. X.; Groisman, E. A. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 491-504.
- (13) McAnoy, A. M.; Wu, C. C.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1498-1509.
- (14) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 437-449.
- (15) Moe, M. K.; Anderssen, T.; Strom, M. B.; Jensen, E. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 46-59.
- (16) Pariza, M. W.; Park, Y.; Cook, M. E. *Prog. Lipid Res.* **2001**, *40*, 283-298.
- (17) Bryant, D. K.; Orlando, R. C.; Fenselau, C.; Sowder, R. C.; Henderson, L. E. *Anal. Chem.* **1991**, *63*, 1110-1114.
- (18) Kerwin, J. L.; Wiens, A. M.; Ericsson, L. H. *J. Mass Spectrom.* **1996**, *31*, 184-192.
- (19) Jensen, N. J.; Tomer, K. B.; Gross, M. L. *Lipids* **1986**, *21*, 580-588.
- (20) Jensen, N. J.; Tomer, K. B.; Gross, M. L. *Lipids* **1987**, *22*, 480-489.
- (21) Vairamani, M.; Mirza, U. A.; Srinivas, R. *Mass Spectrom. Rev.* **1990**, *9*, 235-258.

- (22) Moe, M. K.; Anderssen, T.; Strom, M. B.; Jensen, E. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 2121-2130.
- (23) Moe, M. K.; Strom, M. B.; Jensen, E.; Claeys, M. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1731-1740.
- (24) Harrison, K. A.; Murphy, R. C. *Anal. Chem.* **1996**, *68*, 3224-3230.
- (25) Michaud, A. L.; Lawrence, P.; Adlof, R.; Brenna, J. T. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 363-368.
- (26) Michaud, A. L.; Yurawecz, M. P.; Delmonte, P.; Corl, B. A.; Bauman, D. E.; Brenna, J. T. *Anal. Chem.* **2003**, *75*, 4925-4930.
- (27) Michaud, A. L.; Diau, G.-Y.; Abril, R.; Brenna, J. T. *Anal. Biochem.* **2002**, *307*, 348-360.
- (28) Lawrence, P.; Brenna, J. T. *Anal. Chem.* **2006**, *78*, 1312-1317.
- (29) Thomas, M. C.; Mitchell, T. W.; Blanksby, S. J. *J. Am. Chem. Soc.* **2006**, *128*, 58-59.
- (30) Grimm, R. L.; Hodyss, R.; Beauchamp, J. L. *Anal. Chem.* **2006**, *78*, 3800-3806.
- (31) Folch, J.; Less, M.; Sloane-Stanley, G. H. *J. Biol. Chem.* **1957**, *226*, 497-509.
- (32) Santrock, J.; Gorski, R. A.; Ogara, J. F. *Chem. Res. Toxicol.* **1992**, *5*, 134-141.
- (33) Adachi, J.; Yoshioka, N.; Funae, R.; Nushida, H.; Asano, M.; Ueno, Y. *J. Chromatogr. B* **2004**, *806*, 41-46.
- (34) The "n" nomenclature refers to the position of the double bond as counted from the methyl end of the carbon chain. While this is not standard chemical nomenclature (that numbers bonds from the carboxylate end of the fatty acid), it remains a commonly used classification of fatty acids. In this manuscript the "n" nomenclature is instructive because the values of neutral loss due to chemically induced fragmentation in OzESI-MS are common to lipids with double bonds at the same position in the chain with respect to the methyl terminus. For example, GPCho(16:0/9Z-18:1) and SM(d18:0/15Z-24:1) have differing standard nomenclatures as

shown but are both n-9 lipids and thus exhibit a common neutral losses of 62 Da in their respective OzESI-MS spectra (see Figures 2a and 9b, respectively).

- (35) Gurr, M. I.; Harwood, J. L.; Frayn, K. N. *Lipid Biochemistry*, 5th ed.; Blackwell Science Ltd.: Oxford, UK, 2002.
- (36) Murphy, R. C. *Chem. Res. Toxicol.* **2001**, *14*, 463-472.
- (37) Grosjean, E.; Grosjean, D. *J. Atmos. Chem.* **1999**, *32*, 205-232.
- (38) Keul, H.; Kuczkowski, R. L. *J. Am. Chem. Soc.* **1984**, *106*, 5370-5371.
- (39) Yappert, M. C.; Borchman, D. *Chem. Phys. Lipids* **2004**, *129*, 1-20.
- (40) Oborina, E. M.; Yappert, M. C. *Chem. Phys. Lipids* **2003**, *123*, 223-232.
- (41) Grosjean, E.; Grosjean, D. *Int. J. Chem. Kinet.* **1994**, *26*, 1185-1191.
- (42) Hsu, F.-F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 587-599.
- (43) Han, X. L.; Gross, R. W. *Anal. Biochem.* **2001**, *295*, 88-100.
- (44) Cheng, C. F.; Gross, M. L. *Anal. Chem.* **1998**, *70*, 4417-4426.
- (45) Han, X.; Gross, R. W. *J. Lipid Res.* **2003**, *44*, 1071-1079.

## Scheme Captions

Scheme 1. The proposed mechanism for the formation of the  $m/z$  650 and 698 ions from the ozonolysis of protonated GPCho(16:0/9Z-18:1) in the presence of methanol, the ESI solvent.

## Figure Captions

Figure 1. The MS/MS spectrum of the  $[M+HCO_2]^-$  adduct anion arising from electrospray ionization of a 1  $\mu$ M methanolic solution of (a) GPCho(9Z-18:1/9Z-18:1) and (b) GPCho(6Z-18:1/6Z-18:1) in the presence of formic acid and ammonia.

Figure 2. (a) The OzESI-MS spectrum from a 1  $\mu$ M methanolic solution of GPCho(16:0/9Z-18:1) obtained on a conventional electrospray ionization triple quadrupole mass spectrometer coupled to a commercial ozone generator. (b) The  $m/z$  184 precursor ion spectrum obtained under the same conditions as above. (c) The MS/MS spectrum of the  $m/z$  698 chemically induced fragment ion acquired at a collision energy of 20 eV. The symbols ● and ■ identify the chemically induced fragment ions corresponding to  $\alpha$ -methoxyhydroperoxides and aldehydes, respectively.

Figure 3. The OzESI-MS spectrum of a 1  $\mu$ M methanolic solution of (a) GPCho(9Z-18:1/9Z-18:1) and (b) GPCho(6Z-18:1/6Z-18:1). Both spectra are recorded as  $m/z$ 184 precursor ion scans and thus show only the  $[M+H]^+$  molecular ion and corresponding chemically induced fragment ions. The symbols ● and ■ identify the ozonolysis product ions as  $\alpha$ -methoxyhydroperoxides and aldehydes, respectively.

Figure 4. The OzESI-MS spectrum of a 1  $\mu$ M methanolic solution of GPCho(16:0/9Z,12Z-18:2) recorded as a  $m/z$  184 precursor ion scan and thus showing only the  $[M+H]^+$  molecular ion and corresponding chemically induced fragment ions. The symbols ● and ■ identify the ozonolysis product ions as  $\alpha$ -methoxyhydroperoxides and aldehydes, respectively.

Figure 5. The negative ion OzESI-MS spectra of methanolic solutions of (a) GPA(9Z-18:1/9Z-18:1), (b) GPETn(9Z-18:1/9Z-18:1), (c) GPGro(9Z-18:1/9Z-18:1), (d) GPSer(9Z-18:1/9Z-18:1), (e) GPIIns(9Z-18:1/9Z-18:1) and (f) CL(9Z-18:1/9Z-18:1/9Z-18:1/9Z-18:1). The symbols ● and ■ identify the ozonolysis product ions as  $\alpha$ -methoxyhydroperoxides and aldehydes, respectively. It should be noted that the dioleoyl glycerophospholipids undergo some degree of secondary ozonolysis of both the aldehyde and  $\alpha$ -methoxyhydroperoxide ions to form additional product ions most of which fall below the  $m/z$  range presented here.

Figure 6. (a) The negative ion OzESI-MS spectrum of 10  $\mu$ M methanolic solution of GPETn(16:0p/9Z-18:1). (b) The MS/MS spectrum of the  $m/z$  506 chemically induced fragment ion from (a) acquired at a collision energy of 20 eV.

