1-1-2008

Ozone induced dissociation: elucidation of double bond position within mass-selected lipid ions

Michael C. Thomas
University of Wollongong, mct58@uow.edu.au

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

David G. Harman
University of Wollongong, dharman@uow.edu.au

Jane M. Deeley
University of Wollongong, jmd67@uow.edu.au

Jessica R. Nealon
University of Wollongong, jnealon@uow.edu.au

See next page for additional authors

Ozone induced dissociation: elucidation of double bond position within mass-selected lipid ions

Abstract

Ions formed from lipids during electrospray ionization of crude lipid extracts have been mass-selected within a quadrupole linear ion trap mass spectrometer and allowed to react with ozone vapor. Gas-phase ion–molecule reactions between unsaturated lipid ions and ozone are found to yield two primary product ions for each carbon–carbon double bond within the molecule. The mass-to-charge ratios of these chemically induced fragments are diagnostic of the position of unsaturation within the precursor ion. This novel analytical technique, dubbed ozone-induced dissociation (OzID), can be applied both in series and in parallel with conventional collision-induced dissociation (CID) to provide near-complete structural assignment of unknown lipids within complex mixtures without prior fractionation or derivatization. In this study, OzID is applied to a suite of complex lipid extracts from sources including human lens, bovine kidney, and commercial olive oil, thus demonstrating the technique to be applicable to a broad range of lipid classes including both neutral and acidic glycerophospholipids, sphingomyelins, and triacylglycerols. Gas-phase ozonolysis reactions are also observed with different types of precursor ions including $[M + H]^+$, $[M + Li]^+$, $[M + Na]^+$, and $[M - H]^-$: in each case yielding fragmentation data that allow double bond position to be unambiguously assigned. Within the human lens lipid extract, three sphingomyelin regioisomers, namely SM(d18:0/15*Z*-24:1), SM(d18:0/17*Z*-24:1), and SM(d18:0/19*Z*-24:1), and a novel phosphatidylethanolamine alkyl ether, GPEtn(11*Z*-18:1e/9*Z*-18:1), are identified using a combination of CID and OzID. These discoveries demonstrate that lipid identification based on CID alone belies the natural structural diversity in lipid biochemistry and illustrate the potential of OzID as a complementary approach within automated, high-throughput lipid analysis protocols.

Keywords

Ozone, induced, dissociation, elucidation, double, bond, position, within, mass, selected, lipid, ions, CMMB

Disciplines

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

Publication Details

Thomas, M., Mitchell, T. W., Harman, D. G., Deeley, J. M., Nealon, J. & Blanksby, S. J. (2008). Ozone induced dissociation: elucidation of double bond position within mass-selected lipid ions. Analytical Chemistry, 80 (1), 303-311.

Authors

Michael C. Thomas, Todd W. Mitchell, David G. Harman, Jane M. Deeley, Jessica R. Nealon, and Stephen J. Blanksby

Ozone Induced Dissociation (OzID): a novel method for the elucidation of double bond position within mass-selected lipid ions

Michael C. Thomas, Todd W. Mitchell, David G. Harman, Jane M. Deeley, Jessica R. Nealon, Stephen J. Blanksby*

Department of Chemistry and School of Health Sciences, University of Wollongong, Wollongong NSW, 2522, Australia.

Address reprint requests to Dr Stephen J. Blanksby, Department of Chemistry, University of Wollongong, Wollongong NSW, 2522, Australia. E-mail blanksby@uow.edu.au

Abstract

Ions formed from lipids during electrospray ionization of crude lipid extracts have been mass-selected within a quadrupole linear ion trap mass spectrometer and allowed to react with ozone vapor. Gas phase ion-molecule reactions between unsaturated lipid ions and ozone are found to yield two primary product ions for each carbon-carbon double bond within the molecule. The mass-to-charge ratios of these chemically induced fragments are diagnostic of the position of unsaturation within the precursor ion. This novel analytical technique, dubbed Ozone Induced Dissociation (OzID), can be applied both in series and in parallel with conventional collision induced dissociation (CID) to provide near complete structural assignment of unknown lipids within complex mixtures without prior fractionation or derivatization. In this study, OzID is applied to a suite of complex lipid extracts from sources including human lens, bovine kidney and commercial olive oil, thus demonstrating the technique to be applicable to a broad range of lipid classes including both neutral and acidic glycerophospholipids, sphingomyelins and triacylglycerols. Gas phase ozonolysis reactions are also observed with different types of precursor ions including $[M+H]^+$, $[M+Li]^+$, $[M+Na]^+$ and $[M-H]^-$: in each case yielding fragmentation data that allow double bond position to be unambiguously assigned. Within the human lens lipid extract, three sphingomyelin regioisomers, namely SM(d18:0/15*Z*-24:1), SM(d18:0/17*Z*-24:1) and SM(d18:0/19*Z*-24:1), and a novel phosphatidylethanolamine alkyl ether, GPEtn(11*Z*-18:1e/9*Z*-18:1), are identified using a combination of CID and OzID. These discoveries demonstrate that lipid identification based on CID alone belies the natural structural diversity in lipid biochemistry and

illustrate the potential of OzID as a complementary approach within automated, high through-put lipid analysis protocols.

Introduction

Modern, high throughput lipid analysis techniques are increasingly reliant on tandem mass spectrometry to undertake both the isolation and the structural identification of individual lipids within complex mixtures. In the "shotgun" lipidomic approach a crude lipid extract is subjected to direct analysis by electrospray ionization with little or no prior chromatographic purification.[1,2] An individual lipid is then mass-selected and interrogated by collision induced dissociation (CID) whereby a growing knowledge-base of fragmentation behavior can be brought to bear in the assignment of molecular structure. This approach has proved particularly effective in phospholipid analysis, where the lipids ionize readily and CID data are easily interpreted to yield information on lipid class and the total number of carbons and double bonds of any esterified fatty acids.[3,4] This approach to lipid analysis is rapidly gaining popularity due to its combination of speed and sensitivity. Software packages currently in development also offer great promise for automated interpretation of the large volumes of data generated by the mass spectrometer.[5,6] It is significant to note however, that in most cases conventional CID of lipids does not provide information on the position of carbon-carbon double bond(s). Significantly, the conventional negative ion CID spectra of the $[M-H]^-$ negative ions arising from phospholipids that differ only in the position of a double bond are indistinguishable.[7,8] Most analyses therefore, assign the position of unsaturation purely on the basis of fatty acid natural abundances. For example, if a fragment ion of *m/z* 281 is observed in the CID spectrum of the $[M-H]^-$ ion of a phospholipid, the 18:1 fatty acyl substituent will be inevitably assigned as 9*Z*-18:1 (oleic acid). While oleic acid is a highly abundant fatty acid, other regioisomers such as 6*Z*-18:1 (petroselinic acid) and

11*Z*-18:1 (vaccenic acid) are also found in nature. As such, assumptions of this kind represent a significant limitation of current technologies as isomeric lipids differing only in double bond position can have distinct biochemical and biophysical properties.[9] While traditional gas-chromatography based methods can distinguish regioisomeric fatty acids, this approach requires that all fatty acid-ester bonds are hydrolysed. As a consequence, the assignment of each fatty acid identified to its parent lipid(s) within a complex mixture is impossible without extensive, multidimensional fractionation. Several mass spectrometric approaches to the elucidation of double bond position within intact lipids have been demonstrated but these require either; specialized instrumentation,[10] derivatization prior to analysis,[7,11] sufficient volatility of the parent lipids,[12] and/or isolated lipids or simple mixtures.[8] Ideally, high through-put lipidomic approaches need to incorporate a protocol that, combined with CID, can provide near complete structure elucidation for intact lipids within complex mixtures. The method(s) should; (i) not require chromatographic separation or wet-chemical derivatization prior to analysis, (ii) acquire data quickly (ideally on the mass spectrometric timescale), (iii) provide readily interpretable data that allow *de novo* identification (*i.e.*, spectral comparison to authentic standards should not be required), and (iv) be accomplished on readily available instrumentation (ideally similar to that employed for conventional ESI-MS and CID analysis).

Our previous efforts toward determining double bond position have centred on the use of ozone chemistry in conjunction with mass spectrometry. We have demonstrated that ozone electrospray ionization-mass spectrometry (OzESI-MS), whereby ozonolysis is initiated in the electrospray ionization source of a commercial mass spectrometer,

provides two chemically induced fragment ions for each double bond that can readily identify double bond position.[8,13] While this technology is easy to use and highly effective for individual lipids or simple lipid mixtures, interpretation of OzESI-MS spectra of complex mixtures is exceedingly difficult. This situation arises because ozone induced fragments are often isobaric with other lipid ions and the assignment of fragments to their respective precursor ions becomes ambiguous as the complexity of the mixture increases. To overcome this limitation we have undertaken to expose ionized lipids to ozone vapor within an ion-trap mass spectrometer. Herein we demonstrate that ozone induced dissociation (OzID) can be successfully employed for elucidation of double bond position within intact, unsaturated lipids mass-selected from complex extracts.

Methods

Materials and sample preparation

All synthetic phospholipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and were used without further purification. The triacylglycerol standard, TG(16:0/9*Z*-18:1/16:0), was purchased from Sigma-Aldrich (Castle Hill, Australia). HPLC grade methanol, AR grade chloroform and AR grade sodium acetate were purchased from APS Chemicals (Sydney, Australia). Industrial grade compressed oxygen (purity 99.5%) and ultra high purity helium were obtained from BOC gases (Cringila, Australia). Standard solutions of lipids were prepared in methanol at concentrations of 1 to 10 μM. To aid the formation of sodium adducts 100 to 200 μM sodium acetate was added. Cow kidney was collected from the Wollondilly Abattoir (NSW, Australia) and the phospholipids extracted by homogenisation with chloroform-

methanol (2:1 v/v with 0.01% butylated hydroxytoluene). Normal human lenses were obtained from the Save Sight Institute (Sydney, Australia) and human cataractous lenses from the K.T. Sheth Eye Hospital (Rajkot, India). Phospholipids were extracted with chloroform-methanol (2:1 v/v with 0.01% butylated hydroxytoluene) after homogenisation under liquid nitrogen. Phospholipid extracts were made to approximately 40 μM in 2:1 methanol-chloroform for mass spectrometric analysis. Sodium adducts were observed under standard ESI conditions and could be further enhanced by the addition of 200 μM sodium acetate. Pure Spanish olive oil (Always Fresh) was obtained and diluted to approximately 40 μg/mL in methanol with 100 μM sodium acetate for mass spectrometric analysis.

Ozone generation

A HC-30 ozone generator (Ozone Solutions, Sioux Center, Iowa, USA) was used for the production of ozone. Oxygen pressure was set to 20 psi and the ozone generator set to a power output of 68 (arbitrary units). To produce high concentration ozone, the oxygen flow rate was set at 400-500 mL/min for 20-30 minutes before the flow rate was decreased to between 30-40 mL/min for several minutes prior to ozone collection. The resulting ozone/oxygen mixture (12% v/v by titrimetric analysis) was collected in a 10 mL disposable, ozone-resistant plastic syringe (Livingstone). **Warning:** Ozone is a toxic gas and was produced in a fumecupboard. Excess ozone was destroyed by bubbling through an aqueous solution of sodium thiosulfate, sodium iodide and vitex indicator. Only ozone compatible materials were used. Rubber is not suitable.