Figure 7. The OzESI-MS spectrum of a 1  $\mu$ M methanolic solution of SM(d18:1/9Z-18:1) recorded as a  $m/z$  184 precursor ion scan and thus showing only the  $[M+H]^+$  molecular ion and corresponding chemically induced fragment ions. The symbols ● and ■ identify the ozonolysis product ions as  $\alpha$ -methoxyhydroperoxides and aldehydes, respectively. The ions observed at  $m/z$  743 and 745 were also present in the absence of ozone and are thus not products resulting from in-source ozonolysis.

Figure 8. (a) The OzESI-MS spectrum of 1  $\mu$ M methanolic solution of TG(16:0/9Z-18:1/16:0) with 5 mM ammonium acetate recorded as a  $m/z$  551 precursor ion scan and thus showing only the  $[M+NH_4]^+$  molecular ion and corresponding chemically induced fragment ions. (b) The MS/MS spectrum of the OzESI-MS product ion at  $m/z$  740 (C.E. = 20 eV). (c) The MS/MS spectrum of the OzESI-MS product ion at  $m/z$  788 (C.E. = 20 eV). The symbols ● and ■ identify the ozonolysis product ions as  $\alpha$ -methoxyhydroperoxides and aldehydes, respectively.

Figure 9. (a) The positive ion ESI-MS mass spectrum of a lipid extract obtained from a 50 year-old, cataractous human lens recorded as a  $m/z$  184 precursor ion scan and thus showing only the  $[M+H]^+$  molecular ions of sphingomyelin, dihydrosphingomyelin and phosphatidylcholine lipids present within the extract. (b) The OzESI-MS spectrum of the same extract recorded under the same conditions except for the presence of ozone in the desolvation gas.

Scheme 1.

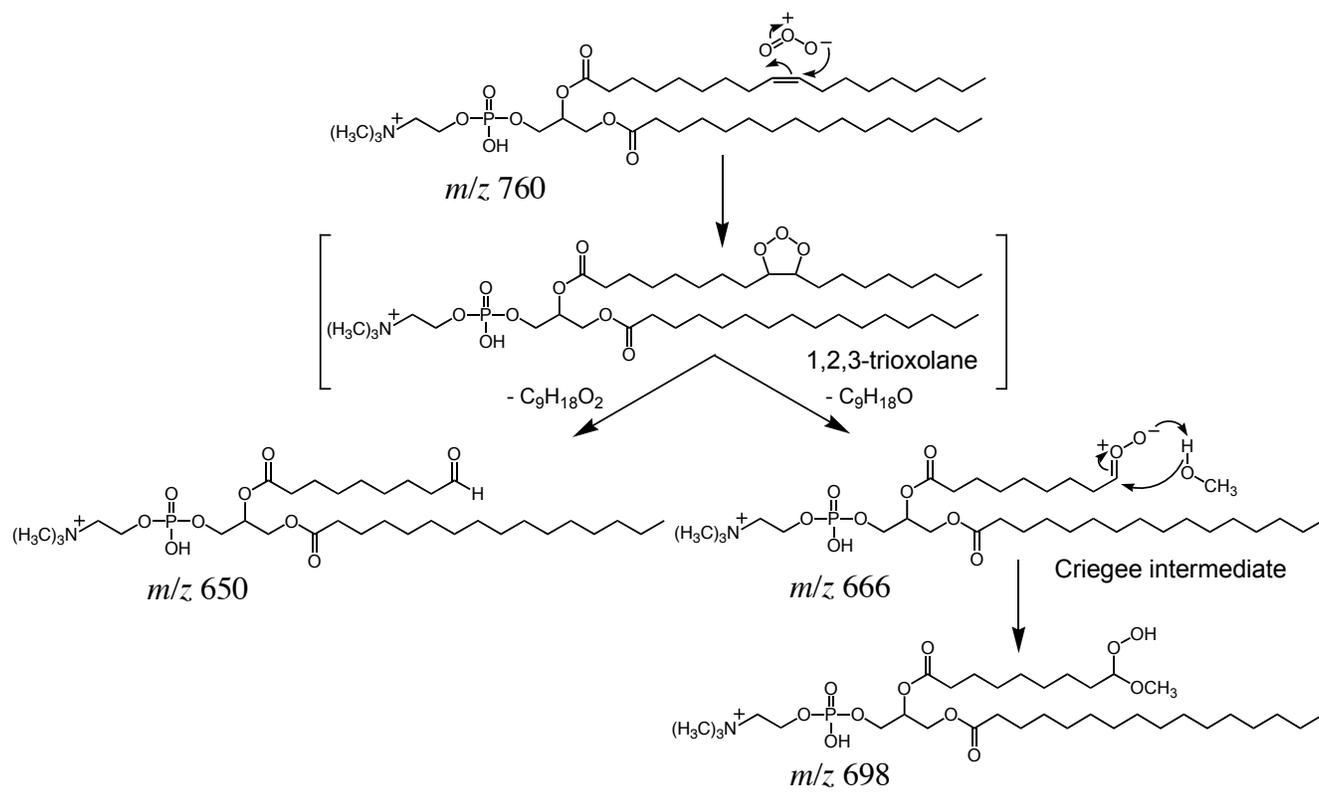


Figure 1.

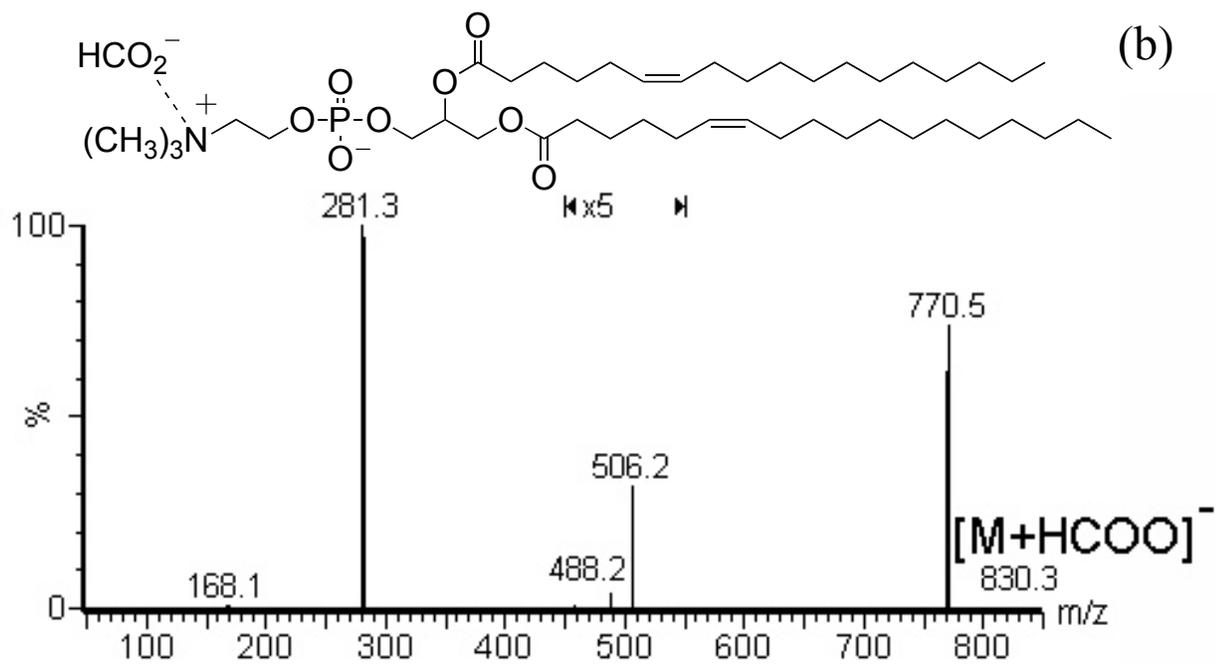
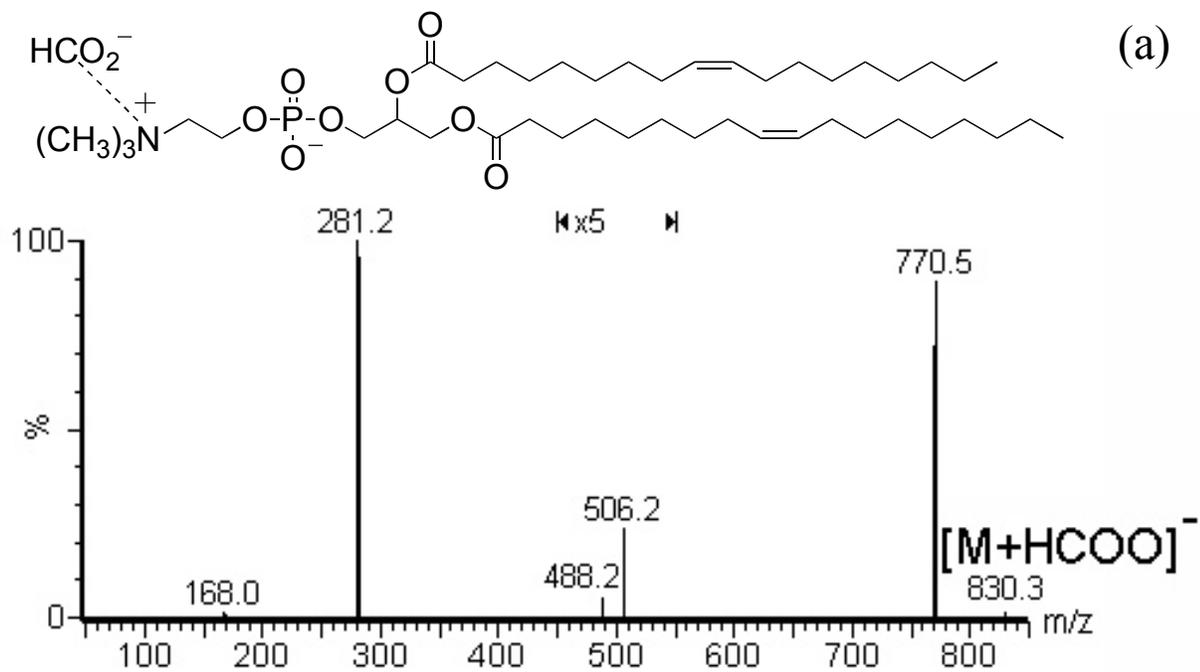


Figure 2.

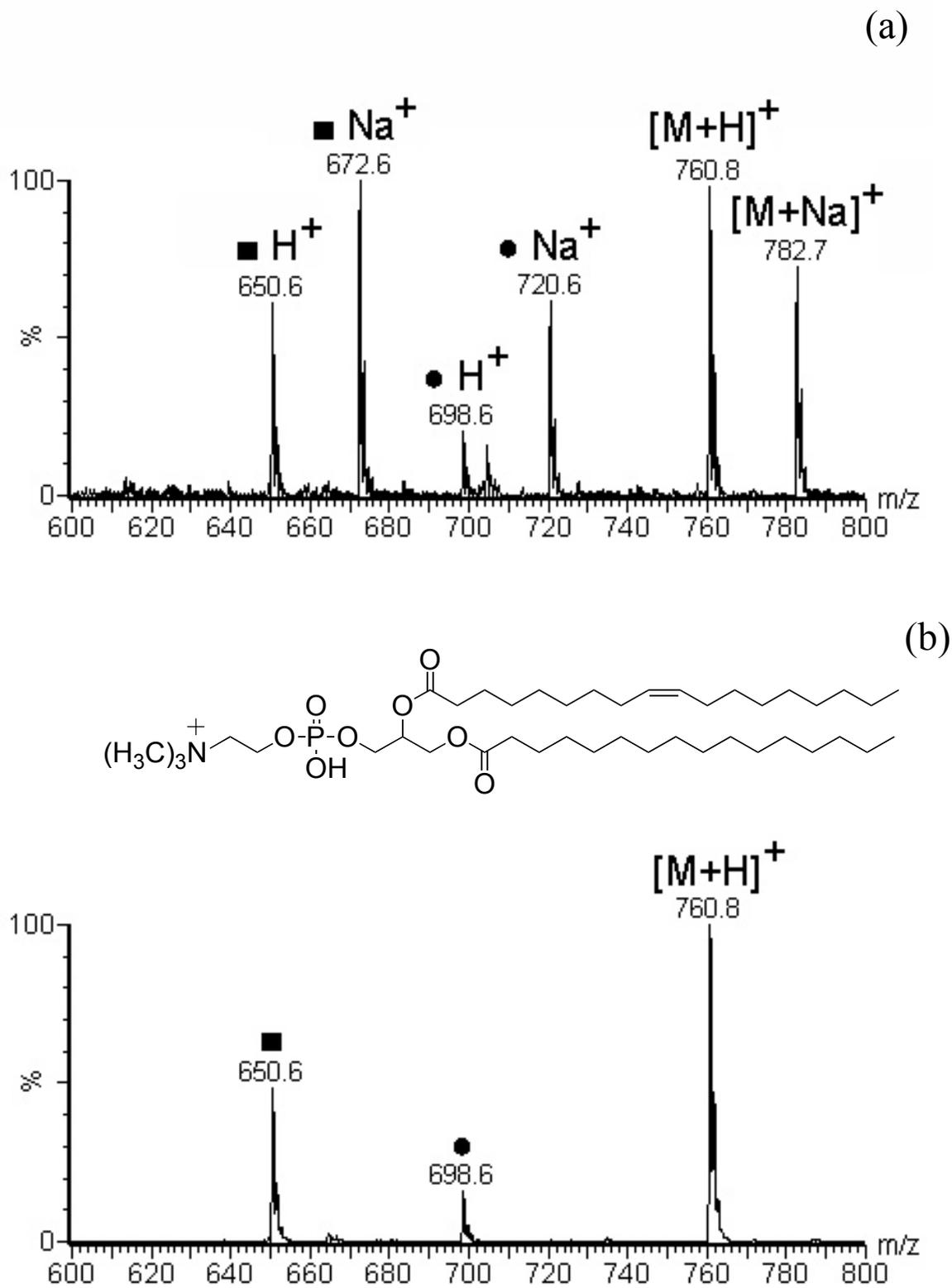


Figure 2(cont).

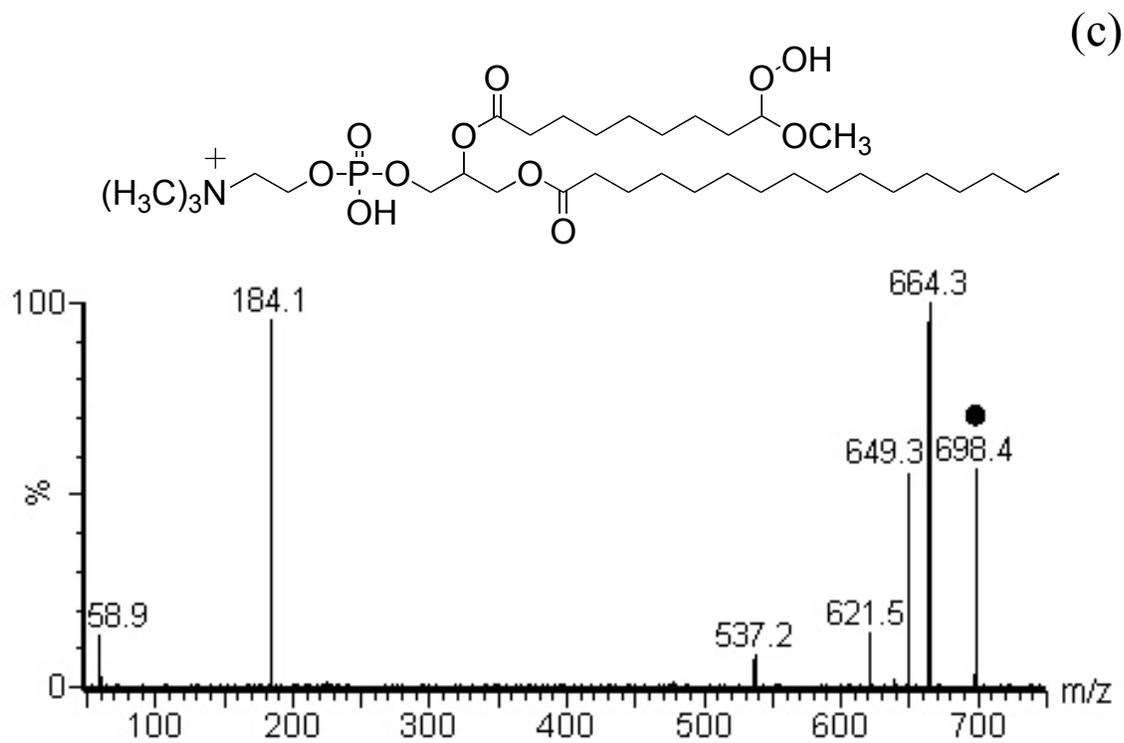


Figure 3.