Instrumentation

OzID experiments were performed using a modified ThermoFinnigan LTQ ion-trap mass spectrometer (San Jose, CA). The instrument modification involved by-passing the helium splitter to make a direct connection between the helium supply and the ion trap with the helium flow rate controlled using a metering flow valve. Ozone was introduced by attaching a plastic syringe containing ozone to a PEEKsil tubing restrictor (100 mm L x 1/16" OD x 0.025 mm ID, SGE) connected to the helium supply line via a shut-off ball valve and T-junction downstream of the metering flow valve. Backing pressure was applied to the syringe using a syringe pump set to 25 μL/min. In the experiments, the helium flow rate was adjusted so that the ion gauge pressure read approximately 0.8 x 10^{-5} Torr with the addition of oxygen and ozone (although this may not be an accurate pressure reading since the ion gauge is calibrated for helium). This measured manifold pressure corresponds to an estimated total pressure of 2.5 mTorr within the ion trap and was found to provide optimal performance in mass accuracy, peak shape and ion abundance. An isolation width of 2-3 Th was used to isolate the ion of interest, and a trapping time of 10 s was used to generate all OzID spectra with the exception of Figure 7d that was acquired with a 30 ms trapping time. For sodiated phosphocholine containing ions, two isolation steps were found to be useful in removing a collision induced fragment ion (59 Da neutral loss) from the spectra. This was done by using an isolation width of 2-3 Th, followed by an isolation at 10 Th with a trapping (activation) time of 10 s. In most cases 50 scans were acquired and averaged to obtain a sufficient signal-to-noise ratio.

Results and Discussion

OzID of Phosphatidylcholine Cations

Electrospray ionization of a methanolic solution of the commercially available phosphatidylcholine standard, GPCho(16:0/9*Z*-18:1), produces an abundant ion at *m/z* 782 corresponding to the $[M+Na]^+$ adduct. Isolation and trapping of this ion within the quadrupole ion-trap mass spectrometer in the presence of ozone vapor for 10 s yields the spectrum shown in Figure 1, which we refer to as an ozone induced dissociation, or OzID, spectrum. These data reveal that the gas phase ion-molecule reaction between the monounsaturated lipid and ozone yields two abundant product ions at *m/z* 672 and *m/z* 688. The formation of the *m/z* 672 ion represents a neutral loss of 110 Da and is identical to previous observations of ozonolysis reactions involving n-9 lipids[14] in protic solvents[15], in model membranes[16] and in the ionization source of various mass spectrometers.[8,13,17] The *m/z* 672 ion is therefore confidently assigned as the sodium adduct of the aldehyde, 2-(9-oxononanoyl)-1-palmitoyl-*sn*-glycero-3-phosphocholine. The second chemically induced fragment ion at *m/z* 688 appears 16 Da to higher mass than the aldehyde, representing an overall neutral loss of 94 Da from the precursor ion. This ion has the correct mass-to-charge ratio for the elusive carbonyl oxide intermediate formed from the decomposition of the primary ozonide according to Criegee's famous mechanism (Scheme 1).[18,19] It is unclear, however, if this ion has the carbonyl oxide moiety or whether rearrangement of the carbonyl oxide results in a more stable isomer such as the carboxylic acid or vinyl hydroperoxide indicated in Scheme 1. The CID spectrum of the *m/z* 672 ion (data not shown) reveals only fragment ions characteristic of

a sodiated phosphatidylcholine[20,21] and thus provides no clues as its structural identity. Work is currently underway to probe the structure of this ion but this falls outside the scope of the current paper as the absence of a definitive molecular structure does not diminish the analytical utility of this fragmentation pathway. Given that the structure of this ion remains ambiguous however, we refer to it herein only as the "Criegee ion" in reference to Criegee's mechanism for ozonolysis.[18,19]

Less abundant ions at *m/z* 613, 629 and 830 are also observed in Figure 1. The latter ion corresponds to an addition of 48 Da to the $[GPCho(16:0/9Z\text{-}18:1)+Na]^+$ precursor ion and is attributed to the secondary ozonide indicated in Scheme 1. The mass defect of the *m/z* 830 ion is slightly larger than that observed for other ions in the same spectrum and may result from dissociation of the labile ozonide during ion-ejection. The low abundance ions at *m/z* 613 and 629 correspond to neutral losses of 59 Da from the *m/z* 672 and 688 ions, respectively. The neutral loss of trimethylamine (59 Da) is a dominant fragmentation resulting from CID of sodium adduct ions of phosphatidylcholines.[20,21] In this case, it is likely that some of the energy liberated from the addition of ozone across the double bond (computed to be *ca*. 55 kcal mol^{-1})[22] is partitioned into the resulting fragment ions and drives subsequent decomposition via loss of trimethylamine. Interestingly, the abundance of the *m/z* 629 ion is greater than the *m/z* 613 ion despite the fact that the abundance of the *m/z* 688 (the putative progenitor of *m/z* 629) is of lower abundance that *m/z* 672. This observation suggests that the Criegee ion at *m/z* 688 ion has more internal energy than the aldehyde at *m/z* 672, perhaps resulting from rearrangement of the former from the reactive carbonyl oxide to a more stable isomer (*cf*. Scheme 1).

[Figure 1]

[Scheme 1]

OzID spectra were also obtained for the $[M+H]^+$ and $[M+Li]^+$ ions of GPCho(16:0/9*Z*-18:1) and are provided as Supporting Information (Figure S1a and S1b). For both cations the major ozonolysis product ions correspond to neutral losses of 110 and 94 Da and thus, by analogy to the sodiated adduct, were assigned to the aldehyde and Criegee ions, respectively. Some subtle differences were observed however, for example, the $[M+H]^+$ ion did not show the loss of 59 Da from the aldehyde and Criegee ions. This observation is consistent with the well known differences in CID behavior of sodiated (or lithiated) and protonated phosphatidylcholines.[21] More interestingly, comparison of the OzID spectra of $[M+H]^+$, $[M+Li]^+$ and $[M+Na]^+$ ions recorded under the same conditions show ozone induced fragment ions formed from the metal adduct ions are *ca*. 5 times more abundant than for the protonated lipid. Higher yields of ozonolysis product ions are indicative of a faster rate of reaction for the lithiated and sodiated adducts and suggest the participation of the charge carrier in the mechanism of the reaction (*i.e.*, this is unlikely to be a charge remote reaction). Based on these observations and their ease of formation, $[M+Na]^+$ adduct ions were used in all further positive ion experiments described herein.

The OzID spectra of the regioisomeric ions, $[GPCho(9Z\text{-}18:1/9Z\text{-}18:1)+Na]^+$ and $[GPCho(6Z\text{-}18:1/6Z\text{-}18:1)+Na]^+$, are shown in Figure 2. For the sodiated GPCho(9*Z*-18:1/9*Z*-18:1) ion the ozone induced fragment ions are observed 110 and 94 Da below the

precursor ion and are thus identical to the neutral losses observed for GPCho(16:0/9*Z*-18:1) and indicative of the presence of an n-9 double bond. Conversely, for the sodiated GPCho(6*Z*-18:1/6*Z*-18:1) ion the OzID ions are observed with neutral losses of 152 and 136 Da. These product ions are 42 Da - or three methylene groups - below the corresponding ions for the n-9 regiosiomer, GPCho(9*Z*-18:1/9*Z*-18:1), and thus confirm the presence of an n-12 double bond. These data demonstrate that OzID can quickly and unambiguously identify double bond position and differentiate between phospholipid regioisomers.

[Figure 2]

OzID of Glycerophospholipid Anions

The OzID spectrum of the $[M-H]^-$ ion of the acidic glycerophospholipid standard, GPGro(9*Z*-18:1/9*Z*-18:1) was acquired using a 10 s reaction time (Figure 3). In negative ion mode only a single isolation step was required since partial dissociation of the anion was not encountered during isolation of the negative ion as had been previously observed for metal-adduct ions of phosphocholine containing lipids. The OzID spectrum of the $[GPGro(9Z\text{-}18:1/9Z\text{-}18:1)\text{-}H]^-$ anion reveals a molecular ion at *m/z* 773 while the ozonolysis products ions are observed at *m/z* 663 and 679. These fragment ions correspond to neutral losses of 110 and 94 Da, respectively, and are directly analogous to those observed in the OzID spectrum of the $[GPCho(16:0/9Z\text{-}18:1)+Na]^+$ cation. The CID spectrum of *m/z* 663 was consistent with those previously reported for acidic phospholipids bearing an aldehydic 9-oxononanoyl radyl (*cf.* Scheme 1).[8,13] CID of the

ozone induced fragment at *m/z* 679 (Supporting Information, Figure S2) reveals a loss of water that could be indicative of either a hydroperoxide or a carboxylic acid moiety (*cf.* Scheme 1). Significantly however, this loss of water accounts for the observation of the *m/z* 661 ion in the OzID spectrum of $[GPGro(9Z\text{-}18:1/9Z\text{-}18:1)\text{-}H]^-$ ion.

[Figure 3]

OzID of Glycerophospholipids From a Cow Kidney Lipid Extract

The selected examples provided thus far, demonstrate that when applied to lipid standards of known structure the OzID spectrum can unambiguously confirm the position of unsaturation. The major advantage of OzID over previous methods for the elucidation of double bond position however, is that the experiment is performed on mass-selected ions and thus should be applicable to the characterization of unknown lipids present in complex mixtures. Ongoing investigations within our laboratory involve the analysis of lipid extracts from a range of biological sources.[23,24] Lipid extracts from bovine kidney are typical of those encountered in our research (and that of many other groups) that include a range of phospholipid classes with varying degrees of unsaturation.

The negative ion ESI-MS spectrum of a bovine kidney lipid extract is shown in Figure 4(a). This spectrum shows the $[M\text{-}H]^-$ of a suite of acidic phospholipids present within the extract. The two most abundant ions in this spectrum are observed at *m/z* 885 and 766 and, based on mass alone, can be tentatively assigned to the polyunsaturated phospholipids GPIns(38:4) and GPEtn(38:4), respectively. The structure of each ion was

further elucidated by the respective negative ion CID spectra that confirm the headgroup assignments and identify the fatty acyl chains to be GPIns(18:0/20:4) and GPEtn(18:0/20:4) (data provided as Supporting Information, Figure S3a and S3b). The position of unsaturation in each of the 20:4 radyls can be determined from the OzID spectra shown in Figures 4(b) and (c). Independent of the distinct headgroups of each phospholipid, both spectra display chemically induced fragment ions that correspond to neutral losses of 188, 172, 148, 132, 108, 92, 68 and 52 Da. For example, in Figure 4(b) high mass fragment ions appear at *m/z* 833 and 817, separated by 16 Da and indicative of a Criegee and an aldehyde ion, respectively. The corresponding neutral losses of 52 and 68 Da suggest that these fragments result from ozonolysis of an n-6 double bond. Subsequent pairs of fragment ions appear to lower mass by consecutive losses of 40 Da, indicative of the C_3H_4 units encountered in skip-conjugated polyunsaturated compounds and thus allowing the assignment of double bonds to the n-6, n-9, n-12 and n-15 positions. These positions of unsaturation unambiguously identify the 20:4 radyl as arachidonic acid as would usually be expected from the high natural abundance of this polyunsaturated fatty acid.[25] Therefore, based on the combination of ESI-MS (Figure 4a), CID (Supporting Information, Figure S3a and S3b) and OzID (Figure 4b and c) spectra these two bovine kidney derived phospholipids can be assigned as GPIns(18:0/5*Z*,8*Z*,11*Z*,14*Z*-20:4) and GPEtn(18:0/5*Z*,8*Z*,11*Z*,14*Z*-20:4), where only the stereochemistry about each of the double bonds is assumed. In this example OzID analysis confirms the expected identity of the unsaturated radyl. In contrast, the examples provided below reveal unexpected regiochemistry, stressing the importance of experimental verification of structure.

Interestingly, the ion abundance ratio of the aldehyde and Criegee ion pairs differs markedly between the OzID spectrum of GPIns(18:0/5*Z*,8*Z*,11*Z*,14*Z*-20:4) (Figure 4b) and GPEtn(18:0/5*Z*,8*Z*,11*Z*,14*Z*-20:4) (Figure 4c). This observation indicates that the nature of the phospholipid headgroup strongly influences the final product branching ratio possibly via base-induced decomposition mechanism. We have previously shown that the gas phase basicity of GPEtn is significantly greater than that of GPIns and as such, differences in product distributions may be anticipated in charge driven fragmentation processes occurring within the phospholipids.[26] These observations combined with the comparative reaction efficiencies for different cation adducts (see above) provide further evidence for the participation of the charge in the ozonolysis reaction.

[Figure 4]

OzID of Abundant Phospholipids Within a Human Lens Lipid Extract

The positive ion ESI-MS spectrum of a lipid extract from a human lens is shown in Figure 5(a) with most of the ions observed corresponding to sodium adducts of either phosphatidylcholines or sphingomyelins. In this spectrum, the two major ions observed are at *m/z* 727 and *m/z* 837 that can be assigned based on mass alone to sodium adducts of the sphingomyelins. CID spectra of these ions identify them as dihydrosphingomyelins with 16:0 and 24:1 fatty acids bound to the sphinganine backbone (data not shown).[27] Based on ESI-MS and CID analyses alone however, the structure of the most abundant unsaturated phospholipid in the human lens would be assigned as the

dihydrosphingomyelin, SM(d18:0/15*Z*-24:1), where the amide linked fatty acid is assumed to be the n-9 nervonic acid (15*Z*-tetracosenoic acid) based on its previous observation in mammalian tissues.[28] Such structure assignment ambiguities are removed by the OzID spectrum of the mass-selected *m/z* 837 ion shown in Figure 5(b). This spectrum reveals three sets of ozonolysis product ions from this monounsaturated lipid indicating the presence of three regioisomers. The most abundant set of ozone induced dissociation ions are observed at *m/z* 727 and 743 representing the neutral losses of 110 and 94 Da. These neutral losses are representative of the anticipated n-9 acyl chain. Another set of ozonolysis products however, are clearly resolved at *m/z* 755 and 771 corresponding to neutral losses of 82 and 66 Da and indicating the presence of a significant abundance of an n-7 acyl chain. The 17*Z*-24:1 fatty acid has been previously identified by Brenna and co-workers in the white matter of a primate brain using covalent adduct chemical ionization tandem mass spectrometry.[29] The spectrum in Figure 5(b) also reveals a further set of ozonolysis product ions of even lower abundance at *m/z* 783 and 799 representing neutral losses of 54 and 38 Da and indicating the presence of a third dihydrosphingomyelin with an n-5 acyl chain. The identification of a novel lipid, SM(d18:0/19*Z*-24:1) within the human lens is consistent with our previous tentative assignment based on ozone electrospray mass spectrometry (OzESI-MS).[8] The combination of ESI-MS, CID and OzID analysis provide strong evidence for three regioisiomeric dihydrosphingomyelins present within the human lens, namely SM(d18:0/15*Z*-24:1), SM(d18:0/17*Z*-24:1) and SM(d18:0/19*Z*-24:1), where once again only the stereochemistry about the double bonds need be assumed. This example serves

to illustrate the potential for wide structural diversity of lipids present in nature and raises intriguing questions about the biological function of these novel compounds.

[Figure 5]

The negative ion ESI-MS spectrum of a lipid extract from a human lens is shown in Figure 6(a) revealing a suite of deprotonated phospholipid ions. While one must be careful in using relative ion abundances as a measure of lipid concentrations,[30] the lipid at *m/z* 728 is clearly a significant lens phospholipid. Based on (i) the mass-to-charge ratio of the anion, (ii) the negative ion CID spectrum (Figure 6b), and (iii) the observation of the corresponding cation in a *m/z* 141 positive ion precursor ion scan (data not shown), this lipid could be assigned to the phosphatidylethanolamines, GPEt(18:0p/18:1) or GPEt(18:1e/18:1), where the *sn*-1 fatty acid is attached via an alkenyl or an alkyl ether linkage, respectively.[31] The OzID spectrum of the $[M-H]^-$ anion at *m/z* 728 (Figure 6c), reveals ozone induced fragment ions at *m/z* 662, 646, 634 and 618. The high mass pair of ions correspond to neutral losses of 66 and 82 Da, characteristic of an n-7 unsaturated carbon chain, while the pair to lower mass correspond to neutral losses of 94 and 110 Da, ascribed to n-9 double bonds. Significantly, no ions were observed corresponding to the neutral losses of either 222 or 206 Da that might be expected from ozone induced cleavage of an 18-carbon alkenyl ether-linked fatty acid. The absence of both such ions is inconsistent with the putative plasmalogen structure, given the expected enhancement of ozone reactivity toward electron rich alkenyl ethers.[8,32] These data are thus more suggestive of an unsaturated alkyl ether phospholipid, *e.g.*, GPEt(18:1e/18:1), where one

of the carbon chains has an n-7 and the other an n-9 double bond. To distinguish these two structural possibilities an MS^3 experiment was performed, wherein the *m/z* 464 CID ion - formed via loss of the esterified 18:1 fatty acid as a ketene from the *m/z* 728 precursor ion (Figure 6b) - was itself mass-selected and allowed to react with ozone. This serial CID-OzID experiment (Figure 6d) yielded fragment ions at *m/z* 398 and 382 via neutral losses of 66 and 82 Da, respectively. These ions can be assigned to ozonolysis of an n-7 double bond on the ether linked 18:1 radyl and strongly suggest an overall assignment of GPEt(11*Z*-18:1e/9*Z*-18:1). The fragment ion at *m/z* 380 is consistent with water loss from the Criegee ion at *m/z* 398, the loss is similar to that observed for Criegee ions formed from the unsaturated phosphatidylglycerol (Figure 3). The complete structural characterization of this unusual lipid is beyond the scope of the current study and will be published elsewhere.

In addition to the analytical utility described above, the OzID spectrum in Figure 6(c) reveals several other interesting features. Firstly, the relative abundance of aldehyde versus Criegee ions shows a clear preference for the latter consistent with other phosphatidylethanolamines discussed earlier (*e.g.*, Figure 4c). Furthermore, with the CID-OzID establishing the different position of unsaturation in each of the two carbon chains, it is interesting to note the relative abundance of the two pairs of fragment ions. The observation that the n-9 ions (*m/z* 634 and 618) are noticeably more abundant than the n-7 ions (*m/z* 646 and 662) may result from differing exposure of the two olefinic sites to ozone in the gas phase conformation of the lipid. If this could be established with certainty such spectral differences might be exploited for assignment of the relative

position of the fatty acid chains on the glycerol backbone. Such nuances in OzID spectra provide fertile ground for future investigations.

[Figure 6]

OzID of Triacylglycerols

Triacylglycerols are an important and abundant class of lipid whose structural complexity makes them challenging targets for analysis. Recent developments by McAnoy *et al.* have demonstrated that the combination of ESI-MS, MS^2 and MS^3 experiments can be used to identify the fatty acid components of mass-selected triacylglycerols and even identify their relative positions on the glycerol backbone.[33] As with phospholipids however, the position of unsaturation within the fatty acid substituents is generally assigned based only on the most naturally abundant fatty acids of the appropriate chain length and degree of unsaturation. Figure 7(a) shows the OzID spectrum of a sodium adduct of the monounsaturated triacylglycerol standard, TG(16:0/9*Z*-18:1/16:0). While ammonium adducts have previously been shown to give more structurally informative fragment ions in MS^2 and MS^3 experiments, these ions proved too fragile for isolation under the experimental conditions for OzID. The $[M+Na]^+$ however, proved sufficiently robust for mass-selection in the ion trap in the presence of ozone and after a 10 s reaction time yielded two structurally diagnostic ions at *m/z* 746 and 762, corresponding to neutral losses of 110 and 94 Da, respectively. These neutral losses are analogous to those observed for n-9 phospholipids (as discussed above) and are consistent with the known structure of this triacylglycerol standard. An abundant

$[M+Na+O_3]^+$ adduct ion is also observed in this spectrum at *m/z* 903 and is assigned to the secondary ozonide by analogy with Scheme 1.

The positive ion ESI-MS spectrum of a dilute methanolic solution of commercial olive oil in the presence of sodium acetate provides a base peak at *m/z* 907 (Figure 7b) corresponding to the sodium adduct of the abundant triacylglyceride TG(18:1/18:1/18:1). The MS^2 spectrum of the ion at *m/z* 907 (see Supporting Information, Figure S4) reveals fragment ions corresponding to neutral losses of 282 and 304 Da characteristic of 18:1 radyls. Mass-selection of the *m/z* 907 ion and exposure to ozone yields the OzID spectrum presented in Figure 7(c) showing two pronounced ozonolysis products at *m/z* 797 and 813 representing the neutral losses of 110 and 94 Da. These data therefore identify the triacylglycerol as TG(9*Z*-18:1/9*Z*-18:1/9*Z*-18:1), where only the stereochemistry about each double bond is assumed.

It is instructive to use this example to explore the speed and sensitivity of the OzID approach in the analysis of extracts. The structurally informative spectrum shown in Figure 7(c) is typical of the OzID spectra presented herein representing the sum of fifty scans, each with a 10 s reaction time. Thus, this experiment requires a total acquisition time of 8 mins and consumes *ca*. 2 nmol of analyte. By mass spectrometric standards this is a low sensitivity experiment and would be too slow for direct integration into on-line liquid chromatography-mass spectrometry protocols. To investigate the limits of sensitivity, the same sample was reanalyzed using only a 30 ms reaction time and the spectrum obtained from 150 such scans (representing a 30 s total acquisition time) is presented in Figure 7(d). Despite the low overall abundance of OzID fragments (requiring some 3000x magnification) the signal-to-noise ratio is excellent and the OzID

ions are clearly identifiable allowing for unambiguous assignment of double bond position within the molecule. The high signal-to-noise ratio is a feature of ion-molecule reaction based methodologies and these data suggest that the speed and sensitivity of OzID can be dramatically improved for future applications.