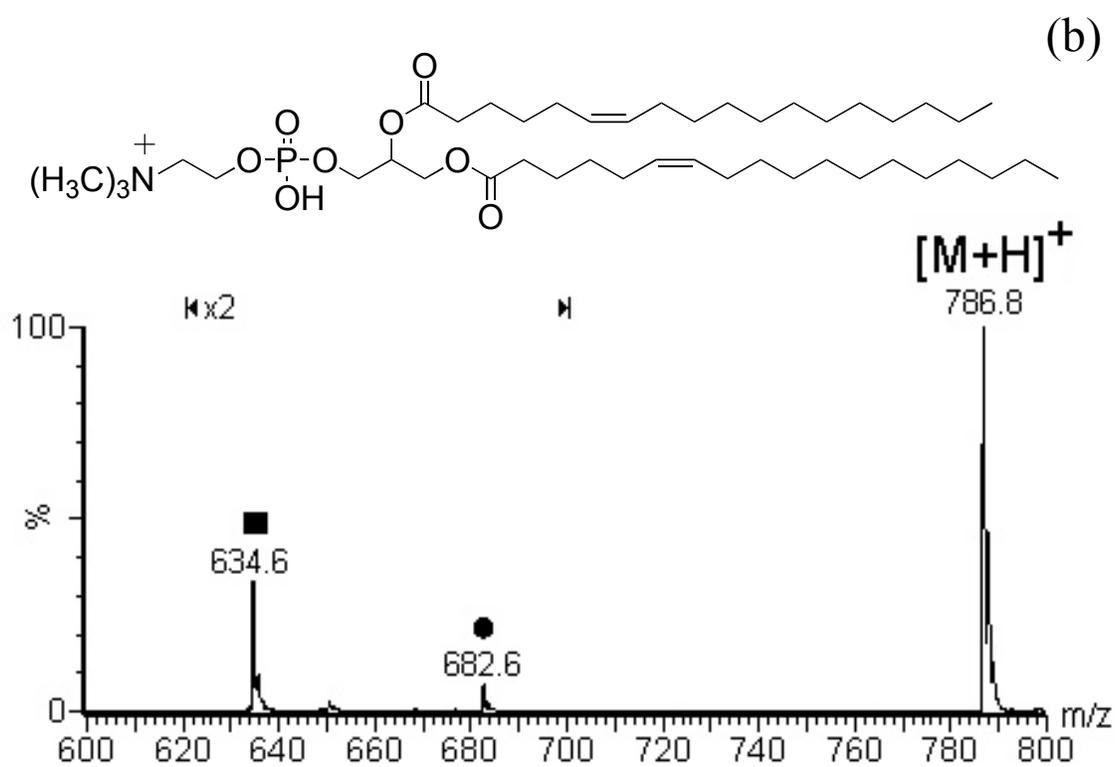
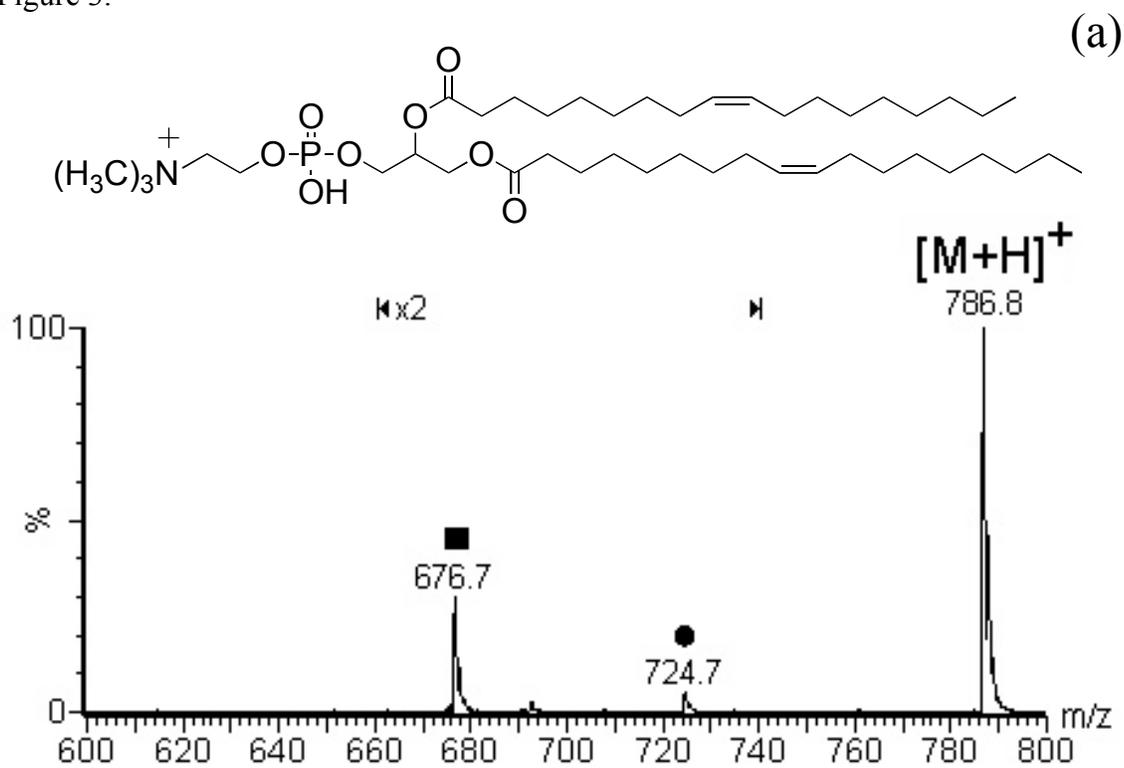


Figure 4.

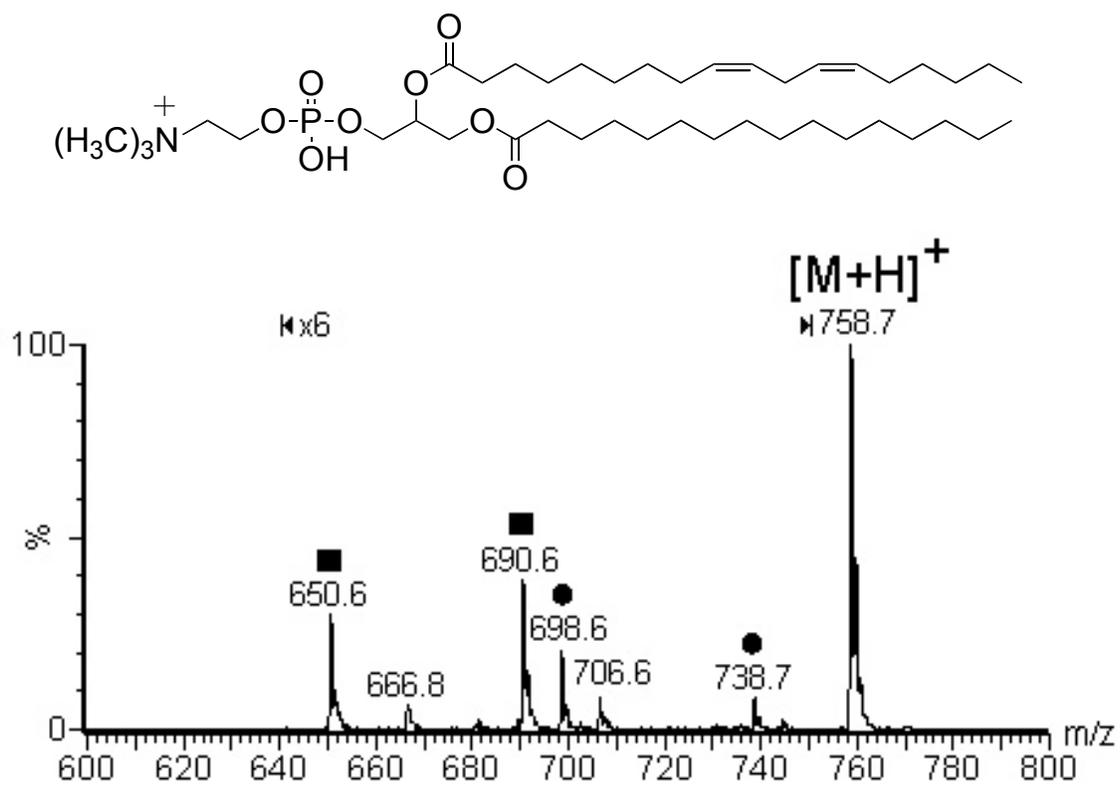


Figure 5.