[Figure 7]

Conclusion

Using a modified linear ion trap mass spectrometer, unsaturated lipid ions were isolated and allowed to react with ozone. The reaction with ozone was found to form two abundant chemically induced fragment ions that appear at mass-to-charge ratios that allow the unambiguous identification double bond position(s). This technique, which we term ozone induced dissociation or OzID, can be applied to a wide range of lipid ions including the deprotonated ions of the acidic phospholipid classes, protonated, lithiated and sodiated adduct ions of phosphocholine containing lipids - including sphingomyelins - and sodium adducts of triacylglycerols. Unlike existing technologies, OzID is demonstrated to be applicable to the analysis of individual lipids isolated only by mass-selection of ions following electrospray ionization of unfractionated lipid extracts. Analysis of naturally occurring lipids using OzID reveals in some instances a surprising structural complexity, *e.g.*, the three regioisomeric sphingomyelins, SM(d18:0/15*Z*-24:1), SM(d18:0/17*Z*-24:1) and SM(d18:0/19*Z*-24:1), identified at the same mass-to-charge ratio in a lipid extract from a human lens. Such discoveries reveal the fascinating diversity of lipid biochemistry and stress the importance of the development of analytical

technologies that provide greater structural insight than conventional CID approaches. The ability of OzID to assign double bond position on the mass-spectrometric time-scale - with acquisition times as low as 30 s (Figure 7d) - suggests a compatibility of this technology with other on-line, automated lipid analysis protocols. Moreover, given the relative simplicity of data interpretation and the ability to carry out *de novo* structure elucidation (*i.e.*, without requiring authentic standards), OzID would be a useful adjunct to the evolving field of computer-based lipid identification.[5,6] The combination of CID and OzID analysis, either in parallel or in series, provides a high degree of structural information. One can imagine that in the not too distant future, such combined analyses - including MS^3 experiments to provide positional identification of fatty acids on the glycerol backbone[33,34] - will allow for the near complete structural characterization of phospholipids by mass spectrometry alone! Assigning stereochemistry about double bonds in intact lipids by on-line mass spectrometric techniques however, represents an exciting challenge for future experimentation.

The data presented, demonstrate that ozone induced dissociation ions display consistent neutral losses across a range of lipid classes and even different modes of ion formation (*i.e.*, $[M-H]^-$, $[M+H]^+$ and $[M+Na]^+$). Interestingly, subtle variation is observed both in; (i) the degree of ozone induced dissociation between different types of cations, and (ii) the relative abundance of aldehyde verses Criegee ions depending on the nature of the phospholipid headgroup (Figure 4). These trends require further investigation, however, they already suggest that the "charge remote" mechanism (Scheme 1) is likely to be an oversimplification of more complex ion-molecule chemistry, involving the participation of the charge moiety and/or the gas phase conformation of the lipid ion.

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 (Grants DP0452849 and LP0455472). M.C.T., J.M.D., and J.R.N. are supported by Australian Postgraduate Awards. The authors acknowledge Professors Paul Else and Roger Truscott for their assistance with obtaining bovine kidney and human lens extracts, respectively. The authors acknowledge the outstanding technical support of Mr Larry Hick and thank Assoc. Prof. Kim Ekroos for helpful discussions and encouragement.

Supporting information available:

Additional spectra as noted in the text. This material is available free of charge via the internet at http://pubs.acs.org.

References:

(1) Han, X.; Gross, R. W. *J. Lipid Res.* **2003**, *44*, 1071-1079.

(2) Han, X.; Gross, R. W. *Mass Spectrom. Rev.* **2005**, *24*, 367-412.

(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) Ejsing, C. S.; Duchoslav, E.; Sampaio, J.; Simons, K.; Bonner, R.; Thiele, C.; Ekroos, K.; Shevchenko, A. *Anal. Chem.* **2006**, *78*, 6202-6214.

(6) Haimi, P.; Uphoff, A.; Hermansson, M.; Somerharju, P. *Anal. Chem.* **2006**, *78*, 8324-8331.

(7) Moe, M. K.; Anderssen, T.; Strom, M. B.; Jensen, E. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 46-59.

(8) Thomas, M. C.; Mitchell, T. W.; Deeley, J. M.; Harman, D. G.; Murphy, R. C.; Blanksby, S. J. *Anal. Chem.* **2007**, *79*, 5013-5022.

(9) Pariza, M. W.; Park, Y.; Cook, M. E. *Prog. Lipid Res.* **2001**, *40*, 283-298.

(10) Bryant, D. K.; Orlando, R. C.; Fenselau, C.; Sowder, R. C.; Henderson, L. E. *Anal. Chem.* **1991**, *63*, 1110-1114.

(11) Harrison, K. A.; Murphy, R. C. *Anal. Chem.* **1996**, *68*, 3224-3230.

(12) Xu, Y. H.; Brenna, J. T. *Anal. Chem.* **2007**, *79*, 2525-2536.

(13) Thomas, M. C.; Mitchell, T. W.; Blanksby, S. J. *J. Am. Chem. Soc.* **2006**, *128*, 58-59.

(14) 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 OzID spectra are common to lipids with double bonds at the same position in the chain with respect to the methyl terminus. For example, GPCho(16:0/9*Z*-18:1) and SM(d18:0/15*Z*-24:1) have differing standard nomenclatures as shown but are both n-9 lipids and thus exhibit a common neutral losses of 110 and 94 Da in their respective OzID spectra.

(15) Finlayson-Pitts, B. J.; Pham, T. T. H.; Lai, C. C.; Johnson, S. N.; Lucio-Gough, L. L.; Mestas, J.; Iwig, D. *Inhal. Toxicol.* **1998**, *10*, 813-830.

(16) Santrock, J.; Gorski, R. A.; Ogara, J. F. *Chem. Res. Toxicol.* **1992**, *5*, 134-141.

(17) Grimm, R. L.; Hodyss, R.; Beauchamp, J. L. *Anal. Chem.* **2006**, *78*, 3800-3806.

(18) Criegee, R. In *Methoden der Organische Chemie*; Houben, J., Weyl, T., Eds.; G. Thieme: Stuttgart, 1952; Vol. VII, pp 6.

(19) Criegee, R. *Angew. Chem., Int. Ed. Engl.* **1975**, *14*, 745-752.

(20) Domingues, P.; Domingues, M. R. M.; Amado, F. M. L.; Ferrer-Correia, A. J. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 799-804.

(21) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 352-363.

(22) Anglada, J. M.; Crehuet, R.; Bofill, J. M. *Chem. Eur. J.* **1999**, *5*, 1809-1822.

(23) Mitchell, T. W.; Ekroos, K.; Blanksby, S. J.; Hulbert, A. J.; Else, P. L. *J. Exp. Biol.* **2007**, *in press*.

(24) Mitchell, T. W.; Turner, N.; Else, P. L.; Hulbert, A. J.; Lee, J. S.; Bruce, C. R.; Hawley, J. A.; Blanksby, S. J. *J. Appl. Physiol.* **2004**, *97*, 1823-1829.

(25) Gurr, M. I.; Harwood, J. L.; Frayn, K. N. *Lipid Biochemistry*, 5th ed.; Blackwell Science Ltd.: Oxford, UK, 2002.

(26) Thomas, M. C.; Mitchell, T. W.; Blanksby, S. J. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 926-939.

(27) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 437-449.

(28) Bettger, W. J.; DiMichelle-Ranalli, E.; Dillingham, B.; Blackadar, C. B. *J. Nutr. Biochem.* **2003**, *14*, 160-165.

(29) Michaud, A. L.; Diau, G.-Y.; Abril, R.; Brenna, J. T. *Anal. Biochem.* **2002**, *307*, 348-360.

(30) Koivusalo, M.; Haimi, P.; Heikinheimo, L.; Kostiainen, R.; Somerharju, P. *J. Lipid Res.* **2001**, *42*, 663-672.

(31) Milne, S. B.; Forrester, J. S.; Ivanova, P. T.; Armstrong, M. D.; Brown, H. A. *AfCS Research Reports [online]* **2003**, *Vol. 1 no. 11*, http://www.afcs.org/reports/v1/DA0011/DA0011.htm, 1 August 2007.

(32) Grosjean, E.; Grosjean, D. *J. Atmos. Chem.* **1999**, *32*, 205-232.

(33) McAnoy, A. M.; Wu, C. C.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1498-1509.

(34) Ekroos, K.; Ejsing, C. S.; Bahr, U.; Karas, M.; Simons, K.; Shevchenko, A. *J. Lipid Res.* **2003**, *44*, 2181-2192.

Figure Captions:

Figure 1 The OzID spectrum of the $[GPCho(16:0/9Z\text{-}18:1)+Na]^+$ adduct ion generated by electrospray ionization of a 1 μM solution of GPCho(16:0/9*Z*-18:1) in methanol with 200 μM sodium acetate. The pair of ions resulting from ozonolysis of the double bond are labeled with ■ and ● indicating aldehyde and Criegee ions, respectively.

Figure 2 The OzID spectra of the $[M+Na]^+$ ions of (a) GPCho(9*Z*-18:1/9*Z*-18:1) and (b) GPCho(6*Z*-18:1/6*Z*-18:1). Both phospholipids were made to a concentration of 1 μM in methanol with 200 μM sodium acetate to aid sodium adduct formation during ESI. The pair of ions resulting from ozonolysis of the double bond are labeled with ■ and ● indicating aldehyde and Criegee ions, respectively.

Figure 3 The OzID spectrum of the $[M-H]^-$ ion of GPGro(9*Z*-18:1/9*Z*-18:1). The precursor ion was generated by electrospray ionization of a 1 μM methanolic solution of GPGro(9*Z*-18:1/9*Z*-18:1). The pair of ions resulting from ozonolysis of the double bond are labeled with ■ and ● indicating aldehyde and Criegee ions, respectively.

Figure 4 (a) The negative ion ESI-MS spectrum of a cow kidney lipid extract (*ca*. 40 μM in 2:1 methanol-chloroform). The OzID spectra of phospholipid anions identified by CID as (b) GPIns(18:0/20:4) and (c) GPEtn(18:0/20:4). The pairs of ions resulting from ozonolysis of each double bond are labeled with ■ and ● indicating aldehyde and Criegee ions, respectively.

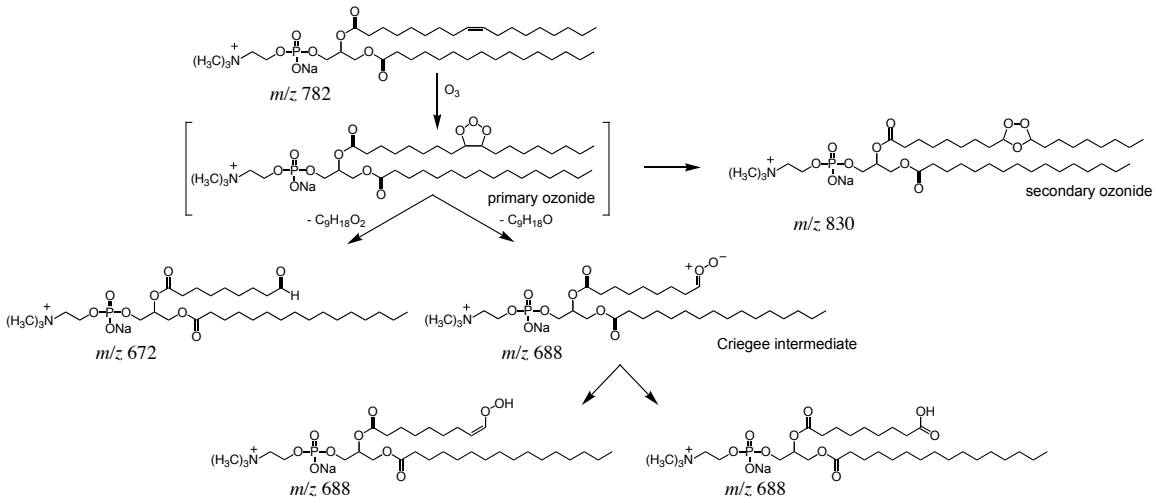
Figure 5 (a) The positive ion ESI-MS spectrum of a human lens lipid extract, all phospholipids within this spectrum appear as sodium adducts. (b) The OzID spectrum of the *m/z* 837 ion, $[SM(d18:0/24:1)+Na]^+$, mass selected from the human lens extract. The ion observed at *m/z* 684 results from a neutral loss of 59 Da (trimethylamine) from the abundant Criegee ion at *m/z* 743 while the ion at *m/z* 885 is assigned to the secondary ozonide resulting from the addition of O_3 (+48 Da). The pair of ions resulting from ozonolysis of double bonds are labeled with ■ and ● indicating aldehyde and Criegee ions, respectively.

Figure 6 (a) The negative ion ESI-MS spectrum of a human lens lipid extract, all phospholipids within this spectrum appear as $[M-H]^-$ anions. The (b) CID and (c) OzID spectra of the *m/z* 728 anion, mass-selected from the human lens extract. (d) The CID-OzID of the mass-selected fragment ion at *m/z* 464 with ozone. In this experiment *m/z* 728 is mass-selected, fragmented under CID, *m/z* 464 is mass-selected and allowed to react with ozone. The pairs of ions resulting from ozonolysis of double bonds are labeled with ■ and ● indicating aldehyde and Criegee ions, respectively.

Figure 7 (a) The OzID spectrum of the sodium adduct of the triacylglycerol, TG(16:0/9*Z*-18:1/16:0), generated from the electrospray of a 1 μM TG(16:0/9*Z*-18:1/16:0) methanolic solution with 100 μM sodium acetate. The ion at *m/z* 903 is assigned to the secondary ozonide resulting from the addition of O_3 (+48 Da). (b) The positive ion ESI-MS spectrum of a commercial sample of olive oil diluted to *ca*. 40 μg

/mL in methanol with 100 μM sodium acetate. It should be noted that in this spectrum the ions are slightly mass-shifted under OzID conditions. As such, the $[TG(18:1/18:1/18:1)+Na]^+$ cation, which has a monoisotopic mass of 907.7731, appears at *m/z* 908.0 (c) The OzID spectrum of *m/z* 907 mass-elected from (b), the sodium adduct of the most abundant triacylglycerol present within the olive oil sample, TG(18:1/18:1/18:1). (d) The OzID spectrum of *m/z* 907 as per (c) except using a 30 ms second reaction time per scan averaged over 30 s. The pair of ions resulting from ozonolysis of the double bond are labeled with ■ and ● indicating aldehyde and Criegee ions, respectively.

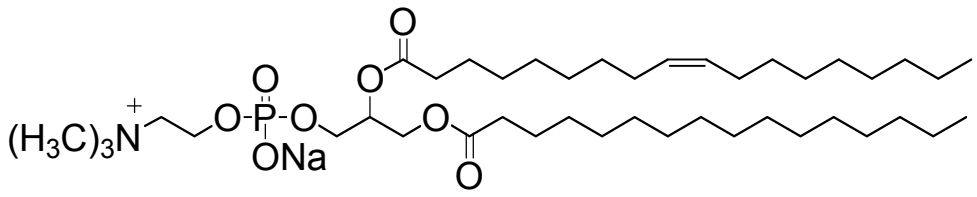
Schemes:



Scheme 1 The proposed mechanism for the gas phase reaction of mass-selected [GPCho(16:0/9*Z*-18:1)+Na]$^+$ ions (*m/z* 782) with ozone in an ion-trap mass spectrometer.

Figures:

Figure 1



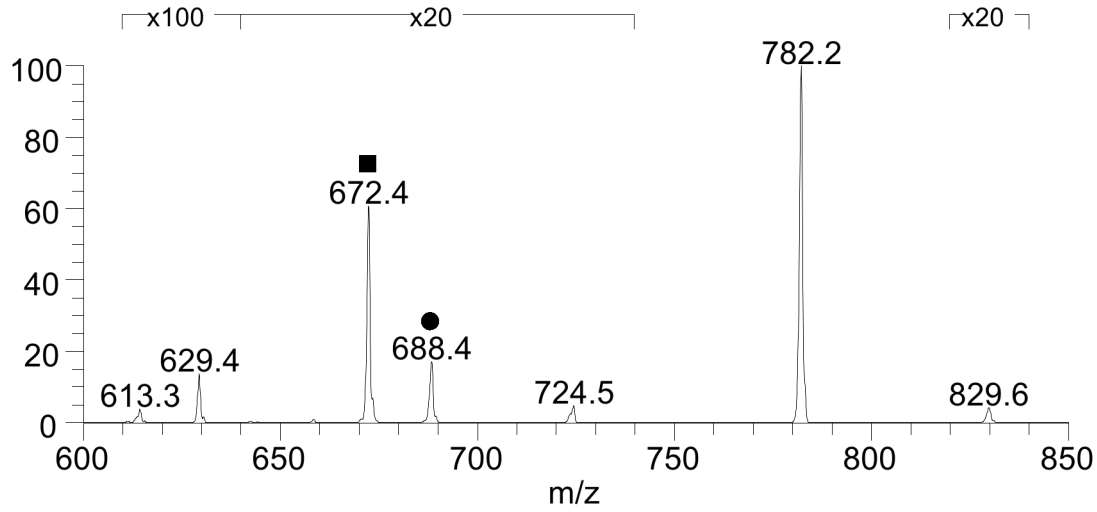
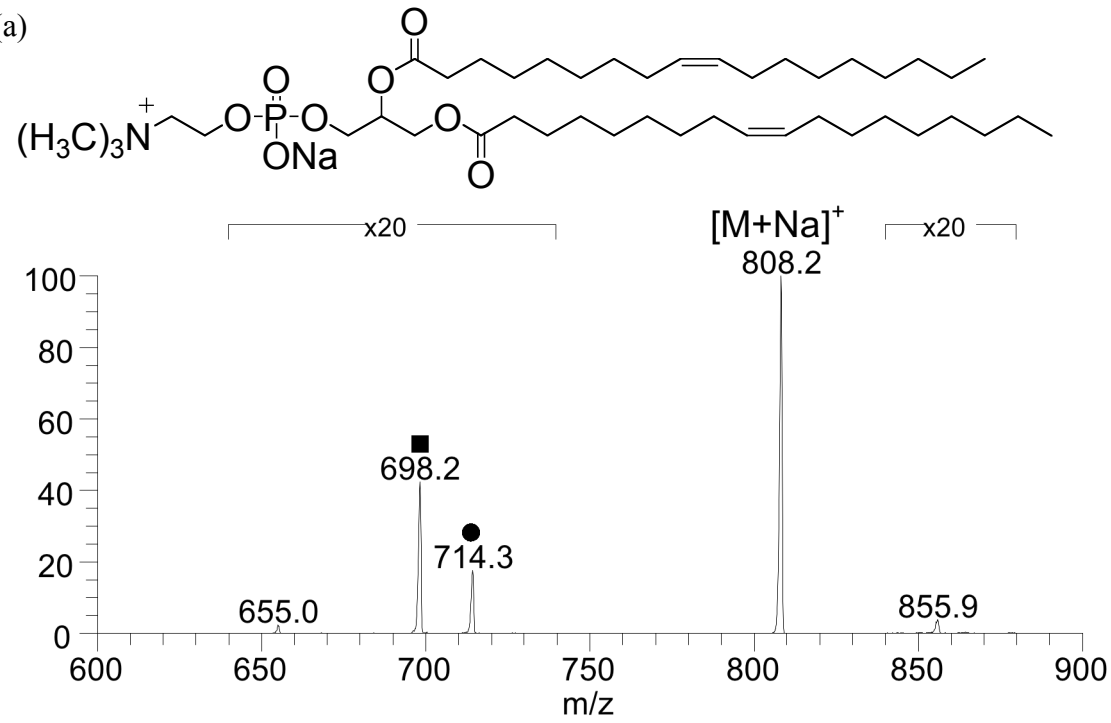


Figure 2

(a)


(H3C)3N+
O-P-O
ONa
x20
[M+Na]+
808.2
x20
100
80
60
40
20
0
698.2
714.3
655.0
855.9
600
650
700
750
800
850
900
m/z

(b)

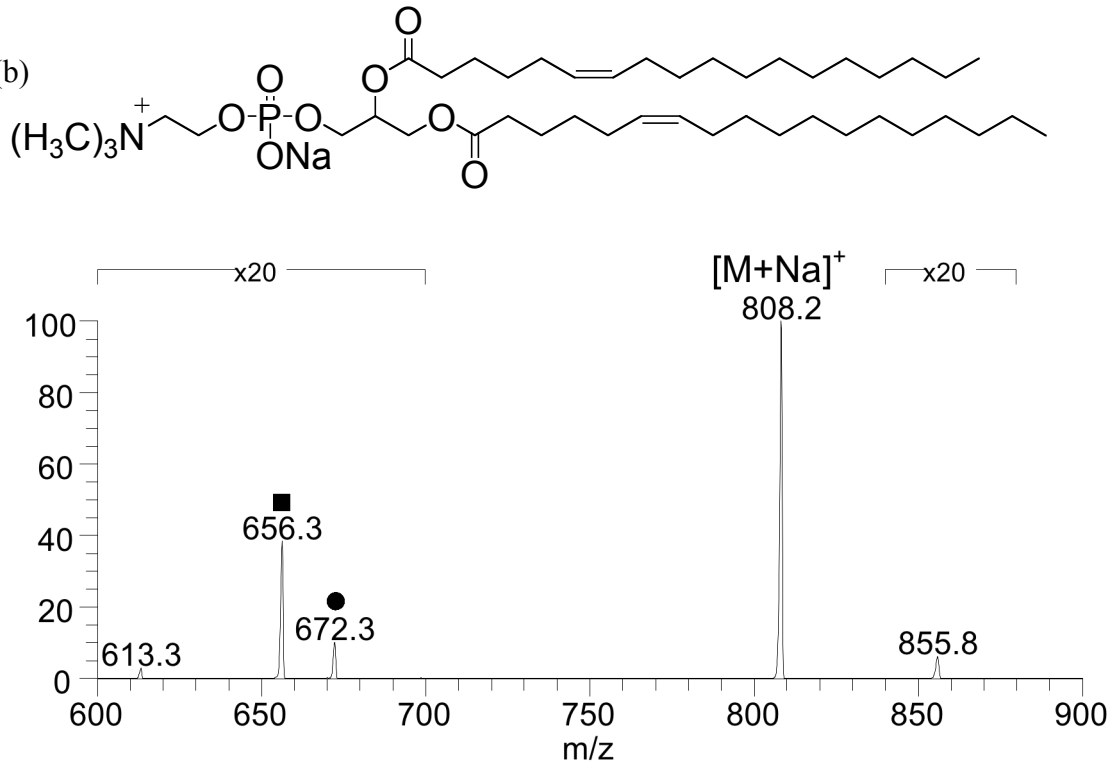

(H3C)3N+
O-P-O
ONa
x20
[M+Na]+
808.2
x20
100
80
60
40
20
0
656.3
672.3
613.3
855.8
600
650
700
750
800
850
900
m/z