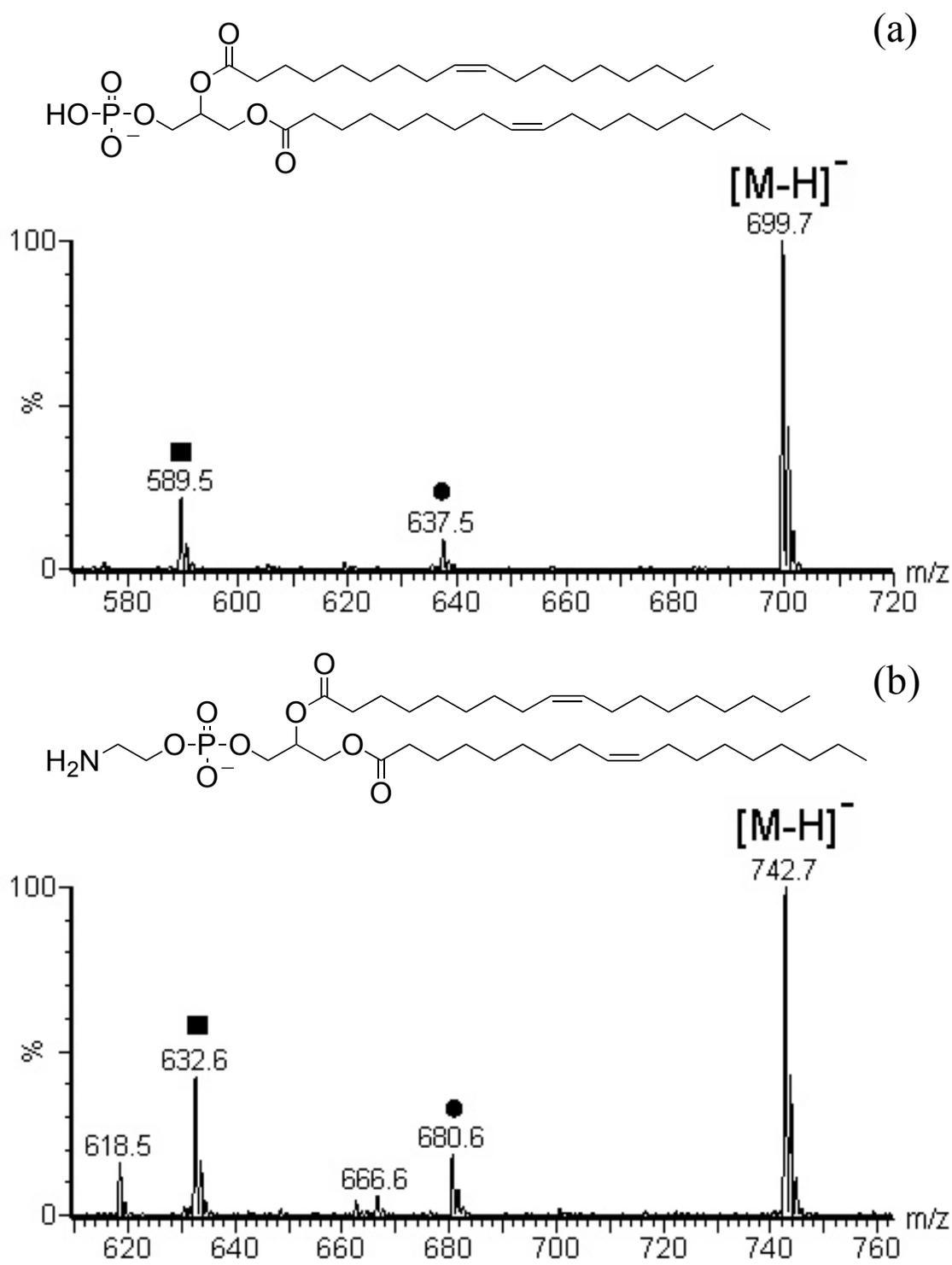


Figure 5(cont).

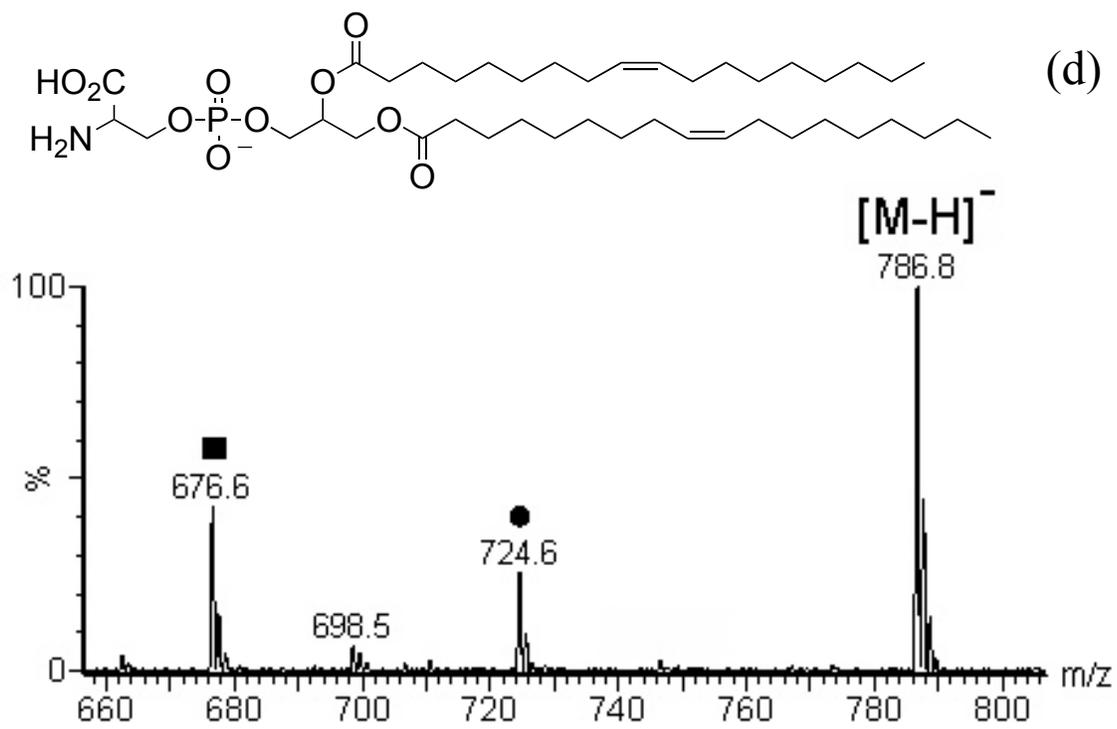
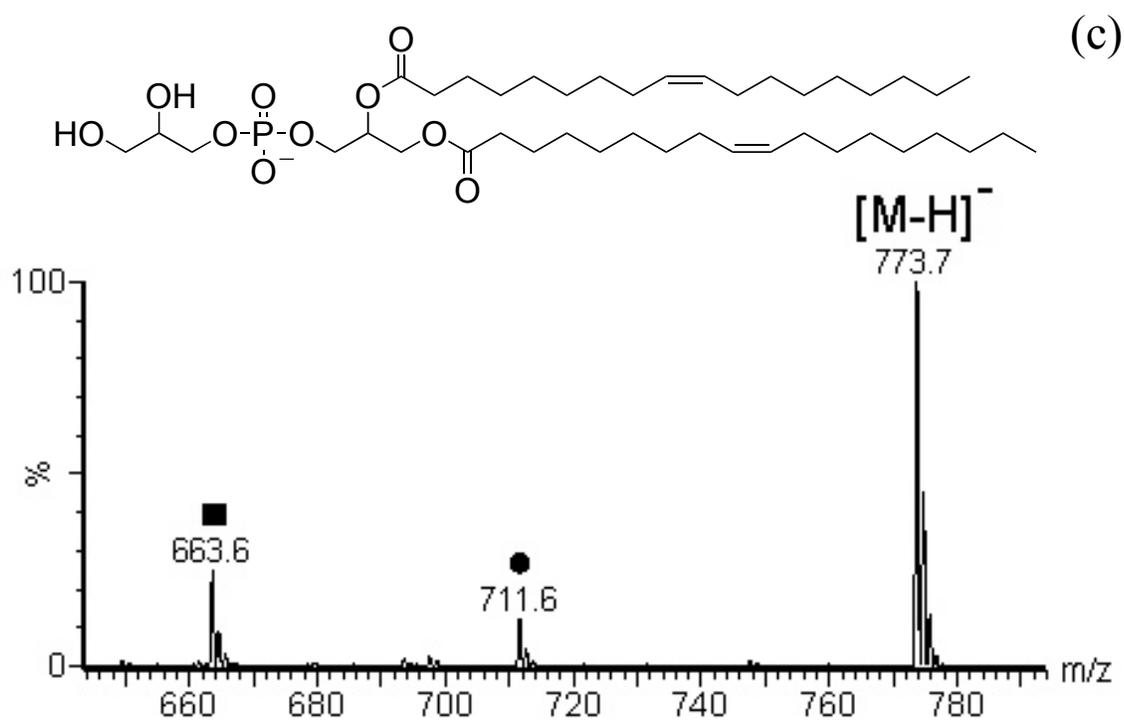