Figure 3

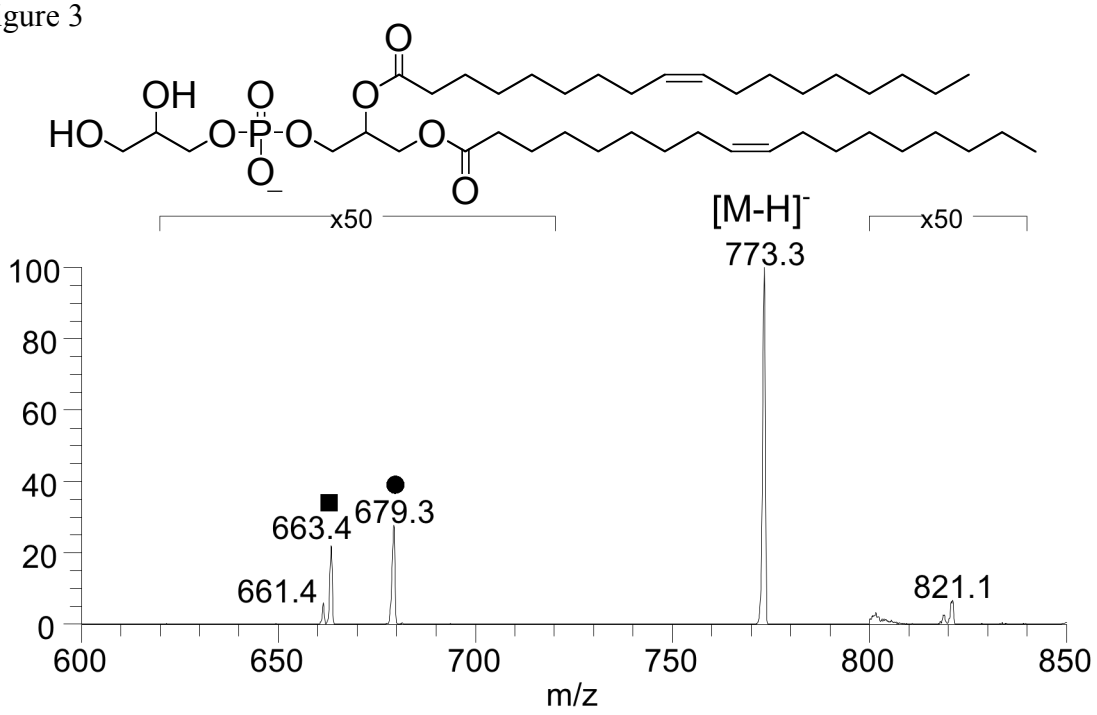
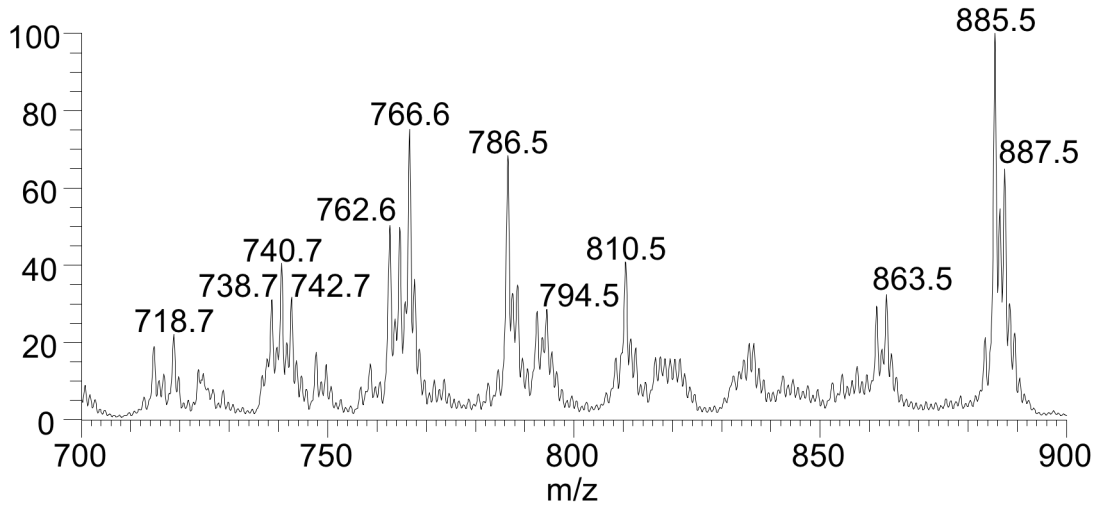
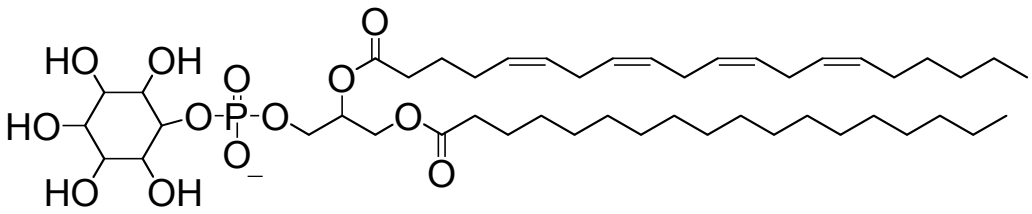

OH
HO
O
O
O
O
O
O
O
O
O
x50
x50
[M-H]-
773.3
100
80
60
40
20
0
663.4
679.3
661.4
821.1
600
650
700
750
800
850
m/z

Figure 4

(a)


100
80
60
40
20
0
700
750
800
850
900
m/z
885.5
887.5
766.6
786.5
762.6
740.7
738.7
742.7
718.7
794.5
810.5
863.5

(b)


HO
OH
HO
O
P
O
O
O
O
O
O
HO
OH

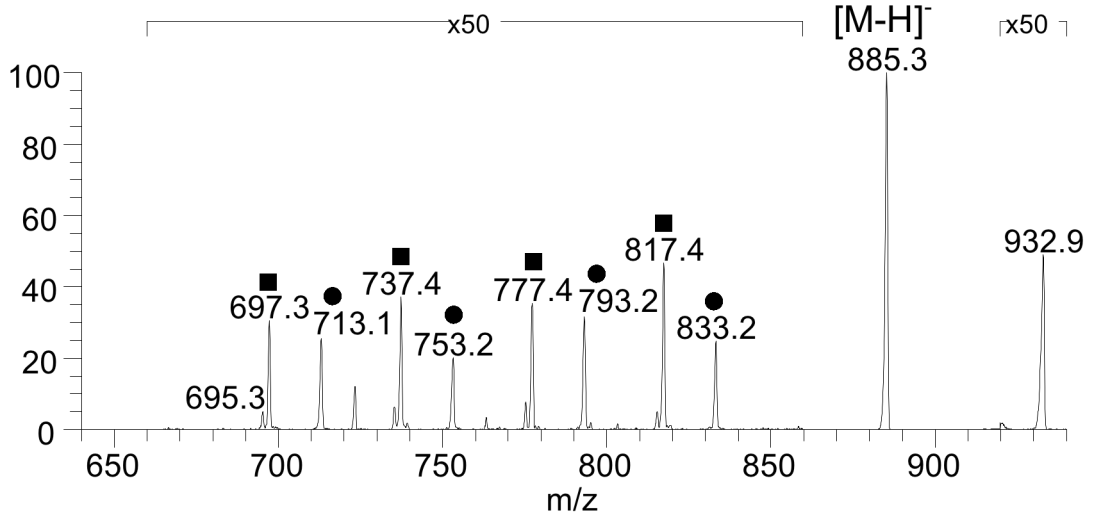
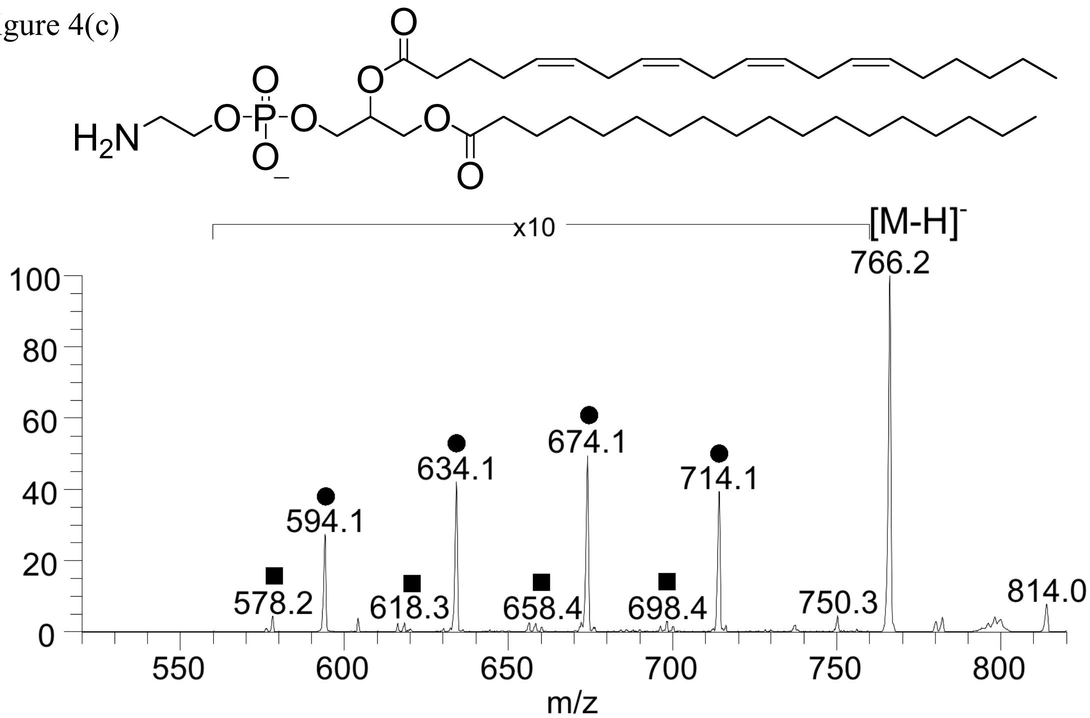

x50
[M-H]-
885.3
x50
100
80
60
40
20
0
650
700
750
800
850
900
m/z
932.9
817.4
737.4
777.4
793.2
697.3
713.1
833.2
753.2
695.3

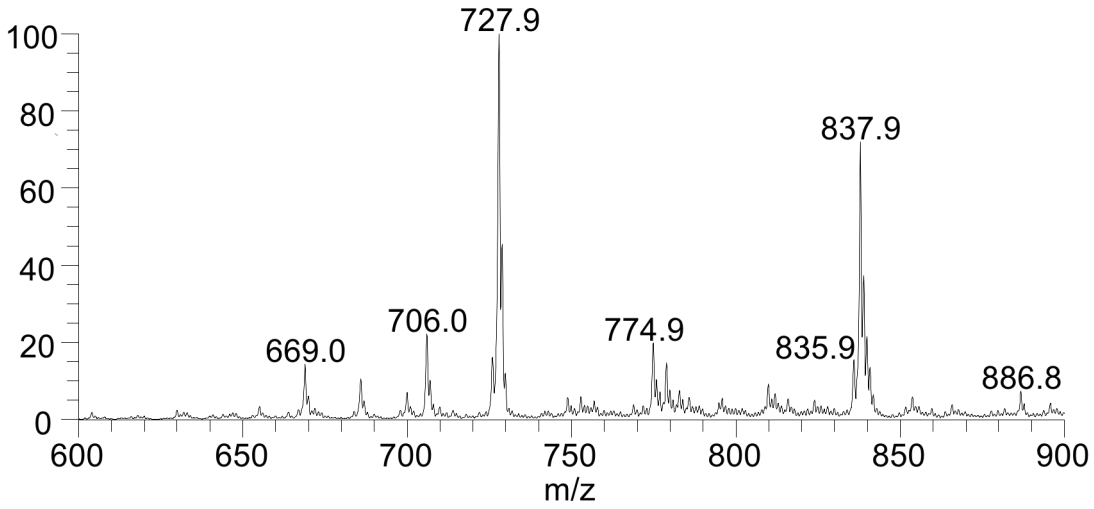
Figure 4(c)



O
O
O
H_2N
O
P
O
O
O
O
O
x10
[M-H]-
766.2
100
80
60
40
20
0
594.1
634.1
674.1
714.1
578.2
618.3
658.4
698.4
750.3
814.0
550
600
650
700
750
800
m/z

Figure 5

(a)


727.9
837.9
706.0
774.9
669.0
835.9
886.8
100
80
60
40
20
0
600
650
700
750
800
850
900
m/z

(b)

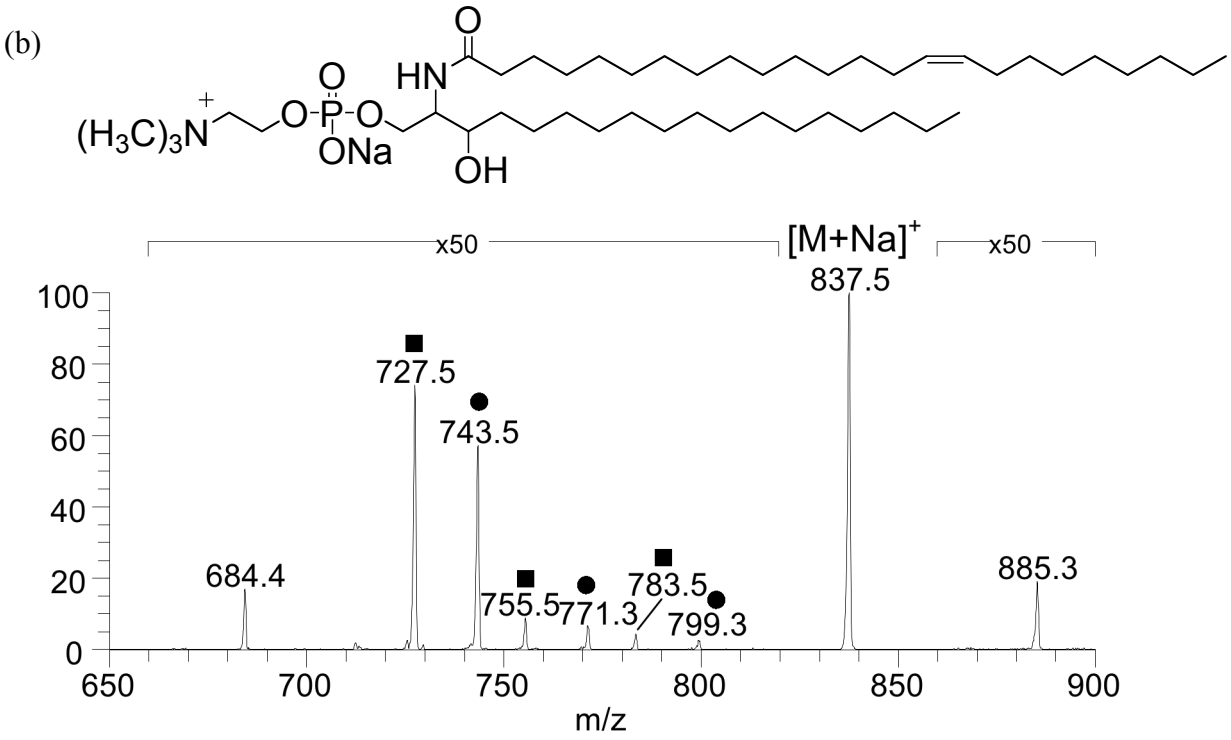

O
HN
O
(H3C)3N
O-P-O
ONa
OH
x50
[M+Na]+
x50
837.5
727.5
743.5
684.4
755.5
771.3
783.5
799.3
885.3
100
80
60
40
20
0
650
700
750
800
850
900
m/z

Figure 6 (a)

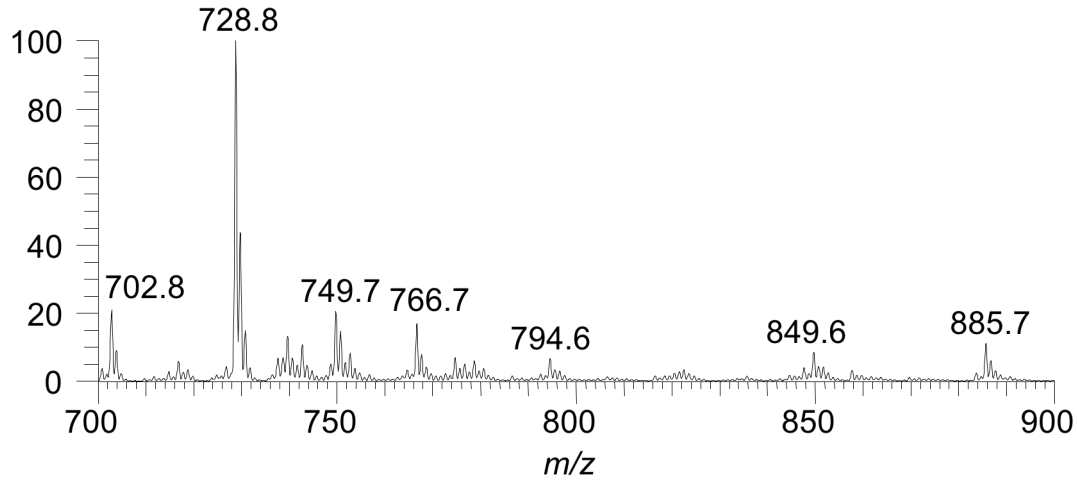


Figure 6(b)

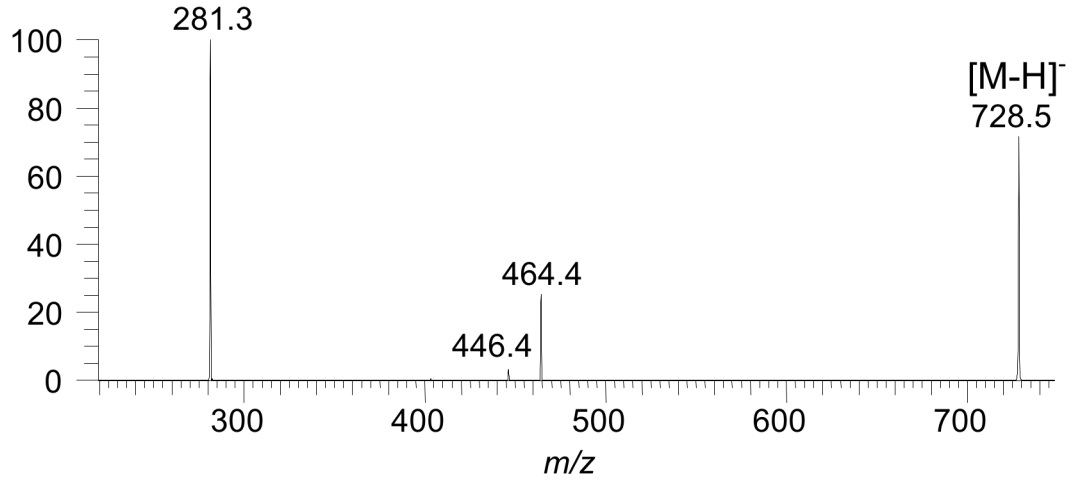


Figure 6(c)

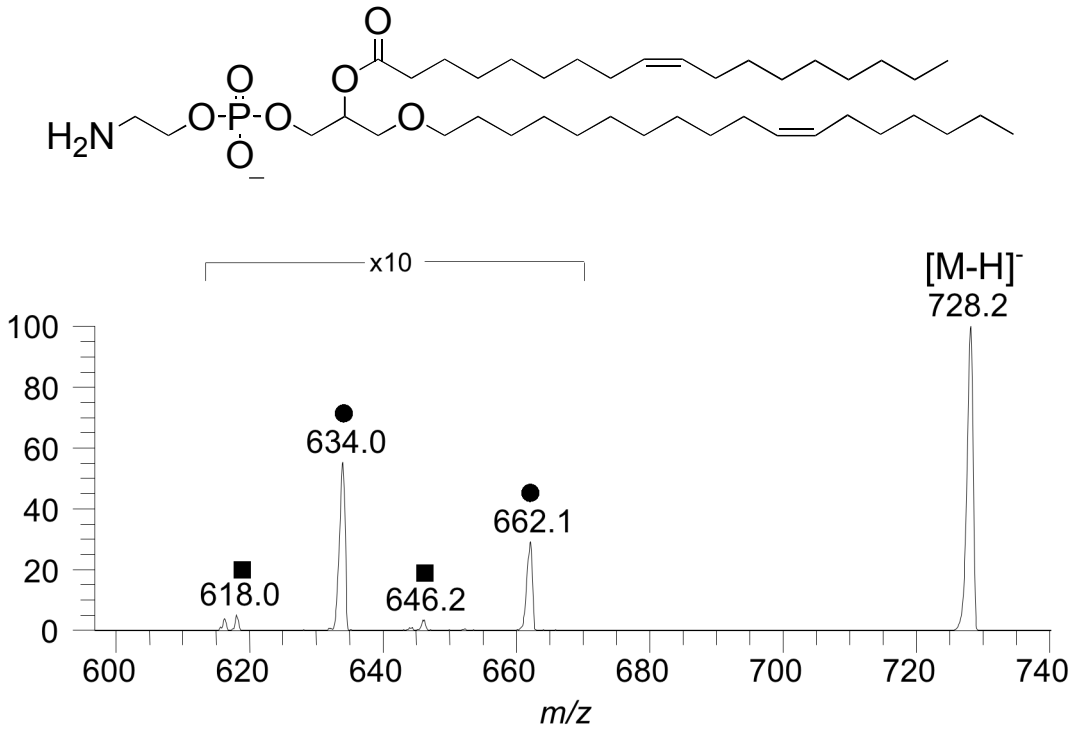

O
O
O
O
H2N
O-P-O
O
O
x10
[M-H]-
728.2
100
80
60
40
20
0
634.0
662.1
618.0
646.2
600
620
640
660
680
700
720
740
m/z

Figure 6(d)