Figure 5(cont).

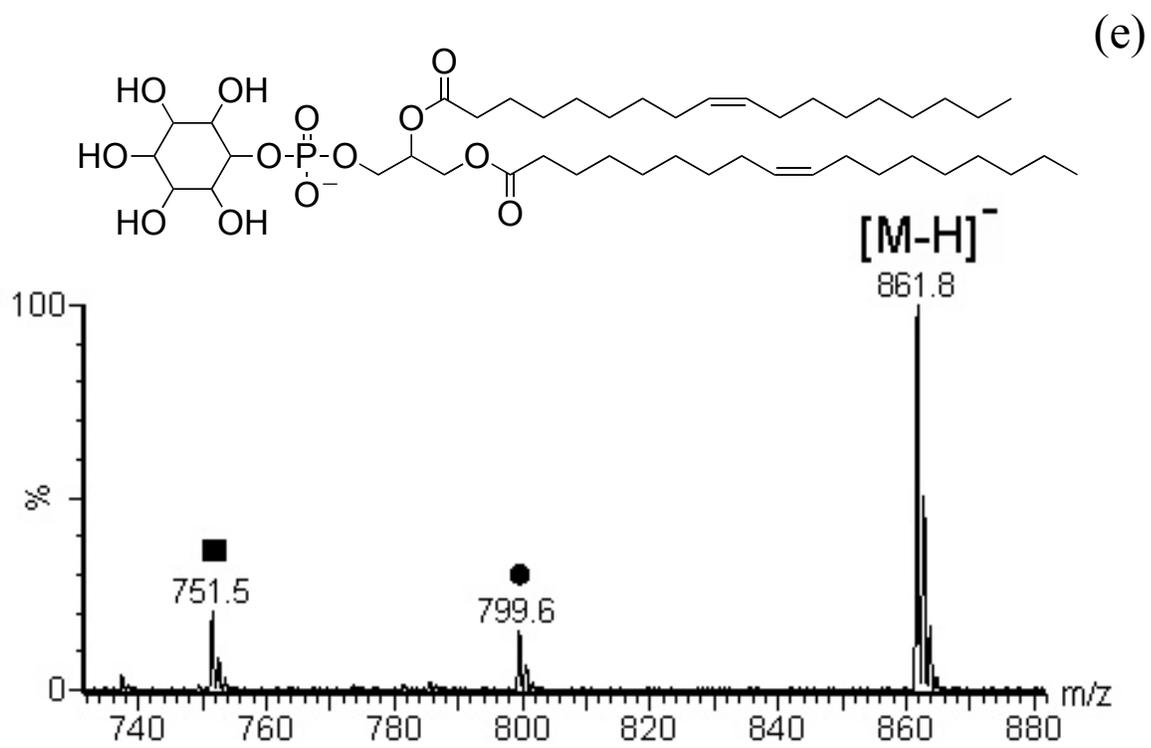


Figure 5(cont).

(f)

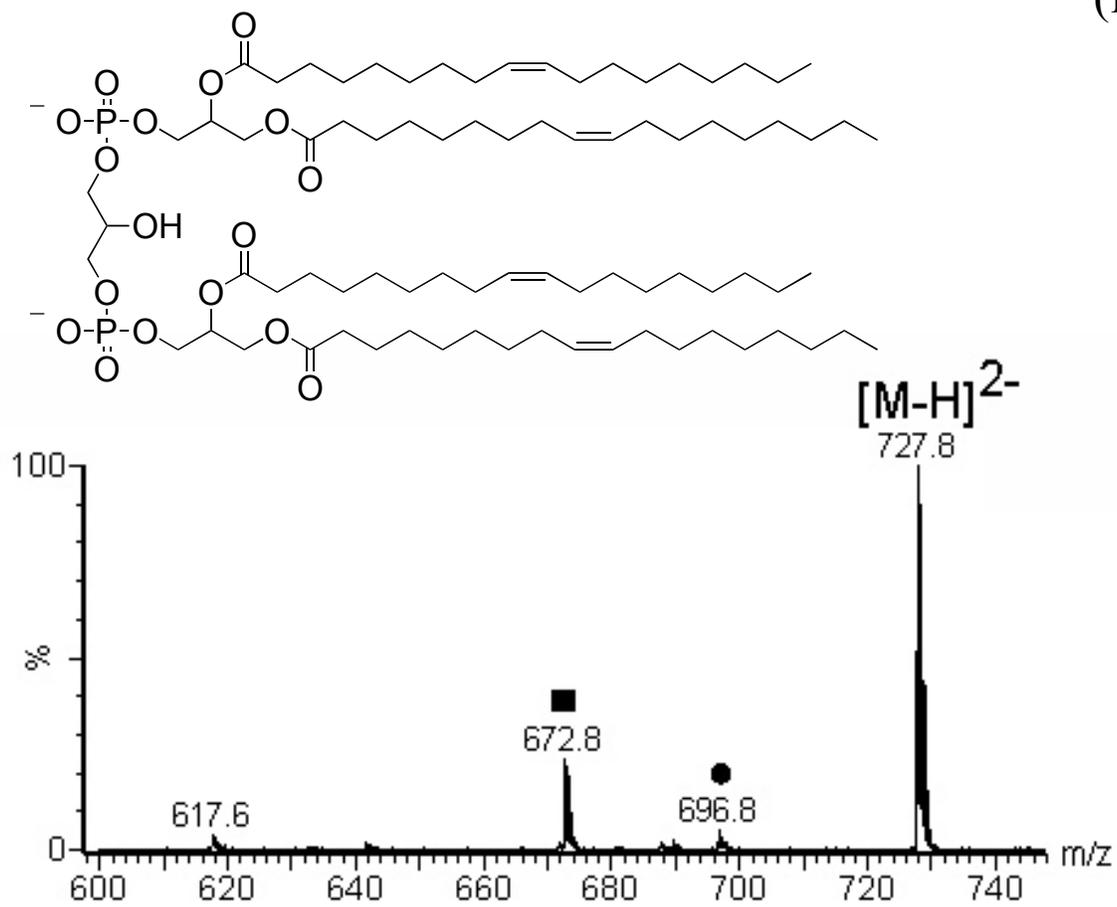


Figure 6.

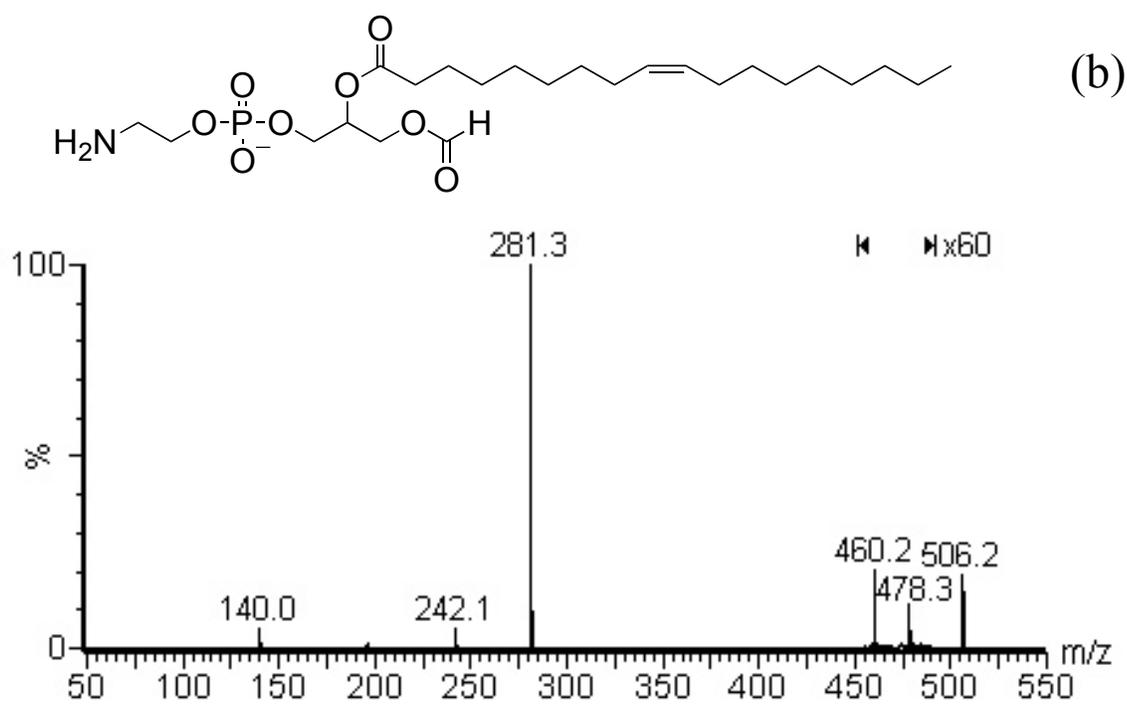
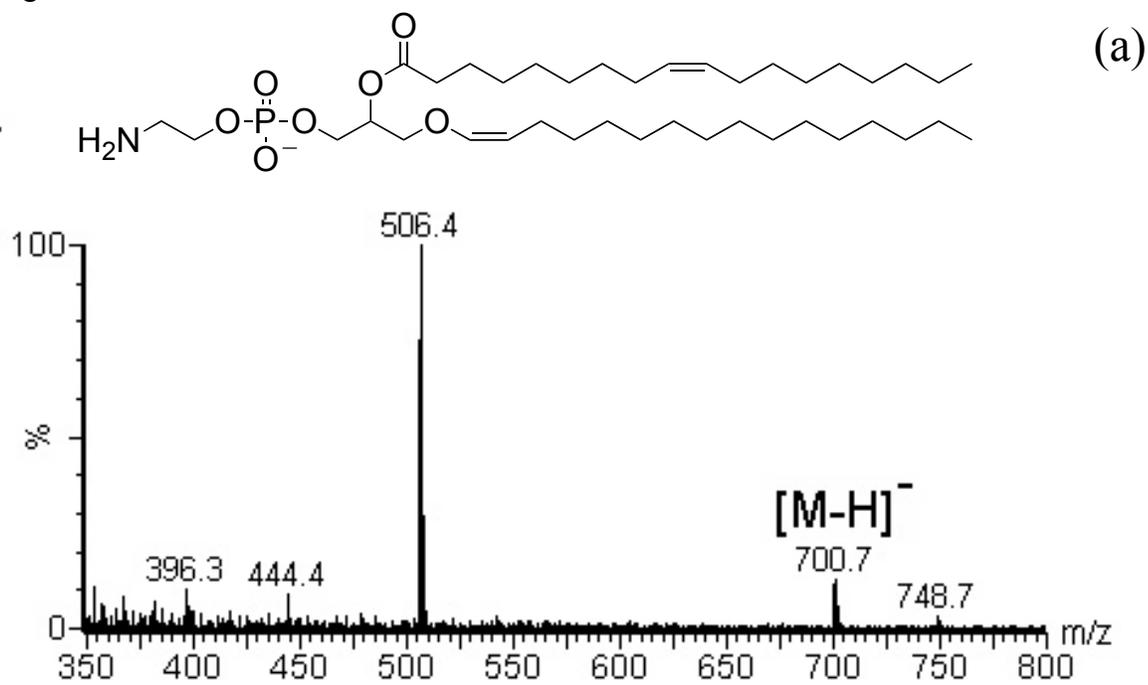


Figure 7.