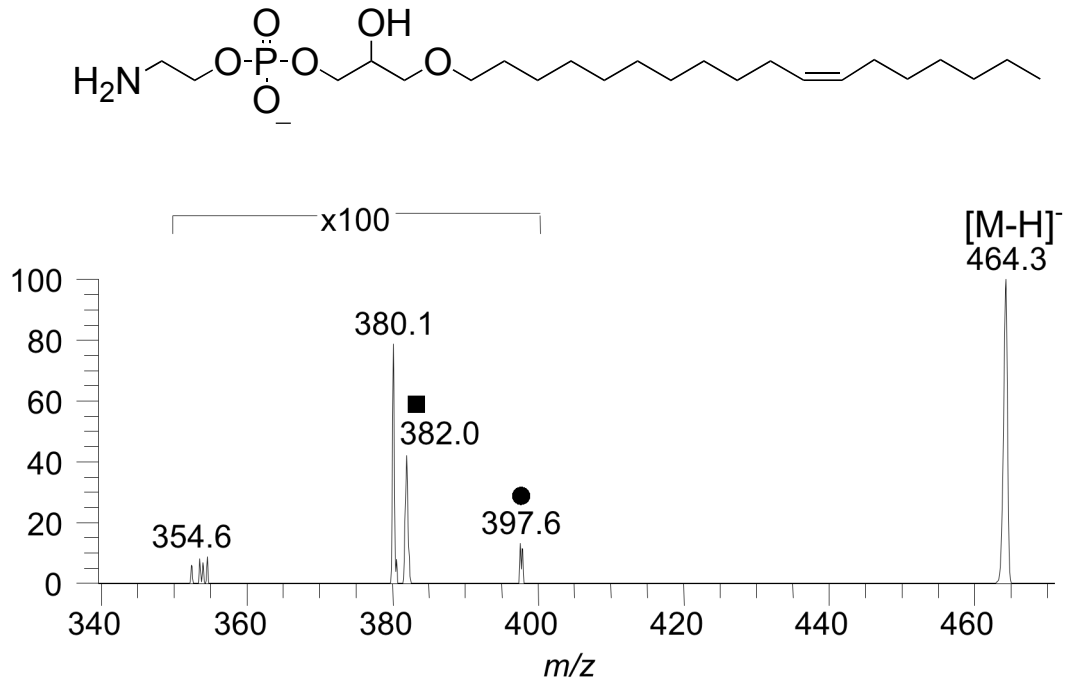
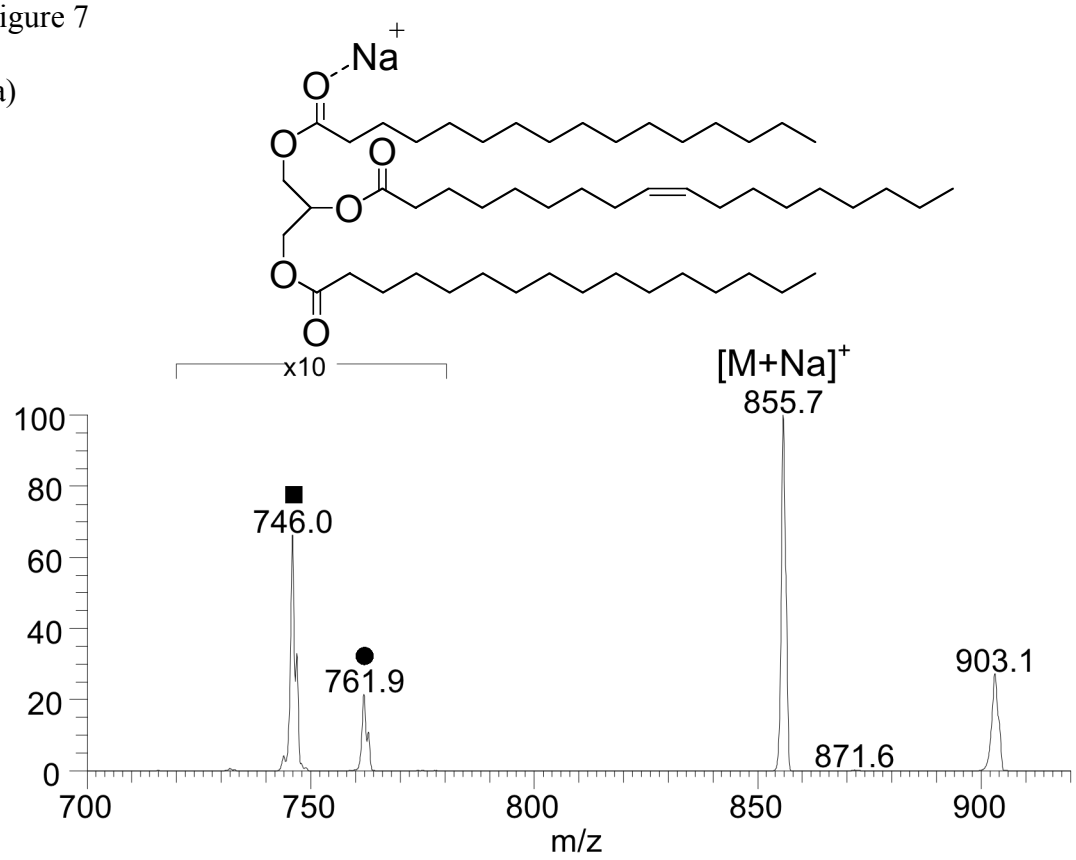

H2N
O
OH
O-P-O
O
O
x100
[M-H]-
464.3
380.1
382.0
397.6
354.6
100
80
60
40
20
0
340
360
380
400
420
440
460
m/z

Figure 7

(a)


Na+
O
O
O
O
O
O
x10
[M+Na]+
855.7
100
80
60
40
20
0
746.0
761.9
903.1
871.6
700
750
800
850
900
m/z

(b)

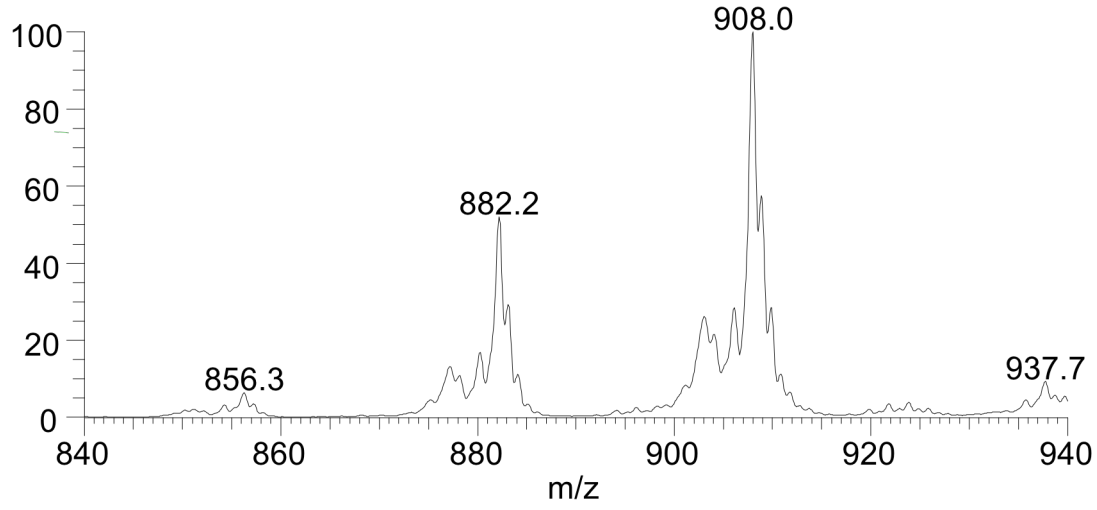
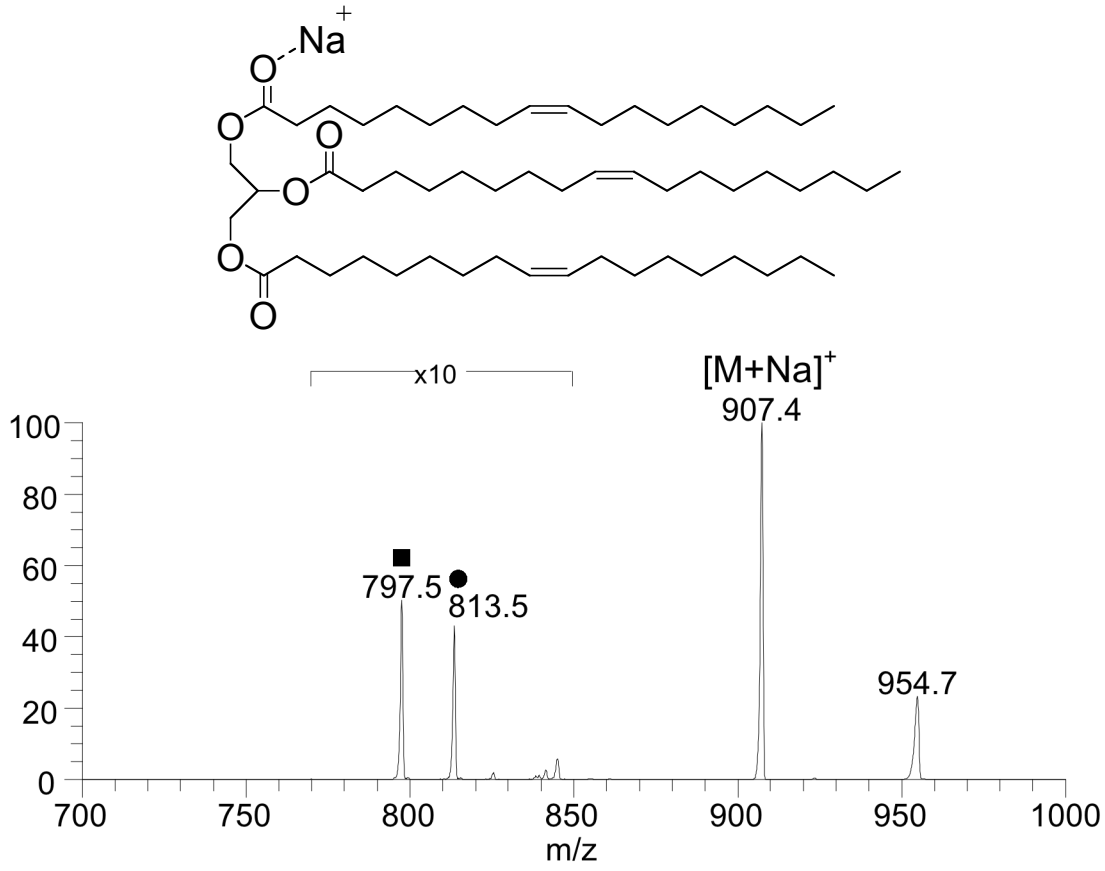
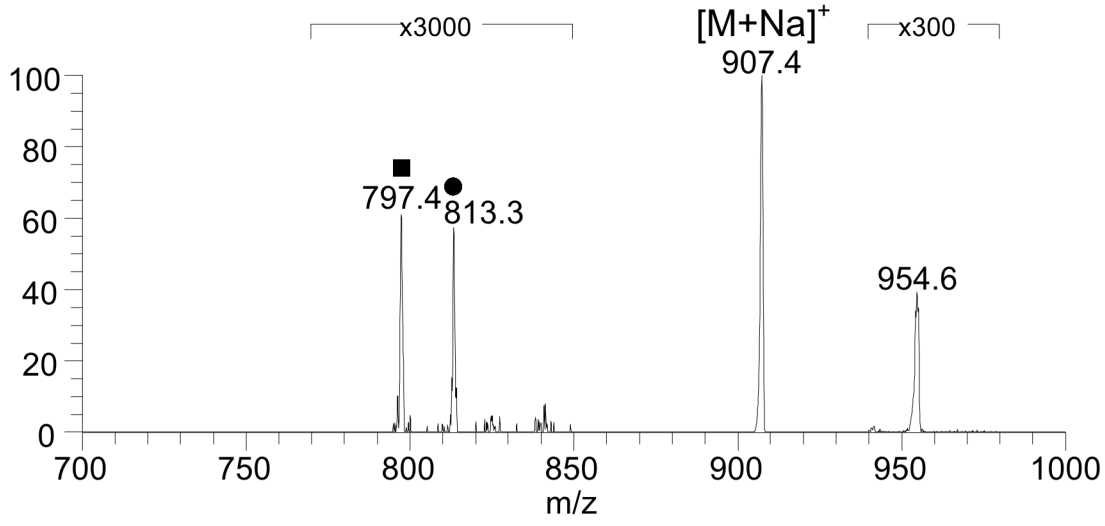

908.0
100
80
60
40
20
0
882.2
856.3
937.7
840
860
880
900
920
940
m/z

Figure 7(c)


Na+
O
O
O
O
O
O
x10
[M+Na]+
907.4
797.5
813.5
954.7
100
80
60
40
20
0
700
750
800
850
900
950
1000
m/z

(d)


x3000
[M+Na]+
907.4
x300
797.4
813.3
954.6
100
80
60
40
20
0
700
750
800
850
900
950
1000
m/z