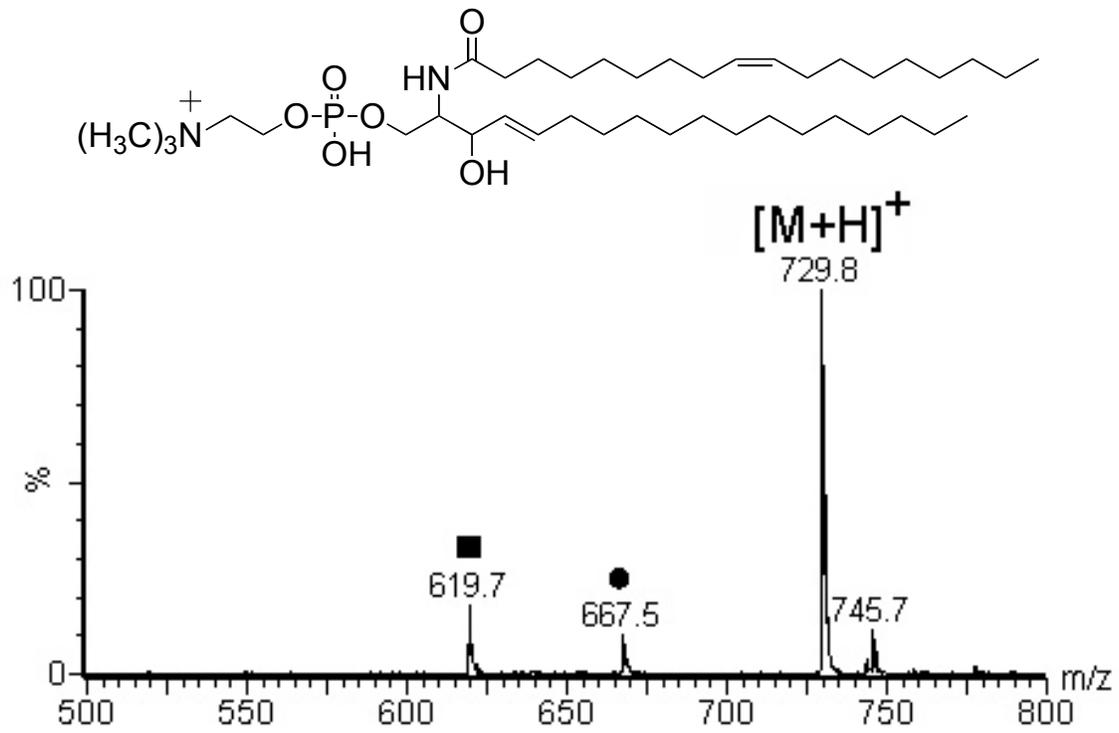


Figure 8

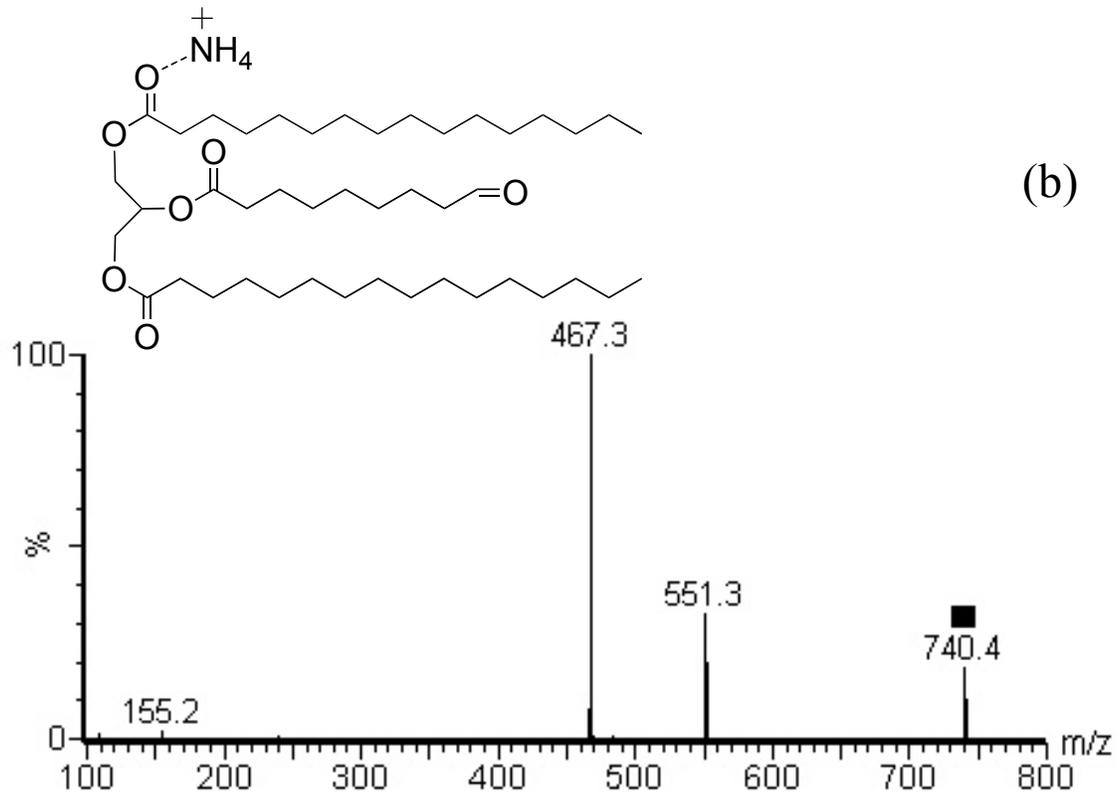
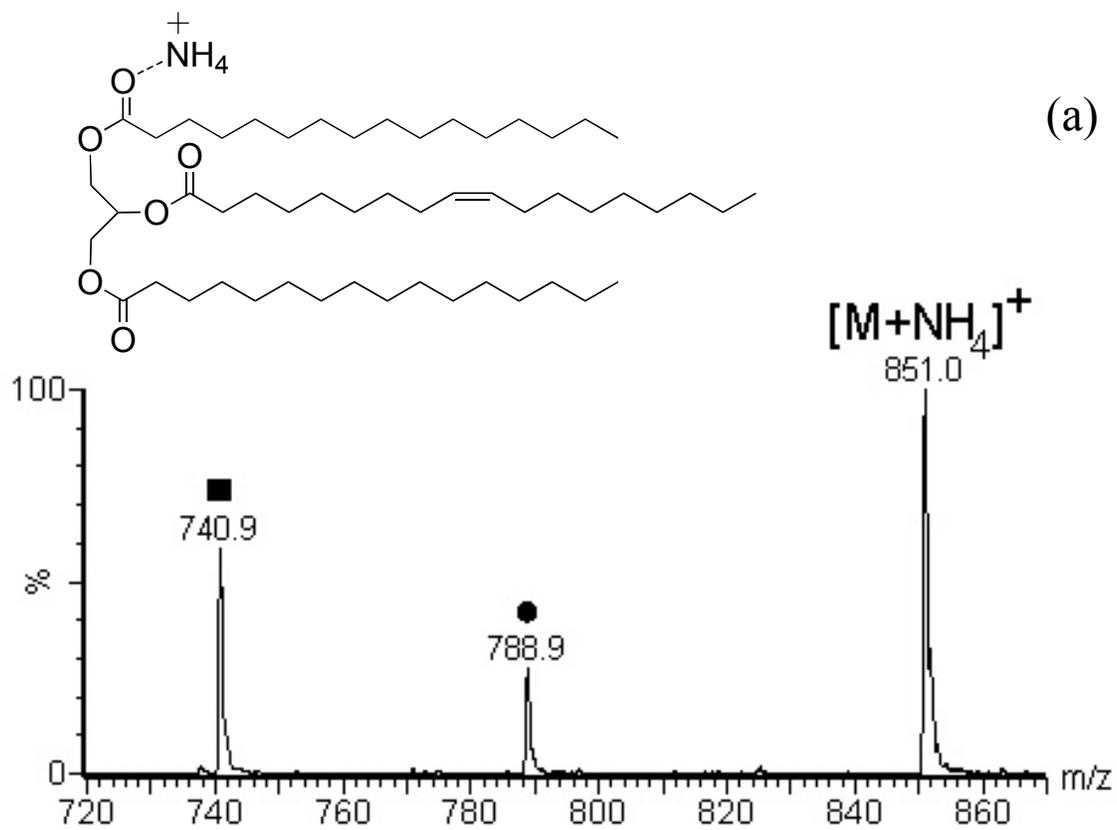


Figure 8(cont).

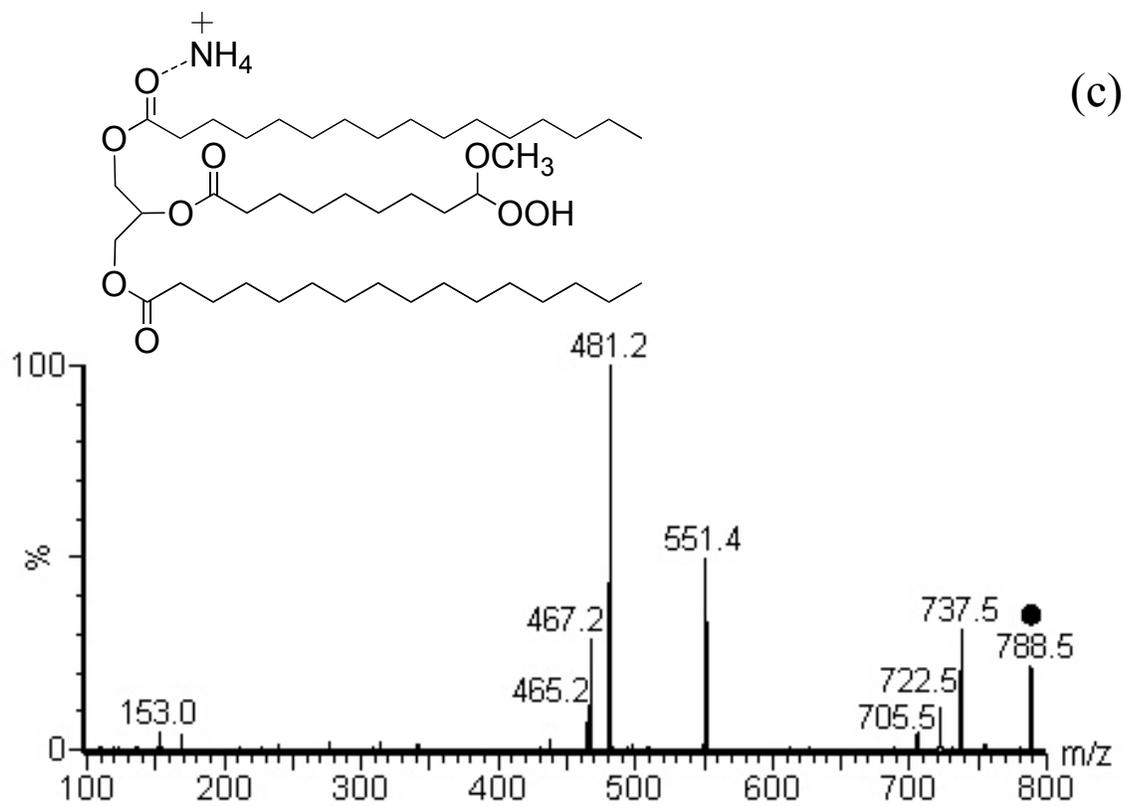


Figure 9.

